Nutrimetabolomics:
An Integrative Action for Metabolomic Analyses in Human Nutritional Studies

Appendix 1

Chapter 4 - Sample Preparation

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4. Sample preparation

4.1. Sample Preparation for GC-MS-based Metabolome Studies

This section provides detailed practical recommendations on how plasma/serum and urine should be prepared for untargeted GC-MS metabolomics analysis. The main points are also summarized in **Box S4.1**.

4.1.1. Cleanup

Labware

Concerning the choice of the labware, sample preparation for GC-based nutritional metabolomics can be divided into two stages. For liquid-liquid extraction, protein precipitation or dilution of urine or plasma/serum samples, microcentrifuge tubes are appropriate because they are resistant against the commonly used solvents like methanol or methanol/water mixtures.[1-4] Likewise, as nutritional metabolomics studies usually target non-volatile sample constituents, liquid handling of biofluids can be done with common air displacement pipets with plastic tips. Only if volatile compounds are of interest, air displacement pipets are not adequate: their operating principle is based on the application of negative pressure which would drive volatile analytes out of the sample.

In contrast, derivatization involves the use of aggressive and volatile solvents and reagents at higher temperatures and thus at higher pressure. Consequently, evaporation and derivatization must be carried out in inert and heat-resistant containers, i.e. glass GC vials, usually with a glass insert (200-300 µL) due to the comparatively low volume of the final sample.[2, 4, 5] Plastic vials containing a glass insert are not recommended as they may soften and release contaminants (e.g. plasticizers) a higher temperatures. As presented in **Box S4.2**, the number of contaminants in blank samples increases drastically if sample preparation steps with increased temperature are performed in plastic tubes compared to glass vials. Two main contaminants coming from plastic tubes are the fatty acids palmitic acid (C16:0) and stearic acid (C18:0). As these fatty acids play an important role in nutrition, a carry-over from the tubes into the samples should be avoided. Finally, the commonly used two-step derivatization procedure (see section 4.1.3) necessitates to re-open the vials after the first reaction to add the silylation reagent. This is quickly and safely possible if tight-closing screw-capped vials are used[2]. In contrast, crimp-capped vials may be damaged during uncapping and the addition of reagents by penetrating the septum may cause leakages and thus inconsistent results. Generally, plastic labware and GC vials should be used only once. Other glass containers used to store solutions should be cleaned with organic solvents of different polarities and of high purity and finally baked out in an oven. Washing with detergents should be avoided. Microliter syringes or their digital counterparts (like the eVol® marketed by SGE/Trajan Scientific) are recommended for the handling of derivatization reagents as they are more inert and enable a quick and precise dispensing of volatile liquids. Microliter syringes need to be cleaned with solvents according to the manufacturer's instructions on a daily basis. As degradation products of the derivatization reagents will nevertheless form brownish deposits in the syringe barrel as well as on the plunger and the needle, a more intense rinsing with diluted acids (e.g. 50 mM hydrochloric acid) is occasionally needed. Generally, syringes with PTFE-tipped, exchangeable plungers as well as exchangeable needles are a practical and sustainable choice for manual derivatization.

Chemicals and Their Handling

Organic solvents need to be at least of HPLC grade, better of GC grade purity. Pyridine is highly hygroscopic and should be dried over molecular sieve for at least 24 h in a desiccator before preparation of the methoximation reagent (MEOX). Molecular sieves usually contain fine abrasion particles which appear as a whitish turbidity in polar solvents and sediment over time. The required volume of the dried solvent should therefore be carefully withdrawn from the top of the container without disturbing the sedimented particles. Methoxylamine hydrochloride is also hygroscopic and should be stored in a desiccator. The amount needed for the preparation of working solutions should be freeze-dried for 12-24 h before use. It has been recommended to prepare the MEOX reagent freshly once a day.[2] Our experience shows that the reagent remains stable for up to one week if the stock solution is divided into one aliquot for each measurement day which are then stored in a desiccator at room temperature. Special care has to be taken to avoid any contamination of pyridine or the MEOX reagent with dichloromethane (often recommended as a rinsing solvent for microliter syringes!) because both solvents react under formation of yellowish products.[6]

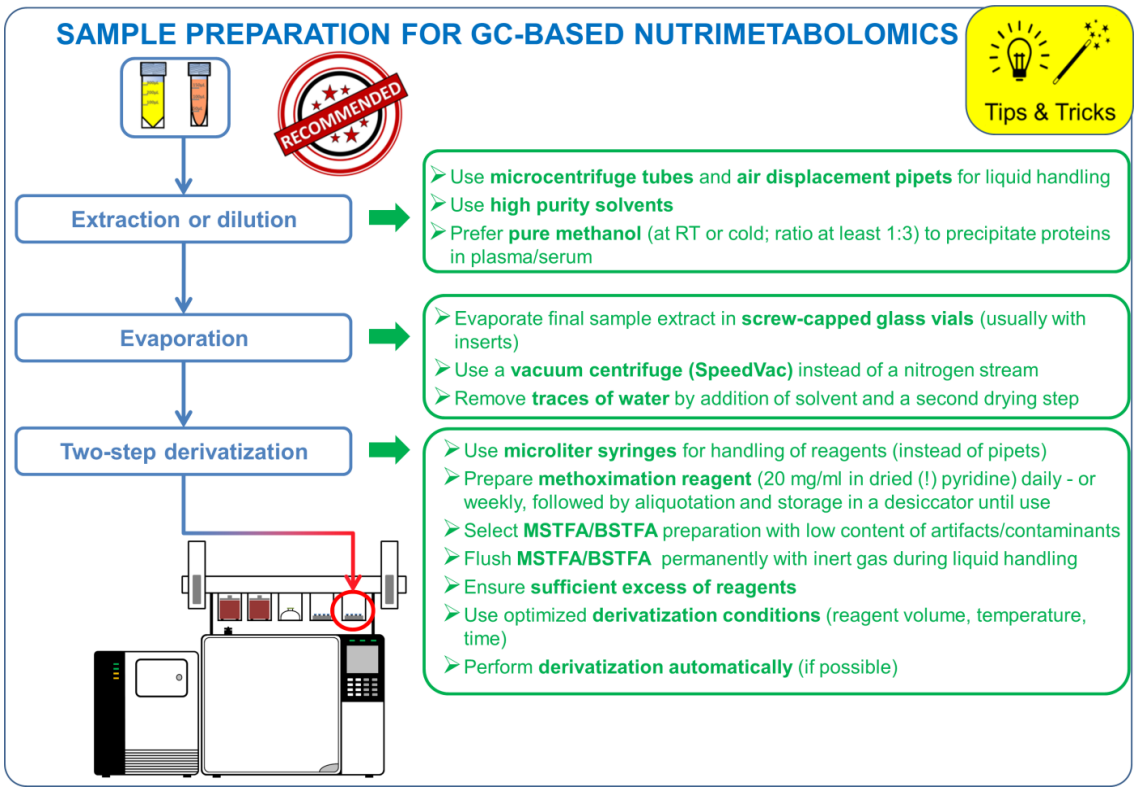
Among the silylation reagents, especially *N*-Methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MSTFA)[1, 2, 5, 7] and *N*,*O*-Bis(trimethylsilyl) trifluoroacetamide (BSTFA),[8-10] often including 1% trimethylchlorosilane (TMCS) as a catalyst, are most popular in the metabolomics community. Recently, Moros et al.[11] compared the performance of the aforementioned silylation reagents for the derivatization of plasma and finally recommended the use of MSTFA. Silylation reagents are highly reactive and must be protected against humidity in order to prevent a degradation of the active reagent and the formation of polysiloxanes.[12] Therefore, silylation reagents should always be kept in the original container (no decanting) which should further be flushed with nitrogen or argon during liquid handling. Once opened, an aliquot of MSTFA or BSTFA should be consumed within 2-3 days. Noteworthy, some commercial preparations of typical silylation reagents may contain considerable amounts of impurities like different hydrocarbons, glycerol, lactic acid or saturated fatty acids. Further, the levels of these impurities may increase during storage (C. Weinert, unpublished data). For this reason, it is advisable to check different preparations of silylation reagents for impurities and to consume each lot within 4-6 months.

Stock solutions of retention index markers – usually *n*-alkanes or saturated fatty acid methyl esters – may be prepared in hexane and pyridine.[2] However, as these solutions are often added to all or selected samples after derivatization, the use of only non-hygroscopic solvents like hexane or heptane may reduce the risk of inconsistencies due to residual water.

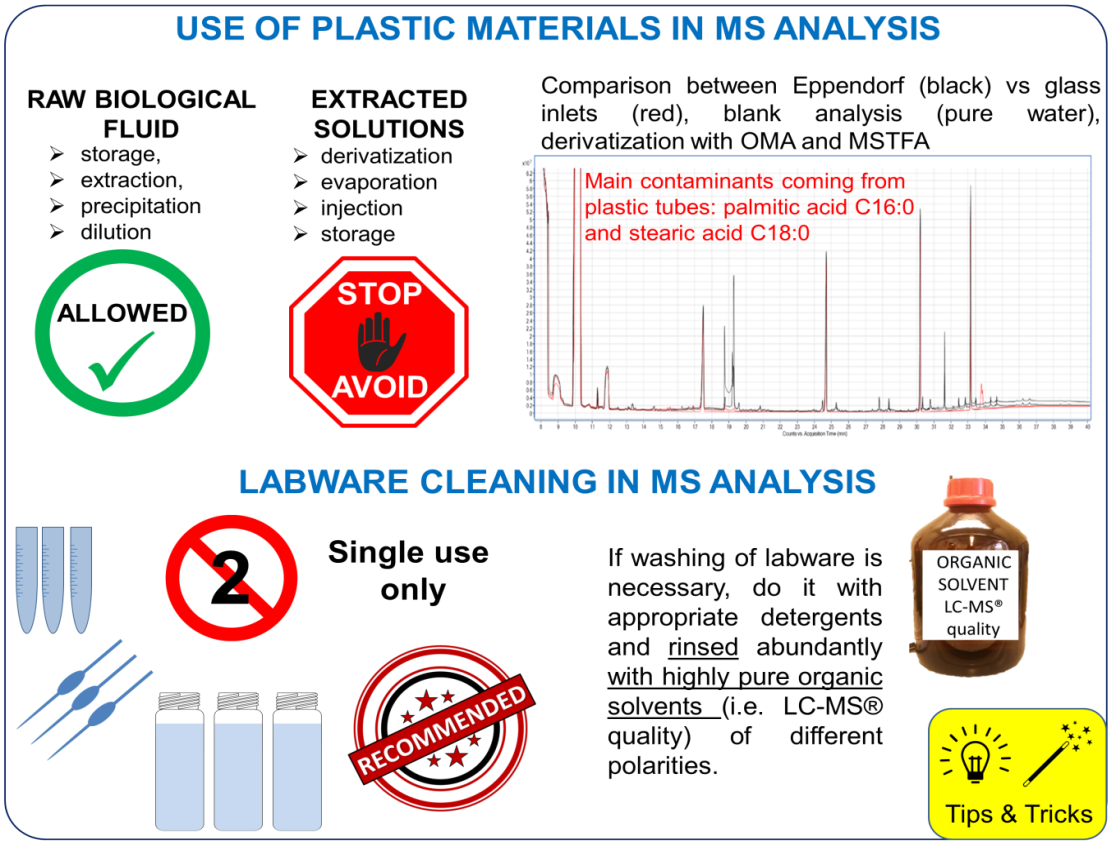
Urine

Extraction/filtration. Extraction steps can be useful if compounds differing in polarity shall be analysed separately.[3] Extraction or filtration of samples may also

help performing targeted approaches by improving separation on specific columns as well as improving data deconvolution due to a reduced number of compounds. However, we do not recommend extraction or filtration steps for untargeted metabolomics as this may cause a loss of analytes.



Box S4.1. GC-MS nutrimetabolomics – sample preparation



Box S4.2. Critical issues in labware cleaning and plastic use in MS-based analysis

Urease treatment. Urea is indeed highly concentrated and thus represents a highly dominant chromatographic peak even in diluted urine samples. For this reason, a urease treatment was often used in recent years[3, 4, 9, 10, 13] and it was shown that a urease treatment may have a beneficial effect on the analysis of urinary metabolite profiles.[14] However, it was also reported that a urease treatment may change the metabolite profile and result in severe artefacts[12, 15] probably due to the side activities of enzyme. It is the authors' opinion that a urease treatment is not necessarily required. If an appropriate deconvolution method is applied, co-eluting compounds will nevertheless be detected. In case of GC×GC-MS, the urea peak is almost completely separated from the other metabolites by application of a second separation mechanism at the chromatography stage. Thus, in conclusion, we do not recommend a urease treatment. However, if a urease treatment is performed, conditions needs to be standardized as much as possible – each sample needs to be incubated at equal temperature and time. For further details see [3, 4, 10, 13, 14, 16].

Plasma/Serum

After urine, blood serum and plasma are the second most used biofluids in metabolomics studies. From the GC perspective, plasma and serum are, in principle, equally suitable. When using EDTA plasma, EDTA complexes with trimethylsilyl (TMS) (EDTA-TMS4) and resulting in a large overloaded peak, which, however, does not interfere with other metabolites. Nevertheless, EDTA consumes the TMS reagent, so that the use of serum may be more appropriate. For protein precipitation, the use of pure methanol[1, 17] or ice-cold methanol[12, 18] has proved to be highly appropriate. Usually, a ratio ranging from 1:3 to 1:5[2, 8, 12, 19] is recommended. Generally, the higher the volume of methanol, the better. However, the final volume of the methanol will depend on the volume of the samples and tubes. The removal of precipitated proteins is achieved by centrifugation, which should be performed at maximum speed in a cooled centrifuge (best at 4°C) for at least 10 minutes.

4.1.2. Evaporation

For derivatization, especially silylation, the sample must be anhydrous. Residues of water and other protic solvents in the sample lead to an increase in the consumption of derivatization reagent and artefactual changes in concentration may occur as a result of the hydrolysis of the derivative formed. Therefore, complete evaporation of the sample extracts is crucial. Drying with a SpeedVac centrifugal evaporator is preferable to the time-consuming drying under nitrogen.[12] We recommend specifying a reproducible time/pressure program. Methanol-water mixtures could be dried without boiling with the protocol shown in **Table S4.1**.

Plasma or serum samples with high sugar content may cause problems with residual water. Such samples can be completely dried by addition of solvents like methanol, dichloromethane, ethyl acetate, or toluene[20] and a subsequent second drying step. Of note, however, phosphorylated metabolites can be degraded during the drying process in a SpeedVac.[12, 19]

Table S4.1. Settings for drying with SpeedVac at 30°C.

Pressure (mbar)	Reduction time (min)	Hold time (min)
900 → 400	1	
400 → 200	1	5
200 → 150	1	5
150 → 100	5	5
100 → 50	20	5
50 → 20	5	30
20 → 0	1	140 (serum), 100 (urine)

4.1.3. Chemical Derivatization

GC enables the separation of volatile and thermally stable metabolites. These include the following chemical classes: ketones, aldehydes, alcohols, esters, furan and pyrrole derivatives, heterocyclic compounds, sulfides, low boilingpoint lipids, isocyanates, isothiocyanates, and hydrocarbons. Chemical derivatization also makes it possible to volatilize mono- and disaccharides, sugar phosphates, amino acids, fatty acids, small peptides, long-chain alcohols, inositols, amines, amides, alkaloids, sugar alcohols and organic acid. Since many metabolites are non-volatile, this time-consuming sample preparation step is necessary, although the measurement variability may increase and artifacts may be formed. Metabolomic studies usually involve a two-step derivatization procedure consisting of an initial methoximation followed by a subsequent silylation. The methoximation protects functional keto groups from tautomerism and decarboxylation of the ketone and inhibits the formation of rings in reducing carbohydrates. In aqueous solutions, carbohydrates are in equilibrium with the cyclic α- and β-semiacetal forms (mutarotation) via the open-chain form (**Figure S4.1**). The complexity of di- and trisaccharides is even greater. The open-chain form is fixed by the reaction of methoxyamine with the carbonyl or aldehyde group of the carbohydrates leading to two isomeric derivatives (*syn* and *anti*). The subsequent silylation then removes the acidic proteins of hydroxyl, amino, carboxylic, amide, thiol and other groups (**Table S4.2**). In most cases, only one derivative is obtained by silylation. Especially in case of amino acids and amines, multiple derivatives can be formed. Further, artefacts may be generated if the derivatization reagent reacts with itself or with the organic solvent. To avoid the formation of artifacts, the use of a weaker silylation reagent is advantageous.[21]

It is still common practice to perform the derivatization of urine or plasma/serum samples manually in a batchwise day-by-day procedure.[2, 3, 5, 7, 19, 22] Due to the comparatively long analysis times, the samples of a given batch then spend usually between 1-24h in the autosampler before injection. During this time, degradation as well as prolonged derivatization reactions may occur and increase the measurement uncertainty. An elegant way to eliminate this problem is to automate derivatization with the help of modern robotic autosamplers as demonstrated by Zarate et al.[23] The consequent application of automated derivatization would certainly make GC-MS-based metabolomics more reproducible and reliable.

Methoximation Conditions

The methoximation (MEOX) reagent is usually prepared in a concentration of 20 mg/mL in dried pyridine and typically between 20-50 µL per sample are required for methoximation.[2-4, 7, 24] Concerning the optimal reaction conditions, no clear recommendation can be given based on the current literature. Typical regimes are:

- 16-24 h at room temperature[1, 3, 7, 11]
- 15 min at 80 °C[2, 25]
- 60-80 min at 40 °C[5, 24]

Table S4.2. Functional groups and their derivatives silylated by MSTFA.

Functional group	TMS-derivative
- OH	- O-Si$(CH_3)_3$
- COOH	- COO-Si$(CH_3)_3$
- NH_2	- NH-Si$(CH_3)_3$
- NH-Si$(CH_3)_3$	- N-[Si$(CH_3)_3$]$_2$
= NH	= N-Si$(CH_3)_3$
- SH	- S-Si$(CH_3)_3$
- SOH	- S-O-Si$(CH_3)_3$
- POH	- P-O-Si$(CH_3)_3$

Recent reports on optimization of methoximation conditions suggest that a slow reaction at room temperature (variant 1) is probably most suitable.[11, 26] However, such long reaction times limit sample throughput. Therefore, a compromise has to be made between organizational considerations and optimal derivatization efficiency. A major point to consider is the amount of sugars in the sample material and the need to prevent the formation of non-methoximated but silylated (and therefore undesirable) sugar derivatives.

Silylation Conditions

As for the methoximation, no consensus can be found in the literature concerning optimal silylation conditions. Silylation is typically performed by addition of 50-100 µL of the silylation reagent followed by incubation for 30-80 min at 40 °C or 15-90 min at 65-80 °C.[2-4, 7-9, 12] Moros et al. recommend a silylation for 2h at high temperatures.[11] A crucial point is that the silylation reagent must be in sufficient excess over the methoximation reagent (which also needs to be silylated) as well as the derivatizable metabolites in the sample. For this reason, reagent volumes as well as derivatization conditions should be optimized for each matrix. An example for a suitable sample/reagent ratio for the derivatization of urine is provided in **Table S4.3**. This protocol has been used for an analysis comprising more than 500 injections. During the measurement series, peak shapes remained constant and there was no need to trim the column.[5, 27] However, the authors observed that the use of 60 µL instead of 40 µL of the diluted urine (in combination with the same reagent volumes) leads to a rapid and pronounced decline in signal intensities after approximately 100 injections, obviously caused by incomplete derivatization.

4.2. Sample Preparation for LC-MS-based Metabolome Studies

Independently of the intended analytical approach (e.g. small molecule metabolomics, lipidomics), serum, plasma, and, to a smaller extent, urine need a cleanup step for the elimination of high molecular weight metabolites such as nucleic acids, polypeptides, and proteins. This step is sometimes referred to as "deproteinization". Such molecules can reduce chromatographic performance by contaminating the ion source and damaging the analytical system, thus shortening column lifetime and requiring frequent cleaning.[28, 29] The cleanup step allows the separation of high- from low-molecular weight compounds, the latter being further investigated (typically <1,500 Da). The cleanup method can have a critical influence on the metabolite content of the extract and on the resulting outcomes of the LC-MS analysis and, therefore, has to be chosen cautiously.[30, 31] This choice will mainly rely on what classes of metabolites are being investigated in order to maximize their extraction. However, for untargeted metabolomics, defining an optimal cleanup method suited for every class of compounds is particularly challenging. Vuckovics et al.[32] addressed this question in a comprehensive review, and recalled that, the optimal cleanup method for untargeted metabolomics has to be unselective, fast, reproducible, and include a metabolism quenching step.

To extract the metabolites of interest, two procedures are currently applied: liquid-liquid extraction (LLE) and solid phase extraction (SPE), the former being the most used.

4.2.1. Liquid-Liquid Extraction Based Cleanup

LLE consists in the addition of an organic solvent to the sample in order to precipitate unwanted high molecular weight compounds. The separation between the two resulting phases is facilitated by centrifuging the mixture, the metabolites of interest being recovered in the supernatant. The solvent, ratio sample/solvent, precipitation duration, centrifugation settings, and temperature have therefore to be precisely defined.

Serum and plasma sample cleanup. For serum or plasma samples the most used organic solvents are acetone, acetonitrile, methanol, ethanol, or isopropyl alcohol. Other methods use acid precipitation with trichloracetic acid, perchloric acid, or sulfosalicylic acid. The efficiency of the different solvents, solvent/solvent mixtures and solvent/sample ratios have been extensively compared. The performance of fourteen extraction methods for LC-MS untargeted metabolomics (using acetone, acetonitrile, methanol or ethanol, separately or in combination as well as three acids) were evaluated by Want and colleagues.[17] These methods were compared by measuring the residual proteins in the extract, the average metabolite intensity, and their coverage of the serum metabolome. Overall, organic solvents appeared to be more efficient than acid-based extraction. Methanol precipitation was the most efficient for metabolites recovery and reproducibility and acetonitrile showed the highest residual standard deviation. The study also highlighted the complementarity of the different methods as a single method could cover, at best, a fifth of the total measured serum metabolome. In addition to testing several organic solvents, Bruce et al.[28] have tested two vortex times (15 or 30 min). The mixtures methanol/ethanol (1:1, v/v) and methanol/ acetonitrile/acetone (1:1:1, v/v/v) at a 4:1 ratio with plasma and with a 15 min vortex time

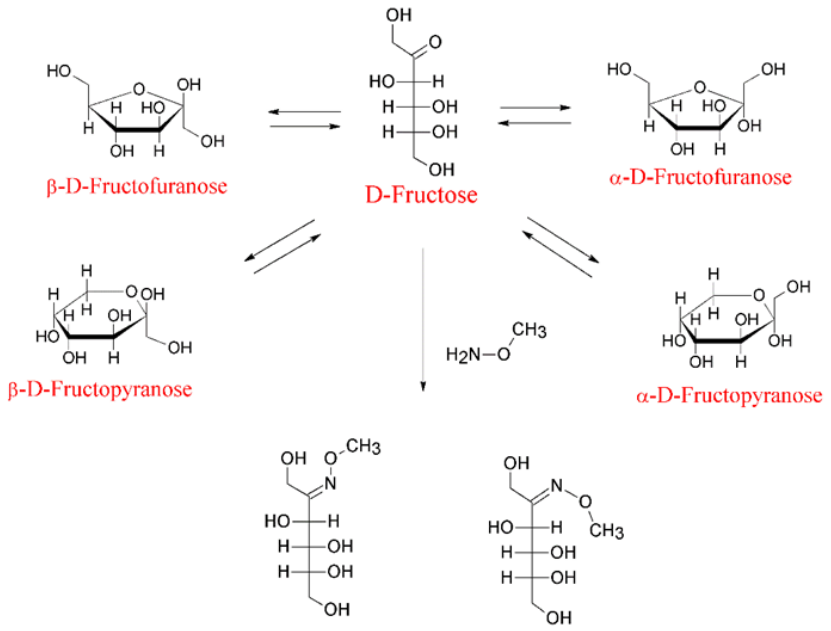


Figure S4.1. Mutarotation and methoximation of D-fructose

Table S4.3. Example for appropriate sample and reagent quantities for the derivatization of urine samples.

Sample or reagent	Quantity	Reaction conditions
Urine	40 µL (after dilution to an osmolality of 60 mosm/kg)	
MEOX reagent	15 µL (20 mg/mL in pyridine)	Heating for 1 h at 40°C
Silylation reagent	50 µL of MSTFA with 1% TMCS	Heating for 1.5 h at 75°C

were the most efficient (in term of the number of extracted markers, data quality and reproducibility, and column lifetime prolongation). Acetonitrile extraction was also compared with methanol for plasma samples cleanup. In a first study, methanol (1:3, v/v sample/solvent ratio) performed better at clustering plasma samples taken two weeks apart and at clustering quality controls. Overall, 1,300 features were noted for samples precipitated with methanol and 2,200 with acetonitrile.[33] Another study compared acetonitrile, methanol, and acetonitrile/ methanol (50:50 v/v) extractions (along with three other sample preparation methods).[34] The acetonitrile-only procedure appeared to give the most information. Detection of amino acids, acylcarnitines, and fatty acids (C8–C18) was higher than with the two other methods, although the repeatability was slightly lower. The methanol-only extraction performed better for bile acids coverage. In this study, the samples/solvent ratio was 1:3 (v/v), vortex time was 2 min, protein precipitation time was 10 min at 4°C, and the mixtures were centrifuged at 10,000 g at 4°C for 10 min. The Human Serum Metabolome Consortium recommended a cleanup using methanol, at a ratio of 3:1 (v/v) to the sample, vortex mixed for 15 s at room temperature, and centrifuged for 15 min at 15,800 g.[35, 36]

Regarding the temperature of extraction, in order to limit additional changes due to the high level of enzymes in blood-derived samples, and in line with the previous sample preparation steps, it is recommended to perform the cleanup step at cold temperatures, *i.e.* using ice-cold solvent, mixing with the sample in cold room at 4°C or on ice, and setting the centrifuge at 4°C. Bruce et al.[28] noted that an extraction temperature of 4°C rather than RT, gave the best results regardless of the solvent used.

It seems that no clear consensus arises from the literature and the researcher might finally choose to test several solvents and ratios with the available LC-MS system, to finally choose the most appropriate method in term of metabolites recovery, handling practicality, and high throughput ability.

Urine sample cleanup. On the contrary, the removal of high molecular weight compounds is less critical for urine samples considering their very low levels (e.g. protein levels of 0.05 to 0.2 g/L in urine compared to 60 to 80 g/L in serum). Therefore, injection of neat urine is commonly used in MS-based untargeted metabolomics.[37, 38] Simple centrifugation following, when necessary, dilution can be applied to neat urine (also called "dilute-and-shoot"). Prior dilution of urine in ultrapure water has the advantage to reduce the influence of high-abundance compounds, such as salts, which can lead to the formation of adducts and cause ion suppression. However, dilution can also reduce the intensity of low-abundance metabolites below the instrument detection limit. Overall, it appears that a greater number of metabolites are observed when urine is used undiluted.[35, 39, 40] Urine centrifugation however is commonly used as it allows the removal of cells or crystals in suspension and can be performed after sample collection (before freezing) or after thawing. We recommend a mild centrifugation for 10 min at 1,800 g and 4°C.[22]

Cleaning-up urine samples using an organic solvent is not common but can be found in literature, notably to extract targeted metabolites.[33, 37, 41, 42] However, we do not recommend it for untargeted metabolomics.

4.2.2. Solid Phase Extraction Based Cleanup

Serum/plasma. Sample cleanup using solid phase extraction (SPE) consists in loading the sample on a sorbent contained in a cartridge that will retain specific metabolites, usually the metabolites of interest. The unwanted compounds are not retained and are directly washed away, while the selected compounds are subsequently removed from the sorbent by solvent elution. By removing interfering compounds and by pre-concentrating the targeted metabolites in a solvent, SPE limits matrix effects, improves repeatability, and increases LC column lifetime. SPE is largely used in targeted metabolomics analysis due to its high selectivity. However, as it reduces metabolite coverage, it is logically rarely applied in untargeted studies.

SPE using a C18 sorbent has nonetheless been suggested for untargeted metabolomics when associated with a C18 chromatographic column.[32, 43] It is likely that the metabolites retained on the sorbent will similarly be retained on the chromatographic column. The procedure would wash away unwanted compounds without impacting too much metabolite coverage. SPE on a C18-bonded phase has been compared with LLE using methanol or acetonitrile for plasma sample cleanup and UPLC-MS analysis.[43] Overall, LLE using acetonitrile and methanol allowed the detection of 2,200 and 1,300 features, respectively (samples were prepared on ice at a 1:3 (v/v) ratio with solvent, followed by a centrifugation at 17,900 g for 10 min), whereas 1,500 features were noted with SPE (C18 96 well plate, activated and eluted with methanol, extract evaporated with N_2 and reconstituted in H_2O/acetonitirle 95:5 (v/v)). LLE with methanol resulted in better metabolite coverage. However, C18 SPE showed clear improvement for clustering the QC samples and the technical replicates. Repeatability (number of features with CV% <20%) was comparable for the three methods, although it was improved with SPE for the most intense ions.

Urine. Considering the less complex metabolic profile of urine compared to blood derived samples, the use of SPE could be considered less relevant. However, the existence of a matrix effect, the need to remove salts, and the ability of SPE to pre-concentrate features with low intensity could justified its use.[44]

The efficiency of several SPE phases to cleanup urine samples has recently been tested for UHPLC-MS analysis.[43] The authors compared raw urine (urine diluted 1:1 by water, vortexed and centrifuged, "dilute and shoot" method), to three alkyl-bonded silica-based phases (C8, C18, C18-OH) and two polymeric materials (Oasis® HLB, Evolute® ABN). Methanol was used for SPE phase activation and elution, eluates were evaporated with N_2, then reconstituted in water/acetonitrile 95:5 (v/v). A total of 1,013 features were detected in the raw sample, of which 44, 49, 58, 74, and 82% were found in C18-OH, C8, C18, Evolute®, and Oasis®, respectively. SPE did not appear to influence the repeatability of the analytical run. The polymeric SPE phases extraction covered a wide variety of metabolites (hydrophilic, lipophillic, and ionizable compounds) while the silica-based SPE was more efficient for moderately hydrophilic and lipophilic compounds.

As for LLE, defining a SPE method that is suitable for every class of compounds is not possible and the choice will rely on the researcher's priorities and evaluation with the available LC-MS system.

4.2.3. Phospholipid Elimination

The other approach using SPE in untargeted metabolomics is to use the sorbent to retain undesired metabolites. This approach is frequently used to specifically remove phospholipids (PLs) from the sample. PLs have been described as the largest source of interference coming from the matrix. Their detrimental effects can be both qualitative and quantitative. Due to their polar nature, PLs will co-elute with polar compounds of interest and, therefore, cause ion suppression, enhance the mass spectrometric signal, lower the number of unique metabolites detected, or interfere with retention times. The loading of PLs-rich samples in a LC system is also known to reduce the column lifetime.[45, 46] The removal of PLs is particularly relevant for serum and plasma samples considering their high concentrations and species variety (approximately 17,000 different types of lipids and fatty acids are present, glycerophosphatidylcholines and phosphatidylcholines being the most abundant).[47, 48]

Various methods are available to remove PLs. Sample deproteinization using organic solvents can partially remove phospholipids (acetonitrile being more efficient than methanol) but the method remains poorly efficient.[47, 49] PLs LLE consisting in diluting the sample with mixtures of chloroform and methanol shows better efficiency. However, the extraction lacks specificity, tends to remove other analytes, and is therefore not recommended for untargeted metabolomics. PLs SPE solutions have shown to be the most efficient and reliable, have the potential for automation, require small volumes of organic solvents, and therefore should be recommended over PLs LLE. PLs SPE columns commonly come in 96 well plates and consist in a C18 stationary silica phase to which zirconia or titanium ions are bonded that will interact with the PLs phosphate group.[50]

On human plasma samples, acetonitrile deproteinization alone has been compared to acetonitrile deproteinization associated with a PLs SPE plate (HybridSPE®). The resulting matrix effects from PLs were 34.8% and 5.1%, respectively.[47, 51] Tulipani et al. have compared two PLs SPE plates on human plasma.[46] Deproteinization by acetonitrile was performed followed by PLs SPE using an Ostro® plate (Waters) or deproteinization. PLs removal was performed simultaneously on a Phree® plate (Phenomenex). The authors followed the respective manufacturer's recommendations. Three sample-to-solvent dilution ratios were also compared for the Phree® plate (1:6, 1:9, 1:12 v/v). Although no clear recommendation can be given regarding the choice of the plate (each plate has advantages depending on the criteria used for extraction performance), the authors indicated that the highest sample dilution factor (1:12, v/v) was surprisingly not associated with a significant loss of metabolite detection or with compromising peak detection and shape, and therefore, was the best compromise between extractive capacity, sample dilution and final organic percentage in the extracts.

Finally, in a previous experiment Tulipani et al.[34] have extensively compared five preparation techniques for plasma and serum samples and LC-MS based metabolomics. The methods included simple deproteinization (using acetonitrile, methanol, or a mixture of both), ultrafiltration (using a 5 kDa filtering membrane), and SPE-mediated PLs removal (acetonitrile deproteinization followed by Ostro® plate PLs removal). The evaluation criteria included extraction repeatability and efficiency, detection of real-life diet related metabolites (amino-acids, alkaloids, acylcarnitines, bile acids…), sample handling (simplicity, speed, compatibility with automation…), as well as their

ability to differentiate samples taken before and after dietary interventions. The authors suggested a combination of deproteinization followed by selective removal of PLs using SPE. By removing both proteins and PLs, this procedure lowered the matrix effect, had the best reproducibility and quickness, and the best coverage for non-lipids metabolites. Acetonitrile deproteinization came as the second choice. The two acetonitrile-based extraction methods gave the most information-rich samples. Simple deproteinization (acetonitrile or methanol) had the advantage to cover low-molecular weight lipids and ultrafiltration was the least effective of the five methods. The article also showed how each method allowed the detection of metabolites that were not covered by the others, highlighting the known fact that there is no ideal sample preparation technique covering every plasma or serum compound.

The choice to remove PLs will ultimately rely on the researcher's priorities. PLs contain valuable biological information, especially for nutritional investigations. Their selective removal will scarify such information for the benefit of low-molecular weight metabolites coverage and data quality. It should therefore, if deemed necessary, be completed with a PLs targeted analysis.

4.3. Sample Preparation for NMR-based Metabolome Studies

Sample preparation for NMR-based metabolomics is significantly less demanding than for the chromatography-based GC-MS and LC-MS technologies as the former requests limited sample manipulation prior to data acquisition. As such, the reader is referred primarily to chapter 3 for general recommendations regarding the sampling, aliquoting, storing, and thawing of samples. Of note, EDTA is not recommended for NMR studies of plasma as it could cover the resonances of many other metabolites.[52] Also, freezing thawed samples for subsequent NMR analysis is not recommended as the spectra may present changes in the spectral linewidth[53] and molecular concentration.[54, 55]

4.3.1. Cleanup for Plasma/Serum Samples

Most of the NMR-based metabolomics studies on plasma/serum are directly performed on the thawed supernatant as is, thus keeping in solution macromolecules and other colloidal species present in the sample.[56] In this case, a specific pulse sequence for NMR acquisition is required, namely the CPMG ('Carr-Purcell-Meiboom-Gill') sequence, aiming at minimizing the signals arising from proteins and other large macromolecules. Some protocols, however, include cleanup steps for serum/plasma samples, physically eliminating large molecules. With this more elaborate sample preparation, the simple 1D-NOESY ('nuclear Overhauser effect spectroscopy') sequence is adopted.[7]

Three main types of cleanup are described:

- Filtration of the serum sample through 3 kDa molecular weight cut off. The 3 kDa filters need to be washed several times with 0.5 mL sterile water, at 4,000 g and 36°C, in order to remove glycerol from the filter membrane until no NMR signal is observed in the filtrate. Generally, 10 washing cycles are employed. The filters should be washed very carefully as they could break. Filters are kept wet at 4°C and then serum is filtered at 10,000 g and 4°C.[57] This method finally uses the buffer system described in **Table S4.4**.
- For elution of the serum samples through SPE plates (e.g. Ostro 96-well plates, Waters)[58] the thawed samples are put into the plate and 1% of formic acid in acetonitrile (3:1 solvent/sample) is added forcefully to precipitate proteins in the well. The plate needs to be incubated at 4°C for 10 min to further precipitate proteins. The collection plate is then inserted at the bottom of the Ostro plate and both are placed above the Positive-Pressure Manifold (Waters). The samples are thus eluted in the collection plate.[34] Eluted samples are finally dried completely and reconstituted with D2O and the buffer system at pH 7.40 in the presence of TSP as internal standard.
- Precipitation of proteins in thawed serum samples with methanol (2:1 solvent/sample) followed by vortexing and incubation at -20°C for 20 min. The solution is then centrifuged at 13,400 g for 30 min, in order to pellet the proteins.[59] The supernatants are decanted into fresh vials and the samples are dried, reconstituted with D2O and the buffer system at pH 7.40 in the presence of TSP as internal standard.

Cleanup steps might be chosen for plasma and serum samples to materially remove the interference of proteins dominating the spectra, which complicate the multivariate data analysis and the interpretation of the spectra as well. However, 3 kDa filtering may cause loss of metabolites adsorbed on the protein surface, while SPE separation and precipitation with organic solvents may introduce errors due to recovery issues. On the other side, CPMG filter may introduce errors when quantitative analyses are performed on samples that use internal standards with signals having different relaxation times than the analytes.

Table S4.4. Buffers for the preparation of samples for NMR spectrometry.

0.07 M sodium phosphate buffer for plasma and serum
0.07 M Na_2HPO_4/D_2O, pH 7.4 (with KOD/D_2O)
0.038 M NaN_3
0.02 M 4,4-dimethyl-4-silapentane-1-sulfonic acid (d6-DSS) (MW 196.34 g·mol^{-1})*
0.02 M 2-chloropyrimidine-5-carboxylic acid (2CLPYR5CA) (MW 158.54 g·mol^{-1})*
For 10 mL buffer
dissolve 0.0993g Na_2HPO_4, 0.022g DSS, 0.0247g NaN_3, and 0.0216g 2C5CA in 8 mL D_2O by ultrasounds
adjust pH to 7.4 with HCl
fill to a final volume of 10 mL with D_2O
add 400 µL of serum to 240 µL of sodium phosphate buffer and mix very well

1.5 M phosphate buffer for urine

1.5M KH_2PO_4/D_2O, pH 7.40 (with KOD/D_2O)

2mM NaN_3

0.1% 3-(trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt (TMSP or TSP) (MW 172.27 g·mol−1)*

For 100 mL buffer

dissolve 10.2 g of KH_2PO_4 in 40 mL D_2O

dissolve 50 mg of TSP in 3-5 mL of D_2O

mix the two solutions and treat by ultrasounds

adjust pH to 7.40 with KOH-tablets or strong KOD-solution (KOH-tablets in D_2O) as the buffer dissolves completely at pH >5

fill the solution to 100 mL with D_2O and mixed very well

add the buffer (10% of the volume of urine sample) to the urine sample and mix carefully

*See section 3.6.3 on internal standard

4.3.2. Sample Preparation

Plasma and Serum Sample Preparation

Serum is largely composed of proteins, which can act as buffer. In spite of this, the use of a buffer is advised. In fact, the CPMG sequence employed for the analysis of serum samples has a filter efficacy correlated to the magnetic permeability and, thus, to the ionic strength of the sample. For this reason, a buffer such as sodium phosphate is needed, in order to make this ionic strength constant among samples. This buffer, in effect, does not form complexes and phosphate is very efficient at pH 7.4 (see **Table S4.4**).

When no cleanup step is employed, samples are simply thawed at 4°C for 60 min. When samples are thawed, the preparation needs to be carried out to completion within 2 hours. Samples are then gently shaken. 500 µL of serum are centrifuged at 18,000 g for 5 min at 4°C, in order to remove possible precipitates. 400 µL of the supernatant are added to 240 µL of sodium phosphate buffer and mixed by inversion or by gentle vortex stirring. 500 µL of the obtained mixture are then put into a 5 mm (or 4.25 mm, depending on the probe bore) disposable NMR tube. The samples in the NMR tubes should to be stored at 4°C until ready for analysis.

Urine Sample Preparation

For urine samples we recommend a 1.5 M phosphate buffer (see **Table S4.4**). The steps for preparing urine samples are the following:

- Thaw samples at 4°C for 60 min. When samples are thawed it is necessary to complete sample preparation within 2 hours
- Gently shake the samples before use
- Centrifuge 630 µL of urine at 18,630 g for 5 min at RT to eliminate residual cells and precipitates
- Add 540 µL of the supernatant to 60 µL of potassium phosphate buffer
- Mix by inversion or gentle vortex stirring
- Put 540 µL of the mixture into high-quality glass NMR tubes, which must be discarded after use, in order to avoid cross-contamination
- Store at 4°C until the samples are analyzed.

Fecal sample preparation

For fecal samples the phosphate buffer use for urine is preferably used and added in the same proportion. The steps for preparing urine samples are the following:

- Vortex 80 mg of stool for 5 min with 1 mL of deionized water
- Centrifuge samples for 10 min at 18,630 g and 4 °C
- Add 540 µL of supernatant to 60 µL of D2O 1.5 phosphate buffer solution
- Centrifuge the samples for 10 min at 18,630 g and 4°C
- Transfer 590 µL of sample into NMR tubes
- Store at 4°C until the samples are analyzed.

References

[1] A. Jiye, J. Trygg, J. Gullberg, A. I. Johansson, P. Jonsson, H. Antti, S. L. Marklund, T. Moritz, *Anal. Chem.* **2005**, 77, 8086.

[2] W. B. Dunn, D. Broadhurst, P. Begley, E. Zelena, S. Francis-McIntyre, N. Anderson, M. Brown, J. D. Knowles, A. Halsall, J. N. Haselden, A. W. Nicholls, I. D. Wilson, D. B. Kell, R. Goodacre, *Nat. Protoc.* **2011**, 6, 1060.

[3] S. Bouatra, F. Aziat, R. Mandal, A. C. Guo, M. R. Wilson, C. Knox, T. C. Bjorndahl, R. Krishnamurthy, F. Saleem, P. Liu, Z. T. Dame, J. Poelzer, J. Huynh, F. S. Yallou, N. Psychogios, E. Dong, R. Bogumil, C. Roehring, D. S. Wishart, *PLoS ONE* **2013**, 8, e73076.

[4] E. C. Y. Chan, K. K. Pasikanti, J. K. Nicholson, *Nat. Protoc.* **2011**, 6, 1483.

[5] C. H. Weinert, B. Egert, S. E. Kulling, *Journal of chromatography. A* **2015**, 1405, 156.

[6] A. B. Rudine, M. G. Walter, C. C. Wamser, *J. Org. Chem.* **2010**, 75, 4292.

[7] N. Psychogios, D. D. Hau, J. Peng, A. C. Guo, R. Mandal, S. Bouatra, I. Sinelnikov, R. Krishnamurthy, R. Eisner, B. Gautam, N. Young, J. Xia, C. Knox, E. Dong, P. Huang, Z. Hollander, T. L. Pedersen, S. R. Smith, F. Bamforth, R. Greiner, B. McManus, J. W. Newman, T. Goodfriend, D. S. Wishart, *PLoS One* **2011**, 6, e16957.

[8] X. Li, Z. Xu, X. Lu, X. Yang, P. Yin, H. Kong, Y. Yu, G. Xu, *Anal. Chim. Acta* **2009**, 633, 257.

[9] K. K. Pasikanti, P. C. Ho, E. C. Chan, *Rapid Commun. Mass Spectrom.* **2008**, 22, 2984.

[10] W. S. Law, P. Y. Huang, E. S. Ong, C. N. Ong, S. F. Y. Li, K. K. Pasikanti, E. C. Y. Chan, *Rapid Commun. Mass Spectrom.* **2008**, 22, 2436.

[11] G. Moros, A. C. Chatziioannou, H. G. Gika, N. Raikos, G. Theodoridis, *Bioanalysis* **2017**, 9, 53.
[12] W. Zou, J. She, V. V. Tolstikov, *Metabolites* **2013**, 3, 787.
[13] A. Jiye, Q. Huang, G. Wang, W. Zha, B. Yan, H. Ren, S. Gu, Y. Zhang, Q. Zhang, F. Shao, L. Sheng, J. Sun, *Anal. Biochem.* **2008**, 379, 20.
[14] B. J. Webb-Robertson, Y. M. Kim, E. M. Zink, K. A. Hallaian, Q. Zhang, R. Madupu, K. M. Waters, T. O. Metz, *Metabolomics* **2014**, 10, 897.
[15] T. Kind, V. Tolstikov, O. Fiehn, R. H. Weiss, *Anal. Biochem.* **2007**, 363, 185.
[16] J. D. Shoemaker, W. H. Elliott, *J. Chromatogr. B* **1991**, 562, 125.
[17] E. J. Want, G. O'Maille, C. A. Smith, T. R. Brandon, W. Uritboonthai, C. Qin, S. A. Trauger, G. Siuzdak, *Anal. Chem.* **2006**, 78, 743.
[18] J. H. Winnike, X. Wei, K. J. Knagge, S. D. Colman, S. G. Gregory, X. Zhang, *J. Proteome Res.* **2015**, 14, 1810.
[19] A. Mastrangelo, A. Ferrarini, F. Rey-Stolle, A. Garcia, C. Barbas, *Analytica Chimica Acta* **2015**, 900, 21.
[20] H. Wang, M. J. Muehlbauer, S. K. O'Neal, C. B. Newgard, E. R. Hauser, J. R. Bain, S. H. Shah, *Metabolites* **2017**, 7.
[21] J. L. Little, *J. Chromatogr. A* **1999**, 844, 1.
[22] L. H. Münger, A. Trimigno, G. Picone, C. Freiburghaus, G. Pimentel, K. J. Burton, F. P. Pralong, N. Vionnet, F. Capozzi, R. Badertscher, G. Vergeres, *J. Proteome Res.* **2017**, 16, 3321.
[23] E. Zarate, V. Boyle, U. Rupprecht, S. Green, S. G. Villas-Boas, P. Baker, F. R. Pinu, *Metabolites* **2017**, 7, 1.
[24] S. O'Hagan, W. B. Dunn, J. D. Knowles, D. Broadhurst, R. Williams, J. J. Ashworth, M. Cameron, D. B. Kell, *Anal. Chem.* **2007**, 79, 464.
[25] P. Begley, S. Francis-McIntyre, W. B. Dunn, D. I. Broadhurst, A. Halsall, A. Tseng, J. Knowles, R. Goodacre, D. B. Kell, *Anal. Chem.* **2009**, 81, 7038.
[26] J. Gullberg, P. Jonsson, A. Nordström, M. Sjöström, T. Moritz, *Anal. Biochem.* **2004**, 331, 283.
[27] M. J. Rist, A. Roth, L. Frommherz, C. H. Weinert, R. Kruger, B. Merz, D. Bunzel, C. Mack, B. Egert, A. Bub, B. Gorling, P. Tzvetkova, B. Luy, I. Hoffmann, S. E. Kulling, B. Watzl, *PLoS One* **2017**, 12, e0183228.
[28] S. J. Bruce, I. Tavazzi, V. Parisod, S. Rezzi, S. Kochhar, P. A. Guy, *Anal. Chem.* **2009**, 81, 3285.
[29] R. E. Patterson, A. J. Ducrocq, D. J. McDougall, T. J. Garrett, R. A. Yost, *J. Chromatogr. B* **2015**, 1002, 260.
[30] B. Álvarez-Sánchez, F. Priego-Capote, M. D. L. D. Castro, *TrAC Trends Anal. Chem.* **2010**, 29, 120.
[31] H. Gika, G. Theodoridis, *Bioanalysis* **2011**, 3, 1647.
[32] D. Vuckovic, *Anal. Bioanal. Chem.* **2012**, 403, 1523.
[33] F. Michopoulos, L. Lai, H. Gika, G. Theodoridis, I. Wilson, *J. Proteome Res.* **2009**, 8, 2114.
[34] S. Tulipani, R. Llorach, M. Urpi-Sarda, C. Andres-Lacueva, *Anal. Chem.* **2013**, 85, 341.
[35] W. B. Dunn, D. Broadhurst, P. Begley, E. Zelena, S. Francis-McIntyre, N. Anderson, M. Brown, J. D. Knowles, A. Halsall, J. N. Haselden, A. W. Nicholls, I. D. Wilson, D. B. Kell, R. Goodacre, C. Human Serum Metabolome, *Nat. Protoc.* **2011**, 6, 1060.
[36] E. Zelena, W. B. Dunn, D. Broadhurst, S. Francis-McIntyre, K. M. Carroll, P. Begley, S. O'Hagan, J. D. Knowles, A. Halsall, I. D. Wilson, D. B. Kell, *Anal. Chem.* **2009**, 81, 1357.
[37] M. A. Fernández-Peralbo, M. D. Luque De Castro, *TrAC Trends Anal. Chem.* **2012**, 41, 75.
[38] A. J. Chetwynd, W. B. Dunn, G. Rodriguez-Blanco, *Adv. Exp. Med. Biol.* **2017**, 965, 19.
[39] T. J. Waybright, Q. N. Van, G. M. Muschik, T. P. Conrads, T. D. Veenstra, H. J. Issaq, *J. Liq. Chromatogr. R. T.* **2006**, 29, 2475.
[40] R. González-Domínguez, R. Castilla-Quintero, T. García-Barrera, J. L. Gómez-Ariza, *Anal. Biochem.* **2014**, 465, 20.
[41] A. D. De Jager, J. V. Warner, M. Henman, W. Ferguson, A. Hall, *J. Chromatogr. B,* **2012**, 897, 22.
[42] R. Fan, R. Ramage, D. Wang, J. Zhou, J. She, *Talanta* **2012**, 93, 383.
[43] F. Michopoulos, H. Gika, D. Palachanis, G. Theodoridis, I. D. Wilson, *Electrophoresis* **2015**, 36, 2170.
[44] M. A. Fernández-Peralbo, M. D. Luque De Castro, *TrAC Trends Anal. Chem.* **2012**, 41, 75.
[45] N. R. Srinivas, *Biomed. Chromatogr.* **2009**, 23, 451.
[46] S. Tulipani, X. Mora-Cubillos, O. Jauregui, R. Llorach, E. Garcia-Fuentes, F. J. Tinahones, C. Andres-Lacueva, *Anal. Chem.* **2015**, 87, 2639.
[47] J. Carmical, S. Brown, *Biomed. Chromatogr.* **2016**, 30, 710.
[48] C. C. Teo, W. P. K. Chong, E. Tan, N. B. Basri, Z. J. Low, Y. S. Ho, *TrAC Trends Anal. Chem.* **2015**, 66, 1.
[49] E. Chambers, D. M. Wagrowski-Diehl, Z. Lu, J. R. Mazzeo, *J. Chromatogr. B* **2007**, 852, 22.
[50] S. Ahmad, H. Kalra, A. Gupta, B. Raut, A. Hussain, M. A. Rahman, *J. Pharm. Bioallied Sci.* **2012**, 4, 267.
[51] V. Pucci, S. Di Palma, A. Alfieri, F. Bonelli, E. Monteagudo, *J. Pharmaceut. Biomed.* **2009**, 50, 867.
[52] P. Bernini, I. Bertini, C. Luchinat, P. Nincheri, S. Staderini, P. Turano, *J. Biomol. NMR* **2011**, 49, 231.
[53] J. D. Bell, P. J. Sadler, A. F. Macleod, P. R. Turner, A. La Ville, *FEBS Lett.* **1987**, 219, 239.
[54] J. Pinto, M. R. Domingues, E. Galhano, C. Pita, C. Almeida Mdo, I. M. Carreira, A. M. Gil, *Analyst* **2014**, 139, 1168.
[55] O. Teahan, S. Gamble, E. Holmes, Jonathan Waxman, J. K. Nicholson, C. Bevan, H. C. Keun, *Anal. Chem.* **2006**, 78.
[56] O. Beckonert, H. C. Keun, T. M. Ebbels, J. Bundy, E. Holmes, J. C. Lindon, J. K. Nicholson, *Nat. Protoc.* **2007**, 2, 2692.
[57] S. Tiziani, A.-H. Emwas, A. Lodi, C. Ludwig, C. M. Bunce, M. R. Viant, U. L. Günther, *Anal. Biochem.* **2008**, 377, 16.
[58] Y. Li, Z. Zhang, X. Liu, A. Li, Z. Hou, Y. Wang, Y. Zhang, *J. Chromatogr. A* **2015**, 1409, 277.
[59] G. A. Nagana Gowda, Y. N. Gowda, D. Raftery, *Anal. Chem.* **2015**, 87, 706.