Analysis of Nucleosides and Nucleotides in Milk and Infant Formula

A thesis

submitted in fulfilment

of the requirements for the degree

of

Doctor of Philosophy in Chemistry

at the

University of Waikato

by

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THE UNIVERSITY OF WAIKATO
Te Whare Wānanga o Waikato

2013

ABSTRACT

Nucleotides have been routinely supplemented to infant formulas due to the important roles they play in metabolism and to replicate the higher concentrations typically found in human milk. A method utilising anion exchange solid-phase extraction clean-up and liquid chromatography was developed for the rapid, routine determination of supplemented cytidine 5′-monophosphate, uridine 5′-monophosphate, inosine 5′-monophosphate, guanosine 5′-monophosphate, and adenosine 5′-monophosphate in bovine milk-based infant formula. Chromatographic analyses were performed using a C_{18} stationary phase with gradient elution, UV detection, and quantitation by an internal standard technique. A single-laboratory validation was performed, with recoveries of 92–101% and repeatability of 1.0–2.3%. An extension study demonstrated the expansion in scope to a wider range of different infant formula products including milk protein and hydrolysate-based products, low and high fat products, soy protein-based and elemental products, adult nutritional and infant formulations, in both ready-to-feed and powder forms.

The development of a method to measure the total potentially available nucleosides (TPAN) in human milk has made an important contribution to further understanding the distribution of nucleosides and nucleotides. This method was applied in a lactation study of bovine milk with colostrum and milk samples collected from two herds over the course of the first month post-partum, pooled within each herd by stage of lactation and the TPAN concentrations were determined. Sample analysis consisted of parallel enzymatic treatments, phenylboronate affinity gel extraction, and liquid chromatography to quantify contributions of nucleosides, monomeric nucleotides, nucleotide adducts, and polymeric nucleotides to the nutritionally available nucleoside 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.

The TPAN method was subsequently applied to the analysis of mature bovine, caprine, and ovine milk. The contributions to TPAN from polymeric nucleotides, monomeric nucleotides, and nucleotide adducts were then calculated. Ovine milk contained the highest concentration of TPAN (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.

A method for the simultaneous analysis of nucleosides and nucleotides in infant formula using reversed-phase liquid chromatography-tandem mass spectrometry was developed. Following sample dissolution, protein was removed by centrifugal ultrafiltration. Chromatographic analyses were performed using a C_{18} stationary phase and gradient elution, with mass spectrometric detection, and quantitation by stable isotope labelled internal standard technique. A single laboratory validation study was performed with recoveries of 80.1–112.9% and repeatability relative standard deviations of 1.9–7.2%. The method was validated for the analysis of bovine milk-based, soy-based, caprine milk-based and hydrolysate-based infant formula.

This work is dedicated to my wife Michelle and to my children, Caitlin, Madelynn, Aidan, and Carys.

“The real voyage of discovery consists not in seeking new landscapes but in having new eyes.” — Marcel Proust, 1923

.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my thesis supervisors; Harvey Indyk, Merilyn Manley-Harris, Lyndsay Main, and Alistair Wilkins.

Harvey, as a work colleague and good friend these last six years, your advice and mentoring has been invaluable. As a scientist, you are a great role model, and in years to come, I would hope that I could emulate the reputation that you hold with your peers.

Merilyn, I thank you once again for offering your time and expertise as my thesis supervisor. In recent years, your duties as departmental chair no doubt kept you very busy. However, whenever I needed guidance you were always available.

Lyndsay and Alistair, while both of you supervised me for only a short while, I am grateful for the knowledge and experience you both have freely shared.

I would like to offer my thanks to Steve Holroyd, my manager for these last six years. Without your support from the outset, this project would not have got off the ground. Your ability to navigate the legal and technical pitfalls for me to pursue part-time study as a Fonterra employee have been invaluable and I wish you continued success in your new role.

I wish to extend my thanks to Bruce Molitor of Abbott Laboratories who was kind enough to provide me with an opportunity to visit his laboratory, and for sharing his time and expertise with TPAN methodology.

Over the course of this study, I have been generously supplied with milk samples. For that, I wish to thank Dee and Rich Syme, Andrew and Jenny McGiven, Colin Prosser, and Derek Knighton.

The learning of a technique such as mass spectrometry does not come over night. I am grateful for the advice and assistance of Grant Abernethy while I developed my skills in operating the LC-MS.

Many thanks go to Roger Kissling for his advice and knowledge with all things statistical and for providing me with insights both practical and theoretical.

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

Finally, I wish to thank my wife Michelle for her patience—finished at last!

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS/ACRONYMS/SYMBOLS

α	statistical level of significance
λ_{max}	wavelength of maximum absorbance
5mCyd	5-methylcytidine
8BrGuo	8-bromoguanosine
Ado	adenosine
ADP	adenosine 5′-diphosphate
AICAR	5-aminoimidazole-4-carboxamide ribonucleotide
AIR	aminoimidazole ribonucleotide
AMP	adenosine 5′-monophosphate
AN	adult nutritional
APCI	atmospheric pressure chemical ionisation
ATP	adenosine 5′-triphosphate
CAIR	carboxyaminoimidazole ribonucleotide
cAMP	adenosine 3′,5′-cyclic monophosphate
CDP	cytidine 5′-diphosphate
CE	capillary electrophoresis
CE-MS	capillary electrophoresis-mass spectrometry
cGMP	guanosine 3′,5′-cyclic monophosphate
CID	collision induced dissociation
CMP	cytidine 5′-monophosphate
CoA	coenzyme A
CoQ	coenzyme Q

CPS-II	carbamoyl phosphate synthetase-II
CRM	certified reference material
CTP	cytidine 5′-triphosphate
CUF	centrifugal ultrafiltration
Cyd	cytidine
DBAA	dibutylammonium acetate
DC	direct current
df	degrees of freedom
DHB	2,5-dihydroxybenzoic acid
DMHA	*N*, *N*-dimethylhexylamine
DNA	deoxyribonucleic acid
EC	Enzyme Commission
EDTA	ethylenediaminetetraacetic acid
EI	electron impact
EPA	Environmental Protection Agency
ERP	expert review panel
ESI	electrospray ionisation
ESI^-	electrospray ionisation in negative mode
ESI^+	electrospray ionisation in positive mode
FAD	flavin adenine dinucleotide
FAICAR	*N*-formamidoimidazole-4-carboxamide ribonucleotide
FGAM	formylglycinamidine ribonucleotide
FGAR	formylglycinamide ribonucleotide
fmol	femtomole (10^{-15} mole)
FO	follow-on formula

GAR	glycinamide ribonucleotide
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
GDP	guanosine 5′-diphosphate
GMP	guanosine 5′-monophosphate
GTP	guanosine 5′-triphosphate
Guo	guanosine
hg	hectogram (1 hg = 100 g)
HiB	*Haemophilus influenzae* type b
HILIC	hydrophilic interaction liquid chromatography
HorRat	Horwitz ratio
HPLC	high performance liquid chromatography
ICH	International Conference on Harmonisation
ICP-MS	inductively coupled plasma-mass spectrometry
IDF	International Dairy Federation
IEG	international expert group
IF	infant formula
Ig	immunoglobulin
IL2	interleukin-2
IMP	inosine 5′-monophosphate
Ino	inosine
IPRPLC	ion pair-reversed phase liquid chromatography
k′	retention factor
LC	liquid chromatography
LC-MS	liquid chromatography-mass spectrometry

LC-UV	liquid chromatography-ultraviolet
LOD	limit of detection
MALDI	matrix assisted laser desorption ionisation
MDL	method detection limit
ME	margin of error
min	minutes
MFGM	milk fat globule membrane
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MWCO	molecular weight cut-off
m/z	mass-to-charge ratio
n	number of replicates
N	theoretical plate number
NAD^+	nicotinamide adenine dinucleotide (oxidised form)
NADH	nicotinamide adenine dinucleotide (reduced form)
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NIL	non-isotopically labelled
NIST	National Institute of Standards and Technology
NK	natural killer
OMP	orotidine 5′-monophosphate
p/h	partially hydrolysed
PACE	pressure assisted capillary electrophoresis

PCA	perchloric acid
PDA	photo diode array
PGC	porous graphite chromatography
P_i	phosphate (inorganic)
PP_i	pyrophosphate (inorganic)
pRSD	predicted relative standard deviation
PRPP	5-phosphoribosyl α-diphosphate
r	repeatability limit
R^2	correlation coefficient
rcf	relative centrifugal force
RF	radiofrequency
RNA	ribonucleic acid
RPLC	reversed phase liquid chromatography
R_s	chromatographic resolution
RSD	relative standard deviation
RTF	ready-to-feed
S/N	signal to noise ratio
SAICAR	*N*-succinylo-5-aminoimidazole-4-carboxamide ribonucleotide
SAX	strong anion exchange
SD	standard deviation
SIL	stable isotope labelled
SIM	selected ion monitoring
SLV	single laboratory validation
SMPR	standard method performance requirement
SPE	solid phase extraction

SPIFAN	Stakeholder Panel on Infant Formula and Adult Nutritionals
T_f	tailing factor
TBAHS	tetrabutylammonium hydrogen sulfate
TCA	trichloroacetic acid
THF	tetrahydrofolate
TIC	total ion current
TMP	thymidine 5′-monophosphate
TMS	trimethylsilyl
TOF	time of flight
TPAN	total potentially available nucleosides
UDP	uridine 5′-diphosphate
UMP	uridine 5′-monophosphate
UHPLC	ultra high performance liquid chromatography
Urd	uridine
UTP	uridine 5′-triphosphate
UV	ultraviolet
XMP	xanthine 5′-monophosphate

1. INTRODUCTION

In recent years, there has been considerable activity in the analysis of bovine milk for bioactive factors that may confer significant improvements to human health. Found in a wide concentration range, from parts per billion to parts per million, these bioactive components influence the physiological development of the neonate.

Nucleosides and nucleotides are a group of structurally related bioactive components present that exhibit a diverse range of nutritional benefits to infants. The presence of nucleotides in human and bovine milk as DNA and RNA was established, with the concentration of RNA more than 20 times higher than DNA (Sanguansermsri *et al*., 1974). The nucleotide content in milk and the health benefits these impart have been fertile topics of research in recent years. The focus of this study is restricted to ribonucleoside and ribonucleotides only, and excludes deoxyribose forms.

Due to the role nucleotides play in metabolism, and in order to resemble more closely the nucleotide profile of human milk, infant formula (unless otherwise specified, infant formula refers to bovine milk-based or whey-based formulas) has been routinely supplemented with nucleotides. However, nucleotide supplementation is still somewhat controversial, particularly when fortified to levels equivalent to the total potentially available nucleoside (TPAN) levels of human milk.

When manufacturing products for infant consumption, analytical methods used to confirm product composition are held to a high standard. The merit of an analytical method is demonstrated through a single laboratory validation (SLV) study whereby a method is shown to be accurate, precise, robust, and fit-for-purpose. Coupled with the commercial requirements for rapid, high capacity sample throughput, there is a distinct need for an accurate and precise rapid, analytical method for the routine product

compliance analysis of nucleotides in a wide range of infant formula. A robust method for the routine analysis of nucleotides was developed and validated as part of this study. A description of this method and results obtained from this research were summarised and presented at the 123rd annual AOAC International conference in 2009 and subsequently published in the Journal of AOAC International (Gill *et al*., 2010) (see Appendix I, Appendix II).

The development of an analytical method to measure TPAN has been an important contribution to further understanding the distribution of nucleosides and nucleotides, which has important implications for infant nutrition. With the increasing trend towards nucleotide supplementation of infant formulas, it is surprising that an analysis of TPAN in bovine milk has not previously been reported. Therefore, an analysis of TPAN in bovine colostrum and milk as well as mature bovine, caprine, and ovine milk forms a part of this study. A description of this method and results obtained from this research were summarised and presented at the 124th annual AOAC International conference in 2010 and subsequently published in the International Dairy Journal (Gill *et al*., 2011; Gill *et al*., 2012b) (see Appendix I, Appendix II).

In recent years, a number of separation modes have been coupled, either online or off-line, to various mass spectrometric techniques in the analysis of nucleosides and nucleotides in biological tissues and fluids. Currently, few of these techniques have been applied to the analysis of milk and infant formula. A part of this study includes the development, optimisation, and validation of a liquid chromatography-mass spectrometry (LC-MS) method for the simultaneous quantitation of nucleosides and nucleotides. A description of this method and results obtained from this research were summarised and subsequently published in Analytical Bioanalytical Chemistry (Gill *et al*., 2013) (see Appendix I, Appendix II).

2. LITERATURE REVIEW

2.1. Nucleotide Biochemistry

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 protein (Carver and Walker, 1995; Cosgrove, 1998; Yu, 1998).

2.1.1. STRUCTURE OF NUCLEOTIDES

Nucleobases are low molecular weight heterocyclic aromatic compounds based on either a purine or pyrimidine structure; **Figure 1a**. These include cytosine, thymine, uracil (pyrimidines) and adenine, guanine, hypoxanthine, xanthine (purines). Nucleosides consist of a purine or pyrimidine base attached via an *N*-glycosidic linkage to β-D-ribofuranose; **Figure 1b**. Nucleotides are *o*-phosphoric acid esters of nucleosides containing one to three phosphate groups on the ribose 2–, 3– or most commonly 5–carbon. Cyclic nucleotides contain a phosphate group that is bonded to two of the ribose hydroxyl groups forming a ring structure; **Figure 2.**

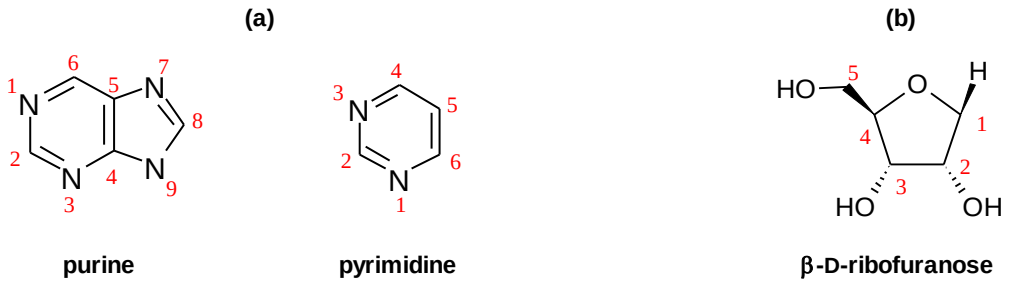

Figure 1. Structure and numbering of purine, pyrimidine and ribose

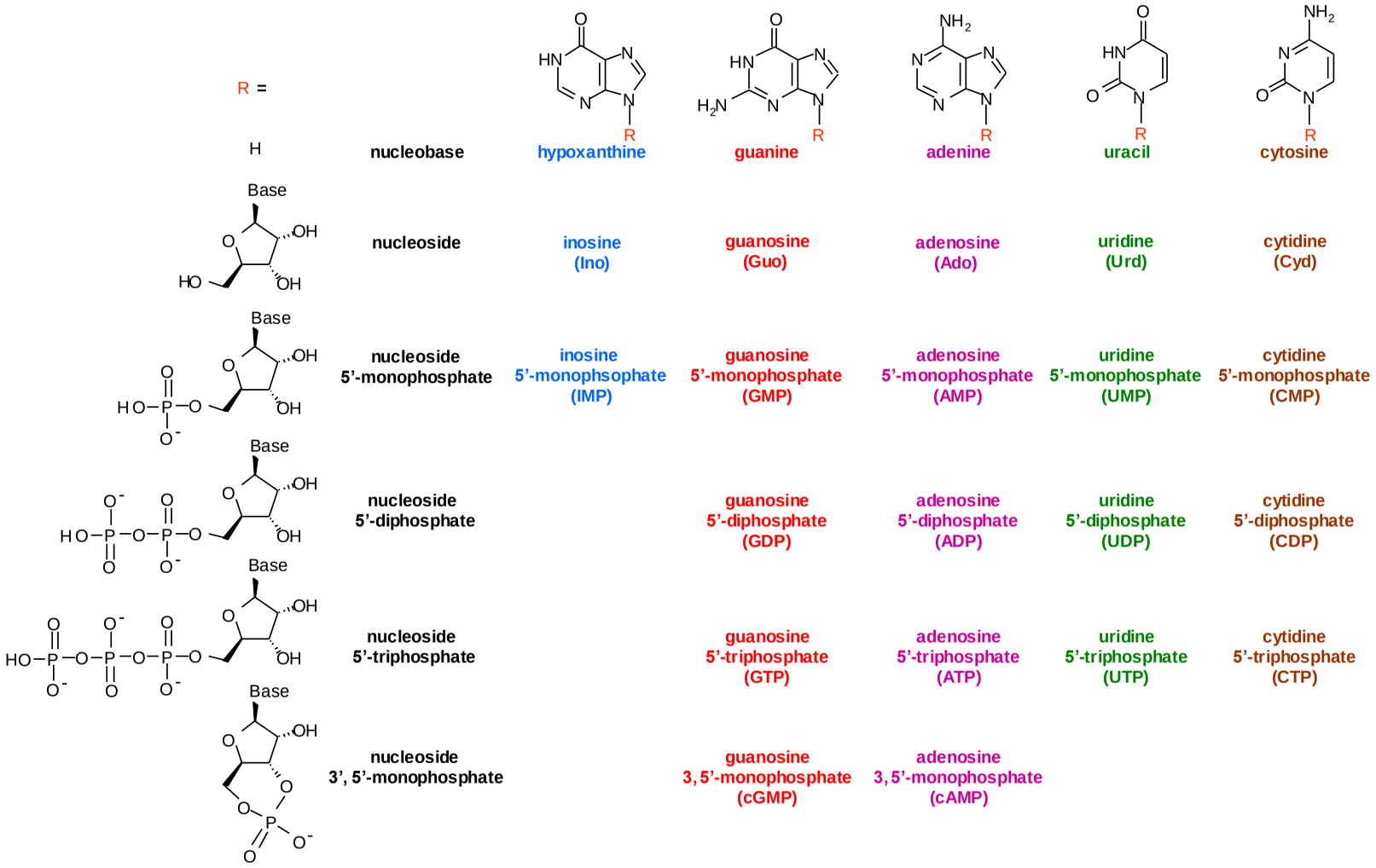

Figure 2. Nucleobase, nucleoside, and nucleotide structure

The chemical behaviour of the polyvalent phosphate group, dominated by its negative charge at physiological pH (5–7) and its chemical stability, confers properties that make nucleotides suitable as building blocks within genetic material (Westheimer, 1987). The presence of high-energy phosphate bonds in adenosine triphosphate (ATP) provides a mechanism whereby chemical energy can be stored and then liberated when needed (Gropper *et al*., 2009).

2.1.2. PHYSICAL PROPERTIES OF NUCLEOTIDES

The acid-base behaviour of a nucleobase is the critical factor that determines its charge, its tautomeric structure and the donation or acceptance of protons. Cytosine and adenine are strongly basic and are protonated at moderately acidic conditions. The dissociation constant for this process is defined as in **Equation 1**. Uracil is weakly acidic and is deprotonated in weakly alkaline conditions. The dissociation constant for this process is defined as in **Equation 2**. Guanine and hypoxanthine are intermediate compounds, and are protonated in strongly acidic conditions, and are deprotonated in weakly alkaline conditions.

$$pK_a = -\log K_a = -\log \frac{[H^+][B]}{[HB^+]} \qquad \text{(Equation 1)}$$

$$pK_a = -\log K_a = -\log \frac{[H^+][B^-]}{[HB]} \qquad \text{(Equation 2)}$$

Those nucleobases with amine groups, adenine, guanine and cytosine, are protonated on the ring nitrogens rather than the free amine group. The positions of (de)protonation and pK_a values of nucleobases are given in **Figure 3**. All of the nucleobases are uncharged from pH = 5–7, that is, at physiological pH.

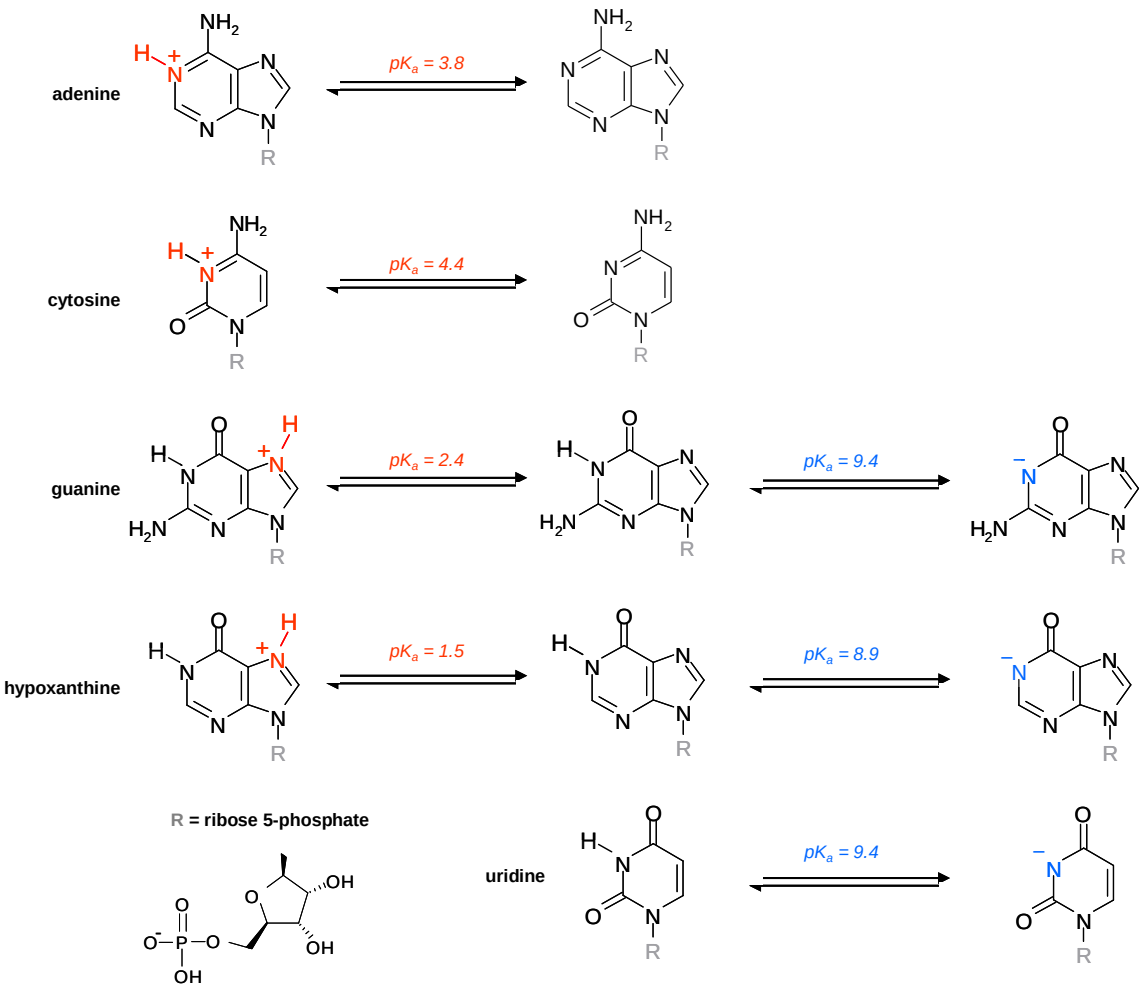

Figure 3. Site and pK_a values for (de)protonation of nucleotides (adapted from Bloomfield et al., 2000; Dawson et al., 1986)

Nucleobases are capable of undergoing tautomeric isomerisation as either keto-enol or amine-imine equilibria. Spectroscopic analysis has shown that the major nucleobases exist primarily (> 99.99%) in their keto or amine forms (Blackburn *et al*., 2006).

The presence of the phosphate group in nucleotides has the effect of making the ring nitrogen atoms more basic as illustrated by a higher pK_a value of the nucleotide compared to the corresponding nucleoside; **Table 1**. The phosphate group of nucleotides possess two ionisable protons. The pK_a values of these are only slightly dependant on the

nucleobases and position of the phosphate on the ribose sugar. The pK_a for the loss of the first and second proton from the phosphate group are ~1 and ~6–7 respectively, and hence, the phosphate group carries a negative charge at physiological pH. It is only at pH > 12 that a proton is removed from the 2′, 3′-diol of the ribose (Blackburn *et al*., 2006; Dawson *et al*., 1986).

Table 1. Nucleoside and nucleotide pK_a values

Base	pK_a	
[site of (de)protonation]	Nucleoside	Nucleotide
adenine [N-1]	3.5	3.8
cytosine [N-3]	4.2	4.4
guanine [N-1, N-7]	1.6, 9.2	2.4, 9.4
uracil [N-3]	9.2	9.4
hypoxanthine [N-1, N-7]	1.2, 8.9	1.5, 8.9

2.1.3. NUCLEOTIDE METABOLISM

In tissues such as the gut and in the immune system in which cells are rapidly turned over, there is a continuous requirement for production of nucleic acids and their constituent nucleotides. To meet cellular demand, nucleotides are supplied via *de novo* synthesis, the salvage pathway, and dietary absorption (Boza and Martínez-Augustin, 2002).

2.1.3.1 Dietary Nucleotides

Nucleotides in the diet are ingested in the form of nucleoproteins, polymeric nucleotides (nucleic acids), nucleotide derivatives, and monomeric nucleotides. These are digested in the gastrointestinal tract by proteases, nucleases, phosphatases and nucleotidases and are available for absorption predominantly as nucleosides; **Figure 4** (Gil *et al*., 2007;

Quan *et al*., 1990; Sonoda and Tatibana, 1978; Thorell *et al*., 1996; Uauy *et al*., 1994).

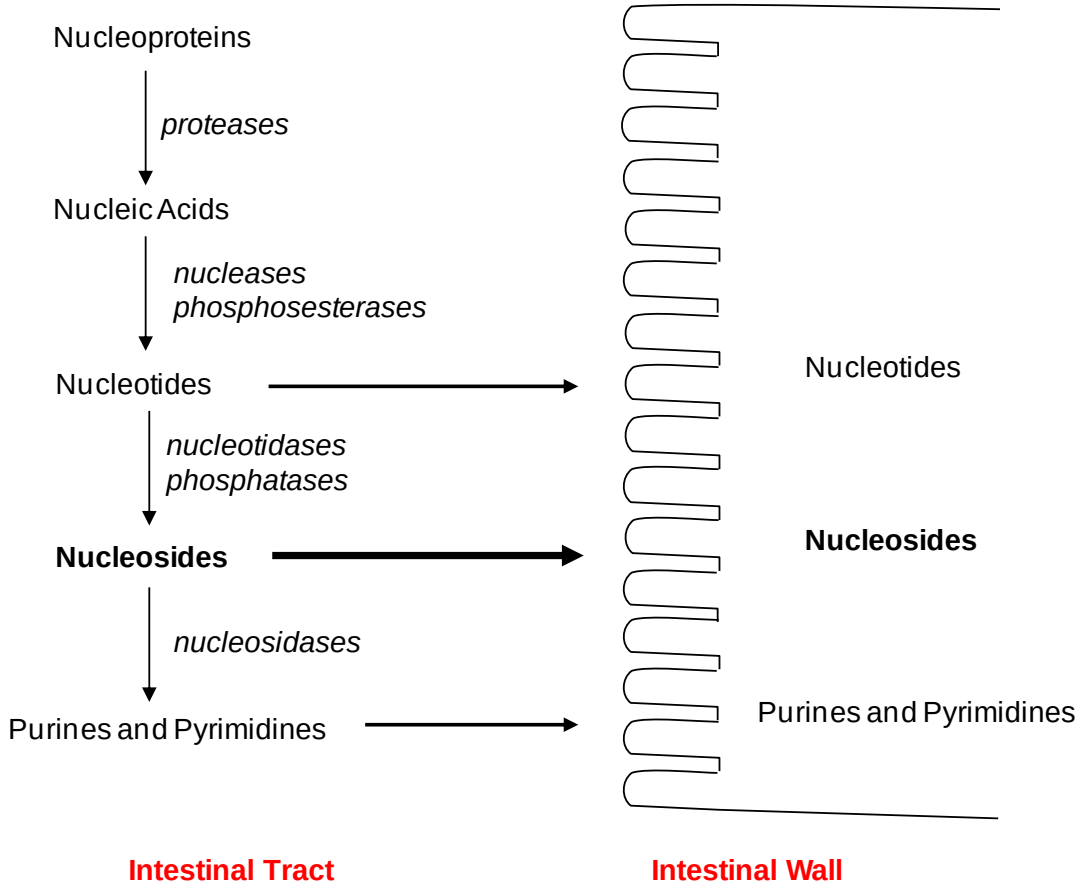

Figure 4. Enzymatic digestion of nucleotides to nucleosides in the gut (from Quan et al., 1990)

Only a small proportion of dietary nucleotides are incorporated directly into nucleotide tissue pools, with the rest converted to uric acid and other metabolites (Quan, 1992).

2.1.3.2 Purine Nucleotide Biosynthesis

The purine nucleotides are all derived from inosine 5′-monophosphate (IMP) (Blackburn *et al*., 2006; Garrett and Grisham, 1999; McMurry and Begley, 2005; NC-IUBMB, 2010). The initial step in *de novo* IMP

synthesis is the formation of 5-phosphoribosyl α-diphosphate (PRPP) from α-D-ribose 5-phosphate and ATP followed by multi-step formation of the purine base; **Figure 5**

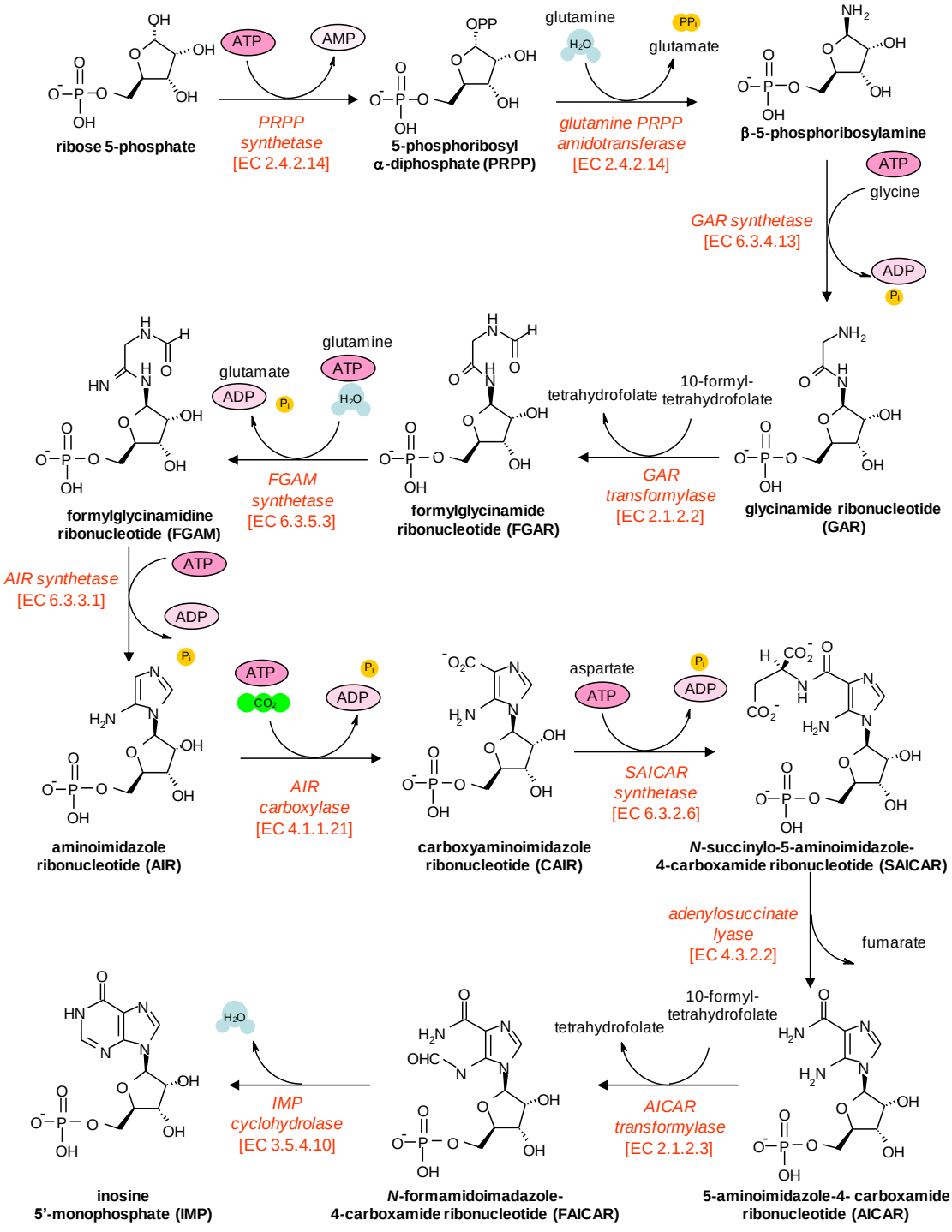

Figure 5. Biosynthesis of IMP from ribose 5'–phosphate (from McMurray and Begley, 2005; Garrett and Grisham, 1999)

The transfer of a diphosphate group from ATP to ribose-5-phosphate forms the α-ribosyl pyrophosphate stereoisomer of PRPP. Glutamine PRPP amidotransferase catalyses the subsequent transfer of an amine group via condensation of glutamine, followed by removal of glutamate from the product and involves an inversion of the configuration of the substituent on the C-1 of the sugar, thereby establishing the β configuration of the forthcoming nucleotide.

Glycinamide ribonucleotide (GAR) is formed by the nucleophilic acyl substitution of glycine with phosphoribosylamine in a reaction catalysed by GAR synthetase. Formylation of the amino group occurs via nucleophilic acyl substitution reaction catalysed by GAR transformylase whereby a formyl group is transferred from 10-formyltetrahydrofolate (10-formyl THF) to form formylglycinamide ribonucleotide (FGAR).

The formation of the amidine formylglycinamidine ribonucleotide (FGAM) by reaction of FGAR with ATP and ammonia (from glutamine) is catalysed by FGAM synthetase. The formation of the imidazole ring to form aminoimidazole ribonucleotide (AIR) is catalysed by AIR synthetase. In vertebrates a single enzyme, AIR carboxylase, catalyses the addition of CO_2 at the C-4 position of the imidazole ring giving carboxyaminoimidazole ribonucleotide (CAIR).

The nucleophilic acyl substitution reaction of aspartate with CAIR to form *N*-succinylo-5-aminoimidazole-4-carboxamide ribonucleotide (SAICAR) is catalysed by SAICAR synthetase. Adenylosuccinate lyase catalyses the elimination of fumarate from SAICAR to 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR). The AICAR transformylase catalysed addition of a formyl group from 10-formyl THF to AICAR produces *N*-formamidoimidazole-4-carboxamide ribonucleotide (FAICAR).

The final step in the synthesis of IMP is the cyclisation of FAICAR catalysed by IMP cyclohydrolase. The *de novo* synthesis of IMP from ribose 5-phosphate requires 7 moles of ATP equivalents, 2 moles of

glutamine, 2 moles of formate (from 10-formyl THF), and 1 mole each of glycine, aspartate, and CO_2 (Blackburn *et al*., 2006; Garrett and Grisham, 1999; McMurry and Begley, 2005; NC-IUBMB, 2010).

From the synthesis of IMP, there is a divergence of pathways converting IMP to either adenosine 5′-monophosphate (AMP) or guanosine 5′-monophosphate (GMP); **Figure 6**.

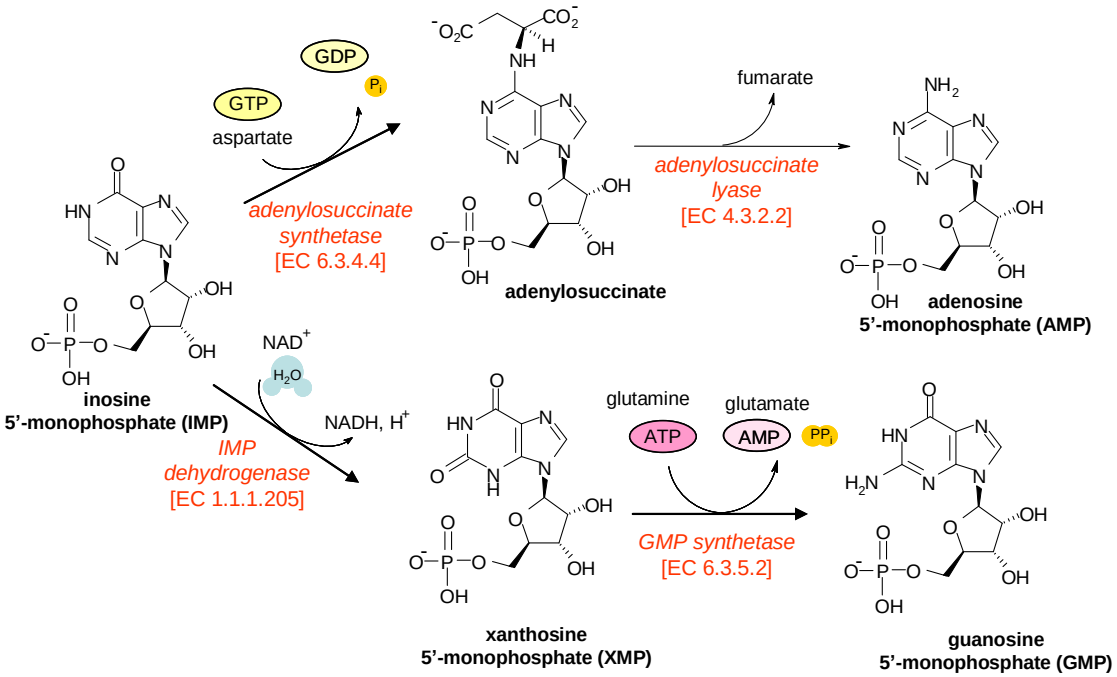

Figure 6. Biosynthesis of GMP and AMP from IMP (from McMurray and Begley, 2005; Garrett and Grisham, 1999)

The biosynthesis of GMP is a two-step process whereby C-2 of the inosine moiety is oxidised, followed by a glutamine dependent amidotransferase reaction replacing the oxygen with an amine group. AMP is also derived from IMP in a two-step sequence whereby aspartate replaces the carbonyl group of inosine, followed by elimination of fumarate (Boza and Martínez-Augustin, 2002; Garrett and Grisham, 1999; NC-IUBMB, 2010).

Purine nucleoside di- and triphosphates are derived from corresponding nucleotide monophosphates via phosphate transfer from nucleotide triphosphates (frequently ATP) catalysed by nucleotide phosphate kinase and nucleoside diphosphate kinase (NC-IUBMB, 2010).

2.1.3.3 Regulation of Purine Nucleotide Synthesis

The first committed step in the *de novo* purine biosynthetic pathway is the replacement of pyrophosphate at C-1 by an amine group from glutamine catalysed by PRPP aminotransferase; **Figure 7**.

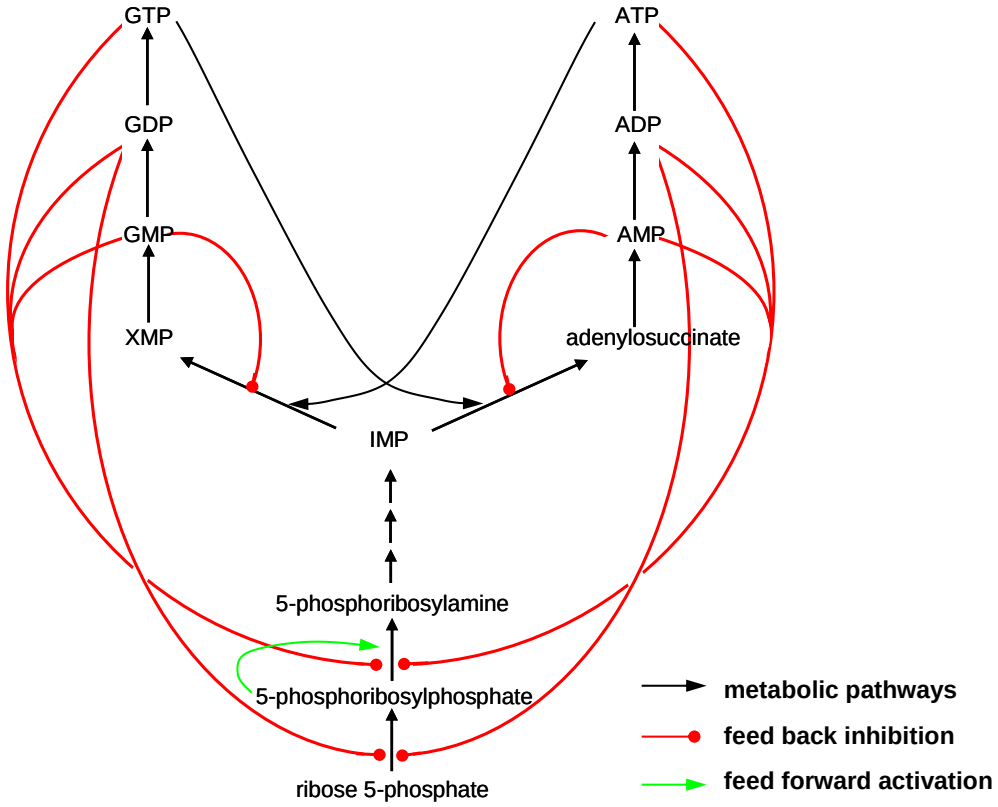

Figure 7. Feed back inhibition in purine synthesis (adapted from Garrett and Grisham, 1999)

This step is regulated by allosteric control by feedback inhibition from adenosine and guanosine nucleotides. Feedback inhibition occurs when an enzyme catalysing an early step in the metabolic pathway is inhibited by the end product of the pathway (Blackburn *et al*., 2006; Kornberg, 1974).

Biosynthesis of GMP and AMP is regulated to recognise which purine nucleotide is in excess and which is in short supply, whereby, the synthesis of AMP requires guanosine triphosphate (GTP) as a co-factor and conversely GMP synthesis requires ATP. This regulation mechanism

ensures that AMP and GMP are in equivalent abundance (Garrett and Grisham, 1999; Kornberg, 1974).

2.1.3.4 Pyrimidine Nucleotide Biosynthesis

The pyrimidine nucleotides are all derived from uridine 5′-monophosphate (UMP). The synthesis of pyrimidines differs significantly from that of purines in that the ring structure is assembled as a free base, not assembled stepwise from PRPP. The biosynthetic pathway for pyrimidines is less complex than that of the purines and begins with the carbamoyl phosphate synthetase-II (CPS-II) catalysed formation of carbamoyl phosphate from bicarbonate, ATP and ammonia (from glutamine); **Figure 8**.

The nucleophilic acyl substitution reaction of carbamoyl phosphate and aspartate, with phosphate as the leaving group forming carbamoyl aspartate, is catalysed by aspartate carbamoyltransferase. Cyclisation catalysed by dihydroorotase then forms dihydroorotate; this is subsequently oxidised to orotate by dihydroorotate oxidase, a flavin-dependent enzyme that uses coenzyme Q, as the ultimate electron acceptor. The orotate phosphoribosyltransferase catalysed condensation of orotate and PRPP with elimination of inorganic pyrophosphate fixes the pyrimidine in the β-configuration to form orotidine 5′-monophosphate (OMP). The final step in UMP synthesis is the decarboxylation of OMP; catalysed by OMP decarboxylase. Cytidine 5′-monophosphate (CMP) is not formed directly from UMP but in a series of reactions via uridine triphosphate (UTP) and cytidine triphosphate (CTP) (Boza and Martínez-Augustin, 2002; Carver and Walker, 1995; Kornberg, 1974; McMurry and Begley, 2005).

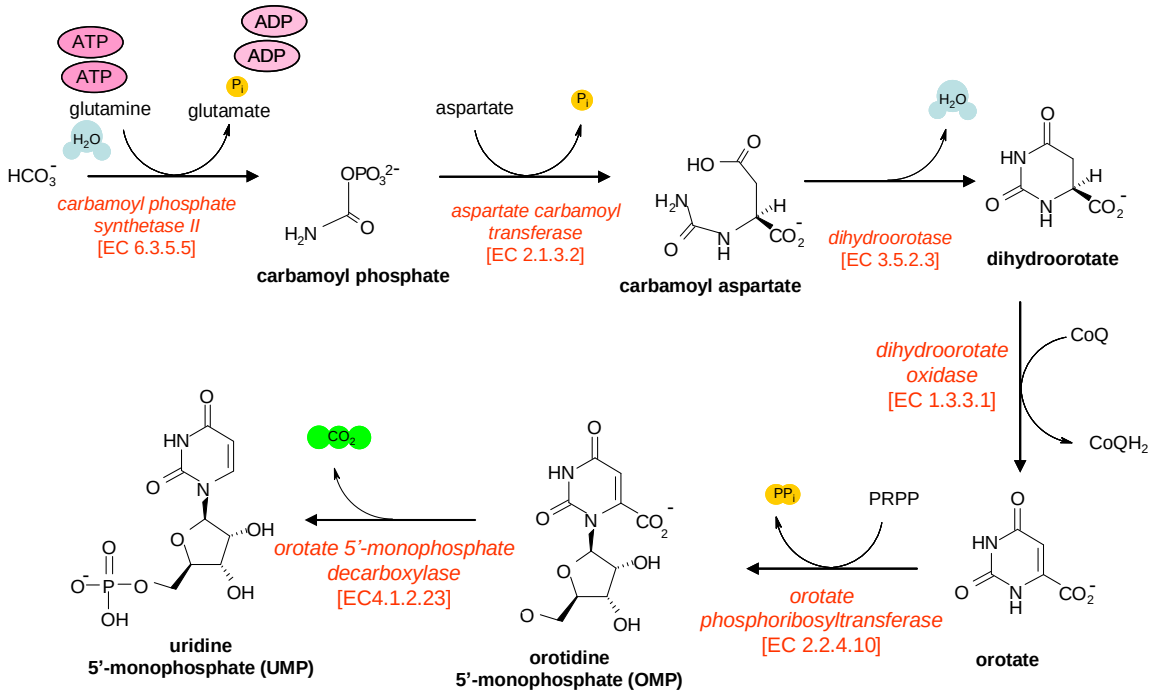

Figure 8. Biosynthesis of UMP from glutamine and bicarbonate (from McMurray and Begley, 2005; Garrett and Grisham, 1999)

UTP is derived from UMP via phosphate transfers from ATP catalysed by nucleoside monophosphate kinase to give uridine diphosphate (UDP), which in turn is phosphorylated to UTP by ATP in a reaction catalysed by nucleotide diphosphate kinase. CTP is derived from amination of UTP catalysed by CTP synthetase (Garrett and Grisham, 1999; Kornberg, 1974; McMurry and Begley, 2005).

The *de novo* synthesis of UMP from bicarbonate and glutamine requires 2 moles of ATP, and 1 mole each of aspartate and PRPP (Blackburn *et al*., 2006; Garrett and Grisham, 1999; McMurry and Begley, 2005; NC-IUBMB, 2010).

2.1.3.5 Regulation of Pyrimidine Nucleotide Synthesis

Regulation of pyrimidine synthesis is controlled in animals at the first step through feedback inhibition of CPS-II by UDP and UTP. ATP and PRPP are allosteric activators, whereby accumulation of these pyrimidine nucleotide precursors signals the need for more pyrimidine nucleotides; **Figure 9** (Garrett and Grisham, 1999; Kornberg, 1974).

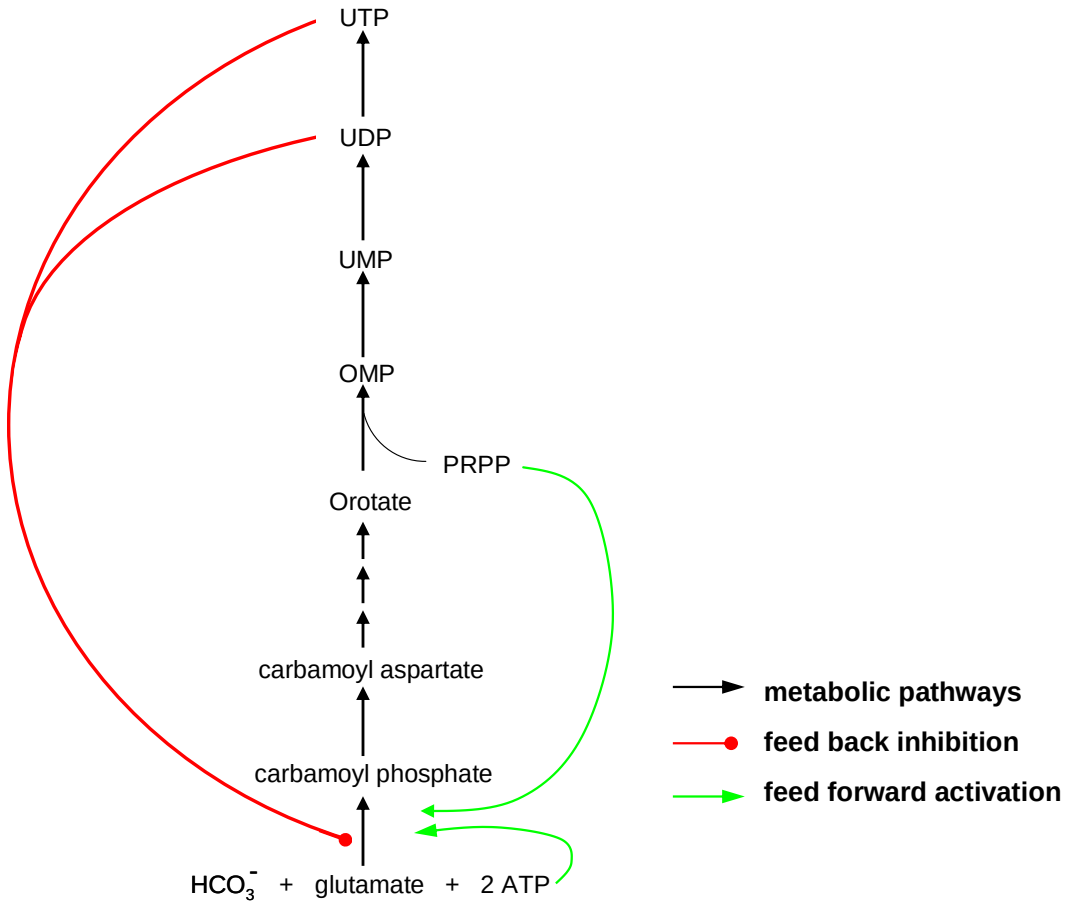

Figure 9. Feed back inhibition in purine synthesis (adapted from Garrett and Grisham, 1999)

2.1.3.6 The Salvage Pathway

Biosynthesis of nucleotides *de novo* is expensive energetically requiring 7 ATP equivalents per mole of IMP, and 4 ATP equivalents per mole of UMP. Salvage and reuse of nucleotides is energetically more efficient

than *de novo* synthesis and requires only 1 mole ATP equivalent per mole UMP or IMP produced. The salvage pathway utilises nucleobases and nucleosides scavenged from dietary sources or left over from cellular metabolism; **Figures 10–11**.

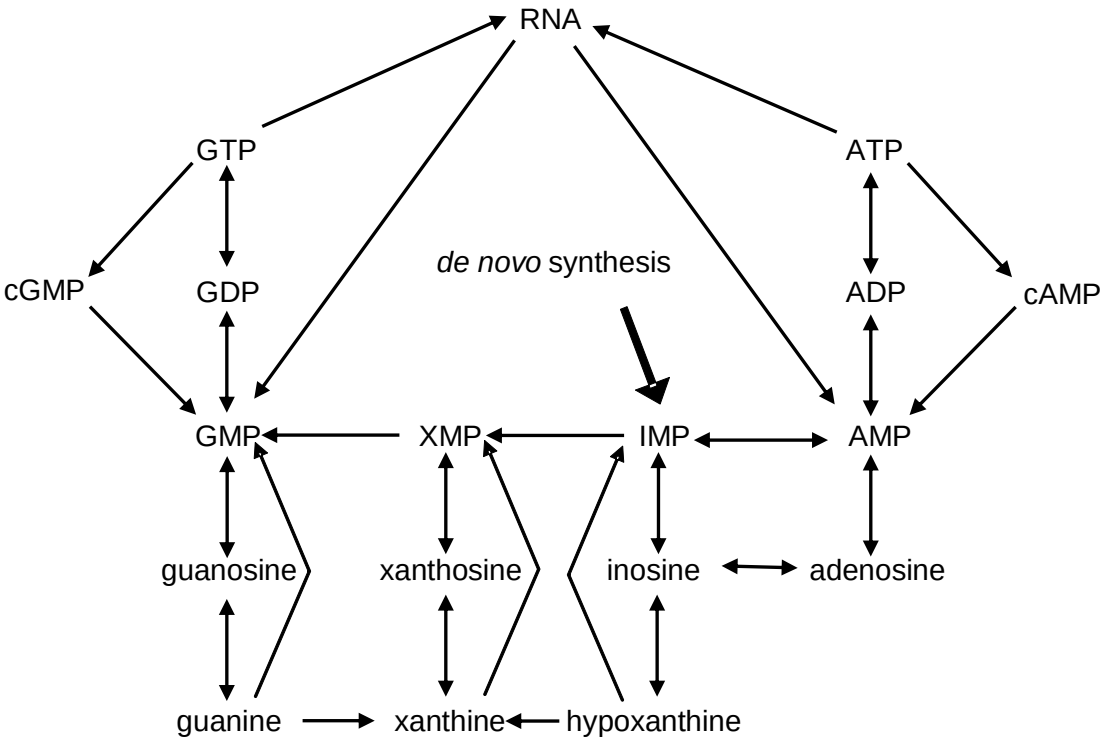

Figure 10. Purine salvage pathways (adapted from la Marca et al. 2006)

Salvage of nucleobases occurs via two mechanisms; one is the direct conversion of a nucleobase to the corresponding nucleotide catalysed by nucleotide pyrophosphorylase enzymes, and the other requires sequential conversion of the nucleobase to the nucleoside catalysed by nucleotide phosphorylase enzymes with subsequent phosphorylation of the nucleoside catalysed by a nucleoside kinase enzyme (Chu, 1991).

Salvage of nucleotides occurs via the interconversion of nucleoside mono-, di-, and triphosphates in reactions catalysed by nucleoside monophosphate kinases and nucleoside diphosphate kinases. In addition, base alteration *via* deamination of AMP→IMP, adenosine→inosine, and

cytosine→uracil catalysed by specific enzymes readily occurs (Kornberg, 1974).

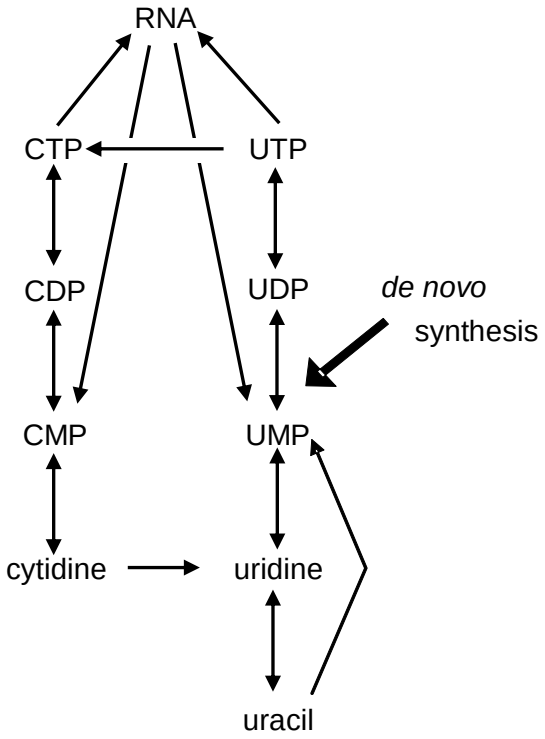

Figure 11. Pyrimidine salvage pathways (adapted from la Marca et al. 2006)

2.1.3.7 Purine Nucleotide Catabolism

Guanosine catabolism begins with GMP conversion by 5′-nucleotidase into guanosine, which in turn is converted into guanine and ribose-1-phosphate by purine nucleoside phosphorylase, with guanine converted into xanthine by guanine deaminase. Adenosine nucleotides are degraded to adenosine by 5′-nucleotidase. In mammals, adenosine is not cleaved to its constituent base adenine, but is instead deaminated to inosine, with subsequent cleavage of the ribose by purine nucleoside phosphorylase to give hypoxanthine, which is oxidised to xanthine. The final step of purine catabolism in primates is the oxidation of xanthine to uric acid, catalysed by xanthine oxidase. In mammals other than primates, uric acid is

oxidised by urate oxidase to allantoin and excreted; **Figure 12** (Garrett and Grisham, 1999; McMurry and Begley, 2005).

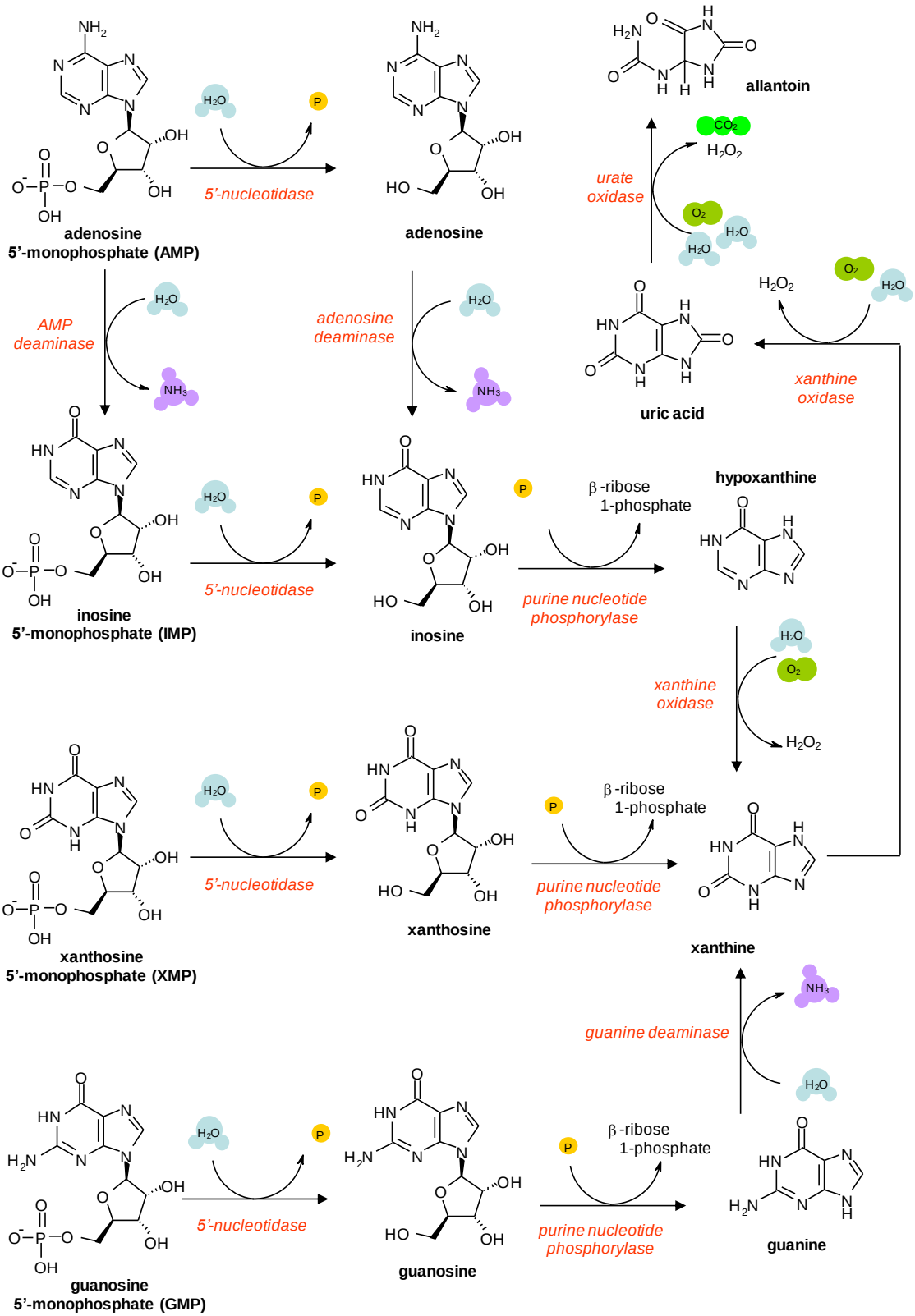

Figure 12. Pathway of purine nucleotide catabolism to uric acid (from McMurray and Begley, 2005; Garrett and Grisham, 1999)

2.1.3.8 Pyrimidine Nucleotide Catabolism

Cytidine catabolism begins with cytidine deaminase catalysed hydrolytic deamination to uridine. Unlike the purine nucleosides, pyrimidine nucleosides undergo ring cleavage. The catabolism of uridine starts with the cleavage of the ribose moiety yielding uracil, which after reduction by NADPH to dihydrouracil, is then hydrolysed to the open-chain β-ureidopropionate, with further hydrolysis and decarboxylation yielding β-alanine, ammonia and carbon dioxide; **Figure 13** (Garrett and Grisham, 1999; McMurry and Begley, 2005).

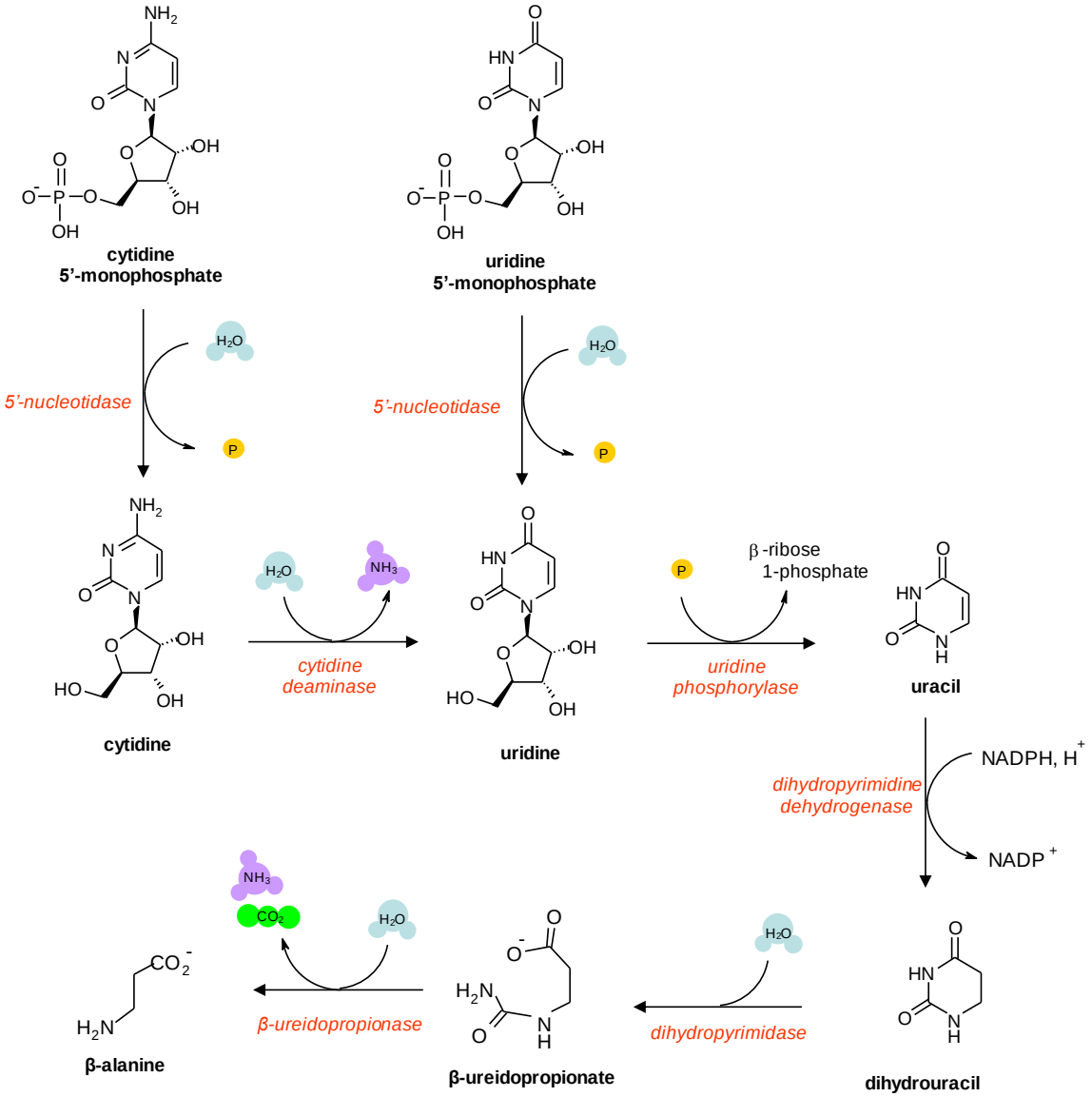

Figure 13. Pathway of pyrimidine nucleotide catabolism to β-alanine (from McMurray and Begley, 2005; Garrett and Grisham, 1999)

2.2. Nucleosides and Nucleotides in Milk

Non-protein nitrogen accounts for approximately 20% of the total nitrogen in human milk but only 2% in bovine milk (Atkinson *et al*., 1980; Donovan and Lönnerdal, 1989). Nucleotides account for between 0.4% and 0.6% of non-protein nitrogen content and between 0.10% and 0.15% of the total nitrogen content of human milk. From 2-12 weeks post-partum, it was found that as lactation advances, the contribution of nucleotide nitrogen to total nitrogen in milk increases (Janas and Picciano, 1982).

The origin of nucleosides and nucleotides in milk; whether they diffuse from the blood into the milk via the blood-milk barrier, or are actively secreted from lactating cells or formed by post-secretory metabolic processes in milk, has not yet been established (Liao *et al*., 2011; Schlimme *et al*., 2000).

The major nucleotide-related compound in bovine milk is orotic acid, a precursor in the synthesis of UMP, although it is not detected in human milk (Gil and Sánchez-Medina, 1982; Gill and Indyk, 2007b; Indyk and Woollard, 2004; Larson and Hegarty, 1979). Bovine milk orotic acid levels increase as lactation progresses, beginning at very low levels in early colostrum to over 200 μmol hg^{-1} in mid-season milk, before reducing in late-season milk (Gill and Indyk, 2007b). This is in contrast to nucleoside and nucleotide levels, which decline rapidly with lactation. High levels of dietary orotic acid leads to severely fatty livers in rats, which appear to be unique in this respect amongst a number of species studied. None of the evidence suggests that orotic acid in milk poses a problem to human consumers (Durschlag and Robinson, 1980).

2.2.1. NUCLEOTIDES IN MILK

Nucleotides were first isolated from human milk by Deutsch and Nilsson in 1960, and since that time, at least 13 acid-soluble nucleotides have been identified (Böhles *et al*., 1998). The expression of nucleotides is highest immediately after parturition with a general trend of decreasing amounts with advancing lactation and with levels stabilising by the third week of lactation in both human and bovine milks (Gil and Sánchez-Medina, 1981; Gill and Indyk, 2007b; Sugawara *et al*., 1995).

This pattern of high concentration in early colostrum followed by a rapid decrease as lactation progresses is analogous to changes in concentration of other bioactive components, such as immunoglobulin. The presence of immunoglobulins in bovine colostrum provides passive immunity to the newborn calf, until maturation of its own immune system (Mehra *et al*., 2006). Elevated levels of nucleotides present in colostrum may be due to their capacity to enhance immune response. Recently, it was shown that dietary UMP affected the immune response of newborn calves and may stimulate humoral and mucosal immunity (Mashiko *et al*., 2009).

It has been generally reported that nucleotides are present in higher amounts in human milk compared to bovine milk (Gill and Indyk, 2007b; Oliveira *et al*., 1999; Sugawara *et al*., 1995). Qualitatively, there is a clear difference in the nucleotide profile between mature human milk and mature bovine milk, the former containing measurable levels of GMP, IMP, UMP, CMP, and AMP, whereas the latter typically contains only CMP and AMP. A survey of the free nucleotide levels that have been reported for milk of both species shows a wide range of results that depend, at least in part, on the various analytical methodologies utilised for quantitation; **Tables 2–3**.

*Table 2. Nucleotides in mature bovine milk (adapted from Gill and Indyk 2007*b*)*

Analyte[a,b] (μmol dL^{-1})					Reference
AMP	CMP	GMP	IMP	UMP	
nd	0.9	nd	–	nd	Kobata *et al*., 1962
nd–0.4	0.9–2.7	nd	–	nd	Johke, 1963
1.8–2.9	1.2–4.9	nd	–	nd	Gil and Sánchez-Medina, 1981[c]
2.0–2.8	1.9–3.3	nd	–	nd	Gil and Sánchez-Medina, 1981[d]
–	0.3	0.2	–	–	Tiemeyer *et al*., 1984[e]
trace	3.0	nd	nd	nd	Ferreira *et al*., 2001[f]
0.1	1.0	nd	0	0.1	Sugawara *et al*., 1995
nd	0.2–0.3	nd	nd	nd	Gill and Indyk, 2007b

[a] Collated results for milks greater than 2 weeks post partum; all results rounded to 1 decimal place
[b] AMP = adenosine 5′-monophosphate; CMP = cytidine 5′-monophosphate; GMP = guanosine 5′-monophosphate; IMP = inosine 5′-monophosphate; UMP = uridine 5′-monophosphate
[c] Ion-exchange chromatography
[d] Enzymatic analysis
[e] Adapted from results reported as μmol L^{-1}
[f] Adapted from results reported as mg dL^{-1}
nd = not detected
– = not measured

*Table 3. Nucleotides in mature human milk (adapted from Gill and Indyk 2007*b*)*

Analyte[a,b] (μmol dL^{-1})					Reference
AMP	CMP	GMP	IMP	UMP	
0.3	3.3	0.2	–	0.4	Kobata *et al*., 1962
1.5–2.6	1.8–2.6	nd–0.3	–	0.7–1.3	Gil and Sánchez-Medina, 1982[c]
0.4–0.5	1.0–1.6	0.3–0.5	0.6–0.8	1.0–1.7	Janas and Picciano, 1982[d]
nd–0.4	0.3–4.3	nd–0.1	nd–0.1	nd–0.3	Sugawara *et al*., 1995
0.2–1.9	4.1–10.6	0–0.6	nd	0.5–2.1	Thorell *et al*., 1996[e]
nd	nd–1.9	nd	nd	0.2–0.5	Gill and Indyk, 2007b
0.4–0.82	0.66–11.4	0–0.34	2.2–5.6	0.1–2.7	Liao *et al*., 2011[f]

[a] Collated results for milks greater than 2 weeks post partum; all results rounded to 1 decimal place
[b] AMP = adenosine 5′-monophosphate; CMP = cytidine 5′-monophosphate; GMP = guanosine 5′-monophosphate; IMP = inosine 5′-monophosphate; UMP = uridine 5′-monophosphate
[c] Adapted from results at 15 days, 1 month, and 3 months post partum
[d] Adapted from results reported as mg dL^{-1} at 4, 8, and 12 weeks post partum
[e] Adapted from range of results reported as μmol L^{-1} at 3–24 weeks post partum
[f] Adapted from range of results reported as μmol L^{-1} from 1 week to 9 months post partum
nd = not detected
– = not measured

Nucleoside diphosphates and nucleotide sugars also contribute to the nucleotide pool in milks of both species (Gil and Sánchez-Medina, 1981, 1982; Janas and Picciano, 1982; Johke, 1963; Sugawara *et al*., 1995). Significant levels of cytidine 5′-diphosphate (CDP), ranging from 6.95–41.1 µmol dL^{-1} were found in a recent study of breast milk from Taiwanese women (Liao *et al*., 2011).

Cyclic nucleotides are also present in human milk although results obtained show a wide range of concentrations from 0.1–0.7 nmol L^{-1} and 0.01–0.15 nmol L^{-1} for adenosine 3′,5′-cyclic monophosphate (cAMP) and guanosine 3′,5′-cyclic monophosphate (cGMP), respectively. Levels of cGMP were highest at the beginning of lactation and tended to decrease over the first fortnight, whereas levels of cAMP were relatively consistent throughout (Skala *et al*., 1981).

The nucleotides levels in human milk are not consistent throughout each day but have been shown to vary in a diurnal rhythm. This rhythmicity has been reported in AMP, UMP, GMP concentrations (Sánchez *et al*., 2009), and cGMP (Skala *et al*., 1981). It is hypothesised that elevated nucleotide levels at night may induce sleep in infants (Sánchez *et al*., 2009).

2.2.2. NUCLEOSIDES IN MILK

Pyrimidine nucleosides are most abundant in mature bovine milk, whereas human milk has significant amounts of both purines and pyrimidine nucleosides; **Tables 4–5**. In both human and bovine milk the nucleoside concentration decreases during the colostral phase and reaches a constant level approximately three weeks post-partum (Schlimme *et al*., 2000).

*Table 4. Nucleosides in mature bovine milk (adapted from Gill and Indyk 2007*b*)*

Analyte[a,b] (µmol dL^{-1})					Reference
Ado	Cyd	Guo	Ino	Urd	
–	0.6	–	–	7.3	Tiemeyer *et al.*, 1984[c]
0–0.4	0.1–1.1	0–0.2	0–0.3	0.4–6.8	Schlimme *et al.*, 1996[d]
nd	0.4–1.6	nd	nd	nd–1.4	Gill and Indyk, 2007b

[a] Collated results for milks greater than 2 weeks post partum; all results rounded to 1 decimal place

[b] Ado = adenosine
Cyd = cytidine
Guo = guanosine
Ino = inosine
Urd = uridine

[c] Adapted from results reported as µmol L^{-1}

[d] Adapted from results reported as µmol L^{-1} for whole lactation period excluding first 3 weeks postpartum

nd = not detected
– = not measured

Table 5. Nucleosides in mature human milk (adapted from Gill and Indyk 2007b)

Analyte[a,b] (µmol dL^{-1})					Reference
Ado	Cyd	Guo	Ino	Urd	
0.5	0.4	0	–	0.5	Topp *et al*., 1993
0.5–1.6	1.2–1.5	–	–	0.4–3.6	Sugawara *et al.*, 1995[c]
0.1–0.5	0.4–0.7	0–0.1	–	0.3–0.8	Leach *et al*., 1995[d]
nd	0.4–1.0	nd	nd	nd–0.3	Thorell *et al*., 1996[e]
0–4.0	0.1–1.6	0.1–1.1	–	0.1–4.4	Schlimme *et al.*, 1996[f]
nd	nd	nd	nd	0.4–0.5	Gerichhausen *et al*., 2000[g]
1.0	7.9	0.8	nd	3.7	Gill and Indyk, 2007b
0–0.12	0.3–2.5	0.01–0.11	0–0.9	0.22–1.5	Liao *et al*., 2011[h]

[a] Collated results for milks greater than 2 weeks post partum, all results rounded to 1 decimal place
[b] Ado = adenosine; Cyd = cytidine; Guo = guanosine; Ino = inosine; Urd = uridine
[c] Adapted from results reported pmol mL^{-1} at 20 d postpartum
[d] Adapted from results reported as TPAN in µg/L for early and late mature milk
[e] Adapted from results reported as µmol L^{-1} at 3-24 wk postpartum
[r] Adapted from results reported as µmol L^{-1} from 15-120 d postpartum
[g] Adapted from results reported as TPAN in µg L^{-1} at 1 and 3 months
[h] Adapted from range of results reported as µmol L^{-1} from 1 week to 9 months post partum.
nd = not detected
– = not measured

In bovine milk, the levels of pyrimidine nucleosides are higher than purine nucleosides. Uridine, while relatively high in early colostrum, decrease by approximately two orders of magnitude within hours post-partum, whereas cytidine reaches a maximum on the second day of lactation (Gill and Indyk, 2007b).

Analysis of human and bovine milk by Schlimme *et al.* (1986a) found at least 10 nucleosides: uridine, cytidine, *N*-1-methyladenosine, inosine, *N*-3-methyluridine, *N*-1-methylinosine, adenosine, *N*-2-methylguanosine, *N*-2-dimethylguanosine, and *N*-6-carbamoyl-threonyladenosine.

Cytidine, uridine, and adenosine are found at similar concentrations in most analyses of human milk, whereas guanosine is at lower concentrations and inosine was found only in one study (Liao *et al*., 2011). Dietary, geographical, and seasonal variations are all likely to have a significant influence upon nucleoside levels in the human milk (Liao *et al*., 2011; Sugawara *et al*., 1995).

2.2.3. TOTAL POTENTIALLY AVAILABLE NUCLEOSIDES IN MILK

Leach *et al*. (1995) developed a method to quantify TPAN sources of human milk. The TPAN method simulates enzymatic conversion of the various sources of nucleosides that occurs during digestion and absorption and hence, has the advantage of reporting a more representative result in terms of infant nutrition.

The TPAN concentrations were determined in milks of both European and American women, with a mean concentration of 18.9 μmol dL^{-1} and 16.1 μmol dL^{-1}, respectively (Leach *et al*., 1995). Similar results (20.3 μmol dL^{-1}) were obtained in a TPAN study of milk from Asian women (Tressler *et al*., 2003). Little variation or trends in TPAN concentrations

were found over different stages of lactation in both studies (Leach *et al*., 1995; Tressler *et al*., 2003). The level of TPAN in human milk is more than twice the levels of nucleotides (Leach *et al*., 1995).

2.3. Nutritional Effects of Nucleotides

In times of rapid growth, the metabolic demand for nucleotides exceeds the capacity of *de novo* synthesis or the salvage pathway, and dietary sources of nucleotides may be essential for continued optimal metabolic function (Yu, 1998). The role nucleotides play in infant nutrition has been reviewed comprehensively by Carver and Walker (1995) and more recently by Michaelidou and Steijns (2006), and by Schaller *et al*. (2007). The role nucleotides play in the immune and gastrointestinal systems was the focus of a recent review by Hess and Greenberg (2012).

2.3.1. IMMUNE RESPONSE

Nucleotide supplemented diets have been associated with enhanced humoral and cellular immune function, both *in vitro* and *in vivo* (Jyonouchi, 1994). The addition of nucleotides to infant formula provides immunologic benefits not available to unsupplemented infant formulas (Schaller *et al*., 2007). In particular, dietary nucleotide supplementation may be important for individuals at increased risk of acquiring infections such as infants, especially those born prematurely (Carver and Walker, 1995).

Results reported from studies on the effect on serum immunoglobulin levels in infants receiving infant formula supplemented with nucleotides compared to those receiving unsupplemented formula are somewhat mixed. Increased levels of serum immunoglobulin (Ig) A in healthy term infants fed nucleotide-supplemented formula (n = 166) were reported by

Yau *et al*. (2003). Navarro *et al*. (1999) reported elevated levels of plasma IgA and IgM in a study of pre-term infants fed nucleotide-supplemented formula (*n* = 14). In contrast to these results, no differences between infants fed nucleotide-supplemented or unsupplemented formula were found in serum levels of IgA and IgG (*n* = 101) (Pickering *et al*., 1998), IgM and IgE (*n* = 166) (Yau *et al*., 2003), and plasma levels of IgG (*n* = 14) (Navarro *et al*., 1999).

The effect of dietary nucleotides has been shown to promote an increase in immune cell proliferation and may facilitate maturation in some lymphocyte populations similar to that of breast-fed infants (*n* = 138) (Buck *et al*., 2004). A study by Carver *et al*. (1991) reported that at two months of age nucleotide-supplemented formula fed infants (*n* = 138) had significantly higher natural killer (NK) cell activity and interleukin-2 (IL2) levels than those receiving unsupplemented formula. The difference between the two groups was only temporary however, as by four months of age no significant differences were found. No effect of nucleotide supplementation on NK cell activity or IL2 levels was found in a similar study of 7-week-old infants (*n* = 98) by Hawkes *et al*. (2006). A study of nucleotides supplemented to soy based infant formula (*n* = 94) resulted in no significant difference measured in NK cell levels in infants measured at 6, 7 and 12 months of age (Cordle *et al*., 2002). Unsupplemented soy-based formula contains high endogenous nucleotide levels rendering nucleotide supplementation unnecessary in contrast to bovine milk based formula. The wide variation in individual results within these studies makes conclusions difficult with respect to any effect of nucleotide supplementation on NK and IL2 levels (Hawkes *et al*., 2006).

Infant responses to immunisation have been used to assess development of the immune system. Infants fed nucleotide-supplemented formula had increased *Haemophilus influenzae* type b (HiB) and diphtheria toxoid humoral antibody responses. Antibody responses to tetanus and polio immunisation were not enhanced by nucleotide supplementation

(Pickering *et al*., 1998). Yau *et al.* (2003) however, found no increase in the response to the HiB vaccine, but did measure an increased risk of upper respiratory tract infections in infants fed nucleotide-supplemented formula. Schaller *et al.* (2004) found a significantly higher response to polio vaccine in nucleotide-supplemented formula fed infants (n = 138) compared to unsupplemented formula.

Nucleotide-supplemented soy based infant formula (n = 94) showed no significant effect on childhood immunisation responses, as evidenced by normal IgA and IgG levels, and normal responses to vaccine antigens compared to breast-fed infants. This may be attributable to the relatively high endogenous nucleotide levels in unsupplemented soy-based formula. However, human milk/formula-fed infants had higher poliovirus neutralising antibody at 12 months than soy-fed infants (Ostrom *et al*., 2002).

A study of diarrhoeal disease in infants from a low socioeconomic group showed infants receiving nucleotide-supplemented formula (n = 141) experienced fewer episodes of diarrhoea, for a shorter duration, with fewer first episodes (Brunser *et al*., 1994). Similar studies have also shown significant reduction in the incidences and severity of episodes of diarrhoea in infants fed nucleotide supplemented infant formula compared to the non-supplemented formula (Pickering *et al*., 1998; Yau *et al*., 2003). Not all studies have shown such effects, however. Neri-Almeida *et al*. (2009) found that there was no therapeutic advantage during episodes of acute diarrhoea from consuming nucleotide-supplemented infant formula (n = 40) compared to unsupplemented formula.

A *meta*-analysis of 15 randomised clinical trials studies comparing nucleotide-supplemented infant formula with non-supplemented formula or breast milk has been reported (Gutiérrez-Castrellon *et al*., 2007). It was concluded that nucleotide-supplemented infant formula was associated with superior response to immunisation with the HiB vaccine, diphtheria toxoid and oral polio vaccine. Additionally, episodes of diarrhoea were

fewer and there was no difference in risk of upper respiratory tract infections.

2.3.2. INTESTINAL MICROFLORA

In a comparison of faecal microflora of breast-fed infants and infants fed either nucleotide-supplemented formula (*n* = 11) or fed unsupplemented formula, breast-fed babies had significantly higher percentage of faecal bifidobacteria and lower percentages of lactobacilli and enterobacteria than either group of formula fed infants. Infants fed nucleotide-supplemented formula had intermediate values that were closer to breast-fed infants (Gil *et al*., 1986a). Conversely, a study by Balmer *et al*. (1994) did not confirm these observations, finding more *Escherichia coli* and less Bifidobacteria in nucleotide-supplemented infants (*n* = 32) than those fed standard infant formula. In their review of prebiotics in human milk, Coppa *et al.* (2006) concluded that based upon available research, prebiotic effects of nucleotides are inconclusive.

Results from a more recent study of infants in the United Kingdom showed an improved composition of gut microbiota in infants fed nucleotide-supplemented formula (*n* = 35), measured as the ratio of Bacteroides-Porphyromonas-Prevotella bacteria to Bifidobacteria, compared to the control formula, and similar to that of breast-fed infants (Singhal *et al*., 2008). In contrast, a study on the effect of nucleotide supplementation in dairy calves, found that nucleotide-supplemented calves (*n* = 23) had the highest incidence of detrimental bacteria (*Clostridium perfringens*) and the lowest incidence of beneficial bacteria (bifidobacteria) of the calves in the study (Kehoe *et al*., 2008).

2.3.3. INTESTINAL GROWTH AND REPAIR

Uauy *et al*. (1990) found increased mucosal protein, DNA, and villous height and disaccharidase activities in the intestine of weanling rats fed a diet over a 2-week period supplemented with 0.8% *w/w* dietary nucleosides (*n* = 10). Moreover, López-Navarro *et al*. (1996) report a reduction in protein synthesis in the small intestine in rats fed nucleotide-free diets (*n* = 10). Following food deprivation dietary nucleotide intake may accelerate normal intestinal response. Although the mechanism for this is not known, it is possible that increased nucleotide levels enhance DNA and RNA synthesis thereby enhancing cell growth and differentiation (Ortega *et al*., 1995).

Dietary nucleotides may also be beneficial following intestinal injury with improved intestinal histology and ultra structure (Bueno *et al*., 1994), and increased DNA, lactase, maltase and sucrase activities (Nuñez *et al*., 1990) in rats fed a nucleotide supplemented diet (*n* = 36) compared to rats fed an unsupplemented diet following diarrhoea. Furthermore, healing of small bowel ulcers in rats was promoted by nucleotide supplementation (*n* = 6–18), which may be attributable to increase in cell proliferation (Sukumar *et al*., 1997).

The intestinal epithelium has a high cell turnover rate and hence requires increased levels of nucleotides as precursors for nucleic acid synthesis (Carver, 1999; Yu, 2002). Exogenous nucleotides may optimise tissue function as endogenous supplies may limit nucleic acid synthesis, particularly during periods of rapid growth, and during recovery from mucosal injury (Carver, 1999).

2.3.4. INFANT GROWTH

Weight gain was observed in weanling rats receiving a low-protein diet supplemented with nucleotides compared to the control group, although this data was unpublished (György, 1971). However, numerous clinical studies on healthy infants have not demonstrated any detectable effect of nucleotide supplementation on weight gain (Carver and Walker, 1995), suggesting that under normal conditions *de novo* nucleotide synthesis is sufficient to sustain normal growth (Cosgrove, 1998). However, a study of term infants born severely small for gestational age (birth weight below the 5th percentile), demonstrated enhanced growth in weight, length, and head circumference in the nucleotide supplemented formula group (n = 39). The improved growth was attributed to tropic effects of nucleotides on the intestinal mucosa previously damaged by intrauterine malnutrition (Cosgrove *et al*., 1996).

A study of severely malnourished infants showed that when fed infant formula with an appropriate calorie and protein content, the impact upon growth and other body composition indicators was favourable regardless of whether nucleotides were supplemented (n = 11) or not (Vásques-Garibay *et al*., 2005).

2.3.5. LIPID METABOLISM

Supplementation of infant formula with nucleotides has been reported to influence plasma lipoprotein concentrations, particularly in pre-term infants (n = 10) (Sánchez-Pozo *et al*., 1994). This differs from results obtained by Villarroel *et al*. (1987), who found no effect of nucleotide supplemented infant formula on serum lipoprotein levels in infants. Siahanidou *et al*. (2004) showed pre-term infants fed nucleotide supplemented infant formula (n = 66) had significantly elevated high-density lipoprotein and

decreased low-density lipoprotein serum levels compared with infants fed unsupplemented formula.

Nucleotide supplementation has also been associated with an increase in long-chain polyunsaturated fatty acids in plasma and erythrocytes in preterm and term infants (DeLucchi *et al*., 1987; Gil *et al*., 1986b; Pita *et al*., 1988) (n = 19, 35, 18, respectively). However, other studies have shown no increase in long-chain polyunsaturated fatty acids production in erythrocytes in term and low birth-weight infants (Gibson *et al*., 2005; Woltil *et al*., 1995) (n = 98, 37, respectively).

2.3.6. IRON ABSORPTION

Inosine and its metabolites, hypoxanthine, xanthine and uric acid positively affect iron absorption in studies on rat intestine (*n* = 3–6) (Faelli and Esposito, 1970). Iron is better absorbed from human milk than cow's milk (McMillan *et al*., 1977), and it has been suggested that the relatively large component of nucleotides in human milk may have a biological effect on iron absorption (Cosgrove, 1998; Janas and Picciano, 1982). However, the addition of nucleotides is reported to have no effect on the iron status of healthy-term infants fed low-iron formula (*n* = 10) (Hernell and Lönnerdal, 2002).

2.4. Infant Formulas

Human milk provides sufficient nourishment for growth as well as providing unique bio-immune factors for protecting infant health. Human milk is therefore considered the "gold standard" for infant nutrition with breast-feeding regarded as one of the most important measures in improving child health (WHO, 1981). However, in cases where breast-feeding is not

preferred, is not possible, or is insufficient, infant formulas are an appropriate substitute to breast-feeding infants during the first year of life. Infant formulas should be formulated to meet dietary needs and promote optimal growth, as well as to minimise stress upon the infants developing organ and enzymatic systems.

Infant formulas fall into one of four broad categories; (1) milk-based, (2) whey-based, (3) milk-protein hydrolysates, and (4) soy-based. The overwhelming majority of pediatric formulas are based on bovine milk or whey, with goat milk-based formula maintaining a niche position in the market. For infants that experience intolerance of milk-based formulas, alternative products based on soy protein or milk-protein hydrolysates are available (Packard, 1982).

2.4.1. NUCLEOTIDE SUPPLEMENTATION

As understanding of the nucleotide composition of bovine and human milk has increased, manufacturers have endeavoured to modify the composition of infant formulas to resemble human milk more closely. Japan (1965) and Spain (1983) were the first countries to allow supplementation of nucleotides to infant formula, with the United States joining them in 1989 (Commission of the European Communities, 1991). Since the early 1990's, nucleotides have been routinely added to infant formulas and to formulas manufactured specifically for pre-term infants since 2002 (Adamkin, 2007). Due to the reported differences between bovine and human milk nucleotide levels, infant formulas are increasingly supplemented with nucleotides to levels equivalent to free nucleotide concentrations in human milk, to a maximum concentration of 5 mg $100kcal^{-1}$ (Aggett *et al*., 2003). While 12 nucleotides or more are present in human milk, supplementation is limited to only GMP, AMP, CMP, IMP and UMP in the form of the readily soluble sodium salts (Commission of the European Communities, 1991).

In recent years, numerous review articles have deliberated the evidence for the efficacy of nucleotide supplementation in infant formulas upon infant health (Adiv *et al*., 2004; Aggett *et al*., 2003; Agostini and Haschke, 2003; Alles *et al*., 2004; Böhles *et al*., 1998; Carver, 2003; Hamosh, 1997; Klein, 2002; Motil, 2000; Riva *et al*., 2005; Schaller *et al*., 2007; Selimoğlu, 2006; Yu, 2002). Despite the purported benefits of nucleotides in infant nutrition, the supplementation of pediatric formulas with nucleotides is controversial (Adiv *et al*., 2004; Lerner and Shamir, 2000; Quan *et al*., 1990; Yu, 2002), as unequivocal clinical evidence supporting supplementation is lacking (Hamosh, 1997; Lteif and Schwenk, 1998). To date there have been no studies evaluating a dose-response relationship between nucleotide concentrations in infant formula and positive effects in infants (Niers *et al*., 2007) and more research is required into the appropriate levels of nucleotide supplementation and to assess the potential benefits (Yu, 2002). However, infant formula products are currently considered safe when supplemented to levels equivalent to the free nucleotide levels of human milk (Gutiérrez-Castrellon *et al*., 2007; Riva *et al*., 2005).

A study by Rueda *et al*. (2002) was undertaken to analyse extracts from an RNA-containing medium exposed to jejunal explants of weaning piglets. Elevated levels of nucleosides found suggested that RNA present in human milk is hydrolysed in the intestinal tract of the breast-fed infant. This gives support to the argument that TPAN concentrations should be considered when formulating nucleotide-supplemented infant formula. The study by Leach *et al*. (1995) is cited as the rationale for advocating higher levels of nucleotide-supplementation in infant formulas, and in some respects has led to disagreement in determining the appropriate level of nucleotide supplementation to infant formulas. Initially, infant formulas were supplemented to levels equivalent to free nucleotide concentrations in human milk, to a maximum concentration of 5 mg $100kcal^{-1}$ (Aggett *et al*., 2003). However, in recent years, fortification of infant formulas with nucleotides to the upper range of TPAN levels in

human milk (16 mg 100kcal^{-1}) has been approved in more than 30 countries (Aggett *et al*., 2003).

In 2004, the Codex Committee on Nutrition and Foods for Special Delivery Uses asked the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition to coordinate the formation of an international expert group (IEG) to find agreement on the optimum content of nutrients, including nucleotides, in infant formulas. The IEG did not find sufficient data to support the optional addition of nucleotides to levels higher than 5 mg 100kcal^{-1}, and therefore recommended this as the maximum total content. The recommended limits for individual nucleotides were 2.5 mg 100kcal^{-1} for CMP, 1.75 mg 100kcal^{-1} for UMP, 1.5 mg 100kcal^{-1} for AMP, 0.5 mg 100kcal^{-1} for GMP, and 1.0 mg 100kcal^{-1} IMP (Koletzko *et al*., 2005).

Current regulations of nucleotide supplementation mandated by Codex have been left in the hands of individual nations (Codex Alimentarius Commission, 1981). The maximum limits for nucleotides in infant formula defined by the IEG are recommended for use within European regulations (European Commission, 2003). In Australasia, minimum limits have been set and maximum limits for each individual nucleotide are as those recommended above, curiously however, the limit for total nucleotides is set at proposed TPAN limits of 3.8 mg 100kJ (16 mg 100kcal^{-1}, 11.2 mg hg^{-1}) (FSANZ, 2007). Currently, nucleotides are not a regulated ingredient in infant formulas as specified in the US Code of Federal Regulations (FDA, 2012).

In the future, it is possible that international agreement will lead to an update of Codex regulations that includes nucleotides. However, coordinated international agreement on nucleotides has been hard to achieve particularly since two of the major parties, the European Union and the United States, have disparate views (Codex Alimentarius Commission, 2006; European Commission, 2003; LSRO, 1998).

2.4.2. ENZYMATIC DEGRADATION OF NUCLEOTIDES

Over 70 endogenous enzymes have been identified in milk and their presence in milk arises from blood plasma through “leaky junctions” between mammary cells, from secretory cell cytoplasm, from the milk fat globule membrane (MFGM), and from somatic cells (Fox and Kelly, 2006). Additionally, microbial contamination of milks introduces its own enzymes. These enzymes, either endogenous or microbially introduced, can influence the stability of nucleotide levels in dairy products. During infant formula production there is a risk that exogenous nucleotides may be degraded by endogenous milk enzymes if the latter are not inactivated by heat prior to nucleotide introduction during manufacture; **Figure 14**.

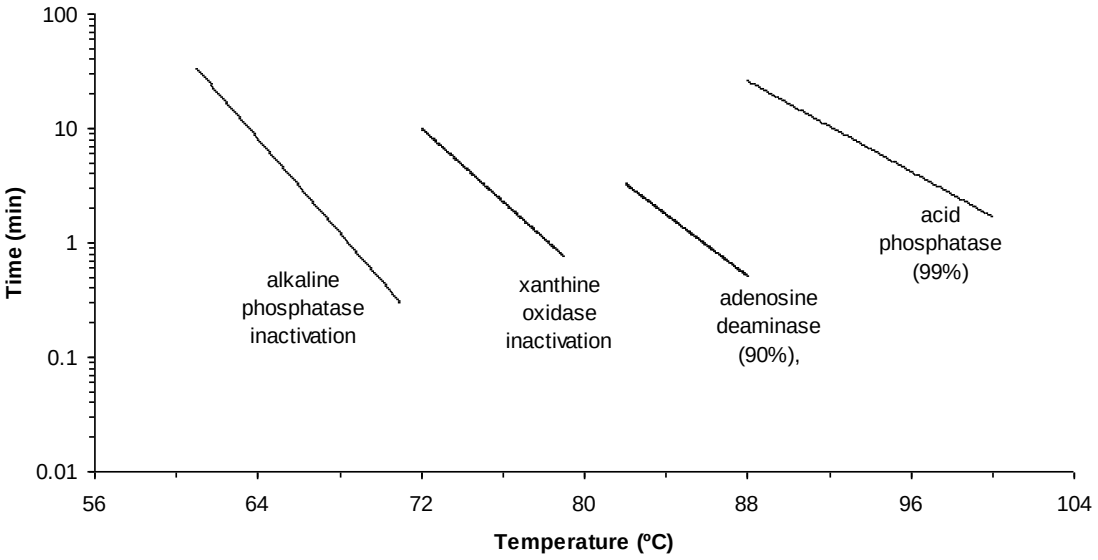

Figure 14. Time-temperature relationship of milk enzyme inactivation (adapted from Federal Dairy Research Centre, 1999; Richardson and Hyslop, 1985)

Alkaline phosphatase [EC 3.1.3.1] is a phosphomonoester hydrolase with a wide range of substrate specificity, including nucleotides. Since its heat stability profile is slightly higher than most pathogenic bacteria, residual bovine milk alkaline phosphatase activity is used as an indicator of the efficacy of pasteurisation. Although alkaline phosphatase may be

inactivated initially, partial reactivation can occur after brief exposures to temperatures of 80–180 ºC followed by storage conditions of 4–40 ºC (Andrews, 1991; Shakeel-ur-Rehman *et al*., 2003). Bacterial contamination of milk can result in the presence of microbial alkaline phosphatase, which is more heat resistant than bovine milk alkaline phosphatase (Karmas and Kleyn, 1990; Pratt-Lowe *et al*., 1987).

Acid phosphatase [EC 3.1.3.2] is also a phosphomonoester hydrolase and although present in milk in significantly lower quantities, acid phosphatase is more resistant to heat treatment than alkaline phosphatase. However, its substrate specificity is reportedly different from that of alkaline phosphatase and it does not appear to hydrolyse aliphatic phosphomonoesters such as adenosine 5′-monophosphate (Andrews, 1991; Shakeel-ur-Rehman *et al*., 2003).

Another phosphomonoester hydrolase for which nucleotides are substrates is 5′-nucleotidase [EC 3.1.3.5]. Both alkaline phosphatase and 5′-nucleotidase are distributed in the MFGM (Andrews, 1991; Shakeel-ur-Rehman *et al*., 2003). Gill and Indyk (2007b) suggested that given that trace levels of intact, endogenous 5′-mononucleotides are found in raw milk, they might be segregated from phosphomonoester hydrolase enzymes in the MFGM, and hence protected as substrates from dephosphorylation

In the analysis of nucleosides and nucleotides in human milk, Thorell *et al.* (1996) reported partial transformation of CMP and UMP to cytidine and uridine and of GMP and AMP to guanine and uric acid. The presence of IMP reported in human milk by Janas and Picciano (1982) was postulated to be an artefact of enzymatic deamination of AMP after sample collection (Aggett *et al*., 2003; Gil and Rueda, 2000; Leach *et al*., 1995; Tressler *et al*., 2003).

In a study of a retail sourced infant formula, the absence of supplemented nucleotides, coupled with an increase in nucleoside levels above those

normally expected in a bovine milk-based product, illustrated that dephosphorylation of nucleotides to nucleosides during manufacture had occurred (Gill and Indyk, 2007b). While equivalent nutritionally, integrity of fortified nucleotides during manufacture is critical from a label claim and quality control perspective. Further enzymatic degradation of nucleosides in infant formulas could lead to a complete loss of nutritional value, or even introduce potentially harmful compounds instead of nutritionally beneficial ones. For example, dietary adenine has been shown to be nephrotoxic in animals when fed at high levels (Brule *et al*., 1988; Story *et al*., 1977) and hence the stability of supplemented AMP or adenosine should be documented throughout infant formula manufacture and storage (Quan *et al*., 1990).

2.5. Analysis of Nucleosides and Nucleotides

Chromatographic analysis of nucleosides and nucleotides has been the subject of review, the focus of which has been on analyses for clinical (Fallon *et al*., 1987; Perrett, 1986; Werner, 1993) and genomic studies (Brown *et al*., 2002). Methods for analysis of nucleosides and nucleotides in milk have been reviewed by Gil and Uauy (1995) and more recently by Gill and Indyk (2007a).

Due to the proliferation of nucleotide-supplemented infant formulas, methods incorporating minimal sample preparation and rapid chromatographic separations have been developed for routine product compliance analysis. In general, the dominant strategy employed in analysis of nucleosides and nucleotides in milk and infant formulas has been protein removal by acid precipitation, with analysis of the crude extract by high performance liquid chromatography (HPLC) with ultraviolet (UV) detection.

2.5.1. SAMPLE EXTRACTION

As milk is a highly complex biological fluid, some form of sample preparation is mandatory to simplify the matrix and facilitate unambiguous signal interpretation. Further precautions may need to be taken before final analysis to ensure both signal fidelity and analyte integrity throughout the analytical process. This is particularly critical in the analysis of raw milk, as nucleotides are susceptible to enzymatic conversions from a variety of endogenous enzymes (e.g., nucleotidases, nucleosidases, and phosphatases), which can rapidly degrade target analytes. Therefore, it is important that following sampling, such potential post-secretory conversion of analytes be inhibited by inactivation of these enzymes immediately upon sample collection by such methods as acid-addition or flash freezing. Depending on the technique and the target analytes, prior separation of cellular and serum material may also be needed.

2.5.1.1 Preparation of Crude Extracts

Extraction of nucleosides and nucleotides from milk is usually achieved following initial protein precipitation with perchloric acid (PCA) or trichloroacetic acid (TCA). Samples are then typically centrifuged and/or filtered, followed by neutralisation of the acid. The use of PCA to obtain protein-free extracts has the advantage that PCA does not absorb UV light, although such extracts reportedly contain more residual UV-absorbing material than TCA extracts (Hernández and Sánchez-Medina, 1981). PCA offers the advantage of removal of poorly soluble salt $KClO_4$ following neutralisation with KOH, however, occurrences of spurious chromatographic peaks from buffer salts, and loss of nucleotides, are risks following perchlorate precipitation (Werner, 1993).

The extraction performed by Kobata *et al*. (1962) consisted of the addition of 2 M PCA. After centrifugation, the precipitate was washed with 0.2 M

perchloric acid and the supernatants combined. Gil and Sánchez-Medina (1981) utilised 1 M PCA and filtered the sample through glass wool after centrifugation. Sow colostrum and milk were prepared for chromatographic analysis using 0.6 M PCA by Mateo *et al.* (2004), and this method was subsequently applied to canine milk and colostrum by Tonini *et al*. (2010). The neutralisation of PCA was achieved with potassium hydroxide (Gil and Sánchez-Medina, 1981; Janas and Picciano, 1982; Krpan *et al*., 2009; Mateo *et al*., 2004; Paubert-Braquet *et al*., 1992; Perrin *et al*., 2001) or potassium carbonate (Oliveira *et al*., 1999) with removal of precipitated potassium perchlorate. Samples for end-point enzymatic analysis were adjusted to pH = 7.4–8.0 with a 0.2 M triethanolamine-0.16 M potassium carbonate solution (Gil and Sánchez-Medina, 1981, 1982; Hernández and Sánchez-Medina, 1981). Thorell *et al*. (1996) removed PCA by extraction with an equal volume of 0.5 M trioctylamine in 1,1,2-trichlorotrifluoroethane (Freon).

Johke and Goto (1962) used a 10% TCA solution to remove proteins from cow milk and goat milk. After centrifugation, the protein residue was homogenised, re-extracted, and the supernatants combined; the removal of excess TCA was accomplished by multiple extractions with diethyl ether. A similar procedure was performed in the analysis of samples of human milk (Sugawara *et al*., 1995). In the analysis of baby foods and infant formulas, a 3% TCA solution was used by Viñas *et al.* (2009). A 10–20% TCA solution employed in the analysis of cyclic nucleotides was neutralised with solid calcium carbonate (Skala *et al*., 1981).

For the extraction of nucleotides from hypoallergenic formulas, an alternative protocol to the PCA extraction used for regular infant formulas was adopted by Perrin *et al*. (2001), whereby 1 M hydrochloric acid was added and the pH was adjusted to 7.0 with sodium hydroxide after centrifugation.

Protein precipitation with acid, without neutralisation, offers the advantage of a rapid, simplified sample preparation. However, there is potential for

losses of nucleotides with long-term storage of the nucleotides in acid (Perrett, 1986). Gill and Indyk (2007b) prepared milk extracts with 3% acetic acid; the extracts were then centrifuged and filtered for immediate chromatographic analysis, with recoveries of 95–105% being reported. Boos *et al*. (1988) adjusted milk samples to pH = 4.0 with concentrated formic acid, stored the samples at -20 ºC for an unspecified time until analysis, and reported recoveries of 95–104%.

In contrast to acid precipitation, alternative methods of deproteination have been described. Tiemeyer *et al*. (1984) added sodium dodecyl sulfate to bovine milk to a final concentration of 1%; the milk was mixed with chloroform to eliminate proteins and lipids and centrifuged, and the upper layer was sampled for analysis. Leach *et al*. (1995) added 1 M sodium hydroxide to pooled milk samples and neutralised to pH = 7.0–7.5 with hydrochloric acid. Topp *et al*. (1993) extracted fat from samples with acetone:dichloromethane (9:1), discarded the supernatant, and extracted nucleosides from the sediment with 70% ethanol. Proteins were then removed by addition of acetone and the supernatant was concentrated by rotary evaporator prior to analysis.

Physical removal of proteinaceous material in infant formulas by use of centrifugal ultrafiltration (CUF) has been reported (Inoue *et al*., 2008; Inoue *et al*., 2010). High molecular weight proteins and large peptides are retained by a semi-permeable membrane but low molecular weight solutes pass through the membrane. This technique offers an advantage over the use of acid in that the risk of nucleotide loss in the protein removal step is reduced. The disadvantages of this technique are that an evaluation of analyte recovery is required and that the tubes tend to be expensive and can significantly increase the cost of analysis per sample.

The preferred sample extraction technique depends on the aim of the analysis. In the first instance, it is necessary to eliminate endogenous enzyme activity, and second to simplify the sample matrix for further analysis. For routine quantitation of nucleotides supplemented to infant

formula, the addition of acid followed by centrifugation of precipitated proteins is straightforward. However, the stability of stored nucleotides at low pH is uncertain, therefore, acid neutralisation is advocated prior to extract storage.

2.5.1.2 Extract Fractionation

Further purification of protein-free extracts prior to analysis has often been recommended, and the early use of charcoal adsorption has been reported (Kobata *et al*., 1962; Rashid, 1973). However, charcoal has variable adsorption characteristics and alternative methods of purifying extracts have been preferred in recent studies.

Phenylboronate Affinity Chromatography

The utilisation of a phenylboronate-modified affinity gel to improve the chromatographic selectivity of nucleosides in urine has been described (Davis *et al*., 1977; Uziel *et al*., 1976). The affinity gel contains a phenylboronic acid bound to various solid supports via a *meta*-amino group. The primary interaction of the phenylboronate functionality is the binding of 1,2 *cis*-diols, such as those found on the C-2 and C-3 of the ribose moiety of nucleosides. This effectively separates ribose forms of nucleosides and nucleotides from similarly related molecules such as deoxyribose forms and cyclic nucleotides, which lack the required 1,2 *cis*-diol moiety. Under alkaline conditions, nucleosides are selectively retained as boronate complexes and released under acidic conditions; **Figure 15** (Liu and Scouten, 2000).

Secondary interactions such as hydrophobic effects, ionic interactions, hydrogen bonding, and charge transfer interactions may also play a role in promoting or retarding boronate *cis*-diol complex formation. The purines tend to bind more strongly than pyrimidines, possibly due to hydrophobic

effects (Liu and Scouten, 2000). Tuytten *et al*. (2007) illustrated that retention on the boronate gel is affected by mechanisms in addition to *cis*-diol complexation. The presence of an exocyclic amine group such as that present in adenosine and guanosine increased retention. The lactam functionality found in uridine or xanthosine gives rise to reduced retention, postulated to be due the presence of an acidic proton, causing the nucleoside to be negatively charged at moderately alkaline pH, leading to electrostatic repulsion.

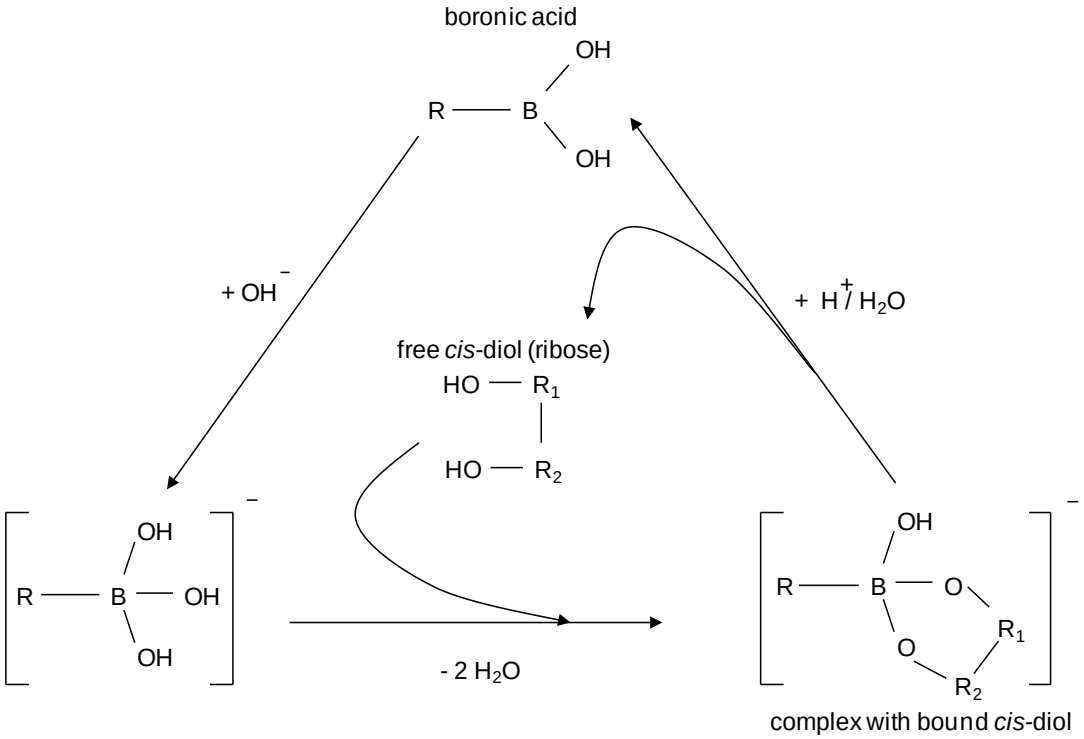

Figure 15. Mechanism of cis*-diol bonding to boronate affinity gel (adapted from Liu and Scouten, 2000)*

The presence of negatively charged phosphate groups adjacent to the ribose in nucleotides may reduce or prevent binding of the *cis*-diol functionality to the gel due to ionic repulsion with negatively charged tetrahedral boronate. The addition of divalent ions, such as Mg^{2+}, has been used to mask the negative charge from the nucleotide phosphate group and lessen this problem (Liu and Scouten, 2000).

Using a commercially available phenylboronate gel, this technique was applied to the analysis of human milk for the determination of nucleosides, with variable recoveries of 58–96% (Topp *et al*., 1993), and TPAN, with recoveries of 76–104% (Leach *et al*., 1995). Perrin *et al*. (2001) found the phenylboronate gel to be unsuitable for use in the quantitative analysis of infant formulas, as only partial recovery of GMP, UMP, cytidine, guanosine, and uridine was achieved, from either infant formula or standard solutions.

Reversed Phase Chromatography

In the analysis of hypoallergenic infant formulas containing partially hydrolysed proteins, chromatographic analysis is more complicated due to the co-elution of peptides under conditions that are suitable for the separation of nucleotides. A solid phase extraction (SPE) clean up procedure prior to chromatography was evaluated, and initial results obtained with a Chromabond-C_{18}ec column showed only unspecified partial recovery of cytidine, guanosine, and adenosine, whereas uridine was not retained on the column (Perrin *et al*., 2001).

Strata-X C_{18} SPE cartridges were used by Contreras-Sanz *et al*. (2012) to purify 12 nucleosides and nucleotides in urine and renal cells. A 25 mM solution of ethanolamine was used to wash the cartridge and 30% methanol in 25 mM ethanolamine was used as elution solvent. Recovery was evaluated at three different pH values of ethanolamine solution (pH = 5.0, 6.0, 7.0). Optimal recovery was obtained at pH = 5.0 and this pH was chosen for use in the final method. Excellent recovery was found values ranged from 98.8% to 104.4%, with the sole exception of uridine, which had recovery of 130.8%. The high recovery was rationalised as being caused by a small peak eluting just prior to, but not resolved from, uridine.

Ion Exchange Chromatography

Early strategies described protein-precipitated milk extracts adsorbed on to Dowex-1 (formate) columns, and elution with increasing concentrations of formic acid, ammonium formate, or sodium formate to determine acid-soluble nucleotides and nucleotide-sugars (Gil and Sánchez-Medina, 1981; Johke and Goto, 1962; Kobata *et al*., 1962). Subsequent removal of formate was achieved by freeze drying (Gil and Sánchez-Medina, 1982), by cation exchange (Johke and Goto, 1962), or by charcoal treatment (Kobata *et al*., 1962).

More recently, a strong anion exchange (SAX) SPE column (Chromabond-SB) was evaluated with a nucleotide-spiked infant formula, with recoveries of individual nucleotides in the range of 92–99%, and the difference between duplicates of approximately 10% (Perrin *et al*., 2001). The use of two SPE columns in series reduced the differences between duplicates to approximately 1%, with an average recovery of 103%. This study further evaluated SAX columns from different manufacturers, and established that two Bakerbond quaternary amine columns in series were optimal with repeatability of 0.8–2.7%, and recovery of individual nucleotides ranging from 93–113%.

2.5.2. CHROMATOGRAPHIC ANALYSIS

Milk of any mammalian species contains a complex mixture of nucleotides, nucleosides, nucleobases, and related molecular species. Physico-chemical analytical techniques rely on the unambiguous separation of these analytes following preliminary crude fractionation of the sample.

Prior to the availability of HPLC systems, final analysis of nucleotides obtained from crude extracts was performed by paper chromatography (Hernández and Sánchez-Medina, 1981; Johke and Goto, 1962; Kobata *et al*., 1962), re-chromatography with a second low-pressure

chromatographic separation, and paper electrophoresis (Kobata *et al*., 1962). While gas chromatography (GC) and capillary electrophoresis (CE) have been employed for the analysis of nucleosides and nucleotides, in recent years, HPLC has become the dominant separation technique used.

2.5.2.1 Gas Chromatography

While chromatographic separation by GC is a rapid and sensitive technique, nucleotides and nucleosides need to be converted to volatile derivatives prior to analysis.

The analysis of 14 nucleosides and modified nucleosides in the urine of cancer patients by gas chromatography-mass spectrometry (GC-MS) was reported by Langridge *et al*. (1993). The nucleosides were isolated via a two-step SPE extraction procedure by means of both C_{18} and phenylboronate columns. Trimethylsilyl (TMS) derivatives of the extracted nucleosides were prepared and analysed using a DB-5 capillary column (30 m x 0.25 mm, 0.25 μm film thickness) with helium as carrier gas.

2.5.2.2 Liquid Chromatography

One of the great advantages of HPLC, particularly reversed-phase liquid chromatohraphy (RPLC) is the considerable number of parameters available to modify separation. These parameters include altering the mobile phase pH, organic solvent content, temperature, and buffer concentration commensurate with the stability of the column. The selection of buffer and its concentration are influenced by a number of factors; the pH desired, buffer solubility, and effects on detection.

For UV, the buffer needs to be transparent at wavelengths suitable for detection of the analyte. Where mass spectrometry (MS) detection is

used, compatible buffers are volatile to reduce maintenance and instrument downtime.

Anion Exchange Chromatography

Anion exchange liquid chromatography is a suitable technique for the separation of nucleotides through exploitation of the charged nature of the phosphate moieties over the operating range of silica (pH = 2–7). The retention behaviour of nucleotides under ion-exchange chromatographic conditions tends to be predictable, as the prevailing mechanisms are largely electrostatic interactions between the negatively charged analyte and the positively charged stationary phase. Thus, by varying pH, buffer ions, and ionic strength, retention can be manipulated (Brown *et al*., 2002).

Separation of nucleotides (nucleoside mono-, di-, and triphosphates) of adenosine, guanosine, inosine, xanthosine, cytidine, uridine, and thymidine was achieved with an SAX column (Partisil 10-SAX) and an acidic phosphate buffer gradient (Hartwick and Brown, 1975). This method was also applied in the analysis of nucleotides (nucleoside mono- and diphosphates) in human milk (Janas and Picciano, 1982). Isocratic elution was used for the analysis of human milk by a similar approach, and good separation of nucleotide was achieved (Paubert-Braquet *et al*., 1992). Viñas *et al.* (2009), used a Tracer Extracil SAX and the mobile phase was a sodium phosphate buffer (10 mM, pH = 3.5) which provided high selectivity for four nucleotides, although the method was not applied to the analysis of samples that included supplemented IMP.

Weak anion exchange chromatography was used for the analysis of a nucleoside analogue by LC-MS (Shi *et al*., 2002). Satisfactory chromatographic separation was achieved for the mono-, di-, and triphosphorylated forms of β-D-2′,3′didehydro-2′,3′-dideoxy-5-fluorocytosine. Inoue *et al.* (2008) used a Capcellpak-NH_2 column with a 50 mM phosphate buffer optimised at pH = 4.0 for the analysis of the nucleotides in infant formula. The column provides a mixed mode of

retention, which in addition to anion exchange properties, has significant reversed-phase characteristics.

Reversed Phase Liquid Chromatography

With the development of robust stationary phases based on porous silica and flexibility in mobile phase optimisation, RPLC, with or without the addition of ion-pair reagents, has become the method of choice for the analysis of nucleosides and nucleotides in milks.

Buffer pH affects analyte retention due to dissociation properties of functional groups, and in its neutral form the analyte is better retained. When pH is near (usually ± 1.5 pH units) the functional group $pK_{a,}$ significant changes in retention are seen. The capacity of a buffer (ability to resist changes in pH) is enhanced at higher concentrations leading to more reproducible separations of compounds. Typically, buffer concentrations range from 10–50 mM. Buffer solubility is important when considering gradient separations since the addition of organic solvent can lead to problems of buffer precipitation (Gloor and Johnson, 1977).

The separation of nucleotides by RPLC is somewhat limited with conventional C_{18} columns due to inherently poor interaction of the polar analytes with the non-polar C_{18} phase resulting in poor retention and resolution. However, by increasing the ionic strength and reducing the pH through the addition of acidic phosphate buffer, nucleotide monophosphates are adequately retained and resolved, with the order of elution typically correlated with hydrophobicity. Organic solvents such as methanol or acetonitrile added to phosphate buffer can facilitate improved resolution (Fallon *et al*., 1987). Additionally, recent advances in column technology, such as hybrid and polymer grafted columns and polar embedded C_{18} phases, offer advantages of suppressed silanol activity, phase stability under highly aqueous conditions, and unique selectivity compared with conventional C_{18} phases (Layne, 2002; Majors, 2004; Majors and Przybyciel, 2002). In contrast, nucleosides lack the charged

phosphate groups present in nucleotides and are therefore relatively well retained on C_{18} phases.

Hypoxanthine, xanthine, guanine, uridine, cytidine, pseudouridine, GMP, and CMP were determined in bovine milk using a μ-Bondapak C_{18} column with isocratic elution of a 0.01 M ammonium phosphate mobile phase adjusted to pH = 6.0 (Tiemeyer *et al*., 1984). Human milk was analysed using a μ-Bondapak C_{18} column with a phosphate buffer:methanol:water linear gradient. Detection of the nucleotides, nucleosides, and nucleobases was possible, although baseline resolution was not always achieved, and a second protocol was necessary to separate CMP from orotic acid (Thorell *et al*., 1996). Quantitation of nucleosides and methylated nucleosides in human milk was achieved with ternary elution gradient of 10 mM ammonium phosphate buffer:methanol:acetonitrile (Topp *et al*., 1993).

Derivatisation of nucleotides, nucleosides, and nucleobases has been reported to improve both reversed phase separation and MS responses. The analytes were derivatised by esterification of free hydroxyl groups using either propionyl or benzoyl acid anhydride. The more hydrophobic derivatives exhibited enhanced retention under reversed phase conditions without the need for ion-pair reagents (Nordström *et al*., 2004).

Elevated nucleoside and modified nucleoside levels are important biomarkers in cancer research, and hence the ability to accurately measure, low nucleoside levels is of critical importance. Therefore, a number of authors have developed methods using LC-MS for the analysis of nucleosides and modified nucleosides in urine. Dudley *et al.* (2004) and Bond *et al*. (2006) used a Spherisorb C_{18} column with 5 mM ammonium acetate and methanol gradient mobile phase system. A similar analytical chromatographic system was used by Cho *et al*. (2006), who also incorporated a column switching technique in order to remove interferences prior to detection.

Nucleosides and nucleobases were analysed by LC-MS in *Cordyceps sinensis* by Fan *et al*. (2006) using a Zorbax Eclipse XDB-C_{18} column and an ammonium acetate buffer:methanol gradient mobile phase system, whereas, Guo *et al*. (2006) used a VP-ODS column with a higher (40 mM) ammonium acetate buffer content for a similar analysis.

A reversed-phase gradient LC-MS method has been reported for the analysis of GMP, AMP and the corresponding cyclic nucleotides. The chromatographic separation of nucleotides is poor however, and the selectivity of the MS detector was used to separately identify and quantify components (Lorenzetti *et al*., 2007).

Gill and Indyk (2007b) developed a method for the simultaneous analysis of nucleotides and corresponding nucleosides in human and bovine milks, milk powders, and infant formulas using RPLC. The separation of nucleotides was achieved predominantly based on increasing hydrophobicity. The elution order for the corresponding nucleosides was the same with the exception that guanosine and inosine were reversed. This procedure used a polymer-grafted silica Gemini C_{18} column and gradient elution with a phosphate buffer:methanol mobile phase, facilitating the simultaneous analysis of nucleosides and nucleotides.

An investigation of RPLC for the analysis of infant formulas and baby foods by Viñas *et al.* (2009) found that nucleotides were not retained on a Zorbax Eclipse XDB-C_{18} column. This is hardly surprising however, since the mobile phases consisted of phosphate buffers with 30–100% acetonitrile.

An LC-MS method for the analysis of supplemented nucleotides in a range of infant formulas was reported by Ren *et al*. (2011). A reversed phase gradient from 100% mobile phase A (0.1% formic acid) to 100% mobile phase B (acetonitrile) was selected (mobile phase transition over 7 minutes), with the best resolution achieved using a Symmetry C_{18} column.

With the commercial availability of ultra high performance liquid chromatography (UHPLC), high-resolution separations with short run times and low solvent consumption are readily available. This technique was applied to the simultaneous analysis of 14 nucleosides and nucleobases in fungi (Yang *et al*., 2007). Ranogajec *et al*. (2010) assessed the retention of nucleosides and nucleotides on five different stationary phases. A narrow-bore Synergy Hydro column was found to be the most efficacious to obtaining sufficient resolution and yet maintaining a relatively short run-time. This chromatographic method was then applied to the analysis of mushrooms.

RPLC was applied to the analysis of nucleosides, nucleotides (nucleotide 5′-monophosphates, 3′-monophosphates, and 2′-monophosphates), and nucleobases in animal feed supplements using an Atlantis T3 C_{18} column with a mobile phase gradient using 0.1% formic acid and 100% methanol. The resolution of analyte peaks was satisfactory, although significant peak tailing was observed (Neubauer *et al*., 2012).

Ion-Pair Reversed Phase Liquid Chromatography

Ion-pair reversed phase liquid chromatography (IPRPLC) has become the prevalent technique for the analysis of nucleotides in milk and pediatric products in recent years. Retention of nucleotides at the appropriate pH is due to strong interactions between the anionic phosphate esters with cationic ion-pair reagents (Brown *et al*., 2002; Werner, 1993). An alternative description of the separation mechanism is the adsorption of the positively charged ion-pair reagent onto the packing material, rendering it similar to an ion-exchange column (Fung *et al*., 2001). At low pH, the charge increases with the number of phosphate residues and hence, in contrast to RPLC, nucleotide monophosphates elute first followed by di- and triphosphates.

While IPRPLC offers significant advantages in chromatographic separation, however when coupled to LC-MS, sensitivity may be

compromised by ion suppression and source contamination. Additionally, simultaneous detection of nucleosides and nucleotides using parallel positive and negative ionisation is not possible due to suppression and interference of added counter ions (Neubauer *et al*., 2012).

A Spherisorb C_{18} column with tetrabutylammonium hydrogen sulfate (TBAHS) as ion-pair reagent and gradient elution was used for the analysis of nucleotides in dairy products (Ferreira *et al*., 2001; Oliveira *et al*., 1999). Sugawara *et al*. (1995) used a Capcellpak C_{18} column with TBAHS for the analysis of nucleotides (nucleoside mono-, di-, and triphosphates) in breast milk from Japanese women. A notable difference in elution under this protocol was the early elution of adenosine nucleotides, the late elution of which can, in other systems, be an impediment in developing assays with shorter run times. A similar chromatographic system was used in the analysis of breast milk from Taiwanese women (Liao *et al*., 2011). Contreras-Sanz *et al*. (2012) used TBAHS as ion-pair reagent with gradient elution of 12 nucleosides and nucleotides from urine and renal cells.

Perrin *et al*. (2001) described a method based on isocratic elution with a mobile phase incorporating tetrabutylammonium dihydrogen phosphate as the ion-pair reagent, where two Nucleosil 120-C_{18} columns in series were required for adequate resolution. A similar mobile phase was used by Krpan *et al*. (2009), although only a single C_{18} column (Supelcosil LC-18T) was sufficient to achieve the required separation.

Alternative ion-pairing salts with more volatility have been employed successfully for MS detection. Tetrabutylammonium bromide was applied in the analysis of cyclic nucleotides in rat brain (Witters *et al*., 1997). In the analysis of nucleotide metabolites in bacteria, hexylamine was utilised as the ion-pair reagent (Coulier *et al*., 2006). The LC-MS analysis of 11 nucleotides in rat tissues was achieved using dibutylammonium formate (DBAA) as the ion-pair reagent (Klawitter *et al*., 2007). Seifar *et al.* (2009) also used DBAA in the LC-MS analysis of nucleotides in cell cultures.

Pentafluorooctanoic acid was used as the ion-pair reagent in the analysis of *Cordyceps militaris* and *C. sinensis* by LC-MS (Yang *et al*., 2010).

The ion-pair reagent most commonly used in studies of nucleotides and related compounds by LC-MS is *N*, *N*-dimethylhexylamine (DMHA) (Auriola *et al*., 1997; Cai, 2001; Cai *et al*., 2002; Cordell *et al*., 2008; Fung *et al*., 2001; Qian *et al*., 2004; Tuytten *et al*., 2002; Viñas *et al*., 2010). Auriola *et al*. (1997) reported that a higher concentration of DMHA was required to increase retention compared to typically used tetrabutylammonium salts and that 10 mM DMHA was required to obtain sufficient retention of nucleotides. Similar concentrations of DMHA were found to be optimal by Qian *et al*. (2004) and Fung *et al*. (2001), 8 mM and 20 mM respectively, to obtain good peak shapes and sufficient resolution. Reduced concentrations of DMHA were necessary when using capillary (0.5 mm) C_{18} columns (Cai *et al*., 2002). Concentrations of DMHA in mobile phase below 0.5 mM have been shown to give poor retention of nucleotides. Retention and peak shapes improved as DMHA concentration increased from 0.5–5 mM with only minor improvements in retention when the concentration was greater than 5 mM (Cordell *et al*., 2008; Tuytten *et al*., 2002).

The effects of DMHA containing mobile phase and pH on retention times and peak shapes on selected nucleotides were examined by Cordell *et al*. (2008). Retention times generally decreased as pH decreased with peak shape degradation at lower mobile phase pH with nucleotides barely retained at pH = 3. A mobile phase of pH = 7 was found to be optimal in terms of retention, resolution, and peak shape and it is this pH which is commonly used in chromatographic systems utilising DMHA as the ion-pair reagent (Cai *et al*., 2002; Fung *et al*., 2001; Qian *et al*., 2004).

Viñas *et al.* (2009) investigated the use of IPRPLC for the analysis of infant formulas and baby foods for nucleotides. A Zorbax Eclipse XDB-C_{18} with phosphate buffer (30 mM, pH = 4.3) containing 0.03% tetrabutylammonium hydroxide and different mixtures of acetonitrile (0–

10%) was trialled. While the best separation was achieved with 100% buffer, peaks were not completely resolved and peak tailing occurred. Variation of conditions such as mobile phase pH from 3.5–6.5; stationary phase, Zorbax Eclipse XDB-C_8; flow rate of 0.25–1.0 mL min^{-1} did not improve separation.

Yamaoka *et al*. (2010) analysed nucleosides and nucleotides in dietary foods and beverages using LC-MS. An Acquity UHPLC HSS T3 column was used with dimethylammonium acetate as ion-pair reagent in an ammonium formate buffer and acetonitrile gradient. While good resolution was obtained for the nucleosides, the nucleotides tended to co-elute based on number of phosphate groups (i.e. the nucleotide monophosphates closely eluted with each other, as did the di- and triphosphate forms). Before each injection, the column was pre-conditioned using a solution containing 0.1% phosphoric acid and 100% acetonitrile mixed 1:1. The pre-conditioning of the column is essential to obtain efficient separation and good resolution of nucleotides. If this pre-conditioning is not done, the peaks of nucleotides, especially triphosphate, will become low and broad (Kaneko, 2011). Severe peak tailing of phosphorylated compounds was investigated by Wakamatsu *et al*. (2005) the cause postulated as the interaction of these compounds with stainless steel components of the analytical system.

Hydrophilic Interaction Chromatography

Hydrophilic interaction liquid chromatography (HILIC) is a separation mode where a polar stationary phase is enriched with a stationary water layer. The more polar a solute, the more it associates with the stationary phase and therefore the later it elutes. That is, retention is analogous to normal phase chromatography and has been described as a “reversed reversed phase” (Hemström and Irgum, 2006).

The analysis of nucleosides and nucleotides using HILIC offers a number of advantages over RPLC and IPRPLC techniques, particularly when

applied to LC-MS analyses. The high organic content of mobile phase enhances spraying and desolvation, thereby increasing signal intensity. The polar nucleotides are well retained without need for ion-pair reagents that can complicate spectra, and there is less need for gradient elution, thereby reducing the impact of variances of mobile phase on ion suppression.

HILIC-MS has been applied to the analysis of nucleosides and nucleotides. Numerous water-soluble cellular metabolites including nucleotides were analysed by HILIC-MS using an aminopropyl column with ammonium acetate (pH = 9.45) and acetonitrile gradient. The polar analytes were effectively separated prior to detection (Bajad *et al*., 2006). The same column and mobile phase was used with a modified gradient by Pucci *et al*. (2009), in the determination of a modified nucleotide, 2′-methylcytidine triphosphate, in rat liver.

The retention of a number of nucleoside mono-, di-, triphosphates and nucleotide adducts was assessed using HILIC with a titania (TiO_2) column. It was found that ligand-exchange and HILIC retention mechanisms were present and that HILIC was favoured at higher acetonitrile concentrations (Zhou and Lucy, 2008).

Tuytten *et al*. (2008) describe an automated online SPE-LC-MS method designed for high throughput clinical laboratories to measure modified nucleosides biomarkers in urine. This method comprised a boronate affinity clean up and HILIC separation followed by MS detection.

The separation of cAMP and ATP was achieved using a zwitterionic HILIC column in an investigation of cAMP agonists (Goutier *et al*., 2010). In method development, a column temperature of 20–50 ºC was found not to affect retention time of the analytes. The ammonium bicarbonate buffer strengths were varied between 1–100 mM at a constant pH of 9.4, with 10 mM selected for final analysis.

The separation of 12 nucleobases and nucleosides was investigated using HILIC by Marrubini *et al*. (2010). Two columns were evaluated, a TSK-gel NH_2-80 column and a ZIC-HILIC. The retention of the analytes was studied by varying the ammonium formate concentration, the acetonitrile content, the pH, and column temperature. The results obtained confirmed the elution order of nucleobases and nucleosides based on their hydrophobicity. Retention and peak shape were influenced by the presence of ammonium formate at different concentrations with increasing retention with increasing salt concentration. Variation in retention due to the mobile-phase pH (3–5) affected the TSK-gel NH_2-80 column more than the ZIC-HILIC column. Column temperature subtly affected retention with increasing temperature resulting in shorter retention times. Gradient elution was necessary to achieve run times shorter than that possible with isocratic elution, with the ZIC-HILIC column providing full resolution of the 12 analytes within a 60-minute run time.

Inoue *et al*. (2010) describe the application of a HILIC-MS method for the analysis of nucleotides in infant formula. A TSK-gel NH_2-100 column was used with ammonium formate buffer and methanol gradient. CMP was weakly retained under this system and GMP and IMP were not resolved.

Phenylboronate Affinity Chromatography

The development of an automated dual column system combining pre-column affinity chromatography and RPLC for the analysis of nucleosides in biological fluids has been reported. With the utilisation of an *m*-aminophenylboronic acid substituted gel and column switching, online dual column clean up and analysis of nucleosides in protein-free extracts was achieved (Schlimme *et al*., 1986b).

Further development of this technique allowed for the analysis of proteinaceous material such as milk (Boos *et al*., 1988; Schlimme and Boos, 1990). With a novel bonded-phase material prepared by immobilisation of phenylboronic acid to a size exclusion gel support, two

different modes of separation based on size exclusion and affinity were exploited and this technique was applied to the analysis of nucleosides in human and bovine milks (Schlimme *et al*., 1997; Schlimme *et al*., 1996). Martin and Schlimme (1997) reported the use of Ca^{2+} and Mg^{2+} ions (50 mM) to reduce the influence of the nucleotide phosphate group in the simultaneous analysis of nucleosides and nucleotides. The recovery of AMP was highest (86–97%), but the recoveries of CMP, GMP, and UMP were much lower and further method optimisation is required. Without the incorporation of these cations, nucleotides remained unbound to the column.

Porous Graphite Chromatography

The use of a porous graphite chromatography (PGC) coupled to MS was used in the analysis of > 40 nucleotide and nucleotide sugars in Chinese hamster ovary cells. The use of PGC alleviated the need for ion-pair reagents, and satisfactory chromatographic performance was found by treatment of the column with reducing agent and HCl (Pabst *et al*., 2010).

2.5.2.3 Capillary Electrophoresis

Nucleotides are readily analysed by CE as they are negatively charged over a wide pH range. CE methods are generally considered to be faster than comparable HPLC methods and use lower quantities of inexpensive buffer salts rather than comparatively large quantities of organic solvents. A review summarising CE analysis of nucleosides and nucleotides in food matrices has been recently published (Chen *et al*., 2010).

The application of CE to analyse nucleotides has been primarily aimed at clinical assays (Grob *et al*., 2003; Qurishi *et al*., 2002). The application of CE to the analysis of nucleotides in human breast milk was reported by Cubero *et al*. (2007).

CE-MS has been used for the analysis of a wide range of metabolites of *Bacillus subtilis*, including nucleosides and nucleotides (Soga *et al*., 2003). A pressure assisted capillary electrophoresis (PACE) method was developed and applied to the analysis of cellular cultures from *Escherichia coli* (Soga *et al*., 2007). The PACE-MS technique used phosphate ions to precondition the capillary to mask silanol groups and prevent the adsorption of multi-phosphorylated analytes. A CE-MS method was applied to the analysis of 12 nucleosides and nucleobases in *Cordyceps sinensis* by Yang *et al*. (2007).

A CE-UV method was developed and applied to infant formula by Ding *et al*. (2011). Excellent resolution of the five nucleotides was obtained with a run time of 48 min. The accuracy of the method was evaluated by comparison with a published RPLC method (Gill and Indyk, 2007b) and by spiked recovery experiments. The results obtained for five different infant formula samples obtained were similar for both methods (**Table 6**).

Table 6. CE and HPLC method comparison (from Ding et al., 2011)

Sample	Method[a]	Measured results (mg hg^{-1})					
		AMP	CMP	GMP	IMP	UMP	Total
1	CE	2.0	10.4	1.4	–[b]	7.3	21.1
	HPLC	1.9	12.3	1.7	–[b]	8.4	24.3
2	CE	3.3	9.1	2.5	–[b]	5.0	19.9
	HPLC	3.3	10.8	2.8	–[b]	5.9	22.7
3	CE	6.3	16.3	10.3	–[b]	9.3	42.2
	HPLC	6.5	18.3	15.3	–[b]	10.0	50.1
4	CE	2.9	11.7	2.3	1.5	4.8	23.1
	HPLC	3.1	12.4	2.3	1.6	5.5	24.8
5	CE	2.4	4.1	–[c]	1.4	2.6	10.5
	HPLC	2.8	4.4	1.0	1.8	3.5	13.5

[a] CE method of Ding *et al*., 2011
HPLC method of Gill and Indyk 2007b
[b] Not detected
[c] Detected but lower than LOQ

2.5.3. ULTRA-VIOLET DETECTION

Pyrimidines and purines readily absorb light in the UV range with maximum absorbances typically between 240 and 270 nm (Cavalieri and Bendich, 1948). However, since the chromatographic pattern of milk extracts is frequently complex, characterisation of putative peaks by retention time with detection at a single wavelength, is generally insufficient for unambiguous identification.

The ratio of the absorbances at 254 and 280 nm, co-chromatography with authentic standards, and enzymatic conversion, were used for confirmation of peak identity of nucleic acid metabolites in bovine milk (Tiemeyer *et al*., 1984). Characteristic peak shifting, or quenching, due to pre-chromatographic chemical or enzymatic treatments can assist in the identification of nucleosides and nucleotides. After a tentative classification of a chromatographic peak, either a substrate-specific enzyme or a reagent known to modify the target analyte selectively is employed. The effect is seen in the subsequent chromatogram whereby the putative peak disappears with the possible appearance of an additional peak elsewhere. Pre-chromatographic modifications by enzymatic (e.g., adenosine deaminase, purine nucleoside phosphorylase) and chemical (e.g., periodate oxidation, Dimroth rearrangement, glyoxal modification, etheno-derivatisation) treatments have been utilised in the identification of nucleosides (Haink and Deussen, 2003; Schlimme *et al*., 1997; Schlimme *et al*., 1996).

In recent years, photodiode array (PDA) detectors have been increasingly employed for the detection and identification of nucleosides and nucleotides in milk (Ferreira *et al*., 2001; Gill and Indyk, 2007b; Oliveira *et al*., 1999; Perrin *et al*., 2001; Thorell *et al*., 1996). The ability to discriminate different peaks over a range of wavelengths is particularly beneficial, by comparison of putative peak spectra with those of authentic compounds and in assessing the chromatographic peak spectral purity.

The use of PDA detectors also offers the advantage of optimal wavelength selection for multiple analytes, so that analyte absorption is maximised and chromatographic interferences may be minimised.

2.5.4. MASS SPECTROMETRIC DETECTION

MS is a powerful analytical technique that can provide both structural information of unknown compounds and can quantify known compounds. MS can be connected online to a variety of separation techniques such as GC, HPLC, and CE (El-Aneed *et al*., 2009).

Ion sources are key components of the mass spectrometer that yield ions from neutral atoms or molecules. Since different ion sources impart different amounts of energy to molecules during ionisation, the choice of ionisation mode is critical to the success of an experiment. A number of ionisation techniques have been coupled to chromatographic techniques to analyse nucleosides and nucleotides and the applications of these in the early to mid 1990's have been summarised by Esmans *et al*. (1998).

In recent years, LC-MS has become widely used for both research and routine use in the pharmaceutical and related industries. Due to the presence of one or more negatively charged phosphate groups, MS detection of nucleotides is frequently performed in the negative mode. However, detection in positive ion mode is used also, particularly for IPRPLC chromatographic methods where $[\text{nucleotide-adduct}]^+$ ions are abundant.

2.5.4.1 Ionisation

Electron Ionisation

Electron ionisation (EI) is the oldest and one of the most commonly used ionisation techniques. Molecules are ionised and fragmented by EI in a reproducible manner and large databases of spectra of known compounds are readily searchable. However, EI is a harsh ionisation technique and is unsuitable for complex biomolecules since the analytes are destroyed. (El-Aneed *et al*., 2009).

Characteristic fragment ions of TMS derivatives of nucleosides were obtained in the analysis of 14 nucleosides and modified nucleosides by GC-MS (Langridge *et al*., 1993). Greater sensitivity and unambiguous identification through characteristic fragmentation of product ions make MS/MS preferable to flame ionisation detection for the analysis of nucleosides by GC (Schram, 1998).

Electrospray Ionisation

Electrospray ionisation (ESI) is an extremely effective technique for analysing polar compounds by MS. Charged droplets are formed by the spraying of solution through an electrically charged needle, with evaporation and coulombic repulsion leading to release of free ions into the gas phase. Since ions are generated directly from samples in solution, ESI is readily compatible with chromatographic separations such as CE and HPLC (El-Aneed *et al*., 2009; Niessen, 1999).

Online chromatographic separation coupled to the sensitivity and selectivity of tandem mass spectrometry (MS/MS) techniques is effective when characterising complex mixtures that are difficult to analyse by standard HPLC-UV analysis. The “soft” ionisation afforded by ESI allows characterisation of non-volatile, thermally labile compounds with minimal fragmentation (Choi *et al*., 2001; Niessen, 1999).

In the LC-MS analysis of a nucleoside triphosphate analogue, ESI^+ was used in order to overcome an interference found in ESI^-, thereby sacrificing sensitivity for selectivity (Shi *et al*., 2002); however, this method suffers from compatibility problems between the LC and MS systems due to the high ionic strength of the mobiles phases.

Ion suppression LC-MS for the analysis of cyclic nucleotides in rat tissue and plant leaves was found to be compatible with ESI (Witters *et al*., 1996). Quantitation of cyclic nucleotides in samples achieved a limit of detection (LOD) in the fmol range.

Analysis of propionyl and benzoyl derivatives of nucleotides, nucleosides and nucleobases has shown that the derivatives possess better ionisation and ESI responses due to increased hydrophobicity and higher surface activity compared to the parent analyte. A lower background noise tends to be found at higher molecular weights resulting in improved signal-to-noise (S/N) (Nordström *et al*., 2004).

DMHA as an ion-pair reagent was used in the development of an LC-MS method for the determination of 12 nucleotides, with ESI^- detection (Tuytten *et al*., 2002). It was reported that a decrease in signal intensity of the $[M-H]^-$ ion was seen as the concentration of DMHA was increased.

ESI^+ is more complex due to a high background from protonated DMHA and the presence of multiple adduct species ($M+H^+$, $M+Na^+$, $M+K^+$, $M+DMHA+H^+$), whereas this is not such a problem in ESI^-. While sensitivity is reduced, since total ion content of each compound is spread over a range of possible ions, the addition of the ion-pair reagent DMHA allows the possibility of enhancing the signal by detecting more easily ionised adduct ions that form between the ion-pairing agent and the nucleotides. Cai *et al*. (2001) reported that the presence of DMHA ion-pair reagent enhanced protonation of nucleotides thereby enhancing ESI^+ sensitivity. This method was applied to the analysis of adenosine nucleotides in cultured cells (Qian *et al*., 2004).

The PACE-MS technique developed for the analysis of cellular cultures (Soga *et al*., 2007) used phosphate during a pre-conditioning phase with the nebuliser gas turned off to avoid contamination of the detector. Preconditioning of an UHPLC-MS system with phosphate was also used prior to the analysis of nucleosides and nucleotides (Yamaoka *et al*., 2010).

Nucleosides in pork were analysed by UHPLC-MS/MS by Clariana *et al*. (2010) with detection of adenosine in ESI^+ mode, and detection of other nucleosides (guanosine, inosine, and uridine) in ESI^- mode. Quantitative product ions were detected from the loss of neutral ribosyl [M-132] group.

Atmospheric Pressure Chemical Ionisation

Atmospheric pressure chemical ionisation (APCI) combined with ESI was used to ionise analytes in a method to determine nucleotides in baby foods (Viñas *et al*., 2010). While ESI is a gentler ionisation technique than APCI and suited to polar compounds, the coupling of ESI and APCI allows for a wider range of compounds to be ionised. In positive ionisation modes, the spectra were more complex with high interference from the DMHA ion-pair reagent in the mobile phase. Intensities of the molecular ions were much higher in negative mode with lower background.

Inductively Coupled Plasma Ionisation

Inductively coupled plasma-mass spectrometry (ICP-MS) is a technique for measuring elemental ions that are generated in hot plasma (6000–8000 ºC). A CE-ICP-MS method was developed for the analysis of nucleotides. The coupling of CE with ICP-MS requires a special interface that introduces the sample to the plasma efficiently and does not degrade resolution achieved by the capillary. Detection limits ranged from 0.036–0.054 $\mu g\ mL^{-1}$ (phosphorus) and recovery ranged from 100–112% (Yeh and Jiang, 2002).

An LC-ICP-MS method was developed and applied to the analysis of nucleotides (nucleoside 5′-monophosphates, 3′-monophosphates, and 2′-monophosphates) in animal feed supplements. Phosphorus was measured as PO^+ by use of a dynamic reaction cell. Detection limits were significantly lower (>1 order of magnitude) using this technique compared to a complementary LC-MS/MS method (Neubauer *et al*., 2012).

Matrix-Assisted Laser Desorption Ionisation

Matrix-assisted laser desorption ionisation (MALDI) uses a pulsed laser to generate ions from analytes embedded in an appropriate solid matrix. MALDI is frequently coupled to a time-of-flight (TOF) detector and MALDI-TOF-MS is popular for analysing both very large molecules as well as low molecular weight compounds. Matrix interferences can be a problem in the analysis of low molecular weight compounds by the MALDI technique due to the similarity of molecular masses of the matrix compound and analyte ions (Hess *et al*., 1998).

MALDI-TOF-MS has been applied to the analysis of nucleosides in urine. The nucleosides were extracted by affinity chromatography with phenylboronic acid gel, and then separated by either RPLC (Kammerer *et al*., 2005) or CE (Liebich *et al*., 2005). The most suitable matrix with high sensitivity was found to be 2,5-dihydroxybenzoic acid (DHB). For measurements with a high mass accuracy a thin layer of α-cyano-4-hydroxycinnamic acid saturated in acetone:ethanol 50:50 *v/v* was used. Using a DHB matrix 18 nucleosides were determined with the LOD from 0.1–10 pmol, with pyrimidines showing a higher LOD than purines (Kammerer *et al*., 2005).

2.5.4.2 Mass Analysers

After ions have been formed by an ion source, mass analysers separate the ions by their mass-to charge ratio (*m/z*) and record relative abundances.

In full-scan mode, a total ion current (TIC) plot records the total intensity summed across the entire range of masses being detected at every point in the analysis. A TIC chromatogram often provides limited information as multiple analytes elute simultaneously, obscuring individual species.

Selected ion monitoring (SIM) is used to record the abundances of specific ions instead of scanning the entire mass spectrum and is used in the quantitative analysis of known compounds to improve sensitivity. Because a narrow mass range is used to collect data, SIM is more selective than full-scan TIC and since more time can be used to acquire a smaller mass range, S/N is improved.

Quadrupole Mass Analyser

Quadrupole instruments consist of four parallel rods, which are connected to direct current (DC) and radiofrequency (RF) generators. By altering the DC and RF potentials, ions of certain *m/z* will be transmitted through the mass analyser to the detector, whereas ions with different *m/z* will possess a different trajectory and ultimately be eliminated prior to detection (El-Aneed *et al*., 2009).

A drawback to using a quadrupole mass analyser is that it is a scanning instrument; that is, it monitors a single *m/z* at any given time, which becomes an issue when scanning across a wide mass range. Since quantitative analyses involve the measurement of known compounds, acquisition of a full mass spectrum is unnecessary, and increased signal can be obtained if specific ions are monitored in SIM mode (Choi *et al*.,

2001). Nucleotides and nucleotides in dietary food and beverages were analysed with a single quadrupole analyser by Yamaoka *et al*. (2010).

A multiple reaction monitoring (MRM) experiment is accomplished by a tandem mass spectrometer. In a triple quadrupole instrument, the first quadrupole selects a specific precursor ion, filtering out other ions with other *m/z*. The selected ions are then collided with a neutral gas in the second quadrupole in a process called collision induced dissociation (CID). Generated product ions are transferred into the third quadrupole where only a specific *m/z* is allowed to pass to the detector, with all other product ions filtered out. Thus, MRM mode works like a double mass filter, and significantly increases S/N and selectivity. MRM is a powerful tool for the identification of particular fragment ions in the determination of the molecular structure of an unknown analyte and also provides confirmation of identity of analyte parent ions of similar mass.

Triple quadrupole instruments are popular instruments that use a number of different scanning modes, which can increase selectivity in studies of known analytes in complex matrices, and can achieve unambiguous identification of unknown analytes. Triple quadrupole mass analysers are the most common detector type used in nucleoside and nucleotide analysis (Cai, 2001; Cohen *et al*., 2009; la Marca *et al*., 2006; Lorenzetti *et al*., 2007; Neubauer *et al*., 2012; Ren *et al*., 2011; Rodríguez-Gonzalo *et al*., 2011; Seifar *et al*., 2009; Shi *et al*., 2002; Soga *et al*., 2003; St Claire, 2000; Tuytten *et al*., 2002; Witters *et al*., 1997; Zhu *et al*., 2001).

Quadrupole Ion Trap Mass Analyser

Quadrupole ion trap mass analysers are highly sensitive instrument and can attain very low detection limits. They consist of a circular ring electrode and two end caps. Ions are trapped and accumulated within the ring and are sequentially ejected with each *m/z* measured. Alternatively, a selected mass can be trapped and undergo CID from gas molecules within the trap producing fragment ions which may be then ejected, or undergo

further CID, producing further fragmentation. Quadrupole ion trap instrumentation was used in the LC-MS/MS quantitation of nucleosides in human urine (Lee *et al*., 2004).

Time of Flight Mass Analyser

A TOF mass analyser accelerates ions through a potential, before they enter a free flight tube in which lighter ions travel faster than heavier ones allowing *m/z* ratios to be measured by arrival times at the detector. In contrast to a quadrupole instrument, a TOF mass analyser is not a scanning instrument instead, the masses of all ions present are determined, and therefore sensitivity is not limited by the mass range making it suitable for qualitative applications (Choi *et al*., 2001).

A TOF mass analyser has been used in the analysis of nucleosides in urine (Kammerer *et al*., 2005; Liebich *et al*., 2005), in the analysis of nucleotide and nucleotide sugars (Pabst *et al*., 2010), in the determination of 16 nucleosides and bases in marine organism extracts (Zhao *et al*., 2011) and in the development of a method for analysis of nucleotides (Tuytten *et al*., 2004), as well as the application of a method for the analysis of nucleotides in baby foods (Viñas *et al*., 2010).

Ion Mobility Spectrometry

Ion mobility spectrometry separates ionised molecules by their arrival time at a detector. The ions traverse a drift tube held filled with a gas at atmospheric pressure, rather than a vacuum. Separation in ion mobility spectrometry occurs in response to ion size, ion shape, and ion charge.

An ion-mobility spectrometer was constructed and coupled to an ESI source in the development of an ion mobility spectrometric method for the determination of 16 nucleotides and nucleosides. Drift times and reduced mobility of nucleotides and nucleosides were determined and detection limits in the pmol range were reported (Kanu *et al*., 2010).

2.5.5. ENZYMATIC ANALYSIS

An enzymatic assay for the determination of individual nucleotides and total nucleotides was developed by Hernández and Sánchez-Medina (1981) based on the method of Keppler (Keppler, 1974). The method was applied to the analysis of cow, goat, sheep (Gil and Sánchez-Medina, 1981), and human milks (Gil and Sánchez-Medina, 1982). Nucleotides were released enzymatically from nucleotide pyrophosphates, nucleotide diphosphates, and nucleotide diphosphate sugars by snake venom phosphodiesterase and quantitatively reacted in a series of enzymatic reactions with measurement of the lactate-dehydrogenase catalysed stoichiometric decrease of NADH at 340 nm (AMP, CMP + UMP, GMP), with UMP determined by enzymatic conversion to UDP-glucose. The recovery of AMP, CMP, GMP, and UMP was estimated at 96% with repeatability between determinations of less than 4%, comparing favourably to an ion-exchange technique (Hernández and Sánchez-Medina, 1981).

Determination of UDP-glucose in milk extracts was performed by a modification of the method of Keppler and Decker (1974), whereby an increase in absorption at 340 nm, due to the stoichiometric reduction of NAD^+ to NADH catalysed by UDP-glucose dehydrogenase, was measured. UDP-galactose was determined by conversion to UDP-glucose catalysed by UDP-glucose-hexose-1-phosphate uridylyltransferase in the presence of glucose-1-phosphate. Free nucleotides were determined similarly, but without the phosphodiesterase hydrolysis step. The recovery of UDP-glucose and UDP-galactose was estimated at 97% with a standard deviation between determinations of approximately 1 nmol mL^{-1} of milk (Hernández and Sánchez-Medina, 1981).

While enzymatic techniques have been superseded by HPLC, enzyme-based methods offer inherent advantages of analyte specificity, and aid in the identification of the multitude of nucleoside and nucleotide related

compounds. In the TPAN analysis of human milks, a number of enzymes have been used to characterise the contributions of different molecular nucleoside sources to infant nutrition. Polymeric nucleotides were hydrolyzed with nuclease, nucleotide adducts were hydrolyzed with pyrophosphatase, and nucleotides were dephosphorylated to nucleosides with phosphatase. The enzymatic reactions employed in the TPAN analyses are illustrated in **Figure 16**.

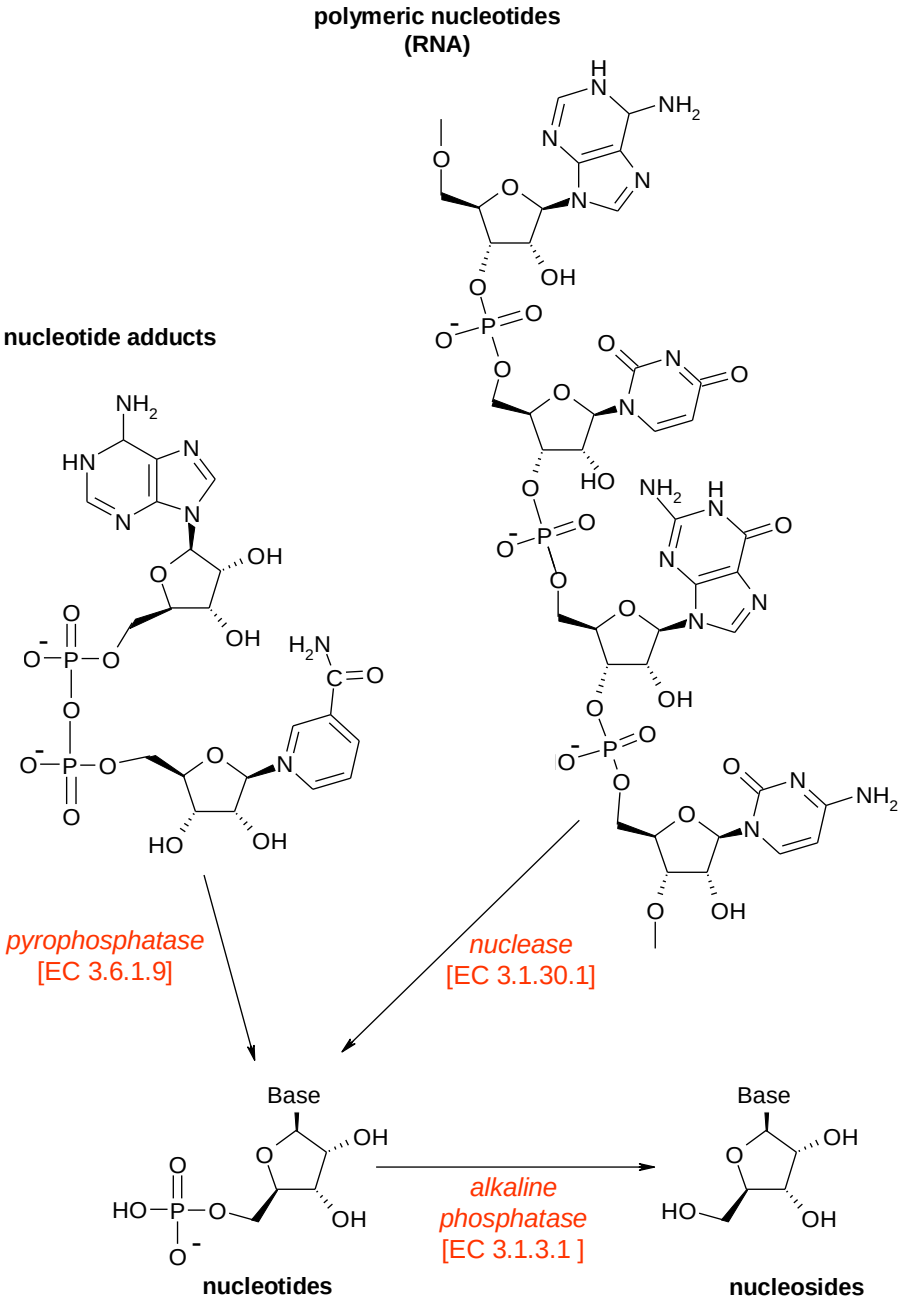

Figure 16. Enzymatic conversion of TPAN to free nucleosides

In this manner, contributions from polymeric nucleotides, monomeric nucleotides, nucleosides, and nucleotide adducts to TPAN were separately estimated (Gerichhausen *et al*., 2000; Leach *et al*., 1995). The recovery of nucleosides ranged from 76% for guanosine to 104% for cytidine, with precision (as repeatability relative standard deviation) of 2.0% for cytidine, guanosine, and adenosine, and 3.6% for uridine (Leach *et al*., 1995).

Luciferase catalyses the oxidative decarboxylation of D-luciferin, and, when ATP is the limiting reagent, the photon count is proportional to the ATP present. In bovine milk, ATP was measured enzymatically using the luciferase-ATP reaction, with light detection by scintillation counter (Richardson *et al*., 1980).

2.5.6. RADIOIMMUNOASSAY

The cyclic nucleotides cAMP and cGMP in milk were determined using a radioimmunoassay technique. This assay is based upon competitive binding between the cyclic nucleotide and an isotopically labelled derivative for a specific cyclic nucleotide antibody (Skala *et al*., 1981; Steiner *et al*., 1972.).

2.5.7. MICROBIOLOGICAL ASSAY

Larson and Hegarty (1977) described a microbiological assay for the determination of orotic acid and pyrimidine nucleotides in ruminant milks. This method is of limited applicability since only pyrimidine nucleotides are measured and they were not individually differentiated.

2.6. Method Validation

2.6.1. SYSTEM SUITABILITY

The aim of a chromatographic analysis is an optimum separation of the analyte(s) from other components in the shortest time practicable. It is essential that the chromatographic separation is functioning in a satisfactory manner for accurate and precise data to be obtained. Therefore, prior to validation experiments it should be established that the HPLC system is suitable for carrying out a particular analysis.

A system suitability study defines a number of parameters used to assess chromatographic performance that may be then evaluated against set criteria. These parameters include resolution, tailing, retention factor, theoretical plates count, and repeatability of peak response and retention time (Bruce *et al*., 1998; CDER, 1994; Shabir, 2003).

2.6.1.1 Resolution

Well-separated peaks with little or no overlap are crucial for dependable quantitative analysis. Resolution (R_s) is a measure of the separation of two peaks and is therefore a critical parameter in assessing the suitability of a chromatographic analysis. It is measured as a function of peak retention times and peak widths. A resolution of 1.5 or greater is acceptable, and a resolution of less than 1.0 is deemed not useable (AOAC International, 2002).

2.6.1.2 Retention Factor

The retention factor (*k'*), also called the capacity factor, is a measure of the retention of the peak of interest compared to a non-retained peak; that is the ratio of time spent for a compound in the mobile phase to the time spent in the stationary phase. Modifying the retention factor is readily achieved by changing the mobile phase. In RPLC, non-polar eluents have higher elution strength than polar eluents. Increasing the non-polar nature of the mobile phase will elute peaks faster and decrease the retention factor. Conversely, an increase in retention factor can be made by increasing the polarity of the mobile phase. A peak should be resolved from interferences in the void volume and a generally accepted value is $k' > 2$ (CDER, 1994).

2.6.1.3 Theoretical Plate Number

Band broadening is the extent to which molecules spread over time within the chromatographic system. A more efficient chromatographic system is one that has less band broadening; this can be expressed numerically as theoretical plate number (*N*). Larger values of *N* correspond to less band broadening and narrower peaks. A generally accepted value for theoretical plate number is for $N > 2000$. Since improvement in resolution is a function of the square root of *N*, a large change in plate number is required to make a small change in resolution. Therefore, increasing the number of theoretical plates is often of less importance than increasing selectivity and retention factor (CDER, 1994).

2.6.1.4 Tailing Factor

Minor peak tailing is a consequence of band broadening, and hence, the trailing part of a peak tends to be wider than the front end. However,

severely tailing peaks negatively affect the accuracy of quantitation due to difficulties in integration. The determination of where a peak ends becomes much more subjective and hence introduces a source of error in peak area estimation. The tailing factor (T_f) is a measurement of peak asymmetry and is calculated by comparing the relative distance of the leading and trailing halves of the peak.

Significant peak tailing can be caused by number of reasons but is most commonly due to sample solvent strength being stronger than the mobile phase, silanol interaction with amines, adsorption of acidic compounds on silica, or void volumes in the column. Once the cause of peak tailing is identified, steps can be taken to minimise it. A recommended value for acceptable peak tailing is $T_f < 2$ (CDER, 1994).

2.6.1.5 Peak Retention Time/Area Repeatability

Numerous factors can affect a chromatographic system; these include column temperature, mobile phase composition, injection volume, pump flow, detector drift, data sampling rates, and even variance in the way peaks are integrated. An assessment of peak area stability is performed to determine whether the system is capable of reporting the same response for the same concentration of analyte. Replicate injections of a standard are analysed to determine the repeatability of peak response (height or area) and retention time. A repeatability of less than 1% is preferable (CDER, 1994).

2.6.2. SINGLE LABORATORY VALIDATION

Method validation is the process of proving that an analytical method is acceptable for its intended purpose (Green, 1996). That is, an evaluation of the method's reliability must be determined by a validation procedure.

Typically, SLV procedures usually involve linearity/range, precision, accuracy, limit of detection/quantitation, robustness/ruggedness, and specificity/selectivity studies (AOAC International, 2002; Bruce *et al*., 1998; CDER, 2001; Eurachem, 1998; Green, 1996; Thompson *et al*., 2002).

2.6.2.1 Linearity

Quantitation requires an understanding of how instrument response varies with concentration. By either internal or external standard technique, a mathematical expression can be devised for calculating unknown analyte concentrations in samples.

The correlation coefficient is widely used as an indication of a linear relationship between two measurements (Bruce *et al*., 1998; Green, 1996; ICH, 1996). However, it can be misleading as a measure of linearity and hence, its use in not recommended and other tests for heteroscedacity are preferred (Analytical Methods Committee, 1988). One of the simplest is a residuals plot where the differences of nominal measured values and that estimated by the calibration line are plotted as a function of concentration. If no structure is seen and random noise is small, then the calibration can be accepted (Bruce *et al*., 1998).

2.6.2.2 Confirmation of Identity

Matrix interferences are usually eliminated by sample extraction procedures. However, residues from the sample matrix through sample preparation procedures may still contain compounds that interfere with the measurement. Where applicable, confirmation of analyte identification can be achieved by comparison of the putative peak in the sample as compared to an authentic standard with respect to retention time (LC), spectral similarity and peak purity (PDA), detection of precursor

(molecular) ion (MS), and detection of characteristic fragment ions (MS/MS).

2.6.2.3 Precision

Repeatability is the smallest expected precision, whereby variability in results is estimated for a single analyst, over a short time frame. Intermediate precision is a useful measure of variability between analysts, over extended time-scale, in the same laboratory. Reproducibility is the largest expected precision, whereby, variation in results is estimated for different analysts on different instruments, in different laboratories, on separate days (Eurachem, 1998). Since reproducibility is measured over different laboratories, its determination requires a collaborative study.

Several official guidelines give procedures for estimating precision (AOAC International, 2002, 2004; CDER, 2001; Eurachem, 1998). The exact procedures for determining repeatability vary, but the principle is the same. A number of replicates are tested under repeatability conditions and is usually expressed as standard deviation (SD) or relative standard deviation (RSD).

The Horwitz ratio (HorRat) has been implemented by a number of international technical and regulatory organisations to assess the acceptability of precision of a particular method. The HorRat is a simple parameter that indicates the suitability of a method by comparing the reproducibility of the method to that of many other methods. The HorRat may be applied to repeatability, although with less reliability. Typically, the repeatability RSD is estimated as one-half to two thirds of reproducibility RSD (Horwitz and Albert, 2006), with limits of 0.3–1.3, although extreme values should be treated with caution (AOAC International, 2004).

2.6.2.4 Accuracy

Accuracy of an analytical method is the closeness of agreement between the accepted true value and the measured value and can be assessed either as bias or recovery (Green, 1996; ICH, 1995; Snyder *et al*., 1997; Thompson *et al*., 2002).

Method bias can be estimated by the analysis of a sample of known concentration, for example a Certified Reference Material (CRM), which can be obtained from standards organisations such as the National Institute of Standards and Technology (NIST). Alternatively method bias can be determined as bias against a method known to be accurate. This is particularly valuable when the method used for comparison is a reference method that has been assessed through a rigorous collaborative study, such as those reference methods published by AOAC International. The bias between methods is determined and a test for significance is performed with the null hypothesis, that there is no difference between methods, rejected if the *p*-value is less than 5%.

In a recovery study, a sample of similar composition to a routine sample is analysed in its original state and after being spiked with a known amount of analyte. An inherent problem with recovery studies as a measurement of accuracy is that the introduced analyte may not behave in the same manner through the analysis as analyte incorporated into the actual samples. For this reason, good recovery may not necessarily be a guarantee of accuracy, but poor recovery is certainly a guarantee of lack of accuracy (Eurachem, 1998; Thompson *et al*., 2002). The acceptable recovery range is dependent upon the sample concentration range and the purpose of the analysis. As a guideline, acceptable limits have been proposed by AOAC International (2002). In general, recoveries of less than 60–70% require further improvement, and recoveries greater than 110% suggest a need for better separation.

2.6.2.5 Robustness

A robustness trial is performed to assess the lack of influence of internal factors on the measured results. The Youden ruggedness trial is an efficient experiment design, where seven factors can be evaluated with only eight analyses of one sample (AOAC International, 2002; Youden and Steiner, 1975). The design is a two-level screening test in which the main effects of the factors are evaluated. Higher order effects that are confounded with the main effects cannot be estimated separately in this design. Typically, in a robustness trial, only the main effects are of concern and factor interactions can be considered negligible (Vander Heyden *et al*., 2001).

The factors selected are those that are most likely to affect the analytical results. They may not be limited to operational factors (explicit in the written procedure), but also include environmental factors (implicit in most procedures). Generally, factors are studied at two extreme levels, with the interval between them equal to the likely variability that will occur during normal application of the method. The choice of interval is a matter of experience; it should be noted however, that the broader the interval, the larger the probability that the factor will exhibit a significant effect. Conversely, the smaller the interval, the more likely the factor is deemed robust at that interval, but the more strictly it needs to be controlled during method use (Dejaegher and Vander Heyden, 2007).

The results of a robustness trial can be interpreted both statistically and graphically. Statistical analysis to identify critical effects consists of a *t*-test, whereby the calculated effect is compared to a critical value at a given level of statistical significance. Graphical interpretation can be assessed by construction of a half-normal plot (measured effects vs. rankit), whereby non-significant effects tend to fall on a straight line through zero, while significant effects deviate from it (Vander Heyden *et al*., 2001).

2.6.2.6 Limits of Detection

When measuring samples at low levels it is important to know the lowest concentration that can be detected by the method. The instrument detection limit is based on visual evaluation of instrument output based on S/N (LOD = 3 x S/N; LOQ = 10 x S/N). The method detection limit (MDL) is a value that defines how easily measurements of an analyte can be distinguished from background noise. The MDL procedure sets the detection limit at the 99% confidence level, minimising false positive errors and is based upon the variability, or precision, between sample replicates run at identical concentrations (EPA, 1999; Su, 1998).

3. RESEARCH

3.1. Nucleotide Analysis by LC-UV

Despite the quantity of published methods, there has been no official internationally accepted reference method for the analysis of nucleotides in milk and infant formulas. This situation has implications for international trade where disputes are possible.

The aim of this study was to validate a simple, rapid, and robust method for routine compliance testing of nucleotide-supplemented infant formula. The method herein describes an SPE sample clean up that avoids the prior need to remove protein, coupled with a binary gradient RPLC system. Due to the multi-step nature of the analysis, the use of internal standard-based quantitation provides additional confidence in analytical results. This technique has been applied to the analysis of bovine milk-based, caprine milk-based, soy-based, and hypoallergenic infant formula.

A description of this method and the results obtained from this research were summarised and presented at the 123rd annual AOAC International conference in 2009 and subsequently published in the Journal of AOAC International (Gill *et al*., 2010) (see Appendix I, Appendix II).

In September 2011, this method was reviewed by an expert review panel (ERP) convened by AOAC International. Based on published SLV data as compared with the standard method performance requirements (SMPR) (Sullivan, 2012) established by the Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN), this method was determined to be acceptable and was approved for Official First Action status and identified as AOAC Official First Action Method 2011.20 (AOAC International, 2012a; Gill *et al*., 2012a) (see Appendix II).

AOAC appointed the author as Study Director to evaluate the performance of the method in an SLV extension study against a set of infant formula and adult nutritional products (SPIFAN kit) that were designed to represent a wide range of different infant formula/adult nutritional products commercially available. The SPIFAN kit covered intact protein and hydrolysate-based products, low and high fat products, soy protein-based, elemental products, used in adult nutritional (AN) and infant formulations, in ready-to-feed (RTF) and powder forms.

In June 2012, an ERP evaluated the SLV extension data against the SMPR and in March 2013, the method was chosen ahead of another (AOAC International, 2012b; Inoue and Dowell, 2012) to continue to the next phase of validation. This will require the determination of method reproducibility via a multi-laboratory collaborative study in order to become an AOAC Official Final Action Method.

3.1.1. EXPERIMENTAL

3.1.1.1 Apparatus

HPLC separation was carried out with an LC-20AT pump, an SIL-20A sample injector unit equipped with a 50 μL injection loop, a DGU-20A5 degasser unit, a CTO-20AC column oven, and a SPD-M20A photodiode array detector. LCSolutions software version 1.22 SP1 was used for instrument control and data processing (Shimadzu, Kyoto, Japan).

Chromatographic separation was achieved with a Gemini C_{18}, 5 μm, 4.6 mm x 250 mm column (Phenomenex, Torrance, CA). UV absorbances for calibration standards were acquired with a model UV-1601 spectrophotometer (Shimadzu) with digital readout to 4 decimal places. A Meterlab PHM210 Standard pH Meter (Radiometer Analytical, Lyon,

France) was used for the determination of pH. Polypropylene centrifuge tubes, 50 mL (Biolab, Auckland, New Zealand), 3 mL disposable syringes (Terumo, Laguna, Philippines), and Minisart 0.2 μm syringe filters with cellulose acetate membranes (Sartorius, Göttingen, Germany) were used for sample preparation.

SPE was performed on a Visiprep 12-port SPE vacuum manifold (Sigma-Aldrich, St. Louis, MO) using Chromabond-SB polypropylene SAX SPE cartridges, 6 mL x 1000 mg (Macherey-Nagel, Düren, Germany).

Prior to use, mobile phases were filtered and degassed using a filtration apparatus with 0.45 μm nylon filter membranes (AllTech, Deerfield, IL).

3.1.1.2 Reagents

AMP (≥ 99%), CMP disodium salt (≥ 99%), GMP disodium salt hydrate (≥ 99%), IMP disodium salt (≥ 98%), and UMP (≥ 99%), thymidine 5′-monophosphate (TMP) disodium salt hydrate (≥ 99%), and potassium bromide (≥ 99%), were purchased from Sigma-Aldrich. Potassium dihydrogen phosphate, orthophosphoric acid, potassium hydroxide, ethylenediaminetetraacetic acid (EDTA), sodium chloride (GR ACS grade or equivalent), and methanol (HPLC grade) were supplied by Merck (Darmstadt, Germany). Water was purified with resistivity ≥ 18 MΩ using an E-pure water system (Barnstead, Dubuque, IA).

A standardising buffer (KH_2PO_4, 0.25 M, pH = 3.5) was made by diluting 34.02 g of KH_2PO_4 in 900 mL of water, adjusting the pH to 3.5 with orthophosphoric acid, and then making the solution to 1 L. An extraction solution (NaCl,1 M: EDTA, 5 mM) was made by dissolving 58.5 g of NaCl and 1.9 g of EDTA in 1 L of water. A wash solution (KBr, 0.3 M) was made by dissolving 3.57 g of KBr in 100 mL of water. The SPE eluent (KH_2PO_4, 0.5 M, pH = 3.0) was made by dissolving 6.8 g of KH_2PO_4 in

90 mL of water, adjusting the pH = 3.0 with orthophosphoric acid, and then making the solution to 100 mL.

Mobile phase A (KH_2PO_4, 0.1 M, pH = 5.6) was made by dissolving 13.6 g of KH_2PO_4 in 900 mL of water, adjusting the pH to 5.6 with KOH solution (25% *w/v*), and then making to 1 L with water. Mobile phase B consisted of 100% methanol. As microbial growth often occurs in phosphate buffers that contain little or no organic solvent at room temperature, the mobile phase was made fresh daily.

3.1.1.3 Standard Solutions

Nucleotide stock standards were prepared as described previously (Gill and Indykb, 2007), with concentrations measured using reported extinction coefficients; **Table 7**. The extinction coefficient of TMP at the UV absorbance maximum (λ_{max}) of 267 nm was determined experimentally as 288.5 dL g^{-1} cm^{-1}.

Table 7. Nucleotide extinction coefficients

Analyte	λ_{max} (nm)	$E_{1cm}^{1\%}$
AMP[a]	257	430.4
CMP[a]	280	398.0
GMP[a]	254	393.3
IMP[a]	249	357.3
UMP[a]	262	313.5
TMP	267	288.5

[a] From Gill and Indyk, 2007b

Stock standards were prepared by accurately weighing approximately 50 mg of each nucleotide into separate 50 mL volumetric flasks and making to volume with water. The concentration of each nucleotide stock standard was determined by diluting 1.0 mL of stock standard to 50 mL

with standardising buffer and measuring the absorbance at the appropriate λ_{max}.

An intermediate standard solution of TMP was made by diluting 4 mL of TMP stock standard into 50 mL of water. A mixed intermediate standard solution of AMP, CMP, GMP, IMP, and UMP was made by diluting 2 mL of each stock standard in a single 50 mL volumetric flask and making to volume with water.

Assay calibration standards were prepared by diluting the two intermediate standards with water to the required concentration. The calibration standards contained a constant concentration of the internal standard TMP (~3 μg mL^{-1}) and variable concentrations (~0.5–7 μg mL^{-1}) of CMP, UMP, GMP, IMP, and AMP.

3.1.1.4 Sample Preparation

Approximately 1 g of infant formula powder was weighed accurately into a 50 mL centrifuge tube and dissolved in 30 mL of extraction solution, 1.0 mL of a TMP intermediate standard (~80 mg mL^{-1}) was added, the tube was capped and vortex mixed. The sample was allowed to stand for 10 min to hydrate before dilution to a final volume of 50 mL with water.

3.1.1.5 Solid Phase Extraction

For each sample, a single SPE cartridge was placed on an SPE vacuum manifold. The columns were conditioned by elution with 4 mL of methanol, followed by elution with 2 x 5 mL of water. The cartridge was loaded with 4 mL of sample solution at a flow rate of < 2 mL min^{-1}. The cartridge was washed (wash solution, 4 mL) to remove interferences. The nucleotides were then eluted (eluent solution, 4 mL) into a test tube. An aliquot of the

eluent was filtered through a 0.2 μm syringe filter into an autosampler vial ready for analysis.

3.1.1.6 Chromatography

Chromatographic separation was achieved using a modification of the procedure described previously (Gill and Indyk, 2007b). Gradients were formed by low pressure mixing of two mobile phases, A and B, with separation of nucleotides achieved using the procedure shown in **Table 8**.

Table 8. Gradient procedure for LC-UV method

Time (min)	Column temperature (°C)	Flow rate (mL min^{-1})	Phase composition[a]	
			%A	%B
0	20	0.5	100	0
5	20	0.5	100	0
14	20	0.5	90	10
15	20	0.5	80	20
35	20	0.5	80	20
36	20	0.5	100	0
50	20	0.5	100	0

[a] Mobile phase A = KH_2PO_4, 0.1 M, pH = 5.6
Mobile phase B = 100% methanol

3.1.1.7 Detection, Identification and Quantitation

The photodiode array detector acquired spectral data between 210 and 300 nm. Integration of peak area was achieved at specific wavelengths: 250 nm (IMP), 260 nm (AMP, GMP, and TMP), 270 nm (CMP and UMP). A linear regression plot of the ratios of peak area against concentration for each nucleotide relative to TMP was generated, and the nucleotide contents in unknown samples were interpolated from this calibration curve; **Equation 3**.

$$\text{Nucleotide (mg dL}^{-1}\text{)} = \frac{A_{NT}}{A_{IS}} \times \frac{1}{L} \times \frac{(C_{IS} \times V_{IS})}{W_S} \times \frac{100}{1000} \quad \text{(Equation 3)}$$

where: A_{NT} = analyte (nucleotide) peak area
A_{IS} = internal standard (TMP) peak area
L = linear regression slope of calibration curve
C_{IS} = concentration of internal standard in sample (µg mL^{-1})
V_{IS} = volume of internal standard in sample (µg mL^{-1})
W_s = weight of sample (g)
100 = unit conversion (from g^{-1} to per hg^{-1})
1000 = unit conversion (from µg to mg)

3.1.1.8 Modifications to Chromatography

As part of the extended SLV evaluation of this method with the SPIFAN kit, minor modifications to the chromatographic procedure were made in the following manner.

The potassium phosphate content in the mobile phase A was reduced, (KH_2PO_4, 10 mM, pH = 5.6), the column temperature and the flow rate were increased, and a more gradual gradient transition to mobile phase B was used; **Table 9**.

Table 9. Modified gradient procedure for LC-UV method

Time (min)	Column temperature (°C)	Flow rate (mL min^{-1})	Phase composition[a]	
			%A	%B
0	40	0.6	100	0
25	40	0.6	80	20
26	40	0.6	100	0
40	40	0.6	100	0

[a] Mobile phase A = KH_2PO_4, 10 mM, pH = 5.6
Mobile phase B = 100% methanol

3.1.2. RESULTS AND DISCUSSION

3.1.2.1 Method Optimisation

Method optimisation consisted of adapting the sample preparation and chromatographic conditions reported previously (Gill and Indyk, 2007b) to accommodate a SPE step for the removal of non-nucleotide interferences, thereby simplifying the chromatographic separation.

Both acid precipitation and CUF techniques to remove protein prior to SPE were initially evaluated. Acid precipitation is a rapid and simple means of removing caseins; however, the low pH of the sample extract may negatively affect SPE retention unless the extract is first neutralised. CUF removes all proteinaceous material above the molecular weight cut-off (MWCO), and the sample remains at physiological pH. However, CUF was found to be an unsatisfactory means of protein removal as it proved to be time consuming, and it was difficult to obtain sufficient permeate for the subsequent SPE step.

Based on these trials, the assumption that it was necessary to remove the protein prior to the SPE was re-considered. The dissolution of a powder sample in the high salt solution was found to be efficacious in producing a uniform sample solution that, when applied directly to the SPE cartridge, did not compromise the recovery of nucleotides. Residual milk protein content in the eluent post-SPE clean up was equivalent to that of an acid-precipitated sample and it is probable that some caseins precipitate and are retained in the SPE cartridge upon addition of the low pH buffer.

The SAX cartridges contain quaternary amine anion-exchange sites, which strongly attract the anionic phosphate moiety of nucleotides. In order to remove the majority of interfering components in the sample, different aqueous wash solutions, containing a variety of anions at a number of

concentrations, were evaluated. Bromide ions were found to be most effective in removing potentially interfering components, such as nucleosides, orotic acid, and uric acid, while retaining nucleotides on the cartridge.

In order to elute the nucleotides from the SAX cartridge, two options were available. One option was to add sufficient acid to lower the pH to the pK_a of the nucleotide phosphate (~pH = 1), thereby neutralising the negative charge and eluting the nucleotides for collection. However, in order to protect the analytical column, neutralisation of the extract would be required prior to HPLC analysis. Alternatively, the addition of anions that have a high affinity for the quaternary amine and added at high ionic strength could be utilised to elute the nucleotides. This was achieved by the addition of 0.5 M phosphate in the eluent, which readily displaces nucleotides bound on the SAX cartridge.

In complex samples that require multiple clean-up steps, internal standard calibration is indispensable in compensating for variation of analyte recovery. Internal standard calibration requires a known amount of the selected compound to be added to each sample, blank and standard. This is done to correct for potential variation of analyte recovery during sample preparation steps. In the selection of an analogous compound suitable for use as internal standard, it is vital that it behaves in a similar manner as the analyte throughout all stages of the analysis. A calibration curve is generated by plotting the ratio of the analyte response to the internal standard response as a function of the concentration of the standards.

The selection of TMP as an internal standard was supported by a number of factors: structural similarity to analyte nucleotides, absence of detectable quantities in infant formulas, retention under desired chromatographic separation, and commercial availability.

3.1.2.2 Method Performance

The use of phosphate as mobile phase buffer has been reported previously (Gill and Indyk, 2007b) and is commonly used in RPLC and IPRPLC methods (Krpan *et al*., 2009; Perrin *et al*., 2001; Viñas *et al*., 2009). The optimal pH for a given mobile phase buffer is at pH ± 1 unit of the pK_a. The mobile phase at pH = 5.6 is outside the optimal range (phosphate pK_{a2} = 7.21) for use as buffer. However, the use of a number of other buffers such as acetate and citrate was found to give poor peak shape and poor repeatability of peak area and retention, a problem that may be attributable to the interaction of stainless steel in HPLC instrument with nucleotides (Tuytten *et al*., 2006; Wakamatsu *et al*., 2005). No such problems were found when using phosphate as mobile phase and the stability of retention time illustrates its suitability for use as a mobile phase buffer.

System Suitability

Chromatographic performance was assessed by replicate analyses (*n* = 6) of a mixed nucleotide standard; **Table 10**. An example of typical chromatography is given in **Figures 17–18**.

Table 10. LC-UV method: system suitability

Parameter	Analyte[a]											
	CMP		UMP		GMP		IMP		TMP		AMP	
Retention time[b]	8.8	(0.2%)[c]	11.8	(0.2%)	19.8	(0.2%)	20.6	(0.1%	25.0	(0.0%)	25.8	(0.0%)
Retention factor, *k′*	0.6	(0.0%)	1.2	(0.8%)	2.7	(0.0%)	2.8	(0.0%)	3.6	(0.0%)	3.8	(0.0%)
Theoretical plates, *N*	6810	(0.9%)	8527	(5.5%)	3369	(3.3%)	60448	(1.2%)	194363	(0.8%)	241749	(0.2%)
Tailing, T_f	1.3	(3.8%)	1.2	(3.3%)	1.0	(0.0%)	1.0	(5.0%)	1.1	(0.0%)	1.1	(3.6%)
Resolution, R_s	–	(–)	6.3	(0.1%)	16.9	(1.1%)	2.2	(0.5%)	15.6	(0.2%)	3.5	(0.6%)
Peak area	142255	(0.5%)	201154	(0.8%)	225242	(0.2%)	122536	(0.7%)	488585	(0.1%)	308754	(0.1%)

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

[b] Retention time (min)

[c] Mean (percent relative standard deviation) of 6 replicates of a mixed nucleotide standard

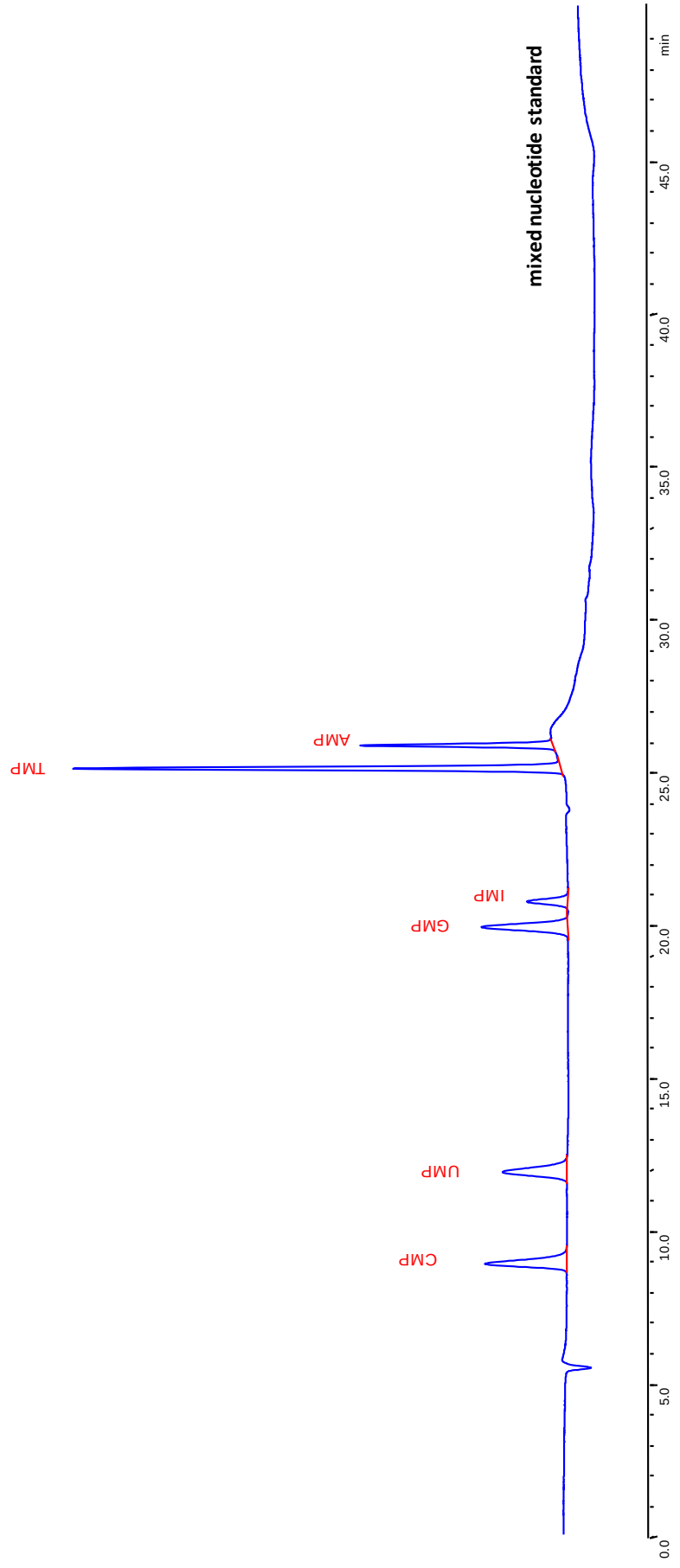

Figure 17. LC-UV chromatography of nucleotide standards (AMP = adenosine 5'-monophosphate; CMP = cytidine 5'-monophosphate; GMP = guanosine 5'-monophosphate; IMP = inosine 5'-monophosphate; UMP = uridine 5'-monophosphate; TMP = thymidine 5'-monophosphate. Chromatography: Gemini C_{18}, 5 µm, 4.6 x 250 mm; mobile phases A = KH_2PO_4 (0.1 M, pH = 5.6); B = methanol (100%), flow rate 0.5 mL min^{-1}, gradient 0–5 min (100% A, 0% B), 14 min (90% A, 10% B), 15–35 min (80% A, 20% B), 36–50 min (100% A, 0% B). UV detection: 260 nm)

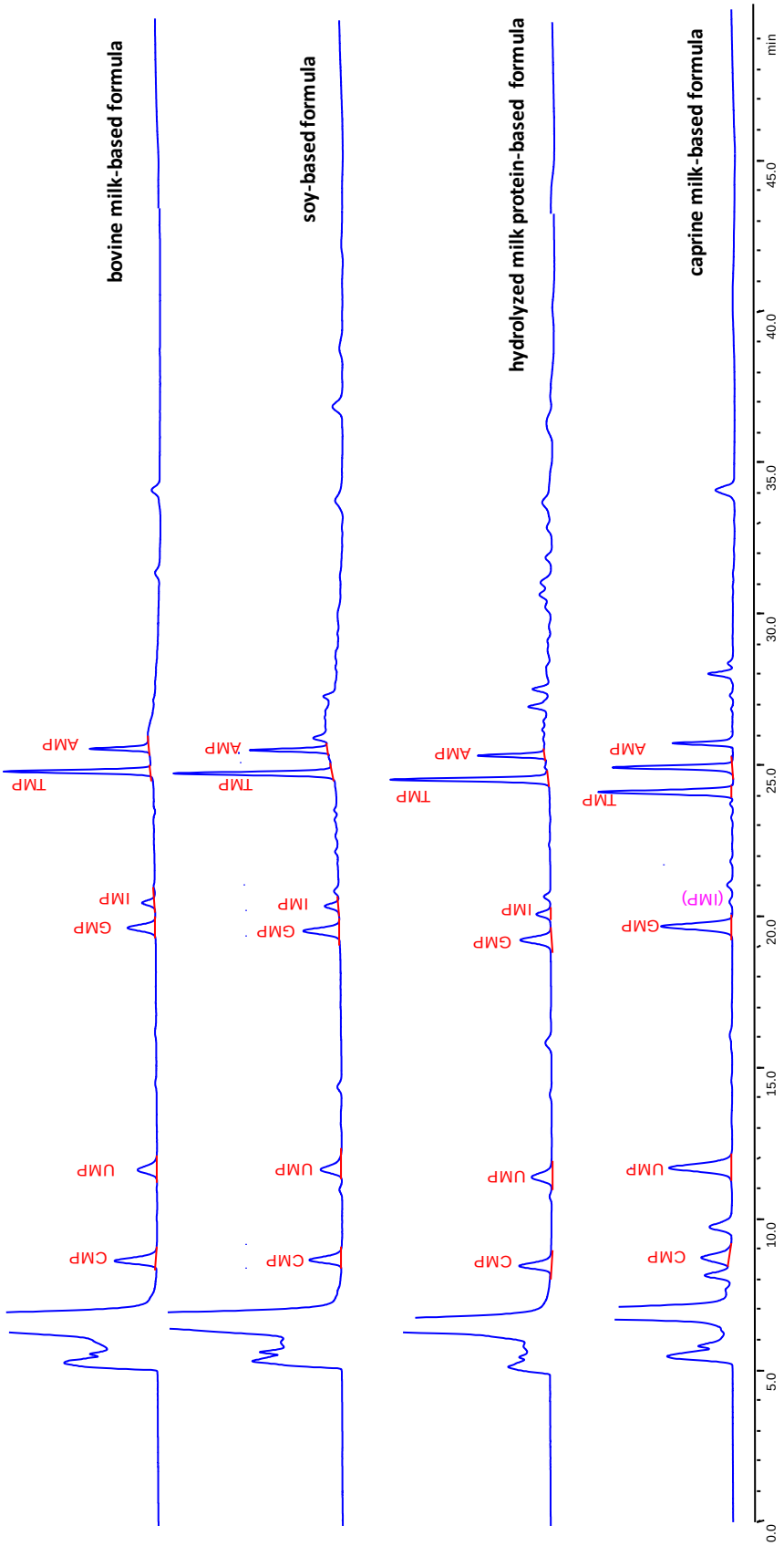

Figure 18. LC-UV chromatography of infant formula samples (AMP = adenosine 5'-monophosphate; CMP = cytidine 5'-monophosphate; GMP = guanosine 5'-monophosphate; IMP = inosine 5'-monophosphate; UMP = uridine 5'-monophosphate; TMP = thymidine 5'-monophosphate. Chromatography: Gemini C_{18}, 5 µm, 4.6 x 250 mm ; mobile phases A = KH_2PO_4 (0.1 M, pH = 5.6); B = methanol (100%), flow rate 0.5 mL min^{-1}, gradient 0–5 min (100% A, 0% B), 14 min (90% A, 10% B), 15–35 min (80% A, 20% B), 36–50 min (100% A, 0% B). UV detection: 260 nm)

Performance within recommended guidelines was achieved, with the exception of the retention factors for CMP and UMP (guideline > 2.0); however, this was deemed acceptable because of uncompromised peak integrity of these two compounds in all samples analysed.

Single Laboratory Validation

A single laboratory validation study was performed on the method and results for linearity, precision, and detection limits are summarised in **Table 11**.

Seven mixed standard nucleotide solutions covering the expected working range were analysed in duplicate. Linearity of dose response was confirmed by least squares regression analysis, with acceptable values obtained for the correlation coefficient. Plots of standard residuals showed no structure and only a small amount of random noise, further demonstrating linearity; **Figures 19–30**.

Repeatability was determined by analysing duplicate pairs (n = 10) of nucleotide-supplemented bovine milk-based infant formula. Intermediate precision was determined from replicate analyses (n = 20) of the same sample tested on five different days by two different analysts. The precision was acceptable, as illustrated by repeatability HorRat of 0.2–0.6, and an intermediate precision RSD of 3.1–9.4%.

The MDL was determined in accordance with EPA (1999) procedures. The concentrations used to generate the MDL (0.52–1.68 mg hg^{-1}) were appropriate to estimate the MDL. The MDL's obtained are approximately an order of magnitude lower than the lowest levels measured in a nucleotide-supplemented infant formula (Gill and Indyk, 2007b).

Table 11. LC-UV method: linearity, detection limit, and precision

Analyte[a]	Range (μg mL^{-1})	Linear regression	R^{2}[b]	MDL[c] (mg hg^{-1})	SD_r[d] (mg hg^{-1})	RSD_r[e] (%)	HorRat[f]	r[g] (mg hg^{-1})	RSD_{iR}[h] (%)
CMP	0.61–8.55	y = 287762x – 2493	0.9999	0.08	0.07–0.18	1.1	0.3	0.4	5.9
UMP	1.12–15.68	y = 146931x – 1839	0.9999	0.08	0.06–0.16	2.8	0.6	0.3	9.4
GMP	1.11–15.55	y = 200342x – 1807	1.0000	0.06	0.02–0.06	2.3	0.4	0.1	5.9
IMP	1.09–15.27	y = 198519x + 3879	1.0000	0.10	0.01–0.04	1.2	0.2	0.1	3.1
AMP	1.25–17.49	y = 255805x + 11862	1.0000	0.19	0.06–0.16	2.5	0.6	0.3	4.7
TMP	1.61–22.54	y = 150494x – 455	1.0000	–	–	–	–	–	–

[a] AMP = adenosine 5′-monophosphate; CMP = cytidine 5′-monophosphate; GMP = guanosine 5′-monophosphate; IMP = inosine 5′-monophosphate; UMP = uridine 5′-monophosphate; TMP = thymidine 5′-monophosphate
[b] R^2 = correlation coefficient
[c] MDL = method detection limit
[d] SD_r = 95% confidence interval for repeatability SD
[e] RSD_r = repeatability RSD% (n = 20)
[f] HorRat = RSD_r/pRSD, where RSD_r = repeatability RSD% and pRSD = $2C^{-0.1505}$
[g] r = repeatability limit = 2.8 x repeatability SD
[h] RSD_{iR} = intermediate precision RSD% (n = 20)
– = Not measured

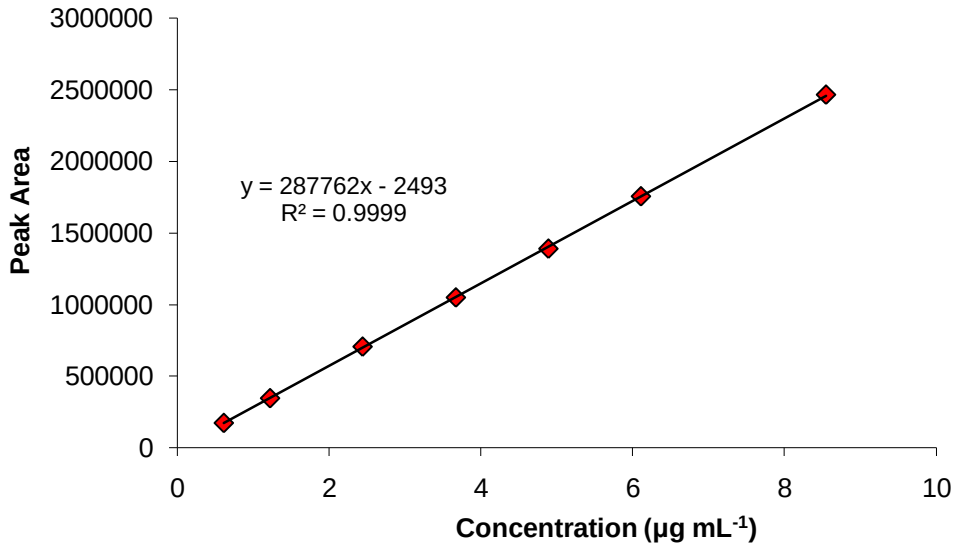

Figure 19. LC-UV method: CMP linear regression plot

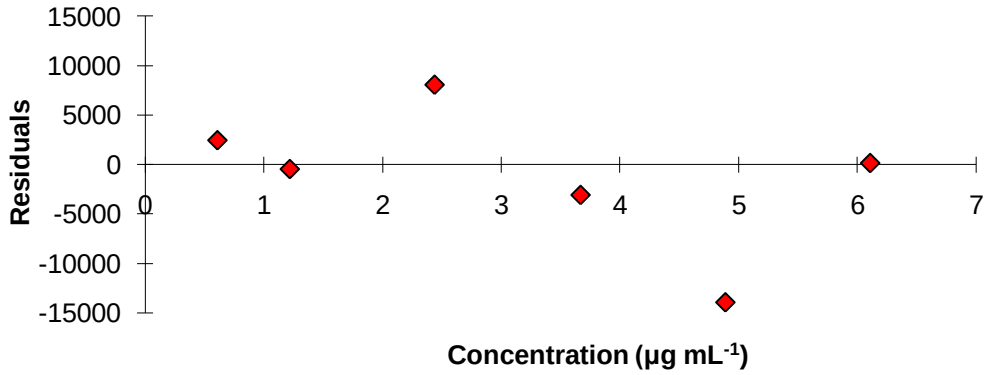

Figure 20. LC-UV method: CMP residuals plot

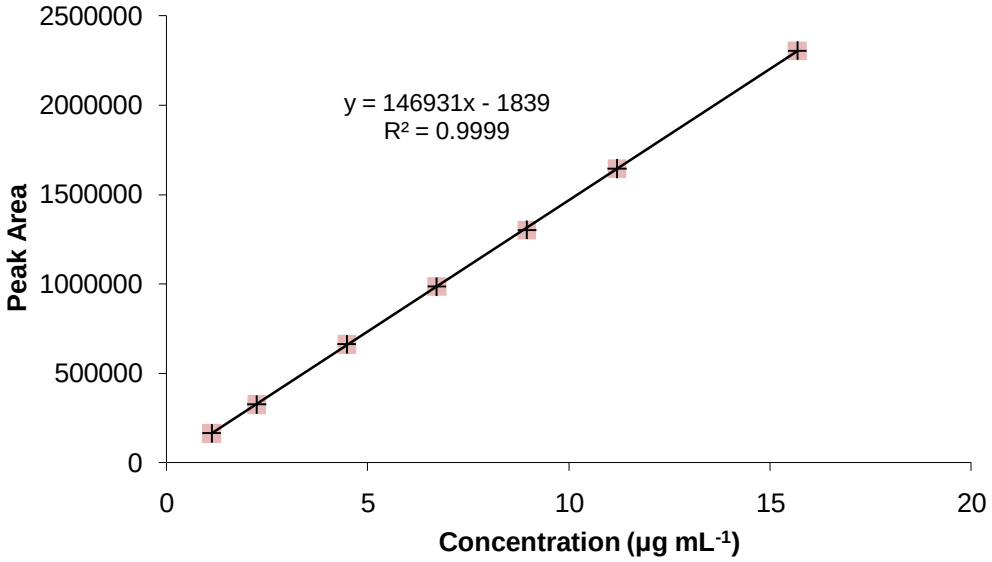

Figure 21. LC-UV method: UMP linear regression plot

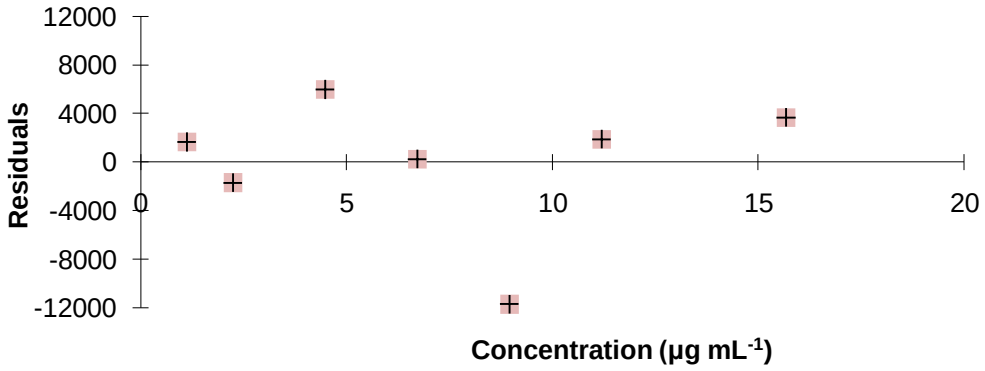

Figure 22. LC-UV method: UMP residuals plot

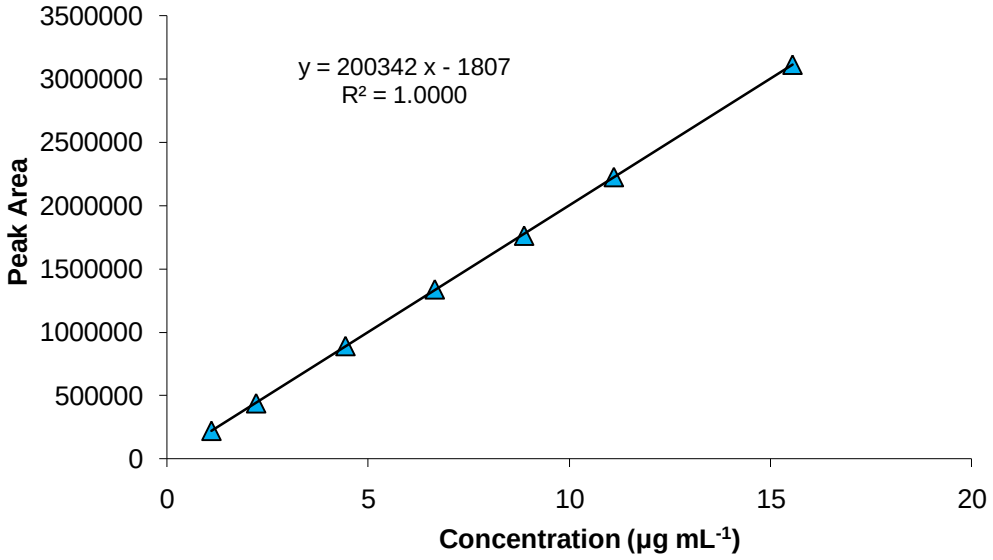

Figure 23. LC-UV method: GMP linear regression plot

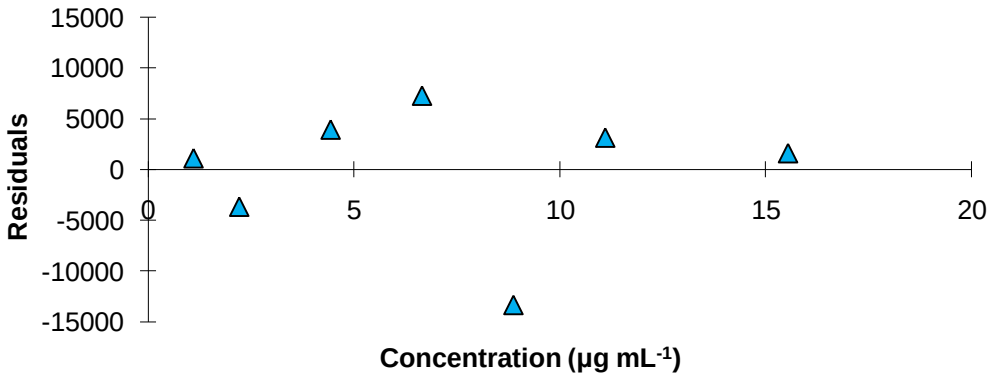

Figure 24. LC-UV method: GMP residuals plot

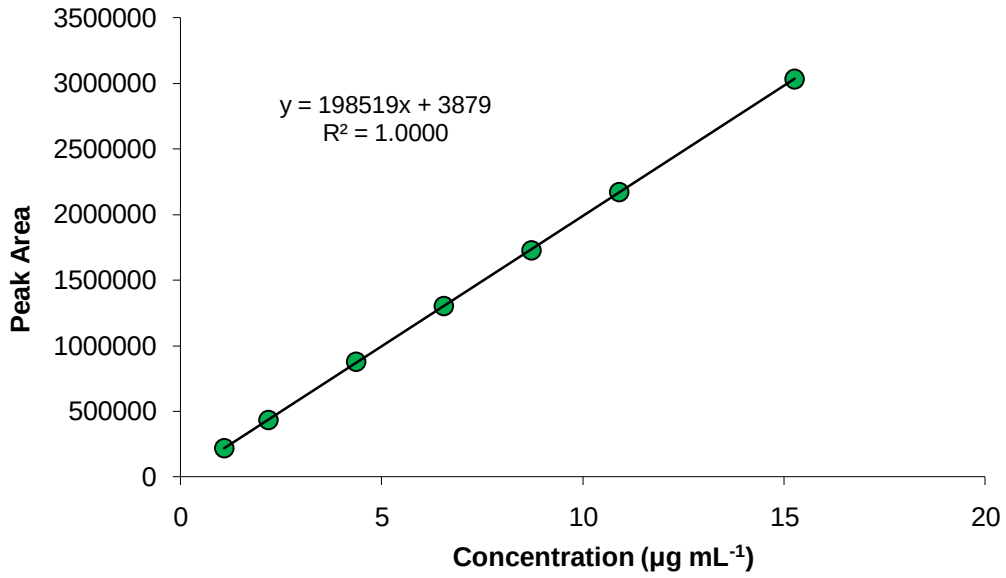

Figure 25. LC-UV method: IMP linear regression plot

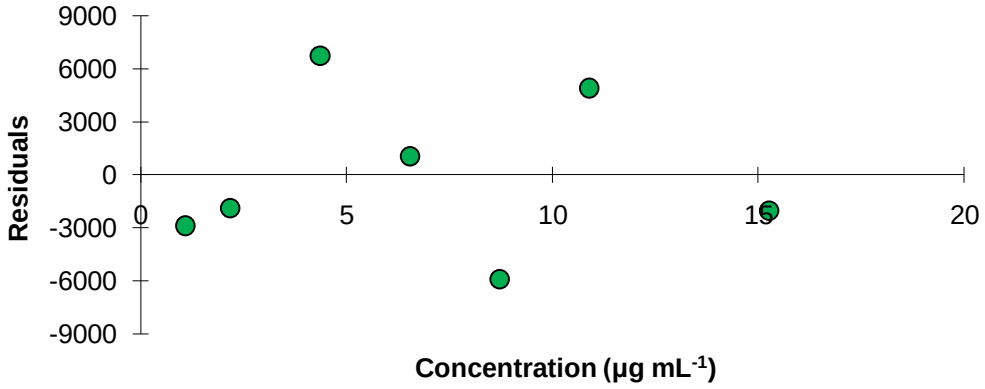

Figure 26. LC-UV method: IMP residuals plot

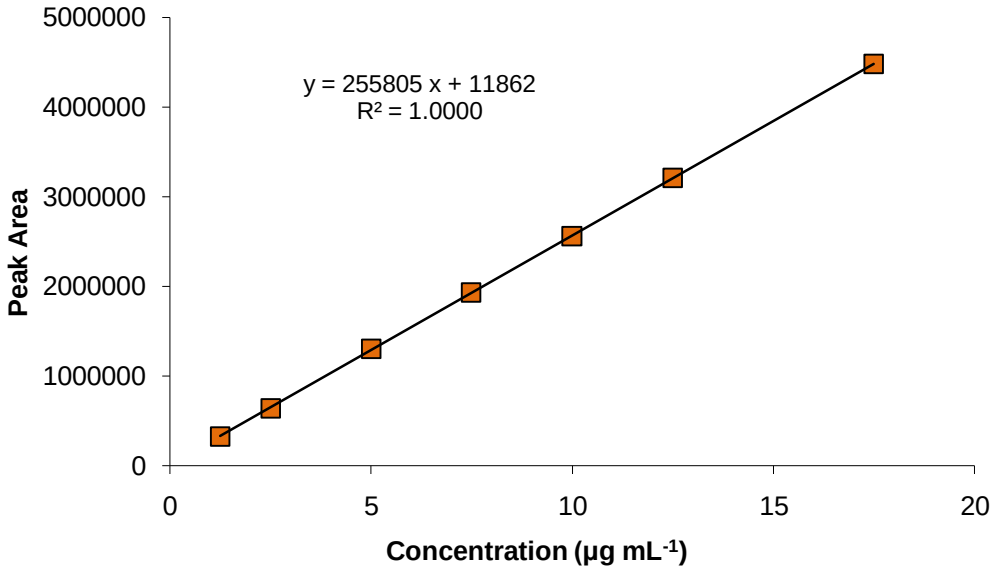

Figure 27. LC-UV method: AMP linear regression plot

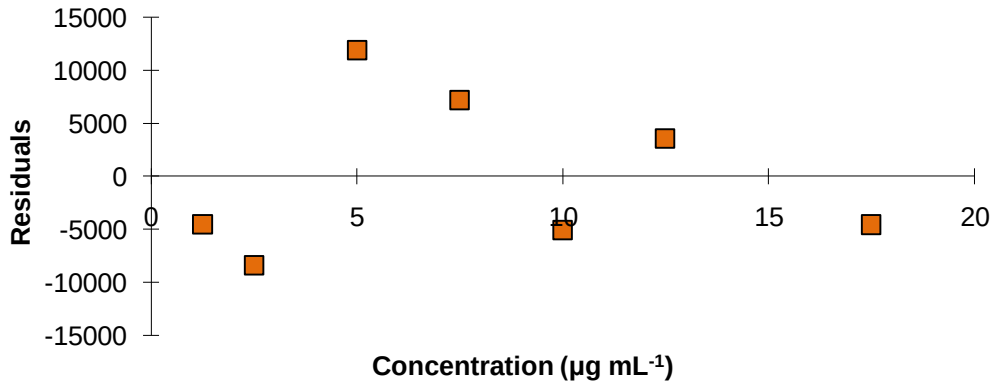

Figure 28. LC-UV method: AMP residuals plot

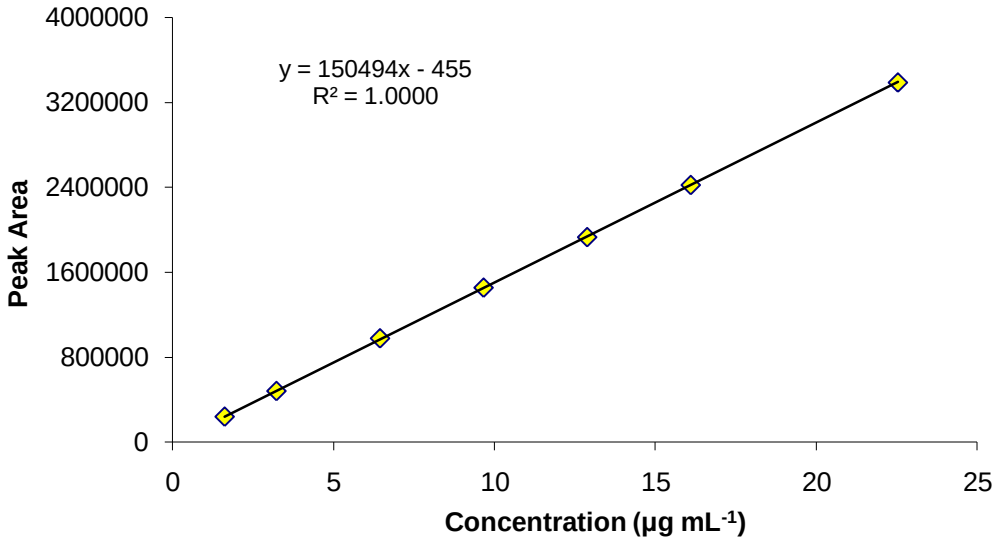

Figure 29. LC-UV method: TMP linear regression plot

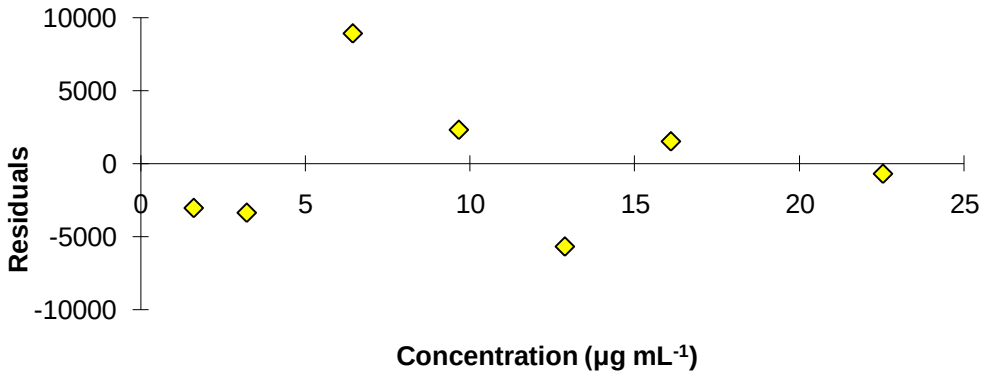

Figure 30. LC-UV method: TMP residuals plot

A robustness trial was performed using seven factors deemed to potentially affect the results and which were evaluated at levels likely to occur during normal use of the method. These were concentration of salt in extraction solution (59 g L^{-1}, 58 g L^{-1}), sample wait time (12 min, 8 min), load volume (4.1 mL, 3.9 mL), wash solution (3.6 g dL^{-1}, 3.5 g dL^{-1}), wash

volume (4.1 mL, 3.9 mL), eluent solution (pH = 3.1, pH = 2.9), and eluent volume (4.1 mL, 3.9 mL). The two factor levels were symmetric around the nominal values from the described analytical procedure, with the interval representing probable experimental error. The seven factors assessed were: initial sample water volume (27 mL, 23 mL); vortex time (40 s, 20 s); wait time (14 min, 6 min); centrifuge volume (4.2 mL, 3.8 mL); centrifuge speed (4000 x *g*, 3000 x *g*); centrifuge time (70 min, 50 min); and a dummy factor. Statistical and graphical interpretation of the results shows that the method to be robust for these factors at the levels studied; **Figures 31–35**.

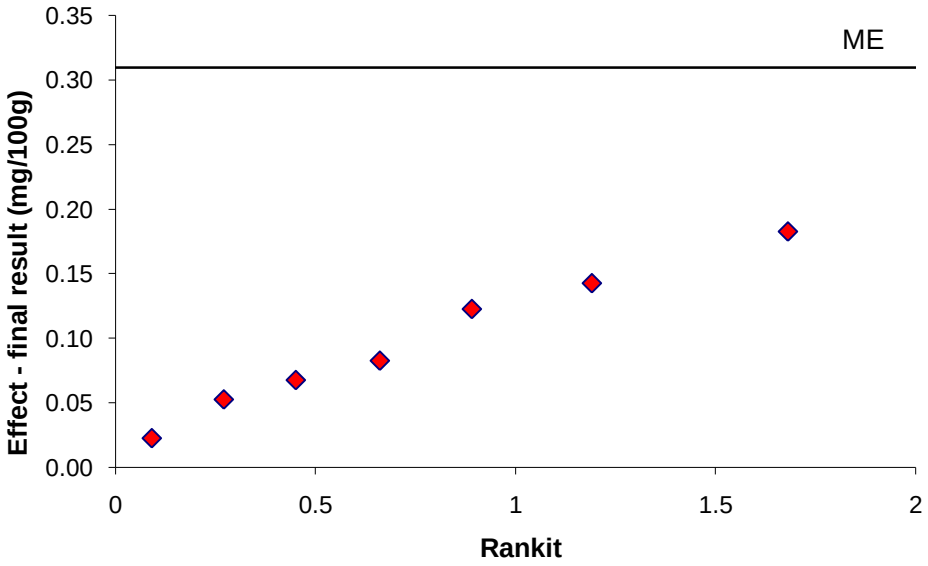

Figure 31. LC-UV method: CMP half-normal plot

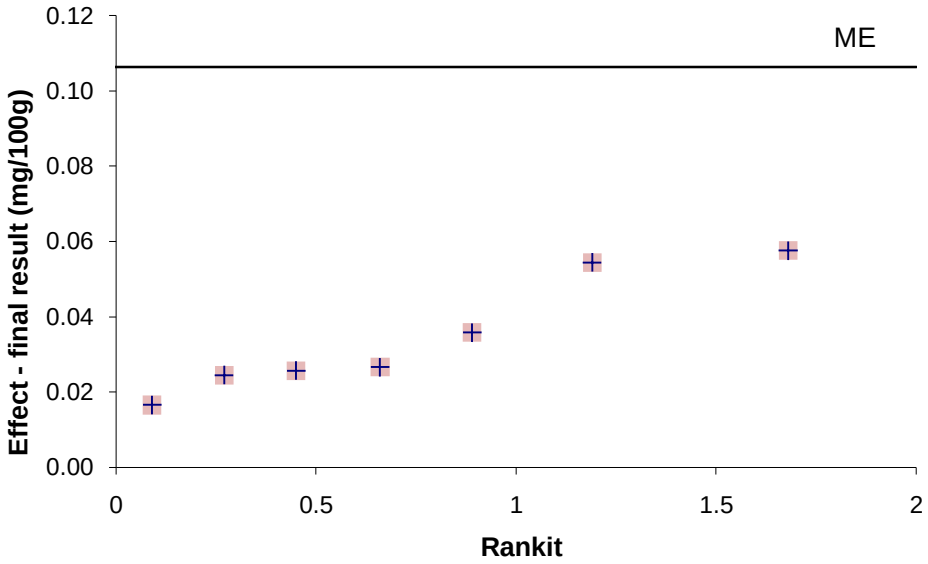

Figure 32. LC-UV method: UMP half-normal plot

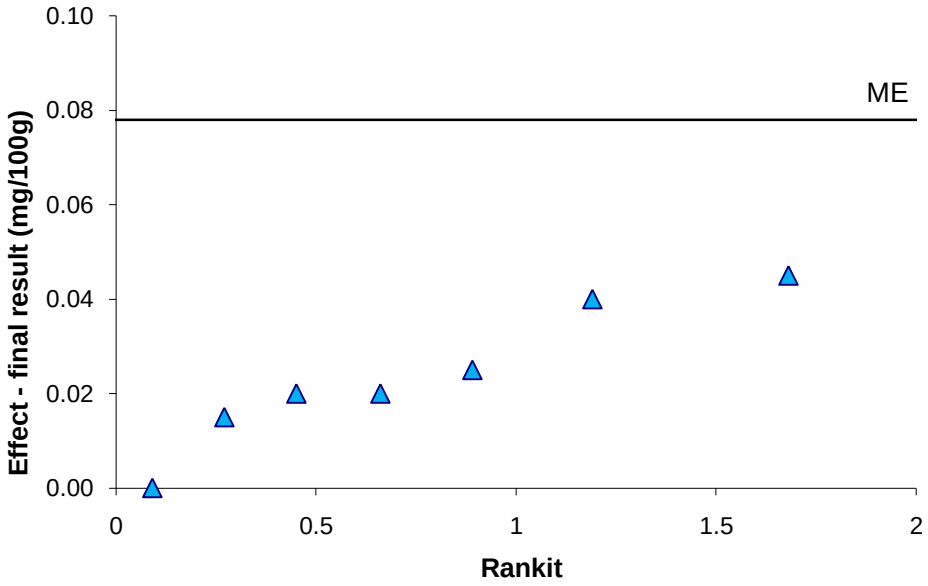

Figure 33. LC-UV method: GMP half-normal plot

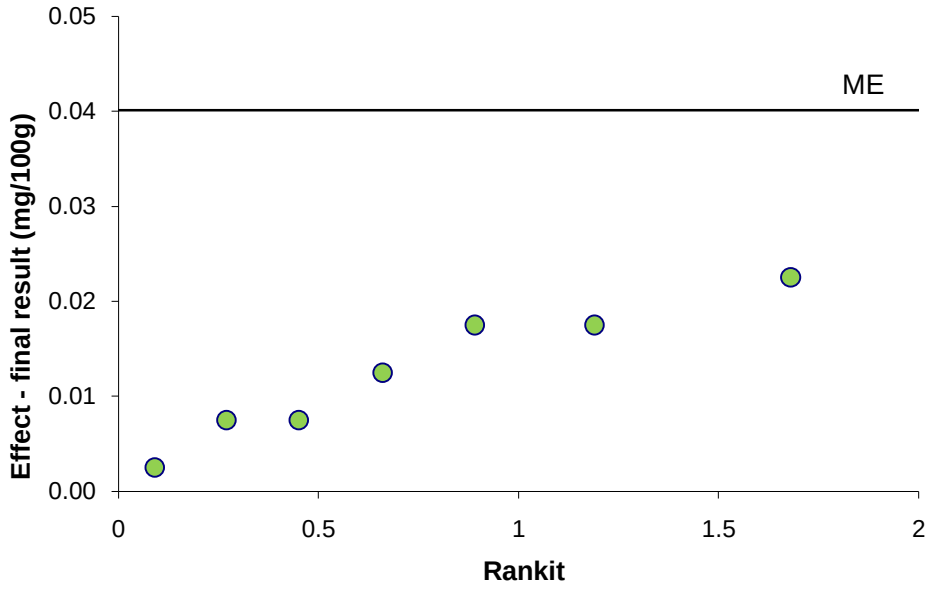

Figure 34. LC-UV method: IMP half-normal plot

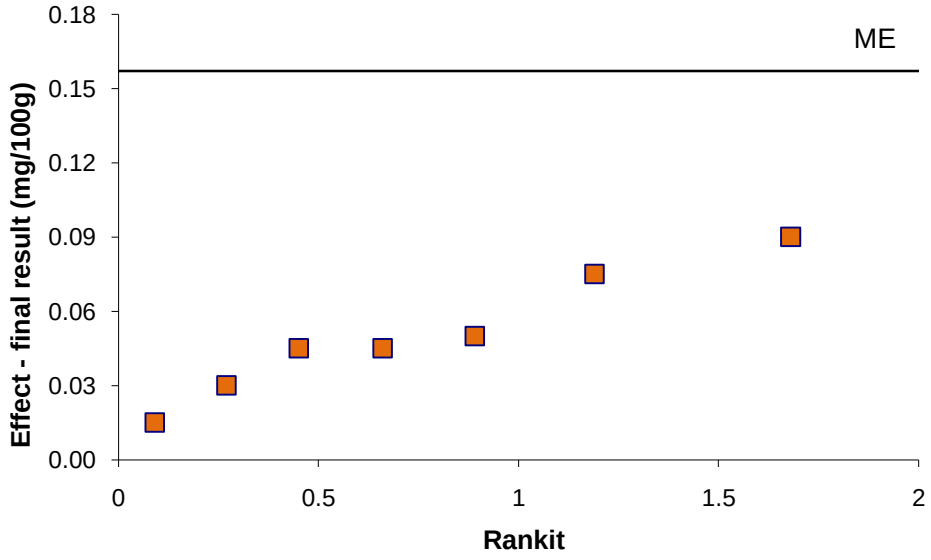

Figure 35. LC-UV method: AMP half-normal plot

Recovery was evaluated at three concentration levels for three different sample matrices: bovine milk-based, soy-based, and hydrolysed milk protein-based infant formula; **Table 12**.

Table 12. LC-UV method: recovery

Sample[a,b]	Recovery (%)											
	CMP		UMP		GMP		IMP		TMP		AMP	
IF bovine milk-based	99	(1.8)[c]	94	(3.3)	98	(2.1)	98	(1.6)	97	(1.3)	100	(2.1)
IF soy-based	98	(3.6)	97	(5.9)	99	(2.4)	97	(4.7)	101	(5.9)	98	(3.6)
IF p/h bovine milk-based	99	(1.9)	92	(5.0)	101	(1.3)	98	(3.8)	100	(3.7)	100	(3.7)

[a] Nucleotides spiked levels at 50%, 100%, 200% of 2.8 mg hg^{-1} (AMP, CMP, GMP, IMP, UMP) and 7.8 mg hg^{-1} (TMP)

[b] p/h = partially hydrolysed

[c] Mean (percent relative standard deviation) of 6 replicates over 3 spiked concentration levels

The recoveries measured were within the limits of 80–115% at the 10 μg g^{-1} level recommended by the AOAC (Horwitz, 2002). Method bias was assessed by testing replicate samples (n = 12) of a nucleotide-supplemented formula by the method described herein and by a method published previously (Gill and Indyk, 2007b). No significant bias was found, with p-values (α = 0.05) calculated to be 0.079, 0.529, 0.676, 0.341, and 0.069 for AMP, CMP GMP, IMP, and UMP, respectively.

3.1.2.3 Nucleotides in Infant Formulas

The method was applied to a number of commercially available bovine milk-based, hydrolysed milk protein-based, caprine milk-based, and soy protein-based infant formulas; **Table 13**. The recoveries determined against label claim were typically found to be > 100%, which is not unexpected due to the practice of overage of fortified ingredients during formulation and production. In the analysis of caprine milk-based infant formula, the presence of significant levels of endogenous nucleotide diphosphates was observed.

3.1.2.4 SLV Extension Study

Modified Chromatography System Suitability

Performance of the modified chromatographic system was assessed by replicate analyses (n = 14) of a mixed nucleotide standard; **Table 14**. An example of typical chromatography is given in **Figures 36–37**. Performance within recommended guidelines was achieved, with the benefits of better resolution between critical pairs of peaks (GMP and IMP, TMP and AMP), an increase in retention of CMP, and an overall reduction in total run time with less interfering peaks near TMP and AMP retention times when compared with the original chromatographic procedure.

Table 13. Nucleotides in infant formula

Sample type[a]	Nucleotide supplemented	Results (mg hg^{-1})									
		CMP[b]		UMP		GMP		IMP		AMP	
Bovine-milk-based IF	Yes	11.6	(116%)[c]	3.7	(95%)	1.7	(106%)	2.0	(125%)	4.5	(145%)
Bovine-milk-based FO	Yes	6.0	(107%)	2.4	(87%)	0.9	(89%)	1.0	(91%)	2.1	(103%)
Bovine-milk-based FO	No	1.0	–	0	–	0	–	0.1	–	0	–
Bovine-milk-based FO	Yes	8.5	(172%)	2.4	(89%)	1.0	(107%)	1.0	(92%)	2.3	(115%)
Bovine-milk-based AN	Yes	17.4	(120%)	4.7	(75%)	8.0	(107%)	0	–	7.2	(130%)
Soy-based IF	No	0.1	–	0.3	–	0.3	–	0	–	0.5	–
Caprine-milk-based IF	No	4.0	–	8.2	–	6.4	–	0.3	–	2.3	–
Bovine-milk-based WMP	No	4.0	–	0	–	0	–	0	–	0	–
Hypoallergenic IF[d]	No	2.6	(101%)	2.6	(92%)	2.7	(96%)	2.6	(96%)	3.1	(100%)
Hypoallergenic IF	No	0	–	0	–	0	–	0	–	0	–

[a] IF = infant formula; FO = follow-on formula; AN = adult nutritional product; WMP = whole milk powder

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

[c] Recovery as percentage of label claim

[d] Hypoallergenic sample spiked with nucleotide mixed standard prior to analysis

Table 14. LC-UV method extension: system suitability

Parameter	Analyte[a]											
	CMP[b]		UMP		GMP		IMP		TMP		AMP	
Retention time[b]	7.4	(1.8%)[c]	9.8	(2.3%)	15.2	(2.8%)	16.6	(2.7%	22.6	(0.6%)	24.0	(0.7%)
Retention factor, *k′*	2.0	(2.5%)	2.9	(3.0%)	5.1	(3.4%)	5.6	(3.1%)	8.1	(0.7%)	8.6	(0.9%)
Theoretical plates, *N*	10827	(2.7%)	12937	(0.8%)	14353	(1.2%)	14740	(3.2%)	95314	(3.1%)	97108	3.2%)
Tailing, T_f	1.2	(3.5%)	1.2	(0.0%)	1.1	(0.0%)	1.1	(3.9%)	1.1	(3.9%)	1.1	(0.0%)
Resolution, R_s	–	(–)	7.5	(2.2%)	12.7	(1.7%)	2.7	(1.9%)	14.8	(4.1%)	4.4	(1.8%)
Peak area	635972	(1.5%)	979695	(1.5%)	1116022	(1.5%)	588552	(1.7%)	768718	(1.5%)	1843061	(1.4%)

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

[b] Retention time (min)

[c] Mean (percent relative standard deviation) of 10 replicates of a mixed nucleotide standard

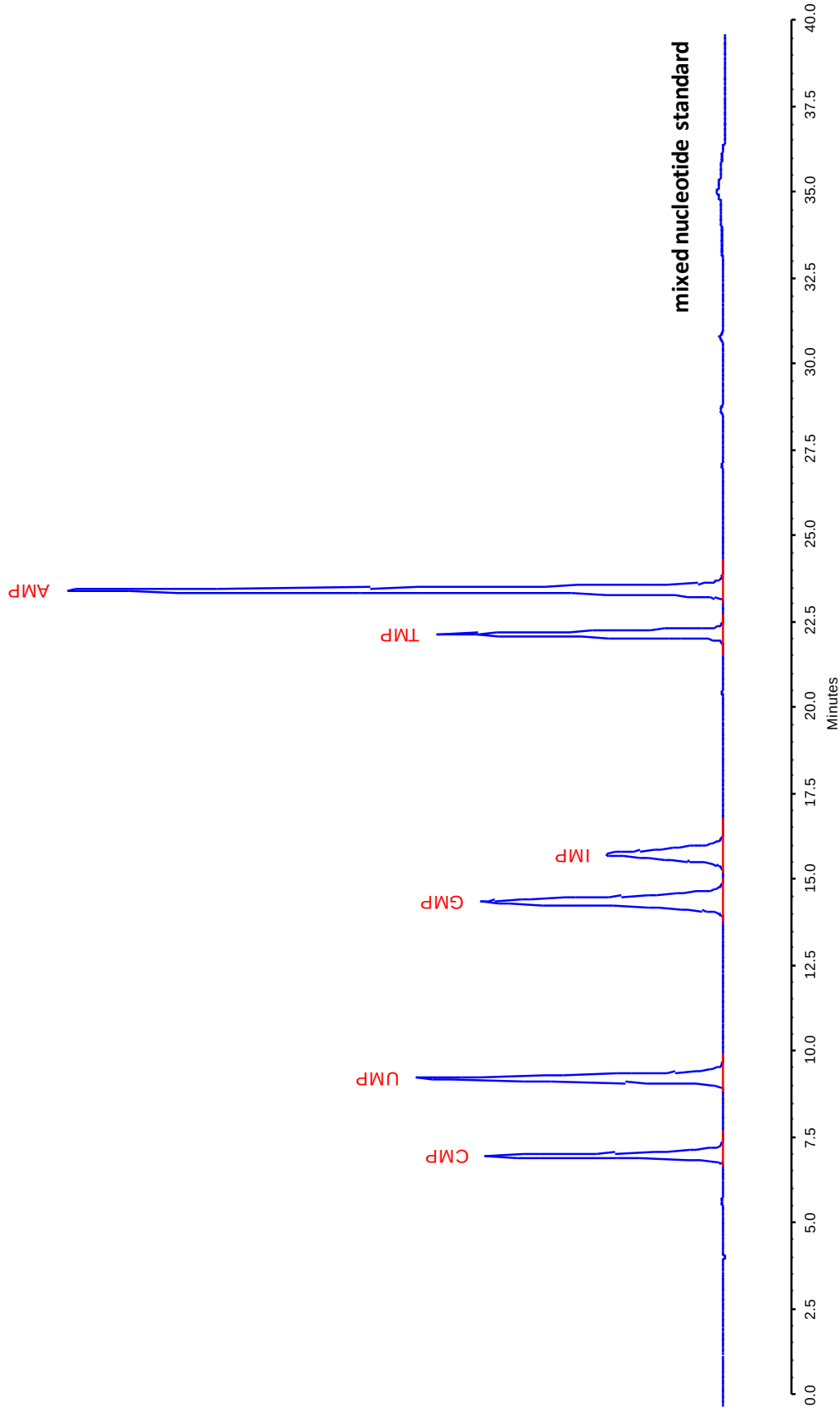

*Figure 36. LC-UV modified chromatography of a nucleotide standard. (**AMP = adenosine 5'-monophosphate; CMP = cytidine 5'-monophosphate; GMP = guanosine 5'-monophosphate; IMP = inosine 5'-monophosphate; UMP = uridine 5'-monophosphate; TMP = thymidine 5'-monophosphate.** Chromatography: Gemini C_{18}, 5 μm, 4.6 x 250 mm; mobile phases A = KH_2PO_4 (10 mM, pH = 5.6); B = methanol (100%); oven temp = 40°C; flow rate 0.6 mL min^{-1}, gradient 0 min (100% A, 0% B), 25 min (80% A, 20% B), 26–40 min (100% A, 0% B). UV detection: 260 nm)*

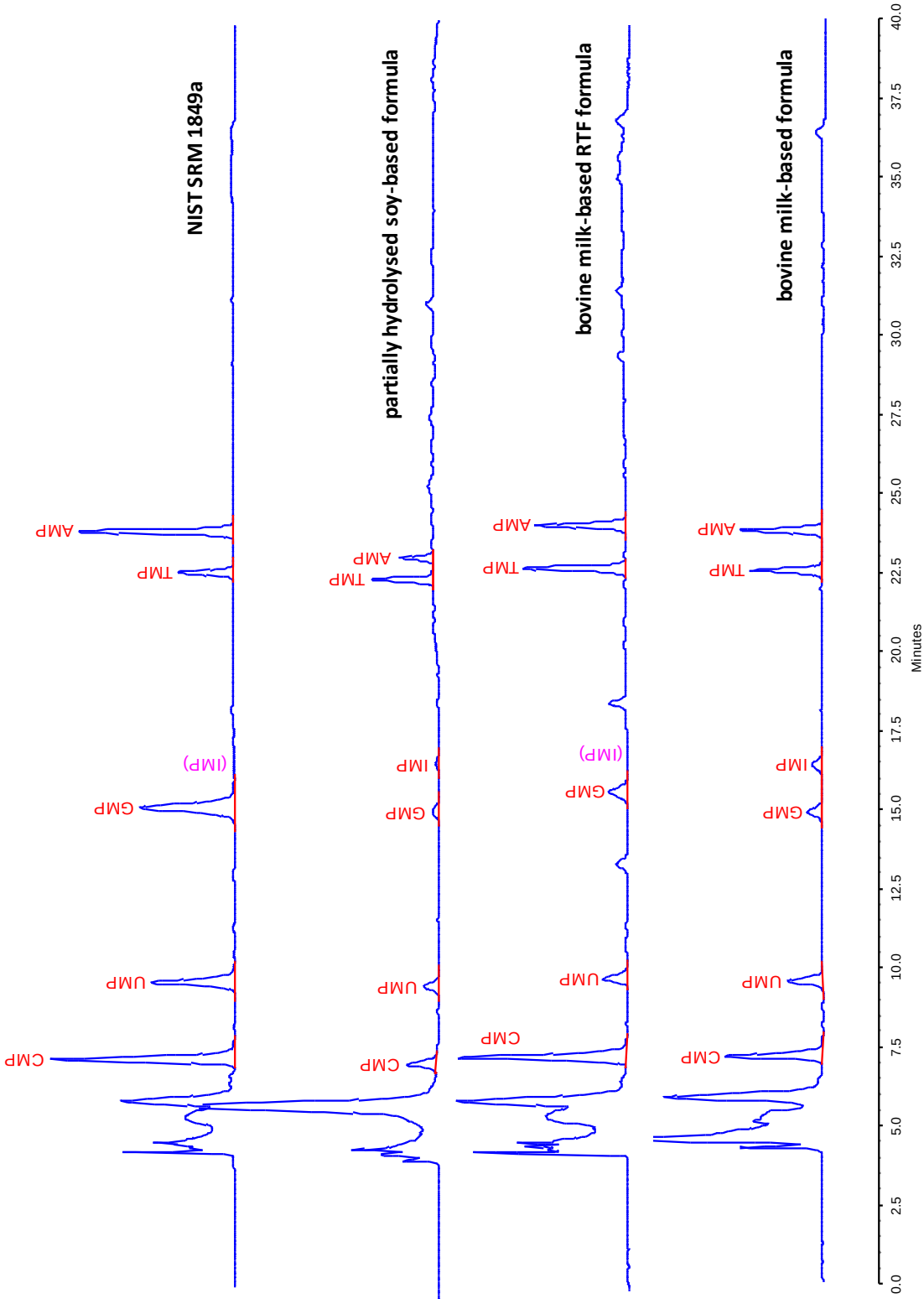

Figure 37. LC-UV modified chromatography of infant formula samples. (AMP = adenosine 5'-monophosphate; CMP = cytidine 5'-monophosphate; GMP = guanosine 5'-monophosphate; IMP = inosine 5'-monophosphate; UMP = uridine 5'-monophosphate; TMP = thymidine 5'-monophosphate. Chromatography: Gemini C18, 5 µm, 4.6 x 250 mm; mobile phases A = KH_2PO_4 (10 mM, pH = 5.6); B = methanol (100 %); oven temp = 40°C, flow rate 0.6 mL min^{-1}, gradient 0 min (100% A, 0% B), 25 min (80% A, 20% B), 26–40 min (100% A, 0% B). UV detection: 260 nm)

Single Laboratory Validation Extension

Eight standards were prepared over the range specified in the Nucleotides SMPR (Sullivan, 2012). Three replicate experiments were performed with standards analysed in random order. The detector response was plotted against concentration and regression analysis performed; **Figures 38–49**.

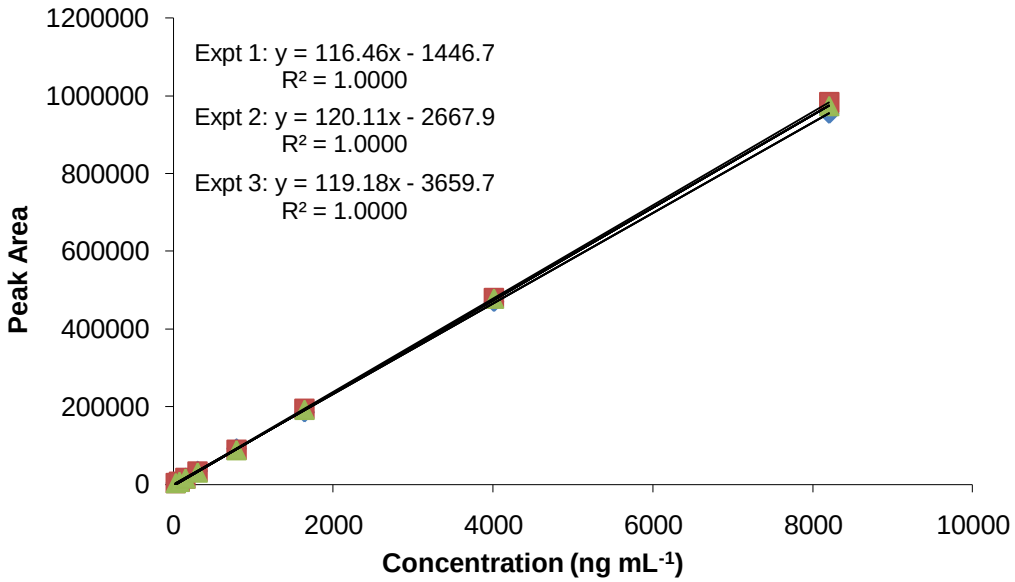

Figure 38. LC-UV method extension: CMP linear regression plot

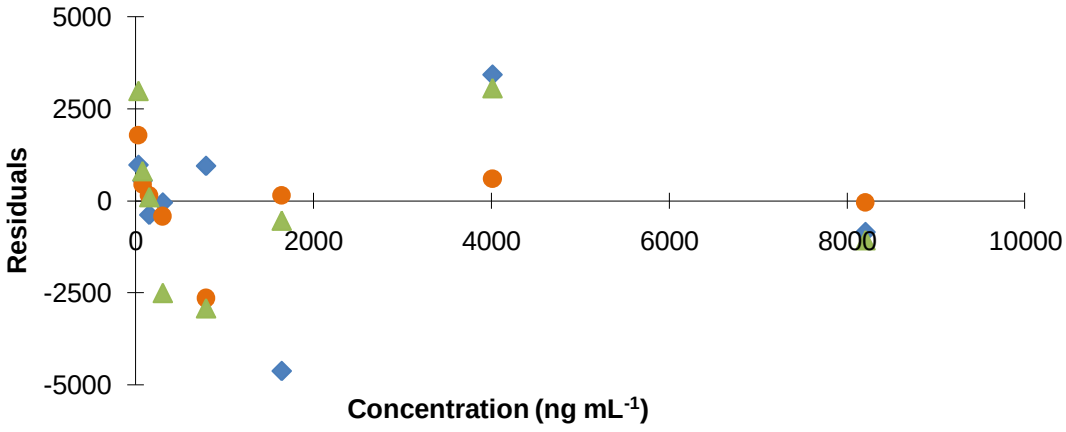

Figure 39. LC-UV method extension: CMP residuals plot

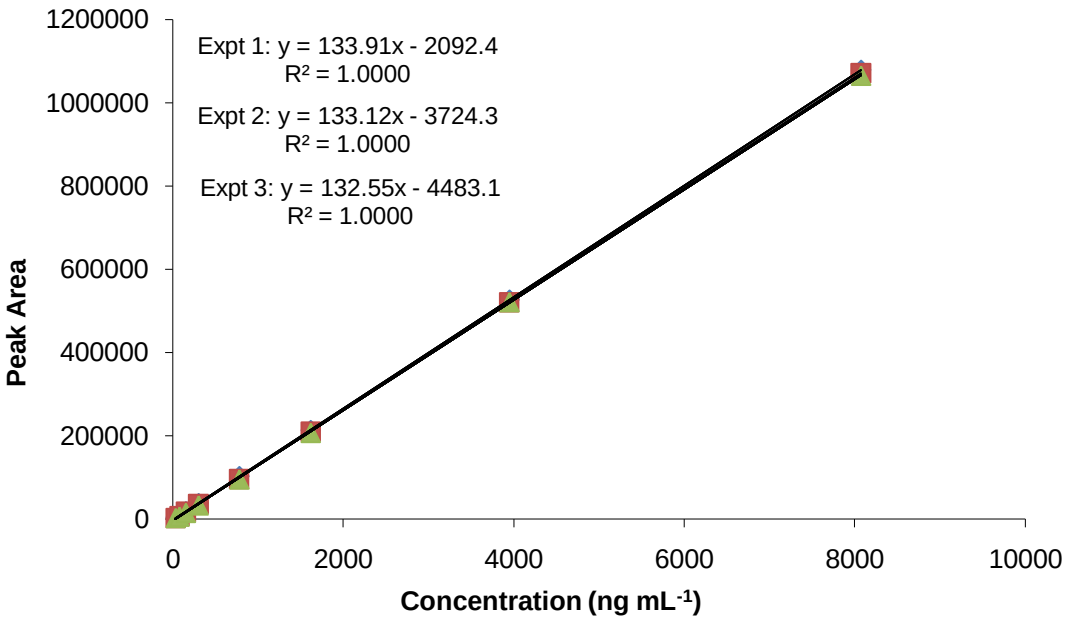

Figure 40. LC-UV method extension: UMP linear regression plot

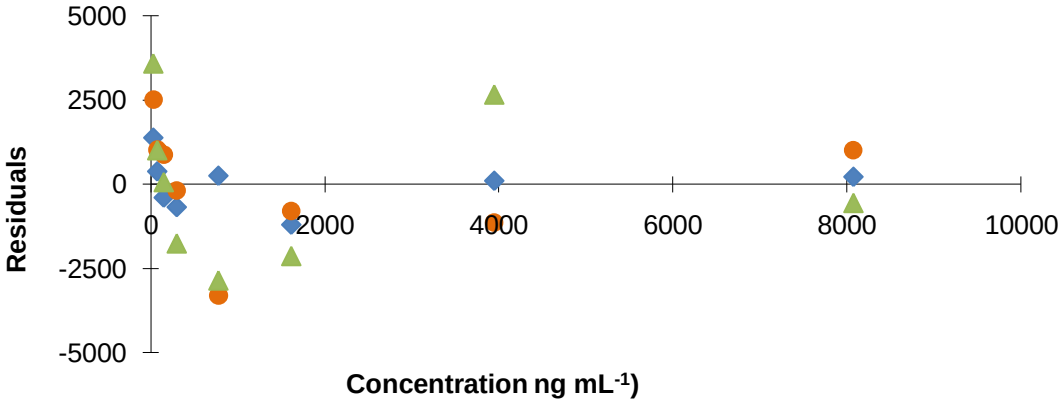

Figure 41. LC-UV method extension: UMP residuals plot

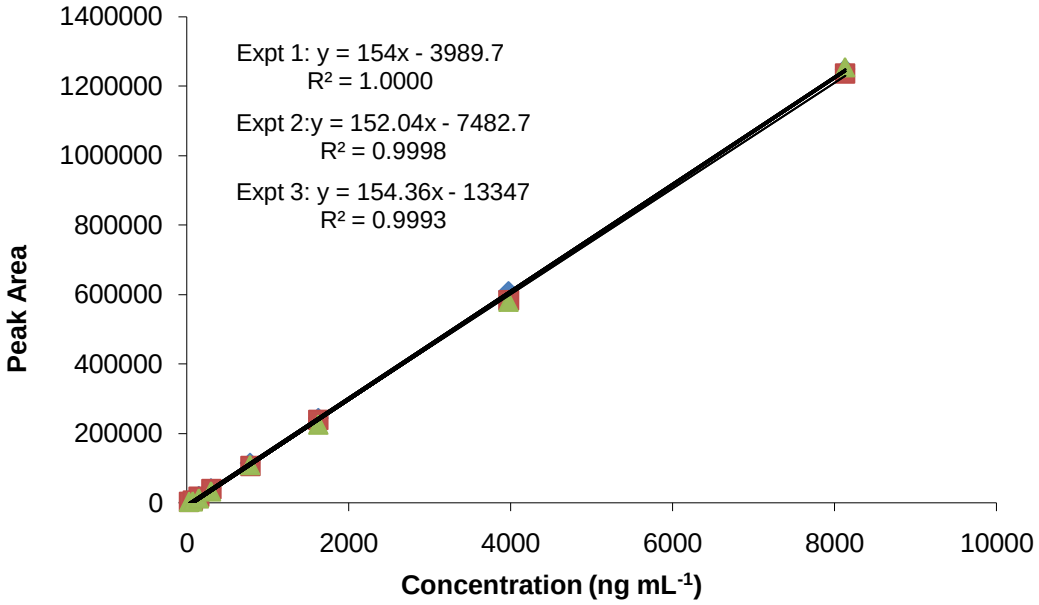

Figure 42. LC-UV method extension: GMP linear regression plot

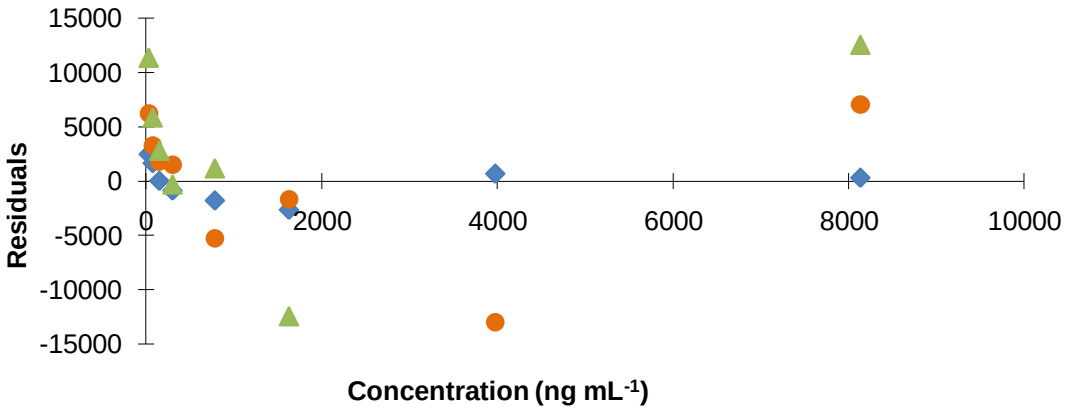

Figure 43. LC-UV method extension: GMP residuals plot

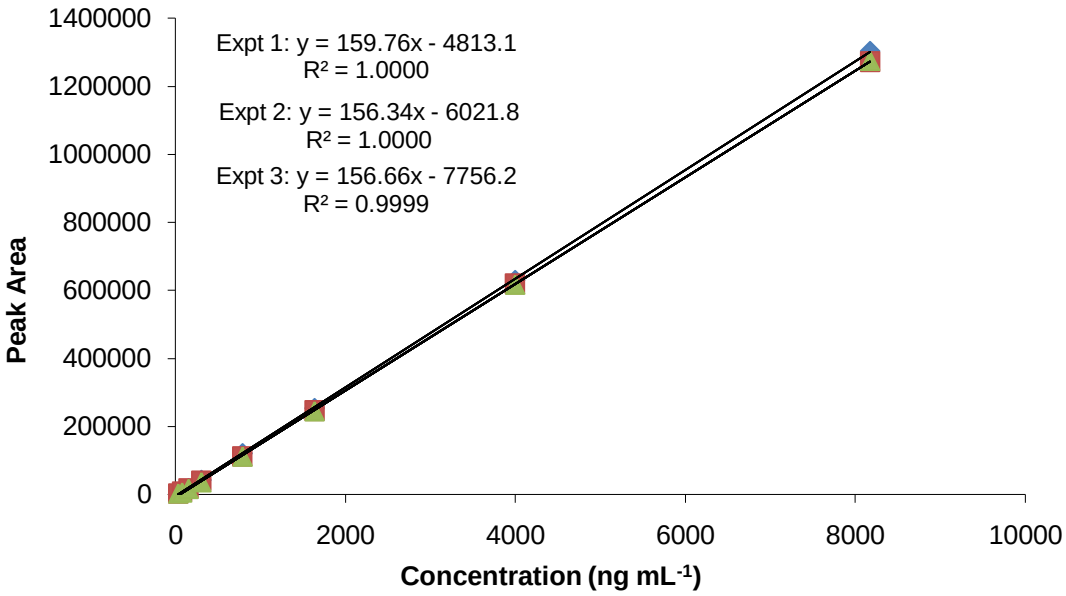

Figure 44. LC-UV method extension: IMP linear regression plot

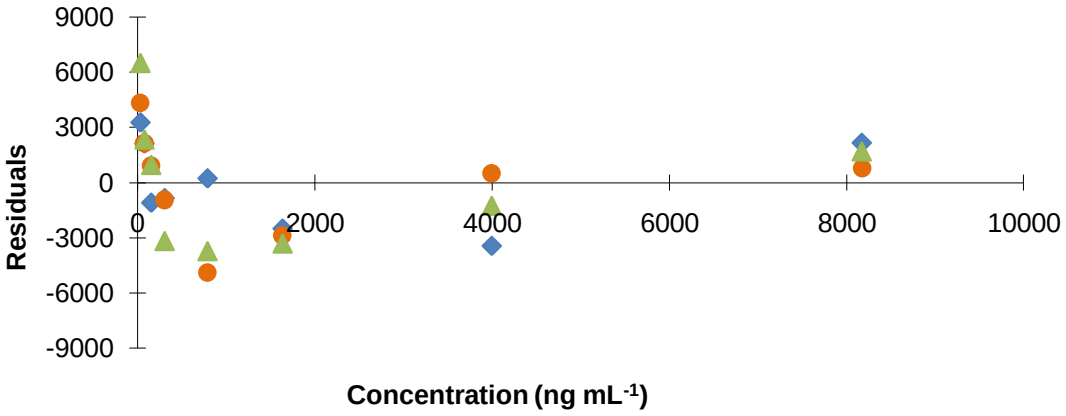

Figure 45. LC-UV method extension: IMP residuals plot

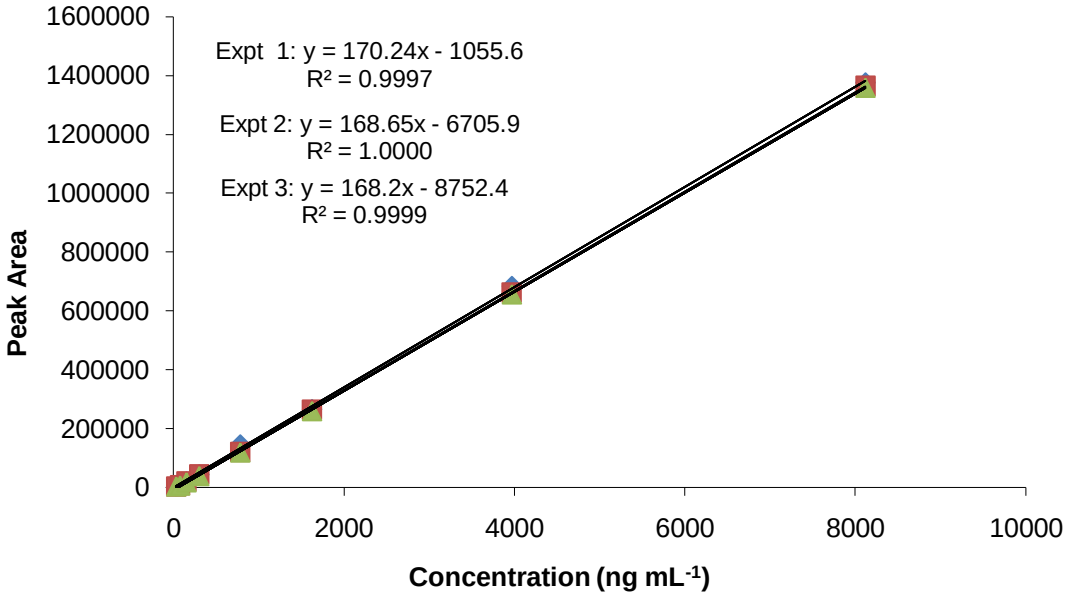

Figure 46. LC-UV method extension: AMP linear regression plot

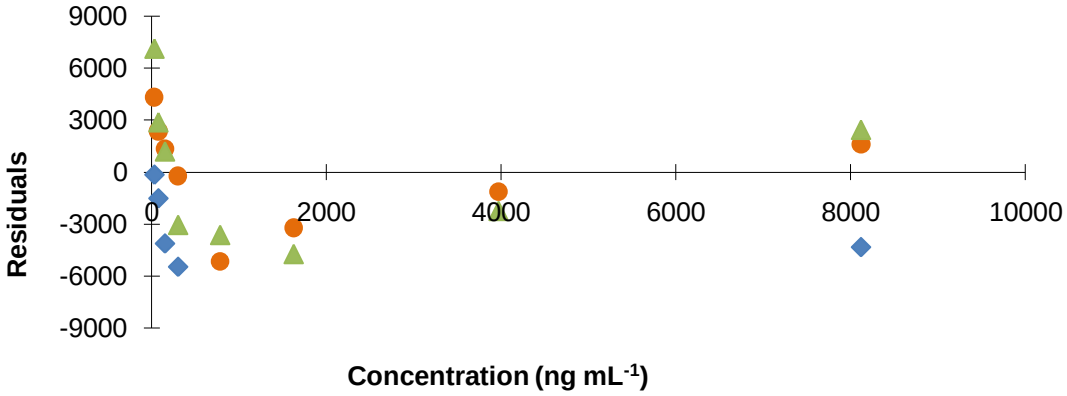

Figure 47. LC-UV method extension: AMP residuals plot

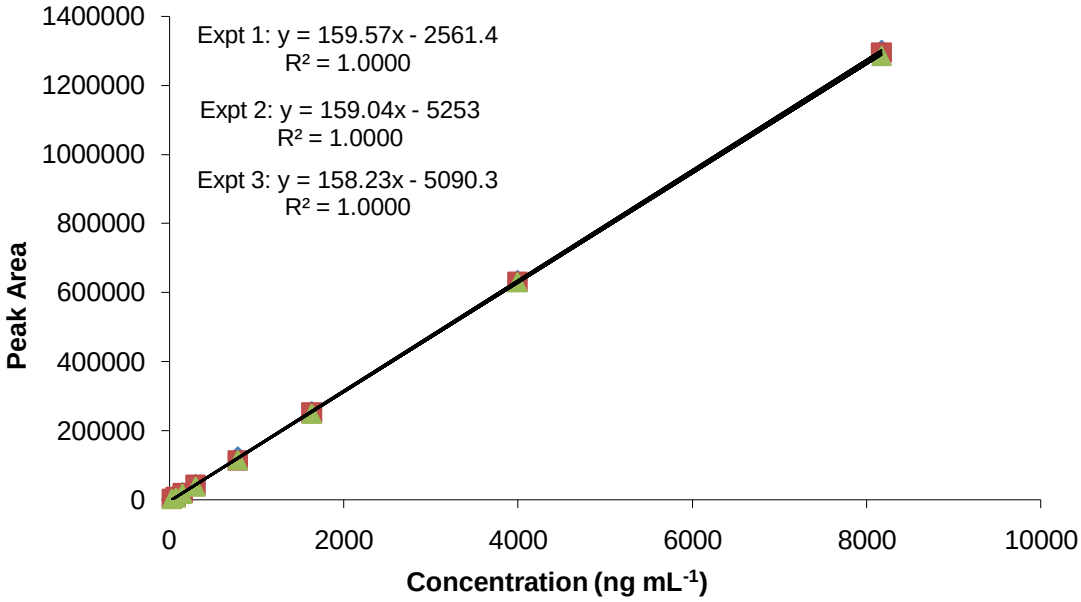

Figure 48. LC-UV method extension: TMP linear regression plot

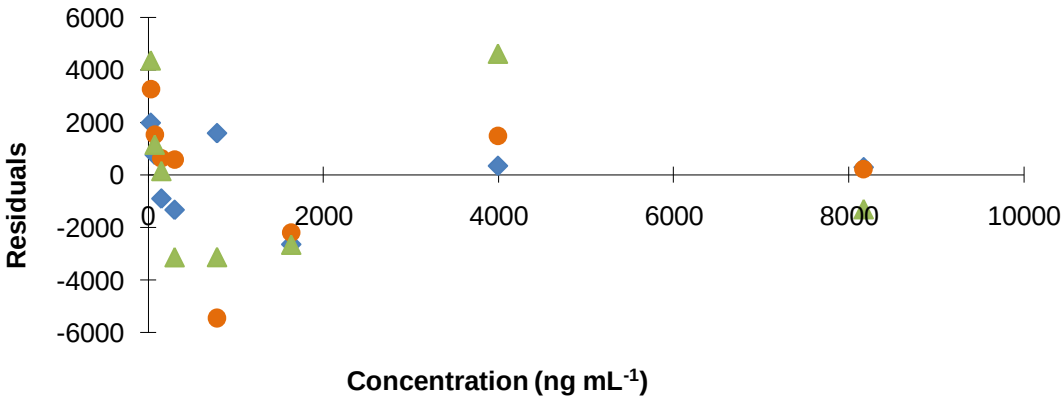

Figure 49. LC-UV method extension: TMP residuals plot

Linearity of dose response was confirmed by least squares regression analysis, with correlation coefficients of >0.9995. Plots of standard residuals showed no structure and only a small amount of random variability, also confirming linearity of the method.

The instrument LOD and LOQ were determined and the standard concentrations were converted to sample concentrations on powder and

RTF liquid basis; **Table 15**. The measured limit of quantitation of 0.017 mg dL^{-1} is less than that specified in the SMPR of 0.02 mg dL^{-1}, and correspond well with the MDLs reported previously (0.06–0.19 mg hg^{-1}) (Gill *et al*., 2010).

Table 15. LC-UV method extension: limit of detection and quantitation

	Analyte[a]				
	CMP	UMP	GMP	IMP	AMP
LOD as dry weight (mg hg^{-1})	0.04	0.04	0.04	0.04	0.04
LOQ as dry weight (mg hg^{-1})	0.13	0.14	0.13	0.13	0.13
LOD as liquid (mg dL^{-1})[b]	0.005	0.005	0.005	0.005	0.005
LOQ as liquid (mg dL^{-1})[b]	0.017	0.017	0.017	0.017	0.017

[a] AMP = adenosine 5′-monophosphate
CMP = cytidine 5′-monophosphate
GMP = guanosine 5′-monophosphate
IMP = inosine 5′-monophosphate
UMP = uridine 5′-monophosphate
[b] RTF calculated on 25g/200mL concentration basis as per SMPR
LOD = limit of detection; LOQ = limit of quantitation

Repeatability was evaluated in various infant formula products and assessed in a sample (low fat adult nutritional powder) spiked at the concentrations specified in the SMPR; **Table 16**.

Repeatability for the method in typical samples ranges for 1.2–4.1% RSD with a HorRat of 0.4–0.9, within the expected range of 0.3–1.3. Repeatability was poorest in the milk-based infant formula RTF liquid, due to the low unfortified concentrations close to the MDL.

For the adult nutritional sample spiked with nucleotides at higher concentrations (1 and 5 mg dL^{-1}) repeatability ranged from 1.1–2.8%, well below the limit of 6% set in the SMPR. The repeatability for the 0.1 mg dL^{-1} concentration was 0.3–2.5% below the limit of 8% set in the SMPR. The lowest concentration (0.02 mg dL^{-1}) is near the limit of quantitation for this method and the poorer repeatability for GMP and AMP (13.1%) reflects this with values above the limit of 10% set in the SMPR.

Table 16. LC UV method extension: repeatability

Sample	Repeatability[a,b]				
	CMP	UMP	GMP	IMP	AMP
Milk-based IF powder	1.2% (0.4)	2.0% (0.6)	1.8% (0.5)	1.8% (0.5)	1.8% (0.6)
NIST1849a CRM	2.1% (0.9)	2.1% (0.8)	2.0% (0.8)	–	1.8% (0.6)
Milk-based IF powder	1.2% (0.4)	1.3% (0.4)	2.0% (0.5)	–	1.7% (0.5)
Milk-based IF RTF liquid[c]	3.1% (0.8)	2.6% (0.5)	3.6% (0.7)	–	4.1% (0.9)
AN powder[d] spiked to 0.16 mg hg^{-1}	5.2% (0.7)	3.2% (0.4)	13.1% (1.8)	5.9% (0.3)	13.1% (1.8)
AN powder[d] spiked to 0.8 mg hg^{-1}	1.6% (0.3)	2.5% (0.4)	0.3% (0.1)	1.7% (0.3)	2.3% (0.4)
AN powder[d] spiked to 8 mg hg^{-1}	1.6% (0.4)	1.3% (0.3)	2.1% (0.5)	1.5% (0.4)	1.8% (0.4)
AN powder[d] spiked to 40 mg hg^{-1}	1.3% (0.4)	2.8% (0.9)	1.1% (0.4)	1.6% (0.5)	2.7% (0.9)

[a] AMP = adenosine 5′-monophosphate; CMP = cytidine 5′-monophosphate; GMP = guanosine 5′-monophosphate; IMP = inosine 5′-monophosphate; UMP = uridine 5′-monophosphate
[b] Percent relative standard deviation (HorRat)
[c] Endogenous levels near the limit of quantitation
[d] Concentration spiked to a low fat adult nutritional powder
IF = infant formula; CRM = certified reference material; RTF = ready-to-feed liquid; AN = adult nutritional
– Not detected

Intermediate precision for the method in nucleotide-supplemented samples was estimated between 3.0–5.7%; **Table 17**. In a nucleotide-unsupplemented infant formula RTF liquid sample, intermediate precision was poorest for UMP with 14.9% RSD, due to the low unfortified concentrations that were measured at or below the limit of quantitation.

Table 17. LC-UV method extension: intermediate precision

Sample	Intermediate precision[a] (RSD%)				
	CMP	UMP	GMP	IMP	AMP
Milk-based IF powder	4.3	5.7	3.8	3.0	4.1
NIST1849a CRM	4.7	5.3	3.3	–	4.5
Milk-based IF powder	4.1	5.1	4.6	–	4.9
Milk-based IF RTF liquid[b]	5.5	14.9	5.3	–	5.8

[a] AMP = adenosine 5′-monophosphate
CMP = cytidine 5′-monophosphate
GMP = guanosine 5′-monophosphate
IMP = inosine 5′-monophosphate
UMP = uridine 5′-monophosphate
[b] Endogenous levels near the limit of quantitation
– Not detected
IF = infant formula
CRM = certified reference material
RTF = ready-to-feed liquid

Recovery was evaluated in a range of different matrices from products in the SPIFAN kit. Recovery was also evaluated in a single SPIFAN matrix (low fat adult nutritional powder) at each of the four concentrations levels as defined in the SMPR; **Table 18**.

Recovery for samples spiked at 50% and 150% of typical concentrations were between 91.6–106.4%. Recovery at three of the concentration levels defined in the SMPR (0.1, 1, 5 mg dL^{-1}) were 92.5–103.4%. The fourth and lowest concentration (0.02 mg dL^{-1}) had recoveries of 91.3–124.7%, outside the 90–110% limit specified. Since this concentration is near the limit of quantitation, higher uncertainty in results and a wider range of recoveries was observed.

Table 18. LC-UV method extension: recovery

Sample	Recovery[a,b] (%)				
	CMP	UMP	GMP	IMP	AMP
Milk-based IF powder[b]	103	102	96.5	103	94.2
AN milk protein-based powder[b]	98.3	92.8	104	99.3	97.7
IF p/h milk-based powder[b]	101	94.1	106	96.5	106
IF p/h soy-based powder[b]	99.3	103	96.7	95.7	97.0
AN low fat powder[b]	103	98.0	102	102	95.3
Child formula powder[b]	100	94.6	100	102	91.6
Infant elemental powder[b]	97.8	100	104	97.9	98.0
IF milk-based powder[b]	99.9	97.3	102	103	97.7
IF milk-based RTF liquid[b]	101	96.9	102	101	101
IF soy-based powder[b]	96.5	97.6	99.2	98.8	106
AN high protein RTF liquid[b]	96.3	98.6	101	101	102
AN high fat RTF liquid[b]	97.2	95.2	100	97.3	98.2
AN powder[c] spiked to 0.16 mg hg^{-1}	115	91.3	102	91.8	125
AN powder[c] spiked to 0.16 mg hg^{-1} [c,d]	96.5	94.8	102	94.0	103
AN powder[c] spiked to 8 mg hg^{-1}	99.8	92.5	101	98.4	99.6
AN powder[c] spiked to 40 mg hg^{-1}	102	91.9	102	99.9	102

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

[b] Mean of independent duplicate measurements for samples spiked at 50% and 150% of typical concentration.

[c] Mean recovery of independent triplicate measurements for spiked low fat adult nutritional powder.

IF = infant formula; AN = adult nutritional; RTF = ready-to-feed liquid; p/h = partially hydrolysed

Bias was evaluated by replicate measurement of the NIST1849a CRM; **Table 19**. Results vary somewhat from the tight limits expressed in the Certificate of Analysis. At the 95% level of confidence, the reference values for UMP, GMP, and AMP are within the confidence interval of the measured data indicating no bias in the measured results, whereas CMP

is outside the confidence interval indicating the there is a bias between the reference value and the measured results.

Table 19. LC-UV method extension: bias vs. CRM values

Sample	Results[a] (mg hg^{-1})			
	CMP	UMP	GMP	AMP
Mean	28.0	12.5	14.9	10.8
Standard deviation	1.3	0.6	0.5	0.5
p–value (α=0.05, *n*=12, *df*=11)	0.01	0.07	0.06	0.11
NIST1849a value[b]	26.8	12.9	14.6	10.5
CRM uncertainty	0.29	0.15	0.11	0.53
Lower confidence limit	27.1	12.1	14.6	10.4
Upper confidence limit	28.8	12.9	15.2	11.1

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

[b] nucleotide values for NIST1849a are reference values only and not certified

α = level of significance, *n* = number of replicates, *df* = degrees of freedom

Selectivity was assessed by determining chromatographic characteristics of retention time stability, peak asymmetry, capacity factor, resolution and peak purity for a nucleotide supplemented milk-based infant formula, and results are shown in **Table 20**.

Table 20. LC-UV method extension: selectivity

Nucleotide	Retention Time (min)	Asymmetry	Capacity Factor	Resolution	3 point Peak Purity
CMP	7.2	1.3	0.8	3.6	0.9995
UMP	9.6	1.2	1.4	6.9	0.9997
GMP	14.9	1.0	2.7	12.5	0.9997
IMP	16.4	1.0	3.1	3.0	0.9996
TMP	22.6	1.0	4.7	15.7	0.9998
AMP	23.9	1.0	5.0	4.6	0.9999

3.2. Total Potentially Available Nucleosides in Milk

The development of the TPAN method has been an important contribution to further understanding of the distribution of nucleosides and nucleotides in human milk. However, the application of the TPAN methodology to the analysis of milk of species other than human has not been published previously. The purpose of the first study was to analyse bovine milk to determine the relative contributions of nucleosides and different nucleotide forms to TPAN and determine how these varied over the first month of lactation. A second study was then undertaken to assess the relative nucleoside and nucleotide contributions to TPAN in bovine, caprine, and ovine milk.

A description of this method and results obtained from this research were summarised and presented at the 124th annual AOAC International conference in 2010 and subsequently published in the International Dairy Journal (Gill *et al*., 2011; Gill *et al*., 2012b) (see Appendix I, Appendix II).

3.2.1. EXPERIMENTAL

The enzymatic digestion and phenylboronate affinity sample clean-up steps of the TPAN analysis was performed in accordance with the method of Leach *et al*. (1995) as demonstrated by Molitor (2008).

Oligonucleotides from RNA are released to nucleotide monophosphates by the phosphodiesterase, nuclease P1; nucleotides from adducts are converted by nucleotide pyrophosphatase; and nucleotides are dephosphorylated to nucleosides by alkaline phosphatase.

Following enzymatic digestion, the free nucleosides are extracted from the reaction mixture using a boronate-derivatised gel and are separated

chromatographically by reversed-phase chromatography. The TPAN value is obtained by summation of nucleosides measured through all enzyme treatments while speciation is determined from results obtained from individual enzyme treatments; **Figure 50**.

3.2.1.1 Apparatus

Milk samples were collected in disposable polypropylene containers (120 mL) and stored in disposable polypropylene centrifuge tubes (50 mL) supplied by Biolab. Measurement of pH was achieved using a Meterlab PHM210 standard pH meter from Radiometer Analytical. Samples were centrifuged using a Varifuge 3.0 centrifuge (Heraeus, Newport Pagnell, UK). Syringe filtering of sample extracts was achieved using 3 mL disposable syringes (Terumo) and 0.22 μm Millex MCE syringe filters (Millipore, Billerica, MA). Sample treatment was performed in glass Kimax tubes (10 mL) with teflon-lined screw caps (Kimble-Chase, Vineland, NJ).

The HPLC system used 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). Class-*vp* software version 6.12 was used for instrument control and data processing. The column used for HPLC analysis was a Prodigy C_{18} column, 5 μm, 4.6 mm x 150 mm (Phenomenex).

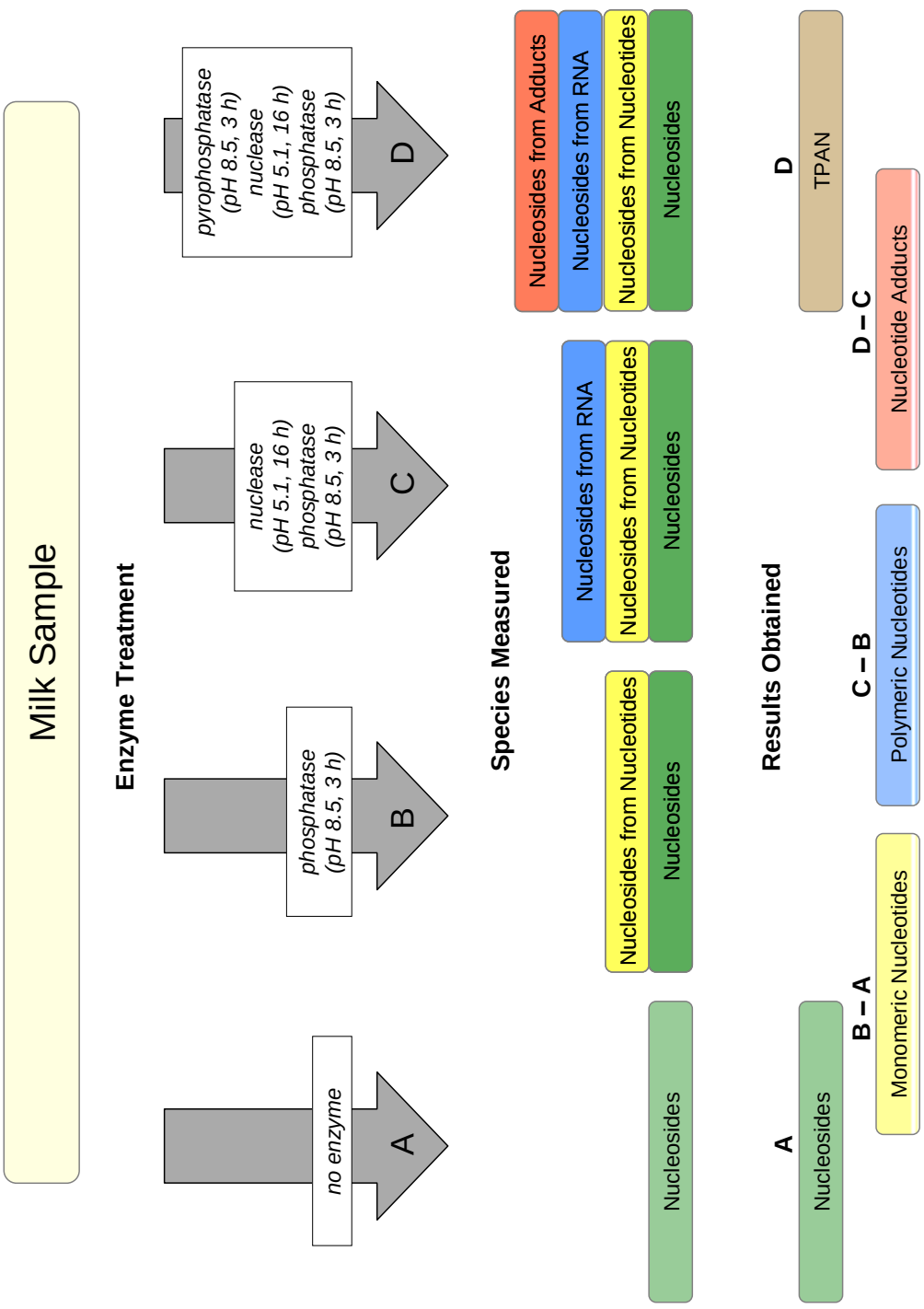

Figure 50. Enzymatic digestion scheme with TPAN speciation

3.2.1.2 Reagents

Ado (≥ 99%), Cyd (≥ 99%), Guo (≥ 98%), Ino (≥ 99%), Urd (≥ 99%), 5-methylcytidine (5mCyd) (≥ 99%), 8-bromoguanosine (≥ 99%), uridine 5′-diphosphoglucose (≥ 98%), RNA, cytidine 5′-diphosphocholine (≥ 99%), β-nicotinamide adenine dinucleotide (≥ 99%), AMP (≥ 99%), CMP disodium salt (≥ 99%), GMP disodium salt hydrate (≥ 99%), UMP (≥ 99%); potassium acetate, zinc sulphate heptahydrate, and magnesium chloride hexahydrate (GR ACS grade or equivalent); pyrophosphatase, nuclease P1, and alkaline phosphatase, were purchased from Sigma-Aldrich. The boronate affinity gel (Affi-gel 601) was purchased from Bio-Rad (Hercules, CA). Glacial acetic acid, sodium hydroxide, dipotassium hydrogen phosphate, 85% phosphoric acid, 37% hydrochloric acid, potassium hydroxide, 30% ammonium hydroxide (GR ACS grade or equivalent), and methanol (HPLC grade) were supplied by Merck. Water was purified to a resistivity ≥18 MΩ using an E-pure water system (Barnstead).

The alkaline phosphatase was tested for possible adenosine deaminase activity before use (Gehrke and Kuo, 1989; Molitor, 2008). A 5 mg dL^{-1} solution of adenosine was incubated with alkaline phosphatase under conditions described below (see 3.2.1.4 below) and no deamination of adenosine to inosine was found (recovery 94.8%).

Solutions containing enzymes were prepared in the following manner: Nuclease P1 solution was made by adding sodium acetate (0.1 M, pH = 5.1, 4 mL) directly to the vial from the supplier containing ~1.2 mg of protein, followed by capping the vial and gently mixing. Pyrophosphatase solution was made by adding ammonium acetate (0.5 M, pH = 8.5, 4 mL) directly to the vial from the supplier containing ~11 mg of protein, followed by capping the vial and gently mixing. Alkaline phosphatase supplied as a suspension in 2.5 M $(NH_4)_2SO_4$.

Stock nucleoside standard solutions (~1 mg mL^{-1}) were prepared by adding ~50 mg of the nucleosides, uridine, cytidine, guanosine, inosine, adenosine, 5-methylcytidine, and 8-bromoguanosine to separate 50 mL volumetric flasks and making to volume with water. A working internal standard solution (~100 µg mL^{-1}) was made by pipetting 5 mL each of 5-methylcytidine and 8-bromoguanosine stock internal standard solution into a 50 mL volumetric flask and making to volume with water.

The boronate affinity gel was hydrated by adding the contents of a 5 g vial of Affi-gel 601 to a 100 mL beaker with a stir bar containing potassium phosphate (0.1 M, 50 mL) and left stirring for two hours. After hydrating, 350 µL aliquots of slurry were transferred to 2 mL snap cap microcentrifuge tubes, which were then stored in the freezer at < -15 ºC for later use.

Mobile phase A ($NaCH_3COO$, 0.05 M, pH = 4.8) was made by dissolving 4.10 g of $NaCH_3COO$ in 900 mL of water, adjusting the pH to 4.8 with acetic acid solution (25% *w/v*), and then making to 1 L with water. Mobile phase B consisted of 100% methanol.

3.2.1.3 Sample Collection and Storage

Milk samples were collected from two herds of Jersey cows from two separate farms (9 km apart) in the eastern Waikato region of New Zealand (**Figure 51**). From each herd, seven cows were selected to provide milk for analysis. The first herd was a winter-milk herd with the first cow calving on the 24th March 2008 (early autumn) and the final one calving on the 18th of April 2008. The second herd was a summer-milk herd with the first one calving on the 1st August 2009 (late winter) and the final one calving on the 7th August 2009. Cows selected for inclusion in this study were chosen because calving occurred between 6:00 and 10:00 am, provided they were healthy cows in their second or subsequent calving and had

normal calvings with no complications. With the exception of the 6-hour sample, sample collection was performed in the morning between 6:00 and 10:00 am, which coincided with regular milking times.

Figure 51. Map of the Waikato region of New Zealand (source: Google Maps)

From each cow, approximately 80 mL of milk/colostrum was collected in a 120 mL disposable container by the farmer. These were collected at various time intervals throughout the first month of lactation, more frequently in the beginning and less so as the month progressed. The scheduled collection times were:

- parturition + 0 hours
- parturition + 6 hours
- parturition + 24 hours
- parturition + 2 days
- parturition + 3 days
- parturition + 5 days

- parturition + 10 days
- parturition + 20 days
- parturition + 30 days

Milk/colostrum samples were collected from the cows at the appropriate time and immediately refrigerated at 4 ºC. The sample was then picked-up from the farm as soon as practicable (within 6 hours), taken to the laboratory, and immediately prepared for storage.

Upon return to the laboratory, each sample mixed by hand, and then divided into four separate 50 mL centrifuge tubes. A 20 mL aliquot was taken for proximate testing, protein by Kjeldahl (IDF, 2001) and total solids by microwave gravimetric analysis (IDF, 1987). A 10 mL aliquot was taken and prepared for storage for later TPAN analysis, with the remainder of milk sample frozen intact at -18 ºC.

In May 2009, samples of bovine milk and caprine milk 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 was supplied from a subset of a flock of sheep in a New Zealand research herd. Upon collection, the samples were taken to the laboratory and immediately prepared for storage for later analysis.

Samples were prepared for storage prior to TPAN analysis by pipetting a 10 mL aliquot of sample, adding NaOH (1 M, 20 mL), vortex mixing, and standing for 30 min, before neutralising to pH = 7.35 ± 0.05 with HCl and making to 50 mL volume and freezing at < -15 ºC.

3.2.1.4 Enzymatic Digestion

Immediately prior to analysis, all milk samples were mixed by hand to ensure sample homogeneity.

Heat Deactivation of Enzymes

Since milk contains a number of endogenous enzymes that can degrade polymeric and monomeric nucleotides, the sample was heated to denature the enzymes thereby eliminating any enzyme activity. To 10 mL glass tubes, 5 mL of milk storage samples were added; the tubes were capped then immersed in a water bath (95 ºC) for 30 min with periodic mixing. The tubes were then removed from water bath and allowed to cool to room temperature for subsequent enzyme treatment. Bovine milk samples from the seven cows in the lactation study were pooled for each post-partum time period prior to analysis.

Determination of TPAN

The sample preparation for the determination of TPAN, requires enzyme digestion with nuclease to release polymeric nucleotides, then enzyme digestion with pyrophosphatase to release nucleotide adducts, and alkaline phosphatase to convert nucleotides to nucleotides; **Figure 50** (enzyme treatment D).

To a 10 mL glass tube from the enzyme deactivation treatment was added: sodium acetate (0.1 M, pH = 5.1, 2 mL), zinc sulphate (0.01 M, 50 μL), nuclease P1 solution (50 μL), and working internal standard solution (100 μL). The tube was capped and incubated at 37 ± 1 ºC overnight for 18 ± 1 hour. After initial incubation ammonium acetate (0.5 M, pH = 8.75, 1 mL), ammonium hydroxide (30%, 50 μL), magnesium chloride (1 M, 50 μL), alkaline phosphatase (50 μL, ~14 units), and pyrophosphatase (50 μL) were added. The tube was capped and incubated at 37 ± 1 ºC for 3 hours. The contents of the tube were transferred to a 25 mL volumetric flask, which was made to volume with potassium phosphate (0.25 M, pH = 10.5). The sample mixture was then cleaned-up on boronate affinity gel (see 3.2.1.5 below).

Determination of Polymeric RNA + Nucleotides + Nucleosides

The sample preparation for the determination of free nucleosides, and nucleosides from RNA and nucleotides, requires enzyme digestion with nuclease to release polymeric nucleotides, then enzyme digestion with alkaline phosphatase to convert nucleotides to nucleosides; **Figure 50** (enzyme treatment C).

To a 10 mL glass tube from enzyme deactivation treatment was added: sodium acetate (0.1 M, pH = 5.1, 2 mL), zinc sulphate (0.01 M, 50 μL), nuclease P1 solution (50 μL), and working internal standard solution (500 μL). The tube was capped and incubated at 37 ± 1 ºC overnight for 18 ± 1 hour. After initial incubation ammonium acetate (0.5 M, pH = 8.75, 1 mL), ammonium hydroxide (30%, 50 μL), magnesium chloride (1 M, 50 μL), and alkaline phosphatase (50 μL, ~14 units) were added. The tube was capped and incubated at 37 ± 1 ºC for 3 hours. The contents of the tube were transferred to a 25 mL volumetric flask, which was made to volume with potassium phosphate (0.25 M, pH = 10.5). The sample mixture was then cleaned-up on boronate affinity gel (see 3.2.1.5 below).

Determination of Nucleotides + Nucleosides

The sample preparation for the determination of free nucleosides and nucleosides from nucleotides requires enzyme digestion with alkaline phosphatase to convert nucleotides to nucleotides; **Figure 50** (enzyme treatment B).

To a 10 mL glass tube from enzyme deactivation treatment ammonium acetate (0.5 M, pH = 8.75, 1 mL), ammonium hydroxide (30%, 50 μL), magnesium chloride (1 M, 50 μL), alkaline phosphatase (50 μL, ~14 units) and working internal standard solution (500 μL) were added. The tube was capped and incubated at 37 ± 1 ºC for 3 hours. The contents of the tube were transferred to a 25 mL volumetric flask that was made to volume

with potassium phosphate (0.25 M, pH = 10.5). The sample mixture was then cleaned-up on boronate affinity gel (see 3.2.1.5 below).

Determination of Free Nucleosides

The sample preparation for the determination of free nucleosides requires no enzyme digestion; **Figure 50** (enzyme treatment A).

To a 10 mL glass tube from enzyme deactivation treatment working internal standard solution (500 μL) was added. The contents of the tube were transferred to a 25 mL volumetric flask that was made to volume with potassium phosphate (0.25 M, pH = 10.5). The sample mixture was then cleaned-up on boronate affinity gel (see 3.2.1.5 below).

3.2.1.5 Boronate Affinity Clean-up

For each sample, a 2 mL snap cap microcentrifuge tube containing the hydrated affinity gel was removed from the freezer and allowed to thaw to room temperature. The microcentrifuge tube was centrifuged at 10,000 x *g* for 2 min; the supernatant was aspirated to waste while avoiding significant gel loss. Phosphoric acid (0.25 M, 1 mL) was added to the microcentrifuge tube to remove any interferences complexed to the boronate moiety. The microcentrifuge tube was vortex mixed for 30 s to re-suspend the gel then centrifuged and the supernatant aspirated to waste. Potassium phosphate (0.25 M, pH = 10.5, 1 mL) was added to the microcentrifuge tube to convert the affinity gel to its basic form. The microcentrifuge tube was then vortex mixed for 30 s to re-suspend the gel then centrifuged and the supernatant aspirated to waste. Potassium phosphate (0.25 M, pH = 10.5, 1 mL) was added once more to the microcentrifuge tube, which was then vortex mixed for 30 s to re-suspend the gel. The microcentrifuge tube was allowed to stand for 15 min with vortex mixing for 30 s every 5 min to convert the gel to the basic form.

The tube was then centrifuged and the supernatant aspirated to waste. The sample extract (1.5 mL) from the enzyme digest was added to the microcentrifuge tube, which was allowed to stand for 15 min with vortex mixing for 30 s every 5 min during which formation of nucleoside-boronate complex occurs. The tube was then centrifuged and the supernatant aspirated to waste. The gel was washed twice by adding potassium phosphate (0.25 M, pH = 10.5, 1 mL), vortex mixing for 30 s, centrifuging, aspirating the supernatant to waste and repeating. To release bound nucleosides phosphoric acid (0.25 M, 1 mL) was added to the tube, which was then vortex mixed for 30 s. The entire contents of the tube were transferred to a syringe with a 0.22 μm filter and filtered into an HPLC vial ready for chromatographic analysis.

3.2.1.6 Chromatography

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 (pK_a = 4.75), thereby offering greater buffer capacity at the desired pH.

An organic solvent component is required in the mobile phase to facilitate the timely elution of nucleosides from the C_{18} column. However, to obtain sufficient resolution between peaks, a gradient elution procedure would be necessary. Gradients were formed by low pressure mixing of two mobile phases, A and B. A number of gradient procedures were evaluated to determine an optimum protocol that would have a relatively short run-time coupled with sufficient resolution between peaks. An optimum separation of nucleosides was achieved using the gradient procedure described in **Table 21**.

Table 21. TPAN method: chromatographic procedure[a]

Time (min)	Phase composition[b]	
	% A	% B
0	95	5
3	95	5
7	75	25
22	75	25
23	95	5
30	95	5

[a] Flow rate 0.7 mL min^{-1}; column temperature 20 °C
[b] Mobile phase A = 0.05 M $NaCH_3COO$, pH = 4.8
Mobile phase B = 100% methanol

3.2.1.7 Detection, Identification and Quantitation

Spectral data were acquired by the PDA detector from 210–300 nm. Peak identification was by retention time and similarity of chromatographic peak spectrum against standards (similarity index >0.99). Chromatograms were integrated at a wavelength of 260 nm and results determined by internal standard technique using 5-methylcytidine; **Equation 4**.

Internal standards were used to account for recovery losses through the enzymatic and phenylboronate affinity clean-up steps. Leach *et al*. (1995) used 5-methylcytidine as internal standard to quantitate adenosine, cytidine, and uridine. The use of 5-methylcytidine gave lower than expected recovery of guanosine; therefore, 1-methylguanosine was used to quantitate guanosine. However, 1-methylguanosine was not commercially available and other modified guanosine alternatives were identified for this study. Trials with 7-methylguanosine were not successful as standard concentrations were not stable and degraded rapidly with time. This was attributed to the susceptibility of 7-methylguanosine to cleavage of the imidazole ring making it unsuitable as an internal standard (Barbarella *et al*., 1991).

$$\text{Nucleoside (mg dL}^{-1}\text{)} = \frac{A_{NT}}{A_{IS}} \times \frac{1}{L} \times \frac{(C_{IS} \times V_{IS})}{V_U} \times \frac{V_T}{V_D} \times \frac{100}{1000} \quad \text{(Equation 4)}$$

where: A_{NT} = analyte (nucleoside) peak area
A_{IS} = internal standard (5mCyd) peak area
L = linear regression slope of calibration curve
C_{IS} = concentration of internal standard (µg mL^{-1})
V_{IS} = volume of internal standard (µg mL^{-1})
V_U = volume of aliquot of diluted sample analysed (mL)
V_T = total volume of diluted sample (mL)
V_D = volume of milk in diluted sample (mL)
100 = unit conversion from mL^{-1} to dL^{-1}
1000 = convert mass result from µg to mg

8-bromoguanosine was selected as an internal standard candidate for guanosine. However, the recoveries for this standard were no better than 5-methylcytidine, therefore quantitation for all four nucleosides was measured against 5-methylcytidine, accepting that lower recoveries were possible for guanosine.

3.2.1.8 Statistical Analysis

Statistical analyses were performed using Minitab version 15.1 (State College, PA). Data obtained in bovine colostrum and milk was analysed by one-way analysis of variance of the response of season (winter-milk, summer-milk) with covariate time (0, 0.25, 1, 2, 3, 5, 10, 20, 30 days post-partum). 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 $\alpha = 0.05$ level. Data obtained in mature bovine, caprine, and ovine milk were analysed by one-way analysis of variance of the response of each species (bovine, caprine, ovine) and Tukey's multiple comparison test.

3.2.2. RESULTS AND DISCUSSION

3.2.2.1 Method Performance

System Suitability

Chromatographic performance was assessed by replicate analyses ($n = 6$) of a mixed nucleotide standard; **Table 22, Figure 52**.

Performance within recommended guidelines was achieved, with the exception of the retention factors for cytidine, uridine, and 5-methylcytidine (guideline > 2.0). The specific nature of the phenylboronate sample clean up provides analytical chromatography relatively free of interferences and therefore, early retention was deemed acceptable.

Recovery

A spiked recovery study was performed on free nucleosides and was assessed through the affinity gel 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 $\mu g\ mL^{-1}$). Recovery was assessed by comparison of peak areas for the spiked and unspiked samples, relative to those of the mixed standard.

Table 22. TPAN method: system suitability

Parameter	Analyte[a]													
	Cyd		Urd		5mCyd		Ino		Guo		Ado		8BrGuo	
Retention time[b]	4.8	(2.4%)c	7.1	(2.8%)	10.0	(3.5%)	13.0	(1.5%)	13.6	(1.2%)	16.1	(1.0%)	18.4	(1.4%)
Retention factor, *k′*	1.4	(4.2%)	2.2	(3.4%)	3.6	(4.7%)	4.9	(1.6%)	5.2	(1.8%)	6.3	(1.2%)	7.4	(1.8%)
Theoretical plates, *N*	6570	(2.1%)	7650	(1.9%)	8193	(2.3%)	57027	13.2%)	81552	(6.1%)	95304	(2.7%)	60082	(3.1%)
Tailing, T_f	1.2	(0.0%)	1.1	(3.9%)	1.0	(0.0%)	1.1	(2.5%)	1.2	(4.1%)	1.2	(0.0%)	1.1	(2.5%)
Resolution, R_s	–	(–)	6.1	(2.2%)	7.6	(3.6%)	9.2	(6.0%)	2.7	(2.7%)	12.6	(1.4%)	9.0	(2.4%)
Peak area	10368	(0.3%)	13955	(0.3%)	96193	(0.3%)	109866	(0.3%)	133288	(0.3%)	187927	(0.3%)	181699	(0.3%)

[a] Ado = adenosine; Cyd = cytidine; Guo = guanosine; Ino = inosine; Urd = uridine; 5mCyd = 5-methylcytidine; 8BrGuo = 8-bromoguanosine
[b] Retention time (min)
[c] Mean (percent relative standard deviation) of 6 replicates of a mixed nucleotide standard

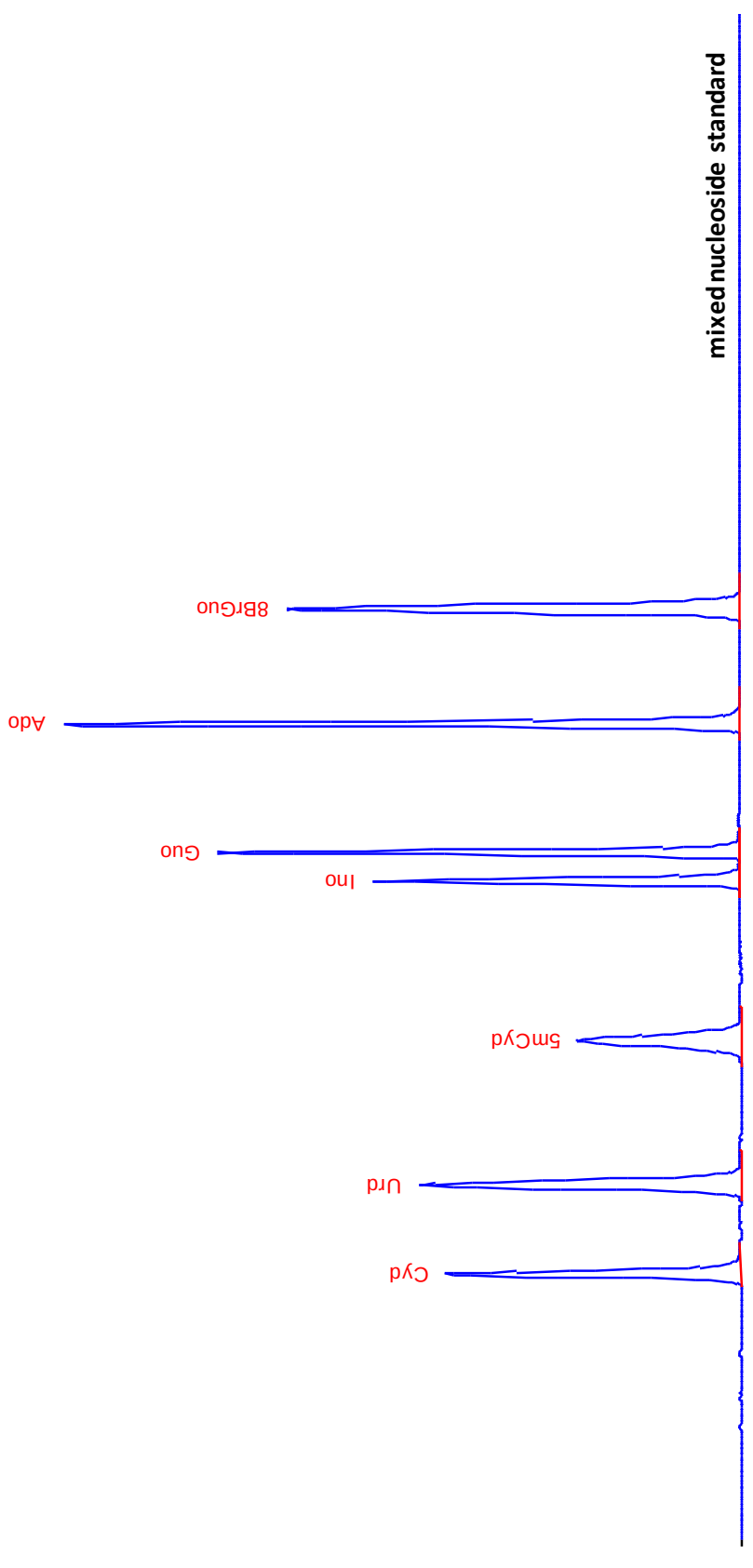

Figure 52. Chromatogram of mixed nucleoside standard (Ado = adenosine; Cyd = cytidine; Guo = guanosine 5; Ino = inosine; Urd = uridine, 5mCyd = 5-methylcytidine, 8BrGuo = 8-bromoguanosine. Chromatography: Prodigy C18, 5 µm, 4.6 x 150 mm; mobile phases A = $NaOCH_2CH_3$ (0.05 M, pH = 4.8); B = methanol (100%), flow rate 0.7 mL min^{-1}, gradient 0–3 min (95% A, 5% B), 7–22 min (75% A, 25% B), 23–30 min (95% A, 5% B). UV detection: 260 nm)

Recovery of nucleosides from the enzymatic digestion was estimated following the protocol described by Leach *et al*. (1995). A solution (TPAN fortified) containing ribonucleosides, nucleotides, 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 hours with KOH (0.2 mol L^{-1}, 50 mL) in order 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, together 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. The recoveries of nucleosides through the affinity clean-up step, and both enzymatic digestion and affinity clean-up is given in **Table 23**.

Table 23. Nucleoside recovery through TPAN sample preparation

Sample Preparation Step	Recovery% (SD)			
	Cyd[a]	Urd	Guo	Ado
Affi-gel clean-up	93.4 (1.1)[b]	92.3 (5.1)	88.3 (4.9)	95.2 (4.2)
Enzymatic digestion and Affi-gel clean up	95.5 (2.8)	101.7 (3.7)	89.2 (2.4)	94.7 (3.0)

[a] Ado = adenosine; Cyd = cytidine; Guo = guanosine; Urd = uridine
[b] Mean (standard deviation) of 3 replicates

The theoretical binding capacity of the Affi-gel is 0.033 milli-equivalents per tube. This is over 40 times the concentration of the highest standard. However, in addition to nucleosides any other components containing a *cis*-diol functionality may also bind to the gel, reducing overall binding capacity available for nucleosides (Molitor, 2008).

3.2.2.2 TPAN in Bovine Colostrum and Milk

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.

The contribution of each nucleobase and form obtained in this study of winter-milk and summer-milk lactation series are summarised in **Tables 24–25**. 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; **Tables 26**. The trend of nucleobase and form over lactation for each season is illustrated graphically in **Figures 53–54**.

Table 24. Total potentially available nucleosides in winter herd milk

Day[a]	Form	Results[b,c] (μmol dL^{-1})				
		Cyd	Urd	Guo	Ado	Total
0	Nucleoside	5.4 (0.1)	57.9 (1.6)	0.3 (0.0)	–	63.6 (1.5)
	Monomeric NT	6.1 (0.3)	143.7 (8.5)	2.8 (0.0)	2.9 (0.2)	156 (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)
	TPAN	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)	–	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)
	TPAN	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)	–	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)
	TPAN	17.5 (0.9)	142.2 (6.6)	8.1 (0.0)	6.5 (0.3)	174 (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)
	TPAN	20.7 (0.0)	99.8 (3.2)	6.2 (0.1)	7.1 (0.3)	134 (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)	103 (1.2)
5	Nucleoside	1.5 (0.3)	21.5 (0.8)	–	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)
	TPAN	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)	–	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)
	TPAN	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)	–	0.1 (0.0)	2.1 (0.3)
	Monomeric NT	3.9 (0.0)	0.1 (0.1)	–	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)
	TPAN	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)	–	0.1 (0.0)	1.5 (0.0)
	Monomeric NT	2.5 (0.0)	0.1 (0.0)	–	0.3 (0.1)	3.0 (0.1)
	NT Adduct	–	–	–	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)
	TPAN	3.4 (0.0)	1.1 (0.0)	0.3 (0.0)	0.6 (0.1)	5.3 (0.1)

[a] Day post-partum ± 2 h
[b] Mean (standard deviation) of duplicate analyses
[c] Cyd = cytidine, Urd = uridine, Guo = guanosine, Ado = adenosine
– = not detected

Table 25. Total potentially available nucleosides in summer herd milk

Day[a]	Form	Results[b,c] (μmol dL^{-1})				
		Cyd	Urd	Guo	Ado	Total
0	Nucleoside	2.6 (0.2)	50.6 (5.8)	2.2 (0.3)	–	55.4 (5.8)
	Monomeric NT	1.5 (0.1)	1.2 (0.0)	0.2 (0.0)	–	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)
	TPAN	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)	–	33.4 (0.5)
	Monomeric NT	0.5 (0.3)	0.4 (0.1)	0.1 (0.0)	–	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)
	TPAN	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)	–	48.5 (1.0)
	Monomeric NT	7.3 (0.1)	4.3 (0.3)	0.3 (0.0)	–	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)
	TPAN	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)	–	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	–	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)
	TPAN	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)	–	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)
	TPAN	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)	–	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)
	TPAN	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)	–	–	3.6 (0.0)
	Monomeric NT	4.1 (0.2)	0.1 (0.0)	–	1.2 (0.1)	5.3 (0.0)
	NT Adduct	0.2 (0.1)	–	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)
	TPAN	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)	–	–	2.1 (0.5)
	Monomeric NT	3.0 (0.2)	0.1 (0.0)	–	0.4 (0.0)	3.4 (0.1)
	NT Adduct	0.1 (0.0)	0.1 (0.0)	–	0.1 (0.0)	0.2 (0.0)
	Polymeric NT	–	0.1 (0.0)	–	0.1 (0.0)	0.1 (0.0)
	TPAN	3.8 (0.2)	1.6 (0.5)	–	0.5 (0.1)	5.9 (0.4)
30	Nucleoside	0.6 (0.0)	1.3 (0.0)	–	–	1.9 (0.0)
	Monomeric NT	1.6 (0.2)	–	–	–	1.6 (0.2)
	NT Adduct	0.1 (0.0)	0.1 (0.0)	–	0.3 (0.0)	0.5 (0.0)
	Polymeric NT	–	–	–	–	0.1 (0.0)
	TPAN	2.3 (0.2)	1.4 (0.0)	–	0.3 (0.0)	4.0 (0.2)

[a] Day post-partum ± 2 h
[b] Mean (standard deviation) of duplicate analyses
[c] Cyd = cytidine, Urd = uridine, Guo = guanosine, Ado = adenosine
– = not detected

Nucleoside Contribution to TPAN

Uridine was the most prevalent nucleoside in both winter and summer milk, with 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 and 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).

Table 26. Significance of rates of decrease through lactation[a]

Form	Results[a,b]				
	Cyd[b]	Urd	Guo	Ado	Total
Seasonal differences (winter vs. summer) between slopes: *p*-values[c]					
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[d]					
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[d]					
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] *p*-value, level of significance = 0.05
[b] Cyd = cytidine, Urd = uridine, Guo = guanosine, Ado = adenosine
[c] Statistical significance means there is evidence of a real difference between seasons
[d] Statistical significance means there is evidence that the levels are actually decreasing

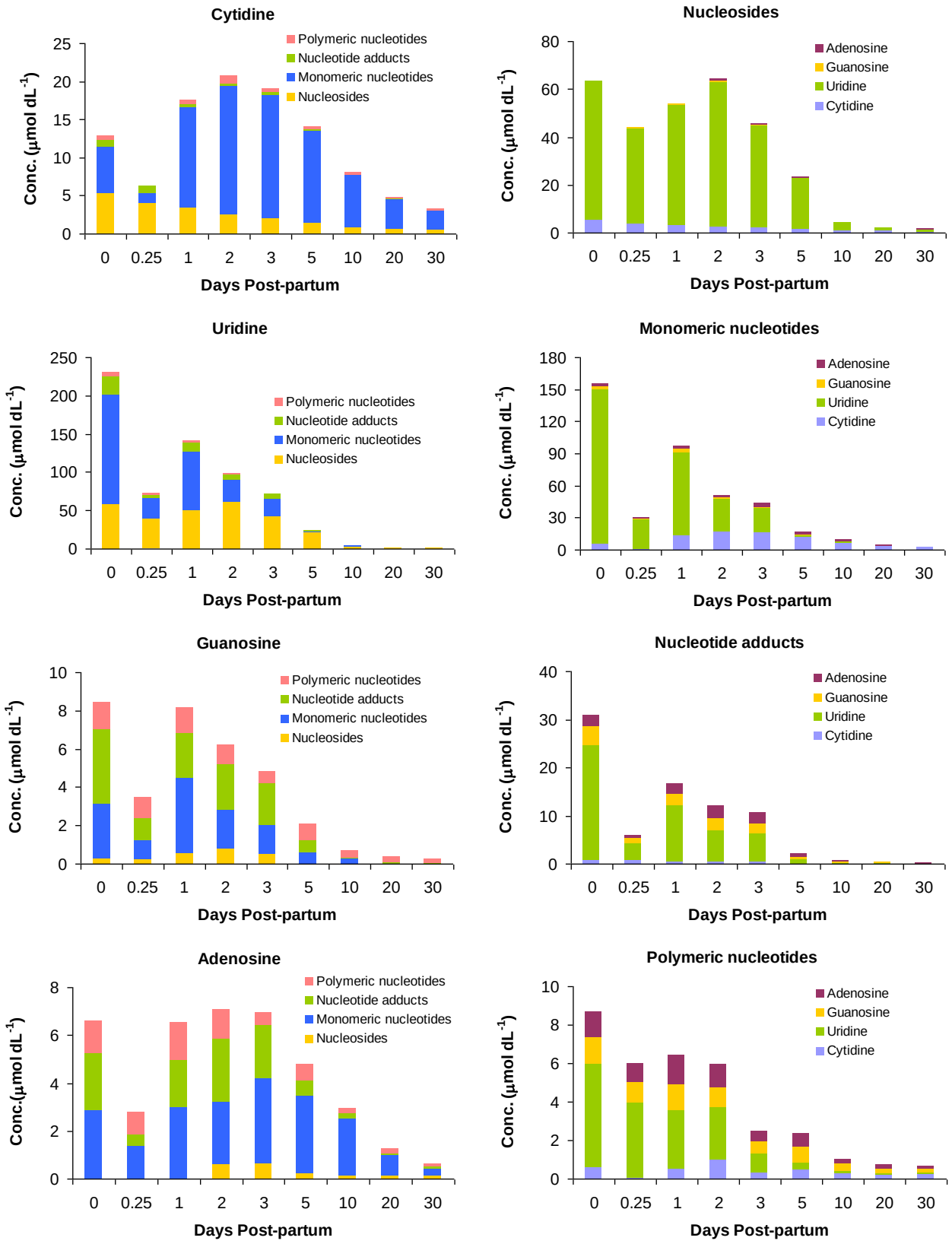

Figure 53. Nucleotides and nucleosides in winter herd milk

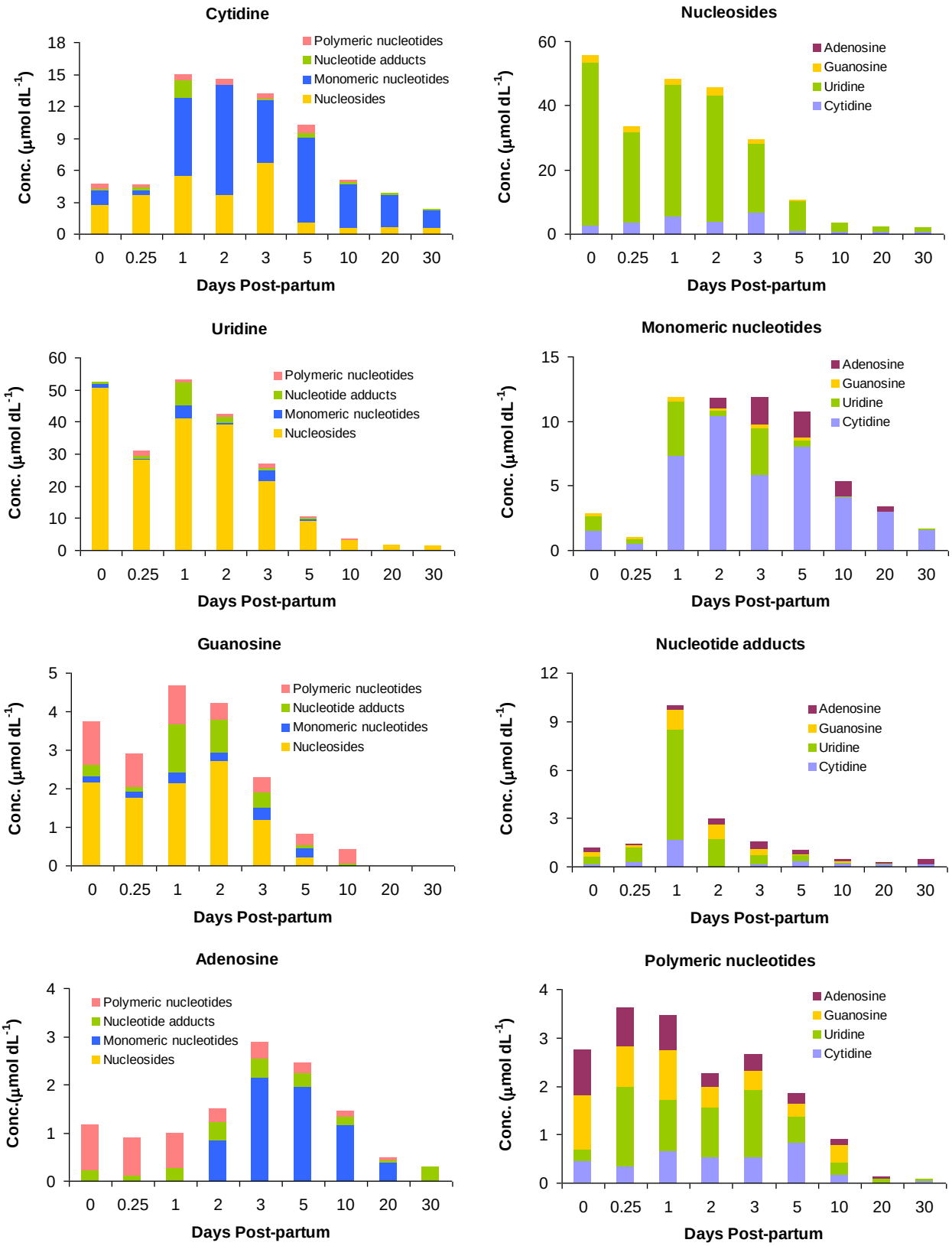

Figure 54. Nucleotides and nucleosides in summer herd milk

Monomeric Nucleotide Contribution to TPAN

Levels of nucleotides measured in this study were generally higher than those reported previously (Gill and Indyk, 2007b); however, there was likely to have been a contribution from multiple phosphorylated forms, which the TPAN analytical method aggregates as a single value. Differences in colostral monomeric nucleotide levels between the two 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 10 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 *et al*., 1991; Linzell and Peaker, 1971). It has been suggested that high levels of uridine and UMP are present in milk, as breakdown products of UDP and UTP, due to their function in the synthesis of lactose (Mateo *et al*., 2004; Schlimme *et al*., 2000). It was proposed that support for this hypothesis is seen by the correlation of decreasing total milk solids and 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 compared to 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 *et al*., 1986; Leach *et al*., 1995; Van Buren *et al*., 1985) and recently, Mashiko *et al*. (2009) demonstrated that dietary UMP affected the immune response of newborn calves.

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 (FAD) and nicotinamide adenine dinucleotide (Fox and McSweeney, 1998; Kanno *et al*., 1991). Utilising enzymatic techniques, UDP hexosamine, UDP hexose and UDP galactose concentrations were measured in bovine colostrum and milk, and 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 (Gil and Sánchez-Medina, 1981).

Polymeric Nucleotide Contribution to TPAN

The concentration of polymeric nucleotides in bovine colostrum was similar to that in human colostrum and milk (Leach *et al*., 1995), 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.

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, whereas uridine was present at considerably higher levels in bovine colostrum and in lower amounts in mature bovine milk compared to results in human milk reported by Leach *et al*. (1995).

The concentrations of the purines also differed, with adenosine levels constant throughout the first month of lactation for both herd milks, 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%) (Leach *et al*., 1995).

Total Potentially Available Nucleosides

In general, the absolute concentrations indicated a distinct difference between the two herds, although the general trends were the same; **Figures 55–56**. 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.

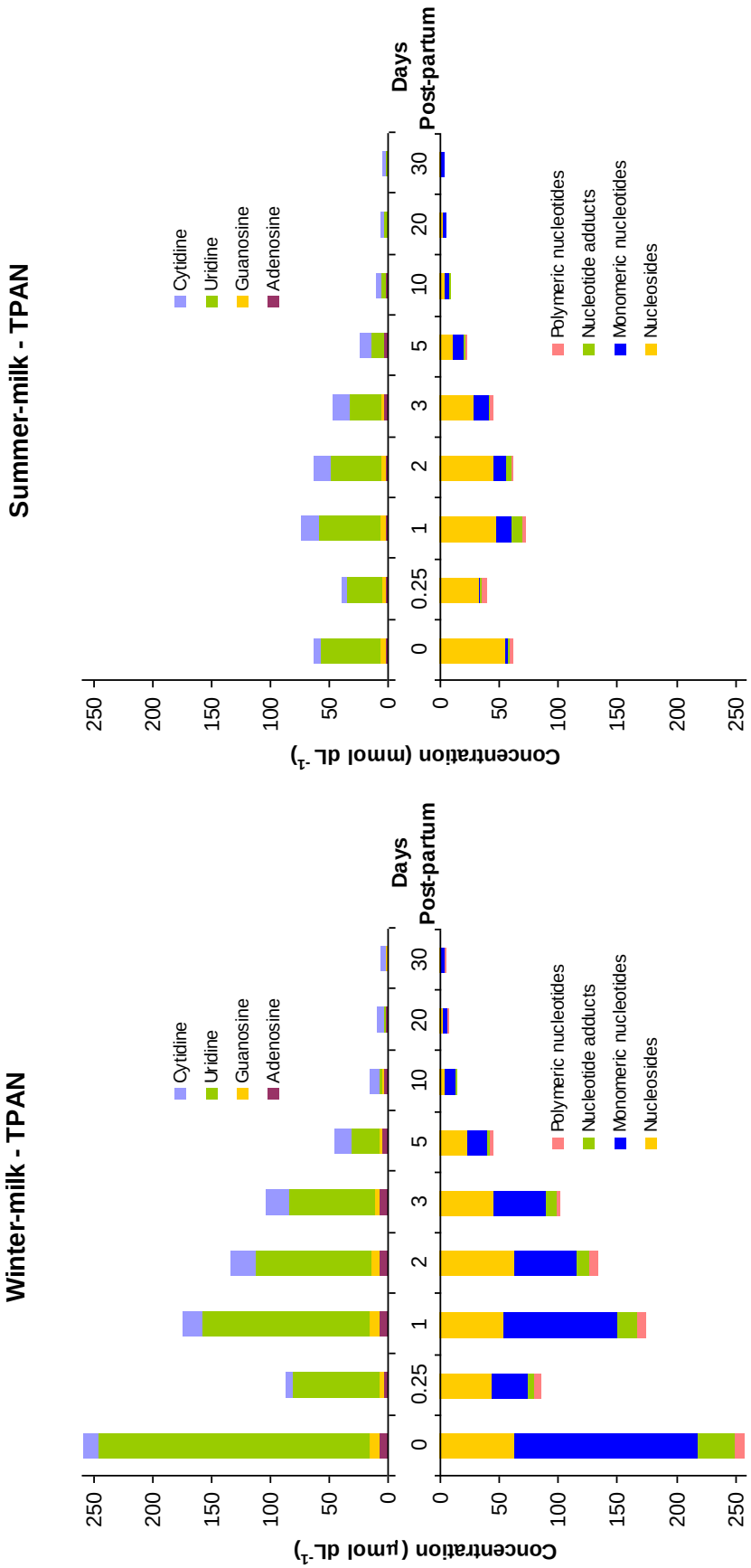

Figure 55. TPAN concentration in winter and summer herd milk

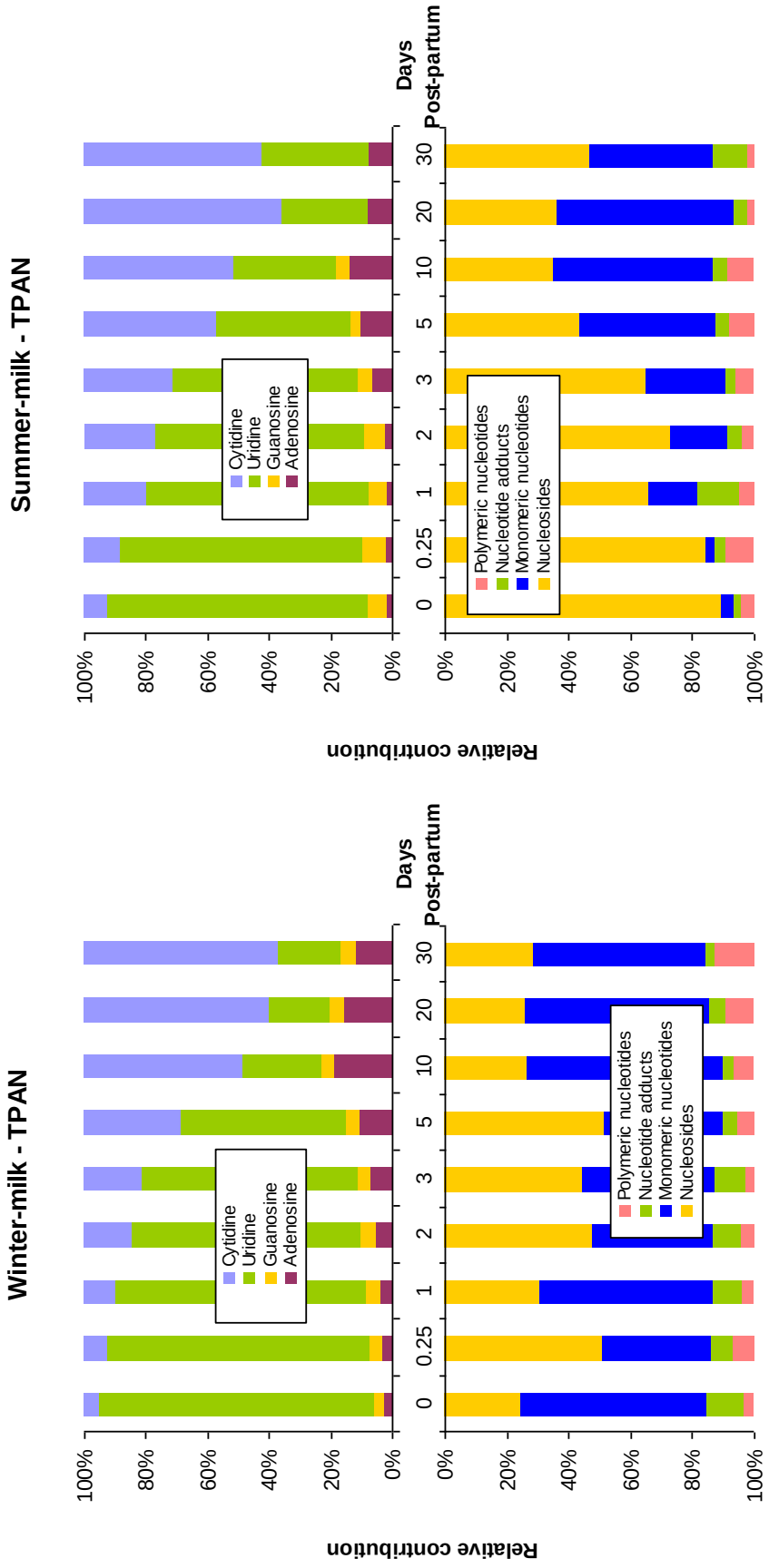

Figure 56. Relative TPAN levels in winter and summer herd milk

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, both herd milks 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 hours post-partum, and such diurnal variation may suggest a plausible rationale given that this sample was uniquely collected in the afternoon.

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 significant 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 (20.3 °C mean air temperature) and a drought with severe soil moisture deficits (>130 mm) in the Waikato. The summer milk herd began calving in late winter 2009, which had the warmest August on record nationally, with above average rainfall in the Waikato (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.

3.2.2.3 TPAN in Bovine, Caprine, and Ovine milk

With 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, the data on endogenous TPAN in milk in this study are timely.

The TPAN concentrations obtained in this study of bovine, caprine and ovine milk are summarised in **Table 27**, and illustrated in **Figure 57**.

Nucleoside Contribution to TPAN

The cytidine concentrations ranged from 0.9–2.3 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 *et al*., 2010).

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%. Ruminant milk contains higher concentrations of total nucleosides than those reported in human milk (Leach *et al*., 1995).

Table 27. TPAN in bovine, caprine, and ovine milk

Species	Form	Results[a] (μmol dL^{-1})				
		Cyd[b]	Urd	Guo	Ado	Total
Bovine	Nucleoside	0.9 (0.1)[a]	1.9 (0.1)	–	–	2.8 (0.3)
	Monomeric NT	3.3 (0.1)	0.5 (0.2)	–	–	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)	–	0.5 (0.0)	0.7 (0.3)
	TPAN	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)	–	–	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)
	TPAN	6.5 (0.3)	59.5 (1.8)	25.0 (0.7)	6.3 (0.3)	97.4 (2.8)
Ovine	Nucleoside	2.3 (0.1)	14.8 (1.1)	0.6 (0.0)	–	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)
	TPAN	9.4 (0.4)	306.8 (9.0)	30.2 (0.3)	27.8 (0.9)	374.1 (9.8)

[a] Mean (standard deviation) of duplicate analyses
[b] Cyd = cytidine, Urd = uridine, Guo = guanosine, Ado = adenosine
– = not detected

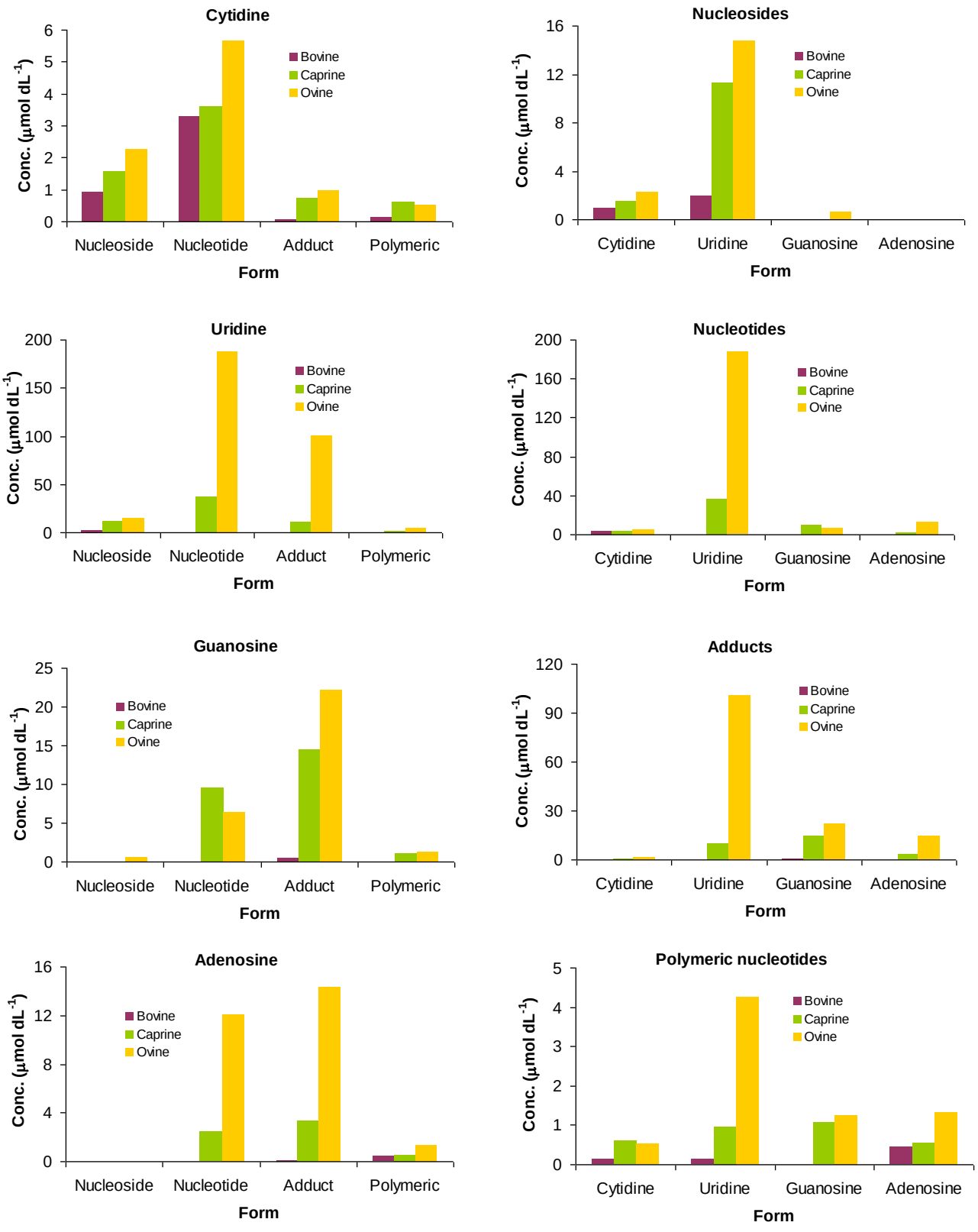

Figure 57. TPAN in bovine, caprine, and ovine milk

Monomeric Nucleotide Contribution to TPAN

The trends in nucleotide concentrations measured in this study were similar to those reported previously in bovine, caprine, and ovine milk (Ferreira *et al*., 2001; Gil and Sánchez-Medina, 1981; Gill and Indyk, 2007b; Martin *et al*., 2005; Plakantara *et al*., 2010). 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. Bovine milk contained significantly lower concentrations of monomeric nucleotides than caprine and ovine milk.

Nucleotide Adduct Contribution to TPAN

The uridine adducts measured in ovine milk were an order of magnitude higher than those in caprine milk and were 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 hexosamine, UDP hexose, 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 FAD and NADH (Fox and McSweeney, 1998; Kanno *et al*., 1991). The adenosine concentrations in bovine milk were much lower than those in caprine milk and ovine milk.

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.

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. 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, whereas caprine and ovine milk contained similar amounts. 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 reported in 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.

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; **Figures 58–59**.

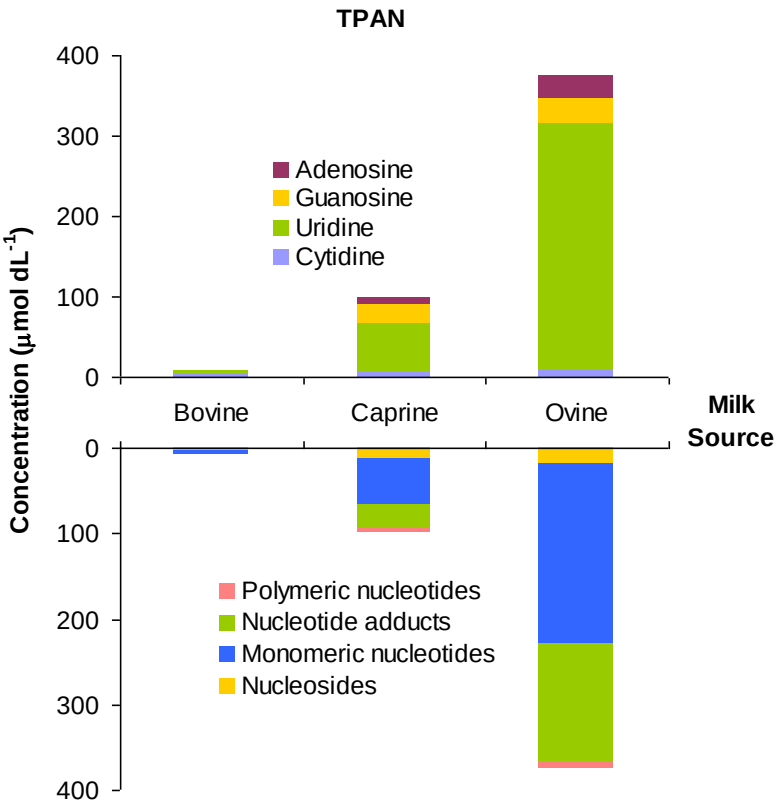

Figure 58. TPAN concentration in bovine, caprine, and ovine milk

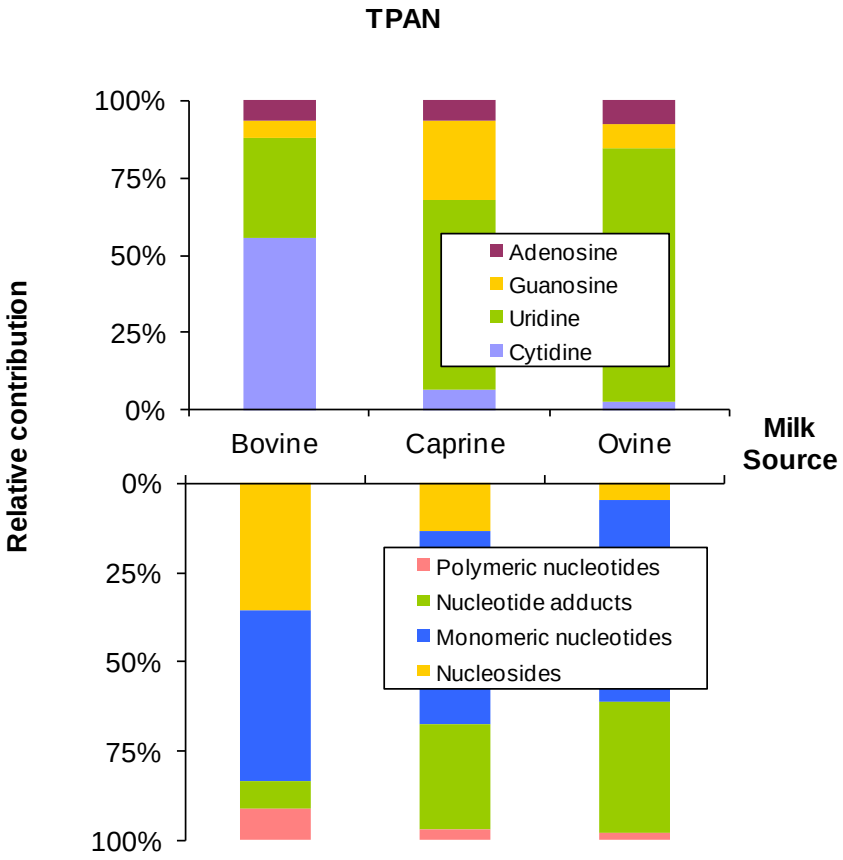

Figure 59. Relative TPAN levels in bovine, caprine, and ovine milk

Ovine milk contained the highest concentrations of nucleosides, free nucleotides, nucleotide adducts, 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 reported in 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 and Sánchez-Medina, 1981; Johke and Goto, 1962), while the nucleotide concentrations in caprine milk have been favourably compared with those in human milk (Prosser *et al*., 2008). Because of this, supplementation of caprine milk-based infant formulas with nucleotides is not necessary, as such products provide similar quantities of free nucleotides to those in nucleotide supplemented bovine milk-based infant formulas. 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 contribution of various forms to TPAN in bovine milk in this study, correspond well to the results for mature milk (days 10, 20, 30) for both winter and summer milk in the preceding study. This is expected since bovine milk collected from a pooled in factory silo will be sourced from cows producing mature milk.

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.

3.3. Nucleoside and Nucleotide Analysis by LC-MS

The analysis of nucleosides as well as nucleotides was identified by the Nucleotides Working Group within SPIFAN as a key tool for compliance testing of nucleotides in infant formulas (Sullivan, 2012). The aim of this study is to develop and validate an accurate and robust method for the simultaneous analysis of nucleosides and nucleotides in fortified infant formulas.

For many compounds, LC-MS is a more sensitive and specific technique than LC-UV. It can analyse compounds that lack a suitable chromophore as well as identifying components in unresolved chromatographic peaks, thereby reducing the need for perfect chromatography. Security of accurate analytical results is enhanced by the use of isotopically labelled internal standards. The method herein describes a simple centrifugal ultrafiltration clean up followed by LC-MS/MS analysis. This method has been validated for the analysis of bovine milk-based, caprine milk-based, soy-based, elemental, and hypoallergenic infant formula.

A description of this method and results obtained from this research were summarised and have been submitted for publication.

3.3.1. EXPERIMENTAL

3.3.1.1 Apparatus

The HPLC system used consisted of a CBM20A system controller, two LC20ADXR pumps for a high-pressure gradient, a CTO20AC column oven, and a SIL20ACXR autosampler equipped with a 50 μL injection

loop, (Shimadzu). The MS/MS system consisted of a 3200 QTRAP quadrupole mass spectrometer with a Turbo V ion source equipped with an ESI probe; Analyst 1.5.1 software was used for instrument control and data processing (ABSciex, Foster City, CA).

UV absorbances for calibration standards were acquired with a model UV-1601 spectrophotometer (Shimadzu) with digital readout to 4 decimal places. An Orion SA520 pH meter (Thermo Scientific, Waltham, MA) was used for the determination of pH.

Chromatographic separation was achieved using a Gemini column, 5 μm, 4.6 mm x 250 mm (Phenomenex). Polypropylene centrifuge tubes were sourced from Biolab and Amicon Ultra-4 3 kDa MWCO centrifugal filter units from Millipore.

3.3.1.2 Reagents

Ammonium acetate (≥ 98%), ammonium bicarbonate (≥ 99.5%), Ado (≥ 99%), Cyd (≥ 99%), Guo (≥ 98%), Ino (≥ 99%), Urd (≥ 99%), AMP (≥ 99%), CMP disodium salt (≥ 99%), GMP disodium salt hydrate (≥ 99%), IMP disodium salt (≥ 98%), and UMP (≥ 99%) were obtained from Sigma-Aldrich. SIL nucleoside standards (chemical purity), $^{13}C_5$ Ado (≥ 97%), $^{13}C_9{}^{15}N_3$ Cyd (≥ 98%), $^{15}N_5$ Guo (≥ 98%), $^{15}N_4$ Ino (≥ 98%), and $^{13}C_9{}^{15}N_2$ Urd (≥ 98%), were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). SIL nucleotide standards (chemical purity), $^{13}C_{10}{}^{15}N_5$ AMP (≥ 90%), $^{13}C_9{}^{15}N_3$ CMP (≥ 90%), $^{13}C_{10}{}^{15}N_5$ GMP (≥ 90%), and $^{13}C_9{}^{15}N_2$ UMP (≥ 90%), were purchased from Sigma-Aldrich; $^{13}C_{10}{}^{15}N_4$ IMP (≥ 90%), was purchased from Medical Isotopes (Pelham, NH, USA). Potassium dihydrogen phosphate, orthophosphoric acid, potassium hydroxide (GR ACS grade or equivalent), and methanol (LC-MS grade) were supplied by Merck. Water was purified with resistivity ≥ 18 MΩ using an E-pure water system (Barnstead).

A standardising buffer (KH_2PO_4, 0.25 M, pH = 3.5) was made as described previously (see *3.1.1.2*). Mobile phase A (NH_4CH_3COO, 10 mM; NH_4HCO_3, 5 mM, pH = 5.6) was made daily by dissolving 0.771 g NH_4CH_3COO and 0.395 g NH_4HCO_3 in 950 mL of water, adjusting the pH to 5.6 with acetic acid solution (10% *w/v*), then making to 1 L with water. Mobile phase B consisted of 100% methanol.

3.3.1.3 Standard Solutions

SIL nucleoside and nucleotide stock standards were prepared by accurately weighing 50 mg each of $^{13}C_5$ adenosine (^{13}C isotope label, subscript number of atoms labelled), $^{13}C_9{}^{15}N_3$ cytidine, $^{15}N_5$ guanosine, $^{15}N_4$ inosine, $^{13}C_9{}^{15}N_2$ uridine, $^{13}C_{10}{}^{15}N_5$ AMP, $^{13}C_9{}^{15}N_3$ CMP, $^{13}C_{10}{}^{15}N_5$ GMP, $^{13}C_{10}{}^{15}N_4$ IMP, and $^{13}C_9{}^{15}N_2$ UMP into separate 50 mL volumetric flasks. To each flask 40 mL of water was added, and then shaken (with gentle warming if necessary) until the standard was completely dissolved, before water was added to volume. Aliquots (~1.5 mL) of SIL stock standards were immediately dispensed into individual cryogenic vials and frozen at -15°C for later use. Prior to analysis cryogenic vials containing each SIL nucleoside and nucleotide stock standard were allowed to thaw to room temperature.

Non-isotopically labelled (NIL) nucleoside and nucleotide stock standards were prepared in the similar manner as described previously (Gill and Indyk, 2007b; see 3.1.1.3) by accurately weighing approximately 50 mg of each nucleotide into separate 50 mL volumetric flasks and making to volume with water. These were refrigerated at 4 °C for up to 1 month.

Estimation of moisture content in nucleosides was performed by the oven moisture method (102 °C) and the concentration was calculated on a dry weight basis. Extinction coefficients at UV absorbance maxima were then determined for each nucleoside. These were compared with the values

previously determined in nucleotides (Gill and Indyk, 2007b), with correction for molecular weights. The values obtained for each nucleoside were in close agreement with those for the corresponding nucleotide. Mean extinction coefficient values (nucleoside and corresponding nucleotide) were calculated and are reported in **Table 28**.

The concentration of each NIL nucleoside and nucleotide stock standard was determined by adding 500 µL of each stock standard into separate 25 mL volumetric flasks, diluting with standardising buffer, and measuring the absorbance at the appropriate λ_{max}.

Table 28. Nucleoside and nucleotide extinction coefficients

Analyte[a]	$E_{1cm}^{1\%}$	λ_{max} (nm)
AMP	428.6	257
Ado	557.0	
CMP	390.9	280
Cyd	519.5	
GMP	392.0	254
Guo	502.8	
IMP	356.5	249
Ino	462.7	
UMP	312.7	262
Urd	415.1	

[a] AMP = adenosine 5′-monophosphate
CMP = cytidine 5′-monophosphate
GMP = guanosine 5′-monophosphate
IMP = inosine 5′-monophosphate
UMP = uridine 5′-monophosphate
Ado = adenosine
Cyd = cytidine
Guo = guanosine
Ino = inosine
Urd = uridine

A mixed SIL intermediate standard was prepared by diluting 2.0 mL of each SIL stock standard into a 25 mL volumetric flask and making to volume with water. A mixed NIL intermediate standard was made by

adding 1.0 mL of each NIL stock standard into a 25 mL volumetric flask and making to volume with water.

Four calibration standards were prepared by pipetting 1.0, 1.0, 0.5, and 0.2 mL of SIL intermediate standard and 2.0, 4.0, 5.0, and 8.0 mL of NIL intermediate standard into 50, 50, 25, and 10 mL volumetric flasks respectively. The calibration standards were then made to volume with water and mixed thoroughly.

3.3.1.4 Sample Preparation

Approximately 5.0 g of infant formula powder was weighed accurately into a 50 mL polypropylene centrifuge tube (Biolab, Auckland, New Zealand) and dissolved in 25 mL of water. To this was added 1.0 mL of SIL intermediate standard and the tube was capped and vortex mixed, followed by 10 min standing to allow sample to hydrate, before dilution to a final volume of 50 mL with water.

A 4.0 mL aliquot of sample solution was added to an Amicon Ultra-4 3 kDa MWCO centrifugal ultrafiltration unit (Millipore, Billerica, MA, USA) and centrifuged at 3500 x g for 60 min. The filter was then removed and discarded and a 1 mL aliquot of filtrate was transferred to an HPLC vial ready for analysis.

3.3.1.5 Chromatography

High-pressure gradients were formed by mixing two mobile phases, A and B, at a constant flow rate of 0.75 mL min^{-1}, **Table 29**.

Table 29. Gradient procedure for RPLC-MS method

Time (min)	Temperature (°C)	Flow rate (mL min^{-1})	Mobile phase composition[a]	
			%A	%B
0.0	40	0.75	100	0
3.5	40	0.75	100	0
10.0	40	0.75	80	20
20.0	40	0.75	80	20
21.0	40	0.75	100	0
35.0	40	0.75	100	0

[a] Mobile phase A = NH_4CH_3COO, 10 mM; NH_4HCO3, 5 mM, pH = 5.6
Mobile phase B = 100% methanol

3.3.1.6 Mass Spectrometry

The mass spectrometer was operated using an ESI source in positive mode with nitrogen utilised as drying and collision gas. The instrumental parameters were set as follows: curtain gas at 30 psi, nebuliser gas GS1 and GS2 at 50 and 70 psi respectively, desolvation temperature at 700 °C, CID gas at medium, and ion spray voltage at 5500 V. MRM transitions and instrument settings for generation of product ions for nucleosides and nucleotides is given in **Table 30–31**.

.

Table 30. LC-MS method: MS/MS conditions for nucleosides

Analyte[a]	Monoisotopic mass[b]	Precursor ions $[M+H]^+$ (*m/z*)	Prominent product ions (*m/z*)	DP[c] (V)	EP[d] (V)	CEP[e] (V)	CE[f] (V)	CXP[g] (V)
Ado	267.10	268.10	136.0	21	8.5	16	39	4.0
Cyd	243.09	244.09	112.0	11	6.5	22	19	4.0
Guo	283.10	284.10	152.0	11	4.0	24	23	4.0
Ino	268.09	269.09	137.0	16	4.5	18	23	4.0
Urd	244.08	245.08	113.0	16	5.0	16	19	4.0
$^{13}C_5$ Ado	272.11	273.11	136.0	21	8.5	16	39	4.0
$^{13}C_9{}^{15}N_3$ Cyd	255.11	256.11	119.0	11	6.5	22	19	4.0
$^{15}N_5$ Guo	288.08	289.08	157.0	11	4.0	24	23	4.0
$^{15}N_4$ Ino	272.07	273.07	141.0	16	4.5	18	23	4.0
$^{13}C_9{}^{15}N_2$ Urd	255.09	256.09	119.0	16	5.0	16	19	4.0

[a] Ado = adenosine; Cyd = cytidine; Guo = guanosine; Ino = inosine; Urd = uridine
[b] Calculated on neutral molecule
[c] DP = declustering potential
[d] EP = entrance potential
[e] CEP = collision cell entrance potential
[f] CE = collision energy
[g] CXP = collision cell exit potential

Table 31. LC-MS method: MS/MS conditions for nucleotides

Analyte[a]	Monoisotopic mass[b]	Precursor ions $[M+H]^+$ (*m/z*)	Prominent product ions (*m/z*)	DP[c] (V)	EP[d] (V)	CEP[e] (V)	CE[f] (V)	CXP[g] (V)
AMP	347.06	348.07	136.0	36	1.0	24	25	4.0
CMP	323.06	324.06	112.0	26	3.5	32	27	4.0
GMP	363.06	364.06	152.0	36	4.5	22	23	4.0
IMP	348.05	349.05	137.0	31	6.0	28	23	4.0
UMP	324.04	325.04	97.0	21	9.5	18	23	4.0
$^{13}C_{10}{}^{15}N_5$ AMP	362.09	363.09	146.0	36	1.0	24	25	4.0
$^{13}C_9{}^{15}N_3$ CMP	335.07	336.08	119.0	26	3.5	32	27	4.0
$^{13}C_{10}{}^{15}N_5$ GMP	378.11	379.08	160.0	36	4.5	22	23	4.0
$^{13}C_{10}{}^{15}N_4$ IMP	361.10	362.07	145.0	31	6.0	28	23	4.0
$^{13}C_9{}^{15}N_2$ UMP	335.06	336.07	102.1	21	9.5	18	23	4.0

[a] AMP = adenosine 5′-monophosphate;
CMP = cytidine 5′-monophosphate
GMP = guanosine 5′-monophosphatel
MP = inosine 5′-monophosphate
UMP = uridine 5′-monophosphate
[b] Calculated on neutral molecule
[c] DP = declustering potential
[d] EP = entrance potential
[e] CEP = collision cell entrance potential
[f] CE = collision energy
[g] CXP = collision cell exit potential

3.3.2. RESULTS AND DISCUSSION

3.3.2.1 Method Development

Sample Preparation

The aim of the sample preparation procedure was to remove co-eluting matrix components without reducing overall recoveries of analytes. Any risk of reduced recovery though sample preparation is mitigated in an LC-MS assay by the use of an SIL standard, therefore, provided sufficient analyte is recovered to ensure than ample signal is obtained, quantitative recovery through sample preparation step is not essential for accurate results.

Initial sample preparation was developed with the intention of using HILIC for quantitative analysis. The pH of the sample was lowered with acetic acid, followed by centrifugation and separating off the supernatant, which was adjusted to pH 6.5 with ammonium hydroxide. An aliquot was added to an HPLC vial with sufficient acetonitrile added to reduce aqueous content to < 30%, thereby facilitating optimal chromatographic separation and eliminate peak splitting. However, it was observed that phase separation was occurring within the HPLC vial. Normally water and acetonitrile are miscible, but phase separation was attributed to the high salt content of the sample extract. This is the principle involved in the QuEChERS (quick, easy, cheap, effective, rugged and safe) technique, typically applied to the analysis of pesticide residues (Anastassiades *et al*., 2003).

The QuEChERS technique was investigated and is based on acetonitrile partitioning whereby water and proteins are removed from the sample by salting out with magnesium sulphate and sodium chloride. This is then followed up with dispersive SPE to remove potential interferences. The

method was developed for the analysis of pesticides in low-fat commodity products, although more recently it has been adapted to intermediate and high-fat products (Lehotay *et al*., 2005). The QuEChERS method covers a wide range of analytes including polar pesticides (Payá *et al*., 2007). The extraction of nucleosides and nucleotides into an acetonitrile/water phase in the sample preparation is potentially attractive, as this can be readily coupled to a HILIC system, which offers a number of advantages over RPLC in the LC-MS analysis of polar compounds. Although the initial use of QuEChERS in this study gave quantitative recovery of nucleosides, the recovery of nucleotides was negligible, as they remained in the aqueous phase and did not partition into the organic phase. The ionic nature of nucleotides therefore makes them unsuitable as candidates for use with the QuEChERS extraction procedure.

The use of CUF for protein removal from infant formula in the analysis of nucleotides was previously described by Inoue *et al*. (2008) and was evaluated in the development of the LC-UV method described previously (see 3.1.2.1). Two brands of CUF tubes were assessed, Vivaspin (GE Healthcare, Little Chelfont, UK) and Amicon, in 4 and 15 or 20 mL sizes and at 3 and 10 kDa. Powder samples were reconstituted at 10 % and 25 % *w/v* and it was found that at either concentration, high centrifuge speeds were required to obtain sufficient filtrate (~1 mL), which necessitated the use of the smaller 4 mL CUF tubes. A higher concentration of powder was considered ideal in order to increase signal in subsequent LC-MS analysis. Because only a small volume of extract was required for LC-MS analysis, the higher concentration sample and lower MWCO tubes were chosen for use as part of the method. For cost reasons, the Amicon Ultra-4, 3 kDa MWCO CUF tubes were selected for continued use in method development and evaluation.

Chromatography

HILIC has been used previously in the analysis of nucleosides and nucleotides, and the use of this technique was evaluated. However, problems associated with nucleotide peak shape and retention were found. An additional concern was the solubility of nucleotides at high organic solvent conditions (> 90%). PGC was also assessed, but similar problems with nucleotide peak shape and retention were found.

The simultaneous chromatographic analysis of both nucleosides and nucleotides in infant formulas has previously been described using LC-UV (Gill and Indyk, 2007b). However, the mobile phase contained a 0.1 M phosphate buffer, which is generally regarded as unsuitable for use in LC-MS. Ammonium acetate (10 mM, pH = 5.6) was chosen to buffer the mobile phase due to its compatibility with LC-MS and its pH buffering range of ~3.8–5.8 which is appropriate given the pKa of various nucleosides and nucleotides. However, significant peak tailing was observed for nucleotide peaks when this buffer was used.

The interaction of phosphorylated compounds with metal surfaces in liquid chromatographic applications has been reported previously (Asakawa *et al*., 2008; De Vijlder *et al*., 2011; Kim *et al*., 2004; Liu *et al*., 2005; Wakamatsu *et al*., 2005). Peak tailing of phosphorylated compounds has been observed in a wide range of liquid chromatographic techniques such that acquiring symmetrical peaks can be extremely difficult (Asakawa *et al*., 2008). Conventional LC-UV nucleotide analyses typically contain phosphate in the mobile phase and no significant peak tailing is observed (Gill and Indyk, 2007b; Perrin *et al*., 2001). It was postulated that the interaction between phosphate compounds and stainless steel may be suppressed by a passive film formed on the stainless steel surface at relatively low phosphate concentrations, thereby obtaining good peak shapes (Wakamatsu *et al*., 2005). Unfortunately, the use of non-volatile buffers such as phosphate in LC-MS is generally not recommended due to a decrease in sensitivity, and contamination of the ion source.

A number of approaches have been employed in order to overcome this problem. Wakamatsu *et al*. (2005) evaluated a pre-treatment phase for the chromatographic system using phosphoric acid prior to switching to a non-phosphate eluent during analysis. This approach was applied to the analysis of nucleosides and nucleotides in dietary foods using 1% phosphoric acid in 50% acetonitrile (0.3 mL min^{-1}, 60 min) to pre-condition the UHPLC system without introduction to the mass spectrometer prior to each measurement Yamaoka *et al*. (2010). While successful in reducing peak tailing, pre-treatment of the chromatographic system came at the cost of a substantial increase in analysis time. The substitution of polyether ether ketone (PEEK) tubing for stainless steel can reduce peak tailing (Wakamatsu *et al*., 2005), however, the removal of all stainless steel is problematic, and PEEK has limited tolerance to back pressure. A highly basic mobile phase (25-50% ammonium hydroxide, pH ≈ 12) was used by Tuytten *et al.* (2006); however, these conditions would be detrimental to silica-based HPLC columns. The addition of EDTA to either sample or the mobile phase was also found to be beneficial in reducing phosphorylated compounds metal interactions (Liu *et al*., 2005); however, as EDTA is not volatile, its use in LC-MS is not ideal.

Several mass spectrometer manufacturers have evaluated the use of phosphate buffers for use with their instruments and shown that modern source designs can handle non-volatile buffers better than older designs (Agilent Technologies, 1998; Applied Biosystems, 2006; Dionex Corporation, 2001; Waters Corporation, 1998). A phosphate-based IPRPLC−MS method was successfully applied to the quantitative analysis of intracellular nucleotides utilising a microbore column to reduce the amount of phosphate introduced to the ion source (St. Claire, 2000).

In the present study, a low phosphate buffer ($NH_4H_2PO_4$ 0.08 mM, pH = 5.6) was evaluated for use with the mass spectrometer. Chromatographic parameters of resolution, retention factor, peak area repeatability, retention time repeatability, plate number, and asymmetry

were evaluated, and with acceptable results obtained; **Table 32**. There was some loss of sensitivity as the analytical run progressed, and a small build-up of buffer on the cone was observed, but did not block the orifice. The method was applied to the analysis of nucleotides in infant formula samples in a validation study; **Table 33**.

Linear response was demonstrated for the ratio of NL/SIL peak areas vs. the ratio of NL/SIL analyte concentration (r^2 = 0.997–0.999). Accuracy and precision were evaluated with both spike recovery (84.2–107.1%) and repeatability (1.5–3.1% RSD) deemed acceptable. A limitation with this approach was that the number of samples within each analytical run was limited due to build-up of phosphate in the ion source, which required regular cleaning.

Asakawa *et al.* (2008) investigated the effect of a number of mobile phase additives on peak tailing and found some that showed positive effects similar to that found with phosphate and EDTA. Of those evaluated, only ammonium bicarbonate is volatile and deemed suitable for use in LC-MS.

Under acidic conditions, only a small fraction of the dissolved CO_2 is present as H_2CO_3. The dissolved CO_2 concentration in water is in equilibrium with the partial pressure of CO_2 in the atmosphere. Acid/base equilibria for ammonium bicarbonate are illustrated in **Figure 60**. This complicates the use of bicarbonate as a buffering agent, therefore bicarbonate was used as an additive to acetate buffered mobile phase.

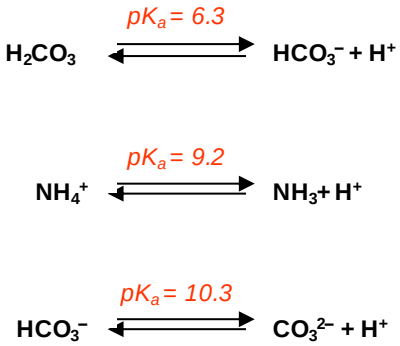

Figure 60. Ammonium bicarbonate equilibria

Table 32. System suitability of phosphate buffer LC-MS method

Parameter	Analyte[a]									
	CMP[b]		UMP		GMP		IMP		AMP	
Retention time[c]	8.2	(3.8%)[b]	10.3	(3.9%)	14.5	(0.9%)	14.8	(1.1%)	17.8	(8.0%)
Retention factor, *k′*	2.3	(4.2%)	3.1	(5.3%)	4.8	(1.0%)	4.9	(2.2%)	6.1	(9.1%)
Theoretical Plates, *N*	14637	(2.9%)	14920	(2.1%)	95641	(27.1%)	91352	(27.6%)	102542	(23.5%)
Tailing, T_f	1.0	(5.0%)	1.0	(5.1%)	0.0	(0.0%)	1.3	(152%)	1.1	(3.4%)
Resolution, R_s	–	(–)	6.9	(2.2%)	16.3	(16.3%)	1.1	(4.7%)	13.3	(16.9%)
Peak area repeatability	206184	(0.9%)	247655	(1.2%)	359479	(1.9%)	225519	(1.1%)	512409	(0.8%)

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

[b] Mean (percent relative standard deviation) of 7 replicates of a mixed nucleotide standard

[c] Retention time (min)

Table 33. Validation of phosphate buffer LC-MS method

Analyte[a]	Range ($\mu g\ mL^{-1}$)	Linear regression	r^2	Recovery (%)	SD_r[b] ($mg\ hg^{-1}$)	RSD_r[c] (%)	HorRat[d]
AMP	0.19–19.40	y = 690641x + 230636	0.9993	107	0.07–0.17	2.6	0.8
CMP	0.15–15.18	y = 345411 x + 141820	0.9977	100	0.13–0.33	2.0	0.7
GMP	0.17–17.24	y = 314862x + 164765	0.9972	96	0.02–0.06	2.4	0.6
IMP	0.13–12.79	y = 420442x + 128924	0.9986	98	0.03–0.09	3.1	0.9
UMP	0.11–11.06	y = 827733x + 294402	0.9969	105	0.03–0.09	1.5	0.5
$^{13}C_{10}$,$^{15}N_5$ AMP	0.18–18.49	y = 596452x + 197077	0.9984				
$^{13}C_9$,$^{15}N_3$ CMP	0.14–13.77	y = 352837x + 126425	0.9983				
$^{13}C_{10}$,$^{15}N_5$ GMP	0.15–14.62	y = 340263x + 144897	0.9936				
$^{13}C_{10}$,$^{15}N_4$ IMP	0.13–13.24	y = 318753x + 219869	0.9892				
$^{13}C_9$,$^{15}N_2$ UMP	0.13–12.51	y = 771057x + 362940	0.9951				

[a] AMP = adenosine 5′-monophosphate
CMP = cytidine 5′-monophosphate
GMP = guanosine 5′-monophosphate
iMP = inosine 5′-monophosphate
UMP = uridine 5′-monophosphate
TMP = thymidine 5′-monophosphate.

[b] 95% confidence interval for repeatability standard deviation.

[c] Repeatability relative standard deviation (RSD_r) = sd/mean x 100 (n = 6)

[d] HorRat = RSD_r/pRSD, where pRSD = $C^{-0.1505}$ at 10 $\mu g\ g^{-1}$ concentration level

The mechanism of how bicarbonate reduces peak tailing was not explained by Asakawa *et al*., however, it is likely that it acts in the same manner to phosphate, which has been postulated to act as a layer on the stainless steel reducing interactions with nucleotides (Wakamatsu *et al*., 2005).

Mass Spectrometry

The optimisation of the MS conditions was performed by infusion of a standard of each nucleoside or nucleotide (~10 µg mL^{-1}) diluted in a mixture of mobile phases A and B (90:10). Initial development focused on ESI^+ for nucleosides and ESI^- for nucleotides, it was found that ESI^+ gave superior response for both analytes, with the $[M+H]^+$ ion most abundant with low levels of potassium adducts. This simplified the analysis given that polarity switching would not be necessary. Conditions for MRM were optimised by selecting individual fragments and adjusting collision energies in order to maximise product ion signal.

Quantitative product ions for nucleosides were obtained from parent ions by loss of neutral ribosyl group (*m/z* = 132). This fragmentation is typical, since a relatively small amount of energy is required to break one (glycosidic) bond, and has been seen in numerous studies (Clariana *et al*., 2010; Kammerer *et al*., 2005; la Marca *et al*., 2006; Lee *et al*., 2004; Tuytten *et al*., 2008). While fragmentation of CMP, GMP, IMP, and AMP resulted in the loss of the ribosyl-phosphate group (212 Da) with detection of the positively charged base, UMP underwent a more complicated fragmentation to obtain a predominant product ion of *m/z* = 97. This product ion for UMP was also reported by Inoue *et al*. (2010). A possible pathway to generate this product ion is given in **Figure 61**.

A similar fragmentation scheme was described by Curtis *et al.* (2010), for the generation of a product ion with *m/z* = 81.0 from the fragmentation of deoxycytidine 5′-monophosphate.

This fragment satisfies a number of conditions:

1) for non-labelled UMP the product ion, *m/z* = 97 is seen, whereas for SIL UMP the corresponding product ion *m/z* = 102; therefore of the 11 isotope labels in parent isotopically labelled UMP, 5 must appear in product ion.

2) the nitrogen rule indicates that the product ion must have either both of the nitrogen's from the precursor ion, or neither of them (Yadav, 2005).

3) since a similar product is not found with uridine, it is probable that the phosphate group is critical to the formation of the product ion.

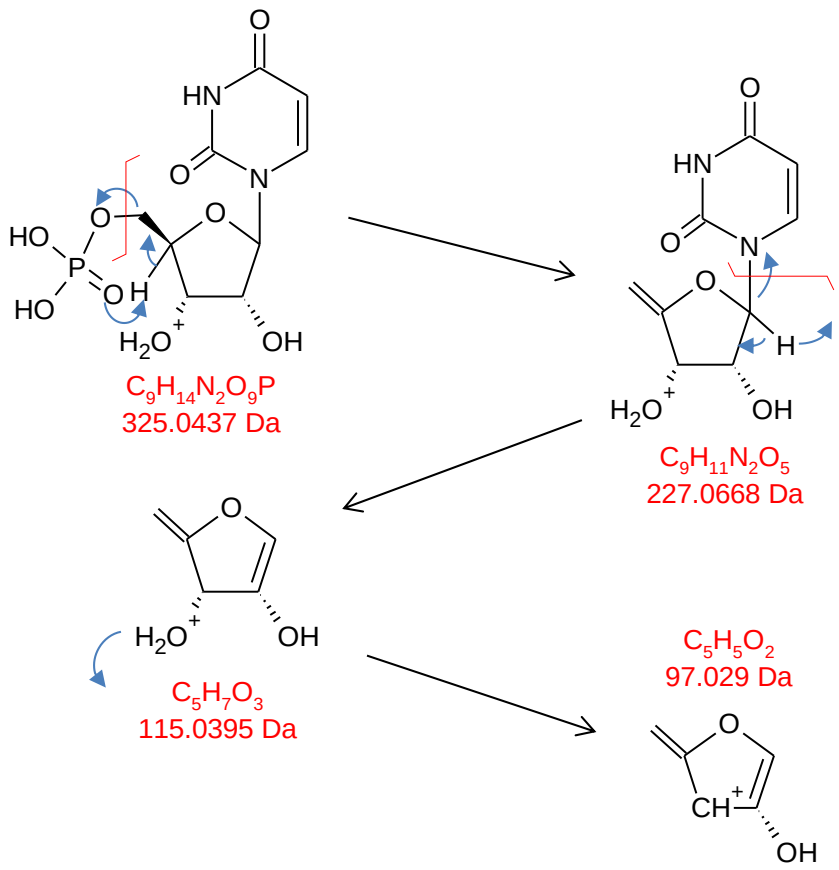

Figure 61. Possible fragmentation of UMP

Using the developed LC-MS/MS method, the simultaneous detection of nucleosides and nucleotides in a standard solution was achieved; **Figures 62–63**.

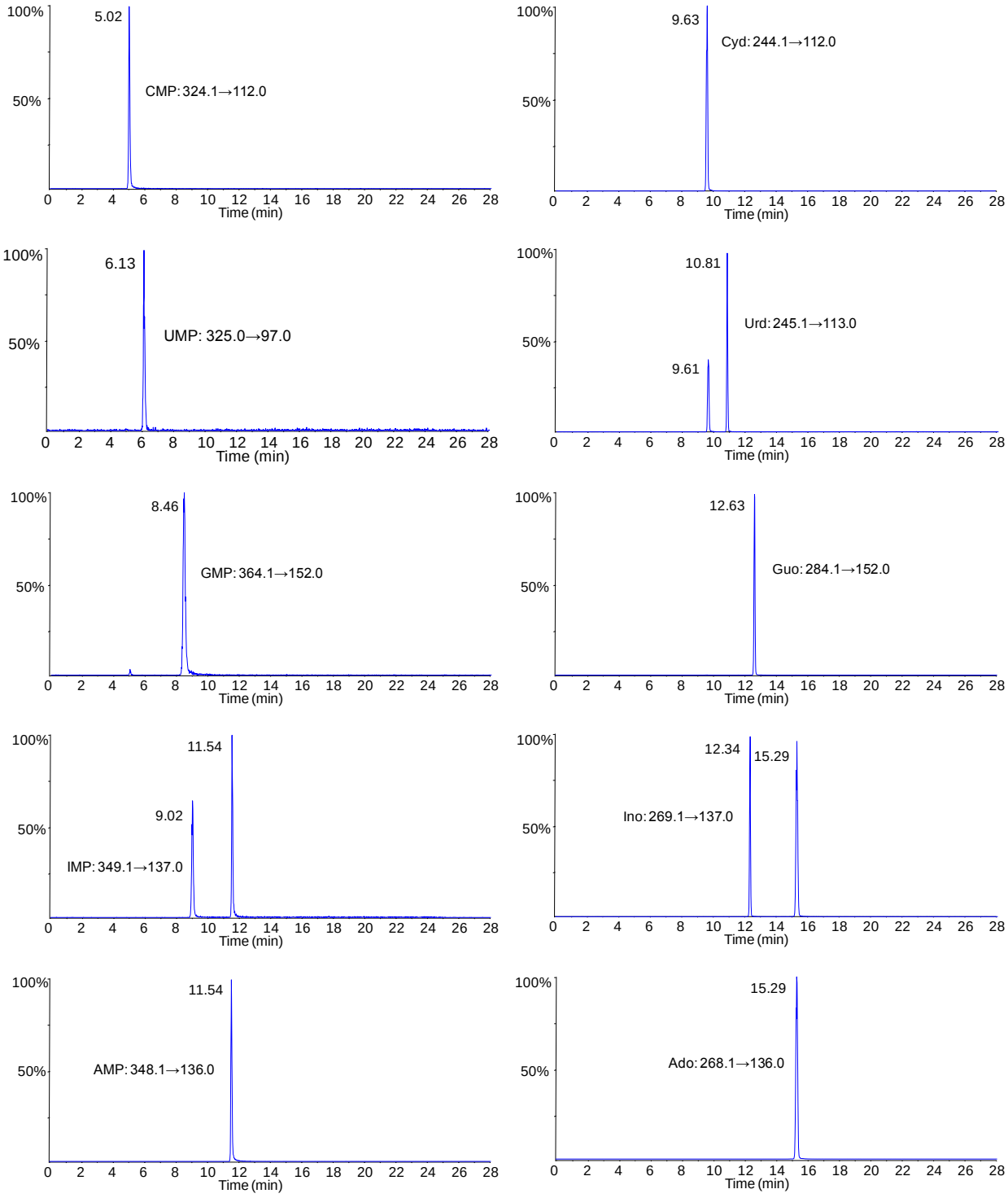

Figure 62. NIL nucleoside and nucleotide MRM chromatograms

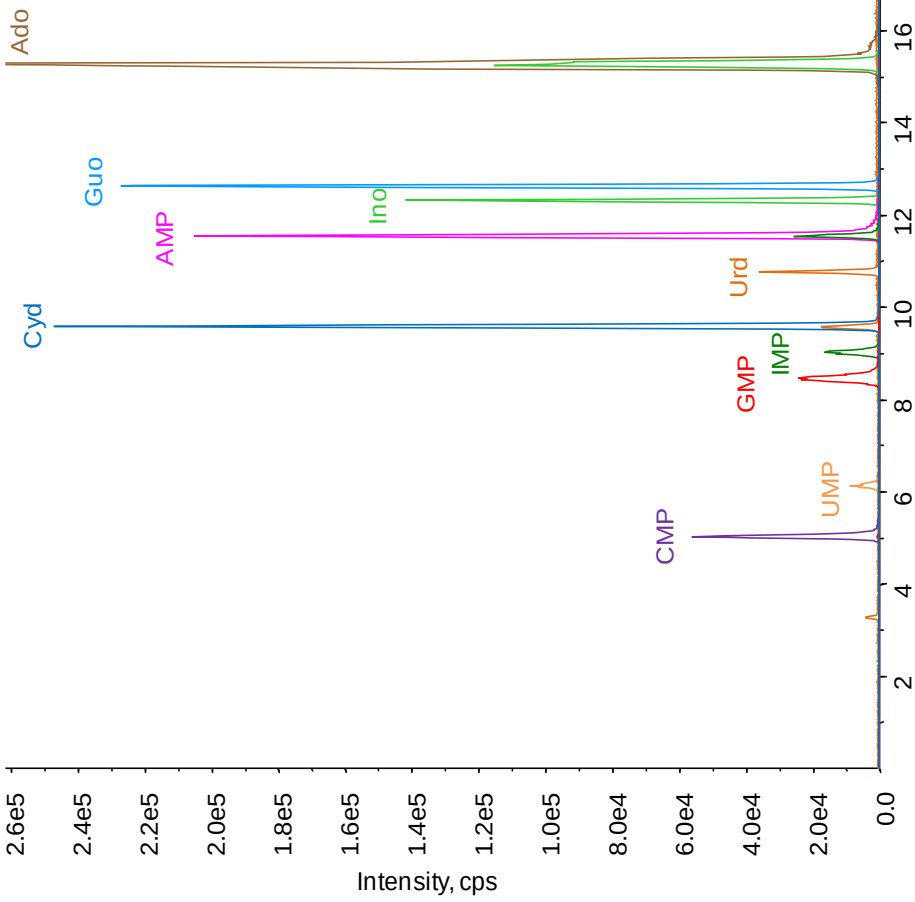

*Figure 63. LC-MS chromatogram of NIL/SIL nucleosides and nucleotides (**AMP = adenosine 5'-monophosphate; CMP = cytidine 5'-monophosphate; GMP = guanosine 5'-monophosphate; IMP = inosine 5'-monophosphate; UMP = uridine 5'-monophosphate;** Ado = adenosine; Cyd = cytidine; Guo = guanosine; Ino = inosine; Urd = uridine 5'-monophosphate;. Chromatography: Gemini C_{18}, 5 µm, 4.6 x 250 mm; mobile phases A = NH_4CH_3COO /NH_4HCO_3 (10 mM/5 mM pH = 5.6); B = methanol (100%); oven temp = 40°C; flow rate 0.75 mL min^{-1}, gradient 0–3.5 min (100% A, 0% B), 10–20 min (80% A, 20% B), 21–35 min (100% A, 0% B). MRM experiment)*

Quantitation

When a compound is introduced into the ion source not all of the molecules are ionised. The ionisation efficiency is the fraction of introduced compound that becomes ionised, and this differs depending upon the chemical structure of the compound, but also due to various source parameters, such as temperature and pressure, within the source that can vary during day-to-day operation, and are impossible to control precisely (Stokvis *et al*., 2005). LC-MS is different from other modes of detection, such as UV and fluorescence, in that co-eluting compounds that are not detected can enhance or suppress the analyte response. Additionally, even similar samples may have different combinations and concentrations of endogenous compounds that yield different ionisation responses of the analyte (Hewavitharana, 2011). A number of strategies can be employed in order to reduce or eliminate matrix effects upon MS signal. However, with complex matrices such as milk products, there is a limit to the effectiveness of sample clean-up strategies.

Matrix matched calibration curves are a common way to compensate for signal suppression or enhancement. However, when a blank matrix is not readily available, this approach becomes less feasible (Lehotay *et al*., 2010).

An internal standard calibration technique is appropriate, since it does not depend upon the absolute response of the analyte, as long as the changes in sensitivity are consistent for the analyte and internal standard. If the analyte and internal standard co-elute, changes in sensitivity of the analyte due to matrix effects are compensated for. However, if the analyte and internal standard have different retention times they can experience different ionisation environments, yielding inaccurate results.

SIL internal standards are forms of the analyte in which several atoms are replaced by isotopes such as ^{13}C, ^{2}H, ^{18}O, or ^{15}N. It is generally accepted that SIL internal standards are chemically and physically identical to the

analyte and therefore they have the same retention time, and since their atomic mass differs by a few Daltons, they are distinguishable from one another by MS making them ideal candidates as internal standards. It should be noted that deuterated internal standards can have stronger bonding to carbon atoms, thereby subtly affecting their physicochemical properties such that they may exhibit different retention times or recoveries (Stokvis *et al*., 2005; Wang *et al*., 2007). For this reason, ^{13}C and ^{15}N labelled nucleosides and nucleotides were selected as internal standards in the development of this method.

3.3.2.2 Method Performance

System Suitability

A high degree of selectivity is afforded by an MRM experiment; however, chromatographic separation is required for critical peaks with similar MRM transitions if accurate quantitation is to be achieved. Chromatographic performance was assessed by replicate analyses ($n = 6$) of a mixed nucleoside and nucleotide standard with satisfactory resolution being obtained between IMP/AMP (6.7), Ino/Ado (6.8), and Cyd/Urd (4.3) critical pairs which differ in mass by < 2 D. While some peak tailing of nucleotides was seen when comparing them with corresponding nucleosides, peak tailing was acceptable and would not impede accurate estimation of peak area for quantitative purposes; **Table 34**.

Single Laboratory Validation

Method validation experiments to determine linearity, detection limits, and precision are summarised in **Table 35**. Linearity was evaluated by least-squares regression analysis, with acceptable values being obtained for the correlation coefficient and with standard residuals plots showing no pattern and only a small amount of random variation; **Figures 64–83**.

Table 34. LC-MS method: system suitability

Parameter	Analyte[a]									
	Cyd	Urd	Guo	Ino	Ado	CMP	UMP	GMP	IMP	AMP
Resolution	1.3 (0.2)	3.5 (0.9)	0.8 (0.2)	2.2 (0.4)	4.6 (0.8)	–	2.8 (0.4)	4.3 (0.6)	0.9 (0.1)	2.8 (0.4)
Capacity factor	2.0 (0.0)	2.4 (0.0)	2.9 (0.0)	2.8 (0.0)	3.8 (0.0)	0.6 (0.0)	0.9 (0.0)	1.6 (0.0)	1.8 (0.0)	2.6 (0.0)
Retention time[c]	9.6 (0.0)[b]	10.8 (0.0)	12.6 (0.0)	12.3 (0.0)	15.3 (0.0)	5 (0.0)	6.1 (0.0)	8.5 (0.0)	9.0 (0.0)	11.6 (0.0)
Tailing factor	1.8 (1.0)	1.2 (0.3)	1.7 (0.5)	1.4 (0.6)	1.8 (0.7)	1.6 (0.3)	1.7 (0.4)	1.6 (0.3)	1.5 (0.3)	1.7 (0.3)
Theoretical plates	11654 (9806)	23515 (5580)	11817 (3764)	25042 (18334)	8065 (3425)	3224 (1359)	3873 (1570)	2548 (419)	4856 (1109)	27472 (9925)
Peak area ratio repeatability[d]	0.42 (0.01)	0.46 (0.02)	0.51 (0.02)	0.55 (0.02)	0.50 (0.01)	0.49 (0.03)	0.36 (0.01)	0.46 (0.01)	5.34 (0.13)	0.51 (0.03)

[a] AMP = adenosine 5′-monophosphate
CMP = cytidine 5′-monophosphate
GMP = guanosine 5′-monophosphate
iMP = inosine = 5′-monophosphate
UMP = uridine 5′-monophosphate
Ado = adenosine
Cyd = cytidine
Guo = guanosine
Ino = inosine
Urd = uridine

[b] Mean (percent relative standard deviation) of 6 replicates of a mixed nucleotide standard

[c] Retention time (min)

[d] Peak area ratio measured as non-labelled nucleoside or nucleotide/stable isotope-labelled nucleoside or nucleotide

Table 35. LC-MS method: linearity, detection limit, and precision

Analyte[a]	Range ($\mu g\ mL^{-1}$)	Linear regression	R^{2}[b]	MDL[c] ($mg\ hg^{-1}$)	SD_r[d] ($mg\ hg^{-1}$)	RSD_r[e] (%)	HorRat[f]	r[g] ($mg\ hg^{-1}$)	RSD_{iR}[h] (%)
Cyd	0.7–58.6	y = 0.737x + 0.1053	0.9996	0.03	0.03 - 0.07	4.8	0.4	0.14	14.4
Urd	0.8–60.9	y = 0.957x - 0.3441	0.9987	0.12	0.05 - 0.13	4.1	0.4	0.25	14.1
Guo	0.7–54.9	y = 0.837x + 0.2553	0.9996	0.01	0.01 - 0.02	6.2	0.4	0.03	7.9
Ino	0.8–62.1	y = 1.059x - 0.0417	0.9982	0.01	0.01 - 0.02	7.2	0.4	0.04	11.2
Ado	0.7–59.2	y = 0.778x + 0.1853	0.9997	0.01	0 - 0.01	14.5[i]	0.8	0.02	–
CMP	0.6–45.3	y = 0.94x + 0.0113	0.9998	0.13	0.61 - 1.6	4.0	0.6	3.09	4.6
UMP	0.5 – 42.6	y = 0.872x - 0.1152	0.9997	0.01	0.34 - 0.89	5.0	0.6	1.72	6.2
GMP	0.6 – 45.7	y = 0.928x + 0.1423	0.9993	0.01	0.15 - 0.4	1.9	0.2	0.77	2.9
IMP	0.6 – 46.8	y = 1.069x + 0.5071	0.9999	0.03	–	–[i]	–	–	–
AMP	0.8–60.6	y = 0.787x + 0.35	0.9986	0.01	0.17 - 0.44	2.8	0.4	0.85	7.8

[a] AMP = adenosine 5′-monophosphate; CMP = cytidine 5′-monophosphate; GMP = guanosine 5′-monophosphate; IMP = inosine 5′-monophosphate; UMP = uridine 5′-monophosphate; Ado = adenosine; Cyd = cytidine; Guo = guanosine; Ino = inosine; Urd = uridine

[b] R^2 = correlation coefficient

[c] MDL = method detection limit

[d] SD_r = 95% confidence interval for repeatability SD (n = 20)

[e] RSD_r = repeatability RSD% (n = 20)

[f] HorRat = RSD_r/pRSD, where RSD_r = repeatability RSD% and $pRSD = 2C^{-0.1505}$

[g] r = repeatability limit = 2.8 x repeatability SD

[h] RSD_{iR} = intermediate precision RSD% (n = 20)

[i] Precision study concentration at or below detection limit

– = Not detected

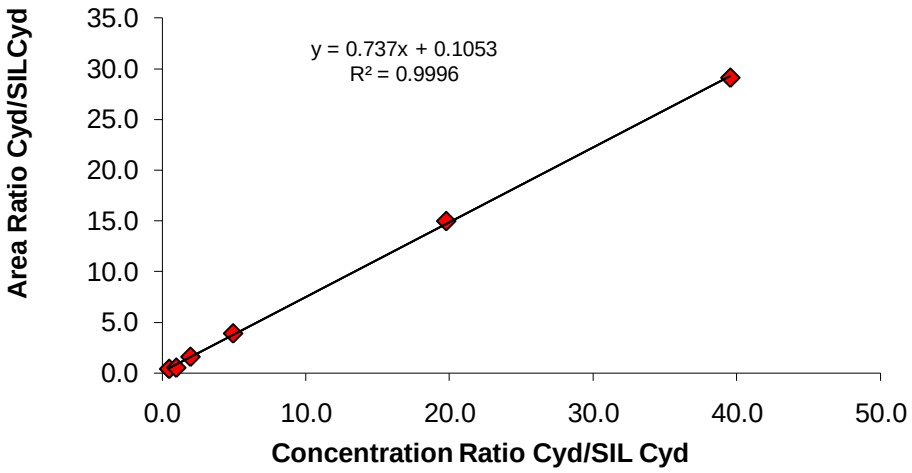

Figure 64. LC-MS method: Cyd linear regression plot

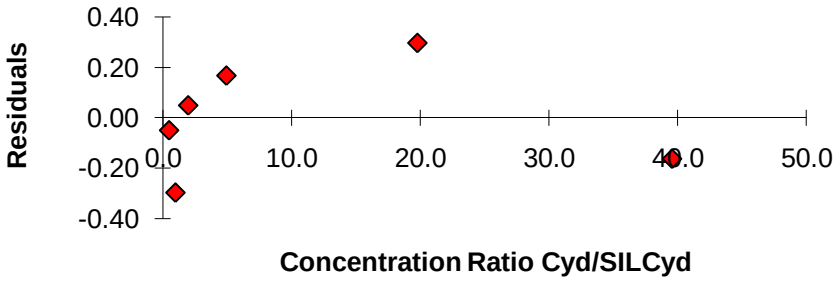

Figure 65. LC-MS method: Cyd residuals plot

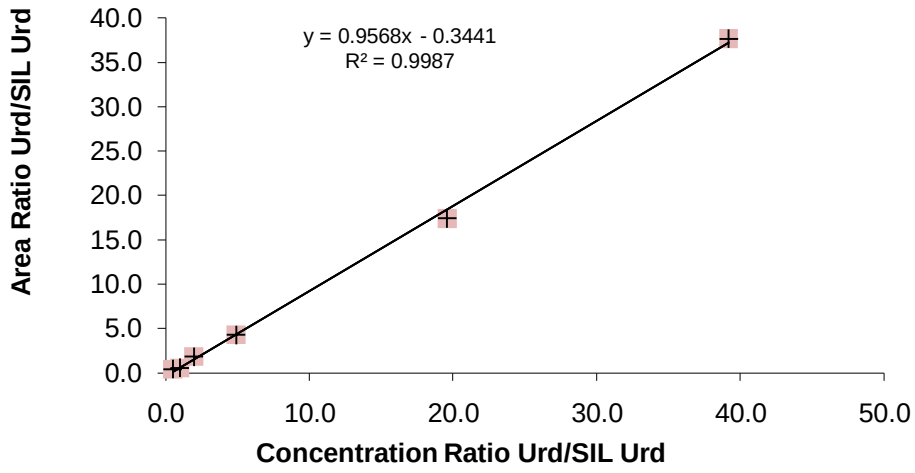

Figure 66. LC-MS method: Urd linear regression plot

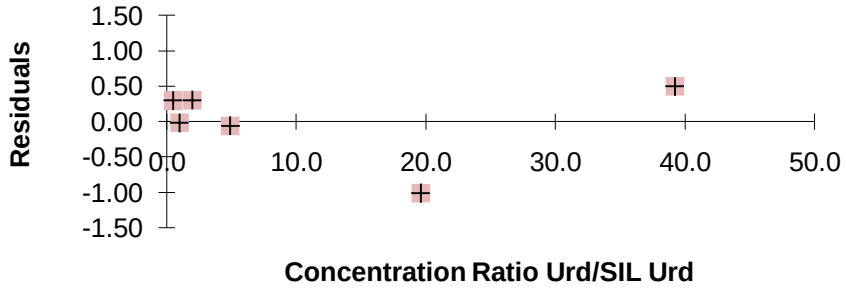

Figure 67. LC-MS method: Urd residuals plot

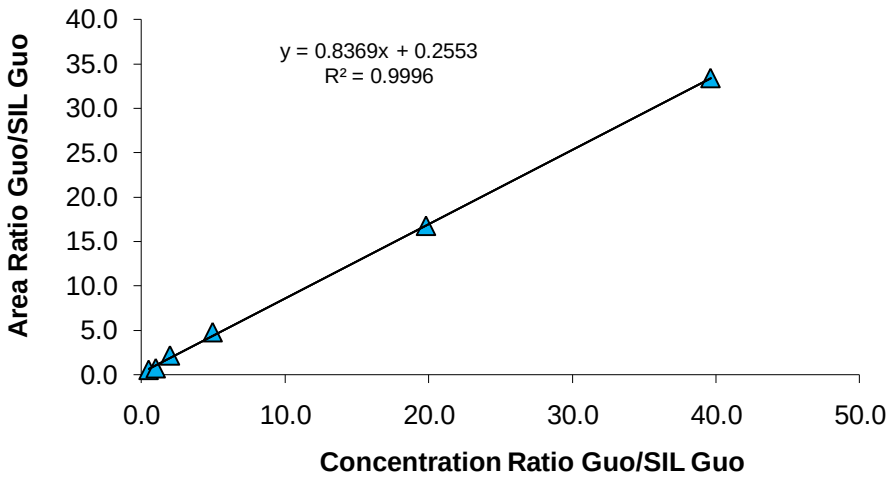

Figure 68. LC-MS method: Guo linear regression plot

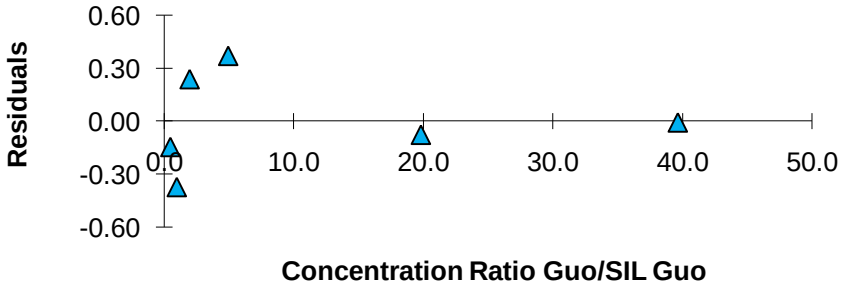

Figure 69. LC-MS method: Guo residuals plot

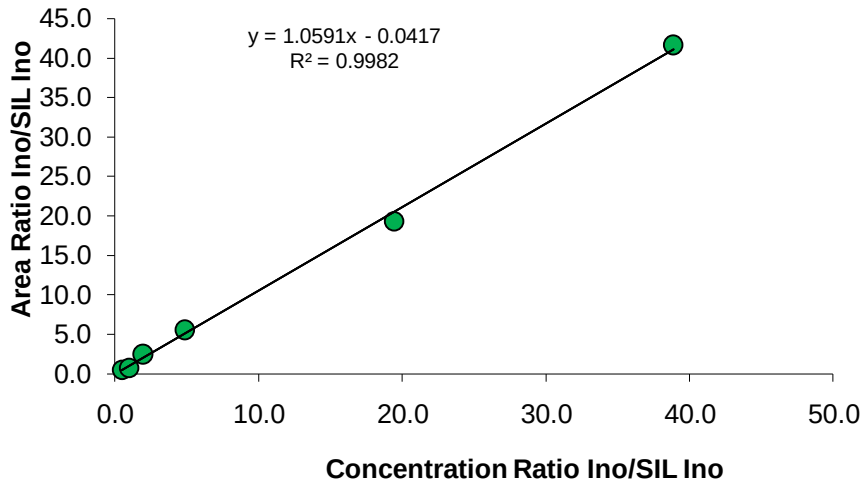

Figure 70. LC-MS method: Ino linear regression plot

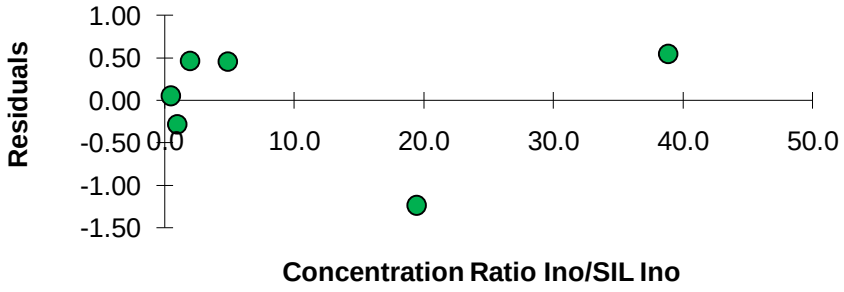

Figure 71. LC-MS method: Ino residuals plot

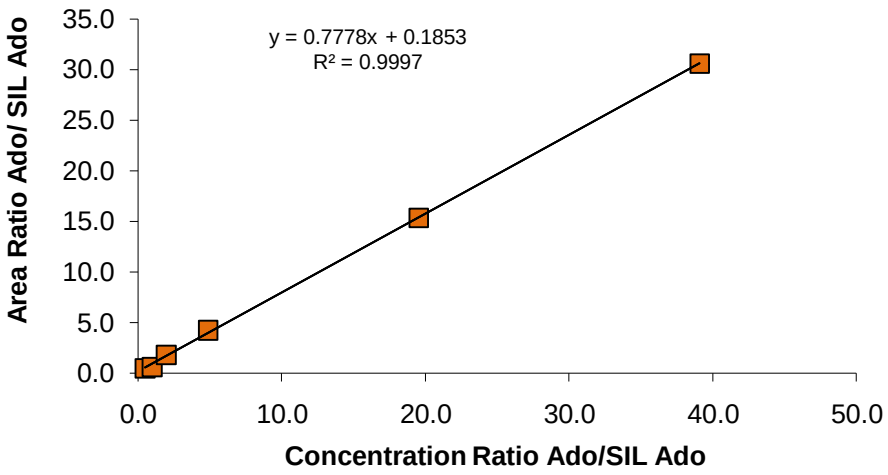

Figure 72. LC-MS method: Ado linear regression plot

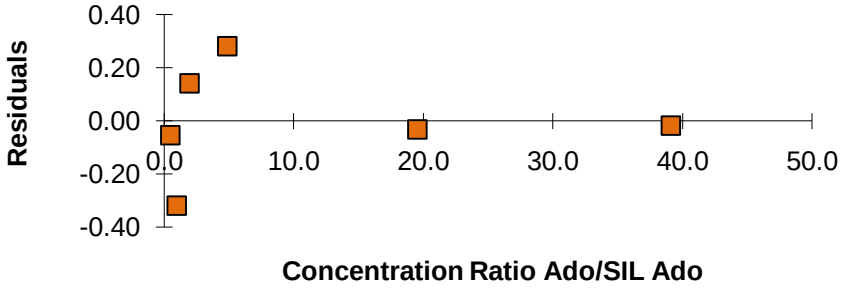

Figure 73. LC-MS method: Ado residuals plot

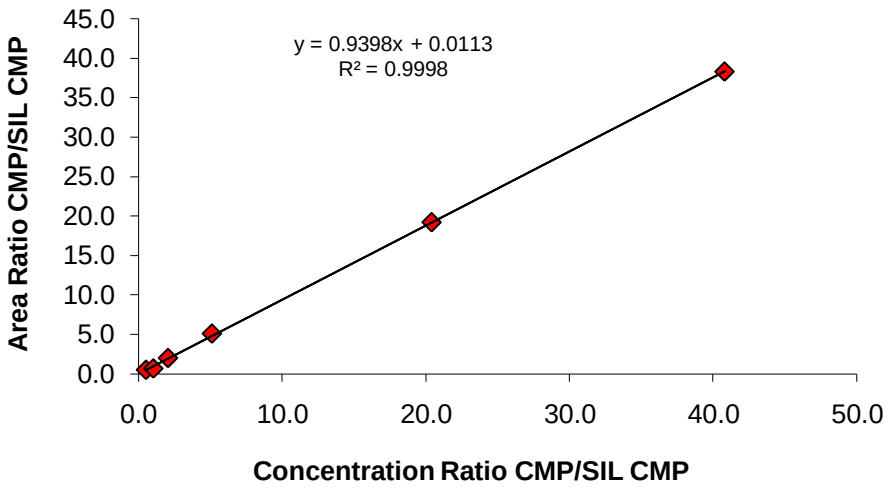

Figure 74. LC-MS method: CMP linear regression plot

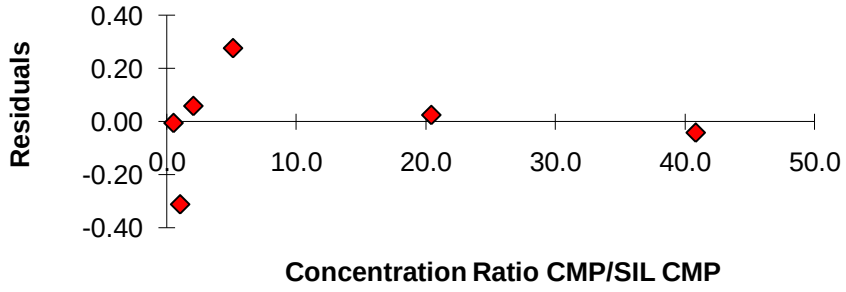

Figure 75. LC-MS method: CMP residuals plot

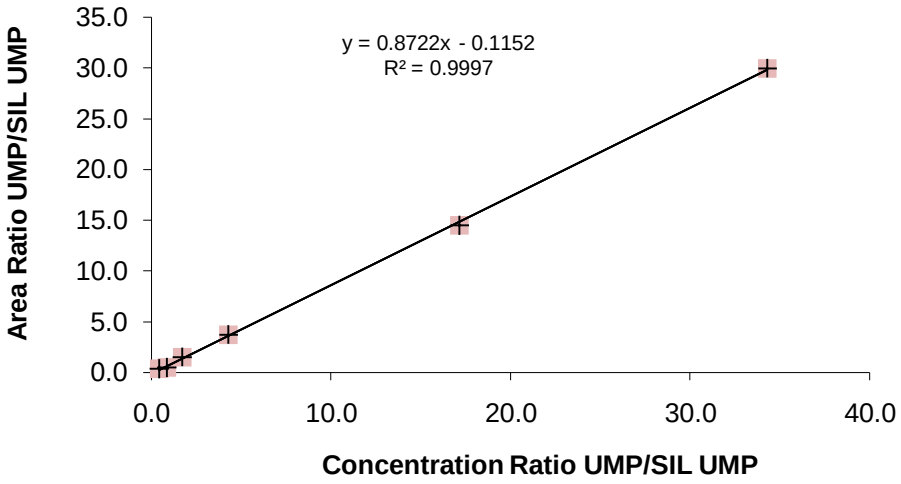

Figure 76. LC-MS method: UMP linear regression plot

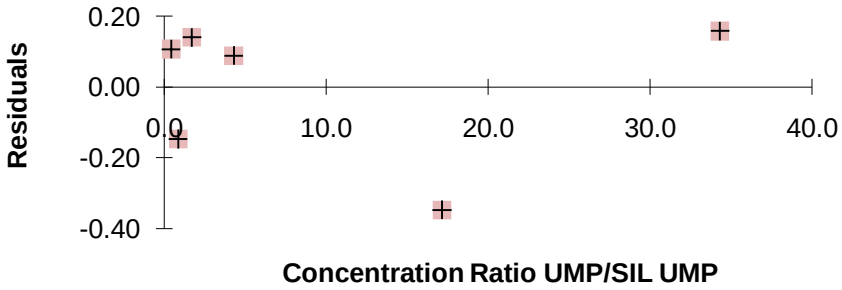

Figure 77. LC-MS method: UMP residuals plot

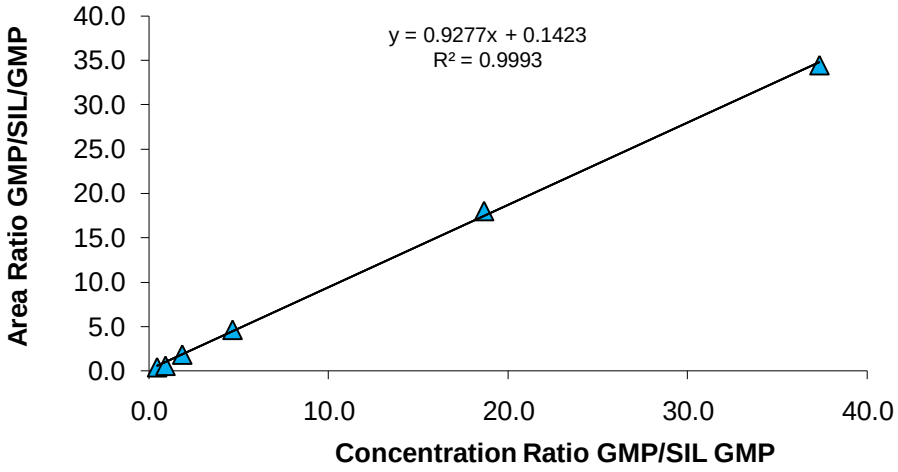

Figure 78. LC-MS method: GMP linear regression plot

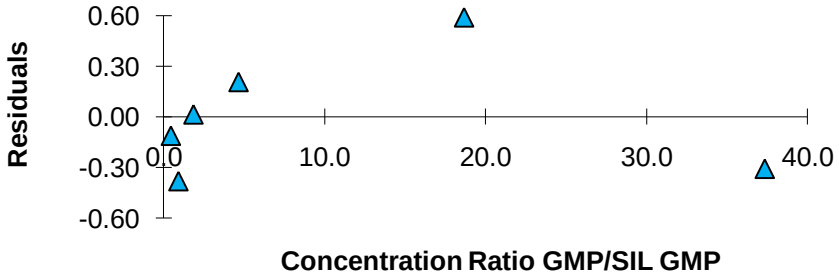

Figure 79. LC-MS method: GMP residuals plot

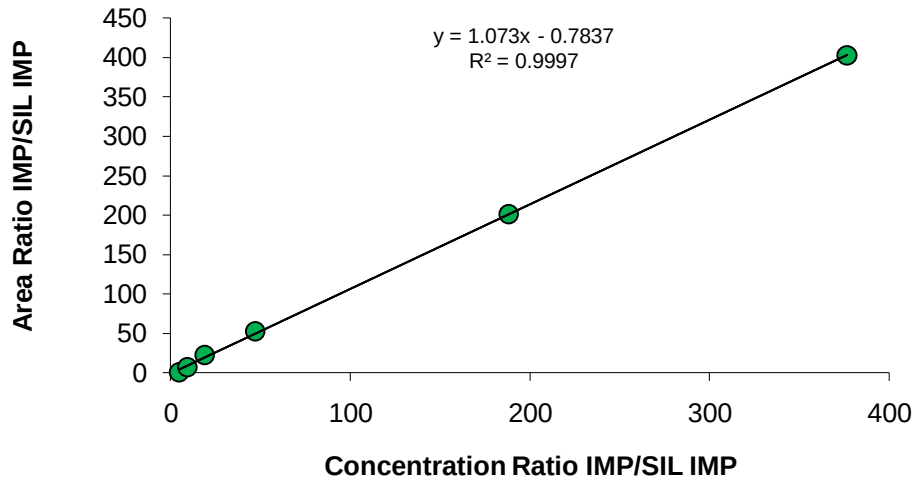

Figure 80. LC-MS method: IMP linear regression plot

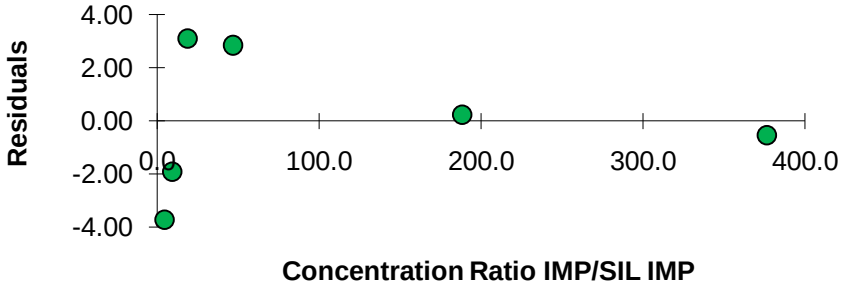

Figure 81. LC-MS method: IMP residuals plot

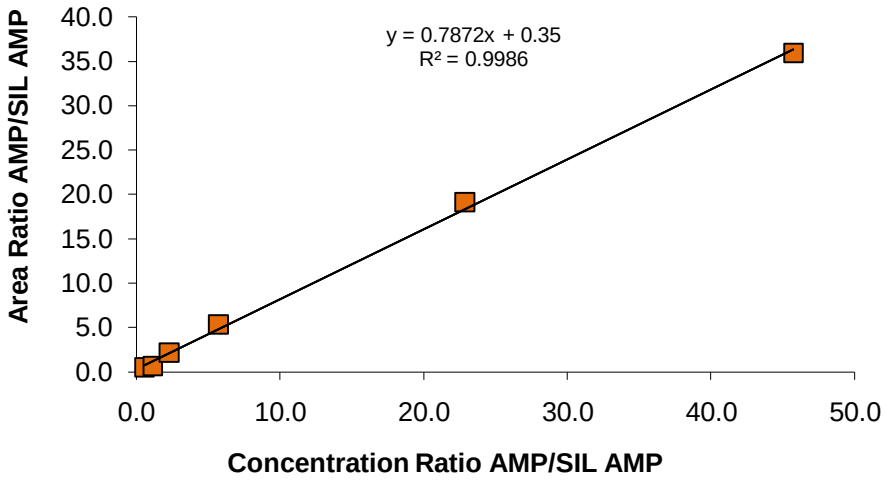

Figure 82. LC-MS method: AMP linear regression plot

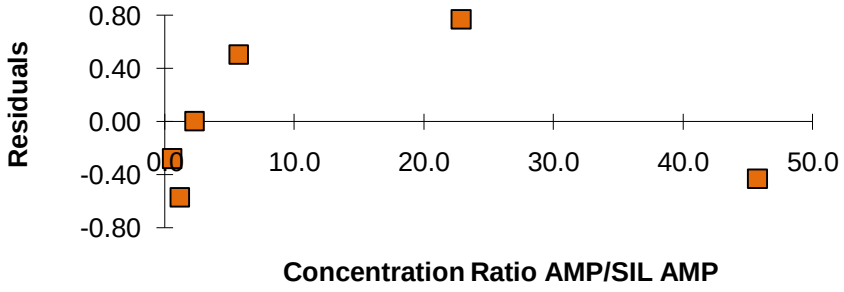

Figure 83. LC-MS method: AMP residuals plot

The detection limits were appropriate as defined by the infant formula industry with the exception of CMP and Urd (Sullivan, 2012). While CMP and UMP limits were higher than those specified, the MDL was two orders of magnitude lower than that found in unfortified milk powder (Gill and

Indyk, 2007b). Precision was evaluated as repeatability from 1.9–14.5% (HorRat = 0.2–0.8) and intermediate precision from 2.9–14.4%.

Accuracy determined as spiked recovery results measured in the six different product types were within the acceptable limits of 80–115% at the 10 µg g^{-1} level as suggested by the AOAC (Horwitz, 2002); **Table 36**. Accuracy estimated as bias was evaluated against reference values for NIST 1849a CRM and against AOAC Method 2011.20; **Tables 37–38**. Although there were statistically significant differences for some of the results, the differences were small enough (0–13%) that they are unlikely to be of practical significance for compliance and labelling requirements.

A robustness trial was performed evaluating seven factors at levels likely to occur during normal use of the method. The seven factors assessed were: initial sample water volume (27 mL, 23 mL); vortex time (40 s, 20 s); wait time (14 min, 6 min); centrifuge volume (4.2 mL, 3.8 mL); centrifuge speed (4000 x g, 3000 x g); centrifuge time (70 min, 50 min); and a dummy factor. The two factor levels were symmetric around the nominal values from the described analytical procedure, with the interval representing probable experimental error. The method was found to be robust for the seven parameters studied with variances in the results obtained not being significantly different from those expected by chance; **Figures 84–93**. Given the method's simplicity, two critical steps are required to ensure the accuracy of the results obtained: accurate measurement of the amount of sample weighed, and accurate addition of the internal standard.

Table 36. LC-MS method: recovery

Sample	Recovery (%)[a]									
	Cyd[b]	Urd	Guo	Ino	Ado	CMP	UMP	GMP	IMP	AMP
IF powder p/h bovine milk-based	95.4[b]	84.9	104.4	94.1	99.3	101.3	81.8	104.8	112.9	95.1
IF Powder p/h soy-based	101.1	98.9	107.2	96.8	100.1	101.8	88.8	101.6	98.4	101.7
Infant elemental powder	98.7	97.2	104.1	98.2	99.0	103.8	91.0	104.8	109.0	98.6
IF Powder bovine milk-based	93.4	86.6	102.6	100.1	97.9	95.7	90.7	102.0	101.3	101.8
IF Powder soy-based	101.7	80.1	107.9	103.0	95.3	101.8	90.3	103.5	94.8	98.7
IF Powder caprine milk-based	96.4	109.1	112.0	100.1	100.5	103.0	97.5	100.9	98.9	110.1

[a] Mean recovery (%) of samples spiked at 50% and 150% of typical concentrations in nucleotide-supplemented infant formula

[b] AMP = adenosine 5′-monophosphate
CMP = cytidine 5′-monophosphate
GMP = guanosine 5′-monophosphate
IMP = inosine 5′-monophosphate
UMP = uridine 5′-monophosphate
Ado = adenosine
Cyd = cytidine
Guo = guanosine
Ino = inosine
Urd = uridine

Table 37. LC-MS method: bias vs. CRM values

Sample	Analyte[a]							
	CMP	UMP	GMP	AMP	CMP+Cyd	UMP+Urd	GMP+Guo	AMP+Ado
Measured results[b]	27.0 (0.99)	12.0 (0.66)	14.8 (0.45)	10.3 (0.29)	28.1 (1.00)	14.4 (0.68)	15.0 (0.45)	10.3 (0.29)
Reference values[c]	26.8 ± 2.9	12.9 ± 1.5	14.6 ± 1.1	10.51 ± 0.5	31.7	15.5	14.6	10.8
Bias (p-value)	0.44	<0.001	0.16	0.03	<0.001	<0.001	0.01	0.05

[a] AMP = adenosine 5′-monophosphate
CMP = cytidine 5′-monophosphate
GMP = guanosine 5′-monophosphate
IMP = inosine 5′-monophosphate
UMP = uridine 5′-monophosphate
Ado = adenosine
Cyd = cytidine
Guo = guanosine
Ino = inosine
Urd = uridine

[b] Mean (standard deviation) of analytical results of NIST 1849a CRM in mg hg^{-1}, (*n* = 12 replicates)

[c] Reference value ± error of NIST 1849a CRM in mg hg^{-1}

Table 38. LC-MS method: bias vs. AOAC Official Method 2011.20

	Bias[a]				
	CMP	UMP	GMP	IMP	AMP
Measured results[b]	12.9 (0.39)	4.1 (0.14)	1.6 (0.04)	0 (0)	3.6 (0.11)
AOAC 2011.20 results[b]	12.3 (0.5)	4.0 (0.21)	1.6 (0.07)	0 (0)	3.2 (0.16)
Bias (*p*-value)	<0.001	0.24	0.44	0	<0.001

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

[b] Mean (standard deviation) of analytical results for bovine milk-based infant formula in mg hg^{-1} (*n* = 12 replicates)

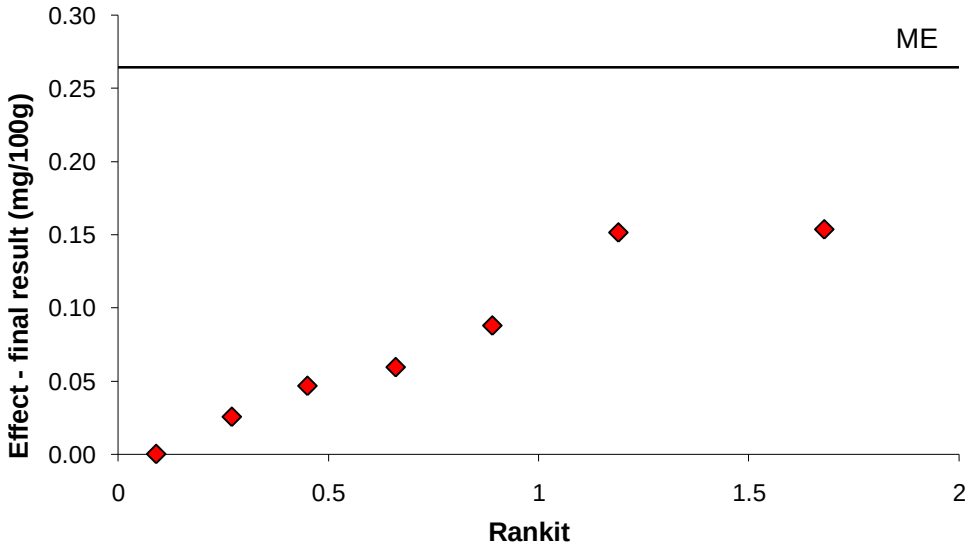

Figure 84. LC-MS method: Cyd half-normal plot

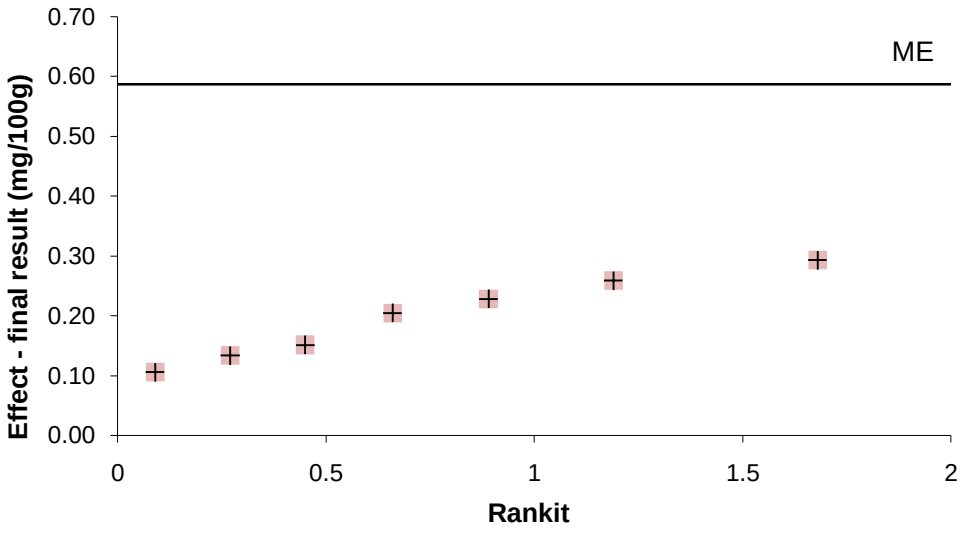

Figure 85. LC-MS method: Urd half-normal plot

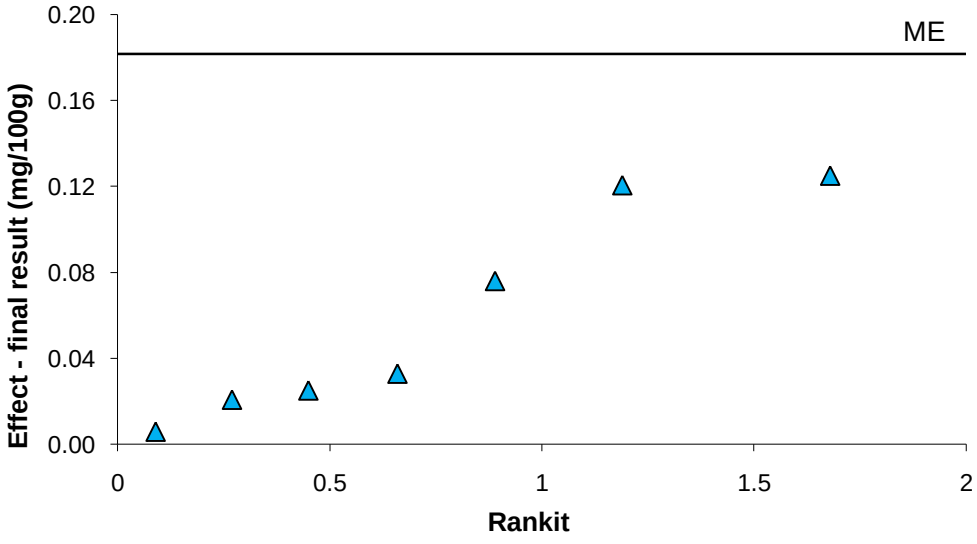

Figure 86. LC-MS method: Guo half-normal plot

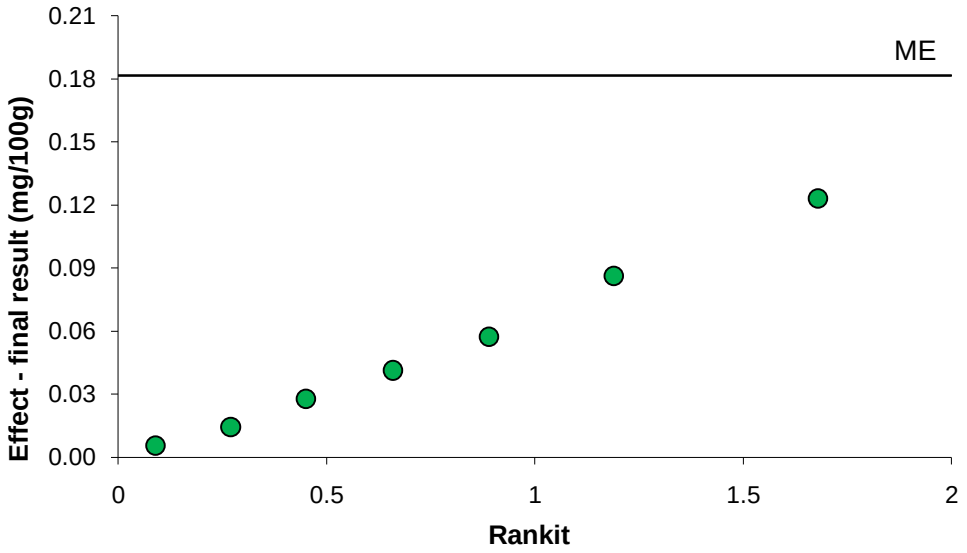

Figure 87. LC-MS method: Ino half-normal plot

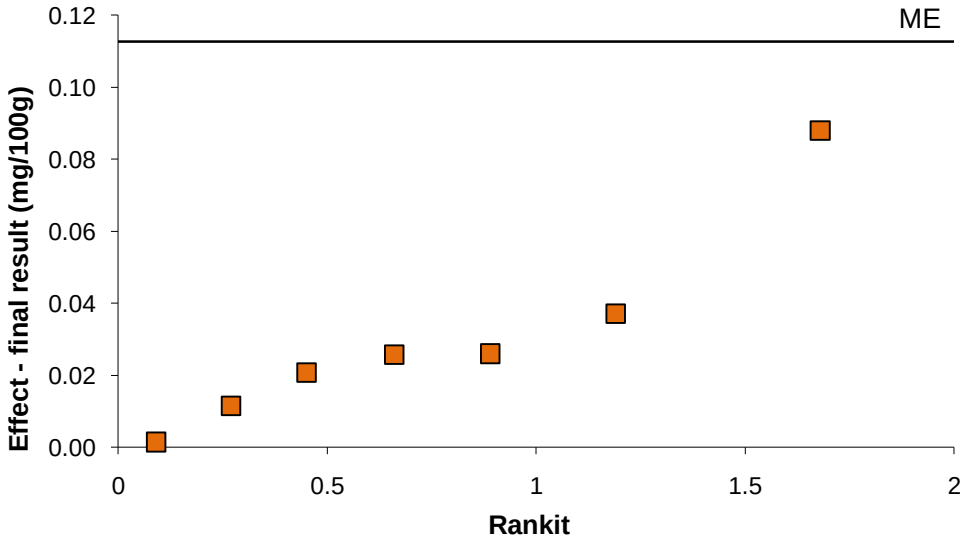

Figure 88. LC-MS method: Ado half-normal plot

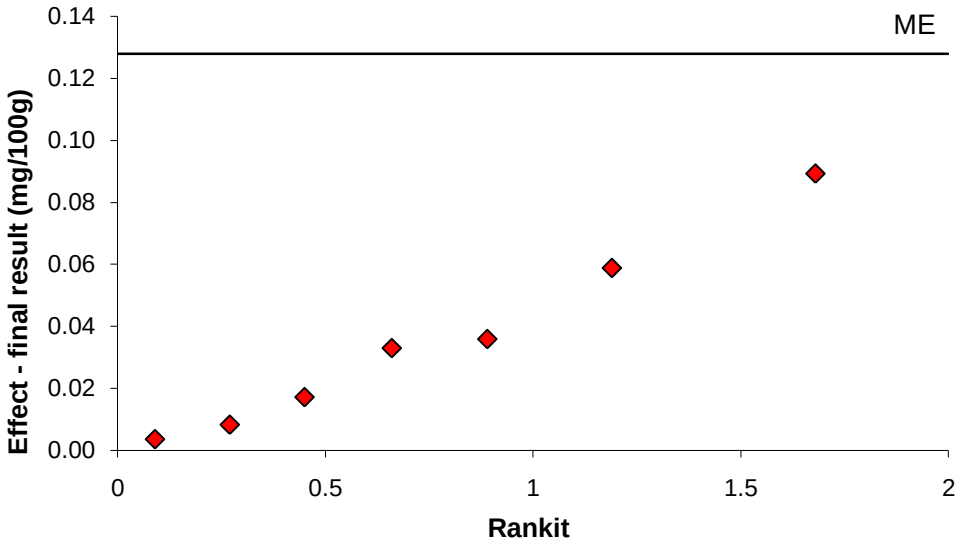

Figure 89. LC-MS method: CMP half-normal plot

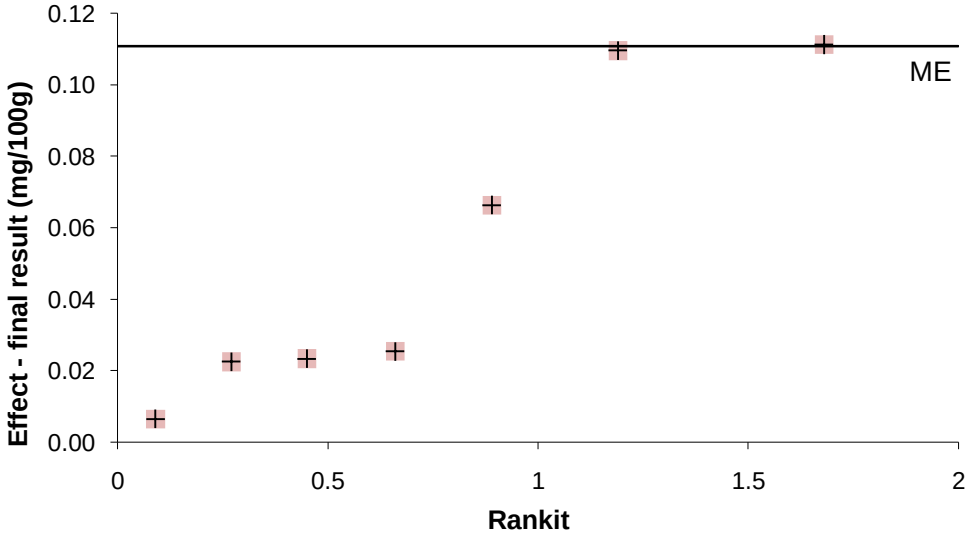

Figure 90. LC-MS method: UMP half-normal plot

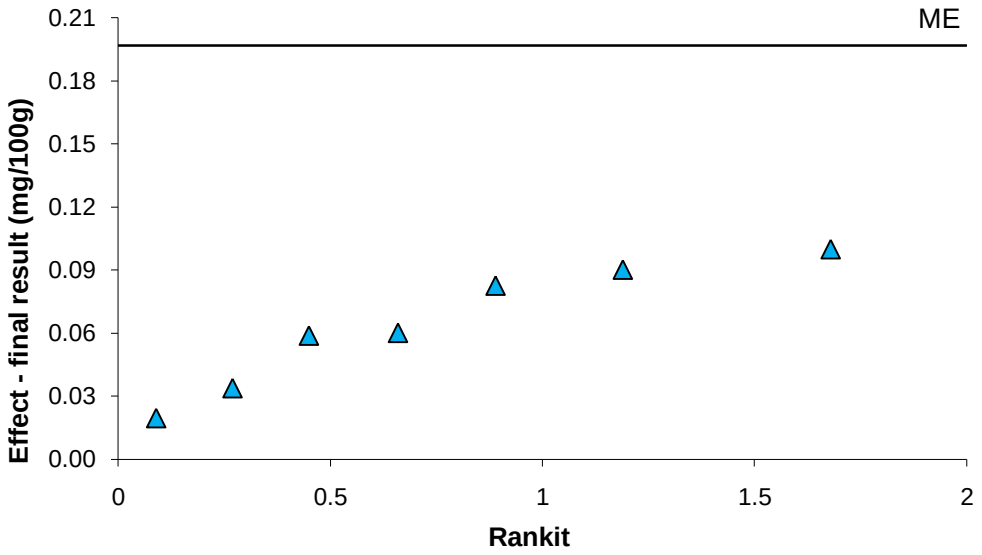

Figure 91. LC-MS method: GMP half-normal plot

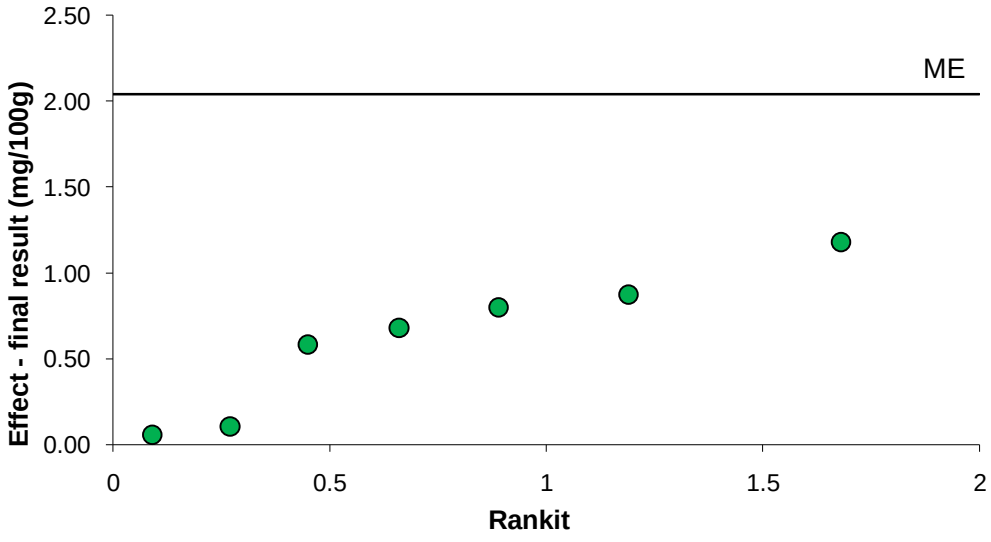

Figure 92. LC-MS method: IMP half-normal plot

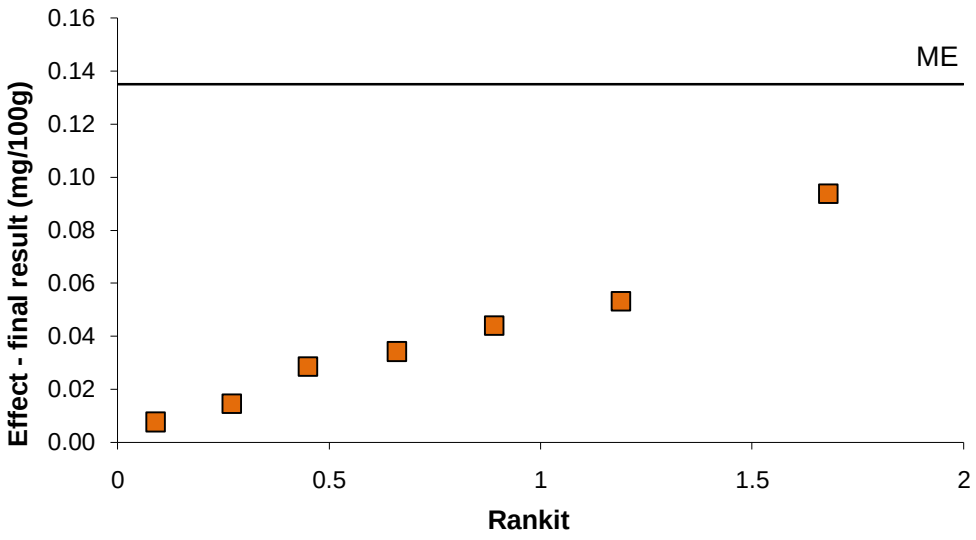

Figure 93. LC-MS method: AMP half-normal plot

4. CONCLUSIONS

LC-UV Method

The optimisation and validation of a simple and rapid method for the routine analysis of nucleotides in infant formulas has been described. The simplicity of analysis is facilitated by the use of SPE without the need for prior protein removal. The use of an internal standard gives additional confidence in the accuracy of the results obtained. The method has been demonstrated to be applicable to the analysis of bovine milk-based, caprine milk-based, soy-based, and hydrolysed milk protein-based infant formulas. An extension study demonstrated the expansion in scope to a wider range of different infant formula products. This method was approved for Official First Action status by by AOAC International and approved for further evaluation of reproducibility via a collaborative study, which will be undertaken in the near future.

TPAN Analysis of Milk

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. The TPAN concentrations in bovine milk and colostrum were studied, with differences in TPAN concentrations between summer-milk and winter-milk herds attributed particularly to variability in uridine and nucleotide concentrations. As lactation progressed, TPAN concentration decreased, as did each of the contributing forms. In a study of mature bovine, caprine, and ovine milk, significant differences among the milk of each species were found. Data obtained for samples collected during the afternoon may be evidence of diurnal rhythmicity of nucleoside and nucleotide production in milk. Further study would be useful in

establishing the extent and possible causes for the diurnal pattern of nucleoside and nucleotide expression in milk.

Numerous climatic and dietary factors could affect TPAN levels in milk. Further study controlling each of these factors would be required to identify those factors that influence nucleoside and nucleotide expression in milk. An expansion of the current study could be undertaken to consider the effects of breed, location, and diet on TPAN expression in milk.

LC-MS/MS Method

It has been identified that a key industry need was an accurate, precise, and robust method for the simultaneous analysis of both nucleotides and nucleosides to ensure food safety to the infant consumer and to provide a reference method for dispute resolution across trade borders. The optimisation and validation of an LC-MS/MS method for the analysis of nucleosides and nucleotides in infant formulas has been described.

The use of SIL internal standards provides confidence in the accuracy of the results obtained. Despite the attributes of tandem MS in facilitating a potentially unequivocal analysis, the technique is challenging with respect to mobile phase conditions and the need for stable isotope labelled standards. The method was demonstrated to be precise and accurate, and has been validated for the analysis of nucleotides and nucleosides in bovine milk-based, soy-based, caprine milk-based and hydrolysed milk protein-based infant formulas.

The infant formula industry is currently evaluating methods to be used as standards for analysing various nutrients, and this method makes a novel and significant addition to those currently under consideration.

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APPENDIX I: POSTER PRESENTATIONS

The Analysis of 5′-mononucleotides in Pediatric Formulas by HPLC.

Poster presented by author during the 123rd Annual AOAC Conference in Philadelphia, PA, 13th–16th September 2009.

The Determination of Total Potentially Available Nucleotides in Bovine Colostrum and Milk.

Poster presented by author during the 124th Annual AOAC Conference in Orlando, FL, 26th–29th September 2010.

Analysis of Nucleosides and Nucleotides in Infant Formula by Liquid Chromatography-Mass Spectrometry.

Poster to be presented by author at a future date.

THE ANALYSIS OF 5'-MONONUCLEOTIDES IN PEDIATRIC FORMULAS BY HPLC

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ABSTRACT

A reversed-phase HPLC method for the routine determination of supplemented 5'-mononucleotides (uridine 5' monophosphate, inosine 5' monophosphate, adenosine 5' monophosphate, guanosine 5' monophosphate, and cytidine 5' monophosphate) in pediatric formulas and milk products is described. Following sample dissolution, potential interferences were removed by strong anion-exchange solid-phase extraction. Chromatographic analyses were performed using a C18 stationary phase with gradient elution, UV detection and with quantitation achieved by an internal standard technique. A single laboratory validation was performed with recoveries of 92 – 101 % and repeatability RSD's of 1.0 – 2.3 %. The method was optimised for the rapid, routine analysis of nucleotide-supplemented bovine milk-based infant and follow-on formulas.

Paper submitted to Journal of AOAC International, 2009.

INTRODUCTION

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 protein.

Nucleotide supplemented infant formulas have been reported to enhance immune response, influence metabolism of fatty acids and improve gastrointestinal tract repair after damage. With the proliferation of nucleotide-supplemented pediatric formulas, robust methods that incorporate minimal sample preparation and rapid chromatographic separations are required for routine product compliance analysis.

This method below describes a simple SPE sample clean-up that avoids the prior need to remove protein, coupled with a binary gradient reversed-phase liquid chromatographic system for the purpose of routine analysis of nucleotide supplemented infant formula. Analytical security is enhanced with an internal standard based quantitation.

METHODOLOGY

SAMPLE PREPARATION

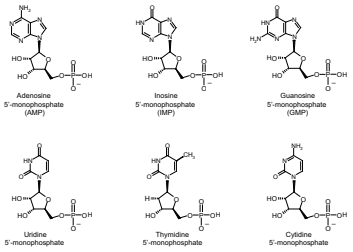
- Dissolve 1.0g powder in 30mL of NaCl/EDTA solution.
- Add 1mL of TMP intermediate standard (~80 µg mL^{-1}), mix well.
- Stand for 10 minutes to ensure complete hydration.
- Dilute to final volume of 50mL with water.

SOLID PHASE EXTRACTION

- For each sample, place a SPE cartridge (Chromabond SB, 6mL x 1000mg) on SPE vacuum manifold.
- Condition cartridge with 4mL MeOH, followed by 2 x 5mL of H_2O.
- Load cartridge with 4mL of sample solution.
- Wash cartridge with 4mL of 0.3M KBr.
- Elute nucleotides with 4mL of 0.5M KH_2PO_4, pH 3.0 into a test tube.
- Filter an aliquot of the eluent through a 0.2 µm syringe filter into an autosampler vial ready for HPLC analysis.

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:	MeOH 100%
Gradient:	Low pressure gradient
Detection:	Photo-diode array, quantify at 250nm (IMP), 260nm (GMP, TMP, AMP) and 270nm (CMP, UMP)
Quanitittation:	Internal standard technique (TMP)
Injection:	50 µL



RESULTS AND DISCUSSION

CHROMATOGRAPHIC PERFORMANCE

Parameters[a]	CMP[b]	UMP	GMP	IMP	TMP	AMP
Retention time	8.8 (0.22%)[c]	11.8 (0.17 %)	19.8 (0.15%)	20.6 (0.10%)	25.0 (0.04%)	25.8 (0.04%)
Capacity factor	0.6 (0%)	1.2 (0.83%)	2.7 (0%)	2.8(0%)	3.6 (0%)	3.8 (0%)
Resolution	–	6.3 (0.07)	16.9 (1.11%)	2.2 (0.45%)	15.6 (0.19%)	3.5 (0.57%)
Tailing	1.3 (3.84%)	1.2 (3.33%)	1.0 (0%)	1.0 (5.00%)	1.1 (0%)	1.1 (3.6%)
Theoretical Plates	6810 (0.87%)	8527 (5.51%)	33692 (3.28%)	60448 (1.22%)	194363 (0.81%)	241749 (0.22%)
Peak Area	142255 (0.51%)	200488 (1.39%)	225242 (0.23%)	122536 (0.75%)w	488585 (0.11%)	308754 (0.05%)

[a] Calculations as defined by US Pharmacopeia.
[b] AMP = adenosine 5'-monophosphate, CMP = cytidine 5'-monophosphate, GMP = guanosine 5'-monophosphate, IMP = inosine 5'-monophosphate, UMP = uridine 5'-monophosphate, TMP = thymidine 5'-monophosphate.
[c] Mean (percent relative standard deviation) of six replicates of a mixed nucleotide standard.

RESULTS OBTAINED FROM COMMERCIALLY AVAILABLE MILK PRODUC

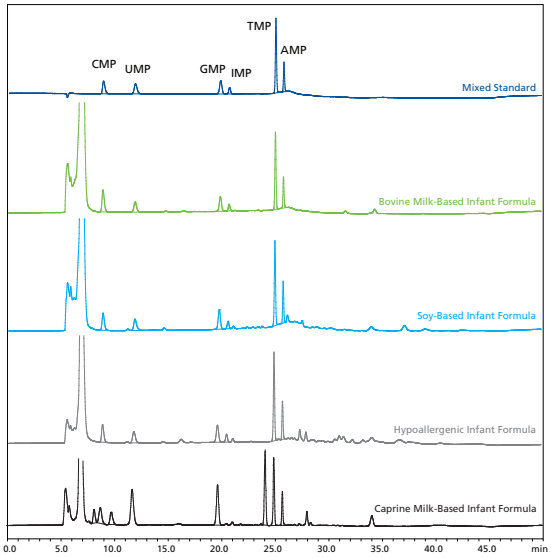
Sample Type[a]	Nucleotide Supplemented	Results (mg/100g^{-1}) CMP[b]	UMP	GMP	IMP	AMP
Bovine milk-based IF	Yes	11.6 (116%)[c]	3.7 (95%)	1.7 (106%)	2.0 (125%)	4.5 (145%)
Bovine milk-based FO	Yes	6.0 (107%)	2.4 (87%)	0.9 (89%)	1.0 (91%)	2.1 (103%)
Bovine milk-based FO	No	1.0	0	0	0.1	0
Bovine milk-based FO	Yes	8.5 (172%)	2.4 (89%)	1.0 (100%)	1.0 (92%)	2.3 (115%)
Bovine milk-based AN	Yes	17.4 (120%)	4.7 (75%)	8.0 (107%)	0 –	7.2 (130%)
Soy-based IF	No	0.1	0.3	0.3	0	0.5
Caprine milk-based IF	No	4.0	8.2	6.4	0.3	2.3
Bovine milk-based WMP	No	4.0	0	0	0	0
Hypoallergenic IF	No[d]	2.6 (101%)	2.6 (92%)	2.7 (85%)	2.6 (96%)	3.1 (100%)
Hypoallergenic IF	No	0.0	0	0	0	0

[a] IF = infant formula, FO = follow-on formula, AN = adult nutritional product, WMP = whole milk powder.
[b] AMP = adenosine 5'-monophosphate, CMP = cytidine 5'-monophosphate, GMP = guanosine 5'-monophosphate, IMP = inosine 5' monophosphate, UMP = uridine 5'-monophosphate.
[c] Recovery as percentage of label claim.
[d] Hypoallergenic sample spiked with nucleotide mixed standard prior to analysis.
[e] IMP not added

METHOD PERFORMANCE

Analytea	Range (mg mL^{-1})	Linear regression	r^2	MDL (mg 100 g^{-1})[b]	RSDr (%)[c]	HorRat[d]	RSD_R (%)[e]	Recovery (%)[f]
AMP	1.25 - 17.49	y = 255805x + 11862	1.0000	0.19	2.0	0.4	4.5	99.6 (2.4%)
CMP	0.61 - 8.55	y = 287762x - 2493	0.9999	0.08	1.0	0.3	6.0	99.7 (1.9%)
GMP	1.11 - 15.55	y = 200342x - 1807	1.0000	0.06	2.1	0.4	5.2	100.5 (1.7%)
IMP	1.09 - 15.27	y = 198519x + 3879	1.0000	0.10	1.4	0.3	3.8	97.8 (2.4%)
UMP	1.12 - 15.68	y = 146931x - 1839	0.9999	0.08	2.3	0.5	8.6	96.5 (3.6%)
TMP	1.61 - 22.54	y = 150494x - 455	1.0000	–	–	–	–	100.1 (3.1%)

[a] AMP = adenosine 5'-monophosphate, CMP = cytidine 5'-monophosphate, GMP = guanosine 5'-monophosphate, IMP = inosine 5'-monophos phate, UMP = uridine 5'-monophosphate, TMP = thymidine 5'-monophosphate
[b] Determined from n replicates at or near the expected detection limit, MDL = $t_{n-1,\alpha=0.99} \times sd$, where n = 10 and α = 0.01.
[c] RSD repeatability = sd/mean x 100 (n = 6)
[d] Horwitz ratio = RSD$_r$ / pRSD$_{eq}$ where pRSD$_{eq}$ = $C^{-0.15}$ at 10 ppm concentration level
[e] RSD intermediate reproducibility = sd/mean x 100 (n = 24)
[f] Mean recovery (percent relative standard deviation) at three levels each for bovine milk-based, soy-based, and hypoallergenic infant formulas[a]



Chromatographic performance was assessed by replicate analyses (n = 6) of a mixed nucleotide standard. Performance within recommended guidelines was achieved with the exception of capacity factor for CMP and UMP, however, this was deemed acceptable due to uncompromised peak integrity of these two compounds in all samples analysed.

Linearity of dose response was confirmed by least-squares regression analysis with acceptable values obtained for correlation coefficient. Plots of standard residuals showed no structure with only a small amount of random noise further demonstrating linearity. Method detection limits determined are well below levels typically supplemented to pediatric formulas. A Plackett-Burman robustness trial demonstrated that the method was found to be robust for seven factors which may have affected final results at levels typically expected during normal use.

Acceptable precision was achieved with HorRat (repeatability) calculated between 0.3 and 0.5. Method accuracy was demonstrated by measurement of recovery well within limits of 80 – 115 % at the 10 µg/g level suggested by AOAC. No evidence of method bias was found between the method described presently and one published previously (Gill and Indyk, 2007, *Int. Dairy J.* 17, 596-605).

The method was applied to a number of commercially available pediatric and nutritional powders. Products included for testing were infant formulas, follow-on formulas and an adult nutritional product. These products included a range of different sources; bovine milk, hydrolysed milk protein, caprine milk, and soy protein.

CONCLUSIONS

A rapid robust analytical method for the analysis of 5'-mononucleotides supplemented to pediatric formulas is described

- Simple sample preparation without need for prior protein removal
- Rapid and thorough removal of interferences via SPE clean-up.
- Increased security of results through analysis by use of internal standard.
- Method applicable to a wide range of pediatric formulas

ACKNOWLEDGEMENTS

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

DETERMINATION OF TOTAL POTENTIALLY AVAILABLE NUCLEOSIDES IN BOVINE COLOSTRUM AND MILK

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INTRODUCTION

Nucleosides and nucleotides are compounds of critical importance to cellular function. Dietary sources of nucleotides are considered conditionally essential for continued optimal metabolic function. Dietary nucleotides are ingested in the form of nucleoproteins, polymeric nucleotides (nucleic acids) and nucleotide adducts as well as free nucleotides.

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 et al. Am. J. Clin. Nutr. 1995, 61, 1224–1230). The analytical method uses a number of enzymatic treatments and incorporates combinations of nuclease, pyrophosphatase and phosphatase enzymes into the sample preparation. 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.

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.

ANALYTICAL TECHNIQUE

Sample Collection
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. Collected samples were initially refrigerated at 4 °C, then taken to the laboratory where endogenous enzymes were chemically inactivated prior to storage at < -15 °C.

Sample Preparation
Each sample was pooled, then split into four 5 mL sub-samples, to each of which internal standard (10 µg, 5-methylcytidine) was added, and then each sub-sample was subjected to a different enzymatic treatment.

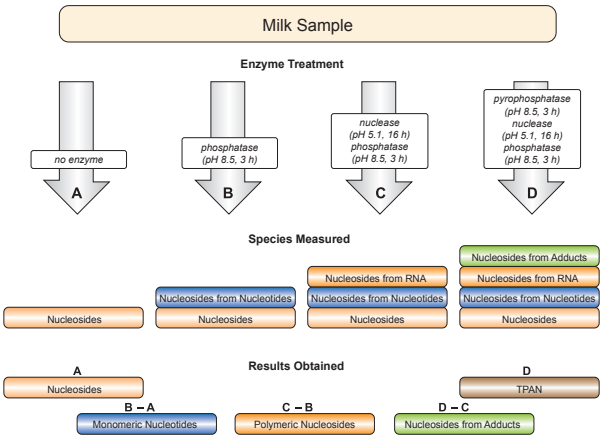

Figure 1: Schematic of TPAN experiments

Solid Phase Extraction
Clean-up of enzymatic extracts was achieved by solid phase extraction using a phenylboronate affinity gel (Bio-Rad), whereby nucleosides are covalently bonded to the gel at high pH, and interferences were then removed with two washings in high pH buffer. The nucleosides were then eluted from the affinity gel at low pH with the addition of phosphoric acid, and filtered ready for analysis.

Chromatographic Analysis
Column: Prodigy C_{18} 5 µm, 4.6 x 150 mm (Phenomenex)
Mobile Phase: (A) $NaCH_3COO$ (0.05 M), pH = 5.6, (B) MeOH (100%)
Flowrate: 0.7 mL/min with low-pressure gradient mixing (A) & (B)
Detection: Photo-diode array 210–300 nm, quantitation at 260 nm
Quantitation: Internal standard technique (5-methylcytidine)

The nucleoside results for each of the four sub-samples allowed the contributions of the different forms (nucleosides, nucleotide adducts, monomeric and polymeric nucleotides) to TPAN to be calculated.

RESULTS AND DISCUSSION

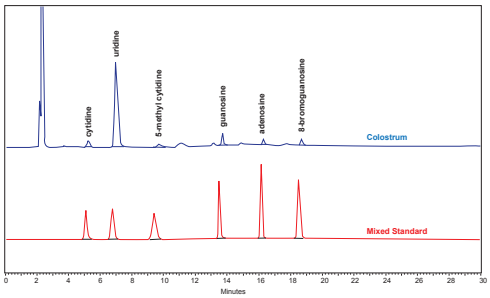

Figure 2: Chromatograms of a mixed standard and colostrum sample

The absolute concentrations indicated a distinct difference between the two herds, although the general trends were the same. High levels of TPAN were found in colostrum, with a decrease in their concentrations as lactation progressed.

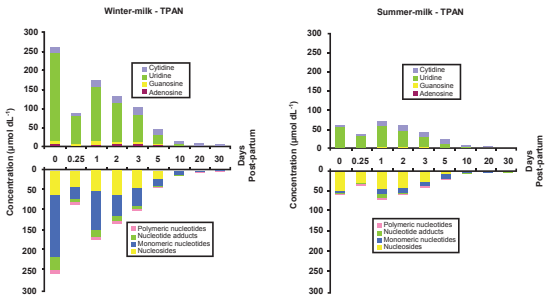

Figure 3: TPAN in bovine milk over the first month of lactation

A comparison of the two herds showed little difference in total free nucleoside content as lactation progressed. Differences in colostral monomeric nucleotide levels between the herds were seen, with the winter-milk herd containing 5–10 times the levels of the summer-milk herd although by the fifth day, nucleotide levels decreased to similar levels between the two herds. The levels of monomeric nucleotides measured in this study were generally higher than those reported previously (Gill and Indyk, Int. Dairy J. 2007, 17, 596–605), most likely due to a significant contribution from multiple phosphorylated forms, which the TPAN analytical method aggregates as a single value.

The pyrimidines (cytidine and uridine) 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. In contrast, concentrations of the purines (guanosine and adenosine) in bovine milk were more consistent through lactation. Purine nucleosides and nucleotides made a relatively small contribution to TPAN (6–20%).

CONCLUSIONS

Bovine milk samples from two herds were studied over the course of the first month of lactation, and total potentially available nucleosides were determined.

- Uridine and uridine nucleotides were the major contributor to TPAN in early colostrum
- Differences in TPAN concentrations between summer milk and winter-milk herds were largely attributable to variability in uridine and nucleotide concentrations.
- TPAN concentration decreased as lactation progressed, as did each of the contributing forms.

ACKNOWLEDGEMENT

The authors thank Rich Syme and Andrew McGiven for supply of the bovine milk samples. Advice on the application of the analytical protocol given by Bruce Molitor (Abbott Laboratories, Columbus, OH, USA) is greatly appreciated. The financial assistance of Fonterra Co-operative Group Limited and of the Tertiary Education Commission in providing an Enterprise Scholarship is gratefully acknowledged.

Analysis of Nucleosides and Nucleotides in Infant Formula by Liquid Chromatography-Mass Spectrometry

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ABSTRACT

A method for the simultaneous analysis of nucleosides and nucleotides in infant formula using reversed-phase liquid chromatography-tandem mass spectrometry is described. Following sample dissolution, protein is removed by centrifugal ultrafiltration. Chromatographic analyses were performed using a C_{18} stationary phase with gradient elution of a ammonium acetate/bicarbonate buffer, MS detection, and quantitation by stable isotope label internal standard technique. A single laboratory validation was performed with recoveries of 80.1–112.9% and repeatability relative standard deviations of 1.9–7.2%. The method has been validated for the analysis of bovine milk- based, soy-based, caprine milk-based and hydrolysed milk protein-based infant formulas.

INTRODUCTION

In view of their physiological benefits, the nucleotides adenosine 5'-monophosphate (AMP), cytidine 5'-monophosphate (CMP), guanosine 5'-monophosphate (GMP), inosine 5'-monophosphate (IMP), and uridine 5'-monophosphate (UMP) are routinely supplemented into infant formula. Although nucleosides are not supplemented into infant formula, dephosphorylation of nucleotides to the corresponding nucleosides, adenosine (Ado), cytidine (Cyd), guanosine (Guo), inosine (Ino), and uridine (Urd), can occur under certain processing conditions.

Analytical chemists representing the infant formula industry have identified as a key need, a suitable method for the simultaneous analysis of both nucleotides and nucleosides. While separate measurements of either form have been published previously, few papers have described the simultaneous analysis of nucleotides and nucleosides for compliance testing of infant formula, and none has done so utilising LC-MS.

A method for the analysis of nucleosides and nucleotides in infant formula using reversed-phase liquid chromatography-tandem mass spectrometry is described. Analytical security is enhanced by using stable isotope labelled internal standard based quantitation. The method has been validated for the analysis of bovine milk-based, soy-based, caprine milk-based and hydrolysed milk protein-based infant formulas.

ANALYTICAL TECHNIQUE

SAMPLE PREPARATION

- Dissolve 5.0 g infant formula in 25 mL of water.
- Add 1 mL of SIL internal standard, mix well.
- Stand for 10 minutes to ensure complete hydration.
- Dilute to final volume of 50 mL with water.
- Ultracentrifuge (Amicon Ultra-4 3000 MWCO) at 3000 x g, 60 mins.

LC-MS/MS ANALYSIS

CHROMATOGRAPHY:

- Chromatography: Shimadzu Prominence LC system
- High pressure gradient (Table 1)
- Column: (Gemini C_{18} 5 µm 110Å 4.6 x 250mm)
- Phase A: 10 mM NH_4CH_3COO, 5 mM NH_4HCO_3, pH = 5.6
- Phase B: MeOH 100%
- Injection: 20 µL

MS DETECTION:

- ABSciex 3200 QTrap
- Tandem mass spectrometry (Table 2)
- Curtain gas (30 psi), Nebulizer gas GS1 (50 psi), GS2 (70 psi), Desolvation temperature (700 °C), CID gas (medium), Ion spray voltage (5500 V)

QUANTITATION:

- Stable isotope labelled internal standard technique

TABLE 1: GRADIENT PROCEDURE FOR CHROMATOGRAPHIC SEPARATION

Time (min)	Flow rate (mL min^{-1})	Phase composition	
		%A	%B
0.0	0.75	100	0
3.5	0.75	100	0
10.0	0.75	80	20
20.0	0.75	80	20
21.0	0.75	100	0
35.0	0.75	100	0

TABLE 2: MS/MS PARAMETERS

Analyte [a]	Precursor ion (M-H)$^-$ (m/z)	Product ions (m/z)	DP [b] (V)	EP [c] (V)	CEP [d] (V)	CE [e] (V)	CXP [f] (V)
CMP	324.1	112.0	26	3.5	32	27	4
SIL CMP	336.1	119.0					
UMP	325.0	97.0	21	9.5	18	23	4
SIL UMP	336.1	102.1					
GMP	364.1	152.0	36	4.5	22	23	4
SIL GMP	379.1	160.0					
IMP	349.1	137.0	31	6	28	23	4
SIL IMP	363.1	145.0					
Cyd	244.1	119.0	11	6.5	22	19	4
SIL Cyd	256.1	112.0					
Urd	245.1	119.0	16	5	16	19	4
SIL Urd	256.1	113.0					
AMP	348.1	136.0	36	1	24	25	4
SIL AMP	363.1	146.0					
Ino	269.1	137.0	16	4.5	18	23	4
SIL Ino	273.1	141.0					
Guo	284.1	152.0	11	4	24	23	4
SIL Guo	289.1	157.0					
Ado	268.1	136.0	21	8.5	16	39	4
SIL Ado	273.1	136.0					

[a] SIL = stable isotope labelled; Ado = adenosine; Cyd = cytidine; Guo = guanosine; Ino = inosine; Urd = uridine; AMP = adenosine 5'-monophosphate; CMP = cytidine 5'-monophosphate; GMP = guanosine 5'-monophosphate; IMP = inosine 5'-monophosphate; UMP = uridine 5'-monophosphate.
[b] DP = declustering potential
[c] EP = entrance potential
[d] CEP = collision cell entrance potential
[e] CE = collision energy
[f] CXP = collision cell exit potential

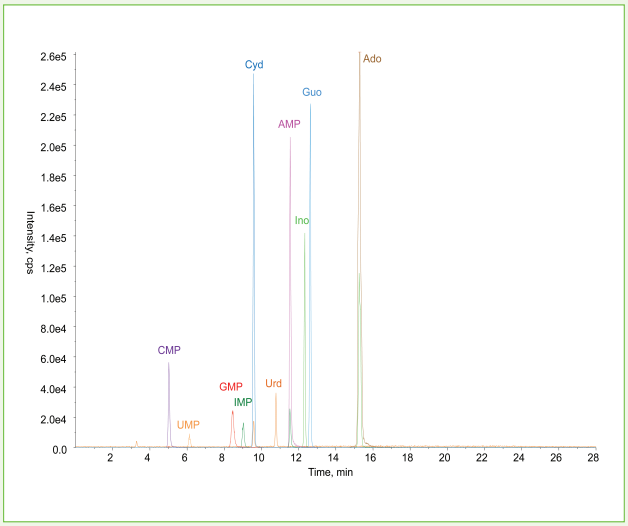

Figure 1. Non-labelled and stable isotopically labelled nucleotide and nucleoside standard mixture; AMP = adenosine 5'-monophosphate, CMP = cytidine 5'-monophosphate, GMP = guanosine 5'-monophosphate, IMP =inosine 5'-monophosphate, UMP = uridine 5'-monophosphate, Ado = adenosine, Cyd = cytidine, Guo = guanosine, Ino = inosine, Urd = uridine

RESULTS AND DISCUSSION

Chromatographic performance was assessed by replicate analyses (n = 6) of a mixed nucleoside and nucleotide standard with satisfactory resolution obtained between IMP/AMP (6.7), Ino/Ado (6.8), Cyd/Urd (4.3) peaks which differ in mass by < 2 Da.

Linearity was evaluated by least-squares regression analysis, with acceptable values obtained for the correlation coefficient and with standard residuals plots showing no pattern and only a small amount of random variation. The detection limits are appropriate as defined by the infant formula industry with the exception of CMP and Urd. While CMP and Urd limits are higher than those specified, the MDL is two orders of magnitude lower than that found in unfortified milk powder. The method was found to be robust and given the method's simplicity, two critical steps are required to ensure the accuracy of results obtained: accurate measurement of the amount of sample weighed, and accurate addition of the internal standard. Precision was evaluated as repeatability from 1.9–7.2% and intermediate precision from 2.9–14.4%. The suitability of these results is demonstrated by a HorRat of 0.2–0.8.

Accuracy determined as spiked recovery results measured in the six different product types were within acceptable limits of 80–115% at the 10 µg g^{-1} level as suggested by the AOAC. Bias was evaluated against reference values for NIST 1849a CRM and against AOAC Method 2011.20 with comparable results obtained.

CONCLUSIONS

An accurate analytical method for the analysis of nucleosides and nucleotides in supplemented to pediatric formulas is described.

- Simple sample preparation
- Enhanced analytical accuracy by use of stable isotope labelled internals standards.
- Method applicable to a wide range of infant formulas including bovine milk-based, soy-based, caprine milk-based and hydrolysed milk protein-based infant formulas.

ACKNOWLEDGMENTS

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

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APPENDIX II: PUBLICATIONS

A Liquid Chromatographic Method for Routine Analysis of 5′-Mononucleotides in Pediatric Formulas

Reprinted from the Journal of AOAC International, Vol.93 (3), Gill, B.D., Indyk, H.E., Kumar, M.C., Sievwright, N.K., Manley-Harris, M., A liquid chromatographic method for routine analysis of 5′-mononucleotides in pediatric formulas, 966–973, copyright (2010), with permission from AOAC International.

Determination of total potentially available nucleosides in bovine milk.

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Determination of total potentially available nucleosides in bovine, caprine, and ovine milk.

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Analysis of 5′-Mononucleotides in Infant Formula and Adult/Pediatric Nutritional Formula by Liquid Chromatography: First Action 2011.20.

Reprinted from the Journal of AOAC International, Vol.95 (3), Gill, B.D., Indyk, H.E., Kumar, M.C., Sievwright, N.K., Manley-Harris, M., Dowell, D. Analysis of 5′-Mononucleotides in Infant Formula and Adult/Pediatric Nutritional Formula by Liquid Chromatography: First Action 2011.20, 599–602, copyright (2012), with permission from AOAC International.

Analysis of nucleosides and nucleotides in infant formula by liquid chromatography-tandem mass spectrometry.

Springer and the original publisher (Analytical Bioanalytical Chemistry, Vol. 405; Gill, B.D., Indyk, H.E., Manley-Harris, M. Analysis of nucleosides and nucleotides in infant formula by liquid chromatography–tandem mass spectrometry. 5311–5319, 2013) is given to the publication in which the material was originally published, with kind permission from Springer Science and Business Media.

FOOD COMPOSITION AND ADDITIVES

A Liquid Chromatographic Method for Routine Analysis of 5 -Mononucleotides in Pediatric Formulas

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An RP-HPLC method for the routine determination of supplemented 5 -mononucleotides (uridine 5 -monophosphate, inosine 5 -monophosphate, adenosine 5 -monophosphate, guanosine 5 -monophosphate, and cytidine 5 -monophosphate) in pediatric formulas and milk products is described. Following sample dissolution, potential interferences were removed by anion-exchange SPE. Chromatographic analyses were performed using a C_{18} stationary phase with gradient elution, UV detection, and quantitation by an internal standard technique. A single-laboratory validation was performed, with recoveries of 92–101% and repeatability RSDs of 1.0–2.3%. The method was optimized for the rapid, routine analysis of nucleotide-supplemented bovine milk-based infant and follow-on formulas.

Nucleotides are compounds of critical importance to cellular function. They operate as precursors to nucleic acids, as mediators of chemical energy transfer and cell signaling, and as integral components of coenzymes in the metabolism of carbohydrates, lipids, and proteins (1–3). Nucleotides are not essential dietary nutrients as they can be synthesized de novo or recovered via salvage pathways. However, in times when the endogenous supply is inadequate, such as during periods of rapid growth or after injury, they may become conditionally essential (1).

Nucleotide-supplemented infant formulas have been reported to enhance immune response (4–6), influence metabolism of fatty acids, and improve gastrointestinal tract repair after damage (1, 7). Infants fed formula supplemented with nucleotides are reportedly less likely to experience diarrhea and have elevated serum immunoglobulin A concentrations (8). Nucleotide-supplemented infant formula has been shown to positively modify the composition of the intestinal microflora, compared with unsupplemented formula (1, 9).

As understanding of the nucleotide composition of bovine milk and human milk has increased, manufacturers have endeavored to modify the composition of infant formulas to resemble that of human milk more closely. Nucleotides have, therefore, been added routinely to infant formulas since the 1980s, and added to formulas manufactured specifically for pre-term infants since 2002 (10). Although more than 12 nucleotides are present in human milk, supplementation is limited to adenosine 5 -monophosphate (AMP), cytidine 5 -monophosphate (CMP), guanosine 5 -monophosphate (GMP), inosine 5 -monophosphate (IMP), and uridine 5 -monophosphate (UMP) in the form of the readily soluble sodium salts (11).

With the proliferation of nucleotide-supplemented pediatric formulas, robust methods that incorporate minimal sample preparation and rapid chromatographic separations have been developed for routine product compliance analysis. Analytical methods for nucleos(t)ides in milk have been reviewed previously by Gil and Uauy (12), and more recently by Gill and Indyk (13). Initial preparation of infant formulas for analysis is usually achieved by acid precipitation of casein proteins from the reconstituted sample (14, 15), although ultrafiltration has also been reported (16). Additional cleanup of sample extracts using ion-exchange SPE has been reported (14).

Over the last decade, LC with UV detection has become the dominant technique for the final determination of nucleotides in milk products following sample preparation. Ion-pair reversed-phase liquid chromatography (IP-RPLC) is frequently used to separate nucleotides and can offer advantages in selectivity and efficiency over RPLC for the separation of charged analytes (14, 17, 18). However, IP-RPLC can require long equilibration times, and ion-pair reagents tend to be corrosive, thereby reducing column life (19). Unmodified reversed-phase chromatography offers the advantage of a simplified mobile phase system and is preferable if acceptable retention and resolution are achieved. Therefore, at an appropriate mobile phase pH, mononucleotides are readily

Received August 16, 2009. Accepted by SG October 2, 2009.
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Table 1. UV absorbance maxima and extinction coefficients for 5 -mononucleotides

Nucleotide[a]	$_{max}$, nm	$E^{1\%}_{1\,cm}$
AMP[b]	257	430.4
CMP[b]	280	398.0
GMP[b]	254	393.3
IMP[b]	249	357.3
UMP[b]	262	313.5
TMP	267	288.5

[a] AMP = adenosine 5 -monophosphate; CMP = cytidine 5 -monophosphate; GMP = guanosine 5 -monophosphate; IMP = inosine 5 -monophosphate; UMP = uridine 5 -monophosphate; TMP = thymidine 5 -monophosphate.

[b] From reference (15).

Table 2. Gradient procedure for chromatographic separation

		Phase composition	
Time, min	Flow rate, mL/min	% A	% B
0	0.5	100	0
5	0.5	100	0
14	0.5	90	10
15	0.5	80	20
35	0.5	80	20
36	0.5	100	0
50	0.5	100	0

retained on a C_{18} column and a methanol gradient is sufficient to remove late-eluting nucleotides (15).

However, despite the quantity of published methods, there is currently no official internationally accepted reference method for the analysis of nucleotides in milk and pediatric formulas, a situation that renders international trade and infant nutrition difficult to standardize.

The aim of this study is to validate a simple, rapid and robust method for routine compliance testing of nucleotide-supplemented pediatric formulas. The method herein describes an SPE sample cleanup that avoids the prior need to remove protein, coupled with a binary gradient RP-HPLC system. Analytical security is enhanced with an internal standard-based quantitation. This technique has been applied to the analysis of bovine milk-based, caprine milk-based, soy-based, and hypoallergenic pediatric formulas.

Experimental

Apparatus

HPLC was carried out with an LC-20AT pump, an SIL-20A sample injector unit equipped with a 50 L injection loop, a DGU-20A5 degasser unit, a CTO-20AC column oven, and an SPD-M20A photodiode array detector (Shimadzu, Kyoto, Japan). Shimadzu LC solutions software Version 1.22 SP1 was used for instrument control and data processing.

Separation was achieved with a Gemini C_{18} column, 5 m, 4.6 250 mm (Phenomenex, Torrance, CA). UV absorbances for calibration standards were acquired with a model UV-1601 spectrophotometer (Shimadzu) with digital readout to four decimal places. A Meterlab PHM210 standard pH meter (Radiometer Analytical, Lyon, France) was used for the determination of pH. Polypropylene centrifuge tubes, 50 mL (Biolab, Auckland, New Zealand), Terumo 3 mL disposable syringes (Terumo Corp., Laguna, Philippines), and Minisart 0.2 m syringe filters with cellulose acetate membranes (Sartorius, Göttingen, Germany) were used for sample preparation.

SPE was performed on a Visiprep 12 port SPE vacuum manifold (Sigma Chemical Co., St. Louis, MO) using Chromabond SB polypropylene strong-anion exchange (SAX) SPE cartridges, 6 mL 1000 mg (Macherey-Nagel, Düren, Germany).

Before use, mobile phases were filtered and degassed using a filtration apparatus with 0.45 m nylon filter membranes (Alltech, Deerfield, IL).

Reagents

Thymidine 5 -monophosphate (TMP), AMP sodium salt, CMP disodium salt, GMP disodium salt, IMP disodium salt, UMP disodium salt, and potassium bromide were purchased from Sigma Chemical Co. Potassium dihydrogen phosphate, orthophosphoric acid, potassium hydroxide, ethylenediaminetetraacetic acid (EDTA), sodium chloride, and methanol were supplied by Merck (Darmstadt, Germany). Water was purified with resistivity 18 M using an E-pure water system (Barnstead, Dubuque, IA).

A standardizing buffer (KH_2PO_4, 0.25 M, pH = 3.5) was made by diluting 34.02 g KH_2PO_4 in 900 mL water, adjusting the pH to 3.0 with orthophosphoric acid, and then making the solution to 1 L. An extraction solution (NaCl, 1 M: EDTA 5 mM) was made by dissolving 58.5 g NaCl and 1.9 g EDTA in 1 L water. A wash solution (KBr, 0.3 M) was made by dissolving 3.57 g KBr in 100 mL water. The SPE eluent (KH_2PO_4, 0.5 M, pH = 3.0) was made by dissolving 6.805 g of KH_2PO_4 in 90 mL water, adjusting the pH to 3.0 with orthophosphoric acid, and then making the solution to 100 mL.

Mobile phase A (KH_2PO_4, 0.1 M, pH = 5.6) was made by dissolving 13.6 g KH_2PO_4 in 900 mL of water, adjusting the pH to 5.6 with KOH solution (25% w/v), and then making to 1 L with water. Mobile phase B consisted of 100% methanol. As microbial growth often occurs in phosphate buffers that contain little or no organic solvent at room temperature, the mobile phase was made fresh daily.

Table 3. Chromatographic performance

Parameters[a]	CMP[b]	UMP[b]	GMP[b]	IMP[b]	TMP[b]	AMP[b]
Retention time, min	8.8 (0.22%)[c]	11.8 (0.17%)	19.8 (0.15%)	20.6 (0.10%)	25.0 (0.04%)	25.8 (0.04%)
Capacity factor	0.6 (0%)	1.2 (0.83%)	2.7 (0%)	2.8 (0%)	3.6 (0%)	3.8 (0%)
Resolution	—	6.3 (0.07)	16.9 (1.11%)	2.2 (0.45%)	15.6 (0.19%)	3.5 (0.57%)
Tailing	1.3 (3.84%)	1.2 (3.33%)	1.0 (0%)	1.0 (5.00%)	1.1 (0%)	1.1 (3.6%)
Theoretical plates	6810 (0.87%)	8527 (5.51%)	33692 (3.28%)	60448 (1.22%)	194363 (0.81%)	241749 (0.22%)
Peak area	142255 (0.51%)	200488 (1.39%)	225242 (0.23%)	122536 (0.75%)	488585 (0.11%)	308754 (0.05%)

[a] Calculations as defined by U.S. Pharmacopeia.

[b] AMP = Adenosine 5 -monophosphate; CMP = cytidine 5 -monophosphate; GMP = guanosine 5 -monophosphate; IMP = inosine 5 -monophosphate; UMP = uridine 5 -monophosphate; TMP = thymidine 5 -monophosphate.

[c] Mean (percent RSD) of six replicates of a mixed nucleotide standard.

Standard Solutions

The extinction coefficient of internal standard TMP at the UV absorbance maximum ($_{max}$) of 267 nm was determined experimentally. The concentrations of analyte nucleotide stock standards were measured using previously reported extinction coefficients (Table 1; 15).

Stock standards were prepared by accurately weighing approximately 50 mg of each nucleotide into separate 50 mL volumetric flasks and filling to volume with water. The concentration of each nucleotide stock standard was determined by diluting 1.0 mL of stock standard to 50 mL with standardizing buffer (KH_2PO_4, 0.25 M, pH = 3.5) and measuring the absorbance at the appropriate $_{max}$.

An intermediate standard solution of TMP was made by diluting 4 mL TMP stock standard into 50 mL water. A mixed intermediate standard solution of AMP, CMP, GMP, IMP, and UMP was made by diluting 2 mL of each stock standard in a single 50 mL volumetric flask and filling to volume with water.

Assay calibration standards were prepared by diluting the two intermediate standards with water to the required concentration. The calibration standards contained a constant concentration of the internal standard TMP (about 3 g/mL) and variable concentrations (about 0.5–7 g/mL) of CMP, UMP, GMP, IMP, and AMP.

Sample Preparation

Approximately 1 g of infant formula powder was weighed accurately into a 50 mL centrifuge tube and dissolved in 30 mL of extraction solution (NaCl,1 M: EDTA 5 mM); 1.0 mL of a TMP intermediate standard (about 80 g/mL) was added, and the tube was capped and vortex mixed. The sample was allowed to stand for 10 min to ensure complete hydration before dilution to a final volume of 50 mL with water.

SPE

For each sample, a single SPE cartridge was placed on an SPE vacuum manifold. The columns were conditioned by

Table 4. Method performance as linearity, detection limit, and precision

Analyte[a]	Range, g/mL	Linear regression	r^2	MDL, mg/100 g[b]	RSD_r, %[c]	$HorRat_r$[d]	RSD_{iR}, %[e]
AMP	1.25–17.49	$y = 255805x + 11862$	1.0000	0.19	2.0	0.4	4.5
CMP	0.61–8.55	$y = 287762x – 2493$	0.9999	0.08	1.0	0.3	6.0
GMP	1.11–15.55	$y = 200342x – 1807$	1.0000	0.06	2.1	0.4	5.2
IMP	1.09–15.27	$y = 198519x + 3879$	1.0000	0.10	1.4	0.3	3.8
UMP	1.12–15.68	$y = 146931x – 1839$	0.9999	0.08	2.3	0.5	8.6
TMP	1.61–22.54	$y = 150494x – 455$	1.0000	—	—	—	—

[a] AMP = Adenosine 5 -monophosphate; CMP = cytidine 5 -monophosphate; GMP = guanosine 5 -monophosphate; IMP = inosine 5 -monophosphate; UMP = uridine 5 -monophosphate; TMP = thymidine 5 -monophosphate.

[b] Determined from n replicates at or near the expected detection limit, MDL = $t_{(n-1,1-\ )}$ SD, where n = 10 and = 0.01.

[c] Relative standard deviation repeatability(RSD_r) = SD/mean 100 (n = 6).

[d] Horwitz ratio = $RSD_r/pRSD_r$, where $pRSD_r = C^{-0.15}$ at 10 ppm concentration level.

[e] RSD intermediate reproducibility = SD/mean 100 (n = 24).

Table 5. Recovery (%) of nucleotides in spiked samples[a]

	AMP	CMP	GMP	IMP	UMP	TMP
Bovine milk-based infant formula	100 (2.10%)[b]	99 (1.82%)	98 (2.14%)	98 (1.63%)	94 (3.30%)	97 (1.34%)
Soy-based infant formula	98 (3.57%)	98 (3.57%)	99 (2.42%)	97 (4.74%)	97 (5.88%)	101 (5.94%)
Hypoallergenic infant formula	100 (3.70%)	99 (1.92%)	101 (1.29%)	98 (3.78%)	92 (5.00%)	100 (3.70%)

[a] AMP = adenosine 5 -monophosphate; CMP = cytidine 5 -monophosphate; GMP = guanosine 5 -monophosphate; IMP = inosine 5 -monophosphate; UMP = uridine 5 -monophosphate; TMP = thymidine 5 -monophosphate.

[b] Mean recovery (percent RSD) of six replicates over three concentration levels.

elution with 4 mL methanol, followed by elution with 2 5 mL water. The cartridge was loaded with 4 mL sample solution at a flow rate of <2 mL/min. The cartridge was washed (KBr, 0.3 M, 4 mL) to remove interferences. The nucleotides were then eluted with SPE eluent solution (KH_2PO_4, 0.5 M, pH 3.0, 4 mL) into a test tube. An aliquot of the eluent was filtered through a 0.2 m syringe filter into an autosampler vial.

Chromatography

Chromatographic separation was achieved using a modification of the procedure described previously (15). Gradients were formed by low pressure mixing of two mobile phases, A and B, with separation of nucleotides achieved using the procedure shown in Table 2.

The photodiode array detector acquired spectral data between 210 and 300 nm. Integration of peak area was achieved at specific wavelengths: 250 nm for IMP; 260 nm for AMP, GMP, and TMP; and 270 nm for CMP and UMP. A linear regression plot of the ratios of peak area against concentration for each nucleotide relative to TMP was generated, and the nucleotide contents in unknown samples were interpolated from this calibration curve.

$$Nucleotide\ \ mg/100g \quad \frac{A_{NT}}{A_{IS}} \quad \frac{1}{L} \quad \frac{C_{IS}}{W} \quad 100$$

where A_{NT} = nucleotide peak area in sample; A_{IS} = TMP peak area in sample; L = linear regression slope of calibration curve; C_{IS} = amount in milligrams of internal standard added; W = weight of sample in grams; and 100 = mass conversion of result to per 100 g.

Method Validation

Seven mixed standard nucleotide solutions covering the expected working range were analyzed in duplicate, and linearity of dose response was evaluated by least-squares regression analysis. A value of 0.997 for the correlation coefficient (r^2) was deemed to be the minimum suitable for acceptable analysis. Plots of standard residuals were assessed as a further test for linearity.

Repeatability was determined by analyzing replicates ($n = 6$) of a nucleotide-supplemented bovine milk-based infant formula. Intermediate precision was determined from replicate analyses ($n = 6$) of the same sample tested on 4 different days by two different analysts.

Method detection limits (MDLs) were determined in accordance with U.S. Environmental Protection Agency procedures (20). The MDL procedure sets the detection limit at the 99% confidence level, minimizing false positive errors.

The robustness of the method was assessed by conducting a Plackett-Burman trial (21), with evaluation of seven factors deemed to potentially affect the final results, at levels likely to occur during normal use of the method. Statistical analysis to identify critical effects consisted of a *t*-test, whereby a calculated *t*-value based on the effect, E_X, and an estimation of the standard error, $(SE)_E$, were compared with a critical value (significance level = 0.05). Graphical interpretation was assessed by construction of a half-normal plot, whereby nonsignificant effects tended to fall on a straight line through zero, whereas significant effects deviated from the straight line. The standard error estimate was used to calculate the margin of error (ME), which was plotted on the half-normal plot to identify the limit above which effects were deemed to be significant (22, 23).

In the absence of a currently available infant formula standard reference material (SRM) with certified levels of nucleotides, method accuracy was determined based on recovery and bias. Recovery was evaluated at three concentration levels for three different sample matrixes: bovine milk-based infant formula; soy-based infant formula; and a hypoallergenic infant formula containing hydrolyzed milk protein. Method bias was assessed by testing replicate samples ($n = 12$) of a nucleotide-supplemented formula by the method described herein and a method published previously (15).

Results and Discussion

Method Optimization

Method optimization consisted of adapting the sample preparation and chromatographic conditions reported previously (15) to accommodate direct SPE for the removal of non-nucleotide interferences, thereby simplifying both the overall analytical scheme and the chromatographic separation.

Both acid precipitation and ultrafiltration techniques to remove protein prior to SPE were initially evaluated. Acid precipitation is a rapid and simple means of removing caseins;

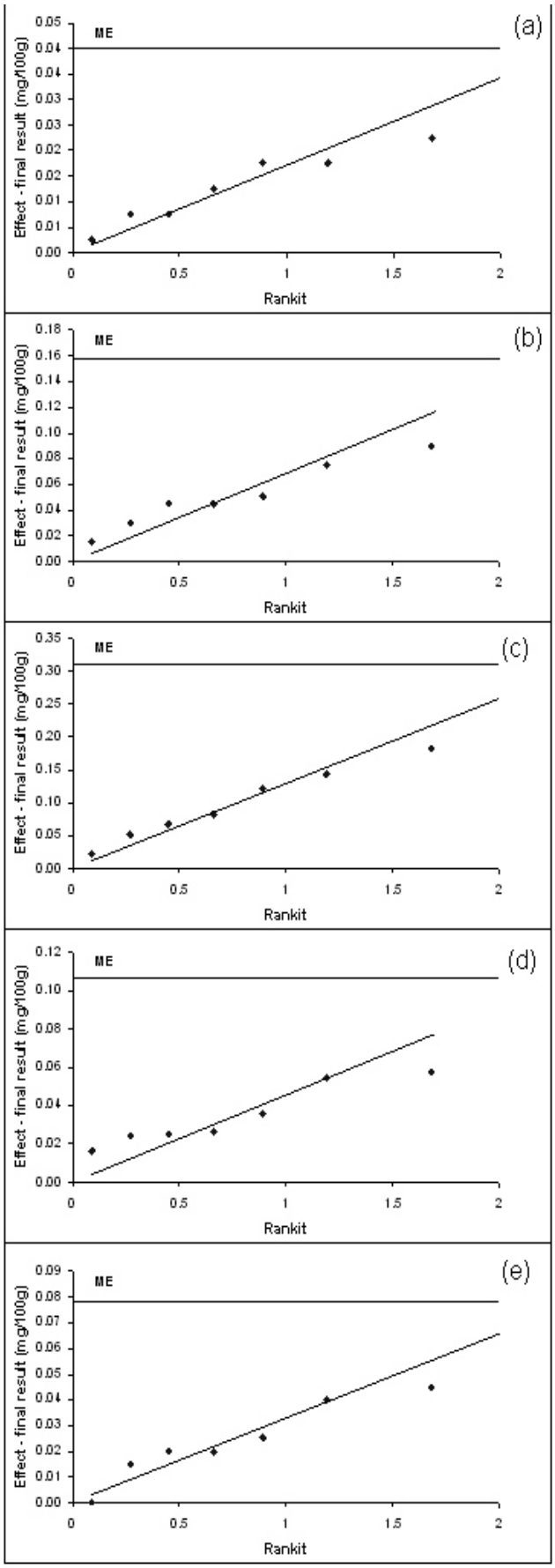

Figure 1. Half-normal plot of results for robustness trial (a) inosine 5 -monophosphate, (b) adenosine 5 -monophosphate, (c) cytidine 5 -monophosphate, (d) uridine 5 -monophosphate, and (e) guanosine 5 -monophosphate. ME = margin of error.

however, the low pH of the sample extract may negatively impact SPE retention unless the extract is first neutralized. Ultrafiltration removes all proteinaceous material above the molecular weight cut-off, and the sample remains at physiological pH, thereby removing a potential neutralization step prior to SPE. However, ultrafiltration was found to be an unsatisfactory means of protein removal as it proved to be time-consuming, difficult to obtain sufficient permeate, and variable in the recovery of individual nucleotides.

Based on these trials, the assumption that it was necessary to remove protein prior to SPE was considered. The dissolution of a powder sample in the high salt solution was found to be efficacious in producing a uniform sample solution that, when applied directly to the SPE cartridge, did not compromise the recovery of nucleotides. Residual milk protein content in the eluent was equivalent to that of an acid-precipitated sample and it is probable that some caseins precipitate in the SPE cartridge with the addition of the low pH buffer.

The SAX cartridges contain quaternary amine anion-exchange sites, which strongly attract the anionic phosphate moiety of nucleotides. In order to remove the majority of interfering components in the sample, different aqueous wash solutions, containing a variety of anions at a number of concentrations, were evaluated. Bromide ions were found to be most effective in removing potentially interfering components, such as nucleosides, orotic acid, and uric acid, while still retaining nucleotides on the cartridge.

In order to elute the nucleotides from the SAX cartridge, two options were available. One option was to add sufficient acid to lower the pH to the pKa of the nucleotide phosphate (approximate pH = 1), thereby neutralizing the negative charge and eluting the nucleotides for collection. However, in order to protect the analytical column, neutralization of the extract would be required prior to HPLC analysis. Alternatively, the addition of anions that have a high affinity for the quaternary amine and added at high ionic strength, could be utilized to elute the nucleotides. This was achieved by the addition of 0.5 M phosphate in the eluent, which readily displaces nucleotides bound on the SAX cartridge.

In multi-step analytical procedures, such as those involving SPE cleanup, there is potential for analyte loss and, hence, the use of an internal standard is considered to be mandatory to obtain consistently accurate and precise results. With an internal standard, it is possible to correct for losses associated with SPE cleanup, either by analyte breakthrough or by incomplete desorption. The selection of TMP as an internal standard was supported by a number of factors: structural similarity to analyte nucleotides; absence of detectable quantities in infant formulas; retention under desired chromatographic separation; and commercial availability.

Method Performance

Chromatographic performance was assessed by replicate analyses ($n = 6$) of a mixed nucleotide standard (Table 3). Performance within recommended guidelines was achieved, with the exception of the capacity factors for CMP and UMP

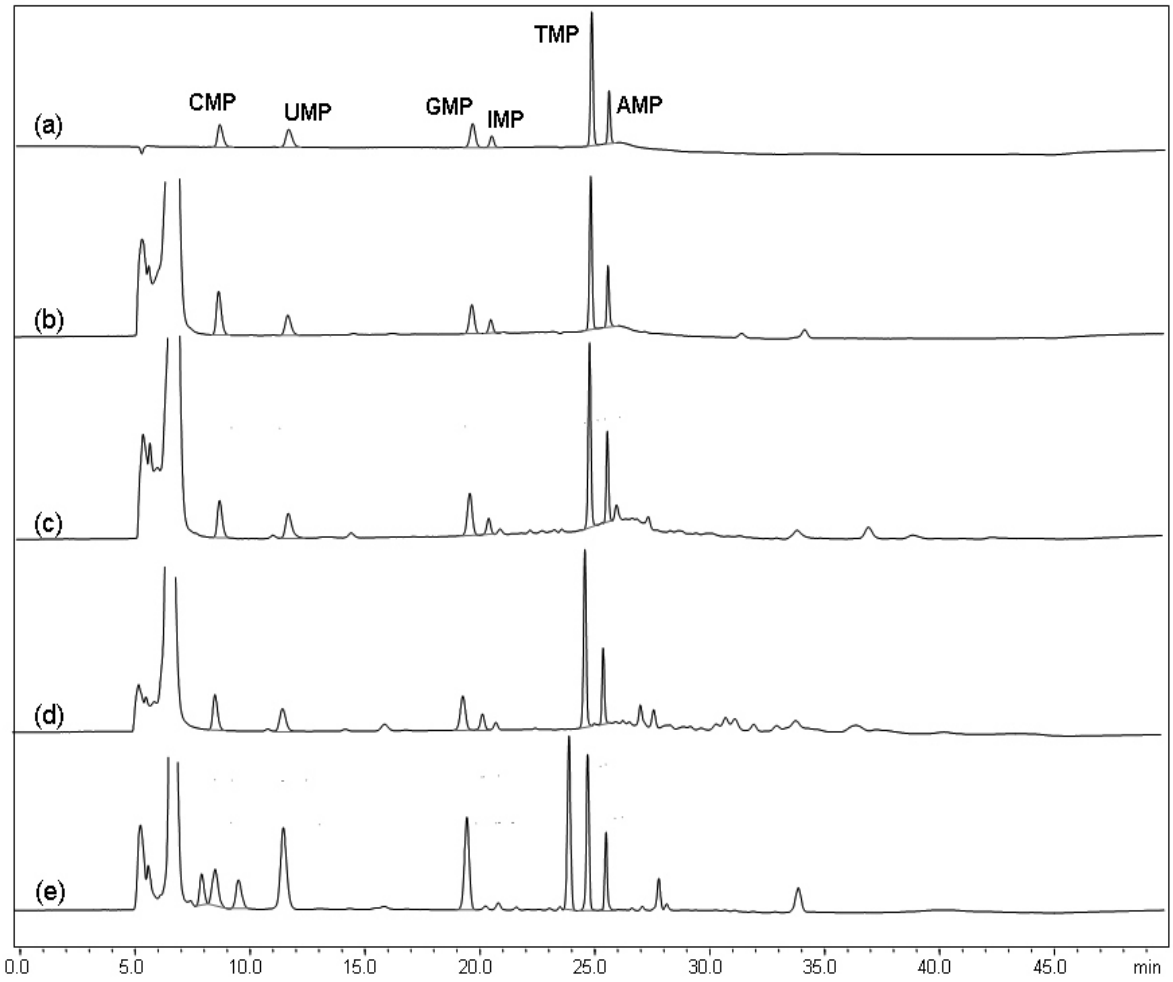

Figure 2. Chromatography of (a) standard mixture of nucleotides, (b) bovine milk-based infant formula, (c) soy-based infant formula, (d) hydrolyzed milk protein-based infant formula, and (e) caprine milk-based infant formula. AMP = Adenosine 5 -monophosphate; CMP = cytidine 5 -monophosphate; GMP = guanosine 5 -monophosphate; IMP = inosine 5 -monophosphate; UMP = uridine 5 -monophosphate; TMP = thymidine 5 -monophosphate. HPLC conditions: column, Gemini C_{18}, 5 m, 4.6 250 mm (Phenomenex); mobile phase A, KH_2PO_4 (0.1 M, pH = 5.6); mobile phase B, methanol (100%); gradient elution, flow rate 0.5 mL/min, 0–5 min (100% A, 0% B), 14 min (90% A, 10% B), 15–35 min (80% A, 20% B), 36–50 min (100% A, 0% B). UV detection: 260 nm.

(guideline >2.0); however, this was deemed to be acceptable because of uncompromised peak integrity of these two compounds in all samples analyzed.

The results from validation studies are summarized in Tables 4 and 5. Linearity of dose response was confirmed by least-squares regression analysis, with acceptable values obtained for the correlation coefficient. Plots of standard residuals showed no structure and only a small amount of random noise, further demonstrating linearity.

The precision was acceptable and similar to what could be expected, as illustrated by a repeatability Horwitz ratio between 0.3 and 0.5, slightly better than the acceptable range of 0.5–2.0, and an intermediate precision of 3.8–8.6% (24).

As the calculated MDL is dependent on the concentration of the replicate samples, the level of analyte in the sample should not exceed 10 times the calculated MDL; nor should it be less than the MDL. The concentrations used to generate the MDL (0.52–1.68 mg/100 g) were appropriate to correctly establish the MDL.

The seven factors assessed in the robustness trial were: concentration of salt solution, sample wait time, load volume, wash solution, wash volume, eluent solution, and eluent volume. The two factor levels were symmetric around the nominal values from the described analytical procedure, with the interval representing experimental error of the equipment used (pipets, volumetric flasks, balances) and an estimated error on the part of the analyst. The method was found to be robust for these factors at the levels studied (Figure 1).

Acceptable recovery is a function of the concentration and the purpose of the analysis. The recoveries measured were well within the limits of 80–115% at the 10 g/g level suggested by AOAC (24).

An estimation of bias between the method described herein and a method published previously (15) showed no bias, with *P* values (95%) calculated to be 0.079, 0.529, 0.676, 0.341, and 0.069 for AMP, CMP GMP, IMP, and UMP, respectively.

Table 6. Nucleotides measured in analysis of commercially available pediatric and nutritional formulas

Sample type[a]	Nucleotide supplemented	Results, mg/100 g				
		CMP[b]	UMP[b]	GMP[b]	IMP[b]	AMP[b]
Bovine-milk-based IF	Yes	11.6 (116%)[c]	3.7 (95%)	1.7 (106%)	2.0 (125%)	4.5 (145%)
Bovine-milk-based FO	Yes	6.0 (107%)	2.4 (87%)	0.9 (89%)	1.0 (91%)	2.1 (103%)
Bovine-milk-based FO	No	1.0	0	0	0.1	0
Bovine-milk-based FO	Yes	8.5 (172%)	2.4 (89%)	1.0 (100%)	1.0 (92%)	2.3 (115%)
Bovine-milk-based AN	Yes	17.4 (120%)	4.7 (75%)	8.0 (107%)	0	7.2 (130%)
Soy-based IF	No	0.1	0.3	0.3	0	0.5
Caprine-milk-based IF	No	4.0	8.2	6.4	0.3	2.3
Bovine-milk-based WMP	No	4.0	0	0	0	0
Hypoallergenic IF	No[d]	2.6 (101%)	2.6 (92%)	2.7 (85%)	2.6 (96%)	3.1 (100%)
Hypoallergenic IF	No	0	0	0	0	0

[a] IF = Infant formula; FO = follow-on formula; AN = adult nutritional product; WMP = whole milk powder.

[b] AMP = Adenosine 5 -monophosphate; CMP = cytidine 5 -monophosphate; GMP = guanosine 5 -monophosphate; IMP = inosine 5 -monophosphate; UMP = uridine 5 -monophosphate.

[c] Recovery as percentage of label claim in parentheses.

[d] Hypoallergenic sample spiked with nucleotide mixed standard prior to analysis.

Method Application

The method was applied to a number of commercially available pediatric and nutritional powders. Products included for testing were infant formulas, follow-on formulas, and an adult nutritional product. These products included a range of different sources: bovine milk, hydrolyzed milk protein, caprine milk, and soy protein (Figure 2). The concentrations of 5 -mononucleotides are given in Table 6. The recoveries determined against label claim, where available, further indicate the reliability of the method. In the analysis of caprine milk-based infant formula, the presence of significant levels of endogenous nucleotide diphosphates was confirmed.

Conclusions

The optimization and validation of a simple, rapid method for the routine analysis of nucleotides in nucleotide-supplemented infant formulas has been described. The simplicity of analysis is facilitated by the use of SPE without the need for prior protein removal. The use of an internal standard gives additional confidence in the accuracy of the result obtained. The applicability of the method has been demonstrated for the analysis of bovine milk-based, caprine milk-based, soy-based, and hydrolyzed milk protein-based infant formulas

Acknowledgments

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

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International Dairy Journal 21 (2011) 34–41

Contents lists available at ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

Determination of total potentially available nucleosides in bovine milk

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ARTICLE INFO

Article history:
Received 22 April 2010
Received in revised form
12 July 2010
Accepted 19 July 2010

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.

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 stabilising 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).

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doi:10.1016/j.idairyj.2010.07.004

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 × 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 μg, 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

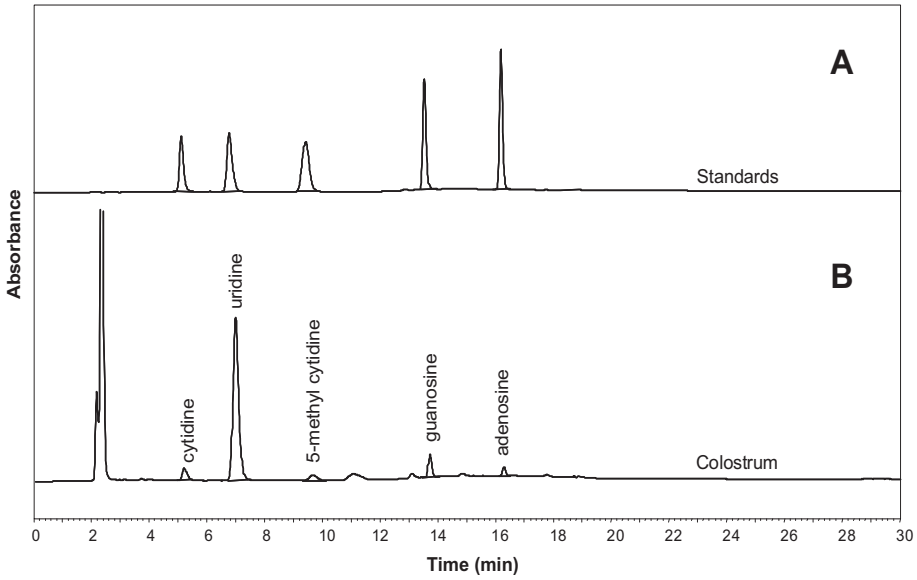

Fig. 1. Chromatograms of a mixed nucleoside standard and colostrum sample. Conditions: mobile phase A: 0.05 M sodium acetate, pH = 4.8; mobile phase B: 100% methanol; gradient elution: flow rate 0.7 mL min^{-1} throughout, 0–3 min (95% A, 5% B v/v), 7–22 min (75% A, 25% B v/v), 23–30 min (95% A, 5% B v/v). UV detection 260 nm.

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 (Fig. 1A). The specificity of the phenylboronate sample

clean-up provides analytical chromatography relatively free of interferences (Fig. 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 Figs. 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 tri-phosphorylated nucleotides), which the TPAN analytical method

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

Day[b]	Form[c]	Cytidine	Uridine	Guanosine	Adenosine	Total
0	Nucleoside	5.4 ± 0.1	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[b]	Form[c]	Cytidine	Uridine	Guanosine	Adenosine	Total
0	Nucleoside	2.6 ± 0.2	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.

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

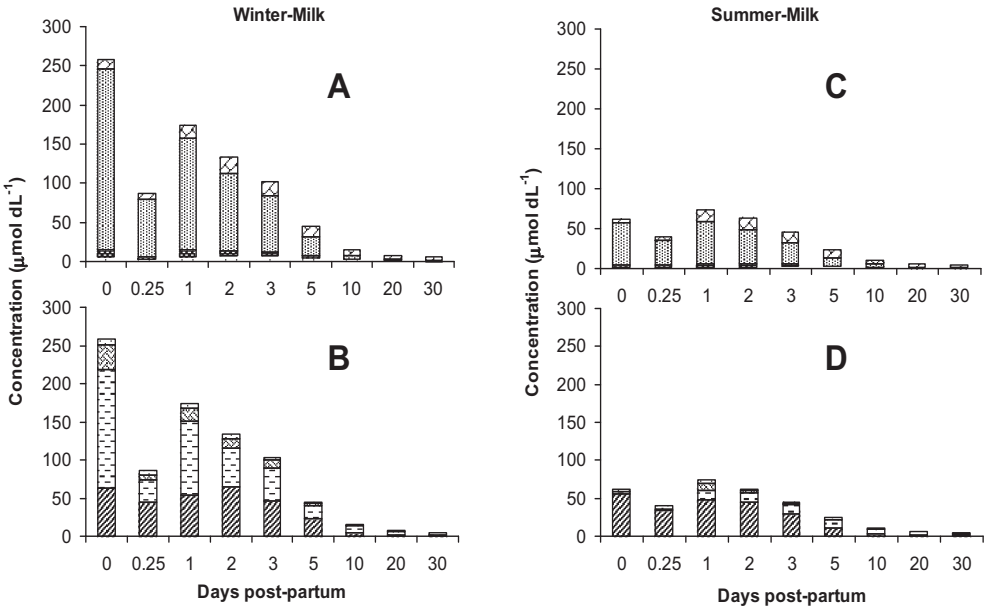

Fig. 2. Total potentially available nucleosides in pooled bovine milk samples from winter-milk (A, B) and summer-milk (C, D) herds over the first month of lactation. A, C: , cytidine; , uridine; , guanosine; , adenosine. B, D: , polymeric nucleotides; , nucleotide adducts; , monomeric nucleotides; , nucleosides.

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

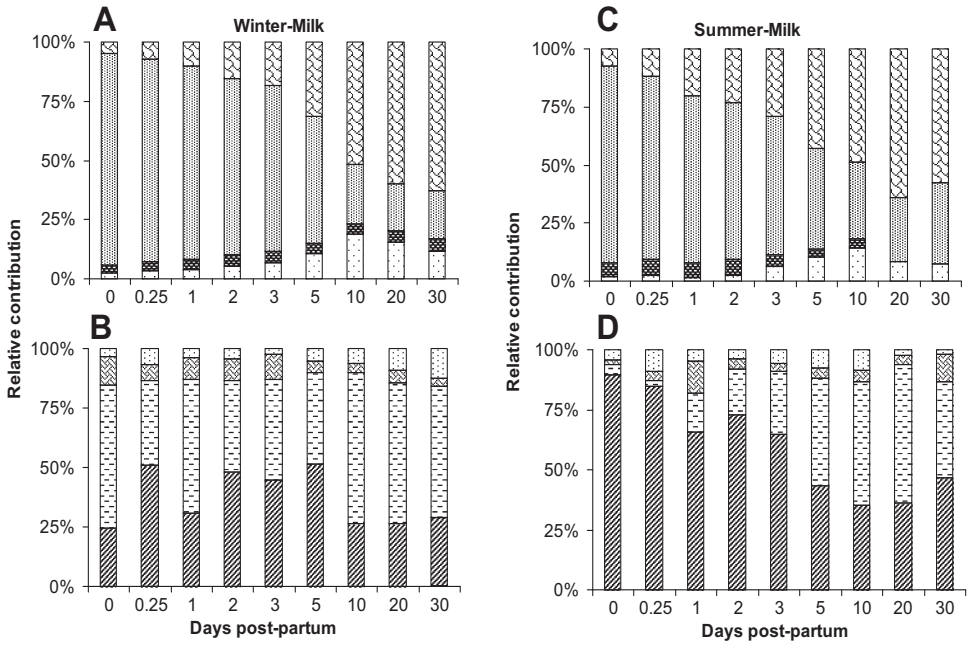

Fig. 3. Percentage contribution to total potentially available nucleosides in pooled bovine milk samples from winter-milk (A, B) and summer-milk (C, D) herds over the first month of lactation. A, C: , cytidine; , uridine; , guanosine; , adenosine. B, D: , polymeric nucleotides; , nucleotide adducts; , monomeric nucleotides; , nucleosides.

Table 3
Significance levels for rates of decrease of nucleosides and nucleotides (NT) 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 $p \leq 0.05$.
[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.

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.

Acknowledgements

The authors thank Rich Syme and Andrew McGiven for supply of the bovine milk samples. We also wish to thank Roger Kissling (Fonterra) for his statistical analysis of results. Advice on the application of the analytical protocol given by Bruce Molitor (Abbott Laboratories, Columbus, OH, USA) is greatly appreciated. The financial assistance of Fonterra Co-operative Group Limited and of the Tertiary Education Commission in providing an Enterprise Scholarship is gratefully acknowledged.

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International Dairy Journal 24 (2012) 40–43

Contents lists available at SciVerse ScienceDirect

International Dairy Journal

journal homepage: www.elsevier.com/locate/idairyj

Determination of total potentially available nucleosides in bovine, caprine, and ovine milk

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ARTICLE INFO

Article history:
Received 25 August 2011
Received in revised form
14 November 2011
Accepted 22 November 2011

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.

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 5′-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, b).

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

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doi:10.1016/j.idairyj.2011.11.011

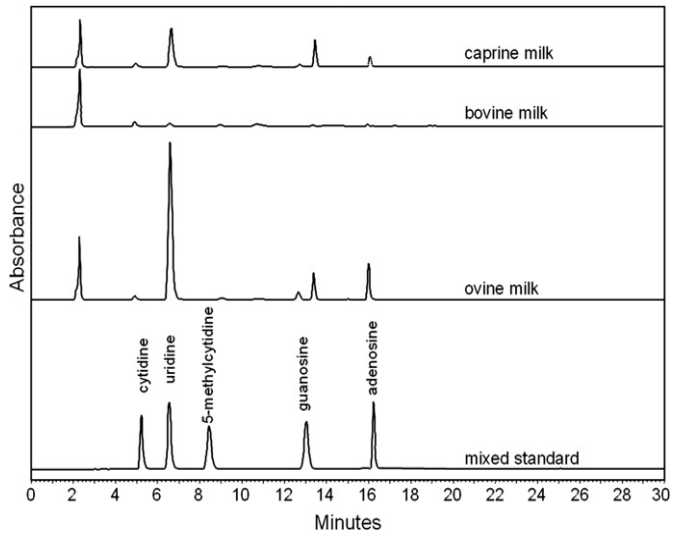

Fig. 1. Chromatograms of a mixed nucleoside standard, and of bovine, caprine, and ovine milk. Conditions: mobile phase A: 0.05 M sodium acetate, pH 4.8; mobile phase B: 100% methanol; gradient elution: flow rate 0.7 mL min^{-1} throughout, 0–3 min (95% A, 5% B, v/v), 7–22 min (75% A, 25% B, v/v), 23–30 min (95% A, 5% B, v/v). UV detection 260 nm.

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 (Fig. 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 Fig. 2. A comparison of the concentration and the relative contribution of each nucleoside, categorised by nucleobase, is shown in Fig. 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).

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 ± 0.7	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.

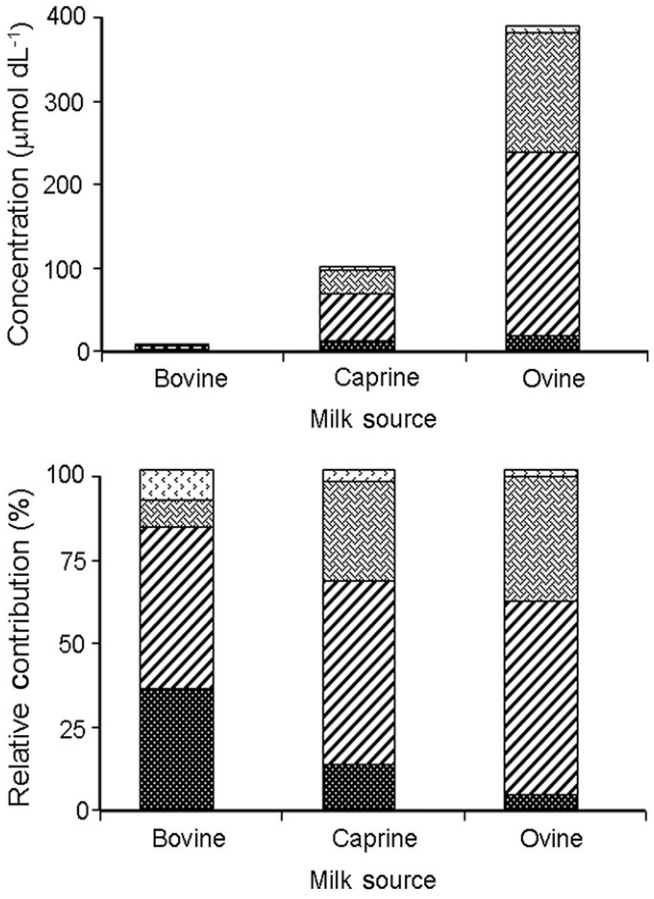

Fig. 2. A comparison of the concentration and the relative contribution of each nucleoside (by phosphorylated form) in bovine, caprine, and ovine milk samples (mean of duplicate analyses): ▩, nucleosides; ▨, nucleotide adducts; ▨, monomeric nucleotides; ⬚, polymeric nucleotides.

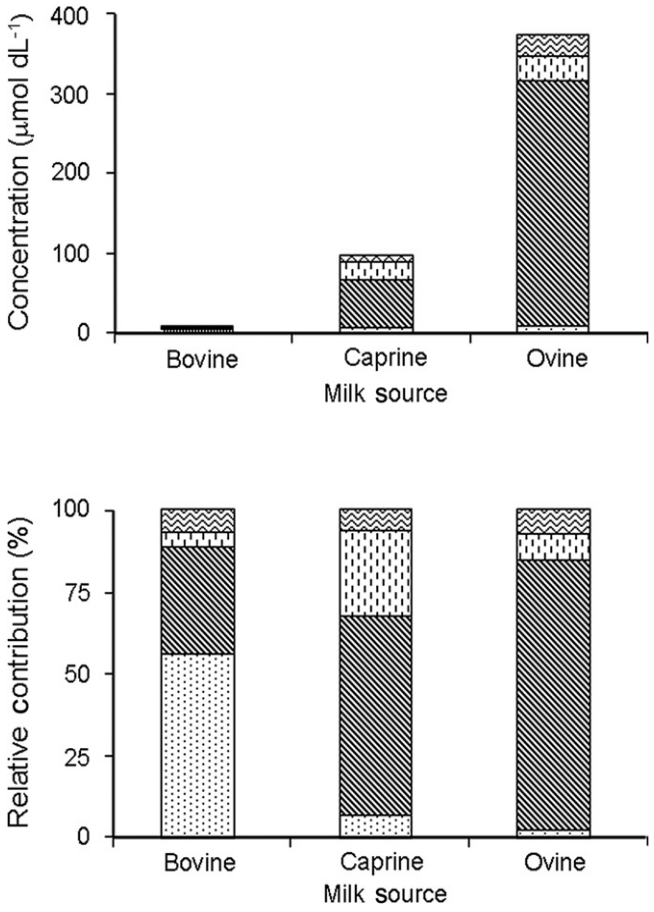

Fig. 3. A comparison of the concentration and the relative contribution of each nucleoside (by nucleobase) in bovine, caprine, and ovine milk samples (mean of duplicate analyses): ▧, uridine; ⬚, guanosine; ▨, adenosine; ⬚, cytidine.

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.

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INFANT FORMULA AND ADULT NUTRITIONALS

Analysis of 5′-Mononucleotides in Infant Formula and Adult/Pediatric Nutritional Formula by Liquid Chromatography: First Action 2011.20

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A method for the routine determination of 5′-mononucleotides (uridine 5′-monophosphate, inosine 5′-monophosphate, adenosine 5′-monophosphate, guanosine 5′-monophosphate, and cytidine 5′-monophosphate) in infant formula and adult nutritionals is described. After sample dissolution and addition of internal standard, potential interferences were removed by anion-exchange SPE followed by HPLC-UV analysis. Single-laboratory validation performance parameters include recovery (92–101%) and repeatability (1.0–2.3% RSD). The method was approved for Official First Action status by an AOAC expert review panel.

Nucleotides are compounds of critical importance to cellular function, and although not essential dietary nutrients, it has been demonstrated that supplementation of pediatric formulas with nucleotides is of benefit in neonatal nutrition. The described method was developed to provide an accurate, rapid, and robust technique for the routine compliance testing of uridine 5′-monophosphate (UMP), inosine 5′-monophosphate (IMP), adenosine 5′-monophosphate (AMP), guanosine 5′-monophosphate (GMP), and cytidine 5′-monophosphate (CMP) in infant formula and adult/pediatric nutritional formula, and was recently reported (1).

In September 2011, the method was reviewed by an AOAC expert review panel and, based on the published single-laboratory validation (SLV) data as compared with the standard method performance requirements (AOAC SMPR **2011.008**; 2) set by the Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN), it was approved for Official First Action status as AOAC *Official Method*℠ **2011.20**.

Submitted for publication February 8, 2012.

The method was approved by the Expert Review Panel on Infant Formula and Adult Nutritionals as First Action. *See* "Standards News," (2011) *Inside Laboratory Management*, September/October issue.

The AOAC Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) invites method users to provide feedback on the First Action methods. Feedback from method users will help verify that the methods are fit for purpose and are critical to gaining global recognition and acceptance of the methods. Comments can be sent directly to the corresponding author.

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DOI: 10.5740/jaoacint.CS2011_20

AOAC Official Method 2011.20
5′-Mononucleotides in Infant Formula and Adult/Pediatric Nutritional Formula

Liquid Chromatography
First Action 2011

(Applicable to the determination of 5′-mononucleotides in infant formula and adult/pediatric nutritional formula.)

Caution: Refer to the material safety data sheets for all chemicals prior to use. Use all appropriate personal protective equipment, and follow good laboratory practices.

A. Principle

Sample is dissolved in high-salt solution to inhibit protein and fat interactions. The 5′-mononucleotides—uridine 5′-monophosphate (UMP), inosine 5′-monophosphate (IMP), adenosine 5′-monophosphate (AMP), guanosine 5′-monophosphate (GMP), and cytidine 5′-phosphate (CMP)—are separated from the sample matrix by strong-anion exchange solid-phase extraction (SPE), followed by chromatographic analysis using a C_{18} stationary phase with gradient elution, UV detection, and quantitation by an internal standard technique using thymidine 5′-monophosphate (TMP).

B. Apparatus

(**a**) *HPLC system*.—Equipped with pump, sample injector unit with a 50 µL injection loop, degasser unit, column oven, and photodiode array detector.

(**b**) *C_{18} column*.—Gemini C_{18}, 5 µm, 4.6×250 mm (Phenomenex, Torrance, CA).

(**c**) *Spectrophotometer*.—Capable of digital readout to 3 decimal places.

(**d**) *pH meter*.

(**e**) *Polypropylene centrifuge tubes*.—50 mL.

(**f**) *Disposable syringes*.—3 mL.

Table 2011.20A. UV absorbance maxima and extinction coefficients for 5′-mononucleotides

Nucleotide	λ_{max}, nm	$E^{1\%}_{1cm}$
Adenosine 5′-monophosphate	257	430.4
Cytidine 5′-monophosphate	280	398.0
Guanosine 5′-monophosphate	254	393.3
Inosine 5′-monophosphate	249	357.3
Uridine 5′-monophosphate	262	313.5
Thymidine 5′-monophosphate	267	288.5

(**g**) *Syringe filters*.—0.2 µm with cellulose acetate membranes.

(**h**) *SPE vacuum manifold*.

(**i**) *Chromabond SB polypropylene strong-anion exchange SPE cartridges*.—6 mL × 1000 mg (Macherey-Nagel, Düren, Germany).

(**j**) *Filter membranes*.—0.45 µm nylon.

C. Reagents

(**a**) *Standards*.—Should be ≥99% pure (Sigma or equivalent). Nucleotide sodium salts or sodium salt hydrates may be substituted if free acid forms are not readily available.

(*1*) *TMP*.—CAS No. 365-07-1.

(*2*) *AMP*.—CAS No. 61-19-8.

(*3*) *CMP*.—CAS No. 63-37-6.

(*4*) *GMP*.—CAS No. 85-32-5.

(*5*) *IMP*.—CAS No. 131-99-7.

(*6*) *UMP*.—CAS No. 58-97-9.

(**b**) *Potassium bromide*.

(**c**) *Potassium dihydrogen phosphate*.

(**d**) *Orthophosphoric acid*.

(**e**) *Potassium hydroxide*.

(**f**) *Ethylenediaminetetraacetic acid*.

(**g**) *Sodium chloride*.

(**h**) *Methanol*.

(**i**) *Water*.—Purified with resistivity ≥18 MΩ.

D. Reagent Preparation

(**a**) *Standardizing buffer (KH_2PO_4, 0.25 M, pH 3.5)*.—Dissolve 34.02 g KH_2PO_4 in 900 mL water and adjust pH to 3.5 with orthophosphoric acid. Dilute to 1 L.

(**b**) *Extraction solution (NaCl, 1 M:EDTA, 5 mM)*.—Dissolve 58.5 g NaCl and 1.46 g EDTA. Dilute in 1 L water.

Table 2011.20B. Nominal concentration of calibration solutions

Calibration solution	AMP, CMP, GMP, IMP, UMP, µg/mL	TMP, µg/mL
1	0.4	3.2
2	0.8	3.2
3	3.2	3.2
4	8	3.2

(**c**) *Wash solution (KBr, 0.3 M)*.—Dissolve 3.57 g KBr in 100 mL water.

(**d**) *Eluent (KH_2PO_4, 0.5 M, pH 3.0)*.—Dissolve 6.805 g KH_2PO_4 in 90 mL water and adjust pH to 3.0 with orthophosphoric acid. Dilute to 100 mL.

(**e**) *Mobile phase A (KH_2PO_4, 10 mM, pH 5.6)*.—Dissolve 1.36 g KH_2PO_4 in 900 mL water and adjust pH to 5.6 with KOH solution (10%, w/v). Dilute to 1 L with water. Make daily as microbial growth often occurs at room temperature in phosphate buffers that contain little or no organic solvent.

(**f**) *Mobile phase B (100% methanol)*.

E. Standard Preparation

See Table **2011.20A** for the UV absorbance maxima and extinction coefficients for 5′-mononucleotides.

(**a**) *Stock standards (~1 mg/mL)*.—(*1*) Accurately weigh approximately 50 mg each nucleotide into separate 50 mL volumetric flasks. (*2*) Add 40 mL water, mix until dissolved, and fill to volume with water.

(**b**) *Purity standards*.—Pipet 1.0 mL each stock standard into separate 50 mL volumetric flasks, make to volume with standardizing buffer (KH_2PO_4, 0.25 M, pH 3.5), and measure absorbance at the appropriate λ_{max} to determine the concentration of each nucleotide stock standard.

(**c**) *Internal standard solution (~80 µg/mL)*.—Dilute 4 mL TMP stock standard into 50 mL water.

(**d**) *Working standard solution (~40 µg/mL)*.—Pipet 2 mL each stock standard (AMP, CMP, GMP, IMP, and UMP) into a single 50 mL volumetric flask and make to volume with water.

(**e**) *Calibration standard solutions*.—*See* Table **2011.20B** for nucleotide concentrations of the calibration standard solutions.

(*1*) *Calibration solution 1*.—Pipet 0.25 mL working standard solution and 1 mL internal standard solution into a 25 mL volumetric flask and make to volume with water.

(*2*) *Calibration solution 2*.—Pipet 0.5 mL working standard solution and 1 mL internal standard solution into a 25 mL volumetric flask and make to volume with water.

(*3*) *Calibration solution 3*.—Pipet 2 mL working standard solution and 1 mL internal standard solution into a 25 mL volumetric flask and make to volume with water.

(*4*) *Calibration solution 4*.—Pipet 5 mL working standard solution and 1 mL internal standard solution into a 25 mL volumetric flask and make to volume with water.

F. Sample Preparation

(**a**) Accurately weigh approximately 1 g powder, or 10 mL liquid milk or ready-to-feed infant/nutritional formula, into a 50 mL centrifuge tube.

(**b**) Dissolve in 30 mL extraction solution (NaCl, 1 M:EDTA 5 mM).

(**c**) Add 1.0 mL TMP intermediate standard (~80 µg/mL).

(**d**) Cap the tube and vortex mix.

(**e**) Allow sample to stand for 10 min to ensure complete hydration.

(**f**) Dilute to a final volume of 50 mL with water.

(**g**) Cap the tube and vortex mix.

G. Extraction

Throughout the extraction procedure, do not let the cartridge run dry but drain to the top of the cartridge bed only. When draining the cartridge the flow rate should be <2 mL/min.

Table 2011.20C. Gradient procedure for chromatographic separation

Time, min	Flow rate, mL/min	Phase A, %	Phase B, %
0	0.6	100	0
25	0.6	80	20
26	0.6	100	0
40	0.6	100	0

(**a**) For each sample, place a single SPE cartridge on a vacuum manifold.

(**b**) Condition the columns by elution with 4 mL methanol followed by elution with 2 × 5 mL water.

(**c**) Load the cartridge with 4 mL sample solution.

(**d**) Wash the cartridge with KBr (0.3 M, 4 mL) to remove interferences.

(**e**) Elute the nucleotides with SPE eluent solution (KH_2PO_4, 0.5 M, pH 3.0, 4 mL) into a test tube.

(**f**) Filter an aliquot (~2 mL) of the eluent through a 0.2 µm syringe filter into an autosampler vial.

H. Chromatography

(**a**) Form gradients by low pressure mixing of the two mobile phases, A and B, with separation of nucleotides achieved using the procedure shown in Table **2011.20C**.

(**b**) Acquire spectral data between 210 and 300 nm by the photodiode array detector with chromatograms monitored at the specified wavelengths below for quantitation.

(*1*) IMP wavelength at 250 nm.

(*2*) AMP, GMP, and TMP wavelengths at 260 nm.

(*3*) CMP and UMP wavelengths at 270 nm.

(**c**) Set column oven to 40°C.

I. Calculations

(**a**) Percentage purity of each nucleotide (as free acid) in purity standard:

$$\text{Purity, \%} = \frac{Abs_{\lambda max}}{E^{1\%}_{1cm}} \times \frac{50}{wtSS} \times \frac{50}{1}$$

where $Abs_{\lambda max}$ = UV absorbance at maximum wavelength; $E^{1\%}_{1cm}$ = extinction coefficient for nucleotide; *wtSS* = weight of nucleotide in stock standard (g); 50 = total volume of stock standard (mL); 50 = total volume of purity standard (mL); 1 = volume of stock standard added to purity standard (mL).

(**b**) Concentration of nucleotide in stock standards:

$$\text{Stock standard, µg/mL} = \frac{wtSS}{50} \times \frac{P\%}{100} \times 10^6$$

where *wtSS* = weight of nucleotide in stock standard (g); 50 = total volume of stock standard (mL); 10^6 = concentration conversion (g/mL to µg/mL); *P%* = purity (%); 100 = mass conversion from % to decimal.

(**c**) Concentration of TMP in internal standard:

$$\text{Internal standard, µg/mL} = SS \times \frac{4}{50}$$

where *SS* = concentration of nucleotide in stock standard (µg/mL); 4 = volume of stock standard in internal standard (mL); 50 = total volume of internal standard (mL).

(**d**) Concentration of nucleotide in calibration standards:

$$\text{Calibration standard, µg/mL} = SS \times \frac{2}{50} \times \frac{VS}{25}$$

where *SS* = concentration of nucleotide in stock standard (µg/mL); 2 = volume of stock standard in working standard (mL); 50 = total volume of working standard (mL); *VS* = volume of working standard in calibration standard (mL); 25 = total volume of calibration standard (mL).

(**e**) Concentration of TMP in calibration standards:

$$\text{Calibration standard, µg/mL} = IS \times \frac{1}{25}$$

where *IS* = concentration of TMP in internal standard (µg/mL); 1 = volume of working standard in calibration standard (mL); 25 = total volume of calibration standard (mL).

(**f**) Determine the linear regression curve for the ratio of peaks areas (nucleotide/TMP; *y*-axis) vs the ratio of concentrations (nucleotide/TMP; *x*-axis) for calibration standards and calculate the slope with the *y*-intercept forced through 0.

(**g**) Interpolate the nucleotide contents in unknown samples from this calibration curve.

For powders:

$$\text{Nucleotide, mg/hg} = \frac{A_{NT}}{A_{IS}} \times \frac{1}{L} \times \frac{(C_{IS} \times V_{IS})}{W_S} \times \frac{100}{1000}$$

For ready-to feed liquids:

$$\text{Nucleotide, mg/dL} = \frac{A_{NT}}{A_{IS}} \times \frac{1}{L} \times \frac{(C_{IS} \times V_{IS})}{V_S} \times \frac{100}{1000}$$

where A_{NT} = nucleotide peak area in sample; A_{IS} = TMP peak area in sample; L = linear regression slope of calibration curve; C_{IS} = concentration of internal standard added to sample (µg/mL); V_{IS} = volume of internal standard added to sample (mL); W_S = weight of sample (g); 100 = mass conversion of result (g to hg); 1000 = mass conversion of result (µg to mg); V_S = volume of sample (mL); 100 = volume conversion of result (mL to dL).

J. Data Handling

Report results in mg/hg to 1 decimal place.

Reference: *J. AOAC Int.* **95**, 599(2012)

Results and Discussion

An SLV of the method previously published (1) indicated that this method is suitable for the routine determination of the 5′-mononucleotide content in milk and milk-based pediatric and adult nutritional products. The validation parameters investigated included linearity and working range, method detection limit, accuracy as recovery and bias, precision as repeatability and intermediate precision, and robustness. Linearity was demonstrated for all five nucleotides with correlation coefficients of >0.9999, and a visual inspection of residual plots. The method detection limits for individual nucleotides ranged from 0.06 to 0.19 mg/kg. The working range for individual nucleotides evaluated was from 0.06 to 17.4 mg/kg. Accuracy was determined as recovery, with values measured from 92 to 101%, within the suggested AOAC limits of 80−115% at the 10 ppm level (3), and no bias was found (*P* values all >0.05) when compared with a

previously published method (4). Precision as repeatability was estimated as 1.0–2.3 %RSD with a range for HorRat of 0.3–0.5 and for intermediate precision of 3.8–8.6 RSD%. A Plackett–Burman robustness study (5) was performed and the seven factors evaluated were shown not to affect the final results within typical experimental variations.

The method was applied to the analysis of a number of commercially available pediatric and nutritional powders. The products used for sampling included infant formula, follow-on formulas, and an adult nutritional product. The range of sources for these products included bovine milk, hydrolyzed milk protein, caprine milk, and soy protein. The method was found to be suitable for use with these various product matrixes.

It is recommended that this method be further examined against a set of infant formula and adult nutritional matrixes developed for this purpose by the SPIFAN community, and its performance evaluated against the SMPRs established by SPIFAN.

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Anal Bioanal Chem (2013) 405:5311–5319
DOI 10.1007/s00216-013-6935-9

RESEARCH PAPER

Analysis of nucleosides and nucleotides in infant formula by liquid chromatography–tandem mass spectrometry

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Received: 28 January 2013 / Revised: 19 March 2013 / Accepted: 20 March 2013 / Published online: 5 April 2013

Abstract A method for the simultaneous analysis of nucleosides and nucleotides in infant formula using reversed-phase liquid chromatography–tandem mass spectrometry is described. This approach is advantageous for compliance testing of infant formula over other LC-MS methods in which only nucleotides or nucleosides are measured. Following sample dissolution, protein was removed by centrifugal ultrafiltration. Chromatographic analyses were performed using a C_{18} stationary phase and gradient elution of an ammonium acetate/bicarbonate buffer, mass spectrometric detection and quantitation by a stable isotope-labelled internal standard technique. A single laboratory validation was performed, with spike recoveries of 80.1–112.9 % and repeatability relative standard deviations of 1.9–7.2 %. Accuracy as bias was demonstrated against reference values for NIST1849a certified reference material. The method has been validated for the analysis of bovine milk-based, soy-based, caprine milk-based and hydrolysed milk protein-based infant formulae.

Keywords Nucleotides · Nucleosides · Infant formula · LC-MS

Introduction

The structure of nucleosides and nucleotides and their importance to infant nutrition have been described previously [1–3]. In view of their physiological benefits, nucleotides are routinely supplemented into infant formulae as sodium salts of adenosine 5′-monophosphate (AMP), cytidine 5′-monophosphate (CMP), guanosine 5′-monophosphate (GMP), inosine 5′-monophosphate (IMP) and uridine 5′-monophosphate (UMP) [4]. Although nucleosides are not supplemented into infant formulae, dephosphorylation of nucleotides to the corresponding nucleosides—adenosine (Ado), cytidine (Cyd), guanosine (Guo), inosine (Ino) and uridine (Urd)—can occur under certain processing conditions [5].

Analytical methods for nucleosides and nucleotides in infant formulae and milk have previously been reviewed [6]. Sample preparation of infant formulae is frequently achieved by acidic precipitation of casein proteins from the reconstituted sample [5, 7]. Alternatively, centrifugal ultrafiltration has also been reported [8] and offers a simple mechanism to remove high-molecular-weight material. Further cleanup of sample extracts using ion exchange solid phase extraction and a phenylboronate affinity gel has been reported [9–11].

Liquid chromatography, i.e. reversed-phase liquid chromatography (RPLC), ion pair RPLC, ion exchange liquid chromatography and hydrophilic interaction liquid chromatography, with UV detection is commonly used for the quantitation of nucleotides in milk products [5, 7, 8, 12–15]. RPLC is easily adapted for the analysis of nucleosides, although the retention of nucleotides is more challenging. However, at the appropriate mobile phase pH, polar nucleotides are retained on a C_{18} column and an organic solvent gradient is able to remove late-eluting nucleosides.

The use of mass spectrometry (MS) offers potential advantages with respect to accuracy and simplicity by incorporating the addition of stable isotope-labelled (SIL) internal standards, whilst the selectivity of tandem MS reduces the need to remove other components that often compromise UV analyses [16–18]. The aim of this study was, therefore, to develop an LC-MS/MS method for the

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simultaneous analysis of nucleosides and nucleotides in infant formulae. The method described involves a simple centrifugal ultrafiltration procedure followed by high-performance liquid chromatography (HPLC) with detection and quantitation by tandem MS. Confidence in analytical accuracy is assured through the use of a SIL standard for each analyte. This technique has been validated for a range of bovine milk-based, caprine milk-based, soy-based and hypoallergenic infant formulae.

Experimental

Reagents

Ammonium acetate (NH_4CH_3COO), ammonium bicarbonate (NH_4HCO_3), AMP sodium salt, CMP disodium salt, GMP disodium salt, IMP disodium salt, UMP disodium salt, and corresponding nucleosides were obtained from Sigma-Aldrich (St. Louis, MO, USA). SIL nucleoside standards—$^{13}C_5$ Ado, $^{13}C_9{}^{15}N_3$ Cyd, $^{15}N_5$ Guo, $^{15}N_4$ Ino and $^{13}C_9{}^{15}N_2$ Urd—were purchased from Cambridge Isotope Laboratories (Andover, MA, USA). SIL nucleotide standards—$^{13}C_{10}{}^{15}N_5$ AMP, $^{13}C_9{}^{15}N_3$ CMP, $^{13}C_{10}{}^{15}N_5$ GMP and $^{13}C_9{}^{15}N_2$ UMP—were purchased from Sigma-Aldrich. $^{13}C_{10}{}^{15}N_4$ IMP was purchased from Medical Isotopes (Pelham, NH, USA).

Potassium dihydrogen phosphate (KH_2PO_4), acetic acid, orthophosphoric acid, potassium hydroxide and methanol were supplied by Merck. Water was purified with resistivity ≥18 M Ω using an E-pure water system (Barnstead, Dubuque, IA, USA).

A standardising buffer (KH_2PO_4, 0.25 M, pH 3.5) was made by dissolving 34.02 g of KH_2PO_4 in 900 mL of water, adjusting the pH to 3.5 with orthophosphoric acid and then making the solution to 1 L. Mobile phase A (NH_4CH_3COO, 10 mM; NH_4HCO_3, 5 mM, pH 5.6) was made daily by dissolving 0.771 g of NH_4CH_3COO and 0.395 g of NH_4HCO_3 in 950 mL of water, adjusting the pH to 5.6 with acetic acid solution (10 %, *w*/*v*) and then making to 1 L with water. Mobile phase B consisted of 100 % methanol.

Standard solutions

SIL nucleoside and nucleotide stock standards were prepared by accurately weighing 50 mg each of $^{13}C_5$ Ado, $^{13}C_9{}^{15}N_3$ Cyd, $^{15}N_5$ Guo, $^{15}N_4$ Ino, $^{13}C_9{}^{15}N_2$ Urd, $^{13}C_{10}{}^{15}N_5$ AMP, $^{13}C_9{}^{15}N_3$ CMP, $^{13}C_{10}{}^{15}N_5$ GMP, $^{13}C_{10}{}^{15}N_4$ IMP and $^{13}C_9{}^{15}N_2$ UMP into separate 50-mL volumetric flasks. To each flask, 40 mL of water was added and then shaken (with gentle warming if necessary) until the standard was completely dissolved before water was added to volume. Aliquots (~1.5 mL) of SIL stock standards were immediately dispensed into individual cryogenic vials and frozen at −15 °C for later use. Prior to analysis, cryogenic vials containing each SIL nucleoside and nucleotide stock standard were allowed to thaw to room temperature.

Non-isotopically labelled (NL) nucleoside and nucleotide stock standards were prepared in a similar manner by accurately weighing approximately 50 mg of each into separate 50-mL volumetric flasks and making to volume with water. These were refrigerated at 4 °C for up to 1 month.

Estimation of the moisture content of nucleosides was performed using the oven moisture method (102±2 °C, 4 h) and the concentration was calculated on a dry weight basis. Extinction coefficients at UV absorbance maxima were then determined for each nucleoside. These were compared with the values previously determined for nucleotides [5], with correction for molecular weight. The values obtained for each nucleoside were in close agreement with those for the corresponding nucleotide. Mean extinction coefficient values (nucleoside and corresponding nucleotide) were calculated by adjusting for molecular weight and are reported in Table 1. The concentration of each nucleoside and nucleotide stock standard was determined by adding 500 μL of each stock standard into separate 25-mL volumetric flasks, diluting with standardising buffer and measuring the absorbance at the appropriate λ_{max}.

A mixed SIL intermediate standard was prepared by diluting 2.0 mL of each SIL stock standard into a 25-mL volumetric flask and making to volume with water. A mixed NL intermediate standard was made by adding 1.0 mL of each NL stock standard into a 25-mL volumetric flask and making to volume with water.

Four calibration standards were prepared by pipetting 1.0, 1.0, 0.5 and 0.2 mL of SIL intermediate standard and 2.0, 4.0, 5.0 and 8.0 mL of NL intermediate standard into

Table 1 Mean extinction coefficients at UV absorbance maxima of nucleosides and corresponding nucleotides

Nucleoside/nucleotide	Extinction coefficient ($E^{1\,\%}$)	λ_{max} (nm)
AMP	428.6	257
Ado	557.0	
CMP	390.9	280
Cyd	519.5	
GMP	392.0	254
Guo	502.8	
IMP	356.5	249
Ino	462.7	
UMP	312.7	262
Urd	415.1	

Ado adenosine, *Cyd* cytidine, *Guo* guanosine, *Ino* inosine, *Urd* uridine, *AMP* adenosine 5′-monophosphate, *CMP* cytidine 5′-monophosphate, *GMP* guanosine 5′-monophosphate, *IMP* inosine 5′-monophosphate, *UMP* uridine 5′-monophosphate

50-, 50-, 25- and 10-mL volumetric flasks, respectively. The calibration standards were then made to volume with water and mixed thoroughly.

Samples

A range of different infant formula samples were evaluated during the validation of the method. These included a partially hydrolysed bovine milk-based powder, a partially hydrolysed soy-based powder, an infant elemental powder, a bovine milk-based powder, a soy-based powder and a caprine milk-based powder.

Sample preparation

Approximately 5.0 g of infant formula powder was weighed accurately into a 50-mL polypropylene centrifuge tube (Biolab, Auckland, New Zealand) and dissolved in 25 mL of water. To this was added 1.0 mL of the SIL intermediate standard and the tube was capped and vortex-mixed. The sample was allowed to stand for 10 min to ensure complete hydration before dilution to a final volume of 50 mL with water.

A 4.0-mL aliquot of sample solution was added to an Amicon Ultra-4 3000 MWCO centrifugal ultrafiltration unit (Millipore, Billerica, MA, USA) and centrifuged at 3,500×*g* for 60 min. The filter was then removed and discarded and a 1-mL aliquot of filtrate was transferred to an HPLC vial ready for analysis.

Instrumentation

The HPLC system used consisted of a CBM20A system controller, two LC20ADXR pumps for high-pressure gradients, a CTO20AC column oven and a SIL20ACXR autosampler equipped with a 50-μL injection loop (Shimadzu, Kyoto, Japan). Chromatographic separation was achieved using a Gemini column, 5 μm, 4.6×250 mm (Phenomenex, Torrance, CA, USA), with a high-pressure gradient elution programme as described in Table 2.

The MS/MS system consisted of a 3200 QTRAP quadrupole mass spectrometer with a Turbo V ion source equipped with an electrospray ionisation (ESI) probe. Analyst 1.5.1 software was used for instrument control and data processing (ABSciex, Foster City, CA, USA). The mass spectrometer was operated in ESI^+ mode with nitrogen utilised as the drying and collision gas. The instrumental parameters were set as follows: curtain gas at 30 psi, nebuliser gases GS1 and GS2 at 50 and 70 psi, respectively, desolvation temperature at 700 °C, collision-induced dissociation gas at medium and ion spray voltage at 5,500 V. Instrument settings and multiple reaction monitoring (MRM) transitions for the generation of product ions for nucleosides and nucleotides are given in Table 3.

Table 2 Gradient procedure for chromatographic separation

Time (min)	Flow rate (mL min^{-1})	Phase composition	
		%A[a]	%B[b]
0.0	0.75	100	0
3.5	0.75	100	0
10.0	0.75	80	20
20.0	0.75	80	20
21.0	0.75	100	0
35.0	0.75	100	0

[a] Mobile phase A: NH_4CH_3COO, 10 mM; NH_4HCO_3, 5 mM, pH 5.6

[b] Mobile phase B: 100 % methanol

Method validation

Six mixed nucleoside and nucleotide solutions covering the expected working range were prepared and linearity was evaluated by least-squares regression analysis of the SIL-corrected response (ratio of NL/SIL analyte peak area versus ratio of NL/SIL analyte concentration). A minimum value of

Table 3 MS/MS parameters

Analyte	Precursor ion $[M-H]^+$ (*m*/*z*)	Product ions (*m*/*z*)	DP (V)	EP (V)	CEP (V)	CE (V)	CXP (V)
CMP	324.1	112.0	26	3.5	32	27	4
SIL CMP	336.1	119.0					
UMP	325.0	97.0	21	9.5	18	23	4
SIL UMP	336.1	102.1					
GMP	364.1	152.0	36	4.5	22	23	4
SIL GMP	379.1	160.0					
IMP	349.1	137.0	31	6.0	28	23	4
SIL IMP	362.1	145.0					
Cyd	244.1	112.0	11	6.5	22	19	4
SIL Cyd	256.1	119.0					
Urd	245.1	113.0	16	5.0	16	19	4
SIL Urd	256.1	119.0					
AMP	348.1	136.0	36	1.0	24	25	4
SIL AMP	363.1	146.0					
Ino	269.1	137.0	16	4.5	18	23	4
SIL Ino	273.1	141.0					
Guo	284.1	152.0	11	4.0	24	23	4
SIL Guo	289.1	157.0					
Ado	268.1	136.0	21	8.5	16	39	4
SIL Ado	273.1	136.0					

SIL stable isotope-labelled, *Ado* adenosine, *Cyd* cytidine, *Guo* guanosine, *Ino* inosine, *Urd* uridine, *AMP* adenosine 5′-monophosphate, *CMP* cytidine 5′-monophosphate, *GMP* guanosine 5′-monophosphate, *IMP* inosine 5′-monophosphate, *UMP* uridine 5′-monophosphate, *DP* declustering potential, *EP* entrance potential, *CEP* collision cell entrance potential, *CE* collision energy, *CXP* collision cell exit potential

0.997 for the correlation coefficient (r^2) was deemed to be suitable. Plots of standard residuals were visually assessed as a further test of linearity.

Repeatability was estimated by analysing replicate pairs (n=9 pairs) of a bovine milk-based infant formula and NIST 1849a. Intermediate precision was determined from replicate analyses (n=6) of a bovine milk-based infant formula and NIST 1849a tested on three different days. Method detection limits (MDLs) were estimated in accordance with US Environmental Protection Agency procedures [19].

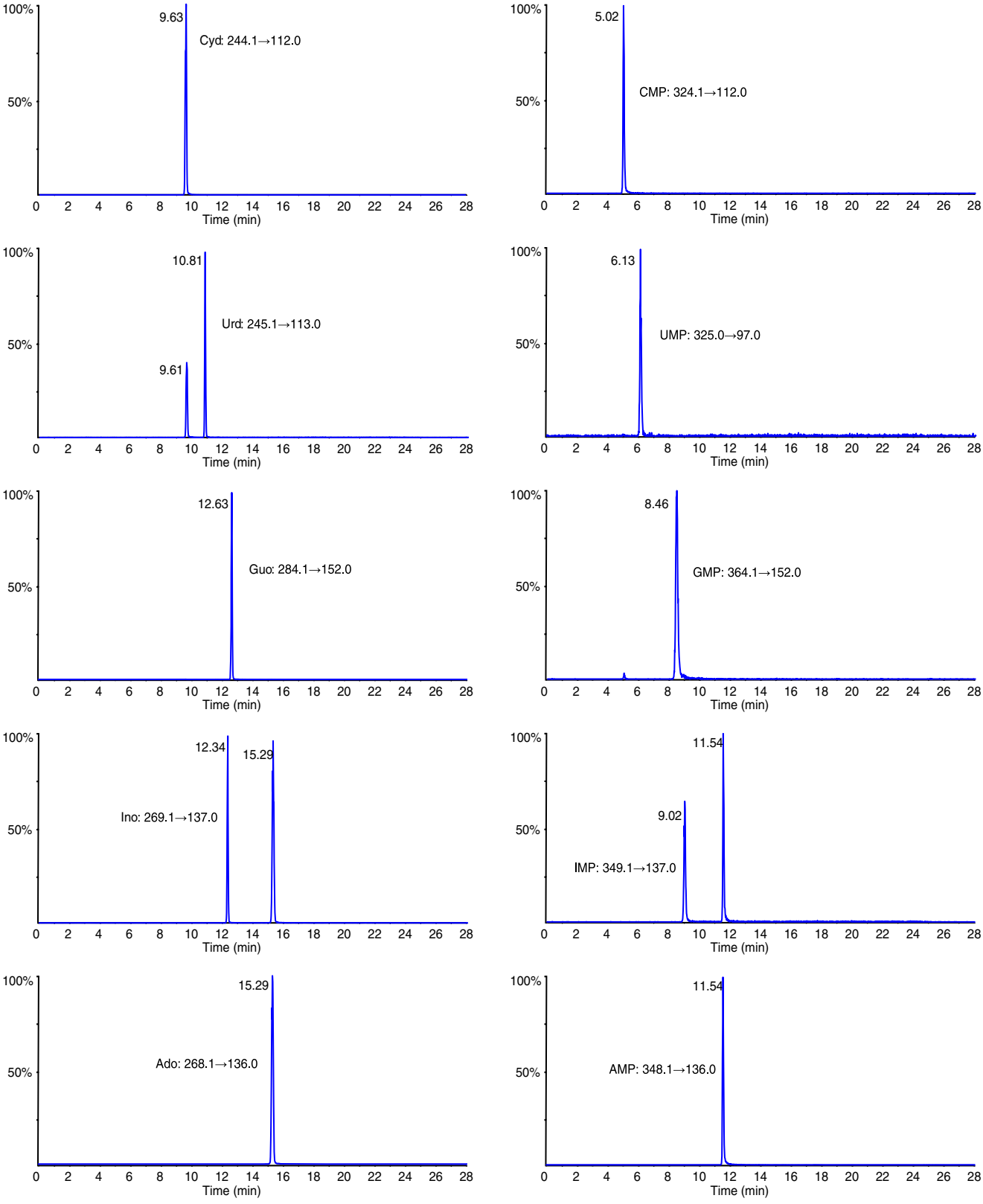

Fig. 1 LC-MS/MS MRM chromatograms of a mixed nucleotide and nucleotide standard solution (~7 μg mL^{-1})

The robustness of the method was assessed by conducting a Plackett–Burman trial [20], as described previously [15]. The seven factors assessed were: initial sample water volume (27 and 23 mL), vortex time (40 and 20 s), wait time (14 and 6 min), centrifuge volume (4.2 and 3.8 mL), centrifuge speed (4,000 and 3,000×*g*), centrifuge time (70 and 50 min) and a dummy factor.

Method accuracy was determined as both recovery and bias. Recovery of both nucleosides and nucleotides was evaluated by spiking a range of sample matrices at 50 and 150 % of the concentration levels typically found in infant formulae. Bias was evaluated by performing a paired *t* test for nucleotides both against reference values of a NIST 1849a powder and against values for a bovine milk-based infant formula tested using AOAC Official Method 2011.20 [21].

Results and discussion

Method optimisation

The simultaneous chromatographic analysis of both nucleosides and nucleotides in infant formulae using LC-UV has previously been described [5]. However, the mobile phase contained a 0.1 M phosphate buffer, which is unsuitable for use in LC-MS. In this study, ammonium acetate (10 mM, pH 5.6) was initially chosen to buffer the mobile phase because of its compatibility with electrospray mass spectrometric detection and a pH buffering range (~3.8–5.8) consistent with nucleoside and nucleotide pK_a values. However, significant peak tailing for nucleotides was observed when this buffer was used.

Conventional LC-UV nucleotide analyses typically contain phosphate in the mobile phase and no significant peak tailing is observed [5, 7]. Unfortunately, the use of non-volatile buffers such as phosphate in LC-MS is generally not recommended because of contamination of the ion source leading to a decrease in sensitivity. Furthermore, the interaction of phosphorylated compounds with metal surfaces in liquid chromatographic applications resulting in peak tailing has been reported [22–26]. Pretreatment of the chromatographic system using phosphoric acid prior to switching to a non-phosphate eluent during analysis [26, 27], the substitution of polyether ether ketone tubing for stainless steel, the use of a high pH mobile phase [28] and the addition of EDTA to the mobile phase [25] have all been employed to overcome this problem.

A number of mass spectrometer manufacturers have evaluated phosphate buffers for use with their instruments and have demonstrated that modern source designs can tolerate the use of non-volatile buffers [29–32]. A phosphate-based ion pair RPLC-MS method was successfully applied to the quantitative analysis of intracellular nucleotides utilising a microbore column to reduce the amount of phosphate introduced to the ion source [33].

In the present study, a low ionic strength phosphate buffer ($NH_4H_2PO_4$ 0.08 mM, pH 5.6) was initially evaluated for compatibility with the mass spectrometer. The chromatographic parameter resolution, retention factor, peak area repeatability, retention time repeatability, plate number and asymmetry were evaluated, with acceptable results being obtained (data not shown). There was some loss of sensitivity as replicate analyses progressed, consistent with a small accumulation of phosphate on the cone. The method was applied to the analysis of nucleotides in infant formula samples in a validation study. Linear response was demonstrated for NL/SIL peak area versus NL/SIL analyte concentration (r^2=0.997–0.999). Accuracy and precision were

Table 4 Chromatographic performance

Parameter	Analyte									
	Cyd	Urd	Guo	Ino	Ado	CMP	UMP	GMP	IMP	AMP
Retention time (min)	9.6 (0.0)[c]	10.8 (0.0)	12.6 (0.0)	12.3 (0.0)	15.3 (0.0)	5 (0.0)	6.1 (0.0)	8.5 (0.0)	9.0 (0.0)	11.6 (0.0)
Capacity factor[a]	2.0 (0.0)	2.4 (0.0)	2.9 (0.0)	2.8 (0.0)	3.8 (0.0)	0.6 (0.0)	0.9 (0.0)	1.6 (0.0)	1.8 (0.0)	2.6 (0.0)
Resolution[a]	1.3 (0.2)	3.5 (0.9)	0.8 (0.2)	2.2 (0.4)	4.6 (0.8)	–	2.8 (0.4)	4.3 (0.6)	0.9 (0.1)	2.8 (0.4)
Tailing factor[a]	1.8 (1.0)	1.2 (0.3)	1.7 (0.5)	1.4 (0.6)	1.8 (0.7)	1.6 (0.3)	1.7 (0.4)	1.6 (0.3)	1.5 (0.3)	1.7 (0.3)
Peak area ratio repeatability[b]	0.42 (0.01)	0.46 (0.02)	0.51 (0.02)	0.55 (0.02)	0.50 (0.01)	0.49 (0.03)	0.36 (0.01)	0.46 (0.01)	5.34 (0.13)	0.51 (0.03)

Ado adenosine, *Cyd* cytidine, *Guo* guanosine, *Ino* inosine, *Urd* uridine, *AMP* adenosine 5′-monophosphate, *CMP* cytidine 5′-monophosphate, *GMP* guanosine 5′-monophosphate, *IMP* inosine 5′-monophosphate, *UMP* uridine 5′-monophosphate

[a] Calculations as defined by US Pharmacopeia

[b] Peak area ratio measured as non-labelled nucleoside or nucleotide/stable isotope-labelled nucleoside or nucleotide

[c] Analysis of six replicates of a mixed nucleoside and nucleotide standard reported as the mean (standard deviation)

Table 5 Method performance as linearity, detection limit and precision

Analyte	Range (μg mL^{-1})	Linear regression[a]	r^2	MDL[b] (mg hg^{-1})	RSD$_r$[c] (%)	HorRat$_r$[d]	RSD$_{iR}$[e] (%)
Cyd	0.7–58.6	y=0.737x+0.1053	0.9996	0.03	4.8	0.4	14.4
Urd	0.8–60.9	y=0.957x−0.3441	0.9987	0.12	4.1	0.4	14.1
Guo	0.7–54.9	y=0.837x+0.2553	0.9996	0.01	6.2	0.4	7.9
Ino	0.8–62.1	y=1.059x−0.0417	0.9982	0.01	7.2	0.4	11.2
Ado	0.7–59.2	y=0.778x+0.1853	0.9997	0.01	–[f]	–	–
CMP	0.6–45.3	y=0.94x+0.0113	0.9998	0.13	4.0	0.6	4.6
UMP	0.5–42.6	y=0.872x−0.1152	0.9997	0.01	5.0	0.6	6.2
GMP	0.6–45.7	y=0.928x+0.1423	0.9993	0.01	1.9	0.2	2.9
IMP	0.6–46.8	y=1.069x+0.5071	0.9999	0.03	–	–	–
AMP	0.8–60.6	y=0.787x+0.35	0.9986	0.01	2.8	0.4	7.8

AMP adenosine 5′-monophosphate, *CMP* cytidine 5′-monophosphate, *GMP* guanosine 5′-monophosphate, *IMP* inosine 5′-monophosphate, *UMP* uridine 5′-monophosphate, *Ado* adenosine, *Cyd* cytidine, *Guo* guanosine, *Ino* inosine, *Urd* uridine

[a] Linear regression plotted as the ratio of peak area of unlabelled analyte to stable isotope-labelled standard versus the ratio of the concentration of unlabelled analyte to stable isotope-labelled standard

[b] Determined from n replicates at or near the expected detection limit: MDL=$t_{(n-1,\ 1-\alpha)}$×SD, where n=7 and α=0.01

[c] Relative standard deviation repeatability (RSD$_r$)=SD of n duplicate pairs/mean×100 (n=9)

[d] Horwitz ratio=RSD$_r$/pRSD$_R$, where pRSD$_R$=$2C^{-0.1505}$ at the 10-ppm concentration level (typical range, 0.3–1.3)

[e] Intermediate reproducibility of six replicates measured on three different days (n=18). RSD%=SD/mean×100

[f] Concentration at or below the detection limit

evaluated, with both spike recovery (84.2–107.1 %) and repeatability relative standard deviation (1.5–3.1 %) deemed to be acceptable. Despite this performance, a limitation with this phosphate-based approach was that the number of samples within each analytical run was limited due to the requirement for regular maintenance of the source.

An alternative chromatographic system was evaluated based on the observations of Asakawa et al. [22], who found a beneficial chromatographic effect with a number of mobile phase additives. Of those evaluated, only ammonium bicarbonate is volatile and considered suitable for use in LC-MS and was therefore incorporated as an additive in the ammonium acetate eluent.

The optimisation of the MS conditions was performed by infusion of a standard of each nucleoside or nucleotide (~10 μg mL^{-1}) diluted in a mixture of mobile phases A and B (90:10). Initial development focused on ESI$^+$ for nucleosides and ESI$^-$ for nucleotides. However, it was found that ESI$^+$ gave superior response for both analytes, with the [M+H]$^+$ ion most abundant and low levels of potassium adduct, thereby simplifying the analysis with the detection of all analytes in positive mode.

The conditions for MRM were optimised by selecting individual fragments and adjusting collision energies to maximise the product ion signal. The most abundant fragment ion observed for nucleosides and nucleotides was

Table 6 Mean recovery of nucleosides and nucleotides in spiked samples at 50 and 150 % of typical concentrations

Sample	Recovery (%)									
	Cyd	Urd	Guo	Ino	Ado	CMP	UMP	GMP	IMP	AMP
IF powder p/h bovine milk-based	95.4	84.9	104.4	94.1	99.3	101.3	81.8	104.8	112.9	95.1
IF powder p/h soy-based	101.1	98.9	107.2	96.8	100.1	101.8	88.8	101.6	98.4	101.7
Infant elemental powder	98.7	97.2	104.1	98.2	99.0	103.8	91.0	104.8	109.0	98.6
IF powder bovine milk-based	93.4	86.6	102.6	100.1	97.9	95.7	90.7	102.0	101.3	101.8
IF powder soy-based	101.7	80.1	107.9	103.0	95.3	101.8	90.3	103.5	94.8	98.7
IF powder caprine milk-based	96.4	109.1	112.0	100.1	100.5	103.0	97.5	100.9	98.9	110.1

AMP adenosine 5′-monophosphate, *CMP* cytidine 5′-monophosphate, *GMP* guanosine 5′-monophosphate, *IMP* inosine 5′-monophosphate, *UMP* uridine 5′-monophosphate, *Ado* adenosine, *Cyd* cytidine, *Guo* guanosine, *Ino* inosine, *Urd* uridine, *IF* infant formula, *p/h* partially hydrolysed

Table 7 Method bias against NIST 1849a reference values

	Analyte							
	CMP	UMP	GMP	AMP	CMP+Cyd	UMP+Urd	GMP+Guo	AMP+Ado
Measured[a] results	27.0 (0.99)	12.0 (0.66)	14.8 (0.45)	10.3 (0.29)	28.1 (1.00)	14.4 (0.68)	15.0 (0.45)	10.3 (0.29)
Reference values	26.8±2.9	12.9±1.5	14.6±1.1	10.51±0.5	31.7	15.5	14.6	10.8
Bias (*p* value)	0.44	<0.001	0.16	0.03	<0.001	<0.001	0.01	0.05

AMP adenosine 5′-monophosphate, *CMP* cytidine 5′-monophosphate, *GMP* guanosine 5′-monophosphate, *UMP* uridine 5′-monophosphate, *Ado* adenosine, *Cyd* cytidine, *Guo* guanosine, *Urd* uridine

[a] Mean (standard deviation) of analytical results of NIST 1849a CRM in milligrams per hectogram (n=12 replicates)

formed by cleaving of the glycosidic bond, resulting in the loss of ribose or ribose + phosphate group and the detection of the positively charged nucleobase. The exception to this was UMP, which underwent fragmentation and rearrangement to generate the *m*/*z* 97.0 ion. A similar fragmentation scheme has been reported for the generation of a product ion with *m*/*z* 81.0 from the fragmentation of deoxycytidine 5′-monophosphate [34].

Using the LC-MS/MS method developed, the simultaneous detection of nucleosides and nucleotides in a standard solution was achieved (Fig. 1).

Method performance

A high degree of selectivity is afforded by an MRM experiment; however, chromatographic separation is required for critical peaks with similar MRM transitions if accurate quantitation is to be achieved. Chromatographic performance was assessed by replicate analyses (n=6) of a mixed nucleoside and nucleotide standard, with satisfactory resolution being obtained between IMP/AMP (6.7), Ino/Ado (6.8) and Cyd/Urd (4.3) critical pairs, compounds which differ in mass by <2 Da (Table 4).

Method validation experiments to determine linearity, detection limits and precision are summarised in Table 5. Linearity was evaluated by least-squares regression analysis, with acceptable values being obtained for the correlation coefficient and with standard residual plots showing no pattern and only a small amount of random variation. The detection limits were appropriate, as defined by the infant formula industry, with the exception of those for CMP and Urd [35]. Although the detection limits of CMP and Urd were higher than those specified, the MDL was two orders of magnitude lower than that found in unfortified milk powder [5]. Precision was evaluated as repeatability (1.9–7.2 %) and intermediate precision (2.9–14.4 %). The suitability of these results was demonstrated by a Horwitz (repeatability) ratio of 0.2–0.6 [36].

The method was found to be robust for the seven method performance parameters studied, with variances in the results obtained not being significantly different from those expected by chance. Given the method's simplicity, two critical steps are required to ensure the accuracy of the results obtained: accurate measurement of the amount of sample weighed and accurate addition of the internal standard.

Accuracy determined as spiked recovery results measured in the six different product types were within the acceptable limits of 80–115 % at the 10-μg g^{-1} level, as suggested by the AOAC [36] (Table 6). Accuracy estimated as bias was evaluated against reference values for NIST 1849a CRM (Table 7) and against an LC-UV method for determining nucleotides in infant formula (AOAC method 2011.20; Table 8). Although there were statistically significant differences for some of the results, the differences were small enough (0–13 %) that they are unlikely to be of practical significance for compliance and labelling requirements.

Conclusions

The optimisation and validation of an LC-MS/MS method for the analysis of nucleosides and nucleotides in infant formulae has been described. The use of SIL internal

Table 8 Method bias against AOAC Official Method 2011.20

	Analyte				
	CMP	UMP	GMP	IMP	AMP
Measured results[a]	12.9 (0.39)	4.1 (0.14)	1.6 (0.04)	0 (0)	3.6 (0.11)
AOAC 2011.20 results[a]	12.3 (0.50)	4.0 (0.21)	1.6 (0.07)	0 (0)	3.2 (0.16)
Bias (*p* value)	<0.001	0.24	0.44	0	<0.001

AMP adenosine 5′-monophosphate, *CMP* cytidine 5′-monophosphate, *GMP* guanosine 5′-monophosphate, *UMP* uridine 5′-monophosphate, *IMP* inosine 5′-monophosphate

[a] Mean (standard deviation) of analytical results for bovine milk-based infant formula in milligrams per hectogram (n=12 replicates)

standards provides confidence in the accuracy of the results obtained. The method has been demonstrated to be precise and accurate and has been validated for the analysis of bovine milk-based, soy-based, caprine milk-based and hydrolysed milk protein-based infant formulae.

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

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APPENDIX III: LC-UV METHOD RAW DATA

Table 39. LC-UV method: resolution

Mixed Standard	Results					
	IMP	GMP	AMP	CMP	UMP	TMP
Rep-1	2.2	17.2	3.5	0.0	6.5	15.6
Rep-2	2.2	16.7	3.5	0.0	6.3	15.6
Rep-3	2.2	16.9	3.5	0.0	6.3	15.7
Rep-4	2.2	16.8	3.5	0.0	6.3	15.6
Rep-5	2.2	16.9	3.5	0.0	6.3	15.6
Rep-6	2.2	16.8	3.6	0.0	6.3	15.6

Table 40. LC-UV method: retention factor

Mixed Standard	Results					
	IMP	GMP	AMP	CMP	UMP	TMP
Rep-1–6	2.8	2.7	3.8	0.6	1.2	3.6

Table 41. LC-UV method: theoretical plate number

Mixed Standard	Results					
	IMP	GMP	AMP	CMP	UMP	TMP
Rep-1	58936	31344	242105	6728	9549	193432
Rep-2	60714	33807	242122	6759	8144	196772
Rep-3	60658	34690	240791	6895	8319	195607
Rep-4	61290	34469	241952	6872	8249	195092
Rep-5	60300	34120	241297	6822	8470	192936
Rep-6	60792	33724	242225	6785	8431	192337

Table 42. LC-UV method: tailing factor

Mixed Standard	Results					
	IMP	GMP	AMP	CMP	UMP	TMP
Rep-1	1.0	1.0	1.2	1.3	1.2	1.1
Rep-2–3	1.1	1.0	1.1	1.3	1.2	1.1
Rep-4	1.0	1.0	1.1	1.4	1.2	1.1
Rep-5	1.0	1.0	1.1	1.4	1.1	1.1
Rep-6	1.0	1.0	1.1	1.3	1.2	1.1

Table 43. LC-UV method: retention time

Mixed Standard	Results					
	IMP	GMP	AMP	CMP	UMP	TMP
Rep-1	20.6[a]	19.7	25.8	8.8	11.8	25.0
Rep-2–6	20.6	19.8	25.8	8.8	11.8	25.0

[a] time (min)

Table 44. LC-UV method: peak area

Mixed Standard	Results					
	IMP	GMP	AMP	CMP	UMP	TMP
Rep-1	123748	225546	309034	141090	201075	489133
Rep-2	121833	225604	308867	142148	200878	488891
Rep-3	121746	225576	308684	141865	199218	489026
Rep-4	121446	225612	308522	143459	203759	488691
Rep-5	122807	224207	308783	142219	199183	488058
Rep-6	123633	224907	308633	142749	202813	487711

Table 45. LC-UV method: CMP linearity

Standard Number	Concentration (µg mL^{-1})	Peak Area		
		Expt-1	Expt-2	Mean
1	0.61	176776	174227	175502
2	1.22	347187	349061	348124
3	2.44	708336	707102	707719
4	3.67	1048784	1052265	1050525
5	4.89	1385545	1395938	1390742
6	6.11	1753167	1758614	1755891
7	8.55	2461364	2467887	2464626

Table 46. LC-UV method: UMP linearity

Standard Number	Concentration (µg mL^{-1})	Peak Area		
		Expt-1	Expt-2	Mean
1	1.12	164979	163776	164378
2	2.24	324607	326570	325589
3	4.48	663277	661509	662393
4	6.72	983556	988010	985783
5	8.96	1298586	1307318	1302952
6	11.20	1641850	1649499	1645675
7	15.68	2301120	2310325	2305723

Table 47. LC-UV method: GMP linearity

Standard Number	Concentration (µg mL^{-1})	Peak Area		
		Expt-1	Expt-2	Mean
1	1.11	221513	221836	221675
2	2.22	439150	439407	439279
3	4.44	893367	889918	891643
4	6.66	1339470	1340023	1339747
5	8.88	1778542	1749201	1763872
6	11.11	2225169	2229101	2227135
7	15.55	3126921	3103321	3115121

Table 48. LC-UV method: IMP linearity

Standard Number	Concentration (μg mL^{-1})	Peak Area		
		Expt-1	Expt-2	Mean
1	1.09	217540	217228	217384
2	2.18	430114	439407	434761
3	4.36	876836	875499	876168
4	6.54	1302257	1304263	1303260
5	8.72	1725020	1733135	1729078
6	10.90	2169873	2175433	2172653
7	15.27	3028787	3037687	3033237

Table 49. LC-UV method: AMP linearity

Standard Number	Concentration (μg mL^{-1})	Peak Area		
		Expt-1	Expt-2	Mean
1	1.25	328042	326090	327066
2	2.50	643253	642699	642976
3	5.00	1299637	1305955	1302796
4	7.49	1940122	1929875	1934999
5	9.99	2558580	2565982	2562281
6	12.49	3202858	3217996	3210427
7	1.25	328042	326090	327066

Table 50. LC-UV method: TMP linearity

Standard Number	Concentration (μg mL^{-1})	Peak Area		
		Expt-1	Expt-2	Mean
1	1.61	237138	240469	238804
2	3.22	479645	481904	480775
3	6.44	977263	978047	977655
4	9.66	1458787	1452497	1455642
5	12.88	1931108	1933364	1932236
6	16.10	2418014	2430058	2424036
7	22.54	3389353	3392650	3391002

Table 51. LC-UV method: precision

Sample	Day Tested	Result (mg hg^{-1})				
		CMP	UMP	GMP	IMP	AMP
Milk-based IF dup-1	1	12.6	4.0	1.9	2.0	4.7
Milk-based IF dup-2	1	12.3	4.0	1.8	2.0	4.5
Milk-based IF dup-1	1	12.4	4.1	1.8	2.0	4.6
Milk-based IF dup-2	1	12.4	4.1	1.8	2.0	4.5
Milk-based IF dup-1	2	12.8	4.1	1.8	2.0	4.4
Milk-based IF dup-2	2	12.7	4.2	1.8	2.1	4.3
Milk-based IF dup-1	2	13.1	4.2	1.9	2.1	4.8
Milk-based IF dup-2	2	12.9	4.4	1.8	2.1	4.7
Milk-based IF dup-1	3	11.1	3.4	1.7	1.9	4.2
Milk-based IF dup-2	3	11.0	3.4	1.7	1.9	4.2
Milk-based IF dup-1	3	11.0	3.5	1.7	1.9	4.1
Milk-based IF dup-2	3	10.9	3.5	1.7	1.9	4.1
Milk-based IF dup-1	4	12.1	3.6	1.7	2.0	4.3
Milk-based IF dup-2	4	12.2	3.8	1.7	2.1	4.3
Milk-based IF dup-1	4	12.2	3.8	1.7	2.1	4.3
Milk-based IF dup-2	4	12.3	3.9	1.8	2.1	4.4
Milk-based IF dup-1	5	11.3	4.6	1.7	2.0	4.2
Milk-based IF dup-2	5	11.7	4.2	1.8	2.0	4.6
Milk-based IF dup-1	5	11.4	4.5	1.5	2.0	4.3
Milk-based IF dup-2	5	11.4	4.5	1.5	2.0	4.3

Table 52. LC-UV method: robustness

Experiment	Results (mg hg^{-1})				
	IMP	GMP	AMP	CMP	UMP
1	1.95	1.66	4.26	11.19	3.65
2	1.99	1.70	4.32	11.38	3.68
3	1.99	1.70	4.34	11.41	3.79
4	2.00	1.75	4.40	11.46	3.70
5	2.01	1.75	4.43	11.53	3.67
6	2.01	1.74	4.43	11.54	3.74
7	2.01	1.78	4.50	11.72	3.69
8	1.99	1.70	4.32	11.38	3.78

Table 53. LC-UV method: method detection limit

Replicate[a]	Results (mg hg^{-1})				
	CMP	UMP	GMP	IMP	AMP
1	1.74	0.56	0.53	0.50	0.72
2	1.75	0.57	0.54	0.53	0.72
3	1.74	0.56	0.53	0.52	0.70
4	1.70	0.56	0.53	0.50	0.69
5	1.65	0.55	0.50	0.49	0.72
6	1.75	0.53	0.53	0.52	0.68
7	1.64	0.57	0.48	0.49	0.64
8	1.56	0.57	0.52	0.54	0.70
9	1.63	0.52	0.54	0.56	0.71
10	1.62	0.49	0.49	0.59	0.73

[a] Milk-based infant formula not fortified with nucleotides

Table 54. LC-UV method: recovery (rep-1)

Sample	Spike Peak Area						Sample Peak Area					
	CMP	UMP	GMP	IMP	AMP	TMP	CMP	UMP	GMP	IMP	AMP	TMP
Unspiked bovine milk-based IF	-	-	-	-	-	-	37663	0	0	0	0	0
Level 1 bovine milk-based IF	34804	31471	44186	41175	60356	96291	72697	28861	43767	40335	61369	93870
Level 2 bovine milk-based IF	68688	64519	87000	81675	120179	193573	105855	59425	86160	80539	119872	188610
Level 3 bovine milk-based IF	137984	127411	176532	165382	240471	388559	172265	117639	167833	159396	230767	373977
Unspiked soy-based IF	-	-	-	-	-	-	3189	0	9230	0	17698	0
Level 1 soy-based IF	34804	31471	44186	41175	60356	96291	37004	33674	52176	42222	76727	103936
Level 2 soy-based IF	68688	64519	87000	81675	120179	193573	66317	57422	93072	73476	129384	173071
Level 3 soy-based IF	137984	127411	176532	165382	240471	388559	138668	123021	183025	158965	251221	386631
Unspiked hypoallergenic IF	-	-	-	-	-	-	0	0	0	0	0	0
Level 1 hypoallergenic IF	34804	31471	44186	41175	60356	96291	34912	29031	45139	43247	64064	102583
Level 2 hypoallergenic IF	68688	64519	87000	81675	120179	193573	66041	55205	86567	78279	120366	187558
Level 3 hypoallergenic IF	137984	127411	176532	165382	240471	388559	134776	111025	174486	159065	237652	374324

Table 55. LC-UV method: recovery (rep-2)

Sample	Spike Peak Area						Sample Peak Area					
	CMP	UMP	GMP	IMP	AMP	TMP	CMP	UMP	GMP	IMP	AMP	TMP
Unspiked bovine milk-based IF	-	-	-	-	-	-	39161	0	0	0	0	0
Level 1 bovine milk-based IF	34712	31022	45714	42833	62706	101386	72842	30944	44241	41112	62478	96811
Level 2 bovine milk-based IF	68666	63202	90209	82349	121981	199497	106801	58229	88738	80602	122479	192339
Level 3 bovine milk-based IF	135134	126239	177198	161994	239951	393617	176667	117761	179263	162556	243485	390893
Unspiked soy-based IF	-	-	-	-	-	-	3418	0	8800	0	16864	0
Level 1 soy-based IF	34712	31022	45714	42833	62706	101386	36934	32026	55300	40155	78376	107516
Level 2 soy-based IF	68666	63202	90209	82349	121981	199497	72758	61579	100139	81977	141107	203935
Level 3 soy-based IF	135134	126239	177198	161994	239951	393617	140510	122551	189164	161647	262745	392570
Unspiked hypoallergenic IF	-	-	-	-	-	-	0	0	0	0	0	0
Level 1 hypoallergenic IF	34712	31022	45714	42833	62706	101386	34914	30184	46127	40735	59445	102727
Level 2 hypoallergenic IF	68666	63202	90209	82349	121981	199497	69145	60322	91806	81296	119995	201160
Level 3 hypoallergenic IF	135134	126239	177198	161994	239951	393617	135927	117764	178983	160967	237712	392510

Table 56. LC-UV method: CMP bias

Replicate Number[a]	Day Tested	CMP Paired Results (mg hg^{-1})	
		Candidate Method	Reference Method[b]
1	1	12.8	12.8
2	1	12.7	12.4
3	1	13.1	13.0
4	1	12.9	12.7
5	1	12.6	13.1
6	1	13.1	12.8
7	2	11.3	11.7
8	2	11.7	11.6
9	2	11.3	11.6
10	2	11.4	11.5
11	2	11.5	11.6
12	2	11.4	11.6

[a] Milk-based infant formula
[b] Gill and Indyk, 2007

Table 57. LC-UV method: UMP bias

Replicate Number[a]	Day Tested	UMP Paired Results (mg hg^{-1})	
		Candidate Method	Reference Method[b]
1	1	4.1	4.3
2	1	4.2	4.2
3	1	4.2	4.4
4	1	4.4	4.1
5	1	4.4	4.4
6	1	4.1	4.4
7	2	4.6	4.0
8	2	4.2	4.0
9	2	4.2	3.6
10	2	4.5	4.0
11	2	4.2	3.7
12	2	4.0	3.7

[a] Milk-based infant formula
[b] Gill and Indyk, 2007

Table 58. LC-UV method: GMP bias

Replicate Number[a]	Day Tested	GMP Paired Results (mg hg^{-1})	
		Candidate Method	Reference Method[b]
1	1	1.8	1.7
2	1	1.8	1.7
3	1	1.9	1.8
4	1	1.8	1.7
5	1	1.7	1.6
6	1	1.8	1.7
7	2	1.7	1.6
8	2	1.8	1.6
9	2	1.5	1.9
10	2	1.5	1.9
11	2	1.7	1.6
1	1	1.8	1.7

[a] Milk-based infant formula
[b] Gill and Indyk, 2007

Table 59. LC-UV method: IMP bias

Replicate Number[a]	Day Tested	IMP Paired Results (mg hg^{-1})	
		Candidate Method	Reference Method[b]
1	1	2.0	2.0
2	1	2.1	2.1
3	1	2.1	2.0
4	1	2.1	1.8
5	1	2.0	1.9
6	1	2.1	2.0
7	2	2.0	2.0
8	2	2.0	2.0
9	2	2.0	2.0
10	2	2.0	2.0
11	2	2.0	2.0
12	2	1.8	2.0

[a] Milk-based infant formula
[b] Gill and Indyk, 2007

Table 60. LC-UV method: AMP bias

Replicate Number[a]	Day Tested	AMP Paired Results (mg hg^{-1})	
		Candidate Method	Reference Method[b]
1	1	4.4	4.2
2	1	4.3	4.2
3	1	4.8	4.4
4	1	4.7	4.2
5	1	4.4	4.2
6	1	4.4	4.2
7	2	4.2	4.3
8	2	4.6	4.4
9	2	4.1	4.3
10	2	4.3	4.3
11	2	4.4	4.2
12	2	4.3	4.2

[a] Milk-based infant formula
[b] Gill and Indyk, 2007

Table 61. LC-UV method extension: resolution

Mixed Standard	Results					
	IMP	GMP	AMP	CMP	UMP	TMP
Rep-1	0.0	7.2	12.4	2.7	15.7	4.4
Rep-2	0.0	7.3	12.4	2.7	15.5	4.4
Rep-3	0.0	7.3	12.4	2.7	15.3	4.4
Rep-4	0.0	7.5	12.6	2.7	15.0	4.3
Rep-5	0.0	7.5	12.6	2.7	14.8	4.4
Rep-6	0.0	7.5	12.7	2.7	14.5	4.4
Rep-7	0.0	7.6	12.7	2.8	14.1	4.4
Rep-8	0.0	7.6	12.8	2.8	14.0	4.4
Rep-9	0.0	7.6	12.9	2.8	14.2	4.5
Rep-10	0.0	7.7	13.0	2.8	14.5	4.6
Rep-11	0.0	7.7	13.1	2.8	14.8	4.5
Rep-12	0.0	7.7	13.2	2.8	15.1	4.6
Rep-13	0.0	7.7	13.4	2.7	15.5	4.6
Rep-14	0.0	7.8	13.6	2.7	15.6	4.6

Table 62. LC-UV method extension: retention factor

Mixed Standard	Results					
	IMP	GMP	AMP	CMP	UMP	TMP
Rep-1	1.9	2.8	4.8	5.4	8.0	8.5
Rep-2	1.9	2.8	4.9	5.4	8.0	8.5
Rep-3	1.9	2.8	4.9	5.5	8.0	8.5
Rep-4	2.0	2.9	5.0	5.5	8.0	8.5
Rep-5	2.0	2.9	5.0	5.6	8.0	8.6
Rep-6	2.0	2.9	5.1	5.7	8.1	8.6
Rep-7	2.0	3.0	5.1	5.7	8.1	8.6
Rep-8	2.0	3.0	5.2	5.8	8.1	8.6
Rep-9	2.0	3.0	5.3	5.8	8.1	8.7
Rep-10	2.0	3.0	5.3	5.9	8.1	8.7
Rep-11	2.0	3.0	5.4	5.9	8.1	8.7
Rep-12	2.1	3.1	5.4	5.9	8.1	8.7
Rep-13–14	2.1	3.1	5.5	6.0	8.1	8.7

Table 63. LC-UV method extension: theoretical plates

Mixed Standard	Results					
	IMP	GMP	AMP	CMP	UMP	TMP
Rep-1	10192	12996	14017	13867	95549	99638
Rep-2	10602	12859	14300	14343	96916	101672
Rep-3	10664	12897	14281	14326	99592	99300
Rep-4	11237	13186	14415	14738	96668	99121
Rep-5	10875	12992	14645	14826	98134	97892
Rep-6	10877	12896	14472	15032	97540	97943
Rep-7	11006	12978	14271	15783	94302	95966
Rep-8	11106	12857	14268	16709	91588	93421
Rep-9	10789	12806	14332	18375	91755	93547
Rep-10	10918	12901	14524	21504	91098	92581
Rep-11	10731	12615	14624	23934	88864	90545
Rep-12	10795	12487	14878	27248	87701	90249
Rep-13	10728	12386	15711	30341	87727	90184
Rep-14	10737	12441	16317	32304	86550	88390

Table 64. LC-UV method extension: tailing factor

Mixed Standard	Results					
	IMP	GMP	AMP	CMP	UMP	TMP
Rep-1	1.2	1.2	1.1	1.1	1.1	1.1
Rep-2	1.3	1.2	1.1	1.1	1.1	1.1
Rep-3–5	1.2	1.2	1.1	1.1	1.1	1.1
Rep-6	1.3	1.2	1.1	1.1	1.1	1.1
Rep-7–8	1.2	1.2	1.1	1.1	1.0	1.1
Rep-9–12	1.2	1.2	1.1	1.0	1.1	1.1
Rep-13	1.2	1.2	1.0	1.0	1.0	1.1
Rep-14	1.3	1.2	1.1	1.0	1.1	1.1

Table 65. LC-UV method extension: retention time

Mixed Standard	Results					
	IMP	GMP	AMP	CMP	UMP	TMP
Rep-1	7.2	9.4	14.5	15.9	22.4	23.7
Rep-2	7.3	9.5	14.7	16.1	22.5	23.8
Rep-3	7.3	9.6	14.8	16.2	22.5	23.8
Rep-4	7.4	9.7	14.9	16.4	22.6	23.9
Rep-5	7.4	9.7	15.1	16.5	22.6	23.9
Rep-6	7.4	9.8	15.2	16.6	22.7	24.0
Rep-7	7.5	9.9	15.4	16.8	22.7	24.0
Rep-8	7.5	9.9	15.5	17.0	22.7	24.1
Rep-9	7.6	10.0	15.6	17.1	22.8	24.2
Rep-10	7.6	10.1	15.8	17.2	22.8	24.2
Rep-11	7.6	10.1	15.9	17.3	22.8	24.3
Rep-12	7.6	10.2	16.0	17.3	22.8	24.3
Rep-13	7.7	10.2	16.1	17.4	22.9	24.3
Rep-14	7.7	10.3	16.2	17.4	22.9	24.3

Table 66. LC-UV method extension: peak area

Mixed Standard	Results					
	IMP	GMP	AMP	CMP	UMP	TMP
Rep-1	638138	982334	1119976	593685	770011	1843228
Rep-2	640022	986420	1127234	597848	773187	1855738
Rep-3	636447	981626	1120259	592765	769603	1842289
Rep-4	645569	993141	1133985	597917	779022	1866522
Rep-5	632147	975974	1111935	585924	766264	1838637
Rep-6	624578	962047	1095944	573769	753739	1809489
Rep-7	653286	1006752	1145159	600617	790462	1892580
Rep-8	637618	981851	1116001	588061	773469	1854067
Rep-9	630436	967112	1099008	580259	758997	1821988
Rep-10	621475	959689	1090717	574672	752423	1806074
Rep-11	630009	967348	1109294	583220	761399	1825315
Rep-12	638728	983285	1128934	591601	771544	1856610
Rep-13	637092	980414	1117724	587872	769926	1842529
Rep-14	616894	946592	1084900	572612	743720	1779084

Table 67. LC-UV method extension: CMP linearity

Standard Number	Concentration (ng mL^{-1})	Peak Area		
		Expt-1	Expt-2	Expt-3
1	30.40	3068	2764	2951
2	75.99	7950	6901	6208
3	151.98	15870	15743	14559
4	303.97	33905	33426	30072
5	790.32	91542	89617	87619
6	1641.44	185087	194640	191435
7	4012.40	469266	479866	477601
8	8207.19	953511	983051	973409

Table 68. LC-UV method extension: UMP linearity

Standard Number	Concentration (ng mL^{-1})	Peak Area Expt-1	Expt-2	Expt-3
1	29.9	3287	2762	3068
2	74.7	8295	7248	6433
3	149.5	17528	17060	15393
4	299.0	37265	35897	33386
5	777.4	102256	96457	95696
6	1614.6	212913	210417	207391
7	3946.7	526514	520532	521321
8	8072.9	1079162	1071946	1065016

Table 69. LC-UV method extension: GMP linearity

Standard Number	Concentration (ng mL^{-1})	Peak Area Expt-1	Expt-2	Expt-3
1	30.1	3139	3356	2685
2	75.3	9279	7244	4161
3	150.6	19241	17251	12724
4	301.3	41543	39812	32870
5	783.3	114857	106351	108758
6	1626.8	243900	238192	225350
7	3976.6	609095	584126	579317
8	8133.9	1248954	1236241	1254776

Table 70. LC-UV method extension: IMP linearity

Standard Number	Concentration (ng mL^{-1})	Peak Area Expt-1	Expt-2	Expt-3
1	30.3	3310	3044	3486
2	75.7	9418	7958	6443
3	151.4	18290	18569	16915
4	302.8	42722	40371	36493
5	787.2	121191	112168	111833
6	1634.9	253892	246719	245055
7	3996.5	630236	619282	617073
8	8174.6	1303341	1272772	1274573

Table 71. LC-UV method extension: AMP linearity

Standard	Concentration	Peak Area		
Number	(ng mL^{-1})	Expt-1	Expt-2	Expt-3
1	30.1	3935	2695	3429
2	75.2	10245	8343	6771
3	150.4	20433	20009	17738
4	300.8	44687	43798	38795
5	782.0	146449	120017	119155
6	1624.1	266211	263989	259700
7	3969.9	685088	661717	656761
8	8120.4	1377034	1364391	1359536

Table 72. LC-UV method extension: TMP linearity

Standard	Concentration	Peak Area		
Number	(ng mL^{-1})	Expt-1	Expt-2	Expt-3
1	30.3	4236	2818	4052
2	75.6	10261	8306	8029
3	151.3	20669	19434	18994
4	302.6	44382	43441	39653
5	786.7	124543	114412	116256
6	1633.8	255504	252406	250776
7	3993.8	635068	631412	631464
8	8169.2	1301278	1294196	1286217

Table 73. LC-UV method extension: recovery (unspiked samples)

Sample + 0 mL Spiked Solution	Spike Concentration (mg hg^{-1})					Results (mg hg^{-1})				
	CMP	UMP	GMP	IMP	AMP	CMP	UMP	GMP	IMP	AMP
Generic milk-based IF powder	0.0	0.0	0.0	0.0	0.0	11.36	3.82	1.87	1.98	4.71
AN milk protein-based powder	0.0	0.0	0.0	0.0	0.0	0.46	0	0	0	0
IF partially hydrolysed milk-based powder	0.0	0.0	0.0	0.0	0.0	9.82	6.08	1.71	0	6.03
IF partially hydrolysed soy-based powder	0.0	0.0	0.0	0.0	0.0	0	0	0.24	0	0
AN low fat powder	0.0	0.0	0.0	0.0	0.0	0.42	0.18	0.47	0	0.55
Child formula powder	0.0	0.0	0.0	0.0	0.0	0.45	0.09	1.84	0	1.60
Infant elemental powder	0.0	0.0	0.0	0.0	0.0	0.38	0	0.06	0	0
IF milk-based powder	0.0	0.0	0.0	0.0	0.0	12.94	4.07	1.73	0	3.55
IF milk-based RTF liquid	0.0	0.0	0.0	0.0	0.0	1.43	0.10	0.18	0	0.37
IF soy-based powder	0.0	0.0	0.0	0.0	0.0	0	0	0.27	0	0
AN high protein RTF liquid	0.0	0.0	0.0	0.0	0.0	0.14	0	0.05	0	0.08
AN high fat RTF liquid	0.0	0.0	0.0	0.0	0.0	0	0	0	0	0

p/h = partially hydrolysed

Table 74. LC-UV method extension: recovery (50% level rep-1)

Sample + 1 mL Spiked Solution	Spike Concentration (mg hg^{-1})					Results (mg hg^{-1})				
	CMP	UMP	GMP	IMP	AMP	CMP	UMP	GMP	IMP	AMP
Generic milk-based IF powder	4.28	1.68	0.83	0.83	1.84	15.93	5.65	2.62	2.92	6.45
AN milk protein-based powder	4.32	1.69	0.83	0.83	1.86	4.80	1.61	0.82	0.84	1.88
IF partially hydrolysed milk-based powder	4.45	1.74	0.86	0.86	1.92	14.41	7.94	2.62	0.86	8.06
IF partially hydrolysed soy-based powder	4.46	1.75	0.86	0.86	1.92	4.47	1.83	1.05	0.86	1.89
AN low fat powder	4.52	1.77	0.87	0.87	1.95	4.99	1.99	1.32	0.92	2.46
Child formula powder	4.50	1.76	0.87	0.87	1.94	5.14	1.76	2.69	0.92	3.38
Infant elemental powder	4.38	1.72	0.85	0.85	1.89	4.63	1.75	0.94	0.86	1.85
IF milk-based powder	4.37	1.71	0.84	0.84	1.88	17.08	5.67	2.64	0.89	5.40
IF milk-based RTF liquid	0.48	0.19	0.09	0.09	0.21	1.93	0.27	0.27	0.10	0.57
IF soy-based powder	4.52	1.77	0.87	0.87	1.95	4.44	1.79	1.08	0.94	2.14
AN high protein RTF liquid	0.48	0.19	0.09	0.09	0.21	0.55	0.24	0.14	0.15	0.31
AN high fat RTF liquid	0.49	0.19	0.09	0.09	0.21	0.50	0.18	0.10	0.10	0.22

Table 75. LC-UV method extension: recovery (50% level rep-2)

Sample + 1 mL Spiked Solution	Spike Concentration (mg hg^{-1})					Results (mg hg^{-1})				
	CMP	UMP	GMP	IMP	AMP	CMP	UMP	GMP	IMP	AMP
Generic milk-based IF powder	4.40	1.72	0.85	0.89	1.89	16.12	5.69	2.75	2.90	6.56
AN milk protein-based powder	4.31	1.69	0.83	0.87	1.85	4.48	1.55	0.89	0.86	1.77
IF partially hydrolysed milk-based powder	4.43	1.73	0.85	0.89	1.91	14.42	7.58	2.60	0.86	8.06
IF partially hydrolysed soy-based powder	4.49	1.76	0.87	0.90	1.93	4.42	1.91	1.02	0.86	1.83
AN low fat powder	4.42	1.73	0.85	0.89	1.90	5.02	1.87	1.38	0.93	2.44
Child formula powder	4.50	1.76	0.87	0.91	1.94	4.89	1.73	2.73	0.93	3.37
Infant elemental powder	4.39	1.72	0.85	0.88	1.89	4.61	1.73	0.91	0.84	1.87
IF milk-based powder	4.40	1.72	0.85	0.89	1.89	17.61	5.64	2.61	0.93	5.52
IF milk-based RTF liquid	0.50	0.20	0.10	0.10	0.21	1.90	0.25	0.24	0.10	0.53
IF soy-based powder	4.46	1.75	0.86	0.90	1.92	4.34	1.76	1.08	0.92	2.04
AN high protein RTF liquid	0.49	0.19	0.09	0.10	0.21	0.54	0.23	0.15	0.16	0.31
AN high fat RTF liquid	0.49	0.19	0.10	0.10	0.21	0.46	0.18	0.09	0.10	0.20

Table 76. LC-UV method extension: recovery (150% level rep-1)

Sample + 3 mL Spiked Solution	Spike Concentration (mg hg^{-1})					Results (mg hg^{-1})				
	CMP	UMP	GMP	IMP	AMP	CMP	UMP	GMP	IMP	AMP
Generic milk-based IF powder	12.97	5.08	2.50	2.61	5.59	24.05	8.64	4.26	4.57	9.96
AN milk protein-based powder	12.95	5.07	2.50	2.61	5.58	13.33	4.50	2.64	2.62	5.48
IF partially hydrolysed milk-based powder	13.37	5.23	2.58	2.69	5.76	23.06	10.80	4.53	2.60	12.04
IF partially hydrolysed soy-based powder	13.32	5.21	2.57	2.68	5.74	13.13	5.17	2.96	2.54	5.61
AN low fat powder	13.30	5.20	2.57	2.68	5.73	14.41	5.35	3.09	2.73	5.86
Child formula powder	13.57	5.31	2.62	2.73	5.85	13.77	5.18	4.46	2.79	6.93
Infant elemental powder	13.11	5.13	2.53	2.64	5.65	13.34	5.13	2.73	2.63	5.57
IF milk-based powder	13.25	5.18	2.56	2.67	5.71	26.35	9.37	4.32	2.75	9.14
IF milk-based RTF liquid	1.46	0.57	0.28	0.29	0.63	2.83	0.62	0.43	0.29	0.94
IF soy-based powder	13.42	5.25	2.59	2.70	5.78	13.04	4.90	2.98	2.68	6.03
AN high protein RTF liquid	1.45	0.57	0.28	0.29	0.63	1.52	0.56	0.33	0.30	0.72
AN high fat RTF liquid	1.48	0.58	0.29	0.30	0.64	1.42	0.57	0.26	0.28	0.58

Table 77. LC-UV method extension: recovery (150% level rep-2)

Sample + 3 mL Spiked Solution	Spike Concentration (mg hg^{-1})					Results (mg hg^{-1})				
	CMP	UMP	GMP	IMP	AMP	CMP	UMP	GMP	IMP	AMP
Generic milk-based IF powder	12.8	4.99	2.46	2.57	5.49	24.0	8.59	4.23	4.54	9.70
AN milk protein-based powder	12.9	5.05	2.49	2.60	5.55	13.3	4.81	2.65	2.65	5.33
IF partially hydrolysed milk-based powder	13.4	5.25	2.59	2.70	5.78	22.9	11.0	4.44	2.62	12.1
IF partially hydrolysed soy-based powder	13.4	5.25	2.59	2.70	5.77	13.4	5.25	2.75	2.62	5.59
AN low fat powder	13.2	5.16	2.55	2.66	5.68	13.5	4.91	3.04	2.69	5.71
Child formula powder	13.5	5.29	2.61	2.72	5.83	13.9	5.13	4.50	2.76	6.94
Infant elemental powder	13.0	5.11	2.52	2.63	5.62	13.3	4.99	2.75	2.60	5.41
IF milk-based powder	13.3	5.19	2.56	2.67	5.71	25.9	9.39	4.06	2.71	8.72
IF milk-based RTF liquid	1.48	0.58	0.29	0.30	0.64	2.96	0.67	0.48	0.31	1.01
IF soy-based powder	13.5	5.27	2.60	2.71	5.80	12.5	4.99	2.99	2.46	6.09
AN high protein RTF liquid	1.45	0.57	0.28	0.29	0.62	1.45	0.55	0.33	0.30	0.71
AN high fat RTF liquid	1.47	0.58	0.28	0.30	0.63	1.38	0.52	0.28	0.28	0.61

Table 78. LC-UV method extension: recovery (SMPR spiked samples)

Sample	Spike Concentration (mg hg^{-1})					Results (mg hg^{-1})				
	CMP	UMP	GMP	IMP	AMP	CMP	UMP	GMP	IMP	AMP
AN low fat powder unspiked	-	-	-	-	-	0.04	0	0	0	0
AN low fat powder spike level-1 rep-1	0.17	0.17	0.17	0.17	0.17	0.22	0.16	0.18	0.15	0.24
AN low fat powder spike level-1 rep-2	0.17	0.17	0.17	0.17	0.17	0.24	0.16	0.19	0.17	0.21
AN low fat powder spike level-1 rep-3	0.17	0.17	0.17	0.17	0.17	0.23	0.15	0.15	0.16	0.19
AN low fat powder spike level-2 rep-1	0.84	0.84	0.84	0.85	0.85	0.87	0.79	0.85	0.81	0.85
AN low fat powder spike level-2 rep-2	0.84	0.84	0.84	0.85	0.85	0.85	0.82	0.86	0.78	0.89
AN low fat powder spike level-2 rep-3	0.84	0.84	0.84	0.85	0.85	0.84	0.78	0.85	0.80	0.89
AN low fat powder spike level-3 rep-1	8.51	8.50	8.51	8.52	8.51	8.39	7.91	8.41	8.24	8.32
AN low fat powder spike level-3 rep-2	8.51	8.50	8.51	8.52	8.51	8.66	7.75	8.75	8.47	8.62
AN low fat powder spike level-3 rep-3	8.51	8.50	8.51	8.52	8.51	8.56	7.94	8.69	8.42	8.48
AN low fat powder spike level-4 rep-1	42.8	42.8	42.8	42.8	42.8	44.1	38.8	44.0	43.3	45.1
AN low fat powder spike level-4 rep-2	42.8	42.8	42.8	42.8	42.8	43.6	40.6	43.2	42.9	42.8
AN low fat powder spike level-4 rep-3	42.8	42.8	42.8	42.8	42.8	42.9	38.6	44.0	42.0	43.4

Table 79. LC-UV method extension: bias (NIST1849a CRM results)

Sample	Results (mg hg^{-1})			
	CMP	UMP	GMP	AMP
NIST 1849a day one, rep-1	27.60	13.00	14.93	10.71
NIST 1849a day one, rep-2	28.68	12.88	15.71	11.15
NIST 1849a day two, rep-1	26.99	13.16	14.25	10.08
NIST 1849a day two, rep-2	27.66	13.17	14.76	10.45
NIST 1849a day three, rep-1	25.97	12.02	14.55	10.52
NIST 1849a day three, rep-2	26.26	12.77	14.34	10.28
NIST 1849a day four, rep-1	27.37	13.12	14.64	10.33
NIST 1849a day four, rep-2	27.41	13.10	14.35	10.30
NIST 1849a day five, rep-1	30.06	12.00	15.43	11.31
NIST 1849a day five, rep-1	28.56	11.56	15.29	11.25
NIST 1849a day six, rep-1	29.77	11.54	15.40	11.44
NIST 1849a day six, rep-2	29.34	11.80	15.15	11.24

Table 80. LC-UV method extension: precision (day one)

Sample	Results[a] (mg hg^{-1})									
	Rep-1						Rep-2			
	CMP	UMP	GMP	IMP	4.21	11.30	UMP	GMP	IMP	AMP
Milk-based IF powder	11.13	4.23	1.76	1.98	10.71	28.68	4.23	1.79	2.08	4.35
NIST CRM 1849a	27.60	13.00	14.93	0.00	3.19	12.68	12.88	15.71	0.00	11.15
Milk-based IF powder	12.56	4.27	1.58	0.00	0.34	1.37	4.17	1.50	0.00	3.32
Milk-based RTF liquid	1.44	0.10	0.17	0.00	0.34	1.37	0.10	0.16	0.00	0.32

[a] Day one, technician one, instrument one

Table 81. LC-UV method extension: precision (day two)

Sample	Results[a] (mg hg^{-1})									
	Rep-1						Rep-2			
	CMP	UMP	GMP	IMP	4.08	10.34	UMP	GMP	IMP	AMP
Milk-based IF powder	10.48	4.12	1.64	1.91	10.08	27.66	3.86	1.69	1.91	4.27
NIST CRM 1849a	26.99	13.16	14.25	0.00	3.50	13.04	13.17	14.76	0.00	10.45
Milk-based IF powder	12.99	4.12	1.73	0.00	0.31	1.27	4.12	1.75	0.00	3.49
Milk-based RTF liquid	1.32	0.10	0.15	0.00	0.31	1.27	0.10	0.14	0.00	0.29

[a] Day two, technician one, instrument one

Table 82. LC-UV method extension: precision (day three)

Sample	Results[a] (mg hg^{-1})									
	Rep-1						Rep-2			
	CMP	UMP	GMP	IMP	4.11	10.22	UMP	GMP	IMP	AMP
Milk-based IF powder	10.12	3.88	1.58	1.89	10.52	26.26	3.94	1.61	1.87	4.14
NIST CRM 1849a	25.97	12.02	14.55	0	3.05	11.93	12.77	14.34	0	10.28
Milk-based IF powder	11.80	4.21	1.55	0	0.30	1.29	4.16	1.58	0	3.12
Milk-based RTF liquid	1.27	0.08	0.15	0	0.30	1.29	0.09	0.14	0	0.30

[a] Day three, technician two, instrument one

Table 83. LC-UV method extension: precision (day four)

Sample	Results[a] (mg hg^{-1})									
	Rep-1						Rep-2			
	CMP	UMP	GMP	IMP	3.99	10.52	UMP	GMP	IMP	AMP
Milk-based IF powder	10.35	3.94	1.61	1.94	10.33	27.41	3.93	1.65	1.92	4.03
NIST CRM 1849a	27.37	13.12	14.64	0	3.03	11.87	13.10	14.35	0	10.30
Milk-based IF powder	11.73	4.19	1.54	0	0.31	1.30	4.22	1.56	0	3.06
Milk-based RTF liquid	1.33	0.09	0.15	0	0.31	1.30	0.09	0.15	0	0.30

[a] Day four, technician two, instrument one

Table 84. LC-UV method extension: precision (day five)

Sample	Results[a] (mg hg^{-1})									
	Rep-1					Rep-2				
	CMP	UMP	GMP	IMP	4.46	11.03	UMP	GMP	IMP	AMP
Milk-based IF powder	11.03	3.71	1.71	1.99	11.31	28.56	3.78	1.73	1.97	4.44
NIST CRM 1849a	30.06	12.00	15.43	0	3.30	11.74	11.56	15.29	0	11.25
Milk-based IF powder	12.18	3.82	1.63	0	0.33	1.27	3.71	1.57	0	3.19
Milk-based RTF liquid	1.38	0.08	0.15	0	0.33	1.27	0.08	0.14	0	0.30

[a] Day five, technician three, instrument two

Table 85. LC-UV method extension: precision (day six)

Sample	Results[a] (mg hg^{-1})									
	Rep-1						Rep-2			
	CMP	UMP	GMP	IMP	4.35	11.43	UMP	GMP	IMP	AMP
Milk-based IF powder	11.12	3.58	1.66	1.96	11.44	29.34	3.56	1.72	2.03	4.47
NIST CRM 1849a	29.77	11.54	15.40	0	3.28	12.81	11.80	15.15	0	11.24
Milk-based IF powder	12.62	3.84	1.57	0	0.34	1.45	3.75	1.58	0	3.31
Milk-based RTF liquid	1.47	0.06	0.15	0	0.34	1.45	0.06	0.15	0	0.34

[a] Day six, technician three, instrument two

APPENDIX IV: TPAN ANALYSIS RAW DATA

Table 86. TPAN chromatography: resolution

Mixed Standard	Results						
	Cyd	Urd	5mCyd	Ino	Guo	Ado	8BrGuo
Rep-1	0.0	6.3	8.1	8.3	2.6	12.8	9.5
Rep-2	0.0	6.4	8.3	7.7	2.6	12.7	9.5
Rep-3	0.0	6.2	7.7	9.1	2.7	12.8	9.1
Rep-4	0.0	6.0	7.5	9.4	2.8	12.6	9.0
Rep-5	0.0	6.1	7.5	9.4	2.8	12.8	9.1
Rep-6	0.0	6.0	7.5	9.5	2.7	12.8	8.9
Rep-7	0.0	6.1	7.5	9.5	2.7	12.7	8.9
Rep-8	0.0	6.1	7.5	9.5	2.8	12.4	8.9
Rep-9	0.0	6.0	7.5	9.5	2.8	12.7	9.0
Rep-10	0.0	6.0	7.4	9.5	2.7	12.5	9.0
Rep-11	0.0	6.0	7.5	9.4	2.7	12.4	8.9
Rep-12	0.0	6.0	7.5	9.4	2.8	12.5	8.9
Rep-13	0.0	6.0	7.4	9.3	2.7	12.3	8.9

Table 87. TPAN method: retention factor

Mixed Standard	Results						
	Cyd	Urd	5mCyd	Ino	Guo	Ado	8BrGuo
Rep-1	1.5	2.4	3.8	5.1	5.3	6.5	7.6
Rep-2	1.6	2.4	4.0	5.1	5.4	6.5	7.7
Rep-3	1.4	2.2	3.6	5.0	5.2	6.3	7.4
Rep-4–5	1.4	2.2	3.5	4.9	5.1	6.3	7.3
Rep-6–7	1.4	2.2	3.4	4.9	5.1	6.3	7.3
Rep-8–9	1.4	2.2	3.5	4.9	5.1	6.3	7.3
Rep-10–12	1.4	2.2	3.5	4.9	5.2	6.3	7.3
Rep-13	1.4	2.2	3.5	4.9	5.2	6.3	7.4

Table 88. TPAN method: theoretical plates

Mixed Standard	Results						
	Cyd	Urd	5mCyd	Ino	Guo	Ado	8BrGuo
Rep-1	6672	7887	8519	70604	89268	96811	56826
Rep-2	6701	7989	8647	74783	94298	93766	56620
Rep-3	6786	7712	8223	61494	83362	98738	62705
Rep-4	6398	7587	8148	52520	78441	94140	61317
Rep-5	6664	7551	8056	53554	80348	98512	62110
Rep-6	6582	7612	8067	50148	79176	98374	61100
Rep-7	6577	7697	8151	50714	79199	96656	60978
Rep-8	6685	7677	8181	52691	75328	94129	61109
Rep-9	6429	7626	8173	53735	80204	97516	60310
Rep-10	6600	7481	8071	55531	79830	94802	60606
Rep-11	6309	7476	8134	54349	80597	92142	59610
Rep-12	6442	7603	8132	55589	81436	91610	58672
Rep-13	6570	7552	8005	55645	78695	91755	59102

Table 89. TPAN method: tailing factor

Mixed Standard	Results						
	Cyd	Urd	5mCyd	Ino	Guo	Ado	8BrGuo
Rep-1	1.2	1.1	1.0	1.1	1.1	1.2	1.1
Rep-2–5	1.2	1.1	1.0	1.1	1.2	1.2	1.1
Rep-6	1.2	1.2	1.0	1.1	1.2	1.2	1.2
Rep-7	1.2	1.1	1.0	1.1	1.2	1.2	1.1
Rep-8	1.2	1.1	1.0	1.1	1.1	1.2	1.1
Rep-9	1.2	1.2	1.0	1.2	1.2	1.2	1.1
Rep-10	1.2	1.1	1.0	1.1	1.1	1.2	1.1
Rep-11	1.2	1.1	1.0	1.1	1.2	1.2	1.1
Rep-12	1.2	1.2	1.0	1.1	1.2	1.2	1.1
Rep-13	1.2	1.1	1.0	1.1	1.1	1.2	1.1

Table 90. TPAN method: retention time

Mixed Standard	Results						
	Cyd	Urd	5mCyd	Ino	Guo	Ado	8BrGuo
Rep-1	5.5	7.4	10.6	13.4	13.9	16.4	18.9
Rep-2	5.6	7.6	10.9	13.5	14.0	16.5	19.1
Rep-3	5.3	7.1	10.1	13.1	13.6	16.2	18.4
Rep-4	5.2	7.0	9.8	12.9	13.5	16.0	18.3
Rep-5	5.2	7.0	9.8	12.9	13.5	16.1	18.3
Rep-6	5.2	6.9	9.7	12.9	13.5	16.0	18.2
Rep-7–9	5.2	7.0	9.8	12.9	13.5	16.0	18.3
Rep-10	5.3	7.0	9.9	13.0	13.5	16.1	18.3
Rep-11	5.3	7.0	9.9	13.0	13.6	16.1	18.3
Rep-12	5.3	7.0	9.9	13.0	13.6	16.1	18.4
Rep-13	5.3	7.1	9.9	13.0	13.6	16.1	18.4

Table 91. TPAN method: peak area

Mixed Standard	Results						
	Cyd	Urd	5mCyd	Ino	Guo	Ado	8BrGuo
Rep-1	101975	136868	94477	107797	130840	184237	177727
Rep-2	105768	141774	98055	111589	135530	190849	183974
Rep-3	105551	142184	97903	111931	135823	191183	184517
Rep-4	100819	135980	93473	107415	130514	184413	177853
Rep-5	101162	136223	93597	107182	130006	183118	176836
Rep-6	102232	137473	94690	108501	131816	186241	180033
Rep-7	101667	136607	94439	107596	130516	183946	177581
Rep-8	103696	139471	96106	109759	133043	187420	181116
Rep-9	103636	139555	96139	109670	132891	187400	180905
Rep-10	100363	134937	93228	106175	128751	181702	176011
Rep-11	106120	142754	98189	112251	136077	192060	186263
Rep-12	112602	151446	104781	119170	144577	203855	197927
Rep-13	103396	138942	95438	109222	132363	186623	181340

Table 92. TPAN raw data: winter herd milk (rep-1)

Sample[a,b]	Enzyme Treatment	Results (mg dL^{-1})			
		Cyd	Urd	Guo	Ado
Day 0	A	1.29	14.43	0.08	0.00
	B	2.82	50.99	0.88	0.74
	C	2.97	51.05	1.25	1.09
	D	3.23	55.29	2.22	1.72
Day 0.25	A	0.94	9.67	0.07	0.00
	B	1.33	17.05	0.36	0.36
	C	1.34	17.81	0.70	0.61
	D	1.52	18.76	0.98	0.77
Day 1	A	0.84	12.31	0.14	0.00
	B	4.07	31.72	1.24	0.84
	C	4.29	31.81	1.64	1.16
	D	4.42	35.87	2.31	1.80
Day 2	A	0.55	14.69	0.22	0.14
	B	4.76	22.61	0.81	0.85
	C	4.99	23.04	1.11	1.19
	D	5.03	24.92	1.74	1.95
Day 3	A	0.46	10.09	0.13	0.14
	B	4.46	16.11	0.58	1.26
	C	4.55	16.41	0.73	1.28
	D	4.58	17.79	1.31	1.90
Day 5	A	0.32	5.40	0.00	0.05
	B	3.33	5.72	0.17	0.94
	C	3.46	5.87	0.39	1.13
	D	3.48	6.08	0.60	1.33
Day 10	A	0.16	0.82	0.00	0.03
	B	1.89	0.91	0.07	0.69
	C	1.90	0.92	0.15	0.75
	D	1.94	0.99	0.18	0.84
Day 20	A	0.13	0.29	0.00	0.04
	B	1.07	0.33	0.00	0.28
	C	1.13	0.35	0.08	0.35
	D	1.14	0.38	0.09	0.37
Day30	A	0.13	0.22	0.00	0.03

B	0.75	0.24	0.00	0.13
C	0.81	0.26	0.06	0.18
D	0.81	0.27	0.07	0.20

[a] Milk/colostrum samples from 7 cows pooled at each sampling time.
[b] Sampling time in days post-partum.

Table 93. TPAN raw data: winter herd milk (rep-2)

Sample[a,b]	Enzyme Treatment	Results (mg dL^{-1})			
		Cyd	Urd	Guo	Ado
Day 0	A	1.31	13.87	0.09	0.00
	B	2.76	47.49	0.88	0.80
	C	2.89	50.04	1.31	1.17
	D	3.07	57.39	2.58	1.81
Day 0.25	A	1.00	9.75	0.07	0.00
	B	1.25	15.52	0.35	0.37
	C	1.27	16.65	0.65	0.61
	D	1.53	17.28	1.01	0.72
Day 1	A	0.87	12.03	0.15	0.00
	B	4.01	30.46	1.30	0.76
	C	4.06	31.85	1.64	1.26
	D	4.11	33.58	2.30	1.68
Day 2	A	0.65	14.81	0.24	0.17
	B	4.66	21.54	0.78	0.87
	C	4.92	22.44	1.06	1.19
	D	5.03	23.82	1.78	1.83
Day 3	A	0.53	10.78	0.16	0.19
	B	4.40	15.62	0.55	0.97
	C	4.47	15.81	0.75	1.23
	D	4.67	17.29	1.41	1.82
Day 5	A	0.41	5.12	0.00	0.07
	B	3.30	5.50	0.16	0.92
	C	3.39	5.53	0.41	1.09
	D	3.40	5.73	0.56	1.22
Day 10	A	0.23	0.76	0.00	0.03
	B	1.84	0.86	0.07	0.64
	C	1.97	0.92	0.19	0.71
	D	1.98	0.94	0.20	0.74
Day 20	A	0.19	0.34	0.00	0.04
	B	1.13	0.34	0.00	0.24
	C	1.17	0.35	0.08	0.27
	D	1.19	0.41	0.11	0.30
Day30	A	0.16	0.18	0.00	0.03

B	0.76	0.23	0.00	0.10
C	0.82	0.25	0.07	0.12
D	0.83	0.26	0.08	0.14

[a] Milk/colostrum samples from 7 cows pooled at each sampling time.
[b] Sampling time in days post-partum.

Table 94. TPAN raw data: summer herd milk (rep-1)

Sample[a,b]	Enzyme Treatment	Results (mg dL^{-1})			
		Cyd	Urd	Guo	Ado
Day 0	A	0.61	13.36	0.66	0.00
	B	0.94	13.64	0.71	0.00
	C	1.05	13.75	1.02	0.25
	D	1.10	13.89	1.11	0.31
Day 0.25	A	0.86	6.76	0.51	0.00
	B	1.04	6.88	0.55	0.00
	C	1.11	7.24	0.81	0.21
	D	1.18	7.43	0.85	0.24
Day 1	A	1.26	10.21	0.63	0.00
	B	3.05	11.20	0.73	0.00
	C	3.23	11.40	1.00	0.24
	D	3.58	12.91	1.33	0.33
Day 2	A	0.82	9.56	0.69	0.00
	B	3.48	9.67	0.75	0.25
	C	3.61	9.92	0.89	0.32
	D	3.61	10.27	1.13	0.43
Day 3	A	1.59	5.53	0.32	0.00
	B	3.15	6.54	0.42	0.50
	C	3.29	6.97	0.53	0.59
	D	3.32	7.11	0.66	0.71
Day 5	A	0.27	2.23	0.11	0.00
	B	2.26	2.33	0.18	0.52
	C	2.43	2.44	0.26	0.58
	D	2.47	2.52	0.29	0.65
Day 10	A	0.15	0.74	0.00	0.00
	B	1.17	0.77	0.00	0.29
	C	1.22	0.84	0.10	0.32
	D	1.29	0.85	0.12	0.37
Day 20	A	0.17	0.44	0.00	0.00
	B	0.86	0.45	0.00	0.11
	C	0.86	0.47	0.00	0.12
	D	0.89	0.49	0.00	0.14
Day30	A	0.15	0.31	0.00	0.00

B	0.57	0.32	0.00	0.00
C	0.57	0.33	0.00	0.00
D	0.60	0.34	0.00	0.08

[a] Milk/colostrum samples from 7 cows pooled at each sampling time.
[b] Sampling time in days post-partum.

Table 95. TPAN raw data: summer herd milk (rep-2)

Sample[a,b]	Enzyme Treatment	Results (mg dL^{-1})			
		Cyd	Urd	Guo	Ado
Day 0	A	0.67	11.37	0.56	0.00
	B	1.05	11.66	0.61	0.00
	C	1.15	11.67	0.93	0.25
	D	1.17	11.77	1.00	0.31
Day 0.25	A	0.90	6.91	0.50	0.00
	B	0.96	6.99	0.54	0.00
	C	1.05	7.44	0.76	0.20
	D	1.10	7.71	0.79	0.22
Day 1	A	1.38	9.78	0.57	0.00
	B	3.14	10.87	0.64	0.00
	C	3.27	11.20	0.94	0.14
	D	3.72	13.03	1.31	0.20
Day 2	A	0.96	9.59	0.84	0.00
	B	3.34	9.67	0.90	0.21
	C	3.46	9.93	1.01	0.28
	D	3.46	10.40	1.27	0.37
Day 3	A	1.66	4.96	0.35	0.00
	B	2.95	5.69	0.43	0.65
	C	3.06	5.95	0.54	0.73
	D	3.09	6.08	0.63	0.84
Day 5	A	0.23	2.27	0.00	0.00
	B	2.15	2.37	0.07	0.53
	C	2.39	2.51	0.15	0.58
	D	2.50	2.61	0.17	0.66
Day 10	A	0.13	0.75	0.00	0.00
	B	1.09	0.78	0.00	0.34
	C	1.12	0.83	0.10	0.37
	D	1.16	0.83	0.11	0.40
Day 20	A	0.17	0.28	0.00	0.00
	B	0.91	0.29	0.00	0.09
	C	0.91	0.31	0.00	0.10
	D	0.95	0.32	0.00	0.12
Day30	A	0.15	0.32	0.00	0.00

B	0.50	0.32	0.00	0.00
C	0.51	0.33	0.00	0.00
D	0.53	0.34	0.00	0.08

[a] Milk/colostrum samples from 7 cows pooled at each sampling time.
[b] Sampling time in days post-partum.

Table 96. TPAN raw data: bovine, caprine, ovine milk (rep-1)

Sample	Enzyme Treatment	Results (mg dL^{-1})			
		Cyd	Urd	Guo	Ado
Bovine[a]	A	0.20	0.44	0.00	0.00
	B	1.03	0.59	0.00	0.00
	C	1.04	0.60	0.00	0.11
	D	1.06	0.62	0.10	0.14
Caprine[a]	A	0.39	2.70	0.00	0.00
	B	1.23	11.64	2.58	0.62
	C	1.42	12.03	2.99	0.81
	D	1.59	14.29	7.09	1.72
Ovine[b]	A	0.54	3.42	0.16	0.00
	B	1.96	48.41	1.94	3.24
	C	2.04	49.43	2.25	3.48
	D	2.29	75.28	8.56	7.44

[a] Pooled samples taken from silos prior to manufacturing.
[b] Pooled sample taken from research herd.

Table 97. TPAN raw data: bovine, caprine, ovine milk (rep-2)

Sample	Enzyme Treatment	Results (mg dL^{-1})			
		Cyd	Urd	Guo	Ado
Bovine[a]	A	0.24	0.49	0.00	0.00
	B	1.02	0.58	0.00	0.00
	C	1.07	0.63	0.00	0.13
	D	1.08	0.64	0.13	0.15
Caprine[a]	A	0.37	2.83	0.00	0.00
	B	1.28	12.05	2.77	0.68
	C	1.39	12.12	2.97	0.78
	D	1.57	14.78	7.09	1.66
Ovine[b]	A	0.56	3.80	0.16	0.00
	B	1.89	50.31	1.93	3.23
	C	2.06	51.37	2.33	3.69
	D	2.28	74.54	8.56	7.40

[a] Pooled samples taken from silos prior to manufacturing.
[b] Pooled sample taken from research herd.

APPENDIX V: LS-MS METHOD RAW DATA

Table 98. LC-MS method: bias (AOAC 2011.20 results)

Sample	Result (mg hg^{-1})				
	CMP	UMP	GMP	IMP	AMP
Bovine milk-based IF rep-1	12.6	4.3	1.6	0.0	3.2
Bovine milk-based IF rep-2	13.0	4.1	1.7	0.0	3.5
Bovine milk-based IF rep-3	11.8	4.2	1.5	0.0	3.1
Bovine milk-based IF rep-4	11.7	4.2	1.5	0.0	3.0
Bovine milk-based IF rep-5	12.2	3.8	1.6	0.0	3.3
Bovine milk-based IF rep-6	12.6	3.8	1.6	0.0	3.3
Bovine milk-based IF rep-7	12.7	4.2	1.5	0.0	3.3
Bovine milk-based IF rep-8	13.0	4.1	1.8	0.0	3.5
Bovine milk-based IF rep-9	11.9	4.2	1.6	0.0	3.1
Bovine milk-based IF rep-10	11.9	4.2	1.6	0.0	3.1
Bovine milk-based IF rep-11	11.7	3.7	1.6	0.0	3.2
Bovine milk-based IF rep-12	12.8	3.7	1.6	0.0	3.3

Table 99. LC-MS method: bias (LC-MS method results)

Sample	Result (mg hg^{-1})				
	CMP	UMP	GMP	IMP	AMP
Bovine milk-based IF rep-1	13.3	4.2	1.6	0.0	3.6
Bovine milk-based IF rep-2	13.2	4.2	1.6	0.0	3.5
Bovine milk-based IF rep-3	12.9	4.1	1.6	0.0	3.5
Bovine milk-based IF rep-4	12.8	4.2	1.6	0.0	3.6
Bovine milk-based IF rep-5	12.7	3.8	1.7	0.0	3.6
Bovine milk-based IF rep-6	12.4	4.0	1.6	0.0	3.6
Bovine milk-based IF rep-7	13.4	4.2	1.6	0.0	3.6
Bovine milk-based IF rep-8	13.5	3.9	1.6	0.0	3.9
Bovine milk-based IF rep-9	12.6	4.2	1.6	0.0	3.6
Bovine milk-based IF rep-10	12.5	4.3	1.6	0.0	3.5
Bovine milk-based IF rep-11	12.7	3.9	1.6	0.0	3.6
Bovine milk-based IF rep-12	12.3	4.2	1.6	0.0	3.7

Table 100. LC-MS method: resolution

Mixed	Results									
Standard	Cyd	Urd	Guo	Ino	Ado	CMP	UMP	GMP	IMP	AMP
Rep-1	1.4	4.3	0.8	2.3	5.6	-	2.7	4.5	0.9	3.3
Rep-2	1.3	3.4	0.6	2.0	4.3	-	2.5	3.8	1.0	2.9
Rep-3	1.0	2.6	0.5	2.2	3.2	-	2.8	4.5	0.9	3.0
Rep-4	1.4	4.7	0.7	2.2	4.5	-	3.3	4.9	0.9	2.9
Rep-5	1.4	3.6	0.9	2.9	5.3	-	3.4	4.8	1.1	2.8
Rep-6	1.0	2.4	1.1	1.7	4.6	-	2.2	3.5	0.8	2.0

Table 101. LC-MS method: retention factor

Mixed	Results									
Standard	Cyd	Urd	Guo	Ino	Ado	CMP	UMP	GMP	IMP	AMP
Rep-1–5	2.0	2.4	2.9	2.8	3.8	0.6	0.9	1.6	1.8	2.6
Rep-6	2.0	2.4	3.0	2.8	3.8	0.6	0.9	1.6	1.8	2.6

Table 102. LC-MS method: retention time

Mixed	Results									
Standard	Cyd	Urd	Guo	Ino	Ado	CMP	UMP	GMP	IMP	AMP
Rep-1	9.6	10.8	12.6	12.3	15.3	5.0	6.1	8.4	9.0	11.6
Rep-2–5	9.6	10.8	12.6	12.3	15.3	5.0	6.1	8.5	9.0	11.6
Rep-6	9.6	10.8	12.7	12.3	15.3	5.0	6.1	8.4	9.0	11.6

Table 103. LC-MS method: theoretical plates

Mixed Standard	Results									
	Cyd	Urd	Guo	Ino	Ado	CMP	UMP	GMP	IMP	AMP
Rep-1	13790	34376	13611	19760	12988	1849	5198	2401	4916	32596
Rep-2	9356	20327	10580	12279	6317	3291	2085	2407	5674	32596
Rep-3	3578	21888	5184	15359	3989	2410	4284	2504	4319	35868
Rep-4	29920	21888	12885	18472	6502	3771	5568	2772	3677	29975
Rep-5	9948	23804	12270	61745	11448	5614	4270	3227	6589	25251
Rep-6	3334	18808	16374	22638	7145	2410	1832	1978	3961	8543

Table 104. LC-MS method: tailing factor

Mixed Standard	Results									
	Cyd	Urd	Guo	Ino	Ado	CMP	UMP	GMP	IMP	AMP
Rep-1	0.8	0.9	1.9	0.8	1.2	2.2	1.5	2.0	1.6	1.6
Rep-2	1.8	1.4	1.3	2.4	1.8	1.5	1.9	1.7	1.4	1.3
Rep-3	2.8	1.0	2.5	2.0	2.9	1.3	1.4	1.3	1.6	1.6
Rep-4	1.0	1.7	1.3	1.1	2.1	1.2	1.7	1.5	1.9	1.6
Rep-5	1.1	1.1	1.9	1.1	1.1	1.6	1.2	1.3	1.0	1.7
Rep-6	3.1	0.9	1.5	1.1	1.6	1.5	2.4	1.7	1.5	2.3

Table 105. LC-MS method: peak area

Mixed standard	Results									
	Cyd SIL Cyd	Urd SIL Urd	Guo SIL Guo	Ino SIL Ino	Ado SIL Ado	CMP SIL CMP	UMP SIL UMP	GMP SIL GMP	IMP SIL IMP	AMP SIL AMP
Rep-1	0.431	0.454	0.535	0.540	0.492	0.495	0.350	0.457	5.109	0.494
Rep-2	0.422	0.488	0.529	0.580	0.513	0.482	0.380	0.458	5.372	0.478
Rep-3	0.429	0.444	0.493	0.558	0.512	0.500	0.345	0.467	5.345	0.530
Rep-4	0.415	0.449	0.503	0.574	0.482	0.529	0.351	0.477	5.317	0.493
Rep-5	0.420	0.449	0.521	0.534	0.506	0.431	0.368	0.460	5.479	0.504
Rep-6	0.425	0.492	0.496	0.542	0.505	0.499	0.378	0.448	5.416	0.557

Table 106. LC-MS method: linearity (nucleoside peak area)

Mixed Standard	Results									
	Cyd	Urd	Guo	Ino	Ado	SIL Cyd	SIL Urd	SIL Guo	SIL Ino	SIL Ado
Level-1	717000	55500	434000	231000	4680000	1710000	132000	830000	440000	9140000
Level-2	2030000	170000	1300000	681750	12510000	3780000	296000	1830000	968000	19990000
Level-3	2400000	229000	1620000	879000	12600000	1490000	125000	753000	354000	6820000
Level-4	4110000	510000	3230000	1680000	19500000	1050000	119000	677000	302000	4570000
Level-5	8040000	1740000	8180000	3900000	32400000	537000	100000	488000	202000	2110000
Level-6	9220000	3710000	13300000	7960000	37300000	317000	98500	398000	191000	1220000

Table 107. LC-MS method: linearity (nucleoside concentration)

Mixed Standard	Concentration (µg mL^{-1})									
	Cyd	Urd	Guo	Ino	Ado	SIL Cyd	SIL Urd	SIL Guo	SIL Ino	SIL Ado
Level-1	0.7	0.8	0.7	0.8	0.7	1.5	1.6	1.4	1.6	1.5
Level-2	1.5	1.5	1.4	1.6	1.5	1.5	1.6	1.4	1.6	1.5
Level-3	2.9	3.0	2.7	3.1	3.0	1.5	1.6	1.4	1.6	1.5
Level-4	7.3	7.6	6.9	7.8	7.4	1.5	1.6	1.4	1.6	1.5
Level-5	29.3	30.5	27.4	31.1	29.6	1.5	1.6	1.4	1.6	1.5
Level-6	58.6	60.9	54.9	62.1	59.2	1.5	1.6	1.4	1.6	1.5

Table 108. LC-MS method: linearity (nucleotide peak area)

Mixed	Peak Area									
Standard	CMP	UMP	GMP	IMP	AMP	SIL CMP	SIL UMP	SIL GMP	SIL IMP	SIL AMP
Level-1	114000	21900	95100	38800	625000	235000	59800	206000	72100	1200000
Level-2	349000	66100	291000	121000	1800000	531000	136000	465000	163600	2650000
Level-3	483000	89600	400000	175000	2220000	243000	58900	212000	77700	1030000
Level-4	1230000	221000	982000	427000	4350000	242000	59500	210000	81200	812000
Level-5	4150000	825000	3880000	1580000	10500000	216000	56900	215000	78400	549000
Level-6	6400000	1810000	6790000	3420000	14300000	167000	60400	197000	84900	398000

Table 109. LC-MS method: linearity (nucleotide concentration)

Mixed Standard	Concentration (µg mL^{-1})									
	CMP	UMP	GMP	IMP	AMP	SIL CMP	SIL UMP	SIL GMP	SIL IMP	SIL AMP
Level-1	0.6	0.8	0.7	0.8	0.7	1.1	1.2	1.2	0.12	1.3
Level-2	1.1	1.5	1.4	1.6	1.5	1.1	1.2	1.2	0.12	1.3
Level-3	2.3	3.0	2.7	3.1	3.0	1.1	1.2	1.2	0.12	1.3
Level-4	5.7	7.6	6.9	7.8	7.4	1.1	1.2	1.2	0.12	1.3
Level-5	22.6	30.5	27.4	31.1	29.6	1.1	1.2	1.2	0.12	1.3
Level-6	45.3	60.9	54.9	62.1	59.2	1.1	1.2	1.2	0.12	1.3

Table 110. LC-MS method: repeatability

Sample	Results (mg hg^{-1})									
	Cyd	Urd	Guo	Ino	Ado	CMP	UMP	GMP	IMP	AMP
Rep-1 dup-1	1.10	2.43	0.19	0.22	0.03	27.3	11.0	13.9	0.00	9.89
Rep-1 dup-2	1.21	2.55	0.19	0.23	0.03	27.2	13.0	14.2	0.00	10.69
Rep-2 dup-1	1.12	2.51	0.19	0.22	0.03	26.7	11.4	14.8	0.00	10.34
Rep-2 dup-2	1.12	2.54	0.19	0.24	0.03	26.3	12.7	15.6	0.00	10.59
Rep-3 dup-1	1.05	2.41	0.17	0.25	0.03	25.5	13.2	14.9	0.00	10.27
Rep-3 dup-2	1.13	2.59	0.19	0.24	0.03	28.7	12.1	15.3	0.00	10.05
Rep-4 dup-1	1.13	2.27	0.18	0.24	0.03	28.7	12.0	14.9	0.00	10.12
Rep-4 dup-2	1.14	2.44	0.18	0.26	0.03	25.9	11.4	14.9	0.00	10.68
Rep-5 dup-1	1.09	2.26	0.19	0.25	0.03	27.4	11.9	14.4	0.00	10.18
Rep-5 dup-2	1.05	2.44	0.19	0.24	0.03	26.5	12.0	14.7	0.00	10.63
Rep-6 dup-1	1.13	2.41	0.18	0.25	0.03	26.8	11.7	14.9	0.00	10.15
Rep-4 dup-2	1.05	2.59	0.18	0.23	0.03	27.5	11.5	15.0	0.01	9.98
Rep-7 dup-1	0.86	1.74	0.17	0.20	0.05	28.1	12.7	14.9	0.00	11.98
Rep-7 dup-2	0.89	1.82	0.17	0.20	0.07	29.4	12.5	14.8	0.00	11.88

Table 110 cont'd. LC-MS method: repeatability

Sample	Results (mg hg^{-1})									
	Cyd	Urd	Guo	Ino	Ado	CMP	UMP	GMP	IMP	AMP
Rep-8 dup-1	1.10	2.43	0.19	0.22	0.03	27.3	11.0	13.9	0.00	9.89
Rep-8 dup-2	1.21	2.55	0.19	0.23	0.03	27.2	13.0	14.2	0.00	10.69
Rep-9 dup-1	1.12	2.51	0.19	0.22	0.03	26.7	11.4	14.8	0.00	10.34
Rep-9 dup-2	1.12	2.54	0.19	0.24	0.03	26.3	12.7	15.6	0.00	10.59
Rep-10 dup-1	1.05	2.41	0.17	0.25	0.03	25.5	13.2	14.9	0.00	10.27
Rep-10 dup-2	0.89	1.82	0.17	0.20	0.07	29.4	12.5	14.8	0.00	11.88

Table 111. LC-MS method: intermediate precision

Sample	Results (mg hg^{-1})									
	Cyd	Urd	Guo	Ino	Ado	CMP	UMP	GMP	IMP	AMP
Day-1 dup-1	1.10	2.43	0.19	0.22	0.03	27.32	11.02	13.94	0.00	9.89
Day-1 dup-2	1.14	2.55	0.19	0.23	0.03	27.19	12.97	14.21	0.00	10.69
Day-1 dup-3	1.12	2.51	0.19	0.22	0.03	26.69	11.38	14.75	0.00	10.34
Day-1 dup-4	1.12	2.54	0.19	0.24	0.03	26.30	12.67	15.59	0.00	10.59
Day-1 dup-5	1.05	2.41	0.17	0.25	0.03	25.46	13.18	14.89	0.00	10.27
Day-1 dup-6	1.13	2.59	0.19	0.24	0.03	28.68	12.10	15.30	0.00	10.05
Day-2 dup-1	0.82	2.10	0.16	0.21	0.03	27.35	11.46	15.12	0.00	12.17
Day-2 dup-2	0.79	2.21	0.15	0.19	0.03	29.15	12.32	15.58	0.00	12.43
Day-2 dup-3	0.80	2.11	0.16	0.21	0.03	29.48	11.42	15.32	0.00	12.17
Day-2 dup-4	0.78	2.07	0.16	0.19	0.03	29.45	11.57	15.00	0.00	12.22
Day-2 dup-5	0.81	2.14	0.18	0.24	0.03	28.43	10.62	15.29	0.00	12.33
Day-2 dup-6	0.84	2.16	0.16	0.20	0.03	28.38	12.11	15.19	0.00	12.41
Day-3 dup-1	0.86	1.74	0.17	0.20	0.05	28.09	12.71	14.85	0.00	11.98
Day-3 dup-2	0.89	1.82	0.17	0.20	0.07	29.37	12.47	14.83	0.00	11.88

Table 111 cont'd. LC-MS method: intermediate precision

Sample	Results (mg hg^{-1})									
	Cyd	Urd	Guo	Ino	Ado	CMP	UMP	GMP	IMP	AMP
Day-3 dup-3	0.93	1.84	0.17	0.18	0.07	29.69	13.03	14.68	0.00	11.68
Day-3 dup-4	0.89	1.81	0.17	0.22	0.05	27.98	12.44	15.14	0.00	11.47
Day-3 dup-5	0.84	1.85	0.16	0.17	0.06	29.75	12.70	14.45	0.00	11.15
Day-3 dup-6	0.92	1.73	0.20	0.19	0.07	29.90	12.97	14.85	0.00	11.78

Table 112. LC-MS method: robustness

Experiment	Results (mg hg^{-1})									
	Cyd	Urd	Guo	Ino	Ado	CMP	UMP	GMP	IMP	AMP
1	4.26	5.37	3.00	3.39	3.13	4.71	1.77	2.58	5.59	3.15
2	4.00	5.38	3.08	3.44	3.10	4.74	1.65	2.64	2.66	3.22
3	3.96	5.89	2.91	3.39	3.24	4.76	1.60	2.57	2.80	3.16
4	3.82	5.85	2.91	3.47	3.24	4.66	1.54	2.51	3.21	3.06
5	3.87	5.08	3.10	3.44	3.26	4.56	1.74	2.56	2.83	3.16
6	4.03	5.86	2.99	3.55	3.20	4.65	1.59	2.54	2.85	3.22
7	3.99	5.66	3.22	3.39	3.18	4.66	1.81	2.53	3.99	3.27
8	4.04	5.46	3.10	3.64	3.17	4.65	1.69	2.31	4.16	3.13

Table 113. LC-MS method: recovery (spiked standard)

Standard	Results (mg hg^{-1})									
	Cyd	Urd	Guo	Ino	Ado	CMP	UMP	GMP	IMP	AMP
50% Spike	1.04	1.13	1.10	1.01	1.26	4.50	1.83	0.97	0.99	1.79
150% Spike	3.01	3.83	3.09	2.99	3.49	13.71	6.04	2.88	3.34	5.28

Table 114. LC-MS method: recovery (unspiked sample)

Sample	Results (mg hg^{-1})									
	Cyd	Urd	Guo	Ino	Ado	CMP	UMP	GMP	IMP	AMP
IF powder p/h bovine milk-based	0.62	1.59	0.09	0.03	0.03	10.01	6.28	2.11	0.00	5.94
IF Powder p/h soy-based	0.10	0.50	0.54	0.01	0.94	0.22	0.15	0.44	0.00	0.40
Infant elemental powder	0.01	0.02	0.02	0.00	0.04	0.00	0.05	0.00	0.00	0.00
IF Powder bovine milk-based	1.09	1.27	0.29	0.18	0.25	11.54	3.67	1.47	0.00	3.02
IF Powder soy-based	0.09	0.52	0.46	0.02	1.13	0.15	0.03	0.25	0.00	0.25
IF Powder caprine milk-based	0.83	9.78	0.40	6.86	0.01	2.97	5.81	0.27	0.31	2.15

p/h = partially hydrolysed

Table 115. LC-MS method: recovery (50% spiked sample)

Sample	Results (mg hg^{-1})									
	Cyd	Urd	Guo	Ino	Ado	CMP	UMP	GMP	IMP	AMP
IF powder p/h bovine milk-based	1.64	2.45	1.21	1.00	1.25	14.73	7.58	3.11	1.15	7.66
IF Powder p/h soy-based	1.13	1.71	1.69	0.94	2.17	4.89	1.81	1.41	0.96	2.20
Infant elemental powder	1.03	1.19	1.16	1.00	1.25	4.86	1.67	1.00	1.08	1.76
IF Powder bovine milk-based	2.03	2.24	1.40	1.25	1.48	15.65	5.36	2.47	0.98	4.87
IF Powder soy-based	1.18	1.38	1.59	1.09	2.28	4.86	1.70	1.27	0.92	1.99
IF Powder caprine milk-based	1.80	10.95	1.63	7.73	1.21	7.78	7.70	1.26	1.33	4.12

p/h = partially hydrolysed

Table 116. LC-MS method: recovery (150% spiked sample)

Sample	Results (mg hg^{-1})									
	Cyd	Urd	Guo	Ino	Ado	CMP	UMP	GMP	IMP	AMP
IF powder p/h bovine milk-based	3.41	5.19	3.42	2.79	3.59	23.42	11.85	5.20	3.64	10.88
IF Powder p/h soy-based	3.22	3.98	3.96	3.07	4.54	13.89	5.43	3.40	3.34	5.85
Infant elemental powder	3.03	3.53	3.27	2.93	3.58	13.67	5.71	3.07	3.63	5.22
IF Powder bovine milk-based	4.01	4.60	3.53	3.03	3.69	25.24	9.06	4.39	3.45	8.32
IF Powder soy-based	3.09	3.73	3.95	3.00	4.58	13.72	5.43	3.21	3.23	5.54
IF Powder caprine milk-based	3.81	14.17	3.88	10.27	3.70	16.54	11.40	3.15	3.46	7.95

p/h = partially hydrolysed