Chapter 2

Recent Developments in Sample Pretreatment Techniques for Mass Spectrometry-based Metabolomics

ABSTRACT

Metabolomics is one of the most rapidly evolving of the omics sciences. For phenotyping individuals, it offers a promising platform that is needed for breakthroughs in biomedical and pharmacological research. A proper sample-pretreatment procedure is required for metabolomics studies to overcome the mismatch of the sample with the analytical method. Within the analytical workflow for metabolomics studies, sample pretreatment is important in order to obtain the meaningful, high-quality quantitative data needed to minimize inconsistencies between laboratories. In this review, we provide an overview of state-of-the-art sample-pretreatment techniques for mass spectrometry-based metabolomics. We pay attention to deproteinization, removal of interfering molecules, liquid-liquid extraction, solid-phase extraction, electromigration-based extraction methods and possibly emerging sample-pretreatment techniques for metabolomics. We give the advantages and the limitations of these techniques for metabolomics, and consider aspects such as automation and high-throughput analysis. Finally, we provide some conclusions and perspectives.

Based on

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INTRODUCTION

Metabolites are key regulators of system homeostasis and provide a functional readout of cellular biochemistry. Metabolomics can be defined as a comprehensive analytical approach for the study of the complete set of metabolites (metabolome), present in a biological system[1]. Interest in metabolomics is rapidly expanding in various research fields, such as cancer research[2], drug discovery and development[3], toxicological, biomedical and clinical research and nutritional research. The major aim of metabolomics is to obtain an answer or insight to a biological question. An analytical workflow comprised of experimental design, sampling, sample pretreatment, data acquisition and data analysis is generally used to address a biological question by a metabolomics approach (Figure 1).

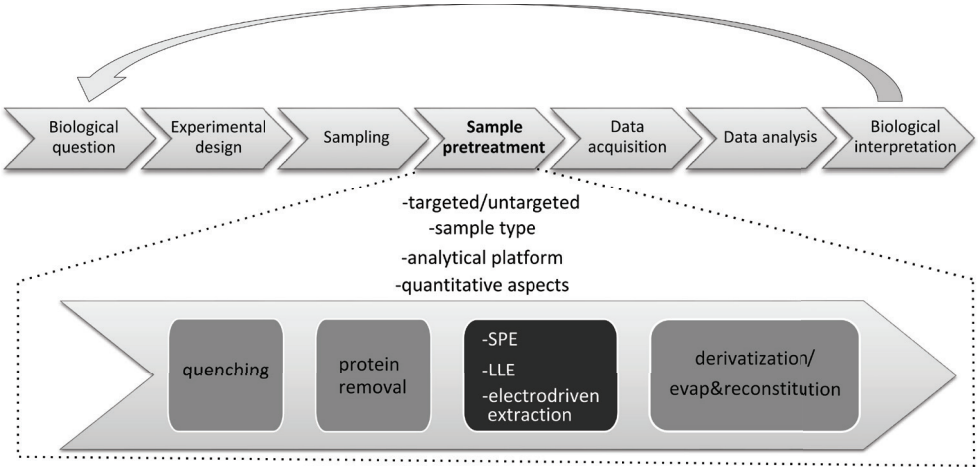

Figure 1: Schematic diagram of a typical analytical workflow for metabolomics studies.

Within the analytical workflow for metabolomics studies, sample pretreatment is a key step as it has a major influence on both the overall coverage and quality of the obtained metabolic profiles and biological interpretation of the data. As such, it may be considered the most error-prone and time-consuming step of metabolomics studies and is maybe the major source of inconsistencies between laboratories[4].

Although technical advances have been made in the fields of bioanalysis and metabolomics in terms of automation and high-throughput analysis, a significant part of the sample pretreatment procedure is still carried out manually[5]. The main challenges of sample pretreatment in metabolomics is the great diversity of chemical structures and physicochemical properties as well as vast differences in dynamic range (up to 9 decades) of the metabolites present in a biological sample[6],[7] and at the same time the lack of universal analytical comprehensive techniques with a sufficient dynamic range and physicochemical coverage. To ensure that the measured metabolic profiles are representative, the sample pretreatment procedures used should allow the reproducible enrichment of all metabolites of interest, and remove those compounds that interfere with the subsequent analytical method in order to preserve the integrity of the analytical system. Moreover, MS analysis can be disturbed by interfering, co-eluting matrix components in the samples resulting in ion suppression effects, especially when using electrospray ionization (ESI)[8]. Sample pretreatment is also required to release metabolites from the sample matrix and transport the analytes to a medium compatible with the analytical instrument. Therefore, there is an urgent need for the development of

reproducible, standardized and quantitative sample pretreatment approaches for biological samples critical for large-scale metabolomics studies and for minimizing inter-laboratory inconsistencies[9].

The purpose of this review is to present the state-of-the-art sample pretreatment methodologies for MS-based metabolomics, not focusing on lipodomics as this is covered including sample pretreatment in another review article by Fiehn *et al*. in this issue of Trends in Analytical Chemistry. In addition, some of the new emerging sample pretreatment techniques, which have not yet been fully evaluated for metabolomics studies, will also be covered. Recent developments in sample pretreatment techniques such as protein removal, liquid-liquid extraction (LLE), solid-phase extraction (SPE) and electromigration-based extraction will be highlighted, and aspects such as automation and high-throughput analysis will be considered. Subsequently, sample pretreatment issues relevant for obtaining reliable quantitative metabolomics data are shortly discussed. Finally, some general conclusions and perspectives are given. As plants represent a particular complex case as plant cell walls are difficult to break and they comprise special biochemical classes, sample pretreatment procedures for plant metabolomics studies are not discussed in this review.

SAMPLE PRETREATMENT FOR METABOLOMICS: CRITICAL ASPECTS

Sample pretreatment is an essential part of a metabolomics study as it has a major effect on the metabolite coverage and the quality of the results obtained. Prior to sample pretreatment, experimental design and pre-analytical steps such as sample collection/storage and stabilization need to be carefully examined and chosen in each metabolomics study (Figure 1); however, these aspects are not covered in this review, but are discussed elsewhere [10]–[14]. Sample pretreatment strategies used in metabolomics prior to separation and detection can include ultrafiltration, protein precipitation, LLE, SPE, derivatization, and evaporation followed by reconstitution[15]. Sample pretreatment finally results in a fraction of a sample that can be analyzed by a subsequent analysis platform. In this section important aspects with regard to the choice of a sample pretreatment technique for metabolomics are considered. First, the difference between targeted and untargeted metabolomics is outlined, followed by a discussion concerning the influence of the sample type and the subsequent analysis platform on the sample pretreatment strategy.

General aspects: targeted versus untargeted metabolomics

In metabolomics, two different approaches may be distinguished, i.e. the targeted and the untargeted detection of metabolites. The targeted approach is focused on the quantitative analysis of preselected metabolites in a biological sample. Targeted metabolomics approaches are commonly driven by a specific biochemical hypothesis, typically focusing on one or more related pathways of interest[16][17]. The untargeted approach involves the profiling of biological samples without having a priori knowledge on the nature and the identity of the measured metabolites. The goal is to obtain qualitative and (semi)-quantitative information, which can be used to compare patterns or fingerprints of metabolites that change in response to normal and abnormal biological processes, genetic alterations and to external stimuli, for example drug exposure[18]. As a consequence, untargeted metabolomics can be used to reveal the involvement of metabolic pathways that may not have been predicted and is therefore often hypothesis-generating or hypothesis-refining.

With regard to sample pretreatment, the analytical requirements for untargeted and targeted

metabolomics studies may be quite different. For untargeted metabolomics studies, the biological sample should preferably be analyzed with minimal pretreatment in order to prevent the potential loss of metabolites, or at least, within a certain wide biochemical window, metabolites should not be removed during pretreatment. The sample pretreatment steps can be quite straightforward, separating low molecular weight (LMW) compounds from proteins and/or lipids using deproteinization and/or delipidation techniques[15]. Therefore in untargeted metabolomics studies of biofluids, non-selective sample pretreatment methods such as "dilute-and-shoot" and solvent protein precipitation are often used, since they enable broad metabolite coverage and high-throughput analysis[18]. Untargeted metabolomics approaches can also be applied to a fractionated sample obtained after e.g., a LLE and/or SPE step.

In contrast to untargeted metabolomics studies, development and optimization of sample pretreatment procedures for targeted metabolomics are often relatively straightforward, as it is known a priori on which metabolite class(es) to focus. Sample pretreatment includes the separation of metabolites from proteins, often followed by LLE and/or off-line/online SPE for the selective isolation and enrichment of the target compounds, and removal of interfering matrix components.

Evaporation and reconstitution is often the final step during sample pretreatment in order to concentrate the analytes by reconstitution in a smaller volume and/or convert the solvent to an analysis-compatible solvent. During such a step, attention should be paid to analyte solubility, and potential oxidation of analytes such as thiol-containing molecules[19].

Sample type

The sample pretreatment strategy also largely depends on the nature of the sample to be analyzed. The protein content of biological samples is an important aspect to consider, as most subsequent analysis methods are not compatible with protein-rich samples such as, plasma, serum, cells and tissue. In contrast, urine, particularly from healthy human individuals, contains relatively low protein amounts, and centrifugation followed by dilution with water is often all that is required prior to analysis[18].

Metabolomics has at first mainly been applied to biofluids and cell samples, but in recent years also tissue samples or biopsies are becoming more studied with metabolomics[20]. For cell samples the quenching procedure including harvesting and washing is critical[21], however we do not cover that in this review. For tissue samples, sampling and homogenization without degradation of metabolites is critical and needs to be added to the sample pretreatment workflow as a first step. However, opposed to biofluids, limited research of (automated) sample pretreatment procedures has been performed for tissue metabolomics[15][22]. Several approaches for sample homogenization such as the manual disaggregation of cold tissue with scissors or a manual homogenizer, mechanical disruption using a bead homogenizer and a cryogenically cooled pestle and mortar on frozen tissue, have been used and are described elsewhere[23], but are beyond the scope of this review. Overall, the choice of solvent seem to have more influence on metabolite coverage than the sample homogenization method[24].

Dried blood spots (DBS) is a sample type which has been used for the screening of inborn errors of metabolism for decades now, and recently DBS samples received increased interest for metabolomics studies[25]. In DBS, blood or biofluid samples are collected on special types of absorbent paper which are then dried, and as such ready for subsequent extraction and analysis. This simple and inexpensive method is easy to automate and is able to store only a few microliters of sample. In addition, it offers a convenient way of storage and

transportation at ambient conditions. Therefore, the use of DBS may have advantages for large-scale epidemiological studies where the pretreatment of blood and the subsequent storage and transport poses logistical challenges. So far only a few studies on untargeted metabolomics have been performed with the use of DBS. Analyte coverage for DBS seem to be comparable with solvent precipitation of whole blood and plasma[25]–[27]. Moreover, the result of stability studies seem to indicate that stability for most metabolites does not seem to be critical when analysis is done within a week of collection[25]. Lately, instrumental manufacturers have developed automated systems which can handle, extract and introduce the DBS extract into the analytical system, thus enabling the use of DBS for large-scale high-throughput profiling studies[28].However, some challenges related to DBS need to be examined, such as the influence of the hematocrit level and the non-uniform distribution of the blood spot on the sampling area[29].

Saliva is another readily available biofluid that may contain metabolites of interest for diagnosis and prognosis of diseases. Nevertheless, there are only a few reports concerning metabolomics. The major challenge in saliva metabolomics is the relatively low abundance of metabolites[30]–[32]. Therefore, in this field there is a need for more sensitive analytical tools which may include sample pretreatment methods that allow analyte enrichment.

Subsequent analytical platform

One major aim of metabolomics is to obtain a comprehensive view of the metabolites present in a biological sample. As no single analytical technique covers the entire spectrum of the human metabolome, various complementary analytical platforms (e.g. gas chromatography-mass spectrometry (GC-MS), liquid chromatography-MS (LC-MS), capillary electrophoresis-MS (CE-MS) and/or direct infusion-MS (DI-MS) should be employed in order to improve metabolite coverage and identification power[33]–[36]. Each of these analytical platforms has different requirements with regard to sample pretreatment. In general, deproteinization is required for each analytical platform, as the presence of proteins can seriously influence precision, accuracy and instrument component lifetime (e.g. LC and GC columns). Moreover, proteins can induce severe matrix effects, may cause clogging of nanoESI emitters and can modify the inner surface of capillaries in CE.

In GC-MS, sample pretreatment involves a number of steps generally including deproteinization, lyophilization and chemical derivatization. Chemical derivatization is often required to decrease the boiling point of many endogenous metabolites. There are a multitude of different chemical derivatization reagents used, although a two-stage process of oximation followed by trimethylsilylation is mostly applied[37]. However, the derivatization process may add more error-prone complex steps in the sample pretreatment method. Furthermore in general, derivatization can impose added requirements to the sample pretreatment procedure in order to clean up compounds that can interfere with the derivatization reactions.

In DI-MS, "dilute and shoot" after deproteinization is the most common approach, including optimization of the dilution factor in order to minimize ion suppression and detector saturation effects. Additionally, prior to DI-MS, a desalting step should be included, because salts can significantly contribute to ion suppression effects[38]. However, to date no general straightforward method is available which separates (endogenous) inorganic ions from the wide range of polar and often ionic metabolites.

SAMPLE PRETREATMENT TECHNIQUES

In this section recent developments in deproteinization techniques, LLE and SPE and emerging

electromigration-driven sample extraction will be discussed. Furthermore, throughout this section delipidation techniques will be discussed.

Protein removal

In metabolomics, the most commonly used methods for protein removal are organic solvent-based protein precipitation (PPT) followed by centrifugation, or membrane-based techniques such as ultrafiltration[4]. It is preferable to use an organic solvent for PPT, since protein denaturation using heat or inorganic acids have been shown to result in lower metabolite coverage[39] and the organic solvent also disrupts binding between many metabolites and proteins. Actually, the latter is also true for many metabolites using inorganic acids, and some inorganic acids extracts metabolites more efficient than organic solvents. However, organic solvent PPT is able to extract both hydrophilic and hydrophobic compounds. Therefore, further clean-up of the extracted samples is often required as the supernatant still contains many components such as lipids which may disturb subsequent MS analysis (i.e. ion suppression) and may reduce lifetime of e.g. LC columns. As a consequence, the combination of protein and lipid removal is often used as an effective strategy to improve metabolite coverage and reproducibility. Moreover, metabolite losses may occur due to co-precipitation with proteins and/or poor solubility in the selected extraction solvent[40].

Over the past few years, the performance of various PPT procedures for metabolomics have been evaluated in terms of protein removal efficiency, metabolite coverage, and precision[20], [37], [39], [41]–[44]. The diverging practices used in these studies reveal that there is still no general consensus on the best PPT procedure that should be used for metabolomics. However, some trends are apparent: precipitation with acetonitrile or acetone seems to perform better in terms of protein removal, whereas PPT with methanol, ethanol or a mixture of both results in improved metabolic coverage and method precision. Therefore, the latter solvents are more often applied[28][15].

In conventional solvent PPT however, it is costly to include centrifugation into robotic automation solutions. Recent innovations have become available overcoming the need of centrifugation after PPT. Surface-functionalized magnetic beads have been recently suggested for sample pretreatment in several fields, addressing large and small molecules. König *et al.* reported a serum protein removal method based on surface-modified MagSi magnetic beads. Proteins were denatured using a proprietary reagent followed by the immobilization of the protein-adsorbing magnetic beads, by applying a magnetic field[45]. Neither centrifugation nor application of pressure is required, but only a time-controlled application of a magnetic field making this technique promising for automated high-throughput applications.

Also recently, solutions have become available for automated solvent PPT in a well-plate format such as the Sirocco™ (Waters), Impact™ (Phenomenex) or HyperSep™ (Thermoscientific). Using these PPT plates the solvent precipitated sample is filtered through a well plate by using a vacuum or pressure. Cao *et al.* demonstrated the automated removal of proteins in 50 µL dog plasma followed by LC-MS analysis, using the Sirocco™ plate[46]. These available solutions could be promising for metabolomics studies, but have not been demonstrated yet. In section 3.3 other recently available well plates are discussed which simultaneously can remove proteins and lipids from a complex sample.

Other protein removing treatments have been developed that do not require a PPT or ultrafiltration step. Turbulent flow chromatography (TFC) is such a promising online technique which enables the direct injection of crude biological samples onto a column packed with large particles (25-50 µm). These large particles can have an additional level of selectivity

using stationary phase functionality. After the sample is injected into a turbo flow column the high flow-rate of 1.5-5.0 mL/min generates turbulent flow conditions inside the column. Small analyte molecules are retained via diffusion into the pores, while proteins are washed to waste. In 2010, Michopoulos *et al.* demonstrated the use of TFC for the protein removal in a metabolomics study of plasma, followed by LC-MS analysis. A comparison between TFC and methanol-based PPT was made and resulted in similar numbers of molecular features (2900), with a somewhat poorer repeatability for TFC. TFC reduced the concentration of phospholipids 10-60 fold, probably because the protein binding compounds (such as certain lipids) were washed away[47]. The principle of TFC has shown potential for the direct analysis of crude serum samples in automated, high-throughput metabolomics studies, however at the price of very high flow-rates and substantial consumption of solvents.

Liquid-Liquid-Extraction

LLE enables the extraction of metabolites into two fractions (aqueous and organic phase) that separately contain polar and apolar compounds and which can be then independently analyzed. In LLE it is challenging to extract polar analytes into an organic phase, therefore in metabolomics LLE is mostly used for sample cleanup purposes, predominantly for the removal of lipids. According to Folch *et al.*[48] and Bligh and Dyer[49], chloroform extraction can be used to recover all major lipid classes, but the use of dichloromethane has been suggested as a less toxic alternative[50]. Recently Matyash *et al.* reported that substituting chloroform with methyl tert-butyl ether (MTBE) also recovered the major lipid classes with the same or better recovery, but with reduced health risks[51]. Moreover, MTBE has a more advantageous phase configuration in which the MTBE phase is at the top and where the precipitated proteins are at the bottom (instead of between the phases using chloroform-based LLE), therefore simplifying online coupling. For these reasons, in the last years several papers have been published comprising LLE with MTBE for metabolomics studies. Chen *et al.* presented an untargeted approach to simultaneously perform metabolomics as well as lipidomics from a small piece of tissue sample (10 mg) based on a MTBE LLE. After the MTBE-based extraction, a mix of the resulting polar and apolar fraction was pooled, evaporated and reconstituted and analyzed with ultra performance (UP)LC-MS. This approach was comparable or superior in yield (3429 vs. 2641 features) and reproducibility (0.3-9.9% vs. 0.5-15.4%) to a standard methanol extraction for the profiling of polar and apolar metabolites[52].

Recently the group of Barbas and co-workers developed a MTBE-based extraction method by performing the whole sample preparation and analysis within and from a single LC-vial, which they coined as in vial dual extraction (IVDE)[53]. The upper MTBE and lower methanol-water phases were successively injected onto an reversed-phase (RP)LC-MS system directly from the vial by the adjustment of the instrument needle height in two separate runs. This way, pretreatment time and analytical variation was reduced. To date, this approach was tested for the direct LC-MS analysis of 20 µL plasma resulting in over 4500 reproducible features, as well as DBS samples for untargeted metabolomics[54], see Figure 2, and also for the application of a biological case [55].

In 2012 Saric *et al.* optimized a single LLE by varying solvent mixtures for comprehensive untargeted metabolic profiling of the aqueous phase from the Fasciola hepatica using a multiplatform approach comprised of RPLC-MS, Hydrophilic Interaction Liquid Chromatography (HILIC)-MS and CE-MS. A choloroform:methanol:water mixture with proportion of 15:59:26 (v:v) delivered the best compromise for all five analytical methods in terms of the number of extracted metabolites and resulted in 142 uniquely identified

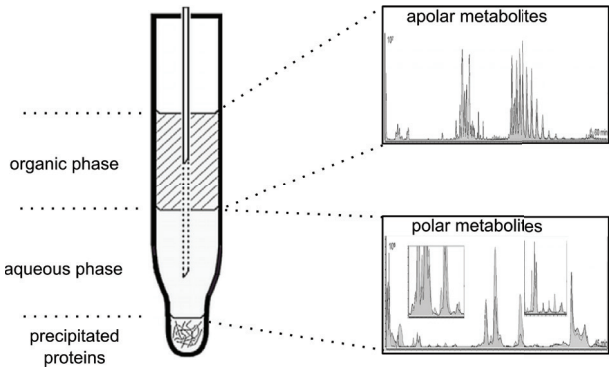

Figure 2: Schematic illustration of the IVDE method comprising the (from bottom to top) precipitated proteins, aqueous and organic MTBE layer. Both organic and aqueous layer are injected on a LC separately for apolar and polar metabolite analysis respectively. Figure adapted with permission from [55].

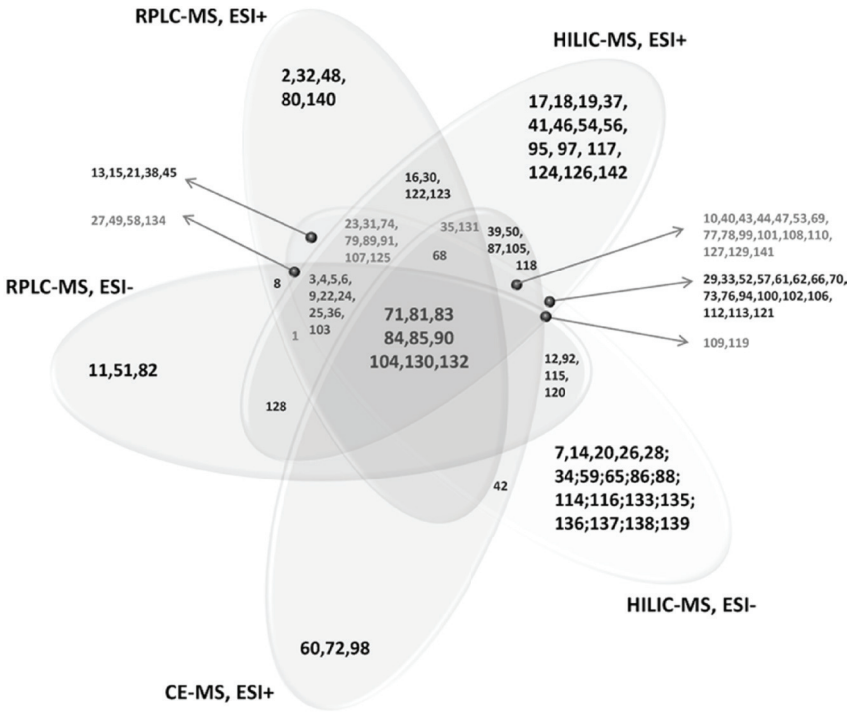

Figure 3: Venn diagram depicting 142 metabolites identified across five analytical platforms after using a single optimized LLE procedure[56].

metabolites, see Figure 3[56].

LLE can also be used for cleaning up samples in order to obtain efficient labeling in metabolomics. Recently, Peng *et al*. reported an improved method for detecting organic acids based on LLE followed by differential isotope p-dimethylaminophenacyl (DmPA) labeling of the acidic metabolites[57]. It is shown that this strategy offers superior performance (3-fold increase in putative detected metabolites) over the method of direct labeling of metabolites in biofluids such as human urine.

Still, conventional LLE as described above has drawbacks being time-consuming and difficult to automate (e.g. in a multi-well format), due to the necessary mixing and often required centrifugation steps.

New LLE approaches have emerged in overcoming automation difficulties, as discussed below. Additionally, these approaches may use less organic solvent than conventional LLE.

Supported Liquid Extraction (SLE), or solid-supported liquid extraction, is an emerging LLE-based technique for metabolite analysis in which an aqueous sample phase is absorbed into a chemically inert, porous, high-surface area diatomaceous earth support. The organic phase is passed through the cartridge and a highly efficient extraction is obtained, therefore bypassing cumbersome mixing and centrifugation steps (Figure 4). As a consequence, the SLE is easier to automate and uses less organic solvent than conventional LLE[58]. Several commercial 96-well plate SLE are available, compatible with automated high-throughput analysis. Although, to date, the analysis of drugs in biofluids is one of the most popular application of SLE, it may also be promising for high-throughput sample clean-up in metabolomics[59][60].

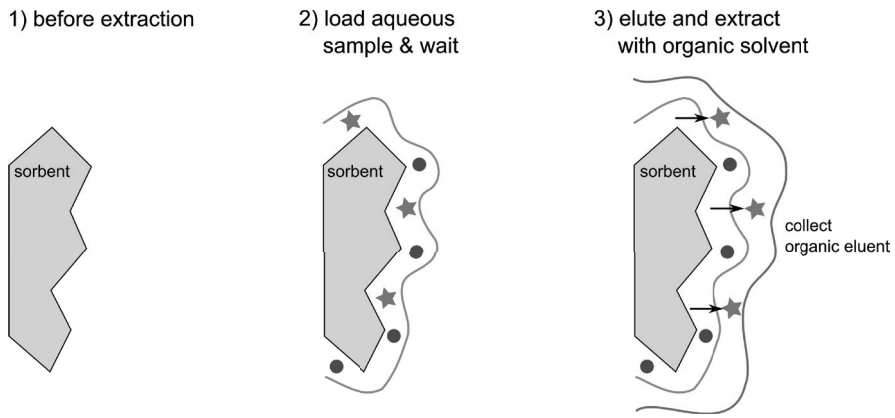

Figure 4: Schematic picture of a typical SLE procedure, modified from[84].

Miniaturization of LLE is another trend such as liquid-phase microextraction (LPME) techniques [61]. In LPME, extraction generally takes place from an aqueous sample phase into a small amount of a water-immiscible organic acceptor phase, and reducing the acceptor-to-donor ratio. Even though this miniaturized approach might be promising in metabolomics studies where limited sample amounts are available, no recent metabolomics applications of LPME techniques have been reported so far.

Another development in LLE is the integration of ultra-sound in sample pretreatment. The application of ultra-sound in LLE can significantly shorten the time required for extraction

of metabolites which increases sample throughput. So far, the potential of this approach has been only demonstrated for a few metabolomics studies [62].

Solid Phase Extraction

SPE is an effective sample pretreatment method for the removal of interfering substances and for the enrichment of analytes. A variety of extraction sorbents is available for SPE; examples are: reversed-phase materials including phenyl groups or polymeric material, weak and strong ion exchange materials including amino groups, mixed mode materials covering for example reversed- phase and weak ion exchange and stationary phases for HILIC separations. Therefore, SPE can address more specific molecular characteristics of target analytes and allows the design of protocols which can be more selective than LLE[5]. However, due to sorbent selectivity, obtaining high metabolite coverage may be challenging, especially in untargeted metabolomics. Therefore, within targeted metabolomics, the combination of SPE and LC systems using reversed-phase sorbents are most often employed for clean-up (desalting and delipidation) and enrichment of relatively hydrophobic analytes. This approach can also be used for analysis of more polar compounds if employing alternative phases (e.g. C_{30}) or ion-pairing reagents[63]. However complementary principles may be more appropriate, such as mixed-mode or HILIC phases. RP SPE-and LC columns are used in most online SPE-LC systems and are now widely commercially available in various dimensions[64].

Recently, Yang *et al.* reported a combined untargeted MTBE-based LLE and SPE (NH_2) method to improve the coverage of the metabolome in plasma caused by effective delipidation. [65]. They separated the sample into five fractions including aqueous species, lipids, fatty acids, neutral lipids and hydrophobic lipids prior to RPLC-MS and HILIC-MS analysis, and detected over 3806 versus 1851 molecular features using methanol extraction only (Figure 5). Improved reproducibility was shown with CV's below 15% for the combined LLE-SPE method, compared to 30% using the methanol method. However, this LLE-SPE method is time-consuming and therefore may be less suitable for high-throughput applications.

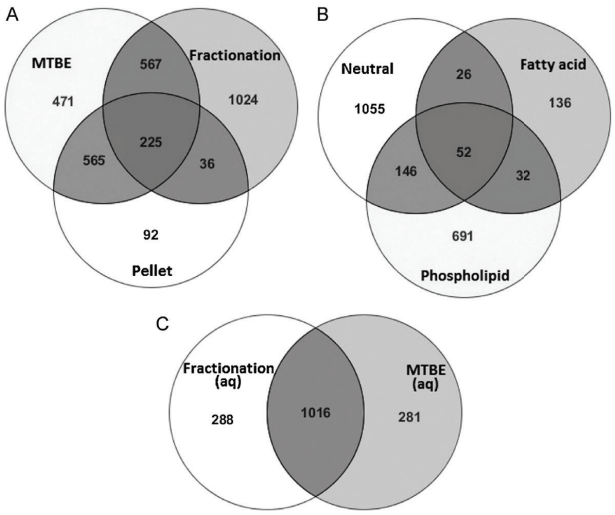

Figure 5: Venn diagrams showing the comparison of MTBE extraction vs MTBE-SPE fractionation method. Extraction was performed and resulting samples were analyzed using positive mode LC-MS by using RPLC (lipids) or HILIC (aqueous). (A) Comparison of lipid fraction, fractionated lipid fractions and pellet fraction; (B) comparison of fractionated fractions; (C) comparison of aqueous fractions. Figure reprinted with permission from[65].

Fully automated SPE has clear advantages over conventional LLE and can outperform manual protocols in terms of reproducibility and sample throughput. Off-line automated SPE sample pretreatment using robotics is available, as well as SPE platforms coupled on-line to MS such as the Agilent Rapidfire system which is a robotic high-throughput SPE-MS system or the Spark Symbiosis™ system which combines SPE and LC in parallel for high sample throughput. These systems could be suitable for rapidly tailoring an efficient targeted metabolite extraction protocol, by employing the various SPE materials that are currently available and optimizing organic solvents, pH or ion strength.

As discussed, delipidation of the sample has gained increased interest, since it can reduce ion suppression effects and can increase column lifetime. Recently, several commercial available lipid depletion plates have become available such as the Hybrid SPE™ (Sigma Aldrich), Ostro™ (Waters), Captiva™ ND (Agilent) and the Phree™ (Phenomenex) 96 well plates which can remove lipids as well as proteins from a sample in a single step. These recent innovations are effective for removing phospholipids and compatible with automation and high sample throughput, but their protocols dilute samples and do not easily allow sample concentration[66].

In SPE recent promising innovations regarding miniaturization, sorbent material and sorbent functionality has appeared in literature and is discussed below.

Also in SPE there is a trend towards miniaturization. One such approach is solid-phase microextraction (SPME) in which a fiber is coated with a thin layer of sorbent material. SPME can integrate sampling, extraction, concentration and sample introduction into a single step[61]. For more details the reader is referred to the review article by Pawliszyn *et al*. in this issue of Trends in Analytical Chemistry. Microextraction by packed sorbent (MEPS) is a more recent miniaturized SPE approach that integrates the sorbent cartridge in a microliter syringe which can be used in e.g. an autosampler. Sample and solvent volume has been scaled down to the microliter level and is therefore very compatible with volume-limited samples. Several MEPS sorbents are available, including reversed- and normal phase, HILIC, mixed mode and ion exchange functionalities[67]. This technique could be very promising in online automated, high-throughput metabolomics studies.

The use of alternative SPE sorbent chemistry based on aptamers could also bring potential to highly selective targeted metabolomics approaches, for example by employing RNA oligomers that have high affinity to small molecule targets including ATP and several amino acids[68]. Another interesting alternative sorbent is the use of immobilized ionic liquids in sample pretreatment for the extraction of polar compounds[69]. However, the potential of these alternative sorbents for metabolomics has not been demonstrated yet.

The use of monolithic material in SPE columns has gained increased interest. This material has several advantages over the use of particle sorbents including low back pressure, high efficiency and a low dead volume[70]. Recently, Abe *et al.* used a spin column, which was packed with a monolithic silica disk, for the extraction of drugs from human urine. In this spin column, the monolithic silica works as a frit, the surface to volume ratio is larger than the use of silica particles and it requires a small volume of extraction solvent [71]. As a consequence this material could be promising for metabolomics, but this has not been demonstrated yet. However, sample loading, washing and elution were accomplished by centrifugation of the spin column, which makes it less suitable for robotic automation.

Carbon nanotubes (CNT) is another SPE sorbent material which also demonstrated to have great potential. CNT are characterized by highly specific surface area, high mechanical strength, chemical stability and strong adsorbance capacity. By modifying the surface of CNT using carboxyl groups, the extraction of highly polar analytes is enabled[72]. As a consequence

CNT could be promising for metabolomics, however it has not been demonstrated yet.

Electromigration-based Extraction Techniques

Electromigration-based extraction utilizes an electric field to induce selective migration of charged compounds. A major part of the metabolome can be targeted with this approach, as many metabolites are charged in solution or can be made charged by adjusting the pH. Electromigration-based sample extraction has received increased attention in the past 5 years[73][74]. In this section, we outline recent developments in two techniques that may be promising for metabolite analysis: electromembrane extraction (EME) and electroextraction (EE). EME is based on an the application of an electric field between a donor phase and an acceptor phase which are separated by a membrane filled with an organic solvent in its pores[75]. Via the membrane, which consists of a polymeric material, analytes are extracted from a sample to an acceptor compartment. The electric field enhances the extraction rate of the analytes and as a consequence it is fast and typically enriches analytes (expressed in concentration units) by one order of magnitude. Strieglerová *et al.* demonstrated the potential of EME for the targeted extraction of endogenous amino acids from human plasma and urine[76]. Here, a simple EME device was coupled with capillary electrophoresis with capacitively coupled contactless conductivity detection. In total, 12 endogenous physiological amino acids were analyzed in human plasma, whole blood, serum and urine with peak area RSDs ranging from 1 to 13%. However, not all endogenous 20 physiological amino acids were recovered[76]. Further optimization of the composition of the liquid phase of the membrane may result in an EME protocol that is capable of extracting a wider physicochemical range of analytes in one run.

EE is an electromigration-based sample enrichment technique in which analytes are concentrated from a large donor phase, through one or more liquid-liquid interfaces, into a small acceptor phase[77]. In the most simple set-up, the donor phase is an organic solvent, which may contain some water to dissolve ions as has been done for ethyl acetate, and the adjacent acceptor phase is an aqueous phase; both phases should be immiscible. It has been shown that EE allows the fast enrichment of metabolites and is promising for high-throughput applications and analysis of low-abundant metabolites [78][79]. The driving force of EE is an electric field that is applied over the liquid-liquid system: in the donor phase a very high field strength is present. When the metabolites enter the aqueous acceptor phase, they enter a very low electric field, their migration speed diminishes and they are enriched in a small volume.

In 2012, Lindenburg *et al.* presented EE of acylcarnitines from human urine. EE took place in a wide-bore PTFE capillary, was coupled online with LC-MS via a switching valve and was automated. Within a few minutes, acylcarnitines at the nM level in urine were enriched up to 1000 times with excellent repeatability (below 12%) in comparison with conventional LC-MS injection, see Figure 6 [78].

Recently, Raterink *et al.* developed a new EE strategy consisting of three liquid phases, so-called 3-phase EE. The extraction in 3-phase EE takes place from the aqueous donor phase, via an organic phase that acts as a filter, into a hanging acceptor droplet. The selectivity of 3-phase EE can be altered by changing the composition of the organic filter phase. After the 3-phase EE procedure was completed, the acceptor droplet was transferred to nanoESI-DI-MS. The 3-phase EE–nanoESI-DI-MS was successfully applied to the targeted analysis of acylcarnitines spiked to human plasma with a good linear response over 2 orders of magnitude and repeatability better than 15%, and could be fully automated. Importantly, it was demonstrated that proteins were excluded from extraction, indicating that 3-phase EE can cope with difficult matrices and requires no prior PPT[79].

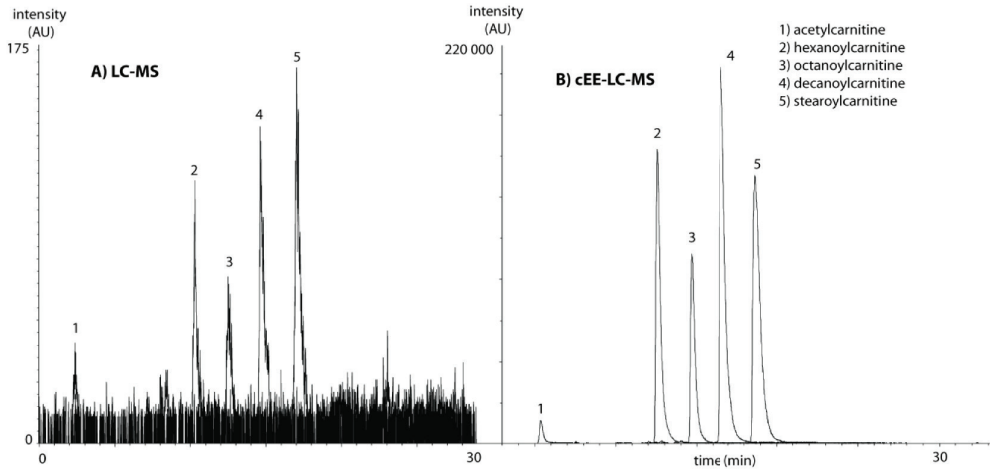

Figure 6: Comparison of (A) LC-MS obtained for a 0.1 µL injection of urine and (B) cEE-LC-MS of 100 µL urine; urine was spiked with acylcarnitines. Figure reprinted with permission from[78].

Until now, it has been shown that electromigration-driven sample extraction methods are in principle promising for metabolomics. Its approach can offer speed, selectivity and excellent potential for both miniaturization and high-throughput analysis. Therefore, we expect that electromigration-based sample extraction will be used as complementary tools for metabolomics studies in the upcoming years. However, an important aspect to study is the applicability of these methods to a wider range of metabolites.

QUANTITATION AND VALIDATION

The systematic evaluation and validation of an extraction procedure for a given biological sample matrix is necessary in order to obtain reliable and meaningful metabolomics data. In this section, attention is paid to some issues that should be considered during sample extraction to obtain reliable quantitative metabolite data.

The performance (i.e. recovery, repeatability, enrichment factors etc.) of the extraction method should be critically examined by using proper internal standards. In untargeted metabolomics, the performance of an extraction method is still often only evaluated in terms of the number of molecular features observed[15]. This strategy may provide useful information, however specific information concerning metabolite enrichment and recovery, or metabolite losses due to e.g. protein binding, is not provided. In this context, we suggest using multiple stable isotopically labeled internal standards, each representing a metabolite (sub)class, during sample extraction in order to evaluate the performance of the method[80][81]. Recently Strassburg *et al*. developed a quantitative (precision RSD ranged from 4% - 15%) and sensitive (down to nM levels) LC-MS/MS method for the profiling of approximately 100 oxylipins in human plasma by using 11 deuterated internal standards which were considered representative for the different compound classes[82]. Method validation in untargeted metabolomics is more challenging than in targeted metabolomics regarding the choice of the internal standard. Another elegant approach to tackle these challenges is the use of 13C-labeled cell extracts, which has been recently used for the quantification of 91 metabolites representing central carbon and energy metabolism[33]. For targeted metabolites, isotopically-labelled

metabolites can be used to correct for potential matrix effects (ion suppression/enhancement) and batch-to-batch effects of the corresponding endogenous metabolites.
At present, there is no consensus yet on the most suited type of sample extraction method that should be used for a certain biological sample matrix or question. Standardization and inter-laboratory comparisons are crucial in order to demonstrate the validity of a sample extraction procedure for metabolic profiling of a particular biological sample, and this would help to compare different metabolomics studies published or even accessible in data repositories. For inter-laboratory comparisons, reference material[83] should be used and preferably absolute concentrations should be provided for metabolites. Especially when reporting relative concentrations, as is often the case in untargeted metabolomics studies, it is crucial to use pooled samples and/or internal standards as quality controls and for correction of variations and possible biases in the overall analytical procedure during studies[81].

CONCLUSIONS AND OUTLOOK

Sample pretreatment is a key step in the metabolomics workflow as it has a major influence on the overall quality of the obtained metabolic profile, metabolite coverage and sample throughput. This review highlights that the development of sample pretreatment for metabolomics is still an active area of research. Although no universal sample pretreatment technique is available for the comprehensive analysis of all metabolites in a given biological sample, the combination of protein and lipid removal is often used as an effective strategy to improve metabolite coverage and reproducibility. In LLE several developments are made towards the compatibility with automation, of which supported liquid extraction is one of the most promising techniques, readily available in a well-plate format. As a consequence, deproteinization and delipidation can be performed by using LLE as well as SPE techniques in a fully automated, high-throughput manner. The recent innovations in automated offline well-plate extraction (including protein precipitation, LLE and SPE) or online extraction (including turbulent flow chromatography) have allowed fast sample cleanup and partly removed the bottlenecks associated with sample pretreatment. All these techniques are very promising for metabolomics, however the effective extraction of polar metabolites is still challenging and the separation of polar analytes from interfering salts is a hurdle for some analytical methods which still needs to be overcome. New emerging electromigration-based sample pretreatment techniques are promising and their compatibility with automation has been demonstrated. Still, more research is needed in order to exploit the full potential of these techniques for metabolomics.
To improve quantitative aspects during sample pretreatment, more research is needed in the use of internal standards, such as 13C-labeled cell extracts. Proper internal standards are crucial to correct for matrix and batch effects and to obtain insights in metabolite enrichment and recovery. In the future, sample pretreatment procedures should not only be automated and fast, but also quantitative and standardized so that metabolomics data of different studies are comparable within and between labs, which would be needed for breakthroughs in biomedical and pharmacological research.

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