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Towards quantitative mass spectrometry-based metabolomics in microbial and mammalian systems

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Metabolome analyses are a suite of analytical approaches that enable us to capture changes in the metabolome (small molecular weight components, typically less than 1500 Da) in biological systems. Mass spectrometry (MS) has been widely used for this purpose. The key challenge here is to be able to capture changes in a reproducible and reliant manner that is representative of the events that take place *in vivo*. Typically, the analysis is carried out *in vitro*, by isolating the system and extracting the metabolome. MS-based approaches enable us to capture metabolomic changes with high sensitivity and resolution. When developing the technique for different biological systems, there are similarities in challenges and differences that are specific to the system under investigation. Here, we review some of the challenges in capturing quantitative changes in the metabolome with MS based approaches, primarily in microbial and mammalian systems.

This article is part of the themed issue 'Quantitative mass spectrometry'.

1. Introduction

Post-genome science is characterized by the parallel analyses of gene products at the level of transcripts, proteins and metabolites, and forms the basis of systems biology. Characterizing metabolomes is central to developing a systems level understanding of cellular function [1–3]. Capturing changes at the level of the metabolome provides a window of opportunity to

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develop an understanding of the biological phenotype observed, and the link between the genotype and the expressed phenotype, in a biological system. Metabolomes represent the final level of '-omic' information that can potentially tell us how an organism organizes itself in expressing the phenotype that is observable.

The field of metabolome analyses is currently developing rapidly for the study of several biological systems including microbial [4–6], plant [7–9] and mammalian systems [10–12]. However, its broad deployment to biotechnology and clinical research and practice is not yet as widespread as desired due to several challenges in the quantitative metabolomics workflow that remain. Absolute quantification of metabolite concentrations, in the true sense, is difficult to achieve in non-targeted metabolomics, and most quantitative measurements are relative changes and are at best semi-quantitative (however, for ease of reference we use the word 'quantitative' in the rest of the article). As the key objective in most metabolomics workflows lies in capturing changes in the metabolome in response to perturbations to the biological system monitored, relative quantifications usually serve the purpose. This can be achieved with the help of external and/or internal standards (ESs and ISs).

Most comprehensive metabolomics workflows employ either nuclear magnetic resonance- or mass spectrometry (MS)-based detections. MS has the advantage of higher speed, versatility and high degree of specificity and sensitivity, which are desired characteristics in quantitative workflows. Given the diversity of chemical characterizations required, only a partial coverage of the total metabolome can be expected to be captured with current technology, although it is often impossible to define what the full metabolome would be in a given organism under a given physiological state. The specificity and sensitivity can be enhanced by hyphenation of MS with high-resolution separation techniques such as gas chromatography (GC), liquid chromatography (LC) or capillary electrophoresis (CE). While enhancing data resolution, these hyphenation techniques bring in additional challenges. In addition to analytical challenges, challenges also exist in controlling the biological variability, sample processing steps (quenching, extraction and derivatization), selection of normalization and quantitative standards, data processing steps and quality control (QC) and validation of all the steps involved in metabolomics pipeline. Overall, in any quantitative MS-based metabolomics, MS is just one part of the integrated workflow; failure or compromise in any step of the overall workflow will invalidate the entire assay. In this review, we assess some of these challenges involved in MS-based quantitative metabolomics with a focus on application to microbial and mammalian systems.

2. Approaches in metabolomics

The two orthogonal approaches used in metabolomics are targeted and non-targeted metabolomics. Targeted metabolomics involves hypothesis-driven experiments and are characterized by obtaining the quantitative data on a predefined set of metabolites with a high level of precision and accuracy. This absolute quantification approach requires not only specialized extraction protocols but also specialized separation and detection techniques in order to identify and quantify a subset of pathway-specific metabolites. Non-targeted metabolomics studies are applied as a hypothesis generation strategy and are characterized by simultaneous qualitative and quantitative measurement of a large number of metabolites in samples. Non-targeted metabolomics uses relative quantification of metabolites, where metabolite spectral patterns and intensities are recorded, statistically compared and used to identify the relevant spectral features that distinguish sample class, and has the potential to provide a panoramic view covering both primary and secondary metabolites. However, the wide diversity of metabolites in terms of their physico-chemical properties presents a major challenge in comprehensively profiling them in a biological system. Hence, analyses of metabolome require an integrated workflow and a number of different approaches. The approaches commonly used in metabolomics are listed in electronic supplementary material, table S1, which aims at investigating subsets of the metabolome depending on the biological question.

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3. Challenges in mass spectrometry-based quantitative metabolomics

Standardization of the quantitative MS-based metabolomics workflow is essential in deriving accurate and meaningful biological interpretations. To explore the great potential of metabolomics, it is essential to first address the challenges involved prior to sampling, during sample preparation and processing, and in data acquisition and analysis.

(a) Challenges prior to sampling

(i) Biological variability

In any quantitative analysis, biological variability can introduce systematic errors. The final reported concentration of metabolites primarily needs to relate to the viable cell population. Viable cells (biovolume) contribute to the metabolome that is relevant and, therefore, represents the proportion of biomass of interest for intracellular metabolite quantification. It is important to ensure this population is sufficiently high in the samples to make appropriate interpretations. In addition, it must be noted that the sampled population provides only a statistical average of the overall population metabolic status, as the cells may not all be in the same physiological state. It is, therefore, important to take note of these in assessing the metabolome for quantitative changes. In cases where single-cell metabolomics can provide valuable information these variations can be accounted for, but the techniques for these are still under development [13]. Optical density and/or cell dry weight is commonly used as the reference to obtain biomass-specific concentration data under the assumption that impact of cell viability and population heterogeneity is negligibly small, thereby introducing systematic errors right from the beginning. Moreover, these errors should be kept constant in any follow-up experiments in order to make the data comparable. In addition, variances resulting from media preparation, inoculum densities or pre-cultivation almost always exceed analytical variance. Therefore, a minimum of five biological replicates is recommended [14] to account for such variances.

(ii) Normalization strategy

As we are interested in changes in metabolite concentration due to biological events and not due to non-biological factors, sample normalization in quantitative metabolomics is crucial in order to minimize the effect of sample variations. Sample normalization in metabolomics is much more complicated compared with that of genomics and proteomics due to the wide physico-chemical diversity of metabolites and this is an understated issue within quantitative metabolomics that can have a significant influence on the interpreted results. To date, consideration of total metabolite concentration or an equivalent metric is not common practice, as it is in proteomics.

Two strategies are commonly used in metabolome normalization, namely pre-acquisition and/or post-acquisition. In the pre-acquisition strategy, the extracted metabolome is normalized to a metric such as biomass that would be expected to have an even influence over all the metabolites extracted for a given sample. In the post-acquisition strategy, individual metabolite signals are normalized for different samples to a metric that is uniformly applicable to all samples post-acquisition, such as the total ion signal intensity of a chromatogram [15]. In MS, varying degrees of ionization efficiencies and ion suppression effects contribute to signal intensities, which often result in non-uniform response for individual metabolites. Hence, it can be argued that more accurate quantitative results can be obtained with pre-acquisition normalization. Moreover, this strategy can also be used to determine the optimal sample injection amount for MS in order to improve the detection of low concentration metabolites. By contrast, the post-acquisition strategy is relatively convenient and simpler to perform, as it does not require additional experimental set-up as required in the pre-acquisition strategy. The selection of appropriate normalization strategy is largely dependent on various factors such as the type of biological system under investigation, required normalization accuracy, convenience, speed and cost, and in some cases use of both strategies may be needed.

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In cellular metabolomics, variation in seeding densities and/or sampling strategies requires normalization of cell extract by cell counting. The haemocytometer is widely used for this purpose in suspension cultures. In the case of adherent cell cultures, cells are detached from their surfaces and harvested either by trypsinization or cell scraping. Both methods often result in loss of cells and changes in metabolic pattern [16], thereby impairing the accuracy of normalization by cell counts. In the case of microbial cells, determining cell counts may be difficult due to their small sizes and colony forming units (CFU) may be used instead. Alternatively, normalization to OD_{600} values could provide a reliable way for quantitative analysis [17]. However, both methods require an additional experimental set-up, making it cumbersome for quantitative metabolomics that is also difficult to apply for adherent cell cultures.

Other conventional methods include normalization to dry cell weight (DCW) and total cellular content of proteins, adenosine triphosphate (ATP) and/or DNA. Normalization to DCW is not ideal, as the method is time consuming, requires large number of samples and introduces relatively large amount of weighing errors [18]. Normalization to protein content using bicinchoninic acid assay or Bradford assay has been widely used, but a better correlation between cell numbers with cellular DNA content than protein content has been shown in some cases [19]. Both approaches require separate experiments and can be time consuming. The classical method for cell proliferation/viability studies includes CFU; however, the overall method is not precise and optimal for slow growing cells. Alternatively, ATP is a key central metabolite to all live cells and intracellular concentration of ATP is fairly constant in living cells, while rapid loss of ATP occurs from dead cells. Hence, ATP quantification using bioluminescence is an attractive solution to conventional CFU enumeration. Moreover, ATP estimation using bioluminescence method can be more rapid, reliable, sensitive, time saving and less expensive compared with conventional methods.

An alternative method for normalization involves the use of specific metabolite biomarkers [20]. However, it is important to note that these biomarkers may be specific to the cell lines under investigation and need to be selectively identified. Normalization of each peak area to the sum of all peak areas has been evaluated [21] as an alternative to normalization to cell count, where the authors reported good linear correlation with this method. Authors also recommended that this method should only be applied when the difference in concentration between two comparative samples is less than twofold, as otherwise the number of false detections would increase to over 10%. Determining the UV absorbance of the sample solution at a specific wavelength as a measure of the total concentration of solute is another concept of sample normalization [15]. This method can be more representative of the overall sample composition, independent of the biological matrix and is performed prior to MS acquisition. Moreover, the method is advantageous to cellular metabolomic studies as it does not require an extra procedure and can be used to correct the concentration variations introduced during the sample preparation steps. This method was further developed [22] into a dansylation metabolite assay, where absorbance of labelled metabolite was measured using simple microplate reader instead of expensive LC-UV systems. Authors have shown a good linear relationship between the UV absorbance values and the cell suspension volume or the protein content.

(b) Challenges in sample preparation

The metabolites of interest can be lost during sample preparation steps, which require careful evaluation and validation using a set of ISs to enable accurate quantification.

(i) Use of internal standards

Commonly used stable isotope-labelled ISs include metabolites labelled with ^{2}H, ^{13}C, ^{15}N and ^{18}O. They possess similar chemical properties to that of the non-labelled metabolites, which result in their partitioning with the associated metabolite throughout the analytical workflow. In addition, they also compensate for any ion suppression effects by matching the ionization

properties of the analyte. This eliminates both sample preparation and instrumental bias and can be used for quantification of metabolites. However, it is important to have sufficient mass difference between the labelled metabolite standard and the associated non-labelled metabolite from the sample in order to avoid isotopic interferences from the naturally occurring metabolite in the quantification of the reference compound [23]. Owing to a wide diversity of metabolites many of which are still uncharacterized, use of isotope-labelled ISs for individual metabolites is not a practical approach. Moreover, availability and expense will have a significant role in their employment. In cases where there are batch to batch variations resulting in the absence or very low concentration of some metabolites of interest in the isotope cell extracts, the use of such ISs might not be valid. In such cases, the use of labelled derivatization reagent might be useful as demonstrated in the past for the absolute quantification of amino and non-amino organic acids in urine and serum samples [24].

The use of a pooled QC sample has been advocated in some cases [23]. This involves generation of the calibration model by analysing the different dilutions of a pooled sample, which can then be used for relative quantification of metabolites. This method can only be applied to samples where matrix effects are minimal. It provides a good way to monitor detector drift, inertness of the analytical column and in calculating the repeatability and precision of response for all metabolites. In the absence of ISs, quantification can be done by spiking or the method of standard additions to the matrix. This method eliminates any chemical or physical bias between the standards and the samples; however, it increases the number of sample determinations required for each sample [25]. Alternatively, ESs can be run independently, where the instrumental response to standard concentration is measured to generate the response curve, which can then be used to calculate the metabolite concentration. This method can only be applied to samples which require minimum preparation and have high degree of reproducibility and good recovery. This method is very good in detecting or correcting for detector drift and in controlling the inertness of the analytical platform. If the sample matrix is not well characterized, this method can have bias from matrix effects. Normalization using optimal selection of multiple ISs (NOMIS) uses the variability information from multiple ISs across multiple samples to find the optimal normalization factor for individual detected metabolites.

Any error by the analyst in weighing, diluting, dispensing or dissolving ISs will be propagated and compromise the integrity of overall assay. Hence, it is important to incorporate the selected ISs at the earliest stage possible such as during quenching or extraction steps. Moreover, it is important to add the optimal amount of IS, as adding too much or too little of IS can increase the variance in the overall assay. The ideal concentration of IS is recommended [25] to be threefold in excess of that of the expected metabolite concentration.

(ii) Quenching

The high turnover rate of intracellular metabolites requires rapid sampling and instantaneous quenching of enzyme activities under mild conditions in order to retain a valid snapshot of the metabolic processes. Quenching with 60% v/v cold methanol at −40°C has been used widely in the past for various biological systems. However, potential problems connected to leakage of intracellular metabolites with this method have been reported [26,27]. Various alternatives to cold methanol quenching such as filter culture methodology, fast filtration, mass balance approach and use of alternative quenching solvents have been evaluated. However, all suggested alternatives [28] have advantages and disadvantages and more importantly cannot be directly applied to a given organism, without prior evaluation. In addition, these alternatives have also been shown to add difficulties in the overall metabolomics workflow.

To minimize metabolite leakage with cold methanol quenching, additives that will buffer the effect and minimize osmotic shock have been suggested [29]. Commonly employed buffer additives involve methanol supplemented with 4-(3-hydroxyethyl)piperazine-1-ethanesulfonic acid, ammonium bicarbonate, tricine or NaCl. Influence of these additives in preserving the membrane integrity and, therefore, in minimizing metabolite leakage is well studied (see

electronic supplementary material, table S2). The concentration of methanol and the quenching temperature [30] can also have an influence on metabolite leakage.

To minimize the influence of exo-metabolome on intracellular metabolite extraction, cells must be rapidly separated from the culture medium after quenching following centrifugation or fast filtration. To minimize carryover effects, it is often essential to introduce an additional washing step in the workflow. Inclusion of a washing step for adherent cultures is easier as it can be performed rapidly prior to quenching. However, influence of washing solutions on metabolite leakage requires careful evaluation prior to their implementation. By contrast, introducing a washing step in the case of suspension cultures is not ideal as it needs to be performed prior to quenching which will result in delaying the quenching time frame.

(iii) Extraction

Owing to the diverse physico-chemical properties of metabolites, the identification of an optimal extraction solvent to quantitatively extract all intracellular metabolites represents a major challenge in metabolomics. In the past, strong acids or alkalis were commonly employed as routine methods for the extraction of acid and alkali stable compounds from animal and plant tissues and microorganisms. Later use of these solvents was limited as they result in lower number of data points with poor reproducibility (as most of the metabolites are unstable at low or high pH conditions) compared with mild extraction solvents [31]. Moreover, these methods are time consuming, as they require neutralization of the sample at later stage. Recently, the compatibility between the extraction solvent and the subsequent analytical platform is gaining more attention and reveals a trend towards selection of more mild extraction conditions such as the use of cold organic solvents (see electronic supplementary material, table S3). However, the selection of an optimal extraction solvent and method seems to be based on the metabolite classes of interest and the biological system under investigation.

Use of biphasic solvent systems such as methanol/chloroform/water mixtures offers several unique advantages over monophasic solvent systems. With such systems, the aqueous methanol–water phase can be used to extract polar metabolites, while the organic chloroform phase can be used to extract non-polar metabolites. Both the phases can be extracted simultaneously and each fraction can be analysed separately with better resolution, following centrifugation. In addition, this method will avoid much of the variations caused by the analysis of both polar and non-polar metabolites from separate samples. The use of chloroform in biphasic solvent systems ensures denaturation of enzymes, thereby halting the metabolism and preventing further degradation or inter-conversion of metabolites [32]. However, implementing these procedures is time consuming and difficult to automate thereby decreasing the scope for high-throughput analyses and overall less suitable for metabolomics investigations. Moreover, considerable loss of metabolites might occur [33], as some of the metabolites might be associated/leftover with the cell debris, which is usually located at the interphase between the polar and non-polar solvents.

For the unbiased analysis of metabolites, it is essential that all metabolites need to be completely, non-selectively and reproducibly extracted by avoiding their degradation and/or conversion to other metabolites. Moreover, the resulting sample matrix should be compatible or amenable to the analytical method of choice. To date, it has not been possible to generate such an extraction solvent. Completeness of extraction cannot be determined theoretically, as no one knows initially the number of metabolites present in the cells; hence determining the extent of metabolite degradation and efficacy of the method should be tested to validate the optimal method. Efficacy can be tested by comparing the different methods for identical biological samples, whereas extent of metabolite degradation and the absence of enzyme activity can be tested by metabolite recoveries, by introducing an isotopically labelled analogue into the extraction solvent. In addition, evaluation based on qualitative (number of peaks) or semi-quantitative manner (peak area or height, normalized intensities) would not be ideal, as both approaches work under the assumption of linearity of response and the absence of matrix effects, which is often not valid for complex cell extracts.

(iv) Derivatization in gas chromatography-mass spectrometry

The two-step derivatization procedure (methoximation followed by silylation with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide) is most commonly used for GC-MS metabolite profiling. However, this method suffers from double derivatization of primary amines, which results in multiple chromatographic peaks that complicate the quantification of metabolites [34]. In addition, determining the optimal duration and temperature for this method is of great significance for quantitative metabolomics. In microbial metabolomics, a set of *n*-alkanes has been used [34] to calculate the derivatization efficiency, where the researchers evaluated several parameters such as choice of derivatization solvents, use of various oximation and silylation reagents, derivatization times and temperature. Moreover, in view of analytical performance of different metabolites which is mainly governed by the stability of the silylation product, authors classified metabolites based on their derivatization efficiencies. In another study [35], influence of storage temperature and duration on stability of the trimethylsilyl derivatives was evaluated in quantifying 28 standard metabolites. Authors recommended −20°C as a suitable temperature for stability of trimethylsilyl derivatives under storage and that analysis should be carried out within 72 h.

The sources of bias in GC-MS-based metabolomics can occur in two forms (types A and B). Type A bias is universal and affects all the metabolites equally. It can be corrected by the addition of an IS, whereas type B bias affects individual metabolites differently. It has been pointed out [36] that the primary source of bias in GC-MS is the sample derivatization step, which introduces both type A and to a greater extent type B bias. In order to avoid the time-dependent bias in derivatization, the use of automated in line derivatization has been proposed [37]. Alternatively, the use of labelled metabolite standards or extracts from organisms (grown on labelled carbon source) has been proposed to calculate derivatization efficiency [4]. However, this approach is very expensive, increases complexity of the deconvolution process, does not address the issue of multiple derivatized peaks for the same metabolite and cannot be applied to biological systems which are difficult to grow *in vitro*. An alternative solution to this has been proposed [24], where the use of isotopically labelled methyl chloroformate derivatization has been advocated. This approach was demonstrated for only two metabolite classes, and also requires evaluation and validation of its applicability to quantify other metabolite classes.

In summary, sufficient derivatizing reagent and optimum conditions are essential for the efficient derivatization of all intracellular metabolites, as incomplete derivatization of compounds with multiple functional groups may result in eluting multiple peaks for the same metabolite. Moreover, the stability of the derivatized extract and metabolite degradation during storage or their decomposition in the analytical system require careful evaluation and validation in different matrices prior to quantification of metabolites.

(c) Challenges with the analytical platform

The choice of analytical platform can have great influence on quantitative data obtained in metabolomics experiments.

(i) High-resolution mass spectrometry

Over the last decade, MS has secured a pinnacle position and holds additional promise for the advancement of quantitative metabolomics based on sensitivity, selectivity, relative cost and depth of coverage. Ionization methods in MS are classified on the basis of the source of the ions. The electron impact (EI) ionization and chemical ionization methods employ gas-phase sources and can be easily coupled with GC, but not with LC. In desorption methods, the sample in either gas or liquid state is converted to gaseous ions and is applicable to analysis of much higher masses, e.g. MALDI and SALDI [38]. Lastly, the spray sources involve ionization of an aerosolized spray, such as atmospheric pressure chemical ionization and electrospray ionization (ESI).

Mass analysers with different resolving powers are widely used in metabolomics. FTICR mass spectrometers are well known to provide higher mass accuracy (less than 1 ppm) and

ultrahigh mass resolution (>1 000 000), but they are very expensive. Q-TOF instruments serve as a cheaper alternative and are capable of providing reasonable mass accuracy, sensitivity and dynamic range [39]. Alternatively, the Orbitrap analyzer uses an electrostatic field to trap ions and has excellent mass accuracy (1–5 ppm) and high resolving power (240 000) [40]. The TOF mass analysers provide greater sensitivity by detecting all ions simultaneously (high-acquisition rates >100 Hz) rather than scanning mass ranges as is the case with many quadrupole instruments. In addition, they provide accurate mass measurement of the molecular ion, with typical mass accuracies of less than 5 ppm and require no prior knowledge of the metabolites to be detected, as would be required for quadrupole and triple quadrupole. In a single sample run, the above-mentioned high-resolution mass spectrometry (HRMS) systems can provide direct structural information from the exact mass (up to level of structural isomers) and the resulting elemental composition of the analyte. Moreover, HRMS can accurately quantify many metabolites within a broad concentration range compared with the multiple reaction monitoring method. Q-exactive MS is an improved version of HRMS which offers excellent detection range, as it can be operated by switching between positive and negative modes with sufficiently fast cycle times [41].

(ii) Hyphenated mass spectrometry platforms

Direct MS analysis has been used in the past for many quantitative analyses; however, it suffers from disadvantages such as ion suppression effects, inability to differentiate isomers and challenges in data interpretation, as unique metabolite ions are difficult to distinguish from adduct and product ions [42]. Therefore, coupling of high-resolution separations (GC, LC or CE) to MS is often essential for accurate quantification of metabolites [43].

GC-MS combines the high separation efficiency of capillary GC with the high sensitivity and resolution of MS. A wide range of volatile and/or derivatized non-volatile metabolites can be analysed qualitatively and quantitatively with high analytical reproducibility and at lower costs compared with LC-MS and CE-MS. GC-MS with EI ionization provides high sensitivity, wide dynamic range and results in production of reproducible spectra and highly transferable EI-MS spectral libraries that allows compound identification through mass spectral library matching. However, single quadrupole mass analysers have nominal mass accuracy and slow scan speed as opposed to QQQ mass analysers. With the use of GC-MS/MS, quadrupole scan speed of up to 20 000 mass units s^{-1} can be achieved, which offers the possibility of direct quantification [44]. Alternatively, GC-TOF-MS offers higher mass accuracy, scan speed and resolution, essential for adequate sampling of high-resolution chromatographic peak widths in the range of 0.5–1 s which also facilitates the implementation of fast GC methods, thus reducing the analysis time and increasing the productivity. For complex biological samples, peak capacities, resolving power and depth of metabolome coverage can be further increased by the use of two-dimensional (2D)-GC (GC × GC) that uses two columns having different stationary phase selectivities and are connected serially. Therefore, two metabolites of similar volatility but different polarity can be separated. In order to acquire sufficient data points across the sharp narrow peaks, 2D-GC is often coupled with TOF-MS. However, the data generated by GC × GC-TOF-MS are large and complex. The recently introduced GC/Q-Orbitrap-MS offers performance characteristics of both Orbitrap- and quadrupole-based isolation for sensitive analyte detection. In addition, it also offers numerous analysis modalities (molecular ion directed acquisition) to facilitate structural elucidation [45], ideal for quantitative metabolomics.

The LC-MS platform offers several advantages over GC-MS, such as operation at lower temperature and does not require chemical derivatization, thus simplifying the sample preparation steps and identification of the metabolites. Detection in both the positive and negative ion mode simultaneously is possible with LC-MS, thus reducing the time required for analysis and reducing bias due to injection errors. The implementation of 2D-LC-MS for metabolomics has lagged behind that of 2D-GC-MS, due to a complicated experimental set-up and loss of sensitivity due to a sample dilution effect in the second dimension [42]. However, the major disadvantage

of LC-MS is ion suppression, which can be overcome to some extent by miniaturization of ESI to nanospray ionization [46]. Another issue is the contamination of the MS source and adduct formation (which have significant consequences on the robustness of the method) and the lack of transferable LC-MS libraries for metabolite identifications [47]. For accurate quantification of metabolites, it is essential to detect these artefacts, prior to normalization of the data. Hydrophilic interaction liquid chromatography (HILIC) separations are the most suitable and are an attractive option for metabolomics. However, there are still many important classes of metabolites which are poorly resolved with HILIC. Therefore, development of a method which can effectively capture a majority of the metabolite classes for a non-targeted metabolomic studies would be beneficial.

In summary, it is also important to determine the optimum analytical factors for accurate quantification of metabolites [48]. To date, there is no single analytical method suitable for detection of all the metabolite classes due to physico-chemical diversity of the metabolites; therefore, parallel application of optimized GC-MS and LC-MS workflows for a given organism would be needed.

(d) Challenges in quantitative data analysis

The resulting data burden arising from the complexity and richness of the metabolome is regarded as one of the major issues. GC-MS and/or LC-MS experiments can generate two general types of data or mass spectral tags: (i) parent mass + chromatographic retention time or (ii) parent mass + fragment mass + chromatographic retention time. The identification of both known and unknown compounds is possible if these properties are properly documented. The processing of raw chromatographic data involves (i) spectral processing, (ii) data analysis, (iii) metabolite identification and quantification, and (iv) biological interpretation.

(i) Spectral processing

Spectral processing involves accurate identification and quantification of the features in the raw spectral data, which are then arranged in a feature quantification matrix (FQM) for subsequent statistical data analysis. For further detailed information on spectral processing steps, we recommend a relevant review article [49]. For post-acquisition feature normalization, refer to §3a(ii).

Quantitative analysis is often challenging as multiple ions may correspond to different fragments from the same molecule, which requires deconvolution methods to assign different ions to the same metabolite. AMDIS is the most promising deconvolution tool for GC-MS, as it can handle huge datasets, has automated processing and provides just one quantitative value per metabolite per sample. However, AMDIS is not compatible with LC-MS or CE-MS, but ESI-LC-MS data can be processed using component detection algorithm. Later freely available software tools have been developed for backfilling missing values obtained from AMDIS-processed GC-MS spectra, producing a data matrix more suitable for subsequent chemometric analysis [50]. In GC × GC-MS analyses, two alternative software platforms, ChromaTOF and parallel factor analysis (PARAFAC), have been used in the past. In the context of quantitation for non-targeted metabolomics, the precision of these deconvolution tools is still lower compared with targeted approaches and requires improvements.

(ii) Data analysis, metabolite identification and spectral databases

Once raw data have been converted to a quantitative description (FQM), one can, in principle, apply chemometric tools. The selection of multivariate analysis in metabolomics is highly dependent on the aim of the study. To define the metabolome more comprehensively via identification of metabolites, it is essential to construct appropriate mass spectral libraries and metabolite databases in order to extract the biological information from the data. Yi *et al.* [51] have provided an extensive review on this aspect, to which readers are referred.

(iii) Validation and quality control

Any quantitative metabolomic workflow ideally should include evaluation of validation parameters such as selectivity, calibration model (linearity and range), accuracy, precision (repeatability and intermediate precision), limits of quantification and additional parameters such as limit of detection, recovery, reproducibility and robustness. These validation parameters can be assessed with the use of appropriate ISs as detailed in §3b(i). The use of isotopically labelled ISs for every metabolite might be the ideal requirement, but its application is dependent on availability and cost considerations. Moreover, the validation performed for one matrix may not be applicable in another, requiring validation to be performed for all the matrices of interest. The most feasible and straightforward approach suggested could be the use of selected isotopically labelled ISs representing different metabolite classes [23]. In the absence of ISs, the accuracy of the analytical method can be determined by determining recovery of the spiked isotopically labelled metabolites to the sample. The variable response of metabolites at particular concentration due to matrix effects can be corrected by determining the ratio of the response of metabolite spiked after extraction and the metabolites in a standard solution.

The QC of the validated analytical method is essential in order to ensure the quality and reliability of the analytical data obtained, which can be achieved with the use of ESs, ISs or combination of both. A better approach could be the use of *in vivo* isotopically labelled microorganisms as ISs, where biological samples are grown on isotopically labelled substrates, resulting in labelling of all the intracellular metabolites. The extract obtained from such a set-up can then be spiked to the extract obtained from non-labelled biological sample. In this way, isotopically labelled ISs can be made available for all intracellular metabolites, for their accurate and reliable quantification. This approach can only be valid if the labelled substrates are available and to their highest labelling efficiency. Moreover, the retention behaviour of labelled and endogenous metabolites is very similar and when silylation is used, and their mass spectra can contain many similar fragments, thereby making the data complicated and difficult to quantify. This limitation can be overcome by the use of HRMS such as LTQ Orbitrap as demonstrated in the past while quantifying central carbon metabolites in *Methylobacterium extorquens* using isotope dilution mass spectrometry (IDMS) [52].

4. Matrix effects and use of isotope dilution mass spectrometry

The biggest bottleneck in quantitative metabolomics is the occurrence of matrix effects, which include artefacts caused by (i) contributions from the biological sample matrix, (ii) loss due to leakage, degradation or inter-conversion of metabolites during sample processing steps, and (iii) instrument-specific negative influences (such as ion suppression), which corrupt the quantification of metabolites. To account for these matrix effects a number of strategies have been suggested such as diluting the sample, using alternative extraction and/or derivatization procedures, cleaning the sample by additional chromatographic steps and compensating the matrix effect by normalization to an IS [48].

The evaluation of matrix effects using set of ISs on quantification of short chain fatty acids, monosaccharides and compounds containing amino groups (not amino acids) in faecal water was studied using GC-MS [53]. Authors pointed out that the pH of the standards mixture is crucial, as pH invariably affects the volatility and solubility of the analytes of interest resulting in matrix effect. Alternatively, the use of ^{13}C-labelled IS at the beginning (after quenching) and at the end of sample processing (prior to analysis) has been demonstrated to account for the matrix effects and in determining the metabolite recoveries in yeast metabolome. ISs added at the beginning determines the efficacy of the extraction protocols and can be used to compensate for the losses (volume losses or partial degradation—not metabolite inter-conversion), whereas the IS added at the end of sample processing can be used to correct analytical artefacts caused by sample matrix effects [54]. Similarly, use of ^{13}C-labelled IS along with GC-IDMS has been proposed to assess the biases (such as leakage and metabolite co-precipitation) related to cold methanol quenching [55].

The presence of high amounts of co-eluents along with the analytes of interest or the presence of salts result in ion suppression. Ion suppression in the sample matrix can be minimized by reducing salt concentration in the resulting ^{13}C-labelled cell extract by exchanging the cultivation medium prior to sampling. Moreover, the labelled substrates are very expensive, requiring development of a small scale set-up with the high yield of ^{13}C-labelled metabolites as demonstrated with *Escherichia coli* for accurate quantification of metabolites using LC-ESI-MS [56]. The authors evaluated the matrix effects using the standard addition method. U-^{13}C-labelled IS and IDMS were also used to quantify amino acids, intermediates of the glycolysis, tricarboxylic acid and pentose phosphate pathways using LC-MS/MS and GC-MS while evaluating the quenching protocols in *Aspergillus niger* chemostat cultures [57]. Dual labelling of metabolites has also been proposed [58] to account for variations in derivatization efficiencies (in LC-MS platform) in different matrices and to eliminate the effect of different matrices on ESI. In another application [59], a quantitative LC-MS approach was developed based on IDMS to quantify siderophores in uropathogenic *E. coli*, where the authors demonstrated the advantages of using IDMS in both structural confirmation and MS-based quantification.

Recently, strategies used to account for matrix effects such as external calibration, IDMS and standards addition with ISs were evaluated and compared, while quantifying selected intracellular metabolites in *E. coli* extract using HILIC-ESI-MS/MS [60]. The linearity and accuracy were found to be similar for all the three strategies. However, matrix effect was evaluated only in the context of chromatographic separation. Moreover, the conclusions were drawn on analysis of specific set of metabolites which might not be valid for other metabolites in the intracellular pool.

So far, IDMS using HRMS coupled to GC or LC seems to be a gold standard for targeted quantitative metabolomics. IDMS is difficult to apply with low resolution MS. EI results in generation of a large number of fragment ions, which requires accurate mass measurements in order to differentiate mass spectral peak pattern between normal and isotopically labelled metabolite. The low resolution MS (GC-EI-MS) with non-targeted tracer fate detection algorithm has been recently proposed [61] for isotopologue ratio normalization, for the automated semi-quantitative analysis of both identified and unidentified metabolites relative to isotopically labelled cell extract. The authors also demonstrated the utilization of labelled yeast extract as a reference for the mammalian metabolome, where complete stable isotope labelling is hard to achieve.

5. Conclusion

MS-based approaches have found widespread interest in quantitative metabolomics. Advances in MS techniques over the years have enabled constructive use of this technique in attempts to capture metabolomic changes in biological systems, quantitatively. While the approach has evolved over the years, there are several challenges that remain in reproducibly capturing quantitative metabolomics changes that enable biological interpretations. Here, we have reviewed some of these challenges in microbial and mammalian systems. There is an increasing drive towards standardized approaches within the metabolomics community, but with the burgeoning interest in deriving quantitative metabolomics data, it is imperative that the associated challenges at each step of the workflow be given due consideration, both in designing experiments and in interpreting the results.

Data accessibility. The datasets supporting this article have been uploaded as part of the electronic supplementary material.

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