Development and application of a liquid chromatographic method for analysis of nucleotides and nucleosides in milk and infant formulas

AUTHORS: Brendon D. Gill, Harvey E. Indyk
Fonterra Co-operative Group Ltd, P.O. Box 7, Waitoa, New Zealand, fonterra.com
brendon.gill@fonterra.com Ph +64 6 889 3989
harvey.indyk@fonterra.com Fax +64 6 887 1502

ABSTRACT

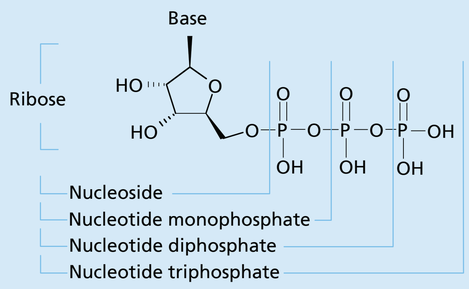
A method for the simultaneous determination in milk of the 5′-mononucleotides adenosine 5′-monophosphate, cytidine 5′-monophosphate, guanosine 5′-monophosphate, inosine 5′-monophosphate and uridine 5′-monophosphate and their corresponding nucleosides is described. Following deproteinisation, the sample extract was analysed by reversed-phase liquid chromatography, whereby 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.

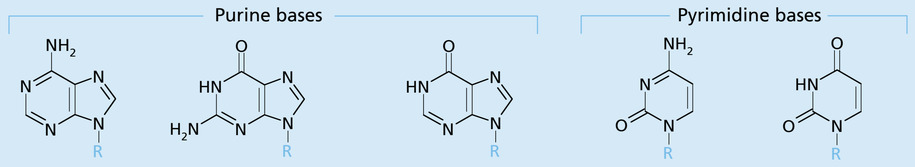
Paper accepted for publication in International Dairy Journal, 2006.

INTRODUCTION

Nucleotides and nucleosides play important roles in biochemical processes and may become conditionally essential when the endogenous supply is inadequate during periods of rapid growth or after injury. Nucleotide supplementation of infant formulas has become increasingly widespread in view of the significant differences in the nucleos(t)ide pool between bovine and human milk.

Although controversial, allowance for the fortification of infant formulas with nucleotides to levels equivalent to the total potentially available nucleosides (TPAN) levels has subsequently been approved in more than 30 countries.





Base	adenine	guanine	hypoxanthine	cytosine	uracil
Nucleoside	adenosine (Ado)	guanosine (Guo)	inosine (Ino)	cytidine (Cyd)	uridine (Urd)
5′-Mononucleotide	adenosine monophosphate (AMP)	guanosine monophosphate (GMP)	inosine monophosphate (IMP)	cytidine monophosphate (CMP)	uridine monophosphate (UMP)

ANALYTICAL TECHNIQUE

Sample Preparation

1 Dissolve 0.5 g powder in 4 mL warm water, mix, add 2.0 mL acetic acid (3% v/v).
2 Stand for 10 min then dilute sample to 10 mL with water.
3 Centrifuge and filter with 0.22 µm filter into HPLC vial.

HPLC Analysis

Column: Gemini C_{18} 5µm 110Å 4.6 x 250mm (Phenomenex)
Mobile Phase A: KH_2PO_4 (0.1M), pH=5.6
Mobile Phase B: KH_2PO_4 (0.1M), 15% v/v MeOH, pH = 5.6
Gradient: 0-30 min 100% A, 65-90 min 100% B, 95-130 min 100% A
Detection: Photo-diode array, quantify at 250nm (IMP, inosine), 260nm (GMP, AMP, guanosine, adenosine, cytidine, uridine) and 270nm (CMP, UMP)
Quanititation: External standard technique
Injection: 50 µL

Method performance parameters

Analyte[a]	Range (µg mL^{-1})	Linear regression	r^2	MDL (mg 100g^{-1})[b]	RSD_r (%)[c]	RSD_{iR} (%)[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	0.9995	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 = $t_{(n-1,\,1-a)}$ x sd, where $n = 7$ and $\alpha = 0.05$.
[c] RSD repeatability = sd/mean x 100.
[d] RSD intermediate reproducibility = sd/mean x 100.
[e] Mean recovery (standard deviation) at three concentration levels tested in triplicate.

RESULTS

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.

There is a potential risk during infant formula production of dephosphorylation of exogenous 5′-mononucleotides yielding the related nucleosides due to residual phosphatase activity that may survive pasteurisation. Sample K showed loss of all exogenous nucleotides with concurrent elevated levels of nucleosides above those normally expected in a milk-based product, demonstrating that dephosphorylation during manufacture had indeed occurred.

In early colostrum the only nucleotides present were GMP and UMP. Cytidine levels were low in pre-colostrum and increased to a maximum yield by the second day post-partum. 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 third week post-partum.

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

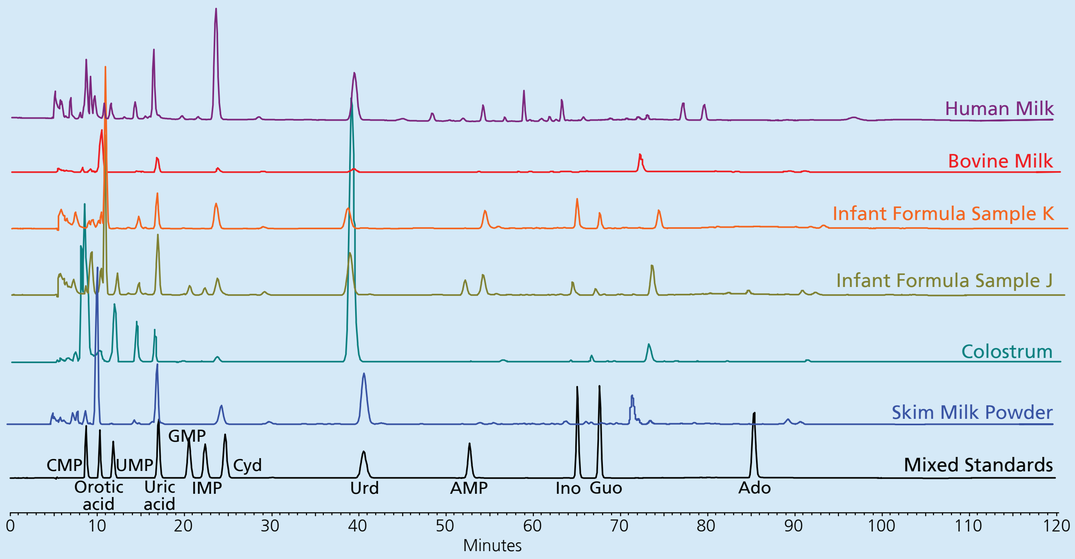
Sample[b]	AMP[c]	CMP	GMP	IMP	UMP	Total nucleotides
A	107.7	120.6	90.7	108.1	110.1	114.2
B	107.9	102.2	93.3	102.3	112.2	104.1
C	109.0	125.6	91.5	107.2	109.6	117.0
D			No Label Claim[d]			113.3
E			No Label Claim			114.4
F	52.0	133.3	57.8	75.0	128.2	95.8
G			No Label Claim			194.5
H			No Label Claim			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) x 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.

Early lactation analysis of nucleotides and nucleosides in the milk of a single cow (µmol 100 mL^{-1})

Day	CMP[ab]	GMP	UMP	Cytidine	Guanosine	Inosine	Uridine
–0.3	nd	0.3	13.3	1.8	0.9	0.3	102.4
0	nd	0.4	8.0	4.6	1.2	0.3	95.9
0.25	nd	nd	0.3	9.7	0.4	0.6	38.1
0.5	nd	nd	nd	6.5	0.4	0.7	6.9
1	nd	nd	nd	11.0	0.5	0.9	4.8
2	1.1	nd	nd	12.5	0.6	1.1	6.4
5	1.9	nd	nd	6.0	0.3	1.2	9.2
10	1.4	nd	nd	3.0	0.3	1.3	3.7
16	0.2	nd	nd	0.4	nd	nd	nd
21	0.3	nd	nd	0.5	nd	nd	nd

[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.
nd = not detected.



CONCLUSIONS

- **A simple gradient reversed-phase HPLC technique for the quantitation of nucleotides and nucleosides in dairy products has been validated.**
- **Acceptable recoveries of nucleotides supplemented in the majority of surveyed infant formulas.**
- **One infant formula demonstrated nucleotide dephosphorylation.**
- **Decreasing trend of nucleoside expression during lactation observed from a maximum during the early colostral phase to a constant level by the third week post-partum.**

ACKNOWLEDGMENTS

The support for this work by Fonterra Co-operative Group Limited is gratefully acknowledged.

2006064-01 07-06