

HPLC analyses of nucleotides in powdered infant formula and liquid infant formula

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1. Introduction

Powdered infant formula and liquid infant formula are essential for providing optimal nutrition to infants. They are manufactured make their composition close to those of breast milk

Nucleotides in breast milk have not been fully characterized in comparison with other key nutrients such as proteins and fats. However, important physiological functions of nucleotides have been revealed in recent years. For example, nucleotides have been reported to improve intestinal development, digestion/absorption, and allergy-prevention of an infant. Furthermore, nucleotides play an important role in the development of lipid metabolism and brain function and are now included in infant formulae as a semi-essential nutrient.

In this poster, the analyses of five nucleotides in powdered infant formula and liquid infant formula using the Nexera XR high performance liquid chromatograph is introduced. The pretreatment method and HPLC analytical conditions are based on ISO 20638:2015(E) ³⁾ published by the International Organization for Standardization (ISO) and the AOAC Official Method 2011.20 ⁴⁾ published by AOAC INTERNATIONAL, a North American organization that standardizes food testing methods and validates analytical methods.

2. Methods

2.1 Analysis of a mixed standard solution

Table 1 shows the analytical conditions. Shim-pack™ GIST C18-AQ, which shows excellent retentions for highly polar compounds, was used as the analytical column. Fig. 1 shows chromatograms of a mixed standard solution of five nucleotides (IMP, AMP, GMP, CMP, and UMP). TMP was used as the internal standard (ISTD). A PDA detector was used and detection wavelengths were 250 nm (IMP), 260 nm (AMP, GMP, TMP), and 270 nm (CMP, UMP).



 $\begin{array}{ccc} \text{System} & : \text{Nexera XR} \\ \text{Column} & : \text{Shim-pack GIST C18-AQ}^{*1} \\ & & & & & & & & & & \\ \text{(250 mm} \times 4.6 \text{ mm I.D., 5 } \mu\text{m)} \\ \text{Flow rate} & : 0.6 \text{ mL/min} \end{array}$

Mobile phase : A)10 mmol/L KH₂PO₄ buffer (pH 5.6) *2

Time program (B.conc) : 0% (0 min)→20% (25 min)→0%(26-40 min)

B)Methanol

Mixer
 Temperature
 Injection volume
 180 μL
 40 °C
 10 μL

Vial : SHIMADZU Shim-Vial™ H 1.5mL Glass *3

Detection (PDA) : Ch 1(IMP): 250 nm,

Ch 2(AMP, GMP and TMP): 260 nm, Ch 3(CMP and UMP): 270 nm *4

- *1 P/N:227-30742-08
- Dissolve 1.4 g KH₂PO₄ in 900 mL water and adjust pH to 5.6 \pm 0.1 with KOH solution(10% w/v). Dilute to 1 L with water.
- *3 P/N:227-34500-01
- *4 IMP: Inosine 5'-monophosphate

AMP: Adenosine 5'-monophosphate

GMP: Guanosine 5'-monophosphate

TMP: Thymidine 5'-monophosphate

CMP: Cytidine 5'-monophosphate

UMP: Uridine 5'-monophosphate

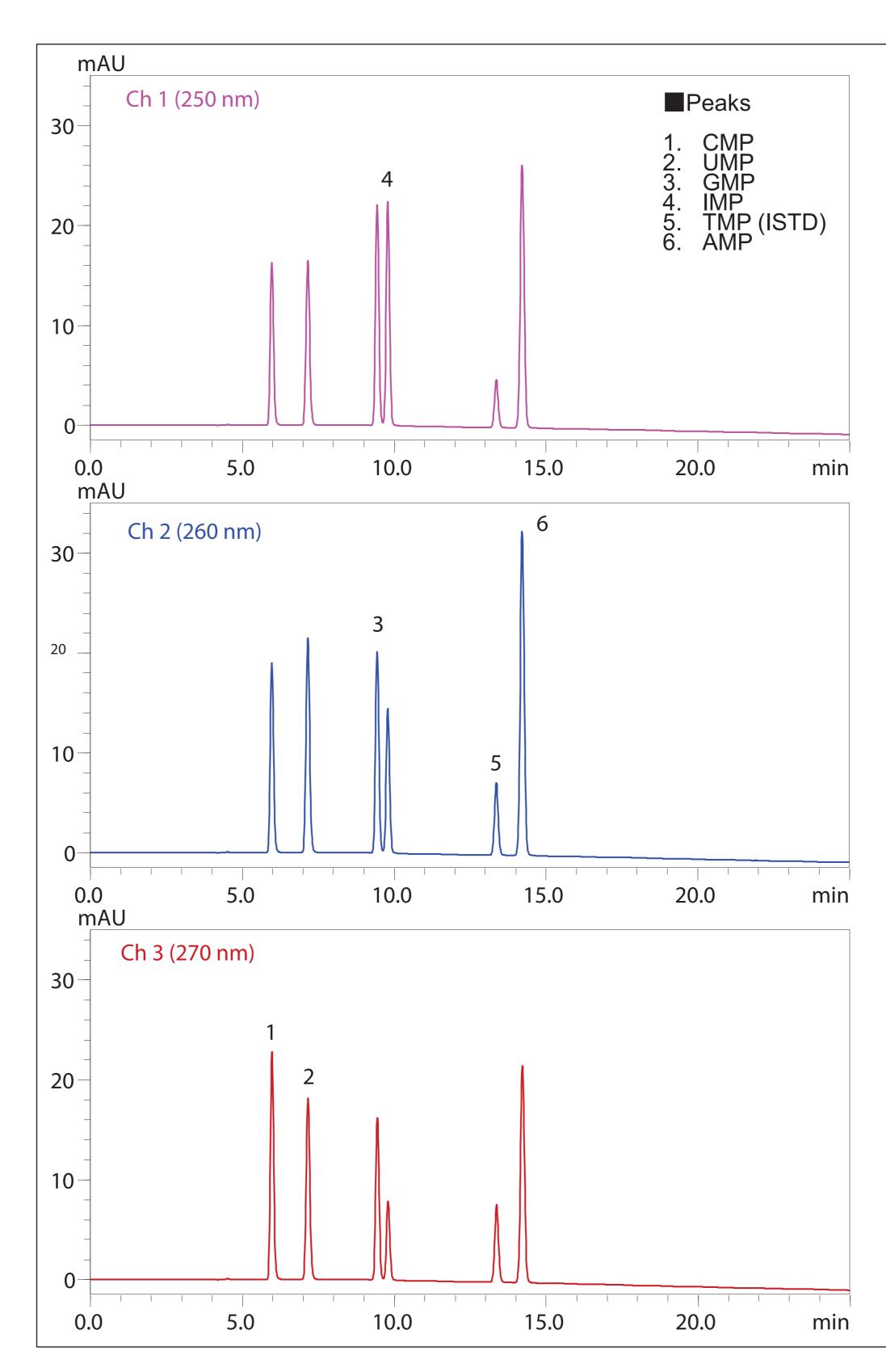


Fig. 1 Chromatograms of the mixed standard solution of the nucleotides (STD 4)

2.2 Calibration curves

The concentrations of the four mixed standard solutions are shown in Table 2. Each reagent used in the standard solutions was the sodium salt or sodium salt hydrate instead of the free acid of the nucleotide, which is difficult to obtain. Absorbance at λmax of each nucleotide in the mixed standard solutions was measured with UV-visible spectrophotometer (UV-1900i) according to ISO and AOAC methods to determine the purity. Concentrations were corrected by multiplying the purity of each nucleotide in standard solution in Table 2.

Table 2 Concentrations of mixed standard solutions (before concentration correction)

	Concentration of each nucleotide (µg/mL)	Concentration of TMP as ISTD (µg/mL)
STD1	0.4	3.2
STD2	0.8	3.2
STD3	3.2	3.2
STD4	8.0	3.2

Table 3 shows the coefficient of determination, slope, and concentration corrected by purity of the calibration curve for each nucleotide prepared from the analytical results of the mixed standard solutions. The calibration curves are based on the internal standard method, and the intercept of the vertical axis (area ratio of the internal standard to each nucleotide) is set to 0 according to ISO and AOAC methods. Good linearities are shown with coefficients of determination of $r^2 = 0.9999$ or higher for the five nucleotides.

Table 3 Coefficient of determination, slope and corrected concentration of each calibration curve

Compound	Linearity (r ²)	Slope	Concentration levels corrected by purity(µg/mL)
CMP	0.9998	1.57	0.2, 0.4, 1.6, 3.9
UMP	0.9998	1.02	0.3, 0.5, 2.2, 5.4
GMP	0.9998	1.04	0.3, 0.5, 2.2. 5.5
IMP	0.99997	2.11	0.2, 0.3, 1.2, 3.0
AMP	0.99997	3.72	0.1, 0.3, 1.2, 2.9

2.3 Repeatabilities

Table 4 shows the relative standard deviations of retention time and peak area of each nucleotide in STD1, the lowest concentration of the mixed standard solutions, in six repeated analyses. For each nucleotide, the relative standard deviation of retention time was less than 0.1%, and the relative standard deviation of area was less than 0.6%, indicating good results.

Table 4 %RSD from six consecutive analyses (STD1)

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Compound	Retention time	Peak area
	(%RSD)	(%RSD)
CMP	0.06	0.33
UMP	0.06	0.32
GMP	0.05	0.28
IMP	0.05	0.52
TMP(ISTD)	0.04	0.11
AMP	0.03	0.19

2.4 Analysis of powdered infant formula and liquid infant formula

Fig. 2 shows the pretreatment method for infant formula and liquid infant formula. Two commercially available powdered infant formulae and one liquid infant formula were pretreated according to Fig. 2 and subjected to HPLC analyses. Fig. 3 shows the chromatograms of powdered infant formula A at 250, 260, and 270 nm. In the pretreatment, the samples were purified using a solid-phase extraction cartridge with a strong anion exchange group in order to reduce the influence of interfering components.

- *5 Dissolve 58.5 g NaCl and 1.5 g EDTA. Dilute in 1L water.
 *6 Polypropylene strong-anion exchange SPE cartridges,
- 6 mL imes 1000 mg , Chromabond SB Dissolve 6.8 g KH $_2$ PO $_4$ in 90 mL water and
- adjust pH to 3.0 with orthophosphoric acid. Dilute to 100 mL.
 *8 TORAST™ Disc, P/N;GLCTD-HPTFE1322

Sample solution preparation	Extraction	
Shake or mix Sample	Place a SPE cartridge	
Weigh approximately 1 g powder or 10 mL	*6 on a vacuum manifold	
liquid milk into 50 mL centrifuge tube	Load 4 mL methanol Load 5 mL water ×2	
Add 30 mL extraction solution*5	Load 4 mL sample solution	
Add 1 mL internal standard (approx. 80	Load 4 mL 0.3 mol/L KBr	
μg/mL)	Place a sample	
Cap the tube and vortex mix	collection tube in manifold	
Allow sample to stand for 10 min	Elute the nucleotides with	
Dilute to 50 mL with	4 mL of eluent solution	
water	Filter the elution	
Cap the tube and vortex mix	through 0.2 µm syringe filter *8	
Fig. 2 Drotrootmont procedure		

Fig. 2 Pretreatment procedure

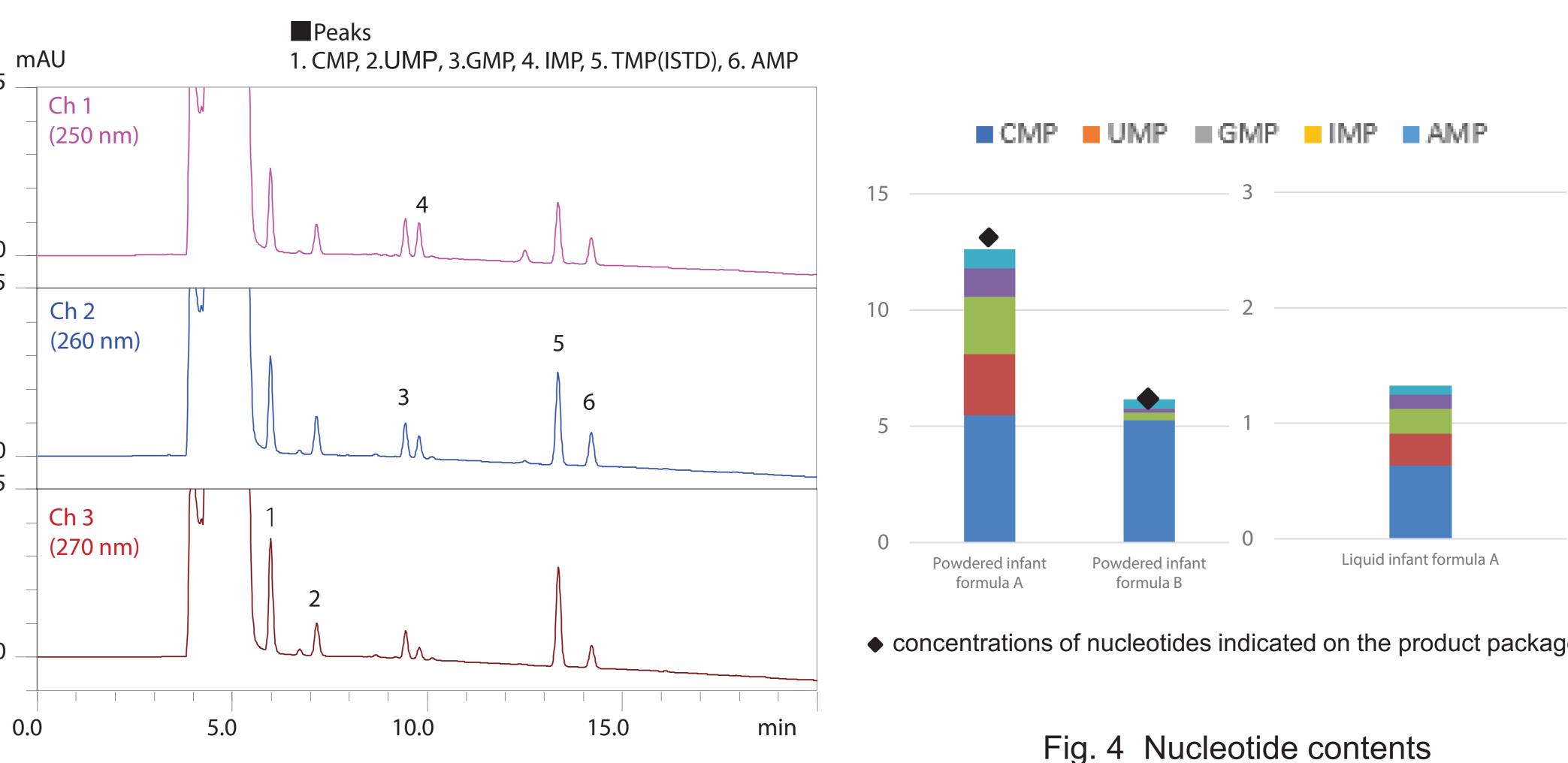


Fig. 3 Chromatograms of powdered infant formula A

in 100 g of powdered infant formula and 100 mL of liquid infant formula

3. Results

Fig. 4 shows the nucleotide contents in 100 g of powdered infant formula and 100 mL of liquid infant formula. Please refer to the ISO and AOAC methods for details on the quantitative determination. The quantitative results obtained in this study (sum of CMP, UMP, GMP, IMP, and AMP) were 90% and 103% of the nucleotide contents indicated on the product package of infant formula A and B, respectively.

Table 5-Table 7 show the relative standard deviations of retention time and peak area for each sample when analyzed six times repeatedly. All the relative standard deviations of retention time were less than 0.1%, and those of peak area were less than 1.1%, showing good results. Six samples spiked with 2 μ g/mL each of CMP, UMP, GMP, IMP, and AMP were prepared and analyzed to evaluate the spike and recoveries. Table 8-Table 10 show the average values of spike and recovery for each sample. The spike and recovery rates ranged from 87% to 105%, being good results.

Table 5 %RSD from six consecutive analyses of powdered infant formula A

powdered infant formula A		
ompound	Retention time	Peak area
	(%RSD)	(%RSD)
CMP	0.07	0.68
UMP	0.06	0.23
GMP	0.06	0.31
IMP	0.05	0.23
MP(ISTD)	0.04	0.39
AMP	0.04	0.50

Table 6 %RSD from six consecutive analyses of powdered infant formula B

powdered illiant formula b			
Compound	Retention time	Peak area	
	(%RSD)	(%RSD)	
CMP	0.02	0.61	
UMP	(Not detected)	(Not detected	
GMP	(Not detected)	(Not detected	
IMP	0.04	1.1	
TMP(ISTD)	0.04	0.37	
AMP	0.05	0.67	

Table 7 %RSD from six consecutive analyses of liquid infant formula A

liquid illiant formula A		
Compound	Retention time (%RSD)	Peak are (%RSD
CMP	0.06	0.51
UMP	0.07	0.36
GMP	0.09	0.58
IMP	0.06	0.20
TMP(ISTD)	0.06	0.43
AMP	0.05	0.45

Table 8 Spike and recovery rates of nucleotides in powdered infant formula A (n=6)

Compound	Average of recovery
	(%)
CMP	97.9
UMP	91.5
GMP	91.6
IMP	90.7
AMP	89.8

Table 9 Spike and recovery rates of nucleotides in powdered infant formula B (n=6)

in powacica imani formula b (ii-		
Compound	Average of recover	
	(%)	
CMP	100.8	
UMP	89.7	
GMP	87.6	
IMP	89.4	
AMP	87.9	

Table 10 Spike and recovery rates of nucleotides in liquid infant formula A (n=6)

Compound	Average of recover	
	(%)	
CMP	99.7	
UMP	88.1	
GMP	105.0	
IMP	92.2	
AMP	93.0	
UMP GMP IMP	99.7 88.1 105.0 92.2	

4. Conclusions

Five nucleotides in infant formula and liquid infant formula were analyzed using Nexera XR high-performance liquid chromatograph. Shim-pack GIST C18-AQ column provided excellent performances both in terms of "pressure fluctuations" and "retention time repeatability" even employing the gradient profile in which 100% aqueous mobile phase delivery interval was included. The influence of interfering components in the sample was suppressed using a solid-phase extraction cartridge.

<References>

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