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Mass spectrometry based metabolomics of volume-restricted *in-vivo* brain samples: Actual status and the way forward



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ABSTRACT

Brain metabolomics is gaining interest because of the aging of the population, resulting in more central nervous system disorders such as Alzheimer's and Parkinson's disease. Most often these diseases are studied *in vivo*, such as for example by analysing cerebrospinal fluid or brain extracellular fluid. These sample types are often considered in pre-clinical studies using animal models. However, the scarce availability of both matrices results in some challenges related to sampling, sample preparation and normalization. Much effort has been made towards the development of alternative, less invasive sampling techniques for collecting small sample volumes (pL till mid μ L range) over the past years. Despite recent advances, the analysis of low volumes is still a tremendous challenge. Therefore, proper preconcentration and sample pretreatment strategies are necessary together with sensitive analysis and detection techniques suitable for low-volume samples. In this review, an overview is given of the state-of-the-art mass spectrometry-based analytical workflows for probing (endogenous) metabolites in volume-restricted *in-vivo* brain samples. In this context, special attention is devoted to challenges related to sampling, sample preparation and preconcentration strategies. Finally, some general conclusions and perspectives are provided.

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1. Introduction to brain metabolomics

As the population is becoming older, central nervous system (CNS) disorders, such as Alzheimer's and Parkinson's disease, are becoming more prominent. The underlying pathological mechanisms of these neurological disorders are not yet well understood, and therefore more research is needed in order to develop novel

drug targets and therapies. Brain metabolomics studies may reveal insights in the mechanisms of these CNS disorders.

Brain metabolomics approaches may be used to profile the (endogenous) small molecules, either in a targeted or untargeted way [1–3]. Regularly, the study objects in *in-vivo* brain metabolomics are samples from small animal models, which result in sample volumes from the picoliter level to about 50 μ L [4–6]. The detection of low metabolite concentrations is even more challenging in such small sample amounts. Therefore, adequate sample preparation and preconcentration steps are necessary, as there is a high need for sensitive techniques. Microextraction techniques for sample pretreatment, such as solid-phase microextraction (SPME) and liquid-phase microextraction (LPME), are emerging. Increased sample throughput can be obtained when coupling these techniques on-line with miniaturized separation techniques [5]. Nano- and micro-liquid chromatography (LC), capillary electrophoresis (CE) and chip-based systems are suitable for small-volume sample analysis. Another often-applied method for low sample amounts in metabolomics is gas chromatography (GC) [7]. GC is, besides LC,

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Abbreviations			
BBB	Blood-brain barrier	ICD	Isotope-coded derivatization
CE	Capillary electrophoresis	IS	Internal standard
CI	Chemical ionization	LC	Liquid chromatography
CMS	Capillary microsampling	LLE	Liquid-liquid extraction
CNS	Central nervous system	LPME	Liquid-phase microextraction
cOFM	Cerebral open flow microperfusion	MOI	Microfluidic open interface
CSF	Cerebrospinal fluid	MS	Mass spectrometry
DA	Dopamine	MCE	Microchip CE
DBS	Dried blood spots	MEPS	Microextraction in a packed syringe
DI-MS	Direct infusion-MS	MIPs	Molecularly-imprinted polymers
DLLME	Dispersive liquid-liquid microextraction	NE	Norepinephrine
DmPABr	Dimethylaminophenacyl bromide	NIST	National Institute of Standards and Technology
ECF	Extracellular fluid	NMR	Nuclear magnetic resonance
EI	Electron ionization	PPPS	Push-pull perfusion sampling
EKS	Electrokinetic supercharging	PT-SPE	Pipette-tip solid-phase extraction
EME	Elektromembrane extraction	μPPPS	Micro Push-pull perfusion sampling
EPI	Epinephrine	SPE	Solid-phase extraction
FASI	Field-amplified sample injection	SPME	Solid phase microextraction
FASS	Field-amplified sample stacking	tITP	Transient-isotachopheresis
GC	Gas chromatography	TFME	Thin-film microextraction
HDI	Hydrodynamic injection	TOF	Time of flight
		VAMS	Volumetric absorptive microsampling

also suitable for on-line coupling with extraction techniques, while on-line derivatization is also possible. More specific, when mass spectrometry (MS) is applied as detection technique, derivatization with isotopes is gaining interest to correct for metabolite variation, resulting from volume mismatch, sample loss and matrix effects [8]. MS is indeed a very suitable detection technique for brain metabolomics, because of its higher detection sensitivity, compared to nuclear magnetic resonance (NMR) which is also often applied in metabolomics. Electrochemical and fluorescence-based detection techniques have also been used for the sensitive analysis of metabolites in brain samples [9,10]. However, electrochemical detection is limited to electroactive substances, whereas fluorescence detection often requires a derivatization step in order to introduce a fluorophore. Therefore, these detection techniques (when used in combination with LC or CE) are more suited for targeted metabolomics studies.

In this review the focus is on the analytical challenges of different small-volume matrices studied in *in-vivo* brain metabolomics. Analytical challenges occurring during sampling, sample preparation and preconcentration are considered in detail, in addition to the challenges associated with coupling miniaturized separation techniques to MS detection. Therefore, emphasis is given on novel miniaturized workflows, while traditional workflows in brain metabolomics will not be discussed. *In-vitro* and *ex-vivo* samples, such as cell lines, organoids and tissue samples, are also excluded from the discussion. More information about challenges related to sample collection, preparation, analysis and data normalization for tissue samples can be found in Refs. [11,12] and for single cells in the following recent reviews [4,13].

2. Volume-restricted sample matrices investigated in *in-vivo* metabolomics

Small sample amounts for *in-vivo* brain metabolomics are caused by a variety of reasons. For example, rodent models, especially mice, allow collection of only small sample volumes (low μL range) from a living animal. Furthermore, brain fluids, such as extracellular fluid (ECF), are scarcely available and are already

volume-limited in larger animal models or human donors. Alternative animal models, such as zebrafish, are also gaining interest for metabolomics studies, leading to even lower sample volumes available for analysis. In order to have sufficient material for metabolomics studies, sometimes a pooling approach is applied. However, pooling will provide an average read-out and therefore information on metabolic changes at the individual level and/or time level, and thus heterogeneity, is lost. In this section, an overview of volume-restricted sample types investigated in *in-vivo* metabolomics, including the trends in their sampling strategies, will be given and discussed.

2.1. Brain fluid

Brain fluids, i.e. ECF and CSF, are of major interest in mental and neurological disorders. Especially CSF sampling, often performed via a lumbar puncture in the brain subarachnoid space, has proven to be an invaluable tool in clinical neurology. However, in animal model studies, CSF is still an underrepresented matrix due to its poor accessibility, invasive sampling and low sampling volumes [6]. Other challenges, related to lumbar puncture, are the presence of non-specific binding of apolar and/or larger molecules to the sampling catheter [14]. To allow serial CSF collection in conscious minipigs, a minimally-invasive method via catheterization of the subarachnoid space was proposed [15]. Šakić described a modified cisternal puncture method in *post-mortem* mice to enhance the quantity (most often still less than 40 μL) and quality of the CSF samples obtained [6].

A technique that is often used to collect brain ECF is microdialysis, an *in-vivo* sampling technique allowing continuous sampling in freely moving animals [16]. Traditionally, the sampling is performed at relatively high flow rates ($\sim 2 \mu\text{L}/\text{min}$) in order to provide adequate temporal resolution (typically 10–20 min) and sufficient sample volumes ($\geq 30 \mu\text{L}$) [17]. However, to get an accurate view on the rapidly changing neurotransmitter levels upon a perturbation, a higher temporal resolution of microdialysis sampling is preferred, providing lower sample volumes (few μL or less). An additional challenge is the high amount of inorganic salts in

microdialysis samples, which makes the analysis with MS detection techniques challenging because of matrix-effect issues. Non-specific binding can also occur at the microdialysis probe [18]. Despite these limitations, microdialysis is still considered the gold standard for brain fluid collection. However, in order to be able to perform more localized measurements, to increase temporal resolution and to minimize tissue damage, promising results are obtained with microfabricated probes [19].

An alternative sampling method, i.e. push-pull perfusion, is regaining interest for neurochemical analysis, mainly due to the introduction of miniaturized probes and advances in microfluidics [19]. In the so-called micro push-pull perfusion sampling (μ PPPS), the sample is withdrawn from one capillary using low flow rates (~ 50 nL/min), while perfusion fluid is infused from another capillary. The main advantage of μ PPPS is its spatial resolution, which is higher than conventional microdialysis sampling (0.06 mm² for microdialysis vs. 0.004 mm² for low-flow push-pull perfusion) [20]. Compared to microdialysis probes, PPS probes typically contain larger pores, making the latter technique better suitable for larger molecules, such as proteins. However, there are some challenges that arise with low flow push-pull sampling, e.g. using low flow rates at high temporal resolution (i.e. a few seconds) results in nanoliter samples, and Taylor dispersion, which is the broadening of sample zones during their transport through a microfluidic channel, also needs to be controlled. The latter issue could be resolved by implementing a segmented flow [19], which has been directly integrated with MS detection (Fig. 1A and B) [21–23], showing great improvements in temporal resolution (few seconds). However, the current push-pull methods, similarly to microdialysis, are still not suitable for the determination of rather apolar analytes.

A recently developed variant of PPS is cerebral open-flow microperfusion (cOFM) [24], which is based on push-pull perfusion, but uses a membrane-free probe to sample brain ECF. The absence of a membrane makes the technique suitable for larger and lipophilic compounds, while membrane-related problems, such as clotting, are prevented [25]. However, this also means that extra sample preparation steps are required before sample analysis. Additionally, the design and material of cOFM probes allow re-establishment of the blood-brain barrier (BBB) integrity, and thus long-time monitoring within brain ECF is possible.

Another *in-vivo* sampling technique that is gaining more interest for monitoring brain chemistry is SPME [26,27]. In (direct immersion) SPME, a thin probe (thickness ~ 200 – 300 μ m) containing a biocompatible outer coating (often a C18 sorbent combined with polyacrylonitrile) is used to extract analytes through passive diffusion (Fig. 1C), directly from brain tissue, plasma or extracellular fluid in a minimally invasive manner. SPME is complementary to microdialysis and PPS, because it can be used to extract hydrophobic analytes, as it has proven to be effective for the analysis of a wide range of metabolites, including low abundance steroids and lipids. Recoveries of highly polar compounds, on the other hand, are still low for SPME [28].

The small size of the SPME probe and the selectivity of its coating allow a highly selective extraction of analytes of interest, isolating them from macromolecules or waste in the sample. Furthermore, for *in-vivo* SPME, the same set-up as for microdialysis can be used, meaning that SPME can be straightforwardly implemented into already existing workflows [29]. Additionally, because of their complementary nature, SPME and microdialysis sampling could be performed in parallel within the same test subject in order to capture as much chemical information as possible [30]. An important limitation of SPME is still the lack of fundamental understanding of the extraction process using biocompatible coatings, which show low recovery of compounds and resulting in a low commercial availability of these coatings [26,31]. However,

progress is ongoing in this area and it is therefore anticipated that SPME sampling will obtain a more prominent place in *in-vivo* brain metabolomics studies.

2.2. Blood

Because of the challenges with extracellular brain fluid and tissue collection, blood samples are more commonly considered for clinical and diagnostic purposes. However, they do not reflect the actual brain concentration. Whole blood collection requires the need of well-trained personnel and results often in large volumes in humans and larger animals (up to 5 mL). Such volumes cannot be sampled from all living animals because of increased animal welfare concerns and the limited available blood volume present per kg body weight (55 – 70 mL/kg body weight). For instance, in mice only a maximum of 20 μ L blood sample is allowed to be taken in a serial sampling strategy [32,33]. Therefore, alternative sampling strategies are necessary for small-volume blood collection. They may also be useful for humans or larger animals because of their less invasive character and possibility of “self-sampling”. Different alternative blood collection and microsampling techniques are introduced, such as dried blood spots (DBS), volumetric absorptive micro-sampling (VAMS) and capillary microsampling (CMS) [5,34]. An important challenge for DBS is the fluctuations in haematocrit content, leading to an uncertain sample volume. VAMS and CMS result in a precise and accurate blood volume by making use of an absorbent polymeric tip (10 , 20 or 30 μ L) or glass capillary (1 – 35 μ L), respectively. CMS is not (yet) widely used in metabolomics but the suitability of VAMS in untargeted metabolomics was already shown by Volani et al. [35]. They evaluated the type of extraction solvent and the sample stability, thereby concluding that the VAMS samples should be stored at -80°C instead of at room temperature. Furthermore, a targeted study with VAMS as a sampling technique was developed and validated for the measurement of 36 metabolites [36]. The extraction of organic acids and amino acids from the VAMS devices was performed with acetonitrile-water ($60:40$, v/v) in only 10 min. Moreover, the internal standard (IS) was added the absorptive tip before blood sampling, allowing to correct for variations in the extraction process and/or potential metabolite changes upon long-term storage [36]. In general, VAMS is not widely applied in metabolomics and even not in brain metabolomics, but it shows a lot of potential as demonstrated by the above studies. More information on DBS, VAMS and CMS can be found in Refs. [5,34].

3. Sample pretreatment and preconcentration

Sample preparation of biological matrices is a crucial step in metabolomics. This step is often most time consuming and labour intensive. The main goal of the sample preparation is to reduce matrix interference, increase selectivity for the metabolites of interest in (semi) targeted analyses, preconcentration and stabilization of the sample, and to get the compounds of interest in the (proper) dynamic range of MS-based metabolomics. These improvements are necessary to overcome the challenges of crowded spectra, sensitivity issues, identification and structure-determination problems. The most easily and quickly applied offline sample preparation methods that are still often applied in brain metabolomics are simple dilution, protein precipitation and centrifugation or filtration. However, matrix effects have a high chance of occurrence using these sample preparation techniques. It is even seen that a more selective sample preparation such as liquid-liquid extraction (LLE) or solid-phase extraction (SPE), can result in an increased coverage in untargeted metabolomics and

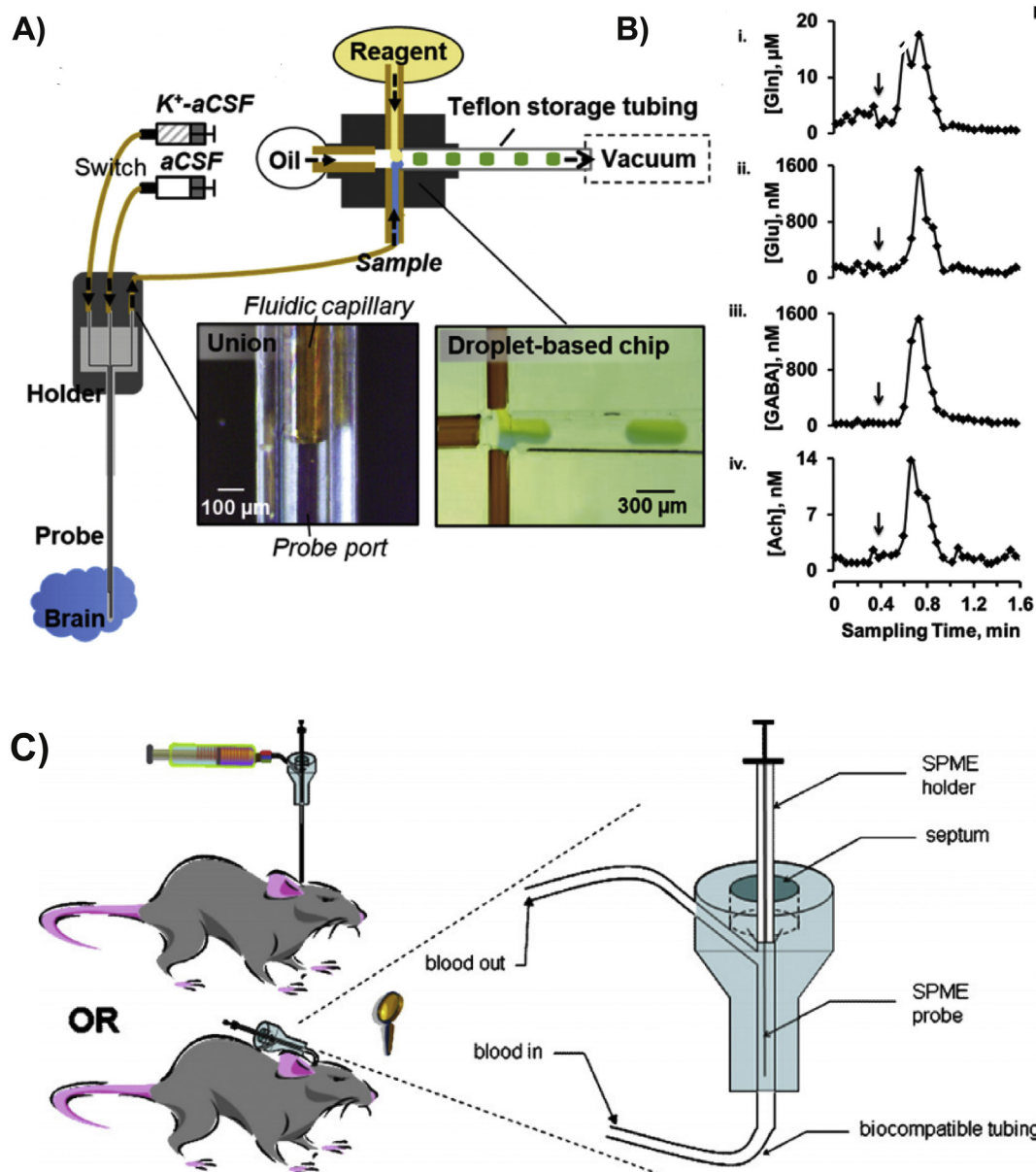


Fig. 1. (A) Monitoring brain chemical dynamics using micro push-pull perfusion sampling, droplet based microfluidics, and direct infusion MS assay, with (A) a schematic overview of a microfabricated push pull probe, and (B) the averaged detection responses as measured by direct infusion MS of glutamine (i), glutamic acid (ii), GABA (iii), and acetylcholine (iv) during a microinjection of high potassium concentrations via the micro probe. Figure is adapted with permission from Ref. [23]. Copyright (2020) American Chemical Society. (C) *In-vivo* SPME study on rats: placement of SPME devices and interface connection to the carotid artery, reused with permission from Ref. [89].

better reproducibility of the method [37] due to the reduced matrix effects.

However, the traditional LLE and SPE techniques often require relatively large sample volumes. Other limitations include the high consumption of organic solvents, use of salts and buffers and waste production. Moreover, the manual operation could be quite laborious and time-consuming. A strategy to make metabolomics workflows better suited for low-volume samples and to overcome the disadvantages of the traditional techniques is to consider the potential of micro-extraction techniques [38–40]. In this context, LPME and SPME approaches were developed, which have been recently reviewed for metabolomics applications by Hemmati et al. [5]. In the present review only the recent advances and (remaining) challenges of microextraction techniques for brain metabolomics

are highlighted. Moreover, alternative miniaturized SPE techniques are briefly discussed.

Another strategy to improve detection sensitivity and selectivity of neurochemicals is to change the physicochemical properties of analytes by performing a derivatization step. Recent advances on this topic are also discussed.

3.1. Micro-extraction techniques and micro-SPE

As already mentioned, two well-known micro-extraction techniques are LPME and SPME. A major advantage of both is their ease of automation, thereby integrating sampling, extraction, concentration and injection, resulting in on-line coupled techniques. Especially in case of *in-vivo* sampling, this on-line coupling is greatly advantageous in terms of minimizing handling errors and

increasing sample stability. In spite of these advantages, SPME and LPME are still often applied off-line.

Different LPME techniques have already been widely discussed in the literature [5,38,41]. However, in brain metabolomics the most often applied LPME technique is dispersive liquid-liquid microextraction (DLLME; Fig. 2A) [5]. The principle of DLLME and its application in metabolomics are discussed in the following reviews [5,38,39]. In general, a small volume of organic solvent mixed with a dispersant solvent is rapidly injected in the aqueous sample, resulting in tiny droplets of extraction solvent in the sample. The major advantage is that analytes can easily penetrate into the solvent resulting in a high preconcentration factor. This technique was for example used by Zheng et al. [34]. They have developed a stable isotope-labelling derivatization-ultrasound-assisted DLLME for the simultaneous determination of monoamine neurotransmitters and their biosynthesis precursors and metabolites in rat brain microdialysis samples [42]. This approach overcomes the severe matrix effects as well as the limitations of isotopic labelling present in traditional dilution approaches. The extraction of hydrophilic compounds with DLLME is still challenging but can be achieved by adapting the pH. Another LPME method in metabolomics is electromembrane extraction (EME). This method has the advantage that the sample clean-up can be selectively tuned by modifying the electrical field. However, the method is already applied for plasma [5,41,43] and for the analysis of spiked neuropeptides in CSF [44]. Those investigations and applications makes EME a very attractive tool for brain metabolomics. A further discussion of the technique and their main objectives with still some points of research are recently discussed by Huang et al. [45].

SPME was previously discussed as an *in-vivo* sampling technique. The possibility of sampling together with applying a sample clean-up and pre-concentration is a main advantage of SPME. Another great possibility is the direct coupling of SPME to MS devices, as has been shown by Nazdrajić et al. [46], where direct immersion SPME was directly coupled to microfluidic open interface (MOI)-MS for direct analysis of metabolites in whole human blood [47]. Furthermore, SPME is more widely applied than LPME as an extraction technique in brain metabolomics [38]. Conventional SPME contains fiber coatings with tuneable structures but shows small absorption capacities and low extraction rates, especially for hydrophilic compounds. Therefore, thin-film microextraction (TFME) was developed. TFME contains a sheet of a flat-film resulting in a higher area-to-volume ratio and consequently a higher uptake rate and extraction recovery, even for hydrophilic compounds. Principles, advantages and applications are discussed in the review of Hemmati et al. [5] and of Olcer et al. [48]. TFME shows a lot of potential but has, to the best of our knowledge, not yet been used in brain metabolomic studies. SPME still has some limitations, such as the instability of the fiber and low recovery, and results in higher variability than LLE and SPE. The same sorbents as for SPE could also be considered in SPME as coating for the fiber. Still, inter-device variability is observed in SPME of commercial and in-house made devices. Therefore, the development of highly reproducible new fiber coating technologies will be of interest to generate more accurate and reproducible results in SPME. Reproducibility could also be encountered by considering the pre-loading of an IS onto the fiber coating to control sampling and matrix related variabilities [49].

In recent years, (more robust) miniaturized SPE techniques (micro-SPE) are upcoming, such as microextraction packed in pipette tips, better known as Pipette-tip solid-phase extraction (PT-SPE) or in syringes (microextraction in a packed syringe (MEPS)). A schematic representation of PT-SPE and MEPS is shown in Fig. 2B and C. Here, the typical steps known in SPE need to be performed such as conditioning, loading, washing and eluting. Both micro-SPE

techniques, PT-SPE and MEPS, contain a small volume of solid-phase sorbent packed in a gas tight syringe or pipette tip and result in higher extraction recoveries than SPME [38,39]. Well-known sorbents are C_{18} or C_8 , while alternatives are mixed mode cation/anion-exchangers and weak anion/cation-exchangers. At the moment, C_{18} and mixed-mode (hydrophilic/lipophilic) extraction phases have been used in brain metabolomics [28,50]. However, different new sorbent materials emerge fast, such as nanoparticles, molecularly-imprinted polymers (MIPs), metallic nanoparticles and metal-organic frameworks [38,41,51]. The MIP cartridges are only used in targeted studies because of their high selectivity [51].

Fractionation of samples provides the possibility to extract each fraction with a different selectivity. However, fractionation is still valuable aliquots is difficult when low sample volumes are acquired. The applicability on low-volume samples (20 μ L plasma) was recently discussed by van der Laan et al. [52]. A comprehensive fractionation approach of serially coupled SPE columns (reversed phase, mixed-mode anion and cation exchanger) was developed and showed reduced ion suppression as well as increased sensitivity for multiple metabolite classes. This highlights an emerging need for multiplatform sample preparation protocols to enhance the metabolome coverage. In the future, the development of pre-fractionation techniques will be more prevalent [40]. The possibility of fraction analyses on different platforms will result in an increased metabolite coverage of studied samples and detection of novel markers with a higher certainty.

3.2. Derivatization strategies

Because sample fractionation prior to multiplatform sample preparations is not always an option, other sample pretreatment approaches can be applied to cover a (large) part of the metabolome. Chemical derivatization, which includes altering the physicochemical properties of analytes by the introduction of a chemical moiety, is an emerging strategy for metabolomics studies [39,53]. It can be used to allow, for instance, the separation and detection of compounds with differences in polarity, acidity/basicity and concentration using the same analytical method. Additionally, derivatization may be used to stabilize metabolites of interest by preventing oxidation, for example, of monoamine neurotransmitters.

Especially for GC-MS based metabolomics studies, chemical derivatization has been applied for several decades in order to improve volatility, separation and detection sensitivity of metabolites [54]. Here, typically a two-step procedure is executed, including oximation and silylation. Often reported issues with regard to this two-step derivatization procedure is the lack of reproducibility during batch derivatization. However, Miyagawa et al. [55] obtained reproducible peak areas for 52 selected metabolites using sequential derivatization and interval injection. Furthermore, silylation is however not suitable for some metabolites, such as amino acids, as unstable silylated derivatives are obtained. Silylation reactions require anhydrous reaction conditions, meaning that no water should be present in the sample. Alternatively, methyl chloroformate can be used as derivatization reagents for alkylation of polyfunctional amines and organic acids. These alkylation reagents are inexpensive and the reaction occurs instantaneously without heat or water exclusion. However, most of them are toxic or hazardous.

Still, in untargeted metabolomics, it is practically impossible to achieve (complete) derivatization for all compounds. Therefore, the optimization of the derivatization steps in GC is still an active research domain [56]. An already developed derivatization approach is known as solid-phase analytical derivatization (SPAD)

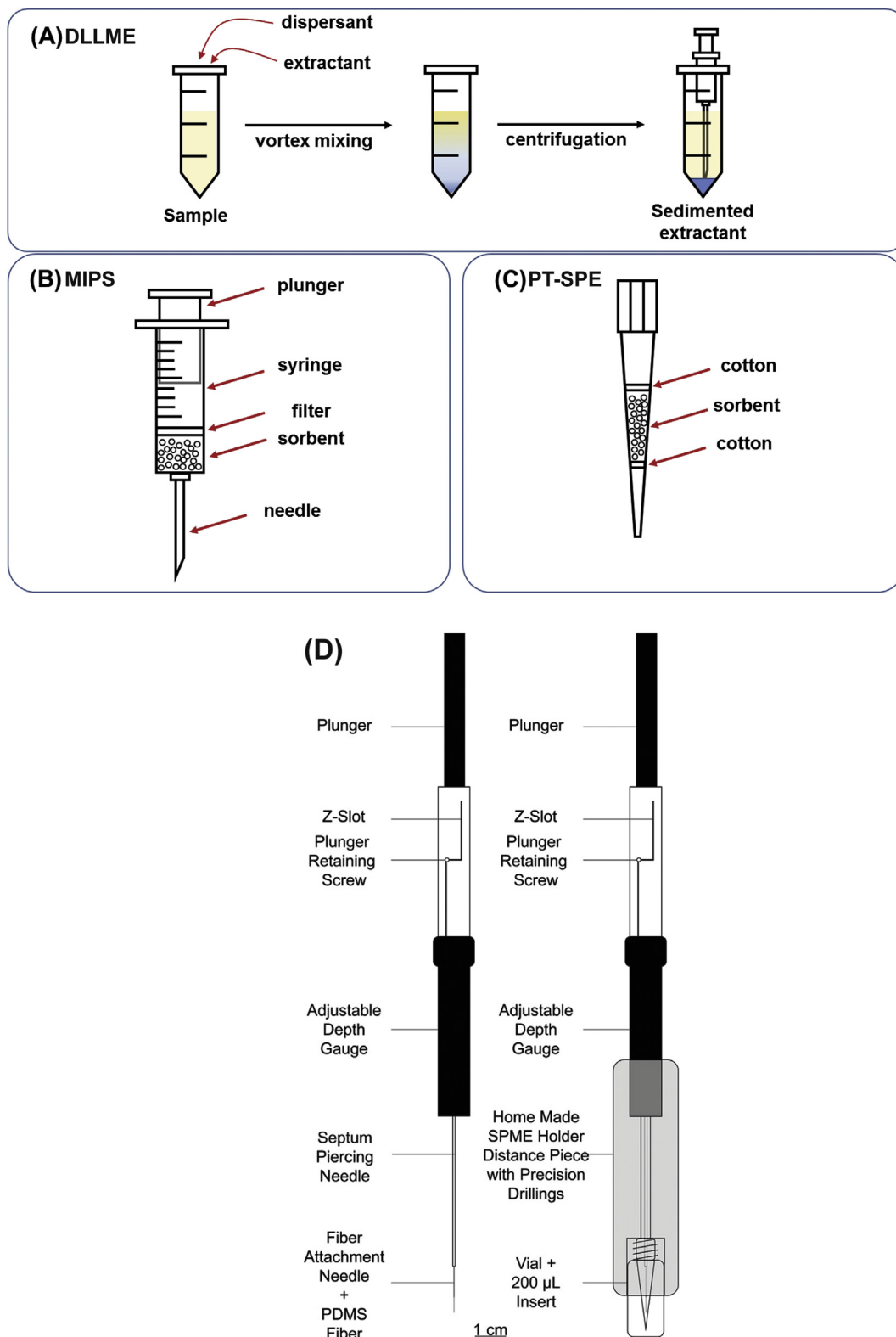


Fig. 2. Schematic overview of miniaturized sample preparation techniques suitable for low-volume samples. A) Dispersive liquid-liquid microextraction, B) Microextraction in a packed syringe and C) Pipette-tip solid-phase extraction. Figures A, B and C re-used with permission from Ref. [39]. D) shows a SPME device with a modified distance piece to get reproducible immersion depths necessary for coupling SPME to GC-MS, reused with permission from Ref. [84].

[57]. Here, derivatization and SPE are combined resulting in less labour intensivity, increased selectivity, automation possibilities and compatibility with a wide range of matrices. Moreover, recent developments in GC derivatization can be found in Ref. [54]. However, an extensive discussion of derivatization agents and procedures is out of the scope of this review and is already discussed in the aforementioned review of Beale et al. [54]. As general drawback, derivatization might lead to incomplete derivatized products, by-products and multiple derivatives which makes the structure elucidation process more complicated. Therefore, optimization and standardization is required for each application.

For LC-MS, chemical derivatizations with for instance dansyl chloride [56], benzoyl chloride [58] and dimethylaminophenacyl bromide (DmPABr) [59] have been successfully applied on low amounts of sample material, showing great potential for future brain metabolomics studies. Recently, a multi-platform study was published where relatively small CSF samples (30 μ L) were derivatized using PITC (phenylisothiocyanate)-derivatization by a commercial Biocrates kit (BIOCRATES Life Sciences AG, Innsbruck, Austria), and analyzed by NMR, LC-MS and GC-MS [60].

An interesting trend in derivatization strategies is isotope-coded derivatization (ICD) [8], where an isotopic labeled version of every derivatized analyte is produced. Using ICD, it is possible to overcome the dependence on isotope labeled analogues, which are not available for all metabolites and which are expensive. Another advantage of introducing a single IS for every metabolite is its large improvement in metabolite quantification [58,59]. A drawback of ICD labelling is that the derivatization usually occurs after the sample pretreatment, which means that for the latter no correction by the IS recovery is made and thus needs to be accurate, efficient and standardized. For more information about ICD we refer to a recent review of El-Magrahbey et al. [8].

4. Mass spectrometry-based approaches for metabolomics of volume-restricted samples

MS is the most suitable detection technique for low-volume samples containing low concentrations of metabolites. Direct infusion-MS (DI-MS) would provide the highest throughput for analysing metabolites in (volume-limited) biological samples. However, a trade-off exists between sample throughput and metabolic coverage. Using DI-MS, matrix effects (i.e., compounds affecting each other's ionization efficiency) may compromise quantification, and in case of ion suppression of low-abundant metabolites it may result in a loss of relevant sample information as these compounds are not detected anymore. Sample preparation is therefore very critical when employing DI-MS for metabolomics studies. Moreover, DI-MS does not allow resolving isomeric compounds with identical fragmentation patterns. Therefore, most often, a separation technique is used before the sample compounds reach the detector. The separation technique needs to be suitable to analyse the low-volume samples with sufficient sensitivity. This brings the discussion towards miniaturized techniques and especially the inclusion of miniaturized columns and capillaries, as applied in LC, CE and GC. These approaches are considered in more detail in the following sections by devoting attention to advantages, trends and challenges for small-volume samples. DI-MS is not covered here as our survey of the scientific literature did not reveal that such an approach has been developed and/or used for metabolite analysis of volume-restricted *in-vivo* brain samples.

4.1. Miniaturized LC-MS and chip-based systems

LC-MS is still the most common applied platform in metabolomics. This technique is suitable to measure polar and apolar

abundant metabolites in different sample matrices. However, due to the high flow rate, sensitivity issues often occur. Miniaturization of the LC part may overcome the sensitivity issues because of the lower flow rate required, resulting in less sample dilution and increased ionization efficiency [37,61,62]. The lower flow rate allows coupling LC with electron ionization (EI). EI is more robust and is less prone to matrix effects than ESI [61,62].

Moreover, low volumes of reagents and samples are also required in miniaturized LC [37,61], which therefore is favourable for the analysis of volume-restricted samples obtained in *in-vivo* brain metabolomics studies. The miniaturization in LC is driven by column adaptations resulting in capillary (i.d. 0.1–0.5 mm) to nano (i.d. < 0.1 mm) columns with reduced column dimensions [61,62].

A further reduction in dimensions is achieved with open tubular columns (0.05–0.005 mm i. d.) [62,63]. The possibilities of open tubular columns are widely studied and extensively described in reviews by Vasconcelos Soares Maciel et al. [62] and Mejía-Carmona et al. [63]. These columns show several advantages, such as higher efficiencies compared to capillary and nano columns, the possibility to work at higher flow rates without an increased pressure and less waste production. Their application in research fields, such as metabolomics, did not reach its climax due to the absence of commercially available open tubular columns for LC [63]. At the moment, companies are still not interested in developing these columns for LC, while research groups made many efforts to prepare their own open tubular columns in order to show their robustness, efficiency and superior performance.

The decreased column dimensions require some instrumental adaptations [37,61,62], which in turn can have beneficial effects. For example, the position of the ESI emitter is closer to the MS inlet in nano liquid chromatography nano electrospray ionization, resulting in an additional gain in sensitivity [37].

Besides this instrumental benefit, some challenges are also related to nano- and capillary LC instruments [37,61]. Indeed, robustness issues, such as retention time shifts and reproducibility issues of the peak intensity, occur in these set-ups. Consequently, the number of papers using nano- or capillary LC for brain metabolomics is limited, while contradictorily it is often applied in brain proteomics [37,61,64]. In a study by Lin et al. [65] nano-LC-MS/MS is applied for proteomics and UHPLC-Time of flight (TOF) for metabolomics, in order to better understand Alzheimer's disease. In case of protein and peptide analysis, sensitivity issues may occur due to the presence of charge state distributions. The use of superchargers could be of interest to overcome this sensitivity problem. A few studies demonstrate the influence of supercharging agents on the charge state of neuropeptides [66,67]. The exact mechanism of supercharging agents is not known yet but their use could be of interest in brain metabolomics to increase sensitivity, especially in the analyses of low volume-samples.

Due to the reduced column dimensions, emitters and connections are more prone to blockages and therefore the sample preparation is crucial in these miniaturized LC set-ups [37,62]. Miniaturized sample preparations and preconcentration steps are already discussed in Section 3. Another, not yet, discussed on-line LC sample preparation approach is a column-switch set-up. This results in a rapid analysis, sample enrichment and purification and an extended coverage of the metabolome. This method was applied in Ref. [68] to study Alzheimer's disease in plasma using a nano-LC system with a nano pre-column for clean-up and preconcentration. The instrumental set-up and a chromatogram are shown in Fig. 3. Actually this type of column-switch set-up is not commonly applied anymore in brain metabolomics. This is the result of other upcoming techniques, such as in-tube SPME, which can be coupled to LC. More information about column switching instrumentation can be found in Ref. [69].

Newer approaches are pillar-array columns and chip-based liquid chromatography. Both are still not commonly applied in metabolomics, although they have several advantages. The efficiency of microchips is better due to a reduction of the void volume problems seen in nano- and capillary LC. However, there are still challenges related to the LC chip devices, such as low reproducibility [62,70]. Researchers try to further integrate all compartments on a chip and some commercial LC-chip devices are already on the market. Examples are the HPLC-Chip/MS devices from Agilent and the more integrated system from Waters, Ion-Key, which will lead to more robust applications. The suitability of the micro-pillar array columns for proteomics is, among others, demonstrated by Tóth et al. [71]. Compared to packed bed columns, the pillar columns resulted in a better identification for peptides and proteins due to reproducible retention times. The technique is considered exceptional because of the low back-pressure, even distribution of compounds, precision of the retention time and the long lifetime of the columns. Therefore, this type of miniaturized and chip-based approaches are of great interest for volume-limited metabolomics studies.

4.2. CE-MS and microchip CE-MS

CE-MS is a microscale analytical technique that has gained popularity for metabolomics studies in the last decade, mainly because of its complementary separation mechanism to chromatographic techniques, such as LC and GC. CE-MS can be considered an attractive tool for the analysis of volume-limited or scarcely available samples as only nanoliter injections are typically employed from just a few microliters of sample. Generally, a classical co-axial sheath-liquid interface is used for coupling CE to MS, but improvements in interfacing designs have emerged over the last decade [72], which mainly resulted in methods with an improved detection sensitivity.

Even though CE-MS is still an underrepresented technique in brain metabolomics studies, it was recently employed for the analysis of low-volume plasma samples from mouse disease models for epileptic seizures [32] and hemiplegic migraine [73], showing its potential for biomarker studies. However, a limitation of the low sample-loading capacity of CE (referring here to capillary zone electrophoresis), especially in conjunction with a classical sheath-liquid interface, is that the detection sensitivity can be compromised. Therefore, a number of on-line sample preconcentration methods have been developed to increase sample-loading volumes without hampering the intrinsic high separation efficiency of CE. Examples of in-capillary preconcentration strategies are pH mediated stacking, dynamic pH junction, field-amplified sample injection (FASI), field-amplified sample stacking (FASS), sweeping, large-volume sample stacking, transient-isotachopheresis and electrokinetic supercharging (EKS) [74]. Preconcentration based on dynamic pH junction already showed to be compatible with high-salt content urine samples, and the technique has recently been optimized for microdialysis samples [75]. In the method, only 5 μL of microdialysate was needed for direct CE-MS analysis, thereby allowing multiple injections from the same sample vial, showing its potential to improve microdialysis sampling times. In another study, preconcentration based on electrokinetic supercharging was successfully coupled with ESI-MS/MS [76], thereby showing an about 5000-fold sensitivity improvement for neurotransmitters analysis as compared to a CE-MS method using a conventional hydrodynamic injection (Fig. 4), reaching LODs as low as 10 pM. However, especially when performing electrokinetic injections, the sample nature is of critical importance for the selectivity and repeatability of injections. Therefore, various chromatographic-based sample preparation systems coupled on-

line or in-line to CE-MS have been developed [77]. Besides improved repeatability, also extra preconcentration could be achieved by adding an extra sample preparation step. With the ability to achieve these low detection limits, CE-MS could play a prominent role in future brain metabolomics studies.

Furthermore, the introduction of novel, high-throughput methodologies employing CE-MS has high potential for brain metabolomics studies. For instance, the introduction of multi-segment injection a few years ago greatly improved the throughput of CE-MS analyses (<5 min per sample), and even though it has not been used yet for brain fluids and/or tissues, it already showed its suitability for the metabolomic analysis in DBS samples [78]. Another study, focusing on increased throughput, presented a method where microdialysis was online coupled to CE in order to investigate branched chain amino acids as possible biomarkers [79]. Temporal resolution of microdialysis sampling was about 60s, and CE coupled to laser-induced fluorescence analysis was performed in less than 30s. This assay shows the potential of online coupling of brain-fluid sampling directly to CE for high-speed brain metabolomics.

Another promising technique for brain metabolomics applications is microchip CE (MCE). In MCE, high-speed separations of ultra-low sample volumes can be performed while maintaining the high separation efficiency of CE. However, MCE coupled to MS is still at its infancy, and without the availability of commercial MCE-MS devices, this will remain an attractive research field [80].

4.3. GC-MS

GC-MS is the most efficient, robust and reproducible analytical platform in metabolomics. The combination with well-established libraries makes the technique the most widely used in metabolomics [39]. Two ionization sources are used to hyphenate GC with MS, EI and chemical ionization (CI) [54]. In metabolomics studies EI is more often applied than CI. As previously discussed, an advantage of using EI is that it is less prone to matrix effects. Another benefit of EI is that it will produce fragments of each analyte and the fragmentation is robust, which makes the data suitable to compare with known databases, such as for example National Institute of Standards and Technology (NIST).

The technique is applicable for the analysis of polar small molecules, for instance neurochemicals, present in different matrices, such as CSF, ECF and blood products (e.g. DBS). The study by Motsinger-Reif et al. [81] for example uses GC-TOF to analyse 30 μL CSF samples to find biomarkers to discriminate between Alzheimer's disease patients and control groups. In general, samples from brain metabolomics have volumes of approximately 40 μL or less. The low-volume samples can easily be analyzed by GC-MS thanks to the small open tubular capillary columns, where only 2 μL are necessary for injection [62].

The above mentioned sample preparation techniques, e.g. SPME and MEPS, can also be used for GC analysis [54,82]. However, chemical derivatization is necessary to increase the volatility and thermal stability of the neurochemical products. Derivatization is already discussed in Section 3, while a more extensive discussion on different derivatization strategies (off-line, in-line and in-liner) and agents are described in Ref. [54]. Still most often a two-step off-line derivatization (including silylation and oximation), performed on approximately 40 μL sample [55,82], is applied in brain metabolomics [83]. The approach could also be suitable for lower volumes by adapting the volumes of derivatization agents. As already mentioned, it is necessary to investigate the most adequate derivatization procedure and combination of agents to obtain the best sensitivity and selectivity for targeted metabolites [54].

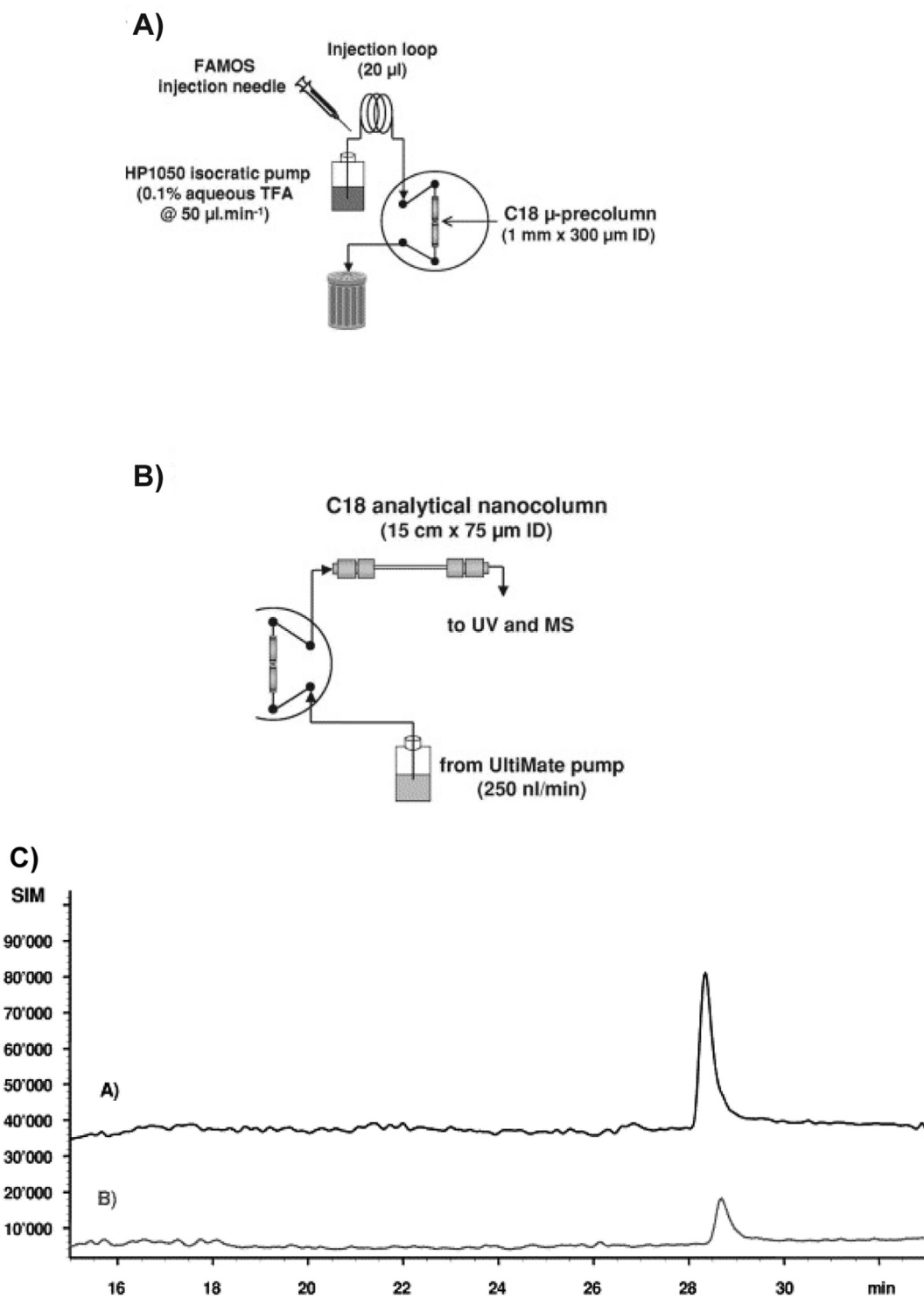


Fig. 3. Column-switch instrumental set-up in capillary LC; (A) sample clean-up, (B) analytical separation and (C) chromatograms of Aβ₁₋₄₀ peptide are shown. In the upper part of (C) A) 20 μL of the solution was injected and compared with B) 10 μL injection. Figures are taken with permission from Ref. [68].

Nowadays there are few novel trends in GC instrumentation or column development. Recent applications consider the more novel sample preparation techniques and preconcentration of low-volume samples. Guntner et al. developed an in-house modified SPME device (Fig. 2D) and coupled it to GC-MS for the analysis of propofol in microdialysates [84].

The future focuses on the development of miniaturized sample preparation/preconcentration methods, of standardized protocols for temperature control in GC-MS analyses and of derivatization processes [83]. The 2D GC technique (GCxGC-MS) may result in an increased peak capacity (resolution power) leading to an improved spectral quality and sensitivity, the advances of GCxGC-MS for

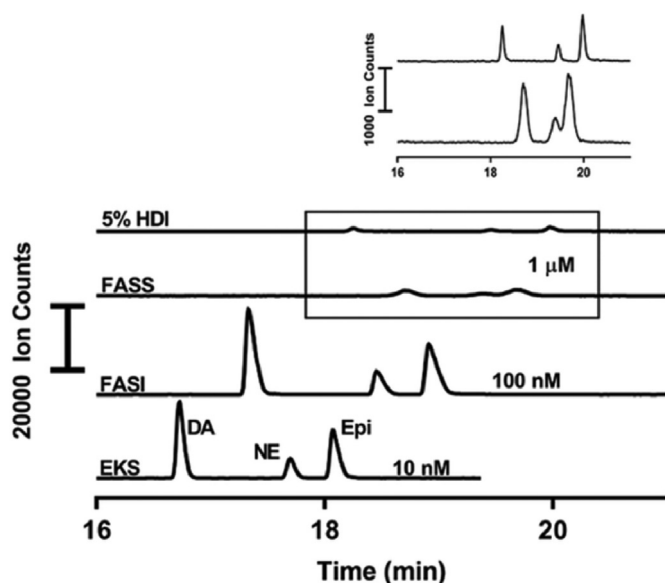


Fig. 4. Comparison of electrokinetic supercharging (EKS) method with conventional hydrodynamic injection (HDI) and other common forms of preconcentration in CE-MS e.g. field-amplified sample injection (FASI) and field-amplified sample stacking (FASS). Figure is reused with permission from Ref. [76].

metabolomics is discussed in the recent review of Higgins Kepler et al. [85]. Increased sensitivity would be of interest for brain metabolomics to reveal low concentrated metabolites in the low volume samples. Although GCxGC-MS is already applied in different metabolomics studies, such as for the profiling of human serum samples of patients with a neurodegenerative disorder [86]. The analysis of the obtained complex data remains very challenging and time-consuming. Therefore, automation of the data analysis procedure needs to be further optimized [87]. The suitability of 2D GC for metabolomics has been demonstrated by Yu et al. [88] who optimized a 2D GC method for different matrices (tissue, serum and urine). The authors showed an increase in metabolite identification with their 2D method due to an improved separation of amongst others lactate and pyruvate. This was also seen in the aforementioned study of Winneke et al. [86]. They compared the analysis of GC-MS with GCxGC-MS, with the result of more detected peaks by the latter method.

5. Conclusions and perspectives

Brain metabolomics is a research area that has greatly gained popularity in last years. The ability to perform *in-vivo* metabolomic profiling provides valuable real-time information about the biological status of the brain, avoiding the risk of metabolite degradation, which happens when analysing *post-mortem* brain tissues. However, *in-vivo* neuromessenger analysis is still found to be a huge analytical challenge, mainly because of the poor accessibility to brain fluids, the highly invasive sampling and the wide range and fast fluctuation of neuromessenger concentrations.

Miniaturization of sampling probes and advances in microfluidics have led to major improvements in spatial and temporal resolution of brain-fluid sampling, drastically reducing tissue damage and sampling invasiveness. The miniaturization of plasma collection and the possibility of self-sampling for patients play also an important role in sample accessibility. As a result of the miniaturization of sampling strategies, which lead to lower sample volumes, miniaturized sample extraction strategies (micro-SPE) and micro-extraction techniques (LPME and SPME) are more

frequently applied for brain metabolomics purposes. In this regard, a trend towards the on-line coupling of micro-extraction techniques to analytical devices is observed, resulting in lower costs and higher throughput.

For the low-volume samples from brain metabolomics studies, microscale separation techniques, such as CE-MS, and nano- and capillary (UHP)LC-MS will probably play a more prominent role in the future. However, there is still room for improvements in the robustness of these techniques. Other approaches to gain a better coverage of the metabolome, such as the coupling of different column chemistries in parallel or in series, and the inclusion of two- and multidimensional techniques are also considered. Multiplatform analysis is the only suitable way to cover in the broadest way the entire metabolome, which is perhaps the most challenging perspective for these limited-volume samples.

Another interesting feature of analyzing small amounts of sample is that large scale animal studies can be reduced as pooling across individual animals will no longer be required to achieve an analyzable sample, thus increasing the 3Rs credentials of metabolomics, i.e. Reduction, Refinement and Replacement of animal testing.

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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References

- [1] C.G. Vasilopoulou, M. Margarity, M.I. Klapa, Metabolomic analysis in brain research: opportunities and challenges, *Front. Physiol.* 24 (2016) 183. <https://doi.org/10.3389/fphys.2016.00183>.
- [2] J. Ivanisevic, G. Siuzdak, The role of metabolomics in brain metabolism research, *J. Neuroimmune Pharmacol.* 10 (2015) 391–395. <https://doi.org/10.1007/s11481-015-9621-1>.
- [3] R. Kaddurah-Daouk, K.R.R. Krishnan, Metabolomics: a global biochemical approach to the study of central nervous system diseases, *Neuropsychopharmacology* 34 (2009) 173–186. <https://doi.org/10.1038/npp.2008.174>.
- [4] A. Ali, Y. Abouleila, Y. Shimizu, E. Hiyama, S. Emara, A. Mashaghi, T. Hankemeier, Single-cell metabolomics by mass spectrometry: advances, challenges, and future applications, *TrAC Trends Anal. Chem.* (Reference Ed.) 120 (2019) 115436. <https://doi.org/10.1016/j.trac.2019.02.033>.
- [5] M. Hemmati, C. Nix, J. Crommen, A.-C. Servais, M. Fillet, Benefits of micro-sampling and microextraction for metabolomics studies, *TrAC Trends Anal. Chem.* (Reference Ed.) 127 (2020) 115899. <https://doi.org/10.1016/j.trac.2020.115899>.
- [6] B. Šakić, Cerebrospinal fluid collection in laboratory mice: literature review and modified cisternal puncture method, *J. Neurosci. Methods* 311 (2019) 402–407. <https://doi.org/10.1016/j.jneumeth.2018.09.025>.
- [7] M.M. Koek, F. Bakels, W. Engel, A. van den Maagdenberg, M.D. Ferrari, L. Coulier, T. Hankemeier, Metabolic profiling of ultrasmall sample volumes with GC/MS: from microliter to nanoliter samples, *Anal. Chem.* 82 (1) (2010) 156–162. <https://doi.org/10.1021/ja9015787>.
- [8] M.H. El-Maghraby, N. Kishikawa, N. Kuroda, Current trends in isotope-coded derivatization liquid chromatographic-mass spectrometric analyses with special emphasis on their biomedical application, *Biomed. Chromatogr.* 34 (3) (2020), e4756. <https://doi.org/10.1002/bmc.4756>.
- [9] J. Van Schoors, J. Viaene, Y. Van Wansseele, I. Smolders, B. Dejaegher, Y. Vander Heyden, A. Van Eeckhaut, An improved microbore UHPLC method with electrochemical detection for the simultaneous determination of low monoamine levels in *in vivo* brain microdialysis samples, *J. Pharmaceut. Biomed. Anal.* 127 (2016) 136–146. <https://doi.org/10.1016/j.jpba.2016.01.015>.
- [10] M.P. Lorenzo, A. Navarrete, C. Balderas, A. Garcia, Optimization and validation of a CE-LIF method for amino acid determination in biological samples, *J. Pharmaceut. Biomed. Anal.* 73 (2013) 116–124. <https://doi.org/10.1016/j.jpba.2012.03.017>.

- [11] C. Gonzalez-Riano, A. Garcia, C. Barbas, Metabolomics studies in brain tissue: a review, *J. Pharmaceut. Biomed. Anal.* 130 (2016) 141–168. <https://doi.org/10.1016/j.jpba.2016.07.008>.
- [12] S. Naz, D.C. Moreira dos Santos, A. Garcia, C. Barbas, Analytical protocols based on LC–MS, GC–MS and CE–MS for nontargeted metabolomics of biological tissues, *Bioanalysis* 6 (12) (2014) 1657–1677. <https://doi.org/10.4155/bio.14.119>.
- [13] M. Qi, M.C. Philip, N. Yang, J.V. Sweedler, Single cell neurometabolomics, *ACS Chem. Neurosci.* 9 (1) (2018) 40–50. <https://doi.org/10.1021/acscemneuro.7b00304>.
- [14] R. Nirogi, V. Kandikere, G. Bhyrapuneni, V. Benade, R. Saralaya, S. Irappanavar, N. Muddana, D.R. Ajjala, Approach to reduce the non-specific binding in microdialysis, *J. Neurosci. Methods* 209 (2) (2012) 379–387. <https://doi.org/10.1016/j.jneumeth.2012.06.010>.
- [15] A. Bergadano, E.M. Amen, B. Jacobsen, S. Belli, A. Vandjour, C. Rapp, C. Senn, A minimally-invasive serial cerebrospinal fluid sampling model in conscious Göttingen minipigs, *J. Biol. Methods* 6 (1) (2019) e107. <https://doi.org/10.14440/jbm.2019.265>.
- [16] J. Bongaerts, D. De Bundel, D. Mangelings, I. Smolders, Y. Vander Heyden, A. Van Eeckhaut, Sensitive targeted methods for brain metabolomic studies in microdialysis samples, *J. Pharmaceut. Biomed. Anal.* 161 (2018) 192–205. <https://doi.org/10.1016/j.jpba.2018.08.043>.
- [17] R.T. Kennedy, Emerging trends in in-vivo neurochemical monitoring by microdialysis, *Curr. Opin. Chem. Biol.* 17 (5) (2013) 860–867. <https://doi.org/10.1016/j.cbpa.2013.06.012>.
- [18] Y. Van Wanseele, A. De Prins, D. De Bundel, I. Smolders, A. Van Eeckhaut, Challenges for the *in vivo* quantification of brain neuropeptides using microdialysis sampling and LC–MS, *Bioanalysis* 8 (18) (2016) 1965–1985. <https://doi.org/10.4155/bio-2016-0119>.
- [19] T. Ngernsutorakul, T.S. White, R.T. Kennedy, Microfabricated probes for studying brain chemistry: a review, *ChemPhysChem* 19 (10) (2018) 1128–1142. <https://doi.org/10.1002/cphc.201701180>.
- [20] F.T.G. van den Brink, T. Phisonkunkasem, A. Asthana, J.G. Bomer, A.M.J.M. van den Maagdenberg, E.A. Tolner, M. Odijk, A miniaturized push-pull-perfusion probe for few-second sampling of neurotransmitters in the mouse brain, *Lab Chip* 19 (8) (2019) 1332–1343. <https://doi.org/10.1039/C8LC01137K>.
- [21] P. Song, N.D. Hershey, O.S. Mabrouk, T.R. Slaney, R.T. Kennedy, Mass spectrometry “sensor” for *in vivo* acetylcholine monitoring, *Anal. Chem.* 84 (11) (2012) 4659–4664. <https://doi.org/10.1021/ac301203m>.
- [22] G. Petit-Pierre, P. Colin, E. Laurer, J. Déglon, A. Bertsch, A. Thomas, B.L. Schneider, P. Renaud, *In vivo* neurochemical measurements in cerebral tissues using a droplet-based monitoring system, *Nat. Commun.* 8 (1) (2017) 1239–1248. <https://doi.org/10.1038/s41467-017-01419-1>.
- [23] T. Ngernsutorakul, D.J. Steyer, A.C. Valenta, R.T. Kennedy, *In vivo* chemical monitoring at high spatiotemporal resolution using microfabricated sampling probes and droplet-based microfluidics coupled to mass spectrometry, *Anal. Chem.* 90 (18) (2018) 10943–10950. <https://doi.org/10.1021/acs.analchem.8b02468>.
- [24] T. Birngruber, A. Ghosh, V. Perez-Yarza, T. Kroath, M. Ratzer, T.R. Pieber, F. Sinner, Cerebral open flow microperfusion: a new *in vivo* technique for continuous measurement of substance transport across the intact blood–brain barrier, *Clin. Exp. Pharmacol. Physiol.* 40 (12) (2013) 864–871. <https://doi.org/10.1111/1440-1681.12174>.
- [25] T. Birngruber, F. Sinner, Cerebral open flow microperfusion (cOFM) an innovative interface to brain tissue, *Drug Discov. Today Technol.* 20 (2016) 19–25. <https://doi.org/10.1016/j.ddtec.2016.07.003>.
- [26] R. Nicolò, F. Fabio, B. Federica, C. Maria, Recent advances in *in vivo* SPME sampling, *Separations* 7 (1) (2020) 6. <https://doi.org/10.3390/separations7010006>.
- [27] M. Huq, M. Tascon, E. Nazdrajić, A. Roszkowska, J. Pawliszyn, Measurement of free drug concentration from biological tissue by solid-phase microextraction: in silico and experimental study, *Anal. Chem.* 91 (12) (2019) 7719–7728. <https://doi.org/10.1021/acs.analchem.9b00983>.
- [28] S. Lendor, M. Olkowicz, E. Boyaci, M. Yu, M. Diwan, C. Hamani, M. Palmer, N. Reyes-Garcés, G.A. Gómez-Ríos, J. Pawliszyn, Investigation of early death-induced changes in rat brain by solid phase microextraction via untargeted high resolution mass spectrometry: *in vivo* versus postmortem comparative study, *ACS Chem. Neurosci.* 11 (12) (2020) 1827–1840. <https://doi.org/10.1021/acscemneuro.0c00270>.
- [29] A. Napylov, N. Reyes-Garcés, G. Gomez-Ríos, M. Olkowicz, S. Lendor, C. Monnin, B. Bojko, C. Hamani, J. Pawliszyn, D. Vuckovic, *In vivo* solid-phase microextraction for sampling of oxylinins in brain of awake, moving rats, *Angew Chem. Int. Ed. Engl.* 59 (6) (2020) 2392–2398. <https://doi.org/10.1002/anie.201909430>.
- [30] E. Boyaci, S. Lendor, B. Bojko, N. Reyes-Garcés, G.n.A. Gómez-Ríos, M. Olkowicz, M. Diwan, M. Palmer, C. Hamani, J. Pawliszyn, Comprehensive investigation of metabolic changes occurring in the rat brain hippocampus after fluoxetine administration using two complementary *in vivo* techniques: solid phase microextraction and microdialysis, *ACS Chem. Neurosci.* 11 (22) (2020) 3749–3760. <https://doi.org/10.1021/acscemneuro.0c00274>.
- [31] S. Lendor, S.-A. Hassani, E. Boyaci, V. Singh, T. Womelsdorf, J. Pawliszyn, Solid phase microextraction-based miniaturized probe and protocol for extraction of neurotransmitters from brains *in vivo*, *Anal. Chem.* 91 (7) (2019) 4896–4905. <https://doi.org/10.1021/acs.analchem.9b00995>.
- [32] K. Segers, W. Zhang, N. Aourz, J. Bongaerts, S. Declerck, D. Mangelings, T. Hankemeier, D. De Bundel, Y. Vander Heyden, I. Smolders, R. Ramautar, A. Van Eeckhaut, CE-MS metabolic profiling of volume-restricted plasma samples from an acute mouse model for epileptic seizures to discover potentially involved metabolomic features, *Talanta* (2020) 121107. <https://doi.org/10.1016/j.talanta.2020.121107>.
- [33] F. Poutout-Belissent, A. Aulbach, N. Tripathi, L. Ramaiah, Reducing blood volume requirements for clinical pathology testing in toxicologic studies—points to consider, *Vet. Clin. Pathol.* 45 (4) (2016) 534–551. <https://doi.org/10.1111/vcp.12429>.
- [34] V. Londhe, M. Rajadhaksha, Opportunities and obstacles for microsampling techniques in bioanalysis: special focus on DBS and VAMS, *J. Pharmaceut. Biomed. Anal.* 182 (2020) 113102. <https://doi.org/10.1016/j.jpba.2020.113102>.
- [35] C. Volani, G. Caprioli, G. Calderisi, B.B. Sigurdsson, J. Rainer, I. Gentilini, A.A. Hicks, P.P. Pramstaller, G. Weiss, S.V. Smarason, G. Paglia, Pre-analytic evaluation of volumetric absorptive microsampling and integration in a mass spectrometry-based metabolomics workflow, *Anal. Bioanal. Chem.* 409 (26) (2017) 6263–6276. <https://doi.org/10.1007/s00216-017-0571-8>.
- [36] M.G.M. Kok, C. Nix, G. Nys, M. Fillet, Targeted metabolomics of whole blood using volumetric absorptive microsampling, *Talanta* 197 (2019) 49–58. <https://doi.org/10.1016/j.talanta.2019.01.014>.
- [37] A.J. Chetwynd, A. David, A review of nanoscale LC-ESI for metabolomics and its potential to enhance the metabolome coverage, *Talanta* 182 (2018) 380–390. <https://doi.org/10.1016/j.talanta.2018.01.084>.
- [38] Z. Niu, W. Zhang, C. Yu, J. Zhang, Y. Wen, Recent advances in biological sample preparation methods coupled with chromatography, spectrometry and electrochemistry analysis techniques, *TrAC Trends Anal. Chem. (Reference Ed.)* 102 (2018) 123–146. <https://doi.org/10.1016/j.trac.2018.02.005>.
- [39] W. Lee, N.H. Park, Y.C. Lee, K.-H. Kim, J. Hong, Advances and challenges in neurochemical profiling of biological samples using mass spectrometry coupled with separation methods, *TrAC Trends Anal. Chem. (Reference Ed.)* 106 (2018) 159–168. <https://doi.org/10.1016/j.trac.2018.07.006>.
- [40] D. Vuckovic, Sample Preparation in Global Metabolomics of Biological Fluids and Tissues, *Proteomic and Metabolomic Approaches to Biomarker Discovery, Elsevier, Oxford, The United Kingdom, 2020*, pp. 53–83.
- [41] M. Abdel-Rehim, S. Pedersen-Bjergaard, A. Abdel-Rehim, R. Lucena, M.M. Moein, S. Cárdenas, M. Miró, Microextraction approaches for bio-analytical applications: an overview, *J. Chromatogr. A* 1616 (2020) 460790. <https://doi.org/10.1016/j.chroma.2019.460790>.
- [42] L. Zheng, X.-E. Zhao, S. Zhu, Y. Tao, W. Ji, Y. Geng, X. Wang, G. Chen, J. You, A new combined method of stable isotope-labeling derivatization-ultrasound-assisted dispersive liquid–liquid microextraction for the determination of neurotransmitters in rat brain microdialysates by ultra high performance liquid chromatography tandem mass spectrometry, *J. Chromatogr. B* 1054 (2017) 64–72. <https://doi.org/10.1016/j.jchromb.2017.03.039>.
- [43] N. Drouin, T. Kloots, J. Schappler, S. Rudaz, I. Kohler, A. Harms, P. Wilhelmus Lindenburger, T. Hankemeier, Electromembrane extraction of highly polar compounds: analysis of cardiovascular biomarkers in plasma, *Metabolites* 10 (1) (2020) 4. <https://doi.org/10.3390/metabo10010004>.
- [44] N. Drouin, S. Rudaz, J. Schappler, Dynamic-electromembrane extraction: a technical development for the extraction of neuropeptides, *Anal. Chem.* 88 (10) (2016) 5308–5315. <https://doi.org/10.1021/acs.analchem.6b00559>.
- [45] C. Huang, Z. Chen, A. Gjelstad, S. Pedersen-Bjergaard, X. Shen, Electromembrane extraction, *Trac. Trends Anal. Chem.* 95 (2017) 47–56. <https://doi.org/10.1016/j.trac.2017.07.027>.
- [46] B. Onat, H. Rosales-Solano, J. Pawliszyn, Development of a biocompatible solid phase microextraction thin film coating for the sampling and enrichment of peptides, *Anal. Chem.* 92 (13) (2020) 9379–9388. <https://doi.org/10.1021/acs.analchem.0c01846>.
- [47] E. Nazdrajić, M. Tascon, D.A. Rickert, G.A. Gómez-Ríos, V. Kulasingam, J.B. Pawliszyn, Rapid determination of tacrolimus and sirolimus in whole human blood by direct coupling of solid-phase microextraction to mass spectrometry via microfluidic open interface, *Anal. Chim. Acta* 1144 (2021) 53–60. <https://doi.org/10.1016/j.aca.2020.11.056>.
- [48] Y. Aray Olcer, M. Tascon, A.E. Eroglu, E. Boyaci, Thin film microextraction: towards faster and more sensitive microextraction, *TrAC Trends Anal. Chem. (Reference Ed.)* 113 (2019) 93–101.
- [49] N. Reyes-Garcés, E. Boyaci, G.A. Gómez-Ríos, M. Olkowicz, C. Monnin, B. Bojko, D. Vuckovic, J. Pawliszyn, Assessment of solid phase microextraction as a sample preparation tool for untargeted analysis of brain tissue using liquid chromatography-mass spectrometry, *J. Chromatogr. A* 1638 (2021) 461862. <https://doi.org/10.1016/j.chroma.2020.461862>.
- [50] N. Reyes-Garcés, M. Diwan, E. Boyaci, G.A. Gómez-Ríos, B. Bojko, J.N. Nobrega, F.R. Bambico, C. Hamani, J. Pawliszyn, *In vivo* brain sampling using a microextraction probe reveals metabolic changes in rodents after deep brain stimulation, *Anal. Chem.* 91 (15) (2019) 9875–9884. <https://doi.org/10.1021/acs.analchem.9b01540>.
- [51] J. Matys, B. Gieroba, K. Józwiak, Recent developments of bioanalytical methods in determination of neurotransmitters *in vivo*, *J. Pharmaceut. Biomed. Anal.* 180 (2020) 113079. <https://doi.org/10.1016/j.jpba.2019.113079>.
- [52] T. van der Laan, A.-C. Dubbelman, K. Duisters, A. Kindt, A.C. Harms, T. Hankemeier, High-throughput fractionation coupled to mass spectrometry for improved quantitation in metabolomics, *Anal. Chem.* (2020). <https://doi.org/10.1021/acs.analchem.0c01375>.

- [53] S. Zhao, L. Li, Chemical derivatization in LC-MS-based metabolomics study, *TrAC Trends Anal. Chem. (Reference Ed.)* 131 (2020) 115988. <https://doi.org/10.1016/j.trac.2020.115988>.
- [54] D.J. Beale, F.R. Pinu, K.A. Kouremenos, M.M. Poojary, V.K. Narayana, B.A. Boughton, K. Kanjia, S. Dayalan, O.A.H. Jones, D.A. Dias, Review of recent developments in GC-MS approaches to metabolomics-based research, *Metabolomics* 14 (11) (2018) 152. <https://doi.org/10.1007/s11306-018-1449-2>.
- [55] H. Miyagawa, T. Bamba, Comparison of sequential derivatization with concurrent methods for GC/MS-based metabolomics, *J. Biosci. Bioeng.* 127 (2) (2019) 160–168.
- [56] X. Luo, L. Li, Metabolomics of small numbers of cells: metabolomic profiling of 100, 1000, and 10000 human breast cancer cells, *Anal. Chem.* 89 (21) (2017) 11664–11671. <https://doi.org/10.1021/acs.analchem.7b03100>.
- [57] E. Takeo, R. Sasano, S. Shimma, T. Bamba, E. Fukusaki, Solid-phase analytical derivatization for gas-chromatography-mass-spectrometry-based metabolomics, *J. Biosci. Bioeng.* 124 (6) (2017) 700–706. <https://doi.org/10.1016/j.jbiosc.2017.07.006>.
- [58] J.-M.T. Wong, P.A. Malec, O.S. Mabrouk, J. Ro, M. Dus, R.T. Kennedy, Benzoyl chloride derivatization with liquid chromatography–mass spectrometry for targeted metabolomics of neurochemicals in biological samples, *J. Chromatogr. A* 1446 (2016) 78–90. <https://doi.org/10.1016/j.chroma.2016.04.006>.
- [59] C.C.W. Willacey, N. Karu, A.C. Harms, T. Hankemeier, Metabolic profiling of material-limited cell samples by dimethylaminophenacyl bromide derivatization with UPLC-MS/MS analysis, *Microchem. J.* 159 (2020) 105445. <https://doi.org/10.1016/j.microc.2020.105445>.
- [60] F. Murgia, L. Lorefice, S. Poddighe, G. Fenu, M.A. Secci, M.G. Marrosu, E. Cocco, L. Atzori, Multi-platform characterization of cerebrospinal fluid and serum metabolome of patients affected by relapsing-remitting and primary progressive multiple sclerosis, *J. Clin. Med.* 9 (3) (2020) 863. <https://doi.org/10.3390/jcm9030863>.
- [61] D.A.V. Medina, E.V.S. Maciel, A.L. de Toffoli, F.M. Lanças, Miniaturization of liquid chromatography coupled to mass spectrometry. 2. Achievements on modern instrumentation for miniaturized liquid chromatography coupled to mass spectrometry, *TrAC Trends Anal. Chem. (Reference Ed.)* 128 (2020) 115910. <https://doi.org/10.1016/j.trac.2020.115910>.
- [62] E. Vasconcelos Soares Maciel, A.L. de Toffoli, E. Sobieski, C.E. Domingues Nazário, F.M. Lanças, Miniaturized liquid chromatography focusing on analytical columns and mass spectrometry: a review, *Anal. Chim. Acta* 1103 (2020) 11–31. <https://doi.org/10.1016/j.aca.2019.12.064>.
- [63] K. Mejía-Carmona, J.S. da Silva Burato, J.V.B. Borsatto, A.L. de Toffoli, F.M. Lanças, Miniaturization of liquid chromatography coupled to mass spectrometry: 1. Current trends on miniaturized LC columns, *TrAC Trends Anal. Chem. (Reference Ed.)* 122 (2020) 115735. <https://doi.org/10.1016/j.trac.2019.115735>.
- [64] S.R. Wilson, H.E. Berg, H. Roberg-Larsen, E. Lundanes, Hyphenations of one-dimensional capillary liquid chromatography with mass spectrometry: state-of-the-art applications, in: P.Q. Trachida, L. Mondello (Editors), *Hyphenations of Capillary Chromatography with Mass Spectrometry*, Elsevier, Amsterdam, The Netherlands, 2020, pp. 319–367.
- [65] W. Lin, J. Zhang, Y. Liu, R. Wu, H. Yang, X. Hu, X. Ling, Studies on diagnostic biomarkers and therapeutic mechanism of Alzheimer's disease through metabolomics and hippocampal proteomics, *Eur. J. Pharmaceut. Sci.* 105 (2017) 119–126. <https://doi.org/10.1016/j.ejps.2017.05.003>.
- [66] Y. Van Wansseele, J. Bongaerts, K. Segers, J. Viaene, D. De Bundel, Y. Vander Heyden, I. Smolders, A. Van Eeckhaut, Assessing mixtures of supercharging agents to increase the abundance of a specific charge state of Neuremedin U, *Talanta* 198 (2019) 206–214. <https://doi.org/10.1016/j.talanta.2019.01.098>.
- [67] J. Bongaerts, K. Segers, L. Van Oudenhove, Y. Van Wansseele, M. Van Hulle, D. De Bundel, D. Mangelings, I. Smolders, Y. Vander Heyden, A. Van Eeckhaut, A comparative study of UniSpray and electrospray sources for the ionization of neuropeptides in liquid chromatography tandem mass spectrometry, *J. Chromatogr. A* 1628 (2020) 461462. <https://doi.org/10.1016/j.chroma.2020.461462>.
- [68] E. Varesio, S. Rudaz, K.-H. Krause, J.-L. Veuthey, Nanoscale liquid chromatography and capillary electrophoresis coupled to electrospray mass spectrometry for the detection of amyloid- β peptide related to Alzheimer's disease, *J. Chromatogr. A* 974 (1) (2002) 135–142. [https://doi.org/10.1016/S0021-9673\(02\)01241-4](https://doi.org/10.1016/S0021-9673(02)01241-4).
- [69] J. Haggarty, K.E. Burgess, Recent advances in liquid and gas chromatography methodology for extending coverage of the metabolome, *Curr. Opin. Biotechnol.* 43 (2017) 77–85. <https://doi.org/10.1016/j.copbio.2016.09.006>.
- [70] F. Haghghi, Z. Talebpour, A.S. Nezhad, Towards fully integrated liquid chromatography on a chip: evolution and evaluation, *TrAC Trends Anal. Chem. (Reference Ed.)* 105 (2018) 302–337. <https://doi.org/10.1016/j.trac.2018.05.002>.
- [71] G. Tóth, T. Panić-Janković, G. Mitulović, Pillar array columns for peptide separations in nanoscale reversed-phase chromatography, *J. Chromatogr. A* 1603 (2019) 426–432. <https://doi.org/10.1016/j.chroma.2019.06.067>.
- [72] P.W. Lindenburg, R. Haselberg, G. Rozing, R. Ramautar, Developments in interfacing designs for CE-MS: towards enabling tools for proteomics and metabolomics, *Chromatographia* 78 (5–6) (2015) 367–377. <https://doi.org/10.1007/s10337-014-2795-5>.
- [73] R. Shyti, I. Kohler, B. Schoenmaker, R.J.E. Derks, M.D. Ferrari, E.A. Tolner, O.A. Mayboroda, A.M.J.M. van den Maagdenberg, Plasma metabolic profiling after cortical spreading depression in a transgenic mouse model of hemiplegic migraine by capillary electrophoresis–mass spectrometry, *Mol. Biosyst.* 11 (5) (2015) 1462–1471. <https://doi.org/10.1039/C5MB00049A>.
- [74] A. Slampová, Z. Malá, P. Gebauer, Recent progress of sample stacking in capillary electrophoresis (2016–2018), *Electrophoresis* 40 (1) (2019) 40–54. <https://doi.org/10.1002/elps.201800261>.
- [75] M. van Mever, K. Segers, N. Drouin, F. Guled, Y. Vander Heyden, A. Van Eeckhaut, T. Hankemeier, R. Ramautar, Direct profiling of endogenous metabolites in rat brain microdialysis samples by capillary electrophoresis–mass spectrometry with on-line preconcentration, *Microchem. J.* 156 (2020) 104949. <https://doi.org/10.1016/j.microc.2020.104949>.
- [76] S.S. Wells, M. Dawod, R.T. Kennedy, CE-MS with electrokinetic supercharging and application to determination of neurotransmitters, *Electrophoresis* 40 (22) (2019) 2946–2953. <https://doi.org/10.1002/elps.201900203>.
- [77] L. Pont, R. Pero-Gascon, E. Gimenez, V. Sanz-Nebot, F. Benavente, A critical retrospective and prospective review of designs and materials in in-line solid-phase extraction capillary electrophoresis, *Anal. Chim. Acta* 1079 (2019) 1–19. <https://doi.org/10.1016/j.aca.2019.05.022>.
- [78] A. Dibattista, N. McIntosh, M. Lamoureux, O.Y. Al-Dibashi, P. Chakraborty, P. Britz-Mckibbin, Temporal signal pattern recognition in mass spectrometry: a method for rapid identification and accurate quantification of biomarkers for inborn errors of metabolism with quality assurance, *Anal. Chem.* 89 (15) (2017) 8112–8121. <https://doi.org/10.1021/acs.analchem.7b01727>.
- [79] R.K. Harstad, M.T. Bowser, High-speed microdialysis–capillary electrophoresis assays for measuring branched chain amino acid uptake in 3T3-L1 cells, *Anal. Chem.* 88 (16) (2016) 8115–8122. <https://doi.org/10.1021/acs.analchem.6b01846>.
- [80] G. Rozing, in: G. de Jong (Editor), *Recent Developments of Microchip Capillary Electrophoresis Coupled with Mass Spectrometry, Capillary Electrophoresis–Mass Spectrometry (CE-MS): Principles and Applications*, Wiley-VCH, Weinheim, Germany, 2016. <https://doi.org/10.1002/9783527693801>.
- [81] A.A. Motsinger-Reif, H. Zhu, M.A. Kling, W. Matson, S. Sharma, O. Fiehn, D.M. Reif, D.H. Appleby, P.M. Doraiswamy, J.Q. Trojanowski, R. Kaddurah-Daouk, S.E. Arnold, Comparing metabolomic and pathologic biomarkers alone and in combination for discriminating Alzheimer's disease from normal cognitive aging, *Acta Neuropathol. Commun.* 1 (2013). <https://doi.org/10.1186/2051-5960-1-28>, 28–28.
- [82] A. Pautova, Z. Khesina, M. Getsina, P. Sobolev, A. Revelsky, N. Beloborodova, Determination of tryptophan metabolites in serum and cerebrospinal fluid samples using microextraction by packed sorbent, silylation and GC–MS detection, *Molecules* 25 (14) (2020) 3258. <https://doi.org/10.3390/molecules25143258>.
- [83] J.-I. Geng, J.-y. Aa, S.-q. Feng, S.-y. Wang, P. Wang, Y. Zhang, B.-c. Ouyang, J.-k. Wang, Y.-j. Zhu, W.-z. Huang, Z.-z. Wang, W. Xiao, G.-j. Wang, Exploring the neuroprotective effects of ginkgolides injection in a rodent model of cerebral ischemia–reperfusion injury by GC–MS based metabolomic profiling, *J. Pharmaceut. Biomed. Anal.* 142 (2017) 190–200. <https://doi.org/10.1016/j.jpba.2017.04.031>.
- [84] A.S. Guntner, S. Stöcklegger, M. Kneidinger, U. Illievich, W. Buchberger, Development of a highly sensitive gas chromatography–mass spectrometry method preceded by solid-phase microextraction for the analysis of propofol in low-volume cerebral microdialysate samples, *J. Separ. Sci.* 42 (6) (2019) 1257–1264. <https://doi.org/10.1002/jssc.201801066>.
- [85] E.A. Higgins Keppler, C.L. Jenkins, T.J. Davis, H.D. Bean, Advances in the application of comprehensive two-dimensional gas chromatography in metabolomics, *TrAC Trends Anal. Chem. (Reference Ed.)* 109 (2018) 275–286. <https://doi.org/10.1016/j.trac.2018.10.015>.
- [86] J.H. Winnike, X. Wei, K.J. Knagge, S.D. Colman, S.G. Gregory, X. Zhang, Comparison of GC-MS and GC \times GC-MS in the analysis of human serum samples for biomarker discovery, *J. Proteome Res.* 14 (4) (2015) 1810–1817. <https://doi.org/10.1021/pr5011923>.
- [87] D. Smith, M.J. McEwan, P. Španěl, Understanding gas phase ion chemistry is the key to reliable selected ion flow tube-mass spectrometry analyses, *Anal. Chem.* 92 (19) (2020) 12750–12762. <https://doi.org/10.1021/acs.analchem.0c03050>.
- [88] Z. Yu, H. Huang, A. Reim, P.D. Charles, A. Northage, D. Jackson, I. Parry, B.M. Kessler, Optimizing 2D gas chromatography mass spectrometry for robust tissue, serum and urine metabolite profiling, *Talanta* 165 (2017) 685–691. <https://doi.org/10.1016/j.talanta.2017.01.003>.
- [89] F.M. Musteata, I. de Lannoy, B. Gien, J. Pawliszyn, Blood sampling without blood draws for *in vivo* pharmacokinetic studies in rats, *J. Pharmaceut. Biomed. Anal.* 47 (4) (2008) 907–912. <https://doi.org/10.1016/j.jpba.2008.03.028>.