JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2009, 57, 7245–7249 7245
DOI:10.1021/jf901726e

Anion Exchange Liquid Chromatography for the Determination of Nucleotides in Baby and/or Functional Foods

Pilar Viñas,[†] Natalia Campillo,[†] Ignacio López-García,[†] Sara Martínez-López,[†] Isabel Vasallo,[‡] and Manuel Hernández-Córdoba*,[†]

[†]Departamento de Química Analítica, Facultad de Química, Universidad de Murcia, E-30071 Murcia, Spain, and [‡]Instituto de Nutrición Infantil (INUI), Grupo Hero España SA, Alcantarilla, Murcia, Spain

A sensitive, selective and solvent-free procedure is proposed for the rapid determination of monophosphate nucleotides (cytidine 5′-monophosphate, uridine 5′-monophosphate, adenosine 5′-monophosphate, and guanosine 5′-monophosphate) in baby foods. The method is based on the deproteinization of foods and direct analysis by anion exchange liquid chromatography (LC). Nucleotides were separated on an SAX anion exchange column with isocratic elution using 0.01 M dihydrogenphosphate buffer (pH 3.5) as mobile phase at a flow-rate of 1 mL min^{-1} and detected by diode-array detection (DAD). The LC method rapidly separated the nucleotides (9 min) and was validated for linearity, detection and quantitation limits, selectivity, accuracy, and precision. The recoveries obtained for spiked samples were satisfactory for all the analytes. The proposed procedure allowed the only authorized nucleotides in infant formulas to be determined and was successfully applied to the analysis of different baby and/or functional food samples, including infant formulas, fermented milk, cereals, and purees.

KEYWORDS: Anion exchange liquid chromatography; 5′-monophosphate nucleotides; baby foods; infant formulas; functional foods

INTRODUCTION

Childhood constitutes the life period when nutritional demands are at their highest. Baby foods combine a wide range of different matrices (*1*): nonfatty baby foods based on fruits and vegetables (fat content lower than 2%), fatty foods based on meat/egg/cheese, and cereal-based foods with different fat contents. Moreover, breast milk and infant formulas are also included. Research in the field of child nutrition has established the optimal requirements of proteins, carbohydrates, fats, vitamins, and minerals for each stage of child growth. More recent advances have allowed one to know the importance of other nutrients, such as fatty acids, lipids, and nucleotides, which take part in the processes of brain development, learning, language, and visual growth. Functional foods satisfy the basic nutritional needs and, moreover, provide health benefits; the range of functional products has strongly increased in recent years (*2*). Dairy functional foods and functional ingredients containing milk represent a growing market (*3*). Indeed, because of their health-giving properties, traditional dairy products (yoghurt, fermented milk, infant formula, or even milk) can themselves be considered functional foods, as well as containing different ingredients for the formulation of other functional foods.

Nucleotides are monomers which constitute the nucleic acids, DNA and RNA. Each nucleotide is formed by three units, a sugar with five carbon atoms (ribose for RNA or desoxirribose for DNA), a nitrogen base (puric or pyrimidic base), and one or more phosphate groups. The functions attributed to nucleotides are principally immune-, lipidic-, and digestive-related, to emulate breast feeding (*4*, *5*). The addition of nucleotides to infant formulas began in Japan in 1965, and in Spain, the first European country to do so, in 1983 (*6*). In total, the maximum concentration authorized is 5 mg/100 kcal (0.26 mg g^{-1}), which is equivalent to the amount of free nucleotides in maternal milk (*7*). Permitted nucleotides are limited to five compounds: cytidine 5′-monophosphate (CMP), 2.5 mg/100 kcal (0.13 mg g^{-1}); uridine 5′-monophosphate (UMP), 1.75 mg/100 kcal (0.09 mg g^{-1}); adenosine 5′-monophosphate (AMP), 1.5 mg/100 kcal (0.08 mg g^{-1}); guanosine 5′-monophosphate (GMP), 0.5 mg/100 kcal (0.026 mg g^{-1}); and inosine 5′-monophosphate (IMP), 1 mg/100 kcal (0.05 mg g^{-1}). However, the latest recommendations in USA have increased the total maximum amount to 16 mg/100 kcal (0.83 mg g^{-1}) in infant formulas and 22 mg/100 kcal (1.13 mg g^{-1}) in infant formulas for premature babies (*8*). These values are based on the total amount of potentially available nucleotides (TPAN) in breast milk. Low weight newborn babies showed a higher growth rate in the first six months of life when fed with infant formula supplemented with nucleotides (*9*). There is no consensus on the concentration at which nutrients should be added to infant formula; however, it seems that the addition of nucleotides is safe and beneficial, especially for premature and low weight newborn babies, who have a low endogenous synthesis capacity. Consequently, supplementation levels need to be monitored.

Nucleotides, like many other biological compounds, are relatively nonvolatile and unstable, and LC has been the preferred

*Corresponding author. Department of Analytical Chemistry, Faculty of Chemistry, University of Murcia, E-30071-Murcia, Spain. Fax: +34868887682. E-mail: hcordoba@um.es.

Published on Web 08/05/2009

method for their analysis in food matrices as a confirmation method or screening test. However, the separation of nucleotides is usually performed by anion-exchange or ion-pairing reversed phase LC because nucleotides have high polarity and an anionic nature. Thus, several LC methods for the separation and determination of nucleotides in infant formulas or biological matrices have been proposed (*10*−*30*). However, there is no information about the levels of nucleotides in other baby foods such as cereals and purees. Techniques for the analysis of nucleotides other than LC include enzymatic assay (*31*), capillary electrophoresis (*32*), and capillary electrochromatography (*33*).

The proposed method uses anion exchange LC with photodiode array detection. This is the first time that the nucleotides, 5′-CMP, 5′-UMP, 5′-AMP, and 5′-GMP, have been determined rapidly in a wide range of baby and/or functional foods, without and with supplementation, including infant formulas, fermented milk, cereals, and purees.

MATERIALS AND METHODS

Apparatus. The LC system consisted of an Agilent 1200 (Agilent, Waldbronn, Germany) binary pump (G1312B) operating at a flow-rate of 1 mL min^{-1}. The solvents were degassed using an online membrane system (Agilent 1100, G1379A). The column was maintained in a thermostatted compartment at room temperature (Agilent 1200, G1316B). The diode array and multiple wavelength detector was an Agilent 1100 (G1315C) operating at three wavelengths of 252, 260, and 271 nm. Separation was performed on a Tracer Extrasil SAX anion exchange column (15 cm × 0.4 cm x 5 μm). The injection (20 μL) was performed using an autosampler (Agilent 1200, G1367C). Solutions were stored in 2 or 10 mL amber glass vials. An EBA 20 (Hettich, Tuttlingen, Germany) centrifuge and an ultrasonic bath (Selecta, Barcelona, Spain) were used for extraction purposes.

Reagents. Analytical-reagent grade acetonitrile and methanol were purchased fron Lab-Scan (Dublin, Ireland). Deionized water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). Four commercially available 5′-monophosphate nucleotides, 5′-CMP, 5′-AMP, 5′-GMP, and 5′-UMP, were obtained as the sodium salts from Sigma (St. Louis, MO, USA). Stock solutions (1000 μg mL^{-1}) were prepared by dissolving the commercial products, without previous purification, in water. All were kept at 4 °C in dark bottles sealed with PTFE/silicone caps for a maximum of two weeks. Working standard solutions were prepared daily by diluting with water. Other reagents used were sodium dihydrogenphosphate and acetic acid (Fluka, Buchs, Switzerland, 99.8%).

Samples. The samples of different type of baby and/or functional foods were supplied from local manufacturers (Hero España, S.A.). These were five types of infant formula (starting, follow-on, prebiotic follow-on, lactum 1, and lactum 2), a breakfast cereal (multicereals with honey), two fermented milk samples (lactis 1 and lactis 2), two puree samples (vegetables and chicken with rice), and two lyophilized puree samples (peach with banana and fruit salad). Recovery experiments were carried out using samples spiked with a standard mixture of nucleotides. The samples were left to equilibrate at room temperature for at least half an hour before starting the extraction procedure.

Analytical Procedure. The samples were prepared by weighing 1 g of the baby food into a 10-mL centrifuge tube and diluting with 6 mL of water. To improve homogenization of lyophilized samples, suspensions were submitted to ultrasounds for 5 min. Then, 0.5 mL of 3% trichloroacetic acid was added, and the sample was left to stand for 15 min to allow precipitation of the proteins. The sample was then centrifuged at 6000 rpm for 10 min at room temperature. The supernatant was recovered and diluted to 10 mL with water in a calibrated flask. Aliquots were filtered through 0.2 μm nylon Millipore chromatographic filters, and 20 μL was injected into the chromatograph using amber vials with the autosampler. The mobile phase used in isocratic conditions was a 0.01 M dihydrogenphosphate buffer (pH 3.5). The flow rate was 1 mL min^{-1} throughout. The diode array detector was set at wavelengths of 252 nm for 5′-GMP, 260 nm for 5′-AMP and 5′-UMP, and 271 nm for 5′-CMP. Analyses were performed in triplicate and injections in duplicate.

Table 1. Effect of Mobile Phase pH on Nucleotide Retention Using a SAX Column

nucleotide	5′-CMP		5′-AMP		5′-UMP		5′-GMP	
pH	t_R	W	t_R	W	t_R	W	t_R	W
3.0	1.97	0.08	3.22	0.13	7.17	0.22	8.37	0.25
3.5	2.75	0.10	4.75	0.17	6.48	0.21	8.10	0.26
4.0	3.12	0.12	5.93	0.22	6.57	0.22	8.38	0.26
5.0	4.33	0.16	6.26	0.21	7.23	0.25	8.03	0.27

RESULTS AND DISCUSSION

Ion Exchange LC Separation. The chromatographic technique was chosen because nucleotides are ionic and very polar organic compounds and, consequently, can be resolved using different techniques such as reversed phase, ionic pairs, or ion exchange chromatography. Reversed phase was first assayed using a Zorbax Eclipse XDB-C_{18} column and different mixtures (30−100% v/v) of acetonitrile (ACN) and phosphate buffer (50 mM, pH 7) as mobile phases. However, because of their polar nature, nucleotides were not retained by the column in any conditions and were always eluted at the void time so that this technique was discarded. Subsequently, taking advantage of the very polar character of the analytes, ion pair chromatography was tried using the same C_{18} column and different mixtures of ACN (0−10% v/v) and a 30 mM buffer phosphate (pH 4.3) containing 0.03% m/v tetrabutylammonium hydroxide. The best separation was achieved using 100% buffer, although the peaks appeared tailed and unresolved. Separation was not improved by varying several experimental conditions, such as the pH of the mobile phase (3.5−6.5), the stationary phase (Zorbax XDB-C_8 eclipse, 5 μm), or the flow rate (0.25−1 mL min^{-1}).

Finally, ion exchange chromatography was selected since nucleotides, being negatively charged, present ionic characteristics. The optimal stationary phase was Tracer Extrasil SAX, a strong anion exchanger generally used for the analysis of small organic molecules, which provided high selectivity and good separation efficiency for nucleotides. The equilibrium conditions depended on variables such as counterion selectivity, pH, temperature, and buffer concentration in the mobile phase. The counterion selectivity depends on the capacity of the resin to discriminate between ions with a similar charge but different geometry. The anions assayed were phosphate, nitrate, acetate, and formiate, and the best results were obtained using phosphate as the counterion. In the analysis of partially ionized samples, the pH of the mobile phase can control the degree of ionization, and an increase in the ions/neutral molecules ratio for the sample could increase sample retention. Thus, the effect of the pH of the mobile phase was studied using different 0.01 M phosphate buffers at pH values ranging from 3 to 5 and a flow rate of 1 mL min^{-1}. **Table 1** shows the variation of both retention time (min) and peak width (W, min) for the analytes as a function of pH. The influence of this variable was more marked for CMP and AMP, which were strongly retained by the column at higher pH values. However, the retention of UMP and GMP were only slightly influenced by pH variations. As can be seen, the best separation of nucleotides was achieved using a pH value of 3.5.

When the influence of the buffer concentration was studied in the 0.01−0.05 M range, retention decreased as the ionic strength increased so that 0.01 M phosphate buffer was selected. When the column temperature was increased to 50 °C, the retention factors slightly decreased for all of the analytes as the temperature increased; thus, room temperature was selected.

Figure 1 shows the chromatogram obtained for a mixture of the four standard nucleotides under the selected chromatographic

conditions. The sequence of the analytes as a function of the eluting time was 5′-CMP (2.75 min), 5′-AMP (4.75 min), 5′-UMP (6.48 min), and 5′-GMP (8.10 min). Retention factors (k) were between 1.7 and 8, separation factors (α) in the 1.3−2.1 range, and resolution (R_S) between 6.9 and 14.8.

Analytical Data and Validation. The method was validated for linearity, detection limit, selectivity, accuracy, and precision. Calibration curves were obtained by least-squares linear regression analysis of the peak area versus analyte concentration using six concentration levels. Quantification was performed by the external standard procedure. The validation parameters, correlation coefficients, and working range for the nucleotides are shown in **Table 2**. The values of r^2 were good ($r^2 > 0.99$), and excellent linearity was obtained for the range studied. The detection limits were calculated on the basis of three times the standard deviation of the intercept of the calibration graphs and the quantitation limits using 10 times the standard deviation of the intercept. These values are also given in **Table 2**. To check the repeatability of the method, 10 replicate analyses of a baby food sample containing 1 μg mL^{-1} of the standards were performed; an RSD of < 3% for peak area values was obtained in all cases (**Table 2**). These values indicate that the precision of the method was satisfactory for the control analysis.

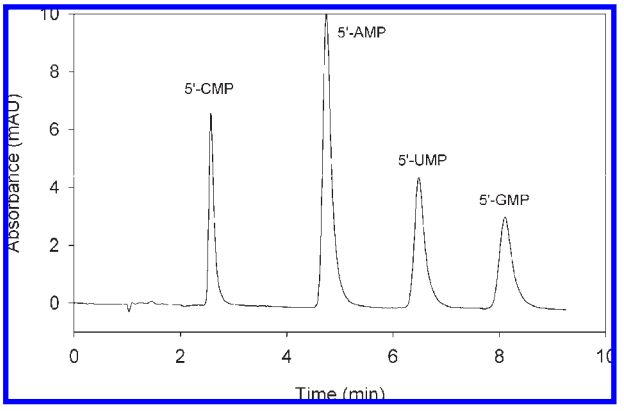

Figure 1. Elution profile obtained for a standard nucleotide mixture using anion exchange LC (260 nm).

The selectivity of the method was judged according to the absence of interfering peaks at the elution times of the analytes for blank chromatograms of different unspiked baby food samples.

For the analysis of samples, different procedures for the extraction of nucleotides from baby foods were compared. The extraction solvent of choice should be able to recover the nucleotides quantitatively and also to precipitate the proteins efficiently. Different experiments were carried out using acetic acid, formic acid, trichloroacetic acid, acetonitrile, and methanol as solvents. The best absolute recovery and best reproducibility for all nucleotides were obtained using extraction with acetic and trichloroacetic acid, probably because of the highly hydrophilic nature of the compounds. Conversely, very low recovery was obtained with the use of the organic solvents. The precipitation of proteins was more complete when using trichloroacetic acid instead of acetic acid. However, the optimal concentration was studied, and the supernatants after filtration remained without turbidity with a concentration of trichloroacetic acid above 3% m/v, which was selected.

The matrix effect of the different baby food samples for the studied nucleotides was evaluated by comparing the slopes of aqueous standards and standard addition calibration graphs for different matrices, obtained by plotting concentration (at five levels) against peak area and following linear regression analysis. **Table 3** shows the data obtained. A statistical study was carried out to compare the slope values using the one-sample t test, which operates by comparing the different slopes obtained from baby foods with the aqueous slope for each analyte. Slopes of the standard addition calibration graphs for the samples were similar to those of aqueous standards ($P > 0.05$) for the four nucleotides, as shown in **Table 3** (values in parentheses correspond to P values), confirming that the matrix did not interfere and that calibration could be carried out using aqueous standards.

To check the accuracy of the proposed method, a recovery study was carried out by fortifying three samples (infant formula, breakfast cereals, and vegetables puree) at two concentration levels corresponding to approximately two and four times the quantitation limits. The recoveries of the nucleotides from spiked samples varied from 88 to 97% with an average recovery ± SD ($n = 24$) of 93 ± 3. The similarity in recoveries obtained for each

Table 2. Analytical Data for Nucleotides Using Anion Exchange LC

parameter	5′-CMP	5′-AMP	5′-UMP	5′-GMP
slope[a] (mL μg^{-1})	26 ± 0.9	43 ± 1.7	40 ± 1.8	43 ± 2.1
ordinate[a]	−0.94 ± 0.04	−2.2 ± 0.09	−0.37 ± 0.02	−2.5 ± 0.11
correlation coefficient	0.9998	0.9998	0.9999	0.9999
working range (ng mL^{-1})	100−2000	50−2000	50−2000	50−2000
detection limit (ng mL^{-1})	40	20	20	20
quantitation limit (ng mL^{-1})	130	65	65	65
repeatability (RSD for peak areas, n = 10)	2.9	1.8	2.5	1.9

[a] Mean ± standard deviation (n = 6).

Table 3. Calibration Slopes for Different Baby Food Samples (mL μg^{-1})[a]

sample	5′-CMP	5′-AMP	5′-UMP	5′-GMP
aqueous standards	26.14	43.55	39.64	43.07
vegetables puree	25.25 (0.90)	43.09 (0.96)	40.10 (0.96)	42.62 (0.96)
milk lactis 1	25.00 (0.87)	41.21 (0.80)	38.52 (0.90)	40.99 (0.82)
milk lactis 2	26.03 (0.99)	44.68 (0.90)	38.14 (0.86)	42.86 (0.98)
supplemented starting milk	25.48 (0.96)	41.34 (0.81)	38.19 (0.87)	39.41 (0.67)
supplemented follow-on milk	24.25 (0.94)	42.57 (0.79)	38.46 (0.97)	42.70 (0.92)
prebiotic follow-on milk	25.58 (0.79)	41.10 (0.91)	39.02 (0.89)	43.99 (0.97)

[a] Values in parentheses correspond to the P values for one-sample t-test.

Table 4. Nucleotide Content[a] in Baby Food Samples (μg g^{-1})

baby food	5′-CMP	5′-AMP	5′-UMP	5′-GMP
milk lactum 1	6.9 ± 0.2	ND	1.1 ± 0.3	3.4 ± 0.1
milk lactum 2	13 ± 0.15	ND	ND	2.9 ± 0.2
supplemented starting milk	134 ± 24	26 ± 0.4	42 ± 3.1	19 ± 1.2
supplemented follow-on milk	122 ± 5	31 ± 0.6	55 ± 6	18 ± 0.9
prebiotic follow-on milk	14 ± 0.8	ND	1.5 ± 0.1	4.0 ± 0.1
fermented milk lactis 1	14 ± 0.7	3.1 ± 0.2	8.4 ± 0.2	10 ± 0.2
fermented milk lactis 2	11 ± 0.4	3.1 ± 0.5	8.1 ± 0.1	11 ± 0.3
breakfast cereals	7.5 ± 1.5	10 ± 1.1	12 ± 1.3	2.7 ± 0.1
chicken with rice puree	2.5 ± 0.1	4.4 ± 0.1	3.7 ± 0.2	6.6 ± 0.2
vegetable puree	2.7 ± 0.1	6.2 ± 0.1	4.1 ± 0.2	6.5 ± 0.3
fruit salad lyophilized puree	17 ± 1.2	30 ± 1.4	11 ± 0.7	13 ± 0.8
peach with banana lyophilized puree	12 ± 0.8	10 ± 0.7	19 ± 0.3	13 ± 0.9

[a] n = 6; ND, not detected.

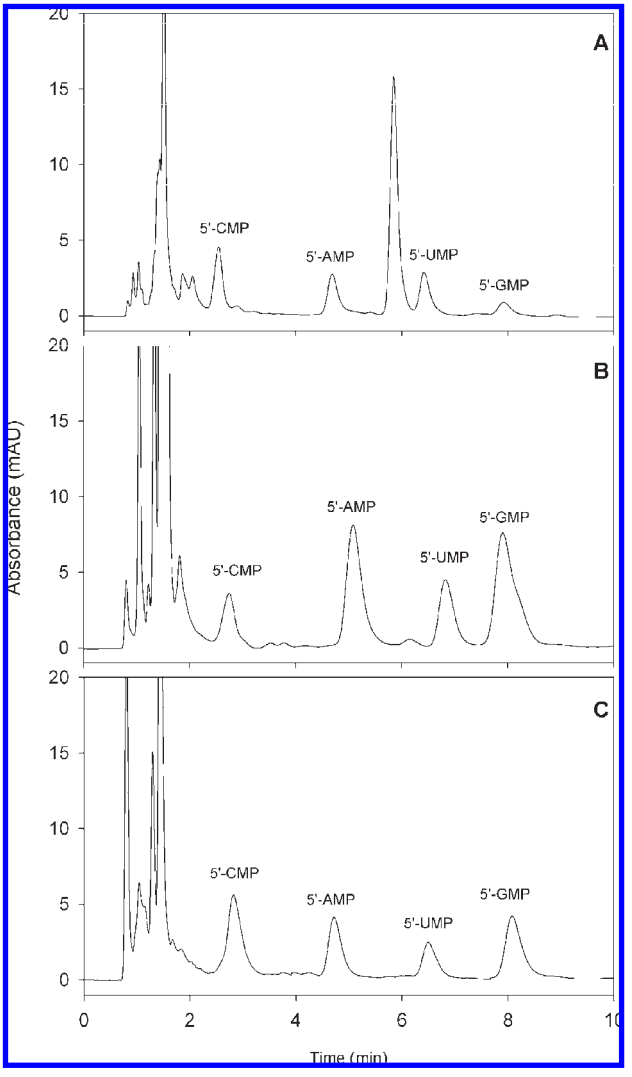

Figure 2. Elution profiles obtained for infant formula (**A**), vegetable puree (**B**), and cereal (**C**) samples using anion exchange LC (260 nm).

nucleotide in the three samples indicates that the matrix had no influence on the method performance.

Analysis of Samples. Typical chromatographic profiles obtained using anion exchange LC for infant formula (**A**), vegetables puree (**B**), and cereal (**C**) samples are shown in **Figure 2**. Similar chromatograms were obtained for the other samples. The profiles demonstrated the absence of interfering peaks. The chromatographic peaks were first identified by comparing the retention data obtained for the standards, the samples, and the different samples spiked with the standards under identical conditions. The average values (mean ± SD) for the retention times of nucleotides in the different baby food samples (n = 13) were as follows: 5′-CMP, 2.75 ± 0.10 min; 5′-AMP, 4.83 ± 0.09; 5′-UMP, 6.58 ± 0.17; and 5′-GMP, 8.05 ± 0.09. These values pointed to very good agreement between the retention data in the different baby foods. The peaks were identified again using the DAD detector to continuously measure the spectrum while the solute passed through the flow-cell, thus confirming the identity and the purity of the peaks. Good agreement was found between the UV spectra of the different peaks obtained for the standards, the samples, and the spiked samples.

Twelve different baby food samples were analyzed corresponding to five infant formulas (starting, follow-on, prebiotic, and supplemented), two fermented milk samples (lactis 1 and 2), breakfast cereal (multicereals with honey), two puree samples (vegetables and chicken with rice), and two lyophilized puree samples (peach with banana and fruit salad). All samples were analyzed in triplicate. **Table 4** shows the results obtained. As can be seen, the highest levels of nucleotides were found in lactum 1 and 2 supplemented infant formulas and were in accordance with label values. The lowest levels were found in the puree samples. Consequently, the levels found in the baby food samples were adequately quantified with no sensitivity problems using the anion exchange LC procedure.

Conclusions. Nucleotides are semiessential functional nutrients, and new analysis methods are needed for their quantification to enable the supplementation of infant formulas with higher amounts of nucleotides. The proposed procedure involving anion exchange LC with DAD detection represents a good alternative since it improves resolution, sensitivity, and the speed of analysis. The sample preparation was very simple and avoided the use of organic solvents and cleanup steps, thus reducing the total analysis time. This solvent-free, economic, and clean procedure allows the reliable control and screening by regulatory agencies of the nucleotides which are authorized for supplementing infant formulas, using instrumentation accessible to almost every laboratory equipped to carry out routine controls on baby foods.

ACKNOWLEDGMENT

We acknowledge Hero España, S.A. for the kind gift of the baby food samples.

LITERATURE CITED

(1) U.S. Food and Drug Administration. In *Pesticide Analytical Manual Vol. I: Multiresidue Methods*, 3rd ed.; U.S. Department of Health and Human Services: Washington, DC, 1994.

(2) Juárez, M.; Olano, A.; Morais, F., Eds.; *Alimentos Funcionales*; Fundación Española para la Ciencia y la Tecnología: Madrid, Spain, 2005.
(3) Playne, M. J.; Bennett, L. E.; Smithers, G. W. Functional dairy foods and ingredients. *Aust. J. Dairy Technol.* **2003**, *58*, 242–264.
(4) Leach, J. L.; Baxter, J. H.; Molitor, B. E.; Ramstack, M. B.; Masor, M. L. Total potentially available nucleosides of human milk stage of lactation. *Am. J. Clin. Nutr.* **1995**, *61*, 1224–1230.
(5) Thorell, L.; Sjöberg, L.; Hernell, O. Nucleotides in human milk: sources and metabolism by the newborn infant. *Pediatr. Res.* **1996**, *40*, 845–852.
(6) Yu, V. Yh. Scientific rationale and benefits of nucleotide supplementation of infant formula. *J. Pediatr. Child Health* **2002**, *38*, 543–549.
(7) Scientific Committee for Food. Minutes of the 100th Plenary Session, March 7−8, 1996. In *Brussels. Directive 96/4/EC*; European Commission of the European Communities: Luxembourg, **1996**; p 7.
(8) Klein, C. J. Nutrient requeriments for preterm infant formulas. *J. Nutr.* **2002**, *132*, S1395–S577.
(9) Cosgrove, M.; Davies, D. P.; Jenkins, H. R. Nucleotide supplementation and the growth of term small for gestational age infants. *Arch. Dis. Child.* **1996**, *74*, 122–125.
(10) Brown, E. G.; Newton, R. P.; Shaw, N. M. Analysis of the free nucleotide pools of mammalian tissues by high-pressure liquid chromatography. *Anal. Biochem.* **1982**, *123*, 378–388.
(11) Burnette, B.; McFarland, C. R.; Batra, P. Rapid isocratic separation of purine nucleotides using strong anion-exchange high-performance liquid chromatography. *J. Chromatogr.* **1983**, *277*, 137–144.
(12) Yokoi, H.; Watanabe, T.; Onishi, H. Simultaneous analysis of 5′-mononucleotides and nucleosides in saline solution by high-performance liquid chromatography. *Agric. Biol. Chem.* **1987**, *51*, 3147–3149.
(13) Hu, W.; Takeuchi, T.; Haraguchi, H. Determination of monophosphate-type nucleotides by high-performance liquid chromatography with phosphorus-selective detection. *Anal. Sci.* **1991**, *7*, 135–138.
(14) Fish, W. W. A method for the quantitation of 5′-mononucleotides in foods and food ingredients. *J. Agric. Food Chem.* **1991**, *39*, 1098–1101.
(15) Thorell, L.; Sjoberg, L. B.; Hernell, O. Nucleotides in human milk: sources and metabolism by the newborn infant. *Pediatr. Res.* **1996**, *40*, 845–849.
(16) Childs, K. F.; Ning, X. H.; Bolling, S. F. Simultaneous detection of nucleotides, nucleosides and oxidative metabolites in myocardial biopsies. *J. Chromatogr., B* **1996**, *678*, 181–186.
(17) Oliveira, C.; Ferreira, I. M.; Mendes, E.; Ferreira, M. A. Development and application of an HPLC/diode array methodology for determination of nucleotides in infant formulae and follow-up milks. *J. Liq. Chromatogr. Relat. Technol.* **1999**, *22*, 571–578.
(18) Schlimme, E.; Martin, D.; Meisel, H. Nucleosides and nucleotides: natural bioactive substances in milk and colostrum. *J. Nutr.* **2000**, *84*, 559–568.
(19) Perrin, C.; Meyer, L.; Mujahid, C.; Blake, C. J. The analysis of 5′-mononucleotides in infant formula by HPLC. *Food Chem.* **2001**, *74*, 245–253.
(20) Tomiya, N.; Ailor, E.; Lawrence, S. M.; Betenbaugh, M. J.; Lee, Y. C. Determination of nucleotides and sugar nucleotides envolved in protein glycosylation by high-performance anion-exchange chromatography: sugar nucleotide contents in cultured insect cells and mammalian cells. *Anal. Biochem.* **2001**, *293*, 129–137.
(21) Kang, D. H.; Lee, J. W.; Row, K. H. Empirical correlation of retention factor of mononucleotides to buffer concentration in RP-HPLC. *J. Liq. Chromatogr. Relat. Technol.* **2001**, *24*, 855–868.
(22) Ferreira, I. M.; Mendes, E.; Gomes, A. M. P.; Faria, M. A.; Ferreira, M. A. The determination and distribution of nucleotides in dairy products using HPLC and diode array detection. *Food Chem.* **2001**, *74*, 239–244.
(23) Aussenac, J.; Chassagne, D.; Claparols, C.; Charpentier, M.; Duteurtre, B.; Feuillat, M.; Charpentier, C. Purification method for the isolation of monophosphate nucleotides from Champagne wine and their identification by mass spectrometry. *J. Chromatogr., A* **2001**, *907*, 155–164.
(24) Tuytten, R.; Lemière, F.; Van Dongen, W.; Esmans, E. L.; Slegers, H. Short capillary ion-pair high-performance liquid chromatography coupled to electrospray (tandem) mass spectrometry for the simultaneous analysis of nucleoside mono-, di- and triphosphates. *Rapid Commun. Mass Spectrom.* **2002**, *16*, 1205–1215.
(25) Mateo, C. D.; Peters, D. N.; Stein, H. H. Nucleotides in sow colostrum and milk at different stages of lactation. *J. Animal Sci.* **2004**, *82*, 1339–1342.
(26) Li, Y. Determination of mononucleotides in extraction product of chlorella by ion exchange chromatography. *Fenxi Huaxue* **2004**, *32*, 1077–1079.
(27) Charpentier, C.; Aussenac, J.; Charpentier, M.; Prome, J. C.; Duteurtre, B.; Feuillat, M. Release of nucleotides and nucleosides during yeast autolysis: kinetics and potential impact on flavor. *J. Agric. Food Chem.* **2005**, *53*, 3000–3007.
(28) Gill, B. D.; Indyk, H. E. Development and application of a liquid chromatographic method for analysis of nucleotides and nucleosides in milk and infant formulas. *Int. Dairy J.* **2007**, *17*, 596–605.
(29) Inovne, K.; Obana, R.; Akiba, T.; Hino, T.; Oka, H. Determination of nucleotides in infant formulae by ion-exchange liquid chromatography. *J. Agric. Food Chem.* **2008**, *56*, 6863–6867.
(30) Cordell, R. L.; Hill, S. J.; Ortori, C. A.; Barrett, D. A. Quantitative profiling of nucleotides and related phosphate-containing metabolites in cultured mammalian cells by liquid chromatography tandem electrospray mass spectrometry. *J. Chromatogr., B* **2008**, *871*, 115–124.
(31) Gil, A.; Sánchez-Medina, F. Acid-soluble nucleotides of cow's, goat's and sheep's milks, at different stages of lactation. *J. Dairy Res.* **1981**, *48*, 35–44.
(32) Qurishi, R.; Kaulich, M.; Müller, C. E. Fast, efficient capillary electrophoresis method for measuring nucleotide degradation and metabolism. *J. Chromatogr., A* **2002**, *952*, 275–281.
(33) Ohyama, K.; Fujimoto, E.; Wada, M.; Kishikawa, N.; Ohba, Y.; Akiyama, S. Investigation of a novel mixed-mode stationary phase for capillary electrochromatography. Part III: Separation of nucleosides and nucleic acid bases on sulfonated naphthalimido-modified silyl silica gel. *J. Sep. Sci.* **2005**, *28*, 767–773.

Received May 22, 2009. Revised manuscript received July 16, 2009. Accepted July 27, 2009. We acknowledge the Spanish MEC (Project CTQ2006-08037/BQU) for financial support. S.M.-L. acknowledges a fellowship from CARM (Spain).