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Towards quantitative mass spectrometry based metabolomics in microbial and mammalian systems

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Summary

Metabolome analyses are a suite of analytical approaches that enable us to capture changes in the metabolome (small molecular weight components, typically<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.

1. Introduction

Post-genome science is characterised by the parallel analyses of gene products at the level of the transcripts, proteins and metabolites, and forms the basis of Systems Biology. Characterising 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 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 organises 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 wide spread 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). Since 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 (NMR) 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 characterisations 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

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techniques such as gas chromatography (GC), liquid chromatography (LC) or capillary electrophoresis (CE). Whilst 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 normalisation 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 characterised 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 specialised extraction protocols but also specialised 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 characterised by simultaneous qualitative and quantitative measurement of a large number of metabolites in samples. Non-targeted metabolomics utilises relative quantification of metabolites, where metabolites 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 physicochemical properties presents a major challenge in comprehensively profiling them in a biological system. Hence analyses of metabolome requires an integrated workflow and a number of different approaches. The approaches commonly used in metabolomics are listed in Supplementary Table 1, which aims at investigating subsets of the metabolome depending on the biological question.

3. Challenges in MS based quantitative metabolomics

Standardisation 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.

3.1 Challenges prior to sampling

3.1.1 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 (CDW) 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.

3.1.2 Normalisation strategy

As we are interested in changes in metabolite concentration due to biological events and not due to non-biological factors, sample normalisation in quantitative metabolomics is crucial in order to minimise the effect of sample variations. Sample normalisation in metabolomics is much more complicated compared to that of genomics and proteomics due to the wide physicochemical 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 normalisation namely pre-acquisition and/or post-acquisition. In the pre-acquisition strategy, the extracted metabolome is normalised 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 normalised 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 ionisation 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 normalisation. 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. In contrast, 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 normalisation strategy is largely dependent on various factors such as the type of biological system under investigation, required normalisation accuracy, convenience, speed and cost, and in some cases use of both strategies may be needed.

In cellular metabolomics, variation in seeding densities and/or sampling strategies requires normalisation of cell extract by cell counting. The haemocytometer is widely used for this purpose in suspension cultures. In 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 normalisation 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, normalisation 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, normalisation to dry cell weight (DCW), total cellular content of proteins, ATP and/or DNA. Normalisation 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]. Normalisation to protein content using BCA 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 experiment and can be time consuming. The classical method for cell proliferation/viability studies include 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 to conventional methods.

An alternative method for normalisation involves 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. Normalisation of each peak area to the sum of all peak areas has been evaluated [21] as an alternative to normalisation 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 two fold, 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 normalisation [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.

3.2 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 internal standards to enable accurate quantification.

3.2.1 Use of internal standards (ISs)

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 ionisation 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]. Due to a wide diversity of metabolites many of which are still uncharacterised, use of isotope-labelled ISs for individual metabolites is not a practical approach. Moreover, availability and expense will have a significant role in its employment. In cases where there are batch to batch variations resulting in 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].

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 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 characterised, this method can have bias from matrix effects. Normalisation using optimal selection of multiple ISs (NOMIS) uses the variability information from multiple ISs across multiple samples to find the optimal normalisation factor for individual detected metabolite.

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 to that of the expected metabolite concentration.

3.2.2 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 minimise metabolite leakage with cold methanol quenching, additives that will buffer the effect and minimise osmotic shock have been suggested [29]. Commonly employed buffer additives involve methanol supplemented with either HEPES, AMBIC, tricine or NaCl. Influence of these additives in preserving the membrane integrity and therefore in minimising metabolite leakage is well studied (see supplementary Table 2). The concentration of methanol and the quenching temperature [30] can also have an influence on metabolite leakage.

To minimise 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 minimise carry-over 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. In 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.

3.2.3 Extraction

Due to the diverse physicochemical 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 alkali 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 were 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 to mild extraction solvents [31]. Moreover, these methods are time consuming, as they require neutralisation 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 use of cold organic solvents (see supplementary Table 3). 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, whilst 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 interconversion of metabolites [32]. However, implementing these procedures is time consuming, 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. Till 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 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, normalised intensities) won't be ideal, as both approaches work under the assumption of linearity of response and absence of matrix effects, which is often not valid for complex cell extracts.

3.2.4 Derivatization in GC-MS

The two-step derivatization procedure (methoximation followed by silylation with MSTFA) 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 TMS derivatives was evaluated in quantifying 28 standard metabolites. Authors recommended -20°C is suitable temperature for stability of TMS derivatives under storage and that analysis should be carried out within 72h.

The sources of bias in GC-MS based metabolomics can occur in two forms (types A & 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, 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 requires evaluation and validation of its applicability to quantify other metabolite classes as well.

In summary, sufficient derivatizing reagent and optimum conditions are essential for the efficient derivatization of all intracellular metabolites, as incomplete derivatization of compound 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 requires careful evaluation and validation in different matrices prior to quantification of metabolites.

3.3 Challenges with the analytical platform

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

3.3.1 High resolution mass spectrometry (HRMS)

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 (APCI) 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 (<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 analyzers provides greater sensitivity by detecting all ions simultaneously (high acquisition rates >100Hz) 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 <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 HRMS's 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 to MRM 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].

3.3.2 Hyphenated MS 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 analyzed qualitatively and quantitatively with high analytical reproducibility and at lower costs compared to LC-MS and CE-MS. GC-MS with EI ionisation 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/second 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 0.5-1s which also facilitates the implementation of fast GC methods, thus reducing the analysis time and increases the productivity. For complex biological samples, peak capacities, resolving power and depth of metabolome coverage can be further increased by the use of 2D-GC (GCxGC) that utilizes 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 GCxGC-TOF-MS is large and complex. The recently introduced GC/Q-Orbitrap-MS offers both performance characteristics of Orbitrap and quadrupole based isolation for sensitive analyte detection. In addition it also offers numerous analysis modalities (molecular ion directed acquisition (MIDA)) 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 reduce 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. 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]. Till date, there is no single analytical method suitable for detection of all the metabolite classes due to physicochemical diversity of the metabolites, therefore parallel application of optimised GC-MS and LC-MS workflows for a given organism would be needed.

3.4 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: 1) parent mass + chromatographic retention time or 2) 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 a) spectral processing b) data analysis c) metabolite identification and quantification and d) biological interpretation.

3.4.1 Spectral processing

Spectral processing involves accurate identification and quantification of the features in the raw spectral data, which is then arranged in a feature quantification matrix (FQM) for subsequent statistical data analysis. For further detailed information on spectral processing steps we recommend relevant review article [49]. For post-acquisition feature normalisation please refer to section 3.1.2.

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 GCxGC-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 are still lower compared to targeted approaches and require improvements.

3.4.2 Data analysis, metabolite identification and spectral databases

Once raw data has 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, which the readers are referred to.

3.4.3 Validation and quality control (QC)

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 (LLOQ) and additional parameters such as LOD, recovery, reproducibility and robustness. These validation parameters can be assessed with the use of appropriate ISs as detailed in section 3.2.1. 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.

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 setup 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 metabolite is very similar and when silylation is used, 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 past while quantifying central carbon metabolites in *Methylobacterium extorquens* using isotope dilution mass spectrometry (IDMS) [52].

4. Matrix effects and use of IDMS

The biggest bottleneck in quantitative metabolomics is the occurrence of matrix effects, which include artefacts caused by a) contributions from the biological sample matrix, b) loss due to leakage, degradation or interconversion of metabolites during sample processing steps and c) instrument specific negative influences (such as ion suppression), which corrupts 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 normalisation 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 group (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, 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 presence of salts result in ion suppression. Ion suppression in the sample matrix can be minimised 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 *E. 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, TCA and PPP pathways using LC-MS/MS and GC-MS while evaluating the quenching protocols in *A. 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 was 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 result in generation of 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 (NTFD) algorithm has been recently proposed [61] for isotopologue ratio normalisation, for the automated semi-quantitative analysis of both identified and unidentified metabolites relative to isotopically labelled cell extract. The authors also demonstrated the utilisation of labelled yeast extract as a reference for the mammalian metabolome, where complete stable isotope labelling is hard to achieve.

5. Conclusions

MS based approaches have found wide-spread 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. Whilst 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 standardised 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.

Additional Information

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Competing Interests
'We have no competing interests.'

Authors' Contributions
RVK and SV conceived the paper. RVK wrote the paper with guidance and supervision from SV. Both authors edited the article.

Supplementary material
The datasets supporting this article have been uploaded as part of the Supplementary Material.

Reference

1. Kell DB, Goodacre R. 2014 Metabolomics and systems pharmacology: why and how to model the human metabolic network for drug discovery. *Drug Discovery Today.* **19**, 171-182. (doi:10.1016/j.drudis.2013.07.014)
2. Saito K, Matsuda F. 2010 *Metabolomics for Functional Genomics, Systems Biology, and Biotechnology*, 463-489. (10.1146/annurev.arplant.043008.092035)
3. Vaidyanathan S, Harrigan GG, Goodacre R. 2005 *Introduction - Metabolome analyses for systems biology*, 1-7. (10.1007/0-387-25240-1_1)
4. Mashego MR, Rumbold K, De Mey M, Vandamme E, Soetaert W, Heijnen JJ. 2007 Microbial metabolomics: past, present and future methodologies. *Biotechnology Letters.* **29**, 1-16. (doi:10.1007/s10529-006-9218-0)
5. Tang J. 2011 Microbial Metabolomics. *Current Genomics.* **12**, 391-403. (doi:10.2174/138920211797248619)
6. Vaidyanathan S. 2005 Profiling microbial metabolomes: what do we stand to gain? *Metabolomics.* **1**, 17-28. (doi:10.1007/s11306-005-1104-6)

7. Fiehn O, Kopka J, Dörmann P, Altmann T, Trethewey RN, Willmitzer L. 2000 Metabolite profiling for plant functional genomics. *Nature Biotechnology.* **18**, 1157-1161.
8. Sumner LW, Mendes P, Dixon RA. 2003 Plant metabolomics: large-scale phytochemistry in the functional genomics era. *Phytochemistry.* **62**, 817-836. (doi:10.1016/s0031-9422(02)00708-2)
9. van der Werf MJ, Overkamp KM, Muilwijk B, Coulier L, Hankemeier T. 2007 Microbial metabolomics: Toward a platform with full metabolome coverage. *Analytical Biochemistry.* **370**, 17-25. (doi:10.1016/j.ab.2007.07.022)
10. Dietmair S, Timmins NE, Gray PP, Nielsen LK, Krömer JO. 2010 Towards quantitative metabolomics of mammalian cells: Development of a metabolite extraction protocol. *Analytical Biochemistry.* **404**, 155-164. (doi:10.1016/j.ab.2010.04.031)
11. Kapoore RV. Mass spectrometry based hyphenated techniques for microalgal and mammalian metabolomics: University of Sheffield; 2014.
12. Mueller D, Heinzle E. 2013 Stable isotope-assisted metabolomics to detect metabolic flux changes in mammalian cell cultures. *Current Opinion in Biotechnology.* **24**, 54-59. (doi:10.1016/j.copbio.2012.10.015)
13. Onjiko RM, Moody SA, Nemes P. 2015 Single-cell mass spectrometry reveals small molecules that affect cell fates in the 16-cell embryo. *Proceedings of the National Academy of Sciences of the United States of America.* **112**, 6545-6550. (doi:10.1073/pnas.1423682112)
14. Sumner LW, Amberg A, Barrett D, Beale MH, Beger R, Daykin CA, et al. 2007 Proposed minimum reporting standards for chemical analysis. *Metabolomics.* **3**, 211-221. (doi:10.1007/s11306-007-0082-2)
15. Wu Y, Li L. 2016 Sample normalization methods in quantitative metabolomics. *Journal of Chromatography A.* **1430**, 80-95. (doi:10.1016/j.chroma.2015.12.007)
16. Kapoore RV, Coyle R, Staton CA, Brown NJ, Vaidyanathan S. 2015 Cell line dependence of metabolite leakage in metabolome analyses of adherent normal and cancer cell lines. *Metabolomics.* **11**, 1743-1755. (doi:10.1007/s11306-015-0833-4)
17. Marcinowska R, Trygg J, Wolf-Watz H, Mortiz T, Surowiec I. 2011 Optimization of a sample preparation method for the metabolomic analysis of clinically relevant bacteria. *Journal of Microbiological Methods.* **87**, 24-31. (doi:10.1016/j.mimet.2011.07.001)
18. Huege J, Krall L, Steinhauser M-C, Giavalisco P, Rippka R, de Marsac NT, et al. 2011 Sample amount alternatives for data adjustment in comparative cyanobacterial metabolomics. *Analytical and Bioanalytical Chemistry.* **399**, 3503-3517. (doi:10.1007/s00216-011-4678-z)
19. Silva LP, Lorenzi PL, Purwaha P, Yong V, Hawke DH, Weinstein JN. 2013 Measurement of DNA Concentration as a Normalization Strategy for Metabolomic Data from Adherent Cell Lines. *Analytical Chemistry.* **85**, 9536-9542. (doi:10.1021/ac401559v)
20. Cao B, Aa J, Wang G, Wu X, Liu L, Li M, et al. 2011 GC-TOFMS analysis of metabolites in adherent MDCK cells and a novel strategy for identifying intracellular metabolic markers for use as cell amount indicators in data normalization. *Analytical and Bioanalytical Chemistry.* **400**, 2983-2993. (doi:10.1007/s00216-011-4981-8)
21. Hutschenreuther A, Kiontke A, Birkenmeier G, Birkemeyer C. 2012 Comparison of extraction conditions and normalization approaches for cellular metabolomics of adherent growing cells with GC-MS. *Analytical Methods.* **4**, 1953-1963. (doi:10.1039/c2ay25046b)
22. Wu Y, Li L. 2014 Dansylation Metabolite Assay: A Simple and Rapid Method for Sample Amount Normalization in Metabolomics. *Analytical Chemistry.* **86**, 9428-9433. (doi:10.1021/ac503359v)
23. Koek MM, Jellema RH, van der Greef J, Tas AC, Hankemeier T. 2011 Quantitative metabolomics based on gas chromatography mass spectrometry: status and perspectives. *Metabolomics.* **7**, 307-328. (doi:10.1007/s11306-010-0254-3)
24. Kvitvang HFN, Andreassen T, Adam T, Villas-Boas SG, Bruheim P. 2011 Highly Sensitive GC/MS/MS Method for Quantitation of Amino and Nonamino Organic Acids. *Analytical Chemistry.* **83**, 2705-2711. (doi:10.1021/ac103245b)
25. Hocart CH. 2010 Mass Spectrometry: An Essential Tool for Trace Identification and Quantification. *Comprehensive Natural Products II: Chemistry and Biology: Modern Methods in Natural Products Chemistry.* **9**, 327-388.
26. Bolten CJ, Kiefer P, Letisse F, Portais JC, Wittmann C. 2007 Sampling for metabolome analysis of microorganisms. *Analytical Chemistry.* **79**, 3843-3849. (doi:10.1021/ac0623888)
27. Villas-Boas SG, Bruheim P. 2007 Cold glycerol-saline: The promising quenching solution for accurate intracellular metabolite analysis of microbial cells. *Analytical Biochemistry.* **370**, 87-97. (doi:10.1016/j.ab.2007.06.028)
28. Shen Y, Fatemeh T, Tang L, Cai Z. 2016 Quantitative metabolic network profiling of Escherichia coli: An overview of analytical methods for measurement of intracellular metabolites. *Trac-Trends in Analytical Chemistry.* **75**, 141-150. (doi:10.1016/j.trac.2015.07.006)
29. Faijes M, Mars AE, Smid EJ. 2007 Comparison of quenching and extraction methodologies for metabolome analysis of Lactobacillus plantarum. *Microbial Cell Factories.* **6**, (doi:10.1186/1475-2859-6-27)

30. Canelas AB, Ras C, ten Pierick A, van Dam JC, Heijnen JJ, Van Gulik WM. 2008 Leakage-free rapid quenching technique for yeast metabolomics. *Metabolomics.* **4**, 226-239. (doi:10.1007/s11306-008-0116-4)
31. Ser Z, Liu X, Ngoc Nu T, Locasale JW. 2015 Extraction parameters for metabolomics from cultured cells. *Analytical Biochemistry.* **475**, 22-28. (doi:10.1016/j.ab.2015.01.003)
32. Madla S, Miura D, Wariishi H. 2012 Optimization of extraction method for GC-MS based metabolomics for filamentous fungi. *Journal of Microbial and Biochemical Technology.* **4**, 5-9. (doi:10.4172/1948-5948.1000064)
33. Dettmer K, Nuernberger N, Kaspar H, Gruber MA, Almstetter MF, Oefner PJ. 2011 Metabolite extraction from adherently growing mammalian cells for metabolomics studies: optimization of harvesting and extraction protocols. *Analytical and Bioanalytical Chemistry.* **399**, 1127-1139. (doi:10.1007/s00216-010-4425-x)
34. Koek MM, Muilwijk B, van der Werf MJ. 2006 Microbial metabolomics with gas chromatography/mass spectrometry (vol 78, pg 1272, 2006). *Analytical Chemistry.* **78**, 3839-3839. (doi:10.1021/ac060521q)
35. Quero A, Jousse C, Lequart-Pillon M, Gontier E, Guillot X, Courtois B, et al. 2014 Improved stability of TMS derivatives for the robust quantification of plant polar metabolites by gas chromatography-mass spectrometry. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences.* **970**, 36-43. (doi:10.1016/j.jchromb.2014.08.040)
36. Mishur RJ, Rea SL. 2012 Applications of mass spectrometry to metabolomics and metabonomics: Detection of biomarkers of aging and of age-related diseases. *Mass Spectrometry Reviews.* **31**, 70-95. (doi:10.1002/mas.20338)
37. Erban A, Schauer N, Fernie AR, Kopka J. 2007 *Nonsupervised construction and application of mass spectral and retention time index libraries from time-of-flight gas chromatography-mass spectrometry metabolite profiles*, 19-38. (10.1007/978-1-59745-244-1_2)
38. de Raad M, Fischer CR, Northen TR. 2016 High-throughput platforms for metabolomics. *Current Opinion in Chemical Biology.* **30**, 7-13. (doi:10.1016/j.cbpa.2015.10.012)
39. Dunn WB. 2008 Current trends and future requirements for the mass spectrometric investigation of microbial, mammalian and plant metabolomes. *Physical Biology.* **5**, (doi:10.1088/1478-3975/5/1/011001)
40. Eliuk S, Makarov A. 2015 *Evolution of Orbitrap Mass Spectrometry Instrumentation*, 61-80. (10.1146/annurev-anchem-071114-040325)
41. Liu X, Ser Z, Cluntun AA, Mentch SJ, Locasale JW. 2014 A strategy for sensitive, large scale quantitative metabolomics. *Journal of visualized experiments : JoVE.* (doi:10.3791/51358)
42. Lei Z, Huhman DV, Sumner LW. 2011 Mass spectrometry strategies in metabolomics. *Journal of Biological Chemistry.* **286**, 25435-25442. (doi:10.1074/jbc.R111.238691)
43. Bedair M, Sumner LW. 2008 Current and emerging mass-spectrometry technologies for metabolomics. *Trac-Trends in Analytical Chemistry.* **27**, 238-250. (doi:10.1016/j.trac.2008.01.006)
44. Savolainen OI, Sandberg A-S, Ross AB. 2016 A Simultaneous Metabolic Profiling and Quantitative Multimetabolite Metabolomic Method for Human Plasma Using Gas-Chromatography Tandem Mass Spectrometry. *Journal of Proteome Research.* **15**, 259-265. (doi:10.1021/acs.jproteome.5b00790)
45. Peterson AC, Hauschild J-P, Quarmby ST, Krumwiede D, Lange O, Lemke RAS, et al. 2014 Development of a GC/Quadrupole-Orbitrap Mass Spectrometer, Part I: Design and Characterization. *Analytical Chemistry.* **86**, 10036-10043. (doi:10.1021/ac5014767)
46. Scalbert A, Brennan L, Fiehn O, Hankemeier T, Kristal BS, van Ommen B, et al. 2009 Mass-spectrometry-based metabolomics: limitations and recommendations for future progress with particular focus on nutrition research. *Metabolomics.* **5**, 435-458. (doi:10.1007/s11306-009-0168-0)
47. Coulier L, Bas R, Jespersen S, Verheij E, van der Werf MJ, Hankemeier T. 2006 Simultaneous quantitative analysis of metabolites using ion-pair liquid chromatography - Electrospray ionization mass spectrometry. *Analytical Chemistry.* **78**, 6573-6582. (doi:10.1021/ac0607616)
48. Buescher JM, Czernik D, Ewald JC, Sauer U, Zamboni N. 2009 Cross-Platform Comparison of Methods for Quantitative Metabolomics of Primary Metabolism. *Analytical Chemistry.* **81**, 2135-2143. (doi:10.1021/ac8022857)
49. Alonso A, Marsal S, Julia A. 2015 Analytical methods in untargeted metabolomics: state of the art in 2015. *Frontiers in bioengineering and biotechnology.* **3**, 23-23. (doi:10.3389/fbioe.2015.00023)
50. Behrends V, Tredwell GD, Bundy JG. 2011 A software complement to AMDIS for processing GC-MS metabolomic data. *Analytical Biochemistry.* **415**, 206-208. (doi:10.1016/j.ab.2011.04.009)
51. Yi L, Dong N, Yun Y, Deng B, Ren D, Liu S, et al. 2016 Chemometric methods in data processing of mass spectrometry-based metabolomics: A review. *Analytica Chimica Acta.* **914**, 17-34. (doi:10.1016/j.aca.2016.02.001)
52. Kiefer P, Portais J-C, Vorholt JA. 2008 Quantitative metabolome analysis using liquid chromatography-high-resolution mass spectrometry. *Analytical Biochemistry.* **382**, 94-100. (doi:10.1016/j.ab.2008.07.010)
53. Gao X, Pujos-Guillot E, Sebedio J-L. 2010 Development of a Quantitative Metabolomic Approach to Study Clinical Human Fecal Water Metabolome Based on Trimethylsilylation Derivatization and GC/MS Analysis. *Analytical Chemistry.* **82**, 6447-6456. (doi:10.1021/ac1006552)

54. Canelas AB, ten Pierick A, Ras C, Seifar RM, van Dam JC, van Gulik WM, et al. 2009 Quantitative Evaluation of Intracellular Metabolite Extraction Techniques for Yeast Metabolomics. *Analytical Chemistry.* **81**, 7379-7389. (doi:10.1021/ac900999t)
55. Zakhartsev M, Vielhauer O, Horn T, Yang X, Reuss M. 2015 Fast sampling for quantitative microbial metabolomics: new aspects on cold methanol quenching: metabolite co-precipitation. *Metabolomics.* **11**, 286-301. (doi:10.1007/s11306-014-0700-8)
56. Weiner M, Troendle J, Schmideder A, Albermann C, Binder K, Sprenger GA, et al. 2015 Parallelized small-scale production of uniformly C-13-labeled cell extract for quantitative metabolome analysis. *Analytical Biochemistry.* **478**, 134-140. (doi:10.1016/j.ab.2015.03.008)
57. Lameiras F, Heijnen JJ, van Gulik WM. 2015 Development of tools for quantitative intracellular metabolomics of Aspergillus niger chemostat cultures. *Metabolomics.* **11**, 1253-1264. (doi:10.1007/s11306-015-0781-z)
58. Feldberg L, Venger I, Malitsky S, Rogachev I, Aharoni A. 2009 Dual Labeling of Metabolites for Metabolome Analysis (DLEMMA): A New Approach for the Identification and Relative Quantification of Metabolites by Means of Dual Isotope Labeling and Liquid Chromatography-Mass Spectrometry. *Analytical Chemistry.* **81**, 9257-9266. (doi:10.1021/ac901495a)
59. Henderson JP, Crowley JR, Pinkner JS, Walker JN, Tsukayama P, Stamm WE, et al. 2009 Quantitative Metabolomics Reveals an Epigenetic Blueprint for Iron Acquisition in Uropathogenic Escherichia coli. *Plos Pathogens.* **5**, (doi:10.1371/journal.ppat.1000305)
60. Teleki A, Sanchez-Kopper A, Takors R. 2015 Alkaline conditions in hydrophilic interaction liquid chromatography for intracellular metabolite quantification using tandem mass spectrometry. *Analytical Biochemistry.* **475**, 4-13. (doi:10.1016/j.ab.2015.01.002)
61. Weindl D, Wegner A, Jaeger C, Hiller K. 2015 Isotopologue ratio normalization for non-targeted metabolomics. *Journal of Chromatography A.* **1389**, 112-119. (doi:10.1016/j.chroma.2015.02.025)