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High performance Liquid Chromatograph Nexera™ XR

Application News

HPLC Analyses of Nucleotides in Powdered Infant Formula and Liquid Infant Formula

Azusa Uchida

User Benefits

- Five nucleotides in powdered infant formula and liquid infant formula can be simultaneously analyzed by HPLC.
- Nucleotides can be determined precisely owing to the optimization of pretreatment procedure and analytical method to reduce the influence of foreign substances with reference to ISO and AOAC official methods.

■ Introduction

Powdered infant formula and liquid infant formula are essential for providing optimal nutrition to infants. They are manufactured to 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.[1),2)] 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 application news, 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.

■ 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).

Table 1 Analytical conditions

System	:	Nexera XR
Column	:	Shim-pack GIST C18-AQ *1 (250 mm × 4.6 mm I.D., 5 µm)
Flow rate	:	0.6 mL/min
Mobile phase	:	A) 10 mmol/L KH_2PO_4 buffer (pH 5.6) *2 B) Methanol
Time program (B.conc)	:	0% (0 min)→20% (25 min)→0%(26-40 min)
Mixer	:	180 µL
Temperature	:	40 °C
Injection volume	:	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
*2 Dissolve 1.4 g KH_2PO_4 in 900 mL water and adjust pH to 5.6 ±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

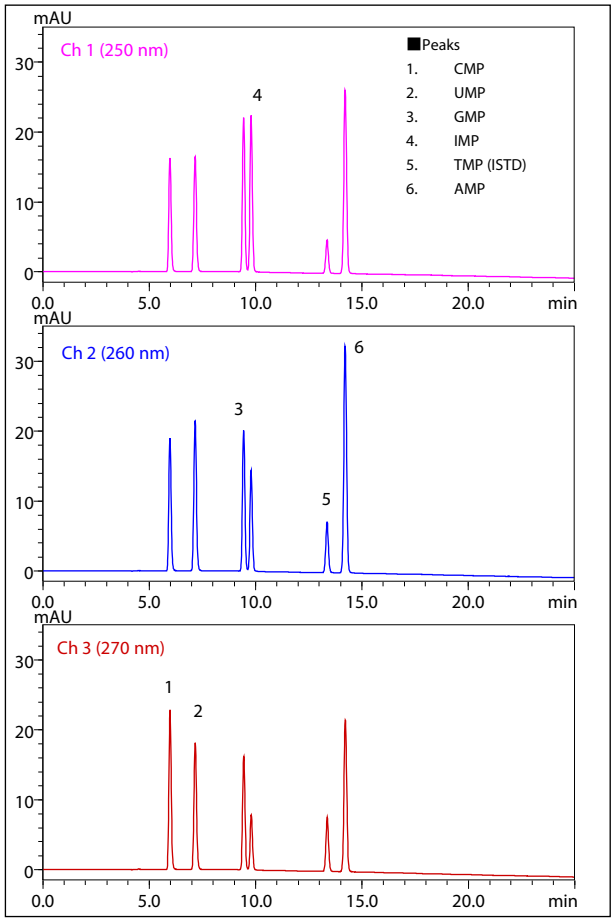

Fig. 1 Chromatograms of the mixed standard solution of the nucleotides (STD 4)

■ 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	0.99998	1.57	0.2, 0.4, 1.6, 3.9
UMP	0.99998	1.02	0.3, 0.5, 2.2, 5.4
GMP	0.99998	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

■ 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)

Compound	Retention time(%RSD)	Peak area(%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

■ 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-Fig. 5 show the chromatograms of each sample 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.

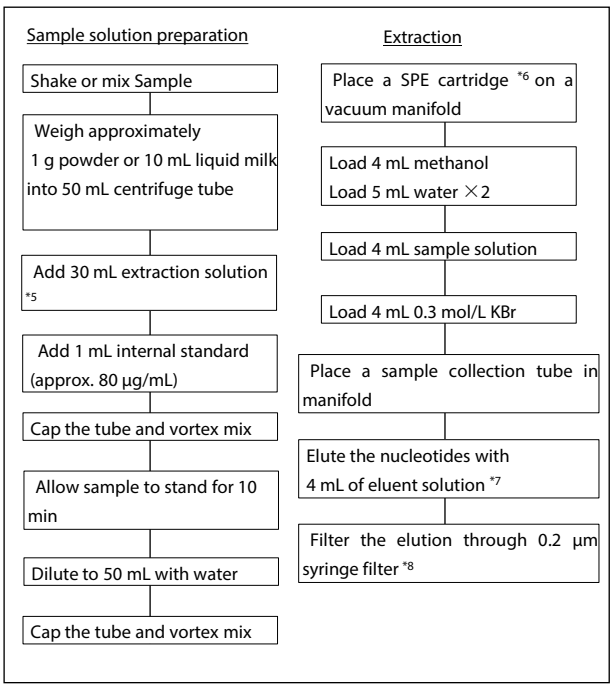

Fig. 2 Pretreatment procedure

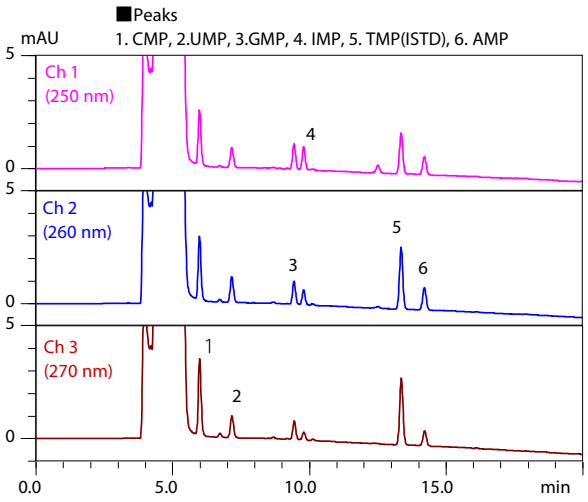

Fig. 3 Chromatograms of powdered infant formula A

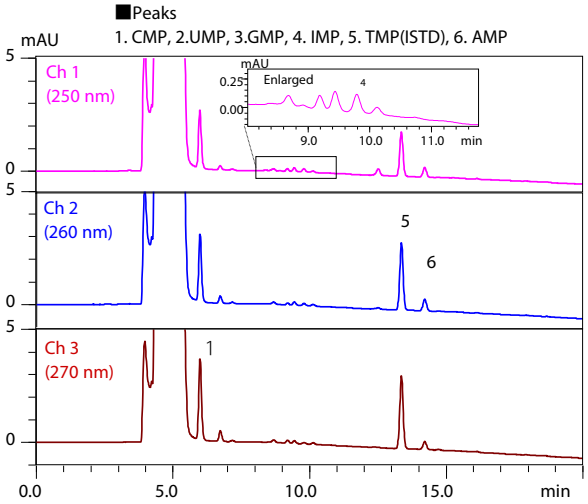

Fig. 4 Chromatograms of powdered infant formula B

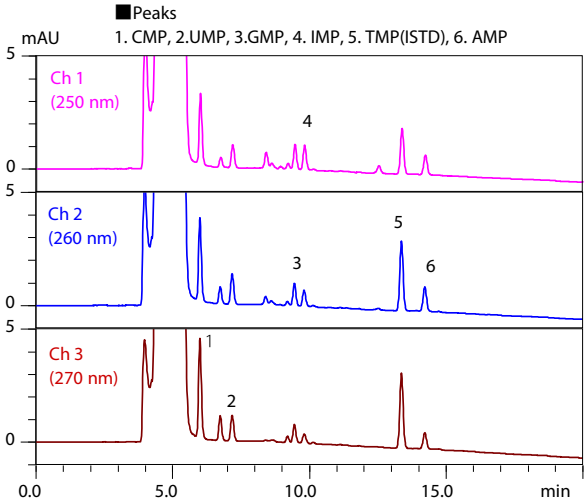

Fig. 5 Chromatograms of liquid infant formula A

*5 Dissolve 58.5 g NaCl and 1.5 g EDTA. Dilute in 1L water.
*6 Polypropylene strong-anion exchange SPE cartridges, 6 mL × 1000 mg , Chromabond SB
*7 Dissolve 6.8 g KH_2PO_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

Fig. 6 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.

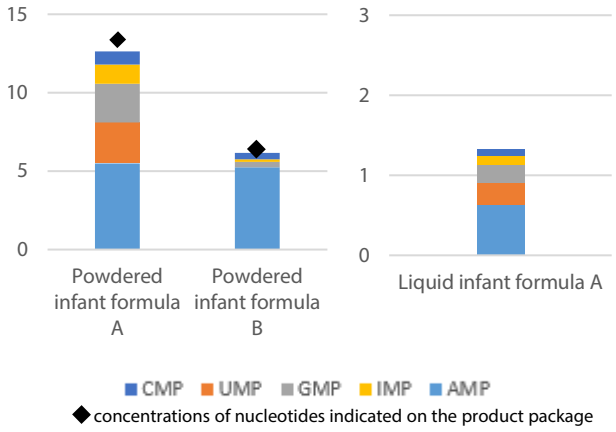

Fig. 6 Nucleotide contents in 100 g of powdered infant formula and 100 mL of liquid infant formula

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.

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

Compound	Retention time(%RSD)	Peak area(%RSD)
CMP	0.07	0.68
UMP	0.06	0.23
GMP	0.06	0.31
IMP	0.05	0.23
TMP(ISTD)	0.04	0.39
AMP	0.04	0.50

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

Compound	Retention time(%RSD)	Peak area(%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

Compound	Retention time(%RSD)	Peak area(%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

Six samples spiked with 2 µg/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 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)

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

■ Conclusion

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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01-00830-EN First Edition: Mar 2025

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