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Short communication

Determination of carnitine, its short chain acyl esters and metabolic precursors trimethyllysine and γ-butyrobetaine by quasi-solid phase extraction and MS/MS detection

Frank Hirche*, Maren Fischer, Janine Keller, Klaus Eder

Institute of Agricultural and Nutritional Sciences, Martin Luther University Halle-Wittenberg, Von-Danckelmann-Platz 2, 06120 Halle/S., Germany

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ABSTRACT

For the investigation of the metabolism and biosynthesis of carnitine, sensitive determination of carnitine and its metabolic precursors, trimethyllysine and γ-butyrobetaine, is required. We present here a new simplified method for the analysis of carnitine, its acetyl- and propyl esters, as well as trimethyllysine and γ-butyrobetaine without need for derivatization reactions by means of normal-phase LC and electrospray ionization tandem mass spectrometry. The limits of quantification were between 5 nM for acetyl carnitine and 70 nM for carnitine. Relative standard deviations in a fivefold determination of standard solutions were between <2% for carnitine and <10% for trimethyllysine. Quantifying the formation of deuterated carnitine from deuterated γ-butyrobetaine, this method is also suitable for the determination of the activity of γ-butyrobetaine dioxygenase in tissues.

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1. Introduction

Carnitine (L-3-hydroxy-4-N,N,N-trimethylaminobutyrate) is an essential metabolite in mammals, which has a number of indispensable functions in intermediary metabolism. The most prominent function lies in its role in the transport of activated long-chain fatty acids from the cytosol to the mitochondrial matrix where β-oxidation takes place. Carnitine biosynthesis involves a complex series of reactions. Lysine in protein peptide linkage provides the carbon backbone of carnitine. It undergoes methylation of the ε-amino group to yield trimethyllysine, which is released upon protein degradation. The released trimethyllysine is further oxidised to γ-butyrobetaine, which is then hydroxylated in liver and kidney by γ-butyrobetaine dioxygenase to form carnitine [1,2]. To date, the regulation of carnitine biosynthesis in mammals is not completely understood. In order to get more insight into carnitine metabolism and synthesis, a sensitive analysis for the determination of the concentrations of free carnitine, carnitine esters and the carnitine precursors trimethyllysine and γ-butyrobetaine is required. Currently, carnitine is mostly analyzed by LC–MS/MS methods [3–8]. The use of multi-reaction mode (MRM) transitions for detection allows the determination of underivatized carnitine and acylcarnitines with sufficient sensitivity [6]. The alternative use of the acid catalyzed esterification of the carboxylic group of carnitines with butanol has the risk of the reduction of the original amount of acylcarnitines [9]. The determination of trimethyllysine by MS/MS is complicated by the existence of the isobaric compound homoarginine with a similar fragmentation behavior. Terada et al. [10] and van Vlies et al. [11] solved this problem by propylation of the carboxylic groups and acetylation of the amino groups prior to the analysis and could thus discriminate both compounds through the number of functional groups.

Vaz et al. [12] reported a MS/MS method for the simultaneous determination of carnitine and its precursors trimethyllysine, 3-hydroxy-trimethyllysine and γ-butyrobetaine which was based on the derivatization of primary, secondary, and tertiary amino groups in the sample by methyl chloroformate and subsequent extraction with ethyl acetate and ion pair chromatography using heptafluorobutyric acid. However, these authors did not determine the concentrations of the short chain carnitine esters such as acetyl- or propionyl carnitine. Other methods which are able to determine carnitine, its short chain esters and its metabolic precursors,

* Corresponding author. Tel.: +49 345 55 22709; fax: +49 345 55 27124.
E-mail address: frank.hirche@landw.uni-halle.de (F. Hirche).

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Table 1
Elution profile for SPE-MS/MS coupling and column regeneration.

Time (min)	Solvent A[a] (%)	Solvent B[a] (%)	Flow (μL/min) pump 1	Diverter valve to API 2000[b]	Time (min) pump 2	Flow (μL/min) pump 2[b]
0.0	100	0	200	Open	0	0
0.5	100	0	200	Closed	0.5	100
1.0	100	0	200	Closed	4.0	100
1.1	100	0	400	Closed	4.1	200
3.0	100	0	400	Closed	6.7	200
3.1	0	100	400	Closed	6.8	20
5.0	0	100	400	Closed	10.5	20
5.1	100	0	400	Closed	10.6	200
6.1	100	0	300	Closed	16.0	200
6.3	100	0	200	Open	16.1	100
10.8	100	0	200	Closed	18.6	100
11.0	100	0	200	Closed	19.0	0
11.2	100	0	300	Closed		
13.0	100	0	450	Closed		
15.0	100	0	550	Closed		
18.7	100	0	550	Closed		
18.8	100	0	200	Open		
19.0	100	0	200	Open		

[a] Solvent A: methanol; solvent B: methanol + water + acetonitrile + acetic acid (100 + 90 + 9 + 1).
[b] In case of “open valve” the eluent from the column was analyzed by the mass spectrometer, in case of “closed valve” the source was flushed with methanol by the second pump.

trimethyllysine and γ-butyrobetaine have not yet been reported. Therefore, it seemed usefully to develop a method which is able to determine concentrations of free carnitine, its esters and precursors which does not need chemical derivatizations and ion pair reagents. Starting from the trial to couple the solid phase extraction on silica gel as proposed by van Kempen and Odle [13] and Janssens et al. [14] and MS experiments, we developed a simple and sensitive method for the measurement of carnitine, short chain acyl carnitines as well as the precursors in the biosynthesis of carnitine trimethyllysine and γ-butyrobetaine by electrospray MS/MS. As examples for its applicability, we present data about concentrations of these compounds in human plasma and pig tissues, and activities of γ-butyrobetaine dioxygenase in various tissues of pigs.

2. Experimental

2.1. Chemicals and reagents

Carnitine, homoarginine, trifluoroacetic acid, and acetone were obtained from Fluka (Buchs, Switzerland); acetyl carnitine was obtained from Lonza (Basel, Switzerland). Dimethyl sulfate-d_6, lysine, trimethyllysine, butyrobetaine, bovine serum albumine, Triton X-100, ascorbic acid, ammonium iron sulfate, α-ketoglutarate, 4-(dimethylamino) butyric acid, propionyl chloride, acetone, acetic anhydride, and ethyl acetate were supplied by Sigma–Aldrich (Deisenhofen, Germany). Iodomethane-d_3 was a product of Acros Organics (Geel, Belgium).

Carnitine-d_3 (N-methyl-d_3) was supplied by Cambridge Isotope Laboratories (Andover, MA), acetyl-(2,2,2)-d_3 carnitine and propionyl-(3,3,3)-d_3 carnitine were products of Larodan Fine Chemicals (Malmö, Sweden). Propionyl carnitine, trimethyllysine-d_9, and γ-butyrobetaine-d_3 were synthesized as described in literature [12,15]. Carnitine-d_3 was acetylated by mixing 20 μL of a 2.9 mM aqueous solution with 200 μL acetic anhydride and incubated at room temperature over night in order to get acetyl carnitine-d_3. Methanol and acetonitrile (both HPLC grade) were purchased from Mallinckrodt Baker (Deventer, Netherlands). Acetic acid, MOPS, potassium chloride, and DTT were from Roth (Karlsruhe, Germany), and potassium phosphate and sodium chloride from VWR International (Darmstadt, Germany). Ultrapure water was obtained by means of a TKA apparatus (TKA, Niederelbert, Germany). Concentration of nondeuterated standard solutions was measured via the nitrogen content (EuroVector, HEKAtech, Wegberg, Germany). The concentrations of stock solutions of

Table 2
Parameters for MS/MS-detection of carnitine and related compounds.

Compound	Mass transition (m/z)	DP (V)	FP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
Carnitine	162.2/103.1	26	370	5	10	23	4
Acetyl carnitine	204.2/85.1	21	370	7.5	14	27	8
Propionyl carnitine	218.2/85.1	21	360	11	12	29	8
γ-Butyrobetaine	146.2/87.0	26	370	7	10	21	0
Trimethyllysine	189.1/84.1	16	370	10.5	12	29	0
Homoarginine	189.1/84.1	16	370	10.5	12	29	0
Homoarginine	189.1/172.1	21	370	11.5	10	19	0
Carnitine-d_3	165.2/103.1	26	370	8	10	25	4
Acetyl-d_3 carnitine	207.2/85.1	16	370	8.5	14	27	8
Propionyl-d_3 carnitine	221.0/85.1	21	280	6.5	14.1	29	2
γ-Butyrobetaine-d_3	149.3/87.2	26	370	10	8	25	0
Trimethyllysine-d_9	198.3/84.1	21	370	10.5	12	29	0
Acetyl carnitine-d_3	207.2/145.2	26	350	10.5	12	21	0
Acetyl-d_3 carnitine	207.2/148.2	26	350	10.5	12	19	0

As internal standards for concentration calculation of the corresponding native compounds carnitine-d_3, acetyl-d_3 carnitine (207.2/85.1), propionyl-d_3 carnitine, γ-butyrobetaine-d_3 and trimethyllysine-d_9 were used. Furthermore acetyl-d_3 carnitine (207.2/85.1) served as internal standard for the determination of carnitine-d_3 during the determination of butyrobetaine dioxygenase activity. General parameters for ionization and fragmentation were: curtain gas = 345 kPa, gas1 = 276 kPa, gas2 = 207 kPa, collision gas = 55 kPa. *Abbreviations*: CE, collision energy; CEP, cell entrance potential; CXP, cell exit potential; DP, declustering potential; EP, entrance potential; FP, focussing potential.

γ-butyrobetaine-d_3 and of carnitine-d_3 were determined spectrophotometrically according to Wall et al. [16].

2.2. Instrumentation and chromatographic conditions

For LC–MS/MS analysis a 1100-er series HPLC with two pumps (Agilent Technologies, Waldbronn, Germany) and a Kromasil 100 Si column, 5 μm, 125 mm × 2 mm (CS-Chromatographie Service, Langerwehe, Germany) at 20 °C coupled to an API 2000 LC–MS/MS-System (Applied Biosystems, Darmstadt, Germany) was used. The eluents were methanol and methanol + water + acetonitrile + acetic acid (100 + 90 + 9 + 1). The eluate was analyzed from 6.3 min to 10.8 min (Table 1). For detection the eluate was partially evaporated at 350 °C and the analytes were ionized by positive ion (5500 V) electrospray (further parameters for API 2000 see Table 2). For instrument control and analysis of the chromatographic data, the program Analyst 1.4.2 (Applied Biosystems, Darmstadt, Germany) was used.

2.3. Sample materials

As test samples human plasma (obtained from three male healthy volunteers) and various tissues (liver, kidney, ileum, brain, spleen, muscle, heart, colon and lung—collected from 4 pigs of a body weight range between 60 and 70 kg) were used.

2.4. Sample preparation

Standard solutions contained 4% (w/v) BSA in 0.9% (w/v) sodium chloride. Samples were prepared in modification to van Kempen and Odle [13]. 20 μL of plasma or standard solution were mixed with 100 μL methanol containing the internal standards (deuterated analoga), incubated for 5 min at 4 °C and centrifuged (16,000 × *g*, 10 min, 4 °C) and the supernatant was used for further analysis. 150 mg tissues were freeze dried, homogenized and extracted with 500 μL water + methanol (2 + 1) first in an ultrasonic bath for 20 min and then under shaking at 50 °C for 30 min. After the centrifugation (13,000 × *g*, 10 min) 20 μL of the supernatant were mixed with 100 μL methanol containing the internal standards, incubated for 10 min and centrifuged (16,000 × *g*, 10 min) before analysis.

2.5. Determination of the activity of γ-butyrobetaine dioxygenase in pig tissues

Tissue homogenates and reaction mixture for the measurement of the enzyme activity were prepared as described by van Vlies et al. [17]. After 5, 10, 15, and 20 min of incubation, a volume of 20 μL was taken from the assays and handled like plasma samples. Activity of γ-butyrobetaine dioxygenase was calculated from the amount of carnitine-d_3 formed during the assay. Acetyl-d_3 carnitine was used as internal standard. Under the reaction conditions used, no enzymatic acetylation of the carnitine-d_3 was detected, based on the mass transition 207.2/145.2 *m*/*z* which is specific for acetyl carnitine-d_3.

3. Results and discussion

Besides free carnitine the two quantitative most important acyl esters and the carnitine precursors trimethyllysine and γ-butyrobetaine had to be analyzed quantitatively. For the isobaric compounds trimethyllysine and homoarginine the daughter ions 84.1, 130.1 *m*/*z* and 84.1, 130.1, 172.1 *m*/*z*, respectively, were observed. The mass transition of 189.1/172.1 *m*/*z* which was specific for homoarginine could be used as control for the development and the stability of the chromatographic process. In difference to the conventional solid phase extraction, which is considered as a binary process, we could observe a sufficient separation of homoarginine (R_t = 6.71 min) and trimethyllysine (R_t = 7.93 min) by an easy two solvent (mixture) extraction (Table 1, Fig. 1). Furthermore, we could regenerate completely our solid phase in the HPLC column, whereas for solid phase extraction disposable columns are used often. Thus we term this procedure “quasi-solid phase extraction”. Due to the chromatographic separation of trimethyllysine and homoarginine, the chemical derivatization reactions as used by Terada et al. [10] and Vaz et al. [12] were not required. Fig. 1 shows chromatograms obtained from a plasma sample spiked with homoarginine. Resulting from the observed retention times, data were collected between 6.3 and 10.8 min. For further optimization the method could be adapted to the combination of two parallel columns (running each for 11 min, Fig. 1) with a column switch valve (Fig. 2) and the use of the second pump (with a modified time table in contrast to Table 1) for reequlibration of the columns.

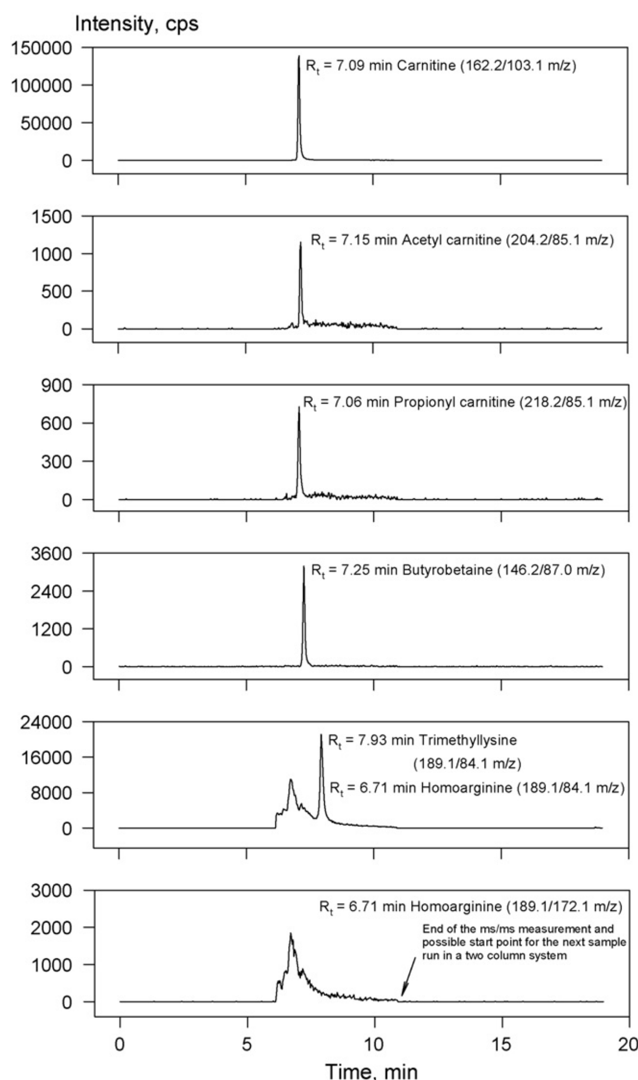

Fig. 1. LC–MS/MS chromatograms of a plasma sample spiked with homoarginine obtained in multi-reaction mode. See text for details.

For an injection of a sample volume of 10 or 40 μL, the limits of quantitation-based on a signal-to-noise ratio of 10—were 250 and 70 nM, respectively, for carnitine and carnitine-d_3, 15 and 5 nM for acetyl carnitine, 30 and 10 nM for propionyl carnitine, 150 and 50 nM for γ-butyrobetaine and 60 and 20 nM for trimethyllysine. In our method, a quasi-solid phase extraction was used for online sample concentrating which resulted in an increased

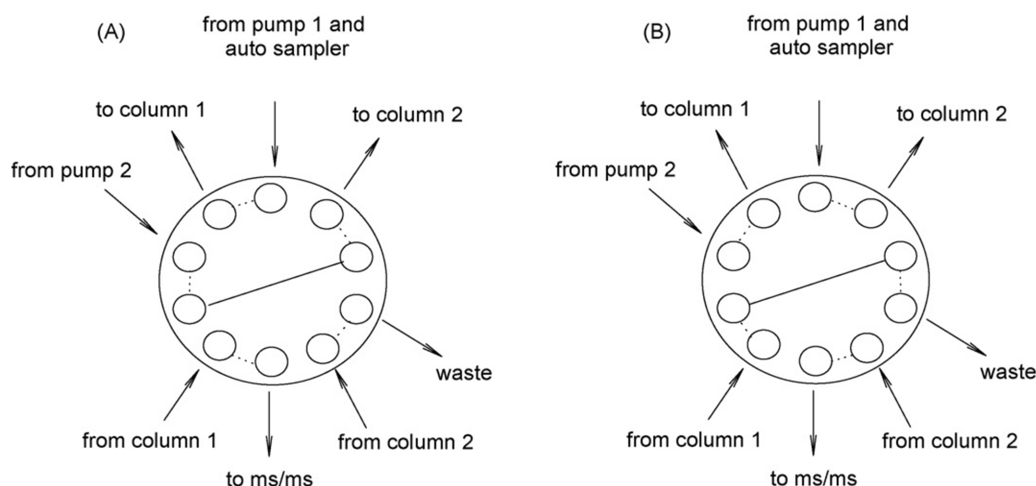

Fig. 2. Proposal for the use of a column switch valve in the discussed two column mode. (A) Separation by column 1 and regeneration of column 2, (B) separation by column 2 and regeneration of column 1. See text for details.

sensitivity. Accordingly, the sensitivity of this method for carnitine, γ-butyrobetaine and trimethyllysine was slightly higher (about three times) than in the method of Vaz et al. [12]. However, sensitivities of about 1 nM as limit of quantitation as described by Jáuregui et al. [7] for long-chain acyl carnitines were not achieved in our method. However, these authors used an API 3000 which is in the multi-reaction mode about 10 times sensitiver as the API 2000 used in our study. When using standard solutions, relative standard deviations (RSD) were <2% for carnitine (in concentrations of 5–150 μM), <2% for acetyl carnitine (in concentrations of 2.5–30 μM), <4% for propionyl carnitine (in concentrations of 0.15–1.5 μM), and <5% for γ-butyrobetaine (in concentrations of 5–80 μM). The RSD for trimethyllysine in a concentration range of 2–15 μM was <10%. Calibration curves showed good linearity between signal and concentration (R^2 = 0.99 or better). For plasma and tissue samples, the RSD were partially higher, particularly if the concentrations were very low such as for propionyl carnitine and acetyl carnitine (Table 3). Nevertheless, sensitivity and precision of the method were acceptable. Recovery rates for carnitine, acetyl carnitine, propionyl carnitine, γ-butyrobetaine, and trimethyllysine in plasma were 112.3 ± 15.4, 107.8 ± 6.3, 96.9 ± 11.1, 96.2 ± 28.8, and 97.2 ± 11.7%, respectively (means ± SD of 10 determinations). Concentrations of free carnitine and carnitine esters in human plasma determined by the presented method, shown in Table 3, are in well agreement with those reported by Minkler and Hoppel [18] (31–49.9, 2.9–4.8, and 0–0.6 μM for carnitine, acetyl carnitine, and propionyl carnitine, respectively). The γ-butyrobetaine concentrations measured in human plasma was, however, lower than reported in literature (1.78 μM [10]), while the concentration of trimethyllysine was similar (0.88 μM [10]). Concentrations of free carnitine and short chain carnitine esters in pig liver and skeletal muscle, presented in Table 3, are similar to those reported by Heo et al. [19] who found concentrations of 73.7 and 759.8 nmol/g for free carnitine and of 2.2 and 193.7 nmol/g for short chain acyl carnitines in liver and muscle, respectively.

Due to its high sensitivity, this method is not only suitable for measuring plasma or tissue concentrations of the respective compounds which are commonly in the μM-range but also for the determination of (deuterated) carnitine formed from (deuterated) γ-butyrobetaine in an assay to determine the activity of γ-butyrobetaine dioxygenase. As shown in Table 4 we could determine the activity of γ-butyrobetaine dioxygenase in various tissues of pig. We could not find out a correlation between the activity of that enzyme and concentrations of the carnitine precursors γ-butyrobetaine and trimethyllysine in the tissues examined (Table 4). As described for rat and mouse by van Vlies et al. [17], we detected a considerable activity of this enzyme in the liver, but no activity in muscle and heart. We also detected a relatively high activity in the kidney and small activities in ileum, brain and spleen. The activity pattern of γ-butyrobetaine dioxygenase activity in var-

Table 3
Concentrations of carnitine, carnitine esters and carnitine precursors in human plasma and pig tissues determined by the method presented[a].

Sample #	Carnitine	Acetyl carnitine	Propionyl carnitine	γ-Butyrobetaine	Trimethyllysine
Plasma (μM)					
1	41.60 ± 0.25	3.74 ± 0.11	0.15 ± 0.01	0.51 ± 0,02	0.50 ± 0.03
2	44.82 ± 1.03	3.99 ± 0.08	0.16 ± 0.01	0.50 ± 0.04	0.51 ± 0.02
3	48.14 ± 1.17	4.17 ± 0.15	0.17 ± 0.01	0.53 ± 0.02	0.56 ± 0.03
Maximum RSD (%)	2.4	4.2	8.3	7.3	6.2
Pig liver (nmol/g wet weight)					
4	62.18 ± 2.93	0.45 ± 0.06	0.18 ± 0.02	5.05 ± 0.39	3.13 ± 0.22
5	36.14 ± 1.83	0.19 ± 0.02	0.13 ± 0.02	3.20 ± 0.14	3.13 ± 0.20
6	34.70 ± 1.54	0.23 ± 0.02	0.12 ± 0.01	3.85 ± 0.17	2.17 ± 0.11
Maximum RSD (%)	5.1	13.4	14.5	7.7	7.0
Pig skeletal muscle (nmol/g wet weight)					
7	475.4 ± 31.5	184.0 ± 9.8	7.55 ± 0.49	160.7 ± 9.7	2.95 ± 0.21
8	477.9 ± 32.6	166.1 ± 5.7	3.05 ± 0.31	126.5 ± 5.5	6.33 ± 0.21
9	358.7 ± 12.8	205.5 ± 4.5	1.50 ± 0.17	137.6 ± 2,8	4.19 ± 0.31
Maximum RSD (%)	6.8	5.3	11.1	6.1	7.5

[a] Three human plasma samples (#1, 2, 3) three pig liver (#4, 5, 6) and pig muscle samples (#7, 8, 9) were analyzed five times each. Data are given as means ± SD of the five determinations.

Table 4
Activities of γ-butyrobetaine dioxygenase and concentrations of γ-butyrobetaine and trimethyllysine in pig tissues.

Tissue	Activity (nmol mg protein^{-1} min^{-1})	γ-Butyrobetaine (nmol g wet weight^{-1})	Trimethyllysine (mol g wet weight^{-1})
Liver	0.393 ± 0.106	5.34 ± 1.22	3.02 ± 0.68
Kidney	2.367 ± 0.926	9.44 ± 0.82	4.46 ± 0.85
Ileum	0.070 ± 0.045	14.93 ± 5.70	2.70 ± 0.84
Brain	0.018 ± 0.009	3.54 ± 2.03	3.85 ± 3.37
Spleen	0.008 ± 0.005	11.39 ± 2.04	9.16 ± 2.26
Skeletal muscle	<0.00125	163.6 ± 23.5	4.71 ± 1.22
Heart	<0.00125	114.5 ± 24.2	4.40 ± 0.37

Activities and concentrations were determined in samples of four pigs. Data are given as means ± SD.

ious tissues of the pig is similar to that of humans described by Rebouche and Engel [20]. In this study we used acetyl-d_3 carnitine as an internal standard in the γ-butyrobetaine dioxygenase activity assay because carnitine-d_9, being the best choice for this [17], was not commercially available. Based on the mass transition 207.2/145.2, we could not detect any enzymatic acetylation of carnitine-d_3 in this assay. Thus, the use of acetyl chain deuterated acetyl carnitine as an internal standard for determination of γ-butyrobetaine dioxygenase activity is acceptable, provided that acetylation of carnitine-d_3 by the enzyme preparation is controlled.

4. Conclusion

The modified direct coupling of the solid phase extraction proposed for the sample preparation [13,14] to a tandem mass spectrometer is suitable for the analysis of carnitine, its short chain esters, and its metabolic precursors trimethyllysine and γ-butyrobetaine in a labour and chemical saving manner. No concentrating steps and chemical derivatization reactions are required. The method has acceptable sensitivities and precisions for the determination of carnitine and related compounds in biological samples and the determination of the activity of γ-butyrobetaine dioxygenase.

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