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MINIREVIEW

Modern analytical techniques in metabolomics analysis†

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Metabolomics is the comprehensive assessment of endogenous metabolites and attempts to systematically identify and quantify metabolites from a biological sample. Small-molecule metabolites have an important role in biological systems and represent attractive candidates to understand disease phenotypes. Metabolites represent a diverse group of low-molecular-weight structures including lipids, amino acids, peptides, nucleic acids, organic acids, vitamins, thiols and carbohydrates, which makes global analysis a difficult challenge. The recent rapid development of a range of analytical platforms, including GC, HPLC, UPLC, CE coupled to MS and NMR spectroscopy, could enable separation, detection, characterization and quantification of such metabolites and related metabolic pathways. Owing to the complexity of the metabolome and the diverse properties of metabolites, no single analytical platform can be applied to detect all metabolites in a biological sample. The combined use of modern instrumental analytical approaches has unravelled the ideal outcomes in metabolomics, and is beneficial to increase the coverage of detected metabolites that can not be achieved by single-analysis techniques. Integrated platforms have been frequently used to provide sensitive and reliable detection of thousands of metabolites in a biofluid sample. Continued development of these analytical platforms will accelerate widespread use and integration of metabolomics into systems biology. Here, the application of each hyphenated technique is discussed and its strengths and limitations are discussed with selected illustrative examples; furthermore, this review comprehensively highlights the role of integrated tools in metabolomic research.

Introduction

Metabolomics is concerned with both targeted and non-targeted analysis of endogenous and exogenous small molecule metabolites (<1500 Da), and presents a promising tool for biomarker discovery.[1,2] It has been used in assessing responses to environmental stress, comparing mutants, drug discovery, toxicology, nutrition, studying global effects of genetic manipulation, cancer, comparing different growth stages, diabetes and natural product discovery.[3–5] Metabolomics is a global metabolic profiling framework which utilizes high resolution analytics (typically NMR and MS) together with chemometric statistical tools such as principal component analysis (PCA) and partial least squares (PLS), to derive an integrated picture of both endogenous and xenobiotic metabolism. These small molecules, including peptides, amino acids, nucleic acids, carbohydrates, organic acids, vitamins, polyphenols, alkaloids and inorganic species act as small-molecule biomarkers that represent the functional phenotype in a cell, tissue or organism.[6] Separation and identification of these small molecules is made possible by the technological advances in metabolomics. These innovational technologies, including accurate measurement of high-resolution MS, NMR, CE, HPLC and UPLC technology, can accomplish detection of metabolites within a few minutes. Measuring such low-molecular-weight metabolites could offer deeper insights into mechanisms of the influence of lifestyle and dietary factors in relation to specific diseases.[7] Metabolites are biological characteristics that are objectively measured and evaluated as indicators of normal biological and pathological processes or pharmacological responses to a therapeutic intervention, widely used in clinical practice for clinical diagnosis.[8–10] Metabolomics has been applied to define metabolites related to prognosis or diagnosis of diseases and could provide greater pathophysiological understanding of disease.

Metabolomics is one functional level tool being employed to investigate the complex interactions of metabolites with other metabolites (metabolism) but also the regulatory role metabolites provide through interaction with genes, transcripts and proteins (*e.g.* allosteric regulation). The application of cutting edge analytical technologies to the measurement of metabolites and the changes in metabolite concentrations under defined conditions have helped illuminate the effects of perturbations in pathways of interest.[11] Owing to the complexity of metabolites

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and the large number of metabolites therein, advanced and high-throughput separation techniques have been coupled to high-resolution MS, but not exclusively, to make these measurements. Technological developments are the driving force behind advances in scientific knowledge. These techniques include NMR, GC-MS, and LC-MS, *etc.*; with these techniques, some favourable outcomes have been gained.[12–16] However, every technique has its advantages and drawbacks; no existing analytical technique can be versatile. NMR has many advantages, but the sensitivity of NMR is relatively poor compared with MS methods, and concentrations of potential biomarkers may be below the detection limit. GC-MS requires sample derivatization to create volatile compounds. Non-volatile compounds that do not derivatize and large or thermo-labile compounds will not be observed in the GC-MS analysis. The recent introduction of UPLC, employing porous particles with internal diameters smaller than 2 μm, in combination with MS, results in higher peak capacity, improved resolution and increased sensitivity compared to conventional HPLC columns, therefore making it even more suitable for a metabolomics approach. Multi-analysis techniques can partially overcome the shortcomings of single-analysis techniques.[17] In data acquisition platforms, NMR, GC-MS, LC/MS^n are the prevalent techniques although at present, none of them is a perfect technique that can meet with the requirements of metabolomics for measuring all metabolites. Recent advances in hyphenated analytical platforms have driven forward the discipline of metabolomics and can analyze a wide range of metabolic components, therefore the most holistic studies using MS or NMR are typically defined as metabolic profiling.[18,19] Hyphenated analysis techniques are very suitable for metabolomics samples analysis, especially by reversed phase separation technology, as the sample can be injected directly into the column without the need for any pretreatment, and the plasma protein can also be simply removed for analysis.

Metabolomics offers a number of benefits compared with other '-omic' strategies, with the most advantageous being its close biological proximity to the phenotype of the system and hence the rapid observation of system perturbations in the metabolome.[20] Although analytical platforms are expensive, costs per sample are low and, when combined with the high-throughput nature of metabolic profiling, these allow metabolic profiling to be applied to screen large sample sets in a high-throughput approach and at low total cost compared with other '-omic' platforms. Typically, as an intermediate phenotype, metabolite signatures capture a unique aspect of cellular dynamics that is not typically interrogated, providing a distinct perspective on cellular homeostasis.[21] Metabolomics focuses on the complex interactions of system components and highlights the whole system rather than the individual parts, providing a distinct perspective on cellular homeostasis.[22,23] Hyphenated techniques have received much attention in recent years and the annual amount of publications in this field has increased significantly over the past decade. This increase in publications is due to improvements in the analytical performance, most notably in the field of NMR and MS analysis, and the increased awareness of the different applications of this growing field.[24–26] Metabolomics methods are mostly focused on the information-rich analytical techniques of NMR spectroscopy and MS.[27] Advances in MS technologies, including direct introduction or in-line chromatographic separation modes, ionization techniques, mass analyzers, and detection methods have provided powerful tools to assess the molecular changes in the metabolome. The applicability of hyphenated analysis techniques in metabolomics research is illustrated by examples of the analysis of biomedical and clinical samples. In this review we present the latest developments of the above mentioned techniques applied in the field of metabolomics; in particular, the strengths and limitations as well as some new trends in the development are discussed with selective illustrative examples.

Measuring the metabolome: current analytical technologies

Metabolomics is a truly interdisciplinary field of science, which combines analytical chemistry, platform technology, MS, and NMR with sophisticated data analysis. It involves the application of advanced analytical tools to profile the diverse metabolic complement of a given biofluid or tissue. Metabolomics offers a platform for the comparative analysis of metabolites that reflect the dynamic processes underlying cellular homeostasis.[28,29] Recent advances in analytical technologies have set the stage for metabolite profiling to help us understand complex molecular processes and physiology. Over the past decade the application of metabolomics has gained ever increasing interest. There are numerous analytical platforms that have been used for metabolomic applications, such as NMR, Fourier transform-infrared spectroscopy (FT-IR) and MS coupled to separation techniques, including NMR, GC-MS, LC-MS, FT-MS and UPLC-MS. Whilst NMR spectroscopy is particularly appropriate for the analysis of bulk metabolites and GC-MS to the analysis of volatile organic compounds and derivatised primary metabolites, LC-MS is highly applicable to the analysis of a wide range of semi-polar compounds including many secondary metabolites of interest. Since LC-MS can avoid chemical derivatization, it is a widely used instrument. MS-based metabolomics offers high selectivity and sensitivity for the identification and quantification of metabolites, and combination with advanced and high-throughput separation techniques can reduce the complexity of metabolite separation, while MS-based techniques require a sample preparation step that can cause metabolite loss. Therefore, parallel application of several techniques, for example GC-MS, LC-MS or NMR, is desirable to study the global metabolome.

Metabolite identification is a key step for metabolomics study. Metabolome analysis may be conducted on a variety of biological fluids and tissue types and may utilize a number of different technology platforms. MS and NMR are among the most emergent technologies in metabolomics, enabling the shortest route toward metabolite identification and quantification. Specialized analytical platforms, such as NMR and MS, are required to interrogate such metabolic complexity. Two of the driving forces are advances in analytical chemistry and our understanding of metabolomics. The advances in LC-MS are extremely impressive, and the speed of analysis has increased even more with recent developments in UPLC. Recent developments in separation sciences have led to the advent of UPLC and MS based technologies showing ever improving resolution of

metabolite species and precision of mass measurements. Each technique has associated advantages and disadvantages. Clearly, no single analytical methodology is ideal for all of the many thousands of metabolites in a biological system; instead, a combination of techniques is needed for analyzing the majority of metabolites in different polarity and molecular weight ranges.[30] Hence, a combination of different analytical technologies can be used to gain a broad perspective of the metabolome.

NMR-based metabolomics

As one of the most common spectroscopic analytical techniques, NMR can uniquely identify and simultaneously quantify a wide range of organic compounds in the micro-molar range. NMR has been introduced to the emerging field of metabolomics where it can provide unbiased information about metabolite profiles. NMR-based metabolomics is able to provide a 'holistic view' of the metabolites under certain conditions, and thus is well-suited and advantageous for metabolomic studies.[31] NMR is straightforward and largely automated and non-destructive, so samples can continue to further analysis. It has been extensively used for metabolite fingerprinting, profiling and metabolic flux analysis. The major limitation of NMR for comprehensive metabolite profiling is its relatively low sensitivity, making it inappropriate for the analysis of large numbers of low-abundance metabolites. Conventionally, within the field of metabolomics of biofluids, NMR has been the technique of choice, due to its ability to measure intact biomaterials nondestructively as well as the rich structural information that can be obtained. Hence, extensive research and significant improvements have been performed using NMR to measure populations of low-molecular-weight metabolites in biological samples. High-resolution NMR could provide an ideal mechanism for the profiling of metabolites within biofluids or tissue extracts. Although it has many advantages, the sensitivity of NMR is relatively poor compared with MS methods, and concentrations of potential biomarkers may be below the detection limit. A number of biofluids such as blood, urine, cerebrospinal fluid, cell culture media and many others can be obtained at a high sampling frequency with minimal invasion, permitting detailed characterisation of dynamic metabolic events.[32] NMR can provide detailed information regarding the structural transformation of a compound as a consequence of metabolism in drug discovery and development.[33] Compared with other techniques, NMR-based metabolomics is becoming a useful tool in the study of body fluids and has a strong potential to be particularly useful for the non-invasive diagnosis of diseases that are very common and pose significant public health problems.

Successful studies have shown that NMR spectroscopy as a particularly information-rich method offers unique opportunities for improving the structural and functional characterization of metabolomes, which will be essential for advancing the understanding of many biological processes.[34] Recently, there has been much interest in the use of high-throughput NMR techniques for the detection of biomarkers. From the perspective of drug discovery, each of these metabolites could fulfill a number of useful functions: disease biomarker, surrogate marker of drug delivery, surrogate marker of drug efficacy and so on. Indeed, NMR is non-selective so that all the low molecular weight compounds are detected simultaneously in a single run, and provides rich structural information which is an important asset to characterize components of complex mixtures.[35] Urine and serum samples after chronic cysteamine treatment were analyzed by NMR-based metabolomics combined with multivariate statistics.[36] A decrease of urine succinate, citric acid, and serum acetoacetate, together with an increase of serum lactate, suggests that chronic cysteamine supplementation results in perturbation of rat energy metabolism. Metabolomics is a powerful tool for investigating any disturbance in the normal homeostasis of biochemical processes. In particular, urine metabolomics provides information on the metabolite phenotype of the human being and therefore is appropriate to study the status of the global system. Jung *et al.* applied an NMR metabolomics approach to investigate the altered metabolic pattern in plasma and urine from patients with cerebral infarctions and in order to identify metabolic biomarkers associated with stroke.[37] The plasma of stroke patients was characterized by an increase in lactate, pyruvate, glycolate, and formate, and by a decrease in glutamine and methanol; the urine of stroke patients was characterized by decreased levels of citrate, hippurate, and glycine. These detected biomarkers were associated with anaerobic glycolysis and folic acid deficiency. It indicated that magnetic resonance methodologies will be paramount in future disease management. However, because of their sensitivity and specificity, these techniques have been currently adequate for use as diagnostic tools in individual patients. Thus, NMR has been used for analysis of metabolites, including analysis of Alzheimer's disease, prostate cancer, amino acids, nucleotides and nucleosides, vitamins, thiols, carbohydrates and peptides.[38,39]

MS

MS is gaining increasing interest in high-throughput metabolomics, often coupled with other techniques such as chromatography-MS techniques. MS has been extensively developed in the past few decades and holds a distinguished position in qualification and separation science. Recent advances in MS-based metabolomics have created the potential to measure the levels of hundreds of metabolites that are the end products of cellular regulatory processes. Due to its high sensitivity and wide range of covered metabolites, MS has become the technique of choice in many metabolomics studies. Its utility derives from its wide dynamic range, reproducible quantitative analysis, and the ability to analyze biofluids with extreme molecular complexity.[40] The aims of developing MS for metabolomics range from understanding the structural characterization of important metabolites to biomarker discovery. MS can be used to analyse biological samples either by direct-injection or following chromatographic separation. Recent developments and improvements in mass accuracy have dramatically expanded the range of metabolites that can be analysed by MS and have improved the accuracy of compound identification. Direct-injection MS can provide a very rapid technique for the analysis of a large number of metabolites, and thus is extensively used for metabolic fingerprinting. However, direct injection of biological samples into MS has some drawbacks including co-suppression and low ionization efficiencies. In this case, to avoid these problems and

to decrease the complexity of the sample mixture, MS is often used as a key hyphenated technique in metabolomics.

Recent research has established MS as a key technique of choice in metabolomics because of its high sensitivity and wide range of covered metabolites. MS analytical tools within metabolomics can profile the impact of time, stress, nutritional status, and environmental perturbation on hundreds of metabolites simultaneously resulting in massive, complex data sets in a global or targeted manner. The high sensitivity and resolution of MS allows for the detection and quantification of thousands of metabolites. Motivated by the success of MS in metabolomics, the analytical community has initiated efforts towards MS-based metabolomics to investigate metabolic biomarkers. MS-based analysis of the metabolome facilitates the reconstruction of metabolic networks, discovery and functional annotation of biomarkers. Multiple analytical techniques, used in a complementary manner, are required to achieve high coverage of the metabolome that is composed of a vast number of small-molecule metabolites that exist over a wide dynamic range in biological samples.[41,42] MS-based technologies are playing a central role in metabolomics research and are widely available to facilitate successful metabolomics studies.

GC-MS

The goal of metabolomics analysis is systematic understanding of all metabolites in biological samples. Many useful platforms have been developed to achieve this goal. Currently, as a core analytical method for metabolomics, GC-MS has been used as a platform in non-targeted analysis, especially for hydrophilic metabolites.[43] Generally, GC-MS-based metabolomics requires a high-throughput technology to handle a large volume of samples and accurate peak identification through the standard retention times and mass spectra. GC-MS has been widely used for metabolomics and can provide efficient and reproducible analysis. For separation on the GC column, GC-MS requires a derivatization reaction to create volatile compounds. Non-volatile compounds are not derivatized and will not be detected in the GC-MS analysis. This limits the applicability to metabolomics. Using this approach, the volatile metabolites can be directly separated and quantified by GC-MS, and it is also possible to simultaneously profile several hundreds of compounds including organic acids, most amino acids, sugars, sugar alcohols, aromatic amines and fatty acids. For others, chemical derivatization is required to make them amenable to GC-MS analysis. Ma *et al.* explored the alteration of endogenous metabolites and identified potential biomarkers using a metabolomic approach with GC-MS in a rat model of estrogen-deficiency-induced obesity.[44] The series of potential biomarkers identified in the present study provided fingerprints of rat metabolomic changes during obesity and an overview of multiple metabolic pathways during the progression of obesity involving glucose metabolism, lipid metabolism, and amino acid metabolism. Kuhara *et al.* devised a more rapid and accurate diagnosis of citrin deficiency patients using the GC-MS urine metabolome.[45] The results show that, together with GC-MS, non-invasive urine metabolomics provides a more reliable and rapid chemical diagnosis of citrin deficiency.

CE-MS

CE-MS is a powerful and promising separation technique for charged metabolites, offering high-analyte resolution, providing information mainly on polar or ionic compounds in biological fluids.[46] CE-MS, as an analytical platform, has made significant contributions in advancing metabolomics research. Metabolomics is a rapidly emerging field of functional genomics research whose aim is the comprehensive analysis of low molecular weight metabolites in a biological sample. CE-MS represents a promising hyphenated microseparation platform in metabolomics, since the majority of primary metabolites are intrinsically polar.[47] Metabolites are first separated by CE based on charge and size, and then selectively detected using MS by monitoring ions over a large range of m/z values. CE-MS provides numerous key advantages over other separation techniques. One of the significant advantages of CE-MS is a short analysis time and very small sample requirement with injection volumes ranging from 1 to 20 nL. This feature makes CE-MS a very promising analytical technique for high-throughput metabolomics. Thus, CE-MS has been used for both targeted and non-targeted analysis of metabolites, including analysis of inorganic ions, organic acids, amino acids, nucleotides and nucleosides, vitamins, thiols, carbohydrates and peptides. Metabolome analysis of human HT29 colon cancer cells was investigated.[48] CE-MS analysis time was less than 20 min per sample and allowed the simultaneous and reproducible analysis of more than 80 metabolites in a single run with a minimum consumption of sample and reagents. Using CE-MS, Sato *et al.* analyzed the dynamic changes in the level of 56 basic metabolites in plant foliage at hourly intervals over a 24 hr period.[49] Adenine nucleosides and nicotinamide coenzymes were regulated by phosphorylation and dephosphorylation. It facilitates an understanding of large-scale interactions among components in biological systems. We conclude that CE-MS is a valid approach for targeted profiling of metabolites.

LC-MS

The metabolome is the set of small molecular mass organic compounds found in a given biological media. Metabolomics refers to the untargeted quantitative or semi-quantitative analysis of the metabolome, and is a promising tool for biomarker discovery. MS and chromatography have been extensively developed in the past few decades and hold a distinguished position in qualification and separation science. The advancement of both HPLC and MS has contributed significantly to metabolomics analysis. MS and HPLC are commonly used for compound characterization and obtaining structural information; in the field of metabolomics, these two analytical techniques are often combined to characterize unknown endogenous or exogenous metabolites present in complex biological samples. With HPLC coupled to MS, there is no need to derivatize compounds prior to analysis. HPLC separations are better suited for the analysis of labile and nonvolatile polar and nonpolar compounds in their native form. Recently, LC-MS techniques have been developed employing a soft ionization approach, making MS more robust for daily use. Furthermore, it should be noted that LC-MS can provide a list of m/z values, retention

times and an estimation of relative abundances of identified metabolites that are not actually identified. Overall, high-resolution and reproducible LC-MS measurement sets up the basis for subsequent data processing and multivariate data analysis. Large-scale metabolomic technologies based on LC-MS are increasingly gaining attention for their use in the diagnosis of human disease.[50] Development of robust, sensitive, and reproducible diagnostic tests for understanding diseases is a worldwide control program. Due to its sensitivity and quantitative reproducibility, LC-MS based metabolomics is a powerful approach to this problem. An LC-MS metabolomics-based diagnostic provides an essential tool and has the potential to monitor the progression of onchocerciasis.[51] LC-MS based nontargeted metabolomics has been thoroughly tested, validated, and applied to screen/identify and validate novel metabolic biomarkers for epithelial ovarian cancer; six key-metabolites were considered as potential biomarker candidates, ready for early stage detection.[52] In a study, an LC-MS method was successfully applied for metabolomic analysis of hydrophilic metabolites in a wide range of biological samples.[53] Classification separation for metabolites from different tissues was globally analyzed by PCA, PLS-DA and HCA biostatistical methods. As a result, a total of 112 hydrophilic metabolites were detected within 8 min of running time to obtain a metabolite profile of the biological samples. Recently, an LC-MS method for targeted multiple reaction monitoring has become a useful tool for the analysis of hundreds of polar metabolites in a complex sample.[54] Targeted bile acid analysis using LC-MS metabolomics demonstrated increased levels of conjugated or unconjugated bile acids and may allow the distinction of different types of hepatobiliary toxicity.[55]

UPLC-MS

UPLC-MS technology is a powerful technique in biomolecular research and can also be used to quantify the activity of signalling and metabolic pathways in a multiplex and comprehensive manner. The recent introduction of UPLC, employing porous particles with internal diameters smaller than 2 μm, in conjuction with MS, results in higher peak capacity, enhanced specificity and high-throughput capabilities compared to conventional HPLC columns, therefore making it even more suitable for a metabolomics approach. Because the optimum linear velocity has a broader range, UPLC also allows a more rapid analysis without loss of resolution. The combination of UPLC with MS detection covers a number of polar metabolites and thus enlarges the number of detected analytes. In view of the recent developments in the separation sciences, the advent of UPLC and MS based technology has shown ever improving resolution of metabolite species and precision of mass measurements. Q-TOF-MS is coupled with UPLC for the analysis and identification of trace components in complex mixtures, as a powerful means to make accurate mass measurement levels of less than 5 ppm, with effective resolution. Given the power of the technology, the UPLC-MS technique represents a promising hyphenated microseparation platform in metabolomics, since the majority of primary metabolites are intrinsically polar. Recently, this technology has rapidly been accepted by the analytical community and hyphenated UPLC-MS has been used for coupling to MS in metabolomic studies to provide a complementary tool widely applied to various fields.

Metabolomics follows the changes in concentrations of endogenous metabolites, which may reflect various disease states as well as systemic responses to environmental, therapeutic, or genetic interventions. To investigate the effect of acupuncture on acute gouty arthritis and search for its mechanism, a metabolomic method was developed and endogenous metabolites were analyzed.[56,57] The plasma samples, when injected onto a reverse-phase 50 × 2.1 mm Acquity 1.7-μm C_{18} column using a UPLC coupled with Q-TOF-MS in positive and negative modes, yielded a data matrix with a total of 2600 features, showing the power of this technology. It indicates that UPLC-MS-based metabolomics can be used as a potential tool for the investigation of the biological effect of acupuncture on acute gouty arthritis. Chaihu-Shu-Gan-San (CSGS), a traditional Chinese medicine (TCM) formula, has been effectively used for the treatment of depression in the clinic.[58] Metabolomics based on UPLC-Q-TOF-MS was used to profile the metabolic fingerprints of urine obtained from a chronic variable stress induced depression model in rats with and without CSGS treatment. This study showed that the urinary UPLC-QTOF-MS approach was a powerful tool to study the efficacy and mechanism of complex TCM prescriptions. A urinary metabolomics method based on UPLC-MS was developed to study characteristics of 'Kidney-Yang Deficiency syndrome' and the therapeutic effects of *Rhizoma Drynariae*.[59] The biochemical changes are related to the disturbance in energy metabolism, amino acid metabolism and gut microflora, which are helpful to further understand 'Kidney-Yang Deficiency syndrome' and the therapeutic mechanism of *Rhizoma Drynariae*. UPLC-MS-based analytical methods were used to pathophysiologically characterize cholestasis.[60] More than 250 metabolites were detected in both plasma and urine. UPLC has been used for assessing the holistic efficacy and synergistic effects of TCM,[61–64] for analyzing toxicity,[65,66] severe childhood pneumonia,[67] nonalcoholic fatty liver disease,[68] diabetes,[69–71] colorectal carcinoma[72] and Alzheimer's disease,[73] and for nutritional studies.[74]

Comprehensive analytical technologies

Metabolomics technology is nowadays widely applied in the field of biology and pharmacy. The integration of MS, NMR, and other modern analytical techniques have accelerated the study of metabolomics. In the field of metabolomics, hundreds of metabolites are measured simultaneously by analytical platforms such as GC-MS, LC-MS, NMR, 2-D-GCxGC and 2-D-GCxGC-TOF-MS, and MALDI-FTICR-MS to obtain their concentration levels in a reliable way. Comprehensive 2-D-GCxGC-MS is a powerful technique which has gained increasing attention over the last two decades, and can provide greatly increased separation capacity, chemical selectivity and sensitivity for complex sample analysis, bringing more accurate information about compound retention times and mass spectra.[75] For the data acquisition platform, NMR, GC-MS and LC-MS are the prevalent techniques, although at present none of them are perfect techniques that can meet with the requirement of metabolomics to measure all metabolites. Multi-analysis techniques can partially overcome the shortcomings of single-analysis techniques. NMR and LC-MS

based metabolomics has great potential for the discovery of biomarkers for diseases.[76] Several safety biomarker candidates were discovered as a result of global metabolic profiling using LC-MS with multivariate data analysis, and they were also confirmed by targeted metabolic profiling using GC-MS and CE-MS.[77] An integrated ionization approach of electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and atmospheric pressure photoionization (APPI) combined with rapid resolution LC-MS has been developed for performing global metabolomic analysis on complex biological samples.[78] This proposed approach provided a more comprehensive picture of the metabolic changes and further verified identical biomarkers that were obtained simultaneously using different ionization methods.

Comprehensive analytical technologies are rapidly becoming a cornerstone of modern life sciences. Two-dimensional GCxGC-MS brings much increased separation capacity, chemical selectivity and sensitivity for metabolomics and provides more accurate information about metabolite retention times and mass spectra.[79] Global metabolome coverage of *Caenorhabditis elegans*, which is widely used as a model organism in many areas of the life sciences, was evaluated by cross-platform combinations by GC-MS, NMR and UPLC/MS.[80] These technologies have proven highly informative for the global analysis of metabolites, providing a powerful approach to study small molecules in order to better understand the implications and subtle perturbations.[81] Targeted and non-targeted NMR, GC-MS and LC-MS methods have identified and quantified 4229 metabolites in the human serum metabolome.[82] GC-MS and LC-MS were integrated to approximate the comprehensive metabolic signature of the malnutrition rat model and to discover differentiating metabolites.[83] Key metabolites indicated the alterations were associated with perturbations in energy metabolism, carbohydrate, amino acid, and fatty acid metabolism, purine metabolism, cofactor and vitamin metabolism, in response to protein and energy malnutrition. These findings showed the integration of GC-MS and LC-MS techniques for untargeted metabolic profiling analysis was promising for nutriology. A combined UPLC-MS and GCxGC-TOF approach could be used to investigate the pathophysiology of irritable bowel syndrome (IBS) by comparing the global mucosal metabolic profiles of IBS patients with those of healthy controls.[84] An LC-MS-NMR innovation platform is demonstrated for the identification of unknown compounds found at low concentrations in complex sample matrixes, providing several-fold higher sample efficiency than conventional flow injection methods.[85] UPLC-MS and GC-MS has aided the identification of a number of biomarkers that have been shown to be both dose- and time-dependent.[86] Systematic untargeted metabolite profiling combined GC-MS and UPLC coupled to FT-ICR-MS (LC-FT-MS) analyse biodiversity on the performance of individual plant species.[87] It revealed 139 significantly changed metabolites (30 by GC-MS and 109 by LC-FT-MS). Taken together, metabolite profiling based GC-MS and LC-FT-MS is a strong diagnostic tool to assess individual metabolic phenotypes in response to plant diversity and ecophysiological adjustment. A novel finding from the hydrophilic interaction ultra performance liquid chromatography (HILIC-UPLC-MS) approach was investigated for the global metabolic profiling of rat urine samples generated in an experimental hepatotoxicity study of galactosamine and the concomitant investigation of the protective effect of glycine.[88] The multiple metabolomics platforms and technologies allowed us to substantially enhance the level of metabolome coverage while critically assessing the relative strengths and weaknesses of these platforms or technologies.[89–91]

Other technologies

The metabolome is characterized by a large number of molecules exhibiting a high diversity of chemical structures and abundances, requiring analytical platforms to reach its extensive coverage. Although NMR, GC-MS, LC-MS and UPLC-MS are most often used for large-scale analysis, metabolomics is not limited to these techniques. Other alternatives include thin-layer chromatography, HPLC with UV/visible absorbance, photodiode array or electrochemical detectors, FT-IR, MALDI-FTICR-MS and a variety of other enzymatic assays. Combined use of multiple techniques or multiple detectors in online or parallel analysis can significantly increase the metabolite coverage and quantification limits and improve identification of metabolites from a single biological sample. The metabolome is characterized by a large number of molecules exhibiting a high diversity of chemical structures and abundances, requiring complementary analytical platforms for extensive coverage. Of these analytical platforms, FT-MS instruments are popular because they provide accurate mass measurements with ppm and even sub-ppm errors, and also high and ultra-high resolving power.[92] MALDI-MS is an emerging analytical tool for the analysis of small molecules with molar masses below 1000 Da. This technique offers rapid analysis, high sensitivity, low sample consumption, a relative high tolerance towards salts and buffers, and the possibility to store samples on the target plate.[93] FTICR-MS has the potential to be a powerful new technique for high-throughput metabolomic analysis. For example, Han and colleagues examined the properties of an FTICR-MS for the identification and quantitation of human plasma metabolites (>400), and for untargeted metabolic fingerprinting.[94] The results demonstrated that FTICR-MS is well-suited to high-throughput metabolomic analysis. API/MS-based technologies, especially those using electrospray ionization, are now very popular.[95] HILIC, although not a new technique, has enjoyed a recent renaissance with the introduction of robust and reproducible stationary phases.[96] It is consequently finding application in metabolomics studies, which have traditionally relied on the stability of reversed phases, since the biofluids analyzed are predominantly aqueous and thus contain many polar analytes. HILIC's retention of those polar compounds and use of solvents readily compatible with MS have seen its increasing adoption in studies of complex aqueous metabolomes.

Conclusion and future perspectives

Analysis of the metabolome with coverage of all of the possible detectable components in the sample, rather than analysis of each individual metabolite at a given time, can be accomplished by metabolic analysis.[97,98] Targeted and/or nontargeted approaches are applied as needed for particular experiments. Monitoring hundreds or more metabolites at a given time requires high-throughput and high-end techniques that enable

screening for relative changes in, rather than absolute concentrations of, compounds within a wide dynamic range. Most of the analytical techniques useful for these purposes use GC or HPLC/UPLC separation modules coupled to a fast and accurate MS. Discovery and validation of biomarkers are exciting and promising opportunities offered by metabolic analysis applied to biological and biomedical experiments. We have demonstrated that integrated techniques (*i.e.* GC-TOF-MS, HPLC/UPLC-RP-MS and HILIC-LC-MS) used for metabolic analysis offer sufficient metabolome mapping, providing researchers with confident data for subsequent multivariate analysis and data mining. This review highlights the importance and benefit of the role of integrated tools in metabolomic research. The recent rapid development of a range of integrated analytical platforms in metabolomics is an ideal strategy and will provide sensitive and reproducible detection of thousands of metabolites in a biofluid sample, accelerating integration of metabolomics into systems biology.

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References

1 J. K. Nicholson and J. C. Lindon, *Nature*, 2008, **455**, 1054–1056.
2 T. A. Clayton, J. C. Lindon, O. Cloarec, H. Antti, C. Charuel, G. Hanton, J. P. Provost, J. L. Le Net, D. Baker, R. J. Walley, J. R. Everett and J. K. Nicholson, *Nature*, 2006, **440**, 1073–1077.
3 X. Wang, H. Sun, A. Zhang, W. Sun, P. Wang and Z. Wang, *J. Pharm. Biomed. Anal.*, 2011, **55**, 859–868.
4 A. Zhang, H. Sun, Z. Wang, W. Sun, P. Wang and X. Wang, *Planta Med.*, 2010, **76**, 2026–2035.
5 A. Sreekumar, L. M. Poisson, T. M. Rajendiran, A. P. Khan, Q. Cao, J. Yu, B. Laxman, R. Mehra, R. J. Lonigro, Y. Li, M. K. Nyati, A. Ahsan, S. Kalyana-Sundaram, B. Han, X. Cao, J. Byun, G. S. Omenn, D. Ghosh, S. Pennathur, D. C. Alexander, A. Berger, J. R. Shuster, J. T. Wei, S. Varambally, C. Beecher and A. M. Chinnaiyan, *Nature*, 2009, **457**, 910–914.
6 A. K. Arakaki, J. Skolnick and J. F. McDonald, *Nature*, 2008, **456**, 443.
7 E. Holmes, R. L. Loo, J. Stamler, M. Bictash, I. K. Yap, Q. Chan, T. Ebbels, M. De Iorio, I. J. Brown, K. A. Veselkov, M. L. Daviglus, H. Kesteloot, H. Ueshima, L. Zhao, J. K. Nicholson and P. Elliott, *Nature*, 2008, **453**, 396–400.
8 O. Yanes, R. Tautenhahn, G. J. Patti and G. Siuzdak, *Anal. Chem.*, 2011, **83**, 2152–2161.
9 D. S. Cao, B. Wang, M. M. Zeng, Y. Z. Liang, Q. S. Xu, L. X. Zhang, H. D. Li and Q. N. Hu, *Analyst*, 2011, **136**, 947–954.
10 D. H. Kim, R. M. Jarvis, Y. Xu, A. W. Oliver, J. W. Allwood, L. Hampson, I. N. Hampson and R. Goodacre, *Analyst*, 2010, **135**, 1235–1244.
11 J. Ding, S. Yang, D. Liang, H. Chen, Z. Wu, L. Zhang and Y. Ren, *Analyst*, 2009, **134**, 2040–2050.
12 R. A. Chylla, K. Hu, J. J. Ellinger and J. L. Markley, *Anal. Chem.*, 2011, **83**, 4871–4880.
13 C. Junot, G. Madalinski, J. C. Tabet and E. Ezan, *Analyst*, 2010, **135**, 2203–2219.
14 P. W. Carr, D. R. Stoll and X. Wang, *Anal. Chem.*, 2011, **83**, 1890–900.
15 L. Lin, Q. Yu, X. Yan, W. Hang, J. Zheng, J. Xing and B. Huang, *Analyst*, 2010, **135**, 2970–298.
16 P. Masson, A. C. Alves, T. M. Ebbels, J. K. Nicholson and E. J. Want, *Anal. Chem.*, 2010, **82**, 7779–7786.
17 C. H. Johnson, E. Karlsson, S. Sarda, L. Iddon, M. Iqbal, X. Meng, J. R. Harding, A. V. Stachulski, J. K. Nicholson, I. D. Wilson and J. C. Lindon, *Xenobiotica*, 2010, **40**, 9–23.
18 A. K. Smilde, M. J. van der Werf, J. P. Schaller and C. Kistemaker, *Analyst*, 2009, **134**, 2281–2285.
19 O. Beckonert, M. Coen, H. C. Keun, Y. Wang, T. M. Ebbels, E. Holmes, J. C. Lindon and J. K. Nicholson, *Nat. Protoc.*, 2010, **5**, 1019–1032.
20 K. Kim, P. Aronov, S. O. Zakharkin, D. Anderson, B. Perroud, I. M. Thompson and R. H. Weiss, *Mol. Cell. Proteomics*, 2009, **8**, 558–570.
21 Y. Minami, T. Kasukawa, Y. Kakazu, M. Iigo, M. Sugimoto, S. Ikeda, A. Yasui, G. T. van der Horst, T. Soga and H. R. Ueda, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 9890–9895.
22 J. Y. Liu, N. Li, J. Yang, N. Li, H. Qiu, D. Ai, N. Chiamvimonvat, Y. Zhu and B. D. Hammock, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 17017–17022.
23 M. Basanta, R. M. Jarvis, Y. Xu, G. Blackburn, R. Tal-Singer, A. Woodcock, D. Singh, R. Goodacre, C. L. Thomas and S. J. Fowler, *Analyst*, 2010, **135**, 315–20.
24 M. Brown, W. B. Dunn, P. Dobson, Y. Patel, C. L. Winder, S. Francis-McIntyre, P. Begley, K. Carroll, D. Broadhurst, A. Tseng, N. Swainston, I. Spasic, R. Goodacre and D. B. Kell, *Analyst*, 2009, **134**, 1322–1332.
25 R. Wei, G. Li and A. B. Seymour, *Anal. Chem.*, 2010, **82**, 5527–5533.
26 R. Shroff, L. Rulísek, J. Doubsky and A. Svatos, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 10092–10096.
27 E. S. Karlsson, C. H. Johnson, S. Sarda, L. Iddon, M. Iqbal, X. Meng, J. R. Harding, A. V. Stachulski, J. K. Nicholson, I. D. Wilson and J. C. Lindon, *Rapid Commun. Mass Spectrom.*, 2010, **24**, 3043–3051.
28 M. P. Stoop, L. Coulier, T. Rosenling, S. Shi, A. M. Smolinska, L. Buydens, K. Ampt, C. Stingl, A. Dane, B. Muilwijk, R. L. Luitwieler, P. A. Sillevis Smitt, R. Q. Hintzen, R. Bischoff, S. S. Wijmenga, T. Hankemeier, A. J. van Gool and T. M. Luider, *Mol. Cell. Proteomics*, 2010, **9**, 2063–2075.
29 J. M. Park, T. Y. Kim and S. Y. Lee, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 14931–14936.
30 H. Tang, C. Xiao and Y. Wang, *Magn. Reson. Chem.*, 2009, **47**, S157–62.
31 J. Wu, Y. An, J. Yao, Y. Wang and H. Tang, *Analyst*, 2010, **135**, 1023–1030.
32 P. Soininen, A. J. Kangas, P. Würtz, T. Tukiainen, T. Tynkkynen, R. Laatikainen, M. R. Järvelin, M. Kähönen, T. Lehtimäki, J. Viikari, O. T. Raitakari, M. J. Savolainen and M. Ala-Korpela, *Analyst*, 2009, **134**, 1781–1785.
33 S. Zhang, G. A. Nagana Gowda, T. Ye and D. Raftery, *Analyst*, 2010, **135**, 1490–1498.
34 H. Winning, E. Roldán-Marín, L. O. Dragsted, N. Viereck, M. Poulsen, C. Sánchez-Moreno, M. P. Cano and S. B. Engelsen, *Analyst*, 2009, **134**, 2344–2351.
35 M. Malet-Martino and U. Holzgrabe, *J. Pharm. Biomed. Anal.*, 2011, **55**, 1–15.
36 G. Liu, Y. Wang, Z. Wang, J. Cai, X. Lv and A. Zhou, *J Agric Food Chem.*, 2011, **59**, 5572–5578.
37 J. Y. Jung, H. S. Lee, D. G. Kang, N. S. Kim, M. H. Cha, O. S. Bang, H. Ryu do and G. S. Hwang, *Stroke*, 2011, **42**, 1282–1288.
38 I. Barba, R. Fernandez-Montesinos, D. Garcia-Dorado and D. Pozo, *J. Cell. Mol. Med.*, 2008, **12**, 1477–1485.
39 K. W. Jordan and L. L. Cheng, *Expert Rev. Proteomics*, 2007, **4**, 389–400.
40 Y. Lee do, B. P. Bowen and T. R. Northen, *BioTechniques*, 2010, **49**, 557–565.
41 K. Hiller, C. M. Metallo, J. K. Kelleher and G. Stephanopoulos, *Anal. Chem.*, 2010, **82**, 6621–6628.
42 D. J. Weston, *Analyst*, 2010, **135**, 661–668.
43 H. Tsugawa, T. Bamba, M. Shinohara, S. Nishiumi, M. Yoshida and E. Fukusaki, *J. Biosci. Bioeng.*, 2011, **112**, 292–298.
44 B. Ma, Q. Zhang, G. J. Wang, J. Y.A, D. Wu, Y. Liu, B. Cao, L. S. Liu, Y. Y. Hu, Y. L. Wang and Y. Y. Zheng, *Acta Pharmacol. Sin.*, 2011, **32**, 270–278.
45 T. Kuhara, M. Ohse, Y. Inoue and A. J. Cooper, *Anal. Bioanal. Chem.*, 2011, **400**, 1881–1894.

46 C. Barbas, E. P. Moraes and A. Villaseñor, *J. Pharm. Biomed. Anal.*, 2011, **55**, 823–831.
47 P. Britz-McKibbin, *Methods Mol. Biol.*, 2011, **708**, 229–246.
48 C. Simó, C. Ibáñez, A. Gómez-Martínez, J. A. Ferragut and A. Cifuentes, *Electrophoresis*, 2011, **32**, 1765–1777.
49 S. Sato, M. Arita, T. Soga, T. Nishioka and M. Tomita, *BMC Syst. Biol.*, 2008, **2**, 51.
50 F. Courant, G. Pinel, E. Bichon, F. Monteau, J. P. Antignac and B. Le Bizec, *Analyst*, 2009, **134**, 1637–1646.
51 J. R. Denery, A. A. Nunes, M. S. Hixon, T. J. Dickerson and K. D. Janda, *PLoS Neglected Trop. Dis.*, 2010, **4**, e834.
52 J. Chen, X. Zhang, R. Cao, X. Lu, S. Zhao, A. Fekete, Q. Huang, P. Schmitt-Kopplin, Y. Wang, Z. Xu, X. Wan, X. Wu, N. Zhao, C. Xu and G. Xu, *J. Proteome Res.*, 2011, **10**, 2625–2632.
53 H. Lv, G. Palacios, K. Hartil and I. J. Kurland, *J. Proteome Res.*, 2011, **10**, 2104–2112.
54 S. Bajad and V. Shulaev, *Methods Mol. Biol.*, 2011, **708**, 213–228.
55 H. Ellinger-Ziegelbauer, M. Adler, A. Amberg, A. Brandenburg, J. J. Callanan, S. Connor, M. Fountoulakis, H. Gmuender, A. Gruhler, P. Hewitt, M. Hodson, K. A. Matheis, D. McCarthy, M. Raschke, B. Riefke, C. S. Schmitt, M. Sieber, A. Sposny, L. Suter, B. Sweatman and A. Mally, *Toxicol. Appl. Pharmacol.*, 2011, **252**, 97–111.
56 P. Masson, K. Spagou, J. K. Nicholson and E. J. Want, *Anal. Chem.*, 2011, **83**, 1116–1123.
57 W. B. Dunn, N. J. Bailey and H. E. Johnson, *Analyst*, 2005, **130**, 606–625.
58 Z. H. Su, S. Q. Li, G. A. Zou, C. Y. Yu, Y. G. Sun, H. W. Zhang, Y. Gu and Z. M. Zou, *J. Pharm. Biomed. Anal.*, 2011, **55**, 533–539.
59 X. Lu, Z. Xiong, J. Li, S. Zheng, T. Huo and F. Li, *Talanta*, 2011, **83**, 700–708.
60 M. Aoki, Y. Konya, T. Takagaki, K. Umemura, Y. Sogame, T. Katsumata and S. Komuro, *Rapid Commun. Mass Spectrom.*, 2011, **25**, 1847–1852.
61 X. Liang, X. Chen, Q. Liang, H. Zhang, P. Hu, Y. Wang and G. Luo, *J. Proteome Res.*, 2011, **10**, 790–799.
62 Y. Lu, X. Liu, X. Liang, L. Xiang and W. Zhang, *J. Ethnopharmacol.*, 2011, **134**, 45–9.
63 P. Wang, H. Sun, H. Lv, W. Sun, Y. Yuan, Y. Han, D. Wang, A. Zhang and X. Wang, *J. Pharm. Biomed. Anal.*, 2010, **53**, 631–645.
64 Y. Lv, X. Liu, S. Yan, X. Liang, Y. Yang, W. Dai and W. Zhang, *J. Pharm. Biomed. Anal.*, 2010, **52**, 129–135.
65 K. Spagou, I. D. Wilson, P. Masson, G. Theodoridis, N. Raikos, M. Coen, E. Holmes, J. C. Lindon, R. S. Plumb, J. K. Nicholson and E. J. Want, *Anal. Chem.*, 2011, **83**, 382–390.
66 H. Lv, L. Liu, Y. Zhang, T. Song, J. Lu and X. Chen, *Mol. BioSyst.*, 2010, **6**, 2056–2067.
67 E. C. Laiakis, G. A. Morris, A. J. Fornace and S. R. Howie, *PLoS One*, 2010, **5**, e12655.
68 J. Barr, M. Vázquez-Chantada, C. Alonso, M. Pérez-Cormenzana, R. Mayo, A. Galán, J. Caballería, A. Martín-Duce, A. Tran, C. Wagner, Z. Luka, S. C. Lu, A. Castro, Y. Le Marchand-Brustel, M. L. Martínez-Chantar, N. Veyrie, K. Clément, J. Tordjman, P. Gual and J. M. Mato, *J. Proteome Res.*, 2010, **9**, 4501–4512.
69 X. Zhao, J. Fritsche, J. Wang, J. Chen, K. Rittig, P. Schmitt-Kopplin, A. Fritsche, H. U. Häring, E. D. Schleicher, G. Xu and R. Lehmann, *Metabolomics*, 2010, **6**, 362–374.
70 H. Tsutsui, T. Maeda, T. Toyo'oka, J. Z. Min, S. Inagaki, T. Higashi and Y. Kagawa, *J. Proteome Res.*, 2010, **9**, 3912–3922.
71 Y. Gu, Y. Zhang, X. Shi, X. Li, J. Hong, J. Chen, W. Gu, X. Lu, G. Xu and G. Ning, *Talanta*, 2010, **81**, 766–772.
72 W. Wang, B. Feng, X. Li, P. Yin, P. Gao, X. Zhao, X. Lu, M. Zheng and G. Xu, *Mol. BioSyst.*, 2010, **6**, 1947–1955.
73 N. J. Li, W. T. Liu, W. Li, S. Q. Li, X. H. Chen, K. S. Bi and P. He, *Clin. Biochem.*, 2010, **43**, 992–7.
74 C. Legido-Quigley, C. Stella, F. Perez-Jimenez, J. Lopez-Miranda, J. Ordovas, J. Powell, F. van-der-Ouderaa, L. Ware, J. C. Lindon, J. K. Nicholson and E. Holmes, *Biomed. Chromatogr.*, 2010, **24**, 737–743.
75 R. E. Mohler, K. M. Dombek, J. C. Hoggard, K. M. Pierce, E. T. Young and R. E. Synovec, *Analyst*, 2007, **132**, 756–767.
76 A. M. Goodpaster, E. H. Ramadas and M. A. Kennedy, *Anal. Chem.*, 2011, **83**, 896–902.
77 P. Gao, C. Lu, F. Zhang, P. Sang, D. Yang, X. Li, H. Kong, P. Yin, J. Tian, X. Lu, A. Lu and G. Xu, *Analyst*, 2008, **133**, 1214–1220.
78 Z. An, Y. Chen, R. Zhang, Y. Song, J. Sun, J. He, J. Bai, L. Dong, Q. Zhan and Z. Abliz, *J. Proteome Res.*, 2010, **9**, 4071–81.
79 S. Castillo, I. Mattila, J. Miettinen, M. Orešič and T. Hyötyläinen, *Anal. Chem.*, 2011, **83**, 3058–3067.
80 F. M. Geier, E. J. Want, A. M. Leroi and J. G. Bundy, *Anal. Chem.*, 2011, **83**, 3730–3736.
81 M. A. Zulyniak and D. M. Mutch, *Curr. Pharm. Biotechnol.*, 2011, **12**, 1005–1015.
82 N. Psychogios, D. D. Hau, J. Peng, A. C. Guo, R. Mandal, S. Bouatra, I. Sinelnikov, R. Krishnamurthy, R. Eisner, B. Gautam, N. Young, J. Xia, C. Knox, E. Dong, P. Huang, Z. Hollander, T. L. Pedersen, S. R. Smith, F. Bamforth, R. Greiner, B. McManus, J. W. Newman, T. Goodfriend and D. S. Wishart, *PLoS One*, 2011 Feb 16, **6**, e16957.
83 Z. Wu, M. Li, C. Zhao, J. Zhou, Y. Chang, X. Li, P. Gao, X. Lu, Y. Li and G. Xu, *Mol. BioSyst.*, 2010, **6**, 2157–2163.
84 K. Kajander, E. Myllyluoma, S. Kyrönpalo, M. Rasmussen, P. Sipponen, I. Mattila, T. Seppänen-Laakso, H. Vapaatalo, M. Oresic and R. Korpela, *World J. Gastroenterol.*, 2009, **15**, 6068–6074.
85 Y. Lin, S. Schiavo, J. Orjala, P. Vouros and R. Kautz, *Anal. Chem.*, 2008, **80**, 8045–8054.
86 C. H. Johnson, A. D. Patterson, K. W. Krausz, C. Lanz, D. W. Kang, H. Luecke, F. J. Gonzalez and J. R. Idle, *Radiat. Res.*, 2011, **175**, 473–484.
87 C. Scherling, C. Roscher, P. Giavalisco, E. D. Schulze and W. Weckwerth, *PLoS One*, 2010, **5**, e12569.
88 K. Spagou, I. D. Wilson, P. Masson, G. Theodoridis, N. Raikos, M. Coen, E. Holmes, J. C. Lindon, R. S. Plumb, J. K. Nicholson and E. J. Want, *Anal. Chem.*, 2011, **83**, 382–390.
89 J. J. van der Hooft, J. Vervoort, R. J. Bino, J. Beekwilder and R. C. de Vos, *Anal. Chem.*, 2011, **83**, 409–416.
90 Y. Chen, R. Zhang, Y. Song, J. He, J. Sun, J. Bai, Z. An, L. Dong, Q. Zhan and Z. Abliz, *Analyst*, 2009, **134**, 2003–2011.
91 R. J. Weber, A. D. Southam, U. Sommer and M. R. Viant, *Anal. Chem.*, 2011, **83**, 3737–3743.
92 C. Junot, G. Madalinski, J. C. Tabet and E. Ezan, *Analyst*, 2010, **135**, 2203–2219.
93 J. J. van Kampen, P. C. Burgers, R. de Groot, R. A. Gruters and T. M. Luider, *Mass Spectrom. Rev.*, 2011, **30**, 101–120.
94 J. Han, R. M. Danell, J. R. Patel, D. R. Gumerov, C. O. Scarlett, J. P. Speir, C. E. Parker, I. Rusyn, S. Zeisel and C. H. Borchers, *Metabolomics*, 2008, **4**, 128–140.
95 E. Werner, J. F. Heilier, C. Ducruix, E. Ezan, C. Junot and J. C. Tabet, *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.*, 2008, **871**, 143–163.
96 S. Cubbon, C. Antonio, J. Wilson and J. Thomas-Oates, *Mass Spectrom. Rev.*, 2010, **29**, 671–84.
97 V. V. Tolstikov, *Methods Mol. Biol.*, 2009, **544**, 343–353.
98 J. L. Ward, J. M. Baker, A. M. Llewellyn, N. D. Hawkins and M. H. Beale, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 10762–10767.

