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Infant formula and adult nutritionals — Determination of free and total choline and free and total carnitine — Liquid chromatography tandem mass spectrometry (HPLC-MS/MS)

Formules infantiles et produits nutritionnels pour adultes — Détermination de la teneur en choline totale et la teneur en carnitine par chromatographie en phase liquide et spectrométrie de masse en tandem (CL-SM/SM)

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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This document was prepared by Technical Committee ISO/TC 34, *Food products*, in collaboration with AOAC INTERNATIONAL. It is being published by ISO and separately by AOAC INTERNATIONAL. The method described in this document is equivalent to the AOAC Official Method 2015.10: *Determination of Free and Total Choline and Free and Total Carnitine in Infant Formula and Adult/Pediatric Nutritional Formula by Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS).*

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Infant formula and adult nutritionals — Determination of free and total choline and free and total carnitine — Liquid chromatography tandem mass spectrometry (HPLC-MS/MS)

1 Scope

This document specifies a method for the determination of total or free choline and carnitine in infant formula and adult nutritionals by liquid chromatography and tandem mass spectrometry (HPLC-MS/MS).

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
adult nutritional
nutritionally complete, specially formulated food, consumed in liquid form, which may constitute the sole source of nourishment, made from any combination of milk, soy, rice, whey, hydrolysed protein, starch and amino acids, with and without intact protein

3.2
infant formula
breast-milk substitute specially manufactured to satisfy, by itself, the nutritional requirements of infants during the first months of life up to the introduction of appropriate complementary feeding

[SOURCE: Codex Standard 72-1981]

4 Principle

Samples are extracted in water for free carnitine and choline. For total carnitine and choline, samples are digested with nitric acid and microwave-assisted heating. Free and total extracts are both diluted with water, mixed with acetonitrile, and analysed using liquid chromatography (LC) with tandem mass spectrometric (MS/MS) detection.

5 Reagents and materials

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and high purity MS grade water or water of equivalent purity.

Non-specific binding can occur with these analytes when using glassware, so plasticware should be used at all times for standard/sample preparation. All laboratory plasticware should be single use whenever possible. Positive displacement pipets are also mandatory for pipetting to avoid contamination and for accuracy with organic solvents.

5.1 Water, purified, MS grade or equivalent purity.

5.2 Acetonitrile, MS grade.

5.3 Ammonium formate, MS grade or equivalent.

5.4 Formic acid, MS grade or equivalent.

5.5 Nitric acid, a mass fraction of 70 %, ACS grade or equivalent.

WARNING — All preparation steps with nitric acid should be performed within a fume hood. The necessary personal protective equipment should be used when handling.

5.6 Isopropanol, MS grade or equivalent.

5.7 Desiccant.

5.8 L-carnitine, **inner salt**, primary reference standard. Store in a desiccator.

5.9 Choline bitartrate, primary reference standard.

5.10 L-carnitine-d_3 HCL, primary reference standard.

5.11 Choline-1,1,2,2-d_4 chloride, primary reference standard.

6 Preparation of solutions

CAUTION — All mobile phase bottles should be rinsed thoroughly prior to use with purified water and isopropanol.

6.1 Mobile phase A

A mixture of 1 part per volume of 5 mmol/l ammonium formate in water and 1 part per volume of acetonitrile, with 0,2 % formic acid.

Weigh 0,32 g ± 0,01g of ammonium formate into a 1 l bottle (7.16) containing 500 ml of purified water. Add a stir bar and then mix on a stir plate until dissolved. Add 500 ml of acetonitrile and 2,00 µl of formic acid to the mobile phase container. Mix on a stir plate until thoroughly mixed, typically for about 2 min. The solution is stable for two weeks when stored at room temperature.

6.2 Mobile phase B

A mixture of 1 part per volume of 30 mmol/l of ammonium formate in water and 1 part per volume of acetonitrile, with 0,2 % formic acid.

Weigh 1,89 g ± 0,01g of ammonium formate into a 1 l mobile phase bottle containing 500 ml of purified water. Add a stir bar and then mix on a stir plate until dissolved. Add 500 ml of acetonitrile and 2,00 µl of formic acid to the mobile phase container. Mix on a stir plate until thoroughly mixed, typically for about 2 min. The solution is stable for two weeks when stored at room temperature.

6.3 HPLC injector wash

Mobile phase B (see 6.2) or as recommended by supplier.

6.4 Preparation of choline and carnitine stock solutions

6.4.1 Stock and working standards are stable for six months when stored in a refrigerator set to maintain 2 °C to 8 °C. Protect standard solutions from actinic light. Alternate weights or volumes may be used to scale these preparations.

6.4.2 Choline stock solution, mass concentration, $\rho \approx 25$ mg/ml.

Weigh 0,520 0 g ± 0,01 g of choline bitartrate into a 20 ml polypropylene container. Dissolve with 10,0 ml of purified water. Correct the final concentration of this solution for purity, moisture, and form to represent free choline ion concentration in solution. The molecular weight (MW) of choline ion is 104,17, The MW of choline bitartrate is 253,25.

6.4.3 Carnitine stock solution, $\rho \approx 25$ mg/ml.

Weigh 0,250 0 g ± 0,01 g of carnitine into a 20 ml polypropylene vial. Dissolve with 10,0 ml of purified water.

Correct for moisture content and purity to represent carnitine concentration.

6.4.4 L-carnitine-d_3 stock solution, IS 1, $\rho \approx 2{,}00$ mg/ml.

Weigh 0,025 g ± 0,001 g of L-carnitine-d_3 HCl into a 20 ml polypropylene vial. Dissolve with 10,0 ml of purified water.

6.4.5 Choline-1,1,2,2-d_4 stock solution, IS 2, $\rho \approx 2{,}00$ mg/ml.

Weigh 0,031 00 g ± 0,001 g of choline-1,1,2,2-d_4 chloride into a 20 ml polypropylene container. Dissolve with 10,0 ml of purified water.

6.5 Preparation of working standard solutions

6.5.1 Prepare the working standard solutions according to Table 1 in polypropylene vials.

Table 1 — Preparation of working standard solutions

Standard solution	Source solution ID	Source concentration mg/ml	Source volume ml	Purified water ml	Final volume ml	Prepared concentration µg/ml	Extracted concentration ng/ml
STD 6	stock solutions (6.4.2, 6.4.3)	25,0	2,00 each	6,00	10,0	5 000	5 000
STD 5	stock solutions (6.4.2, 6.4.3)	25,0	1,60 each	6,80	10,0	4 000	4 000
STD 4	stock solutions (6.4.2, 6.4.3)	25,0	0,800 each	8,40	10,0	2 000	2 000
STD 3	STD6	5,00	1,00	9,00	10,0	500	500
STD 2	STD3	0,500	0,400	9,60	10,0	20	20
STD 1	STD3	0,500	0,200	9,80	10,0	10	10
NOTE Alternate masses or volumes may be used to scale these preparations.							

6.5.2 Mixture of L-carnitine-d_3 and choline-1,1,2,2-d_4 internal standard working solution (IWS), $\rho \approx 200$ µg/ml.

Transfer 10 ml of IS 1 solution (6.4.4) and 10 ml of IS 2 solution (6.4.5) into a 100 ml tube (7.13). Add 80 ml of purified water to bring to a final volume of 100 ml.

7 Apparatus

7.1 HPLC system, Prominence (Shimadzu, Kyoto, Japan)[1] or equivalent.

7.2 MS/MS system, API 4000 with Electrospray Ionization (ESI) (ABSciex, Framingham, MA)[1] or equivalent.

7.3 Mass spectrometry software, Analyst (ABSciex)[1] or equivalent.

7.4 Analytical column, Zorbax 300-SCX, 3,0 mm × 50 mm, 5 µm (Agilent, Santa Clara, CA)[1] or equivalent.

7.5 Microwave, a commercial microwave designed for laboratory use, with a closed-vessel system and controlled temperature ramping capability. Use manufacturer-recommended vessels, i.e. MARS6[1] or equivalent.

7.6 Microwave turntable, liner, and cap., MARSXpress[1], 55 ml PFA Teflon®[1], 40 position (CEM)[1] or equivalent.

7.7 Vortex mixer.

7.8 Analytical balance, precision to 0,001 g.

7.9 Horizontal shaker.

7.10 Magnetic stir plate.

7.11 Positive displacement pipets.

7.12 Repeater positive displacement pipet.

7.13 Polypropylene tubes, assorted sizes

7.14 Digestion vessels for microwave digestion.

7.15 Graduated polypropylene tube, e.g. Digitube®[1] or equivalent.

7.16 Glass containers, 1 l to2 l bottle.

7.17 Syringe filters, 0,45 µm PTFE (polytetrafluorethene).

7.18 Disposable syringes, 3 ml.

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1) This is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute and endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

7.19 Graduated cylinders, assorted sizes.

7.20 Magnetic stir bars.

7.21 Autosampler vials and caps, 1,5 ml silanized crimp top.

7.22 Microcentrifuge tubes, 1,5 ml polypropylene.

7.23 Bottle top dispenser, 5 ml acid resistant.

7.24 Desiccator, glass.

8 Procedure

8.1 Sample preparation

8.1.1 Samples needing reconstitution

Weigh 10,00 g ± 0,500 g of sample in a suitable disposable cup or beaker. Add purified water to bring the total mass (including the powder mass) to 100,00 g ± 1,00 g. Add a stir bar and stir as fast as possible without causing the sample to splatter. Stir for at least 10 min, but no longer than 30 min.

Masses may be adjusted as needed to accommodate different powder types and levels of analytes.

8.1.2 Analysis of free carnitine and choline

Weigh 0,1 g to 1,0 g of ready-to-feed (RTF) or reconstituted sample into a tared 50 ml tube (7.15) depending on the concentration of each analyte (to fall within calibration curve). A reagent blank, a reagent blank + internal standard (ISTD), and working standards shall be included for each analysis and treated the same as samples through the analysis.

Add 50 µl of each working solution to separate 50 ml tubes (7.15). The final nominal concentrations for these after they have gone through the sample preparation (diluted 1 000 times) are listed in 6.4 and 6.5 to be used for construction of the calibration curve.

Add 50 µl of mixed internal standard working solution (6.5.2) to each sample, working standards solutions and reagent blank + ISTD.

Dilute to 25 ml with purified water, cap, and thoroughly mix the sample on a horizontal shaker. This sample extract is stable for three days when stored refrigerated and protected from light.

If further dilution of samples is required, dilute the samples to appropriate concentrations using the reagent blank + ISTD using polypropylene vessels to do the dilutions.

Transfer a 0,5 ml aliquot of extract into a microcentrifuge tube, along with 0,5 ml of acetonitrile. Mix well.

Filter samples through a syringe filter (7.17) into a silanized injection vial. Standards do not need to be filtered through the syringe filter. This sample extract is stable for three days when stored refrigerated and protected from light.

8.1.3 Analysis of total carnitine and choline

Weigh 0,1 g to 1,0 g of RTF or reconstituted sample into a tared microwave digestion vessel (7.14) depending on the concentration of each analyte (to fall within calibration curve).

For viscous (high fat, high protein) ready to feed samples, perform a pre-dilution by weighing 1,0 g of sample and adding purified water to a final mass of 5,0 g into a suitable plastic container. Mix well prior to weighing into the 55 ml vessel (7.14).

A reagent blank, a reagent blank + ISTD, and working standards shall be included for each analysis and are treated the same as samples through the analysis.

Add 50 µl of each working solution to separate 50 ml tubes (7.15). The final nominal concentrations for these after they have gone through the sample preparation (diluted 1 000 times) are listed in 6.4 and 6.5 to be used for construction of the calibration curve.

Add 50 µl of mixed internal standard working solution (IWS, 6.5.2) to each sample and reagent blank + ISTD.

Add 5 ml of purified water and 2,5 ml of a volume fraction of 70 % nitric acid with a bottle top dispenser (7.23). Cap tightly or use a capping station. Mix the sample by either vortexing or inverting.

Insert the vessels into their appropriate sleeves and into the turntable. Microwave the samples following the following conditions:

— power = 1 000 W;

— ramp to temperature: 10 min;

— hold time: 40 min;

— temperature: 120 °C.

Allow the vessels to complete the cooling process in the microwave before removing the caps to prevent the loss of sample through pressure release.

Quantitatively transfer the contents of the vessels into 50 ml tubes (7.15) using purified water, and dilute to a volume of 25 ml with purified water.

If further dilution of samples is required, dilute the samples to appropriate concentrations using the reagent blank + ISTD and polypropylene vessels to do the dilutions.

Transfer a 0,5 ml aliquot of extract into a silanized injection vial, along with 0,5 ml of acetonitrile. Mix well.

Filter samples through a 0,45 µm PTFE syringe filter into a microcentrifuge tube. Standards do not need to be filtered through the syringe filter.

Cap and then mix well by shaking or vortexing the vials. Prepared sample extracts in injection vials are stable for 24 h while stored 2 °C to 8 °C and protected from light.

8.2 Instrument parameters

8.2.1 HPLC parameters

The parameters given in Tables 2 and 3 apply to the Zorbax 300-SCX column. See Annex A for typical chromatograms.

Table 2 — HPLC parameters

Column	Zorbax 300-SCX, 3,0 mm × 50 mm, 5 µm
Mobile phase A	mixture of 1 part per volume of 5×10^{-3} mol/l ammonium formate in water and 1 part per volume of acetonitrile, with 0,2 % formic acid
Mobile phase B	mixture of 1 part per volume of 30×10^{-3} mol/l of ammonium formate in water and 1 part per volume of acetonitrile, with 0,2 % formic acid
Injection volume	1 µl to 10 µl
Runtime	4,2 min

Table 3 — Gradient programme

Time min	**Flow rate** ml/min	**Phase B** %
0	1,00	0
1,0	1,00	0
1,5	1,00	100
2,5	1,00	100
3,0	1,00	0
4,2	1,00	0

8.2.2 LC-MS parameters

The MS/MS settings in Tables 4 and 5 may be modified except for ionization type and mode to obtain optimum chromatography and sensitivity. Exact mass ions may vary slightly from instrument to instrument because of unit resolution of quadrupole mass spectrometers.

Table 4 — LC-MS parameters

LC-MS model	**Sciex 4000**	**Sciex 6500**
Ionization mode	Positive ion electorspray (ESI+)	Positive ion electorspray (ESI+)
IonSpray voltage	1 000 V	2 000 V
Turbo IonSpray temperature	550 °C	550 °C
Entrance potential (EP)	10 V	10 V
Collision gas (CAD)	nitrogen, 5,0	nitrogen, 5,0
Curtain gas (CUR)	nitrogen, 20,0	nitrogen, 20,0
Nebulizing gas (GS1)	nitrogen, 60,0	nitrogen, 60,0
Nebulizing gas (GS2)	nitrogen, 60,0	nitrogen, 60,0
Needle position	Y = 5 mm, X = 5 mm	Y = 5 mm, X = 5 mm