Validation and application of an ultrahigh-performance liquid chromatographic-Orbitrap mass spectrometric method for the simultaneous detection and quantification of volatile and non-volatile organic acids in human faecal samples

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Abstract

A simple and selective ultrahigh-performance liquid chromatographic-Orbitrap mass spectrometric (UHPLC-HR-MS) method was developed and validated for the simultaneous detection and quantification of short-chain fatty acids (SCFAs) such as acetic, propionic, butyric, isobutyric, valeric, isovaleric, 2-methyl-butyric (IS) and lactic, pyruvic and succinic acid in human faecal samples. A simple extraction procedure with 0.001 % formic acid in water was performed on 40 samples. The extracts were centrifuged and analyzed by UHPLC-HR-MS on a sub-2 µm column using gradient elution; meanwhile, the same samples were analyzed by GC-FID and HPLC-UV as reference methods. The UHPLC-HR-MS method showed a recovery of 83-105 %, a repeatability of 2.2-8.3 % and an intermediate precision of 2.9-9.4 %. The LOD and LLOQ were in the range of 0.04-0.23 and 0.2-0.5 µg/ml, respectively. Regarding the SCFAs, statistical analysis showed a good correlation between the data obtained by UHPLC-HR-MS and those provided by GC-FID ($p > 0.05$). On the contrary, the LC-UV data were not in agreement with those obtained by UHPLC-HR-MS determination ($p < 0.05$). To the best of our knowledge, this is the first method available for the simultaneous extraction and quantification of SCFAs, lactic, pyruvic and succinic in faecal samples by UHPLC-HR-MS.

Keywords: Short-chain fatty acids; organic acids; UHPLC; high-resolution mass spectrometry; faeces; humans

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1. Introduction

The intestinal microbiota has been proposed as an additional organ of the human body that performs numerous functions, ranging from immunomodulation to improvement of nutrient bioavailability and competitive exclusion against potential detrimental microorganisms [1]. Therefore, the modification of the intestinal microbial ecosystem may potentially induce functional changes that could affect the health of the host with the consequent onset of pathologies. Irritable bowel syndrome (IBS) is one of the most common gastrointestinal disorders in the industrialized World [2]. The prevalence of IBS is approximately 10-15% [2]. The diagnosis is based on internationally accepted symptom-based criteria after exclusion of organic diseases [3]. Extensive research has been carried out to find valid and reliable biomarkers for IBS, but so far, none has been judged as satisfactory for the use in daily practice [4]. The association between IBS and gut microbiota has been demonstrated in several studies and efforts have been made to characterize the abnormal microbiota in patients with IBS [5]. The results seem inconsistent but some microbiota metabolites such as short-chain fatty acids (SCFAs), which have extensive immunological and regulatory functions, appear to be the link in the host-microbe interactions [6]. The relation between SCFAs and functional bowel disorders has not been extensively investigated, but immunological activation detected in subjects with IBS could be associated with the changes in SCFAs [7- 9]. Approximately 83% of SCFAs in the human colon is represented by butyric, acetic and propionic acid [10]. Their concentration in the intestinal lumen ranges from 60 to 150 mmol/kg [11], and the acetate-propionate-butyrate balance is relatively constant, with a typical ratio of 60:25:10 [12]. Among these, butyrate appears to be a potential new IBS therapy [10, 13-14]. In order to isolate SCFAs and other organic acids from complex matrices such as faeces, several sample preparation procedures have been developed and applied including filtration [15], liquid-liquid [16] or solid-phase extraction [17]. The

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determination of SCFAs in faecal samples is generally performed by gas chromatography in combination with flame-ionization [18] or MS detection without [19] or with derivatization to improve chromatographic behaviour or to increase molecular weight [20]. Alternatively, SCFA determination can be performed by capillary electrophoresis [21] and liquid chromatography coupled to refractive index [22-23], conductivity [24], electrochemical [15], spectrophotometric [25-26] or mass spectrometer [27] detector. More recently, Raposo and colleagues [23] published a multi-laboratory study regarding the quantitative determination of volatile fatty acids in aqueous matrix by GC-FID and HPLC coupled to conductivity, RI and UV. Short-chain fatty acids have no chromophore group, thus their detection occur only at short ultraviolet wavelengths (200-210 nm), and this can compromise method selectivity. To overcome this problem, several methods have been developed including derivatization of the organic acids with consequent formation of products absorbing at higher UV wavelengths [28]. This increases the specificity but also the time-consuming.

The aim of this study was to develop a simple and cost-effective method based on UHPLC coupled to Orbitrap Mass Spectrometer (HR-MS) for the quantification of SCFAs (C2-C5) and other organic acids such as lactic, pyruvic and succinic in faecal samples. The selected analytes and their deprotonated ions are shown in Table 1. The increases specificity and sensitivity of HR-MS has allowed simplifying the sample preparation procedure permitting the routine use of the developed and validated UHPLC-HR-MS method. Moreover, the method was applied for the quantitative determination of SCFAs and organic acids in human faecal samples, and the observed outcomes were compared with those obtained by using LC-UV and GC-FID assay method reported in literature.

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2. Experimental

2.1 Chemicals

Analytical grade pyruvic (Pyr), lactic (LA), acetic (AA), succinic (SA), propionic (PA), methyl-malonic (MMA), butyric (BA), isobutyric (iBA), 2-methylbutyric (2-MBA, IS), valeric (VA), isovaleric (iVA), oxaloacetic (OxA) and acetoacetic (AcA) acid were used as standards (Sigma-Aldrich, Milan, Italy), and their purity was higher than 98%. Acetonitrile, methanol and formic acid LC-MS grade were from Sigma. Water was obtained from a MilliQ apparatus (Millipore, Milford, MA). Human faecal samples (n=40) were a kind gift of prof. Guglielmetti (University of Milan, DeFENS).

2.2. Sample preparation

Approximately 100 mg of faecal sample was suspended in 2 ml of a solution 0.001 % HCOOH in water containing 2-MBA (IS, 50 µmolar). After vortexing for 1 min, the suspension was centrifuged at 10,000-x g for 1 min, and the supernatant was collected into a 5 ml flask. The residue was treated with 2 ml of a solution 0.001 % HCOOH in water containing 2-MBA (50 µmolar), vortexed for 1 min and centrifuged at 10,000-x g for 1 min. The supernatant was pooled; the volume was adjusted to 5 ml with 0.001 % HCOOH in water containing 2-MBA (50 µmolar) and the solution stored at -20°C until analysis.

2.3. UHPLC-HR-MS determination

The analysis was carried out on an Acquity UHPLC separation module (Waters, Milford, MA, USA) coupled with a model Exactive Orbitrap MS through an HESI-II probe for electrospray ionization (Thermo Scientific, San Jose, CA, USA) set in negative ion mode. The ion source and interface conditions were: spray voltage -3.0 kV, capillary -27 V, tube lens -80 V, skimmer -16 V, sheath gas flow-rate 35, auxiliary gas flow-rate 10, heater and capillary temperature 120 and 320 °C, respectively. A 1.8 µm HSS T3 column (150x2.1 mm,

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Waters) was used for separation at a flow-rate of 0.2 ml/min. The eluents were 0.001% HCOOH in MilliQ-treated water (solvent A) and CH_3OH:CH_3CN (1:1, v/v, solvent B). Five µl of the sample were separated by the UHPLC using the following elution gradient: 0% B for 4 min, 0-15% B in 6 min, 15-20 % B in 5 min, 20 % for 13 min and then return to initial conditions in 1 min. The column and samples were kept at 30 and 10 °C, respectively. The UHPLC eluate was analyzed in full scan MS in the range $(m/z)^-$ 50-150 u. The resolution, AGC target, maximum ion injection time and mass tolerance were 50 K, 1E6, 100 ms and 2 ppm, respectively. The ion with *m/z* 91.0038 and 112.9858 u, corresponding to the formic acid dimer $[2M-H]^-$ and formic acid dimer Na adduct $[2M+Na-2H]^-$, respectively, has been used as lock mass. The MS data were processed using Xcalibur software (Thermo Scientific). Lactic, AA, PA, BA, iBA, VA and iVA mother solutions (1mg/ml) were prepared by dissolving 0.1 ml of standard in 100 ml of water. Mother solution for Pyr, OxA, AcA, MMA and SA was prepared by dissolving 20 mg of standard powder in 20 ml of water. The working solutions in 0.001% formic acid were prepared in the range of 1.9-255 µmolar for PA, BA, iBA, iVA, VA, 4.2-315 µmolar for LA, AA, SA, 5.7-51.1 and 51.1-214.8 µmolar for Pyr (Table 2).

2.4 Method validation

2.4.1 Sensitivity, selectivity and Matrix Effect (ME)

Calibration curves were constructed for each standard at six concentration levels and 2-MBA was used as an internal standard to correct for the loss of analytes during sample preparation. Six independent determinations were performed at each concentration and regression analysis was employed to determine the linearity of the calibration graphs. LLOQ was defined by the lowest injected inter-day concentration whose RSD% resulted to be lower than 20 % [29]. LOD was defined by the lowest concentration the assay can differentiate

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from background levels (S/N ratio > 5). Selectivity was evaluated by comparing the retention time and accurate mass of each analytes in faecal samples, standard solutions and spiked faecal samples. The possible interference from other organic acids such as MMA, OxA and AcA was also evaluated. Matrix effect (ME) was evaluated as described by Garcia-Villalba et al. [19]. Briefly, the response of the standards in 0.001 % HCOOH (0.5-21, µg/ml, n=6) was compared with that of the same compounds in faecal extract spiked with standards at the same concentration levels. Unspiked samples were also analyzed to determine the initial amount of each compound present in the sample. All peak areas were normalized by the area of the IS and the percentage ME was calculated as follows: %ME = [(A-B)/C] x 100

Where, A: Area of the analytes in the post extraction spiked sample, B: Area in the unspiked sample, C: Area in 0.001 % formic acid solution. The ratio of the calibration graph slopes have also been used for the evaluation of the %ME using the following equation: %ME = (m1/m2) x 100. Where, m1: slope of the analytes in the post-extraction sample, m2: slope of the analytes in 0.001 % HCOOH.

2.4.2 Accuracy

In order to optimize the extraction conditions, different quantities of faeces, from 20 to 200 mg, were suspended in 4 ml of a solution 0.001 % HCOOH in water containing IS (5 µg/ml). The suspension was vortexed for 1 min, centrifuged at 10,000-x g for 1 min, and the supernatant was moved to a flask and the volume set to 5 ml by a solution of 0.001 % HCOOH in water containing IS (5 µg/ml). The accuracy of the procedure was determined by a recovery test according to the published method [30]. Briefly, three faecal samples were spiked with different amounts of the analytes (2-10-20 µg). The spiked samples were extracted under optimised conditions, and the recovery rates calculated. Each sample was

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extracted and analysed in triplicate. Every ten analysis, a standard solution was injected to verify the mass spectrometer response.

2.4.3. Precision

Intra- and inter-day precision of the assay were verified by analyzing the same faecal sample four times for five consecutive days. Precision was confirmed by evaluating standard deviations of the amounts and of retention times.

2.4.4. Robustness study

Two analysts evaluating the amounts of the analytes in a faecal sample estimated the ruggedness of the proposed UHPLC-HR-MS method. Each analyst performed twelve tests and standard and extract solutions were injected in triplicate. Robustness was estimated by varying several chromatographic conditions such as flow-rate -0.05,+0.2 ml/min, column temperature ±5 °C, organic strength 0-0.1 % HCOOH in water, pH 3-7, capillary voltage ±3 V, skimmer ±2 V and tube lens ±5 V. Data were analyzed by Wilcoxon test considering significant a level of $p>0.05$.

2.4.5. Stability studies

The faecal and standards solutions were stored at -20 °C and 4 °C, respectively, and their stability evaluated up to 30 days. The 24-h stabilities of all analytes were measured in three faecal extracts and standard solutions at the UHPLC-HR-MS autosampler temperature of 10 °C.

189 2.5. Quantitative analysis of SCFAs and organic acids by reference methods

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191 *2.5.1. LC-UV analysis*

192 Quantification of SCFAs in faecal samples was carried out using external calibration
193 standard curves method. Six levels of concentration ranging from 0.3 to 10.4 mg/ml were
194 used.

195

196 *2.5.2. GC-FID analysis*

197 Faecal samples were extracted and analysed according to Zhao et al. [18]. Calibration
198 solutions were in the range 0.1-5.2 mg/ml.

199

200 2.6. Statistical analysis

201 Wilcoxon and t test was performed by Statistica 10 software (Statsoft Inc., Tulsa, OK, USA)
202 and other calculations were carried out using Excel software. Analytes amount were
203 expressed as µmoles/100 mg of wet faeces.

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3. Results and discussion

3.1 Optimization of UHPLC-MS conditions

In a preliminary phase, different sub-2μm columns (150x2.1 mm, C_{18} and PFP) were tested in order to optimise the condition of separation. The columns tested were from Waters (BEH, BEH Shield, HSS, HSS-SB and HSS-T3), Phenomenex (Kinetex, Kinetex-XB and Kinetex PFP) and Thermo (Hypersil Gold and Gold PFP). The separation efficiency and peak shape obtained through the C_{18} columns were better than the PFP columns and for the separation of SCFAs, pyruvic, lactic and succinic acid, the HSS T3 C_{18} column achieved the best performance. Regarding MS conditions, the determinations were carried out in negative ion mode, as the analytes signal was much higher than that in positive ion mode. For all the analytes, the MS analysis showed the presence of deprotonated molecular ion $[M\text{-}H]^-$. Critically remarkable, for SA and MMA an ion with *m/z* of 72.0295 u, corresponding to $[M\text{-}CO_2\text{-}H]^-$, was present even at lower voltage. This indicates that these compounds could easily lose a carboxyl group giving PA (see Supplementary material 1). Consequently, chromatographic separation is mandatory to avoid an overestimation of the amount of PA in the faecal specimen. In the chromatographic conditions used, methylmalonic, SA and PA were well separated and their retention time was 6.1, 7.4 and 10.3 min, respectively. Oxaloacetic acid also easily lost a carboxyl group giving Pyr (see Supplementary material 1). Thus, also in this case the chromatographic separation was mandatory. Pyruvic and OxA had retention time of 4.5 and 9.1 min, respectively. Acetoacetic acid did not produce interfering. Therefore, the obtained data seem to suggest that some dicarboxylic acids, losing spontaneously a carboxyl group, may interfere with the quantification of some SCFAs, LA and Pyr.

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3.2 Sample preparation for UHPLC-HR-MS analysis

Two subsequent extractions were preliminarily applied to extract the analytes present in the faecal samples. The extracted amount following the first and second extraction was 97.1±1.1 and 2.9±0.4 %, respectively. Thus, for the routine analysis one extraction (5 mL) was performed. The extraction procedure was linear in the range of 20-200 mg of faeces, thus about 100 mg were used for the routine analysis of SCFAs and organic acids. When this amount was not available, the extraction was carried out in order to obtain a suspension containing 20 mg faeces/ml.

3.3 Method validation

3.3.1 Sensitivity, selectivity and matrix effect

The calibration curves showed a coefficient of determination (R^2) ranging from 0.9981 to 0.9992. Analytes had a narrow linear range (approximately 0.032-0.185 mMolar) which encompassed the amounts present in most samples. Only for some samples containing high concentrations of acetic acid was necessary a 10-fold dilution.

Selectivity was confirmed by comparing the retention time of each analyte in standard solution with those of faecal extracts and extracts spiked with standards. The %RSD of the retention times was lower than 0.9%. Peak identity was also verified evaluating the $[M-H]^-$ with a tolerance of 2 ppm. The method of standard addition was used to correct the loss of analytes during sample preparation and their quantification performed by external calibration.

Percentage ME values calculated with peak area and calibration slopes method were in the range of 93-105 % and 0.93-1.05, respectively. Thus, the interference of the matrix with the analytes signal in the faecal samples was not reliable.

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255 *3.3.2 Accuracy*

256 The percentage mean recovery values of the extraction for the analytes from spiked faecal
257 samples was in the range of 83-105 % and the %RSD was lower than 8.7 (Table 2).

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259 *3.3.3 Precision*

260 The intra- (n=4) and inter-day precision (n=5) was evaluated analyzing in triplicate the
261 spiked samples. Repeatability and intermediate precision resulted to be in the range of 2.2-
262 8.3 % and 2.9- 9.4 %, respectively (Table 2).

263

264 *3.3.4 Robustness study*

265 Regarding robustness, slight variations in pH and column temperature do not change the
266 peak shape and resolution, and moderate variations in capillary (±3V), skimmer and tube
267 lens voltage did not influence significantly ($p>0.5$) the quantification of the analytes. On the
268 contrary, increments of the flow-rate or the percentage of formic acid in the eluent caused a
269 drastic decrease of the peak relative to AA and PA and a more modest reduction of the signal
270 related to other SCFAs. In particular, an increase of 0.003 % in the percentage of formic acid
271 caused a reduction of the peak intensity for AA and PA of 70 and 50 %, respectively; while
272 for BA and iBA the reduction was about 25 %. To achieve a significant reduction of the
273 peaks of VA and iVA, formic acid concentration was increased up to 0.005 %. For Pyr, LA
274 and SA, the augment of the percentage of formic acid in the eluent caused an improvement
275 of the shape of the peak and an increase of the response. A reduction in the percentage of
276 formic acid in the eluent or the replacement with water caused a broadening of the peak
277 shape and a decrease of its intensity. Regarding flow-rate, an increase caused a loss of
278 resolution for AA, Pyr and LA, which resulted not base-separated and the intensity of the
279 signal relative to AA and PA decreased up to 50 and 30 %, respectively. Thus, the proposed

method was found to be robust and rugged but particular attention should be paid to the formic acid percentage in the eluent.

3.3.5 Stability studies

The mean of AA and other analytes recovered from the faecal extracts stored at -20 °C for up 30 days was 90 and 95 %, respectively. Standard solutions stored at -20 °C for up 30 days showed a slight reduction of about 1.9 and 1.3 % for AA and other analytes, respectively. All the analytes in standard solutions and faecal extracts resulted stable in the autosampler at 10 °C overnight (RSD<4.3 %).

3.4 Quantitative analysis of SCFAs, LA, SA and Pyr in human faecal samples by UHPLC-HR-MS, GC-FID and LC-UV

The efficiency of the proposed UHPLC-HR-MS method was tested by analyzing the levels of SCFAs, LA, SA and Pyr in faecal samples from humans. Figure 1 shows a typical UHPLC-HR-MS chromatogram of a faecal extract. The separation of all the analytes was completed within 30 min using an UHPLC column with a flow-rate of 0.2 ml/min, which was a balance between ionization and column performance.

In addition, in some faecal extracts were found ions with *m/z* 89.0244 u, corresponding to LA, but with retention times higher than LA (Fig. 1, peak B and C). The peak B spectra showed also the presence of an abundant ion with *m/z* 161.0451 u, which formula brute ($C_6H_9O_5$) corresponds to a dimer of the LA (error <3ppm). The peak C spectrum contains an unidentified ion with *m/z* 301.0543 u. The concentrations of the analytes were all within the linear range for all the samples analyzed and the results are shown in Table 3.

In general, as might be expected, a high inter-individual variability in the content of faecal SCFAs was observed (RSD 33-100 %). The high variability in the levels of the SCFAs are

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likely strongly regulated by many different factors such as diet, environment, microbiota composition and activity, gut transit time and health of the intestine [31-32].

Nevertheless, our data indicated that the most abundant SCFAs detected in the faecal samples were AA, PA and BA, representing approximately 48, 18 and 9 %, respectively, of the total SCFAs in human samples. These data, together with the content of iBA, VA and iVA, are in agreement with those reported in the literature [18-19, 33].

Regarding the comparison with LC-UV and GC-FID method, the amounts of SCFAs did not match those given by the traditional LC-UV analysis ($p<0.05$, Supplementary material 2). Lacking chromophores, SCFAs and organic acids do not have a typical UV spectrum; for this reason, their identification in LC-UV was only based on time retention, which could easily overestimate the actual amount of such compounds in faecal sample.

This could explain the high values of SCFAs in faeces reported by some authors who have carried out the measurement using LC-UV technique. In particular, some authors found about 10-fold higher stool SCFA levels [34] than others. On the contrary, the amounts of SCFAs obtained performing the analysis by HR-MS showed a good correlation with data obtained using GC-FID technique ($p>0.05$). Therefore, the two methods are comparable and the HR-MS technique can be applied for the simultaneous detection and quantification of volatile and non-volatile organic acids in human faecal samples.

4. Conclusion

To the best of our knowledge, this is the first validated UHPLC-HR-MS method for the simultaneous detection of AA, PA, BA, iBA, VA, iVA, LA, Pyr and SA in human faeces. The validation showed good results for recovery, repeatability, LOD, LOQ, linearity and stability. Moreover, the UHPLC coupled with an Orbitrap detector makes the method more sensitive and specific. The method was successfully applied to analyse the faecal samples

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and an internal standard used to assess the loss of analytes during the extraction step. Being easy to use and offering satisfactory chromatographic performances, such procedures make these methods particularly suitable for the routine analysis of SCFAs and organic acids in faecal samples.

Conflict of interest and funding sources

The authors declare that they have no conflict of interest.

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Legends to the figures

Figure 1. Typical UHPLC-HR-MS chromatogram of a faecal extract sample.

The extracted ions are: 1, Lactic $(m/z)^-$ 89.0244; 2, Pyruvic 87.0087; 3, Acetic 59.0138; 4, Succinic 117.0193; 5, Propionic 73.0295; 6, Butyric 87.0451; 7, isoButyric 87.0451; 8, 2-methylButyric (IS) 101.0608; 9, isoValeric 101.0608; 10, Valeric acid 101.0608 u.

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472 **Table 1**. Brute formula and characteristic deprotonated ions $[M-H]^-$ of SCFA, LA, Pyr and

473 SA and possible interfering compounds.

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Analyte	RT (min)	Structure	$[M-H]^-$
Acetic acid (AA)	4.8	CH_3-COOH	59.0138
Propionic acid (PA)	10.3	CH_3-CH_2-COOH	73.0295
Butyric acid (BA)	15.8	CH_3-CH_2-CH_2-COOH	87.0451
isoButyric acid (iBA)	16.0	$(CH_3)_2$-CH-COOH	87.0451
Valeric acid (VA)	26.8	CH_3-CH_2-CH_2-CH_2-COOH	101.0608
isoValeric acid (iVA)	24.5	$(CH_3)_2$-CH-CH_2-COOH	101.0608
Lactic acid (LA)	4.3	CH_3-CH(OH)-COOH	89.0244
Pyruvic acid (Pyr)	4.5	CH_3-C(O)-COOH	87.0087
Succinic acid (SA)	7.4	HOOC-CH_2-CH_2-COOH	117.0193
I.S. (2-MBA)	18.6	CH_3-CH_2-CH_2-(CH_3)-COOH	101.0608
Possible interfering		Structure	$[M-H]^-$
Acetoacetic acid (AcA)	8.1	CH_3-C(O)-CH_2-COOH	101.0244
methyl-malonic acid (MMA)	6.1	HOOC-CH(CH_3)-COOH	117.0193
Oxaloacetic acid (OxA)	9.1	HOOC-C(O)-CH_2-COOH	130.9985

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Table 2. Limit of detection (LOD), linear range, accuracy and precision for the UHPLC-HR-MS analysis of six SCFAs and three non-volatile organic acids in human faeces. Accuracy and precision were evaluated using faeces spiked with three different amounts of standard compounds.

	External calibration[a]		Spiked faecal samples					
Analyte	LOD	Range	LOQ[b]	Added µmoles	Recovery (%)	RSD (%)	Intra-day RSD% (n=4)	Inter-day RSD% (n=5)
Pyr	2.5	5.7-51.1 51.1-214.8	0.027	0.023	94	5.2	4.8	5.4
				0.114	102	4.6	6.8	7.4
				0.227	94	4.4	5.7	6.9
LA	1.9	5.5-210.0	0.026	0.022	94	4.0	3.6	4.4
				0.111	94	3.8	6.7	7.4
				0.222	96	4.0	7.5	8.7
AA	3.8	8.3-315.0	0.039	0.033	83	7.4	2.2	2.9
				0.167	84	6.9	4.7	5.6
				0.333	87	6.4	3.3	4.3
SA	1.3	4.2-160.2	0.020	0.017	91	5.9	6.2	7.2
				0.085	96	5.6	8.3	9.4
				0.169	94	5.5	7.2	8.1
PA	0.7	2.7-255.4	0.013	0.027	92	6.9	3.5	4.1
				0.135	96	6.3	6.3	7.0
				0.270	105	4.2	6.9	6.8
BA	0.5	2.3-214.8	0.011	0.023	96	4.4	2.4	3.0
				0.114	102	3.9	2.7	3.1
				0.227	102	3.6	2.6	3.3
iBA	0.6	2.3-214.8	0.011	0.023	95	8.7	3.2	3.4
				0.114	94	6.3	3.5	3.6
				0.227	100	5.2	3.4	3.8
iVA	0.5	1.9-185.3	0.009	0.020	95	3.2	5.1	5.8
				0.098	102	3.5	4.9	5.7
				0.196	94	3.1	5.7	6.1
VA	0.4	1.9-185.3	0.009	0.020	95	3.6	4.3	4.5
				0.098	92	3.5	4.6	4.1
				0.196	102	3.3	4.7	4.4

[a]µMolar, [b](µmoles/100mg)

The mean value and %RSD were calculated from four measurements. The faecal sample initially contains 0.03, 0.07, 1.31, 0.04, 0.12, 0.81, 0.27, 0.23 and 0.21 µmoles/100mg of Pyr, LA, AA, SA, PA, BA, iBA, VA and iVA.

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484 **Table 3.** Amount and relative percentage of the analytes detected in the human faecal
485 samples (n=40) by UPLC-HR-MS method.

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Analyte	µmoles / 100mg				Relative %	
	min	max	media	s.d.	media	s.d.
Pyr	0.02	0.04	0.03	0.01	0.5	0.3
LA	0.00	0.37	0.07	0.07	1.2	1.8
AA	0.71	10.10	4.24	2.12	48.0	12.2
SA	0.10	1.30	0.21	0.19	2.9	2.0
PA	0.22	5.41	1.70	1.15	18.5	7.0
BA	0.11	4.60	1.20	1.03	11.4	6.7
iBA	0.00	1.10	0.19	0.18	8.9	6.6
iVA	0.20	1.10	0.37	0.19	4.6	2.0
VA	0.10	3.60	1.16	0.85	4.1	2.5

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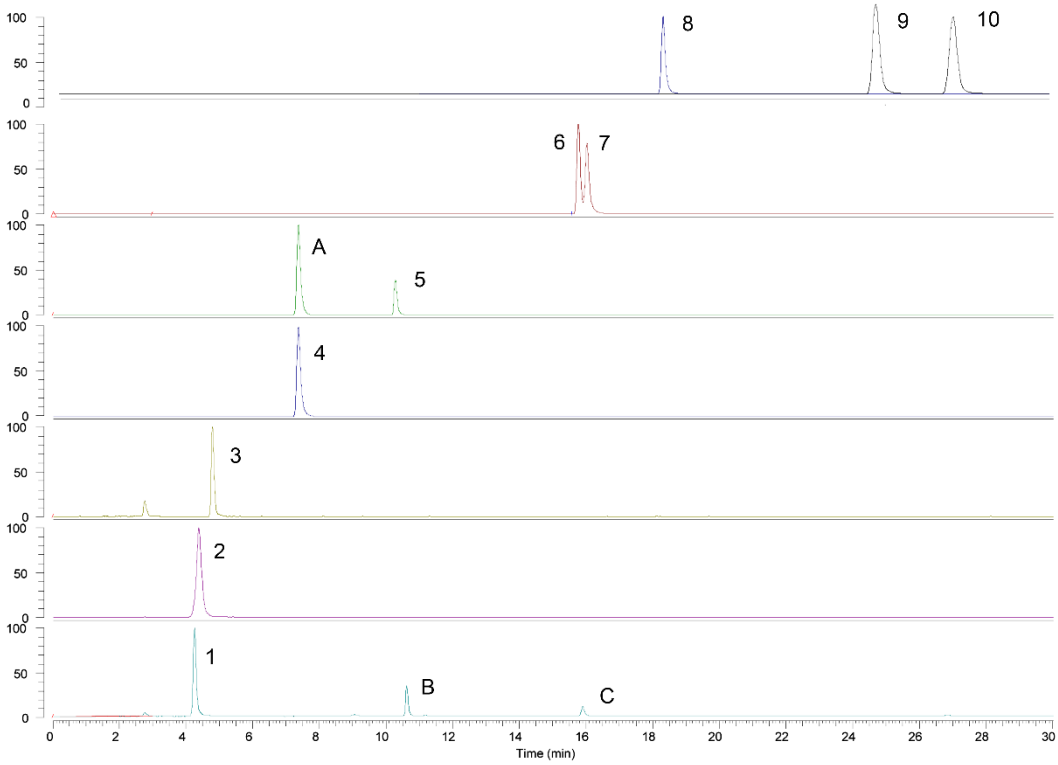
Figure 1



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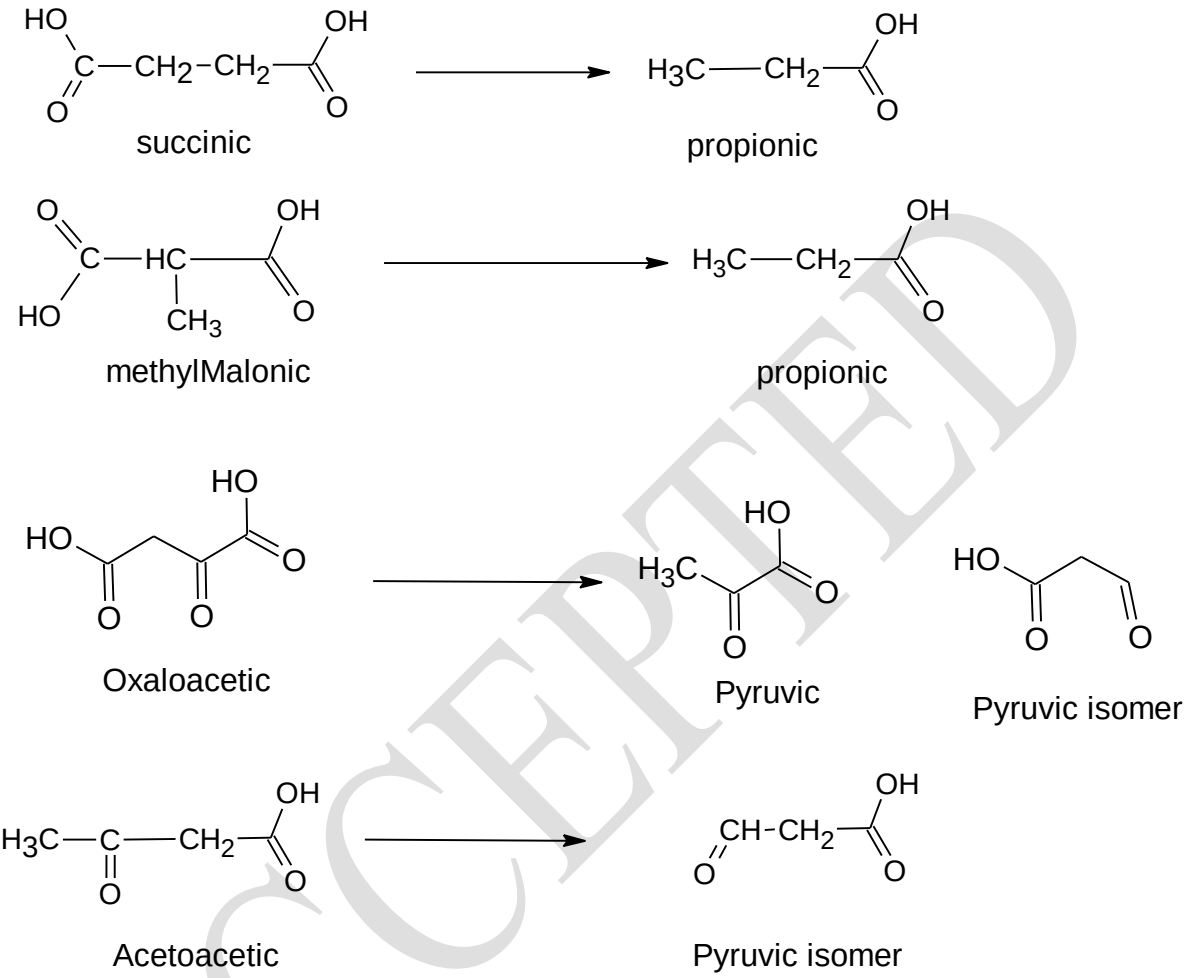
Supplementary 1

Molecular structures and product ions of possible interfering compounds.



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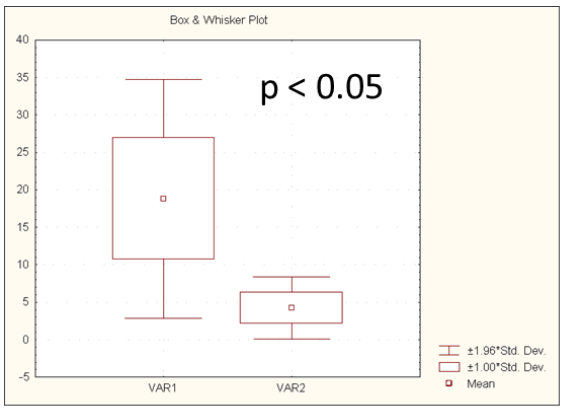
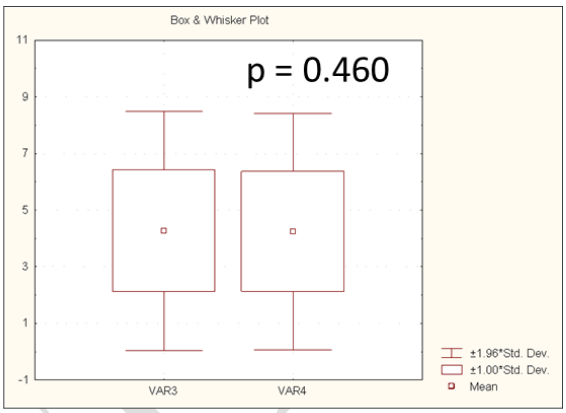
Supplementary 2

Statistical comparison of the results obtained by UHPLC-HR-MS with those provided by GC-FID and LC-UV method.

Acetic acid

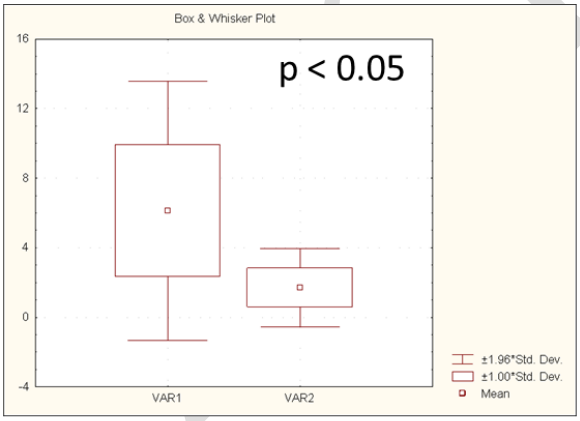


VAR 1: LC-UV data, VAR 2: HR-MS data

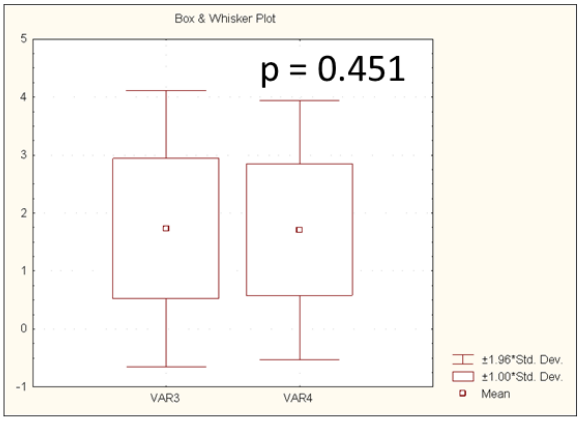


VAR 3: GC-FID data, VAR 4: HR-MS data

Propionic acid

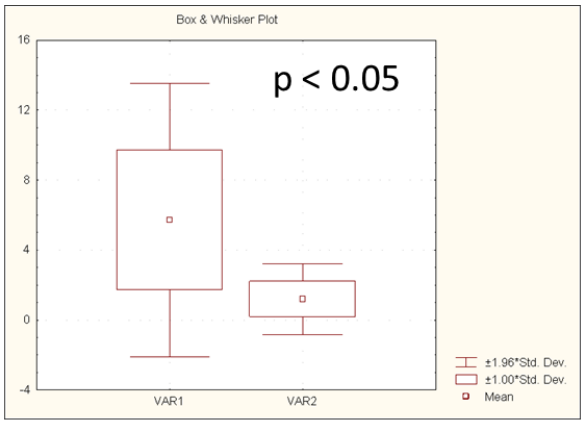


VAR 1: LC-UV data, VAR 2: HR-MS data

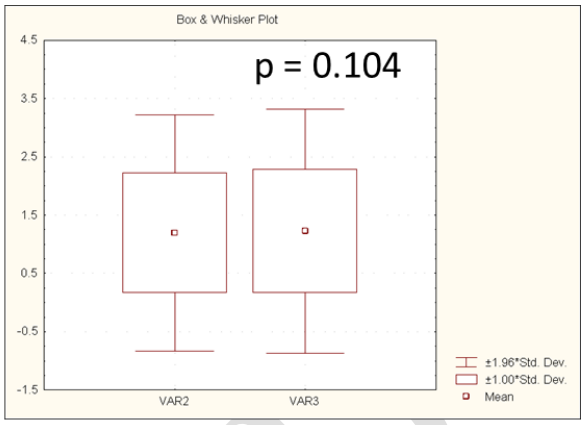


VAR 3: GC-FID data, VAR 4: HR-MS data

508 **Butyric acid**



VAR 1: LC-UV data, VAR 2: HR-MS data



VAR 3: GC-FID data, VAR 4: HR-MS data

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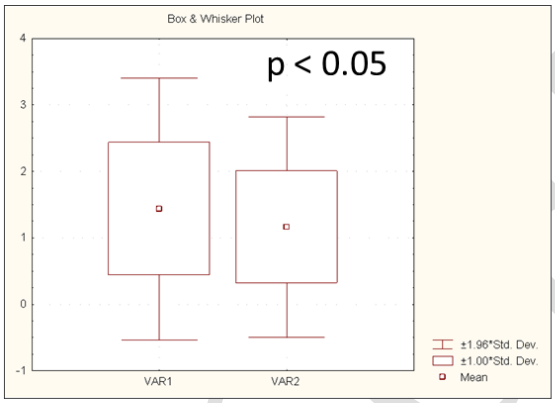
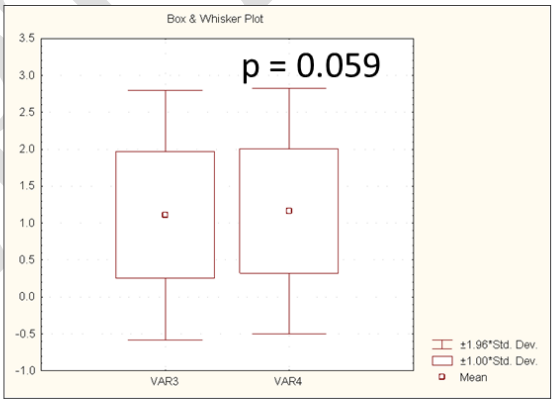
511 **Valeric acid**



VAR 1: LC-UV data, VAR 2: HR-MS data



VAR 3: GC-FID data, VAR 4: HR-MS data

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513 **Graphical Abstract**

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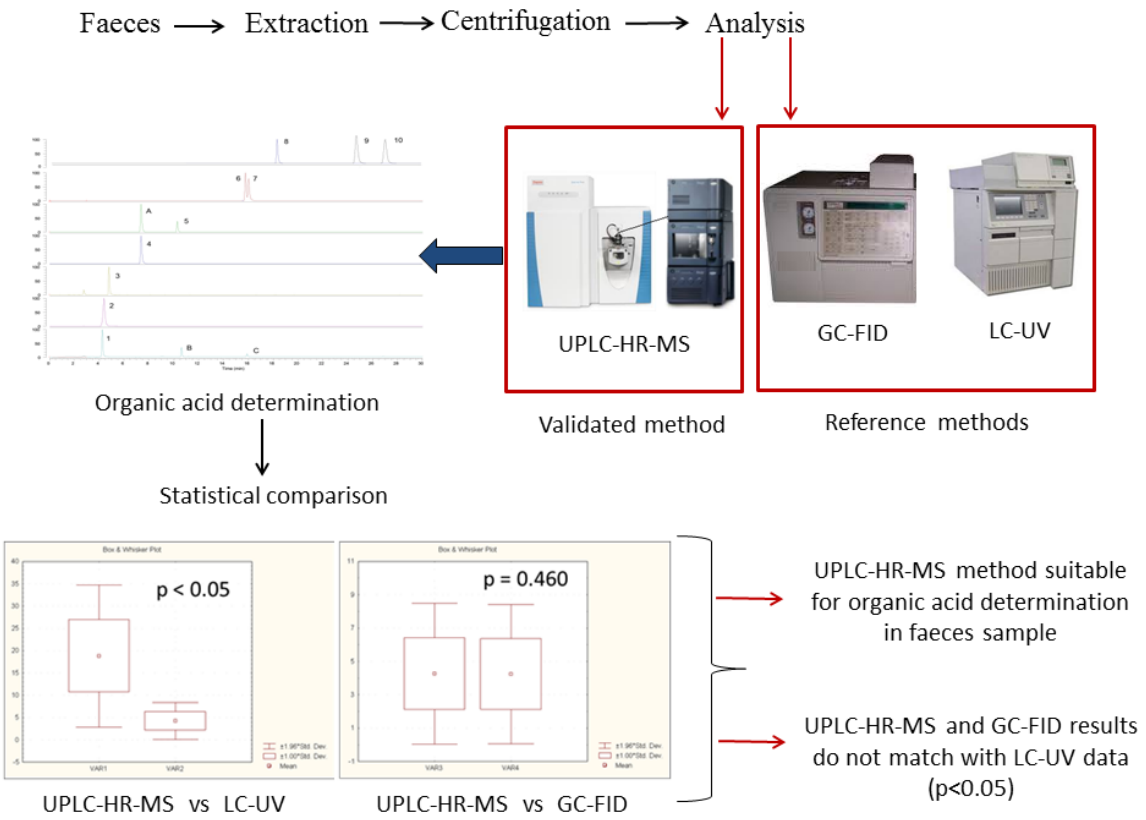

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