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Milk and milk products — Determination of amino acids in infant and adult/paediatric nutritional formulas and dairy products

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Contents

Page

Forewords

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, and the International Dairy Federation (IDF), in collaboration with AOAC INTERNATIONAL and ISO/TC 34, *Food products*, Subcommittee SC 4, *Cereals and pulses*. It is being published jointly by ISO and IDF, and separately by AOAC INTERNATIONAL.

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.

IDF (the International Dairy Federation) is a non-profit private sector organization representing the interests of various stakeholders in dairying at the global level. IDF members are organized in National Committees, which are national associations composed of representatives of dairy-related national interest groups including dairy farmers, dairy processing industry, dairy suppliers, academics and governments/food control authorities.

ISO and IDF collaborate closely on all matters of standardization relating to methods of analysis and sampling for milk and milk products. Since 2001, ISO and IDF jointly publish their International Standards using the logos and reference numbers of both organizations.

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This document was prepared by the IDF *Standing Committee on Analytical Methods for Composition* and ISO Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 5, *Milk and milk products*, in collaboration with AOAC INTERNATIONAL and ISO/TC 34, *Food products*, Subcommittee SC 4, *Cereals and pulses*. It is being published jointly by ISO and IDF, and separately by AOAC INTERNATIONAL.

The work was carried out by the IDF/ISO Action Team C51 of the *Standing Committee on Analytical Methods for Composition* under the aegis of its project leader Mr C. Fuerer (CH).

Milk and milk products — Determination of amino acids in infant and adult/paediatric nutritional formulas and dairy products

1 Scope

This document specifies a method for the quantitative determination of total amino acids using 6-aminoquinolyl-N-hydroxy-succinimidyl carbamate (ACQ) derivatization followed by ultra-high-performance liquid chromatography (UHPLC) separation and ultraviolet (UV) detection. It specifies a method for the determination, in one single analysis, of the following amino acids: alanine, arginine, aspartic acid (combined with asparagine), cystine (dimer of cysteine, combined with cysteine), glutamic acid (combined with glutamine), glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tyrosine and valine.

This method does not apply to the determination of tryptophan.

This method is applicable to infant and adult/paediatric nutritional formulas, dairy products and other matrices such as cereals. It was validated in infant formulas (milk- and soy-based, including partially hydrolysed and elemental products), toddler formula, adult nutritional powder, UHT skimmed milk, whey powder, sodium caseinate, whole milk powder, bran pet food, dry pet food and breakfast cereal (see Annex A for details).

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 terminology databases for use in standardization at the following addresses:

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

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

3.1
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]

3.2
adult/paediatric 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

4 Principle

Proteins are hydrolysed in 6 mol/l hydrochloric acid (HCl) for 24 h at 110 °C in the presence of phenol, 3-3′-dithiodipropionic acid (DDP) and norvaline. Phenol is added to prevent halogenation of tyrosine.

Norvaline is added as an internal standard. DDP is added to convert cystine and cysteine to S-2-carboxyethylthiocysteine (XCys), as described in Reference [1], and the resulting derivative can be separated from other amino acids for quantification.

After neutralization, amino acids and converted XCys are derivatized with AQC. Derivatized amino acids are separated using reversed phase UHPLC with UV detection at 260 nm.

NOTE Fluorescence detection can be used provided equivalence has been demonstrated.

During acid hydrolysis, glutamine (Gln) and asparagine (Asn) are converted to glutamic acid (Glu) and aspartic acid (Asp), respectively. Thus, Glu values represent the combined values of Glu and Gln, and Asp values represent the combined values of Asp and Asn. Cys2 values represent the combined values of cysteine and cystine since both are converted to XCys by DDP.

5 Reagents

Use only reagents of recognized analytical grade.

Commercial references are only a guideline. Equivalent chemicals or materials can be used provided their equivalence has been demonstrated. Before using chemicals, refer to the safety data sheets and ensure that the safety precautions are applied.

5.1 AccQ·Tag™ Ultra Derivatization Kit (Waters 186003836[1]).

As an alternative derivatizing buffer, di-sodium tetraborate decahydrate (CAS Registry Number©[2] 1303-96-4) can be used.

As an alternative tagging reagent, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate (CAS 148757-94-2) can be used.

5.2 AccQ·Tag™ Ultra Eluent A concentrate (Waters 186003838[1]).

As alternative reagents, acetonitrile, gradient grade for liquid chromatography (LC) (CAS 75-05-8), and formic acid (CAS 64-18-6) can be used.

5.3 AccQ·Tag™ Ultra Eluent B (Waters 186003839[1]).

As alternative reagents, acetonitrile, gradient grade for LC (CAS 75-05-8), formic acid (CAS 64-18-6) and ammonium formate (CAS 540-69-2) can be used.

5.4 Phenol (CAS 108-95-2).

5.5 3,3′-Dithiodipropionic acid (CAS 1119-62-6).

5.6 Amino acid standard solution, containing the following 17 amino acids at 2,5 µmol/ml each (except L-cystine at 1,25 µmol/ml) in 0,1 mol/l HCl: L-alanine, L-arginine, L-aspartic acid, L-cystine, L-glutamic acid, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tyrosine and L-valine.

5.7 L-cystine (CAS 56-89-3).

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 an endorsement by ISO or IDF of the product named. The alternative reagents listed in this document have been shown to lead to the same results.

2) CAS Registry Number® is a trademark of CAS corporation. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of the product named. Equivalent products may be used if they can be shown to lead to the same results.

5.8 Norvaline (CAS 6600-40-4).

5.9 Sodium hydroxide pellets, reagent grade (CAS 1310-73-2).

5.10 Sodium hydroxide solution (CAS 1310-73-2), substance concentration c = 1 mol/l.

5.11 Sodium hydroxide solution (optional) (CAS 1310-73-2), c = 6 mol/l.

5.12 Hydrochloric acid fuming 37 % (CAS 7647-01-0), c = 12 mol/l, GR grade for analysis.

5.13 Hydrochloric acid solution (CAS 7647-01-0), c = 1 mol/l.

5.14 Hydrochloric acid solution (CAS 7647-01-0), c = 0,1 mol/l.

5.15 Laboratory water, with a resistivity of 18,2 MΩ-cm (ultra-pure water).

6 Reagents and standard preparation

6.1 General

6.1.1 Sodium hydroxide (NaOH) solutions, c = 6 mol/l, c = 0,2 mol/l and c = 0,05 mol/l.

For the c = 6 mol/l solution, weigh out 24 g of sodium hydroxide (5.9) into a 100 ml volumetric flask. Dissolve in about 80 ml of water. Allow to cool down and dilute to the mark with water. Optional: use a commercially available equivalent (5.11).

For the c = 0,2 mol/l solution, pipet 20 ml of 1 mol/l NaOH (5.10) into a 100 ml volumetric flask and make up to the mark with water.

For the c = 0,05 mol/l solution, pipet 5 ml of 1 mol/l NaOH (5.10) into a 100 ml volumetric flask and make up to the mark with water.

6.1.2 Hydrochloric acid (HCl) solution, c = 0,2 mol/l.

Pipet 20 ml of 1 mol/l HCl (5.13) into a 100 ml volumetric flask and make up to the mark with water.

6.1.3 DDP solution, mass concentration ρ = 10 g/l (in NaOH, c = 0,2 mol/l).

Into a 50 ml volumetric flask, weigh out 500 mg of DDP and make up to the mark with 0,2 mol/l NaOH (6.1.1).

6.1.4 Phenol/HCl solution, ρ = 1 g/l (in HCl, c = 12 mol/l).

Into a 100 ml volumetric flask, weigh out 100 mg of phenol and make up to the mark with 12 mol/l HCl (5.12).

6.1.5 AccQ·Tag™ Ultra Derivatization kit.[1)]

Prepare the reagents included in the kit following the manufacturer's instructions.

6.1.6 AccQ·Tag™ Ultra Borate buffer (reagent 1).[1)]

Ready-to-use solution. An alternative reagent is sodium tetraborate in water solution (ρ = 50 g/l). Into a 100 ml volumetric flask, weigh 5 g of sodium tetraborate decahydrate, dissolve and make up to the mark with water.

6.1.7 AccQ·Tag™ Ultra reagent (vial 2A and 2B).[1)]

Reconstitute AccQ·Tag™ Ultra reagent (vial 2A) according to the manufacturer's instructions as follows:

a) Preheat a heating block to 55 °C.

b) Tap vial 2A lightly before opening to ensure all AccQ·Tag™ Ultra reagent powder is at the bottom of the vial.

c) Rinse a clean micropipette by drawing and discarding 1 ml of AccQ·Tag™ Ultra reagent diluent from vial 2B (ready-to-use solution). Repeat twice.

d) Draw 1,0 ml from vial 2B and transfer it to the AccQ·Tag™ Ultra reagent powder in vial 2A. Cap the vial tightly.

e) Vortex mix for approximately 10 s.

f) Heat vial 2A on top of the preheated heating block until the AccQ·Tag™ Ultra reagent powder is dissolved. Do not heat the reagent for longer than 10 min.

Once reconstituted, the AccQ·Tag™ Ultra reagent concentration is approximately 10 mmol/l. Store the reconstituted AccQ·Tag™ Ultra reagent in a desiccator at room temperature for up to one week.

CAUTION — AccQ·Tag™ Ultra reagent reacts with atmospheric moisture. Seal the container tightly when not in use. Do not refrigerate. Do not use discoloured reagent, especially if it is yellow or green.

The following alternative reagent can be used. Into a 4 ml vial, weigh out approximately 3,0 mg to 4,0 mg of 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate. Continue with step c) above using LC-grade acetonitrile instead of the AccQ·Tag™ Ultra reagent diluent.

6.2 Norvaline (Nva) internal standards

6.2.1 Nva stock solution, c = 10 mmol/l.

Weigh 117,16 mg Nva into a 100 ml volumetric flask and make up to the mark with 0,1 mol/l HCl (5.14).

6.2.2 Nva solution, c = 2,5 mmol/l.

Pipet 2,5 ml of Nva stock solution (6.2.1) into a 10 ml volumetric flask and make up to the mark with 0,1 mol/l HCl (5.14).

Store both Nva solutions at −20 °C for up to six months as 2 ml aliquots.

6.3 Cystine calibration standards

6.3.1 Cystine stock solution, c = 10 mmol/l.

Weigh 240 mg cystine into a 100 ml volumetric flask and make up to the mark with 0,05 mol/l NaOH (6.1.1). Store this solution at −20 °C for up to three months as 1 ml aliquots.

6.3.2 Cystine solution, c = 1 mmol/l.

Add 900 µl of 0,05 mol/l NaOH (6.1.1) to 100 µl of cystine stock solution (6.3.1). Prepare this solution freshly for each analysis.

6.4 Amino acid (AA) calibration standards (with exception of cystine)

6.4.1 AA stock solution, *c* = 2,5 mmol/l.

Amino acid standard solution is ready-to-use and contains 2,5 mmol/l of each amino acid (although present in this solution, cystine is not used for quantification and is prepared separately, see 6.3).

Store this calibration standard stock solution at −20 °C for up to six months as 250 µl aliquots.

6.4.2 AA solution 1, *c* = 0,5 mmol/l.

Add 600 µl of 0,1 mol/l HCl (5.14) to 150 µl of AA stock solution (6.4.1). Prepare this solution freshly for each analysis.

6.4.3 AA solution 2, *c* = 0,05 mmol/l.

Add 900 µl of 0,1 mol/l HCl (5.14) to 100 µl of AA solution 1 (6.4.2). Prepare this solution freshly for each analysis.

6.5 Chromatography solvents (mobile phases)

6.5.1 Eluent A (Solvent A).

Prepare Eluent A from AccQ·Tag™ Ultra Eluent A concentrate as follows:

a) Measure 850 ml of water into a 1 l graduated cylinder.

b) In a separate graduated cylinder, measure 150 ml of AccQ·Tag™ Ultra Eluent A concentrate.

c) Add the concentrate to the water and mix thoroughly.

Eluent A concentrate, once opened, shall be stored tightly capped at around 4 °C. Dilute Eluent A is stable for one week at room temperature.

As an alternative for Eluent A concentrate, the following solution can be used. Mix 840 ml of 200 mmol/l ammonium formate solution (12,61 g ammonium formate in 1 l of water), 110 ml of acetonitrile and 50 ml of formic acid. Prepare Eluent A from the concentrate as described above.

6.5.2 Eluent B (Solvent B).

AccQ·Tag™ Eluent B is supplied as a working solution. No additional preparation is required. Eluent B, once opened, shall be stored tightly capped at around 4 °C for no longer than one month.

As an alternative for Eluent B, the following solution can be used. Add 13,2 ml formic acid to 1 l acetonitrile.

6.5.3 Wash solvents.

a) The weak needle wash solvent is 50 ml/l acetonitrile in water.

b) The strong needle wash solvent is 950 ml/l acetonitrile in water.

c) The seal wash solvent is 500 ml/l acetonitrile in water.

7 Apparatus

7.1 UHPLC system that sustains a pressure of approximately 62 MPa and can achieve baseline separation of the amino acids[3].

7.2 Chromatography column, ACQUITY UPLC™ BEH C18 Column, 130 Å, 1,7 µm, 2,1 mm × 150 mm (Waters 186002353[1]) or equivalent, provided baseline separation of the amino acids is achieved.

7.3 Adjustable micropipettes, of volume 10 µl, 20 µl, 200 µl and 1 000 µl and tips.

7.4 Vortex mixer.

7.5 Analytical balance, with a precision of 0,1 mg.

7.6 Heating block, able to maintain a temperature of 55 °C ± 2 °C.

7.7 Laboratory oven, able to maintain a temperature of 110 °C ± 2 °C.

7.8 Syringe filter, 0,45 µm Polyvinylidene fluoride (PVDF) Millex®-HV (e.g. Millipore SLHV013NL[1] or equivalent).

7.9 Syringes, of 2 ml volume.

7.10 Borosilicate glass tubes (e.g. Pyrex), of 10 ml volume with screw cap.

7.11 Microtubes, of volume 1,5 ml and 2 ml.

7.12 Vial with screw cap, of volume 4 ml.

7.12.1 Glass screw neck total recovery vial, 12 mm × 32 mm (Waters 186000384C[1] or equivalent).

8 Sample analysis

8.1 Sample preparation

Prepare different sample types differently. Powder infant and adult/paediatric nutritional formulas shall be reconstituted in water first. Other samples are used without reconstitution.

Reconstitute powder infant and adult/paediatric nutritional formula samples by adding 25 g powder to 200 g water and mix thoroughly. Weigh 220 mg ± 20 mg reconstituted powders into a 10 ml glass tube with screw cap. Report the sample mass to 0,1 mg.

For ready-to-feed infant and adult/paediatric nutritional formula and liquid dairy samples, weigh 220 mg ± 20 mg liquid into a 10 ml glass tube with screw cap. Report the sample mass to 0,1 mg.

For dairy powder and cereals samples, weigh 50 mg ± 5 mg dairy powder samples or 100 mg ± 10 mg cereals samples into a 10 ml glass tube with screw cap. Report the sample mass to 0,1 mg.

To each tube, add water, DDP, HCl, Nva and phenol/HCl according to Table 1.

3) Agilent 1260 Infinity II[1], Waters Acquity[1], Waters Acquity-I[1], Waters Acquity-H[1] and Thermo Fisher Scientific 3000 RS[1] have been successfully used during the multi-laboratory study.

Add the phenol/HCl solution under the hood. Sparge the tube a minimum of approximately 5 s with a stream of nitrogen to displace oxygen. Close tubes with screw caps and vortex. Make sure the caps are perfectly clean (i.e. devoid of any particle) to ensure tightness and avoid evaporation during hydrolysis.

Table 1 — Preparation of the sample tubes

Solution	Reconstituted infant formulas and adult nutritionals, ready-to-feed formulas and liquid dairy samples	Dairy powders	Cereals
Sample, mg	220	50	100
Water, µl	880	750	1 000
DDP solution (6.1.3), µl	600	600	600
0,2 mol/l HCl (6.1.2), µl	600	600	600
Nva stock solution (6.2.1), µl	200	500	200
Phenol/HCl solution (6.1.4), µl	2 500	2 500	2 500

8.2 Cystine calibration standards preparation

Table 2 describes how to prepare calibration standards for converted cystine at 0 pmol/µl to 10 pmol/µl with Nva at 10 pmol/µl (all are final concentrations after derivatization).

Add the phenol/HCl solution under the hood. Sparge the tube approximately 5 s with a stream of nitrogen to displace oxygen. Close tubes with screw caps and vortex. Make sure the caps are perfectly clean (i.e. devoid of any particle) to ensure tightness and avoid evaporation during hydrolysis.

Table 2 — Final cystine concentration after derivatization

Solution	10 pmol/µl	5 pmol/µl	2,5 pmol/µl	1 pmol/µl	0,5 pmol/µl	0 pmol/µl
Cystine solution, µl	200[a]	100[a]	50[a]	200[b]	100[b]	0
Water, µl	900	1 000	1 050	900	1 000	1 100
DDP solution (6.1.3), µl	600	600	600	600	600	600
0,2 mol/l HCl (6.1.2), µl	600	600	600	600	600	600
Nva stock solution (6.2.1), µl	200	200	200	200	200	200
Phenol/HCl solution (6.1.4), µl	2 500	2 500	2 500	2 500	2 500	2 500

[a] 10 mmol/l cystine stock solution (6.3.1).

[b] 1 mmol/l cystine solution (6.3.2).

8.3 Hydrolysis (of samples and cystine standards)

Place tubes in an oven at 110 °C ± 2 °C for 24 h ± 0,5 h.

8.4 Neutralization and dilution (of samples and cystine standards)

Take the tubes out of the oven. Allow hydrolysates to cool down and particles to settle down prior to taking an aliquot. When transferring aliquots, pipet about 1 cm below the top of the liquid. Perform neutralization under the hood.

For infant formula, liquid dairy and cereal samples as well as converted cystine standards, transfer 0,2 ml of each hydrolysate (samples and converted cystine standards) into a 1,5 ml microtube, add 0,2 ml of 6 mol/l NaOH (6.1.1) and then 0,4 ml of 0,1 mol/l HCl (5.14). Mix well and filter through a 0,45 µm membrane filter into another 1,5 ml microtube.