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Review Article

Analytical techniques for biomass-restricted metabolomics: An overview of the state-of-the-art

Bingshu He, Wei Zhang, Faisa Guled, Amy Harms, Rawi Ramautar^{*}, Thomas Hankemeier^{*}

Division of Systems Biomedicine and Pharmacology, Leiden Academic Centre for Drug Research, Leiden University, The Netherlands

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ABSTRACT

Biomedical and clinical questions increasingly deal with biomass-restricted samples. To address these questions with a metabolomics approach, the development of new microscale analytical techniques and workflows is needed. Over the past few years, significant efforts have been made to improve the overall sensitivity of MS-based metabolomics workflows to enable the analysis of biological samples that are low in metabolite concentration or biomass. In this paper, factors that are crucial for the performance of biomass-restricted metabolomics studies are discussed, including sampling and sample preparation methods, separation techniques and ionization sources. Overviews of MS-based miniaturized metabolomics studies reported over the past five years are given in tables, with information provided on sample type, sample preparation volume, injection volume, separation techniques and MS analyzers. Finally, some general conclusions and perspectives are given.

1. Introduction

Metabolomics has become an important tool in biological and clinical research and is particularly promising for biomarker discovery studies. Currently, reversed-phase liquid chromatography-mass spectrometry (RPLC-MS), gas chromatography-mass spectrometry (GC-MS) and nuclear magnetic resonance spectroscopy (NMR) are used as the main analytical tools in metabolomics, providing the performance needed for the analysis of hundreds to thousands of samples [1,2]. However, these well-established analytical tools require a relatively large amount of sample for work-up and injection or detection (especially in case of NMR), thereby limiting their applicability to those biological and clinical problems that inherently deal with (very) low metabolite concentrations or biomass. For instance, one of the most crucial endocannabinoids, anandamide, possesses a concentration in the range from 0.5 to 2.7 pM in human cerebrospinal fluid (CSF) [3], and prohormone thyroxine and 3,3',5-triiodothyronine are present in concentrations from 1.4 to 2.3 pg/mL in biological samples [4]. The development of miniaturized RPLC-MS methods was required in order to determine these trace-level compounds [5,6]. Besides addressing issues with low metabolite concentrations, another analytical challenge is the study of biological and clinical questions intrinsically dealing with low

amounts of starting material, such as exosomes, primary cells, single cell analysis [7], zebra fish, and samples from 3D microfluidic cell culture systems (Fig. 1). Improved or new analytical techniques are therefore needed to enable the study of these questions with metabolomics.

Over the last few years, considerable efforts have been dedicated to improving the overall sensitivity of MS-based metabolomics workflows to enable the analysis of samples that are limited in metabolite concentration or biomass. Fig. 2 shows a typical analytical workflow used for metabolomics. In this work, specific attention will be given to the developments in sampling, sample preparation and separation-based MS approaches for biomass-restricted metabolomics studies. General factors dictating the sensitivity of MS-based approaches are shortly considered including the development of miniaturized ionization sources. An overview of recently developed micro- and nanoscale separation techniques coupled to MS and their applications to biomass-restricted metabolomics studies is provided in table format. Selected examples will be highlighted to illustrate the utility of miniaturized methods for biomass-restricted metabolomics with emphasis given on some analytical performance metrics. Finally, some general conclusions and perspectives are provided.

^{*} Corresponding authors at: Biomedical Microscale Analytics, Leiden Academic Centre for Drug Research (LACDR), Leiden University, Einsteinweg 55, 2333 CC Leiden, The Netherlands (R. Ramautar). Division of Systems Biomedicine and Pharmacology, Leiden Academic Centre for Drug Research (LACDR), Leiden University, Einsteinweg 55, 2333 CC Leiden, The Netherlands (T. Hankemeier).

E-mail addresses: r.ramautar@lacdr.leidenuniv.nl (R. Ramautar), hankemeier@lacdr.leidenuniv.nl (T. Hankemeier).

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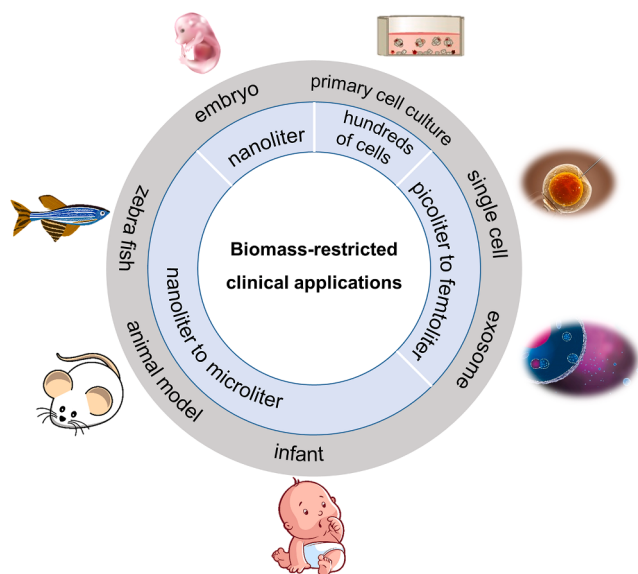


Fig. 1. An illustration of biomass-restricted applications requiring microscale separation techniques for performing metabolomics studies. *Blood and sweat samples are only considered in case of infants.

2. Sampling and sample preparation strategies for biomass-restricted metabolomics

The volume-mismatch between the volume-limited biological samples and the minimum volume requirements for sampling and/or sample preparation could be considered as one of the main analytical challenges for biomass-restricted metabolomics studies. The commonly used animal models in biological and biomedical studies, such as mouse, guinea pig and zebra fish, have restrictions in terms of the amount of body fluid or tissue available for experimental work, resulting in samples which are rather hard to analyze with conventional analytical methods and workflows employed in metabolomics. Thus, sampling and sample preparation methods for these kinds of samples should be scaled-down enough to minimize sample loss and provide as much sample as possible for the follow-up analysis.

2.1. Developments in sampling strategies

In order to obtain samples from volume-limited experimental subjects, recent advancements in sampling methods endeavor to acquire microliter to nanoliter volumes with micromanipulation techniques. One of the promising sampling techniques for limited sample volume is microdialysis sampling, which was designed to continuously obtain samples from extracellular regions of living tissues. Since the collected samples can be analyzed by any appropriate analytical technique [8], microdialysis sampling has been used in studies on brain diseases including Alzheimer's disease, Parkinson's disease, Huntington's disease, traumatic brain injury and epilepsy [9,10]. However, due to the relatively large size of probe, the first applications of microdialysis sampling suffered from poor spatial resolution. To overcome this weakness, a low-flow push–pull strategy was proposed. By using this

strategy, there is less tissue damage than normal microdialysis, as artificial CSF would be pushed into the same sampling position with low flow rate (50 nL/min). This low-flow push–pull perfusion design contributed to a higher spatial resolution and provided more information from intact tissues than previous methods for neurochemical monitoring [11]. For application to other tissues, Weisenberger et al. developed an online microdialysis-capillary electrophoresis method for the *in vivo* monitoring of amino acids from adipose tissue. This microdialysis probe was constructed with two fused silica capillaries inserted into a hollow fiber and gave a sampling region of 3 mm. Laser-induced fluorescence (LIF) detection was used in this method together with CE, after derivatization with 20 mM 4-fluoro-7-nitrobenzofurazan/250 μ M hydrochloric acid in 50% methanol. As a result, 12 amines were detected with only 22 s per analysis in inguinal adipose tissue. This method has been successfully assessed by administering an insulin stimulation via tail vein injection to record dynamic, *in vivo* changes in amino acid metabolism with good reproducibility [12]. Schoors et al. developed a sensitive method by coupling the microdialysis sampling probe with ultra-high pressure liquid chromatography-electrochemical detection (UHPLC-ECD). A 1 mm I.D. column was used for the simultaneous determination of monoamines, dopamine, noradrenaline and serotonin with lower limit of quantification (LLOQ) of 100–150 pM in material-limited samples, such as rat hippocampus, prefrontal cortex and striatum [13]. The direct connection with sensitive analytical techniques such as LC-MS, immunoassay, and capillary electrophoresis with laser-induced fluorescence (CE-LIF) [10] is conducive to higher temporal and spatial resolution.

Apart from its application in microdialysis, the pull–push strategy was also used on a dual-probe microfluidic chip developed by Huang et al. Instead of artificial matrix, extraction solvents or ionization buffer were pushed for the sample extraction in dried spot samples and liquid-phase samples, providing better conditions for ambient MS ionization. The sampling procedure of dried spots takes about 10 s for each spot with 500 nL/min flow rate, and sample volume was 100 nL for liquid-phase sampling. While they used reserpine solution to validate these two methods, the detection limit with dried spot sampling device was 0.4 pg with RSDs ranges from 8.9 to 31.5% in all tested concentrations. As for liquid-phase sampling, the sampling volume was 100 nL, limit of detection was 41 nM for reserpine, the peak area RSD of 63 reserpine droplets was 15.6%. By integrating the micro-sampling probe, electrospray emitter probe and online mixer (for derivatization) on one glass microchip, this technique was applied on an analysis for *in situ* evaluation of residual pesticide on apples and also an evaluation of nanoliter-scale ugi-type reactions for 8 compounds [14]. This study demonstrated the versatility of the micro sampling method and its promising utility in sampling nanoliter samples.

When it comes to single cells, direct infusion mass spectrometry analysis with integrated micro sampling probes is preferable since the nanoliter (or even lower) level sample amount would impose a tremendous challenge on sample preparation and/or transfer. Moreover, the biochemical heterogeneity of each cell could be preserved as much as possible by using this approach [15]. Pipette-based micromanipulation and chip-based sampling strategies were normally used prior to MS analysis [16]. In this context, Liu et al. recently designed a “T-probe” for the sampling of intracellular samples in single mammalian cells followed by direct infusion nanoESI analysis (see Fig. 3). Cytoplasm was sampled



Fig. 2. Typical analytical workflow used for metabolomics studies.

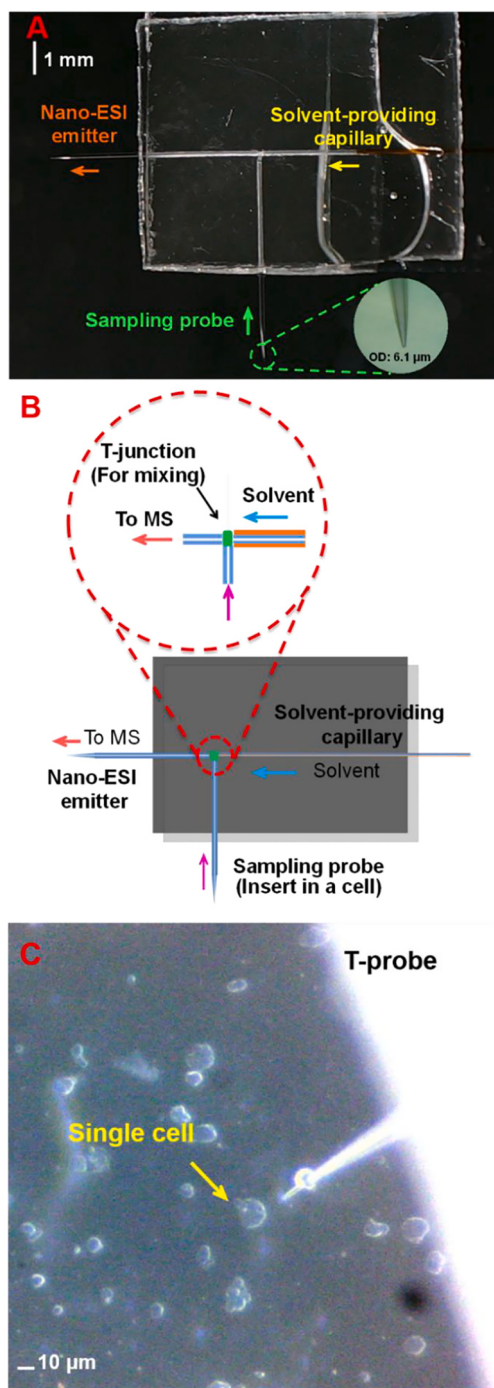


Fig. 3. Utilizing the T-probe for the single cell MS experiments. (A) Photo of a T-probe. Inset: a zoomed-in photo of the sampling probe tip. (B) Illustration of the working mechanism and fluid flow directions in the T-probe. (C) Photo illustrating the insertion of the T-probe tip into a cell [ref. 17].

through the sampling capillary and injected directly through the nano-ESI emitter into an LTQ Orbitrap XL MS together with sampling solvents. Four standard compounds including two lipids, one anticancer drug and a peptide were selected to validate this method, the limit of detection ranges from 0.1 to 10 nM. Although the limited sampling volume from one HeLa cell (1.2 to 4.3 pL) may affect the compound coverage, this method was applied on two cell groups with or without irinotecan treatment, and the multivariate analysis of metabolic profiles showed significant differences between these two groups. Some biomarkers that could be used to evaluate treatment efficacy were identified, indicating

the potential utility of this method in pharmaceutical studies [17]. CE-MS is well matched with single cell sampling devices because of its nanoscale injection volume. Onjiko et al. developed a sampling system containing a pulled capillary, called a “micropipette”, with a tapered tip (about 20 μm tip inner diameter) to aspirate cytoplasm from cells, coupled to a motorized three-axis micro-manipulator to control the movement of micropipettes with 20 nm resolution. Around 10–15 nL cytoplasm was collected from a single live frog embryo cell and extracted with 4 μL of solvent. The cell extract was deposited in a microvial, together with cell debris and precipitates, and analyzed by CE-MS. Results showed approximately 230 different molecular features were observed, and 70 compounds including spermidine, thiamine, and choline were identified [18]. This method enabled triplicate sampling and analysis from one cell within 5 s without influencing cell division from the 8-cell stage embryo to the 16-cell stage. The RSD of 4 to 7 biological replicates was around 22% with only 10 nL injected. In order to look into the *in situ* information from a single cell, it is crucial for the sampling method to preserve the physiological environment of the cell during the metabolomics study, so the results reflect the real metabolic situation in a living organism. Guillaume-Gentil et al. used fluidic force microscopy to extract cytoplasmic metabolites from a single HeLa cell under subpicoliter resolution (0.8 to 2.7 pL) without perturbing the biochemical environment or damaging its viability. The picoliter level sample was released as a 95 μm spot on a coated chip for matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The advantage of this fluidic force microscopy was its unique pyramidal geometry probe with small size aperture (400 nm), which could prevent membrane damage while extracting all the soluble intracellular molecules. With the assistance of 9-aminoacridine as MALDI matrix, 20 different metabolites including ribonucleotides, activated sugars, amino acids and glutathione were identified in at least 2 out of 4 cytoplasmic samples [19]. With these single cell sampling methods, heterogeneity could be addressed during the metabolomics analysis, which provided valuable information for the understanding of disease mechanisms and metabolic pathways of other important biological processes.

As for vulnerable research subjects such as newborns and the elderly, several dried blood spot (DBS) sampling techniques have already been applied for decades in the screening of inborn errors of metabolism and disease diagnosis [20,21]. Improvements to this technique which minimize discomfort and improve quantitation are found in novel micro/nano-scale minimally invasive sampling methods such as volumetric absorptive microsampling (VAMS) which are now available for clinical and metabolomics research. The volumes sampled by these devices vary from 10 to 30 μL for plasma, urine or oral fluid [20,22], depending on the tip size.

Generally, sample loss is often observed during the sample transfer in sampling and sample preparation procedures regardless of the adopted strategy. The loss of target compounds can be corrected for through proper use of internal standards in metabolomics analysis. However, corrections will not be possible if the concentration is already below the detection limit. Therefore, it is crucial to find strategies to avoid sample loss during extraction and increase the sensitivity of analytical methods as much as possible.

Although it is possible to collect samples with limited volume, it is still challenging to perform efficient sample preparation in especially sub-microliter samples without dilution. Choices have to be made whether it is more important to provide enough volume for following analysis or if concentrating targeted compounds to reach the detection limits is required. Pooling several volume-limited samples together is normally used to provide enough starting material, but this is not an ideal option in metabolomics studies since it only provides an average read-out instead of reflecting individual differences due to diseases or drug response. In order to determine the internal drug exposure in the blood of zebrafish larvae, Van Wijk et al. developed a sampling method with pulled glass capillary needles under microscope. In this method,

around 1 nL of blood could be obtained from zebrafish larvae. However, in order to reach measurable levels of paracetamol and its main metabolites, 15–35 samples were pooled together for sample preparation for analysis using a UHPLC-MS method where they were quantified with sub-picomole levels [23]. The same pooled strategy was also commonly used in many other zebrafish studies [24,25] due to the minimal material for the required sensitivity. Unfortunately, the metabolic heterogeneity of different organisms is overlooked in this way.

2.2. Developments in sample preparation strategies

After choosing a suitable sampling method, development of sample preparation strategies for especially volume limited samples is the next important step for biomass restricted sample analysis. Sample transfers between tubes or vials should be avoided as much as possible for an ideal miniaturized sample preparation procedure to prevent unnecessary sample loss. Microvials or nanovials are recommended during sample preparation for better sampling of small volumes [18]. Protein precipitation, LLE and SPE methods are still the most used sample preparation procedures in bioanalysis, including metabolomics [26,27].

Protein precipitation and LLE strategies can be used for many volume-limited applications. By using volatile liquids in these two strategies, relatively large amounts of organic solvents can be evaporated and then the sample can be reconstituted in only a small amount of solvent compatible with the follow-up separation technique. Obviously, when the starting amount is lower than the reconstitution volume, there would still be a dilution effect with these steps. The extent to which compounds can be enriched with an evaporation step is sometimes limited by solubility, and for good quantification, methods should avoid supersaturation of abundant components, while still concentrating the low abundance compounds.

Ideally, human and animal studies would be able to collect real-time metabolomics data. In animal studies where samples are collected post mortem or when biopsies are taken in clinical setting, there is a risk that the metabolome may degrade between collection and sample preparation and analysis. One potential solution to this issue could be *in vivo* solid phase micro extraction (SPME) which is gaining more interest in metabolomics. In SPME, a thin probe is often coated with a C18 sorbent in union with polyacrylonitrile to increase biocompatibility. This probe is then inserted directly into tissue of a (sedated) organism to sample the metabolome in a minimized invasive manner, as no tissue is removed. The total sample “mass” is just the metabolites adsorbed to the C18 SPME material. Collecting sample in this manner is non-depleting and thus collects sample in real time during normal cellular metabolism while causing minimal metabolomics disruption [28]. *In vivo* SPME has proven to be effective for the analysis of a wide range of metabolites including low abundance steroids and lipids. These studies demonstrate particularly good recoveries for non-polar analytes as a result of the non-polar nature of the C18 phase being used as a sorbent [29]. Vasiljevic et al. introduced a solid-phase microextraction (SPME) minitip featuring a tip apex (1 mm) coated with polyacrylonitrile (PAN) and N-vinylpyrrolidone-co-divinylbenzene (also known as HLB) particles for the extraction of compounds from several sample-limited matrix types. In one application, this minitip was used in the quantification of several drugs of abuse in 1 μ L human blood with LODs from 0.1 to 2.5 ng/mL. After static extraction, the blood sample was desorbed in 3 μ L MeOH: ACN:FA (80:20:0.1) and transferred to a nanoESI sprayer. The minitip was also used for untargeted metabolomic profiling of single caviar eggs. The study of 4 different types of caviar eggs (6 replicates per type) with LC-HRMS showed the SPME minitip method was able to distinguish samples based on metabolomics profiles. 149 significant metabolites including eicosapentaenoic acid, L-tryptophan, and retinoic acid were detected. Although the repeatability of SPME minitip method was still not satisfying (20–30 RSD%) due to the deviations during the coating procedure, it is indeed promising as an integrated method of sampling and sample preparation for volume limited samples [30]. By combining

SPME with a miniaturized probe, another study developed a promising technique for the *in vivo* sampling and sample preparation of neurotransmitters from macaque brain. After thorough optimization of probe shape, desorption solvent and extracting phase, the SPME probe was coated with in-house synthesized HLB particles with a total diameter less than 200 μ m. Validation of this method was carried out by using brain surrogate matrix, LOQ from 25 ng/mL to 20 μ g/mL were reached with a 20% RSD value. When the method was applied on a macaque brain, 3 brain areas (prefrontal cortex area, premotor cortex area, and caudate nucleus head) were sampled simultaneously in consecutive triplicates, each extraction procedure in brain took 20 min. Several compounds including dopamine, serotonin, glutamate and taurine were quantified after extraction. Meanwhile, untargeted analysis revealed the possibility of detecting a wide polarity range of endogenous metabolites in brain sample using a SPME-based miniaturized *in vivo* sampling method (Fig. 4) [31].

Other than its use for sampling endogenous metabolites, a SPME method was also reported for the quantification of doxorubicin in pig lung tissue. The quantification abilities of SPME in intact lung tissue, *ex vivo* SPME homogenized samples and solid-liquid extraction showed no significant difference. The LOD for doxorubicin was 2.5 μ g/g in tissue. Therefore, without extra sample preparation steps or significant invasive injury to the organism, *in vivo* SPME could be a future solution as a rapid quantitative method for monitoring and adjusting drug dosages during chemotherapy [32].

3. MS-based separation techniques for biomass-restricted metabolomics

For miniaturized metabolomics studies with biomass restricted samples, after effective microsampling and sample preparation procedures, proper chromatographic or electrophoretic separation methods and sensitive electrospray mass spectrometry (ESI-MS) are crucial aspects that determine the overall sensitivity of MS-based analytical methods.

When a sample is analyzed by LC-MS, the flow rate controls the amount of sample that reaches the ion source per unit time. In fact, sensitivity loss is likely to happen when there's limited current and the charge on each sample droplet is lower than the concentration of metabolites and causes insufficient ionization, hence the signals of compounds with lower proton affinity or surface activity could lose the competition for charge to compounds with a fixed charge or with higher proton affinity. With micro or nano flow rates, smaller droplets with higher surface-to-volume ratio will reduce ion suppression, thus not only increasing the detection sensitivity, but also broadening the coverage of metabolites [33,34]. To assess the influence of flow rate on ESI-MS detection sensitivity, a mixture of n-octyl-glucopyranoside ($c = 10^{-6}$ mol/L) and turanose ($c = 10^{-5}$ mol/L) dissolved in methanol/water (30:70, v/v) was infused at various flow rates. At flow rates of a few nanoliters per minute, ion suppression effects were virtually absent while at flow rates above 50 nL/min ion suppression increased up to fivefold [35]. A higher sensitivity for fructose 6-phosphoric acid was also observed from nano-flow injection analysis (nano-FIA), indicating nano-flow rates give better analytical sensitivity than higher flow rates. In this study, the peak areas of 22 metabolites were 7.6 to 66 times higher with nano-FIA compared to the conventional flow [36].

In a miniaturized analysis, the typical inner diameters of columns are decreased to below 1 mm for micro-LC, and to 75 μ m for nano-LC. Tubings and connectors in LC system are also narrowed down to avoid too much dead volume, reduce analysis time and increase sensitivity for the analysis of biomass-restricted samples (Table 1) [37–39]. However, the tradeoff here is that smaller inner diameters generate higher back pressure and therefore require more complex instrumentation. Recently developed micro Pillar Array columns (μ PAC) could offer a solution to this problem. Their highly ordered pillars containing an outer porous shell grafted with C18 groups could limit backpressure while enhancing

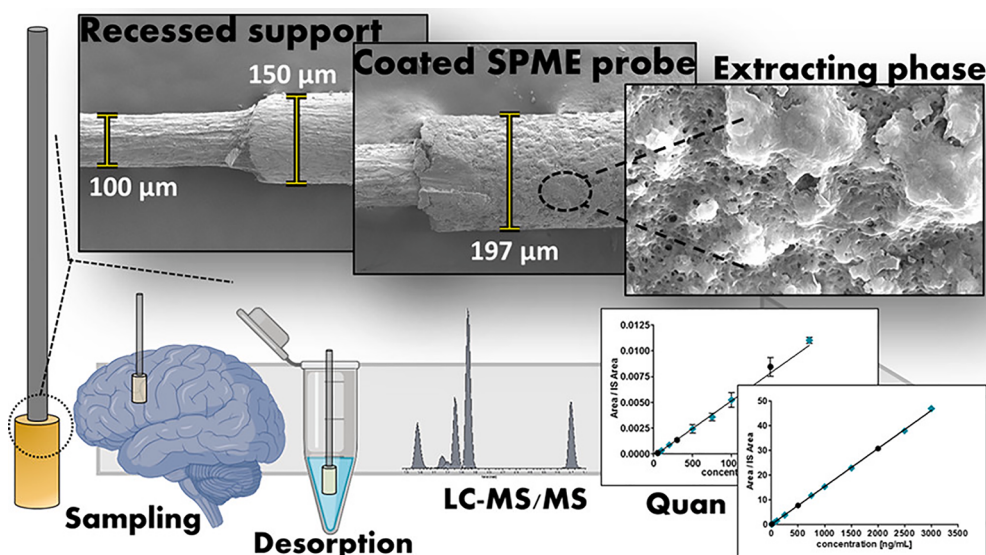


Fig. 4. Solid Phase Microextraction-Based Miniaturized Probe and Protocol for Extraction of Neurotransmitters from Brains in Vivo [ref. 31]

Table 1

Analytical characteristics of standard and micro- to nanoscale analytical separation techniques [54–56].

	HPLC-MS	micro-LC-MS	nano-LC-MS	CE-MS
flow rate	0.5–1 mL/min	500–4000 nL/min	up to 500 nL/min	20–100 nL/min
column i.d.	1–4.6 mm	75 μm–1 m	up to 75 μm	–
injection volume	above 5 μL	up to 2 μL	up to 1 μL	up to 20 nL
Sensitivity	nM to μM level	pM level	fM level	Low nM range
Application	Limited volume/low concentration	Less limited volume/high concentration		

chromatographic performance [40].

Downscaling of the LC method improves sensitivity and increases the coverage of analyzed compounds. A nanoscale ion-pair reversed-phase HPLC-MS method was developed to analyze highly polar metabolites in the low femtomolar down to hundreds of attomolar range in solvent as well as in cell extracts. Compared to previous HPLC-MS study, the sample amount required per injection was 1000 times lower with nano flow rate, indicating very low LODs could be reached with small matrix sample volume [41]. By adding the metal chelating agent ethylene diamine tetraacetic acid (EDTA) to the sample solution, Myint et al. managed to improve peak shapes of multiply charged anionic compounds with nano-LC/MS. The method was applied on cell extracts, mouse brain tissues, human plasma and CSF with detection limits of 0.19 to 2.81 pM [42]. Furthermore, the trap-and-elute strategy which was first developed for proteomics studies also became a classic strategy in metabolomics study, which further improves the sensitivity of miniaturized LC methods [43].

Apart from LC systems, CE-MS is also highly suited for biomass-restricted metabolomics because of its low sample injection volume. A sheath liquid interface is most commonly used for electrical contact in CE-MS. However, the addition of the sheath liquid flow dilutes the sample, decreasing sensitivity. In contrast, sheathless interfaces couple the CE to the MS without dilution for higher sensitivity. An example using a standard sheath liquid CE-MS method, Zhang et al. analyzed limited mouse plasma samples (10 μL) and detected 44 polar components [44]. Through the application of field amplified sample injection (FASI), Liao et al. obtained enhanced detection sensitivity for cationic

metabolites and identified some important metabolites in single neurons from *A. californica* [45]. Examples using a sheathless CE-MS interface demonstrating better sensitivity are shown in the work of Zhang et al. which adopted transient-isotachopheresis (tITP) as the preconcentration technique when analyzing the sample derived from an extract of 500 HepG2 cells with a sheathless CE-MS method, and uncovered more than 24 cationic metabolites by injecting merely the content of 0.25 cell [46]. With the use of a thin-walled tapered emitter in CESI-MS, Kawai et al. obtained satisfying repeatability of migration time (1.5%) and peak areas (6.8%) after fifty consecutive analyses on 20 amino acids [47]. By further incorporating a dual pre-concentration strategy, the authors acquired LOD improvement of up to 800 fold compared with normal sheathless CE-MS. The metabolic profiling of single HeLa cells by this approach led to the quantitation of 20 and detection of 40 metabolites (Fig. 5). The versatility of CE-MS in interfaces [48,49], injection modes [50,51], and preconcentration strategies [52] renders CE-MS a promising tool for the analysis of biomass-restricted samples.

Lower flow rates require better ionization environments from electrospray ion sources. Recent developments on ion sources include providing more stable structures for continuous spray, incorporating the column oven directly into the ion source to reduce band broadening, avoiding possible leakage and minimizing dead volume, and by optimizing the tip inner diameter of spray needles for smaller droplets to improve ionization and transfer efficiency [53]. Improved spray emitters were designed and are commercially available for micro/nano-ESI-MS, such as the PicoTip™ emitter from New Objective and the stainless steel NanoTip emitter from Thermo Fisher. Instead of using single nozzle emitter, a chip-based multinozzle emitter (M3 emitter) was produced by NewOmics. By combining multiple emitters on one chip, this emitter splits the sample flow into multiple smaller streams to generate even and smaller droplets, which enhances the ionization efficiency. All these spray emitters are provided with various inner diameter for both micro and nano flow rate, they have proven to be robust and sensitive in several studies [54–56]. Furthermore, the angle between the spray needle and ionization interface was adjusted to gain more sensitivity (Table 2). Some of the commercially available ion sources performed well in both proteomics and metabolomics studies. Taki et al. performed a robust analysis on 17 highly polar metabolites using nano-flow injection analysis (nano-FIA) with a CaptiveSpray Ionization (CSI) source, which was initially designed for protein analyses, and presented results in good repeatability for small molecular compounds with 1000 nL/min flow rate [36].

Decades have been passed since the introduction of miniaturized

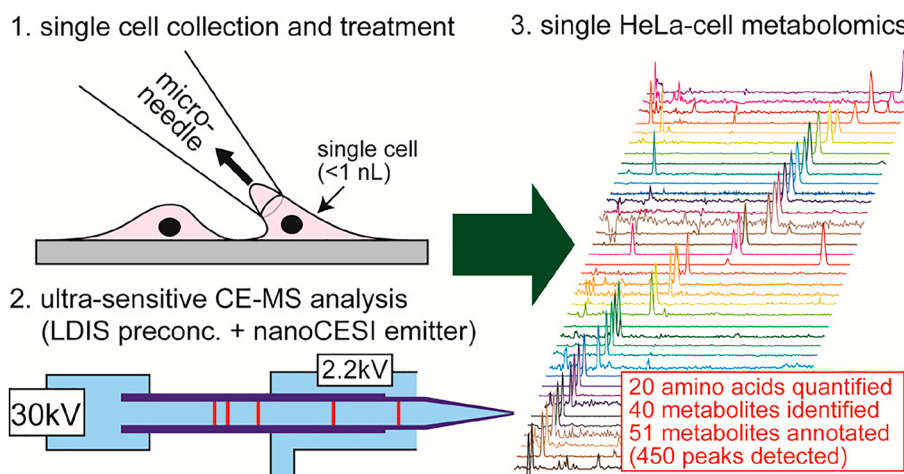


Fig. 5. Brief procedure of single HeLa-cell metabolomics by capillary electrophoresis–mass spectrometry with a thin-walled tapered emitter and large-volume dual sample preconcentration [ref. 44].

Table 2

Current commercially available micro- or nanospray ion sources.

Ion source name	Flow rate	Emitter i.d.	remarks
Thermo fisher Nanospray Flex	50–500 nL/ min	1–30 μ m	Provides stable electrospray
Agilent Nanospray	100–900 nL/ min	–	Offers three choices of spray orientation
Waters NanoLockSpray Exact Mass	up to 1 μ L/ min	–	Enables valid exact mass measurement and improves mass accuracy
Sciex OptiFlow Turbo V	micro: 1–200 μ L/ min nano: 100–1000 nL/min	20 μ m, 25 μ m, 50 μ m	Switch between nanoflow and microflow in minutes
Waters ionKey/MS	1–50 μ L/min	150 μ m/300 μ m iKey Separation Device	Integrates microflow directly into the source Provides an increased level of sensitivity, ease-of-use
Shimadzu Nexera Mikros	1–500 μ L/ min	20 μ m	Connected with UF-link column oven to avoid dead volume.
Sciex Nanospray III	30–1000 nL/ min	5–30 μ m	Possessed an X-Y-Z positioning unit that can be used to position the emitter tip relative to the curtain plate.
Bruker CaptiveSpray	nano-flow	–	A vortex gas that sweeps around the emitter spray tip for better desolvation. The direct connection to the inlet capillary making the source truly Plug-and-Play.

techniques, various of improvements regarding to micro/nano flow LC and comparable columns, nanospray ionization sources and spray emitters have been achieved. Current MS-based separation techniques for biomass-restricted metabolomics are able to analyze trace level compounds, as well as nanoliter level liquid sample or even single cell matrix. Although these techniques have only been used in academic studies so far, by coupling with efficient sampling and sample preparation methods, they are promising to contribute to future pharmaceutical and clinical research.

4. Applications

With the advantages of high sensitivity and high throughput,

analytical techniques for biomass-restricted samples have been applied in relevant metabolomics studies, such as food quality tests, biomedical and clinical studies. A selection of recent studies of recent micro/nano-LC-based metabolomics studies is given in Table 3, which provides information about the type of samples and compounds analyzed, volumes for sample preparation and injection, separation techniques and MS analyzers employed. Table 4 shows some metabolomics studies using CE-MS during 2019 to April 2021, more applications from previous years can be found in reference [38,57]. Representative application examples with both analytical techniques in metabolomics are discussed.

Lipids have been shown to be important in understanding many diseases including Alzheimer's disease, kidney diseases and cardiovascular diseases [58–60]. Several lipidomics studies were carried out with miniaturized methods for the identification and quantification of important lipids. The total ion chromatograms of yeast lipidomics profiles with good separation and response are shown in Fig. 6. The coverage and sensitivity of lipids measured utilizing a nanoLC-MS method clearly increased over those obtained using standard flow rates with 447 lipids from the core phospholipid lipid classes (PA, PE, PC, PS, PG, and PI) identified. The stability of retention time and repeatability of some targeted compounds were evaluated with 25 replicate measurements from one extract. Results showed the average retention time standard deviation was 5.2 ± 2.3 s, and RSDs of most compounds peak area were below 15% [33]. Another lipidomics study on rabbits with non-alcoholic fatty liver disease managed to quantify approximately 300 lipids within 20 min using nanoflow UPLC-MS/MS, revealing that non-alcoholic disease was highly associated with high-cholesterol diet and high-cholesterol diet combined with inflammation [61]. Byeon et al. performed comprehensive lipid profiling in plasma and urine samples from Fabry disease patients with nanoflow LC-MS/MS and 129 plasma lipids and 111 urinary lipids were identified. The results showed currently used enzyme replacement therapy influenced lipids in plasma more than those in urine [62].

As a group of crucial hormones involves in inflammation, immune functions and gender development, steroid hormones are of low abundance in biological matrices. Márta et al. established a sensitive and robust microflow UHPLC-MS/MS method for the simultaneous determination of 13 different steroid molecules in human plasma, the LODs ranged from 0.008 to 0.178 ng/mL with a repeatability less than 8% RSD for all the compounds [63]. A metabolite profiling of fecal extracts using nanoflow UHPLC-nanospray ESI-MS method revealed the presence of trace levels of eicosanoid and sex steroid signaling compounds in the presence of other compounds with high abundance like major bile acid metabolites. Furthermore, researchers applied this method to feces from

Table 3

Overview of applications using miniaturized analytical techniques from January 2015 to April 2021 (micro/nano-LC-MS).

Compounds	Sample matrix	Sample prep volume	Injection volume	Separation technique	Column ^a	flowrate	LOD ^b	MS analyzer	Ref.
Endocannabinoids	human CSF	600 µL CSF/ethanol mixture (200 µL/400 µL)	8 µL	Agilent 1100/1200 series nano-LC system	Agilent Polarishr-Chip 3C18 (75 µm × 15 cm, 3 µm)	2 µL/min	0.28–146.9 pM	Agilent 6460 triple quadrupole MS system	[3]
thyroid hormones 3,3',5-triiodothyronine (T3) prohormone thyroxine (T4)	egg yolk	50–150 mg	5 µL	Thermo Easy-nLC1000	trapping column C18 (100 µm × 2 cm, 5 µm) separation column C18 (75 µm × 15 cm, 5 µm)	300 nL/min	T3 5.9 aM T4 3.5 aM	Thermo TSQ Vantage MS	[4]
profiling	single HeLa cells	n.s.	0.1 µL	Thermo UltiMate 3000 RSLCnano system	nanoLC column (100 µm × 18 cm, 3 µm)	600 nL/min	0.02–38 fmol	Shimadzu LCMS-8060	[7]
irinotecan, leucine encephalin, PC (18:1/16:0), TG (16:0/18:1/16:0)	single cell extraction	–	n.s.	T-probe	–	200 nL/min	0.1–10 nM	Thermo LTQ Orbitrap XL MS	[17]
17 highly polar metabolites	rodent serum/plasma samples	45 µL	2 µL	ParadigmMS4 pump system	–	1000 nL/min	0.11–2.2 µg/mL	Sciex TripleTOF 5600 Captivespray ion source	[36]
lipids	yeast extraction	500 µL of yeast suspension (≈6.7 × 10 ⁸ cells)	1 µL	Thermo UltiMate 3000 system	Ascentis Express C18 (100 µm × 30 cm, 2.7 µm)	600 nL/min	n.s.	Thermo QExactive Plus MS	[33]
polyphenols and related compounds	red wine	n.s.	n.s.	Sciex Eksigent MicroLC 200 Plus UHPLC System	Kinetex C18 (100 µm × 5 cm, 2.6 µm)	50 µL/min	n.s.	SCIEX TripleTOF 5600	[34]
lipids	rabbit hepatic tissue	10 mg	n.s.	Waters nanoACQUITY UPLC	C18 (75 µm × 7 cm, 3 µm)	400 nL/min	n.s.	Thermo LTQ Velos ion trap MS	[61]
lipids	human plasma and urine	50 µL plasma 2 mL urine	4 µL	Agilent model 1200 capillary pump system	C18 (75 µm × 6 cm, 3 µm)	300 nL/min	n.s.	Thermo LTQ Velos ion trap MS	[62]
steroid hormones	human plasma	90 µL	n.s.	Sciex Eksigent MicroLC 200 Plus UHPLC System	trap column ProntoSIL 120 C18H (0.5 × 10 mm, 5 µm) separation column HALO Fused-Core Phenyl Hexyl (0.5 × 50 mm, 2.7 µm)	40 µL/min	0.01–1 ng/mL	Sciex QTRAP 6500 with Turbo V Source	[63]
amine/phenol submetabolome	breast cancer cell extraction	100 cells 1000 cells 10,000 cells	n.s.	micro LC: Thermo UltiMate 3000 UHPLC nano LC: Waters NanoAcquity UPLC	micro : Eclipse Plus C18 (2.1 mm × 10 cm, 1.8 µm) nano: Acclaim PepMap 100 trap column (75 µm × 2 cm, 3 µm) and Acclaim PepMap RSLC C18 (75 µm × 15 cm, 2 µm)	n.s.	n.s.	micro: Bruker Maxis II QTOF nano: Bruker Impact HD Q-TOF Captivespray ion source	[65]
small molecular drugs	dried blood spot and plasma	15 µL blood for dried blood spot 50 µL plasma	0.1–2 µL	Waters nanoACQUITY UPLC system	capillary scale LC on the ceramic microfluidic device BEH C18 (0.3 × 100 mm, 1.7 µm)	12 µL/min	n.s.	Waters Xevo TQS MS	[69]
profiling	human faeces	10 g	0.5 µL	Waters nanoAcquity UHPLC	Waters nanoAcquity HSS-T3 (100 µm × 10 cm, 1.8 µm)	700 nL/min	–	Waters Xevo G2 TOF MS	[64]
endocannabinoids	serum	50 µL	10 µL	Waters M-class UPLC	iKey with a post-column addition (PCA) channel (Peptide BEH C18	2 µL/min	0.36–4.02 pg/mL	Waters Xevo TQ-S tandem MS	[70]

(continued on next page)

Table 3 (continued)

Compounds	Sample matrix	Sample prep volume	Injection volume	Separation technique	Column ^a	flowrate	LOD ^b	MS analyzer	Ref.
profiling	cell extraction	n.s.	2 µL	Eksigent nanoLC system	150 µm × 5 cm, 1.7 µm) Self-packed columns (15 cm in length): 5 µm Magic C18-AQbeads (New Objective), PicoFrit column (360 µm OD × 100 µm ID, 15 µm Tip ID)	positive 400 nL/min negative 500 nL/min	–	Thermo LTQ-Orbitrap hybrid MS	[71]
modified nucleosides in RNA	mammalian cells and tissues	n.s.	n.s.	Thermo EASY-nLC II	precolumn (150 µm × 70 mm, 5 µm) separation column Zorbax SB-C18 column (75 µm × 250 mm, 5 µm)	300 nL/min	around 10 amol	ThermoLTQ XL linear ion trap MS with nanoelectrospray ion source	[72]
lipids	mouse serum, heart, and kidney tissues	100 µL serum 8 mg kidney/heart	untargeted analysis: 3 µg lipid extract targeted analysis: 2 µg lipid extract	untargeted analysis: Thermo Dionex Ultimate 3000 RSLCnano System targeted analysis: Waters nanoACQUITY UPLC system	home-made fused silica tubing column (100 µm ID, 7 cm length), the end portion (~5 mm) was filled with 3 µm of 100 Å Watchers® ODS-P C-18 particles, the rest (6.5 cm) was packed with 1.7-µm XBridge® BEH.	1 µL/min for loading, 300 nL/min for analytical column	0.011 pmol to 0.099 pmol in serum; 0.006 pmol to 0.141 pmol in kidney; 0.010 pmol to 0.119 pmol in heart	untargeted analysis: Thermo LTQ Velos ion trap MS targeted analysis: Thermo TSQ Vantage triple-stage quadrupole MS	[73]
Betalains	rat plasma	250 µL	n.s.	Eksigent LC200	Eksigent HALO C18 column (0.5 mm × 10 cm, 2.7 µm)	25 µL/min	2.00 to 5.74 nM	Sciex QTRAP 5500	[74]

a) The column sizes are all expressed as I.D. × Length, Particle size.

b) LOD = limit of detection (S/N = 3); n.s.: not specified in paper.

colorectal cancer patients, and the results indicated that signaling metabolites as well as other key metabolic pathways are potentially related to this disease [64]. Concentration restricted lipids could be detected and quantified in many studies with miniaturized analytical methods, which shows the advantage of down-scaling analysis for better sensitivity for diseases diagnose and treatments in the future.

Among volume-restricted samples, microfluidic cell culture such as organ-on-chip has become a research focus in recent years. High sensitivity of miniaturized analytical techniques laid the foundation for the analysis of small number cells and promoted the development of related studies. By using nano-LC-MS/MS, Luo et al. developed a method based on high-performance chemical isotope labeling for the analysis of 100, 1000 and 10,000 cells, resulting in acquiring over two thousand peak pairs of metabolites in the amine/phenol submetabolome, and more than half of them could be identified [65]. For targeted metabolomics, Junaid et al. were able to detect several signaling lipids in conditioned cell medium sample from blood vessels-on-a-chip upon exposure of to TNFα with less than 1 µL injection [66]. CE-MS has proven to be a key microscale method for biomass-restricted samples, and nanomole level of LOD could be reached with only a few nanoliters injection volume [46]. Zhang et al. developed a highly sensitive and efficient sheathless CE-MS method for the profiling of nucleotides, which are difficult to analyze with conventional analytical techniques, including adenosine triphosphate, adenosine diphosphate and adenosine monophosphate in 50 000 down to 500 HepG2 cells, with the LODs in matrix ranging from 0.1 to 0.9 nM [67]. A sample limited tissue example is from Sánchez-López et al. who performed an interesting study by analyzing 20 µm-

thick kidney sections from a mouse model of polycystic kidney disease using CE-MS. Injections were performed from a modified vial containing only 2 µL sample. The profiling covered more than 100 metabolic features with acceptable repeatability and could distinguish the experimental groups, which highlighted the use of biomass-restricted samples for metabolomics studies [68].

Meanwhile, non-invasive sampling methods yielding only a few microliter samples are expected to become the preferred strategy for clinical studies and applications. Rainville et al. successfully employed an integrated capillary scale (300 µm i.d.) ceramic microfluidic LC-MS/MS method for the quantitative analysis of pharmaceutical compounds in low volume human plasma and dried blood spot samples. In addition, this method showed 11–38 fold increase in sensitivity for different drugs compared to conventional LC-MS/MS methods [69].

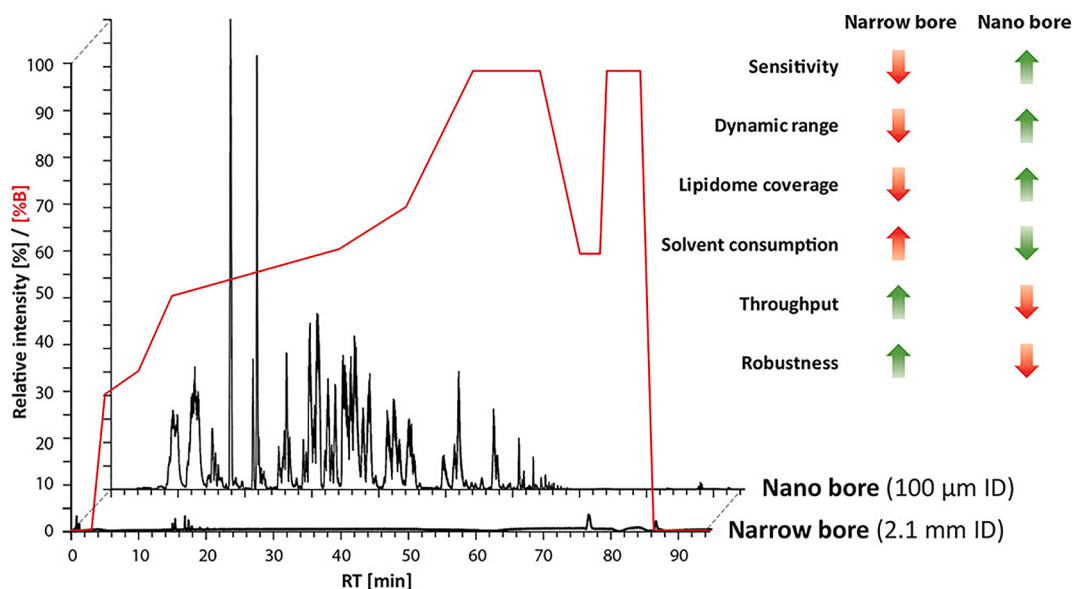
Details of several miniaturized metabolomics applications can be found in Table 3 [3,4,7,17,33,34,36,61–65,69–74] and Table 4 [44,46,67,68,75–84]. Low flow rates ranging from 200 nL/min to 50 µL/min were used to achieve high sensitivity and increase coverage of metabolome. For samples that are low in metabolite abundance but are not of limited volume, the trap-and-elute strategy was often used. In general, highly sensitive LC-MS with micro- or nano-flow and CE-MS methods were developed and applied for the analysis of biomass-restricted samples such as low number cells, dry blood spots, tissue sections and many other matrices.

Table 4

Overview of applications of miniaturized analytical techniques from January 2019 to April 2021 (CE-MS).

Compounds	Sample matrix	sample prep volume	BGE	sample pretreatment	MS analyser	LOD ^a	Ref.
Anionic and cationic metabolites	human urine and plasma	20 µL	1 M formic acid with 15 % acetonitrile (pH 1.8); 50 mM ammonium bicarbonate (pH 8.5)	centrifugation and dilution for urine; Ultrafiltration using 3-kDa filter for plasma	Agilent 6230 TOF-MS	n.s.	[75]
Cationic metabolites	mammalian cells	5000, 2500, 1000 and 500 cells	16 mM ammonium acetate (pH 9.7)	Ultrafiltration using 3-kDa filter	Sciex TripleTOF 6600 MS	0.1 to 0.9 nM	[67]
Cationic metabolites	mouse kidney sections	20 µm-thick kidney sections	10% (v/v) acetic acid (pH 2.3)	extraction in 80:20 MeCN: water (v/v)	Bruker UHR-QqTOF maXis Impact HD MS	n.s.	[68]
Anionic metabolites	cell medium	200 µL	0.8 M formic acid in 10% methanol	centrifugation	Agilent 6224 TOF-MS	n.s.	[76]
Anionic and cationic metabolites	Neonatal dried blood spot and sweats	about 15 µL blood	1 M formic acid, 15% v/v acetonitrile (pH 1.8); 50 mM ammonium bicarbonate (pH 8.5)	ultrafiltration using 3-kDa filter	Agilent 6550/6230 TOF-MS	n.s.	[77]
Cationic metabolites	HepG2 cells	500 and 10,000 cells	10% acetic acid	Ultrafiltration using 5-kDa filter	Sciex TripleTOF 5600 + MS	ranging from 1.4 to 92 nM (except for aspartic acid, 417 nM)	[43,78]
Anionic and cationic metabolites	human serum	50 µL	n.s.	protein precipitation and ultrafiltration using 5-kDa filter	TOF-MS	n.s.	[79]
Cationic metabolites	Macrophages	5 × 10 ⁶ cells	1 M formic acid in 10% methanol (v/v)	quenching, disruption and centrifugation	Agilent 6224 TOF-MS	n.s.	[80]
Cationic metabolites	human urine	10 µL	500 mM formic acid (pH 1.55)	dilution	Agilent 6410 Triple Quadrupole tandem MS	0.23–5.45 µM	[81]
Anionic and cationic metabolites	Freeze-dried muscle tissue	2 mg	1 M formic acid with 15 vol % acetonitrile (pH 1.8) 50 mM ammonium bicarbonate (pH 8.5)	modified Bligh-Dyer extraction	Agilent 6230 TOF-MS	n.s.	[82]
Anionic and cationic metabolites	human brain tissues	50 mg	50 mM ammonium acetate (pH 9.0)	Ultrafiltration using 5-kDa filter	Agilent 6210 TOF-MS	n.s.	[83]
Cationic metabolites	extracellular fluid of HK-2 cells	100 µL	1 M formic acid (pH 1.8)	Protein precipitation	Agilent 6530 TOF-MS	n.s.	[84]
Cationic metabolites	seizure mouse plasma	10 µL	water with 10% acetic acid (V/V)	Bligh and Dyer extraction and ultrafiltration using 5-kDa filter	Agilent 6230 TOF-MS	n.s.	[41]

a) LOD = limit of detection (S/N = 3); n.s. : not specified in paper.

**Fig. 6.** Total ion chromatograms from a yeast lipidomics study using two columns with 100 µm and 2.1 mm inner diameters (red line: LC gradients) [ref. 33]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

5. Conclusions and perspectives

To deal with the desired sensitivity and coverage of analytical metabolomics technologies for biomass-restricted samples in biomedical and clinical studies, some developments have been realized in MS-based metabolomics workflows. Even more importantly, over the past 5 years, optimizations of separations and ionization techniques, and hardware including columns, LC pumps and ionization sources have been made in micro and nano flow methods, yielding higher sensitivity for biomass-restricted samples not only for metabolomics but also other fields. Delicate parts such as micro- or nano-volume connectors, unions and tubings in miniaturized LC-MS systems have been designed for lower dead volume and improved the system robustness. We are convinced that the advances in analytical technologies and metabolomics will allow to further increase sensitivity and robustness for ultrasamples by further optimization of sampling and sample preparation procedures. Sampling methods for volume limited samples are required to avoid sample loss and preserve the biological heterogeneity in each sample as much as possible, and several strategies have been reported for this. After that, efficient sample preparation methods suitable for volume and especially concentration limited samples are also essential to gain higher sensitivity during analysis. While protein precipitation, liquid-liquid extraction and solid phase extraction are classic strategies for metabolomics sample preparation, current tools such as vials and sample transfer methods have to be further optimized for biomass-restricted samples. In summary, where development of sensitive LC/CE-MS techniques has been advanced for over recent years, the development of sample handling for small samples has progressed less for small molecules. Therefore, future developments should put more emphasis on miniaturized sampling and sample preparation methods in order to further increase the sensitivity and strengthen the robustness of miniaturized analytical methods.

Declaration of Competing Interest

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

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