A Liquid Chromatographic Method for Routine Analysis of 5′-Mononucleotides in Pediatric Formulas

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Abstract

An RP-HPLC method for the routine determination of supplemented 5′-mononucleotides (uridine 5′-monophosphate, inosine 5′-monophosphate, adenosine 5′-monophosphate, guanosine 5′-monophosphate, and cytidine 5′-monophosphate) in pediatric formulas and milk products is described. Following sample dissolution, potential interferences were removed by anion-exchange SPE. Chromatographic analyses were performed using a C_{18} stationary phase with gradient elution, UV detection, and quantitation by an internal standard technique. A single-laboratory validation was performed, with recoveries of 92–101% and repeatability RSD of 1.0–2.3%. The method was optimized for the rapid, routine analysis of nucleotide-supplemented bovine milk-based infant and follow-on formulas.

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Introduction

Nucleotides are compounds of critical importance to cellular function. They operate as precursors to nucleic acids, as mediators of chemical energy transfer and cell signaling, and as integral components of coenzymes in the metabolism of carbohydrates, lipids, and proteins (1–3). Nucleotides are not essential dietary nutrients as they can be synthesized de novo or recovered via salvage pathways. However, in times when the endogenous supply is inadequate, such as during periods of rapid growth or after injury, they may become conditionally essential (1).

Nucleotide-supplemented infant formulas have been reported to enhance immune response (4–6), influence metabolism of fatty acids, and improve gastrointestinal tract repair after damage (1, 7). Infants fed formula supplemented with nucleotides are reportedly less likely to experience diarrhea and have elevated serum immunoglobulin A concentrations (8). Nucleotide-supplemented infant formula has been shown to positively modify the composition of the intestinal microflora, compared with unsupplemented formula (1, 9).

As understanding of the nucleotide composition of bovine milk and human milk has increased, manufacturers have endeavored to modify the composition of infant formulas to resemble that of human milk more closely. Nucleotides have, therefore, been added routinely to infant formulas since the 1980s, and added to formulas manufactured specifically for pre-term infants since 2002 (10). Although more than 12 nucleotides are present in human milk, supplementation is limited to adenosine 5′-monophosphate (AMP), cytidine 5′-monophosphate (CMP), guanosine 5′-monophosphate (GMP), inosine 5′-monophosphate (IMP), and uridine 5′-monophosphate (UMP) in the form of the readily soluble sodium salts (11).

With the proliferation of nucleotide-supplemented pediatric formulas, robust methods that incorporate minimal sample preparation and rapid chromatographic separations have been developed for routine product compliance analysis. Analytical methods for nucleos(t)ides in milk have been reviewed previously by Gil and Uauy (12), and more recently by Gill and Indyk (13). Initial preparation of infant formulas for analysis is usually achieved by acid precipitation of casein proteins from the reconstituted sample (14, 15), although ultrafiltration has also been reported (16). Additional cleanup of sample extracts using ion-exchange SPE has been reported (14).

Over the last decade, LC with UV detection has become the dominant technique for the final determination of nucleotides in milk products following sample preparation. Ion-pair reversed-phase liquid chromatography (IP-RPLC) is frequently used to separate nucleotides and can offer advantages in selectivity and efficiency over RPLC for the separation of charged analytes (14, 17, 18). However, IP-RPLC can require long equilibration times, and ion-pair reagents tend to be corrosive, thereby reducing column life (19). Unmodified reversed-phase chromatography offers the advantage of a

simplified mobile phase system and is preferable if acceptable retention and resolution are achieved. Therefore, at an appropriate mobile phase pH, mononucleotides are readily retained on a C_{18} column and a methanol gradient is sufficient to remove late-eluting nucleotides (15).

However, despite the quantity of published methods, there is currently no official internationally accepted reference method for the analysis of nucleotides in milk and pediatric formulas, a situation that renders international trade and infant nutrition difficult to standardize.

The aim of this study is to validate a simple, rapid and robust method for routine compliance testing of nucleotide-supplemented pediatric formulas. The method herein describes an SPE sample cleanup that avoids the prior need to remove protein, coupled with a binary gradient RP-HPLC system. Analytical security is enhanced with an internal standard-based quantitation. This technique has been applied to the analysis of bovine milk-based, caprine milk-based, soy-based, and hypoallergenic pediatric formulas.

Experimental

Apparatus

HPLC was carried out with an LC-20AT pump, an SIL-20A sample injector unit equipped with a 50 mL injection loop, a DGU-20A5 degasser unit, a CTO-20AC column oven, and an SPD-M20A photodiode array detector (Shimadzu, Kyoto, Japan). Shimadzu LC solutions software Version 1.22 SP1 was used for instrument control and data processing.

Separation was achieved with a Gemini C_{18} column, 5 µm, 4.6 x 250 mm (Phenomenex, Torrance, CA). UV absorbances for calibration standards were acquired with a model UV-1601 spectrophotometer (Shimadzu) with digital readout to four decimal places. A Meterlab PHM210 standard pH meter (Radiometer Analytical, Lyon, France) was used for the determination of pH. Polypropylene centrifuge tubes, 50 mL (Biolab, Auckland, New Zealand), Terumo 3 mL disposable syringes (Terumo Corp., Laguna, Philippines), and Minisart 0.2 µm syringe filters with cellulose acetate membranes (Sartorius, Göttingen, Germany) were used for sample preparation.

SPE was performed on a Visiprep 12 port SPE vacuum manifold (Sigma Chemical Co., St. Louis, MO) using Chromabond SB polypropylene strong-anion exchange (SAX) SPE cartridges, 6 mL × 1000 mg (Macherey-Nagel, Düren, Germany). Before use, mobile phases were filtered and degassed using a filtration apparatus with 0.45 µm nylon filter membranes (Alltech, Deerfield, IL).

Reagents

Thymidine 5'-monophosphate (TMP), AMP sodium salt, CMP disodium salt, GMP disodium salt, IMP disodium salt, UMP disodium salt, and potassium bromide were purchased from Sigma Chemical Co. Potassium dihydrogen phosphate, orthophosphoric acid, potassium hydroxide, ethylenediaminetetraacetic acid (EDTA), sodium chloride, and methanol were supplied by Merck (Darmstadt, Germany). Water was purified with resistivity > 18 MΩ using an E-pure water system (Barnstead, Dubuque, IA).

A standardizing buffer (KH_2PO_4, 0.25 M, pH = 3.5) was made by diluting 34.02 g KH_2PO_4 in 900 mL water, adjusting the pH to 3.0 with orthophosphoric acid, and then making the solution to 1 L. An extraction solution (NaCl, 1 M: EDTA 5 mM) was made by dissolving 58.5 g NaCl and 1.9 g EDTA in 1 L water. A wash solution (KBr, 0.3 M) was made by dissolving 3.57 g KBr in 100 mL water. The SPE eluent (KH_2PO_4, 0.5 M, pH = 3.0) was made by dissolving 6.81 g of KH_2PO_4 in 90 mL water, adjusting the pH to 3.0 with orthophosphoric acid, and then making the solution to 100 mL.

Mobile phase A (KH_2PO_4, 0.1 M, pH = 5.6) was made by dissolving 13.6 g KH_2PO_4 in 900 mL of water, adjusting the pH to 5.6 with KOH solution (25% w/v), and then making to 1 L with water. Mobile phase B consisted of 100% methanol. As microbial growth often occurs in phosphate buffers that contain little or no organic solvent at room temperature, the mobile phase was made fresh daily.

Standard Solutions

The extinction coefficient of internal standard TMP at the UV absorbance maximum (λ_{max}) of 267 nm was determined experimentally. The concentrations of analyte nucleotide stock standards were measured using previously reported extinction coefficients (Table 1; 15).

Stock standards were prepared by accurately weighing approximately 50 mg of each nucleotide into separate 50 mL volumetric flasks and filling to volume with water. The concentration of each nucleotide stock standard was determined by diluting 1.0 mL of stock standard to 50 mL with standardizing buffer (KH_2PO_4, 0.25 M, pH = 3.5) and measuring the absorbance at the appropriate λ_{max}.

An intermediate standard solution of TMP was made by diluting 4 mL TMP stock standard into 50 mL water. A mixed intermediate standard solution of AMP, CMP, GMP, IMP, and UMP was made by diluting 2 mL of each stock standard in a single 50 mL volumetric flask and filling to volume with water.

Assay calibration standards were prepared by diluting the two intermediate standards with water to the required concentration. The calibration standards contained a constant concentration of the internal standard TMP (about 3 mg/mL) and variable concentrations (about 0.5–7 mg/mL) of CMP, UMP, GMP, IMP, and AMP.

Sample Preparation

Approximately 1 g of infant formula powder was weighed accurately into a 50 mL centrifuge tube and dissolved in 30 mL of extraction solution (NaCl,1 M: EDTA 5 mM); 1.0 mL of a TMP intermediate standard (about 80 mg/mL) was added, and the tube was capped and vortex mixed. The sample was allowed to stand for 10 min to ensure complete hydration before dilution to a final volume of 50 mL with water.

Solid Phase Extraction

For each sample, a single SPE cartridge was placed on an SPE vacuum manifold. The columns were conditioned by elution with 4 mL methanol, followed by elution with 2 × 5 mL water. The cartridge was loaded with 4 mL sample solution at a flow rate of < 2 mL/min. The cartridge was washed (KBr, 0.3 M, 4 mL) to remove interferences. The nucleotides were then eluted with SPE eluent solution (KH_2PO_4, 0.5 M, p 3.0, 4 mL) into a test tube. An aliquot of the eluent was filtered through a 0.2 µm syringe filter into an autosampler vial.

Chromatography

Chromatographic separation was achieved using a modification of the procedure described previously (15). Gradients were formed by low pressure mixing of two mobile phases, A and B, with separation of nucleotides achieved using the procedure shown in Table 2.

The photodiode array detector acquired spectral data between 210 and 300 nm. Integration of peak area was achieved at specific wavelengths: 250 nm for IMP; 260 nm for AMP, GMP, and TMP; and 270 nm for CMP and UMP. A linear regression plot of the ratios of peak area against concentration for each nucleotide relative to TMP was generated, and the nucleotide contents in unknown samples were interpolated from this calibration curve.

$$Nucleotide(mg/100g) = \frac{A_{NT}}{A_{IS}} \times \frac{1}{L} \times \frac{C_{IS}}{W} \times 100$$

A_{NT} = nucleotide peak area in sample;

A_{IS} = TMP peak area in sample;

L = linear regression slope of calibration curve;

C_{IS} = amount in milligrams of internal standard added;

W = weight of sample in grams; and

100 = mass conversion of result to per 100 g.

Method Validation

Seven mixed standard nucleotide solutions covering the expected working range were analyzed in duplicate, and linearity of dose response was evaluated by least-squares regression analysis. A value of 0.997 for the correlation coefficient (r^2) was deemed to be the minimum suitable for acceptable analysis. Plots of standard residuals were assessed as a further test for linearity.

Repeatability was determined by analyzing replicates (n = 6) of a nucleotide-supplemented bovine milk-based infant formula. Intermediate precision was determined from replicate analyses (n = 6) of the same sample tested on 4 different days by two different analysts.

Method detection limits (MDLs) were determined in accordance with U.S. Environmental Protection Agency procedures (20). The MDL procedure sets the detection limit at the 99% confidence level, minimizing false positive errors.

The robustness of the method was assessed by conducting a Plackett-Burman trial (21), with evaluation of seven factors deemed to potentially affect the final results, at levels likely to occur during normal use of the method. Statistical analysis to identify critical effects consisted of a t-test, whereby a calculated t-value based on the effect, E_X, and an estimation of the standard error, $(SE)_E$, were compared with a critical value (significance level $\alpha = 0.05$). Graphical interpretation was assessed by construction of a half-normal plot, whereby non-significant effects tended to fall on a straight line through zero, whereas significant effects deviated from the straight line. The standard error estimate was used to calculate the margin of error (ME), which was plotted on the half-normal plot to identify the limit above which effects were deemed to be significant (22, 23).

In the absence of a currently available infant formula standard reference material (SRM) with certified levels of nucleotides, method accuracy was determined based on recovery and bias. Recovery was evaluated at three concentration levels for three different sample matrixes: bovine milk-based infant formula; soy-based infant formula; and a hypoallergenic infant formula containing hydrolyzed milk protein. Method bias was assessed by testing replicate samples (n = 12) of a nucleotide-supplemented formula by the method described herein and a method published previously (15).

Results and Discussion

Method Optimization

Method optimization consisted of adapting the sample preparation and chromatographic conditions reported previously (15) to accommodate direct SPE for the removal of non-nucleotide interferences, thereby simplifying both the overall analytical scheme and the chromatographic separation.

Both acid precipitation and ultrafiltration techniques to remove protein prior to SPE were initially evaluated. Acid precipitation is a rapid and simple means of removing caseins; however, the low pH of the sample extract may negatively impact SPE retention unless the extract is first neutralized. Ultrafiltration removes all proteinaceous material above the molecular weight cut-off, and the sample remains at physiological pH, thereby removing a potential neutralization step prior to SPE. However, ultrafiltration was found to be an unsatisfactory means of protein removal as it proved to be time-consuming, difficult to obtain sufficient permeate, and variable in the recovery of individual nucleotides.

Based on these trials, the assumption that it was necessary to remove protein prior to SPE was considered. The dissolution of a powder sample in the high salt solution was found to be efficacious in producing a uniform sample solution that, when applied directly to the SPE cartridge, did not compromise the recovery of nucleotides. Residual milk protein content in the eluent was equivalent to that of an acid-precipitated sample and it is probable that some caseins precipitate in the SPE cartridge with the addition of the low pH buffer.

The SAX cartridges contain quaternary amine anion-exchange sites, which strongly attract the anionic phosphate moiety of nucleotides. In order to remove the majority of interfering components in the sample, different aqueous wash solutions, containing a variety of anions at a number of concentrations, were evaluated. Bromide ions were found to be most effective in removing potentially interfering components, such as nucleosides, orotic acid, and uric acid, while still retaining nucleotides on the cartridge.

In order to elute the nucleotides from the SAX cartridge, two options were available. One option was to add sufficient acid to lower the pH to the pKa of the nucleotide phosphate (approximate pH = 1), thereby neutralizing the negative charge and eluting the nucleotides for collection. However, in order to protect the analytical column, neutralization of the extract would be required prior to HPLC analysis. Alternatively, the addition of anions that have a high affinity for the quaternary amine and added at high ionic strength, could be utilized to elute the nucleotides. This was achieved by the addition of 0.5 M phosphate in the eluent, which readily displaces nucleotides bound on the SAX cartridge.

In multi-step analytical procedures, such as those involving SPE cleanup, there is potential for analyte loss and, hence, the use of an internal standard is considered to be mandatory to obtain consistently accurate and precise results. With an internal standard, it is possible to correct for losses associated with SPE cleanup, either by analyte breakthrough or by incomplete desorption. The selection of TMP as an internal standard was supported by a number of factors: structural similarity to analyte nucleotides; absence of detectable quantities in infant formulas; retention under desired chromatographic separation; and commercial availability.

Method Performance

Chromatographic performance was assessed by replicate analyses (n = 6) of a mixed nucleotide standard (Table 3). Performance within recommended guidelines was achieved, with the exception of the capacity factors for CMP and UMP (guideline > 2.0); however, this was deemed to be acceptable because of uncompromised peak integrity of these two compounds in all samples analyzed.

The results from validation studies are summarized in Tables 4 and 5. Linearity of dose response was confirmed by least-squares regression analysis, with acceptable values obtained for the correlation coefficient. Plots of standard residuals showed no structure and only a small amount of random noise, further demonstrating linearity.

The precision was acceptable and similar to what could be expected, as illustrated by a repeatability Horwitz ratio between 0.3 and 0.5, slightly better than the acceptable range of 0.5–2.0, and an intermediate precision of 3.8–8.6% (24).

As the calculated MDL is dependent on the concentration of the replicate samples, the level of analyte in the sample should not exceed 10 times the calculated MDL; nor should it be less than the MDL. The concentrations used to generate the MDL (0.52–1.68 mg/100g) were appropriate to correctly establish the MDL.

The seven factors assessed in the robustness trial were: concentration of salt solution, sample wait time, load volume, wash solution, wash volume, eluent solution, and eluent volume. The two factor levels were symmetric around the nominal values from the described analytical procedure, with the interval representing experimental error of the equipment used (pipettes, volumetric flasks, balances) and an estimated error on the part of the analyst. The method was found to be robust for these factors at the levels studied (Figure 1).

Acceptable recovery is a function of the concentration and the purpose of the analysis. The recoveries measured were well within the limits of 80–115% at the 10 mg/g level suggested by AOAC (24). An estimation of bias between the method described herein and a method published previously (15) showed no bias, with *P*-values (95%) calculated to be 0.079, 0.529, 0.676, 0.341, and 0.069 for AMP, CMP GMP, IMP, and UMP, respectively.

Method Application

The method was applied to a number of commercially available pediatric and nutritional powders. Products included for testing were infant formulas, follow-on formulas, and an adult nutritional product. These products included a range of different sources: bovine milk, hydrolyzed milk protein, caprine milk, and soy protein (Figure 2). The concentrations of 5’-mononucleotides are given in

Table 6. The recoveries determined against label claim, where available, further indicate the reliability of the method. In the analysis of caprine milk-based infant formula, the presence of significant levels of endogenous nucleotide diphosphates was confirmed.

Conclusions

The optimization and validation of a simple, rapid method for the routine analysis of nucleotides in nucleotide-supplemented infant formulas has been described. The simplicity of analysis is facilitated by the use of SPE without the need for prior protein removal. The use of an internal standard gives additional confidence in the accuracy of the result obtained. The applicability of the method has been demonstrated for the analysis of bovine milk-based, caprine milk-based, soy-based, and hydrolyzed milk protein-based infant formulas

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Table 1. UV absorbance maxima and extinction coefficients for 5′-mononucleotides

Nucleotide[a]	λ_{max}, (nm)	$E^{1\%}$
AMP	257	430.4
CMP	280	398.0
GMP	254	393.3
IMP	249	357.3
UMP	262	313.5
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.

[b] From reference (15).

Table 2. Gradient procedure for chromatographic separation

Time, min	Flow rate (mL/min)	Phase Composition	
		% A	% B
0	0.5	100	0
5	0.5	100	0
14	0.5	90	10
15	0.5	80	20
35	0.5	80	20
36	0.5	100	0
50	0.5	100	0

Table 3. Chromatographic performance

Parameters[a]	CMP[b]	UMP[b]	GMP[b]	IMP[b]	TMP[b]	AMP[b]
Retention time, min	8.8 (0.22%)c	11.8 (0.17%)	19.8 (0.15%)	20.6 (0.10%)	25.0 (0.04%)	25.8 (0.04%)
Capacity factor	0.6 (0%)	1.2 (0.83%)	2.7 (0%)	2.8 (0%)	3.6 (0%)	3.8 (0%)
Resolution	—	6.3 (0.07%)	16.9 (1.11%)	2.2 (0.45%)	15.6 (0.19%)	3.5 (0.57%)
Tailing	1.3 (3.84%)	1.2 (3.33%)	1.0 (0%)	1.0 (5.00%)	1.1 (0%)	1.1 (3.6%)
Theoretical plates	6810 (0.87%)	8527 (5.51%)	33692 (3.28%)	60448 (1.22%)	194363 (0.81%)	241749 (0.22%)
Peak area	142255 (0.51%)	200488 (1.39%)	225242 (0.23%)	122536 (0.75%)	488585 (0.11%)	308754 (0.05%)

[a] Calculations as defined by U.S. Pharmacopeia

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

[c] Mean (percent RSD) of six replicates of a mixed nucleotide standard

Table 4. Method performance as linearity, detection limit, and precision

Analyte[a]	Range (μg mL^{-1})	Linear regression	r^2	MDL[b] (mg hg^{-1})	RSD_r[c] (%)	$HorRat_r$[d]	RSD_{iR}[e] (%)
AMP	1.25-17.49	y = 255805x + 11862	1.0000	0.19	2.0	0.4	4.5
CMP	0.61-8.55	y = 287762x - 2493	0.9999	0.08	1.0	0.3	6.0
GMP	1.11-15.55	y = 200342x - 1807	1.0000	0.06	2.1	0.4	5.2
IMP	1.09-15.27	y = 198519x + 3879	1.0000	0.10	1.4	0.3	3.8
UMP	1.12-15.68	y = 146931x - 1839	0.9999	0.08	2.3	0.5	8.6
TMP	1.61-22.54	y = 150494x - 455	1.0000	–	–	–	–

[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

[b] Determined from n replicates at or near the expected detection limit, MDL = $t_{(n-1,\,1-\alpha)}$ x SD, where n = 10 and α = 0.01

[c] Relative standard deviation repeatability(RSDr) = SD/mean x 100 (n = 6)

[d] Horwitz ratio = RSDr/pRSDr, where pRSDr = $C^{-0.15}$ at 10 ppm concentration level

[e] RSD intermediate reproducibility = SD/mean x 100 (n = 24)

Table 5. Recovery (%) of nucleotides in spiked samples[a]

Sample	AMP[a]	CMP	GMP	IMP	UMP	TMP
Bovine milk-based infant formula	100 (2.10%)[b]	99 (1.82%)	98 (2.14%)	98 (1.63%)	94 (3.30%)	97 (1.34%)
Soy-based infant formula	98 (3.57%)	98 (3.57%)	99 (2.42%)	97 (4.74%)	97 (5.88%)	101 (5.94%)
Hypoallergenic infant formula	100 (3.70%)	99 (1.92%)	101 (1.29%)	98 (3.78%)	92 (5.00%)	100 (3.70%)

[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

[b] Mean recovery (percent RSD) of six replicates over three concentration levels

Table 6. Nucleotides measured in analysis of commercially available pediatric and nutritional formulas

Sample Type[a]	Nucleotide Supplemented	Results (mg/hg)				
		CMP[b]	UMP	GMP	IMP	AMP
Bovine milk-based IF	Yes	11.6	3.7	1.7	2.0	4.5
Bovine milk-based FO	Yes	6.0	2.4	0.9	1.0	2.1
Bovine milk-based FO	No	1.0	0	0	0.1	0
Bovine milk-based FO	Yes	8.5	2.4	1	1	2.3
Bovine milk-based AN	Yes	17.4	4.7	8.0	0	7.2
Bovine milk-based IF	Yes	11.4	2	3.3	0	0.8
Soy-based IF	No	0.1	0.3	0.3	0	0.5
Caprine milk-based IF	No	4.0	8.2	6.4	0.3	2.3
Bovine milk-based WMP	No	4.0	0	0	0	0
Hypoallergenic IF	Noc	2.6	2.5	2.7	2.6	3.1
Hypoallergenic IF	No	0	0	0	0	0

[a] IF = Infant formula; FO = follow-on formula; AN = adult nutritional product; WMP = whole milk powder

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

[c] Recovery as percentage of label claim in parentheses

[d] Hypoallergenic sample spiked with nucleotide mixed standard prior to analysis

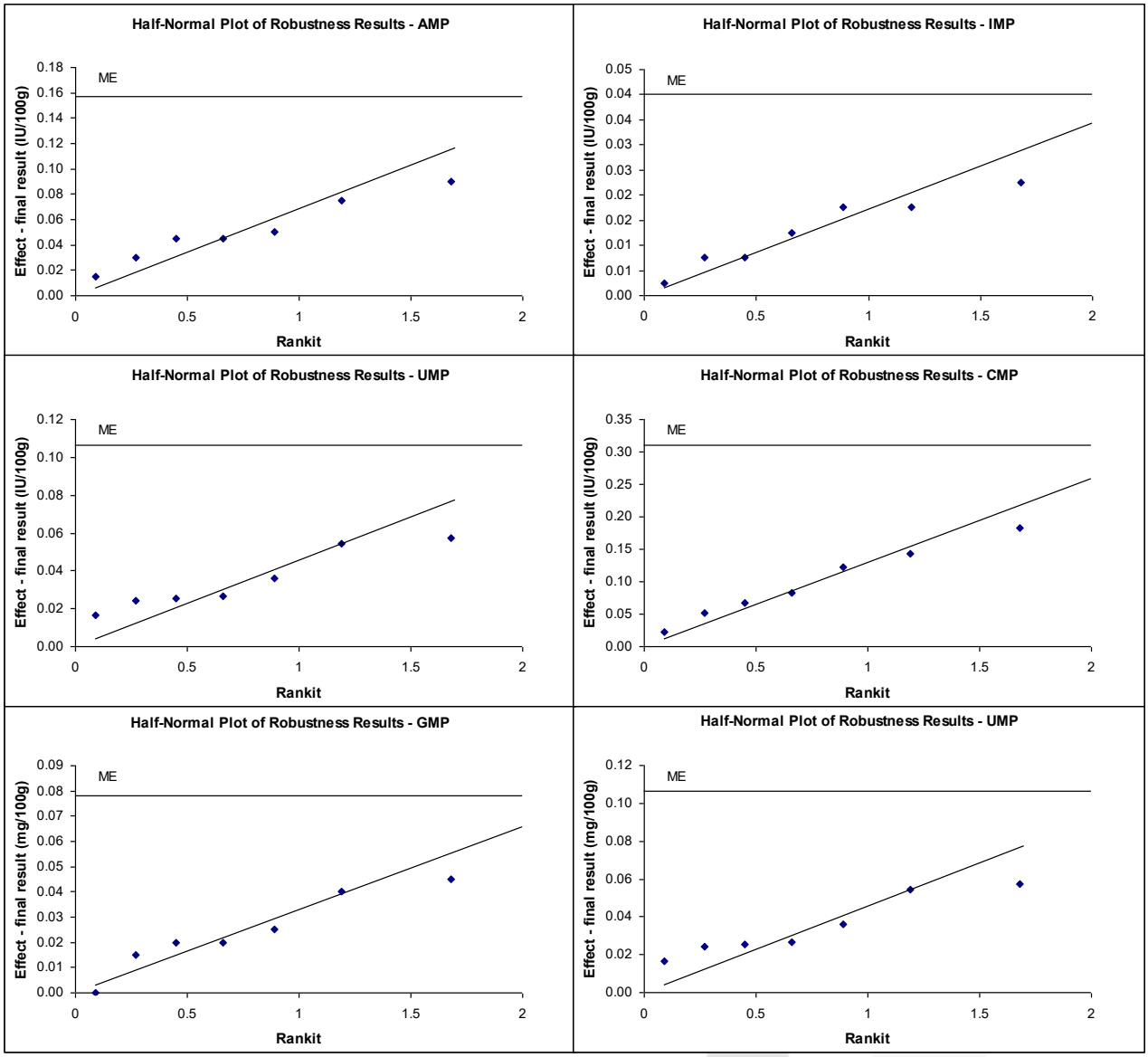

Figure 1. Half-normal plot of results for robustness trial; ME = margin of error

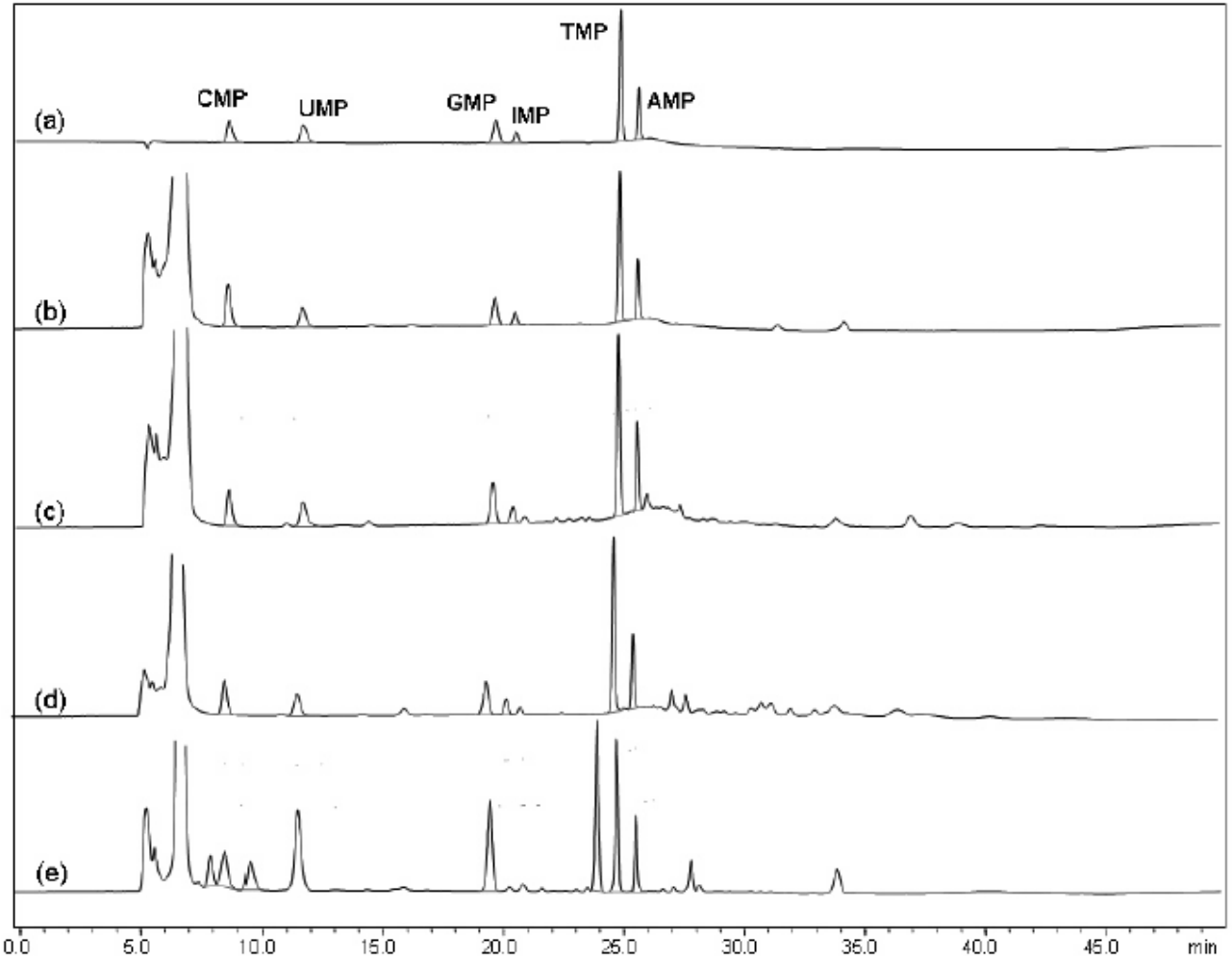

Figure 2. Chromatography of (a) standard mixture of nucleotides, (b) bovine milk-based infant formula, (c) soy-based infant formula, (d) hydrolyzed milk protein-based infant formula, and (e) caprine milk-based infant formula. AMP = Adenosine 5′-monophosphate; CMP = cytidine 5′-monophosphate; GMP = guanosine 5′-monophosphate; IMP = inosine 5′-monophosphate; UMP = uridine 5′-monophosphate; TMP = thymidine 5′-monophosphate. HPLC conditions: column, Gemini C18, 5 µm, 4.6 × 250 mm (Phenomenex); mobile phase A, KH_2PO_4 (0.1 M, pH = 5.6); mobile phase B, methanol (100%); gradient elution, flow rate 0.5 mL/min, 0–5 min (100% A, 0% B), 14 min (90% A, 10% B), 15–35 min (80% A, 20% B), 36–50 min (100% A, 0% B). UV detection: 260 nm.