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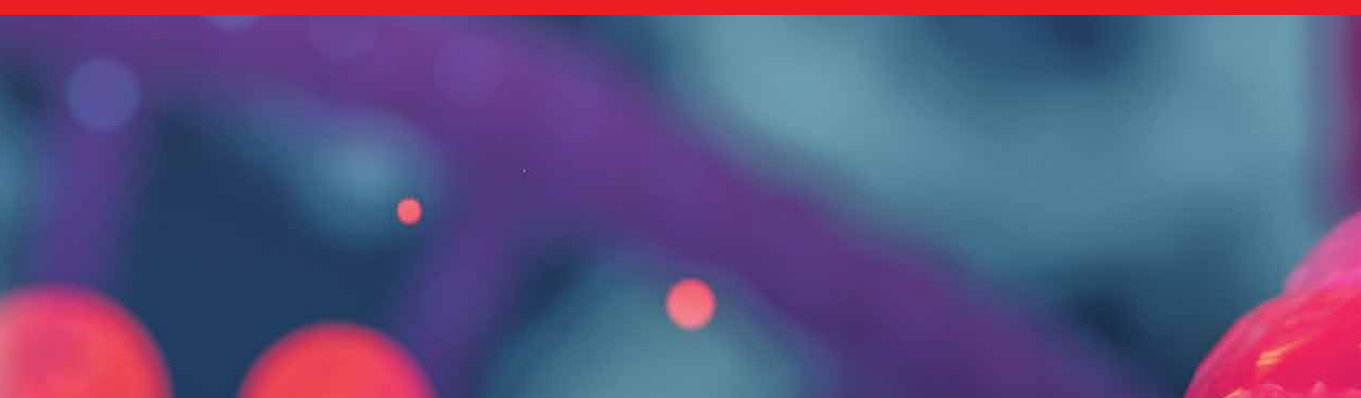
Metabolomics

Methodology and Applications in Medical Sciences and Life Sciences

Edited by Xianquan Zhan



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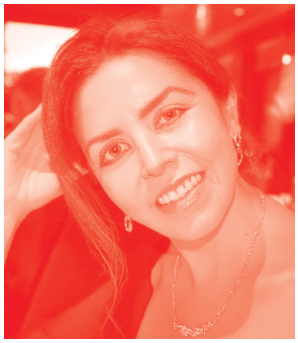

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Meet the editor

Xianquan Zhan received his MD and Ph.D. in Preventive Medicine from the West China University of Medical Sciences. He received his post-doctoral training in oncology and cancer proteomics at Central South University, China, and the University of Tennessee Health Science Center (UTHSC), USA. He worked at UTHSC and the Cleveland Clinic during 2001-2012, and achieved the rank of associate professor at UTHSC. Currently, he is a full professor at Central South University and Shandong First Medical University, as well as an advisor to MS/Ph.D. graduate students and postdoctoral fellows. Dr. Zhan is a fellow of the Royal Society of Medicine and the European Association for Predictive, Preventive & Personalized Medicine (EPMA), a national representative for EPMA, and a member of the American Society of Clinical Oncology (ASCO) and the American Association for the Advancement of Sciences (AAAS). He is editor in chief of the *International Journal of Chronic Diseases & Therapy*, associate editor of *EPMA Journal* and *BMC Medical Genomics*, and guest editor of *Frontiers in Endocrinology*, *Mass Spectrometry Reviews*, *EPMA Journal*, and *Oxidative Medicine and Cellular Longevity*. He has published more than 140 articles, twenty-five book chapters, five books, and two US patents in the field of clinical proteomics and biomarkers.

Contents

Preface

The center of multiomics is moving from genome-centered studies to phenome-centered studies in life sciences and medical sciences. Metabolomics, as the important aspect of phenomics, is the methodology and theory for studying the metabolome, including identifying its biochemical and molecular characteristics, characterizing interactions among different metabolites or between metabolites and genetic/environmental factors, and evaluating biochemical mechanisms related to given conditions such as different pathophysiological processes. The metabolome contains all metabolites derived from sugars, lipids, proteins, and nucleic acids in a given biological system, tissue, cell, or body fluid. The metabolites in a metabolome interact mutually in enzymatic reaction systems to form metabolic network systems. Alteration in the metabolome is associated with multiple factors, including genetic, environmental, internal, external, drug, or dietary factors. Currently, the studies on variations in the metabolome are insufficient. Metabolomics includes a targeted approach based on selected/multiple reaction monitoring (SRM/MRM) and an untargeted approach based on nuclear magnetic resonance (NMR) or mass spectrometry (MS). The development of high-throughput, high-sensitivity, and especially high-reproducibility approaches is necessary to maximize the coverage of variations in the metabolome. Studies on metabolomic variations result in the discovery of effective biomarkers to clarify molecular mechanisms of a disease and determine reliable therapeutic targets. These studies also benefit precise prediction, diagnosis, and prognostic assessment in the context of predictive, preventive, and personalized (3P) medicine, as well as contribute to life sciences.

This book focuses on the advancements in the concept and methodology of metabolomics, including sample preparation, targeted metabolomics based on SRM/MRM, untargeted metabolomics based on NMR or MS, and applications of metabolomics in the research and practice of medical sciences and life sciences. Chapter 1 addresses the concept and importance of metabolomics and its methodology, including targeted and untargeted approaches. Chapter 2 addresses the versatility of metabolomics analysis due to the enormous variety of samples and the absence of strict barriers between quantitative and qualitative analyses. The chapter also highlights untargeted metabolomics using the metabolomics of opposite Antarctic cryptoendolytic communities as an example, and targeted metabolomics using urine during childbirth as an example. Chapter 3 suggests that pharmacometabolomics is a new horizon in personalized medicine, highlighting potentials and advantages in designing innovative and personalized drug treatment. Chapter 4 examines the theory and methodology of volatiomics of natural products from plants, flowers, fruits, microorganisms, and animals. Chapter 5 presents new insights into seagrass metabolomics in marine-based drug discovery through the study of its bioactive products. Chapter 6 discusses the metabolic profiling of transgenic tobacco plants that synthesize bovine interferon-gamma for optimizing the quality and quantity of plant-synthesized interferon-gamma. Chapter 7 discusses the use of gas chromatography (GC)-MS-based metabolomic changes in wood-inhabiting filamentous fungi during ontogenesis.

This book contains only a fraction of the very important metabolomic studies in medical sciences and life sciences. However, the studies included serve to stimulate and encourage researchers to study and practice metabolomics, especially to discover effective biomarkers, novel therapeutic targets/drugs, and patient stratification for diseases. Moreover, the extensive studies on metabolomics in life sciences, in turn, promote and serve research and clinical practice in medicine.

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Chapter 1

Introductory Chapter: Metabolomics

Xianquan Zhan, Jingru Yang, Shu Zheng, Nannan Li and Na Li

1. Introduction

The center of multiomics is being moved from genomics to phenomics (**Figure 1**) [1]. Proteome and metabolome are two main components of phenome, and are equally important. The concept and development of proteoforms significantly enrich the content of a proteome. A book entitled "Proteoforms: Concept and Applications in Medical Sciences" has been published focusing on proteomics at the proteoform level [3]. It is driving the editor to edit another book focusing on metabolomics to discuss (i) the methodology of metabolomics, including sample preparation, targeted metabolomics, and untargeted metabolomics based on nuclear magnetic resonance (NMR) or mass spectrometry (MS), and (ii) applications of metabolomics in the research and practice of life science and medical science.

Metabolomics is an important aspect of phenomics, which is the theory and methodology to study metabolome, including identification of biochemical and molecular characteristics of metabolome, characterization of interactions among

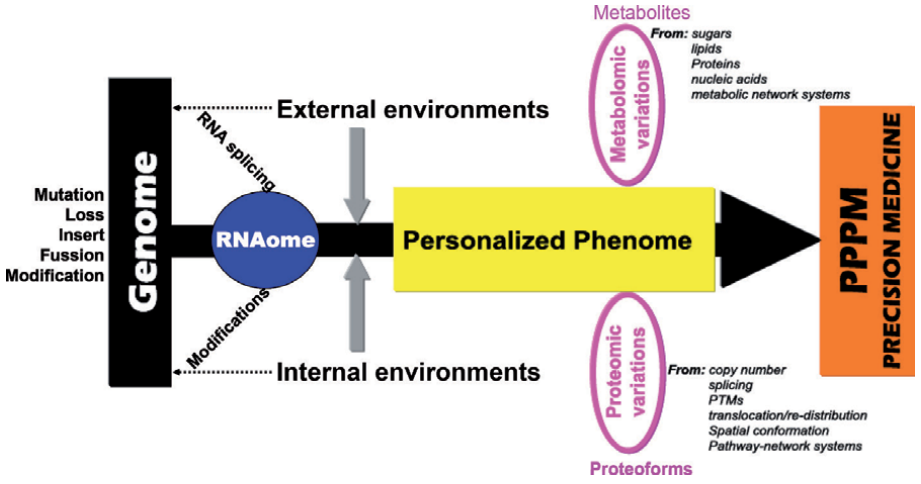

Figure 1.
The imbalance contribution of multiomics to clinical practice. RNAome includes messenger RNAs (mRNAs) and non-coding RNAs (ncRNA). Multiple modifications extensively occur at three different levels of DNAs, RNAs, and proteins to systematically regulate physiological and pathological processes. The center of multiomics is being moved from genomics to phenomics, especially proteomics and metabolomics. PTMs = post-translational modifications. PPPM = predictive, preventive and personalized medicine (3P medicine). Modified and upgraded from Zhan et al. [1], with permission from Elsevier publisher, copyright 2018; and reproduced from Li et al. [2], with permission from Wiley publisher, copyright 2021.

different metabolites or between metabolites and genetic/environmental factors, and evaluation of biochemical mechanisms related to a given condition such as different pathophysiological processes [1]. Metabolome contains all metabolites derived from nucleic acids, proteins, lipids, and sugars in a given cell, tissue, biological system, or body-fluid. The metabolites in a metabolome interact mutually in enzymatic reaction systems to form metabolic network systems. The metabolomic variation is associated with multiple factors, including genetic, environmental, internal, external, drug, or dietary factors [1]. Currently the studies on metabolomic variations are much insufficient in the width and depth of metabolomics. It is necessary to develop high-sensitivity, high-throughput, and high-reproducibility methodology for maximizing the coverage of metabolomic variations. The studies on metabolomic variations directly result in the discovery of effective biomarkers to clarify molecular mechanisms of a disease, determine reliable therapeutic targets, and discover reliable biomarkers for precise prediction, diagnosis, and prognostic assessment in the context of predictive, preventive and personalized medicine (3P medicine, PPPM).

2. Importance of metabolomic variations in medical science

Metabolome contains all metabolites derived from nucleic acids, proteins, lipids, and sugars in a given cell, tissue, biological system, or body-fluid [4–6]. The metabolites in a metabolome interact mutually in enzymatic reaction systems to form metabolic network systems [5]. The change of metabolites is associated with multiple factors, including internal, external, genetic, environmental, drug, or dietary factors. Metabolomics is the theory and methodology to study metabolome, including identifying biochemical and molecular characteristics of metabolome, characterizing interactions among different metabolites or between metabolites and genetic/environmental factors, and evaluating biochemical mechanisms related to a given condition such as different pathophysiological processes [7]. Metabolomic variations can reflect the status of physiological and pathological processes, monitor the progression of a disease, and predict and assess the drug effects compared to the baseline of metabolic profiles, which benefits for disease stratification, and personalized/precise medicine in the context of PPPM [8].

3. Samples used to measure metabolomic variations

The biological samples are very intricate that are used to measure metabolomic variations, including extracts from different cells, tissues, and body-fluids (**Table 1**). Urine and serum/plasma [6, 17, 18] are the most commonly used body-fluids to analyze metabolome for different diseases because these samples are very easily available and are easy to be prepared, without any injury. In addition, tears [19] are the good samples for analyzing metabolome of an eye disease, exhaled air [20, 21] for pulmonary and airway diseases or other diseases, saliva [22] for oral diseases, synovial fluid [23] for arthritis, and cerebrospinal fluid (CSF) [24] for neurological systems disease. Generally speaking, there are many biological samples that are suitable for metabolomics analysis of a disease. The metabolomics studies based on these different samples can directly or indirectly reflect the status of a disease, which may use to understand the molecular mechanism of a disease, and discover therapeutic targets and reliable biomarkers to predict, diagnose, and prognostically evaluate a disease.

Biological sample	Methods	Main results	References
HeLa cells	Gas cluster ion beam-secondary ion MS (GCIB-SIMS)	Purinosomes comprise nine enzymes that act synergistically, channeling the pathway intermediates to synthesize purine nucleotides, increasing the pathway flux, and influencing the adenosine monophosphate/guanosine monophosphate ratio.	[9]
Carcinoma and adjacent normal tissues	UHPLC-Orbitrap MS	This method enables targeted profiling of over 400 biologically important metabolites covering 92 metabolic pathways	[10]
Sweat	GC–MS and LC–MS/MS	As most of the identified metabolites are involved in key biochemical pathways, this study opens interesting possibilities to the use of dry sweat as a source of metabolite markers for specific disorders.	[11]
Urine and plasma	HPLC-ESI-qTOF-MS	A total of 31 and 38 metabolites in plasma and urine, respectively, showed significant differences between healthy volunteers and Sjögren's Syndrome patients and were proposed for their identification.	[12]
Cerebrospinal fluid (CSF)	GC–MS and LC–MS/MS	A total of 274 CSF-derived metabolites were common to the discovery and replication cohorts in cancer-related fatigue.	[13]
Saliva	UHPLC-qTOF-MS	The study identified and classified a total of 211 endogenous and exogenous salivary metabolites. The results reveal a distinct metabolite profile of dog and human saliva as 25 lipid compounds were identified only in canine saliva and eight dipeptides only in human saliva.	[14]
Sputum	LC–MS/MS	The KEGG analysis revealed that the glycerophospholipid metabolism pathway was downregulated in severe COPD. Due to the critical role of glycerophospholipid metabolism in oxidative stress, significant negative correlations were discovered between glycerophospholipid metabolites and three oxidative stress products (SOD, MPO, and 8-iso-PGF2α). The diagnostic values of SOD, MPO, and 8-iso-PGF2α in induced sputum were found to exhibit high sensitivities and specificities in the prediction of COPD severity.	[15]
Blood	1D 1H NMR spectroscopy methods	This has led to the absolute quantitation of nearly 70 metabolites in serum and plasma and nearly 80 in whole blood.	[16]

Table 1.
Examples of different types of biological samples used for metabolomics analysis.

4. Methods used to measure metabolomic variations

The appropriate analytical methods for metabolomics are important to detect, identify, and quantify metabolomic variations in a given condition; for example, a disease status versus control, which are mainly classified into targeted metabolomics [25] and untargeted metabolomics [26]. (i) The targeted metabolomics [25] is to mainly quantify hypothesis-driven known metabolite variations in a metabolome (such as

metabolites derived from one or more unknown metabolism pathways) between or among research groups, and then use multivariate statistical analysis to establish mathematical models [27]. This mathematical model then is used to discriminate Diseases from healthy controls, treatment from untreatment, or different stages of diseases. The often used methods for targeted metabolomics are the selected/multiple reaction monitoring (SRM/MRM) analysis with an optimized sample extraction and liquid chromatography-mass spectrometry (LC–MS) conditions using the triple quadrupole mass spectrometry (QqQ-MS) [28]. (ii) The untargeted metabolomics [26] is an none hypothesis-driven approach to globally detect, identify, and quantify metabolite variations in a metabolome in a biological system without any bias, which will benefit the understanding molecular mechanism of a disease, discover new therapeutic targets/drugs and metabolite biomarkers for effective prediction, diagnosis, and prognosis. The often used methods for untargeted metabolomics are the mass spectrometry (MS)-based methods [6, 29], and nuclear magnetic resonance (NMR)-based methods [30, 31] (**Figure 2**). (a) MS-based methods have ion mobility coupled with MS (IM-MS) that can measure time, mass-to-charge (m/z) and intensity variables [1], capillary electrophoresis coupled with MS (CE-MS) that can measure time, m/z and intensity variables [29, 32, 33], gas chromatography coupled with MS (GC–MS) that can measure time, m/z and intensity variables [29, 34], liquid chromatography coupled with MS (LC–MS) that can measure retention time (RT), m/z and intensity variables [26, 29, 35], and direct injection coupled with MS (DI-MS) that can measure m/z and intensity variables [1]. IM-MS is to use a buffer gas and a uniform or periodic electric field for separation of ions based on size and shape of the ions, followed by MS analysis. This is a very high throughput and high selectivity method, which can easily separate isomeric and isobaric compounds. CE-MS is to use electro kinetics for

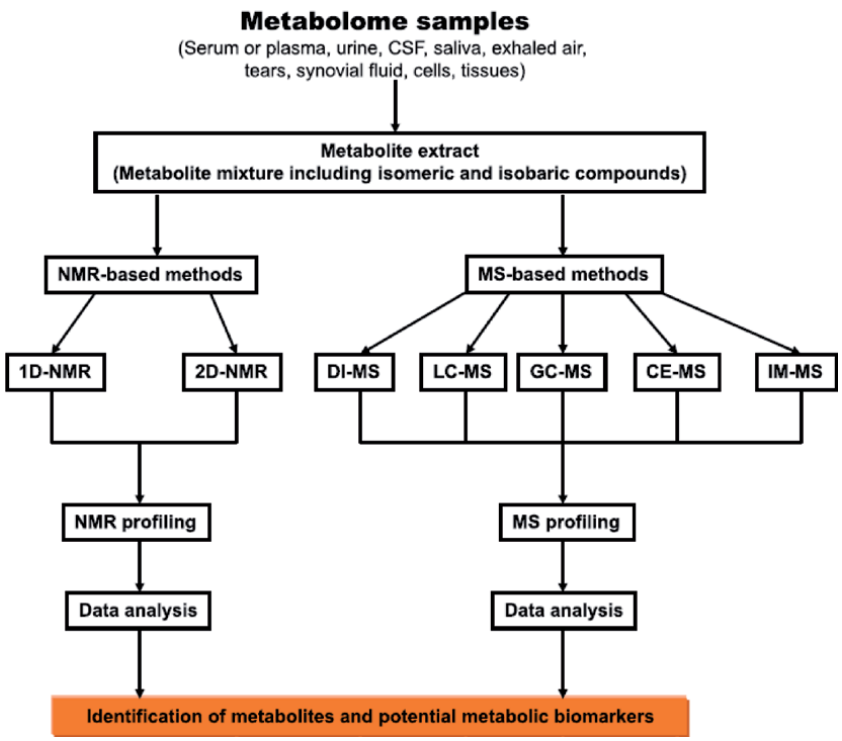

Figure 2.
The main metabolomic strategies for identification of metabolite profiling and discovery of biomarkers. Reproduced from Zhan et al. [1], with permission from Elsevier Publisher, copyright 2018.

separation of polar molecules, followed by MS analysis. This is a very good method to analyze polar molecules in aqueous samples for measurement of inorganic and organic anions, with low running costs and relatively low throughput. GC–MS is to use gas chromatography for separation of molecules, followed by MS analysis. This method is suitable for a polar and volatiles compounds, whose advantages are availability of universal database for identification, high sensitivity, and high reproducibility; and whose disadvantages are only detection of a polar and volatile compounds, requirement of derivatization of polar compounds, low ionization discrimination, and requirement of higher amount of samples. LC–MS is to use liquid chromatography for separation of molecules, followed by MS analysis. This method is suitable for polar to hydrophobic compounds, whose advantages are requirement of minimal amount of samples, high sensitivity, high throughput, and flexibility in column chemistry widening the range of detectable compounds; and whose disadvantages are requirement of high ionization discrimination, lack of large metabolite databases, and requirement of specific chromatographic conditions for very polar molecules. DI-MS is to use the nanospray source directly coupled with MS, which does not require chromatography separation, whose advantages are low sample volume requirement, high sensitivity, high-throughput, and low cost; and whose disadvantages are requirement of high ionization discrimination, significant ion suppression phenomenon, and inability to separate isomers and isbaric species. (b) NMR-based methods have one-dimensional, two-dimensional, and three-dimensional NMR methods (1D-NMR, 2D-NMR, and 3D-NMR) [31], which is to use the interaction of spin active nuclei (13C, 1H, 31P, 19F) in the electromagnetic fields for obtaining structural, chemical, and molecular environment information [30, 31], whose advantages are non-destruction of sample, minimal sample preparation, high reproducibility, relative high throughput, availability of molecular dynamic and compartmental information with diffusional methods, and availability of databases; and whose disadvantages are low sensitivity, overlapping of metabolites, and high instrumentation cost [36]. MS-based methods and NMR-based methods are complimentary for metabolomics analysis, and both will produce very complex data. The processing, analysis, and annotation of data are very important and crucial steps to discover the potential and important metabolic biomarkers [37, 38]. However, compared to the NMR-based metabolomics, MS-based metabolomics has a relatively low cost, high sensitivity and resolution, and very good analytical performance to measure the metabolomic variations for PPPM or PM practice [39].

5. Applications of metabolomics in life science and medical science

Metabolome is the important content of phenome. Metabolomics conducts qualitative and quantitative analysis of all small molecule metabolites in organisms, and searches for the relative relationship between metabolites and physiological and pathological changes. The subjects are mostly small molecules with molecular weights of less than 1,000. With the development of high throughput technology, the study of living organisms has developed from single small molecule to multi-omics; such as genomics, transcriptomics, proteomics, metabolomics. Multiomics reflects molecular changes in a disease or biological process, and molecules that can be identified can be used as valuable biomarkers. Metabolites are substances produced or consumed through the metabolic process. Metabolites are the final expression products subject to genetic control and environmental influence. Imprints with genomic, transcriptomic, epigenetic and environmental effects are called "associations between genotypes and phenotypes" [40]. Metabolomics has been extensively applied in fields of medical science and life science (**Table 2**). It has important applications in medicine and life sciences, agriculture, food safety and so on. Metabolites,

Metabolomics methods	Biological samples	Main discoveries	References
1D-NMR	Plasma samples from SARS-CoV-2 rRT-PCR-positive patients (n = 15, with multiple sampling timepoints) and age-matched healthy controls (n = 34, confirmed rRT-PCR negative), together with patients with COVID-19/ influenza-like clinical symptoms who tested SARS-CoV-2 negative (n = 35).	The study observed four plasma cytokine clusters that expressed complex differential statistical relationships with multiple lipoproteins and metabolites. These included the following: cluster 1, comprising MIP-1β, SDF-1α, IL-22, and IL-1α, which correlated with multiple increased LDL and VLDL subfractions; cluster 2, including IL-10 and IL-17A, which was only weakly linked to the lipoprotein profile; cluster 3, which included IL-8 and MCP-1 and were inversely correlated with multiple lipoproteins. IL-18, IL-6, and IFN-γ together with IP-10 and RANTES exhibited strong positive correlations with LDL1–4 subfractions and negative correlations with multiple HDL subfractions.	[41]
2D-NMR	Different aging regimes (crust from dry-aged beef, inner edible flesh of dry-aged beef, and wet-aged beef striploin)	NMR-based multivariable analyses could be used to distinguish the method, degree, and doneness of beef aging.	[42]
3D-NMR	*E. coli* cell lysate	For 19 of the 25 model metabolites, "Structure of unknown metabolomic mixture components by MS/NMR" yielded complete structures that matched those in the mixture independent of database information.	[43]
DI-MS	A parasite–host cell system	The study applied a metabolic fingerprinting approach to evaluate metabolic changes induced by six different (candidate) drugs in a parasite–host cell system.	[44]
LC–MS	*P. aeruginosa* (35 clinical strains)	Those clinical strains that differed in their virulence and biofilm phenotype also had pronounced divergence in their metabolomes, as underlined by 332 features that were significantly differentially abundant with fold changes greater than 1.5 in both directions.	[45]
GC–MS	Embryonic zebrafish	A total of 87 important endogenous metabolites such as citric acid and hypoxanthine were identified by universal databases or standards among 270 extracted metabolites, which consisted of sugars, amines, amino acids, nucleotides, fatty acids, and sterols.	[46]
CE-MS	Plasma samples of acute corneal seizure mouse model.	Both electrically induced seizures showed decreased values of methionine, lysine, glycine, phenylalanine, citrulline, 3-methyladenine and histidine in mice plasma. However, a second provoked seizure, 13 days later, showed a less pronounced decrease of the mean concentrations of these plasma metabolites, demonstrated by higher fold change ratios.	[47]

Metabolomics methods	Biological samples	Main discoveries	References
IM-MS	Breast cancer plasma samples	Analysis of the resulting data showed that phosphatidylcholines, triglycerides and diglycerides exhibited lower expression and phosphatidylserine showed increased expression in the breast cancer samples compared to those of healthy subjects. The coefficients of variation, determined by reference to the QC data, for all of the features identified as potential markers of disease, were 6% or less.	[48]

Table 2.
Examples of different metabolomics applied in life science and medical science.

as the end products of gene expression, have been implicated in many diseases. For example, metabolomics has great potential for diabetes research, metabolic markers hold the potential to detect diabetes-related complications already under subclinical conditions in the general population [49]. Metabolomics is used to identify key disease-related metabolic changes and disease-progression-related changes, and defining metabolic changes during AD disease trajectory and its relationship to clinical phenotypes provided a powerful roadmap for drug and biomarker discovery [50]. Carmen Peña-Bautista's work shows that the untargeted analysis carried out in human plasma samples from early Alzheimer's disease patients and healthy individuals, and the use of sophisticated statistical tools, identified some metabolic pathways and plasma biomarkers [51]. Nina P Paynter's work shows metabolomics also has important applications in cancer. The processes of life accompany metabolism, such as glycolysis, protein synthesis and metabolism. These fundamental features of cellular metabolism are reprogrammed in cancer cells to support their pathological levels of growth and proliferation. Metabolic reprogramming in malignant cells is likely the result of the multifactorial effects of genomic alterations (i.e. mutations of oncogenes and tumor suppressors), the tumor microenvironment (which imposes metabolic stress caused by compromised nutrients and oxygen availability), and other influences [52]. These changes may be the result of changes in the genome or environmental impacts and a variety of other factors. We need to understand the complete breadth of metabolic abnormalities in cancer because some metabolic changes provide opportunities to develop novel therapeutic targets and predictive biomarkers [52]. As mentioned in Yousra Ahmed-Salim's study, generally, combinations of more than one significant metabolite as a panel, in different studies, achieved a higher sensitivity and specificity for diagnosis than a single metabolite [53]. Metabolomics has become the most powerful platform for studying tissue samples. A common application of metabolomics is the discovery of biomarkers for diagnosis or prediction of treatment sensitivity and prognosis. For example, Yousra Ahmed-Salim et al. conducted a systematic review of the application of metabolomics in the treatment of ovarian cancer. The most frequently described metabolite difference between the biological fluids and tissues of patients with ovarian cancer and those of healthy controls have been in phospholipids [53]. Su et al. interrogated metabolomics and gene-expression from the NCI-60 cell lines to study relationships between metabolite and transcripts [54]. They observed that the metabolome can distinguish cancer subtypes and that metabolite levels correlate well with gene expression under strong correlation models [54]. In conclusion, metabolomics can more accurately determine pathophysiological changes of diseases and identify effective biomarkers through

the high-throughput study of metabolites in organisms with abundant sources of samples, so as to further understand the molecular mechanism of diseases. Thus, it is beneficial to the prevention, diagnosis and treatment of diseases.

6. Conclusion

Metabolomics as the important aspect of phenomics is emerging as the frontier field in life science and medical science. Many biological samples have been used to measure metabolomic variations, including extracts from different cells, tissues, organisms, and body-fluids (for example, urine, serum/plasma, tear, exhaled air, saliva, synovial fluid, CSF, and sputum). Metabolomics is classified into targeted metabolomics and untargeted metabolomics. Targeted metabolomics is used to analyze the known metabolite profiling with SRM/MRM methods. Untargeted metabolomics is used to globally analyze the unknown metabolite profiling with NMR-based methods (1D-NMR, 2D-NMR, and 3D-NMR) and MS-based methods (DI-MS, LC–MS, GC–MS, CE-MS, and IM-MS). Metabolomics has been extensively applied in the research and practice of life science and medical science. However, currently the studies on metabolomic variations are much insufficient in the width and depth. The development of high-sensitivity, high-throughput, and high-reproducibility methodology is needed to maximize the coverage of metabolomic variations for clarification of molecular mechanism of a disease, determination of effective therapeutic targets, and discovery of reliable biomarkers for prediction, diagnosis, and prognostic assessment in the context of PPPM practice.

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Author's contributions

X.Z. conceived the concept, designed the manuscript, wrote and critically revised the manuscript, coordinated and was responsible for the correspondence work and financial support. J.Y., S.Z., N.L., and N.L. participated in the literature analysis, and wrote partial manuscript.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations.

Acronyms and abbreviations

CE-MS	Capillary electrophoresis coupled with mass spectrometry
CSF	Cerebrospinal fluid
DI-MS	Direct injection coupled with mass spectrometry

GC–MS	Gas chromatography coupled with mass spectrometry
IM-MS	Ion mobility coupled with mass spectrometry
LC–MS	liquid chromatography coupled with mass spectrometry
LC	Liquid chromatography
MS	Mass spectrometry
m/z	Mass-to-charge
NMR	Nuclear magnetic resonance
QqQ-MS	Triple quadrupole mass spectrometry
PPPM	Predictive, preventive and personalized medicine
PTM	Posttranslational modification
SRM/MRM	Selected/multiple reaction monitoring
1D-NMR	One-dimensional NMR
2D-NMR	Two-dimensional NMR
3D-NMR	Three-dimensional NMR
3P medicine/PPPM	Predictive, preventive and personalized medicine

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Chapter 2

From Targeted Quantification to Untargeted Metabolomics

Veronica Lelli, Antonio Belardo and Anna Maria Timperio

Abstract

Metabolomics is an emerging and rapidly evolving technology tool, which involves quantitative and qualitative metabolite assessments science. It offers tremendous promise for different applications in various fields such as medical, environmental, nutrition, and agricultural sciences. Metabolomic approach is based on global identification of a high number of metabolites present in a biological fluid. This allows to characterize the metabolic profile of a given condition and to identify which metabolites or metabolite patterns may be useful in the discrimination between different groups. The use of one mass spectrometry (MS) platform from targeted quantification to untargeted metabolomics will make more efficient workflows in many fields and should allow projects to be more easily undertaken and realized. Metabolomics can be divided into *non-targeted* and *targeted*. The first one can analyze metabolites derived from the organisms comprehensively and systematically, so it is an unbiased metabolomics analysis that can discover new biomarkers. Targeted metabolomics, on the other hand, is the study and analysis of specific metabolites. Both have their own advantages and disadvantages, and are often used in combination for discovery and accurate weight determination of differential metabolites, and allow in-depth research and analysis of subsequent metabolic molecular markers. Targeted and non-targeted metabolomics are involved in food identification, disease research, animal model verification, biomarker discovery, disease diagnosis, drug development, drug screening, drug evaluation, clinical plant metabolism and microbial metabolism research. The aim of this chapter is to highlight the versatility of metabolomic analysis due to both the enormous variety of samples and the no strict barriers between quantitative and qualitative analysis. For this purpose, two examples from our group will be considered. Using non-targeted metabolomics in opposite Antarctic *cryptoendolytic* communities exposed to the sun, we revealed specific adaptations. Instead, through the targeted metabolomics applied to the urine during childbirth, we identified a different distribution of specific metabolites and the metabolic differences allowed us to discriminate between the two phases of labor, highlighting the metabolites most involved in the discrimination. The choice of these two approaches is to highlight that metabolomic analysis can be applied to any sample, even physiologically and metabolomically very distant, as can be microorganisms living on Antarctic rocks and biological fluids such as urine.

Keywords: urine, metabolite, HPLC–MS, fungus, biomarkers, system biology

1. Introduction

1.1 Metabolomics: the medicine of the third millennium

Metabolomics is a discipline thanks to whose analysis it is possible to ascertain the presence of biochemical imbalances caused by the lack of nutrients that are the basis of the functions of our body. With this research it is therefore possible to identify the true causes of any chronic disease and restore the biochemical balance of our body.

The use of "omics" sciences, especially metabolomics, has been having positive implications in recent years in the main actions of daily life, as monitoring the metabolism helps to keep energy levels, sleep and body weight under control. The operation is very simple: by measuring the metabolites present in the body, problems are identified and action is taken in a targeted and relevant way. An action of extraordinary effectiveness if you think about the current situation, for example in the food sector; nutrition today is very rich in calories but poor in nutrients, with the real risk of being overfed, but undernourished (**Figure 1**).

It is essential to remember that this is not an alternative medicine, but is complementary to other disciplines: in the face of even important pathologies, such as neoplasms, it will support the oncologist, improving the responses to cancer treatment and helping to defeat the disease without interfering with the pathways of oncological treatment. A normal blood draw or a simple urine sample is sufficient to check the metabolites. An extraordinarily precise picture of the situation will emerge, a sort of fingerprint of our body and of how many external factors,

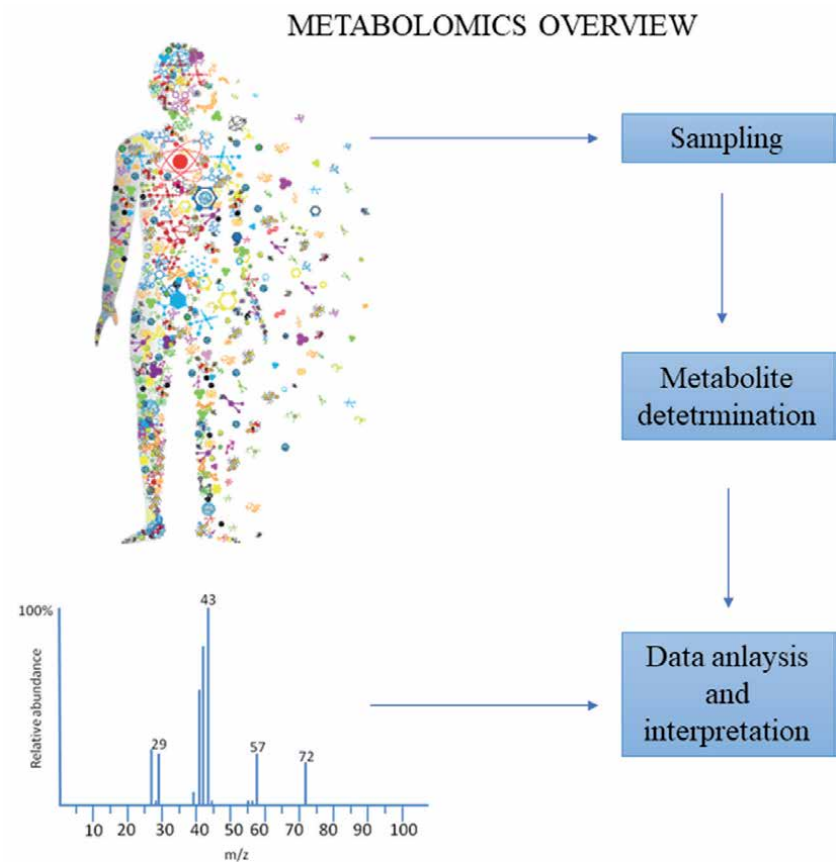

Figure 1.
Metabolomic workflow.

which we can define as part of the so-called epigenetics, can directly influence the chemical aspect. The ultimate goal is to bring the body back to perfect efficiency, taking into account that the body tends by itself, by its nature, to the best possible state of health. However, when the body has difficulty repairing the damage or is facing a progressive problem, which does not have time to fix or lacks adequate resources, metabolomics comes to its rescue. Our body must be considered like a car: to function at its best, the best fuel is needed together with winning strategies of suitable equipment. This discipline, which is progressively affirming itself, has set itself the task of identifying the optimal conditions to support the architrave of human existence.

2. Targeted and untargeted metabolomics

The metabolome is the final downstream product of the genome and consists of all low molecular weight molecules (metabolites) in a cell, tissue or organism [1, 2]. The metabolic profile can provide a complete picture of that cell's physiology. As emerging data suggest an important role for the microbiome and its metabolic products, the potential size of the metabolome is often highly controversial. Given the variety of chemical classes and physical properties that characterize metabolites and the dynamic range of metabolite concentrations over large orders of magnitude, a wide range of analytical techniques are required for metabolomics research. Metabolomics aims to identify and quantify multiple molecules in the context of physiological stimuli or in disease states. The "omics" revolution of the 1980s and 1990s provided new methodologies for the study of interactions on a global level and offered an alternative means of investigation to the more reductionist one in molecular biology. Omics is a field that aims to study the abundance and/or structural characterization of a wide range of molecules in organisms in distinct scenarios. In the clinical field, high-throughput omics techniques are used for disease characterization to better predict the clinical course of organisms and to evaluate the efficacy of existing or developing therapies [3]. In food science, for example, omics plays a significant role in trying to improve human nutrition [4]. On the other hand, concerning the environment, omics studies aim to evaluate the alterations that organisms could undergo after exposure to environmental stressors [5, 6]. In recent years, a variety of omics subdisciplines have emerged (eg Fluxomics, lipidomics, glycomics, foodomics, interactomics and metallomics), demonstrating that omics is a continuously evolving discipline and among all these platforms, metabolomics is becoming increasingly popular [7].

The first definition of metabolomics dates back to the 1990s, describing techniques aimed at identifying existing metabolites within a cell, tissue or organism during a genetic alteration or physiological stimulus [8, 9]. Metabolomics has been shown to be complementary to other omics techniques, thus identifying - called silent phenotypes - genes that when perturbed have no apparent influence on physical characteristics or behavior [10]. The metabolomic approach can be conducted in two distinct ways; non-targeted approach and targeted approach [11].

The reason for this differentiation is due to the different types of data generated in these two approaches, which must be handled accordingly (**Figure 2**). Targeted studies focus research on a number of known metabolites, while non-targeted studies allow for a more comprehensive evaluation of metabolomic profiles. Most of the methodologies used in the first targeted studies only allowed for the identification of a limited number of metabolites. However, recent targeted methodologies allow for the creation of large-scale metabolic profiles, including hundreds of compounds. However, the number of compounds analyzed in non-targeted studies is even greater. This is because entire datasets, including thousands of metabolic signals, need to be processed, and of these, few are finally identified as candidate biomarkers.

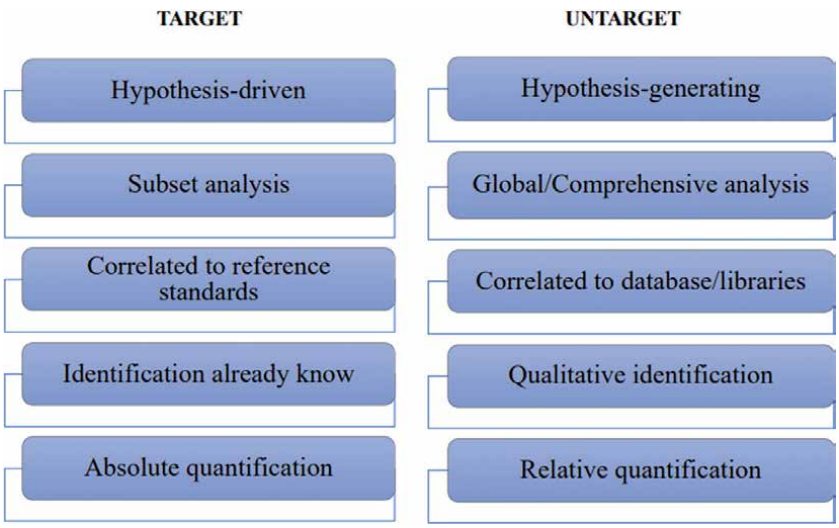

Figure 2.
Targeted and untargeted approach.

3. Untargeted approaches

Untargeted approaches provide the most correct path to detect unexpected changes in metabolite concentrations. The goal is to maximize the number of metabolites detected and thus provide the opportunity to observe unexpected changes. However, a single analytical method cannot detect all metabolites in a biological system. It is therefore necessary to combine multiple analytical approaches (such as complementary HPLC methods) to maximize the number of metabolites detected and improve metabolome coverage.

Sample preparation in non-targeted studies consists of extracting the metabolites from the biological sample in a suitable solvent for analytical analysis. The extracted sample is analyzed with an appropriate analytical method (for example, LC–MS). The result of the mass spectrometry analysis is a chromatogram and the peak area of each metabolite is used as a parameter in the statistical analysis to define the concentration differences between the different biological samples measured. This is called relative quantification as there is no comparison with calibration curves constructed from chemical standards. The use of calibration curves is indispensable for full quantification. The biological significance of each metabolite is determined during data analysis and metabolite identification, and biological interpretation is performed at the end of the experimental pipeline. Currently, one of the main limitations in non-targeted approaches is the identification of metabolites. It may not be possible to identify the metabolites highlighted in the statistical analysis as significant changes between biological classes in the study. The identification of metabolites is currently one of the hot topics of metabolomics.

4. Targeted approaches

Targeted studies investigate a relatively limited and specific number of metabolites. At the start of the study, before data acquisition is performed, the metabolites are chemically characterized and biochemically annotated. Targeted methods have greater selectivity and sensitivity than non-targeted methods. A targeted study

can only be performed if a genuine chemical standard of the metabolite is available. The quantification of metabolites is performed using internal and chemical standards to construct calibration curves for each of the metabolites under study. Sample preparation in targeted studies applies methods that can be optimized to retain metabolites of interest and to remove other biological species and analytical artifacts that are not performed via downstream analysis.

Therefore, data analysis strategies for non-targeted studies require very extensive chromatogram processing. A large number of data analysis strategies are found in the literature, but none of them can be considered the optimal choice in all cases, which makes data analysis an open task in bioinformatics research. In fact, the field of MS-based metabolomics is quite young and new methods, software and platforms are regularly published or updated.

The data are produced by chemical–physical investigation techniques such as magnetic resonance spectroscopy, chromatography and mass spectrometry applied to biofluid samples or suitably selected solid tissues. These methodologies find today numerous possibilities of use in the field of medical sciences where there are numerous variables detectable on human and animal subjects that present a specific pathology. This approach is valid both for the description of existing pathologies and for the identification of pre-pathological stages. The use of metabolomic methods can help provide an overall - holistic - view of the problem, highlighting the relationships between variables and their relative importance, and can also highlight differences and similarities between samples. Considering individual biological processes as isolated processes expresses a reductionist view of vital functions, an abstraction that at times makes it possible to considerably simplify the problem under consideration but which inevitably leads to models of limited value.

5. Metabolomics techniques

There is a range of analytical chemistry tools applied in metabolomics research.

Each instrument has advantages and limitations, and no single instrument or instrumental method can detect all of the metabolites present in a metabolome, but multiple instrumental methods or multiple different instruments are required to provide the largest number of metabolites detected.

Several analytical techniques have been developed for each of the omics platforms, including techniques based on DNA microarray and RNA sequencing [12], nuclear magnetic resonance (NMR) spectroscopy [13, 14] and mass spectrometry (MS) [15, 16]. NMR and MS are the most used in the field of metabolomics. High resolution proton NMR spectroscopy (1H-NMR) has proven to be one of the best technologies for examining biofluids and studying intact tissues, as the result is a complete signal profile of metabolites without separation, derivatization and preselected measurement parameters [17, 18]. On the other hand, MS methods, both by direct injection [19] and coupled with chromatographic techniques [20], have also evolved into an excellent technology for metabolomics due to their ability to analyze low molecular weight compounds in biological systems. These two approaches (NMR and MS) are complementary and the integration of both technologies can provide more comprehensive information in the field of metabolomics.

Applications of metabolomics have expanded in line with genomics, proteomics and transcriptomics with the aim of determining gene function in microbes [21], plants [22] and animals [23]. Many different applications are also used today, such as the determination of metabolic biomarkers that change as an indicator of the presence of a disease or in response to a pharmacological intervention or the determination of the effect of biochemical or environmental stress on plants or

microbes, genetically modified plants [24], bacterial characterization [25], health status assessments [26] and metabolic engineering [27].

5.1 Gas chromatography–mass spectrometry (GC–MS)

GC–MS is a combined system in which volatile and thermally stable compounds are first separated from the GC and then the eluted compounds are detected by electron impact mass spectrometers. During the run, aliquots of derivatized samples (injection volumes of 1 µl or less) are analyzed using split and splitless techniques on different polarity GC columns (DB-5 or DB-50 or similar capillary columns in stationary phase are most commonly used). These provide both high chromatographic resolution of compounds and high sensitivity (typical limits of detection are concentrations of pmol or nmol). Quantification is provided by external calibration or response ratio (metabolite peak area/internal standard peak area). The coverage of the metabolome is largely characterized by the volatility of the non-derivatized or derivatized sample components. The identification of metabolites is provided by matching the retention time and mass spectrum of the sample peak with those of a pure compound previously tested on the same or a different instrument under identical instrumental conditions [28]. Since the electron impact mass spectrometer provides the standard fragmentation of molecular ions during ionization, structural identification can be performed through the interpretation of fragment ions and fragmentation patterns.

5.2 Liquid chromatography-mass spectrometry (LC–MS)

LC–MS provides separation of metabolites by liquid chromatography followed by electrospray ionization (ESI) or, less typically, atmospheric pressure chemical ionization (APCI) [29]. This technique differs from GC–MS for several reasons (lower assay temperatures and unsolicited sample volatility) and this simplifies sample preparation. In most non-pharmaceutical applications, samples are prepared after intracellular extraction and/or protein precipitation by dilution in an appropriate solvent. The chemistry and HPLC column size used will affect chromatographic resolution and sensitivity. Analytical columns do not provide the chromatographic resolving power to separate these complex samples and run times of 10 minutes followed by chemometric instruments are used to extract the chromatographically unsolved data and classify the differences between the samples [30]. The application of very high-pressure chromatography systems can improve the chromatographic resolution. The most common column chemicals used today are reverse phase C 18 or C 8 columns. However, for polar metabolites injected on these columns, the retention of these metabolites is minimal, thus reducing the volume of interpretive data. To overcome this problem, other chemical columns are needed, such as HILIC [31] and other weak ion exchange chemicals. The sample then, once the chromatographic separation has been carried out, reaches the source of the mass spectrometer. Electrospray instrumentation operates in positive and negative ion modes (as separate experiments or by polarity switching during analyses) and detects only those metabolites that can be ionized by adding or removing a proton or adding another species of ions. Metabolites are generally detected in one but not both ion modalities, so broader metabolic coverage can be achieved by analysis in both modalities. Quantification is performed by external calibration. ESI does not cause molecular ion fragmentation as observed in electron impact mass spectrometers, thus it does not allow direct identification of metabolites by comparing ESI mass spectra, as ESI mass spectral libraries are not commonly available (as in the case by GC–MS). However, with the use of accurate mass measurements

and/or tandem MS (MS/MS) to provide collision induced dissociation (CID) and correlated mass spectra (MS/MS), correct identification of metabolites can be achieved [30].

5.3 Direct injection mass spectrometry (DIMS)

DIMS is shown as a high-throughput screening tool (hundreds of samples per day with a run time generally of 1 minute). The extracts of the crude sample are injected or infused into an electrospray mass spectrometer and results in a mass spectrum per sample, which is representative of the composition of the sample. The ionization capacity of the metabolite determines metabolic coverage, as for LC–MS. The applications of DIMS are mainly concentrated in the microbial [32] and plant areas.

5.4 Fourier transform infrared spectroscopy (FT-IR)

Vibrational spectroscopy techniques such as Fourier transform infrared spectroscopy (FT-IR) and Raman spectroscopy have been used to analyze metabolic changes in biological samples. The methodologies consist of passing ultraviolet or infrared light through a sample before it is detected. The vibrations and rotations of the bonds relating to different chemical groups resulting from the interaction of the sample with ultraviolet or infrared light are mainly measured.

The problem with these techniques is the inability to detect each metabolite separately; instead, it is a specific technique for single molecules that will absorb ultraviolet or infrared light at specific wavelengths. In FT-IR, a metabolic fingerprint is taken with a single absorption spectrum collected for each sample and consisting of information for many metabolites. The result is similar to data produced by direct infusion mass spectrometry where a single mass spectrum is collected rather than an absorption spectrum for each sample. The metabolic fingerprints produced in these approaches lack the sensitivity of mass spectrometry but are a useful tool for high throughput screening since the FT-IR analysis time is approximately one minute per sample. To date, the vast majority of metabolomic studies undertaken using vibrational spectroscopy have been performed with FT-IR spectroscopy. However, the work was done using Raman and, in terms of metabolomics, this is an emerging technology with significant potential for metabolite monitoring [33].

5.5 Nuclear magnetic resonance (NMR)

Nuclear magnetic resonance (NMR) spectroscopy applies the magnetic properties of atomic nuclei in a metabolite. Only some atoms are active NMRs and include 1H, 13C and 31P. Proton (1H) NMR spectroscopy is the most frequently applied in metabolomics. The technique works by inserting a liquid sample into a small internal diameter tube (about 5 mm), where it is pulsed with a range of radio frequencies that cover all possible energies required to excite the selected type of nuclei. Nuclei absorb energy at different radio frequencies depending on their chemical environment and then the release of this energy is measured, forming what is called a free induction decay (FID). This FID is converted from a time domain data set to a frequency domain - using a Fourier transformation - and an NMR spectrum is constructed as the absorption energy plotted against the peak intensity. The NMR spectrum (in particular the chemical shift) depends on the effect of the shielding by the electrons orbiting the nucleus. The chemical shift for 1 H NMR is determined as the difference (in ppm) between the resonance frequency of the observed proton and that of a reference proton present in a reference compound (for 1 H NMR experiments, tetramethyl-silane in solution, fixed at 0 ppm).

The intensity of the signal depends on the number of identical nuclei and the presence of complex samples does not interfere with the measured intensity, as does the suppression of ionization with electrospray ionization. NMR spectroscopy is a high-speed fingerprinting technique. Raw samples are mixed with a solution of the reference compound added to an NMR probe (usually less than 2 mL), inserted into the instrument and analyzed. NMR probes are generally based on a large volume of μl and this adds constraints to the required sample volume. However, the introduction of 1 mm μl probes allowed to analyze volumes of 2 μl, thus enabling invasive sampling of smaller volumes of study subjects, which is important for small animal studies [10]. Spectra are complex and contain thousands of metabolic signals. For data processing, the spectrum is generally divided into chemical shift ranges with widths of 0.02–0.04 ppm. All signals in this bucket are added together. Chemical changes can be assigned to specific metabolites and the pure metabolite can be added for further clarification. However, the spectrum model is generally used in sample classification, similar to that used for FT-IR and DIMS.

6. Examples of metabolomics studies

6.1 Urine metabolomics

The metabolic profile of biofluids has emerged as an important tool in the diagnosis of numerous diseases that remain silent until late progression of the disease [34]. Because urine is such a rich source of biomarkers, the metabolic profile is a promising tool for assessing therapeutic efficacy. Urine collection is also non-invasive, does not require patient preparation and substantially improves compliance. Recent results clearly demonstrate the potential of urine metabolomics in diagnosis by providing new insights into the biochemistry of its pathophysiology [35]. The qualitative/quantitative analysis on the urine of pregnant women between two different stages of labor called OL (out of labor) and IL-DP (in labor in the dilation phase), using as a technique the ultra-performance liquid chromatography of hydrophilic interaction coupled with mass spectrometry (HILIC-UPLC–MS), a highly sensitive, accurate and unbiased approach, are an example [36]. The list of metabolites is shown in **Table 1**. The urinary metabolites showing the greatest differences belong to the steroid hormone, in particular conjugated estrogens and amino acids, much of this difference being determined by fetal contribution. The increased excretion of conjugated estrogens in the DP stage may confirm the coordinated role played between fetus, mother and placenta during labor. It is reasonable to consider this terminal phase of pregnancy not only as a mechanical event linked to the increase in uterine contractions, but as a more complex process. **Figure 3** shows the major compounds excreted in the urine (such as Estradiol Glucoronide, Estrone 3-Sulphate and Estriol Glucuronate) which are downstream of a more intricate process. These compounds originate from an interconnected metabolism between mother-fetus and placenta involving steroid hormones. As seen in **Figure 3**, the metabolites excreted can come directly from the mother, or with the contribution of both the placenta and the fetus. In the latter case, the excretion of metabolites occurs through the degradation of intermediates, in particular the hormone pregnenolone.

6.2 Antarctic cryptoendolithic communities

Antarctic *cryptoendolite* communities are microbial ecosystems that dominate the biology of most ice-free areas in mainland Antarctica. These are complex and

Compounds	Molecular weight	Percentage
3 hydroxy2-methyl-1H-quinolin-one	175.05	3% - up
19 chloro19-Chloro-3beta-hydroxyandrost-5-en-17-one = dehydroepiandrosterone	365.1695	95% - up
Androst-5-ene-3beta,17beta-diol = androsterone	290.1736	21% - down
Androsterone	290.1736	21% - down
Dehydroepiandrosterone Sulfate	369.0992	92% - down
Dehydroepiandrosterone	288.1873	3% - down
Pregnanediol	321.2145	25% - down
3-Hydroxy-1-methylestra-1,3,5(10),6-tetraen-17-one	283.1551	63% - up
Tetrahydrocortisone	365.1592	88% - down
Estrone 3 sulfate	351.1085	>100% - up
Estrone gluconoride	447.1790	87% - down
Estradiol 17 beta 3 gluconoride	449.1945	42% - up
Ser	105.09	59% - down
Val	117.15	35% - up
His	155.15	23% - up
Arg	174.20	11% - up
Cys	121.16	47% - up
Ala	89.09	47% - down
Glu	147.13	85% - up
Gln	146.14	25% - up
Leu	131.17	38% - up
Lys	146.19	60% - up
Ile	131.17	38% - up
Thr	119.12	18% - up
Phe	165.19	11% - down
Tyr	181.19	12% - down

Table 1.
Estrogen and amino acid amount extracted from the urine in the two stages of pregnancy (OL and IL-DP). The table refers to the relative concentration of the metabolites calculating as a percentage of the total compounds by comparing the intensity of deconvolution of each compound.

self-supporting assemblies formed by the association of autotrophic and heterotrophic microorganisms such as Bacteria Chlorophyta and Fungi, which live at the limit of their physiological adaptability and this represent the only possibility of survival before extinction. They live inside the pores of the rocks creating an environment that protects them from environmental stress, they are extremely tolerant and remarkably resistant to stress and finally they adapt perfectly to the lithic life (**Figure 4A**).

The study was conducted on the basis of the different solar exposure, verifying how this factor influences the production of key metabolites and therefore on the adaptation strategies implemented by these microorganisms to survive in extreme conditions (**Figure 4B**). The result is the presence of 331 altered and differentially expressed metabolites [37]. All intermediates of melanogenesis are found in the highest concentration in south facing rocks (**Table 2**). Organisms have developed

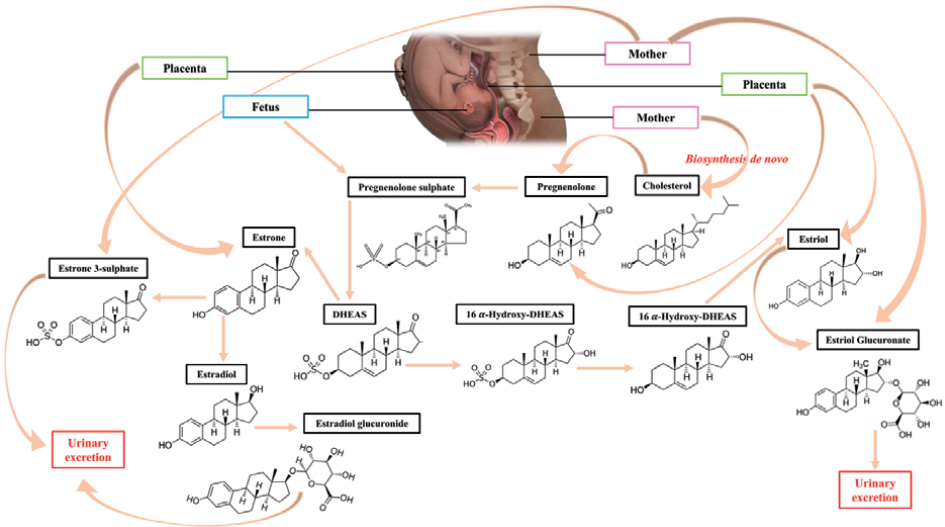

Figure 3.
A coordinated cycle between mother fetus and placenta through the biosynthesis of steroid hormones.

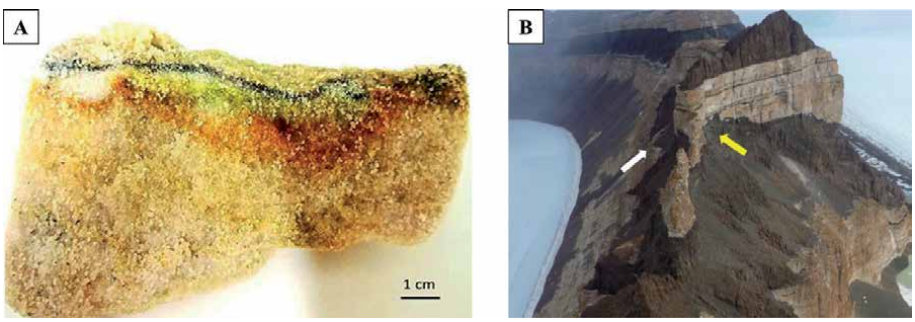

Figure 4.
*A - Communities stratified on rocks. B - Antarctic cryptoendolite communities. The yellow arrow indicates the north-facing surface, while the white arrow indicates the south-facing surface. (**Figure 4B** comes from Coleine et al. [37].*

Compound	Molecular weight	Regulation
Gentisyl alcohol	140.14	Down
Hypoxanthine	136.11	Down
6- Methoxymellein	208.21	Down
Allantoin	158.12	Down
DOPA	197.18	Up
5,6-Hydroxy indole	133.15	Up
Anthocyanin 3-O-β-D glucoside	449.38	Up
Plastoquinone	749.2	Up
Xanthine	152.11	Down
Uric acid	168.11	Down
Tyrosine	181.19	Up

Compound	Molecular weight	Regulation
Dopaquinone	195.17	Up
Cysteinyldopa	316.33	Down
Dopachrome	193.16	Up
5,6-Dihydroxyindole	149.15	Up
Indole 5,6- quinone	147.13	Up

Table 2.
Regulation of metabolites in Antarctic cryptoendolithic *communities exposed to the south in the absence of sun compared to the north in the presence of minimal solar radiation.*

metabolic profiles responding to the condition of deprivation of sunlight, in fact in fungi, melanin performs functions such as photoprotection, energy conversion, protection from thermal stress, metal chelation, resistance to drying and cell strengthening. Therefore, every organism that lives in non-optimal conditions has the ability to modify its biological functions and its structures according to external dynamics. The adaptation analysis allowed to demonstrate how the environment, and in particular the solar exposure, can strongly influence the olic phenotype of these microbial communities, demonstrating how the direct incidence of sunlight in one case, or their absence in the other, it has determined the acquisition of various adaptations by the colonizing species aimed at favoring their growth and guaranteeing their survival in such a hostile environment.

7. Conclusion

Starting from a holistic approach, with a high-throughput non-targeted metabolomics without prior knowledge of the metabolome, it is possible to find biologically relevant metabolites (potential biomarkers), characteristic of targeted metabolomics as shown by the two examples described in this chapter. In the case of urinary metabolites hundreds of metabolites are represented but the major differences between OL and IL-DP belong to the steroid hormone, in particular to conjugated estrogens and amino acids. These compounds could predict the time to delivery in pregnant women.

As for the Antarctic *cryptoendolytic* communities, among the candidates to be considered biomarkers, the precursor metabolites of the melanin and allantoin pathways emerged as these were the most affected by exposure to the sun. These paths can be considered directly involved in the response to environmental pressure. In conclusion, both targeted and non-targeted metabolomics are then used in combination for the detection and accurate weighting of differential metabolites. Furthermore, the types of metabolites that are recovered are influenced by the extraction and the analytical method chosen. In this chapter the main devices used have been summarized, subsequently the results will require computational tools to identify and correlate the metabolites among the samples to examine their connection in the metabolic pathways in relation to the phenotype.

Conflict of interest

The authors declare no conflict of interest.

Abbreviations

CID	Collision induced dissociation
DIMS	Direct Injection Mass Spectrometry
FID	Free induction decay
FT-IR	Fourier Transform Infrared Spectroscopy
GC–MS	Gas Chromatography–Mass Spectrometry
HILIC	Hydrophilic interaction chromatography
HPLC	High performance liquid chromatography
IL-DP	In labor in the dilation phase
LC–MS	Liquid Chromatography-Mass Spectrometry
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
OL	Out of labor

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Chapter 3

Pharmacometabolomics: A New Horizon in Personalized Medicine

Abdul-Hamid Emwas, Kacper Szczepski, Ryan T. McKay, Hiba Asfour, Chung-ke Chang, Joanna Lachowicz and Mariusz Jaremko

Abstract

Pharmacology is the predominant first-line treatment for most pathologies. However, various factors, such as genetics, gender, diet, and health status, significantly influence the efficacy of drugs in different patients, sometimes with fatal consequences. Personalized diagnosis substantially improves treatment efficacy but requires a more comprehensive process for health assessment. Pharmacometabolomics combines metabolomic, genomic, transcriptomic and proteomic approaches and therefore offers data that other analytical methods cannot provide. In this way, pharmacometabolomics more accurately guides medical professionals in predicting an individual's response to selected drugs. In this chapter, we discuss the potentials and the advantages of metabolomics approaches for designing innovative and personalized drug treatments.

Keywords: Personalized Medicine, Pharmacometabolomics, Metabolomics, NMR, metabolites

1. Introduction

Conventional drugs are developed as standard treatments for all patients diagnosed with particular diseases regardless of any differences between those patients. Consequently, this universal approach comes with a high degree of uncertainty regarding the treatment outcome. It is well-established that individuals can be differentially affected by the same disease due to factors such as general health status, genetics, gender, diet habits, smoking, alcoholic intake, *etc.* [1, 2]. The global COVID-19 pandemic has demonstrated clearly that a single disease can have different outcomes in different people, and the choice of therapeutic strategies needs to be calibrated to an individual rather than using a standard protocol for heterogenous populations. Indeed, the increasing incidence of treatment failure, especially with life threatening diseases such as cancer relapse, evidences a need for personalized drug regimens.

Each pathological state in humans affects multiple organs/systems and leads to the perturbation of metabolites and protein concentration levels. Thus, analysis of biomarkers (such as unique metabolites or proteins) is an effective way to monitor human health [3, 4]. Biomarkers can be used for disease prediction, diagnosis, and to screen the efficacy of treatment intervention. For example, the glucose level in blood is a biomarker of diabetes and can be used to monitor

drug efficacy [5–7]. **Table 1** summarizes the most prominent examples of protein biomarkers discovered recently.

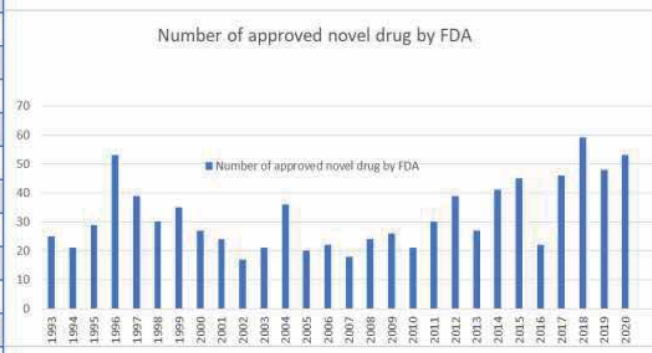
Among all '-omics' approaches, metabolomics is the most effective of qualifying and quantifying the perturbation of metabolite concentrations under external and internal factors. Thus, joining metabolomics with other '-omic' sciences (e.g. genomics) is essential for a comprehensive understanding of disease onset and pathogenesis, and provides a better diagnosis and treatment.

The total number of endogenous metabolites (although it is not completely determined yet) in human bio-fluid and tissues are lower than the total number of expressed proteins, giving metabolomics an extra advantage in monitoring disease pathology. Moreover, the perturbation of metabolite levels in human bio-fluids is usually greater than that of protein concentrations, providing an easy and clearer bio-marker role [18–20]. For instance, cancer leads to changes in affected cells, which cause an up-regulation in metabolite concentration levels during carcinogenesis [21]. For example, increased lactate levels have long been associated with different types of cancer [22]. Recently, the development of computational methods, such as bioinformatics and human metabolome databases establishing large scale bio-banks and computer programs, have facilitated the employment of metabolomics in stratified medicine. Pharmacometabolomics is a new subset of the metabolomics field aiming to predict the response of an individual to a drug or to develop optimized treatment strategies based on previous knowledge of subject

Protein biomarkers	**Useful for:**	**Ref**
Apolipoprotein H, ApoCI, Complement C3a, Transthyretin, ApoAI	Prediction of recurrence-free survival in women with estrogen receptor-negative tumors	[8]
S100 calcium-binding protein B, Neuron-specific enolase, Glial fibrillary acidic protein, Ubiquitin Carboxy-terminal hydrolase-L1, Tau, Neurofilament-light	Prediction of outcome and severity in traumatic brain injury patients	[9]
S100A9, ThioredoxiN, Cadherin-related family member 2	Diagnosis (presence) of cholangiocarcinoma	[10]
TFF1, ADAM (male only), BARD (female only)	Early diagnosis of gastric cancer	[11]
Acidic nuclear phosphoprotein 32 family member B, Thrombospondin-4, Cardiac muscle troponin T, Glucocorticoid-induced TNFR-related protein, NAD-dependent deacetylase sirtuin-2	Creating new utrophin modulation strategies that could help patients with Duchenne muscular dystrophy	[12]
C-reactive protein, S100A8, S100A9, S100A12	Prognosis of the severity of rheumatoid arthritis.	[13]
S100A4, S100A8, S100A9, Carbonic anhydrase I, Annexin V	Diagnosis of urinary bladder cancer and prognosis of patient outcome.	[14]
Gelsolin, Fibronectin, Angiotensinogen, Haptoglobin	Detection of lymph node metastasis of oral squamous cell carcinoma.	[15]
Neurotrophic factor, Angiotensinogen, Insulin-like growth factor binding protein 2, Osteopontin, Cathepsin D, Serum amyloid P component, Complement C4, Prealbumin (transthyretin)	Diagnosis of Alzheimer's disease in Han Chinese.	[16]
Alpha-2-macroglobulin, Chromogranin-A, Glutathione pertidase 3	Obtaining qualitative and quantitative assessments of radiation exposure.	[17]

Table 1.
Examples of biomarkers and their use in medicine.

Year	Number of approved novel drug by FDA
1993	25
1994	21
1995	29
1996	53
1997	39
1998	30
1999	35
2000	27
2001	24
2002	17
2003	21
2004	36
2005	20
2006	22
2007	18
2008	24
2009	26
2010	21
2011	30
2012	39
2013	27
2014	41
2015	45
2016	22
2017	46
2018	59
2019	48
2020	53



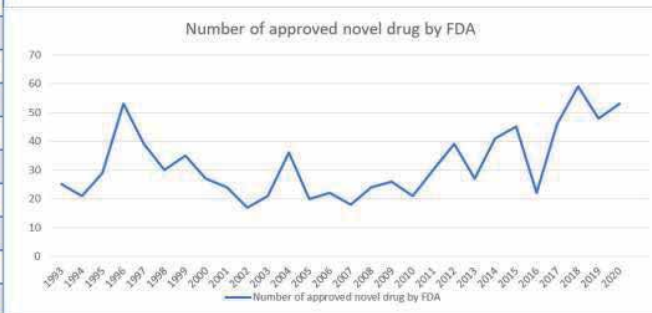


Figure 1.
Number of novel drugs approved annually by the FDA between 1993 and 2020 with graphical representation.

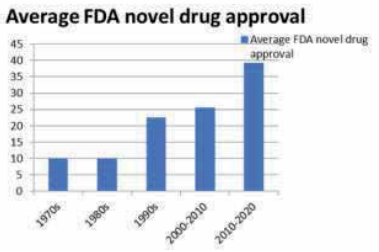
metabolomics information (individual's metabolic profile). One should keep in mind that aerosolized treatment would never lead to the discovery of a novel drug for each individual subject. Indeed, the number of new drugs is almost constant in the last decades (**Figure 1**).

In this chapter, we briefly introduce metabolomics along with common metabolomics analytical platforms regarding the development of a personalized medicine approach and factors that will empower advances in personalized medicine.

2. FDA approved drugs since 1975

Over the past few decades, pharmaceutical product intervention has improved significantly resulting in more saved lives and enhanced public health. The annual number of newly approved drugs applicable for human use has varied greatly over the years (**Figure 1**). The Food and Drug Administration (FDA) is an agency within

Years	Average FDA novel drug approval per year
1970s	10
1980s	10
1990s	20-25
2000-2010	25.6
2010-2020	39.2



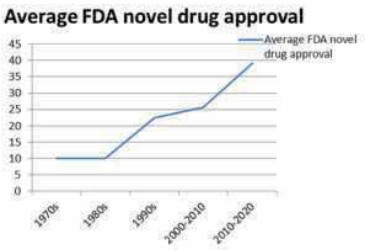


Figure 2.
Average numbers of novel drug approvals by the FDA over the last five decades with graphical representation.

the United States. One of its primary responsibilities is the approval of human pharmaceutical products based on safety and efficacy. Regulating and managing the human pharmaceutical industry and the approval of new drugs is the responsibility of the Center for Drug Evaluation and Research (CDER) [23].

The FDA catalog contains most of the approved drug products since 1939. However, since 1998 a complete human drug database is available, known as the Orange Book, which includes patient information, drug labels, and drug reviews. The Orange Book is considered a comprehensive detailed list of all pharmaceutical products approved in the U.S. by the FDA. However, studying the number of pharmaceutical products approved annually is not straightforward. First, the number of approved human drugs was not accurate before 1981, as the Orange Book did not report pharmaceutical drug approval data until after 1981, including new molecular entities (NMWE), the pharmaceutically active ingredient, drug dosage form, combination, formulation, and indication [24]. In addition, the Orange Book excludes any withdrawn drug or 'no-longer marketed' pharmaceutical products due to either drug efficacy concerns or safety concerns. Below, the reader can find **Figure 1** summarizing the number of FDA-approved novel human drugs per year from 1993 to 2020 [24, 25].

As is apparent from **Figure 1**, the year 2020 represents the second-highest number of FDA-approved novel human drugs over the past twenty years (53 drugs), while 2018 was the year when the highest number of drugs were approved by the FDA (57 drugs). In 2017, only 46 drugs were approved [26].

The average rate of new drug approvals by the FDA has increased over the years (**Figure 2**). Before 1950, the average annual drug approval was less than four, while the average annual drug approval in the 1960s and 1970s was 10. However, in the 1980s the average approval rate increased to more than twenty per year. It has continued to increase to reach more than twenty-five approvals per year from 2000 to 2010. Over the last several years there have been further increases, reaching an average of more than 39 approved compounds per year from 2010 till 2020 [26, 27]. The average novel drug approval by the FDA over the decades is listed in **Figure 2**.

3. Metabolomics

Metabolomics is defined as "the measurement of metabolite concentrations and fluxes and secretion in cells and tissues in which there is a direct connection between the genetic activity, protein activity, and the metabolic activity itself" [28]. It is a

relatively new field and is employed in a wide range of applications that monitor biological systems [3, 29, 30]. Integrating metabolomics with other '-omics', including proteomics, transcriptomics, and genomics, provides an exhaustive description of the biological system under study. Metabolomics provides a snapshot of the metabolite dynamics, and is a powerful tool when investigating numerous perturbations in biological systems, including pathophysiological events, environmental stimuli, and genetic modifications [31–34]. Moreover, metabolomics investigates every perturbation in metabolite compositions and/or concentrations, and it has already been applied in different fields such as biomedicine, environmental science, nutrition and diet studies, microbiology, and drug toxicology, as well as marine and plant sciences [35–39].

Metabolomics is usually classified into two main categories: (1) untargeted, and (2) targeted. Untargeted metabolomics is focused on the entire pool of "detectable" metabolites in a biological sample and makes no assumptions about metabolite(s) or class of metabolites, nor their concentrations. Untargeted metabolomics relies on fingerprinting approaches, where a group or different classes of samples (e.g., healthy control vs. pathological samples) are compared, and where absolute metabolite quantifications are not necessary. In contrast, targeted metabolomics focuses both on the identification and quantification of a specific number of metabolites. Targeted metabolomics approaches are relevant for drug development, where the drug mechanism (including drug absorption and drug distribution) needs to be precisely monitored.

The choice of proper analytical technique(s) in metabolomics is the crucial step, and particularly targeted metabolomics requires accurate metabolite quantification. Metabolomics applies different analytical techniques, including mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy, Fourier transformed infrared (FT-IR) spectroscopy, and high-performance liquid chromatography (HPLC). Among them, NMR spectroscopy and MS spectrometry are the most common and powerful analytical tools [40, 41].

3.1 Analytical techniques in metabolomics

Similar to other '-omics' disciplines, metabolomics uses different analytical platforms, separately or in combination (two or more techniques) [32, 42]. Although several analytical platforms are employed in metabolomics studies, including FT-IR spectroscopy [43–45], HPLC [46, 47], NMR spectroscopy [48–53], and MS [54–57] combined with gas or liquid chromatography [58–62], MS and NMR are the most common approaches [3, 50, 63–65]. There is no single optimum analytical technique that can elucidate all metabolites equally. Each analytical method has its advantages and limitations. For example, NMR is a non-destructive and highly reproducible technique where metabolic pathways or metabolic flux can be studied by using isotopic nuclei (such as ^{13}C and ^{15}N NMR), thus monitoring the flow of compounds through metabolic pathways [66–69].

Nevertheless, it has two main drawbacks that must be kept in mind: inherently low sensitivity and potential signal overlap. Different technical approaches have been developed to overcome these two drawbacks, contributing to the development of new and more efficient NMR techniques. For example, dynamic nuclear polarization (DNP) can be used to increase the NMR signal enhancement [70, 71], and the use of cryoprobes and the introduction of ultra-high magnetic fields (e.g., 1GHz) helps to overcome the low sensitivity limitation [72, 73]. The peak overlap problem can be minimized by the use of the highest magnetic fields and multi-dimensional NMR methods such as HSQC, TOCSY, COSY, and HMBC techniques [66, 74–78].

As stated, no singular analytical technique can perform a complete quantification and identification of all the metabolites in one analysis. Therefore, in addition to one and two-dimensional NMR experiments, different complementary techniques are required, such as liquid chromatography-mass spectrometry (LC–MS) and gas chromatography–mass spectrometry (GC–MS), which help to maximize the number of identified and quantified metabolites [32, 65, 79, 80].

For instance, the human urine metabolome was analyzed by Wishart *et al.* with several different analytical tools (ICP-MS, NMR, GC–MS, DFI/LC–MS/MS, HPLC) to facilitate the detection of the highest possible number of human urine metabolites. Among all metabolites, 209 were identified by NMR, 179 by GC–MS, 127 by DFI/LC–MS/MS, 40 by ICP-MS, and 10 by HPLC [81].

Based on the ability to separate and detect a wide range of metabolites, LC–MS is one of the most widely used tools for carrying out metabolite profiling studies [82–86]. LC–MS combines HPLC and mass spectrometry, and provides a powerful analytical tool for the separation, identification, and quantification of metabolites in a studied sample [65, 87–90]. HPLC separates molecules based on different physical and chemical properties such as charge, polarity, molecular size, and affinity towards column matrices [91–94]. Thus, different successful chromatography methods have been developed, such as reversed-phase (RP) gradient chromatography [85, 86, 95, 96]. To obtain the best separation, and presumably the highest number of detected metabolites, each sample can be analyzed twice using RP and normal phase chromatography. Moreover, the column switching approach of 2-dimensional analysis in an "orthogonal" combination of hydrophilic interaction liquid chromatography (HILIC) and RP-L, in conjunction with utilizing different electro spray ionization (ESI) modes can also be used [85, 86, 97–99]. In addition to using different separation methods and/or ionization methods, LC–MS is inherently far more sensitive than NMR and enables researchers to detect secondary metabolites at lower concentrations [100, 101]. The drawback occurs with the consistency of the separation performance. For example, columns can degrade non-linearly over time, requiring constant monitoring, determination of effect(s), and compensation in the final analysis. Solvent purity, pump performance, and injector consistency can all come into play. The inclusion of quality control samples at the beginning, end, and randomly inserted into the experimental samples should allow the compensation and quality control of any introduced confounder(s), but adds material costs, extends batch run times, and introduces complexity to the analyses.

3.2 Development of '-omics' in personalized medicine approach

Over the last decades, various fields of bioresearch (genetics, genomics, proteomics, and metabolomics) have quickly evolved and revealed mechanisms of diseases, and most importantly delivered new therapeutic outcomes. Although the current tenet regarding the uniformity of the drug response seems to be widely accepted, it does not take into account the individual differences. Individuals may not respond in the same way to the pharmacological treatments or present minor and serious side effects. For example, antidepressants [102], statins [103, 104], or antipsychotic drugs [105] have been shown to have reduced effects on some individuals, even to the extent that only a quarter of patients can achieve a functional remission of the disease [105]. Pharmaceutical treatments are ineffective for 30 to 60% of patients [106]. Moreover, a significant number of patients may develop adverse drug reactions (ADR) related to their treatment, with the incidence of fatal ADR being 0.32% [107]. In order to minimize the negative effects of pharmaceutical treatments, and at the same time optimize the drug therapy in terms of its

efficiency, a more personalized approach has been proposed, which assesses various factors prior to the treatment through the application of the different '-omics' [108].

This approach is not entirely new, as some characteristics (age, weight, co-morbidity, family history, and biochemical parameters) are already commonly considered. However, technological progress allows us to analyze individuals in more detail – from different genes, and single-nucleotide polymorphism (SNPs) genomics, to small, biologically active molecules (proteomics, metabolomics) and even the metabolic pathways of individuals (metabolomics, fluxomics) [109, 110]. In addition, personalized medicine not only takes into account the physiological status of a person's body - it also considers the unique, psychosocial situation of the individual, which may have a direct effect when a given health condition manifests in that individual and how he/she will respond to treatment [111]. Although these aspects are taken into consideration for a more complete picture of a person's medical status, separate approaches could also be used to focus on precise problems. For example, a fairly new field called pharmacogenomics tries to assess and validate the impact of human genetic variation on drug responses [112, 113]. Currently, we know that inherited variations in approximately 20 genes can affect around 80 medications and the way the body responds to them [114]. Another young field that has become a prominent branch of metabolomics is pharmacometabolomics, which is the subject of this review.

Personalized medicine has already shown its value in therapies to combat diabetes and cancer [115–119]. For example, the management of blood glucose in diabetes requires proper medication, for which the dosage and efficiency is suited to the individual patient. The efficacy of one of the drugs used in type 2 diabetes, metformin, has been associated with polymorphisms in several genes, specifically solute carrier family (SLC) 22 (an organic cation transporter) member 1 (*SLC22A1*), *SLC22A2*, *SLC47A1*, organic cation transporter 1 and 2 (*OCT1* and *OCT2*), and the gene encoding for multidrug and toxin extrusion 1 protein [MATE1] [115, 120]. Sulfonylureas which are another class of drugs used to treat type 2 diabetes, have been shown to have a variable response effect depending on the genomic profile of the patient, *e.g.*, the variant 'TT' at rs12255372 in the *TCF7L2* gene results in a weaker response compared to the 'GG' version [116, 121]. Those genetic factors are usually not considered when therapy is administered, despite the fact that the information they provide can have direct and substantial effects on therapy optimization and the success of treatment.

Similar benefits from personalized medicine have been observed in the treatment of various types of cancer. One of the best examples that highlights recent progress is breast cancer. Based on the biomarkers present in tumors, such as the estrogen receptor, progesterone receptor, antigen Ki-67, human epidermal receptor 2 [122], and mutations in genes such as Breast cancer gene 1 and 2 (*BRCA1*, *BRCA2*), which are related to carcinogenesis [123], breast cancer can be divided into different subgroups [122]. Each of the cancer types has its own characteristics and requires a specific, more personalized approach to maximize treatment efficacy while minimizing the adverse effects [122, 124]. The decision regarding which therapy to choose becomes even more complicated when we also consider the genetic profile of an individual (the susceptibility to the treatment) [118, 122, 125]. For example, different variants of CYP2D6 (cytochrome P450, family 2, subfamily D, polypeptide 6), which interacts with tamoxifen (a standard drug used in steroid receptor positive breast cancer) have been shown to have direct impacts on the treatment. The impaired version of the protein could also be associated with the recurrence of breast cancer [118, 122]. On the other hand, a personalized approach could also be used in a preventive way. As an example, genetic testing with a focus on the identification of potential, carcinogenic mutations in the *BRCA1* and *BRCA2*

genes could be used to create a proactive strategy (MRI, chemoprevention, bilateral mastectomy), thus significantly decreasing the chances of developing a more severe disease [126].

3.3 Metabolomics databases

The demand for functional and inclusive metabolomics databases is driven by the need for fast data analysis including metabolite identification, quantification, and subsequent interpretation of complex metabolite data, and possibly from multiple instrument sources. As a result of collective efforts in this area, several different databases have been established, including the Human Metabolome Database (HMDB) (https://hmdb.ca/) [127, 128], Platform for RIKEN Metabolomics (PRIMe) [129], Biological Magnetic Resonance Data Bank (BMRB) [130], and the Madison Metabolomics Consortium Database (MMCD) [131]. The existing information on the human urine metabolome was published recently with detailed information on each reported metabolite, including concentration perturbation at normal and disease-related levels (http://www.urinemetabolome.ca). The human urine metabolome along with the human serum metabolome represent a significant development and resource for researchers, which may be critical when employing metabolomics approaches in clinical applications including stratified medicine. Furthermore, the human metabolome database serves as a cross-referencing and benchmarking tool for general metabolomics studies, including metabolite identification, quantification, and newly discovered disease biomarkers. The Madison-Qingdao Metabolomics Consortium Database (http://mmcd.nmrfam.wisc.edu/) contains information on more than 20,000 compounds, including NMR and MS data that are valuable in the identification and quantification of metabolites present in biological samples [131]. Among different freely available metabolomic databases, the HMDB (www.hmdb.ca) [128, 132] (University of Alberta, Canada, David Wishart group) is becoming the de-facto standard reference for the metabolomics community. The HMDB contains information on 74,462 metabolite entries gathered and summarized from literature-derived data and also contains an extensive collection of experimental metabolite concentration information compiled from hundreds of MS and NMR metabolomics analyses performed on urine, blood, and cerebrospinal fluid samples. The data entries encompass a wide range of information, including structural, chemical, clinical, and biological information for many of the reported metabolites.

In 2012, the MetaboLights database (http://www.ebi.ac.uk/metabolights) [133] was established for the online storage of metabolomic experiments, associated raw data, and metadata, to interrogate databases of collected experimental information in publications. This database was first developed and maintained by the European Bioinformatics Institute [134], and later it has been endorsed and developed by the COSMOS consortium [135]. The continuous development of metabolomics databases alongside the uninterrupted advancements in software and supercomputer capabilities may lead to better clinical practices, including diagnosis, disease prognosis, and, ultimately, effective personalized treatments.

3.4 Biobanks and their impact on personalized medicine studies

Over the past decade, several high-capacity biobanks have been established to serve as baseline research and clinical studies tools in use by scientific institutions, clinics, private companies, and regulators at both national and international levels, encompassing a high number (*i.e.*, millions) of samples necessary for medical research. Furthermore, the standardization of sample collection and storage

conditions will help reduce sample collection bias and overcome the limitations afforded by variations between different studies, protocols, and practices. Biobanks usually also collect relevant data, such as whole-genome, genotype, geographic location, dietary preference(s), proteomic, and medical image information [136–138]. In addition to national registries, the incorporation of existing electronic health records (EHRs) is becoming more common, making large biobank datasets more applicable for a greater number of users [139, 140]. The availability of this additional information, combined with the collection of multiple samples over longer periods from the same individual, facilitates improved interpretation of experimental data and provides controls for possible confounders. Establishing large scale national and international biobanks therefore is an essential step and a valuable resource for clinical practitioners and in the development of public health policies, in addition to being crucial for the development of personalized treatments. These megabanks have the capacity to store samples from the same person over the course of many years, which in the future may be collected from childhood and followed up with the periodic collection of new samples throughout life [141].

As biobanks represent a major resource in large-scale global studies, we believe that the impact of metabolomics approaches will become ever more important in future medical research and public health efforts, including personalized health care and stratified medicine.

3.5 Pharmacometabolomics

As mentioned, pharmacometabolomics is a fairly new addition to the '-omics" family of studies. One of the pioneering works that helped create this novel field of science was carried out in 2006 by Clayton *et al.* on paracetamol [142]. Their main goal was to check if the metabolite profile of an animal, prior to the administration of a drug, would allow for the prediction of the metabolism of a drug as well as its toxic effects on an animal. For this purpose, the team collected urine samples from 65 rats, both before and after the administration of paracetamol. Later, samples were analyzed by ^{1}H 1D NOESY NMR spectroscopy. After analyzing the spectra, researchers identified four paracetamol-related metabolites, specifically paracetamol sulphate, paracetamol glucuronide, mercapturic acid derived from paracetamol, and paracetamol. Compared to the histopathological results obtained from the same rats, a substantial model for predicting post-dose histology of the liver could not be established, but they discovered a relationship between the pre-dose metabolic profile of urine and the histological outcome. The main factors predicting that relationship were identified as taurine, trimethylamine-N-oxide (TMAO), and betaine, where higher pre-dose levels of taurine indicated less damage to the liver while higher levels of TMAO and betaine were associated with greater damage [142]. This pioneering work paved the way for the establishment of organizations focused on pharmacometabolomics, such as the Pharmacometabolomics Research Network (PMRN), where the main aim is to "integrate the rapidly evolving science of metabolomics with molecular pharmacology and pharmacogenomics" [143]. So far, PMRN has produced numerous publications, many of them pertaining to lifestyle disorders and diseases. One example concerns the lipidomic response to treatment with simvastatin [144]. The authors of this paper identified metabolites that could predict the outcome of treatment with simvastatin – phosphatidylcholine, including 18 carbon fatty acids with two double bonds at the n6 position, cholesterol esters with 18 carbon fatty acids with one double bond at the n7 position, and 18 carbon-free fatty acids with three double bonds at the n3 position [144]. Additionally, the authors discovered a group of metabolites that may help to predict the changes of C-reactive protein (CRP)

after the treatment - five of them were plasmalogens (a specific group of glycerophospholipids containing a vinyl ether moiety at the sn-1-position of the glycerol backbone) [145], and the others were phosphatidylcholines and cholesterol esters [144]. Another interesting study worth mentioning is related to changes in lipids levels in schizophrenia and upon treatment with antipsychotics [146]. The authors measured the changes in the lipid profiles of patients before and after treatment with olanzapine, risperidone, and aripiprazole. They discovered that treatment

Type of Biomarker	Definition	Example
Diagnostic Biomarker	Biomarker used to detect or confirm the presence of disease or to identify individuals with a subtype of the disease.	Sweat chloride can be used to confirm cystic fibrosis [148].
Monitoring Biomarker	Biomarker measured constantly to assess the status of the disease or for evidence of exposure to (or effect of) a medical product or an environmental agent.	HIV-RNA can be used as a monitoring biomarker to measure and guide treatment with antiretroviral therapy (ART) [149].
Pharmacodynamic/Response Biomarker	Biomarker used to show a biological response of an individual exposed to a medical product or an environmental agent.	Serum LDL cholesterol can be used for evaluating response to a lipid-lowering agent in patients with hypercholesterolemia [150].
Predictive Biomarker	Biomarker used to identify individuals who will experience positive or negative outcome from exposure to a medical product or an environmental agent.	Mutations in BRCA 1/2 genes can be used to identify women with platinum-sensitive ovarian cancer that will most likely respond to PARP inhibitors [151].
Prognostic Biomarker	Biomarker used to identify the likelihood of a clinical event such as disease recurrence or progression.	Mutations in BRCA 1/2 genes can be used to evaluate the likelihood of a future second breast cancer in patients currently diagnosed with one [152].
Safety Biomarker	Biomarker used for indicating the likelihood or presence of a toxic effect, measured before or after the exposure to a medical product or an environmental agent.	Hepatic aminotransferases and bilirubin can be used to evaluate potential hepatotoxicity [153]
Susceptibility/Risk Biomarker	Biomarker used for the estimation of a chance of disease or other medical condition in an individual who currently does not have clinically apparent disease or condition.	Mutation in BRCA 1/2 genes can be used to identify individuals with a predisposition to develop breast cancer [154].

Table 2.
Types of biomarkers with examples of practical applications. Based on the BEST (Biomarkers, EndpointS, and other Tools) resource by the FDA-NIH Biomarker Working Group [147].

with olanzapine and risperidone increased the levels of 50 lipids, raised the concentration of triacylglycerols and generally decreased free fatty acids. Moreover, the concentration of phosphatidylethanolamine that is suppressed in patients with schizophrenia was raised by all three drugs [146].

Presently, most of the pharmacometabolomics studies focus on identifying specific biomarkers related to administered medications. Those biomarkers can provide information ranging from predicting patient treatment response, to monitoring the changes during the treatment, or evaluating the end effects of treatment (i.e., if the patient responded positively or negatively to the therapy) (see **Table 2** and **Figure 3**). Examples of pharmacometabolomic studies are shown in **Table 3**.

The successful isolation of a metabolite that may become a biomarker depends on the type of sample and the approach. In addition to easily and commonly accessed samples like urine and blood serum, pharmacometabolomics studies can also utilize feces, saliva, human breast milk, and even breath [175–177]. Samples are usually collected before, during, and after the treatment, and can be further divided by type of response from an individual (*e.g.*, mostly positive, mostly negative, or intermediate) [175, 178]. After obtaining data from a set of samples using various techniques adapted to the particular type [36, 175, 178], a database for each individual is created, with metabolites detected and identified before and after the treatment [178]. Lastly, a statistical analysis is applied to obtain information ranging from differences that can distinguish good and poor responders prior to the treatment, to changes in metabolites due to drug application that can be correlated with response phenotypes and assumptions of pathways connected to variants of response [178].

For example, Wikoff and colleagues [179] investigated atenolol-induced changes in Caucasians and African Americans. Atenolol is a beta-adrenergic receptor blocker used in a first line antihypertensive treatment. However, various patients responded quite differently. The main objective of this study was to obtain metabolic signatures of atenolol treatment that provided insight into racial differences in response to beta blockers. They found that atenolol has a strong impact on fatty acids in blood serum, but the results were different for different groups (*e.g.*, effects of treatment were highly significant in Caucasians but minimal in African Americans). Furthermore, the authors examined associations between oleic acid and SNPs on the 16 genes encoding lipases. They discovered that a SNP in the *LIPC* (rs9652472) and *PLA2G4C* (rs7250148) genes were associated with the change in oleic acid concentration in Caucasians and African Americans, respectively [179]. Another example of utilizing a combined approach

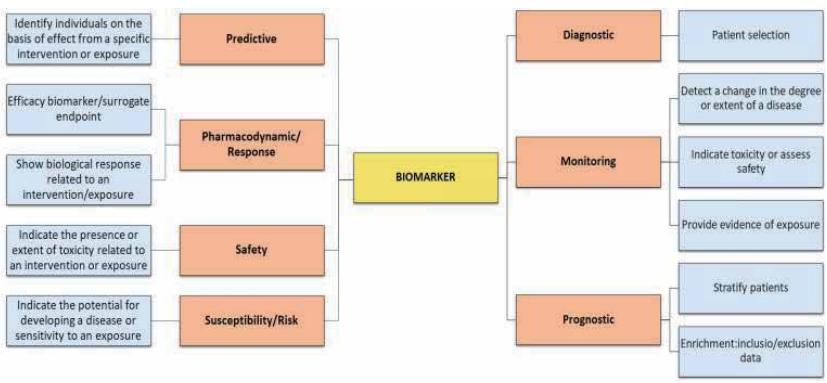


Figure 3.
A brief description of biomarkers of specific use in the drug development process. Based on "Context of use (COU) for a biomarker" by U.S. Food and Drug Administration [155].

Chemical Compound	Goal of the study	Main changes in metabolites post exposure	Conclusions	Ref
Aspirin	To investigate the mechanism of aspirin resistance.	↑ Inosine, adenosine, guanosine ↓ Hypoxanthine, xanthine	• Higher levels of adenosine and inosine were observed in the group categorized as "poor responders". • A pharmacogenomics approach pinpointed an SNP in the adenosine kinase (ADK) intronic variant - rs16931294, where the G allele of this variant was associated with poor response to the treatment.	[156]
	To define pathways implicated in variation of response to treatment with a focus on metabolites containing an amine functional group.	↑ O-Phosphoethanolamine, serotonin ↓ Glycylglycine, L-aspartic acid, L-glutamic acid, L-leucine, L-phenylalanine, L-serine, ethanolamine, glycine, ornithine, taurine, L-asparagine, L-valine, beta-alanine, L-lysine, L-histidine, L-tyrosine, L-glutamine	• The changes in metabolite profiles of healthy individuals treated with low dosage of aspirin cannot be directly attributed to COX-1 inhibition. • Increased levels of serotonin correlated with higher post-aspirin platelet reactivity.	[157]
	To investigate: • The effects of low-dose aspirin therapy on the oxylipid metabolic pathways, • the sex differences in aspirin-induced oxylipid changes, and • potential association of oxylipid on aspirin-induced inhibition of platelet aggregation.	↑ 13,14-dihydroPGF2 ↓ TXB2, 12-HHTrE, 11-HETE, 5-HETE, 12-HETE, 8-HETE, 15-HETE, 9-HODE, 13-HODE, 5-HETrE, 5-HEPE, 12-HEPE, 15-HEPE, 9-HOTrE, EpOMEs, DiHOMEs, DiHETrEs, 20-HETE.	• Aspirin does not show any sex-specific effects on oxylipid levels. • Aspirin decreases almost all of the oxylipids measured in the samples. • Several LA-derived oxylipid (3-HODE, 9-HODE, 12,13-DiHOME, and 12,13-EpOME) metabolites might contribute to the variability of non-COX1-mediated response to aspirin.	[158]
	To assess the metabolic pathways affected by aspirin administration that are potentially involved in cardiovascular and antitumoral protection.	↑ 3-methylglutarylcarnitine ↓ L-histidine, hydantoin-5-propionate, 4-imidazolone-5-propanoate, N-formimino-L-glutamate, xanthosine, L-glutamine, 5-aminoimidazole-4-carboxamide-1-β-D ribofuranoside, butyryl-L-carnitine, tiglylcarnitine, isovalerylcarnitine, heptanoylcarnitine,	• Aspirin decreases the levels of glutamine and metabolites involved in histidine and purine metabolism. • The ability of aspirin to increase the β-oxidation of fatty acids and decrease glutamine levels implicates reduced synthesis of acetyl-Co-A that could help explain aspirins potential anticancer effects.	[159]

Chemical Compound	Goal of the study	Main changes in metabolites post exposure	Conclusions	Ref
Aspirin eugenol ester (AEE)	To evaluate the protective effect of AEE on paraquat-induced acute liver injury (ALI) in rats.	↑ L-histidine, D-asparagine, L-phenylalanine, pipecolic acid, acetylglycine, N-(2-methylpropyl)acetamide, inosine, xanthosine, melatonin radical, ophthalmic acid, glutamylarginine, S-(PGJ2)-glutathione, L-octanoylcarnitine, lysoPC(P^{-16}:0), argininic acid, N-undecanoylglycine, chenodeoxyglycocholic acid, ↓ Glycerophosphocholine, hypoxanthine, nonyl isovalerate, glutamylleucine, pipecolic acid, deoxycholic acid glycine conjugate, dephospho-CoA, taurochenodesoxycholic acid, lysoPC(14:1), PA(22:2), cholic acid, 5,9,11-trihydroxyprosta-6E,14Z-dien-1-oate, lysoPE(18:2), lysoPE(20:4), lysoPE(16:0)	• AEE shows protective effects against PQ-induced ALI. • The mechanisms in which aspirin eugenol ester protects against the effects on PQ-induced ALI are correlated with antioxidants that regulate amino acid, phospholipid, and energy metabolism metabolic pathway disorders and attenuate liver mitochondria apoptosis.	[160]
	To identify the different proteins and small molecules in plasma to explore the mechanism of action of AEE against thrombosis.	↑ Oleamide, palmitic amide, linoleic acid, L-acetylcarnitine, creatine, proline betaine, arachidonic acid ↓ L-carnitine, L-methionine, L-proline, L-pipecolic acid, allantoin, palmitic acid, citric acid, L-tryptophan*	• Metabolomics results suggested that the therapeutic mechanism of action of AEE (as well as for aspirin and eugenol) could be involved with energy metabolism, amino acid metabolism, and fatty acid metabolism. • A total number of 38 (AEE), 41 (aspirin) and 54 (eugenol) proteins were differentially regulated in rats treated with those compounds.	[161]
Busulfan	To investigate biomarkers for predicting busulfan optimal dosage.	↑ Deferoxamine-derived metabolites ↓ Carnitine C9:1, carnitine C12:1-OH, phenylacetylglutamine**	• Busulfan metabolism is decreased in patients with high ferritin levels and reduced liver function.	[162]
Gemcitabine	To investigate potential predictive biomarkers for the efficacy of gemcitabine-based chemotherapy while obtaining the most optimal therapeutic results in patients with pancreatic cancer.	A total number of 38 and 26 different metabolites were identified between the gemcitabine resistant and gemcitabine sensitive pancreatic carcinomas from whom four of them: 3-hydroxyadipic acid, D-galactose, lysophosphatidylcholine (LysoPC) (P-16:0) and tetradecenoyl-L-carnitine, were significantly different between the carcinoma types.	• 3-hydroxyadipic acid, D-galactose, lysophosphatidylcholine (LysoPC) (P-16:0) and tetradecenoyl-L-carnitine could be used as biomarkers for evaluating the efficacy of chemotherapy in pancreatic carcinoma.	[163]

Chemical Compound	Goal of the study	Main changes in metabolites post exposure	Conclusions	Ref
Isoniazid (INH), Rifampicin (RIF), Pyrazinamide (PZA), Ethambutol (EMB) - DOTS treatment program	To identify metabolites that describe the changes related to tuberculosis therapy	↑ Dodecyl acrylate, pyrazinamide, 1,6-hexylene glycol, ribitol, 1-decene, 2,4-dimethylbenzaldehyde, 2,6-dimethylnonane, 3,4-dihydroxybutyric acid, 5-hydroxyindoleacetic acid, alfa-isosaccharinic 1,4-lactone, beta-Isosaccharinic 1,4-lactone, decane, fumaric acid, hippuric acid, N-formylglycine, sebacic acid, threonic acid, undecane, urea, 3-ethyl-4-methyl-1Hpyrrole-2,5-dione, D-lyxose, phosphoric acid, ↓ Pyrazinoic acid, ethylene glycol, oleic acid, 5-oxoproline, citric acid, ethyl ester, cumene, hemimellitene, hexadecane, indane, isocumene, o-ethyltoluene, oxalic acid, p-ethyltoluene, sorbose, vannilic acid, cyclobutanamine***	• Metabolite markers that are associated with oxidative stress decline between weeks 2 and 4 of treatment – a sign of patient recovery. • During the tuberculosis therapy several enzymes (CYP2E1, CYP3A4, alcohol dehydrogenase, aminocarboxymuconate-semialdehyde decarboxylase) undergo inhibition in a time-dependent manner. • During treatment, the urea cycle is upregulated, and the production of insulin is altered.	[164]
Paclitaxel	To investigate the association between pretreatment metabolome, early treatment-induced metabolic changes, and the development of paclitaxel-induced peripheral neuropathy for breast cancer patients.	↑ Pyruvate, alanine, threonine, phenylalanine, tyrosine, asparagine, lysine, o-acetylcarnitine, proline, lactate, glutamine, leucine ↓ 3-hydroxy-butyrate, 2-hydroxybutyrate****	• Pre-treatment levels of histidine, phenyl-alanine, and threonine may predict severity of potential peripheral neuropathy.	[165]
	To investigate metabolite signatures prior to the treatment, in order to explain the variability of paclitaxel-induced pharmacokinetics.	↑ Creatinine, glucose, lysine, lactate ↓ Betaine	• Pre-treatment levels of creatinine, glucose, lysine, lactate and betaine could be associ-ated with variability of paclitaxel-induced pharmacokinetics	[166]

Chemical Compound	Goal of the study	Main changes in metabolites post exposure	Conclusions	Ref
Irinotecan	To identify metabolite changes that could have potential implications on the mechanism of action of irinote and could serve as biomarkers for efficiency of a treatment.	↑ N-α-acetyllysine, 2-aminoadipic acid, asymmetric dimethylarginine, cystathionine, propionylcarnitine,L-acetylcarnitine, malonylcarnitine, valerylcarnitine, thymine, uracil, xanthine	• The increased levels of purine and pyrimidine nucleobase metabolites could be the result of purine/pyrimidine nucleotide degradation (break of double stranded DNA in cancer cells) as a response to the treatment with irinotecan. • The increased levels of acylcarnitines and amino acid metabolites could reflect dysfunction of mitochondria and oxidative stress in the liver.	[167]
Docetaxel (DTX)	To evaluate the response of MCF7 tumor cells to high (5uM) and low (1 nM) doses of DTX.	For high dosage (5uM): ↑ Phosphoethanolamine, cytidinediphosphocholine, polyunsaturated fatty acid, ↓ Phosphatidylcholine, glycerophosphocholine, glycerophosphoethanolamine, total glutathione, glutamate, arginine, lysine, lactate, acetate, For low dosage (1 nM): ↑ Phosphoethanolamine, cytidinediphosphocholine, homocysteine, aspartate, ↓ Phosphatidyl-choline, glycerophosphocholine, hypotaurine, taurine, total glutathione, arginine, alanine, threonine, lysine, acetate,	• Both dosages result in inhibition of phosphatidylcholine biosynthesis and decreased levels of glutathione. • The mechanisms responsible for decreased glutathione levels are different. At high dosage, the extensive consumption and precursor starvation was the main reason, while for low dosage, it was the inhibition of trans-sulfuration that inhibited glutathione biosynthesis.	[168]
Metformin	To identify urinary markers of metformin responses in patients with type 2 diabetes mellitus.	↑ Myoinositol, hypoxanthine ↓ Citric acid, pseudouridine, p-hydroxyphenylacetic acid, hippuric acid*****	• Citric acid, myoinositol and hippuric acid have the potential to become biomarkers that could predict the response to metformin in patients with type 2 diabetes mellitus.	[169]

Chemical Compound	Goal of the study	Main changes in metabolites post exposure	Conclusions	Ref
Simvastatin	To investigate the metabolic changes connected with the increased risk of developing hyperglycemia as an adverse response to simvastatin.	↑ Glucose, glutamic acid, alanine, ↓ Lauric acid, myristic acid, linoleic acid, glycine, palmitoleic acid, 3-hydroxybutanoic acid, aminomalonate, oleic acid, N-methylalanine******	• Patients showing a mild resistance to insulin tend to develop full insulin resistance after simvastatin treatment. • Branched-chain amino acids, and other metabolites such as ketoleucine, hydroxylamine and ethanolamine could predict type 2 diabetes mellitus risk following simvastatin therapy.	[170]
Olanzapin	To reveal the pharmacodynamics and mechanism of action of olanzapine.	↑ Tyrosine, succinic acid semialdehyde, homovanillic acid, 3,4-dihydroxyphenylacetic acid, L-asparagine ↓ 5-hydroxytryptamine, –5- hydroxyindoleacetic acid, L-3,4-dihydroxyphenylalanine, γ-aminobutyric acid, kynurenine, kynurenine acid, tryptophan, glutamic acid, taurine, acetylcholine	• Olanzapin alters glycerophospholipid metabolism, sphingolipid metabolism and the citrate cycle.	[171]
Losartan	To predict inter-individual variations in the metabolism of losartan.	↑ Lipid CH3 (LDL/VLDL), lipid CH2 (LDL), lactate, citrate, creatine, α-glucose ↓ Lipid CH3 (HDL), creatinine, choline, glycine, phosphorylcholine	• Identification of 11 potential biomarkers from whom lactic acid, creatinine, glucose, and choline showed a good score for prediction of metabolic processes of losartan.	[172]
Midazolam, Ketoconazole, Rifampicin,	To predict biomarkers related to midazolam sum of the clearance related to the induction and inhibition of CYP3A.	↑ 6β-hydroxycortisol/cortisol, 6β-hydroxycortisone/cortisone, 16α-hydroxy-DHEA/DHEA, 16α-hydroxyandrostenedione/androstenedione, 4-hydroxyandrostenedione/androstenedione, 7β-hydroxy-DHEA/DHE,6β-hydroxyandrostenedione/androstenedione, 2-hydroxyestrone/estrone, 2-hydroxyestradiol/estradiol, 11β-hydroxyandrosterone/androsteron, 11β-hydroxyandrostenedione/androstenedione ↓16α-Hydroxytestosterone/testosterone, 11β-Hydroxytestosterone/testosterone*******	• Urinary DHEA levels, 7β-hydroxy-DHEA:DHEA ratios, 6β-hydroxycortisone: cortisone ratios could be used to predict sum of the clearance for midazolam	[173]

Chemical Compound	Goal of the study	Main changes in metabolites post exposure	Conclusions	Ref
DA-9701 (extract from *Pharbitis nil* seed and *Corydalis yanhusuo* tube)	To monitor the changes of endogenous metabolites in order to understand better the mechanism of action.	For 0–4 h after exposure: ↑ Uric acid, L-acetylcarnitine ↓ Azelaic acid, ophthalmic acid, suberic acid, ε-(γ-glutamyl)-lysine, pimelic acid For 12–24 h after exposure[********]: ↑ Ophthalmic acid, pimelic acid, suberic acid, azelaic acid, ↓ Uric acid, ε-(γ-glutamyl)-lysine, L-acetylcarnitine	• Application of DA-9701 affects purine metabolic pathway, lipid, fatty acid metabolism and lipid peroxidation. DA-9701 improves gastrointestinal motility.	[174]

[*] *Rats treated with AEE versus model.*
[**] *Patients from high busulfan concentration-time curve (high-AUC) compared with low-AUC group.*
[***] *The differences between 2 weeks and 4 weeks of treatment.*
[****] *Pre-treatment levels of metabolites compared to 24 hrs after the first infusion.*
[*****] *Differences between responders and non-responders.*
[******] *Type of association between post-treatment metabolites levels and post-treatment insulin measures.*
[*******] *Fold change of mean urinary metabolite ratios in the induction phase.*
[********] *When compared to mean fold-changes of 0–4 hours exposure.*

Table 3.
Examples of pharmacometabolomic studies.

is the evaluation of aspirin response variability during antiplatelet therapy [180]. Lewis and colleagues identified that metabolites related to aspirin (salicylic acid and 2-hydroxyhippuric acid) were significantly increased, but exposure to aspirin also changed the levels of purines, fatty acids, glycerol metabolites, amino acids, and carbohydrate-related metabolites. Moreover, a substantial difference could be observed between good and poor responders in purine metabolites - higher levels of inosine and adenosine were observed in poor responders after aspirin intervention. Later, the authors identified 51 SNPs in the *ADK* gene region that had associations with platelet aggregation in response to aspirin exposure, the strongest of which was the rs16931294 variant. To confirm their findings, the authors compared their results to previously obtained metabolomic data and observed that rs16931294 was significantly associated with adenosine monophosphate, xanthine, and hypoxanthine levels before aspirin exposure. When compared with post-exposure results, this SNP was strongly associated with levels of inosine and guanosine [180]. The examples presented above [142, 144, 146, 179, 180] as well as other available literature [36, 175, 178] demonstrate the importance of pharmacometabolomics in drug design studies. Combined with other approaches, *e.g.*, pharmacogenomic, pharmacometabolomics can greatly contribute to our understanding of individual differences in responses to drug treatment and thus directly aid us in the development of new generations of drugs. There is also potential for significantly extending our understanding of health sustenance and disease development, and thus reduce drug-dependent therapies. Perhaps not the most profitable news for the pharma industry, but good news for health workers and the general population who will be able to identify at risk individuals and indeed tailor health management strategies to prevent and/or reduce the impact of disease.

4. Future perspectives

An intense research on '-omics' approaches, devoted to human health, led to the development of pharmacometabolomics, which is a new horizon in personalized medicine. Numerous research data on metabolomics, genomics, and transcriptomics can be combined and compared with health records around the world due to potent databases and biobanks collecting data and samples. Nowadays, software and informatics systems with sophisticated algorithms of artificial intelligence allow for deeper analyses of pharmacometabolomics data, and transform general medicine into a personalized approach.

The analytical techniques, databases, and biobanks presented here are the general trends, which need to be further developed. The sensitivity of the analytical platforms needs to be improved, and additional ameliorations related to time and overall costs must be done. Particular attention must be paid to the standardization of study protocols. The number of data and samples deposited in databases and biobank must be extended.

Up to now, major efforts in pharmacometabolomics have been concentrated on research aspects and method validation for medical applications. The results presented here show undoubtedly that pharmacometabolomics is key for personalized medicine and needs to be transferred 'from bench to bedside'. Nevertheless, medical personnel can source from pharmacometabolomics only if the data are presented in a simple and comprehensive way. In the future, more effort is needed to increase the broad awareness of pharmacometabolomics among patients and healthcare system staff, and to introduce the benefits of pharmacometabolomics into clinical practice.

5. Conclusion

Human genetics and lifestyle variation directly influence pharmacological treatments, whose effect can be enhanced positively or negatively in some individuals over the statistical population used in clinical trials.

This chapter has described pharmacometabolomics as an innovative tool capable of assisting researchers and frontline medical personnel in establishing personalized therapeutic strategies. Pharmacometabolomics can be used to personalize treatment type, dosage, duration, and to monitor metabolites' profiles during pharmacotherapy. The existing '-omics' and health records databases, and biobanks of human fluid samples and tissues are a precious resource for pharmacometabolomics, which identify biomarkers of therapeutic effects over a disease course. The metabolomics databases are increasing their data pool every day, and are priceless for researchers combining '-omics' knowledge for better and personalized pharmacotherapy.

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Conflict of interest

The authors declare no conflict of interest.

Abbreviations

ADK	Adenosine kinase
ADR	Adverse drug reactions
AEE	Aspirin eugenol ester
ALI	Acute liver injury
ART	Antiretroviral therapy
BMRB	Biological Magnetic Resonance Data Bank
BRCA1	Breast cancer gene 1
BRCA2	Breast cancer gene 2
CDER	Center for Drug Evaluation and Research
CYP2D6	Cytochrome P450 2D6
DTX	Docetaxel
EHRs	Existing electronic health records
EMB	Ethambutol
ESI	Electro spray ionization
FDA	Food and Drug Administration

FT-IR	Fourier transformed infrared spectroscopy
HILIC	Hydrophilic interaction liquid chromatography
HMDB	Human Metabolome Database
HPLC	High-performance liquid chromatography
INH	Isoniazid
MATE1	Multidrug and toxin extrusion 1
MMCD	Madison Metabolomics Consortium Database
MS	Mass spectrometry
NMR	Nuclear magnetic resonance spectroscopy
OCT1	Organic cation transporter 1
OCT2	Organic cation transporter 2
PMRN	Pharmacometabolomics Research Network
PRIMe	Platform for RIKEN Metabolomics
PZA	Pyrazinamide
RIF	Rifampicin
RP	Reversed-phase gradient chromatography
SLC22A1	Solute carrier family 22 member 1
SLC22A2	Solute carrier family 22 member 2
SLC47A1	Solute carrier family 47, member 1
SNP	Single-nucleotide polymorphism
TMAO	Trimethylamine-N-oxide

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Chapter 4

Volatilomics of Natural Products: Whispers from Nature

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Abstract

Volatilomics studies the emission of volatile compounds from living organisms like plants, flowers, animals, fruits, and microorganisms, using metabolomics tools to characterize the analytes. This is a complex process that involves several steps like sample preparation, extraction, instrumental analysis, and data processing. In this chapter, we provide balanced coverage of the different theoretical and practical aspects of the study of the volatilome. Static and dynamic headspace techniques for volatile capture will be discussed. Then, the main techniques for volatilome profiling, separation, and detection will be addressed, emphasizing gas chromatographic separation, mass spectrometry detection, and non-separative techniques using mass spectrometry. Finally, the whole volatilome data pre-processing and multivariate statistics for data interpretation will be introduced. We hope that this chapter can provide the reader with an overview of the research process in the study of volatile organic compounds (VOCs) and serve as a guide in the development of future volatilomics studies.

Keywords: volatile organic compounds (VOCs), microbial volatile organic compounds (mVOCs), static headspace, dynamic headspace, SPME, PDMS-patches, GC–MS, metabolomics workflow

1. Introduction

Volatilomics indicates the qualitative and quantitative study of the volatilome, defined as the complex blend of volatile organic compounds (VOCs) originating from different biosynthetic pathways and emitted by living organisms [1]. VOCs are small molecules (below 500 Da), with hydrophobic character, low boiling points, and high vapor pressure at ambient temperature. Unconjugated volatiles can freely diffuse across membranes to be released from flowers, fruits, and vegetative tissues into the atmosphere and from roots into the soil to be perceived at short and long-distance. Therefore, plants and animals use VOCs for chemical communication with the surrounding ecosystem, and plants also use them as attractors for pollinators and defense against herbivory and biotic and abiotic stress [2–5].

The study of VOCs of plants has focused not only on the qualitative and quantitative composition of the volatile fraction but on the bioactive compounds as well as flavors and fragrances [6, 7]. Similarly, the understanding of fruits' sensorial attributes is of great interest as quality control, as well as in the determination of origin mark, and the performance of ecological studies aimed at the establishment

of the relationship between the ripening stage and the incidence of fruit diseases for insect or microorganism attack [8–10].

Microorganisms produce a plethora of important microbial volatile organic compounds (mVOCs), that play an essential role in inter- and intra-kingdom connections. The study of mVOCs has allowed, for example, to detect terpenes, compounds normally associated with plants, also in fungi and bacteria [11]. Also, these compounds are related to ecological interactions between living organisms found in the soil, including the rhizosphere [12].

In addition, several studies of VOCs from animals not only have allowed decoding the signal of the animal chemical communication but also have demonstrated the potential use of that knowledge in early disease's diagnostics. For example, recent studies have shown novel practice for the detection of biomarkers to identify the intoxication using unusual biological fluids like ear wax, being fast, economic, and noninvasive bioanalysis, with minimal sample preparation and very versatile to identify the first signals of intoxication [13, 14].

Differently to the genomes, the volatilome changes continuously across time, and its composition depends on external and internal factors, such as the environmental conditions, and/or the physiological state [15]. Therefore, the study of the volatilome is not a simple task and the researchers in this area entail multiple challenges derived from the chemical complexity of the samples and the superposition of VOCs signals as proper of the ecosystems. Thus, sensitive yet unbiased methodologies are needed to provide researchers with comprehensive and accurate representations of a plant species' volatile metabolome.

However, current methodologies are limited in their ability to isolate, and even more critically to identify, many of the compounds present in each sample. In volatile metabolomics, the emitted metabolites are already isolated from tissues, they need to be temporarily trapped, and eventually preconcentrated, in a way that allows them to be released unadulterated for separation and identification.

A variety of technologies have been developed. In these methods, the sample of interest is enclosed in a collection chamber and the released volatiles present in the airspace surrounding the sample, headspace (HS), are trapped onto an adsorbent. And are subsequently analyzed by gas chromatography in combination with mass spectrometry (GC–MS) as the method of choice for volatilomics.

Hence, in the next sections of this chapter, we will provide an overview of the volatilome study process, including the main practical and theoretical aspects of volatiles capture, sample preparation, and the main analytical techniques employed to monitor VOCs, together with the chemoinformatics tools used for volatilome dereplication, elucidation, annotation, and interpretation of data.

2. Volatiles collection

Sample acquisition in volatilomics experiments requires consistency, therefore due to the high variability of chemical structures, concentrations levels, sample types, and physiological variations, other variables different than metabolites (addressed as meta-variables from now on) should be controlled or at least carefully monitored in order to evaluate their effect on the study outcome. Some important variables that should be taken into account include replicate number, taxonomic identification, geographic location, phenotypic or phylogenetic variant, sample weight, phenotypic characteristics, sex, developmental stage, health status, collection date, and time. Photographs should be taken. A useful reference for registering meta-variables is the ReDU Sample Information Template. (https://docs.google.com/spreadsheets/d/1v71bnUd8fiXX51zuZIUAvYETWmpwFQj-M3mu4CNsHBU/

edit?usp=sharing) [16] build by the collaborative Global Natural Products Social Networking (GNPS) (https://gnps.ucsd.edu/ProteoSAFe/static/gnps-splash.jsp) [17] where researchers can add new meta-variables and share their data in an open-source and collaborative environment.

2.1 Plants

The plant volatilome is defined as the complex blend of essential oils (EOs) and VOCs fed by different biosynthetic pathways and emitted by plants, constitutively and/or after induction, as a defense strategy against biotic and abiotic stress. Plants have a vast diversity in their range of metabolites and their concentrations, as there are hundreds of thousands of metabolites in different categories. As such, there is no single analytical technique that has the capability of extracting and detecting the whole metabolome [18].

Plant volatile emissions are linked to the physiological status of the emitter, therefore special care must be taken to control the plant-growing environment as well as all variables concerning the developmental stage of the plant to limit unwanted fluctuations in metabolism that might affect collected. These include the time of day, photoperiod, temperature, humidity, water conditions, collection site altitude, plant age, climate, and soil type so that a careful experimental design is recommended. Whenever possible, growth chambers must be used for plant cultivation and volatile collection [19, 20]. EOs and VOCs can be extracted and analyzed from both fresh and dried plant materials. When using fresh material, particular attention must be paid to the health status of plants, since microbial and other infections may alter metabolites production. Plants must not show necrotic areas and be at the same developmental stage if comparative analyses are needed. Since the content of water may vary, it is a good practice to use some of the fresh material to calculate the dry matter percentage [21].

Since volatile emissions from many plant species vary with respect to the time of day, and different organs in the plant are known to produce and/or accumulate different profiles of secondary metabolites, collection strategies should consider volatile sampling over an extended period of time and from the investigated organ or entire plant, to prevent unintentional exclusion of volatile components in the sampled mixture. Also, when running VOCs analyses from living plants it must be remembered that rooted plants in pots respond differently than cuttings, and that soil in pots may contain microorganisms that can produce VOCs [22, 23]. Once a plant part is collected, at least two herbarium samples should be prepared and identified or authenticated by a taxonomist. One of these voucher specimens should be deposited in a local national herbarium. A card with details of the place, altitude, environment, and photographs should be attached to the herbarium sample, in case a recollection of the plant material is necessary. Although depositing herbarium samples is a basic step in performing phytochemical investigation, many researchers in the past neglected this step and thus were unable to reproduce their work [23–25].

2.2 Flowers

Living flowers change their volatile profile in a continuous way that depends on intrinsic and extrinsic factors. Once cut, flowers undergo rapid deterioration and loose volatiles. Flower volatiles allow discrimination between different plants and attract insects for pollination when they are released. The amount of emission is not uniform through time, with some differences between diurnal and nocturnal emission levels, and between reproduction phases. The volatile compounds emitted

by flowers are mainly aliphatics, terpenoids, benzenoids, and phenylpropanoids. Flower volatiles require special methods for their isolation with preconcentration and can be obtained from the air surrounding the living or excised flower, or from the flower tissues themselves. The selected extraction technique determines the composition of the isolated volatiles mixture [26, 27].

2.3 Fruits

Fruits are very complex samples, rich in a great number of different classes of metabolites, including volatile, semi-volatile, and no volatile compounds. The flavor is one of the most important characteristics to value the quality of fruit. Volatile and semi-volatile compounds usually are responsible for aroma fruit, and their study has conducive to identify both positive and negative sensory attributes [28]. VOCs are produced in trace amounts, and although they are easily perceptible by the human nose, their sampling and monitoring can be challenging at an analytical level [29]. The volatile fraction of fruits is composed of hundreds of different chemical substances that can vary according to the type of fruit, but the emitted compounds can be grouped according to the chemical function mainly into esters, alcohols, aldehydes, ketones, lactones, and terpenoids [29]. Moreover, VOCs emitted by fruit depend on the production conditions (cultivars, state of maturity, post-harvest treatment, and storage) the sample format (whole fruit, sliced, wet, dry), and the type of analysis (in-field or in-lab). Capturing volatiles in-situ is a challenge, as small amounts of VOCs are released and diffuse in a large volume of air, which requires highly efficient sampling techniques to capture them. Solid-phase microextraction (SPME) and solid-phase extraction (SPE) are usually the most profitable techniques for the capture of fruit volatiles in-situ. Once the volatile compounds are retained in an adsorbent material, their storage and transport are facilitated. On the other hand, in laboratory capture of VOCs from fruits, can be efficiently performed by solvent or gas-based extraction techniques, such as Soxhlet, simultaneous distillation extraction, purge and trap, and headspace, among others.

2.4 Microorganisms

Analysis of mVOCs is commonly performed under controlled culture media, temperature, and agitation. Also, the percentage of humidity and exposure to UV–visible light among other growing conditions should be taken into account. In order to account for reproducibility of the experiments, laboratory tests on microorganisms must be performed using international reference strains e.g.: American Type Culture Collection (ATCC), instead of clinical or field isolations, or even strains isolated and saved in the research group for a long time. Because the emission of VOCs can vary in terms of presence or absence, and in terms of fluctuation in concentration, throughout the life span of the microorganisms (which can be from a few hours to days), it is advisable to perform analyses both in the exponential or logarithmic growth phase, as well as in the stationary phase [12, 30, 31]. During the exponential phase, the microorganism is reactivating its biosynthetic pathways after having been in a state of latency. Therefore, in this stage, there is generally a high concentration of some metabolites that are part of the first stages of the biosynthetic pathways, which can later diminish and disappear in the exponential phase. The stationary phase is achieved when the initial metabolic processes have been reached and occurs when the survival process of the species begins [32]. The metabolic changes produced in these two stages of microbial culture are fundamental to understanding and solving research questions [33, 34]. The determination of

each of the culture phases is commonly done with a measurement of the absorption of light in the visible region between 500 and 650 nm for liquid growth medium. This is achieved by counting the colony-forming units (CFU) in the solid medium. The sampling time for analysis of mVOCs must coincide with those obtained in the growth curves, correctly differentiating the exponential and stationary phases.

2.5 Animals

For conducting volatile sampling from animals, the specimens could be either raised in captivity at controlled vivaria or extracted from their natural environments. Proper training in animal manipulation is an important aspect to be fulfilled before performing animal experimentation, as well as an approved permit by the Institution in charge to validates the procedures. Also, when animals are to be collected in their habitats, it is necessary to review if a Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) permit is needed for protected species. A specialist should validate taxonomic identification and, in those cases, where sample collection involves euthanization of specimens that should be registered at a recognized Museum, and voucher numbers should be annotated and published on the research paper. In the same way, as other organisms could be sampled by different methods, almost all animals could be sampled in vivo, but in some cases, tissue extraction could be preferred for guaranteeing detection of less abundant metabolites. Some techniques applied for VOCs analysis from terrestrial arthropods [35–39], aquatic organisms [40–42], mammals [43–48], birds [49, 50], reptiles [51, 52], fishes [53], and amphibians [37, 38, 54–57] include headspace-adsorbent traps, polydimethylsiloxane (PDMS) patches, swabs and stir bar sorptive extraction (SBSE).

3. Volatiles extraction

Sample preparation is one of the most important steps in the analytical process. The goal of sample preparation is to efficiently isolate target analytes from potential interferences and to extract as many VOCs as possible to provide a true representation of the studied system.

Some steps of pre-treatment of the sample are necessary in order to minimize the manipulation of the sample and avoid its modification, to clean-up the sample efficiently, and to quench metabolic reactions that could cause degradation and decomposition. To date, two different types of headspace sampling, static and dynamic, are widely used for volatilomics investigation.

Static headspace sampling is a passive technique for VOCs collection, where no air is circulated for the concentration of the volatiles on a sorbent matrix [18]. As a result, the background noise is drastically reduced due to the absence of a continuous airflow that can contain impurities that could mask compounds released at trace amounts. In static headspace methods, samples are typically sealed inside a container or bag, where the volatiles are released and, in the more traditional version of the technique, the headspace is sampled directly using a gas-tight syringe and transferred to the Gas Chromatography (GC) injection port. When the analytes are present at trace level, it might be necessary to carry out static headspace methods with special techniques to concentrate volatiles during collection and reduce the dilution of the sample during desorption in the GC inlet. In such a context, SPME stands out as the most versatile strategy for volatile capture from the sample headspace in static mode. Nowadays, SPME is the leading technique in the analysis of volatiles of biological origin because it uses a fiber coated with a sorbent phase to

combine extraction and pre-concentration compounds. SPME fibers are available in a wide range of coatings that allow the sampling of compounds of different polarities and volatilities. Considering that the goal of volatilomics profiling is to analyze as many metabolites as possible, the use of divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibers is the most suited to increase the number of analytes that can be trapped on the fiber because it can allow capture VOCs in a wide range of polarity and molecular weight [58].

This type of coating contains a layer of CAR particles underneath a layer of DVB particles. Because the ability of adsorbent coatings to extract a particular analyte strongly depends on the size of the pores, larger analytes will be retained in the outer DVB layer, while the smaller analytes will migrate through this layer and are retained by the inner layer of CAR. On the contrary, if the study targeted only on the most volatile fraction, PDMS/CAR would be an appropriate choice of coating, since the micropores of the CAR retain smaller analytes better than other coatings, although introducing a high degree of discrimination towards high-molecular-weight compounds.

On another hand, although other coatings, such as PDMS, polyacrylate (PA), and Carbowax (CW), are also commercially available, their use in volatilomics is quite scarce due to the higher selectivity towards certain classes of polarities [58, 59].

From a practical point of view, SPME is a versatile technique for in-field sampling as a non-destructive strategy for the study of the volatiles emitted ex-vivo, for example, by grapes. In this case, an aluminum wire cage can be used to support a polymeric film to enclose a whole cluster of grapes, and SPME fiber is introduced through a port fitted with a silicone septum (**Figure 1a**).

Also, an interesting strategy for speeding up the volatiles' uptake is vacuum-assisted SPME. For example, in-field sampling of volatiles from a single grape berry, a modified screw top, and a 2 mL glass vial can be used for fiber exposition. A syringe is usually used to create a negative pressure to hold the sampling device with the SPME sealed onto the sample surface (**Figure 1b**).

This type of coating contains a layer of CAR particles underneath a layer of DVB particles. Because the ability of adsorbent coatings to extract a particular analyte strongly depends on the size of the pores, larger analytes will be retained in the outer DVB layer, while the smaller analytes will migrate through this layer and are retained by the inner layer of Carboxen. Conversely, if the study targeted only on the most volatile fraction, PDMS/CAR would be an appropriate choice of coating, since the micropores of the CAR retain smaller analytes better than other coatings, although introducing a high degree of discrimination towards high-molecular-weight compounds.

Although SPME generally exhibits better extraction efficiency as the polarity of the compound decreases, these three coatings can provide balanced metabolome

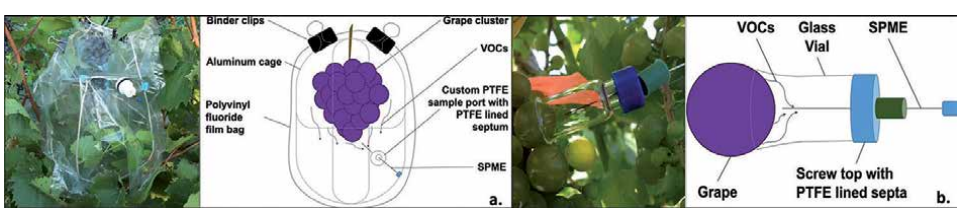

Figure 1.
Sampling handling techniques of VOC's fruit. a) Ex-vivo sampling of volatiles from the whole cluster of grapes by SPME; b) Ex-vivo sampling of volatiles from a single grape berry by SPME. Adapted from [10] under the Creative Commons Attribution License.

coverage as long as most polar analytes are present at reasonable concentration levels. Absorbent coatings, such as PDMS, PA, and CW, were rarely employed in profiling studies. These coatings display selectivity based on polarity, resulting in poor metabolomic coverage. The second case is dynamic headspace sampling, which offers a highly concentrated sample that can be desorbed into a solvent at volumes suitable for multiple analyses. To date, it is the most frequently used technique in all areas of plant volatile analysis. Dynamic headspace sampling collects a much larger quantity of compounds at higher concentrations because the continuous stream of air allows the sorbent to act as a filter trapping the volatiles.

Also, push and pull headspace sampling, two examples of dynamic headspace sampling, allow to avoid problems often encountered with the sealed systems used in static headspace and closed-loop stripping methods including heat, water vapor, and, in the case of plants, ethylene accumulation that can affect not only sampling efficiency but also plant physiology. Among the several methods, closed-loop stripping systems have broad utility for the collection of volatiles: volatiles are collected during continuous circulation of HS air inside closed chambers in which air circulation pumps are connected to supporting columns or coated supports [22].

As an example, SPME is a versatile technique for in-field sampling handling as a non-destructive strategy for the study of the volatiles emitted ex-vivo, for example, by the whole cluster of grapes. In this case, an aluminum wire cage can be used to support a polymeric film to enclosing a whole cluster of grapes, and SPME fiber introduced through a port fitted with silicone septa (**Figure 1a**) [60]. Also, an interesting strategy for speed up the volatile's uptake is vacuum-assisted SPME. For example, in-field sampling of volatiles from a single grape berry, a modified screw top, and a 2 mL glass vial can be used for fiber exposition. A syringe is usually used to create a negative pressure to hold the sampling device with SPME sealed unto the sample surface (**Figure 1b**) [60].

Alternatively, to the SPME, some liquid-phase microextractions (LPME), such as the single drop microextraction (SDME) or the hollow fiber liquid-phase microextraction (HF-LPME), can also provide efficient and profitable volatiles recoveries in the headspace static mode. For example, SDME is a technique based on a few microliters of solvent, in which volatiles can be capture in a small drop of extraction solvent-exposed to the headspace of the sample [20, 59]. In the same way, to address the drawbacks of the drop instability, the extraction solvent can be deposited into the lumen of a porous fiber HF-LPME, improving the extraction kinetics by use of a bigger transference surface or by the incorporation of an acceptor solvent into the membrane pores (Supported Liquid Membrane, SLM). Although the use of hazardous organic solvents can be considered a drawback, nowadays those solvent-based extractions can be performed with environmental-friendly alternatives, such as ionic liquids, deep eutectic solvents, or supramolecular solvents, among others.

The second type of headspace sampling is the dynamic headspace (DHS) method. It encompasses strategies in which VOCs are captured in a sorbent-packed trap by passing a continuous flow of inert dry gas through the sample. In this way, the emission of VOCs speeds up by the continuous renovation of the headspace fraction. After extraction, concentrated VOCs can be desorbed from the sorbent-packed trap with a suitable solvent or via thermal desorption. Besides, DHS address some drawbacks of the static modes such as the accumulation of water vapor or highly concentrated compounds, which presence can affect extraction efficiency. Two examples of dynamic headspace sampling which allow avoiding some drawbacks of the static mode, e.g., heat and water vapor accumulation that can affect not only sampling efficiency but also plant physiology, are closed-loop stripping and push and pull methods. These systems collect VOCs in sorbent-packed traps or coated devices, via the continuous circulation of gas inside closed circuits [22].

In addition to headspace sampling techniques, some sui generis approaches can combine two methods from different groups, for example, solvent-assisted flavor evaporation (SAFE). SAFE is an exhaustive extraction technique based on the high volatility rather than the polarity of the target compounds. In this case, a crude-extract from dry sample pieces is prepared with an appropriate solvent, such as dichloromethane, and then added into the dropping funnel and passed through a specific distillation chamber. Extraction takes place at high vacuum, and low-temperature conditions (20–30°C), and VOCs are collected in a cooled extraction vessel [61]. Other techniques including in this group are simultaneous extraction-distillation (SDE) and/or liquid–liquid extraction (LLE). Nevertheless, those can be subjected to some drawbacks, like the use of hazardous solvents, as well as the requirement of high temperatures and long extraction times, with potential formation of artifacts and degradation of some compounds.

Finally, volatile compounds also can be obtained for direct collection of the secretions of odoriferous glands or via non-invasive strategies using PDMS patches or swabs [22]. These techniques are especially useful in the monitoring of VOCs from animals. For example, obtaining the animal skin volatilome on PDMS patches is an excellent option [62]. Patches could be prepared by cutting a Silicone Elastomer Sheet (Goodfellows mfr. No. 942-965-49, Coraopolis, PA) and then carefully fix it on the animal skin with Tegaderm® dressings or water block clear Band-aids®. Alternatively, this procedure could be modified by gently swabbing the skin with or without previous stress-induced secretion. PDMS patches also can be placed into an animal enclosure and used without direct contact for capturing the volatiles that emanates in the headspace.

4. Volatilome profiling: separation and detection

Currently, gas chromatography coupled to mass spectrometry (GC–MS) is the primary analytical technique for the elucidation of the volatilome profile from natural sources. In gas chromatography analytes elute according to their volatility carried by a gas, usually Helium, through a coated fused silica capillary using a temperature gradient. Separation occurs based on the differential partition between the gas phase and the coating and the eluting peaks will give a response in the detector. The sample is vaporized in the injection system before it enters the column.

Several injection systems can be used to introduce the sample onto the column. Split injection allows transferring to the column only controlled sample amounts and prevent overloading of the column, thanks to a split valve at the base of the hot injector that divides the flow between column and waste in a fixable ratio. High-concentration samples can easily overload the GC column, resulting in all active sites on the column becoming occupied and leading to additional analytes not being retained and therefore to poor chromatographic resolution. For trace analysis, the injector can be used in splitless mode, which allows the entire volume of sample vaporized in the injector to reach the column. An alternative to the split/splitless interface is the programmed temperature vaporizer (PTV). Samples are injected onto a cool (40–60°C) PTV where they are trapped and concentrated on different sorbent materials before the inlet is rapidly heated to desorb the sample onto the column.

Different selectivity and sizes of columns have been used for GC–MS–based metabolomic analysis. The most used phase is 5% phenyl, 95% methyl siloxane, which offers a sufficiently generic selectivity, optimal for metabolomic applications where analytes with a wide range of volatilities have to be separated. Capillary columns of 25 to 30 m will provide the highest resolution and are available in most

phases. An important point for all capillary GC–MS work is the need to condition the column prior to running valuable samples. Sangster et al. have recommended that several quality control samples be run at the beginning of a sample batch to condition the column [63]. Care also needs to be taken to randomize the injection sequence in order not to compromise subsequent statistical analysis.

In GC–MS ionization of analytes is mainly produced by electron ionization (EI) or chemical ionization (CI), while ion separation is obtained by mass analyzers operating on different principles. In EI, analytes that elute from the GC column are vaporized into the ion source and collide with an electron beam at 70 eV. As a result of the high energy imparted by electrons to the vaporized molecules, characteristic fragmentation occurs, providing structural information. EI is very robust and highly reproducible between instruments, and spectral libraries are available that can be used to search for the identities of unknown compounds based on m/z and intensity ratios of the observed fragment ions. A disadvantage of EI is that fragmentation is usually so efficient that the intensity of the molecular ion can be extremely low or even lost. For CI, a reagent gas, such as methane or ammonia, is introduced into the source of the mass spectrometer. Protonated gas ions, produced by the collision with electrons originating from an electron beam, ionize the analytes eluting from the column after vaporization into the ion source. Significantly less energy than in EI is transferred to the analytes, and as a result, the dominant ion is usually the molecular ion.

Mass spectrometer based detectors are mainly used in metabolomic analysis and can be grouped according to the spectral information they provide, i.e., low-resolution instruments such as quadrupole mass spectrometer (qMS), ion-trap mass spectrometer (IT-MS), and high-speed time-of-flight mass spectrometer (TOF-MS) give nominal molecular weights and fragmentation of an analyte, while high-resolution instruments (high-resolution TOF-MS and hybrids) give the precise elemental composition of nominal masses. The single quadrupole mass analyzer is widely used and relatively inexpensive. The ions move along the axis of four parallel rods to which a direct current (DC) and an alternating current (AC) voltage are applied. These voltages affect the trajectory of ions traveling down the flight path between the rods in a way that only ions of a given m/z are transmitted at a given point in time. Scan speeds are rather low on quadrupole instruments, therefore considering the very high separation power of GC with peak widths of only a few seconds, it will be difficult to acquire several spectra across the width of a typical peak on a single quadrupole instrument. Time-of-flight (TOF) instruments are the most common mass analyzers in GC–MS–based metabolomics. The ions are accelerated in an electric field in which ions with the same charge will have the same kinetic energy, but different velocity depending on their mass-to-charge ratio (m/z). Successively, the ions enter a field-free region (flight tube) where they separate based on their m/z. TOF instruments are characterized by the fastest scan rate among all mass analyzers: a significant number of spectra can be acquired across each peak, leading to higher sensitivity and better spectral quality.

GC–MS has very high sensitivity and can therefore be used for the analysis of less commonly encountered samples that might only be available in trace amounts. Monodimensional GC–MS analysis provides suitable resolving-power for the analysis of relatively simple mixtures of VOCs. Nevertheless, volatilome samples can be very complex mixtures, involving a diverse plethora of chemical structures in a wide range of polarities, so that the restricted chromatographic resolution commonly limits the identification via MS to the more abundant compounds. Complex mixtures can be better resolved by employing comprehensive two-dimensional gas chromatography–mass spectrometry (GCxGC–MS), which has been defined as "...an orthogonal two-column separation, with complete transfer of a solute from

the separation system 1 (column 1) to the separation system 2 (column 2), such that the separation performance from each system (column) is preserved" [64]. In GC × GC, two columns with different polarity—usually a nonpolar column in the first dimension and a moderately polar column for the second one—are run in series. Analytes eluting from the first dimension (1D) column are trapped, focused, and then rapidly injected, as a narrow band of few milliseconds, in the second dimension (2D) column, then the eluting peaks are detected by MS. The transfer process is actuated by a modulator, a thermal or valve-based focusing system. Each single modulator cycle takes a fixed time (4–8 s) and each fraction, injected online into the second column must be analyzed in a time equal to that of the successive modulation. The challenge is to avoid continuously transmitting analyte onto the second column, which would lead to a loss of resolution. A solution to this problem is to make the separation on the second column much faster than the separation on the first column. The volume of data generated is significantly larger than the one obtained in a one-dimensional analysis. However, this approach allows for better separation of the number of components in the sample. Although single qMS instruments are cheaper, can provide very low LODs via selected ion monitoring (SIM), and can provide maximum acquisition rates (20,000 amu/s) suitable for metabolic profiling, TOF has become the preferentially MS analyzers for GCxGC volatilome analysis. TOF-MS instruments are capable of full-spectrum collection rates up to 500 Hz with improved sensitivity. Besides the high-resolution mass spectrometry (HRMS) provide accurate mass data, which increases the identification confidence and allows to annotate molecular formulas for unknown compounds, being especially useful in untargeted metabolomic studies.

Metabolite identification remains a major complication. Although EI generates highly reproducible fragmentation spectra, only a relatively small percentage of metabolites can be identified by searching databases, mainly because these have traditionally been a repository of EI spectra of synthetic organic compounds. Only recently, the number of metabolite spectra started to increase. A more powerful identification method involves comparing both EI/CI spectra and retention indices obtained from analyzing a reference compound under identical analytical conditions. If commercial standards are not available, metabolite identification can be cumbersome.

Retention indexes (RI) were first introduced by Kováts [28] for isothermal analysis and then by Van den Dool [65] for temperature-programmed analysis (linear retention indices, LRIs) and are calculated vs. a homologous series of linear hydrocarbons run in the same GC conditions as samples. RI can also be automatically calculated using the Automated Mass Spectral Deconvolution and Identification System (AMDIS), freely available from the National Institute of Standards and Technology (NIST) at this site (http://www.amdis.net/).

In order to achieve the identification of unknown compounds, their background-subtracted EI spectra are searched against EI libraries (such as the NIST library) to achieve identification. Values of *m/z* values and relative ion intensities in a spectrum are matched against spectra in a reference library [the most used database of EI spectra is the NIST database (http://www.nist.gov/srd/nist1.htm)]. The database search will usually return a list of possible hits, ranked by the probability of the match. Although, even if a match is exceedingly high, the metabolite should still not be considered as identified.

The high variability of data obtained from the investigated matrix composition makes it hard to indicate a universal approach to quantitatively evaluate the volatilome composition. The most widely used approaches are: (a) relative percentage abundance, (b) internal standard normalized percentage abundance, and (c) "absolute" or true quantitation of one or more target components, with or without a validated method.

Relative percentage abundance can be applied only to evaluate relative component ratios within the same sample. Internal standard normalized percentage abundance is the ideal approach when a group of samples is compared: raw data must first be corrected vs. analyte response factors to the detector, then normalized vs. an internal standard. Percentage abundance must be calculated vs. the sum of the areas of a fixed number of selected components, found in all the samples. The quantitation of marker components is obtained from the chromatographic area in SIM mode vs. an internal (or external) standard and calculated via a calibration curve constructed from amounts of pure standards in the selected concentration range.

Some common non-separative techniques used in the study of volatilome using mass spectrometry are selected-ion flow-tube mass spectrometry (SIFT-MS) and proton-transfer-reaction mass spectrometry (PTR-MS). These techniques are focused on the use of soft chemical ionization, allow on-line detection of VOCs with low levels of detection without the need for pre-concentration or sample preparation, which facilitates obtaining reproducible results. For example, Vendel and co-workers [66], used SIFT-MS and HS-SPME-GC–MS for the analysis of strawberry aroma. Although both techniques provided similar results in the study of the fruit ripening, the SIFT-MS analysis was about 11 times faster than HS-SPME-GC–MS. Moreover, SIFT-MS showed low detection limits, so that the postharvest analysis can be easily performed by the analysis of individual fruit. Capellin and collaborators [67] developed a similar study was using PTR-TOF-MS to study the volatilome of clones belonging to three types of apple. They concluded that PTR-TOF-MS is a very useful tool for volatilome studies once this technique allows obtaining a rapid and non-invasive fingerprint of the VOCs profile from single apple fruits.

With an alternative focus, the chromatographic system can be coupled to an olfactometer detector to identify the aroma-active compounds present in a determinate volatilome. This type of analysis allows determining the compounds which generate a positive response to the electronic noise detector, obtaining their identification by comparison of the mass spectrum, retention index, and odor descriptions with reference compounds. Using gas chromatography-olfactometry-mass spectrometry (GC-O-MS), Zhu and co-workers [68] studied the volatile profile of three cultivars of mulberries, establishing benzaldehyde, ethyl butanoate, (E)-2-nonenal, 1-hexanol, hexanal, methional, 3-mercaptohexyl acetate, and 3-mercapto-1-hexanol as the main compounds responsible for the characteristic aroma of mulberry.

5. Volatilome data processing

Once the raw data have been acquired following chromatographic separation and mass spectrometry analysis, the large amount of data generated needs to be processed following a standardized procedure that includes data conversion, pre-processing, pre-treatment, and metabolite annotation [69]. An additional step, sharing data derived from any metabolomics analysis, currently is optional for researchers but highly recommended.

5.1 Extract raw files from instruments and proceed to data conversion

Data processing starts with a set of raw data files for different samples. Usually, default vendor formats from instruments need a conversion. A useful toolkit compatible with several instruments formats is ProteoWizard (http://proteowizard.sourceforge.net/download.html) [70]. Open-source formats usually supported by many software packages are Network Common Data Form (NetCDF) [71], Extensible Markup Language (mzXML) [72], and Mass Spectrometry Markup

Language (mzmL) [73]. Each file is processed to an easily accessible and more informative data table, where rows represent samples and columns represent different features from volatilome. Values from this matrix represent intensity values of peak area/height, standing for relative concentration. The data should be checked for missing values and possible outliers.

5.2 Set parameters to perform data pre-processing

Pre-processing involves setting different filters to recognize signals from noise, select masses or intensities to perform feature detection, and finally adjust the retention time shifts parameters needed to align features throughout all samples. The aim of pre-processing is to minimize the number of false positives features and to establish quantitative procedures for discarding less reliable signals with low signal-to-noise ratio, or low prevalence within a similar set of samples [74].

5.3 Choose the best method to perform data pre-treatment

Pre-treatment or data correction is one of the most important steps from data analysis because systematic and technical variation could obscure relevant biological patterns. The variation in the data resulting from a metabolomics experiment is the sum of the induced variation and the total uninduced variation [75]. Some sources of variation could be controlled by researchers through a careful experimental design. In other cases, this variation is very difficult to control. Natural variation in the metabolism of an organism can cause 5000-fold differences in signal intensities for different metabolites, or sampling could not be performed on the exact conditions for all samples, sample work-up varies naturally between batches, and analytical errors are always present. This variation could be accounted for using different classes of corrections that include centering, scaling, transformation, and normalization of raw data and several methods are available to do so (e.g., autoscaling, pareto scaling, range scaling, vast scaling, log transformation, and power transformation, normalization by sum, normalization by a reference sample). The selection of the most appropriate method depends on the hypothesis to be tested and the statistical behavior of the data matrix. Before applying pre-treatment methods, it is required to check if data is fit for analysis. For example, performing the treatment may enhance the results of a clustering method (if the hypothesis is related to comparison of similarities), while obscuring the results of a Principal Component Analysis (PCA) (if in contrast, the hypothesis is related with determining redundancy between metabolites) [75].

5.4 Metabolite annotation

The analysis by comparison with pure standards of different family of compounds is advisable, in order to compare the retention rates of the compounds. However, the characterization of a certain metabolite that there are no pure standards, its determination can be done by comparison with homologues of a certain family of compounds, which the detailed analysis of the fragmentation pattern. Metabolite annotation is still challenging despite all efforts made for establishing specialized databases with mass spectral properties of different metabolites. Annotation and identification levels for metabolites were defined by the Chemical Analysis Working Group of the Metabolomics Standards Initiative (MSI). Level 1 indicates compromise identified compounds, level 2 is used for putatively annotated compounds, level 3 is used for putatively characterized compound classes,

and level 4 is used for unidentified or unclassified metabolites that still can be differentiated and quantified based upon spectral data. Dark matter, also called "unknown unknowns", represents the majority of metabolites analyzed on a metabolomics experiment, because instruments collect much more information than it is currently possible to annotate [76]. It is estimated that an average of only 2% of the data can be annotated. This is even a most common problem in metabolomics analysis from animals because many databases are specialized in human-derived metabolites, or some molecular structures from animals have been solved but are absent from the reference databases. Analysis from non-model organisms tends to have a higher number of truly novel compounds, called "unknown unknowns" [77]. As it is impossible to collect spectra for every molecule in the universe, computer-generated (in silico) spectral prediction algorithms are also recommended during metabolite annotation such as CSI:FingerID (https://www.csi-fingerid.uni-jena.de/) and Competitive Fragmentation Modeling-ID (CFM-ID, https://cfmid.wishartlab.com/) for analyzing fragmentation patterns. For volatilome analysis NIST (https://www.mswil.com/software/spectral-libraries-and-databases/nist20/) and Wiley (https://www.mswil.com/software/spectral-libraries-and-databases/wiley-spectral-libraries/wiley-gcms-libraries/) electronic collections are the most used mass spectra databases. The Dictionary of Natural Products (DNP) (http://dnp.chemnetbase.com/faces/chemical/ChemicalSearch.xhtml;jsessionid=DBE98AD72918A1607A7E739064D0DB21), Pherobase (https://www.pherobase.com/), Human Metabolome Database (HMDB) (https://hmdb.ca/), METLIN (https://metlin.scripps.edu/landing_page.php?pgcontent=mainPage), MassBank Japan (http://www.massbank.jp/), MassBank Europe (https://massbank.eu/MassBank/), MassBank North America (https://mona.fiehnlab.ucdavis.edu/), Supernatural II (http://bioinf-applied.charite.de/supernatural_new/index.php), ChEMBL (https://www.ebi.ac.uk/chembl/), Mass Spectral and GC Data of Drugs, Poisons, Pesticides, Pollutants, and Their Metabolites (https://www.wiley.com/en-gb/Mass+Spectral+and+GC+Data+of+Drugs%2C+Poisons%2C+Pesticides%2C+Pollutants%2C+and+Their+Metabolites%2C+5th+Edition-p-9783527342877) and vocBinBase (https://bitbucket.org/fiehnlab/binbase/src/master/) are other useful resources. When compound annotation is not possible and only chemical class could be assigned to a metabolite it is recommended to employ the comprehensive, and computable chemical taxonomy from Classyfire (http://classyfire.wishartlab.com/). See [78] for a review focused on mass spectral databases for LC/MS- and GC/MS-based metabolomics. For the analysis of mVOCs, in 2014 was developed a software that allows the characterization of mass spectra obtained in microorganisms. It was updated in 2018 with more than 2000 compounds from more than 1000 species, which is called mVOC database 2.0 (http://bioinformatics.charite.de/mvoc) [79]. With this tool a more precise characterization of the different volatilome of the microbes studied at present is achieved.

6. Select the best statistical analysis for the research question and coherent with data pre-treatment

Select the univariate statistics according to the variables of interest. T-test, U-test, and analysis of variance (ANOVA) are the most common univariate statistics employed for data mining in volatilomics. As datasets usually include a large number of features, the significance level should be determined appropriately to reduce the number of false positives and false negatives. For reducing false positive, family wise error rate (FWER) correction, such as a Bonferroni correction, is a conservative approach, in which the p-values are multiplied by the number of

comparisons. In contrast, for reducing false negatives, false discovery rate (FDR) correction is a highly sensitive method [80].

7. Select the best suitable multivariate statistics

Multivariate statistical methods are very powerful at summarizing large and multidimensional data generated from volatilomics. Exactly as for pre-treatment methods, multivariable analysis should be chosen carefully and selected coherently with the hypothesis of interest and methods used for data pretreatment. Unsupervised approaches and supervised approaches differ in how samples are grouped within the multivariate calculations. Unsupervised solely have access to the matrix to find features useful for grouping and categorizing the samples. Clustering methods, such as hierarchical clustering (HCA), K-means clustering, self-organizing maps, principal component analysis (PCA) are among this group. Once the data have been analyzed by unsupervised methods, supervised methods (e.g. partial least squares discriminant analysis (PLD-DA), artificial neural networks, and evolutionary algorithms) should be applied for further evaluation [81]. Supervised methods have access to qualitative or quantitative traits (e.g., specie, location, body size, tissue type) and the matrix of measurements and can classify samples. Volcano plots have also recently been used to identify significantly covarying metabolites in binary comparisons. Volcano plots show each features' statistical significance, p-value, on the y-axis, and fold change along the x-axis [82].

7.1 Determine if network inference provide better insights about data interpretation

Correlation networks is a visualization tool that summarizes positive and negative correlations found between samples that represent different biological process [69]. Molecular networking organizes metabolite features from a volatilomics analysis into a connectivity network based on similarities in molecular fragmentation patterns obtained from mass spectrometry [82]. This analysis cluster families of molecules through vector correlations between fragment ions and enhance the interpretation of volatilome differentiation using a chemically informed visualization. Also, it enhances the annotation process with experimental and in silico databases [83]. When it is possible to combine Volatilomic and Genomic analysis, molecular networking can also be useful to prioritize features by linking observed natural products to their cognate biosynthetic gene clusters and gene cluster families [82].

7.2 Whenever possible, share data in public repositories

Recently, many researchers have shared raw data files on open repositories, and this has motivated computer scientists to develop modern algorithms for facilitating the comparison of MS spectra obtained in different conditions [78]. This comparison still needs human inspection from experts trained in mass spectrometry fragmentation patterns, because is not an automatic process. Some examples of sites that allow raw experimental data to be shared in public repositories include MetaboLights (http://www.ebi.ac.uk/metabolights/), the Metabolomics Workbench (https://www.metabolomicsworkbench.org/), XCMS Online (https://xcmsonline.scripps.edu/landing_page.php?pgcontent=mainPage), MetabolomeExpress (https://www.metabolome-express.org/), GNPS (https://gnps.ucsd.edu/ProteoSAFe/static/gnps-splash.jsp) and the Metabolomic Repository Bordeaux (http://services.cbib.u-bordeaux.fr/MERYB/).

8. Conclusions

Current technological advances in sample collection, extraction techniques, volatile profiling, and data processing allow that the analysis of an invisible world where VOCs mediates different ecological processes could recover a more accurate picture of the complex chemical communication that occurs in nature. Different combinations of procedures need to be followed by researchers with the aim to answer specific scientific questions or hypotheses. Microextraction techniques emerge as tools for increasing extraction efficiency and at the same time facilitating faster extraction times without the environmental impact of large volume solvent wastes. Gas chromatography has played a fundamental role to detect volatile compounds often present as trace levels. Mass spectrometry has proved to be the preferred technique for the structure elucidation of new compounds and annotation of known VOCs. Current improvements in data analysis allow to extract of more biologically relevant information from a single study and to standardize procedures for evaluating hypothesis properly. All these steps are of paramount importance to evaluate both the ecological function of these compounds and the economic value in the medical, agricultural, flavor, and fragrance industry.

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Conflict of interest

The authors declare no conflict of interest.

Abbreviations

1D	First-Dimension
2D	Second-Dimension
AC	Alternating Current
AMDIS	Automated Mass Spectral Deconvolution and Identification System
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
CAR	Carboxen
CFM-ID	Competitive Fragmentation Modeling-Id
CI	Chemical Ionization
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora

CW	Carbowax
DC	Direct Current
SDE	Simultaneous Extraction-Distillation
DHS	Dynamic Headspace
DNP	Dictionary of Natural Products
DVB	Divinylbenzene
EI	Electron Ionization
EOs	Essential Oils
FWER	Family Wise Error Rate
FDR	False Discovery Rate
GC	Gas Chromatography
GC–MS	Gas Chromatography–Mass Spectrometry
GC-O-MS	Gas Chromatography-Olfactometry-Mass Spectrometry
GNPS	Global Natural Products Social Networking
GCxGC	Comprehensive Two-Dimensional Gas Chromatography
HCA	Hierarchical Clustering
HF-LPME	Hollow Fiber Liquid-Phase Microextraction
HMDB	Human Metabolome Database
HRMS	High-Resolution Mass Spectrometry
HS	Headspace
IT-MS	Ion-Trap Mass Spectrometer
LLE	Liquid–Liquid Extraction
LPME	Liquid-Phase Microextractions
LRI	Linear Retention Indices
MS	Mass Spectrometer
MSI	Metabolomics Standards Initiative
mVOCs	Microbial Volatile Organic Compounds
m/z	Mass-To-Charge Ratio
mzmL	Mass Spectrometry Markup Language
mzXML	Extensible Markup Language
NetCDF	Network Common Data Form
NIST	National Institute of Standards and Technology
PA	Polyacrylate
PCA	Principal Component Analysis
PDMS	Polydimethylsiloxane
PLD-DA	Partial Least Squares Discriminant Analysis
PTR-MS	Proton-Transfer-Reaction Mass Spectrometry
PTV	Programmed Temperature Vaporizer
RI	Retention indexes
qMS	Quadrupole Mass Spectrometer
SAFE	Solvent Assisted Flavor Evaporation
SBSE	Stir Bar Sorptive Extraction
SDME	Single Drop Microextraction
SIFT-MS	Selected-Ion Flow-Tube Mass Spectrometry
SIM	Selected Ion Monitoring
SLM	Supported Liquid Membrane
SPE	Solid-Phase Extraction
SPME	Solid-Phase Microextraction
TOF-MS	Time-of-Flight Mass Spectrometer
VOCs	Volatile Organic Compounds

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Chapter 5

Seagrass Metabolomics: A New Insight towards Marine Based Drug Discovery

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Abstract

Metabolomics is one of the new field of "Omics" approach and the youngest triad of system biology, which provides a broad prospective of how metabolic networks are controlled and indeed emerged as a complementary tool to functional genomics with well-established technologies for genomics, transcriptomics and proteomics. Though, metabolite profiling has been carried out for decades, owing to decisive mechanism of a molecule regulation, the importance of some metabolites in human regimen and their use as diagnostic markers is now being recognized. Plant metabolomics therefore aims to highlight the characterization of metabolite pool of a plant tissue in response to its environment. Seagrassses, a paraphyletic group of marine hydrophilous angiosperms which evolved three to four times from land plants back to the sea. Seagrasses share a number of analogous acquired metabolic adaptations owing to their convergent evolution, but their secondary metabolism varied among the four families that can be considered as true seagrasses. From a chemotaxonomic point of view, numerous specialized metabolites have often been studied in seagrasses. Hence, this chapter focus the metabolome of seagrasses in order to explore their bioactive properties and the recent advancements adopted in analytical technology platforms to study the non-targeted metabolomics of seagrasses using OMICS approach.

Keywords: seagrass, metabolomics, OMICS, non-targeted, drug discovery

1. Introduction

Over the past decades, metabolomics has emerged as a valuable tool for the comprehensive profiling and metabolic networks in the biological system. Pauling et al. [1] coined the term metabolomics which was first used in 1998 and even up to 2010 metabolomics was considered as an emerging one in the science field. Reports were documented on the complete genome ([2]; Yu et al., 2002), transcriptome [3] and proteome studies [4–6], but in recent years metabolome analyses using mass spectrometry (MS) - based platforms attracted attention. Even though, metabolite profiling have been carried out for decades, due to ultimate mechanism of a molecule regulation as constituents of metabolic pathways, the prominence of some metabolites in human regimen and their use as diagnostic markers is now being recognized [7].

Currently, metabolomics is a powerful tool for characterizing the metabolites and their metabolic pathways which provides a clear metabolic picture of biological samples. Metabolites are small molecules with diverse structures that are chemically transformed during the cellular metabolism [8]. The number of metabolites is expected to be significantly lowered than the number of genes, mRNAs and proteins which reduce the sample complexity. So far, the total number of metabolites in the plant kingdom is estimated to exist between 100000 to 200000, which make the task more challenging to detect more diverse group of metabolites [9]. Plant metabolomics therefore aims to highlight the characterization of metabolite pool of a plant tissue in response to its environment [10–13]. Since, metabolomics is a balanced approach that obtains inclusive information on the cell's, tissues or organisms metabolite content with low molecular weight, their configuration likely to be changed owing to diverse environmental conditions which reproduces different genetic background [14, 15].

Recent reports on the plant metabolome bought huge challenges to analytical technologies that have been used in current plant metabolomics programs. Some analytical approaches comprise metabolite profiling, metabolite target analysis and metabolite fingerprinting which can be employed according to focus of the research and research questions [16, 17]. Metabolite profiling does not certainly determine the absolute concentrations of metabolites; rather their comparative levels within a structurally related predefined group. Targeted metabolite analysis aims to determine the absolute concentration of metabolites using specialized extraction protocols with an adapted separation and detection methods [18]. Metabolite fingerprinting generally not used to detect individual metabolites, but rather it provides a fingerprint of all compounds which can be measured for sample comparison and discrimination analysis by non-specific rapid analysis of crude metabolite mixtures. However, single analytical technology is not enough to cover the whole metabolome owing to the metabolic diversity and their broad dynamic range in cellular abundance. Accordingly, different extraction techniques and combinations of analytical methods are often employed in order to acquire diverse group of metabolite coverage.

2. Mass spectrometry-based metabolomics analysis

Historically, metabolite concentrations were achieved either by spectrophotometric assays capable of detecting single metabolites or by simple chromatographic separation of mixtures with low complexity. However, over the past decade several methods with high accuracy and sensitivity have been established for the analysis of highly complex mixtures of compounds [19–21]. These methods include gas chromatography - mass spectrometry (GC–MS), liquid chromatography - mass spectrometry (LC–MS), fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) and capillary electrophoresis - mass spectrometry (CE-MS). In addition, NMR coupled with chromatography have found great efficacy in addressing specific issues with respect to medical fields [22, 23] and conceivably more important to the unequivocal determination of metabolite structures [24]. However, NMR shows relatively low sensitivity and hence can be used for profiling the diverse group of metabolites from complex mixtures. The pros and cons of mass-spectrometric based metabolomics is given in **Table 1**.

Gas Chromatography - Mass Spectrometry assists the identification and robust quantification of few hundred metabolites in a single plant extracts, which results in inclusive coverage of the central pathways of primary metabolism [25]. GC–MS has a major advantage than other methods that it has long been used for profiling the

Metabolomics Technology	Advantages	Disadvantages
GC–MS	• Well established chromatographic-mass spectrometric technology • Less expensive • Easy to perform • Better stability and reproducibility • Universal database for metabolite identification is available	• Time consuming • Can only analyze volatile compounds upon derivatization • Not able to identify the unknown compounds
LC–MS	• Highly sensitive • Method for sample pre-treatment is simple • Wide coverage of metabolite could be identified • Scanning range for different ions is usually less • Different columns can be used depends on the polarity of compounds	• Less reproducibility • Highly Expensive • Difficult to identify highly polar and charged metabolites
FT-ICR-MS	• High resolution mass spectrometric technique • Extemely high mass accuracy • High acquisition rates • Highly flexible	• Involves series of steps • Use large super conducting magnets • Highly expensive than GC–MS and LC–MS

Table 1.
Advantages and disadvantages of mass-spectrometric based metabolomics.

metabolites and therefore it has stable protocols for machine setup, their maintenance with chromatogram evaluation and interpretation. Though, single analytical system cannot cover the whole metabolome, GC–MS has a quite broad coverage of compounds classes including organic and amino acids, sugars, sugar alcohols, lipophilic compounds and phosphorylated intermediates [26]. During method validation, recovery experiments of all measurable compounds have been done and for unknown compounds, recombination experiments were executed to determine the recovery rates in which the extracts of two plant species are evaluated independently and also with mixtures [27, 28]. Liquid chromatography-based methods offer numerous advantages such as detection of broad range of metabolites, as they suffer from the lower reproducibility of retention time. In addition, they are more susceptible to ion suppression effects due to the predominant use of electrospray ionization, which renders the precise quantification more difficult [29–31].

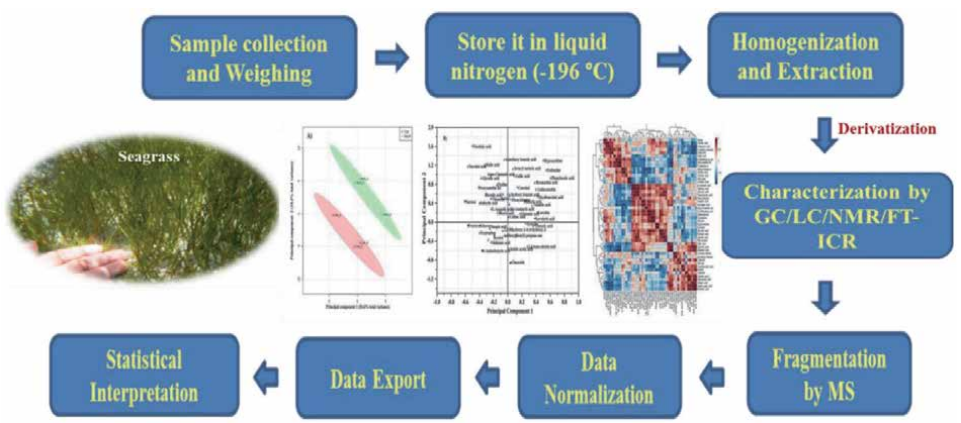

Figure 1.
Schematic overview of MS based metabolomics.

FT-ICR-MS and CE-MS has been reported to be worth mentioning, where FT-ICR-MS has unsurpassed mass accuracy thereby allows the researcher to obtain an idea about the chemical composition of the specific compounds. In case of CE-MS, the low-abundance metabolites can be detected and affords good chromatographic separation [32, 33]. Of these techniques, GC–MS is mostly preferred for the separation of low molecular weight metabolites which can be either volatile or can be converted into volatile and thermally stable compounds via chemical derivatization prior to the analysis [34]. The experimental procedure for GC–MS based metabolomics analysis is represented in **Figure 1**.

3. Derivatization

Derivatization is a process by which a compound is chemically modified to produce a new compound that has properties which are more amenable to specific analytical procedure. Samples analyzed by gas chromatography requires derivatization in order to make them suitable for analysis. Derivatization procedure imparts volatility, decreases the adsorption in the injector, increases the stability of compounds; improve the resolution and detectability between coeluting compounds and overlapping which assist in structure determination [35]. A good derivatizing reagents and the procedure should produce the compound of interest with desired chemical modification and be efficient, reproducible and non-hazardous (www.piercenet.com). For GC, derivatization reaction can be done by three basic types: silylation, acylation and alkylation. Silylating reagents react with compounds containing active hydrogen and are most frequently used in GC. Acylating reagents react with compounds having high polar functional groups such as amino acids or carbohydrates. While alkylating reagents target the active hydrogen's on amines and acidic hydroxyl group [36].

4. Seagrasses

Seagrasses, a marine hydrophilus angiosperm live entirely in an estuarine or in the marine environment and nowhere else [37]. Seagrass ecosystem act as a breeding and nursery ground for numerous organisms and also help in promoting the commercial fisheries. It is considered to be one of the most productive ecosystems that retain the structural complexity and biodiversity shed light to some researchers to describe seagrass community as marine representation of the tropical rainforests [38]. Currently, seagrasses are assigned to four families Hydrocharitaceae, Cymodoceaceae, Posidoniaceae and Zosteraceae (den [39, 40]). According to angiosperm Group III System, all four families occurred exclusively to monocot order Alismatales [41]; while Les and Tippery [40] favored to treat the same clades as a subclass Alismatidae. The family hydrocharitaceae comprises of three genera namely *Enhalus, Halophila and Thalassia* and the Cymodoceaceae encompasses the highest variety of genera includes Amphibolis, *Halodule, Syringodium, Cymodocea* and *Thalassodendron*. Likewise, the Zosteraceae comprises of *Zostera, Heterozostera, Nanozostera, Phyllospadix* which are exclusive marine organisms and the family Posidniaceae are monogeneric [42–44]. Therefore, seagrasses can be considered as a unique ecological group occurring worldwide in different climatic zones and sharing their metabolic features with their terrestrial counterparts [37]. However, their metabolism must have undergone several adaptations to survive and colonize in shorelines and oceans worldwide [37]. The seagrass ecosystems and their functions were given in **Table 2**.

Seagrass and Seagrass beds	Properties
Common names	Eelgrass, turtle grass, tape grass, shoal grass, and spoon grass
Families: 4	• Hydrocharitaceae • Posidonaceae • Zosteraceae • Cymodoceaceae
Total Species: 72	In India: 14 species exist
Habitat	Found in salt and brackish water
Depth	1 meter - 58-meter depth
Seagrass Parts	
Leaves • Chloroplast • Lacunae • Cuticle • Viens	Photosynthesis Help for buyoncy Exchange oxygen and carbondioxide in the water column Transport nutrients throughout the plants
Rhizome	Stabilize the seagrass beds under wave action
Roots	Absorb nutrients from the soil and transport to the plants
Growth and reproduction	Sexual reproduction and asexual clonal growth
Biodiversity • Small bodied	Small thin leaves Small rhizome "Guerilla" strategy Short lived with fast turnover Low biomass Abundant flowering Many small seeds and seed bank
• Large bodied	Large thick leaves Large rhizome "Phalanx" strategy Long-lived with slow turnover High biomass and holds space Patchy flowering Few large seeds and seeds germinate rapidly
Ecosystem benefits	Lungs of the sea Creation of Living Habitat Foundation of Coastal Food Webs Blue Carbon

Table 2.
Seagrass ecosystem and their benefits.

5. Seagrass metabolome

Seagrasses share a number of analogous acquired metabolic adaptations owing to their convergent evolution, but their secondary metabolism varies among the four families that can be considered as true seagrasses. During the period of ancient Tethys Sea, approximately 90 million years ago surrounded by Africa, Gondwana-land, and Asia, the terrestrial like species returned to the sea and thus explaining the "terrestrial-like" chemical profile of seagrass. From a chemotaxonomic viewpoint, numerous secondary metabolites have been often studied in seagrasses. The metabolome of seagrasses may differ with respect to geographical location, substrates and other physiological factors includes wide fluctuations in the salinity which are prone to synthesize novel metabolites with defined physiological, biochemical, defense and ecological roles [45, 46]. Preliminary suggestions confirmed

that seagrasses have pharmaceutically potent bioactive secondary metabolites [47], that are directed to prove to be a lead molecule for drug discovery [48]. The status of metabolomic study in seagrasses reported so far is tabulated in **Table 3**.

Seagrass	Methods used	Derivation method	Results	Potential application	Reference
Zostera marina Zostera noltii	GC/TOF	Trimethyl silylation	Adaptivemechanisms are involved through metabolic pathways to dampen the impacts of heat stress	Sucrose, fructose, and myo-inositol were identified to be the most responsive metabolites of the 29 analyzed organic metabolites.	Gu et al. [49]
Cymodocea nodosa	GC-QTOF-MS	Trimethyl silylation	Growth promoting metabolites (sucrose, fructose, myo-inositol, heptacosane, tetracosane, stigmasterol, catechin and alpha-tocopherol) were lower close to the zone, whereas metabolites involved with stress-response (alanine, serine, proline, putrescine, ornithine, 3,4-dihydroxybenzoic acid and cinnamic acid) were identified	Metabolomic fingerprinting of seagrass provides opportunities for early detection of environmental degradation in marine ecological studies	Kock et al. [50]
Halodule pinifolia	GC–MS	Trimethylsilyl etherification	GC–MS analysis revealed the presence of thirty-five compounds which include flavonoids, sugars, amino acids and plant hormones	Study has explored a newer marine source, *H. pinifolia* for RA, which is an emerging potential preclinical chemical entity	Jeyapragash et al. [51]
Zostera marina	GC–MS	Trimethyl silylation	Decreased carbohydrate decomposition products and tricarboxylic acid (TCA) cycle intermediate products, indicating that the energy supply of the eelgrass may be insufficient at high temperature	composition of the membrane system of eelgrass may change at high temperature and implying that high temperature may cause the membrane system to be unstable	Gao et al. [52]
Halodule pinifolia	GC–MS	Trimethyl silylation	98 metabolites in wild and 125 metabolites in SCC were identified. 77 primary and secondary metabolism pathways in wild, while 73 metabolism pathways in SCC were reported	Baseline information on *H.pinifolia* metabo-lism in the marine and artificial environments	Jeyapragash et al. [53, 54]

Seagrass	Methods used	Derivation method	Results	Potential application	Reference
Zostera muelleri	NMR	Trimethyl-silylation	Several potential bioindicators of low-light stress: a reduction of soluble sugars and their derivatives, glucose, fructose, sucrose and myo-inositol, N-methylnicotinamide, organic acids and various phenolic compounds were identified	Metabolomics measurements may be useful bio-indicators of low-light stress in seagrass	Griffith et al. [55]
Halodule pinifolia	GC–MS	Trimethyl-Silylation	Three thermo-protective metabolites such as trehalose (sugar), glycine betaine (amino acid) and methyl vinyl ketone (organic acid) were profiled from *H. pinifolia* (45°C) and is the first report on the occurrence of glycine betaine and methyl vinyl ketone from seagrasses and other aquatic species so far	Facilitate the further research on identifying gene to metabolite networks for an effective management of seagrass conservation by genetic manipulation	Jeyapragash et al. (2021)

Table 3.
Status of metabolomics studies in seagrasses.

Primary metabolites from seagrasses reported to be similar, to that of any other terrestrial angiosperms [56]. Despite the higher phenol content, seagrasses found to be rich source of protein which alleviates the chronic problem of protein deficiency in developing countries like India [47]. In addition, seagrasses are a rich source of secondary metabolites such as simple phenolic compounds, phenylmethane and phenylethane derivatives, flavonoid and volatile derivatives with high commercial value [38]. Jeyapragash et al., reported that the plant growth regulators enhance the production of flavonoid production in the callus and cellular suspension cultured cells of seagrass *H. pinifolia* (**Figure 2**) Though, quantum of research has been published with respect to metabolome of seagrasses, there is still a dearth of knowledge as compared to the terrestrial plants. Seagrass offers remarkable opportunities to derive new commercially valuable phytochemicals when compared to algae [57, 58]. Therefore, the metabolite content from seagrasses constitutes another treasure of the ocean. Henceforth, the knowledge on metabolomics analysis from seagrass is decisive for understanding the complete metabolite picture and to explore their bioactive properties.

Seagrasses, the only higher plants solely living in the marine habitats and are ultimate importance for marine ecological systems close to the shorelines. Several studies dealt with the function of seagrasses as primary producers, shelter and food for fish, turtles and invertebrates as well as spawning areas for these organisms [59–61]. The reviews existing on seagrasses with different focus than the present one deal in more detail with other aspects of the ecological role of seagrasses,

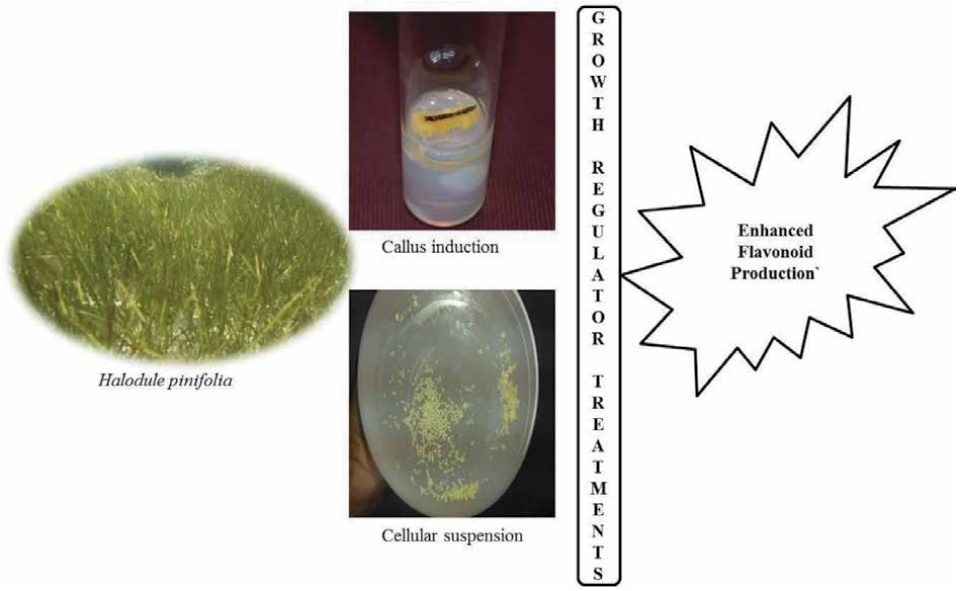

Figure 2.
Growth regulators mediated flavonoid production in callus and cellular siuspenison of H. pinifolia.

particulary the metabolite classes which are very few and primitive. Seagrasses reported to share the most features of primary and secondary metabolites with respect from the Alismatales order which live in land and freshwater habitats [62]. Kannan and Kannal [63] and Pradheeba et al. [56] reported that primary metabolites such as carbohydrate, protein and lipid content from seagrasses acts as a rich source of nutritional value and was eveidenced by the obvious increase in the carbohydrate content o*f E. acoroides, T. hemporichii* [63], *C. rotundata* [56] and lipid content of *C. nodosa* [64]. Higher protein content form seagrasses have also been reported in *E. acoroides* [63], *Ruppia cirrhosa* [64], *C. serrulata, S. isoetifolium, H. ovalis, H. pinifolia* [47], *C. rotundata* and *H. uninervis* [56] that are found to accumulate manximum concentration than the seaweeds [47].

Secondary metabolism occurs in seagrasses depends on the season and environmental conditions and was reported as a rich source of diverse natural products from simple to conjugated phenolic compounds such as phenolic acids, flavones, tannins and lignins [65, 66]. It was also reported that *P. oceanica* harbors compounds ranging from simple phenol derivatives, phenylmethane, phenyethane and phenypropane derivatives [67]. Athiperumalsami *et al*. [47] reported the occurrence and absence of alkaloids, antroquinones, catechins, coumarins, flavonoids, phenols, saponins, quinones, tannins and secondary metabolites in the tropical seagrass *Cymodocea serrulata*, *Halodule pinifolia* and *Syringodium isoetifolium*. Heglmier and Zindron [62] reported 51 natural products in *P. oceanica* that includes phenols, flavones, phenylmethane, ethane derivatives. Though reports availed on the seagrass derivatives, limited study dealt with the chemistry of secondary metabolites of seagrasses [62, 68]. The list of metabolites profiled and the metabolic pathways from wild seagrasses are tabulated in **Table 4** and **Figure 3**.

The presence of sulphated flavones was reported to be accumulated in *Halophila* and *Thalassia* species [69] and in *Z. marina* [70], but not found in *Syringodium* spp. and *P. oceanica*. Mc Millan [71] found the presence of either flavones or phenolic acid in 43 segrass species while the sulphated flavones found specifically in five species namely, *Zostera, Phyllospadix, Enhallus, Thallasia* and *Halophila* species.

Compound Name	Molecular Formula	Molecular weight (g/ mol)	Exact Mass (g/mol)
D-Glucose	$C_6H_{12}O_6$	180.156	180.063
Maltose	$C_{12}H_{22}O_{11}$	342.297	342.116
D-Fructose	$C_6H_{12}O_6$	180.156	180.063
Sucrose	$C_{12}H_{22}O_{11}$	342.297	342.116
Inositol	$C_6H_{12}O_6$	180.156	180.063
Methyl alpha-D-Glucopyranose	$C_7H_{14}O_6$	194.183	194.079
D-Galactose	$C_6H_{12}O_6$	180.156	180.063
Lactose	$C_{12}H_{22}O_{11}$	342.297	342.116
L-Rhamnose	$C_6H_{12}O_5$	164.157	164.068
D-Ribose	$C_5H_{10}O_5$	150.13	150.053
Adenosine-2′:3′- cyclic monophosphate	$C_{10}H_{14}N_5O_7P$	347.224	347.063
N-Acetyl-Î-D-glucosamine	$C_8H_{15}NO_6$	221.209	221.09
Aspartyl-Leucine	$C_{10}H_{18}N_2O_5$	246.263	246.122
Glycine	$C_2H_5NO_2$	75.067	75.032
Threonine	$C_4H_9NO_3$	119.12	119.058
Valine	$C_5H_{11}NO_2$	117.148	117.079
Proline	$C_5H_9NO_2$	115.132	115.063
Alanine	$C_3H_7NO_2$	89.094	89.048
Thiamine	$C_{12}H_{17}N4OS^+$	265.355	265.112
Methionine	$C_5H_{11}NO_2S$	149.208	149.051
Phenylanaline	$C_9H_{11}NO_2$	165.192	165.079
Tyrosine	$C_9H_{11}NO_3$	181.191	181.074
Methyl Pyroglutamate	$C_6H_9NO_3$	143.142	143.058
Glutamic acid	$C_5H_9NO_4$	147.13	147.053
Vanillic acid	$C_8H_8O_4$	168.148	168.042
Oxalic acid	$C_2H_2O_4$	90.034	89.995
gamma-Aminobutyric acid	$C_4H_9NO_2$	103.121	103.121
Citrate	$C_6H_5O_7^{-3}$	189.099	189.004
Stearic acid	$C_{18}H_{36}O_2$	284.484	284.272
Hexadecanoic acid	$C_{16}H_{32}O_2$	257.422	257.244
Potassium Gluconate	$C_6H_{11}KO_7$	234.245	234.014
Nicotinic acid	$C_6H_5NO_2$	123.111	123.032
Phosphoric acid	H_3PO_4	97.994	97.977
Sodium Pyrophosphate	$Na_4P_2O_7$	265.9	265.871
Acetamide	C_2H_5NO	59.068	59.037
Decanedioic acid	$C_{12}H_{22}O_4$	230.304	230.152
Indoleaceteic acid	$C_{10}H_9NO_2$	175.187	175.063
1-Napthaleneacetic acid	$C_{12}H_{10}O_2$	186.21	186.068

Compound Name	Molecular Formula	Molecular weight (g/ mol)	Exact Mass (g/mol)
4-Hydroxybenzaldehyde	$C_7H_6O_2$	122.123	122.037
2,4-dihydroxybenzaldehyde	$C_7H_6O_3$	138.122	138.032
3,4-Dihydroxybenzoic	$C_7H_6O_4$	154.121	154.027

Table 4.
List of metabolites identified from wild seagrasses.

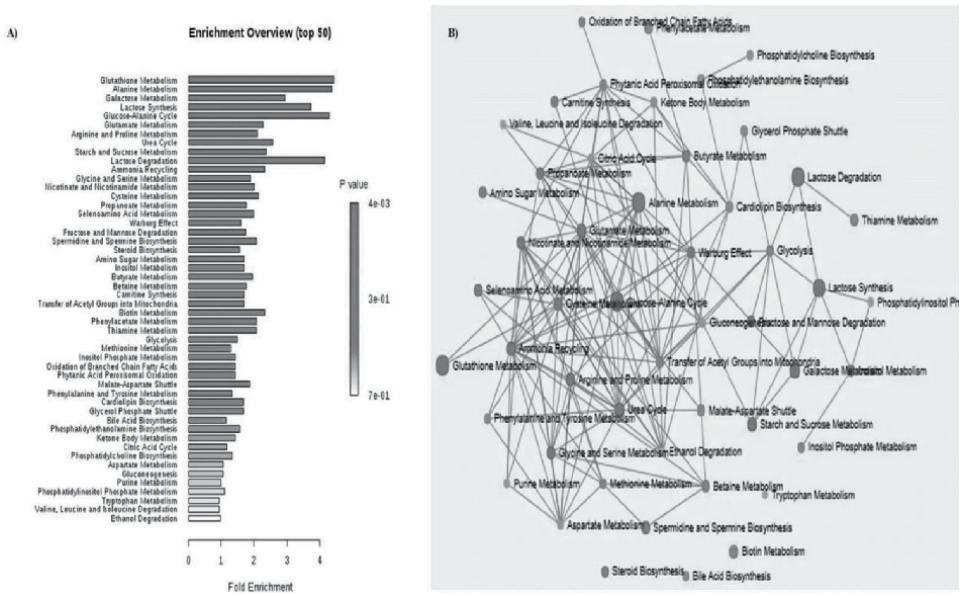

Figure 3.
Distribution of metabolic pathways of differential metabolites derived from wild seagrass *(A) and their active networks (B) (adapted from [53, 54]).*

The sulphated flavonoids have also been traced out in *Halophila ovalis* and *Thalassia testudinum* [72]. It was found that the flavonoid glycosides and acyl derivatives in *P. oceanica* and 15 flavonoid derivatives in *Halophila johnsonii* [73]. Similar to flavonoids and phenolic compounds, the sterol composition has also been reported from temperate seagrasses than the tropical seagrass. The presence of long cahin fatty acids in *P. oceanica* [74] and α-hydroxy fatty acids in *Z. mulleri* [75]. The polar lipids and fatty acids from *Z. marina* and Phyllospadix iwatensis [76], phospholipids and glycolipids from *Z. marina* [77] and steroids, fatty acids from *Z. japonica* [78] confirms the prevalence of volatile compounds in segrasses.

Jeyapragash et al. [53] investigated the systematic identification and characterization of metabolic changes in wild and SCC of *H. pinifolia* using GC–MS based metabolomics approach. It was found that the wild sample accumulated 98 metabolites, while SCC with 125 metabolites along with their relative abundance. The metabolites profiled from wild and SCC was used to map their biochemical pathways. Interestingly, the accumulated metabolites in wild were spanned with 77 primary and secondary metabolism pathways and 73 pathways in SCC. Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway analysis confirmed insightful biochemical alterations occurred in wild and SCC such as glutathione metabolism, glutamate metabolism, phenylalanine and tyrosine metabolism in wild

with significant variations in citric acid metabolism, glycolysis, gluconeogenesis, oxidative phosphorylation related pathways of SCC respectively. Random forest analysis helped to group top 15 metabolites and their correct classification. The data obtained will provide baseline information on *H. pinifolia* metabolism in the marine and artificial environments. Furthermore, it helps to study the stress responsive mechanisms of seagrasses in the marine environment which would further aid in the dissection stress tolerance mechanism.

In addition, Jeyapragash et al. [79] reported the heat stress responsive metabolomics analysis of seagrass *H. pinifolia* in the marine environment. Since, ocean warming is a major global concern in the marine environment, the study focussed to understand the tolerance mechanism of seagrass *Halodule pinifolia* under temperature stress (24, 29, 37, and 45°C) using OMICS approach. Ecophysiologiacal responses such as net photosynthesis (ΔF/F'm) and dark respiration (Fv/Fm) were also studied. Results found that photosynthetic efficiency (ΔF/F'm) significantly reduced due to heat stress, while the rate of dark respiration rate increased as compared to the control (24°C), respectively. Metabolomics study revealed that heat stress could cause huge metabolic alterations with respect to sugar, amino acids and organic acids. Interestingly, thermo-protective compounds such as trehalose (sugar), glycine betaine (amino acid) and methyl vinyl ketone (organic acid) were accumulated from *H. pinifolia* (45°C) which was the first report on the occurrence of glycine betaine and methyl vinyl ketone from seagrasses and other aquatic species so far. These findings would help the research groups to focus on the gene to metabolite networks mechanism for an effective management of seagrass conservation by genetic manipulation.

6. Seagrass cell suspension culture

Plants are considered as the factories of chemical compounds produced in order to carry out their biochemical pathways for survival and propagation [80]. All plants produce secondary metabolites which gained importance in pharmaceutical applications since ancient periods. The plant-based drug discovery gained importance with the development of anti-infectious and anti-cancer drugs which contributes to new bioactive molecules that are being isolated for the treatment of other diseases such as diabetes and obesity [81]. However, the important plant derived drugs are obtained commercially by the extraction from their respective plants. Currently, the natural plant habitats are vanishing due to environmental and geo-political instabilities and so making it very difficult to procure important secondary metabolites and in the process many potential bioactive compounds have been left undiscovered. Plant cell culture is considered as a promising alternative approach for producing the bioactive compounds that are challenging to be obtained by chemical synthesis or plant extraction [82]. Plant cell culture studies have been carried out on the basis of the totipotent nature, in which the cell has the full set of genes necessary for secondary metabolisms [83]. The production of secondary metabolite via plant tissue culture have been commercialized sincelate 1950s, when atropine from the roots of *Atropa belladonna* was accumulated in roots and callus [84]. The list of metabolites profiled and their metabolic pathways from SCC of seagrasses were shown in **Figure 4** and tabulated in **Table 5**.

Improved plant cell culture techniques made possible to increase the target metabolite production under *in-vitro* conditions. However, agronomically focussed biotechnology is capable to make use of plant tissue culture; such methods are not usually available to marine biologist and notably, *in-vitro* culture of seagrasses has been far more problematic due to lack of the suitable culturing conditions [85].

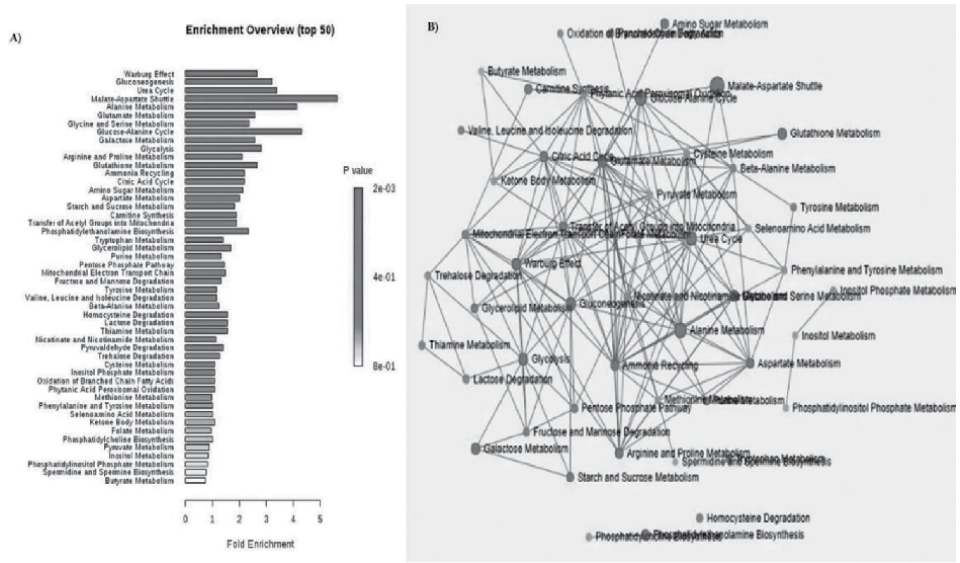

Figure 4.
Distribution of metabolic pathways of differential metabolites derived from SCC of seagrass *(A) and their active networks (B) (adapted from [53, 54]).*

Available literature has brought that 14% of seagrasses are at an elevated risk of extinction under IUCN red list of threatened species [86]. Though numerous studies have been addressed the in-vitro culture of seagrass [38, 87–93], no report exist on the metabolomics analysis from suspension cultured cells of seagrass. *In-vitro* propagation techniques of different seagrass species for restoration and protocol for seagrass protoplast isolation are prevailing [94–98]. Among all these protocols, Carpeneto *et al*. [96] was successful in the cell wall regeneration of protoplast from *Posidonia oceanica* and *Cymodocea nodosa.* Recently, establishment of cell suspension culture has been achieved in *C. nodosa* [99], *Halodule pinifolia, C. rotundata* and *C. serrulata* [38]. Jeyapragash et al., [53, 54] reported that the metabolites synthesized from seagrass *H. pinifolia* in the marine and artificial environment will be highly similar with a total of 98 metabolites in wild and 125 metabolites in SCC respectively and however the cellular suspension accumulated the higher content. The study suggested that the use of seagrass cellular suspension for metabolomics engineering will provide a new facet for novel metabolite identification and characterization. Nevertheless, there lay famine knowledge in the metabolite accumulation pattern and their different biotechnological and pharmaceutical applications. Enhancement of metabolite biosynthesis can also be achieved via the precursors or elicitor treatments of plant cells (**Figure 5**). Precursors are the compound which act as intermediate in or at the beginning of biosynthetic route, treatments using the same stands a good chance of increasing the yield of the desired product. Exogenous supply of biosynthetic precursors to the culture medium induces the high yield of targeted products (Whitmer *et al*., 1998; [100]).

Plant also synthesizes the secondary metabolites to protect themselves in response to various environmental stresses. It might be physical, chemical or a biological factor which induces the higher secondary metabolism known as elicitors. The use of elicitors in cell suspension cultures has been developed to enhance the yield of secondary metabolites, wherein elicitation of target compounds can be induced by the addition of trace number of elicitors [101]. Biotic and abiotic

Query	Match	HMDB	PubChem
Rosmarinic acid	$C_{18}H_{16}O_8$	360.318	360.085
Caffeic acid	$C_9H_8O_4$	180.159	180.042
p-Coumaric acid	$C_9H_8O_3$	164.16	164.047
Protocatacheuic acid	$C_7H_6O_4$	154.121	154.027
p-Anisic acid	$C_8H_8O_3$	152.149	152.047
Vanillic acid	$C_8H_8O_4$	168.148	168.042
Naringenin	$C_{15}H_{12}O_5$	272.256	272.068
4-hydroxybenzoic acid	$C_7H_6O_3$	138.122	138.032
Fructose-6-phosphate	$C_6H_{13}O_9P$	260.135	260.03
Glucose-6-phosphate	$C_6H_{13}O_9P$	260.135	260.03
Glucose	C6H12O6	180.156	180.063
Phosphoenol pyruvic acid	$C_3H_5O_6P$	168.041	167.982
Pyruvic acid	$C_3H_4O_3$	88.062	88.016
Citric acid	$C_6H_5O_7^{-3}$	189.099	189.004
Fumaric acid	$C_4H_4O_4$	116.072	116.011
3-PGA	$C_3H_7O_7P$	186.056	185.993
Ketoglutaric acid	$C_5H_6O_5$	146.098	146.022
Malic acid	$C_4H_6O_5$	134.087	134.022
Succinic acid	$C_4H_6O_4$	118.088	118.027
Mannose	$C_6H_{12}O_6$	180.156	180.063
Oxaloacetic acid	$C_4H_4O_5$	132.071	132.071
Sucrose	$C_{12}H_{22}O_{11}$	342.297	342.116
D-Fructose	$C_6H_{12}O_6$	180.156	180.063
Raffinose	$C_{18}H_{32}O_{16}$	504.438	504.169
Trehalose	$C_{12}H_{22}O_{11}$	342.297	342.116
Turanose	$C_{12}H_{22}O_{11}$	342.297	342.116
Mannitol	$C_6H_{14}O_6$	182.172	182.079
Inositol	$C_{12}H_{22}O_{11}$	342.297	342.116
Xylitol	$C_5H_{12}O_5$	152.146	152.146
Alanine	$C_3H_7NO_2$	89.094	89.048
Aspargine	$C_4H_8N_2O_3$	132.119	132.053
Aspartic acid	$C_4H_7NO_4$	133.103	133.038
Glutamic acid	$C_5H_9NO_4$	147.13	147.053
Glycine	$C_2H_5NO_2$	75.067	75.032
Proline	$C_5H_9NO_2$	115.132	115.063
Serine	$C_3H_7NO_3$	105.093	105.043
Threonine	$C_4H_9NO_3$	119.12	119.058
Valine	$C_5H_{11}NO_2$	117.148	117.079
2,4-dihydroxybenzoic acid	$C_7H_6O_3$	138.122	138.032
2-hydroxybutyric acid	$C_4H_8O_3$	104.105	104.047

Query	Match	HMDB	PubChem
Gamma-aminobutyric acid	$C_4H_9NO_2$	103.121	103.121
Dimethylamine	$(CH_3)_2NH$	45.085	45.058
Ethanolamine	C_2H_7NO	61.084	61.053
Thiamine	$C_{12}H_{17}N_4OS^+$	265.355	265.112
Nicotinic acid	$C_6H_5NO_2$	123.111	123.032
Pyridoxine	$C_8H_{11}NO_3$	169.18	169.074
Phenylanaline	$C_9H_{11}NO_2$	165.192	165.079
Tryrosine	$C_9H_{11}NO_3$	181.191	181.074
Shikimic acid	$C_7H_{10}O_5$	174.152	174.053
Acotinic acid	$C_6H_6O_6$	174.108	174.016
Xylonic acid	$C_5H_{10}O_6$	166.129	166.048
Ascorbic acid	$C_6H_8O_6$	176.124	176.032
Guanine-2′3’-cyclic monophosphate	$C_{10}H_{12}N_5O_7P$	345.208	345.047
Pantothenate	$C_9H_{16}NO_5$	218.229	218.103
Sphingosine	$C_{18}H_{37}NO_2$	299.499	299.28
N-acetylglucosamine	$C_8H_{15}NO_6$	221.209	221.09
Aspartyl leucine	$C_{10}H_{18}N_2O_5$	246.263	246.122
2-hydroxy glutaric acid	$C_5H_8O_5$	148.114	148.037
Glyceric acid	$C_3H_6O_4$	106.077	106.027
Chlorogenic acid	$C_{16}H_{18}O_9$	354.311	354.095
Rhamnose	$C_6H_{12}O_5$	164.157	164.068
Guanosine monophosphate	$C_{10}H_{15}N_5O_{11}P_2$	443.202	443.024
Ribose	$C_5H_{10}O_5$	150.13	150.053
Adenosine-2′3’-cyclic Monophosphate	$C_{10}H_{14}N_5O_7P$	347.224	347.063
Dihydroquercetic acid	$C_{15}H_{12}O_7$	304.254	304.058
Adenosine-2-Monophosphate	$C_{10}H_{14}N_5O_7P$	347.224	347.063
p-hydroxybenzoic acid	$C_7H_6O_3$	138.122	138.032
Quinic acid	$C^7H^{12}O^6$	192.167	192.063
Tryptophan	$C^{11}H^{12}N^2O^2$	204.229	204.09
Pyroglutamic acid	$C_5H_7NO_3$	129.115	129.043
Salicylic acid	$C_7H_6O_3$	138.122	138.032
Methionine	$C_5H_{11}NO_2S$	149.208	149.051
Lactic acid	$C_3H_6O_3$	90.078	90.032
Isovaleric acid	$C^5H^{10}O2$	102.133	102.068
2-oxyglutaric acid	$C_5H_6O_5$	146.098	146.022
2-hydroxyisobutyric acid	$C_4H_8O_3$	104.105	104.047
1-methylnicotinic acid	$C_7H_8NO_2^+$	138.146	138.056
Hypoxanthine	$C_5H_4N_4O$	136.114	136.039

Query	Match	HMDB	PubChem
Indole Acetic acid	$C_{10}H_9NO_2$	175.187	175.063
Naptheline acetic acid	$C_{12}H_{10}O_2$	186.21	186.068

Table 5.
List of metabolites identified from suspension cultured cells seagrasses.

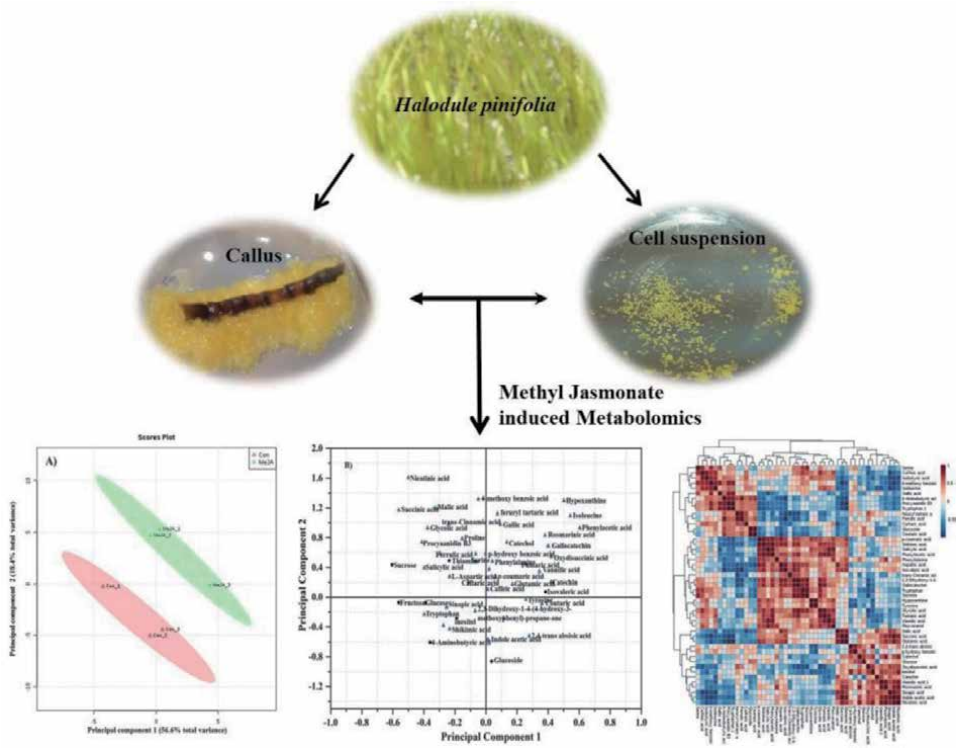

Figure 5.
Elicitor induced comparative metabolomics of wild and cellular suspension of seagrass.

elicitors are available which depends on the target compounds that need to be synthesized.

7. Seagrasses—a source for marine based drug discovery

Ravn *et al*. [102] reported that phenolic acids such as *p*-coumaric acid, caffeic acid, ferulic acid, protocatacheuic acid, *p*-hydroxybenzoic acid, vannillic acid, gentisic acid found predominant in *Halophila ovalis, Thalassia hemprichii, Halodule* sp., *Cymodocea* spp., *Enhalus acoroides, Syriingodium isoetifolium* and other seagrass species. In addition, the pharmaceutically potent rosmarinic acid has been isolated from *Z. marina* [102] *Z. noltii* [103], *H. pinifolia* [51] and also from the detritus of *Z. noltii* and *Z. marina* [57]. Along with rosmarinic acid, caffeic acid and chlorogenic acid found to be present in some trace amounts, since caffeic acid acts as a precursor for roamarinic acid in the shikimic acid biosynthesis. It was also been reported that the predominance of caffeic acid was accumulated in the leaves of *P. oceanica* [104] and *Thalassodendron ciliatum* [105]. Jeyapragash *et al*. [51], profiled 45 metabolites

from *Halodule pinifolia*, which includes caffeic acid, coumaric acid, chlorogenic acid and rosmarinic acid which found predominant than other compounds. Recently, biofilm associated, multidrug resistant *Pseudomonas aeruginosa* infection remain a challenging problem in the clinical field since the conventional antibiotic therapy are largely inefficient and new approaches are needed. Inactivating the QS virulence mechanism with anti-infective agent is an attractive approach to prevent bacterial infections without resistance development [54]. Seagrass *Halodule pinifolia* (Miki) Hartog has been shown to exhibit potential antimicrobial activities against *P. aerugonasa* PAO1. Preliminary screening on antibiofilm activity showed that the methanolic extract of *H. pinifolia* exhibited potential inhibition of biofilm formation (96%) as compared to the control respectively. Eight bioactive compounds such as 4-hydroxybenzoic acid, rosmarinic acid, 4-methoxybenzoic acid, *p*-coumaric acid, protocatacheuic acid, caffeic acid, naringenin, vanillic acid, were profiled. Of these compounds, 4-methoxybenzoic acid (4-MBA) showed maximum bacterial growth inhibition that act as a lead molecule with minimum inhibitory concentration (MIC). Furthermore, 4-MBA at MIC concentration reduced the virulence factors and down regulated the level of QS mediated virulence transcripts. The study suggests that seagrasses may act as a newer source for the marine based drug discovery and may act as anti-infective agent against biofilm-mediated harmful pathogens.

8. Conclusion

To summarize, experiments in seagrass metabolomics to date helped us to validate a vast array of metabolites and their alterations in response to various stress mechanisms. This approach has previously enabled to recognize a large number of metabolites whose accumulation is affected upon the exposure of organisms under stress conditions. Nevertheless, despite the many advancements that have been achieved in this field, much work is still needed to identify the seagrass metabolites and their novel metabolic pathways connected to stress response and their tolerance mechanism and to interpret the extensive organization and interaction among gene to metabolite networks. This chapter provides knowledge on the systematic identification and metabolic characterization of seagrass metabolites using metabolomics approach. The bioactive potential of compounds derived from seagrasses paves a way to lead as potential inhibitors of many harmful pathogens in the pharmaceutical sectors and therefore, seagrass explored as newer marine source for the development of plant-based drugs. Further, in-vitro cultures of seagrass afford an alternate model for the up-regulation of enhanced bioactive compound synthesis. Moreover, various stress related metabolomics approach of wild seagrasses should be studied in order to derive diverse group of bioactive metabolites as much as possible, so as to fill the knowledge gap of seagrass metabolites and step forward towards the commercialization of bioactive natural products from seagrasses.

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Conflict of Interest

The authors declare that there is no conflict of interest.

Abbreviations

GC–MS	Gas Chromatography–Mass Spectrometry
LC–MS	Liquid Chromatography Mass Spectrometry
FT-ICR-MS	Fourier Transform- Ion Cyclotron Resonance-Mass Spectrometry
SCC	suspension cultured cells
NMR	Nuclear Magnetic Resonance
4-MBA	4-methoxy benzoic acid
MIC	minimum inhibitory concentration
KEGG	Kyoto Encyclopaedia of Genes and Genomes
IUCN	International Union for conservation of Nature
CE-MS	Capillary Electrophoresis-Mass Spectrometry

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Chapter 6

Metabolic Profiling of Transgenic Tobacco Plants Synthesizing Bovine Interferon-Gamma

Vladislav V. Yemelyanov, Roman K. Puzanskiy, Mikhail S. Burlakovskiy, Lyudmila A. Lutova and Maria F. Shishova

Abstract

Interferon-gamma belongs to a large family of cytokines – multifunctional secreted proteins involved in animal non-specific immune response. Previously inbred lines of *Nicotiana tabacum* L. plants harboring a heterologous gene of bovine interferon-gamma *Bt-sIFNG* under the control of a constitutive 35S CaMV promoter have been created by *Agrobacterium*-mediated genetic transformation. The antiviral and immunomodulatory activities of plant-produced interferon-gamma in bovine cell culture and laboratory animals (mice) were observed. A state-of-the-art GS-MS technique has been used to identify the possible effect of the transformation on the plant's metabolome. Total profiles included 350 metabolites from leaves, among which 150 substances were identified up to their class and 80 up to the exact metabolite. Metabolite profiling revealed that plants able to synthesize interferon-gamma are characterized by a higher level of amino acids and other substances involved in nitrogen metabolism. In transgenic plants intensification of the secondary metabolism was also detected. Some alterations were distinguished in plant metabolome depending on cultivation conditions.

Keywords: interferon-gamma, *Nicotiana tabacum*, transgenic plant, metabolome

1. Introduction

Metabolic alterations triggered by biotic and abiotic factors in the environment are the basis of a plant development and adaptation. They are closely related to the shifts in primary as well as secondary metabolism that are responsible for synthesis and accumulation of different regulatory and defensive metabolites [1–3]. Metabolic profiling is a useful tool for distinguishing these alterations. Powerful approaches to provide metabolomic investigations are based on recent technology such as GC–MS (gas chromatography–mass spectrometry), LC–MS (liquid chromatography–MS), CE–MS (capillary electrophoresis–MS), and FI-ICR–MS (Fourier transform ion cyclotron resonance–MS), combined with mass spectrometry (MS) and NMR (nuclear magnetic resonance spectroscopy) [4–6]. Detection of metabolites is followed by multivariate statistical analysis of the accumulated datasets. Qualitative and quantitative changes in metabolic profiles

during development and under stress conditions are analyzed by metabolomics, a discipline known as a part of systems biology.

Aside from native metabolic alterations, metabolome profiling is used to detect the changes in plant metabolism brought about by genetic engineering. Such improvements in metabolites serve to elevate the economic importance and environmental sustainability of agricultural plants by increasing herbicide tolerance and resistance to pests and pathogens. Plants possess a high biochemical potential of synthesizing an enormous number of various natural substances: fatty acids, phenolics, terpenoids, alkaloids, glucosinolates and other biologically active compounds; many of which are of great value to pharmacology [7]. Advances in metabolomics have enabled the decoding of many metabolic networks and provide for the active genetic engineering of pathways. These offer the opportunity to manipulate the biosynthesis of valuable, biologically active substances of interest, and to create "design" biochemicals. They also can serve to considerably alter the secondary metabolism thus improving a plant's capacities to synthesize new substances or, on the contrary, reduce their toxicity by switching off such metabolic pathways [8–10]. This is a direct effect of genetic engineering on the metabolome. Nevertheless, activation of one key enzyme will not always result in facilitation of the whole metabolic pathway. Thus metabolic profiling is an important tool to evaluate results of such genetic modification. The technology of genetic engineering is widely used in modern agriculture. Frequently plants are genetically engineered to improve their productivity or tolerance to adverse environment. Among the modified crops are maize, cotton, soybean, canola, rice, tomato, potato, among others [2, 11–13]. Other promising approaches are the use of biotech plant systems as a platform for the production of various heterologous proteins for the pharmaceutical industry [7, 14, 15]. The major advantage of plants as bioreactors, aside from their autotrophic type of nutrition, is that the edible plant with the recombinant protein of interest can be eaten directly, skipping rather expensive procedure of purifying the target protein. The production of immunogenic fragments of recombinant antigens in a bioreactor plant is called "plant-based vaccine". Apart from vaccines, biotech plants are used to synthesize numerous antibodies, cytokines, hormones, and other proteins [7, 14–16].

Social, ethical, economic, and ecological norms demand that biotech crops be subjected to intense scrutiny [17]. A very important question is whether accumulation of a large amount of "foreign protein" could be considered a stress factor by the engineered plant and be accompanied by an accumulation of compounds that would pose an ancillary potential risk. A series of investigations were focused on the evaluation of differences in the metabolic profiles of transgenic plants in comparison with wild types (WT). The main conclusion is that metabolism after transgenesis suffered only insignificant alterations or about the same in comparison to that of WT plants [12]. Moreover, results indicate that environmental variations usually produce greater major differences in metabolome composition than genetic modifications. To uncover a possible problem, we used inbred lines of *Nicotiana tabacum* L. plants synthesizing bovine interferon-gamma to study the effects of genetic transformation on the plant metabolome. It must be noted that interferon-gamma is a powerful stimulator of the immune system against pathogens and the tumors of various origins and has no enzymatic activity. Therefore its possible effect on metabolism would be indirect.

2. Material and methods

2.1 Plant material

In this study two separate inbred lines of tobacco harboring a heterologous gene of bovine interferon-gamma *Bt-sIFNG* under the control of a constitutive 35S

CaMV promoter were used. These lines were created earlier by the *Agrobacterium*-mediated genetic transformation of *Nicotiana tabacum* L., cv. Trabzon [18]. The transformation generated six independent kanamycin-resistant transgenic plants (T_0), of which only two became the founders of homozygous inbred lines: InterB.6 and Inter311.2 (T_1). They demonstrated stable inheritance and expression of the transgene insertion and presence of a biologically active heterologous interferon-gamma protein in plant tissues [18]. T_4 generation of transgenic plants, InterB.6.13.8-1 and Inter311.2.7.2-1, and WT tobacco (as a control) was used for the study. The Inter311.2.7.2-1 plant and WT were grown *in vivo* in soil culture in a greenhouse at a temperature of 22–25 °C with a 16 h photoperiod. InterB.6.13.8-4 and WT were cultivated *in vitro* in a MS0 medium [19] supplemented with sucrose (20 g/L) in sterile conditions under the same temperature and light conditions.

2.2 RNA extraction

Total plant RNA was extracted from tobacco leaves with a Pure-ZOL™ reagent according to the manufacturer's protocol (Bio-Rad Laboratories, Hercules, CA, USA). Samples were then treated with DNase I (Thermo Fisher Scientific, Waltham, MA, USA) (5 U/sample). After that the RNA was precipitated with ethanol to remove residual DNase I. Purified RNA was dissolved in sterile water and stored at −80 °C until analysis.

2.3 Reverse transcription

Two μg of total RNA were taken for cDNA synthesis using MMLV RT kit (Evrogen, Moscow, Russia) in accordance with manufacturer's protocol. cDNA samples were precipitated with 0.1 M sodium acetate in ethanol, diluted with sterile deionized water, aliquoted and stored at −80 °C until analysis.

2.4 PCR analysis

The PCR reaction mixture included Taq-polymerase (Evrogen), a mixture of 2 μM dNTP, magnesium-containing Taq-buffer, cDNA template (100–200 ng), the forward and reverse primers sIFNG-1 (5'-AGGAGTATGGACATCATCAAGCA-3′) and sIFNG-2 (5'-AGTCGTCGACCGGAATTTGA-3′) for *Bt-sIFNG* (product size 105 b.p.), and EF-1 (5'-CAAGCGGTCATTCAAGTATGC-3′) and EF-2 (5'-TGTCCAGGACGATCAATCACA-3′) for tobacco *Nt-EF-1α* gene (product size 135 b.p.) [20]. The tobacco house-keeping gene was used as a control present in both WT and transgenic plants. The primers were ordered from Evrogen. Amplification of the fragments corresponding to *sIFNG* and *Nt-EF-1α* genes was performed using the following program:

95°C, 2 min —
{95°C, 20 s,
60°C, 30 s,
72°C, 30 s} — 32 cycles,
72°C, 1 min
in CFX96 Touch Deep Well Real-Time PCR Detection System (Bio-Rad Laboratories).

2.5 Agarose gel electrophoresis

Electrophoresis was performed in a 1% agarose gel in a TAE buffer with the addition of ethidium bromide (0.5 μg/mL) for 40 min at a voltage of 120 V. PCR fragments were visualized in UV light.

2.6 Sample preparation for metabolome analysis

Average samples of 3–5 leaves were prepared in four biological replicates. The plant material (0.2 g) was frozen in microtubes with liquid nitrogen and ground three times in a Tissue Lyser LT (Quiagen, Düsseldorf, Germany) bead mill with metal balls 5 mm in diameter (50 hits/s, 2 min) and subjected to a single-stage extraction with two mL of methanol. After the centrifugation at 15,000 g for 15 min at 4 °C the supernatant was collected and evaporated in a refrigerated CentriVap centrifugal concentrator (Labconco, Kansas City, MO, USA) at 10 °C. The dried residue was dissolved in pyridine with tricosane (nC23) as an internal standard. The samples were then supplied with the silylating agent BSTFA: TMCS 99:1 (Sigma-Aldrich, St. Louis, MO, USA) and derivatizated at 90 °C for 20 min [21, 22].

2.7 Gas chromatography and mass spectrometry (GC–MS)

An Agilent 5860 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) with Agilent 7893 autosampler, and Agilent ChemStation E.02.02.1431 software were used for analysis. The samples were separated on a J&W HP-5MS capillary column 30 m long, 0.25 mm in diameter, stationary phase film (95% dimethylpolyoxane, 5% diphenyl), thickness 0.1 mm. The helium gas constant flow was 1 mL/min and the inlet temperature was 250 °C. The temperature parameters of the oven included an initial temperature of 70 °C and a linear increase to 320 °C at the rate of 4 °C/min. Chromatograms were registered with an Agilent 5975C mass selective detector. Metabolite profiling was performed using equipment of the Center for Molecular and Cell Technologies of Research park of St. Petersburg State University.

2.8 GC/MS data processing

GC–MS data was processed using the PARADISe program (Department of Food Science Faculty of Science, University of Copenhagen, Denmark, [23]) coupled with NIST MS Search (National Institute of Standards and Technology (NIST), USA). In addition, the AMDIS (Automated Mass Spectral Deconvolution and Identification System, NIST, USA) was used. The following mass-spectrometer libraries were applied: NIST2010, library of the Resource Center of the Science Park "Center for Molecular and Cell Technologies" (St. Petersburg State University), the Golm Metabolome Database (GMD) and MoNA (Massbank of North America). Retention index (RI) was determined by calibration with standard alkanes.

2.9 Data analysis and visualization

Data analysis was performed in the environment of the R language 3.6.3 "Holding the Windsock" [24]. Data were normalized by internal standard (nC23) as well as by sample median. The data were log-transformed and standardized. Metabolite that was not detected but present in other replicated samples was considered a technical error and missing values were imputed. Missing data imputation was performed by KNN (k-nearest neighbors) with an *impute* R package [25]. The heatmap was made with ComplexHeatmap [26]. PCA (Principal Component Analysis) was realized with *pcaMethods* [27]. OPLS-DA was performed in the *ropls* package [28].

For enrichment analysis, the *fgsea* package was used [29]. For the statistical ranking factor, loadings of the predictive components from OPLS-DA models were used. Pathways associated with this metabolite set were extracted from KEGG with the *KEGGREST* package using *Nicotiana tabacum* as a reference organism [30]. The resulting lists of metabolites for pathways were manually curated. Metabolites identified

just up to class were included in the pathways related to these groups. Pathways were mapped within the Cytoscape software environment [31]. Graph nodes were assigned to KEGG pathways, edges represent the presence of common metabolites.

3. Results and discussion

Previous studies generated two different families of transgenic tobacco harboring the heterologous gene of bovine interferon-gamma *Bt-sIFNG* [18, 32]. One homozygous *Bt-sIFNG* plant was identified in the Inter311.2 family at T_1 generation. It established the founder of the 311.2 inbred line of transgenic *Bt-sIFNG* tobacco. The other was obtained in T_2 generation of the InterB.6.13 family, leading to a second inbred line of tobacco synthesizing interferon-gamma. In the current study, plants from the T_4 generation of transgenic lines, Inter311.2.7.2-1 and InterB.6.13.8-1, as well as wild type (WT) tobacco for control were used. To demonstrate the transgenic nature of 311.2.7 and B.6.13, RNA was extracted and reverse transcribed. RT-PCR was performed with primers for the tobacco gene *Nt-EF-1α*, encoding housekeeping protein elongation factor 1α, and for the *Bt-sIFNG* gene, encoding bovine interferon-gamma. It was clear that PCR products corresponding to *Nt-EF-1α* were observed for cDNAs from all tested plants, both WT and transgenic. RT-PCR products with primers for *Bt-sIFNG* gene were found in transgenic plants only (**Figure 1**).

Therefore plants of transgenic inbred lines used in the study were shown to possess and express *Bt-sIFNG* gene insertion. These plants synthesize heterologous interferon-gamma protein at about 1 to 1.5 μg per g of fresh weight of plant tissue [18]. The biological effects of plant-produced bovine interferon-gamma demonstrated antiviral activity in bovine cell culture and the induction of immune response in mice, manifested in qualitative alteration of peripheral blood lymphocytes and overall level of Ig G [18].

The production of recombinant human interferon-gamma expressed in *Escherichia coli* resulted in accumulation of 0.35 g/g of dry weight [33]. Similarly *E. coli* culture produced human interferon-gamma with a speed 3 mg/L in hour [34]. Apart from bacteria, different yeast systems based on transformed lines of *Pichia pastoris* and *Saccharomyces cerevisiae* are used for interferon production of up to 300 mg/L [34]. Several examples using insect or even mammalian cell lines have also been designed. Thus in spite of all the economic costs for production and further purification, all of are characterized by rather intensive protein synthesis.

Another interferon-producing system on the basis of rice suspension culture was shown to accumulate intracellular human interferon-gamma protein of up to

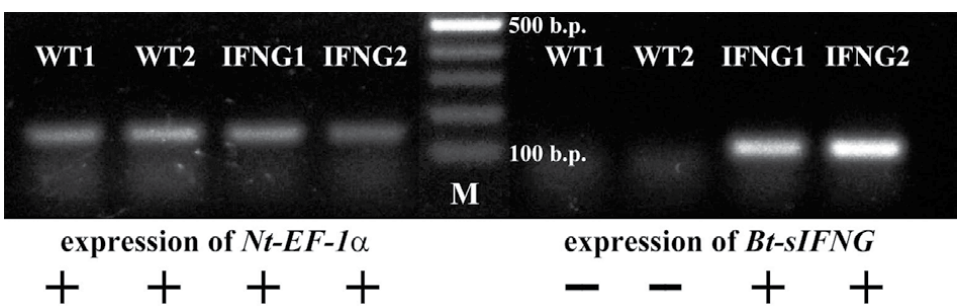

Figure 1.
Electrophoresis of RT-PCR products of cDNAs obtained from WT plant grown in a pot (WT1), or in vitro (WT2), transgenic 311.2.7.2-1 plant, grown in a pot (IFNG1), and transgenic B6.13.8-1 plant, cultivated in vitro (IFNG2), with primers for tobacco gene Nt-EF-1α, or heterologous gene Bt-sIFNG. M - DNA ladder (100–500 b.p.).

699.79 ng/g of cells [35]. The most effective genetic construction used was starvation-inducible endogenous rice αAmy3 promoter.

An example of intensive synthesis of functionally active endogenous plant protein in leaf cells is the accumulation of the major photosynthetic enzyme – RUBISCO (D-Ribulose-1,5-bisphosphate carboxylase/oxygenase), which is claimed to be the most abundant plant protein on earth, even if recently the exact value of the carboxylase has come under revision [36]. The amount of the RUBISCO protein ranged from 30 to 50% of total soluble protein in leaf cell.

Synthesis of protein due to heterologous expression might seriously affect amino acid balance and/or cause alterations in carbohydrates as sources of energy. But along with that, an unregulated accumulation of "foreign" protein inside a plant cell could become a stress signal itself which would lead to production of defensive metabolites. For example, in microbial-based bioreactor systems overproduction of recombinant protein led to formation of an insoluble protein bodies in cytosol and development of oxidative stress as was shown in *E. coli* [37], *P. pastoris* and *S. cerevisiae* [38]. Thus it is appropriate to assume that the tobacco plant metabolism might be shifted due to transgenesis. Earlier comprehensive metabolic analysis of transformed plants showed different alterations in various biochemical processes, nevertheless there was not a clearly established link between gene manipulation and changes in metabolism [12]. The aim of this work was to distinguish possible rearrangements of tobacco leaf cells' metabolic profiles due to transformation with a heterologous gene of bovine interferon-gamma *Bt-sIFNG* under the control of a constitutive 35S CaMV promoter. It was decided that additional attention should be focused on two types of cultivation (in pot soil culture and *in vitro* sterile conditions) – both suitable for transgenic plants growing to estimates of their effect on metabolic alterations.

Metabolite profiles of tobacco leaves were performed by GS-MS analysis. In total profiles included 350 metabolites (**Figure 2**). 80 of these were identified up to the exact metabolite and 150 were identified up to the metabolite class. Metabolic profiles were characterized by a wide variety of carbohydrates and their derivatives (about 90), including pentoses, hexoses and oligosaccharides and their derivatives such as sugar alcohols and sugar acids. This pattern is typical for profiling plant metabolites. The profiles were completed with 19 amino acids, including proteinogenic ones; about 20 carboxylic acids, mainly energy metabolism intermediates; and quite a few fatty acids and their derivatives (only 8). Moreover, some secondary metabolites were also identified.

Simple unsupervised dimension reduction methods (PCA, **Figure 3**) showed differences in tobacco leaf metabolome due to both the type of growth and to genetic status. Metabolite profiles are visualized in the score space of the first two principal components (PC). Metabolomes were grouped along to PC1 according to the type of growth (35% dispersion) and were grouped along with PC2 accordingly to the absence/presence of *Bt-sIFNG* gene insertion (28% dispersion). Similar metabolite profile grouping was also characterized by a correlation of metabolite content. Spearman's correlation coefficient was used as a measure of distance (1-r) and multidimensional scaling (MDS) to reduce the dimension (**Figure 4**). The obtained results highlighted the importance of both factors, namely the method of plant cultivation and its transgenic status.

Further comparison of metabolite profiles of WT and Inter311.2.7.2-1 transgenic tobacco plants grown in pots was detailed by supervised methods such as PLS-DA. The predictive component of PLS-DA has a 31% rate of dispersion (R2Y = 0.99, Q2Y = 0.83). So the metabolic shifts prompted by the transgenic construct were significant. A bar plot of factor loadings of the predictive component from OPLS-DA is presented in **Figure 5**. Positive values correspond to higher content in the WT. According to results, leaves of the WT tobacco plant contain a higher

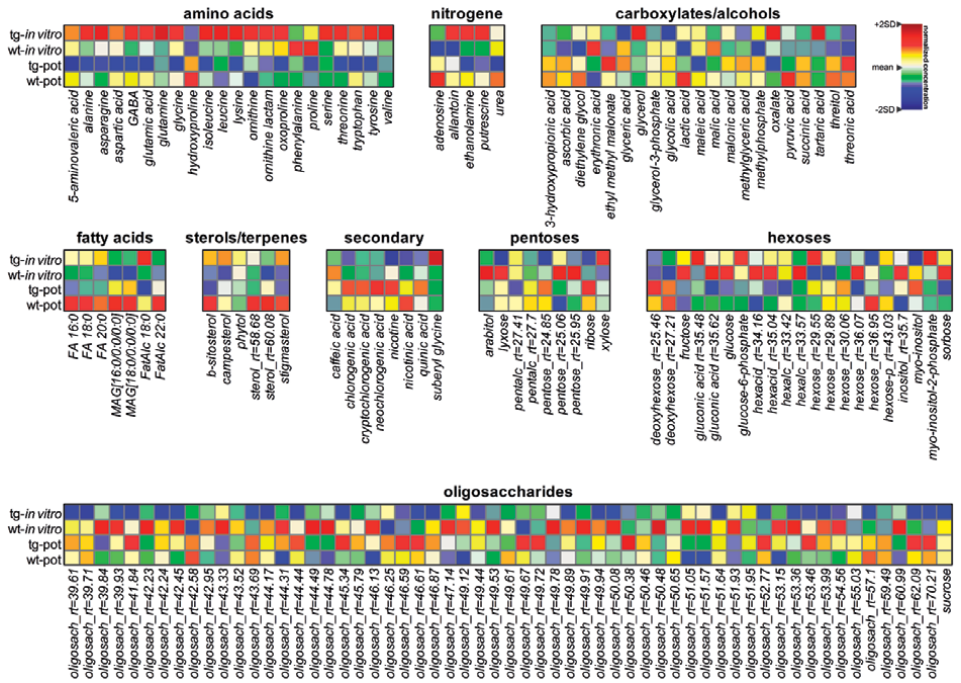

Figure 2.
Heatmap of metabolite content in WT and transgenic (tg) tobacco plants grown in pot or in vitro culture (logarithmic, normalized to median and standardized values).

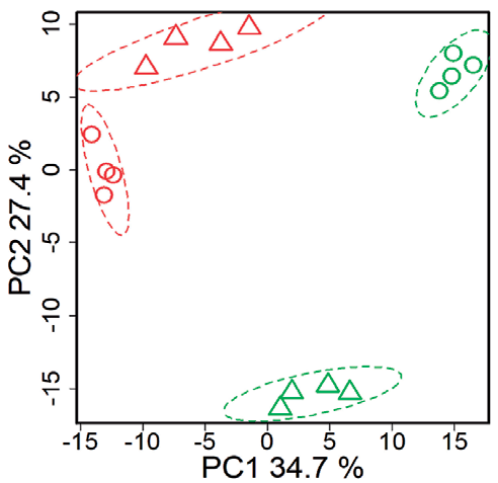

Figure 3.
Representation of metabolite profiles of WT (Δ) and transgenic (o) tobacco in low-dimensional spaces. PCA score plot, % - percent of variance, ellipses - 90% CI. red - pot cultivation, green - in vitro cultivation.

amount of lipophilic compounds, including acylglycerols, sterols, and some fatty acids. WT was also characterized by a higher content of amino acids, amines and carboxylates. Leaves of transgenic plants accumulated high levels of oligosaccharides. Subsequent enrichment analysis revealed metabolic processes that occurred in WT and transgenic tobacco plants (**Figure 6**). In agreement with the bar plot, the results of enrichment analysis of WT plants indicated an intensive wide spectrum of metabolic pathways responsive for the balance of amino acids, carboxylates, lipophilic metabolites, and others. Furthermore, biotech tobacco was characterized

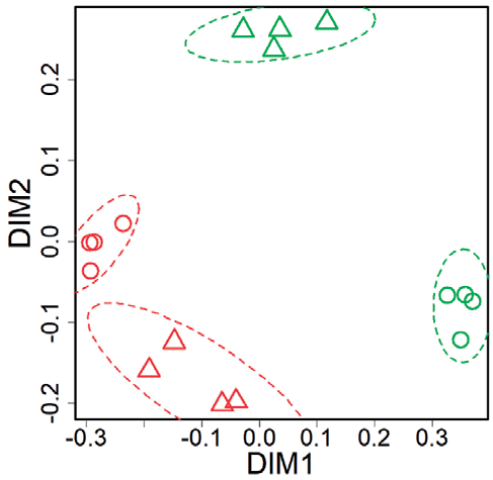

Figure 4.
Representation of metabolite profiles in low-dimensional spaces. Metabolite profiles in the space revealed using MDS with 1-rho as a measure of distance, where rho is Spearman's correlation coefficient, ellipses - 90% CI. Δ - wt, o - transgenic, red - pot culture, green - in vitro cultivation.

by an intensification of only the carbohydrate metabolism pathways. Thus the procedure of growing a plant in soil pot culture exerted significant effects on its metabolism. Such cultivation activated different aspects of WT plant metabolism involving the developmental activity of young leaves. Surprisingly, the presence of an interferon-gamma synthesis-encoding heterologous construct intensified carbohydrate metabolism. The possible higher amount of amino acids required for excessive synthesis of heterologous protein were not distinguished, perhaps because of the intensification of protein synthesis itself.

Another pair of plants were grown in sterile conditions with the application of *in vitro* technology that provides a full supply of required nutrients. Therefore the next step was a similar comparative analysis of metabolite profiles of WT and InterB.6.13.8-1 transgenic plants cultivated *in vitro*. The OPLS-DA provided showed that 65% of the dispersion was associated with the predictive component (R2Y = 1.0, Q2Y = 0.98). Thus the influence of the genotype is more pronounced under *in vitro* conditions. The bar plot of factor loadings of the predictive component from OPLS-DA is presented in **Figures 2** and 7. Leaves of the WT plant accumulated different sugars, oligosaccharides, a number of amino acids, and lipophilic compounds. The transgenic plant, in contrast to pot cultivation, was characterized by a higher diversity of metabolite classes. Metabolic profiles consist of a large number of amino acids, carboxylates, sterols (stigmasterol, β-sitosterol and campesterol), as well as other lipophilic compounds. Nonetheless, enrichment analysis proved activation of only those pathways related to amino acids in transgenic plants and pathways of sugar metabolism in WT plants (**Figure 8**).

Taken together data obtained for WT and transgenic plants grown in pots and *in vitro* were used for SUS-plot construction (**Figure 9**). Unexpectedly, the effect of transformation was the opposite at different conditions of plant cultivation. It might be assumed that the rich conditions of *in vitro* growth cause more intensive metabolic alterations due to genetic modification. These more "comfortable" conditions for plant development might enhance the intensification of heterologous gene expression and interferon-gamma synthesis. This supposition is indirectly confirmed by higher accumulation of PCR-products of the *Bt-sIFNG* transcript in the leaves of transgenic tobacco grown *in vitro* (**Figure 1**).

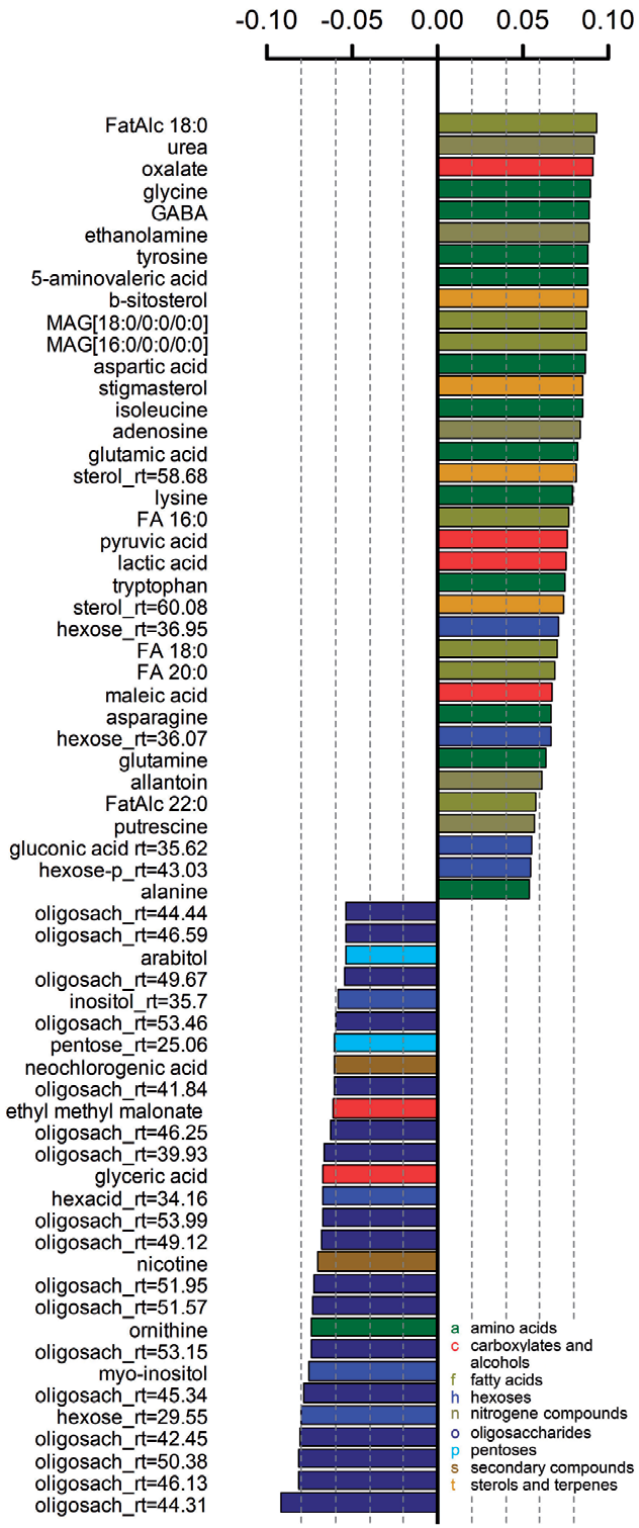

Figure 5.
Differences between metabolomes of WT and transgenic plants cultivated in pots. Barplot of factor loadings of the predictive component from OPLS-DA. Positive values correspond to a higher content in the wild type. Colors mirror chemical class (legend same as in **Figures 7** *and* **9***). Abbreviations: rt. - retention time, oligosacch -oligosaccharides, FA - fatty acid, FatAlc - fatty alcohols, hexacid, pentacid, hexalc, pentalc - sugar acids and alcohols, MAG - monoacylglycerols.*

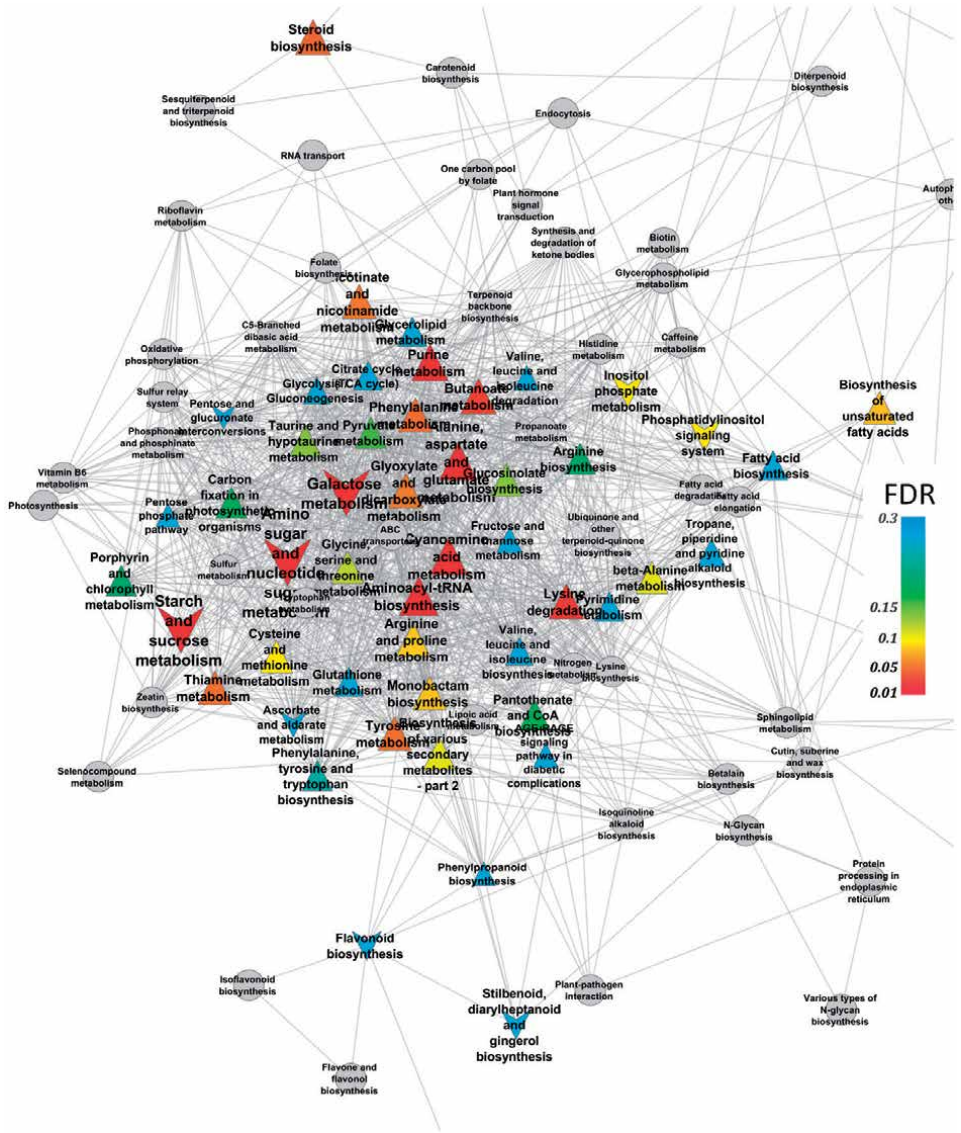

Figure 6.
Enrichment analysis based on predictive component loadings from OPLS-DA model for WT and transgenic tobacco plants cultivated in pots. The network of biochemical pathways (nodes) of tobacco, if the paths have common metabolites, then they are connected by edges. Colors mark significance of enrichment (FDR - false discovery rate), size reflects level of enrichment (|NES| - normalized enrichment score), upward direction - activation (NES > 0) in WT, downward - repression (NES <0) in WT.

An additional criterion that would be of an interest is the crosslink between different metabolic pathways. Thus one can expect some correlative alterations in metabolite content. Changes in the correlations of the metabolite content may reflect systemic metabolic changes. Therefore, we examined the frequency distribution of the correlation coefficients in plants of different genetic status, grown under different conditions. Based on the analysis of the frequency distribution of the Pearson correlation coefficient (**Figure 10**), it was determined that in transgenic plants the number of strong correlations increases when cultivated in a pot in comparison with *in vitro* conditions. The observed effect was absent in WT plants. This might reflect the more stressful cultivation conditions for transgenic plants in pot culture. It may be assumed that the increase in correlations is associated with some kind of coordinated response to stress.

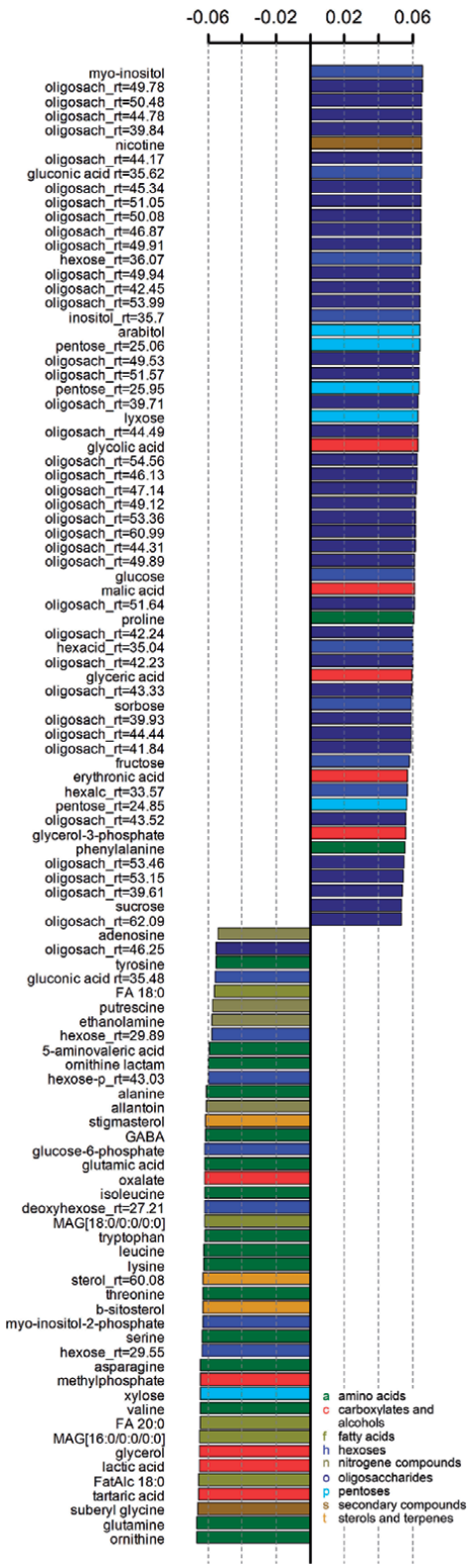

Figure 7.
Differences between metabolomes of WT and transgenic plants cultivated in vitro. Barplot of factor loadings of the predictive component from OPLS-DA. Positive values correspond to a higher content in the wild type. Colors mirror chemical class. Legend and abbreviations as in **Figure 5**.

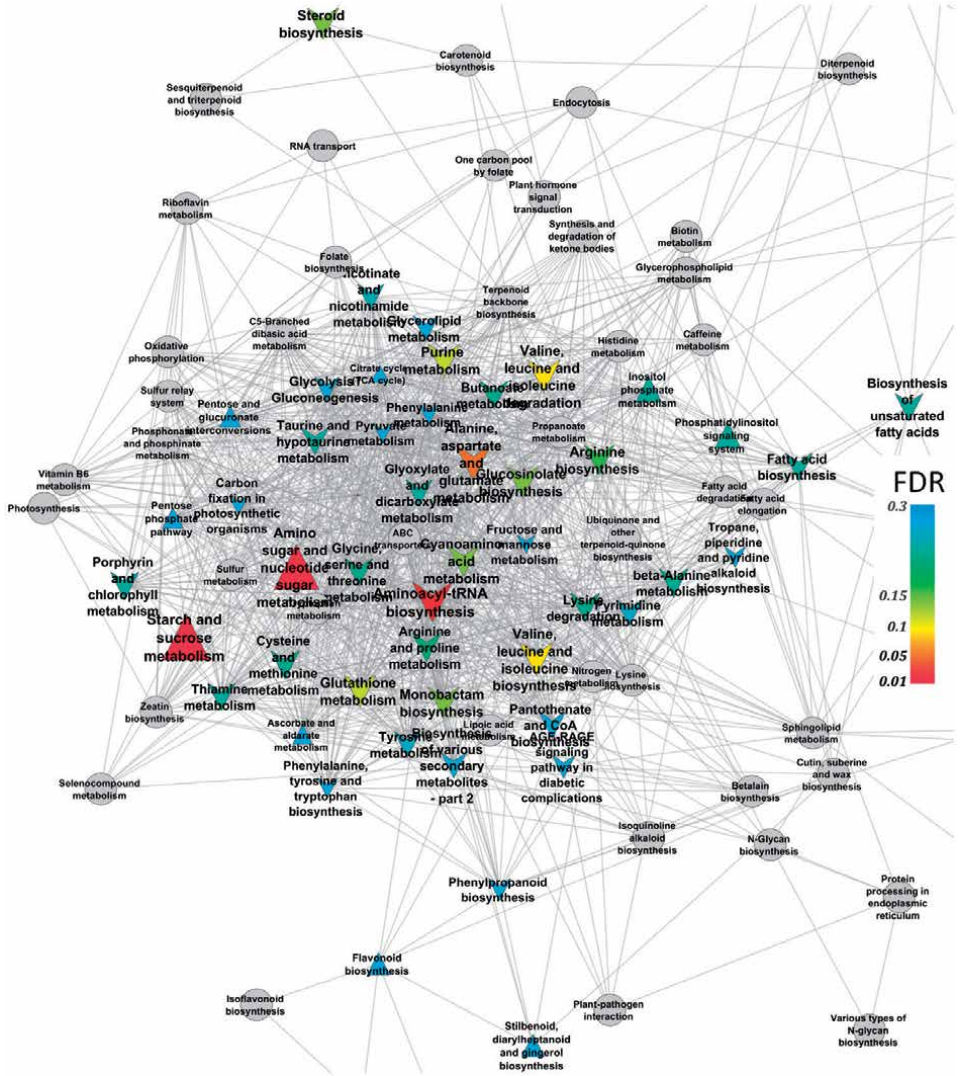

Figure 8.
Enrichment analysis based on predictive component loadings from OPLS-DA model for WT and transgenic plants cultivated in vitro. The network of biochemical pathways (nodes) of tobacco, if the paths have common metabolites, then they are connected by edges. Colors mark significance of enrichment (FDR), size reflects level of enrichment (|NES|), upward direction - activation (NES > 0) in WT, downward - repression (NES <0) in WT.

Analysis of selected literature sources revealed that the majority of work on genetically-modified plant metabolome was carried out with plants expressing heterologous genes encoding different enzymes [12]; i.e. these types of transgenes directly affect the metabolome by enhancing pathways operated by the target enzyme protein. The bovine interferon-gamma used in this study belongs to cytokines and has no enzymatic activity. Therefore, its effects on metabolism would be indirect. There is no doubt that introduction of excessively synthesized "foreign" protein would interfere with the cellular metabolism at least at the level of consumption of an additional amount of amino acids and energy (i.e., sugars and carboxylates). The results obtained showed accumulation of oligosaccharides in the transgenic plant Inter311.2.7.2-1 (compared to WT), grown in soil pot culture (**Figures 2** and **5**) and stimulation of amino acid and sterol metabolism in InterB.6.13.8-1 cultivated *in vitro* in Murashige-Skoog medium (**Figures 2** and **7**).

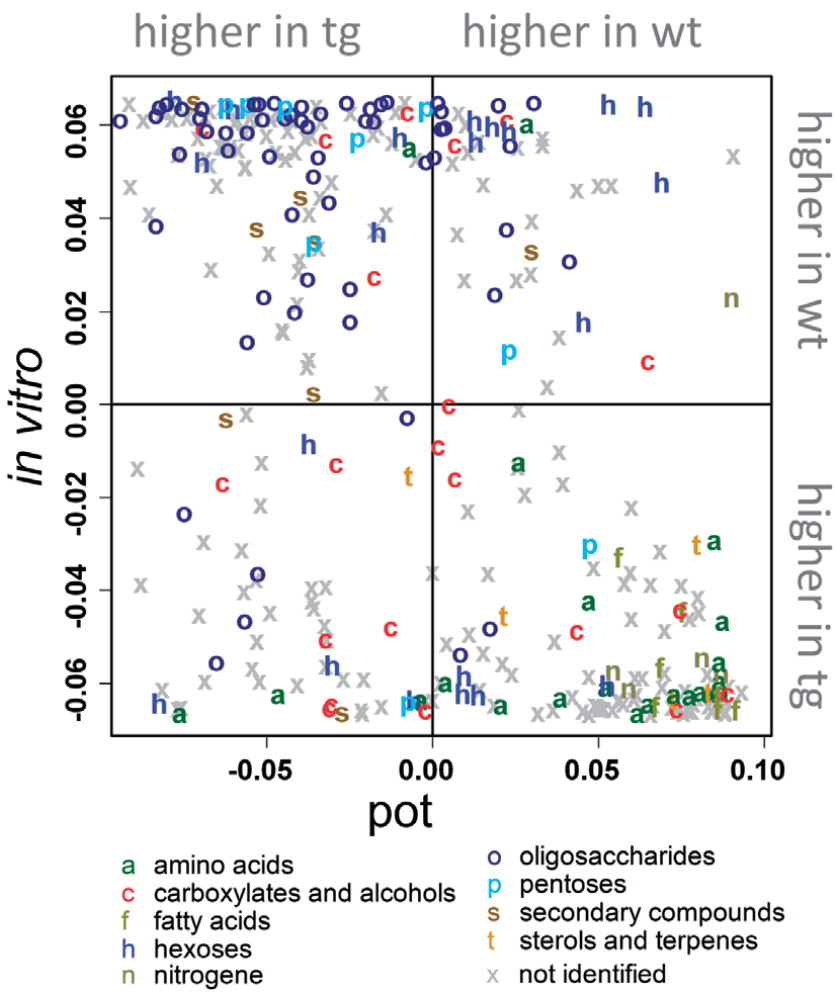

Figure 9.
Comparative analysis of the transformation effects in vitro and in the pot culture. SUS-plot of predictive loadings of OPLS-DA models for WT and transgenic (tg) plants comparison during pot and in vitro cultivation. Colored letters represent chemical classes.

We did not succeed in finding any report on effects of heterologous interferon-gamma on the metabolome of its bioreactor organism, neither of plant, nor of animal origin. Exogenously applied human interferon-gamma led to accumulation of some amino acids (Ala, Tyr, etc.) and depletion of pyruvate and lactate in the culture of human mesenchymal stem cells [39]. Despite the fact that tobacco is a non-target organism for bovine interferon-gamma, its synthesis in InterB.6.13.8–1 grown *in vitro* also led to significant accumulation of amino acids (Lys, GABA, Trp, Asp, Asn, Ser, Glu, Gln, Gly, Tyr, Ile, Leu, Val, Ala, Thr and oxoproline); levels of lactic and pyruvic acids were more or less the same in transgenic plants as in those compared with WT (**Figures 2** and 7).

There are quite a few studies of the metabolome of transgenic plants synthesizing non-enzymatic heterologous protein which can be compared with the system producing bovine interferon-gamma and they mostly concern different types of Bt-toxin expressing plants [12, 13, 40]. It was shown that both cultivation conditions and gene modification induced similar alterations of metabolomes. Moreover, in many cases the growing conditions or developmental stage of plants had a greater effect on the metabolome than the presence of a transgenic insert.

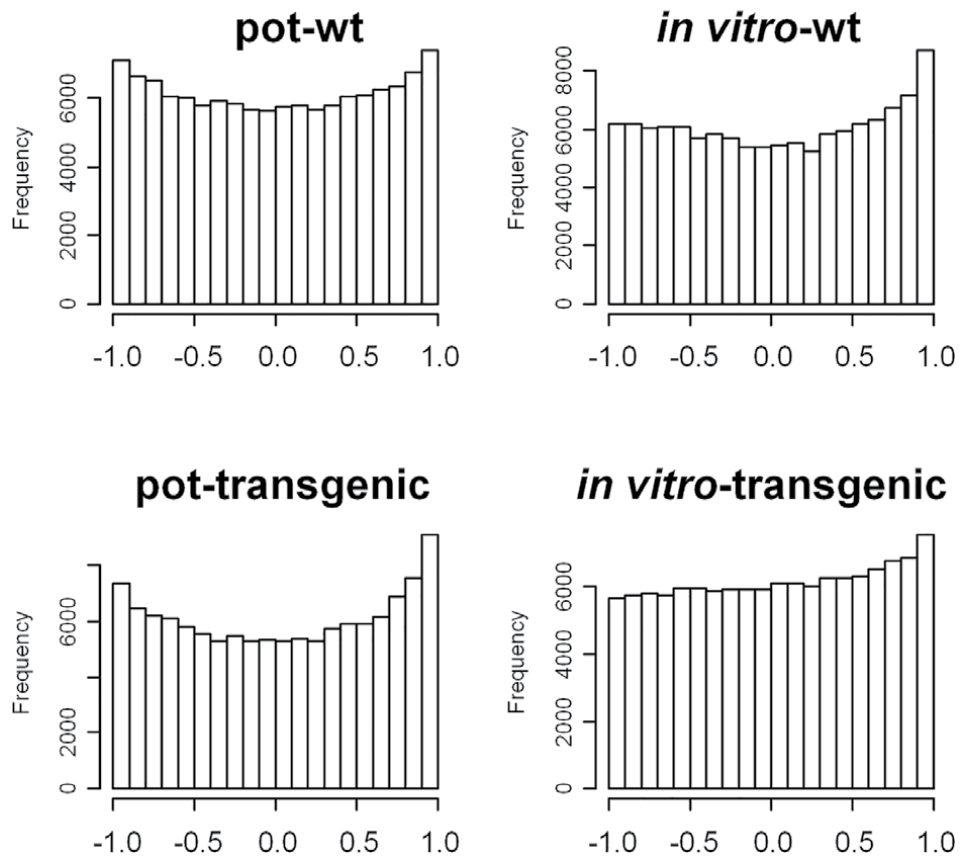

Figure 10.
Influence of transformation and cultivation method on the correlation of metabolite content. Histograms of frequencies of Pearson's correlation coefficient.

So further investigations of biotech plant metabolomics are strongly required, especially those focused on the metabolomics of bioreactor plants.

4. Conclusion

Taken together, the data of this investigation clearly showed that metabolic profiles are dynamic parameters which characterize plant development. Metabolic profile changes in tobacco plants specialized in the synthesis of bovine interferon-gamma were discovered. The presence of heterologous of *Bt-sIFNG* gene insertion itself did not cause development of intracellular stress in transgenic tobacco. In fact, even more intensive metabolic alterations were determined to be dependent on the type of plant growing conditions. It was assumed that soil conditions might be considered as somewhat stressful for transgenic bioreactor plants in comparison with cultivation *in vitro*. This could cause depletion of the target gene transcript accumulation and thus indirectly indicate active regulation of "foreign" protein synthesis by the host transgenic plant. The results obtained are in accordance with data in the literature illustrating the priority of growing conditions and developmental stage above transgenic status in the determination of metabolic intensity. Nevertheless, it is too early in the resolution of this question to make firm conclusions and further investigation is still needed.

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Conflict of interest

The authors declare no conflict of interest.

Abbreviation

FDR	false discovery rate
GC–MS	gas chromatography–mass spectrometry
MDS	multidimensional scaling
NES	normalized enrichment score
OPLS-DA	orthogonal partial least squares discriminant analysis
PC	principal component
PCA	principal component analysis
PCR	polymerase chain reaction
PLS-DA	partial least squares discriminant analysis
RI	retention index
SUS	shared and unique structures
T_X generation	generation of transgenic plants
T_0	transformed plants
T_1	first generation, obtained after self-pollination of T_0 plants
T_2	second generation, obtained after self-pollination of T_1 plants, etc.
WT	wild type

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Chapter 7

Metabolomic Changes in Wood Inhabiting Filamentous Fungi during Ontogenesis

Katerina V. Sazanova, Nadezhda V. Psurtseva and Alexey L. Shavarda

Abstract

GC–MS-based metabolomic profiling of different strains of basidiomycetes *Lignomyces vetlinianus, Daedaleopsis tricolor* and *Sparassis crispa* were studied. On different stages of growth in the methanol extracts of mycelium the different compounds including amino acids, organic acid of TCA cycle, sugars, fatty acids, sugar alcohols, and sugar acids were detected. Changes in the metabolite network occurring with age of the mycelium of *L. vetlinianus* and *D. tricolor* are discussed. The exponential phase of mycelium growth is characterized by pronounced differences during of growth, which manifests itself both in the analysis of specific compounds and in the modeling of the statistical model of the metabolic network. The metabolomic network in the stationary growth phase is less susceptible to changes over time, and is also characterized by a lower dispersion of samples from one aging group. For some compounds, including biotechnologically significant ones, targeted analysis by GC–MS was performed. 4, 6-dimethoxy-phthalide (4, 6-dimetoxy-1 (3H) -isobenzofuranone) was isolated from the mycelium of *Lignomyces vetlinianus*, accumulating in the mycelium in the form of large aggregates. The accumulation of sparassol and other orsellinic acid derivatives in *Sparassis crispa* culture under various conditions is described.

Keywords: metabolomic profiling, metabolite network, gas chromatography–mass spectrometry, wood inhabiting fungi, ontogenesis, phthalide

1. Introduction

The interactions and interconversions of small biomolecules that form the metabolic network play an important, if not decisive, role in the development and ontogenetic processes of living organisms [1]. It is well known that in addition to the wide variability of the content of each metabolite in any biological object in the course of its vital activity, the transition from one stage of ontogenesis to the next is associated with a number of systemic changes in the qualitative and quantitative composition of metabolites and their ratios [2, 3], i.e. "Metabolic state" (metabolome). Thus, in the last decade in physiological research, the study of the dynamics of the metabolome of the system with the help of metabolic profiling taking into account the time factor has become increasingly important. The results of research in the field of metabolomics largely depend on the instrumental approach,

detection, methods of quantitative assessment and localization of metabolites [4]. To implement this approach, it is necessary to analyze complex mixtures using methods such as NMR (nuclear magnetic resonance), GC–MS (gas chromatography–mass spectrometry), LC–MS (liquid chromatography-mass spectrometry), CE-MS (capillary electrophoresis-mass spectrometry) [5, 6]. The application of a modern analytical platform in biology has two main results: metabolomic profiling and the dynamics of metabolic networks. The general purpose of metabolomic profiling is to document the set of metabolites [7]. This task has an independent meaning, since characterizes the molecular resources of the system, i.e. its potential in the context of the concept of biodiversity. For physiology, of particular interest is not so much the annotated metabolome as the dynamics of its correlation structure in the process of development and vital activity. Our laboratory has long been using classical methods of multivariate statistics ("-omic" approaches) with a categorical load in the form of a time scale.

Metabolome analysis represents a tool that finds common application in all aspects investigations, and hence it represents a focal point in studies of fungal physiology [8]. Filamentous fungi have a very active and diverse metabolism resulting in the production of a broad range of metabolites. Fungal chemodiversity is important source of the new compounds for biotechnology and medicine. This makes fungi very promising and interesting object for metabolomic studies, both for the study of the fundamental regularities of the metabolic network, and for the targeted analysis of biotechnologically significant compounds. Below a brief introductions of basidial fungi being an object of this investigation is presented.

Common in Russia bracket fungus *Daedaleopsis tricolor* (Bull.) Bondartsev & Singer (*Polyporaceae*, *Polyporales*, *Agaricomycetes*) is often considered as a color variation of *D. confragosa* (Bolton) J. Schröt. It causes a white rot of dead deciduous and coniferous trees (**Figure 1**). It is known as medicinal fungus producing bioactive compounds with antibiotic [9], antiviral [10], antihypertensive [11] and anti-tumor [12] effects.

Figure 1.
Fruiting bodies of Daedaleopsis tricolor *in nature.*

Pleurotoid xylotrophic fungus originally have been described from Poland as *Pleurotus vetlinianus* Domański but in 2008 was found in Russia (**Figure 2**). Its taxonomy was settled as *Lignomyces vetlinianus* (Domański) R.H. Petersen &

Figure 2.
Fruiting bodies of Lignomyces vetlinianus *in nature.*

Figure 3.
Fruiting bodies of Sparassis crispa *in nature.*

Zmitr. (Resupinataceae, Agaricales, Agaricomycetes), and culture characters were studied [13]. It was noticed then that some strains growing in Petri plates produced agglomerates of light colored crystals formed on colony mats, which were later identified as phthalides [14]. Since this species is not studied well, its physiological and biochemical properties are of quite interest.

Xylobiontic aphyllophoroid basidiomycete *Sparassis crispa* (Wulfen) Fr. (*Sparassidaceae*, *Polyporales*, *Agaricomycetes*) grows in the southern part of the Holarctic, parasitizing on the roots of coniferous trees (**Figure 3**). It belongs to rare species. *S. crispa* has the ability to produce various biologically active substances. Many compounds synthesized by *S. crispa* are well studied, as a rule, these are substances with therapeutic properties [15, 16]. Nevertheless, the physiological and biochemical characteristics of *S. crispa*, which determine its ecological specificity, have not been sufficiently studied, which makes it possible to consider this species as an interesting object for ecological and physiological studies.

The aim of this study is to investigate the dynamic changes in the metabolomic profile of presented species of fungi during their cultures growth using GC–MS analysis.

2. Materials and methods

2.1 Cultivation and cultural studies

The following strains from the Komarov Botanical Institute Basidiomycetes Culture Collection were used for the study: *Daedaleopsis tricolor* LE-BIN 2266, *Sparassis crispa* LE-BIN 043, LE-BIN 2902, LE-BIN 2967 and *Lignomyces vetlinianus* LE-BIN 2335, LE-BIN 2339, LE-BIN 3253.

For culture characters, the strains were cultivated in Petri plates 90 or 60 mm in diam. on beer-wort agar (BWA) (4% beer-wort, Severnie pivovarni, Russia; 2% agar, Difco) at 25 °C. Linear growth was measured every other day for *D. tricolor* and every week for *S. crispa* and *L. vetlinianus* strains (n = 4). Macromorphology of *D. tricolor* was described at 6 weeks, of *S. crispa* and *L. vetlinianus* – at 8 weeks. Micromorphology was studied under Zeiss Axio Imager A1, Axio Scope A1 and Stemi 2000-CS (Zeiss, Germany) using transmitted light.

For metabolomic analysis the strains were also grown in Petri plates: *S. crispa* and *D. tricolor* on BWA, whereas *L. vetlinianus* on malt extract agar (1,5% MEA, Oxoid, England) at 25 °C. Mycelium samples for the analysis of *D. tricolor* were taken on the 3rd, 7th, 14th, 21st and 35th days of growth. Analysis of *L. vetlinianus* mycelium on the 7-th, 14-th, 21-st, 35-th and 50-th days of growth was performed. *S. crispa* strains were analyzed on the 7-th, 14-th, 28-th and 60-th days. Mycelium was scraped from Petri plates and was dried for biomass evaluation.

Quenching and extraction of mycelium and fruiting bodies were carried out based on method described by Gummer with co-authors [17].

2.2 *Quenching*

Quenching of cellular metabolism was performed by 50% of cold methanol (-30°C).

2.3 Extraction

After quenching wet mycelium were ground to powder by a liquid N_2. Samples were extracted with 1.5 mL cold methanol (–25 °C) and chloroform (independently), vigorously mixed and centrifuged (10 min, 400 x g) at room temperature.

The supernatant was transferred to a new vial and the precipitated pellet was re-extracted with 1.5 mL of cold methanol. All extracts were drying by a rotary evaporator.

For a more detailed study of the accumulation of individual compounds in cultures, extraction by chloroform was applied. Crystals collected from the lids of the Petri dish and the surface of the *S. crispa* mycelium and aggregates from the *L. vetlinianus* mycelium were dissolved in chloroform and analyzed by GC–MS.

2.4 GC-MS analysis

GC–MS –based metabolomic analysis was carried out according to the standard derivatization scheme using bis—3-methyl-silyl-3-F-acetamide (BSTFA, Sigma). Silylated samples were analyzed using an Agilent 6850 gas chromatograph interfaced with 5975C mass selective detector. An HP5-MS capillary column (30 m x 0.25 mm inner diameter; film thickness of 0.25 um) was used with helium as a carrier gas at a constant rate of 1 ml/min. The temperatures of the injector and MS source were maintained at 320 °C and 230 °C, respectively. The column temperature program consisted of injection at 70 °C with an increase of 4 °C/min up to 320 °C followed by an isothermal hold at 320 °C for 15 min. Tricosane (10 ug) was used as an internal standard for the quantification of analytical results in semiquant mode, excluding sensitivity coefficients. The samples (0.5 ul) were injected in splitless mode using direct Ultra Inert Liner (Restek). The mass spectrometer was operated in the electron impact mode with an ionization energy of 70 eV. The scan mass range was set from 50 to 1000 Da at 1.27 scans per second. The data were processed and quantified with the AMDIS software (http://www.amdis.net). Compounds were identified through comparison with the retention characteristics and mass spectra of authentic standards, reported mass spectra, and the mass spectral library of the GC–MS data system (NIST 2010). The sum of the extracted ion chromatograms of the ions associated with a compound was used for quantification by UniChrom software (http://www.unichrom.com).

2.5 Statistical analysis

Statistical analysis was carried out using Microsoft Excel 2016 and Metaboanalyst.

3. Results and discussion

3.1 Growth and cultural characteristics of the fungi

D. tricolor LE-BIN 2266 grew faster than strains of the other studied species. Average growth rate on BWA – 5,5 ± 0.5 mm/d. The dynamics of biomass change is shown on the **Figure 4**. On BWA the advancing zones of colonies even, hyphae surface or raised and fringed. Colony mat wooly with long aerial interwoven hyphae around inoculum then more or less radially ordered (**Figure 5**), white, with age becoming yellowish with brown droplets of exudate and crustose hazel or brown areas. Fruiting in culture sometimes observed. Aerial hypha 1–5 μm wide, branched with regular clamps, skeletal hypha long rare branched thick walled, 2–3 μm wide (**Figure 6**). Laccase is positive.

L. vetlinianus strains showed rather slow growth rate that varied depending on strain and used medium [13, 14]. In our study, average growth rate on BWA was 1.0 ± 0.3 mm/d, 2.0 ± 0.9 mm/d and 2.9 ± 0.6 mm/d for the strains LE-BIN 2335,

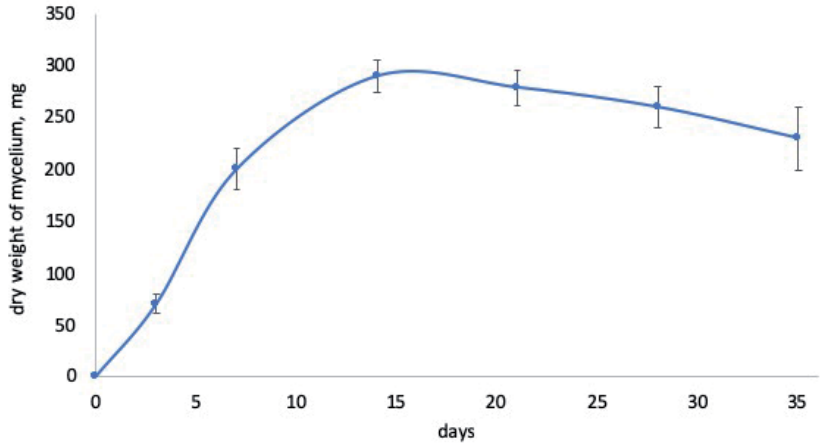


Figure 4.
Dynamic of biomass accumulation of D. tricolor *LE-BIN 2266.*

Figure 5.
Colony of D. tricolor *LE-BIN 2266 on BWA, 6 weeks. Bar = 10 mm.*

LE-BIN 2339 and LE-BIN 3253 respectively. The dynamics of biomass change is shown on the **Figure 7**. Characters of colonies were also variable depending on strain and used medium. On BWA the advancing zones of colonies even, hyphae surface or submerged. Colony mat wooly consisting of thin spread on the surface zonate aerial mycelium with more or less expressed radial fibrous hyphal bands (**Figure 8**), with age abundant fruiting [13] and conglomerates of crystals appear on the colonies (**Figures 9** and **10**). Aerial hypha monometic, 1.5–5 μm wide, branched with regular clamps (**Figure 11**). Laccase is positive.

S. crispa strains grew the most slowly in comparison with the other cultures in this study. In our experiments average growth rate of the strain LE-BIN 2902 on BWA was 0.5 ± 0.1 mm/d. The dynamics of biomass accumulation for involved in the experiment three strains is shown on the **Figure 12**.

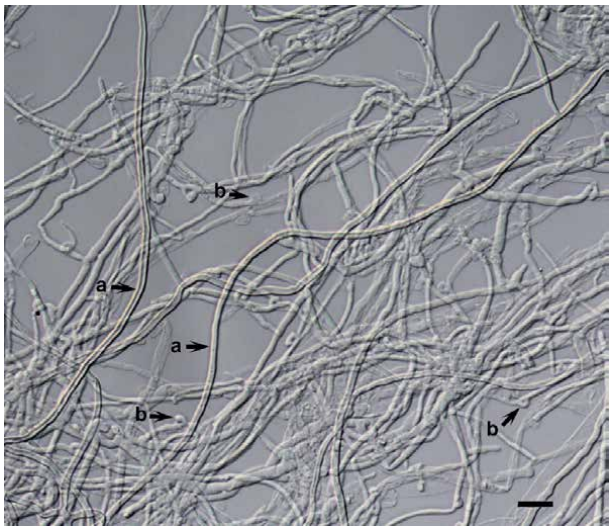


Figure 6.
Micromorphology of D. tricolor *LE-BIN 2266. Arrows indicate: a – Skeletal hypha; b – Clamp connections. Bar = 10 μm.*

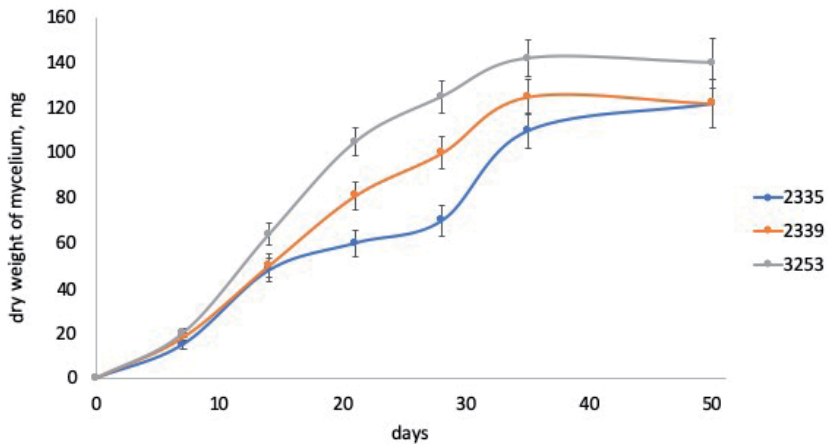


Figure 7.
Dynamic of biomass accumulation of L. vetlinianus *LE-BIN strains.*

Colony of LE-BIN 2902 on BWA is presented on **Figure 13**. The advancing zone of the colony is even, or slightly waved, marginal hyphae distant, appressed and submerged. Colony mat first downy later becoming wooly with aerial interwoven hyphae, white, creamy to grayish. Odor is absent.

Transparent crystals of various size and form can be observed on mycelium or on edges and lids of plastic plates (**Figure 14**). Over time, the number and size of such crystals increased. Aerial hypha irregular 2.0–5.0 μm wide with clamps and numerous swellings up to 10–15 μm in diam. Often arranged in chains (**Figure 15**). Single and grouped crystals were observed under the microscope. Laccase is negative.

3.2 Chemical diversity of metabolites detected by GS-MS in fungal mycelium

The results of metabolomic analysis showed that the GC–MS method allows to detect of many low molecular weight metabolites, among which are identified:

Figure 8.
Colony of L. vetlinianus *LE-BIN 2339 on BWA, 8 weeks. Bar = 10 mm.*

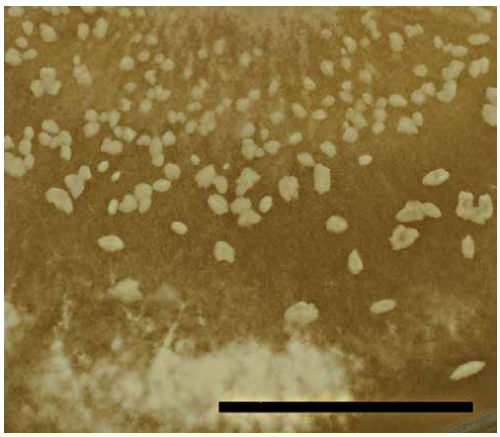


Figure 9.
Conglomerates of crystals in mycelium of L. vetlinianus *LE-BIN 2335. Bar = 10 mm.*

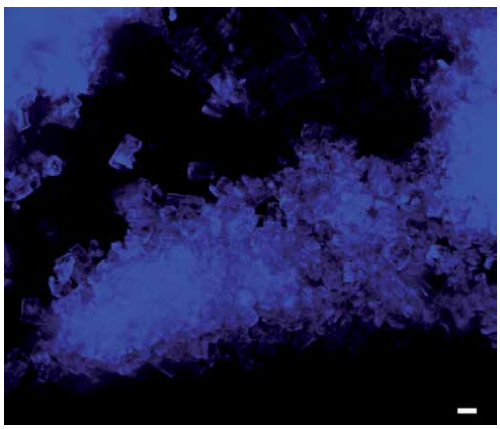


Figure 10.
Conglomerates of crystals in mycelium of L. vetlinianus *LE-BIN 2335 under the microscope in UV light. Bar = 10 μm.*

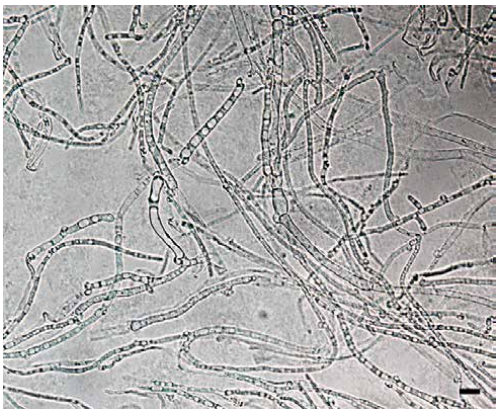


Figure 11.
Micromorphology of L. vetlinianus *LE-BIN 2335. Bar = 10 μm.*

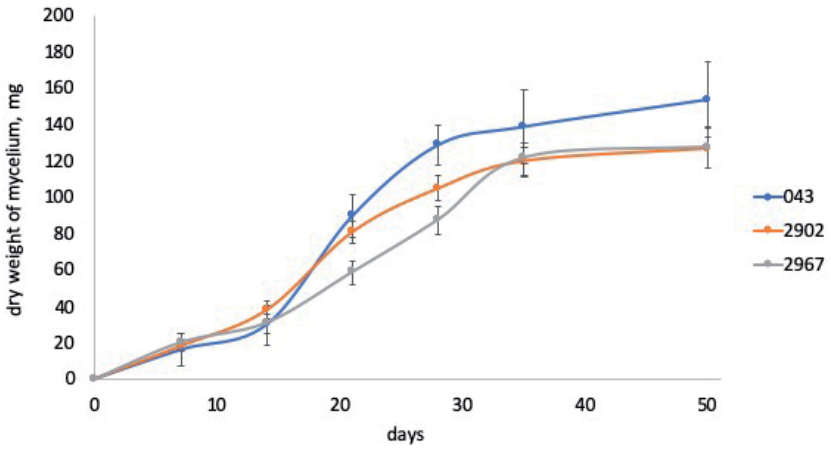


Figure 12.
Dynamic of biomass accumulation of S. crispa *LE-BIN strains.*

Figure 13.
Colony of S. crispa *LE-BIN 2902 on WBA, 8 weeks. Bar = 10 mm.*

amino acids, TCA (tricarboxylic acid) cycle, sugar-acids, fatty acids, monosaccharides, di- and trisaccharides, poliols, hydroxypropionic acid, cyclic metabolites, including phenolic compounds, glyceric acid, pyruvic acid, nicotinic acid,

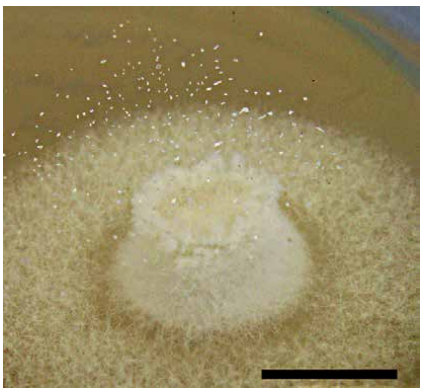


Figure 14.
Crystals on the lid of petri plate with S. crispa. *Bar = 10 mm.*

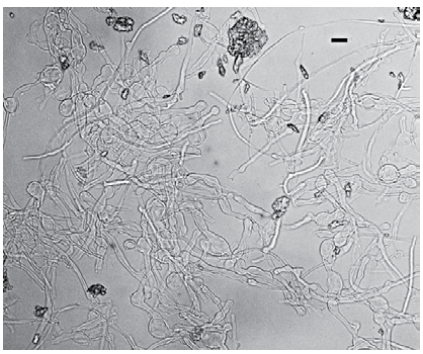


Figure 15.
Micromorphology of S. crispa *LE-BIN 2902. Bar = 10 μm.*

phosphate, methyl phosphate, glyceraldehyde, glycerol-3-P, phosphoglyceric acid (PGA).

The qualitative composition of most of the metabolites was generally similar for the three studied species of fungi. *S. crisra* mycelium contained very high quantitative of sugar that made it difficult to analyze a number of other compounds in the metabolomic profile. For this reason we do not present a detailed matrix of metabolites and statistical models for this species. The main differences between species, bur show the main differences between species only by cyclic compounds.

Specific phenolic compounds (sparassol, methyl ester of sparassol and methyl ester of orsellinic acid) were found in the *S. crispa* mycelium, as well as compounds similar to orsellinates in structure with molecular weights of 298 and 356. In some *S. crispa* cultures, on the mycelium surface and on the inner surface of Petri dish lids were found numerous clear crystals (**Figure 14**). These crystals were identified by GC–MS as methyl 2-hydroxy-4-methoxy-6-methyl benzoate (sparassol). The most intense sparassol formation was observed in the LE-BIN 2902 strain.

The mycelium of *L. vetlinianus* distinguished by the accumulation of furoic acid, cyclohexanone-3-carboxylic acid, 3-oxo-1-cyclohexene-1-carboxylic acid, and 4, 6-dimetoxy-1 (3H) -isobenzofuranone. The fastest growing strain LE-BIN 3253 also contained cyclopentene-3-carboxylic acid, in contrast to the other two *Lignomyces* strains. Numerous crystals-like aggregates glowing in UV were noticed in the plates with *L. vetlinianus* cultures (**Figures 9**, **10**). They were picked up from the plates and identified by GS-MC as clusters of 4,6-dimethoxy-phthalide or 4, 6-dimetoxy-1 (3H) -isobenzofuranone, also found in mycelium.

3.3 Changes in the metabolomic profile of fungi during ontogenesis

The analysis of metabolites was carried out on the following stages of mycelium growth: the onset of growth, the formation of differentiated mycelium, the log phase, the stationary growth phase, and aging.

General patterns of changes in the metabolic network in the ontogeny of fungal mycelium were visualized using the method of principal components (PC). The dynamics of changes in the metabolomic profile of fungi had its own characteristics both at the level of the species and the strain. *D. tricolor* was characterized by the separation of the initial growth stage (day 3) and the exponential growth stage (day 7) (**Figure 16a**). All other points formed a single cluster.

A similar pattern was observed for the statistical model of the dynamics of the *L. vetlinianus* LE-BIN 2339 metabolomite network in ontogenesis (**Figure 16b**). The stages of the exponential growth phase (7, 14, and 21) were distributed in the space, and 35th and 50th days were grouped together.

The distribution of points characterizing the development of *L. vetlinianus* LE-BIN 2335 strain (**Figure 16c**) can be described as a cyclical trend, in which the

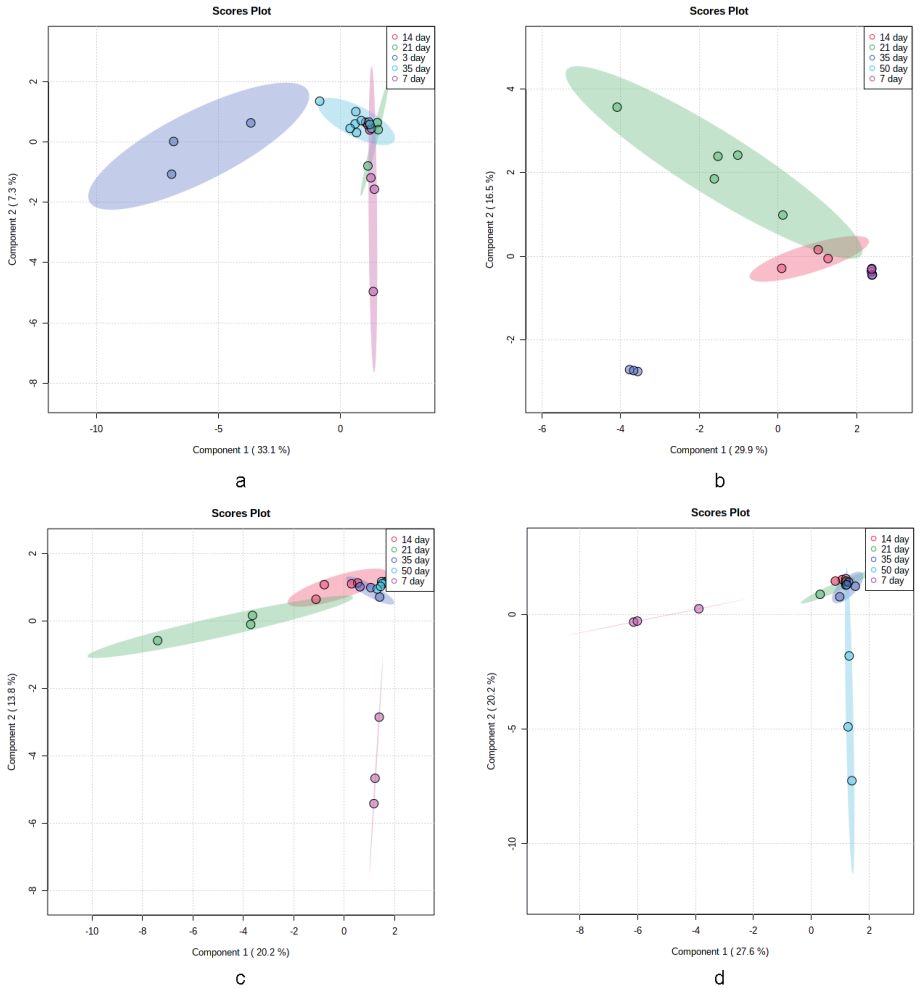


Figure 16.
Principal component analysis (PCA) of the metabolomic profile of fungi in ontogenesis: a – D. tricolor *LE-BIN 2266; b* - L. vetlinianus *LE-BIN 2339; c* - L. vetlinianus *LE-BIN 2335; d* - L. vetlinianus *LE-BIN 3253.*

7th and 50th days of growth formed a single cluster, and other stages of growth were distributed sequentially.

The distribution pattern of the points visualizing the mycelium metabolome in the fast-growing *L. vetlinianus* LE-BIN 3253 strain (**Figure 16d**) differed by an almost unexpressed discrepancy at 14, 21, and 35 days of growth. At the same time, the distribution of points describing the beginning of growth (7th day) and aging (50th days) was very different. Judging by the dynamics of changes in the mycelium biomass, the stationary phase of growth is reached only on day 35, as in other *L. vetlinianus* strains; nevertheless, the beginning of exponential growth, i.e. 7th day is distinguished by the most contrasting metabolic differences.

Thus, the isolation of the initial stage of exponential growth was general for all studied fungi. For all strains, with the exception of *L. vetlinianus* LE-BIN 3253 points characterizing the exponential growth phase on different days were distributed into separate clusters. Joint grouping of 14–21 and 35 days of growth *L. vetlinianus* LE-BIN 3253 into a single cluster is probably associated with striking changes in the metabolic network on the 7th and 50th days.

In systems biology, approaches are being developed to model the behavior of biological systems consisting of a large number of interacting variables, the dynamics of which is determined by numerous linear or non-linear relationships [18]. The method based on nonlinear time series analysis provides a global view of the dynamics of biological systems. A point in multidimensional space represents the state of a system with n-set of variables. Temporary changes in the system, formed by many points, converge to specific regions of space. The importance of this approach lies in providing a global view of the dynamics of a biological object, the experimental study of which is carried out using "-omic" approaches and assumes the factor of time [19].

According to the our results, the metabolomic analysis by GC–MS with subsequent processing of the metabolomic matrix by multivariate statistics methods makes it possible to reveal the biochemical changes of fungal mycelium during ontogenesis. The exponential phase of mycelium development is characterized by pronounced differences during of growth, which manifests itself both in the analysis of specific compounds and in the modeling of the statistical model of the metabolic network. The metabolomic network in the stationary growth phase is less susceptible to changes over time, and is also characterized by a lower dispersion of samples from one aging group.

The distribution of the main metabolites, depending on the stage of development are shown on the heat maps (**Figure 17**). At the initial stages of *D. tricolor* growth, amino acids and organic acids of the TCA cycle accumulated in the undifferentiated mycelium. Subsequently, at the stage of differentiated growth (7th - 21st days of growth), the concentrations of these compounds decreased and slightly increased on the 35th day of growth. On the 14th day of growth, the amount of glucose and some unidentified monosaccharides, as well as trisaccharides increased. On the 21st day of growth, mainly fructose, arabinose, sugar acids and some unidentified monosaccharides with retention time (Rt) 21–22 minutes accumulated. On the 35th day, the total amount of carbohydrates significantly decreased, but the accumulation of phosphate increased.

The dynamics of changes in the metabolomic profile of *L. vetlinianus* was very different from *D. tricolor*. While in *D. tricolor* the maximum concentrations of most metabolites were observed at the beginning of the exponential growth phase, in *L. vetlinianus* the concentration changes of small molecules varied greatly at all growth stages. The initial stage of *L. vetlinianus* growth was characterized by active accumulation of monosaccharides (glucose, fructose and mannose) and a number of unidentified sugars (Rt 20–22 min). Amino acids

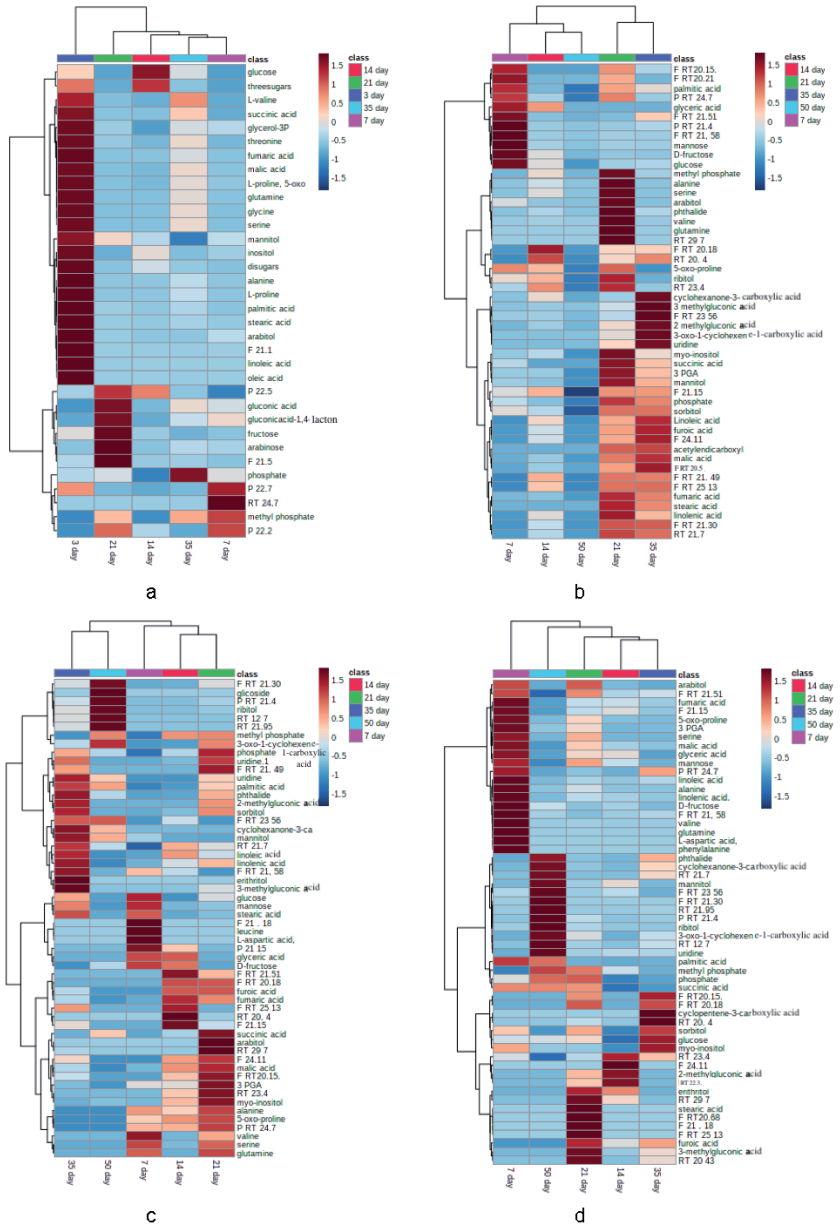


Figure 17.
Heat map of metabolites changes in the ontogenesis of fungi: a- D. tricolor *LE-BIN 2266; b -* L. vetlinianus *LE-BIN 2339; c -* L. vetlinianus *LE-BIN 2335;* d - L. vetlinianus *LE-BIN 3253 (for unidentified metabolites: Rt – Retention time, P- pyranose, F – Furanose).*

accumulated in young cultures of *L. vetlinianus* LE-BIN 2339 and LE-BIN 3253. In *L. vetlinianus* LE-BIN 2335, the maximum amino acid content was found on the 21st day of growth. In general, the strain LE-BIN 2335 with the least intensive growth was characterized by a very poor metabolomic profile on the 50th day of growth, while the faster-growing strains accumulated glycosides, sugars, and cyclic compounds on the 50th day of growth.

Cyclic compounds are mainly dominated in stationary phase of growth. But in strain *L. vetlinianus* LE-BIN 2335, the maximum content of phthalide was recorded in the mycelium on the 21st day of growth, and then it decreased. Perhaps this is due to

the excretion of phthalide into the medium or to the uneven distribution of phthalide clusters on the mycelium. In *L. vetlinianus* LE-BIN 3253, which forms the smallest number of aggregates, the maximum of phthalide concentration in the mycelium was recorded on the 50th day of growth. The isolation of the 50th day into a single cluster is probably associated with a significant accumulation of phthalide and other cyclic compounds from the mycelium of this strain. Fatty acids dominated mainly at the end of the exponential and the beginning of the stationary growth phase. Di- and trisaccharides dominated in the young (7 days-old) and in aging (50 days-old) cultures.

The strain differences of *L. vetlinianus* in the composition of the metabolomic profile, described above, are illustrated on the 7th and 35th days of growth, that is, in the exponential and stationary growth phases in **Figure 18**.

In the methanol extracts of *S. crispa* amino acids (alanine, valine, isoleucine, serine, threonine, proline), sugar alcohols (glycerol, ribitol), and sugars (fructose, glucose, melibiose, sucrose), organic acids and phosphate predominated at the beginning of the log phase (day 7) of growth. On the 14th day of growth, as in *D. tricolor*, the amount of monosaccharides (Rt 18–22 min) increased. At the same time, the qualitative composition of other metabolites in the *S. crispa* mycelium practically did not differ on the 7th and 14th days of growth. With the onset of the stationary growth phase (21 days), the total amount of sugars decreased.

On the 28th day of growth, the composition of the metabolomic profile of *S. craspa* did not differ much from the three-week-old cultures, but the total quantitative content of amino acids was lower than in younger cultures by 30–35%.

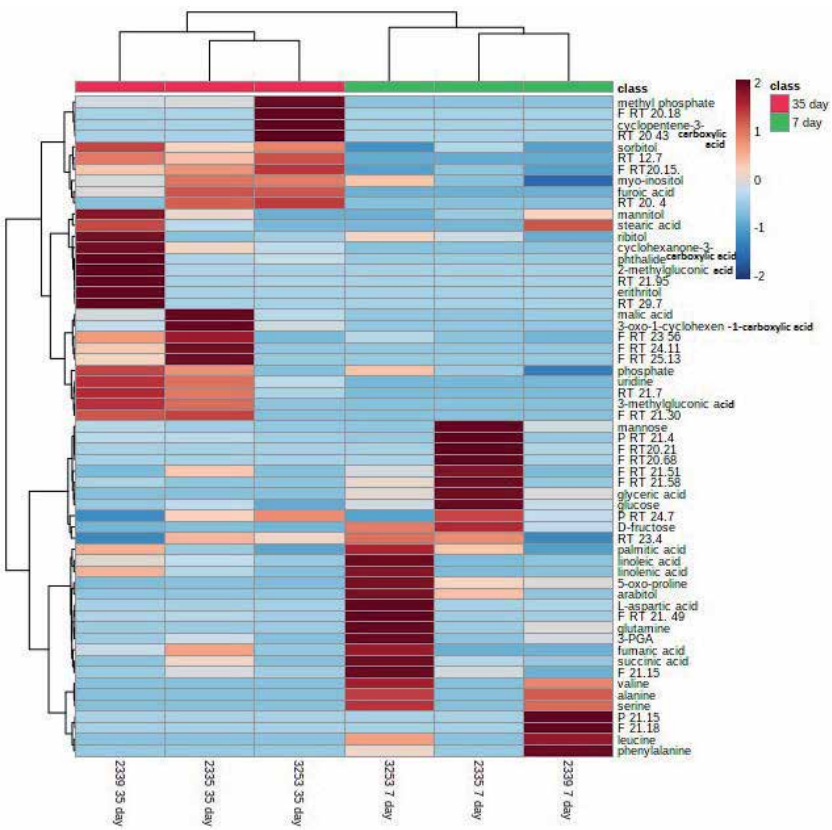


Figure 18.
Heat map of metabolites of L. vetlinianus *strains at different stages of ontogenesis (for unidentified metabolites: Rt – Retention time, P- pyranose, F – Furanose).*

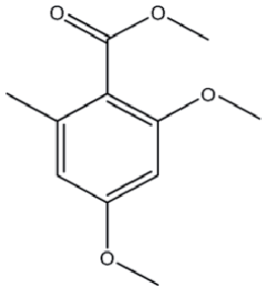
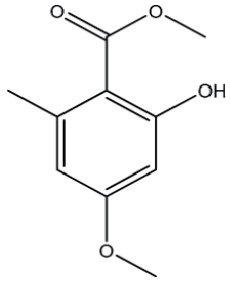
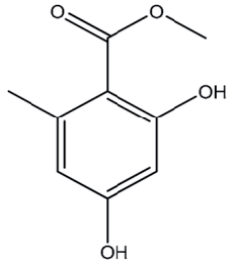
S. crispa **strain number**	**Cultivation time (days)**	**Compounds (μg/g mycelium)**		
		Methyl ester of sparassol	**Sparassol**	**Methyl ester of orsellinic acid**
				
LE-BIN 043	21	—	875.0 ± 97.3	4375.5 ± 802.8
	35	509.2 ± 55.4	2598.5 ± 505.1	124.1 ± 67.5
LE-BIN 2902	21	—	905.0 ± 108.4	4013.5 ± 604.1
	35	7.6 ± 2.2	4915.7 ± 678.2	2706.3 ± 303.3
LE-BIN 2967	21	—	181.9 ± 20.9	197.6 ± 19.1
	35	17.6 ± 4.4	1638.1 ± 80.8	424.9 ± 54.3

Table 1.
Composition of phenolic compounds in the mycelium of S. crispa *strains at different cultivation periods.*

The composition of phenolic compounds synthesized by *S. crispa* and their change with the age of culture is presented in the **Table 1**. In strains 2902 and 043, methyl-orsellinate dominated in the mycelium on the 21st day of growth; its amount was 4.5–6 times higher than the amount of sparassol. In *S. crispa* LE-BIN 2967, the concentrations of these compounds were the same. On the 35th day of growth in strain 2902, the amount of sparassol increased by 4 times, the amount of methyl orsellinate decreased by 35%. In strain LE-BIN 2967, the content of sparassol increased 9.5 times, and methyl orsellinate increased 2 times. In *S. crispa* LE-BIN 043, the amount of sparassol increased by 3 times, while the amount of methyl-orsellinate, on the contrary, decreased by more than 10 times. Also, all cultures with age in the medium accumulated methyl ester of sparassol and compounds with molecular weights of 298 and 356.

Phenolic compounds found in the culture of *L. vetlinianus* and *S. crispa* are derivatives of orsellinic acid and are widespread in fungi of various taxonomic groups. In recent studies, much attention is paid to the biosynthesis, as well as the biological activity of these compounds [20, 21]. Orsellinates are believed to be involved in allelopathic interactions, exhibiting a rather weak antifungal effect [16, 22, 23]. Orsellinic acid itself is formed in the acetate-malonate pathway and is an intermediate for many phenolic compounds [24]. Many natural phthalides display a variety of biological activities. It was stated that phthalides are responsible for numerous bioactivities, however their exact mechanism of action is not studied yet [25]. Phthalides are known to be responsible for antimicrobial, antifungal, cytotoxic, enzyme inhibiting, antitumor and plant hormone like activities [26, 27]. Also there are some results suggested that phthalides acted as inhibitors of oxidative and inflammatory stress.

4. Conclusion

According to the results, the metabolomic analysis by GC–MS with subsequent processing of the metabolomic matrix by multivariate statistics methods makes it possible to reveal the species and strain specificity of biochemical changes in fungal mycelium during ontogenesis. The exponential phase of mycelium growth is characterized by pronounced differences during of growth, which manifests itself both in the analysis of specific compounds and in the modeling of the statistical model of the metabolic network. The metabolomic network in the stationary growth phase is less susceptible to changes over time, and is also characterized by a lower dispersion of samples from one aging group. High concentrations of phthalide accumulated in *L. vetlinianus* and phenolic compounds synthesized by *S. crispa* revealed in some LE-BIN strains under certain cultivation conditions allow considering these strains as promising objects for biotechnology and the study of phenolic compounds metabolism in fungi.

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changes in the correlation structure of metabolic networks in the process of growth and development of fungi and plants from the viewpoint of systemic biology", using equipment at The Core Facility Centre at the Komarov Botanical Institute RAS. The study of the statistical regularities of the metabolic networks of fungi was carried out within the framework of Grant of the President of the Russian Federation for state support of young Russian scientists № MK-799.2021.1.4 "The metabolomics of communities of microorganisms of lithobiontic systems".

Conflict of interest

The authors declare no conflict of interest.

Abbreviations

BSTFA	bis—3-methyl-silyl-3-F-acetamide
BWA	beer-wort agar
CE-MS	capillary electrophoresis-mass spectrometry
F	furanose
GC–MS	gas chromatography–mass spectrometry
LC–MS	liquid chromatography-mass spectrometry
MEA	malt extract agar
NMR	nuclear magnetic resonance
P	pyranose
PC	principal components
PCA	principal component analysis
PGA	phosphoglyceric acid.
Rt	retention time
TCA	tricarboxylic acid

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Metabolomics is the methodology and theory to study the metabolome, including targeted approaches based on selected/multiple reaction monitoring (SRM/MRM) and untargeted approaches based on nuclear magnetic resonance (NMR) or mass spectrometry (MS). The metabolome contains all metabolites derived from sugars, lipids, proteins, and nucleic acids in a given biological system, tissue, cell, or body fluid in a metabolic network system. Metabolomic variations directly link to molecular mechanisms of a disease, reliable therapeutic targets, and effective biomarkers for prediction, diagnosis, and prognostic assessment of disease. This book presents new advances in the concept and methodology of metabolomics, as well as applications of metabolomics in the research and practice of medical and life sciences.

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