Best practices for experimentation and reporting in mass spectrometry based metabolomics

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Abstract:

Mass spectrometry-based metabolomics approaches can enable detection and quantification of tens to 10,000's of metabolite features simultaneously. However, compound identification and reliable quantification is greatly complicated due to the chemical complexity and dynamic range of the metabolome. Simultaneous quantification of many metabolites within complex mixtures can additionally be complicated by ion suppression, fragmentation, and the presence of isomers. As a result, in the absence of necessary controls, misinterpretation of metabolomics data can easily occur. Here, we present guidelines to ensure reliable peak annotation and more robust quantification as a means to enable high quality reporting of mass spectral metabolomics-derived data.

Introduction

Metabolomics, the large-scale study of the metabolic complement of the cell [1-3], is now a mature science which has been practiced for over 20 years [4, 5]. Indeed, it is now a commonly-used experimental systems biology tool with demonstrated utility in both fundamental and applied aspects of plant, microbe and mammalian research[6-19]. Among the many thousands of studies published in this area over the last twenty years notable highlights [6-12] are briefly described in **Supplementary Note 1**.

Despite the insight afforded by such studies, the nature of metabolites particularly their diversity (both in chemical structure and dynamic range of abundance[13, 14]) remains a major challenge with regard to our ability to provide adequate coverage of the metabolome that can complement the genome, transcriptome and proteome. Despite these comparative limitations enormous advances have been made with regard to the number of analytes we can acquire accurate quantitative information and a vast number of studies have yielded important biological information and biologically active metabolites across the kingdoms of life [15]. We have previously estimated that upwards of 1 million different metabolites occur across the tree of life with between 1000 and 40 000 being estimated to occur in a single species [5]. However, to date, even the most comprehensive methods are not able to approach comprehensitivity. The above-mentioned features of chemical diversity and broad dynamic range in cellular abundance currently prohibit the possibility of extracting and measuring all metabolites using single extraction and analytical procedures[16, 17]. Consequently, many different extraction techniques and combinations of analytical methods have been developed in an attempt to achieve adequate metabolite coverage. This renders the establishment of good working practices [18-23], more difficult than those associated with RNAseq [24-27] [24]. Furthermore, rigorous standards are needed for normalization of metabolomics data [28, 29]. This is exacerbated by the breadth of aims associated with the measurement of metabolites which encompasses; targeted metabolite analysis, metabolite profiling, flux profiling, metabolomics-scale analysis and metabolite

fingerprinting techniques [4, 30-32]. Given the myriad of aims and methodologies, as well as the rapid turnover times of metabolites, we argue it is particularly important to define clear guidelines for acquisition and reporting of metabolite data since there are many potential sources of misinterpretation. This is of course not the first time such guidelines have been suggested with several insightful papers published on this topic [14, 33 34] and a considerable number of long established metabolome databases including MetaboLights[35],[36-38] and the Metabolome Workbench (https://www.metabolomicsworkbench.org/) also driving this field. A more detailed description of these guidelines and repositories as well as of more recent developments is provided in **Supplementary Note 2.** Although the detailed standards sets out by the metabolomics standards initiative and these repositories are laudable and clearly represent the gold-standard of metabolomics reporting it is notable that the number of published metabolomics studies both far exceeds the number following these in their entirety and those submitting their data to the metabolome databases. There are probably several reasons underlying this. First, few journals currently mandate that data is stored in one of the metabolomics repositories. Secondly, unlike the situation 20 years ago, or even when the work of the metabolomics initiative was first published some 13 years ago [33 39-41], metabolomics experiments are often a component, rather than the entire study. Aligned to this fact is that many groups outsource their metabolomics workflow to companies or similar service providers and do not always have either the experience to provide, or even full access to the raw data. In parallel requiring reviewers to comment on all aspects of multi-omics in the absence of clear guidelines regarding what may not be their key area of competence is a big ask. Finally, and perhaps most tellingly is the difficulty in reporting chromatogram level information with several weeks and often several attempts required to fulfil the criteria of the major metabolomics repositories

Our aim here is to present a simplified reporting workflow with the hope of capturing more of the missing information. By contrast to the suggestions of the metabolomics standards initiative [33 39-41] and the major repositories mentioned above we propose to perform this at the level of the processed data (supported by the provision of representative chromatograms allowing the assessment of metabolite identifications), rather than the raw chromatograms. A similar suggestion was made to the Plant research community in 2011[42]. However, it is notable that of the 172 citations accrued by this publication as of September 2020 153 were related to plants). We have therefore in the following rewritten and updated these suggestions to (i) be more globally applicable and (ii) to reinforce our contention that quantitation control experiments should be regarded as mandatory and can aid in determining how problematic the effects of ion suppression are in an experiment. To this end we highlight potential sources of error and provide recommendations for ensuring the robustness of the metabolite data obtained and reported. Our article will include clear guidelines for sampling, extraction and storage, metabolite identification and reporting. It will stress the need for recombination and recovery experiments aimed at checking both qualitative metabolite identifications and their quantitative recovery. In addition, it will provide a suggestion for a stricter nomenclature for metabolite annotations which

would improve reporting by removing much of the ambiguity concerning the quality of metabolite annotation that is currently apparent in many metabolomics studies. Indeed this article aims at covering all aspects of reporting from sample extraction through to data evaluation but will not detail downstream computational evaluation of the acquired datasets despite there being several important advances in this regard [43, 44] [45] [46, 47] [48] [49]. These tools and their application are outlined in **Supplementary Note 3**.

Such efforts are necessary to allow the inter-laboratory comparisons of datasets which, as has been demonstrated for transciptomics, provides huge statistical power and deeper biological insights as well as opening a route for better integration with other datasets [50-52]. Whilst nuclear magnetic resonance (NMR) and capillary electrophoresis mass spectrometry (CE-MS) both have their advocates and have clear advantages in structure elucidation and sensitivity, respectively, given that the majority of metabolomics studies rely on the complementary techniques of chromatography hypenated with mass spectrometry (MS, either gas chromatography (GC) or liquid chromatography (LC) respectively) we will therefore focus this paper on such techniques.

As mentioned above, metabolites exhibit tremendous chemical diversity, thus differing from nucleic acids and proteins which are constructed via genetically encoded polymerization of a small number or building blocks [3, 53, 54]. Alongside their dynamic range in concentration and half-life this chemical complexity presents a range of challenges when attempting comprehensive profiling of the cellular small molecule complement. These difficulties are sufficient that no single analytical technique, let alone no single protocol can capture all metabolites. Taken together with our incomplete understanding of the spontaneous chemical reactions of the cell [55], this means that we cannot even be certain as to what constitutes the full metabolite complement of a given system. That said, our current capabilities for detection and quantification of metabolites fall a long way short of being comprehensive. To illustrate this point, currently combinations of the most comprehensive methods are able to quantify 700 of the 3700 metabolites predicted to be present in *E.coli* [56, 57], 500 of the 2680 metabolites predicted to be present in yeast [58, 59], 8000 of the 114100 metabolites predicted to be present in in human [60] and only 14000 of the over 400000 metabolites predicted to be present in the plant kingdom [5, 61, 62]. The pursuit of ever-greater coverage of the metabolome is likely to remain the grand challenge of metabolomics for some time. However, there are nonetheless a number of other important topics that should considered. Amongst these critical issues is how to ensure deep coverage while retaining high data quality and annotation confidence.

Sampling, quenching, metabolite extraction and storage

The very first (and particularly vital) step in the metabolomics workflow (Figure 1 and 2) is the rapid stopping or quenching metabolism and extracting the metabolites in a manner which produces a stable extract which is quantitatively reflective of the endogenous metabolite levels present in the original living cell. This is especially important in highly metabolically active systems such as cells and tissues but less so

in biofluids such as serum plasma or urine samples [14]. Indeed, there is no one method to fit all cases, with specific sampling, quenching, and extraction needed for each tissue type. That said the certain evaluations of quality are universally applicable and our aim here is to provide clear instructions on how to apply them. Quenching needs to satisfy two criteria: (i) it needs to completely terminate all enzyme and chemical activities and (ii) it needs to avoid the perturbation of existing metabolite levels during harvesting. Details regarding specific considerations that need to be taken into account for quenching the metabolism of various species are provided in **Supplementary Note 4**. The efficiency of quenching can be followed either by controlled comparisons of various extraction methods[33] or alternatively by determining the abundance of (stable-isotope labelled) standards spiked into the quenching solvent (see recovery and recombination experiments). For tissues, where possible, quick excision and snap-freezing in liquid nitrogen is recommended with subsequent storage of deep-frozen tissue at constant -80 °C until the first application of extraction solvent. However, for bulky tissue, submersion in liquid nitrogen is not sufficient since the center of the tissue is cooled too slowly. In such cases freeze-clamping - where tissue is almost instantaneously squashed flat between two pre-frozen metal blocks (known as a Wohlehberger clamp) is preferred [42, 63, 64]. Irrespective of the quenching method, downstream steps of these processes also warrant caution. For example, improper freeze drying and lack of storage in sealed containers can generate artifactual geometric isomers of pigments [42]. Freeze drying is also unsuitable when volatile components are of interest. Whilst the appropriate means of storage is strictly dependent on the stability of the class of targeted metabolites under study, it is not recommended to store samples between 0 and 40 °C. At these temperatures, substances can become concentrated in a residual aqueous phase [42]. It is therefore, recommended, where necessary, to store completely dry residues for as short a time as possible before their analysis. In addition, great care must be taken to ensure that metabolism remains quenched during thawing. This is particularly pertinent for extracts containing secondary metabolites where degradative enzymes often retain their activities which, if not kept in check, may result in the consumption or conversion of certain metabolites with a concomitant appearance of new compounds or breakdown products [65]. Similar issues are apparent with respect to both experimental growth media and the initial extraction solvents used. Growth media often needing to be removed via multiple wash steps in order to reduce the effects of ion suppression during the subsequent MS analysis, and the solvent used for initial extraction may need to be exchanged due to incompatibility with the instrumentation used for the metabolite analysis *per se*. Two pitfalls are apparent here; (i) loss of metabolites in the washing process and (ii) solvent removal leads to concentration of the metabolites and thereby an acceleration of chemical reactions occurring between them. Thus, considerable caution is advised in method optimization to ensure that extraction and handling methods allow adequate quantitative representation of cellular metabolites. In some instances, such as the analysis of volatile or semi-volatile compounds, sample extraction and handling should only be performed on fresh material. In either case, we recommend that the adoption of recovery and recombination experiments (see *Recovery and recombination experiments*) be mandated either when a substantially

novel metabolomics technique is introduced or when a novel cell type, tissue or organism is studied.

Sample replication and randomization

The nature and number of biological, technical and analytic replicates is a further issue worthy of consideration. Before employing any new extraction protocol or analytical procedure and when working with new biological materials it is essential to perform extensive pilot experiments in order to assess fully the technical variation which is necessary to design a statistically sound experiment. To avoid misunderstanding, we refer readers to the definitions of each type of replicate provided in [42]. While analytical replicates, i.e. the repeated injection of the exact same extract, are useful in assessing machine performance, technical replicates which encompass the entire experimental procedure allow a far more comprehensive assessment of any experimental variance in data generation [42]. Indeed, such analyses are essential for the establishment of a new extraction or processing procedure, a new analytic technique or the optimization of a new instrument. Beyond these cases, biological replication is considerably more important and should involve at least four but preferably more replicates, although the required number of replicates depends on the desired statistical power, effect size and actual variances[66]. Care must be taken to harvest such replicates in a highly uniform manner. For plants, this can also mean harvesting at the same time of day and under the same environmental conditions. In many instances, a full and independent repeat of a biological experiment is advisable[67]. There are different stages where technical replicates can be made; at the sampling, the quenching, the extraction and the analysis independently the entire process. From experience the extraction step is the most critical of these. Whether technical replication is needed in support of biological replication is highly dependent on their relative magnitudes in cases in which the biological variation greatly exceeds technical variation it is sensible to sacrifice the latter to increase the former. Pilot experiments with new systems are highly recommended to evaluate biological and technical variation and hence determine how many samples and how many replicates are needed for final statistical robustness[66]

Careful spatiotemporal randomization of biological samples throughout workflows is equally essential. If a set of samples is analyzed in a non-random order, treatment and control samples or time-points may end up getting measured under very different conditions, and as a result interpretation can be confounded by sample age or shifting instrument performance, potentially occluding biological variation between sample groups, or worse, creating artefactual differences. This is particularly important in large-scale metabolic profiling that characterizes studies of natural variation of metabolism such as genome wide association studies [10, 68-71]. Here, even weeks of instrument time may be required. Clear best practice guidelines for such large-scale studies have been presented elsewhere [72-76], so we will not dwell on them here. Irrespective of the size of the experiment, the use of quality control samples and batch correction are also essential [77]. Such experimental controls help monitor instrument performance and stability and thereby data quality. These controls ensure that missing

data or peaks with low signal to noise ratios occur. Either mixtures of authentic metabolite samples at defined concentrations or dry-stored aliquots of a broadly-shared and appropriately standardized biological extract (for example multi kilogram extracts of Arabidopsis, *E. coli,* yeast or human cell lines) could likely serve as broadly useful reference samples. This would likely enhance accurate quantifications and render rendering the utility of metabolite databases more effective [78-82]. Pooled QC sample allows for evaluation (and correction) of run-order and batch effects within a study, but not necessarily across experiments like the reference material.

Quantification

The aforementioned details of extraction, storage and replication are equally applicable for ensuring the accuracy of any method of metabolite quantification, including those that target single metabolites (Figure 2). The remainder of this article will address issues that are, at least partially, restricted to untargeted metabolomics approaches. There are several essential aspects that need consideration here. First, it is essential to ensure that the levels of all metabolites of potentially interest can be detected and ideally, can be measured within linear range of detection. This is most readily achieved through the analyses of independent dilutions of each extract. Additionally, for experiments that begin with intact tissues it is important to ensure complete tissue disruption. In the case of cellular studies one must further take into consideration whether to limit the study to the endogenous cellular metabolites or whether to also assess the exo-metabolome. For these controls, and many others, we provide a list of reporting recommendations in the section on transparency in measurement, metabolite annotation and documentation below. Most frequently metabolomics data are provided as relative quantities (i.e. relative quantification is performed) with respect to a reference sample. This is in contrast to NMR based studies which usually provide absolute concentrations (i.e. absolute quantification) with peak intensities are directly proportional to concentrations and directly comparable across different peaks and samples. In contrast, the relative intensities of LC-MS and GC-MS peaks representing different compounds do not directly correlate to their absolute concentrations. This is due to the differential ionization efficiencies of the different metabolites within a complex mixture. To address this issue, standard curves can be used to determine how signal intensity responds as a function of analyte concentrations and moreover the range of linearity of this relationship[14]. This is of course dependent on the availability of validated pure standards. Whilst relative values are highly useful in many contexts and indeed are the only way of expressing the levels and changes of non-annotated analytes, absolute values have much greater utility being able to determine enzyme binding site occupancies, the thermodynamics of metabolic reactions[14, 83], and the molecular dynamics underlying the flow of atoms through a metabolic network [84-88]. A further advantage of the methods used for absolute quantification is that they can be readily adapted into a means of quality control for both quantification and the correctness of peak annotation e.g. through

thermodynamics[89]. However, obtaining standard curves for many thousand metabolites in a complex mixture is currently not always practical.

Recovery and recombination experiments

Recovery experiments in which authentic standard compounds are added to the initial extraction solvent in order to assess losses during extraction, storage and handling were vigorously championed in the 70s-90s [90, 91] and can provide persuasive evidence that the data reported provide a valid reflection of cellular metabolite compositions [42]. Recent examples have been provided that validate methods in microbial, plant and mammalian systems [92-94]. However, the metabolomics community has been relatively slow in adopting these control procedures. This is partially explained by the lack of commercially available and/or simple to synthesize standards. Indeed, this is *de facto* the case for unknown analytes for which this approach is impossible. Fortunately, there is an alternative approach – that of extract recombination – which circumvents this practical limitation. In this approach the extract of a novel tissue is characterized by combination with that of a well characterized reference material such as one from *E. coli*, *A. thaliana* or human biofluids. Such experiments not only provide information concerning the appropriateness of the extraction buffer but additionally allow an assessment of so-called matrix effects caused by ion suppression [95-97]. These experiments additionally allow a quantitative assessment of the reliability of known peaks[98]. A schematic representation of recovery and metabolic recombination experiments is presented in Figure 3. We suggest that for known metabolites, recovery or metabolic recombination experiments are carried out for each new tissue or species type. It is clear that for any metabolomics-scale study certain metabolites will have poor recoveries. While this does not preclude the reporting of their values it is important that this is documented in order to allow the readers discretion in their interpretation. Recoveries of 70-130% are acceptable with anything deviating beyond this range representing a metabolite whose quantification should be subject to further testing. For example even a 50 % recovery - if reproducible and linear - could be deemed acceptable (Figure 3). The importance of such control experiments is perhaps best illustrated with cases in which they were not carried out. Anecdotally, there are several examples in the literature wherein the metabolite data reported cannot be reflective of their cellular content; e.g. since for example zero levels have even been reported for metabolites which, if representative of cellular levels, would indicate that the cells tested were inviable.

A further aspect of quantitation is the basis on which this is expressed for tissue samples data is often provided per gram fresh or dry weight, whilst for body fluids this is often provide per volume. The case of cell metabolomics is more complicated given that cell size is often variable values are therefore often provided per mg protein, or based on cell counts. The basis on which both absolute and relative metabolite levels is provided is of fundamental importance - for example values on a fresh weight basis can be dramatically influenced by the osmotic potential of the cell -yet is often not given enough consideration by the community.

Ion suppression

Despite the selectivity and sensitivity of mass spectrometry techniques, there are considerable challenges with regard to reproducibility and accuracy when analyzing complex samples. These problems are not insurmountable but require that extra care is taken when interpreting results. Ion suppression is a general problem in LC–MS analyses due to matrix effects influencing the ionization of coeluting analytes, affecting precision and accuracy of their quantification, or preventing less abundant metabolites from getting detected at all [95, 97, 99]. As mentioned above the best method of assessing it is in the mixture of two independent extracts in a recombination experiment (see Figure 3) and subsequent assessment as to whether the metabolites detected can be quantitatively recovered[100]. Essentially, within this process co-eluting analytes compete for the ionization energy, resulting in incomplete ionization. Therefore, observation a decreased ion count for an analyte may be due either to decreased concentration of the analyte itself or increased concentrations of co-eluting analytes. It is critically important to consider these effects during method validation to ensure the quality of the analysis. While there is no universal solution to the ion suppression problem, assessing the effects of ion suppression affords greater confidence in the accuracy of the results. However, there are several strategies that can help minimize ion suppression[96]. Among these improving sample preparation and chromatographic selectivity are currently the most effective ways. In some situations, using suitable cleanup procedures depending on sample type and analyte properties may allow to remove co-eluting components. This might involve simple dilution of extracts [65] or of the growth media the samples are derived from[65] or optimization of various steps of sample workup, including sonication, solvent partitioning, filtration, centrifugation, and protein precipitation[101]. In addition, solid phase extraction (SPE) using appropriate absorbents has been demonstrated as an effective method to reduce matrix effects. Furthermore, it is possible to adjust chromatography conditions so that peaks of interest are not eluting in regions of suppression, for example, modifying mobile phase composition or gradient conditions can aid chromatographic separation and thereby improve performance. Careful selection of the ion source and column polarity is an alternative strategy to reduce ion suppression, for example, APCI is less to matrix effects compared to ESI. In addition, using APCI can also reduce interference effects [14]. It has been demonstrated that ion suppression is often less severe for negatively ionized compounds than for positively charged ones[102] . Finally, even though the above-mentioned strategies may not suffice to completely remove the effects of ion suppression in complex samples, the extent of the problem can at least be quantified via the carrying out control experiments as described in the Recovery and Recombinations section above.

Peak misidentification

The orthogonal use of chromatography (either gas or liquid based) with mass spectrometry and in some cases also MS/MS fragmentation patterns provides great specificity [103] [104]. Indeed current high-end instruments detect on the order of 10,000 or 100,000 features, however, these include a large number of adduct and isotope peaks. Bioinformatics tools for analyte identification take this into account and even use commonly observed adducts as a means of identifying analytes (discussed in detail below). Nonetheless, there are three common problems that contribute to mis-identification – the third of which being relevant more for LC- than GC-MS. Firstly, *isomers* – compounds with identical molecular formula but distinct structures – are common in nature. Important examples from primary metabolism include hexose-phosphates/inositol phosphates, citrate/isocitrate, glucose/fructose and alanine/sarcosine. High-resolution MS alone may not suffice to discriminate between these and other sets of isomers, especially when fragmentation patterns are similar, and some types of isomers may not separate well on conventional reverse-phase HPLC. To improve separation, reversed-phase ion pairing chromatography, HILIC, and other chromatographic methods can be used[14]; another option is chemical derivatization prior to chromatography [14]. In cases where isomers cannot be separated this needs to be clearly stated since they may display highly different biological functions. Secondly, the presence of *overlapping compounds* may prevent detection of some metabolites. Whilst the increasingly high resolution of mass spectrometers has mitigates this issue to some extent, the resolving power of many current instruments is insufficient to separate ions differing in mass by less than 5 ppm[14]. This problem, however, is only acute when chromatography is also unable to separate analytes that cannot be separated on the basis of their masses. The third major hurdle is that of *in-source degradation products* these are byproduct ions of electrospray ionization owing to simple loss of water, carbon dioxide of hydrogen phosphate, more complicated molecular rearrangements, and the attachments of other ions. In-source degradation reduces the intensity of the metabolite parent ion and the resulting fragment ions may confound analysis of other co-eluting compounds, e.g. if they have the same molecular formula as the molecular ion of another metabolite[14]. We provide examples of these from our own work in Supplementary Figure 1. This example demonstrates the need for careful manual curation of all peak assignments, which, however, is often not feasible when annotating several 100 or 1000 metabolites (Figure 4). In ambiguous cases, the exact identification of a peak can often be best demonstrated via comparative biochemical approaches, e.g. by analyzing the metabolome in known mutants which can be anticipated to lack certain metabolites [22, 105] or the incubation of a purified peak with known enzymes or chemical treatments [93]. This can also be combined with other approaches such as using authentic standards for isomer annotation [106] and dual labelling approaches[107]

Transparency in measurement, metabolite annotation/documentation

In order to fully exploit metabolomics data they need to be comparable between different laboratories. Indeed several comparative studies have been published as we detail in **Supplementary Note 5.** In addition to comparability at a quantitative level clear metabolite ontologies are also needed to ensure that metabolites are annotated in a common fashion see **Supplementary Note 6.** Indeed both issues are important enough to merit a further article related to post data-acquisition of metabolomics data. Furthermore to ensure that methods can be readily adopted by others, a wealth of detailed information is required. However, currently this detailed descriptions of sample preparation and analytical procedures is often (at least partially) absent in publications, especially in cases where metabolomics is not the primary focus of the published work. We recommend the following items should be considered as mandatory components of any methods section for metabolomics experiments. Mass spectrometry: ionization source and type of detection mode, MS method, scan number and speed, MS/MS parameters, including resolution settings and the energy used for fragmentation (Box1). The description of the chromatography methods must include a detailed description of the compositions of the mobile phase, column properties, temperature, flow rate, and injection volume. Extensive recommendations have been made before [42 39]; however, we believe that this list will need to be revisited frequently due to improvements in instrumentation and other aspects of the metabolomics workflow. If unsure, imagine that your twin is sitting on a different continent in front of similar instrumentation, and has to configure the equipment in a comparable manner. Increasingly, there is software support to extract such information from raw data files converted into e.g. the mzML file format [49] (see figure 4 C).

Considering the number of possible pitfalls in the correct annotation and quantification of metabolites in metabolomics approaches the current general level of reporting in the literature is not entirely satisfactory (Figure 4 and 5). Given restrictive journal word limits and the fact that scientific reports tend to be highly concise, it is perhaps not surprising that authors do not refer to compounds as “the metabolite which we putatively annotate as X’’ within the text of their articles. That said, there is nothing to preclude highly detailed reporting of the exact nature of the annotation within the Supplementary Data associated to a paper, either being co-published or made available through separate web resources. Databases such as MetaboLights[65] and Metabolomics Workbench[108] can be used for this purpose, and indeed have been adopted as a requirement for many journals. We also recommend a streamlined and hence simpler reporting within the Supplementary Data and Figure 4. Whilst this is similar to that previously suggested for plant analyses[42] we have updated reporting recommendations to ensure broader applicability and relevance. To simplify the adoption of these recommendations we supply Supplementary Tables 1 and 2 online as template Microsoft Excel spreadsheets. Supplementary Table 1 contains a list of simple questions regarding the reporting of metabolite data whilst Supplementary Table 2 provides recommendations for supplementary data to be provided on the presentation of a typical GC- or LC-MS experiments. Once used to filling out such Tables it is our experience that it takes between 30 and 60 minutes to complete the process which is at least one, if not two, order(s) of magnitude less time than required

for fulfilling the requirements of the data repositories. We believe that a more widespread adoption of these recommendations will enhance the quality of reporting of metabolite data, advance community efforts to improve the annotation of metabolomes, and finally to facilitate the exchange and inter-comparability of metabolite data from different laboratories. These effort will also facilitate comparison of metabolomics data sets obtained from different species, supporting the renaissance of comparative biochemistry.

Summary

In summary, we have here presented a range of recommendations to improve quality and cross-laboratory comparison of metabolic datasets. These range from sampling and metabolite extraction, quantification, peak misidentification, to transparency in measurement and documentation for which a data- rather than chromatogram- centric approach is suggested.We would anticipate that their adoption will offer several advantages: (i) importantly they will provide the reader with the ability to assess the quality of the data reported and as such allow greater confidence in the conclusions drawn; (ii) they will allow researchers a simple route to gain information needed to aid them in annotating their own experimental output and (iii) they will allow the facile comparison of data obtained via multiple laboratories. A recent exemplary documentation of a metabolomics experiment is provided by the study of Price et al [109], who evaluated metabolite levels in under-studied crop species providing an extensive database of the underlying data. Greater adoption of reporting of this type either using the simple reporting table here or the similar one proposed by Dorrestein and co-workers (for a comparison of these Tables please see **Supplementary Note 7**) have the potential to elucidate general aspects of the metabolic response. For example simple studies based on data collected as suggested in our earlier plant-based reporting standards were interrogated in order to identify specific and general metabolic responses to abiotic stress [110]. Such studies have been greatly enhanced via recent the computational approaches of Dorrestein and co-workers [43, 45, 111], which highlighted the power of combining metabolomics datasets in analyzing the effect of the microbiome on the human metabolome[111] , facilitating the discovery of antiviral compounds[43] and evaluation of likely poisonous metabolites in dart frog skin [45]. Further development of both chromatogram-dependent and chromatogram independent computational metabolomics will likely aid greatly in such endeavors, it is, however, beyond the scope of our current article**.** Expansion of such approaches, including both experimental and computational scientists, will facilitate the generation of pan-metabolome databases which will undoubtedly open of new horizons for metabolomics in all kingdoms of life.

Box 1. Information required for transparency in measurement and metabolite annotation/documentation

Chromatography	Ref.
• Instrument description: manufacturer, model number, software and version. • Separation conditions: Column parameters (model, number, thickness, diameter, length, particle size) • Separation method: Mobile-phase composition and modifiers, flow rate, gradient program, column temperature, pressure, temperature, injection; split or splitless, injection cycle time	39, 42
Mass spectrometry	
• Instrument type and parameters: model, software and version • Type of ionization (ESI, EI, APCI, or others), positive or negative polarity. Other ionization parameters (voltage, gas, vacuum, temperature). • Mass analyzer: (TOF, Orbitrap, ion-trap, FT-ICR, etc.), hybrid or single mass analyser used for the experiment. Collision energy used for fragmentation. • Instrument performance (resolution, sensitivity, mass accuracy, scan rates) • Acquisition mode (full scan, MSMS, SIM, MRM, ddMS, etc.) • Detector	39, 42
Metabolite documentation (minimum Ontology)	
• Details are represented in figure 4 and supplementary tables 1 and 2. Included minimum proposed reporting data: retention time, theoretical monoisotopic mass, the ion $(M-H)^-$ and or $(M+H)^+$, m/z detected in the experiment, m/z error (in ppm), MS/MS. fragments, obtained from the ion $(M-H)^-$ and/ or$(M+H)^+$, metabolite name, compound class. • For known compound we proposed to add: international identifiers (such as: HMDB, KEGG, PubChem, KNApSAcK, etc.). • Quantified data (peak intensity, area, etc.) across the experiment must provide as .xls or .text file as supplementary table. • Representative chromatogram/s allowing the assessment of metabolite identifications.	42, 112
More extensive Ontology*	
• Check requirements for repository submission. • Data formats, such as NetCDF for MS data. • International metabolite identifiers. • Data availability, free available, published or not. • Summary about the experiment. • Authentic or reference spectral used for identification. • Code or other information used for analysis if available. • In the case of submission downstream data (results), the minimum structure for table format and experiment must be provided, see Hoffmann, et al., 2019 for example. • In the case of submission data to GNPS for molecular networking for example, see Jarmusch et al., 2020.	111, 113 35, 47, 114

*Recommendation in case submitting the raw data or downstream results to repositories databases (e.g: metaboLights, Metabolomics Workbench, MetaPhen, GNPS etc.)

Main Figures

Figure 1. Metabolomics workflow

Metabolomics involves several basic steps: including sample preparation and extraction, followed by metabolite separation and detection, and data analysis. Steps: (1) describes the basic sample preparation and extraction in metabolomics studies across many applications; (2) analyte separation; metabolite separation on a column (chromatography) such as gas chromatography (GC), liquid chromatography (LC) or capillary electrophoresis (EC); (3) ionization: metabolites ionized at an ion source; (4) separated by a mass anlyzer as they fly or oscillate based on their m/z and (5) detected. Metabolites (from the chromatography) can be identified based on a combination of retention time (RT) and mass spectrometry (MS) signatures.

Figure 2: Workflow for typical mass spectrometry–based metabolomics.

Figure 3. Recovery test

A mixture of extract of leaves, from Arabidopsis and Lettuce. Peaks were measured by GC-MS (a) and LC-MS (b) in leaves of Arabidopsis and lettuce, were used for recovery tests. The percentage recovery was estimated using the theoretical concentration of extracts mixture. Recovery test was carried out with Arabidopsis (A) and Lettuce (B) extracts (0.2 mg FW µl−1) of leaves. Extracts from “A” and “B” were mixed at 50:50 ratio [(A:B). The percentage recovery was estimated for evaluation using theoretical concentration of extracts mixture, [(level in leaves (A) × A%) + (level in leaves (B) × B%)]/100. Dashed lines indicate 70-130 % acceptance range. Compounds in gray are statistically outside this range.

Figure 4. Workflow for metabolic data processes and downstream result documentation:

Structure elucidation workflow of data acquisition, processing and annotation. Simple design for metabolic data documentation and how could be linked to mzTab[49] tool to facilitate data representation, sharing and deposit to public repositories.

Figure 5. Metabolite annotation and documentation :

Structure elucidation workflow of metabolite identification. MS/MS fragmentation provides information about compound structure. Metabolite annotation; this can be achieved using reference compounds, MS^2 analysis, nuclear magnetic resonance (NMR), photodiode array (PDA) detector for UV–VIS spectra detection will add support to metabolite annotation. In addition, database searching and molecular formula calculation.

The Figure shows an example of the MS and MS/MS spectra comparison of rutin (a flavonoid glycoside) revealed a 611 *m/z* peak in MS scan, and two major fragments of 611 *m/z* in MS/MS fragments providing information about chemical moieties; loss of

rhamnose (-146 *m/z*) and glucose (162 *m/z)*. The figure provides the recommendations for metabolic data to be included in a typical LC-MS experiment.

Supplementary Figures

Supplementary figure 1. Examples of mis-annotation

Chromatograms and mass spectrum $(M+H)^+$ ions of dipeptide Ser-Tyr (a) and tyrosine (b) measured by liquid chromatography-mass spectrometry (LC-MS) showing the mis-annotation of metabolites. Both dipeptide Ser-Tyr and tyrosine are present in the Arabidopsis samples and share identical retention time (RT=2.88). The 182.08 *m/z* fragment in source of Tyr-Lys shares elemental composition with tyrosine and thus can lead to erroneous annotation.
Similarly, the dipeptide Tyr-Lys 310.17 *m/z* (c) and a small molecule defense compounds pipecolic acid 130.09 *m/z* (d) are both present in the Arabidopsis samples. Both compounds share identical retention time (RT1.05). One of the in source fragments of Tyr-Lys 130.09 *m/z* shares elemental composition with pipecolic acid, which might lead to mis-annotation.

Glossary

APCI: atmospheric pressure chemical ionization (APCI) Chemical ionization of a sample that is a gas or nebulized liquid, using an atmospheric pressure corona discharge.

API: atmospheric pressure ionization (API): Ionization process in which ions are formed from atoms or molecules at atmospheric pressure.

EI: electron ionization (EI): also referred to as "electron impact" ionization technique that removes one or more electrons from an atom or molecule through interactions with electrons.

ESI: electrospray ionization (ESI): ionization process in which either cations or anions in solution are transferred to the gas phase via formation and desolvation at atmospheric pressure of a stream of highly charged droplets that result from applying a potential difference between the tip of the electrospray needle containing the solution and a counter electrode.

GC-MS: gas chromatography–mass spectrometry (GC-MS) is a chromatographic analytical technique for detection of compounds with separation being performed in the gaseous phase.

LC-MS: liquid chromatography–mass spectrometry (LC-MS) is a chromatographic analytical technique for detection of compounds, with separation being performed in the liquid phase.

m/z: Mass-to-charge ratio (m/z): a dimensionless quantity formed by dividing the ratio of the mass of an ion to the unified atomic mass unit, by its charge number.

Matrix effects: (or ion suppression) phenomenon in mass spectrometry in which the ionization efficiency of a analyte is lowered by the presence of a different species. Typically the ionisation mechanism is suppressed, meaning that a lower response than expected is observed

MS/MS: Acquisition of the spectra of the product ions or precursor ions of *m/z* selected ions, or of precursor ions of a selected neutral mass loss.

Orbitrap: is an ion trap mass analyzer that traps ions in an orbital motion around the spindle. The image current from the trapped ions is detected and converted to a mass spectrum using the Fourier transform of the frequency signal.

TOF: Time-of-Flight (TOF) a mass analyzer that separates ions of different *m/z* by their time of travel through a field-free vacuum region after having been given the same kinetic energy.

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