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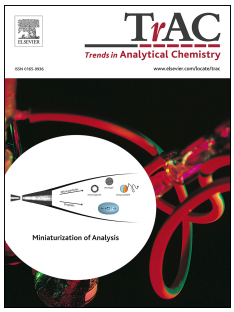

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Novel technologies for metabolomics: more for less

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ABSTRACT

The human metabolome provides a direct physiological read-out 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.

Keywords: metabolomics; high-throughput; nuclear magnetic resonance spectroscopy; mass spectrometry; sample preparation; on-line coupling; automation; microfluidics; ion mobility; precision medicine

Abbreviations:

AIF: all ion fragmentation

CSS: collisional cross section

DART: direct analysis in real time

DESI: desorption electrospray ionisation

DLLME: dispersive liquid-liquid micro extraction

DMS: differential mobility separation

DTIMS: drift tube ion-mobility separation

EESI: electrospray-assisted laser desorption/ionisation

FAIR: findable, accessible, interoperable and reusable

LC×LC: comprehensive two-dimensional liquid chromatography

NDLLME: non-dispersive liquid-liquid micro extraction

NIMS: nanostructure-initiator mass spectrometry

OPP: open port probe

SPME: solid-phase microextraction

SWATH: sequential window acquisition of all theoretical fragment ion spectra

TWIMS: travelling-wave ion-mobility separation

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1. 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 Database [1] 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 µM) 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 read-out 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 replication [2]. 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 repositories[1].

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 ^{1}H-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.

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

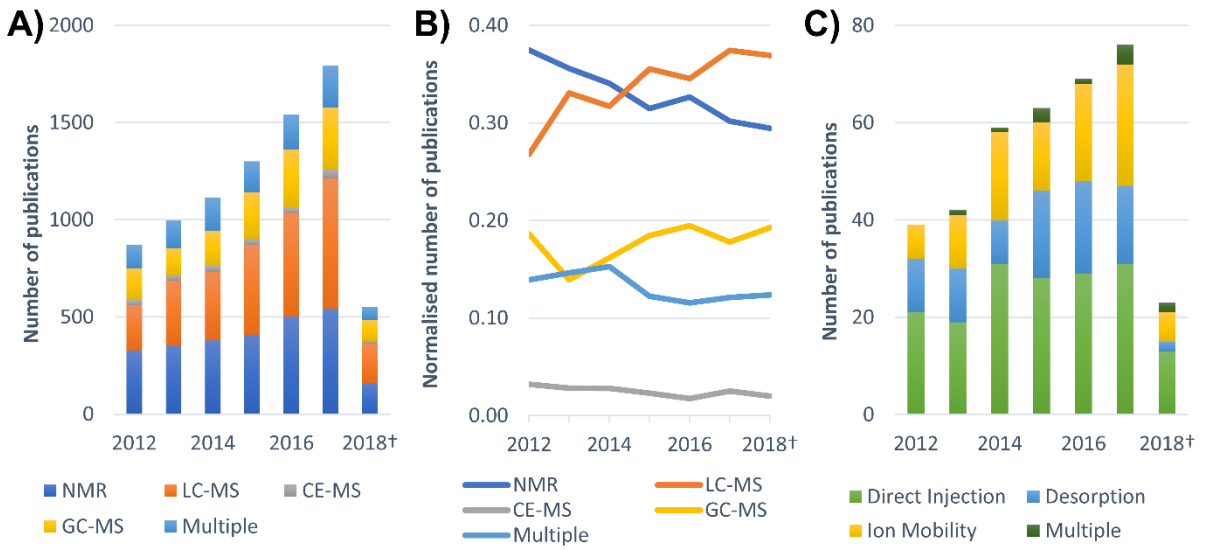
[1] Data extracted on October 31st, 2018. Studies with no metabolites reported were excluded.

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.

2. DISCUSSION

2.1 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-McKibbin [4]. 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.



153
154 *Figure 1. Literature survey of publications with metabolom* OR metabonom* in the title,*
155 *abstract, or keywords in the period 2012-2018† (up to May 7th, 2018) on Web of Science: (A)*
156 *number of publications mentioning the use of the main techniques in metabolomics: NMR,*
157 *LC-MS, GC-MS and CE-MS, or multiple; (B) relative number of publications (trend plot) of*
158 *the main techniques in metabolomics; (C) number of mass spectrometry-related publications*
159 *mentioning the use of fast sample introduction methods (direct injection and ambient*
160 *desorption ionisation), ion-mobility separation, or a combination of these. Further details*
161 *can be found in the Supplementary Information.*

162

163 **2.2 NMR**

164 One-third of the recent academic publications in metabolomics report NMR as the used
165 technique (Figure 1B). NMR is highly reproducible, suitable for high-throughput analysis,
166 cost-efficient, and still unrivalled when it comes to quantitation, or structural identification of
167 unknown compounds. Compared to GC-MS and LC-MS, NMR offers complimentary
168 information for the analysis of more-abundant metabolites that are difficult to ionise or would
169 require derivatisation, or are at very high concentrations. Its main downside is that it lacks
170 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 probes [7], and emerging techniques such as high-temperature superconducting coils [8], microcoil-NMR probes [9], 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 µL 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 µL 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 system [10]. 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 $^{15}N_2$ magnetization at room temperature and longer-lived $^{15}N_2$ singlet spin order. Theis *et al.* reported over 10 000-fold enhancements generating detectable NMR signals for over an hour [11]. 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.

2.3 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 lipidomics [13]. 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.

2.3.1 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

220 several small-volume SPE cartridges (mixed mode, graphitic carbon, and HILIC) [13]. This
221 automated SPE systems results in a significant gain in throughput. However, the packed-bed
222 cartridges still require relatively large elution volumes which limits the upconcentration for
223 low-abundant metabolites.

224 Solid-phase microextraction (SPME) has been introduced to address some of these
225 challenges. A polymer-coated fibre with a variety of available surface functionalities can be
226 inserted directly into the vial headspace, into the liquid sample, or can even be exposed *in*
227 *vivo*. The fibre is then transferred to an analytical platform for desorption and analysis. Direct
228 coupling of SPME to MS remains challenging [15], and a promising development has
229 addressed this via an Open Port Probe (OPP) interface [16]. One of the advantages of eluting
230 directly into the probe is that dilution is minimised. While this workflow still includes time-
231 consuming steps such as equilibration of the fibre (30 min) and sample-extraction (up to 5
232 min), it has potential for high throughput (less than 20 s per sample) since these steps can be
233 automated and performed in parallel. SPME for metabolomics is fairly new and its major
234 drawback is relatively low metabolite coverage. It has proven its value in small-scale studies
235 with various biofluids, and with the ongoing development of sorbent material we expect its
236 application in larger studies with broader metabolite coverage to follow soon [17].

237

238 **2.3.2 Liquid-Liquid Micro Extraction (LLME)**

239 LLE enables the fractionation of hydrophilic and hydrophobic metabolites in two immiscible
240 phases (*i.e.* aqueous and organic). As samples for metabolomics are mostly aqueous (*e.g.*
241 body fluids), LLE is used for either removal or extraction of hydrophobic metabolites (*e.g.*
242 Bligh & Dyer). Theoretically, high preconcentration factors can be achieved by extracting
243 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 extraction [18]. DLLME methods still require a phase-separation step which proves to be challenging to automate for high-throughput analysis [19]. In nDLLME, 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 3 orders of magnitude upconcentration in several minutes [20]. While the extraction kinetics of nDLLME are slightly slower compared to DLLME, this is compensated by its greater potential for automation [21].

2.3.3 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 injection [22].

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-phase [23] or three-phase [24] on-chip EE, whereby in the latter case the intermediate organic layer acts as a

268 filter for hydrophobic metabolites. Automated three-phase EE into a 2-µL droplet directly
269 coupled to nanoESI-MS has been used for metabolomics to extract acylcarnitines in 3 min, as
270 shown in Figure 2A [25]. Similarly, electro-membrane extraction (EME) utilises a membrane
271 impregnated with organic solvent as a filter. Recent developments to perform EME in parallel
272 in a multiwell-format (as shown in Figure 2B) are a significant step towards large-scale
273 automation [26].

274 Alternatively, preconcentration techniques can be used to further improve sensitivity for low-
275 abundant analytes, complementary to prior enrichment steps. Two developments for
276 controlled solvent reduction feature vacuum-assisted membrane evaporation [27] and
277 machine-vision controlled evaporation from a pendant droplet [28]. The former achieved a
278 10-fold solvent reduction in 60 min, whereas the latter achieved 10-fold reduction in a few
279 minutes without significant loss of volatiles.

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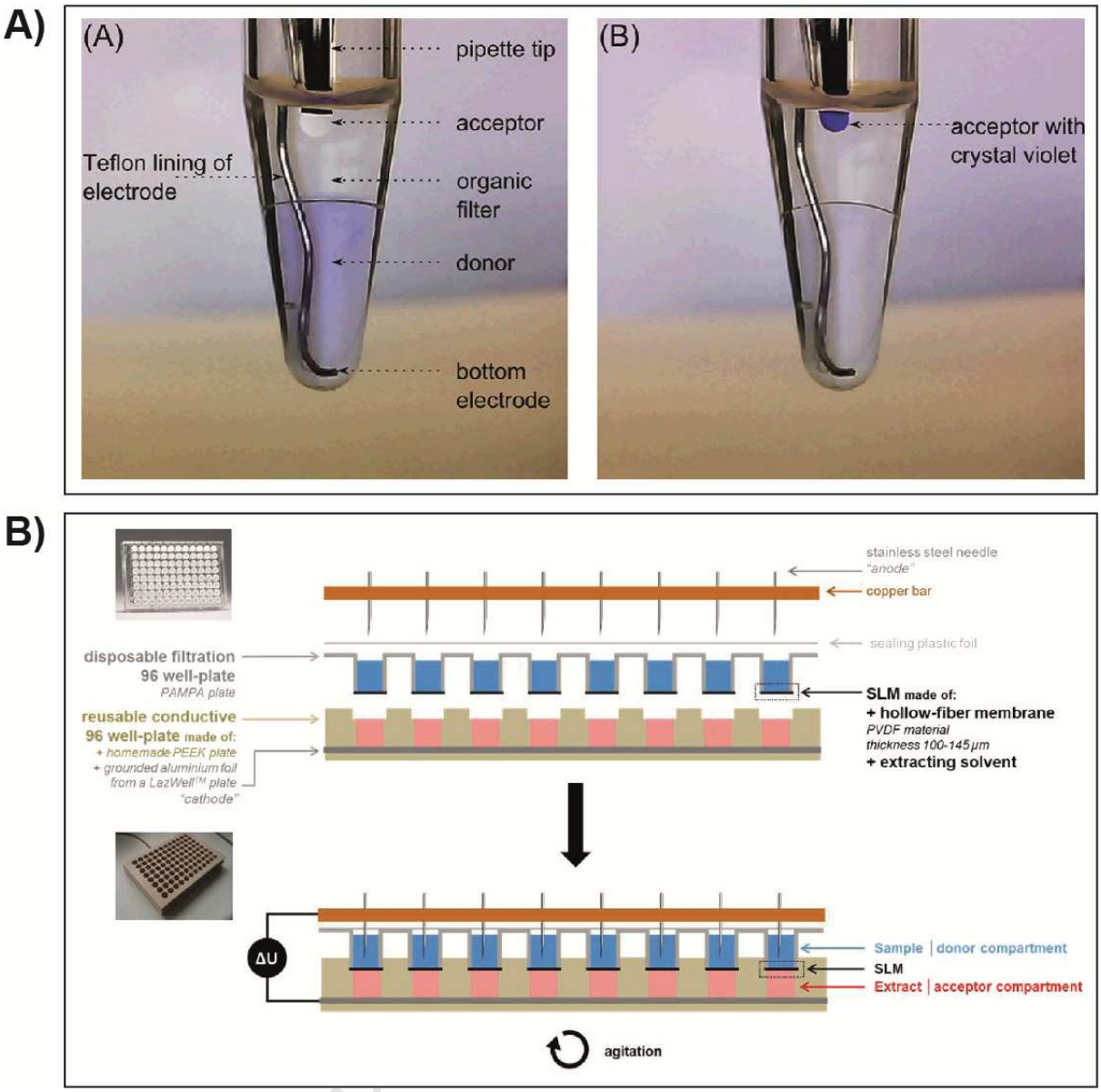

Figure 2. Two examples of electro-driven sample preparation. A) Three-phase droplet electroextractions. Video stills of crystal violet subjected to three-phase EE at t=0 and t=3 min after applying the voltage. Reprinted with permission from [25]. *Copyright 2013 American Chemical Society. B) Schematic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

2.4 Sample separation prior to MS

2.4.1 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 formats [31]. Secondly, a trend towards decreasing mobile-phase viscosity can be observed, including strategies such as the use of elevated mobile-phase temperature (>60 °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 columns [33] as shown in Figure 3 or silica-based monolithic

316 columns with higher permeability [34] has been typically explored for high-efficiency
317 separation of complex samples. However, it can also be used to gain separation power in
318 gradient separations of only several minutes [35].

319 While a plethora of chromatographic columns and conditions is available, finding the right
320 combination for an application can be a daunting task. Kinetic plots as introduced by Desmet
321 and co-workers are a powerful tool to compare the performance of chromatographic systems
322 [36]. These plots take into account information about the interplay between pressure, mobile-
323 phase velocity and plate height, and their effects on the performance of different columns, in
324 order to find the best possible combination of particle size, column length and temperature. If
325 higher separation efficiencies and deeper metabolite coverage are necessary, multi-
326 dimensional separations such as online comprehensive two-dimensional liquid
327 chromatography (LC×LC) can be used. Its two main advantages are greater selectivity by the
328 use of two independent (orthogonal) retention mechanisms targeting different sample
329 dimensions, and higher resolving power as the total peak capacity can be approximated by
330 the product of the individual peak capacities. However, using LC×LC to its full potential can
331 be difficult as design challenges include selecting orthogonal separation mechanisms, and
332 solving eluent-compatibility issues between the two dimensions [37]. We expect that the use
333 of predictive software-tools and optimisation algorithms to establish optimal conditions [38]
334 will increase the use of LC×LC in metabolomics in the coming years.

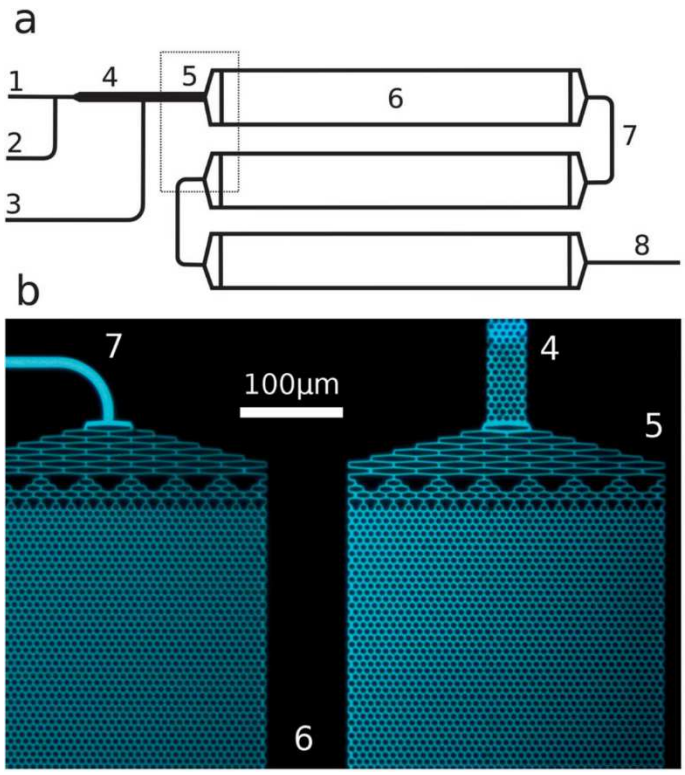

Figure 3. Perfectly-ordered micropillar-array LC column for increased separation efficiency. A) Schematic overview of the different zones of a microfluidic device containing pillar-array columns. 1: inlet mobile phase, 2: inlet sample phase, 3: outlet sample phase, 4: injection box, 5: inlet distributor, 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 [33] *with permission of The Royal Society of Chemistry.*

2.4.2 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 sensitivity [40]. 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 detection [39]. 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 throughput [41]. 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 magnitude [42]. 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.

2.4.3 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 popularity [44].

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 hold stationary against a moving gas instead of the other way around [47].

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 components [50]. 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.

2.5 Sample introduction for MS

2.5.1 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 3 samples per second for high-throughput analysis of small samples [54]. Ambient MS techniques have recently been reviewed by Clendinen *et al*. with respect to their (increasingly popular) use in metabolomics [55]. 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-workers [56], 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.

2.5.2 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 effects [57]. Furthermore, downscaling the system offers advantages such as improved sensitivity with concentration-dependent detectors, and increased ionisation efficiency of electrospray-ionisation mass

449 spectrometry (ESI-MS) due to the low flow rates [58]. Examples of commercially-released
450 devices include the IonKey (Waters) and HPLC-Chip (Agilent) chipLC-ESI devices, and the
451 ZipChip (908 Devices) chipCE-ESI device. A recent temperature-gradient chip-HPLC
452 approach demonstrates the advantage of low thermal mass of chips for rapid and extreme
453 temperature cycling. This system reduced the separation time by a factor of 2 down to 20 sec.
454 for six analytes (Figure 4B) [59].

455 Alternatively, digital microfluidics platforms allow for fast and accurate manipulation of
456 small volumes of sample and reagent, such as addition of internal standards, and can be
457 directly interfaced to MS with an on-chip emitter tip [60]. Moreover, droplet or digital
458 microfluidics shares its discrete and array-based traits with desorption/ionisation techniques,
459 making it an attractive match for desorption-based MS such as nanostructure-initiator mass
460 spectrometry (NIMS) as an advanced, high-throughput technique [61]. Electro-driven
461 separations also benefit greatly from the short distance between separation and detection with
462 microfluidic chips, as it eliminates the need for long capillaries that inherently increase
463 separation times. This was for example demonstrated for the analysis of pharmaceuticals [62]
464 and amino acids [63] in under 3 min.

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

468

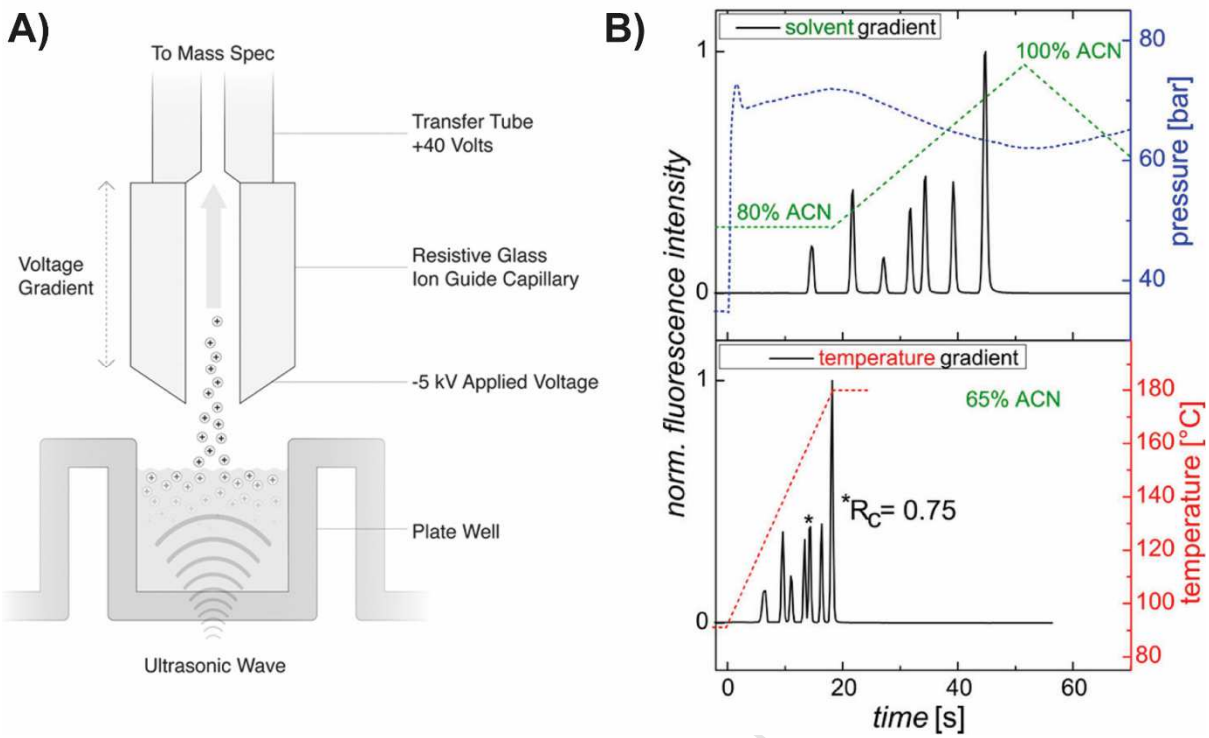

Figure 4. Two novel developments to increase throughput in sample introduction and separation. A) Schematic overview of ADE-MS high-throughput interface, whereby (sub)nL droplets of sample are acoustically ejected into the mass spectrometer through a transfer tube. Combined, these droplets form an equivalent flow of low µL/min-range. Reprinted from [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* [59]. *Copyright 2017 American Chemical Society.*

2.6 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 structure [64]. 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.

2.7 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])

508 which can perform one or more of these tasks. The BioContainer and the PhenoMeNal
509 consortium address incompatibility by creation and online deposition of software containers
510 that remove incompatibilities caused by dependencies of the installation or the version [65].
511 Additionally, these containers can reproduce data analysis with the exact same software
512 conditions.

513 A related second challenge is to make research data Findable, Accessible, Interoperable and
514 Reusable (FAIR) so that data of different studies can be combined [66]. An important tool to
515 realise FAIR metabolomics data is the introduction and increasing use of metabolomics data
516 repositories, such as the MetaboLights database (Europe) [67] and Metabolomics Workbench
517 (United States) [68]. To facilitate their use by the international community, efforts are being
518 made to allow easy sharing and exchanging of data between repositories as exemplified by
519 the creation of the online resource MetabolomeXchange
520 (http://www.metabolomexchange.org), which lists the available (meta-)data sets from the
521 various repositories. An additional advantage of these repositories is that it stimulates the
522 metabolomics community towards the highly necessary data reporting standards [69].
523 Standardisation allows to combine multiple datasets within the field of metabolomics to
524 increase statistical power, but also to integrate data with the other –omics fields for more
525 exhaustive analysis of biological phenomena.

526 A third challenge is the computational power required to analyse the data sets and files with
527 extraordinarily high information density. To illustrate this, May and McLean calculated
528 theoretically that peak capacities exceeding 10^{12} could be achieved when coupling three-
529 dimensional MS imaging with ion mobility and TOF-MS, with a peak-production rate of
530 around 10^{6} s^{-1} [70]. To address this challenge, the metabolomics society is increasingly
531 making use of cloud-computing technologies, such as XC-MS Online and OpenMSI, wherein

data-intensive computational work is distributed across a network of computers [70]. 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.

3. 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 this 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.

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HIGHLIGHTS

- Overviews trends towards faster, cheaper and more comprehensive metabolomics
- Features automation and miniaturisation to increase throughput and reduce costs
- Reports progress in fast separations and sample introduction for mass spectrometry
- Describes emergence of open-source and independent, automated, data analysis tools
- Substantiates the increasing power of metabolomics as a tool in precision medicine