Combined Nuclear Magnetic Resonance Spectroscopy and Mass Spectrometry Approaches for Metabolomics

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Metabolomics is based on cutting-edge analytical methods that provide a "snapshot" of all the detectable metabolites, small molecules generally weighing under 1200 Da, present in complex biological samples. It presents a wide scope of applications and has allowed considerable progress to be made in health and disease research,[1,2] pharmaceutical sciences,[3–5] personalized medicine,[6–10] microbiome research,[11,12] but also in food and nutrition,[13–16] agriculture,[17,18] marine environmental research[19,20] or exposome research.[21–23] Metabolomics can be undertaken through two different approaches. Untargeted methods aim at capturing a broad view of all the metabolites present in a biological sample, without any *a priori*, up to the limit of the accessible metabolites which depends on the detection limit of the applied analytical method, the physicochemical properties of the analytes, as well as the sample handling or preparation applied. An untargeted assay often aims to identify one or several new biomarkers of a particular phenotype, which can be for instance markers of interest of a specific disease, or markers of effect following an exposition to a physical or a chemical stress or a therapeutic treatment, and to elucidate their structures. It can also help to build a model capable to predict a specific condition, such as in foodomics. Once untargeted analyses have been performed and that effect biomarkers have been discovered, or if there are known exposure biomarkers of interest (e.g., xenobiotic such a specific drug or a chemical contaminant and their metabolites), a targeted approach will make it possible to perform a quantitative analysis of those compounds. In the case of biomarker discovery, quantitative insights through targeted analysis are often needed to validate that a metabolite is indeed a real biomarker. Halfway between untargeted and targeted approaches stands a slightly different strategy, where compounds from the same class of metabolites or a particular biochemical pathway (e.g*.,* bile acids or amino acid metabolism) need to be broadly captured and if possible in a quantitative way but when the rest of the metabolome is not relevant to the research problem.

Currently, the two main analytical techniques used to apply those approaches are nuclear magnetic resonance (NMR) spectroscopy, based most of the time on the detection of ^{1}H or ^{13}C nuclei, and mass spectrometry (MS), often coupled with separation techniques such as liquid or gas chromatography (LC or GC), capillary electrophoresis (CE), or ion mobility (IM). NMR spectroscopy is a noninvasive technique, as the sample can be recovered and used in a following experiment (providing that the sample preparation needed for NMR analysis such as D_2O addition, does not interfere with the following experiment). In contrast, MS analysis, due to the nature of the technique, is destructive but, as only relatively small volumes are required (as low as few microliters of an often very diluted sample), this does not necessarily cause problems. NMR spectroscopy also presents the advantage of providing accurate quantitative results, making possible the quantitation of multiple analytes with a single internal or external reference.[24] Furthermore, ^{1}H NMR spectroscopy has the strong advantage

of being a robust and reproducible technique, both through time and between laboratories.[25] However, the main drawback of NMR spectroscopy is its relative lack of sensitivity, together with ubiquitous signal overlap in the ^{1}H NMR spectrum of biological samples, which limits the identification of metabolites and the discovery of significant biomolecular changes and biomarkers. Recent progress has been made to overcome such limitations. The overlap issue has been solved by including the acquisition of 2D NMR data sets in metabolomics workflows to reduce signal overlap while providing crucial information to elucidate the structure of metabolites.[26,27] While 2D NMR suffers from long acquisition times, numerous methodological developments made it possible to reduce acquisition times to a few minutes for biological samples, and the quantitative issues associated with 2D NMR have also been addressed through pulse sequence developments or calibration strategies.[28–30] More recently, proof-of-concept developments have been addressing the sensitivity issues of NMR metabolomics, notably through hyperpolarization strategies such as dissolution dynamic nuclear polarization (d-DNP)[31–33] or para-hydrogen induced polarization (PHIP).[34,35] These techniques offer an unprecedented boost in NMR sensitivity by several orders of magnitude; however, they are very recent and not yet implemented in routine for metabolomics.

MS-based techniques are the most widely implemented strategies for metabolomics purposes (Figure 1), especially UPLC-MS with electrospray ionization (ESI), thanks to the greater sensitivity that this technique offers. This fact has been reinforced in recent years thanks to the development of high-resolution (HR)-MS techniques and the possibility to determine the accurate mass of a compound. However, these techniques are somewhat less robust than NMR ones, and although targeted assays are generally comparable between laboratories,[36,37] untargeted methods require careful quality control (QC, biological pool sample) procedures to assess robustness and repeatability over time.[38–41] Also, the important sensitivity that MS and especially HRMS techniques offer comes with its drawback, such as ion suppression. However, using multiple ionization modes (positive and negative) for ESI and several chromatographic systems (reversedpPhase [RP] and hydrophilic interaction chromatography [HILIC]), as well as any other strategy enabling signals deconvolution such as IM,[42] are clear advantages to increase metabolite coverage.

Metabolomics greatly benefits from the tremendous progress made in both MS and NMR in the past couple of decades, in terms of sensitivity, resolution, and rapidity, but also from advances in statistical analysis and bioinformatics methods. However, most of these recent advances are costly, time-consuming, and require advanced technical skills, which makes them not easily accessible. Furthermore, none of the analytical methods existing today allow a full capture of the metabolome. This is due to multiple factors, such as sensitivity limitations, loss of metabolites during sample collection, handling, preparation, and analysis (e.g., nonretention/nonelution, ionization efficiency,

signal overlapping…). In consequence, the use of both routine NMR spectroscopy and MS-based techniques through an integrated platform is a sensible and powerful option to maximize metabolome coverage, facilitate metabolite identification and biomarkers discovery, and build more robust models through the use of multiple data set integrations.[43,44]

The combined use of NMR and MS has been long exploited for the structural characterization of new metabolites of interest, especially in the natural products field.[45] Analysis of 1D and 2D NMR spectra to extract chemical shift and coupling information, as well as direct infusion MS to obtain an exact *m*/*z* ratio and fragmentation patterns, help to identify and structurally define new metabolites following several steps of purification. In metabolomics, many published studies relied on only one of the two techniques, mainly for opportunistic reasons, i.e., researchers focusing on metabolomics studies were using the closest available technique in their laboratory or institute. Figure 1 shows that the proportion of MS versus NMR in metabolomics has been increasing over time, mainly for cost and sensitivity reasons. However, Figure 1 also sheds light on a still modest but substantial increase of the number of studies that make use of both techniques, suggesting that the combination of MS and NMR for metabolomics could be highly valuable. This review focuses on the description of such powerful combination, which can be done through various ways. NMR and MS can be combined at the hardware level through physical association of the two techniques. However, in most cases, it relies on integrating the respective data sets in a common chemometric software and multivariate statistical analysis pipeline. Such integration can be performed at different levels, through cross-comparison, correlation, or multiblock integration. In the review herein, we describe the principles of such combination, highlighting how it has provided a considerable paradigm shift in metabolomics in the past few years. The benefits of gathering these techniques in postmetabolomics workflow through more targeted approaches to improve metabolite identification, quantitative assays, and fluxomic analysis are also exposed, before discussing the perspectives of integrating several metabolomics and omics methods in general. Individual methodological advances made in NMR or MS metabolomics are excluded from the scope of this review, but they have been thoroughly reviewed recently[46,47] and will certainly benefit to combined MS and NMR approaches.

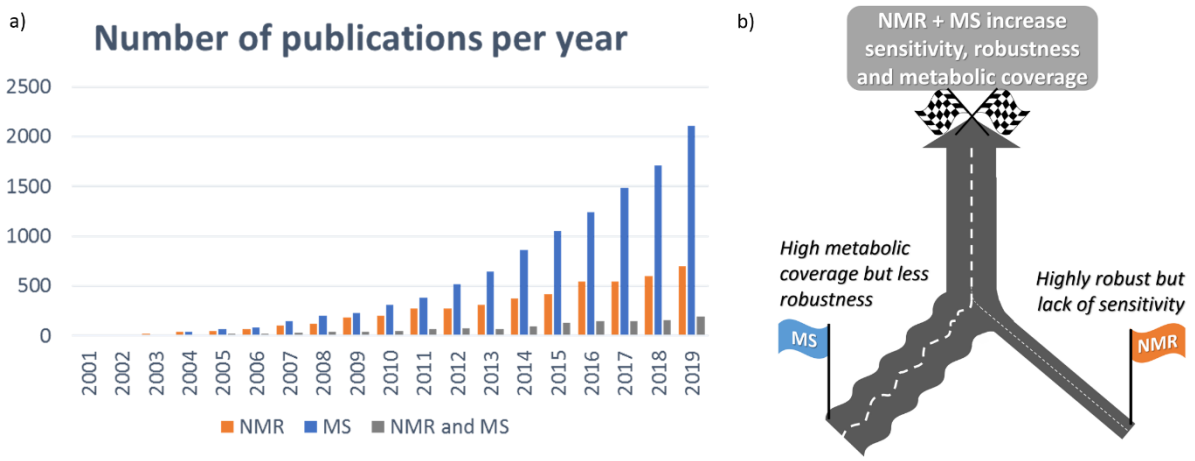

Figure 1. (a) Results obtained by searching through *pubmed.ncbi.nlm.nih.gov* in [Title/Abstract], the following terms: "(nuclear magnetic resonance OR NMR) AND (metabolomics OR metabonomics OR metabolic profiling)", shown in orange; "(mass spectrometry OR MS) AND (metabolomics OR metabonomics OR metabolic profiling)", shown in blue; "(nuclear magnetic resonance OR NMR) AND (mass spectrometry OR MS) AND (metabolomics OR metabonomics OR metabolic profiling)", shown in gray. Research was done on September 18, 2020. (b) Schematic representation of the advantages and the drawbacks of NMR and MS-based analytical methods and the benefits of combining them.

NMR and MS Hardware Combination

As stated above, the combination of several NMR (e.g., 1D/2D, $^{1}H/^{13}C$) or MS platforms (e.g., LC/GC, ESI+/-, RP/HILIC) increases the metabolic coverage, and the combined use of both NMR and MS platforms can also help toward that same objective. Such a combination was initially applied in the natural products research field, in order to help with structural elucidation, through an off-line platform based on the comparison of NMR chemical shifts and coupling constants as well as HRMS to obtain exact *m/z* and fragmentation patterns. This hyphenation led to the development of online platforms including both NMR and MS hardware, often preceded by an LC system. This type of system found applications in drug metabolism research or drug discovery from natural products, especially to help with dereplication, consisting of identifying known natural compounds from active fractions to avoid spending time on compounds which had already previously been discovered.[48] The hyphenated use of LC-MS-NMR was achieved thanks to postcolumn splitters, which send 10% of the outgoing flow from the LC column to the MS system and the remaining 90% to the NMR system.[44] A commercial NMR-MS interface was also developed, composed of a splitter controlled by the operator and a double dilutor.[44] The latter allows, on the one hand, the prevention of an extensive use of deuterated solvent as the sample is mixed in D_2O just before entering the NMR system rather than before entering the LC system and, on the other hand, to dilute the sample in the appropriate solvent for ionization and MS detection.[44]

Three different ways exist to set up such a combined platform, namely, through a continuous-flow mode, a stop-flow mode or a storage mode (Figure 2).[49] By using the dynamic continuous-flow mode, the sample already mixed with deuterated solvent is sent to the LC system before flowing separately and continuously in the MS and the NMR systems. One of the first examples of this continuous-flow mode was made by Shockcor and co-workers in 1996 to analyze a urine sample from an individual administrated with paracetamol, in order to identify with more confidence paracetamol metabolites and urinary endogenous compounds.[50] Phenylacetylglutamine, not previously detected by ^{1}H NMR spectroscopy alone due to spectral overlapping but usually detected by HPLC-MS and confirmed by the use of a standard, was well identified thanks to the good resolution obtained by this HPLC-NMR-MS system.[50] The NMR data set made it possible to clearly define which paracetamol-glucuronide isomer was observed, and this would not have been possible by the unique use of HPLC-MS or HPLC-MS/MS alone without comparison to an internal standard. However, this technique is limited by the time-evolving LC gradient composition which induces a bias in the NMR measurement. Indeed, this leads to an evolution of the position of the solvent peaks which makes it difficult to maintain an efficient solvent signal suppression over time.[49] Most importantly, the short residence time of nuclear spins in the NMR detection cell strongly limits signal averaging, which in turns impacts the resulting NMR signal-to-noise ratio (SNR). This issue can be addressed by using a static analysis such as the stop-flow mode, during which a valve pauses the LC flow when a peak is detected or selected and that the corresponding analyte has reached the NMR detection cell. This approach provides enough time for the NMR measurement to be performed with a satisfactory SNR. However, stop-flow broadens the LC peaks thus limiting the chromatographic resolution. As a consequence, a storage mode has often been preferred, either performed through an online or off-line setup. For this mode, the different fractions coming out from the LC system can be collected and stored in a loop while the NMR analysis is running. The sample collection can also be done in a cartridge, most of the time a solid-phase extraction (SPE) cartridge, which receives 95% of the LC eluent (the other 5% being sent toward the MS system) and which efficiently retains and concentrates analytes before NMR characterization, preventing an extended use of deuterated solvent.[51]

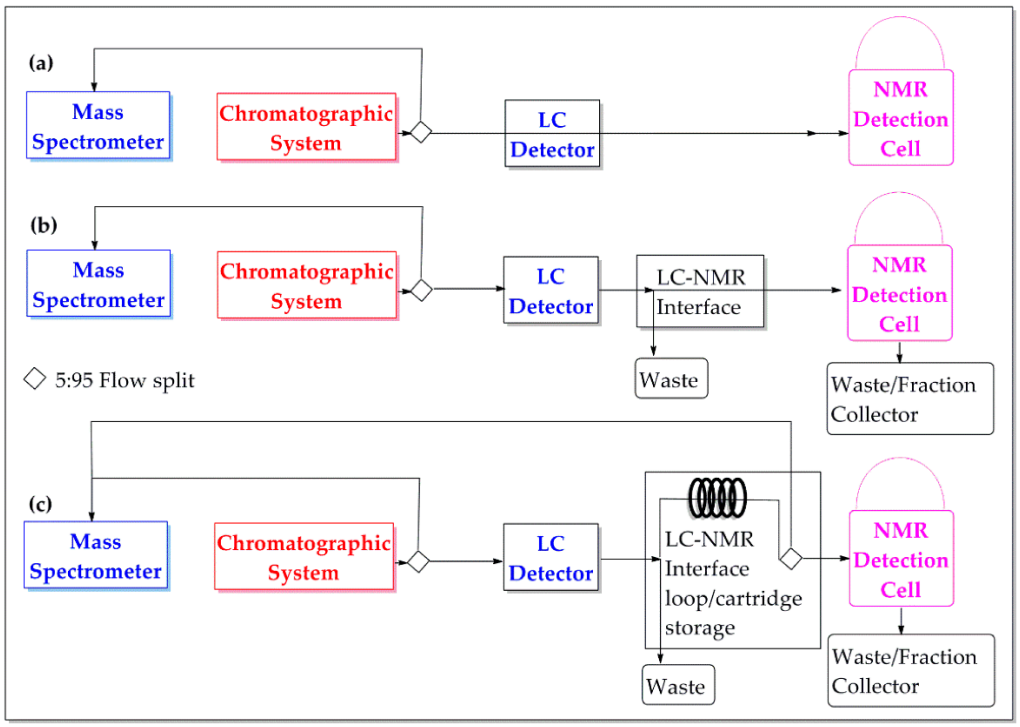

Figure 2. Schematic setups of the different LC–NMR working modes with parallel mass spectrometer (MS) detection: (**a**) online/continuous-flow mode, (**b**) stop-flow mod,e and (**c**) loop/cartridge storage mode. Figure reproduced from ref. [49] under Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

LC-MS-SPE-NMR has been elegantly applied to help with the structural elucidation of urinary phenolic compounds in humans following tea consumption, once classical off-line LC-Orbitrap Fourier transform MS (LC-FTMS) and 1D ^{1}H NMR analysis were performed separately to select features of interest for further characterization.[52] This approach efficiently provides comprehensive structural confirmation of the fragmentation patterns of the selected feature, while simultaneously providing quantitative data based on the ^{1}H NMR spectroscopy part of the system. This hyphenated system has found successful applications in natural product research, especially when it comes to structural elucidation or to differentiate isomeric or isobaric compounds.[49] However, several drawbacks still limit its widespread use, such as the bulky and expensive equipment, associated with a limited sensitivity. The latter drawback has pushed toward the development of integrated microprobe technologies and capillary separation.[49,53] Lin and co-workers reported an LC-MS-NMR platform with microscale instruments, namely, a nanoSplitter LC-MS and a microdroplet NMR, for increased sensitivity.[54] The use of a highly sensitive instrument such as a nanoelectrospray MS, requested only 2% of the LC column eluent, driving away the rest to a UV-guided collection for concentration prior to be stored waiting for an off-line NMR analysis. This setup limited the use of deuterated solvent as it was added just before the NMR analysis, which can thus be done retrospectively once the LC-MS analysis have been performed. However, it reintroduced an additional step of sample handling prior to NMR

analysis. And although the sensitivity was further optimized in this application by using a microcoil NMR probe,[54] the robustness of such equipment is questionable,[53] which is a major bottleneck to create high-quality databases aiming to accelerate structural characterization of low concentration analytes in complex matrices.[55]

Beyond its ability to help with structural elucidation in natural product research and the efforts made to minimize sample handling and preparation while increasing its sensitivity, the hyphenated LC-MS-NMR system did not receive the success expected by some in the past decade. The known limitations of such a platform, namely, the incompatibility of the solvents for MS and NMR or the extended use of expensive deuterated solvent, the low flow rate for efficient ionization and MS detection, and the long acquisition time requested for a sufficient NMR sensitivity,[56] were not overcome. As such, it seems unlikely that such a system will be further encouraged by the metabolomics community, especially when promising computed-based combination solutions are being developed.

NMR and MS Data Set Combination for Metabolomics Analysis

Cross-Comparison of NMR and MS Data Sets to Increase Metabolic Coverage

Although good convergence was found between different techniques in interlaboratory studies,[57] individual analytical techniques do not necessarily cover the same types of metabolites. The parallel use of NMR and MS methods can highly improve the quality of metabolomics studies in a variety of ways. The most obvious case where the combination of the two techniques can be beneficial is the increase of metabolic coverage.[58] This was illustrated, for instance, in a study aiming to investigate the biomolecular processes behind the mycotoxins production of cereals infected by the plant pathogen *Fusaium graminearum*.[59] In this work, NMR spectroscopy and LC-QTOF-MS (based on a reversed-phase stationary phase) analyses were performed to measure polar and semipolar compounds, respectively. In total, 15 amino acids or derivatives, 3 sugars and polyols, 4 tricarboxylic acid (TCA) organic acid derivatives and 4 nucleosides and nucleotides were identified or putatively annotated by NMR or 2D NMR, while 55 sesquiterpenes and 10 polyketides were highlighted by MS or MS/MS.[59] None of the metabolites identified or annotated by one technique were claimed to be identified by the other. More studies with key numbers highlight the advantage of combining NMR and MS method to increase the metabolic coverage. Goulitquer et al. combined one ^{1}H NMR, one GC-MS, and seven LC-MS data sets to explore the changes induced in the metabolome and the lipidome of human gastric cancer cells following treatment with anticancer drugs.[60] The LC-MS data sets were acquired on three different instruments (UHPLC-LTQ-Orbitrap, UHPLC-Exactive, UPLC-HRMSe Q-TOF) and provided analyses in both positive and negative modes. Out of the 111 metabolites and lipids annotated, only 9 were

common to the LC-MS and the GC-MS data sets, 4 between the LC-MS and the NMR, 2 between the GC-MS and the NMR, and 6 were concordant between the three platforms. A recent study successfully attempted to capture the broadest picture of the human serum metabolome.[61] To do so, five analytical platforms were used, namely, ^{1}H NMR, GC-MS, LC-ESI-MS/MS, TLC/GC-FID-MS and DI-MS. Over 3500 distinct metabolites were identified, and from those, only 29 were commonly identified by NMR and GC-MS, 13 by NMR and DI-MS, 14 between GC-MS and DFI-MS, 8 between the three analytical methods just cited, and 53 between DFI-MS and TLC/GC-FID-MS.[61] It should be noted that the spectacular effort made through this study was complemented with an extensive literature research, called "bibliomic", which found 665 other serum metabolites already reported in the literature but not detected by the five analytical platforms applied. Quantitative data were also reported for a portion of the over 4000 metabolites, showing acceptable agreement between the concentrations obtained from the different analytical methods but still with some exceptions.[61] In a similar way, the combined use of NMR, FIA-MS/MS, GC-MS, and LC-HRMS was applied to explore the skeletal muscle metabolome,[62] in order to assess their performance as well as different sample extraction protocols. Here again, only 2 metabolites were commonly detected by the four analytical methods, 2 metabolites common to LC-HRMS, GC-MS, and NMR, 3 common ones between FIA-MS, GC-MS, and NMR, and 4 common ones between LC-HRMS, FIA-MS, and NMR (Figure 3).[62] The GC-MS covered 7 unique metabolites, against 13 ones for the NMR, 26 for the FIA-MS and 58 for the LC-HRMS. Although these numbers suggest that GC-MS was the less efficient tool to study such samples,[62] some of the 7 metabolites detected by GC-MS could be of crucial importance to understand key metabolic pathway alterations or biomarker discovery.

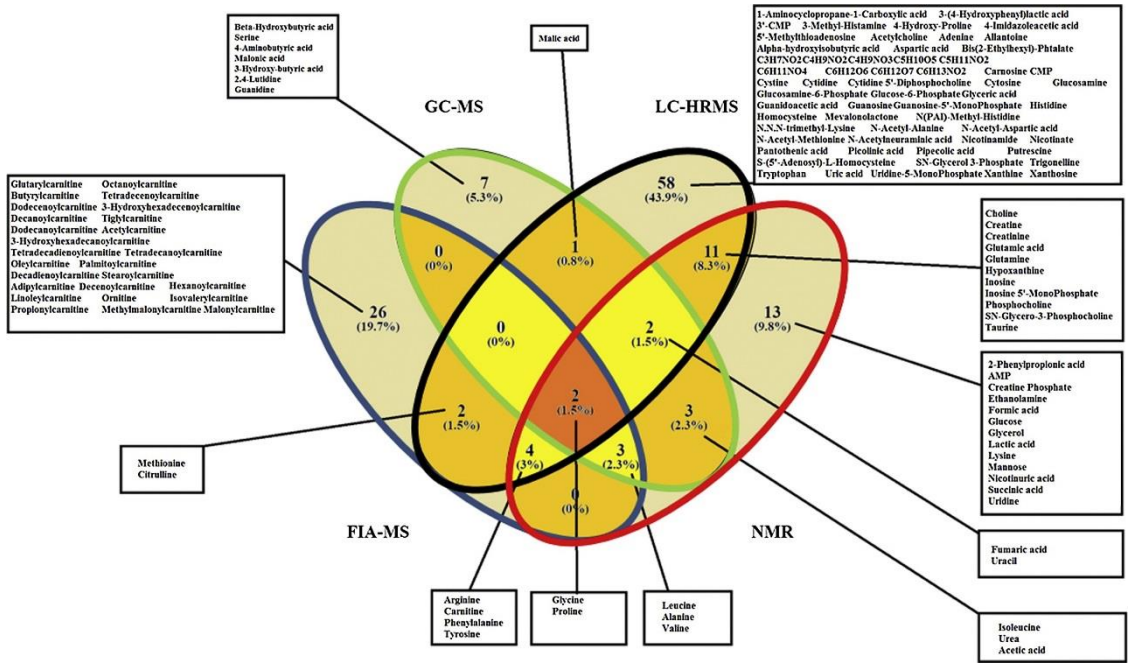

Figure 3. Venn diagram representing specificity and overlap of metabolites reliably detected by each analytical method. Red, blue, green, and black circles represent metabolites analyzed respectively, by NMR, FIA-MS, GC-MS, and LC-HRMS. Crossed zone represents overlaps between methods. Figure reprinted from J. Pharm. Biomed. Anal., Vol. 148, Bruno, C.; Patin, F.; Bocca, C.; Nadal-Desbarats, L.; Bonnier, F.; Reynier, P.; Emond, P.; Vourc'h, P.; Joseph-Delafont, K.; Corcia, P.; Andres, C. R.; Blasco, H. The Combination of Four Analytical Methods to Explore Skeletal Muscle Metabolomics: Better Coverage of Metabolic Pathways or a Marketing Argument?, p 273-279 (ref [62]). Copyright 2018, with permission from Elsevier.

Several examples have shown how increasing the metabolic coverage with a second metabolomics approach can help gaining a deeper understanding of a biological process. For instance, Allwood et al. used this strategy to understand fragrance and maturity development in five melon cultivars,[63] mainly depending on volatile organic compounds (VOCs). As it is often the case to measure VOCs, GC-MS was used, with thermal desorption. Optimization of the sampling method based on the use of a polydimethylsiloxane membrane allowed the detection of 58 VOCs.[63] Principal component analysis (PCA), heatmap, and relative changes highlighted clear differences between five melon cultivars, which were further supplemented with quantitative measures of amino-acids, known precursors of certain VOCs, by ^{1}H NMR spectroscopy. Reverse correlations between amino acids concentrations and VOCs levels were observed, clearly showing that consumption of amino acids to produce VOCs directly influence melon fragrance and maturity.

Still, contradictory results are sometimes encountered when both techniques are applied to the same matrix. For instance, different concentrations can be measured in the NMR and the MS data set, or a compound can be detected by one technique and not by the other, which is actually the reason why using both in combination increases the metabolic coverage. A typical example of this problematic has been reported by Atherton et al. in 2006,[64] where metabolic profiling of several tissues from control or peroxisome proliferator-activated receptor-alpha (PPAR-alpha) null mice were explored by using ^{1}H NMR spectroscopy, high-resolution magic angle spinning (HR-MAS) ^{1}H NMR spectroscopy, GC-MS, and LC-MS. In this study, significant changes were observed in the cardiac metabolic profile of the control vs muted mice by ^{1}H NMR, HR-MAS ^{1}H NMR, and GC-MS, but the order of magnitude of these changes were different from one method to the other. Indeed, as ^{1}H NMR spectroscopy has limited sensitivity, only the most concentrated compounds appeared significantly different in the PCA model, while the number of metabolites significantly impacting the GC-MS partial least-square discriminant analysis (PLS-DA) model were 5 times more numerous.[64] This was explained by the fact that metabolites with hydroxyl and amine functional groups were easily detected by GC-MS, even though they might not be the most concentrated metabolites. This constitutes a perfect representation of how difficult it is to catch, within a complex biological sample, metabolites present in a broad variety of concentration, polarity, and mass range.[64] On a side note, in order to increase the number of metabolites detected by both NMR and MS techniques, an elegant approach is to use a smart isotope

tag, such as ^{15}N-cholamine, which present the advantage of being a sensitive isotope for NMR and of having a permanent charge for MS efficient detection.[65] Chemical derivatization with this smart tag allows the simultaneous detection of carboxylic acid derivatives without ambiguity. However, this approach is restricted to metabolites containing a carboxyl group and thus covers a limited part of the metabolome.

Correlation of NMR and MS Data Sets

Rather than taking into consideration the different variables within a sample, correlation analysis is based on taking into consideration the intensities of the same variable across different samples.[66] The first correlation tools that appeared in metabolomics focused on the 2D correlation of vibrational spectroscopic data, such as IR or Raman.[67] Numerous statistical tools for spectroscopic correlation arose from it but mainly for the interpretation of NMR data sets.[68] One of the most common tools is based on statistical total correlation spectroscopy (STOCSY), which correlates signals showing similar variations across samples within 1D ^{1}H NMR spectra, in order to better extract individual metabolite spectral patterns and facilitate the identification of biomarkers.[69] It was further adapted to several other statistical tools,[68] such as Het-STOCSY, to correlate heteronuclear NMR signals, STOCSY-editing, which aimed to correlate only the endogenous compounds without taking into consideration the exogenous ones, or also statistical heterospectroscopy (SHY), which aimed to correlate signals from ^{1}H NMR with LC-MS ones. Through this tool, the intrinsic covariance of the NMR chemical shifts and the *m*/*z* signal intensities of the same features is analyzed to help biomarker discovery and achieve a deeper understanding of the biological alterations due to a specific drug treatment or disease. The efficiency of the SHY method was illustrated through a proof-of-concept study measuring the effect of hydrazine treatment in rat urine samples.[70] Prior to the correlation, the data sets need to be formatted. As such, a cubic spline was used to smooth the NMR spectra, and MS spectra were binned to produce 2D histograms which were further summed by a specific retention time window to create pseudo direct infusion spectra and prevent the loss of the LC-MS signals eluting closely to the chromatographic dead volume.[70] Subsequent normalization was required to take into consideration the different dilution factors of the urinary metabolites. Correlation coefficients were calculated by using a Pearson correlation and visualized according to a specified cutoff.[70] This powerful tool was then applied to human urine samples within an epidemiological study where the studied population was not controlled or selected.[71] Even so, the use of therapeutic treatment was easily detected in the samples, and the additional use of MS^{E} (the combined used of low and high collision energy to simultaneously detect the precursor and the fragments *m*/*z*) data allowed the annotation of unreported drug metabolites (Figure 4). Together with the detection of more common endogenous metabolites, it

showed that SHY is a useful statistical tool to explore the xenometabolome and its effect on metabolic phenotypes.[71]

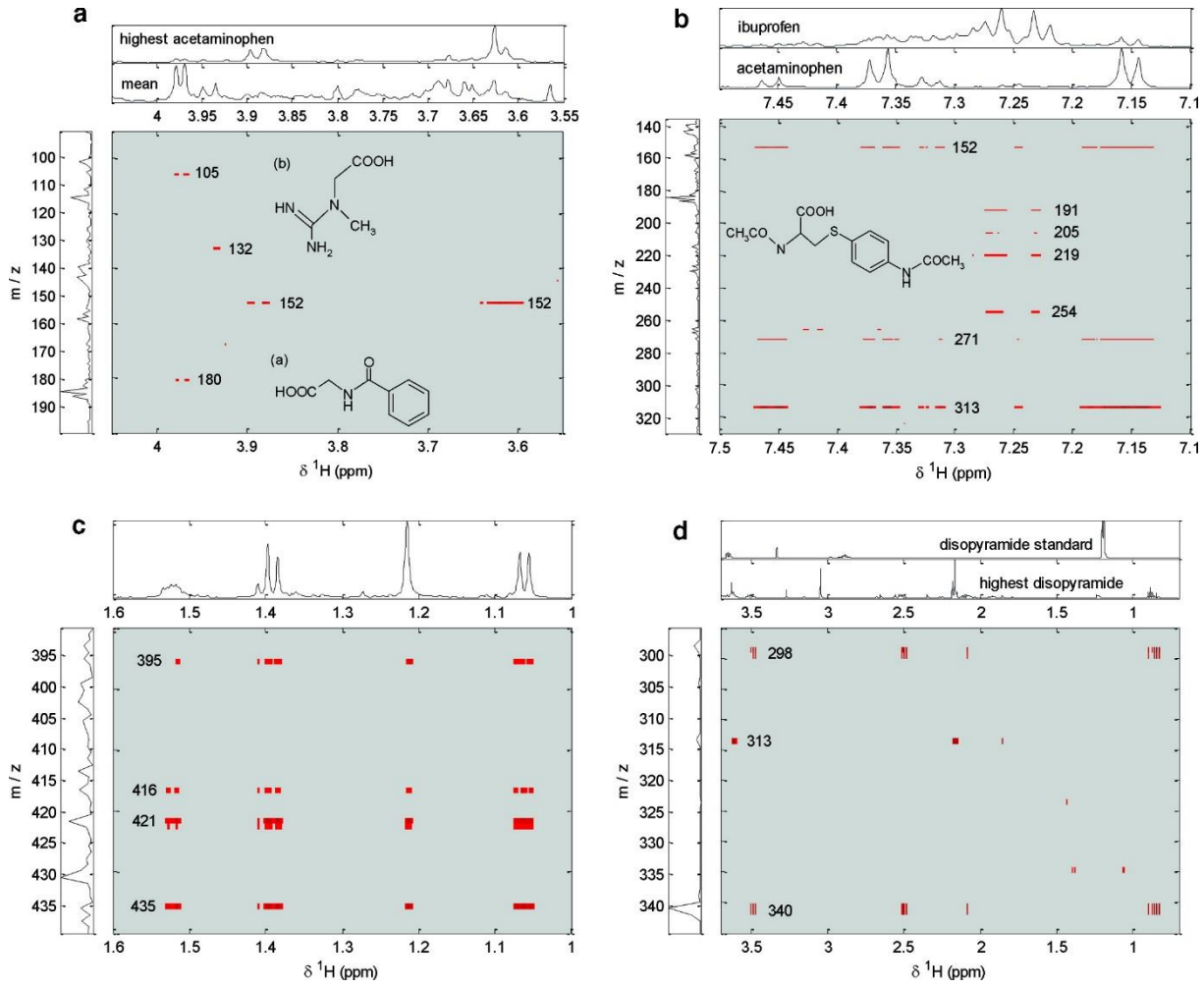

Figure 4. SHY plots: (a) Correlation (cutoff 0.7) of a hippurate NMR signal (doublet at 3.97 ppm) with the hippurate parent ion (m/z 180, neutral molecule shown as inset a) and a fragment due to cleavage of glycine in-source (m/z 105). Also shown are correlations due to creatine (singlet at 3.93 ppm, m/z 132, neutral molecule shown as inset b) and acetaminophen-related signals (doublet at 3.89 ppm, multiplet at 3.62 ppm, m/z 152). The inset NMR spectra are the mean spectrum and the spectrum for the sample with the highest concentration of acetaminophen. The inset MS spectrum is the mean. (b) Correlation (cutoff 0.7) between aromatic NMR signals for acetaminophen and ibuprofen metabolites and various nominal m/z values. Those for acetaminophen can be immediately identified as belonging to the unmodified drug (m/z 152), its cysteinyl conjugate (m/z 271), and its acetylcysteinyl conjugate (m/z 313, neutral molecule shown as inset). Those for ibuprofen require investigation of the MSE spectra. The inset NMR spectra are for those samples having the highest respective NMR intensities, and the inset mass spectrum is the sum of the corresponding mass spectra. Some trace of previous acetaminophen usage is present in the NMR spectrum illustrating ibuprofen. (c) Correlation (cutoff 0.8) between some aliphatic NMR signals for ibuprofen metabolites and m/z values in a higher range than given in Figure 1b. The inset spectrum is for the sample having the highest ibuprofen intensity. Correlations due to the first 13C isotope can be distinguished for the strong signal at m/z 421. (d) Correlation (cutoff 0.8) of disopyramide-related NMR signals with m/z values for disopyramide (m/z 340) and its known metabolite N-dealkyldisopyramide (m/z 298). The first 13C isotope correlations are visible. The inset NMR spectra are for a disopyramide standard (top) and for the urine sample giving the highest relevant signal intensities (bottom). The inset mass spectrum is for the same urine sample. It is clear that the urine NMR signals do not match the NMR signals for the disopyramide

standard (although in UPLC-MS an exact match to the standard was obtained). The NMR signals must therefore be due to an unidentified disopyramide metabolite, a covarying endogenous metabolite, or an additional (unknown) drug that was being taken by the single subject who was taking disopyramide. Figure reproduced from Crockford, D. J.; Maher, A. D.; Ahmadi, K. R.; Barrett, A.; Plumb, R. S.; Wilson, I. D.; Nicholson, J. K. Anal. Chem. 2008, 80 (18), 6835–6844 (ref [71]). Copyright 2008 American Chemical Society.

SHY also found applications in other fields than toxicology, as illustrated by Marti et al., who used this statistical tool to assess the authenticity and geographical metabolic differences of cold-pressed lemon oil.[72] In this study, the NMR/MS correlation complemented by the use of geranial and neral isomer standards, helped to determine the presence of both isomers but showed that the geranial one was present in the samples at a higher concentration.[72] SHY was also used in the natural product field to identify bioactive compounds while avoiding a time-consuming isolation process.[73] To do so, microfractionation of samples prior to LC-MS (positive and negative ionization mode) and NMR analyses were performed, as well as bioactivity assays. The selection of active NMR compounds, often made difficult by the important overlap with inactive compounds, was facilitated by the correlation with LC-MS signals, making SHY a powerful tool for the deconvolution of natural products.[73] Correlation analyses between NMR and MS data sets can also be used to confirm the annotations of the discriminative features previously identified by PCA applied to one of the methods, as it was done to study biological processes underlying the urine samples of patients with inborn errors of metabolism.[74] In this particular example, unsupervised PCA analysis was first performed on a NMR data set and the features that significantly impacted the distribution of the samples were annotated. Second, NMR spectra and MS spectra, from a DESI-MS data set, were bucketed into the same number of bins (594) to obtain a square matrix that was subjected to a Pearson correlation.[74] With this approach, a common list of discriminant features can be annotated with more confidence when a MS feature is positively correlated to an NMR annotation. Furthermore, it can also help to gain a better understanding of the biochemical reactions lying behind a specific condition, as a negative correlation can be explained by the consumption of the precursor compound and the production of another compound.[74] However, since the metabolic coverage of two analytical methods is different, it makes sense to assume that a list of common features between the NMR and the DESI-MS data sets will capture only a restricted portion of the urinary metabolome and that important biomarkers could be missed. Still, statistical analysis based on correlation coefficients can be easily implemented while allowing a straightforward interpretation of the results.

Although SHY seems to be the most widely correlation tool used in metabolomics to correlate NMR and MS data sets, another statistical correlation tool based on networks was also reported. In a first example, correlation networks were used to visualize the relationships between melon fruit analytes that were identified and for some quantified by various analytical techniques (namely ^{1}H NMR

spectroscopy and GC-MS analyses of polar compounds, HPLC analysis of lipophilic isoprenoids, untargeted LC-MS analysis of semipolar compounds, untargeted GC-MS analysis of volatile compounds and elemental profiling for mineral elements).[75] This extensive metabolic profiling allowed the identification or annotation of about 1932 features and 15 mineral elements. Within these features, only a small proportion were detected by several analytical methods, which proves the essential need of using several complementary analytical techniques to increase metabolic coverage. Following feature selection based on a two-way ANOVA, Spearman correlation coefficients were calculated and a cutoff (> 0.90) was applied, which set the number of features or mineral elements in the correlation network at 715.[75] A network cartography was then created based on the Fruchterman-Reingold algorithm, where analytes were represented by the nodes and the Spearman correlation coefficients by the distance of the link between the nodes. This correlation network analysis made it possible to identify clusters of metabolites which were coregulated, to establish global changes in metabolic composition, and to highlight the association between primary and secondary metabolites with minerals or volatile compounds.[75] This approach was not developed to help with structural elucidation but rather to obtain a broader picture of the biological process and better understand metabolic interactions. It also applies to other kind of biological interactions; indeed, correlation networks were used to explore gene-metabolite association in tomato fruit, for instance.[76]

Multiblock Fusion

The use of multiblock data integration, or data fusion, has been increasing in omics sciences for a couple of decades and this approach can be applied at different levels.[66] Low-level data fusion consists of combining the preprocessed individual blocks at the data level without performing any variable selection prior to modeling the resulting block and in interpreting the global outcome. Although this is a straightforward way to approach data integration, careful consideration must be taken regarding scaling and normalizing the individual data sets. Indeed, because of the sensitivity and robustness differences existing between analytical methods (e.g., NMR or HRMS), the analytical response for a single compound will vary from one data set to another. Data set manipulation to overcome this obstacle can give too much weight to similar variables (e.g., isotopes and fragments from the same metabolite or metabolites from the same pathway), as it is the case when the individual data sets are autoscaled. Scaling can thus also be performed by considering sub-blocks of similar variables, which weight the influence of sub-blocks according to their size. Either way, low-level data fusion has been described as an approach which provides only limited useful information in metabolomics.[66] Furthermore, because the totality of each individual data set is being integrated as is, the size of the resulting data matrices before modeling is considerable. As such, mid-level data fusion considers only the most discriminant features highlighted by block-wise statistical analyses of the individual blocks,

which can be complemented by applying an additional technique to further reduce the dimensionality of the integrated matrix.[66] Also, assessing separately each data set through mid-level data fusion highlighted metabolites detected by several methods, which could be over-represented in the global model and which may introduce bias in biomarker discovery. Finally, data fusion can also be applied through a high-level approach, where individual blocks are preprocessed and modeled separately, as for a mid-level approach, but where the global output (e.g., the predictive algorithms) of each of the individual model is integrated, rather than their discriminant features.[66] It is important to mention that statistical model validation is crucial in metabolomics in order to properly assess the performance of the model without overfitting it,[77,78] but although several validation tools exist, no common agreement has been found regarding which one is the most suitable.[79–81] The predictive power of the combined output obtained following a high-level approach is highly expected to be equal or higher than the predictive power of the best performing individual model, and thus the error rate is also expected to be reduced.[82] However, the predictive performance of the global output will increase more importantly if the classifiers used present similar discriminative performance, which is often the case in metabolomics.[82] In such cases, integration of the individual output through correlation networks, as presented in the Correlation of NMR and MS Data Sets section, might be a solution to jointly interpret individual results while preserving the predictive performance of the individual analysis.[66] In a way, correlation networks can be considered as a high-level data fusion approach but from which the biological interpretation can often be complicated by an extensive visualization output.

Following the selection of the data integration approach, several modeling techniques are available and can be applied for individual data set analysis (for mid- or high-level approaches, before data fusion of the selected features or the individual global outputs, respectively) or by global analysis (for low- or mid-level approaches once data fusion has been made).[66] The resulting models naturally highlight possible association between variables from different data sets to improve biological interpretation but also serve to assess the contribution of each individual data set to the global model. Sequential multiblock analysis, as unsupervised single block multivariate statistical analysis, aims to describe the general trend of the matrix and is based on the calculation of one single component at a time followed by a deflation procedure to calculate the next one. Several sequential multiblock methods exist, from the simplest which are SUM-PCA or consensus PCA (CPCA) to more complex such as hierarchical PCA (HPCA), generalized PCA (GPCA), multivariate component models or multiple factor analysis.[66,83] Similarly, several modeling tools exist to apply predictive supervised analysis, such as PLS regression or discriminant analysis, orthogonal-PLS (O-PLS or O2-PLS), hierarchical PLS (HPLS) or multiblock PLS (MBPLS).[66,84] Those statistical methods allow the combination of data sets from different analytical methods, whatever the size of the different blocks. In metabolomics, however,

even though the numbers of variables can change from one NMR to one MS block for instance, it is preferred to have the same number of objects (or samples) for all blocks. The following paragraphs illustrate how such data fusion and data modeling approaches have maximized the potential of combining NMR and MS data in metabolomics.

In a first example, HPCA was applied to the study of three melon cultivars by ^{1}H NMR spectroscopy and by GC-MS, but not only to fuse both data sets.[85] Indeed, in this example, classical PCA on ^{1}H NMR managed to discriminate the samples coming from different spatial positions in the melon fruit, but it failed when the PCA was based on the GC-MS data set. As such, the authors assigned each of the three melon cultivars as an individual block and applied HPCA on them, which successfully highlighted metabolic differences linked to the spatial positions of the samples.[85] Furthermore, a HPCA model was also built on the combination of both analytical techniques and showed the robustness of this statistical tool as the compounds detected by both NMR and GC-MS were located at similar positions of the loading plots.[85] In another plant study exploring tomato fruits and leaves,[86] the integration of ^{1}H NMR, LC-MS, and GC-MS data was directly done through a low-data level fusion approach, regardless of their individual performance but further association was made with correlation networks to facilitate the interpretation of the biological pathway regulations.

In a second example, ^{1}H NMR spectroscopy and two HRMS instruments (TOF and Orbitrap) were used to analyze honey samples from different botanical origins.[87] PCA and PLS-DA were applied on each of the individual data sets, before PCA modeling based on mid-level data fusion was performed from two different angles. The first one was based on the fusion of the PCA scores of each of the data sets, to prevent any loss of information, and the second one was based on the fusion of selected variables from the individual PLS-DA models, to remove any irrelevant information. Both data fusion approaches performed better than the individual models in term of discriminative power and sample misclassification.[87] The mid-level data fusion between NMR and HRMS-Orbitrap with variable selections had the best discrimination of all the models reported, without misclassification, while the mid-level data fusion between NMR and HRMS-Orbitrap without variable selections led to misclassification. The fusion of NMR with HRMS-TOF data with our without variable selection did not misclassify the samples but underperformed the discrimination of the samples with variable selection compared to the fusion of the NMR and HRMS-Orbitrap data sets.[87]

Another study focused on the metabolic profiles of plasma samples from patients with stable carotid atherosclerosis versus healthy subjects using GC-MS and ^{1}H NMR spectroscopy.[88] The individual PCA models showed good separation of the samples but only along the third principal component, which proved the presence of discriminative variables irrelevant to the pathology characterization. As

462 in single block multivariate analyses, PLS, or orthogonal signal correction (OSC), also called OPLS,
463 provided a better sample separation since the sample classification is included in the model.
464 Supervised analyses were thus performed, and the PLS-DA and OPLS-DA models of both individual data
465 sets did present a higher discriminative power compared to PCA models. Low-level data fusion was
466 then performed, and the supervised analysis of the resulting data set performed as well as the
467 individual supervised models.[88] Here, the application of the combined NMR and MS supervised analysis
468 could thus be questioned, but a Pearson correlation between the metabolites scores obtained from
469 the predictive component of the combined OPLS-DA model allowed a broader understanding of the
470 metabolic pathway alteration than if only one technique would have been used for statistical analyses.

471 Even if supervised methods often enable a better separation than unsupervised analyses, they can
472 sometimes fail, and combining several analytical technologies might enhance the discriminative
473 performance of a model and help to highlight specific biomarkers. For instance, Gu et al. used ^{1}H NMR
474 spectroscopy and direct analysis in real time (DART)-MS to discriminate serum samples from patients
475 with breast cancer from healthy controls.[89] No distinct separation between the samples were observed
476 in the PCA models of each of the analytical data sets, although a slight grouping along the first principal
477 component was observed for the ^{1}H NMR PCA model. Furthermore, both PLS-DA and OSC-PLS-DA
478 models based on each of the individual data sets did misclassify an important number of samples,
479 which was clearly beyond the acceptance rate when it comes to health applications.[89] As such, PLS-DA
480 and OSC-PLS-DA models were rebuilt by setting the Y dummy matrix, the classification variable, to the
481 first principal component of the ^{1}H NMR PCA model, which performed slightly better than the DART-
482 MS PCA model, and the X matrix to the DART-MS data set, which was more sensitive. These models
483 both performed better than the individual supervised models, with a major preference for the OSC-
484 PLS-DA which resulted in a lowest misclassification score due to the removal of confounding factors
485 following the orthogonal signal correction.[89] Another study, which aimed to differentiate the
486 extraction protocols of cold-pressed lemon oil, supervised modeling analyses, namely, MB-PLS-DA and
487 consensus (C)-OPLS-DA, elegantly showed the benefits of using orthogonal projection to improve the
488 separation between samples.[90] In this illustration, a low-data level fusion of untargeted data sets
489 obtained by ^{1}H NMR, GC-FID and LC-MS in positive and negative ionization modes was used (Figure
490 5).[90] The supervised analyses then showed a much better separation, and thus interpretability, when
491 it came to the C-OPLS-DA compared to the MB-PLS-DA, although their predictive performance was
492 similar. It is important to note that even if the data matrix resulting from the fusion of these four data
493 sets was extensive, high-level data fusion successfully discriminated the same samples according to
494 their geographical origins in another study,[91] but not according to their extraction processes.
495 Therefore, low-level data fusion can be a useful alternative when other modelling tools failed, at the

condition to take particular care for the scaling of the different datasets. As illustrated in another study applying low-level data fusion, both of the ^{1}H NMR and direct infusion (DI)-ESI-MS data sets were scaled first to unit variance and second by the square root of the block variable count, in order to ensure fairness in the consideration of each block.[92] Other key elements were to note in this report, as a thorough optimization of the sample preparation in order to prevent important sample handling. This allowed the analysis of one single sample by both ^{1}H NMR and DI-ESI-MS, and optimization of the DI-ESI-MS protocol was undertaken to limit the matrix effect.[92] Also, the backscaled ^{1}H NMR and DI-ESI-MS loading plots obtained from the MB-PLSDA, which outperformed the single block PLS, were complemented by additional MS accurate mass and MS/MS experiments to compare with the NMR signals and facilitate metabolite identification.[92] Overall, this study provides a complete illustration of how to combine NMR and MS data sets from sample preparation, data set acquisition, multivariate analyses and metabolite identification.

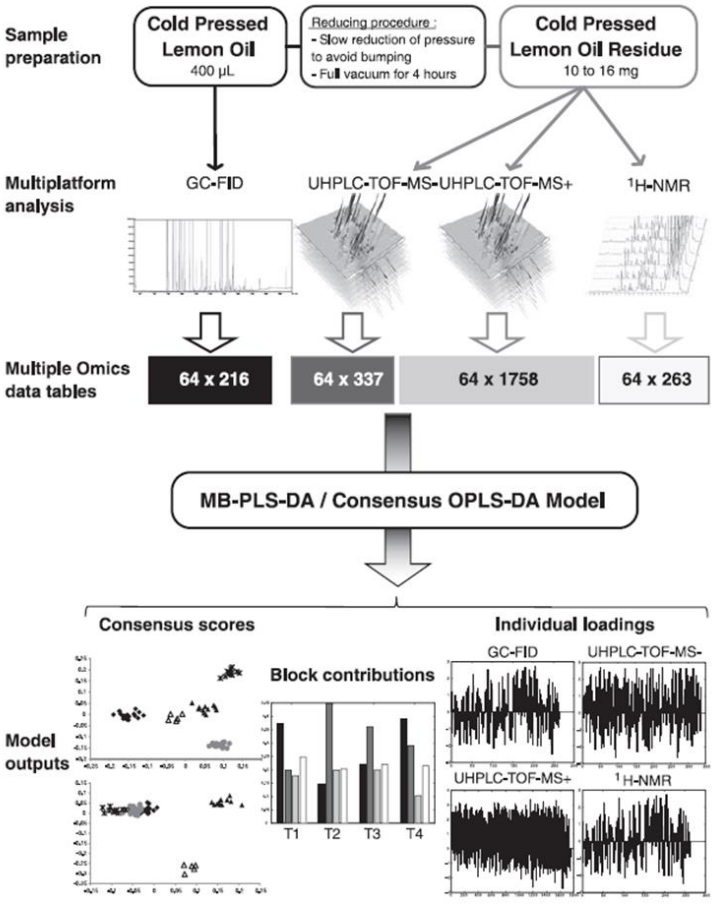

Figure 5. Methodology used to integrate metabolomic data from multiple analytical platforms for a comprehensive characterization of lemon essential oils. Figure reproduced from Integrating Metabolomic Data from Multiple Analytical Platforms for a Comprehensive Characterization of Lemon Essential Oils, Mehl, F.; Marti, G.; Merle, P.; Delort, E.; Baroux, L.; Sommer, H.; Wolfender, J.-L.; Rudaz, S.; Boccard, J. Flavor Fragr., Vol. 30, Issue 2 (ref [90]). Copyright 2015 Wiley.

Multiblock data fusion is thus starting to be anchored in the metabolomics landscape, and some attempts are made to refine and bring some originality into those methods and to increase their application scope. For instance, C-PLS-DA and C-O-PLS-DA have been applied to integrate MS with two NMR data sets, one ^{1}H data set and one 2D J-resolved (J-res) NMR.[93] Also, data fusion can be based on multiple kernel learning (MKL). This approach was applied to explore plasma metabolic alterations in three different chronic diseases for example, namely, acute coronary syndrome, breast and colon cancers.[94] Serum samples were analyzed by NMR and LC-MS, and the resulting data sets were fused with the metadata of the patients, which aimed to represent their lifestyle. The MKL fusion model increased the performance of the individual models when it came to the acute coronary syndrome, but slightly underperformed compared to the individual NMR model for the breast cancer condition, and none of the individual or fusion model performed well regarding the colon cancer conditions.[94] This could be due to the increase presence of confounding variables in the matrix, which complicated the selection of discriminative variables. Several methods were actually developed to optimize variable selection. Deng and co-workers proposed one based on backward variable elimination from PLS-DA models and combined with Monte Carlo cross validation (MCCV-BVE-PLSDA).[95] This method could be considered as half way between low-level and mid-level data-fusion, as all the variables were considered during the first iteration and since only the most predictive variables were kept during the last iteration. A similar approach had been already proposed, called a Sparse multiblock PLS regression (Sparse MBPLSR), implemented with a cross model validation in order to ensure the reliable and stable variable selection for biomarker discovery.[96] In a more recent report, the variable selection before obtaining the final PLS-DA model was made in three steps. First, a curation step was applied to remove all the redundant variables. Second, PCA and PLS-DA were performed and the resulting variables were further filtered by performing different kind of statistical analyses, namely one-way ANOVA Sparse PLS, or least absolute shrinkage and selection operator (LASSO). Third, PLS-DA was performed on each of the resulting new subset of selected variables.[97] All the PLS-DA following variable selection performed better than the PLS-DA before variable selection and the models which had the best performance were the one based on the variables selected by the Sparse PLS and the LASSO techniques.[97] Further matrix reduction can be performed but it depends on operator willingness to discard information without affecting biomarker discovery or biological pathway understanding. In any case, although a 2014 review mentioned that the multiblock fusion of NMR and MS techniques followed by supervised analyses was not common in metabolomics studies,[66] we hope to have shown that since it has gained great interest as it maximizes the complementarity between both analytical techniques. Although multiblock analyses do not always end up giving better results than single block analysis and that it is important to keep a critical eye on the usefulness of this hyphenation, it has already found various application fields.

Postmetabolomics Analysis: Reaching the Aim and Going Beyond

Identification

While increasing the metabolic coverage is of the utmost importance in metabolomics, it might be even more crucial to identify the metabolites covered. Indeed, metabolite identification is clearly seen as a major bottleneck in the field of metabolomics, especially in MS-based techniques. Thanks to the robustness of ^{1}H NMR spectroscopic analysis, NMR databases are easier to produce and more trustworthy than MS databases. Generally, signals are compared and matched to experimental or theoretical spectra registered in in-house or online databases. If a compound of interest cannot be identified, it is an unknown compound annotated at a level 4 of confidence, the lowest according to the criteria used by the Metabolomics Standards Initiative (MSI).[98,99] If the chemical class of the compound can be determined, the annotation level is 3. If a compound can be determined by comparison to a database, it is a level 2 of annotation. Finally, if the compound matches with at least two orthogonal parameters (e.g., the *m/z* and the retention time) compared to an authentic standard which has been spiked into a sample, then the level of confidence is 1 and the term "identification" can be used (although care still needs to be taken in the case of isomeric compounds, but stereochemistry should soon be taken into consideration through a new and revised reporting standards which are being discussed in the community with the Metabolite Identification Task group of the Metabolomics Society leading on this initiative [Personal communication, Prof. Warwick Dunn, cochair Metabolite Identification task group]), in contrast to the term "annotation" for levels 2, 3, and 4.[99] Also sometimes, subconfidence groups in the level 2 annotation emerge, 2b being given if the annotation to a specific metabolite is based on one orthogonal parameter (e.g., *m/*z or δ values matching to database) and 2a if the annotation is based on two orthogonal parameters (e.g., *m/z* values and retention time or *m/*z and δ values matching to databases) without spiking the corresponding authentic standard. Hence, annotating a compound by using both NMR and MS analytical techniques provides more confidence in the annotation level and facilitates structural elucidation of unknowns.

Identification of unknowns is particularly difficult in plant metabolomics due to the lack of chemical standards to confirm the identification of a new metabolite. Initial strategies developed were thus based on accurate mass measurements by HRMS, allowing to obtain chemical formula and matching them to possible chemical structures available in databases, before comparing their fragmentation pattern.[100] Successful candidates were confirmed following purification and NMR analysis for structural characterization. This method could be limited by the fact that the lack of sensitivity of NMR measurements could restrict the annotation of new metabolites or biomarkers. The use of capillary

NMR to overcome this limitation was seen as an alternative,[101] but the process was not less time-consuming and labor-intensive. More recent strategies have thus been proposed to increase the identification of unknowns by relying on the complementarity between NMR and MS. The one that attracted the most attention in metabolomics is the so-called SUMMIT, for Structures of Unknown Metabolomics Mixture components by MS/NMR.[102] Its principle relies on HRMS measurements of the accurate masses of the different analytes present within a complex sample in order to determine their molecular formulas. From those, all the possible scaffolds which can correspond to these formulas are predicted, and the list can be extensive. A 1D or 2D NMR spectra is then predicted for each of the predicted scaffolds. These predicted NMR spectra are then compared to experimental HSQC NMR spectra, previously deconvoluted into ^{13}C-^{1}H HSQC chemical shifts of each metabolite by combining information from 2D NMR experiments. The possible scaffold hits are finally ranked according the level of concordance between the predicted and the experimental spectra.[102] This method was initially developed to prevent time-demanding steps such as sample separation/purification or interrogating metabolic databases and was successfully applied to the identification of previously known compound from *E. coli* as a proof of principle.[102] However, subsequent publications showed that the approach could be further improved by the interrogation of databases, making the method more general and efficient.[103,104] In the future, using the SUMMIT strategy could also help guiding retrospectively to a specific MS platform *ad hoc* or encourage the use of multiple MS platform to increase the chance to detect metabolites by both MS and NMR. Recently after the introduction of SUMMIT MS/NMR, another strategy called NMR/MS Translator was proposed by the same group, as a tool which could be used prior to SUMMIT MS/NMR.[105] Following the 1D or 2D NMR spectral acquisition, NMR/MS Translator questions NMR databases and from the obtained hits, it calculates isotopes, adducts, and fragments. From those, MS spectra are reconstructed and compared to the upstream acquired MS^1 experimental spectra.[105] Interrogating databases is limited by the fact that the hits will depends on how well a database is furnished, and yet there have always been a lot of disparities from one database to another.[58] Therefore, authors highlighted that the eye confirmation of the resulting annotations by an operator should always be applied to prevent false identification, as reported with NMR/MS Translator for 11 urinary metabolites. However once known metabolites are identified, unknown metabolites are easily distinguished and SUMMIT MS/NMR can come to help (Figure 6),[103] for which automation efforts have been pursued.[104] While these approaches are very elegant, they imply that metabolites need to be detected by both NMR and MS measurements. However, as already explained in Cross-Comparison of NMR and MS Data Sets to Increase Metabolic Coverage the metabolic coverage between NMR and MS measurements is limited by different parameters such as the low sensitivity of NMR or for MS techniques the ionization efficiency, the choice of the chromatographic phase, and the ionization mode. Therefore, methods such as SUMMIT or NMR/MS translator are restricted to

compounds that can be detected by both the NMR and MS techniques. As such, physical or chemical derivatization[103] prior to analysis might give access to an increased number of unknowns that would not have been commonly detected by NMR and MS otherwise. Although the associated sample handling could be more time-demanding, it would lead to promising perspectives to uncover more unknown metabolites.

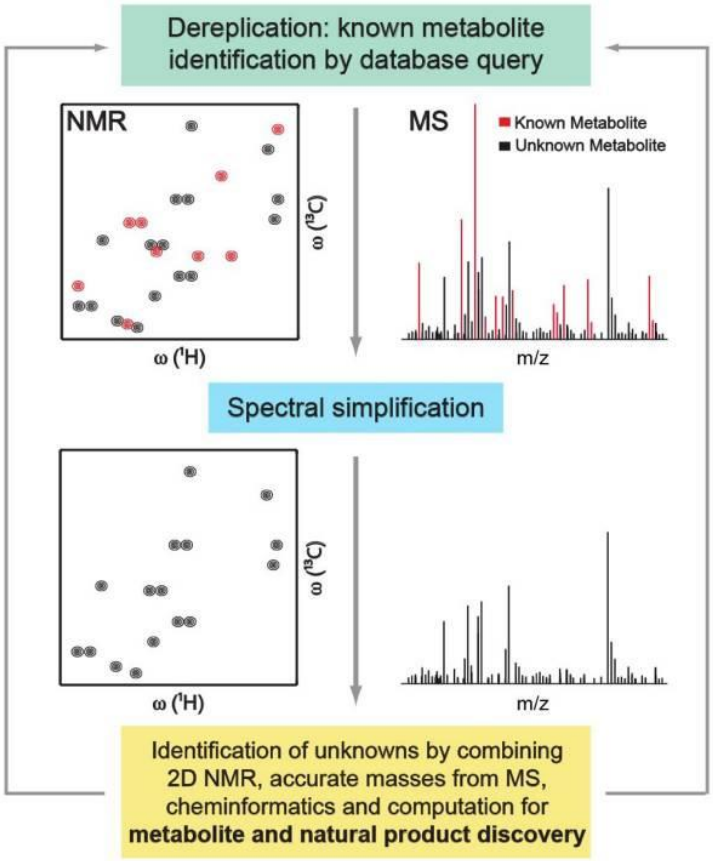

Figure 6. Integrated metabolomics workflow for the identification of known and unknown metabolites in complex mixtures. Combined use of metabolomics databases with experimental NMR and MS spectra (e.g., NMR/MS Translator[105]) allows the rapid identification of a maximal number of known metabolites present in the mixture, while unidentified signals are used as fingerprints of unknowns. Next, structures of unknown metabolites can be elucidated or vastly narrowed down by the combined use of multidimensional NMR, MS, cheminformatics, and computation (e.g., SUMMIT MS/NMR[102]). Figure reprinted from Curr. Opin. Biotechnol., Vol. 43, Bingol, K.; Brüschweiler, R. Knowns and Unknowns in Metabolomics Identified by Multidimensional NMR and Hybrid MS/NMR Methods, p 17-24 (ref [103]). Copyright 2017, with permission from Elsevier.

Quantitation

Quantitative information about metabolites constitutes an invaluable asset to obtain a deeper understanding of the biological reactions and processes occurring in an organism, as changes in metabolite concentrations reflect changes in protein concentrations or gene expressions. For this reason, efforts are being made to collect quantitative data and incorporate them in databases, as it

was done for the human serum metabolome.[61] Quantitative data also helps toward more robust, discriminant, and predictive statistical models.[106] NMR- and MS-based quantitation methods are well established.[106,107] NMR quantitation is relatively straightforward and lies on the use of a single internal standard such as TSP (sodium d_4-3-(trimethylsilyl)-propionate), provided that data are acquired and processed under proper conditions known as qNMR.[108] For complex mixtures where peak overlaps prevent accurate quantitation, quantitative procedures based on 2D NMR have also been developed.[109] MS quantitation is more time-demanding as it requires further development to target the metabolites of interest and free from ion suppression phenomenon. Most robust targeted assays lie on either the combined use of stable isotope labeled (SIL) internal standards and calibration curves, especially in regulatory science,[110] or on differential $^{12}C/^{13}C$ isotope labeling using for instance isotope reagent such as ^{13}C-dansyl chloride to label metabolites.[111] However, SIL internal standards are not always available and costly. Chemical analogues of the metabolites of interest can be used as internal standards but this is a less reliable approach and results must be interpreted with care. The idea of combining both NMR and MS techniques to obtain more robust quantitative assays and overcome the difficulties encountered through MS-based assays is relatively recent.[106] A first approach has been described as an "NMR-guided-MS quantitation".[112] In this approach, NMR absolute quantitation of the metabolites of interest was performed for a serum sample which was randomly selected to be the reference sample. The concentrations obtained were then set as references for the multiple reaction monitoring transitions of the metabolites in the MS experiment, during which the rest of the samples were analyzed.[112] Without the use of internal standards, 30 serum metabolites were successfully quantified and the correlation between the concentration obtained by the NMR-guided-MS approach with the one obtained by NMR were above 0.92 for most metabolites. Metabolite concentrations which showed poor correlation between the NMR-guided-MS approach and NMR draw special attention to the fact that ion formation is not always stable during MS measurements (which might be due to ion suppression or ionization efficiency due to e.g., source clogging) and that matrix effect can skew the results.[112] To alleviate this problem, the same group proposed to combine NMR and MS techniques with chemical derivatization through a so-called qNMR-MS method.[113] As for the NMR-guided-MS method, NMR was first used to obtain metabolite concentrations of a randomly assigned reference sample. This sample was then derivatized with SIL internal standards and mixed with the remaining study samples, which had been derivatized with unlabeled internal standards. The comparison between the labeled and the unlabeled signals allowed absolute quantitation by MS, making it possible to account for matrix effects.[113] Results showed excellent agreement between a classical internal standards methods and proved that the matrix effect, which can be important in complex biological samples such as serum, is well corrected in the qNMR-MS method compared to the NMR-guided-MS one (Figure 7).[113]

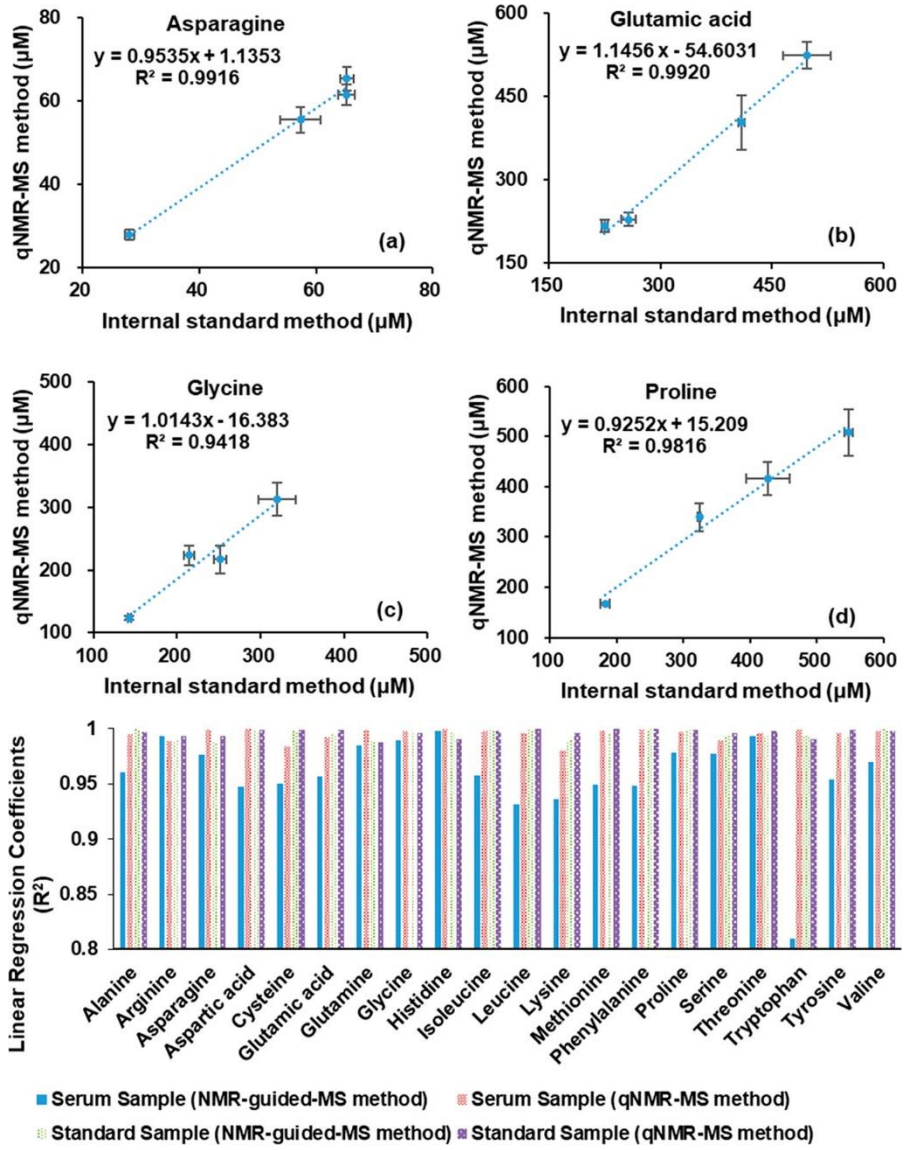

Figure 7. Linear regression equations for four amino acids in human serum samples: (a) asparagine, (b) glutamic Acid, (c) glycine, and (d) proline. Coefficients of determination (R^2) values show excellent agreement between qNMR-MS and internal standard methods. Coefficients of determination (R^2) for quantitating amino acids in human serum and standard samples. Each sample was tested using two quantitation calculation methods: NMR-guided-MS and qNMR-MS. NMR-guided-MS uses the peak area of each amino acid derivative to quantify, while qNMR-MS uses the peak area ratio between labeled and unlabeled MS peaks of each amino acid derivative to quantify metabolites. Figure reproduced from Fei, Q.; Wang, D.; Jasbi, P.; Zhang, P.; Nagana Gowda, G. A.; Raftery, D.; Gu, H. Combining NMR and MS with Chemical Derivatization for Absolute Quantification with Reduced Matrix Effects. Anal. Chem. 2019, 91 (6), 4055–4062 (ref [113]). Copyright 2019 American Chemical Society.

Stable-Isotope Resolved Metabolomics (SIRM)

Stable-isotope resolved metabolomics (SIRM) is an atom-based approach which aims to measure, following a stimulus, metabolic pathways alterations, and fluxes, which explains that it is also called fluxomics. This field is based on stable isotope tracers which are used to label precursor molecules, making it possible to measure metabolic reactions and quantify the metabolite byproducts based on

690 the measurement of the tracer atoms. The combined use of NMR and MS analytical techniques for
691 SIRM is an evidence, as NMR spectroscopy can distinguish isotopomers, compounds which have the
692 same number of heavy isotopes but at different positions, while MS can quantify the isotopologues,
693 isomers that differ by their isotope composition. The most commonly used stable isotope-labeled
694 precursor in fluxomics is U-^{13}C-glucose, but several others are available and can be selected according
695 the metabolic pathway of interest.[114] Some studies actually used multiple labeled precursors in order
696 to cover more metabolic pathways.[115] SIRM is thus a very promising tool for clinical applications such
697 as understating metabolic disturbances in diseases, pharmaceutical[116] and toxicological research, and
698 highlighting novel drug targets, especially because experiments can be performed *in vitro* through cell
699 cultures, on *ex vivo* tissue models, or *in vivo* human or animal models.[117] It helped for instance to
700 understand the mechanism of action of a lithium therapeutic treatment for bipolar disorders.[115] It is
701 also of foremost interest for cancer research,[118,119] such as this *in vivo* application for lung cancer
702 patients infused with ^{13}C-glucose which highlighted an upregulation of the glycolysis and Krebs cycle
703 activity, as well as the unexpected pyruvate carboxylation, compared to the noncancerous tissues
704 surrounding the tumor.[120] The most used MS technologies in SIRM are GC-MS and FT-ICR-MS, the first
705 one being more affordable but the second being more sensitive, having higher reoslution, and allowing
706 high-throughput workflows to be applied more easily.[121] In NMR, fast 2D or 3D methods are generally
707 used to accurately quantify the populations of all isotopomers, given the complex spectra resulting
708 from complex metabolite mixtures with multiple isotopic patterns.[122–124] The combination of 2D NMR
709 with FT-ICR-MS highlighted for instance precursor metabolites for the synthesis of specific
710 glycerophospholipids while obtaining accurate m/z which facilitate identification, within a relatively
711 rapid workflow.[125] However, SIRM is a rapidly growing field and LC-MS has also been applied in
712 combination with NMR to develop a workflow allowing the flux analysis of isoprenoids in yeast, which
713 could be further applied to other organisms.[126] Upon further progresses made in terms of
714 computational tools and high-throughput workflow (Figure 8)[127] for fluxomics analysis, the
715 combination of NMR and MS within this field will for sure unravel the understanding of numerous
716 metabolic pathways within a large scope of applications.

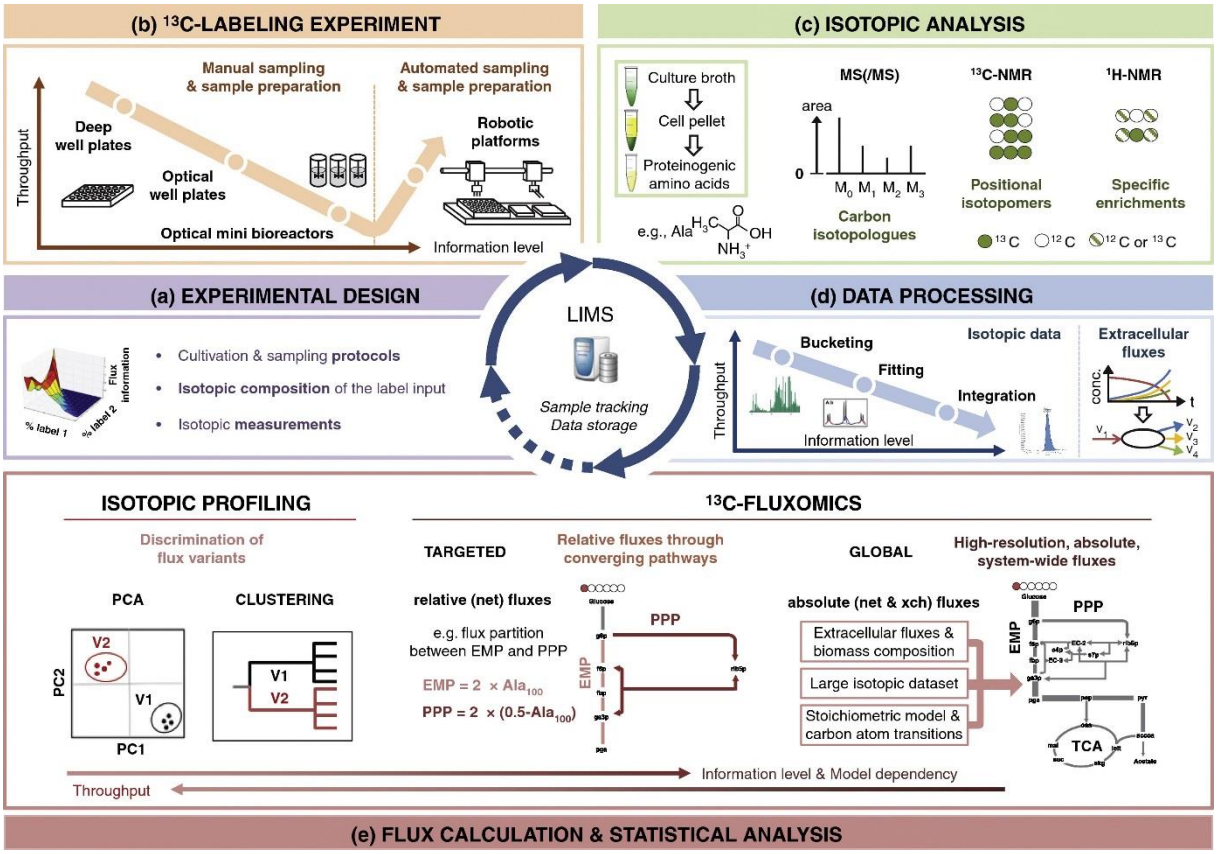

Figure 8. Overview of a high-throughput ^{13}C-fluxomics workflow. (a) Experimental design allows maximizing the flux information depending on the biological question addressed by the study. Besides defining appropriate cultivation conditions (medium composition, temperature, pH, among others), the label input must be optimized according to the isotopic data that can be measured, the metabolic systems investigated, and the experimental costs. (b) Robotic and manual systems are available to perform ^{13}C-labeling experiments and sample preparation. A trade-off between throughput and fine control of growth is determined by the cell cultivation system used and by the degree of workflow automation. (c) NMR-based and/or MS-based isotopic analyses are then carried out to measure the ^{13}C-incorporation into metabolites. (d) Data processing is necessary to extract meaningful isotopic information from the raw data. In contrast to isotopic profiling or targeted ^{13}C-fluxomics, global ^{13}C-fluxomics also requires measuring extracellular (production and consumption) fluxes from the time-course variations of extracellular concentrations. (e) Finally, different computational approaches for flux calculation and statistical analysis are applied according to the purpose of the investigations (i.e., isotopic profiling, targeted fluxomics or global fluxomics) and the level of biological knowledge required to extract the flux information. Figure reprinted from Curr. Opin. Biotechnol., Vol. 43, Heux, S.; Bergès, C.; Millard, P.; Portais, J.-C.; Létisse, F. Recent Advances in High-Throughput 13C-Fluxomics, p 104-109 (ref [127]). Copyright 2017, with permission from Elsevier.

Future Perspectives

Public Databases

The previous sections clearly showed that metabolite identification relies on comparing experimental spectral data to those present in various databases. With Metabolomics being a rapidly growing field, databases have been expending as well. Common examples are the Human Metabolome

Database,[128] the human serum metabolome,[61] METLIN,[129] the Biological Magnetic Resonance Data Bank,[130] LipidMaps,[131] or the Yeast Metabolomics Database.[132] However, as indicated by their names, these databases are either instrument-, organism-, or compound class-specific. Furthermore, high-quality spectra are sometimes missing, which limits annotation confidence, or experimental conditions are not reported, which makes the spectral comparison more difficult and less trustworthy. Therefore, such databases do not always make good use of the MS/NMR complementarity. To overcome this issue, MetaboLights, the first metabolomics open-access repository regardless of the species or the analytical technique used, was released in 2012.[133,134] It aimed to offer a repository where compound structure, spectral reference, biological concentration, location, and role as well as raw data could be submitted, stored, shared with the metabolomics community, and reused. However, automatic reporting of metabolomics data is not always performed by the community, although it has improved in the past couple of years.[135] Undoubtedly, this has been helped by initiatives such as COSMOS (Coordination of Standards in MetabOlomicS), which aimed to promote data reporting according to specific standards, in order to facilitate data exchange and dissemination.[136] An experimental workflow has been proposed to guide members of the metabolomics community in their standardization efforts (Figure 9) and which ensure FAIR (Findable, Accessible, Interoperable and Reusable) data sharing.[137] This is of paramount importance for the future of metabolomics and even if it goes without saying that this should be applied for both NMR and MS experiments; even taken separately, it is obvious that it will also help toward further combined use of NMR and MS analysis by gathering both spectral data for a same compound.

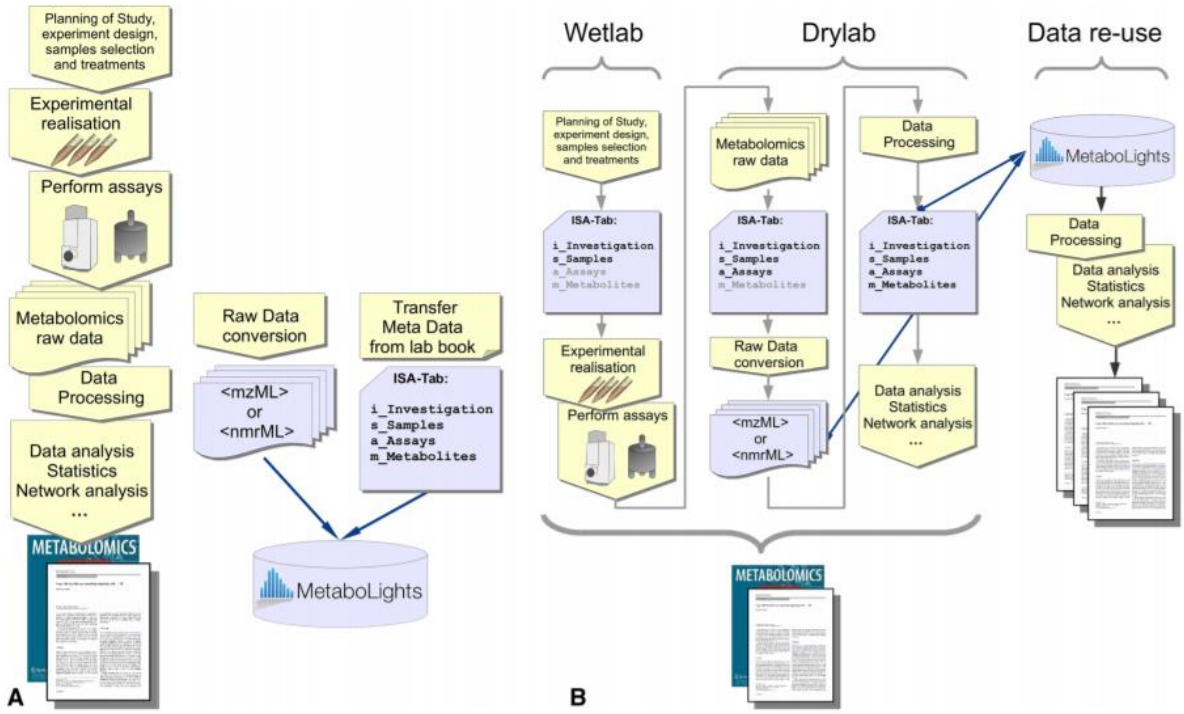

Figure 9. Experimental workflows in metabolomics. Shown in light blue are the relevant parts where data standards come into play. Annotated data deposition in open repositories allow for data reanalysis and reuse. (a) Traditional workflow using tools which do not depend on data standards, and where data annotation and data publication happen together with manuscript submission. (b) Fully standard embedded workflow, where data annotation is part of the standard operational procedures, data processing can use open software, and data publication is an integral part of the dissemination. Figure reproduced from ref. [137] under Creative Commons Attribution 4.0 International License (http://creativecommons.org/licenses/by/4.0/).

Integration to Other Platforms (Outside Metabolomics)

The new bioinformatic tools allowing the combination of different analytical methods will certainly not stop their boundaries to the metabolomics field. A growing interest has been noticed for the integration of untargeted metabolomics data with DNA sequencing techniques for instance, to investigate the microbiome and its role in health and disease or in pharmaceutical studies to investigate impact that drugs can have on the human metabolic profile.[138] Integrating multiple omics data from genomics, transcriptomics, proteomics, and metabolomics as well as epigenomics or pharmacogenomics provides a deeper understanding of complex biological mechanisms linked to a specific condition. Although computational challenges persist with respect to the integration of large omics data sets together and their biological interpretation, there is a will to go toward that goal in the omics community,[139] and some tools, such as the R package mixOmics, have already been developed.[140] Omics integration is thus already attracting attention in a number of different fields, either to have more insight into the human health risks linked to environmental chemical exposition,[141] ecological interactions through chemical signals,[142] plant biology through integration of transcriptome and metabolome data sets,[143] or many others. Some examples cited in this review did actually combine transcriptomics and NMR- and MS-based metabolomics to study tomato fruit composition and used correlation analyses to highlight associations between genes and metabolites, to finally visualize them in a common network.[76] Proteome and metabolome data sets were also combined in the same aim of studying tomato fruit composition.[144] This combination successfully promoted biomarkers discovery in diseases with a case study based on encephalomyelitis[145] and promises further applications to toxicological studies following a recent published workflow.[146] Parallel developments in computational tools and databases, together with the pursuit of better sensitivity and resolution for NMR and MS methods, will undoubtedly increase the level of biological information that can be accessed (Figure 10).

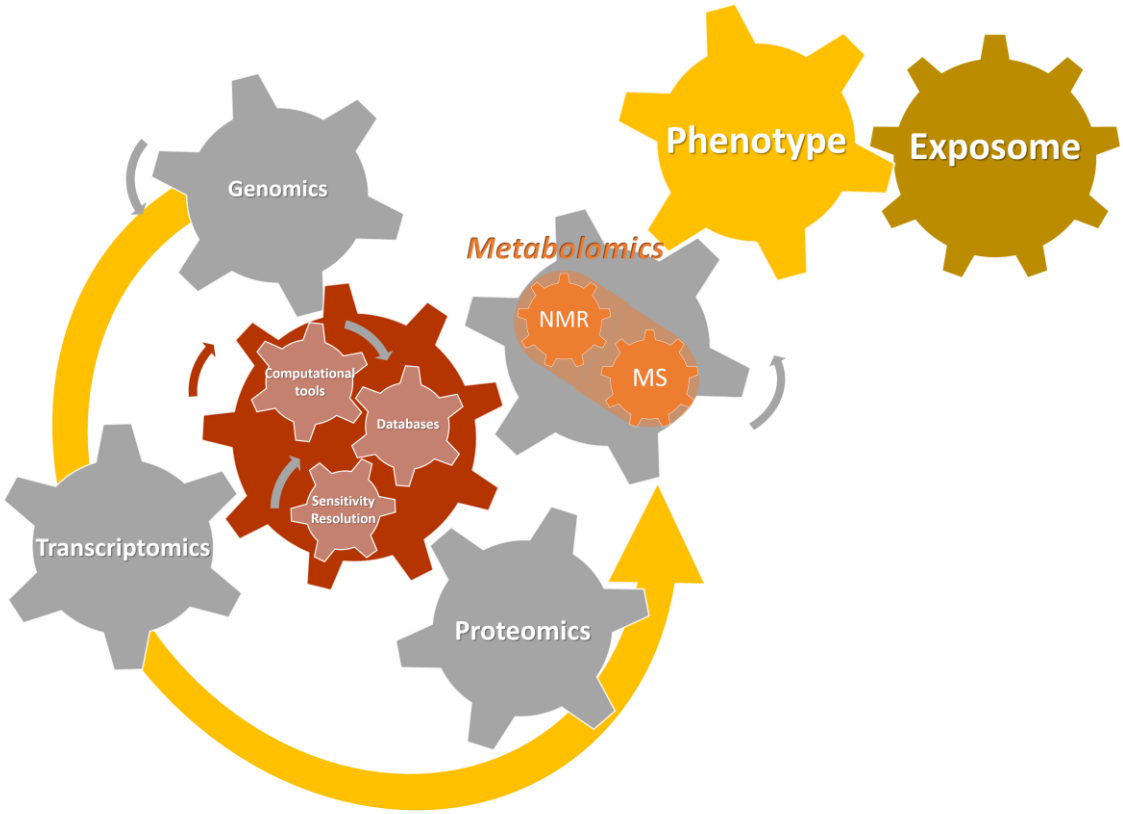

Figure 10. Schematic representation of systems biology, from which the heart relies on current progress being made in computational tools for multiomics integration, expanding databases, and increased sensitivity and resolution of current analytical instruments, which will allow new discovery in genomics, transcriptomics, proteomics, and finally metabolomics, which is the closest field which can explain an organism phenotype and which the exposome has constant impact.

Conclusion

In this review, various ways to combine NMR spectroscopy and MS technologies for metabolomics have been discussed, from hardware combination to computational tools, highlighting how it made it possible to access crucial information at the postmetabolomics level. It seems clear that metabolomics is a growing and dynamic field which will greatly benefit from NMR and MS combination, in particular from the computational point of view. Indeed, hardware combination seems a bit outdated when it comes to metabolomics but will probably remain relevant in natural product research. In terms of computational combination of NMR and MS-based metabolomics, the solutions to do so are clearly multiplying, and the massive improvements which have been made in the recent years guarantee that the use of correlation and data fusion approaches will spread. This will allow one to obtain more robust statistical models and will definitely help biomarkers discoveries and a more profound understanding of biological systems. This will be even truer if efforts in integrating metabolomics with other omics are maintained to obtain deeper capture of the biological processes lying under an organism phenotype and of its constant interaction with its environment, the so-called exposome.[147]

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Biographies

Dr. Marine Letertre studied chemistry at the Universities of Rennes and Nantes, where she received her Master Diploma in 2015. During her studies, she acquired a rich international experience with internships in Ireland and New-Zealand. Then she did a Ph.D at Imperial College London, where she applied NMR and LC-MS methods to investigate the two-way interactions existing between the gut microbiota and host drug metabolism. After obtaining her Ph.D degree in 2020, she joined the Corsaire metabolomics platform as a facilitator. In 2021, she is joining the CEISAM research institute in Nantes as a post-doctoral fellow to work on the complementarity of NMR and MS for metabolomic applications in collaboration with the National Veterinary College of Nantes. In 2020 she was elected Chair of the Early-career Member Network of the Metabolomics Society.

Dr. Gaud Dervilly studied biochemistry and food science at AgroParisTech (former ENSIA), where she received her engineer Diploma and Ph.D. degree in 1997 and 2001, respectively. She further holds an habilitation degree in Analytical Chemistry form Nantes University since 2007. She is head deputy of INRAE research unit within the National Veterinary College of Nantes, France. For 20 years, her research activity has been devoted to Chemical Food Safety issues. She is responsible for the management of research projects related to the modelling of contaminants transfer along the food chain and the evaluation of consumer's chemical exposure. Her competences range from targeted mass spectrometric approaches to more global and non-targeted strategies, such as metabolomics, to study the effects of chemical exposure and related biomarkers discovery, in a risk assessment perspective.

Prof. Patrick Giraudeau studied physics and chemistry at the University of Nantes, where he received his Ph.D. degree in 2008. He worked for one year each as a postdoctoral researcher in the Department of Chemical Physics at the Weizmann Institute of Science (Israel). In 2009, he became an associate professor at the University of Nantes, where he became a full professor in 2017. In 2014, he became a fellow of the *Institut Universitaire de France*, and received a consolidator grant from the European Research Council in 2018. His research activities at the CEISAM research institute are focused on the development of quantitative NMR methods for the analysis of complex mixtures, including applications to metabolomics and fluxomics. Research highlights include the development of fast

multi-dimensional quantitative experiments at high fields and also on benchtop spectrometers, as well as recent investigations in dissolution dynamic nuclear polarization.

The authors declare no competing financial interest.

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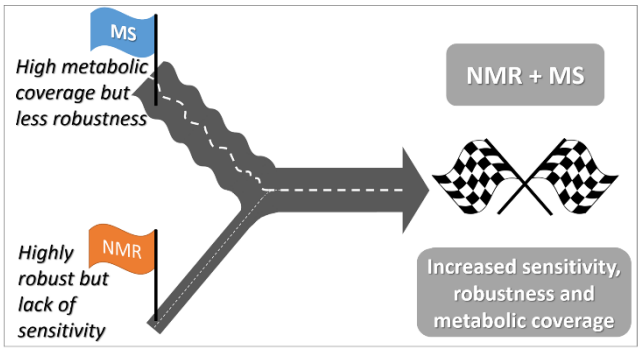

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