Analysis of Nucleotide 5′-Monophosphates in Infant Formulas by HPLC-UV: Collaborative Study, Final Action 2011.20

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Abstract

A collaborative study was conducted on AOAC First Action Method 2011.20: 5′-Mononucleotides in Infant Formula and Adult/Pediatric Nutritional Formula. After the successful analysis of National Institute of Standards and Technology (NIST) 1849a Standard Reference Material (SRM) as a practice sample, 12 laboratories participated in the analysis of duplicate samples of six different infant formula products. The samples were dissolved in high-salt solution to inhibit protein and fat interactions, with the nucleotides [uridine 5′-monophosphate (UMP), inosine 5′-monophosphate (IMP), adenosine 5′-monophosphate (AMP), guanosine 5′-monophosphate (GMP), and cytidine 5′-monophosphate (CMP)] 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 technique using thymidine 5′-monophosphate. For nucleotide supplemented products, precision is within the Standard Method Performance Requirements^{SM} (SMPR) 2011.008 target reproducibility limit of ≤ 11%, with the reproducibility RSD (RSD_R) estimated at 7.1–8.7% for CMP, 7.9–9.0% for UMP, 2.8–7.7% for GMP, 5.5–10.3% for IMP, and 2.7–6.2% for AMP, and Horwitz ratio (HorRat) values of 0.9–1.0 for CMP, 0.9–1.0 for UMP, 0.3–0.7 for GMP, 0.6–1.0 for IMP, and 0.3–0.7 for AMP.

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Introduction

Nucleotides and nucleosides play important roles in cellular function as precursors to nucleic acids, as intermediaries in the transfer of chemical energy, and as critical components of coenzymes involved in carbohydrate, lipid, and protein metabolism. Although nucleotides are not essential dietary components as they can be synthesized *de novo*, they may be conditionally essential when the endogenous supply is insufficient, such as during periods of rapid neonatal growth. In recognition of their nutritional importance, infant formulas are increasingly supplemented with nucleotides. As neonates are dependent on a single dietary source for an extensive period, it is important that reliable analytical methods be available to accurately estimate the nucleotide content in infant formulas (1).

In view of the absence of an internationally accepted analytical method, nucleotides were identified by the AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) as a priority for which a reference method was urgently needed. The SPIFAN Nucleotides Working Group developed Standard Method Performance Requirements (SMPR, 2011.008) for assessing merits of proposed nucleotide methods and established reproducibility limits of ≤ 11% in the range of 1–5 mg/hg reconstituted product, and ≤ 16% for 0.1 mg/hg reconstituted product.

We previously developed and performed a single laboratory validation (SLV) study on an HPLC-UV method that incorporated SPE and internal standardization for the routine estimation of nucleotide 5'−monophosphates in milk and pediatric products (2). In September 2011, this HPLC-UV method was reviewed by an AOAC expert review panel (ERP) and, based on published SLV data, was approved for Official First Action status as AOAC Method 2011.20 (3, 4). The method subsequently underwent a comprehensive SLV study using a set of infant formula and adult nutritional products (SPIFAN Kit) that were selected as a representative subsample of the wide range of commercially available products, and the results were compared with the SMPR (5, 6). This SLV study was approved by the ERP in June 2012, and the method was recommended to advance to collaborative study for evaluation of reproducibility.

Collaborative Study

Although 19 laboratories initially indicated their interest to take part in this study, a number later withdrew primarily because of the timing of the study and difficulties with importation of the samples. Participating laboratories included those representing regulatory agencies, infant formula manufacturers, contract analytical services, and food research institutes. Prior to commencement of the study, each collaborator received a detailed study protocol to allow familiarization with the technique and an opportunity to communicate any difficulties. The NIST 1849a (National Institute of Standards and Technology, Gaithersburg, MD) Standard Reference Material (SRM) was selected as a

practice sample to allow the laboratories to begin preliminary method evaluation. The distribution of the samples for this collaborative study was complicated because of the implementation of strict importation regulations by many countries; ultimately, only 12 laboratories from five countries were able to participate.

The SPIFAN Kit was unsuitable for use in this collaborative study because few of the included products were fortified with nucleotides; therefore, alternative sources of samples were required. Infant formula products (lactose-free, starch-based, hydrolysate-based, soy-based, and two whey-based) were sourced from manufacturing sites in Europe for subsampling and distribution, and each was pooled, mixed, subsampled into duplicate sachets (10 coded as blind-coded duplicates, two uncoded as a duplicate), sealed, and dispatched to the participating laboratories. The starch-based sample was uncoded because of the need for special handling during sample preparation. With the exception of the soy-based infant formula, all products had been supplemented with nucleotides during their manufacture.

Homogeneity of the nucleotides dispersed in the samples was assessed by replicate analyses of test samples from separate sachets (n = 5). Statistical analysis was on the basis of a paired *t*-test to establish significant difference between results obtained from different sachets. No bias was found between any sachets for any of the nucleotides, and the precision obtained was that expected for the concentration levels in these products (data not shown). On this basis, the samples were deemed to be fit for use in the collaborative study.

Upon completion of analysis of the samples, the collaborators were required to submit raw data as sample weights, UV absorbances of standard solutions, and peak areas for standards and samples, as well as the final results of nucleotide concentrations in the samples. Participants were also invited to add any relevant comments based on their experience in the use of the method.

All data were statistically analyzed using the AOAC protocol for overall mean, intra-laboratory repeatability (S_r), repeatability RSD (RSD_r), interlaboratory reproducibility (S_R), reproducibility RSD (RSD_R), and Horwitz ratio (HorRat; 7). Cochran ($P = 0.025$, one-tail) and Grubbs (single and double, $P = 0.025$, two-tail) tests were utilized to determine outliers.

The method protocol sent to the collaborating laboratories was as described in AOAC First Action Method 2011.20, with minor modifications to the nucleotide extinction coefficients (6) and to the sample preparation for starch-based products, based upon recommendations made by the ERP.

AOAC Official Method 2011.20

5′-Mononucleotides in Infant Formula and

Adult/Pediatric Nutritional Formula HPLC-UV

First Action 2011

Final Action 2015

(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 λ_{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]	λ_{max}, (nm)	E1%
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):

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

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

$\text{Abs}_{\lambda max}$ = 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:

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

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):

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

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):

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

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):

$$\text{CS, } \mu\text{g/mL} = \text{WS} \times \frac{V_{WS}}{25}$$

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 CSs 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.

Results and Discussion

The initial phase of method evaluation within the participating laboratories involved the analysis of a practice sample. The NIST 1849a SRM was selected for this purpose for a number of reasons: (1) as it was readily available in most laboratories, the method setup and evaluation could commence without receipt of shipped samples; (2) participants could evaluate method implementation in their laboratory against certified values; and (3) it provided additional confidence that there was no significant bias in method performance among all participants.

Precision and bias were evaluated for NIST 1849a practice samples as defined by the AOAC ERP (8). All participating laboratories provided acceptable data for the practice sample (Table 1) and, when the test sample set had been received, participants could begin the analysis at their earliest convenience.

Upon completion of the analyses, each participant reported the results accompanied by calibration regression parameters and a description of any method deviations. All 12 laboratories returned acceptable standard calibration parameters based on linear regression correlation coefficients (r^2: 0.9971–1.0000). The analytical results submitted by the participants were collated (Tables 2–6) and statistically analyzed (Tables 7–11). In some instances, statistical outliers were identified, but, where deemed to be reasonable to do so, these were retained in the data set for calculation of the method precision.

As the soy-based infant formula was not fortified with nucleotides and contained endogenous levels only, the precision for this sample was poor, as expected at concentrations near or below the method detection limit (2). The mean nucleotide concentrations in the supplemented infant formula powders were in the ranges 5.4–11.4 mg/hg for CMP, 3.5–4.2 mg/hg for UMP, 1.1–1.7 mg/hg for GMP, 1.7–2.5 mg/hg for IMP, and 3.3–4.7 mg/hg for AMP. The RSD_r values obtained were in the ranges 1.1–2.7% for CMP, 1.5–5.4% for UMP, 1.6–3.9% for GMP, 1.4–2.8% for IMP, and 1.3–3.9% for AMP. The RSD_R values obtained were in the ranges 7.1–8.7% for CMP, 7.9–9.0% for UMP, 2.8–7.7% for GMP, 5.5–10.3% for IMP, and 2.7–6.2% for AMP. In all instances of nucleotide-supplemented infant formulas, the repeatability and the reproducibility were within limits set in the SMPR for nucleotides (6). Acceptable reproducibility was also demonstrated, with HorRat values for the method in the ranges 0.9–1.0 for CMP, 0.9–1.0 for UMP, 0.3–0.7 for GMP, 0.6–1.0 for IMP, and 0.3–0.7 for AMP (recommended range 0.5–2.0; 9).

A summary of each laboratory's performance was sent to participants, along with an invitation to make comments on the performance of the method in their laboratory. In general, comments were positive with respect to the use of the method and intra-laboratory performance. Laboratory 3 recommended that EDTA used be standardized to the salt form. It was noted by Laboratory 5 that, if the pH of the mobile phase was higher by > 0.3 pH units, the elution sequence changed for AMP and TMP. Some concerns were expressed by Laboratory 7 regarding the value of the extinction coefficient for CMP. Follow-up work was undertaken, and the extinction coefficient used for CMP was verified by Laboratory 7 after an investigation with the supplier of the standard. Laboratory 9 recommended a 5 min centrifugation of the samples prior to the SPE step. Laboratory 10 suggested adding a reduced amount of extract to the SPE cartridge to make the method more applicable to various product matrixes.

The method has demonstrated its compliance with the applicability statement of SMPR 2011.008 (6), and it has been shown in this collaborative study to be suitable for the analysis of nucleotides in a wide range of supplemented infant formulas. The method has been demonstrated to be unsuitable for samples containing endogenous nucleotide levels only. Nucleosides are an optional nutrient defined by the SMPR and are not determined with this method. Although the method may be applicable to adult nutritional products, such products are generally not fortified with nucleotides because they are not considered to be an essential dietary nutrient for adults.

Conclusions

A collaborative study of the AOAC First Action 2011.20 HPLC-UV method for the analysis of nucleotides in infant formula was undertaken. The method was applied to a number of different infant formula

matrixes and demonstrated acceptable reproducibility precision for nucleotide-supplemented infant formulas.

Recommendation

A study report summarizing the outcomes of this collaborative study was submitted with the recommendation that AOAC First Action Method 2011.20 be accepted as a SPIFAN-endorsed AOAC Final Action Method. The AOAC ERP evaluated the collaborative study data in September 2014, and endorsed the recommendation, which was subsequently approved by the Official Methods Board in November 2014.

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(9) Official Methods of Analysis (2012) 19th Ed., AOAC INTERNATIONAL, Rockville, MD, Appendix D

Table 1. Bias and precision results for NIST 1849a practice sample

Statistic	CMP	UMP	GMP	IMP	AMP
Total number of laboratories	12	12	12	12	12
Total number of replicates	24	24	24	24	24
Mean ($\overline{x}$)	28.1	11.8	15.1	0	10.9
Certified value (μ)	26.8	12.9	14.6	n/a	10.5
Uncertainty (U_{CRV})	2.9	1.5	1.1	n/a	0.53
Coverage factor (k)	2.57	2.57	2.57	n/a	2.57
Nominal bias	5.00%	-8.90%	3.20%	n/a	3.50%
Students test-statistic (t_{Stat})	1.16	1.92	1.05	n/a	1.63
Degrees of freedom (DF)	5.6	5.4	6.1	n/a	7.3
p-value	0.30	0.11	0.34	n/a	0.15
Repeatability standard deviation (SD_r)	0.46	0.30	0.38	n/a	0.22
Reproducibility standard deviation (SD_R	1.36	0.59	0.68	n/a	0.47
Repeatability relative standard deviation (RSD_r)	1.6	2.5	2.5	n/a	2.1
Reproducibility relative standard deviation (RSD_R)	4.8	5	4.5	n/a	4.4
HorRat value	0.7	0.6	0.6	n/a	0.6

CMP = cytidine 5′-monophosphate, UMP = uridine 5′-monophosphate, GMP = guanosine 5′-monophosphate, IMP = inosine 5′-monophosphate, AMP = adenosine 5′-monophosphate

Table 2. Collaborative study data for cytidine 5′-monophosphate in infant formulas

Lab Number	NIST 1849a		Lactose-free		Starch-based		Hydrolysate-based		Soy-based[a]		Whey-based		Whey-based	
1	27.88	27.97	11.09	11.12	10.75	10.73	9.61	9.63	0.49	0.47	5.20	5.25	5.17	5.21
2	29.60	29.10	9.52	9.48	10.49	9.43	10.3	10.10	0.00	0.00	4.55	4.40	3.94[b]	3.56[b]
3	27.46	26.90	10.71	10.50	9.90	9.98	9.60	9.53	0.00	0.00	6.23	5.98	5.05	4.81
4	29.66	30.45	12.40	12.40	11.90	12.30	10.70	10.90	1.00	1.10	6.00	6.10	6.20	6.00
5	28.77	28.65	12.84	13.12	11.92	11.92	10.91	10.20	0.58	0.78	5.86[c]	6.41[c]	6.24	6.14
6	27.80	28.00	11.68	11.76	11.27	11.20	10.23	10.21	0.88	0.89	5.48	5.43	5.47	5.41
7	27.55	29.25	11.28	11.00	11.28	11.22	9.49	8.56	0.56	0.46	5.30	5.26	5.27	5.15
8	27.65	27.88	11.65	11.52	11.10	10.93	8.93	9.19	0.44	0.48	5.41	5.39	5.36	5.41
9	28.21	28.17	11.53	11.56	2.77[d]	3.08[d]	9.96	9.96	0.59	0.47	5.44	5.47	5.44	5.46
10	29.50	29.60	11.86	11.95	11.44	11.44	9.83	9.98	0.66	0.62	5.58	5.59	5.63	5.60
11	24.54	25.27	11.60	11.90	11.03	11.84	10.30	10.10	0.90	0.00	5.50	5.20	5.30	5.50
12	28.08	27.53	10.78	10.94	9.91	9.79	9.03	9.09	0.30	0.35	5.13	5.23	4.80	4.83

[a] Product not fortified with cytidine 5′-monophosphate

[b] Identified as Grubbs outlier; results removed from data set for statistical analysis

[c] Identified as Cochran outlier; results kept in data set for statistical analysis

[d] Problems were identified by participants and as Grubbs outlier results excluded from data set for statistical analysis

Table 3. Collaborative study data for uridine 5′-monophosphate in infant formulas

Lab Number	NIST 1849a		Lactose-free		Starch-based		Hydrolysate-based		Soy-based[a]		Whey-based		Whey-based	
1	11.75	11.58	3.94	3.95	4.03	4.14	4.30	4.22	0.29	0.32	3.47	3.51	3.60	3.60
2	11.30	11.10	3.75	3.83	3.91	3.78	4.00	3.70	0.00	0.00	3.25	3.26	3.26	3.11
3	10.59	10.51	3.27	3.26	3.14[b]	3.11[b]	3.59	3.38	0.00	0.00	2.97	2.90	2.70	3.11
4	12.14	12.00	4.00	4.00	3.90	3.90	4.40	4.40	0.20	0.20	3.60	3.60	3.60	3.80
5	10.91	11.29	3.51	3.34	3.44[c]	4.29[c]	4.11	4.31	0.19	0.00	3.78	3.93	3.85	3.78
6	11.60	11.80	4.32	4.30	4.23	4.24	4.56	4.51	0.28	0.25	3.87	3.84	3.83	3.87
7	12.26	12.36	3.93	4.00	4.02	3.89	4.25	4.47	0.32	0.28	3.70	3.56	3.49	3.73
8	12.04	12.57	3.66	3.80	4.04	3.77	3.63	3.58	0.38	0.38	3.64	3.66	3.69	3.60
9	12.25	11.79	3.97	4.03	1.19[d]	0.00[d]	4.33	4.28	0.35	0.22	3.58	3.55	3.67	3.64
10	11.80	11.90	3.84	3.88	3.91	3.93	4.51	4.11	0.22	0.23	3.53	3.59	3.60	3.61
11	12.05	12.45	3.50	3.80	3.73	3.91	4.00	3.70	0.00	0.00	3.00	3.00	3.10	3.10
12	11.50	12.59	4.28	4.05	3.93	4.17	4.53	4.60	0.27	0.29	3.75	3.87	3.78	3.89

[a] Product not fortified with uridine 5′-monophosphate

[b] Identified as Grubbs outlier; results kept in data set for statistical analysis

[c] Identified as Cochran outlier; results kept in data set for statistical analysis

[d] Problems were identified by participants and as Grubbs outlier results excluded from data set for statistical analysis

Table 4. Collaborative study data for guanosine 5′-monophosphate in infant formulas

Lab Number	NIST 1849a[a]		Lactose-free		Starch-based		Hydrolysate-based		Soy-based[a]		Whey-based		Whey-based	
1	14.31	14.40	1.39	1.39	1.59	1.60	1.33	1.35	0.26	0.32	1.01	1.01	0.99	1.02
2	15.80	15.50	1.50[b]	1.40[b]	1.78	1.78	1.40	1.40	0.00	0.00	1.06	1.05	0.70[c]	1.02[c]
3	15.15	14.86	1.44	1.40	1.63	1.63	1.60	1.58	0.00	0.00	1.13	1.13	1.12	1.17
4	14.77	15.36	1.50	1.50	1.70	1.70	1.40	1.40	0.00	0.00	1.10	1.10	1.10	1.10
5	14.92	14.93	1.45	1.45	1.67	1.69	1.44	1.33	0.25	0.41	1.07	1.09	1.08	1.02
6	14.60	14.70	1.40	1.45	1.63	1.60	1.48	1.39	0.23	0.25	1.06	1.03	1.05	1.05
7	13.97	15.63	1.43	1.40	1.65	1.60	1.25[a]	1.04[a]	0.23	0.20	0.98	0.98	0.91	1.01
8	14.68	14.84	1.45	1.46	1.67	1.62	1.33	1.34	0.37	0.54	1.06	1.05	1.05	1.06
9	15.12	15.08	1.47	1.45	0.00[d]	0.00[d]	1.41	1.40	0.29	0.3	1.05	1.08	1.08	1.09
10	15.30	15.30	1.48	1.50	1.72	1.79	1.36	1.41	0.36	0.35	1.07	1.06	1.08	1.06
11	16.73	16.69	1.50	1.50	1.78	1.75	1.40	1.40	0.00	0.00	1.10[b]	1.00[b]	1.00	1.10
12	14.60	14.41	1.42	1.40	1.64	1.58	1.39	1.38	0.49	0.45	1.03	1.00	1.02	1.04

[a] Product not fortified with guanosine 5′-monophosphate

[b] Identified as Cochran outlier; results kept in data set for statistical analysis

[c] Identified as Grubbs outlier; results removed from data set for statistical analysis

[d] Problems were identified by participants and as Grubbs outlier results excluded from data set for statistical analysis

Table 5. Collaborative study data for inosine 5'-monophosphate in infant formulas

Lab Number	NIST 1849a		Lactose-free		Starch-based		Hydrolysate-based		Soy-based[a]		Whey-based[a]		Whey-based[a]	
1	0.00	0.00	1.52	1.53	1.51	1.49	2.32	2.34	0.21	0.13	0.03[b]	0.02[b]	0.02[b]	0.05[b]
2	0.00	0.00	1.87	1.82	1.82	1.84	2.50	2.50	0.00	0.00	0.00	0.00	0.00	0.00
3	0.00	0.00	1.64	1.63	1.58	1.59	2.46	2.44	0.00	0.00	0.00	0.00	0.00	0.00
4	0.00	0.00	1.70	1.70	1.70	1.70	2.95[b]	3.90[b]	0.00	0.00	0.00	0.00	0.00	0.00
5	0.57	0.32	1.77	1.73	2.05	2.07	2.52	2.54	0.29	0.60	0.00[c]	0.22[c]	0.00	0.00
6	0.00	0.00	1.58	1.59	1.55	1.56	2.37	2.35	0.16	0.13	0.00	0.00	0.00	0.00
7	0.00	0.00	1.46	1.54	1.47	1.44	2.40[e]	2.21[e]	0.00	0.00	0.00	0.00	0.00	0.00
8	0.00	0.00	1.63	1.61	1.61	1.61	2.38	2.42	0.22	0.26	0.00	0.00	0.00	0.00
9	0.00	0.00	1.61	1.63	0.00[d]	0.00[d]	2.39	2.41	0.00	0.00	0.00	0.00	0.00	0.00
10	0.00	0.00	1.66	1.68	1.63	1.63	2.38	2.43	0.13	0.13	0.00	0.00	0.00	0.00
11	0.00	0.00	1.80[e]	1.60[e]	1.80[e]	1.71[e]	2.50	2.50	0.00	0.00	0.00	0.00	0.00	0.00
12	0.00	0.00	1.60	1.59	1.62	1.59	2.78	2.74	0.89	0.76	0.00	0.00	0.35[b]	0.24[b]

[a] Product not fortified with inosine 5'-monophosphate

[b] Identified as Grubbs outlier; results removed from data set for statistical analysis

[c] Identified as Cochran outlier; results removed from data set for statistical analysis

[d] Problems were identified by participants and as Grubbs outlier results excluded from data set for statistical analysis

[e] Identified as Cochran outlier; results kept in data set for statistical analysis

Table 6. Collaborative study data for adenosine 5′-monophosphate in infant formulas

Lab Number	NIST 1849a[a]		Lactose-free		Starch-based		Hydrolysate-based		Soy-baseda		Whey-based		Whey-based	
1	10.69	10.75	3.33	3.32	3.45	3.48	4.96	4.95	0.57	0.97	3.69	3.69	3.68	3.70
2	11.80	11.60	3.27	3.09	3.53	3.52	5.10	4.70	0.71	1.00	3.15	3.06	2.58[b]	3.09[b]
3	11.15	10.89	3.29	3.32	3.38	3.42	4.79	4.76	0.00	0.00	3.35	3.52	3.41	3.35
4	11.05	11.23	3.40	3.40	3.60	3.70	5.10	5.20	0.60	0.60	3.70	3.60	3.70	3.70
5	10.84	11.22	3.52	3.45	3.56	3.79	4.88	4.89	0.41	0.59	3.48	3.52	3.52	3.49
6	10.70	10.70	3.35	3.35	3.52	3.46	4.75	4.72	0.44	0.43	3.46	3.40	3.43	3.40
7	9.59	10.42	3.32	3.28	3.59	3.58	4.57[a]	3.84[a]	0.51	0.50	3.64	3.51	3.63[b]	3.48[b]
8	10.82	10.90	3.43	3.41	3.55	3.42	4.32	4.51	0.74	0.86	3.42	3.39	3.36	3.39
9	10.34	10.28	3.26	3.27	0.00[c]	0.00[c]	4.67	4.65	0.63	0.61	3.39	3.39	3.35	3.32
10	11.30	11.30	3.42	3.43	3.58	3.54	4.79	5.01	0.86	0.88	3.68	3.71	3.73	3.72
11	11.25	10.79	3.40	3.30	3.56	3.75	5.10	4.70	0.00	0.00	3.60	3.50	3.60	3.60
12	10.69	10.59	3.24	3.27	3.46	3.42	4.79	4.77	0.54	0.55	3.67	3.70	3.30	3.43

[a] Product not fortified with inosine 5′-monophosphate

[b] Identified as Cochran outlier; results kept in data set for statistical analysis

[c] Problems were identified by participants and as Grubbs outlier results excluded from data set for statistical analysis

Table 7. Collaborative study results for cytidine 5′-monophosphate in infant formulas

Infant Formula	Labs	n	Mean (mg hg^{-1})	S_r (mg hg^{-1})	S_R (mg hg^{-1})	RSD_r (%)	RSD_R (%)	HorRat
Lactose-free	12 (0)	24	11.42	0.12	0.89	1.1	7.8	1.0
Starch-based	11 (1)	22	10.99	0.30	0.81	2.7	7.4	0.9
Hydrolysate-based	12 (0)	24	9.72	0.26	0.69	2.7	7.1	0.9
Soy-based[a]	12 (0)	24	0.50	0.19	0.34	38.5	67.1	5.3
Whey-based	12 (0)	24	5.47	0.15	0.48	2.7	8.7	1.0
Whey-based	11 (1)	22	5.43	0.09	0.43	1.6	7.9	0.9

[a] Product not fortified with cytidine 5′-monophosphate

Table 8. Collaborative study results for uridine 5′-monophosphate in infant formulas

Infant Formula	Labs	n	Mean (mg hg^{-1})	S_r (mg hg^{-1})	S_R (mg hg^{-1})	RSD_r (%)	RSD_R (%)	HorRat
Lactose-free	12 (0)	24	3.84	0.09	0.30	2.4	7.9	0.9
Starch-based	11 (1)	22	3.88	0.21	0.31	5.4	8.4	0.9
Hydrolysate-based	12 (0)	24	4.15	0.13	0.36	3.1	8.7	1.0
Soy-based[a]	12 (0)	24	0.19	0.05	0.14	25.0	72.0	5.0
Whey-based	12 (0)	24	3.52	0.05	0.31	1.5	8.8	0.9
Whey-based	12 (0)	24	3.54	0.11	0.32	3.2	9.0	1.0

[a] Product not fortified with uridine 5′-monophosphate

Table 9. Collaborative study results for guanosine 5′-monophosphate in infant formulas

Infant Formula	Labs	n	Mean (mg hg^{-1})	S_r (mg hg^{-1})	S_R (mg hg^{-1})	RSD_r (%)	RSD_R (%)	HorRat
Lactose-free	12 (0)	24	1.45	0.03	0.04	1.8	2.8	0.3
Starch-based	11 (1)	22	1.67	0.03	0.07	1.6	4.2	0.4
Hydrolysate-based	12 (0)	24	1.38	0.05	0.11	3.9	7.7	0.7
Soy-based[a]	12 (0)	24	0.22	0.05	0.18	22.9	82.7	5.8
Whey-based	12 (0)	24	1.05	0.02	0.04	2.2	4.1	0.4
Whey-based	11 (0)	22	1.05	0.04	0.05	3.4	5.2	0.5

[a] Product not fortified with guanosine 5′-monophosphate

Table 10. Collaborative study results for inosine 5′-monophosphate in infant formulas

Infant Formula	Labs	n	Mean (mg hg^{-1})	S_r (mg hg^{-1})	S_R (mg hg^{-1})	RSD_r (%)	RSD_R (%)	HorRat
Lactose-free	12 (0)	24	1.65	0.05	0.10	2.8	6.1	0.6
Starch-based	11 (1)	22	1.66	0.02	0.17	1.4	10.3	1.0
Hydrolysate-based	11 (1)	22	2.46	0.04	0.13	1.8	5.5	0.6
Soy-based[a]	12 (0)	24	0.16	0.07	0.25	43.7	156.2	10.5
Whey-based[a]	10 (2)	20	nd	–	–	–	–	–
Whey-based[a]	10 (2)	20	nd	–	–	–	–	–

[a] Product not fortified with inosine 5′-monophosphate

Table 11. Collaborative study results for adenosine 5′-monophosphate in infant formulas

Infant Formula	Labs	n	Mean (mg hg^{-1})	S_r (mg hg^{-1})	S_R (mg hg^{-1})	RSD_r (%)	RSD_R (%)	HorRat
Lactose-free	12 (0)	24	3.34	0.05	0.09	1.4	2.7	0.3
Starch-based	11 (1)	22	3.54	0.08	0.11	2.1	3.0	0.3
Hydrolysate-based	12 (0)	24	4.73	0.19	0.30	3.9	6.2	0.7
Soy-based[a]	12 (0)	24	0.54	0.11	0.30	20.4	55.7	4.6
Whey-based	12 (0)	24	3.51	0.06	0.18	1.7	5.0	0.5
Whey-based	11 (1)	22	3.51	0.05	0.15	1.3	4.3	0.5

[a] Product not fortified with adenosine 5′-monophosphate