Clinica Chimica Acta 489 (2019) 169–176

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/cca

Metabolomic analysis—Addressing NMR and LC-MS related problems in human feces sample preparation

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ARTICLE INFO

Keywords:
Feces
Metabolomics
Sample preparation
Extraction
HPLC-MS
NMR

ABSTRACT

Metabolomics is a well-established field in fundamental clinical research with applications in different human body fluids. However, metabolomic investigations in feces are currently an emerging field. Fecal sample preparation is a demanding task due to high complexity and heterogeneity of the matrix. To gain access to the information enclosed in human feces it is necessary to extract the metabolites and make them accessible to analytical platforms like NMR or LC-MS.

In this study different pre-analytical parameters and factors were investigated i.e. water content, different extraction solvents, influence of freeze-drying and homogenization, ratios of sample weight to extraction solvent, and their respective impact on metabolite profiles acquired by NMR and LC-MS.

The results indicate that profiles are strongly biased by selection of extraction solvent or drying of samples, which causes different metabolites to be lost, under- or overstated. Additionally signal intensity and reproducibility of the measurement were found to be strongly dependent on sample pre-treatment steps: freeze-drying and homogenization lead to improved release of metabolites and thus increased signals, but at the same time induced variations and thus deteriorated reproducibility.

We established the first protocol for extraction of human fecal samples and subsequent measurement with both complementary techniques NMR and LC-MS.

1. Introduction

"We are what we eat". This slogan more and more moves into focus of medicinal research. One major player in modulating human's state of health is the intestinal microflora. The human gut is home to > 100 trillion microbes including bacteria belonging to 1200 different species [1]. A high phylogenetic diversity of mainly anaerobic bacteria characterizes the human gut microbiota [2]. Gut health is associated with a symbiosis of the gut microbiota, also called normobiosis which is characterized by the dominance of beneficial bacteria over harmful ones [3]. An imbalance of this equilibrium which is called dysbiosis, is presumed to be the cause for the development of several diseases [4].

Human gut microbiome is a complex ecosystem involved in strong interactions with its host. Those interactions include the immune system, affect multiple host organs and are mediated by a vast array of signaling pathways [5]. Thus, numerous diseases like e.g. inflammatory bowel disease (Crohn's disease, ulcerative colitis), cardiovascular diseases, cancer (gastrointestinal tumours), autism, allergic diseases, lung infection, diabetes, obesity, and fatty liver disease are attributed to a dysbiosis of the gut microflora [4,6–9]. Many of these diseases are associated with a depletion of the variability of bacteria [10].

In the course of gut microbiota's activity many different small molecules are produced and subsequently resorbed by the host. These metabolites include substances that are essential for the host, since they cannot be produced endogenously, as well as substances with both, beneficial and harmful effects on the host [5,11]. The metabolite composition depends on various factors like host's state of health and diet as well as the number and diversity of microbial species. In

Abbreviations: CV, coefficient of variation; MeOH, methanol; MeCN, acetonitrile; EtOAc, ethyl acetate

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http://dx.doi.org/10.1016/j.cca.2017.10.029
Received 17 August 2017; Received in revised form 12 October 2017; Accepted 29 October 2017
Available online 31 October 2017
0009-8981/

addition to bacterial metabolites various host endogenous metabolites like e.g. signaling peptides or bile acids, which are excreted into the intestine for fat digestion and subsequently are in large part resorbed, can be found in the gut. All these metabolites, together with water, undigested food residues, microbiota, fats, inorganic substances, and proteins constitute feces [5,12]. Despite the fact that the large diversity of microbial and human metabolites comprised in feces makes it an interesting matrix that provides valuable information on host's state of health and host-microbiota-interactions, up to now feces is not that much in focus of metabolomic investigations as other body fluids such as plasma/serum or urine.

In metabolomics many different analytical technologies have been used for metabolite detection in which nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS), usually hyphenated to chromatographic separation techniques like liquid (LC-MS) or gas (GC–MS) chromatography, are most prevalent [13–15] and platform specific methods for sample preparation, data acquisition and processing are well established [16–18]. Either analytical platform has its own characteristic advantages and constraints but neither method is capable of detecting all metabolites present in complex matrixes like human body fluids [14].

It is general knowledge that in metabolomic investigations independent of the analytical method chosen, pre-analytical considerations like sample collection, storage, and preparation must be as consistent as possible. Theoretically, in metabolomics the ambitious aim is to have as little sample preparation as possible since any additional step might introduce undesired variability and bias [19]. Introduction of any bias during sample collection and pre-treatment cannot be balanced later on by any analytical method or data processing and might invalidate the outcome of the respective study [16]. In practice modern analytical techniques are highly sensitive and hence require sufficient sample clean-up in order to improve the measurement results and as precautionary measures to protect the equipment. Furthermore, addition of substances is required for sample preservation, mass or chemical shift calibration, inter-sample adjustments of e.g. pH or monitoring of accuracy and reproducibility [16–18,20]. Other than serum or plasma and urine, for which minimal sample preparation, like precipitation of proteins, centrifugation to remove solids, and dilution are sufficient, [16–18,21] feces needs substantial sample pre-treatment in order to extract the metabolites from the complex matrix into a sample solution that can be analyzed, in a reliable and reproducible way. For sample preparation in metabolomic investigations generally and particularly for metabolite extraction, robust methods, preferably thorough extraction, good reproducibility, and non-selective coverage of the whole metabolome are desired. At the same time simplicity is mandatory in order to meet the requirements of high throughput in large-scale studies [4,19,20]. As one can easily comprehend, in complex matrices like feces it is difficult to fulfil all these requirements at once and hence reasonable compromises have to be made. As reviewed elsewhere [4] up to now only few publications dealt with this multifaceted topic of fecal sample preparation and only few methods are established. Additionally for analysis mostly single platform approaches, either NMR spectroscopy [4,20,22–24] or LC-MS [25–32], were used. Thus we aimed to develop a straight-forward sample preparation methodology for human fecal samples for comprehensive metabolomic analyses using NMR spectroscopy and LC-MS as complementary analytical techniques. Specifically, fecal samples from two groups, healthy people and patients with intestinal diseases were used and different parameters and factors were determined i.e. water content, different extraction solvents, influence of freeze-drying and homogenization, ratios of sample weight to extraction solvent, impact on metabolic profiles acquired by both NMR and LC-MS while benefits were weighted against drawbacks. Finally, reproducibility of different methods was assayed and a simple protocol that can be used for NMR as well as for LC-MS was established. To our knowledge, this is the first sample preparation procedure of fecal biomatrix that facilitates the analysis of one extract with both NMR and LC-MS.

2. Experimental procedures

2.1. Sample collection

Fecal samples (n = 13) were collected at the Gastroenterology Department of Medical University Innsbruck from patients suffering from different inflammatory bowel diseases (either ulcerative colitis or Crohn's disease). Patients were in different states of their disease from acute flares to long-term remission and under different medication. The local ethics committee approved collection and investigation of the samples. For comparison purposes, fecal samples (n = 13) from apparently healthy volunteers were collected at the University of Innsbruck. All samples were refrigerated upon delivery and stored at − 80 °C until processing.

2.2. LC-MS analysis

High resolution LC-MS experiments were performed using a 1200 UHPLC liquid chromatograph (Agilent, Waldbronn, Germany) equipped with an auto-sampler at 4 °C, an automatic injector, a column oven set to 40 °C and a diode array detector (DAD) (190–600 nm) coupled to a micrOTOF-Q II operated by Hystar software (Bruker Daltonics, Bremen, Germany). Nitrogen used as nebulizer and dry gas was produced on site by a nitrogen generator (PeakScientific Instruments, Fountain Crescent, UK). The stationary phase used for LC-MS analysis was a 100 × 3 mm 3.5 μm Zorbax Eclipse XDB C18 (Agilent), guarded with a 4 × 2 mm C18 guard column (Phenomenex, Aschaffenburg, Germany). Analytes were eluted with a mobile phase consisting of 0.1% formic acid in LC-MS grade water (A) and 0.1% formic acid in LC-MS grade acetonitrile (B) and the following gradient: 0 min, 99% A; 5 min, 99% A; 45 min, 2% A; 60 min, 2% A at a flow rate 0.3 mL/min. The injection volume was 10 μL. The column was equilibrated between runs with 99% A for 10 min. MS experiments were performed in positive and negative ESI mode with the following parameters: endplate offset, 500 V capillary energy, 3500 V; nebulizer gas, 20.3 psi; dry gas, 8.0 L/min at a dry temperature of 220 °C; scanning range, m/z 50–1000; spectra rate, 2 Hz. High-resolution mass calibration was facilitated by injecting 10 mM NaOH in isopropanol/water (1:1, v/v) fortified with 0.1% formic acid at the beginning of each analysis. The exact mass of the sodium formate clusters was used as mass calibrant.

2.3. LC-MS data processing

For processing raw centroid LC-MS data were transformed to netCDF-format using the export tool in Hystar and further processed with XCMS package in R (Bioconductor, version 1.50.1) [33]. Features were detected, grouped, retention time corrected and missing data filled with "*centWave*", "obiwarp" or the respective default algorithms and parameters were adjusted according to the raw data: ppm = 25.25, peak width interval from 21.6 to 65 s, signal-to-noise-threshold = 10, noise threshold = 300, prefilter = 5 × 500, band width/bw = 0.88, minfrac = 0.8, mzwid = 0.0414, and for all other parameters default values were used. In this way a data matrix of samples analyzed vs. detected features and corresponding m/z values, retention times, and intensity values was produced. Isotope peaks, fragments, and adducts were treated as separate metabolite features.

2.4. NMR analysis

NMR spectra were acquired using an Avance II 600 MHz spectrometer (Bruker Biospin, Rheinstetten, Germany), equipped with a Bruker 5 mm CryoProbe Prodigy TCI probe head with Z-gradient. Samples were measured in automation using a Bruker BACS-60 sample changer operated by IconNMR and Topspin 3.2 (Bruker Biospin). Probe head temperature was adjusted to 300.0 ± 0.1 K using a 99.8% deuterated methanol sample, water suppression was calibrated using a sample containing 2 mM sucrose, 0.5 mM TSP, and 2 mM NaN_3 in 90% D_2O, and for quantification of metabolites via the ERETIC method a standard sample provided by Bruker was measured as described elsewhere [34]. Prior to measurement temperature of every sample was equilibrated to 300 K for 5 min and automatic tuning and matching, locking to D_2O-signal, shimming, and pulse calibration were performed. For every sample a 1D ^{1}H-NOESY with water suppression via pre-saturation, 32 scans, a receiver gain of 90.5, a relaxation delay d1 of 4.0 s, a mixing time d8 of 0.01 s, and an acquisition time of 2.726 s was acquired. Additionally ^{1}H–*J*-Res, COSY, HMBC and HSQC-spectra were acquired for peak annotation and substance identification in selected samples using standard pulse sequences and experimental settings.

2.5. NMR data processing

Raw data were automatically Fourier-transformed, phase and baseline corrected. For data processing Topspin 3.2 (Bruker) and for annotation of NMR peaks to compounds Chenomx Profiler (Chenomx Inc., version 8.1) and Human Metabolome Database HMDB (Version 3.6) [35] were used.

2.6. Determination of water content

For determination of water content in fecal samples from patients with different gastrointestinal diseases (n = 13) and healthy volunteers (n = 13) aliquots of 0.5–1.0 g thawed feces were weighed into tared tubes, frozen to − 80 °C and freeze-dried for 24 h to constant weight. Afterwards the loss of water was determined from the difference between original and dry weight divided by the original weight.

2.7. Comparison of different extraction solvents

Fecal samples (n = 3) were freeze-dried for 36 h, pooled, and homogenized with the dismembrator for 10 min at 1500 rpm. Sample aliquots of 30 mg (n = 3 per solvent) were weighted into Eppendorf tubes, 500 μL of the extraction solvent (water, methanol, methanol/water (4:1), acetonitrile, acetonitrile/water (1:3), ethyl acetate or a biphasic system of chloroform/methanol/water (3:1:2)) were added, mixing applied (10 min ultra-sonication followed by 10 min vortex mixing), and centrifuged (15 min at 4 °C and 13,000 rpm). After centrifugation supernatants were collected, dried under a stream of nitrogen at ambient temperature and weighted to determine the extraction yield. This extraction process was repeated two more times for each sample. Dried residues were dissolved in water/acetonitrile (4:1) to a concentration of 0.5 mg/mL filtered through syringe filters and analyzed by LC-MS. Additionally dried residues were dissolved in a 75 mmol/L buffer containing 10.05 g $Na_2HPO_4 \times 7\ H_2O$, 0.2 g NaN_3 and 0.4 g TSP in 400 mL water and 100 mL D_2O at a pH of 7.4 [34] to a final concentration of 1 mg/mL and 650 μL of this solution analyzed by NMR. A graphical visualization of all sample preparation protocols is given in Fig. 1.

2.8. Comparison of direct extraction and extraction after freeze-drying and homogenization

Fecal samples (n = 3) were divided into two aliquots of 450 mg each. One 450 mg aliquot per sample was freeze-dried for 36 h followed by homogenization for 10 min at 1500 rpm in the dismembrator. The other 450 mg aliquots were not dried or homogenized. Afterwards all samples were extracted with 900 μL of 75 mmol/L buffer by ultra-sonication for 10 min, followed by 10 min vortex mixing, and centrifugation for 15 min at 4 °C and 13,000 rpm. After centrifugation supernatants were collected and analyzed by LC-MS and NMR.

2.9. Determination of feces-solvent-ratio for extraction

300 mg aliquots of one fecal sample were diluted with water in ratios of 1:2, 1:5 and 1:10 (w:v), vortex mixed for 10 min and extracted with ultra-sonication for 20 min. After two freeze-thaw cycles consisting of 30 min at − 80 °C and 30 min at ambient temperature, the extracts were briefly vortex mixed and the solid particles sedimented by centrifugation at 14000 rpm for 20 min. 540 μL of the supernatants were taken, mixed with 60 μL of a 1.5 mol/L buffer containing 20.41 g KH_2PO_4, 13.00 g NaN_3 (2 mmol/L) and 0.1 g TSP in 100 mL D_2O at a pH of 7.4 and analyzed by NMR [34].

2.10. Optimized direct extraction

300 mg of stool samples (n = 6) were weighed into Eppendorf tubes, diluted with 1.5 mL water and vortex mixed for 10 min. After 20 min of ultra-sonication samples are subjected to two freeze-thaw-cycles consisting of − 80 °C for 30 min followed by thawing to ambient temperature for another 30 min. The thawed samples are again briefly vortex mixed and solid components sedimented by centrifugation at 14000 rpm for 20 min. The clear supernatant is taken and aliquoted for further analyses: 100 μL are directly analyzed by LC-MS, 540 μL are mixed with 60 μL of a 1.5 mol/L buffer containing 20.41 g KH_2PO_4, 13.00 g NaN_3 (2 mmol/L) and 0.1 g TSP in 100 mL D_2O at a pH of 7.4 and analyzed by NMR [34].

3. Results and discussion

3.1. Heterogeneity of fecal matrix

Feces are a highly heterogeneous matrix and variances become obvious at first sight. Within one sample the major source of inhomogeneity is the irregular scatter of undigested food residues. Between samples from different individuals the most obvious variations are sample color, caused by varying concentrations of dyes from food as well as excreted hepatic metabolites like e.g. bilirubin derivatives, and sample consistency. According to the Bristol scale, from stool consistency conclusions on nutrition and health state of the individual can be drawn [36]. A lack of drinking water or dietary fiber changes stool consistency just like a variety of diseases and have a strong impact on fecal water content [37].

As shown in Fig. 2 the water content in different samples, determined by weighing of the sample before and after freeze-drying, strongly varies. Average water content in all samples is 76.0% whereupon it is significantly lower in samples from healthy (72.4%, n = 13) compared to diseased (79.6%, n = 13) people (*t*-test: $p = 0.04$). Not only inter-group, but also intra-group variations are quite high: in healthy people water content between 61.0% and 84.3% and in diseased people even between 62.4% and 96.2% was observed which is in good accordance with literature [37]. These strong inter-individual variations of water content make it problematic to compare metabolite concentrations: In samples from people with diarrhea metabolites are innately more diluted than in solid samples with lower water content. This makes it more difficult to detect low concentrated metabolites in samples with higher water content. This possible loss of low abundant and the reduced signal intensities of all metabolites might lead to misinterpretation of the obtained data. Therefore, the different water contents urgently have to be taken into account during sampling, sample preparation, measurement, and data processing.

We are aware that the selection of samples used for method development might influence the outcome. Hence we have considered different diseases, states of treatment, age, and sex when we approached individuals to donate samples. For development of every sample preparation protocol we have selected subsets from our sample pool to obtain a matrix as balanced and representative as possible. Additionally we used pooled samples alongside with individual samples to minimize

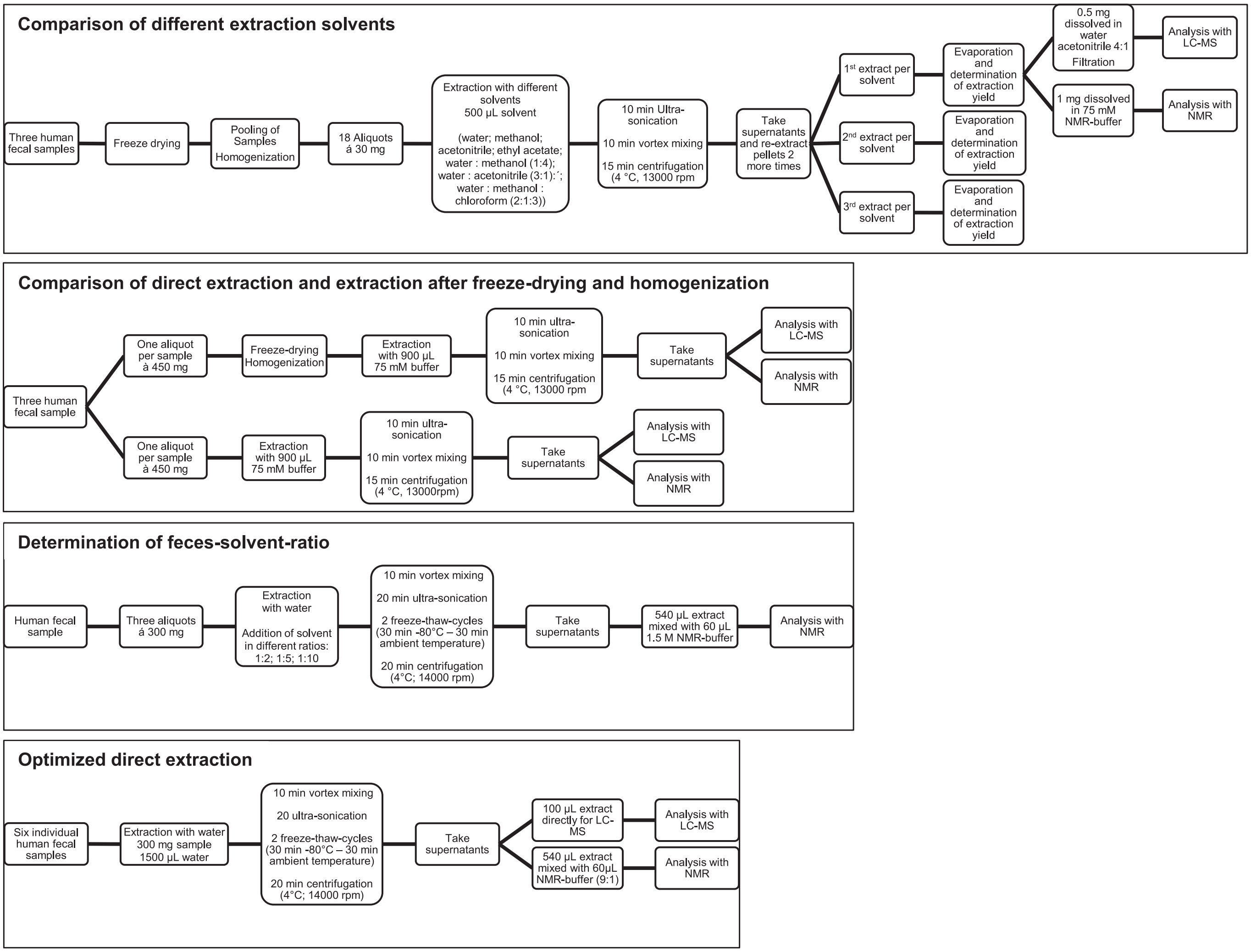

Fig. 1. Flow chart showing the workflows and parameters from different sample preparation protocols: comparison of different solvents (top), comparison of direct extraction to extraction after freeze-drying and homogenization (second from top), optimization of feces-solvent-ratio (second from bottom), and the optimized direct extraction (bottom).

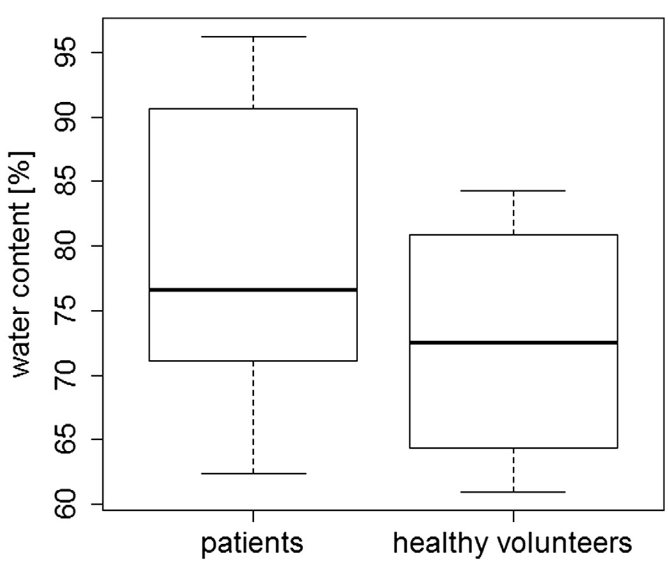

Fig. 2. Comparison of water content in stool samples from 13 healthy people and 13 patients suffering from different intestinal diseases determined by freeze-drying for 24 h. Average water content: 76.0% (79.6% in patients and 72.4% in healthy people) ranging from 62.4–92.2% in patients and 61.0–84.3% in healthy people.

the bias introduced by different states of health, medication or other factors.

As already mentioned, apart from high inter individual variations strong intra sample inhomogeneity can be observed. In order to minimize the consequential bias care has to be taken that sampled aliquots are representative for the matrix. Customarily averaged samples are obtained by homogenization of the material prior to sampling. However, most common homogenization methods as grinding or mixing are difficult to apply to fresh fecal samples.

3.2. Drying and homogenization

A two-step procedure of freeze-drying and grinding of the fecal material allows reproducible sample homogenization and provides the possibility to use accurate amounts of sample in subsequent analysis steps. This allows direct comparison of different samples not only qualitatively but also quantitatively and makes the analytical result independent from the individual sample water content. Additionally sample surface is enlarged and metabolites are better accessible to the extraction solvent what consequently increases signal intensities and peak areas in NMR and LC-MS.

The extraction solvent defines the cluster of the metabolome to be imprinted based on the polarity and generally physicochemical properties of both metabolites and solvent. Thus, for an untargeted metabolomics analysis, the solvent system which enables extraction of the

Table 1
Comparison of mean (n = 3) extraction yields achieved with different solvents Three extractions per solvent. Ratio feces/solvent 30 mg/500 µL.

	1st			2nd			3rd			total		
	Mean [mg]	CV [%]	Yield [%]	Mean [mg]	CV [%]	Yield [%]	Mean [mg]	CV [%]	Yield [%]	Mean [mg]	CV [%]	Yield [%]
H_2O/MeOH	7.5		25	2.4		8.0	1.0		3.3	10.9		36
H_2O/MeCN	6.9		23	1.9		6.3	1.5		5.0	10.3		34
$CHCl_3$/H_2O/MeOH (aq.)	5.8		19	2.3		7.7	0.9		3.0	9.0		30
$CHCl_3$/H_2O/MeOH (org.)	3.9		13	1.3		4.3	0.3		1.0	5.5		18
H_2O	6.4	1.9	21	1.3	46	4.2	0.6	36	2.0	8.3	6.0	28
MeOH	4.2	3.9	14	1.1	44	3.6	0.6	22	1.9	5.8	11	19
EtOAc	3.4	8.7	11	1.2	40	3.9	0.9	18	3.0	5.5	5.7	18
MeCN	1.6	20	5.3	0.8	20	2.7	0.7	20	2.3	3.1	7.0	10

higher number of metabolites is preferable. On the other hand, in targeted metabolomics e.g. lipidomics the solvent system which extract certain group(s) of metabolites is more suitable. Thus, multiple solvent systems were tested in the current work using freeze-dried and homogenized fecal samples as staring material. Specifically, water, methanol, acetonitrile, ethyl acetate, mixtures of methanol/water (4:1), acetonitrile/water (1:3) and a biphasic system of chloroform/methanol/water (3:1:2) were tested. The samples were extracted three times with the respective solvent by ultra-sonication and vortex mixing. After centrifugation supernatants were taken, independently evaporated, and the dry residue weight determined in order to assess extraction efficiency. The results, summarized in Table 1, show that for all solvents approximately 2/3 of the metabolites are extracted in the first passage, roughly 1/5 in the second and the rest in the third. It could be shown that one single extraction is not exhaustive, which is in accordance with the literature [20]. Most efficient extraction was achieved under protic conditions, that is, by using water or aqueous mixtures of protic organic solvents for extraction (Fig. 1, Supplementary information).

Generally, it has to be considered that different solvents extract different (classes of) metabolites. In our opinion water as extraction solvent is a suitable compromise. We have not observed any metabolite class to be completely lost by extraction with water. On the contrary, if using organic solvents for extraction the more hydrophilic products are lost and overall extraction efficiency decreases. Taking into account that microbial bioactivity contributes more to hydrophilic substance classes and that food residues ending up in the intestinal route of excretion as feces are rich in lipophilic substance classes (e.g. cell wall constituents of the microbiome or food) and high molecular weight compounds (e.g. seeds, fibers), choosing a extraction regimen with an emphasis on hydrophilic fraction of the metabolome might be more relevant than investigating the hydrophobic counterpart. Hence we are convinced that an approach that uses water as extraction solvent covers the broadest range of relevant microbiome related metabolites with the least effort invested.

Exhaustive extraction is desirable in metabolic investigations to provide high level of reproducibility and to increase signal intensity particularly of low abundant metabolites. Especially in NMR, an inherently insensitive analytical platform, but also in LC-MS sufficiently high concentrations of metabolites are necessary to facilitate metabolite detection and identification. Multiple extraction of a sample is capable of extracting thoroughly but it is very laborious and pooling of repeated extracts with decreasing metabolite concentrations, as recommended by Wu et al. [20], leads to dilution of metabolites and consequently further decreased signal intensities. Thus, a compromise has to be found in order to achieve high signal intensities and good reproducibility. In our study water as extraction solvent showed sound extraction yields and decent extraction efficiency. The high number of detected features implied a good coverage of the metabolome; excellent data reproducibility (low coefficient of variation) was found both for LC-MS feature intensities (Fig. 3) as well as NMR signal integrals. Therefore a single extraction with water is our method of choice and was used for all further analyses.

Differences in extraction yield and coverage of the metabolome strongly vary between solvents/mixtures. Solvents like ethyl acetate or chloroform, pure methanol and acetonitrile only show moderate extraction yields and manly cover the lipophilic constituents of the fecal metabolome. In absence of water mainly lipids and other lipophilic components are extracted whereas water-soluble components like e.g. sugars, amino or other organic acids, and hydrophilic conjugates of lipophilic substances remain in the matrix (see Supplementary information, Fig. 1). Thus these solvents are suitable for special questions, like investigations of the lipophilic constituents, and for fractionation of fecal extracts precisely because organic and aqueous extracts (like the biphasic system) complement one another in coverage of the metabolome. Contrary to pure methanol and acetonitrile, their aqueous mixtures showed good extraction yields and coverage of the metabolome, but their applicability in both NMR and LC-MS is limited. Our goal is to achieve one single extract than can be analyzed with both techniques to minimize effort and costs per sample. However, these mixtures are incompatible with the analytical platforms or lead to considerable higher effort associated with introduction of further variations and bias. Any organic solvent used for extraction is interfering in both techniques – in LC organic solvents lead to poor peak shape and shifting of retention times especially in the beginning of the chromatographic run, in NMR the un-deuterated solvent proton signal is too high and thus might interfere with signals from metabolites, and impairs the detection of low concentrated metabolites. Removing the organic solvents e.g. by evaporation and reconstituting the residue in water for further analyses is laborious and it remains uncertain if all the metabolites are re-dissolved. Using deuterated solvents for extraction would solve the problems in NMR but this approach is rather expensive and deuterium from the solvent could exchange protons which leads to wrong masses and isotope pattern in high resolution MS and consequently to wrong results in identification of metabolites. For these reasons we decided not to use (mixtures of) organic solvents for extraction although they showed excellent results in extraction. Having a closer look on the obtained data it became obvious that one single technique is not sufficient for a holistic overview over the metabolite composition of human feces. NMR as an inherently insensitive technology is only able to detect highly concentrated components. On the other hand NMR is able to detect any substance containing at least one proton and it is able to distinguish between substances that are hard to separate in RP-LC-MS like different hexoses (isobaric), as long as they differ in shift of at least one signal. We found that NMR and LC-MS complement one another and thus provide a more comprehensive view of our fecal metabolome.

Due to their different underlying principles of operation the two analytical platforms have different requirements on the samples investigated: In NMR spectroscopy addition of D_2O for field lock, usually high concentrated phosphate buffers to minimize sample pH variations (below 0.1 pH units) [20], sodium azide to prevent microbial growth, and TSP for shift referencing are required. For NMR based

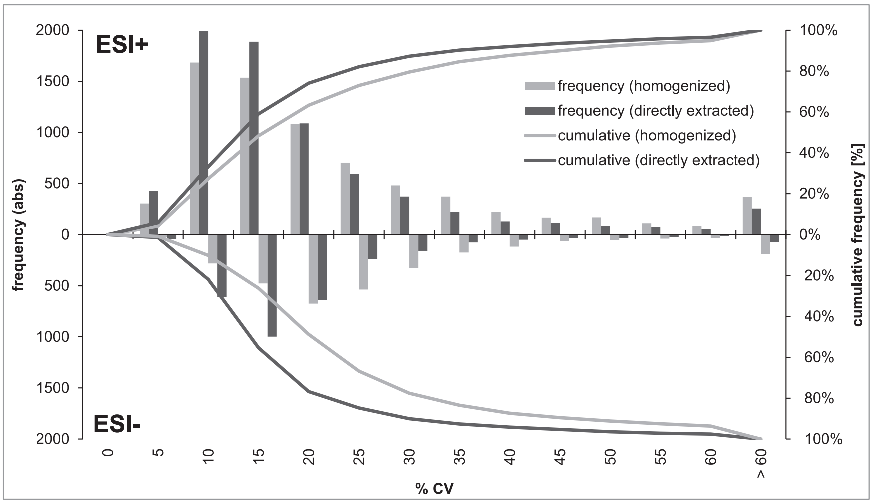

Fig. 3. Frequency distribution plots comparing chromatographic features CV's of LC-ESI(+)-MS (pointing upwards) and LC-ESI(−)-MS (pointing downwards) from samples extracted after lyophilization and homogenization (n = 6) and directly extracted samples (n = 6). Similar absolute numbers of features were detected with both extraction methods (ESI(−) ~2980 ESI(+) ~7280 features). Better reproducibility, indicated by higher number of features with lower CV's, is achieved by direct extraction.

metabolomics, samples are usually extracted directly with the buffer but this is incompatible with LC-MS. In mass spectrometry non-volatile components in injection solvent might lead to ion-yield attenuation (ion suppression or ion enhancement) or may precipitate in the ion-source and/or block the ESI-spray. Additionally for separation of metabolites usually reversed phase chromatography has to be used and solvents with little eluting strength (like water) are preferred as injection solvents. Other than strong eluting solvents (like methanol or acetonitrile) weak solvents do not lead to peak broadening, shifting or splitting and hence provide better and more reproducible chromatograms. For comprehensive investigations with different analytical platforms hence either a different sample extraction for every analytical method would be necessary, or, as we recommend, one extraction with a certain solvent (mixture) followed by treatment of the extract as it is required for the respective technique.

In our case, extraction with water was the easiest way to achieve effective and reproducible extraction, the extract could be used directly for LC-MS and needed only minimal further treatment for NMR, namely addition of phosphate buffered D_2O, containing TSP and sodium azide. Adding a strong buffer (1.5 mol/L) to the extract in a ratio of 1:9 (V_{buffer}:$V_{extract}$) enables pH stabilization, filed lock and referencing but does not lead to excessive dilution of metabolites. In our opinion this is the best way to achieve comprehensive metabolic profiles with highest gain in information and optimum results in both complementary techniques, combined with comparatively simple sample preparation.

The big advantages of this procedure, with drying and grinding of the samples, is firstly the possibility to compensate strongly varying water contents and thus use same amounts of sample for analyses and secondly enhanced release of metabolites and thus increased signal intensities in both analytical methods. On the other hand this workflow is very time-consuming and laborious and contains many steps each of which is prone to introduce variations and bias. This becomes obvious when chromatograms of samples extracted after freeze-drying and homogenization are compared to samples, which were extracted without any other pre-treatment. As shown in Fig. 3 coefficients of variation of detected features were significantly lower in samples that were only extracted compared to samples that underwent more sample preparation steps. Increased CV's in measurement results indicate a worse reproducibility of the entire sample preparation. Such a method should not be used for metabolomic investigations due to the fact that it strongly affects the results of the study.

Another critical step is freeze-drying of the samples. After freeze-drying, homogenization, extraction, and measurement at the NMR it became obvious that some metabolites were lost during drying which is in accordance with the findings of Saric et al. [23]. As shown in Supplementary Fig. 2 especially components with a high vapor pressure like short chain fatty acids propionate, butyrate, valerate and their corresponding isoforms, but also some amines like e.g. trimethylamine or trimethylamine-*N*-oxide and alcohols like e.g. methanol were volatized and their NMR signals significantly reduced or completely lost after drying. This most likely occurs no matter which evaporation-technique, freeze-drying, heating or drying with a gas stream, is used and thus is a further source of bias, which affects the study outcome. To overcome these limitations direct extraction of fecal samples should be used.

3.3. Direct extraction as fecal water

For direct extraction we used approximately 300 mg of feces, added water in a ratio 1:5 (w/v) and extracted the metabolites by vortex mixing and ultra-sonication. After lysing the cells by two freeze-thaw-cycles and centrifugation the supernatant was used directly for LC-MS and after mixing of the extract with the deuterated 1.5 M phosphate buffer in a ratio of 9:1 for NMR.

Direct extraction protocols similar to this one are most often used in literature, [4,20,22–24,26–32,38,39] since they are much easier and faster than protocols with extensive drying and homogenization [40,41]. Other than in most of the methods described in literature we used only pure water for extraction in order to achieve applicability in comprehensive investigations with both analytical platforms NMR and LC-MS. As discussed above, water is our extraction solvent of choice since it shows good extraction efficiency, coverage of the metabolome, reproducibility of the extraction, and the aqueous extracts can be analyzed by both techniques. Without any further treatment the aqueous extract can be injected to the LC-system since it is a suitable injection solvent providing excellent peak shape even in early eluting peaks in RP-chromatography. Dilution of the extract prior to injection in our case was not necessary since almost all detected features showed signal intensities in the optimum range of our instrument. Thus, saturation of the detector and loss of mass accuracy are minimized whereas signal intensities are still high enough to detect even low concentrated metabolites. For NMR analyses of the extracts addition of a deuterated buffer is essential. Using this 1.5 M buffer minimizes pH variances between samples and thus reduces shift of signals (see Supplementary Fig. 3) from compounds with pK_a values close to the relevant pH region. In this manner alignment of peaks across samples, identification, and quantitation of substances are facilitated. Based on the strength of the buffer only low amounts have to be added to the fecal extract (9:1 extract:buffer) for pH adjustment. Thus, the samples do not get needlessly diluted allowing detection of metabolites appearing in concentrations close to the instrument detection limits.

In metabolomic investigations various feces to extraction solvent were used: no matter which analytical platform (NMR or LC-MS) was employed, commonly ratios of 1:2 (w:v) to 1:10, in some cases even up to 1:50 were used [4]. It is comprehensible that higher amounts of feces and lower volumes of solvent used for extraction lead to increased signal intensities (see Supplementary Fig. 4) and hence are preferentially used. However, in our study we found that ratios of 1:2 (w:v) are more difficult to process and in some cases sufficient amounts of extract could not be recovered after centrifugation due to limited initial quantity or sample consistency. Additionally in NMR analyses signal to noise ratios were lowered and signals broadened which led partially to overlapping signals that were more difficult to annotate to the respective metabolites (see Supplementary Fig. 5). Increasing the amount of water used for extraction to a ratio of 1:5 (w:v) solved the problems during sample processing, improved spectral quality in NMR, and still provides adequate signal intensity in both NMR and LC-MS to detect high numbers of features. Even higher ratios of feces to solvent like 1:10 (w:v) in contrast lead to more diluted extracts and the numbers of detected features decreased.

When signals from directly extracted samples are compared to freeze-dried and ground samples it becomes obvious that the absolute number of features in both methods is almost the same (direct extraction: 7289 (ESI +), 2988 (ESI-); after freeze-drying and homogenization 7280 (ESI +), 2980 (ESI-)). This indicates that both methods cover the same range of the metabolome and no metabolites are lost by selecting either method. However huge differences become obvious in signal intensities: samples that were dried and ground prior to extraction showed considerable higher signal intensities in both NMR and LC-MS (see Supplementary Fig. 2). Grinding of the freeze-dried material increases the surface accessible to the extraction solvent and additionally facilitates release of intracellular metabolites during extraction. However, by direct extraction of the fecal material a slurry is obtained that cannot be ground. Applying a protocol as e.g. suggested by Monleón et al. [24], in which the cells are lysed by two freeze-thaw-cycles, lead to increased signal intensities after direct extraction of fecal samples. Especially sugars and amino acids showed elevated levels which indicate that metabolites from gut microbiota and undigested food residues are released.

Direct extraction, more than extraction after sample drying, requires a representative aliquot of the sample. After drying, samples can be homogenized by grinding e.g. in a ball mill where homogenous powders are obtained. In fresh or thawed fecal samples this is much more difficult: undigested food residues or fiber are scattered irregularly all over the sample and there is no easy way to achieve a homogenous distribution particularly in solid samples with low water content. Using larger amounts of raw material usually is less prone to variations introduced by intra-sample inhomogeneity and thus leads to more reproducible results. To evaluate reproducibility of LC-MS measurements Wilson and coworkers in a recent publication on quality control in metabolic profiling demand feature intensity coefficients of variation (CV) after repeated preparation and measurement of a single (quality control) sample to be $\leq 20\%$ for high and $\leq 30\%$ for low concentrated metabolites [42]. Using a minimum of 300 mg of fecal sample, as we did in our study, results in representative extracts for further analyses. As shown in Fig. 3 and Supplementary Fig. 3 with our extraction, sample preparation, and measurement methods we obtain reproducible metabolic profiles meeting the above mentioned criteria: in ESI(+) 74.0% and 87.2% and in ESI(−) even 76.7% and 90.1% of the features showed CV's of below 20% or 30%. Up to now no comparable data on reproducibility of fecal sample preparation methods were published but our results are in good accordance with data published for urine [43].

In our study we could show that by using water as solvent for direct extraction of human fecal samples reproducible results can be obtained that allow for detection of high numbers of features in both techniques NMR and LC-MS. However, one major challenge of this method remains the handling of strong inter-sample variations of water content. Other than in dried samples, where same amounts of material can be used for extraction, in direct extraction varying water contents have a strong impact on the results. To handle this challenge some kind of data normalization prior to multivariate statistical analysis is mandatory. This can be done either by determining the water content independently from profiling experiments and multiplying integrals or peak intensities with the corresponding factor or by using mathematical algorithms as reviewed elsewhere [44–46]. But still it has to be considered that especially low concentrated metabolites might be overlooked in diluted samples.

4. Conclusion

Due to the high demands of LC-MS and NMR concerning the quality of the samples on the one hand and the heterogeneity and complexity of fecal material on the other hand, sample preparation is a crucial and a challenging step in a metabolomics workflow. Fecal samples cannot be used directly for either analytical technique and hence metabolites have to be extracted prior to analysis. For extraction a variety of solvents can be used each of which with its own characteristics and coverage of the metabolome. In our study water as extraction solvent yielded the best results in terms of coverage of the metabolome, number of detected features as well as reproducibility. Additionally the extract can be used for LC-MS analysis without further treatment and, after addition of a deuterated buffer, for NMR spectroscopy. Therefore, water in our study is the solvent of choice for fecal sample extraction.

The differing contents of water between samples and the intrinsic inhomogeneity can be overcome by drying of the sample followed by homogenisation. This procedure leads to enhanced release of metabolites and thus to higher signals in measurement but it has to be taken into account that it is more laborious, time consuming, leads to loss of volatile components and therefore is highly susceptible to introduction of variations and bias. This can be averted by direct extraction of the fecal material without any further pre-treatment. The latter method showed lower signal intensities for many features but it is still sufficient for detection. In order to increase signal intensities a step for lysing cells, e.g. by repeated freezing and thawing, can be introduced into sample treatment. Since less work steps are required in this approach probability for introduction of inaccuracies is reduced which becomes apparent in reduced coefficients of variation of the measurement results.

Several methods for preparation of fecal samples for metabolomic investigations are available each of which with its own benefits and drawbacks. These have to be considered when setting up a metabolomics workflow and the methods complying best with the objective of the study have to be selected.

Funding

This research was supported by an excellence initiative (Competence Centers for Excellent Technologies—COMET) of the Austrian Research Promotion Agency FFG (Research Center of Excellence in Vascular Ageing—Tyrol, VASCage; K-Project No. 843536 funded by the Austrian Ministry for Transport, Innovation and Technology, Austrian Ministry of Science, Research and Economy, Vienna, Salzburg, and Standortagentur Tirol).

Acknowledgements

We thank Bruker Biospin for support in setting up the NMR experiments and the voluntary donors for stool samples.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2017.10.029.

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