INTERNATIONAL STANDARD

ISO 20638

First edition
2015-11-01

Infant formula — Determination of nucleotides by liquid chromatography

Formules infantiles — Détermination de la teneur en nucléotides par chromatographie liquide

Reference number
ISO 20638:2015(E)

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Foreword

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The committee responsible for this document is 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 International Standard is equivalent to the AOAC Official Method 2011.20: *Nucleotides in infant formula*.

Infant formula — Determination of nucleotides by liquid chromatography

WARNING — The use of this International Standard can involve hazardous materials, operations and equipment. This International Standard does not purport to address all the safety problems associated with its use. It is the responsibility of the user of this International Standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.

1 Scope

This International Standard specifies a method for the quantitative determination of 5′-mononucleotides in infant formula in solid (i.e. powders) or liquid (i.e. ready-to-feed liquids and liquid concentrates) forms using liquid chromatography.

2 Terms and definitions

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

2.1
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 Principle

The sample is dissolved in high-salt solution to inhibit protein and fat interactions. The 5′-mononucleotides — uridine 5′-monophosphate (UMP), inosine 5′-monophosphate (IMP), adenosine 5′-monophosphate (AMP), guanosine 5′-monophosphate (GMP), and cytidine 5′-monophosphate (CMP) — are separated from the sample matrix by strong-anion exchange solid-phase extraction (SPE), followed by chromatographic analysis using a C18 stationary phase with gradient elution, UV detection, and quantitation by an internal standard technique using thymidine 5′-monophosphate (TMP).[1]

4 Reagents and materials

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

4.1 Standards, ≥ 99 % pure (Sigma[1)] or equivalent). Nucleotide sodium salts or sodium salt hydrates may be substituted if free acid forms are not readily available.

4.1.1 TMP, thymidine 5′-monophosphate, CAS No. 365-07-1.

4.1.2 AMP, adenosine 5′-monophosphate, CAS No. 61-19-8.

4.1.3 CMP, cytidine 5′-monophosphate, CAS No. 63-37-6.

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 of the product named. Equivalent products may be used if they can be shown to lead to the same results.

4.1.4 GMP, guanosine 5′-monophosphate, CAS No. 85-32-5.

4.1.5 IMP, inosine 5′-monophosphate, CAS No. 131-99-7.

4.1.6 UMP, uridine 5′-monophosphate, CAS No. 58-97-9.

4.2 Potassium bromide (KBr).

4.3 Potassium dihydrogen phosphate (KH_2PO_4).

4.4 Orthophosphoric acid (H_3PO_4).

4.5 Potassium hydroxide (KOH).

4.6 Ethylenediaminetetraacetic acid, disodium salt dihydrate (EDTA).

4.7 Sodium chloride (NaCl).

4.8 Methanol (CH_3OH).

4.9 Reagent preparation

4.9.1 Standardizing buffer (KH_2PO_4, c = 0,25 mol/l, pH = 3,5). Dissolve 34,0 g KH_2PO_4 (4.3) in 900 ml water and adjust pH to 3,5 with orthophosphoric acid (4.4). Dilute to 1 l.

4.9.2 Extraction solution (NaCl, c = 1 mol/l, EDTA c = 4 mmol/l). Dissolve 58,5 g NaCl (4.7) and 1,5 g EDTA (4.6). Dilute in 1 l water.

4.9.3 Wash solution (KBr, c = 0,3 mol/l). Dissolve 3,6 g KBr (4.2) in 100 ml water.

4.9.4 Eluent solution (KH_2PO_4, c = 0,5 mol/l, pH = 3,0). Dissolve 6,8 g KH_2PO_4 (4.3) in 90 ml water and adjust pH to 3,0 with orthophosphoric acid (4.4). Dilute to 100 ml.

4.9.5 Mobile phase A (KH_2PO_4, c = 10 mmol/l, pH = 5,6). Dissolve 1,4 g KH_2PO_4 (4.3) in 900 ml water and adjust pH to 5,6 ± 0,1 with KOH solution (10 % m/v). Dilute to 1 l with water. Make daily as microbial growth often occurs at room temperature in phosphate buffers that contain little or no organic solvent.

4.9.6 Mobile phase B, 100 % methanol (4.8).

4.10 Standard preparation

4.10.1 Stock standard solutions, ρ approximately 1 mg/ml. Accurately weigh approximately 50 mg each nucleotide 5′-monophosphate into separate 50 ml volumetric flasks. Add 40 ml water, mix until dissolved, and make to volume with water.

4.10.2 Purity standard solutions. Pipette 1,0 ml each stock standard (4.10.1) into separate 50 ml volumetric flasks, make to volume with standardizing buffer (4.9.1), and measure absorbance at the appropriate λ_{max} to determine the concentration of each nucleotide stock standard. See Table 1 and References [1] and [2].

Table 1 — UV absorbance maxima and extinction coefficients for nucleotide 5′-monophosphates

Nucleotide 5′-monophosphate	λ_{max} nm	$E^{1\%}_{1cm}$
Adenosine 5′-monophosphate	257	428,6
Cytidine 5′-monophosphate	280	390,9
Guanosine 5′-monophosphate	254	392,0
Inosine 5′-monophosphate	249	356,5
Uridine 5′-monophosphate	262	312,7
Thymidine 5′-monophosphate	267	288,5

4.10.3 Internal standard solution, ρ approximately 80 µg/ml. Dilute 4 ml TMP stock standard (4.10.1) into 50 ml water.

4.10.4 Working standard solution, ρ approximately 40 µg/ml. Pipette 2 ml each stock standard (4.10.1) (AMP, CMP, GMP, IMP, and UMP) into a single 50 ml volumetric flask and make to volume with water.

4.10.5 Calibration standard solutions. See Table 2 for nominal nucleotide concentrations of the calibration standard solutions.

4.10.5.1 Calibration standard 1. Pipette 0,25 ml working standard (4.10.4) and 1 ml internal standard (4.10.3) into a 25 ml volumetric flask and make to volume with water.

4.10.5.2 Calibration standard 2. Pipette 0,5 ml working standard (4.10.4) and 1 ml internal standard (4.10.3) into a 25 ml volumetric flask and make to volume with water.

4.10.5.3 Calibration standard 3. Pipette 2 ml working standard (4.10.4) and 1 ml internal standard (4.10.3) into a 25 ml volumetric flask and make to volume with water.

4.10.5.4 Calibration standard 4. Pipette 5 ml working standard (4.10.4) and 1 ml internal standard (4.10.3) into a 25 ml volumetric flask and make to volume with water.

Table 2 — Nominal concentration of calibration standards

Calibration solution	Concentration of each nucleotide: AMP, CMP, GMP,IMP, UMP µg/ml	Concentration of TMP µg/ml
1	0,4	3,2
2	0,8	3,2
3	3,2	3,2
4	8,0	3,2