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Multi-Compartment SCFA Quantification in Human

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Abstract

Short-chain fatty acids (SCFA) play an important role in human biochemistry. They originate primarily from the digestive system through carbohydrates microbial fermentation. Most SCFA produced in the colon are absorbed by the intestinal wall and enter the bloodstream to be distributed throughout the body for multiple purposes. At the intestinal level, SCFA play a role in controlling fat storage and fatty acid metabolism. The effects of these beneficial compounds therefore concern overall health. They facilitate energy expenditure and are valuable allies in the fight against obesity and diabetes. SCFA are also involved in the regulation of the levels of several neurotransmitters such as GABA (γ-aminobutyric acid), glutamate, serotonin, dopamine, and norepinephrine. Their role is also highlighted in many inflammatory and neurodegenerative diseases such as Alzheimer's disease (AD) or Parkinson's disease (PD). To have a realistic picture of the distribution of SCFA in different biological compartments of the human body, we propose to study SCFA simultaneously in five human biological samples: feces, saliva, serum, cerebrospinal fluid (CSF), and urine, as well as in Dried Blood Spot (DBS). To evaluate their concentration and repeatability, we used 10 aliquots from pooled samples, analyzed by 3-nitrophenylhydrazine (3-NPH) derivation and liquid chromatography coupled with high sensitivity mass spectrometry (LC-QqQ-MS). We also evaluated the SCFA assay on Dried Blood Spot (DBS). In this work, we adapted the pre-analytical parts for each sample to be able to use a common calibration curve, thus facilitating multi-assay quantification studies and so being less time-consuming. Moreover, we proposed new daughter ions from the same neutral loss (43 Da) to quantify SCFAs, thus improving the sensitivity. In conclusion, our methodology, based on a unique calibration curve for all samples for each SCFA, is well-suited to quantified

them in a clinical context.

Keywords

LC-MS, 3-Nitrophenylhydrazine, Short-Chain Fatty Acids, Human Biological Samples, Quantification

1. Introduction

Increasing evidence that short-chain fatty acids (SCFA) play an important role in the maintenance of the gut and in human biochemistry [1] meaning metabolic health. Short-chain fatty acids are small molecules that originate in the digestive system through microbial fermentation of carbohydrates [2]. Most SCFA produced in the colon are absorbed by the intestinal wall and enter the bloodstream to be distributed throughout the body for multiple purposes. The literature highlights the involvement of SCFA in intestinal permeability, lipid metabolism, insulin sensitivity, and inflammation [3]. SCFA can bind to G-protein-coupled receptor (GPR41/43) in the colon leading to the activation of intestinal gluconeogenesis thus affecting satiety and glucose homeostasis [4] [5] or with cellular receptors to regulate glucose storage, fatty acid oxidation or fuel the tricarboxylic acids (TCA) cycle [6]. In a recent review, You *et al.* [7] discuss first how SCFA regulate intestinal function to improve obesity and the possible pathways related to obesity pathological processes and secondly discuss dietary management strategies to enrich SCFA-producing bacteria and whether fecal bacteria transplantation therapy to restore the composition of the gut to regulate SCFA can help prevent or improve obesity. SCFA are also involved in the regulation of the levels of various neurotransmitters such as GABA (γ-aminobutyric acid), glutamate/glucose or dopamine [8] [9].

Due to their significant involvement in a wide variety of biochemical pathways, the role of SCFA in pathologies has been intensively studied [3] [10], especially in neurodegenerative diseases such as Alzheimer's disease (AD) or Parkinson's Disease (PD) [2] [11]. Yilmaz *et al.* [12] described a higher propionic acid concentration in saliva of AD patient compared to healthy ones. Wu *et al.* [13] have studied two levels of progression of AD: healthy control, mild amnestic cognitive impairment, and AD patient. They found a decreased concentration of SCFA in the stool, ranging from healthy patients to more affected ones. For PD, the role of the microbiota has also been studied. Although the exact role of SCFA in PD is still unclear Sampson *et al.* [14] have proposed a way of explaining their implications with neurodegenerative diseases. SCFAs may cross the blood brain barrier (BBB) and altered cells in the central nervous system (CNS): Mitchell *et al.* [15], have studied the influx of fatty acids in brain and concluded SCFA cross the BBB because of albumin-binding, intracellular-binding and fatty acids transporters as FATP-1, FATP-4 and FAT/CD36 [15]. The modification of SCFA levels was also described in Autism Spectrum Disorders (ASD) [16] [17],

Colon Cancer [18], Crohn's Disease [19] or cardiovascular diseases [20]. Despite the crucial role of SCFA and their ubiquitous repartition in biological systems, the quantification of SCFA in the different biological compartments is not systematic to describe an individual.

To analyse SCFA, a number of technologies can be used: 1) NMR is a quantitative and robust method but lacks sensitivity for some applications; 2) Capillary electrophoresis requires pretreatment of the sample leading to poor repeatabilities; 3) Enzymatic detection is a very selective technique that able to identify isomers. Unfortunately, the inhibitors naturally present in biological samples can interfere with the enzymatic detection, limiting its possibilities [3] [21].

Through the years, chromatography coupled with mass spectrometry (MS) has become the analysis of reference for the quantification of SCFAs. Gas chromatography (GC) and liquid chromatography (LC) are used and both generally involve derivatisation [22]: a preanalytical step to improve the detection of compounds. GC-MS has long been the method of choice because of the low instrument cost and the robustness/sensitivity. For SCFA in GC-MS, the most common derivative agents are based on silylation, esterification, or alkylation [23]. For LC-MS analysis, precolumn derivatization has been promoted because native SCFA have a poor ionisation efficiency and the ion suppressions occurring in biological samples make their analysis tough. Known labels are dimethylamino phenacyl bromide (DmPA-Br) [24] [25] which is used to label carboxyl, thiol and amine functions; Dansyl-Chloride (Dns-Cl) [26] for phenols, hydroxyls, and amines; Girard's reagents for carbonyls [27] and 3-nitrophenylhydrazine (3-NPH) labels carbonyls, phosphoryls and carboxylic acids. 3-NPH, brought to the forefront by Han *et al.* [28], is the reference label for the study of SCFA in biological samples, especially feces, due to the high increase in ionisation efficiency. In addition, 3-NPH is compatible with aqueous solutions facilitating the step of chemical derivation in biological samples thus avoiding a drying step improves the reproducibility of the method. 3-NPH is largely analysed in the negative ionisation mode, producing less background noise compared to the positive mode. Liebisch *et al.* [29] have quantified SCFA from 2 to 4 carbons in feces.

Given the implications of SCFAs in many diseases, several studies have reported quantification of SCFA in one or more biological samples [29] [30] [31] [32] [33]. Lastly, Valdivia-Garcia *et al.* [34] have developed a robust method to quantify SCFA in 5 biological samples (feces, duodenal content, stoma content, urine, and serum) and, more especially, they have used a unique calibration curve for feces, duodenal content and stoma content. This all-in-one calibration curve approach is interesting when investigating SCFA from different biological compartments to describe the impact of SCFA distribution on pathologies.

In this work, we propose absolute quantification of SCFA using to 6 heterogenous human biological samples (feces, saliva, serum, cerebrospinal fluid (CSF), urine and dried blood spot (DBS)) using 3-NPH derivatization and LC-MS quantification using a unique calibration curve with a dynamic range covering all these biological concentrations. Our aim was to facilitate multi-assay

quantification studies to reach a more global view of the SCFA biological distribution. We adapted the preanalytical preparation for each sample and, in order to gain sensitivity, we proposed a new transition with a specific neutral loss of 43 Da to quantify SCFA.

2. Materials & Methods

2.1. Reagents & Solutions

All chemical compounds were purchased from Sigma-Aldrich acetic acid (C2, >99.5%), propionic acid (C3, >99.5%), butyric acid (C4, >99%), isobutyric acid (IC4, >99.5%), valeric acid (C5, >99%), isovaleric acid (IC5, >99%), decanoic acid (>99%), formic acid (FA, AnalaR Normapur grade), 3-nitrophenylhydrazine hydrochloride (3NPH-HCl, >98%), n-(3-dimethylaminopropyl)-n'-ethylcarbodiimide (EDC, >97%) and pyridine (>99.5%). Internal standards were purchased from Toronto Research Chemicals: acetic-$^{13}C_2$-acid, butyric-d_7-acid, valeric-d_9-acid and decanoic-$^{13}C_4$-acid. Solvents such as MeOH (Hipersolv Chromanorm, >99.9%), H_2O LC-MS Grade (Lichrosolv), ACN (HiperSolv Chromanorm, >99.9%) were purchased from VWR.

2.2. Sample Preparation

2.2.1. Ethics

All samples were obtained in accordance with French legislation (Loi Jardé). In French hospitals with a research mission, the use of sample leftovers for research purposes is based on the principle of no opposition. According to Loi Jardé, no requirement of Ethical Committee or Institutional Review Board was required because no new sampling was required. This law also stipulates that patients must be fully informed of this information/practice and patients must have the possibility, at any time, of objecting to the use of their samples in research. The analyses were always performed in accordance with confidentiality rules. Data were coded without mention of first and last names, and the results were produced in a way that does not allow patients to be identified. Patients did not invoke their right to have a right of access, rectification, portability and limitation of the processing of data and/or biological samples.

2.2.2. Feces

Feces samples were freshly collected from 5 healthy volunteers in ethanol and homogenised to quench metabolism and stabilise the metabolome [29]. Then, samples were stored at −80˚C until analysis. For the analyses, samples returned to room temperature during 4 h and then 400 µL were aliquoted twice with a wide bore cone. One is for the quantification process; other one will be dried 10 h at 50˚C under vacuum with a SpeedVac™ (Thermo Fisher Scientific, Illkirch-Graffenstaden, France) to determine the amount of dried matter per aliquot for the normalization process. The repeatability of the sampling of 400 µL aliquots of homogenate aliquots and the drying process to determine the dry matter have been studied. For the derivatization process, the samples were centrifuged (10 min,

20,000 g, 4˚C) and then 50 µL of supernatant was diluted and derivatised.

2.2.3. Saliva

Samples were collected from 10 patients and pooled in tubes adapted to the collection of saliva (Salivette®, SARSTEDT S.A.R.L., Marnay, France) and they were stored at −80˚C under 1 h. For analysis, samples were kept at room temperature for 4 h and then vortexed during 30 s. Then 50 µL of the sample was extracted with 450 µL of cold MeOH. After 30 s of vortex, and 10 min of rest, the sample was centrifuged (20,000 g, 10 min, 4˚C). 50 µL of supernatant was diluted 5 times and then derived.

2.2.4. Serum

Blood samples were collected from 10 patients by venipuncture in dry tubes. They were centrifuged at 2000 g for 10 min, and then the serum was collected, aliquoted in 100 µL fractions and frozen at −80˚C until analysis. Samples were excluded if there was hemolysis, jaundice, or lipemia. 50 µL of pooled serum samples were extracted with 150 µL of cold methanol. After shaking for 30 s, they are left to stand for 10 min and then centrifuged (20,000 g, 10 min, 4˚C). 50 µL of supernatants were derived.

2.2.5. Dried Blood Spot (DBS)

Capillary blood was spotted on filter papers PerkinElmer 226® (PerkinElmer, Greenville, USA). After collection, the samples were allowed to dry at room temperature, protected from light, for 3 h. The DBSs were aliquoted into calibrated punches (3.0 mm disc) containing 3 µL of blood (2020 Annual Summary Report of the Quality Assurance Program) in a 1.5 mL Eppendorf tube and stored at room temperature, without light exposure. To avoid any significant change in metabolomic composition, DBS were analyzed within a month as previously recommended [35]. Punches were extracted with 120 µL of cold methanol and extracted during 20 min of planar agitation. 50 µL of supernatant was derived.

2.2.6. Urine

Urine samples were freshly collected from 10 patients and centrifuged at 2000 g for 10 min. Before any further step, creatinine concentrations were measured by the enzyme method (Cobas®, Roche Diagnostics). The urine samples were then aliquoted and stored at −80˚C. For analysis, 100 µL of cold MeOH/pyridine (4:1 v:v) were added to 100 µL of urine samples. After shaking for 30 s and before centrifugation (20,000 g, 10 min, 4˚C), a 10 min rest time was applied. Then 50 µL of supernatants were derived.

2.2.7. Cerebrospinal Fluid (CSF)

CSF samples were collected from 20 patients in polypropylene tubes (SARSTEDT SARL, Marnay, France) and centrifuged at 4000 g for 10 min at 4˚C. The supernatants were aliquoted into polypropylene tubes (Sorenson Bioscience, Bernolsheim, France) and frozen at −80˚C. For analysis, 100 µL of sample was extracted with 100 µL of cold MeOH. After shaking for 30 s, they were left to stand

for 10 min and then centrifuged (20,000 g, 10 min, 4˚C). 50 µL of supernatants were derived.

2.3. LC-MS Analysis

LC-MS instruments are described below. Further information concerning electrospray ionisation (ESI) parameters, LC-MS characteristics of each SCFA, and the calibration curve is available in supplementary data (**Tables S1-S4**).

2.3.1. Derivatization

For 50 µL of sample (or for calibration curve points), 50 µL of an aqueous internal standard mixture containing acetic-$^{13}C_2$-acid (for acetic acid and propionic acid), butanoic-d_7-acid (for butyric acid and isobutyric acid) and valeric-d_9-acid (for valeric acid and isovaleric acid), all at the concentration is 1 nM, were added to samples. Then, 50 µL of a 2.5% pyridine solution in methanol, 50 µL of EDC solution (n-(3-dimethylaminopropyl)-n'-ethylcarbodiimide) in methanol at 105 mM and 50 µL of an aqueous/methanol solution (1:1) of 3-NPH (3-nitrophenylhydrazine) at 175 mM were added. The derivatization solution acted for 1 h at 4˚C. The reaction was quenched by adding 250 µL of acidified water (1% formic acid). The derivatization reaction is described in detail and a mechanism is proposed (**Figure S1**). The stability of 3-NPH-SCFA derivatives, in aqueous solution or in biological sample has been previously established [36] [37] [38] [39] and therefore not investigated here.

2.3.2. Chromatography

Chromatography was carried out on a Waters Acquity I-Class UPLC Class with a column Phenomenex Kinetex 1.7 µm XB – C18 (150 mm × 2.10 mm) and kept at 55˚C (Waters, Guyancourt, France). The solvent system is composed of mobile phase A [0.1% formic acid in water] and of a mobile phase B [0.1% formic acid in acetonitrile]. Chromatographic separation started with a flow rate of 0.6 mL/min at 85 %A, until 2 min. Then %A decreased to 80%, then to 75% at 4 min. At 6 min %A is down to 60%, 50% at 8 min and then 15% at 9 min. From 9.5 to 12.4 min, %A is 0% and then from 12.5 to 14.5 min a loop on the starting condition. The injection volume was 5 µL. Carryover has been evaluated by comparing theoretical and experimental concentrations of a low-level solution, from the calibration curve, after the injection of the highest level from calibration curve (**Table S1**).

2.3.3. Mass Spectrometry

Analyses were performed on a triple-quadrupole Xevo TQ-XS mass spectrometer (Waters, Guyancourt, France), operated in MRM mode with negative electrospray ionisation modes (Zspray; ESI -). Experimental masses of parent ions and daughter ions of every standard, injected alone, were investigated through a high-resolution spectrometer before the optimization of the MRM parameters on the triple quadrupole (QqQ) for the quantification methods. To validate the fragmentation patterns specific to 3-NPH-derived fatty acids, decanoic acid and

decanoic-$^{13}C_4$-acid were also fragmented. Specific daughter ions, which can be used for quantification, were then fragmented at 5, 10, 20, 30, 40 and 50 eV to determine the optimal range of fragmentation energy. Then each ion was fragmented every 2 eV in its optimum range to determine the fragment and collision energy that give the best S/N ratio. ESI parameters are available in the supplementary data (**Table S4**).

2.3.4. Data Processing

The acquired data were processed using the MassLynx TargetLynx module (Waters, Guyancourt, France). Quantification was evaluated by plotting the peak area ratio of each SCFA to the corresponding internal standard.

3. Results & Discussion

3.1. New Fragmentation Patterns

All compounds derived with 3-NPH have common fragments: 137 m/z and 152 m/z. The investigation for these specific fragments, to enable quantification, was performed in high resolution on a UPLC Ultimate WPS-3000 system (Dionex, Germany) coupled to a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Chromatographic conditions were the same as described above. Once the fragmentation candidates had been found using the high-resolution apparatus (Q-Exactive, Q-Orbitrap), the fragmentation conditions were optimised on the high-sensitivity apparatus that will be used for quantification (XEVO TQ-XS, triple quadripole). The previously mentioned fragments are generally those used for LC-MS quantification. However, upon successive injection of the compounds in high resolution, another fragmentation pattern has emerged: Loss of 43 Da relative to the mass of the parent ion (**Figure 1**).

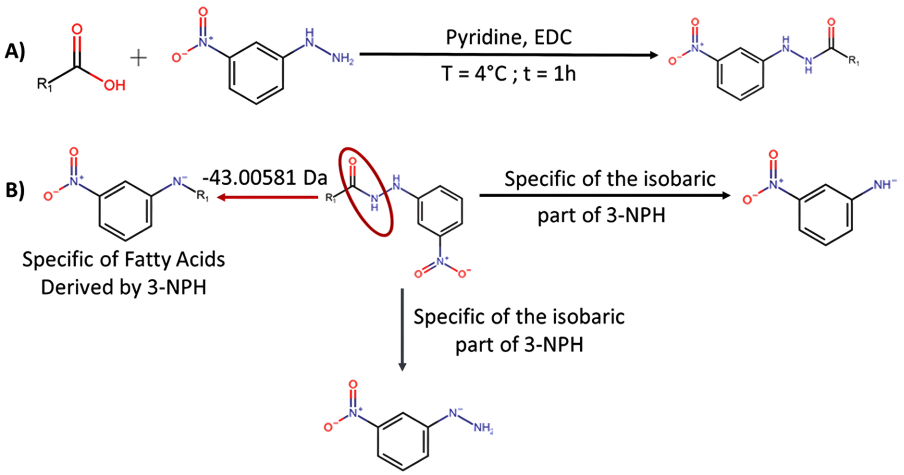


Figure 1. (A) Reaction of 3-NPH derivatization & (B) Main fragments of a fatty acid derivatized with 3-NPH.

The fragment resulting of this loss could also be observed on medium chain fatty acids. The loss formula has been verified using several ^{13}C linear fatty acids

where the carbon that carries the acid function is a ^{13}C, such as decanoic acid and decanoic-$^{13}C_4$-acid (Figure 2), validating the specificity of this fragment in fatty acids. This fragment is the one giving the best S/N ratio and peak intensity so the highest specificity is illustrated with valeric acid in Table 1.

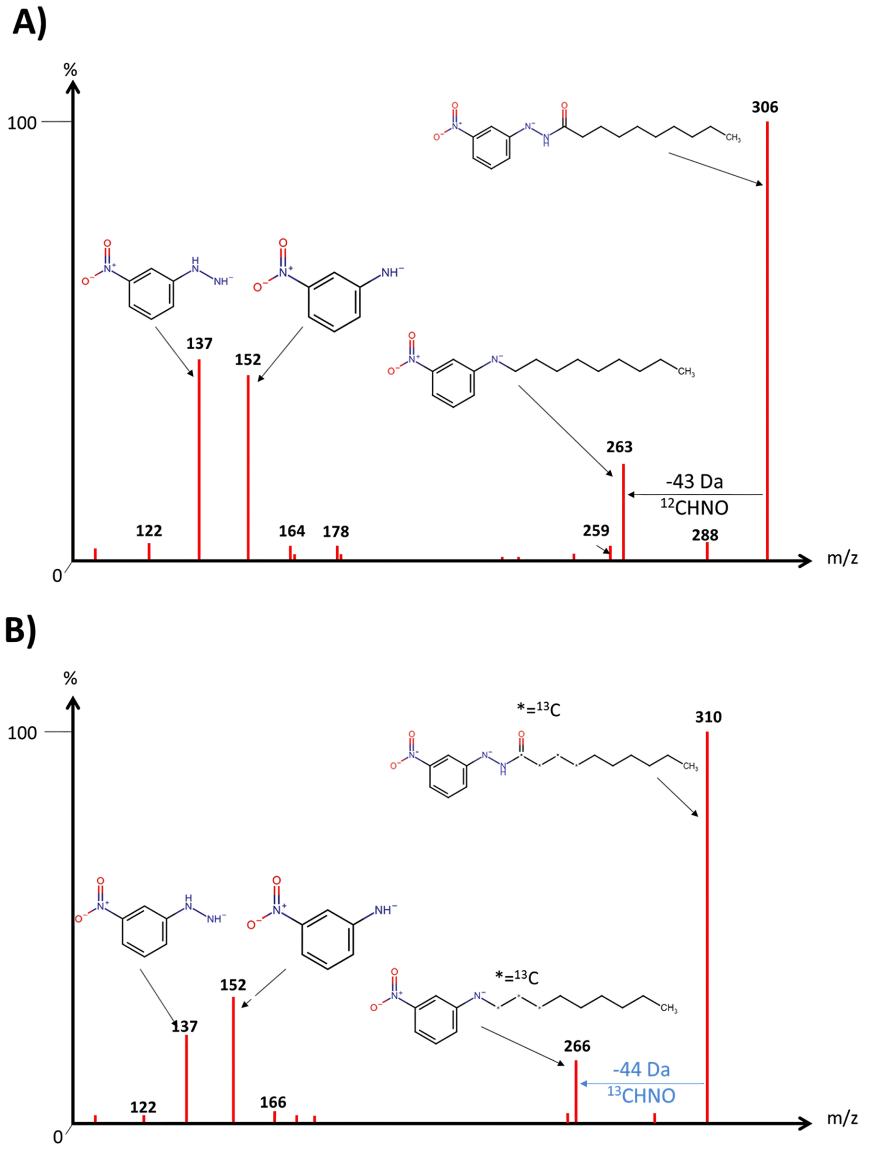


Figure 2. Fragmentation pattern study on low sensibility Xevo TQ-XS (A) decanoic acid (B) decanoic-$^{13}C_4$-acid.

Table 1. Comparison of the 3 main fragments of derived valeric acid at the concentration of 46,500 nM in aqueous solution.

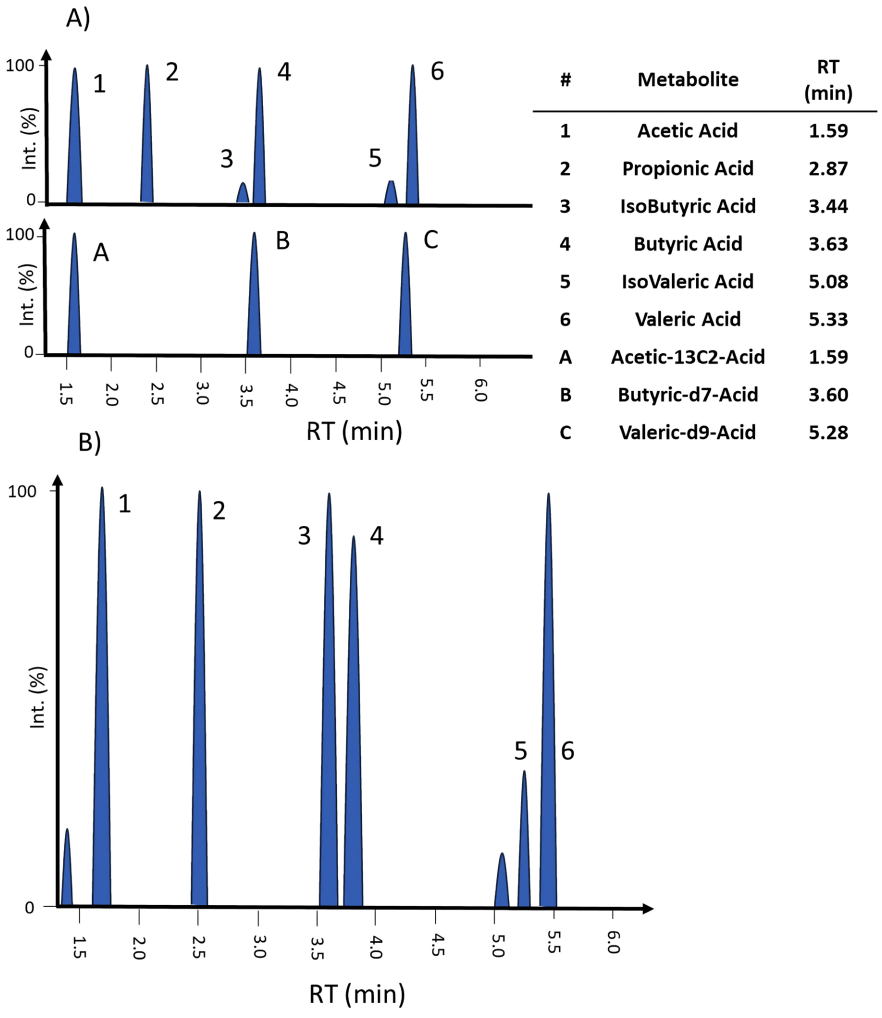
Fragment	CE optimum (eV)	S/N	Intensity (u.a.)
137 m/z	14	1920	3.61×10^8
152 m/z	14	10,835	5.34×10^7
193 m/z*	14	15,071	1.99×10^8

*: Loss of 43 Da.

The ion produced by the loss of 43 Da was therefore chosen as the daughter ion, except for the case of acetic acid, where the small size of the carbon chain should prevent rearrangement. The S/N ratio has been calculated with MassLynx after smoothing of the chromatogram (Window Size Scan = 2/Number of Smooth = 2). The noise region was 2 min long, after the peak of interest, and the method of calculation was the mean noise. This gain in specificity will make it possible to quantify SCFAs in matrices where they are poorly concentrated and, therefore, would have been lower than the limit of quantification (LOQ) with another specific fragment.

3.2. Quantitative Data for 5 Human Biological Samples and DBS

Chromatographic peaks of 3-NPH-derived SCFA elute between 1 min and 7 min. There is no acquisition before 1 min because the excess of derivatization reagents elutes in this interval and will cause heavy pollution of the apparatus. Typical standard solution chromatograms and some typical matrix chromatograms are available in Figure 3.



#	Metabolite	RT (min)
1	Acetic Acid	1.59
2	Propionic Acid	2.87
3	IsoButyric Acid	3.44
4	Butyric Acid	3.63
5	IsoValeric Acid	5.08
6	Valeric Acid	5.33
A	Acetic-13C2-Acid	1.59
B	Butyric-d7-Acid	3.60
C	Valeric-d9-Acid	5.28

Figure 3. Example of chromatograms (A) calibration curve (B) DBS.

The quantitative data are summarized in Table 2. The intra-day repeatability and the inter-day repeatability have been evaluated with 10 aliquots of distinct pooled samples, prepared and analyzed independently. The first one (n_1) was used for intra-day analysis and the second one (n_2) for inter-day analysis. The inter-day injections were carried out within 1 month.

Table 2. Intra-day ($n_1 = 10$) and inter-day ($n_2 = 10$) repeatabilities of SCFAs quantification in 6 biological samples, from 2 different pools.

		Feces		Saliva		Serum		DBS		CSF		Urine	
Metabolite	Variability	Mean (nM/mg of dry matter)	CV (%)	Mean (µM)	CV (%)	Mean (nM)	CV (%)	Mean (nM)	CV (%)	Mean (nM)	CV (%)	Mean (nmol/mmol of creatinine)	CV (%)
Acetate	Intra-Day	59.35	5.66	14,357	4.71	57,349	7.65	128,742	7.42	24,414	4.48	3459	7.75
	Inter-Day	71.3	7.71	14,323	10.26	64,280	7.48	148,383	7.79	24,467	7.89	3416	4.39
Propionate	Intra-Day	28.42	7.09	3851	5.06	4804	6.62	4285	7.82	232	7.81	59.9	10.32
	Inter-Day	35.74	8.13	3915	11.14	5174	15.48	4709	7.52	208	12.03	44.4	17.98
Butyrate	Intra-Day	23.5	6.43	332	4.22	12,971	7.26	1235	8.73	<LOQ	N/A	59.6	5.35
	Inter-Day	28.32	8.4	365	7.95	14,477	8.69	1349	11.08	<LOQ	N/A	42.3	20.44
Isobutyrate	Intra-Day	4.08	7.37	81	4.07	921	8.58	626	8.30	278	7.40	18.7	13.11
	Inter-Day	5.13	9.22	85	7.76	1035	13.72	689	12.21	299	8.91	16.2	14.51
Valerate	Intra-Day	6.08	6.91	3.1	8.06	433	9.70	2243	8.36	<LOQ	N/A	<LOD	N/A
	Inter-Day	7.05	8.07	2.8	12.86	452	8.85	2621	7.50	<LOQ	N/A	<LOD	N/A
Isovalerate	Intra-Day	3.34	6.66	78	5.90	309	15.53	360	12.29	96.7	16.25*	15.2	15.59
	Inter-Day	3.82	8.92	77	5.97	374	14.44	419	12.44	101.1	21.15*	17.6	11.95

LOQ: Concentration where S/N was ≥10; LOD: Concentration where S/N was ≥3; *: above the LOQ but below the dynamic range of concentration.

3.2.1. Feces

In feces, we can notice that as the chain length increases, the concentration decreases. In literature, most of the studies normalized the concentrations of each metabolite in feces to the wet matter. Han *et al.* [28] quantified SCFA with isotope-coded derivatization with $^{13}C_6$-3-NPH on 6 patients. The concentration ranges for acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid and isovaleric acid normalized to the wet matter were respectively 23.2 - 58.3, 5.0 - 14.4, 4.4 - 11.9, 0.08 - 2.26, 0.19 - 2.94 and 0.05 - 3.23 nmol/mg wet matter. As we normalized the concentration to the dried matter, we cannot compare our results with those of Han *et al.* Indeed, the percentage of humidity was not measured, thus impeding the conversion. Liebisch *et al.* [29] quantified acetic acid, propionic acid and butyric acid in feces with a dried weight normalization, on 22 healthy volunteers. Respectively, the concentration ranges are 102 - 1210, 19 - 340 and 18 - 370 nmol/mg_{DW}. Except for the concentration of acetic acid, our results are in the same range as Liebisch's.

Homogenising the samples in ethanol has the effect of stabilising the sample. Without ethanol quenching, at the time of collection, patient samples need to be at the laboratory within 4 h before storage at −80˚C. During the time between collection and storage, metabolism is still running, affecting the dosage of metabolites. Moreover, the stools samples are not homogeneous and the sampling could be impactful. So, with the aim of avoiding these known drawbacks, we develop these preanalytical steps in EtOH. Our method is inspired by the colorectal cancer screening kit that uses organic solvent [40] [41]. The ethanol stops the enzymatic reactions and stabilises the sample. In return, we cannot normalize our data with the quantity of wet matter as in many papers or with the data provided by HMDB [42]. However, the coefficients of variation are contained, for both intra-day and inter-day repeatabilities. This EtOH sample collection leads to lower variability and longer-term storage that are more suitable for assays.

3.2.2. Saliva

The quantification of SCFA in saliva by 3-NPH derivatization-LC-MS is uncommon. Nagatomo *et al.* [43] quantified 12 SCFA in saliva of healthy mouse and Sjögren syndrome (SS) mouse by LC-MS with derivatization of 2-picolylamine. SCFA concentrations are clearly higher in SS mouse. However, concentrations of SCFA in mouse saliva are different from human values. Methods for quantification of metabolites in human saliva are more frequently GC-MS based.

Kawase *et al.* [44] quantified SCFA by GC-MS in saliva from 5 patients (men & women) who suffered from various periodontal diseases with a method validated according to the Food & Drug Administration criteria. In periodontal disease, the concentration of SCFA could be higher because of the gingival pathogens [45] [46]. The concentrations are between 1812 - 4117 µM for acetic acid, 185 - 836 µM for propionic acid, 12 - 162 µM for butyric acid, 3 - 50 µM for isobutyric acid, 3 - 21 µM for valeric acid and 2 - 26 µM for isovaleric acid. Acetic acid, propionic acid, butyric acid and isovaleric acid are less concentrated than in our samples. One of the explanations could be the time of sampling. Indeed, we looked at the SCFA concentration during a full circadian cycle and we noted the concentration was much higher at waking time or in early morning than during the rest of the day (data not shown). Therefore, we conclude that sampling time is really impactful in saliva-based studies.

The quantification of SCFA in saliva remains infrequent but can bring information on the oral microbiota, especially with the ability of our method to quantify compounds at low concentrations. For example, Szczeklik *et al.* [47] have highlighted differences in SCFA concentration between people with gastro-oesophageal reflux disease. Ho *et al.* [48] have highlighted the decreasing levels of SCFA in people with peanut allergy. Our method is appropriate for such a use.

3.2.3. Serum

Valdivia-Garcia *et al.* [34] quantified propionate and isovalerate in serum at 170

nM and 570 nM. Two points are important: the difference in propionic acid concentration and the fact that they have not detected acetate, butyrate, isobutyrate, and valerate in a pooled mixture purchased from Sigma-Aldrich. Many articles use fragments 137 m/z and 152 m/z as daughter ions because they are specific for 3-NPH labelling [49]. Our analysis has highlighted the loss of 43 Da of the derived SCFA, which gave a highly specific fragment with better S/N ratios. This may explain the discrepancy between their results and ours for propionate. Dei Cas *et al.* [50] quantified some SCFA and MCFA (Medium Chain Fatty Acids) in 54 serum from healthy patients also with 137 m/z fragment. The biological concentration determined are: 7831 - 13,415 nM for butyrate, 5606 - 10,260 nM for isobutyrate, 470 - 764 nM for valerate and 6511 - 16,106 nM for isovalerate. Butyrate and valerate correspond to the concentrations found here, whereas other SCFA are more concentrated by a factor of 10. This may be explained by the high heterogeneity of the 54 patients, which has been highlighted by Dei Cas *et al.* [50].

3.2.4. Dried Blood Spot (DBS)

Although to our knowledge, there is no literature on SCFA assays on DBS, targeted analysis has been developed on other compounds such as MCFA [51] or carnitines [49]. For their part, Ingels *et al.* [52] have developed the specific quantification of hydroxybutyric acid using spot derivatization by GC-MS analysis.

There is growing interest in DBS for several reasons: less invasive than blood sampling, participative medicine is increasing either for pathology follow-up or for the diagnosis of older people. DBS are easily storable and stable over time. Several studies have explored the potential and the qualities of DBS for the early detection of pathologies. For example, Barone *et al.* [53] have studied acylcarnitine modifications in children with ASD. Wang *et al.* [54] have worked on breast cancer detection and Zytkovicz *et al.* [55] on metabolic disorders in newborns. Although DBS are already used for the early detection of pathologies as a newborn screening, the long-term stability of SCFA in DBS should be evaluated in future studies. However, our method enables the quantification of SCFA in DBS and may be used to explore a potential dysfunction of this metabolism in a wide range of pathologies.

3.2.5. Cerebrospinal Fluid (CSF)

Wishart *et al.* [56] have characterized the CSF metabolome using NMR, GC-MS & LC-MS, creating the CSF Metabolome, currently integrated and completed in HMDB. The concentration ranges given are 31 - 171 µM for acetic acid, 0 - 6 µM for propionic acid, 0 - 2.8 µM for butyric acid, 0 - 3.6 µM for isobutyric acid and 0 - 2.7 µM for isovaleric acid. Our results fit these data however the CV of isovaleric acid is slightly higher than 20%. This variability can be explained by the fact that the raw data obtained was below the dynamic range but above the LOQ. Moreover, in biological studies, and more particularly in the study of a disease, the aim is to be able to diagnose it among a cohort of healthy and pathological

patients. In the case of neurodegenerative diseases, abnormal SCFA production can be detected by the digestive system [57] [58]. Given the close connection between the CSF and the BBB (and more generally the CSF and the brain), it would be interesting to study SCFA in the CSF in the case of neurodegenerative diseases or intestinal disorders [59] [60]. They are rarely quantified in CSF even for neurodegenerative disease because urine, serum and feces are actually the matrices of choice for their availability. However, the CSF investigation may better reflect the "metabolic state" of the brain in diseases.

3.2.6. Urine

In urine, valeric acid concentrations is not detected and this result seems to be in agreement with the data available on HMDB [42] for adults. Valdivia-Garcia *et al.* [34] have produced data for 4 groups: Crohn's disease, Pouchitis, Ulcerative Colitis, and control groups. On the control group, acetate, propionate and isobutyrate have not been detected. This result is not that surprising. In fact, urine is known to be a highly variable matrix. In our results, except for acetic acid, others SCFA concentrations are weakly concentrated, which can explain the higher CVs. However, this variability must still be low enough to differentiate pathological patients; for example, patients with acidosis are likely to have different concentrations of SCFA than healthy ones [61]. In animals, acidosis is strongly correlated with SCFA levels [62] [63]. Hanstock *et al.* [62] have studied the link between acid accumulation in the hindgut of rats following the fermentation of commonly consumed carbohydrate sources on behavioural parameters indicating anxiety and aggression. As a result, they have predicted that acid accumulation in the hindgut of rats can cause behavioural problems.

3.2.7. Global

Analysing multiple biological matrices and quantifying metabolites can be difficult due to the variations of the large variations of the matrix from sample to sample and the highly variable background concentrations that have been checked in the Human Metabolome Database (**Table S5**). We have adapted the method of Valdivia-Garcia *et al.* [34] to match the diversity of our biological samples. The calibration curve was extended to take into account the SCFA concentrations in the matrices studied. To have a single curve, the preanalytical steps have been adapted. In particular, the dilutions used during the extraction step. The specific usual fragments recognised as coming from a compound derived from 3-NPH are 137 m/z and 152 m/z. As our study focused on a restricted family of compounds, we were able to highlight a new fragment that resulted from the loss of 43 Da from parent ion. The high specificity of this fragment makes it more sensitive. Using the fragment that lost 43 Da results in better LOQs. Combined with our dilutions, this fragment makes it possible to combine highly concentrated and low-concentration matrices within the same calibration curve with minimal matrix effect. The matrix effect in 3-NPH-derived biological samples has been intensively studied. For Liebisch *et al.* [29], the matrix effects of SCFA in feces ranged from 3% to 6.5%. In the Valdivia-Garcia study [34] on

urine, feces, and serum, the highest matrix effect was found for isovalerate, especially in urine, but it is otherwise less than 15% in absolute value.

We provide reference values for saliva, CSF, and DBS. There is a marked lack of data for these samples, whereas they present some advantages. DBS is an alternative to serum tests. This type of sampling provides access to a population with limited access to healthcare while allowing monitoring. The oral microbiota is rich and studying the evolution of SCFA in saliva according to the time of day could provide information on the circadian rhythm. CSF is a rare matrix and is not one of the usual samples. However, SCFAs are ubiquitous in all living compartments. Studying the CSF could give us access to information from the brain, under the guise of understanding these exchanges. This "one calibration curve for all" method along with the neutral loss of 43 Da means that studies investigating the role of SCFA are no longer restricted to specific matrices and can be proposed as a "multi-compartment" description to evaluate the global biological dysregulation or diversity of SCFA (Figure 4).

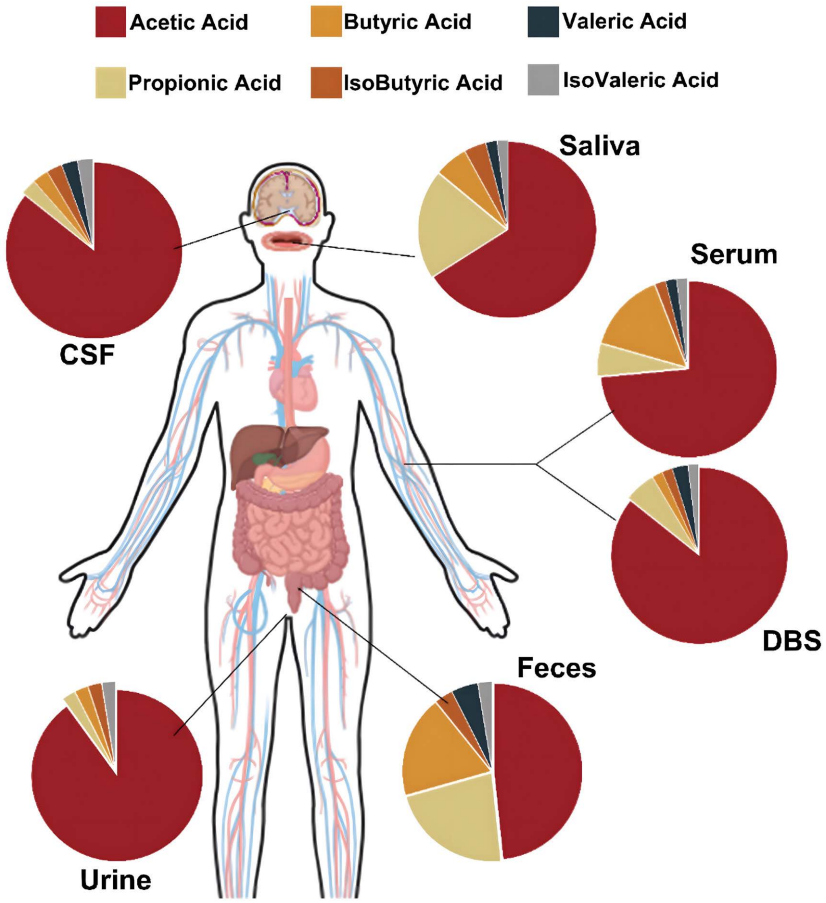


Figure 4. Pie charts illustrating the proportion of each SCFA by biological samples and DBS.

4. Conclusion

SCFAs have been extensively and intensively studied by 3-nitrophenylhydrazine

derivatization-LC-MS² because of their implication in metabolism and diseases. In our study, we present a quantification method with specific preanalytic steps for 6 samples (CSF, feces, saliva, serum, urine, and DBS) using the same derivatization process, a single calibration curve, and a highly specific fragment for derived SCFA. This method makes it possible to include new types of samples in future clinical studies of SCFA. Moreover, the results were obtained according to the same procedure allowing the direct comparison of metabolite balances between biological compartments. This work also proposes new SCFA concentration ranges for CSF and DBS. Future work is planned on the multicompartment quantification of SCFA in different diseases.

Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary Data

S1. Method Validation

S1.1. Linearity of the Method—Limit of Quantification (LOQ)

The calibration curves were obtained by injecting seven standard solutions. The regression parameters of slope, intercept and correlation coefficient were calculated by weight (1/x) linear regression. Linearity was assumed when the regression coefficient was greater than 0.995.

The limit of quantification (LOQ) corresponded to the concentration of derived metabolite producing a signal-to-noise ratio (S/N) equal than to 10. The concentration of each LOQ was determined by diluting the less concentrated point of the calibration curve. The S/N was calculated using MassLynx in RMS method. The region selected for the "Noise" was 2 min long and always selected after the peak of interest on the chromatogram.

S1.2. Intra Assay Accuracy and Precision

For the calibration curve, the intra-day precision and accuracy were evaluated using 10 independent replicates for each level of QC (low, medium, high) injected the same day. The inter-day precision and accuracy were evaluated with one independent injection per QC level on 10 separate days. The intra-day and inter-day precisions were restricted to less than 15% for medium and higher levels whereas it is less than 20% for low level. Same criteria were applied for the accuracy. Accuracy was defined as the ratio of the difference between the spiked concentration and the observed concentration to the spiked concentration, while precision was defined by the coefficient of variance (CV%) as the ratio of the standard deviation to the mean. For matrices, the intra-day and inter-day precision have been evaluated with two separate pools.

S1.3. Carry Over

Carryover is a potential source of error with automatic sampler, especially with high concentration. In the aim to evaluate this possible contamination between two samples, standard solutions must be injected one after the other: low level from the calibration curve—highest level from calibration curve—low level a second time. The comparison of the integration between the concentrations will allow the evaluation of carry-over.

$$\text{Carry Over}\left(\text{CO}\%\right) = \frac{\text{Mean of Low Level \#2} - \text{Mean of Low Lovel \#1}}{\text{Mean of Low Level \#1}} * 100$$

S2. Tables

Table S1. Retention time, fragmentation parameters of SCFA and range of concentrations of the calibration curve.

Metabolite	RT (min)	Parent Ion (m/z)	Daugther Ion (m/z)	CE (-eV)	Cone Voltage (V)	Internal Standard	Dynamic Range of Concentration (nM)	LOQ_a[2] (nM)	CO (%)
Acetic Acid	1.67	194.1	152.0[1]	14	20	Acetic-$^{13}C_2$-Acid	638 - 425,000	0.42	4.47
Propionic Acid	2.50	208.1	165.1	16	20	Butyric-d_7-Acid	300 - 200,000	0.27	6.53
Isobutyric Acid	3.60	222.1	179.1	14	20	Butyric-d_7-Acid	30 - 20,000	0.04	13.22
Butyric Acid	3.73	222.1	179.1	14	20	Butyric-d_7-Acid	338 - 225,000	0.42	0.62
Isovaleric Acid	5.25	236.1	193.1	14	20	Valeric-d_9-Acid	18 - 12,000	0.12	15.76
Valeric Acid	5.50	236.1	193.1	14	20	Valeric-d_9-Acid	140 - 46,500	0.10	2.67
Acetic-$^{13}C_2$-Acid	1.67	196.1	152.0[1]	14	20	-	-	-	-
Butyric-d_7-Acid	3.72	229.1	186.1	14	20	-	-	-	-
Valeric-d_9-Acid	5.44	245.1	202.1	14	20	-	-	-	-

1: For acetic acid, and its internal standard, the loss of 43Da is not possible; 2: Concentration where S/N was ≥10.

Table S2. Intra-day and inter-day repeatabilities of the calibration curve, prepared in water.

Metabolite	Concentration (nM)	Intra-Day (n = 10)			Inter-Day (n = 10)		
		Mean + sd (nM)	CV (%)	Accuracy (%)	Mean + sd (nM)	CV (%)	Accuracy (%)
Acetic Acid	17,000	16,955 + 1058	6.24	0.27	17,551 + 1448	8.25	3.24
	170,000	166,246 + 3599	2.17	2.21	169,252 + 8945	5.28	0.44
	340,000	342,841 + 4555	1.33	0.84	333,227 + 10,447	3.14	1.99
Propionic Acid	8000	8228 + 635	7.71	2.85	8136 + 684	8.4	1.7
	80,000	79,569 + 1948	2.45	0.54	78,613 + 3832	4.87	1.73
	160,000	159,870 + 2076	1.3	0.08	156,731 + 9277	5.92	2.04
Butyric Acid	9000	8879 + 488	5.5	1.35	8884 + 732	8.24	1.29
	90,000	90,360 + 1947	2.16	0.4	88,579 + 4656	5.26	1.58
	180,000	182,728 + 2579	1.41	1.52	174,105 + 9579	5.5	3.27
Isobutyric Acid	800	803 + 59	7.35	0.42	767 + 63	8.21	4.07
	8000	7997 + 162	2.02	0.04	7864 + 425	5.4	1.69
	16,000	16,237 + 266	1.64	1.48	15,616 + 826	5.29	2.4
Valeric Acid	1860	1871 + 115	6.16	0.6	1833 + 156	8.51	1.45
	18,600	18,270 + 279	1.53	1.77	17,909 + 825	4.6	3.71
	37,200	37,471 + 682	1.82	0.73	36,356 + 1998	5.5	2.27
Isovaleric Acid	480	468 + 29	6.19	2.5	479 + 43	8.99	0.21
	4800	4791 + 96	2	0.19	4609 + 167	3.62	3.98
	9600	9555 + 201	2.11	0.47	9559 + 725	7.59	0.43

Repeatabilities are based on 10 injections of each of the three levels of calibration curve QC. The inter-day injections were carried out within a maximum of 1 month.

Table S3. LC gradient.

#	Time (min)	Flow (mL/min)	%A	%B
1	0	0.6	85	15
2	2	0.6	80	20
3	4	0.6	75	25
4	6	0.6	60	40
5	8	0.6	50	50
6	9	0.6	45	55
7	9.5	0.6	0	100
8	12.4	0.6	0	100
9	12.5	0.6	85	15
10	14.5	0.6	85	15

Table S4. ESI parameters.

Polarity	NEG
Capillary Voltage	3 kV
Desolvatation Temperature	450˚C
Source Temperature	150˚C
Nebuliseur Gas Flow	7.0 bar
Cone Gas Flow Temperature	150˚C
Desolvatation Gas Flow	1200 L/hr

Table S5. HMDB concentrations of SCFA in biological samples.

Metabolite	Serum (μM)	Urine (μmol/mmol of cretinine)	Feces (nmol/g of wet matter)	Saliva (μM)	CSF (μM)
Acetic Acid	20 - 60	2.5 - 106.0	12,800 - 103,400	20 - 25,000	20 - 170
Propionic Acid	0.5 - 1.5	0.2 - 5.7	4500 - 27,800	<1 - 6000	0 - 6
Butyric Acid	0.3 - 1.5	0.5 - 81.0	4000 - 53,000	5 - 5000	0.2 - 8.2
Isobutyric Acid	0.7 - 4.4	0.86 - 4.02	-	15 - 1760	0.0 - 3.6
Valeric Acid	0.3 - 1.2	0.0 - 8.5	600 - 3800	0 - 200	-
Isovaleric Acid	0.3 - 2.7	0.8 - 66.0	300 - 6000	15 - 100	0.0 - 2.7

S3. Figures

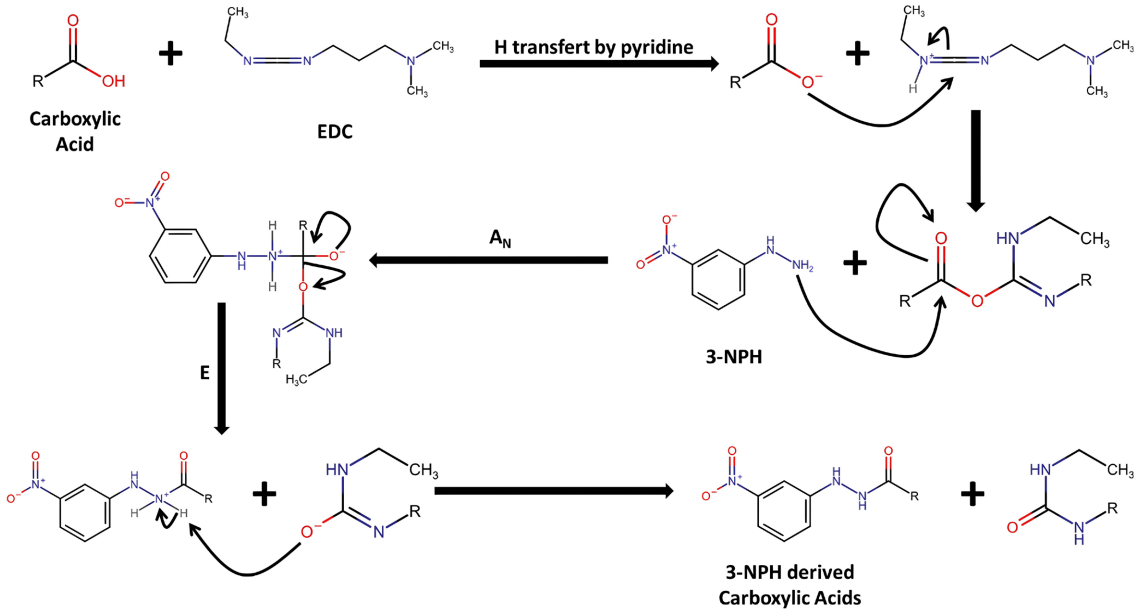

Figure S1. Mechanism of derivation of carboxylic acids with pyridine, EDC & 3NPH.