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LATEST RESEARCH INTO QUALITY CONTROL

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Meet the editor



Isin Akyar graduated from School of Medicine, Cukurova University, Adana, Turkey in 1989. She finished her Medical Microbiology Specialty training in Gazi University, Ankara, Turkey in 1999. She joined the Acibadem Labmed Clinical Laboratories in Istanbul as a Specialist of Microbiology in 2004. She became Coordinator of Microbiology in 2007. Since 2004 she has had several Quality Control trainings. She works in the first accredited laboratory according to ISO 15189 for clinical laboratories in Turkey. In 2008 she joined the Department of Medical Microbiology at Acibadem University in Istanbul. In 2011 she was promoted to serve as an Assistant Professor. Her special interests are laboratory quality control, molecular microbiology, parasitology and proteomics studies. Currently she is working as both Microbiology Coordinator and Assistant Professor. She has been an Associate Editor for the Journal of Acibadem University Science of Health since 2009.

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Preface

Quality control has an emerging importance in every field of life. Quality control is a process that is used to guarantee a certain level of quality in a product or service. It might include whatever actions a business deems necessary to provide for the control and verification of certain characteristics of a product or service. Most often, it involves thoroughly examining and testing the quality of products or the results of services. The basic goal of this process is to ensure that the products or services that are provided meet specific requirements and characteristics, such as being dependable, satisfactory, safe and fiscally sound. There are some standards which guarantee quality control. In those standards you've got to document everything and track it. You should write what you do, do what you write. Groups that engage in quality control typically have a team of workers who focus on testing a certain number of products or observing services being done. The goal of the quality control team is to identify products or services that do not meet a company's specified standards of quality. If a problem is identified, the job of a quality control team or professional might involve stopping production or service until the problem has been corrected. Depending on the particular service or product as well as the type of problem identified, production or services might not cease entirely. There should be well organized procedures and management for ensuring quality control.

With the improvement of technology everyday we meet new and complicated devices and methods in different fields. Quality control should be performed in all of those new techniques.

In this book "Latest Research Into Quality Control" our aim was to collect information about quality control in many different fields such as:

Quality Control in general: SOPs

Quality Control in Clinical Laboratory Medicine

Quality Control of Herbal Medicine

Quality Control in Food Science

Quality Control in Pharmaceuticals

Quality Control in Radiology and Clinical Imaging

Quality Control in Energy

Quality Control in Cosmetics

The aim of this book is to share useful and practical knowledge about quality control in several fields with the people who want to improve their knowledge.

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Quality Control of Herbal Medicine

Quality Control of *Rheum* and *Cassia* Species by Immunological Methods Using Monoclonal Antibodies Against Sennosides

Osamu Morinaga and Yukihiro Shoyama

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/51272>

1. Introduction

Recently, medical usage of Japanese traditional medicine has been expanded by reaching aging society and increasing various chronic diseases. Therefore, the demand of crude drugs prescribed for Japanese traditional medicine has been increased. However, over 90% of crude drugs are imported in our country, and those over 70% are supplied by the collection of wild species. It is well known that the natural resources bring the difficulty of quality control depending on collection season, cultivation place, a variety of species and so on. The other problem, shortage of crude drug comes up. For these general environment, micropropagation and clonal propagation systems using tissue and cell culture were investigated in this laboratory.

Sennoside A (SA) and B (SB) have the strong catharsis activity and contained in rhubarb and senna (Figure 1) [1]. The concentration of sennosides in rhubarb and senna is variously dependent on the genetic heterogeneity of species, differences in soil condition and climate influence. Sennosides are metabolized by intestinal bacteria to rheinanthrone which acts in the intestines as a direct purgatives [2, 3] and functions as similar to a natural prodrug (Figure 2). Despite the rising availability of a number of synthetic cathartics, sennoside-containing prescriptions are still among the most widely used today, and their importance is increasing.

Rhubarb, the rhizome and root of *Rheum* spp. (Polygonaceae), is an important drug in traditional Japanese herbal medicine as well as in western medicine since ancient times. It was already recorded in *Chinese Materia Medica* 2000 years ago. It is used in many traditional Japanese herbal medicines prescribed with other herbal medicines for the syndrome of stasis of blood, as an anti-inflammatory, sedative agent and as a stomachic. Furthermore, it is widely

used as cathartics in Japan. The main purgative principles of rhubarb have proved to be sennosides [1], identical with those isolated from senna leaves, and rheinosides, which were also isolated as purgatives of rhubarb, together with various kinds of phenolics, like tannins, stilbenes, naphthalenes and lindleyin. The quality of rhubarb is severely regulated by Japanese Pharmacopoeia as rhubarb contains SA of over 0.25% dry weight in root [4].

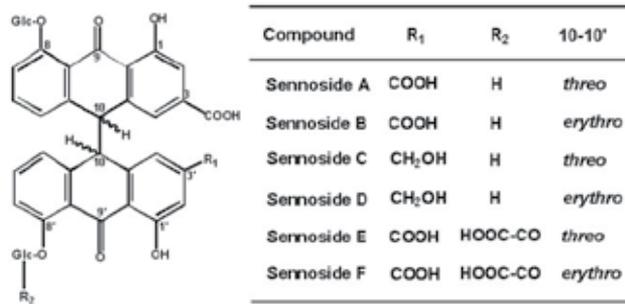


Figure 1. Structures of sennosides

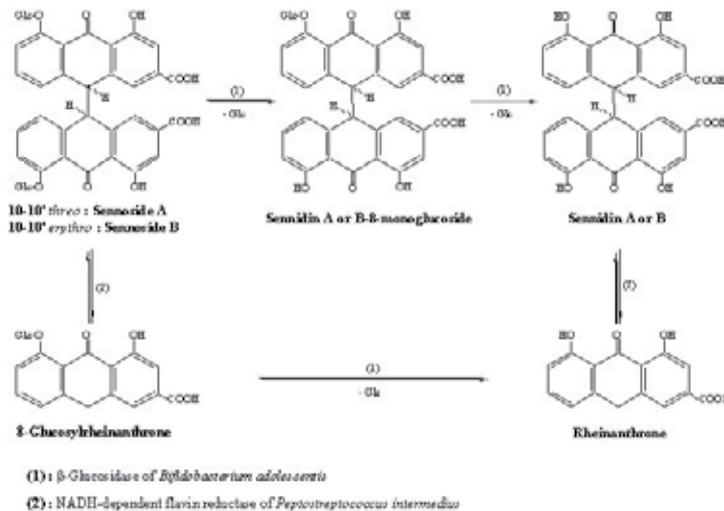


Figure 2. Metabolic pathways of sennosides by intestinal bacteria.

Senna, the leaf and pod of *Cassia* spp. (Leguminosae), is one of the most popular in herbal remedies and in health food industry. It has been widely used in cathartics for the relief of constipation prescribed with other health teas and dieter's teas in Japan, and often used as natural dietary supplements for enhancement of bloodflow and metabolism in USA, Europe and Australia. These pharmaceutical properties are due to sennosides, which are contained

in *Cassia acutifolia* Delile and *Cassia angustifolia* Vahl. *C. angustifolia* listed in Japanese Pharmacopeia, and the quality is severely regulated as senna contains total sennosides (SA and SB) of over 1.0% dry weight in leaf [4].

In the breeding research on the plant, a lot of stages are required as follows : dedifferentiation, extension of mutation by the mutagen, redifferentiation, analysis of the redifferentiated plant, mass propagation of the higher yielding plant and transplanting to soil. Therefore, it is very important to study a large number of plant samples in the phytochemical field and a small sample size *in vitro* for the breeding of *Rheum* and *Cassia* species yielding high concentration of sennosides. Many analytical approaches have been investigated for the determination of sennosides in plant extracts. Among these methods, the use of high-performance liquid chromatography (HPLC) appears most frequently and widely today. However, when the assay of very low concentration of sennosides in the regenerated plantlets is needed, the HPLC method is not appropriate and efficient.

Recently, the immunological assay method is widely developed for the purpose of analysis for a small amount of constituent. In general immunological methodologies in particular enzyme-linked immunosorbent assay (ELISA) have promoted the development of higher sensitive assay system.

On the one hand, monoclonal antibodies (MAbs) have many potential uses in addition to immunological methods in plant sciences. MAbs are superior to polyclonal antibodies (PAb) in the antigenic specificity and stability. Therefore, immunoassay using MAbs against pharmacologically active compound having small molecular weight has become an important tool for the studies on receptor binding analysis, enzyme assay and quantitative and/or qualitative analytical techniques in plants owing to its specific affinity, and possesses an extremely high possibility in the phytochemical analysis. Up to now, immunological approach for assaying quantities of sennosides in *C. angustifolia* using PAb against SB has been investigated by Atzorn *et al* [5]. However, since no success with MAbs against SA and SB has been reported, objectives of this work are shown as following.

1. Production of MAb against SA, its characterization and use for ELISA.
2. Production of MAbs against SB, their characterization and use for ELISA.
3. Establishments of a new eastern blotting, double staining and immunohistochemical staining using anti-SA and SB MAbs.

2. Production of MAb against SA, its characterization and use for ELISA

2.1. Preface

In the immunologically analytical methodology, there are two measuring methods using the antiserum (polyclonal antibody ; PAb) and MAb in general. PAb is a heterogeneous mixture of antibody molecules arising from a variety of constantly evolving B lymphocytes. Therefore, PAb can often show high affinity because different antibody populations react with the

variety of epitopes that characterize the antigen. On the other hand, there are some problems of PAb that the extensive cross-reactivity occurs between the antibody and the multiple antigens which have the same antigenic determinant, and it is impossible to supply for identical antibody permanently. In the meantime, MAb is produced from a single B lymphocyte and can react with one antigenic determinant of the specific antigen. Besides MAb has identical specificity and affinity. There are some advantages that the complete purity of the immunized antigen is not required and the hybridoma cells can be preserved as freeze stock, and it is possible to get MAb depending on necessary respond.

There are several formats for ELISA like direct ELISA, competitive ELISA, sandwich ELISA and competitive ELISA according to the immune complexes formed during manipulation. Analysis of low molecular weight compound by immunoassay is still limited to competitive format.

Quality control of the Japanese herbal medicine is necessary because it is believed that approximately 70% of these crude drugs prescribed are collected from natural resource. Furthermore, since MAbs become necessary for the assay of concentrations of active constituents in our on-going plant biotechnological projects, we have already produced MAbs against natural compounds such as forskolin [6], solamargine [7], opium alkaloids [8], marijuana compounds [9], glycyrrhizin [10], crocin [11], ginsenoside Rb1 [12] and Rg1 [13], and developed individual competitive ELISAs. An immunological approach for assaying quantities of sennosides using a PABs has been investigated by Atzorn *et al.*[5]. However, since no result of MAb related to sennosides has been reported yet, anti-SA MAb was produced as described [14].

2.2. Experimental

2.2.1. Chemicals and immunochemicals

SA was purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). 1-Ethyl-3-(3'-dimethylaminopropyl)-carbodiimide HCl (EDC) was purchased from Nacalai Tesque Inc. (Kyoto, Japan). BSA and HSA were provided by Pierce (Rockford, IL, USA). Peroxidase-labeled anti-mouse IgG was provided by Organon Teknika Cappel Products (West Chester, PA, USA). Enriched RPMI1640-Dulbecco's-Ham's F12 (eRDF) medium and RD-1 additives (containing 9 µg/mL insulin, 20 µg/mL transferrin, 20 µM ethanolamine, 25 µM sodium selenite) were purchased from Kyokuto Pharmaceutical Industrial Co., Ltd. (Tokyo, Japan). Hypoxanthine-aminopterin-thymidine (HAT) additives were obtained from Sigma Chemical Company (St. Louis, MO, USA). Fetal calf serum (FCS) was purchased from Cambrex Corporation (Walkersville, MA, USA). All other chemicals were standard commercial products of analytical grade. Samples of various rhubarb roots were purchased from the Tochimotoenkaido Corporation (Osaka, Japan).

2.2.2. Extraction of various rhubarb samples

Dried samples (30 mg) of various rhubarb roots were powdered, and then extracted five times with MeOH containing 0.1% (w/v) NH_4OH (0.5 mL) with sonication, filtered using a Cosmonice Filter W (0.45 μm Filter Unit, Nacalai Tesque Inc., Kyoto, Japan), and the combined extracts were diluted with 10 mM NaHCO_3 to prepare a solution suitable for the ELISA.

2.2.3. Synthesis of antigen conjugates

To SA (6 mg) dissolved in 1 mL of tetrahydrofuran-20 mM phosphate buffer of pH 5.5 (7:3), 0.3 mL of 20 mM phosphate buffer (pH 5.5) containing 6 mg of EDC was added. Then, 0.3 mL of 20 mM phosphate buffer (pH 5.5) containing 6 mg of BSA was added, with stirring at room temperature for 14 hr. The reaction mixture was dialyzed five times against H_2O , and then lyophilized to give 5.8 mg of SA conjugate (SA-BSA). SA-HSA conjugate was also synthesized in the same manner.

2.2.4. Determination of hapten density in SA-carrier protein conjugate by matrix-assisted laser desorption/ionization (MALDI)-time of flight (TOF) mass spectrometry

The hapten number in the SA-carrier protein conjugate was determined by MALDI-TOF mass spectrometry as previously described [15]. A small amount (1-10 pmol) of antigen conjugate was mixed with a 10^3 -fold molar excess of sinapinic acid in an aqueous solution containing 0.15% trifluoroacetic acid (TFA). The mixture was subjected to a JEOL Mass Spectrometers (JMS) time-of-flight (TOF) mass monitor (model Voyager Elite, PerSeptive Biosystems Inc., Framingham, MA, USA) and irradiated with a N_2 laser (337 nm, 150 ns pulse). The ions formed by each pulse were accelerated by a 20 kV potential into a 2.0 m evacuated tube and detected using a compatible computer as previously reported [15].

2.2.5. Competitive ELISA for SA

SA-HSA (five molecules of SA per molecule of HSA) (100 μL , 1 $\mu\text{g}/\text{mL}$) dissolved in 50 mM carbonate buffer (pH 9.6) was adsorbed to the wells of a 96-well immunoplate then treated with 300 μL S-PBS for 1 hr to reduce non-specific adsorption. Fifty μL of various concentrations of SA or samples dissolved in 10 mM NaHCO_3 solution were incubated with 50 μL of MAbs solution (0.218 $\mu\text{g}/\text{mL}$) for 1 hr. The plate was washed three times with T-PBS, and then incubated with 100 μL of a 1:1000 dilution of POD-labeled anti-mouse IgG for 1 hr. After washing the plate three times with T-PBS, 100 μL of substrate solution [0.1 M citrate buffer (pH 4) containing 0.003% H_2O_2 and 0.3 mg/mL of ABTS] was added to each well and incubated for 15 min. The absorbance was measured by a micro plate reader at 405 nm and 490 nm.

The cross-reactivities (CR) of sennosides and related compounds were determined as following.

$$\text{CR}(\%) = \frac{\mu\text{g}/\text{mL of SA yielding } A/A_0=50\%}{\mu\text{g}/\text{mL of compound under investigation yielding } A/A_0=50\%} \times 100$$

where A is the absorbance in the presence of the test compound and A_0 is the absorbance in the absence of the test compound.

2.3. Results and discussion

2.3.1. Direct determination of SA-carrier protein conjugate by MALDI-TOF mass spectrometry

In general, the low molecular weight compounds (hapten) like plant secondary metabolite have no immunogenicity. Therefore, it should be conjugated with some high molecular compound like protein resulting in immunogenic. The specificity of immunoassay method is dependent on the site of linkage between hapten and carrier protein moiety, and enumeration of hapten in immunogen conjugate. SA-BSA and SA-HSA conjugates were synthesized as immunogen and the immobilization antigen for ELISA, respectively. Figure 3 shows the typical synthetic pathway of SA-BSA conjugate. The commonly used methods to link carboxyl group and amino group in a hapten or carrier involve activation by carbodiimides, isobutylchloroformate or carbonyldiimidazole. Carbodiimides react with carboxyl groups to form an unstable *O*-acetylisourea intermediate, which reacts with amines to form amide bonds. EDC can be used commonly as a carbodiimide. In this case, carrier protein combined directly to antigen as indicated in Figure 3.

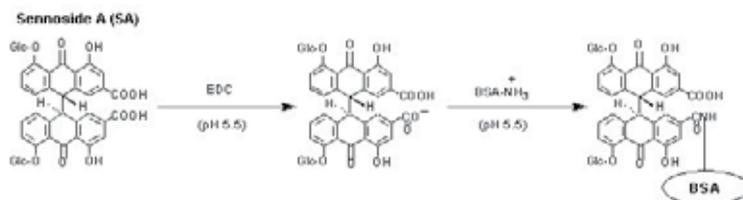


Figure 3. Typical synthetic pathway of SA-BSA. Carboxyl group of SA was activated by EDC and subsequently combined to amino residues of lysine and/or arginine on the protein to form amide bond.

Figure 4 shows the MALDI-TOF mass spectrum of the antigen, SA-BSA conjugate. A broad peak coinciding with the conjugate of SA and BSA appeared from m/z 68,500 to 73,500 centering at around m/z 70,600. Using experimental results and a molecular weight of 66,433 for BSA, the calculated values of SA component (MW 862) are 4,218 resulting in the range of two to eight molecules of SA (five on average) conjugated with BSA. In general eight to twenty five molecules of hapten conjugated with carrier protein in the conjugate were sufficient for immunization. Therefore, the hapten number was estimated to be sufficient for immunization because an antigen conjugate having a similar hapten number was sufficient for immunization in a previous study [10]. The number of SA contained in the SA-HSA conjugate was also determined to be around five molecules by its spectrum.

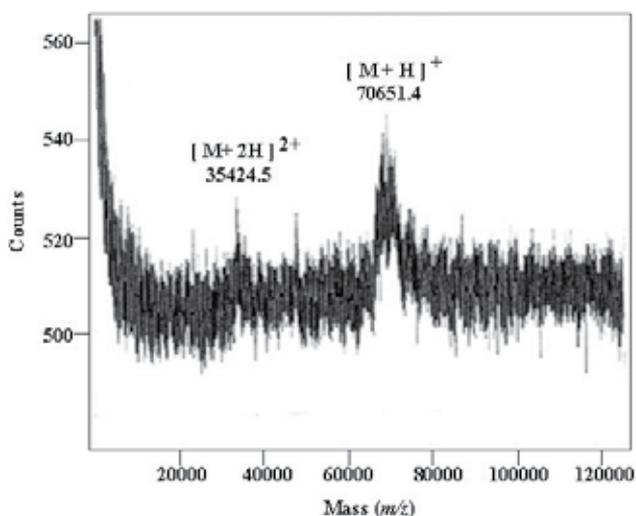


Figure 4. Direct determination of SA-BSA by MALDI-TOF MS. $[M+H]^+$, $[M+2H]^{2+}$ are single and double protonated molecules of SA-BSA, respectively.

2.3.2. Production and characteristic of MAb against SA

After the cell fusion and HAT selection, hybridoma producing MAb reactive to SA was obtained, and classified into IgG₁ which had *k* light chains. Refined MAb was confirmed to be IgG compared to the MALDI-TOF MS measurement. The molecular weight of MAb was 151,396 calculated [16]. The reactivity of IgG type MAb 6G8 was tested for varying the antibody concentration and for performing a dilution curve in direct ELISA. The antibody concentration of 0.218 $\mu\text{g/mL}$ showed the absorbance at 0.8 in direct ELISA, therefore it was selected for the competitive ELISA.

2.3.3. Assay sensitivity and assay specificity

The free MAb 6G8, following incubation with competing antigen, was bound to the polystyrene microtitre plates precoated with SA-HSA. Under these conditions, the full measuring range of the assay extended from 20 to 200 ng/mL as indicated in Figure 5.

SA is a unique anthraquinone having individual double of carboxylic acid-, hydroxyl-, carbonyl- and *O*-glucosyl-groups at C-3, C-1, C-9 and C-8 positions in a molecule, respectively. Moreover, SA possessed a *threo*-configuration between C-10 and C-10' positions as indicated in Figure 6. Therefore, a MAb should detect all these functions, and also the stereochemical recognition is needed for this complicated compound. Since the newly established ELISA against SA is expected to be applied for phytochemical investigations involving crude plant extracts, the assay specificity was checked by determining the cross-reactivities of MAb with various related compounds. The cross-reactivities of the MAb was examined by competitive ELISA.

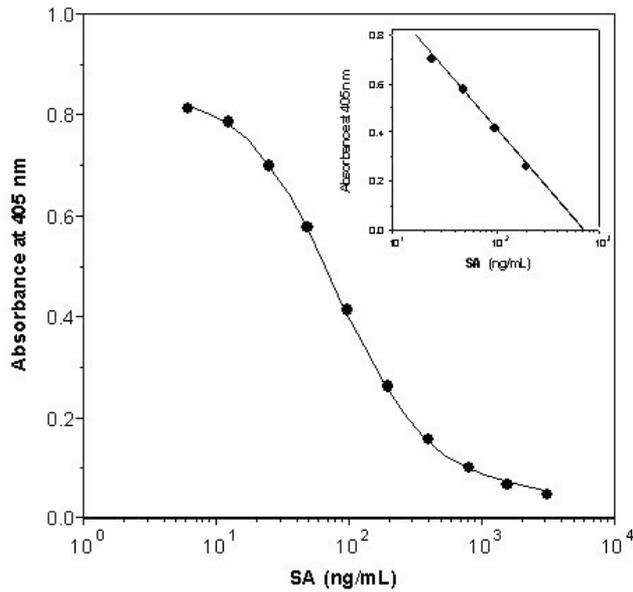


Figure 5. Calibration curve for SA.

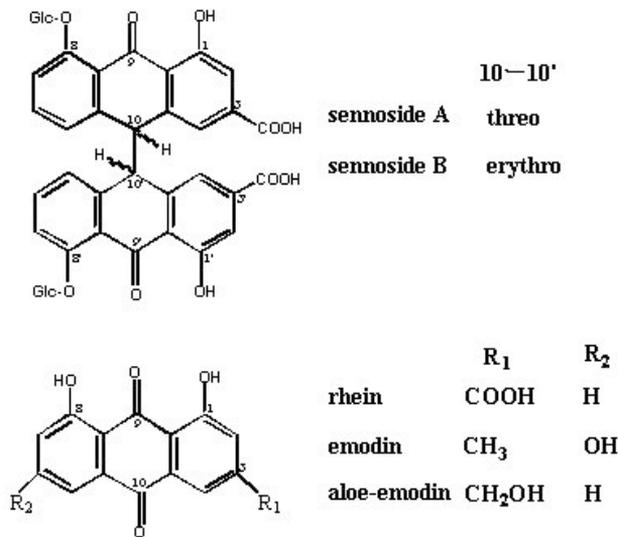


Figure 6. Chemical structures of SA, SB and its structurally related compounds.

Table 1 indicates the cross-reactivities of anti-SA MAb against related anthraquinone, anthrone and phenol carboxylic acid. MAb 6G8 cross-reacted with rhein and SB weakly; 0.28 and 0.35%, respectively. However, the other related anthraquinone and anthrone did not

have appreciable cross-reactivities. From these results it is suggested that a basal structure of rhein and sugar moiety caused immunization. In addition the most important property of MAb 6G8 is an ability of stereochemical recognition because the differences of structure between SA and SB are only the stereochemical configuration at the C-10 and C-10' positions. Therefore, it is suggested that *threo*-configurational structure of bisanthrone is indispensable as an immunodominant molecule for reactivity of MAb 6G8.

Compound	Cross-reactivities (%)
Anthraquinone and anthrone	
sennoside A	100
sennoside B	0.28
rhein	0.35
emodin	< 0.04
aloe-emodin	< 0.04
barbaloin	< 0.04
1,4-dihydroxy-anthraquinone	< 0.04
Stilbene	
rhaponticin	< 0.04
Phenol carboxylic acid	
gallic acid	< 0.04
vanillic acid	< 0.04
caffeic acid	< 0.04
homogentisic acid	< 0.04

Table 1. Cross-reactivities (%) of MAb-6G8 against sennosides and other compounds.

2.3.4. Correlation of results of SA determination in crude extracts of rhubarb roots between HPLC and ELISA using MAb 6G8

The ELISA was utilized to measure the concentrations of SA in various rhubarb (Table 2). Oshio and Kawamura determined sennoside concentrations in various crude rhubarbs by HPLC [17]. More recently Seto *et al.* reported the comparative concentrations of sennosides determined by HPLC in various commercial rhubarbs [18]. They required a larger sample size compared to the newly established ELISA due to some pretreatments because the crude materials contained several kinds of phenolics such as tannins, stilbens, naphthalen derivatives and lindleyin as previously indicated.

Table 2 shows the SA concentrations in various rhubarbs. Shinshu Daio bred by crossing *R. palmatum* and *R. coreanum* in order to increase the concentration of SA in Japan, contained the highest SA; 13.69±0.69 µg/mg dry wt. Ga-wo which was estimated to be high grade, con-

tained 6.62 ± 0.42 $\mu\text{g}/\text{mg}$ dry wt. The other three species showed almost the same concentrations of SA, around 3.3 $\mu\text{g}/\text{mg}$ dry wt. These results are in good agreement with the previous reports [18]. The correlation between results from ELISA and HPLC is reasonable except for Kinmon Daio. The concentration analyzed by HPLC was very low compared to the others. The reason is still obscure although individual peaks separated by HPLC were analyzed by ELISA.

Sample	Concentration ($\mu\text{g}/\text{mg}$ dry wt. powder)	
	ELISA	HPLC
Shinshu Daio	13.69 ± 0.69	12.28 ± 0.41
Ga-wo	6.62 ± 0.42	6.93 ± 0.02
Kinmon Daio	3.34 ± 0.02	0.85 ± 0.04
Itto-Ga-wo (powder)	3.27 ± 0.20	3.69 ± 0.32
Itto-Ga-wo (refuse)	3.43 ± 0.16	3.69 ± 0.28

Table 2. SA concentrations in various rhubarb samples. Data are the means of triplicate assays.

3. Production of MAbs against SB, their characterization and use for ELISA

3.1. Preface

SB is a very important natural bioactive component of rhubarb and senna as well as SA. Total sennoside (SA and SB) concentrations are important, when rhubarb and senna are used as a raw material of medical supply and traditional Japanese herbal medicine for the purgative effect.

A number of methods for the quantification of SB have been published, most of which have been performed by HPLC [17]. Immunological approaches for assaying quantities of sennosides and SA using PAb and MAb have been investigated by Atzorn *et al.* [5] and by us [14], respectively. However, no success with MAb against SB has been reported. In here, production of anti-SB MAb and the competitive ELISA using anti-SA and SB MAbs for the direct determination of SA and SB in various samples are described [19].

3.2. Experimental

3.2.1. Plant materials

Samples of various rhubarb roots were purchased from the Tochimotoenkaido Corporation (Osaka, Japan). Samples of leaves of *Cassia* plants were collected in Thailand. Traditional Japanese prescriptions were procured from Tsumura & Co. (Tokyo, Japan). Dietary supplements (health teas and dieter's teas) were purchased from drug and department stores.

3.2.2. Sample preparation

Dried samples (30 mg) of various rhubarb roots, *Cassia* plant leaves, traditional Japanese prescriptions and dietary supplements were powdered, and then extracted five times with MeOH containing 0.1% (w/v) NH_4OH (0.5 mL) with sonication, filtered using a Cosmonice Filter W (0.45 μm Filter Unit, Nacalai Tesque Inc., Kyoto, Japan), and the combined extracts were diluted with 10 mM NaHCO_3 to prepare a solution suitable for the ELISA.

3.2.3. Synthesis of antigen conjugates

To SB (6 mg) dissolved in 1 mL of tetrahydrofuran-20 mM phosphate buffer of pH 5.5 (7:3), 0.3 mL of 20 mM phosphate buffer (pH 5.5) containing 6 mg of EDC was added. Then, 0.3 mL of 20 mM phosphate buffer (pH 5.5) containing 6 mg of BSA was added, with stirring at room temperature for 14 hr. The reaction mixture was dialyzed five times against H_2O , and then lyophilized to give 5.5 mg of SB-BSA conjugate. SB-HSA conjugate was also synthesized in the same manner.

3.2.4. Determination of hapten density in SB-carrier protein conjugate by MALDI-TOF mass spectrometry

The hapten number in the SB-carrier protein conjugate was determined by MALDI-TOF mass spectrometry as previously described [15].

3.2.5. Competitive ELISA for SB

SB-HSA (four molecules of SB per molecule of HSA) (100 μL , 1 $\mu\text{g}/\text{mL}$) dissolved in 50 mM carbonate buffer (pH 9.6) was adsorbed to the wells of a 96-well immunoplate then treated with 300 μL S-PBS for 1 hr to reduce non-specific adsorption. Fifty μL of various concentrations of SB or samples dissolved in 10 mM NaHCO_3 solution were incubated with 50 μL of MAbs solution (0.121 $\mu\text{g}/\text{mL}$) for 1 hr. The plate was washed three times with T-PBS, and then incubated with 100 μL of a 1:1000 dilution of POD-labeled anti-mouse IgG for 1 hr. After washing the plate three times with T-PBS, 100 μL of substrate solution [0.1 M citrate buffer (pH 4) containing 0.003% H_2O_2 and 0.3 mg/mL of ABTS] was added to each well and incubated for 15 min. The absorbance was measured by a micro plate reader at 405 nm and 490 nm.

3.3. Results and discussion

3.3.1. Direct determination of SB-carrier protein conjugate by MALDI-TOF mass spectrometry

It is well known that hapten number in an antigen conjugate is important for immunization against low molecular weight compounds. Figure 7 shows the MALDI-TOF mass spectrum of the antigen, SB-BSA conjugate. A broad peak coinciding with the conjugate of SB and BSA appeared from m/z 67,300 to 70,700 centering at around m/z 68,900. Using experimental results and a molecular weight of 66,433 for BSA, the calculated values of SB component

(MW 862) are 2,500 resulting in the range of one to five molecules of SB (three on average) conjugated with BSA. This conjugate, although having a relatively low hapten number, proved sufficiently immunogenic in agreement with our previous results [10]. The number of SB contained in the SB-HSA conjugate was also determined to be around four molecules by its spectrum.

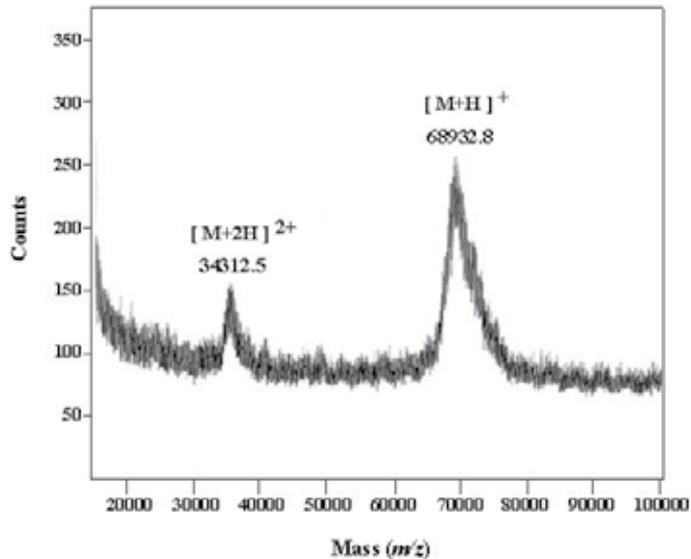


Figure 7. Direct determination of SB-BSA by MALDI-TOF MS.

3.3.2. Production and characteristics of Mabs against SB

The immunized BALB/c mice yielded splenocytes which were fused with P3-X63-Ag8-653 myeloma cells by the routinely established procedure in this laboratory [6]. Hybridoma producing MAbs reactive to SB were obtained, and classified as IgG1 (5G6, 7H12) and IgG2b (5C7) which had *k* light chains. The reactivity of IgG type MAb 7H12 was tested by varying the antibody concentration and by performing a dilution curve in direct ELISA. The antibody concentration (0.121 $\mu\text{g}/\text{mL}$) at which the absorbance was about 1.0 in direct ELISA was selected for competitive ELISA.

3.3.3. Assay sensitivity and assay specificity

The free MAb 7H12 following competition was bound to the polystyrene microtitre plates precoated with SB-HSA. Under these conditions, the full measuring range of the assay extends from 0.5 ng/mL to 15 ng/mL as indicated in Figure 8 and the ELISA using a MAb 7H12 is more sensitive than those using MAb 5C7 and 5G6.

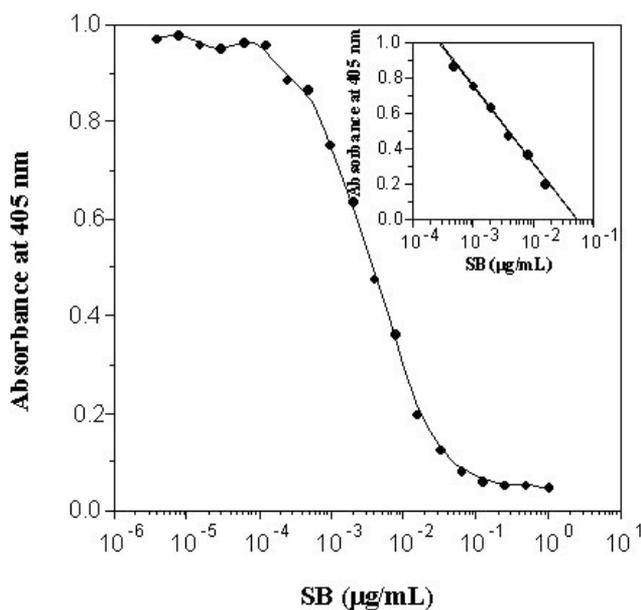


Figure 8. Calibration curve for SB.

SB is a unique anthraquinone having individual double-carboxylic acid-, hydroxyl-, carbonyl- and *O*-glucosyl-groups at C-3, C-1, C-9 and C-8 positions in the molecule, respectively. Moreover, SB possesses an *erythro*-configuration between C-10 and C-10' positions. Therefore, MABs should distinguish all these functional groups, and also recognize the stereochemistry of this complicated compound. Since the newly established ELISA against SB is expected to be used for phytochemical investigations involving crude plant extracts, the assay specificity was checked by determining the cross-reactivities of the MABs with various related compounds. The cross-reactivities of MABs were examined by the competitive ELISA. Table 3 indicates the cross-reactivities of anti-SB MABs against related anthraquinone, anthrone and phenol carboxylic acid. MAB 7H12 has weak cross-reactivities with SA (2.45%) and rhein (0.012%). However, the other related anthraquinone and anthrone did not have appreciable cross-reactivities. From these results it is suggested that the epitope consists of a basal structure of rhein and sugar moiety. In addition the most important property of MAB 7H12 is its ability to distinguish between SB and SA, which differ only in the stereochemical configuration at the C-10 and C-10' positions. Therefore, it is suggested that *erythro*-configurational structure of bisanthrone is indispensable as an immunodominant molecule for the reactivity of MAB 7H12. So the ELISA using a MAB 7H12 possesses apparently high sensitivity and specificity for SB. Because we have also prepared an anti-SA MAB having a weak cross-reactivity with SB (0.28%) as already discussed, these two MABs make it possible to investigate stereochemical recognition precisely.

Compound	Cross-reactivities (%)		
	7H12	5G6	5C7
Anthraquinone and anthrone			
sennoside B	100	100	100
sennoside A	2.45	2.30	8.53
rhein	0.012	0.030	0.007
emodin	< 0.004	< 0.023	< 0.006
aloe-emodin	< 0.040	< 0.023	< 0.006
barbaloin	< 0.004	< 0.023	< 0.006
1,4-dihydroxy-anthraquinone	< 0.004	< 0.023	< 0.006
Stilbene			
rhaponticin	< 0.004	< 0.023	< 0.006
Phenol carboxylic acid			
gallic acid	< 0.004	< 0.023	< 0.006
vanillic acid	< 0.004	< 0.023	< 0.006
caffeic acid	< 0.004	< 0.023	< 0.006
homogentisic acid	< 0.004	< 0.023	< 0.006

Table 3. Cross-reactivities of anti-SB MAbs against various compounds.

3.3.4. Correlation of results of SB determination in crude extracts of rhubarb roots between HPLC and ELISA using MAb 7H12

The concentrations of SB in various rhubarb samples were determined by ELISA (Table 4). Shinshu Daio, bred by crossing *R. palmatum* and *R. coreanum* in order to increase the level of SB concentration in Japan, contained the highest SB level of 6.01 ± 0.18 $\mu\text{g}/\text{mg}$ dry wt. Ga-wo, estimated to be high grade in the traditional Japanese medicine, contained SB level of 3.14 ± 0.27 $\mu\text{g}/\text{mg}$ dry wt. These results are in good agreement with previous reports [18]. The correlation between results from ELISA and HPLC is also good.

Sample	Concentration ($\mu\text{g}/\text{mg}$ dry wt. powder)	
	ELISA	HPLC
Shinshu Daio	6.01 ± 0.18	6.15 ± 0.59
Ga-wo	3.14 ± 0.27	3.80 ± 0.16
Kinmon Daio	0.35 ± 0.01	0.38 ± 0.02
Itto-Ga-wo (powder)	1.44 ± 0.12	1.52 ± 0.18
Itto-Ga-wo (refuse)	1.42 ± 0.07	1.40 ± 0.11

Table 4. SB concentrations in various rhubarb samples. Data are the means of triplicate assays.

3.3.5. Determination of concentrations of SA and SB in various *Cassia* species

The concentrations of SA and SB in leaves of various *Cassia* species were determined by ELISA using anti-SA and SB MAbs (Table 5). The results indicate that *C. angustifolia* contains 4.56 ± 0.25 $\mu\text{g}/\text{mg}$ dry wt. powder of SA and 5.10 ± 0.15 $\mu\text{g}/\text{mg}$ dry wt. powder of SB indicating higher amounts of SA and SB compared to the other species. *C. alata* contains 1.19 ± 0.12 $\mu\text{g}/\text{mg}$ dry wt. powder of SA and 1.16 ± 0.15 $\mu\text{g}/\text{mg}$ dry wt. powder of SB. *C. fistula* (A)~(D) contain 0.10-2.04 $\mu\text{g}/\text{mg}$ dry wt. powder of SA and 0.13-2.05 $\mu\text{g}/\text{mg}$ dry wt. powder of SB, respectively.

Sample	Concentration ($\mu\text{g}/\text{mg}$ dry wt. powder)		
	Sennoside A	Sennoside B	Total sennosides
<i>Cassia angustifolia</i>	4.56 ± 0.25	5.10 ± 0.15	9.66 ± 0.40
<i>C. alata</i>	1.19 ± 0.12	1.16 ± 0.15	2.35 ± 0.27
<i>C. bakeriana</i>	0.40 ± 0.03	0.44 ± 0.02	0.84 ± 0.05
<i>C. fistula</i> (A)	1.14 ± 0.08	0.75 ± 0.08	1.89 ± 0.16
<i>C. fistula</i> (B)	2.04 ± 0.32	1.52 ± 0.12	3.56 ± 0.44
<i>C. fistula</i> (C)	1.90 ± 0.16	2.05 ± 0.24	3.95 ± 0.40
<i>C. fistula</i> (D)	0.10 ± 0.01	0.13 ± 0.00	0.23 ± 0.01
<i>C. mimosoides</i>	$(1.30 \pm 0.24) \times 10^{-2}$	$(1.88 \pm 0.29) \times 10^{-4}$	$(1.32 \pm 0.24) \times 10^{-2}$
<i>C. floribunda</i>	$(2.78 \pm 0.11) \times 10^{-3}$	$(1.04 \pm 0.03) \times 10^{-4}$	$(2.88 \pm 0.11) \times 10^{-3}$
<i>C. surattensis</i>	$(1.15 \pm 0.18) \times 10^{-2}$	$(2.44 \pm 0.17) \times 10^{-4}$	$(1.17 \pm 0.18) \times 10^{-2}$
<i>C. tora</i>	$(2.13 \pm 0.21) \times 10^{-3}$	$(3.64 \pm 0.21) \times 10^{-5}$	$(2.17 \pm 0.23) \times 10^{-3}$
<i>C. siamea</i>	$(4.45 \pm 0.14) \times 10^{-3}$	$(1.87 \pm 0.13) \times 10^{-3}$	$(6.32 \pm 0.27) \times 10^{-3}$

Table 5. Total sennoside concentrations in leaves of various *Cassia* species. Data are the means of triplicate assays.

4. Establishments of a new eastern blotting, double staining and immunohistochemical staining using anti-SA and SB MAbs

4.1. Preface

Thin-layer chromatography (TLC) is most widely used for detection, separation and monitoring of small molecular compounds like sennosides. If the direct TLC immunostaining with MAb can be done, this procedure must be contributive to the development of structural analysis of small molecular compounds. However, this procedure cannot be used for the direct detection of small molecular compounds on a TLC plate because the silica gel is sloughed off from the plate and the compounds on the plate are easily washed out without fixing during treatment. If the compounds are transferred from the TLC plate to a plastic

membrane with hydrophobic properties and immobilized on the membrane, these difficulties can be solved. Therefore, I examined the transfer of sennosides from a TLC plate to a plastic membrane. Towbin *et al.* first reported the transfer of glycosphingolipids using nitrocellulose membranes [20]. However, since its transfer efficiency was poor and reproducible results were not obtained, I tested various plastic membranes and transfer conditions resulting in a polyvinylidene difluoride (PVDF) membrane to be the best [21]. The membrane is very stable against heating and various organic solvents in addition to retaining sennosides with high efficiency. I named this new method as eastern blotting (EB), because theoretically same methodology compared to previous EB except the way of sennoside-BSA conjugation for fixing sennosides on the membrane [22]. I communicate here the EB procedure for sennosides and its application for analytical survey of sennosides [23].

4.2. Experimental

4.2.1. Chemicals and immunochemicals

Polyvinylidene difluoride (PVDF) membranes (Immobilon-N) were purchased from Millipore Corporation (Bedford, MA, USA). Glass microfiber filter sheets (GF/A) were purchased from Whatman International Ltd. (Maidstone, England). All other chemicals were standard commercial products of analytical grade.

4.2.2. EB and Double staining

Sennosides were applied to a TLC plate and developed with 1-propanol-ethyl acetate-water-acetic acid (40:40:30:1, by volume). The developed TLC plate was dried and then sprayed with a blotting solution mixture of isopropanol-methanol-water (1:4:16, by volume). It was placed on a stainless steel plate and then covered with a PVDF membrane sheet. After covering with a glass microfiber filter sheet, the whole assembly was pressed evenly for 70 s with a 120 °C hot plate as previously described with some modifications [24, 25]. The PVDF membrane was separated from the TLC plate and dried.

The blotted PVDF membrane was dipped in 20 mM carbonate buffer solution (pH 9.6) containing BSA (1%) and EDC (20 mg/mL), and stirred at room temperature for 14 hr. After washing the PVDF membrane twice with T-PBS for 5 min and then treated with S-PBS for 3 hr to reduce non-specific adsorption. The PVDF membrane was washed with T-PBS twice for 5 min, and then immersed in anti-SA MAb (6G8) and stirred at room temperature for 3 hr. After washing the PVDF membrane twice with T-PBS for 5 min, a 1:1000 dilution of POD-labeled goat anti-mouse IgG in PBS containing 0.2% of gelatin (G-PBS) was added and stirred at room temperature for 1 hr. The PVDF membrane was washed twice with T-PBS and water, then exposed to 1 mg/mL 4-chloro-1-naphthol-0.03% H₂O₂ in PBS solution which was freshly prepared before use for 10 min at room temperature. The protocol of the EB technique is shown in Figure 9.

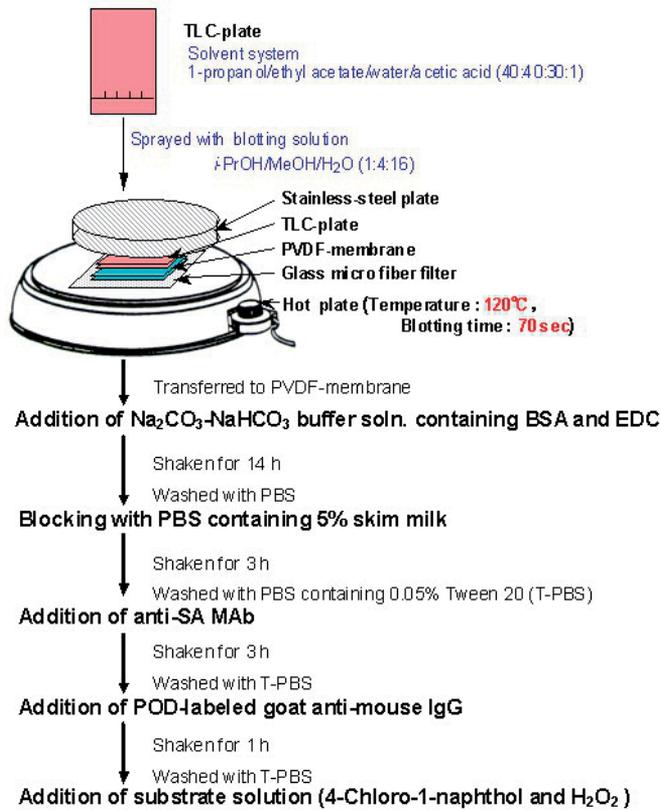


Figure 9. Eastern blotting protocol.

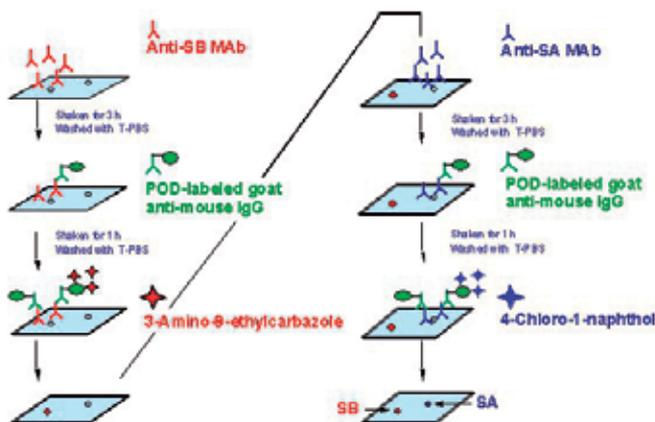


Figure 10. Double staining protocol.

For successive staining by anti-SB MAb (7H12), the PVDF membrane stained by anti-SA MAb was treated in the same way as anti-SA MAb (6G8) except that it was exposed to 2 mg/10 mL 3-amino-9-ethylcarbazole-0.03% H₂O₂ in acetate buffer (0.05 M, pH 5.0) containing 0.5 mL of *N,N*-dimethyl formamide. The protocol of double staining is shown in Figure 10.

4.2.3. EB for immunohistochemical staining of SA

A piece of PVDF membrane was placed on a glass microfiber filter sheet. A sliced fresh rhubarb root was placed on the PVDF membrane, and they were pressed together evenly for 1 hr. The blotted PVDF membrane was stained using the same procedure described for the EB method.

4.3. Results and discussion

4.3.1. EB of SA using anti-SA MAb

Previously we established a new immunostaining method named as eastern blotting for several glycosides like solasodine glycosides [21], ginsenosides [26, 27] and glycyrrhizin [22, 28] by using individual MAbs. In this methodology we separated the sugar moiety in a molecule into two functions, the epitope part and fixation ability part on a membrane after blotted to a PVDF membrane from a TLC plate, since small molecular compounds can not be fixed on the membrane. Although I followed the previous methodology for SA, unfortunately staining was not succeeded. Therefore, a new blotting method onto a PVDF membrane from the developed TLC plate is required. SA was transferred to the PVDF membrane by the same way as previously described, and treated with EDC solution followed by the addition of BSA as indicated in Figure 9. This reaction enhanced the fixation of SA via SA-BSA conjugate on the PVDF membrane and the pathway was indicated diagrammatically in Figure 11. When the blotted PVDF membrane was incubated in the absence of EDC, it was essentially free of immunostaining (data not shown).

Figure 12 shows the EB of sennosides and other structurally related compounds using anti-SA MAb (A) and the H₂SO₄ staining (B). The EB indicated only limited staining of SA as shown in Figure 12A, lane 7. Moreover, the EB method was considerably more sensitive than that of H₂SO₄ staining. Since anti-SA MAb cross-reacts against SB and rhein as 0.28 and 0.35%, respectively, they can be stained very weakly by anti-SA MAb, as described in the previous section. Previously Fukuda *et al.* succeeded the EB of ginsenoside Rb1 by using anti-ginsenoside Rb1 MAb resulting in staining together with ginsenoside Rc, Rd, Re and Rg1 [26, 27]. The difference between the newly established EB and the previous methodology is combine system of sugar moiety to PVDF membrane. The sugar moiety in ginsenosides was oxidatively cleaved to release aldehyde groups which were conjugated with a protein to fix on a PVDF membrane. Since it was evident that a part of sugar moiety in ginsenoside Rb1 was immunized, the cleavage of sugar moiety by NaIO₄ expanded its cross-reactivity against other ginsenosides resulting in possibility of staining for ginsenoside Rc, Rd, Re and Rg1, though their cross reactivities are weak. On the other hand, the newly established EB in here does not hinder around sugar moiety in SA. Therefore, strength of staining for SA, SB and rhein was proportional to their cross-reactivities as described in ELISA.

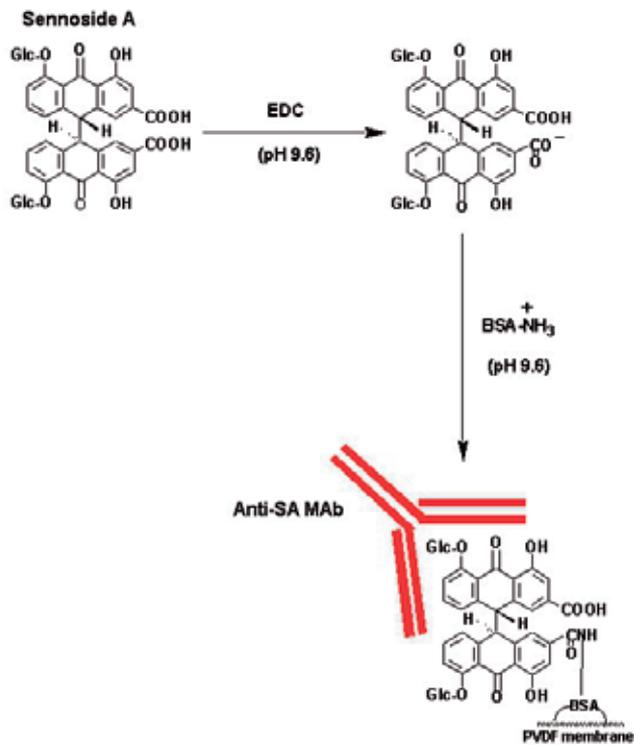


Figure 11. Schematic diagram illustrating the eastern blotting of SA onto the PVDF membrane and the detection using anti-SA MAb.

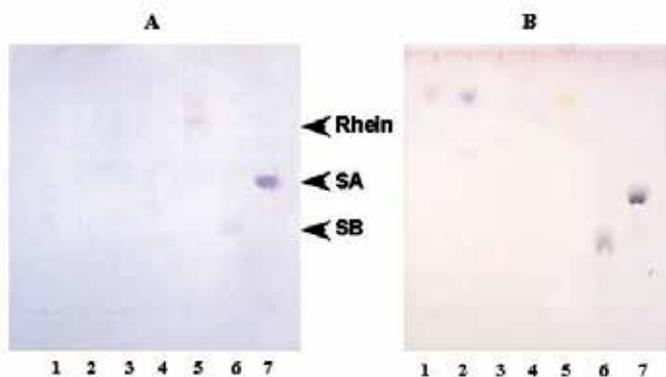


Figure 12. Eastern blotting of sennosides and related compounds stained by anti-SA MAb (A). B shows a TLC plate stained by 10% H₂SO₄. Lanes 1, 2, 3, 4, 5, 6 and 7 indicate rhaponticin, barbaloin, aloe-emodin, emodin, rhein, SB and SA (3 μg), respectively.

4.3.2. Double staining of sennosides using anti-SA and SB MAbs

Previously, I used 4-chloro-1-naphthol for staining of SB. However, since it could not function well for SB, the combination of 4-chloro-1-naphthol and 3-amino-9-ethylcarbazole was selected to improve double staining of sennosides as indicated in Figure 10. SA and SB were stained clearly by the purple and red color, respectively (Figure 13). From this result both antibodies can distinguish stereochemical configurations, *threo* and *erythro* between C-10 and C-10' positions in a molecule on PVDF membrane stained as double coloring, respectively.

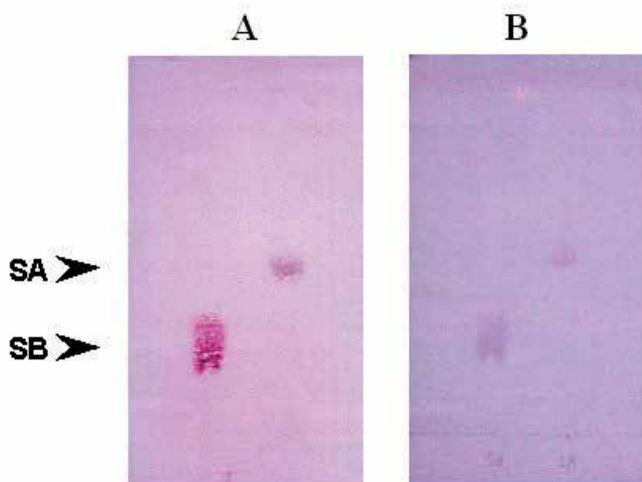


Figure 13. Double staining of sennosides using eastern blotting technique (A). B shows a result of H_2SO_4 staining. Red and purple colors were stained by anti-SB and SA MAb, respectively.

4.3.3. Detection of SA and SB in various *Cassia* species using double staining with a new EB technique

The crude extracts of various *Cassia* species were analyzed by the newly developed double staining system and TLC stained with H_2SO_4 as shown in Figure 14. Although H_2SO_4 staining (Figure 14B) detected many spots including probably sugars and different types of anthraquinone glycosides in various *Cassia* species, double staining (Figure 14A) detected clearly SA and SB, and very weakly other sennosides except appearance of chlorophylls around top. Band 1 indicated a purple color that means a *threo*-configuration between C-10 and C-10' positions detected by EB using anti-SA MAb as shown in Figure 14A. Moreover, its R_f value indicated that band 1 has one sugar moiety and a CH_2OH group instead of $COOH$ group in a molecule. I surveyed the previous papers regarding sennosides in senna [1]. Judging from these evidences, I suggested that band 1 is sennoside C (SC) having *threo*-configuration as indicated previously [1]. Band 2 was easily suggested to be *erythro*-configu-

ration from its red color. The R_f value clearly showed that band 2 includes one sugar moiety having a HOOC-CO group. From these results I supposed that band 2 is sennoside F (SF) that has *erythro*-configuration as indicated previously [1]. The double staining by EB indicates that *C. angustifolia*, *C. alata*, *C. bakeriana* and *C. fistula* contain a higher concentration of sennosides compared to the other species. This result has a good agreement with that of ELISA. The limit of detection by the double staining method was confirmed to be 48 $\mu\text{g/mL}$ of both SA and SB.

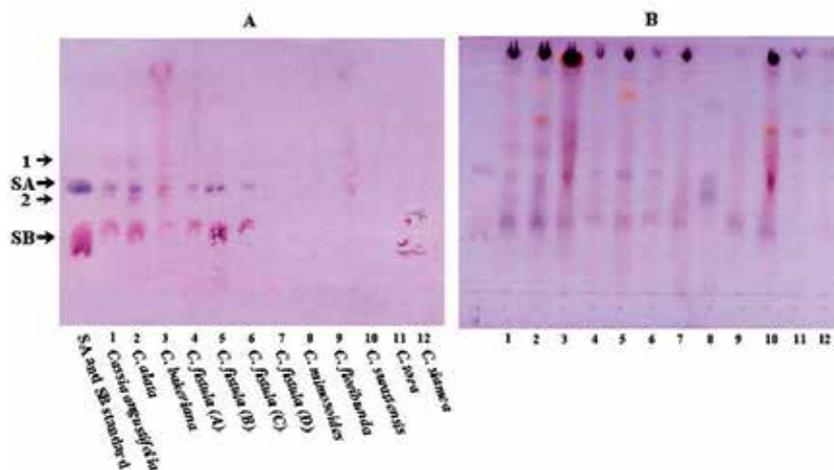


Figure 14. Double staining of SA and SB in various *Cassia* species (A). B shows a result of H_2SO_4 staining. Lefthand lane indicates SA (4 μg) and SB (3 μg). Lanes 1–12 indicate various *Cassia* species (3 μL).

4.3.4. Validation of EB for immunohistochemical staining of SA

As an other application of the EB method, the immunohistochemical staining of SA in rhubarb root, was investigated. A sliced fresh rhubarb root was placed on the PVDF membrane, and they were pressed together evenly for 1 hr. The blotted PVDF membrane was stained using the same procedure described for the EB method. Figure 15II illustrates the immunohistochemical staining of SA in fresh Hokkai Daio root. The phloem and cambium contained a higher concentration of SA compared to other tissues, pith and bud. To confirm this result, I analyzed these tissues individually by ELISA and HPLC. The concentrations of SA were determined by ELISA to determine 64.4 ± 4.5 , 48.1 ± 8.2 , 15.0 ± 1.6 and 1.8 ± 0.3 ng/mg fresh wt. in phloem, cambium, pith and bud, respectively. This result was a good agreement with those of HPLC resulting in 58.4 ± 2.6 , 49.0 ± 3.9 and 13.3 ± 0.5 ng/mg fresh wt. in phloem, cambium and pith, respectively.

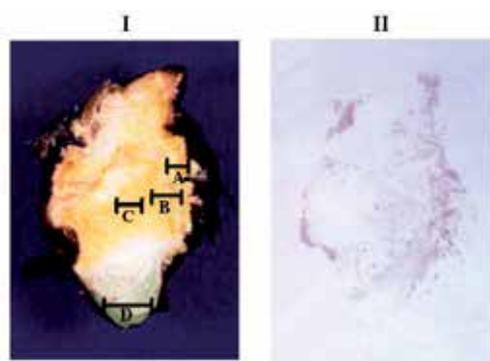


Figure 15. Immunohistochemical staining of SA using anti-SA MAb in rhubarb root. I, cross section of Hokkai Daio root; II, direct eastern blotting on PVDF membrane of a cross section of Hokkai Daio root. A, Phloem; B, Cambium; C, Pith; D, Bud, respectively.

5. Conclusion

The recent developments of molecular biosciences and their biotechnological applications have opened up many new avenues of pharmaceutical areas. MAbs have many potential uses in addition to immunological methods to plant sciences. Therefore, immunoassay system using MAbs against pharmacologically active natural products having low molecular weight have become an important tool for the studies on receptor binding analysis, enzyme assay, and quantitative and/or qualitative analytical techniques in plants owing to their specific affinity.

In order to analyze the stereochemical isomers, SA and SB in plants, medicaments, prescriptions, health foods and patients' sera, I have produced MAbs against them. These MAbs have the most important ability to distinguish between SA and SB, which differ only in the stereochemical configuration at the C-10 and C-10' positions, respectively. Moreover, they have no detectable cross-reaction with the other related anthraquinone and anthrone.

Analytical systems of SA and SB by competitive ELISA using anti-SA and SB MAbs were established. These ELISA systems are capable of measuring SA and SB in complex matrices without any pretreatments. Furthermore, these ELISA methods are approximately 2,000 times for SA and 10,000 times for SB more sensitive than that of HPLC method.

The newly developed EB methodology can be theoretically expanded for all compounds having carboxylic acid such as phenol carboxylic acids, glucuronides, furthermore compounds having only a carboxylic group in a molecule. A new double staining with EB method for sennosides using anti-SA and SB MAbs was established. SA and SB were stained purple and red color, respectively. This system visualized sennosides on a PVDF membrane. In fact, SA and SB in the crude extracts of various *Cassia* species were distinguished by their coloring and R_f values. Moreover, it could make it possible to survey the natural resour-

ces of sennosides and quickly determine their structures. Furthermore, EB also can be used for the survey of distribution of SA and/or SB in the *Rheum* specimen by immunohistochemical staining.

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Applications of Anti-natural Compound Immunoaffinity Purification on Quality Control

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Additional information is available at the end of the chapter

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1. Introduction

Worldwide demand of herbal medicines has increased in recent years owing to rising interest in the health benefits. Among with this, the quality control of plant extracts and plant-derived medicines is growing in importance to ensure their efficacy and safety. Effective quality control of the traditional Chinese medicines (TCM) and plant crude extracts requires the rapid and sensitive methods for separation and quantification of bioactive compounds. Various methods have been employed for the separation and quantification of certain constituents in medicinal plants or herbal medicines. However, the current methods in use are not necessarily optimal approaches. For example, separation and quantification of glycyrrhizin (GC), the main active constituent in licorice (*Glycyrrhiza* spp.), have been used gas chromatography, high performance liquid chromatography (HPLC) and micellar trokinetic chromatography and so on [1,2]. Commercial purification of GC typically progressed through several steps, including crystallization, column chromatography, and liquid partitioning. These current methods are not sufficiently approaches because of insufficient sensitivity and reproducibility, large consumption of organic solvent for extraction and analysis, and long analysis time.

Immunoassay systems using monoclonal antibody (MAb) against drugs and small molecular weight bioactive compounds have become an important tool for studies on receptor binding assays, enzyme assays, and quantitative and qualitative analytical techniques both *in vivo* and *in vitro* studies. Although immunoaffinity purification against higher molecule analyte such as peptides and proteins are widely used in the research and commercial ways, there are too few cases of immunoaffinity purification targeting a small molecule compound such as natural compounds. Our laboratory has prepared many kinds of MAbs against

naturally occurring bioactive compounds such as terpenoids [3-5], alkaloids [6,7], saponins [8-12], and phenolics [13-16], and developed several applications. One of the applications by using MAbs is immunoaffinity column conjugated with anti-natural compound-specific MAbs and work by specifically binding and removing the target compounds. We have been establishing several affinity columns against a kind of terpenoid, forskolin [17], solasodine glycosides [18], ginsenosides Rb₁ [19], and GC [20]. Application of an immunoaffinity column to isolate and concentrate a natural compound may decrease the amount of solvent consumption and the number of purification steps, shorten analysis time, and simplify sample analysis compared to traditional cleanup techniques.

In this chapter, we focus on the immunoaffinity purification to separate and concentrate the target bioactive compounds from the crude extract. Our approaches effectively succeeded one-step purification of target compounds by MAb-conjugated immunoaffinity column, which leads to high-sensitivity detection and isolation of target compounds. In addition, the immunoaffinity column can prepare the knockout (KO) extract which contains all components except an antigen molecule, and KO extract will be useful for the pharmacological investigation to reveal the real effects of bioactive compound in the crude extract. The information in this chapter may provide new insight into quality control of plant-derived medicines.

2. Preparation of anti-ginsenoside Rb₁ immunoaffinity column and its application

Ginseng, the root of *Panax ginseng*, has been an important component in traditional medicines for more than 1000 years in Eastern Asia. It is now one of the most extensively used alternative medicines all over the world and appears in the pharmacopoeias of several countries. The biological and pharmacological activities of ginseng have been reported to have anti-aging, anti-cancer, anti-inflammation, anti-diabetics, anti-stress, maintenance of homeostasis, and to affect on central nervous system and immune function [21]. The bioactive components responsible for ginseng actions are ginsenosides, which are triterpenes saponins that possess a dammarane skeleton with sugar moieties [22]. Up to now more than 60 kinds of ginsenosides have been isolated from *Panax* genus [23]. It is well-known that the concentrations of ginsenosides vary in the ginseng root or the root extracts depending on the method of extraction, subsequent treatment, or even the season of its collection [24,25]. Due to the importance of ginseng, a number of researches has been carried out to develop the methods for the identification, quantification and quality control of ginsenosides in raw plants materials, extracts and commercial products. Currently, analytical and preparative HPLC are commonly used to quantify and purify the individual ginsenosides from ginseng [26]. However, isolation of ginsenosides by HPLC requires the repeated purification steps, including cumbersome handling and lengthy analysis times, and may result in the decrease of the final yield. Thus, the developed approaches are required for quality control of ginseng in the field of TCM.

Ginsenoside Rb₁ (G-Rb₁) is one of the main ginsenosides responsible for many pharmaceutical actions of ginseng [27]. G-Rb₁ has various biological activities, including facilitating acquisition and retrieval of memory [28], scavenging free radicals [29], inhibition of calcium over-influx into neurons [30], and preserving the structural integrity of the neurons [31].

In order to develop efficient quality control of ginseng, we have prepared anti-G-Rb₁ MAb, set up of enzyme-linked immunosorbent assay (ELISA), and a new immunostaining method named Eastern blotting [8,32]. Furthermore, we established an immunoaffinity column against G-Rb₁ and its application for one-step isolation from crude extract of ginseng root [19, 32]. Herein we describe the preparation of anti-G-Rb₁ immunoaffinity column and its applications for identification and concentration of G-Rb₁.

2.1. Preparation of MAb and immunoaffinity column against G-Rb₁

2.1.1. Analytical methodology for determination of hapten number in antigen, hapten-carrier protein conjugate

The first step for the MAb production is the synthesis of a hapten-carrier protein conjugate. Bovine serum albumin (BSA) conjugated with G-Rb₁ was produced for the preparation of specific MAb in mouse [8]. There had been no direct and appropriate methods for the determination of haptens conjugated carrier proteins without differential UV analysis, radiochemical or chemical methods. Therefore, immunization by the injection of hapten-carrier protein conjugate was unreliable. Wengatez *et al.* determined the hapten density of immuno-conjugates by matrix-assisted UV laser desorption/ionization (MALDI) mass spectrometry [33]. We also reported the direct analytical method of hapten and carrier protein conjugates by a MALDI tof mass spectrometry using internal standard [3-16]. Figure 1 shows the MALDI tof mass spectra of G-Rb₁-BSA conjugate. A broad peak coinciding with the conjugate of G-Rb₁ and BSA appeared from *m/z* 70,000 to 90,000 centering at around *m/z* 79,469. Using experimental results and a molecular weight of 66,433 for BSA, the calculated values of G-Rb₁ component (MW1,109) are from 3,327 to 23,289 resulting in the range of 3 to 21 (12 in average) molecules of G-Rb₁ conjugated with BSA [8]. This method is suitable for characterization of conjugates between small molecule natural compound and carrier protein conjugates.

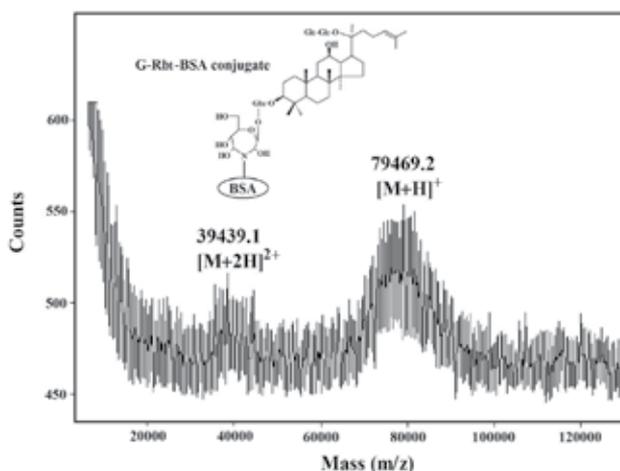


Figure 1. Direct detection of G-Rb₁-BSA conjugate by MALDI tof mass spectrometry. [M+H]⁺, [M+2H]²⁺ are single and double protonated molecules of G-Rb₁-BSA, respectively.

2.1.2. Preparation of anti-G-Rb₁ MAb and ELISA as an assay system

A hybridoma-producing MAb reactive to G-Rb₁ was obtained by general procedure and classified into IgG2b which had κ light chains [8]. The reactivity of IgG type MAb, 9G7 was tested by varying antibody concentration and by performing a dilution curve. The antibody concentration was selected for competitive ELISA. The free MAb following competition is bound to polystyrene microtiter plates precoated with G-Rb₁-human serum albumin (HSA). Under these conditions, the full measurement range of the assay extends from 20 to 400 ng/mL. The cross-reactivity against G-Rc and G-Rd, which possess a diglucose moiety attached to the C-3 hydroxy group, were weak compared with G-Rb₁ (0.024 and 0.020 %, respectively). G-Re and G-Rg₁ showed no cross-reactivity (less than 0.005 %). It is evident that the MAb reacted only with a small number of structurally related G-Rb₁ molecules, and very weakly and did not react with other steroidal compounds.

2.1.3. Preparation of anti-G-Rb₁ immunoaffinity column and appropriate buffer systems for separation of G-Rb₁

The purified IgG (10 mg) was treated by NaIO₄ to give dialdehyde group in sugar moiety which was coupled to Affi-Gel Hz hydrazide gel resulting in a hydrozone-type immunoaffinity gel [32]. The immunoaffinity gel was packed into plastic mini-column (Figure 2). Due to examine the optimal conditions of adsorption and elution, 400 μ g of G-Rb₁ was dissolved in phosphate buffered saline (PBS) and loaded on anti-G-Rb₁ affinity column. After washing with washing buffer (20 mM PB containing 0.5 M NaCl), various buffer solutions for elution were loaded on the column, and then the recovery efficiency was determined by ELISA. The G-Rb₁ concentration was somewhat increased by eluting with a 20 mM phosphate buffer containing 0.5 M KSCN and 10 % MeOH. When the 20 mM phosphate buffer was changed to 100 mM AcOH buffer (pH 4), the elution ability reached the optimal level. Although 20 % MeOH could enhance the elution of G-Rb₁, higher MeOH concentration of over 20 % was ineffective. Thus, 100 mM AcOH buffer containing 0.5 M KSCN and 20 % MeOH could be used as an elution buffer in the immunoaffinity chromatography.

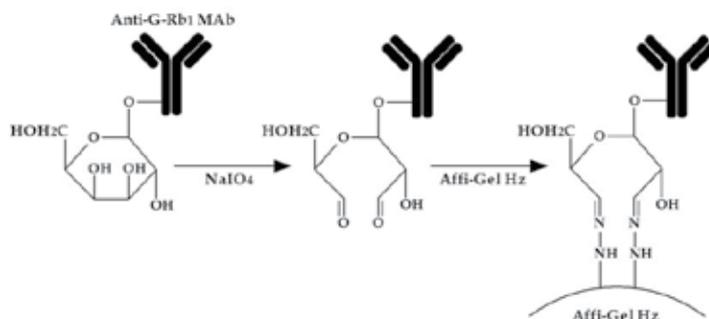


Figure 2. Preparation of anti-G-Rb₁ immunoaffinity column

2.2. Purification of G-Rb₁ by immunoaffinity column

2.2.1. One-step purification of G-Rb₁ from crude extract of *P. ginseng* roots by anti-G-Rb₁ immunoaffinity column

A crude extract (3.8 mg) of *P. ginseng* roots was loaded onto anti-G-Rb₁ immunoaffinity column. The column was washed with the washing buffer (fractions 1-20), and then eluted with elution buffer (fractions 21-40). As shown in Figure 3, the fractions 1-8 contained the overloaded G-Rb₁, which determined by ELISA. The other ginsenosides such as G-Rg₁, Rc, Re and Rd were also detected in these fractions by Eastern blotting procedure. After washing, a sharp peak was observed around fractions 21-24 of elution buffer, which contained G-Rb₁. However, these eluted fractions were still contaminated by a small amount of malonyl-G-Rb₁ as detected by Eastern blotting. The malonyl-G-Rb₁ has almost the same cross-reactivity with G-Rb₁ [32]. Therefore, the eluted fractions were treated with a mild alkaline solution (0.1 % KOH in MeOH) at room temperature to give pure G-Rb₁ [19]. Overcharged G-Rb₁ in washing solution (fractions 1-8) was repeatedly loaded and finally isolated in pure form. The anti-G-Rb₁ MAb was stable during all procedures, and the immunoaffinity column showed almost no decrease in capacity (20 µg of G-Rb₁/ml gel) after repeated use more than 10 times under same conditions, as reported for a one-step purification of forskolin from a crude extract of *Coleus forskohlii* root [17].

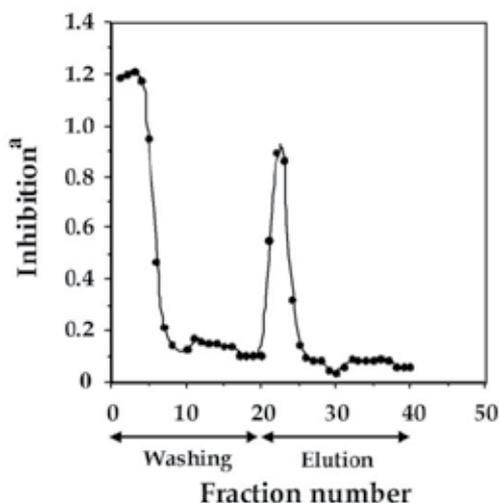


Figure 3. Elution profile of *P. ginseng* crude extract separated by anti-G-Rb₁ immunoaffinity column. The concentration of G-Rb₁ in each fraction was monitoring by ELISA using anti-G-Rb₁ MAb. Individual fraction (2 mL) were assayed by ELISA. ^aInhibition = (A₀-A)/A₀; A₀ is the absorbance in the absence of the test compounds. A is the absence in the presence the test compounds.

This methodology is effective for the rapid and simple purification of G-Rb₁ and may open up a wide field of comparable studies with other families of saponins for which an acceptable method for one-step separation has not yet been developed. Furthermore, to separate the

total ginseng saponins, a wide cross-reactive MAb against ginsenoside, like anti G-Re MAb which showed wide cross-reactivity, could be designed [34]. A combination of immunoaffinity column, Eastern blotting and ELISA could be used to survey low concentrations of ginsenoside Rb₁ of plant origin and/or in experimental animals and human. In fact we have succeeded in the isolation of G-Rb₁ from a different plant, *Kalopanax pictus* Nakai, which was not known previously to contain ginsenosides, using this combination of methods [35].

2.2.2. Isolation and determination of unknown compounds related to G-Rb₁ by anti-G-Rb₁ immunoaffinity column

Several species of ginseng are known to exist and contain different amount and kinds of ginsenosides. *P. japonicus* is distributed in Japan and China and it is morphologically different from the other *Panax* species. Yahara *et al.* indicated that G-Rb₁ was not detected in *P. japonicus*, and isolated oleanane-type saponins called chikusetsusaponins and determined their structures [36]. Morita *et al.* reported the varieties of saponins in *P. japonicus* by chemical analysis. These results suggested that the concentration of G-Rb₁ might be trace level in *P. japonicas* [37]. We previously analyzed the G-Rb₁ concentration in several ginseng roots by ELISA using anti-G-Rb₁ MAb and HPLC after pre-treatment under mildly alkaline condition [32]. As shown in Table 1, G-Rb₁ concentrations of *P. ginseng*, *P. notoginseng* and *P. quinquefolius* were correlated between ELISA and HPLC. However, the G-Rb₁ of *P. japonicus* was higher concentrations compared with HPLC and previous reports [37]. This data suggest that anti-G-Rb₁ MAb using ELISA has the cross-reactivity with some unknown compounds contained in *P. japonicus*.

Sample		G-Rb ₁ concentration ($\mu\text{g}/\text{mg}$ dry weight powder)	
		ELISA	HPLC
<i>P. ginseng</i>	White ginseng	5.49 \pm 0.75	4.96 \pm 0.05
	Red ginseng	3.57 \pm 0.62	3.93 \pm 0.34
	Fibrous ginseng	64.44 \pm 3.64	69.75 \pm 1.45
<i>P. notoginseng</i>		47.08 \pm 3.34	42.39 \pm 1.39
<i>P. quinquefolium</i>		48.51 \pm 1.79	47.96 \pm 1.04
<i>P. japonicus</i>		1.37 \pm 0.34	0.63 \pm 0.06

Table 1. G-Rb₁ concentration in various ginseng samples

To clarify the unknown compounds bound to anti-G-Rb₁ MAb, the crude extract of *P. japonicus* was concentrated by immunoaffinity column using anti-G-Rb₁ MAb. The crude root extract was loaded on the column and washed with the washing Buffer, followed by the elution buffer as already indicated. Figure 4 shows the H₂SO₄ staining (A) and the Eastern blotting (B) profiles of the washing fractions 1-4 and eluted fraction 5. Fraction 1 is first elut-

ed fraction by the washing buffer, and showed many spots, indicating chikusetsusaponins, similar to the original extract of *P. japonicus*. After washing, the column was eluted by elution buffer (fraction 5), and then one spot was detected. As shown in Figure 4B, Eastern blotting indicated two different spots in washing fraction (Compound 1) and eluted fraction (Compound 2). These compounds bound with anti-G-Rb₁ MAb have a dammarane saponin having protopanaxadiol as a framework.

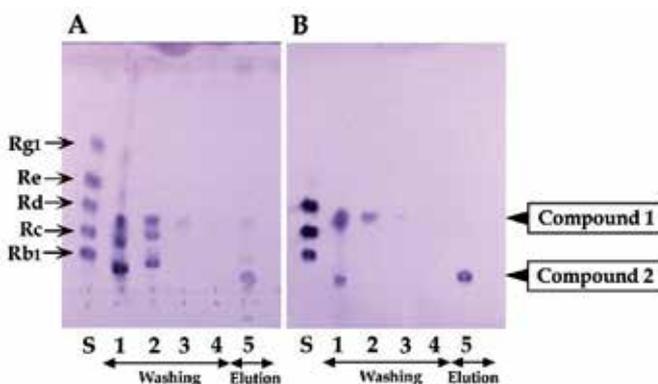


Figure 4. Purification and determination of ginsenosides of *P. japonicus* by the anti-G-Rb₁ immunoaffinity column. TLC (A) and Eastern blotting (B) profiles of the separated fractions from *P. japonicus* crude extract by the anti-G-Rb₁ immunoaffinity column. Lane S indicates the standard of ginsenosides (G-Rd, G-Rc, G-Rb₁, G-Rg₁, and G-Re). Lane 1-4 and Lane 5 were the washing fractions and the eluted fraction, respectively.

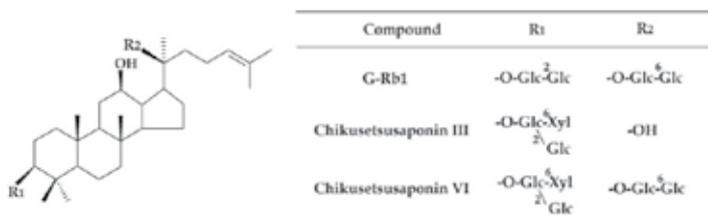


Figure 5. Chemical structures of G-Rb₁ and chikusetsusaponins purified from *P. japonicus* by the anti-G-Rb₁ immunoaffinity column.

Compound 1 has three sugar moieties in a molecule because that the R_F value closes to that of G-Rd, indicating that this compound is chikusetsusaponin III (Figure 5). Finally, we identified that this compound as chikusetsusaponin III in a direct comparison with authentic sample [32]. Another unknown spot, compound 2 appeared in fraction 5 of the eluted fraction. Thus, compound 2 has a similar molecular structure and high cross-reactivity with G-Rb₁, and seems to be related ginseng saponin having protopanaxadiol as an aglycone. Moreover, compound 2 might have the same sugar fragments and possess five sugar moiety compared with G-Rb₁, as indicated by their R_F value. From these evidences compound 2

might be chikusetsusaponin III-20-*O*-gentiobiose, chikusetsusaponin VI (Figure 5), which has 5 sugars in a molecule in good agreement with the R_F value previously reported [38] and we confirmed that compound 2 is chikusetsusaponin VI by the direct comparison with authentic sample.

These data suggested that the anti-G-Rb₁ immunoaffinity column could isolate some unknown structurally resemble compounds having cross-reactivity against anti-G-Rb₁MAB. Therefore, this purification system will be applied to survey new compounds related to target compound of MAB. In our previous studies, we demonstrated the immunoaffinity purification against all solasodine glycosides from crude extract by one-step purification. In this case, all solasodine glycoside have almost same cross-reactivity against anti-solamargine MAB [12].

2.2.3. Preparation of G-Rb₁ knockout extract by anti-G-Rb₁ immunoaffinity column

The capacity of this anti-G-Rb₁ immunoaffinity column is 20 µg of G-Rb₁/ml gel [32]. By loading the samples not to exceed the binding capacity against G-Rb₁, this immunoaffinity column becomes possible to remove all G-Rb₁ from crude ginseng extract. Figure 6 showed H₂SO₄ staining of TLC of the purification steps by the immunoaffinity column. Lane 1 and 2 were spotted the standard of ginsenosides (G-Rd, G-Rc, G-Rb₁, G-Rg₁, and G-Re). Lane A, B, and C were the crude extract, the washing fraction, and the eluted fraction, respectively. In the crude extract (lane A), all spots of ginsenosides were clearly detected. On the other hand, the washing fraction (lane B) contained all of the ginsenosides in the crude extract except G-Rb₁. Furthermore, the spot of G-Rb₁ was detected in the eluted fractions (lane C). These data strongly indicated that G-Rb₁ molecule in the ginseng extract can be eliminated by an anti-G-Rb₁ immunoaffinity column and the washing fractions was knockout only by the antigen molecule, G-Rb₁. Thus, we named the washing fractions a knockout (KO) extract [39,40]. This KO extract may be useful for the determination of real pharmacologically active principle in the TCMs.

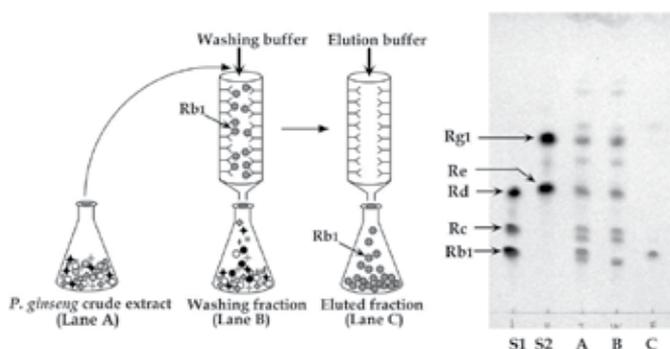


Figure 6. Preparation of G-Rb₁-KO extract from *P. ginseng* crude extract using anti-G-Rb₁ immunoaffinity column. Lane S1 and S2 indicate the standard of ginsenosides (G-Rd, G-Rc, G-Rb₁, G-Rg₁ and G-Re). Lane A, B, and C were the crude extract, the washing fraction, and the eluted fraction, respectively.

3. Glycyrrhizin-knockout extract and its application for *in vitro* assay

Licorice (*Glycyrrhiza* spp.) is also important crude drug used in over 70 % of the TCMs and Japanese Kampo medicines. It is prescribed with other herbal medicines as a demulcent in the treatment of sore throats, an expectorant for coughs and bronchial catarrh, an antitussive, a taste-modifying agent for relieving pain, an anti-inflammatory agent for anti-allergic reactions, rheumatism and arthritis, a prophylactic for liver disease and tuberculosis and adrenocorticoid insufficiency [41-43]. Accumulated evidence indicated that GC, a main saponin component of licorice, is one of the biologically active compounds. It has been reported that GC exhibits numerous pharmacological effects such as anti-inflammation, anti-ulcer, anti-tumor, anti-allergy, and hepatoprotective activities [44,45]. Clinically, GC has been used to treat patients with chronic hepatitis [46,47]. Although GC is supposed to be a major active principle in licorice crude extract, a number of studies by HPLC profiles suggested that licorice has many other bioactive components, including flavonoids, isoflavonoids and chalcones [43,48]. Biological studies showed that various flavonoid glycosides and their aglycones of licorice exhibit anti-inflammatory, anti-oxidative, anti-microbial, superoxide scavenging, and anti-carcinogenic activities [43,48]. In order to confirm the role of GC in TCM, we previously purified GC from TCM using an immunoaffinity column conjugated with anti-GC MAb [20]. In this section, we describe the preparation of GC-KO extract and its application for functional analysis of GC in licorice crude extract.

3.1. Preparation of GC-KO extract by anti-GC immunoaffinity column and the characterization of GC-KO extract

Our previous study demonstrated the preparation of anti-GC MAb [11]. The cross-reactivities of the anti-GC MAb against glycyrrhetic acid-3-*O*-glucuronide and glycyrrhetic acid were 0.585 % and 1.865 %, respectively. The other related compounds (deoxycholic acid, ursolic acid, and oleanolic acid) were all less than 0.005 %. Moreover, we established competitive ELISA and Eastern blotting method using anti-GC MAb [11,49].

The immunoaffinity column against GC was prepared by coupling the purified 60 mg of the anti-GC MAb to 25 ml of an Affi-Gel Hz gel [11]. To eliminate GC from licorice extract, 12 mg of licorice crude extract (GC content: 1275.0 μ g) in loading buffer (5 % MeOH) was applied on the anti-GC MAb immunoaffinity column, and then the loading buffer was continuously circulated through the column to enhance the binding efficiency. After overnight circulation at 4 °C, the unbound fraction was separated. The column was washed with washing buffer (5 % MeOH) and then eluted with elution buffer (20 mM phosphate buffer containing 30 % MeOH). After separation, each fraction was deionized and the solvent was lyophilized. Figure 7 showed the recovery ratio of GC checked by ELISA. In the unbound fraction, 3.50 μ g of GC (0.27% of the applied GC) was detected. On the other hand, 1269.26 μ g of GC (99.55% of the applied GC) was obtained in the bound fraction. These data indicate that the anti-GC column could eliminate 99.55 % of the loading GC. Thus, we named this unbound fraction "GC-knockout (GC-KO) extract" [50].

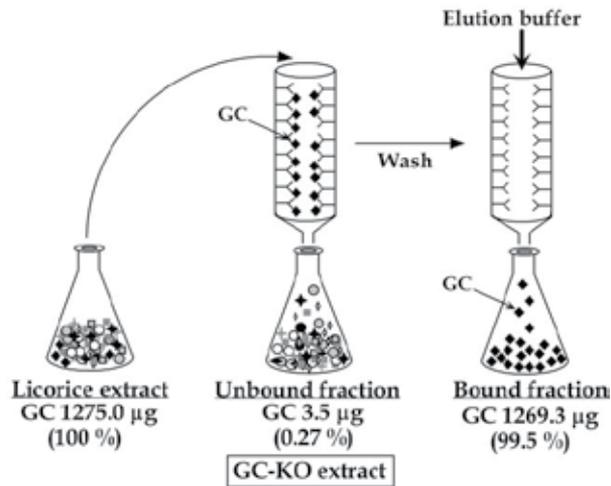


Figure 7. Preparation of GC-KO extract from licorice extract by anti-GC immunoaffinity column.

To further characterize GC-KO extract, the TLC analysis and Eastern blotting were performed [50]. As shown in Figure 8A, several spots including GC were detected in licorice extract (Lane B). However, the spot of GC was completely disappeared in GC-KO extract, although all other spots were clearly detected (lane C). Eastern blotting by anti-GC MAb indicated that GC was detected in licorice extract (Figure 8B, lane B), but the spot of GC was disappeared in GC-KO extract (Figure 8B, lane C). Therefore, these data suggest that GC was specifically eliminated from licorice extract by anti-GC MAb immunoaffinity column.

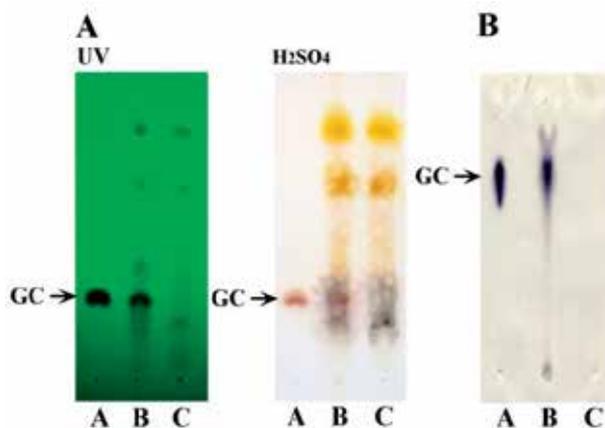


Figure 8. TLC profiles (A) and Eastern blotting by anti-GC MAb (B). Lane A, B, and C indicate GC, licorice extract, and GC-KO extract, respectively.

3.2. *in vitro* Assay by GC-KO extract prepared by anti-GC immunoaffinity column

Nitric oxide (NO), synthesized by NO synthase (NOS) from L-arginine, is an important regulatory/modulatory mediator for several physiological processes [51]. However, during inflammatory process, a large amount of NO is produced by inducible NOS (iNOS) stimulated by bacterial lipopolysaccharide (LPS) and inflammatory cytokines participates in the pathogenesis of inflammatory diseases [52]. Overproduced NO synthesized by iNOS triggers a variety of inflammatory diseases including sepsis, psoriasis, arthritis, multiple sclerosis, and systemic lupus *erythematosus* [53]. Therefore, inhibiting NO production by blocking iNOS expression may be useful strategy to treat a variety of inflammatory diseases.

In LPS-treated mouse RAW264 macrophages, licorice extract inhibited NO production and iNOS expression. At 100 µg/mL of licorice extract, iNOS protein and mRNA were completely suppressed [50].

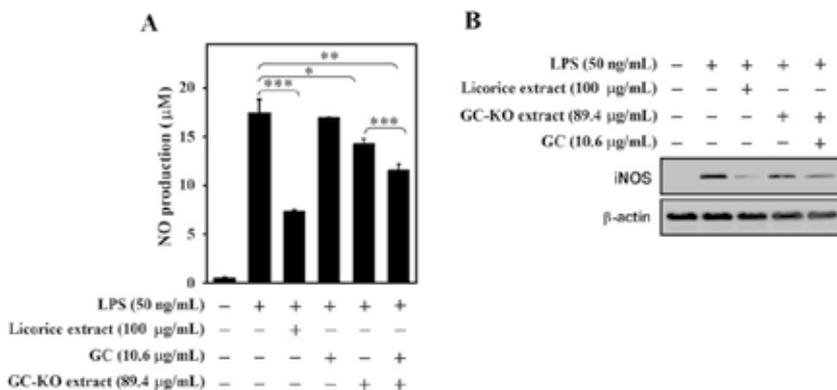


Figure 9. Effect of GC-KO extract and the combination of GC-KO extract and GC on NO production (A) and iNOS protein expression (B) in LPS-treated RAW264 cells. Each bar indicates the mean S.D. of four individual experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ indicate significant differences from the LPS alone

We next examined the inhibitory effect of GC alone, GC-KO extract and the combined treatment with GC and GC-KO extract on NO production [50]. Since 100 µg of licorice extract contains 10.6 ± 0.618 µg of GC, the cells were pre-treated with licorice extract (100 µg/ml), GC-KO extract (89.4 µg/ml), or the combination of GC-KO extract (89.4 µg/ml) and GC (10.6 µg/ml). Figure 9A indicated that the treatment of licorice extract led to a marked suppression of NO production as compared to LPS treatment [inhibition ratio (IR) 57.7%]. Interestingly, the inhibitory effect of GC-KO extract was lower (IR 17.8%) compared with licorice extract although GC alone could not block NO production as indicated above. On the other hand, the combined treatment with GC-KO extract and GC significantly improved the inhibitory ability (IR 33.5%). To determine whether the combinational effect of GC-KO extract and GC was related to iNOS expression, we performed Western blotting. As shown in Figure 9B, the treatment of GC-KO extract diminished the inhibitory ability of LE on iNOS expression, and addition of GC to GC-KO extract could improve it. These data suggest that GC alone cannot

suppress iNOS expression, but combinational inhibition of iNOS expression may occur when GC coexists with the other constituents contained in licorice extract. The *in vitro* and *in vivo* analysis by using KO extract prepared by immunoaffinity column is a useful approach for determination of potential function of natural compound on *in vitro* and *in vivo* assays.

4. Conclusion

In this chapter, we introduce the unique strategy of one-step purification of target compounds from crude extract by anti-natural compound specific MAb-conjugated immunoaffinity column. The immunoaffinity column conjugated with anti-G-Rb₁ MAb could purify the G-Rb₁ from *P. ginseng* extract, and the washing fraction contained all compounds except only G-Rb₁, which was named G-Rb₁-KO extract. By the use of the cross-reactivity of MAb, the anti-G-Rb₁ immunoaffinity column can identify new unknown compounds related to target compound of MAb and determine their structures. Furthermore, our data suggest that the combination of the immunoaffinity column and ELISA by using MAb provided a reliable and high sensitivity analysis for target compound in various TCMs and crude extract. We also demonstrated the *in vitro* assay by using GC-KO extract prepared by anti-GC immunoaffinity column from licorice extract. The KO extract may be able to support the pharmacological investigation for finding out a really active compound in a TCM and crude drug.

Acknowledgements

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Standard Operating Procedures (SOP) for the Spectrophotometric Determination of Phenolic Compounds Contained in Plant Samples

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Additional information is available at the end of the chapter

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1. Introduction

The quality control of raw materials and products from plants is one of the topics most discussed by universities and health surveillance agencies. One of the primary tools used to ensure the reliability of production processes is the use of Standard Operating Procedures (SOPs). SOPs sequentially describe the steps of a particular methodology so that it can be reproduced by different analysts, which minimises variations in their implementation and improves the standardisation of the final product.

Several techniques, such as high performance liquid chromatography, gas chromatography and mass spectrometry, can be used in SOPs to control the quality of plant phenolic compounds [1,2]. However, these compounds have a characteristic spectrum produced by the double bonds in the aromatic rings and substituent positions that facilitates their identification and the development of spectrometric analytical techniques is easily accomplished. In this sense, spectrophotometric methods are more practical, reproducible and inexpensive than other techniques and are therefore favoured for the development of analytical methodologies for such determinations.

In addition to producing compounds such as carbohydrates, lipids, proteins and nucleic acids directly involved in their essential growth functions, plants have an arsenal of enzymes capable

of producing, processing and accumulating several other substances not necessarily related to the maintenance of their life [3]. All of these reactions can be defined as secondary metabolism, the products of which provide advantages for both survival and species perpetuation in the plant's ecosystem [4]. However, this protection has a cost for the plant because metabolic resources that could increase its biomass are used to produce these compounds. In addition to protection, secondary metabolites perform important ecological functions such as inhibiting the germination and growth of other plants, attracting both pollinators and seed-dispersing animals and providing chemical defences against microorganisms [5].

Phenolic compounds, which have one or more hydroxyl groups linked to an aromatic ring, stand out from other classes of plant secondary metabolites because they are widely distributed and have various ecological functions that are scientifically proven to have numerous pharmacological activities and are well represented by tannins, flavonoids and coumarins.

1.1. Tannins

Tannins are water soluble phenolic compounds with a molecular weight between 500 and 3000 Daltons and may be chemically classified into two groups: hydrolysable tannins and condensed tannins [6,7]. Hydrolysable tannins are connected by ester-carboxyl linkages, which undergo hydrolysis under acidic and basic conditions [8]. Figure 1 presents an example of hydrolysable tannin (gallotannin), connected through a polyol (usually β -D-glucose) with the hydroxyl group esterified by gallic acid. Polyphenols connected with ellagic acid are called ellagitannins [9].

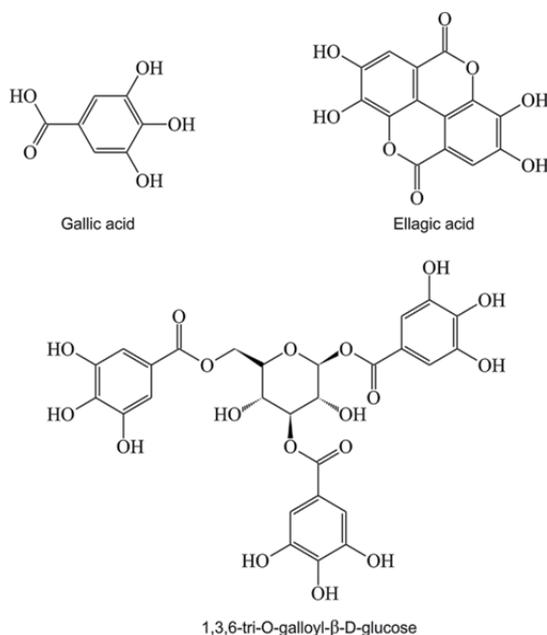


Figure 1. Structure of gallic acid, ellagic acid and 1,3,6-tri-O-galloyl- β -D-glucose, a hydrolysable tannin.

Condensed tannins, also known as proanthocyanidins (Figure 2), can contain dozens of units of flavan-3-ols (catechin) or flavan-3,4-diols (leucoanthocyanidins). These units have a complex structure and are resistant to hydrolysis; however, they can be soluble in aqueous organic solvents because of their structure [7].

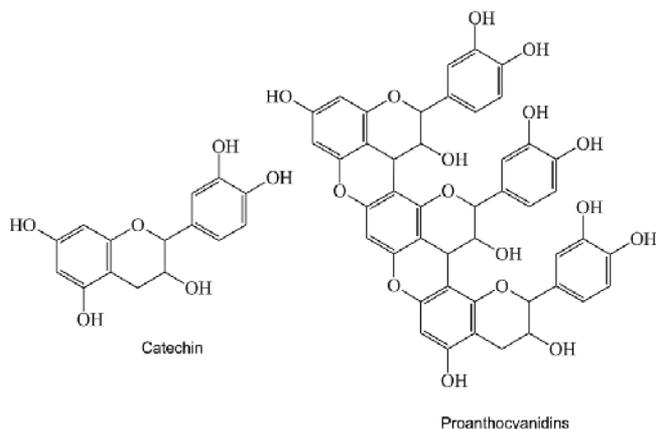


Figure 2. Basic structures of catechin and proanthocyanidins (condensed tannin).

Hydrolysable and condensed tannins may occur in the same plant simultaneously. However, the hydrolysable tannins are characteristic of Magnoliopsida herbaceous and woody plants and are restricted to certain taxonomic families. Ellagitannins have been used as taxonomic markers, particularly for Hamamelidaceae, Dillenidaceae and Rosaceae. Condensed tannins have been identified in all plant groups, including Gymnosperms and Pteridophytes [10].

These secondary metabolites were initially identified by their astringent taste and capacity to bind proteins, which allows for the precipitation and formation of complexes with collagen skin fibres to increase their resistance to water and heat. Chemically speaking, hydrophobic interactions and hydrogen bonds between the phenolic groups in tannins and some macromolecules explain these features. However, the stability of the formed complexes only results after the formation of covalent bonds via the oxidation of tannins by quinones [11].

Since antiquity, plants containing tannins have been used medicinally as anti-inflammatory, antimicrobial, antitumor and antiviral agents and to treat both wound and burns [7]. Tannins are also used to manufacture beverages and process animal skin into leather. Some researchers have shown that tannins protect plants against attack by herbivores and pathogens [12].

Although the use of tannin in the tanning industry has become restricted, interest in studying the ingestion of foods containing tannins to prevent diseases such as atherosclerosis or certain types of cancer has increased because of various epidemiological studies. Some studies report that the complexation of tannins with proteins gives them an important role in controlling bacteria, fungi and insects [13-15]. Other studies examined the inhibitory action of the enzyme reverse transcriptase [16] and the anticarcinogenic activity associated with

green tea and diets rich in fruits containing tannins [17]. It is generally believed that the pharmacological activity of tannins occurs via their complexation with metal ions, antioxidant activity or the ability to complex with macromolecules.

Maytenus ilicifolia (Schrad.) Planch. (Celastraceae), popularly known as “espinheira-santa”, is a species native to Brazil traditionally used to treat digestive disorders and the literature cites tannins as compounds that act to protect the stomach by helping to treat ulcers and gastritis [18-20]. In addition to these activities, *M. ilicifolia* has other pharmacological applications, such as anticancer, antimicrobial and antioxidant activity and treatment of the central nervous system [21]. For these reasons, *M. ilicifolia* was chosen as a reference species in this study.

1.2. Flavonoids

Flavonoids comprise a group of natural substances with great structural diversity and there are currently more than nine thousand known flavonoids that do not occur in humans but can be found in various plant parts such as the leaves, fruits, bark, roots, stems and flowers [22,23]. Flavonoids (Figure 3) are composed of a simple skeleton containing two phenol rings connected by a propionic chain; where ring A is the acetate derivative and both ring B and the three-carbon bridge are derived via a shikimate pathway, which may be associated with carbohydrates (heterosides), un associated (aglycones) or polymerised further (anthocyanins) [24].

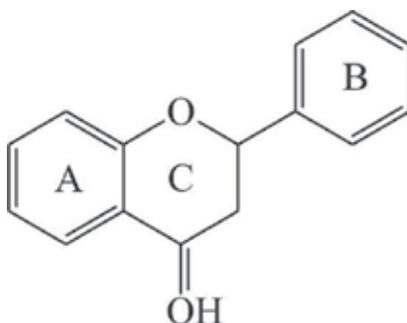


Figure 3. Basic structure of flavonoids.

This class of metabolites has several biological functions, such as defence against both herbivores and pathogens, the perpetuation of the species by attracting seed dispersing animals, protection from ultraviolet rays and allelopathy [24,25]. Flavonoids also possess important pharmacological properties, such as antioxidant, antiinflammatory, anti-thrombogenic, antimicrobial, anticancer, antidiabetic and hypocholesterolemic activities [23,26].

Studies show that flavonoids are chemical markers responsible for various pharmacological activities performed by the genus *Bauhinia* [27-29]. *Bauhinia forficata* Link (Fabaceae), popularly known as “pata-de-vaca”, is a tree native to Brazil that prevails in the phytogeographical area of Mata Atlântica [30] and can also be found in Cerrado. Pata-de-vaca is used in folk

medicine as a hypoglycemic, diuretic and antihypertensive agent [31,32]. Several plant-based products are sold in open markets, pharmacies and natural product stores [33]. Though not officially recommended, herbal products from the leaves of pata-de-vaca (*Bauhinia* L.) are popularly used for therapeutic purposes. This plant was used in our study as a reference species for flavonoids.

1.3. Coumarins

Coumarins are lactones of *O*-hydroxy-cinnamic acid derived from trans-cinnamic acid via oxidation-reduction and isomerisation to produce 1,2-benzopyrone. Coumarins are divided into simple coumarins, furanocoumarins, pyranocoumarins, dimeric coumarins and chromones (Figure 4) [34]. The difference between these classes is the position of the radical in the coumarin lactone ring, which varies between C-6, C-7 and C-8. Chromones represent a group of natural isomers to coumarin that may be linearly and angularly prenylated at C-6 and C-8, respectively, in furanochromones and pyranochromones, respectively [34].

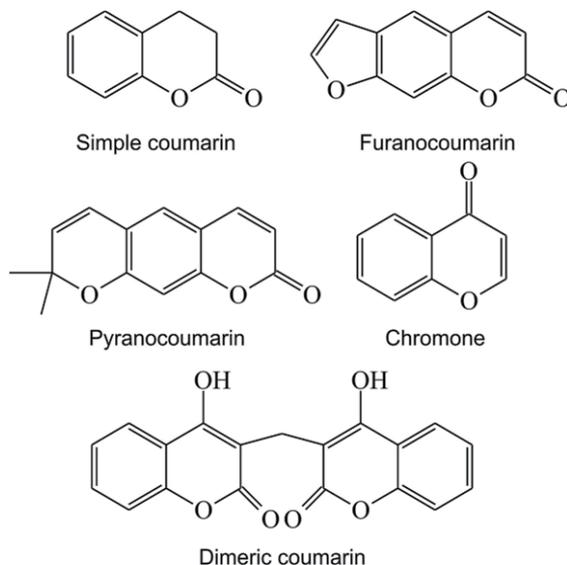


Figure 4. Basic structure of coumarins and chromones.

Coumarins are used as antioxidants, anti-HIV drugs, antispasmodics, spasmolytics, hypolipidemics, hypotensives and vasodilating agents [34]; however, they are also used in food flavouring, perfumes, tobacco and cosmetic products [35]. It is estimated that the daily human exposure to coumarins from cosmetics and perfumes is 0.04 mg/kg/day [36].

Coumarins are a class of secondary metabolites widely distributed throughout the Plantae kingdom and found in Fungi and Bacteria as well [37]. In plants, coumarins are found frequently in the families of Apiaceae, Rutaceae and Asteraceae, and less frequently in the families of Fabaceae, Oleaceae, Moraceae and Thymeleaceae [38].

Mikania glomerata Spreng. (Asteraceae), popularly known in Brazil as "guaco", has existed in the Brazilian Pharmacopoeia since 1929 and is used as an expectorant to treat respiratory problems. Most herbal products are marketed as some form of syrup; however, they are produced from fluid extracts and tinctures [39,40]. Chemical studies of this species show coumarins to be a major component that can be used as a chemical marker for the quality control of products based on guaco [41-43].

Coumarins have a characteristic UV spectrum due to the nature and position of their substituents, which facilitates both their identification and the development of analytical spectrophotometric techniques [11]. For these reasons, *M. glomerata* was chosen as the reference species to quantify the coumarin level.

2. Problem statement

Despite extensive literature presenting various analytical methods, the development of an SOP is often difficult for three reasons: 1) the work does not detail the difficulties and adjustments required to implement the methodology, 2) the steps are not clearly presented for reproduction and 3) the limits of interpretation are not discussed. One criterion recommended by the National Sanitary Surveillance Agency (Agência Nacional de Vigilância Sanitária - ANVISA) in Brazil for the standardisation of herbal drugs is the active compound content or chemical class, which is the total concentration of tannins for products based on *M. ilicifolia* (espinheira-santa) [44], flavonoids for *B. forficata* (pata-de-vaca) [45,46] and coumarins for *M. glomerata* (guaco) [44].

Thus, this paper presents research protocols adopted by our research group to study the levels of tannins, flavonoids and coumarins from plant extracts and the experimental application of these SOPs to analyse products sold in markets (pharmacies and natural product stores) as phytomedicines¹ or plant drugs² with high commercial value.

3. Standard operating procedures (POP)

The following SOPs describe the chemical classes to be analysed and the chemical basis of the methods. They provide a detailed list of all the reagents required for the preparation and describe the experimental procedure to be followed. Finally, there is a list of references used in the development of the SOP.

1 "All medicine is obtained using solely active raw vegetables. It is characterised by knowledge of the effectiveness and risks of their use, as well as the reproducibility and consistency of its quality. Its efficacy and safety are validated through ethnopharmacological surveys of use, documentation, technical and scientific publications or clinical trial phase 3"[44].

2 "Medicinal plant or their parts, after collection processes, stabilisation and drying and can be full, erasures, crushed or powdered" [44].

3.1. Standard operating procedure for the quantification of tannins

Description: Tannins are phenolic compounds with the ability to bind macromolecules, especially proteins. This class of compounds has traditionally been divided into two groups: hydrolysable and condensed tannins. Hydrolysable tannins are characterised by a central polyol, usually β -D-glucose, containing hydroxy groups esterified with gallic acid and ellagic acid. Condensed tannins are oligomers or polymers formed by the condensation of two or more molecules of flavan-3-ol or flavan-3,4-diol [3] [7].

Principle of the method: The phenolic compounds in the sample are oxidised using the Folin-Ciocalteu reagent. This reagent is a mixture of phosphotungstic and phosphomolybdic acids that are reduced by the oxidation of phenolic compounds in a mixture of tungsten and molybdenum oxides. The blue colour produced by the oxides has a maximum absorption at 760 nm and is proportional to the total phenolic concentration [47]. The tannin content is calculated as the difference between the total and waste phenol content.

Reagents:

- (1) Tannic acid (0.1 mg/mL, w/v): Dissolve 10 mg of tannic acid in 100 ml of distilled water.
- (2) Folin-Ciocalteu reagent (10%, v/v): Dilute 5 ml of Folin-Ciocalteu reagent with 45 mL of distilled water.
- (3) Sodium carbonate Na_2CO_3 (7.5%, w/v): Dissolve 7.5 g of Na_2CO_3 in 100 ml of distilled water. If necessary, solubilising the solution on a heating plate and magnetic stirrer.
- (4) Methanol (80%, v/v): Dilute 800 ml of methanol with 200 ml of distilled water.

The reagent volume is sufficient to examine a maximum of 100 analyses.

Preparation of samples: The powdered sample (500 mg) should be extracted with 50 mL of 80% methanol for 30 minutes on a hot plate. The extract should be filtered through filter paper into a 50 mL volumetric flask and the volume should be completed using the same solvent. The final extract concentration will be 10 mg/mL. If the products are liquid, they must be evaporated. The dried extract should be dissolved in 80% methanol for a final concentration of 1 mg/mL.

Calibration curve: The calibration curve must be prepared using 100-500 μL aliquots of the tannic acid solution, 500 μL of the Folin-Ciocalteu solution and 1 mL of the sodium carbonate solution. The final volume should be adjusted to 10 mL with distilled water. The final tannic acid concentration will be 1-5 $\mu\text{g}/\text{mL}$.

Measurement procedure: To quantify the total phenol concentration (that is, all of the phenols present in the sample), 500 μL of the extract must be transferred to a test tube. Next, 500 μL of the Folin-Ciocalteu solution, 1 mL of the sodium carbonate solution and 8 ml of distilled water are added. The samples remain at room temperature for 30 minutes. The spectrophotometer should be adjusted to a wavelength of 760 nm and the equipment must be rinsed with distilled water. To quantify the phenol waste, (i.e., the phenols present in the sample except for the proteins precipitated with the tannin) 500 mg of casein is weighed and

transferred into a 25 ml Erlenmeyer flask before adding 5 ml of the extract and 5 ml of water distilled. After two hours (time required for the complexing of the tannins to the total protein), the extracts are filtered into a 10 mL volumetric flasks and its volume is adjusted with distilled water. The phenols are considered to be equal to the residue from the total phenol. These assays are performed at least in triplicate and the total phenolic content is expressed as milligrams of tannic acid equivalents per gram of sample or extract (mg TAE/g).

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Amorim E. L. C, Nascimento J. E., Monteiro J. M., Peixoto Sobrinho T. J. S, Araújo T. A. S., Albuquerque U. P. A simple and accurate procedure for the determination of tannin and flavonoid levels and some applications in ethnobotany and ethnopharmacology. *Functional Ecosystems and Communities* 2008; 2(1) 88-94 [47].

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3.2. Standard operating procedure for the quantification of flavonoids

Description: Flavonoids are the most important and diverse phenolic compounds. Most flavonoids have 15 carbon atoms and consist of two phenol rings connected by a chain of three carbons to form a tricyclic compound [24].

Principle of the method: The aluminium ion (Al^{3+}) is reacted with the flavonoids in the sample to form the stable flavonoid- Al^{3+} complex, which has a yellow colour and intensity proportional to the flavonoid concentration. This reaction causes a bathochromic shift and intensification in the absorption, which can be measured without influence from other phenolic compounds present in the sample [48].

Reagents:

- (1) Rutin (0.1 mg/mL, w/v): Dissolve 10 mg of rutin in 100 ml of methanol.
- (2) Acetic acid solution (60%, v/v): Dilute 30 ml of acetic acid with 20 ml of methanol.
- (3) Pyridine Solution (20%, v/v): Dilute 40 ml of pyridine with 160 ml of methanol.
- (4) Aluminium chloride solution AlCl_3 (5%, w/v): Dissolve 5 g AlCl_3 in 100 mL of methanol. If necessary, complete dissolution via magnetic stirring.
- (5) Methanol (80%, v/v). Dilute 80 ml of methanol with 20 ml of distilled water.

The reagent volume is sufficient to examine a maximum of 100 analyses.

Preparation of samples: The powdered sample (500 mg) should be extracted with 50 mL of 80% methanol on a hot plate for 30 minutes. The extract should be filtered through filter paper into a 50 mL volumetric flask and the volume should be adjusted with the same solvent. The final extract concentration will be 10 mg/mL. Liquid products must be evaporated. The dried extract should be dissolved in 80% methanol to obtain a final concentration of 1 mg/mL.

Calibration curve: The calibration curve must be prepared using 100-1000 μL aliquots of the rutin solution, 500 μL of the acetic acid solution, 2 mL of the pyridine solution and 1 ml of the reagent aluminium chloride solution. The final volume should be adjusted to 10 mL with 80% methanol. The final rutin concentration will be 1-10 $\mu\text{g}/\text{mL}$.

Measurement procedure: To quantify the flavonoids, 500 μL of the extract should be transferred to a test tube. Next, 500 μL of the acetic acid solution, 2 mL of the pyridine solution, 1 ml of the reagent aluminium chloride solution and 6 ml of 80% methanol will be added. The samples remain at room temperature for 30 minutes. The spectrophotometer should be adjusted to a wavelength of 420 nm and the equipment must be rinsed with distilled water. The test shall be performed at least in triplicate and the flavonoid content is expressed as milligrams of rutin equivalents per gram of sample or extract (mg RE/g).

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Peixoto Sobrinho T. J. S, Silva C. H. T. P., Nascimento J. E., Monteiro J. M., Albuquerque U. P., Amorim E. L. C. Validação de metodologia espectrofotométrica para quantificação dos flavonóides de *Bauhinia cheilantha* (Bongard) Steudel. Brazilian Journal of Pharmaceutical Sciences 2008; 44 (4) 683-689 [48].

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3.3. Standard operating procedure for the quantification of coumarins

Description: Coumarins are lactones of *O*-hydroxy-cinnamic acid and are metabolites of phenylalanine. 1,2-benzopyrone is the simplest coumarin representative, others being furocoumarins, pyranocoumarins, dimeric coumarins and chromones [34].

Principle of the method: The Borntrager reaction is based on the solubility of free coumarin derivatives in polar organic solvents and the solubility of their soluble alkali phenolates. Coumarin absorbs at 280 nm; however, ionisation of phenolic hydroxyls in the molecule by alkaline hydroxide causes a bathochromic deviation to 320 nm, which is proportional to the coumarin concentration [40].

Reagents:

- (1) 1,2-benzopyrone (1 mg/mL, w/v): Dissolve 10 mg of coumarin in 10 ml of distilled water.
- (2) Lead acetate (5%, w/v): Dissolve 2,5 g of lead acetate in 50 ml of distilled water.
- (3) Hydrochloric acid solution, HCl (0.1 M, v/v): Dilute 10 ml of concentrated hydrochloric acid with 1000 ml of distilled water.
- (4) Methanol (80%, v/v): Dilute 80 ml of methanol with 20 ml of distilled water.

The reagent volume is sufficient to examine a maximum of 100 analyses.

Preparation of samples: The powdered sample (500 mg) should be extracted with 50 mL of 80% methanol for 30 minutes on a hot plate. The extract should be filtered through filter paper

into a 50 mL volumetric flask and the final volume should be adjusted with the same solvent. The final extract concentration will be 10 mg/mL. Liquid products must be evaporated. The dried extract should be dissolved in 80% methanol for a final concentration of 1 mg/mL.

Calibration curve: The calibration curve must be prepared using 50-500 μL aliquots of the coumarin solution, 2 ml of distilled water and 500 μL of the lead acetate solution. The sample should be shaken and the final volume should be adjusted to 10 mL with distilled water before transferring 2 mL of this solution to a new test tube and adding 8 mL of hydrochloric acid solution. The final concentration of rutin will be 1-10 $\mu\text{g/mL}$.

Measurement procedure: To quantify the coumarins, 500 μL of the extract should be transferred to a test tube. Next, 2 ml of distilled water and 500 μL of lead acetate solution will be added. The sample is shaken and then 7 ml of distilled water are added before transferring 2 mL of this solution to a new test tube and adding 8 mL of hydrochloric acid solution. The samples remain at room temperature for 30 minutes. The spectrophotometer should be adjusted to a 320 nm wavelength and the equipment must be rinsed with distilled water. The test should be performed in at least triplicate and the total coumarin content is expressed as milligrams of coumarin equivalents per gram of the sample extract (mg CE/g).

References:

Kuster R. A. M., Rocha L. A. M. A. Cumarinas, coronas e cantinas. In: Simões CMO, Schenkel EP, Gosmanm, G, Mello JCP, Mentz LA, Petrovick PR. (ed.) Farmacognosia: da planta ao medicamento. Porto Alegre: Universidade Federal do Rio Grande do Sul; 2004, p. 537-556 [34].

Osório O. K., Martins J. L. S. Determinação de cumarina em extrato fluido e tintura de guaco por espectrofotometria derivada de primeira ordem. Brazilian Journal of Pharmaceutical Sciences 2004; 40 (4) 481-486 [40].

4. Results

Analysis of the active component levels in raw plant materials and phytomedicines is essential for the safety and efficacy of pharmaceutical products [49]. The quantification of active compounds in herbals is still only incidentally performed due to the presence of active phytocomplexes plants and their extracts [50], which complicates their analysis. Through this framework, the use of standardised extracts focusing on specific groups of active components ensures the chemical homogeneity of the product, which improves product quality [51]. The compounds selected for this quality adjustment process should be the same as the assets in the product [52].

In this way, five products containing *M. ilicifolia* (all plant drugs), four containing *B. forficata* (all plant drugs) and five containing *M. glomerata* (three plant drugs and two fluid extract) sold in pharmacies and health food stores in Recife/PE, Northeast Brazil were obtained.

Analysis of the active component concentrations were conducted as listed in the described standard operating protocols (SOPs) and the results are presented below.

4.1. Calibration curves

To quantify the active components, calibration curves with increasing concentrations proportional to their absorbance were constructed. A correlation equation was obtained from these curves (generally linear) of the type $y = ax + b$, where y corresponds to the absorbance of the sample and x the concentration. To convert the absorbance values (nm) to sample concentration ($\mu\text{g/mL}$), it is necessary to place the sample absorbance into the equation as y . The correlation coefficient (R^2) shows the ability of the method to provide directly proportional results between the analyte concentration and the device response. The interval between the lowest and highest scalar values, which is also called the linearity range, should be determined with both precision and accuracy. The correlation coefficient must be equal to or greater than 0.98.

The calibration curve constructed for tannic acid and tannins and used to quantify *M. ilicifolia* demonstrated a correlation equation of $y = 0.067x + 0.01$ and a correlation coefficient of $R^2 = 0.996$ (Figure 5).

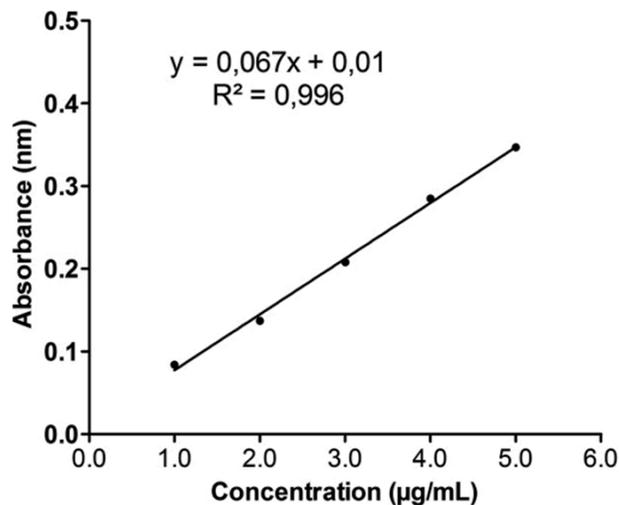


Figure 5. Calibration curve constructed using tannic acid concentrations of 1-5 $\mu\text{g/mL}$ at 760 nm used to quantify the tannin content of *Maytenus ilicifolia* (Schrad.) Planch.

A calibration curve was constructed from rutin to quantify flavonoids in products from *B. forficata* and yielded the correlation equation $y = 0.022 + 0.0039x$ and correlation coefficient $R^2 = 0.991$ (Figure 6).

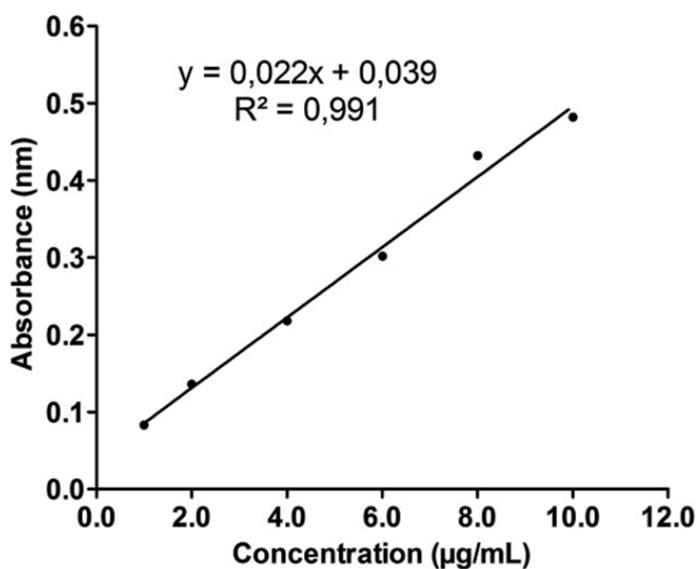


Figure 6. Calibration curve constructed from rutin for concentrations of 1-10 µg/mL at 420 nm used to quantify the flavonoids content in *Bauhinia forficata* Link.

The correlation equation and coefficient obtained from the calibration curve used to analyse coumarins in products containing *M. glomerata* were $y = 0.049x + 0.031$ and $R^2 = 0.994$ (Figure 7), respectively.

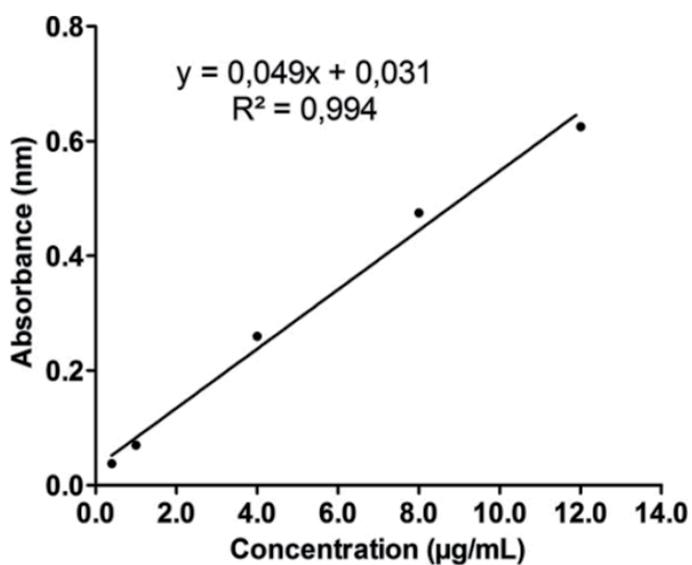


Figure 7. The calibration curve constructed using 1,2-benzopyrone in concentrations of 1-12 µg/mL using 320 nm excitation to quantify the coumarin content in *Mikania glomerata* Spreng.

4.2. Content of active principles

The results of quality control of tannins of *M. ilicifolia*, flavonoids of *B. forficata* and coumarins of *M. glomerata* are shown in Table 1.

Specie / Marker	Sample	Concentration \pm SD (mg/g)	CV (%)
<i>Maytenus ilicifolia</i> Tannins	MI1	12.57 \pm 2.15a	17.12%
	MI2	4.04 \pm 0.23b	5.75%
	MI3	5.61 \pm 0.55bc	9.76%
	MI4	7.72 \pm 0.84c	10.84%
	MI5	11.81 \pm 1.00a	8.44%
<i>Bauhinia forficata</i> Flavonoids	BF1	4.89 \pm 0.11a	2.33%
	BF2	7.27 \pm 0.39a	5.41%
	BF3	50.38 \pm 5.36b	10.64%
	BF4	65.98 \pm 3.62c	5.49%
<i>Mikania glomerata</i> Coumarins	MG1	3.06 \pm 0.20a	6.67%
	MG2	5.17 \pm 0.59b	11.40%
	MG3	6.80 \pm 0.24c	3.46%
	MG4	1.63 \pm 0.20d	12.50%
	MG5	4.49 \pm 0.20b	4.55%

Table 1. Results of quality control of tannins, flavonoids and coumarins contained in products based on *Maytenus ilicifolia* (Schrad.) Planch., *Bauhinia forficata* Link and *Mikania glomerata* Spreng. respectively, sold in pharmacies in Recife/PE, Northeast of Brazil.

Values are mean \pm standard deviation. Values followed by the same letter in column are not statistically different (n = 6, p<0.05).

Analysis of variance (ANOVA) is one way to indicate significant differences (p<0.01) for the drugs of *M. ilicifolia*, which is made from five plants. Samples Mi1 (12.57 \pm 2.15 mg TAE/g) and Mi5 (11.81 \pm 1.0 mg TAE/g) both had higher concentrations of tannins and were not significantly different, whereas at least three of the other samples showed tannins (Figure 8). Comparing the average tannin concentration from different samples showed a low coefficient of variation (CV = 37.61%).

Analysis of four products containing *B. forficata* showed a significant difference (p<0.01). The sample Bf4 presented the highest flavonoid concentration (65.98 \pm 3.62 mg RE/g), whereas samples Bf1 and Bf2 had the lowest concentrations (4.89 \pm 0.11 and 7.27 \pm 0.39 mg RE/g, respectively) with a content approximately 13 times lower. These results indicate that there is no standardisation regarding the flavonoid concentrations in products (Figure 9).

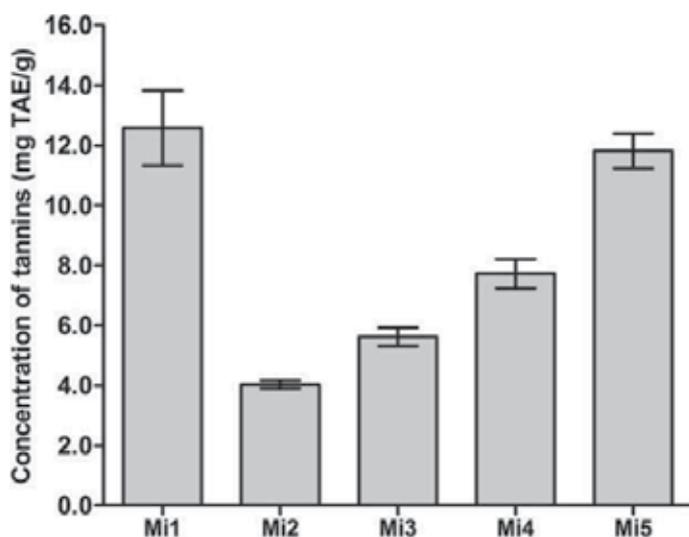


Figure 8. Concentration of tannin (mg TAE/g) contained in products from *Maytenus ilicifolia* (Schrad.) Planch. Sold in Recife/PE, Brazil.

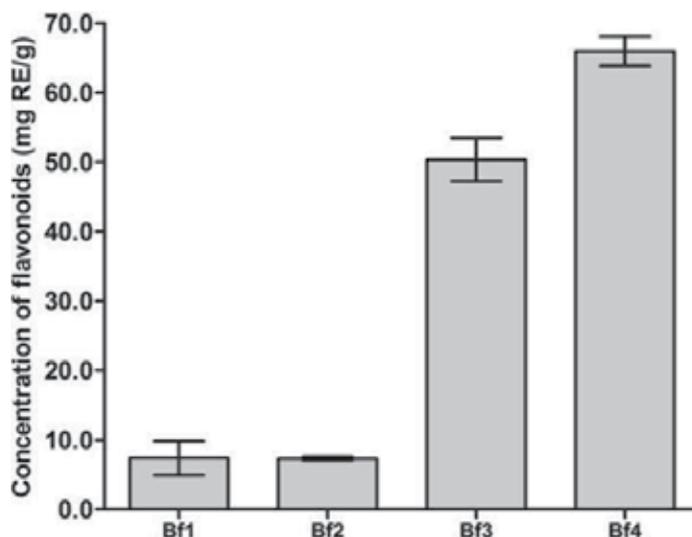


Figure 9. Concentration of flavonoids (mg RE/g) contained within *Bauhinia forficata* link. sold in Recife/PE, Brazil.

Of the five products from *M. glomerata*, three were plant drugs and two were fluid extracts. The plant drugs were extracted with ethanol as recommended by the Brazilian Pharmacopoeia 4th Edition to remove discrepancies from the results [53]. Both the extracts and liquids were evaporated to dryness. The one way ANOVA showed significant differences between the products ($p < 0.01$) and sample Mg3 had the highest level (6.80 ± 0.24 mg CE/g), whereas

sample Mg4 had the lowest level (1.63 ± 0.20 mg CE/g), with an approximately four times lower coumarin concentration.

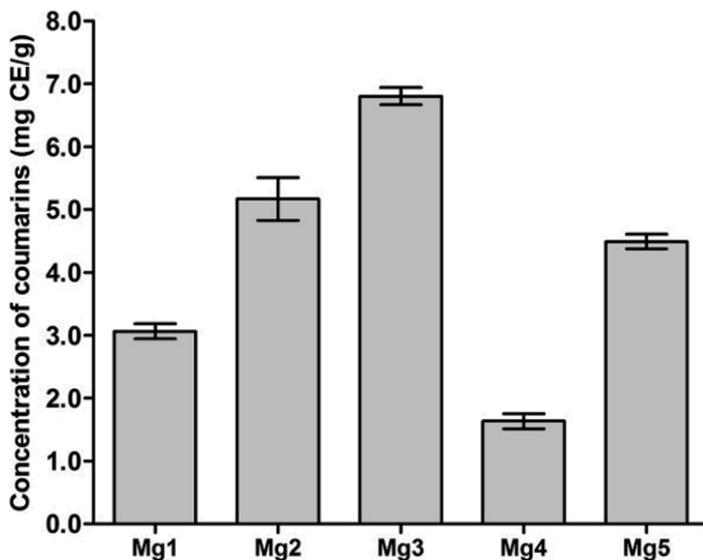


Figure 10. Concentration of coumarins (mg CE/g) contained in products from *Mikania glomerata* Spreng. sold in Recife/PE, Brazil.

The quantitative analysis of raw vegetables and phytomedicines is a fundamental quality control process that leads to security, stability, consistency and effectiveness in the produced phytomedicines [49]. It is important to emphasise the need for standardisation in analysing herbal medicines to determine the concentration of their active components in raw vegetable materials as well as for species identification.

5. Conclusion

This chapter provides easily reproducible standard operating procedures (SOPs) for the quality control of raw materials and herbal plants to ensure a minimal standard of quality in products sold. The implementation of these SOPs allows for the analysis of samples sold in establishments in Recife/PE and reveals an inconsistency in the concentration of tannins, flavonoids and coumarins within these products.

The low level of these metabolites may alter their effectiveness and more rigorous quality control and standardisation of these products is required to prevent compromising their therapeutic activity.

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Microbial Quality of Medicinal Plant Materials

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Additional information is available at the end of the chapter

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1. Introduction

The use of medicinal plants is continually expanding worldwide. The increasing search for therapeutic agents derived from plant species is justified by the emergence of diseases, yet without proper treatment, and the growth of scientific knowledge about the herbal medicines as important treatment alternatives. Therefore, the quality and safety of herbal preparations are also of great concern [1]. The reference [2] explained that quality is the basis of reproducible efficacy and safety of herbal drugs, and to ensure the standard of research on herbal medicines, the quality of the plant materials or preparations is of utmost importance. With the ever increasing use of herbal medicines and the global expansion of the herbal medicines market, safety has become a concern for both health authorities and the public in many countries. This is because many contaminants and residues that may cause harm to the consumers have been reported [3].

The microbial load of plants is the result of a series of influences (Figure 1). By their origin, herbal drugs are subject to contamination by microorganisms from soil, air and water may be present potentially pathogenic microorganisms to man. Microbial contamination of medicinal herbal can be influenced by environmental factors such as temperature, humidity and extent of rainfall during pre-harvesting and post-harvesting periods, handling practices and the storage conditions of crude and processed medicinal-plant materials. In order to improve the purity and safety of the products, observation of basic hygiene during preparation, standardization of some physical characteristic such as moisture content, pH and microbiological contamination levels are desirable [1,4,5].

The presence of microbial contaminant in non sterile pharmaceutical products can reduce or even inactivate the therapeutic activity of the products and has the potential to adversely affect patients taking the medicines. As herbal medicinal products are complex mixtures

which originate from biological sources, great efforts are necessary to guarantee a constant and adequate quality. Manipulation and processing factors largely determine the microbiological quality of the final products [6]. Previous studies have confirmed the presence of potential contaminants in herbal preparations [7-10]. Thus, manufacturers should ensure the lowest possible level of microorganisms in the raw material, finished dosage forms and the packaging components to maintain appropriate quality, safety and efficacy of the natural products [9].

This review intends to contribute to knowledge regarding the microbial contamination of medicinal plants by considering the influence of different commonly used pharmaceutical preparation techniques on the microbiological status of the products. Finally, quality standards will be discussed, considering the main guidelines of microbial quality control and through quality assurance measures such as good manufacturing practices (GMP) for herbal medicines.

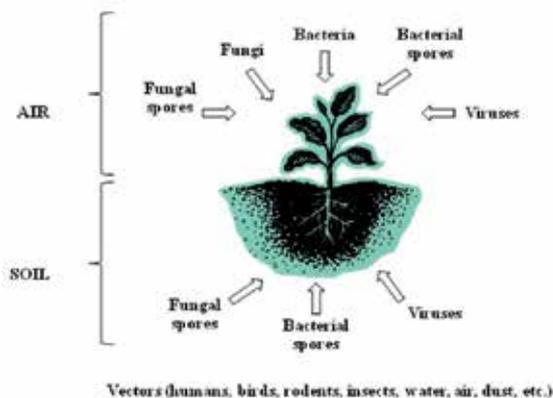


Figure 1. Influence of environmental factors and possible pathways of microbial contamination of medicinal herbs (adapted from [4]).

2. Common microbial contaminants associated with medicinal plants

The growing, harvesting and manipulation methods usually applied cannot avoid microbial contamination of the plant material which therefore reflects the environmental conditions as well as the specific hygiene during the diverse treatments [4]. Biological contamination refers to impurities in medicinal herbs and their preparations and products, and may involve living microbes such as bacteria and their spores, yeasts and moulds, viruses, protozoa, insects (their eggs and larvae), and other organisms. However, products of microbial metabolism such as toxic, low-molecular-weight metabolites from moulds are important chemical

contaminants [11]. The main microbial contamination of plant materials, in general, are attributed to total aerobic mesophilic, enterobacterial, yeast and mould [4].

The presence of higher numbers of spores bacteria could be explained by the fact that some of these organisms (e.g. *Bacillus* and *Clostridium* spp.) produce spores which are resistant to harsh processing, elevated heat and dry conditions. Therefore, they can survive for a long time on the product in a dormant state. *Bacillus cereus* and *Clostridium perfringens* are recognized as having potential pathogenicity and have been incriminated in food poisoning [12]. Although bacterial endospores and fungal spores can be regarded as the two dominating groups of contaminants associated with medicinal plants, a broad diversity of bacterial, fungal cells and viruses can be found either in or on the plant material [4]. *B. cereus* and *C. perfringens* were isolated from chamomile and other herbs by [13].

Although enterobacteria can be found in nature, this family possesses some indicative value towards faecal contamination. The presence of enterobacteria and *E. coli* reflect the situation regarding faecal contamination [7]. Together with the group of coliforms, it can be taken as an indicator for undesirable hygiene conditions, although this conclusion has to be related to the magnitude of viable count measured [4]. *Staphylococcus aureus* is not common contaminant of this type of plant material and relatively rarely found. However, contamination could provide amount of enterotoxin produced by *S. aureus*, depending on the specific nature of the individual [11].

Herbal medications are likely to be contaminated with a wide variety of others potentially pathogenic bacteria. In a study whose was evaluated the bacterial contamination of powdered herbal medicinal preparations sourced from identified herbal retail outlets in different parts of Kaduna, Nigeria, the results showed that a number of herbal remedies were contaminated with *Salmonella typhi* and *Shigella* spp., besides *E. coli* and *S. aureus* [1]. In addition, the presence of pathogenic bacteria like *B. cereus*, *Aeromonas hydrophila*, *Shigella* spp., *Enterobacter agglomerans*, *E. cloacae*, *Vibrio fluvialis*, *Pasteurella multocida*, *S. epidermidis*, *Acinetobacter iwoffii*, *Klebsiella* spp., *B. subtilis* and *Pseudomonas aeruginosa*, and fungi *Rhizopus stolonifer* also were observed to be present in plant samples analyzed recently [14,15].

Because they are widespread in the atmosphere, moulds are common natural contaminants of medicinal herbs. It is known that, under favourable conditions, some fungi can synthesize toxic metabolites – mycotoxins. Among the known mycotoxins, the most toxic one is aflatoxin synthesised by species of *A. flavus* and *A. parasiticus*, and a minor number of other fungi [10]. Contamination by *A. flavus*, the most famous aflatoxin producer, is common in medicinal plant and herbal tea [16]. *A. flavus* colonization does not necessarily reduce yield, but causes economic losses by contaminating with aflatoxin [17]. In a study of 91 medicinal herb samples in Brazil [18], were found that 50 % of aerial part samples were contaminated with fungi. Samples of medicinal plants were evaluated by [5] for the fungal contamination, and results indicated that predominant mycoflora (89.9% of the isolates) corresponded to genera *Aspergillus* and *Penicillium*, which are extremely important from the mycotoxicological standpoint. The fungal contamination of powdered herbal medicinal preparations sourced from some herbal retail outlets in some parts of Nigeria was evaluated by [19] and

the results showed that all of the herbal preparations had the presence of fungal contaminants with predominance of *Aspergillus* spp. and *Penicillium* spp., but *Mucor* spp., *Candida* spp., *Trichosporium* spp., also were found. The fungal deterioration adversely affects the chemical composition of the raw materials and thereby decreases the medicinal potency of herbal drugs [20].

The risk of the presence of microorganisms in a plant product depends on this finality of the use, its nature and its potential damage that may be caused to the consumers. Considering natural flora, current production conditions and the need to warrant the quality and the safety of these products, monographs establish a maximum fungal contamination limit for products that contain raw material of natural origin [5]. Although high fungal loads may be accepted due to the natural origin of those products, they indicate the potential for spoilage and mycotoxigenesis.

3. Influence of different preparation techniques on the microbiological quality

The production of an herbal medicine generally involves the steps in which a vegetable is subjected to unfavorable conditions to survival of microorganisms. Next, we introduce some of these processes and their influence on the microbial load.

3.1. Drying process

Drying is basically defined as the decreasing of plant moisture content, aimed at preventing enzymatic and microbial activity, and consequently preserving the product for extend shelf life [21]. Drying is the most common and fundamental method for post-harvest preservation of medicinal plants because it allows for the quick conservation of the medicinal qualities of the plant material in an uncomplicated manner. This process may also contribute to facilitate the marketing of plants, because drying results in reduction of the weight and volume of the plant with positive consequences for transport and storage [21,22].

The optimization of the drying process contributes to physical, chemical and microbiological stability of the medicinal herbs. The choice of drying conditions depend on the moisture content of tissue at harvest, the plant parts used, and the temperature best suited for preservation of the requested ingredients. For this reason, adequate dryers are needed, using temperature, velocity and humidity values for drying air that provides a rapid reduction in the moisture content without affecting the quality of the active ingredients of medicinal plants [21].

Medicinal plants can be dried in a number of ways: in the open air (shaded from direct sunlight); placed in thin layers on drying frames, wire-screened rooms or buildings; by direct sunlight, if appropriate; in drying ovens/rooms and solar dryers; by indirect fire; baking;

lyophilization; microwave; or infrared devices. When possible, temperature and humidity should be controlled to avoid damage to the active chemical constituents. In the case of natural drying in the open air, efforts should be made to achieve uniform drying of medicinal plant materials and so avoid mould formation [23].

Spray drying technique has been widely used to obtain dried extracts presenting better technological characteristics and greater concentration of biological active constituents. This method is widely used in the pharmaceutical industry, despite the high temperature drying (100°C to 200°C), the contact time between the material to be dry and hot air is extremely fast, less than 1 minute, theoretically is not enough to remove the microorganisms [24]. Comparative microbiological analysis of drug pulverized, extraction in liquid phase and the the spray drying extraction, using *Phyllanthus niruri* L., revealed that there is a significant reduction in microbial load, caused by the extraction in liquid process, while the spray dryer, despite the high temperature, did not affect the microbial load [25].

Drying at high temperature decreases the total aerobic microbial count in herbs. Water is a significant component of biological materials. Drying methods can lower the water activity to the level required for preventing growth of *Aspergillus* species and also for ensuring quality of medicinal herbs which may get destroyed upon over drying [10]. Exposure of herbs to microwaves and warm-air ovens can be efficient to reduce the microbial load, but they are not recommend to medicinal herbs containing volatile oils. The reference [26] evaluated both method of drying of plant, and reduction the microbial load present on the plants was observed but the effect on the volatile oil profile was profound by microwave drying, and warm drying air revealed that at temperatures >60°C, most of the volatile constituents were lost.

Other methods such as freeze-drying, oven drying and tray drying have been previously used to preserve medicinal herbs but to date there is little information in the literature on the effect of these drying conditions on the decrease of microbial loads [27].

Once drying is complete, plants are packaged in preparation for shipping or other further processing.

3.2. Extraction methods

Water is almost universally the solvent used to extract activity. At home, dried plants can be ingested as teas (plants steeped in hot water) or, rarely, tinctures (plants in alcoholic solutions) or inhaled via steam from boiling suspensions of the parts. Dried plant parts can be added to oils or petroleum jelly and applied externally. Poultices can also be made from concentrated teas or tinctures [28]. These kinds of preparations are usually called medicinal teas and are prepared using natural plants collected, dried and packaged without an effective hygienic and sanitary control. In addition, there can be microbiological contamination and controlling microbial contamination can be difficult in aqueous extracts [13].

Environmental dust settled on different parts of the plant and other contaminations can carry very significant amounts of bacterial and moulds spores [13]. However, those drugs which are subjected to cold water extraction (herbal maceration) may host a considerable amount of microbes, and the extraction procedure carried out at ambient temperature usually enables microbial multiplication [4]. The application of hot water extraction usually compensates for microbiological contaminations, since it can be expected that boiling water markedly reduces the viable counts by several log units and also inactivates possible pathogens [4]. However, bacterial spores of the Bacillaceae family are resistant to thermal treatment usually applied in infusion preparation, and this thermal shock may stimulate spore germination. Some of these bacteria like *B. cereus* and *C. perfringens* are recognized as having potential pathogenicity and have been incriminated in food poisoning [12]. Thus, in extractions using only water, hot or cold, as extractor liquid, the stability of the extract becomes compromised and the risk of microbiological contamination increases significantly. This contamination can compromise the quality and integrity of the plant material itself, as well as products arising from its use [29-30].

In addition to extraction temperature, the choice of extraction solvent is another important factor to prevent microbial contamination. The aim of an extraction process should be, of course, to provide for the maximum yield of substances and of the highest quality (concentration of target compounds and pharmacological power of the extracts). For extraction of active phytochemicals, the most commonly used solvents are methanol, ethanol, hexane, chloroform and diethyl ether [31]. Herbal extraction which made by ethanol or methanol extraction should, in general, provide good hygiene conditions, but the result depends on the alcoholic concentration applied [4].

3.3. pH influence

The pH value is one of the main factors influencing the quality of medicine. It always controls many chemical and microbiological reactions [32]. When the pH value is low (presence of acidic substances), the bacterial count could be low, but at neutral or higher pH the level of contamination of the herbal preparations could be observed to be higher. This suggests that a neutral or alkaline pH favoured high contamination levels of the herbal preparations. This agrees with the observation that bacterial growth is optimal at more or less neutral pH, around pH 5-8.5 [1].

3.4. Storage

Most pre-storage processing of plant material, such as that involving drying, heat, cooling and packaging, can prevent the degradation of plant material during storage [33]. Storage of medicinal herbs is an important part in the process production. During storage, due the factors in the outside world and their own physical and chemical properties of the interaction, gradually occurring physical, chemical and biological changes. Prolonged storage in poorly

ventilated storehouse usually increases sample moisture content in the bulk due to heat exchange capacity, rendering herbs more susceptible to molds growth and toxin production. Fungi are the predominant contaminants of herbs, but most such microbial populations are probably regarded as commensal residents on the plant that survived drying and storage. Most fungi are present on plants, which develop after harvest if relative humidity is not controlled during storage [34-35].

Moulds are responsible for biodeterioration of a number of substrates including raw materials of some medicinal plants. These moulds reduce raw herbal drugs shelf life and market value. The fungal deterioration adversely affects the chemical composition of the raw materials and thereby decreases the medicinal potency of herbal drugs [20]. Samples of herbal parts stored for sale in markets located in Ibadan, Nigeria were analysed for mycoflora associated with their storage and twenty eight fungal species were isolated, showing that herbal drug plant pieces are hazardous for human health [36]. Some samples of herbal raw materials have been reported to contain aflatoxin. The reference [37] determined the incidence of toxigenic fungi and their mycotoxins on 152 dried medicinal and aromatic herbs from Argentina, which are used as raw material for drugs. *A. flavus* and *A. parasiticus* were the predominant species isolated, and high aflatoxin concentrations were detected. There is a potential risk for mycotoxins contamination, especially during prolonged storage in poorly conditions without temperature and moisture control that usually render medicinal plants more susceptible to moulds growth and mycotoxins production [5,10,20].

The reduction of plant enzyme activity and inactivation of microorganisms is achieved by drying. Dried plant materials tend to be hygroscopic (readily absorbing moisture) and must be stored under controlled humidity. Rehydration can lead to the decomposition of the bioactive metabolites by enzymes from microorganisms or the plant itself. Significant contamination by bacteria and fungi suggest inadequate storage facilities and poor hygienic practice during preparation of these medicinal plants. The storage processes of such products are stages during which it is important to avoid even further contamination [38].

Studies on long-term stability of dried herbal teas and preparations are rare. In a study of [20] was examined the deterioration of herbal drug samples which were stored for 6-9 months by traders after collection. Some of the contaminated materials were found to be deteriorated by toxigenic strains of *A. flavus* and contain aflatoxin B1 which was above the permissible limit. In a study of [38], dried *P. lanceolata* leaves were exposed to atmospheres of different relative humidity (75, 45 and 0%) for 24 weeks and was evaluated the chemical changes of the compounds of interest. It was shown that exposure to water results in loss of bioactive molecules of *P. lanceolata* dried leaves, and that colonising fungi are the key contributors to this loss. The fungal deterioration adversely affects the chemical composition of the raw materials and thereby decreases the medicinal potency of herbal drugs. Biodeterioration of herbal products samples by associated fungi during storage has drawn attention regarding quality maintenance of these products [35,38-40].

It is common practice for herbalists to prepare herbal medicines and store them in a refrigerator. However, in previous study the effect of microbial contaminants on active com-

pounds of African plant extracts was assessed and indicated that after 25 days of storage in low temperature there may be little or no active compounds due to spontaneous biodegradation by naturally-occurring microbes [41]. The World Health Organization (WHO) recommends that whenever required and when possible, fresh medicinal plant materials should be stored at appropriate low temperatures, ideally at 2-8°C; frozen products should be stored at less than -20°C.

Processed medicinal plant materials should be packaged as quickly as possible to prevent deterioration of the product and to protect against unnecessary exposure to potential pest attacks and other sources of contamination.

4. Decontamination of plant materials

Attempts have always been made to decontaminate and preserve these medicinal plants so as to get more safe, natural and potent medicines. The number of methods has been tried for decontamination such as heat treatment, UV irradiation and fumigation. However, volatility and heat sensitivity of the delicate flavor and aroma components of the medicinal plants do not permit the use of heat treatment [42].

Low penetration power of UV radiations makes this irradiation method unsuitable [42]. Fumigation with gaseous ethylene oxide brings down the microbial burden but this method is now prohibited or restricted in many countries due to the carcinogenic nature of one of its residue in treated medicinal plants [43,44]. Various disinfectant technologies have been suggested which include electromagnetic radiations, photodynamic pulsing, ultrahigh pressure and CO₂ treatment [42].

Gamma irradiation is now getting recognition throughout the world as a phytosanitary treatment of herbal materials. It improves the hygienic quality of various herbal materials and reduces the losses due to microbial contamination and insect damage [45]. Besides, it is a fast, safe, convenient, eco-friendly method which reduces the reliance on chemical fumigants and preservatives currently used by industries. The chances of recontamination are also reduced, as it can be done after packaging [46]. Some studies showed that the exposition of plant samples to different doses of gamma radiation can result in reduction in total bacterial counts and also indicated that the microbial load could be decreased by increasing the radiation-absorbed dose. These studies indicate that gamma irradiation is an effective treatment for microbial decontamination of medicinal plants [42,47,48].

Certain plants contain natural barriers and antimicrobial substances which exert typical inhibitory effects on microbial growth and stability. It has been estimated that around 1400 herbs and spices may possess antimicrobial agents of different chemical nature as oils, peptides, liquid and organic extracts [4]. Some medicinal herbs contain essential oils which act as natural antimicrobials and may inhibit mould development and mycotoxin production [11]. Different studies have demonstrated the effectiveness of antimicrobials and their effec-

tive compounds to control or inhibit the growth of pathogenic and spoilage microorganisms [49-51].

5. Microbial quality parameters

The most widely accepted and used technique is that recommended by WHO for total count of microorganisms in plant materials. According to the methodology of the WHO, 10 g of sample should be suspended in 90 ml of buffer sodium chloride-peptone, adjusting the pH to 7.0. To count total aerobic bacteria, sample should be plated in duplicate, using the official technique of sowing depth on casein-soybean digest agar, and then incubated at 30-35°C for 48h. To count yeast and mold, the technique employed is the sowing depth in Sabouraud-dextrose plus a solution of 10% tartaric acid to obtain pH 3.0 to 3.5. The dilution is plated in duplicate and incubated at 20-25°C for 5 days [52]. Analysis of specific pathogens, Enterobacteriaceae and other Gram negative bacteria (*E. coli*, *Salmonella* sp., *P. aeruginosa* and *S. aureus*) consists of specific methods of cultivation and through biochemical and serological tests. The specification of WHO for total aerobic microorganisms is not more than 10^7 CFU/g for the plant material for use as teas and infusions and at most 10^5 CFU/g for internal use. The specification of WHO for yeasts and molds are at most 10^4 CFU/g for the plant material for use as teas and infusions and at most 10^3 UFC/g for internal use. High counts of fungi are a risk because of the possibility to produce mycotoxin, such as aflatoxin, which is a carcinogen toxin. The WHO also recommends a test to detect the possible presence of aflatoxins, which are highly dangerous contaminants in any material of plant origin.

In Brazil, despite the large consumption of products derived from plants, products sold and consumed were not subject to any kind of quality control. In 1995, the Ministry of Health instituted the ordinance MS/SNVS No. 6, January 31, 1995 [53] that regulated the registration of herbal products for commercial purposes. Then came the Resolution RDC No. 17 [54] and, more recently, the RDC No. 48 [55] which confirms definitely that are herbal medicines and thereby rescues the need for the existence of safety studies, efficacy and quality, prior to the registration of these products. The Resolution RDC No. 48 of March 16 of 2004 [55] recommends that the contamination analysis on herbal medicines must be in accordance with pharmacopoeial specifications.

Both the Brazilian Pharmacopeia [56], as the United States Pharmacopeia [57] draw the following specifications for products for oral use: 10^4 aerobic bacteria/g or mL, 10^2 fungi/g and absence of *Salmonella* spp, *E. coli* and *S. aureus*. However, the Brazilian Pharmacopeia also indicates the detection of other indicators of increased risk for oral administration, such as *P. aeruginosa*, *B. cereus*, *Enterobacter* spp, *C. albicans*, *A. flavus* and *A. parasiticus*. High microbial loads are indicative of the possibility of potentially pathogenic microorganisms.

In Europe the evaluation of microbial contamination of medicinal plants has increasingly become an integral part of Good Agricultural Practice (GAP) and Hazard Analysis and Critical Control Point (HACCP) concepts [33]. The limits of microbial contamination given in European Pharmacopoeia [58] for herbal medicinal products to which boiling water is added before use are: total aerobic bacteria (10^7 CFU/g), fungi (10^5 CFU/g); for herbal medicinal products to which boiling water is not added before use are: total aerobic bacteria (10^5

CFU/g), fungi (10^4 CFU/g); Enterobacteria and other Gram-negative organisms (10^3 CFU/g); *E. coli* and *Salmonella* sp. should be absent. In general, the tests used to verify the presence of microorganisms in plant drugs and microbial limits show no significant variation and follow the recommendations used for non-sterile pharmaceutical products (Table 1).

	United States Pharmacopoeia ^a	European Pharmacopoeia ^b	WHO ^c	Brazilian Pharmacopoeia ^d
Aerobic bacteria	$10^5 / 10^4 / 10^2$	$10^7 / 10^5$	* / $10^7 / 10^5$	$10^7 / 10^5 / 10^4$
Mold and yeast	$10^3 / 10^2 / 10$	$10^5 / 10^4$	$10^5 / 10^4 / 10^3$	$10^4 / 10^3 / 10^2$
Enterobacteria and other Gram negative bacteria	$10^3 / * / *$	* / 10^3	* / $10^4 / 10^3$	$10^4 / 10^3 / 10^2$
<i>E. coli</i>	absent	$10^3 /$ absent	$10^4 / 10^2 / 10$	absent
<i>Salmonella</i>	absent	* / absent	* / absent / absent	absent

^a United States Pharmacopoeia: The first value represents dried or powdered botanicals and botanicals to be treated with boiling water before use; The second value represents tinctures, powdered botanicals extracts, fluid extracts and nutritional supplements with botanicals; The third value represents infusions/decoctions. ^b European Pharmacopoeia: Herbal medicinal products consisting solely of one or more herbal drugs (whole, reduced or powdered); the first value represents herbal medicinal products to which boiling water is added before use; The second value represents herbal medicinal products to which boiling water is not added before use. ^c WHO: The first value represents contamination of "crude" plant material intended for further processing; The second value represents for plant materials that have been pretreated (e.g. with boiling water as used for herbal teas and infusions) or that are used as topical dosage forms; The third value represents For other plant materials for internal use. ^d Brazilian Pharmacopoeia: The first value represents herbal drugs to which boiling water is added before use; The second value represents herbal drugs to which the extractive process made in cold temperature; The third value represents final products for oral use. * Limits are not specified.

Table 1. Recommended microbial limits for herbal drugs (values in CFU/g).

6. Conclusion

Microbial contamination can lead to impaired performance of the product due to disruption of the stability of the formulation, modification of physical characteristics and appearance and lead to inactivation of the active ingredients and excipients in the formulation and also cause loss of confidence in the company. Herbalists should be trained to apply Good Manufacturing Practices, good harvesting practices and the safe handling and storage of herbal medicinal products. Further studies are recommended for herbal products to establish other contaminants and ways in which the contaminants can be reduced to recommended levels. The microbial loads should be established and the contaminants isolated and identified. In addition, alternative methods such as treatment with ethylene oxide or radiation with ionic rays lead to decontamination effects. These methods can be seen as a compromise between ensuring the microbiological safety of the product and avoiding consumer's risk and special

legal permissions are required in many countries. It is evident that more detailed studies of plant species popularly used are needed in order to ensure the quality, an important concept for providing the wished security and reliability for its use.

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Application of ISO 9001 Industrial Standard to Herbal Drug Regulation

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Additional information is available at the end of the chapter

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1. Introduction

We noted earlier [1] that 1978 was the turning point in current public perception of traditional medicine (TM) following the famous WHO declaration at Alma-Ata. That declaration ushered in a positive attitude that paved the way for the present global popularity of TM, especially herbal medicine. We noted earlier also [2,3] that whereas herbal remedies are called dietary supplements in the US, thereby shifting emphasis away from their medicinal attributes, the Dietary Supplement Health Education Act of 1994 [4], which occasioned the shift, actually helped to promote herbal medicine in the US, albeit indirectly, through the innovative provision it made for user information [5,6]. A similar situation obtained in Europe, where the net effect of the laws and rules passed in 2004 on herbal remedies had been to promote their production and use [7, 8]. In terms of trade and economics of herbal drugs, the following fact is notable: Although, Asia contributed only US\$ 7.3 billion to herbal world trade in 1999 [9], by 2005, a mere 6 years, China's contribution alone rose to US\$ 14 billion [10]. This stupendous growth was due to policies and programmes that favoured herbal medicine – the cornerstone of Traditional Chinese Medicine (TCM). Similar situations as in China held sway in Japan, South Korea and the Indian sub-continent, where government policies also favoured herbal medicine. However, in many developing countries like Nigeria, a totally different picture obtained, not because policies were expressly against herbal medicine, but in these countries there had been a lingering absence of proper policies and laws supportive of traditional remedies. Another key fact on the political economy of herbal drugs is that: Although, about 80% of people in developing countries depended on herbs, these countries contributed only 7.2% to herbal drug trade in 1999. By contrast, the developed nations, where people relied less on herbs, contributed 55.2%. Asia, less Japan and

South Korea, contributed 37.6%. Equally interesting is the comparison of Brazil with Nigeria. Both are rich in medicinal plants and have high populations that depend substantially on herbs. But, while herbs contributed an unknown amount to the Nigerian economy in 2007, in Brazil it contributed US\$ 160 million. By contrast, Nigeria's entire federal budget for health in 2007 was a mere US\$ 800 million [2]. These findings earlier led us [11,12] to conclude that developing countries need strategies that will enhance the regulation of herbal drugs and promote their trade. The present article is an attempt to enunciate one of such strategies. It is particularly of note that the superior performance of Brazil in comparison with Nigeria indicates that with proper policies and strategies, herbs can indeed contribute substantially to any economy.

2. Methodology: Determinative Review of ISO 9001 and the Mandates of Nigeria's and Europe's DRAs

2.1. ISO 9001:2008 industrial standard – A synopsis

ISO 9001:2008 industrial standard or quality management system (QMS) is a document of about 30 pages with 8 clauses, published by and obtainable from the International Organization for Standardization (ISO), Basle, Switzerland, or from any of its national affiliates. The standard is designed to be met by any organization that i) needs to demonstrate its ability to consistently provide product or service that meets both customer and applicable statutory and regulatory requirements (collectively legal requirements); ii) aims to enhance customer satisfaction by effectively and continually improving its QMS; and iii) plans to provide continual assurance of conformity to customer and applicable legal requirements. These aims/ approaches (often called "QMS requirements" or "quality procedures") are generic and intended to be applicable to all organizations regardless of type, size and product provided. Wherever any requirement cannot be applied due to the nature of an organization and its product, such can be considered for exclusion. But wherever exclusions are made, claims of conformity to the standard are not acceptable unless such exclusions are limited to requirements within clause 7 of the standard, and such exclusions do not affect the organization's ability, or responsibility, to provide product that meets customer and applicable legal requirements. ISO 9001:2008 defines the minimum requirements for a well managed organization. In other words, noncompliance to an ISO 9001:2008 requirement puts at risk an organization's ability to consistently and efficiently satisfy the expectations of its customers/ stakeholders.

2.2. The six QMS requirements or "The Six Quality Procedures"

These procedures or requirements, as one may choose to call them, actually refer to sub-clause 4.1 (General requirements) under clause 4 (Quality Management System) of ISO 9001:2008. The sub-clause prescribes that organizations shall establish, document, implement, and maintain a QMS, and continually improve its effectiveness. To do so means that the organization shall operate its QMS with a view to carrying out (or meeting) the following six procedures (or requirements): determine the processes needed for the QMS, and their

application throughout the organization; determine the sequence of the processes and their interactions; determine the criteria and methods for operating and controlling the processes; determine and ensure the availability needed resources and supporting information; check, measure and analyze the processes, where applicable; and implement actions to achieve planned results and continual improvement of the processes.

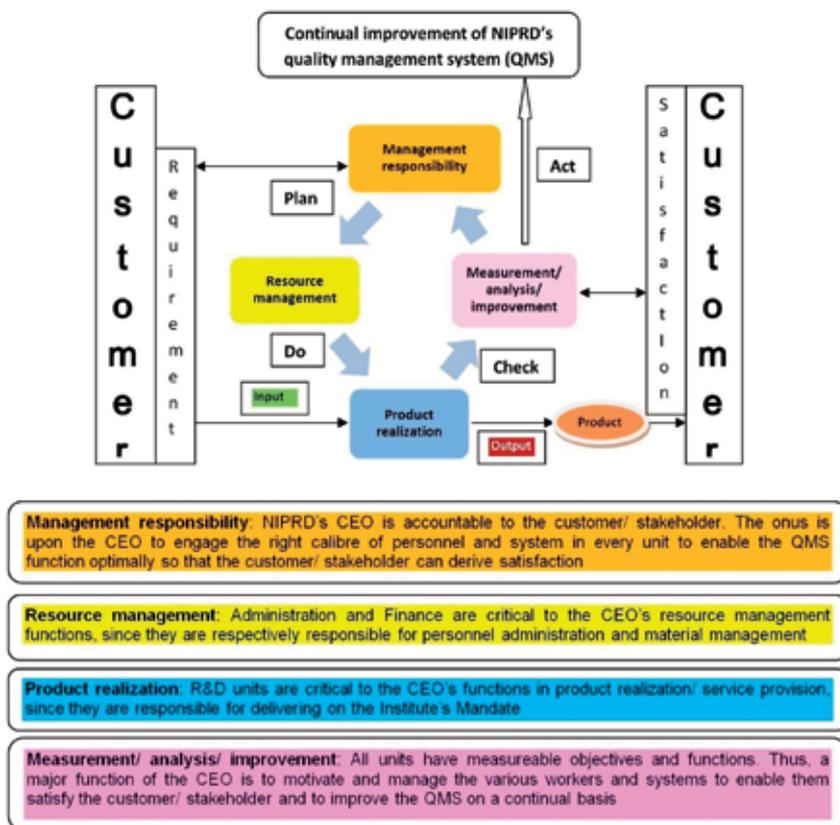


Figure 1. NIPRD's core business in the context of Plan-Do-Check-Act process-based QMS.¹

The organization shall manage the processes above in accordance with ISO 9001:2008 requirements. It shall also define the type and extent of control to be applied to any outsourced process that can affect product conformity to requirements. ISO 9001:2008 specifically notes as follows:

¹ Management responsibility corresponds to clause 5 of ISO 9001; while Resource management, Product realization and Measurement/ analysis/ improvement correspond to clauses 6, 7 and 8 respectively.

1. Processes needed for the QMS include the processes for management activities (clause 5), provision of resources (clause 6), product realization (clause 7), and measurement, analysis, and improvement (clause 8).
2. An outsourced process is a process the organization needs for its QMS, and which the organization chooses to have performed by an external party.
3. Ensuring control over outsourced processes does not absolve the organization of the responsibility to conform to customer and legal requirements.

The type and extent of control applied to an outsourced process can be influenced by factors such as: the potential impact of the outsourced process on the organization's capability to provide product that conforms to requirements; the degree to which the control over the process is shared; and the capability of the organization in achieving the necessary control via the application of sub-clause 7.4 (Purchasing). Philosophically, ISO 9001:2008 is formulated on the basis of management by objectives (MBO) and draws upon eight quality management principles. Ideally therefore, quality assurance covers activities in research, development, production and documentation. It embraces the rule: "do it right the first time". It involves regulating the quality of raw materials, the state of production line and works-in-progress, the product and related management processes. One of the most widely used paradigms for quality assurance management (QAM) is the "Shewhart cycle", also called "PDCA approach", meaning, "Plan-Do-Check-Act" [13,14]. The foregoing is illustrated in Figure 1 using NIPRD QMS processes as an example.

2.3. The eight quality management principles that underlie ISO 9001:2008

Like other ISO standards (Example: ISO 9004 - *Managing for Sustained Success*), ISO 9001:2008 is based on 8 quality management principles that are aligned with the philosophy and objectives of most quality award programmes in the world's most industrialized nations. The 8 principles are associated with the following themes:

1. Customer focus.
2. Leadership.
3. Involvement of people.
4. Process approach to management.
5. System approach to management.
6. Continual improvement.
7. Factual approach to decision making.
8. Mutually beneficial supplier relationships.

2.4. Key terminologies of ISO 9001:2008

2.4.1. Process approach to management

A process is an activity or operation that receives inputs and converts them to outputs. Practically all activities or operations involved in generating a product or providing a service are processes. For an organization to function, it must define and manage several inter-linked processes. Most often, the output of one process becomes the input into the next process. The systematic identification and control of the various processes employed within an organization, and the interactions between such processes, is termed “process approach” to management. Thus process approach to management is a way of obtaining a desired result, by controlling activities and related resources as a process. Process approach is a key element of all ISO 9000 standards, including ISO 9001:2008.

2.4.2. System approach to management

System approach to management is based on the premise that the efficiency and effectiveness with which an organization achieves its quality objectives are contributed and enhanced by identifying, understanding and managing all the interrelated processes within the organization as a system

2.4.3. Quality policy

Quality policy is a formal statement from the management of an organization that is linked to the nature of its business and its plans to meet the needs of its customers/ stakeholders. The policy is designed to be understood and followed at all levels and by all staff.

2.4.4. Quality objective

Quality objective is the factual or tangible basis upon which quality policy and plans for implementing the quality programmes of an organization are built. Quality objective should be SMART (ie: specific, measurable, achievable, realistic and time-bound). Each staff of the organization is expected to work towards measurable objectives.

2.4.5. Decision

Decision simply means the selection of one or more options from a multitude of options in tackling a given organizational task. As far as the QMS is concerned, an organization should make SMART decisions based on recorded data. An example of a SMART decision is: The QMS must be audited and evaluated regularly for conformance and effectiveness, so as to assure quality and continual improvement.

2.4.6. *Traceability*

Traceability is concerned with and refers to the fact that typically, recorded data are meant to show how and where raw materials and products were processed, in order to allow products and problems to be traced to their sources.

2.4.7. *Product realization*

Product realization refers to the scenario in which, when developing a new product, an organization plans the stages of development, with appropriate testing at each stage. The organization tests and documents whether the product meets design requirements, legal requirements, and user or customer needs.

2.4.8. *Quality plan*

Quality plan refers to a document specifying the QMS processes (including the product realization processes), and the resources to be applied to a specific product or project.

2.4.9. *Monitoring and measurement*

Monitoring and measurement refer to the scenario in which an organization must regularly review its performance through meetings and internal audits, and determine whether the QMS is working and what improvements can be made. The organization must have a documented procedure for internal audits and a procedure for dealing with past problems and potential problems. It must keep records of these activities and the resulting decisions, and monitor their effectiveness. It must have documented procedures for dealing with actual and potential non-conformances (problems involving suppliers, customers, or internal problems).

2.4.10. *Continual improvement*

Continual Improvement refers to the scenario in which an organization 1) makes sure no customer uses a bad product, 2) determines what to do with a bad product, 3) deals with the root cause of problems, and 4) keeps records to use as a tool to improve the QMS.

2.4.11. *Customer requirements*

Customer requirements refer to the attributes that the buyer of a product (or user of a service) wants. The core business of an organization is to determine customer requirements and to meet them, in accordance with sub-clause 5.2 (Customer focus).

2.4.12. *Drug Regulatory Agencies (DRAs)*

Drug regulatory agencies (DRAs) are organizations set up by the State on behalf of the general public with a Mandate to regulate drugs and related products and services. The Mandate of some DRAs may include production and distribution of certain goods like vaccines and orphan drugs. Either the State or the general public can be regarded as customer, stakeholder or shareholder. DRAs like all other organizations must have a system for communi-

cating with customers or stakeholders about product information, inquiries, contracts, orders, feedback, and complaints. All DRAs are “service providers” but some produce and even distribute certain specific items, as mentioned above. Nigeria’s National Agency for Food and Drug Administration and Control (NAFDAC) is a national DRA, while the European Medicines Evaluation Agency is a regional DRA.

2.4.13. Mandate

Mandate is a piece of legislation or instruction from a constituted authority to another constituted authority or body to carry out a named task. DRAs are mandated by the State to regulate drugs and health related products.

2.5. The new industrial revolution and the aim of this chapter

It is well established that the high state of development in the chemical/ pharmaceutical industrial sector in the US, Japan, South Korea, Britain, Germany and other European countries owes much to the powerful synergy between regulatory legislations, industrial standards and a focused political will. It is also manifest that the rapid, all-round industrial revolution in China in the past decade or so owes much to China’s embrace of ISO standards, especially ISO 9001, as shown in Table 1 after a recent [15].

Country	Ranking	No. certificates	Pertinent remark
China	1	257,076	Relies mostly on ISO standard.
Italy	2	130,066	Relies mostly on ISO standard
Japan	3	68,484	Relies only partly on ISO standard
Spain	4	59,576	Relies substantially on ISO standard
Russia	5	53,152	Relies substantially on ISO standard
Germany	6	47,156	Relies only partly on ISO standard
UK	7	41,193	Relies only partly on ISO standard
India	8	37,493	Relies substantially on ISO standard
South Korea	9	28,935	Relies substantially on ISO standard
US	10	23,400	Relies only partly on ISO standard

*Source ISO Survey 2009 [15]. Most countries have their own national standards in addition to ISO standards. For example the UK is well known for its industrial standards pre-sufixed by BSI (British Standards Institution).

Table 1. The top 10 countries in ISO certification in 2009.

It must be stated that countries like Japan, Germany, Britain and US use their own national standards in addition to those of ISO. Based on the foregoing, we state that the specific aim of this article is: To examine the QMS requirements of ISO 9001:2008 and the requirements

for regulating herbal drugs in Nigeria (a developing economy) and Europe (a developed economy), with a view to devising a framework that will better regulate herbal drugs and facilitate their trade worldwide. Such a framework will greatly benefit developing countries like Nigeria that are yet to benefit optimally from their comparative advantage in the abundance of spices, herbs and medicinal plants. In addition, marketers and users of herbs in consumer nations like the US, Canada, Germany, UK and France, where consumption now runs in to billions of US dollars, will also profit greatly from an improved and regularized world trade in herbs.

3. Results & discussion: A Framework for Efficient Herbal Drug Regulation (HDR)

3.1. Justification for establishing national or regional DRAs

Most or all countries have a national or regional agency that regulates the production, distribution and use of drug products. The process of regulation commences with the registration of the producer, the product, the distributor and in some cases the user. In some countries drugs, foods and dietary supplements are regulated by the same body (eg: Nigeria's NAFDAC and US-FDA). The EU's EMEA however regulates only drug products. States or regions need to have DRAs in order to ensure order in the production, distribution and use of drugs. Without DRAs utter chaos and pandemonium will result in production (eg: manufacturers will do as they please without a uniform control), distribution (distributors and suppliers will do as they choose without a uniform order) and use (prescribers and users will do as they think without a uniform regime), which would allow incidences of counterfeit and expired drugs in drug distribution chain, drug abuse and emergence of drug resistant disorders, especially infective conditions like malaria and TB.

3.2. Comparative analysis of Nigeria's and EU's requirements for herbal drug regulation

A careful scrutiny of the requirements for registering and regulating herbal drugs in Europe and in Nigeria reveals their basic similarity, as shown in Table 2.

European Union (EU) – regulated by EMEA		Nigeria – regulated by NAFDAC	
Type of data	Details of data required	Regulatory aspect	Requirement
Product information:	These include: name, strength, dosage form, list of excipients, shelf life, posology, indications, contraindications, and special precautions. These are used as basis for inserts or advertisement, which must undergo a process called "readability".	Legal status of applicant - manufacturer or marketer	Applicant must be certified by the Corporate Affairs Commission as a business. A marketer must show evidence of Power of Attorney.

European Union (EU) – regulated by EMEA		Nigeria – regulated by NAFDAC	
Type of data	Details of data required	Regulatory aspect	Requirement
Quality control data:	These include: production must be in a GMP compliant, product must be produced with validated formula and method, there must be a product specification, stability studies must be carried out in the container proposed for marketing for purposes storage/ shelf-life, and dossiers must be provided for starting materials and finished product.	Analytical status of the product for registration.	The product must have: certificate of analysis, dossier containing data on ingredients, method of analysis, stability, dosage and safety precautions.
Refer to GMP requirements for production.			
Safety data requirements	The data may be assembled from: animal or human studies, review of potential drug-drug interactions, side effects and contraindications. Others include: recognized monographs, data special groups - children, the elderly and mothers.	Pre-registration inspection of premises. premises must be GXP compliant. Marketers	Manufacturing, storage and distribution must provide convincing evidence of GXP
Traditional use evidence	Evidence that the product has been in use as medicine for 30 years or more (the last 15 must be in the EU. Notably, there is no requirement to prove efficacy (De Smet, 2005).	Post marketing surveillance plan/ report	Applicant may be required to provide a plan for reporting on the use of the product and of any adverse reactions.
-	-	Others, such as fees and waivers.	Fees are required at several stages of the registration but waivers are not expressly stated, thereby negating the concept and need for transparency (see Table 3 for extra requirements).

*The Table was drawn based on data gathered from references including Goldman [5]; De Smet [7, 8]; Ann Godsell Regulatory [16]; and various NAFDAC leaflets, including Akunyili [17]. Note that the requirements for registration in Nigeria are not necessarily less tasking, but their lack of explicitness can be a booby trap and a leeway for non-transparency. The necessity for explicitness and transparency is very important because some years ago the Director General of China’s drug regulatory agency was sentenced to death for alleged corrupt practice [18]. In 2000 the entire Management of NAFDAC was sacked in similar grey circumstances.

Table 2. Requirements for herbal registration compared between EU and Nigeria.

But, while the EMEA approach is technically more explicit, though not necessarily more exerting than NAFDAC’s, the latter is administratively much more cumbersome, and therefore more liable to inefficiency and abuse. Table 3 shows the extra bureaucratic demands of NAFDAC. We stated earlier that, although, 80% of people in developing countries like Nigeria depended on herbs, these countries contributed only 7.2% to herbal drug trade in 1999. By contrast, the developed nations, where people relied less on herbs, contributed 55.2%. This scenario is explained by the fact herbal drugs are better regulated in developed regions like the China, India, Japan and South East Asia, the EU and North America [2].

3.3. Justification for selecting ISO 9001:2008 for this study

Although most of the OECD countries and other highly industrialized economies, where herbal drugs are well regulated, have their own national standards, all do embrace ISO standards, especially ISO 9001:2008. For example, the British Standards Institution (BSI) is well known and widely adopted in many other countries worldwide, and although international in application, such national standards do not bear the tag “international”. By contrast, the ISO family of standards bear the tag “international”. ISO 9001:2008 is an international standard designed to address systemic change (ie: a change that affects an organization as a whole). The global popularity of ISO 9001:2000 - the predecessor of ISO 9001:2008, is attributable to the following factors: a) major purchasers require their suppliers to hold ISO 9001 certification [15, 19]; b) studies indicate significant financial benefits for organizations certified to ISO 9001 [19,20]; and c) similar superior operational performance of ISO certified firms has been severally confirmed [21-24]. As just noted, ISO 9001:2008 is an update of ISO 9001:2000, and we have selected it for this study by reason of its popularity and versatility, and because it is a process-based QMS that addresses systemic change affecting whole organizations like a national or regional drug DRA, like Nigeria’s National Agency for Food and drug Administration and Control (NAFDAC), the US Food and Drug Administration (US-FDA) and the European Medicines Evaluation Agency (EMA).

S/No	Extra requirement	Remark
1	Five (5) copies of the product dossier.	Probably unreasonable
2	Three (3) packs of the products samples.	Probably reasonable
3	Notarized original copy of the duly executed Power of Attorney from the product manufacturer.	Clearly unreasonable for all categories of applicants
4	Certificate of Manufacture issued by the competent health or regulatory authority in country of origin and authenticated by the Nigerian Mission in that country. Where there is no Nigerian mission, The British High Commission or an ECOWAS country Mission will authenticate.	Probably unreasonable for all categories of applicants
5	If contract-manufactured, Contract Manufacturing Agreement, properly executed and notarized by a Notary Public in the country of manufacture.	Clearly unreasonable for all categories of applicants
6	Current World Health Organization Good Manufacturing Practice Certificate for the manufacturer, authenticated by the Nigerian Mission.	Clearly unreasonable for all categories of applicants
7	Certificate of Pharmaceutical Products (COOP) duly issued and authenticated.	Clearly unreasonable for all categories of applicants
8	Current Superintendent Pharmacists license to practice issued by the Pharmacists Council of Nigeria (PCN).	Only probably reasonable

S/No	Extra requirement	Remark
9	Premises Registration License from PCN	Only probably reasonable
10	Certificate of Registration of brand name with trademark registry in the Ministry of Commerce here in Nigeria; Letter of invitation from manufacturer to inspect factory abroad, stating full name and location of plant.	Probably unreasonable for all categories of applicants
11	The applicable fee payable only if documents are confirmed to be satisfactory	Likely to be abused if the amount is high. The fee should be a token amount paid by all applicants
12	Nutraceuticals, medical devices and other regulated drug products have similar requirements, with minor variations. Specific details can be obtained from NAFDAC.	A sketch of the minor variations should be provided in print no matter how brief. Any information provided by NAFDAC should be printable for sake of transparency

*The information on NAFDAC were drawn from leaflets and NAFDAC's website (2010): www.nafdacnigeria.org/ The remarks are informed by current affairs and public perception of NAFDAC's role and activities including the wholesale reorganization of its Management in 2000.

Table 3. NAFDAC's extra requirements for registering herbal medicines.

3.4. A systematic review of the eight clauses of ISO 9001:2008 in relation to DRAs

3.4.1. A synopsis of the Mandate of DRAs and the eight clauses of ISO 9001:2008

ISO 9001:2008 is the most widely used QMS standard, with over a million certificates issued worldwide. Alas, it was revealed at the SON-NIPRD course in 2011, that only two public institutions in Nigeria have ISO 9001 certification! Yet, as stated earlier, ISO 9001:2008 defines the minimum requirements for a well managed organization. The standard is published by the International Organization for Standardization (ISO), Basle, Switzerland. National accreditation bodies like the Standards Organization of Nigeria (SON) provide accreditation to registrars who issue the ISO 9001 certificates to those they audit. ISO 9001:2008 is set out in eight clauses designated clauses 1 to 8. The structure and salient points/ directing principles of the clauses are tabulated below. A copy of ISO 9001:2008 is a prerequisite for this study. Similarly required, is a grasp of the requirements for registering and regulation herbal drugs in a developed economy like Europe; and in a developing country like Nigeria, as depicted in Table 2. It is well known that herbal drugs are better regulated in the developed than in developing countries. Table 3 suggests that undue bureaucracy or needlessly cumbersome requirements can hinder efficient regulation. Tables 4-13 show the structure and salient points/ directing principles of the 8 clauses.

Clause	Title and subtitles, with remarks	Salient points/ directing principles/ application to DRAs
1	<p>1. Scope</p> <p>1.1 General</p> <p>1.2 Application</p>	<p>ISO 9001:2008 can be used to establish, and to update a DRA's QMS. A DRA, like other parastatals or private organizations must consider its unique operational environment and the dynamics and risks associated therewith.</p>
2	<p>2. Normative references</p> <p>(eg: ISO 9000:2005 is devoted to <i>QMS Fundamentals and Vocabulary</i>; and ISO 9004:2009 is devoted to <i>Managing for Sustained Success</i>)</p>	<p>A normative reference implies, unless otherwise stated, that the most recent versions of the separate documents should be referenced. DRAs would benefit immensely from such key references and compendia such as the <i>International Pharmacopoeia</i> and others like the <i>BP</i> and <i>USP</i>, and the WHO manual on <i>Quality Control Methods for Medicinal Plant Materials</i>.</p>
3	<p>3. Terms and definitions</p> <p>(see section 2.4 of this article on "Key terminologies of ISO 9001:2008)</p>	<p>The term "product" may also mean "service". "Legal requirements" means "statutory and regulatory requirements". Most DRAs are service providers only, while others may produce and distribute certain specialized health products.</p>

*The Table is to be studied side by side with the contents of ISO 9001:2008 and Table 2, which is on regulatory requirements of DRAs.

Table 4. Clauses 1-3 of ISO 9001:2008 in relation to DRAs.

Clause	Title and subtitles, with remarks	Salient points/ directing principles/ application to DRA
4	<p>4. Quality Management System (QMS)</p> <p>4.1 General requirements</p> <p>4.2 Documentation requirements:</p> <p>4.2.1 General – QMS documents must include: quality policy, quality objective, quality manual, documented procedures/ records specified by ISO 9001:2008, and documents/ records determined by the organization to be relevant for effective planning, operation and control of the QMS.</p> <p>4.2.2 Quality manual – this should include the scope of the QMS, SOPs and a description of the QMS processes.</p> <p>4.2.3 Control of documents - the documents required by the QMS must be established and controlled. This means that SOPs are to be established to define the controls needed.</p> <p>4.2.4 Control of records – records are a special type of documents and must be established and controlled. Here too, SOPs are to be established to define the controls needed.</p> <p>Note:1) A document is a piece of written, printed, or electronic matter that provides information or evidence. It may or may exist</p>	<p>Clause 4.1 implies that the DRA must identify, manage and document the processes that make up its QMS – ie: the DRA must address the so called "Six Quality Procedures" and generate relevant documents, including: 1) quality manual, 2) quality policy, 3) quality objective, 4) process flowchart, and 5) work instructions. The DRA can achieve this by using a management strategy called "process approach", which means that it must manage: 1) the processes that make up its organization, 2) the interaction between these processes, and 3) the inputs and outputs that glue these processes together. The quality manual should: 1) describe how the QMS processes interact; 2) define the scope of the QMS (it should explain any reductions in the scope of the QMS and justify all exclusions/ reductions); and 3) document all procedures in the QMS or refer to them. It is most crucial that the DRA prepares, establishes and maintains a quality manual.</p> <p>The DRA must establish SOPs to define the controls needed: 1) to approve, review, update and re-approve documents prior to use; 2) to ensure that changes, current status, relevant versions of documents are identified; and 3) to prevent the unintended use of obsolete documents. The DRA must establish records to provide: 1) evidence that operations conform to QMS requirements; and 2) evidence that operations of the QMS are effective. Records must be ensured to be legible, readily identifiable and retrievable.</p>

Clause	Title and subtitles, with remarks	Salient points/ directing principles/ application to DRA
	in a permanent form. 2) A record is a permanent document of something that is kept for evidence or information. It specifically bears the history of events or arrangements, and is preserved in a lasting form.	

*The Table is to be studied side by side with the contents of ISO 9001:2008 and Table 2, which is on regulatory requirements of DRAs.

Table 5. Clause 4 of ISO 9001:2008 in relation to DRAs.

Clause	Title and subtitles, with remarks	Salient points/ directing principles/ application to DRA
5	<p>5 Management Responsibility</p> <p>5.1 Management commitment</p> <p>5.2 Customer focus – the organization must ensure that its purpose/ focus (inclusive of customer/ stakeholder requirements) is understood and determined.</p> <p>5.3 Quality policy – this should be: 1) appropriate to the purpose of the organization; 2) focused on meeting requirements and continual improvement; 3) used as a framework for quality objectives; 4) publicized and understood at appropriate levels; and 5) reviewed for continuing suitability .</p> <p>5.4 Planning</p> <p>5.4.1 Quality Objectives</p> <p>5.4.2 QMS Planning</p> <p>5.5 Responsibility, Authority, and Communication</p> <p>5.5.1 Responsibility and Authority</p> <p>5.5.2 Management Representative</p> <p>5.5.3 Internal Communication – it is crucial that the organization ensures that appropriate communication processes regarding the effectiveness of the QMS are established and implemented.</p> <p>5.6 Management Review</p> <p>5.6.1 General</p> <p>5.6.2 Review Input – includes audit results, public feedback, process performance, status of preventive/ corrective action, follow-up from previous management review,</p>	<p>The DRA must be committed to developing and implementing a QMS, as well as, a commitment to continually improve the effectiveness of the QMS. The DRA can do this by 1) communicating the importance of meeting “legal and customer requirements”; 2) establishing a quality policy and quality objectives; 3) conducting management reviews; and 4) by ensuring the availability of necessary resources. The “legal and customer requirements” of a DRA are implicit in its Mandate – which may be an act, law or decree. In planning, the DRA must 1) ensure that quality objectives are established at the relevant functions and levels within the Agency; 2) ensure that quality objectives are measurable and consistent with the quality policy; and 3) ensure that planning for the QMS meets the general requirements (clause 4.1) and quality objectives (clause 5.4.1), as well as, maintains the integrity of the QMS.</p> <p>In as much as operations must be carried out the DRA must ensure that the responsibilities and authorities for such are defined and communicated appropriately. It is essential that a member of top management, irrespective of other duties, be appointed (as Quality Manager) and given the responsibility to: 1) ensure that the needed processes are established, implemented, and maintained; 2) report to top management on the performance of the QMS; 3) report to top management on any need for improvement; and 4) ensure the promotion of awareness of Agency’s Mandate. Most DRAs have a public relation office.</p> <p>For a DRA to be effective it must review its QMS at planned intervals to: 1) ensure an effective QMS; 2) assess possible opportunities for improvement; 3) evaluate the need for any changes; and 4) consider the need for changes to the policy and objectives. The DRA must of course maintain records of reviews as per clause 4.2.4. For a DRA, the inputs for review must include information on: 1) results of audits; 2) feedback from government and the public, eg - incidences of counterfeit drugs; 3) status of preventive and corrective actions, eg – incidences of drug abuse; 4) follow-up actions from earlier reviews; 5) changes that can affect the QMS; and 6) recommendations for improvement.</p>

Clause	Title and subtitles, with remarks	Salient points/ directing principles/ application to DRA
	changes that can affect the QMS, and recommendations for improvement.	
	5.6.3 Review Output - includes decisions/ actions related to: 1) improvement of the QMS; 2) improvement in meeting Mandate;	
	and 3) resource needs	

*The Table is to be studied side by side with contents of ISO 9001:2008 and Table 2, which is on regulatory requirements of DRAs.

Table 6. Clause 5 of ISO 9001:2008 in relation to DRAs.

3.4.2. *The immediate historical antecedent of NAFDAC and the continuing relevance of ISO 9001*

Nigeria's NAFDAC was created by decree in 1992/93 following the ethylene glycol disaster of 1991/92 in Langtang General Hospital, Plateau State, where ethylene glycol was used in the place of propylene glycol in preparing paracetamol elixir. The glycol had been purchased from a hitherto popular pharmacy shop located at Masalachin-Jumai Street, Jos. Prior to 1992/93, a department in the Federal Ministry of Health handled food and drug administration in Nigeria. Alas, in 2009 another ethylene glycol disaster occurred in Lagos. In this latter disaster a hitherto popular brand of paediatric mixture ("My Pikin") was found to contain ethylene glycol that had been purchased from an unregulated source. It is important to note that whereas the glycol implicated in the Lantang disaster was purchased from pharmaceutically regulated source, the glycol in the case of the Lagos disaster was purchased from a company that dealt in industrial chemicals associated with automobiles and cooling systems. The occurrence of this kind of disaster within less than two decades is matter of concern that calls for a more efficient programme for regulating drugs and industrial chemicals in developing countries. The US-FDA after whose image and likeness NAFDAC was created is known for efficiency mostly because it is supported by proper laws and strong institutions. Better laws and stronger institutions, including DRAs, are required to avoid or minimize this kind of disaster as seen in Nigeria. It seems instructive to mention the "Tylenol case" in the US, and how that case led to a new legislation. Between late September and early October 1982, seven persons in Chicago died after taking capsules of Tylenol (a brand of paracetamol), to which cyanide crystals had been added. The crystals had apparently been introduced into the capsules by someone who had removed bottles of Tylenol from several drugstores and then replaced them on the shelves. It took an intensive investigation by a team of over 100 agents, including FDA staff, to discover the mischief, which led to a 1982-legislation that required all over-the-counter drugs and medicines sold in the county (and later elsewhere in the US and beyond) carry manufacturers' seals which broken would be obvious. The rapid conclusion of the investigation led by the Illinois Attorney General himself (Tyron C. Fahner) and the dispatch with which the new law was issued collectively testify to the inner workings of strong institutions – which developing nations lack.

Clause	Title and subtitles, with remark	Salient points/ directing principles/ application to DRAs
6	<p>6 Resource management</p> <p>6.1 Provision of resources</p> <p>6.2 Human resources</p> <p>6.2.1 General</p> <p>6.2.2 Competence, Training, and Awareness</p> <p>6.3 Infrastructure</p> <p>Like any other public outfit the DRA must determine, provide, and maintain infrastructure like buildings, workspace and associated utilities, and essential support services.</p> <p>6.4 Work Environment</p> <p>"Work environment" implies conditions under which work impacts the DRA's Mandate. is performed, and includes physical aspects like weather and noise pollution.</p>	<p>The DRA must determine and provide the resources needed: 1) to implement, maintain and continually improve the effectiveness of its QMS; and 2) to enhance the fulfilment of its Mandate.</p> <p>By virtue of its role as a highly specialized agency, the DRA must ensure that all staff irrespective of department whose work can impact DRA's Mandate are competent based on appropriate education, skills, experience and abide by their professional ethics.</p> <p>This implies that the DRA must: 1) determine the competency of staff; 2) provide training as needed; 3) evaluate the effectiveness of the actions taken on training and skills acquisition ; 4) inform staff of their relevance within the QMS; 5) ensure staff know their contributions to achieving quality objectives; and 6) maintain staff records of education, training, skill, and experience in accordance with clause 4.2.4.</p> <p>The DRA must, of course, provide appropriate work environment for all staff whose work</p>

*The Table is to be studied side by side with contents of ISO 9001:2008 and Table 2, which is on regulatory requirements of DRAs.

Table 7. Clause 6 of ISO 9001:2008 in relation to DRAs.

3.4.3. *The making of stronger DRAs and the need for clearer demarcation of responsibilities*

In most countries where DRAs are not a department of the Ministry of Health, they exist as a parastatal or as a special department within the Ministry (as in Japan), with conditions of service being slightly more favourable than in the rest of the Ministry. The idea is to give special incentives to the staff on account of hazards perceived to be peculiar to the job. In Nigeria, NAFDAC is well housed both at the federal and state levels and the staff earn about the same remuneration as the universities and research institutes. In most countries the DRAs have well equipped offices and laboratories, and those DRAs that produce and distribute goods are equipped with the necessary plant and storage facilities.

3.4.4. *The inevitability of confusion in the absence of regulatory standardization*

In Nigeria, NAFDAC previously handled certain aspects of manufacture/ distribution of vaccines until certain developments (or rather controversies over quality/ effectiveness of polio vaccines during the late 1990s/ early 2000s) led, first to the creation of a National Programme on Immunization (NPI); and latter to the transfer of the same functions from NPI back to the Federal Ministry of Health. It is obvious from the foregoing that institutions like NAFDAC and NPI would have performed better had they been certified. It seems also that one of the keys to ending the cycle of poverty and underdevelopment in some countries is to ensure that elite institutions like the DRAs are certified to appropriate ISO standards. Certifications of agencies like the Health Insurance Scheme and the Pension Commission will definitely reduce perceive current levels of corruption in such institution.

Clause	Title and subtitles, with remark	Salient points/ directing principles / application to DRAs
7	<p>7 Product realization</p> <p>7.1 Planning of product realization Product realization typically implies that manufacturers 1) plan and develop the QMS processes needed for product realization; 2) keep the planning consistent with other requirements of the QMS; 3) document the plan in a suitable form; and 4) determine through the planning, the following: a) quality objectives and product requirements; b) need for processes, documents, and resources; c) verification (establishment of truth/ confirmatory evidence), validation (formal registration/ obtainment of official sanction), monitoring, measurement, inspection, and test activities; d) criteria for product acceptance; and e) records providing evidence that the processes and resulting product meet requirements. Since DRAs regulate manufacturers they too must be acquainted with clause 7. NOTE 1: Recall that "quality plan" (2.4.8 of this article) is a document specifying the processes, and the resources to be applied to a specific product, project, or contract.</p> <p>NOTE 2: An organization can apply the requirements of sub-clause 7.3 (vide infra) to the development of product realization processes.</p> <p>7.2 Customer-Related Processes</p> <p>7.2.1 Determination of Requirements Related to the Product</p> <p>7.2.2 Review of Requirements Related to the Product</p> <p>7.2.3 Customer Communication</p> <p>The intensity and scope of communication depends on the product and the associated mandate. Thus the DRA must determine and implement the necessary arrangements for communicating with stakeholders on aspects like 1) product information; 2) inquiries and contracts; 3) customer/ stakeholder feedbacks -positive or negative</p>	<p>Some DRAs produce/ store/ distribute specialized and non-profit products like vaccines and orphan drugs. For such, all aspects of clause 7 apply. The DRAs of developed economies concentrate on regulating manufacturers, distributors and use of products. Different processes are involved in drug regulation but these often have some aspects in common.</p> <p>For example, the process of registering a manufacturer and that of registering a product are essentially the same, but they differ in their aims, point of action, who by, and so on. Some of the processes involved in "planning of service realization", which is the core business of a typical DRA, require a wide range of differing concepts, technicalities, approaches, specializations, and so on. For example, although the technical aspects of producing tablets of aspirin, diazepam, B-complex, erythromycin, and orphan drugs may be similar, the modes of their regulation and distribution are different. Given the involved Mandate of DRAs, different strategies must be developed to grapple with the differing nuances and intricacies associated with the regulation of the five products. Typically, questions that have answer buried in culture/ society rather than the lab do arise in drug regulation. Why, for example, despite the similarities between NAFDAC and EMEA, it is impossible to buy erythromycin or diazepam over-the-counter in Europe but not in Nigeria? To what extent do political, social and economic factors affect "planning of service realization" in different social environments? It well known that regulatory strategies that work in Europe often fail to work outside despite obvious legislative similarities between nations.</p> <p>The customers/ stakeholders of a typical DRA are the general public, manufacturers, suppliers and the government. DRAs that engage in production and distribution must determine customer requirements, which invariably include specified and unspecified but desirable attributes. Such DRAs must also determine the legal requirements applicable to the product. Other desirable requirements, including post-delivery activities like maintenance services, may be considered. DRAs that produce or distribute would normally review the product requirements before committing to supply in order to: 1) ensure that product requirements are defined; 2) resolve any requirements differing from those previously expressed; and 3) ensure its ability to meet the requirements. In the same vein when a DRA plans a regulatory strategy or legislation the plan should be graduated and made reasonable to its purpose and scope and with reference to the operating socioeconomic environment. The DRA must maintain the results of reviews, and any subsequent follow-up actions in accordance with 4.2.4. When the requirements are not documented, they must be confirmed before acceptance. But if product requirements are changed, the DRA must ensure relevant documents are amended and relevant personnel are made aware of the changed requirements.</p> <p>NOTE: In some situations a formal review is impractical for each order. In such cases reviews can cover relevant product information such as catalogues or adverts.</p>

*The Table is to be studied side by side with contents of ISO 9001:2008 and Table 2, which is on the regulatory requirements of DRAs.

Table 8. Clause 7 of ISO 9001 in relation to DRAs (Product planning and Customer-Related processes).

Clause	Title and subtitles, with remarks	Salient points/ directing principles/ application to DRAs
7	<p>7.3 Design and Development</p> <p>7.3.1 Design/ Development Planning</p> <p>DRAs that produce or distribute must plan and control product design/ development. They must determine 1) the stages of design/development; 2) appropriate testing, review and validation for each stage; and 3) responsibility/authority for design/ development.</p> <p>7.3.2 Design /Development Inputs</p> <p>In designing/ developing a physical good or a service, the DRA must determine the needed inputs and keep records as per 4.2.4. The inputs must include: 1) functional and performance requirements; 2) applicable legal requirements; 3) applicable information derived from similar designs; and 4) requirements essential for design and development.</p> <p>7.3.3 Design/ Development Outputs</p> <p>Where applicable, DRAs must document the outputs of the design/ development process in a form suitable for verification against the inputs to the process. The outputs must 1) meet or match design and development input requirements; 2) provide information for purchasing, production and service; 3) contain or reference product acceptance criteria; 4) define essential characteristics for safe and proper use; 5) be approved before their release</p> <p>7.3.4 Design/ Development Review</p> <p>7.3.5 Design/ Dev. Verification</p> <p>7.3.6 Design/ Dev. Validation</p> <p>Validation activities are performed in accordance with 7.3.1 to confirm that the resulting product is capable of meeting the requirements for its specified application or intended use.</p> <p>7.3.7 Control of Des./ Dev Changes</p> <p>For either physical goods or policy, DRAs must 1) identify design and development changes and maintain records as per 4.2.4; 2) review, verify, validate and approve changes before implementation; 3) evaluate the changes in terms of their effect on constituent parts (raw material) and products (or policies) already delivered (or implemented).</p>	<p>The same principle followed in planning a physical product is followed in planning a service. The interfaces between the different groups involved must be managed to ensure effective communication/ clear assignment of responsibility. Design and development review, verification and validation have distinct purposes. They can be conducted and recorded separately or in any combination, as the DRA deems suitable for the product or the type of service.</p> <p>A DRA would review the selected inputs for adequacy and resolve any incomplete, ambiguous, or conflicting requirements. Examples of application inputs include: (1) applicable information derived from similar designs; and (2) requirements essential for design and development. If a DRA is designing a policy to curb drug abuse in a particular locality, useful inputs for the design would include statistics like 1) the age, gender and occupation of abusers; 2) the type of drugs abused; and 3) the success rate of similar policies elsewhere. NOTE: Information for production and service can include details for product preservation.</p> <p>A DRA must perform systematic reviews of design and development at suitable stages in accordance with planned arrangements (7.3.1) so as to: 1) evaluate the ability of the results to meet requirements; and 2) identify problems and propose necessary actions. Reviews must include representatives of the functions concerned. Results of reviews and subsequent follow-up actions must be maintained as per 4.2.4. A DRA would perform design and development verification in accordance with 7.3.1 to ensure that output meets the design and development input requirements; and maintain the results of such verification and subsequent follow-up actions. When practical and desirable, validation must be completed before delivery or implementation of the product. Results of the validation and of subsequent follow-up actions must be maintained as per 4.2.4. Just as some DRAs produce or distribute physical products, some DRA have their own testing facilities while others contract out such tests. Thus the purchase needs of DRAs differ with their Mandate. However, whenever purchasing is indicated the DRA must 1) ensure that purchased items conform to specified purchase requirements (Note: The type and extent of control applied to the supplier and purchased product depends upon the effect of the product on the subsequent realization processes or the final product); 2) evaluate and select suppliers based on their ability to supply goods in accordance with requirements; 3) establish the criteria for selection, evaluation, and re-evaluation; and 4) maintain the results of such evaluations and subsequent follow-up actions in accordance with sub-clauses 4.2.3 and 4.2.4.</p>

*The Table is to be studied side by side with contents of ISO 9001:2008 and Table 2, which is on the regulatory requirements of DRAs.

Table 9. Clause 7 of ISO 9001:2008 in relation to DRAs (Design and Development).

3.4.5. Some causes and signs of a malfunctioning DRA

Once the staff recruitment system can be skewed to favour persons, a serious non-compliance exists. Once the purchase processes can be demonstrated to have vested interest, a serious flaw exists in the QMS. Once there is a convincing evidence of maladministration, arbitrary treatment of personnel or executive high handed, a serious condition against performance exists.

Clause	Title and subtitles, with remarks	Salient points/ directing principles/ application to DRAs
7	<p>7.4 Purchasing</p> <p>7.4.1 Purchasing Process</p> <p>7.4.2 Purchasing Information</p> <p>All organizations irrespective of type of business will have cause to purchase a multitude of goods for the business. Such goods are to meet their purposes criteria and processes must be developed their purchase. Thus purchasing information should contain: 1) explicit description of goods; 2) approval criteria for the goods, procedures, processes, and associated equipment or accessories; and 3) profession/ qualification of staff associated with the goods.</p> <p>7.4.3 Verification of Purchased Product</p> <p>7.5 Production and Service Provision</p> <p>7.5.1 Control of Production and Service Provision</p> <p>A producing/ distributing DRA must plan and carry out production and service provision under controlled conditions, which include: 1) availability of data on needed inputs; 2) availability of necessary work instructions; 3) availability/ usability of essential equipment; 4) availability/ usability of monitoring and measuring equipment; 4) ability to implement monitoring and measurement activities; and 5) ability to implement product release, delivery, and post-delivery activities. DRAs that do not produce/ distribute must nevertheless have possess the ability to ensure that manufacturer/ distributors have all it takes to adequately meet QMS requirements.</p> <p>7.5.2 Validation of Processes for Production and Service Provision</p> <p>7.5.3 Identification and Traceability</p> <p>7.5.4 Customer Property</p>	<p>Whether a DRA produces/ distributes it will have cause to purchase various items of commerce hence QMS requirements for purchases are required. Like other organizations a DRA would require and ensure the adequacy of the specifications of items to be purchased before communicating the purchasing information to the supplier. Typically a DRA would establish and implement inspection or other necessary activities for ensuring that purchased goods meet the specified purchase requirements. If a DRA or its customers/ stakeholders propose to verify a good or service at the supplier's location, the intended verification arrangements/ method must be stated in the purchasing information.</p> <p>It is typically pertinent that a DRA 1) validates any production or service provision that subsequent monitoring cannot verify. Such validations include processes where deficiencies may become apparent only after product use or service delivery; 2) demonstrates through the validation the ability of processes to achieve the planned results; and 3) establishes validation arrangements including, as applicable: a) criteria for process review and approval, b) approval of equipment, c) qualification of staff, d) use of defined methods and procedures, e) requirements for records, and f) re-validation. DRAs that produce/ distribute or have their own test facilities must 1) identify, where appropriate, the product by suitable means during product realization; and 2) identify the product status with respect to monitoring and measurement requirements throughout product realization. DRAs may require that manufacturers of herbal products have the following where necessary and feasible: a) chemically defined reference active crude extract (RACE), b) chemically defined marker substance (DMS) and TLC, HPLC or GC-MS fingerprints of RACE and DMS. Since traceability is a key requirement, DRAs need to enforce manufacturers to have the means of controlling the unique identification of the product at various stages of development, and of course maintain records.</p> <p>Obviously, DRAs that produce/ distribute products or run test laboratories/ facilities must exercise care with any customer property under their control. They must record and promptly report any loss or damage to the customer. NOTE: Customer property may be physical or otherwise.</p> <p>As a standard practice, a DRA would: 1) assess and record the validity of prior results if the equipment/ method are found not to conform to requirements; 2) maintain records of the results of calibration and verification; and 3) confirm or re-confirm the ability of any software or programme used for monitoring or measurement before its initial use. To ensure the validity of results, a DRA would normally:</p> <p>1. Calibrate and/or verify the measuring equipment at specified intervals or prior to use.</p>

Clause	Title and subtitles, with remarks	Salient points/ directing principles/ application to DRAs
	7.5.5 Preservation of Product Preservation of product broadly includes: 1) identification, basis). 2) handling, 3) packaging, 4) storage, and 5) protection	2. Calibrate the equipment to national or international standards (or record other appropriate 3. Adjust or re-adjust as necessary. 4. Identify the measuring equipment in order to determine its calibration status
	7.6 Control of Measuring and Monitoring Equipment As may be applicable, a DRA would: 1) Determine the type of monitoring and measurements to be made, and the equipment/ method to be used in providing evidence of conformity 2) Use and control the monitoring and measuring devices in order to ensure that measurement capability is consistent with monitoring and measurement requirements.	5. Safeguard equipment from improper adjustments. 6. Protect equipment from damage and deterioration

*The Table is to be studied side by side with contents of ISO 9001:2008 and Table 2, which is on the regulatory requirements of DRAs.

Table 10. Clause 7 of ISO 9001:2008 in relation to DRAs (Purchasing/ Production/ Control of Equipment).

Clause	Title and subtitles, with remarks	Salient points/ directing principles/ application to DRAs
8	8 Measurement, analysis and Improvement 8.1 General A producing/ distributing DRA would plan and implement necessary, determine through planning the need for, use of, and extent of use of applicable the monitoring, measurement, analysis, and improvement methods, including statistical techniques. DRAs should view customer/ stakeholder perception processes it needs to: 1) demonstrate conformity to product requirements; 2) ensure conformity of the QMS to planned arrangements; and 3) continually improve the effectiveness of the QMS. Non-producing/ distributing DRAs must have the ability to ensure that manufacturers/ distributors comply. 8.2 Monitoring and measurement 8.2.1 Customer Satisfaction DRAs must routinely: 1) monitor information on customer/ stakeholder perception as to whether it is meeting its Mandate; and 2) define the methods for obtaining and using that information. 8.2.2 Internal Audit	Given the overwhelming importance of measurement, analysis and improvement to the Mandate of DRAs, a DRA would typically want to be sure, thorough and effective in the application of clause 8. To ensure effectiveness therefore, the DRA would routinely, or as may be necessary, determine through planning the need for, use of, and extent of use of applicable as a key performance measurement of its QMS. For producing and/ or distributing DRAs especially, monitoring customer/ stakeholder perception can be obtained from: 1) customer/ stakeholder satisfaction surveys; 2) customer data on delivered product quality; 3) user opinion surveys; 4) lost business analysis; 5) compliments; 6) warranty claims; and 7) dealer reports. For thoroughness and effectiveness a producing/ distributing DRA must: 1) plan the audit program; 2) consider the status and importance of the audited areas; 3) consider the results of prior audits; 4) define the audit criteria, scope, frequency, and methods; and 5) select and use impartial and objective auditors. Non-producing/ distributing DRAs must have the ability to ensure that manufacturers/ distributors comply. To institute thoroughness and effectiveness, producing/ distributing DRAs must: 1. Maintain records of the audits and their results. 2. Ensure control of the audited areas. 3. Take actions without undue delay to eliminate detected nonconformities and their causes. 4. Verify through follow-up actions.

Clause	Title and subtitles, with remarks	Salient points/ directing principles/ application to DRAs
	Internal audits must be conducted at planned intervals so that DRAs can determine if their QMS: 1) conforms to requirements of ISO 9001:2008; 2) conforms to planned arrangements as per sub-clause 7.1; and 3) is effectively implemented and maintained. In order to thoroughly address staff responsibilities and the requirements to be met by the audit exercise, DRAs must establish: 1) a documented procedure for planning audit; 2) a documented procedure for conducting audits; and 3) a documented procedure for recording and reporting audit results.	Again, non-producing/ distributing DRAs must have the ability to ensure that manufacturers/ distributors comply. NOTE: ISO 19011 (Audit guidance) should be consulted for further enlightenment quality auditing.

*The Table is to be studied side by side with contents of ISO 9001:2008 and Table 2, which is on the regulatory requirements of DRAs.

Table 11. Clause 8 of ISO 9001:2008 in relation to DRAs (Monitoring and measurement).

3.4.6. Remediation of a malfunctioning DRA

A national or regional DRA is a critical factor in socioeconomic development and wellbeing in at least two ways: i) by “guaranteeing the health of the nation” (as trumpeted in NAF-DAC’s adverts); and by supporting the emergence of responsible manufacturers of regulated products. It is well known that the US-FDA more than any US organization has made the US the world leader in manufacture of health products. The prominence of India and China in world drug trade owes much to the vibrancy and relative efficiency of their DRAs. There is therefore a critical need for DRAs to be vibrant and responsible. The gravity with which China views the role of her DRA can be gauged by the death sentence passed on the Director General in 2007 for accepting a bribe [15].

Clause	Title and subtitles, with remarks	Salient points/ directing principles/ application to DRAs
8	<p>8.2.3 Monitoring and Measurement of Processes</p> <p>1) Apply suitable methods to monitor and, where applicable, measure the QMS processes.</p> <p>2) Confirm through these methods the continuing ability of each process to satisfy its intended purpose.</p> <p>3) When the planned results are not achieved, take correction and corrective action, as appropriate.</p> <p>8.2.4 Monitoring and Measurement of Product</p>	<p>Producing/ distributing DRAs need to 1) apply suitable methods for monitoring and measuring QMS processes; and 2) confirm through these methods the continuing ability of each process to satisfy its intended purpose.</p> <p>Non-producing/ distributing DRAs must have the ability to ensure that manufacturers/ distributors comply.</p> <p>NOTE: When determining “suitable” methods, consideration is given to the type and extent of monitoring or measurement for each process in relation to its impact on product conformity and on the effectiveness of the QMS.</p> <p>To better fulfil their Mandate producing/ distributing DRAs must 1) monitor and measure product characteristics so as to verify if product requirements are being met; 2) carry out the monitoring and measurements at the appropriate stages of product realization in accordance with planned arrangements; and 3) maintain evidence of conformity with the acceptance criteria.</p>

Clause	Title and subtitles, with remarks	Salient points/ directing principles/ application to DRAs
	1) Monitor and measure product characteristics to verify if product requirements are being met.	It is again stressed that non-producing/ distributing DRAs must have the ability to ensure that manufacturers/ distributors comply.
	2) Carry out the monitoring and measuring at the appropriate stages of product realization in accordance with planned arrangements (see 7.1).	DRAs must ensure that product release and service delivery cannot proceed until all planned arrangements (see 7.1) have been satisfactorily completed, unless otherwise approved by a relevant authority, and where applicable, the customer. Where applicable, DRAs must deal with the nonconforming product by one or more of the following ways:
	3) Maintain evidence of conformity with the acceptance criteria.	1. Take action to eliminate the detected nonconformity.
	4) Record the person responsible for authorizing release of product for delivery to the customer.	2. Authorize its use, release, or acceptance by concession. 3. Take action to preclude its original intended use or application.
	8.3 Control of nonconforming product	4. Take action appropriate to the effects, or potential effects, of the nonconformity when nonconforming product is detected after delivery or use has started
	DRAs must: 1) Ensure any nonconforming product is identified and controlled to prevent its unintended use or delivery.	To better fulfil their Mandate, DRAs must: 1) Ensure that any nonconforming product is identified and controlled to prevent its unintended use or delivery. 2) Establish a documented procedure to define the controls and related responsibilities and authorities for dealing with nonconforming product.
	2) Establish a documented procedure to define the controls and the related responsibilities/authorities for dealing with nonconforming product.	
	DRAs must maintain records of the nature of the nonconformity, and any subsequent actions, (including any concessions). When the nonconformity is corrected, DRAs must re-verify it to prove or show evidence of conformity.	

*The Table is to be studied side by side with contents of ISO 9001:2008 and Table 2, which is on the regulatory requirements of DRAs. Although a DRA may not possess certain facilities for measurements and monitoring, it should possess the ability or the means necessary to ensure that manufacturers/ distributors possess and use them in accordance with approved QMS guidelines.

Table 12. Clause 8 of ISO 9001 in relation to DRAs (Product characteristics/ Control of nonconformities).

In Nigeria, the entire NAFDAC Management was sacked on alleged acts of corruption in 2000. It seems to us that the following are essential for a DRA to perform optimally:

1. The laws creating/ amending a DRA should be well articulated as is the case with the US-FDA.
2. DRAs should be so well funded as not to rely on a plethora of frivolous fees as with NAFDAC.
3. Staffing of DRAs must be transparent - competence and integrity must be the decisive criteria.
4. DRAs should be audited frequently, at least yearly or twice yearly.
5. DRAs should have a Board of Governors to whom the Management reports.

6. Parliamentary health committees should view DRAs as critical to socioeconomic well-being of the nation.

Clause	Title and subtitles, with remarks	Salient points/ directing principles/ application to DRAs
8	<p>8.4 Analysis of data</p> <p>Whether producing/ distributing or not, as a rule, DRAs must determine, collect, and analyze appropriate relevant sources in their analyses. data to demonstrate the suitability and effectiveness of their QMS, as well as, evaluate where continual improvement of the QMS can be made. DRAs cannot enforce compliance among manufacturers/ distributors in an aspect of quality management in which they are themselves deficient.</p> <p>8.5 Improvement</p> <p>8.5.1 Continual Improvement</p> <p>8.5.2 Corrective Action</p> <p>The standard practice for organizations is that:</p> <p>1. Wherever a nonconformity or breach is detected, corrective action must be taken to eliminate the cause of the nonconformity and to prevent its recurrence.</p> <p>2. Wherever corrective action is taken by an organization, such action must be such as is appropriate to the effects of the problem caused by the nonconformity or breach.</p> <p>8.5.3 Preventive Action</p> <p>Organizations must:</p> <p>1. Determine in advance the action that needs to be taken to eliminate the causes of potential non-conformity, in order to prevent its occurrence.</p> <p>2. Ensure that preventive actions are appropriate to the anticipated effects of the potential problem.</p>	<p>DRAs should as a matter of practice:</p> <p>1. Include the primary data generated by monitoring and measuring activities, and from other DRAs</p> <p>2. Analyze such primary data to provide secondary data on:</p> <p>a. Customer satisfaction as per 8.2.1.</p> <p>b. Conformity to product requirements as per 8.2.4.</p> <p>c. Characteristics and trends of processes and products, including opportunities for preventive action as per 8.2.3, 8.2.4, and 8.5.3.</p> <p>d. Suppliers as per 7.4</p> <p>DRAs are to continually improve the effectiveness of their QMS through:</p> <p>1. Quality policy</p> <p>2. Quality objectives</p> <p>3. Audit results</p> <p>4. Analysis of data</p> <p>5. Corrective and preventive action</p> <p>6. Management review</p> <p>For thoroughness and effectiveness, DRAs must establish a documented procedure (SOP) for corrective action. Such a procedure must define requirements to:</p> <p>1. Review nonconformities (including customer complaints).</p> <p>2. Determine the causes of nonconformities.</p> <p>3. Evaluate the need for actions to prevent recurrence.</p> <p>4. Determine and implementing the needed action.</p> <p>5. Maintain records of the results of the action taken.</p> <p>6. Review the effectiveness of corrective action taken</p> <p>For thoroughness and effectiveness, DRAs must establish a documented procedure for preventive action. Such a procedure must define requirements to:</p> <p>1. Determine potential nonconformities and their causes.</p> <p>2. Evaluate the need for actions to prevent occurrence.</p> <p>3. Determine and implementing the needed action.</p> <p>4. Maintain records of the results of the action taken.</p> <p>Review the effectiveness of preventive action taken</p>

*The Table is to be studied side by side with contents of ISO 9001:2008 and Table 2, which is on the regulatory requirements of DRAs. It must be stressed once again that if DRAs are to persuade manufacturers/ distributors to comply with the provisions of this and other clauses of ISO 9001:2008 industrial standard, they too must be conversant with and adept in them.

Table 13. Clause 8 of ISO 9001:2008 in relation to DRAs (Analysis of data/ Improvement).

3.4.7. Further remarks on clauses 7 and 8 of ISO 9001:2008

Although the principles of clauses 7 and 8 apply to all organizations, they are strictly speaking, the deeds and stuff intended for high profile institutions with elaborate concern and facilities for design and R&D, and with tall entrepreneurial ambition. Such organizations include the most successful pharmaceutical and biotechnology companies of the US, Europe, Japan and India; NASA, aircraft manufacturers, international airlines and 5-star hospitality concerns. However, in as much as DRAs must regulate the work and product of advanced pharmaceutical manufacturers, the onus is upon the DRAs themselves to be conversant with the entire provisions of these clauses and be as intellectually equipped as the manufacturer. This explains why it is often desirable that regulators have a stint in both academia and industry. In many countries, especially the US and India, top rate biomedical facilities/ institutions and personnel are to be found in the following four circles: i) the DRAs (eg: US-FDA); ii) health research institutions (eg: NIH); iii) the universities/ R&D institutions patronized by the DRAs; and iv) big transnational drug manufacturers (eg: Pfizer). We once again refer to the Nigeria polio vaccine controversy of the late 1990s/ early 2000s mentioned earlier, and ask the following question: When a DRA produces or distributes product as is the case in many developing economies, who regulates the DRA? Can subsequent revisions of ISO 9001 or some other ISO standard provide an answer?

4. Conclusions

It is evident from the foregoing that all the eight clauses of ISO 9001:2008 apply to the Mandate of DRAs. However, most of what appears in clauses 7 and 8, the lengthiest of the clauses, relates more pertinently to high stake pharmaceutical manufacturers that have elaborate R&D than they do to the average DRA, which nevertheless should be thoroughly acquainted with the clauses. Some DRAs like the US-FDA and EMEA that have advance laboratories or access to such or that heavily fund R&D must be guided by the rigorous provisions of clauses 7 and 8. Needless to say, those DRAs that produce/ distribute products must be similarly guided to the extent of their relevance to the scope and size of their operations. The US-FDA, Japan's Ministry of Health and Social Services and EMEA are certified to appropriate performance standards and are known for their efficiency. By contrast NAFDAC and other developing national DRAs are not similarly certified and are less well known for efficiency, considering the rampancy of counterfeit drugs and other ills in their drug delivery systems. The DRAs of China, India and Southeast Asian countries compare quite well in many aspects with those of Europe, Canada and the US, and are by far more efficient than those of many African and South American countries. From the foregoing, and in view of the historical and international dimensions of phytotherapy, especially its galloping global patronage in recent times [1,2,11,12], it is necessary that there to be a minimum global standard to which DRAs should be certified. We propose ISO 9001 because of its global popularity, applicability and suitability. The standard provides the general climate for DRAs to efficiently discharge their Mandate. We project that a carefully planned application of ISO 9001 to herbal drug regulation will improve the production, distribution and usage of herbal drugs.

It will also boost the economy of developing economies that rely to a large extent on herbal drugs. But since the DRAs of many developing economies produce/ distribute certain products, there is a need for subsequent revisions of ISO 9001 to take cognisance of the question of who regulates the regulator that produces/ distributes? In the meantime we recommend that the Minister/ Secretary of Health and/ or the Parliamentary Committees of Health take note of this significant lacuna.

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Quality Control in Food Science

QA: Fraud Control for Foods and Other Biomaterials by Product Fingerprinting

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Additional information is available at the end of the chapter

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1. Introduction

Fraud can be generally defined as “the intentional deception made for personal gain or to damage another individual”. In particular, *food fraud consists in the deliberate misdescription in order to deceive the consumers about the real nature of the product or of any of its ingredients*. It results in the mismatch between what a food product is and what it is claimed to be. Food fraud is a broad term that also involve criminal acts such as tax-avoidance and smuggling. In the following we will mainly discuss about economically motivated adulteration and mislabelling i.e. food fraud issues falling in one of the following categories: 1) the substitution of an ingredient with a cheaper alternative (e.g., substitution of ethanol with methanol in wine or proteins with melamine in milk powders), 2) misdescription of the real nature of the product or one of its ingredients (e.g. counterfeiting, conventional products that are sold as added value products such as organic, fair trade, biodynamic), 3) incorrect quantitative ingredient declaration and 4) implementation of non-acceptable process practices such as irradiation, heating or freezing (e.g. thawed fish sold as fresh).

Food fraud can be implemented in any step of the food chain but it is mainly a food industry issue. In criminology, there are 3 elements of fraud opportunity (the crime triangle): victim, fraudster and guardian [1]. The typical set-up is that where final consumers play the role of victims, food industry (but in general food producers, processors, traders or retailers) plays the role of the fraudsters and governmental control authorities, non-governmental and certification organizations play the role of the guardian. However, food producers can also be victim or guardian. They are victims when, for example, their products are counterfeited or simulated and guardian when they implements QA systems for the assessment of the authenticity of the raw materials. Nowadays food fraud represents a major problem that costs the EU food industry and governments hundreds of millions of euros every year. But the

problem that food fraud poses is not merely an economic one: It is also a problem of public health because the adulteration can pose toxicological and hygienic risks to purchasers and consumers. In 2008, for example, Chinese milk was adulterated with melamine, a hazardous chemical, to increase milk nitrogen content causing 900 infants to be hospitalized with six deaths. Several other such examples can be given.

Since no one likes to be swindled, neither producers, traders, importers, retailers, and consumers, fraud prevention and detection is an important issue. Nowadays, authenticity of ingredients or products is mainly warranted by paper trailing. Analytical tests which can help to confirm the authenticity of ingredients/products compose a very useful complementary approach to paper trailing.

2. Fingerprinting approach: generalities and tools

Traditional strategies for the food fraud control have relied on the determination of the amount of a marker compound or compounds and the comparison of the obtained values for the test material with those established for the genuine material. The presence of an undesired adulterant can be uncovered by checking for its presence in the food material whereas the compliance of the food composition with the established legislative standards or with the amount of an ingredient as declared on the label can be simply proved by measuring the target compound or compounds. However, some aspects of food authenticity such as the geographical origin, the farming management systems (organic, free range..), or the application of some specific processes cannot be dealt with those traditional approaches. No single marker exists for the unequivocal authentication of an organic egg or a Dutch specialty cheese. Furthermore, based on conventional target analyses, an adulteration can be detected only if the adulterant is known beforehand and explicitly searched for by the analyst. Traditional quality control strategies are not designed to look for a near infinite number of potential contaminants so that new adulterants will not be unveiled until their presence in food is first acknowledged. For those reasons a more holistic approach is needed that is based on the measurement and the evaluation of several compounds at once, i.e. a fingerprinting approach. Moreover, in industrial and laboratory settings, there is always the need of implement screening methods that are able to reliably identify, in large numbers of samples, those that are potentially non-compliant before more detailed and accurate analysis with confirmatory methods are performed. A fingerprinting approach may, in many cases, provide rapid and high-throughput analyses well suited for screening purposes.

Fingerprint refers to the characteristic spectrum or image of a test material which can be related to its properties and thus to its authenticity in the same way as a human fingerprint is specific of a certain person and unequivocally identify him/her. The term thus recalls a comprehensive description of a test material that is carried out in a non-selective (or untargeted) way. Fingerprints can be generated through many analytical techniques. They can be obtained from chromatograms, spectroscopic measurements, spectral measurements or any other specific signal of complete spectra (Figure 1).

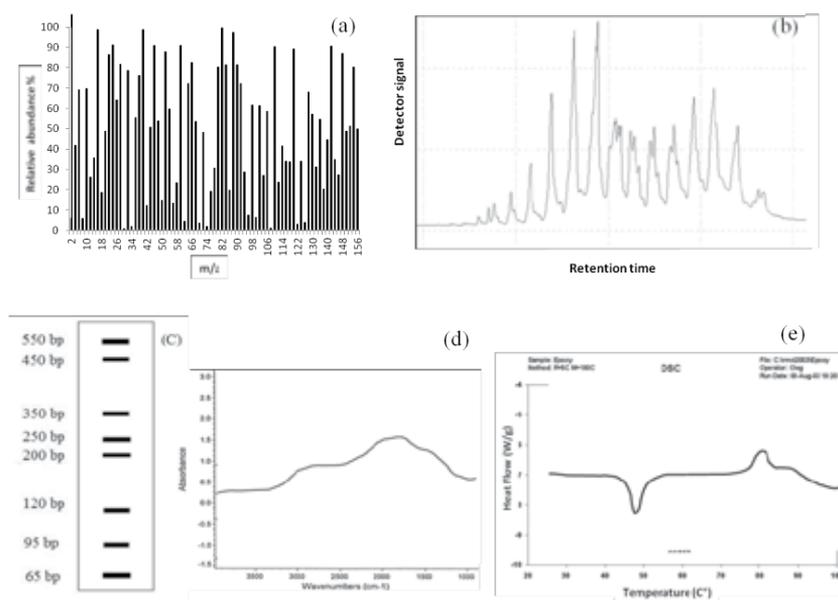


Figure 1. Analytical fingerprints: A mass spectrum (a), a chromatogram (b), a schematic representation of a DNA fingerprint on gel electrophoresis (c), an infrared spectrum (d) and a thermogram (e).

In a mass spectrum (Figure 1a), a collection of m/z and relative signal intensities is a chemical fingerprint of the material. Similarly, a chromatographic profile (Figure 1b) is a fingerprint of a more or less broad class of constituents of the material. The specific distribution of the restriction fragments of a selected DNA sequence on the electrophoretic gel (Figure 1c) is a genetic fingerprint of the test material. A NIR spectrum (Figure 1d) is a representation of the interaction of a test material with the infrared radiation whereas a thermogram (Figure 1e) is a representation of its interaction with thermal energy. (a) and (b) can be referred to as *chemical fingerprints*. They may be composed of as many groups of compounds as possible or alternatively of a specific group of compounds which requires higher level of purification and a selective extraction from the sample. (d) and (e) can be referred to as *physical fingerprints* even though chemical information can be obtained as well.

A fingerprinting approach implies that the whole information contained in the fingerprint (or a selected part of it) is used to infer about the properties of the system under study. To do that, a special statistical tool is necessary, i.e. chemometrics. Chemometrics can be defined as the science of extracting chemically relevant information from multivariate data by using statistical techniques to reduce the dimensionality of the dataset. It offers a tool to graphically summarise the analytical data to reveal relationships between samples and to detect characteristic patterns that can be used to identify a certain material. As a first step, an exploratory analysis is carried out in order to investigate the natural relations between the samples. This is carried out by so called unsupervised pattern recognition techniques because they do not require any prior knowledge of the properties of the samples. Examples of such techniques

are: Hierarchical cluster analysis (HCA), cluster analysis (CA) and principal component analysis (PCA). PCA is the most widespread of those explorative tools. In a PCA model the original variables are transformed in new uncorrelated variables that arise from the linear combination of the original variables: the principal components (PCs). A number of PCs are extracted in sequence with each principal component accounting for the maximum of the residual variance in the data. The PCs extraction stops when most of the variance in the original data (typically around 90%) is explained. The new set of PCs define therefore a new space where the contribution of each original variables to each PC can be easily represented and the relationships between the original samples highlighted (Figure 2).

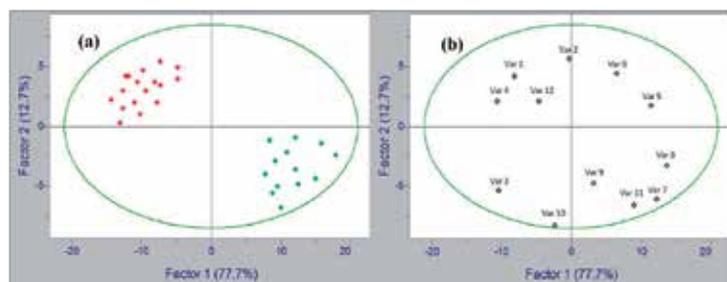


Figure 2. Plot of the first two dimensions in a typical PCA model. In a scores plot (panel a), samples are plotted in the space defined by the PCs. Similarities and differences between samples can be highlighted (in this case, two well separated groups of samples, red and green are apparent). In a loading plot (panel b) each variable (analytical response) is plotted on the new space defined by PCs. When score plot and loading plots are superimposed, information on the effect of the variable on samples properties can be obtained. When a variable is plotted close to a sample, this indicates that this variable shows relatively high concentration in this particular sample compared to the other samples. In the figure, variables 1,4,12 are higher in the samples of the red group and variables 6,7 and 11 are higher in the green group.

As a further step, multivariate methods are applied to either classify a certain product or quantify a certain property of the product. In the *classification models*, information about the class membership of the samples to a certain group (class or category) is used to classify new unknown samples in one of the known classes on the basis of its pattern of measurements. Classification models are useful, for instance, for the authentication of organic products or of geographical origin. Supervised pattern recognition techniques are used such: k nearest neighbours (kNN), soft independent modelling of class analogy (SIMCA), partial least square regression discriminant analysis (PLS-DA), linear discriminant analysis (LDA), support vector machine (SVM) and artificial neural network (ANN). Classification models may either build a delimiter between the classes so that they always assign a new object to the class to which it most probably belongs (suitable for limited and defined number of possible classes) or build a model for each class studied and then evaluate the fitting of new objects to each model (suitable for unlimited number of classes). In the *regression models*, a functional relationship is established between some quantitative sample property, the dependent variable, and a multivariate independent variables such as a raw chromatogram or a IR spectrum and the model is used to predict the property of interest in the unknown sample. Regression model are used, for instance, to quantify the level of adulteration in a food sample. For the build-

ing of regression models, multivariate regression methods such as principal component regression (PCR) and partial-least square regression (PLSR) are used.

The building of a classification (or regression) model comprises four essential steps: (1) Selection of a training set, which consist of objects of known class membership (or known quantitative values of a sample property) for which variables are measured. (2) Variable selection. Variables that contain information for the aimed classification are retained, whereas those encoding noise and/or with no discriminating power are eliminated. (3) Building of a model using the training set. A mathematical model is derived between the selected variables measured on the training set and their known categories (or quantitative values of the sample property). (4) Validation of the model. The model is validated in order to evaluate the reliability of the classification achieved either using an independent test set of samples (external validation) or the training set (cross validation).

The fingerprinting techniques are gaining more and more popularity over the past years thanks to advancements in the analytical instruments that are able to generate enormous amount of data at once and the application of chemometrics techniques. Herewith, fingerprinting techniques are classified in five broad categories according to the kind of fingerprint that can be obtained: *Mass spectrometry (MS) fingerprinting, chromatographic fingerprinting, electrophoretic fingerprinting, spectroscopic fingerprinting, and other fingerprinting*. This classification is shown in Table 1.

MS fingerprinting	Chromatographic fingerprinting	Electrophoretic fingerprinting	Spectroscopic fingerprinting	Other fingerprinting
PTR-MS	LC (HPLC, LC-MS..)	CE (CZE, CIF...)	NMR	DSC
ICP-MS	GC (GC-FID, GC-MS..)	Gel electrophoresis (isoelectric focusing, DNA electrophoresis...)	Fluorescence spectroscopy	Electronic nose
IR-MS			IR (NIR, MIR, FTIR)	Microarray technologies, reverse PCR
Direct infusion and ambient MS (ESI, MALDI-TOF, DART...)				

Table 1. Classification of fingerprinting techniques. For abbreviations, see text.

3. MS fingerprinting

MS is a powerful analytical technique that measures the mass-to-charge ratio of ions. The samples are first ionised, the ions are separated and their relative abundance assessed based

on the intensity of the ions flux. MS produces therefore a mass spectrum representing the fingerprint of the sample components (Figure 1a). A number of different MS set-ups are possible based on the ionisation technique and the mass analyser used. MS can be used alone or they can be coupled with separation techniques. In this section, the stand-alone MS techniques will be dealt with in details whereas the application of MS as coupled with separation techniques will be dealt with in the next sections.

Stand-alone MS fingerprinting techniques that proved to be very useful for the fraud control and prevention are: proton transfer reaction MS (PTR-MS), inductively coupled MS (ICP-MS), isotope ratio mass spectrometry (IRMS), and direct infusion MS techniques.

Proton Transfer Reaction Mass Spectrometry (PTR-MS)

PTR-MS is a relatively new technique that is rapidly gaining popularity in the food analysis. PTR-MS allows quantitative on-line monitoring of volatile organic compounds. The volatile compounds are softly ionized by means of hydroxonium ions that are generated in an external ion source operating in pure water vapour. Only the volatile compounds that have a higher affinity for the ions are protonated and then accelerated by an electric field to the reaction chamber where they are separated and quantified. Because of this soft chemical ionisation the fragmentation of the parent compounds is limited and the interpretation of the spectra are much easier. Other major advantages of this technique are the great sensitivity with detection limits as low as few part per trillion, volume (pptv) and the possibility to monitor the food samples in real time, without any work up procedure. As a result, a fingerprint of all the volatile compounds comprised in a well definite mass range is obtained. The main disadvantage of this technique is that compounds are characterized only via their masses which is insufficient for their unequivocal identification.

PTR-MS has been extensively used in several aspects of food fraud control. It proved, for example, very successful for the geographical authentication of foods. The EU has long recognized the importance of differentiating food products on a regional basis. The normative framework introduced by the EU comprises the EU Regulations 509/2006 and 510/2006 and the EU Regulation 1898/2006. The EU Regulation introduced three geographical indications to a food product: protected designation of origin (PDO), protected geographical indication (PGI) and traditional specialities (TSG). In a study of 2008, the geographical origin (country) of butter samples was successfully predicted in 88% of the cases based on PTR-MS fingerprint and PLS-DA [2]. Recently, volatile fingerprint generated by PTR-MS has been used to discriminate between the Boeren Leidse specialty cumin cheeses with EU PDO from other 29 cumin cheese manufactured in the Netherlands [3]. The volatile fingerprint coupled with a PLS-DA model allowed the correct classification of 96% of the traditional boeren leidse cheese samples and 100% of the other commercial cheese samples. Another typical added value that is protected by the EU regulations is represented by the monovarietal extra virgin olive oil (EVOO), i.e. oil that is produced out of just one variety of olive trees. Frauds can be committed by mixing the more valuable monovarietal virgin olive oil with cheaper oils or by mixing different monovarietal olive oils. Volatile fingerprint of virgin olive oil obtained by PTR-MS and subjected to PLS-DA proved successful in discriminating among 5 different monovarietal EVOO from Spain with 100% sensitivity (% of objects of the modelled class

correctly accepted by the model) and specificity (% of object, extraneous from the modelled class, correctly refused by the model) close to 100% [4].

In conclusion, PTR-MS is a rapid and low cost analytical technique that can be also fully automated and implemented on-line. Recently, the coupling of the time of flight (TOF, see below in this section) mass analyser to PTR-MS instruments has generated PTR-TOF-MS which is characterized by a high sensitivity with limits of detection down to few pptv and a high time resolution. The technique has been recently applied to discriminate among PDO labelled hams from Spain and Italy [5].

Inductively coupled plasma mass spectrometry (ICP-MS)

ICP-MS provides quantitative measurements of a wide range of metals and non-metals (inorganic elements) at trace and ultratrace concentration level (ppt). In this technique, the sample (even solid or liquid) is decomposed to neutral elements in a high-temperature argon plasma and the single elements are separated based on their mass/charge ratio and analysed. The great advantage of this technique compared to others (e.g. atomic spectroscopy) is that more than one element can be analysed at once so that a multi-elemental fingerprint is obtained in a very fast and sensitive way. The multi-elemental composition of animal and vegetal tissues can provide valuable information on the characteristics of the soil where a crop has been cultivated and on plants composition of the animal diet. The multi-element fingerprint is thus a valuable marker of the geographical origin of food. For instance, the authenticity of Tropea red onion, an onion Italian variety that achieved the PGI certification by the European Union as "Cipolla Rossa di Tropea Calabria" can be proved by means of multi-elemental analysis by ICP-MS and multivariate statistics [6]. All the statistical models applied (LDA, stepwise LDA, SIMCA, ANN), allowed a success rate of prediction >90% for the genuine samples. Moreover, the availability of nutrients from the soil strictly depends on the fertilization strategies and the pest and weed control management systems. In organic farming synthetic fertilizers are not permitted and the pest and weed control is based exclusively on natural products. It has been thus proposed that the multi-elemental fingerprint might be a marker for organically cultivated crops as compared to conventionally cultivated ones. Laursen *et al.* managed to discriminate between organic and conventional wheat, barley and faba beans (but not potatoes) based on the profile of 25 elements measured by ICP-MS [7].

Isotope ratio mass spectrometry (IRMS)

IRMS is a technique that can measure the ratio of the stable isotopes of the constituents of a biological material. Light elements like carbon, nitrogen, hydrogen, oxygen and sulphur stable isotopes ratios are most frequently assessed with this technique. Those ratios vary according to specific food production factors and geo-climatic conditions. Carbon stable isotope ratio depends, for example, from the plant composition of ruminant diets and can then be used to authenticate feeding regime or the farming management system (organic, free-range). Nitrogen stable isotope ratio is on the other hand depending on the type of fertilizers used in agriculture and is thus much useful for the authentication of farming practices for vegetal products and crops. Oxygen isotope ratio is instead highly dependent on the

distance from the ocean and the altitude above sea level and could then be used for the authentication of the geographical origin of a food product.

The stable isotope fingerprint has been successfully used for the authentication of geographical origin and the farming practice. Normally the data are measured for many different elements and analysed with multivariate statistics. As an example, Fontina PDO cheese can be discriminated with good success from other cheeses based on stable isotopes $^2\text{H}/^1\text{H}$, $^{13}\text{C}/^{12}\text{C}$, $^{15}\text{N}/^{14}\text{N}$ and $^{34}\text{S}/^{32}\text{S}$ and PCA analysis [8]. However, frequently the isotope ratios (or a selection of them) are combined with other markers (elements) to improve the accuracy of the classification models. IRMS is often combined with ICP-MS for simultaneous elemental analysis. Stable isotope analysis combined with multi-elemental analysis has proven ideally suited to determine geographical origin of foods. The most accurate measurements of the isotope ratios is obtained by dual inlet (DI) IRMS. However, the purchasing and operating costs of a DI-IRMS instrument coupled with the time-consuming sample preparation are major disadvantages for the diffusion of this technique. The introduction of continuous flow (CF) IRMS instruments offers on-line, rapid and automated sample preparation, greater cost-effectiveness and easier interfacing with other preparation techniques.

Direct infusion mass spectrometry

Direct infusion MS techniques are based on the direct injection of the sample in the ion source without or with small sample pre-treatment. This allows for rapid analysis suited for high-throughput screenings. Electrospray ionisation (ESI), matrix assisted laser desorption ionization (MALDI) and direct analysis in real time (DART) are typical ionization techniques used for direct infusion MS. They are coupled with a variety of mass analysers, e.g. time of flight (TOF), Fourier Transform Ion Cyclotron Resonance (FT-ICR), single quadrupole (Q) and ion trap (IT) in many different set-ups.

ESI is a typical soft ionisation technique that is particularly suited for the determination of the molecular mass of large molecules (proteins, peptides, polysaccharides, triglycerides), because the ionisation does not bring about the fragmentation of the molecule. The liquid in which the analyte is contained is dispersed by electrospray to a fine aerosol. The droplets shrink as the solvent evaporates till solvated ions desorb from their surface. ESI-MS has proven to be very helpful in the authentication of vegetable oils. Lipid composition of vegetable oils depends on their botanical origin and the way they are processed. Fatty acids (FAs) and/or triglycerides (TGs) profile can thus help authenticate the type of oil, its origin, its quality grade and potential adulteration. Direct infusion ESI-MS has been for example used to predict the olive oil quality according to European Union marketing standards based on fatty acids and LDA analysis [9]. In the same research, the percentage of either EVOO and VOO in binary mixture with other lower grade oils was predicted with 5–11% average prediction errors by using PLS and multilinear regression (MLR). Samples were 1:50 diluted in an alkaline 85:15 (v/v) propanol/methanol mixture and directly infused into the MS system. Triglycerides analysis has some advantage over the analysis of the fatty acids profile for authentication or fraud control. Indeed, different oils can have specific TG fingerprint despite showing the same fatty acids composition. The triglyceride profiles, obtained using Q-TOF-ESI-MS was used to predict adulteration of olive oils with other vegetable oils.

The adulteration with hazelnut oil was predicted at a level of 10% v/v [10]. This adulteration is difficult to detect at levels below 20% by conventional methods due to the compositional similarity between the two oils. Similarly, PCA and HCA methodologies, applied to the ESI(+)-MS data were able to readily detect adulteration of EVOO with ordinary olive oils, at levels as low as 1% w/w [11]. Mono-, di- and triglycerides together with vitamins and antioxidants were detected and quantified with this method. Direct infusion ESI-MS has been used to authenticate other food commodities. The chemical fingerprint generated by direct infusion ESI-Q-TOF-MS in the negative mode can be used to discriminate between genuine whisky from Scotland and US, from counterfeited whisky produced in Brazil [12] and between alembic (the most valuable) and industrial cachaças (Brazilian sugarcane spirit) as well as the fraudulent addition of sucrose to the spirit [13]. Finally, direct-infusion ESI-QqQ-TOF-MS and atmospheric pressure photoionization (APPI)-QqQ-TOF-MS have been used for Iberian ham typification. APPI is a soft ionization technique based on a photoionisation mechanism. Five types of Iberian hams were successfully classified. Applying a PLS-DA model [14].

MALDI is another soft ionisation technique that proved very useful in the analysis of macromolecules, especially proteins. In MALDI the molecules are desorbed from the support matrix and ionised by means of a UV laser beam in a complex process mediated by the support matrix itself. MALDI is mainly coupled with a time-of-flight (TOF) mass analyser which separate the ions based on their flying time to the detector, which on turns depends on their m/z ratio. An example of application of MALDI-TOF-MS for authentication issues is represented by the fast method developed by Wang *et al.* for the fingerprinting of honey proteins [15]. The mass spectra were used to build up a database library to be used for authentication purpose. The protein fingerprint was thus successfully used to authenticate the geographical origin of commercial honeys produced in the US and other countries. In a similar fashion, peptide fingerprinting obtained by MALDI-TOF has been converted in a biological bar code for the authentication of high quality Campania white wines [16].

An innovative technique for food fingerprinting is represented by the direct analysis in real time (DART)-MS. DART is an ambient ionisation technique i.e. in which ions are formed outside the mass spectrometer without sample preparation or separation. The samples, either gaseous, liquid or solid are ionised in open air under ambient conditions. This means that organic compounds can be directly, and in real time, determined without time-consuming analytical protocols and thus with high sample throughput. DART coupled with TOF-MS has been used to obtain the fingerprint of the triglycerides from olive oil [17]. This method, coupled with LDA allowed the discrimination between EVOO, olive oil and olive oil pomace and the detection of hazelnut oil in EVOO at 6% v/v. DART-TOF-MS with solid phase micro extraction (SPME) pre-concentration of the analytes has been also reported to allow discrimination between trappist and non-trappist beers based on volatiles and phenolic compounds [18]. A combination of DART-TOF-MS and chemometrics was used for animal fat (lard and beef tallow) authentication [19]. TGs and polar compounds were extracted and analyzed. Mass spectral records were processed by PCA and stepwise LDA. The LDA model developed using TAG data enabled the classification of lard and beef tallow samples but also detection of admixed lard and tallow at adulteration levels of 5 and 10% w/w.

Additional ambient ionisation techniques have been recently proposed for authentication and fraud control by product fingerprinting. For instance, easy sonic spray ionisation (EASI)-MS fingerprinting of fatty acids and phenolic compounds have been used for the authentication of olive oil geographical origin [20].

4. Chromatographic fingerprinting

Chromatographic techniques aim at resolving complex mixtures in well separated compounds. Based on the detection system, each single compound generates a signal that can be used for the qualitative and quantitative analysis of the mixture. The graphical representation of such signal as a function of time is referred to as a chromatogram and can be thought of as the fingerprint of one or more classes of compounds occurring in the sample. Different strategies are available to obtain multivariate data matrices from chromatographic analyses (Figure 3). The fingerprint can be composed by the set of concentrations of the separated compounds based on an identification/calibration/quantification procedure as depicted in the path (a) of Figure 3. Alternatively, the fingerprint can be represented by the set of peak areas/heights (b). In this case the identification of each single peak is not necessary. Finally, it can be represented by the whole chromatogram that is handled as a continuous signal (c). In this case, the multivariate dataset is composed by as many variables as the sampling points the chromatogram is made up of (each data point of the chromatogram represents an individual variable). However, the application of chemometrics on raw chromatographic data requires specific data pre-processing techniques. In fact, problems related to the peak alignment or baseline shifts are particularly critical when a raw chromatogram is used as a data set.

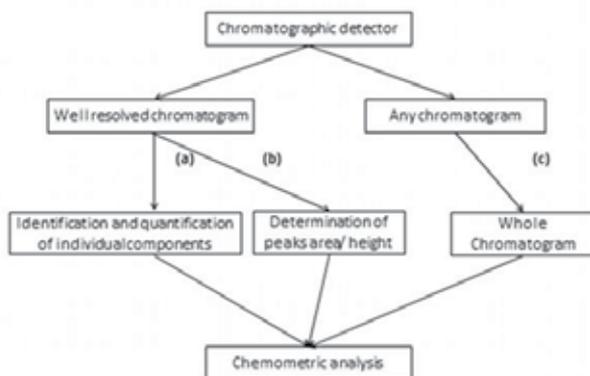


Figure 3. Schematic representation of the strategies to obtain multivariate dataset from a chromatogram for chemometric analysis.

Liquid chromatography

In liquid chromatography, the mixture components are separated as they pass through a column based on their selective partition behaviour between a stationary phase (column material) and a mobile liquid phase. Depending on the type of stationary phase, compounds can be separated based on their size, charge, molecular mass, hydrophobicity etc. The most popular LC technique is high performance liquid chromatography (HPLC) that is a straightforward, robust and reproducible technique. HPLC has been used for the analysis of a wide range of food compounds such as vitamins, proteins, carbohydrates, TGs, additives, secondary plant metabolites. A typical example of the application of HPLC based fingerprint for fraud control is the authentication of the organic eggs by means of the carotenoids profile [21]. Carotenoids are a group of fat-soluble pigments that occur in the egg yolk in concentrations of about 10 mg kg⁻¹. In animals carotenoids are entirely of dietary origin. Since the feeding regime of organic hens is clearly different from that of conventionally reared hens, the carotenoids fingerprint in eggs were used to discriminate between production systems. The carotenoids profile was determined by HPLC with UV detection, the single carotenoids quantified and the resulting concentrations used to build up a classification model by kNN. Almost all the conventional eggs and all the organic ones were correctly classified in external validation. The robustness of the method has been recently improved by testing eggs produced in several EU and non-EU countries. An example of HPLC fingerprint based on raw chromatographic data is in [22]. The authors applied PLS to the full TGs chromatogram profiles of vegetable oils to predict the % of olive oil in the mixtures with errors not exceeding 10%. Liquid chromatography can also be coupled with MS which allows higher resolution and higher sensitivity for metabolites occurring in relatively low amount. As an example, an untargeted method for proteins analysis based on LC-QTOF-MS has been developed which allowed to detect the fraudulent addition of cheaper vegetal proteins (from soy and pea) to skimmed milk powders based on the different peptides profile [23].

Gas Chromatography

In gas chromatography, the mixture is first vaporised in a heated chamber and then the mixture components are separated as they travel through the column transported by the flow of an inert gas (helium, nitrogen or hydrogen) based on their selective interaction with the column material. GC is a very popular separation technique mainly used for the analysis of volatile compounds. However a wide spectrum of compounds can be rendered volatile by proper derivatisation and thus analysed by GC. The analysis of fatty acids and triglycerides is usually carried out by GC with flame ionisation detector (FID) previous derivatisation in fatty acids methyl esters (FAME) and TG trimethylethers, respectively. FID is a general detector capable of high sensitivity and robustness. Fatty acids composition of animal tissues and animal products strongly depends on the feeding regime. FAs composition of fish muscle fat is affected by animal diet/feeding, the geographical area of catch and the marine conditions and is thus different between farmed and wild fishes. The discrimination between wild and farmed Atlantic salmon (*Salmo salar* L.) and Wild Turbot (*Psetta maxima*) has been reported based on FAs analysis and chemometrics [24-25]. The fatty acid fingerprint obtained by GC-FID followed by PLS-DA analysis has been also reported for the authentica-

tion of organic eggs and of organic feeds [26-27]. In the last case 90% of the analysed samples were correctly classified in their proper group in external validation. GC-FID can also be used for the TG profiling. TG fingerprinting by GC-FID has been for example reported for the authentication of three fat classes (animal fats, fish oils, recycled cooking oils) [28]. The TGs fingerprint was subjected to multivariate analysis (PLS-DA) and allowed the correct classification of 96% of the fat samples.

GC coupled with MS represents the method of choice for the analysis of volatile compounds because of its high reproducibility. On the other hand, GC-MS analysis requires careful sample cleaning and is quite expensive and time-consuming. The volatile fingerprint of coffee obtained by GC-TOF-MS after SPME has been reported for the geographical authentication of coffee [29]. SPME preconcentration of volatiles followed by GC-MS analysis coupled with PCA analysis allowed the detection of adulteration of ground roasted coffee with roasted barley [30]. The adulteration is detectable at level of 1% w/w in mixtures of dark roasted barley and coffees. Metabolomics studies can be also fruitfully performed by GC-MS. The fingerprint of a large range of metabolites obtained by GC-MS has been used to discriminate between mechanical separated meat (MSM) from hand-deboned meat [31]. MSM could be detected in raw meat mixtures down to a level of 10%.

5. Electrophoretic fingerprinting

Electrophoretic techniques are able to separate a complex mixture under a spatially uniform electric field, based on electrophoretic mobility of its components that depends, in turn, from their hydrodynamic properties and charge. Positively charged molecules move towards the anode and negatively charged molecules towards the cathode at a different rate based mainly on their mass to charge ratio. Smaller molecules move faster than larger ones.

Gel electrophoresis

In a gel electrophoresis, a gel is used as a medium for the movement of the charged particles under the applied electric field. Agar and polyacrylamide are typical medium used in gel electrophoresis. Proteins and nucleic acid fragments are usually separated by gel electrophoresis. Gel electrophoresis is of major importance for the genomic fingerprint of a sample material. Genomic fingerprints are obtained when properly amplified targeted or untargeted DNA or RNA fragments are separated by electrophoresis thus providing patterns that can be associated to sample properties (specie, variety and the like). Unlike the fingerprints discussed in the previous (and the next) sections, DNA fingerprint shows somehow different characteristics. The single features of the fingerprint are not quantitative variables (physical or chemical variables allowed to take on quantitative values, e.g. area of a peak in a chromatogram, signal intensity for a m/z or absorbance at a fixed wavelength in a IR spectra) but rather categorical variables, i.e. electrophoretic bands that can be either present or absent (see Figure 1 (c)). The sample identification is thus mainly carried out by checking for the presence (or absence) of one or more target bands. Multivariate analysis of the DNA fragments patterns is rarely performed.

Genomic fingerprinting mainly relies on polymerase chain reaction (PCR) based techniques. PCR is based on the amplification of a target DNA sequence by means of a thermostable DNA polymerase. The process consists of several cycles where the DNA molecule is denatured, specific primers (small DNA sequences) anneal to the target DNA sequence and the DNA polymerase synthesizes a new DNA fragment delimited by the two primers. In each cycle the number of DNA molecules increases exponentially. A PCR-derived fingerprint can be obtained in different ways. In PCR-RFLP (restriction fragment length polymorphism) the amplified region is digested with an endonuclease and the resulting DNA fragments are separated by electrophoresis and properly visualised. The pattern of fragment represents a fingerprint of the DNA sequence that has been amplified. In multiplex PCR, two or more DNA fragments are simultaneously amplified by means of different target primer pairs, separated by electrophoresis and visualised. In RAPD (random amplification of polymorphic DNA) random DNA fragments are amplified by means of arbitrarily created primers. After separation, the DNA fragments will give rise specific patterns on the gel. Finally, in single-strand conformation polymorphism (SSCP), DNA sequences are amplified, denatured and the resulting single strand DNA molecules separated by electrophoresis based on their specific secondary structures.

PCR-based fingerprinting techniques have been widely used for species identification. For instance, the identification of ten species of salmon genus in a wide range of commercial products can be accomplished by PCR-RFLP based on the amplification of a specific region of the mitochondrial *cytochrome b* gene followed by polyacrylamide gel electrophoresis (PAGE) [32]. Similarly, PCR-RFLP has been used to identify 15 species of gadoid fishes based on the amplification of a small region of the *cytochrome b* gene and three restriction enzymes [33]. Gadidae family is one of the most commercially important fish family comprising species as Atlantic cod (*Gadus morhua*), the pollack (*Pollachius pollachius*) and the haddock (*Melanogrammus aegleus*). Duplex PCR targeting the *cytochrome b* gene can be used to detect cow milk in buffalo mozzarella at a level of 1%. Buffalo mozzarella is labelled with PDO and can be produced only with pure water buffalo milk (*Bubalus bubalis*) [34]. In an original approach, the multiplex PCR fingerprint of the 16S and 23S rDNA genes of the lactic bacteria naturally occurring in milk has been used to discriminate the geographical origin of PDO mozzarella cheese [35]. The PCR fingerprint was subjected to cluster analysis (neighbour-joining algorithm) which allowed a fair discrimination of the samples.

Genomic fingerprinting shows a unique potential for the species or variety authentication in food products. The introduction of PCR has notably increased the potential of this approach. However, compared to other fingerprinting techniques, genomic fingerprinting is relatively time-consuming and labour-intensive. Its applicability to fraud issues other than genetic identification is limited. Furthermore, food processing may degrade the DNA molecule and lower its recovery thus negatively affecting the results of a analysis when applied to heavily processed foods.

Capillary electrophoresis

Capillary electrophoresis (CE) is the electrophoretic technique that shows a notable potential for food fraud detection based on product fingerprint. CE is a family of separation techni-

ques that separate charged analytes based on their electrophoretic mobility: capillary zone electrophoresis (CZE), capillary isoelectric focusing (CIF), capillary gel electrophoresis, capillary electrochromatography. An electric field is applied to the ends of a capillary column. The ions migrate through the column in the same direction pulled by the electrosmotic flow and are separated based on their electrophoretic mobility. The signal that is generated when the mixture components are detected as they are eluted from the column is referred to as a capillary electropherogram. Multivariate dataset can be obtained from electropherogram in the same way as depicted in Figure 3. However, only strategy (a) has been used so far for authentication and fraud control purposes. CE are capable of rapid, low cost and high resolution analysis with little consumption of mobile phase. Main disadvantages of the technique are the low reproducibility (compared to other separation techniques) and low sensitivity that makes CE not suitable for the analysis of compounds occurring in trace amounts. CE represents a good alternative for the multiple detection of inorganic and organic acids. Many fruits and vegetables is rich in organic acids occurring in varying quantities in different fruits types, giving each fruit a unique organic acid profile. These profiles can be thus used to authenticate a vegetable product or identify the addition of another fruit type. For example the organic acids content measured by CE and LDA has been used to classify Spanish white wines [36].

6. Spectroscopic fingerprinting

Spectroscopy is the study of the interaction between a material and radiated energy. The graphical representation of such interaction is what is referred to as a spectrum i.e. a plot of the response of interest as a function of the wavelength or the frequency of the radiation used (see Figure 1d). Such a spectrum is by its very nature a fingerprint of the target material and contains information that are multivariate in nature. The extraction of the chemically relevant information from such a fingerprint requires the application of multivariate statistical techniques. The whole spectrum is used (or part of it) to obtain a multivariate dataset for further chemometric analysis in the same way as described for raw chromatograms (see Figure 3c). Spectral fingerprinting can be used either to classify and discriminate between samples or to quantify a certain compounds. According to the nature of the radiating energy (infrared, visible, ultraviolet, x-rays) and the nature of the interaction between energy and matter (absorbance, emission, scattering, resonance) different kind of spectra can be obtained. In the following we will mainly focus on nuclear magnetic resonance (NMR), fluorescence spectra and infrared (IR) spectra.

NMR

NMR spectra are generated by the absorption of radiofrequency radiation by atomic nuclei with non-zero spin in a strong magnetic field. Such absorption is affected by the surroundings of the atomic nucleus so that precise information about the molecular structure of a sample can be obtained. The atomic nuclei with non-zero spin that are most frequently used in NMR are ^1H , ^{13}C even though ^{15}N , ^{17}O , ^{19}F and ^{31}P can be also employed.

Generally, NMR is superior to other spectroscopic technique because of the much richer and more detailed information that can be gathered from the NMR spectra, at least with high resolution instruments that use frequencies above 100 MHz. Those information can be used for the simultaneous quantitative determination of a number of compounds without any prior separation. Furthermore the NMR spectrum can be considered a molecular fingerprint of the test material and subjected to multivariate analysis. The main disadvantage of this technique is the elevated costs of the instruments and the running costs. Nowadays, low resolution NMR instruments are available that use frequency ranging from 10 to 40 MHz. Those instruments are much cheaper and easy to use but do not provide the same detailed information as the high resolution instruments. NMR instruments are also capable of good accuracy but the sensitivity is lower compared to MS.

A recent study on the quality control of cola beverages using NMR is exemplar of the potential application of this technique for food authentication and fraud control [37]. ^1H NMR spectroscopy was used to discriminate with high success between premium and discount cola brands. This is important in the light of possible counterfeiting. The whole NMR spectra were used in combination with PCA. In addition, the information contained in specific regions of the NMR spectra combined with multivariate calibration (PLS) allowed the quantification of several cola ingredients (caffeine, aspartame, acesulfame-K, and benzoate) which concentration must comply with regulatory limits. NMR has been also used for the authentication of the geographical origin of olive oils. ^1H NMR spectra of the bulk olive oil, its corresponding unsaponifiable fraction, and a subfractions of the unsaponifiable fraction (alcohol, sterol, hydrocarbon, and tocopherol fractions) were used to classify olive oils according to their origin [38]. The unsaponifiable fraction had to be extracted to avoid the signal to be masked by that from the TGs in the bulk oil. The adulteration of virgin olive oil with a wide range of seed oils can be detected at level as low as 5% by means of combined ^1H and ^{31}P NMR spectra and discriminant analysis provided that the virgin olive oil are fresh (as reflected by their high 1,2-diglycerides to total diglycerides ratio) [39]. In this case the multivariate analysis was performed on 13 compositional parameters derived from the spectra rather than on the whole NMR spectral fingerprint. ^{13}C NMR spectra have been used for the authentication of fish and fish products. Discrimination between farmed and wild salmon is possible based on the NMR spectra of the muscle lipids and neural networks (PNN) and support vector machines (SVM) multivariate analysis [40]. Using the peak intensities of 12 selected chemical shifts an excellent discrimination is obtained by using PNN and SVM (98.5 and 100.0%, respectively). The authentication of different gadoid species was also achieved based on the NMR spectra of muscle lipids and Bayesian belief networks (BBN) with successful classification of 100% [41]. However, ^1H NMR spectroscopy can also provide useful information for the authentication of wild fish. In Figure 4, the PLS-DA scores plot for the ^1H NMR data measured in the authors' group on frozen, smoked and canned salmons both wild and farmed is presented. The score plot shows a clear separation of the two groups in distinct regions of the three dimensional plot. The results of the classification model (leave 5 out internal validation) were extremely positive with 100% of the wild samples (29 samples) and almost 100% of the farmed samples (60 out of 62 samples) correctly classified.

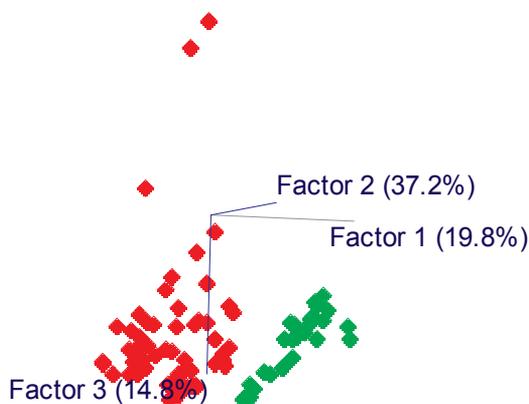


Figure 4. PLS-DA scores plot for the ^1H NMR data on wild and farmed salmon (frozen, canned and smoked). Red=farmed salmons; green=wild salmons

Fluorescence spectroscopy

Fluorescence spectra are normally obtained by exciting the test material. With radiations at a fixed wavelength and recording the intensity of the emitted radiation over a range of wavelengths. However, a 3D spectrum can be obtained by recording the emission spectra at different excitation wavelengths: the so called fluorescence excitation-emission matrix (EEM) which provides more information about the fluorescent compounds occurring in the sample. In synchronous fluorescence scan (SFS) the whole fluorescence landscape can also be achieved by scanning the excitation and the emission wavelengths simultaneously keeping a fixed wavelength interval (the so-called offset). Products that contains natural (or added) fluorophores are suitable for Fluorescence spectroscopy. Tryptophan, tyrosine and phenylalanine residues are fluorophores typically present in a variety of foods along with vitamin A, riboflavin (vit B2), NAD, NADH and compounds originating from Maillard reaction/lipid oxidation. Fluorescence spectra provide information about the amount of those compounds and on the way the fluorophore environment interacts with them. Traditionally, the fluorescence spectra have been treated by means of univariate approaches i.e. taking advantage of one specific wavelength or the derived fluorescence peak features. Nowadays the multivariate information contained in the fluorescence spectra is processed by chemometric techniques. When EEMs or SFS spectra are produced, decomposition methods such as two-way PCA, TUCKER and parallel factorial analysis (PARAFAC) are necessary to extract information from such a multi-way dataset (the data can be arranged in a cube instead of a

matrix as in standard multivariate data sets). In the right-angle fluorescence spectroscopy the incidence angle between the excitation and the emission radiation is 90° . Only liquids and diluted solutions can be analysed and an attenuation of the signal intensity at high absorbance (> 0.1) is observed. To overcome this problem the front-face fluorescence (FFF) has been developed where only the surface of the material is analysed and the incidence angle is around 56° to minimise the artefacts from the excitation radiation reflected or scattered by the sample. Solid and powdered samples as well as bulk liquids can be analysed by FFF.

Fluorescence spectra are a promising tool to verify the egg freshness. Albumen samples stored for 1,2,3 and 4 weeks can be discriminated by means of the Maillard reaction products fluorescence and factorial discriminant analysis (FDA) with high success rate [42]. Vitamin A fluorescence together with FDA allows discrimination among egg yolk samples stored for different times [43]. Similarly, the freshness of fish can be predicted based on NADH and tryptophan fluorescence spectra. NADH fluorescence spectra can be also considered as a promising tool for the discrimination between frozen-thawed fish and fresh fish. The NADH emission spectra show a typical maximum at 455 nm in fresh fish and at 379 nm in frozen-thawed fish. The multivariate analysis (FDA) of the NADH spectrum allowed the correct classification of 100% of the analysed samples [44]. The authenticity of edible oils has also been extensively investigated by fluorescence spectroscopy. Chlorophyll and vitamin E are important fluorophores in olive oils and contribute greatly to oil colour and stability during storage. Refining processes decreases the content of chlorophyll and vitamin E with a corresponding change in the fluorescence spectrum. However, the discrimination between virgin and refined olive oil is mainly based on the fluorescence of lipid oxidation products (more abundant in the less resistant refined oils). A fast screening method has been also developed to detect adulteration of EVOO with olive-pomace oil [45]. It is based on the EEMs and it is able to detect adulteration at a level of 5%. Similarly, the discrimination between olive oils according to their overall acidity are also possible with fluorescence spectroscopy [46]. In this case, the maximum differentiation between the oils was obtained in the region 429-545 nm of the spectrum and allowed 100% correct classification of lampante olive oil (acidity $>3.3\%$, not edible) from virgin olive oil with lower acidity ($<3.3\%$). Finally, SFS with multiple regression analysis has been reported to for the detection of adulteration of EVOO with olive oil to a level as low as 8.4% when a 80 nm wavelength interval is used [47].

The great advantage of fluorescence spectroscopy is the rapidity, the limited costs and the non-destructive nature of the analysis. The sensitivity is also much greater than that of other techniques because the fluorescence signal has in principle no background.

Infrared spectroscopy

Infrared spectra are produced by measuring the intensity of the absorbance of infrared light by a sample as function of the wavelength. The absorption of infrared light is ascribed to transitions in the vibrational energies of the molecules contained in a sample. Each functional group of a molecule shows characteristic IR absorption at specific frequency ranges regardless of the interaction of the functional group with the rest of the molecule. However, interaction between atoms within a molecule may sometimes affect the position of characteristic bands in a IR spectra depending on the surroundings of the functional group. IR

spectra can thus provide qualitative information about the nature of the functional group present in a food sample and quantitative information on their amount. When the effect of all the functional groups is taken together, the whole spectrum represents a molecular fingerprint that can be used to verify the nature of the sample. The IR region of the electromagnetic spectrum can be divided in 3 portion: The far IR (FIR, $400\text{-}10\text{ cm}^{-1}$) has the lower energy and induces rotational transitions in the molecules. The mid IR (MIR, $4000\text{-}400\text{ cm}^{-1}$) induces fundamental vibrational transitions in the molecules. The near IR region (NIR, $14000\text{-}4000\text{ cm}^{-1}$) also induces transitions in the vibrational energies of the molecules. However, the transitions of the vibrational energy induced by the NIR portion of the spectrum are more complex than those induced by the MIR region. Overtones (transitions from the fundamental vibrational level over two or higher energy levels) and combination modes (arising from the interaction of two or more vibrations taking place simultaneously in different functional groups) give rise to very complex bands in the NIR spectrum that can give more complex structural information than MIR. On the other hand, NIR spectra are less selective than MIR spectra because of the superposition of different overtones and combination bands. A raw spectrum contains background information and noise beside valuable information. To remove those interferences as well as those coming from scattering, to normalise the effect of particle size and light distance, pre-processing methods such as smoothing, derivative, standard normal variate transformation (SNV), multiplicative scatter correction (MSC) or wavelet transforms (WT) are required. Recently, the introduction of the Fourier transform technique in IR (FTIR) has further increased the application range of the IR spectroscopy in the food field. In such a case the spectrum is obtained by mean of an interferogram in which multiple frequencies are measured simultaneously. The resulting interferogram is then deconvoluted using proper algorithms in order to have the original spectrum. The advantages of that technology is a faster analysis and a higher throughput and a better alignment of spectrum obtained by repetitive scans.

IR spectroscopy have been successfully applied to detect adulteration of juices, purees and syrups with cheaper juice concentrates. Adulteration of orange juice with orange pulp wash, grapefruit juice or synthetic sugars/acids mixture can be detected at a level as low as 50 g/kg by NIR [48]. Similarly, the adulteration of strawberry or raspberry juice with apple juice can be detected at level $> 10\%$ by transmittance NIR coupled with PLS [49]. MIR spectra have been used to detect adulteration of pure pomegranate juice with grape juice (2%-14% v/v) [50] and to predict the percent fruit content in strawberry jam [51]. Adulteration of honey and maple syrup can also be detected by NIR and MIR spectroscopy. NIR and FTIR have been successfully applied for the detection and quantification of cane and beet sugars in maple syrup [52]. Attenuated total reflectance (ATR)-FTIR coupled with LDA and PLS was used to discriminate the type of adulterant in three different honey varieties. A success rate of prediction of 100% was achieved for honey samples adulterated with 7-25% w/w of simple (glucose, fructose, sucrose) and complex (beet and cane invert) sugars [53]. NIR and MIR have also been employed for the authentication of lard and fats. Lard adulteration can be detected in cake [54] and in chocolate [55]. The adulteration of shortening with lard can be detected at levels ranging from 0 to 100% and a standard error of calibration (SEC) of 1.75 by using the regions $1.117\text{-}1.097\text{ cm}^{-1}$ and $990\text{-}950\text{ cm}^{-1}$ of the NIR spectrum. Adulteration of

olive oils has also attracted much attention due to the economic value of the product. Adulteration of EVOO with palm oil can be detected by FTIR and PLS in concentration varying from 1.0 to 50.0% w/w [56]. The region 1500-1000 cm^{-1} of the MIR spectra was used for the regression model. The adulteration of EVOO with sunflower, corn, soyabean and hazelnut oil can be detected at level as low as 5% by using FTIR and LDA [57]. In this latter case, the normalized absorbance of peaks and shoulders areas were used in the model as predictors. The standard of identity for butter require that no vegetal oil nor margarine is added to the product. The presence of margarine can be detected by NIR coupled with PLS in the range 0-100% with a standard error of calibration after validation (SECV) <1.2% [58]. IR spectroscopy has been also widely used to predict and control meat quality. The discrimination between fresh and frozen-thawed beef can be accomplished by IR spectroscopy due to modification of the myofibrillar proteins and the corresponding change in their water holding capacity [59].

Infrared spectroscopy is a well-established technique for fast, high-throughput and non-destructive analysis of food and other biological samples. The analysis can be easily implemented on-line, can be automated and does not requires trained personnel to be carried out. It is little expensive and environmental friendly since does not require solvents, chemicals and does not produce waste. However, even though the analysis per se (collection of the spectrum) is fast, post-processing (pre-processing of the spectra and model building) of the input data can be laborious and time-consuming. The calibration models are usually built against reference analytical methods so that the measurement errors accumulated and the total predictive error increases. Finally, the classification or regression models are theoretically valid only on the instruments with which the training and the calibration has been carried out. The transfer of a multivariate model to other instruments affects its precision and accuracy compared to the original ones.

A case study on NIRS and adulteration.

As an example of the potential of the NIR Spectroscopy for the detection of adulteration, the results of an investigation that has been carried out in the authors' research group will be shown. NIR spectra were used to detect the presence of nitrogen replacers in milk powders. The compositional standards for milk powders require that the amount of milk proteins in milk solids-not-fat should be at least equal to 34% m/m, unless declared. The low prices of some nitrogen containing compounds make them attractive as potential adulterants to increase the level of apparent proteins in milk powders. The Kjeldahl method (official reference method for proteins content) measures the total amount of N irrespective of whether it comes from proteins or not. Expensive and time-consuming analytical methods such as enzyme-linked immunosorbent assay (ELISA), LC-MS/MS and GC-MS/MS are necessary for confirmatory analysis of melamine and its analogues in milk powders. To prove the potential of NIR spectroscopy for the detection of such adulteration, 33 skim milk powders were randomly adulterated with adulterants ammonium chloride, caprolactam, diammonium phosphate and polyvinylpyrrolidone (PVP) in order to produce an increment of 0.10, 0.50, 1.00 and 2.00% in the (apparent) proteins content of the milk powder. The samples were measured by NIR spectroscopy and the spectra subjected to PLS-DA analysis. A few milk

powders were randomly selected and adulterated by melamine, ammeline and urea to test the robustness of the predictive models.

A PLS-DA model was first developed to predict the type of adulterant. The training set consisted of 80% genuine milk powders (26 samples) and 80% adulterated samples (19 samples from each adulterant) which were randomly selected. The remaining 20% of the samples, and those adulterated with the non-modelled adulterants melamine, ammeline and urea were used for external validation. The success rate of prediction was 100% in cross-validation and 78% for the external validation set (Table 2). Three out of 6 samples adulterated with melamine, ammeline and urea were correctly predicted as adulterated.

Class item	Genuine powder	+ NH ₄ Cl	+ caprolactam	+ (NH ₄) ₂ HPO ₄	+ PVP	No match
PLS-DA model based on training set						
Genuine powder	26	-	-	-	-	-
+ NH ₄ Cl	-	19	-	-	-	-
+caprolactam	-	-	19	-	-	-
+ (NH ₄) ₂ HPO ₄	-	-	-	19	-	-
+PVP	-	-	-	-	19	-
External validation						
Genuine powder	7	-	-	-	-	-
+ NH ₄ Cl	-	4	-	-	1	-
+caprolactam	-	-	3	-	1	1
+ (NH ₄) ₂ HPO ₄	-	-	-	4	-	1
+PVP	-	-	-	-	3	2
External validation based on melamine, ammeline and urea						
+melamine	-	-	-	-	-	2
+ammeline	2	-	-	-	-	-
+ urea	1	-	-	-	-	1

Table 2. Prediction results of PLS-DA model for the type of adulterant in milk powders

A PLS-DA model was then developed to discriminate generally between genuine and adulterated samples. The training set and the validation set were built in the same way as previously described. The PLS-DA scores plot is presented in Figure 4. The prediction results are reported in Table 3.

In external validation only one genuine sample was wrongly predicted as adulterated. One adulterated sample was incorrectly predicted as genuine (PVP added at its lowest concen-

tration) and 3 adulterated samples could not be classified. Samples adulterated with melamine, ammeline and urea were all correctly predicted as adulterated.

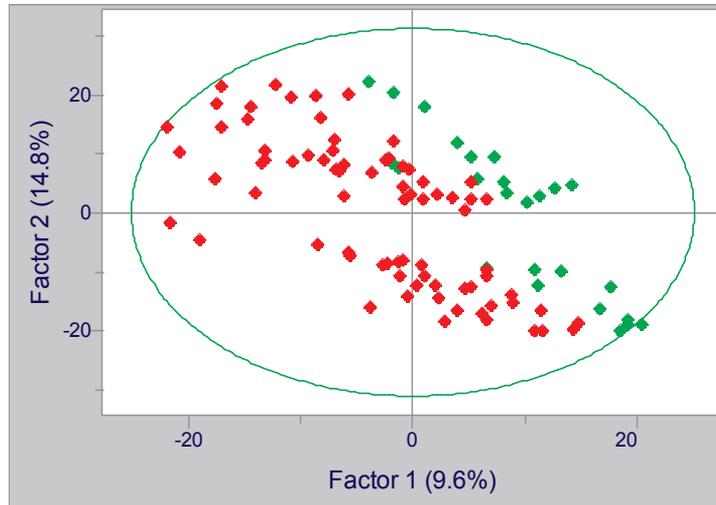


Figure 5. PLS-DA scores plot of IR spectra for genuine and adulterated milk powders. Red = adulterated powders; green=genuine powders.

Class Item	Genuine powder	Adulterated powder	No match
Internal validation			
Genuine powder	24	2	-
Adulterated powder	0	68	8
External validation			
Genuine powder	6	1	0
Adulterated powder	1	22	3

Table 3. Prediction results of PLS-DA model for genuine versus adulterated milk powders.

NIRS combined with chemometrics proved to be a promising tool for the cost-effective detection of adulteration of milk powders with a range of nitrogen replacers. The reliability of the classification models can be improved by the careful selection of those regions of the NIR-visible spectrum which showed the best discrimination power between genuine and adulterated samples.

7. Other fingerprintings

Differential scanning calorimetry (DSC)

Differential scanning calorimetry is a thermoanalytical technique that measures the amount of heat required to increase the temperature of a sample relative to a reference material. When the amount of heat absorbed/released is plotted as a function of temperature a thermal spectrum of the sample is obtained from which kinetic and thermodynamic information such as the heat capacity and the enthalpy of any phase transition (fusion, evaporation, glass transition etc) the sample undergoes in the temperature span of the experiments can be determined. Those physical properties depends in turn on compositional and structural properties of the samples.

DSC has been mainly applied for the authentication of fats/oils. The level of adulteration of canola oil with lard, beef tallow and chicken fat as well as that of virgin coconut oil with palm kernel oil (PKO) and soybean oil can be predicted by the analysis of the DSC thermogram and stepwise multilinear regression (SMLR) [60]. The melting profiles of cow, goat, sheep, camel, horse and water buffalo milk fat samples were also determined by DSC. Differences in the DSC profiles for the fat fraction of milk of different species can be attributed to their fatty acid/TAG composition. In general, the melting point of the fats decreases with decreasing chain length and increasing degree of unsaturation of the fatty acids in the milk. The thermograms were subjected to PCA analysis which showed a clear distinction of the four milk samples analysed. The loadings plots of the heat flow data showed that the data are most influenced between the temperatures of 13° and 24° C [61].

Sensor technology

Sensor technology is often referred to as electronic nose technology. In this technology the volatile compounds present in the headspace of a sample are detected by an array of semi-selective sensors. Normally each sensor is sensitive to all the volatile but each in a peculiar way. There are many sensors for EN instruments such as metal oxide sensors, conducting polymer sensors, quartz crystal membrane sensors, or mass spectrometers (MS-EN) available on the market. The sensor's response is then transformed in a spectrum that represents a fingerprint of the volatile compounds of the tested sample. Electronic nose analysis are often cheaper and faster than GC analysis and the sample preparation is usually quite simple. The technique, however, does not have the same sensitivity of other techniques. Furthermore, single volatile compounds cannot be identified and the signal is sensitive to water vapour.

Electronic nose has been used mainly for the detection of adulteration in fats/oils. Rapid detection of pork and lard in food samples for halal authentication (compliance with Islamic dietary rules) are reported with electronic nose [62]. Under Jewish and Islamic dietary laws, foods containing porcine-based ingredients such as lard are strictly prohibited from consumption. Electronic nose and chemometric analysis was applied for the detection of adulteration of olive oil samples with sunflower and olive-pomace oil at levels as low as 5% [63]. Application are also reported for the authentication of the geographical origin of Italian wines [64], and emmenthal cheese [65].

Transcriptomics

Transcriptomics is a post-genomic technique that consists in the simultaneous measurements of all the transcripts (mRNA molecules) in a given organism, or of a specific subset of transcripts present in a particular cell type. Unlike genome, transcriptome varies according to the environmental conditions and represents the genes that are actively expressed in a certain cell at a certain time. Transcriptome is usually obtained by DNA microarray technologies and reverse PCR. The set of all the mRNA produced (and hence of the genes actively expressed) represents a fingerprint of that target cell/organism and can thus use for fraud detection and authentication purposes. At the present the potential of transcriptomics in this respect is still underexplored.

8. Fingerprinting options for other biomaterials

Biofuels are an important environmental-friendly alternative to fossil fuels. The term biodiesel refers primarily to FAME obtained after transesterification of triglycerides with methanol. The methyl esters can be produced from many different triglyceride sources, primarily rapeseed oil but also sunflower oil, soybean oil, palm oil, linseed oil, tallow, and used frying oil. Blends of biodiesel with conventional petrodiesel fuel represent a common utilization of biodiesel. The ability to predict retail biodiesel blend percent composition is important to detect adulterations. It has been reported that biodiesel from different sources can be discriminated by direct infusion ESI-MS and multivariate statistics [66]. In addition, the % of rapeseed or salmon biodiesel in petrodiesel in concentrations ranging from 0.5% to 10% can be predicted with good accuracy by applying a PLS model. Similarly, the feedstock source of blends of biodiesel and conventional diesel, as well as the % composition of the blend can be predicted applying a kNN and a PLS model respectively to the total ion current chromatograms from gas chromatography–quadrupole mass spectrometry (GC–qMS) using a polar column [67]. The precision of the prediction was between 4-5%. Furthermore, the addition of residual oil (non-transesterified residual vegetable oil) is one of the easiest ways of adulterating biofuels. Synchronous fluorescence combined to LDA can be used to discriminate between diesel oils, biodiesels and biodiesel adulterated with residual oil and the % of residual oil can be predicted with good accuracy when a PLS model is used [68].

Perfume counterfeiting is an illegal practice that causes huge economical loss to the perfume industry and pose potential health risk to consumers who might be exposed to harmful chemical from counterfeited products. Traditionally, the quality control analyses for perfume focuses on volatile and semi-volatile compounds and are performed by GC based techniques. Recently, more straightforward methods have been proposed for the fast authentication of premium perfumes and detection of adulteration/counterfeiting. A fingerprint of the polar compounds can be achieved by direct infusion EASI-MS (see section 3) [69]. The samples are sprayed onto a glass rod and directly exposed to the ionisation source of the MS system. An almost instantaneous, simple and reproducible fingerprint of the polar compounds in the product is obtained that allows a complete discrimination between au-

thentic and counterfeited products. A fast discrimination between authentic and counterfeited products can be also obtained by fingerprinting of the polar compounds by ESI-MS in the positive ion mode and chemometric analysis previous extraction of few μL of the sample in a 1:1 methanol/water solution [70]. Even more recently, a fast, simple and low-cost method for the authentication of perfume based on a commercial electronic olfactory system (EOS) equipped with thin film metal oxide semiconductors has been proposed [71]. The PCA analysis of the R/R_0 values (resistance of the sensor in the presence of the volatile compounds relative to that of the sensor balanced in air) generated by 6 sensors based on different metal oxide semiconductors can unequivocally discriminate between authentic and counterfeited perfumes. The prior removal of ethanol from the samples is necessary for the correct discrimination of the samples.

Essential oils are also widely employed for their fragrance in perfumery but also in cosmetics and household products. One of the most common fraud is the blend of valuable essential oils with other less valuable alternatives. As an example, the valuable Rosewood essential oil, obtained from the trees of *Aniba rosaeodora* Ducke and employed in fine perfumery, can be blended with the far cheaper synthetic linaol, obtained by re-distillation of Rosewood leaf oil. The ESI-MS fingerprint in the positive ions mode of the polar compounds extracted by an acidified 1:1 water methanol solution can easily detect adulteration of Rosewood oil with synthetic linaol at concentration as low as 10% v/v [72]. A PCA model is necessary to extract the relevant information from the fingerprint.

Direct infusion MS can be used to authenticate wood as well. Venturi easy ambient sonic spray ionization (V-EASI)-MS fingerprint of a very simple methanolic extract of wood chips or directly acquired from the freshly scratched wood surface may help to control the illegal logging and trade of the noble Mahogany tropical wood and its falsification [73]. V-EASI-MS is a novel ambient ionization MS technique characterized by sonic spray ionization and a self-pumping system based on the Venturi effect. It allows the direct analysis of solid or liquid samples. Ionization is assisted by compressed nitrogen and the apparatus is thus free of electrical discharge, thermal interferences since no heating, voltage or radiation is used. The introduction of fast and high-throughput analytical techniques for wood authentication is especially valuable since the classical controls are based on time-consuming morphological evaluations. Recently an original and fast approach for the authentication of wood species has been proposed. It is based on the analysis of the volatile compounds measured by a low-cost conductive polymer-based portable electronic nose formed by an array of only three gas sensors and/or the elemental fingerprint measured by laser-induced breakdown spectrometry (LIBS) which performs a multielemental and direct analysis even in solid samples [74].

The assurance of quality of herbal supplements and medicines is a major concern for the phytopharmaceutical and the food industry. The identification of the herbal drug and the presence of adulterant is a mandatory test to ensure the quality, the efficacy and the safety of a medical preparation or an herbal supplement. Among a variety of quality control methods, chromatographic fingerprinting has gained more and more attention recently and have been used to authenticate a large number of herbal products. They are accepted by many international organization for the quality control of herbal medicine but are relatively time-consuming. More recently, spectroscopic techniques have been explored to rapidly authenti-

cate herbal products. For instance, different species of *Echinacea*, e.g., *E. purpurea*, *E. angustifolia*, and *E. pallida* are used for commercial preparations to prevent or cure the common cold, flu, and several other diseases due to their nonspecific stimulating effect of the immune system. It is also well known that *Echinacea* preparation are commonly adulterated with roots of *Parthenium integrifolium* L. This adulteration can be detected by NIRS at a minimum of 10% of adulteration [75]. The method requires just the milling of the sample and can be carried out within 1 minute.

The list of potential applications of products fingerprinting is not limited to the cases discussed above and many other examples may be provided. In the authors' research group for instance, PTR-MS and ICP-MS are used for the authentication of the geographical origin of flower bulbs. Counterfeiting of pharmaceuticals is another area where the application of fingerprinting techniques has proved of great help. The topic is so huge that we would address the interested readers to specialized publications.

9. Conclusions

Product fingerprinting combined with chemometrics represents a valuable tool for fraud detection and control for food products and other biomaterials. A fingerprinting approach is particularly useful:

- For the authentication of products for which target analyses based on specific markers are not available
- For the detection of adulteration based of yet unknown adulterants
- For a fast and high-throughput screening of the samples before more elaborated confirmatory analysis are applied.

At the same time a fingerprinting approach may substantiate nutritional, sensory or other product qualities.

LC, GC, and IR spectroscopy are already common instrumental platforms available in most QA laboratories and they will continue to provide valuable support for food fraud prevention. IR and other spectroscopic techniques have the great advantage of providing fast, high-throughput and non-destructive analyses with limited costs. They can be easily automated and adapted for *in-line* or *in-situ* analysis which makes these techniques well suited for implementation in the industrial setting. MS and NMR are not as common in QA laboratories, principally because of their high costs but they may become more important in routine QA testing because of their superior performances. However, even though the costs of MS and NMR instruments is still very high, the cost per sample can be very low if a high samples turnover can be achieved.

From an analytical point of view, a further improvement is expected in the future from the broader application of multi-dimensional separation techniques such as GCxGC or LCxLC which provide enhanced resolution and an higher number of peaks. Ultra-performance liq-

uid chromatography (UPLC) and CE may also provide rapid separation with limited costs. The application of novel (or still underexploited) ambient ionisation MS techniques that allow the rapid analysis of liquid and solid samples with little, if any, preparation will be particularly valuable in the future.

The applicability and the reliability of a fingerprint approach also depends from the correct and tailored usage of the relevant and appropriate chemometric tools. For the development of regression and/or classification models, special care should be devoted to ensure:

- The representativeness of the classes considered, in order to cover all the possible source of variability for the class at stake.
- A robust validation of the model (external validation to be preferred over internal validation).
- The use of the appropriate chemometric tools depending on the problem at stake. Whereas a few pattern recognition techniques are frequently used (PLS-DA, SIMCA, LDA..), some other such as classification and regression trees (CART), quadratic discriminant analysis (QDA) are still underexploited despite the good results that they can provide.

Another key aspect is represented by the validation of methods based on fingerprinting and chemometrics that is essential for their application in a commercial context. Whereas standards exist for the validation of regular analytical methods (see for instance, Commission Decision 2002/657/EC and ISO 17025), internationally accepted protocols for the validation of methods based on fingerprinting techniques and chemometric classification models are lacking at the present. Such protocols should indicate the performance characteristics that have to be checked and the criteria to be met in order to verify the compliance of the method with the performance characteristics.

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Principle of Meat Aroma Flavors and Future Prospect

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Additional information is available at the end of the chapter

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1. Introduction

The population growth fact of the world has been much quickly increasing through the years. As reported by the United Nations Population Fund (UNFPA) the estimated world population of 6.1 billion in the year 2000 and reached to 7 billion in the year 2011, increased 0.9 billion people only after 10 years. The population increases always proportionally accompany to the consumption demands in which including foods. Calculating the global meat consumption only and based on the data collected from IFPRI/ FAO/ILRI by Delgado et al (1999) [1] suggested that global production and consumption of meat will continue to raise from 233 million metric tons in the year 2000 to 300 million metric tons in 2020. On the other hand, income growth of people in most of the countries especially in the developed countries has been significantly increasing in the recent years. Combination of the large populations together with a high-income that will give a big pressure for the food producers in general and meat producers in particular. As a consequence, higher income growth in countries has led to an increase in living standards and changes in consumer diets to include a higher proportion of meat and meat products. While, productivity and provision of meats on the markets has been limited and rising costs of production resulting in not keeping pace with the strong growth in demand, that has caused a rise in meat prices.

Although, a strong demand for meat amounts but consumers are getting quite fastidious to choose meat and meat products since consumer's preference for meat buying is strongly based on quality, freshness and hygiene. Quality factors are very important in the meat purchasing behavior of consumers including marbling (intramuscular fat tissues), texture, color, tenderness and especially flavor characteristics.

Aroma flavor characteristics of cooked meat in particular play the most important level in eating quality of meat, acceptance and preference by consumers. The aroma flavor charac-

teristics of cooked meats are derived from volatile flavor components which derive from thermally induced reactions occurring during heating via the four pathways including (1) Maillard reaction of amino acid or peptides with reducing sugars, (2) Lipid oxidation, (3) interaction between Maillard reaction products with lipid-oxidized products and (4) vitamin degradation during cooking [2]. Aroma flavor is perceived through the nostrils (orthonasal aroma) it gives the first impression of a certain food. When the food is placed in the mouth, the volatile flavor compounds will be transferred through the pharynx to the olfactory receptors (retronasal aroma). It has been reported that flavors together with other sensory attributes such as tenderness and juiciness are specially considered the most important criterion of acceptability and the palatability of meat that affects consumer's purchasing decisions [3,4]. It has been well known that all volatile flavor components are organic and they have low molecular weight [5]. The chemical structures of volatile flavor classes are varied widely including aldehydes, ketones, hydrocarbons, pyrazines, acids, esters, alcohols, nitrogen and sulfur-containing compounds and other heterocyclic compounds as well. Due to the differences in chemical structures therefore their volatility is also quite different.

Many factors have been found to be as influences on the aroma flavors of cooked meat. Rabe et al (2003) [6] found that among all food constituents, lipids generally have the greatest influence on production of aroma flavor components, as they not only reduce the vapour pressure of most flavor compounds. Otherwise, Kinsella (1990) [7] showed that aroma compounds are more lipophilic than hydrophilic therefore fats act as a solvents for aroma compounds reducing their volatility. In addition to these effects, other factors such as diets, breed, sex, chiller ageing, meat pH, cooking conditions which all also affect the flavor [8, 9, 40, 11]

With the crucial importance of aroma flavor of meat for the acceptance and preference of consumers and as well as the factors influencing the generation of aroma flavor compounds as mentioned above, the present chapter aims to highlight the basic information regarding aroma flavor components in terms of mechanisms of formation pathways; current techniques being used for detection; factors that affect aroma flavors; and final ideas and as well as suggestions are also given out to improve flavor quality attributes according to criterion of acceptability, satisfaction and the palatability for consumer.

2. Meat aroma flavor

2.1. The importance of volatile flavor compounds in contributing to the flavor characteristics of cooked meat

Flavor characteristics of cooked meat are directly detected by the nose (i.e., olfactory receptors) before and during chewing. Raw meat has little aroma and only blood-like taste, meat develops its aroma flavor characteristics during cooking as the result of complex interaction of precursors derived from both the lean and fat compositions of meat generating volatile flavor compounds that contribute to meat flavor [12]. To date, approximately thousands of volatile flavor compounds have been detected and identified in cooked meat. There is a large number of these compounds contributing to the flavor characteristics of cooked meat have been identified in previous works [13, 14, 15, 16, 17, 18, 19].

As mentioned above, regarding the chemical structures of volatile flavor classes, among that the heterocyclic compounds especially those containing sulfur are the important flavor compounds produced in the Maillard reaction providing savory, meaty, roasty and boiled flavor characteristics. While, lipid-degraded- compounds which give 'fatty' aromas to cooked meat and compounds which determine some of the aroma flavor differences between meats from different species [20]. The individual volatile compounds have been found to determine distinct aroma flavors of cooked meat represent; dimethylsulfide, 2-butanone, ethyl acetate, 2- and 3-methylbutanal, 2-heptanone, dimethyl trisulphide and nonanal were detected as key flavor compounds of cooked Irish Angus beef, while methional, 2,4-nonadienal and bezothiazole were characterized as meaty, oily notes in cooked Belgian Blue, Limousin and Aberdeen Angus beefs [21]. Kerscher & Grosch, (1997) [22] reported that 2-furfurylthiol, 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone and 2-methyl-3-furanthiol were the most important odorants of boiled beef. 2-ethyl-3,5-dimethyl pyrazine and 2,3-diethyl-5-methylpyrazine possess roasty, caramel-like, burnt and earthy notes of roasted beef [23]. Other carbonyl compounds such as methional, E-2-undecenal, E-2-dodecenal, decanal, heptanal and 2-methylbutanal also were found to be associated with roasty, sweet, fruity and fatty odor notes of cooked beef [14,17]. Also, a great number of studies considered on the objective volatile flavor components in cooked pork, chicken, lamb, ham and etc... have been documented over the last years [24, 25, 26]. In fact, although thousands of volatile compounds identified but not all of them are important because their high odor detection threshold, only some of them play a significant role in the overall aroma flavor characteristics of cooked meat. An aroma flavor compound with its distinct odor note can be defined as its flavor dilution factor indicating that at the lowest concentration at which the compound still can be detected by the sense of smell. Some represent volatile flavors active-compounds have been detected in cooked meats by using gas chromatography-olfactometry technique (GC-O) are showed in Table 1.

Compound name	Aroma flavor characteristics
<i>Aldehydes</i>	
Methional	Cooked potato, meaty
E,2-nonenal	Fatty
E,E,2,4-decadienal	Fatty
Benzenacetaldehyde	Sweet, honey
E,E,2,4-nonedial	Fatty
Decanal	Sweet, fruity, like aldehydes, roasty
Heptanal	Fruity, fatty, sweet, oil
Nonanal	Sweet, fatty, green
Undecanal	Sweet, pungent, green
E,2-heptenal	Fatty
E,2-heptenal	Fatty

Hexanal	Green, fatty
E,2-hexenal	Green
E,Z,2,6-nonadienal	Cucumber
Undecanal	Sweet, pungent, green
2-methylbutanal	Pungent, sweet, roasty
E,2-undecenal	Sweet, fruity, fatty
2,E-dodecenal	Sweet, fruity, roasty, pungent
Ethanol	Grilled (weak), acetaldehyde-like
3-methylbutanal	Meaty, fish, rotten, aldehyde, valeric acid, fatty
Octanal	Green, lemon, citrus, aldehyde
E,E,2,4-heptadienal	Aldehyde, green, broth, spicy
Propanal	caramel, sweet, alcoholic, "cooked", broth, spicy
Butanal	smoky, fish, amylic, aldehyde-enal or dienal
<i>Ketones</i>	
2-octanone	Fruity, musty
2-decanone	Fruity, musty
2-dodecanone	Fruity, musty
1-octen-3-one	fresh, mushrooms, pungent, rubbery
3-octanone	Fruity, nutty, moldy, fatty, earthy
2,5-dimethyl-4-hydroxy-3(2H)-furanone	Roasted almonds, sweet
4,5-dihydro-5-propyl-2(3H)-furanone	Fruity, fatty, sweet, pungent, roasty
2,3-butanedione	Sweet, buttery
2-heptanone	Citrus grapefruit, limonene, floral, cheese
2,3-pentanedione	buttery, lemon-like, sweet, fruity
2-nonanone	Hot milk, soap, green, fruity, floral
3-octen-2-one	Nut, crushed bug, earthy, spicy, sweet, mushroom,
6-Methyl 2-heptanone	Cloves, menthol
2-undecanone	Fruity
2,2,6-Trimethylcyclohexanone	Mint, acetone
<i>Alcohols</i>	
1-octen-3-ol	Mushroom
Cyclobutanol	Roasted

1-heptanol	Fragrant, woody, oily, green, fatty, winey, sap
1-hexanol	Woody, cut grass, chemical-winey, fatty, fruity
2-Ethyl 1-hexanol	Resin, flower, green
1-octanol	Penetrating aromatic odor, fatty, waxy, citrus, oily,
2-Octen-1-ol	Green citrus
1-pentanol	Mild odor, fuel oil, fruit, balsamic
Propanol	Alcoholic
<i>Hydrocarbons</i>	
Ethylbenzene	Pungent, aromatic, fragrant, roasty
1-undecen	Fatty, burnt, nutty, rubbery
Hexane	Faint peculiar odor
(Z)-3-Octene	Fruity, old apples
Pentane	Very slight warmed-over flavor, oxidized
Styrene	Penetrating odor, sweet smell
Tridecane	Alkane
Tetradecane	Alkane
Ethylbenzene	Aromatic, fragrant, roasty
<i>Pyrazines</i>	
2-ethyl-3,5-dimethylpyrazin	Burnt, fragrant, meaty, green
2-ethenyl-3,6(5)-dimethylpyrazine	Sweet, cooked rice, fatty
2-ethyl-3,6-dimethylpyrazine	Burnt, roasty
2,3-diethyl-5-methylpyrazine	meaty, roasty, fragrant, sweet
2,5-dimethylpyrazine	Fried rice, popcorn, pungent, green
2-ethenyl-5(6)-methylpyrazine	Roasty break-like, cooked rice, coffee-like
2,5-dimethylpyrazine	Fried rice, popcorn, pungent, green
2-ethyl-5-methylpyrazine	Fruity, sweet, pungent
2-ethenyl-5(6)-methylpyrazine	Smoky, roasty, break-like, cooked rice, popcorn
2-ethyl-3,6-dimethylpyrazine	Burnt, pungent, roasty
2-ethenyl-3,6(5)-dimethylpyrazine	Pungent, sweet, cooked rice, fatty
2,3-diethyl-5-methylpyrazine	Meaty, roasty, fragrant, sweet
2-isopentyl-3,6-dimethylpyrazine	Sweet, fragrant, fatty, fruity, pungent
Sulfur & nitrogen containing compounds	
2-furfurylthiol	Roasty

2-acetyl-1-pyrroline	Roasted, sweet
2-formyl-5-methylthiophene	Sulfurous
2-methyl-3-furanthiol	Meaty, sweet, sulfurous
Benzylthiol	Sulphurous
2,4-dimethylthiazole	Rubbery, moldy, fruity, pungent
2-acetylthiazole	Roasted
Dimethyltrisulfide	Fragrant, musty, roasty, rubbery
2-acethylthiophene	Sulphurous, sweet
Bis(2-methyl-3-furyl)disulfide	Meaty-like
Benzothiazole	Metallic
Dimethyldisulfide	Moldy, pungent, rubbery, onion-like
2,4-dimethylthiazole	Rubbery, moldy, fruity, pungent
4,5-dimethylthiazole	Smoky, roasty, fragrant, nutty
2-methylchinoxaline	Aromatic, roasted, nutty, sweet, fruity, fatty
3-mercapto-2-butanone	Fried onion, sulfury, cooked meat
2-mercapto-3-pentanone	Brothy, mashed potatoes meaty, roast meat
2-[(methylthio)methyl]furan	Brothy, spices, roast, fatty
3-[(2-furanylmethyl)dithio]-2-butanone	onion, burnt rubber, burnt wood

Table 1. The representative volatile flavor compounds with their aroma flavor characteristics found in cooked meat. [References: 13, 14, 20, 15, 27]

2.2. Precursors of meat flavor

Earlier studies on meat flavor, researchers recognized that the low molecular weight, water-soluble compounds and fats in meat constituents are the most important precursor of aroma flavor characteristics of cooked meat [28, 29]. The flavor precursor of meat namely, free sugars, free amino acids, peptides, vitamin, sugar phosphate, nucleotide-bound sugars and nucleotides [30, 31, 32, 33], all of them are able to either participate the Maillard reaction or oxidation/degradation and interaction on heating to generate volatile flavor compounds then create the final aroma flavor characteristics of cooked meat. It is suggested that these precursor components found to contribute to the development of meaty flavor, while the adipose tissues and intramuscular fat not only occupy an important role in development of flavor characteristics of cooked meat but also contribute to the characteristic-specific species flavors. This means that the distinct flavor characteristics between the meats from different species are due to the intramuscular fat content and not from water-soluble precursor compounds. The details on flavor precursors of meat found in the past years are showed in Table 2. However, researchers found that the roles of these flavor precursors in the development of flavor characteristics

of cooked meat are not similar. Macey et al (1964) [28] found some sugars present in beef such as glucose, fructose, mannose and ribose, in that ribose was the most heat-labile sugar among these whereas fructose was the most stable. Among the amino acids present in meat, cysteine and selenocysteine are two sulfur-containing amino acids, the reaction of these with other sugars lead to formation of many sulfur-containing flavor compounds [34], while the reaction of other non-sulfur containing amino acids with sugars dominated by the nitrogen-containing products such as pyrazines [72]. In the recent years, researchers have found that the flavor precursor components in meats are influenced by several factors. Koutsidis et al (2008) [31] indicated that diets significantly affected the reducing sugars in beef *longissimus lumborum* muscle, higher total reducing sugars was obtained in beef from concentrate feeding group compared to the grass silage feeding group whereas beef from cattle fed with grass silage had higher level of free amino acids. When the beef was chiller aged for several days at chilling condition resulted in several times increase in free sugars such as ribose, free amino acids also increased with conditioning especially phenylalanine, methionine, lysine, leucine and isoleucine were the amino acids showing the greatest increase with conditioning time [32]. Meinert et al (2009) [35] have found that feeding, fasting and post-mortem ageing factors significantly influenced the concentration of flavor precursors of beef *longissimus dorsi* muscle. Additionally, the recent works also showed that fat-supplemented diets had large effect on the fatty acid compositions, for instance, dietary linseed oil and soybean oil significantly increased the contents of C18:3 and C18:2 in the neutral lipids and phospholipids in both *longissimus* and *biceps brachii* muscles [36], and subsequently influence the volatile flavor compounds of cooked beef [19, 25, 37].

Flavor precursors	Names in detail	Reference
Free amino acids	Systine; systeine; glycine; lysine; alanine; valine; isoleucine; leucine; threonine; serine; proline; asparagines; aspartic acid; methionine; glutamic acid; phenylalanine; glutamine; ornithine; histidine; tyrosine; tryptophan; arginine.	[38,39,40, 31,32,32]
Reducing sugars	Ribose; glucose; xylose; starch; mannose; fructose; maltose; mannose 6-phosphate, glucose 6-phosphate; fructose 6-phosphate; ribose 6-phosphate.	[38,39,72, 41,31,32]
Fats/ lipids	Triglycerides and phospholipids Oleic acid (C18:1n-9) Linoleic acid (C18:2n-6) Linolenic acid (C18:3n-3) and etc.	[42,19, 43,34]
Vitamin	Thiamin	[33,44]
Nucleotides and peptides	Glutathione; carnosine inosine; inosine monophosphate; inosine 5'-monophosphate; guanosine 5-monophosphate; creatine; creatinine; Hypoxanthine and etc.	[45,44, 31,32]

Table 2. The representative precursors of meat flavor.

2.3. Pathways for the formation of volatile flavor compounds

2.3.1. Maillard reaction

Maillard reaction, a non-enzymatic browning which plays an important role in generation of volatile flavor compounds and appearances of the cooked foods, it is due to most of important volatile flavor compounds found in cooked foods are originated from this reaction. Otherwise, Maillard reaction also can produce antioxidative components and toxicological implications as well. However, in the present chapter we are focusing on the Maillard reaction in relation to aroma flavor characteristics, particularly the formation of volatile flavor compounds in cooked meat. Maillard reaction was firstly mentioned in the early time, 1912 by Maillard [46] since he wanted to investigate the browning reaction between glucose and glycine. After that many studies focused on determining the fundamentals and mechanisms of this reaction [47, 48, 49].



Figure 1. General stages of Maillard reaction showing the formations of flavor compounds (based on van Boekel, 2006) [51].

The Maillard reaction is taken place with the participation of reducing sugars (e.g., ribose, glucose) and free amino compounds (e.g., amino acids, amines, peptides, proteins, ammonia) at certain heating condition to produce the Maillard products, and usually this reaction is divided into three main stages. In which the firstly initial stage starts with a condensation between a reducing sugar and an amino group, the loss of water from this molecule produces an amine that is able to cyclise resulting in formation of an N-glycosylamine (a sugar attached to NR_2 group) or called Amadori product. The next intermediate stage involves the rearrangement and decomposition of the Amadori product to release amino group and sugar fragmentation. The final stage of Maillard reaction is leading to dehydration, fragmenta-

tion, polymerization and cyclization reactions. A general scheme of the Maillard reaction is given in Figure 1.

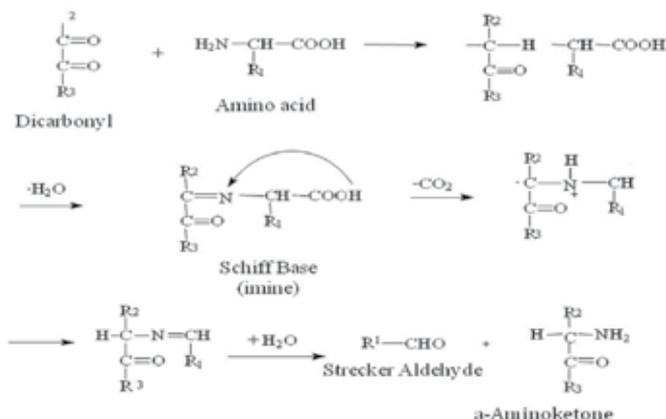


Figure 2. Strecker degradation mechanisms, a part of Maillard reaction

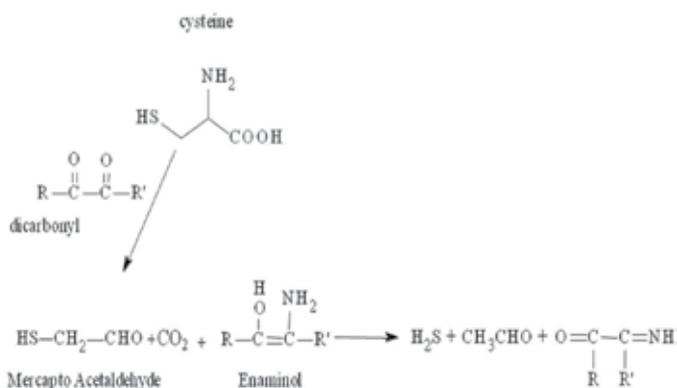


Figure 3. The formation of H₂S from the Strecker degradation of cysteine

Among events occurring in the Maillard reaction, Strecker degradation is one of the quite important events, in which amino acids are undergone degradation processes (oxidative deamination and decarboxylation) in the presence of a dicarbonyls compound formed from Maillard reaction. The Strecker degradation processes lead to formation of aldehydes (e.g., fufural) and aminoketone (Figure 2). Especially the other important intermediate products such as H₂S, NH₃, etc are also formed from the Strecker degradation by sulphur-containing amino acids such as cystein and systine (Figure 3); all of these intermediate products can further react with other compounds or with each other to produce low and high molecular weight end flavor compounds.

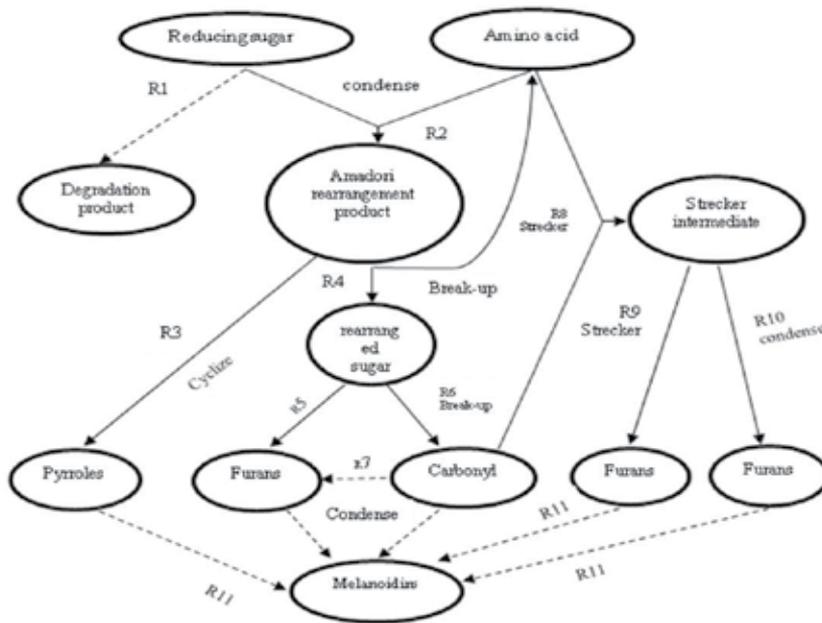


Figure 4. Kinetic scheme of flavor formation by Maillard reaction (Jousse et al., 2002) [50]

Flavor class	Characterized Flavor/aroma notes	Remark
Pyrazines	Cooked, roasted, toasted, baked cereals	
Alkylpyrazines	Nutty, roasted	
Alkylpyridines	Green, bitter, astringent, burnt	Unpleasant flavor
Acetylpyridines	Cracker-like	
Pyrroles	Cereal-like	
Furan, furanones, pyranone	Sweet, burnt, pungent, caramel-like	
Oxazoles	Green, nutty, sweet	
Thiophenes	Meaty	Formed from heated meat by the reaction of cysteine and ribose

Table 3. Some representative classes of flavor compounds formed from the Maillard reaction (based on van Boekel, 2006) [51].

The formation of volatile flavor compounds in the Maillard reaction largely depend on the reactants (e.g., the nature of reducing sugars and amino acids participated) and also the cat-

alytic condition (e.g., heating temperature, moisture, pH). For the type of reducing sugars and amino acids which determine the kinds of flavor compounds generated for instance, many sulfur-containing flavor compounds are formed from the Maillard reaction between cysteine and ribose [34] whereas, the nitrogen-containing compounds (e.g., pyrazines) dominated in the Maillard reaction containing glucose and lysine [72]. Therefore, it should be noted that nature of reactants will require the kinds of Maillard products. For the catalytic condition of Maillard reaction, it usually influences the kinetics of flavor compound generation by Maillard reaction in that depending on each catalytic condition (temperature, pH and etc) will determine the yields and also kinds of Maillard products. The kinetic of flavor compound formation resembles the scheme in Figure 4 with 11 determining steps [50]. Based on the kinetic scheme it shows that there are many chemical classes of flavors are formed via the Maillard reaction, some of the representative classes associated with odor notes are showed in Table 3.

2.3.2 Lipid oxidation and degradation

Lipids and fatty acids play an important role in direct and indirect generating the volatile flavor compounds and some of them contributing to the aroma flavor characteristics of cooked meat. Therefore, the levels of fat contents and as well as fatty acids of meats should be concerned, and it has been reported that the fatty acids of meat are influenced by several factors but almost are the pre-harvest factors such as diets, feed regimes and breeds [52, 53, 54]. Based on our surveillance it seems that the fatty acid profiles significantly vary across the breeds even these breeds are fed with the same diets [55, 56, 57]. Both adipose tissue and intramuscular fat contents are constituted by fatty acids including saturated and unsaturated fatty acids which all are capable to get oxidized and degraded under a certain condition to create a prolific number of volatile flavor compounds [2]. Hundreds of volatile flavor compounds derived from lipid degradation have been found in cooked meat including aliphatic hydrocarbons, aldehydes, ketones, alcohols, carboxylic acids and esters. In general, the odor detection threshold values for the lipid-derived compounds are much higher than those for the sulfur and nitrogen-containing heterocyclic compounds which are formed from the water-soluble precursors via the Maillard reaction. Therefore, the aroma significance of many of these lipid-derived compounds is not as great as that for relatively low concentrations of the heterocyclic compounds. However, certain classes of compounds such as particular aldehydes included saturated and unsaturated aldehydes which containing from 6 to 10 carbons in the structures are major volatile components of all cooked meats and, therefore, they probably play an important part in meat aroma [20]. The oxidation of subcutaneous fat, adipose tissues and intramuscular fat occur in raw meat and continues under the catalysis of many factors such as metals, oxygen, light, heating and etc.

Among the oxidation-induced factors for instance, lights (e.g., ultraviolet) is thought to be thermodynamically capable of production of free radicals directly in lipids, the principles of light-absorbing groups of lipids are double bonds, peroxide bonds and carbonyls which subsequently under the other steps to generate volatiles. And other factors such as oxygen, lipoxygenase, metals and etc which all also affect the lipid oxidation however that is another

er concern, in the present work we only consider on the heat effect that similar to cooking condition to induce the oxidation and degradation of fatty acids in producing volatile flavor compounds of cooked meat. The degrees of heating temperatures have been reported to affect variously lipid oxidation, in that high heating temperatures (e.g., frying, roasting) can have highly sufficient energies to break the single bonds (e.g., C-C or C-H) in the acyl back bonds to generate a lot of lipid alkyl radicals that participate the radical chain formation of oxidation [58]. Lower heating temperatures have lower energies which can break O-O bonds in traces of ROOH. Mottram (1985) [59] also stated that meat is cooked under boiled and lightly roasted conditions, lipid oxidation products dominated the detected compounds, and many of among them such as aldehydes, alcohols, ketones and lactones which have sufficiently low odor threshold to be contributors of meat aroma flavors.

Early work of Mottram et al (1982) [60] found that lipid has a considerable role in meat flavor, when the adipose tissue is added to lean meat does not affect the lipid-derived flavor compounds. A later study by Mottram and Edwards (1983) [42] found that the removal of intramuscular fats and phospholipids from beef caused marked differences in flavor compounds and sensory characteristics as well. So that the intramuscular fat contents (marbling fats) and membrane lipids are the main source of volatile flavor components and make species-specific flavors. However, it has been demonstrated that high levels of lipids especially polyunsaturated fatty acid contents (PUFA) cause undesirable aroma flavors due to their PUFA-derived products lower or inhibit the formation of some heterocyclic Maillard products [42]. This phenomenon has recently been elucidated by researchers when they used model systems. In the model systems containing cysteine, ribose and lipid (e.g., lecithin or individual fatty acids) the concentrations of heterocyclic compounds and especially sulfur-containing compounds were lower several times compared with the model system without lipid content [43, 38, 61, 34]. However, the interaction between the lipid-derived products with Maillard products to form volatile flavor components has been much considered in the previous studies and thought as the important pathway for formation of flavor compounds.

2.3.3. *Thiamin degradation*

Thiamin is considered as a source of meat flavor generated on heating. Researchers found that the thermal degradation of thiamin produces some ended and intermediate flavor compounds [62,63]. It was assumed that thermal degradation of thiamin is a quite complex reaction including various degradation pathways to produce interesting flavor compounds in which most of them contain one or more sulfur and/or nitrogen atoms, and many of them are heterocyclic structures. The thermal degradation of thiamin under the basic condition to produce several flavor compounds is illustrated in Figure 5.

It was reported that the primary products of thermally-degraded thiamin including 4-methyl-5-(2-hydroxyethyl)thiazole which subsequently responds for formation of thiazoles and other sulfur compounds such as 5-hydroxy-3-mercaptopentan-2-one which then gives some sulfur-containing compounds such as thiophenes and furans as well [62]. Heating temperature and pH conditions have been showed to affect the degradation products of thiamin. At pH 5.0 and 7.0 the 2-methyl-3-furanthiol and bis (2-methyl3-furyl) disulfide (meaty aroma)

and thiophenes were the dominant aroma volatile compounds. But the levels of these meaty compounds decrease when increasing pH to 9.0 [64]. Similarly, a recent study by Dreher et al (2003) [65] also showed that the most significant thiamin thermal degradation products in the model reaction of orange juice containing 0.024 mM thiamin are 2-methyl-3-furanthiol and bis(2-methyl-3-furyl) disulfide produce intense meaty aromas. Otherwise, some other aroma-active compounds also were found such as 4, 5-dimethylthiazole (skunky, earthy), 3-thiophenethiol (meaty, cooked), 2-methyl-4, 5-dihydro-3(2H)-thiophenone (sour-fruity, musty, green), 2-acetylthiophene (burnt), 2-formyl-5-methylthiophene (meaty), and 2-methyl-3-(methylthio) furan (meaty).

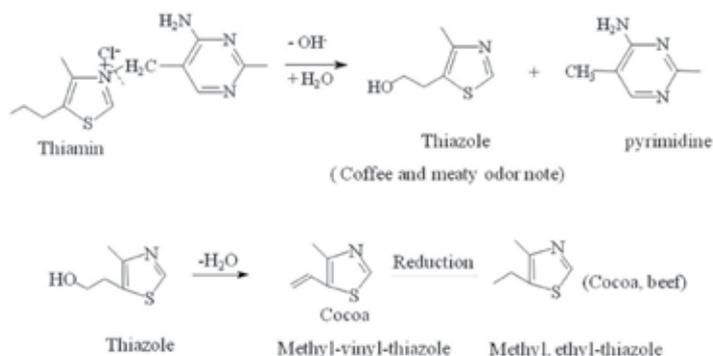


Figure 5. The thermal degradation of thiamin under basic condition

2.3.4. Interaction between lipid-oxidized products with Maillard products

The interaction between oxidized lipids and amino acids or proteins is very complex, in term of a consequence of the contribution of both lipid hydroperoxide and its secondary-oxidized products. This interaction may imply both the formation of physical complexes between the oxidized lipids and the amino acids or protein and the formation of various types of covalent bonds. Protein polymerization produced by reaction with peroxy free radicals generated during lipid peroxidation is known to occur during nonenzymatic browning [66, 67, 68]. However, in term of flavor study, the interaction between lipid-oxidized products (secondary products) with amino acids or proteins is the most concerned. Lipid-oxidized products are generic terms used to describe a mixture of aldehydes, alcohols, ketones and other products obtained by the decomposition of lipid hydroperoxides. Although it is not widely recognized, this decomposition does not necessarily imply the breakage of the lipid chain, and the formation of covalent bonds in the reaction between long chain oxidized lipids and amino acids and proteins has been described [69, 70]. This is a consequence of the existence of fatty acids that produce a complex and diverse mixture of lipid oxidation products that are able to react with the different reactive protein residues.

In the Maillard reaction, amino acids can undergo the Strecker degradation process that subsequently generates some reactive radicals such as ammonia, hydrosulfide and etc which al-

so are able to further react with the secondary oxidized products of lipid to produce volatile flavor compounds such as thiols, thiophenes, thiazoles and etc as showed in Figure 6.

The interaction between lipid and Maillard reaction have extensively been studied in a number of studies using model systems containing amino acids and sugars in the presence of lipid [43, 61, 39, 34]. In these studies, cysteine and ribose were used for Maillard reaction and in the presence of phospholipids from various sources including egg-yolk and beef. The reaction mixtures produced a lot of aroma volatiles which dominated by sulfur-containing components especially heterocyclics such as thiols and thiophenes. These studies also observed that the presence of phospholipids made a great reduction in amounts of these compounds. Famer and Mottram (1990) [61] also noted that beef-originated triglyceride has much less influence on amounts of heterocyclics than the phospholipids from beef do. The study also found that the addition of beef triglyceride to the Maillard reaction did not influence the sulfurous and rubbery aroma but when beef phospholipids were added resulting in higher meaty aroma note whereas the sulfurous notes were less. However, the Maillard reaction systems containing phospholipids usually had lower level of some meaty compounds especially 2-methyl-3-furanthiol this is due to the lipid limits generation of these compounds and only maintain theme at an optimum level in the reaction mixture.

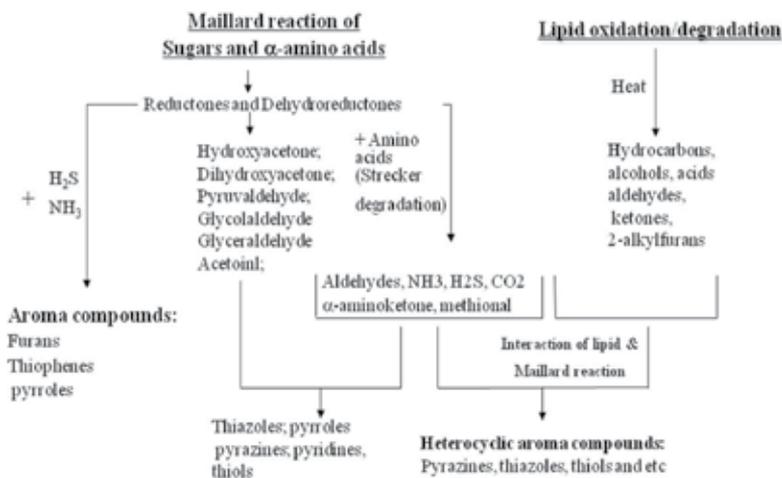


Figure 6. The interaction between lipid-oxidized products with Maillard products.

In general, in Maillard reaction mixtures containing lipids produce a lot of lipids-derived volatile compounds such as aldehydes, furans, hydrocarbons, alcohols and ketones. Furthermore, the reaction mixtures also containing the Maillard products such as H_2S , NH_3 , etc. Which all are able to interact with each other to form new heterocyclic aroma volatile compounds as the consequences of the interactions between lipids with Maillard products. The most abundant compounds have been detected as results of the interactions are thiophene class such as 2-pentylthiophene, 2-hexylthiophene and thiol class such as 2-thiophenethiol, 2-furylmethanethiol, 2-methyl-3-furanthiol and etc [38, 61]. A recent study by Elmore and

colleagues (2002) [34] concluded that breakdown products of polyunsaturated fatty acids especially are *n*-3 acids have a shorter chain length have lower odor thresholds will also be present at relatively high concentrations and are more reactive. These breakdown products will affect meat flavor by interacting with the Maillard reaction reducing levels of meaty aroma compounds, such as sulfur-substituted thiophenes and furans. As *n*-3 PUFAs are readily oxidized, they could initiate the free radical oxidation of more saturated acids, increasing levels of breakdown products of *n*-6 and *n*-9 fatty acids, which may also alter the aroma compounds of the cooked meat.

3. The factors affect aroma flavors

3.1 Effect of diets

Diet is as an important indicator to show the growth rate, performance, reproducibility effects and as well as meat quality of cattle. There has been an existed hypothesis of meat flavor changes due to feeding diets in which some works stated a large difference in meat flavor characteristics of the same cattle breed but fed on different diets. Early work by Melton (1983) [71] stated that steers fed with corn-based diets had more intense beef flavor (desirable flavor) than the same age steers fed based pasture or Bermuda pellets. A later study of Melton (1990) [10] found that the less desirable flavor of meat from cattle is mainly caused by several grass species. Conversely, no significant difference in flavors existed between the grass and grain diets-fed animals [73]. The less desirable flavors were also seen on meats from the hay diets-fed animals compared to corn silage diets [74], while Oltjen et al (1971) [75] showed the opposite results. It has been hypothesized that majority of flavor effects due to feeding of forages is mainly due to changes in fatty acid compositions. Fishy off-flavor was significantly higher in meat from grass-finished cattle with increasing unsaturated fatty acids [76]. Recently, researchers have attempted to higher level of PUFA in meat aiming to increase the health benefits by using the fat supplemented-diets (e.g., linseed, sunflower oil and fish oil) to cattle [77, 78], although these works have achieved an increase in several benefit fatty acids however, the detrimental effects on meat flavor characteristics appeared due to higher levels of PUFA [79]. A large number of studies regarding the effect of diets on volatile flavor compounds of cooked meat have been performed. Melton (1983) [71] also noted that the greatest difference in the flavors of meat from cattle fed on grass and grain-based diets is due to fatty acid concentration and type as fatty acids are the primary source of carbonyl. Suzuki and Bailey (1985) [80] indicated that higher concentrations of pentanoic, heptanoic, octanoic, nonanoic, decanoic, and dodecanoic acids were formed in the meat fat from grass-fed animals while heptanal, 2,3-octanedione, 3-hydroxyoctan-2-one, 2-decenal, 2-tridecanone, hexadecane, heptadecane, octodecane, d-dodecalactone, phyt-1-ene, neophytadiene, phyt-2-ene, an isomer of neophytadiene, 2-heptadecanone, dihydrophytol, and phytol with the terpenoids in much higher concentration due to rumen-fermented chlorophyll. Individual volatile flavor compounds like 4-heptanal, 2, 4-heptadienal and 2, 6-nonadienal (derived

from C18:3n-3) and hexanal, 2-heptanal and 2, 4-decadienal (derived from C18:2n-6) found to be higher concentration in meats from grass and grain-fed animals, respectively [81]. Elmore et al (1997) [82] also reported that cooked meat from the animals that had been fed fish oil had considerably higher concentrations of saturated and unsaturated aldehydes than meat from the control. While, Descalzo et al (2005) [83] found that some classes of volatile flavor components affected by diets in which aldehydes increased in meat from concentrate diets-fed animals. In general, we can see that diets have a large influence on meat flavors due to directly affect the meat contents especially the intramuscular fat contents which play an important role in interaction and generation of volatile flavor compounds. On the other hand, it is worth noting that the uses of fat –supplemented diets to feed cattle may result in increases of important polyunsaturated fatty acids (e.g., n-3 fatty acids, DHA, EPA) which known to positively affect on consumers health however, a negative effect on meat flavors may appear due to these fatty acids not only produce some unexpected volatile compounds but also inhibit production of other Maillard products.

3.2. Effect of breeds and sex

Researchers have reported that breed also affects volatile flavor components and then influence overall flavor notes of cooked meat. Elmore et al (2000) [25] stated that fifty-four compounds were affected by breed, 75% of which was Maillard reaction products. Over 40 compounds were present at higher levels in the Soay breed than in the Suffolk breed. Other sulfur-containing compounds present at higher levels in the Suffolks than the Soays were bis-(2-furylmethyl) disulfide and 2-methyl-4,5-dihydro-thiophene and the differences in sulfur and nitrogen-containing compounds could contribute to flavor differences between the two breeds. A study on pork flavors as affected by breeds also have found that twenty-three among the detected flavor compounds were significantly affected by breed. Based on sensory analysis indicated that cooked *longissimus* muscle from hybrid breed (Duroc x Landrace x Large White) had the lowest pork flavor intensity and flavor-liking compared with the Chinese indigenous breeds. Laiwu and Dahuabai breeds showed the highest pork flavor intensity and flavor-liking in cooked *longissimus* muscle [36]. In beef, Insausti and colleagues (2005) [84] also found the differences in volatile flavor compounds among the breeds were considerable and may contributed to the perception of flavor differences in the cooked beef. Particularly, level of dimethyl sulfide probably related to cauliflower notes, was highest for meat from the Pirenaica breed. While, levels of the sulfur-compounds in cooked beef from the Asturiana breed were low-intermediate and potentially related to blood and liver notes and unpleasant flavors.

For the sex effect, it has been reported that meat from bulls has a strong livery and blood flavors while meat from heifers has a strong characteristic flavor. The differences could be associated with the differences in amounts of certain volatile compounds such as hydrocarbons, aldehydes, alcohols and ketones [85]. On the other hand, the differences in meat flavors between bulls and heifers could be explained by the genetic control of animal development and production of sex hormones and their influence the lipid composition which affects the kinds of volatile flavor compounds [86]. Overall, it may be assumed that

the differences in meat flavors existing between breeds or sexes are probably due to the differences in the levels of flavor precursors especially the fat contents which large affect the formation of aroma flavor compounds and also interact with other contents in determining flavor characteristics of cooked meat.

3.3. Effect of chiller ageing

Ageing has been become a universal method widely used to improve eating quality of meat (e.g. tenderness, juiciness, flavor). Un-aged beef has a weak, bland odor while aged beef has a strong, savory, roasted odor. Ageing of meat makes an increase in fatty flavor characteristics however; long term ageing (e.g., > 3 weeks) could cause a decrease in positive flavor notes and increase liver-like aroma, bloody, bitter and off-flavor [87, 85, 88]. Ismail et al (2008) [89] stated that ethanol was responsible for the increase in alcohols caused by the microbial growth in beef during storage furthermore, the levels of aldehydes significantly increased after 7 days of storage. Beef from various muscles including gluteus medius, rectus femoris, vastus lateralis, vastus medialis, teres major, complexus, serratus ventralis, psoas major and longissimus dorsi of heifer carcasses were chiller aged for 7 or 14 days the results showed that flavor-active volatiles included nonanal, 2,3-octanedione, pentanal, 3-hydroxy-2-butanone, 2-pentyl furan, 1-octen-3-ol, butanoic acid, pentanal and hexanoic acid which all often associated with lipid oxidation were affected by enhancement and ageing in the various muscles [90]. Additionally, ageing of beef achieved an increase in characteristic flavor and also aftertaste intensity, making an appreciable improvement of its flavor. After slaughter, loss of circulatory competency results in the accumulation of metabolic by-products, including lactic acid, in the muscle, that induces pH decline. The endogenous enzymes (e.g., cathepsins B and L) are activated at near pH 5.4. Spanier and Miller (1993) and Spanier et al (1990) [91, 92] suggested that these thiol proteinases can hydrolyze more peptide bonds than any other group of enzymes, are redistributed during ageing period. Proteolytic enzyme activity is temperature-dependent; some enzymes retain high activity levels even at cooking temperatures. The combined effect of postmortem ageing and cooking, via enzyme redistribution and activity can influence the production of aroma flavor compounds. Toldrá and Flores (2000) [93] stated that enzymes known primarily for textural changes (e.g., μ - and m-calpain) during the postmortem period affect flavors by producing peptides, but it was observed that these enzymes correlate with increases in rancid, sour and salty flavors. The ageing conditions (e.g., oxygen availability, temperature, humidity and aging time) under which beef is aged influences the ultimate flavors of the meat particularly ageing in a higher oxygen environment cause a burnt, toasted off-odor. In addition, dry-ageing increases beef flavor attributes more than ageing in vacuum or in carbon dioxide [94, 95]. Based on the results reported in the previous studies it could be concluded that chiller ageing of meat resulted in increases of most of flavor compounds however a long ageing period (e.g., > 3 weeks) may negatively influences the flavor quality of cooked meat due to increase in amounts of some unexpected compounds which associated with undesirable flavors and decrease in the some important compounds which associated with desirable flavors.

3.4. Effect of cooking temperature and pH conditions

Cooking temperature is one of the important factors impacting the development of flavors through the Maillard reactions and lipid oxidation. Amino acids can undergo Strecker degradation to produce Strecker products. Degradation of sulfur-containing amino acids (e.g., cysteine, cystine and methionine) generates sulfur that contributes to subsequent processes of Maillard reaction. These compounds can react with amines and amino acids to produce a number of flavor-contributing compounds and potent cooked meat odorants such as pyrazines, oxazoles, thiophenes, thiazoles and other heterocyclic sulfur containing compounds [20]. It was well seen that cooking temperatures affect these reactions and then determine flavor characteristics, for instance the stewed meat lacks flavors of the roasted products because of stewed meat has a water activity of approximately 1.0 and not exceed temperature of 100°C while roasted meat has dried surfaces and temperature may exceed 100°C therefore, the conditions like low water activity and high surface temperature will increase production of flavor compounds which give roasted odor notes rather than meat is stewed. Ames et al (2001) [40] concluded that the amounts of most volatile flavor compounds increased with cooking temperature. Cooking at lower temperatures (<165 °C) versus higher temperatures (>180 °C) results in differences in the concentrations of a number of compounds such as 2, 4, 5-trimethyl- 3-oxazoline; 2, 4-dimethyl-5-ethyl-3-oxazoline; 2, 5- dimethyl-4-ethyl-3-oxazoline; 2, 4-dimethyl-3-thiazoline; 2, 4, 5-trimethyl-3-thiazoline [96]. Previous works found that a strong relationship existing between cooking temperature, concentration of free amino acids, carnosine, pyrazines and hexanol, and roasted, burnt and beefy flavor intensity [97, 98]. Cooking beef generates urea content which can also reduce sulfur-containing compounds generating important nitrogen-containing compounds like pyrazines and thiazoles in which pyrazines are formed mostly on the surface of meat and having nutty and roasty odor notes [99]. In general, the higher degree of heating, the higher the concentration of aliphatic aldehydes, benzenoids, polysulfides, heterocyclic compounds and lipid-derived volatiles. Ketones, alcohols sulfur-containing components make smaller contributions.

pH is one of the important factors that influence the kind of volatile flavor compounds formed in the Maillard reaction, and then determine the final flavor characteristics of cooked food. Madruga and Mottram (1995) [8] showed that as pH increases, color and polymeric compounds increase and nitrogen-containing compounds like pyrazines are favored, therefore it was assumed that higher ultimate pH in meat from grass-fed animals may favor the formation of thiazoles and thiophenones due to the availability of amino acid degradation products while decreasing other sulfur volatiles that favor lower pH. A number of early studies have been performed to investigate the effect of pH changes on volatile flavor compounds using model systems (El'Gde et al., 1966; Shu et al., 1985; Meynier and Mottram, 1995) [100, 101, 102]. These studies found that high pH values also favor the formation of many volatile compounds but other compounds are only favored at low pH condition. Meynier and Mottram (1995) [102] used meat-like model systems containing amino acids and ribose on different pH 4.5 and 6.5, results showed that nitrogen-containing compounds such as pyrazines were detected at higher pHs. While, dimethyldisulphide and methional

showed decrease as the pH increased, and an increase in the disulphide was observed. It was observed that a large number of sulphur-containing compounds such as 2-methyl-3-fur-anthiol a strong meaty aroma, whose formation was greatly favored by lower pH condition. Ames et al (2001) [40] used model reactions containing cysteine and reducing sugar at varied pH conditions 5.5, 6.5 and 7.5, results showed that amounts of most of compounds increased with pH especially are pyrazines. Cerny and Biffod (2007) [103] recently found that pH determined strongly which volatile flavors were formed and to what extent. In general, based on the results of the previous studies which all found that pH condition strongly influence the formation of flavor components.

3.5. Effect of irradiation on meat flavors

Irradiation is a food safety technology designed to eliminate disease-causing germs from foods. Depending on the dose levels of irradiation applying on the raw meat and poultry, or ready-to-eat meats that can eliminate bacteria commonly found such as *E. coli*, *Salmonella* and *Listeria*; virus; or parasites. However, irradiation may result in off-odors and flavors. The odors vary with the type of meat, temperature during irradiation, oxygen exposure during and/or after the irradiation process, packaging and presence of antioxidative substances [104]. Most of studies have reported that the aroma flavors of irradiated meat associated with rotten egg, sweet, bloody, cooked meat, barbecued corn, burnt, sulfur, metallic, alcohol, acetic acid, liver-like serummy and bloody [105, 106, 107]. Irradiation can initiate or promote lipid oxidation resulting in undesirable off-odors and flavors [108, 109]. Jo and Ahn, (2000) [110] showed that reactions of sulfur-containing amino acids with radiolytic products of water appear to be the source of hydrogen sulfide and other volatile sulfur-containing compounds which contribute to off-flavor. On the other hand, irradiation may result in the formation of free radicals from unsaturated fatty acids at double bond positions [109]. An increase in lipid peroxidation products such as hexanal and (E)-4,5-epoxy-(E)-2-decenal in combination with a loss of desirable meaty odorants (4-hydroxy-2,5-dimethyl-3(2H)-furanone and 3-hydroxy-4,5-dimethyl-2(5H)-furanone) result in development of warmed over flavor of cooked, refrigerated beef [16]. However, the effects of irradiation on aroma flavors are also depended on: (1) Dose levels of irradiation, it has been demonstrated that the dose levels of irradiation influence variedly on volatile flavor components of cooked meat, as reported by Jo and Ahn (2000) [110] who indicated some of hydrocarbons included 1-heptene and 1-nonene increased with irradiation dose immediately after irradiation of beef. A similar observation also was reported by Yong et al (2000) [111] who indicated that among the 150 flavor compounds identified in beef the cyclodecene, (E)-2-hexenal, nonene and 2-nonenal showed an increase in a dose-dependent fashion. For the effect of irradiation on chicken flavors, Yong et al (2000) [112] showed that among the 129 identified volatile flavor compounds the cyclotetradecene, 2-methylpentanal and 4-methylcyclohexene were formed specifically in response to irradiation, and level of cyclotetradecene increased in a dose-dependent fashion; (2) Oxygen presence, the presence of oxygen around meats during irradiating can diffuse into the meats, and then results in radiolytic changes which precipitate oxidation and unacceptable secondary breakdown products. As well known, lipid oxidation needs oxygen presence to produce oxidized-products such as aldehydes, Nam and Ahn

(2003) [113] indicated that irradiation of meat in aerobic packaging promoted production of aldehydes such as propanal and hexanal which is assumed as a good indicator of lipid oxidation. The similar observation also was reported by Nam et al (2001) [114] who also showed that irradiation increased TBARS values and off-flavor in aerobically-packaged pork (3) Temperature effect, temperature during irradiating meats has a large effect on aroma flavors of irradiated meat because temperature affects what radiolytic products are formed and what ratios [104]. Using lower temperature during irradiation of meat by freezing meat before irradiation can reduce detrimental effects via retarding autoxidation and extending shelf life; (4) pH effect, it has also been demonstrated that the ultimate pH of meat at the time of irradiation influences lipid oxidation. Nam et al (2001) [114] recently showed that irradiation increased lipid oxidation of normal and pale-soft-exudative (low pH group) muscles, whereas dark-firm-dry (high pH group) muscle was very stable and resistant to oxidative changes. Therefore, to minimize the detrimental effects of irradiation on aroma flavor characteristics we can modify atmosphere packaging by using vacuum packaging (anaerobic packaging) or replacement with inert gases (i.e. nitrogen, helium, hydrogen, carbon dioxide) to eliminate oxygen. Reducing the temperature (freezing) prior to irradiation and addition of antioxidants. Vacuum packaging retains irradiation-generated sulfur-containing compounds, however re-packaging meat in oxygen-permeable materials allows for dissipation of these flavor compounds.

4. Warm-off flavor and liver-like off flavor in cooked meat

Warm-off flavor and liver-like off flavor are undesirable flavors that result from the flavor changes and deterioration in meats that have been pre-cooked, chilled-stored and reheated. The warm-off flavor includes odors and tastes commonly described as stale, cardboard-like, painty, rancid, bitter and sour [115], and together with liver-like off flavor they both are the main factors that negatively affect eating sensory quality, purchase, economic impact of meat industry, and consumer complaint. Researchers have found that warm-off flavor appearing in cooked meat is mainly caused by oxidation of membrane phospholipids [116,115]. A recent report of Byrne et al (2001) [117], which also demonstrated that warm-off flavor associated with the development of lipid oxidation derived nuance off-flavor and odor notes such as rancid-like flavor and linseed oil-like odor, in association with a concurrent decrease in cooked pork meat-like flavor. The development of warm-off flavor usually results in loss of meaty flavor due to mask by lipid-oxidized products. Additionally, processes which involve any action that disrupts the muscle fiber membrane, such as chopping, restructuring, or heating which all can enhance warm-off flavor of meat product [118]. Previous works also suggested that reactions involving sulphhydryl-disulfide interchanges in proteins and the degradation of sulfur-containing heteroatomic compounds, leading to a decrease in the "meatiness" of freshly cooked meat may also be an integral part of warm-off flavor [119,120]. For the liver-like off flavor of cooked meat, it was hypothesized that since foodservice preparation traditionally cooked the meat quickly and then held the product in warming ovens until the food was presented to the consum-

er these conditions might promote the liver-like flavor [27]. James and Calkins, (2005) [121] also hypothesized that the slower cooking and longer hold time allow the undesirable volatile flavor compounds to dissipate.

5. The current techniques used for extraction and detection of aroma flavor components

Up to present time, various techniques have been designed, combined with gas chromatography and mass spectrometry (GC/MS) or Flame ionizing detector (GC/FID) and applied to evaluate volatile flavor components in cooked meat. Of which, simultaneous steam distillation-extraction (SDE), dynamic headspace entrainment on Tenax TA, and solid-phase micro-extraction (SPME) are the techniques widely used for the extraction of volatile compounds in cooked meat [19, 122, 123, 85, 90, 124, 18,125]. SDE is a simple technique which involves small volumes of solvent, efficient stripping of volatiles and quantitative recovery of many compounds. The sample is dispersed in water which is heated to boiling. The steam that is generated carries volatiles with it into a section of the apparatus where the steam condenses in the presence of extracting solvent vapor. The co-condensation of volatile-laden steam and extracting solvent results in an effective extraction of volatiles [123]. The Dynamic headspace entrainment on Tenax has been used in the studies regarding cooked meat volatile flavor compounds since the 1980s. This technique probably has been used more than any other aroma extraction technique for the analysis of meat aroma and continues to be widely used. The action mechanism of this technique involving purging the headspace of a sample with a purified inert gas (e.g., nitrogen or helium), followed by collection of the volatiles onto a trap containing a suitable adsorbent, which will retain the volatile analytes carried there by the purge gas. Finally, the volatiles of meat samples collected on this trap are desorbed onto a GC or GC-MS column using a modified injection port. In the recent years, SPME technique has been widely adopted and considered as an alternative to isolate volatile flavor components in cooked meat. In SPME, the needle is coated with an absorbent material (e.g. CAR/PDMS), is placed above the cooked meat samples. Volatiles will migrate from the sample matrix to the needle coating and be absorbed. Volatile components will then be desorbed from the needle coating by inserting the needle in GC injection port.

The extraction techniques as mentioned above in combination with GC/MS or GSC/FID can help researchers to tentatively detect the volatile flavor compounds in experimented meat samples but it could not identify the aroma flavors or odor characteristics of detected compounds. It would be advantageous to combine two or more different techniques, such as gas chromatography (GC) and olfactometry, the combination of measuring odor notes is called gas chromatography-olfactometry (GC/O). Gas chromatography-olfactometry (GC-O) is a bioassay that measures human response to odorants separated by gas chromatography. The superior sensitivity and selectivity of human olfaction make GC-O a powerful and meaningful tool for flavor chemistry. In the recent year, GC-O is one of the main techniques which have been used to determine intensity of aroma (odor) characteristics of volatile compounds in cooked meat [15, 21, 125].

6. Conclusion and Implication

In order to have a cooked meat product with its desirable aroma flavors as expectation of consumer, it is important to understand how aroma flavors are derived, the mechanisms by which flavor components are generated, and the factors affect formation of flavor compounds then determine the final aroma flavor characteristics of cooked meat. Regarding the effects of factors on aroma flavors of cooked meat and to minimize the detrimental effects it is suggested if increasing the polyunsaturated fatty acids (e.g. C18:3n-3, DHA, EPA) to increase nutritional benefits to the consumer by using fat-supplemented diets however the undesirable flavors may result. Because the breakdown products of these fatty acids have a shorter chain length therefore are more volatile and they affect meat flavors by interacting with the Maillard reaction results in reducing levels of meaty aroma compounds such as sulfur-substituted thiophenes. Therefore, diets, feeding regimes, welfare and management of animals should be taken into account. Cooking conditions such as temperature, holding time and cooking methods play an important role in determining the formation volatile flavor compounds. In general, it has been demonstrated that cooking meat at high temperature (by roasting, grilling) will produce better aroma flavor characteristics due to the important Maillard products are formed. In addition to the cooking effect, it is suggested that a slow cooking and longer hold time can allow the undesirable volatile flavor compounds to dissipate, thus reduce warm-off flavor. Irradiation of meat can eliminate pathogens however, off-flavor may result therefore, and to minimize the detrimental effect of this method we can lower temperature during irradiation of meat by freezing meat before irradiation. Modifying atmosphere packaging by using vacuum packaging (anaerobic packaging) or replacement with inert gases (i.e. nitrogen, helium, hydrogen, carbon dioxide) to eliminate oxygen in meat during irradiation are also the alternatives. Chiller ageing of meat should be applied to improve eating quality however should not age for a long time (3 week period in maximum is encouraged) because chiller ageing meat for a too long period may result in flavor deterioration and decreasing desirable flavors.

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Novel Analytical Tools for Quality Control in Food Science

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Additional information is available at the end of the chapter

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1. Introduction

Due to the fast technological and data treatment advancements new insights into food can be considered. The application of these novel analytical techniques belongs to the responsibility of food chemists and analysts. Thereby, an increase in efficiency is based on an improved lower limit of detection (LOD), selectivity to separate analytes of interest and speed of analysis.

High-performance liquid chromatography (HPLC) belongs to the traditional separation techniques applied to a broad range of hydrophilic and hydrophobic ingredients in both the reversed-phase (RP) [1] as well as normal-phase (NP) [2] mode. In a conventional HPLC system the inner diameter of the separation column, which is the core of the separation unit, is 4.6 mm. During the last decade miniaturization down to 20 μm allowed to increase on one side the sensitivity and on the other side speed of analysis could be enhanced dramatically. Therefore, novel stationary phases mainly based on polymers have been designed and brought to the market to enable both the separation of low and high-molecular weight analytes [3]. As an alternative separation technique capillary electrophoresis (CE), which separates analytes due to their different ion mobility based on charge and molecular weight in an electric field within a fused silica capillary having an inner diameter of approximately 200 μm can be applied [4]. Thereby, the appearance of the electroosmotic flow (EOF) can influence the separation efficiency by either speeding up the separation process or by improving the resolution. Capillary electrochromatography (CEC) is a hybrid technique of both HPLC and CE in which both pressure and an electrical field are applied and enables extreme high resolution. The drawback of this separation method is the fact, that real samples can hardly be analysed due to the disturbance by the matrix [5]. In many cases the analyte of interest is only available in very low con-

concentrations. Therefore, selective enrichment and purification steps are the method of choice, which can be accomplished by solid-phase extraction (SPE). Therefore, a material designed for a special analytical question is filled into a cartridge or pipette tip and the sample of interest is put onto the material in liquid form [6]. In the following, analytes of interest can interact with the functional groups of the stationary phase and compounds being not of interest can simply be washed away. In the final elution step, only some micro liters of liquid are required to elute the analytes of interest from the stationary phase being available in relatively high concentrations for the following analytical steps. The following analytical procedure can be either a separation or spectroscopic method. Spectroscopic methods at this stage of the analytical procedure either include mass spectrometry (MS) and/or vibrational spectroscopy, respectively. In MS most of the samples are analysed applying electrospray ionization (ESI) as an interface with different types of mass detectors including e.g. time of flight (TOF), ion trap, ion cyclotron and quadrupoles. As an alternative, matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF/MS) can be applied for the determination of high molecular weight compounds including proteins, peptides and lipids. For the analysis of low molecular ingredients < 1000 Da the so called matrix-free laser desorption ionization (mf-LDI) MS technique must be applied [7]. Vibrational spectroscopy in the field of food analysis is mainly applied in the mid ($400 - 4000 \text{ cm}^{-1}$) as well as in the near infrared ($4000 - 12000 \text{ cm}^{-1}$) of the electromagnetic spectrum. In combination with chemometrical algorithms these methods can be used for the authentication of the material on one hand, on the other hand quantitative analysis allows to control selected quality parameters [8].

In the following a systematic analytical approach is introduced, which allows combining the different analytical techniques in a synergistic manner to get deeper insights into the composition and origin of food samples.

2. Systematic analytical approach

The key technologies described in the above chapter can be combined according to the scheme depicted in Figure 1. In this approach extraction of the material for the further analytical steps and individual procedures can be linked to sample enrichment/purification, separation, vibrational spectroscopy and mass spectrometry followed by database analysis. The different parts are described in the following sub-chapters.

2.1. Sample enrichment/purification

In many cases interesting analytes are only available in extremely low concentrations and/or in very complex matrices, respectively. Therefore, pre-concentration steps based on solid-phase extraction (SPE) can be very helpful. Nano-materials such as nanotubes, fullerenes, diamond offer excellent physiochemical properties due to a high ratio of surface to size, which results in a high capacity and allows analyte detection with high sensitivity down to the femtomole range in the case when mass spectrometry is applied for

detection. Especially carbon nano materials can be easily further derivatised with a number of different functional groups including reversed-phase (RP), normal-phase (NP), ion exchange (IEX), immobilized affinity (IMAC) and so on depending on the specific demand. As an alternative they can be incorporated into a polymer matrix for highly selective extraction by certain compound characteristics. For the practical handling pipette tips have been tested to be most suitable and this special type of SPE is called “hollow monolithic incorporated tip” as it has an open flow channel in the middle enabling an easy pipetting procedure. For the highly efficient pre-concentration of phosphopeptides nano particular TiO_2 , ZrO_2 and mixtures thereof are incorporated into a polymer matrix as depicted in Figure 2 [9]. By this technique hundreds of microliters can be flushed over the system and finally elution of the desired compounds to be analysed is carried out with only a few microliters causing a dramatic increase in concentration from which further analytical investigations can benefit due to the easier handling of the systematic investigation.

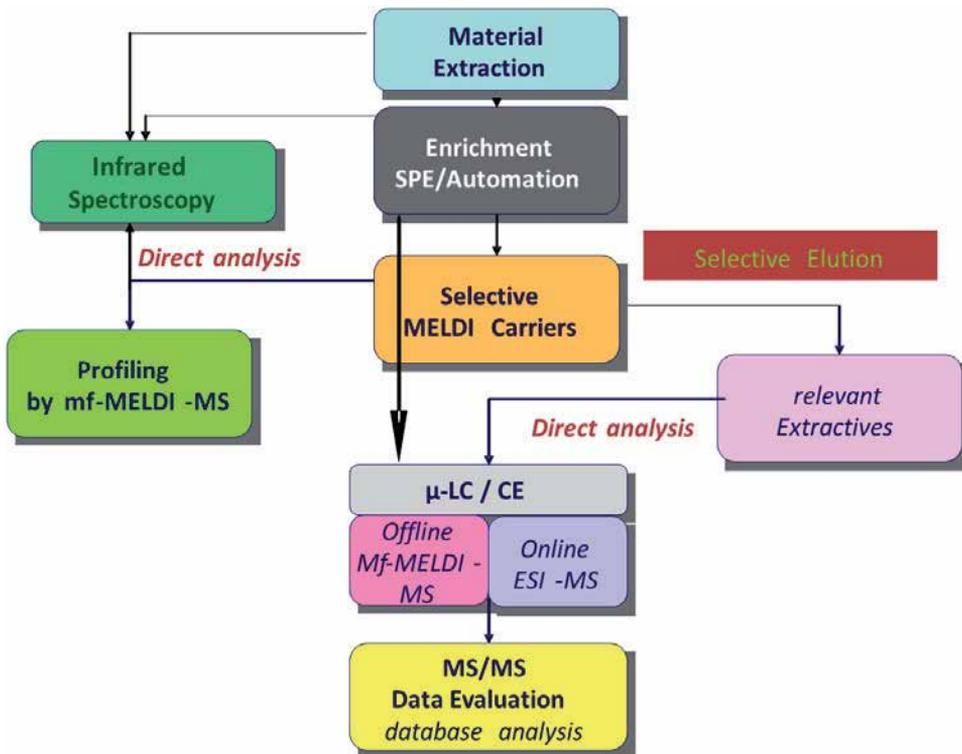


Figure 1. Multidimensional analytical approach

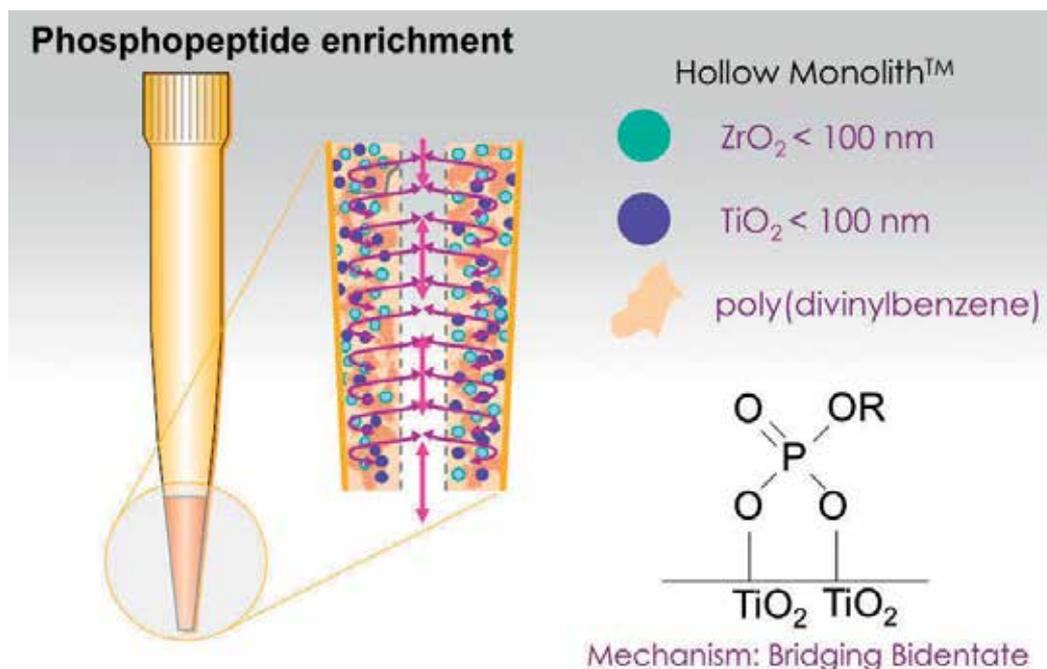


Figure 2. TiO_2 , ZrO_2 incorporated into a polymer matrix for phosphopeptide enrichment

Immobilisation of such polymers into pipette tips with trypsin can be used for fast digestion of peptides and proteins within only a few minutes ensuring high capacity and sequence coverage (Figure 3) even in the high-throughput mode using robotic pipetting systems [10]. In comparison to this quite young approach the conventional digestion procedure lasts approximately 24 hours and doesn't show in any case better results by higher sequence coverages. For this reason this approach is of high interest for the routine analysis and/or diagnostics, respectively. As a carrier glycidylmethacrylate-co-divinylbenzene (GMA/DVB) polymerized in pipette tips was chosen. The major advantages of in-tip digestion are easy handling and small sample amount required for analysis. Microwave-assisted digestion was applied for highly efficient and time saving proteolysis. Adaption to an automated robotic system allowed fast and reproducible sample treatment. Investigations with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) and liquid chromatography coupled to electrospray-ionization mass spectrometry (LC-ESI/MS) attested high sequence coverages (SCs) for the three standard proteins, myoglobin (Myo, 89%), bovine serum albumin (BSA, 78%) and alpha-casein (α -Cas, 83%). Compared to commercially available trypsin tips clear predominance concerning the digestion performance was achieved. Storability was tested over a period of several weeks and results showed only less decrease (<5%) of protein sequence coverages. The application of microwave-assisted in-tip digestion (2 minutes) with full automation by a robotic system allows high-throughput analysis (96 samples within 80 minutes) and highly effective proteolysis.

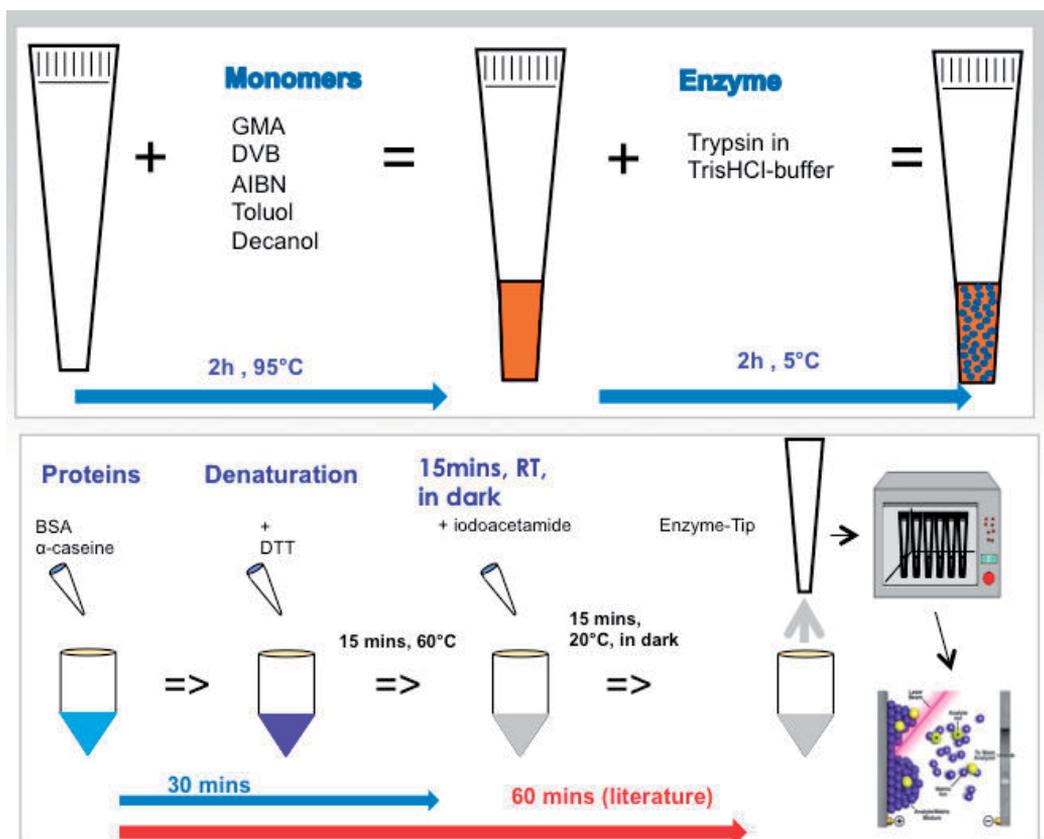


Figure 3. Trypsin immobilized pipette tips for high-throughput analysis of peptides

2.2. Selective MELDI-carriers

Material enhanced laser desorption ionisation (MELDI) is a method, which is based on the conventional matrix assisted laser desorption ionisation time of flight mass spectrometric (MALDI-TOF/MS) detection with the significant difference that before LDI MS step a selective enrichment procedure is carried out for the distinct analysis of a certain compound class. Compared to other similar techniques in this field, this approach benefits from the physical properties of the material itself (pore size, surface area, capacity, etc.) and its chemical derivatisation/functionalisation. In the past this technique was proven to be highly efficient for the analysis of biomarkers following an optimised strategy (Figure 4). In the first step a selected material including e.g., nanotubes, fullerenes, nano-crystalline diamond, polymers, cellulose, etc., which are derivatised with functional groups (C18, IMAC (immobilised metal affinity chromatography), IEX and others) is activated and the serum sample of interest is incubated. During this step, selective binding of molecules according to their functional group is achieved and finally undesired components can be washed away applying an optimised protocol. In the next step the incubated material is put onto a conventional

steel target used in MALDI-TOF/MS, a matrix substance is added (e.g., sinapinic acid) and finally the mass spectrum is generated by the laser desorption ionisation process. The result is a mass spectrum being characteristic for a patient and/or the nutrition profile. Multivariate analysis (MVA) can be applied for further data analysis and interpretation, a clustering into certain stages of an illness can be achieved, respectively. From the mass spectrum potent biomarker molecules can be selected and identified by further analytical steps. The biomarker itself and/or the profile of the corresponding mass spectrum can be used for the screening of certain diseases, stages therefrom, allergies, nutrition effects and so on [7].

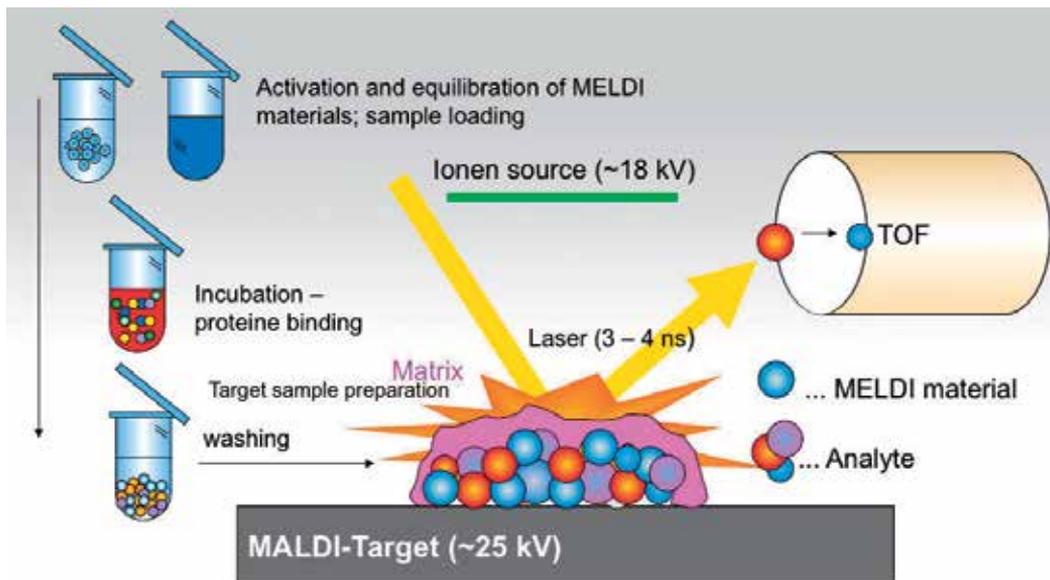


Figure 4. Principle of material enhanced laser desorption ionization (MELDI)

For the analysis of low-molecular weight compounds ($MW < 1000$ Da) the conventional MELDI approach is replaced by the matrix-free (mf) MELDI approach for which the addition of a matrix substance is not required so that no disturbing peaks appear. In this approach a conventional steel target with a 50 nm thick titanium oxide layer can be applied fulfilling all requirements for a successful laser desorption ionization process [11].

As an alternative the incubated analytes of interest can be selectively eluted from the functionalised carrier material and further analysed by liquid chromatography (LC) or capillary electrophoresis (CE).

2.3. Liquid chromatography, capillary electrophoresis and electrochromatography

Novel materials used in miniaturised liquid chromatography (μ -LC) are mainly polymer based, e.g. poly(1,2-bis(p-vinylphenyl)ethane). These polymers possess the huge advantage that chemical (composition of the polymer) and physical parameters including mainly po-

rosity can be adjusted [12]. Extensive investigations on polymerisation time and temperature have been carried out enabling a tailored design of micro-, meso- and macro-pore distribution [13, 14]. This results in the applicability of such capillaries with an inner diameter between 20 and 200 μm for even the separation of high- and low-molecular weight compounds. These capillaries can be highly successfully applied analysing peptides, proteins, oligonucleotides, DNA fragments as well as “small molecules” such as phenols, flavonoids, catechins, acids etc. Figure 5 shows as an example the separation of olive oil ingredients. This separation is characterised by a very high ratio of flow to back pressure, which is of high interest to perform extremely rapid Coupling to mass spectrometry enables a highly efficient analysis even of crude samples offering all the possibilities of collision induced dissociation (CID) and database search [15].

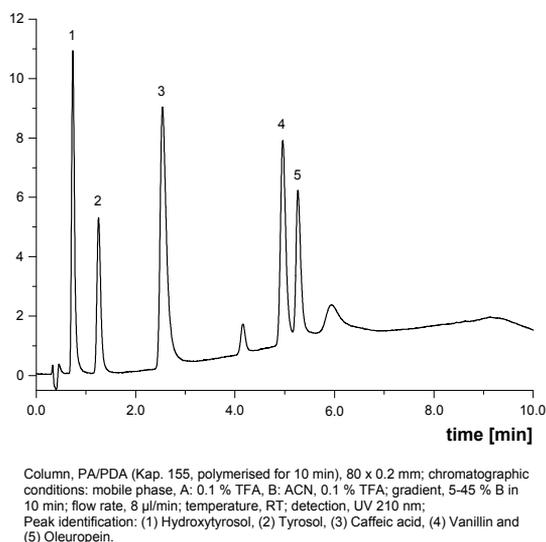


Figure 5. Separation of olive oil ingredients using a monolithic capillary column. Conditions: capillary 80 x 0.2 mm; mobile phase, A: 0.1% TFA; B: CAN; gradient, 5-45%B in 10 min; Flow rate 8 $\mu\text{l}/\text{min}$; temperature, RT; detection, UV 210 nm. Peak assignment, (1) hydroxytyrosol, (2) tyrosol, (3) caffeic acid, (4) vanillin, (5) oleuropein.

As an alternative separation method capillary electrophoresis (CE) and /or electrochromatography (CEC) can be applied. In CE separation of analytes is achieved due to their different ion mobility based on charge and molecular weight in an electric field within a fused silica capillary having an inner diameter of approximately 200 μm [4]. As has already been remarked the electroosmotic flow (EOF) has a main influence on the separation and can be used for speeding up. In CEC both an electrical field and high pressure are applied resulting in high resolution. This technique can be applied to check the identification and purity of standards compounds with very high efficiency. For the reproducible separation and analysis of food ingredients such as phenols, acids, peptides, lipids, coating of the capillary's inner wall was shown being advantageous as irreversible analyte adsorption by free hydroxyl-groups from the silanole of the fused silica capillary can be avoided. Latex-diol

and fullerene coated capillaries were successfully introduced and as a detection system on-line hyphenation to MALDI-TOF/MS was shown to be highly efficient not only for the investigation of flavonoids but also for peptides, especially phosphorylated (Figure 6) [16, 17]. This system can be used for the investigation of the casein profile in milk offering the advantage over all other more classical analysis tools that in this case also higher phosphorylated species can be separated and detected. From the ratio of different phosphorylation degrees several interpretations concerning the quality but also the origin of the milk can be carried out.

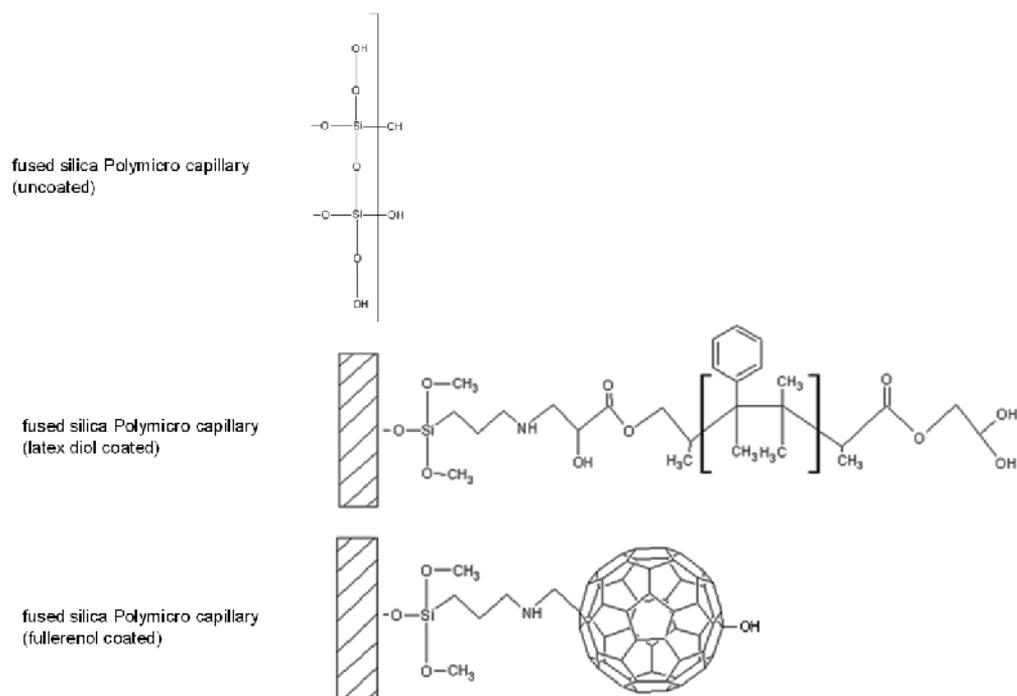


Figure 6. Inner capillary wall coatings applied in CE and CEC

2.4. Vibrational spectroscopy

For quality control both mid- (MIR, $400 - 4000 \text{ cm}^{-1}$) and near-infrared (NIR, $4000 - 12000 \text{ cm}^{-1}$) can be conducted. In MIR fundamental stretching and bending vibrations occur, in NIR the corresponding overtones and combination vibrations are detected. This means that NIR-spectra can contain a lot of more vibrational information, which is an advantage for the analysis of highly complex samples. Therefore, during the last decade several applications in the field of food analysis were developed in the NIR region. Samples can be analysed either in transmission, reflectance and interactance mode (Figure 7) so that liquid as well as solid samples can be investigated. Due to the quite broad bands compared to MIR, chemometrical spectra treatment is required for establishing adequate calibration models and to

analyse data. These are mainly multivariate (MVA) methods allowing to correct baseline, atmospheric noise etc. For qualitative analysis in most cases principal component analysis (PCA), for quantitative partial least square regression (PLSR) are applied [18].

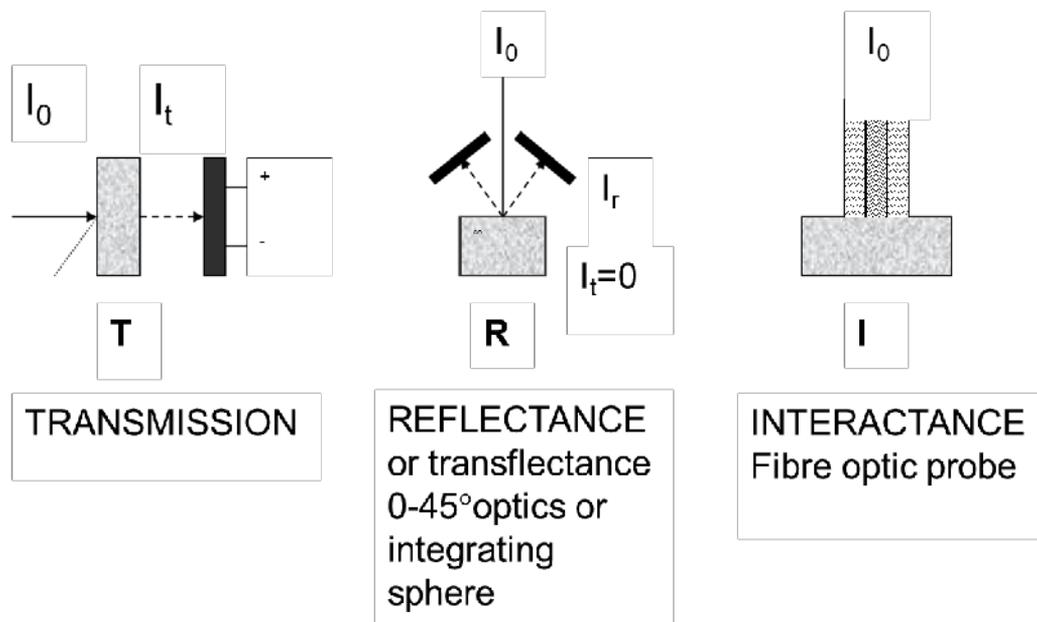


Figure 7. Sample measurement modes in NIR

An impressive example for the successful implementation in the food related production is the quality control of wine. It has been shown that NIR can be used to identify grapes, vines, age by qualitative (Figure 8) and its ingredients (acids, carbohydrates, pH etc.) simultaneously, non-invasively within a few seconds by quantitative analysis [19]. Another big advantage of this method can be found by the fact that the sample is not destroyed and can therefore be used for further purposes including following analytical steps.

Quantitative NIRS methods, which allow determining the carbohydrate, total acid, tartaric acid, malic acid, pH in grape variety and the polyphenol content in grapes were established [19]. The method can control the quality already at a very early stage during the wine production and allows improvement of its quality by this. Grapes of 12 different vines (*Weißburgunder*, *Chardonnay*, *Ruländer*, *Silvaner*, *Müller Thurgau*, *Gewürztraminer*, *Sauvignon*, *Lagrein*, *Grossvernatsch*, *Blauburgunder*, *Cabernet*, *Merlot*) were harvested in autumn 2000 and squeezed. The obtained grape variety was thermo stated at 23°C and analyzed quantitatively by NIRS in the transfection mode using an optical thin layer thickness of 1 mm. In order to establish a calibration model 252 spectra of samples with lower and upper concentration as a reference were recorded. 76 % of all spectra were randomly used for calibration, 24% for validation. Data preparation was carried out in order to minimize technical influences,

which mainly cause a drift in baseline. Quantitative analysis was carried out by partial least square regression (PLSR).

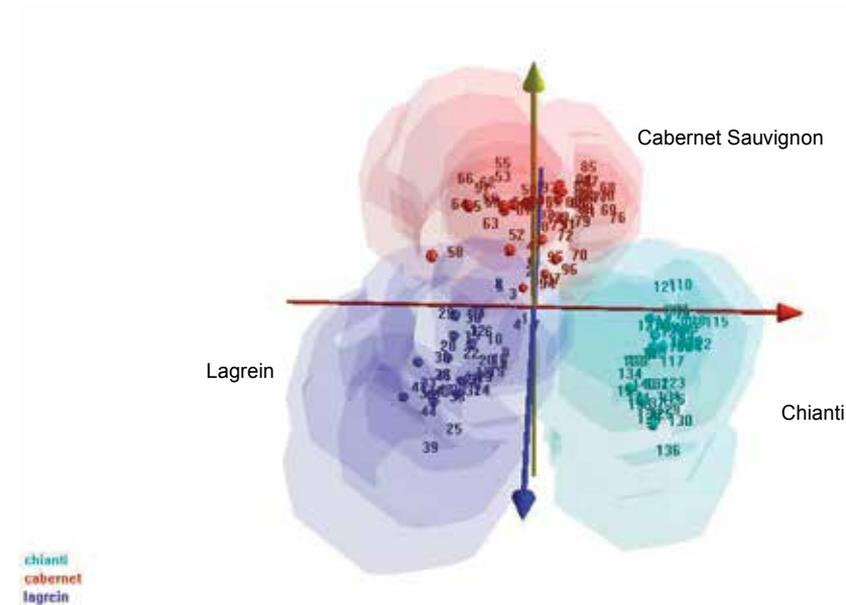


Figure 8. Factor plot of 141 spectra of different wines (Lagrein, Chianti, Cabernet Sauvignon). Conditions: Normalisation, 1. derivative; wavenumber range, 4500 - 10000 cm^{-1} ; thickness 3 mm; scans, 10; temperature, 23°C.

Carbohydrates. Data preparation comprised normalization between 0 and 1 and following calculation of the first derivative using a wavenumber range from 4500 - 7548 cm^{-1} . The PRESS function showed that 3 factors were needed for the calculation of the model. Calculation with 3 factors resulted in a good conformity between SEE and SEP. Linear regression between true and predicted values resulted in a value for the correlation coefficient of $R^2=0.99$ for calibration and $R^2=0.99$ for validation. Results for SEE and SEP: 0.13° KMW and 0.11 °KMW, the BIAS value is 2.30×10^{-15} .

Total acids. PLSR in a concentration range between 5 and 11 g/l included normalization between 0 and 1, full multiplicative scatter correction (MSC) and calculation of the 1st derivative (Taylor 3 points) between 4500 and 7548 cm^{-1} . 3 factors were necessary to obtain a minimum for PRESS and an agreement between SEE (0.60 g/l) and SEP (0.61 g/l) of nearly 100%. The highly linear model allows determining the total acid content with a prediction error of 0.61 g/l.

Tartaric acid. After normalization, performing of the 1st derivative over between 4500 and 7308 cm^{-1} , four factors were used for creation of the highly linear model depicted in Figure 6c with $R^2=0.91$ for calibration and $R^2=0.87$ for validation). Despite the small concentration range between 3.1 and 6.7 g/l used for calibration this system allows to determine the tartaric acid content with a prediction error of 0.15 g/l.

ric acid content in grape variety with an absolute error of estimation of 0.40 g/l and prediction of 0.54 g/l.

Malic acid. Malic acid often shows 2-5 times higher values compared to tartaric acid. Calibration between 2.9 and 7.0 g/l after normalization between 0 and 1 and calculation of a second smoothed derivative was carried out using three factors, SEE and SEP showing acceptable agreement. Absolute values for SEE, and BIAS were 0.43 g/l and -4.25×10^{-15} . Straight line for calibration showed a linearity of $R^2=0.89$ and allowed a prediction of the malic acid content with an absolute error of 0.55 g/l.

pH. Normalization and calculation of the smoothed 2nd derivative between 4500 and 7308 cm^{-1} showed an optimum for BIAS at five factors. Despite the narrow calibration range of pH 3.09 - 3.74 the calibration equation shows a R^2 of 0.82.

In order to enable the determination of these parameters with only one single measurement, simultaneous analysis of the carbohydrate, total acid, tartaric acid, malic acid content and pH was achieved by performing normalization (between 0 and 1) and calculating its 2nd derivative (Taylor 3 points). Four factors over a wavenumber range from 4500 to 7308 cm^{-1} showed 73-100% agreement between SEE and SEP. Linear regression showed high linearity for each investigated parameter with slightly lower values for R^2 . Compared to the above-described single analysis this method allows a quantitative analysis of all parameters at once within a few seconds. Values for SEP are slightly increased (Table 1).

Polyphenols mainly influence taste, sensory properties and color of a wine. Therefore, a rapid method to analyze its quantity is important. The method according to Folin - Ciocalteu was used as a reference method (see Materials and Methods). Gallic acid-1-hydrate was used as reference standard in a concentration range from 0 to 4.93 $\mu\text{g/ml}$ with equidistant steps. 24 gallic acid-1-hydrate solutions in a concentration range between 0.442 and 7.08 mg/ml were measured in the transmission mode threefold and in random order by NIRS. Evaluation using PLSR was achieved by dividing 72-recorded spectra randomly into a calibration (54 spectra) and validation (18 spectra) set. Data pretreatment comprised normalization between 0 and 1 and calculation of the 1st derivative (Savitzky-Golay) between 4008-7512 cm^{-1} . Using three factors, the PRESS function showed a minimum and a good agreement between SEE (0.45 mg/ml) and SEP (0.46 mg/ml). Linear regression between predicted and true values allowed to predict the gallic acid-1-hydrate concentration between 0 and 7 mg/ml with $R^2=0.98$.

In order to determine the total polyphenol concentration 30 must samples were measured in the transmission mode threefold and in random order. 90 spectra were divided into 72 calibration and 18 validation spectra. Normalization and performing of the 1st derivative allowed minimizing shifts in the baseline. 4 factors were necessary to obtain a minimum for the PRESS function and to get a maximum agreement of SEE and SEP. Linear regression allowed correlating true and predicted values with a R^2 of 0.97. Compared to the traditionally used Folin - Ciocalteu method in a winery, which is very time-consuming and expensive due to the usage of different chemicals, the NIRS method is very simple, precise and incomparably fast.

Parameter	Unit	SEE		SEP		BIAS	
		a	b	a	b	a	b
Carbohydrates	KMW	0.13	0.21	0.11	0.19	2.30×10^{-15}	3.33×10^{-16}
Total acids	g/l	0.60	0.43	0.61	0.53	7.17×10^{-15}	-1.08×10^{-14}
Tartaric acid	g/l	0.40	0.41	0.54	0.55	-1.08×10^{-14}	-3.43×10^{-15}
Malic acid	g/l	0.43	0.49	0.55	0.65	-4.25×10^{-15}	-2.44×10^{-15}
pH		0.07	0.09	0.06	0.09	-1.26×10^{-15}	-7.15×10^{-15}

Note. a Single analysis; b Simultaneous analysis

Table 1. Prediction results for the determination of the carbohydrate, total acid, tartaric acid, malic acid content and pH

Quality control of coffee ingredients including caffeine, theobromine and theophylline [20] and of food additives deriving from the highly interesting field of Traditional Chinese Medicine (TCM) [21] can be carried out in a similar way. Thereby, emphasis must be put onto the calibration method for which the above mentioned techniques can be applied as a reference. A new analytical method based on near infrared spectroscopy (NIRS) for the quantitation of the three main alkaloids caffeine (Caf), theobromine (Tbr) and theophylline (Tph) in roasted coffee after discrimination of the rough green beans into Arabic and Robusta was established. This validated method was compared to the most commonly used liquid chromatography (LC) connected to UV and mass spectrometric (MS) detection. As analysis time plays an important role in choosing a reference method for the calibration of the NIR-spectrometer, the non-porous silica-C18 phase offers a very fast method. Coupling of the optimised LC method to a mass spectrometer (MS) via an electrospray ionisation (ESI) interface not only allowed to identify Caf, Tbr and Tph by their characteristic fragmentation pattern using collisionally induced dissociation (CID), but also to quantitate the content of the three analytes, which was found to be 6% higher compared to UV-detection. The validated LC-UV method was chosen as a reference method for the calibration of the NIRS system. Analysis of 83 liquid coffee extracts in random order resulted for Caf and Tbr in values for S.E.E. (standard error of estimation) of 0.34, 0.40 g/100 g, S.E.P. (standard error of prediction) of 0.07 and 0.10 g/100 g with correlation coefficients of 0.86 and 0.85 in a concentration range between 0.10 and 4.13 g/100 g. Compared to LC the lower limit of detection (LOD) of the NIRS-method is found at 0.05 g/100 g compared to 0.244–0.60 ng/100 g in LC, which makes it impossible to analyse Tph by NIRS.

The possibility to hyphenate a MIR/NIR spectrometer to a microscope unit allows determining the distribution of active ingredients within a tissue sample down to a resolution of 1.2 μm [22]. A "hyperspectral cube" is recorded with the dimensions of the sample on the x- and y-axis and the absorbance on the z-axis from which the image can be extracted (Figure 9).

Fourier Transform Infrared (FTIR) spectroscopic imaging and mapping techniques have become essential tools for the detection and characterization of the molecular components of

biological tissues and the modern analytical techniques enabling molecular imaging of complex samples. These techniques are based on the absorption of IR radiations by vibrational transitions in covalent bonds and their major advantage is the acquisition of local molecular expression profiles, while maintaining the topographic integrity of the tissue by avoiding time-consuming extraction, purification and separation steps. These new techniques enable global analysis of biological samples with high spatial resolution and provide unique chemical-morphological information about the tissue status. With these non-destructive examination methods it is possible to get qualitative and quantitative information of heterogeneous samples.

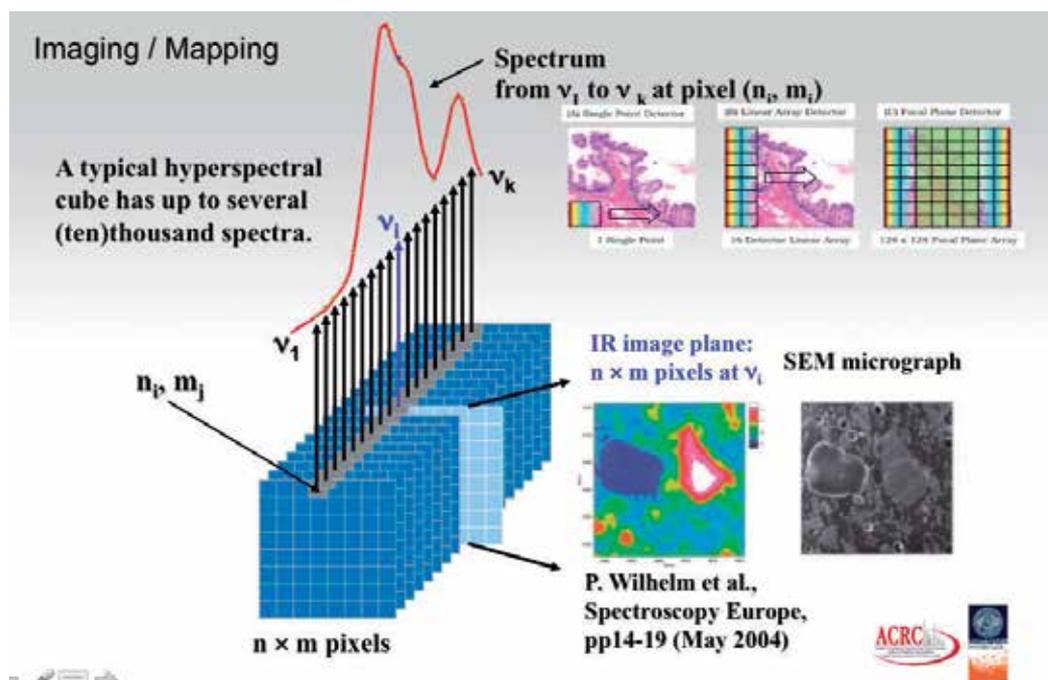


Figure 9. Principle of hyperspectral cube

Additionally, MALDI-TOF/MS imaging can be applied from the same sample of interest to get knowledge concerning the molecular weight distribution. This method is also suitable for studying the effect of nutrition onto different kinds of diseases, e.g. prostate cancer.

3. Conclusions

The techniques described can be applied according to the scheme depicted in Figure 1. This systematic analytical strategy allows getting multifacial knowledge and insights into food and samples derived therefrom.

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Quality Control in Pharmaceuticals

Microbial Quality Concerns for Biopharmaceuticals

Farzaneh Lotfipour and Somayeh Hallaj-Nezhadi

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/52114>

1. Introduction

Finding an appropriate definition or a clear classification for biologically occurring pharmaceutical products is a complicated task because of overlapping borders and consequent misconceptions in this area. Indeed, numerous definitions and classifications for this category of products have been proposed so far, and different points of view for this concept can be found in research literature, business, industry, and even the general public [1, 2].

To obtain a better view of biopharmaceutical concept, first, it is necessary to know the present definitions for the main constituents of the word, that is, pharmaceutical product and biological product.

According to the WHO, a finished pharmaceutical product (FPP) is “A finished dosage form of a pharmaceutical product, which has undergone all stages of manufacture, including packaging in its final container and labeling.” [3]

An active pharmaceutical ingredient can be defined as “A substance used in a finished pharmaceutical product (FPP), intended to furnish pharmacological activity or to otherwise have direct effect in the diagnosis, cure, mitigation, treatment or prevention of disease, or to have direct effect in restoring, correcting or modifying physiological functions in human beings.” [3] Hence, in brief, it can be said that any material, regardless of its origin or structure, with treatment, diagnosis, or prevention applications and passing regulatory requirements, is a pharmaceutical product.

On the other hand, the FDA definition for biological products is as follows: “Biological products or biologics are medical products made from a variety of natural sources (human, animal or microorganism). Like drugs, some biologics are intended to treat diseases and

medical conditions or to prevent or diagnose diseases.” [4] Consequently, any product of biological origin with treatment, diagnosis, or prevention applications is a biological product.

A biotechnology-derived product is another concept that should be taken into consideration. It is defined by Walsh as “any pharmaceutical product used for a therapeutic or in vivo diagnostic purpose, which is produced in full or in part by either traditional or modern biotechnological means.” [5]

On comparing the definitions for a biotechnology-derived product and a biological product, both of which should be of biological origin, it is obvious that the key element in the former definition is the application of biotechnological means for production.

In practice, the regulatory requirements needed for a biotechnology-derived product and a biological product are methodologically different from the pharmaceutical product due to their biological essence. For example, the determination of adventitious agents such as viruses, transmitting spongiform encephalopathy (TSE), and mycoplasma are included in most of the related guidelines and pharmacopeias for a biotechnology-derived product and a biological product.

Finally, a biopharmaceutical is defined by Walsh as “A protein or nucleic acid based pharmaceutical substance used for therapeutic or in vivo diagnostic purposes, which is produced by means other than direct extraction from a native (non-engineered) biological source.” This definition that will be used in the present chapter for biopharmaceuticals includes all pharmaceutical products produced by modern biotechnology techniques as well as nucleic acid (DNA or RNA) based pharmaceutical products for gene therapy. Hence, the overlapping area between biotechnology-derived products and biopharmaceuticals is the application of modern biotechnological means in their production. However, the differentiating area can be the application of traditional biotechnological means for the production of biotechnology-derived products. In addition, nucleic acid-based pharmaceutical products that are categorized as biopharmaceuticals are not biotechnology-derived products [6]. Figure 1 illustrates these overlapping and differentiating areas. Moreover, some examples of products in these categories are shown in Table 1.

Biological products	Biotechnology-derived product by:		Biopharmaceuticals
	Traditional technology	Modern technology	
Blood and blood products	Therapeutic proteins from natural sources	Recombinant proteins	Recombinant proteins
Human cells and tissues	Antibiotics fully or partially from microorganisms	Monoclonal antibody produced by hybridoma technology	Nucleic acid-based pharmaceutical products for gene therapy

Table 1. Some examples of products related to biological, biotechnology-derived, and biopharmaceutical products.

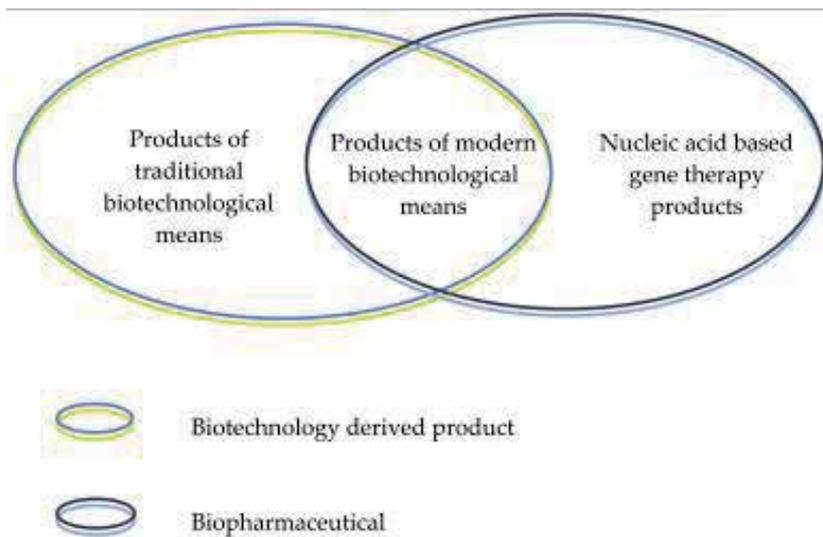


Figure 1. The schematic representation for biotechnology-derived products and biopharmaceutical categories and the overlapping areas between them.

2. Methods for biopharmaceutical production

2.1. Production of an original recombinant system

2.1.1. Recombinant DNA technology

The main category of biopharmaceuticals is manufactured via recombinant DNA technology. Indeed, recombinant DNA technologies are enabling techniques that manipulate and engineer different gene fragments and which have been introduced less than 50 years ago by the revolutionary invention of Polymerase Chain Reaction (PCR) by Kary Mullis [7].

DNA and RNA extraction from different cell types, cutting DNA fragments using restriction endonucleases, joining DNA fragments by DNA ligases, PCR to amplify gene fragments, cloning of the gene fragments into different vectors, introduction of recombinant constructs into proper hosts, protein expression, extraction, and purification are some of the most widely used means in recombinant protein production.

Figure 2 schematically represents the summarized process of production of a recombinant protein. As can be seen from the chart, first, the gene of interest should be isolated and amplified from the original cell. According to the type of the cell, it can be done through direct total DNA extraction followed by a PCR using proper primers to obtain the gene in prokaryotes. On the other hand, in eukaryotes, due to the existence of introns and some modifications that occur in the transcribed mRNA, the process is considerably complicated. Introns are non-coding sequences which are removed after transcription versus coding sequences

that are called *exons*. In addition, mRNA is more modified by the addition of a methylated guanine (CAP) on its 5' end and a poly-adenine tail on its 3' end. After these modifications, mature mRNA is exported to the cytoplasm in order to start the translation process. Consequently, to obtain a gene of interest in eukaryotes, the mature mRNA should be extracted from the cell, and the complementary DNA should be synthesized followed by amplification of the gene by PCR using proper primers. However, in both cases (prokaryotes and eukaryotes), the short genes can be obtained by a solid-phase synthesis process.

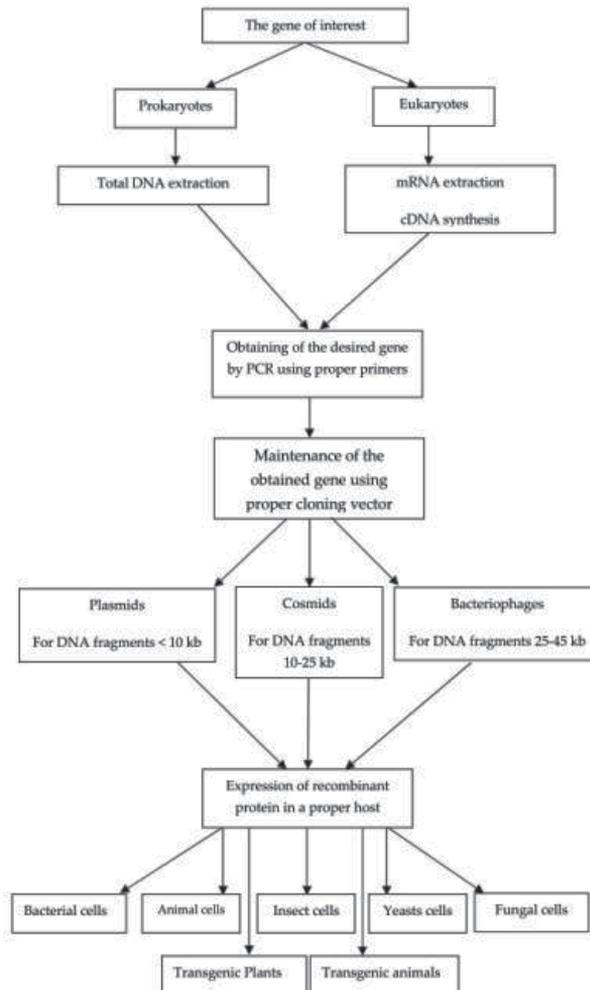


Figure 2. Schematic representation of recombinant protein production process

Based on their sizes, the obtained genes can now be introduced into a proper vector for maintenance, replication, or expression purposes. Plasmids, cosmids, and bacteriophages are the most important cloning vectors that are classified according to the size of the DNA

fragment that can be inserted into them. Ultimately, the new recombinant construct should be introduced into an expression system for production. Different classes of expression systems with their certain merits and disadvantages are available and range from cell systems such as bacterial, animal, fungal, and yeast cells to transgenic systems such as transgenic plants and animals [7, 8].

2.1.2. Monoclonal antibody production

2.1.2.1. Classical hybridoma technology

Each specific antibody is secreted by a specific B cell and could recognize a specific region on the antigen that is called *epitope*. Each antibody-secreting B cell could be used as a source of an antibody of interest if it is isolated and cultured in vitro. Nevertheless, B cells are not considered a satisfactory source, as they are not immortalized and cannot survive for a long time. The main approach for overcoming this problem is cell hybridization, which includes the fusion of antibody-secreting B cells with tumor cells (such as mouse myeloma cells) proliferating ever more. The classical hybridoma technology was first introduced by Georges Kohler and Cesar Milstein in the mid 1970s for the generation of immortalized hybridoma cells that could grow in cell culture for a long time and produce the desired monoclonal antibodies [9, 10]. The basic process (Figure 3) includes the immunization of a mouse with the desired antigen. The mouse was then sacrificed, and B lymphocytes secreting antibodies that were selective for the specific epitope on the antigen were isolated from the spleen. The spleen is considered the most ready source for antigen-specific lymphocytes that provides access to a large number of antibody-secreting cells [1]. The isolated B cells were subsequently fused with immortal mouse myeloma cells. The resultant hybridoma cells were then separated from the unfused cells by culturing in specific cell culture media. The cell culture media for the hybridoma growth and production of monoclonal antibodies have been reviewed in detail by Bols et al. [11].

In general, for the successful fusion of hybridomas, the cells are grown in HAT selection medium. The selection medium is called *HAT*, as it has Hypoxanthine, Aminopterin, and Thymidine. This is because a mutation in either the hypoxanthine-guanine phosphoribosyltransferase (HGPRT) or the thymidine kinase (TK) gene of the cells would cause their death in the HAT medium. Generally, a TK-deficient cell (TK-negative mutant) is resistant to bromodeoxyuridine (BrdU), and an HGPRT-deficient cell (HGPRT-negative mutant) is resistant to 6-thioguanine (6-TG) and 8-azaguanine. Hence, in order to make myeloma cells sensitive to HAT (unable to grow in HAT media), they are treated with one of these drugs before their passage to HAT media.

Normal cells can synthesize the required nucleotides in two pathways: (1) the main one or de novo biosynthetic pathway, and (2) the alternative one or the salvage pathway (when the main pathway is blocked).

Aminopterin (a folic acid analog that inhibits dihydrofolate reductase) blocks the activation of tetrahydrofolate, which is required for the synthesis of nucleotides via the de novo synthetic pathway, and, therefore, the main pathway is blocked. Thus, in aminopterin-treated

cells (HGPRT⁺ and TK⁺), the synthesis of nucleotides shifts to the salvage pathway only if hypoxanthine and thymidine are supplied in the medium. HGPRT and TK, the two enzymes, are required for the salvage pathway, and they catalyze the synthesis of purine and thymidylate from hypoxanthine and thymidine substrates, respectively.

Since unfused myeloma cells lack HGPRT or TK, they cannot use the salvage pathway. Thus, the unfused myeloma cells get killed in the HAT medium, as both biosynthetic pathways are blocked. Normal unfused B cells die in the HAT medium, as they are not immortalized and cannot grow for a long time. Nevertheless, the fusion of normal B cells with the HGPRT⁺ or TK⁺ myeloma cells allows the hybridoma cells to grow in HAT medium, as the B cells provide the necessary enzymes for growth of the hybridoma cells.

Hence, the HAT selection medium offers an ideal environment for the isolation of fused myeloma and B cells (hybridoma cells) from unfused myeloma cells and unfused B cells, as this medium allows only the hybridoma cells to survive in the culture.

The production of monoclonal antibodies could be accomplished by ascites (ascitic fluid) production (in vivo) or by cell culture (in vitro) methods. In the in vivo method, hybridoma cells are injected intraperitoneally into mice. The peritoneum serves as a growth chamber for the injected cells. These cells could secrete a high-titered solution of desired antibodies as they grow in the cavity. Finally, the produced antibodies are extracted from the ascitic fluid accumulated in the peritoneal cavity [6]. The antibody concentrations typically range between 1 and 15 mg/ml. The in vivo method offers a very high concentration of monoclonal antibody that often does not need more concentration procedures. Nevertheless, monoclonal antibodies produced by this technique may be contaminated by considerable levels of mouse proteins and other contaminants that might require more complicated, subsequent downstream purifications. The other disadvantage of the ascites production is related to animal welfare issues, as these could cause distress in mice.

Currently, more than 90% of monoclonal antibodies are produced by in vitro techniques [12] that use large-scale manufacturing plants containing several 10,000-L or larger culture bioreactors [13]. The in vitro method of monoclonal antibody production decreases the use of mice and also avoids the need for experienced personnel for animal handling. Regardless of the privileges and importance of the in vitro methods of antibody production, there are some situations in which this method is not applicable; for instance:

1. Hybridoma cells do not adapt well to in vitro conditions.
2. Downstream purification methods cause protein denaturation or decreased antibody activity.
3. The cell line cannot maintain the production of monoclonal antibodies.
4. When hybridoma cells are contaminated with infectious agents (such as yeasts or fungi), the cells must often be passed through mice. Since removal of the organisms cannot be accomplished by current antimicrobial drugs, thus the in vivo method may save a valuable hybridoma.

5. When in vitro methods result in monoclonal antibodies that are glycosylated at positions different from those harvested from mouse ascites, they affect antigen-binding capacity as well as biological functions [12].

Taken together, the cell culture technique is a method of choice for large-scale monoclonal antibody production due to the simplicity of the cell culture and financial considerations without ethical concerns that are related to animal use.

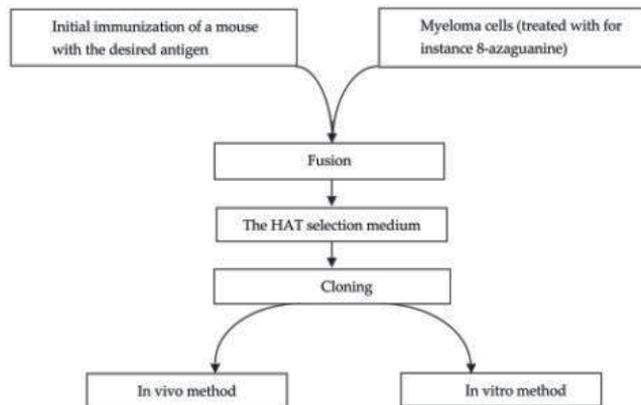


Figure 3. The diagram of the monoclonal antibody production via classical hybridoma technology

2.1.2.2. Chimeric and humanized antibodies

In 1986, about 10 years after the conception of monoclonal antibody technology, Orthoclone OKT3 was approved by the FDA for use in patients with acute rejection of a transplanted kidney [14]. Unfortunately, early clinical applications of murine monoclonal antibodies were disappointing. This was due to the fact that monoclonal antibodies produced via the classical method are of murine origin and are, therefore, immunogenic to human subjects. In general, patients receiving an antibody exhibit HAMA responses (human anti-mouse antibodies) within two weeks. Multiple infusions of murine monoclonal antibodies significantly enhance the HAMA reactions [6]. In addition, the immune system eliminates the murine monoclonal antibody molecule. Thus, murine monoclonal antibodies demonstrate short serum half lives after administration to humans. Furthermore, the other main difficulty related to murine monoclonal antibodies is the poor recognition of the Fc region by human effector systems of complement and Fc receptors.

Thus, new strategies that are used for producing humanized mouse antibodies that are less immunogenic have been discovered. The first strategy includes the production of functional specific recombinant IgG molecules consisting of mouse variable regions and human constant regions; these are known as chimeric antibodies. Taken together, in the chimeric antibody, 8 out of 12 domains are of human origin (constant regions of the heavy and light chains) (C_H and C_L) [15]. Chimeric antibodies exhibit reduced HAMA responses compared

with mouse antibodies, but the affinity and the selectivity are the same. Furthermore, since the Fc region contains human sequences, the activation of Fc-mediated immune effector functions is allowed.

To further minimize the antigenicity of murine antibodies, humanized antibodies were developed. For their generation, hyper-variable complementarity-determining regions (CDRs) of the specific murine antibody are transferred to a fully human framework. In comparison with the mouse antibodies, humanized antibodies suggest a lower occurrence of HAMA responses.

Further efforts have been invested in the development of technologies that generate fully human monoclonal antibodies. One of the approaches entails the development of transgenic mice, in which a repertoire of human immunoglobulin germline gene segments is inserted into the mouse genome. After the immunization of these mice, they produce fully human antibodies, which can subsequently be separated with the classical hybridoma technology [15].

Figure 4 illustrates the schematic structures of mouse, chimeric, humanized, and human antibodies.

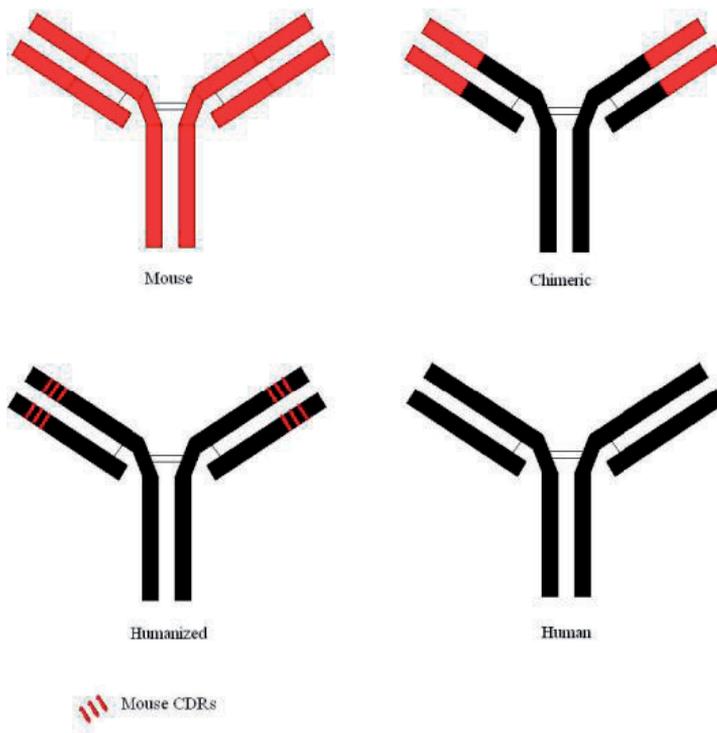


Figure 4. The structure of mouse (shown in red color), chimeric, humanized (shown in black color), and human antibodies. Chimeric antibodies comprise mouse variable regions and human constant regions. Humanized antibodies consist of murine hyper-variable complementarity-determining regions (CDRs) that are grafted to fully human framework.

2.2. Mass production of a recombinant product

Mass production of recombinant products can be achieved in a process that is divided into two main sections called *upstream* and *downstream* processing, as schematically depicted in Figure 5.

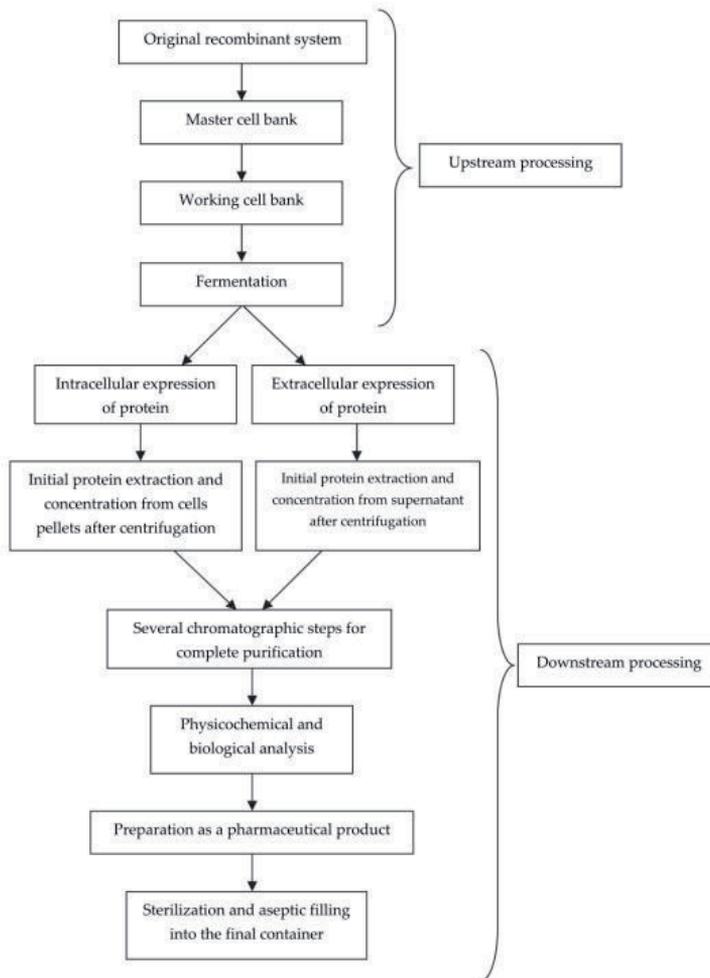


Figure 5. Mass production of a recombinant product.

The first step, the upstream processing step, is the mass production of a recombinant protein via the fermentation process. The original recombinant system that is used for the expression of the recombinant protein (i.e. in the form of a recombinant plasmid maintained in a suitable host cell) forms the cell deposit in a cell banking system. There are two levels of cell deposits in the cell banking system: The first line is called *master cell bank*, which is prepared

directly from a culture of the original recombinant system that includes several hundred stored ampoules.

The second line that is referred to as the *working cell bank* is produced from a single master cell bank ampoule. Each ampoule from the working cell bank is thawed and used to seed the fermentor for the production of a batch. Obviously, the fermentation process for various recombinant systems, such as bacterial fermenting systems or animal cell systems, is quite different and needs its own certain requirements.

On the other hand, *downstream processing*, which is the next step, refers to the purification of the mass produced protein. The first step in downstream processing is the initial extraction and concentration of the product, which depends on the situation of the expressed protein; that is, it should be extracted from the pellet cells for intracellular proteins or from the supernatant for extracellular proteins after centrifugation.

The second stage entails (1) several chromatographic steps that complete the purification of the product; (2) the potency test; (3) the addition of suitable excipients; (4) sterilization; (5) filling of the product in its final form (liquid or solid) into the final container before sealing; and labeling [6].

3. Sources of biological contamination of biopharmaceuticals

In line with conventional pharmaceutical products, the main sources of biological contamination in biopharmaceuticals can be related to raw materials and the production environment. Indeed, the biological contaminant content of any pharmaceutical product is a representative of their starting materials and the production environment flora.

3.1. Raw materials

Animal origin materials, such as cell culture media, sera, and supplements that are extensively used in biopharmaceutical production, are of high contamination risk. These materials can be considered the main source for the contamination of biopharmaceuticals with adventitious agents such as TSEs, viruses, and mycoplasmas. Therefore, they should be supplied from reliable resources, and special attention should be paid to their quality control procedure. It should be ensured that all raw materials, especially those of high risk, gain quality specifications for current good manufacturing practice.

Standard methods for sterilization of cell culture media, sera, and supplements should be established according to the properties of the materials. Due to the heat-labile nature of the majority of materials used in biopharmaceutical production, autoclaving is usually replaced with alternative strategies such as filter-sterilization or less frequently high-temperature, short-time treatment strategies. In spite of the routine filter-sterilization procedure that uses 0.22 μm , it is usually performed with 0.1- μm membrane filters due to the risk of contamination with adventitious agents.

Furthermore, high-temperature, short-time treatment strategies are sometimes employed for the elimination of biological contaminants from small solutes such as vitamins and amino acids [16].

Another important raw material that is used in the production of any pharmaceutical product, including biopharmaceuticals, is water, which can be considered an important source for contamination, with water-borne bacteria such as *Pseudomonas* spp., *Alcaligenes* spp., *Flavobacterium* spp., *Chromobacter* spp., and *Serratia* spp. Water for pharmaceutical purposes is discussed in detail in the USP [17]. Due to the fact that the intended administration of biopharmaceuticals in the majority of cases is via injection, Water for Injection (WFI) which is sterile and apyrogen is routinely used in this area.

3.2. Production environment

Pharmaceutical products' contamination may occur from the transformation of microorganisms from the production environment to the product. The production environment includes air, surfaces, instruments, equipments, and personnel.

The main groups of microorganisms that are isolated from air are the spore-forming bacteria (*Bacillus* spp. and *Clostridium* spp.), the non-sporing bacteria *Staphylococcus* spp., *Streptococcus* spp., and *Corynebacterium* spp.), the molds (*Penicillium* spp., *Cladosporium* spp., *Aspergillus* spp., and *Mucor* spp.), and the yeast (*Rhodotorula* spp.). These contaminants may be air borne or can be initiated from process equipment or personnel [16]. Consequently, environmental monitoring programs in a production environment are essential actions. Furthermore, the critical operations in biopharmaceutical production should be performed in controlled environments or clean rooms. A clean room is a place with high control of the entrance of particles via the establishment of some air filters called *high-efficiency particulate air (HEPA) filters*. HEPA filters made from a microglass material with a pleated construction system provide a large surface area that efficiently filters the incoming air and generates a constant air motion. Based on the permitted quantity of viable microorganisms and particulates, various classes of clean rooms can be established using HEPA filters with required efficiencies.

According to the *EC Guide to Good Manufacturing Practice for Medicinal Products (EC GGMP)*, four grades for clean rooms are available, such as grade A, B, C, or D, based on the number of viable microorganisms and particulates (Table 2).

Clean room grade	Maximum permitted number of particles/m ₃	Maximum permitted CFU of viable microorganisms/m ₃ in air sample
A	3500	<1
B	3500	10
C	350000	100
D	3500000	200

Table 2. Clean room grades according to the number of viable microorganisms and the number of particulates

Critical operations such as inoculum preparation and aseptic filling are generally performed in the highest air grade (A); however, less critical operations can be performed in lower grades or even non-classified air.

In addition to the establishment of suitable filters in the clean rooms, special attention should be paid to the position, type, and texture of surfaces, floors, and fixtures. They should be made from smooth and chemically stable materials. In addition, a distinct transfer lock area should exist before entry to the clean room for sanitization of materials and personnel or garment changing. Furthermore, all doors should be interlocking [6].

4. Hazards of biological contamination of biopharmaceuticals

Similar to other pharmaceuticals, biological contamination of biopharmaceuticals may perhaps cause product spoilage. It may result in product metabolization by microorganisms, and, therefore, lead to a decrease in biopharmaceutical potency. The product spoilage may also provide a potential health hazard to patients and lead to outbreaks of infections that may cause additional complications. In addition, microbial-derived agents secreted in products such as endotoxins can be hazardous to a patient's health.

5. Determination of biological contaminants

5.1. Bacteria and fungi

Bacteria and fungi can be considered important contamination sources for all kinds of pharmaceutical products, including biopharmaceuticals; hence, the control of them is of critical importance. The control of both bacteria and fungi is considered to be worthy of mandatory tests for nearly all kinds of pharmaceuticals in pharmacopoeias. All the related tests and procedures are covered in detail in the major pharmacopoeias such as USP and EP [17, 18].

Since almost all the biopharmaceuticals are administered intravenously, general sterility testing must be carried out for these products. Basically, sterility testing can be defined as "a test that evaluates whether a sterilized pharmaceutical product is free of contaminating microorganisms." The European Pharmacopoeia (2002) proposes two media for sterility testing:

(1) fluid mercaptoacetate medium (also known as *fluid thioglycollate medium*), which is mainly appropriate for the culture of anaerobic organisms at 30–35°C; and (2) soyabean casein digest medium, which is used for the culture of both aerobic bacteria at 30–35°C and fungi at 20–25°C.

Two main methods are used for sterility tests: (1) direct inoculation of the test samples in the media mentioned earlier; or (2) filtration of the test material through a sterile membrane filter with a pore size of 0.45 µm; then, the filter containing any microorganism present in the fluids is divided aseptically, and portions are transferred to the media.

The eradication of bacteria and fungi from the products is generally carried out via inactivation and sterile filtration.

5.2. Endotoxins

Since most of the biopharmaceuticals are administered intravenously, finished-product biopharmaceuticals must be sterile and free from pyrogenic substances. The endotoxin limit for the intravenous administration of pharmaceutical and biological products is 5 endotoxin units (EU)/kg of body weight/hour by all pharmacopoeias [19]. Hence, the detection and removal of pyrogenic substances, especially endotoxins (lipopolysaccharides in the cell wall of gram-negative bacteria), are necessary to ensure safety of biopharmaceutical products. Currently available methods for endotoxin detection include the U.S. Pharmacopeia rabbit test and the *Limulus* amoebocyte lysate (LAL) test [7].

The rabbit pyrogen test entails measurements of the rise in body temperature of rabbits after an intravenous injection of a test substance. The presence of pyrogens of all kinds can be tested using this method. However, this method suffers from a number of disadvantages and limitations: (1) Endotoxin tolerance occurs after repeated use of rabbits; (2) variations in the response depending on sex, age, and species; (3) differences between the responses of rabbits and humans to various pyrogen types; and (4) the rabbit pyrogen test is inadequate for sera, radiopharmaceuticals, chemotherapeutics, analgesics, cytokines, immunosuppressive agents, and others [20].

Accordingly, the use of the rabbit pyrogen test has been reduced. Nowadays, the most widely used endotoxin detection systems are based on the highly sensitive LAL test. It is based on the coagulation cascade of the blood of a horseshoe crab, *Limulus polyphemus*, which is induced by lipopolysaccharide. The currently known methods for lipopolysaccharide detection entail (1) gel-clot assay, (2) turbidimetric LAL technique, and (3) the chromogenic LAL technique.

The gel-clot assay is a limit test that provides simple positive or negative results. The LAL reagent is introduced to a sample, and the test material is considered endotoxin positive if a gel is formed via a clotting reaction.

The turbidimetric and the chromogenic LAL techniques are quantitative tests. The former is based on the fact that turbidity increases as a result of the precipitation of the clottable protein that is related to endotoxin concentration in the sample. The optical density is read by a spectrophotometer at either a fixed time (for the end-point method) or progressively (for the kinetic assay) as turbidity develops.

The chromogenic LAL technique makes use of a synthetic substrate which contains an amino acid sequence similar to that of the clottable protein, coagulogen, in order to detect endotoxin. The enzyme cleaves a yellow-colored substance from the chromogenic substrate, and the color intensity produced is proportional to the amount of endotoxin present in the sample.

Endotoxins are temperature and pH stable, and, therefore, their removal is one of the most challenging issues. Numerous techniques are used to reduce endotoxin contamination of biopharmaceuticals, including ion-exchange chromatography, sucrose gradient centrifugation, gel filtration chromatography [19], affinity adsorption [21], charged membrane/depth filtration, and ultrafiltration [22].

5.3. Viruses

Owing to the risks of transmission of adventitious agents to patients, the different cell levels should be studied for the absence of these agents. Among the adventitious agents, special attention should be paid to viruses that are capable of contaminating the original species. Generally, the virological safety of biopharmaceuticals includes several levels of control at various manufacturing stages, including 1 - rigorous screening of cell banks (both master cell bank and working cell bank) for viruses; 2 - screening of each cell culture harvest for adventitious agents; and 3 - a demonstration that the purification process can clear potential adventitious agents [15].

The detection of viruses in cell lines can be carried out via various techniques. The commonly used methods of detecting viral infections include

- co-cultivation assays (specific in vitro tests),
- in vivo assays,
- antibody production in animals (MAPs, RAPs, or HAPs),
- immunoassays for viral specific proteins,
- Transmission Electron Microscopy (TEM),
- Polymerase Chain Reaction (PCR).

For the co-cultivation assays (specific in vitro tests), the cells used for production, or culture supernatant, or the final product are incubated with the detector cells. The detector cell lines are susceptible to different viruses and are used to detect desired viruses via monitoring subsequent cytopathic effects, hemadsorption, morphological changes, or other signs of viral infection. The detector cells usually contain humans, primates, and cells from the same species.

The in vivo assay can be performed by the inoculation of cells or cell lysates into animals, including newborn and adult mice, guinea pigs, rabbits, or embryonated chicken eggs to detect viruses. The animals are consequently monitored for any abnormality.

Species-specific viruses potentially present in rodent cell lines can be examined using assays for antibody production in the animals. The MAP, RAP, and HAP (mouse, rat, and hamster antibody production assays, respectively) tests involve an injection of the test article into the animals. The inoculated animals are bled after four weeks, and the sera are tested for the presence of the antibodies against the specific viral antigens. For instance, Hantaan virus, Lactic Dehydrogenase virus, and Sendai virus have been screened using MAP.

An immunoassay for viral-specific proteins can be undertaken through production of the relevant antibodies after an injection of a virus of interest into animals. Currently commercially available immunoassays are able to detect various viruses.

Another method that is used for virus detection is TEM (Transmission Electron Microscopy). TEM is a quantitative assay that is based on the visualization and morphological identification of virus particles in samples [23].

Nevertheless, more sensitive methods, such as the PCR identification methods, can be employed for the detection of sequences of the viruses [16].

Since the biopharmaceuticals can be originated from mammalian cell lines with a high risk of endogenous retroviruses, on one hand, and these products may be infected with adventitious viruses through processing, on the other hand, virus inactivation and removal steps in the purification process are required [24]. These entail gamma irradiation, low pH treatment, or virus filtration.

Indeed, ensuring the absence of virus contamination in biopharmaceuticals is challenging. For instance, a limited number of commercial poultry vaccines were contaminated by avian leukosis virus even after routine quality assurance procedures. In addition, reovirus was found as a contaminant in urokinase. On the whole, sourcing and testing alone cannot guarantee the virological safety of biopharmaceuticals owing to some limitations: the limit of sensitivity for cell culture and PCR tests and also due to the fact that cell culture or *in vivo* tests are not able to detect all known kinds of potential contaminants [25]. Thus, practical methods are required for the virological safety of biopharmaceuticals, which involve the inclusion of risk assessment as well as management policies.

5.4. Mycoplasma

Mycoplasmas are the smallest free-living and self-replicating organisms in nature that are sized between 50 and 500nm. They lack a rigid cell wall and, consequently, are highly pleomorphic from round to filamentous. They are filterable and penicillin-resistant forms. Furthermore, their membrane contains sterol and due to this, mycoplasmas require the addition of serum or cholesterol to the growth medium. They grow on special media in aerobic or anaerobic conditions with optimum growth at 37°C and pH 7.0 and form with a “fried egg” morphology on agar media (Figure 6).

Mycoplasma contamination of cell culture systems for the production of mycoplasmas is a critical problem due to its effect on various parameters within the cell culture system. Mycoplasma contaminates cell cultures approximately without any sign, and it persists for a long time. Indeed, mycoplasma-positive cell cultures can be considered the major source of biopharmaceutical infection, and they should be discarded or effectively decontaminated. Taken together, mycoplasma-positive cell cultures pose a serious problem and should be effectively detected and eradicated [26].

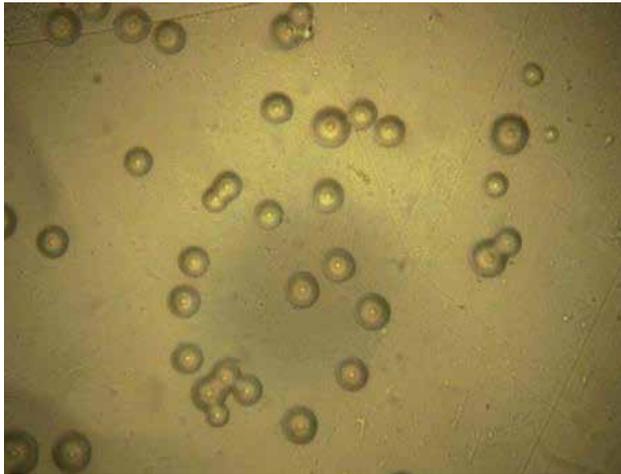


Figure 6. Mycoplasma colonies with fried egg morphology on mycoplasma agar medium. Picture was taken at Mycoplasma Reference laboratory, Razi vaccine and serum research institute, Iran

Different methods are used in international pharmacopoeias and guidance for detecting mycoplasma in biological test samples, mainly categorizing them as direct assay by microbiological culture, indirect assay by indicator mammalian cell culture, and PCR.

Direct assay by microbiological culture: The principle of detection is based on the growth of mycoplasma on supporting agar and liquid media (broth). First, the test sample is introduced into a special broth culture, is incubated for an appropriate time, and, consequently, it is sub-passaged to plate agar. After the required incubation period, the presence of mycoplasma colonies is observed microscopically in the agar plates (Figure 6).

Indirect assay by indicator mammalian cell culture: The indirect method requires the co-cultivation of the test sample with an indicator cell line for two to three days. Typically, VERO cells with a large cytoplasm area around the nucleus were used. Consequently, the cells were stained using a DNA binding stain (such as Hoechst stain) that binds specifically to DNA and is observed via fluorescent microscopy. Due to the affinity of mycoplasmas for the mammalian cell membrane, mycoplasmas appear as granules surrounding the nucleus.

Mycoplasma PCR: In this method, detection is carried out using specific oligonucleotide primers for the amplification of mycoplasma DNA. This method is specially recommended for detecting contamination with the non-cultivable strains of *M. hyorhinis* [27].

On the whole, it is advisable to use two different methods in the detection of mycoplasmas in order to allow for the differentiation between false-positive and false-negative results.

5.5. DNA

The importance of DNA contamination detection in biopharmaceuticals is related to the fact that the DNA from some sources such as hybridoma cell lines in monoclonal antibody production may act as active oncogenes. These kinds of DNA contaminants can be introduced

and expressed in human cells and result in the initiation of cancer cells. According to guidelines, the acceptable level of residual DNA in recombinant products is 10 pg per therapeutic dose. DNA hybridization studies that use radiolabeled DNA probes with a specific nucleic acid sequence constitute one of the most widely used methods for the detection of DNA contaminants in the product to a nanogram (ng) range [6]. The important steps involved in DNA hybridization are shown in Figure 7.

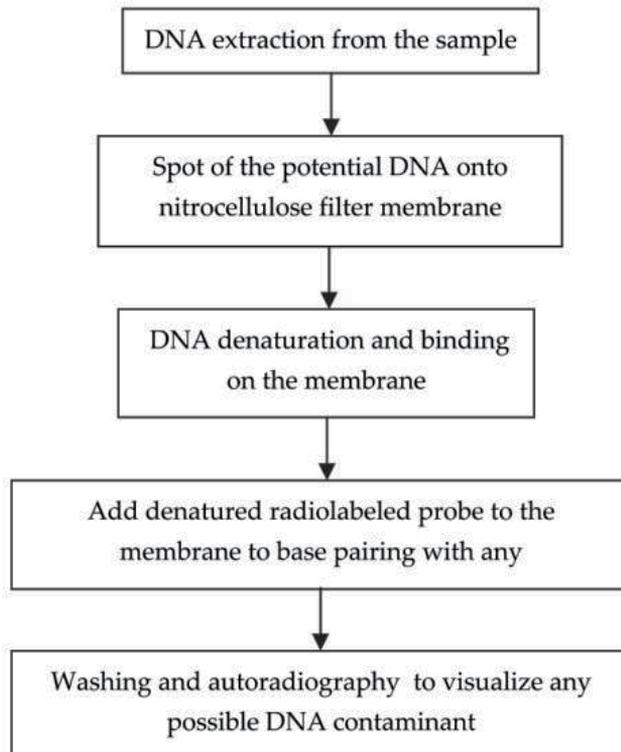


Figure 7. The main steps of the DNA hybridization procedure

5.6. Cross-contamination

Cell cultures may be infected with other cell types due to the use of contaminated items or operator mistakes. Also fail of the sterilization process can be another reason. The detection of cross-contamination is very challenging due to the fact that macroscopic and microscopic properties of the original and contaminant cells are commonly the same. Cross-contamination in the production of biopharmaceuticals would prove to be disastrous and terrible.

Various tests for detecting cross-contaminations can be applied; however, a product-specific identity test will be the best choice [16].

6. Summary

Microbial quality control plays a prominent role in the manufacture of safe and effective biopharmaceuticals. The main sources of microbial contamination can be related to raw materials and the production environment. The main categories of raw materials that are involved in the manufacturing of biopharmaceuticals with a high risk of contamination are those of animal origin such as cell culture media, sera, and supplements. The production environment includes air, surfaces, instruments, equipments, and personnel. All these can be considered the main source for the contamination of biopharmaceuticals with adventitious agents such as viruses, bacteria, fungi, transmitting spongiform encephalopathy, and mycoplasma. The use of contaminated biopharmaceuticals causes product spoilage, which may lead to (i) metabolization of the therapeutic agents by microorganisms, thus bringing about a decrease in the potency of the therapeutic agent; (ii) a potential health hazard to patients as a result of either infectious diseases or microbial-derived agents such as endotoxins that are secreted into products.

Various methods are used for detecting and eliminating different biological contaminants that are used in the manufacturing of biopharmaceuticals. Generally, bacteria and fungi can be detected by standard sterility testing or macroscopic and microscopic characteristics, as well as biochemical tests. In addition, viruses can be detected via a number of methods such as co-cultivation assays (specific *in vitro* tests), *in vivo* assays, antibody production in animals (MAPs, RAPs, or HAPs), immunoassays for viral specific proteins, TEM, or PCR. The detection of endotoxin can be carried out using the pharmacopeial rabbit test or LAL test. The available approaches for the detection of mycoplasma include direct assay using special culture media, indirect assay by mammalian cells, and DNA staining, as well as PCR. Furthermore, DNA hybridization is a widely used approach for the detection of DNA contaminants in biopharmaceuticals. The best method for cross-contamination detection includes a product-specific identity test.

With regard to the collection of tests for biological quality control of biopharmaceuticals summarized in this chapter, it is obvious that various sets of methods are available in different guidelines and pharmacopeias which are complicated and problematic. The development and compilation of harmonized guidelines for biological quality control of biopharmaceuticals is a critical necessity that can facilitate the control of the safety of these ever-increasing products.

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New Approachs in Drug Quality Control: Matrices and Chemometrics

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Additional information is available at the end of the chapter

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1. Quality control

Quality control refers to the process of quality evaluation that focuses on the internal measurement of the quality of a process, institution, product, service, or other. Often used interchangeably with *quality management* and *quality assurance* [1-3].

2. Drug quality control

Quality Assurance plays a very important role in making sure that the GMP standards are met and products comply with the international quality standards. The main functions carried out by drug quality control are:

- Approval of raw materials
- Monitoring of manufacturing processes
- Approval of finished products
- Documentation of technical information
- Implementation of cGMP

Manufacturing processes are monitored and controlled by testing of raw materials, in-process parameters. Final active pharmaceutical ingredients and dosage forms are tested for specified parameters before release. Analytical testing is carried out with highly sophisticated instruments: viz. HPLC, GC, IR, UV spectrophotometer mettler titrators, particle size analyzer etc.

All the analytical test procedures and manufacturing procedures are well documented and revision is undertaken as per specified protocol. Analytical methods are validated to give the reproducible results. Stability study as per stability protocol is considered to be very important area of Quality Assurance.

Automated systems are becoming increasingly important tools for appropriate monitoring and controlling of the pharmaceutical packaging process. Solutions for comprehensive quality assurance or production data acquisition and evaluation are just as important as applications that meet the legislative requirements of different countries in terms of serial numbering and the unique marking of products.

Quality control involves many phases, such as sample collection, measuring, analysis of results, and the approval/rejection of the batch. Nonetheless, the most important thing is the continuity and systematization of the quality control.

Effective process validation contributes significantly to assuring drug quality. The basic principle of quality assurance is that a drug should be produced that is fit for its intended use. This principle incorporates the understanding that the following conditions exist: Quality, safety, and efficacy are designed or built into the product.

Quality cannot be adequately assured merely by in-process and finished-product inspection or testing.

Process validation is defined as the collection and evaluation of data, from the process design stage through commercial production, which establishes scientific evidence that a process is capable of consistently delivering quality product. Process validation involves a series of activities taking place over the lifecycle of the product and process. Usually, process validation includes three stages:

- **Process Design:** The commercial manufacturing process is defined during this stage based on knowledge gained through development and scale-up activities.
- **Process Qualification:** During this stage, the process design is evaluated to determine if the process is capable of reproducible commercial manufacturing.
- **Process Verification:** Ongoing assurance is gained during routine production that the process remains in a state of control [1-3].

3. New approaches in drug quality control

3.1. Matrices

3.1.1. Residue analysis of pharmaceuticals in the aquatic environment

Residue analysis of pharmaceuticals in the aquatic environment has attracted considerable interest during the last few years.

Traces of such compounds have been detected in surface water samples from all countries where pharmaceuticals are widely in use.

Pharmaceutically active compounds have captured the attention of the scientific community because such pollutants result not primarily from manufacturing but from widespread, continual use in human and veterinary clinical practice. The biological activity of these compounds can lead to adverse effects in aquatic ecosystems and potentially have an impact on drinking-water supplies [4].

In the human body, pharmaceuticals can be transformed to one or more metabolites and excreted as a mixture of parent compound and metabolites, in which the parent compound is often the minor component. However, some drugs are poorly metabolized and are excreted unchanged. The degree of metabolism depends on a number of parameters, including age, gender and ethnicity, the constitution of the patient and the time of administration. Drug-drug interactions caused by enzyme induction or inhibition, as well as enhanced metabolism due to previous exposure, can also influence the pharmacokinetics of drugs [5].

Both the parent compound and the metabolites enter the aquatic environment once they are excreted from the human body. Monitoring studies in the environment have demonstrated the discharge of pharmaceuticals and their metabolites through municipal wastewater-treatment plants (WWTPs). Although unchanged drugs can undergo biochemical transformations during sewage treatment, some studies indicate that the absence of pharmaceutical compounds in treated water does not necessarily imply their complete removal. In most instances, human drugs are metabolized in the body to more polar compounds that are more likely to pass through the WWTP. In some cases, pharmaceuticals and their human metabolites can be microbially degraded in the activated sludge treatment.

Knowledge of the formation of stable metabolites in WWTPs is also important in order to understand the environmental fate of the parent compound. Once in the environment, these compounds can be transported and distributed in rivers, streams, and possibly further biodegraded. For most pharmaceuticals and their biotransformation products, these pathways in the aquatic environment are largely unknown, and investigations into their occurrence in environmental compartments are still rare.

Studies have been carried out to investigate their fate not only in surface waters, but also in sediment and soil environments. By nature, most pharmaceuticals are designed to be at least moderately water-soluble and to possess half-lives in the human body in the range of hours. Because human and microbial degradates will generally coexist with their parent compounds in the environment, indicators that summarize all the information on parent substances and degradates would be important instruments for decision-making and assessment [6].

Progress in instrumental analytical chemistry has resulted in the availability of methods that allow a monitoring of these pollutants at ng levels.

Improvements in detection limits over the past years have mainly been due to sophisticated mass spectrometric detection techniques. Furthermore, robust sample preparation and pre-concentration protocols have contributed significantly to the achievements observed so far.

Nowadays it is a well-established fact that pharmaceutical drugs used during medical treatment may partly be excreted in an un-metabolized form, enter municipal sewage systems, and can even survive the passage through the sewage treatment plant. Therefore, sewage treatment plant effluents are the major source for introduction of pharmaceuticals into the aquatic environment. Furthermore, pharmaceuticals employed in veterinary medicine may be introduced into soil (and eventually into water) via manure, or may find a direct way into the aquatic system when used in fish farms.

Unfortunately, the consequences of continuous presence of low concentrations of pharmaceuticals for the ecosystem are still not fully known.

In many cases, the analytical procedures for residue analysis of pharmaceutical drugs nowadays available includes a pre-concentration and clean-up step by solid-phase extraction or related techniques, followed by chromatography in combination with mass spectrometry (MS) as detector.

Although GC-MS may still be the perfect technique for certain classes of pharmaceuticals, high-performance liquid chromatography (HPLC) hyphenated with atmospheric pressure ionization-MS has established itself as the better choice for simultaneous determination of pharmaceuticals of widely differing structures.

The concentration levels of pharmaceuticals found in environmental water samples are generally too low to allow a direct injection into a chromatographic system. Therefore, efficient pre-concentration steps are necessary which should also result in some sample clean-up. One of the most widely used sample treatment technique for residue analysis of pharmaceuticals in water is the extraction of the analytes by means of a solid sorbent.

This extraction procedure can be based on multiple equilibria between the liquid phase and the sorbent filled into a small cartridge (solid-phase extraction, SPE), or on a single equilibrium (sorptive extraction) [7-23].

3.1.1.1. Solid-phase extraction

Pharmaceuticals of adequate hydrophobicity can easily be pre-concentrated using any reversed-phase material such as alkyl-modified silica or polymer-based materials. Deprotonation of acidic compounds and protonation of basic compounds should be suppressed to ensure sufficient hydrophobicity of the analytes. Therefore, acidic pharmaceuticals should be pre-concentrated under acidic conditions, whereas basic analytes should be pre-concentrated at an alkaline pH. Alternatively, mixed-mode SPE materials can be used which exhibit both reversed-phase and cation-exchange properties due to the presence of sulfonic acid groups on the hydrophobic surface of the particles. Using acidified sample solutions, acidic and neutral analytes would be extracted by hydrophobic interactions, whereas protonated basic analytes would interact via ion exchange mechanisms.

A recent review has summarized new SPE materials that can improve the recoveries for polar analytes. These materials are mainly polymeric sorbents that improve the retention of polar compounds either by novel functional groups in the polymeric structure (resulting in a

hydrophilic–hydrophobic balance material) or by considerably increased surface area. Some of these new materials have turned out to be well suited for multi-class analysis of pharmaceuticals in water samples. Nowadays, one of the most widely used sorbent is a copolymer of divinylbenzene and vinylpyrrolidone [7-23].

3.1.1.2. Sorptive extraction

Sorptive extraction based on a single partitioning equilibrium of analytes between the aqueous sample and a solid sorbent includes solid-phase microextraction (SPME), stir-bar sorptive extraction (SBSE), and several related variants. Originally, these techniques were based on polydimethylsiloxane (PDMS) as material for trapping trace analytes from a water sample due to partitioning between the aqueous matrix and the PDMS phase. Besides PDMS, some alternative sorptive materials have become commercially available recently, such as polyacrylates, copolymers of PDMS with divinylbenzene, copolymers of polyethylene glycol with divinylbenzene, and mixtures of carboxen (an inorganic adsorbent) with PDMS or divinylbenzene [7-23].

3.1.1.3. Sample pre-concentration procedures for sediment and sludge samples

Extraction of pharmaceuticals from sediment and sludge is generally done by blending the sample with an organic solvent or with mixtures of aqueous buffers and organic solvents.

Ultrasonication is frequently applied to assist the extraction process.

Additional clean-up steps for the extract may be necessary employing SPE or liquid–liquid extraction. Somewhat more advanced procedures are based on pressurized liquid extraction (accelerated solvent extraction) which may need less time and less solvent consumption [7-23].

3.1.1.4. Derivatization of the compounds

Various groups of pharmaceuticals can be derivatized to make them suited for GC analysis. Typical derivatization reagents for acidic pharmaceuticals include pentafluorobenzylbromide, methyl chloromethanoate, methanol/BF₃, or tetrabutylammonium salts (for derivatization during injection). Phenazone-type drugs have been derivatized by silylation using *N*-*tert*-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA). Silylation procedures are also commonly used for synthetic estrogens [7-23].

3.2.1. Some latest researches in this area

3.2.1.1. Pharmaceuticals in the aquatic environment: a critical review of the evidence for health effects in fish

The authors review the current data on the presence and reported biological effects in fish of some of the most commonly detected pharmaceuticals in the aquatic environment; namely nonsteroidal anti-inflammatory drugs (NSAIDs), fibrates, beta-blockers, selective serotonin

reuptake inhibitors (SSRIs), azoles, and antibiotics. Reported biological effects in fish in the laboratory have often been shown to be in accordance with known effects of pharmaceuticals in mammals. Water concentrations at which such effects have been reported, however, are generally, between microg L⁻¹ and mg L⁻¹, typically at least 1 order of magnitude higher than concentrations normally found in surface waters (ng L⁻¹). There are exceptions to this, however, as for the case of synthetic oestrogens, which can induce biological effects in the low ng L⁻¹ range. Although generally effect levels for pharmaceuticals are higher than those found in the environment, the risks to wild fish populations have not been thoroughly characterised, and there has been a lack of consideration given to the likely chronic nature of the exposures, or the potential for mixture effects. As global consumption of pharmaceuticals rises, an inevitable consequence is an increased level of contamination of surface and ground waters with these biologically active drugs, and thus in turn a greater potential for adverse effects in aquatic wildlife [24].

3.1.1.2. Human Pharmaceuticals, Hormones and Fragrances: The Challenge of Micropollutants in Urban Water Management

The observed concentrations of pharmaceuticals and personal care products (PPCPs) in raw wastewater confirm that municipal wastewater represents the main disposal pathway for the PPCPs consumed in households, hospitals and industry. In sewage treatment plant effluents most PPCPs are still present, since many of these polar and persistent compounds are being removed only partially or, in some cases, not at all. Treated wastewater therefore represents an important point source for PPCPs into the environment. After passing a sewage treatment plant the treated wastewater is mostly discharged into rivers and streams or sometimes used to irrigate fields. If drinking water is produced using resources containing a substantial proportion of treated wastewater (e.g. from river water downstream of communities) the water cycle is closed and indirect potable reuse occurs. Human Pharmaceuticals, Hormones and Fragrances provides an overview of the occurrence, analytics, removal and environmental risk of pharmaceuticals and personal care products in wastewater, surface water and drinking water. [25].

3.2.1.2. Factors affecting the concentrations of pharmaceuticals released to the aquatic environment

Although recent research has demonstrated that pharmaceuticals are widely distributed in the aquatic environment, it is difficult to assess the threat that they

pose to drinking water supplies or their rate of attenuation in natural systems without an adequate understanding of the sources of contamination. To identify pharmaceutical compounds of significance to water supplies in the United States, the authors have reviewed available data on the use of prescription drugs. Results of our analysis indicate that approximately 40 compounds could be present in municipal wastewater effluent at concentrations above 1,000 ng/L and at least 120 compounds could be present at concentrations above 1 ng/L. Important classes of prescription drugs include analgesics, beta-blockers, and antibiotics. Analysis of a group of the most commonly used pharmaceuticals in the United States indicates that they are ubiquitous in wastewater effluents. Authors have detected concentra-

tions ranging from approximately 10- 3,000 ng/L for high use pharmaceuticals such as beta-blockers (*e.g.*, metoprolol, propranolol) and acidic drugs (*e.g.*, gemfibrozil, ibuprofen). The concentration of pharmaceuticals in effluent from conventional wastewater treatment plants is similar. Advanced wastewater treatment plants equipped with reverse osmosis systems reduce concentrations of pharmaceuticals below detection limits. In addition to removal during biological wastewater treatment, pharmaceuticals also are attenuated in engineered natural systems (*i.e.*, treatment wetlands, ground water infiltration basins). Preliminary evidence suggests limited removal of pharmaceuticals in engineered treatment wetlands and nearly complete removal of pharmaceuticals during ground water infiltration [26].

3.2.1.3. A preliminary ecotoxicity study of pharmaceuticals in the marine environment

Environmental fates and effects of pharmaceuticals in the aquatic environment have been the focus of recent research in environmental ecotoxicology. Worldwide studies of common over-the-counter pharmaceuticals have reported detectable levels in the aquatic environment, but there are few studies examining impacts on marine habitats. These drugs can affect the functions of various vertebrates and invertebrates. The stability of two pharmaceuticals, cyclizine (CYC) and prochlorperazine (PCZ), in seawater was examined under light and dark conditions, as well as the toxicity of these compounds to larvae of the barnacle *Balanus amphitrite*, which is a cosmopolitan marine organism found in most of the world's oceans. CYC was very stable under all the tested conditions. On the other hand, PCZ degraded in light but not in the dark, and was more stable in seawater than fresh water. For the barnacle larvae, the LC50 of prochlorperazine was 0.93 microg/mL and the LC50 for CYC was approximately 0.04 microg/mL [27].

3.2.1.4. Estrogenic activity of pharmaceuticals in the aquatic environment

In the last years pharmaceuticals have aroused great interest as environmental pollutants for their toxic effects towards non target organisms. This study wants to draw attention to a further adverse effect of drugs, the endocrine interference. The most representative drugs of the widespread classes in environment were investigated. The YES-test and the E-screen assay were performed to detect the capability of these substances to bind the human estrogenic receptor alpha (hER alpha) in comparison with 17beta-estradiol. Out of 14 tested pharmaceuticals, 9 were positive to YES-assay and 11 were positive to E-screen assay; in particular, Furosemide and the fibrates (Bezafibrate, Fenofibrate and Gemfibrozil) gave the maximal estrogenic response. Tamoxifen showed its dual activity as agonist and antagonist of hER alpha [28].

3.2.1.5. Colloids as a sink for certain pharmaceuticals in the aquatic environment

The occurrence and fate of pharmaceuticals in the aquatic environment is recognized as one of the emerging issues in environmental chemistry and as a matter of public concern. Existing data tend to focus on the concentrations of pharmaceuticals in the aqueous phase, with limited studies on their concentrations in particulate phase such as sediments. Furthermore, current water quality monitoring does not differentiate between soluble and colloidal phas-

es in water samples, hindering our understanding of the bioavailability and bioaccumulation of pharmaceuticals in aquatic organisms. In this study, an investigation was conducted into the concentrations and phase association (soluble, colloidal, suspended particulate matter or SPM) of selected pharmaceuticals (propranolol, sulfamethoxazole, meberverine, thioridazine, carbamazepine, tamoxifen, indomethacine, diclofenac, and meclofenamic acid) in river water, effluents from sewage treatment works (STW), and groundwater in the UK. Colloids were isolated by cross-flow ultrafiltration (CFUF). Water samples were extracted by solid-phase extraction (SPE), while SPM was extracted by microwave. All sample extracts were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) in the multiple reaction monitoring.

Five compounds propranolol, sulfamethoxazole, carbamazepine, indomethacine, and diclofenac were detected in all samples, with carbamazepine showing the highest concentrations in all phases. The highest concentrations of these compounds were detected in STW effluents, confirming STW as a key source of these compounds in the aquatic environments. The calculation of partition coefficients of pharmaceuticals between SPM and filtrate, between SPM and soluble phase, and between colloids and soluble phase showed that intrinsic partition coefficients are between 25% and 96%, and between 18% and 82% higher than relevant observed partition coefficients values, and are much less variable. Secondly, K_{coc} values are 3–4 orders of magnitude greater than K_{oc} values, indicating that aquatic colloids are substantially more powerful sorbents for accumulating pharmaceuticals than sediments. Furthermore, mass balance calculations of pharmaceutical concentrations demonstrate that between 23% and 70% of propranolol, 17–62% of sulfamethoxazole, 7–58% of carbamazepine, 19–84% of indomethacine, and 9–74% of diclofenac are present in the colloidal phase.

The results provide direct evidence that sorption to colloids provides an important sink for the pharmaceuticals in the aquatic environment. Such strong pharmaceutical/colloid interactions may provide a long-term storage of pharmaceuticals, hence, increasing their persistence while reducing their bioavailability in the environment.

Recommendations and perspectives from this study:

Pharmaceutical compounds have been detected not only in the aqueous phase but also in suspended particles; it is important, therefore, to have a holistic approach in future environmental fate investigation of pharmaceuticals. For example, more research is needed to assess the storage and long-term record of pharmaceutical residues in aquatic sediments by which benthic organisms will be most affected. Aquatic colloids have been shown to account for the accumulation of major fractions of total pharmaceutical concentrations in the aquatic environment, demonstrating unequivocally the importance of aquatic colloids as a sink for such residues in the aquatic systems. As aquatic colloids are abundant, ubiquitous, and highly powerful sorbents, they are expected to influence the bioavailability and bioaccumulation of such chemicals by aquatic organisms. It is therefore critical for colloids to be incorporated into water quality models for prediction and risk assessment purposes [29].

4. Chemometrics

Chemometrics is the science of extracting information from chemical systems by data-driven means. It is a highly interfacial discipline, using methods frequently employed in core data-analytic disciplines such as multivariate statistics, applied mathematics, and computer science, in order to address problems in chemistry, biochemistry, medicine, biology and chemical engineering.

Chemometrics is applied to solve both descriptive and predictive problems in experimental life sciences, especially in chemistry. In descriptive applications, properties of chemical systems are modeled with the intent of learning the underlying relationships and structure of the system (i.e., model understanding and identification). In predictive applications, properties of chemical systems are modeled with the intent of predicting new properties or behavior of interest. In both cases, the datasets can be small but are often very large and highly complex, involving hundreds to thousands of variables, and hundreds to thousands of cases or observations.

Chemometric techniques are particularly heavily used in analytical chemistry and metabolomics, and the development of improved chemometric methods of analysis also continues to advance the state of the art in analytical instrumentation and methodology. It is an application driven discipline, and thus while the standard chemometric methodologies are very widely used industrially, academic groups are dedicated to the continued development of chemometric theory, method and application development [30-33].

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Quality Control of Formulated Medicines

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Additional information is available at the end of the chapter

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1. Introduction

A pharmaceutical drug is technically obtained or prepared for prophylactic, curative, palliative or diagnostic purposes. The final product must meet quality standard, be safe and effective. In Brazil, there is a high demand for formulated drugs. This is mainly due to their lower price compared to manufactured drugs, evidenced by the rapid growth – an increase of 350% from 1998 to 2010.

Even after the ANVISA's (Agency National Health Surveillance) establishment of the new handling standards to be followed by the magistral pharmacies, several serious cases, including death reports, caused by the consumption of formulated drugs have recently become public [1-5].

Other problems related to this subject occurred in 2004, when deaths caused by manipulated medicines of low therapeutic index (clonidine and levotiroxine) led ANVISA to modify the regulation for manipulated medicines. The concentration of each compound was not totally assured and the contamination by impurities, not included in the original formula, were present in the final product.

In a previous work, we have also observed the presence of impurities – such as metals - in different kinds of medicines. The ingestion of metals, even at low levels, can be very harmful to humans. Besides this, the long-term uptake of some drugs is also risky. This should require attention and surveillance from the public health-related agencies [6-10].

The quality and safety of drugs must follow the specifications described in the official compendia - among them, the pharmacopoeias. Medicines cannot contain impurities or

other substances that endanger the patient's health. According to the second edition of the Brazilian Pharmacopoeia National Formulary [1], to ensure safety, efficacy and quality of the handled products it is necessary correct calculations, exact measurements, and adequate conditions and procedures of preparation. The prudent judgment of the pharmacist, who must be a qualified professional for this purpose, is another fundamental aspect. Additionally, an appropriate profile with a proven stability must be sought in the literature [11-14].

The requirements of sanitary legislation and quality control of raw materials for magistral solid preparations are:

- raw material: character sensory, solubility, pH determination, melting point, density, weight and volume; analysis report of manufacturer/supplier;
- raw material of vegetal origin: organoleptic characters, solubility, pH determination, melting point, density, weight and volume, evaluation of vendor analysis report;
- manipulated product: solid dosage forms: description, appearance and organoleptic characteristics, determination of average weight

The legislation also determines that all pharmacies must perform analyzes every two months of at least one of the formulas containing drug(s) ≤ 25 mg of drugs. The priority is to those that contain ≤ 5 mg of drugs. The legislation establishes special quality control requirements for preparations of substances with low therapeutic index, like hormones, antibiotics and cytotoxic drugs, homeopathic products, and sterile products. The raw materials used in sterile preparations must also be analyzed [15-17].

The Legislation on Good Practices for Handling does not require impurity tests for the raw materials received by the pharmacies. It is only necessary to check the certificate of a qualified supplier – issued in accordance with methods described in the pharmacopoeia, which are only suitable for the detection of some elements (Ag, As, Bi, Cd, Hg, Mo, Pb, Sb, and Sn) [18].

Quality control tests for the products handled do not include detection and quantification of impurities. Moreover, the analysis required for formulated preparations allow limited conclusions about the quality of the process, since they do not testify the homogeneity of the active principle directly, but only as to the uniformity of filling of the capsules. So, a particular formulation can have the acceptance criteria for average mass, standard deviation and coefficient of variation but not the uniformity of this active content in the capsules [19].

Periodic reviews performed every two months for formulated drugs do not statistically have significant value, so that a reliable conclusion about the quality of formulated drugs can not be reached [20]. The analysis of thirty batches of 20 mg of Sinvastatin medicine manipulated in pharmacies of Belo Horizonte, showed that only fourteen of them, met the quality standard required by pharmacopoeia. Thus, the therapeutic efficacy of 53% of the analyzed products can not be totally dependable [21].

In this study, the quality of medicines Omeprazole and Enalapril Maleate from five (5) different magistral pharmacies was evaluated according to the methodology described in pharmacopoeia. The analyses for mass determination, identification of active principle, content, content uniformity and related compounds were performed.

The target drugs, Omeprazole and Enalapril Maleate, were chosen because of their representativeness of consumption and availability of related reference data in the pharmacopoeias.

In order to evaluate the presence and concentration of chemical elements, the technique used in this study was neutron activation analysis (NAA), applying the k_0 -standardization method [23-25]. The neutron activation analysis is a very sensitive and reliable multielemental technique, suitable for determination of the elements such as: As, Ba, Br, Ca, Ce, Cl, Co, Cr, Eu, Fe, Hf, Mg, Mn, Na, Sb, Sc, Sm, Ti and Zn, in different drugs [14]. The technique is based on the principle that when the material is irradiated by neutrons, some elements with suitable nuclear characteristics become radioactive isotopes. Thus, the concentration of each element can be determined by counting the respective radiation emitted by the corresponding radionuclide [26].

The results described here are part of a wider project which also includes the analyses of Fluoxetin and Sinvastatin medicines and will be published briefly.

2. Quality control of formulated drugs

2.1. The pharmacopoeia

The 5th edition of the Brazilian Pharmacopoeia [27] defines quality control as: "The set of measures to ensure, at any time, the batch production of medicines and other products that meet the standards of identity, activity, content, purity, efficacy and safety." According to Resolution RDC N^o. 67, October 8, 2007 [15], which provides the Technical Regulation establishing the Good Handling Practices in Pharmacies (Good Compounding Practices) quality control of magistral and officinal preparations, is given by the completion of at minimum, the tests described in Table 1, according to the Brazilian Pharmacopoeia or other Official Compendium recognized by the National Health Surveillance Agency (ANVISA).

Results of tests must be recorded in the same order of handling, in addition to other relevant information. The pharmacist must evaluate the results to approve or not the preparation for dispensing. Each pharmacy is responsible for the quality of magistral preparations that handles, keeps, transports and dispenses. Raw materials should be checked in its receipt and moved to quarantine soon after, until the release of the reports of quality control. In the absence of pharmacopoeia monograph, the scientific literature should be used as a reference, and only with the lack of literature, the specification provided by the supplier may be used. All results must be written and stored [15].

Preparation	Test
Solid	Description, appearance, organoleptic characteristics, average mass
Semi-solid	Description, appearance, organoleptic characteristics, pH (where applicable), mass
Non-sterile liquid	Description, appearance, organoleptic characteristics, pH, mass or volume before filling

Table 1. Tests for quality control of magistral drugs

However, some studies also show that the rule of Good Practices on Handling does not answer and does not guarantee the quality of compounded drugs [20].

2.2. Analyses performed

The analyses of quality control were performed at the Laboratory for Quality Control of Chemical Physics Drug, and Cosmetic Sanitizing of the Ezequiel Dias Foundation (FUNED). The following tests were performed [15,27,28]:

- Aspect;
- Identification ;
- Labeling;
- Content;
- Related compounds;
- Dosage uniformity;
- Unit Change in mass;

The test of aspect is just a visual description of the product to be analyzed, coloration of the capsule and its content.

The test of identification allows determining the presence of the active principle in the product analyzed. It is performed through the high performance liquid chromatography (HPLC) [28].

The analysis of content aims to verify whether the drug has a dose of active ingredient on the label provided and used to quantify the active ingredient in the product analyzed. This test is performed according to the pharmacopoeia for each product, and may be performed in the ultraviolet and visible spectrophotometry, by high performance liquid chromatography, among other methods. The test uses usually ten to twenty capsules and each capsule analyzed separately, but the "pool" of these. There are limits specified in the monograph, which should be within the active drug, usually 90 to 110%. Results below the limit can result in ineffective therapy and above, intoxication, depending on the drug analyzed [28].

The analysis of related compounds determines the amount of by-products of synthesis of the substance and / or its degradation products and / or contaminants from the process of

obtaining the substance which can be normally found within a specified limit. This test is done only when specified in the pharmacopoeia.

The variation of the mass allows checking the uniformity of mass between units within a batch. For products in hard capsules should be weighed individually, twenty units, the contents of each one should be removed, properly cleaned and reweighed. The mass content of each capsule is determined by mass difference between the full and the empty capsule. Then the average mass of the contents can be determined. For hard capsules, the limit of variation is $\pm 10\%$ of the mass corresponding to less than 300 mg. If the mass corresponds to 300 mg or more, the maximum range is $\pm 7.5\%$. It cannot be tolerated more than two units outside the limits specified in the official compendia, but none can be above or below twice the percentages indicated [27].

The uniformity of dosage units evaluate the uniformity of distribution of active component units in a single batch can be determined by two methods: mass variation and content uniformity. The mass variation test is only applicable in specific cases. The test for content uniformity is based on the content of each active ingredients in a number of unit doses in order to determine whether the content is within specified limits, being applicable in all cases [28].

3. Methodology

3.1. High performance liquid chromatography

In this study the identification tests, content, related compounds and content uniformity was performed by high performance liquid chromatography (HPLC) according to the specifications of literature [28]. The chromatograph Shimadzu detector was coupled to molecular absorption spectrophotometry in the ultraviolet-visible Perkin Elmer Lambda 25 model, Class-VP software. All chemical reference substances (SQR) were purchased from USP (The United States Pharmacopeia).

To analyze the Enalapril Maleate, L7 C₈ column (4.6 mm x 25 cm x 5 mm) was used. Isocratic elution was performed with a buffer monobasic sodium phosphate pH 2.2 /acetonitrile at a ratio of 75:25. Solvents and solutions were degassed in ultrasonic bath (Elma Transsonic Digitals) and filtered through a Millipore membrane of 0.45 micrometers. Chromatography was performed at 50°C, flow rate of 2 mL.min⁻¹, with injections of 50 µL, detection at 215 nm and running time of 30 min. The calculations were based on the content of the samples obtained areas of the areas of the SQR of Enalapril Maleate. For related compounds the content of diketopiperazine compounds and enalaprilat was also calculated.

For omeprazole, L7 C₈ column (4.6 mm x 15 cm x 5 mm) was used. Elution was performed by mixing two solutions – solution A (6 g of glycine in 1500 mL water, pH 9) and solution B (acetonitrile and methanol, 85:15 ratio) – as shown in Table 3. Solvents and solutions were degassed in ultrasonic bath (Elma Transsonic Digitals) and filtered through a Millipore membrane of 0.45 micrometers. Chromatography was performed with a flow of 1,2 mL.min⁻¹, with injections of 10 µL, detection at 305 nm and running time of 30 min. The cal-

culations were based on the content of the samples obtained areas of the areas of the SQR of omeprazole.

Time (minutes)	Solution A (%)	Solution B (%)	Elution
0 – 20	88 → 40	12 → 60	Linear gradient
20 – 21	40 → 88	60 → 12	Linear gradient
21 – 25	88	12	Isocratic

Table 2. Parameters of elution of the HPLC analysis of Omeprazole

3.1.1. Results and discussion

a. Appearance, Identification and Labeling

Both, Omeprazole and Enalapril Maleate samples, showed similar aspects as their samples: hard capsule containing white pellets for Omeprazole, and hard capsule containing white powder varying only the color of the hard capsule used by each pharmacy. All samples, Omeprazole and Enalapril Maleate, were satisfactory for labeling and identification, confirming that the identity of the material was in accordance with the label from its packaging. In addition, all labels contain information provided by RDC Resolution N^o 67, October 8, 2007 [15]:

1. Name of the prescriber;
2. Name of the patient;
3. Registration Number of the formulation;
4. Data handling and shelf life;
5. Formulation components and their quantities;
6. Number of units;
7. Dosage;
8. Identification of pharmacy, full address and federal registration;
9. Name and professional register of the responsible person.

b. Related substances (Enalapril Maleate)

The test for related substances is performed only when described in the pharmacopeia of the compound to be analyzed, or another official compendium regulated by ANVISA [28]. For the Enalapril Maleate it is specified that no more than 5% of diketopiperazine and enalaprilat can be found in the final product. All samples of Enalapril Maleate were satisfactory for this analysis and the results are shown in Table 5.

Pharmacy	Enalaprilat (%)	Diketopiperazine (%)	Enalaprilat + Diketopiperazine (%)
A	0.66	0.82	1.48
B	0.16	0.07	0.23
C	0.53	0.87	1.40
D	0.05	0.001	0.055
E	1.18	2.77	3.95

Table 3. Content of related compounds of Enalapril Maleate

c. Content

The reference values for the content of both drugs should not be less than 90% nor exceed 110% of the declared value, 10 mg and 20 mg for omeprazole and enalapril, respectively. The results for content of active ingredient are described in Table 6.

It can be observed that four from the five samples of omeprazole were unsatisfactory; two of them with content above the permissible and the other two with the content below. For the samples of enalapril, two were unsatisfactory, one exceeding the limit and the other with recommended content lower than expected, as showed in Table 6.

Pharmacy	Omeprazole (10 mg/caps)	Enalapril Maleate (20 mg/caps)
A	(11.3 ± 0.4) mg/caps or 112.7% declared	(18.5 ± 0.2) mg/caps or 92.6% of declared
B	(8.4 ± 2.2) mg/caps or 84.4% declared	(20.5 ± 0.1) mg/caps or 102.7% declared
C	(6.9 ± 2.6) mg/caps or 68.7% declared	(18.5 ± 0.1) mg/caps or 92.5% declared
D	(11.2 ± 0.1) mg/caps or 111.5% declared	(11.2 ± 4.3) mg/caps or 56.0% declared
E	(11.0 ± 0.2) mg/caps or 109.6% declared	(16.9 ± 1.2) mg/caps or 84.7% do declared

Table 4. Final content of Omeprazole and Enalapril Maleate

d. Uniformity of the dosage unit

All Omeprazole samples were considered unsatisfactory for uniformity of the dosage unit. Three samples were satisfactory for Enalapril Maleate. The results for uniformity of dosage unit are described in Table 7 as contained in the final analysis report issued by FUNED. Variations in dose uniformity should not exceed 15% [28].

e. Mass Variation

The acceptable limit for the analysis of variation in mass of capsules, weighing less than 300 mg is $\pm 10\%$ above the average mass, and it is tolerable no more than two units outside the specified limit and any unit may be above or below twice the percentages indicated. Thus, only a sample of Omeprazole was considered unsatisfactory. The results for the samples of Omeprazole and Enalapril Maleate are presented in Tables 8 and 9, respectively [27].

Pharmacy	Omeprazole (10 mg/caps)	Enalapril Maleate (20 mg/caps)
A	18.7%	7.8%
B	24.0%	14.2%
C	47.9%	10.6%
D	15.6%	14.0%
E	15.8%	46.5%

Table 5. Dose uniformity of the capsules of Omeprazole and Enalapril Maleate

Pharmacy	average weight (mg/caps.)	Lower	Higher
*A	220.4 \pm 1.2	2.4	1.6
B	226.8 \pm 5.8	9.5	8.7
C	210.0 \pm 4.9	9.7	7.5
*D	119.0 \pm 2.1	6.5	10.9
**E	120.7 \pm 2.9	10.9	7.9

*One unit above the limit. ** Two units above the limit. Caps, capsules

Table 6. Variation (%) in mass of the capsules of Enalapril Maleate (20 mg/caps)

Pharmacy	average mass (mg/caps.)	Lower	Higher
*A	108.3 \pm 3.0	7.1	15.1
B	195.4 \pm 1.8	4.6	3.0
C	175.9 \pm 3.0	9.1	4.7
**D	107.0 \pm 7.0	15.3	40.8
***E	105.0 \pm 2.1	10.6	7.2

*Two units above the limit. ** Four units above the limit. Unsatisfactory. *** One unit above the limit.

Table 7. Variation (%) in mass of the capsules of Enalapril Maleate (20 mg/caps)

From the ten samples analyzed, seven were rated as *unsatisfactory*, considering the analysis of aspect, mass variation, identification, related substances, uniformity of dosage units, content and labeling.

It was observed that, if only the official established procedures (description, appearance, organoleptic characteristics and average mass) were considered from the seven samples rated as unsatisfactory, just one would be classified in this status. The remaining six samples would erroneously be rated satisfactory, meaning that would be approved for human consumption [18].

Some factors may cause deviations, inherent to the handling process of drugs in capsules, such as the loss of substance during the grinding, mixing and filling the capsules. Miscalculations and weight of the formulation components, errors inherent to the operator and the use of damaged equipment may also compromise the process and therefore the quality of the final product [29].

The results of Omeprazole and Enalapril Maleate were analyzed by ANOVA followed by Tukey's test for uniformity of content and unit dose. Results were considered significantly different at $p < 0.05$.

3.2. Neutron activation analysis

3.2.1. Material and methods

All samples of Omeprazole and Enalapril Maleate were purchased in the market of the Belo Horizonte, state of Minas Gerais, Brazil from five (5) different magistral pharmacies.

Due to operational reasons, Jožef Stefan Institute (JSI) performed analyses only from three (3) different pharmacies from the sampling group. Due to same operational reasons, the JSI did not analyze the short half-lives radionuclides of elements like Al, Cl, Mg, Mn and Ti.

The samples of Omeprazole and Enalapril Maleate performed by the JSI were packed in polyethylene capsules in plastic bottle containing 20 capsules each. Whole powder mass from 20 capsules was taken to prepare homogenized samples, which was transferred in clean polyethylene bottle. In the samples of Omeprazole performed by CDTN, just one the mass of one capsule taken randomly was considered by each sample. The difference of procedures carried out by both Institutes was due to operational reasons.

Both institutes CDTN/CNEN and JSI followed the same procedure to prepare the samples. The aliquots of each sample were manually crushed or ground using an agate mortar with pestle, whenever necessary, to avoid any contamination. In most cases, unless the amount of material did not allow it, two replicates were taken and weighed in polyethylene vials. It is relevant to emphasize that no additional chemical sample preparation was performed. At CDTN/CNEN and IJS, the samples were irradiated together with several Al-0.1% Au disks as neutron flux monitors, according to the k_0 -standardisation method procedure [23,24,30].

Table 10 shows the characteristics of the applied technique such as the parameters f (thermal to epithermal fluxes ratio) and the α (parameter which measures the epithermal flux devia-

tion from the ideal (1/E) distribution), needed for the k_0 -method, the irradiation times and gamma spectrometry systems at each Institute.

3.2.2. Results and discussion

The obtained results of NAA from the medicines Omeprazole and Enalapril Maleate are showed in the Tables 11 to 13.

The technique applied was suitable for determining 20 chemical elements – Al, Br, Ca, Cl, Co, Cr, Fe, Mg, Mn, Na, Sb, Sc, Sm, Sr, Ta, Th, Ti, U and Zn – in a large range of concentration, without any chemical process. The elements Cl, Fe, K, Mg, Mn, Na, and Zn could be expected in this kind of samples. Other elements, not considered essential, for the human being such as As and Sr, found in lower concentration compared to Cl, Fe, K, Mg, Mn, Na, and Zn can also represent a health problem in a long term consumption. Even essential elements were determined but in high concentrations, like Fe may be toxic.

High concentration of elements such as Cl, Ca, Mg, Na and Ti are expected because they are frequently components of excipients in the preparation of pellets. The presence of Mg is due to the excipients usually used: magnesium is a component of magnesium stearate ($\text{Mg}[\text{C}_{18}\text{H}_{35}\text{O}_2]$), a lubricant for tablets and capsules and opadry, coloring agent, respectively [26]. Mg also is present in magnesium silicate ($\text{Mg}_3\text{SiO}_4(\text{OH})_2$) Na is a component of sodium laurilsulfate, ($[\text{CH}_3(\text{CH}_2)_{10}(\text{CH}_2\text{O})(\text{SO}_3)\text{Na}]$) and sodium bicarbonate NaHCO_3 . Ca is added as excipient as calcium phosphate and Ti as titanium dioxide, TiO_2 . Fe comes from red iron oxide, used as excipient as well [10,13]. The impurities such as Br, Co, Cr, Hf, La, Sb, Sc, Sm, Sr, Ta, Th and U, are probably original from the raw material and/or from the process of production and manipulation of the medicine. All elements determined not foreseen in the original formula can be considered as impurities.

	INSTITUTE	
	CDTN/CNEN	JSI
Thermal Flux (neutrons $\text{cm}^{-2} \text{s}^{-1}$)	6.4×10^{11}	1.1×10^{12}
k ₀ -standardisation parameters		
f	20.4	28.6
α	0.197	- 0.011
Irradiation time (h)	8	13
Detector nominal efficiency (%)	50	40
Software used for:	Genie 2000	Genie 2000
Acquisition spectra	(CANBERRA)	(CANBERRA)
Spectra Analysis	HyperLab	HyperLab
Concentration calculation	Kayzero for Windows, V.2.42	Kayzero for Windows, V.2.42
Sample mass (mg)	200-250	240-250

Table 8. Experimental information of neutron activation analysis

The data presented in Tables 12, 13 and 14 cannot be compared directly because the samples analyzed are not from the batch, but the results are, in general, very similar. Most results determined by the CDTN in one capsule of Omeprazole taken randomly were also determined by the JSI in the homogenized samples, except for the elements Br, Cr and La. The concentrations of the elements determined by both institutes have, in general, the same magnitude.

The discussion about toxicity levels and possible consequences for humans being is very difficult, due to the low concentration of the elements and limitations on the studies available in the literature. For most trace elements, there are just some available data on acute and chronic toxicity in experimental animals, not sufficient data to assess the risks to the human health on a long term daily intake [22].

4. Conclusion

The obtained results of samples of omeprazole and enalapril from five different magistral pharmacies of Belo Horizonte, Brazil, confirm the concern about the quality and safety for consumption of formulated medicines. They represent a preliminary part of a more complete investigation, still under way.

From the ten samples analyzed, seven were considered unsatisfactory. Most of the problems found through analyses Omeprazole and Enalapril Maleate medicines, like the variation of active principle mass, mass variation and dosage unit, come from the inadequacy of procedures for handling the ingredients in the pharmacy.

Problems can also be caused by the quality of the raw material used and inefficient or inexistence of test for checking the material. Diversified impurities reinforce the hypothesis that these elements are not controlled by the quality system. It also suggests that quality control over the purity of medicines in general should be established, as well as the concentration limits for the impurities, at least for some elements like As, Cd, Cu, Hg, Pb and Sn, already foreseen for food in the Brazilian legislation.

The possible harmful and/or toxicological effects for the human health as a consequence of long term use of the formulated medicines represent an important concern for the authorities of the public health system. Recent cases of contamination and death in Brazil due to the consumption of inadequate formulated medicines has been enhancing the debate about the quality of the magistral pharmacy.

In conclusion, the results point out the necessity of prompt and efficient actions by the authorities of the health public system to assure the quality of formulated medicines. The aim of this work is just to provide evidences in order to contribute with this initiative.

Pharmacy												
Element	A		B		C		D		E			
Al	532	± 20	260	± 10	305	± 11	452	± 17	335	± 12		
Br	DL		0.60	± 0.03	DL		DL		DL			
Ca	17740	± 793	9379	± 446	11230	± 512	19600	± 880	14260	± 190		
Cl	806	± 48	313	± 18	262	± 18	490	± 30	514	± 31		
Co	0.5	± 0.1	0.3	± 0.1	0.3	± 0.1	DL	±	0.10	± 0.01		
Cr	11.7	± 0.5	7.9	± 0.3	6.7	± 0.3	11.6	± 0.5	13	± 1		
Fe	65	± 5	64	± 11	47	± 10	49	± 11	51	± 5		
Mg	4643	± 192	449	± 28	390	± 28	998	± 57	697	± 47		
Mn	2.8	± 0.3	1.4	± 0.1	DL		1.8	± 0.2	2.2	± 0.2		
Na	8134	± 326	4003	± 144	3918	± 140	6838	± 241	6718	± 247		
Sb	0.09	± 0.01	0.41	± 0.02	0.04	± 0.01	0.06	± 0.01	0.13	± 0.01		

Table 9. Elemental concentration (mg.kg⁻¹) for Omeprazole (CDTN/CNEN)

Pharmacy												
Element	A		B		C		D		E			
Sc	0.03	± 0.01	0.03	± 0.01	0.01	± 0.01	0.02	± 0.01	0.02	± 0.01		
Sm	DL		0.02	±	DL		0.02	± 0.01	DL			
Sr	DL		DL		DL		21	± 4	DL			
Ta	0.02	± 0.01	0.14	± 0.01	DL		0.15	± 0.01	0.09	± 0.01		
Ti	2748	± 105	1483	± 55	897	± 34	2124	± 79	1907	± 71		
U	DL		0.22	± 0.01	DL		DL		0.4	± 0.1		
Zn	3.4	± 0.4	1.8	± 0.3	DL		2.9	± 0.4	2.5	± 0.3		

* DL – Lower than the Detection Limit

Table 10. Elemental concentration (mg.kg⁻¹) for Omeprazole (CDTN/CNEN)

Pharmacy												
Element	A		B		C							
Br	0.19	± 0.01	0.17	± 0.01	0.22	± 0.01						
Ca	16453	± 592	14908	± 540	12817	± 467						
Ce	DL		DL	±	0.09	± 0.01						

Pharmacy									
Element	A			B			C		
Co	0.18	±	0.01	0.016	±	0.001	0.51	±	0.02
Cr	0.52	±	0.03	0.26	±	0.02	0.59	±	0.04
Fe	102	±	4	53	±	2	265	±	9
Hf	0.020	±	0.001	0.009	±	0.001	0.021	±	0.002
La	0.019	±	0.002	0.030	±	0.004	0.040	±	0.002
Mo	0.34	±	0.05	DL	±		DL	±	
Na	6616	±	232	5988	±	210	4836	±	169
Sb	0.014	±	0.001	0.012	±	0.001	0.011	±	0.001
Sc	0.013	±	0.005	0.010	±	0.001	0.022	±	0.001
Sm	DL	±		DL	±		0.0052	±	0.0003
Sr	22.3	±	1.1	17.4	±	1.0	9.0	±	1.0
Ta	0.34	±	0.01	0.076	±	0.003	0.42	±	0.02
Th	0.012	±	0.002	DL	±		0.027	±	0.002
U	0.09	±	0.01	0.14	±	0.01	0.053	±	0.004
Zn	0.60	±	0.1	0.4	±	0.1	0.7	±	0.1

* DL – Lower than the Detection Limit

Table 11. Elemental concentration (mg.kg⁻¹) for Omeprazole (JSI)

Pharmacy									
Element	A			B			C		
Br	0.13	±	0.01	0.47	±	0.02	0.24	±	0.02
Cr	0.07	±	0.01	0.15	±	0.01	0.10	±	0.01
Na	12540	±	439	271	±	10	72480	±	2538
Sc	0.0009	±	0.0001	0.0007	±	0.0001	0.0054	±	0.0002
Sb	0.09	±	0.01	0.41	±	0.02	0.04	±	0.01
Th	DL	±		DL	±		0.015	±	0.001
Zn	0.49	±	0.04	DL	±		0.015	±	0.001

* DL – Lower than the Detection Limit

Table 12. Elemental concentration (mg.kg⁻¹) for Enalapril Maleate (JSI)

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Quality Control in Radiology and Clinical Imaging

Quality Assurance in Diagnostic Medical Exposures in Ghana - A Medical Physicist's Perspective

Stephen Inkoom

Additional information is available at the end of the chapter

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1. Introduction

It is well known that medical exposure procedures such as diagnostic radiology, nuclear medicine and radiotherapy remains the largest source of man made exposure to ionising radiation and continues to grow substantially. This makes the role of quality assurance (QA), an important tool in medical exposure procedures. This paper reviews the future of quality assurance in diagnostic medical exposures in Ghana from the perspective of a Medical Physicist, since a viable QA programme must be developed under the guidance and supervision of a medical physicist who is qualified in this area of expertise by education, training and experience. The Medical Physicist is expected to give guidance and supervision to the Technologists and other staff to execute the programme but should be prepared to perform higher level QA procedures as required. The focus of this review is on diagnostic radiology since it is the dominant mode of medical exposure as compared to nuclear medicine and radiotherapy procedures in Ghana as per the database of the Regulatory Authority Information System "(RAIS)" of the Radiation Protection Institute. It is also worth noting that most of the issues under consideration for discussion mirrors similar conditions in many developing countries. The main goal of a diagnostic quality assurance programme is to make sure that radiation doses to patients, staff and public are as low as reasonable achievable (ALARA) consistent with high quality diagnostic images of patients. An adequate diagnostic QA program involves periodic checks of all major components in the respective diagnostic imaging modalities. On the other hand, an optimum QA programme for any individual diagnostic facility will depend on some items such as the type of procedures performed, type of equipment utilized, patient workload, etc. The current scope of diagnostic imaging procedures in Ghana covers conventional, fluoroscopy, dental, computed tomography, interventional procedures and nuclear medicine scans. Interventional radiology procedures performed are

quite few but the future looks promising in this field. The performance of QA practices are done on three fronts; namely at the hospital, equipment engineers and the Regulatory Authority (RA). The hospital based QA are done mainly by the Radiographic Technologist through their routine equipment warm ups and minor quality checks. Equipment Engineers perform engineer related QA checks through installation and acceptance testing, performance tests and periodic preventative maintenance procedures. On the other hand, the RA is largely in charge of major QA procedures through it's on site safety assessment inspections by assessing the compliance of the equipment within regulatory requirements. This is largely so because the RA has the technical expertise and equipment. Due to the expansion of diagnostic imaging procedures in medicine coupled with rapid technological advances, the availability of qualified and trained personnel is crucial if the desired quality is to be achieved. Some measures have been put in place for human resource development, but there is room for improvement. On the way forward, there is a strong need for the establishment of National Quality Control Centre for Diagnostic Radiology. This body must be equipped with the requisite state of the art equipment, highly qualified and trained personnel in order to coordinate all QA activities in the country. Such a body can initiate some guidelines on the minimum instrumentation requirements for all imaging modalities. Nevertheless, a good QA programme is not a guarantee for the assurance of the radiation safety of patients, staff and public. What is also needed is a separate radiation safety programme, which is very essential in every diagnostic imaging facility and must also be under the direction of a qualified expert in radiation protection.

2. Overview of quality assurance

The 2008 United Nations Scientific Committee on the Effects of Atomic Radiation (UNSCEAR) report on medical exposures from the assessment of the global population dose from medical exposures for the period 1997-2007 indicates that medical exposure remains the largest source of man made exposure to ionizing radiation and continues to grow substantially. (UNSCEAR, 2008). A summary of the annual per caput effective dose to the global population due to all sources of ionizing radiation is illustrated in Table 1.

It is evident that diagnostic examinations result in a per caput effective dose of 0.66 mSv, while medical exposures now contribute around 20% of the average annual per caput dose to the global population. Medical exposures are defined as; (i) exposure of patients as part of their medical diagnosis or treatment; (ii) exposure of individuals as part of health screening programmes; and (iii) exposure of healthy individuals or patients voluntarily participating in medical, biomedical, diagnostic or therapeutic research programmes. These exposures include diagnostic radiology, nuclear medicine and radiation therapy (Fig. 1), out of which diagnostic radiology accounts for the largest contribution. Diagnostic radiology generally refers to the analysis of images obtained using x-rays. In nuclear medicine, a radiopharmaceutical is administered to the patient and concentrates primarily in a specific region of the body which allows: (i) external imaging of the body to evaluate structure and/or function, and (ii) or delivery of a large radiation dose to control a specific disease. Radiation therapy

involves the use of intense radiation beams and high-activity sources for the treatment of many types of cancer.

Source	Annual per caput effective dose (mSv)	Contribution (%)
Natural background	2.4	79
Diagnostic medical radiology	0.62	20
Diagnostic dental radiology	0.0018	<0.1
Nuclear medicine	0.031	1.1
Fallout	0.005	<0.2
Total	3.1	100

Source: UNSCEAR 2008 report on medical radiation exposures. (UNSCEAR, 2008).

Table 1. Sources of ionizing radiation and the annual per caput effective dose to the global population.

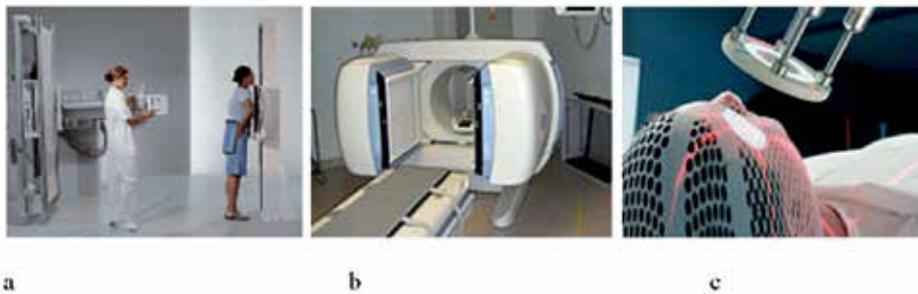


Figure 1. An illustration of (a) diagnostic radiology, (b) nuclear medicine and (c) radiation therapy procedures.

This makes the role of (QA), an important tool in medical exposure procedures. Quality assurance procedures must aim at to produce images of optimal diagnostic quality while ensuring that the radiation exposures to patients, staff and the public are kept as low as practicable. In achieving this goal, QA procedures if well implemented would ensure that any problem in the imaging chain will be dealt without a compromise in the diagnostic quality of the images. The World Health Organization (WHO), (WHO, 1982), indicates that achieving adequate diagnostic information and least possible exposure of the patient to radiation should be done at the lowest possible cost. An adequate diagnostic QA program involves periodic checks of all major components in the respective diagnostic imaging modalities. On the other hand, an optimum QA programme for any individual diagnostic facility will depend on some items such as the type of procedures performed, type of equipment utilized, patient workload, etc. Any QA procedure must be in agreement with the relevant national and international legislation or regulations on the protection and safe uses of ionising radiation. Another form of QA is to establish clinical audit, which is an essential

tool for quality improvement in any diagnostic centre. For instance, the European Council directive (Commission of the European Communities. 1997) defines clinical audit as: “a systematic examination or review of medical radiological procedures which seeks to improve the quality and the outcome of patient care, through structured review whereby radiological practices, procedures, and results are examined against agreed standards for good medical radiological procedures, with modifications of the practices where indicated and the application of new standards if necessary.”

This paper reviews the future of quality assurance in diagnostic medical exposures in Ghana from the perspective of a Medical Physicist, since a viable QA programme must be developed under the guidance and supervision of a medical physicist who is qualified in this area of expertise by education, training and experience. The Medical Physicist is expected to give guidance and supervision to the Technologists and other staff to execute the programme but should be prepared to perform higher level QA procedures as required.

3. Current status of quality assurance in diagnostic medical exposures

The types of diagnostic medical exposure procedures in Ghana are;

- Plain radiography
- Mammography
- Fluoroscopy
- Computed Tomography
- Dental

In addition to the above procedures for diagnosis, some hospitals perform interventional or invasive procedures on a limited scale. On the other hand, the types of medical exposure in nuclear medicine procedures are the use of gamma camera and single photon emission computed tomography (SPECT/CT) for imaging various organs. Plain radiography (screen-film and digital systems) is the dominant mode of all the diagnostic medical procedures, accounting for more than 80% of the total contribution of all the imaging modalities. (RAIS, 2011).

The performance of QA practices are done on three fronts; namely at the hospital, equipment/service engineers and the (RA). The hospital based QA are done mainly by the Radiographers/Radiologic Technologist through their routine equipment warm ups and minor quality checks. In this scenario, a qualified Medical Physicist with the requisite expertise must supervise such QA procedures. Unfortunately, there are not many Medical Physicists in diagnostic departments as compared to radiation therapy centres. Equipment/service engineers perform engineer related QA checks through installation and acceptance testing, performance tests and periodic preventative maintenance procedures as well as when there is equipment down time. On the other hand, the RA is largely in charge of major QA procedures through it's on site safety assessment inspections by assessing the compliance of the

equipment within regulatory requirements. This is largely so because the RA has the requisite technical expertise and equipment.

In Ghana, Medical Physicists are engaged in service, teaching, research and administration activities. They perform some of the tasks outlined by the International Organisation for Medical Physics (IOMP) in its definition of who a Medical Physicist is and the roles and responsibilities (International Organisation for Medical Physics, 2010) such as:

- teaching principles of medical physics to physicians, residents, graduate students, medical students, technologists, and other health care professionals by means of lectures, problem solving, and laboratory sessions.
- conducting research into various human disorders, illnesses and disabilities, develop instrumentation, mathematical analysis and applications of computers in medicine; investigating biophysical techniques associated with any branch of medicine. Research is very important for advancement of medical physics as a profession and science.
- responsible for ensuring the quality, safety testing and correct maintenance of all radiation emitting devices in order to get an accurate diagnosis of illnesses. Medical Physicists also involved in the formulation of radiation protection guides and procedures specific to clinical environment and producing protocols to minimize radiation exposure of patients, staff and the general public.
- in administration, they supervise and manage radiation workers and other health professional workers.
- participating in and contributing to the development and implementation of national and
- prepares guidance on education and training drawing-up standards and guidance relating to medical devices.
- preparing, publishing and presenting scientific papers and reports

Ghana is involved in several International Atomic Energy Agency (IAEA) Technical and Research Projects. Some of the Projects in which Medical Physicists are involved are:

- RAF/9/033 - Strengthening Radiological Protection of Patients and Medical Exposure Control.
- RAF/9/034 - Establishment of National Capabilities for Response to a Radiological and Nuclear Emergency.
- RAF/9/035 - Education and Training in Support of Radiation Protection Infrastructure.
- RAF/9/032 - Development of Technical Capabilities for the Protection of Health and Safety of Workers Exposed to Ionizing Radiation.
- RAF/9/027 - National Regulatory Control and Occupational Radiation Protection Programmes.
- RAF/9/031 - Strengthening National Regulatory Infrastructure for the Control of Radiation Sources.

- GHA/6/015 - Upgrading and Expansion of Radiotherapy and Nuclear Medicine Services
- INT/6/054 - Strengthening Medical Physics in Radiation Medicine
- RAF/2/008 - Strengthening and Expanding Radiopharmacy Services in Africa (AFRA)
- RAF/6/032 - Promoting Regional and National Quality Assurance Programmes for Medical Physics in Nuclear Medicine (AFRA II-7)
- RAF/6/041 - Supporting the Development of Comprehensive National Cancer Control Programmes
- RAF/6/044 - Medical Physics in Support of Cancer Management (AFRA II-8)
- RAF/6/045 - Enhancing Accessibility and Quality in the Care of Cancer Patients (AFRA II-10)

The main objectives of some of the projects are discussed. For instance in RAF/9/033, the objectives are to upgrade / strengthen radiological protection of the patient in medical exposures due to:

- i. Diagnostic Radiology and Interventional Radiological procedures
- ii. Nuclear Medicine procedures
- iii. Radiotherapy practice

The objectives of other projects are as follows:

- GHA/6/015 - To consolidate existing radiotherapy and nuclear medicine facilities at two leading Teaching Hospitals located in the southern part of the country, and establish a third one in the northern part to cater for the diagnosis, curative and palliative treatment of cancer patients and the efficient diagnosis and management of other diseases.
- INT/6/054 - To promote the recognition of medical physics in radiation medicine and to harmonize educational material in order to ensure safe and effective diagnosis and treatment of patients.
- RAF/2/008 - To strengthen radiopharmacy in support of in vivo and in vitro nuclear medicine in Africa.
- RAF/6/032 - To improve the effectiveness and safety of nuclear medicine procedures by providing support for design and implementation of quality assurance (QA) programmes and by establishing training and education programmes in medical radiation physics, focusing on aspects related to the application of nuclear medicine techniques.
- RAF/6/041 - To assist Member States in performing comprehensive cancer capacity need assessments and national cancer strategic planning via collaboration with IAEA, WHO, and other partners under the Programme of Action for Cancer Therapy (PACT) umbrella.
- RAF/6/044 - To strengthen national and regional medical physics capabilities to ensure efficient support of cancer management in AFRA Member States and to sustain quality as-

urance/quality control (QA/QC) programmes, including the promotion of safety culture and innovative practices in dosimetry.

- RAF/6/045 - To establish national and regional networks in clinical radiation oncology. To interact with National Organizations with the aim to promote the comprehensive management of commonest cancers. Support academic education, training and accreditation, patients and personnel safety in radiotherapy improvement of documentation of clinical outcomes through regular patient assessment.

4. Regulatory guidelines for quality assurance procedures

The National Competent/Regulatory Authority in Ghana charged with the responsibility for authorization and inspection of practices using ionizing radiation sources and radioactive materials is the Radiation Protection Board (RPB) (Radiation Protection Instrument LI 1559, 1993). However, the operational functions of the RPB are carried out by RPI, which was established in 2000 to provide scientific and technical support for the enforcement of the provisions in LI 1559. Details about how the RA was established and the main activities have been described elsewhere (Inkoom et al, 2011). There are plans to establish a new Regulatory Body to regulate the peaceful uses of nuclear energy and technology which will be independent of any governmental agency. Currently, the RA is answerable to the Ghana Atomic Energy Commission (GAEC) which is a promoter for the peaceful uses of nuclear energy and technology and also plays the role of a regulator. However, the new RA is expected to be only a regulator and not a promoter of the application of nuclear technology.

5. Human resource development

The categories of Radiographic Staff available in Ghana are Radiologists, Medical Physicists, Biomedical Engineers and Radiographers/X-ray Technicians. Most of our Radiologists were trained overseas until the last few years when local training of Radiologists started and the accreditation is given by either the Ghana College of Surgeons or the West African College of Physicians and Surgeons. Similarly, the other professionals were also trained overseas. Currently, the School of Allied Health Sciences (SAHS), College of Health Sciences (CHS) of the University of Ghana (UG) is responsible for churning out medical and dental technical graduates in physiotherapy, medical laboratory science and radiography. There are plans to establish another Allied Health University and some private institutions are also running some of the programmes. A Post-Graduate School of Nuclear and Allied Sciences which was established jointly by the GAEC and UG, in co-operation with the IAEA is training the Medical Physicists, Radiation Protection Professionals, Nuclear Engineers, etc. at the National and Sub-Regional levels.

6. Recent trends in quality assurance

The increasing expansion of diagnostic imaging procedures in medicine coupled with rapid technological advances makes the availability of qualified and trained personnel to be very crucial if the desired quality is to be achieved. This comes with a lot of challenges to the medical imaging community. This offers practitioners the opportunity to continually undergo retraining and other continuous professional development programmes in their respective fields. Also with the emergence of picture archiving and communication system (PACS) in many hospitals, there is the need for the development of appropriate on-line QA procedures and in incorporating them into hospital PACS systems. Special attention must also be given to the emergence of digital technology over the last decade as one of the greatest technological advances in medical imaging. This new technology poses a great challenge in medical imaging, requiring re-training of staff on the safe use of equipment and radiation protection issues. In Ghana for instance, the RPI of GAEC, in collaboration with the IAEA, has in the previous years developed a lot of expertise in the training of occupationally exposed workers in Ghana and the rest of Africa, spanning a period of almost two decades (Boadu et al. 2011). This local expertise in training can be tapped. In this regard, a critical review of all QA procedures that were developed for screen-film systems needs special attention.

7. The way forward

Various practitioners in the medical imaging community must brace themselves in order to face challenges of technological developments. With the advent of digital radiography: advances in computed radiography, direct digital radiography, digital subtraction angiography, new digital receivers, image processing techniques, computer applications in radiology and PACS offers enormous challenges. The advantages of digital technology: post-processing capabilities, decreased costs, multiple viewing options, electronic transfer, possibilities of archiving, wide dynamic range of flat panel detectors and increased detection quantum efficiency has led to a high demand of this technology by the medical imaging community. Therefore, the development of the requisite human resource must be continued and sustained in order to deal with the challenges.

There is a strong need for the establishment of National Quality Control Centre for Diagnostic Radiology. This body must be equipped with the requisite state-of-the-art equipment, highly qualified and trained personnel in order to coordinate all QA activities in the country. Such a body can initiate some guidelines on the minimum instrumentation requirements for all imaging modalities. With the training of more Medical Physicists and Radiation Protection Professionals, it is expected that they would take up positions

in all major hospitals which have a myriad of imaging modalities. The Ghana Society of Medical Physics, RA, Ministry of Health and other stakeholders must initiate procedures for the establishment of Medical Physics Departments in such hospitals. This would give the necessary recognition to the profession of Medical Physics in Ghana, which has been given recognition by the International Labour Organization (ILO) in its International Standard Classification of Occupations (ISCO) (ILO, 2008). With this recognition, Medical Physics has been accepted as modern applied branch of physics. Clinical audit should also be incorporated in the overall QA procedures in the country.

As the uses of ionizing radiation continue to increase in medicine, it is also expected that the services of Medical Physicist would increase. As such, more physicists would be required to be trained in subsequent years. Appropriate accreditation bodies charged with issuing accreditation certificates, for a period of years must be put in place to regulate the profession of Medical Physics and maintain international standards of practice.

8. Conclusion

The role of an effective QA programme in any diagnostic department cannot be overemphasized especially if the desired quality of producing good diagnostic images and the least radiation exposure are to be achieved. Nevertheless, a good QA programme is not a guarantee for the assurance of the radiation safety of patients, staff and public. What is also needed is a separate radiation safety programme, which is very essential in every diagnostic imaging facility and must also be under the direction of a qualified expert in radiation protection or a Medical Physicist expert. With significant contributions in clinical service, education, and research, Medical Physics continues to grow in importance both as a profession and as science, driven by the technological developments of societies in general and medicine in particular.

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Quality by Design and Risk Assessment for Radiopharmaceutical Manufacturing and Clinical Imaging

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Additional information is available at the end of the chapter

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1. Introduction

Radiopharmaceuticals have been widely used in many clinical and nonclinical applications, such as *in vivo* and non-invasive diagnosis or treatment of human diseases. The quality of radiopharmaceuticals administered for a patient is primarily related for the radiation dose delivered to achieve optimizing diagnostic imaging or therapeutic efficacy. Radiopharmaceuticals with different half-lives (short, medium, and long), decay modes (alpha, beta, gamma, and electron capture), and biochemical properties (of ligands) can determine their utilities in medicine. Moreover, chemical and radiochemical impurities in a radiopharmaceutical can produce a serious trouble of diagnosis or treatment. Therefore, different requirements, regulations, and instrumentations for ensuring their high quality and high safety have been developed in many countries.

There are only few years for the progress of “Quality by Design (QbD)” in International Conference on Harmonisation (ICH) Guidelines, e.g. ICH Q8, ICH Q9, and ICH Q10 [1-3]. According to the requirement of ICH Q8, quality can not be tested into products; i.e., quality should be built in by design, i.e. QbD. Enhanced QbD approach to pharmaceutical development can improve the product and process knowledge.

In this chapter, we provide a harmonized framework of QbD for manufacturing and clinical applications of radiopharmaceuticals in accordance with the requirements and guidelines of U.S. Food and Drug Administration (FDA), International Atomic Energy Agency

(IAEA), World Health Organization (WHO) and European Association of Nuclear Medicine (EANM). The attributes of the components in the quality system (QA/QC), including organization, staffing and personnel, facilities, instrumentation and equipment, operation procedure, radiopharmaceuticals, protocol and conduct of a study or a treatment, records and reports, and audit framework were further characterized. Assessments and comparisons of critical quality attributes (CQAs) for assuring accurate radioactive dosimetry calculation in the efficiency tracing of absolute activity measurement and patient- and technologist-related risks for nuclear medicine imaging including Positron Emission Tomography (PET), Computed Tomography (CT), PET/CT, and Single Photon Emission Computed Tomography (SPECT) were identified.

2. Quality system design based on the Requirements and Guidelines

2.1. Quality policy and system

The quality system by design for radiopharmaceuticals and clinical imaging techniques is aimed to maintain and improve the qualified service for the patients, fulfill the regulatory requirements, optimize the safety and efficacy for patient care, demonstrate a proper equipment operating condition, and obtain a reliable quantitative performance in both diagnostic and therapeutic nuclear medicine procedures [4,5]. The pursuit of excellence in quality system is not a single action over a short period, instead, it is achieved through the whole life cycle of instruments, analytical methods or education for example, from planning and procurement to decommissioning based on advanced technology [6]. Continuous quality improvement implies a commitment to continuously struggle to advance based on state-of-the-art information and techniques developed by the nuclear medicine and metrology community at large [5].

Implementation of a quality system must be in accordance with the quality policy, i.e. the overall quality intentions and direction of an organization, as formally expressed by top management. And quality system includes the structure, responsibilities, and procedures for implementing quality management. An integrated infrastructure of quality policy and system design is demonstrated as in Figure 1, which is mainly developed from the European Standard EN 28402 proposed by Bergmann *et al.* [7]. The attributes of the components in the quality sub-system (QA/QC), e.g. organization, personnel, facilities, instrumentation, operation procedures, preparation of radiopharmaceuticals, protocol and conduct, records and reports, and audit or inspection, were further integrated and classified in this article.

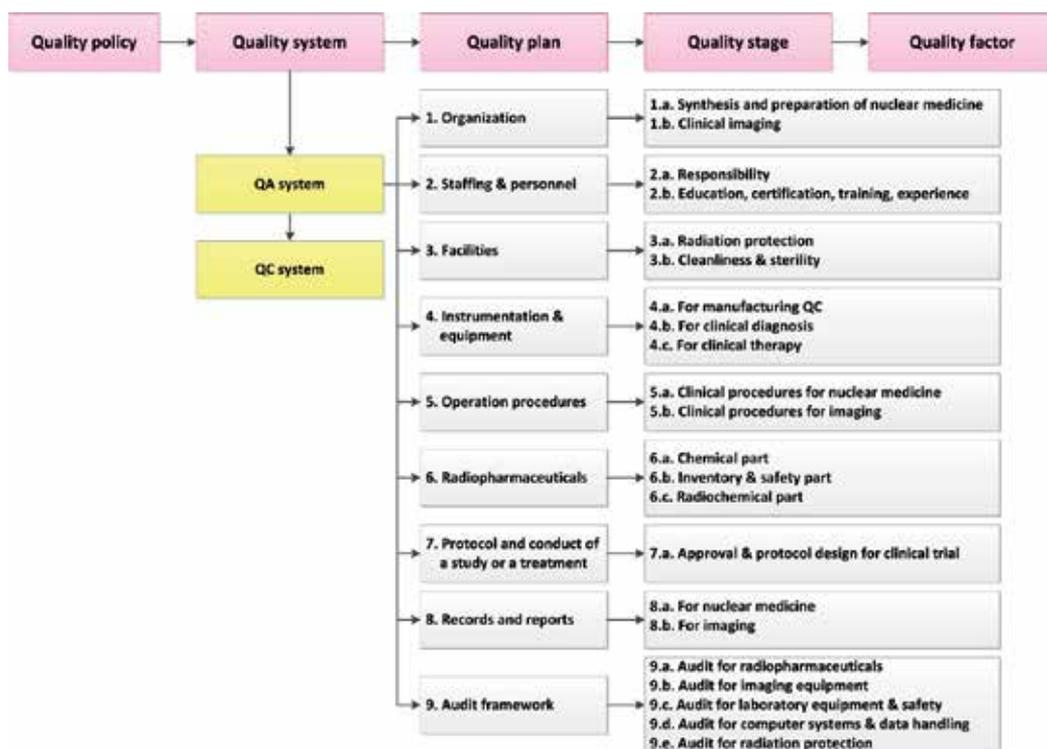


Figure 1. Quality policy and system for the radiopharmaceuticals [7].

2.2. Quality plan and key factors

2.2.1. Organization

The organization of quality system could be grouped into two categories: (a) synthesis and preparation of nuclear medicine and (b) clinical imaging as shown in Figure 2 [8-10]. For synthesis and preparation of nuclear medicine, three important guidelines were considered [11-13]. Basically, preparation of “classical” radiopharmaceuticals in “kit” procedures and in a “distinct chemical” procedures for PET radiopharmaceuticals are distinguished as two different parts [11].

For the clinical imaging, the major differences PET and SPECT in QbD are related to the properties and applications of a radiotracer. The most commonly used nuclides for PET imaging, such as carbon-11, oxygen-15, nitrogen-13, and fluorine-18, exhibit shorter half-life and more complicated labelling technology than that for SPECT imaging (Table 1)[14-31]. For example, the short half-lives of radionuclides used in PET modality allow for better de-

tection sensitivity over a given period of time. This is because radiotracers with shorter half-lives can be injected in higher activities to the patient without posing any additional radiation damage to the patient (since overall accumulation over time remains the same) leading to the increased detectable radiation over a shorter time. Moreover, arguments that the natural occurrence of PET isotopes in biologically active molecules (as opposed to heavy isotopes used in SPECT) results in a less challenging task of synthesizing physiologically useful tracers in PET modality [32,33]. In general, PET generally has a higher resolution, higher sensitivity, and a better quantitation capability than SPECT. However, SPECT is more practical as a routine procedure [18] and is more cost-effective for the system setting or maintain than a PET facility [8].

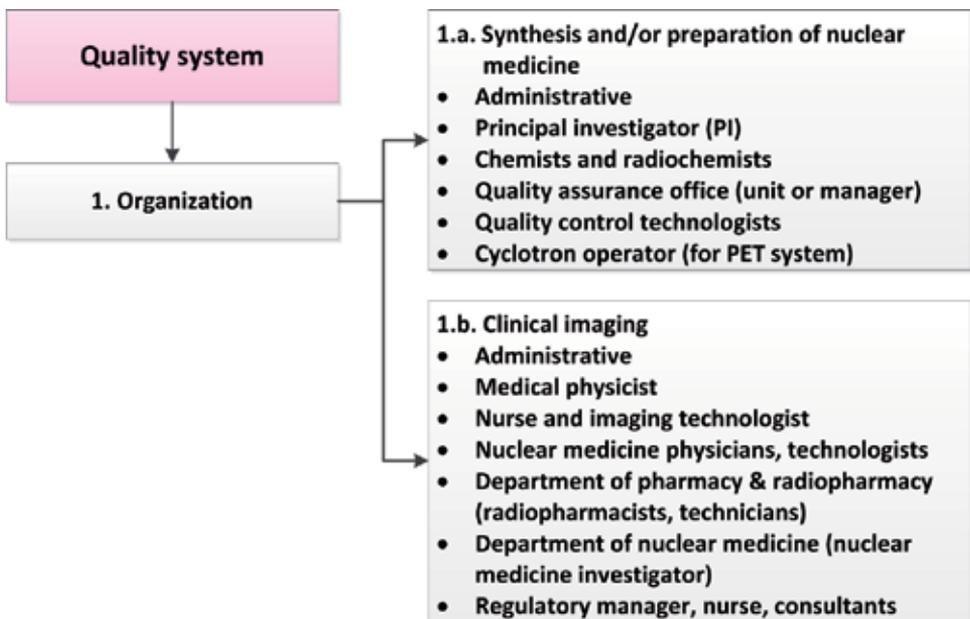


Figure 2. Quality system of organization [8-10].

Agents	Isotope	Half-life ($t_{1/2}$)	Radiopharmaceutical	Applications
PET imaging agents	C-11	20.4 min	C-11-raclopride	D2/D3 dopamine receptor
			C-11-MADAM	Serotonin transporter
	N-13	9.96 min	N-13-ammonia	Blood flow (ventricle)
	O-15	2.07 min	O-15 water	Myocardium perfusion, brain perfusion
	Ga-68	68 min	Ga-68-DOTA	Neuroendocrine tumours

F-18	109.8 min	F-18-fallypride	D2/D3 dopamine receptor
		F-18-FDG	Oncology imaging, metabolism of glucose in tumors, brain and myocardium
		F-18-NaF	Osseous metastasis
Cu-64	12.7 h	Cu-64-ATSM	Tumor hypoxia
I-124	4.12 d	I-124-FIAU	HSV1-tk expression
		I-124-HMFGI (IgG ₁)	Breast ductal carcinoma
SPECT imaging agents	6 hr	Tc-99m-HMPAO	Brain perfusion
		Tc-99m-ECD	Brain perfusion
		Tc-99m-TRODAT-1	Dopamine transporter
		Tc-99m-Prostascint	Prostate cancer
		Tc-99m-CEA	Colon cancer
		Tc-99m-Depreotide	Lung cancer
		Tc-99m-Annexin-V	Acute myocardial infarction, chemotherapy response monitoring, apoptosis of lung tumors
		Tc-99m-sestambi	Myocardium perfusion
		Tc-99m-MAG3	Kidney perfusion
		Tc-99m-DTPA	Kidney perfusion
		Tc-99m-DMSA	Kidney perfusion
SPECT imaging agents	13 hr	I-123-Iomazenil	Benzodiazepine (γ -aminobutyric acid) receptor
		I-123-IBZM	D2/D3 dopamine receptor
		I-123-iodobenzofuran	D2/D3 dopamine receptor
		I-123-epidepride	D2/D3 dopamine receptor
		I-123-FP- β -CIT	Dopamine-transporter
		I-123-ADAM	Serotonin transporter
		I-123-IMP	Brain perfusion
		I-123-Nal	Thyroid
In-111	2.8 d	In-111-Zevalin	Non-Hodgkin's lymphoma

		In-111-Octreotide		Somatostatin receptor (Neuroendocrine tumors)
	TI-201	3.04 d	TI-201	Myocardium perfusion
	Ga-67	3.3 d	Ga-67 citrate	Non-Hodgkin's lymphoma
Therapy agents	Sm-153	1.95 d	Sm-153 EDTMP	Metastatic bone pain palliation
	Sr-89	50.5 d	SrCl ₂	Palliative treatment of bone cancers and for prostate cancer
	P-32	14.28 d	Orthophosphate	Metastatic bone pain palliation
	Re-186	3.78 d	Re-186-HEDP	Metastatic bone pain palliation
	Re-188	17 h	Re-188-bisphosphonate	Metastatic bone pain palliation
	Y-90	64.14 h	Y-90 Ibritumomab Tiuxetan	B-cell non-Hodgkin's lymphoma
	I-131	8 d	I-131 Tositumomab	B-cell non-Hodgkin's lymphoma
	Lu-177	6.7 d	Lu-177-DOTA-Tyr ³ - Octreotate	Small cell lung cancer
	Ho-166	1.1 d	Ho-166-DOTMP	Multiple myeloma
	Sn-117m	13.6 d	Sn-117m-DTPA	Metastatic bone pain palliation
At-211	7.2 h	At-211-81C6	Glioblastoma multiforme tumors	

Table 1. Some examples of radiopharmaceutical classification and applications [14-31]. ADAM: 2-((2-((dimethylamino)-methyl) phenyl)thio)-5-iodophenylamine; DTPA: diethylenetriaminepentaacetic acid; ECD: ethyl cysteinate dimer; FDG: fluoro-deoxy-glucose; FIAU: 1-(2-fluoro-2-deoxy-β-D-arabinofuranosyl)-5-[1-124]iodouracil; FP-β-CIT: N-propyl-2-beta-carboxy-methoxy-3-beta(4-iodophenyl)-nortropane; HMPAO: hexamethyl propylene amine oxime; IBZM: iodobenzamide.

2.2.2. Staffing and personnel

Facilities should have written staff and personnel responsibilities and requirements. Two types of staff in the requirements for synthesis and preparation of nuclear medicine and clinical imaging are necessary [4]:

- a. Personnel for synthesis and preparation of nuclear medicine may include such as facility management, administrative staff, study director (SD), principal investigator (PI), production chemists, QA manager or quality assurance unit (QAU), radiochemists, QC chemists, cyclotron operators, and technologists.
- b. Personnel for PET and SPECT imaging examination may include such as facility management, administrative staff, medical physicists, nurses, referring physicians, nuclear medicine physicians, radiopharmacist, radiochemists, radiation protection® officer, engineers, QA manager or QAU, and technologists.

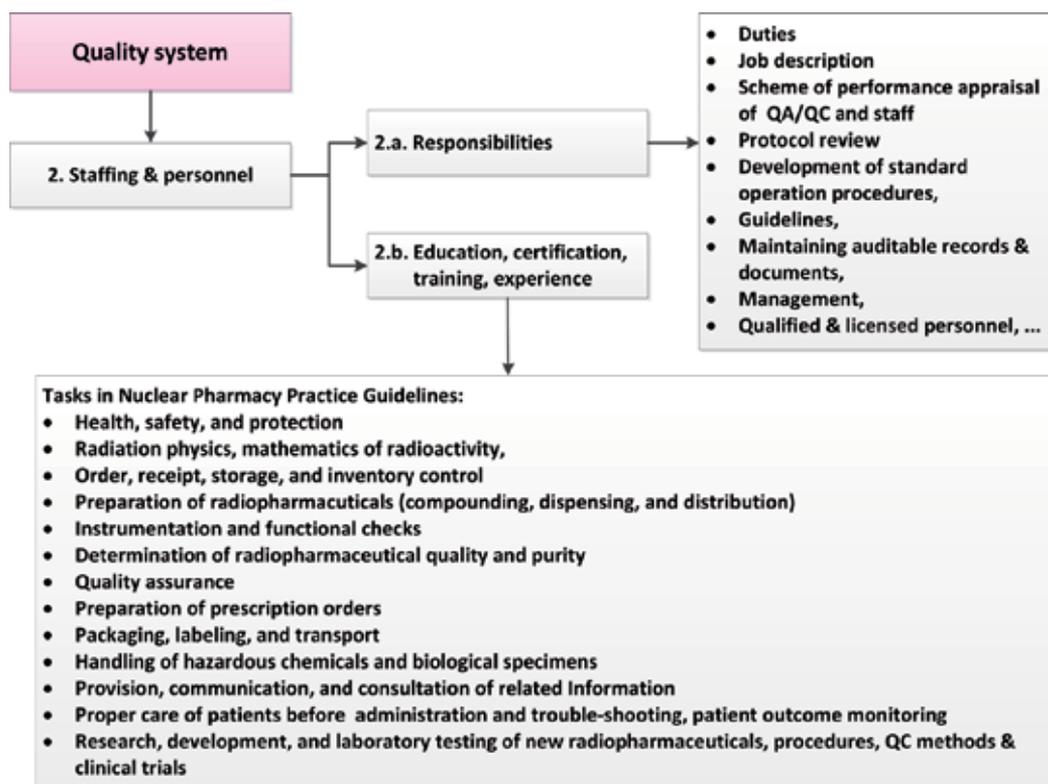


Figure 3. Quality system of staffing and personnel [9,34,35].

The responsibilities for staffing and personnel in a quality system are classified in Figure 3 [9,34,35] and briefly introduced below [4,6,7,36-38]:

- a. Facility management: ensure the requirements, guidelines, and practices are complied within facility, sufficient qualified personnel, appropriate facilities, equipment, and materials are available, ensure that personnel clearly understand the functions they are to perform and appropriate and technically valid Standard Operating Procedures (SOPs) are established and followed, ensure that there is a QA manager or QAU with designated personnel and their responsibility is being performed, ensure that for each study an individual with the appropriate qualifications, training, and experience is designated by the management as the SD and PI, ensure that an individual is identified as responsible for the management of the archive.
- b. Administrative staff: represent the first encounter a patient has with the centre. They receive the patients according to the established protocols. In collaboration with the medical and technical staff, they are responsible of the application of the procedures for scheduling studies.

- c. SD and PI: they are responsible for approving, conducting, documenting, recording and archiving the overall of the study and for its final report.
- d. Nuclear medicine physicians: responsible for quality encompasses the general services of the centre. In particular, supervises all patient care and management procedures and all clinical protocols. In addition, he/she supports and enforces the QA/QC of equipment, establish clinical review and auditing.
- e. QA manager or QAU: all those planned and systematic actions necessary to provide adequate confidence that a product or service will satisfy given requirements for quality, express the closeness with which the outcome of a given procedure approaches some ideal, free from all errors and artefacts. Quality assurance embraces all efforts made to this end.
- f. Radiopharmacist and Radiochemists (Nuclear pharmacy): they are responsible for compounding, dispensing, quality assessment, patient monitoring, drug use review, new drug development and evaluation, product selection and performance evaluation, pharmacokinetic modeling, drug information and educational services. They are also responsible for the performance of acceptance testing and organization/supervision of routine calibration and QC of all radiopharmacy equipment; QC of chemicals, enriched materials, precursors, and kits; QC of radiopharmaceuticals products and batch release.
- g. Cyclotron operators: they are in charge of the daily operations, take part in the acceptance test of the cyclotron and related equipment, and are responsible for calibration and QC procedures for equipment.
- h. Production chemists: synthesis and preparation of nuclear medicine.
- i. QC chemists: the restriction of QC persons is independent of the production operations or must have independent oversight of these duties. The operational techniques and activities that are used to fulfill requirements for quality and are used in reference to the specific measures taken to ensure that one particular aspect of the procedure is satisfactory.
- j. Medical physicists: specialized in nuclear medicine and responsible for the performance of acceptance testing and organization/supervision of routine calibration and QC of imaging and radiation measurement equipment, including radiation protection instrumentation.
- k. Radiation protection officer: ensure the radiation safety for patient, staffing, and environmental.
- l. Engineers and Technologists: contribute to the preparation of clinical examination protocols and the performance of patient examinations according to the established protocols, involved in the performance of routine calibration and QC of scanners.

- m. Nurses: manage and care of the patient, collaborate in preparing protocols of patient management and information material as well as in checking the operation of other institutional services.

In IAEA, quality manager is responsible for the entire quality management system supervision, the authority to enforce it and act on its findings, and should be involved in the evaluation and periodic review of the results [5,6]. But, in EANM, the responsibility for overseeing the preparation operations of a qualified radiopharmaceutical is called QAU [11,12].

2.2.3. Facility

In a PET facility, it should include the facility for (a) PET/CT scanner, (b) cyclotron, and (c) radiopharmacy. The location of the facility is a very important issue for the flow of patients, materials, and radiation protection. According to the risk of radiation exposure, two areas are planned [4]:

- a. low risk area, cold area or uncontrolled area is the area of offices, reception, waiting room, consulting room, cleaning utilities room or store, and
- b. high risk area, hot area or controlled area is the area of hot laboratory, preparation, injection and uptake room, toilet, control and scanning room, post-examination waiting room, reporting room, and waste disposal room.

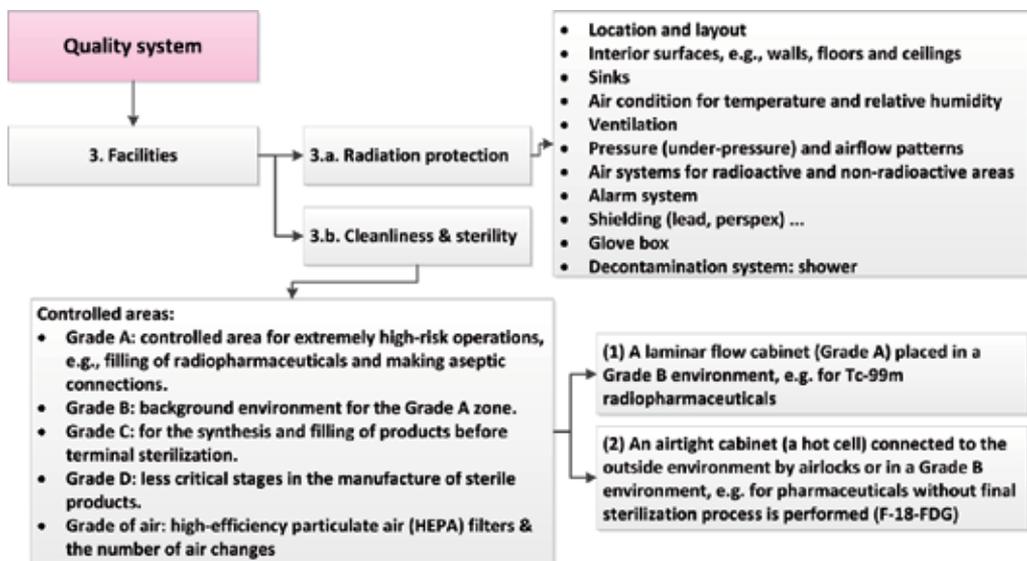


Figure 4. Quality system of facilities [34].

More considerations for the requirements of radiation protection and cleanliness are summarized in Figure 4 [34].

2.2.4. Instrumentation and equipment

The instrumentation and equipment in the quality system are summarized in Figure 5 [34,38]. Apparatus and equipment for the purposes of manufacturing QC, diagnosis, and therapy, including validated computerized systems, used for the generation, storage and retrieval of data, and for controlling environmental factors relevant to the study should be suitably located and of appropriate design and adequate capacity. Apparatus used in a study should be periodically inspected, cleaned, maintained, and calibrated according to SOPs. Records of these activities should be maintained. Calibration should be traceable to national or international standards of measurement [39].

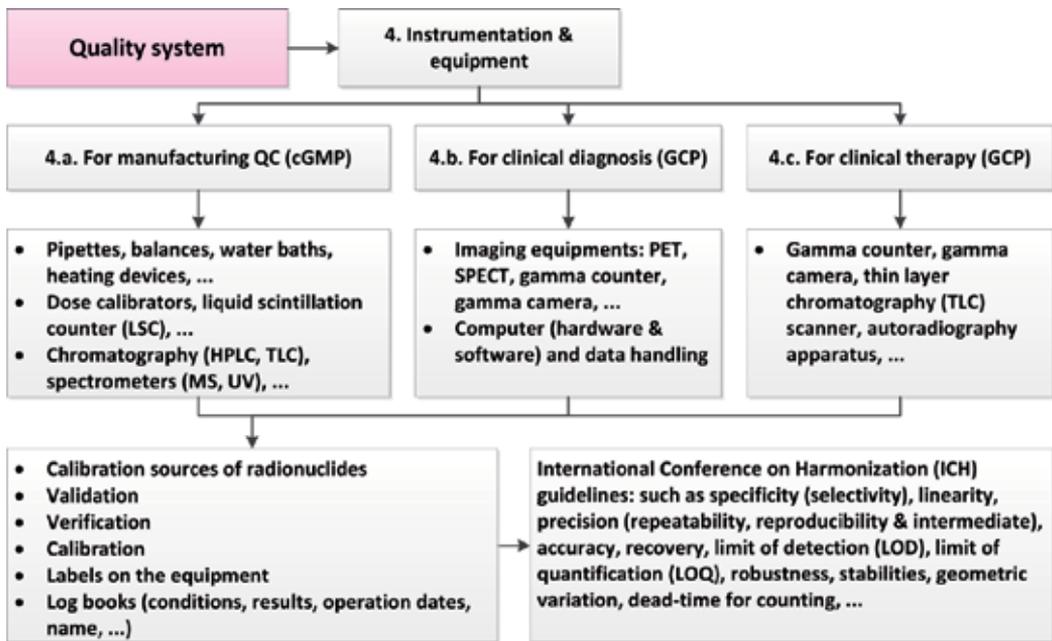


Figure 5. Quality system of instrumentation and equipment [34,38].

Performance tests and operation verification for the nuclear medicine units are achieved daily, weekly, monthly, quarterly, or annually by a qualified medical physicist, a qualified nuclear medicine technologist, or a medical physicist in training, with management by a qualified medical physicist. The tests results of intrinsic or system spatial resolution, uniformity, center of rotation, sensitivity, energy resolution, counting rate parameters, multiple-window spatial registration, formatter and video display, linearity, leak test, overall system performance for imaging systems, interlocks, dose calibrators, thyroid uptake and counting systems must be reviewed and documented in an annual survey report in accordance with the ACR Technical Standard for Medical Nuclear Physics Performance Monitoring of Nuclear Medicine Imaging Equipment [37].

2.2.5. Operation procedures

A test facility should have written SOPs approved by facility management for ensuring the quality and integrity of the data generation. Deviations from SOPs related to the manufacturing, study, or treatment should be documented and should be acknowledged by the study director, the principal investigator, the medical physician, quality assurance personnel and/or radiopharmacist. The historical file of different version of all SOPs should be well recorded and stored. The requirements of SOPs for nuclear medicine manufacturing and imaging are summarized in Figures 6 and 7 [11,12,40-44].

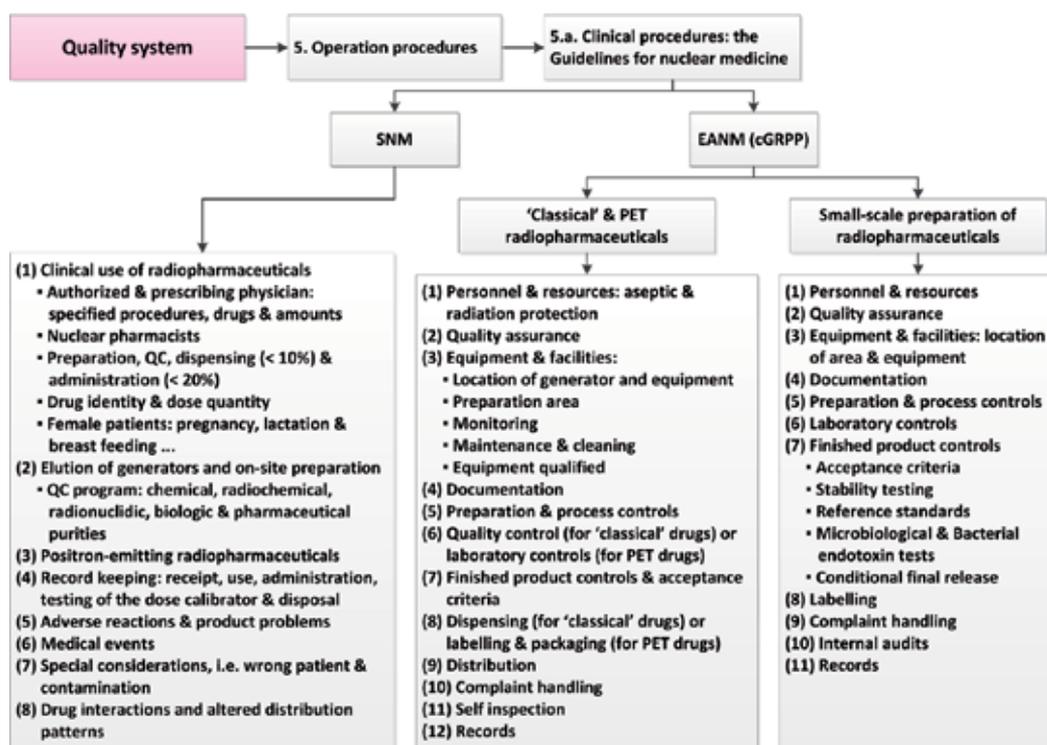


Figure 6. Quality system of clinical operation procedures for nuclear medicine [11,12,40].

2.2.6. Radiopharmaceuticals

(a) Manufacturing of radiopharmaceuticals

Manufacturing and quality control plans for radiopharmaceuticals are indicated in Figure 8 [34,40,45]. Radiopharmaceuticals might be manufactured or prepared in hospital radiopharmacies, centralized radiopharmacies, nuclear centers, institutes, industrial manufacturers, or PET centers in accordance with the requirements of good manufacturing practices (GMP) or Current Good Radiopharmacy Practice (cGRPP) [11-13,34].

Two categories of radiopharmaceuticals are classified in EANM Radiopharmacy Committee according to the significant difference of preparation procedures, i.e. “kit” and PET radiopharmaceuticals. Also, significant consideration in the “Guidelines on Current Good Radiopharmacy Practice (cGRPP) in the Preparation of Radiopharmaceuticals” is proposed by EANM Radiopharmacy Committee. Two types of preparation methods, i.e. in “classical” procedure and in “synthetical” procedure, have been distinguished in cGRPP [11]. According to WHO guideline, radiopharmaceuticals are divided into four categories including ready-to-use, radionuclide generators, “kits” for the labelled with a radioactive component, and precursors used for radiolabelling other substances before administration (e.g. samples from patients) [13].

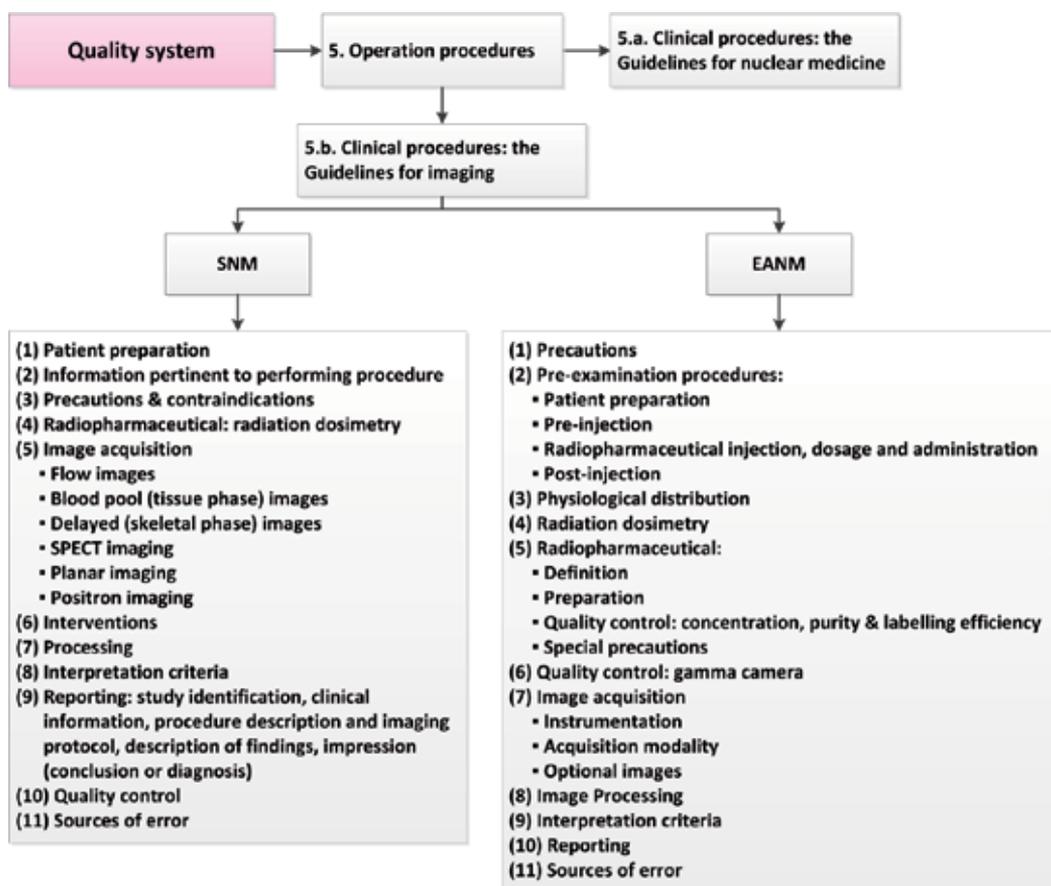


Figure 7. Quality system of clinical operation procedures for imaging [41-44].

Clinical investigations of radiopharmaceuticals can be approved by FDA as “legend drugs.” The investigational radiopharmaceutical drug service (IRDS) is responsible for establishing study-specific procedures for radiopharmaceutical drug, including preparation, storage, dis-

pening and destruction of investigational drugs within the hospital [9]. Manufacturing or preparation of radiopharmaceuticals must follow the FDA 21CFR Part 212 “Current Good Manufacturing (cGMP) for PET drugs,” USP Chapter <797> “Pharmaceutical Compounding-Sterile Preparations,” USP Chapter <823> “Radiopharmaceuticals for Positron Emission Tomography - Compounding,” and U.S. FDA Guidance: PET Drugs - Current Good Manufacturing Practice (CGMP) [10].

(b) Quality control of radiopharmaceuticals

Three essential parts i.e. chemical, inventory, and radiochemical QC diagrams for radiopharmaceuticals are also indicated in Figure 8 [30,50,45].

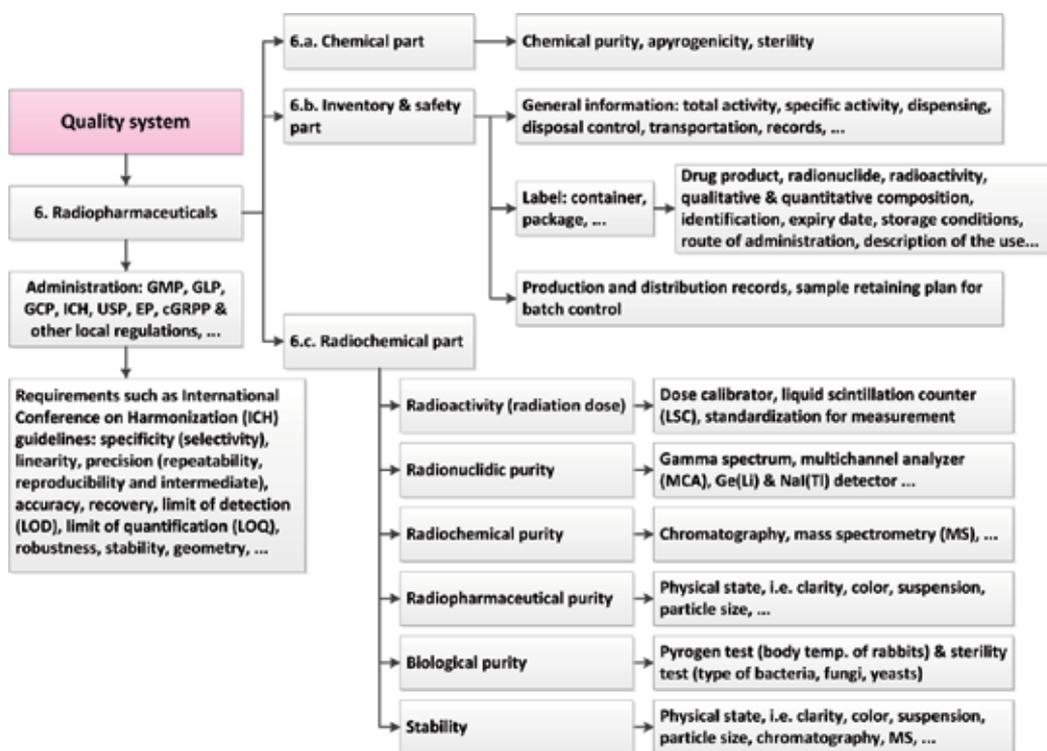


Figure 8. Quality system for radiopharmaceutical manufacturing and quality control [34,40,45].

Method developments for the chemical and radiochemical analysis of starting material, intermediates, precursor used for the radiolabelling, active pharmaceutical ingredient (APIs or drug substance), and finished product (drug product or finished dosage form) are essential requirements of Chemistry, and Manufacturing and Controls (CMC). In the applications of investigational new drug (IND), New Drug Application (NDA), and Abbreviated New Drug Application (ANDA), information on the CMC has to be filed as per 21 CFR 312.23(a) for a drug substance and drug product. The contents for the CMC sections of the

EU and U.S. are very much the same. However, the sequence and titles of the sections are quite different [46,47].

According to International Conference on Harmonization (ICH) guidelines, the parameters for the validation of analytical methods should basically include specificity (selectivity), linearity, precision (repeatability, reproducibility and intermediate), accuracy, recovery, limit of detection (LOD), limit of quantification (LOQ), robustness, and stability. However, instrument validation parameters for the radioactivity measurement or isotopic analysis, such as dose calibrator or liquid scintillation spectrometry, are partially different.

Radiopharmaceuticals are usually used before all quality control testing has been completed. The implementation of and compliance with the quality assurance program are therefore essential. Principal responsibilities of QA/QC are detailed by WHO and De vos et al., including preparation of detailed instructions for each test and analysis, ensuring the adequate identification, ensuring equipment and process validation, release or rejection of materials, evaluation of the quality and stability of the finished products, expiry dates, storage conditions, control procedures, specifications, and records keeping [13,34].

2.2.7. Protocol and conduct

Protocol for a medicine manufacturing study or imaging examination should be evaluated according to the purposes of a study, a treatment, or a clinical trial. Safety issue, such as algorithm proposed by ASNC for maximal benefit in patient radiation exposure must be included [33].

For each study and treatment, a written plan or protocol should exist prior to the initiation of the study. The protocol should be approved by dated signature of the study director, principal investigator or medical physician, facility management, sponsor and verified by quality assurance personnel and/or radiopharmacist. The study and treatment should be conducted in accordance with the study plan or protocol by using a unique identification to each study.

Clinical protocol should be evaluated based on the patient characteristics (e.g. patient history of disease or ability to complete the examination) and complexity of clinical situation in accordance with the current statements and guidelines [33]. For instance, advantages and disadvantages of assessing myocardial perfusion with PET, as compared to SPECT imaging, was reported and concluded that use of very short half-life tracers injected at very high activities, as well as the introduction of increasingly fast scintillators technology, which in turn has allowed reduction of random coincidences and introduced the possibility of time-of-flight (TOF) PET are expected to further contribute to high sensitivity imaging capabilities of PET [32].

An example for approving of protocol design for a clinical trial is shown in Figure. 9 [10,47]. Two pathways for the clinical studies of investigational radiopharmaceuticals are called Ra-

dioactive Drug Research Committee (RDRC) and IND. For an investigational medical product (IMP, investigation only), if there are adequate data from literature or original assessments that no pharmacologic effects are likely in humans, and the chosen radioactivity is small enough to result in the total radiation absorbed dose, clinical trial can be approved by National Competent Authority (NCA) and Ethical Committee (EC) in EU or approved by RDRC in U.S.. Otherwise, it is approved by EC in EU or approved by FDA in U.S., depending on the phase of drug development [47].

The FDA allows certain unique applications by the local RDRC, consisting of at least five individuals and three individual specialists in nuclear medicine, in formulate radioactive drugs, and in radiation safety, to approve and monitor for the use of radiopharmaceuticals in humans without IND approval. This is due to the low potential for toxicity of radiopharmaceuticals that are typically administered in tracer quantities. Requirements to establish a local RDRC at one's institution is outlined in regulation 21 CFR 361.1. And RDRC has to submit an annual report to the FDA as part of the procedures for maintaining an active and approved RDRC program [48].

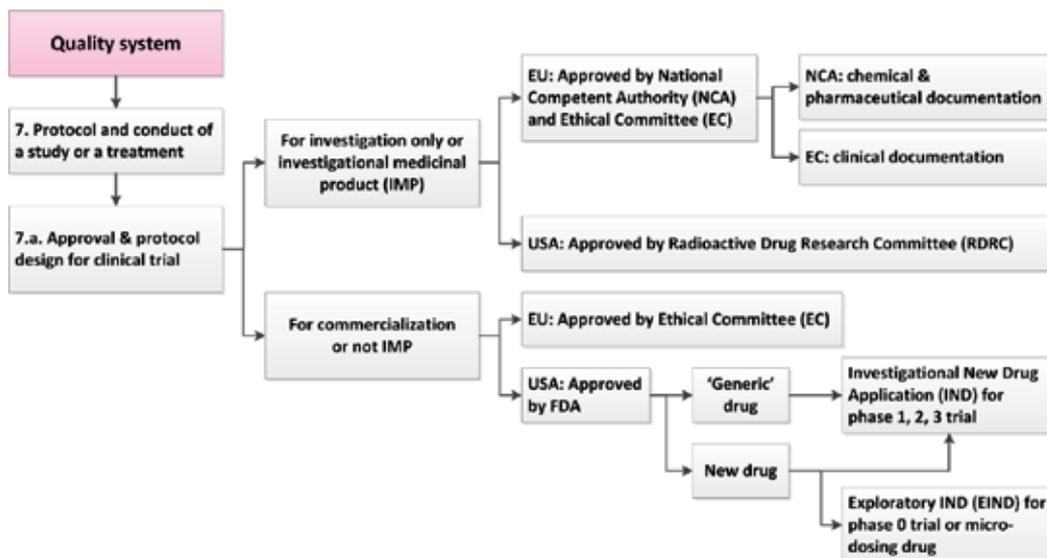


Figure 9. Quality system of protocol and conduct for a study or a treatment [10,47].

2.2.8. Records and reports

Records and reports for the manufacturing of radiopharmaceuticals and imaging trial or testing are summarized in Figure 10 [7,9,40-44]. All records and reports should be maintained at the radiopharmaceutical laboratory or another location that is accessible to respon-

sible officials and to government employees designated to perform inspections [11,12]. Storage of records must ensure safekeeping for many years. Archive facilities of independent locations should be provided for the secure storage and retrieval of study plans, raw data, final reports, samples of test items and specimens. Archive conditions, e.g. fireproof, waterproof, and insect prevention are designed for protecting contents from untimely deterioration [38].

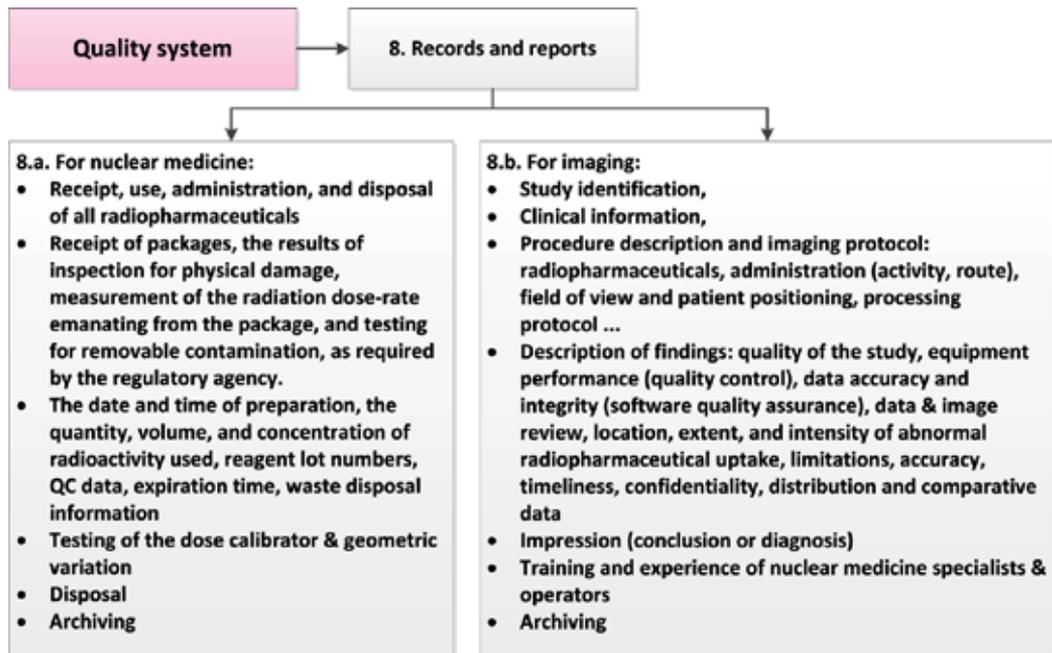


Figure 10. Quality system of records and reports [7,9,40-44].

2.2.9. Audit framework

Laboratory inspections and study audits should be established for periodical monitoring compliance with GLP, GCP, or GMP principles, study protocol, and SOPs [9,38]. Audits for radiopharmaceutical drug products typically begin by confirming the clinical site is appropriately licensed and authorized to receive, possess, store, handle, prepare and administer radiopharmaceuticals. The audit framework of quality system for radiopharmaceuticals, imaging equipment, laboratory equipment, safety, computer systems, data handling, and radiation protection are displayed in Figure 11 [9,39].

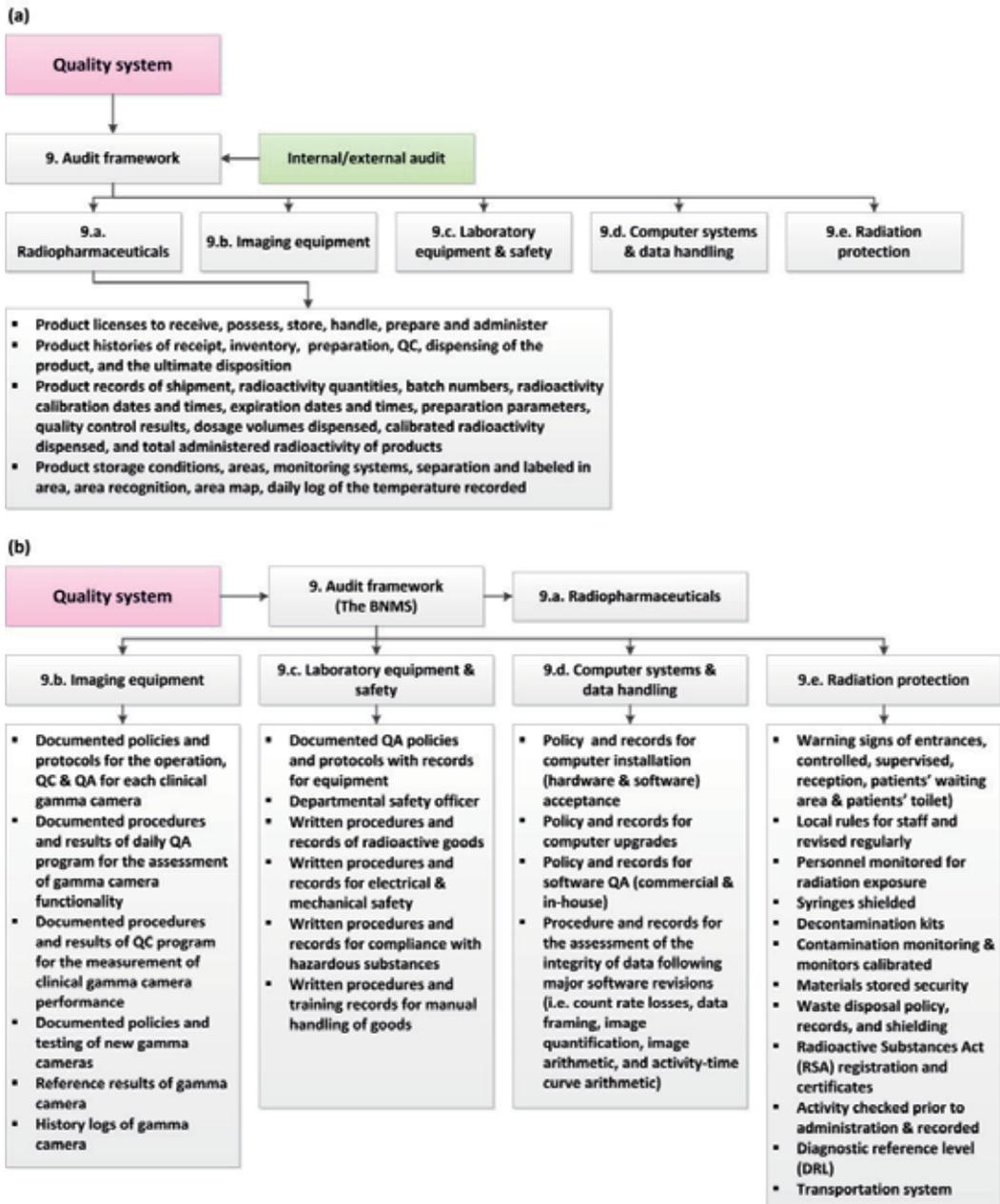


Figure 11. Quality system of audit framework. Audit (a) for radiopharmaceuticals manufacturing, and (b) for imaging equipment, laboratory equipment, safety, computer systems, data handling, and radiation protection. [9,39]

3. Quality evaluation and sources of uncertainty

3.1. Radiopharmaceuticals

3.1.1. Standardization: principle and applications

Quality control for the quantification of radiopharmaceutical activity is critical for accurate dosimetry calculations, from whole body to cell microscopy. Tumor uptake of radiopharmaceutical need to be correlated with tumor response and to be related to the tumor radiation absorbed dose. [14]

Isotope	Major decay modes	Method for standardization	Detection efficiency (ϵ)	Activity accuracy & uncertainty (U)	Ref.
-	β , γ , β - γ , EC, EC- γ , α , and mixed decay nuclides	LSC and C/N method	$\epsilon_{\text{pure } \gamma}$: $\sim 100\%$ $\epsilon_{\text{pure EC}}$: $< 75\%$ $\epsilon_{\text{EC-}\gamma}$: $\sim 100\%$ ϵ_{α} : $\sim 100\%$	U: 0.2-0.5% (pure β) U: 0.2-0.5% (β - γ)	51
Co-57	EC decay to Fe-57	$4\pi\beta$ - γ coincidence method	ϵ : $\sim 75\%$	U: 2%	50
Ge-68/Ga-68		γ spectrometer	To ground state: β^+ (87.85%), EC (8.92%)	U: 1%	52
1. Ge-68	EC decay to Zn-68	x -ray spectrometer	To 1077 keV: β^+ (1.29%), EC (1.93%)		
2. Ga-68	β^+ , EC, γ decay to Zn-68	LSC Calibrated IC	Annihilation radiation: 178.29% γ -ray to 1077 keV: 3.22%		
Sn-117m- DTPA	Decay to Sn-117	$4\pi\beta$ LS and $4\pi\gamma$ methods		U_C : 0.60% (for DTPA by LS) U_C : 2.43% (for DTPA by NaI(Tl))	17
Cs-131	EC decay to Xe-131	Coincidence methods: L Auger electrons plus L X-rays and K X-rays	-	U: 1%	53
Cs-134	β^- and γ decay to Ba-134	LSC	ϵ : $\sim 95\%$	3191 ± 8 kBq/g (0.54%)	54

		4πβ-γ coincidence method	ε: 65-87%	3194 ± 12 kBq/g (0.88%)	
		4πγ method	ε: ~83%	3174 ± 25 kBq/g (2.09%)	
		C/N method			
TI-201	γ decays to Hg-201	High-pressure IC 4πγ coincidence method	γ ray (167.4 keV) probability: 0.1000 ± 0.0006	7.207 ± 0.033 (NIST) 7.197 ± 0.027 (NPL) 7.116 ± 0.050 (PTB)	55
TI-204	β ⁻ decay (97.4%) to Pb-204 and EC decay (2.6%) to Hg-204	Windowless 4π-CsI(Tl) -sandwich spectrometer, LSC, PPC	4πβ-γ coincidence method and Cs-134 _{EC} (~ ε _{AEC}): 50 - 100%	ε _β : 71 - 91% U _C : 0.76%	56
Pb-210	β decay to Bi-210 (t _{1/2} 5.103 d), Po-210 (t _{1/2} 138.4 d), and α decay to Pb-206	4πβ-γ coincidence method Germanium γ spectrometry		U _C : 2.7%	58

Table 2. Some examples of absolute standardization of radiopharmaceuticals and related radioisotopes [17,50-58]

The theoretical counting efficiency, i.e. counts/disintegration or counts per minute/disintegration per minute (cpm/dpm), for a radionuclide can be used to examine the absolute activity, in disintegration or disintegration per minute (dpm) of the radionuclide. Different efficiency tracing methods has been developed for more than six decade by characterizing the effects of sample volume, medium composition (matrix), pulse discrimination conditions, photomultiplier voltage, amplifier gain, and luminophor concentration on counting efficiency of a radioactive species [49]. The use of 4πβ scintillation counting and 4πβ-γ coincidence counting for the standardization of certain electron capture (EC) nuclides with simple decay schemes is established since 1952 [49] and 1957 [50].

Some examples of absolute standardization of radiopharmaceuticals and related radioisotopes are shown in Table 2 [17,50-58]. Below, we introduce different tracing methods, including (a) efficiency tracing (and extrapolation) method using a non-H-3 standard solution, (b) CIEMAT-NIST (C/N) efficiency tracing method, (c) non-extrapolation tracer method, (d) coincidence method by a 4πβ-γ system, (e) triple to double coincidence ratio (TDCR) method, and (f) 4πγ counting method.

(a) The efficiency tracing (and extrapolation) method using a non-H-3 standard solution

The efficiency tracer techniques, using Co-60, Cs-134, C-14, Cr-51, Mn-54 or Am-241 standard solution for the standardization of the β - γ nuclides were developed. The 4π liquid scintillation (LS) consisted of the extrapolation of the 4π counting rate to the zero discrimination level for the standardization of the Tl-204 (97.6% β emission and 2.4% electron capture) solution was carried out for efficiency tracing using a Co-60 standard solution received in the framework of the 1997 BIPM comparison was carried out by Sahagia et al. [59]. A germanium spectrometer was calibrated for the standardization of Pb-210 using Am-241 as a normalizing agent has been proposed [58]. Instead, Dias et al. chose Cs-134 as an efficiency tracer to standardize Tl-204 as well as a $4\pi\beta$ - γ coincidence system for the calibration [57]. This method can be also successfully used for the standardization of radionuclides such as Ir-192, Zn-65, Mn-54, with the detection of the β rays, Auger electrons, X rays, in the proportional counter (PC) [60]. Efficiency tracing with C-14 and zero detection threshold techniques with H-3 as tracers was applied for standardization of various β -emitting radionuclides, e.g. C-14, Cl-36, and Tl-204 using LS spectrometer [61].

Recently, different methodologies were proposed. Koskinas et al. developed a “dual-tracers”, e.g. Cr-51 and Mn-54 procedure followed by the Laboratório de Metrologia Nuclear (LMN) for the standardization of EC nuclide, i.e. Fe-55. The efficiency was obtained by selecting a γ -ray window set at 320 keV (Cr-51) and at 834 keV (Mn-54) [62]. The activity of EC radionuclides is usually determined by 4π (proportional counter, PC)- γ coincidence counting and by an efficiency extrapolation method. However, an alternative method, called “wet extrapolation method”, utilizes an absorption change during the drying of a water droplet added onto the source surface, variation of the PC detection efficiency can be achieved. Slopes of extrapolation curves and resulting activity values obtained are compared for several radionuclides (Mn-54, Ce-139, Y-88, and Co-57) [63].

(b) The CIEMAT-NIST (C/N) efficiency tracing method

CIEMAT/NIST (C/N) method, developed by Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT), Spain and the National Institute of Standards and Technology (NIST), U.S. is used for standardization of radionuclides with Liquid Scintillation (LS) Spectrometry by calculating the counting efficiency of the radionuclide to be assayed and using H-3 as a tracer [61]. C/N program is suitable used for the calculation of the efficiency of nuclides decayed by β , β - γ , EC, EC- γ and nuclides with mixed decay [51]. The basic principle of C/N LS efficiency tracing method is a combination of a theoretical calculation of the counting efficiency and an experimental determination of correction factors in three steps [61,64]:

- Count rates (cpm) and the quench-indicating parameters (QIPs, i.e. tSIE) are determined for a set of samples of the nuclide to be measured, and for a set of H-3 standard samples, with a different quench. The tSIE values were calculated using the Ba-133 source inside of the instrument. By combining these data, a corresponding H-3 efficiency is obtained for each sample of the nuclide.
- The universal curve of Figure of Merit (FOM) as a function of tSIE was plotted. The efficiency of the nuclide is theoretically calculated as a function of the efficiency of the tracer nuclide H-3.

- This relation is used in conjunction with the measured data to calculate the efficiency for the nuclide and an activity value in dpm for each single measurement.

The parameters of emitters in different decay modes used for the C/N calculations are summarized as follows [51]:

- Pure β emitters (Sr-89, Sr-90, Y-90, and K-40): atomic number Z of the radionuclide, the mass number A, the endpoint energy E_{Max} , and the shape parameters.
- Pure γ emitters (Nb-93m): the efficiency is nearly 100%.
- $\beta+\gamma$ emitters, if the radionuclide has significant levels with half-lives in the order of the coincidence resolving time or the dead time of the equipment, a C/N calculation is not possible.
- Pure EC emitters: the input parameters are the capture probabilities, PK; PL; PM, and the atomic parameters for the rearrangement: the fluorescence yields ω_K and ω_L (averaged), the probabilities of the X-rays (PKL, PKX, and PLX) and their average energies (EKL, EKX, and ELX), the emission probabilities of the Auger electrons (PKLL, PKLX, PKXY, and PLXY) and their average energies (EKLL, EKLX, EKXY, and ELXY).
- EC+ γ emitters (Co-57, Se-75, Sr-85, and Ba-133): the calculation method is the same as for $\beta+\gamma$ nuclides.
- The efficiency of LSC systems with respect to alpha radiation is in each case very close to unity. A tracer method is not necessary.

(c) The non-extrapolation tracer method

An alternative called “non-extrapolation tracer method” was proposed by Steyn et al. in 1979, where Fe-55 was used as a tracer to establish the figure-of-merit (FOM) of the detection system for the calculation of counting efficiency [65]. The liquid scintillation method, for the determination of absolute activity of Mn-54 and Zn-65 from $4\pi(LS)e-\gamma$ data by direct calculation without efficiency extrapolation was performed. The non-extrapolation LS method relies on determining the probability of the γ -ray interacting with the scintillator solution, is described and validated by measurements made on Co-60 [66].

(d) The coincidence method by a $4\pi\beta-\gamma$ system

Coincidence method comes from the additional coincidence channel, which records a disintegration event when it is detected in both β - and γ -channels. Typically, the system for absolute standardization is usually consisted of a gas-flow or pressurized proportional counter with 4π geometry as the α , β , electrons or X-ray detector and coupled to a pair of NaI(Tl) scintillation counters or a semiconductor detector, as γ detectors. The $4\pi\beta-\gamma$ coincidence technique has been considered a primary standardization method due to its high accuracy and because it can obtain the radionuclide activity depending only on observables quantities [57,67].

Alternatively, solid or liquid scintillation counters (LSC) are used in place of gas-flow proportional counters. Advantages of using LSC counting in the 4π channel are that self-absorption does not occur, leading to Auger electrons being detected with relatively high

efficiency; source preparation is easy; and the source geometry is highly reproducible. The latter leads to good reproducibility of the counting efficiency of the X-rays and Auger electrons, which in turn gives rise to consistent results amongst the counting sources. The efficiency data can generally be fitted with a linear function, particularly in the high-efficiency region, or by a low-order polynomial expression, giving rise to reliable extrapolated activity values [68].

Several examples for the applications of the coincidence method by a $4\pi\beta\text{-}\gamma$ system are such as standardization of Ho-166m using the normal gas flow $4\pi\beta\text{-}\gamma$ coincidence method [69], standardization of Tl-204 using Cs-134 as tracer and a $4\pi\beta\text{-}\gamma$ coincidence system was used for the calibration [57], directly measured of radionuclides with EC decay schemes, e.g. I-125, Ir-192, Zn-65, and Ce-139 by a LS coincidence extrapolation technique [68], and standardization of Fe-55 using a “dual-tracers” method coupled with a $4\pi\beta\text{-}\gamma$ coincidence calibration system [62].

(e) The triple to double coincidence ratio (TDCR) method

The TDCR method was first developed at the R.C., Poland and at the LNHB, France. The equipment consists in a detection unit, provided with three photomultipliers (PMs), acted by the light emitted in the vial containing the radioactive solution dissolved in a liquid scintillator, and the electronic unit [60]. TDCR, allowing the observation of three kind of double coincidences (2-photodetectors) and triple coincidence (3-photodetectors) method in LSC, is a fundamental measurement method suitable to the standardization of pure-beta emitters, i.e. H-3, C-14, P-32, Ni-63, Tc-99, Tl-204 and some low energy electron-capture emitters, i.e. Fe-55 [59,60,70,71]. Detection efficiency variation can be achieved using techniques of chemical quenching, coaxial grey filters and PM tubes defocusing. The two former processes reduce the mean quantity of light emitted and the later reduces the detection probability [71]. Basically, the specific experimental parameter (K) is equal to the ratio of the triple coincidences counting rate (N_T) to the sum of double coincidences counting rate (N_D). Determination of a counting efficiency (ε_D) for each counting point (N_D) leads to the activity of the source (N_0). The efficiency functions ε_T and ε_D are nonlinear functions for a particular emitter and counting system [70].

Two innovative TDCR instrumentations were developed:

- The TDCR method of LSC is well established for measuring the activity of pure beta emitting and electron capture radionuclides. Recently, a new TDCR counting system was designed by the National Physical Laboratory (NPL) for activity assays of low-energy, pure β -emitting radionuclides and EC nuclides. Three photomultiplier tubes (PMT) were arranged in the optical chamber as well as a NaI(Tl) detector was mounted below the optical chamber. The detector allows $4\pi\beta\text{-}\gamma$ coincidence measurements to be performed in parallel [72].
- Radionuclides such as P-32, Sr-89, Y-90, Tl-204, and Rh-106 were successfully studied using an in-house built new TDCR-Čerenkov counter developed by Kossert. Since Čerenkov counting acts as natural discrimination for α emitters and low-energy β emitters, some potential radioactive impurities or progenies will not disturb the measurements. Two standard sources, e.g. Cl-36 and P-32 were used to determine the free parameter and to

calculate the Čerenkov counting efficiencies. Since Čerenkov counting is more sensitive to changes in the computed β spectra, the method was extensively used to investigate β shape factor functions [73].

(f) The $4\pi\gamma$ counting method.

An ionization chamber system referring to a long living and stable standard source is very adequate for the comparison of γ -ray emitting radio-nuclides. In most cases Ra-226 sealed sources have been used as the reference because the Ra-226 sources were widely used in radiotherapy [69]. Zimmerman et al. standardized and compared solution of Sn-117m by $4\pi\beta$ liquid scintillation (LS) spectrometry and $4\pi\gamma$ γ -ray spectrometry (NaI(Tl) and high-purity germanium detectors). Massic activities were measured for determining the dose calibrator factor settings [17].

3.1.2. Uncertainty of measurement

Examples for the evaluation of detection efficiency (ϵ), activity accuracy, and measurement uncertainty (U) of absolute activity of radiopharmaceuticals and related radioisotopes are shown in Table 2. Components of combined uncertainty were further summarized in this section.

(a) Uncertainty for the efficiency tracing (and extrapolation) method using a non-H-3 standard solution

Components of combined uncertainty in the activity determination include counting statistics, background, dead time, weighing, decay scheme parameter, half-life, and extrapolation of efficiency curve [57]. Source of uncertainty evaluated by Woods et al. in the absolute standardization of low energy β emitter, i.e. Pb-210 are counting, background, half life, β dead time, γ dead time, resolving times, choice of fit, count rate dependence, dead time formula, weighing, separation time, extrapolation range, contaminants, and reproducibility [58].

(b) Uncertainty for the C/N efficiency tracing method

Component of uncertainty in the standardization of Re-186 by the C/N method of LS efficiency tracing with H-3 include source preparation, scintillator stability, dead time, liquid-scintillation measurements, uncertainty due to H-3 reference standard, EC/ β^- branching ratio, spectral distributions for EC and β^- branches. [74]

The contributions to the uncertainty of the value of the specific activity are volatility of H₂ [GeCl₆] during the preparation of solid sources for coincidence measurements, drop masses, counting statistics, background variation, accidental coincidences and dead time losses, Compton continuum of the 1077 keV peak included in the γ window around the 511 keV peak, decay scheme correction factor, correction factor for non-vanishing ϵ EC, impurities and half-life uncertainty, and detection of 511 keV quanta in the β detector due to its γ sensitivity [52].

The components contributing to the uncertainty of $4\pi\beta$ - γ coincidence method were estimated as follows: counting statistics and background variation, instrumental corrections, impurities, half-life uncertainty, decay scheme correction factor, and mass of droplet. Standard

deviation of LSC composed of the following contributions: counting statistics, background variation, scintillator stability, comparison with H-3 tracer, instrumental corrections (dead time), dilution factor, droplet mass, radioactive impurities, half-life uncertainty, main decay data, uncertainty of the ϵ calculation due to the K-L model, capture probabilities P_K , P_L , fluorescence yields, ω_K , ω_L , spectral distribution of β particles, and average energy of weak Auger electrons [75].

Source of the uncertainty: counting statistics, mass, dead time, background, timing, chemical effects (adsorption, sample spread, impurities), input parameters and statistical model, quenching, kB influence, decay scheme parameters, and pulse shape discriminator setting. [76]

(c) Uncertainty for the non-extrapolation tracer method

The quoted total uncertainty (1σ) of 0.85% comprised mainly the components due to counting statistics (0.28%), afterpulsing (0.40%) and the evaluated decay-scheme data (0.63%). ϵ_M : double tube detection efficiency of Mn-54, ϵ_M^* : reduced Mn-54 efficiency due to quenching caused by the addition of the Fe-55 aliquot [65].

(d) Uncertainty for the coincidence method by a $4\pi\beta\text{-}\gamma$ system

Uncertainty components assayed by Koskinas et al. for the standardization of Eu-152 were counting statistics, weighing, dead time, impurities, half life, extrapolation of efficiency curve [77].

(e) Uncertainty for the TDCR method

The main source of uncertainty of TDCR method comes from the model describing the non-linearity of the scintillator due to the ionization quenching phenomenon [71]. Type A standard uncertainty, i.e. counting statistics and type B standard uncertainty, i.e. extrapolation (interception uncertainty), spurious pulses, nonuniformity of sources, tracer activity, E. C. correction, dead-time, background, half-life, weighing were evaluated by Sahagia et al. [59].

(f) Uncertainty for the $4\pi\gamma$ counting method.

Construction of an ionization chamber efficiency curve is not a straightforward process as the curve has to be extracted from experimental calibration points analytically. The efficiency curve is implicitly contained in individual radionuclide coefficients and these are obtained experimentally or by Monte Carlo modelling or calculated back from the efficiency curve. Due to this variety, the interpretation and intercomparison of different efficiency curves is often hard and transferring individual radionuclide calibration coefficients between ionization chambers of different constructions is not a simple process [78].

3.1.3. International measurement program

One of the most important components in the quality system of radiopharmaceuticals is to establish the measurement traceability to international standards for ensuring the accurate and consistent of measurement results [5]. Traceability of activity measurements is the critical part in the production and use of unsealed radioactive sources in nuclear medicine. The U.S. Nuclear Regulatory Commission (NRC) defines a medical event as a patient receiving

an injected activity greater than 20% different from the prescribed dosage. The Society of Nuclear Medicine (SNM) guidelines also recommend that the measurement be within 10% of the prescribed dosage. Moreover, the instruments being used are capable of accurate measurements to within 5% [79]. Therefore, programs for the establishment and dissemination of activity measurement standards in nuclear medicine are held in many countries.

International comparison of standard sources and solutions, such as P-32, Mn-54, Zn-65, Ir-192, Tl-204, and Am-241, which is organized by the International Committee of Weights and Measures (CIPM), the EUROMET system, the former COMECOM, and bilateral comparisons, has been held since 1962 [60].

South Africa's national radioactivity measurement standard is maintained by the National Metrology Laboratory (NML) of the Council for Scientific and Industrial Research (CSIR). Standardizations are undertaken by a number of direct methods utilizing liquid scintillation counting (LSC) [80].

Comparisons of activity measurements for I-131, Tl-201 and Tc-99m with radionuclide calibrators were organized in Cuba since 2002. During 2002, the Radionuclide Metrology Department of the Isotope Center (CENTIS-DMR) has organized several comparisons with various radionuclides in order to obtain information on the quality of the activity measurements during production and administration of radiopharmaceuticals in Cuba [81].

The Australian Radiation Protection and Nuclear Safety Agency (ARPANSA) conducts a series of Radiopharmaceutical Quality Assurance Test Program under a Memorandum of Understanding (MOU) between ARPANSA and the Therapeutic Goods Administration (TGA). For example, in 2005, 46 batches of 24 different types of radiopharmaceuticals, e.g., ready to use radiopharmaceuticals and kits for the preparation of Tc-99m were tested. Two percent in 46 batches of radiopharmaceuticals tested was failure to meet full specifications [82].

International comparison program of national metrological institutes for the standardization of Fe-55, which is a suitable radionuclide standard for X-ray spectrometers, was held by the Comité Consultative pour les Etalons de Mesures des Rayonnements Ionisants (CCEMRI) of the Bureau International des Poids et Mesures (BIPM) [62]. National Metrology Institute of Japan - Advanced Industrial Science and Technology (NMIJ/AIST, Japan) and National Institute of Ionizing Radiation Metrology (ENEA-INMRI, Italy) have been involved in recent years, particularly those relevant in the frame of the international cooperation coordinated by the BIPM and the International Committee for Radionuclide Metrology (ICRM). Particular research activities are devoted on the field of the nuclear safety, nuclear medicine and environmental radionuclide measurements. [83]. International comparisons held by BIPM also can be traced by laboratories such as National Institute for Physics and Nuclear Engineering (Romania) [59], Laboratório de Metrologia Nuclear (Brazil) in collaboration with the Laboratório Nacional de Metrologia das Radiações Ionizantes, from Rio de Janeiro [57], Radiation Safety Systems Division, Bhabha Atomic Research Centre (India) [61], and Electro-technical Laboratory (ETL) (Japan) [69].

The Ce-139 measurements formed part of a regional comparison organized by the Asia Pacific Metrology Programme (APMP) [68].

The National Institute of Standards and Technology (NIST) maintains a program for the establishment and dissemination of activity measurement standards in nuclear medicine, i.e. Ga-67, Y-90, Tc-99m, Mo-99, In-111, I-125, I-131, and Tl-201 for more than ten years. These standards are disseminated through Standard Reference Materials (SRMs), Calibration Services, radionuclide calibrator settings, and the NIST Radioactivity Measurement Assurance Program (NRMAMP, formerly the NEI/NIST MAP). For over 3600 comparisons, 96% of the participants' results differed from that of NIST by less than 10%, with 98% being less than 20%. The percentage of participants results within 10% of NIST ranges from 88% to 98% [79].

Measurements from a variety of types of detectors including, ionization chambers, radionuclide calibrators, solid state detectors, Ge detectors, NaI(Tl) detectors, liquid scintillation counters (LSC), Cherenkov counting, and proportional counter are reported [79].

3.2. Nuclear medicine imaging

3.2.1. PET, CT, PET/CT, and SPECT imaging

PET, CT, PET/CT, and SPECT are non-invasive imaging tools and applied for creating two dimensional (2D) cross section images of three dimensional (3D) objects. PET and SPECT can potentially provide functional or biochemical information by measuring distribution and kinetics of radiolabelled molecules, whereas CT visualizes X-ray density in tissues in the body. The PET imaging in oncology has been migrating from the use of dedicated PET scanners to the use of PET/CT tomographs. This is due to the advantages that PET/CT offers over dedicated PET. One of these advantages is that the integration of PET and CT imaging into a single scanning session allows excellent fusion of the acquired data. Although these nuclear medicine imaging tools provide many advantages and applications in diagnosing diseases clinically, they also poses some challenges and induce artifacts and quantitative errors that can affect the image quality.

3.2.2. Risks of artifact in PET, CT, and SPECT imaging

Artifacts and pitfalls can arise at any stage in the process of nuclear medicine imaging and can be grouped into issues related to the (i) patient, (ii) the equipment, or the technologist.

(a) Patient-related risks:

In PET/CT, the patient-related artifacts commonly found are due to metallic implants, truncation, and respiratory motion (or patient motion). These artifacts occur because the CT scan is used to replace a PET transmission scan for the purpose of attenuation correction of the PET data.

Metallic implants, such as dental fillings, hip prosthetics, or chemotherapy ports, cause high CT numbers and generate streaking artifacts on CT images due to their high photon absorption [85,86]. This increase CT numbers causes correspondingly high PET attenuation coefficients, resulting in an overestimation of the PET activity and thereby to a false-positive PET finding.

In PET/CT, truncation artifacts occur due to the difference in size of the field of view between the CT (50 cm) and PET (70 cm) tomographs [87,88] and frequently seen in large patients or patients scanned with arms down, such as in the case of melanoma and head and neck indications. When a patient extends beyond the CT field of view, the extended part of the anatomy is truncated and consequently is not represented in the reconstructed CT image. Truncation also causes streaking artifacts at the edge of the CT image, leading to an overestimation of the attenuation coefficients used to correct the PET data. This increase in attenuation coefficients creates a rim of high activity at the truncation edge, resulting in the misinterpretation of the PET scan.

The most prevalent artifact in PET/CT imaging is respiratory motion during scanning. The artifact is due to the discrepancy between the chest position on the CT image and the chest position on the PET image. PET images are acquired over time periods (time frames) that can vary from a few seconds to tens of minutes. Therefore, during such time periods various motions may have significant effects on the PET images. Both respiratory and contraction induced heart motions have major effect (source of error) on PET imaging of cardiac and thoracic regions. Some equipment, e.g., dose calibrators for the measurements of quantitative measurements is calibrated against or traceable to a reference source of whole body tomographs [89]. Because of the long acquisition time of a PET scan, it is acquired while the patient is freely breathing. The final image is hence an average of many breathing cycles. On the other hand, a CT scan is usually acquired during a specific stage of the breathing cycle. This difference in respiratory motion between PET scans and CT scans results in breathing artifacts on PET/CT images. Several literatures have described this problem [90-91]. The artifacts resulted from respiratory motion or patient motion is also commonly found in myocardial perfusion SPECT. This is because that SPECT requires that the object of interest remains constant for the duration of the acquisition [92-93]. Visually detectable patient motion has been reported in 36% of clinical studies in one study [94] and 43% in another [95].

Source of clinical problems of the patients were also indicated by Hladik III, including (i) special patient populations, e.g., pregnant or breast-feeding women, pediatric and geriatric patients, patients requiring dialysis, incontinent, catheterized or miscellaneous patients, (ii) insufficient patient care, education, and preparation, e.g. insufficient patient instruction, shielding or protection in exposure and contamination problems, pregnancy testing, withholding xanthine-containing foods and drug-drug interaction prior to imaging, delay in the administration or imaging, metal implants of patient, (iii) improper behavior of patient, e.g., excessive movement, contamination from incontinence, attenuation from jewelry, prostheses, or implants, etc., and (iv) unexpected altered biodistributions may be undetectable, adverse reactions or untoward effects, [96]

(b) Equipment- or technologist-related risks:

There are several patient-related artifacts and interpretation pitfalls that can potentially compromise nuclear medicine imaging, as discussed above. In order to minimize or identify these artifacts, technologists play an important role in recognizing and correcting them. For example, technologists should ask patients to remove all metallic objects before imaging and should document the location of non-removable metallic objects to minimize or identify the

artifacts from metallic implants. In PET/CT imaging, it is crucial for technologists to carefully position patients at the center of the field of view and with arms above head to reduce truncation artifacts. Moreover, in order to minimize the artifacts from respiratory motion and produce accurately quantifiable images, it is also essential that technologists instruct patients about breath-hold techniques before the scanning session.

Moreover, sources of clinical problems of error medication also include fail of (i) patient identification, (ii) dosage prescription and administration, (iii) radionuclide administration, (iv) radiopharmaceutical prescription and administration in kinetics or finished product purity testing, (v) interventional medications, (vi) injection technique, (vii) radiopharmaceutical labelled, (viii) preparation or execution of diagnostic or therapeutic procedure, and (ix) radiation protection [7,96].

QC performed on nuclear medicine cameras provides the confidence to technologists and physicians that a scan supplies an accurate representation of the radioisotope distribution in the patient. The instrumentation for nuclear medicine imaging is more complex than that used for whole-body and planar imaging, and requires careful quality control to ensure optimum performance. According to the standards, the main performance parameters are divided into two groups. The first group includes basic intrinsic measurements: spatial resolution in axial and transaxial directions, sensitivity, count rate capabilities by measuring the system dead time and the generation of random events at different radioactivity levels, and scatter fraction of γ rays emitted by the annihilation of positron. The second group includes measurements of the accuracy of corrections for physical effects, specifically: uniformity correction, scatter correction, attenuation correction, and count rate linearity correction. Other possible tests to be added to the list of acceptance or performance tests such as: noise equivalent count rate, partial volume and spillover, motion artefacts, image quality test, and PET/CT image co-registration [89].

Nuclear medicine imaging increases the accuracy of diagnosis by combining anatomic information with functional imaging. It is highly dependent on a host of technical considerations. Knowledgeable technologists can minimize or reduce artifacts and other potential problems with image acquisition and, in that way, produce better-quality images.

4. Conclusion

Implement of ICH QbD for the radiopharmaceutical manufacturing and imaging technology can be harmonized to a globalized framework in accordance with the regulations and requirements of U.S. FDA, IAEA, WHO and EANM. The attributes of the components in the quality unit (QA/QC), including the aspects of organization, staffing and personnel, facilities, instrumentation and equipment, operation procedure, radiopharmaceuticals, protocol and conduct of a study or a treatment, records and reports, and audit were reviewed and indentified. Critical quality attributes (CQAs) for assuring accurate radioactive dosimetry calculation in the efficiency tracing of absolute activity measurement and in the patient- and technologist-related risks for nuclear medicine imaging (PET, CT, and SPECT), i.e. potential

sources of error or uncertainty, were elucidated. Although there still have many hard-to-controlled quantitative errors and artifacts that can eventually affect the quality of imaging, therapeutic efficacy, or safety, it is important for the facility staffs to be aware and continual improvement of these quality factors. By reducing uncertainty and risk or increasing process knowledge and product understanding resulting from QbD can significantly improve the efficiency of manufacturing processes.

5. Abbreviations

API Active pharmaceutical ingredient

ARPANSA Australian Radiation Protection and Nuclear Safety Agency

BNMS British Nuclear Medicine Society

BIPM Bureau International des Poids et Mesures (France)

CT Computed Tomography

CFR Code of Federal Regulations (U.S.)

CMC Chemistry, and manufacturing and controls

CQAs Critical quality attributes

CSIR Council for Scientific and Industrial Research

CGMP Current Good Manufacturing Practice

CGRPP Current Good Radiopharmacy Practice (EU)

EC Ethical Committee (EU)

EANM European Association of Nuclear Medicine (EU)

EDQM European Directorate for the Quality of Medicines & HealthCare

EIND Exploratory IND (FDA, U.S.)

FDA U.S. Food and Drug Administration

HPLC High-performance liquid chromatography

ICH International Conference on Harmonisation

IMP Investigational Medicinal Product (for drugs used in clinical trials of EU)

IND Investigational new drug

IAEA International Atomic Energy Agency

IRDS Investigational Radiopharmaceutical Drug Service

LSC Liquid scintillation counting

MA Marketing authorization (EU)

MS Mass spectrometry

MOU Memorandum of Understanding

NRC Nuclear Regulatory Commission

NCA National Competent Authority (EU)

PET Positron emission tomography

QA Quality assurance

QC Quality control

QP Qualified persons who are professional responsible for the release of a drug in Europe

QbD Quality by design

RPR Responsible person for the small-scale preparation of radiopharmaceuticals

RDRC Radioactive Drug Research Committee (FDA, U.S.)

SPECT Single photon emission computed tomography

SSRP Small-scale "in-house" radiopharmaceutical

SOP Standard operating procedure

TGA Therapeutic Goods Administration

TLC Thin layer chromatography

USP United States Pharmacopeia

WHO World Health Organization

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Unified Procedures for Quality Controls in Analogue and Digital Mammography

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Additional information is available at the end of the chapter

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1. Introduction

Breast cancer is the most commonly diagnosed cancer in women [1]. Current attempts to control breast cancer concentrate on early detection by means of massive screening campaign, via periodic mammography and physical examination, because ample evidence indicates that such screening indeed can be effective in lowering the death rate [2]. Early diagnosis of breast cancer plays a leading role in reducing the mortality and improving the prognosis of this disease [3].

Mammography consists in imaging the female breast using X-rays with low contrast (to keep the delivered dose low), but at the same time high resolution (especially used for early detection).

The goal of mammography is to achieve the image quality required for a given detection task, while ensuring that the patient-absorbed dose is kept as low as reasonably achievable [4]. As practised now, it normally requires a dedicated X-ray tube with special anode materials such as molybdenum or rhodium, small focal spots, operating at a tube voltage around 25 to 32 kV, and carefully chosen films and screens in dedicated cassettes. Stationary or moving grids are used as in other branches of plain film radiography. Present-day mammography can be described as a low-dose procedure [5]. In recent years, advances in screen-film technology and film-processing techniques have contributed to major improvements in the quality of mammographic images. At present, two distinct mammographic techniques exist:

- Analogue mammography in which the image is recorded on a film.

- Digital mammography in which the image is digitalised.

The production of analogue or digital mammography images is based on two distinct concepts of image formation [6].

The analogue image is a continuous representation of spatial and intensity variations of the X-ray pattern transmitted by the tissue under analysis. Traditionally, the mammographic image is analogue, obtained using conventional screen-film image receptors as the standard detector [7]. The advantages of screen-film mammography are: high spatial resolution and low contrast sensitivity achieved through improvements in X-ray tube design, screen-film combinations, grids, and film processing [8]. Thus, analogue mammography permits high image quality, low patient dose, and most importantly, the ability to detect small, nonpalpable breast cancers.

In digital systems, image acquisition and display are two independent processes [4]. In such systems images are captured as a digital signal, making electronic transfer and storage of images possible. Digital systems offer a large dynamic range of operation, improving visualization of all areas of the breast and increasing exposure latitude. Also, the digital format allows grayscale adjustment to optimize contrast for any imaging task.

In addition, with the digitalization of the diagnostic image, new medical applications have now emerged, such as Computer-Aided Diagnosis (CAD), stereo mammography, tomosynthesis, contrast medium imaging and dual energy imaging [7].

For a successful screening function the mammograms should contain sufficient diagnostic information to be able to detect breast cancer, using a radiation dose as low as reasonably achievable (ALARA principle). In this context, it is necessary to establish and actively maintain regular and adequate Quality Assurance (QA) procedures that take into account medical, organisational and technical aspects. The QA procedure should include periodic tests to ensure accurate target and critical structure localization. Such tests are referred to as Quality Controls (QC). They are fundamental for the QA procedure because they help ascertain that the equipment performs consistently at a high quality level.

However, whilst the requirement for standardisation is impelling, the Italian legislation (D.L.vo 187/00) is not keeping pace with the advances in mammographic technology. Indeed, at present both analogue and digital formats are used in an un-regulated way, without introducing a proper regulation especially for digital mammography. As a consequence, the QA protocols have been adapted ad hoc to the new digital technology, thus resulting in multiple protocols, some of which valid only for specific machines, resulting in high costs of operation.

On the other hand, at the European level, QA procedures for both analogue and digital mammography systems have been properly addressed and defined, [European guidelines for quality assurance in mammography screening – 4th Edition, Section 2]. In both cases, in fact, the QC of the physical and technical aspects must guarantee the best possible diagnostic information obtainable and image quality stability, within the limits imposed by the ALARA principle.

However, for the case of digital systems the imaging chain can be divided into three independent parts, as cited in [9]:

- a. Image acquisition, including X-ray generation system, image receptor and (in some systems) image receptor corrections;
- b. Image processing software;
- c. Image presentation, including monitor, imaging presentation software, printer and viewing box.

To produce images with adequate quality, each part of the imaging chain must function within the limits dictated by the standards of screen-film mammography [9], although the definition of such limits for digital systems is still in progress.

In the EUREF protocol it is assumed that digital mammography should perform at least as screen-film mammography.

In this context, a unified protocol is proposed here that can be used with either analogue or digital mammography systems, with the view of reducing the volume of verification procedures to test the operation of such equipment. The advantage of the proposed protocol is that it can be applied as is to both analogue and digital mammography. The results obtained from the application of this protocol to analogue and digital mammography are presented in Section 3, with particular emphasis on image quality. The remaining part of this Section is dedicated to a review of mammographic techniques.

1.1. Screen-film mammography

In screen-film mammography, the film is used as the medium for both image acquisition and display. However, whilst providing excellent spatial resolution in high contrast structures, screen-film mammography has limited detection capability for low-contrast lesions in dense breasts [10]. On phantoms, the highest spatial resolution can be as high as 15–20 lp/mm but with a very low associated contrast. In addition, noise can limit the reliability of detection, especially for the small or subtle structures [11]. Although considerable advances in film-screen mammography have occurred over the past 20 years, some inherent limitations to further technical improvement exist [12]. One such limitation results from the trade-off between dynamic range (latitude) and contrast resolution (gradient) [13]. The relationship between X-ray exposure, image density, and contrast is illustrated by the Hurter and Driffield (H&D) sigmoid curve (Fig. 1) which uniquely characterises a given type of screen-film system under specific conditions [14].

Because of the sigmoid shape of the characteristic curve, the range of X-ray exposures over which the film display gradient is significant, i.e., the image latitude, is limited. The parts of the H&D curve where the slope is flat indicate poor contrast (i.e. over- or under-exposed images) [12, 16].

In screen-film mammography, the automatic exposure control (AEC) has the critical role of ensuring that the appropriate amount of radiation reaches the image receptor to produce a target optical density on the processed film [16]. In AEC systems, an ion chamber or other radiation detector is placed beneath the film cassette and connected electrically to the exposure time control circuit. When a pre-set amount of radiation has been detected, the expo-

sure is automatically terminated. Other limitations of film-screen mammography include (a) noise caused by the random fluctuation of X-ray quantum absorption by the fluorescent screen and the film emulsion, which can limit the detection of subtle structures, (b) the trade-off between spatial resolution and detection efficiency of the film and screen, and (c) the inefficiency of rejection of scatter radiation by the mammographic grid [12].

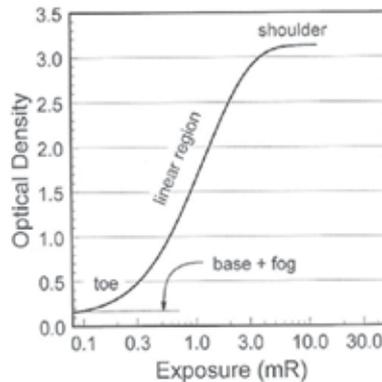


Figure 1. The Hurted & Driffield (H&D) curve describes optical density (OD) vs. the logarithm (base 10) of exposure [15].

1.2. Digital mammography

Digital mammography is an emerging technology, first approved in January 2000 [17], in which the image acquisition, display and storage functions can be performed independently, allowing for optimisation of each function. It offers several potential advantages including wider dynamic range, improved contrast, increased signal to noise ratio for overcoming the limitations of the film–screen combination (limited latitude, limited display contrast, low detection efficiency and noise), and therefore, increasing the sensitivity and specificity of breast cancer detection [18, 19]. Moreover, digital images offer a variety of new and improved applications. The digital image will provide image archiving and retrieval advantages over film, and will facilitate the use of computer-aided diagnosis [11, 20]. Other advanced applications made possible through digital imaging, such as dual energy and 3D tomosynthesis are expected to further improve diagnostic sensitivity and specificity.

In particular, Full Field Digital Mammography (FFDM) offers the promise of revolutionizing the practice of mammography through its superior dose and contrast performance [20]. In FFDM the screen-film is substituted by a fixed or removable digital detector. The digital image is obtained by sampling the X-ray pattern at discrete increments of spatial position and image signal intensity. Any digital image is a 2-dimensional grid of picture elements (pixels), which is defined by its size and bit depth. The size of an image is given by the length by width (in pixels) product. The bit depth is the number of shades of gray that can be displayed [1].

In a digital imager a detector absorbs the X-rays and produces an electronic signal at each pixel. The signal is then translated into a digital value by an analog-to-digital converter

(ADC). Once the digital image is stored in the computer memory it can be displayed with contrast independent of the detector properties [12].

Digital mammography systems, unlike screen-film mammography systems, allow manipulation of fine differences in image contrast by means of image processing algorithms [10]. The physical properties of the digital image (contrast, resolution and noise) can vary noticeably according to the detection technology used. There are two methods of image capture used in digital mammography that represent different generations of technology: indirect conversion and direct conversion [20].

Indirect conversion digital detectors uses a two step process for X-rays detection, similar to screen-film [1].

Direct conversion should not be confused with “direct readout”, which is a capability of all electronic detectors.

Fully digital mammography (FDM) detectors are the final class of detectors. These detectors are sealed units that are permanently mounted to a mammography system. FDM detectors are electronic devices that directly capture X-ray images. In general, such devices require that a new mammography system be installed [8].

1.2.1. Photostimulable phosphors (Computed Radiography systems)

Computed Radiography (CR) is at this moment the most common digital radiography modality in radiology departments, in place of conventional screen film systems [21].

CR for mammography system employ as the X-ray absorber a storage photostimulable phosphor imaging plate (typically BaFBr:Eu⁺², where the atomic energy levels of the europium activator determine the characteristics of light emission), that replaces the traditional screen-film combination [22]. In this case, the removable detector or the Imaging Plate (IP) is inserted as a cassette in a conventional mammography unit. So, the IP can be used in a standard mammography machine without modification [7].

X-ray absorption mechanisms are identical to those of conventional phosphors. The peculiarity here is that the useful optical signal is not derived from the light that is emitted in prompt response to the incident radiation, but rather from the subsequent emission.

CR digital phosphor plates have shown promise in mammographic imaging because of the wide exposure latitude and linear response [23].

The potential advantages of this technology are the small detector-element size, the fact that the plates can be used also in conventional mammography units, the ease of having multiple plate sizes, and the relatively low cost. In addition the plates are reusable since they can be readily erased optically [8].

However scattering of the light within the phosphor causes the release of traps over a greater area of the image than the size of the incident laser beam. This results in loss of spatial resolution [24].

1.2.2. Optical detector

The detector consists of a phosphor screen, a charged coupled device (CCD) camera, and a fiberoptic taper to couple the light from the screen to the camera. It now represents the most widely used digital mammography technique for cassette-free imaging [8].

The imaging performance of these systems depends on a number of factors, including the characteristics of the phosphor screen, the choice of CCD and the method used to optically couple the phosphor to the CCD.

A CCD is an integrated circuit formed by depositing a series of electrodes, called 'gates' on a semiconductor substrate to form an array of metal-oxide-semiconductor (MOS) capacitors [22].

CCDs are particularly well suited to digital radiography because of their high spatial resolution capability, wide dynamic range and high degree of linearity with incident signal.

1.2.3. Flat panel

The active matrix flat panel technology is the most promising digital radiographic technique [25, 26].

The active matrix detector is based on large glass substrates on which imaging pixels are deposited.

This flat panel plate consists of a matrix of approximately 5 million photodiodes that form the readout for each image. The charge produced on the diode in response to light emitted from the phosphor surface is collected and digitized [1].

2. Quality Control (QC)

QCs are fundamental to guarantee that the radiological equipment performs consistently, with standard and constant physical and technical operational parameters.

The technological advances of the past ten years have revolutionised imaging techniques for diagnostics. As a consequence, QC procedures need to be updated to suit the new technologies and related protocols. This is particularly true for mammographic equipment, for which the physical parameters to be monitored to guarantee high-quality imaging are identified in specific documents.

The European Protocol for "Quality Control of the Physical and Technical Aspects of Mammography Screening" [9] gives guidance on physical, technical and dose measurements, and the periodicity of the corresponding tests to be performed as part of mammography screening programmes.

On the other hand, in the case of the Italian regulation, the relevant legislation (D.L.vo 187/00) was approved before the commercialisation of CR and digital mammography. Therefore, guidelines and procedures for CR and digital mammography are missing.

This shortcoming is particularly relevant in the case of mammography because it is well known that both image quality and breast dose depend on the equipment used and the radiographic technique employed.

For a complete and accurate estimate of image quality and delivered dose, the following components and system parameters should be monitored [9]:

- X-ray generation and exposure control system
- Bucky and image receptor
- Film processing (for screen-film systems)
- Image processing (for digital systems)
- System properties (including dose)
- Monitors and printers (for digital systems)
- Viewing conditions

		Screen-film mammography	Digital mammography
X-ray generation	X-ray source	Focal spot size	
		Source-to-image distance	
		Alignment of X-ray field/image receptor	
		Film/bucky edge	
		Radiation leakage	
		Output	
Tube voltage	Reproducibility		
	Accuracy		
	HVL		
AEC	Central opt. dens. control settings	Exposure control steps: central value	
	Opt. dens. control step	Exposure control steps: difference per step	
	Target opt. dens. control settings	-----	
	Short-term reproducibility	Short-term reproducibility	
	Long-term reproducibility	Long-term reproducibility	Variation in SNR

	Screen-film mammography	Digital mammography	
	Object thickness and tube voltage compensation	Object thickness and tube voltage compensation CNR per PMMA thickness	
	Adjustable range	-----	
	Spectra	-----	
	Correspondence between AEC sensors	-----	
Back-up timer and security cut-off			
Compression		Compression force	
		Maintain force for 1 minute	
		Compression force indicator	
		Compression plate alignment, symmetric	
Bucky and image receptor	Anti scatter grid	Grid system factor	
	Screen-film	Inter cassette sensitivity variation (mAs) -----	
		Inter cassette sensitivity variation (OD range) -----	
		Screen-film contact -----	
	Response function	-----	Linearity
		-----	Noise evaluation
	Missed tissue at chest wall side	-----	Variation in mean pixel value (on image)
	detector	-----	Variation in SNR (on image)
	homogeneity	-----	Variation in mean SNR (between images)
		-----	Variation in dose (between images)
	Detector element failure	-----	Number of defective dels
		-----	Position of defective dels
Uncorrected dels	-----	Number of uncorrected defective dels	
	-----	Position of uncorrected defective dels	

	Screen-film mammography	Digital mammography
Inter plate sensitivity variations	-----	Variation in SNR
	-----	Variation in dose
Dosimetry	Glandular dose per PMMA thickness	
Image quality	Threshold contrast visibility	
	Exposure time	
	Spatial resolution	MTF and NPS
	-----	Scanning time
	-----	Geometric distortion
	-----	Artifact evaluation
	-----	Ghost image factor

Table 1. Operational parameters relevant to analogue and digital mammographs [9].

Some of the above components are suitable only for analogue systems, others only for digital ones, and some are common to both systems although requiring dedicated QC procedures.

With reference to QCs for mammography, the EU legislation is subdivided in two parts: Section 2a for screen-film mammography, Section 2b for digital mammography. In both cases, several measurements should be undertaken by medical physicists. The components that are common to both analogue and digital mammographic systems are listed in Table 1 with corresponding operational parameters specific for the two cases. As expected, the methodology to be used for QC in the two different cases are substantially different particularly with respect to image quality monitoring.

For example, in the case of traditional, analogue mammography, spatial resolution and threshold contrast visibility can be used to uniquely characterise the image quality. On the other hand, in digital mammography image quality is assessed by monitoring the Modulation Transfer Function (MTF), Noise Power Spectrum (NPS) and Nyquist frequency. MTF represents the efficiency or fan imaging system in reproducing subject contrast at various spatial frequencies [7, 20, 27]. The Nyquist frequency, instead, indicates the maximum spatial resolution that can be visualized in an image. NPS provides information on noise at different spatial frequencies. In digital mammography, in fact, spatial resolution is obtained from MTF and Nyquist frequency.

The combination of MTF and NPS gives the Detective Quantum Efficiency (DQE), regarded as the best overall indicator of the image quality of digital radiographic systems. DQE is the efficiency with which a detector uses the incident photons to form an image [28]. Systems with higher DQE can produce higher quality images, at the same dose. Further, there are also other parameters that need to be monitored in digital techniques to defined the image quality. These are listed in Table 2.

Metric	Performance attribute
MTF	Resolution properties of the image/detector/system
NPS	Noise properties of the image/detector/system
DQE	SNR transfer properties of the detector
eDQE	SNR transfer properties of the system
Dark noise	Noise in the absence of signal
Uniformity	Signal uniformity in the absence of an object
Exposure Indicator	Accuracy of exposure indication by the system
Linearity	Exposure response behavior of the system
High-contrast resolution	Ability of the system to represent high-contrast patterns
Low-contrast resolution	Ability of the system to represent low-contrast patterns
Distortion	Geometrical accuracy of images
Artifact	Non-uniform artifactual features in the images
Ghosting	Appearance of shadows of prior images on subsequent images
Throughput	Speed by which a system can sequentially capture images
Normal exposure	Target exposure values for clinical use reflecting system speed

Table 2. List of parameters for digital image quality control [27].

The problem is to define a unified protocol that can be applied to any (analogue, CR, digital) type of mammographic system.

On the basis of procedures developed previously [29, 30], and to minimise problems arising from the use of different QC procedure to monitor different physical parameters for analogue and digital mammographs it is proposed here to monitor only parameters related to the beam at the output of the RX tube. The resulting QC procedure is then flexible and applicable universally to any type of mammograph.

The only additional pieces of equipment needed to execute the proposed QC is a phantom coupled to a solid-state exposure meter (PHAN-EX).

The phantom is a 4.5 cm thick block of PMMA, simulating a standard breast, including details simulating those of clinical interest (micro-calcification, tumoral mass, fibrous structures). This is coupled to an RX exposure meter composed of a photodiode and a digital counter, thus capable of measuring the exposure and the quality of the mammographic image [31]. The proposed protocol was tested on different (analogue and digital) mammographs, to assess its versatility and accuracy, independent of the physical characteristics of the mammographic system. Results on AEC tests obtained from the implementation of the proposed protocol implemented on analogue and digital mammographs, are presented and discussed in Section 3.

Acoustic and light signaling	<p>Acceptance test, status test and constancy test.</p> <p>The acoustic and light signals should function properly.</p> <p>Operating procedure: The test of acoustic and light signalling will be performed with the exposure.</p> <p>Frequency: Daily</p>
Security cut-off	<p>Acceptance test, status test and constancy test.</p> <p>The security cut-off should function properly.</p> <p>Operating procedure: To verify the correct functioning of the security cut-off produce an exposure with a high mAs value and report the measured dose. Then, produce a second exposure releasing the switch before the set time and report the new measured dose. This value had to be considerably smaller than the previous one.</p> <p>Frequency: Daily</p>
Source-to-image distance	<p>Acceptance test.</p> <p>Manufacturers specification, typical ≥ 600 mm.</p> <p>Operating procedure: if the focal spot is indicated, measure the distance between the focal spot indication mark on the tube housing and the top surface of the bucky. Add the distance between bucky surface and the top of the image receptor to the resulting value. Alternatively, calculate the source-to-image distance by the magnification of an object of known dimension.</p>
Long-term reproducibility	<p>Constancy test.</p> <p>Deviations from the reference value of exposures $\leq \pm 2\%$.</p> <p>Operating procedure: the long term reproducibility of the AEC system is calculated by determining the deviation of the exposures obtained from the phan-ex and from the reference value (45 mm PMMA test block), with the exposure meter accurately placed on the plate holder. The measured counts per second (cps) should be recorded.</p> <p>Frequency: Yearly</p>
Short term reproducibility	<p>Acceptance test, status test and constancy test.</p> <p>Deviations from the mean value of exposures $< \pm 5\%$.</p> <p>Operating procedure: the short term reproducibility of the AEC system is calculated by the deviation of the 3 routine exposures (45 mm PMMA test block) of the phan-ex, with the exposure meter accurately placed on the plate holder. The measured counts per second (cps) should be recorded.</p> <p>Frequency: Yearly</p>
Object thickness compensation	<p>Acceptance test, status test and constancy test.</p> <p>Deviations from the reference value of exposures (45 mm PMMA test block) $\leq \pm 15\%$.</p> <p>Operating procedure: the object thickness compensation of the AEC is calculated by determining the deviation of exposures of the phan-ex detector, accurately placed on PMMA plates of 30, 45 and 60 mm thickness, from the reference value (45 mm PMMA) at 28 kV. The measured counts per second (cps) should be recorded.</p> <p>Frequency: Yearly</p>
Tube voltage compensation	<p>Acceptance test, status test and constancy test.</p> <p>Deviations from the reference value of exposures (45 mm PMMA test block, imaged at 28 kV) $\leq \pm 15\%$.</p>

Operating procedure: the tube voltage compensation of the AEC is calculated by imaging the 45 mm PMMA test block, setting the tube voltage at 26 kV, 28 kV and 30 kV, with the exposure meter of the phan-ex accurately placed on the test block. The measured counts per second (cps) should be recorded.

Frequency: Yearly

Difference per step	<p>Acceptance test, status test and constancy test.</p> <p>All the deviation in the measured exposures between successive steps: 0.1 - 0.2 per step.</p> <p>Operating procedure: The optical density control step can be determined by placing the phan-ex on a 45 mm PMMA plate and taking an exposure at all possible steps, setting the operating voltage at 28 kV. The measured counts per second (cps) should be recorded.</p> <p>Frequency: Yearly</p>
Uniformity	<p>Acceptance test, quality control.</p> <p>Parallel to the axis tube, the exposure value should decrease by 30-35 % at a height of 12 cm from the chest wall. Perpendicularly to the axis tube, a typical value of exposure decrease is < 7% from the centre of the X-ray field to 10 cm, for each side.</p> <p>Operating procedure: Beam uniformity can be determined by positioning the exposure meter on a 45 mm PMMA plate, first at the centre of the PMMA plate and, successively, at the top, right, bottom and left of the test block. Image the plate and report the measured counts per second (cps).</p> <p>Frequency: Yearly</p>
Spatial resolution (at high frequency)	<p>Acceptance test, status test and constancy test.</p> <p>Spatial resolution should be ≥ 12 line pairs per mm (lp/mm)</p> <p>Operating procedure: It can be estimated by imaging two resolution lead bar patterns, up to 20 line pairs per mm (lp/mm) each, placed on a 45 mm-thick PMMA plate. Image the patterns using a Mo/Mo target-filter combination at 28 kV.</p> <p>Frequency: Yearly</p>
Threshold contrast visibility	<p>Acceptance test, status test and constancy test.</p> <p>Minimum detectable contrast for a 5-6 mm detail < 1.3%.</p> <p>Operating procedure: It can be estimated by imaging a suitable phantom containing 5-6 mm circular details. The phantom is accurately placed on a 45 mm PMMA plate. Image the phantom using a Mo/Mo target-filter combination at 28 kV.</p> <p>Frequency: Yearly</p>
Alignment of X-ray field/image receptor	<p>Acceptance test, status test and constancy test.</p> <p>X-rays must cover the film by no more than 5 mm outside the film parallel to the axis tube, laterally X-rays must totally cover the film.</p> <p>Operating procedure: The alignment of the X-ray field and image receptor at the chest wall side can be determined by using two loaded cassettes and two X-ray absorbers. Produce an exposure</p> <p>Frequency: Every three months</p>
Tube Voltage Accuracy	<p>Acceptance test, status test.</p> <p>Accuracy for the range of clinically used tube voltages (25 –31 kV): $< \pm 1$ kV.</p> <p>Operating procedure: The equipment should be tested over the range of clinically used settings (typically 25 – 31 kV) at intervals of 1 kV. To determine the tube voltage accuracy, the kV-meter</p>

should be accurately placed. The resulting measured kV should be recorded. After having assessed that the differences between measured and nominal tube voltage values are within 1 kV, the exposures can be repeated at 1 kV intervals, after positioning the exposure meter, by recording the resulting counts per second (cps).

Constancy test.

Accuracy for the range of clinically used tube voltages (25–31 kV): measured mGy vs nominal kV curve should be within the error bar.

Operating procedure: Adequately position the exposure meter and report the counts per second (cps) measured at intervals of 1 kV.

Frequency: Yearly

<p>Tube Voltage Reproducibility</p>	<p>Acceptance test, status test and constancy test.</p> <p>Reproducibility (at 28 kV): $< \pm 0.5$ kV.</p> <p>Operating procedure: To determine tube voltage reproducibility, accurately position the kV-meter and make at least three exposures at a fixed tube voltage that is normally used clinically (e.g. 28 kV). When the deviation from the mean value is $< \pm 0.5$ kV and repeat the exposures, after positioning the exposure meter, and record the resulting counts per second (cps).</p> <p>Constancy test.</p> <p>Reproducibility (at 28 kV): $< \pm 2$ %.</p> <p>Operating procedure: Adequately position the exposure meter. Make at least three exposure at a fixed tube voltage that is normally used clinically (e.g. 28 kV) and report the measured counts per second (cps).</p> <p>Frequency: Yearly</p>
<p>Exposure time</p>	<p>Acceptance test, status test and constancy test.</p> <p>Exposure time needed to image a 45 mm PMMA phantom: < 2 sec.</p> <p>Operating procedure: After accurately positioning the PMMA phantom and the sensor, the time for a routine exposure is measured.</p> <p>Frequency: Yearly</p>
<p>Reference dose</p>	<p>Acceptance test, status test and constancy test.</p> <p>Entrance dose: ≤ 10 mGy (40 mm PMMA test block); ≤ 12 mGy (45 mm PMMA test block); ≤ 20 mGy (50 mm PMMA test block).</p> <p>Operating procedure: Accurately position the exposure meter on the PMMA test block of known thickness. Report the counts per second (cps) measured at the entrance.</p> <p>Frequency: Yearly</p>
<p>Output rate</p>	<p>Acceptance test, status test and constancy test.</p> <p>Output rate must be < 7.5 mGy/s (at the focus-to-film distance).</p> <p>Operating procedure: The output rate should be measured using a Mo/Mo target-filter combination at 28 kV, in the absence of scatter material and attenuation, and reporting the counts per second (cps). After calculating the exposure value, calculate the output rate at a distance equal to the focus-to-film distance (FFD).</p> <p>Frequency: Yearly</p>
<p>Average glandular dose (AGD)</p>	<p>Acceptance test, quality control.</p> <p>AGD (45 mm PMMA): < 2 mGy.</p>

Operating procedure: After determining the tube load (mAs) necessary to image the phan-ex, accurately position the exposure meter on the 45 mm PMMA test block and report the measured counts per second (cps), without backscattering. After calculating the exposure value, calculate the output rate at a distance equal to the focus-to-film distance (FFD) and convert this value into the average glandular dose.

Frequency: Yearly

Grid system factor	<p>At acceptance and when dose or exposure time increases suddenly.</p> <p>Grid system factor must be ≤ 3.</p> <p>Operating procedure: The grid system factor can be estimated by accurately positioning the phan-ex and measuring counts per second (at 28 kV), without compression, and with and without the grid system.</p>																	
Grid imaging	<p>Acceptance test, status test and constancy test.</p> <p>No significant non uniformity</p> <p>Operating procedure: image the bucky at the lowest position of the AEC-selector, without PMMA. Verify the image uniformity.</p> <p>Frequency: Yearly</p>																	
Back-up timer	<p>Acceptance test, quality control.</p> <p>The back-up timer should function properly.</p> <p>Operating procedure: Make an exposure of a 1 mm lead sheet and verify if the AEC system terminates the exposure.</p> <p>Frequency: Yearly</p>																	
Half Value Layer (HVL)	<p>Acceptance test, status test and constancy test.</p> <p>For 28 kV Mo/Mo target-filter combination the HVL must be between 0.30 and 0.40 mm Al equivalent.</p> <p>Operating procedure: Position the exposure detector at the reference ROI (since the HVL is position-dependent) on top of the bucky. Place the compression device halfway between focal spot and detector. Select a Mo/Mo target/filter combination, 28 kV tube voltage and an adequate tube loading (mAs-setting), and expose the detector directly. The filters can be placed on the compression device and must intercept the whole radiation field. Use the same tube load (mAs) setting and expose the detector through each filter.</p> <p>Frequency: Yearly</p>																	
Focal spot size	<p>At acceptance and when resolution has changed, quality control.</p> <p>For 28 kV Mo/Mo target-filter combination, focal spots size are reported in the following table.</p> <table border="1" data-bbox="524 1379 1030 1556"> <thead> <tr> <th rowspan="2">Focal spot size</th> <th colspan="2">Reference values</th> </tr> <tr> <th>Length (cm)</th> <th>Width (cm)</th> </tr> </thead> <tbody> <tr> <td>1 × 1</td> <td>0.1 ± 0.15</td> <td>0.1 ± 0.15</td> </tr> <tr> <td>2 × 2</td> <td>0.2 ± 0.3</td> <td>0.2 ± 0.3</td> </tr> <tr> <td>3 × 3</td> <td>0.45 ± 0.65</td> <td>0.3 ± 0.45</td> </tr> <tr> <td>4 × 4</td> <td>0.6 ± 0.85</td> <td>0.4 ± 0.6</td> </tr> </tbody> </table> <p>Operating procedure: Produce a magnified image of the pinhole and measure, on the image, the length and the width, in cm. Repeat for all available focal spots.</p> <p>Frequency: Yearly</p>	Focal spot size	Reference values		Length (cm)	Width (cm)	1 × 1	0.1 ± 0.15	0.1 ± 0.15	2 × 2	0.2 ± 0.3	0.2 ± 0.3	3 × 3	0.45 ± 0.65	0.3 ± 0.45	4 × 4	0.6 ± 0.85	0.4 ± 0.6
Focal spot size	Reference values																	
	Length (cm)	Width (cm)																
1 × 1	0.1 ± 0.15	0.1 ± 0.15																
2 × 2	0.2 ± 0.3	0.2 ± 0.3																
3 × 3	0.45 ± 0.65	0.3 ± 0.45																
4 × 4	0.6 ± 0.85	0.4 ± 0.6																
Compression force	Acceptance test, status test and constancy test.																	

Maximum automatically applied force: 130 - 200 N.
 Operating procedure: The compression force can be estimated using a compression force test device or a bathroom scale.
 Frequency: Yearly

Compression plate alignment	Acceptance test, status test and constancy test. The difference between the measured distances at the left and right side of the compression paddle should be ≤ 5 mm for symmetrical load. Operating procedure: The alignment of the compression device at maximum force can be visualized and measured when a piece of foam-rubber is compressed. Frequency: Yearly
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Table 3. Proposed protocol for mammography QC and technical specification of the parameters to be monitored.

3. Results and discussion

The chosen protocol can be used equally for acceptance, status and constancy tests. It was successfully implemented for both analogue and digital mammographs.

In particular, it was implemented for constancy tests of all parameters relevant to the exposure, utilising the same phantom-exposure meter pair.

In addition to the protocol, Table 3, the QC report worksheet is proposed in which the raw results (counts per second, cps) can be reported, Fig. 2-3. The raw data is then elaborated to estimate the entrance dose.

The proposed protocol and QC report were tested on different (analogue and digital) mammographs, to assess their versatility and accuracy, independent of the physical characteristics of the mammograph.

As an example, the AEC test results obtained for a digital mammographic system are reported in Figs. 4-6 to show that the same protocol can also be used on digital instruments.

The results obtained from the object thickness compensation are represented in Fig. 4. In particular, in Fig. 4 (a), the value of the dose (mGy) normalised to the tube load value (mAs) for the reference PMMA test block thickness (45 mm), is constant and within the error bar (± 15 %). The dose as a function of the PMMA plate thickness is presented in Fig. 4 (b). This curve shows that, with increasing dose, the normalised dose is constant, indicating the correct operation of the AEC system.

The results obtained from the tube voltage compensation are presented in Fig. 5 (a). Differently from the previous test, where the tube voltage was kept constant (28 kVp) varying only the tube load, in this type of test two parameters are varied: tube voltage and tube load. Therefore, in this test the parameter chosen to assess the tube voltage compensation is the logarithm (base 10) of the dose. Also in this case the results show that the logarithm of the dose is within the limit values (± 15 % calculated for a reference tube voltage of 28 kVp and for a 45 mm PMMA test block).

TEST RESULTS

DATE / /

QC REPORT

(A) ACCEPTANCE TEST
 (B) STATUS TEST
 (C) CONSTANCY TEST

Monitored parameters

2) **Acoustic and light signalling [(A), (B), (C)]**
 Acoustic signal functions properly: yes no
 Light signal functions properly: yes no

3) **Security cut-off [(A), (B), (C)]**
 Exposure value: _____ cps;
 Terminated exposure: _____ cps;

3) **Source-to-image distance [(A), (B)]**
 Nominal value: manufacturer's specification: _____ cm
 Focus indication to Bucky: _____ cm
 Bucky to cassette (or receiver image): _____ cm
 Source-to-image distance: _____ cm

4) **AEC-system [(A), (B), (C)]**

- Long term reproducibility [(C)] and short term reproducibility [(A), (B), (C)]
 Limit: $\leq 2\%$ (long term reproducibility)
 $\leq 5\%$ (short term reproducibility)
- Difference per step [(A), (B), (C)]
 Limit: 0.1 – 0.2 per step

PMMA test block: 45 mm; Tube voltage: 28 kVp;

Step	Tube load (mAs)	Counts per second (cps)
-2		
-1		
0		
+1		
+2		

5) **Uniformity [(A), (B), (C)]**
 Limit: $< 30-35\%$ (parallel to the axis tube)
 $< 7\%$ (perpendicular to the axis tube)

Exposure meter position	Counts per second (cps)
Centre	
Top	
Right	
Bottom	
Left	

6) **Spatial resolution [(A), (B), (C)]**
 Limit: ≥ 12 lp/mm

Target-filter combination: Mo/Mo
 Tube voltage: 28 kVp Tube load: _____ mAs
 Resolution: _____ lp/mm

PMMA test block: 45 mm; Tube voltage: 28 kVp;

exposure	Tube load (mAs)	Counts per second (cps)
1		
2		
3		
4		
5		

- Object thickness compensation [(A), (B), (C)]
 Limit: $\leq 15\%$

Tube voltage: 28 kVp;

PMMA test block (mm)	Tube load (mAs)	Counts per second (cps)
30		
45		
60		

- Tube voltage compensation [(A), (B), (C)]
 Limit: $\leq 15\%$

PMMA test block: 45 mm;

Nominal tube voltage (kV)	Tube load (mAs)	Counts per second (cps)
26		
28		
30		

7) **Threshold contrast visibility [(A), (B), (C)]**
 Limit: $\geq 1.3\%$

Target-filter combination: Mo/Mo
 Tube voltage: 28 kVp Tube load: _____ mAs

Diameter disc: _____ mm Contrast: _____ %
 Diameter disc: _____ mm Contrast: _____ %
 Diameter disc: _____ mm Contrast: _____ %
 Diameter disc: _____ mm Contrast: _____ %

8) **Alignment of X-ray field/image receptor [(A), (B), (C)]**
 Limit: < 5 mm (chest)
 totally cover the film (otherwise)

Left: _____ mm
 Nipple: _____ mm
 Right: _____ mm
 Chest: _____ mm

9) **Tube voltage [(A), (B), (C)]**

- Accuracy [(A), (B), (C)]
 Limit: ≤ 1 kVp [(A); (B)]
 measured mGy vs nominal kV curve should be within the error bar [(C)]

Nominal tube voltage (kVp)	25	26	27	28	29	30	31
Measured tube voltage (kVp)							
Counts per second (cps)							
Tube load (mAs)							

- Precision [(A), (B), (C)]
 Limit: ≤ 0.5 kVp [(A); (B)]
 $\leq 2.0\%$ [(C)]

Nominal tube voltage (kVp)	28	
Measured tube voltage (kVp)		
Counts per second (cps)		
Tube load (mAs)		

Figure 2. QC report worksheet for raw data recording (part 1).

10) Exposure time [(A), (B), (C)]
 Limit: ≤ 2 second
 PMMA test block: 45 mm
 Tube voltage: 28 kVp; AEC settings: _____
 Exposure time: _____ sec

11) Reference dose [(A), (B), (C)]
 Limit: ≤ 10 mGy (40 mm PMMA test block)
 ≤ 12 mGy (45 mm PMMA test block)
 ≤ 20 mGy (50 mm PMMA test block)
 PMMA test block: _____ mm;
 Tube voltage: _____ kVp Tube load: _____ mAs
 Counts per second: _____ cps

12) Output rate [(A), (B), (C)]
 Limit: > 7.5 mGy/s (at the focus-to-film distance)
 Tube voltage: 28 kVp; Tube load: _____ mAs Exposure time: _____ sec
 FFD: _____ cm Counts per second: _____ cps

13) Average glandular dose [(A), (B), (C)]
 Limit: ≤ 2 mGy (45 mm PMMA test block)
 Tube voltage: 28 kVp Tube load: _____ mAs
 FFD: _____ cm Tube output = _____ cps/mAs

14) Anti scatter grid [(A), (B), (C)]
 • Grid system factor [(A)]
 Limit: ≤ 3

Grid	Counts per second (cps)	Tube load
Present		
Absent		

• Grid imaging [(A), (B), (C)]
 Artefacts are present: yes no
 Description of artefacts: _____

15) Back-up timer [(A), (B), (C)]
 Exposure terminates by exposure limit:
 yes no

16) Half-Value Layer (HVL) [(A), (B), (C)]
 Limit: 0.3 mm Al ≤ HVL ≤ 0.4 mmAl (Mo/Mo filter-tube combination)
 Tube voltage: 28 kVp; Tube load: _____ mAs;
 no filter 0 mm cps = _____
 filter 1 _____ mm cps = _____
 filter 2 _____ mm cps = _____
 filter 3 _____ mm cps = _____
 filter 4 _____ mm cps = _____
 filter 5 _____ mm cps = _____

17) Focal spot size [(A), (B), (C)]
 Limits:

Nominal focal spot size (mm)	Length (cm)	Width (cm)
1 x 1	0.1 - 0.15	0.1 - 0.15
2 x 2	0.2 - 0.3	0.2 - 0.3
3 x 3	0.45 - 0.65	0.3 - 0.45
4 x 4	0.5 - 0.85	0.4 - 0.6

Limits:

Nominal focal spot size (mm)	Measured Length (cm)	Measured Width (cm)
1 x 1		
2 x 2		
3 x 3		
4 x 4		

18) Compression force [(A), (B), (C)]
 Limit: maximum automatically-applied force 130-200 N
 Measured compression force: _____ N
 Compression force after 1 min: _____ N

19) Compression plate alignment [(A), (B), (C)]
 Limit: ≤ 5 mm

	Left (mm)	Right (mm)
Rear		
Front		

Figure 3. QC report worksheet for raw data recording (part 2).

The dose radiated by the AEC system as a function of the tube voltage is presented in Fig. 5 (b), as measured with the phan-ex. From the results of Fig 5 (b) it is noticed that as the tube voltage increases, the dose decreases, further confirming that the AEC system is functioning correctly.

Results from the test on the “difference per step” are reported in Fig. 6. Also in this case, the logarithm of the dose was calculated at each step. The obtained values are within the limit values (0.2 – 0.4 as the step difference was 2), Fig. 6 (a). The corresponding values of the dose per step are reported in Fig. 6 (b).

For the short-term reproducibility test, exposure values were measured, from which the average dose value was determined with respect to the tube load supplied by the AEC system (mGy/mAs), Fig. 7, to show the proposed unified protocol is equally applicable to analogue and digital mammographic system.

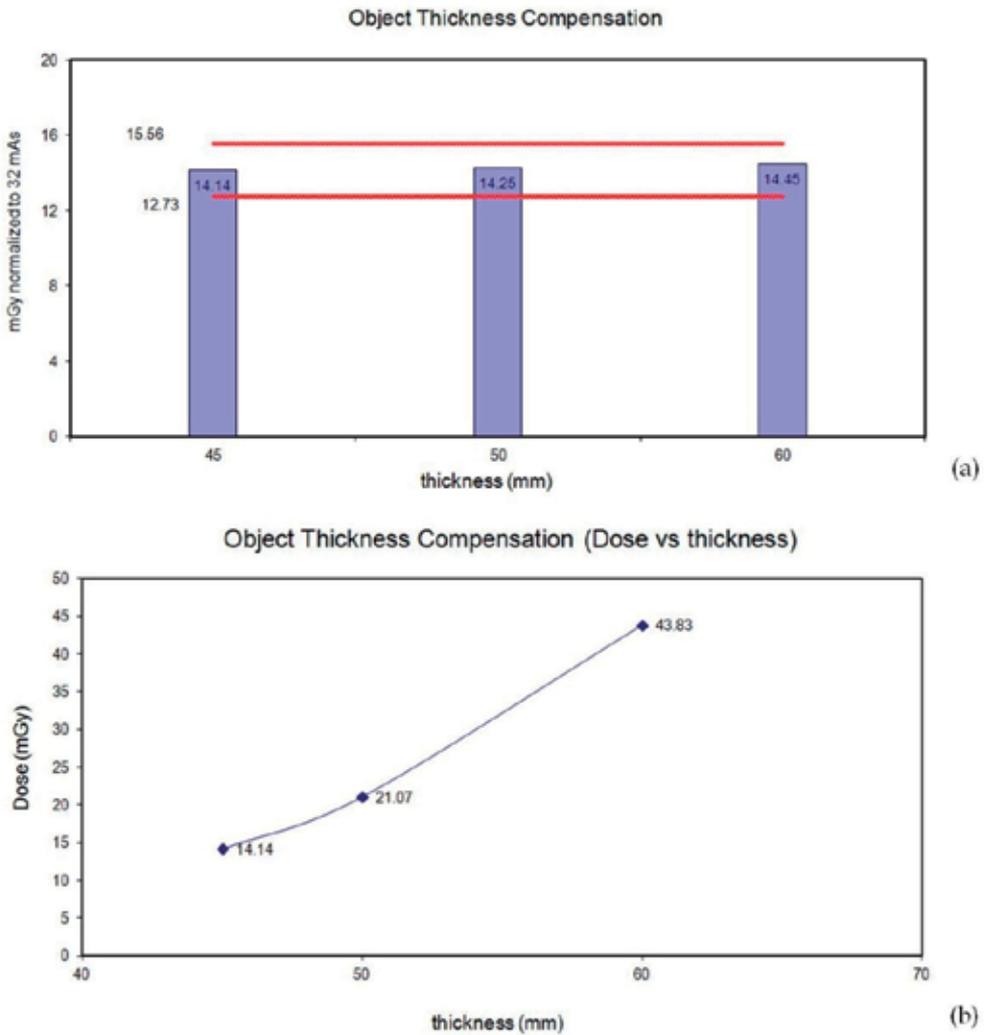


Figure 4. Results of object thickness compensation test: (a) the dose (mGy) normalised to the tube load value (mAs); (b) the dose (mGy) as a function of PMMA plate thickness.

The use of the phan-ex, coupled with the proposed protocol, is useful also to verify parameters related to the exposure such as tube voltage precision and accuracy, and exposure time.

Most importantly the proposed protocol permits the evaluation of the functional parameters of the instruments by utilising a single phantom, thus significantly reducing the number of additional dedicated equipment and simplifying the task of the Medical Physics Expert.

The results obtained from raw data analysis obtained following the proposed protocol were found to be consistent with those obtained from standard procedures [32-35], thus highlighting the usefulness and versatility of the proposed unified protocol to test all relevant param-

eters in analogue and (direct or indirect) digital instruments. The simplification is even more relevant in the latter type of mammographs for which the QC procedures currently used present considerable difficulties in the interpretation of the measurement protocols.

The applicability of the proposed phantom can be further extended to the measurement of parameters other than those relevant to the exposure even for the next generation of mammographs which are still under development. One such instrument is the SYRMEP, equipped with a Si-based microstrip detector and a synchrotron X-Ray source characterised by superior performance with respect to typical X-Ray tubes [36].

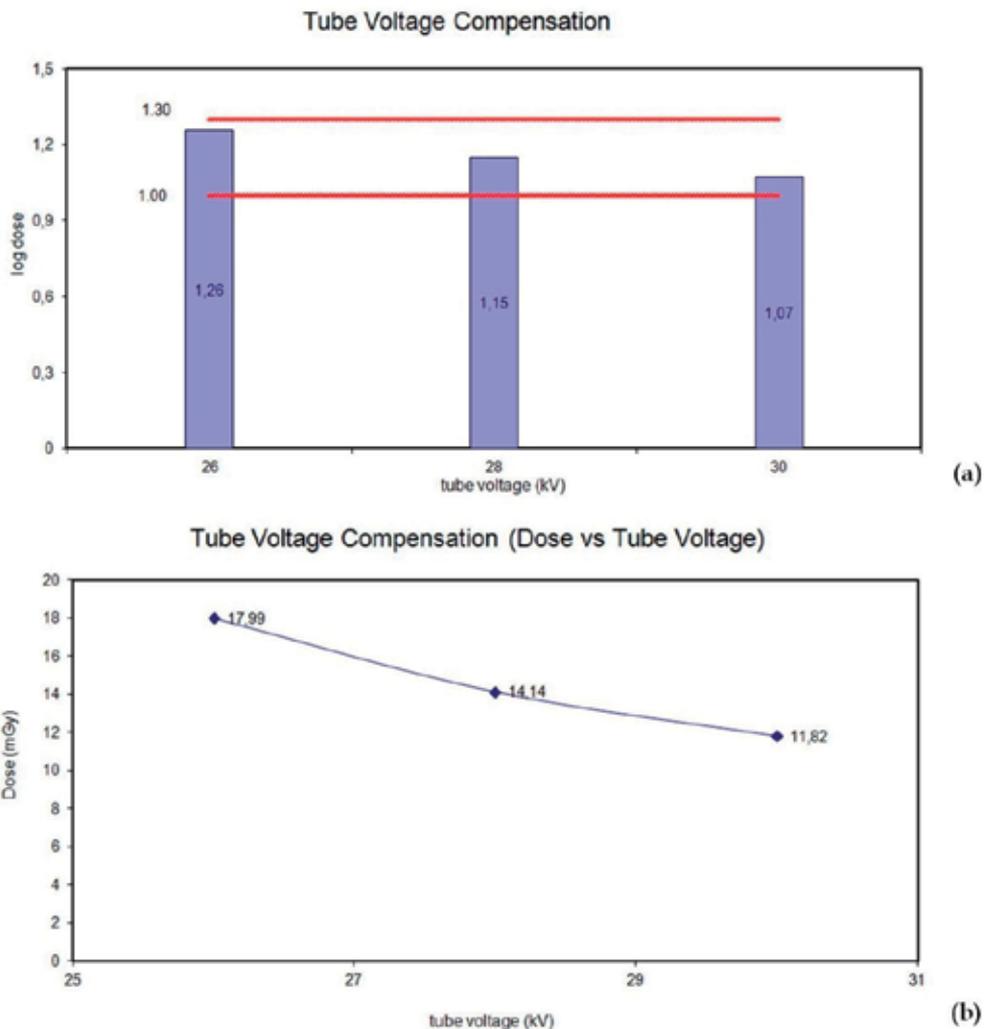


Figure 5. Results of tube voltage compensation test: (a) logarithm of the dose, red lines representing the limit values ($\pm 15\%$) with respect to 28 kVp reference tube voltage; (b) the dose (mGy) as a function of tube voltage (kVp).

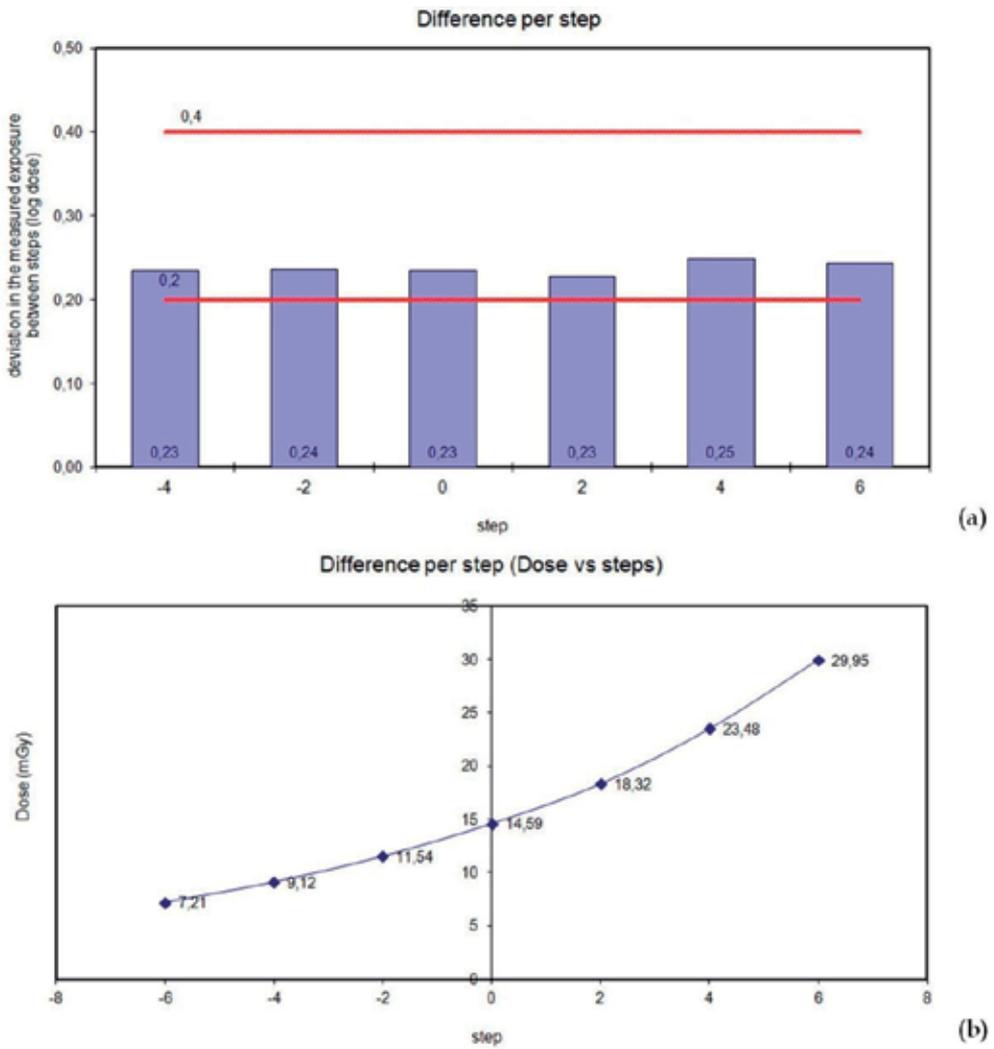


Figure 6. Results of difference per step test: (a) logarithm of the dose, red lines representing the limit values (0.2 – 0.4 per step); (b) the dose (mGy) per step.

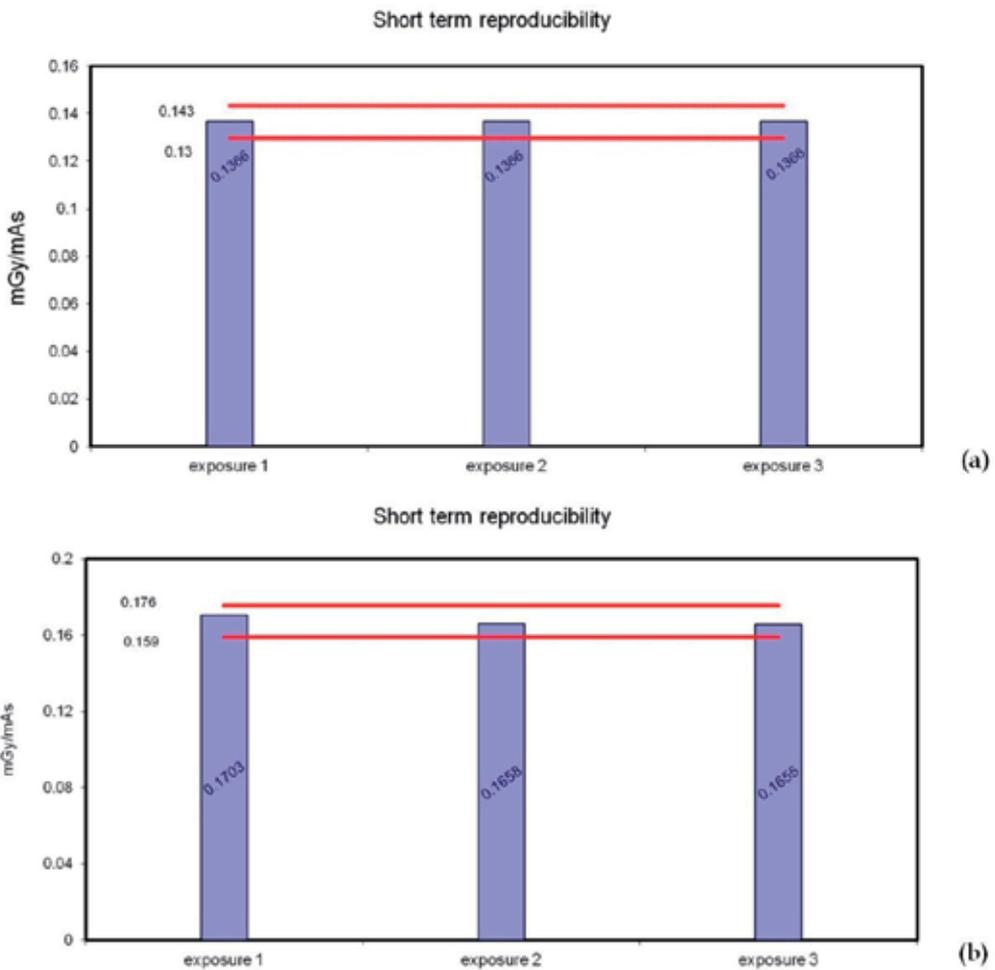


Figure 7. Results of short term reproducibility test, dose to tube load ratio (mGy/mAs) for three different exposures : (a) digital, (b) analogue mammograph;

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Quality Control in Energy

The Quality Management of The R&D in High Energy Physics Detector

Xuemin Zhu and Sen Qian

Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/51434>

1. Introduction

Particle physics, also recognized as high energy physics, is a basic subject focusing on the research of the elementary elements of materials and their mutual actions. One distinguished characteristic of particle physics study is that the experimental equipments involved are always huge and special ones. Therefore, big science projects, including the R&D of large detectors, are usually required in high energy physics experiments. Those projects are complicated systematic engineering, involving many front and technology fields. It is impossible for a single institute to finish those large projects by its own. Cooperation among different institutes or organizations is necessary for big science projects in particle physics, especially international ones.

1.1. The Institute of High Energy Physics [1]

The Institute of High Energy Physics (IHEP) is the biggest and comprehensive fundamental research center in Chinese Academy of Science. The major research fields of IHEP are particle physics, accelerator physics and technologies, radiation technologies and application, Particle physics experiments and Accelerator physics and technology are two of the leading research areas. The main research facilities at IHEP include Beijing Electron Positron Collider (BEPC) and Beijing Spectrometer (BES), DayaBay Neutrino Experiment, Chinese Spallation Neutron Source, etc. IHEP has extensive cooperation with all high energy physics laboratories and participates in many important particle physics experiments in the world.

1.2. The Beijing Electron Positron Collider [2] and the Beijing Spectrometer [3]

The Beijing Electron Positron Collider (BEPC) consists of the injector, the storage ring, the transportation line, the Beijing Spectrometer (BES), the Beijing Synchrotron Radiation Facili-

ty (BSRF) and the computer center. Beijing Spectrometer (BES) is a general purpose magnetic spectrometer in the South IP of the storage ring. The general layout of the BEPC is shown in Fig.1.



Figure 1. The aircscape of the BEPC.

BEPC started construction in 1984 and the first electron-proton collider was produced in Oct. 1988. BEPCII was installed in 2003 and finished five years later in 2009. IHEP establishes comprehensive and long-term cooperation with high energy laboratories and universities all over the world, especially in USA, Japan and Europe. With the international cooperation, IHEP have gained huge success in 30 years. For example, IHEP took part in the research of CMS and ATLAS detectors of Large Hadron Collider (LHC), which is the world's largest, highest-energy particle accelerator and the collider at the beginning of 21 centuries, built by CERN [4]. BESIII is also organized by IHEP and participated by 51 institutions and universities around the world, 34 from Asian, 12 from Europe and 5 from USA.

1.3. The Daya Bay Neutrino Experiment [5]

The Daya Bay Neutrino Experiment is a neutrino-oscillation experiment designed to measure the mixing angle θ_{13} using anti-neutrinos produced by the reactors of the Daya Bay Nuclear Power Plant (NPP) and the Ling Ao NPP.

The Daya Bay Neutrino Experiment is a major international joint research program, mainly organized by China working closely with researchers from other countries. In terms of both money and people, it is among the largest scientific collaborations between US and China. More than 200 scientists from China, include Hong Kong and Taiwan, the US, Russia, the Czech Republic are involved in the Daya Bay experiment. During the cooperation, China is in charge of the laboratory construction, R&D of Anti-neutrino detector (AD), Gd-loaded

Liquid Scintillator, Muon Veto Detector, Readout Electronic and Data acquisition system (DAQ) etc. While America is in responsible of the construction of water Cherenkov detector and so on.

Scientists from the Chinese Academy of Sciences (CAS) and the U.S.-based Brookhaven National Laboratory and the Lawrence Berkeley National Laboratory will participate in the underground experiment. An international funding commission comes into existence in the funding agency to discuss fee issues and instruct the experiment process and fee management through experimental supervision organization. The project management of the Daya Bay Neutrino Experiment adopts the advanced and mature modern management idea used for managing large international joint project and big science experimental research project. An international cooperation group is built and management rules are made. Besides, a co-operation group commission is founded, during which executive board and spokesperson is elected for overall supervision of the whole project. The Daya Bay Neutrino Experiment is initiated in 2007 and finished in 2012.

1.4. Chinese Spallation Neutron Source [6]

Chinese Spallation Neutron Source (CSNS) is designed to build a device with the power of proton beam reaching up to 100 kW effective and the flux of pulsed neutrons coming out top in the world, along with other three spallation neutron sources built in America, Japan and British. CSNS is also a large cooperative project, supported by Chinese Academy of Sciences and Guangdong government. The normal operation for uses is foreseen in 2018. IHEP is the main construction institution in the project with the Institute of Physics Chinese Academy of Sciences as the co-operation unit. The construction team bring together three generations of outstanding scientific and technical researchers in China. An international CSNS neutron technology advisory committee is set up for reviewing the key experimental work. The experts of the advisory committee are from well-known laboratories in America, Japan, Germany, Australia and other countries.

2. Introduction of Quality Management of Scientific Projects in IHEP

During the process of big science project and research, IHEP has significant advantages in accelerator physics and technology, human resources, international cooperation and academic exchange. IHEP owns mature model and advanced experience in the quality management of scientific projects.

2.1. Project Management System

Before 2011, the project manager is responsible for the big science project management in IHEP. International cooperation group is formed and fees are under the sponsors' supervision and review. There is a perfect project management system, though without quality management system meeting international standards.

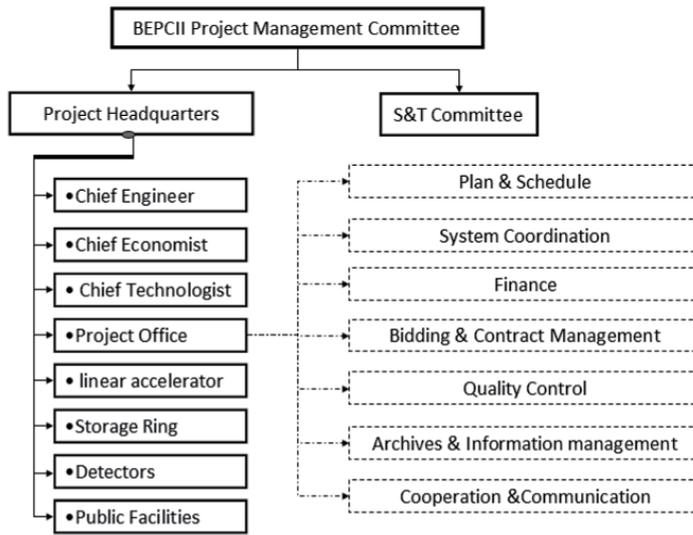


Figure 2. The Organization Chart of the BEPCII Project Management.

In the project management system shown in Fig.2, special-purpose management mechanism such as fund, purchasing, quality, safety and archive is established, with clear responsibilities and authorities. Besides, the internal communication mechanism and interface management mechanism are also set up. CPM Plan is adopted for fund and schedule management. To ensure the quality of the project, during the design and development process, experts are always invited for evaluation. And an international council committee is asked for review in terms of major international cooperation projects.

In fact, the requirements of the project management system have already displayed in the ISO 9001 quality management system. Though without a systematic quality manual and standards and lack of resource, purchasing and archive management. In the project management system, quality management is more focused on the management of various test guidelines and processing of key parts (including outsourced progress)

2.2. Quality Management Systems

The BEPCII project headquarters has placed great important on the quality management and published “BEPCII project management file” in 2002. In the file, responsibilities and rights of personnel, fund management, file number, document signing and alteration, early stages management, bidding and purchasing are described in detail.

At the beginning of 2005, during the construction of BEPCII project, the headquarters built a quality management system according to GB/T19001-2000(idt ISO9001:2000). Although the system doesn’t get a national certification, it is completely in accordance with standards of quality management system requirements and it has played a very good effect. In 2009,

BEPCII completed the construction task successfully by time, with high quality and budget under control.

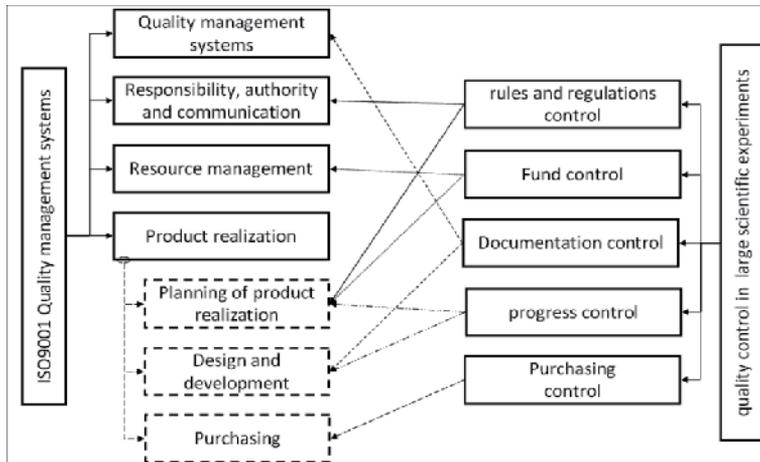


Figure 3. The Relationship between the quality control system in large scientific experiments and ISO9001 Quality management systems.

As described in Fig.3, The quality control in large scientific experiments corresponds with ISO9001 Quality management systems, which is classified according to the production. While, the quality control in large scientific experiments is classified according to the type of different work. The ISO9001 Quality management system is widely adopted by corporations all over the world and it's more normative.

In 2011, IHEP passed a national quality management certification system: GB/T19001-2008(idt ISO9001:2008). After two years' development of quality management system from its very beginning to being passed, it has confirmed that IHEP has the ability to produce scientific production meeting requirements.

The set up of quality management system makes the project management procedure standard, and promotes the overall management level in IHEP. The clients' needs are fully met and the quality management of IHEP joined the line of international standard management. The role played by quality management in the scientific research, especially in the big science project, is invaluable and imponderable.

3. Quality management in R&D of BESIII detector

The project of BESIII detector began its research and development, according to the scientific project management system and quality management system, like other big science projects.

3.1. Mechanism Management

BESIII detector R&D is part of BEPCII project. So the quality management of the detector research is responsible by the project director. As a whole, BESIII carries out the management system of BEPCII project headquarters strictly and makes some special mechanism to form a mechanism with a clear hierarchy. Quality technician are employed in the project.

BESIII detector R&D project has outlined the responsibilities and rights of each person in charge with an appropriate staffing in the organization. The communication methods of the total and sub system and record control requirements are defined.

The director in charge of sub system is responsible for the implementation of the BESIII research plan, management, arrangement of related resources and coordination with scientific and technical issues. Each division leading person is specifically responsible for the respective task implementation plan. Members in the project cooperate with each other closely at reaching difficult goals. The whole project has the characteristic of unified task, defined responsibilities, reasonable arrangement and integrated resources.

The high energy physics experiment is a complex project, and the communication in different study cells seems more important. The Task Control Form is widely used in study works, and the forms are preserved and archived as records of the system.

Subject				
Send to				
From		Date		
Serial No.		Pages		
Attached				
C.C.				
Content				
Jointly Sign				
validation	Prepared by	Checked by	Examined by	Approved by
Signature				
Date				

Figure 4. The task control form used by different teams.

Researchers in the project communicate with each other in time and have a regular meeting each or twice a week, to make sure the project is under schedule control and discuss some technical problems. Meeting minutes are kept as a reference. Sub-system will report the progress of the project and accept an inspection and evaluation regularly.

3.2. Fund Management

Fund management is important for the whole management of scientific project. Appropriate fund use a basis for carrying out any high energy physics experiment smoothly. As for the R&D of BESIII detector, the experiment design and development planning will affect the rationality of the budget and fund use directly. They are also the important contents in the requirements of the quality management system

Funds come from Chinese Academy Sciences (CAS) allocation and self-provided funds in the BESIII project. At the end of the year, expenses are counted and reported to CAS and the project will receive examination and evaluation.

3.3. Control of documents and records

Control of documents and records is critical whether for scientific project management or for quality management. For high energy physic experiments, large and complicated equipments are usually involved. During the project design and scheme phase, rules for documents and records reserved need to be made clearly and principles for numbering and signing the documents and records need to be described specifically.

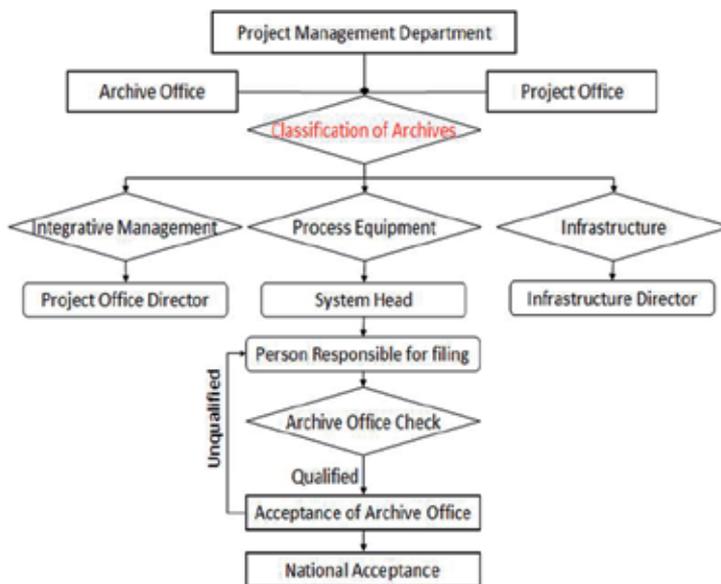


Figure 5. The workflow of archive management.

BEPCII project builds up a special mechanism of file control. Because BESIII is part of the whole project, the rules of document management are in accordance with the requirements of BEPCII. *Documents and records* need to be signed according to the regulations, in accord-

ance with the whole project and effective as well. *Documents and records* need to be preserved and archived on a regular basis.

There are several characteristics in archive management work, especially for the high energy experiments. Firstly, this work must be arranged by the project management department at the beginning of the project. Secondly, the document and records which need to be preserved must be clearly described and the responsibility should be defined at the first time. Thirdly, the archive office, the project office and each member working for the project should cooperate to get the work done quickly and perfectly.

All the quality documents of the whole process of each single detector, from design, research, test, and acceptance are preserved according to the regulations. Technical specifications, interface of different tasks, diagrams, test reports are archived as written documents. Regular meeting minutes are kept also as archives. Those *Documents and records* can be used to track and follow the quality of the product in the whole process.

The running cycle of big science project, just like its construction cycle, is as long as to last more than ten years. Therefore, control of documents and records is very essential for the running and maintenance of the big science project, as an important prop and support.

3.4. Schedule Management

BESIII project has followed Critical Path Method (CPM) to control the schedule of the whole project. The plan in the CPM is in detail and convenient for check. It is easy for revision according to the actual process and make sure it is updated in time within the system.

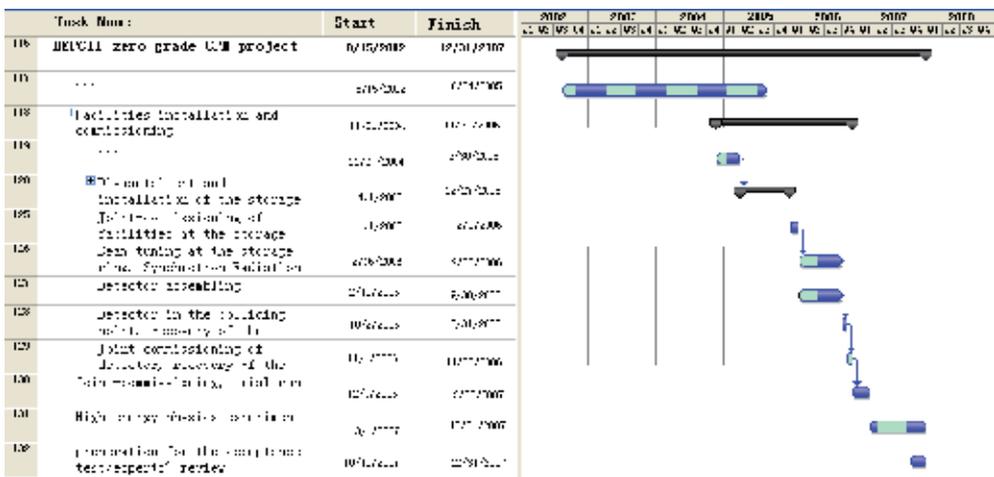


Figure 6. BEPCII zero-grade CPM project (partly,2002).

In order to give a better control of the schedule, CPM is classified. Any sub-system could make its own play and updates in time following the step of the total plan. Therefore inter communication plays an important role in the schedule management. In a word, CPM is a

further refinement of the time arrangement of the project design report and makes the management of the project construction effective.

The CPM project is highly in accord with the practical progress and BEPCII zero-grade CPM project is modified frequently. The BEPCII project was finished in 2008 and was finally checked in 2009.

3.5. Purchasing Management

The R&D of large detectors is involved with bulk purchase. In the BESIII project, purchasing management rules are made according to the relevant laws and regulations on acquisition. Purchasing and approval process are defined clearly. Bidding is strictly adopted in the project to save research money. An appropriate regulation in the purchasing process is a guarantee for carrying out the project under the budget..

Abroad purchase has a long life cycle, heads of procurement need to do significant preparatory work in advance, and the heads should be quite familiar with the procurement procedures in order to complete the purchase in time. The purchasing department published the flowchart to facilitate the work.

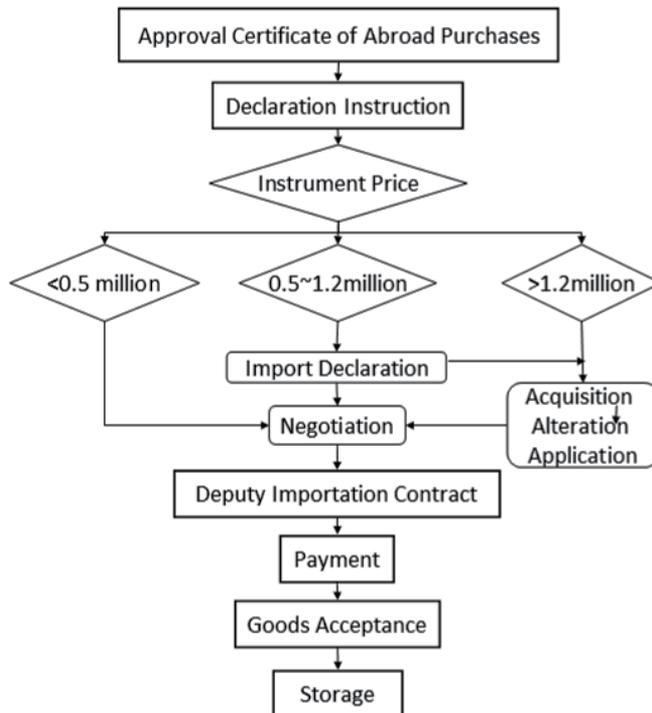


Figure 7. Abroad purchase flowchart.

4. BESIII-MUC Quality Management in R&D of BESIII-MUC Detector

4.1. Introduction of BESIII Detector and MUC Detector

The Beijing Spectrometer (BESIII) is designed to measure the properties of the particles produced in the collisions of electrons and positrons at BEPCII. The physics goal of the BESIII experiment is to conduct high statistics and highly precise studies on a number of physics topics in this energy region, including light hadron spectroscopy, charmonium spectra, charm meson decay properties, QCD, tau physics, rare decays, search for glueballs and other non-pure quark states [3].

The BESIII detector will consist of a 1 T superconducting solenoid magnet, a high precision main drift chamber (MDC), Time-Of-Flight counters (TOF), a CsI crystal Electromagnetic Calorimeter (EMC) and a muon identifier chamber (MUC) that is integrated in the iron magnetic field return yoke [7]. The muon identifier is the outer most subsystem of the BESIII detector [8], which is constructed by resistive plate chambers (RPCs, shown in Fig.8.a). 962 RPC are used in the whole MUC detector, which consists of 136 RPC superlayer modules (SM, shown in Fig.8.b). And the Fig.8.c shows the status of the MUC detector when it was finished it's barrel part assemblage. The Fig.8.d shown the designed construct of the BESIII MUC detector with the endcap and barrel parts.

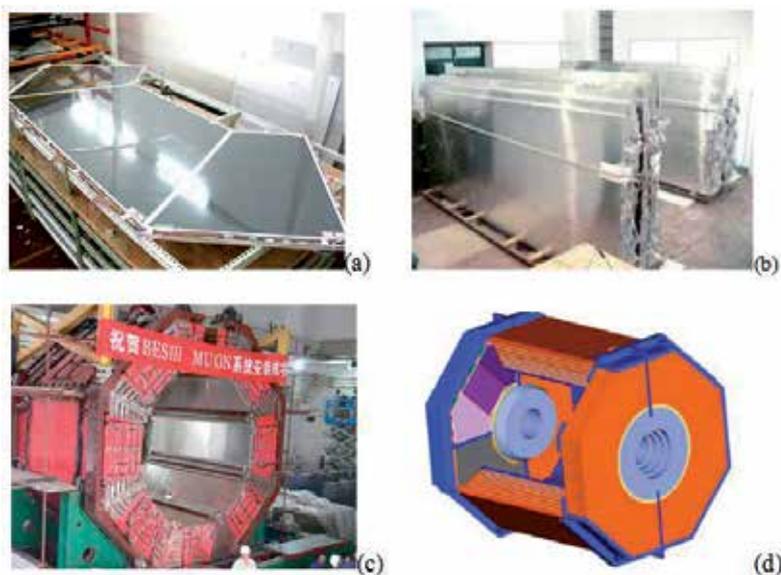


Figure 8. a). The RPC moduls, (b). The Suplayer Modul, (c). The overview the barrel part of the MUC detector after it's assemblage, (d) The construct of the BESIII MUC detector.

4.2. Quality Management in the R&D of BESIII-MUC Detector

The whole process of R&D of MUC detector include the design of the basic unit RPC, properties investigation, bulk production, SM design; design of MUC detector, installation debugging, running and maintenance.

Throughout the research process, the project director managed the project scientifically and effectively, with each research aspect considered carefully, comprehensively and deeply, and made some achievements. From the pre-research in 2003 to the formal data collection in 2009, more than 30 papers have been published by the research group of MUC, covering the whole research process.

7 papers have been published in *NIMA*, as follows:

1. A new surface treatment for the prototype RPCs[9],
2. Cosmic ray test results on resistive plate chamber for the BESIII experiments [10]
3. The Design and Mass Production on RPC for the BESIII Experiment [11]
4. A monitor for the composition of the gas mixture of BESIII muon chambers [12]
5. First results of the RPC commissioning at BESIII [13]
6. The BESIII Muon Identification System [14]
7. An underground cosmic-ray detector made of RPC [15]

8 papers have been published in *Chinese Physics C*, as follows:

1. Cosmic Ray Test Station for BES^{III} RPC [16]
2. Research and Development of Large Area Resistive Plate Chamber [17]
3. A Study of RPC Gas Composition using Daya Bay RPCs [18]
4. Quality control and database on RPC for the BES^{III} experiment [19]
5. Test of BES^{III} RPC in the avalanche mode [20]
6. Performance Study of RPC Prototypes for the BES^{III} Muon Detector [21]
7. Study of the RPC-Gd as thermal neutron detector [22]

	Design	Performance Test	Mass Production	Research Work	Application
RPC	1 b	2 a c e f	3 d	4	7 g
SM			6		
MUC			6	5	

Table 1. The analysis of the manuscripts published by MUC group.

As shown in Table 1, it is not difficult to come into conclusion that the whole R&D of MUC detector applied scientific project quality management, which promotes the research work. In the phase of initial RPC research, the key point is on the study of the detector’s performance test. It is the phase for building a standard quality management. After the acceptance of RPC and project review, mass production and SM reassembling come into being. In this phase, scientific quality control and management play a key role.

A perfect quality tracking system is established in each session, from the production and test of RPC, assembling and test of modules, to the installation and debugging of MUC detector, to ensure the supervision of the performance of detector is plausible.

Especially for the mass production of RPC and SM, before research and test, a database is built for storage related data and affording date support for quality control and final running & maintenance.

4.3. Summary

All the requirements such as verification, validation, monitoring, measurement, inspection and test activities specific to the detector are described in the design report of the detector in detail. The report plays the same role as in making a particular quality control plan.

□ ■	Design	Prototype production	Mass production	Assemble	Debug	Running
critical characteristic	■	□				
major characteristic	■	■	□			
critical process		■	■			
article inspection	■	■	■			■
quality improvement	■	■		■		
effectiveness			■	■		
traceability			■	■	■	■
preventive action		□	□		■	■
corrective action					■	
quality plan			□	■		

Table 2. Quality management/control factor distribution of MUC detector.

The design report of the detector divide the R&D process into several phases, including concept design, project design, sample trail-manufacture, product research and production, test,

installation and debugging. In each phase, review and identification is defined. For important phases, such as aging test, assembly test and system test, detailed guidelines and instructor are written. As shown in Table 2, during the outsourcing process, key parts are defined, and acceptance rules are also clearly described. Control point is set up and design files are carried out strictly to ensure the product quality. More detailed could be found in table 2 for summary.

5. Significance of Scientific Quality Management in Research

5.1. Promote Scientific Projects

We could come into conclusion that scientific quality management can promote scientific projects to proceed successfully, in the following ways:

The schedule of the project could be arranged and controlled well, especially the adoption of CPM, which could provide a time map for the whole project. Throughout the four years' successful implementation and of BEPCII project, CPM plays an important role in the project acceptance in due. CPM was adjusted in time according to the project status, thus effective management and restriction was formed for all the related sub systems in the project.

The project has been implemented within the budget and cost was controlled. Purchasing procedures and approval process were strictly described, which played a role for the fair use of the fund.

Documents and records were kept in detail, as reference in the project to find the source of old problems and avoid new problems. Especially for those big scientific projects which will last more than ten years, files about interface management and quality management and various records are significant for the running and maintenance in the following work. They also act as important reference for the future project construction in high energy physics.

5.2. Promote Scientific Research

Scientific quality management could promote scientific research effectively. At the same time, as the development of scientific research, cooperation among researchers will be increased. It is good for the communication and exchange in the area of quality management and promotes the refining of the quality management system thus.

Experiences in big scientific project are good for the growth of young researchers. With participation in the R&D of big science equipment under quality management system, researchers will learn how to organize and manage scientific programs or projects in future.

In an ongoing scientific project managed launched by IHEP, researchers are from participants in BESIII or DayaBay. Although it is non-international, at the beginning the project is managed as required in strict quality management, just like that in big science project. As the development of the project, communication and cooperation among other institutions

both at home and abroad have increased. To coordinate the partnership among different organizations and unites, cooperation group is formed. As shown below, a formality management system and strictness organization is built, which lays a solid foundation for the sustainable development of cooperation group and joint research work in future, whose Organization Chart shown in fig.9 for example.

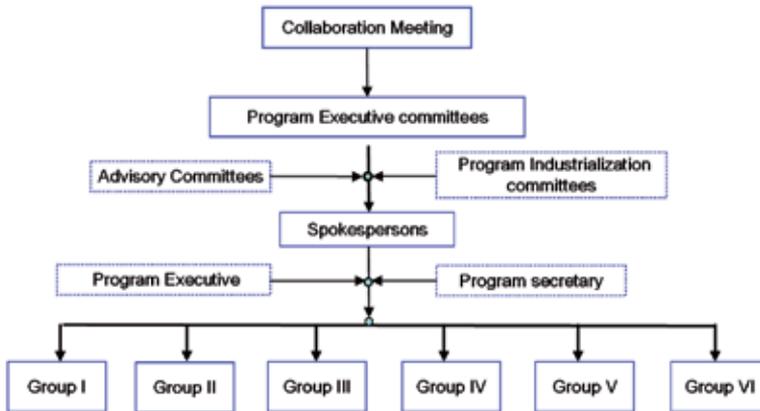


Figure 9. The organization of the BEPCII

6. Conclusion

Quality management plays a significant role both in project management and in the scientific research. With a scientific and comprehensive quality management system, big science project will be duly executed. The level of scientific projects will be greatly improved by the application and popularization of national and international quality standards.

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Quality Control in Cosmetics

Cosmetics' Quality Control

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Additional information is available at the end of the chapter

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1. Introduction

The quality of a cosmetic product, in the same way as to other kind of products, is initially defined by the manufacturer that chooses the features that a product should present. On the other hand, the quality control of a product aims to verify if all of these defined features are in accordance with the standard definitions and if it will be maintained during the shelf life of the product (Shewhart, 1980).

The quality control of cosmetics is important to ensure the efficacy and safety of products and its raw-materials. Due to the rapid growth that cosmetic industries have exhibit all over the world, efficient, low cost and rapid methods to assay cosmetics' quality control are a priority. Some current techniques used by the cosmetic industry can be applied to the evaluation of cosmetics' quality control in an efficient manner, such as: rheology, sensory analysis and small angle X-ray scattering (SAXS).

Sensory analysis is a powerful tool, since there is no equipment able to measure the human feelings. It applies experimental design and statistical analysis to obtain information about a product in relation to what people feel when use or consume a product, in other words, it is used to indicate consumer acceptance of a particular product. It can be understood as the discipline that interprets, assess and measures characteristics of a product, after stimulating people in relation to their vital senses, as vision, touch, smell and taste (Stone et al., 1992). It is widely used in food industry and recently, it has also been applied in the cosmetic industry (Almeida et al., 2008; Aust et al., 1987; Backe et al., 1999; Lee et al., 2005; Parente et al., 2005; Wortel et al., 2000).

The sensory analysis can be applied in the research and development of a new cosmetic (Isaac et al., 2012a), in controlling the manufacturing process to evaluate raw-materials quality and, even, to make possible the substitution of a raw-material of a product that is traditional in the market without changes in the product's features (Meilgaard et al., 1991; Muñoz et al., 1993).

The application of sensory analysis could be related to the product control, referring to the storage, packaging and maintenance of sensory quality in relation to time and temperature (Muñoz et al., 1993), since these factors can change a sensory attribute that the product present originally (Zague, 2008) and people who participates of the sensorial panel could realize the changes in the sensorial attributes. Another function of this important tool is to performance comparative tests between competing products.

Another tool that could be applied to evaluate cosmetics' quality control is the rheology, which studies the flow and deformation of fluids. It has been used in research laboratories and industries as a tool for characterizing ingredients and products, and to predict the performance of products and consumer acceptance.

Rheology has been widely used because, by means of this tool, the researcher can determine physicochemical properties of a product. Constructing a rheogram, it is possible to check the flow curve, evaluate if there is a yield stress and a hysteresis area, which appears to be related to the release of drugs and actives. It is also possible to construct a creep and recovery curve obtaining information about viscoelasticity of each system.

Specifically, in relation to the quality control of cosmetics, specifically, rheology can be applied to help in determining the stability of products by means of the apparent viscosity measured periodically in a determined period exposing the samples to stress conditions (high and low temperatures, solar irradiation), and to monitor the flow characteristics during the shelf life or in the stability assay of a product.

The SAXS technique have being used for the analysis of cosmetics, in order to evaluate the presence of liquid crystalline structures, called liquid-crystals, which are known to increase the stability of formulations becoming, therefore, desirable in cosmetics (Makai et al., 2003).

Combining these three tools, it is possible to test the quality of cosmetics with a rich range of data, and obtain a deep characterization of the system. The results contribute to determining product use, or even, they provide indication of what need to be done to develop a product with predetermined characteristics.

2. Sensory analysis

Sensory analysis is defined by Piana et al. (2004) as the examination of a product through the evaluation of the attributes perceptible by the five sense organs (organoleptic attributes), such as color, odor, taste, touch, texture and noise, allowing the establishment of the organoleptic profile of diverse products, including cosmetics.

The sensory analysis was first applied to the food industry, but the high advance in other areas, such as the cosmetic and pharmaceutical industries, and the important data obtained with the sensory analysis, demanded this useful technique to describe what the consumers feel.

An important advantage of the use of sensory analysis in the quality control of a cosmetic product is that it yields a complex analysis in relation to all sensorial attributes that a product could present, it means that, the volunteer who participates of the sensorial panel is able to give information about the fragrance, the sensation, the appearance, the consistence, and other features that this person experience when use such product. The description of these characteristics by means of equipment would be an arduous work and would provide not sufficient or not valuable data when compared to the data provided by the human senses. Beyond that, the acquisition of this equipment could be of high cost when compared to the sensory analyses' costs (Ross, 2009).

The association of data obtained from sensory analysis and instrumental analysis (especially physicochemical analysis) provides great information and a more complete profile of the product (Ross, 2009).

Nowadays, there are companies specialized in perform sensory analysis of cosmetic products, and thus, they could be contracted to perform this study for cosmetic industries that don't have a sector trained to do it.

The sensorial performance of cosmetics is essential to the acceptance of consumers (Almeida et al., 2008; Fouéré et al., 2005; Lee et al., 2005; Proksch, 2005), thus, especial attention should be given to this subject.

The sensorial features of a formulation are mainly related to the raw-materials and package (Dooley et al., 2009). The raw-materials influence directly in what the consumer feels when applies the cosmetic. The emollients, for example, are raw-materials of marked influence in the tactile sense (Parente et al., 2008; Gorcea and Laura, 2010). Other raw-materials are available at the market and are commercialized to be used in formulations as sensorial modifiers. The main representatives of this kind of product are the silicones and Polymethyl Methacrylate (Ozkan et al., 2012).

The package influences in the first impression of the consumer about a product, since the first sense used to choose a cosmetic in the market is the vision. After, the smell is used too. The tact is not involved in the first purchase attitude, but it will define if a consumer will become a loyal consumer.

In this context, it is possible to verify that the sensorial features of a cosmetic are of great importance in the success of it in the market.

Thus, the sensorial analysis could help a company to define the attributes that a product should or not present beyond the characteristics and intensity of these attributes.

Another point is that these desired sensorial characteristics should be maintained during the cosmetic shelf life. To obtain that, the raw-materials used should be of good quality, the

manufacture practices should be appropriate, the preservatives used need to be efficient and the formulation should be stable.

In conclusion, the sensorial analysis is an indispensable technique to help the formulator to evaluate the quality of its new product, in relation to its sensorial characteristics and to its stability, testing if the product will keep the nice sensorial feelings that transmit to the consumer during the time of use. This tool is helpful to the research and development area of a company which aims to obtain good quality products of high acceptance by the consumers. The suitable application of sensory evaluation could avoid the outlay of a company with the launching of a product in the market that was rejected by the volunteers of the preliminary study.

Currently, the sensorial analysis have gained more scientific rigor due to the need to offer to the consumers products that meet their expectations and due to the high competition between the major industries of this sector.

To perform the sensorial analysis with rigor and organization, the laboratory destined to it must have the following areas:

A room destined to the analyst who leads the team (Figure 1a)

A conference room (Figure 1b)

A room for the samples preparation (Figure 1c)

An area to the analyses with the volunteers (Figure 1d)

The laboratory should be located in an easy access place.

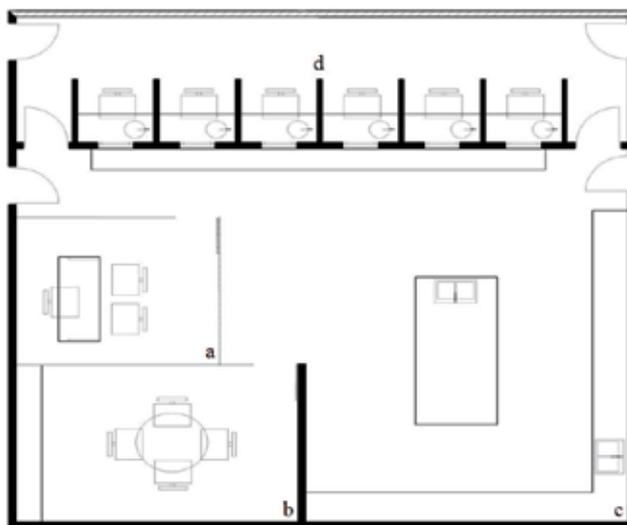


Figure 1. An example of layout of a sensorial analysis laboratory (Isaac et al., 2012a).

The area where will be performed the analyses should be divided in individual cabins (Figure 2) with a window, where the analyst must offer the samples to the volunteer, sink and faucet, to the volunteer use when necessary (Isaac et al., 2012).



Figure 2. An example of layout of sensorial analysis cabine (Isaac et al., 2012a).

The cabins must be ventilated and odor free, to avoid interferences in the analyses. The temperature and humidity should be controled around 22 °C and at 45% of humidity (Isaac et al., 2012).

It is recommended that the walls and furniture of the rooms are colored with neutral and light colors to not disturb the attention of the volunteers and to not interfere in the attributes analyzed by the vision, such as color and appearance of the product.

The volunteers should not smoke, should be healthy, with ease of memorization and communication.

In the study, the volunteers judges could be an experienced judge or not, depending on the kind of evaluation and the answers that the professional team needs to obtain. In the case of utilization of sensorial analysis in the quality control of a cosmetic, usually the volunteers are regular users of the product in analysis, since they need to be familiarized with the characteristics of the product and have sensibility to perceive slight modification on it. When the aim of the sensorial analysis is to evaluate the acceptance of a product that should be launched in the market, it is recommended that the volunteers are potential users of this new product, orienting the formulator to make changes in the formulation and guiding the company to evaluate if the costs of the product launch are recommended or not.

There are four different methods to perform the sensorial analysis that are most used, they are: affective, discriminative, descriptive (Aust et al., 1987) and methods to evaluate the effective of the product.

Independent of the method of sensory analysis suitable for each evaluation, the professional team should use printed questionnaires to obtain the answers from each volunteer. The use of printed questionnaires avoids the contact between the professional and the volunteers preventing that the professional is biased in his responses, beyond that, it facilitates the data collection.

In the elaboration of these questionnaires the professional team should use suitable lexicons for each class of product, for example, the lexicons used to the evaluation of lip products are different from that used for corporal lotions (Dooley et al., 2009). Some researches had developed suitable lexicons for different classes of cosmetic products (Civille and Dus, 1991; Wortel and Wiechers, 2000; Dooley et al., 2009). The manner as the volunteer is questioned is fundamental to obtain the information required from them. An inadequate formulary could invalidate a sensory evaluation. It is interesting also, that a description of all descriptors attributed to the formulation being provided to the volunteer, for example: "Thickness: Viscosity of the cream when picking up from the container", "Ease of spreading: Ease of rubbing the sample over the skin", "Absorption: Ease of absorption of the product through the skin", "Residue: Amount of product left on the skin after application" (Parente et al., 2010).

The affective methods represent the consumer opinion and evaluate how much consumers like or dislike a product. It is a quantitative method that is performed in order to know the consumers preferences (Aust et al., 1987). This technique could be applied in the development of new products and when it is necessary to replace a constituent of a formulation without loss of the product quality. It could be performed in two different ways: offering two different samples to the volunteer asking him about what sample he prefers between them or using a hedonic scale for the volunteer attributes grades of intensity of its acceptance in relation to the sample.

The hedonic scale either can be presented to the panel of evaluators in different manners, as shown in Figure 3.

The affective methods provide quantitative data and allow more than one attribute in each sample being evaluated at the same time.

The discriminative test is better represented by the Triangular test. It allows differentiating one between three different samples and is very useful in shelf life studies and in the quality control of cosmetics. The ideal is to perform this evaluation with twelve to forty volunteers, who will receive the three samples and should indicate the different one between them (Zenebon et al., 2008).

The descriptive tests provide a broad sensory description about the product that is being evaluated (Almeida et al., 2008), helping to predict the consumer acceptance and what consumers think about such product (Almeida et al., 2006; Aust et al., 1987).

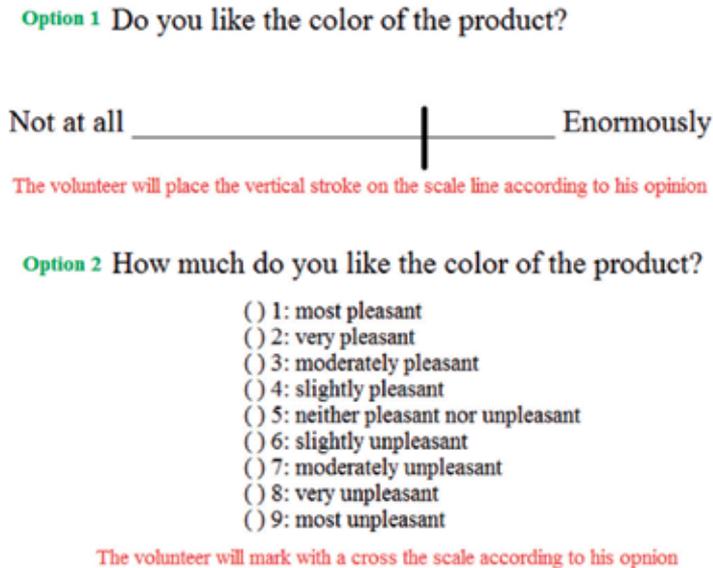


Figure 3. Examples of presentation of hedonic scale (Olshan et al., 2000; Barkat et al., 2003).

The tests to evaluate the effective of the products should be performed in true conditions of use and the volunteer may use only the product that is being assessed. These tests could be conduct by the evaluation of dermatologists, by the evaluation of volunteers, and even, by the measurement of one parameter by un equipment, such as the equipment that measures hydration, sebum and transepidermal water loss, to define if a product is really effective. Based on these clinical evaluations, a company could create an efficacy claim to the product (Wortel and Wiechers, 2000).

The sensory analysis could be also applied when a cosmetic industry needs to replace a raw-material of a commercialized product without changes in the performance of it. This replacement could be originated by many factors, such as the reduction of costs, problems with the firm who provides this raw-material problems with same raw material which causes irritation, comedogenicity or other problems that affect the consumer. In this field, the sensorial analysis helps the formulator, who proposes different raw materials as substitute, to evaluate if the consumer will notice the adaptation in the cosmetic product.

The statistical analysis is indispensable in the sensory studies. The sensory analysis data should be evaluated transforming them in scores which allows the application of statistical analysis to calculate the mean and standard deviation of the results, and the determination if the difference between the scores obtained is statistically significant. Graphics, tables and preference maps could be elaborated with the results obtained to facilitate the analysis of the data by the professional team.

The sensory analysis is especially indispensable in the industries of fragrances and perfumes, and because of that, high-resolution instrumental methods for evaluation of flavor

and aroma have been developed and between them are the breath analysis via mass spectrometry (Dijksterhuis and Piggott, 2001; Ross, 2009). Instrumental measurements are thought to be objective, representing an independent fact or truth, however, the human smell sense is irreplaceable, being considered by Ross (2009) not necessarily valid because instrumental methods cannot account for the complexity of human perception.

Nevertheless, rheological studies have been applied to objectify the sensations when cosmetic emulsions are applied to the skin (Brummer and Godersky, 1999).

3. Rheology

Rheology is a tool widely applied in the food, petrochemical and pharmaceutical industries, but to the cosmetic industry it is incipient yet. Until now, the majority of cosmetic industries use viscometers to guarantee that the viscosity of different batches of a product is maintained.

This chapter was elaborated in order to show that many other rheological characteristics could be used to evaluate and to predict the stability of cosmetic products and could be applied to compare competing products in the market and to assay if a change in the composition will cause alterations that could be perceived by the consumer.

First, it is necessary to define the three parameters of most importance in rheology: shear stress, shear rate and viscosity. Shear stress can be defined as a force applied in an area. Shear rate is the ratio of the velocity of material to its distance from a stationary object (Naé, 1993). The shear rate can be calculated by the ratio between the velocity and the layer or film thickness. In a lipstick application, for example, with a velocity estimated in 5 cm/s and a layer thickness of 0.1 mm, the ratio (shear rate) is $5 \cdot 10^2 \text{ s}^{-1}$. Finally, the viscosity can be defined as the resistance to flow. Thus, a viscous product presents smaller flow than others.

Concluding, rheology is the study of deformation and flow of materials under external forces. Some equations and the units of these parameters are (Naé, 1993):

$$\sigma = F / A \quad (1)$$

Where:

σ = shear stress (Pa = $\text{kg} \cdot \text{m}^{-1} \cdot \text{s}^{-2}$)

F = force (N or $\text{kg} \cdot \text{m} \cdot \text{s}^{-2}$)

A = area (m^2)

The viscosity can be defined as the ratio between shear stress and shear rate:

$$\eta = \sigma / \dot{\gamma} \quad (2)$$

Where:

η = viscosity

τ = shear stress (Pa)

$\dot{\gamma}$ = shear rate (s^{-1})

Since the unit of shear stress is Pa and the unit of deformation is s^{-1} , the unit of viscosity is Pa.s. These parameters are involved in scientific measurements of rotational assays.

Using controlled shear rate and measuring shear stress is possible to carry out rotational assays, and determine flow curves and describe the models: Newtonian or non-Newtonian and, among the last one, plastic, pseudoplastic, dilatant, tixotropic and reopetic fluids. Newtonian fluids are materials that present constant viscosity, independent of time and temperature. These materials present flow curves with proportionality between shear stress and shear rate. The Figure 4 represents the flow curve of a Newtonian material.

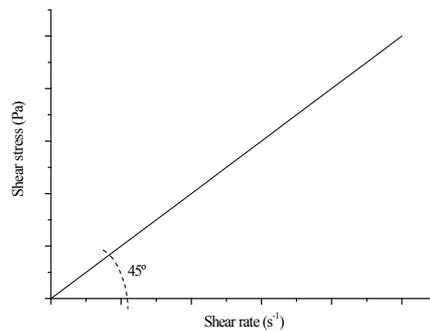


Figure 4. Flow curve of a Newtonian material.

In the case of non-Newtonians materials, this proportionality between shear stress and shear rate does not happen.

If in the beginning of the flow curve there is an increasing in the shear stress but the shear rate is equal to zero, and after to it is verified a Newtonian flow, this material is called plastic. This initial shear stress with shear rate equal to zero is called yield value and it represents the shear stress necessary for the material flow. The Figure 5 represents a plastic material. The yield value is related to the energy required to deform the material sufficiently so that they can flow. The value of the yield stress can be determined by measuring the deformation of the material as a function of the applied stress (Abdel-Rahem et al., 2005).

For non-Newtonian materials time-dependents, if the viscosity decreases with the shear rate, the material is called pseudoplastic and if the viscosity increases, the material is called dilatant. On the other hand, if the material is time-independent, it will be called tixotropic if the viscosity decreases with the shear rate or reopetic if the viscosity increases with the shear rate.

rate (Naé, 1993). When the ascending and the descending curves of the flow curve do not overlap it shows thixotropy which is a desirable feature for cosmetics and semisolid drug carriers for topical application (Lippacher et al., 2004). The Figures 6, 7, 8 and 9 represent the flow curves of non-Newtonian materials (Naé, 1993).

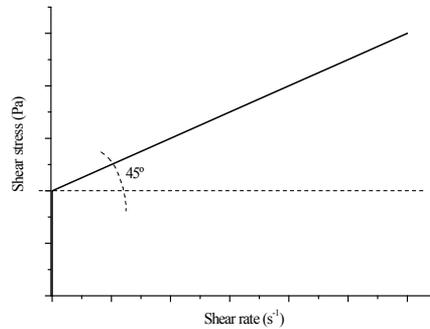


Figure 5. Flow curve of a plastic material.

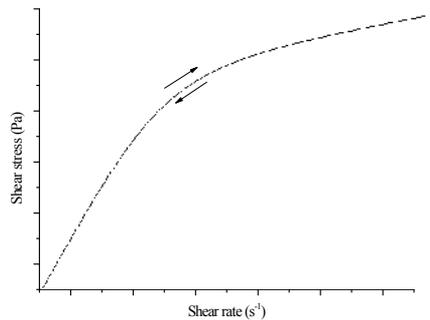


Figure 6. Flow curve of a pseudoplastic material.

For screening purposes and in the initial phases of the formulation development, the rheological tests proved to be very useful for the study of stability.

In a stability assay to determine the shelf life of a recently developed product, the formulation should be exposed to stress conditions, such as storage at $-5\text{ }^{\circ}\text{C}$, $45\text{ }^{\circ}\text{C}$, and cycles of $-5\text{ }^{\circ}\text{C}$ during 24 hours followed by exposure to $45\text{ }^{\circ}\text{C}$ during more 24 hours. This procedure is done in order to induce the appearance of instability signals in the formulations, where can be cited the darkening of the formulation, the precipitation of a constituent, the phase separation in the case of emulsions, and other signals. These stressing conditions are kept for a period around 2 or 3 months.

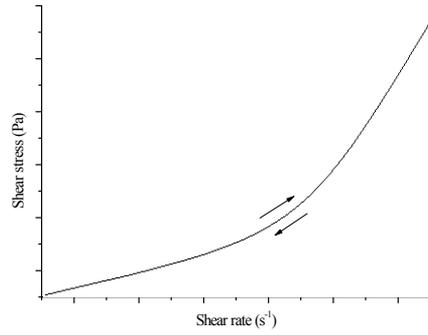


Figure 7. Flow curve of a dilatant material.

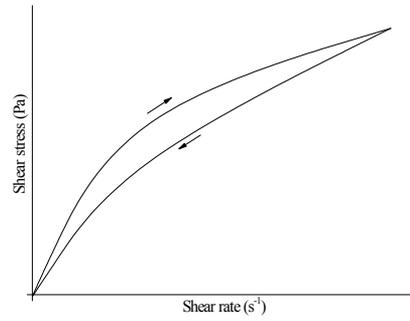


Figure 8. Flow curve of a thixotropic material.

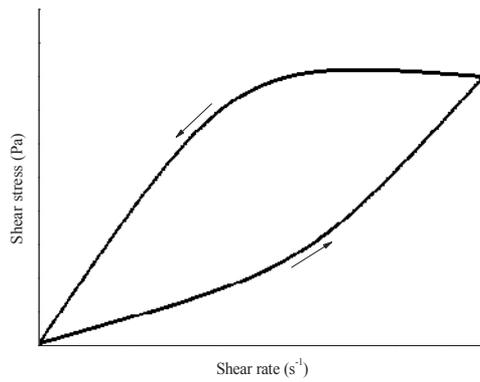


Figure 9. Flow curve of a reopetic material.

It is usually measured the viscosity of the stressed formulations periodically during the stability assay. It could be done by means of a viscometer or by using a rheometer.

With a viscometer, it is possible to carry out rotational assays or measurements by steady-state flow. On the other hand, the rheometer allows the development of oscillatory assays or dynamic measurements (Biradar, 2009).

When using an oscillatory rheometer it is necessary to carry out a flow curve assay and determine the apparent viscosity of the formulation in a defined shear rate. It is recommended to use the higher shear rate in the ascendant curve of the flow curve, since in this point the sample is in a suitable condition, it means that the formulation is not starting to flow and is not excessively sheared (Figure 10).

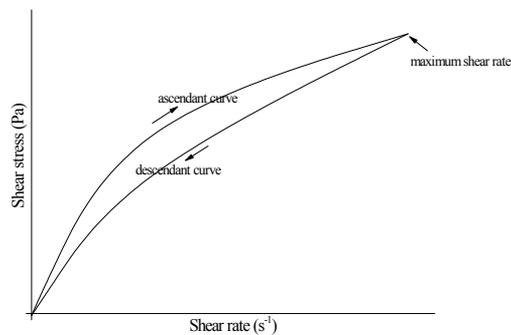


Figure 10. Example of a flow curve indicating the condition to the measurement of apparent viscosity.

In some papers, the flow curves have been plotted as viscosity as a function of shear stress instead of the traditional approach of plotting it versus shear rate because has been previously found that such curves are more discriminating and give better results for evaluation (Roberts, 2001; Samavati, 2011).

After obtaining, periodically, the minimum apparent viscosity of the samples exposed to stress conditions during a period, they should be compared with the initial value, and also compared the viscosity values of the control with the samples exposed to stress conditions, which allows the verification of the increase, decrease or maintenance of this attribute of the formulations.

Further exploiting the same assay, it is possible to calculate the hysteresis area of the formulation in each flow curve performed during the stability assay. The hysteresis loop areas can be obtained through a three-step experiment: upward curve, plateau, downward (Benchabane and Bekkour, 2008) and represents a way to measure, indirectly, the spreadability of the formulation, so it is possible to define if the formulation losses or gains easiness on spreadability during the shelf life. How much bigger is the hysteresis area, higher is the spreadability.

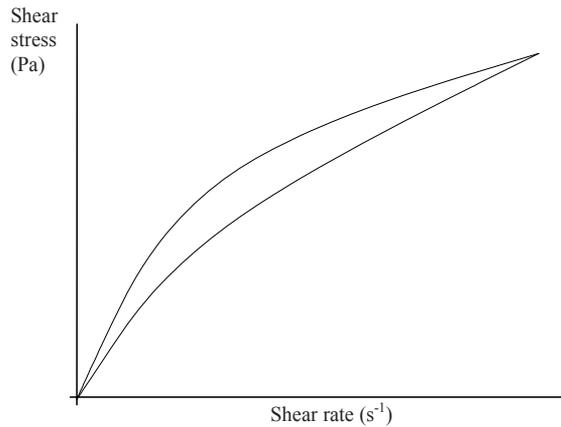


Figure 11. Flow curve with hysteresis area.

Using the flow curve is possible to compare two samples in relation to its hysteresis area and viscosity. A simple way to verify what formulation have a higher viscosity is by simple observation of the rheogram, since the curve that forms a bigger inclination in relation to the x axis of the graphic is the one with higher viscosity. On Figure 12 is showed an example of it, where sample 2 is more viscous than sample 1. It happens because the tangent of the angle formed is correspondent to the viscosity of the formulation in each shear rate.

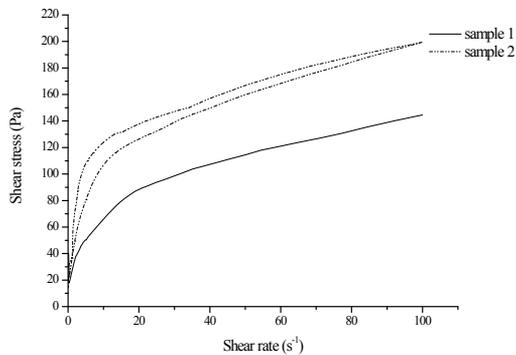


Figure 12. Comparison between flow curves of different samples (a thixotropic and a plastic fluid).

Beyond the different sensorial features caused by the differences in viscosity is known that the viscosity of emulsified systems is one of the factors that retards or avoids the phase separation processes. The coalescence of dispersed phase can be due to the emulsifier agent and can be related to an instability because of low viscosity of dispersed phase (Corrêa & Isaac, 2012). This low viscosity can occur because of high shear stress (Samavati et al., 2011).

In general, for emulsions systems, the continuous phase is shear thinning, which means that its viscosity decreases with the increasing on shear rate and viscoelastic, which means that it has viscous and elastic components (Tadros, 2004).

An example of the verification of differences in viscosity and thixotropy between two samples is shown on Figure 13.

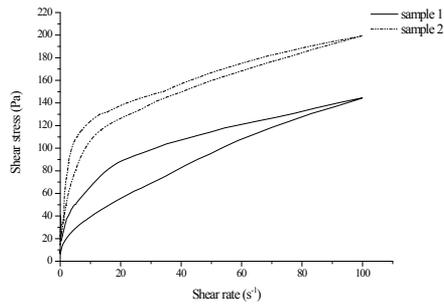


Figure 13. Comparison between flow curves of different samples.

Sample 1 is less viscous but more thixotropic than formulation 2. This simple verification gives to the analyst wide information, depending on what he needs.

Lescanne et al. (2004) studied organogels and aging properties of them. Organogels can be obtained by precipitation processes. These authors verified that, when aggregates are formed by the cooling rate, can be observed a elastic behavior, however, these aggregates can be aligned in the direction of the flow without lost the structure and when the flow is stopped, the aggregates are quickly rearranged and it inducing an thixotropic behavior. When the hot solution is introduced between the flat and the conical plates of the rheometer cell it is cooled to 5 °C with a cooling rate of 20°C/min, during the first hour of the gel life, it was measured the elastic properties of a gel as a function of time just after the cooling. Five minutes after its formation, the gel was submitted to a periodic stress (0.5 Pa) at a constant frequency ($f = 1\text{Hz}$). The authors showed that the shear moduli are constants and the aging phenomenon did not modify the elastic properties at least in a period of 1 h. However, when more than a week of aging is waited the samples lost most of its elastic properties.

The flow curve is a rotational assay, but using a rheometer it is possible to perform oscillatory assays too. Among the oscillatory assays are stress sweep and the frequency sweep assays.

The elastic (storage) modulus G' and the viscous (loss) modulus G'' are determined as a function of frequency or stress. The elastic modulus is a measure of energy stored and recovered per cycle of deformation and represents the solid-like component of a viscoelastic material. If a sample is elastic or highly structured then the elastic modulus will be high. The

viscous modulus is a measure of the energy lost per cycle and represents the liquid-like component. If a sample is viscous the viscous modulus will be high.

In the stress sweep analyses, the structure of the sample is progressively destroyed by applying oscillations with an increasing stress amplitude at a fixed frequency (Callens et al., 2003). The linear viscoelasticity region occurs over that region of strain where the complex modulus is independent of the strain (Hemar, 2000). The linear viscoelastic region is determined by the maximum stress which can be applied without affecting G' and G'' . Furthermore, the relative magnitude of the moduli is a qualitative indication for the structure in the sample. Two different situations can occur: $G' > G''$ for a network consisting of secondary bonds and $G' \leq G''$ for a physically entangled polymer solution (Callens et al., 2003).

Frequency sweep tests are performed in the linear viscoelastic region of each sample, keeping the structure of the system intact during the measurement. By performing such small stress amplitude oscillations at a whole range of frequencies, the type of network structure can be revealed. The main difference between a network of secondary bonds and one of physical entanglements is located in the low frequency range: in an entangled network the polymers can disentangle if the available time is long enough (low frequency). In a network with secondary bonds the bonds are fixed irrespective of the time scale. This results for an entangled solution in a limiting slope of 2 for G' and 1 for G'' at low frequency in a log-log plot of moduli versus frequency, while at intermediate frequency a plateau develops. For a network of secondary bonds an almost constant value of G' and G'' is observed over the whole frequency range, with the value of G' exceeding that of G'' (Callens et al., 2003; Madsen et al., 1998).

The stress sweep is important to evaluate the linear viscoelastic region of a sample that is a range of shear stress in which the formulation does not suffer profound alterations on its structure, being not disrupted. When a shear stress of the linear viscoelastic region is applied in an oscillatory assay, only the intermolecular and interparticle forces are being evaluated (Martin, 1993). To determine the linear viscoelastic region, the oscillating stress sweeps are carried out for the most extreme values. These measurements are used to determine where the rheological properties are independent of the applied stress and to identify the critical rheological properties (Tuarez, 2011).

Knowing the values of shear stress that do not cause the disrupt in the formulation by means of the stress sweep, the analyst could perform a frequency sweep of the formulation. The frequency sweep is carried out in a constant shear stress found in the linear viscoelastic region. With this assay it is possible to evaluate the elastic or storage modulus (G') and the viscous or loss modulus (G''). The cosmetic excipients most used, emulsions and gels, are often viscoelastic samples. The viscoelastic samples when evaluated by means of the frequency sweep present G' and G'' values. When the G' value is higher than G'' it is an indicative that the formulation is more elastic than viscous. It is a characteristic of gels.

Emulsions which exhibits G' values higher than G'' (Figure 14) are described as more stable than formulations with G'' values higher than G' (Figure 15), since they tends to recovery its initial structure faster and more efficiently than the others, and are less susceptible to the

gravitational forces which retards or avoids the coalescence process and the phase separation of emulsions (Alam and Aramaki, 2009). So, the G' values higher than G'' in emulsions is a desirable feature, being an indicative of stability of the cosmetic system.

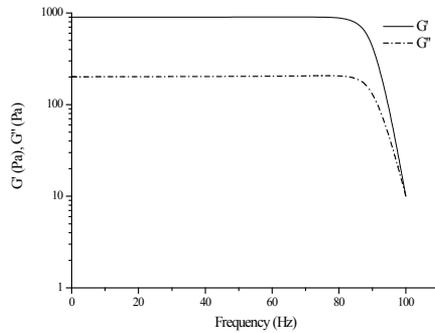


Figure 14. A frequency sweep example ($G' > G''$).

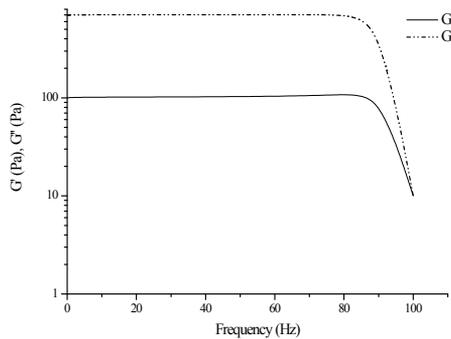


Figure 15. A frequency sweep example ($G'' > G'$).

Another assay that could be conducted using an oscillatory rheometer is the creep and recovery assay. It is done by submitting the samples to a constant shear stress during a period, and after, removing this shear stress and monitoring the formulation in relation to the deformation (measured by the compliance - J) during the same period. The compliance parameter is the resulting strain divided by the applied stress (Koop, 2009; Toro-Vazquez et al., 2010). If the compliance parameter is the relationship between strain and the applied stress, the strain is dimensionless and stress is measured in Pa, then, the compliance can be measured in $1/\text{Pa}$.

In the example showed on the Figure 16 the samples were submitted to a shear stress during 300 seconds, and after removing this shear stress it was monitored during more 300 seconds.

Analyzing the result obtained in the first 300 seconds is verified that sample 1 exhibited lower compliance values than sample 2, which represents a higher difficult on being deformed than sample 1. The difficult on being deformed is always linked to higher viscosity values.

In the second part of the assay, where the shear stress imposed to the sample is removed, represented in the graphic by the time 301 to 600 seconds, is verified the viscoelastic properties of the samples. Formulations that are able to recovery its initial structure or part of it exhibit a gradually decrease in the compliance values. On Figure 17 there is an example of a formulation that is not a viscoelastic sample, it means that it do not exhibits storage modulus, and is not able to recovery its structure when the shear stress is ceased.

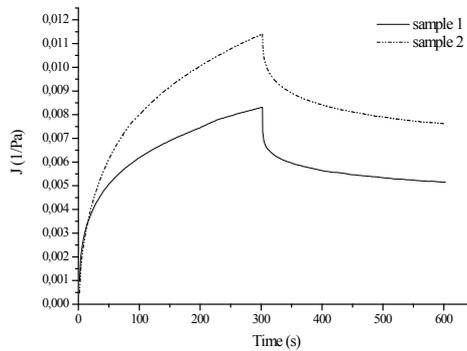


Figure 16. A creep and recovery example of viscoelastic samples.

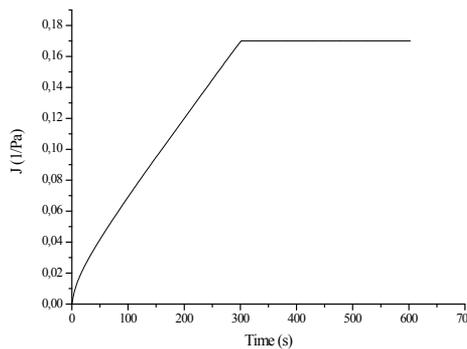


Figure 17. A creep and recovery example of a non-viscoelastic sample.

In addition, the rheology can be used to evaluate the stability over the time by dynamic and oscillatory rheological measurements (Péñzes et al., 2004; Vasiljevic et al., 2006) and the release of active principles. According to Martinez et al. (2007), the transdermal absorption of

topically administered drugs depends on the rate of release and the permeability of them into the skin and also of the viscosity of the formulation (Martinez et al., 2007).

Thus, it is possible to say that different categories of products should present peculiar rheological properties inherent to its application (Gregolin et al., 2010).

In this way, the rheology can influence the diffusion coefficient, altering the release and permeation of cosmetics active substances (Welin-Berger et al., 2001; A-sadutjarit et al., 2005; Vasiljevic et al., 2006). Some authors have related the influence of rheological characteristics on the release profiles and consequently in the permeation of active substances in the skin; thus, the addition of thickening agents or attainment of a weak-gel because of physical entanglement of polymer chains must be considered in the choice of cosmetics bases (Spiclin, et al., 2003). Thus, rheology can help in the assay of release and permeation in the skin. Some studies have been published about it.

So, in a short way, the rheology is a valuable tool that helps in the quality control of cosmetics, being used in the stability tests, in the comparison between competing samples, in the comparison between an original product and a product with an alteration in a constituent, and in the development of new products, aiming to develop cosmetic with rheological characteristics which indicate stability.

4. Small Angle X-ray Scattering (SAXS)

The use of this technique in determining the quality control of a cosmetic is closely related to the stability of the product, which could be improved with the presence of liquid crystals.

Liquid crystals are described as a state of matter between solids and liquids, it means that, they are fluid like liquids but are organized like solids, being called mesophases (Marsh, 1973; Kelker and Hatz, 1980; Müller-Goymann, 2004). These organization contributes to the highly stability of systems.

The formation of liquid crystals in emulsions could be induced by some components present in this system, such as surfactants (Müller-Goymann, 2004). So, what happens is that it is possible to find a peculiar system that is not a simple emulsion and not a genuine liquid crystal, but an emulsified system that contains liquid crystals, commonly lamellar structures, that are formed around of the inner phase of the emulsion (Oka et al., 2008), making difficult the coalescence, flocculation and the separation of the oily and water phases, what makes the system formed more stable than a simple emulsion (Figures 18 and 19). Flocculation is defined as the formation of aggregates of droplets of an emulsion under the influence of interparticle colloidal forces which are net attractive (Dickinson, 1992) and the formation of lamellar structures avoid or prevent the occurrence of this phenomenon. The formation of lamellar structures is essential to obtain emulsified oil/water systems finely dispersed, with balanced hydrophilic-lipophilic properties, resulting in minimal interfacial tension between aqueous and oily phases, thus contributing to the stability of the system (Engels et al., 1995). Previous studies have also shown that it is possible to make correlation between SAXS and

rheological analysis, since were verified that the thicker the interlamellar water layers, the higher the viscosity of the cream (Eccleston et al., 2000). Thus, liquid crystals could be responsible by the emulsion stabilization and by the increasing in the viscosity (Klein, 2002), being the presence of this structures desirable in cosmetic emulsions which could be an indicative of quality of them.

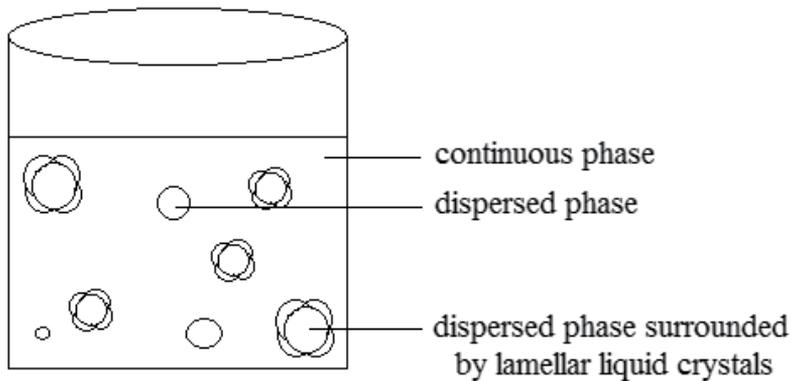


Figure 18. Scheme of a cosmetic emulsion containing liquid crystals.

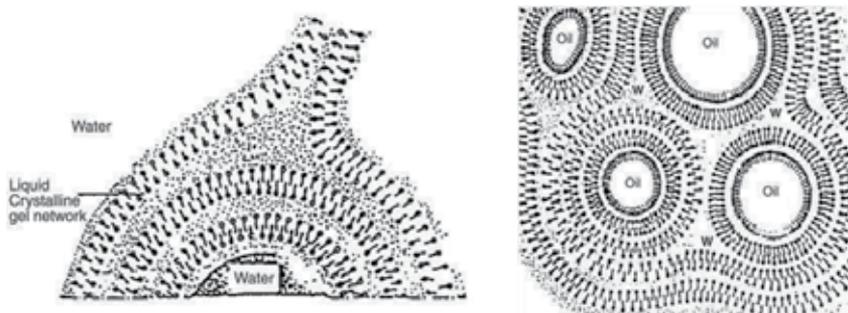


Figure 19. Schemes of the microscopic visualization of lamellar gel networks surrounding emulsion droplets proposed by Klein (2002).

This kind of structure is more commonly found in cosmetics due to the high diversity of components used in it in order to obtain a moisturizer, emollient, humectant, good sensory and, above all, stable cosmetic. In other pharmaceutical forms, usually are used a less diversified composition, which gives a system easier to understand, described as emulsion or liquid crystal, or even, a gel, a suspension, etc. The quantity of these lamellar structures, found in cosmetic emulsions, probably is dependent of three main factors: the raw-materials, the amount of it used and the process of preparation, where should be cited, the temperature and the speed of agitation.

In cosmetics, other kinds of systems could be used, such as genuine liquid crystals aiming to explore its characteristics of controlled delivery systems.

There are different kinds of liquid crystals and different classifications, but this chapter has not the function of describe them, since it have been done by many authors (Bechtold, 2005; Formariz et al., 2005; Atkins and Jones, 2006), the aim was to demonstrate the importance of these structures in the maintenance of the cosmetics' quality. Nevertheless, according to the literature data (Klein, 2002) and to our experience in this subject, it is possible to say that the lamellar arrangement is the most commonly found in cosmetic emulsions.

An initial analysis of the presence of liquid crystals in a cosmetic emulsion could be done using a polarized light microscope, but it should be confirmed and better analyzed by means of Small Angle X-Ray Scattering. When a microscope slide containing a sample of the system is studied and it presents structures that reflect the incident light, it is an evidence of the presence of liquid crystals (Figure 20). So, they should be submitted to SAXS analysis to confirm this expectation (Savic et al., 2011).

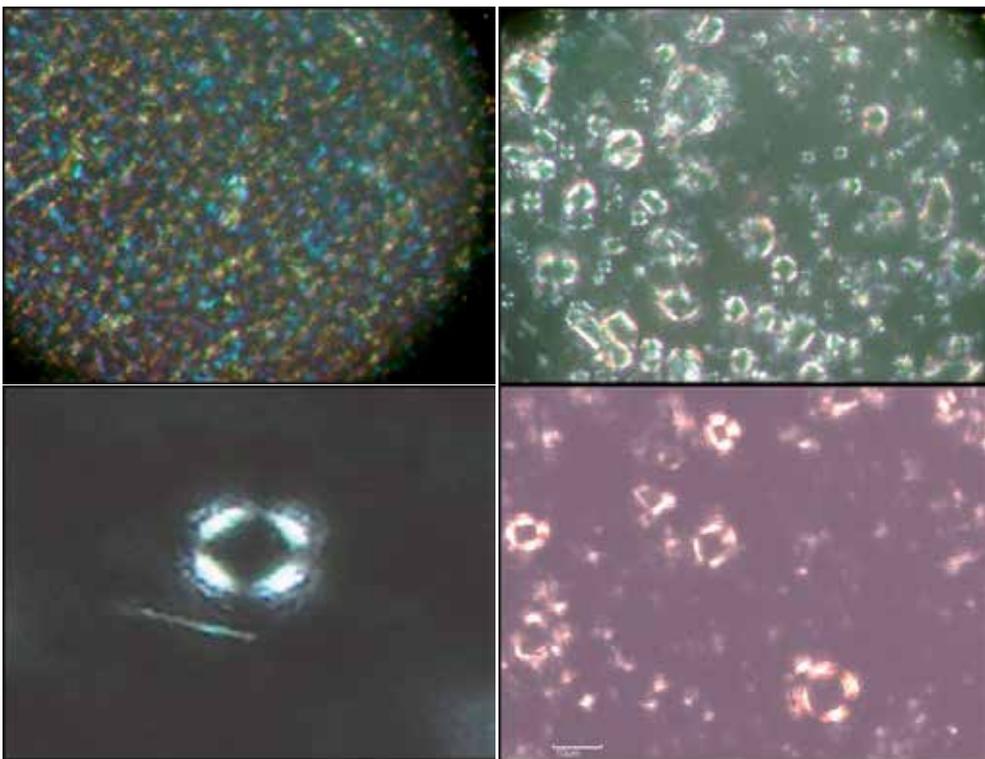


Figure 20. Photomicrographs of liquid-crystal present in emulsions evaluated by polarized light microscope.

The SAXS method requires a synchrotron light source that is formed by means of a particle accelerator, and using a monochromatic beam, that is used to irradiate the sample. After

that, the scattering of the rays in small angle should be analyzed (Glatter and Kratky, 1982; Urban, 2004; Koch, 2010). Liquid crystals can be analyzed by SAXS since they are able to disperse the X-rays focused on it. In the SAXS line is used an X-rays detector and an multichannel analyzer to capture the intense of the SAXS measures ($I(q)$) in function of the modulus of the scattering vector (q) (Glatter and Kratky, 1982; Molina et al., 2006; Koch, 2010).

Analyzing the data obtained (Figure 19), the d value obtained represents the distance between the particles able to scatter the X-rays. It is calculated by the equation: $d = 2\pi / q_{max}$, where q_{max} is the maximum intensity of scattering (Craievich, 2002). The relation between the d values obtained indicates the type of arrangement found in the system (Glatter and Kratky, 1982; Craievich, 2002; Alexandridis et al., 1998).

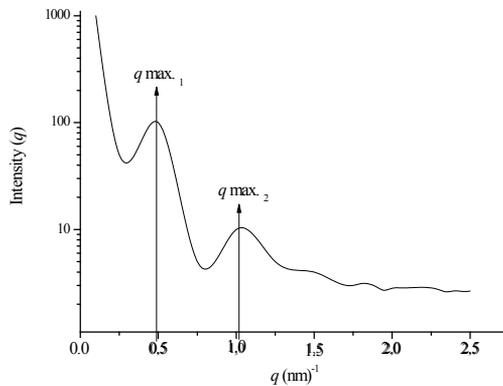


Figure 21. Hypothetical SAXS curve.

In the case of the hypothetical curve showed in Figure 19, d_1 / d_2 would result in 2, which describe lamellar structures (Alexandridis et al., 1998).

Beyond the advantages already mentioned, in a research conducted by Moaddel and Friberg (1995), the authors showed that the presence of lamellar liquid crystals in an emulsion avoids the water evaporation rate in this system, thus contributing in another way to the stability and maintenance of the cosmetic quality.

According to the advantages obtained with the presence of liquid crystals, these mesophases can be of great importance to the Cosmetic Industry in the development of very stable cosmetics and, the SAXS technique, an efficient tool to confirm the presence of these desirable structures that helps in the maintenance of cosmetics' quality control.

Camerel et al. (2003) pointed the importance in correlate the microstructure of a colloidal suspension with its rheological behavior to define its better use in industry and in life, beyond that, according to these authors there are few reports correlating these analyses.

Our research group has invested in researches to assess the stability of cosmetics (Isaac et al., 2008); evaluating of the influence of the addition of thickening agents in creams using rheological measurements (Isaac et al., 2012a); evaluating the thickeners' influence on the rheological properties of a cosmetic (Isaac et al., 2012b,c); proposing alternative methods to assay the efficacy and safety of them (Chiari et al., 2012a; Chiari et al., 2012b) and using of the sensory analysis in the cosmetics development (Isaac et al., 2012a) which, in different points of view of what was demonstrated in this chapter, also influence in the product quality.

5. Conclusion

This chapter aimed to show the facility that some simple or advanced techniques already used, sometimes to other finalities, could offer to the quality control of cosmetic products. The sensory analysis, rheology and SAXS technique have earned attention due to the important contribution that they can offer to the cosmetic area.

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Sops: What Are They Good For?

Standard Operating Procedures (What Are They Good For ?)

Isin Akyar

Additional information is available at the end of the chapter

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1. Introduction

Standardization is defined as an activity that gives rise to solutions for repetitive application to problems in various disciplines. Generally, the activity constitutes the process of establishing (determining, formulating, and issuing) and implementing standards. Thus, standards are the perfect result of a standardization activity and inside the context of quality systems consist of quality documents or documents related to the quality system. High levels of quality are important to accomplish Company business objectives. Quality, a source of competitive benefit, should stay a symbol of Company products and services. High quality is not an additional value; it is an important elementary necessity. Each employee in all organizational units is responsible for guaranteeing that their work processes are effective and continually getting better. Top management should provide the training and an appropriate motivating environment to support teamwork both inside and across organizational units for employees to advance processes. Ultimately, everyone in an institution is responsible for the quality of its products and services. An institution in the role of a sponsor of clinical trials can best achieve its business objectives by establishing and managing robust quality systems with their integral quality documents including standard operating procedures (SOPs) (Manghani, K. 2011). The Quality Management system must evolve by trial and error, with enlarging experience, by group discussions and with changing understanding. In the beginning, attention will be focused on basic operational SOPs, afterwards moving to record keeping (as more and more SOPs are issued) and filling gaps as practice admits missing links in the chain of Quality Assurance. Essentially problems will turn up. One way to react to them is to talk with people in other laboratories who have faced similar problems. It

should not be forgotten that Quality Management is a tool rather than a goal. The goal is quality performance of the laboratory. The philosopher Kant saw autonomy as self-government originating from morality, with morality proceeding from knowledge and self-discipline. Conger & Kanungo noted that an appropriate level of authority, discretion, formalization, and rule structure is a requirement for worker empowerment, which we see as consistent with the concept of self-government. Merriam-Webster defined autonomy as 'the quality or state of being self-governing; especially: the right of self-government; self-directing freedom and especially moral independence'. Necessitated SOP use will be absolutely related to the sense of self-determination experienced by workers. Worker participation in SOP advancement and clarification controls the affiliation between required SOP use and the sense of self-determination experienced by workers.

Standard Operating Procedures (SOP) is a process document that describes in detail the way that an operator should perform a given operation. SOPs involve the purpose of the operation, the equipment and materials required, how to perform the set-up and operations required for the process, how to perform the maintenance and shutdown operations carried out by the worker, a description of safety issues, trouble-shooting, a list of spare parts and where to find them, illustrations, and checklists. The SOP is one of many process documents which is needed for consistent operation of a given process, with other documents involving process flow charts, material specifications, and so forth.

The purpose of SOPs today is to guarantee that all workers are performing tasks in the same way, which is a needed condition to get expected output from the process. When all workers perform their tasks constantly, it becomes possible to run controlled experiments to test the impact of changing various process parameters. When a process change is shown to improve process performance, SOPs are updated and workers are trained to the new procedures. All over the process, it is adorable to involve workers in SOP development and to praise worker ideas for the SOP improvement. For constant organizational advance, organized processes need to be constantly improved, hence necessitating ideas from those workers using those procedures. Ideas are not creative simply because they deviate from organized knowledge; ideas are creative when they are novel and suitable to the task at hand. Workers may have many ideas; nevertheless, what they choose to do with their ideas will depend on various organizational and individual-difference factors. The most important factor, however, for the advancement of creative behaviours is worker intrinsic motivation— a *sine qua non* of worker creative contribution. By the help of confirmatory factor analysis, the Spreitzer construct validated the four dimensions of intrinsic motivation (i.e. psychological authorization): (a) Competence (example item includes 'I am confident about my ability to do my job'); (b) Meaning (example item includes 'The work I do is very important to me'); (c) Impact (example item includes 'I have a great deal of control over what happens in my department'); (d) Self-determination (example item includes 'I can decide on my own how to go about doing my work'). Furthermore, Spreitzer argued and empirically established that an antecedent condition to innovation (i.e. creativity) and effectiveness is intrinsic motivation (De Trevil et al. 2005).

2. Overview

The quality documents constitutes of Company policies, quality management plan, SOPs, working instructions, conventions, guidelines, forms, templates, logs, tags and labels. They are organized by consensus and approved by a nominated body and they provide for common and repeated use, rules, guidelines or characteristics for activities or their results with a view to promote transparency, consistency, reproducibility, interchangeability and to facilitate communication. The hierarchy and types of quality documents relevant to quality systems will depend upon Company business objectives and business model. SOPs are Level 2 quality documents and, along with other related quality documents, guarantee the efficacy and effectiveness of quality systems (Manghani, K. 2011). Standard operating procedures (SOPs) are a vital component in any quality management system (Hattamer-Apostel, R. 2001). Every good quality system is based on its Standard Operating Procedures (SOPs) (Saxena). The advancement and use of SOPs are a necessary part of a successful quality system as it supplies individuals with the information to carry out a job adequately, and aids precision in the quality and integrity of a product or end-result (United States Environmental Protection Agency, 2007). They assign all processes involved in an organization (Frank, D. 2010). A quality system is defined as the organizational structure, responsibilities, processes, procedures and resources for implementing quality management (Manghani, K. 2011).

Standard Operating Procedures are sets of instructions having the force of a directive, covering those features of operations which lend themselves to a definite or standardized procedure without loss of effectiveness (Saxena).

The purpose of a SOP is to reach out the operations correctly and always in the same manner. A SOP should be available at the place where the work is done". SOPs assist the progress of constant application of processes and procedures so even when there are changes in personnel, organizations avoid inconsistencies and safety risks (Frank, D. 2010). Standard operating procedures or SOPs are written step-by-step procedures that quality control (QC), quality assurance (QA), and production units use in order to assure the accuracy and precision of the quantitative experimental results and materials that they generate and provide in support of other units. SOP's are needed to guarantee the continuity of processes to obtain quality performance and quality products/preparations (Natural Resources Management and Environment Dept.). SOP's are alive documents that detail written instructions describing specific steps to follow in all activities under defined conditions (Jain, SK. 2008). They are used to accomplish standardization when performing specific functions and is used to set out the way practice and procedures necessitated to be performed. SOPs are written instructions and records of procedures agreed and adopted as standard practice (Cardiff University, 2009). SOP's are necessary to guarantee the progression of processes to accomplish quality performance and quality products/preparations (Jain, SK. 2008). A Standard Operating Procedure (SOP) document is a routine or repetitive activity followed by an organization. SOPs describe both technical and administrative operational elements of an organization that would be managed under a Quality Assurance Project Plan and under an organization's Quality Management Plan (Almeida S.L.), (United States Environmental Protection Agency 2001).

SOPs are determined to be specific to the organization whose activities are defined and assist that organization to maintain their quality control and quality assurance processes (United States Environmental Protection Agency 2001).

All organizations, businesses, etc. should have SOPs (Jain, SK. 2008). SOPs support employees with the information necessitated to perform their jobs regularly and help guarantee consistency in the quality of performance (Frank, D. 2010). SOPs are used by the governmental agencies, private industry, and academic laboratories by scientists and engineers from all of the science, technology, engineering, and mathematical disciplines. SOPs can also be intensely valuable in academic laboratories and can be employed anytime there is process that likely more than one person will use in a research group (Natural Resources Management and Environment Dept.). SOPs are mainly associated with specific documentation necessities. It should not be forgotten that "If you don't document, it didn't happen!" (Jain, SK. 2008). The International Conference on Harmonization Good Clinical Practice (ICH GCP) guideline ascertains SOPs as "detailed, written instructions to achieve uniformity of the performance of a specific function". SOPs must be well written in order to supply an efficacious control of good clinical practice (GCP) and prevent errors from occurring, thereby lessening waste and rework. Poorly written SOPs are a source of misinformation. To be user friendly, they should be absolute, unambiguous and must be written in plain language. SOPs are controlled documents and are best written by persons involved in the activity, process or function that is required to be specified or covered in the SOP. SOPs must be reviewed prior to their approval for release, for adequacy, completeness and compliance with Company standards and all applicable legal, ethical and regulatory requirements. They must be checked out and updated as necessitated over their life cycle and any changes made to the SOPs must be re-approved. They must bear a revision status on them and their distribution must continually be documented and controlled. When obsolete SOPs are needed to be held for any purpose, they should be suitably identified to prevent unintended use. Only relevant SOPs in their current version must be available at points of use and must remain legible. SOPs are mandatory for the implementation of GCP and other GxPs, namely, cGMP (Good Manufacturing Practice) and GLP (Good Laboratory Practice), within the scope of quality systems; therefore, it is well said that without SOPs there are no GxPs: no SOPs, no quality systems, and no GxPs (Manghani, K. 2011). SOPs are necessary for a clinical research organization whether it concerns a pharmaceutical company, a sponsor, a contract research organization, an investigator site, an Ethics Committee or any other party involved in clinical research to achieve maximum safety and efficiency of the performed clinical research operations. It is therefore a must that all people and sites involved in clinical studies (both at the sponsor and at the investigative sites) have suitable SOPs in place so as to conduct clinical research and to ensure compliance with the current regulations.

The presence of these quality documents is important when regulatory inspections (FDA, EMEA) take place since the most frequent reported deficiencies during inspections are the lack of written SOPs and/or the failure to adhere to them. The risk of GMP non-compliance is high at organizations with a poor suitability of specific SOPs and also if at all they are

achievable the staff or the people for whom they were written are not either following them. It therefore becomes very essential for the personnel to be trained on these SOPs so that they are absolutely aware of why and how SOPs can play important role in fulfilling the specific organizational requirements from WHO, FDA, EMEA or other national health authorities. Health authorities world wide like the FDA or EMEA expect pharmaceutical, cosmetic and food producers to describe their manufacturing processes in written SOPs (GMP7.com). An organization's SOP manual is an important training document and provides workers with increased confidence, motivation and a sense of achievement (Frank, D. 2010). A SOP is a compulsory instruction. If deviations from this instruction are allowed, the conditions for these should be documented including who can give permission for this and what exactly the complete procedure will be. The original should rest at a secure place while working copies should be authenticated with stamps and/or signatures of authorized persons. The advancement and use of SOPs are a basic part of a successful quality system. It supplies information to perform a job regularly, and constantly in order to access pre-determined specification and quality end-result.

SOP clarifies the followings; what is the objective of SOP (Purpose), what are applicability and use of SOP (Scope)?, who will perform tasks (Responsibility), who will ensure implementation of procedure (Accountability), how tasks will be performed (Procedure).

Responsibility	Responsible
Identifying the need for development or revision of a standard operating procedure (SOP) and to convey that need to their immediate supervisor and/or the QA Manager (QAM).	Staff
An individual SOP to include sufficient detail that the process or procedure can be followed by another person when needed.	Author
Requesting peers to review the SOP to determine whether it contains sufficient detail.	Author
Reviewing and approving the SOP prior to its use.	Immediate supervisor and the QA Manager
Ensuring that the procedure or process follows the details noted in the individual SOP and to detail in writing when the SOP or a component of that SOP has not been followed.	Staff and the QAM
Ensuring that all routine operations and activities in their area are documented by SOPs.	Manager
Overseeing the appropriate preparation, numbering, retention, indexing, revision, and use of SOPs.	QAM
(United States Environmental Protection Agency, 2007).	

Table 1. Responsibility distribution in SOP.

Procedures are not an end in themselves - they do not ensure good performance or results. More important are well-designed systems and processes, qualified employees, and a motivating company culture. Procedures provide process people – environment but do not create processes, qualified people, or a good working environment (Jain, SK. 2008). The responsibility distribution in a SOP is shown in Table 1.

3. Purpose

The purpose of SOP is to assign the procedures for the preparation, approval, distribution, amendment and storage of Standard Operating Procedures (Cardiff University, 2009). The purpose or objective of the procedure should express and expand well written title (Jain SK., 2008). SOPs serve as frame for organizational action – support direction and structure. They tell what, how, when, why, and who. (Iowa State University, 2010). In order to be active, SOPs need to define not only what needs to be, but who is qualified to carry it out, and under what conditions the procedure can be performed reliably (Levine D.I., 2010). They should aid constant conformance support data quality. They should be determined to be specific to the organization and assist that organization to obtain their quality control and quality assurance processes and ensure compliance (Almeida S.L.) SOPs specify the commonly recurring work processes that are to be conducted or followed inside an arrangement. They approve the way activities are to be performed to alleviate constant conformance to technical and quality system necessities and to provide data quality. They may define, for example, basic programmatic actions and technical actions such as analytical processes, and procedures for maintaining, calibrating, and using equipment. If not written appropriately, SOPs are of limited value. Additionally, the best written SOPs will fail if they are not followed. Therefore, the use of SOPs needs to be checked out and re-enforced by management, alternatively the direct supervisor. Current copies of the SOPs also need to be readily accessible for reference in the work areas of those individuals absolutely carrying out the activity, either in hard copy or electronic format, otherwise SOPs serve little purpose (United States Environmental Protection Agency, 2007).

4. Benefits

The improvement and use of SOPs promotes quality through consistent implementation of a process or procedure within the organization reduced work effort, along with advanced data comparability, credibility, and legal defensibility (Almeida S.L.) The details in an SOP *standardize* the process and support step-by-step how-to instructions that enable *anyone* within your operation to perform the task in a consistent manner (Iowa State University, 2010). They abbreviate difference and advance quality through constant impact of a process or procedure inside the organization, although there are temporary or permanent personnel changes.

SOPs can signify agreement with organizational and governmental needs and can be used as a part of a personnel training program, since they should supply detailed work instructions. It minimizes opportunities for miscommunication and can address safety concerns.

When historical data are being estimated for current use, SOPs can also be very important for reconstructing project activities when no other references are accessible. Besides, SOPs are commonly used as checklists by inspectors when auditing procedures. Eventually, the benefits of a valid SOP are decreased work effort, along with developed comparability, credibility, and legal defensibility.

The advancement and use of SOPs is a basic part of a successful quality system. It supplies individuals with the information to perform a job regularly and aids constancy in the quality and integrity of a product or end-result through constant implementation of a process or procedure inside the arrangement.

SOPs can also be used as a part of a personnel training program, hence they should support detailed work instructions. When historical data are being assessed for current use, SOPs can be beneficial for reconstructing project activities. Additionally, SOPs are commonly used as checklists by inspectors when auditing procedures. Finally, the benefits of a valid SOP are minimized work effort, together with improved data comparability, credibility, and legal defensibility. SOPs are necessary even when published methods are being administered because cited published methods may not include appropriate information for conducting the procedure in-house.

For example, if the SOP is written for a standard analytical method, the SOP should designate the procedures to be followed in greater detail than appear in the published method, detailing how, if at all, the SOP differs from the standard method and any options, changes or alterations that the organization follows (United States Environmental Protection Agency, 2007). The significance regularly set up and managed quality control and quality assurance systems with their integral well-written SOPs and other quality documents for the achievement of Company business objectives cannot be ignored. They serve as a passport to success by assisting the Company to accomplish high-quality processes, procedures, systems, and people, with eventual high-quality products and services and enhancement of the following: Customer satisfaction, and therefore, customer loyalty and repeat business and referral; timely registration of drugs by eliminating waste and the requirement for rework; operational results such as revenue, profitability, market share and export opportunities; alignment of processes with achievement of better results; understanding and motivation of employees toward the Company quality policy and business objectives, as well as participation in continuous quality improvement initiatives; and confidence of interested parties in the effectiveness and efficiency of the Company as demonstrated by the financial and social gains from Company performance and reputation (Manghani, K. 2011). Benefits of SOPs are shown in Table 2.

Benefit	Explanation
To provide people with all the safety, health, environmental and functional information necessitated to perform a job properly.	Placing value only on production while disregarding safety, health and environment is costly finally. It is better to train employees in all aspects of doing a job than to face accidents, fines and litigation later
To guarantee that production operations are performed constantly to obtain quality control of processes and products.	Consumers, from individuals to companies, want products of consistent quality and specifications. SOPs specify job steps that help standardize products and consequently quality.
To guarantee that processes continue uninterrupted and are completed on a prescribed schedule.	By following SOPs, you help to guarantee against process shut-downs caused by equipment failure or other facility damage
To guarantee that no failures occur in manufacturing and other processes that would harm anyone in the surrounding community.	Following health and environmental steps in SOPs guarantees against spills and emissions that threaten plant neighbors and create community outrage
To guarantee that acknowledged procedures are followed in compliance with company and government regulations.	Well-written SOPs help to guarantee that government regulations are satisfied. They also show a company's good-faith intention to operate perfectly. Failure to write and use good SOPs only signals government regulators that your company is not serious about compliance.
To serve as a training document for teaching users about the process for which the SOP was written.	Thorough SOPs can be used as the basis for supplying standardized training for employees who are new to a particular job and for those who need re-training.
To serve as a checklist for co-workers who observe job performance to reinforce proper performance.	The process of actively caring about fellow workers involves one worker coaching another in all aspects of proper job performance. When the proper procedures are outlined in a good SOP, any co-worker can coach another to help improve work skills.
To serve as a checklist for auditors.	Auditing job performance is a process similar to observation mentioned in the previous item only it usually involves record keeping. SOPs should serve as a strong basis when detailed audit checklists are developed.
To serve as an historical record of the how, why and when of steps in an existing process so there is a factual basis for revising those steps when a process or equipment are changed.	As people move from job to job inside and between companies, unwritten knowledge and skills disappear from the workplace. Regularly maintained written SOPs can chronicle the best knowledge that can serve new workers when older ones move on.
To serve as an explanation of steps in a process so they can be reviewed in accident investigations.	Although accidents are unfortunate, view them as opportunities to learn how to improve conditions. A good SOP gives you a basis from which to being investigating accidents

(Jain, SK. 2008)

Table 2. Benefits Of SOPs.

5. Writing style

SOPs should be written in a step-by-step, easy-to-read format by subject-matter experts who know the processes and the structure of the organization (Frank, D. 2010). They should be written by individuals aware of the activity and the organization's internal structure. These individuals are basically subject-matter experts who actually perform the work or use the process. A team accession can also be followed, particularly for multi-tasked processes where the experiences of a number of individuals are critical (United States Environmental Protection Agency, 2007).

Well-written SOPs should first shortly define the purpose of the work or process, involving any regulatory information or standards that are suitable to the SOP process, and the scope to show what is covered. Any specialized or different terms either in a separate definition section or in the suitable discussion section should be explained.

The information presented should be clear and easy to understand. The active voice and present verb tense should be used. SOP shall be simple and short. Information should be transported clearly and absolutely to remove any doubt as to what is needed. Flow charts should be used to illustrate the process being defined (Jain SK., 2008), (United States Environmental Protection Agency, 2007). (United States Environmental Protection Agency, 2001), (Almeida S.L.). It may be helpful to include additional experts to help gather information and to review, test and approve draft SOPs (Frank D., 2010).

The most commonly used method of task analysis is *Hierarchical Task Analysis (HTA)*. Operating instructions should be close to the user and kept up to date. The following issues should be considered in evaluating operating procedure documentation:

1. There should be no easier, more dangerous opportunities than following the procedure.
2. There should be an appropriate QA system in place to guarantee that the procedures can be kept up to date and that any errors are rapidly detected and corrected.
3. The procedures should not be needlessly prescriptive. The best way of guaranteeing that procedures do not become overly prescriptive is through involving the operator during the design stage.
4. Procedures should contain information on the necessities for the wearing of personal protective equipment during the task.
5. Any risks to the operator should be documented at the start of the procedure, based on a risk assessment of the task.
6. An appropriate method of coding each procedure should be used.
7. Each time a procedure is produced it should be dated.
8. There should be no uncertainty between which procedures apply to which situations.
9. Procedures do not always have to be paper based.

10. At the start of the procedure an overview of the task should be provided.
11. Prerequisites should be presented clearly at the start of the procedure to guarantee that the operator can check that it is safe to proceed.
12. The most important information on the page should be defined and this should be designed to be the most prominent information.
13. Separate headings should be used to discriminate apparently between sub tasks.
14. Any warnings, cautions or notes should be placed immediately prior to the instruction step to which they refer.
15. Language should be kept as simple as possible, i.e. use nomenclature familiar to the operator.
16. The nomenclature should be consistent with that on controls or panels.
17. Symbols, colours, and shapes used for graphics should conform to industry standards (Health and Safety Executive).

6. Preparation of SOP

When actualizing a SOP one can choose number of different ways to organize and format them. There are some factors which determine what type of SOP to use or create: How many decisions will user need to make during process? How many steps and sub steps are there in procedure? Routine procedures that are short and necessitate few decisions can be written using simple steps format. Long procedures consisting of more than 10 steps, with few decisions should be written along with graphical format or hierarchical steps. Procedures that necessitate many decisions should be written along with flow chart. Requirement for document identification and control, accountability and traceability responsibility must be involved with every SOP; this can be obtained by supporting constant format.

The need for an SOP or the revision of an existing one should be identified by informing the appropriate supervisor. Written instructions on standardized procedures supply guidance to guarantee that activities are conducted in a constant way, hence leading to reliable product and service quality. SOPs should be prepared in full compliance with guidelines and organizations and must mirror current organizational practices (Hattamer-Apostel, R. 2001). Ideally, SOP's should be written by teams that involve some or all of the following people: Those who will perform the job, those who will perform maintenance on equipment involved in an SOP, engineers or others who design equipment and processes, technical initiator, safety personnel, environmental personnel, equipment manufacturers (Jain, SK. 2008).



Figure 1. A SOP should be written by a team in that field.

7. Implementing SOP

The most substantial step for administering the SOP in working area, train or retrain the user. Every one should follow the procedure accurately with each and every step in detail, it is very significant to train the user otherwise individual may interpret meaning in different ways.

The trainer should share the reason WHY, SOP must performed correctly while training the user. People can follow better when they understand significance of procedure. Trainer should explain and demonstrate how each step in the SOP will be performed and should assure them this will increase Quality of product by providing safety and accuracy which will ultimately increase the confidence of the user.

The people in the writing team can write or edit parts of an SOP independently and then one person can combine the individual contributions. After combination the SOP should be circulated the draft SOP for review among the initiator before editing a final draft for review by supervisors and subsequent supervised testing by employees. Ideally a writing team should meet at least once in the beginning of a project to establish writing objectives, targets and responsibilities, but then can work semi-independently with one person serving as co-

ordinator. SOPs should be checked out by several people qualified to assess the SOP in terms of its completeness and clarity of subject matter.

SOPs should at least mention:

- a.* who can or should make which type of SOP;
- b.* to whom proposals for a SOP should be submitted, and who estimates the draft;
- c.* the procedure of approval;
- d.* who decides on the date of implementation, and who should be informed;
- e.* how revisions can be made or how a SOP can be withdrawn.

It should be organized and recorded who is responsible for the proper distribution of the documents, the filing and administration (e.g. of the original and further copies). Finally, it should be indicated how frequently a valid SOP should be periodically evaluated (usually 2 years) and by whom. Only officially issued copies may be used, only then the use of the proper instruction is guaranteed (United States Environmental Protection Agency, 2007).

8. SOP Review and approval

SOPs should be reviewed (that is, validated) by one or more individuals with appropriate training and experience with the process (Almeida S.L.).

It is especially helpful if draft SOPs are completely tested by individuals other than the original writer before the SOPs are finalized. The completed SOPs then must be checked out and approved by peer reviewers, the QA Manager, and appropriate management prior to the use of the SOP. A set format in styling, information necessitated, and a numbering system is required, as well as biannual or annual review to ensure that the procedure is up-to-date. An archival system is needed to ensure that an historical record can be maintained and only current SOPs are available for staff use (United States Environmental Protection Agency, 2007).

The finalized SOPs should be approved as described in the organization's Quality Management Plan or its own SOP for preparation of SOPs. Generally the immediate supervisor, such as a section or branch chief, and the organization's quality assurance officer review and approve each SOP. Signature approval indicates that an SOP has been both reviewed and approved by management. When practical, use of electronic signatures, as well as electronic maintenance and submission, is an acceptable substitution for paper.

SOP general form defines an integrated system of management activities involving planning, implementation, documentation, assessment, and improvement to ensure that a process, or item, is of the type and quality needed for the project (United States Environmental Protection Agency, 2007), (Jain, SK. 2008).

9. Revising SOPS

If the SOP does not definitely describe the procedure, then the SOP must be revised. Any change in the procedure must be included into the SOP. Nevertheless, prior to any change to the SOP, management must be advised of, and approve, the change.

If there are some errors in the finalized SOPs, such as typographical errors, printing errors, e.g., wrong page numbers or misaligned sentences) or any errors that do not affect the scope of the procedure, they may be correctly immediately and reprinted. These types of errors do not require full SOP revision, thus a revision number will not be generated and management approval is not needed. If the error occurs on the signature page then the signature page will be resigned. These types of corrections will be traceable since the historical file will reflect all corrections including typographical errors. Specifically, the historical SOP file will contain both the SOP with the correct page(s) as well as the page(s) containing the error. The page with the error will not be removed from the historical file. Additions can be made to an SOP via a clarification or an addendum. Explanations and addenda must be attached to the appropriate SOP until such time that the SOP can be revised. Usually, the revision will be organized during the biannual review process. When the SOP is revised, the revision number is updated. Revisions, explanations, and addenda are prepared by appropriate personnel, but must be approved by management. An SOP can be eliminated when it is no longer applicable. Management must approve the elimination of an SOP. Two or more SOPs can be consolidated; in this case one SOP supersedes the other, but management approval is required for consolidation of procedures. The signed revised SOP must be sent to the historical file for archiving (United States Environmental Protection Agency, 2007).

10. Frequency of revisions & Reviews

SOPs necessitate to remain current to be useful. The review process should not be overly cumbersome to encourage timely review. Therefore, whenever procedures are changed, SOPs should be updated and re-approved. If desired, only the pertinent section of an SOP can be modified and indicate the change date/revision number for that section in the Table of Contents and the document control notation.

SOPs should also be reviewed systematically on a periodic basis, e.g. every 1-2 years, to ensure that the policies and procedures remain current and suitable, or to decide whether the SOPs are even needed. The review date should be added to each SOP that has been reviewed. If an SOP defines a process that is no longer followed, it should be removed from the current file and archived (Almeida S.L.) The frequency of review should be indicated by management in the organization's Quality (Jain, SK., 2008).

11. Checklists

SOPs should describe how the checklist is to be prepared or on what it is to be based (Almeida S.L.)

Many activities use checklists to guarantee that steps are followed in order. Checklists are also used to document completed actions. Any checklists or forms involved as part of an activity should be referenced at the points in the procedure where they are to be used and then attached to the SOP (United States Environmental Protection Agency, 2007).

In some cases, detailed checklists are prepared specifically for a given activity. In those cases, the SOP should describe, at least generally, how the checklist is to be prepared, or on what it is to be based. Copies of specific checklists should be then maintained in the file with the activity results and/or with the SOP.

Many activities use checklists to guarantee that steps are followed in order. Checklists are also used to document completed actions. Any checklists or forms involved as part of an activity should be referenced at the points in the procedure where they are to be used and then attached to the SOP. In some cases, detailed checklists are prepared specifically for a given activity. In those cases, the SOP should describe, at least generally, how the checklist is to be prepared, or on what it is to be based. Copies of specific checklists should be then maintained in the file with the activity results and/or with the SOP. Remember that the checklist is not the SOP, but a part of the SOP (Jain SK., 2008).

12. Document Control

Each organization should develop a numbering system to systematically identify and label their SOPs, and the document control should be described in its Quality Management Plan. Usually, each page of an SOP should have control documentation notation. A short title and identification (ID) number can serve as a reference designation. The revision number and date are very useful in identifying the SOP in use when reviewing historical data and is critical when the requirement for observable records is included and when the activity is being reviewed (United States Environmental Protection Agency, 2007).

13. SOP Document Tracking and Archival

The organization should sustain a master list of all SOPs. This file or database should show the SOP number, version number, date of issuance, title, author, status, organizational division, branch, section, and any historical information regarding past versions. The QA Manager (or designee) is usually the individual responsible for sustaining a file listing all current quality-related SOPs used inside the organization. If an electronic database is used, automatic "Review SOP" notices can be sent. Note that this list may be used also when audits are being considered or when questions are raised as to practices being followed within the organization.

The Quality Management Plan should indicate the individual (s) responsible for assuring that only the current version is used. That plan should also designate where, and how, outdated versions are to be maintained or archived in a manner to prevent their continued use, as well as to be available for historical data review.

Electronic storage and retrieval mechanisms are generally easier to access than a hard-copy document format. For the user, electronic access can be limited to a read-only format, thereby protecting against unauthorized changes made to the document (United States Environmental Protection Agency, 2007).

14. SOP General Format

This term describes an integrated system of management activities involving planning, implementation, documentation, assessment, and improvement to ensure that a process, or item, is of the type and quality needed for the project (Levine, D.I. 2010).

How should a SOP be organized? A SOP should be organized to ensure ease and efficiency in use and to be specific to the organization which develops it. There is no one 'correct' format; and internal formatting will vary with each organization and with the type of SOP being written.

How much detail needs to be included in a SOP? A SOP should be written with sufficient detail so that someone with a basic understanding of the field, can successfully reproduce the activity or procedure when unsupervised (United States Environmental Protection Agency, 2007).

The QA systems in place will be covered in general by 'standard operating procedures' (SOP) and will be made up of the following essential components:

SOPs should be organized to guarantee ease and efficiency in use and to be specific to the organization which develops it. There is no one "correct" format; and internal formatting will vary with each organization and with the type of SOP being written. Where possible break the information into a series of logical steps to avoid a long list. The level of detail provided in the SOP may differ based on, e.g., whether the process is critical, the frequency of that procedure being followed, the number of people who will use the SOP, and where training is not routinely available. A generalized format is discussed next (Levine, D.I. 2010).

Organization shall have SOP on Preparation, approval, revision and control of standard Operating Procedure for more excellent control and management of SOPs. Before finalizing and distributing SOPs, organizations should get the documentation reviewed and validated by people with training and experience on the processes. If the SOP does not definitely define the procedure, then the SOP must be revised. Any change in the procedure must be included into the SOP. After all, prior to any change to the SOP, management must be advised of, and approve, the change. Finalized SOPs, containing typographical errors, printing errors, e.g., wrong page numbers or misaligned sentences) or any errors that do not act on the scope of the procedure may be corrected immediately and reprinted.

An organization's SOPs should be written in a format that is tailored to the organization type and its unique requirements. In general, administrative/programmatic SOPs will consist of five elements: Title page, Table of Contents, Purpose, Procedures, Quality Assurance/Quality Control, and Reference (Frank, D. 2010). General SOP format is shown in Table 3.

Element	Explanation
Title page	<p>The SOP should be arranged to guarantee ease and efficiency in use and to be specific to the organization (Almeida S.L.) Each SOP produced will be issued with a unique SOP number for reference purposes. This will be located in the table on the front page and in the footer of the document. This number will state where the SOP originated, the year it was produced, the SOP number and also state the version number. The SOP reference and effective date should be included in the footer on each page of the SOP. The first page or cover page of each SOP should contain the following information: a title that clearly identifies the activity or procedure, an SOP identification (ID) number, date of issue and/or revision, the name of the applicable agency, division, and/or branch to which this SOP applies, and the signatures and signature dates of those individuals who prepared and approved the SOP. Electronic signatures are satisfactory for SOPs obtained on a computerized database. (Jain SK., 2008), (United States Environmental Protection Agency, 2001), (Almeida S.L.), (Frank D., 2010). The Author shall be the individual primarily responsible for writing the SOP.</p> <p>Chapter pages: Chapter pages can help divide content by area or task type. Chapter pages serve as mini title pages introducing each section and indicate dates for the most recent revisions.</p> <p>Title – a clear, brief title describing the aim of the SOP and the conditions under which it can be accurately used (Levine D.I., 2010).The title should use directive language to declare what is being done to what (United States Environmental Protection Agency, 2007). Each SOP should be given a unique name which captures the significance of the practice described (Levine D.I., 2010).</p>
Table of Contents	<p>Table of contents is not required if SOP is three pages or less.</p> <p>A table of contents may be necessitated for quick reference, particularly if the SOP is long, for locating information and to designate changes or revisions made only to certain sections of an SOP. Denotes changes or revisions made only to certain sections of a SOP (Almeida S.L.), (United States Environmental Protection Agency, 2007).</p>
Definitions	<p>There should be a part defining any words, phrases, or acronyms having special meaning or application (United States Environmental Protection Agency 2001).</p>
Purpose	<p>Each chapter should first briefly describe the purpose of the work or process, including any regulatory information or standards that are appropriate to the process (Levine, D.I. 2010). It is recommended to include criteria for the control of the described system during operation.</p>
Procedures	<p>In general there are four major types of procedure: Procedures that supply general operating guidance; an aid to meeting operating aims; mandatory and prescribe behaviour; and used as a training tool.</p> <p>The key to any program striving for quality is the set of Standard Operating Procedures (SOPs) that describe how work is to be done. The procedure section will identify how the aims will be achieved. This will clearly indicate a step by step description of how the procedure to be followed. Steps should include products and equipment required, possible obstacles, personnel qualifications and safety considerations. For lengthy process descriptions, a flow chart might be necessary to define processes that often involve interferences or variances.</p>

Procedures (continued) If calculations are involved in analyzing the data, then an example of the calculation should be provided. Figures and tables showing laboratory apparatus, representative data, etc. can be included here (Levine, D.I. 2010).

Once the necessity for a particular SOP is organized, it should be drafted immediately. SOPs are drafted by laboratory or supervisory staff qualified to perform the procedure. Next the SOP is reviewed by other staff, where possible, and then approved by the QA Manager (QAM) and management, such as immediate supervisor. Circulation to staff members for review/comment is advisable prior to acquiring management approval. The SOPs should be written to define study methods or procedures in sufficient detail so as to guarantee the quality and integrity of the data or procedure to be followed. When writing SOPs, the detail used may include both procedural requirements (exact instructions) and guidance information (general information) on the procedure. Procedural requirements must be followed accurately, while guidance information is used to help perform the procedure; it is not a mandatory requirement and, therefore, it does not have to be followed exactly. Procedural requirements can be distinguished from guidance elements, based on the context they are used. Office standard format for margins, font, and font size should be followed. Official SOPs will have a colored header and footer on each page, dated signatures on the front title page, and be printed on ivory colored paper with a watermark. An outline format should be used and include alpha and/or numeric characters are to be included to indicate levels of information (United States Environmental Protection Agency, 2007).

A SOP should be written as soon as the need for a standard written procedure for an activity is required (Cardiff University, 2009). How much someone knows about an entire process or job affects the way he or she does that job. Incorporate safety, health and environment into the traditional how-to-operate or how-to-do steps. Based on best practice/standards, the procedure should be written in specific detail to ensure that the procedure can be repeated in a reproducible fashion to include the order of steps that should be followed, the times allowed for each step (as needed) and the temperatures at which the steps are performed.

It should be kept in mind that many people do not read all the steps before starting on step one. ASOP should be written as long as necessary for a specific job. People tend to ignore long SOPs because they cannot remember more than 6 to 12 steps. If the SOP goes beyond 10 steps, the following solutions should be considered; The long SOPs should be broken into several logical sub-job SOPs, an accompanying shortened SOP should be written that lists only the steps but not detailed explanations of those steps, and the long-form SOP should be made as a training document or manual to supplement the shorter sub-job SOPs mentioned earlier (Jain, SK. 2008).

All SOPs before implementation or after revision will be approved by the management committee before implementation. Previous versions of all documentation will be stored electronically, with only the current versions available in the biorepository file.

All SOPs will be checked out on an annual basis by the management committee. Protective equipment that should be worn by staff when performing the procedure described. A list of the equipment needed to perform the procedure. All materials and supplies should be recorded. The date the procedure was first introduced as well as the date of the most recent version. The date format should be based on the ddmmyyy system where d represents day, m represents month and y represents year.

Procedures (continued)	<p>Personnel Qualifications/Responsibilities (identifying any special qualifications users should have such as certification or training experience and/or any individual or positions having responsibility for the activity being described)(United States Environmental Protection Agency 2001)</p> <p>Any related SOPs (of operations used in the present SOP); possible safety instructions should be added.</p> <p>Generally there are four types of procedure: (Health and Safety Executive).</p> <p>General operating guidance procedures</p> <p>Procedures that aid providing procedures to meeting operating aims</p> <p>Mandatory and behaviour prescribing procedures.</p> <p>Training tool procedures</p> <p>Scope and Applicability—under what specific conditions can this protocol be used reliably; are there any known interferences or other limitations on the protocol’s effective use?</p> <p>Introduction—Appropriate background information on the system, methods, and instruments are used. The background section should plan the procedure and the specific aim of the SOP.</p> <p>Materials and Supplies—There should be a list of any reagents involving names of suppliers used in this procedure. If the suppliers are obscure sources, a list of addresses and contact information should be supplied as well. Cautions – If there are some specific health and safety precautions they should be considered. For example, should gloves be worn? If so, what kind? How should spills, if they occur, be cleaned up? Are there any special procedures that should be followed in order to safely dispose of waste? (Levine, D.I. 2010). Some SOPs should be written for people who perform under different interpersonal circumstances, people who work alone, two or more people who work together as a team, for people who will control other people doing a job, for people who not familiar with rules generally understood by your employees (Jain, SK. 2008).</p> <p>Well-written SOPs should first briefly describe the purpose of the work or process, including any regulatory information or standards that are appropriate to the SOP process, and the scope to indicate what is covered. Diagrams and flow charts should be used to help to break up long sections of text and to briefly summarize a series of steps for the reader(Almeida S.L.)</p> <p>The age, education, knowledge, skill, experience and training, and work culture of the individuals should be considered who will be performing the SOP steps.</p> <p>Criteria, checklists, or other standards should be applied during the procedure such as citing the document as guidance for reviewing SOPs Records Management (specifically, e.g., as forms to be used and locations of files.</p> <p>Once writing of an SOP have been completed, there should be several workers test it and give you feedback.</p>
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Health and Safety Warnings	Primarily for technical SOPs
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Cautions	Primarily for technical SOPs
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Interferences	Primarily for technical SOPs
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Quality Assurance/Quality Control The preparation of appropriate QC procedures (self-checks, such as calibrations, recounting, reidentification) and QC material (such as blanks - rinsate, trip, field, or method; replicates; splits; spikes; and performance evaluation samples) that are needed to display successful performance of the method should be defined.

All SOPs should be checked out annually to certify all SOPs are in line with current processes, guidelines and regulations. They should be checked out in regards with the review date assigned and recorded on the front page of each SOP. The designated individuals will approve all SOP amendments. All significant amendments will be required 2 months in advance of the annual review. Any control steps and provisions for review or oversight should be defined prior to acceptance of the product or deliverable. This can involve test plans such as verification and validation plans for software or running a "spell-check" program on the finished document (United States Environmental Protection Agency 2001).

Finally, next all appropriate QA and quality control (QC) activities for that procedure should be defined, and list any cited or significant references (United States Environmental Protection Agency, 2007). Before finalizing and distributing SOPs, organizations must get the documentation reviewed and validated by people with training and experience on the processes. Additionally, it is a good idea to have the SOPs tested by staff who will be asked to comply with them. By following these steps, the author can identify missing information or needed revisions. Once SOPs are approved, they should be made readily available to facility management, building occupants and cleaning employees. The SOP will require final approval and authorization. The signature on an SOP will authorize the associated forms which should show an identical issue date to the SOP. When an SOP is issued and become effective, adequate time is required for training purposes. Finally, SOPs must remain current, so they should be updated and re-approved at least annually or whenever procedures change. Though the SOP development process takes time and effort, it can provide significant improvement to a cleaning organization's operational results and workers' understanding and job performance (Frank, D. 2010).

All SOPs are reviewed by the applicable supervisor at least every two years in order to maintain their relevancy. Names of those individuals who have reviewed and approved the SOP for use in the laboratory. Signatures and dates should be supplied whenever possible as well. For those SOPs which do not necessitate a revision, documentation attesting to that fact must be submitted to the QAM who in turn initials and dates the table located at the bottom of the title page of the original SOP (United States Environmental Protection Agency, 2007). All SOPs require version control to ensure that individuals are using the correct version of SOP. It is good to practice to assign a document a version number, in the format N. n where N represents a finalized document and n represents draft versions. Each new, approved and finalized document a major version number... should be assigned. When taking a document for revision or as draft, assign a new minor version. During the review cycle assign each new revision of the draft the next minor version, upon approval/finalization of the document assign the next major version (United States Environmental Protection Agency 2001), (Natural Resources Management and Environment Dept.).

References References relating to the development of the SOP are required to be listed. These may include other SOPs, regulatory guidelines and published papers etc. Documents listed in the SOP must be recorded in the appendices and listed accordingly (United States Environmental Protection Agency 2001).

Contact list	It should involve contact details for relevant individuals such as author of document.
Appendices	This section should list appendices of other SOPs referenced in the document, or related to the procedure.
Distribution	<p>Once approved the original paper SOP folder, it will also contain supporting documentation relating to each approved SOP for referencing purposes. An electronic copy of the SOP will be held. Approved SOPs will be distributed in hard copy to PDs and will be published. The paper version of the abandoned SOP will be filled into the archived SOP folder. All SOPs will be checked out and approved annually before it is superseded, unless a specific reason for a 6 month review can be justified. All SOPs must be kept for the duration of the project.</p> <p>When the Sop fulfils all the necessary requirements it is printed. The author hands over the manuscript (or the floppy disk with text) to the SOP administrator who is responsible for the printing. The number of copies is decided by him/her and the author. Copying SOPs is forbidden. Extra copies can be obtained from the SOP administrator. The author (or his successor) signs all copies in the presence of the administrator before distribution. As the new copies are distributed the old ones (if there was one) are taken in. For each SOP a list of holders is made. The holder signs for receipt of a copy. The list is kept with the spare copies. Users are responsible for proper keeping of the SOPs. If necessary, copies can be protected by a cover or foil, and/or be kept in a loose-leaf binding.</p> <p>Appropriate SOPs will be placed in green binders to be found in a designated spot in each work area, e.g., laboratory, equipment rooms, the library, etc., and shall be available to staff and managers. These binders will not be located in the supervisor's office. Removal of an individual SOP requires completion of the sign-out located on the insider of the binder. The binder must not be removed from its designated spot by anyone other than the QAM or laboratory director. It is the responsibility of the QAM to update each binder as individual SOPs are revised. The staff is required to read any revised SOP within 7 working days of issuance if the SOP is applicable to their work. Reading of the updated SOP requires signature on the SOP review sheet (United States Environmental Protection Agency, 2007).</p>
Archiving	<p>Proper archiving is essential for good administration of SOPs. All operating instructions should be kept up-to-date and be accesible to personnel. Good Laboratory Practice requires that all documentation pertaining to a test or investigation should be kept for a certain period. SOPs belong to this documentation. An historical file is created for each SOP that is approved by management and will be maintained in the company's archives by the QAM. The historical file will consist of the original signed SOP and all subsequent modifications thereof. Official SOPs will have both colored header and footer lines, and be printed on watermarked ivory colored paper. All copies of the original will be black and white, initialed, numbered, and placed in the appropriate binder located in each office. If a procedure is incorporated into another SOP (superseded), a copy of the superseded version is placed in the historical file of both SOPs (United States Environmental Protection Agency, 2007).</p>

Table 3. General SOP Format.

15. Types of SOP

Several categories and types of SOPs can be distinguished. The name "SOP" may not always be appropriate, e.g., the description of situations or other matters may better designated *protocols*, *instructions* or simply *registration forms*. Also *worksheets* belonging to an analytical procedure have to be standardized (to avoid jotting down readings and calculations on odd pieces of paper) (Almeida S.L.)

Some of the most important SOP types:

- Fundamental SOPs. These give instructions how to make SOPs of the other categories.
- Methodic SOPs. These describe a complete testing system or method of investigation.
- SOPs for safety precautions
- Standard procedures for operating instruments, apparatus and other equipment.
- SOPs for analytical methods.
- SOPs for the preparation of reagents.
- SOPs for receiving and registration of samples.
- SOPs for Quality Assurance.
- SOPs for archiving and how to deal with complaints.

Generally the SOPs may be written for any repetitive technical activity, as well as for any administrative procedure (Almeida S.L.).

SOPs may be written for any repetitive technical activity, as well as for any authoritative or functional programmatic procedure, that is being followed inside an organization. General guidance for preparing both technical and administrative SOPs follows and examples of each are located in the Appendix (United States Environmental Protection Agency, 2007).

16. Guidelines for Technical SOP Text

Technical SOP and Administrative SOP are typical structures of SOPs. Technical and administrative SOPs need to involve the specific steps aimed at initiating, coordinating, and recording and/or reporting the results of the activity, and should be tailored only to that activity.

A technical SOP is a standard operating procedure which involves environmental data generation, manipulation, or accumulation, e.g., an analytical process. Technical SOPs can be written for a wide variety of activities.

Examples are SOPs instructing the user how to perform a specific analytical method to be followed in the laboratory or field (such as field testing using an immunoassay kit), or how to collect a sample in order to preserve the sample integrity and representativeness (such as collection of samples for future analysis of volatile organic compounds or trace metals), or how to conduct a bioassessment of a freshwater site. Technical SOPs are also needed to cover ac-

tivities such as data processing and evaluation (including verification and validation), modeling, risk assessment, and auditing of equipment operation. Citing published methods in SOPs is not always acceptable, because cited published methods may not contain pertinent information for conducting the procedure-in-house. Technical SOPs need to include the specific steps aimed at initiating, coordinating, and recording and/or reporting the results of the activity, and should be tailored only to that activity. Technical SOPs should fit within the framework presented here, but this format can be modified, reduced, or expanded as required.

17. Guidelines for Administrative or Fundamental Programmatic SOP

An administrative SOP is a standard operating procedure which does not include environmental data manipulation activities, e.g., how to conduct an inspection. As with the technical SOPs, these SOPs can be written for a wide variety of activities, e.g., reviewing documentation such as contracts, QA Project Plans and Quality Management Plans; inspecting (auditing) the work of others; determining organizational training needs; developing information on records maintenance; validating data packages; or describing office correspondence procedures.

Administrative SOPs need to include a number of specific steps aimed at initiating the activity, coordinating the activity, and recording and/or reporting the results of the activity, tailored to that activity. For example, audit or assessment SOPs should specify the authority for the assessment, how auditees are to be selected, what will be done with the results, and who is responsible for corrective action. Administrative SOPs should fit within the framework presented here, but this format can be modified, reduced, or expanded (United States Environmental Protection Agency, 2007).

18. Conclusion

Eventually, SOPs serve as a fundamental means of communication for all levels of the organization. Not only do they include employees departmentally, but they also allow management and employees to gain a cross-functional view of the organization. This attitude encourages employees to think about how process change may affect other functional areas. A good system forces employee to think through processes and examine how procedure might influence product, personnel, production, and equipment. *It should not be forgotten that the "Best written SOPs will fail if they are not followed"* (Hattamer-Apostel, R. 2001), (Jain, SK. 2008).

What happens to workers' intrinsic task motivation and creativity when they are required to follow SOPs in completing their tasks? Job design and work motivation theory literatures have suggested a negative relationship; the OM literature has suggested a positive relation. We suggest that the discussion has been hindered by differences in conceptualizing required SOP use, by not explicitly incorporating the multidimensional nature of intrinsic motivation into the analysis, by an ambiguous definition of autonomy, and by ignoring important contextual moderators. When these three elements are included in the discussion, we showed that the relationship between required SOP use and intrinsic motivation could theoretically

be positive. Finally, our model highlights the importance of worker participation. Production pressures, high capacity utilization, and lack of management – especially supervisor – support are likely to reduce opportunities for worker participation, and hence lower intrinsic motivation and creativity (De Trevil et al. 2005).

Example SOP

TITLE: Preparation of the Perfect Cup of Coffee by the Drip Method

Date of Preparation: 11/29/05; *Date of Revision:* N/A; *Revision No.:* N/A

Submitted by: Ay Dot Student; *Approved by:* Professor Ex

Purpose: Provide an example of a standard operating protocol or SOP that can be appreciated by undergraduate research students from all academic disciplines.

Scope and Applicability: The following protocol can be used wherever quality coffee beans, good drinking water, and a drip coffee maker are available.

Introduction: Coffee is the beverage of choice of many college students. Properly prepared the beverage provides an invigorating and revitalizing effect. One of the most frequently used methods of preparation is the drip method. In this method, water, heated to near boiling temperatures, is slowly added to finely ground coffee beans held in a filter unit. The coffee beverage is collected below the filter unit in a glass carafe. Today this procedure is frequently accomplished using a semi-automated process in an electronic coffee maker. The procedure below outlines a reliable method for preparing drip coffee using any commercial-ly available drip coffee maker, high quality ground coffee beans, and filtered water.

References: For information on coffee beans, the standard methods of preparation of coffee, and recipes see:

Materials and Supplies: Freshly ground Starbucks® coffee (any flavor you prefer; medium grind works best with most commercial coffee makers), commercial 4-c drip coffee maker including filter (gold mesh preferred but high quality paper filter may be used), good quality drinking water (Polar Springs®, Brita®-filtered, or similar quality source recommended), coffee cup, and additives (as desired: sugar or sugar alternative, cream or milk).

Cautions: Hot coffee can scald and burn. Water is an electrical conductor. If spills occur during the brewing process, wait until the brewing process is complete, turn off the electricity, and disconnect the unit from the electricity before attempting to clean up any spills. Accidental spills may be cleaned up with a kitchen sponge and dish washing detergent such as®. Used coffee grounds can be disposed of in the regular trash. Be sure to carefully read the directions that accompanied your coffee maker unit before attempting to use it. In particular, it is important to find out if your unit has (1) a pause feature that will allow you to remove the carafe while the coffee is brewing; and (2) an auto-off feature that turns off the heater unit located beneath the carafe at a set time after the coffee has been brewed.

Personnel Qualifications: No special knowledge or training is required to make coffee. However, due to the potential risk of burns, it is recommended that anyone performing this procedure who is less than ten years old be actively supervised by an adult.

Protocol

1. Make sure that the coffee maker is off. Locate water reservoir unit on coffee maker and carefully add 4-cups of clean drinking water to the reservoir. Note that the outside or inside of most quality coffee makers' water reservoir units are marked for the user's convenience.
2. Locate the coffee filter assembly on the coffee unit. If you are preparing the standard 4-c carafe of coffee, carefully measure one coffee measure of ground coffee into your units coffee filter assembly. Note that one standard coffee measure is equivalent to 1/8-c of coffee. Close the coffee filter assembly.
3. Plug in the coffee maker and turn the unit on. Wait until the carafe located beneath the coffee filter unit is filled with coffee. Note that some units may have a "pause" feature that will allow you to temporarily remove the carafe and pour a cup of coffee while the unit is working. If you are unfamiliar with your unit, be sure to wait until the unit is done filtering before attempting to remove the carafe.
4. If coffee spills beneath the base of the carafe unit, be sure to turn off the unit and disconnect the electricity before attempting to clean up the spill.
5. Pour yourself a cup of coffee. Most coffee units will keep the carafe warm for a set period of time before turning off automatically. Some however, do not turn off automatically. Be sure to read your coffee maker's instructions beforehand. If in doubt, be sure to turn off the electricity to your unit after the brewing process is complete. (Levine D.I et al, 2010)

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Quality Control in Clinical Laboratory Medicine

Postmortem DNA: QC Considerations for Sequence and Dosage Analysis of Genes Implicated in Long QT Syndrome

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Additional information is available at the end of the chapter

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1. Introduction

Long QT syndrome is a rare disorder of cardiac ion channels, characterised by a prolonged QT interval and T-wave abnormalities on electrocardiogram (ECG) and the occurrence of the ventricular tachycardia *torsade de pointes*. Sodium, potassium or calcium channels present in heart muscle may be affected, altering the regulation of electrical current in the cells [1-3]. Individuals with this condition will be predisposed to cardiac events such as arrhythmias and polymorphic ventricular tachycardia, which may lead, if untreated, to sudden cardiac death [2,3]. Thirteen genes are associated with the condition, and hundreds of mutations have been identified [3-5]. Currently, more than 95% of the pathogenic mutations listed in disease databases (Gene Connection For the Heart, <http://www.fsm.it/cardmoc/>; online Human Gene Mutation Database, www.hgmd.cf.ac.uk/) are sequence variants (including point mutations and small insertions or deletions), but the importance of whole or multi-exon deletions and duplications has more recently been recognised [6] and it is now recommended to use both sequence and dosage techniques in order to provide comprehensive analysis [3].

In New Zealand, the majority of specimens referred for Long QT syndrome diagnostic testing are retrieved after death. Postmortem specimens are often difficult to handle as they are usually either tissue samples or severely haemolysed blood. The extracted DNA is frequently of low quality, due to the presence of unwanted material such as short fragments produced by degradation and chemical modifications from oxidation and hydrolysis processes [7]. As a result, only short sequences can be reliably amplified [7]. Moreover, capillary-based

sequencing, the gold standard first-line diagnostic test for Long QT syndrome, is very sensitive to the presence of contaminants, such as proteins, RNA and residual salt. The presence of such contaminants leads to poor quality electropherograms for analysis, which tend to be compromised by the presence of dye blobs, C-shoulders and a variable degree of baseline noise (Figure 1). Although the QC requirements for array comparative genomic hybridisation (aCGH), which can be used to detect whole exon deletion and duplication mutations, are less stringent than those for sequencing, contamination or degradation of sample DNA can lead to suboptimal efficiency of labelling and hybridisation. Such difficulties arising in the practical procedure mean that the analysis of postmortem DNA can be time-consuming and challenging, and obtaining high quality data within a reasonable timeframe can be extremely difficult.

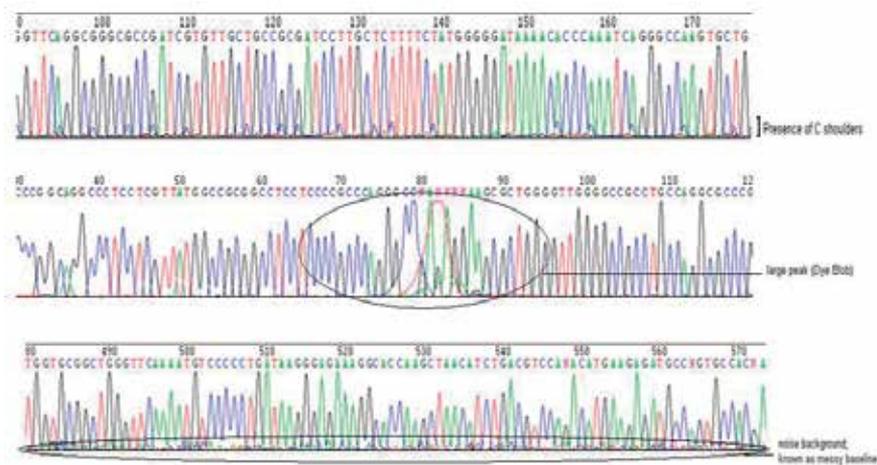


Figure 1. Electropherograms showing poor quality sequence data.

Long QT syndrome affects 1 in 2000 individuals [8] and contributes to 15-25% of sudden unexplained death in 1-40 year olds [9,10] and 10% of sudden unexplained deaths in infancy (SIDs) [11]. As a consequence, it is an important differential diagnosis to be considered in all cases of autopsy negative young sudden death. Molecular genetic testing is essential to make a postmortem diagnosis, given that screening for the electrocardiogram characteristics is no longer possible after death. Historically the turnaround time for diagnosis has been up to six months, due to the large number of genes to be analysed and the difficulties inherent in dealing with postmortem specimens (as detailed above). However, the demand by coroners for diagnostic laboratories to undertake more rapid analysis has been steadily increasing. A protocol tailored specifically to the treatment of postmortem specimens is necessary to meet this demand. Within our laboratory, we have successfully developed a robust process for sequence and dosage analysis of postmortem samples and have achieved an excellent turnaround time of 6-12 weeks. Here, we address the critical QC parameters that should be considered in order to obtain high quality data for rapid, accurate analysis. The discussion

presented below concerns both sequence analysis and dosage analysis. The former uses conventional sequencing technology, while the latter involves the simultaneous high resolution screening of deletion and duplication mutations in multiple cardiac genes as opposed to the more conventional multiplex ligation dependent probe amplification (MLPA) technique, which many diagnostic laboratories still use [6].

2. Materials and methods

2.1. Sequencing

2.1.1. Primer design

We have used two approaches to design primers flanking each of the coding exons of the genes *KCNQ1* (LQT1), *KCNH2* (LQT2), *SCN5A* (LQT3), *KCNE1* (LQT5), *KCNE2* (LQT6), and *KCNJ2* (LQT7), including at least 50 base pairs of the flanking intronic regions. The first used the primer design program called PrimerQuest (Integrated DNA Technologies Inc; <http://www.idtdna.com/Scitools/Applications/Primerquest/Advanced.aspx>). This program allows the region in a sequence file to be specified, against which primers are designed to flank the targeted region. The designed primers for each exon are then checked *in silico* for annealing characteristics using the Macintosh-based program, Amplify. Finally, all primers were checked for single nucleotide polymorphisms (SNPs) using the software tool available from the National Genetic Reference Laboratory, Manchester (<http://ngrl.man.ac.uk/SNPCheck/SNPCheck.html>). This bioinformatics program uses the current National Center for Biotechnology Information (NCBI) build of the human genome and the current release of the Single Nucleotide Polymorphism database (dbSNP) to identify the position in the sequence where the primers bind and to detect any known SNPs at these sites

Following the above approach, we developed an alternate design protocol [12,13]. Each mRNA sequence of interest was identified through the public UCSC genome browser page <http://genome.ucsc.edu>. This website provides a direct link to ExonPrimer for the design of primers specific to the mRNA sequence. ExonPrimer uses exon position information provided by the UCSC genome browser in combination with the primer design tool Primer3 to create primer pairs according to set parameters, while avoiding pairing to homologous regions within the genome. Exon and amplicon size are provided and multiple alternative primer sets are given. Following primer design, all primers were checked for single nucleotide polymorphisms (SNPs), as described above. Following a negative SNP check the primer sequences were evaluated using the UCSC genome browser to confirm the identification of single amplicons. Each primer was then tailed with an M13 sequence and manufactured by Integrated DNA Technologies Inc or Life Technologies. In this way, primers are designed to allow amplification of all exons of interest and the corresponding splice sites using a single set of PCR conditions such that PCR (and subsequent sequencing) can be performed under identical conditions within a 96-well PCR plate.

2.1.2. DNA extraction

Genomic DNA (gDNA) was extracted from peripheral blood leucocytes (EDTA blood samples) using the Genra Puregene DNA Extraction kit (Qiagen), according to the manufacturer's instructions.

A standard phenol/chloroform protease protocol was used to extract gDNA from postmortem tissue specimens. A small section (2mm x 2mm x 4mm) is usually cut from frozen tissue and diced as finely as possible using a scalpel blade. The tissue is placed into a 1.5ml microcentrifuge tube with 450µl of 1x TES Buffer (1M NaCl, 0.5M Tris-HCl, 10mM EDTA), 60µl of 20µg/µl Proteinase K (Roche) and 10µl of 10% SDS, and incubated overnight with vigorous shaking. Following digestion, an equal volume of phenol is added and the sample is vortexed vigorously. Once homogenous, the sample is centrifuged to separate the layers and the top aqueous layer is removed and transferred to a fresh 1.5ml centrifuge tube. An equal volume of chloroform is then added to the aqueous layer. This is vortexed, centrifuged, and the aqueous (top) layer again transferred to a fresh tube. A 2x volume of 100% ethanol is added to the aqueous layer to precipitate the DNA, followed by centrifugation at 13,000rpm and the supernatant is removed. The pellet is then washed with 70% ethanol and re-centrifuged for 2 minutes at 13,000rpm. The supernatant is again removed, and the pellet air dried prior to re-suspension in TE buffer (10mM Tris-HCl, 1mM EDTA, pH 7.0-8.0).

The quality and quantity of extracted gDNA is measured using a NanoDrop ND-1000 Spectrophotometer.

2.1.3. PCR

PCR amplification is performed in a final 25µl reaction volume with the following reagents: Faststart buffer (Roche Applied Science), 2mM MgCl₂ (Roche Applied Science), M13-tailed forward and reverse primers at 0.8µM each (synthesised by Integrated DNA Technologies Inc), 0.4mM dNTPs (GE Healthcare Ltd), 1 unit Faststart Taq DNA polymerase (Roche Applied Science) and 5µl GC-rich solution (Roche Applied Science). 50ng of gDNA is included in each reaction. PCR amplification is carried out with the following conditions: denaturation at 95°C for 5 minutes, followed by 35 cycles of 94°C for 45 seconds, 60°C for 30 seconds and 72°C for 30 seconds, with a final extension of 72°C for 10 minutes.

2.1.4. Sequencing (Figure 2)

5µL of each PCR is cleaned with ExoSAP-IT (Affymetrix, USB) prior to bidirectional DNA sequencing using M13 forward and reverse primers and Big-Dye Terminator v3.0 (Applied Biosystems Ltd). 20µl of each sequenced product is manually purified using the CleanSEQ Sequencing Purification System (Agencourt Bioscience). Four different drying times prior to elution (20 minutes, 24 hours, four days and seven days) were assessed to establish an optimal drying time for generating high quality sequencing data. 15µL of purified product was then subjected to capillary electrophoresis using the Applied Biosystems model 3130xl Genetic Analyzer.

The analysis of sequence traces is performed using Variant Reporter v1.0 (Applied Biosystems). Variant Reporter uses advanced algorithms and quality metrics to automate the detection of variants and to streamline the analysis process.

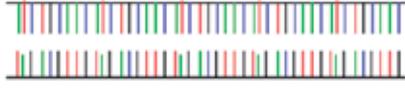
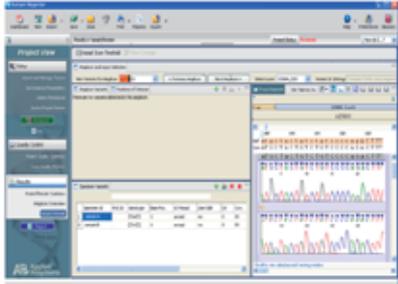
Sources of samples	 Blood taken in EDTA from peripheral blood or  Frozen postmortem tissue samples											
DNA extraction	 DNA quality $A_{260/280}$ ratio : 1.8 - 2.0 $A_{260/230}$ ratio : 2.0 - 2.2											
PCR amplification	 PCR conditions: <table style="display: inline-table; vertical-align: middle;"> <tr> <td>95°C</td> <td>5'</td> <td rowspan="5" style="font-size: 2em; vertical-align: middle;">}x35</td> </tr> <tr> <td>94°C</td> <td>45"</td> </tr> <tr> <td>60°C</td> <td>30"</td> </tr> <tr> <td>72°C</td> <td>30"</td> </tr> <tr> <td>72°C</td> <td>10'</td> </tr> </table> 50ug genomic DNA Faststart Buffer 2mM MgCl ₂ 0.8uM Primers 1U Faststart Taq DNA Polymerase 5uL GC rich solution 0.4mM dNTPs	95°C	5'	}x35	94°C	45"	60°C	30"	72°C	30"	72°C	10'
95°C	5'	}x35										
94°C	45"											
60°C	30"											
72°C	30"											
72°C	10'											
Product cleanup	 Cleanup using EXOSAP-IT 37°C - 15' 80°C - 15'											
Sequencing	 2.5ng per 100bp of amplified product – sequenced using BigDye											
Sequencing Product Purification	 Purified using CleanSEQ Purification System 20ul sequencing product 10ul CleanSEQ reagent Two washes of 100ul 85% Ethanol											
Product Drying prior to Elution	<table border="1" style="width: 100%; text-align: center;"> <tr> <td style="width: 25%;">20 mins</td> <td style="width: 25%; background-color: yellow;">24 hours</td> <td style="width: 25%;">4 days</td> <td style="width: 25%;">7 days</td> </tr> </table>	20 mins	24 hours	4 days	7 days							
20 mins	24 hours	4 days	7 days									
Elution	 Dried sequencing product eluted in 15ul 0.1mM EDTA and loaded on an Applied Biosystems model 3130xl Genetic Analyzer											
Analysis using Variant reporter												

Figure 2. Flowchart of the sequencing method used in our laboratory.

2.2. Array comparative genomic hybridization (aCGH)

A Roche NimbleGen 12x135K Custom CGH Array was used for dosage analysis. This bespoke CGH array has been designed to screen for dosage changes within the genes responsible for LQT1-12 (*KCNQ1*, *KCNH2*, *SCN5A*, *ANK2*, *KCNE1*, *KCNE2*, *KCNJ2*, *CACNA1C*, *CAV3*, *SCN4B*, *AKAP9*, and *SNTA1*), the LQT-associated genes *GPD1L*, *KCNE3*, *SCN1B*, *SCN3B*, *CACNB2*, and the CPVT1 (*RYR2*) and CPVT2 (*CASQ2*) genes.

Two hundred and fifty nanograms of gDNA are processed according to the manufacturer's instructions (NimbleGen Array User's Guide: CGH and CNV Arrays v8.0; <http://www.nimblegen.com>). In brief, extracted gDNA from samples and Promega controls are denatured in the presence of a Cy3- (test) or Cy5- (control) labelled random primers and incubated with the Klenow fragment of DNA polymerase, together with dNTPs (5mM of each dNTP), at 37°C for 2 hours. The reaction is terminated by the addition of 21.5µL of 0.5M EDTA, prior to isopropanol precipitation and ethanol washing. Following quantification, the test and sex-matched control samples are combined in equimolar amounts and applied to one of the twelve arrays on a microarray slide. Hybridisation is carried out in a Roche NimbleGen Hybridisation Chamber for a period of 48 hours. Slides are washed and scanned using a NimbleGen MS 200 Microarray Scanner. Array image files (.tif) produced by the MS 200 Data Collection Software are imported into DEVA v1.2.1 (Roche NimbleGen Inc) for analysis. Each genomic region exhibiting a copy number change within one of the LQT genes of interest were examined using the UCSC genome browser (<http://genome.ucsc.edu/>) to determine the location and significance of the change.

3. Results

In order to overcome the historical difficulties faced when performing Long QT syndrome testing using postmortem specimens and meet the demand by coroners for rapid results, we addressed the following parameters:

3.1. DNA purity

Since poor quality gDNA leads to suboptimal PCR amplification affecting downstream applications, the purity of gDNA is an important criterion for success in generating high quality sequencing data [14,15]. A NanoDrop ND-1000 spectrophotometer was used to measure the quality and quantity of the extracted gDNA. The ratio of absorbance at 260nm and 280nm (A260/280 ratio) is used to assess the purity of gDNA, which should be in the range of 1.8 to 2.0 to be accepted as pure gDNA. The ratio of absorbance at 260nm and 230nm (A260/230 ratio) is used as a secondary measure of nucleic acid purity, and for pure gDNA should be in the range of 2.0-2.2 [16]. Postmortem gDNA isolated using a standard phenol/chloroform protease protocol may contain residual phenol, chloroform or ethanol. These contaminants inhibit the activity of DNA polymerase in downstream applications (Figures 3-5) [14], so the purity of the gDNA must be strictly monitored. If a suboptimal A260/280 or A260/230 ratio indicates that the gDNA is of low quality, a secondary cleanup process

should be considered. In our laboratory, we perform this cleanup using another phenol extraction and ethanol precipitation to further purify the gDNA sample [17].

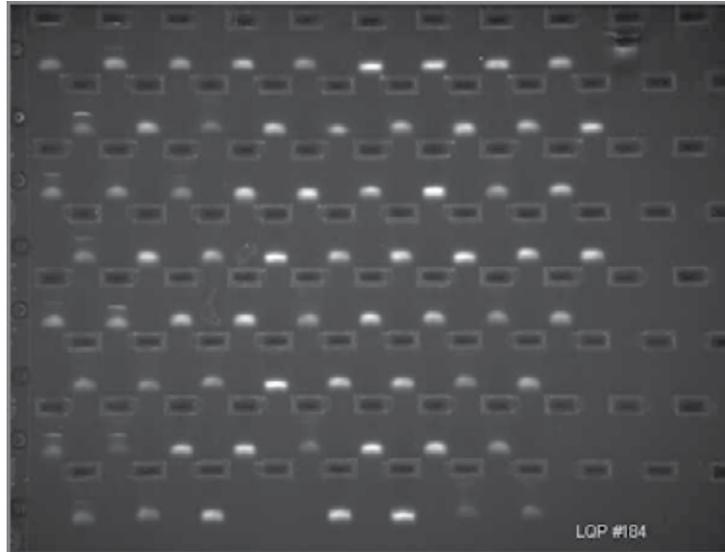


Figure 3. Agarose gel image of PCR amplification from an impure DNA with an acceptable $A_{260/280}$ ratio (1.88) but an acceptable $A_{260/230}$ ratio (2.5). NOTE: very few exons amplified effectively and strong primer dimers are visible.

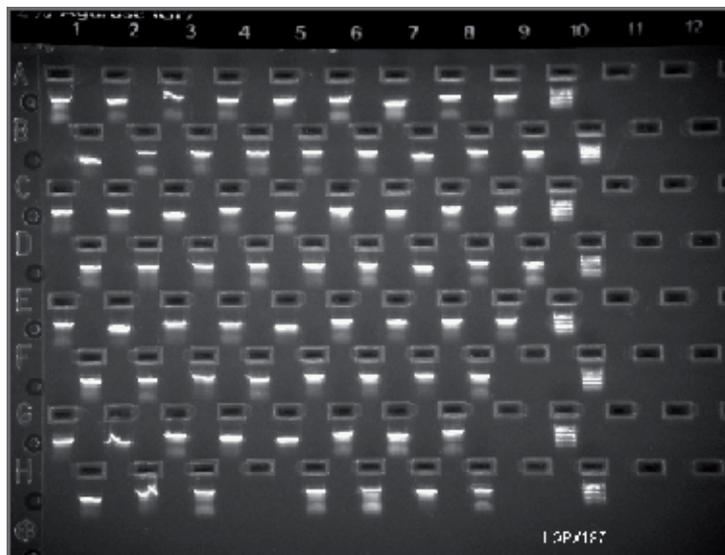


Figure 4. Agarose gel image of PCR products amplified from the same DNA sample after purification. NOTE: successful amplification and significantly reduced primer dimers.

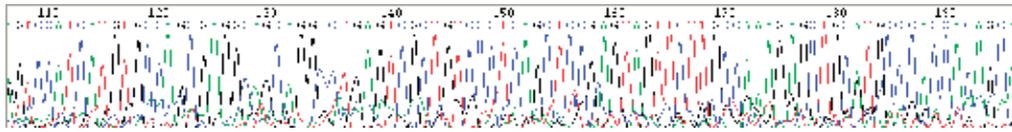


Figure 5. Electropherogram generated from an impure DNA sample with an acceptable $A_{260/280}$ ratio (1.95) but an unacceptable $A_{260/230}$ ratio (0.95). NOTE: significant baseline noise and data unable to be analysed.

3.2. Amount of DNA template used in a sequencing reaction

The extent of dilution of the cleaned PCR product prior to sequencing determines the amount of DNA template used in the sequencing reaction, which can affect the data quality for analysis [15]. The presence of excessive DNA template in a sequencing reaction will lead to rapidly progressive signal loss on the electropherogram (Figure 6), while using insufficient DNA template in a sequencing reaction will result in weak signal strength and a loss of peak shape (Figure 7), causing difficulties in basecalling and accurate analysis [15]. In order to obtain high quality data with good signal strength, the amplified product should be diluted in ddH₂O to as close as possible to 2.5ng per 100bp.

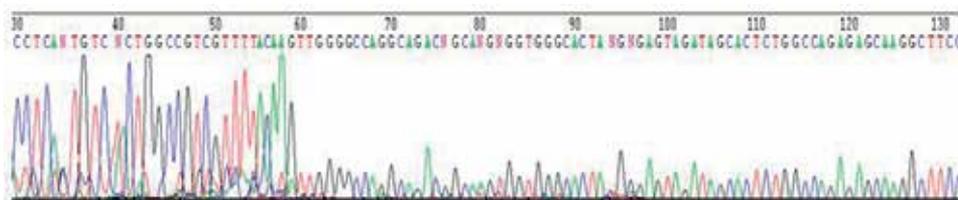


Figure 6. Electropherogram begins with strong high peaks, which fade rapidly.

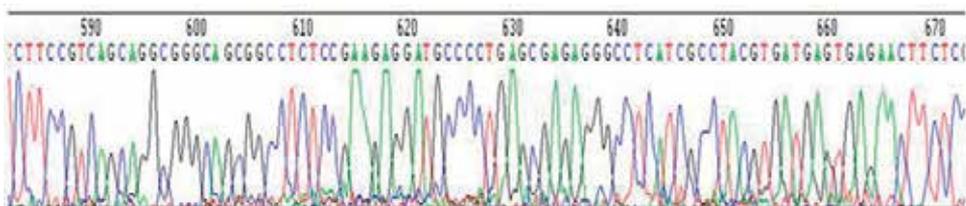


Figure 7. Electropherogram with increased background noise and loss of sharp peak shape.

3.3. CleanSEQ treatment of sequencing reactions

The sequenced product is purified using an Agencourt CleanSEQ system, which uses SPRI (Solid Phase Reversible Immobilization) magnetic bead-based technology. According to the manufacturer's recommendation, the sequencing product should be cleaned using undiluted CleanSEQ reagent, but in our experience, postmortem DNA samples should be cleaned using CleanSEQ reagent diluted 1:2 in ddH₂O (figures 8,9).

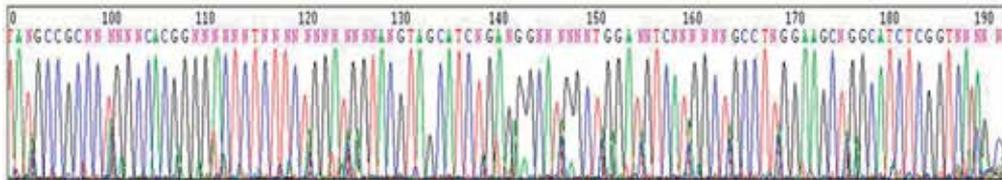


Figure 8. Electropherogram obtained from sequencing product cleaned with 10µl of undiluted CleanSEQ reagent: serious baseline noise and incorrect basecalling.

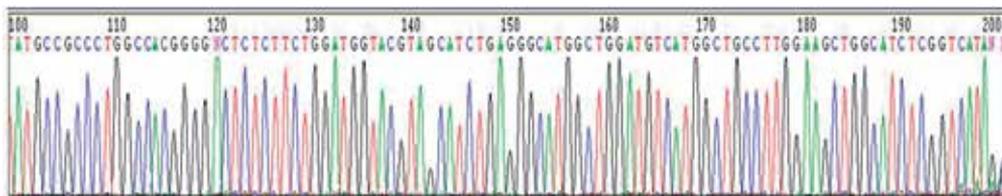


Figure 9. Electropherogram obtained from sequencing product cleaned using diluted CleanSEQ reagent: good quality trace.

3.4. Drying of DNA-bound beads prior to elution

The length of drying time following ethanol washing and prior to elution of the sequencing product from the CleanSEQ beads is one of the critical parameters for obtaining high quality sequence data. We assessed 4 different drying periods: 20 minutes, 24 hours, four days and seven days.

A drying time of 20 minutes, as recommended by the manufacturer, is frequently associated with the presence of large 'dye blobs', most probably as a result of residual ethanol in the eluted product (Figure 10); this problem resolves if drying time is extended to 24 hours (Figure 11). However, variable baseline noise on the electropherogram will be evident if the drying time exceeds four days (Figure 12); further increased background noise and loss of resolution occur when the drying time exceeds seven days (Figure 13).

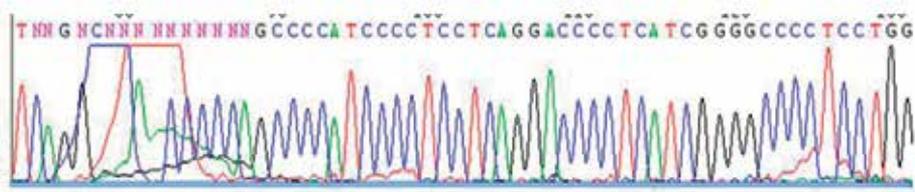


Figure 10. Electropherogram of purified products dried for 20 minutes prior to elution; presence of large dye blob.

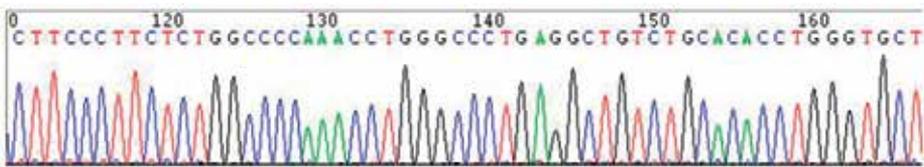


Figure 11. Electropherogram of purified products dried for 24 hours prior to elution; free of dye blobs, minimal baseline noise.

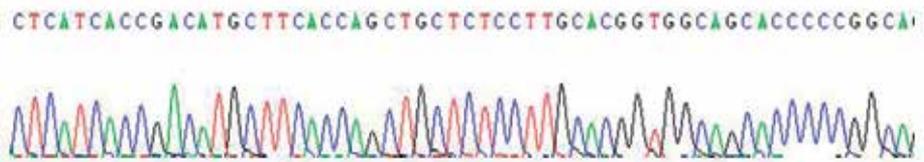


Figure 12. Electropherogram of purified products dried for 4 days prior to elution; presence of shoulders and messy baseline.

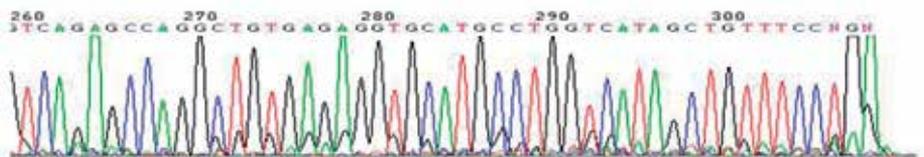


Figure 13. Electropherogram of purified products dried for 7 days prior to elution; extremely messy baseline and early loss of resolution.

4. Discussion

4.1. DNA quality

4.1.1. Sequence analysis

The gDNA used in a PCR should be as pure as possible in order to optimise the quality of the template that will be used in downstream applications. The purity of DNA is assessed in our laboratory using a NanoDrop ND-1000 spectrophotometer. It is important to consider both A260/280 and A260/230 as poor DNA quality will affect downstream applications [16,18]. An unacceptable A260/280 or A260/230 ratio indicates the presence of contaminants in the DNA; an abnormal A260/230 indicates the presence of residual phenol or other chemical from the extraction process, while an abnormal A260/280 most frequently indicates the presence of protein [16,18]. If any of the ratios appear to be abnormal, DNA purification should be considered before processing any further.

4.1.2. Dosage analysis (aCGH)

The quality requirements for gDNA used in an aCGH assay are not as stringent as those for a sequencing assay. An awareness of the presence of significant degradation is important; however, partial compensation for this can be made by increasing the volume of gDNA used in the amplification and labelling step. In our laboratory, we have found that doubling the volume of gDNA when degradation is visible on the 'check gel' (present in lanes 5-8 of Figure 14) is an effective counter-measure. The adequacy of amplification is measured using a NanoDrop ND-1000 spectrophotometer in order to allow the hybridisation of equimolar amounts of test and control DNA to the array slide. A post-amplification concentration of greater than 2500ng/ μ l is ideal for further processing; a sample with a post-amplification concentration of less than 1500ng/ μ l is unlikely to produce good quality data for analysis. Therefore, if there is sufficient volume of gDNA available, a repeat amplification with an increased volume of template gDNA should be undertaken.



Figure 14. Agarose gel (2%) check of gDNA quality prior to aCGH.

4.2. Amount of DNA template used in a sequencing reaction

The DNA template should be diluted to 2.5ng per 100bp prior to sequencing. Incorrect quantification and dilution will alter the amount of input DNA template in a sequencing reaction, which can lead to problematic data for analysis. In our laboratory, we use Variant Reporter™ (Applied Biosystems Ltd) for automated analysis of sequence data. The signal strength of the sequence data is automatically assessed. Data with good signal strength is above the value of 200 in the Variant Reporter™ software. Sequence data with weak signal strength, a value below 100, indicates insufficient DNA template has been used in the sequencing reaction. Noisy background and a loss of sharp peak shape are also commonly evident. A repeat treatment with EXOSAP-IT and less extensive dilution of product is necessary to avoid inaccurate analysis using sequence data with unacceptable quality. In contrast, when excess DNA template is used in a sequencing reaction, a rapidly progressive signal loss is seen on the electropherogram. In this instance, trace quality can be improved by diluting the eluted product with water and reloading the sample on the capillary sequencing platform (we use an Applied Biosystems model 3130xl Genetic Analyzer).

4.3. CleanSEQ treatment of sequencing reactions

Agencourt CleanSEQ is routinely used to purify the sequenced products. According to the manufacturer's recommendation, 20µl of sequencing product should be cleaned with 10µl of CleanSEQ reagent. However, we found that this leads to an excess of CleanSEQ beads in relation to the amount of sequence product, therefore unwanted short fragments (e.g unincorporated dNTPs and excess primers) were able to bind to beads during purification, and the quality of sequence data generated was consequently reduced. We found that diluting the CleanSEQ beads 1:2 with ddH₂O leads to much higher quality data.

4.4. Drying of DNA-bound beads prior to elution

This drying step is a critical QC consideration that significantly affects sequence quality. When the purified product is dried for 24 hours prior to elution, the subsequent electropherograms are free of dye blobs, C-shoulders and baseline noise, and consistently pass all QC metrics in Variant Reporter.

A drying time of 20 minutes, as suggested by manufacturer, was found to be problematic, causing the presence of dye blobs. Dye blobs are a common artefact due to either the presence of excess unincorporated dye or residual ethanol following product purification [15]. Previously, operators have focused on purifying sequenced products using an accu-

rate final concentration of ethanol in an attempt to achieve consistency of evaporation/drying between sequencing runs. Ethanol is highly volatile and it is therefore difficult to achieve consistency - even the short period of time in which the vessel containing the ethanol is open to allow access can result in a decrease in concentration. Here, we showed that the issue of dye blobs can be resolved by simply extending the drying time to 24 hours, allowing adequate time for all ethanol to evaporate completely, despite any minor differences in the concentration of ethanol used.

On the other hand, we found that the quality of sequence data is adversely affected if the drying time is extended to more than 4 days. This suggests that the stability of purified sequencing products is another factor that affects data quality. High quality sequence data was obtained with a drying time of 24 hours; however, the sequences were suboptimal when elution and electrophoresis were carried out at day 4 or day 7 of drying. This indicates that purified sequencing product is most stable for the first 24 hours and that consideration of the sample stability should be taken into account when aiming for high quality data.

4.5. Analysis parameters

4.5.1. Sequencing

All sequence traces are analysed in our laboratory using Variant Reporter Software v1.0 (Applied Biosystems). The current CMGS best practice guidelines for Sanger sequence analysis in diagnostic laboratories [19] recommend a PHRED score of at least 20 for bidirectional data (corresponding to 99% confidence that the base is called correctly), and a PHRED score of at least 30 for unidirectional data (99.9% confidence that each base is called correctly). The procedure we describe above produces bidirectional sequence data that meet our laboratory's even more stringent analysis criteria: a Variant Reporter trace score of 35, corresponding to a false base call rate of 0.031%.

4.5.2. Dosage analysis

In order to provide simultaneous dosage analysis of all the Long QT syndrome genes, we developed a custom designed Roche NimbleGen 12x135K CGH array. As part of the validation of this array we analysed twenty patients with known copy number abnormalities [20]. Analysis of the data from these individuals with known copy number changes allowed precise threshold criteria to be developed. It was determined that a \log_2 ratio ≤ -0.4 over 6 contiguous probes is indicative of a deletion, and a \log_2 ratio of ≥ 0.4 over 15 contiguous probes indicative of a duplication.

4.6. Other parameters

It is important to monitor other QC parameters when it comes to sequence analysis of Long QT specimens: the freshness of the Polymer (POP-7) and the usage of the capillaries in the Applied Biosystems model 3130xl Genetic Analyzer.

Polymer (POP-7) is used to separate DNA fragments on genetic analyzers and the polymer remains stable for up to 7 days [15]. The capillary in the genetic analyzer should be replaced after 1000 injections. Within our laboratory, we perform a regular weekly maintenance of the analyzer, and the capillary is replaced when 600 injections are reached. From past experience, both the freshness of polymer and the usage of the capillary play a vital role in the data quality. Electropherograms with reduced resolution and peak shape are produced when either of these two parameters is suboptimal. In order to achieve high quality data, both freshness of polymer and usage of the capillary should be closely monitored.

5. Conclusions

We have successfully established a robust method for processing postmortem specimens for Long QT diagnostic testing in a timely manner. The electropherograms in Figure 15 are indicative of the high quality data routinely produced, despite the limitations inherent in the types of specimens that are referred. The blood sample used to extract gDNA for sequencing in this example was heavily haemolysed on arrival at the laboratory.

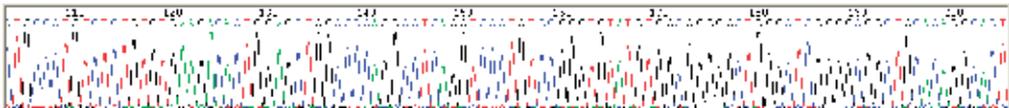


Figure 15. High quality sequence data generated from a haemolysed blood specimen; flat baseline along with distinct and evenly spaced peaks allow accurate basecalling.

The QC parameters described above should be monitored closely in order to consistently achieve optimal results (Figure 16). Although the basic procedure is essentially the same as that routinely used for sequence and aCGH analysis, the poor quality of postmortem specimens as a source of template DNA mean that particular attention needs to be paid to each step, in particular the critical initial assessment of DNA quality, the addition of the appropriate volume of template DNA to the sequencing reaction, and the drying time of the beads used in the purification procedure prior to elution.

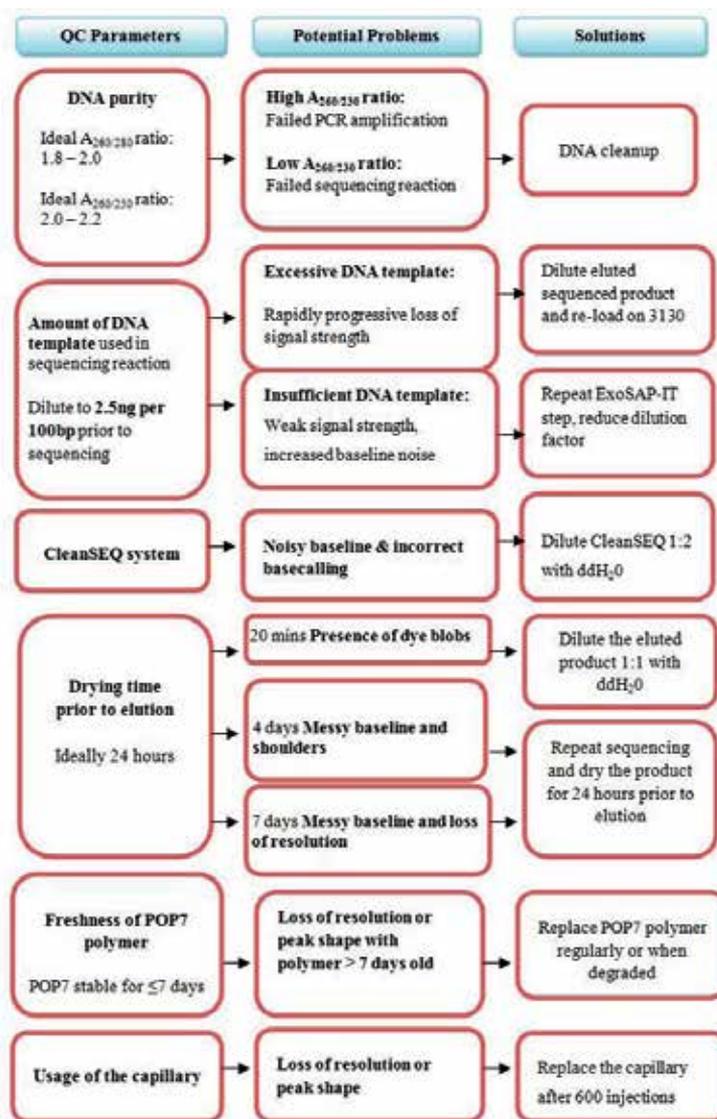


Figure 16. Flowchart of potential QC problems and their solutions.

Acknowledgements

We acknowledge the assistance of Dr Andrew Dodd in the initial design of primers against the coding regions of the LQT genes, and Mr Daniel Lai for his assistance in optimizing aspects of our amplicon clean-up.

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Quality Assurance in Antimicrobial Susceptibility Testing

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Additional information is available at the end of the chapter

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1. Introduction

Most of the clinically important bacteria causing infections in humans are capable of exhibiting resistance to antimicrobial agents commonly used for the treatment. Therefore, upon isolation of the organism in the clinical microbiology laboratory, characterization frequently also employs tests to detect its antimicrobial susceptibility. Thus, the report produced by clinical microbiology laboratory for the physician, also includes organism's susceptibility profile to different antimicrobials along with its identification [1]. Antimicrobial susceptibility testing (AST) is performed on bacteria that are isolated from clinical specimens to determine if the bacterial etiology of concern can be killed or inhibited by antimicrobial drugs that are potential choices for therapy, at the concentrations of the drugs that are attainable at the site of infection using the dosing regimen indicated in the drug product's labeling. The results of AST are generally reported with interpretive categories. The category "susceptible" indicates that the bacteria are inhibited by the usually achievable concentrations of antimicrobial agent when the dosage recommended to treat the site of infection is used. The "intermediate" category defines the bacteria for which the response rates to usually attainable blood and tissue levels of antimicrobial agent are lower compared to susceptible isolates. The intermediate category plays the role of a buffer zone between the susceptible and resistant categories, but also indicates a number of other possibilities; the antimicrobials which are concentrated at the site of infection may be regarded as options for treatment (e.g., nitrofurantoin for the urinary tract infections). The "resistant" category, however, defines the bacteria which are not inhibited by the usually achievable concentrations of the agent with normal dosage regimens and that the clinical efficacy of the agent against the isolate may not be sufficient [2]. Clinicians consider these interpretations to determine which antimicrobial agent might be effective in treating the particular patient. The primary role of routine microbiology laboratories is to provide accu-

rate and timely antimicrobial susceptibility test results for guiding the treatment of infectious diseases. In order to achieve that, the microbiologist should inform the clinician about whether an infectious agent is present in the patient's specimen and which antimicrobial agent should provide the optimum therapy. Although the importance of antimicrobial susceptibility testing is well established, the procedure itself is very sensitive to changes in the environment and test conditions. Therefore, it is crucial that each variable in the procedure should be standardized and carefully controlled. With more reliable susceptibility results, infectious disease specialists and public health leaders can be able to recognize emerging resistance and novel resistance patterns. Additionally, the results of AST can be applied to define the agent of choice for empirical therapy, establish institutional or nationwide policies for prescribing of antibiotics, conduct epidemiological studies or resistance surveillance, and to evaluate the efficacy of newly developed agents. Owing to numerous variables that may affect the results, rigorous quality control is of utmost importance for susceptibility testing. Properly performed quality control would aid in providing accurate, reproducible and timely results. In this chapter the components of a quality assurance program for antimicrobial susceptibility testing will be highlighted.

2. Overview of the antimicrobial susceptibility testing methodologies

Fleming was first to report the inhibitory effect of penicillin on agar by observing a zone of growth inhibition of staphylococcal colonies grown next to a *Penicillium* contaminant on an agar plate. Fleming also made two significant contributions to the field of AST in the 1920s. In 1924, he introduced the use of the ditch plate technique for evaluating antimicrobial qualities of antiseptic solutions [3]. Fleming's second contribution to modern AST was the development of broth dilution technique using turbidity as an end-point determination [4]. Filter paper disks incorporating penicillin were utilized by Vincent & Vincent for assaying this newly discovered compound in 1940s [5]. Agar dilution AST method was also described in the 1940s [6]. At an early stage, it was realized that there were many variables affecting AST methods [7]. In 1961, World Health Organization (WHO) published a report on standardization of AST methodology [8]. The broad application of AST was introduced to clinical laboratories by the efforts of Bauer, Kirby and co-workers, with the method known as Kirby-Bauer disk diffusion method which is still the most widely used AST technique in the world [9]. Bergeron & Ouellette highlighted the shortcomings of the phenotypic approach to AST and concluded that different bacterial species have different susceptibilities to the same antibiotic, and that there is no international agreement on breakpoints for interpretation of antimicrobial susceptibility tests [10]. The need for developing standardized AST methods became a necessity soon after antibiotics became commercially available. During World War II, following penicillin, other antibiotics were discovered and used. Although these new antibiotics were regarded as "wonder drugs" at the time of their introduction, emergence of resistant strains followed. With the emergence of bacterial resistance to antimicrobials and the changing properties of different bacteria to different classes of antimicrobials, the need for the performance of AST on pathogens became a practical necessity.

Nationwide attempts were made to standardize AST methodologies; Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) (USA) [11], Werkgroep Richtlijnen Gevoeligheidsbepalingen (Netherlands) [12], Comité de l'Antibiogramme de la Société Française de Microbiologie (France) [13], the Swedish Reference Group for Antibiotics (Sweden) [14], Deutsches Institut für Normung (Germany) [15], the British Society for Antimicrobial Chemotherapy (UK) [16], they all published guidelines to improve the methodology and interpretation of AST. Recently, the European Committee on Antimicrobial Susceptibility Testing (EUCAST), a non-profit organization under the auspices of European Society of Clinical Microbiology and Infectious Diseases (ESCMID), developed and published AST guidelines. Breakpoint and QC tables for disk diffusion and minimum inhibitory concentration (MIC) testing can freely be accessed on organization's website [17].

In clinical laboratories, widely adopted AST methods are disk diffusion and broth dilution methods. In disk diffusion method, disks impregnated with antimicrobial agents are used. The disks are placed onto agar plates which are preinoculated with the suspension of the microorganism being tested. The basic principle of the disk diffusion method is the diffusion of the antimicrobial agent into the medium which occurs when the disks come into contact with the moist surface of the plate. The concentration of the agent reduces logarithmically as the distance from the disk is increased. After the incubation period the plates are observed for the circular inhibition zone created around the disk which is due to the inhibitory effect of the antimicrobial agent on the microorganism. Within the zone the concentration of the agent is sufficient to inhibit growth, whereas at the point where the concentration of the agent is no longer enough to inhibit growth, the organism is able to grow and forms a lawn of bacteria around the disk. To interpret the test results, the radius of the inhibition zone is measured and compared against the predefined values provided by the guidelines [18]. The most widely used guidelines are the CLSI and EUCAST guidelines [2, 17]. CLSI divides the results into three categories for most of the organism-agent combinations; susceptible, intermediate and resistant, whereas EUCAST uses only two categories, susceptible and resistant.

In the dilution methods, however, the susceptibility of the microorganisms to antimicrobial agents is determined whether in tubes (macrobroth dilution method) or in microtube wells molded into a plastic plate (microbroth dilution method). Both broth dilution methods use the same principle; first serial two-fold dilutions of the antimicrobial agent to be tested are made in the tubes/wells containing broth, and then same amount of bacterial suspension is distributed on each tube/well. At the end of the incubation period, the tubes/wells are examined for turbidity which is the indicator of bacterial growth in broth. The tubes/wells remain clear where the concentration of the agent is high enough to inhibit the bacterial growth, whereas at lower concentrations of the agent, the bacteria may grow which causes the tube/well become turbid. The lowest concentration of antimicrobial agent that prevents the *in vitro* growth of bacteria is defined as the minimal inhibitory concentration (MIC) [18]. As in the disk diffusion method, the MIC values are compared against the predefined values provided by the guidelines and their interpretive category is determined and reported.

3. Quality assurance program for antimicrobial susceptibility testing

Clinical microbiology laboratories are an integral part of the total healthcare delivery system. Quality assurance (QA) is the overall process by which a laboratory can verify that a laboratory does its job well. While QA and quality control (QC) share the similar purposes, their meanings and functions are different [19]. QA can be defined as the overall program by which the quality of the test results can be guaranteed [20]. It evaluates and ensures that procedures provide relevant and timely data in the delivery of healthcare services. QA is primarily concerned with broader measures and monitors the performance of laboratory in total and covers all three phases of testing; pre-analytical, analytical and post-analytical. QC, in the other hand, is responsible for monitoring of the analytical phase of testing only and ensures that the daily tests are working properly [21]. QC and QA, only together provide measures for controlling how correct the tests are being performed because QC by itself often does not detect problems in time to prevent harmful results. For example, if >5% of *Enterobacter*, *Serratia*, or *Citrobacter* isolates are susceptible to ampicillin, it likely indicates a problem with insufficient inoculum [22]. Although daily or weekly QC test results are in acceptable limits, such an error can be overlooked until enough data have been accumulated and evaluated which can sometimes take weeks.

Standard processes are required to establish quality measures to be monitored. Standardization of AST has been achieved by CLSI, and in part by EUCAST. The processes defined in CLSI guidelines help clinical laboratories to perform QC tests, measure their results and provide corrective action recommendations covering a broad spectrum of error types. Each laboratory should establish its own quality requirements for testing processes. Only with established quality goals, laboratories can determine whether acceptable quality is being achieved, identify processes that are not performing satisfactorily and are in need of improvement, or to plan new processes to reach a specified level of quality [21]. And to ensure that all the established quality goals are achieved, a comprehensive QA program should be functional in a clinical laboratory.

The major components of a comprehensive QA program for AST, with the relative amount of effort required to be spent on each component given in parantheses, can be listed as follows [23]:

- Clinically relevant testing strategies (15%)
- Testing of reference QC strains (15%)
- Technical competency (15%)
- Organism antibiogram verification (15%)
- Supervisor review of results (15%)
- Procedure manual (10%)
- Cumulative antibiogram (5%)

- Proficiency surveys (5%)
- Other (5%)

The goals of the QC program as set by the CLSI [24, 25] includes to monitor the following:

- the precision (repeatability) and accuracy of AST procedures
- the performance of reagents used in the tests
- the performance of persons who carry out the tests and read the results

The continuous monitorization of the performance is best achieved, but not limited to, by the testing of QC strains.

3.1. Developing relevant antimicrobial susceptibility testing strategies

Only organisms likely to be the cause of an infection should be tested for antimicrobial susceptibility which necessitates the differentiation should be done between the normal flora that resides at the site of the infection and the actual organism causing the infection. Some important factors are to be considered to decide which bacterium or bacteria from a clinical specimen must be included in the AST; such as the body site from which the organism was isolated, the presence of other bacteria and the quality of the specimen from which the organism was grown, the host's status, the ability of the bacterial species to cause infection at the body site from which the specimen was obtained, etc. [1, 26].

3.2. Selecting antimicrobials to test and to report

Each laboratory is unique in its capability, resources, level of experience or institutional needs. Therefore, the decision of which antimicrobials to test depends on each laboratory's specifications and cannot be generalized. The decision involves the opinions of infectious diseases specialist and the pharmacist and should also be in concordance with the hospital formulary. Generally, a laboratory defines 10 to 15 antimicrobial agents for routine testing against various organisms or organism groups, which is called antimicrobial panel or battery. In CLSI's M100 documents Table 1A (Suggested Groupings of Antimicrobial Agents With FDA Clinical Indications That Should Be Considered for Routine Testing and Reporting on Nonfastidious Organisms by Clinical Microbiology Laboratories in the United States) is a valuable source of information to refer to when such tables are to be created at the local level [2]. Because the identity of the bacterial isolate is often not known at the time the AST is performed, some drugs, which are inappropriate to report for that particular isolate, may be tested. These results, however, should be suppressed in the final report.

The goal of the clinical microbiology laboratory is to create a report which will direct the clinician to use the least toxic, most cost-effective and most clinically effective agent that is available. This is accomplished by using the selective-reporting protocol provided by the CLSI. CLSI categorizes antimicrobial agents generally into four groups, Group A, B, C and U. Group A includes the primary agents whose results to be reported first. The results of

Group B drugs should be selectively reported because these are generally broader spectrum agents. However, if the isolate is resistant to the primary agents, the patient cannot tolerate drugs in Group A, the infection has not responded to the therapy with the primary agents, a secondary agent would be a better clinical choice for the particular infection or that the patient has organisms isolated from another site also, and a secondary agent might be more appropriate for treating both organisms, then the results of Group B drugs can be reported [26]. Group C includes alternative or supplemental agents for special cases; such as resistant strains, for patients allergic to primary drugs, for treatment of unusual isolates or for epidemiological purposes. And finally, Group U, includes the agents that are used only or primarily in the treatment of urinary tract infections (e.g., nitrofurantoin, norfloxacin).

Selective-reporting, also called cascade-reporting, improves the clinical relevance of the reports produced and minimizes the selection of multiresistant strains by avoiding the use of broad spectrum agents when narrow spectrum option is susceptible.

3.3. Standardization of the antimicrobial susceptibility testing methodology

The procedural steps of each method must be followed strictly in order to obtain reproducible results. Standardization of AST methodology helps to optimize bacterial growth conditions so that the inhibition of growth can be attributed to the antimicrobial agent and the effects of nutrient limitations, temperature differences or other environmental conditions can be eliminated. And it also optimizes conditions for maintaining antimicrobial integrity and activity so that the failure to inhibit bacterial growth can be attributed to the organism's resistance mechanisms [1].

The standardized components of AST include:

Bacterial inoculum size: Preparation of the inoculum is one of the most critical steps in any susceptibility test method. Inoculum suspensions are prepared using either a log-phase or direct-colony suspension. When direct-colony suspension method is used, 4 to 5, fresh (16- to 24-hour old) colonies, rather than a single colony, should be selected to minimize the possibility of testing a susceptible colony only and missing the resistant mutants dispersed in other colonies. McFarland turbidity standards are used to standardize the number of bacteria in the inoculum. McFarland standards can be prepared by adding specific volumes of 1% sulfuric acid and 1.175% barium chloride to obtain a barium sulfate solution with a specific optical density. The most commonly used is the McFarland 0.5 standard, which provides turbidity comparable with that of a bacterial suspension containing approximately 1.5×10^8 CFU/mL (CFU: colony-forming unit). Once standardized, the inoculum suspensions should be used within 15 minutes of preparation. False-susceptible results may occur if too few bacteria are tested, and false-resistant results may be the outcome of testing too many bacteria [26].

Growth medium: The most frequently used growth media are Mueller-Hinton broth and Mueller-Hinton agar. The standardized variables regarding these media should include; its formulation, pH, cation concentration and thymidine content, thickness of agar (disk diffusion test), and supplements such as blood and serum.

Incubation conditions (atmosphere, temperature, duration): Different organisms require different incubation conditions. Moreover, some antimicrobial agents require different incubation length or temperature than the other disks used for the same organism (e.g., oxacillin with *Staphylococcus* spp.). The user should refer to CLSI M100 tables which give detailed testing conditions for each organism or organism group [2].

Antimicrobials concentrations to be tested: The contents of antimicrobial disks in disk diffusion test and concentrations of antibiotic solutions to be tested in dilution tests are also included in CLSI documents [2].

3.4. Quality control testing with reference quality control strains

Routine QC testing with a range of QC strains is the backbone of the internal QC testing. QC strains are well characterized organisms with defined susceptibility or resistance mechanisms to the antimicrobial agent(s) tested. Testing of QC strains helps to concurrently monitor the performance of the test and ensures that the test is being performed properly. The results obtained with the QC strains should be in predefined, acceptable ranges; for disk diffusion test, between the predefined inhibition zone diameters, and for MIC tests in predefined MIC ranges. If deviations from the acceptable limits are observed, it indicates unacceptable performance and the source(s) of the error should be investigated. CLSI recommends to use various QC strains for different aspects of AST. The list of QC strains can be found in the M100 tables which are updated on a yearly basis. Because of the introduction of new drugs, the changes effecting the existing drugs, or the emergence of new resistance mechanisms which should be investigated by the laboratory, the users are always referred to the latest update available. The QC strains recommended by CLSI are divided in two as being regular „QC strains“ and „supplemental QC strains“. Each laboratory performing AST with CLSI's reference methods should include QC strains in regular QC tests, however, the supplemental strains are only required if they are used to assess a new test, for training new personnel, investigation of special susceptibility or resistance characteristics, etc., and are not required to be included in the routine QC of AST [2].

CLSI's European counterpart, EUCAST, also publishes guidelines for the use of QC strains for AST, however, compared with the comprehensive battery of QC strains suggested by the CLSI, EUCAST is limited to six QC strains at the moment [27]. The guidelines of EUCAST are continuously evolving and on areas where EUCAST's experience is not able to cover yet, EUCAST does not refrain from making referrals to relevant CLSI documents. However, one big difference between the QC strains recommended by CLSI and EUCAST is that, EUCAST's recommendation for *Haemophilus influenzae* NCTC 8468 in contrast to CLSI's *H. influenzae* ATCC® 49247. The strain EUCAST chose as a QC strain is susceptible to β -lactam antibiotics whose inhibition zones are easier to read than the ATCC® strain which is a β -lactamase negative, ampicillin resistant (BLNAR) strain. The suggested QC strains by CLSI with their specifications are listed in Table 1 [2].

QC Strain	Test(s), for which strain is primarily used
<i>Escherichia coli</i> ATCC® 25922	Disk diffusion and MIC of <i>Enterobacteriaceae</i> , <i>Pseudomonas aeruginosa</i> , <i>Acinetobacter spp.</i> , <i>Burkholderia cepacia</i> , <i>Stenotrophomonas maltophilia</i>
	MIC of other non- <i>Enterobacteriaceae</i>
	Screening and confirmatory tests for ESBLs (negative)
	Disk diffusion and MIC of <i>Neisseria meningitidis</i> (for ciprofloxacin, nalidixic acid, minocycline, and sulfisoxazole)
<i>Escherichia coli</i> ATCC® 35218	Disk diffusion and MIC for β -lactam/ β -lactamase inhibitor combination drugs of <i>Enterobacteriaceae</i> , <i>Pseudomonas aeruginosa</i> , <i>Acinetobacter spp.</i> , <i>Burkholderia cepacia</i> , <i>Stenotrophomonas maltophilia</i> , <i>Staphylococcus spp.</i>
	MIC for β -lactam/ β -lactamase inhibitor combination drugs of other non- <i>Enterobacteriaceae</i>
	Testing of amoxicillin-clavulanic acid for <i>Haemophilus spp.</i>
<i>Klebsiella pneumoniae</i> ATCC® 700603	Screening and confirmatory tests for ESBLs (positive)
<i>Klebsiella pneumoniae</i> ATCC® BAA-1705	Confirmatory test for suspected carbapenemase production in <i>Enterobacteriaceae</i> (MHT positive)
<i>Klebsiella pneumoniae</i> ATCC® BAA-1706	Confirmatory test for suspected carbapenemase production in <i>Enterobacteriaceae</i> (MHT negative)
<i>Pseudomonas aeruginosa</i> ATCC® 27853	Disk diffusion and MIC of <i>Pseudomonas aeruginosa</i> , <i>Acinetobacter spp.</i> , <i>Burkholderia cepacia</i> , <i>Stenotrophomonas maltophilia</i>
	MIC of other non- <i>Enterobacteriaceae</i>
<i>Staphylococcus aureus</i> ATCC® 25923	Disk diffusion of <i>Staphylococcus spp.</i> and <i>Enterococcus spp.</i>
	Screening test for β -lactamase production of <i>Staphylococcus aureus</i> group and coagulase negative <i>Staphylococci</i> (negative)
	Screening test for <i>mecA</i> -mediated oxacillin resistance using cefoxitin in <i>Staphylococcus aureus</i> group and coagulase negative <i>Staphylococci</i> (<i>mecA</i> negative; disk diffusion susceptible)
	Screening test for inducible clindamycin resistance in <i>Staphylococcus aureus</i> group and coagulase negative <i>Staphylococci</i> with disk diffusion (D-zone test) (negative)
	Screening test for high-level mupirocin resistance in <i>Staphylococcus aureus</i> group (<i>mupA</i> negative; disk diffusion susceptible)
<i>Staphylococcus aureus</i> ATCC® 29213	MIC of <i>Staphylococcus spp.</i>

QC Strain	Test(s), for which strain is primarily used
	Screening test for β -lactamase production in <i>Staphylococcus aureus</i> group and coagulase negative <i>Staphylococci</i> (positive)
	Screening test for oxacillin resistance in <i>Staphylococcus aureus</i> group (susceptible)
	Screening test for <i>mecA</i> -mediated oxacillin resistance using cefoxitin in <i>Staphylococcus aureus</i> group (<i>mecA</i> negative; MIC susceptible)
	Screening test for inducible clindamycin resistance in <i>Staphylococcus aureus</i> group, coagulase negative <i>Staphylococci</i> and <i>Streptococcus</i> spp. β -hemolytic group with broth microdilution (no growth)
	Screening test for high-level mupirocin resistance in <i>Staphylococcus aureus</i> group (<i>mupA</i> negative; MIC susceptible)
<i>Staphylococcus aureus</i> ATCC® 43300	Screening test for oxacillin resistance in <i>Staphylococcus aureus</i> group (resistant)
	Screening test for <i>mecA</i> -mediated oxacillin resistance using cefoxitin in <i>Staphylococcus aureus</i> group (disk diffusion and MIC) and coagulase negative <i>Staphylococci</i> (disk diffusion) (<i>mecA</i> positive)
<i>Staphylococcus aureus</i> ATCC® BAA-976	Screening test for inducible clindamycin resistance in <i>Staphylococcus aureus</i> group, coagulase negative <i>Staphylococci</i> and <i>Streptococcus</i> spp. β -hemolytic group with broth microdilution (no growth)
<i>Staphylococcus aureus</i> ATCC® BAA-977	Screening test for inducible clindamycin resistance in <i>Staphylococcus aureus</i> group, coagulase negative <i>Staphylococci</i> and <i>Streptococcus</i> spp. β -hemolytic group with broth microdilution (growth)
<i>Staphylococcus aureus</i> ATCC® BAA-1708	Screening test for high-level mupirocin resistance in <i>Staphylococcus aureus</i> group (<i>mupA</i> positive; disk diffusion and MIC resistant)
<i>Enterococcus faecalis</i> ATCC® 29212	MIC of <i>Enterococcus</i> spp.
	Screening test for vancomycin MIC ≥ 8 $\mu\text{g}/\text{mL}$ in <i>Staphylococcus aureus</i> group (susceptible)
	Screening test for high-level aminoglycoside resistance in <i>Enterococcus</i> spp. (disk diffusion, broth microdilution, agar dilution: susceptible)

QC Strain	Test(s), for which strain is primarily used
	Screening test for vancomycin resistance in <i>Enterococcus</i> spp. (agar dilution: susceptible) checking that medium is acceptable for testing sulfonamides, trimethoprim, and trimethoprim/sulfamethoxazole
<i>Enterococcus faecalis</i> ATCC® 51299	Screening test for vancomycin MIC ≥ 8 $\mu\text{g}/\text{mL}$ for <i>Staphylococcus aureus</i> group (resistant)
	Screening test for high-level aminoglycoside resistance in <i>Enterococcus</i> spp. (broth microdilution, agar dilution: resistant)
	Screening test for vancomycin resistance in <i>Enterococcus</i> spp. (agar dilution: resistant)
<i>Haemophilus influenzae</i> ATCC® 49247	Disk diffusion and MIC of <i>Haemophilus</i> spp. (BLNAR; β -lactamase negative, ampicillin resistant)
<i>Haemophilus influenzae</i> ATCC® 49766	Disk diffusion and MIC of <i>Haemophilus</i> spp. with selected cephalosporins (β -lactamase positive)
<i>Haemophilus influenzae</i> ATCC® 10211	Checking growth capabilities of medium used for disk diffusion and MIC tests for <i>Haemophilus</i> spp.
<i>Neisseria gonorrhoeae</i> ATCC® 49226	Disk diffusion and MIC of <i>Neisseria gonorrhoeae</i> (CMRNG; chromosomally mediated (penicillin) resistant <i>N. gonorrhoeae</i>)
<i>Streptococcus pneumoniae</i> ATCC® 49619	Disk diffusion and MIC of <i>Streptococcus pneumoniae</i> (penicillin intermediate), <i>Streptococcus</i> spp. β -hemolytic group <i>Streptococcus</i> spp. viridans group and <i>Neisseria meningitidis</i>
	Screening test for inducible clindamycin resistance in <i>Streptococcus</i> spp. β -hemolytic group with disk diffusion (D-zone test) and broth microdilution (negative)
<i>Bacteroides fragilis</i> ATCC® 25285	MIC of anaerobes
<i>Bacteroides thetaiotaomicron</i> ATCC® 29741	MIC of anaerobes
<i>Clostridium difficile</i> ATCC® 700057	MIC of anaerobes
<i>Eubacterium lentum</i> ATCC® 43055	MIC of anaerobes

Table 1. Quality Control Strains Suggested for Antimicrobial Susceptibility Testing by CLSI

3.5. Selection, obtaining and maintenance of reference QC strains

When selecting QC strains for routine internal QC testing; the strains that most closely resemble the patient's isolate should be tested [23]. This will provide that the drugs planned to be tested for the patient can be concomitantly tested with the QC strain. Additionally, same materials and testing conditions used for the clinical isolates can be evaluated. Before obtaining the QC strains, laboratories should decide which strains do fit best to the laborato-

ry's procedures. For example, if a laboratory does not perform Modified Hodge Test (MHT) to confirm suspected carbapenemase production in *Enterobacteriaceae*, the *Klebsiella pneumoniae* ATCC® BAA-1705 (MHT-positive) and *Klebsiella pneumoniae* ATCC® BAA-1706 (MHT-negative) strains are not necessary for that particular laboratory. QC organisms susceptible to the tested antimicrobials are generally used but resistant QC strains are also necessary when testing for special resistance mechanisms.

The QC strains can be obtained from various suppliers and in many formats. What important is, no matter in what format the strain has been received, the initial reconstitution should be performed according to supplier's recommendations. For long term storage, stock cultures can be stored in a suitable stabilizer (e.g., trypticase soy broth with 10 to 15% glycerol, 50% fetal calf serum in broth, defibrinated sheep blood or skim milk) at -20°C or below (preferably at -60°C or below). To obtain working control cultures, subcultures from the permanent stock culture are made onto agar plates. Isolated colonies (4 to 5) are selected and subcultured to an agar slant (trypticase soy agar slants for non-fastidious organisms and chocolate agar slants for fastidious organisms) and incubated overnight. These working cultures on agar slants are stored at 2 – 8°C, for no more than three successive weeks. New working control cultures should be prepared at least monthly from permanent stock cultures. Prior to QC testing, growth from an agar slant is subcultured to agar plates and incubated overnight. To use for QC testing, 4 to 5 isolated colonies from the plate are selected. A new working culture should be prepared each day the QC test is being performed [2, 23].

Working control cultures can be used to monitor precision (repeatability) and accuracy of the AST as long as no significant change in the mean zone diameter or MIC value, not attributable to faulty methodology, is observed. Laboratories usually do not have problems with the maintenance of susceptible QC strains owing to the stability of these strains, however, QC strains with particular resistance mechanisms are harder to maintain since they may be less genetically stable. Repeated subcultures can cause the loss of resistance mechanisms and unsatisfactory performances can be experienced. Documented problems have arisen with the QC strains which carry their specific resistance mechanism on a plasmid (e.g., *E. coli* ATCC® 35218 and *K. pneumoniae* ATCC® 700603) [2]. Suboptimal storage conditions and repeated cultures may cause the spontaneous loss of the plasmid encoding the β -lactamase and off-the-limit results may be encountered.

3.6. Frequency of QC testing

Appropriate QC organisms should be tested daily for all antimicrobial agents routinely included in the antimicrobial battery until a laboratory achieves "satisfactory performance". CLSI makes the definition of "satisfactory performance" as obtaining unacceptable results in no more than 1 out of 20 or 3 out of 30 results obtained in consecutive test days for each antimicrobial agent/organism combination. Once this satisfactory performance is obtained, a laboratory can convert from daily QC testing to weekly QC testing. As long as all QC test results are within the acceptable limits, the laboratory can continue weekly testing, however on occasions when a modification in the test is made, consecutive QC testing is required (Table 2., adapted from reference 2).

Day(s)*	Modification in the Test
1	Start to use new shipment or lot number of disks/MIC panels or prepared agar plates
	Start to use disks from a new manufacturer
	Expand or reduce the dilution range in MIC testing
	Repair of instrument that affects the AST results
5	Start to use prepared agar plates (disk diffusion), broth or agar (MIC) from a new manufacturer
	Convert inoculum preparation/standardization method from visual adjustment of turbidity to use a photometric device which has its own QC protocol
	Update of the software which affects the AST results
20 or 30	Use new method for MIC test (e.g., convert from visual reading to instrument reading of panel, convert from overnight to rapid MIC test)
	Use new manufacturer of MIC test
	Change method of measuring zones in disk diffusion test (e.g., start using an automated zone reader)
	Convert inoculum preparation/standardization method to a method that is dependent on user technique

* Number of days of consecutive QC testing required

Table 2. Required Quality Control Frequency after Modifications in the Test

For both, disk diffusion and MIC testing, addition of any new antimicrobial agent to the existing panel requires 20 or 30 consecutive days of satisfactory testing before it can be tested on a weekly schedule.

3.7. Corrective action

Corrective action is defined as the “action to eliminate the cause of a detected nonconformity or other undesirable situation” [28] and in regard to AST, is needed whenever any of the weekly QC results are not within the acceptable limits. The factors causing for the deviation in the results are various but can be divided in two as being results due to identifiable errors and results with no error identified [24, 25]. Identifiable errors, also named obvious errors, are easy to detect and also easy to correct. Most usual reasons causing for identifiable errors include; use of the wrong disk, use of the wrong QC strain, contamination of the strain or media, use of the wrong incubation temperature or conditions. If the reason causing the out-of-range results is one of the identifiable errors, the test must be carried out again the day the error is observed. If results of the repeat test are in acceptable limits, no further correc-

tive action is necessary. On the other hand, if the reason causing for the error cannot be identified, the test must be carried out again the day the error is observed, preferably with a new working culture or subculture, but should also be monitored for a total of five consecutive test days. During five consecutive days, if all results are within the acceptable limits no additional corrective action is required. However, if any of the results are outside the acceptable limits, additional corrective action is required. At this point, a systematic error, rather than a random should be suspected and the components of AST should be thoroughly investigated. The reasons include; wrong measurement, clerical errors, problems in the adjustment of turbidity, past expiration date materials, failure in providing proper growth conditions (temperature, atmosphere), improper storage of disks, contamination of QC strain, loss of characteristics, inoculum prepared from an old plate (> 24 hours), etc.. In order to start to routine QC testing, satisfactory performance for another 20 or 30 consecutive days is required once the reason causing the error is detected and corrected.

When an out-of-range QC results necessitates a corrective action, the factors listed in Table 3 should be considered for troubleshooting (Table 3., adapted from references 24 and 25).

QC Strain	Use of the wrong QC strain
	Improper storage
	Inadequate maintenance (e.g., use of the same working culture for >1 month)
	Contamination
	Nonviability
Testing supplies	Changes in the organisms (e.g., mutation, loss of plasmid)
	Improper storage or shipping conditions
	Contamination
	Use of a defective agar plate (too thick or too thin)
	Inadequate volume of broth in tubes or wells
	Use of damaged plates, panels, cards, tubes (e.g., cracked, leaking)
Testing process	Use of expired materials
	Use of the wrong incubation temperature or conditions
	Inoculum suspensions were incorrectly prepared or adjusted
	Inoculum prepared from a plate incubated for the incorrect length of time
	Inoculum prepared from differential or selective media containing anti-infective agents or other growth-inhibiting compounds
	Use of wrong disk/reagents, ancillary supplies
	Improper disk placement (e.g., inadequate contact with the agar)
	Incorrect reading or interpretation of test results
Transcription error	
Equipment	Not functioning properly or out of calibration (e.g., pipettes)

Table 3. Factors Frequently Causing Out-of-range Results

3.8. Documentation of the quality control test results

Results from all QC tests should be documented on a QC log sheet [23]. On this log sheet information regarding the following are required: the date, the technician who performed the test, antimicrobial agents used (potency, lot, expiration date, etc.), media used (lot, expiration date, etc.). Once the log sheet has been filled by the technician who performed and read the test, a second technician, or the supervisor, should check the results. Also, corrective actions taken, if any, and their outcomes should be noted.

A useful and simple way of monitoring QC results is to use the Shewhart diagram, in which the daily readings are plotted on a chart with upper and lower control limits marked [29]. It provides the visual assessment of the results but can also provide in depth information if a more formal mathematical approach is followed [20]. An example of presenting daily QC results on a Shewhart diagram is given in Figure 1. The famous rules of Westgard and Klee [30] can be easily adopted to the QC of disk diffusion test in which the control diameters are treated as mean ± 2 SD [20].

One QC result lies outside the limits (Westgard rule 1_{2s}): It is a warning, whether it's a random error or the beginning of an emerging problem. Routine test results for that day may be reported if there is no other evidence of problems in the current tests. It does not require corrective action by itself, unless the result is far out of range or there are other indications of a problem.

Two consecutive QC results are outside the limits in the same side of the mean of the range (Westgard rule 2_{2s}): Indicates an error in the test methodology (a systematic error), corrective action is required.

Ten consecutive QC results falling on one side of the mean (Westgard rule 10_x): Results may be accepted but this likely indicates a systematic problem which should be acted on.

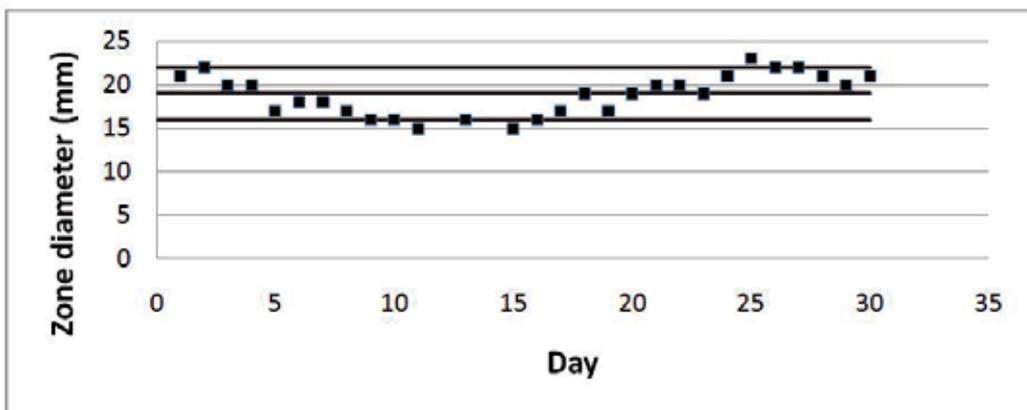


Figure 1. Example for daily disk diffusion QC results for *Escherichia coli* ATCC® 25922 vs. ampicillin plotted on a Shewhart diagram (acceptable zone limits: 16 – 22 mm).

3.9. Organism - Antimicrobial susceptibility test result verification

One of the most widely used supplemental QC measure is the use of susceptibility test results to verify results generated on patient results. Species with „typical“ antibiograms are useful in verification of the identification as well as the susceptibility results. CLSI suggests some results to be confirmed before they are reported, these mostly include rare resistance phenotypes. The rare resistance phenotypes are divided in three categories; Category I; not reported or only rarely reported to date, Category II; uncommon in most institutions, and Category III; may be common, but is generally considered of epidemiological concern. Since category I includes the least encountered and most significant results, it is highly important to detect these results before being reported unnoticed and to follow the necessary steps for the verification. Unusual resistance phenotypes which require confirmation are given in Table 3 (adapted from reference 2).

Category	Observed susceptibility result
I	NS to carbapenems, extended-spectrum cephalosporins or fluoroquinolones in <i>H. influenzae</i>
	NS to extended-spectrum cephalosporins, meropenem or minocycline, R to ampicillin or penicillin in <i>N. meningitidis</i>
	NS to linezolid or vancomycin in <i>S. pneumoniae</i>
	NS to ampicillin, penicillin, extended-spectrum cephalosporins, daptomycin, ertapenem, meropenem, linezolid or vancomycin in β -hemolytic group <i>Streptococcus</i>
	NS to daptomycin, ertapenem, meropenem, linezolid, or vancomycin, R to quinupristin-dalfopristin in viridans group <i>Streptococcus</i>
II	I or R to carbapenems in <i>Enterobacteriaceae</i>
	I or R to 3rd generation cephalosporins or fluoroquinolones in <i>Salmonella</i> and <i>Shigella</i> spp.
	R to colistin/polymyxin in <i>A. baumannii</i>
	I or R to colistin/polymyxin in <i>P. aeruginosa</i>
	I or R to trimethoprim-sulfamethoxazole in <i>S. maltophilia</i>
	R to amoxicillin-clavulanic acid, R to ampicillin without accompanying β -lactamase production in <i>H. influenzae</i>
	NS to extended spectrum cephalosporins in <i>N. gonorrhoeae</i>

Category	Observed susceptibility result
	I to ampicillin, penicillin, I or R to rifampin, NS to azithromycin in <i>N. meningitidis</i>
	R to linezolid, NS to daptomycin for <i>Enterococcus</i> spp.
	NS to daptomycin, R to linezolid, I or R to quinupristin-dalfopristin, vancomycin MIC = 4 µg/mL or vancomycin MIC ≥ 8 µg/mL for <i>S. aureus</i>
	NS to daptomycin, I or R to quinupristin-dalfopristin or vancomycin, R to daptomycin in coagulase-negative <i>Staphylococcus</i> spp.
	I or R to fluoroquinolone, imipenem, meropenem, quinupristin-dalfopristin, rifampin in <i>S. pneumoniae</i>
	I or R to quinupristin-dalfopristin in β-hemolytic group <i>Streptococcus</i>
III	R to amikacin, gentamicin, and tobramycin in <i>Enterobacteriaceae</i>
	I or R to extended spectrum cephalosporins in <i>E. coli</i> , <i>Klebsiella</i> spp. or <i>P. mirabilis</i>
	I or R to carbapenem in <i>A. baumannii</i>
	R to amikacin, gentamicin, and tobramycin, or carbapenem in <i>P. aeruginosa</i>
	I or R to fluoroquinolone in <i>N. gonorrhoeae</i>
	I or R to chloramphenicol or fluoroquinolone in <i>N. meningitidis</i>
	R to vancomycin or high-level aminoglycoside in <i>Enterococcus</i> spp.
	R to oxacillin in <i>S. aureus</i>
	R to amoxicillin, penicillin or extended spectrum cephalosporins in <i>S. pneumoniae</i> using nonmeningitis breakpoints

NS; nonsusceptible, I; intermediate, R; resistant

Table 4. Unusual Resistance Phenotypes Which Require Confirmation

The general approach to be followed is, for all three categories, to confirm the identification of the organism and the AST. If the results are confirmed, the infection control should be informed about the case.

3.10. Real-time review of results

Accuracy of the susceptibility test results should be continuously monitored. This is mostly accomplished by daily reviewing of the data that is being produced. Profiles which are likely, somewhat likely, somewhat unlikely and nearly impossible should be identified, whether manually or with the help of a software programmed to recognize different patterns of susceptibility data [1]. Prompt recognition of unusual resistance or inconsistent susceptibility helps the laboratory to timely confirm the susceptibility results. In order to confirm the results, first step is to exclude the transcriptional and reading errors and make sure of the purity of the inoculum which has been tested. If no errors are found in the previous steps, the identification of the organism should be confirmed and the susceptibility test be repeated, preferably with another method. In cases where no errors are detected and the unusual resistance is confirmed, the clinician may be warned and measures can be taken to limit the spread of this unusual resistance.

3.11. Education

Education is an important component of the QA process. Having knowledge about the methods also provides the understanding of their limitations and pitfalls. A well-educated technician may timely recognize atypical results and is aware of the approach to follow for the resolution and avoidance of errors [20]. A very efficient way of training in-service personnel is the end-point interpretation control [24, 25]. Laboratory workers, who perform AST, are provided with a set of selected disk diffusion plates and are asked to read the results. The recorded results are then compared by an experienced reader, e.g., the laboratory director, and the individual performances of each technician is evaluated and if necessary, corrected. It significantly helps to minimize variation in the interpretation of zone sizes among laboratory workers.

3.12. External quality assessment

In external quality assessment (EQA) programs, a central laboratory distributes test strains with known susceptibility profiles to all participant laboratories. Each participating laboratory tests and reports the results to the central laboratory. Once all the results are returned from participants, the central laboratory evaluates the results and prepares a feedback report. The benefit of participating in such program is that each individual laboratory can assess its own performance compared with other laboratories, at national and international levels, it functions as an educational tool, and also provides the evidence of performance required by the accrediting bodies. On the other hand, the number of strains distributed in a year is relatively small, which brings the disadvantage of the rare errors going unnoticed [20]. Also, in contrast to internal QC, which is capable of acting on problems encountered on daily basis, it takes quite a while for the EQA feedback reports to be sent to the participating laboratories, thus corrective action is delayed.

3.13. Internal quality assessment

Internal quality assessment (IQA) is a complementary activity to EQA in which routine tests are repeated on the same day as the original, but this time, with the identity of the specimen

blinded. After the reports are produced, the results are compared and discrepancies noted. This activity helps to monitor the precision and accuracy of the test procedure and may highlight problem areas not detected by other QC methods. It monitors not only the performance of the test and reagents, but also the performance of the persons carrying out the tests [20]. The EQA and the IQA are complementary activities, while IQA focuses on monitoring a single laboratory on a daily basis, EQA compares the performance of different laboratories and is important for maintaining long-term accuracy of the AST methods employed [21].

3.14. Proficiency testing programs

They are a type of EQA in which simulated patient specimens are sent to participating laboratories. Again, the reports are produced by each laboratory, and returned to the central laboratory for evaluation. In the United States, government mandates that clinical laboratories be accredited and licensed. The government and licensing agencies are using proficiency testing as an objective method for the accreditation of laboratories [21]. In 1988, the U.S. Congress passed the Clinical Laboratory Improvement Amendment (CLIA '88) which mandated proficiency testing (PT) as a major part of the laboratory accreditation process [31]. The initial CLIA '88 proposal called for two PT specimens per year but final legislative rule, published in 2003, expanded this to study five samples three times per year. The definition of failure is defined as two of five incorrect results on two of the three consecutive PT surveys [32].

4. Quality control of automated antimicrobial susceptibility test systems

According to the work load and the resources a laboratory has, a laboratory can choose to use one of many types of commercial automated antimicrobial susceptibility test systems. Most of these systems use the principle of turbidimetric detection of bacterial growth in a broth medium by use of a photometer which periodically examines the test wells [26]. The most widely used systems in the world are VITEK 2 System (bioMérieux Vitek, Hazelwood, MO), BD Phoenix System (BD Diagnostic Systems, Sparks, MD), MicroScan WalkAway SI (Siemens Healthcare Diagnostics, Sacramento, CA) and TREK Sensititre (ARIS 2X, Trek Diagnostic Systems, Cleveland, OH). Each device has its own QC procedure and commercial susceptibility testing devices are not addressed in CLSI standards. CLSI only describes methods regarding generic reference procedures, however these reference methods are used by the US Food and Drug Administration before clearance is given to a commercial system for marketing in the US to evaluate its performance.

5. Conclusion

Although great improvement has been done in AST methodology and automated susceptibility systems have been introduced which provide same-day results, it should be considered that there are still many variables not covered by the standard methods. First of all, the

laboratory test conditions are far different from *in vivo* conditions where the organism and the antimicrobial agent do actually interact. Factors, such as bacterial inoculum size, pH, cation concentration and oxygen tension differ greatly depending on the site of infection [1]. In spite of all these limitations, the clinical microbiology laboratory should follow the most up-to-date guidelines to serve the patients in the best possible way. With a well constructed QA program in operation, a laboratory should aim to ensure that the right test is carried out on the right specimen, and that the right result and right interpretation is delivered to the right person at the right time.

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The Investigation of Gene Regulation and Variation in Human Cancers and Other Diseases

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Additional information is available at the end of the chapter

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1. Introduction

Dynamic regulation of genes is an important part of the cell life cycle in health and disease. The regulation includes the variety and alteration of genome and gene expression, and the concept such as quality of genome will be useful to predict and assess the developmental stages of the cells, disease status and drug sensitivity. Recent technologies and worldwide sequencing projects have revealed 26,383 annotated genes in the 2.91-Gigabase human genome [1,2]. The main molecular functions of the annotated genes, as categorized by Gene Ontology (GO), are enzyme, signal transduction, nucleic acid binding, cell adhesion, chaperone, cytoskeletal structural protein, extracellular matrix, immunoglobulin, ion channel, motor, structural protein of muscle, protooncogene, select calcium binding protein, intracellular transporter, and transporter [1,3]. Despite a wealth of knowledge, the function of 42% of the annotated genes remains unknown [1]. When the human genome sequence was published in 2001 [1], there were a predicted 39,114 genes, of which 59% were of unknown function. According to the International Human Genome Sequencing Consortium, the number of identified genes is approximately 32,000, of which 51% show a match within InterPro, a database that integrates diverse information about protein families, domains, and functional sites [2-5]. In 2001, InterPro combined sequence and pattern information from four databases (PRINTS, PROSITE, Pfam, Prosite Profile); however, it now includes information from an additional eight databases (SMART, ProDom, PIRSF, SUPERFAMILY, PANTHER, CATH-Gene3D, TIGRFAM, and HAMAP) [2,4-16]. In [2], the InterPro entries are collapsed into 12 broad categories: cellular processes, metabolism, DNA replication/modification, transcription/translation, intracellular signaling, cell-cell communication, protein folding and degradation, transport, multifunctional proteins, cytoskeletal/structural, defense and immunity, and miscellaneous function. The

rate of single nucleotide polymorphism (SNP) variation has been reported as 1 in 1250 base pairs [1] and more than 1.4 million SNPs have been identified [2] (Table 1).

Size of the genome	2.91 Gbp	[1]
Number of annotated genes	26,383	[1]
Main molecular functions of annotated genes	enzyme, signal transduction, nucleic acid binding, cell adhesion, chaperone, cytoskeletal structural protein, extracellular matrix, immunoglobulin, ion channel, motor, structural protein of muscle, protooncogene, select calcium binding protein, intracellular transporter, transporter	[1]
Percentage of annotated genes with unknown function	42%	[1]
Number of hypothetical and annotated genes	39,114	[1]
Percentage of hypothetical and annotated genes with unknown function	59%	[1]
Number of identified genes	approx. 32,000	[2]
Percentage of matches with InterPro	51%	[2]
Rate of SNP variation	1/1250 bp	[1]
SNPs identified	more than 1.4 million	[2]

Table 1. Genomic and gene characteristics revealed by the Human Genome Project.

Among the databases combined in InterPro (Table 2), PRINTS, PROSITE, and Pfam contain protein families in which the homology between each protein is predicted by the degree of sequence similarity [8]. The others—SMART, ProDom, PIRSF, SUPERFAMILY, PANTHER, CATH-Gene3D, TIGRFAM, and HAMAP [4-16]—have unique characteristics and URLs, and have been developed sharing information among each other and incorporating information from GO. In detail, PRINTS is a collection of diagnostic protein family “fingerprints”, which are groups of conserved motifs, evident in multiple sequence alignments [6]; PROSITE is a protein domain database for functional characterization and annotation that consists of documentation entries describing protein domains, families, and functional sites as well as associated patterns and profiles to identify them [7]; Pfam contains collections of protein families, each represented by multiple sequence alignments and hidden Markov models, available via servers in the UK, the USA, and Sweden [8]; SMART (Simple Modular Architecture Research Tool) is an online resource for the identification and annotation of protein domains and the analysis of protein domain architectures [9]; ProDom is a comprehensive set of protein domain families generated automatically from the UniProt database [10]; PIRSF is a classification system that reflects evolutionary relationships among full-length proteins and domains [11]; SUPERFAMILY is a database of structural and functional annotation for all proteins and genomes [12]; PANTHER is a classification system that clas-

lifies genes by their functions using published experimental evidence and evolutionary relationships to predict function even in the absence of direct experimental evidence [13]; CATH-Gene3D is a comprehensive database of protein domain assignments for sequences from the major sequence databases [14]; TIGRFAM is a collection of protein family definitions built to aid high-throughput annotation of specific protein functions [15]; and HAMAP is composed of two databases: the proteome database and the family database, and of an automatic annotation pipeline mainly focused on microbial proteomes [16]. Hidden Markov models are usually used for the database algorithm.

Database Name	Context	URL	Reference
InterPro	integrative predictive models of protein families, domain and functional sites of multiple databases such as PRINTS, PROSITE, Pfam, SMART, ProDom, PIRSF, SUPERFAMILY, PANTHER, CATH-Gene3D, TIGRFAM, and HAMAP	http://www.ebi.ac.uk/interpro/	[4], [5]
PRINTS	a collection of diagnostic protein family "fingerprints" which are groups of conserved motifs, evident in multiple sequence alignments	http://www.bioinf.manchester.ac.uk/dbbrowser/PRINTS/index.php	[6]
PROSITE	a protein domain database for functional characterization and annotation which consists of documentation entries describing protein domains, families and functional sites as well as associated patterns and profiles to identify them	http://prosite.expasy.org/	[7]
Pfam	a database of collection of protein families, each represented by multiple sequence alignments and hidden Markov models, available via servers in the UK, the USA and Sweden	http://pfam.sanger.ac.uk/http://pfam.janelia.org/http://pfam.sbc.su.se/	[8]
SMART	an online resource for the identification and annotation of protein domains and the analysis of protein domain architectures, of which abbreviation is Simple Modular Architecture Research Tool	http://smart.embl.de/	[9]
ProDom	a comprehensive set of protein domain families automatically generated from the uniProt knowledge Database	http://prodom.prabi.fr/prodom/current/html/home.php	[10]
PIRSF	the classification system which reflects evolutionary relationships of full-length proteins and domains	http://pir.georgetown.edu/pirsf/	[11]
SUPERFAMILY	a database of structural and functional annotation for all proteins and genomes	http://supfam.org/SUPERFAMILY/	[12]
PANTHER	the classification system which classifies genes by their functions using published scientific experimental evidence and evolutionary relationships to predict function even in the absence of direct experimental evidence	http://www.pantherdb.org/	[13]
CATH-Gene3D	a comprehensive database of protein domain assignments for sequences from the major sequence databases	http://gene3d.biochem.ucl.ac.uk/	[14]

Database Name	Context	URL	Reference
TIGRFAM	a collection of protein family definitions built to aid in high-throughput annotation of specific protein functions	http://www.jcvi.org/cgi-bin/tigrfams/index.cgi	[15]
HAMAP	a system which composed of two databases, the proteome database and the family database, and of an automatic annotation pipeline	http://hamap.expasy.org/	[16]

Table 2. Database information.

2. Gene regulation

2.1. Gene markers for cancer and cancer stem cells

Several molecular markers of cancer have been identified [17]. Metastatic cancer cells can transfer into bodily fluids through the cellular epithelia, which enables the detection of cancer markers in bodily fluids such as blood plasma, urine, or saliva [17]. The different types of cancer markers include genomic DNA point mutations, microsatellite alterations, promoter hypermethylation, viral sequences, aberrant chromosomal copy number, chromosomal translocations, deletions, or loss of heterozygosity, telomere extension, alterations in RNA or protein expression, and mitochondrial DNA mutations [17].

Molecular markers of cancer include *TP53* (encoding p53), which has been shown to be mutated in head and neck, lung, colon, pancreatic, and bladder cancer [17,18]; colon, lung, esophagus, breast, liver, brain, reticuloendothelial tissue, and hematopoietic tissue cancers [19]; and bladder cancer [20]. Mutation of the epidermal growth factor receptor (*EGFR*) gene is an important predictive/prognostic factor for EGFR-tyrosine kinase inhibitor therapy in non-small cell lung cancer [21]. *RAS* oncogene mutations have been identified in colorectal tumors [22]. Microsatellites, which are tandem iterations of simple di-, tri-, or tetranucleotide repeats, have been reported to be unstable in some inherited diseases and in some types of cancer [23], including head and neck, lung, breast, and bladder cancer [17,23].

The expression levels of the cell cycle-related proteins p21 (*CDKN1A*), p53 (*TP53*), cyclin D1 (*CCND1*), and aurora kinase A (*AURKA*) may be used as prognostic markers to predict recurrence in stage II and stage III colon cancer [24]. In addition, markers of the epithelial–mesenchymal transition (EMT)—such as reduced expression of keratins, a switch from E-Cadherin to N-Cadherin, and enhanced migration in D492M cells—might be a useful marker in breast cancer [25]. Furthermore, expression of the stem cell markers cytokeratins 15 and 19 was altered in squamous cell carcinoma: cytokeratin 15 levels were decreased and the localization of cytokeratin 19 was altered [26]. *KLK3*, which encodes prostate-specific antigen, a member of the kallikrein family of serine proteases, is a biomarker for prostate cancer detection and disease monitoring [27,28]. Mitochondrial DNA mutations have been associated with bladder, head and neck, lung, colorectal, and pancreatic cancer [29–32] (Table 3).

Highly parallel identification of cancer-related genes using small hairpin RNA screening has revealed that the expression of known and putative oncogenes, such as *EGFR*, *KRAS*, *MYC*, *BCR-ABL*, *MYB*, *CRKL*, and *CDK4* that are essential for cancer proliferation, is altered in cancer cells [33]. Other genes such as *PTPN1*, *NF1*, *SMARCB1*, and *SMARCE1* have been identified as essential for the imatinib response of leukemia cells, and *TOPOIIA* expression is involved in resistance to etoposide, an anti-topoisomerase II agent, in small cell lung cancer [33-36].

Marker	Cancer Type	Reference
<i>TP53</i> mutation	head and neck cancer	[18]
	bladder cancer	[20]
	lung cancer (small cell lung cancer and non-small cell lung cancer); breast, colon, esophagus, liver, bladder, ovary, and brain cancers; sarcomas, lymphomas, and leukemias	[19]
<i>EGFR</i> mutation	non-small cell lung cancer	[21]
<i>RAS</i> mutation	colorectal tumors	[22]
DNA microsatellite alterations	bladder cancer	[23]
alteration in cell cycle mRNA expression	colon cancer	[24]
alteration in cytokeratin mRNA expression	squamous cell carcinoma	[26]
alteration in kallikrein mRNA expression	prostate cancer	[27]
mitochondrial DNA mutations	bladder cancer, head and neck cancer, lung cancer	[29]
	colorectal tumors	[30], [32]
	pancreatic cancer	[31]

Table 3. Genomic markers of cancer.

2.2. Genes related to cell proliferation

Cyclins, which regulate the cell cycle, play important roles in cell proliferation and the uncontrolled cell proliferation that is the most important factor in tumorigenesis [37]. Tumor cells accumulate mutations that result in constitutive mitogenic signaling and defective responses to anti-mitogenic signals that contribute to unscheduled proliferation [38]. In cancer, unscheduled proliferation, genomic instability, and chromosomal instability are the three major factors in cell cycle dysregulation [38]. Regulation of the cell cycle is mainly conducted by complexes of cyclins and cyclin-dependent kinases [38]. Cyclin D1 in cell migration and proliferation is temporo-spatially separated by its biphasic expression induced by thrombin, a G protein-coupled receptor agonist, which is mediated by nuclear factor of activated T cells c1 (*NFATC1*) and signal transducer and activator of transcription 3 (*STAT3*) [39]. Cyclin D1 regulates kinase activity and the G₁-S phase tran-

sition in the cell cycle; deregulated cyclin D1 expression is well documented in breast, colon, and prostate cancers [39,40]. The expression of cyclin D1 is regulated by several factors including cytokines such as interleukin 3 and interleukin 6 *via* STAT3 and STAT5, or extracellular matrix factors such as collagen, fibronectin, and vitronectin, which activate focal adhesion kinase upon integrin clustering, and hepatocyte nuclear factor 6 [41]. Cyclin D1 is a crucial regulator of Wnt- and Notch-regulated development [41,42]. The binding of Wnt to its receptor, Frizzled, causes release of β -catenin to translocate from the cytoplasm to the nucleus, where it forms a complex with the ternary complex factor and/or the lymphoid enhancer-binding factor [41,43]. Cyclin D1 is induced by overexpression of β -catenin, which is a major component of adherens junctions that link the actin cytoskeleton to members of the cadherin family of transmembrane cell–cell adhesion receptors. It plays an important role in linking the cytoplasmic side of cadherin-mediated cell–cell contacts to the actin cytoskeleton [43]. β -catenin is upregulated in colorectal cancer, which is considered to trigger cyclin D1 gene expression followed by uncontrolled progression of the cell cycle [43]. In addition, β -catenin plays another role in signaling that involves transactivation, in complex with transcription factors of the lymphoid enhancing factor family in the nucleus [43]. The pathway involving β -catenin/LEF1 and elevation of cyclin D1 might be crucial for tumorigenesis [43]. Inhibiting EglN2, a member of the EglN (also called PHD or HPH) family of prolyl hydroxylases that regulates the heterodimeric transcription factor hypoxia-inducible factor (HIF), causes a decrease in the expression of its interaction partner cyclin D1 in cancer cells and impairs the cells' ability to proliferate *in vivo* [44].

Progression of the eukaryotic cell cycle is driven by cyclin-dependent protein kinases (CDKs), which are binding partner of cyclins. The CDK oscillator acts as the primary organizer of the cell cycle [45]. Phosphorylation of cyclin-Cdk complexes is one of the primary mechanisms of cell cycle regulation [46]. Cyclins are degraded by ubiquitin-mediated proteolysis [46]. The ubiquitylation and degradation of cyclin 1 and cyclin 2 are mediated by the SCF complex, a multi-subunit ubiquitin ligase that contains Skp1, a member of the cullin family (Cdc53) and an F-box protein, as well as a RING-finger-containing protein [46]. CDKs including CDK1, CDK2, CDK4, CDK6, and CDK11 have various functions that have been investigated using loss-of-function, target validation, and gain-of-function mouse models [38]. CDK1 is a mitotic CDK, also known as cell division control protein 2 (CDC2). It is one of the master regulators of mitosis as it controls the centrosome cycle as well as mitotic onset; deficiency in CDK1 results in embryonic lethality in the first cell divisions [38,47]. CDK2, CDK4, and CDK6 are interphase CDKs that are not essential for the mammalian cell cycle; they are, however, required for the proliferation of specific cell types [38]. Deficiency in CDK2, CDK4, and CDK6 caused mid-gestation embryonic lethality because of hematopoietic defects [38,47].

2.3. Genes related to cell differentiation

Inhibitor of differentiation 1 (Id1) is associated with the induction of cell proliferation and invasion [48], as well as the invasive features of cancer and the EMT [48]. The *HOX* genes

encode homeodomain-containing transcription factors involved in the regulation of cellular proliferation and differentiation during embryogenesis [49]. The expression of HOXA1, which plays an important role in proliferation, apoptosis, adhesion, invasion, the EMT, and anchorage-independent growth, was significantly increased in oral squamous cell carcinoma compared with in healthy oral mucosa [49], and it might be a useful prognostic marker for patients with this disease [49].

Wnt/ β -catenin signaling controls skeletal development and differentiation [50]. The initiating step of skeletal development is mesenchymal condensation, during which mesenchymal progenitor cells are at least bipotential [50]. Osteochondral progenitor cells differentiate into osteoblasts instead of chondrocytes when Wnt/ β -catenin signaling is activated [50]. *In vitro* models using human pluripotent stem cell-derived neural progenitor cells have been used to examine whether G11778A-mutated mitochondrial DNA, which is associated with Leber's hereditary optic neuropathy, might be involved in the differentiation of neural progenitor cells into neurons, oligodendrocytes, and astrocytes [51]. The differentiation of neural progenitor cells can be visualized by staining for the neuronal marker class III beta-tubulin [51]. Alternative splicing of exons play an important role in cellular differentiation and pathogenesis [52]. Alternative splicing in colorectal cancer and renal cell cancer samples has been analyzed by the Bioinformatics Exon Array Tool (BEAT, <http://beat.ba.itb.cnr.it/>) using an Affymetrix GeneChip Exon Array [52]. When the dataset was analyzed using GO terms, the cell differentiation (GO:0030154)-related gene delta-like 1 (*Drosophila*) (*DLL1*) was found to be involved in colorectal cancer [52].

2.4. Genes related to apoptosis

Cell proliferation and death are regulated by various molecules. Recently, microRNAs have been revealed to play important roles during death receptor-mediated apoptosis (programmed cell death) [53]. Transfection with miR-133b caused a proapoptotic effect on tumor necrosis factor alpha (TNF α)-stimulated HeLa cells [53]: the expression of apoptosis regulatory proteins such as transgelin 2 (TAGLN2), myosin, heavy chain 9, non-muscle (MYH9), cytoskeleton-associated protein 4 (CKAP4), polypyrimidine tract binding protein 1 (PTBP1), glutathione-S-transferase pi 1 (GSTP1), and copine III (CPNE3) were down-regulated compared with in control cells [53]. The BCL protein family plays a major role in regulation of the apoptotic cascade [54]. BCL2-associated protein (BAX) promotes apoptosis and delays disease progression, and has been associated with longer disease-free survival in patients with a number of gastrointestinal cancers, such as esophageal, stomach, small intestine, and colon cancer; moreover, high BCL6 expression is correlated with worse prognosis in patients with other gastrointestinal tumors, such as esophageal adenocarcinoma [54]. There are two major cell death pathways that transduce the effects of various death inducers: the extrinsic death pathway that is mediated through cell death receptors of the TNF receptor family, such as the Fas receptor; and the intrinsic death pathway that proceeds through mitochondria [55]. The expression of apoptosis signal-regulating kinase (ASK1), which plays an important role as a mitogen-activated protein kinase kinase kinase in apoptosis signaling, is in-

creased in gastric cancer [56]. Furthermore, the levels of cyclin D1 and phosphorylated JNK were higher in gastric cancer than in non-tumor epithelium [56]. ASK1 may play a role in the development of gastric cancer [56].

2.5. Detection of cell proliferation or apoptosis

Several methods have been suggested for the diagnosis of cancer [57]. Protein markers for cancer include prostate-specific antigen for prostate cancer, CA125 for ovarian cancer, carcinoembryonic antigen for colon cancer, human chorionic gonadotropin for trophoblastic cancer, and α -fetoprotein for hepatocellular carcinoma and germ cell tumors [57]. Assays to detect telomerase activity in clinical samples include the TRAP (telomere repeat amplification protocol) assay, which involves protein extraction and subsequent primer-directed PCR amplification of telomere extensions [57].

Assays for the detection of kinases that regulate cell growth, proliferation, differentiation, and metabolism have been developed [58]. The assay technology includes fluorescence polarization to detect protein phosphorylation, scintillation proximity to detect protein dephosphorylation by phosphatases, fluorescence resonance energy transfer to detect protein cleavage or modification, immunosorbent assays to detect phosphorylation state, luciferase-based ATP detection to detect the kinase-dependent depletion of ATP, luminescent oxygen channeling to detect phosphorylation, time-resolved fluorescence resonance energy transfer to detect phosphopeptide formation, and enzyme fragment complementation to detect molecular interactions with kinases [58,59]. Cell proliferation can also be determined by the tetrazolium hydroxide (XTT) cell proliferation assay, in which absorbance is measured by an ELISA reader under 490-nm-wavelength light (Biological Industries) [60].

Cell proliferation assays and apoptosis assays have been used to examine the effects of inhibitors on cancer cells [61]. The cell proliferation of Neuro-2A cells, neuroblastoma cells, can be determined using the CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay reagent (Promega) [61]. A colony formation assay using Neuro-2A cells was used to determine the effect of an inhibitor of GSK-3 β [61]. In this experiment, colonies were allowed to form for 10 days, after which the cells were fixed with 70% ethanol and stained with 1% methylene blue. Apoptosis was then measured by flow cytometry using an Annexin V-allophycocyanin (APC) /propidium iodide (PI) detection kit (BD PharMingen) [61]. Apoptosis was also determined using 4',6-diamidino-2-phenylindole (DAPI) staining, observing apoptotic nuclear morphology, and immunoblotting with antibodies to β -catenin, X-linked inhibitor of apoptosis, and BCL2 [61]. Cell cycle analysis using PI to quantify the proportions of cells in the G₁/G₀ or G₂-M phases was used to examine cell cycle status [61].

Viable cells can be determined using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assays [62]. Absorbance at 570 nm is used to detect the incorporation of MTT. Apoptosis can also be determined by caspase activation using an anti-poly ADP-ribose polymerase (PARP) antibody [62]. Viable cells can also be determined using a 3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) kit (Promega) [63]. The terminal transferase dUTP nick end labeling (TUNEL) assay is commonly used to detect apoptosis [63]. Harvested cells are resuspended in DNA labeling solution

consisting of TdT reaction buffer, TdT enzyme, and BrdUTP, then stained with PI to detect a fluorescein isothiocyanate-labeled anti-BrdU antibody [63]. Cell viability and proliferation assays were used to validate internal tandem duplication mutations in *FLT3* as a therapeutic target for human acute myeloid leukemia [64]. Cell viability and proliferation can be determined using a Vi-cell XR automated cell viability analyzer (Beckman Coulter) [64].

3. Genomic variation in disease

3.1. Genome-wide association studies in cancer

Despite extensive research efforts for several decades, the genetic basis of common human diseases such as cancers remains largely unknown [65]. Genome-wide association studies (GWAS) have emerged as an important tool for the discovery of genomic regions that harbor genetic variants conferring risk for various cancers [66,67]. Family-based linkage studies and studies comprising tens of thousands of gene-based SNPs can also assay genetic variation across the genome [68], but the National Institutes of Health guidelines for GWAS require a sufficient density of genetic markers to capture a large proportion of the common variants in the study population, measured in enough individuals to provide sufficient power to detect variants of modest effect [67]. The recent success of GWAS can be attributed to the convergence of new technologies that can genotype hundreds of thousands of SNPs in hundreds or thousands of samples [66,69].

GWAS have been conducted in the five of the most common cancer types: breast, prostate, colorectal, lung, and melanoma (Table 4) and have identified more than 20 novel disease loci, confirming that susceptibility to these diseases is polygenic [70]. For many years, human genetics has been used to map rare mutations with large effect sizes in families or genetically homogeneous populations, such as *BRCA1/BRCA2* mutations in Ashkenazi women with breast cancer and ovarian cancer [71]. A number of SNPs have now been associated with breast cancer; for example, a SNP in intron 2 of the *FGFR2* gene, which encodes a receptor tyrosine kinase that is amplified and overexpressed in 5–10% of breast tumors [72,73], and SNPs on chromosomes 16q and 5q. The locus on 16q contains a gene *TNRC9* and a hypothetical gene *LOC643714*. The function of *TNRC9* is unknown but the presence of an HMG box motif suggests that it might act as a transcription factor. The 5q locus includes *MAP3K1*, which encodes a protein involved in signal transduction (but not previously known to be involved in cancer) and two other genes: *MGC33648* and *MIER3*. In addition, several of the breast cancer loci appear to be associated with specific subtypes of the disease. In particular, the *FGFR2* association is strongly associated with estrogen receptor-positive breast cancer, while the *TNRC9* SNP is associated with both estrogen receptor-positive and -negative breast cancer [74,75]. It is surprising that none of the strongest associations map to regions harboring estrogen/progesterone genes in women of European background, particularly because a GWAS in Asian women reported a convincing association with markers near the estrogen receptor alpha (*ESR1*) gene [76]. In prostate cancer, the first and most important region to emerge was 8q24. This region was first associated with prostate cancer through

linkage studies by the deCode group, was followed up by association analyses [77], and has been confirmed in subsequent GWAS [78-81]. Another signal, on chromosome 10q13, points to a variant in the promoter of the *MSMB* gene, which encodes the PSP94 protein; this is now under intense investigation as a biomarker for prostate cancer [80,81].

In general, the susceptibility alleles discovered thus far are common—that is, with a frequency in one or more population of >10%, and each allele confers a small contribution to the overall risk of the disease. For nearly all regions conclusively identified by GWAS, the effect sizes per allele are estimated at <1.3. It was not anticipated that GWAS in certain cancers would yield many novel regions when other cancers strongly associated with particular environmental exposures have yielded so few regions. For example, prostate cancer, breast cancer, and colon cancer have been associated with 29, 13, and 10 regions of the genome, respectively, while there are only three associated regions for lung cancer in smokers, and three for bladder cancer despite analysis of sufficiently large data sets [67]. Several GWAS for lung cancer have identified the same locus on 15q25, suggesting that this is an important susceptibility locus for this disease [82-87]. This locus contains the nicotinic acetylcholine receptor subunit genes *CHRNA3* and *CHRNA5*, suggesting that susceptibility may be mediated through smoking behavior [86,87].

GWAS represent an important advance in discovering genetic variants influencing disease but have important limitations. There is a high potential for false-positive results, they do not yield information on gene function, they are insensitive to rare and structural variants, they require large sample sizes, and incur possible biases because of case and control selection and genotyping errors [88]. Clinicians and scientists must understand the unique aspects of these studies and be able to assess and interpret GWAS results for themselves and their patients. However, at present these studies mainly represent a valuable discovery tool for examining genomic function and clarifying pathophysiological mechanisms. However, through GWAS, the identification of variants, genes, and pathways involved in multiple cancers offers a potential route to new therapies, improved diagnosis, and better disease prevention [65].

Cancer type	Reference	Year	Platform [SNP passing QC]	Ethnic group	Initial sample size		Replication sample size		
					Cases	Controls	Ethnic groups	Cases	Controls
Breast cancer	[89]	2012	Affymetrix [555,525]	Korean	2,273	2,052	Korean	4,049	3,845
	[90]	2012	Affymetrix [690,947]	Chinese	2,918	2,324	Chinese	6,838	6,888
							Han Chinese	1,297	1,585
							Taiwan Chinese	1,066	1,065
							Korean	5,038	6,869
						Japanese	1,934	1,875	

Cancer type	ReferenceYear	Platform [SNP passing QC]	Ethnic group	Initial sample size		Replication sample size			
				Cases	Controls	Ethnic groups	Cases	Controls	
	[91]	2012	Affymetrix [613,031]	Chinese	1,950	-	Chinese	4,160	-
	[92]	2012	Illumina [470,796]	Japanese	240	-	Japanese	222	-
	[93]	2011	Affymetrix [684,457]	East Asian	2,062	2,066	East Asians	15,091	14,877
	[94]	2011	Affymetrix [782,838]	European	302	321	European	1,153	1,215
	[95]	2011	Illumina [-296,114]	British	1,694	2,365	British	7,317	8,124
							European	1,145	1,142
	[96]	2010	Illumina [285,984]	Swedish & Finnish	617	4,583	European	1,001	7,604
	[97]	2010	Affymetrix [592,163]	European	899	804	European	1,264	1,222
	[98]	2010	Illumina [285,984]	European	2,702	5,726	European	7,386	7,576
	[99]	2010	Illumina [582,886]	UK	3,659	4,897	European	12,576	12,223
	[100]	2010	Illumina [528,252]	British	1,145	-	British	4,335	-
	[101]	2009	Illumina [528,173]		1,145	1,142		8,625	9,657
	[102]	2009	Affymetrix [up to 607,728]	Chinese	1,505	1,522	Chinese	1,554	1,576
	[103]	2008	Affymetrix [200,220]		30	30	-	-	-
	[104]	2008	Affymetrix [492,900]	Ashkenazi Jewish	249	299	Ashkenazi Jewish	1,193	1,166
	[105]	2007	Affymetrix [70,897]	Framing-ham	1,345	-	-	-	-
	[106]	2007	Perlegen [205,586]		390	634		26,646	24,889
	[107]	2007	Illumina [528,173]		1,145	1,142		1,176	2,072

Cancer type	Reference	Year	Platform [SNP passing QC]	Ethnic group	Initial sample size		Replication sample size		
					Cases	Controls	Ethnic groups	Cases	Controls
Prostate cancer	[108]	2012	Illumina [509,916]	European	1,176	1,101	European	1,964	3,172
	[109]	2012	Affymetrix & Illumina [1,117,531] (imputed)		4,723	4,792	-	-	-
	[110]	2011	NR [2.6 million] (imputed)	European	6,621	6,939	European	22,957	23,234
							Japanese	285	298
							Chinese	135	135
							African	112	298
							American	7,140	5,455
	[111]	2011	Illumina [571,243]	European	2,782	4,458	European	8,217	6,732
	[112]	2011	Illumina [1,047,198]	African American	3,425	3,290	African American	1,275	1,695
							Senegalese	86	414
							Ghanaian	271	968
							Barbadian	246	253
	[113]	2011	Affymetrix [387,384]	European	202	100	European	1,122	1,167
	[114]	2010	Affymetrix [419,613]	Caucasian	222	415	Caucasian	500	155
[115]	2010	Illumina [510,687]	Japanese	1,583	3,386	Japanese	3,001	5,415	
[116]	2009	Illumina [541,129]	European	1,854	1,894	European, Chinese, Japanese, African American, Latino, and Hawaiian	19,879	18,761	
[117]	2009	Illumina [310,520]	Icelandic	1,968	35,382	European	11,806	12,387	
[118]	2008	Illumina [541,129]	European	1,854	1,894		3,268	3,366	

Cancer type	Reference	Year	Platform [SNP passing QC]	Ethnic group	Initial sample size		Replication sample size		
					Cases	Controls	Ethnic groups	Cases	Controls
	[119]	2008	Illumina [527,869]		1,172	1,157		3,941	3,964
	[120]	2007	Affymetrix & Illumina [60,275]		1,235	1,599		1,242	917
	[121]	2007	Affymetrix [70,897]	Framingham	1,345		-	-	-
	[122]	2007	Illumina [316,515]		1,453	3,064	East Asia	1,210	2,445
	[123]	2007	Illumina [538,548]		1,172	1,157		3,124	3,142
Colorectal cancer	[124]	2011	Illumina [378,739]	European	2,906	3,416	European	8,161	9,101
	[125]	2010	Illumina [-550,000] (imputed)	European	3,334	4,628	European	14,851	15,569
	[126]	2010	Affymetrix [460,945]	German	371	1,263	German Czech	4,121 794	7,344 815
	[127]	2008	Illumina [-548,586]		1,902	1,929		4,878	4,914
	[128]	2008	Illumina [541,628]		981	1,002		16,476	15,351
	[129]	2008	Illumina [547,647]		922	927		17,872	17,526
	[130]	2007	Illumina [547,647]		940	965		7,473	5,984
	[131]	2007	Illumina [547,647]		930	960		7,334	5,246
	[132]	2007	Affymetrix & Illumina [99,632]		1,257	1,336		6,223	6,443
Lung cancer	[133]	2011	Affymetrix [906,703]	Chinese	2,331	3,077	Chinese	6,313	6,409
	[134]	2011	Illumina [307,260]	White	327		European	587	

Cancer type	Reference	Year	Platform [SNP passing QC]	Ethnic group	Initial sample size		Replication sample size		
					Cases	Controls	Ethnic groups	Cases	Controls
	[135]	2011	Illumina [620,901] (pooled)	Italian	600	-	Italian	317	-
	[136]	2010	Affymetrix [265,996]	Han Chinese	245	-	Han Chinese	305	-
	[137]	2010	Affymetrix [246,758]	Korean	621	1,541	Korean	804	1,470
	[138]	2010	Illumina [542,050]		377	377		511	1,007
	[139]	2009	Illumina [515,922]	European	5,739	5,848	European	7,561	13,818
	[140]	2009	Illumina [511,919]	European	1,952	1,438	European	5,608	6,767
	[141]	2008	Illumina [317,498]		1,154	1,138		2,724	3,694
	[142]	2008	Illumina [306,207]		10,995 smokers			4,848 smokers	
	[143]	2008	Illumina [310,023]		1,926	2,522		2,513	4,752
	[144]	2007	Affymetrix [~116,204] (pooled)	Italian	338	335	Norwegian	265	356
Melanoma	[145]	2011	Illumina [594,997]	European	2,804	7,618	European	5,551	7,449
	[146]	2011	Illumina [5,480,804] (imputed)	European	2,168	4,387	European	5,193	15,144
	[147]	2011	Illumina [818,977]	European	1,804	1,026	European	6,483	23,324
	[148]	2011	Illumina [491,227]	European	156	2,150	NR	-	-
	[149]	2009	Illumina [~317,000]	European	1,539	3,917	European	2,312	1,867

Table 4. Summary of GWAS for the five of the most common types of cancer.

3.2. Genetic risk score in cancer and diabetes

Type 2 diabetes mellitus and cancers are major health problems worldwide [150,151]. The recent increase in the prevalence of these diseases is largely attributable to environmental factors. However, convincing evidence shows that genetic factors may play an important role in these diseases [152,153]. Recent GWAS have led to the identification of a series of SNPs that are robustly associated with either the risk of diabetes or cancers [151,154-159]. For type 2 diabetes mellitus, common SNPs have been identified in the *PPARG*, *KCNJ11*, and *TCF7L2* genes, and have been widely replicated in populations of various ethnicities [160-162]. Other potential new loci include *HHEX*, *CDKAL1*, *CDKN2A/B*, *IGF2BP2*, *SLC30A8*, and *WFS1* [65,155-159,163,164]. A number of SNPs have been identified as associated with breast cancer risk, including *FGFR2*, *CASP8*, *ERBB4*, *TAB2*, *BARX2*, *TMEM45B*, *ESR1*, *FGFR2*, *TNRC9*, *MAP3K1*, *MGC33648*, *MIER3*, and *RAD51L1* [74,75,151] (Table 5).

Combining multiple loci with modest effects into a global genetic risk score (GRS) might improve the identification of those at risk for common complex diseases such as type 2 diabetes and cancers [165-167]. Several studies have developed methods to predict the risk of certain diseases, such as coronary heart disease, type 2 diabetes, and breast cancer, aggregating information from multiple SNPs into a single GRS [151,168,169]. For example, in the Atherosclerosis Risk in Communities study, the aggregation of multiple SNPs into a single GRS was responsible for improving the prediction of coronary heart disease incidence [168]. In a study that used a GRS to determine the risk of type 2 diabetes in US men and women, individuals in the highest quintile of GRS had a significantly increased risk of type 2 diabetes compared with those in the lowest quintile; however, the addition of a GRS to the conventional model consisting of lifestyle risk factors only increased the area under the curve by only 1% (AUC=0.78). In this instance, the GRS was determined to be useful only when combined with the body mass index or a family history of diabetes [169]. For breast cancer, a GRS was created using 14 SNPs previously associated with breast cancer, and was substantially more predictive of estrogen receptor-positive breast cancer than of estrogen receptor-negative breast cancer, particularly for absolute risk [151]. Further studies are needed to confirm whether a GRS improves disease risk prediction.

The GRS is calculated on the basis of reproducible tagging of SNP-associated loci reaching genome-wide levels of significance. The GRS can be created by two methods: a simple count method (count GRS) and a weighted method (weighted GRS) [169,170]. Both methods anticipate each SNP to be independently associated with risk. An additive genetic model is used for each SNP, applying a linear weighting of 0, 1, or 2 to genotypes containing 0, 1, or 2 risk alleles, respectively. This model is known to perform well even when the true genetic model is unknown or wrongly specified [171]. The count model assumes that each SNP in the panel contributes equally to the disease risk and is calculated by summing the values for each of the SNPs. The weighted GRS is calculated by multiplying each B-coefficient, the estimates resulting from an analysis carried out on variables that have been standardized, by the number of corresponding risk alleles (0, 1, or 2).

Disease	Reference	Year	Ethnic group	Participants	No. of SNPs	Genes found from GWAS
Type 2 diabetes	[165]	2008	Framingham	2,377 diabetic patients	18	NOTCH2 (rs10923931), BCL11A (rs10490072), THADA (rs7578597), IGF2BP2 (rs1470579), PPARG (rs1801282), ADAMTS9 (rs4607103), CDKAL (rs7754840), VEGFA (rs9472138), JAZF1 (rs86475), SLC30A8 (rs13266634), CDKNA/2B (rs10811661), HHEX (rs1111875), CDC123, CAMK1D (rs12779790), TCF7L2 (rs7903146), KCNJ11 (rs5219), INS (rs689), DCD (rs1153188), TSPAN8, LGR5 (rs7961581)
	[169]	2009	European	2,809 diabetic patients & 3,501 health controls	10	WFS1 (rs10010131), HHEX (rs1111875), CDKAL1 (rs7756992), IGF2BP2 (rs4402960), SLC30A8 (rs13266634), CDKN2A/B (rs10811661), TCF7L2 (rs12255372), PPARG (rs1801282), KCNJ11 (rs5219)
Breast cancer	[151]	2010	UK	10,306 breast cancer patients & 10,393 controls	14	FGFR2 (rs2981582), TNRC9 (rs3803662), 2q35 (rs13387042), MAP3K1 (rs889312), 8q24 (rs13281615), 2p (rs4666451), 5pas (rs981782), CASP8 (rs104548), LSP1 (rs3817198), 5p (rs30099), TGFB1 (rs198/2073), ATM (rs1800054), TNRC9 (rs8051542), TNRC9 (rs12443621)

Table 5. Studies using a genetic risk score for cancers and diabetes, comprising SNPs identified in GWAS.

3.3. Cancer Cell Line Encyclopedia

The Cancer Cell Line Encyclopedia (CCLE) has made predictive modeling of anticancer drug sensitivity a realistic proposition, by determining genomic markers of drug sensitivity in cancer cells [172,173]. The CCLE contains information from 947 human cancer cell lines including data on gene expression, chromosomal copy number, and massively parallel sequencing data. It has been used to identify genetic, lineage-specific, and gene expression-based predictors of drug sensitivity [172]. This has revealed, for example, that the plasma cell lineage is correlated with sensitivity to IGF1 receptor inhibitors, aryl hydrocarbon receptor (*AHR*) expression is associated with MEK inhibitor efficacy in *NRAS*-mutant lines, and *SLFN11* expression is associated with sensitivity to topoisomerase inhibitors [172]. Genomic markers of drug sensitivity in cancer cells have also been systematically identified using the Genomics of Drug Sensitivity in Cancer database (<http://www.cancerRxgene.org>) [173]. These databases will enable to overview genome quality.

4. Conclusion

There are dramatic changes in the genomes of cancer cells, which vary according to cancer subtype. Integrative and wide investigations of cancer cell genomes have revealed mutations and alterations in gene expression that are associated with the disease. Databases that include abundant data related to gene and protein conformation, gene expression, and genomic mutations enable the construction of dynamic cellular simulations and disease models. New sequencing tools such as next-generation sequencing will reveal new horizons in the prediction of disease and drug sensitivity, which play an important role in personalized medicine. Appropriate translation of the abundance of information to clinical practice is one of most important future challenges for medicine. The quality of genome would be one of the important factors for detecting the development of the disease.

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Quality Control Considerations for Fluorescence *In Situ* Hybridisation of Paraffin-Embedded Pathology Specimens in a Diagnostic Laboratory Environment

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Additional information is available at the end of the chapter

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1. Introduction

Paraffin FISH testing is the application of the fluorescence *in situ* hybridisation (FISH) methodology to formalin fixed paraffin embedded sections (FFPE), and has proven a powerful tool for both histopathologists and cytogeneticists. Pathologists use the method to confirm or exclude a histological diagnosis, to differentiate between tumour subtypes, or as a confirmatory tool where the tissue morphology is poor or the immunohistochemistry (IHC) staining is uninformative [1]. Similarly, cytogeneticists find it useful when the tissue sample is insufficient or unsatisfactory for conventional culture methods, or when such methods fail to yield a result. The method can also be used to confirm abnormalities found in other tissue samples. Paraffin testing has a further advantage over conventional cytogenetic and molecular testing methods, as it can localize the anomaly within specific cells or tissue areas, and this provides the ability to study anomalies at a single cell level [2,3], unlike DNA techniques that pool DNA from hundreds of different cells [1,3].

Compared to FISH testing on conventional suspension samples (Figure 1), paraffin FISH can be labour intensive and highly variable due to differing fixation times between samples and referring histology labs, and the interpretation may be limited due to truncation of signal and overlapping cells [1,4].

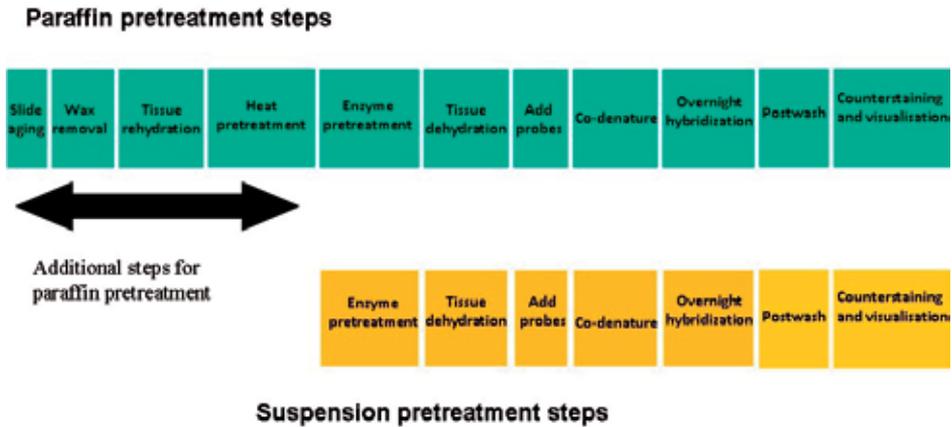


Figure 1. A comparison of the paraffin pre-treatment process with the conventional FISH pre-treatment process on suspension samples.

For these reasons, it must be considered separately from the conventional suspension FISH method, and while it can be used as either a stand-alone technique, or an adjunct to conventional cytogenetics techniques [5], it must be noted that due to the use of interphase nuclei, a prior knowledge of the anomaly of interest is required.

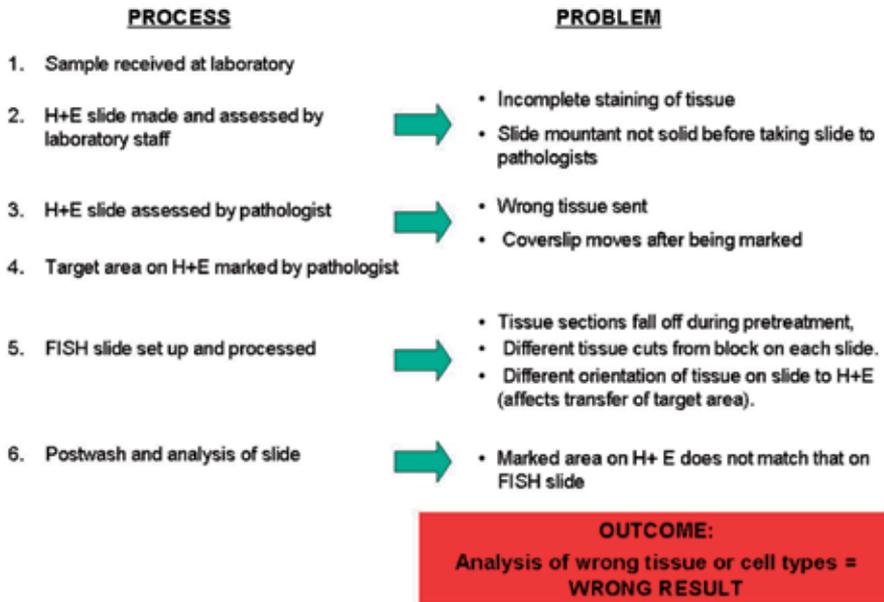


Figure 2. Errors that occur during the paraffin pre-treatment process.

The basic premise of the method involves establishing the area of interest for testing on the H+E stained pathology slide, and transferring this area to an unstained paraffin slide, which is then

pretreated, probed and co-denatured using the traditional FISH methodology [6,7]. However, one of the most crucial factors for paraffin analysis is the assessment of the correct target area before beginning the procedure – without this, an erroneous result may occur (Figure 2), which may be costly to patients if it results in the appropriate treatment being withheld [1].

For this reason, robust internal and external quality control procedures are required for diagnostic paraffin FISH testing and the exclusion of non-target tissue before analysis decreases the likelihood of an incorrect result due to an analysis error [1]. This protocol therefore aims to provide a guide to some of the considerations and troubleshooting that are necessary when using the method for diagnostic medical testing. It is adapted from the method used by the Diagnostic Genetics Department, LabPlus at Auckland City Hospital, New Zealand. There are a number of variations to the basic FISH method that can be used depending on the nature and number of samples being processed, and new technology has also been developed to automate the process (Xmatrix, Abbott Molecular). In this protocol however, we have suggested extra steps that are designed to help improve the quality of the testing procedure for diagnostic use. Probes used for diagnostic testing are commercially available and may be downloaded and gathered from the websites of companies such as Abbott Molecular, Cytocell, Zytovision or Kreatech Diagnostics.

2. Method

One slide (2-5 micron thickness usually) is needed per probe or probe set, and if a haematoxylin and eosin (H+E) slide is not provided by pathologists, an extra slide must also go through the deparaffinisation steps before staining with the Shandon Rapid-Chrome™ Frozen Section Staining kit (alternatively the individual stain kit components can be made from powder).

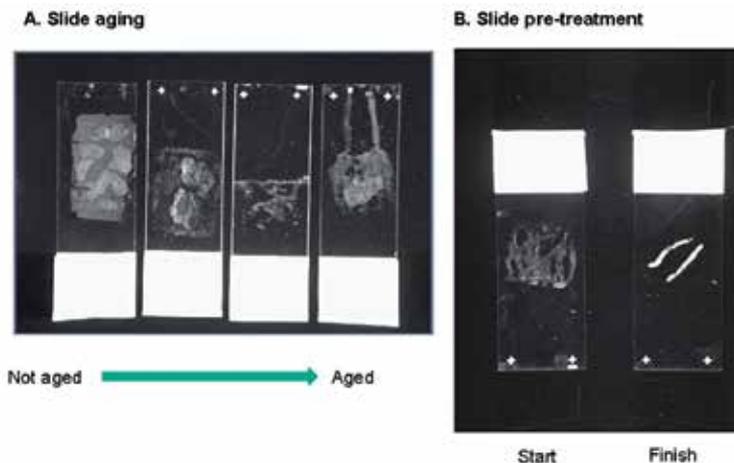


Figure 3. Slide pretreatment steps for paraffin FISH. (A) Appearance of unstained paraffin slides after aging in a 60°C oven - note melted or "bubbled" appearance. (B) Unstained paraffin slides and after the pre-treatment steps.

1. Deparaffinisation (approx. 60 minutes); see Figure 3
 - a. Leave slide/s on the hotplate/in the oven at approximately 65°C for 30-60 minutes for aging (Figure 3).
 - b. Perform deparaffinization by placing slide/s in xylene for at least 10 minutes in the fume hood, with intermittent shaking.
 - c. Rehydrate slide/s by placing them for 2 minutes in each of 100%, 80%, and 70% ethanol solutions, followed by deionised water at room temperature.
2. Haematoxylin and Eosin (H+E) slides; see Figure 4



Figure 4. A haematoxylin and eosin (H+E) stained slide with the target area for analysis marked by a pathologist.

- a. Take rehydrated slide/s and stain using the Shandon Rapid-Chrome™ Frozen Section Staining kit and mount the slide using Shandon Mount.
 - b. Leave slides on the hotplate for at least 30 min to dry the mountant.
 - c. Check slides for stain quality under a light microscope.
 - d. Take slide/s to pathologist for marking (Figure 5).
3. Heat Pre-treatment (approx. 30 minutes)
 - a. Add 35µl of heat pre-treatment solution (Invitrogen Tissue Pre-treatment Kit) to the slide/s, cover with a 22x22mm (or bigger sized cover slip) glass cover slip and seal with rubber cement. Alternatively slides can be heat-pre-treated in coplin Jar at 95°C or pressure cooker.
 - b. Heat slide/s on the thermal cyler for 15-60 minutes at 95°C (The time is dependent on the type of tissues and length of formalin fixation).

- c. On completion, immerse slide/s with cover slip in deionised water to cool down and gently remove the cover slip.
 - d. Wash briefly in a coplin jar of deionised water at room temperature and drain off excessive water.
4. Enzyme Digestion (approx. 40 minutes).
- a. Add an appropriate amount (~15µl) of enzyme reagent (Invitrogen Tissue Pre-treatment Kit) to the slide/s, depending on the size of hybridisation area, and cover with a square of parafilm.
 - b. Incubate slide/s for 15-45 minutes in a humidified chamber at 37°C (This time is dependent on the type of tumours and length of formalin fixation).
 - c. Remove cover slip/s and wash briefly in a coplin jar of deionised water at room temperature.
 - d. Dehydrate slide/s for 2 minutes each in each of 70%, 80% and 100% ethanol solutions and air dry at room temperature. Please note that a different ethanol series is used for the dehydration steps to avoid reagent contamination issues.
 - e. Check the tissue morphology of the pre-treated slide looks the same as that of the H+E.
 - f. The pre-treated paraffin slide/s should then be carefully matched against the marked H&E slide/s, and the area for testing transferred to the pre-treated slide/s using a marker pen initially, followed by the diamond-tipped engraver. This means that the area can still be visualised after the post-wash steps.

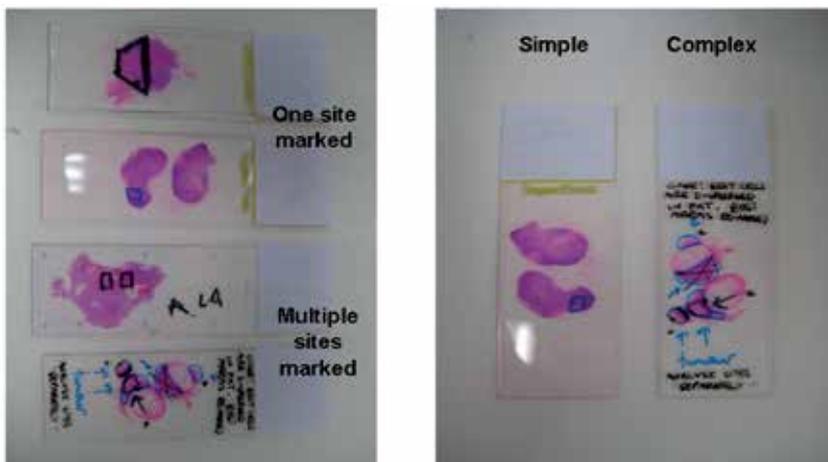


Figure 5. Haematoxylin and eosin (H+E) stained slides marked with the target area for analysis. This reduces the volume of probe necessary and ensures that non-target tissue is excluded as much as possible before the FISH analysis procedure.

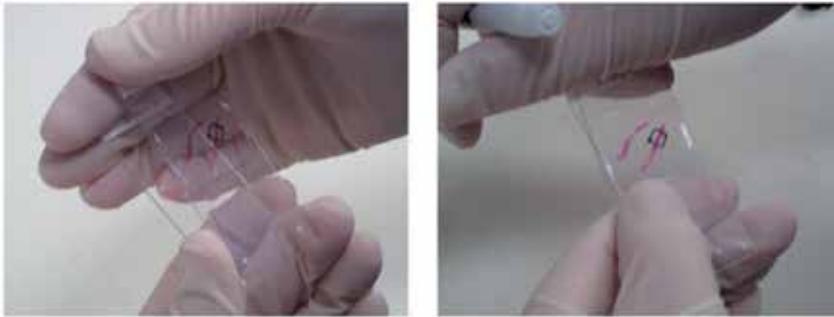


Figure 6. Transfer of target area for analysis from the H+E slide to the pre-treated FISH slide prior to the probing steps.

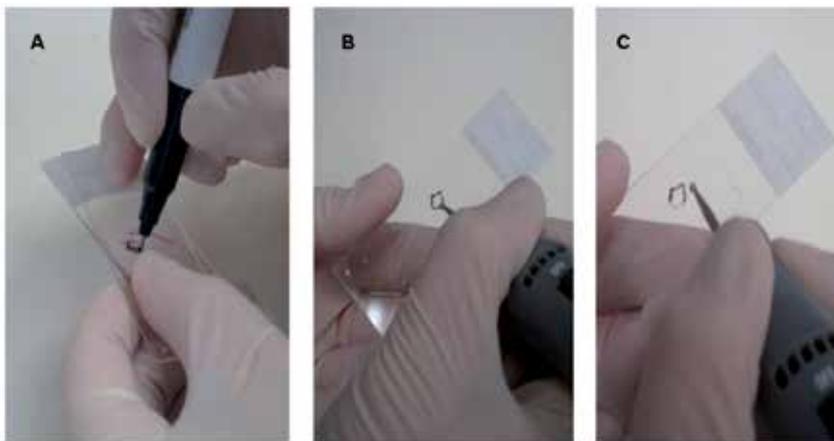


Figure 7. Engraving of target area on to the pre-treated paraffin FISH slide. (A) Draw target area onto bottom of slide with fix-resistant pen. (B & C) Engrave marked area onto bottom of slide using diamond-tipped engraver to keep area visible after post-wash steps.

5. Probe preparation (approx. 10 minutes)

Use Ready-To-Use probes or refer to the probe preparation protocol outlined by the manufacturer.

6. Co-denaturation and hybridization (approx. 25 minutes)

- a. Apply an appropriate amount (2-10 μ l) of probe mix to the hybridization site marked on each slide, depending on the size of cover slip being used, and seal with rubber cement. Leave the slide/s in the incubator or in a drawer at room temperature for a few minutes to allow the rubber cement to dry before placing them in the thermal cycler.
- b. Denature slide/s together with probe mix for 10-20 min at 85°C or 5-10min at 95°C.
- c. After co-denaturation, slide/s may be placed in a humidified box in the incubator at 37°C for at least 12-16 hours, usually no more than 72 hours.

7. Post Hybridization Wash (5 Minutes)
 - a. Briefly soak slide/s in 2xSSC and gently remove rubber cement.
 - b. Wash slide/s in 0.4xSSC/0.03% Tween 20 (or NP40) at 72°C for 2 min.
 - c. Place slide/s in 2xSSC/0.01% Tween 20 (or NP40) for 1 min.
 - d. Briefly drain slide/s, apply DAPI counter stain and put cover slip on.
 - e. Visualize FISHed-slide/s under fluorescence microscope.

When using indirectly labelled commercial probes that require antibody detection, signal detection must be done according to the manufacturer's instructions.

8. Analysis and interpretation; see Figure 8.
 - a. With a pathologist's consultation, check the H+E slide on a transmitted light microscope to assess whether the sample contains a mixture of cell types, as this may affect the interpretation of the FISH signal pattern.
 - b. Check the paraffin FISH slide on a fluorescence microscope using the 10x objective to ensure the area marked on the slide approximately matches that on the H+E slide.
 - c. Using two observers, analyse a minimum of at least 8 representative sites within the marked region (a minimum of 4 different areas per observer), scoring only cells that show both the target and control loci. Analysis of areas of areas where the cells are not overlapped is preferable, and a third analyst is required where there is discordance between two observers.

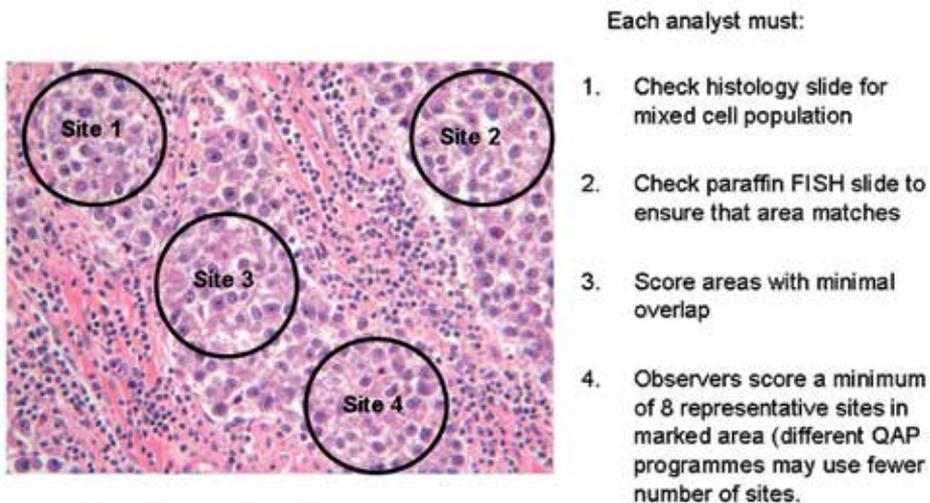


Figure 8. Analysis principles for paraffin FISH slides.

3. Troubleshooting

Problem: Unclear whether slides have been aged before arrival, as repeating this step may decrease the hybridization efficiency of the probe.

[Step 1]

Solution: Although some waxes do not change in appearance, pre-aged slides generally have a bubbled or melted appearance of the wax compared to the smooth appearance of non-aged slides in general (N.B: some wax types do not change in appearance so this is a rule of thumb only).

Problem: The use of xylene to remove the wax from around the sample is not ideal as xylol is highly toxic.

[Step 1]

Solution: An alternative to xylene is HemoDe from Scientific Safety Solvents.

Problem: Finding that the wrong tissue was sent by the referring laboratory.

[Step 2]

Solution: Ask for a copy of the pathology report to be sent with all samples, and get pathologists to ring the referring laboratory to request the appropriate sample for testing.

Problem: Incomplete staining of the H+E slide causing correct target area to be missed by pathologist.

[Step 2]

Solution: Slides should be quality checked before taking them to a pathologist. Check the stain by eye to see if there are obvious colour differences across the slide – if one of the stains has been missed in an area it will appear either a dull purple (eosin missed) or a dull pink (haematoxylin missed or there is a problem with the pH of the bluing reagent) compared to the rest of slide. If there are any doubts, ask a histopathology technologist for assistance.

Problem: Cover slip moves after the slide has been marked because mountant is not completely hardened. This causes the target area to move.

[Step 2]

Solution: Leave the slides on the hotplate for a longer period of time, or change mountant to a faster drying version such as Entellan (Note: it is not possible to remove the Entellan with methanol after it has been cover slipped, hence why DPX is the preferred mountant).

The Rapid-Chrome™ Frozen Section Staining kit uses Shandon Mount; however alternatives such as Entellan are available.

Problem: Disappearance of tissue on slide during dehydration steps.

[Steps 3 and 4]

Solution: The ethanol series (in step 1) is necessary to rehydrate the tissue for the enzyme solution to act on, and may cause the tissue to become translucent, however it will become white again once the slide is dehydrated.

Problem: Scratching or loss of tissue during washing steps. Small tissue samples (e.g. core biopsies) may become fragile during the pretreatment steps and fall off the slide.

[Steps 3 and 4]

Solution: As the tissue becomes soft during pre-treatment it may easily fall off or get scratched; coplin jars of deionised water can be used to dip slides into rather than the more aggressive use of squirter bottles or running tap water (do not leave the pre treated slide in water for a long time, especially for a core biopsy or a tiny sample). The size of the tissue gives a good indication as to the fragility of the tissue, so this should be taken into account before beginning the pre treatment steps. Increasing the ageing step may also help to fix the tissue to the slide better, although it may also decrease the hybridization efficiency of the probe to the sample. Alternatively, skipping the heat pretreatment step and doing a reduced enzyme treatment on the sample may combat this.

Problem: The tissue does not look the same as the H+E slide after dehydration steps.

[Step 4]

Solution: This can either be due to loss of tissue during pretreatment or different cuts through the tissue block. Untreated slides should be closely examined to find one that appears to match the pretreated slide and a new H+E slide created using this slide. See also steps for reducing the loss of tissue during pretreatment.

Problem: Transfer of area is difficult due to a slight difference in the morphology of the tissue in different layers of the tissue section, or different orientation of tissue on pre-treated slide to that of the H+E slide.

[Step 4]

Solution: If the morphology of tissue on the pre-treated slide looks different to that of the H+E slide, check it against the remaining untreated slides to see if it looks like tissue has been lost during the pre-treatment procedure. If tissue has been lost, simply start the procedure over again with a new slide. If the morphology of the tissue appears different between the untreated slides, ask a pathologist for help selecting an appropriate slide to pre-treat, and try to find two similar untreated slides. Pre-treat one and make the other into an H+E slide to allow for more accurate marking.

Problem: There is more than one target area marked on slide – is more probe required?

[Step 4 and 5]

Solution: Assess the size of the areas – if there are several small areas, the total volume of probe does not need to be increased, simply aliquot the volume of probe equally over the different areas and place a small cover slip over each. More than one aliquot of probe is only required if the areas are greater than can be covered by a 13mm diameter cover slip.

Problem: The hybridisation buffer for a probe runs out.

[Step 5]

Solution: As hybridisation buffers are all fairly similar, it is fine to use the buffer of similar probe as a substitution. Alternatively, hybridization mix can be made up:

Hybridization mix

(10% dextran sulphate, 50% formamide in 2xSSC, 0.1% SDS, pH 7.0)

1. Mix 12.5ml formamide, 2.5ml 20xSSC pH7.0 and 10ml MilliQ water. Adjust pH to 7.0 with HCl then transfer to a 50ml Falcon tube.
2. Add 2.5mg dextran sulphate and place on a roller mixer at room temperature for 1-2 hours.
3. Add 25µl Tween 20 and invert to mix.
4. Aliquot 500µl into sterile eppendorf tubes. Store at -20°C and use a fresh aliquot each time.

Problem: A thermal cycler is not available for use.

[Step 6]

Solution: Denaturation of the slide(s) can be done separately using 70% formamide/2xSSC, as it gives better quality denaturation although the downside is that it is highly toxic. The hybridisation steps can also be done adequately in a programmable system (e.g. Thermobyte).

Problem: The cover slip is hard to remove before the post wash steps.

[Step 7]

Solution: Place slide in 2xSSC solution and agitate gently after removing the rubber cement, and then remove cover slip. If the cover slip is still stuck to slide, slide the blade of a scalpel under one corner of the slide and lift gently before immersing the slide in a 2xSSC solution and agitating it gently. This may need to be repeated several times if the cover slip remains stuck.

Problem: Weak or patchy signal quality.

[Step 8]

Solution: This can be difficult to fix, as it primarily occurs as a result of poor handling and fixation of tissue prior to receiving the sample for FISH testing [8,9]. Different tissue samples may require the pretreatment times to be varied [10]. The heat pretreatment buffer prepares the tissue for the enzyme to act on and the enzyme degrades the cellular material away from the DNA, in order to allow the probe to anneal to the chromatin. Variation of either or both these times is effective, and the steps may be repeated on the probed slide to reduce the need for lengthy pretreatment times on a new slide. Bone samples such as trephines may show poor hybridization efficiency of the probe, and require hydrogen chloride treatment, unless the sample has already been decalcified prior to arrival.

Poor signal quality may also be a result of incorrect post wash stringency. There is an alternative wash technique that uses 50% formamide/2xSSC to increase the stringency of the wash. However, this is not always ideal, as it significantly increases the length of the post wash, and also uses formamide which is extremely toxic [11].

Problem: High levels of cross hybridization due to non-specific binding of probes.

[Step 8]

Solution: This is due to incorrect stringency of the post wash [1]. For a quick fix, slides can be rewashed using the quick wash procedure reported here, or alternatively washing at a higher temperature or use of a different post wash procedure can be tried [11].

Problem: Cells only show one signal colour.

[Step 8]

Solution: Only cells showing both the control and target loci should be scored (e.g. 2R2G), so if both the control probe and the probe for the region of interest are on the same chromosome, it is most likely to be due to poor hybridisation of one of the probes. First check to see using single colour filters whether the signal colour is present but weak – if it is, repeat the pre-treatment and hybridisation steps again on the same slide (for a shorter time e.g. 15/15 buffer: enzyme treatment).

Problem: Using an indirectly labeled probe and can't get a good signal quality.

[Step 8]

Solution: In most cases, amplification with only a primary antibody is necessary, and further amplification can also increase the level of background on the slide(s). However if the signal is not bright enough, carefully remove the cover slip, rinse slide in 1xPBS (or SSC) and perform further amplification steps with secondary or tertiary antibodies as many times as necessary. After adding each antibody, slides should be covered with parafilm and incubated in a humidified chamber at 37°C for 5 minutes before being washed in 4xSSC/0.05% Tween20 for 2 minutes. Then mount with 8µl Vectashield antifade solution with DAPI.

Problem: Distinguishing between real signal and background or 'rubbish' on slide.

[Step 8]

Solution: Look at the signal intensity on single colour filters – rubbish generally appears to be brighter and shinier compared to real signals, and background will appear fuzzy and indistinct compared to real signal. High background may be due to the slides not being properly sealed with rubber cement during the pretreatment steps, as this allows the solution to evaporate and the tissue to dry out.

Problem: High background on the slides when analyzing.

[Step 8]

Solution: High background may be due to insufficient removal of material during the pre-treatment steps. With high case numbers, solutions can become contaminated, therefore the

solutions in the pretreatment steps need to be changed regularly, and it pays to have an additional coplin jar of 100% ethanol to dip the slides into after the xylol step in order to reduce contamination from the xylol solution. Alternatively, background may be due to the cover slip not being sealed properly during the pretreatment and co-denaturation steps, causing the tissue to dry out. By placing the slide in the incubator to allow the rubber cement to dry before these steps, this effect can be reduced. The use of a glass coverslip rather than a plastic coverslip also helps, as plastic acts as an insulator, and therefore will hold the temperature and increase the drying of the tissue.

The use of detergents in the post wash steps also helps to solubilize proteins, and if Tween20 is not effective, then NP-40 can also be used.

Problem: There is a mixed cell population in the marked target area (e.g. Tumour cells with non-target lymphocytes also present); see Figure 9.

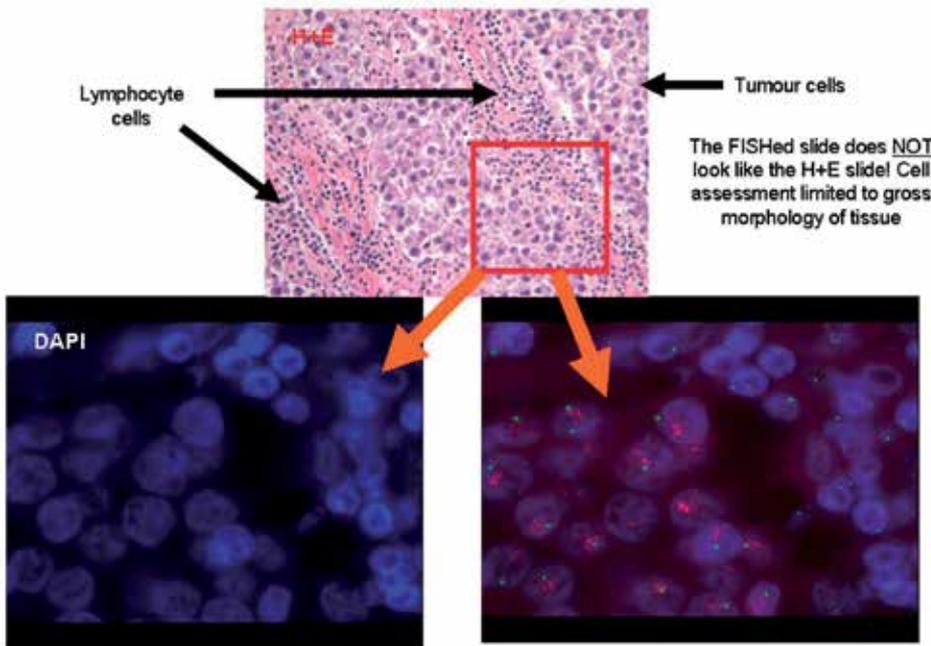


Figure 9. The analysis of slides with mixed tissue populations.

[Step 8]

Solution: Check the H+E slide first before analysing the FISH slide to see whether there is clustering of cell types, or differences in morphology between the different cell types. Then scan the marked target area on the FISH slide using the 10x objective to find areas which appear to be targeted cells and switch to a higher objective for confirmation and then analyse using appropriate filter. Consideration of accidental analysis of non-target cells must also be taken into account when interpreting such cases, therefore increasing the number of cells or sites analysed will increase the accuracy of the analysis. Alternatively, it may be pos-

sible to get a pathologist to mark several smaller sites containing only target cells, as this reduces the risk of error before beginning the analysis.

Problem: Target area marked is very small, so it is difficult to test a variety of areas.

[Step 8]

Solution: While this makes analysis difficult, switching to the 10x objective and moving the stage to a different position will reduce the likelihood of reanalyzing the same cells. Numerical scoring is also preferable in such a case, as it provides a reliable basis for interpretation.

Problem: The cells are highly dispersed or highly clustered, making analysis difficult.

[Step 8]

Solution: Select good areas where the cells are not overlapping using the DAPI filter and use numerical scoring of individual cell signal patterns (this may mean increasing the number of sites examined if the cells are widely dispersed). If a gene rearrangement probe is being used, it may be sufficient just to report the presence or absence of a rearrangement without doing individual cell analysis.

Problem: Distinguishing between real loss and gain of signal compared to artefact.

[Step 8]

Solution: If the target abnormality is either a gain (trisomy/tetrasomy) or loss (deletion) of a signal, it pays to establish thresholds using normal control slides to estimate the level of artefactual gain or loss of signal, and to check the manufacturer's product information to see if splitting of the probe or non-target binding/polymorphisms are common with the probe. The variance in the signal patterns can also be checked – if the percentage of cells showing a 1R2G signal pattern is roughly equivalent to those showing a 2R1G signal, then it is reasonable to assume that it is due to artefactual truncation of signal.

Problem: There is discordance between analysts.

[Step 8]

Solution: Get a third analyst to score the sample. If two analysts have similar results, discard the third analysis, or if all three give different results, take an average of all three results to allow robust interpretation. If the three results differ hugely, it is preferable to confirm the result with a secondary probe where possible, or request a repeat sample from another block. Where the interpretation is still not clear, the case can be reported as inconclusive or failed.

Problem: A low level abnormality, multiple clones or mosaicism is suspected.

[Step 8]

Solution: Where the result is not straightforward use quantitative scoring and use appropriate thresholds for interpretation. Paraffin FISH is not the most suitable method of detection for these cases, although methods that involve taking thicker slices of the section have been developed [12].

4. Conclusion

The role of pathologists is crucial to the analysis of paraffin FISH sections from the beginning of the process. They can help to eliminate very basic laboratory errors, such as identifying whether incorrect tissue has been sent prior to processing the slides, and can also help to identify the appropriate target tissue within the paraffin section prior to analysing the sample, so that inappropriate tissues can be reduced or eliminated. When analysing products of conception, the fetal component can be very small compared to the maternal component, and without guidance of pathologists, an erroneous result may occur. Similarly, in breast cancer samples, it is important to eliminate areas of contained carcinoma (*in situ* components such as DCIS and LCIS) and lymphocytes, as these may result in false positive or negative results, which can be deleterious if treatments such as Herceptin are then withheld from the patient. Some samples such as lymphomas or graft versus host disease may require extensive guidance from pathologists as knowledge of the disease characteristics will allow for highly targeted analysis. In follicular lymphoma, the follicles need to be identified so that centrocytes and centroblasts are targeted for analysis, and normal lymphocytes and reactive cells are avoided when analysing the sample (Swerdlow et al. 2008). For this reason, it is best to include a variety of areas to get a representative result. It should be noted that external quality assurance programmes may differ in the number of sites required for analysis. Generally speaking, fewer sites are required, if initially the non-target tissue is eliminated.

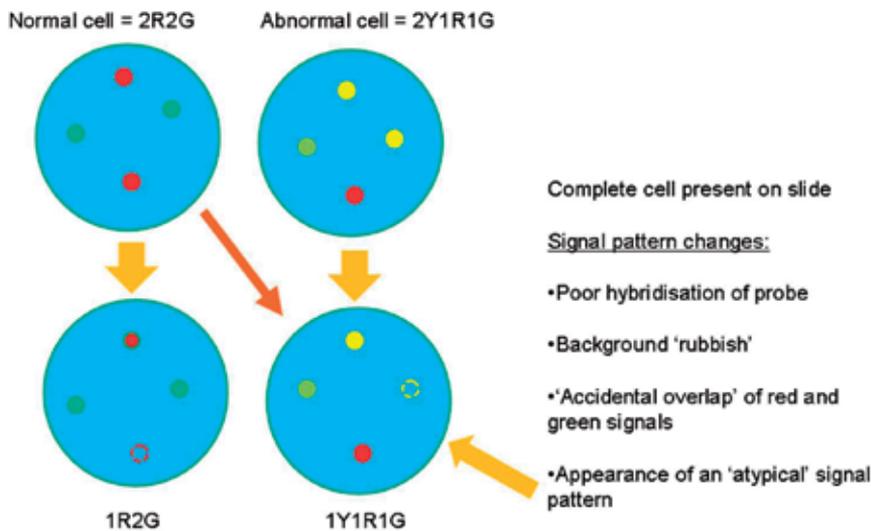


Figure 10. Artefactual signal changes on suspension FISH slides.

Despite such assistance however, care must also be taken during the analysis of paraffin samples, as in many cases it is impossible to completely remove the non-target tissue from the area of interest. It is therefore important to check the H+E slide before beginning the

analysis, as this will give an indication as to whether the sample is made up solely of target tissue, or whether it contains a mixture of target and non-target tissue that must be taken into account when making the final interpretation.

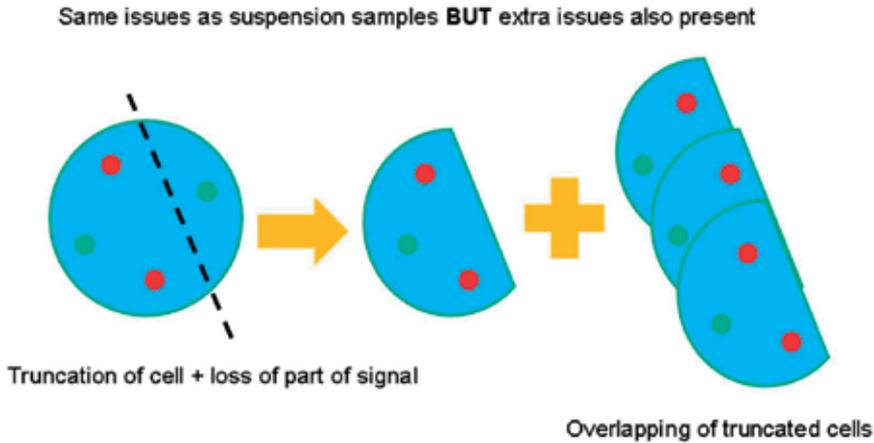


Figure 11. Artefactual considerations for paraffin FISH samples - truncation and overlapping of cells in specimen.

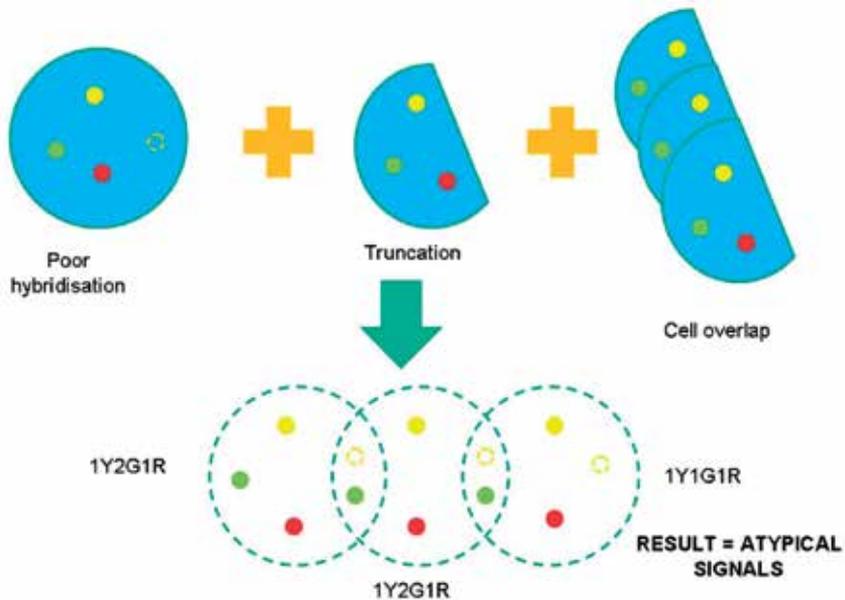


Figure 12. The need for thresholds for paraffin FISH analysis.

Due to both the potential for analysis of the incorrect target cells as outlined, and the artefactual variation that can arise when using the FISH technique [13], it is necessary to establish

robust thresholds to guide the interpretation of results. Signal pattern changes can occur due to poor hybridization of probe, background 'rubbish-autofluorescence' or 'accidental overlap' of red and green signals (Figure 10).

These can lead to the appearance of false or atypical signal patterns; therefore thresholds need to be established to distinguish between false positives and negatives. Paraffin analysis requires higher thresholds than those for suspension cultures, as there is the additional complication of overlap and truncation of cells [1,12], causing artefactual gain or loss of signals (Figures 11 and 12).

Thresholds are of particular importance when dealing with cases that show atypical, non-target (e.g. unexpected loss or increase of copy number instead of a gene rearrangement) or low level abnormalities, or those where mosaicism or multiple clones appear to be present, as it is unclear in most cases as to how they may impact on patient treatment. While paraffin FISH is usually not the most appropriate way to deal with such cases, but when tissue is scarce or has already been processed, it can sometimes be the only option for testing. Numerical scoring of the tissue in such cases will give an indication of the major signal pattern(s) and the level of variation inherent in the tissue, particularly in tumours where there can be concurrent increase in the ploidy level, together with loss or gain of the target loci. This will allow a judgment to be made about whether the variation is likely to be artefactual or not, as false aneuploidies will show almost equivalent levels of loss between target and control loci.

Due to the potential complexities of paraffin analysis, the use of both cytogenetic and pathology external quality control programs such as the College of American Pathologists (CAP) and Australasian Society of Cytogeneticists (ASoC) is recommended, as it allows quality issues to be addressed from both the cytogenetic and pathology perspectives. This provides a balanced perspective on the degree of analytical stringency that is required prior to releasing result.

Appendices

Materials

Reagents

Biotin and Digoxigenin

Bovine serum albumin (BSA) Deionised water

Enzyme reagent (Invitrogen cat #00-8401)

Ethanol (70%, 80% and 100%)

Heat pre-treatment solution pH7.0 (Invitrogen cat #00-8401)

Hybridisation buffer

Non ionic detergent: NP40 (Vysis 30-80482). Store in -20°C.

Phosphate Buffered Saline (PBS)

0.01% pepsin/HCl solution

Purified H₂O

DNA probes

Shandon Rapid-Chrome™ Frozen Section Staining kit

2X SSC/0.1% Tween20

0.4xSSC/0.3%Tween20 solution

2xSSC/0.01% Tween20 solution

Vectashield antifade mounting solution with 1.5µg/ml DAPI (Vector laboratories Cat # H-1200). Store in the dark at 4°C.

Xylol

Equipment

Atlas cooler box

Blotting paper

Centrifuge –Heraeus Biofuge Pico

Coverslips (13mm diameter round, 22x22mm and 24 x 50mm)

Diamond pen or diamond-tipped engraver –Easy Marker Engraver (Taiwan)

Eppendorf tubes

Fix-resistant marker pen

Fluorescence microscope - Zeiss Axio Imager.M1 microscope, Zeiss Axioplan microscope, Olympus BX60 fluorescence microscope

Glass coplin jars

H&E slide

Hotplate

Humidified box

Incubator – Contherm Scientific NZ

Parafilm

Pipettes (2 ul and 100ul)

Pipette tips

Poly-lysine slides (with tissue sections of 2-5µm thickness)

Rubber cement – Weldtite Vulcanising Rubber Solution

Safety goggles

Scalpel

Scissors

Slide drying racks

Thermal cycler – MJC Research PTC- 100 and PTC-200 Peltier Thermal Cyclers

Transmitted light microscope (Zeiss)

Fine tweezers (2 pairs)

Water bath – Grant Instruments (Cambridge)

Recipes

Biotin- and Avidin-conjugated antibodies

Store antibodies as 20µl aliquots at 4°C in sterile eppendorf tubes. Do not freeze.

Texas Red Avidin DCS (Biotek/Vector Laboratories Cat #A-2016).

Add 0.5ml of MilliQ water to 1mg lyophilised antibody for a final concentration of 2mg/ml.

Fluorescein Avidin DCS (Cell sorter grade), (Biotek/Vector Laboratories Cat #A-2011).

2mg/ml stock solution aliquotted at 20µl and stored in the dark at 4°C. Dilute 1:400 in 4xSSC/1% BSA immediately prior to use.

Biotinylated goat anti-avidin D (Biotek/Vector Laboratories Cat #BA0300).

Add 1ml of MilliQ water to 0.5mg lyophilised antibody for a final concentration of 0.5mg/ml.

Bovine Serum albumin (BSA)

1% BSA in 4xSSC. Dissolve 0.25g of BSA (Sigma A-7030) in 25ml 4xSSC pH 7.0. Store at 4°C for up to 1 month.

FITC– conjugated anti-digoxigenin antibodies

Store antibodies as 50µl aliquots at 4°C in sterile eppendorf tubes. Do not freeze.

Anti-digoxigenin-fluorescein, FAB fragments (Boehringer Mannheim Cat #1207741).

Add 1ml of MilliQ water to 200µg lyophilised antibody for a final concentration of 0.2mg/ml.

Rabbit fluorescein anti-sheep IgG(H+L) (Biotek/Vector Laboratories Cat #FI-6000).

Add 1ml of MilliQ water to 1.5mg lyophilised antibody for a final concentration of 1.5mg/ml.

Goat fluorescein anti-rabbit IgG(H+L) (Biotek/Vector Laboratories Cat #FI-1000).

Add 1ml of MilliQ water to 1.5mg lyophilised antibody for a final concentration of 1.5mg/ml.

Ethanol 100% Molecular biology grade.

Ethanol 80% Mix ethanol absolute (molecular biology grade) and distilled water in a 4:1 ratio (v/v).

Ethanol 70% Mix ethanol absolute (molecular biology grade) and distilled water in a 7:3 ratio (v/v).

Hydrochloric acid (HCl)

0.2M HCl. Add 2.4ml of 5N HCl to 60mls of MilliQ water.

0.01N HCl. Add 1mL of 5N HCl to 499mLs of distilled water. Store at room temperature for up to 1 year.

Phosphate buffered saline (PBS)

1xPBS. Ca⁺⁺ and Mg⁺⁺ free. Dissolve 8.0g sodium chloride, 0.2g potassium chloride, 2.89g Na₂HPO₄.12H₂O and 0.2g KH₂PO₄ in order in 750ml of MilliQ water. Adjust the volume to 1 litre and autoclave. Store at room temperature.

Pre-treatment reagents for paraffin embedded tissue – Zymed (Invitrogen) Spotlight™HER2 CISH kit (84-0146)

Reagent A. 1 litre of heat pretreatment solution, pH 7.0 (Ready-To-Use).

Reagent B. 5 ml of enzyme pretreatment reagent (Ready-To-Use).

Saline sodium citrate (SSC)

20xSSC (7.0). Dissolve 175.3g sodium chloride and 88.2g trisodium citrate in 800ml MilliQ water. (or use SSC that comes with the Vysis kits; add 4 bottles to make 1L), pH to 7.0 and adjust the final volume to 1 litre. Autoclave and store at room temperature.

4xSSC (pH7.0). Add 200ml of 20xSSC to 700ml MilliQ water. pH to 7.0 and adjust the final volume to 1 litre. Autoclave and store at room temperature.

4XSSC/0.05% Tween20. Add 500µl Tween20 to 1 litre of 4xSSC. Mix well.

2XSSC/0.1% NP40. Add 1mL of NP40 to 1L of 2XSSC (pH7.0)

2xSSC (pH7.0). Add 100ml 20xSSC (pH 7.0) to 800ml MilliQ water. pH to 7.0 and adjust the final volume to 1 litre. Autoclave and store at room temperature.

1xSSC (pH 7.0). Add 50ml of 20xSSC (pH 7.0) to 950ml of milliQ water. Adjust the pH to 7.0, autoclave and store at room temperature.

0.4XSSC/0.3% NP40 (Quickwash buffer). Add 20ml of 20xSSC and 3ml of NP40 to 900ml MilliQ water. Adjust the pH to 7.0 and final volume to 1 litre. Store at room temperature.

Caution

All reagents are potentially hazardous. Appropriate safety procedures must be followed when handling these materials. Avoid contact with skin and mucous membranes, and heating of slides should be performed in a fume hood, as formalin fixed specimens may produce toxic fumes when heated during processing. For more information consult the Hazardous Substances Data Bank (HSDB) - <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB>.

Formamide: perform steps involving formamide in hood to avoid inhalation of fumes

Xylene: perform steps involving xylene in hood to avoid inhalation of fumes

Commercial probes and hybridisation buffer solutions: Wear gloves at all times, and when co-denaturing probes use a fume hood, as formamide may be present in probe mixtures and give off toxic fumes.

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Quality Control of Biomarkers: From the Samples to Data Interpretation

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Additional information is available at the end of the chapter

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1. Introduction

The recent advances in biotechnology and the improved understanding of disease's mechanisms and pathophysiology have strongly shifted the treatment paradigm of empiric knowledge to targeted therapy. Science has enhanced its ability to guide application of new and existing treatments with development, assay verification, biological validation and application of biomarkers; however, in order to be successful, it is needed a thorough understanding of the relationship between the choice of a biomarker and its influence on the treatment effects. [1]

Current biochemical and molecular biological knowledge states that genetic information flows from genomic DNA to mRNA transcripts, which are then translated to proteins; this class of molecules, which also include enzymes, directly influence the concentrations of their substrates and products, which are integrating parts in several tightly-controlled metabolic pathways. Finally, the existence and multiple interactions of these low-molecular weight metabolites within a cell, tissue, or organism, generates a phenotype. [2]

Metabolome, the link between phenotype and genotype, is the last comprehensive grouping for downstream products of the genome and contemplates the total complement of all the low-molecular weight molecules (metabolites) in a cell, tissue, or organism, required for growth, maintenance, or basal function in any given specific physiological state. [3] The potential size of the metabolome is arguable, as studies suggest more and more that an important role is played by residing microflora and its metabolic products. [2]

The monitoring of metabolite changes has been the primary indicator of disease, and has made it possible to diagnose it in individuals. For that reason, the measurement of metabo-

lites has become an essential part of clinical practice. Employing a wide range of biological fluids, such as blood (including both plasma and serum), saliva, cerebrospinal fluid (CSF), synovial fluid, urine, semen, and tissue homogenates have ensured the widespread use of metabolites as a very powerful diagnostic tool. [4]

Despite significant advances in analytical technologies the past few years, the discovery of metabolomic biomarkers in biological fluids still remains a challenge. As discussed, metabolome plays an important role in biological systems, hence, are attractive candidates to understand disease phenotypes. [5-6] It represents a diverse group of low-molecular weight structures including lipids, amino acids, peptides, nucleic acids, organic acids, vitamins, thiols, carbohydrates and a few others. [7]

Biomarkers are defined as “characteristics that are objectively measured and evaluated as indicators of normal biological processes, pathogenic processes or pharmacological responses to therapeutic intervention”. They can be categorized as biomarkers of exposure, biomarkers of effect and biomarkers of susceptibility. [8] Those characteristics are informative for clinical outcome and can be broadly understood as prognostic or predictive biomarkers. [9-10]

Along the variety of chemical classes and physical properties that constitute metabolites, as well as the dynamic range of metabolite concentrations across large orders of magnitude, it becomes clear why it is necessary to employ an extensive array of analytical techniques in metabolomic research, for it represents a comprehensive method for metabolite assessment. [11-12]

Enabling the parallel assessment of the levels of a broad number of endogenous and exogenous metabolites, it has been demonstrated to have great impact on investigation of physiological status, diseases diagnosis, biomarker discovery and identification of disrupted pathways due to disease or treatment. [13-14]

2. Mass Spectrometry and Biomarkers

2.1. Mass spectrometry in metabolomics

Nowadays, mass spectrometry is one of the most promising approaches for quantifying and qualifying known and unknown specific molecules within a very complex sample, and for elucidating the structure and chemical properties of different compounds. A mass spectrometer consists of three major components: (1) Ion Source: For producing gaseous ions from the substance being studied, some examples are electron impact (EI), chemical ionization (CI), electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), atmospheric pressure photon ionization (APPI), thermospray ionization (TSI), among others; (2) Analyzer: For resolving or separating ions according to their mass-to-charge ratios, some analyzer examples are: quadrupole, time of flight, ion traps, Fourier transform ion cyclotron resonance, orbitrap, among others; (3) Detector system: For detecting the ions and recording the relative abundance of each of the resolved ionic species, for example: electron multiplier, microchannel plate detector, Daly detector, Faraday cup, among others. The mass spectrom-

etry technique relies on the capacity of converting neutral molecules into gaseous ions, with or without fragmentation, which are then characterized by their mass to charge ratios (m/z) and relative abundances. The introduction of a sample into the system, which can be a gas chromatography or liquid chromatography system is necessary to allow the study of different structures and ionic forms.

Historically, most studies with metabolites have been performed with a combination of high resolution capillary gas chromatography, combined with electron impact ionization mass spectrometry (GC-MS). This configuration allowed, for decades, the separation and identification of key micromolecules from complex mixtures, including fatty acids, amino acids, and organic acids in biofluids, generating diagnostic information for several metabolic disorders in qualitative and quantitative pathways [15-16].

Despite its age, GC is still a very useful and informative technique that seems to be far away from retirement; however, there are some limitations in relation to the size and metabolite types that can be analyzed by this technique, and the extensive sample preparation for this purpose. This resulted in the use of nuclear magnetic resonance (NMR) as a tool for metabolite profiling; however, besides the richness of information about molecular structures obtained by this approach, NMR has low sensitivity, allowing just the most abundant compounds to be identified. In contraposition of GC-MS and NMR, the mass spectrometry with a high performance liquid chromatographic system (LC-MS), and the possibility of tandem mass spectrometry (LC-MS) as post-source fragmentation, especially after soft ionization techniques, offers the possibility of analyzing a wide range of polar and medium polarity compounds with good quantification, sensibility and reproducibility [16].

According to Birkemeier et al. (2005) [17], the metabolomic approaches are in dynamic development and a diversity of synonyms have been suggested, such as metabonomics, metabolite profiling (fingerprinting), among others. Several analytical platforms have been introduced, including spectroscopies using diverse electromagnetic wavelengths, like metabolite profiling with the use of infrared spectroscopy (IR), near infrared (NIR), or ultraviolet (UV), besides gas chromatography coupled to mass spectrometry (GC-MS), liquid chromatography with electrospray ionization mass spectrometry (LC-ESIMS), capillary electrophoresis with mass spectrometry (CE-MS) or liquid chromatography with nuclear magnetic resonance (LC-NMR), and these are only a few examples of the technologies involved with metabolomic studies. There is not a single approach to analyze the wide range of chemically different biomolecules, but it is important to choose the technology that fits better to your target molecules [17].

Hollywood et al. (2006) [18] have summarized the main metabolomic strategies:

1. Metabolomic target analysis, which is a more restrict approach. For example, the metabolites originated from a particular enzymatic system after any kind of biotic or abiotic disturbance.
2. Metabolite profiling, which is focused in a group of specific metabolites, for example, lipids associated to a determined metabolic pathway; or related with clinical and pharmaceutical analyses, to map drug metabolism in an organism. This strategy can be also applied with

other approaches, e.g.: *a.* “Metabolite fingerprinting”, this approach is used in order to classify samples based both in their biological relevance to the organism, and in their origin. The fingerprinting technology is fast, but not necessarily gives specific information about metabolites. *b.* “Metabolite footprinting”, exometabolome or secretome, this is similar approach to the fingerprinting, however the target now is a non-invasive analysis, in order to identify the extracellular metabolites. This technique is generally employed to the study of culture cells, with the advantage of not needing to extract the metabolites, and not having to interrupt the metabolism in a given moment before the analysis. Otherwise, this technique can be used for analysing the secretion of any organism, including the secretome of human embryos before *in vitro* fertilization, with the purpose of finding viable embryos and general disease biomarkers.

3. Metabolomics itself, which is the comprehensible analysis of the whole metabolome (all the measurable metabolites), under a specific analysis condition. This term is frequently mistaken with metabonomics, a technique that focus in a wider profile of metabolites involved with different metabolic pathways interacting under the effect of some external stimuli, including diseases, drugs, toxins, among other.

2.2. MALDI AND MALDI-Imaging

Matrix-assisted laser desorption/ionization (MALDI) is an ionization method with common applications to high mass biomolecules, being a key technique in mass spectrometry (MS), and more traditionally to the proteomics field. MALDI-MS is extremely sensitive, easy-to-apply, and relatively tolerant to contaminants [19]. Its high-speed data acquisition and large-scale, off-line sample preparation has made it once again the focus for high-throughput proteomic analyses. These and other unique properties of MALDI offer new possibilities in applications such as rapid molecular profiling and imaging by MS [19].

More recently, there is a growing focus on the use of MALDI ionization system to the analysis of small molecules, however it is important to take into consideration that the coupling of LC-MALDI is a more delicate issue than the coupling of HPLC with other ionization sources such as ESI, because MALDI, based on desorption of molecules from a solid surface layer, is a priori not compatible with LC or CE [20]. A simple alternative to this limitation is the automatic deposition of fractions from a chromatographic separation on a MALDI-TOF target. More advanced techniques have been developed recently: electrospray deposition, electrically mediated deposition, rotating ball inlet, continuous vacuum deposition, and continuous off-line atmospheric-pressure deposition. The current interfacing improvements will surely expand the use of LC-MALDI in the metabolomic area [20,21].

Another good advantage of MALDI ionization is the possibility of obtaining tissue imaging. This is a new technology that allows the simultaneous investigation of the content and temporal/spatial distribution of molecules within a tissue section, enabling to find the exact localization of any biomarker of interest for the prediction of pathologies and for the discovery of future secondary complications originated from different metabolic disease [22].

One of the most common applications for this new approach besides the well described proteomics application is the identification of membrane lipids, which have been successfully analyzed by different authors for several biological tissues. MS imaging of cryosections of mature cotton embryos revealed a distinct, heterogeneous distribution of molecular species of triacylglycerols and phosphatidylcholines, the major storage and membrane lipid classes in cotton embryos. Other lipids were imaged, including phosphatidylethanolamines, phosphatidic acids, sterols, and gossypol, indicating the broad range of metabolites and applications for this chemical visualization approach [23].

There are several possibilities for MALDI imaging technology; however applications to the study of small molecule biomarkers are becoming an interesting novel possibility for this ionization method, mainly when considering the development of new matrices which generate low noise levels in the low m/z range of the spectra. Bnabdellah et al. (2009) [24] have described the detection and identification of 13 primary metabolites (AMP, ADP, ATP, UDP-GlcNAc, among others), directly from rat brain sections by chemical mass spectrometry imaging. Matrix-assisted laser desorption/ionization tandem mass spectrometry (MALDI-MS/MS) was combined with 9-aminoacridine as a powerful matrix in this study.

Metabolite distribution via imaging mass spectrometry (IMS) is an increasingly utilized tool in the field of neurochemistry. As most previous IMS studies analyzed the relative abundances of larger metabolite species, it is important to expand its application to smaller molecules, such as neurotransmitters [25]. However, it has been pointed out two technical problems that must be resolved to achieve neurotransmitter imaging, the lower concentrations of bioactive molecules, compared with those of membrane lipids, require higher sensitivity and/or signal-to-noise (S/N) ratios in signal detection, and the rapid molecular turnover of the neurotransmitters; thus, tissue preparation procedures should be performed carefully to minimize *postmortem* changes [25].

Furthermore, matrix-assisted laser desorption/ionization (MALDI) imaging mass spectrometry has attracted great interest for monitoring drug delivery and metabolism. Since this emerging technique enables simultaneous imaging of many types of metabolite molecules, MALDI-IMS can visualize and distinguish the parent drug and its metabolites. As another important advantage, changes in endogenous metabolites in response to drug administration can be mapped and evaluated in tissue sections [26].

Another applications of MALDI and MALDI imaging to the study of small molecule biomarkers are the use of the method for detecting drug-related degradation products [27] analysis of drugs from intact biological samples and crude extracts, a method that can be applied to rapid drug screening and precise identification of toxic substances in poisoning cases and *postmortem* examinations [28], the application of MALDI imaging mass spectrometry to the study of elevated nigral levels of dynorphin neuropeptides in L-DOPA-induced dyskinesia in rat model of Parkinson's disease [29], it is also possible to point out the recent advances in the field of lipidomics and oxidative lipidomics based on the applications of mass spectrometry and imaging mass spectrometry as they relate to studies of phospholipids in traumatic brain injury [30] and the using of proteomic or lipidomic signatures for discovery and spatial mapping of molecular disturbances within the microenvironment of chronic wounds using MALDI imaging technology [31].

2.3. Orbitrap

The orbitrap mass analyzer is a powerful and relatively new technology, which operates in the absence of any magnetic or *rf* fields. In this analyzer, ion stability is achieved only due to ions orbiting around an axial electrode. Orbiting ions also perform harmonic oscillations along the electrode with frequency proportional to $(m/z)^{-1/2}$. These oscillations are detected using image current detection and are transformed into mass spectra using fast FT, similarly to FT-ICR [32]. In an orbitrap, ions are injected tangentially into the electric field between the electrodes and trapped because their electrostatic attraction to the inner electrode is balanced by centrifugal forces. Thus, ions cycle around the central electrode in rings. In addition, the ions also move back and forth along the axis of the central electrode. Therefore, ions of a specific mass-to-charge ratio move in rings which oscillate along the central spindle. The frequency of these harmonic oscillations is independent of the ion velocity and is inversely proportional to the square root of the mass-to-charge ratio (m/z). The entire instrument operates in LC/MS mode (1 spectrum/s) with nominal mass resolving power of 60 000 and uses automatic gain control to provide high-accuracy mass measurements, within 2 ppm using internal standards and within 5 ppm with external calibration. The maximum resolving power exceeds 100 000 (Full Width at Half-Maximum – FWHM). Rapid, automated data-dependent capabilities enable real-time acquisition of up to three high-mass accuracy MS/MS spectra per second [32,33].

Some recent applications of this mass analyzer in the search of biomarkers include the on-tissue digestion of proteins followed by detection of the resulting peptides, taking advantage of the high resolution obtained. Trypsin was applied by a spraying device for MALDI imaging experiments in a LTQ-Orbitrap mass spectrometer. The mass accuracy under imaging conditions was better than 3 ppm RMS. This allowed for confident identification of tryptic peptides by comparison with liquid chromatography/electrospray ionization tandem mass spectrometry (LC/ESI-MS/MS) measurements of an adjacent mouse brain section [34].

Another possible application for this mass analyzer is the monitoring of metabolites in human urine, approximately 970 metabolite signals with repeatable peak areas could be putatively identified in human urine, by elemental composition assignment within a 3 ppm mass error. The ability of the methodology for the verification of non-molecular ions, which arise from adduct formation, and the possibility of distinguishing isomers could also be demonstrated. Careful examination of the raw data and the use of masses for predicted metabolites produced an extension of the metabolite list [35].

Orbitrap mass analyzer has been also successfully applied to the monitoring of environmental contamination. The use of pharmaceuticals in livestock production is a potential source of surface water, groundwater and soil contamination. A rapid, versatile and selective multi-method was developed and validated for screening pharmaceuticals and fungicides compounds, in surface and groundwater, in one single full-scan MS method, using benchtop U-HPLC-Exactive Orbitrap MS at 50,000 (FWHM) resolution. It demonstrates that the ultra-high resolution and reliable mass accuracy of Exactive Orbitrap MS permits the detection of pharmaceutical residues in a concentration range of 10-100 ng.L⁻¹, applying a post-target screening approach, in the multi-method conditions [36].

Other recent applications of orbitrap mass analyzer in the search of biomarkers include: the analysis of serotonin and related compounds in urine and the identification of a potential biomarker for attention deficit hyperactivity/hyperkinetic disorder [37,38]; the quantitative profiling of phosphatidylethanol molecular species, which are a group of aberrant phospholipids formed in cell membranes in the presence of ethanol by the catalytic action of the enzyme phospholipase D on phosphatidylcholine in human blood, by liquid chromatography high resolution mass spectrometry performed on an LTQ-Orbitrap XL hybrid mass spectrometer equipped with an electrospray ionization source operated in negative ion mode [39]; frozen sections (12 μm thick) of an *ex vivo* tissue sample set comprising primary colorectal adenocarcinoma samples and colorectal adenocarcinoma liver metastasis samples were analyzed by negative ion desorption electrospray ionization (DESI), with spatial resolution of 100 μm using a computer-controlled DESI imaging stage mounted on a high resolution orbitrap mass spectrometer. DESI-IMS data were found to predominantly feature complex lipids, including phosphatidyl-inositols, phosphatidyl-ethanolamines, phosphatidyl-serines, phosphatidyl-ethanolamine plasmalogens, phosphatidic acids, phosphatidyl-glycerols, ceramides, sphingolipids, and sulfatides among others, were identified based on their exact mass and MS/MS fragmentation spectra [40]; among several other applications of this promising technology to the discovery of important biomarkers in different biological systems, taking advantage of the high resolution and speed for LC-MS of this new analytical system.

2.4. Gas Chromatography

Gas chromatography (GC) can be understood as the chromatographic technique in which a gas is the mobile phase and, since 1952, when the first paper in this field was published, GC has always been considered simple, fast and applicable to the separation of many volatile materials, especially petrochemicals, for which distillation was the preferred method of separation at that time. Now, GC is a very important technique, and global market for instruments is estimated around to US\$ 1 billion or over 30,000 instruments annually [41].

Chromatography is the separation process of a mixture into individual components; through the separation process, each component in the sample can be identified (qualitatively) and measured (quantitatively). There are several kinds of chromatographic techniques with their corresponding instruments, and gas chromatography is one of those techniques. GC is used for compounds that are thermally stable and volatile - or that can become volatilizable. Because of its simplicity, sensitivity and effectiveness in separating components, GC is one of the most important tools in chemistry. The principle of basic operation of this instrument involves the evaporation of the sample in a heated inlet port (injector), separation of the components in a mixture employing a prepared column specially and detection of each component by a specific detector. At the end of the process, the amplified detector signals are often recorded and evaluated by integrator software, calculating the analytical results. The sample is introduced into a stream of inert gas, the carrier gas, and transported through the column by its flow. The column can be a packed column or a capillary column, depending on the properties of the sample. As the gas flow passes through the column, the components of the sample move in velocities that are influenced by the degree of interaction of each component with the stationary phase in the column. Consequently, the different

components are separated. Since the processes are temperature-dependent, the column is usually contained in a thermostat-controlled oven. Once that the components are eluted from the column, they can be quantified by a suitable detector and/or be collected for further analysis. There are some types of detectors and the choice of the ones depends on the type of components that will be detected and measured. The most common detectors are: flame ionization detectors (FIDs), thermal conductivity detectors (TCDs), electron capture detectors (ECDs), alkali flame ionization detectors – also called nitrogen/phosphorous detectors (NPDs), flame photometric detectors (FPDs) and photo ionization detectors (PIDs). Several of these are further described in separate leaflets [41,42].

GC is a widely used method for separating and analyzing organic compounds. There are a variety of applications for gas chromatography in every laboratory and in different processes within several industries. In chemical, petrochemical and pharmaceutical industries we can have measurements of any kind of organic compounds, such as process control as well as product control. Also for environmental measurements: aromatic pollutants in air and water, detection and measurement of pesticides, etc. Beside the wide application of GC, there are a few examples of applications on which this analysis technique plays an important role [43,44,45,46].

The detection of reliable biomarkers is a major research activity within the field of proteomics and a growing trend on metabolomics. A biomarker can be a single molecule or set of molecules that can be used to differentiate between normal and diseased states and can be separated and detected by Gas Chromatography - Mass Spectrometry (GC/MS). This combined technique is used to identify the presence of different substances in a given sample.

Kuhara et al. (2011) [47] has used a GC/MS-based approach to investigate the metabolome in urine of patients whom had been previously diagnosed with citrin deficiency. In this noninvasive technique, urine metabolic profiling provided should assist in the rapid and more reliable differential chemical diagnosis of citrin deficiency from other hyperammonemic syndromes.

Another application of GC/MS in biomarker analysis is its application on the studies of volatile organic compounds (VOCs). These compounds are exhaled in breath and provide valuable information about the human health status. The composition of the breath is variable and depends on the disease's characteristics; for example, a sweetened smell indicates diabetes, while the odor of rotten eggs, which are caused by sulfur-containing compounds, suggests liver problems [48,49]. Rudnicka (2011) [50] employed solid phase micro-extraction technique and gas chromatography coupled to time of flight in mass spectrometry (GC-TOF/MS) for the analysis of VOCs on exhaled air from patients with lung cancer and healthy persons. The total number of identified compounds in breathing samples equal 55 and the compound that enables as an indication of lung cancer was isopropyl alcohol.

These studies show how highly important and relevant are the studies on the use of chromatographic techniques for biomarker analysis and identification. It shows a wide range of applications in a field not yet fully developed, which still may be a very suitable area for new ideas and uses for the next couple decades.

3. Statistical and chemometrical analysis of biomarkers

In metabolomics, as well as in other branches of science and technology, there is a steady trend towards the use of more variables (properties) to characterize observations (e.g., samples, experiments, time points). Often, these measurements can be arranged into a data table, where each row constitutes an observation and the columns represent the variables or factors we have measured (e.g., wavelength, mass number, chemical shift, etc). This development generates huge and complex data tables, which are hard to summarize and overview without appropriate tools. Recently, with development of “omics” technologies (metabolomics, proteomics, foodomics, genomics, etc), the adoption of chemometric methods has been playing a very important role in planning and analyzing the obtained results. That includes efficient and robust methods for modeling and analysis of complex chemical or biological data tables that produce interpretable and reliable models capable of handling incomplete, noisy, and collinear data structures. These methods include principal component analysis (PCA) and partial least squares (PLS). It is also completely important to emphasize that chemometrics also provides a straightforward way to collect relevant information through statistical experimental design (SED) [51,52,53].

Multivariate statistical analysis such as Principal Components Analysis (PCA) is probably the most widely used technique for analyzing metabolomics. PCA technique is robust and objective and it is an appropriate way to reduce data sets containing high numbers of variables. By reducing the number of original variables to a smaller number of independent variables, this approach highlights fundamental differences between groups of variables. PCA has been extensively used in metabonomics literature. Despite apparent satisfying published results, the known large sensitivity of PCA to noise can suggest that improvements are expected with more robust methods to identify biomarkers in noisy data. Moreover, the traditional use of PCA remains highly questionable: biomarkers are identified from the loadings of the two first principal components, while the two first components do not necessarily contain the most relevant variations between altered and normal spectra. Sometimes, the results of the initial unsupervised analysis are confirmed by a second supervised analysis. This one employs classification methods as Partial Least Squares (PLS), SIMCA and neural networks, allowing firstly to separate normal and altered spectra, and secondly to identify more robust biomarkers [54,55].

Other data analysis methods frequently employed for disease diagnosis and biomarker identification in metabolomics are Univariate Testing, Soft independent modeling of class analogy (SIMCA), Linear discriminant analysis (LDA), Partial least squares discriminant analysis (PLS-DA), Orthogonal projection to latent structures discriminant analysis, (OPLS-DA), Neural networks (NN), Self organizing maps (SOM) and Support vector machines (SVM). Regardless of the chosen method, both statistical and biological validations are critical. Multivariate methods are of special importance to metabolomics since one biomarker often will not be sufficiently specific for a given condition by itself. There is a wide range of methods and it is natural that this can seem confusing to the non-specialist. The literature has already shown in previous works that it is more important that the chosen method is used correctly than the methodology itself. The reason for this is that all methods are data-driven, and since the parameter definition is through pre-processing, the contained features are static. Many statisti-

cal methods will highlight the same metabolites with similar classification ability. It is clear, however, that pre-processing and scaling of the data can lead to dramatically different results, both with regard to chosen biomarkers and classification ability of the model [53].

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Quality control has an emerging importance in every field of life. Quality control is a process that is used to guarantee a certain level of quality in a product or service.

It might include whatever actions a business deems necessary to provide for the control and verification of certain characteristics of a product or service. With the improvement of technology everyday we meet new and complicated devices and methods in different fields. Quality control should be performed in all of those new techniques. In this book “Latest Research Into Quality Control” our aim was to collect information about quality control in many different fields. The aim of this book is to share useful and practical knowledge about quality control in several fields with the people who want to improve their knowledge.

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