Determination of Total Potentially Available Nucleosides in Bovine, Caprine, and Ovine Milk

Brendon D. Gill[1*], Harvey E. Indyk[1], and Merilyn Manley-Harris[2]

[1] Fonterra Co-operative Group Ltd, P.O. Box 7, Waitoa, New Zealand

[2] Department of Chemistry, University of Waikato, Private Bag 3105, Hamilton 3240, New Zealand

* Corresponding author

Abstract

The total potentially available nucleosides (TPAN) in bovine, caprine, and ovine milk were analyzed through the sequential application of phosphatase, pyrophosphatase, and nuclease enzyme treatments prior to high performance liquid chromatographic analysis of released nucleosides. The contributions to TPAN from polymeric nucleotides, monomeric nucleotides, and nucleotide adducts were then calculated. Ovine milk contained the highest concentration of TPAN, i.e., 374.1 µmol dL^{-1}, with lower concentrations in caprine milk (97.4 µmol dL^{-1}) and bovine milk (7.9 µmol dL^{-1}). Ovine milk contained the highest concentrations of each of the different nucleoside and nucleotide forms, and bovine milk contained the lowest.

Gill, B.D.; Indyk, H.E.; Manley-Harris, M. (2012) Determination of total potentially available nucleosides in bovine, caprine, and ovine milk. International Dairy Journal 24, 40–43. doi.org/ 10.1016/j.idairyj.2011.11.011

1. Introduction

The dietary significance of nucleosides and nucleotides, the forms in which they can exist in milk, their role as semi-essential nutrients in the human diet and their analytical determination in bovine milk have been described in a recent study that formed the basis for the developments currently presented (Gill, Indyk, & Manley-Harris, 2011).

Dietary nucleotides have been shown to beneficially affect intestinal growth, gut microflora, and liver growth and repair, and clinical studies have shown that infant formula supplementation with nucleotides provides a benefit to neonatal immune function when compared with unsupplemented formulae (Boza & Martínez-Augustin, 2002; Schaller, Buck, & Rueda, 2007; Yu, 2002). Nucleotides have been routinely supplemented in bovine milk-based infant formulae since the 1980s in recognition of the purported health benefits of nucleotide supplementation and the lower concentrations of free nucleotides in bovine milk compared with human milk (Adamkin, 2007). In recent years, nucleotide supplementation of infant formulae to TPAN concentrations (72 mg L^{-1}) has been approved in more than 30 countries (Aggett, Leach, Rueda, & MacLean, 2003).

Enzymatic methods were used by Gil and Sánchez-Medina (1981) to measure nucleotides in bovine, ovine, and caprine milk. Nucleoside 50-monophosphates were released enzymatically from nucleoside precursors using snake venom phosphodiesterase and were quantitatively reacted in a series of enzymatic reactions with an NADH end-point at 340 nm. In recent years, nucleosides and nucleotides have most commonly been analyzed by protein removal using acid precipitation, followed by high performance liquid chromatography ultraviolet (HPLC-UV) analysis of the crude or fractionated extract (Ferreira, Mendes, Gomes, Faria, & Ferreira, 2001; Gill & Indyk, 2007a, Gill & Indyk, 2007b).

The content of free nucleosides and nucleotides have been studied in milk of a number of mammalian species, including human, bovine, caprine, and ovine, and the concentration and relative proportions of their free forms in the milk of different species has been reported to vary (Gil & Sánchez-Medina, 1981; Gill & Indyk, 2007a; Johke & Goto, 1962; Martin, Clawin-Rädecker, Lorenzen, Ziebart, & Barth, 2005; Schlimme et al., 1997). However, measurement of the concentrations of free nucleosides and monomeric nucleotides does not account for the significant nucleotide adducts or polymeric nucleotides that are also nutritionally available to the neonate of mammalian species.

To determine the total potentially available nucleosides (TPAN) in human milk and to characterize the contributions of different molecular TPAN sources to infant nutrition, a combined multi-enzyme method incorporating a boronate extract clean-up followed by HPLC-UV analysis was developed (Leach, Baxter, Molitor, Ramstack, & Masor, 1995; Liu & Scouten, 2000). This analytical strategy allows specific contributions to the TPAN pool from polymeric nucleotides, monomeric nucleotides, nucleosides, and nucleotide adducts to be estimated. Recently, this technique was applied to a lactational study of bovine colostrum and milk given the importance of this component in infant formula production (Gill et al., 2011).

Given the global importance of large domesticated ruminants to human nutrition, the purpose of the current study was to provide a comparative assessment of the TPAN contents of mature bovine, caprine, and ovine milk and to differentiate the contributing nucleoside and nucleotide forms for each species.

2. Materials and methods

Materials, instrumentation and methods were as described in Gill et al. (2011). Sample collection and statistical analysis in the present study are detailed below.

2.1. Sample collection

In May 2009, samples of bovine milk (mixed Holstein-Friesian and Jersey) and caprine milk (Saanen) were collected directly from tanker silos prior to processing at two manufacturing sites in the Waikato region of New Zealand. A mature ovine milk sample (East Friesian) was supplied from a flock of sheep from the Southland region of New Zealand. Upon collection, the samples were taken to the laboratory and immediately prepared for storage in the same manner as previously described (Gill et al., 2011).

2.2. Statistical analysis

The experimental data were statistically analyzed by one-way analysis of variance (ANOVA) of the response of each species (bovine, caprine, ovine) and Tukey's multiple comparison test (Minitab v.15, State College, PA).

3. Results and discussion

3.1. Chromatographic analysis of sample extracts

Chromatographic performance was evaluated on the basis of retention factor, peak symmetry, peak resolution, and area repeatability, and was deemed to be acceptable from replicate analyses ($n = 6$) of a mixed nucleoside standard (Figure 1).

3.2. TPAN in bovine, caprine, and ovine milk

The results of the TPAN analysis of the milks of the three species are given in Table 1. A comparison of the concentration and the relative contribution of each nucleoside source is illustrated in Figure 2.

A comparison of the concentration and the relative contribution of each nucleoside, categorised by nucleobase, is shown in Figure 3.

3.2.1. Nucleoside contribution to TPAN

The cytidine concentrations ranged from 0.9 to 2.3 µmol dL^{-1} and were comparable among the milk of the three species, as were the relatively low concentrations of both adenosine and guanosine. In contrast, uridine was present in higher concentrations in both caprine milk (11.3 µmol dL^{-1}) and ovine milk (14.8 µmol dL^{-1}), differentiating these milks from bovine milk (1.9 µmol dL^{-1}). This dominance of uridine in ovine milk and caprine milk has been reported previously (Martin et al., 2005; Plakantara, Michaelidou, Polychroniadou, Menexes, & Alichanidis, 2010).

Nevertheless, the higher nucleoside concentrations in caprine and ovine milk represented only minor contributions to TPAN, whereas the contribution of nucleosides to the TPAN of bovine milk was >30%. It is noteworthy that ruminant milk contains higher concentrations of total nucleosides than those reported in human milk (Leach et al., 1995).

3.2.2. Monomeric nucleotide contribution to TPAN

The trends in nucleotide concentrations measured this study were similar to those reported previously in bovine, caprine, and ovine milk (Gil & Sánchez-Medina,1981; Gill & Indyk, 2007a; Martin et al., 2005; Plakantara et al., 2010). Bovine milk contained significantly lower concentrations of monomeric nucleotides than caprine and ovine milk. The cytidine nucleotide concentration ranges were comparable among the three species, as were the nucleotide concentration ranges for both adenosine and guanosine, which were at similarly low concentrations. The concentrations of uridine nucleotides varied greatly among the milk of the three species, with the range spanning 0.5–187 µmol dL^{-1}, with the lowest concentration in bovine milk and the highest concentration in ovine milk. The uridine nucleotide concentration of 37.2 µmol dL^{-1} in caprine milk is similar to those reported previously (Ferreira et al., 2001; Plakantara et al., 2010), and although these studies did not report substantially higher levels of uridine nucleotides in ovine milk, the value of 187.4 µmol dL^{-1} in the present study is similar to the levels reported in early lactation ovine milk reported by Gil and Sánchez- Medina (1981). Elevated uridine nucleotide levels may, in part, be rationalised on the basis of their role in lactose biosynthesis (Arthur, Kent, & Hartmann, 1991; Linzell & Peaker, 1971) and their potential immunological properties (Kulkarni, Fanslow, Rudolph, & Van Buren, 1986; Van Buren, Kulkarni, Fanslow, & Rudolph, 1985) as has been noted previously (Gill et al., 2011).

3.2.3. Nucleotide adduct contribution to TPAN

The range of concentrations of nucleotide adducts in the milk of the three species was similar to that of nucleotides, with the lowest concentration in bovine milk and the highest concentration in ovine milk. The uridine adducts measured in ovine milk were an order of magnitude higher than those in caprine milk and three orders of magnitude higher than those in bovine milk. Similar results were obtained in mature milk by Gil and Sánchez-Medina (1981) in their determination of UDP hexose, UDP hexosamine, and UDP galactose in the milk of the three species. The concentrations of guanosine adducts measured were 0.4, 14.5, and 22.1 µmol dL^{-1} in bovine, caprine, and ovine milk, respectively. Nucleotide adducts contributed significantly (> 30%) to TPAN in caprine and ovine milks, whereas their contribution to TPAN in bovine milk was ~10%.

The result for guanosine adducts compared well with the aggregate of guanosine-sugar adduct concentrations previously reported at 1 month (Martin et al., 2005). Similar concentrations of adenosine adducts were found, presumably derived from flavin adenine dinucleotide and NADH (Fox & McSweeney, 1998; Kanno, Shirahuji, & Hoshi, 1991). The adenosine concentrations in bovine milk were much lower than those in caprine milk and ovine milk.

3.2.4. Polymeric nucleotide contribution to TPAN

Polymeric nucleotides showed the least difference among the milk of the three species and, as with the other nucleoside forms, polymeric uridine from ovine milk was most abundant and was comparable to the concentration in human milk (Leach et al., 1995). Given the overwhelming concentration of uridine in ovine milk from monomeric nucleotides, it is possible that polymeric uridine concentrations were elevated as a consequence of calculation by difference.

3.2.5. Nucleobase contribution to TPAN

The pyrimidines, cytidine and uridine, were present primarily as monomeric nucleotides in the milk of the three species. This was in contrast to the purines, guanosine and adenosine, which were predominantly present as adducts in the milk of each of these species (Table 1). Cytidine and cytidine nucleotides were the most prevalent forms in bovine milk; similar results were obtained in the TPAN analysis of human milk (Leach et al., 1995). In contrast, uridine was the dominant nucleobase in caprine and ovine milk. The total cytidine concentration was lowest in bovine milk and highest in ovine milk. The concentrations of total uridine, guanosine, and adenosine were lowest in bovine milk and highest in ovine milk. The concentrations of total uridine, guanosine, and adenosine of human milk (Leach et al., 1995) were higher than those measured in bovine milk but much lower than those of caprine milk and ovine milk.

3.2.6. Total potentially available nucleosides

The TPAN concentrations in the milk of the three species varied markedly, with ovine milk having the highest concentrations and bovine milk having the lowest concentrations. Ovine milk contained the highest concentrations of nucleosides, nucleotide adducts, free nucleotides, and polymeric nucleotides, as well as the highest contribution from each nucleobase. Similarly, bovine milk contained the lowest concentrations of all forms of nucleosides and nucleotides, with caprine milk being intermediate. The TPAN concentration of human milk (Leach et al., 1995) is higher than that measured in bovine milk but much lower than those of caprine milk and ovine milk.

Previous studies on nucleotides in both bovine and caprine milk have shown higher concentrations of free nucleotides and related compounds in the latter (Gil & Sánchez-Medina, 1981; Johke & Goto, 1962), while the nucleotide concentrations in caprine milk have been favourably compared with those in human milk (Prosser, McLaren, Frost, Agnew, & Lowry, 2008). Because of this, supplementation of caprine milk-based infant formulae with nucleotides is not necessary as such products provide similar quantities of free nucleotides to those in nucleotide-supplemented bovine milk-based infant formulae. However, this present study showed that, when TPAN concentrations were calculated, caprine milk contained 97.4 μmol dL^{-1}, i.e., more than four times greater than the highest TPAN concentration reported in human milk (Leach et al., 1995).

The TPAN concentration in bovine milk measured in the present study was most comparable with the concentration in human milk, as reported by Leach et al. (1995). Bovine milk contained cytidine and uridine nucleosides and nucleotides in approximately equal molar proportions, whereas ovine and caprine milk were dominated by uridine and uridine nucleotides.

4. Conclusions

Despite the increasing awareness of the nutritional benefit of nucleotides in infant nutrition, and the proliferation of milk of various species being used as replacements for breast milk, a comparative study of TPAN across three ruminant species has not been previously reported.

The TPAN concentrations in bovine, caprine, and ovine milk were studied and significant differences among the milk of each species were found. The highest concentration of TPAN was found in ovine milk, with significantly lower concentrations in caprine milk and bovine milk. Ovine milk contained the highest concentrations of each of the individual nucleoside and nucleotide forms, whereas bovine milk contained the lowest. Bovine milk contained cytidine and uridine nucleosides and nucleotides in approximately equal molar proportions, whereas ovine milk and caprine milk were dominated by uridine and uridine nucleotides.

Acknowledgements

The authors thank Colin Prosser (Dairy Goat Co-operative, New Zealand) and Derek Knighton (AgResearch, New Zealand) for the supply of samples of caprine and ovine milks. We also wish to thank Roger Kissling (Fonterra, Co-operative Group, New Zealand) for his statistical analysis of the results. Advice on the application of the analytical protocol given by Bruce Molitor (Abbott Laboratories, USA) is greatly appreciated.

The financial assistance of Fonterra Co-operative Group and of the New Zealand Tertiary Education Commission in providing an Enterprise Scholarship is gratefully acknowledged.

References

Adamkin, D.H. (2007). Controversies in neonatal nutrition: docosahexanoic acid (DHA) and nucleotides. *Journal of Perinatology*, 27, S79–S82.

Aggett, P., Leach, J.L., Rueda, R., & MacLean Jr., W.C. (2003). Innovation in infant formula development: a reassessment of ribonucleotides in 2002. *Nutrition*, 19, 375–384.

Arthur, P.G., Kent, J.C., & Hartmann, P.E. (1991). Metabolites of lactose synthesis in milk from women during established lactation. Journal of Pediatric *Gastroenterology and Nutrition*, 13, 260–266.

Boza, J.J., & Martínez-Augustin, O. (2002). Role and function of nucleotides in infant nutrition. *Nestle Nutrition Workshop Series*, 47(Suppl.), 165–184.

Ferreira, I.M.P.L.V.O., Mendes, E., Gomes, A.M.P., Faria, M.A., & Ferreira, M.A. (2001). The determination and distribution of nucleotides in dairy products using HPLC and diode array detection. *Food Chemistry*, 74, 239–244.

Fox, P.F., & McSweeney, P.L.H. (1998). *Dairy Chemistry and Biochemistry*. New York, NY, USA: Kluwer Academic/Plenum Publishers.

Gil, A., & Sánchez-Medina, F. (1981). Acid-soluble nucleotides of cow's, goat's and sheep's milks, at different stages of lactation. *Journal of Dairy Research*, 48, 35–44.

Gill, B.D., & Indyk, H.E. (2007a). Development and application of a liquid chromatographic method for analysis of nucleotides and nucleosides in milk and infant formulas. *International Dairy Journal*, 17, 596–605.

Gill, B.D., & Indyk, H.E. (2007b). Determination of nucleotides, nucleosides and nucleobases in milks and pediatric formulas: a review. *Journal of AOAC International*, 90, 1354–1364.

Gill, B.D., Indyk, H.E., & Manley-Harris, M. (2011). Determination of total potentially available nucleosides in bovine milk. *International Dairy Journal*, 21, 34–41.

Johke, T., & Goto, T. (1962). Acid-soluble nucleotides in cow's and goat's milk. Journal of Dairy Science, 45, 735–741.

Kanno, C., Shirahuji, K., & Hoshi, T. (1991). Simple method for separate determination of three flavins in bovine milk by high performance liquid chromatography. *Journal of Food Science*, 56, 678–681.

Kulkarni, A.D., Fanslow, W.C., Rudolph, F.B., & Van Buren, C.T. (1986). Effect of dietary nucleotides on response to bacterial infections. *Journal of Parenteral and Enteral Nutrition*, 10, 169–171.

Leach, J.L., Baxter, J.H., Molitor, B.E., Ramstack, M.B., & Masor, M.L. (1995). Total potentially available nucleosides of human milk by stage of lactation. *American Journal of Clinical Nutrition*, 61, 1224–1230.

Linzell, J.L., & Peaker, M. (1971). Mechanism of milk secretion. *Physiological Reviews*, 51, 564–597.

Liu, X.-C., & Scouten, W.H. (2000). Boronate affinity chromatography. In P. Bailon, G. K. Ehrlich, W.-J. Fung, & W. Berthold (Eds.), *Affinity Chromatography: Methods and Protocols* (pp. 119–128). Totowa, NJ, USA: Humana Press.

Martin, D., Clawin-Rädecker, I., Lorenzen, P.C., Ziebart, M., & Barth, K. (2005). Ribonucleoside contents in sheep and goat milk. *Kieler Milchwirtschaftliche Forschungsberichte*, 57, 21–32.

Plakantara, S., Michaelidou, A.M., Polychroniadou, A., Menexes, G., & Alichanidis, E. (2010). Nucleotides and nucleosides in ovine and caprine milk during lactation. *Journal of Dairy Science*, 93, 2330–2337.

Prosser, C.G., McLaren, R.D., Frost, D., Agnew, M., & Lowry, D.J. (2008). Composition of the non-protein nitrogen fraction of goat whole milk powder and goat milk-based infant and follow-on formulae. *International Journal of Food Sciences and Nutrition*, 59, 123–133.

Schaller, J.P., Buck, R.H., & Rueda, R. (2007). Ribonucleotides: conditionally essential nutrients shown to enhance immune function and reduce diarrheal disease in infants. *Seminars in Fetal and Neonatal Medicine*, 12, 35–44.

Schlimme, E., Martin, D., Meisel, H., Schneehagen, K., Hoffmann, S., Sievers, E., et al. (1997). Species-specific composition pattern of milk ribonucleosides and -nucleotides: chemical and physiological aspects. *Kieler Milchwirtschaftliche Forschungsberichte*, 49, 305–326.

Van Buren, C.T., Kulkarni, A.D., Fanslow, W.C., & Rudolph, F.B. (1985). Dietary nucleotides, a requirement for helper/inducer T lymphocytes. Transplantation, 40, 694–697.

Yu, V.Y.H. (2002). Scientific rationale and benefits of nucleotide supplementation of infant formula. *Journal of Paediatrics and Child Health*, 38, 543–549.

Table 1. Total potentially available nucleosides in bovine, caprine, and ovine milk[a]

Milk	Form	Cytidine	Uridine	Guanosine	Adenosine	Total
Bovine	Nucleoside	0.9 ± 0.1	1.9 ± 0.1	nd	nd	2.8 ± 0.3
	Monomeric NT	3.3 ± 0.1	0.5 ± 0.2	nd	nd	3.8 ± 0.3
	NT adduct	0.1 ± 0.0	0.1 ± 0.0	0.4 ± 0.1	0.1 ± 0.0	0.6 ± 0.0
	Polymeric NT	0.1 ± 0.1	0.1 ± 0.1	nd	0.5 ± 0.0	0.7 ± 0.3
	Total base	4.4 ± 0.2	2.6 ± 0.2	0.4 ± 0.1	0.5 ± 0.0	7.9 ± 0.5
Caprine	Nucleoside	1.6 ± 0.1	11.3 ± 0.4	nd	nd	12.9 ± 0.3
	Monomeric NT	3.6 ± 0.2	37.2 ± 0.8	9.4 ± 0.5	2.4 ± 0.2	52.7 ± 1.7
	NT adduct	0.7 ± 0.0	10.1 ± 1.2	14.5 ± 0.1	3.4 ± 0.1	28.7 ± 1.2
	Polymeric NT	0.6 ± 0.2	1.0 ± 0.9	1.1 ± 0.5	0.5 ± 0.2	3.2 ± 1.9
	Total base	6.5 ± 0.3	59.5 ± 1.8	25.0 ± 0.78	6.3 ± 0.3	97.4 ± 2.8
Ovine	Nucleoside	2.3 ± 0.1	14.8 ± 1.1	0.6 ± 0.0	nd	17.6 ± 1.2
	Monomeric NT	5.7 ± 0.3	187.4 ± 4.4	6.3 ± 0.0	12.1 ± 0.0	211.4 ± 4.0
	NT adduct	0.9 ± 0.1	100.4 ± 7.8	22.1 ± 0.2	14.4 ± 0.6	137.8 ± 8.7
	Polymeric NT	0.5 ± 0.3	4.3 ± 0.1	1.2 ± 0.3	1.3 ± 0.6	7.3 ± 1.2
	Total base	9.4 ± 0.4	306.8 ± 9.0	30.2 ± 0.3	27.8 ± 0.9	374.1 ± 9.8

[a] Values (μmol dL^{-1}) are means ± standard deviation of duplicate analyses: NT = nucleotide; nd = not detected

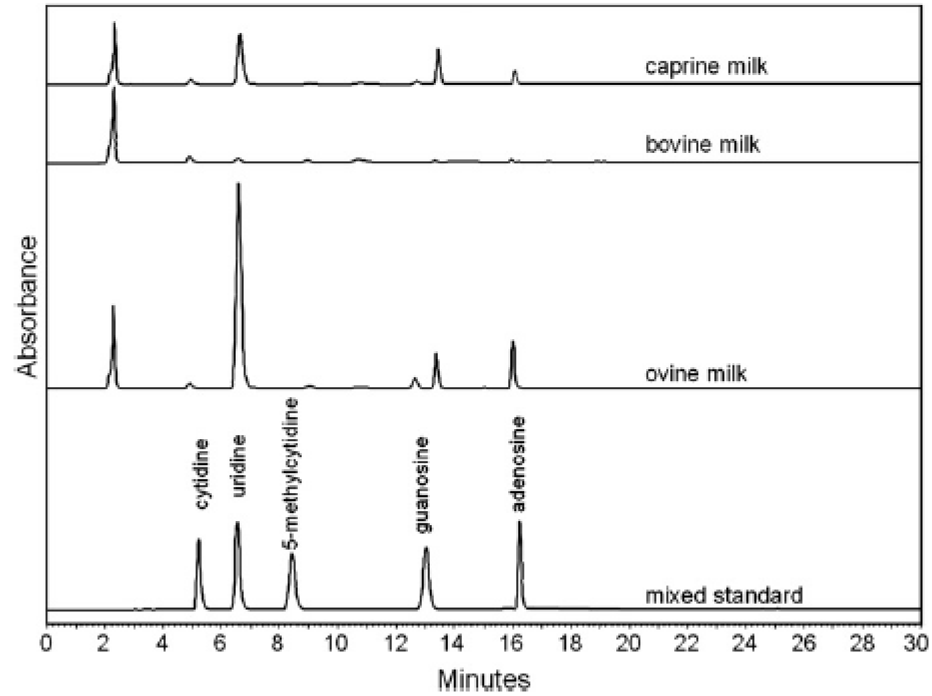

Figure … and ovine milk. Condit… thanol; gradient elution: flow rate 0.7 mL min^{-1} throughout, 0–5 min (95% A, 5% B, v/v), 7–22 min (75% A, 25% B, v/v), 23–30 min (95% A, 5% B, v/v). UV detection 260 nm

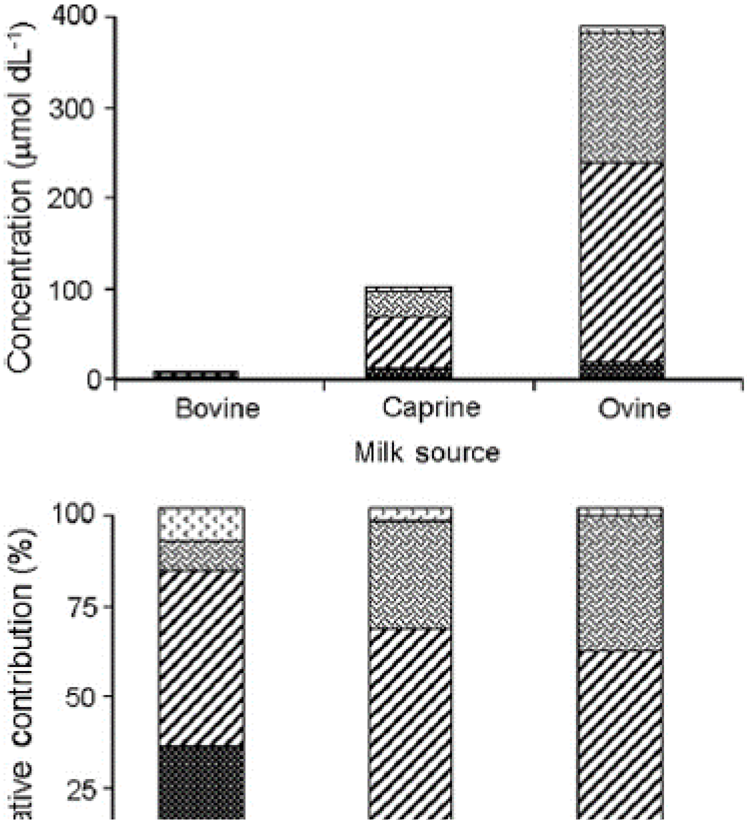

Figure 2. A comparison of the concentration and the relative contribution of each nucleoside (by phosphorylated form) in bovine, caprine, and ovine milk samples (mean of duplicate analyses):
▩ nucleosides; ▨ nucleotide adducts; ▩ monomeric nucleotides; ⁘ polymeric nucleotides

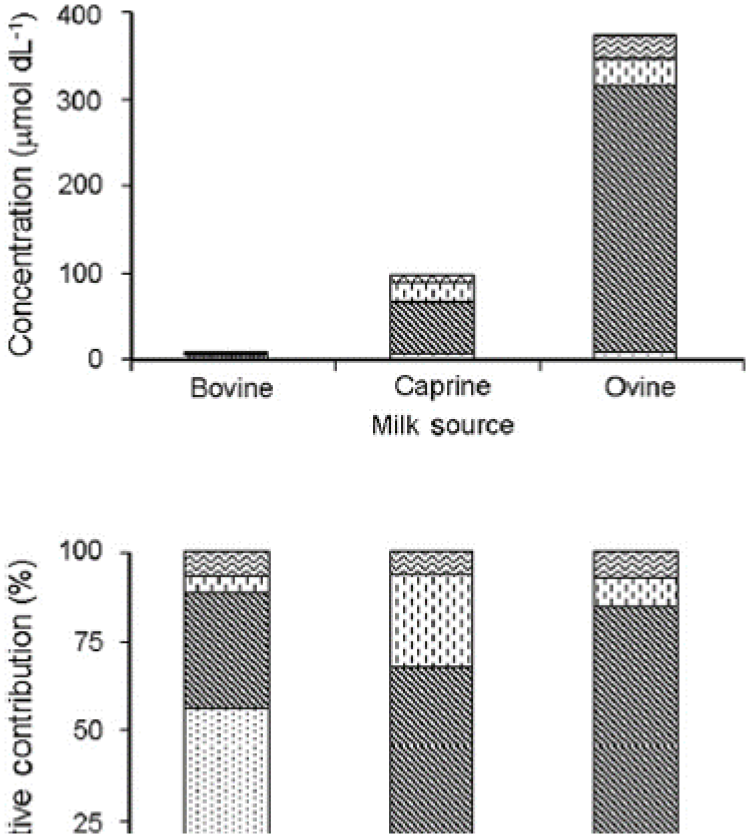

Figure 3. A comparison of the concentration and the relative contribution of each nucleoside (by nucleobase) in bovine, caprine, and ovine milk samples (mean of duplicate analyses): uridine; guanosine; , adenosine; cytidine