# Analysis of 5′-Mononucleotides in Infant Formula and Adult/Pediatric Nutritional Formula by Liquid Chromatography: First Action 2011.20

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## Abstract

A method for the routine determination of 5′-mononucleotides (uridine 5′-monophosphate, inosine 5′-monophosphate, adenosine 5′-monophosphate, guanosine 5′-monophosphate, and cytidine 5′-monophosphate) in infant formula and adult nutritionals is described. After sample dissolution and addition of internal standard, potential interferences were removed by anion-exchange SPE followed by HPLC-UV analysis. Single-laboratory validation performance parameters include recovery (92–101%) and repeatability (1.0–2.3% RSD). The method was approved for Official First Action status by an AOAC expert review panel.

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# Introduction

Nucleotides are compounds of critical importance to cellular function, and although not essential dietary nutrients, it has been demonstrated that supplementation of pediatric formulas with nucleotides is of benefit in neonatal nutrition. The described method was developed to provide an accurate, rapid, and robust technique for the routine compliance testing of uridine 5′-monophosphate (UMP), inosine 5′-monophosphate (IMP), adenosine 5′-monophosphate (AMP), guanosine 5′-monophosphate (GMP), and cytidine 5′-monophosphate (CMP) in infant formula and adult/pediatric nutritional formula, and was recently reported (1).

In September 2011, the method was reviewed by an AOAC expert review panel and, based on the published single-laboratory validation (SLV) data as compared with the standard method performance requirements (AOAC SMPR 2011.008; 2) set by the Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN), it was approved for Official First Action status as AOAC Official Method 2011.20.

**AOAC Official Method 2011.20**

**5′-Mononucleotides in Infant Formula and**

**Adult/Pediatric Nutritional Formula HPLC-UV**

First Action 2011

(Applicable to the determination of nucleotide 5′-monophosphates in infant formula and adult/pediatric nutritional formula.)

*Caution:* Refer to the material safety data sheets for all chemicals prior to use. Use all appropriate personal protective equipment and follow good laboratory practices.

## A. 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 SPE, followed by chromatographic analysis using a $C_{18}$ stationary phase with gradient elution, UV detection, and quantitation by an internal standard (IS) technique using thymidine 5′-monophosphate (TMP).

## B. Apparatus

(1) HPLC system.—Equipped with pump, sample injector unit with a 50 µL injection loop, degasser unit, column oven, and photodiode array detector.

(2) $C_{18}$ column.—Gemini $C_{18}$, 5 µm, 4.6 × 250 mm (Phenomenex, Torrance, CA) or equivalent.

(3) Spectrophotometer.—Capable of digital readout to 3 decimal places.

(4) pH meter.

(5) Centrifuge.

(6) Amicon ultra centrifuge tubes.—MWCO 3k, 4 mL (Millipore-Carrigtwohill, Co. Cork, Ireland) or equivalent.

(7) Polypropylene centrifuge tubes.—50 mL.

(8) Disposable syringes.—3 mL.

(9) Syringe filters.—0.2 µm with cellulose acetate membranes.

(10) SPE vacuum manifold.

(11) Chromabond SB polypropylene strong-anion exchange SPE cartridges.—6 mL × 1000 mg (Macherey-Nagel, Düren, Germany) or equivalent.

(12) Filter membranes.—0.45 µm nylon.

## C. Reagents

(1) Standards.—Should be ≥99% pure (Sigma, St. Louis, MO, or equivalent). Nucleotide sodium salts or sodium salt hydrates may be substituted if free acid forms are not readily available.

   (1) TMP.—CAS No. 365-07-1.

   (2) AMP.—CAS No. 61-19-8.

   (3) CMP.—CAS No. 63-37-6.

   (4) GMP.—CAS No. 85-32-5.

   (5) IMP.—CAS No. 131-99-7.

   (6) UMP.—CAS No. 58-97-9.

(2) Potassium bromide (KBr).

(3) Potassium dihydrogen phosphate ($KH_2PO_4$).

(4) Orthophosphoric acid ($H_3PO_4$).

(5) Potassium hydroxide (KOH).

(6) Ethylenediaminetetraacetic acid, disodium salt dihydrate (EDTA).

(7) Sodium chloride (NaCl).

(8) Methanol (MeOH).

(9) Water.—Purified with resistivity ≥ 18 MΩ.

## D. Reagent Preparation

(1) Standardizing buffer ($KH_2PO_4$, 0.25 M, pH 3.5).—Dissolve 34.0 g $KH_2PO_4$ in 900 mL water and adjust pH to 3.5 with $H_3PO_4$. Dilute to 1 L.

(2) Extraction solution (NaCl 1 M, EDTA 4 mM).—Dissolve 58.5 g NaCl and 1.5 g EDTA in 1 L water.

(3) Wash solution (KBr, 0.3 M).—Dissolve 3.6 g KBr in 100 mL water.

(4) Eluent solution ($KH_2PO_4$, 0.5 M, pH 3.0).—Dissolve 6.8 g $KH_2PO_4$ in 90 mL water and adjust pH to 3.0 with $H_3PO_4$. Dilute to 100 mL.

(5) Mobile phase A ($KH_2PO_4$, 10 mM, pH 5.6).—Dissolve 1.4 g $KH_2PO_4$ in 900 mL water and adjust pH to 5.6 with KOH solution (10% w/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.

(6) Mobile phase B.—100% MeOH.

## E. Standard Preparation

See Table 2011.20A for the UV absorbance maxima and extinction coefficients for nucleotide 5′-monophosphates.

(1) Stock standard solutions (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 fill to volume with water.

(2) Purity standard solutions.—Pipette 1.0 mL each stock standard into separate 50 mL volumetric flasks, make to volume with standardizing buffer ($KH_2PO_4$, 0.25 M, pH 3.5), and measure absorbance at the appropriate $\lambda_{max}$ to determine the concentration of each nucleotide stock standard.

(3) Internal standard solution (approximately 80 µg/mL).—Dilute 4 mL TMP stock standard in 50 mL water.

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

(5) Calibration standard solutions.—See Table 2011.20B for nominal nucleotide concentrations of the calibration standard solutions.

   (1) Calibration standard 1.—Pipette 0.25 mL working standard and 1 mL internal standard into a 25 mL volumetric flask and make to volume with water.

   (2) Calibration standard 2.— Pipette 0.5 mL working standard and 1 mL internal standard into a 25 mL volumetric flask and make to volume with water.

   (3) Calibration standard 3.— Pipette 2 mL working standard and 1 mL internal standard into a 25 mL volumetric flask and make to volume with water.

(4) Calibration standard 4.— Pipette 5 mL working standard and 1 mL internal standard into a 25 mL volumetric flask and make to volume with water.

**Table 2011.20A. UV absorbance maxima and extinction coefficients for nucleotide 5′-monophosphates**

| Nucleotide[a] | $\lambda_{max}$, (nm) | $E^{1\%}$ |
|---|---|---|
| AMP | 257 | 428.6 |
| CMP | 280 | 390.0 |
| GMP | 254 | 392.0 |
| IMP | 249 | 356.5 |
| UMP | 262 | 312.7 |
| TMP | 267 | 288.5 |

[a] AMP = adenosine 5′-monophosphate; CMP = cytidine 5′-monophosphate; GMP = guanosine 5′-monophosphate; IMP = inosine 5′-monophosphate; UMP = uridine 5′-monophosphate; TMP = thymidine 5′-monophosphate

## F. Sample Preparation

(1) Shake or mix sample container prior to opening.

(2) Accurately weigh approximately 1 g powder or 10 mL ready-to-feed/liquid milk infant formula/adult nutritional product into a 50 mL centrifuge tube.

(3) Add 30 mL extraction solution (NaCl 1 M, EDTA 4 mM).

(4) Add 1.0 mL TMP IS (approximately 80 µg/mL).

(5) Cap the tube and vortex mix until powder dissolved.

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

(7) Dilute to a final volume of 50 mL with water.

(8) Cap the tube and vortex mix.

(9) For starch-based products, transfer 2 × 4 mL prepared sample to two separate ultra centrifuge tubes and centrifuge at 3500 × g for 60 min, and then pool filtrates from both tubes.

**Table 2011.20B Nominal concentrations of calibration standards**

| Calibration solution | Concentration of AMP, CMP,GMP, IMP, UMP (µg/mL)[a] | Concentration of TMP (µg/mL)[a] |
|---|---|---|
| 1 | 0.4 | 3.2 |
| 2 | 0.8 | 3.2 |
| 3 | 3.2 | 3.2 |
| 4 | 8.0 | 3.2 |

[a] AMP = adenosine 5′-monophosphate; CMP = cytidine 5′-monophosphate; GMP = guanosine 5′-monophosphate; IMP = inosine 5′-monophosphate; UMP = uridine 5′-monophosphate; TMP = thymidine 5′-monophosphate

## G. 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.

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

(2) Condition the columns by adding 4 mL methanol and draining to the top of the cartridge bed, followed by adding two aliquots of water (5 mL each) and draining to the top of the cartridge bed.

(3) Load the cartridge with sample solution (4 mL) and drain to the top of the cartridge bed.

(4) Wash the cartridge to remove interferences with wash solution (KBr, 0.3 M, 4 mL) and drain to the top of the cartridge bed.

(5) Place a sample collection tube in the SPE manifold.

(6) Elute the nucleotides with eluent solution ($KH_2PO_4$, 0.5 M, pH 3.0, 4 mL) into a sample collection tube and completely drain the cartridge.

(7) Filter an aliquot (approximately 2 mL) eluent through a 0.2 µm syringe filter into an autosampler vial.

## H. Chromatography

(1) Form gradients by low pressure mixing of the two mobile phases, A and B, with separation of nucleotides achieved using the procedure shown in Table 2011.20C.

(2) Acquire spectral data between 210 and 300 nm using the photodiode array detector with chromatograms monitored at the specified wavelengths below for quantitation.

  (1) IMP wavelength at 250 nm.

  (2) AMP, GMP, and TMP wavelengths at 260 nm.

  (3) CMP and UMP wavelengths at 270 nm.

(3) Set column oven to 40 °C.

**Table 2011.20C. Gradient procedure for chromatographic separation**

| Time, min | Flow rate (mL/min) | Phase Composition | |
|---|---|---|---|
| | | % A | % B |
| 0 | 0.6 | 100 | 0 |
| 25 | 0.6 | 80 | 20 |
| 26 | 0.6 | 100 | 0 |
| 40 | 0.6 | 100 | 0 |

## I. Calculations

(1) Concentration of nucleotide in stock standard (SS):

$$\text{SS, µg/mL} = \frac{wtSS}{50} \times \frac{PS\%}{100} \times 10^3$$

where

wtSS = weight of nucleotide in stock standard (mg),

50 = total volume of SS (mL),

$10^3$ = concentration conversion (mg/mL to µg/mL),

PS% = percent purity, and

100 = mass conversion (% to decimal).

(2) Percentage purity of each nucleotide (as free acid) in purity standard (PS):

$$\text{Purity, \%} = \frac{\text{Abs}_{\lambda max}}{E^{1\%}_{1cm}} \times \frac{50}{\text{wtSS}} \times \frac{50}{1} \times 1000$$

where

$Abs_{\lambda max}$ = UV absorbance at maximum wavelength,

$E^{1\%}$ = extinction coefficient for nucleotide,

wtSS = weight of nucleotide in stock standard (mg),

50 = total volume of stock standard (mL),

50 = total volume of purity standard (mL),

1 = volume of stock standard added to purity standard (mL), and

1000 = mass conversion from mg to g.

(3) Concentration of TMP in IS:

$$\text{IS, µg/mL} = \text{SS} \times \frac{4}{50}$$

where

SS = concentration of TMP in stock standard (µg/mL),

4 = volume of TMP stock standard in IS (mL), and

50 = total volume of IS (mL).

(4) Concentration of nucleotides in working standard (WS):

$$\text{WS, µg/mL} = \text{SS} \times \frac{2}{50}$$

where SS = concentration of nucleotide in stock standard (µg/mL),

2 = volume of nucleotide stock standard in working standard (mL), and

50 = total volume of working standard (mL).

(5) Concentration of TMP in calibration standards (CS):

$$\text{CS, µg/mL} = \text{IS} \times \frac{1}{25}$$

where

IS = concentration of nucleotide in IS (µg/mL),

1 = volume of IS in calibration standard (mL), and

25 = total volume of calibration standard (mL).

(6) Concentration of nucleotides in calibration standard (CS):

$$\text{CS, µg/mL} = \text{WS} \times \frac{V_{ws}}{25}$$

where

WS = concentration of nucleotide in working standard (µg/mL),

$V_{WS}$ = volume of working standard in CS (mL), and

25 = total volume of CS (mL).

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

(8) Interpolate the nucleotide contents in unknown samples from this calibration curve.

(1) For powders:

$$\text{Nucleotide, mg/hg} = \frac{A_{NT}}{A_{IS}} \times \frac{1}{L} \times \frac{(C_{IS} \times V_{IS})}{W_S} \times \frac{100}{1000}$$

(2) For ready-to-feed liquids:

$$\text{Nucleotide, mg/dL} = \frac{A_{NT}}{A_{IS}} \times \frac{1}{L} \times \frac{(C_{IS} \times V_{IS})}{V_S} \times \frac{100}{1000}$$

where

$A_{NT}$ = nucleotide peak area in sample,

$A_{IS}$ = TMP peak area in sample,

L = linear regression slope of calibration curve,

$C_{IS}$ = concentration of IS added to sample (µg/mL),

$V_{IS}$ = volume of IS added to sample (mL),

$W_S$ = weight of sample (g),

1000 = mass conversion of result (µg to mg),

$V_S$ = volume of sample (mL), and

100 = mass or volume conversion of result (g to hg; mL to dL).

## J. Data Handling

Report results in mg/hg or mg/dL to 1 decimal place.

## K. Reference

Gill et al. (2012) *J. AOAC Int.* 95, 599–602

# Results and Discussion

An SLV of the method previously published (1) indicated that this method is suitable for the routine determination of the 5'-mononucleotide content in milk and milk-based pediatric and adult nutritional products. The validation parameters investigated included linearity and working range, method detection

limit, accuracy as recovery and bias, precision as repeatability and intermediate precision, and robustness. Linearity was demonstrated for all five nucleotides with correlation coefficients of > 0.9999, and a visual inspection of residual plots. The method detection limits for individual nucleotides ranged from 0.06 to 0.19 mg/kg. The working range for individual nucleotides evaluated was from 0.06 to 17.4 mg/kg. Accuracy was determined as recovery, with values measured from 92 to 101%, within the suggested AOAC limits of 80−115% at the 10 ppm level (3), and no bias was found (*P*-values all > 0.05) when compared with a previously published method (4). Precision as repeatability was estimated as 1.0–2.3 %RSD with a range for HorRat of 0.3–0.5 and for intermediate precision of 3.8–8.6 RSD%. A Plackett−Burman robustness study (5) was performed and the seven factors evaluated were shown not to affect the final results within typical experimental variations.

The method was applied to the analysis of a number of commercially available pediatric and nutritional powders. The products used for sampling included infant formula, follow-on formulas, and an adult nutritional product. The range of sources for these products included bovine milk, hydrolyzed milk protein, caprine milk, and soy protein. The method was found to be suitable for use with these various product matrixes.

It is recommended that this method be further examined against a set of infant formula and adult nutritional matrixes developed for this purpose by the SPIFAN community, and its performance evaluated against the SMPRs established by SPIFAN.

# References

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