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Strategy for nuclear-magnetic-resonance-based metabolomics of human feces

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1 **Abstract:**

2 Metabolomic analyses of fecal material are gaining increasing attention because the gut microbial
3 ecology and activity have an impact on the human phenotype and regulate host metabolism. Sample
4 preparation is a crucial step, and in this study we recommend a methodology for extraction and
5 analysis of fresh feces by NMR-based metabolomics. The evaluation of extraction solvents showed
6 that buffer extraction is a suitable approach to extract metabolic information in feces. So, the effects
7 of weight-to-buffer (Wf:Vb) combinations and the effect of sonication and freeze-thaw cycles on
8 the reproducibility, chemical shift variability, and signal to noise ratio (SNR) of the ^1H NMR
9 spectra were evaluated. Based on our results, we suggest that fresh fecal extraction with a Wf:Vb
10 ratio of 1:2 may be the optimum choice to determine the overall metabolite composition of feces. In
11 fact, more than 60 metabolites have been assigned in the NMR spectra obtained from the fresh fecal
12 buffer extract, and assignments of the lipophilic signals are also presented. To our knowledge, some
13 of the metabolites are reported here for the very first time employing ^1H NMR spectroscopy on
14 human fecal extracts.

15

16 **Keywords:** Nuclear magnetic resonance, metabolomics, gut, human feces, metabolites

17

18 **Introduction**

19 Metabolomics is a holistic approach to study systematic metabolic changes in biological samples. In
20 general, nuclear magnetic resonance (NMR) and mass spectrometry (MS) are the analytical
21 techniques used to observe and quantify metabolites from biological samples, and each technique
22 has its own advantages and disadvantages. NMR-based metabolomics has emerged as a reliable
23 high throughput analytical technique that has been extensively applied to a variety of biological
24 samples such as urine, plasma, tissues, saliva, milk, seminal fluid, and sputum.¹⁻⁴ Presently, urine
25 and plasma are the most common sample types included in metabolomics studies. However,
26 metabolomics on fecal material is gaining increased attention because gut ecology has an impact on
27 the human phenotype and gut-microbe cross talk is regarded as one of the key determinants in the
28 regulation of host metabolism.⁵⁻¹¹ Because feces comes into direct contact with the colon and
29 sample collection is non-invasive, feces is the most biologically most relevant sample material for
30 metabolomics studies aiming to explore gut microbial activity and thereby gut health status. To
31 date, NMR studies focused on fecal metabolomics have been applied to gain insight into metabolic
32 effects involved in dietary interventions^{12,13} and to study gut diseases such as inflammatory bowel
33 disease,^{14,15} ulcerative colitis,¹⁴ and colorectal cancer.¹⁶ An increased understanding of the
34 metabolic activity in the colon may contribute to the identification of new biomarkers that could be
35 related to health or disease status and molecular regulation of the complex gut system.

36

37 In order to extract as much information as possible from a complex fecal sample, optimization and
38 standardization of an analytical methodology are essential. Although several studies have been
39 reported on fecal metabolomics,^{12,14-16} few studies have focused on optimizing the NMR
40 methodology for fecal extracts and detailed spectral assignments have rarely been reported.¹⁷⁻¹⁹
41 Two of the most important factors to consider in the optimization of an extraction method are (i) the

42 production of reproducible NMR spectra with low inter-sample chemical shift variability and (ii)
43 high signal-to-noise ratio (SNR). In addition, the extraction method should preferably be rapid,
44 robust, (and suitable) and compatible with high-throughput analyses. Previously, fecal sample
45 extractions have been performed by centrifugation,²⁰ D₂O,²¹ distilled water,¹⁶ buffers,^{14,22} and
46 mixed solvent extractions on lyophilized samples.¹⁸ Lyophilization is advantageous, as it minimizes
47 the effect of solvent dilution (water content) on the feces. However, lyophilization affects the
48 concentration of volatile metabolites, such as short chain fatty acids and succinate.^{18,19} Therefore, to
49 reduce the loss of volatile metabolites and minimize the steps required during extraction; fresh fecal
50 samples are considered the preferred choice for fecal metabolomics studies. Buffer extraction might
51 be the most suitable approach to extract metabolic information as it reduces inter-sample pH
52 variation during extraction. In order to obtain reproducible NMR spectra with lower inter-sample
53 chemical shift variability and high SNR, the choice of extraction solvents must be evaluate and the
54 fecal weight-to-buffer volume (Wf:Vb) must therefore be optimized.

55

56 In previous studies, different extraction solvents were implied for extraction^{16,18-20} and Wf:Vb ratios
57 of 1:50,¹⁴ 1:5¹⁶, 1:10,²³ 1:2 and an unknown ratio^{15,17,22} have been applied, but none of the studies
58 report the rationale for their choice of solvent and the weight-to-buffer volume. Wu et al.²⁴ have
59 recommended the use of a Wf:Vb ratio of 1:10 and the pooling of the first two extracts. It is also
60 noteworthy that the recommendation (Wf:Vb of 1:10) was solely based on evaluation of chemical
61 shift inconsistency and the experiment was conducted in fecal material from mice. Saric et al.
62 showed that the fecal metabolome varies between species and, more specifically, the human
63 metabolome contained lower levels of lactate and amino acids compared to mice and rats.¹⁹
64 Thereby, this finding urges further investigation of the choice of fecal weight-to-buffer ratio to
65 extract metabolites from fresh human feces in metabolomic analyses. Besides that, the fecal

66 metabolomics studies have focused on the analysis of intracellular bacterial metabolites; therefore,
67 different sample pretreatment techniques have been used to homogenize fecal samples. However,
68 the presence of different residual food components in the feces might affect the extraction.
69 Therefore, it is of interest to compare the effects of sample pre-treatment and the choice of weight-
70 to-buffer ratio during metabolic profiling from fresh human samples. In the present study we aimed
71 to compare the effect of different solvents during extraction, weight-to-buffer combinations on the
72 reproducibility, chemical shift variability, overall metabolite composition and SNR of the NMR
73 spectra from healthy human feces. In addition, we analyzed the consistency of fecal metabolite
74 composition upon different sample treatment techniques (sonication and freeze-thaw cycles) and
75 suggest an optimized protocol for NMR-based metabolomics analyses of human feces.

76 **Materials and methods**

77

78 **Sampling and sample material**

79 Fecal material from nine healthy volunteers was used for the study. The study protocol was
80 approved by the Ethics and Research Committee and conducted according to the guidelines in the
81 Declaration of Helsinki. The fecal material was collected from all subjects in a falcon tube
82 immediately after defecation. The samples were then stored at -20 °C prior to analysis. Initially,
83 fecal samples from four healthy volunteers were used to optimize the fecal-to-water buffer ratio
84 during extraction. Samples from an additional five subjects were included to elucidate the
85 reproducibility of the method and the stability over time.

86

87 **Fecal metabolite extraction**

88 Fecal water was extracted to ratios of 1:2, 1:5, and 1:10 (weight of fresh feces-to-buffer) in 0.75 M
89 phosphate buffered saline (PBS, pH 7.4). The samples were homogenized by whirl mixing for 2
90 min, and then aliquots were centrifuged at 10,000 g for 15 minutes at 4 °C (Eppendorf 5471, USA).
91 The supernatants were carefully removed and stored in Eppendorf tubes at -80 °C until analysis. To
92 determine recovery, feces from one individual (n = 3) was spiked with 100 µL of standards maleic
93 acid, dimethyl sulfone, and succinic acid (0.05 M). Fecal water was extracted to ratios of 1:2, 1:5,
94 and 1:10 (w/v) as described earlier. The recovery values of dimethyl sulfone, and succinic acid were
95 referenced to maleic acid. In addition, to investigate the influence of solvent during fecal
96 metabolites extraction, feces from one individual has been extracted in duplicate with distilled
97 water, D₂O extraction, (adapted from Monleón et al.¹⁶) and using combination of
98 methanol/chloroform/water (section A, supplementary information).

99 **Freeze thaw and sonication treatment**

100 To compare the effect of sample pretreatment on the fecal NMR profile; we extracted feces
101 simultaneously with/without freeze-thaw and sonication treatment. During freeze thaw whirl-mixed
102 samples were frozen to $-18\text{ }^{\circ}\text{C}$ for 10 minutes and immediately thawed at $4\text{ }^{\circ}\text{C}$ for 10 min in
103 Eppendorf tubes (Eppendorf 5471, USA). This treatment was replicated for 3 cycles. Sonication
104 was conducted between each freeze thaw cycle (Branson 5210, USA). The samples were placed in
105 Eppendorf tubes and sonicated for three consecutive cycles of 2 min on and off between each freeze
106 thaw cycle.

107 **pH measurement**

108 The pH values of the fecal extracts diluted to 1:2, 1:5, and 1:10 (w/v) in 0.75 M phosphate buffered
109 saline (PBS, pH 7.4) were measured at room temperature ($22\text{ }^{\circ}\text{C}$) before NMR analysis, using pH
110 electrodes for NMR sample tubes (IKATRON®, Germany).

111 **^1H NMR spectroscopic analyses**

112 Fecal water samples extracted in PBS were thawed and centrifuged at 10,000 g for 15 minutes at
113 4°C (Eppendorf 5471, USA). A volume of 500 μL of clear supernatant was transferred to a 5 mm
114 NMR tube, and 100 μL of deuterium oxide (D_2O) containing 0.025 mg/mL of 3-(Trimethylsilyl)
115 propionic acid- d_4 sodium salt (TSP) was added as a lock solvent. One-dimensional NMR
116 experiments were carried out using a Bruker Avance III 600 MHz spectrometer (Bruker,
117 Rheinstetten, Germany) equipped with a 5 mm triple resonance (TXI) probe at 298 K. A standard
118 Bruker noesypr1d ($90^{\circ}\text{-t}_1\text{-}90^{\circ}\text{-d}_{\text{mix}}\text{-}90^{\circ}\text{-FID}$) sequence was used to suppress signals from water
119 molecules, where t_1 is a 4 μs delay time and d_{mix} is the mixing time. Acquisition parameters for the
120 spectra were 64 scans, a spectral width of 7288 Hz collected into 32K data points, an acquisition
121 time of 2.24 s and an inter-scan relaxation delay of 5 s. The Free Induction Decay (FID) obtained

122 was multiplied by 0.3 Hz of exponential line broadening before Fourier transformation. The spectra
123 were referenced to TSP (chemical shift 0 ppm), phased, and baseline corrected in Topspin 3.0
124 software (Bruker, Rheinstetten, Germany). SNR was measured using the built-in graphical interface
125 in Topspin 3.0, where alanine (1.47 ppm) was considered to be the signal, and spectra at lower
126 frequency than 0 ppm were considered to represent the noise region. Relative quantification of
127 selected ^1H resonance was performed by integration of peak areas using Topspin 3.0 (Bruker).
128 Assignments of ^1H NMR signals were carried out using Chenomx NMR Suite 7.7 (Chenomx,
129 Canada) according to the Human Metabolome Database,²⁵ and literature.^{14,18,26} In addition,
130 heteronuclear single quantum coherence (HSQC) and total correlation spectroscopy (TOCSY) 2D
131 NMR techniques were applied to confirm the identity of the assigned metabolites (see Figures S1 to
132 S6 in supplementary material). The HSQC experiment was acquired with a spectral width of 7288
133 Hz in the ^1H dimension and 27164 Hz in the ^{13}C dimension, a matrix with size of 4096 $\times$ 1024, 512
134 transients per increment and a relaxation delay of 2 s. The TOCSY spectrum was acquired with
135 acquired with a spectral width of 7288 Hz in the both ^1H dimension, a matrix with size of
136 2048 $\times$ 512, and an inter-scan relaxation delay of 2s. In addition, two-dimensional NMR
137 experiments were carried out using a Bruker Avance III 800 MHz spectrometer equipped with a 5-
138 mm ^1H observe cryoprobe (Bruker Biospin, Rheinstetten, Germany). The HSQC experiment was
139 acquired with a spectral width of 11961 Hz in the ^1H dimension and 33276 Hz in the ^{13}C dimension,
140 a matrix with a size of 4096 $\times$ 4096, 512 transients per increment and a relaxation delay of 1.5 s. The
141 TOCSY spectrum was acquired with a spectral width of 9578 Hz in both ^1H dimensions, a matrix
142 with a size of 8192 $\times$ 1536, and an inter-scan relaxation delay of 1.8 s.

143

144 **Multivariate Data analysis**

145 ¹H NMR spectra were imported to Matlab R2010b (The Mathworks, Inc., USA), and the
146 misalignments of the spectra were corrected using the icoshift algorithm, based on the correlational
147 shifting of spectral intervals.²⁷ The spectrum with the highest correlation to the rest of the spectra in
148 the matrix was used as a reference. The 9.5 to 12 ppm and 0.5 to -1.2 ppm regions and the region
149 containing residual water resonance (4.8. to 4.7 ppm) were removed from the aligned spectra. The
150 spectra were normalized to unit area and pareto-scaled before principal component analysis (PCA)
151 using the PLS Toolbox (Eigenvector Research, USA) in MATLAB20010b.

152

153 **Results and discussion**

154 The ^1H NMR spectra obtained from fresh fecal samples revealed the presence of a wide range of
155 metabolites. Figure 1 shows a representative spectrum of a fecal sample extracted in PBS buffer.
156 The obtained ^1H NMR spectra contained resonances from short chain fatty acids (predominantly
157 acetate, propionate, and butyrate), branched-chain fatty acids (iso-valerate, iso-butyrate), biogenic
158 amines (trimethylamine and dimethylamine), organic acids (succinate, fumarate), carbohydrates
159 (glucose predominant), and amino acids (leucine, isoleucine, valine, tyrosine, phenylalanine, and
160 others). Figure 1 and the numbering scheme outlined in Table 1 summarize the assigned resonances
161 of the fresh fecal samples. To the best of our knowledge, we report here for the first time signals
162 from the metabolites xylose, arabinose, nicotinate, orotic acid, malic acid, xanthine, thymine, and
163 acetoin in the fecal extract using ^1H NMR spectroscopy.

164 **Evaluation of extraction solvents**

165 The influence of solvent: water, D_2O , PBS buffer, and mixed solvent (methanol/chloroform/water)
166 on extraction of the fecal metabolites was evaluated by assessing the metabolite compositions in the
167 NMR profile obtain from the different extracts. This analysis revealed that the signals from n-acetyl
168 compounds, lipid residue, and some unknown anomeric hydrogen in carbon signals 5.0-5.5 ppm
169 were preferentially extracted in the methanol extract (Figure S7, supplementary information). In
170 contrast, the resonance from trimethylamine appeared only in the ^1H NMR spectra of the extracts
171 from distilled water, D_2O , or PBS buffer. Signals from SCFA, amino acids, and organic acids were
172 similar in the four extracts, however, recoveries differed.

173 The methanol/chloroform/water extraction is time-consuming, labor-intensive, expensive, and toxic
174 (hazardous) compared to the one-solvent extractions. As PBS buffer extraction reduces inter-
175 sample pH variation during extraction, we suggest PBS buffer as the best choice for extraction

176 solvent. However, the lipid-containing organic (chloroform) phase in the
177 methanol/chloroform/water provides complementary information about the non-polar metabolites in
178 the feces, which presently has not been investigated thoroughly by using NMR-based
179 metabolomics. The NMR signals recorded on chloroform phase in the non-polar extracts were
180 assigned to different lipid classes/functional groups. Our result indicates the presence of unsaturated
181 fatty acids, triacylglycerides, glycerol backbone of phospholipid, phosphatidylcholine, alkyl chains,
182 and acyl saturated chains in the proton NMR spectra of the lipid extracts. These signals
183 predominantly from the saturated and unsaturated fatty acids are comparable to the lipid signals in
184 the other body fluid such as plasma and the tissue samples. Assignments of ^1H NMR lipid signals
185 were carried out using the American Oil Chemist Society (AOCS) Lipid library ²⁸ and literature.
186 ^{29,30} In future, examination of these fatty acid compositions in feces might have great potential to
187 reveal mechanistic interplay between the gut microbiota and lipid metabolism.

188

189 **Effect of varying the Wf:Vb ratio during extraction**

190 Theoretically, concentrated samples are beneficial in NMR spectroscopy as SNR will increase with
191 increasing amount of compound. However, in metabolomics concentrated samples at the same time
192 retain a challenge as they potentially may lack signal stability due to pH variations and
193 intermolecular interactions. Therefore, in the present study the possible tradeoffs of chemical shift
194 stability and signal intensity were investigated in a detailed comparison of three buffer ratios
195 (dilutions) and two sample treatments. Three different buffer dilutions (1:2, 1:5, and 1:10) and the
196 effect of sample treatments (sonication and freeze-thaw cycles) during fecal metabolite extraction
197 were evaluated. The effects of sonication and freeze-thaw cycles were less pronounced when
198 compared to the effect of varying buffer concentration on the fecal metabolite extraction (Figure 3).
199 The signal to noise ratio (SNR) measured from the fecal extracts with different Wf:Vb ratios clearly

shows that 1:2 extracts have higher SNR in comparison to 1:5 and 1:10 extracts (Figure 3), which can be ascribed to the dilution of metabolites during extraction in the 1:5 and 1:10 samples. Visual inspection of the spectra (scaled by the dilution factor) showed that the intensities of the metabolites and the noise in the spectra were different in the 1:2 extract when compared to the 1:5 and 1:10 extracts (Figure 4). The signals from nicotinate, UDP glucose, tryptophan, bile acids are masked by higher contribution from noise in the 1:5 and 1:10 extracts compared to the 1:2 extract (Figure 4). In contrast, the signal from the n-acetyl compound had lower signal intensity in the 1:2 extract compared to 1:5 and 1:10 (data not shown). The lower intensity of n-acetyl compound in 1:2 extract can likely be ascribed to saturation of metabolites during extraction. In addition to signal intensity of metabolites in the NMR spectrum, the recovery of a metabolite during extraction is essential. Consequently, in order to measure recovery, feces were spiked with maleic acid, dimethyl sulfone, and succinic acid. The results showed that the recovery yield of these standards is minor influenced by dilution factor (Table 2). Nevertheless, the overall results implicate that the Wf: Vb affects the detection and quantification of certain metabolites when extracted with a high buffer volume (Figure 4). Thereby, based on our finding it is likely that proper choice of solvent and the weight-to-buffer volume during feces extraction might have an impact in the outcome/understanding of the nutritional metabolomics study.

217

218 **Inter-sample pH consistency**

219 In general, the pH of human feces can range from 6.8 to 7.0, and the variation usually depends on
220 diet, xenobiotic intake and/or the health status of the individual. For effective subsequent data
221 mining, inter-sample pH consistency remains a critical problem because this variability can result in
222 chemical shift variations of the metabolites. Although some peak alignment techniques can
223 minimize the chemical shift inconsistency in the data after NMR acquisition, chemical shift

224 variations still create ambiguity for overlapping and unknown signals.³¹ Therefore, the most
225 efficient strategy is to minimize such inter-sample pH variations during sample preparation i.e.,
226 prior to data acquisition. In the present study, we measured the inter sample pH variation for the
227 three different buffer concentrations (1:2, 1:5, and 1:10) and sample treatments (sonication and
228 freeze-thaw). Differences in pH between the samples due to sample treatment were minor in
229 comparison with the effects of the Wf:Vb ratio of buffer (Figure S8, supplementary information).
230 The pH of the fecal sample increased with an increase in the Wf:Vb ratio of buffer. The samples
231 extracted with a Wf:Vb ratio of 1:10 and 1:5 showed more stable pH in the range of 7.30 to 7.45,
232 while the samples extracted with a 1:2 Wf:Vb ratio varied in pH from 7.20 to 7.40. Even though the
233 pH was marginally more stable between the samples extracted with 1:5 and 1:10 Wf:Vb ratios, the
234 SNR was reduced more than 2-fold and, the variations in the chemical shifts for the samples
235 extracted at 1:2 were sufficiently low to be easily overcome by the application of an alignment
236 method such as ico-shift,²⁷ FOCUS (based in the Recursive UN referenced Alignment of Spectra
237 algorithm)³² or binning of the sample during data pre-processing (Figure S9, supplementary
238 information). For the purpose of NMR-based metabolomics of the feces, it is essential to extract all
239 the metabolites from fecal samples with substantially higher signal intensity, i.e., reasonable SNR.
240 Thus, it was concluded that a Wf:Vb ratio of 1:2 is the optimum ratio during extraction under the
241 conditions tested. It can be expected that the optimum Wf:Vb ratio will depend on the physico-
242 chemical properties of the fecal material. It is therefore recommended to use the Bristol stool
243 scaling (BSS)³³ of the feces prior to analysis; so that the physico chemical properties of the stool
244 samples are taken in consideration.

245

246 **Reproducibility**

247 To address the suitability of a methodology to profile metabolites in metabolomics studies, the
248 reproducibility of the method should be evaluated. Pareto-scaled normalized NMR spectra obtained
249 at three different time points from 5 individuals (extracted with a Wf:Vb ratio of 1:2) were analyzed
250 by multivariate data analysis to elucidate the reproducibility of the method. The PCA score plot in
251 Figure 5 shows that the individual samples (represented by the colors) were clustered together
252 depicting the reproducibility of the extraction methodology. In addition, the data from this study
253 also revealed distinct inter-individual variation supporting the fact that the sample preparation
254 method was capable of detecting and identifying metabolic differences among individuals.
255 Intriguingly, the samples from two subjects (depicted as green and cyan) did not cluster together as
256 observed for the other three subjects (Figure 5). During extraction, the presence of undigested food
257 residuals were clearly observed in these samples compared to the rest of other samples. The
258 presence of these food residuals could affect the NMR profile of the fecal extract. Therefore, it may
259 be worthwhile to perform BSS which could for instance indicate that the observed differences
260 within the samples may be due to larger variation in the stool consistency of the individuals.

261

262 To eliminate inter-individual differences and assess the reproducibility performance of the
263 metabolites, extraction was done in triplicate using different Wf: Vb ratio (1:2, 1:5, and 1:10) from
264 the feces of same individual. The coefficient of variation (% CV) of eleven different metabolites
265 was determined (Figure 6). The majority of the metabolites show low and similar % CV
266 independent of the buffer dilutions. However, fumarate ($\approx 55\%$) and formate ($\approx 18\%$) displayed
267 considerably higher % CV when extracted at 1:5 and 1:10 dilution ratio respectively, when
268 compared to metabolites extracted with the 1:2 dilution ratio. This could possibly be ascribed to the
269 difference in the pH between the samples extracted in Wf: Vb ratio of 1:10 and 1:5, and 1:2 as seen

270 previously. This difference most probably affected the recoveries of fumarate and formate,
271 indicating they were preferentially extracted in lower pH. Considering the recovery would be
272 different for specific class of compound, the main purpose for metabolic profiling/metabolomics
273 study is to obtain reproducible spectra that can be on compared with each other. Intriguingly, based
274 on our results, it is also noteworthy that Wf: Vb ratio of 1:2 showed reproducible result during
275 extraction under the conditions tested. Thus, we suggest that the Wf: Vb ratio affects the
276 reproducibility of certain metabolites when extracted with a diluted buffer.

277

278 **Fecal NMR profile stability study**

279 The NMR experiments may last from a few minutes to hours of acquisition time. During NMR
280 acquisition, the metabolites might change over time due to either enzymatic or non-enzymatic
281 processes. Thus, it is essential to elucidate spectral changes that might occur over time. Therefore,
282 ¹H NMR spectra were acquired over a period of 12 hours by repeating the acquisition at regular
283 intervals of 2 hours (Figure S10 and S11). The obtained spectra were analyzed by overlaying the
284 spectra from all acquired time points. The results showed that the metabolite profile of the fecal
285 extracts remained stable over the 12 h period.

286

287 **Conclusions**

288 Fecal-based metabolomics is a rapidly emerging discipline. An optimized sample preparation is
289 crucial for metabolomics studies, and here we suggest buffer extraction might be the most suitable
290 approach to extract metabolic information in the feces. The study demonstrates that a Wf:Vb ratio
291 of 1:2 might be optimal quality for ¹H NMR spectroscopic data for metabolomics data of human
292 feces, while the effects of sonication and freeze-thaw were less pronounced of the condition tested.

293 More than 60 metabolites have been assigned from the ^1H NMR spectra obtained, and to our
294 knowledge, some of the metabolites are reported here for the very first time using NMR spectra
295 acquired on fecal extracts.

296

297 **Acknowledgement**

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302 800 MHz spectra NMR spectra were obtained at the Danish Instrument Center for NMR
303 spectroscopy of Biological Macromolecules.

304 **Supporting Information Available**

305 Supplementary method section on metabolite extraction methods and Figure S1 to S11. This
306 information is available free of charge via the Internet at <http://pubs.acs.org/>

307

Figure Captions

Figure 1. Representative ^1H NMR spectrum of a human fecal sample extracted in PBS buffer. A) 0.7 to 4.55 ppm; B) 4.9 to 8.55 ppm C) 8.6 to 9.8 ppm. The aromatic region in the spectrum (8.6 to 9.8 ppm) has been magnified three times as compared to the region B. The inserts show additional signals detected in spectra obtained on other human fecal samples. Keys to the figure are given in Table 2.

Figure 2. Representative ^1H NMR spectrum of a human fecal sample extracted in organic (chloroform) phase in the methanol/chloroform/water extract. A) 5.7 to 4.7, double bond section; UFA = unsaturated fatty acids; TAG = triacylglycerides, Glyc-B = glycerol backbone of phospholipid. B) 4.4 to 2.8 regions; PC = phosphatidylcholine. C) Alkyl and Acyl saturated chains

Figure 3. Average SNR ratios from the fecal extracts with different Wf: Vb and obtained with sonication and without sonication, respectively (n=4). The alanine signal at 1.44 ppm was used for the calculation of SNR. Error bars show standard deviations.

Figure 4. Representative ^1H NMR spectra of human fecal extracts with varying Wf: Vb ratios A) 1:10 B) 1:5 C) 1:2.

Figure 5. PCA scores plot showing reproducibility for fecal extracts with Wf: Vb ratio 1:2. Each symbol represents an individual. The number 1, 2, 3 illustrates the samples from same individual collected during 3 different time periods.

Figure 6. The coefficient of variation (% CV) of eleven different metabolites from NMR measurement of the fecal extract (three different buffer dilutions 1:2, 1:5, and 1:10).

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Figure 6.

Table 1. ¹H NMR Chemical Shifts and Assignments of Metabolites Detected in Human Fecal Extracts

No.	Compound	Proton Chemical shift in ppm and multiplicity
1	2-methyl butyrate ^a	0.86 (t), 1.05 (d), 1.38 (m) , 1.5 (m), 2.21 (m)
2	caprylate	0.85 (t), 1.27(m), 1.53 (m), 2.16(t)
3	valerate	0.88 (t), 1.31(m), 1.54 (m), 2.18 (m)
4	n-butyrate	0.91 (t), 1.56(t), 2.16 (t)
5	leucine	0.96 (d), 0.97(d), 1.7 (m)
6	iso-leucine	0.94 (t), 1.02 (d), 1.27 (m)
7	valine	0.99 (d), 1.05(d), 2.3 (m)
8	propionate	1.06 (t), 2.19 (m)
9	methyl succinate	1.08 (d), 2.11 (dd), 2.51 (d), 2.61 (m)
10	iso butyrate	1.12 (d), 2.40 (m)
11	ethanol	1.18 (t), 3.6 (q)
12	3-methyl 2oxovalerate ^a	0.89 (t), 1.10 (d), 1.46 (m), 1.70 (m), 2.93 (m)
13	threonine	1.33 (d), 3.57 (d), 4.24 (m)
14	lactate	1.31 (d), 4.1 (q)
15	acetoin	1.37 (d), 2.20 (s), 4.42(q)
16	iso-caproate	0.88 (d), 1.45 (m), 1.49 (m), 2.19 (t)
17	alanine	1.48 (d), 3.78 (q)
18	cadavarine	1.46 (m), 1.73 (m), 3.02 (m)
19	lysine	1.40 (m), 1.70 (m), 3.03 (t), 3.72 (t)
20	unknown singlet	1.81 (s)
21	thymine	1.87 (s), 7.39(s)
22	arginine	1.68 (m), 1.91 (m), 3.23 (t), 3.76 (t)
23	acetate	1.92 (s)
24	n-acetylglutamate	2.02 (s), 2.05 (m), 4.1 (m)
25	iso-valerate	2.06 (d), 1.98 (m), 0.91 (d)
26	glutamate	2.14 (m), 2.46 (m), 3.78 (m)
27	succinate	2.41 (s)
28	glutamine	2.12 (m), 2.44 (m), 3.75 (t)
29	5 aminopentionate ^a	3.02 (t), 2.21 (t), 1.69 (m), 1.65 (m)
30	methionine	2.15 (m), 2.63 (t), 3.85 (dd)
31	sarcosine ^a	2.72 (s), 3.6 (s)
32	aspartate	2.69 (dd), 2.82 (dd), 3.91 (dd)
33	trimethylamine	2.87 (s)
34	3-phenylpropionate ^a	2.87 (t), 2.47 (t), 7.26 (t), 7.32 (d), 7.36 (t)
35	malonate	3.11 (s)
36	choline	3.27 (s), 3.53 (m)
37	proline	1.98 (m), 2.06 (m), 2.34 (m), 3.32 (dt), 3.34 (m)
38	ornithine	1.72 (m), 1.82 (m), 1.93 (m), 3.03 (t), 3.77 (t)
39	glucose	3.40 (t), 3.53 (dd), 3.71 (t) 3.73 (dd), 3.83(m), 5.24(d)
40	glycerol	3.64 (m), 3.78 (m)
41	glycine	3.57 (s)
42	ribose	3.52 (m), 3.60(m), 3.91 (m), 4.10 (m), 4.20 (dd), 4.92 (d), 5.25 (d),

43	dihydroxyacetone	4.41(s)
44	xylose	5.19 (d), 4.57 (d), 3.92(dd), 3.89(dd), 5.18(d), 3.31(d)
45	galactose	3.54(dd), 3.65(dd), 3.72(m), 3.82(m), 4.06(t), 5.26(d)
46	uracil	5.78 (d), 7. 49 (d)
47	fumarate	6.52 (s)
48	tyrosine	6.91 (d), 7.20 (d)
49	histidine	3.16 (dd), 3.23 (dd), 3.98 (dd), 6..99 (d), 7.83 (s)
50	phenylalanine	3.54 (s), 7.31 (m), 7.37 (m)
51	xanthine	7.94 (s)
52	hypoxanthine	8.12 (s), 8.21 (s)
53	formate	8.46 (s)
54	nicotinate	7.56 (dd), 8.28 (m), 8.66 (dd), 8.94 (d)
55	p-cresol	2.25 (s), 6.87 (m), 7.18(d),
56	putresine	1.77 (m), 3.06 (m)
57	urocanic acid ^a	6.38 (d), 7.27 (d), 7.87 (s)
58	dimethylamine	2.74 (s)
59	3 hydroxyphenylacetate ^a	3.56 (s), 6.93 (m), 7.21 (m)
60	tryptophan	7.19 (m), 7.31 (m), 7.53 (d), 7.76 (d)
61	bile acid	0.78 (m)
62	UDP glucose ^a	5.62 (dd), 6.0 (m),
63	glutarate	1.8 (t), 2.19 (t)
64	malic acid	2.33 (dd), 2.65 (dd), 4.28(dd)
65	orotic acid	6.19 (s)
66	n-acetyl group's	2.04 (s).7,88 (s)
67	ethanolamine	3.12 (t), 3.80 (t)
68	allantion ^a	5.38 (s)
69	glycerophosphocholine	3.19 (s)
70	arabinose	5.24(d), 4.52 (d), 3.95(m), 3.89(m), 3.52(dd)

^a Assignment with best matched signals

Table 2. Comparison of Yields of Standard Compounds with Different Wf: Vb during Extraction

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Standards	Wf:Vb	Wf:Vb	Wf:Vb
	1:2	1:5	1:10
Sucinate	3.06±0.02	3.11±0.04	3.06±0.09
Dimethylsulphone	3.51±0.05	3.48±0.02	3.50±0.05

Means (±) SD determined from the integrals of the compounds relative to that of maleic acid

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