Available online at www.sciencedirect.com

ScienceDirect

Current Opinion in Chemical Biology

New analytical methods focusing on polar metabolite analysis in mass spectrometry and NMR-based metabolomics

James McCullagh and Fay Probert

Abstract

Following in the footsteps of genomics and proteomics, metabolomics has revolutionised the way we investigate and understand biological systems. Rapid development in the last 25 years has been driven largely by technical innovations in mass spectrometry and nuclear magnetic resonance spectroscopy. However, despite the modest size of metabolomes relative to proteomes and genomes, methodological capabilities for robust, comprehensive metabolite analysis remain a major challenge. Therefore, development of new methods and techniques remains vital for progress in the field. Here, we review developments in LC-MS, GC−MS and NMR methods in the last few years that have enhanced quantitative and comprehensive metabolome coverage, highlighting the techniques involved, their technical capabilities, relative performance, and potential impact.

Addresses

Department of Chemistry, University of Oxford, Mansfield Road, Oxford, OX1 3TA, UK

Corresponding author: McCullagh, James (james.mccullagh@chem.ox.ac.uk)

(McCullagh J.)

Current Opinion in Chemical Biology 2024, **80**:102466

This review comes from a themed issue on **Omics - Metabolomics (2023)**

Edited by **James McCullagh** and **Hector Keun**

For complete overview of the section, please refer the article collection - Omics - Metabolomics (2023)

Available online 20 May 2024

https://doi.org/10.1016/j.cbpa.2024.102466

Keywords

Mass spectrometry, NMR, IC-MS, HILIC-MS, Methods, Mixed mode, Pure-shift NMR, Ultrafast NMR, Quantification, Hyphenated methods.

Introduction

Metabolomics has risen to take a strategically and functionally important place amongst the 'omics' sciences, however, due to the ongoing challenge of comprehensive and quantitative metabolite coverage and identification, new analytical methods still have the potential to significantly impact the field. Metabolomics has led to important developments in our understanding of metabolic systems across a wide range of organisms and how the metabolome interacts with the genome, transcriptome and proteome in the molecular processes of life. Nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry coupled with liquid or gas chromatography (LC-MS and GC−MS) are most widely used in metabolomics (although electrophoresis and other spectroscopies, including Raman spectroscopy, also have an important role to play). However, unlike genomics (and to a lesser extent proteomics) where the analytical approaches have matured into more unified technological solutions, a broad range of platforms and methods are still used widely in metabolomics and technical developments can have significant positive impact.

NMR and MS have advantages and disadvantages as platform technologies for metabolic profiling. Although inherently quantitative and non-destructive, the complexity of NMR spectra can lead to time-consuming interpretation and relatively low sensitivity prohibits characterisation of the majority of metabolites in complex systems. Despite the significantly higher sensitivity of LC-MS and GC−MS methods, quantitative analysis of metabolites with broad physiochemical properties, remains a major challenge. A range of chromatographic approaches are used in contemporary metabolomics for characterising subsets of the metabolome, but none are universally comprehensive and the main analytical platform methods are currently insufficient to provide full metabolome coverage.

This review focuses on selected recent technical and methodological developments in NMR and hyphenated techniques, with specific focus on those that have improved quantitative metabolome coverage and made important analytical contributions. It focusses across the main LC-MS, GC−MS, and NMR platforms where

Given the role as guest editor, James McCullagh had no involvement in the peer review of the article and has no access to information regarding its peer-review. Full responsibility for the editorial process of this article was delegated to Hector Keun.

recent advances in the characterisation of metabolites have been made. A particular focus is given to polar metabolites enabling the profiling of primary metabolic pathways. The technical merits and deficiencies of new methods are summarised (rather than details of their applications) providing an overview of current method capabilities and where technical challenges still lie. This review therefore provides a concise update on selected new methods in the field of metabolomics focussed on mass spectrometry and NMR platforms.

New mass spectrometry and separation science methods

A major analytical challenge in MS-based metabolomics is the comprehensive analysis of metabolites representing a wide range of polarities and structural isomers. Triple quadrupole technologies are typically used in targeted biomarker studies whilst 'time of flight' and Orbitrap technologies are utilised in untargeted (discovery) applications. There have been progressive advances in these technologies including higher speed, sensitivity and linear range of both triple quadrupole systems and orbitrap technologies enhancing quantitation and metabolite coverage capabilities. Incorporation of ion-mobility has provided orthogonal separation capacity as well as developing the effectiveness of data-independent tandem mass spectrometry methods. Notably recent developments in ion-source and extraction technologies have also enhanced spatial metabolomics, providing a new level of sensitivity and metabolite coverage. However, one of the main technical challenges in metabolomics is the limitations of current separation science capabilities in providing comprehensive metabolome coverage using hyphenated techniques. GC or LC separation, followed by direct mass spectrometry measurement remains one of the most effective approaches, however, the relatively narrow physiochemical selectivity of most chromatographic stationary phases makes it difficult to gain broad metabolome coverage. Multiple LC-MS and/or GC−MS methods can be applied but this is not ideal as it leads to considerably greater analysis time, sample quantity requirements and results in non-contiguous, partially overlapping datasets. Highly polar and ionic metabolites remain some of most difficult to characterise (with potentially significant impact as they are well-represented across primary and secondary metabolism). Therefore, more comprehensive and robust methods, integrating highly polar and ionic metabolites (ideally using a smaller number of methods), remain an important method development challenge, as does the need to provide comprehensive, robust, and quantitative coverage of metabolites across interconnected pathways, not just well-characterised metabolite classes or defined polarity ranges.

Reversed-phase (RP) LC-MS is widely used in metabolomics due to its robustness, but poorly separates more polar and ionic metabolites. In contrast, HILIC-MS (as well as mixed-mode and ion-pairing chromatographic methods) is more suitable for higher polarity metabolite analysis but less reproducible and more selective. The chromatographic approaches used in a study, the number of methods applied, and their robustness can therefore significantly affect metabolite coverage, quality of the data, and the overall effectiveness of untargeted or semi-targeted studies in both looking metabolome-wide for biomarkers and altered metabolic pathways. New methods and combinations of methods can therefore have a significant impact on the success of metabolomics studies, particularly when used as discovery tools. Recent developments in chromatographic technologies, and novel chromatographic combinations, have helped integrate broad coverage whilst mitigating some of the challenges inherent to highly polar and ionic metabolite analysis.

Mixed-mode methods

'Mixed-mode' approaches combine distinct stationary phases to provide multiple mechanisms of analyte retention which can facilitate analysis of a broader range of metabolites with different physiological properties. Sagi-Kiss and colleagues recently developed a complementary approach to highly polar and ionic metabolite analysis combining amine derivatisation (AccQTag) for high sensitivity analysis of anionic metabolites with tributylamine ion-pairing-MS for anionic metabolome coverage [1]. They characterised approximately 450 metabolite standards and applied the method to the study of sex differences in liver extracts [1]. The method requires two sequential analyses with multiple sample injections, increasing the investment needed in analysis time and sample quantities. Due to the targeted nature of the derivative the cation analysis is largely limited to primary and secondary amines and the use of the ion-pairing reagent tributylamine can lead to persistent contamination [2]. Despite these limitations, this innovative method demonstrates that new mixed-mode methods can help characterise highly polar and ionic metabolites, providing an alternative separation capability to HILIC-MS.

Taking the mixed-mode approach in a different direction Nakatani et al. recently combined HILIC with anion-exchange chromatography in a single chromatographic run (unified-HILIC/AEX/(HR)MS/MS) to maximise coverage of highly polar metabolites [3]. They reported an almost 40% increase in compound-feature coverage compared to HILIC alone in a novel, two-step, single injection chromatographic separation. Over 400 metabolites in Hela cell extracts, including nucleic acids, TCA cycle intermediates and amino acids, were detected. However, an absence of sugar mono and di-phosphates was noted. Although the approach focussed on anionic metabolites only, it clearly

demonstrated the added benefit of combining chromatographic approaches and the limitations of using HILIC alone to gain highly polar and ionic metabolite coverage.

Mixed-mode chromatographic approaches, using a single stationary phase, have also been developed that combine an RP-LC mechanism with embedded ionisable groups that retain more polar and ionic metabolites [4]. Whilst applied less frequently in contemporary untargeted metabolomics studies, there is potential to maximise metabolite coverage across a wider range of physiochemical space using these approaches. For example, Hodek and colleagues developed a mixed-mode method (RP-AX-LC-MS/MS) to characterise TCA cycle intermediates, medium to long chain fatty acids, and other metabolites in human blood plasma. This fast method provided efficient coverage of the central carbon metabolome, successfully analysing metabolites across a broad polarity range [5]. Pushing the boundaries of mixed-mode separations further, Xing and co-workers recently incorporated an ion-pairing dimension to a mixed-mode gradient separation using a polyvinyl alcohol stationary phase [6]. In another study, retention of almost 400 metabolite standards included the monosaccharides glucose and fructose, a class of compounds notoriously difficult to resolve and characterise in the context of discovery metabolomics [7].

Derivatisation

Chemical derivatisation can be used to both enhance sensitivity and chromatographic selectivity. This has a long history in GC−MS applications where it also serves to ensure metabolites are sufficiently volatile, enabling gas phase separation. For LC-MS, the range of suitable derivatising agents is far greater, in part because they do not need to also ensure metabolites are volatile. It is worth noting that derivatisation does not universally enhance metabolite coverage, as the presence of specific functional groups are a pre-requisite for derivatisation, tending to make it a more selective approach. The instability of derivatives ounce formed, can also be a problem with certain structures more unstable than others and the potential for poly-derivatisation making metabolite identifications more challenging. These issues make untargeted coverage particularly difficult using derivatisation approaches. Nevertheless, multiple functional groups can be further functionalised by a single derivative and multiple derivatives can also be used with the same sample. There continues to be important methodological developments that explore derivatisation approaches to enhance coverage in metabolomics applications.

Capellades and co-workers developed a GC-chemical ionisation-MS technique (GC−CI−MS) using MTSBSTFA derivatisation which provided protonated molecular ions using isobutane as a CI reagent gas [8]. They showed this outperformed methane which is typically used in CI and demonstrated increased sensitivity and greater isotopologue coverage in isotope tracer studies. Other notable derivatisation-based methods recently developed include a targeted 60 compounds silyl derivative method [9]. Despite the separation efficiency of GC, and generally taller, narrower chromatographic peaks, coverage and sensitivity remain a potential disadvantage due to the narrower range of derivatives and reliance on electron ionisation which is inherently less sensitive that electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCi), both commonly used in LC-MS approaches. As a result, a greater proportion of more sensitive, higher coverage, LC-MS derivatisation methods have been recently developed for metabolomics applications. For example, Willacey et al. observed that the majority of derivatising agents used in LC-MS based metabolomics focus on amines, phenols and thiol functional groups leaving a major gap for carboxylic acids [10]. They used dimethylaminophenacyl bromide (DmPABr) to simultaneously derivatise carboxylic acids, thiols and amines and demonstrated targeted quantitation of 64 metabolites in urine. Several other novel derivatives and derivatisation approaches, with specific application to analysis of metabolites involved in primary metabolism, have also been developed recently [11,12].

By far the most time-consuming aspect of any method, where a chromatographic technique is coupled with mass spectrometry, is the length of the chromatographic separation. It was therefore a significant breakthrough when Nemkov and co-workers developed a 3-min, high throughput quantitative method for analysis of metabolites found in central carbon and nitrogen metabolic pathways, demonstrating analysis of over 400 metabolite standards [13].

Ion-exchange chromatography-MS

Most new methods in metabolomics involve the incremental development of established LC-MS techniques and stationary phases that have been used in the field for some time. It is rarer to find a new chromatographic technique being introduced. Ion-exchange chromatography coupled directly to mass spectrometry (IC-MS) used in metabolomics applications is therefore of particular interest. The coupling of IC with MS via the development of online electrolytic ion-suppression technology has enabled the high salt and extreme pH mobiles phases used to be compatible with electrospray ionisation mass spectrometry and our recent review includes an overview of IC-MS applications in metabolomics [14]. In 2020, our group reported an IC-MS method characterising over 400 highly polar and ionic metabolites from cell extracts and demonstrated untargeted coverage of thousands of

compound-features in glioblastoma cancer cells [15]. There is a growing number of publications that have demonstrated anion-exchange-MS is a highly reproducible, sensitive and robust platform that can be used for both targeted and untargeted metabolomics; to date mainly focussed on the anionic metabolome [16–20]. Comprehensive coverage of metabolites found in multiple primary metabolic pathways has been demonstrated, including glycolysis, pentose phosphate pathway, TCA cycle, purine and pyrimidine metabolism and a wide range of carboxylic acids and phosphorylated metabolites in cell extracts. Our work has shown that IC-MS can provide superior analytical performance when compared to HILIC-MS in both targeted and untargeted validation experiments for a range of classes of highly polar and ionic metabolite [15]. However, the electrolytic suppressor technology removes counter-ions preventing the characterisation of certain compounds, including amino acids and other zwitterions. It also requires anion and cation modes to be run independently. To date very little research has been published using cation-exchange in the context of metabolomics. Nevertheless, anionic IC-MS provides an important methodological development for metabolomics applications and may see an increasing number of applications in the future [21–28].

New NMR methods

While the rate of publications focusing on NMR metabolomics continues to grow, the number of studies lags significantly behind metabolomics studies utilising MS techniques. This is largely due to the reduced sensitivity relative to MS, complexity and overcrowding of NMR spectra making interpretation challenging, and the non-trivial quantification of overlapping signals. However, significant advances in NMR methods have been made to address these issues in recent years.

Resolution

Whilst chromatographic separation is not needed for NMR quantification, the complexity of NMR spectra of biological samples can make metabolite assignment and quantification challenging. Recent advances in pure shift approaches overcome this by collapsing ^{1}H multiplet patterns to singlets [29]. While the use of pure-shift NMR in metabolomics studies is still relatively new, several promising applications of pure-shift methods to biological samples have recently emerged. The improved resolution obtained is of particular benefit in the analysis of complex mixtures of unknown compounds such as those obtained from extracts of natural products [30–34]. It is reasonable to assume that improved resolution will lead to improved statistical analysis of metabolomics data. While direct comparisons of downstream multivariate analysis of traditional and pure-shift spectra are limited, there is evidence that reducing peak crowding results in improved downstream analysis [35]. Recently, incorporation of a NOESY-presat block with pure-shift NMR provided sufficient water suppression [36], essential for measurements of biological samples, for analysis of extracellular matrices with improved interpretation.

Although it should be noted that pure-shift methods suffer from a loss of sensitivity, the power of the technique lies in improved peak assignments, down-stream statistical analysis, and interpretation. Indeed, ultrahigh-resolution spectra more readily lend themselves to automated peak fitting and quantification algorithms which will likely streamline NMR analysis in future.

2D NMR can also be used to alleviate crowding in complex mixtures by spreading the signals throughout a second, more well dispersed chemical shift range. Indeed, ^{1}H–^{1}H TOCSY and ^{1}H–^{1}H COSY methods improve performance over 1D NMR in the analysis of mouse urine [37], cell culture, and human serum [38]. However, the increased time required to acquire 2D data is problematic when hundreds-thousands of samples are required and where samples may degrade during data acquisition. The development of non-uniform sampling (NUS) and ultrafast NMR pulse programmes overcome this limitation and recent studies have demonstrated that they can be reliably applied to biological samples, paving the way for the increased use of 2D NMR in metabolomics studies.

It has been shown that NUS ^{1}H–^{13}C HSQC of plasma extracts have markedly improved sensitivity compared to traditional uniform sampling methods [39]. Furthermore, NUS ^{1}H–^{1}H TOCSY, ^{1}H–^{1}H COSY methods have been reproducibly applied to the analysis of plant extracts [40–42]. Schatzlein *et a*l recently demonstrated that ultrafast ALSOFAST-HSQC could be successfully applied to ^{13}C-enriched cancer cells [43] which, coupled with the improvements in this method, developed by Schulze-Sunninghausen and co-workers, to study compounds at natural abundance [44] could lead to exciting opportunities to apply these methods to a range of disease models without the need for isotope enrichment.

Quantification

Although many NMR pulse programmes have been developed to quantify compounds with high accuracy and precision, those typically used in metabolomics do not lend themselves to quantification due to the need to suppress macromolecule signals, significant peak overlap, and the necessity to minimise acquisition times. The use of line shape fitting methods which simultaneously correct the baseline, particularly in the study of plasma and serum samples, has become common in recent years and is found in commercial software. However, these approaches require *a priori* assumptions

of the chemical shift, line width, relative concentration and quantity of each overlapping peak of interest requiring a library of reference NMR spectra and so are used principally for targeted analysis. Furthermore, such methods fail if the spectra deviate significantly from these assumptions, which can be a problem when analysing samples from patients with severe disease where the composition of metabolites in the mixture may be greatly perturbed or if an unknown metabolite is present. An ingenious approach to overcome this limitation is to deconvolute signals in the time domain leading to NMR parameter estimation requiring no prior assumptions or user input [45–47]. While the application of these methods to metabolomics studies remains limited, the use of the Bayesian-based CRAFT (completed reduction to amplitude-frequency table) method has been shown to allow rapid analysis and improve discrimination in plant cultivar studies [48] and to improve the quantification of several lipid species in rodent serum, liver, heart, and adipose tissue samples [49,50].

An area which remains in its infancy, but is likely to rapidly gain in popularity, is the application of artificial intelligence (AI) methods to spectral processing, peak deconvolution and assignment. Very recently the development of neural network-based approaches to deconvolute frequency domain NMR data has shown promise on real biological samples in both 1D [51,52] and 2D spectra [53]. Furthermore, developments in AI assignment may improve 2D NMR metabolomics workflows in future [54,55].

Sensitivity

Improvements in NMR instrumentation consistently expand the lower limits of detection of NMR metabolomics data. Indeed, the recently developed 1.2 GHz NMR spectrometer offers vast improvements in sensitivity and resolution when compared to lower field spectrometers [56,57] and is expected to double the number of metabolites detectable in biofluid samples [58]. Simultaneously, advances in benchtop NMR instrumentation are paving the way for biofluid analysis in primary care settings [59–63]. Hyperpolarisation [64–67] and the use of paramagnetic probes [68] offer another avenue to significantly improve sensitivity. Although the cost of these methods is decreasing, the advanced technology and NMR expertise required for many of the methods currently limits their use in the wider-spread metabolomics community.

Combined NMR and mass spectrometry (hyphenated methods)

The combination of NMR and mass spectrometry has been used for a long time in the structural characterisation of new metabolites but their combination is much rarer in untargeted applications. This is in part due to the significantly more complex datasets produced and, in practice, many studies utilise the most convenient platform available to them. It is rarer that both are available simultaneously, with the required expertise and there is a significant additional cost and time requirement to performed combined analysis. Nevertheless, in large part due to the challenges that currently face comprehensive coverage in metabolomics, NMR and MS platforms have begun to be combined in metabolomics studies [69]. Integration of NMR and MS methods can occur at different levels. E.g. via integration of the physical instrumentation (rare) through to combining the respective datasets in a post-metabolomics workflow (increasingly common). An important but neglected area is the development of sample preparation and analysis approaches which enhance the information combinatorial analysis can provide. For example, solid-phase extraction (SPE)-NMR methods coupled with mass spectrometry allow unknown compounds to be identified more efficiently and simultaneously validated on both systems [70,71]. Studies have shown that combining NMR and MS approaches is able to increase the depth of metabolome coverage and provide data representing a broader physiochemical range. Finally, combination of MS and NMR techniques for metabolomics imaging is highly complementary and currently being applied, particularly to identify pathology, disease biomarkers and prognostic markers [72].

Conclusions and outlook

Metabolomics remains analytically challenging and in this review we have focussed on recently developed NMR, GC–MS and LC-MS methods that enhance metabolomics capabilities. Developments in the last few years have provided important innovations but also highlighted comprehensive quantitative profiling of metabolomes, using a single technology, is still an aspiration rather than the reality. No single method or platform can currently capture even the majority of metabolites found in mammalian, plant and microorganism systems. MS and NMR remain the major platforms used in metabolomics (usually independently applied) and new methods aimed at overcoming the technical challenges still inherent in discovery metabolomics are critical and enabling as demonstrated by excellent new methods developed over the last few years. Finally, the combination of MS and NMR methods is still relatively rare in metabolomics, leaving room for new applications to exploit their complementary capabilities for comprehensive metabolomic profiling.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

Acknowledgements

Probert is supported by a Dorothy Hodgkin Early Career Development Fellowship in Chemistry in association with Somerville College.

References

Papers of particular interest, published within the period of review, have been highlighted as:

* of special interest

1. * Sagi-Kiss V, Li Y, Carey MR, Grover SJ, Siems K, Cirulli F, Berry A, Musillo C, Wilson ID, Want EJ, Bundy JG: **Ion-pairing chromatography and amine derivatization provide complementary approaches for the targeted LC-MS analysis of the polar metabolome**. *J Proteome Res* 2022, **21**:1428–1437.

The authors combined ion-pairing chromatography (IPC) and reversed-phase chromatography (RPLC) of 6-aminoquinoloyl-N-hydroxysuccinidimyl carbamate derivatization of metabolite extracts to improve retention and coverage of the polar metabolome. They demonstrated a significant increase to 91% coverage of a panel of 500 metabolites when combining the methods and demonstrated its application in mouse liver metabolite extracts.

2. Franey T: **A volatile ion-pairing chromatography reagent for an LC-MS mobile phase**. *LC-GC N Am* 2003, **21**:54 [+].

3. Nakatani K, Izumi Y, Takahashi M, Bamba T: **Unified-hydrophilic-interaction/anion-exchange liquid chromatography mass spectrometry (Unified-HILIC/AEX/MS): a single-run method for comprehensive and simultaneous analysis of polar metabolome**. *Anal Chem* 2022, **94**:16877–16886.

4. McCullagh JS: **Mixed-mode chromatography/isotope ratio mass spectrometry**. *Rapid Commun Mass Spectrom* 2010, **24**: 483–494.

5. Hodek O, Argemi-Muntadas L, Khan A, Moritz T: **Mixed-mode chromatography-mass spectrometry enables targeted and untargeted screening of carboxylic acids in biological samples**. *Anal Methods* 2022, **14**:1015–1022.

6. Xing G, Sresht V, Sun Z, Shi Y, Clasquin MF: **Coupling mixed mode chromatography/ESI negative MS detection with message-passing neural network modeling for enhanced metabolome coverage and structural identification**. *Metabolites* 2021, **11**.

7. Li Y, Liang J, Gao JN, Shen Y, Kuang HX, Xia YG: **A novel LC-MS/MS method for complete composition analysis of polysaccharides by aldononitrile acetate and multiple reaction monitoring**. *Carbohydr Polym* 2021, **272**, 118478.

8. Capellades J, Junza A, Samino S, Brunner JS, Schabbauer G, Vinaixa M, Yanes O: **Exploring the use of gas chromatography coupled to chemical ionization mass spectrometry (GC-CI-MS) for stable isotope labeling in metabolomics**. *Anal Chem* 2021, **93**:1242–1248.

9. Phelippe M, Coat R, Le Bras C, Perrochaud L, Peyretaillade E, Kucma D, Arhaliass A, Thouand G, Cogne G, Goncalves O: **Characterization of an easy-to-use method for the routine analysis of the central metabolism using an affordable low-resolution GC-MS system: application to Arthrospira platensis**. *Anal Bioanal Chem* 2018, **410**:1341–1361.

10. Willacey CCW, Naaktgeboren M, Lucumi Moreno E, Wegrzyn AB, van der Es D, Karu N, Fleming RMT, Harms AC, Hankemeier T: **LC-MS/MS analysis of the central energy and carbon metabolites in biological samples following derivatization by dimethylaminophenacyl bromide**. *J Chromatogr A* 2019, **1608**, 460413.

11. Li P, Su M, Chatterjee M, Lammerhofer M: **Targeted analysis of sugar phosphates from glycolysis pathway by phosphate methylation with liquid chromatography coupled to tandem mass spectrometry**. *Anal Chim Acta* 2022, **1221**, 340099.

12. Meyer M, Montero L, Meckelmann SW, Schmitz OJ: **Comparative study for analysis of carbohydrates in biological samples**. *Anal Bioanal Chem* 2022, **414**:2117–2130.

13. Nemkov T, Hansen KC: **D'Alessandro A: a three-minute method for high-throughput quantitative metabolomics and quantitative tracing experiments of central carbon and nitrogen pathways**. *Rapid Commun Mass Spectrom* 2017, **31**:663–673.

14. Ngere JB, Ebrahimi KH, Williams R, Pires E, Walsby-Tickle J, McCullagh JSO: **Ion-exchange chromatography coupled to mass spectrometry in life science, environmental, and medical research**. *Anal Chem* 2023, **95**:152–166.

15. Walsby-Tickle J, Gannon J, Hvinden I, Bardella C, Abboud MI, Nazeer A, Hauton D, Pires E, Cadoux-Hudson T, Schofield CJ, McCullagh JSO: **Anion-exchange chromatography mass spectrometry provides extensive coverage of primary metabolic pathways revealing altered metabolism in IDH1 mutant cells**. *Commun Biol* 2020, **3**:247.

16. * Winter H, Kaisaki PJ, Harvey J, Giacopuzzi E, Ferla MP, Pentony MM, Knight SJL, Sharma RA, Taylor JC, McCullagh JSO: **Identification of circulating genomic and metabolic biomarkers in intrahepatic cholangiocarcinoma**. *Cancers* 2019, **11**.

This study reports the development and validation of an untargeted anion-exchange - tandem mass spectrometry (IC-MS/MS) method, characterising 431 anionic metabolite standards. It demonstrated comprehensive characterisation of metabolites found in central carbon metabolism including sugar mono and di-phosphate and nucleotides but not amino acids. Application was illustrated via the analysis of isocitrate dehydrogenase 1 (IDH1) mutant and wild-type glioblastoma cell extracts.

17. Riffelmacher T, Clarke A, Richter FC, Stranks A, Pandey S, Danielli S, Hublitz P, Yu Z, Johnson E, Schwerd T, *et al.*: **Autophagy-Dependent generation of free fatty acids is critical for normal neutrophil differentiation**. *Immunity* 2017, **47**:466–480 e465.

18. Schulthess J, Pandey S, Capitani M, Rue-Albrecht KC, Arnold I, Franchini F, Chomka A, Ilott NE, Johnston DGW, Pires E, *et al.*: **The short chain fatty acid butyrate imprints an antimicrobial program in macrophages**. *Immunity* 2019, **50**:432–445 e437.

19. Si-Hung L, Troyer C, Causon T, Hann S: **Sensitive quantitative analysis of phosphorylated primary metabolites using selective metal oxide enrichment and GC- and IC- MS/MS**. *Talanta* 2019, **205**, 120147.

20. Favara DM, Zois CE, Haider S, Pires E, Sheldon H, McCullagh J, Banham AH, Harris AL: **ADGRL4/ELTD1 silencing in endothelial cells induces ACLY and SLC25A1 and alters the cellular metabolic profile**. *Metabolites* 2019, **9**.

21. Haythorne E, Lloyd M, Walsby-Tickle J, Tarasov AI, Sandbrink J, Portillo I, Exposito RT, Sachse G, Cyranka M, Rohm M, *et al.*: **Altered glycolysis triggers impaired mitochondrial metabolism and mTORC1 activation in diabetic beta-cells**. *Nat Commun* 2022, **13**:6754.

22. Kadir AA, Stubbs BJ, Chong CR, Lee H, Cole M, Carr C, Hauton D, McCullagh J, Evans RD, Clarke K: **On the interdependence of ketone body oxidation, glycogen content, glycolysis and energy metabolism in the heart**. *J Physiol* 2023, **601**:1207–1224.

23. Zois CE, Hendriks AM, Haider S, Pires E, Bridges E, Kalamida D, Voukantsis D, Lagerholm BC, Fehrmann RSN, den Dunnen WFA, *et al.*: **Liver glycogen phosphorylase is upregulated in glioblastoma and provides a metabolic vulnerability to high dose radiation**. *Cell Death Dis* 2022, **13**:573.

24. Reinbold R, Hvinden IC, Rabe P, Herold RA, Finch A, Wood J, Morgan M, Staudt M, Clifton IJ, Armstrong FA, *et al.*: **Resistance to the isocitrate dehydrogenase 1 mutant inhibitor ivosidenib can be overcome by alternative dimer-interface binding inhibitors**. *Nat Commun* 2022, **13**:4785.

25. Liu S, Abboud MI, John T, Mikhailov V, Hvinden I, Walsby-Tickle J, Liu X, Pettinati I, Cadoux-Hudson T, McCullagh JSO, Schofield CJ: **Roles of metal ions in the selective inhibition of oncogenic variants of isocitrate dehydrogenase 1**. *Commun Biol* 2021, **4**:1243.

26. Skaripa-Koukelli I, Hauton D, Walsby-Tickle J, Thomas E, Owen J, Lakshminarayanan A, Able S, McCullagh J, Carlisle RC, Vallis KA: **3-Bromopyruvate-mediated MCT1-dependent metabolic perturbation sensitizes triple negative breast cancer cells to ionizing radiation**. *Cancer Metabol* 2021, **9**:37.

27. Honarmand Ebrahimi K, Vowles J, Browne C, McCullagh J, James WS: **ddhCTP produced by the radical-SAM activity of RSAD2 (viperin) inhibits the NAD(+) -dependent activity of enzymes to modulate metabolism**. *FEBS Lett* 2020, **594**: 1631–1644.

28. Sarac H, Morova T, Pires E, McCullagh J, Kaplan A, Cingoz A, Bagci-Onder T, Onder T, Kawamura A, Lack NA: **Systematic characterization of chromatin modifying enzymes identifies KDM3B as a critical regulator in castration resistant prostate cancer**. *Oncogene* 2020, **39**:2187–2201.

29. Castanar L: **Pure shift NMR: past, present, and future**. *Magn Reson Chem* 2018, **56**:874–875.

30. Lee M, Nurse P: **Cell cycle control genes in fission yeast and mammalian cells**. *Trends Genet* 1988, **4**:287–290.

31. Lopez JM, Cabrera R, Maruenda H: **Ultra-Clean Pure Shift (1)H-NMR applied to metabolomics profiling**. *Sci Rep* 2019, **9**:6900.

32. Stark P, Zab C, Porzel A, Franke K, Rizzo P, Wessjohann LA: **PSYCHE-A valuable experiment in plant NMR-metabolomics**. *Molecules* 2020, **25**.

33. Watermann S, Schmitt C, Schneider T, Hackl T: **Comparison of regular, pure shift, and fast 2D NMR experiments for determination of the geographical origin of walnuts**. *Metabolites* 2021, **11**.

34. Bo Y, Feng J, Xu J, Huang Y, Cai H, Cui X, Dong J, Ding S, Chen Z: **High-resolution pure shift NMR spectroscopy offers better metabolite discrimination in food quality analysis**. *Food Res Int* 2019, **125**, 108574.

35. Santacruz L, Hurtado DX, Doohan R, Thomas OP, Puyana M, Tello E: **Metabolomic study of soft corals from the Colombian Caribbean: PSYCHE and (1)H-NMR comparative analysis**. *Sci Rep* 2020, **10**:5417.

36. Bertho G, Lordello L, Chen X, Lucas-Torres C, Oumezziane IE, Caradeuc C, Baudin M, Nuan-Aliman S, Thieblemont C, Baud V, Giraud N: **Ultrahigh-resolution NMR with water signal suppression for a deeper understanding of the action of anti-metabolic drugs on diffuse large B-cell lymphoma**. *J Proteome Res* 2022, **21**:1041–1051.

37. Van QN, Issaq HJ, Jiang Q, Li Q, Muschik GM, Waybright TJ, Lou H, Dean M, Uitto J, Veenstra TD: **Comparison of 1D and 2D NMR spectroscopy for metabolic profiling**. *J Proteome Res* 2008, **7**:630–639.

38. Féraud B, Govaerts B, Verleysen M, de Tullio P: **Statistical treatment of 2D NMR COSY spectra in metabolomics: data preparation, clustering-based evaluation of the Metabolomic Informative Content and comparison with 1H-NMR**. *Metabolomics* 2015, **11**:1756–1768.

39. Zhang B, Powers R, O'Day EM: **Evaluation of non-uniform sampling 2D (1)H-(13)C HSQC spectra for semi-quantitative metabolomics**. *Metabolites* 2020, **10**.

40. Marchand J, Martineau E, Guitton Y, Le Bizec B, Dervilly-Pinel G, Giraudeau P: **A multidimensional (1)H NMR lipidomics workflow to address chemical food safety issues**. *Metabolomics* 2018, **14**:60.

41. Feraud B, Martineau E, Leenders J, Govaerts B, de Tullio P, Giraudeau P: **Combining rapid 2D NMR experiments with novel pre-processing workflows and MIC quality measures for metabolomics**. *Metabolomics* 2020, **16**:42.

42. Jiang L, Howlett K, Patterson K, Wang B: **Introduction of a new method for two-dimensional NMR quantitative analysis in metabolomics studies**. *Anal Biochem* 2020, **597**, 113692.

43. Schatzlein MP, Becker J, Schulze-Sunninghausen D, Pineda-Lucena A, Herance JR, Luy B: **Rapid two-dimensional ALSOFAST-HSQC experiment for metabolomics and fluxomics studies: application to a (13)C-enriched cancer cell model treated with gold nanoparticles**. *Anal Bioanal Chem* 2018, **410**:2793–2804.

44. Schulze-Sunninghausen D, Becker J, Koos MRM, Luy B: **Improvements, extensions, and practical aspects of rapid ASAP-HSQC and ALSOFAST-HSQC pulse sequences for studying small molecules at natural abundance**. *J Magn Reson* 2017, **281**:151–161.

45. Krishnamurthy K: **CRAFT (complete reduction to amplitude frequency table)–robust and time-efficient Bayesian approach for quantitative mixture analysis by NMR**. *Magn Reson Chem* 2013, **51**:821–829.

46. Rubtsov DV, Waterman C, Currie RA, Waterfield C, Salazar JD, Wright J, Griffin JL: **Application of a Bayesian deconvolution approach for high-resolution (1)H NMR spectra to assessing the metabolic effects of acute phenobarbital exposure in liver tissue**. *Anal Chem* 2010, **82**:4479–4485.

47. Hulse SG, Foroozandeh M: **Newton meets Ockham: parameter estimation and model selection of NMR data with NMR-EsPy**. *J Magn Reson* 2022, **338**, 107173.

48. * Tang F, Krishnamurthy K, Janovick J, Crawford L, Wang S, Hatzakis E: **Advancing NMR-based metabolomics using complete reduction to amplitude frequency table: cultivar differentiation of black ripe table olives as a case study**. *Food Chem* 2023, **405**, 134868.

The authors describe an alternative approach to the FT to extract spectral parameters from time-domain NMR signals which relies on regularizes non-linear optimization. They demonstrate that their method extracts meaningful parameters from 1D NMR spectra and provide an open-source python package which is fully intergrated in to Bruker Topspin software so that the method can be easily implemented by the NMR community.

49. Johnson H, Yates T, Leedom G, Ramanathan C, Puppa M, van der Merwe M, Tipirneni-Sajja A: **Multi-tissue time-domain NMR metabolomics investigation of time-restricted feeding in male and female nile grass rats**. *Metabolites* 2022, **12**.

50. Johnson H, Puppa M, van der Merwe M, Tipirneni-Sajja A: **CRAFT for NMR lipidomics: targeting lipid metabolism in leucine-supplemented tumor-bearing mice**. *Magn Reson Chem* 2021, **59**:138–146.

51. Schmid N, Bruderer S, Paruzzo F, Fischetti G, Toscano G, Graf D, Fey M, Henrici A, Ziebart V, Heitmann B, *et al.*: **Deconvolution of 1D NMR spectra: a deep learning-based approach**. *J Magn Reson* 2023, **347**, 107357.

52. Huang Y, Zhao J, Wang Z, Orekhov V, Guo D, Qu X: **Exponential signal reconstruction with deep hankel matrix factorization**. *IEEE Transact Neural Networks Learn Syst* 2023, **34**:6214–6226.

53. Li DW, Hansen AL, Yuan C, Bruschweiler-Li L, Bruschweiler R: **DEEP picker is a deep neural network for accurate deconvolution of complex two-dimensional NMR spectra**. *Nat Commun* 2021, **12**:5229.

54. * Du H, Gu X, Chen J, Bai C, Duan X, Hu K: **GIPMA: global intensity-guided peak matching and alignment for 2D (1)H-(13) C HSQC-based metabolomics**. *Anal Chem* 2023, **95**:3195–3203.

The authors describe a novel method for NMR peak picking and deconvolution using a deep neural network trained on synthetic spectra. The performance of the new method is demonstrated on 2D NMR spectra of proteins as well as mouse urine metabolomics samples providing strong evidence that this method could be used to improve automation and standarziation of metabolomics analysis in future.

55. Kim HW, Zhang C, Cottrell GW, Gerwick WH: **SMART-Miner: a convolutional neural network-based metabolite identification from (1) H-(13) C HSQC spectra**. *Magn Reson Chem* 2022, **60**: 1070–1075.

56. Wishart DS, Cheng LL, Copie V, Edison AS, Eghbalnia HR, Hoch JC, Gouveia GJ, Pathmasiri W, Powers R, Schock TB, *et al.*: **NMR and metabolomics-A roadmap for the future**. *Metabolites* 2022, **12**.

57. Luchinat E, Barbieri L, Cremonini M, Banci L: **Protein in-cell NMR spectroscopy at 1.2 GHz**. *J Biomol NMR* 2021, **75**: 97–107.

58. Wishart DS, Rout M, Lee BL, Berjanskii M, LeVatte M, Lipfert M: **Practical aspects of NMR-based metabolomics**. *Handb Exp Pharmacol* 2023, **277**:1–41.

59. Edgar M, Percival BC, Gibson M, Jafari F, Grootveld M: **Low-field benchtop NMR spectroscopy as a potential non-stationary tool for point-of-care urinary metabolite tracking in diabetic conditions**. *Diabetes Res Clin Pract* 2021, **171**, 108554.

60. Izquierdo-Garcia JL, Comella-Del-Barrio P, Campos-Olivas R, Villar-Hernandez R, Prat-Aymerich C, De Souza-Galvao ML, Jimenez-Fuentes MA, Ruiz-Manzano J, Stojanovic Z, Gonzalez A, *et al.*: **Discovery and validation of an NMR-based metabolomic profile in urine as TB biomarker**. *Sci Rep* 2020, **10**, 22317.

61. Ruiz-Cabello J, Sevilla IA, Olaizola E, Bezos J, Miguel-Coello AB, Munoz-Mendoza M, Beraza M, Garrido JM, Izquierdo-Garcia JL: **Benchtop nuclear magnetic resonance-based metabolomic approach for the diagnosis of bovine tuberculosis**. *Transbound Emerg Dis* 2022, **69**:e859–e870.

62. Finch N, Percival B, Hunter E, Blagg RJ, Blackwell E, Sagar J, Ahmad Z, Chang MW, Hunt JA, Mather ML, *et al.*: **Preliminary demonstration of benchtop NMR metabolic profiling of feline urine: chronic kidney disease as a case study**. *BMC Res Notes* 2021, **14**:469.

63. Alonso-Moreno P, Rodriguez I, Izquierdo-Garcia JL: **Benchtop NMR-based metabolomics: first steps for biomedical application**. *Metabolites* 2023, **13**.

64. Ausmees K, Reimets N, Reile I: **Understanding parahydrogen hyperpolarized urine spectra: the case of adenosine derivatives**. *Molecules* 2022, **27**.

65. Ribay V, Dey A, Charrier B, Praud C, Mandral J, Dumez JN, Letertre MPM, Giraudeau P: **Hyperpolarized (13) C NMR spectroscopy of urine samples at natural abundance by quantitative dissolution dynamic nuclear polarization**. *Angew Chem Int Ed Engl* 2023, **62**, e202302110.

66. Dey A, Charrier B, Martineau E, Deborde C, Gandriau E, Moing A, Jacob D, Eshchenko D, Schnell M, Melzi R, *et al.*: **Hyperpolarized NMR metabolomics at natural (13)C abundance**. *Anal Chem* 2020, **92**:14867–14871.

67. Judge PT, Sesti EL, Price LE, Albert BJ, Alaniva N, Saliba EP, Halbritter T, Sigurdsson ST, Kyei GB, Barnes AB: **Dynamic nuclear polarization with electron decoupling in intact human cells and cell lysates**. *J Phys Chem B* 2020, **124**:2323–2330.

68. Honrao C, Teissier N, Zhang B, Powers R, O'Day EM: **Gadolinium-based paramagnetic relaxation enhancement agent enhances sensitivity for NUS multidimensional NMR-based metabolomics**. *Molecules* 2021, **26**.

69. Letertre MPM, Dervilly G, Giraudeau P: **Combined nuclear magnetic resonance spectroscopy and mass spectrometry approaches for metabolomics**. *Anal Chem* 2021, **93**:500–518.

70. * Ghosh R, Bu G, Nannenga BL, Sumner LW: **Recent developments toward integrated metabolomics technologies (UHPLC-MS-SPE-NMR and MicroED) for higher-throughput confident metabolite identifications**. *Front Mol Biosci* 2021, **8**, 720955.

This review explores the combination of NMR and MS approaches in metabolomics from the combination of data produced using completely independent sample preparation and analysis workflows through to approaches which align workflows and even integrate the physical techniques themselves.

71. Wagner L, Zargar M, Kalli C, Fridjonsson EO, Ling NNA, May EF, Zhen J, Johns ML: **Solid-phase extraction nuclear magnetic resonance (SPE-NMR): prototype design, development, and automation**. *Ind Eng Chem Res* 2020, **59**:20836–20844.

72. Zhong AB, Muti IH, Eyles SJ, Vachet RW, Sikora KN, Bobst CE, Calligaris D, Stopka SA, Agar JN, Wu CL, *et al.*: **Multiplatform metabolomics studies of human cancers with NMR and mass spectrometry imaging**. *Front Mol Biosci* 2022, **9**, 785232.