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From chromatogram to analyte to metabolite. How to pick horses for courses from the massive web-resources for mass spectral plant metabolomics

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Abstract:	The grand challenge currently facing metabolomics is the expansion of the coverage of the metabolome from a minor percentage of the metabolic complement of the cell towards the level of coverage afforded by other post-genomic technologies such as transcriptomics and proteomics. In plants this problem is exacerbated by the sheer diversity of chemicals that constitute the metabolome with the number of metabolites in the plant kingdom generally being considered to be in excess of 200 000. In this review we focus on web-resources that can be exploited in order to improve analyte and ultimately metabolite identification and quantification. There is a wide range of available software that not only aids in this but also in the related area of peak alignment, however, for the uninitiated choosing which program to use is a daunting task. For this reason we provide an overview of the pros and cons of the software as well as comments regarding the level of programing skills required to effectively exploit their basic functions. In addition the torrent of available genome and transcriptome sequences that followed the advent of next-generation sequencing has opened up further valuable resources for metabolite identification. All things considered, we posit that only via a continued communal sharing of information such as that deposited in the databases described within the article are we likely to be able to make significant headway towards improving our coverage of the plant metabolome.
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Review:

From chromatogram to analyte to metabolite. How to pick horses for courses from the massive web-resources for mass spectral plant metabolomics

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Abstract

The grand challenge currently facing metabolomics is the expansion of the coverage of the metabolome from a minor percentage of the metabolic complement of the cell towards the level of coverage afforded by other post-genomic technologies such as transcriptomics and proteomics. In plants this problem is exacerbated by the sheer diversity of chemicals that constitute the metabolome with the number of metabolites in the plant kingdom generally being considered to be in excess of 200 000. In this review we focus on web-resources that can be exploited in order to improve analyte and ultimately metabolite identification and quantification. There is a wide range of available software that not only aids in this but also in the related area of peak alignment, however, for the uninitiated choosing which program to use is a daunting task. For this reason we provide an overview of the pros and cons of the software as well as comments regarding the level of programing skills required to effectively exploit their basic functions. In addition the torrent of available genome and transcriptome sequences that followed the advent of next-generation sequencing has opened up further valuable resources for metabolite identification. All things considered, we posit that only via a continued communal sharing of information such as that deposited in the databases described within the article are we likely to be able to make significant headway towards improving our coverage of the plant metabolome.

Keywords: Arabidopsis, bioinformatics, crop species, GC-MS, LC-MS, peak identification, peak annotation.

Background

Metabolomics emerged in the late 1990s with the term coined in a review of Steven Oliver [1]. However, the 2000 paper by Fiehn and co-workers wherein gas chromatography (GC) coupled to mass spectrometry (MS) defined the chemical composition of a morphological and metabolic mutant of the model plant *Arabidopsis thaliana* [2]; in doing so they were able to describe changes in the level of 326 analytes. This work thus greatly extended on the early metabolite profiling study of Sauter et al. [3] , which presented the technology as a means of putative classification of mode-of-action of pesticides. Thus the advent of metabolomics in plants arguably preceded that in microbes and mammals although the approach was rapidly adopted in these communities also [2, 4-6]. During the next two decades metabolomics had one considerable advantage over profiling technologies such as transcriptomics and proteomics in that it is not directly reliant on the genome sequence and during this time the species scope of metabolomics rapidly expanded such that it was no longer merely a tool for identifying biomarkers of cellular circumstance but additionally one of the cornerstones of systems biology and an approach which could provide mechanistic insight into metabolic regulation [7-11] . This advantage has subsequently disappeared following the widespread adoption of next-generation sequencing and the lack of linear relationship between the genome and the metabolome now represents part of the problem in identification of unknown analytes [12] . This is nicely exemplified by the fact that computation of the size of the metabolome on genome information as attempted by Nobeli and co-workers in 2003 for the E. coli metabolome and [13] rendered values far smaller than the number of metabolites actually measured to date [14]. Whilst the size of the metabolome for prokaryotes has been estimated at a couple of thousand, that of the plant kingdom dwarves these numbers with estimates ranging between 200 000 and 1 million metabolites [15]. Within the last two decades metabolomics has been employed to address a wide range of important questions in plant biology including pathway structure [15], the influence of metabolism on growth [8, 16], plant ecology [17], various aspects of plant genetics including evolution and the domestication syndrome [18-20] as well as detailed characterizations of the metabolic response to biotic and abiotic stressors [21, 22].

In this review, we discuss two topics. The first is the availability of tools to aid in chromatogram evaluation. Since we last reviewed this in 2009 [23], the number of resources has exploded as has their diversity in type. In 2009 a number of pathway, analytical standards, analytical samples and literature databases were available. In the intervening period additional sites providing information on experimental and *in silico* mass fragmentation, isotopic labeling, pathway predicted metabolites, integration of metabolomics with other platforms and mass spectrometry imaging have become available. For each resource we will briefly outline functionality and provide illustrative examples of their utility. The second is to review the current status of the broad variety of plant metabolomics databases. In this respect we list sources of archived data and their respective volumes of data. We also briefly discuss recent meta-analysis which illustrate

that despite current hurdles regarding comparability of data there is great potential for cross-study comparisons on metabolite responses in determining common responses between either genetic or environmental perturbations of metabolism. Finally, we will provide an outlook as to how the grand challenge of comprehensitivity will best be met and how the power of archived plant metabolic responses will be best exploited in the future.

It is not the scope of this review to discuss the theoretical details of every procedure or to document the subtle differences between the many similar tools referred to here. We rather aim to provide a general idea of the importance and challenges of each step in the metabolomics workflow and to summarize the major functions of each tool while referring to the more comprehensive literature supporting them. We attempt to classify all the resources in a simple and logical manner in order to facilitate understanding of the main functionalities of each one. It is, however, important to mention that while few of the tools presented here provide a complete workflow, most of them are able to perform multiple complementary functions somewhat blurring any initiative to accord their functions specific classifications. Other important information that we include here is how these tools can be accessed. This is usually performed either via command-line-or graphical-user-interface (GUI), the former provides flexibility and facilitating integration, automation and development, while the latter was developed to be intuitive and friendly for unexperienced users. Finally, it is important to highlight that the active developments in the field result in frequently outdated and discontinued resources. While many groups keep releasing new upgraded versions of their tools, it is often the case that the projects are just discontinued and the tools are kept available online. We tried to represent this by including the most recent references as well as the last update dates for each of the resources in supplementary table 1. All these features considered allow the researcher to access the information required to choose the “winning horse” under the conditions or “course” in which they are racing. Finally it is also important to highlight that these tools are constantly being updated, integrated and discontinued, and while we ensured that all the links provided here were functioning at the time of writing, it is impossible to ensure that to be the case in the future.

Sample preparation and data acquisition

The metabolomics workflow (Figure 1) starts with sample preparation including extraction and often coupled to pre-treatment and chemical derivatization, followed by data acquisition which will depend on the chromatographic system, ionization source and analyzer. Optimization of sample preparation and data acquisition can considerably improve the analysis and is particularly interesting for plant metabolomics where matrix complexity is very high; nevertheless this step is often skipped over in favor of standardization and simplicity which allow for greater sample throughput. Methods for chromatography mass spectrometry based optimization are well developed and usually rely on statistical designs collectively known as Design of Experiments (DoE) [24].

While some studies have detailed its application in plant metabolite extraction [25] and liquid chromatography (LC) analysis [26], very few software tools were developed so far focusing on this kind of approach for metabolomics data. That said a couple of interesting software are MUSCLE [27], a tool for the automated optimization of targeted LC-MS/MS analysis that was shown to significantly shorten analysis times and increase analytical sensitivities of targeted metabolite analysis, and FragPred [28], which uses experimental fragmentation from a database to select common fragmentation products that minimize uncertainty about metabolite identities in large-scale multiple reaction monitoring (MRM) experiments, have been published and appear to be highly promising.

Data processing

Raw mass spectrometry chromatograms are three dimensional data consisting of a distribution of m/z intensities over the time. Processing this data requires filtering, detecting and integrating relevant features, aligning signals across different samples, extracting compound mass spectra and normalizing the data, all with the final goal of simplifying and hence facilitating data interpretation.

Feature detection and peak alignment are the initial steps for extracting information from raw data and corresponds to the process in which relevant signals are identified and quantified across samples, having peak alignment as one of the big challenges to overcome, particularly for LC-MS where retention time is more prone to fluctuations in relation to GC-MS. The many different approaches available to perform these steps of data processing were recently reviewed by [29, 30], and some of the most popular algorithms for feature detection and peak alignment were compared in different works [31, 32]. Most software somehow integrate both steps in the same pipeline to generate a report of signal intensities over samples from raw data, and many of them also include some resource for data analysis and peak annotation that will be discussed later in more detail. In the following section we will detail the available tools for this step, adopting a similar approach in all subsequent sections also (the details of the programs are all given in additional file 1). MetAlign [33] is a versatile tool that performs well with both LC-MS and GC-MS and allows direct conversion from and to vendor formats while most other tools need an extra software for this step. It additionally provides a series of functionalities through other tools that are developed by the same group and integrate directly in the output of MetAlign. XCMS appears to be the most cited software for LC-MS data processing, it was developed for R and implements different algorithms for feature detection and alignment suitable for different kinds of data, while it can be argued that the software requires familiarity with programming and lacks resources for simple data inspection, its platform is, nevertheless, powerful and easily integrated with other tools and its extensive community of users provide a great resource for troubleshooting. Moreover, a great number of other tools are built upon the functions of XCMS [34]. Amongst these, TracMass 2 [35], a MATLAB software which provides a GUI in a

modular suite, was developed to provide immediate graphical feedback of every step of the processing pipeline, its benchmark paper compared the complexity of different algorithms highlighting the importance of low complexity when dealing with large data files and demonstrating it to be more efficient than MZmine 2 (see below for discussion of this software) and comparable to XCMS, two of the most popular current data processing tools. The particularities of TracMass algorithm makes it more suitable for detecting mass traces in the low mass region that can be missed by other approaches. iMet-Q [36], a C# software with a GUI whose algorithm includes automatic detection of charge state and isotope ratio of detected peaks and was developed to minimize the amount of necessary input parameters significantly facilitates the pipeline for new users. GridMass [37] is a 2D feature detection algorithm implemented in MZmine 2 that is faster than other algorithms and potentially improves detection of low-intensity masses. MSFACTs [38], was one of the first tools developed for peak alignment, it uses peak tables or raw data in the ASCII format as input being limited only to the chromatographic domain, this approach can, however, now be considered outdated when compared with many other resources currently available. MET-IDEA [39] is a more recent and flexible tool, developed by the same group as MSFACTs, for feature detection and alignment with a friendly interface developed in .NET platform. Its features include visualization of integrated peaks and manual integration and display of mass spectra, which can be very helpful for quick data inspection. EasyLCMS [40] is a web application tool with focus on calibration and calculation of targeted metabolite concentration in terms of µmol using algorithms developed for MZmine 2. IDEOM [41] is a metabolomics pipeline using functions from XCMS and MZmatch from an Excel GUI. It also includes automated annotation based on an internal database of exact mass and retention time that can be update by users according to the machine. Massifquant [42] is a feature detection algorithm integrated into XCMS based on a Kalman filter for the detection of isotope trace, this approach was shown to be particularly useful for low-intensity peaks. MET-COFEA [43] is a C++ software accessed via a GUI that implements a novel mass trace based extracted-ion chromatogram extraction that copes better with drifts in the mass trace. It additionally uses compound-associated peak clusters instead of individual features for alignment (this clustering process is an important step to extract metabolite information and simplify data as it will be discussed below). MET-Xalign [44] is an extension for MET-COFEA that can potentially align compounds of samples from different experiments, a hard task for metabolomics datasets that is not approached by most other tools. apLCMS [45], is an R package for high mass accuracy LC-MS, which tries to be user friendly by providing a file-based operation and a wrapper function for a single command line batch process of LC-MS data, however, still requires quite some computational knowledge to operate. xMSanalyzer [46] is an R package for improving feature detection that integrates with existing packages such as apLCMS and XCMS, it systematically re-extracts features with multiple parameter settings and merges data to optimize sensitivity and reliability. Yamss [47] is a recently developed R package focused in providing high-quality differential analysis implementing a method based on bivariate approximate kernel density estimation for peak

identification. In addition to the tools mentioned above there are a few tools for data processing that exclusively perform peak detection or alignment such as peak-grouping-alignment [48], an approach where information from grouping peaks within samples improve alignment across samples, and PTW [49] a fast alignment algorithm based on a variation of parametric time warping working on detected features rather than on complete profile data. In addition, cosmiq (http://www.bioconductor.org/packages/devel/bioc/html/cosmiq.html) is a peak detection algorithm to improve detection of low abundant signals that can be easily integrated with XCMS. These algorithms represent an important effort in improving the existing approaches but they are much less accessible since they need to be integrated with other tools that usually perform similar functions and in some instances this requires quite advanced computational skills.

It is important to note the significant differences between GC-MS and LC-MS which are intrinsic to the features of each system, and can be summarized as a much higher efficiency and stability in GC over LC separation followed by a very stable fragmentation in traditional GC ion sources in contrast with the typical atmospheric pressure ionization employed with LC. This significantly influences the processes of peak alignment and spectra annotation, and while most of the tools developed with a focus towards LC-MS can also be used for processing GC-MS data, there are many developed with a particular focus on processing GC-MS data, making use of different strategies for peak alignment and integrating metabolite annotation by matching spectra to libraries. AMDIS [50], developed with the support of U.S. Department of Defense, is one of the most popular GC-MS processing tools, it automatically extracts component mass spectra from GC-MS data and uses it for search in mass spectral libraries, a disadvantage of this software is that the output requires extensive treatment to be used for further analysis. However Metab [51], an R package based on functions of XCMS was developed to automate the pipeline for analysis of GC-MS data processed by AMDIS dealing with the issue of its output data. MetaQuant [52] is a tool that uses retention index to define metabolites but it depends on other deconvolution software like AMDIS to extract mass spectra. Both MetaboliteDetector [53] and TagFinder [54] provide an efficient pipeline performing deconvolution, peak detection, compound identification, alignment based on Kovats retention index using alkane mix and quantification, and provide an interactive user interface facilitating use by unexperienced users. They do however require several manually input and data check steps that are time consuming and negate truly high throughput. TargetSearch [55] uses similar approaches to process data, identify and quantify targeted metabolites based on retention time index and spectra matching of multiple correlated masses but it is highly automated and efficient thus allowing the processing of large sample sets. PyMS [56] is an alternative to the previously mentioned interactive software, providing similar functions but being particularly suitable for scripting of customized processing pipelines and for data processing in batch mode working in Python. MET-COFEI (http://bioinfo.noble.org/manuscript-support/met-cofei/) uses reconstructed compound

spectra instead of individual peaks to align signals across samples, which is expected to improve peak information for downstream analyses, it also match spectrum against an user-specific library. TNO-DECO [57] uses a segmentation approach to allow the performance of simultaneous deconvolution of multiple chromatographic MS files in a semi-automated fashion in MATLAB, thereby eliminating peak alignment. By contrast, MetaMS [58] is a pipeline for high-throughput GC-MS processing based on XCMS for peak detection and alignment and CAMERA for compound spectra extraction. Compound spectra is further annotated based on match with a database. This tool may be convenient for users that already implement XCMS analysis of other data, but this kind of processing is not optimal for GC-MS when compared with other processing types. Maui-VIA [59] implements a graphical interface that facilitates visual inspection of identifications and alignments providing faster interaction with the data. eRah [60] is an R tool that integrates a novel spectral deconvolution method using multivariate techniques based on blind source separation, alignment of spectra across samples without the need of internal standards for calculating retention indexes, quantification, and automated identification of metabolites by spectral library matching, in a fully automated pipeline, even though internal standards are not necessary they are still recommended to increase reliability in metabolite identification. The software ADAP-GC 3.0 [61] uses a deconvolution algorithm based on hierarchical clustering of fragment ions, the updated version is incorporated into the MZmine 2 platform and addressed issues from the first version such as fragment ions that are produced by more than one co-eluting components, and improved sensitivity and robustness. Finally, MetPP [62] is a processing tool that includes normalization and statistical analysis but is directed towards data emanating from GC×GC-TOF MS system.

Extracting compound mass spectra is another important step of data processing that reduces data complexity by many orders of magnitude by identifying m/z signals that belong to the same compound and provide essential information for further metabolite annotation through the reconstructing of mass spectra. While this process is usually integrated in GC-MS tools for feature detection, alignment and annotation, as mentioned above, there are many approaches to deal with LC-MS data such as the ones employed by CAMERA [63] a package developed in R to extract compound spectra, annotate isotopes and adducts, and propose compound mass as an extension to XCMS, it is easy to use in combination with this software and provides a significant reduction on data complexity. AStream [64] is another R package very similar to CAMERA but using a simpler algorithm for grouping the peaks. ALLocator [65], is a web based workflow that applies centwave from XCMS for feature detection followed by spectra deconvolution either by CAMERA or by the ALLocatorSD algorithm which is optimized for dealing with the particularities of ^{13}C labeled data by grouping mirrored isotopes (lighter isotopologues from feeding experiment). MSClust [66], has the same general features as the others but it was developed in the C++ language and it is optimized to work with the output files of MetAlign. RAMClustR [67] was developed in MATLAB and implemented in R, accepting directly the output of XCMS. The authors suggest

the use of a workflow consisting of data acquisition under both low and high collision energy as a way to improve the quality of the spectra generated by feature clustering and provide a data format that can be submitted directly to the MassBank Database and NIST MSSearch program. By contrast, RAMSY [68] uses average peak ratios and their standard deviations rather than correlation to allow the recovery of compound spectra, the performance of this approach is typically better than the results from correlation methods, furthermore, the script for MATLAB is available or it can be run from a web interface with a .csv table as input.

The last step of data processing, data normalization, is essential for further data analysis in order to remove bias introduced by sample preparation from meaningful biological variation. Most methodologies rely either on the use of internal standards statistical means for normalization. Most data normalization procedures are usually integrated in data analysis tools, but there are few examples of more specialized tools such as MetTailor [69] that uses a dynamic block summarization method for correcting misalignments reducing missing data and apply an RT-based local normalization procedure, or Normalyzer [70] that uses twelve different well known normalization methods and compares the results based on different parameters. IntCor [71] that corrects for peak intensity drift effects based on variance analysis, MetNormalizer [72] which allows normalization and integration of multiple batches in large scale experiments using support vector regression, and EigenMS [73] which detect bias trends in the data and eliminates them using single value decomposition are also highly useful. All of these software are implemented in R, however, with the exception of Normalyzer which can be also used in a web interface they all require considerable familiarity with this programing language. A couple of other tools that help to extract specific information previous to data analysis include the program SpectConnect [74], that identifies conserved metabolites in GC-MS datasets, and MathDAMP [75], a Mathematica package for Differential Analysis of Metabolite Profiles highlighting differences within raw LC-MS and GC-MS datasets.

A common feature of mass spectrometry data is the presence of multiple peaks for individual fragments resulting from the distribution of natural isotopes which are particularly interesting and explored in stable isotope labeling experiments. There are a few tools for correcting and extracting label enrichment from processed data such as Corrector [76], IsoCor [77] and ICT [78]. These tools are very similar all being based on the same matrix calculation. Corrector was developed to work on the output of TagFinder but data processed with most other tools can be easily arranged in a similar table format. IsoCor provides a GUI with a few different options including corrections for the label input whereas ICT includes features to process data from tandem MS. Nevertheless most data processing pipelines available are not particularly efficient for dealing with this kind of experiment, to fill this gap there are some specialized tools like mzMatch–ISO [79], integrated in the mzMatch pipeline. This software is capable of targeted and untargeted processing of labeled datasets and the output includes a set of plots summarizing the pattern of labelling

observed per peak allowing users to quickly explore data. MetExtract [80] which relies on a mixture of cultures from the same organism under natural and labeled media to select signals that show a clear pattern of isotopic enrichment. However, the approach requires the labeled fraction to be fully labeled and the tracer to be highly pure to get the proper isotopic distributions. X13CMS [81] and geoRge [82], both run on the R platform using GC-MS output, the former algorithm iterates over MS signals in each mass spectra using the mass difference due to the label, while the latter uses statistical testing to distinguish Spectral peaks originated from labeled metabolites resulting in significant less false positives. The MIA program [83] detects isotopic enrichment in GC-MS datasets in a non-targeted manner, providing an easy GUI to visualize mass isotopomer distributions (MID) of the detected fragments as barplots including confidence intervals and quality measures, tools for differential analysis of relative mass isotopomer abundance across samples and network assembly based on pairwise similarity of MID that can reveal related metabolites.

Another important feature of many mass spectrometry systems is their capability of performing tandem mass spectrometry. While this can significantly improve data in many ways, it adds another level of complexity for data processing. A very common use of tandem MS is to increase selectivity and accuracy in targeted analysis and MRMAnalyzer [84], MMSAT [85] and MRMPROBS [86] are useful tools developed for processing data from multiple reaction monitoring experiments. MMSAT [85] is a web tool that takes mzXML files as the input, it is able to automatically quantify MRM peaks but lacks metabolite identification capability. By contrast, MRMPROBS [86] detects and identifies metabolites automatically, providing a user-friendly GUI for data analysis. The algorithm has one limitation that it needs at least two transitions per metabolite in order to discriminate the target metabolite form isomeric metabolites and the background noise. Similarly, MRMAnalyzer [84] is an R tool allowing processing, alignment, metabolite identification, quality control check and statistical analysis of large datasets that transforms data in “pseudo” accurate m/z, in order to use the centwave algorithm from XCMS for peak detection. Untargeted metabolomics analysis can also take advantage of tandem MS, particularly for compound annotation, and there are few resources for dealing with the complexity of such experiments such as decoMS2 [87], an R package for deconvoluting MS2 spectra eliminating contaminating fragments without the need of sacrificing sensitivity in favor of sensibility by narrowing the window of isolation for collision-induced dissociation (CID) during data acquisition. This approach requires MS2 data to be acquired under low and high collision energies to solve the mathematical equations potentially reducing sensitivity of the method. MS-DIAL [88] and MetDIA [89] both deal with Data-independent acquisition (DIA) data, an interesting approach for untargeted metabolomics that acquire MS2 spectra for all precursor ions simultaneously with the complication that it uses larger isolation windows, hence increasing the probability of contamination in the MS2, and it loses the relation between precursor and fragment ions. MS-DIAL addresses these problems by a mathematical deconvolution based on GC-MS processing tools in a fully untargeted

manner, whilst achieving the metabolite identification through a spectrum-centric library matching. MS-DIAL is applicable to both data-independent and data-dependent MS/MS fragmentation methods in LC-MS and GC-MS. By contrast, MetDIA [89] uses algorithms from XCMS for peak detection and alignment combined with a targeted approach based on matching metabolites in a library to the detected peaks, thus achieving higher sensitivity and specificity on metabolite identification and wider metabolite coverage.

A trade-off for most of the more flexible and powerful resources presented here is that they have multiple parameters that need to be optimized, and recently a number of tools try to assist in evaluating and automatizing this process. In this context IPO [90] was developed to perform automatic optimization of XCMS parameters based on design of experiment , Credentialing Features [91] optimize detection based on regular and 13C-enriched , MetaboQC [92] is a quality control approach that evaluates alignment and suggests optimal parameters for feature detection based on discrepancies between replicate samples , and SIMAT [93] allows the selection of the optimal set of fragments and retention time windows for target analytes in GC-SIM-MS based analysis.

Data analysis

Metabolomics datasets are usually characterized by high dimensionality, heteroscedasticity (i.e. the variance in errors is not constant across the dataset) and differences of orders of magnitude across metabolite concentrations and fold changes, making it challenging to extract and visualize useful information from processed data. There are numerous approaches for data scaling, reduction, visualization and statistical analysis particularly useful for analyzing metabolomics data, many of them very well established such as analysis of variance (ANOVA), hierarchical cluster analysis (HCS), principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) to mention just a few. There are many general statistical software capable of performing most of these functions, but also a variety of software tools exist combining procedures relevant to metabolomics in a single pipeline and thus facilitating the workflow such as DeviumWeb (https://github.com/dgrapov/DeviumWeb), BioStatFlow (http://biostatflow.org/), MetaboLyzer [94], metaP-Server [95], Fusion (https://fusion.cebitec.uni-bielefeld.de/Fusion/login) , Pathomx [96], MSPrep [97], MixOmics (http://mixomics.org/) and COVAIN [98].

Other interesting and somehow more specialized tools include RepExplore [99] which exploits information from technical replicate variance to improve statistics of differential expression and abundance of omics datasets, KMMDA [100] and Metabomxtr [101] which deal with the troublesome issue of missing metabolite values, the former through a kernel-based score test and the later through mixed-model analysis. Similarly, PeakANOVA [102] identifies peaks that are likely to be associated with one compound and uses them to improve accuracy of quantification, a particularly useful approach for experiments with limited sample size. SPICA [103], is a tool that aims at extracting relevant information from

noisy data sets by analyzing ion-pairs instead of individual ions. MetabR [104], normalizes data using linear mixed models and tests for treatment effects with ANOVA. By contrast MPA-RF [105] combines random forests with model population analysis for selecting informative metabolites. Qcscreen [106], helps to verify data consistency, measurement precision and stability of large scale biological experiments.

Metabolite annotation

Metabolite annotation is often considered the most challenging step and as such represents a major bottleneck for metabolomics studies. Even though the gold standard for structural characterization remains NMR characterization of the pure compound [107, 108], MS based metabolomics offers many advantages including lower cost, higher sensitive and throughput, and it can be easily hyphenated with chromatography while still providing considerable structural information. As a consequence great efforts have been made to improve mass spectrometry based metabolite annotation, and a battery of interesting tools were developed with this goal in mind. The great interest from metabolomics and mass spectrometry communities even culminated with the creation of the "Critical Assessment of Small Molecule Identification" (CASMI) contest. The idea of the contest is to challenge multiple approaches and rank their performance over a series of categories [109, 110]. Structural information is normally extracted from mass of molecular ion in high-resolution MS (HRMS) which can provide the molecular formula and fragmentation pattern. It is important to note that most strategies for metabolite annotation rely heavily on information retrieved from databases of molecular formulas, spectra and pathways which will be discussed in more detail below.

The most common tools are based on matching spectra or exact masses from unknown compounds against spectral data deposited in some database. One example using this approach is MetaboSearch [111], which accepts either a list of m/z or the output of CAMERA as input and searches against four major metabolite databases, Human Metabolome DataBase (HMDB), Madison Metabolomics Consortium Database (MMCD), Metlin, and LipidMaps. Similarly, PUTMEDID-LCMS [112] developed in the Taverna Workflow Management System, also integrates a step of compound mass spectra extraction to define a molecular formula from high resolution m/z that is then matched against a predefined list of molecular formulas to annotate compounds. MetAssign [113] is integrated in mzMatch and it considers the uncertainty related with metabolite annotation using a Bayesian clustering approach to assign peak groups, this approach has the advantage of providing a quantitative values for uncertainty/confidence in the outputs that can be used in further analysis. The program SIRIUS [114] is a Java-based software that combines high accuracy mass with isotopic pattern analysis to distinguish even molecular formulas in higher mass regions. Furthermore it also analyses the fragmentation pattern of a compound using fragmentation trees that can be directly uploaded to CSI:FingerID (described below) via a web service. MFSearcher [115] is a tool that efficiently searches high accuracy masses

against a database of pre-calculated molecular formulas with fixed kinds and numbers of atoms that are further queried against different databases, HR3 [116] is a similar tool for molecular formula calculation and query in external databases. It uses different sets of rules for heuristic filtering of candidate formulas instead of a pre-calculated database which makes it slightly slower than MFSearcher, but HR3 includes compounds with atoms that are not present in MFSeacher's list as well as considering matches to the isotopic pattern within its annotations. MS-FINDER [117] is a C# program with a GUI providing a constraint-based filtering method for selecting structure candidates. The workflow begins with molecular formulas from precursor ions being determined from accurate mass, isotope ratio, and product ion information. Next, structures of predicted formulas are retrieved from databases, MS/MS fragmentations are predicted and the structures are ranked considering bond dissociation energies, mass accuracies, fragment linkages, and, most importantly, nine hydrogen dissociation rules. MS-FINDER provides an interesting theoretical background from which to interpret MS/MS spectra and its comparison to database matches. Additionally it was shown to be able to predict with 91.8% accuracy over 80% of the manually annotated metabolites in test samples [117]. MS2Analyzer [118] is a java software for identifying neutral losses, precursor ions, product ions and m/z differences from MS2 spectra based on a list of predefined transitions. These features are essential for structure elucidation using mass spectrometry and the software provides a fast and high-throughput platform for extracting this data. MS2LDA [119] is based on latent Dirichlet allocation (LDA), an algorithm originally used for text mining that was adapted to generate a list with blocks of co-occurring fragments and losses providing results similar to MS2Analyzer but without the need of user specified precursor/product transitions.

Another level of biologically relevant information is added by many tools that incorporate pathway information to assist annotation and interpretation of results such as Metabolome searcher [120], a web-based application to directly search genome-constructed metabolic databases which includes MetaCyc with data on plant metabolism. MassTRIX [121] is a web interface that takes a mass peak list from HRMS as input and matches them against KEGG compounds database returning a pathway map with the matches, organisms can be selected and the output represents organism-specific and extra-organism items differentially colored to assist interpretation. MetabNet [122] is an R package to perform targeted metabolome wide association study of specific metabolites. This approach uses the correlation of all mass signals with the targeted metabolite across samples to build networks that can be visualized in pdf or exported to Cytoscape. This can be a very useful approach to identify related compounds and associate them to metabolic pathways. Similarly, ProbMetab [123] is an R package for probabilistic annotation of compounds based on the method developed by Rogers et al. (2009) [124] that incorporates information on possible biochemical reactions between the candidate structures to assign higher probabilities to compounds that form substrate/product pairs within the same sample. MI-Pack [125], implemented in python, calculates differences in mass between all molecular

formulas annotated from HRMS and compares them to known substrate/product pairs from KEGG, but matches are considered based on the error between experimental and theoretical masses compared to a threshold defined by a calculated mass error surface. PlantMAT [126] is a particularly interesting tool specifically for the investigation of plant specialized metabolism, which uses an approach based on common metabolic building blocks to predict combinatorial possibilities of phytochemical structures used for annotation and as such is a highly effective way to search the chemical space surrounding a (set of) metabolite(s)

Another more recent and promising approach made possible by the huge amount of data available uses algorithms, mostly based on machine learning, to predict molecular properties of unknown compounds from its tandem mass spectra. All the tools listed below provide similar web interfaces for putative metabolite identification differing mainly on the algorithms used to perform the identification and the overall performance. MetFrag [127] retrieves candidate structures either from databases based on exact mass or from user specified structure-data files (SDF), a data format based on MDL Molfile with focus on caring structural information. Candidate structures are fragmented using a bond dissociation approach and fragments are compared with the input spectra scoring matches based on a series of rules. The candidates can also be filtered to facilitate the analysis based on relevant factors such as metabolite origin, composition, LC retention time and metadata from the databases. Besides the Java web-interface a command line version and an R package are provided which are more suitable for batch processing and integration with other tools. In a very similar approach MolFind [128] retrieves candidates from databases based on exact mass, filters them by comparing experimentally measured retention index, ECOM50 (the energy in eV required to fragment 50% of a selected precursor ion) and drift time (for ion mobility MS) with predicted ones, and analysis CID of the best candidates using MetFrag. CFM-ID [129] is based on competitive fragmentation modeling, a probabilistic generative model that uses machine learning to learn its parameters from data. It can be used to predict spectra of known chemical structures, to annotate peaks in the spectra of a known compound or to predict candidate structures for an unknown compound by ranking candidates in terms of how closely the predicted spectra match the input. MAGMa [130], extends prediction based on substructure assignment by creating hierarchical trees of predicted substructures capable of explaining MS^n data, where each level takes into account the restrictions imposed by the assignment of precursor and subsequent fragmentation. FingerId [131] developed a model based on a large dataset of tandem MS from MassBank and uses a support vector machine to predict the molecular fingerprint of the unknown spectra and compare this with the fingerprint of compounds in a large molecular database. CSI:FingerID [132] is a more recent tool based on fingerID that includes computation of fragmentation tree achieving one of the best search performances. Besides the web interface it can be also queried directly through Sirius but it currently does not support batch mode. CSI:IOKR was the last CASMI winner approach for the category “Best Automatic

Structural Identification—In Silico Fragmentation Only" [110]. It is based on the integration of CSI:FingerID with an Input Output Kernel Regression (IOKR) machine learning approach to predict the candidate scores [133]. CSI:IOKR outperforms other approaches in metabolite identification rate while considerably shortening running time, nevertheless, it is still not available as an implemented workflow. Finally MetFusion [134] is a Java web tool that combines spectra database matching against MassBank with the prediction based annotation provided by MetFrag.

Data interpretation

Interpretation of omics data is usually complicated by the amount and complexity of data. There are many tools to assist metabolomics data interpretation, particularly for its visualization by mapping metabolites into pathways and providing biological context, and for the integration with data from different platforms (e.g. transcriptomics, proteomics see Tohge et al. (2015) [15] for details). As for metabolite annotation, these tools usually rely upon knowledge stored in metabolite and pathway databases, and many of them include some kind of statistical analysis such as pathway enrichment and correlation analysis.

Visualization tools provide a simple mean of representing and mapping metabolic changes in tools like PATHOS [135], PathWhiz [136] and iPath [137]. They can often provide some kind of pathway structure analysis such as PathVisio [138], FunRich [139], BiNChE [140] and MPEA [141] that uses pathway enrichment analysis and PAPi [142] that calculates pathway activity scores to represent the potential metabolic pathway activities, and performs statistical analysis to investigate differences in activity between conditions. Tools like InCroMAP [143], IIS [144], KaPPA-View4 [145], MapMan [146], ProMeTra [147] which is integrated with MeltDB 2.0, Paintomics [148], VANTED [149], MBROLE [150] and IMPaLA [151] go one step further and integrate metabolomics processed data with other omics platforms, particularly transcriptomics, providing analysis and visualization of large integrated datasets to assist data interpretation.

Few tools try to actually use mass spectra features to build the networks, which can also improve annotation of unknown compounds. MetaNetter [152] uses raw high-resolution data and a list of potential biochemical transformations to infer metabolic networks. MetaMapR [153] builds chemical and spectral similarity networks based on annotated and unknown compounds. ChemTreeMap [154] uses annotated structures and a computational approach to produce hierarchical trees based on compound similarity to assist visualization of chemical overlap between molecular datasets and the extraction of structure–activity relationships. MetFamily [155], groups metabolites in families based on an integrated analysis of MS1 abundances and MS/MS facilitating further data interpretation. MetCirc [156] is an R tool particularly useful for comparative analysis from cross-species and cross-tissue experiments through computation of similarity between individual MS/MS spectra and visualization of similarity based on interactive graphical tools, and TrackSM [157] is a

Java tool that uses molecular structure similarities to assign newly identified biochemical compounds to known metabolic pathways.

Databases

It must be clear from previous sections that mass spectrometry based metabolomics, particularly metabolite annotation and data interpretation, relies heavily upon data from characterized mass spectra, molecular properties of analytes and metabolic pathways. While all the different techniques offer a lot of flexibility, metabolomics struggles with standardization and a great volume of metadata when compared with other omics techniques and still lags behind most of them in terms of public repositories of published data. Nonetheless there are a wealth of databases with useful information for mass spectrometry based plant metabolomics and we try to summarize some of the most relevant and the structure and functionalities of resources available.

Chemspider [158], PubChem [159], ChEBI [160], ChEMBL [161],ChemBank [162], HMDB [163], MMCD [164] and MMsINC [165] are all large databases of small molecules with information such as chemical structure, molecular formula and molecular/exact mass, many of these databases complement each other and data exchange between them is very common, nevertheless it is important to be aware of the sources of data in each one of them and to which extent these data is curated, Chemspider for instance has more than 58 million structures automatically retrieved from over 450 different sources, with only a fraction of this being manually curated by registered users while the majority of data only went through some sort of automatic curation and elimination of redundant entries. Overall such huge databases are particularly useful for looking for physico-chemical properties of identified metabolites and checking for possible candidates based solely on their mass.

There are a few plant specific databases with curated information on chemical composition and distribution across different plant species as well, namely KNApSAcK [166] with information of more than 50,000 metabolites, and chemical composition of over 22,000 species, the Universal Natural Products Database (UNPD) [167], with 229358 metabolite structures Flavonoid viewer [168] with 6,902 molecular structures of flavonoids from 1,687 plant species, Dr. Duke's Phytochemical and Ethnobotanical Databases (https://phytochem.nal.usda.gov/phytochem/search) with information on 29,585 chemicals of 3,686 medicinal plants, BioPhytMol [169] a resource on anti-mycobacterial phytomolecules and plant extracts holding 2,582 entries including 188 plant families, comprised of 692 genera and 808 species, and 633 active compounds and plant extracts identified against 25 target mycobacteria, and EssOilDB [170] with 123,041 essential oil records from 92 plant families. These are very interesting resources for screening chemical composition of specific species and analyzing chemical distribution species wide, and all of the data in these databases is manually curated. From all this resources KNApSAcK is particularly useful not only for the large amount of data but also for providing an easy platform to access and extract information quickly.

Databases providing mass spectra of pure compounds under controlled conditions developed to allow search for common spectra features for the identification of unknown compounds are an essential resource for MS based identification of metabolites. As previously mentioned the great stability and reproducibility of GC-MS generates reliable fragmentation patterns and relative retention indexes that are very efficient for metabolite annotation by spectra matching. NIST is a very popular commercial library for GC-MS annotation, that also provide free access to some data through NIST Chem WebBook (http://webbook.nist.gov/chemistry/), containing mass spectra of 33,000 compounds. SDBS (http://sdbs.db.aist.go.jp/sdbs/cgi-bin/cre_index.cgi) with 25,000 mass spectra is the database from the National Institute of Advanced Industrial Science and Technology (AIST) from Japan. Both of them are limited in the fact that they do not offer an interface for spectra matching and the user have limited access to data, so those are only useful for checking the spectra of targeted compounds. Some more interesting freely-accessible plant specific GC-MS libraries include the Golm metabolome database [171] with a total of 26,590 spectra and 4,663 analytes at the time this article was written and the VocBinBase [172] includes 1,537 unique mass spectra at the time this article was written. Both of these databases can be downloaded and integrated to processing tools for metabolite annotation based on spectra matching. Also worth mentioning is fiehnLib (http://fiehnlab.ucdavis.edu/projects/fiehnlib), however, access of the spectral data is highly limited for this resource.

One of the greatest efforts in the field of metabolomics has been directed to the development of databases of mass spectra obtained from LC-MS analysis. The higher flexibility of this technique compared to GC-MS in terms of the chemical space that it can analyze comes with the drawback of a high sensitivity to multiple factors that can influence mass spectra quality and reproducibility. LC-MS databases are usually characterized by the greatest volume of metadata that accompanies the analytical data, and a more complex structure for search based in spectra features when compared to GC-MS databases. Some large general LC-MS databases include MassBank [173], a public repository of mass spectra with 41,092 spectra of 15,828 compounds obtained by 26 different systems (at the time of writing). This database is very accessible allowing search by submitted spectra or simply by typing in spectral features, mass or targeted compound name, it furthermore allows users to directly extract spectra during data processing through many tools like RAMClustR, RMassBank and Mass++. METLIN [174] currently contains 961,829 molecules from which 200,000 have in silico MS/MS data. Additionally over 14,000 metabolites were analyzed and mass spectra at multiple collision energies in positive and negative ionization mode obtained. METLIN also integrates isoMETLIN [175] that allows the search of isotopologues for all METLIN metabolites based on m/z and isotopes of interest, and includes experimental data on hundreds of isotopic labeled metabolites that can be used to obtain information of precursor atoms in the fragments, both databases can be accessed after free registration and searching by mass is fast and easy with the advantage that it allows the user to select

possible adducts and spectra conditions and search directly the mass observed in the spectra. T3DB [176], is a database for toxin data, many of which are plant secondary metabolites, with MS, MS-MS and GC-MS spectra of 3,600 common toxic substances (at the time of writing). mzCloud is a new database with a more complex organizing structure that can improve and facilitate data interpretation, currently with 6,255 compounds analyzed in different conditions totalizing 1,913,621 spectra arranged in 9,896 tree structures. It allows the user to easily navigate through different spectra of a single compound through its tree structure and also includes visualization of predicted molecular formula of the fragments in the spectra (https://www.mzcloud.org/). Finally the recently developed MoNA (http://mona.fiehnlab.ucdavis.edu/) is intended to be a centralized, collaborative database of metabolite mass spectra and metadata, currently containing over 200,000 mass spectral records from experimental and in-silico libraries from different sources. The search is limited to name, compound class, molecular formula or exact mass of the metabolite, it can be filtered by type of spectra, and the results are presented as a single list of individual interactive spectra next to the metadata making it easy to navigate through different spectra. The great diversity of phytochemicals observed in plants represent an important portion of all these numbers, and a few plant specific databases are available such as Spektraris [177], a LC-MS of about 500 plant natural products that integrates accurate mass – time tag to incorporate retention time relative to an internal standard in a similar fashion as it is usually done for GC-MS based annotation, therefore, in order to use this feature it is necessary to analyze samples with addition of the same internal standard used when developing the database entries. It is important to highlight that this kind of approach is much less effective for LC-MS where relative retention time is prone to larger variation. MS-MS Fragment Viewer (http://webs2.kazusa.or.jp/msmsfragmentviewer/) is a very small and not very frequently updated database containing FT-MS, IT- and FT-MS/MS spectral data on 116 flavonoids. ReSpect [178] is a collection of MSn spectra data from 9,017 phytochemicals from literature and standards with searching functionalities very similar to MassBank, and WEIZMASS [179], a metabolite spectral library of high-resolution MS data from 3,540 plant metabolites that uses a probabilistic approach to match library and experimental data with the MatchWeiz software. WEIZMASS is available for implementation in R as a pipeline for metabolite identification which can be easily integrated with data processing. While this is a much less accessible tool for general use compared with other web based databases the results obtained are far more considerable and the effort required in its use is, therefore, more than compensated by the gains which it affords.

A very common issue encountered in data from mass spectrometry is the presence of a variety of contaminants from sample preparation and analysis that can be challenging for data interpretation. MaConDa [180] provides a very useful database of common contaminants and adducts in mass spectrometry, containing over 200 contaminant records with origin of the contaminant, its mass and the adducts formed. MaConDa can be downloaded in different formats or accessed via the web browser.

Compound spectra databases are essential for identification of metabolites by mass spectrometry, but a significant effort has also been directed towards the development of repositories of experimental data on specific samples to facilitate dereplication studies and data analysis. These databases are often restricted to specific species, as it is the case for AtMetExpress [181], a LC-MS database of Arabidopsis with data on 20 different ecotypes and 36 developmental stages which allows users to download raw and processed data as well as query using mass chromatogram features in the web platform and visualize annotation and distribution of selected features. MeKO [182], is a GC-MS database of 50 Arabidopsis KO mutants. All raw data can be downloaded as netCDF files and results from data analysis can be visualized in a very informative summary in the web browser that shows plant phenotypes, differentially accumulated metabolites indicated in a pathway map and log fold changes for most significantly changed metabolites. MoTo DB [183] is a LC-MS database of *Solanum lycopersicum* with information of annotated metabolites where the user can search for specific masses or a range of masses. The database is based on accurate mass and the user therefore does not have access to raw data and chromatograms. NaDH [184], a platform for integration and visualization of different omics datasets of *Nicotiana attenuata* including LC-MS data on 14 different tissues, allows search for spectra based on name and m/z and provides some interesting tools for data interpretation easily accessible directly from the metabolite entry including metabolite-metabolite and metabolite-gene coexpression analysis and visualization of metabolite expression across different tissues in a bar chart or eFP browser interface. The Optimas-DW software [185], is a data collection for maize data of 15 different experiments, the interface for metabolites allows easy browsing through all the metabolites and visualization of values for individual experiments in a table format but no access to raw data, and the SoyMetDB [186], a metabolomics database for soybean, with GC-MS and LC-MS data of four different tissues under two different conditions, which has a simple interface that provide search by metabolite name or browsing through the whole dataset, metabolite entries provide m/z, retention time as well as an apparent defunct link to a pathway viewer. Similar databases with relative broader spectra include the plant specific KOMIC Maket [187] currently warehousing LC-MS data on 74 samples from 17 species, in which the user can search for peaks and browse through samples and the interface shows retention times, m/z and annotation details classifying the annotation based on a grading system. MS2T [188] is an MSMS library created using a function for automatic Tandem MS acquisition from over 150 samples from 10 different plant species, the web platforms allows search by retention time, m/z and spectra similarity. PMR [189], is a database for plants and eukaryotic microorganisms which includes the earlier database of medicinal plants MPMR [190] and currently comprises of GC-MS and LC-MS data on 24 species from different sources and experiments including different tissues and developmental stages. It has an easy and clear interface with summary of all the experiments once an individual species is selected including metadata and annotated metabolites. It additionally allows the download of all the results in csv format in the form of peak tables and it has some basic tool for comparative analysis where volcano plots can

be generated comparing different experiments. By contrast, the more general databases Bio-MassBank (http://bio.massbank.jp/), a repository of LC-MS and GC-MS data from biological samples, in contrast with the original MassBank in this database most of the data is tagged as “Unknowns” or are just putative metabolites, searching functions are similar to the original database but it includes a samples section where it is possible to access all the experiments available. MassBase (http://webs2.kazusa.or.jp/massbase/) is a large repository providing raw and processed mass chromatograms on 46,398 samples of over 40 species, including several plants, analyzed by LC-MS, GC-MS and CE-MS. Metabolomics Workbench [191] is a repository of a variety of metabolomics experiments containing over 60,000 entries, including raw and processed MS data, a section with detailed protocols for the experiments, and web tools for analysis and interpretation that can be used with any uploaded data. Similarly, Metabolights [192], is a cross species repository containing data from 190 mass spectrometry based metabolomics studies that is currently recommended as repository of experimental data by many journals, all experimental data can be downloaded from an ftp server and data submission is powered by the use of ISA software that assists in the reporting and management of metadata. MetabolomeXchange [193], is a data aggregation system that allows users to efficiently explore experimental metabolomics data from different databases including MetaboLights and Metabolomics Workbench providing an RSS feeding service to allow users to get updates over the datasets available. Similarly, GNPS [194], a plant natural product knowledge base for community-wide organization and sharing of raw, processed or identified tandem mass spectrometry data currently comprising of 221,083 MS/MS spectra from 18,163 unique compounds. The platform allows users to upload data and provides a series of tools for analysis and interpretation based on the data from the database.

As previously mentioned, many resources that are particularly useful for data interpretation organize the data in pathways based on literature data, and often also provide tools for data visualization and interpretation. Many of these databases contain either generic pathways or combine different organisms, some examples are KEGG [195], which includes 504 pathway maps with 17,891 compounds and 10,419 reactions for 4,607 different organisms, representing data in an interactive interface that links the entries to a great amount of external resources being one of the most popular sources of information on metabolic pathways One of the greatest issues of KEGG leading many user to misinterpreting their data is that it displays all genes in generic pathway maps of which some are characterized only by similarity, resulting in pathways that are not present in the analysed organism being represented. By contrast, WikiPathways [196], is a wiki-style website with 2,471 community curated pathways of 28 different organisms. Its interactive interface is similar to KEGG providing link with external resources for metabolites and enzymes. Similarly, kpath [197], is a database that integrates information related to metabolic pathways with 74,180 pathways 13,153 reactions and 37,029 metabolites providing tools for pathway visualization, editing and relationship search. BioCyc [198], is a collection of 9,387 Pathway/Genome Databases,

and MetaCyc [198] is the largest curated database of experimentally elucidated metabolic pathways containing 2,491 pathways from 2,816 different organisms. KBase [199], meanwhile, is a data platform with data on plants and microbes that allow users to upload their own data and integrates data and tools for systems biology including 1,470 metabolic pathways with 33,773 reactions and 27,838 compounds, genome data on 60 different plant species and tools for assembly, annotation, metabolic modeling, comparative analysis, phylogenetic analysis and expression analysis. There are also a significant amount of plant specific data organized in databases like KaPPA-View4 [145], containing 153 pathways with 1,427 compounds and 1,434 reaction from 10 species, allowing users to upload their own data and is able to represent gene-to-gene and metabolite-to-metabolite relationships as curves on a metabolic pathway maps to help in data interpretation. PlantCyc (http://www.plantcyc.org/) provides access to manually curated or reviewed information about metabolic pathways in over 800 pathways of 350 plant species, usefully the platform provides "evidence codes" to clearly indicate the type of support associated with each database item. MetaCrop [200], is a pathway database containing information about seven major crop plants and two model plants that allows integration of experimental data into metabolic pathways, as well as the automatic export of information for the creation of detailed metabolic models. Similarly, MetNetDB [201], contains integrative information on metabolic and regulatory networks of Arabidopsis and Soybean with metabolism, signalling, and transcriptional pathways being fully integrated into a single network and manually curated subcellular localization is represented in the pathway maps. The network information can be exported to other applications for network analysis such as exploRase, and Cytoscape/FCM. Like MetNetDB, Gramene [202] is an integrated data resource for comparative functional genomics in crops and model plants that host pathway databases for rice, maize, Brachypodium, and sorghum as well as providing mirrors for MetaCyc and PlantCyc data. It is worth mentioning a few resources that are focused on the reactions within the pathways offering detailed curated metabolic reactions, namely BioMeta [203], whose contents are based on the KEGG Ligand database with a large number of chemical structures corrected with respect to constitution and reactions' stereochemistry being correctly balanced. BKM-react [204] is a non-redundant biochemical reaction database containing 18,172 unique biochemical reactions retrieved from BRENDA, KEGG, and MetaCyc databases that were matched and integrated by aligning substrates and products. Similar to this MetRxn [205], also integrates information from BRENDA, KEGG and MetaCyc, combining also Reactome.org and 44 metabolic models in a standardized description of metabolites and reactions where all metabolites have matched synonyms, resolved protonation states, and are linked to unique structures, and all reactions are balanced.

Together with the development of many prediction tools previously mentioned we watched in the last years the development of some interesting *In Silico* databases that are extremely useful for *de novo* metabolite identification such as MINE [206], a database developed by the integration of an algorithm called Biochemical Network Integrated Computational

Explorer (BNICE) and expert-curated reaction rules to predict chemical structures product of enzyme promiscuity, MetCCS [207] a database and algorithm for prediction of Collision Cross-Section values for metabolites in ion mobility mass spectrometry, a technique increasingly used to assist metabolite elucidation based on the drift speed of the ion that is proportional to its cross section, and the plant specific ISDB [208] an *in silico* database of natural products generated using CFM-ID [129] with input from the commercial Dictionary of Natural Products.

Other programs of interest

The complexity of metabolomics data experiments, particularly in terms of sample number and metadata pushed the development of many tools for experiment and metadata management, and while many of these functions are integrated in some of the databases previously discussed there are a few specialized tools such as QTREDS [209] and MASTR-MS [210], that are LIMS based software for assisting in organizing experimental design, metadata management and sample data acquisition , MetaDB [211] a web application for Metabolomics metadata management with interface to MetaMS data processing tool, and Metabolonote [212], a metadata database/management system.

The enormous amount of data available for metabolomics raises many questions regarding how to easily access and unify all this data, taking into account the vast chemical space explored in these experiments. Many tools have been developed with the purpose of facilitating access to chemical data spread in the literature, from the development of identifiers to reduce duplication of information such as the SPLASH [213] hash designed for the MoNA database, to tools like Metmask [214], for managing different identifiers, Chemical Translation Service (CTS) [215], for translation of chemical identifiers, PhenoMeter [216] for querying databases based on metabolic phenotype and Metab2MeSH [217] for a more efficient literature search that automatically annotate compounds with the concepts defined in MeSH providing a fast link between compound and the literature.

Different vendors usually export their data in proprietary formats which complicates data transfer across different platforms. Most proprietary software are able to convert files to .cdf format, but some tools from which the most popular is msConverter from Proteowizard (http://proteowizard.sourceforge.net/) can handle conversion from/to different formats including mzXML. mzTab is another format proposed by the Proteomics Standards Initiative targeting researchers outside of proteomics, it is supposed to contain the minimal information required to evaluate the results of a proteomics experiment making it more accessible to non-experts, jmzTab [218] is a java application that provides reading and writing capabilities and conversion of files to mzTab. The PeakML [219] file format is an initiative developed by the creators of mzMatch to enable the exchange of data between analysis software by representing peak and meta-information from each step in an analysis pipeline, as a proof of concept the R-package ‘mzmatch.R’ was developed to extend XCMS functionalities for storing and reading data in PeakML format.

All equipment for mass spectrometry comes with their own software for data visualization and some basic analysis but those are usually not designed to deal with the complexities of metabolomics datasets. There are some interesting open source alternatives such as BatMass [220] and Mass++ [221] for data visualization, and for generating images from raw data like SpeckTackle [222] that provides several pre-defined chart types easy to integrate into web-facing resources and RMassBank [223] capable of automatically generating MassBank records from raw MS and MS/MS data.

Mass spectrometry imaging is a relative young technique that has being growing fast in importance providing high resolution special distribution of small molecules in molecular histology [224]. Few tools have been developed so far, namely EXIMS [225] for data processing and analysis, and OpenMSI [226], a web-based visualization, analysis and management tool.

Lipidomics data requires a very specialized pipeline and therefore many tools were developed exclusively for this kind of analysis however we will only briefly summarize these here. ALEX [227], MRM-DIFF [228], LICRE [229], LipidXplorer [230], LIMSA [231], VaLID [232], LOBSTAHS [233], Lipid-Pro [234], LDA [235] and LipidQA [236] are all tools for processing, annotating and analyzing lipidomics data. Lipids databases include LIPID MAPS [237], LIPIDBANK [238], LipidBlast [239], and in silico generated lipids database LipidHome [240], SwissLipids [241] and ARALIP (http://aralip.plantbiology.msu.edu/pathways/pathways).

Future perspectives

Many of the resources presented here were fruit of the efforts in setting the theoretical background for each step in the data processing and analysis workflow. However, more recent efforts are moving towards the development of integrated tools, which are often developed by the integration of already well established tools into a single pipeline in an attempt to accelerate the process and in a few cases providing an easier interface. XCMS online, for example, is a web platform providing most of the function from XCMS with additional capabilities for interactive exploratory data visualization and analysis in a much easier interface than the original software [242], HayStack [243], is a web platform that uses XCMS to process data and automatically generates total ion current chromatograms (TIC) and base peak chromatograms as well as offering an easy way of plotting extracted ion chromatograms (EIC) and some basic statistical tools such as PCA scores plot, volcano plots, and dendrograms for group comparisons, SMART [244] is an R package that combines different tools such as XCMS and CAMERA with a series of common statistical approaches to provide an integrated pipeline for data processing, visualization, and analysis. MZmine 2 [245] is another very popular tool with over 1000 citations, it was originally developed for LC-MS data processing but it became one of the most popular platforms for development of integrated tools in Java providing a user-friendly, flexible and extendable software constantly updated and with a set of modules covering most steps of LC-MS processing and

data analysis workflow including several option of visualization tools. MetSign [246] is a MATLAB package providing tools for spectra deconvolution, metabolite putative assignment by matching m/z and peak isotopic distribution against its own database, peak list alignment, a series of normalization algorithms, statistical significance tests, unsupervised clustering, and time course analysis, all in a modular and interactive design presented with a wizard to facilitate the analysis workflow. MultiAlign [247] is a software developed in the .NET platform using C++ and C# originally for proteomics but that can also be used for metabolomics comparative analysis, its functionalities include feature detection, alignment, several plotting options, normalization, and basic statistical comparisons, Metabolome Express [248] works as a web server to process, interpret and share GC/MS metabolomics datasets, whilst MAIT [249] is an R package aiming at providing an end-to-end programmable metabolomics pipeline with emphasis in metabolite annotation and statistics, it uses XCMS for peak detection, an approach based on CAMERA combined with an user defined table of biotransformations followed by database search for metabolite annotation and a series of statistical tests to identify statistically significant features containing the highest amount of class-related information. By contrast, MAVEN [250] is a software for data processing, analysis and visualization with some interesting features for pathway-based visualization of isotope-labeling data that can be helpful for the interpretation of this kind of experiment. MeltDB [251] is a java web based platform that integrates different algorithms for data processing, compound identification by spectra matching statistical analysis, data visualization and integration with transcriptomics and proteomics datasets via the ProMeTra software. It provides a tool for saving peaks of reference compounds directly in the MeltDB database, and allows storage and sharing of projects within the web server. MetaboAnalyst [252] is another java web platform with data processing and a comprehensive set of data analysis tools, it includes most common approaches for statistical analysis as well as modules for functional enrichment analysis, metabolic pathway analysis, time series and two-factor data analysis, biomarker analysis, sample size and power analysis, integrated pathway analysis, and image and report generation. The program mzMatch [219] is a popular Java toolkit for processing, filtering, and annotation, with particular focus on integration of processed data across different platforms and providing a customizable modular pipeline to facilitate the development and integration of different tools. It includes many other tools previously described here like mzmatchISO and metAssign and it is based entirely in the PeakML file format. The MarVis-Suite [253] is a software for the interactive ranking, filtering, combination, clustering, visualization, and functional analysis of transcriptomics and metabolomics data sets, the clustering algorithm is based on one-dimensional self-organizing maps (1D-SOMs), and the software additionaly provides functions for metabolite annotation and pathway reconstruction. MetMSLine [254] is an R package that works with processed data providing a series of statistical analysis steps focusing on biomarker discovery combined with metabolite annotation based on exact mass matching against a target list of metabolites and MassCascade [255] is a Java library that takes advantage of the KINIME workflow

environment facilitating integration with other tools and making the tool user friendly, the core library contains a collection of data processing algorithms, a visualization framework and metabolite annotation functions, while the plug-in for KNIME allows easy integration with other statistical workflows. MASSyPup [256] does not actually integrate different procedures but it does provides an easy platform for accessing many different tools in the form of a Linux distribution that can be run directly from different media without installation.

It is clear from this review the infinity of choices for performing a variety of functions and the fast pace by which they change and get outdated; hence it is an arduous task to keep updated of all of them. Some research groups, engaged in the development of metabolomics tools, have their own repositories like KOMICS [257], MetaOpen (http://metaopen.sourceforge.net/) and PRIMe [258], while OMICtools [259], NAR online Molecular Biology Database Collection and the Bioinformatics Links Directory provide unified repositories but still covering only a small portion of all the resources available. Tools developed for R have the advantage of counting with some well-established platforms such as Biocunductor [260] or CRAN. Nevertheless, with the rapid development of new tools it is of great interest for the metabolomics community to develop classification systems and repositories to catalog and provide a platform for submission, curation and feedback facilitating users' access to the most appropriate and updated resources for each aim. Another clear observation that can be made from the proceeding sections is that the number of tools for analysis by far exceeds that of the number of data repositories whilst metabolomics is clearly difficult to fully standardize this is still a great shame. There are a number of clear reporting standards that should aid in this respect [261], furthermore, both the existing databases and carefully compared meta-analysis [22, 262], demonstrate that such approaches are indeed highly powerful in the enhancement of biological understanding. As such we feel that it is an urgent priority to focus efforts on the improvement of this feature of computational metabolomics since it will aid not only in the expansion of our coverage of the metabolite complement of the plant cell but also in the equally important task of interpreting the biological function of the individual metabolites themselves.

Abbreviations

ADAP	Automated data analysis pipeline for untargeted metabolomics
AIST	National institute of advanced industrial science and technology
ALEX	Analysis of lipid experiments
AMDIS	Automated mass spectral deconvolution & identification system
ANOVA	Analysis of variance
apLCMS	Adaptive processing of high-resolution LC-MS data
ARALIP	Arabidopsis acyl-lipid metabolism
ASCII	American standard code for information interchange
BKM-react	BRENDA-KEGG-MetaCyc-reactions

BNICE	Biochemical network integrated computational explorer
CAMERA	Collection of algorithms for metabolite profile annotation
CDF	Common data format
CFM-ID	Competitive fragmentation modeling for metabolite identification
ChEBI	Chemical entities of biological interest
CID	Collision-induced dissociation
Cosmiq	Combining single masses into quantities
COVAIN	Covariance inverse
CRAN	Comprehensive r archive network
CSI:FingerID	Compound structure identification: FingerID
CTS	Chemical translation service
DIA	Data independent acquisition
DoE	Design of experiments
EIC	Extracted ion chromatograms
EssOilDB	Essential oil database
EXIMS	Exploring imaging mass spectrometry data
FT	Fourier transform
FTP	File transfer protocol
FunRich	Functional enrichment analysis tool
GC	Gas chromatography
GMD	Golm metabolome database
GNPS	Global natural products social molecular networking
GUI	Graphical user interface
HCS	Hierarchical cluster analysis
HMDB	Human metabolome database
HRMS	High resolution mass spectrometry
ICT	Isotope correction toolbox
IIS	Integrated interactome system
iMet-Q	Intelligent metabolomic quantitation
IMPaLA	Integrated molecular pathway level analysis
InCroMAP	Integrated analysis of cross-platform microarray and pathway data
IOKR	Input output kernel regression
iPATH	Interactive pathways explorer
IPO	Isotopologue parameter optimization
ISDB	In-silico MS/MS database
IT	Ion trap
KaPPA - view	Kazusa plant pathway viewer
KEGG	Kyoto encyclopedia of genes and genomes
KMMDA	Kernel machine approach for differential expression analysis of mass spectrometry-based metabolomics data
KomicMarket	Kazusa omics data market
kpath	Khaos metabolic pathways
LC	Liquid chromatography
LDA	Latent Dirichlet allocation
LDA	Lipid data analyzer
LIMS	Laboratory information management system

LIMSA	Lipid mass spectrum analysis
LOBSTAHS	Lipid and oxylipin biomarker screening through adduct hierarchy sequences
m/z	Mass-to-charge ratio
MaConDa	Mass spectrometry contaminant database
MAGMa	Ms annotation based on in silico generated metabolites
MAIT	Metabolite automatic identification toolkit
MarVis-Suite	Marker visualization suite
MathDAMP	Mathematica package for differential analysis of metabolite profiles
MAVEN	Metabolomic analysis and visualization engine
MBRole	Metabolites biological role
MeKO	Metabolite profiling database for knock-out mutants in arabidopsis
MetCCS	Metabolite collision cross-section predictor
MET-COFEA	Metabolite compound feature extraction and annotation
MET-COFEI	Metabolite compound feature extraction and identification
MET-IDEA	Metabolomics ion-based data extraction algorithm
METLIN	Metabolite link
MetNetDB	Metabolic network exchange database
MFSearcher	Molecular formula searcher
MIA	Mass isotopolome analyzer
MID	Mass isotopomer distributions
MINE	Metabolic in silico network expansion databases
MI-Pack	Metabolite identification package
MMCD	Madison metabolomics consortium database
MMSAT	Metabolite mass spectrometry analysis tool
Mona	Massbank of north america
Moto DB	Metabolome tomato database
MPA-RF	Model population analysis - random forests
MPEA	Metabolite pathway enrichment analysis
MPMR	Medicinal plant metabolomic resources
MRM	Multiple reaction monitoring
MRM-DIFF	Multiple reaction monitoring based differential analysis
MRMPROBS	Multiple reaction monitoring based probabilistic system
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MS2T	MS/MS spectral tag
MS-DIAL	Mass spectrometry – data independent analysis
MSFACTs	Metabolomics spectral formatting, alignment and conversion tools
MUSCLE	Multi-platform unbiased optimization of spectrometry via closed-loop experimentation
NaDH	Nicotiana attenuata data hub
NIST	National institute of standards and technology
OpenMSI	Open mass spectrometry imaging
PAPi	Pathway activity profiling
PCA	Principal component analysis
PlantMAT	Plant metabolite annotation toolbox
PLS-DA	Partial least squares discriminant analysis

PMR	Plant/eukaryotic and microbial systems resource
PRIMe	Platform for RIKEN metabolomics
PTW	Parametric time warping
RAMSY	Ratio analysis of mass spectrometry
ReSpect	RIKEN MSn spectral database for phytochemicals
RSS	Rich site summary
RT	Retention time
SDBS	Spectral database for organic compounds
SDF	Structure-data files
SIM	Single ion monitoring
SIRIUS	Sum formula identification by ranking isotope patterns using mass spectrometry
SMART	Statistical metabolomics analysis - an r tool
SOM	Self-organizing maps
SoyMetDB	Soybean metabolome database
SPICA	Selective paired ion contrast
SPLASH	Spectral hash
T3DB	Toxin and toxin target database
TIC	Total ion current
TOF	Time-of-flight
UNPD	Universal natural product database
VaLID	Visualization and phospholipid identification
VANTED	Visualization and analysis of networks containing experimental data
vocBinBase	Volatile compound binbase
yamss	Yet another mass spectrometry software

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LPS and TN reviewed the literature and prepared supplementary table and figure. LPS, TT and ARF wrote the manuscript. All the authors contributed revising the manuscript.

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Figure 1 Typical mass spectrometry based metabolomics workflow.

Additional file 1.xls Summary of resources for mass spectrometry based metabolomics.

Figure 1

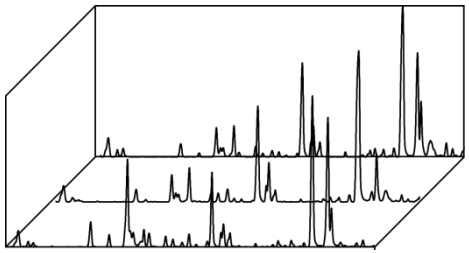
METABOLOMICS WORKFLOW

Sample Preparation

Data Acquisition

Processing

- Feature detection
- Alignment
- Quantification
- Spectra deconvolution
- Normalization

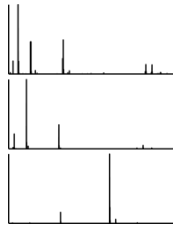


Features

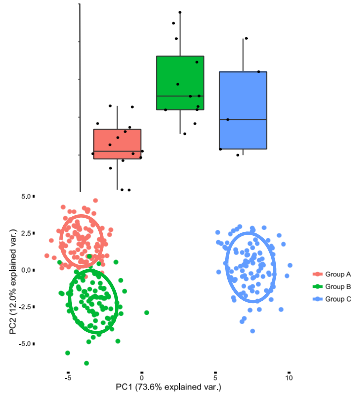
Samples

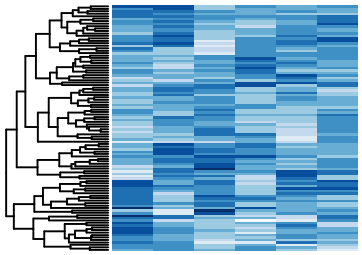
Intensities

Compound Spectra



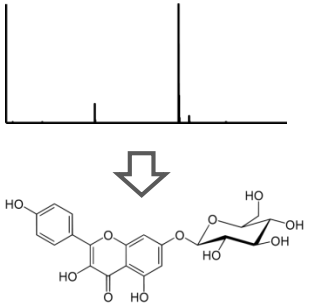
Statistical Analysis





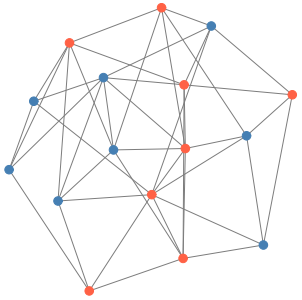
Annotation

- Exact mass
- MS^n
- Spectra matching
- *In silico* prediction



Interpretation

- Network structure
- Pathway enrichment
- Integration



Databases

- Compounds
- Mass spectra
- Samples
- Pathway