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Simultaneous measurement of plasma concentrations and ^{13}C-enrichment of short-chain fatty acids, lactic acid and ketone bodies by gas chromatography coupled to mass spectrometry

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Abstract

A new method has been developed for the simultaneous measurement, in a reduced plasma sample, of concentration and ^{13}C-isotopic enrichment of acetic, propionic, butyric, lactic, acetoacetic and beta-hydroxybutyric acids by gas chromatography coupled to mass spectrometry. After plasma deproteinisation, a diethylic extraction and a *N-tert.*-butyldimethylsilyl-*N*-methyltrifluoroacetamide derivatisation were performed. Both diethyl extraction and derivatisation procedures were optimised using the central composite designs methodology. The optimised method provides good linearity, intra-day and within-day repeatability. Except for beta-hydroxybutyric (49 μM) and acetoacetic acid (5 μM), detection limits were ranging between 0.2 and 0.7 μM allowing uses of this method for colonic metabolism studies.

Keywords: ^{13}C Enrichment; Short-chain fatty acids; Lactic acid; Ketone bodies

1. Introduction

Many in vitro studies have shown that short-chain fatty acids (SCFA) and related compounds (ketone bodies (KB) and lactic acid) are involved in many metabolic process and particularly colonic cell metabolism. End-products of the microbial cæco-colonic fermentation of carbohydrates, SCFA—mainly acetic, propionic and butyric acids—are known to have beneficial properties for the hindgut [1]. Also indicators of bacterial activity, their quantification in many biological matrices is of great interest in nutrition, bacteriological and also environmental studies [2]. The lactic acid, that is produced by gut microflora as well as by the host, is frequently quantified for diagnosis of metabolic diseases [3]. Mainly produced in the liver, ketone bodies (betahydroxybutyric and acetoacetic acids) are also synthesised in the colonic cells from butyric acid, providing energy for the colonocyte and sub-

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strates for its cellular building [4]. Up to now, all these different molecules have been quantified separately using individual time consuming procedures which require a large volume of sample.

As the SCFA are indispensable for the colonic health maintenance [5–9], the understanding improvement of their in vivo colonic metabolism appears to be of great interest in nutrition as well as in medicine. In order to investigate this metabolism, we developed a rat model using ^{13}C-enriched SCFA infusions (sodium salt) in the large intestine. Infused acetic and propionic acids cross the mucosa and directly reach the portal vein whereas butyric acid (the main fuel for the colonic cells [10,11]) is partly oxidised into KB in the colonocyte [4]. To estimate the colonic mucosal metabolism, we needed to measure the concentrations and the ^{13}C-enrichments of SCFA, KB and lactic acid in portal plasma samples. These determinations had to be performed simultaneously on a few volume of plasma, as the chosen animal model allowed only restricted blood sampling. Furthermore, measurements of isotopic enrichment imposed the use of a mass selective detector.

Generally, plasma determination of SCFA is carried out by several techniques such as enzymatic (only for acetic acid) [12–14], liquid chromatography [15,16], gas chromatography (GC) [17,18] and gas chromatography–mass spectrometry (GC–MS) [19–21]. Despite the fact that a study has reported a case of direct plasma GC injection [22], proteins are usually excluded from the plasma by acidic treatment with perchloric [23], metaphosphoric [24] or sulfosalicylic [21,23,24] acids. Then, the deproteinised plasma can be injected directly in the GC column [24]. Most often, after deproteinisation, SCFA are directly extracted with dichloromethane [25] or diethyl ether [17,18,20,21], or sometimes by vacuum distillation [26,27]. Then, except in few studies [24,28], the injection is usually preceded by a derivatisation step using some reagents such as bromoacetophenone [25], difluoroaniline [29], phenyldiazomethane [30], methylderivatives [19,31] and butylderivatives [19] or 1-(*tert*.-butyldimethylsilyl)imidazole (TBDMS) derivatives [18,20,21]. Risks of contamination and ghost peaks are sometimes limiting factors during SCFA analysis especially the one of acetate [20,24].

Plasma lactic acid and KB determinations by GC–MS technique are less documented, their quantification being generally based on enzymatic techniques. However, phenylbutyryl [32], methyl derivatives of lactic acid [3] and TBDMS derivatives of KB [33–35] have been quantified by GC–MS.

Our aim was to develop the most accurate method allowing the simultaneous determination of plasma SCFA, lactic acid, and KB by GC–MS in a small volume of plasma, using TBDMS derivatives which have been described to be appropriate for KB determinations by GC–MS [33–35].

Several preliminary assays have indicated that both extraction and derivatisation steps were the most critical one. Consequently, the aim of the present work was to optimise them by using the central composite design (CCD) methodology, this taking into account the simultaneous variation of several variables. Based on an enlarged two-levels full factorial design, it allows the realisation of three-dimensional response-surface to determine the optimal conditions for the studied factors (in our case: extraction and derivatisation procedures), using the minimal number of assays [36,37]. Then, the optimised method has been validated.

2. Experimental

2.1. Chemicals

All molecules of interest as sodium acetate, sodium propionate, sodium butyrate, sodium (±)3-hydroxybutyrate (β-OH-butyrate), lithium lactate(DL), and lithium acetoacetate were obtained from Sigma–Aldrich (St Quentin-Fallavier, France). Hydrochloric acid (37% in water, w/v), 5-sulfosalicylic acid, diethyl ether, *N-tert*.-butyldimethylsilyl-*N*-methyltrifluoroacetamide, 2-ethylbutyric and 4-methylvaleric acids were also purchased from Sigma–Aldrich. [1-^{13}C] acetic and propionic acids (sodium salts) were obtained from Euriso-top™ (Gif sur Yvette, France) while [1-^{13}C] butyric acid (sodium salt) was from Mass Trace™ (Woburn MA, USA). A sterile water for medical irrigation (B-Braun, Melsungen, Germany) was used for preparation of the solutions to avoid risks of acetate contamination.

2.2. Biological materials

Rat (Harlan, Gannat, France) blood samples were taken out in an heparinized tube according to the French animal welfare rules. After centrifugation (2100 *g*, 10 min) plasma samples were collected and deproteinised with 10% (v/v) of a 20% (w/v) 5-sulfosalicylic acid solution. Then, samples were centrifuged at 19120 *g* for 45 min and the supernatant was frozen at −80 °C until analysis.

2.3. Analytical procedure

Then, 400 μl of deproteinised plasma were transferred into a 4 ml clear vial with solid cap with PTFE liner (Supelco, St Quentin-Fallavier, Fance) and spiked with 25 μl of a solution containing two internal standards (IS): 2-ethylbutyric acid (200 m*M*)(IS 1) and 4-methylvaleric acid (2 m*M*)(IS 2). After acidification with 10 μl of hydrochloric acid (37%), a double extraction procedure was performed with diethyl ether. The volume of diethyl ether [V_{ether}] and the vortexing duration [$T_{agitation}$] were two of the variables optimised with CCD. After the vortex step, the two phases were separated by centrifugation (765 *g*, 3 min). The upper organic layer was transferred into another clear vial and a new ethyl extraction was performed on the aqueous layer. After complete recovery of the organic layer, 500 μl were placed in a chromatographic vial and added, at room temperature, with *N-tert*.-butyldimethylsilyl-*N*-methyltrifluoroacetamide (MTBSTFA) as derivatisation reagent (volume of MTBSTFA [$V_{MTBSTFA}$] and derivatisation duration [$T_{derivatisation}$] were the last two variables optimised with CCD).

2.4. Gas chromatography–mass spectrometry conditions

Derivatisated samples (2 μl) were injected into a gas chromatograph (HP 5890) coupled with a mass spectrometer detector (HP-5989A, Hewlett-Packard, Palo Alto, CA, USA). Analysis were carried out in a splitless mode on an OV-1 capillary column (30 m×0.25 mm, 0.25 μm film thickness, Interchim, Montluçon, France) using electronic impact (70 eV) as ionisation mode and selected ion monitoring acquisition mode. The column head-pressure was 12 p.s.i. Injector, source and quadrupole temperatures were 250, 280 and 150 °C, respectively. The GC oven was programmed as follow: 40 °C held for 0.1 min, increased to 70 °C at 5 °C min^{-1}, 70 °C held for 3.5 min, increased to 160 °C at 20 °C min^{-1} and finally increased to 280 °C for 3 min at 35 °C min^{-1}. The total run time was 20.53 min. Retention times, Dwell times and *m*/*z* monitored for each molecules are shown in Table 1.

The reaction of MTBSTFA with active hydrogen atoms on oxygen of acetic, propionic, butyric acids (and of the two internal standards) formed TBDMS derivatives. Acetoacetic and β-OH-butyric acids [34], as well as lactic acid, were consequently bi-derivatives. As previously described [34], a double peak was observed for acetoacetic acid, corresponding to isomers due to 2,3 double bond of derivatives [34].

In each case, the monitored ion (Table 1) corres-

Table 1
Retention times, Dwell time and monitored ion for each target molecule

Molecule	Molecular weight (g/mol)	Retention time (min)	Dwell (ms)	*m*/*z*
Acetic acid	60	6.5	200	117[a], 118
Propionic acid	74	9.7	210	131[a], 132
Butyric acid	88	11.8	210	145[a], 146
Lactic acid	90	15.7	130	261[a], 262, 263
β-OH-butyric acid	104	16.3	130	275[a], 276, 277
Acetoacetic acid	102	16.5 and 16.7	130	273[a], 274, 275
Internal standard 1 (IS1)	116	13.9	430	173[a]
Internal standard 2 (IS2)	116	14.2	430	173[a]

[a] $[M-57]^+$.

ponded to the loss of a *tert*.-butyl fragment [34] from the molecular ion ($\Delta m = 57$ units).

For acetic, propionic and butyric acids, the ions at m/z 118, 132 and 146 have also been monitored to evaluate the ^{13}C-enrichment. According to the known colonic metabolism of the butyric acid [4], it appeared that lactic, β-OH-butyric and acetoacetic acids could be ^{13}C-enriched on any, one (1 or 3) or two (1 and 3) carbon atoms. Consequently, the mass (M), (M + 1) and (M + 2) have been monitored as it has been described in Table 1.

2.5. Method optimisation with experimental design

Four different experimental variables have been optimised using the central composite experimental design method. The first experimental design (CCD1) concerned the volume of diethyl ether [V_{ether}] and the vortexing duration [$T_{agitation}$] during the extraction step, whereas the second one (CCD2) focused on the volume of MTBSTFA [$V_{MTBSTFA}$] and duration [$T_{derivatisation}$] during the derivatisation step.

On the basis of preliminary results, the range of V_{ether}, $T_{agitation}$, $V_{MTBSTFA}$, $T_{derivatisation}$ were chosen between 1 and 6.5 ml, 5 and 25 min, 30 and 70 μl, 0.5 and 4 h, respectively. Details about the ranges used in CCD1 and CCD2 are described in Table 2. The −1, 0 and +1 values represented the low, central and high levels chosen for each factor whereas $-x$ and $+x$ corresponded to minimal and maximal extreme levels called ''star points''. Thirteen assays were randomly conducted for each CCD. Four experiments corresponded to the low and high levels of the factors and referred to a complete 2^2 factorial design. Four ''star points'' assays allowed the study of the response outside of the complete 2^2 factorial design limits and were necessary to calculate the three-dimensional response-surfaces. Finally, five repetitive trials in the centre point estimated the response variability and the effects significance. The studied response was the ratio of each analyte to I.S.

2.6. Calculations

For acetic, propionic and butyric acids, concentration values were calculated considering the ratio of (M) + (M + 1) areas to the corresponding internal standard, while lactic, β-OH-butyric and acetoacetic acids concentration values were calculated with the ratio of (M) + (M + 1) + (M + 2) areas to the corresponding internal standard. Depending on their physiological plasma levels, acetic, propionic, butyric, β-OH-butyric and acetoacetic acids concentrations values were calculated in using I.S.1 whereas I.S.2 was used for lactic acid.

The ^{13}C-enrichment was calculated with the following ratios:

[1-^{13}C] acetic, propionic and butyric acids: (M + 1) to (M) + (M + 1)

[1-^{13}C] or [3-^{13}C] β-OH-butyric, acetoacetic and lactic acids: (M + 1) to (M) + (M + 1) + (M + 2)

[1,3-^{13}C] β-OH-butyric, acetoacetic and lactic acids: (M + 2) to (M) + (M + 1) + (M + 2)

Calculations of the three-dimensional response-surfaces and analysis of variance related to the estimated linear and quadratic effects and their interactions were performed using Statistica Software v.5 (Statsoft, Maison-Alfort, France).

Table 2
Factor levels of the central composite design CCD 1 and CCD 2

Central composite design 1						Central composite design 2					
Factor	Level					Factor	Level				
	$-x$	−1	0	+1	$+x$		$-x$	−1	0	+1	$+x$
V_{ether}	1	2	3.5	5	6.5	$V_{MTBSTFA}$	30	40	50	60	70
$T_{agitation}$	5	10	15	20	25	$T_{derivatisation}$	0.5	1	2	3	4

Table 3
Experiments and results obtained for the central composite design 2

Assay	$V_{MTBSTFA}$ (μl)	$T_{derivatisation}$ (h)	Relative signal intensity (Analyte/internal standard)					
			Acetic acid	Propionic acid	Butyric acid	Lactic acid	β-OH-butyric acid	Acetoacetic acid
1	40	1	0.3093	1.0508	2.4541	1.4404	2.6013	1.2839
2	40	3	0.3537	0.8486	2.1759	2.3080	1.8435	1.8939
3	60	1	0.2253	0.8417	0.8670	2.0210	2.5423	1.1428
4	60	3	0.2779	0.8285	2.3606	2.6294	2.2527	1.6592
5	30	2	0.4220	1.1295	2.4504	2.0492	1.9739	1.3628
6	70	2	0.2265	0.8474	0.8391	2.0984	2.5767	0.9653
7	50	0.5	0.2335	0.8377	2.2117	0.9893	3.1576	1.5250
8	50	4	0.3252	0.8411	2.2397	2.9788	1.9755	0.8802
9	50	2	0.3129	0.9390	2.5578	2.7920	2.4674	1.0609
10	50	2	0.2946	0.8408	2.4111	2.8103	2.1505	1.1400
11	50	2	0.3026	0.8301	2.3269	2.6808	2.1363	1.1830
12	50	2	0.3103	0.8581	2.3910	3.1363	2.0653	1.2852
13	50	2	0.3324	0.8678	2.4061	3.2960	2.2445	1.2634

3. Results

3.1. Method optimisation

3.1.1. Volume of diethyl ether and agitation duration

Concerning the first CDD, no statistical significant effect was found for the variables V_{ether} and $T_{agitation}$. Nevertheless, some tendencies appeared, which confirmed the preliminary observations. Effectively, the optimal diethyl ether volume for the extraction tended to be between 3.5 and 5 ml, while the vortex duration required to obtain a higher simultaneous extraction of the six compounds was on the centre point (15 min). After having confirmed that two successive extractions with 2 ml diethyl ether each provided better extraction efficiency than a single one using 4 ml of solvent, we fixed the final conditions as follows: the optimised simultaneous extraction of the six analytes was based on two successive extractions with 2 ml diethyl ether each followed by 15 min of agitation.

3.1.2. Volume of MTBSTFA and derivatisation duration

As in CCD1, the ratio of each analyte to the internal standard was compared between 13 experiments (Table 3).

An analyse of variance was performed in order to estimate the linear and quadratic effects of the two factors ($[V_{MTBSTFA}]$ and $[T_{derivatisation}]$) and their interaction (Table 4).

Table 4
Estimation of the linear and quadratic effects of each factor and their interaction

		Estimated effect					
		Acetic acid	Propionic acid	Butyric acid	Lactic acid	β-OH-butyric acid	Acetoacetic acid
$V_{MTBSTFA}$	Linear	−9.201[a]	−4.185[a]	−5.000[a]	0.628	3.043[a]	−0.929
	Quadratic	0.950	3.268[a]	−4.674[a]	−2.510[a]	0.378	−0.032
$T_{derivatisation}$	Linear	5.241[a]	−1.374	2.265	4.145[a]	−6.445[a]	0.193
	Quadratic	−3.194[a]	−0.008	−2.064	−3.307[a]	3.484[a]	−0.182
Interaction	Linear	0.422	2.090	4.239[a]	−1.137	1.299	−0.121

[a] Indicate a significant effect with $P<0.05$, ANOVA.

Effects attributed to [$V_{MTBSTFA}$] and [$T_{derivatisation}$] (Table 4) differed between the six analytes (those who have a positive sign indicate that the response increases when increasing the value of the considered factor whereas it is the opposite when they have a negative sign). This method being intended to mainly allow the study of the colonic metabolism of butyric acid in rats, we consequently focused the optimisation on the butyric acid. We then verified that the chosen parameters allowed the analysis of the other analytes in satisfactory conditions. So, the choice of the optimal [$V_{MTBSTFA}$] and [$T_{derivatisation}$] was based on the response surface obtained for butyric acid (Fig. 1).

The volume of MTBSTFA used for the formation of all TBDMS derivatives ($V_{MTBSTFA}$) was fixed at 50 μl. The determination of the optimal delay between derivatisation and GC injection ($T_{derivatisation}$) was difficult because of the variability between the different analytes: some of them increased (lactic acid for example) whereas others rapidly decreased (β-OH butyric acid). When considering the response-surface of each analyte, we found that the best duration of derivatisation for the simultaneous analysis of the six compounds was 1 h. A specimen chromatogram is indicated in Fig. 2.

3.2. Method validation

This method was then validated under the experimental optimised conditions (extraction with 2× 2 ml of diethyl ether followed by a 15 min agitation/ 50 μl of MTBSTFA and 1 h of derivatisation) on standards samples. The validation protocol focused on the measurement of the linearity, repeatability, reproducibility, accuracy, detection and quantification limits.

The linearity was assessed by plotting the ratio of the concerned molecule to the corresponding internal standard against the molecule concentration. For all the six analytes, results were linear in the range of physiological values in rat plasma (Table 5).

The repeatability, expressed as the signal relative standard deviation (RSD), was evaluated on the basis of eight analysis on a same single sample during the same day (intra-day repeatability), and on different days (within-day reapeatability). As indicated in Table 5, intra-day repeatability was tested at three different levels of concentration for each analyte (low, mean, high levels) whereas the within-day reapeatability corresponded to the mean level of concentration. Acceptance level considering RSD less than 5% were all satisfactory (Table 5).

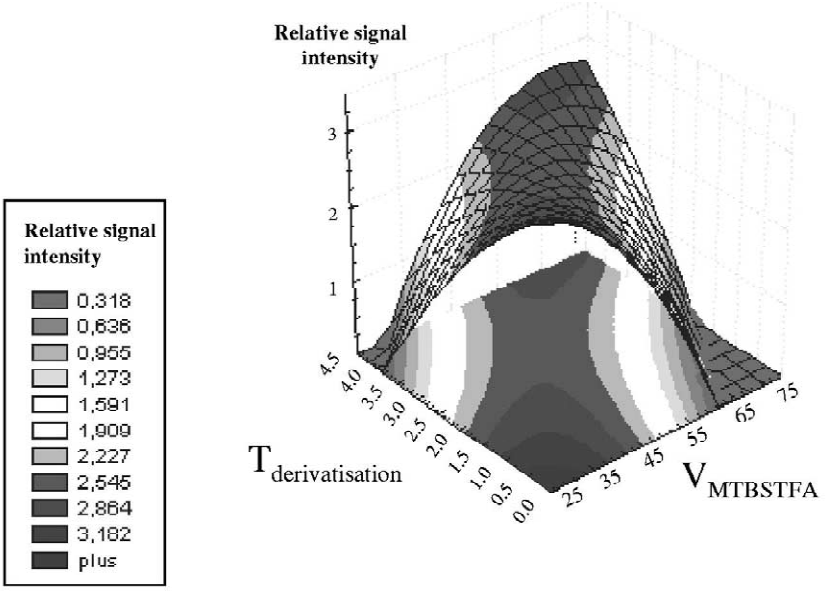

Fig. 1. Three-dimensional response–surface plots of $V_{MTBSTFA}$ vs. $T_{derivatisation}$ obtained for butyric acid.

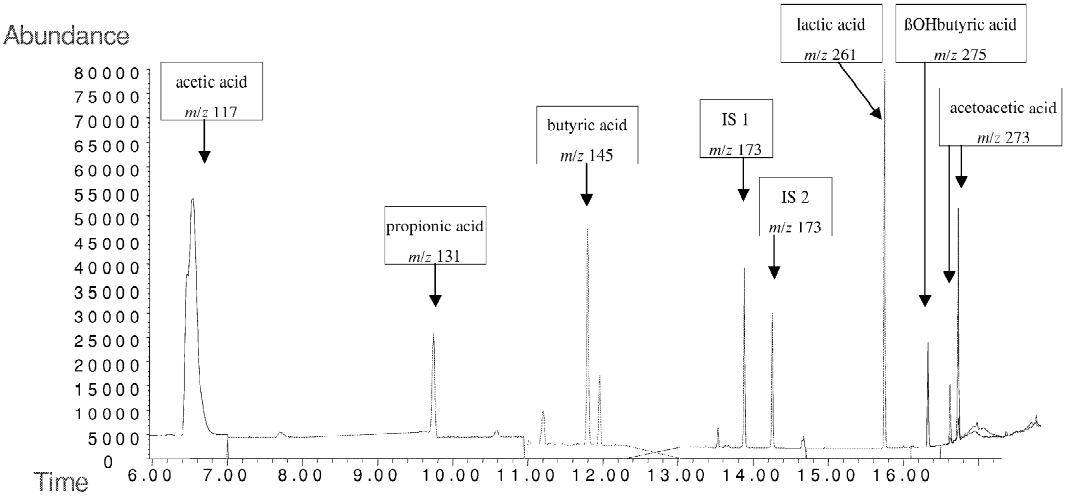

Fig. 2. Single ion monitoring chromatogram of TBDMS derivatives of acetic acid ($m/z=117$), propionic acid ($m/z=131$), butyric acid ($m/z=145$), lactic acid ($m/z=261$), beta-OH-butyric acid ($m/z=275$), acetoacetic acid ($m/z=273$) and internal standards (IS1 and I.S.2, $m/z=173$) using impact electronic gas chromatography–mass spectrometry analysis.

The method accuracy was estimated by adding known quantities of each analyte in one sample and calculating each percentage of recovery. Low and high levels of added quantities were tested (Table 5).

The limit of detection (LOD) and the limit of quantification (LOQ) corresponding to a signal-to-noise value of 3 and 10, respectively, were evaluated for the six analytes (Table 5).

Calibration curves for [1-^{13}C] acetic, [1-^{13}C] propionic and [1-^{13}C] butyric acids shown linear results: $r^2=0.981$ from 0 to 20% (isotopic enrichment) for [1-^{13}C] acetic, $r^2=0.997$ from 0 to 20% for [1-^{13}C] propionic acid and $r^2=0.993$ from 0 to 40% for [1-^{13}C] butyric acid.

All these data support the suitability of the present method for its application in physiological studies.

Table 5
Results of the validation of the method

Validation parameters	Analyte (acids)					
	Acetic	Propionic	Butyric	Lactic	βOH-butyric	Acetoacetic
Linearity interval (μ*M*)	0-3500 ($r^2=0.999$)	0-1100 ($r^2=0.998$)	0-1000 ($r^2=0.999$)	0-1700 ($r^2=0.995$)	0-3500 ($r^2=0.971$)	0-3500 ($r^2=0.985$)
Intra-day repeatability RSD, % (concentration, μ*M*)	4.8% (100) 4.3% (1500) 4.7% (3200)	2.6% (50) 2.4% (500) 3.2%(1000)	2.7% (50) 2.6% (500) 2.4% (1000)	3.3% (100) 3.0% (850) 3.8% (1500	3.9% (100) 3.7% (1500) 4.0% (3200)	3.8% (100) 3.4% (1500) 3.9% (3200)
Within-day repeatability (RSD, %)	4.0	3.8	2.9	4.7	4.3	4.3
Limit of detection (μ*M*)	0.2	0.3	0.3	0.7	49.0	5.0
Limit of quantification (μ*M*)	0.8	1.0	1.0	2.5	73.0	64.1
Accuracy: added known quantity in nmoles (and recovery in %)	17.6 (96.2) 52.4 (96.1)	17.6 (99.2) 52.4 (96.9)	17.4 (98.8) 34.9 (102.6)	737 (104.3) 2210 (97.2)	856 (103.7) 1713 (96.2)	2500(100.3) 5000 (96.7)

4. Discussion

Up to now, SCFA and KB were currently quantified separately by different methods, hence time consuming and requiring large sample volume. In order to study, using a rat model, the colonic metabolism of SCFA and among them, butyric acid—the preferred energy providing substrate for the colonic epithelial cells [10,11]—using stable isotopes, we were aiming to develop and validate a new method for simultaneous measurements of concentrations and ^{13}C-enrichments of acetic, propionic, butyric acids (SCFA), lactic acid, β-OH-butyric and acetoacetic acids in only 400 μl of deproteinised plasma sample.

Due to the different physico–chemical properties of the six target molecules, this method could not determine each of them in optimal conditions. As a result, we had to choose the best compromise for the simultaneous determination of SCFA, lactic acid and KB by GC–MS.

The selected parameters provided linear, sensitive, repeatable and reproducible results after a rapid and easy sample treatment. Moreover, the repeatability for propionic and butyric acids was higher than previous GC–MS methods [21]. The limit of detection of KB seemed to be about 100 times less important in comparison with other methods (10 nmoles for β-OH butyric acid) [35]. Additionally, the rapidity and simplicity of the sample treatment associated with a short-time GC–MS run, allowed the analysis of 55 plasma samples per day. Part of the considerable time gain was related to the derivatisation duration which was only of 1 h, whereas TBDMS ketone bodies derivatives were previously obtained after incubation at room temperature overnight [33] or 12–24 h [34]. Preliminary assays concluded that heating samples during the derivatisation step worsened results especially for KB. Therefore, the derivatisation temperature was not included in the CCD model. Meanwhile, the laboratory ambient temperature was maintained at 20±2 °C during the derivatisation procedures.

We did not observe any memory effect or ghost peaks (even for acetic acid [24]) in our derivatisation conditions, and no cleaning solution run was required after heating the column for 3.5 min at 280 °C. In order to avoid the risk of acetate contamination, it is necessary to work in a reserved place in which acetic reagents are forbidden, utilise selected chemical reagents only and finally, making blanks samples before each batch of analysis.

We have specifically used two internal standards to avoid difficulties induced by the high heterogeneity of concentration levels of SCFA, lactic acid and KB in rat plasma. This last point should be adapted for other quantifications. The determination of ^{13}C-enrichments was based on [1-^{13}C] acetic, propionic and butyric acids calibration curves. The [1-^{13}C] butyric acid metabolism in the colonic cell is known to provide [1 or 3-^{13}C] KB or [1,3-$^{13}C_2$] KB. But, to our knowledge, these ^{13}C-isotopic KB are not all available and must be synthesised from isotopic enriched ethylacetoacetate [34,38,39]. As far as the ^{13}C-enrichment of KB is concerned, only comparisons between different groups of animals will be done in our future study. Consequently, these types of comparative (and not quantitative) studies do not require isotopic calibration curves. Nevertheless, this point will have to be adapted if this method is used for other types of studies.

With this present method, we dispose at present of a reliable mean to study in vivo the way in which the rat colonic mucosa is able to utilise butyric acid from digestive origin for its own requirements, and secondly, to follow butyric acid different colonic metabolic pathways.

In conclusion, we have used in the present work, the Central Composite Design method to obtain a rapid optimisation of the simultaneous analysis of plasma concentration and ^{13}C-enrichment of SCFA, lactic acid and KB by GC–MS in a restricted volume of sample. This method seems suitable for stable isotopes studies on SCFA in vivo metabolism on small animal models, this topic being of great interest in nutrition.

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