



Universiteit
Leiden
The Netherlands

Innovative sample preparation and handling strategies for automated and high-throughput metabolomics

Miggiels, A.L.W.

Citation

Miggiels, A. L. W. (2024, March 12). *Innovative sample preparation and handling strategies for automated and high-throughput metabolomics*. Retrieved from <https://hdl.handle.net/1887/3721725>

Version: Publisher's Version

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/3721725>

Note: To cite this publication please use the final published version (if applicable).

Chapter 2

Novel Technologies for Metabolomics: More for less

Paul Miggiels, Bert Wouters, Gerard JP van Westen, Anne-Charlotte Dubbelman, Thomas
Hankemeier

Trends in Analytical Chemistry (2019)

Volume 120, November 2019 115323

Abstract

The human metabolome provides a direct physiological readout of an individual's actual health state and includes biomarkers that may predict disease or response to a treatment. The discovery and validation of these metabolomic biomarkers requires large-scale cohort studies, typically involving thousands of samples. This analytical challenge drives novel technological developments to enable faster, cheaper, and more comprehensive metabolomic analysis: *more for less*. This review summarises recent (2012-2018) developments towards this goal in all aspects of the analytical workflow, in relation to NMR but primarily to mass spectrometry (MS). Recent trends include miniaturisation and automation of extraction techniques, online coupling to fast analysis methods including direct infusion ion mobility MS, integrated microfluidic devices, and sharing and standardizing metabolomics software and data. The technological advances in metabolomics support its widespread application, integration with other -omics fields, and ultimately disease prediction and precision medicine.

Introduction

Metabolomics is defined as an analytical approach for the identification and quantification of metabolites, i.e. small molecules with a molecular weight <1500 Da. The Human Metabolome Database¹ currently contains over 114,000 metabolite entries such as peptides, lipids, amino acids, nucleic acids, carbohydrates and organic acids, with a wide dynamic concentration range from high abundance (>1 μ M) to relatively low abundance (<1 nM). The metabolome is influenced by internal and external factors, and therefore reflects the actual health status of an individual. A full readout of the metabolome provides a wealth of information that can be used to identify metabolic profiles that predict disease risk, disease progression, and treatment outcome. Studies have shown that usually thousands of samples are necessary to find novel metabolic biomarker profiles, and even more for validation and replication². The number of samples increases even further when we want to identify early disease biomarkers using population-based metabolic profiling. To realise the potential of metabolomics for disease prediction and precision medicine, large-scale data acquisition is needed with sample sizes typically in the order of thousands. The impact and advantages of profiling such large cohorts, and the effects on study design, were recently elaborately reviewed by Zampieri et al.³

Metabolomics currently uses two main techniques: nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). Mass spectrometry is hereby often coupled with a separation technique, such as liquid chromatography (LC), gas chromatography (GC) or capillary electrophoresis (CE). As recently reported for the human metabolome database for 2018 (HMDB 4.0)¹, the number of metabolites with experimentally measured spectra for NMR, MS/MS, and GC-MS was 1494 (24%), 2265 (36%), and 2544 (40%), respectively. On a per-analysis basis, MS provides a broader coverage than NMR; the average number of reported metabolites per analysis is 37 (NMR) and 197 (MS) for human samples in the MetaboLights and Metabolomics Workbench repositories¹.

Nonetheless, NMR is currently more established for high-throughput analysis than MS in terms of analysis time and cost per sample. Typically 3-8 min is required for a ¹H-NMR profile at 10-25 €/sample versus 5-30 min for an LC-MS profile at 30-150 €/sample, while often more than one LC-MS profile is acquired. The wider coverage and potential gain in throughput expressed as metabolites/minute is higher with MS due to its sensitivity, which explains why more novel technologies are being developed in this field as compared to NMR.

¹ Data extracted on October 31st, 2018. Studies with no metabolites reported were excluded.

Concerning sample throughput for MS, direct introduction of a sample into the ionisation chamber of an MS would yield the highest throughput. However, a trade-off exists between sample throughput and metabolite coverage. Introducing mixtures of compounds of different structures, concentrations, proton affinities, etc. into the ionisation chamber leads to matrix effects; compounds affect each other's ionisation efficiency. This may compromise quantification, and in case of ion suppression of low-abundant analytes it may result in a loss of relevant sample information as these analytes are not detected anymore. Additionally, it is problematic when using MS only to identify isomeric compounds with identical fragmentation patterns (e.g., enantiomers or diastereoisomers), or to discriminate precursor ions from identical fragment ions formed by in-source fragmentation (e.g., adenosine triphosphate fragmenting in-source into adenosine diphosphate). The key to minimizing these effects is to use sample preparation and/or sample separation prior to MS. However, these steps usually increase both analysis time and costs.

The analytical challenge posed by high-throughput metabolomics drives technological developments into the direction of more exhaustive analysis using smaller samples and shorter analysis times at a lower cost-per-sample: *more for less*. This review intends to identify recent developments and trends for metabolomics from 2012 onwards, that are promising for higher sample throughput without compromising metabolite coverage or vice versa. We will start with a short literature overview on the 6-year trends of the more established metabolomics techniques. After a brief discussion on recent NMR developments, we will discuss promising developments spanning the entire mass spectrometry-based workflow, from sample preparation, separation, and introduction for MS, to data acquisition and data analysis.

Discussion

Literature overview

A literature survey of publications on metabolomics in the period 2012-2018 (Figure 1A) shows the growing importance and adaptation of metabolomics, continuing a trend that was previously observed by Kuehnbaum and Britz-McKibbin⁴. The projected number of publications for 2018 where the analytical technique is specified is expected to reach approximately 2200. The trends in Figure 1B show that LC-MS has recently overtaken NMR as the dominant technique reported in peer-reviewed publications, while CE-MS remains a rather niche technique. Furthermore, Figure 1C shows the growing use of fast sample introduction techniques for MS (e.g., direct injection and ambient desorption) and ion-mobility separations.

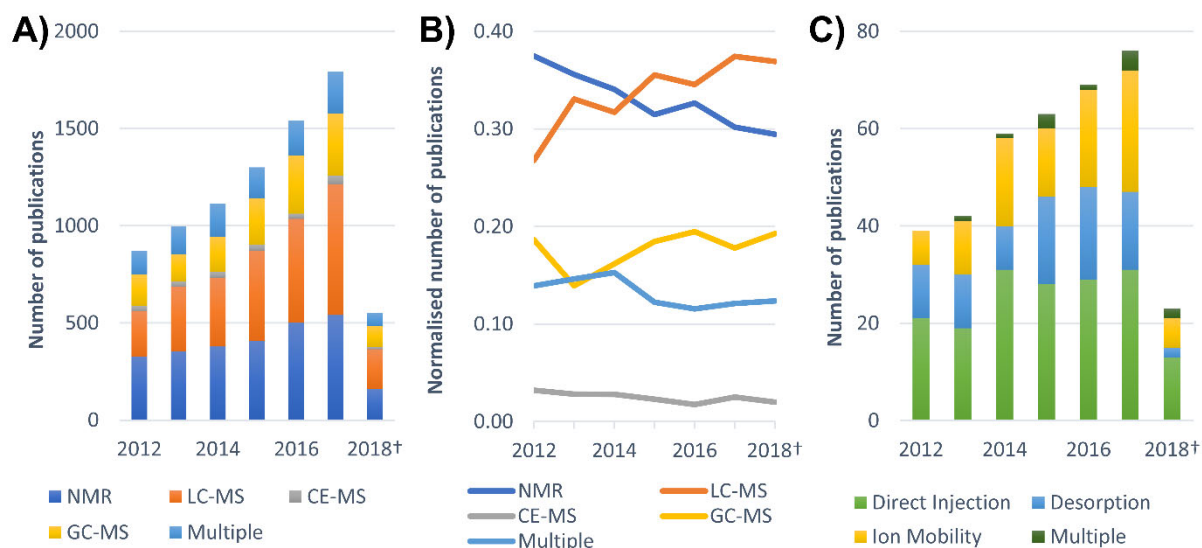


Figure 1. Literature survey of publications with metabolom* OR metabonom* in the title, abstract, or keywords in the period 2012-2018 (up to May 7th, 2018) on Web of Science: (A) number of publications mentioning the use of the main techniques in metabolomics: NMR, LC-MS, GC-MS and CE-MS, or multiple; (B) relative number of publications (trend plot) of the main techniques in metabolomics; (C) number of mass spectrometry-related publications mentioning the use of fast sample introduction methods (direct injection and ambient desorption ionisation), ion-mobility separation, or a combination of these.

NMR

One-third of the recent academic publications in metabolomics report NMR as the used technique (Figure 1B). NMR is highly reproducible, suitable for high-throughput analysis, cost-efficient, and still unrivalled when it comes to quantitation, or structural identification of unknown compounds. Compared to GC-MS and LC-MS, NMR offers complimentary information for the analysis of more-abundant metabolites that are difficult to ionise or would require derivatisation, or are at very high concentrations. Its main downside is that it lacks sensitivity compared to MS.

The future of NMR-based metabolomics has been reviewed by Markley *et al.*⁵. Strategies to improve NMR sensitivity include established techniques such as the introduction of higher field magnets (operating at frequencies of 1.2 GHz or higher)⁶ and cryogenically-cooled NMR probes⁷, and emerging techniques such as high-temperature superconducting coils⁸, microcoil-NMR probes⁹, and hyperpolarization. As the costs for higher field (>600 MHz) NMR systems and cryo-probes are significant, microcoil NMR presents a cost-efficient approach to increase sensitivity especially for biomass-limited samples. Currently, with 600 MHz NMR systems using 150 μ L NMR probes, metabolites can be detected in the low nanomole-range with sufficient resolution for the detection of 50-100 metabolites, whereas with a 30 μ L cryoprobe sub-nanomole amounts can be detected.

Recently, 1 μL and sub- μL microcoil NMR probe heads have been developed, which are able to detect compounds down to 25 picomole with a 400 MHz NMR system¹⁰. Alternatively, hyperpolarization may offer a cost-efficient approach to improve NMR sensitivity. A recently developed method, SABRE-SHEATH, overcomes the short spin lifetimes (typically seconds) of biologically-relevant molecules by direct hyperpolarization of $^{15}\text{N}_2$ magnetization at room temperature and longer-lived $^{15}\text{N}_2$ singlet spin order. Theis et al. reported over 10,000-fold enhancements generating detectable NMR signals for over an hour¹¹. A current limitation is that molecular tags, e.g., diazirines, are selective for certain classes only. A different recent trend is the use of compact low-field NMR instruments which use permanent magnets in the range of 1-2 T, as reviewed by Blümich et al.¹² These compact and low-cost systems lower the threshold for the use of NMR in a range of applications including real-time reaction monitoring and quality control at an industrial site or research laboratory, albeit at the expense of sensitivity, and the need for proper sample preparation.

Sample preparation prior to MS

Mass-spectrometric analysis of complex samples poses a challenge due to matrix-related background noise and ion suppression, especially in the case of direct sample introduction. In case of untargeted metabolomics, sample preparation should be generic; therefore dilution or simple protein precipitation is often applied. On the other hand, when doing targeted metabolomics, selective extractions and stabilisation of metabolites can be more favourable. These extraction methods, including solid-phase extraction (SPE) and liquid-liquid extraction (LLE) can provide enrichment of the analytes from the matrix in a fraction of the time and costs of sample separation methods such as LC. Although these methods are robust, their preconcentration capacity is often limited, especially for complex or biomass-limited samples. Furthermore, their selectivity is often mentioned as a risk for bias, however it can be utilised to fractionate metabolite classes for subsequent analysis, for example shotgun lipidomics¹³. Recent trends in sample preparation include miniaturisation to improve preconcentration factors and extraction time while reducing reagent consumption (e.g., microextractions)¹⁴, automation, and the rise of fast electro-driven extractions.

Solid-Phase Extractions

Hyphenation of SPE and MS has potential for high-throughput analysis with deeper metabolite coverage. A wide variety of different solid phases is available (e.g., hydrophobic, mixed-mode, charged surfaces) which allow the enrichment of various metabolite classes. However, conventional packed-bed SPE cartridges often have limited preconcentration power due to their poor separation efficiency and the cumbersome elution step. The sample volume, biomass loaded and complexity

dictate the required bed capacity – and therewith the minimal elution volume – which limits the preconcentration factor.

Zhang et al. achieved sample-to-sample cycle times of 15 s with an automated SPE-IMS-MS setup based on a commercially-available automated SPE device (RapidFire, Agilent), using several small-volume SPE cartridges (mixed mode, graphitic carbon, and HILIC)¹³. This automated SPE systems results in a significant gain in throughput. However, the packed-bed cartridges still require relatively large elution volumes which limits the upconcentration for low-abundant metabolites.

Solid-phase microextraction (SPME) has been introduced to address some of these challenges. A polymer-coated fibre with a variety of available surface functionalities can be inserted directly into the vial headspace, into the liquid sample, or can even be exposed *in vivo*. The fibre is then transferred to an analytical platform for desorption and analysis. Direct coupling of SPME to MS remains challenging¹⁵, and a promising development has addressed this via an Open Port Probe (OPP) interface¹⁶. One of the advantages of eluting directly into the probe is that dilution is minimised. While this workflow still includes time-consuming steps such as equilibration of the fibre (30 min) and sample-extraction (up to 5 min), it has potential for high throughput (less than 20 s per sample) since these steps can be automated and performed in parallel. SPME for metabolomics is fairly new and its major drawback is relatively low metabolite coverage. It has proven its value in small-scale studies with various biofluids, and with the ongoing development of sorbent material we expect its application in larger studies with broader metabolite coverage to follow soon¹⁷.

Liquid-Liquid Micro Extraction (LLME)

LLE enables the fractionation of hydrophilic and hydrophobic metabolites in two immiscible phases (i.e., aqueous and organic). As samples for metabolomics are mostly aqueous (e.g., body fluids), LLE is used for either removal or extraction of hydrophobic metabolites (e.g., Bligh & Dyer). Theoretically, high preconcentration factors can be achieved by extracting into a small volume, but for practical reasons often relatively large volumes are still used. Developments in liquid-liquid micro extraction (LLME), whereby samples are extracted in microliter-range volumes of extraction solvent, have overcome these practical issues and have significantly improved extraction kinetics and preconcentration factors.

LLME approaches can be categorized as dispersive and non-dispersive (DLLME and nDLLME, respectively). In DLLME, a liquid is used to disperse the small-volume extraction solvent in order to drastically increase the effective surface area, resulting in near-instantaneous extraction¹⁸. DLLME methods still require a phase-separation step which proves to be challenging to automate

for high-throughput analysis¹⁹. In DLLME, analytes are extracted into a small-volume acceptor phase, e.g., a droplet. Droplet-based or continuous-flow approaches have been realised on microfluidic chips, which could lead to automated and parallelised extraction. On-line droplet-based three-phase LLME was demonstrated with almost three orders of magnitude upconcentration in several minutes²⁰. While the extraction kinetics of DLLME are slightly slower compared to LLME, this is compensated by its greater potential for automation²¹.

Emerging preconcentration technologies

Electro-driven extractions (EE) target the polar, charged analytes which constitute a large part of the human metabolome. Although EE has been around for many years, its use is not as widespread as SPE and LLE. It is exclusively applicable to chargeable metabolites and as it is based on electro-migration it has mostly been associated with CE. Recent developments increase the versatility of EE for hyphenation to LC, GC or direct injection²².

EE offers tuneable selectivity and shows potential for increased throughput and high preconcentration factors. By using a small-volume acceptor phase, EE can offer simultaneous enrichment and preconcentration. This has been demonstrated with two-phase²³ or three-phase²⁴ on-chip EE, whereby in the latter case the intermediate organic layer acts as a filter for hydrophobic metabolites. Automated three-phase EE into a 2- μ L droplet directly coupled to nanoESI-MS has been used for metabolomics to extract acylcarnitines in 3 min, as shown in Figure 2A²⁵. Similarly, electro-membrane extraction (EME) utilises a membrane impregnated with organic solvent as a filter. Recent developments to perform EME in parallel in a multiwell-format (as shown in Figure 2B) are a significant step towards large-scale automation²⁶.

Alternatively, preconcentration techniques can be used to further improve sensitivity for low-abundant analytes, complementary to prior enrichment steps. Two developments for controlled solvent reduction feature vacuum-assisted membrane evaporation²⁷ and machine-vision controlled evaporation from a pendant droplet²⁸. The former achieved a 10-fold solvent reduction in 60 min, whereas the latter achieved 10-fold reduction in a few minutes without significant loss of volatiles.

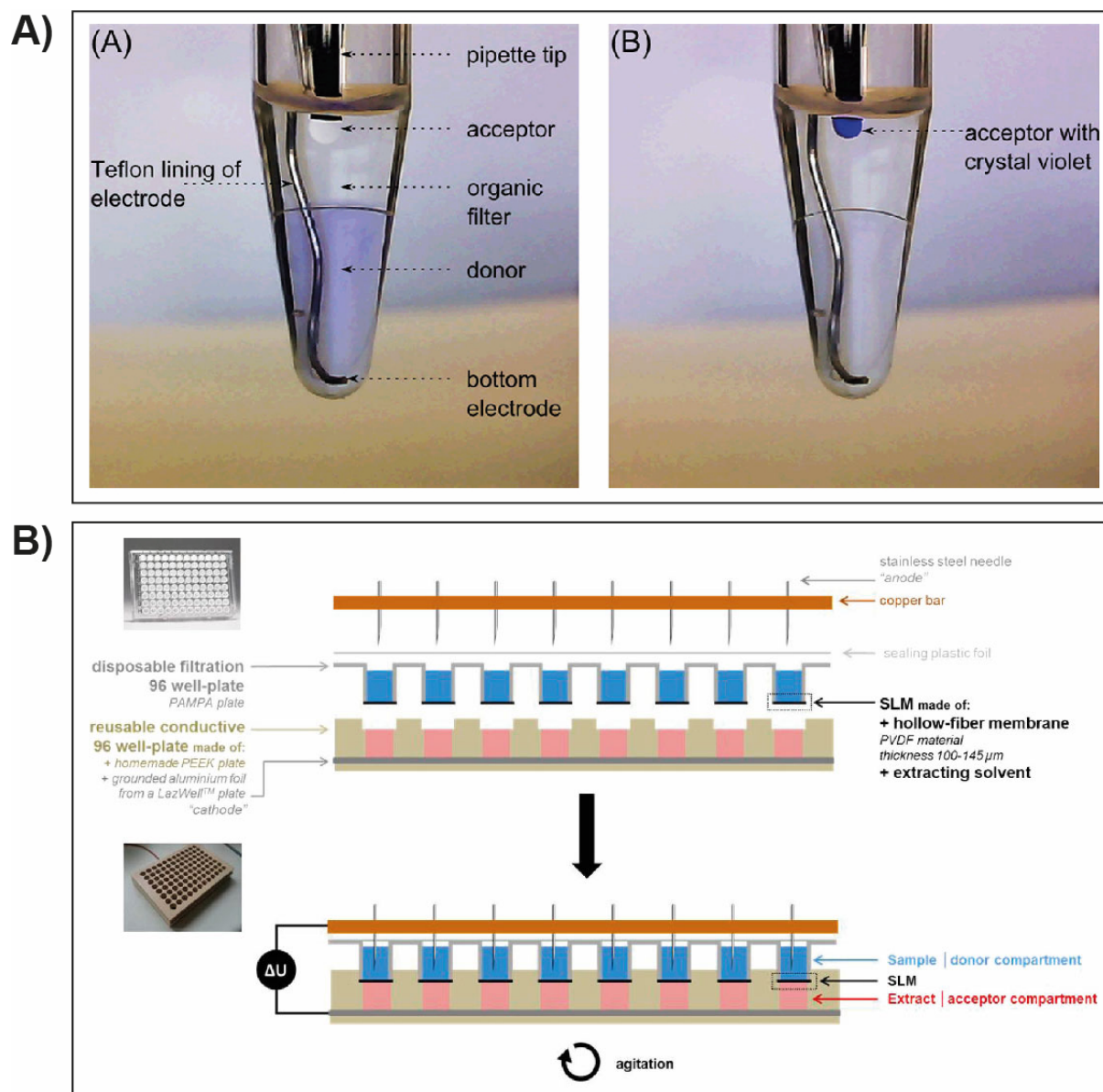


Figure 2. Two examples of electro-driven sample preparation. (A) Three-phase droplet electroextractions. Video stills of crystal violet subjected to three-phase EE at $t=0$ and $t=3$ min after applying the voltage. Reprinted with permission from Raterink et al.²⁵. Copyright 2013 American Chemical Society. (B) Schematic overview of a parallel-electromembrane extraction (Pa-EME) device using filtration 96-wells plate. Reprinted with permission from Drouin et al.²⁶. Copyright 2017 American Chemical Society.

Sample separation prior to MS

LC-MS

Compared to GC and CE, LC is the most popular separation strategy to hyphenate with MS in metabolomics research (Figure 1A). The development of novel LC technologies aiming to increase both sample throughput and metabolite coverage has accelerated since the introduction of the first commercially-available ultra-high pressure liquid chromatography (UHPLC) systems in 2004,

allowing high operating pressures (now up to 1400 bar) and flow rates (now up to 5 mL/min). A number of excellent reviews have recently been written on this topic^{29,30}.

Several ongoing trends can be distinguished, in the first place towards use of smaller (sub-2 μm) particle-size packed columns. These lead to higher separation efficiency due to a reduction in eddy diffusion and decreased resistance to mass transfer and shorter analysis times due to higher optimal flow velocity. While reversed-phase (RP) separation remains the most popular technique, complementary separation mechanisms such as hydrophilic interaction chromatography (HILIC), ion-exchange, and chiral LC are also becoming available in sub-2 μm particle size formats³¹. Secondly, a trend towards decreasing mobile-phase viscosity can be observed, including strategies such as the use of elevated mobile-phase temperature ($>60\text{ }^{\circ}\text{C}$), separation modes based on mobile phases with a high organic content (e.g., HILIC) or a low-viscosity (co-)solvent (e.g., CO_2 for supercritical fluid chromatography, SFC)³². With respect to the pressure limits of the LC system, lowering the viscosity of the mobile phase allows to achieve higher flow rates resulting to shorter analysis times. Additionally, the increase in solute diffusivity at higher temperatures leads to a higher optimal flow velocity and enhanced mass transfer kinetics. A third trend is the optimisation of column technology, including the use of core-shell particles which offer an increased efficiency due to reduction of eddy diffusion, longitudinal diffusion, and an improvement in mass-transfer resistance, even at high linear flow rates. Likewise, the use of perfectly-ordered pillar-array columns³³ as shown in Figure 3 or silica-based monolithic columns with higher permeability³⁴ has been typically explored for high-efficiency separation of complex samples. However, it can also be used to gain separation power in gradient separations of only several minutes³⁵.

While a plethora of chromatographic columns and conditions is available, finding the right combination for an application can be a daunting task. Kinetic plots as introduced by Desmet and co-workers are a powerful tool to compare the performance of chromatographic systems³⁶. These plots take into account information about the interplay between pressure, mobile-phase velocity and plate height, and their effects on the performance of different columns, in order to find the best possible combination of particle size, column length and temperature. If higher separation efficiencies and deeper metabolite coverage are necessary, multi-dimensional separations such as online comprehensive two-dimensional liquid chromatography ($\text{LC}\times\text{LC}$) can be used. Its two main advantages are greater selectivity by the use of two independent (orthogonal) retention mechanisms targeting different sample dimensions, and higher resolving power as the total peak capacity can be approximated by the product of the individual peak capacities. However, using $\text{LC}\times\text{LC}$ to its full potential can be difficult as design challenges include selecting orthogonal separation mechanisms, and solving eluent-compatibility issues between the two dimensions³⁷. We expect that

the use of predictive software-tools and optimisation algorithms to establish optimal conditions³⁸ will increase the use of LC×LC in metabolomics in the coming years.

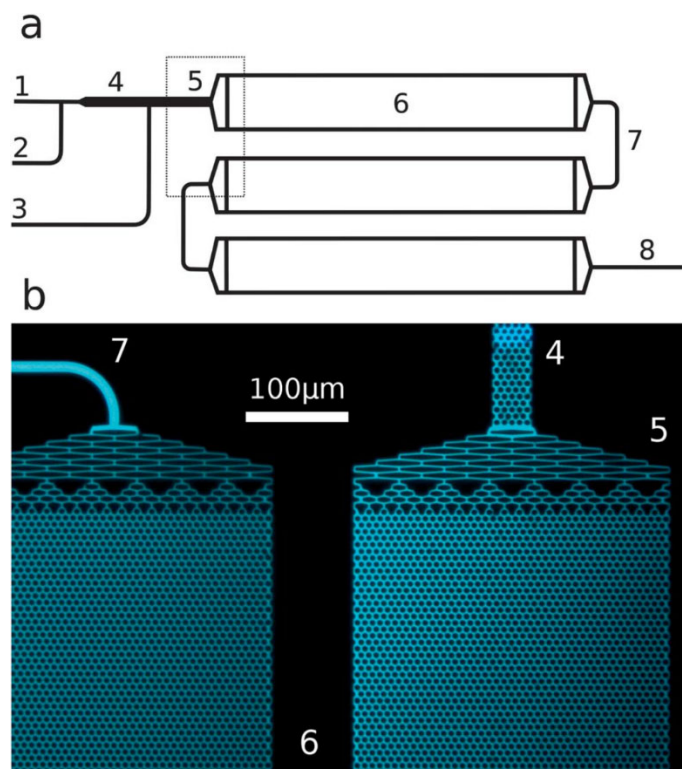


Figure 3. Perfectly-ordered micropillar-array LC column for increased separation efficiency. (A) Schematic overview of the different zones of a microfluidic device containing pillar-array columns. 1: inlet mobile phase, 2: inlet sample phase, 3: outlet sample phase, 4: injection box, 5: inlet distributor, 6: channel track, 7: connecting turn, 8: mobile phase outlet. (B) Optical fluorescence microscope image of the box at the channel section in A. Reproduced from Callewaert et al.³³ with permission of The Royal Society of Chemistry.

CE-MS

Capillary electrophoresis is predominantly hyphenated to mass spectrometry via ESI with a sheath-liquid interface. However, interfacing CE and ESI-MS with sufficient sensitivity and robustness has proven to be challenging. Current developments in bioanalysis applications mostly focus on increasing sensitivity, as was recently reviewed by Ramautar et al.³⁹. Sheathless ESI-MS interfacing was introduced to reduce sample dilution and background noise, resulting in more information per sample and pM-range levels of detection. Other approaches successfully reduced the sheath-liquid flow to tackle sheathless interfacing issues, with minimal loss in sensitivity⁴⁰. Sheath-liquid CE-MS is still advantageous for stability, and platinum (alloy) emitter tips are currently being adapted to overcome corrosion issues and further boost robustness for large-scale application. On-line coupling of SPE-CE-MS further aids to remove matrix compounds and improve the limits of detection³⁹. Additionally, the isocratic nature of CE separations allows for multi-segment or

overlapped injections, whereby multiple samples are injected in a single run and separated simultaneously to improve throughput⁴¹. The samples can be uniquely identified afterwards based on their mass spectra. Finally, CE allows for unique electrophoretic sample preconcentration methods generally referred to as “stacking”. A recent review showed that developments in stacking methods have led to sensitivity gains of several orders of magnitude⁴². CE has superior separation power and speed compared to LC-MS, and advances in MS-interfacing have brought sensitivity up to par. However, stability issues still prevent large-scale adaptation.

Ion-mobility separations

With the increasing number of commercially-available systems enabling ion-mobility separation (IMS), this gas-phase electrophoretic technique is an upcoming trend in metabolomics. It separates ions based on their shape, size, and charge. The measured drift time can be converted into the collisional cross section (CSS), a unique physicochemical property of an ion. A review by Ewing et al.⁴³ comprehensively describes the various technologies within ion-mobility separation, which are either dispersive (keeping all ions for MS analysis) or selective (excluding ions based on mobility). For metabolomics, the dispersive techniques drift-tube ion mobility (DTIMS) and travelling-wave ion mobility (TWIMS) have most commonly been applied over the last decade, but recently also selective techniques such as differential mobility separation (DMS) are gaining in popularity⁴⁴. While dispersive techniques are inherently more suitable for untargeted analysis, the selective techniques provide better orthogonality to mass spectrometric data. Recent developments in ion mobility aimed to increase the separation resolution and reduce the loss of ions. An example is prolonging the drift tube (and with that the resolution) using Structures for Lossless Manipulations (SLIM). When used in a smart arrangement, e.g., serpentine, which could even be combined with ion escalators and elevators to create multi-level SLIM devices, the length of the ion path can be increased without the need to increase the travelling-wave voltage with tube length^{45,46}. Another interesting approach increasing the separation resolution was introduced with Trapped Ion Mobility Spectrometry whereby ions are held stationary against a moving gas instead of the other way around⁴⁷.

Adding ion-mobility separation to LC-MS or SPE-MS analysis is appealing as the separation occurs in the milliseconds time scale, is orthogonal to e.g., LC and SPE, and is (low-) orthogonal to MS. As such, it can increase the peak capacity of regular LC separations and provide an extra identifier for metabolite identification^{48,49}. Additionally, ion mobility has the potential to separate isomers based on their shape and has even been applied to separate chiral components⁵⁰. Ion-mobility separation has been used for metabolomics studies, albeit limited in sample size. Examples include its application for the characterization of metabolic changes in colorectal cancer by Williams et al.

and for the comparison of the metabolome of different cells involved in cancer development by Paglia *et al.*^{51,52}. For more widespread use in the metabolomics field, ion mobility has a few limitations still to overcome. It can for example reduce sensitivity and increases file-sizes. For example, the ion-mobility MS method of Williams *et al.* needed 1.5 min in addition to each 3.5 min of data acquisition per sample to allow the large data files to be saved. In addition, if used in combination with LC ion mobility complicates data analysis by the additional dimension it provides. As it requires a longer scan time, the number of data points per peak (and consequently the peak capacity in ultrafast chromatographic separations) is limited and the separation will not aid the quantitative analysis as it occurs after the ionisation.

Sample introduction for MS

Direct sample introduction

For direct sample introduction, we can distinguish between direct infusion (DI) and flow-injection analysis (FIA). In DI-MS, a sample is continuously introduced into the ionisation chamber with a pump. While DI maintains the sample concentration, it suffers from large sample consumption and the need for extensive rinsing in between samples. For FIA, a sample plug is injected into a continuous fluid stream towards the MS. While consuming less sample and allowing drastically increased throughput, FIA does face a trade-off in sensitivity between dilution and ion suppression. Ion suppression can be reduced by mild dilution to gain sensitivity, however too much dilution will again result in loss of sensitivity. Additionally, any hypothesis has to be confirmed with subsequent targeted MS analysis if the signal cannot be uniquely identified due to limited separation or in-source fragmentation.

The biggest challenge related to direct sample introduction remains ion suppression, and as a result the relatively low number of metabolites that can be detected and quantified. As an alternative to direct sample introduction, a sample can be introduced by various forms of ambient desorption directly from sample surfaces, categorised as *i*) spray- or jet-based ambient ionisation techniques (e.g., DESI, desorption electrospray ionisation), *ii*) electric discharge-based ambient ionisation techniques (e.g., DART, direct analysis in real time), *iii*) ambient gas-, heat- or laser-assisted desorption/ionisation techniques (e.g., EESI (electrospray-assisted laser desorption/ionisation), and *iv*) acoustic or ultrasonic waves (e.g., surface acoustic wave nebulisation (SAWN) and acoustic droplet ejection (ADE))⁵³. In this latter category, the ADE-MS interface (Figure 4A) acoustically eject (sub)nL sample droplets from a microtiter plate directly to the MS at a rate of up to three samples per second for high-throughput analysis of small samples⁵⁴. Ambient MS techniques have recently been reviewed by Clendinen *et al.* with respect to their (increasingly popular) use in

metabolomics⁵⁵. They reported the advantages and limitations of eight ambient MS techniques that have recently been developed and applied to metabolomics. In general, the advantages of ambient MS are the reduced need for sample preparation, suitability for high-throughput analysis (seconds per sample), high sensitivity (sub-ng detection limits), and the quantitative capabilities (accuracy and precision) provided that proper internal standards are used. Disadvantages include its sensitivity to matrix effects, emphasising the need to use standards for reliable quantification. Ambient desorption directly from sample surfaces is still an emerging technique and has not yet been used for large cohort metabolic profiling. A promising ambient mass-spectrometric imaging development was recently reported by Abliz and co-workers⁵⁶, describing the use of air flow-assisted desorption electrospray ionization (AFADESI) for a sensitive and spatial *in-situ* analysis of tumor metabolism from lung cancer tissue samples and multivariate statistical analysis (MVSA) for identification of diagnostic biomarkers. This development could enable imaging analysis to be used as a molecular pathological tool for rapid, label-free histopathological diagnosis and image-guided surgery.

Hyphenation of microfluidic devices with MS

Integrated microfluidic devices for sample preparation, separation and/or introduction are emerging in the field of bioanalysis. Miniaturisation allows the incorporation of different components, e.g., the stationary phase and an ESI-tip, into a microfluidic platform with low dead-volume connections to minimise band-broadening effects⁵⁷. Furthermore, downscaling the system offers advantages such as improved sensitivity with concentration-dependent detectors, and increased ionisation efficiency of electrospray-ionisation mass spectrometry (ESI-MS) due to the low flow rates⁵⁸. Examples of commercially-released devices include the IonKey (Waters) and HPLC-Chip (Agilent) chipLC-ESI devices, and the ZipChip (908 Devices) chipCE-ESI device. A recent temperature-gradient chip-HPLC approach demonstrates the advantage of low thermal mass of chips for rapid and extreme temperature cycling. This system reduced the separation time by a factor of 2 down to 20 sec. for six analytes (Figure 4B)⁵⁹.

Alternatively, digital microfluidics platforms allow for fast and accurate manipulation of small volumes of sample and reagent, such as addition of internal standards, and can be directly interfaced to MS with an on-chip emitter tip⁶⁰. Moreover, droplet or digital microfluidics shares its discrete and array-based traits with desorption/ionisation techniques, making it an attractive match for desorption-based MS such as nanostructure-initiator mass spectrometry (NIMS) as an advanced, high-throughput technique⁶¹. Electro-driven separations also benefit greatly from the short distance between separation and detection with microfluidic chips, as it eliminates the need for

long capillaries that inherently increase separation times. This was for example demonstrated for the analysis of pharmaceuticals⁶² and amino acids⁶³ in under 3 minutes.

Considering these developments, and the availability of commercial solutions, it is clear that integrated microfluidics tools are promising for bioanalysis of low-volume samples. In the coming years, it could prove itself for large-scale, exhaustive, high-throughput metabolomics.

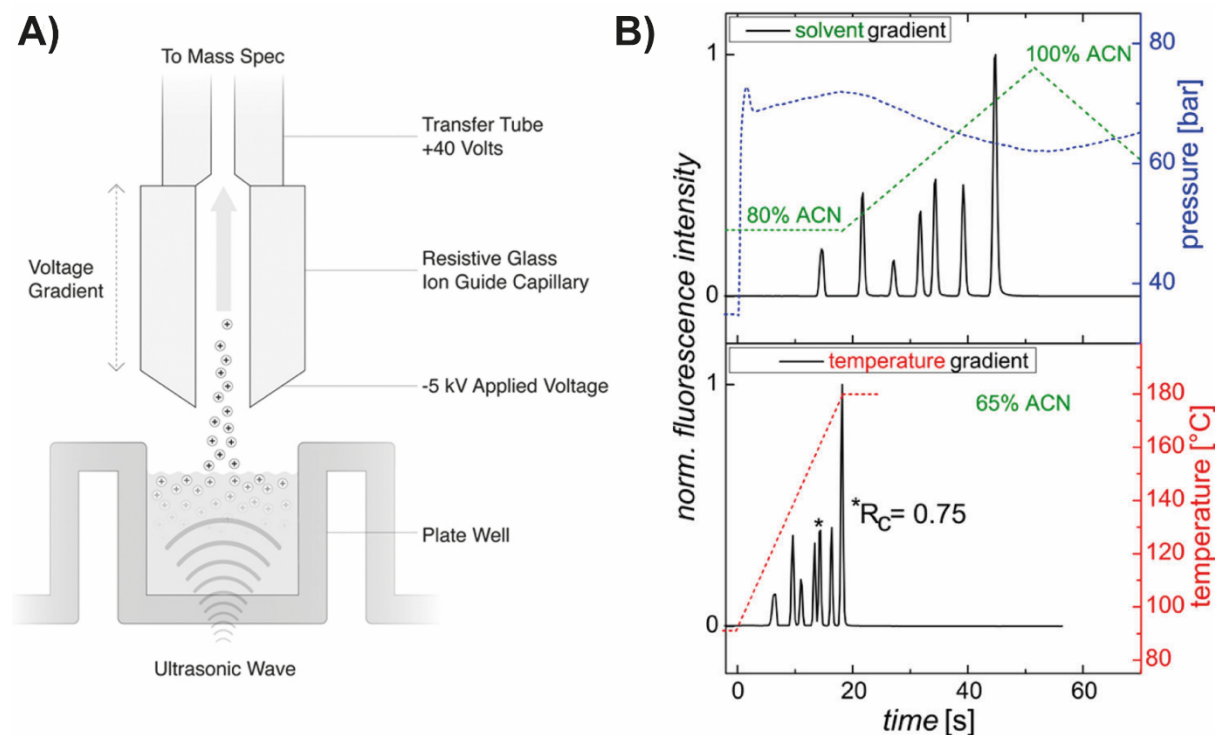


Figure 4. Two novel developments to increase throughput in sample introduction and separation. (A) Schematic overview of ADE-MS high-throughput interface, whereby (sub)nL droplets of sample are acoustically ejected into the mass spectrometer through a transfer tube. Combined, these droplets form an equivalent flow of low $\mu\text{L}/\text{min}$ -range. Reprinted from Sinclair et al.⁵⁴ with permission by SAGE Publications, Inc. (B) Chromatogram of temperature-gradient chip-HPLC shows more than twofold decrease in separation time. Reprinted with permission from Heiland et al.⁵⁹, copyright 2017 American Chemical Society.

MS data acquisition

The trend to achieve more for less in mass spectrometry-based metabolomics is reflected by the increasing use of high-resolution mass spectrometry (HRMS) for combined targeted and untargeted analysis and for simultaneous quantitative and qualitative analysis. Accordingly, development of MS data-acquisition protocols relates to this by offering possibilities for data-dependent and data-independent acquisition (DDA and DIA), whereby apart from a TOF-MS scan also MS/MS scans are being collected to obtain more information about the molecular structure⁶⁴.

In DDA, an MS/MS event can be triggered based on intensity, isotope pattern, or mass defect. Although the quality of the produced spectra is generally good, its drawbacks include missing interesting targets due to the stochastic nature of chosen selection criteria, and increasing the duty cycle time, thereby decreasing the number of data points per peak and affecting quantitative analysis. As a result, DIA techniques are more attractive for untargeted metabolomics. Within these data-independent protocols, a distinction can be made between those fragmenting all ions at once (e.g., all ion fragmentation (AIF), MS^E) and those consecutively fragmenting selected precursor ion windows (sequential window acquisition of all theoretical fragment ion spectra, SWATH). The latter generally produce cleaner MS/MS spectra, but at a cost of the duty cycle time.

Data (pre-)processing, analysis and exchange

Finally, the acquired metabolomics data needs to be translated to useful biological insights, a process that includes data pre-processing, peak annotation, post-processing, statistical analysis and pathway analysis. The increased data-acquisition rates, data complexity and study size bring new challenges for the process of data processing, analysis and exchange, similar to other fields that face increasing data quantities, such as high-throughput microscopy.

Metabolomics data comes in a wide variety, from NMR spectra to direct-injection MS or multidimensional separation-MS data. One challenge is the incompatibility of data formats between different processes or different software versions. Besides data processing and analysis with (non-complimentary) vendor software, we see an increasing availability of open-source and free-to-use software tools (comprehensively reviewed by Spicer et al.⁶⁵) which can perform one or more of these tasks. The BioContainer and the PhenoMeNal consortium address incompatibility by creation and online deposition of software containers that remove incompatibilities caused by dependencies of the installation or the version⁶⁵. Additionally, these containers can reproduce data analysis with the exact same software conditions.

A related second challenge is to make research data Findable, Accessible, Interoperable and Reusable (FAIR) so that data of different studies can be combined⁶⁶. An important tool to realise FAIR metabolomics data is the introduction and increasing use of metabolomics data repositories, such as the MetaboLights database (Europe)⁶⁷ and Metabolomics Workbench (United States)⁶⁸. To facilitate their use by the international community, efforts are being made to allow easy sharing and exchanging of data between repositories as exemplified by the creation of the online resource MetabolomeXchange (<http://www.metabolomexchange.org>), which lists the available (meta-)data sets from the various repositories. An additional advantage of these repositories is that it stimulates the metabolomics community towards the highly necessary data reporting standards⁶⁹.

Standardisation allows to combine multiple datasets within the field of metabolomics to increase statistical power, but also to integrate data with the other –omics fields for more exhaustive analysis of biological phenomena.

A third challenge is the computational power required to analyse the data sets and files with extraordinarily high information density. To illustrate this, May and McLean calculated theoretically that peak capacities exceeding 10^{12} could be achieved when coupling three-dimensional MS imaging with ion mobility and TOF-MS, with a peak-production rate of around 10^6 s^{-1} ⁷⁰. To address this challenge, the metabolomics society is increasingly making use of cloud-computing technologies, such as XC-MS Online and OpenMSI, wherein data-intensive computational work is distributed across a network of computers⁷⁰. A downside of this solution is the additional time required for data uploading and downloading.

With all these challenges it is easy to overlook the primary, seemingly trivial challenge: peak integration. This pivotal process in metabolic data analysis often still requires laborious manual checks to ensure that no noise has been misinterpreted as a peak and that peaks have been correctly integrated for quantitation. We envision that with increased availability of metabolomics data, algorithms can be better trained to this end and can benefit from self-learning tools. The alternative is to rely on vast amounts of data, whereby the power of using large numbers compensates errors caused by noise.

Conclusion

Metabolomics is a rapidly expanding field, yet its broad implementation is still hampered by low sample throughput and high cost per analysis. In this review, we have discussed recent technological developments and trends for metabolomics towards faster and more exhaustive analysis using smaller samples at a lower cost-per-sample: *more for less*. For the workhorse techniques, NMR and LC-MS, the developments mostly focus on gaining sensitivity (e.g., microcoil NMR and hyperpolarization) and faster/more efficient separations (e.g., UHPLC, reduced viscosity, improved column technologies). Developments in sample preparation are focused on miniaturisation, increased efficiency, and automation for online coupling to MS. Hyphenation of sample separation with MS still provides deeper metabolite coverage compared to direct injection MS, and through the emergence of microfluidic chips with an integrated separation column and electrospray emitter, speed and costs can be drastically improved. Likewise, we observe an increased use of ion mobility for ultra-fast separations. Combined with fast sample preparation,

this may lead to increased metabolite coverage in a relatively short analysis time. The increasing possibilities of data sharing initiatives, together with standardised data reporting, aid the development of fully-automated (open-source) data analysis platforms required to process the vast amounts of data acquired, and to integrate these data with other omics fields to enable novel biological insights.

We are convinced that by recent developments for all steps in the analytical workflow, metabolomics is on a fast lane towards wider implementation, comparable to the rise of genomics. Widespread integration of the omics fields may then pave the way to disease prediction and precision medicine, using metabolomics data obtained far more cost-efficiently than today.

Acknowledgements

This work received funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme under grant agreement No 667375, and from the Netherlands Organisation for Scientific Research (NWO) in the framework of the Technology Area COAST (MI3 project). Gerard JP van Westen would like to thank NWO domain TTW (VENI project 14410) for funding. The authors thank Michael van Vliet for his help in extracting the data from the MetaboLights and Metabolomic Workbench repositories.

References

1. Wishart, D. S. *et al.* HMDB 4.0: The human metabolome database for 2018. *Nucleic Acids Res.* **46**, D608–D617 (2018).
2. Beger, R. D. *et al.* Metabolomics enables precision medicine: “A White Paper, Community Perspective”. *Metabolomics* **12**, 149 (2016).
3. Zampieri, M., Sekar, K., Zamboni, N. & Sauer, U. Frontiers of high-throughput metabolomics. *Curr. Opin. Chem. Biol.* **36**, 15–23 (2017).
4. Kuehnbaum, N. L. & Britz-Mckibbin, P. New advances in separation science for metabolomics: Resolving chemical diversity in a post-genomic era. *Chemical Reviews* vol. 113 2437–2468 (2013).
5. Markley, J. L. *et al.* The future of NMR-based metabolomics. *Curr. Opin. Biotechnol.* **43**, 34–40 (2017).
6. Moser, E., Laistler, E., Schmitt, F. & Kontaxis, G. Corrigendum: Ultra-high field NMR and MRI-the role of magnet technology to increase sensitivity and specificity *Frontiers in Physics* vol. 5 1–15 (2017).
7. Jézéquel, T. *et al.* Absolute quantification of metabolites in tomato fruit extracts by fast 2D NMR. *Metabolomics* **11**, 1231–1242 (2015).
8. Ramaswamy, V. *et al.* Development of a¹³C-optimized 1.5-mm high temperature superconducting NMR probe. *J. Magn. Reson.* **235**, 58–65 (2013).
9. Saggiomo, V. & Velders, A. H. Simple 3D Printed Scaffold-Removal Method for the Fabrication of Intricate Microfluidic Devices. *Adv. Sci.* **2**, 1500125 (2015).
10. Fratila, R. M., Gomez, M. V., Sýkora, S. & Velders, A. H. Multinuclear nanoliter one-dimensional and two-dimensional NMR spectroscopy with a single non-resonant microcoil. *Nat. Commun.* **5**, (2014).
11. Theis, T. *et al.* Direct and cost-efficient hyperpolarization of long-lived nuclear spin states on universal ¹⁵N₂-diazirine molecular tags. *Sci. Adv.* **2**, 1–8 (2016).
12. Blümich, B. Introduction to compact NMR: A review of methods. *TrAC Trends Anal. Chem.* **83**, 2–11 (2016).
13. Zhang, X. *et al.* SPE-IMS-MS: An automated platform for sub-sixty second surveillance of endogenous metabolites and xenobiotics in biofluids. *Clin. Mass Spectrom.* **2**, 1–10 (2016).

14. Ocaña-González, J. A., Fernández-Torres, R., Bello-López, M. Á. & Ramos-Payán, M. New developments in microextraction techniques in bioanalysis. A review. *Analytica Chimica Acta* vol. 905 8–23 (2016).
15. Deng, J., Yang, Y., Wang, X. & Luan, T. Strategies for coupling solid-phase microextraction with mass spectrometry. *TrAC - Trends Anal. Chem.* **55**, 55–67 (2014).
16. Gómez-Ríos, G. A. *et al.* Open Port Probe Sampling Interface for the Direct Coupling of Biocompatible Solid-Phase Microextraction to Atmospheric Pressure Ionization Mass Spectrometry. *Anal. Chem.* **89**, 3805–3809 (2017).
17. Bojko, B. *et al.* Solid-phase microextraction in metabolomics. *TrAC - Trends Anal. Chem.* **61**, 168–180 (2014).
18. Mansour, F. R. & Khairy, M. A. Pharmaceutical and biomedical applications of dispersive liquid–liquid microextraction. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **1061–1062**, 382–391 (2017).
19. Alexovič, M., Horstkotte, B., Šrámková, I., Solich, P. & Sabo, J. Automation of dispersive liquid–liquid microextraction and related techniques. Approaches based on flow, batch, flow-batch and in-syringe modes. *TrAC - Trends Anal. Chem.* **86**, 39–55 (2017).
20. Gao, W. *et al.* Trace analysis of three antihistamines in human urine by on-line single drop liquid-liquid-liquid microextraction coupled to sweeping micellar electrokinetic chromatography and its application to pharmacokinetic study. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **904**, 121–127 (2012).
21. Alexovič, M., Horstkotte, B., Solich, P. & Sabo, J. Automation of static and dynamic non-dispersive liquid phase microextraction. Part 1: Approaches based on extractant drop-, plug-, film- and microflow-formation. *Anal. Chim. Acta* **906**, 22–40 (2016).
22. Oedit, A., Ramautar, R., Hankemeier, T. & Lindenburg, P. W. Electroextraction and electromembrane extraction: Advances in hyphenation to analytical techniques. *Electrophoresis* **37**, 1170–1186 (2016).
23. Schoonen, J. W. *et al.* Continuous-flow microelectroextraction for enrichment of low abundant compounds. *Anal. Chem.* **86**, 8048–8056 (2014).
24. Campos, C. D. M., Park, J. K., Neužil, P., da Silva, J. a. F. & Manz, A. Membrane-free electroextraction using an aqueous two-phase system. *RSC Adv.* **4**, 49485–49490 (2014).

25. Raterink, R.-J., Lindenburg, P. W., Vreeken, R. J. & Hankemeier, T. Three-Phase Electroextraction: A New (Online) Sample Purification and Enrichment Method for Bioanalysis. *Anal. Chem.* **85**, 7762–7768 (2013).
26. Drouin, N., Mandscheff, J. F., Rudaz, S. & Schappler, J. Development of a New Extraction Device Based on Parallel-Electromembrane Extraction. *Anal. Chem.* **89**, 6346–6350 (2017).
27. Fornells, E. *et al.* Evaporative membrane modulation for comprehensive two-dimensional liquid chromatography. *Anal. Chim. Acta* **1000**, 303–309 (2018).
28. Schoonen, J.-W. *et al.* Solvent Exchange Module for LC-NMR Hyphenation Using Machine Vision-Controlled Droplet Evaporation. *Anal. Chem.* **85**, 5734–5739 (2013).
29. De Vos, J., Broeckhoven, K. & Eeltink, S. Advances in Ultrahigh-Pressure Liquid Chromatography Technology and System Design. *Anal. Chem.* **88**, 262–278 (2016).
30. Fekete, S., Schappler, J., Veuthey, J. L. & Guilleme, D. Current and future trends in UHPLC. *TrAC - Trends Anal. Chem.* **63**, 2–13 (2014).
31. Kohler, I. & Giera, M. Recent advances in liquid-phase separations for clinical metabolomics. *J. Sep. Sci.* **40**, 93–108 (2017).
32. Jones, M. D. *et al.* Ultra high resolution SFC-MS as a high throughput platform for metabolic phenotyping: Application to metabolic profiling of rat and dog bile. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **966**, 200–207 (2014).
33. Callewaert, M. *et al.* Integration of uniform porous shell layers in very long pillar array columns using electrochemical anodization for liquid chromatography. *Analyst* **139**, 618–625 (2014).
34. Rigobello-Masini, M., Pentead, J. C. P. & Masini, J. C. Monolithic columns in plant proteomics and metabolomics. *Anal. Bioanal. Chem.* **405**, 2107–2122 (2013).
35. Song, Y. *et al.* Rapid quantitative method for the detection of phenylalanine and tyrosine in human plasma using pillar array columns and gradient elution. *Amino Acids* **48**, 1731–1735 (2016).
36. Broeckhoven, K. & Desmet, G. The future of UHPLC: Towards higher pressure and/or smaller particles? *TrAC - Trends in Analytical Chemistry* vol. 63 65–75 (2014).
37. Pirok, B. W. J., Gargano, A. F. G. & Schoenmakers, P. J. Optimizing separations in online comprehensive two-dimensional liquid chromatography. *J. Sep. Sci.* **41**, 68–98 (2018).

38. Pirok, B. W. J., Pous-Torres, S., Ortiz-Bolsico, C., Vivó-Truyols, G. & Schoenmakers, P. J. Program for the interpretive optimization of two-dimensional resolution. *J. Chromatogr. A* **1450**, 29–37 (2016).
39. Ramautar, R., Somsen, G. W. & de Jong, G. J. CE–MS for metabolomics: Developments and applications in the period 2014–2016. *Electrophoresis* vol. 38 190–202 (2017).
40. Liu, J.-X., Aerts, J. T., Rubakhin, S. S., Zhang, X.-X. & Sweedler, J. V. Analysis of endogenous nucleotides by single cell capillary electrophoresis-mass spectrometry. *Anal. Novemb.* **21**, 5835–5842 (2014).
41. Kuehnbaum, N. L. Multisegment Injection-Capillary Electrophoresis-Mass Spectrometry: A High-Throughput Platform in Metabolomics for Assessment of Lifestyle Interventions in Human Health. (2014).
42. Malá, Z., Šlampová, A., Křivánková, L., Gebauer, P. & Boček, P. Contemporary sample stacking in analytical electrophoresis. *Electrophoresis* vol. 36 15–35 (2015).
43. Ewing, M. A., Glover, M. S. & Clemmer, D. E. Hybrid ion mobility and mass spectrometry as a separation tool. *Journal of Chromatography A* vol. 1439 3–25 (2016).
44. Liu, C. *et al.* Fast quantitation of opioid isomers in human plasma by differential mobility spectrometry/mass spectrometry via SPME/open-port probe sampling interface. *Anal. Chim. Acta* **991**, 89–94 (2017).
45. Deng, L. *et al.* Ultra-High Resolution Ion Mobility Separations Utilizing Traveling Waves in a 13 m Serpentine Path Length Structures for Lossless Ion Manipulations Module. *Anal. Chem.* **88**, 8957–8964 (2016).
46. Ibrahim, Y. M., Hamid, A. M., Cox, J. T., Garimella, S. V. B. & Smith, R. D. Ion Elevators and Escalators in Multilevel Structures for Lossless Ion Manipulations. *Anal. Chem.* **89**, 1972–1977 (2017).
47. Michelmann, K., Silveira, J. A., Ridgeway, M. E. & Park, M. A. Fundamentals of trapped ion mobility spectrometry. *J. Am. Soc. Mass Spectrom.* **26**, 14–24 (2014).
48. Baglai, A. *et al.* Comprehensive lipidomic analysis of human plasma using multidimensional liquid- and gas-phase separations: Two-dimensional liquid chromatography–mass spectrometry vs. liquid chromatography–trapped-ion-mobility–mass spectrometry. *J. Chromatogr. A* **1530**, 90–103 (2017).

49. Zhang, X., Quinn, K., Cruickshank-Quinn, C., Reisdorph, R. & Reisdorph, N. The application of ion mobility mass spectrometry to metabolomics. *Curr. Opin. Chem. Biol.* **42**, 60–66 (2018).
50. Yu, X. & Yao, Z. P. Chiral recognition and determination of enantiomeric excess by mass spectrometry: A review. *Analytica Chimica Acta* vol. 968 1–20 (2017).
51. Williams, M. D. *et al.* Characterizing metabolic changes in human colorectal cancer. *Anal. Bioanal. Chem.* **407**, 4581–4595 (2015).
52. Paglia, G. *et al.* Ion mobility derived collision cross sections to support metabolomics applications. *Anal. Chem.* **86**, 3985–3993 (2014).
53. Li, L.-P. *et al.* Applications of ambient mass spectrometry in high-throughput screening. *Analyst* **138**, 3097 (2013).
54. Sinclair, I. *et al.* Novel Acoustic Loading of a Mass Spectrometer: Toward Next-Generation High-Throughput MS Screening. *J. Lab. Autom.* **21**, 19–26 (2016).
55. Clendinen, C. S., Fernández, F. M., Monge, M. E. & Fernández, F. M. Ambient mass spectrometry in metabolomics. *Analyst* 3101–3117 (2017).
56. Li, T. *et al.* In situ biomarker discovery and label-free molecular histopathological diagnosis of lung cancer by ambient mass spectrometry imaging. *Sci. Rep.* **5**, 1–12 (2015).
57. Desmet, G. & Eeltink, S. Fundamentals for LC Miniaturization. *Anal. Chem.* **85**, 543–556 (2013).
58. Wang, X. *et al.* Microfluidics-to-mass spectrometry: A review of coupling methods and applications. *J. Chromatogr. A* **1382**, 98–116 (2015).
59. Heiland, J. J., Lotter, C., Stein, V., Mauritz, L. & Belder, D. Temperature Gradient Elution and Superheated Eluents in Chip-HPLC. *Anal. Chem.* **89**, 3266–3271 (2017).
60. Kirby, A. E. & Wheeler, A. R. Microfluidic origami: a new device format for in-line reaction monitoring by nanoelectrospray ionization mass spectrometry. *Lab Chip* **13**, 2533 (2013).
61. Heinemann, J. *et al.* On-chip integration of droplet microfluidics and nanostructure-initiator mass spectrometry for enzyme screening. *Lab Chip* **17**, 323–331 (2017).
62. Dietze, C., Hackl, C., Gerhardt, R., Seim, S. & Belder, D. Chip-based electrochromatography coupled to ESI-MS detection. *Electrophoresis* **37**, 1345–1352 (2016).

63. Li, X., Xiao, D., Sanders, T., T'chounwou, P. B. & Liu, Y. M. Fast quantification of amino acids by microchip electrophoresis-mass spectrometry Amino Acid Analysis. *Anal. Bioanal. Chem.* **405**, 8131–8136 (2013).
64. Fenaille, F., Barbier Saint-Hilaire, P., Rousseau, K. & Junot, C. Data acquisition workflows in liquid chromatography coupled to high resolution mass spectrometry-based metabolomics: Where do we stand? *Journal of Chromatography A* vol. 1526 1–12 (2017).
65. Spicer, R., Salek, R. M., Moreno, P., Cañueto, D. & Steinbeck, C. Navigating freely-available software tools for metabolomics analysis. *Metabolomics* vol. 13 1–16 (2017).
66. Wilkinson, M. D. *et al.* Comment: The FAIR Guiding Principles for scientific data management and stewardship. *Sci. Data* **3**, 160018 (2016).
67. Haug, K. *et al.* MetaboLights - An open-access general-purpose repository for metabolomics studies and associated meta-data. *Nucleic Acids Res.* **41**, 781–786 (2013).
68. Sud, M. *et al.* Metabolomics Workbench: An international repository for metabolomics data and metadata, metabolite standards, protocols, tutorials and training, and analysis tools. *Nucleic Acids Res.* **44**, D463–D470 (2016).
69. Creek, D. J. *et al.* Metabolite identification: are you sure? And how do your peers gauge your confidence? *Metabolomics* **10**, 350–353 (2014).
70. May, J. C. & McLean, J. A. Advanced Multidimensional Separations in Mass Spectrometry: Navigating the Big Data Deluge. *Annual Review of Analytical Chemistry* vol. 9 387–409 (2016).

