Development and Application of a Liquid Chromatographic Method for Analysis of Nucleotides and Nucleosides in Milk and Infant Formulas

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Abstract

A method for the simultaneous determination in milk of the 5′-mononucleotides adenosine 5′-monophosphate, cytidine 5′-monophosphate, guanosine 5′-monophosphate, inosine 5′-monophosphate and uridine 5′_monophosphate and their corresponding nucleosides is described. Following deproteinisation, the sample extract was analysed by reversed-phase liquid chromatography, whereby chromatographic separation was achieved using a polymer grafted silica C_{18} column, gradient elution with a simple binary mobile phase and UV detection by photodiode array. Performance parameters included recoveries of 95.5–105.2% and precision evaluated as 3.42–6.38% relative standard deviation. The described technique has been applied to the analysis of bovine and human milk, a range of commercial bovine milk-based infant and follow-on formulas, a seasonal study of skim milk powders and an assessment of alkaline phosphatase influence on nucleotide retention.

Keywords

Bovine milk; Nucleotides; Nucleosides; Human milk; High-performance liquid chromatography; Alkaline phosphatase

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1. Introduction

Nucleotides and nucleosides play important roles in biochemical processes. In addition to being precursors in nucleic acid synthesis, nucleotides reportedly enhance immune response (Carver, Pimentel, Cox, & Barness, 1991; Pickering et al., 1998; Schaller et al., 2004), influence metabolism of fatty acids, contribute to iron absorption in the gut and improve gastrointestinal tract repair after damage (Carver & Walker, 1995; Sanchez-Pozo & Gil, 2002; Schlimme, Martin, & Meisel, 2000). Nucleotides and nucleosides can be synthesised de novo or via salvage pathways and thus are not essential dietary nutrients. However, they may become conditionally essential when the endogenous supply is inadequate during periods of rapid growth or after injury (Carver & Walker, 1995). In view of the significant differences in the non-protein nitrogen nucleos(t)ide pool between bovine and human milk, infant formulas are increasingly supplemented with nucleotides, despite gastrointestinal dephosphorylation to nucleosides, the preferred form for intestinal absorption (Aggett, Leach, Rueda, & MacLean, 2003; Thorell, Sjöberg, & Hernell, 1996).

Early clinical studies employed infant formulas containing nucleotides supplemented to levels based on the free nucleotide and nucleoside content of human milk. It is on this basis that the European Union has approved the use of free nucleotides up to a maximum concentration of 5mg $100kcal^{-1}$ (Aggett et al., 2003). However, the measurement of free nucleotide and nucleoside levels does not account for polymeric nucleotides, nucleoproteins or nucleos(t)ide derivatives. When all total potentially available nucleoside (TPAN) sources of nucleotides present in human milk were determined, it was confirmed that the total nucleotides in human milk that were available to the infant were underestimated by over 50% (Leach, Baxter, Molitor, Ramstack, & Masor, 1995). Thus, allowance for the fortification of infant formulas with nucleotides at TPAN levels has subsequently been approved in more than 30 countries (Aggett et al., 2003), including Australasia with a maximum limit of 3.8 mg $100kJ^{-1}$ (16 mg $100kcal^{-1}$) (Food Standards Australia New Zealand (FSANZ), 2006). The benefits of nucleotide supplementation of infant formula to TPAN levels remain however, controversial (Yu, 1998) and indeed, a recent study reported both a decreased incidence of diarrhoea and an increased risk of respiratory tract infections associated with such paediatric products (Yau et al., 2003).

Alkaline phosphatase (ALP) is a phosphomonoesterase with a wide range of substrate specificity, including 5′-mononucleotides. Further, the 5′-mononucleotides are substrates for endogenous nucleotidases and nucleosidases that may also be present and can generate nucleosides and nucleobases respectively. Bovine milk ALP is present in raw milk and since its heat stability profile closely matches that necessary for satisfactory pasteurisation, residual ALP activity is used as an indicator of the efficacy of high temperature short-time (HTST) pasteurisation. However, under short

exposures to temperatures of 80–180 °C and storage conditions of 4–40 °C, partial reactivation of the enzyme can occur. Bovine milk acid phosphatase is also a phosphomonoesterase and although present in milk in significantly lower amounts, is more resistant to heat than ALP. However, its substrate specificity is reportedly different from that of ALP and it does not seem to hydrolyse aliphatic phosphomonoesters such as adenosine 5′-monophosphate (Andrews, 1991; Shakeel-ur-Rehman, Fleming, Farkye, & Fox, 2003). In addition, bacterial contamination of milk can result in the presence of microbial ALP, which is more resistant to heat than bovine milk ALP (Karmas & Kleyn, 1990).

Due to residual ALP activity that may occasionally survive post-pasteurisation, there is a potential risk during infant formula production of dephosphorylation of exogenous 5′-mononucleotides yielding the related nucleosides. There is currently no official reference method for the analysis of nucleotides in infant formulas. Thus, an analytical method for the analysis of nucleotide supplemented infant formulas involving simultaneous determination of both nucleotides and nucleosides is ideally suited both to assess the integrity of nucleotides during infant formula manufacture and to establish compliance with regulatory nucleos(t)ide levels.

Techniques for the analysis of nucleotides other than liquid chromatography include enzymatic assay (Gil & Sanchez-Medina, 1981), capillary electrophoresis (Qurishi, Kaulich, & Müller, 2002), capillary electrophoresis-inductively coupled plasma mass spectrometry (Yeh & Jiang, 2002) and capillary electrochromatography (Ohyama et al., 2005).

Ion-pair reversed-phase chromatography (IP-RPC) is currently the most commonly used technique utilised for the separation of nucleotides. Excellent resolution can be achieved because the ionic nature of the phosphate esters facilitates strong interactions with cationic ion-pair reagents at the appropriate pH (Brown, Robb, & Gelhart, 2002; Werner, 1993). Thus, IP-RPC has been employed to determine the nucleotide content in various mammalian milks and dairy products (Ferreira, Mendes, Gomes, Faria, & Ferreira, 2001; Mateo, Peters, & Stein, 2004; Oliveira, Ferreira, Mendes, & Ferreira, 1999; Perrin, Meyer, Mujahid, & Blake, 2001; Sugawara, Sato, Nakano, Idota, & Nakajima, 1995), in food ingredients (Fish, 1991) and in blood (Cichna et al., 2003).

The retention and the resolution of nucleotides in the absence of ion-pair reagents are limited with conventional C_{18} columns due to poor interaction of the highly polar analytes with the apolar C_{18} phase under the required conditions of low organic modifier content (Brown et al., 2002). Recent developments in polar embedded C_{18} phases, hybrid columns and polymer grafted silica columns offer advantages over conventional C_{18} phases, such as suppressed silanol activity leading to superior peak shape for basic compounds, phase stability under highly aqueous conditions and unique selectivity (Layne, 2002; Majors, 2004; Majors & Przybyciel, 2002).

Nucleosides lack the charged phosphate groups present in nucleotides and are therefore relatively well retained on C_{18} phases without the requirement for ion-pair reagents. Nucleosides have been determined in various mammalian milks (Leach et al., 1995; Schlimme et al., 1997; Thorell et al., 1996), in serum (Xu, Enderle, Liebich, & Lu, 2000), in urine by RPC (Vidotto, Fousert, Akkermann, Griesmacher, & Müller, 2003) and in urine by high-performance liquid chromatography–electrospray ionisation mass spectrometry (Dudley et al., 2004).

The method herein describes a simple binary elution protocol using RPC with a polymer-grafted column to separate both nucleotides and nucleosides, thereby avoiding the inherent disadvantages of diminished column lifetime and long equilibration times when using gradient ion-pair techniques. The described technique has been applied to the analysis of bovine and human milk, a range of commercial paediatric formulas, skim milk powders across a production season and an assessment of ALP influence on nucleotide retention.

2. Materials and methods

2.1. Apparatus

The HPLC system consisted of a SCL-10Avp system controller, LC-10ADvp pump, FCV-10ALvp low pressure gradient unit, SIL-10AF sample injector unit equipped with a 50 µL injection loop, DGU-14A degasser unit, CTO-10ASvp column oven and a SPD-M10Avp photodiode array detector (Shimadzu, Kyoto, Japan). Instrument control and data processing were implemented using Shimadzu Class-VP version 6.12.

The column selected for use was a Gemini C_{18}, 250 × 4.6 mm, 5 µm (Phenomenex, Torrance, CA, USA). UV absorbances for calibration standards were acquired with a model UV-1601 spectrophotometer (Shimadzu, Kyoto, Japan) with digital readout to 4 decimal places. A Meterlab PHM210 Standard pH Meter (Radiometer Analytical, Lyon, France) was used. Samples were centrifuged in a Sorvall Instruments Econospin (Dupont, Wilmington, DE, USA). Polypropylene centrifuge tubes, 15 mL calibrated (Corning Incorporated, Acton, MA, USA), Terumo 3 mL disposable syringes (Terumo (Philippines) Corporation, Laguna, Philippines) and Minisart 0.2 µm syringe filters with cellulose acetate membranes (Sartorius, Göttingen, Germany) were used for sample preparation.

Prior to use, mobile phases were filtered and degassed using a filtration apparatus with 0.45 mm nylon filter membranes (AllTech, Deerfield, IL, USA). ALP was measured using the Paslite ALP test for dairy products and a Charm Lum-K luminometer (Charm Sciences Inc., Lawrence, MA, USA).

2.2. Reagents

Adenosine 5′-monophosphate (AMP) sodium salt, cytidine 5′-monophosphate (CMP) disodium salt, guanosine 5′-monophosphate (GMP) disodium salt, inosine 5′-monophosphate (IMP) disodium salt, uridine 5′-monophosphate (UMP) disodium salt, adenosine, cytidine, guanosine, inosine, uridine, orotic acid and uric acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Potassium dihydrogen phosphate (KH_2PO_4), orthophosphoric acid (H_3PO_4), potassium hydroxide (KOH) and acetic acid were supplied by Merck (Darmstadt, Germany). Water was purified with resistivity >18MΩ using an E-pure water system (Barnstead, Dubuque, IA, USA).

2.3. Standards

Because of the hygroscopic nature of mononucleotide salts, the moisture content was determined by the Karl Fischer method and the concentration was calculated on a dry weight basis. Extinction coefficients at UV absorbance maxima were determined experimentally for the routine calibration of nucleotide stock standard concentrations (Table 1).

Stock standards were prepared by accurately weighing approximately 50 mg of each nucleotide or nucleoside into separate 50 mL volumetric flasks and adding approximately 40 mL of water. To promote dissolution, 1–2 drops of 50% w/v KOH solution were added to GMP or guanosine stock standard as necessary. Volumes were made to 50 mL and stock standards were stored at 4 °C for up to 2 weeks. The concentration of each nucleotide stock standard was determined by diluting 1.0 mL of stock standard to 50 mL with 0.25 M phosphate buffer (34.02 g of KH_2PO_4 dissolved in 900 mL of water, pH adjusted to 3.5 with H_3PO_4 and then made up to 1 L) and measuring the absorbance at the appropriate λ_{max}.

Mixed working standard solutions (3–16 mg mL^{-1}) containing nucleotides and nucleosides were prepared by diluting the appropriate stock standards with water to the required concentration.

2.4. Sample collection

Three commercially available infant formulas and eight follow-on formulas supplemented with nucleotides were sourced through retail outlets and stored at room temperature until analysis. These products were bovine milk-based, but excluded both hypoallergenic and soy-based formulas. Skim milk powders were prepared monthly across a production season from pooled pasteurized herd skim milk using a pilot-scale spray drier. The skim milk powders were collected, sealed in laminated sachets and stored at 20 °C until analysis. Human milk samples (approximately 30 mL) were individually collected by mothers at the end of regular feeding and were immediately chilled to 4 °C and delivered to the laboratory within 24 h. Sample preparation occurred within 12 h of collection. Bovine

colostrum and milk samples (approximately 40 mL) were collected from a single Jersey cow in its fourth calving and were immediately chilled to 4 °C. Sample preparation occurred within 24 h of collection.

2.5. Sample preparation

Liquid milk samples were gently inverted by hand and 5.0 mL was transferred to 15 mL centrifuge tubes. Approximately 0.5 g of skim milk powder was weighed accurately into 15 mL centrifuge tubes and dissolved in 4 mL of water (20 °C). Acetic acid (2.0 mL, 3% v/v) was added to each sample, which was mixed, allowed to stand for 15 min, diluted to 10.0 mL with water, mixed and then centrifuged for 10 min at approximately 4000 × g, 10 min, 20 °C. An aliquot of the supernatant was filtered through a 0.2 µm syringe filter into an autosampler vial.

2.6. Chromatography

Gradients were formed by low pressure mixing of two mobile phases, A and B. Mobile phase A: 13.6 g of KH_2PO_4 dissolved in 750 mL of water, pH adjusted to 5.6 with KOH (20% w/v) and made to 1 L with water. Mobile phase B: 13.6 g of KH_2PO_4 dissolved in 750 mL of water, pH adjusted to 5.6 with KOH (20% w/v), 150 mL of methanol added and made to 1 L with water. Simultaneous separation of nucleotides and nucleosides was achieved using the gradient procedure shown in Table 2. When the analysis required measurement of nucleotides only, mobile phase A was used isocratically, with a run time of 70 min.

The photodiode array detector acquired spectral data between 200 and 350 nm. Peak identification was by co-chromatography and similarity of chromatographic peak spectrum against authentic standards, as estimated by a similarity index of > 0.99. Wavelengths selected for routine integration were: 250 nm for IMP and inosine; 260 nm for AMP, GMP, adenosine, guanosine, cytidine and uridine; 270 nm for CMP and UMP.

Multilevel calibration (n = 3) was performed using linear least-squares regression, with quantification of nucleotides and nucleosides estimated by the external standard technique. At the end of each sample sequence, the column was rinsed with > 20 column volumes of water and stored in acetonitrile:water (65:35), resulting in a stable column performance over numerous subsequent analyses (n > 40 injections).

2.7. Influence of ALP on nucleotide retention

Skim milk was pasteurised in a pilot-plant scale pasteuriser at either 68 or 82 °C for 15 s, simulating sub-optimum and exhaustive pasteurisation conditions, respectively. Each sample was cooled to

room temperature and tested for residual ALP activity using the Charm Paslite ALP test. In duplicate, 5 mL samples were then spiked with a known amount of UMP (158 mg) and were left to stand for 3 min before sample preparation, as described, prior to nucleos(t)ide analysis. The percentage recovery of added UMP was then calculated after accounting for the endogenous UMP contribution.

3. Results

3.1. Method optimisation

Initial strategies considered for sample preparation and HPLC conditions included optimisation of the ion-pair reagent (tetrabutylammonium hydroxide) concentration, mobile phase ionic strength, pH and column type, as reported previously for the quantification of orotic acid in milk (Indyk & Woollard, 2004). These trials, conducted isocratically, exhibited three impediments to unambiguous analysis: (1) poor resolution of GMP and IMP, (2) late elution of AMP relative to other nucleotides and (3) inadequate resolution of several pyrimidine species due to poor retention. Unfortunately, there was no single elution protocol that delivered optimum simultaneous separation of nucleotides and nucleosides without resorting to a gradient elution scheme. Due to inherently long column reconditioning times between each such analysis, a non ion-pair phase was found to be preferable for routine use. In view of these observations, an alternative column type was identified and selected. The Gemini column, combined with the presently described elution scheme, provided a superior resolution in the absence of an ion-pair reagent and overcame previously identified limitations, and was therefore selected for single laboratory validation.

The selected detection wavelengths were ideally at, or proximal to, the λ_{max} of each species. However, a detection wavelength for CMP and UMP of 270 nm represents a compromise between maximum signal acquisition and discrimination of closely eluting interferences. For clarity, Figure 1 illustrates single channel chromatography obtained at 260 nm for a nucleotide and nucleoside standard mixture.

3.2. Method performance

The performance of the method was determined as summarised in Table 3. Dose response linearity was confirmed by least-squares regression analysis of multilevel calibration standards. In the absence of a currently available infant formula standard reference material (SRM) with certified levels of nucleotides, method accuracy was confirmed by evaluating recovery using the standard additions method. Intra-run precision, as repeatability, was determined using seven replicate analyses of a sample of a 5'-mononucleotide fortified infant formula spiked with known concentrations of corresponding nucleosides. Intermediate precision was determined from analyses of a sample of a

5′-mononucleotide fortified infant formula over different days. The method detection limits (MDL) were determined in accordance with US Environmental Protection Agency procedures.

A Youden ruggedness trial testing seven factors (amount of sample, pH of mobile phase, pH of standards, temperature of water, time standing, centrifuge speed, volume of acetic acid) with the potential to affect quantitative results showed that the pH of the standards was the most important parameter for control during sample preparation.

Method performance was further evaluated through analysis of a nucleotide supplemented milk-based nutritional powder by the described procedure and independent methods at two different laboratories. The respective results as mean (μg g^{-1}), relative standard deviation were: CMP 301, 6.7% (311, 3.8%); UMP 139, 5.6% (150, 3.1%), GMP 150, 8.8% (150, 15.7%) and AMP 112, 10.8% (107, 3.6%).

3.3. Method applications

The potential for dephosphorylation of exogenous 5′-mononucleotides during product manufacture was assessed. Milk that was exhaustively pasteurised showed a reduction in ALP levels well below the regulatory limit of 350 mU L^{-1}, with added UMP remaining intact and quantitatively recovered. However, sub-optimal pasteurization did not completely denature endogenous ALP, resulting in a quantitative conversion of UMP substrate to uridine. It was interesting to note that a very small proportion (< 1%) of UMP remained, consistent with levels in raw milk, despite the presence of residual ALP (Table 4).

A range of commercially available 5′-mononucleotide supplemented infant and follow-on formulas was tested, with the results summarised in Table 5. It is apparent that the majority of such products are manufactured at close to label claim.

Animal husbandry techniques in New Zealand differ drastically from those in other regions in the world, as cows graze year round almost exclusively on grass, with the exception of the winter months when the grass is supplemented with silage. Such practices allow natural changes in herd milk to be monitored over a season. This was accomplished in this study by analysing low heat skim milk powder manufactured across a production season (Table 6).

Figure 2 illustrates the analysis of early lactation milk samples for a single cow from 8 h pre-partum to 31 days post-partum, with the results also being summarized in Table 7. A significant peak eluted at 73 min, and its UV spectrum is shown, although its identity was not established by mass spectrometry. In order to assist identification of minor unknown peaks, a number of candidate compounds were investigated, including creatinine, niacinamide, pyridoxal, nucleobases and tri-nucleotides.

Human breast milk from three mothers was analysed for nucleotides and nucleosides and although a high variance between individuals was noted, all three samples shared an absence of purine nucleotides, inosine and orotic acid (Table 8).

4. Discussion

This method was primarily developed for the compliance testing of free nucleotides. Sample preparation was straightforward and protein precipitation was simply achieved by the addition of acid, as used by others (Ferreira et al., 2001; Indyk & Woollard, 2004; Perrin et al., 2001), in contrast to the more complex multi-enzyme treatment required for the determination of TPAN (Leach et al., 1995). With respect to chromatographic conditions, the Gemini column, a grafted polymer column designed to be stable over a large pH range, was also confirmed as being stable against phase collapse under 100% aqueous conditions. As a precautionary note, although it has been reported that adsorption of phosphorylated compounds may occur in stainless steel chromatographic systems under acidic conditions (Tuytten et al, 2006), no evidence of such losses was observed in this study.

In 2005, regulations that lowered the ALP limit for demonstrating acceptable HTST pasteurisation from 500 to 350 mUL^{-1} came into effect in the USA. It is expected that the statutory limit in Europe will soon be lowered to the same level (Micciche, 2005). However, as standard HTST pasteurisation treatment may not completely eliminate ALP activity, exogenous 5'-mononucleotides may be potentially vulnerable to dephosphorylation either by residual ALP or by ALP reactivated post-pasteurisation (Thorell et al., 1996).

In raw milk, the presence of trace levels of intact, endogenous 5'-mononucleotides requires explanation given the high levels of ALP activity. In an experiment to determine the influence of ALP on nucleotide retention, as reported presently, there was a complete loss of added UMP to raw milk, but partial retention of low, but measurable, endogenous levels. This suggests that such inherent nucleotides are protected as substrates from dephosphorylation, although by mechanisms that are currently poorly understood. In this respect, ALP is reportedly associated with microsomal particles, and is concentrated in the milk fat globule membrane (MFGM), where it may be segregated from potential nucleotide substrates until disruption of the MFGM upon homogenization and further processing (Shakeel-ur-Rehman et al., 2003).

The recoveries of nucleotides in commercial paediatric formulas tested presently generally complied with label claim, observations supporting both the validity of the analytical method and integrity of manufacture. However, sample K showed significant loss of all exogenous nucleotides and concurrent elevated levels of nucleosides above those normally expected in a milk-based product, demonstrating that dephosphorylation during manufacture had occurred. This result illustrates the nutritional and

regulatory importance of an analytical method that measures nucleosides as well as nucleotides in paediatric products.

Skim milk powder produced over a full season showed a general reduction in nucleotide and nucleoside levels as the season progressed. CMP was the most abundant nucleotide in early season milk powder, whereas trace levels only of the other nucleotides were detected, and no nucleotides were quantifiable by late summer. Cytidine and uridine were present in elevated concentrations in early season, but were expressed at constant levels by mid season.

In early bovine colostrum, the only nucleotides detected were GMP and UMP. Cytidine levels were low in precolostrum and increased to a maximum yield by the second day post-partum. Uridine and UMP were the most abundant nucleos(t)ides in early colostrum, consistent with previous reports (Gil & Sanchez-Medina, 1981; Gil & Uauy, 1995; Schlimme et al., 2000). Thus, uridine concentrations decreased from a maximum at 8 h pre-partum to trace levels by day 10. The observed trend of decreasing expression of nucleosides during lactation continued from a maximum during the early colostral phase and reached a constant level by the 3rd week post-partum, similar to observations reported elsewhere (Schlimme et al., 1997).

In mature cows' milk, CMP was the only nucleotide quantifiable, cytidine and uridine were at similar levels, and the purine nucleosides were not detected. The presence of CMP only has been reported (Ferreira et al., 2001), whereas others have detected AMP and CMP at similar levels (Gil & Sanchez-Medina, 1981) and uridine at levels higher than for other nucleosides (Schlimme et al., 1997).

In human milk, pyrimidine nucleotides only were detected; comparable levels of pyrimidine nucleotides as well as measured levels of purine nucleotides have been reported by others (Ferreira, 2003; Gil & Sanchez-Medina, 1982; Janas & Picciano, 1982; Sugawara et al., 1995; Thorell et al., 1996). Cytidine was the dominant nucleoside, with lesser amounts of uridine, adenosine and guanosine, and similar results have been summarised by Schlimme et al. (2000). The human diet is highly variable and is likely to have a significant bearing upon variability in nucleotide and nucleoside expression in the milk of human individuals. Further, geographical and seasonal variations of nucleotide and nucleoside levels in human milk have been reported (Sugawara et al., 1995).

The present comparison of mature human and bovine milk indicates a similar qualitative profile. The dominance of cytidine, uridine and CMP in the milk of both species and lower quantities of both nucleotides and nucleosides in bovine milk agree well with previously published results (Barness, 1994; Sugawara et al., 1995).

The described chromatographic protocol also allows for the quantification of orotic acid, an intermediate in the de novo synthesis of pyrimidines, and uric acid, an end product of purine metabolism. Seasonal variation in milk powder and early lactation levels of orotic and uric acids were found to be comparable with those reported by Indyk and Woollard (2004) and the absence of orotic acid in human milk has been reported also by others (Ferreira, Gomes, & Ferreira, 1998; Indyk & Woollard, 2004; Janas & Picciano, 1982; Larson & Hegarty, 1979; Thorell et al., 1996). Indeed, orotate is the major nucleospecies in bovine milk, and is apparently poorly salvageable by human infants (Barness, 1994).

5. Conclusions

With the increasing trend towards humanising bovine milk-based infant formulas and the mandatory requirement to comply with regulatory guidelines, the ability to reliably and routinely quantify both nucleotides and nucleosides simultaneously is becoming strategically important. The method described offers this facility with a simple gradient reversed-phase technique without requiring commonly used ion-pair reagents, thereby avoiding the inherent disadvantages of diminished column lifetime and long equilibration times.

The applicability of the method has been demonstrated for intact milk protein infant formulas, skim milk powders and human milk.

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

Nucleotide[a]	λ_{max} (nm)	E1%
AMP	257	430.4
CMP	280	398.0
GMP	254	393.3
IMP	249	357.3
UMP	262	313.5

[a] AMP = adenosine 5′-monophosphate, CMP = cytidine 5′-monophosphate, GMP = guanosine 5′-monophosphate, IMP = inosine 5′-monophosphate, UMP = uridine 5′-monophosphate.

Table 2. Gradient procedure

Time (min)	Flowrate (mL min^{-1})	Phase Composition	
		%A	%B
0	0.5	100	0
35	0.5	100	0
65	0.5	0	100
90	0.5	0	100
95	0.5	100	0
130	0.5	100	0

Table 3. Method performance parameters

Analyte[a]	Range (μg mL^{-1})	Linear regression	r^2	MDL (mg hg^{-1})[b]	RSDr (%)[c]	RSDiR (%)[d]	Recovery (%)[e]
AMP	0.13–62.7	y = 221439x-92221	0.9992	0.16	4.22	6.77	97.5 (1.3)
CMP	0.15–74.7	y = 155708x-60787	0.9994	0.53	3.50	3.51	103.5 (5.6)
GMP	0.11–53.4	y = 195055x-70658	0.9991	0.05	5.47	5.49	95.5 (1.3)
IMP	0.10–50.3	y = 206933x-63652	0.9993	0.10	3.42	5.40	99.0 (1.2)
UMP	0.09–43.5	y = 173977x-40626	0.9994	0.17	3.66	8.47	100.5 (2.5)
Adenosine	0.16–80.0	y = 301728x-122321	0.9994	0.08	6.38		105.0 (2.7)
Cytidine	0.18–90.7	y = 201324x-92407	0.9994	0.29	5.74		102.9 (2.7)
Guanosine	0.16–82.1	y = 210766x-85202	0.9994	0.17	5.79		102.1 (3.9)
Inosine	0.16–79.8	y = 241890x-90296	0.9992	0.47	6.08		105.2 (2.9)
Uridine	0.15–76.8	y = 178262x-69800	0.9994	0.68	4.39		103.7 (3.4)

[a] AMP = adenosine 5′-monophosphate, CMP = cytidine 5′-monophosphate, GMP = guanosine 5′-monophosphate, IMP = inosine 5′-monophosphate, UMP = uridine 5′-monophosphate

[b] Determined from n replicates at or near the expected detection limit, MDL = $t_{(n-1,\ 1-a)} \times$ sd, where n = 7 and α = 0:05

c RSD repeatability = sd/mean × 100

d RSD intermediate reproducibility = sd/mean × 100

e Mean recovery (standard deviation) at three concentration levels tested in triplicate

Table 4. Uridine 5′-monophosphate recovery associated with residual alkaline phosphatase activity

Sample	Alkaline phosphatase (mU L^{-1})	Endogenous UMP[a] (mg mL^{-1})	Retained UMP[b] (mg mL^{-1})	Recovery[c] (%)
Raw milk	800000	0.43	0.19	-0.8
Milk pasteurized 68 °C/15 s	9310	0.18	0.15	-0.1
Milk pasteurized 82 °C/15 s	9	0.18	31.92	100.1

[a] UMP = uridine 5′-monophosphate

[b] Each 5 mL sample was spiked with 0.5 mL of a 317 mg mL^{-1} UMP standard solution

[c] Recovery (%) = (retained−endogenous)/spike × 100

Table 5. Nucleotide recoveries[a] as percentage of label claim in a range of infant formulas and follow-on formulas

Sample[b]	AMP[c]	CMP	GMP)	IMP	UMP	Total Nucleotides
A	107.7	120.6	90.7	108.1	110.1	114.2
B	107.9	102.2	93.3	102.3	112.2	104.1
C	109.0	125.6	91.5	107.2	109.6	117.0
D			No label claim[d]			113.3
E			No label claim			114.4
F	52.0	133.3	57.8	75.0	128.2	95.8
G			No label claim			194.5
H			No label claim			97.4
I	99.6	96.1	88.6	101.5	107.7	98.4
J	101.1	100.1	94.6	108.0	114.8	102.9
K	0	29.3	0	0	0	10.7

[a] Recovery (%) = (Measured/Label Claim) × 100

[b] Samples A–H were follow-on formulas and samples I–K were infant formulas

[c] AMP = adenosine 5′-monophosphate, CMP = cytidine 5′-monophosphate, GMP = guanosine 5′-monophosphate, IMP = inosine 5′-monophosphate, UMP = uridine 5′-monophosphate

[d] Samples D, E, G and H declared total nucleotide levels only

Table 6. Seasonal analysis of nucleotides and nucleosides in skim milk powder (µmol hg^{-1})[a]

Month	AMP[a,b]	CMP	UMP	Adenosine	Cytidine	Guanosine	Inosine	Uridine	Orotic	Uric
August	0.3	14.1	0.5	0.4	33.0	2.4	ndc	104.8	158.1	101.1
September	0.3	13.8	0.4	0.2	21.2	3.4	4.5	71.4	177.8	107.8
October	0.3	9.3	0.6	0.3	12.0	nd	nd	27.4	219.0	96.7
November	0.3	6.3	0.4	0.3	10.7	nd	nd	20.5	227.1	87.3
December	nd	3.9	0.6	nd	13.1	nd	2.9	19.0	259.1	82.0
January	nd	4.4	nd	0.3	4.4	nd	nd	10.1	265.8	79.7
February	nd	3.3	nd	nd	5.3	nd	1.7	9.2	230.5	63.9
March	nd	nd	nd	nd	9.6	nd	2.3	11.7	250.4	66.0
April	nd	nd	nd	nd	8.8	5.0	3.0	12.2	214.2	56.1
May	nd	nd	nd	nd	10.0	4.8	2.8	19.2	213.0	60.6

[a] AMP = adenosine 5′-monophosphate, CMP = cytidine 5′-monophosphate, UMP = uridine 5′-monophosphate

[b] Measurable levels of guanosine 5′-monophosphate and inosine 5′-monophosphate were not detected

[c] nd = not detected.

Table 7. Early lactation analysis of nucleotides and nucleosides in the milk of a single cow (µmol dL^{-1})

Day	CMP[a,b]	GMP	UMP	Cytidine	Guanosine	Inosine	Uridine	Orotic	Uric
-0.3	nd[c]	0.3	13.3	1.8	0.9	0.3	102.4	nd	10.8
0	nd	0.4	8.0	4.6	1.2	0.3	95.9	nd	11.3
0.25	nd	nd	0.3	9.7	0.4	0.6	38.1	nd	14.3
0.5	nd	nd	nd	6.5	0.4	0.7	6.9	nd	11.5
1	nd	nd	nd	11.0	0.5	0.9	4.8	0.9	8.1
2	1.1	nd	nd	12.5	0.6	1.1	6.4	4.1	9.3
5	1.9	nd	nd	6.0	0.3	1.2	9.2	8.5	9.2
10	1.4	nd	nd	3.9	0.3	1.3	3.7	9.2	9.8
16	0.2	nd	nd	0.4	nd	nd	nd	8.9	7.3
21	0.3	nd	nd	0,5	nd	nd	nd	9.2	4.1
31	0.2	nd	nd	1.5	nd	nd	1.4	9.3	6.2

[a] CMP = cytidine 5'-monophosphate, GMP = guanosine 5'-monophosphate, UMP = uridine 5'-monophosphate

[b] Measurable levels of adenosine 5'-monophosphate, inosine 5'-monophosphate and adenosine were not detected.

[c] nd = not detected

Table 8. Mean values of human breast milk samples (µmol dL^{-1})

Day	AMP[a]	CMP	GMP	IMP	UMP	Adenosine	Cytidine	Guanosine	Inosine	Uridine
Mean (n = 3)	0	1.3	0	0	0.4	1.0	7.9	0.8	0	3.7
Min	0	0	0	0	0.2	0.4	6.3	0.5	0	3.1
Max	0	1.9	0	0	0.5	2.0	9.3	1.0	0	4.9
SD	0	1.1	0	0	0.2	0.9	1.5	0.3	0	1.0
RSD (%)	–	86.7	–	–	43.3	93.3	19.1	37.6	–	26.4

[a] AMP = adenosine 5′-monophosphate, CMP = cytidine 5′-monophosphate, GMP = guanosine 5′-monophosphate, IMP = inosine 5′-monophosphate, UMP = uridine 5′-monophosphate

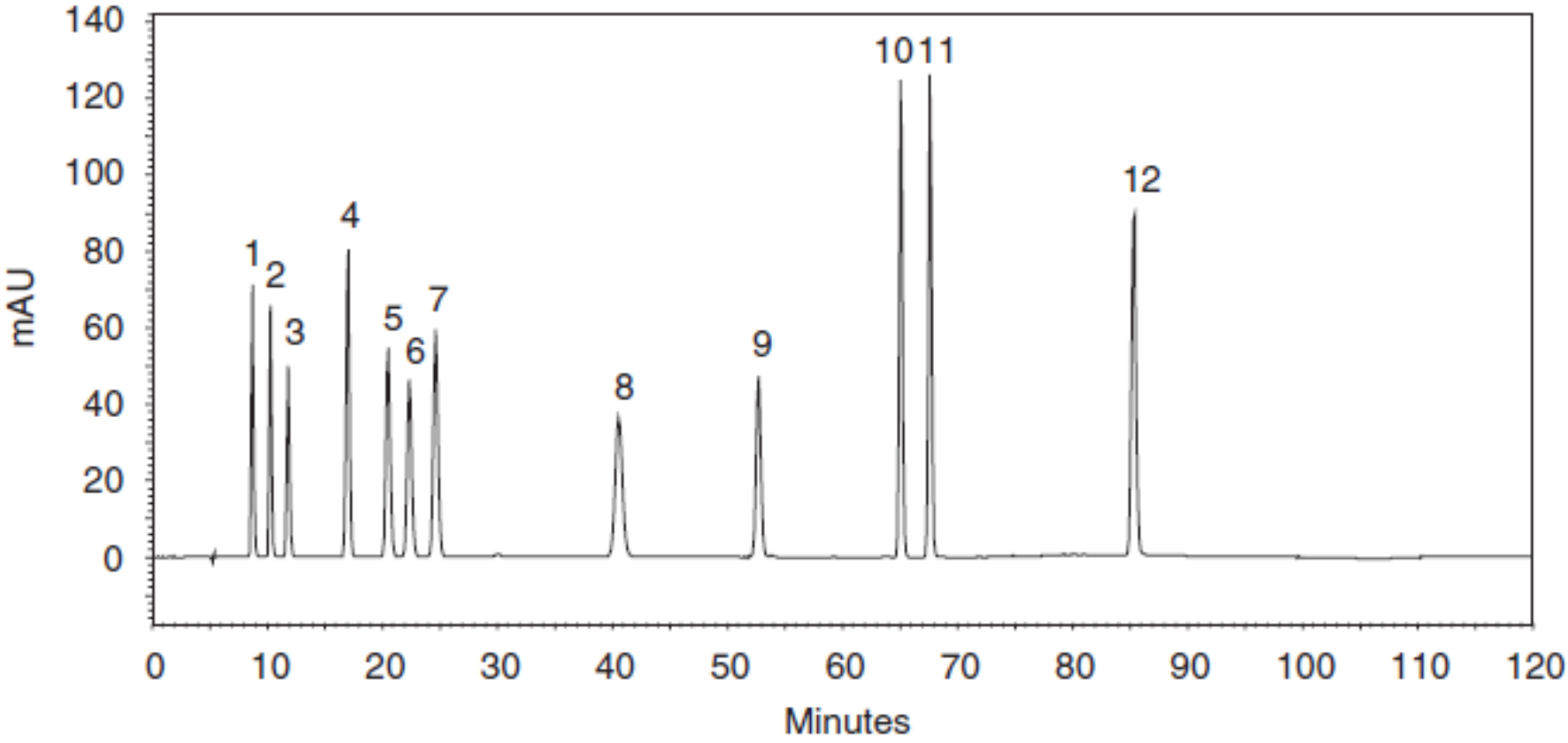

Figure 1. Chromatography of a standard mixture of: (1) 27.8 mM cytidine 5′-monophosphate, (2) 61.9 mM orotic acid, (3) 20.0 mM uridine 5′-monophosphate, (4) 59.7 mM uric acid, (5) 17.9 mM guanosine 5′-monophosphate, (6) 19.4 mM inosine 5′-monophosphate, (7) 44.8 mM cytidine, (8) 37.8 mM uridine, (9) 20.4 mM adenosine 5′-monophosphate, (10) 35.7 mM inosine, (11) 34.8 mM guanosine, (12) 35.9 mM adenosine. Conditions: mobile phase A: KH_2PO_4 (0.1 M), pH = 5.6; mobile phase B: KH2PO4 (0.1M), 15% methanol, pH = 5.6; gradient elution: flowrate 0.5 mL min^{-1} throughout, 0–30 min (100% A, 0%B), 65–90 min (0%A, 100%B), 95–130 min (100%A, 0%B). UV detection 260 nm

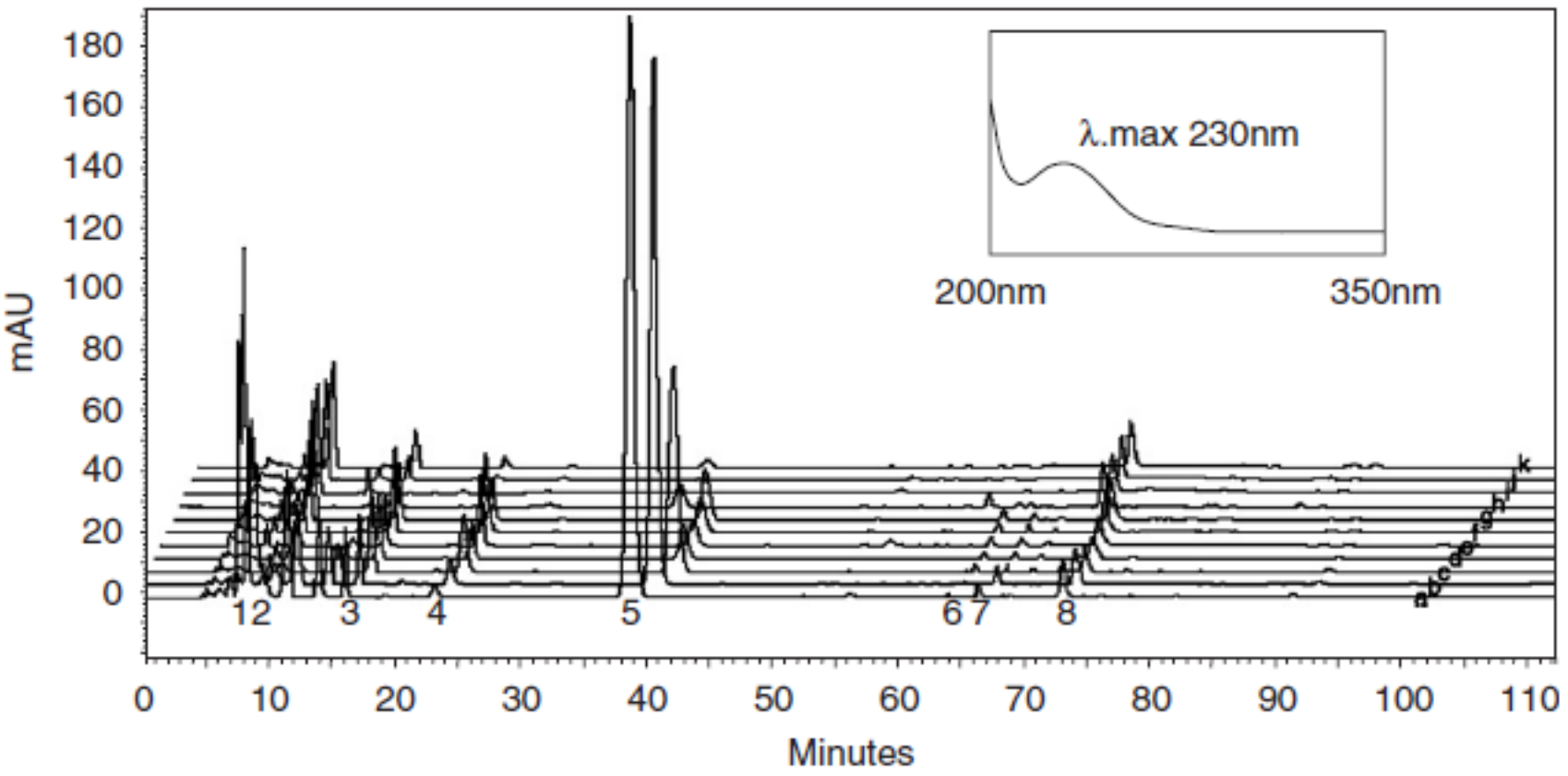

Figure 2. Chromatography of early lactation milk of a single cow: (a–k) 0.3, 0, 0.25, 0.5, 1, 2, 5, 10, 16, 21, 31 days post-partum respectively. (1) cytidine 5'-monophosphate, (2) orotic acid, (3) uric acid, (4) cytidine, (5) uridine, (6) inosine, (7) guanosine, (8) unknown peak (UV spectrum shown in inset). Conditions: mobile phase A: KH_2PO_4 (0.1 M), pH = 5.6; mobile phase B: KH_2PO_4 (0.1 M), 15% methanol, pH = 5.6; gradient elution: flow rate 0.5 mL min^{-1} throughout, 0–30 min (100%A, 0%B), 65–90 min (0%A, 100%B), 95–130 min (100%A, 0%B). UV detection 260 nm