Determination of Total Potentially Available Nucleosides in Bovine Milk

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Abstract

Bovine colostrum and milk samples were collected from two herds over the course of the first month post-partum, pooled for each herd by stage of lactation and total potentially available nucleosides were determined. Sample analysis consisted of parallel enzymatic treatments, phenylboronate clean-up, and liquid chromatography to quantify contributions of nucleosides, monomeric nucleotides, nucleotide adducts, and polymeric nucleotides to the available nucleosides pool. Bovine colostrum contained high levels of nucleosides and monomeric nucleotides, which rapidly decreased as lactation progressed into transitional milk. Mature milk was relatively consistent in nucleoside and monomeric nucleotide concentrations from approximately the tenth day post-partum. Differences in concentrations between summer-milk and winter-milk herds were largely attributable to variability in uridine and monomeric nucleotide concentrations.

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1. Introduction

Nucleosides are low molecular weight compounds consisting of a purine or pyrimidine base (e.g., adenine, cytosine, guanine and uridine) attached via a β-glycosidic linkage to a ribose sugar (ribonucleosides). Nucleotides are *o*-phosphoric acid esters of nucleosides containing one to three phosphate groups on C-2, C-3 or most commonly C-5 of the ribose (ribonucleotides).

Nucleotides are compounds of critical importance to cellular function. They operate as precursors to nucleic acids, as mediators of chemical energy transfer and cell signalling, and as integral components of coenzymes in the metabolism of carbohydrates, lipids and proteins (Carver & Walker, 1995; Cosgrove, 1998).

Nucleotides can be synthesised *de novo* or recovered via salvage pathways and thus are not essential dietary nutrients. However, during periods of rapid growth or after injury, when the metabolic demand for nucleotides exceeds the combined capacity of de novo synthesis and the salvage pathway, dietary sources of nucleotides are considered to be conditionally essential for continued optimal metabolic function (Carver & Walker, 1995; Yu, 1998). Dietary nucleotides are ingested in the form of nucleoproteins, polymeric nucleotides (nucleic acids) and nucleotide adducts as well as free nucleotides. These are digested in the gastrointestinal tract by proteases, nucleases, phosphatases and nucleotidases, and are available for absorption predominantly as nucleosides (Quan, Barness, & Uauy, 1990; Uauy, Quan, & Gil, 1994).

Dietary nucleotides have been shown to increase immune response in infants (Carver, Pimentel, Cox, & Barness, 1991; Pickering et al., 1998), to influence metabolism of long chain fatty acids and to enhance gastrointestinal tract repair after damage, when compared with nucleotide-unsupplemented diets (Carver & Walker, 1995; Gil, Corral, Martínez, & Molina, 1986). Dietary supplementation of infant formula with nucleotides has also been reported to beneficially modify the composition of intestinal microflora (Uauy et al., 1994), to elevate serum immunoglobulin concentrations and to reduce incidences of diarrhoea (Yau et al., 2003).

The expression of nucleosides and nucleotides in bovine milk is highest immediately after parturition with a general decreasing trend in concentration with advancing lactation, with levels stabilizing by the third week of lactation (Gill & Indyk, 2007b; Gil & Sánchez-Medina, 1981; Schlimme, Martin, & Meisel, 2000; Sugawara, Sato, Nakano, Idota, & Nakajima, 1995). This pattern of high concentration in early colostrum followed by a rapid reduction as lactation progresses is analogous to changes of other bioactive components, such as immunoglobulins.

In general, the dominant strategy employed in analysis of free nucleosides and nucleotides in colostrum and milk has been protein removal by acid precipitation, followed by HPLC-UV analysis of

the crude or fractionated extract (Ferreira, Mendes, Gomes, Faria, & Ferreira, 2001; Gill & Indyk, 2007a, 2007b; Sugawara et al., 1995).

Early clinical studies employed infant formulas containing nucleotides supplemented to levels based on estimates of the free nucleotide content of human milk (Aggett, Leach, Rueda, & MacLean, 2003). However, the measurement of free nucleotide levels does not account for nucleosides, polymeric nucleotides or nucleotide adducts that are also nutritionally available to the infant. In order to determine the total potentially available nucleosides (TPAN), an analytical protocol to characterise the contributions of different molecular nucleoside sources to infant nutrition was developed (Leach, Baxter, Molitor, Ramstack, & Masor, 1995). The development of this protocol has been an important contribution to further understanding the distribution of nucleosides and nucleotides and their implications for infant nutrition. The analytical method uses a number of enzymatic treatments incorporating combinations of nuclease, pyrophosphatase and phosphatase enzymes into the sample preparation. In this manner, contributions from nucleoside precursors to TPAN in human milk have been estimated, and it was reported that the nutritionally relevant concentrations of nucleosides and nucleotides in human milk had been underestimated by approximately 50% when compared with free nucleotide concentrations only (Gerichhausen, Aeschlimann, Baumann, Inäbnit, & Infanger, 2000; Leach et al., 1995; Tressler et al., 2003).

Bovine milk is almost exclusively used in the manufacture of infant formula intended to substitute for human breast milk, and since the levels of TPAN in bovine milk have not been previously reported, the purpose of the current study was to evaluate bovine milk TPAN levels and variation over the first month of lactation.

2. Materials and methods

2.1. Apparatus

The high-performance liquid chromatography (HPLC) system consisted of an 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 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 was a Prodigy C_{18} column, 5 µm, 4.6 x 150 mm (Phenomenex, Torrance, CA, USA). Prior to use, mobile phases were filtered and degassed using a filtration apparatus with 0.45 µm nylon filter membranes (AllTech, Deerfield, IL, USA). Solid phase extraction of nucleosides was performed using Affi-gel 601 (Bio-Rad, Hercules, CA, USA).

2.2. Reagents

Adenosine, cytidine, guanosine, uridine, 5-methylcytidine, uridine 5′-diphosphoglucose, RNA, cytidine 5′-diphosphocholine, β-nicotinamide adenine dinucleotide, adenosine 5′-monophosphate (AMP), cytidine 5′-monophosphate (CMP), guanosine 5′-monophosphate (GMP), uridine 5′-monophosphate (UMP) nuclease P1, pyrophosphatase, and alkaline phosphatase were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Potassium dihydrogen phosphate, orthophosphoric acid, hydrochloric acid, sodium hydroxide and potassium hydroxide were supplied by Merck (Darmstadt, Germany). Water was purified with resistivity > 18 MΩ using an E-pure water system (Barnstead, IA, USA).

2.3. Sample collection

Milk and colostrum samples were collected from seven cows from each of two Jersey herds from two separate farms in the eastern Waikato region of New Zealand. Samples from a winter-milk herd were collected over a 1 month period in late March 2008 and samples from a summer-milk herd were collected over a 1 month period in early August 2009. Cows selected for inclusion in this study were in general good health, in their second or subsequent calving and had experienced normal calvings without complications. With the exception of the 6 h sample, sample collection was performed between 6:00 and 10:00 am, which coincided with regular morning milking times.

From each cow, approximately 80 mL of sample was collected in a 120 mL disposable container. These samples were collected at various time intervals throughout the first month of lactation, with a frequency that reduced as the month progressed.

Collected samples were refrigerated at 4 °C, picked up from the farm as soon as practicable (within 6 h), taken to the laboratory and immediately prepared for storage. NaOH (1 M, 20 mL) was added to a 10 mL sample aliquot and mixed, and the sample was then left to stand for 30 min, neutralised to pH = 7.35 ± 0.05 with HCl and made to 50 mL volume before freezing at < 15 °C.

2.4. Sample analysis

Samples from the seven cows at each time period post-partum were pooled for analysis, and enzymatic hydrolysis and boronate affinity extraction were performed as described by Leach et al. (1995). Each pooled sample was tested in duplicate with the mean and standard deviation calculated.

Samples were enzymatically hydrolysed using nucleotide pyrophosphatase, nuclease P1 and bacterial alkaline phosphatase (Sigma Chemical Co., St. Louis, MO, USA). Each pooled sample was split into four 5 mL sub-samples, to each of which internal standard (10 mg, 5-methylcytidine) was added, and each sub-sample was subjected to a different enzymatic treatment. The first treatment had no added

enzymes and innate nucleosides only were therefore measured. The second treatment involved phosphatase (pH = 8.5, 3 h), which dephosphorylated monomeric nucleotides to nucleosides. The third treatment incorporated nuclease (pH = 5.1, 16 h) and phosphatase (pH = 8.5, 3 h), which hydrolysed polymeric nucleotides to monomeric nucleotides, which were subsequently dephosphorylated to nucleosides. The fourth treatment consisted of nuclease (pH = 5.1, 16 h), pyrophosphatase and phosphatase (pH = 8.5, 3 h), which converted all nucleoside precursors (polymeric and monomeric nucleotides, and nucleotide adducts) to free nucleosides.

Clean-up of enzymatic extracts was achieved by solid phase extraction using a phenylboronate affinity gel as described by Leach et al. (1995), whereby nucleosides were covalently bonded to the gel at high pH, and interferences removed with two washings of high pH buffer. The nucleosides were eluted from the affinity gel at low pH by the addition of phosphoric acid (0.25 M) and filtered ready for analysis (Liu & Scouten, 2000).

2.5. Chromatographic analysis

The initial chromatographic protocol was a modification of a reversed-phase system described by Gill and Indyk (2007b), using phosphate buffer and a methanol gradient. As optimum separation of nucleosides was achieved at pH = 4.8, phosphate was replaced with acetate (pKa = 4.75), thereby offering greater buffer capacity at the desired pH.

An organic solvent component is required in the mobile phase to facilitate elution of nucleosides from the C_{18} column. However, to obtain sufficient resolution between peaks, a gradient elution procedure was necessary. A number of gradient procedures were evaluated to determine an optimum protocol that had a relatively short run-time coupled with sufficient resolution between peaks. An optimum separation of nucleosides was achieved at a flow rate of 0.7 mL min^{-1} with gradients formed by low pressure mixing of two mobile phases, A (0.05 M sodium acetate, pH = 4.8) and B (100% methanol) (0–3 min, 95:5, v/v, A:B; 7–22 min 75:25, v/v, A:B; 23–30 min 95:5, v/v, A:B).

The photodiode array detector acquired spectral data between 210 and 300 nm. Peak identification was by co-chromatography and similarity of the chromatographic peak spectrum to authentic standards, as estimated by a similarity index of > 0.99. Chromatograms were integrated at a wavelength of 260 nm and results were determined by an internal standard technique using 5-methylcytidine.

The contributions of the different forms (nucleosides, nucleotide adducts, monomeric and polymeric nucleotides) to TPAN were calculated in the manner described by Leach et al. (1995) using Excel spreadsheet software (Microsoft, Redmond, WA, USA).

2.6. Recovery

A spiked recovery study was performed on free nucleosides and was assessed through the affinity gel sample clean-up. A stored pooled milk sample was spiked with a single mixed standard containing cytidine, guanosine, uridine, adenosine and 5-methylcytidine (95.0–135.0 μg mL^{-1}). Recovery was assessed by comparison of peak areas for the spiked and unspiked samples, relative to those of the mixed standard.

Recovery of nucleosides from the enzymatic digestion was estimated following the protocol described by Leach et al. (1995). A solution (TPAN-fortified) containing ribonucleosides, 5'-mononucleotides, nucleotide adducts and RNA was prepared for a spiked recovery study. A solution (TPAN-digest) was made from an aliquot (5 mL) of the TPAN-fortified solution that was hydrolysed for 20 h with KOH (0.2 mol L^{-1}, 50 mL) to convert polymeric RNA to monomeric nucleotides. The pH of the solution was adjusted to 9.0 with HCl and then incubated with alkaline phosphatase and nucleotide pyrophosphatase to convert adducts and monomeric nucleotides to nucleosides. The concentration of nucleosides in the TPAN-digest solution was determined by HPLC and was used to calculate the TPAN content in the TPAN-fortified solution.

A stored pooled milk sample was then spiked (in triplicate) with an aliquot of the TPAN-fortified solution and, along with unspiked sample replicates, was analysed and TPAN concentrations determined. Recovery was assessed by comparison of the difference in results for the spiked and unspiked samples, divided by the TPAN concentration of the TPAN-fortified solution.

2.7. Statistical analysis

The experimental data were analysed by one-way analysis of variance (ANOVA) of the response of season (winter-milk, summer-milk) with covariate time (0, 0.25, 1, 2, 3, 5, 10, 20, 30 days postpartum). All results (X) were transformed $\log_{10}(1 + X)$, so that the postulated model was an exponential decrease in levels with time, with the initial levels and the rates of decrease dependent upon season. The "exponential decay" model was found to provide a better fit than a linear or quadratic model in time. For hypothesis testing, significance was evaluated at the $P < 0.05$ level. Statistical analyses were performed using Minitab version 15.1 (State College, PA, USA).

3. Results and discussion

3.1. Recovery

The recoveries of nucleosides (recovery ± standard deviation) through the affinity gel clean-up were as follows: cytidine (93.4 ± 1.1%), uridine (92.3 ± 5.1%), guanosine (88.3 ± 4.9%), adenosine

(95.2 ± 4.2%), and 5-methylcytidine (92.6 ± 2.3%). Recoveries measured through the enzymatic digestion and subsequent affinity gel clean-up were cytidine (95.5 ± 2.8%), uridine (101.7 ± 3.7%), guanosine (89.2 ± 2.4%) and adenosine (94.7 ± 3.0%). These recovery values were acceptable for the quantitative analysis of nucleosides at concentrations typical of bovine milk samples (AOAC, 2002).

3.2. Chromatography

Chromatographic performance evaluated as resolution, peak tailing, retention factor, and peak area repeatability, was deemed acceptable by replicate analyses (n = 6) of a mixed nucleotide standard (Figure 1A). The specificity of the phenylboronate sample clean-up provides analytical chromatography relatively free of interferences (Figure 1B).

3.3. Total potentially available nucleosides in bovine milk

The TPAN concentrations and contribution of each nucleobase and form obtained in this study of winter-milk and summer-milk lactation series are summarised in Tables 1 and 2 and illustrated graphically in Figures 2 and 3. For each parameter (each base within each form), comparisons of the initial levels and rates of decrease were made between seasons and whether each seasonal slope differed from zero (Table 3).

3.3.1. Nucleoside contribution to total potentially available nucleosides

Uridine was the most prevalent nucleoside, at levels of ~50 μmol dL^{-1} in colostrum, but these levels were not sustained beyond the third day post-partum and rapidly decreased to levels similar to those of cytidine and guanosine, at 1–3 μmol dL^{-1}. Adenosine was present at much lower levels but these low levels were maintained throughout the lactation period for both seasons milk.

The nucleoside levels measured in this study were consistent with those reported previously (Gill & Indyk, 2007b). Although nucleosides were present at higher concentrations in bovine colostrum than in mature bovine milk, they rapidly decreased to levels similar to that in mature human milk, as reported by Leach et al. (1995).

3.3.2. Monomeric nucleotide contribution to TPAN

Levels of nucleotides measured in this study were generally higher than those reported previously (Gill & Indyk, 2007b); however, there was likely to have been a significant contribution from multiple phosphorylated forms (cyclic-, mono-, di- and triphosphorylated nucleotides), which the TPAN analytical method aggregates as a single value. Differences in colostral monomeric nucleotide levels between the herds were evident, with the winter-milk herd initially containing 5–10 times the levels of the summer-milk herd. However, by the fifth day, nucleotide levels decreased to approximately 15 μmol dL^{-1} in both herds, somewhat lower than those reported in human milk (Leach et al., 1995).

The high initial uridine nucleotides levels and subsequent rapid decrease in concentration seen in winter-milk was absent in summer-milk which maintained constant levels throughout lactation. Cytidine and adenosine nucleotides are stable throughout lactation for both seasons. The most abundant nucleotides in bovine colostrum were based on uridine; however, as colostrum transitioned into mature milk, cytidine nucleotides became the dominant form.

Uridine nucleotides are critical components in the biosynthesis of lactose. As lactose is a major osmotic component of milk, there is a correlation between the amount of lactose and the volume of milk produced (Arthur, Kent, & Hartmann, 1991; Linzell & Peaker, 1971). It has been suggested that high levels of uridine and UMP are present in milk, as breakdown products of uridine diphosphate (UDP) and uridine triphosphate (UTP), due to their function in the synthesis of lactose (Mateo, Peters, & Stein, 2004; Schlimme et al., 2000). It has been proposed that support for this hypothesis is seen by the correlation of decreasing total milk solids and 5'-UMP concentrations in sow's milk as lactation progresses (Mateo et al., 2004). However, as colostrum contains higher total milk solids and lower lactose levels (on a dry weight basis) than mature milk (Heng, 1999), a reduced proportion of uridine nucleotides than in mature milk might be expected based on this proposal. Alternative reasons must therefore be sought to account for the higher relative proportions of uridine nucleotides in colostrum. It has also been suggested that uridine accounts for many of the immunological properties of nucleotides in colostrum (Kulkarni, Fanslow, Rudolph, & Van Buren, 1986; Leach et al., 1995; Van Buren, Kulkarni, Fanslow, & Rudolph, 1985) and, more recently, Mashiko et al. (2009) demonstrated that dietary UMP affected the immune response of newborn calves.

3.3.3. Nucleotide adduct contribution to TPAN

The results for uridine adducts in the present study ranged from not detected to 23.7 μmol dL^{-1} in the winter-milk herd and from not detected to 6.8 μmol dL^{-1} in the summer-milk herd, with a rapid reduction in concentration after the third day post-partum. Guanosine adducts measured ranged from not detected to 3.9 μmol dL^{-1} in the winter-milk herd and from not detected to 1.2 μmol dL^{-1} in the summer-milk herd. Similar levels of adenosine adducts were found, presumably derived from flavin adenine dinucleotide and nicotinamide adenine dinucleotide (Fox & McSweeney, 1998; Kanno, Shirahuji, & Hoshi, 1991). Utilising enzymatic techniques, Gil and Sánchez-Medina (1981) measured UDP hexosamine, UDP hexose and UDP galactose concentrations in bovine colostrum and milk, which ranged from not detected to ~104 μmol dL^{-1}. Levels were highest at 27 and 78 h and much lower or absent in subsequent stages of lactation. Guanosine diphosphate fucose was also reported at 27 and 78 h, at levels of 6.7 and 4.1 μmol dL^{-1}, respectively.

3.3.4. Polymeric nucleotide contribution to TPAN

The concentration of polymeric nucleotides in bovine colostrum was similar to that in human colostrum and milk, however, with advancing lactation, the levels in bovine milk decreased below those in human milk. Both cytidine and uridine contributions to polymeric nucleotides are steady throughout lactation for summer-milk, whereas the higher initial levels of polymeric uridine shows distinct decrease in concentration as lactation progresses in winter-milk.

3.3.5. Nucleobase contribution to TPAN

Differences in the contributions of each nucleobase from the various nucleoside and nucleotide forms were found. The pyrimidines differed markedly from each other through lactation. Whereas the quantities of cytidine and cytidine nucleotides were relatively constant throughout, uridine and uridine nucleotides levels varied considerably. Cytidine concentrations were similar to those in human milk reported by Leach et al. (1995), whereas uridine was present at considerably higher levels in bovine colostrum and in lower amounts in mature bovine milk.

The concentrations of the purines also differed with adenosine levels throughout the first month of lactation for milk from both herds, whereas guanosine showed a significant decrease in levels for both herds. The quantities of both guanosine and adenosine, and their respective nucleotides were slightly higher in bovine colostrum than in human colostrum and milk, but concentrations were lower as colostrum transitioned to mature milk. In bovine milk, purine nucleosides and nucleotides made a relatively small contribution to TPAN (6–20%), whereas human milk purine nucleosides and nucleotides consistently represent a greater proportion of TPAN (> 30%).

3.3.6. Total potentially available nucleosides

In general, the absolute concentrations indicated a distinct difference between the two herds, although the general trends were the same. Winter had higher initial levels of TPAN but the rate of decrease was greater, such that the seasonal differences in TPAN concentration found in colostrum were largely absent in mature milk.

TPAN levels in winter-milk colostrum were attributable largely to significantly higher amounts of uridine nucleotides compared with summer-milk colostrum; however, by the tenth day, milk from both herds showed similar TPAN levels. The TPAN levels in bovine colostrum were higher than those in both human colostrum and milk, however, after transition to mature milk, the TPAN levels were lower than those reported in human milk (Leach et al., 1995).

It has been reported that nucleotides in human milk exhibit a circadian rhythmicity (Sánchez et al., 2009). Anomalous results for uridine and uridine nucleotides were found in bovine colostrum samples collected from both herds at 6 h post-partum, and such diurnal variation may suggest a plausible rationale given that this sample was uniquely collected in the afternoon.

The levels and distribution of TPAN in mature bovine milk are important in the manufacture of infant formulas, particularly when formulating to TPAN regulatory limits. If all endogenous forms of nucleosides and nucleotides that contribute to TPAN are not accounted for prior to nucleotide supplementation, possible over fortification could occur during the manufacture of bovine milk-based infant formula.

3.4. Herd conditions

Although the feeding practices were similar on both farms, it is possible that seasonal or pasture differences could have had a significant effect on the nucleoside precursors expressed in the milk of each herd. Prior to calving, the cows' diet was extensive grass grazing supplemented with maize silage and palm kernel, and after calving, intake of grass and palm kernel increased with inclusion of whey permeate. One uncontrolled variable that may have had a profound influence is the climate. Calving for the winter-milk herd began in the early autumn of 2008, which followed a summer characterised by a La Niña weather pattern that contributed to record high temperatures and a drought with severe soil moisture deficits in the Waikato region of New Zealand. The summer-milk herd began calving in late winter 2009, which had the warmest August on record, although rainfall was normal (National Institute of Water and Atmospheric Research [NIWA], 2010). In addition to obvious climatic factors, other factors could have affected TPAN levels in both herds, such as the conditions under which the cows were raised and fed, tolerance to stress, sunlight exposure and other environmental factors. Further study controlling each of these factors would be required to identify those factors that influence nucleoside and nucleotide expression in milk. Limitations of the current study could be expanded upon in future experiments that consider the effects of breed, location and diet on TPAN expression in milk.

4. Conclusions

Nucleosides and monomeric nucleotides were the dominant forms of TPAN in bovine milk and colostrum, whereas nucleotide adducts and polymeric nucleotides contributed relatively little. Uridine and uridine nucleotides were the major contributor to TPAN in early colostrum, and cytidine and cytidine nucleotides dominated later in lactation. Differences in TPAN concentrations between summer-milk and winter-milk herds were largely attributable to variability in uridine and nucleotide concentrations. As lactation progressed, TPAN concentration decreased, as did each of the contributing forms.

With the increasing trend towards nucleotide supplementation of bovine milk-based infant formulas, and the need for compliance with TPAN regulatory limits, the data presented in this study provide a greater understanding of the contributions of endogenous nucleosides and nucleotides in bovine milk.

In addition, colostrum is increasingly being used as a dietary supplement and the high level of TPAN present may be nutritionally significant.

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Table 1. Nucleosides and nucleotides in bovine milk from a winter-milk herd (µmol dL^{-1})[a]

Day[a]	Form	Cytidine	Uridine	Guanosine	Adenosine	Total
0	Nucleoside	5.4 ± 0.1[b]	57.9 ± 1.6	0.3 ± 0.0	nd	63.6 ± 1.5
	Monomeric NT	6.1 ± 0.3	143.7 ± 8.5	2.8 ± 0.0	2.9 ± 0.2	155.5 ± 8.7
	NT Adduct	0.9 ± 0.2	23.7 ± 9.0	3.9 ± 0.8	2.4 ± 0.0	30.9 ± 9.6
	Polymeric NT	0.6 ± 0.0	5.4 ± 7.2	1.4 ± 0.2	1.4 ± 0.1	8.7 ± 7.4
	Total Base	13.0 ± 0.5	230.7 ± 6.1	8.5 ± 0.9	6.6 ± 0.2	258.7 ± 6.8
+0.25	Nucleoside	4.0 ± 0.2	39.8 ± 0.2	0.2 ± 0.0	nd	44.0 ± 0.4
	Monomeric NT	1.3 ± 0.4	26.9 ± 4.7	1.0 ± 0.0	1.4 ± 0.0	30.6 ± 5.0
	NT Adduct	0.9 ± 0.2	3.2 ± 0.9	1.1 ± 0.2	0.5 ± 0.1	5.8 ± 0.6
	Polymeric NT	0.1 ± 0.0	3.9 ± 1.1	1.1 ± 0.1	0.9 ± 0.0	6.0 ± 0.9
	Total Base	6.3 ± 0.0	73.8 ± 4.3	3.5 ± 0.1	2.8 ± 0.1	86.4 ± 4.3
+1	Nucleoside	3.5 ± 0.1	49.8 ± 0.8	0.5 ± 0.0	nd	53.9 ± 0.7
	Monomeric NT	13.1 ± 0.3	77.5 ± 2.8	4.0 ± 0.2	3.0 ± 0.2	97.5 ± 3.2
	NT Adduct	0.4 ± 0.2	11.9 ± 6.8	2.4 ± 0.2	2.0 ± 0.6	16.5 ± 7.6
	Polymeric NT	0.5 ± 0.5	3.0 ± 3.8	1.3 ± 0.2	1.5 ± 0.5	6.4 ± 3.6
	Total Base	17.5 ± 0.9	142.2 ± 6.6	8.1 ± 0.0	6.5 ± 0.3	174.4 ± 7.9
+2	Nucleoside	2.5 ± 0.3	60.4 ± 0.4	0.8 ± 0.0	0.6 ± 0.1	64.2 ± 0.8
	Monomeric NT	16.9 ± 0.6	30.4 ± 3.4	2.0 ± 0.1	2.6 ± 0.0	51.6 ± 4.2
	NT Adduct	0.3 ± 0.2	6.7 ± 1.4	2.4 ± 0.2	2.6 ± 0.3	12.0 ± 1.3
	Polymeric NT	1.0 ± 0.1	2.7 ± 1.3	1.0 ± 0.1	1.2 ± 0.1	6.0 ± 1.3
	Total Base	20.7 ± 0.0	99.8 ± 3.2	6.2 ± 0.1	7.1 ± 0.3	133.8 ± 3.4
+3	Nucleoside	2.0 ± 0.2	42.7 ± 2.0	0.5 ± 0.1	0.6 ± 0.1	45.9 ± 2.4
	Monomeric NT	16.2 ± 0.4	22.2 ± 3.4	1.5 ± 0.2	3.6 ± 0.9	43.5 ± 4.9
	NT Adduct	0.4 ± 0.5	5.9 ± 0.3	2.2 ± 0.2	2.3 ± 0.1	10.7 ± 0.9
	Polymeric NT	0.3 ± 0.1	1.0 ± 0.3	0.6 ± 0.1	0.5 ± 0.6	2.5 ± 0.4
	TPAN	19.0 ± 0.2	71.8 ± 1.5	4.8 ± 0.2	7.0 ± 0.2	102.6 ± 1.2
+5	Nucleoside	1.5 ± 0.3	21.5 ± 0.8	nd	0.2 ± 0.0	23.3 ± 0.5
	Monomeric NT	12.1 ± 0.3	1.4 ± 0.1	0.6 ± 0.0	3.3 ± 0.1	17.4 ± 0.3
	NT Adduct	0.1 ± 0.0	0.8 ± 0.0	0.6 ± 0.2	0.6 ± 0.2	2.2 ± 0.4
	Polymeric NT	0.5 ± 0.1	0.4 ± 0.4	0.8 ± 0.1	0.7 ± 0.1	2.4 ± 0.5

	Total Base	14.1 ± 0.3	24.2 ± 1.0	2.1 ± 0.1	4.8 ± 0.3	45.2 ± 1.7
+10	Nucleoside	0.8 ± 0.2	3.2 ± 0.2	nd	0.1 ± 0.0	4.1 ± 0.0
	Monomeric NT	6.9 ± 0.3	0.4 ± 0.0	0.2 ± 0.0	2.4 ± 0.1	9.9 ± 0.4
	NT Adduct	0.1 ± 0.1	0.2 ± 0.2	0.1 ± 0.0	0.2 ± 0.1	0.6 ± 0.4
	Polymeric NT	0.3 ± 0.4	0.1 ± 0.1	0.4 ± 0.1	0.2 ± 0.0	1.0 ± 0.6
	Total Base	8.0 ± 0.1	3.9 ± 0.2	0.7 ± 0.1	3.0 ± 0.2	15.6 ± 0.2
+20	Nucleoside	0.7 ± 0.2	1.3 ± 0.1	nd	0.1 ± 0.0	2.1 ± 0.3
	Monomeric NT	3.9 ± 0.0	0.1 ± 0.1	nd	0.8 ± 0.1	4.8 ± 0.2
	NT Adduct	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.4 ± 0.2
	Polymeric NT	0.2 ± 0.1	0.1 ± 0.0	0.3 ± 0.0	0.2 ± 0.1	0.7 ± 0.2
	Total Base	4.8 ± 0.2	1.6 ± 0.1	0.4 ± 0.0	1.3 ± 0.2	8.0 ± 0.1
+30	Nucleoside	0.6 ± 0.1	0.8 ± 0.1	nd	0.1 ± 0.0	1.5 ± 0.0
	Monomeric NT	2.5 ± 0.0	0.1 ± 0.0	nd	0.3 ± 0.1	3.0 ± 0.1
	NT Adduct	nd	nd	nd	0.1 ± 0.0	0.2 ± 0.1
	Polymeric NT	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.7 ± 0.1
	Total Base	3.4 ± 0.0	1.1 ± 0.0	0.3 ± 0.0	0.6 ± 0.1	5.3 ± 0.1

[a] Values are given as the mean ± standard deviation of duplicate analyses; nd = not detected

[b] Day post-partum ± 2 h

[c] NT = nucleotide

Table 2. Nucleosides and nucleotides in bovine milk from a summer-milk herd (µmol dL^{-1})[a]

Day[a]	Form	Cytidine	Uridine	Guanosine	Adenosine	Total Form
0	Nucleoside	2.6 ± 0.2[b]	50.6 ± 5.8	2.2 ± 0.3	nd	55.4 ± 5.8
	Monomeric NT	1.5 ± 0.1	1.2 ± 0.0	0.2 ± 0.0	nd	2.8 ± 0.2
	NT Adduct	0.1 ± 0.1	0.5 ± 0.1	0.3 ± 0.0	0.2 ± 0.0	1.1 ± 0.2
	Polymeric NT	0.4 ± 0.0	0.3 ± 0.3	1.1 ± 0.0	0.9 ± 0.0	2.7 ± 0.3
	Total Base	4.7 ± 0.2	52.5 ± 6.1	3.7 ± 0.3	1.2 ± 0.0	62.1 ± 6.2
+0.25	Nucleoside	3.6 ± 0.1	28.0 ± 0.4	1.8 ± 0.0	nd	33.4 ± 0.5
	Monomeric NT	0.5 ± 0.3	0.4 ± 0.1	0.1 ± 0.0	nd	1.0 ± 0.5
	NT Adduct	0.2 ± 0.0	0.9 ± 0.2	0.1 ± 0.0	0.1 ± 0.0	1.4 ± 0.1
	Polymeric NT	0.3 ± 0.0	1.7 ± 0.3	0.8 ± 0.1	0.8 ± 0.0	3.6 ± 0.2
	Total Base	4.7 ± 0.2	31.0 ± 0.8	2.9 ± 0.2	0.9 ± 0.1	39.4 ± 0.3
+1	Nucleoside	5.4 ± 0.4	40.9 ± 1.2	2.1 ± 0.2	nd	48.5 ± 1.0
	Monomeric NT	7.3 ± 0.1	4.3 ± 0.3	0.3 ± 0.0	nd	11.9 ± 0.2
	NT Adduct	1.6 ± 0.3	6.8 ± 0.9	1.2 ± 0.1	0.3 ± 0.1	10.0 ± 1.2
	Polymeric NT	0.6 ± 0.1	1.1 ± 0.4	1.0 ± 0.1	0.7 ± 0.3	3.4 ± 0.0
	Total Base	15.0 ± 0.4	53.1 ± 0.4	4.7 ± 0.1	1.0 ± 0.3	73.8 ± 0.4
+2	Nucleoside	3.7 ± 0.4	39.2 ± 0.1	2.7 ± 0.4	nd	45.6 ± 0.9
	Monomeric NT	10.4 ± 0.8	0.4 ± 0.1	0.2 ± 0.0	0.9 ± 0.1	11.8 ± 1.0
	NT Adduct	nd	1.7 ± 0.4	0.9 ± 0.0	0.4 ± 0.0	2.9 ± 0.4
	Polymeric NT	0.5 ± 0.0	1.0 ± 0.0	0.4 ± 0.1	0.3 ± 0.0	2.3 ± 0.0
	Total Base	14.5 ± 0.4	42.3 ± 0.4	4.2 ± 0.4	1.5 ± 0.2	62.6 ± 0.2
+3	Nucleoside	6.7 ± 0.2	21.5 ± 1.6	1.2 ± 0.1	nd	29.4 ± 1.3
	Monomeric NT	5.8 ± 0.8	3.6 ± 0.8	0.3 ± 0.0	2.1 ± 0.4	11.9 ± 1.2
	NT Adduct	0.1 ± 0.0	0.5 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	1.5 ± 0.1
	Polymeric NT	0.5 ± 0.1	1.4 ± 0.5	0.4 ± 0.0	0.3 ± 0.0	2.7 ± 0.6
	Total Base	13.2 ± 0.7	27.0 ± 3.0	2.3 ± 0.1	2.9 ± 0.4	45.3 ± 3.4
+5	Nucleoside	1.0 ± 0.1	9.2 ± 0.1	0.2 ± 0.3	nd	10.4 ± 0.3
	Monomeric NT	8.0 ± 0.2	0.4 ± 0.0	0.2 ± 0.0	2.0 ± 0.1	10.7 ± 0.1
	NT Adduct	0.3 ± 0.2	0.4 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	1.0 ± 0.3
	Polymeric NT	0.8 ± 0.2	0.5 ± 0.1	0.3 ± 0.0	0.2 ± 0.1	1.9 ± 0.2

	Total Base	10.2 ± 0.1	10.5 ± 0.3	0.8 ± 0.3	2.4 ± 0.0	24.0 ± 0.1
+10	Nucleoside	0.6 ± 0.1	3.0 ± 0.0	nd	nd	3.6 ± 0.0
	Monomeric NT	4.1 ± 0.2	0.1 ± 0.0	nd	1.2 ± 0.1	5.3 ± 0.0
	NT Adduct	0.2 ± 0.1	nd	0.1 ± 0.0	0.2 ± 0.1	0.5 ± 0.2
	Polymeric NT	0.2 ± 0.1	0.2 ± 0.0	0.4 ± 0.0	0.1 ± 0.0	0.9 ± 0.1
	Total Base	5.0 ± 0.4	3.4 ± 0.0	0.4 ± 0.0	1.5 ± 0.1	10.3 ± 0.4
+20	Nucleoside	0.7 ± 0.0	1.5 ± 0.5	nd	nd	2.1 ± 0.5
	Monomeric NT	3.0 ± 0.2	0.1 ± 0.0	nd	0.4 ± 0.0	3.4 ± 0.1
	NT Adduct	0.1 ± 0.0	0.1 ± 0.0	nd	0.1 ± 0.0	0.2 ± 0.0
	Polymeric NT	nd	0.1 ± 0.0	nd	0.1 ± 0.0	0.1 ± 0.0
	Total Base	3.8 ± 0.2	1.6 ± 0.5	nd	0.5 ± 0.1	5.9 ± 0.4
+30	Nucleoside	0.6 ± 0.0	1.3 ± 0.0	nd	nd	1.9 ± 0.0
	Monomeric NT	1.6 ± 0.2	nd	nd	nd	1.6 ± 0.2
	NT Adduct	0.1 ± 0.0	0.1 ± 0.0	nd	0.3 ± 0.0	0.5 ± 0.0
	Polymeric NT	nd	nd	nd	nd	0.1 ± 0.0
	Total Base	2.3 ± 0.2	1.4 ± 0.0	nd	0.3 ± 0.0	4.0 ± 0.2

[a] Values are given as the mean ± standard deviation of duplicate analyses; nd = not detected

[b] Day post-partum ± 2 h

[c] NT = nucleotide

Table 3. Significance levels for rates of decrease of nucleosides and nucleotides in bovine milk[a]

Form	Cytidine	Uridine	Guanosine	Adenosine	Total Form
			Seasonal differences (winter vs. summer) between slopes: p-values[b]		
Nucleoside	< 0.001	< 0.001	< 0.001	0.600	< 0.001
Monomeric NT	0.310	< 0.001	< 0.001	0.007	< 0.001
NT Adduct	0.048	< 0.001	< 0.001	0.002	< 0.001
Polymeric NT	0.303	0.002	< 0.001	< 0.001	< 0.001
Total Base	0.676	< 0.001	< 0.001	0.107	< 0.001
			Non-zero slope (summer): p-values[c]		
Nucleoside	< 0.001	< 0.001	< 0.001	1.000	< 0.001
Monomeric NT	0.168	0.182	0.384	0.002	0.207
NT Adduct	0.437	0.051	0.097	0.552	0.030
Polymeric NT	0.196	0.233	< 0.001	0.001	< 0.001
Total Base	0.769	< 0.001	< 0.001	0.386	< 0.001
			Non-zero slope (winter): p-values[c]		
Nucleoside	< 0.001	< 0.001	0.048	0.316	< 0.001
Monomeric NT	0.511	< 0.001	< 0.001	0.905	< 0.001
NT Adduct	0.019	< 0.001	< 0.001	0.001	< 0.001
Polymeric NT	0.399	0.001	< 0.001	< 0.001	< 0.001
Total Base	0.408	< 0.001	< 0.001	0.053	< 0.001

[a] Level of significance *P*-value <= 0.05

[b] Statistical significance means there is evidence that there is a real difference between seasons

[c] Statistical significance means there is evidence that the levels are actually decreasing

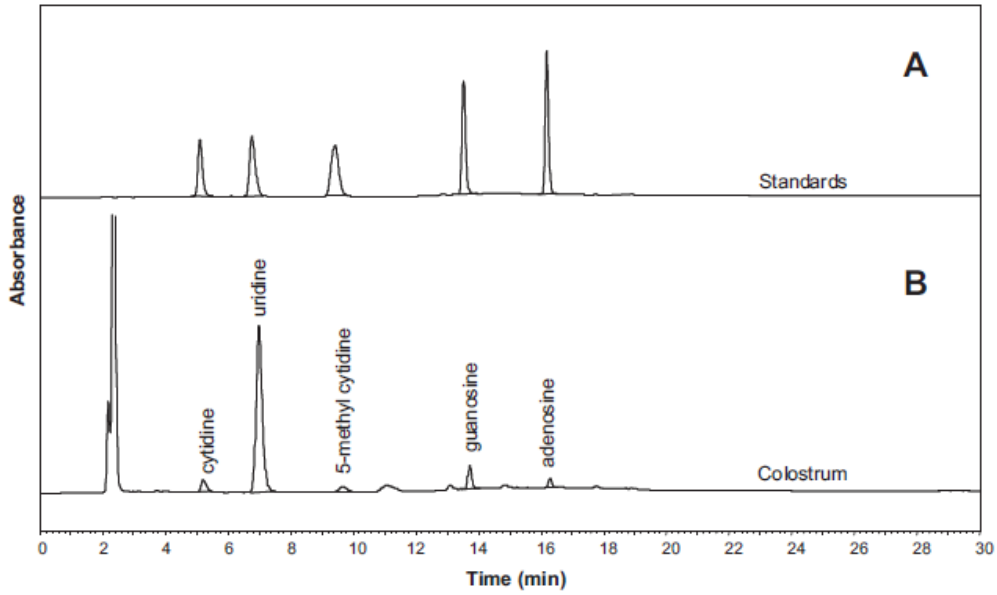
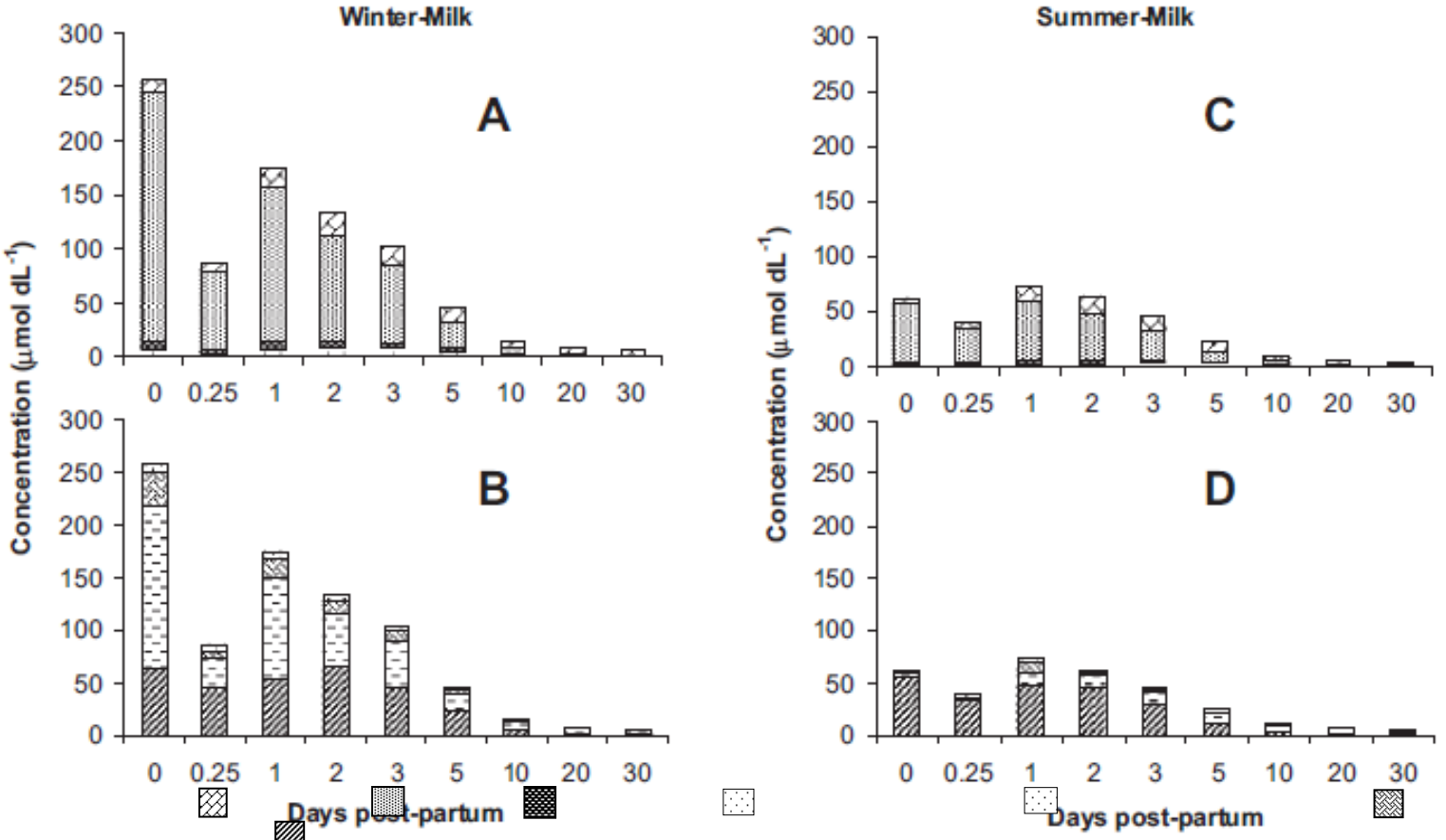


Figure **itions: mobile phase** **ion: flow rate 0.7 mL min^{-1} throughout, 0–3 min (95% A, 5% B v/v), 7–22 min (75% A, 25% B v/v), 23–30 min (95% A, 5% B v/v). UV detection 260 nm**



Figu **r the first month of** **eotide adducts;**

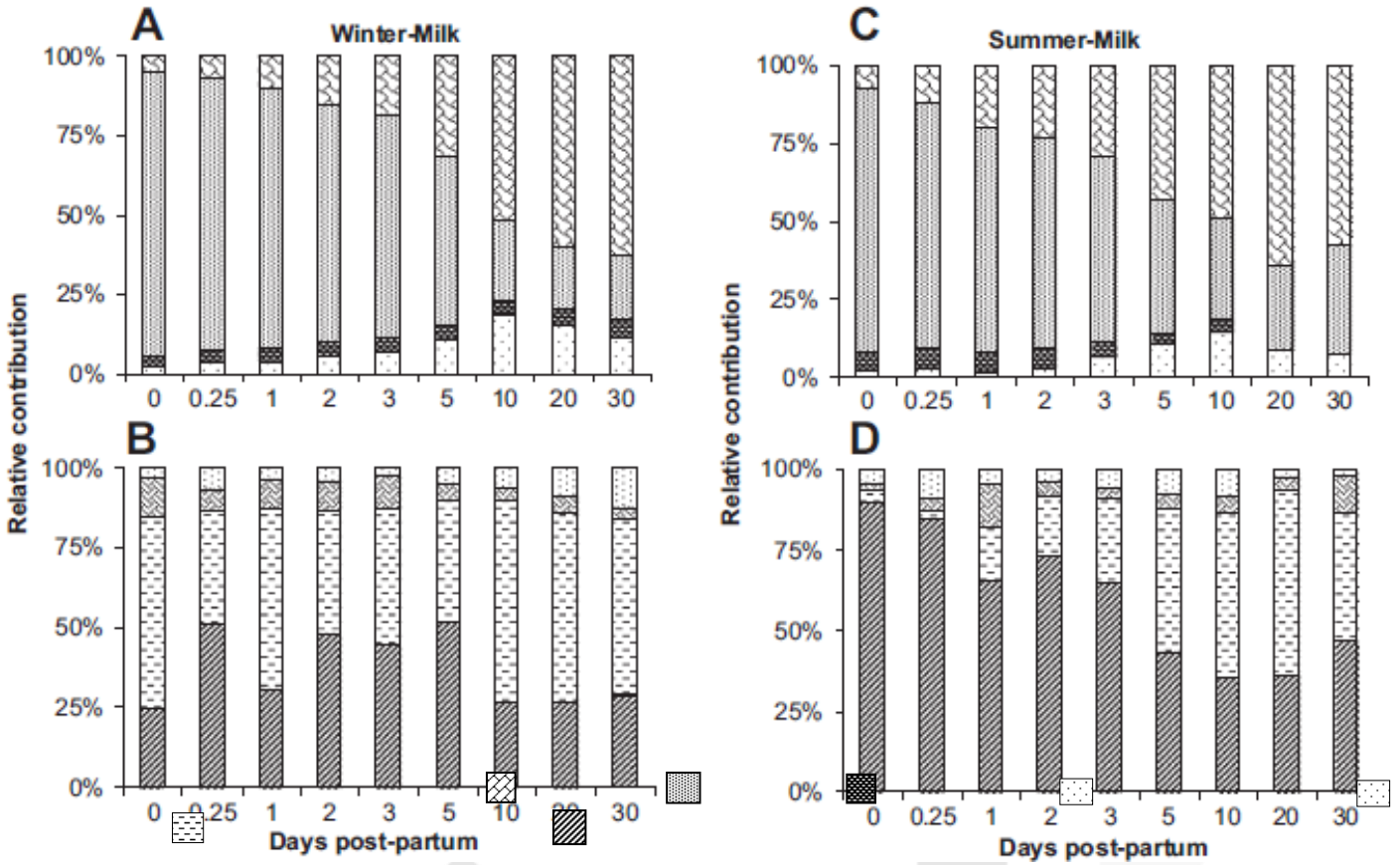


Figure 3. P summer-milk (C, D) herds ove neric nucleotides; nucl