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The analysis of 5′-mononucleotides in infant formulae by HPLC

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Abstract

A method is described for the determination of four 5′-mononucleotides (cytidine 5′-monophosphate, uridine 5′-monophosphate, adenosine 5′-monophosphate and guanosine 5′-monophosphate) in infant formulae. Nucleosides which may be formed during processing can also be analysed simultaneously. This method is based on deproteinisation of samples and direct analysis by ion-pair HPLC using two Nucleosil 120-C_{18} columns in series, followed by diode-array detection. This method gives good recoveries of 5′-mononucleotides from spiked infant formula products. However, some chromatographic interferences were observed when analysing hypoallergenic infant formulae containing hydrolysed proteins which made peak quantification difficult. To overcome this problem a strong anion-exchange solid-phase extraction (SPE) column was used. Four SPE columns from different suppliers were evaluated, but the best recoveries of all four 5′-mononucleotides and highest reproducibility of results were obtained with Baker-bond® quaternary amine columns. Nucleosides, which may occur in very low concentrations in hypoallergenic products, are not retained on the SPE columns and so cannot be analysed by this technique.

Keywords: Infant formulae; 5′-mononucleotides; HPLC

1. Introduction

Nucleotides are the primary units of nucleic acids (RNA, DNA) which control reproduction, growth and metabolism of living systems. They are made up of a cyclic nitrogen-containing base (purine or pyrimidine), a sugar (pentose), and at least one phosphate group (Fig. 1). Recently there has been an increasing interest in the nutritional aspects of dietary nucleotides. They may play an important role in human physiology, especially in infants, and it has been reported that they are implicated in polyunsaturated fat metabolism, immunological systems and differentiation of intestinal cells. Dietary nucleotides seem to influence several aspects of neonatal development such as modulate lipoprotein metabolism via their involvement in the synthesis of phospholipids, modify the composition of the intestinal microflora, improve gastrointestinal tract repair after damage, and participate in the immunity response mediated by T-cells (Barnes, 1994; Boza, 1998; Carver, 1994; Gil & Sanchez-Medina, 1981; Gil & Uauy, 1995; Kulkarni, Rudolph, & van Buren, 1994; Leach, Baxter, Molitor, Ramstack, & Masor, 1995; Schlimme et al., 1997; Thorell, Sjöberg, & Hernell, 1996; Uauy, Quan, & Gil, 1994; Ynekubo et al., 1998). Moreover, rapidly growing tissues such as the intestinal epithelium and lymphoid cells lack significant capacity for 'de-novo' synthesis of nucleotides and require exogenous sources of these compounds (Oliveira, Ferreira, Mendes, & Ferreira, 1999).

Human milk was investigated by Sugawara, Sato, Nakano, Idato, and Nakajima (1995) and three nucleosides — cytidine, uridine and adenosine — and six nucleotides — cytidine 5′-monophosphate (5′-CMP), uridine 5′-monophosphate (5′-UMP), adenosine 5′-monophosphate (5′-AMP), guanosine 5′-monophosphate (5′-GMP), inosine 5′-monophosphate (5′-IMP) and cytidine 5′-diphosphate (5′-CDP) were quantified.

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The authors suggested that nucleosides and nucleotides found in human milk might play some important role in the development of infants. On the other hand the total nucleotide content has been found to be much lower in bovine milk and bovine milk-based infant formula than in human milk, although orotic acid is present in significant quantities in cow's milk and is a precursor of pyrimidines (Sugawara et al., 1995; Thorell et al., 1996).

In order to ensure that infants gain the same nutritional benefits from infant milks and formulae as those from human milk, nucleotide supplementation is permitted by regulatory agencies in the USA, Europe and Japan, as well as some other countries. These countries have accepted the safety of the practice of nucleotide addition to infant formulae, because these compounds are present in human milk and, thus, there is a high likelihood that they are of benefit to infants.

Supplementation levels in starter and follow-up infant formulae need to be monitored. Until recently, analysis of nucleotides was carried out by enzymatic methods or by ion-exchange chromatography (Gil & Sanchez-Medina, 1981). An ion-pair HPLC and diode array detection was recently published by Oliveira et al. (1999) for determination of nucleotides in infant formulae and follow-up milks which permitted the analysis of four nucleotides: 5′-AMP, 5′-CMP, 5′-GMP and 5′-UMP.

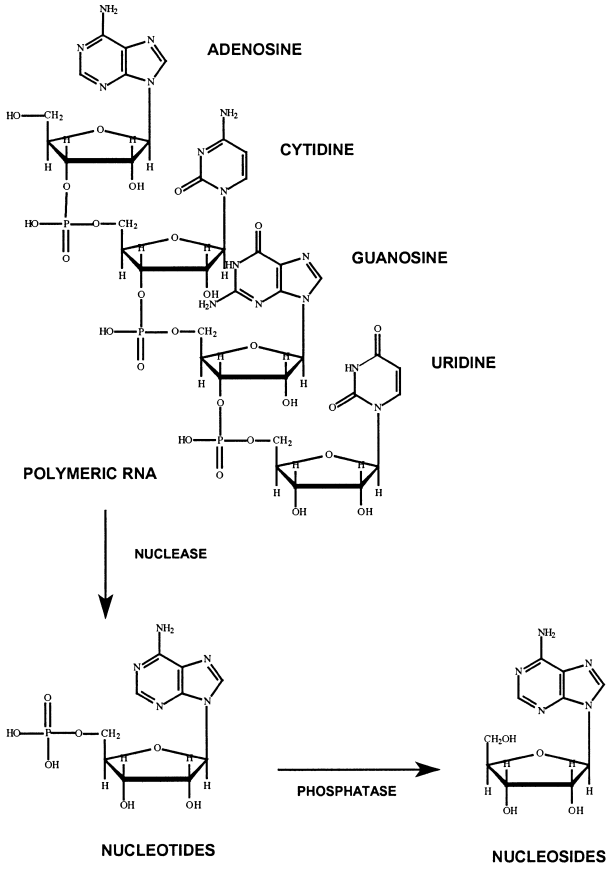

Fig. 1. Relationships between RNA, nucleosides and nucleotides.

The chromatographic separation obtained was not optimal and better chromatographic conditions were reported by Sugawara et al. (1995) using a 50-cm reversed phase C_{18} column with a gradient formed with 25 mM tetrabutylammonium hydrogen sulphate — 20 mM potassium phosphate (pH 3.5) and methanol. An improved version of the latter HPLC method was adopted in the present work, with two 25-cm Nucleosil C_{18} HPLC columns coupled in series, which enables the separation of 5′-AMP, 5′-CMP, 5′-IMP, 5′-GMP, 5′-UMP, orotic acid, adenosine, cytidine, guanosine, inosine, and uridine.

This chromatographic separation is adequate for analysis of nucleotides and nucleosides in regular infant formula. The analysis of hypoallergenic (HA) infant formulae, which contain hydrolysed proteins, was found to be more difficult due to co-elution of peptides with the 5′-mononucleotides. Integration of the peak areas and subsequent data treatment is time consuming and may be inaccurate. In practice two chromatographic runs with the eluent at different pH's were often necessary to obtain accurate results. To overcome these difficulties two examples of clean-up procedures described in the literature were evaluated. Leach et al. (1995) proposed a solid phase extraction (SPE) procedure for nucleotides in human milk, after their enzymatic hydrolysis to nucleosides, using a boronate-polyacrylamide gel with an affinity for adjacent *cis*-hydroxyl groups (*cis*-diols). This polyacrylamide gel is also recommended for the extraction of nucleotides such as 5′-AMP and 5′-GMP by the supplier. Preliminary experiments carried out in our laboratory demonstrated that this support was not suitable for the quantitative analysis of nucleotides in infant formulae. Indeed, 5′-GMP, 5′-UMP, cytidine, guanosine and uridine were only partially extracted from either an infant formula powder or from a standard solution of nucleotides and nucleosides.

Another procedure was reported by Macherey-Nagel and Co (1998) involving SPE extraction of nucleosides from aqueous solutions using C_{18} columns Chromabond® C18ec and another procedure for nucleotides using strong anion-exchange (SAE) columns Chromabond® SB. Preliminary experiments carried out on standard solutions using the C18ec columns showed that cytidine, guanosine and adenosine were only partially retained on this adsorbent while uridine was not retained. On the other hand, the four nucleotides were quantitatively extracted from the standard solution using the SAE columns, with recoveries higher than 90%. An investigation was thus made to compare the efficiency of SPE columns from several suppliers for the extraction of four 5′-mononucleotides from HA formulae.

Therefore, this study reports a robust HPLC protocol for the determination of free nucleotides in regular and HA infant formulae which is appropriate for routine compliance monitoring.

2. Experimental

2.1. Equipment

HPLC system Shimadzu (Kyoto, Japan) equipped with a low-pressure gradient solvent delivery system LC-10AD, an UV-Vis photodiode array detector SPD-M10AV, an automatic sample injector SIL-10A, a column oven CTO-10A and a CLASS-LC10 data software.

Two HPLC columns Nucleosil 120-C_{18}, 5 μm, (250×4 mm) and HPLC filters, Chromafil® 0.45 μm, ∅ 25 mm were purchased from Macherey-Nagel (Oensingen, Switzerland). Multiposition electromagnetic stirrer Variomag, with 15 stirring position, from Merck (Les Acacias-Geneva, Switzerland) was used. A centrifuge model Sigma 3K 15 with cooling system, 11,000 ***g***, was obtained from Fischer Scientific (Wohlen, Switzerland) with Nalgene 50-ml FEP tubes. Solid phase extraction vacuum manifold Visiprep 12-port model as obtained from Supelco (Buchs, Switzerland). SPE columns Bakerbond® spe Quaternary AmineN$^{\oplus}$, 3 ml, 500 mg from J.T. Baker (Deventer, Holland), SPE columns Chromabond® SB, anion exchanger, 3 ml, 500 mg from Macherey-Nagel and SPE columns Sep-Pak® Vac Acell™ Plus QMA, 6 ml, 1 g from Waters (Rupperswil, Switzerland) were used. SPE columns Mega Bond Elut® SAX, 6 ml, 1 g from Varian were purchased from Stehelin and Cie (Basel, Switzerland).

2.2. Reagents

Adenosine 5′-monophosphate disodium salt was from BDH and purchased from Chemie Brunschwig (Basel, Switzerland). Cytidine 5′-monophosphate disodium salt, guanosine 5′-monophosphate disodium salt, and uridine 5′-monophosphate disodium salt, adenosine, cytidine, guanosine, uridine, orotic acid and tetrabutylammonium dihydrogen phosphate were purchased from Sigma (Buchs, Switzerland).

Hydrochloric acid solution (1 mol/l), methanol (gradient grade for chromatography), *ortho*-phosphoric acid 85%, perchloric acid 70%, sodium dihydrogen phosphate monohydrate, potassium hydroxide and sodium hydroxide solution (1 mol/l) were purchased from Merck (Les Acacias-Geneva, Switzerland).

Water was purified using a Milli-Q system from Millipore (Le Mont-sur-Lausanne, Switzerland).

2.3. Procedure

2.3.1. Preparation of standard solutions

For each standard nucleotide, the moisture content was previously determined by the Karl-Fischer method and concentrations were calculated on a dry-weight basis.

A stock standard solution containing 0.6, 0.4, 0.15 and 0.10 mg/ml of 5′-CMP-Na_2, 5′-UMP-Na_2, 5′-AMP-Na_2 and 5′-GMP-Na_2, respectively, was prepared in water and stored at 4°C for a maximum of 2 weeks.

A stock solution of nucleosides containing 0.3, 0.2, 0.1 and 0.1 mg/ml of cytidine, uridine, adenosine, guanosine was prepared in water, and stored at 4°C for a maximum of 2 weeks.

A day of use, the standard working solutions containing nucleotides and/or nucleosides were prepared by diluting the stock solution with water to the required concentration followed by filtration through a 0.2-μm membrane filter.

2.3.2. Preparation of samples

Just prior to analysis 5 g of powdered products were dissolved in approximately 35 ml water at 50°C, then cooled to room temperature and diluted to 50 ml with water in a volumetric flask.

Ready-to-feed formulae (RTF) were homogenised by shaking prior to analysis. While concentrates were prepared as follows: 10 ml of homogenised concentrates were weighed, diluted with an equal volume of water and mixed thoroughly before analysis.

2.3.3. Extraction of the nucleotides and nucleosides for regular infant formulae

Fifteen millilitres of dissolved powder solution or RTF formula or diluted concentrate were transferred to a centrifuge tube and diluted with exactly 15 millilitres of 13% (v/v) perchloric acid. The mixture was stirred for 15 min and then centrifuged at 5000 ***g*** for 20 min at 4 °C. Twenty-five millilitres of the supernatant were introduced into a beaker and the pH adjusted to about 4.0 with 5 M KOH. Then, the sample was diluted to 50 ml with water and left in an ice bath for at least 1 h to precipitate all the potassium perchlorate. A few millilitres of the supernatant were filtered through a 0.45-μm membrane filter before analysis.

The standard working solutions 2.3.1 were prepared by adding perchloric acid and KOH in the same manner as for the samples.

2.3.4. Extraction of the nucleotides for HA formulae

Twenty millilitres of dissolved powder solution or RTF formula or diluted concentrate were transferred to a centrifuge tube and 400 μl 1 M HCl were added. The mixture was shaken vigorously and centrifuged at 11,000 ***g*** for 20 min, at 4°C. An aliquot of 15 ml was transferred to a beaker containing 10 ml water and the pH was adjusted to 7.0 with 1 M and 0.1 M NaOH. The solution was then quantitatively transferred to a 50 ml volumetric flask and diluted to volume with water.

2.3.5. Clean-up procedure for HA formulae using SPE columns

One or two 500-mg SPE columns (Chromabond® SB or Bakerbond® Quaternary Amine N$^{\oplus}$) in series or one 1-g SPE column (Mega Bond Elut® SAX or Sep-Pak® Vac Acell™ Plus QMA) containing silica modified with

quaternary ammonium groups were placed on a SPE vacuum manifold. The columns were conditioned by eluting twice with 6 ml of water. Ten millilitres of sample solution were passed through the prepared columns, at a flow rate of maximum 1 ml/min. The columns were rinsed with 6 ml of water and then dried by aspirating air through for 5 min. The nucleotides were then eluted with 7.0 ml of 0.05 M hydrochloric acid into a 10 ml volumetric flask, at a flow rate of maximum 1 ml/min. The pH of the eluate was raised to a value close to the pH of the mobile phase by adding 350 μl 0.5 M NaOH and the solution was diluted to volume with water. A few millilitres of this solution were filtered through a 0.45-μm membrane filter before HPLC analysis.

2.4. HPLC analysis

Standard and sample solutions were analysed under the following conditions:

Stationary phase:	Two HPLC-columns Nucleosil 120-C_{18}, 5 μm, 250×4 mm, in series
Flow rate:	0.5 ml/min
Oven temperature:	40°C
PDA detector:	detection wavelength 257 nm for 5′-UMP, 5′-GMP and 5′-AMP, adenosine guanosine and uridine, and 278 nm for 5′-CMP and cytidine; spectrum acquisition range between 200 and 300 nm
Injection volume:	25 μl
Run time:	65 min
Mobile phase:	3.45 g sodium dihydrogen phosphate and 339 mg tetrabutylammonium dihydrogen phosphate were dissolved in 900 ml water. Seventy millilitres of methanol were added and the pH adjusted to 4.3 with 1% (v/v) phosphoric acid. Then the solution was made up to 1000 ml with water.

Each 5′-mononucleotide and nucleoside was identified by comparing retention times and photodiode array spectra for standards and samples. Purity of each peak was checked so as to exclude any contribution from interfering peaks. Quantification was then carried out by comparing the areas of the corresponding peaks.

At the end of each working day the whole chromatographic system was rinsed with water — methanol 95:5 (v/v) for 30 min.

3. Results

3.1. Chromatography

The chromatographic conditions were optimised to separate the various nucleotides and nucleosides (Fig. 2). A single reverse phase Nucleosil C_{18} column did not provide sufficient resolution for all the compounds of interest and a better separation was obtained with two columns in series. A column temperature of 40°C was necessary to separate uridine and 5′-CMP from interfering substances. The pH of the eluent was fixed exactly to 4.3 to obtain an optimum separation of the nucleotides and nucleosides (including 5′-IMP and inosine) within a reasonable run time thus avoiding an unsuitable enlargement of the last peaks. A typical chromatogram for a fortified starter formula is shown in Fig. 3.

Since the SPE cleanup effectively eliminates the interfering peptides, thus improving the peak integration and chromatography, the results for 5′-mononucleotides were more accurate than those obtained using the standard procedure without SPE cleanup (Table 1). A typical chromatogram for a HA infant formula with SPE cleanup is compared with one obtained using the standard extraction procedure without cleanup (Figs. 4 and 5).

3.2. Method performance (regular infant formulae)

Recovery of the method was determined by spiking three different products with 5′-mononucleotides (Table 2).

Table 1
Determination of 5′-mononucleotides in hypo-allergenic infant formulae: influence of cleanup with two 500-mg Bakerbond® quaternary amine columns in series

	Concentration of nucleotide, in mg/kg (mean of two determinations)							
	5′-CMP-Na_2		5′-UMP-Na_2		5′-GMP-Na_2		5′-AMP-Na_2	
Product	Without cleanup	With SPE cleanup	Without cleanup	With SPE cleanup	Without cleanup	With SPE cleanup	Without cleanup	With SPE cleanup
Starter HA formula (C)	178[a]	157	96	96	21	23	33	34
Starter HA formula (D)	128	134	42	49	22	25	45[a]	50
Starter HA formula (E)	189	189	144[a]	135	13	13	18	18
Starter HA formula (F)	189	189	144[a]	138	14	13	20	19
Starter HA formula (G)	192	191	145	138	12	13	18	19
Starter HA formula RTF	21	20	12	12	2.8	2.8	5	5

[a] Not baseline separated from interferences.

Table 2
Recovery (%) of added 5′-mononucleotides from infant formulae powders

Product	Spike (mg/kg)	5′-AMP-Na_2	5′-CMP-Na_2	5′-GMP-Na_2	5′-UMP-Na_2
Starter infant formula (A)	80	98	107	95	98
Starter infant formula (B)	40	98	98	106	113
Starter infant formula (C)	40	100	105	107	110

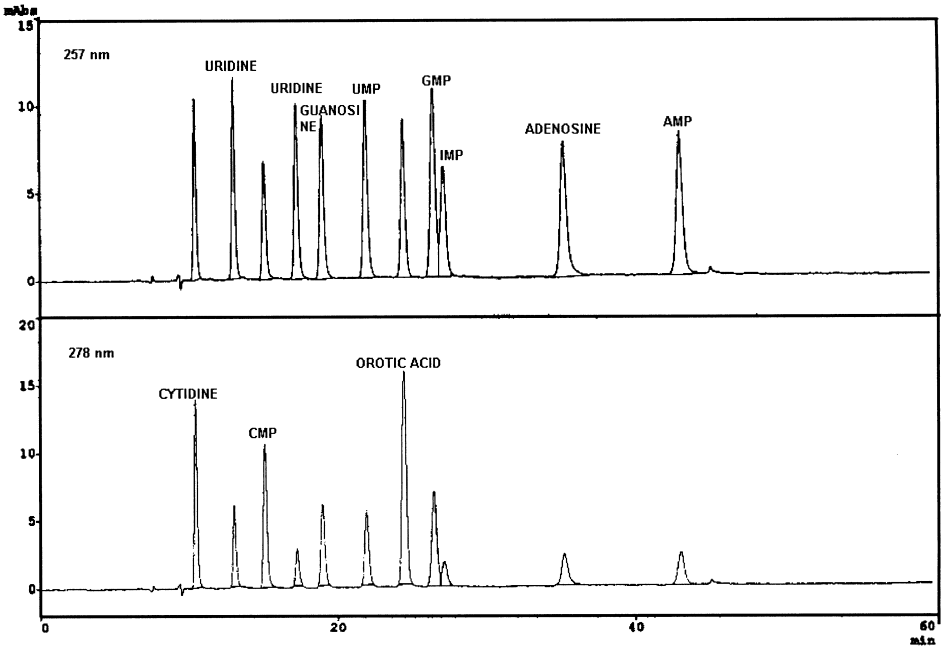

Fig. 2. Chromatogram of a mixed standard solution of 5′-mononucleotides (4 μg/ml of each), nucleosides (2 μg/ml of each) and orotic acid (4 μg/ml).

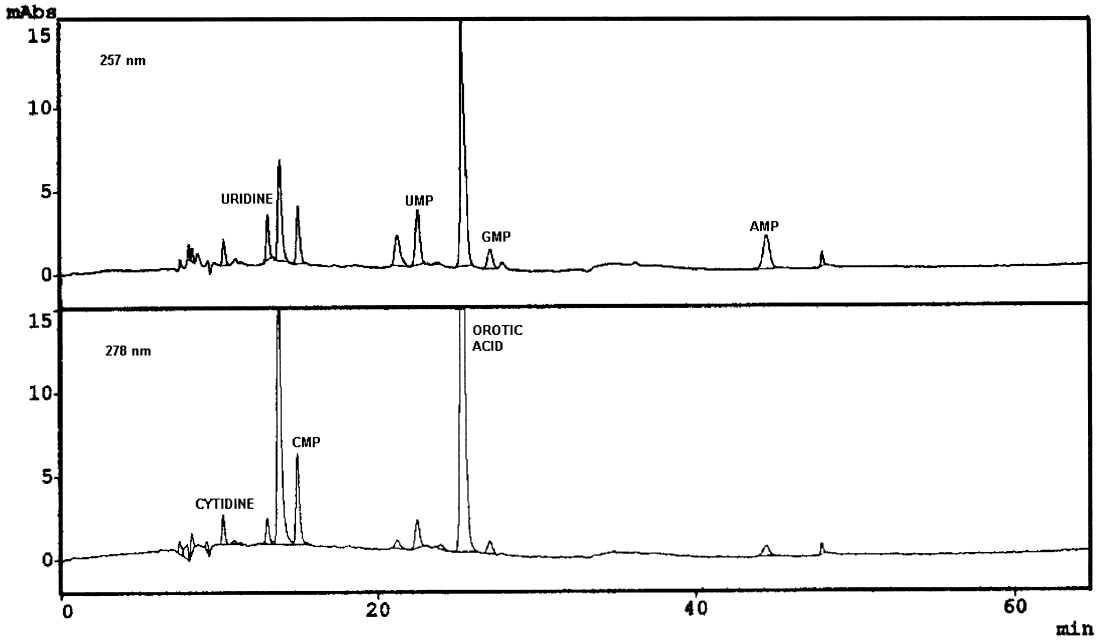

Fig. 3. Chromatogram of a fortified starter formula.

Recoveries in the range 95–113% were obtained which are considered to be satisfactory. Repeatability was checked by analysing a fortified whey-casein based formula five times. The following relative standard deviation (RSD) values were obtained: 5′-AMP, 1.1%; 5′-CMP, 0.5%; 5′-GMP, 1.3%; 5′-UMP, 3.1%; Orotic acid, 1.1%.

For nucleosides similar recovery experiments were performed and the results obtained are shown in Table 3. Recoveries were in the range 95–106% for all four nucleosides. The repeatability of measurements (RSD) was 1.7, 1,9, 0.7 and 4.1% for adenosine, cytidine, guanosine and uridine, respectively.

3.3. Method performance (HA products)

SPE columns, silica modified with quaternary ammonium groups, from four suppliers were tested.

Initial recovery tests using a standard solution of 5′-mononucleotides were made using Chromabond® SB columns. The recoveries were in the range of 92–99% for all four 5′-mononucleotides.

The performance of the Chromabond® columns was then checked by analysing an unfortified infant formula powder spiked with the four nucleotides. Even if recoveries were satisfactory (higher than 93%), the difference between duplicates was not better than 13 and 10%, respectively of the mean values for 5′-UMP and 5′-CMP. Further tests showed that these results were clearly improved by using two SPE columns in series. Indeed, the difference between duplicates was lowered to about 1% of the mean value with an average recovery of 103%.

The performances of the Bakerbond® spe Quaternary AmineN⊕, Mega Bond Elut® SAX and Sep-Pak® Vac Acell™ Plus QMA columns were compared with those of the Chromabond® SB columns (Table 4). The values obtained for a fortified infant formula, using two Bakerbond® columns in series, were equivalent to those obtained with two Chromabond® columns. The Mega Bond Elut® columns yielded results about 18% lower for the 5′-CMP, and moreover the repeatability was unsatisfactory. The Sep-Pak® QMA columns yielded results equivalent to those obtained with the Chromabond® and Bakerbond® columns for 5′-CMP, 5′-UMP, and 5′-GMP but not for 5′-AMP. Indeed, the results for 5′-AMP were up to 13% higher using the Sep-Pak® QMA columns. This was due to an interference that co-eluted with the peak of 5′-AMP, which could not be separated by changing the pH of the HPLC mobile phase.

The Chromabond® and Bakerbond® SPE columns were further evaluated under repeatability and reproducibility conditions. Repeatability was checked by carrying out six replicate analyses on one infant formula

Table 3
Recovery (%) of added nucleosides from infant formulae powders

Product	Spike (mg/kg)	Adenosine	Cytidine	Guanosine	Uridine
Starter infant formula (D)	40	95	102	100	106
Soya-based formula (concentrate, RTF)	10	104	96	96	105

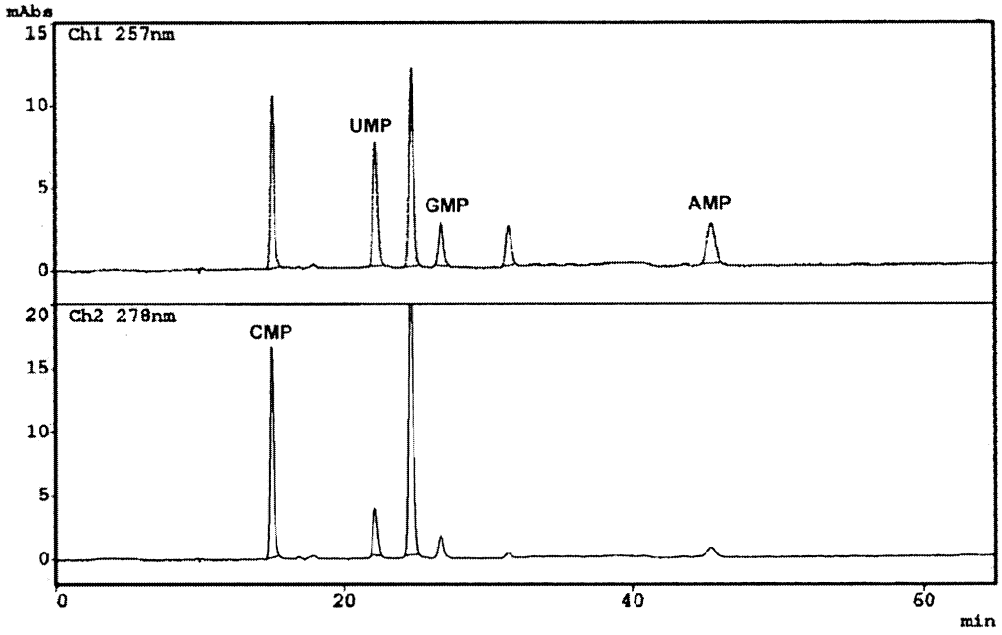

Fig. 4. Chromatogram of a HA infant formula with SPE cleanup.

RTF and one infant formula powder, using two 500-mg columns in series from the same batch. For Chromabond® the average relative standard deviation (RSD) was 0.4, 0.8, 1.6 and 2.1% for 5′-CMP, 5′-UMP, 5′-GMP and 5′-AMP, respectively. However, analyses carried out on three different days showed that the Chromabond® columns were not able to ensure day-to-day repeatability of the analysis of 5′-UMP in infant formula RTF. Indeed, the RSD of repeatability varied from 0.7 to 12.4% and the mean value decreased 15% (from 12.4 to 10.5 mg/kg). Repeatability experiments involving two 500-mg Bakerbond® columns in series gave better results. The RSD ranged from 0.8% to 2.7% and the average RSD values were 1.2, 1.0, 2.3 and 1.6% for 5′-CMP, 5′-UMP, 5′-GMP and 5′-AMP, respectively. The intermediate reproducibility was checked by analysing, in duplicate, on six days the same product. The RSD values were 0.8, 1.7, 2.7 and 2.2% for 5′-CMP, 5′-UMP, 5′-GMP and 5′-AMP, respectively.

Since the Bakerbond® columns were the most effective in terms of repeatability and reproducibility of results they were adopted for the final method. Finally, recovery tests were made by analysing two infant formula products spiked with different levels of nucleotides (see Table 5). Recovery values ranged from 93 to 113% and the average recovery was 98, 101, 102 and 101% for 5′-CMP, 5′-UMP, 5′-GMP and 5′-AMP, respectively.

Table 4
Comparison of the performances of four different SPE columns for the analysis of 5′-mononucleotides in fortified HA infant formulae

	Concentration of 5′-mononucleotides, in mg/kg (mean of two determinations)			
SPE Column	5′-CMP-Na2	5′-UMP-Na2	5′-GMP-Na2	5′-AMP-Na2
Chromabond® SB column (two 500-mg)	20.2	12.2	2.9	4.8
Bakerbond® Quaternary Amine column (two 500-mg)	20.3	12.4	2.8	4.7
Mega Bond Elut® SAX column (1-g)	16.7	12.1	2.8	5.0
Sep-Pak® QMA column (1-g)	20.4	12.2	2.7	5.3

Table 5
Recovery (%) of added 5′-mononucleotides from HA infant formulae powders

Product	Spike (mg/kg)	5′-CMP-Na_2	5′-UMP-Na_2	Spike (mg/kg)	5′-GMP-Na_2	5′-AMP-Na_2
Starter HA infant formula (A; unfortified)	165	101	106	165	100	103
	50	97	99	50	113	99
Starter HA infant formula (B; unfortified)	185	97	99	55	93	101

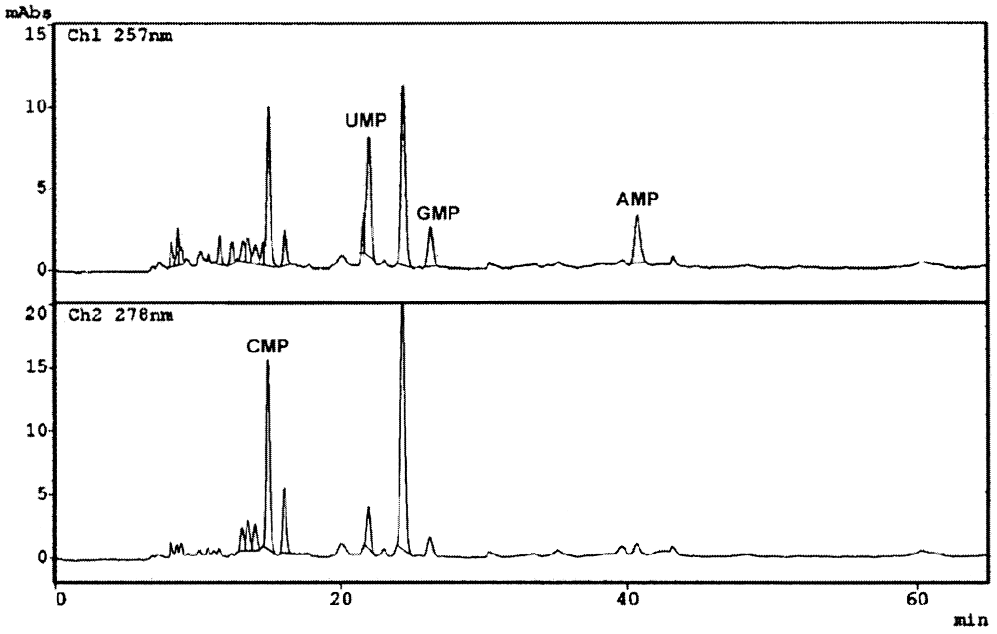

Fig. 5. Chromatogram of a HA infant formula without SPE cleanup.

Table 6
Linearity parameters for each nucleotide (disodium salt) and nucleoside: linear least mean square regression equations and correlation coefficients

Nucleot(s)ide	Concentration range (μg/ml)	Equation	Correlation coefficient (r)
Adenosine	0.12–11	$y = 106\,559\,x - 12\,230$	0.9998
5′-AMP	0.15–15	$y = 76\,737\,x - 10\,853$	0.9999
Cytidine	0.04–9	$y = 51\,572\,x + 172$	0.9997
5′-CMP	0.06–15	$y = 37\,671\,x - 904$	0.9999
Guanosine	0.06–10	$y = 105\,592\,x - 5965$	0.9998
5′-GMP	0.06–16	$y = 97\,695\,x - 4048$	0.9999
Uridine	0.04–10	$y = 83\,300\,x - 3072$	0.9999
5′-UMP	0.07–19	$y = 66\,609\,x - 2193$	0.9999

Table 7
Determination of nucleotides and nucleosides in fortified infant formulae[a]

Product	Adenosine	5′-AMP	Cytidine	5′-CMP	Guanosine	5′-GMP	Uridine	5′-UMP	Total
Starter HA formula (C)	n.d.	29	n.d.	157	n.d.	19	n.d.	85	290
Starter HA formula (D)	n.d.	40	n.d.	113	n.d.	20	n.d.	37	210
Starter HA formula (E)	n.d.	16	n.d.	166	n.d.	12	n.d.	127	321
Starter HA formula (F)	n.d.	18	n.d.	166	n.d.	13	n.d.	127	324
Starter HA formula (G)	n.d.	16	n.d.	169	n.d.	11	n.d.	127	321
Starter HA formula (H)	n.d.	29	4	148	n.d.	23	n.d.	91	295
Starter HA formula RTF	n.d.	4	1	19	n.d.	3	n.d.	11	38
Soya-based infant formula RTF	26	n.d.	24	n.d.	16	n.d.	15	n.d.	81
Starter formula (E)	n.d.	21	5	108	n.d.	11	17	63	225
Starter formula (F)	n.d.	26	15	61	n.d.	13	22	45	182
Starter formula (G)	n.d.	24	15	63	n.d.	12	20	43	177
Starter formula (H)	14	n.d.	134	3	19	n.d.	146	n.d.	316
Starter formula RTF	n.d.	4	4	17	1	2	9	11	48

[a] Results expressed in nucleotide equivalent (free acid form) in mg/kg. n.d., not detected.

3.4. Linearity

The linearity of the photodiode array detector response for each compound was tested by analysing standard solutions containing each 5′-mononucleotide (disodium salt) and nucleoside. Linear calibration curves were obtained by plotting the peak area against the concentration of the respective compound, in the range of concentrations given in Table 6.

4. Discussion

It has been reported (Thorell et al., 1996) that sterilisation and spray drying of milk significantly lower the 5′-CMP, 5′-AMP and orotic acid concentrations. In addition, incubation of human milk for 24 h at 23°C yields a partial transformation of 5′-CMP and 5′-UMP to cytidine and uridine and of 5′-GMP and 5′-AMP to guanine and uric acid. It is known that nucleotides are converted to nucleosides by nucleotidase and phosphatase hydrolysis. Some typical analytical data is given in Table 7 from the analysis of nucleosides and 5′-mononucleotides in various fortified infant formulae. For powdered regular infant formulae, the conversion rate of 5′-mononucleotides to nucleosides was variable. The concentrations of nucleosides (cytidine) in HA infant formulae were found to be very low whereas, in a soy-based RTF formula, the nucleotides were completely converted into their nucleoside forms.

In infant formulae the conversion of added 5′-mononucleotides to nucleosides is not an issue since nucleosides are the preferred form for absorption by the intestinal mucosa (Thorell et al., 1996). However, the total amount of nucleotides and nucleosides must be determined to check the correct addition of 5′-mononucleotides.

5. Conclusions

A simple reliable analytical method has been developed for the routine determination of nucleotides in infant formula. For regular infant formulae a simple sample preparation is adequate involving precipitation of proteins with perchloric acid solution and filtration. Nucleosides formed during processing of the products can also be separated and quantitatively analysed. For hypoallergenic (HA) infant formulae, an extra clean-up step with two SPE columns (Bakerbond® Quaternary Amine $N^{\oplus}$) was used to remove interfering peptides. This step markedly improved the chromatographic analysis and also the accuracy of the results for

5′-mononucleotides. Quantitative recoveries of all four 5′-mononucleotides were obtained from various HA infant formulae with 5′-mononucleotides.

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