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Infant formula — Determination of nucleotides by liquid chromatography

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

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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

5 Apparatus

Usual laboratory glassware and equipment and, in particular, the following.

5.1 HPLC system, equipped with pump, sample injector unit with a 50 µl injection loop, degasser unit, column oven, and photodiode array detector.

5.2 C18 column, Gemini[2)] C18, 5 µm, 4,6 mm × 250 mm (Phenomenex[2)]).

5.3 Spectrophotometer, capable of digital readout to 3 decimal places.

5.4 pH-meter.

5.5 Centrifuge.

5.6 Centrifuge tubes, Amicon Ultra MWCO 3k, 4 ml (Millipore)[2)].

5.7 Polypropylene centrifuge tubes, capacity 50 ml.

5.8 Disposable syringes, capacity 3 ml.

5.9 Syringe filters, 0,2 µm with cellulose acetate membranes.

5.10 SPE vacuum manifold.

5.11 Polypropylene strong-anion exchange SPE cartridges, 6 ml × 1 000 mg, Chromabond SB[2)].

5.12 Filter membranes, 0,45 µm nylon.

6 Sample preparation

a) Shake or mix sample container prior to opening.

b) Accurately weigh approximately 1 g powder or 10 ml ready-to-feed/liquid milk infant formula into a 50 ml centrifuge tube.

c) Add 30 ml extraction solution (4.9.2).

d) Add 1,0 ml TMP internal standard (4.10.3).

e) Cap the tube and vortex mix until powder dissolved.

f) Allow sample to stand for 10 min to ensure complete hydration.

g) Dilute to a final volume of approximately 50 ml with water.

h) Cap the tube and vortex mix.

i) For starch based products, transfer 2 × 4 ml of prepared sample to two separate ultra centrifuge tubes and centrifuge at 3 500*g* for 60 min, then pool filtrate from both tubes.

2) 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.

7 Procedure

7.1 Extraction

Throughout the extraction procedure, do not let the cartridge run dry but drain to the top of the cartridge bed only.

When draining the cartridge, the flow rate should be < 2 ml/min.

a) For each sample, place a single SPE cartridge on a vacuum manifold.

b) Condition the columns by adding with 4 ml methanol and draining to top of the cartridge bed; followed by adding 2 lots of water (5 ml each) and draining to top of cartridge bed.

c) Load the cartridge with 4 ml of sample solution and drain to the top of the cartridge bed.

d) Wash the cartridge to remove interferences with 4 ml of wash solution (4.9.3) and drain to the top of the cartridge bed.

e) Place a sample collection tube in SPE manifold.

f) Elute the nucleotides with 4 ml of eluent solution (4.9.4) into a sample collection tube and completely drain the cartridge.

g) Filter an aliquot of approximately 2 ml of the eluent through a 0,2 µm syringe filter into an autosampler vial.

7.2 Chromatography

a) Form gradients by low pressure mixing of the two mobile phases, A and B, with separation of nucleotides achieved using the procedure given in Table 3.

Table 3 — Gradient procedure for chromatographic separation

Time min	Flow rate ml/min	Mobile Phase A %	Mobile Phase B %
0	0,6	100	0
25	0,6	80	20
26	0,6	100	0
40	0,6	100	0

b) Acquire spectral data between 210 nm and 300 nm by the photodiode array detector with chromatograms monitored at the specified following wavelengths for quantitation.

   1) IMP: wavelength at 250 nm.

   2) AMP, GMP, and TMP: wavelength at 260 nm.

   3) CMP and UMP: wavelength at 270 nm.

c) Set column oven to 40 °C.

Example chromatograms can be found in Annex A.

8 Calculations

8.1 Calculate the percent purity of each nucleotide (as free acid) in purity standard solution (4.10.2), using Formula (1):

$$\text{Purity},\% = \frac{\text{Abs}_{\lambda\text{max}}}{\text{E}^{1\%}_{1\text{cm}}} \times \frac{50}{m\text{SS}} \times \frac{50}{1} \times 1\,000 \quad (1)$$

where

$\text{Abs}_{\lambda\text{max}}$ is the UV absorbance at maximum wavelength;

$\text{E}^{1\%}_{1\text{cm}}$ is the extinction coefficient for nucleotide;

mSS is the mass of nucleotide in stock standard (mg);

50 is the total volume of stock standard (ml);

50 is the total volume of purity standard (ml);

1 is the volume of stock standard added to purity standard (ml);

1 000 is the mass conversion from mg to g.

8.2 Calculate the concentration of nucleotide in stock standard solution (SS) (4.10.1), using Formula (2):

$$\text{SS } (\mu\text{g/ml}) = \frac{m\text{SS}}{50} \times \frac{\text{PS}\%}{100} \times 10^3 \quad (2)$$

where

mSS is the mass of nucleotide in stock standard (mg);

50 is the total volume of SS (ml);

10^3 is the concentration conversion (mg/ml to µg/ml);

PS% is the percent purity;

100 is the mass conversion (% to decimal).

8.3 Calculate the concentration of TMP in internal standard (IS) (4.10.3), using Formula (3):

$$\text{IS } (\mu\text{g/ml}) = \text{SS} \times \frac{4}{50} \quad (3)$$

where

SS is the concentration of TMP in stock standard (µg/ml);

4 is the volume of TMP stock standard in internal standard (ml);

50 is the total volume of internal standard (ml).

8.4 Calculate the concentration of nucleotides in working standard (WS) (4.10.4), using Formula (4):

$$\text{WS (µg/ml)} = \text{SS} \times \frac{2}{50} \tag{4}$$

where

SS is the concentration of nucleotide in stock standard (µg/ml);

2 is the volume of nucleotide stock standard in working standard (ml);

50 is the total volume of working standard (ml).

8.5 Calculate the concentration of TMP in calibration standards (CS) (4.10.5), using Formula (5):

$$\text{CS (µg/ml)} = \text{IS} \times \frac{1}{25} \tag{5}$$

where

IS is the concentration of nucleotide in internal standard (µg/ml);

1 is the volume of IS in calibration standard (ml);

25 is the total volume of calibration standard (ml).

8.6 Calculate the concentration of nucleotides in calibration standards (CS) (4.10.5), using Formula (6):

$$\text{CS (µg/ml)} = \text{WS} \times \frac{V_{\text{ws}}}{25} \tag{6}$$

where

WS is the concentration of nucleotide in working standard (µg/ml);

V_{WS} is the volume of working standard in calibration standard (ml);

25 is the total volume of calibration standard (ml).

8.7 Determine the linear regression curve for the ratio of peak areas (nucleotide/TMP; y-axis) vs. the ratio of concentrations (nucleotide/TMP; x-axis) for calibration standards and calculate the slope with the y-intercept forced through 0.

8.8 Interpolate the nucleotide contents in unknown samples from this calibration curve.

a) For powders:

$$\text{Nucleotide, mg/100 g} = \frac{A_{\text{NT}}}{A_{\text{IS}}} \times \frac{1}{L} \times \frac{\left(C_{\text{IS}} \times V_{\text{IS}}\right)}{m_{\text{s}}} \times \frac{100}{1\,000} \tag{7}$$

b) For ready-to-feed liquids:

$$\text{Nucleotide, mg/100 ml} = \frac{A_{\text{NT}}}{A_{\text{IS}}} \times \frac{1}{L} \times \frac{\left(C_{\text{IS}} \times V_{\text{IS}}\right)}{V_{\text{s}}} \times \frac{100}{1\,000} \tag{8}$$

where

A_{NT} is the nucleotide peak area in sample;