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Comparison of LC-MS/MS and Enzymatic Methods for the Determination of Total Choline and Total Carnitine in Infant Formula and Milk Products

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Abstract

Background: Choline and L-carnitine are classified as pseudo-vitamins because of their conditionally essential status. As they are involved in multiple physiological metabolic pathways in the human body, they are routinely fortified in infant and adult nutritional formulas. *Objective*: The performance of an LC-MS/MS method for the analysis of choline and carnitine, compared with enzymatic methods in routine use for the analysis of total carnitine and total choline, is described. *Method*: Powder samples were reconstituted, with release of carnitine and choline facilitated by both acid and alkaline hydrolysis and the extract analyzed by LC-MS/MS. Quantitation was by internal standard technique using deuterium labelled carnitine and deuterium-labelled choline. *Results*: Method range, specificity, sensitivity, precision, recovery, accuracy, and ruggedness were assessed for milk powders, infant formulas, and soy- and milk-based nutritional products. Spike recoveries of 94.0–108.4% were obtained for both total carnitine and choline, and no statistical bias ($\alpha = 0.05$) between measured results and certified values (choline: $P = 0.36$; free carnitine: $P = 0.67$) was found for NIST 1849a certified reference material (NIST1849a). Precision, as repeatability relative standard deviation (RSD), was 2.0% RSD_r for total carnitine and 1.7% RSD_r for total choline. Equivalent results for total choline and total carnitine were obtained by LC-MS/MS and enzymatic methods (n = 30). *Conclusions*: The described LC-MS/MS method is fit for purpose for routine product compliance release testing environments. This validation study has confirmed that alternative enzymatic assays can be used with

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confidence in laboratories in which LC-MS/MS platforms are unavailable. *Highlights*: An LC-MS/MS method was evaluated and found to be fit-for-purpose for routine product compliance release testing of infant formula. The LC-MS/MS method was compared with enzymatic methods for the analysis of total carnitine and total choline. Alternative enzymatic assays can be used with confidence in laboratories in which LC-MS/MS platforms are unavailable.

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Introduction

Choline (2-hydroxyethyltrimethyl ammonium) and L-carnitine [(3R)-3-hydroxy-4-(trimethylammonio)butanoate], although assembled from endogenous *de novo* synthesis, are classified as pseudo-vitamins because of their conditionally essential status, and are involved in multiple physiological metabolic pathways in the human body (1, 2). As the human neonate undergoes a period of rapid growth with limited capacity for de novo synthesis, infant formulas are commonly supplemented with both micronutrients in order to emulate human breast milk levels, provide sufficient nutrients for growth, and comply with regulatory requirements (3–5).

Choline and carnitine are present in mammalian milk in both free and multiple bound forms, predominantly either phosphorylated (choline) or acylated (carnitine) (1–3, 6). Although the free forms are water soluble and may be readily extracted for analysis, the bound forms require more exhaustive extraction protocols. This generally involves high temperature acid and/or alkaline hydrolysis when an estimate of total analyte is required, as is typical for product regulatory compliance testing.

Many analytical methods for the separate determination of either free or total choline (7–16) and carnitine (4, 5, 17–22) in milk, infant formula, clinical tissues, and foods have been reported, predominantly by enzymatic, electrophoretic, or chromatographic techniques, although biosensor techniques have also been described (23–25). Conventional chromatography is limited by the need for separate method protocols, prior analyte derivatization, and/or ion pair reagents in order to enhance retention and detection response under reversed-phase conditions. Until recently, enzymatic methods have most commonly been routinely applied for infant formula compliance purposes and, although successful, are manipulative and time consuming and require separate determinations.

With the proliferation of a range of LC-MS platforms, and because of their similar molecular structures and physicochemical properties, this technique has increasingly been exploited for the simultaneous determination of both free and total choline and carnitine contents in foods, utilizing reversed-phase, hydrophilic interaction chromatography, or strong cation exchange column separation modes (26–30), with two recently adopted by AOAC International as Official First and Final Action Methods, subsequent to single- or multi-laboratory study (31, 32). For the regulatory compliance of pediatric

formula foods, the sample treatments utilized in these methods are generally optimized for the quantitation of total choline and carnitine.

Many laboratories, including that of the authors, have routinely utilized both the current Official AOAC Method 999.14 for choline and a separate and similar enzymatic spectrophotometric method for the determination of carnitine in infant formula (4, 8, 9, 17). Prior to method migration in our laboratory, an optimized LC-MS/MS protocol for the simultaneous determination of choline and carnitine was compared directly with the currently utilized routine enzymatic methods. This study reports on this comparison of methods as a component of single-laboratory validation (SLV).

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Experimental

Enzymatic Methods

Enzymatic-spectrophotometric methods for total choline and total carnitine have been previously reported in detail and are described only briefly here (4, 9).

Bound choline was released by acid hydrolysis at 70 °C for 3 h. After pH adjustment, the residual choline phospholipids were cleaved with phospholipase D and free choline was subjected to choline oxidase with the liberation of H_2O_2. In the presence of peroxidase, phenol is oxidized and a quinonimine chromophore is formed with 4-aminoantipyrine. The end-point absorbance was measured at 505 nm and the choline content, as choline hydroxide, was calculated by interpolation from a multi-level calibration (5–25 μg/mL).

Following sequential acid extraction and alkaline hydrolysis, carnitine was measured by the carnitine acetyltransferase initiated acetylation of carnitine. The stoichiometric release of coenzyme A (from added acetyl coenzyme A) was monitored by its subsequent reaction with dithiobenzoate through reaction rate (dA/min) measurement after 2 min at 412 nm, with quantitation by multi-level external standardization (1.6–6.4 μg/mL).

LC-MS/MS Method

Sequential acid and alkaline hydrolysis effected the release of total carnitine and total choline, and the extract was analyzed by LC-MS/MS, with quantitation achieved utilizing deuterium labelled internal standards.

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Apparatus

(a) HPLC system.—Nexera X2 UHPLC system consisting of two LC-30AD pumps, an SIL-30AC autosampler, a CTO-20AC column oven, a CBM-20A control module, and a DGU- 20A5R degasser unit (Shimadzu, Kyoto, Japan).

(b) Mass spectrometer.—6500 QTrap triple quadrupole detector with Analyst software version 1.6 (Sciex, Foster City, CA).

(c) HPLC column.—Zorbax 300-SCX, 3.0 × 50 mm, 5 μm (Agilent, Santa Clara, CA).

(d) Autoclave.—SX-300E high-pressure steam sterilizer (Tomy Digital Biology, Tokyo, Japan).

(e) Centrifuge tubes.—Polypropylene, 50 mL (ThermoFisher, Waltham, MA).

(f) Cryogenic freezer.—- Set to −80 °C.

(g) Analytical balance.—Mettler-Toledo (Columbus, OH) AE 260 analytical delta range (± 0.1 mg) or equivalent, calibrated with National Institute of Standards and Technology (NIST; Gaithersburg, MD) traceable calibration weights.

(h) Syringes.—3 mL Luer-lock (Hapool, Shandong, China).

(i) Syringe filters.—PTFE, 0.2 μm pore size × 13 mm id (Merck Millipore, Carrigtwohill, Cork, Ireland).

(j) Cryogenic vials.—2 mL Nalgene (ThermoFisher, Waltham, MA).

(k) Horizontal shaker.

(l) Schott bottles.—500 mL, 1 L.

(m) Disposable plastic container.—200 mL.

(n) Measuring cylinder.—500 mL.

(o) Volumetric flasks.—25 mL, 50 mL, 100 mL.

(p) Pipettes.—20 mL, 200 mL, 1 mL.

(q) Vortex mixer.

(r) Microcentrifuge tubes.

(s) Water bath.—Temperature controlled at 60 °C, and ambient.

(t) Mobile phase filter membranes.—0.20 μm, nylon or PTFE.

(u) HPLC vials.—Amber, 1 mL with Teflon-coated caps.

Reagents

(a) Ammonium formate (NH4CHOO).—LC-MS grade.

(b) Formic acid (HCOOH).—LC-MS grade.

(c) Ammonium hydroxide.—Reagent grade (28–30% v/v).

(d) L-Carnitine inner salt.—Purity ≥ 99.0% (Sigma-Aldrich, St. Louis, MO).

(e) L-Carnitine (trimethyl-*d9*).—Purity ≥ 98.0% (Cambridge Isotope Laboratories, Andover, MA).

(f) Choline bitartrate.—Purity ≥ 99.0% (Sigma-Aldrich, St. Louis, MO).

(g) Choline chloride (trimethyl-*d9*).—Purity ≥ 98.0% (Cambridge Isotope Laboratories, Andover, MA).

(h) Hydrochloric acid (HCl).—Reagent grade (36% v/v).

(i) Methanol.—LC-MS grade.

(j) Perchloric acid (HClO4).—Reagent grade.

(k) Sodium perchlorate (NaClO4).—Reagent grade.

(l) Water (H2O).—Reagent grade (≥ 18 MΩ).

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Solutions

(a) Methanol solution (50% v/v).—Mix water (250 mL) and methanol (250 mL) in 500 mL Schott bottle.

(b) Sodium perchlorate solution (1 M, pH = 3.5).—Add sodium perchlorate (6.1 ±0.1 g) to a 100 mL Schott bottle. Dissolve with magnetic stir bar, add perchloric acid to adjust pH to 3.5. Filter through 0.20 µm filter membrane and degas.

(c) Mobile phase (17.5 mM ammonium formate: methanol, 1:1 v/v). Add ammonium formate (1.11 ± 0.05 g) and 450 mL water to a 500 mL volumetric flask. Dissolve buffer, add formic acid (2 mL), and make to 500 mL with water. Transfer to 1 L Schott bottle and add 500 mL methanol. Filter through 0.20 µm filter membrane and degas (expiry: 1 week).

Standards

(a) Carnitine-*d9* stock solution (~200 mg/mL).—Transfer contents of 5 mg vial of d9-L carnitine into a 25 mL volumetric flask and add 20 mL Methanol Solution. Shake to dissolve and make to volume. Transfer (1.2 mL) to separate cryogenic vials and store at −80 °C for up to 1 year.

(b) Choline-*d9* stock solution (800 mg/mL).—Weigh ~40 mg of d9 choline chloride into a 50 mL volumetric flask and add 45 mL Methanol Solution. Shake to dissolve and make to volume. Transfer (~1.2 mL) to separate cryogenic vials and store at −80 °C for up to 1 year.

(c) Carnitine stock solution (250 mg/mL).—Dry 75 mg L-carnitine in 102 °C oven for 2 h; cool to ambient in desiccator. Weigh approximately 25 mg into a 100 mL volumetric flask and add 90 mL Methanol Solution. Shake to dissolve and make to volume. Store at 2–8 °C for up to 1 month.

(d) Choline stock solution (2000 mg/mL).—Dry approximately 250 mg choline bitartrate in 102 °C oven for 2 h; cool to ambient in desiccator. Weigh ~200 mg into a 100 mL volumetric flask and add 90 mL Methanol Solution. Shake to dissolve and make to volume with methanol (50%) solution. Store at 2–8 °C for up to 1 month.

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(e) Internal standard solution.—Thaw one vial each of carnitine-d9 and choline-d9 stock solutions to ambient temperature. Transfer 1.0 mL of each into a single 50 mL volumetric flask and make to volume with Methanol Solution. Prepare weekly.

(f) Working standard solution.—Transfer 1.0 mL each of carnitine and choline stock solutions into a single 50 mL volumetric flask and make to volume with Methanol Solution. Prepare weekly.

(g) Calibration standards.—Pipette 50, 200, 500, 1000, or 2000 mL Working Standard Solution into separate 25 mL volumetric flasks, to each 25 mL volumetric flask, pipette 50 μL Internal Standard Solution, and dilute to volume with Methanol Solution. Prepare fresh. Transfer an aliquot of each to separate HPLC vials ready for analysis.

Samples

A certified reference material (SRM 1849a, NIST, Gaithersburg, MD), and locally obtained infant formulas, follow-on formulas, whole milk, skim milk, and buttermilk powders were selected for method comparison.

Sample Preparation

Powder samples were reconstituted by accurately weighing approximately ~12.5 g powder into a 200 mL container. Water (100 g) was added, the final weight was recorded, and the mixture was vortex mixed then placed in a 60 °C water bath for 10 min with periodic shaking to dissolve the powder. The container was cooled in an ambient water bath for 10 min and mixed to ensure homogeneity.

For analysis of total carnitine and total choline, liquid milk or reconstituted powder sample (3.0 g) was accurately weighed in a centrifuge tube and the weight was recorded. Internal standard solution (1.0 mL) was added, followed by water (5 mL) and HCl (36% v/v, 2.5 mL); the tube was then capped and the sample was vortex mixed. Samples were autoclaved at 120 °C for 60 min and then cooled in an ambient water bath. Ammonium hydroxide (28–30% v/v, 3.5 mL) was added to each tube, which was then capped, vortex mixed, and stored for 30 min in the dark. After vortex mixing, an aliquot of sample extract (0.5 mL) was transferred into a centrifuge tube, water (10 mL) was added, and the sample was vortex mixed. An aliquot (1 mL) was filtered through a 0.22 μm syringe filter into a microcentrifuge tube and the extract (0.5 mL) was transferred into an HPLC vial containing LC-MS grade methanol (0.5 mL). The vial was capped and vortex mixed prior to LC-MS/MS analysis. For analysis of free analytes, sample preparation was achieved by aqueous extraction only.

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LC-MS/MS Instrumental Conditions

(a) Column temperature.—40 °C.

(b) Chiller temperature.—10 °C.

(c) Injection volume.—0.5 μL.

(d) Flow rate.—0.8 mL/min.

(e) Switching valve.—On (1.0 min to source; 4.5 min to waste).

(f) Detection.—Triple quadrupole MS configured for low mass with electrospray ionization in positive mode (ESI+). Settings as presented in Tables 1 and 2.

System Suitability

The chromatographic system was equilibrated for 15 min, with water (1 mL) injected to ensure that the system was contaminant free. Amid-range calibration standard was injected ten times to confirm system resolution and response stability. Authenticity of sample analyte peaks was ensured by comparison to retention time and precursor/product ion transitions for authentic standards.

Calculations

For each analyte, a linear multilevel least-squares regression calibration plot of peak area ratio against concentration ratio for non-labelled and labelled internal standard was constructed, with the y-intercept forced through zero. The concentrations of total choline and total carnitine in powder samples are given by the following equation:

$$\text{Result} = \frac{A_{NV}}{A_{IS}} \times \frac{1}{L} \times \frac{(C_{IS} \times V_{IS})}{M_S} \times \frac{100}{1000}$$

where

Result = concentration of total choline or total carnitine in the sample (mg/hg);

A_{NV} = peak area of choline or carnitine in the sample;

A_{IS} = peak area of *d9*-carnitine or *d9*-choline in the sample;

L = slope of the calibration curve;

C_{IS} = concentration of *d9*-carnitine or *d9*-choline in the internal standard (mg/mL);

V_{IS} = volume of internal standard spiked to the sample (mL);

M_S = mass of powder in the reconstituted sample (g);

1000 = concentration conversion factor (mg/g to mg/g);

100 = mass conversion factor (mg/g to mg/hg).

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Results and Discussion

Method Development

The optimized method described was developed based on previously validated international reference methods, AOAC Method 2014.04 (31) and AOAC Method 2015.10 (32),with a few key modifications: (i) alternative deuterated internal standards were selected; (ii) autoclave rather than microwave conditions were used; (iii) to avoid potential explosive safety concerns, acid hydrolysis was performed with hydrochloric acid rather than nitric acid (33).

Sample preparation was similar to AOAC Method 2014.04, whereby acid hydrolysis was complemented by a subsequent mild alkaline hydrolysis to release remaining acid-soluble acylcarnitines. This strategy has been reported to provide a reliable estimate of the total carnitine content in milk, as the sum of acid-soluble free and short-chain acylcarnitines, with the acid insoluble long-chain esters present at negligible levels in bovine milk (4). With respect to choline, the described sample preparation protocol is known to be suitable for the estimation of both free choline and choline bound as acetylcholine, phosphatidylcholine, lysophosphatidylcholine, sphingomyelin, and glycerophosphocholine, although the minor contribution in milk and infant formulas of acid-resistant phosphocholine may not be quantitatively recovered (8).

Instrumental strong cation-exchange chromatography and detector conditions for quantitation were comparable with AOAC Method 2015.10, with minor modifications: (i) isocratic mobile phase conditions; (ii) use of switching valve; (iii) selected multiple reaction monitoring conditions incorporated in order to optimize the method for routine use.

Method Validation

The LC-MS/MS method described for the simultaneous determination of carnitine and choline in milk powders, infant formulas, and soy and milk-based nutritional products has been subjected to comprehensive SLV that has included a comparison of the candidate method with alternative existing enzymatic methods, and a formal estimation of uncertainty.

The chromatographic method incorporates valve switching at 1.5 and 4.5 min in order to divert both unretained and later eluting components to waste, with a total chromatographic run time of 8 min. The chromatography obtained for the NIST 1849a SRM sample, where carnitine and its deuterium-labeled form co-elute at 1.7 min followed by choline and its co-eluting deuterated form at 3.1 min, is illustrated in Figure 1.

Detector linearity was evaluated by generating routine multi-level calibration curves (0.01–0.42 μg/mL carnitine and 0.07–2.68 μg/mL choline) and analyzing by least-squares regression. In

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addition, the concentration range was extended a further one order of magnitude to identify the point at which the calibration deviates from a linear fit. A mean value of the calibration correlation coefficient (r^2) for both analytes over 3 days was 0.9998, and the mean recovery of standard solutions run as independent samples with results back-calculated was 99.3–104.1%.

In the absence of samples containing carnitine and choline at levels close to the expected detection limits, the instrumental LOD and LOQ were estimated from the residual standard deviation of the y-intercept and slope derived from multiple (n = 10) calibration curves (34), and were 0.002 µg/mL and 0.006 µg/mL, respectively, for carnitine, and 0.02 µg/mL and 0.07 µg/mL, respectively, for choline. When expressed as sample powder contents, the LOD and LOQ values (0.25 mg/hg and 0.76 mg/hg, respectively, for carnitine, and 2.84 mg/hg and 8.60 mg/hg, respectively, for choline) are at least one order of magnitude lower than levels typically found in any product evaluated, but do provide confidence for application to potential blended products that may contain lower target analyte content.

Seven pediatric formula products, including the NIST SRM 1849a, were tested in replicate for repeatability (r) and intermediate reproducibility (iR) by two analysts and two instruments over 6 days. Across all samples, the relative standard deviations (RSD%) were RSD_r: 2.0% (HorRat: 0.4), RSD_{iR}: 3.2% for total carnitine, and RSD_r: 1.7% (HorRat: 0.4), RSD_{iR}: 2.5% for total choline. Method uncertainty was also estimated by the addition of variances using first principles (α = 0.05, k = 2), with values of ±15.2% and ±12.0% for total carnitine and choline, respectively (35).

The effect of sample matrix on ionization efficiency (IE) was estimated as IE = (A−B)/A×100, where A and B are the mean peak areas of the internal standard in the mid-range calibration standard solution and the sample extract, respectively. Although ionization suppression was confirmed for carnitine (~20%) and was insignificant for choline (2%), the variation in IE was consistent for both analytes (RSD = 3.8%) across all product types investigated. The use of isotope-labelled internal standards compensates for this inherent issue in LC-MS/MS analysis. Primary transition responses for native choline and carnitine analytes were confirmed to be negligible for the labelled internal standards and, when internal standards were absent from samples, negligible response was observed at the primary transitions of the labelled forms, indicating response specificity. Response specificity was further confirmed through the routine monitoring of secondary ion transitions, yielding consistent quantifier: qualifier response ratios, with an overall precision RSD_{iR} of < 2.5%.

Bias against a certified reference material for free carnitine and choline was evaluated by replicate analyses (n = 20) of NIST 1849a, with differences between the measured value and the certified value determined with the mean and standard deviation of the differences, and the *t*-test statistic and

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P-values calculated (36). No statistical bias (α = 0.05) between the measured results and the certified values (choline: *P* = 0.36; carnitine: *P* = 0.67) was found.

Spike recovery experiments were performed on six different product types spiked with two concentration levels (40%, 80% endogenous levels) and were analyzed over 3 different days with recoveries of 94.0–108.4% obtained for both total carnitine and choline. The recovery of added O-acetyl-carnitine was also investigated and yielded values of 90.8–108.4%, demonstrating quantitative conversion of short-chain carnitine esters during hydrolysis. The inclusion of an alkaline hydrolysis step to extract short-chain carnitine esters yielded modest improvements in total carnitine recovery compared to acid digestion alone.

The ruggedness of a method is the resistance to change in results when minor modifications in experimental conditions that may occur during routine method implementation are intentionally introduced. A seven-factor Plackett-Burman trial (37, 38) using the NIST SRM 1849a sample was performed to evaluate sample weight (0.2 g, 0.4 g), digestion acid volume (2 mL, 3 mL), digestion alkali volume (2 mL, 5 mL), alkaline digest time (20 min, 40 min), autoclave time (30 min, 90 min), post digest dilution volume (3 mL, 7 mL), and a dummy factor, with binary levels for each variable selected either side of the optimized values. The derived half-normal plots are presented in Figures 2 and 3 and graphically distinguish significant from insignificant effects for each parameter, whereby nonsignificant effects tended to fall on a straight line through zero, whereas significant effects deviated from the straight line and above the calculated margin of error (ME) (39, 40). Of the seven factors tested, minor deviations from the optimized conditions were demonstrated to not significantly affect the performance of the method for either choline or carnitine.

Comparison between LC-MS/MS and Enzymatic Methods

Prior to the ready availability of LC-MS instrumentation, enzymatic methods for choline and carnitine, as used in this study, were commonly used to quantify these nutrients in foods, including infant formulas and milk products (26, 27, 30), and many laboratories continue to use them. Although sensitive and specific, they are labor intensive, and the analytes cannot be determined simultaneously. Method accuracy and potential analytical bias were therefore further evaluated by direct comparison of the analytical performance of these alternative enzymatic methods (4, 9) against the described and validated LC-MS/MS method (Tables 3 and 4). A paired *t*-test shows a statistically significant bias (a¼0.05) for both total carnitine (*P* < 0.001) and choline (P < 0.001), although the difference was of minor practical significance as the nominal bias was 4.5 and 5.4%, respectively. Analytical equivalence of the two techniques is illustrated in Bland-Altman plots and linear regression of results obtained by the LC-MS/MS and enzymatic methods for total choline and total carnitine (n = 30), as shown in

Figures 4 and 5 and linear regression between the methods (y = 1.0507x, r^2 = 0.9599 for choline; y = 1.0348x, r^2 = 0.9764 for carnitine). The fact that independent analytical techniques, utilizing significantly different detection principles, yielded equivalent results indicates that both deliver a reliable estimate of the true value. The favorable method comparison data reported here provide confidence that both LC-MS/MS and enzymatic methods may be routinely deployed with confidence.

Conclusions

The described LC-MS/MS method has been comprehensively validated in a single laboratory and demonstrated to be suitable for the quantitative estimation of total carnitine and choline in infant formula, unfortified skim milk, and unfortified whole milk products, and is fit-for-purpose for routine product compliance release testing laboratories. The described method, based on both AOAC First Action Method 2014.04 and AOAC Final Action Method 2015.10, incorporates modifications that have proven to be reliable in the determination of total choline and carnitine in dairy products.

Compared with alternative enzymatic methods, the principal advantages of the LC-MS/MS method are a significant reduction in analysis time and simultaneous analyte determination. Nonetheless, this validation study has confirmed that alternative enzymatic assays can be used with confidence in laboratories in which LC-MS/MS platforms are unavailable.

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Table 1. Mass spectrometry compound parameters

Analyte	Precursor ion, m/z	Product ions, m/z	DP, V[a]	EP, V[b]	CE, V[c]	CXP, V[d]	Dwell time, ms
Carnitine	162.1	103.2[e]	41	10	23	9	150
		58.0[f]	41		61	9	100
d9-Carnitine	171.1	66.0[g]	60		59	11	150
Choline	103.9	58.0[e]	50	10	40	9	150
		45.1[f]	50		30	9	100
d9-Choline	112.9	66.0[g]	60		38	8	150

[a] DP = Declustering potential
[b] EP = Entrance potential
[c] CE = Collision energy
[d] CXP = Collision cell exit potential
[e] Quantifier ion
[f] Qualifier ion
[g] Internal standard

Table 2. Mass spectrometry instrument settings

Instrument parameter	Value
Ionization mode	ESI^+
Curtain gas	35 psi
Nebulizer gas GS1	40 psi
Heater gas GS2	40 psi
Collision gas	N_2
Source temperature	550 °C
Ion spray voltage	1500 V

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Table 3. Comparison of LC-MS/MS and enzymatic method results for total carnitine[a]

Sample	n[b]	LC–MS/MS method mean (standard deviation)	Enzymatic method mean (standard deviation)
NIST[c]	6	15.5 (0.4)	16.0 (0.4)
BMP[d]	1	30.5 (–)e	30.9 (–)
IF1[f]	3	8.3 (0.2)	9.2 (0.2)
IF2	6	10.7 (0.8)	12.0 (0.3)
FO1[g]	12	14.0 (0.8)	15.5 (3.1)
FO2	1	24.8 (–)	24.9 (–)
FO3	1	25.6 (–)	25.4 (–)
FO4	1	11.2 (–)	11.0 (–)
FO5	2	13.5 (1.8)	13.5 (2.3)
FO6	1	12.9 (–)	12.7 (–)
SMP[h]	1	25.3 (–)	25.1 (–)
WMP[i]	1	14.5 (–)	14.4 (–)

[a] carnitine results in mg/hg (standard deviation)
[b] n = number of replicates
[c] NIST CRM 1849a
[d] BMP = buttermilk powder
[e] – = standard deviation not available
[f] IF = infant formula
[g] FO = follow-on formula
[h] SMP = skim milk powder
[I] WMP = whole milk powder

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Table 4. Comparison of LC-MS/MS and enzymatic method results for total choline[a]

Sample	n[b]	LC–MS/MS method mean (standard deviation)	Enzymatic method mean (standard deviation)
NIST[c]	6	104.0 (2.3)	105.8 (5.7)
BMP[d]	1	226.5 (–)e	235.0 (–)
IF1[f]	3	147.5 (0.2)	158.3 (0.2)
IF2	6	173.8 (0.8)	183.0 (0.3)
FO1[g]	12	154.6 (0.8)	166.7 (3.1)
FO2	1	166.7 (–)	167.0 (–)
FO3	1	160.2 (–)	164.0 (–)
FO4	1	86.5 (–)	83.0 (–)
FO5	2	130.6 (1.8)	128.0 (2.3)
FO6	1	113.8 (–)	115.0 (–)
SMP[h]	1	118.1 (–)	134.0 (–)
WMP[i]	1	90.5 (–)	96.0 (–)

[a] carnitine results in mg/hg (standard deviation)
[b] n = number of replicates
[c] NIST CRM 1849a
[d] BMP = buttermilk powder
[e] – = standard deviation not available
[f] IF = infant formula
[g] FO = follow-on formula
[h] SMP = skim milk powder
[I] WMP = whole milk powder

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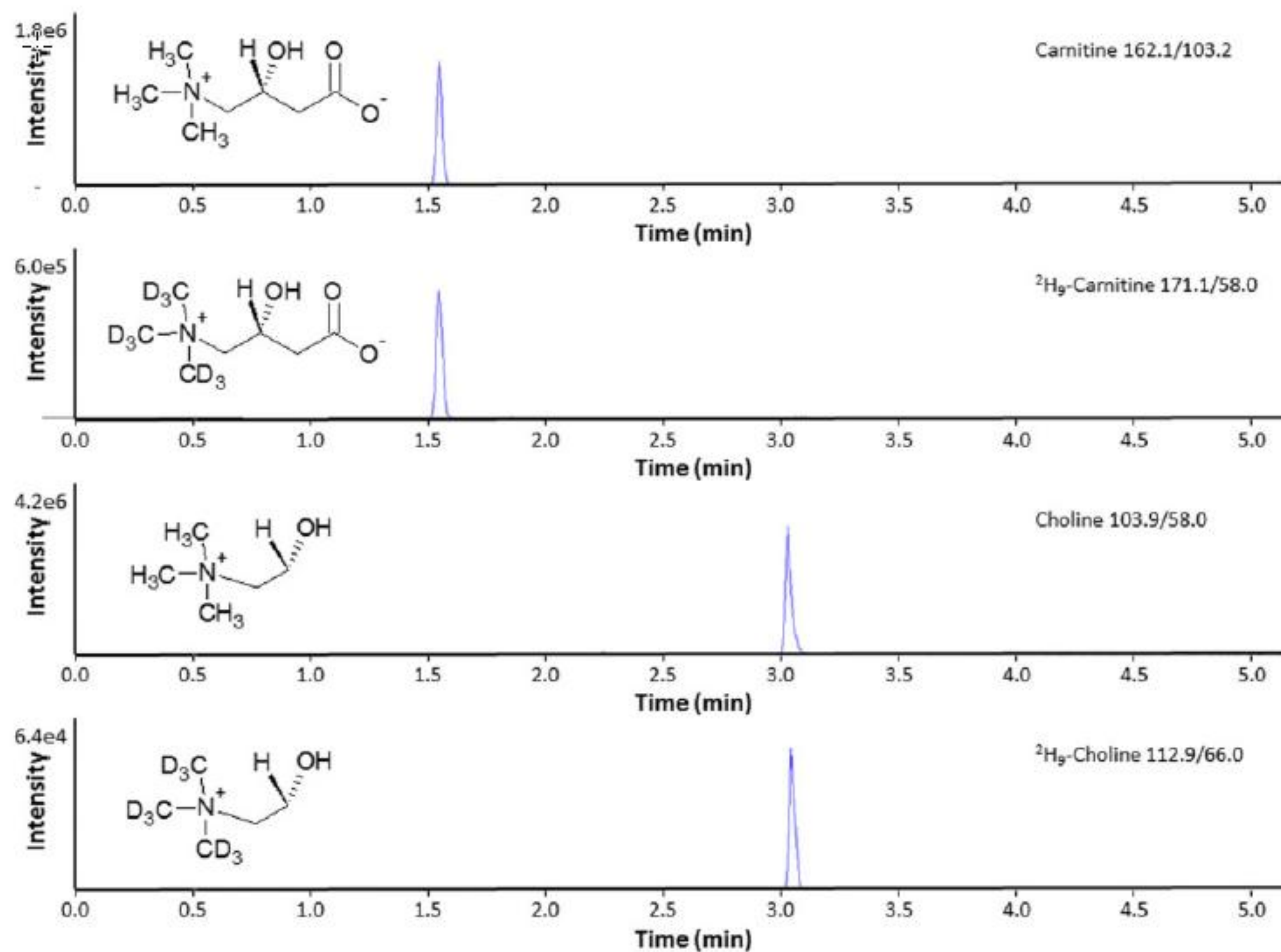

Figure 1. Chromatograms of MRM transitions for carnitine, d9-carnitine, choline, and d9-choline for mixed standard

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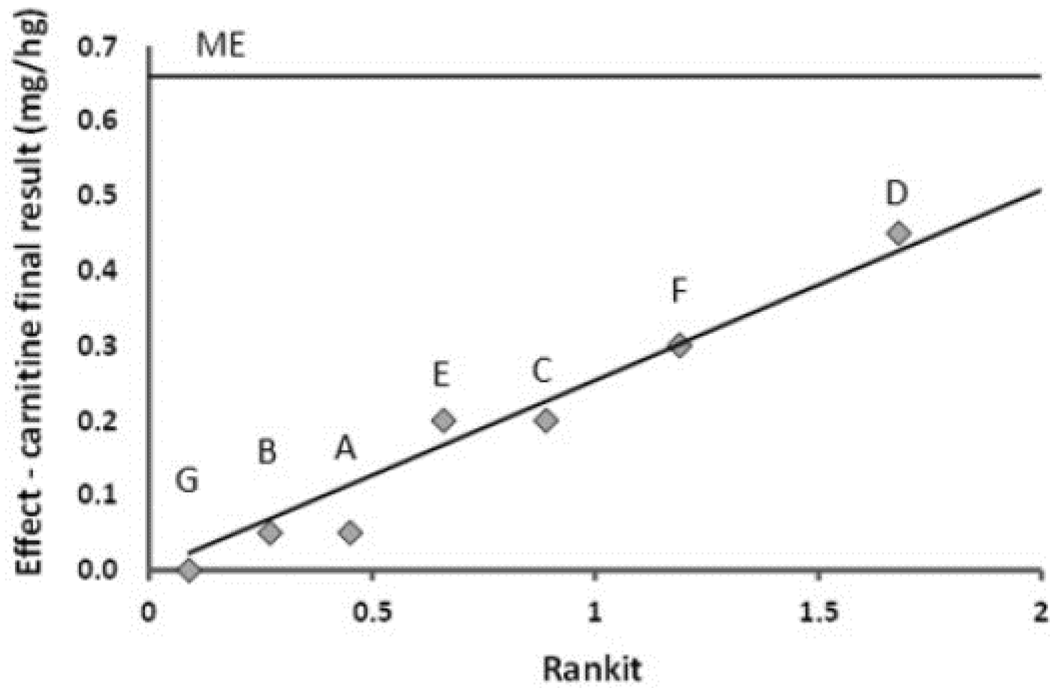

Figure 2. Half-normal plot of carnitine ruggedness experiment (A = sample weight, B = digestion acid volume, C = digestion alkali volume, D = alkaline digest time, E = autoclave time, F = post digest dilution volume, G = dummy factor, ME = margin of error)

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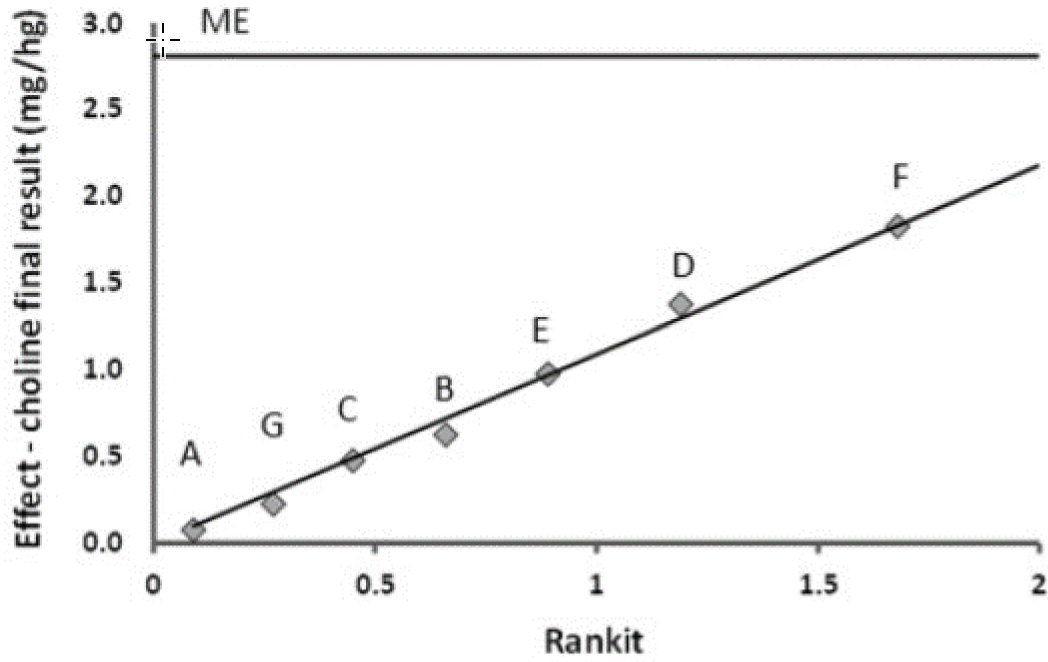

Figure 3. Half-normal plot of choline ruggedness experiment (A = sample weight, B = digestion acid volume, C = digestion alkali volume, D = alkaline digest time, E = autoclave time, F = post digest dilution volume, G = dummy factor, ME = margin of error)

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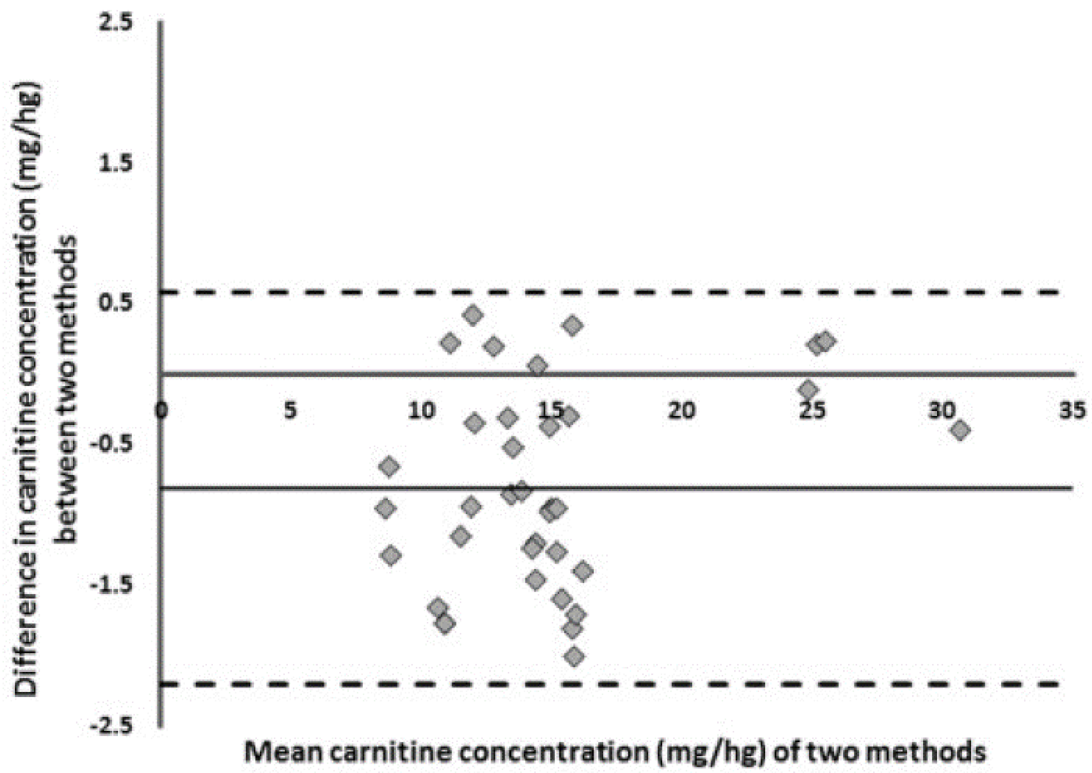

Figure 4. Bland-Altman plot for carnitine, comparing LC MS/MS and enzymatic methods (LoA = limit of agreement)

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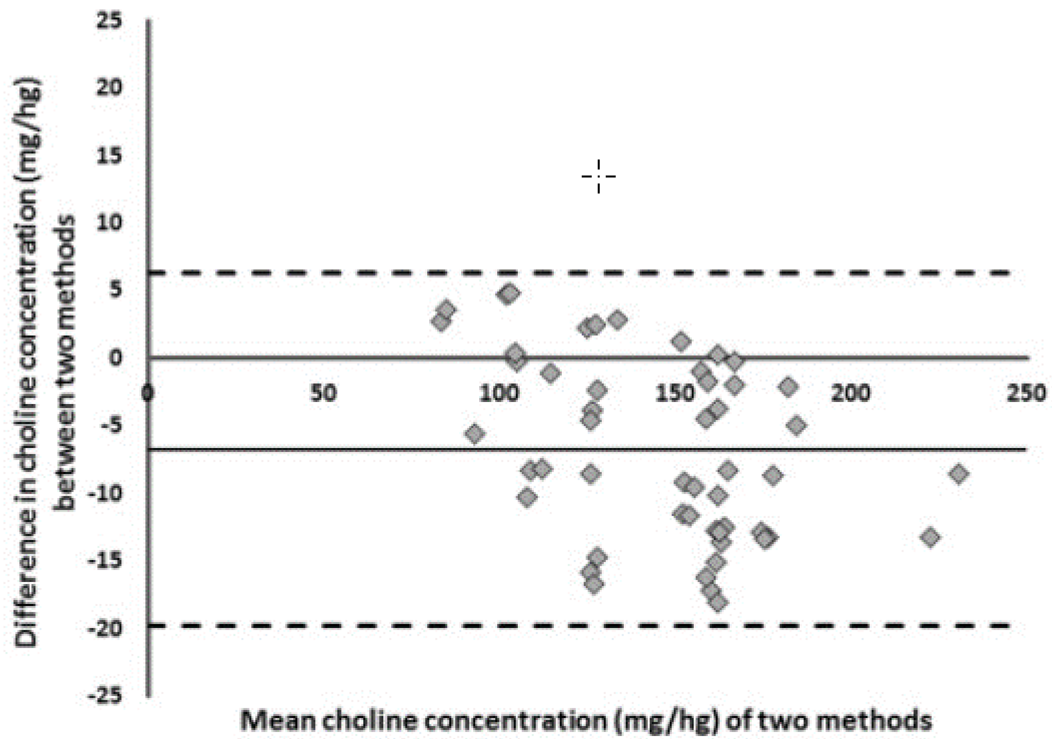

Figure 5. Bland-Altman plot for choline, comparing LC-MS/MS and enzymatic methods (LoA = limit of agreement)