Determination of Nucleotides and Nucleosides in Milks and Pediatric Formulas: A Review

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Abstract

Nucleotides and nucleosides play important roles as structural units in nucleic acids, as coenzymes in biochemical pathways, and as sources of chemical energy. Milk contains a complex mixture of nucleotides, nucleosides, and nucleobases, and because of the reported differences in their relative levels in bovine and human milks, pediatric formulas are increasingly supplemented with nucleotides. Liquid chromatography is the dominant analytical technique used for the quantitation of nucleospecies and is commonly applied using either ion-exchange, reversed-phase, or ion-pair reversed-phase modes. Robust methods that incorporate minimal sample preparation and rapid chromatographic separations have been developed for routine product compliance analysis. This review summarizes the analytical techniques used to date in the analysis of nucleospecies in bovine and human milks and infant formulas.

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Introduction

In recent years, there has been a great deal of interest in the study of bovine milk for bioactive factors that may be significant to the improvement of human health. Found in a wide range of concentrations from parts per billion to parts per million, bioactive components, such as nucleotides, growth factors, and vitamins, influence the physiological development of newborns (1). The influence of nucleotides on pediatric growth and nutrition and their composition in milk are productive areas of research. A number of analytical tools have been used to characterize the specific nucleos(t)ide composition of milks, the review of which forms the basis of this article.

Nucleobases are heterocyclic compounds which include cytosine, thymine, and uracil (pyrimidines) and adenine, guanine, hypoxanthine, and xanthine (purines). Nucleosides consist of a purine or pyrimidine base attached to a sugar (ribose or deoxyribose). Numerous derivatives of nucleosides, particularly methylated derivatives, occur naturally. Nucleotides are o-phosphoric acid esters of nucleosides that contain 1, 2, or 3 phosphate groups on the 2-, 3-, or, most commonly, 5-ribose carbon (Figure 1). Nucleotides form polymers such as RNA and are incorporated as adducts with sugars and within coenzymes such as FAD, NADH, and coenzyme A. Cyclic nucleotides also exist, where a phosphate group is bonded to two of the (deoxy)ribose hydroxyl groups, forming a ring structure. A large variety of nucleotides and nucleosides are found in milk, the profile of which is species dependent (2–4).

The chemical behavior of the polyvalent phosphate group, dominated by its ionization at physiological pH and its chemical stability, confers properties that make nucleotides suitable as building blocks within genetic material (5). In addition to forming the structural units of genetic information, nucleotides and nucleosides play important roles as coenzymes in biochemical pathways and as sources of chemical energy (6–8). Given the quantitative predominance of RNA over DNA in cells (9) and in milk (10), research on metabolically active nucleos(t)ides has largely been restricted to ribose forms; therefore, only ribonucleos(t)ides are covered in this review.

Physiological/Nutritional Role

Nucleotides are not considered essential dietary nutrients and can be synthesized de novo or via salvage pathways. However, they may become conditionally essential when the endogenous supply is inadequate, such as during periods of rapid growth or after injury (6, 7, 11). Nucleotide-supplemented diets are reported to exhibit enhanced immune response in infants, as compared to unsupplemented diets (12–14). Nucleotides influence metabolism of long-chain fatty acids and improve gastrointestinal tract repair after damage (6, 12, 15, 16). A number of studies have also shown significant reduction in the incidences and severity of episodes of diarrhea in infants fed

nucleotide-supplemented compared to non-supplemented infant formula (17–19). Nucleotide-supplemented infant formula has also been shown to positively modify the composition of the intestinal microflora, emulating this attribute of human milk (20). The role nucleotides play in infant nutrition has been reviewed comprehensively by Carver and Walker (6), and more recently by Schaller et al. (21).

Contribution in Milk

The non-protein nitrogen pool accounts for approximately 20% of total nitrogen in human milk, but only 2% in bovine milk (22). Nucleotides contribute between 0.4 and 0.6% of non-protein nitrogen and between 0.10 and 0.15% of the total nitrogen content of human milk, with an increase in the ratio of nucleotides to total nitrogen with advancing lactation (12, 23). The expression of nucleos(t)ides is highest immediately after parturition, with a general trend of decreasing concentration with advancing lactation in both bovine milk and human milk (2, 24–28).

It has been generally reported that nucleotides are present in higher amounts in human milk than in bovine milk (26, 28, 29). Qualitatively, there is a clear difference in the nucleotide monophosphate profile between mature bovine milk and mature human milk, the former containing measurable levels of guanosine 5′-monophosphate (GMP), inosine 5′-monophosphate (IMP), uridine 5′-monophosphate (UMP), cytidine 5′-monophosphate (CMP), and adenosine 5′-monophosphate (AMP), whereas the latter 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 used for quantitation (Tables 1 and 2). Nucleotide diphosphates and nucleotide sugars also contribute to the nucleotide pool in milks of both species (23–26, 30, 31).

In addition to free nucleosides, a number of other sources are available to the breast-feeding infant, such as nucleoproteins, polymeric nucleotides (nucleic acids), and nucleos(t)ide derivatives, which are digested in the infant's gastrointestinal tract by proteases, nucleases, phosphatases, and nucleotidases to yield physiologically available nucleosides (15, 32–35). Compared with the free nucleotide levels in human milk, the nucleoside equivalents available to the infant were underestimated by over 50% when all total potentially available nucleoside (TPAN) sources were determined (36). However, to the authors' knowledge, a direct comparison of the TPAN composition of human and bovine milks has not been reported.

Geographical and seasonal variations in the nucleotide and nucleoside levels that have been reported suggest that highly variable dietary habits impact on the qualitative and quantitative expression of nucleos(t)ides in human milk (26). In the case of ruminant species, herd feeding and animal husbandry

practices around the world are quite different and may contribute to geographical differences in the nucleos(t)ide levels expressed in bovine milks.

The predominant nucleotide-related compound in bovine milk is orotic acid, a precursor intermediate in pyrimidine synthesis. However, orotic acid is poorly salvageable by human infants (9) and is essentially absent in human milk for reasons that are currently not well understood (23, 25, 28, 37–39).

Two comprehensive reviews of compositional, nutritional, and biochemical aspects of endogenous nucleotides and nucleosides in bovine and human milks have been published (3, 4).

Pediatric Formulas

Bovine milk is the basis for the overwhelming majority of pediatric formulas, despite goat milk and soy protein finding a minor niche in this market. In view of the reported differences between the nucleotide levels in bovine milk and human milk, pediatric formulas are increasingly supplemented with nucleotides, a practice that is subject to regulatory controls by individual national bodies as defined by Codex (40). Despite gastrointestinal dephosphorylation to nucleosides (16, 32–34), which are the main form for intestinal absorption, supplementation is accomplished exclusively with 5'-mononucleotides.

Infant formulas were initially supplemented to levels equivalent to the free nucleotide and nucleoside concentration in human milk, up to a maximum concentration of 5 mg/100 kcal. In recent years, fortification of modern pediatric formulas with nucleotides to TPAN levels has subsequently been approved in more than 30 countries (41).

Despite the purported benefits of nucleotides in infant nutrition, the supplementation of pediatric formulas with nucleotides is controversial (8, 35, 42–44), as there is a lack of reproducibility in many of the findings of the beneficial effects of nucleotide supplementation in newborns (45). However, these pediatric formulas are currently considered to be safe (8, 16), although one recent study reported an increased risk of upper respiratory tract infection in infants fed nucleotide-supplemented formula (19).

Over 70 indigenous enzymes have been identified in milk (46). A number of these can influence the stability of nucleotide levels in dairy products. Thus, during pediatric formula production, there is a potential for exogenous nucleotide monophosphate degradation by indigenous milk enzymes. An absence of supplemented nucleotides, coupled with an increase in nucleoside levels above those normally expected in a bovine milk-based product, illustrates that dephosphorylation of nucleotides can occur in commercial pediatric formulas, attributable to the presence of residual active alkaline

phosphatase remaining after ineffective heat treatment (28). Further, Thorell et al. (47) have reported partial transformation of CMP and UMP to cytidine and uridine and GMP and AMP to guanine and uric acid in human milk. The presence of IMP reported in human milk by Janas and Picciano (23) has been postulated to be an artifact of enzymatic deamination of AMP after sample collection (36, 41, 48, 49). Similar enzymatic degradation of nucleotides added in the manufacture of pediatric formulas may be possible.

Analytical Techniques

Chromatographic analyses of nucleos(t)ides have been reviewed previously, the focus of which has generally been methods for use in clinical (50–52) and genomic (53) studies. Analytical methods for nucleos(t)ides in milk have been reviewed previously by Gil and Uauy (4), and the methods surveyed in this current review are summarized in Table 3.

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 sample integrity throughout the analytical process. This is particularly critical in the analysis of raw milk, as nucleos(t)ides 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 is 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.

Preparation of crude extracts.—Extraction of nucleos(t)ides from milk is usually achieved following initial protein precipitation with perchloric acid (PCA) or trichloroacetic acid (TCA), with the nucleos(t)ides remaining in the supernatant. Samples are then typically centrifuged and/or filtered, followed by neutralization 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 (54). Occurrences of spurious chromatographic peaks from buffer salts, and loss of nucleotides, are additional risks following perchlorate precipitation (50).

The extraction performed by Kobata et al. (31) involved the addition of 2 M PCA and, after centrifugation, the precipitate was washed with 0.2 M PCA and the extracts were combined. Gil and Sánchez-Medina (24) used 1 M PCA and filtered the sample through glass wool after centrifugation.

PCA was neutralized with potassium hydroxide (23, 24, 55, 56) or potassium carbonate (29) 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 (24, 25, 54). Thorell et al. (47) removed PCA by extraction with an equal volume of 0.5 M trioctylamine in 1,1,2-trichlorotrifluoroethane (Freon).

Johke and Goto (57) used a 10% TCA solution to remove proteins from cow milk and goat milk. After centrifugation, the protein residue was homogenized and re-extracted, the supernatants were combined, and excess TCA was removed by multiple extractions with diethyl ether. A similar procedure was performed in the analysis of samples of human milk (26). A 10–20% TCA solution used in the analysis of cyclic nucleotides was neutralized with solid calcium carbonate (58).

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. (55), 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 neutralization, 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 (51). Gill and Indyk (28) prepared unneutralized 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. (59) adjusted milk samples to pH 4.0 with concentrated formic acid, stored the samples at -20°C until analysis, and reported recoveries of 95–104%.

In contrast to acid precipitation, alternative methods of deproteination have been described. Tiemeyer et al. (60) added sodium dodecyl sulfate to bovine milk to a final concentration of 1% (w/v), mixed the milk with chloroform to eliminate proteins and lipids, and, after centrifugation, sampled the upper layer for analysis. Leach et al. (36) added 1 M sodium hydroxide to pooled milk samples and neutralized them to pH 7.0–7.5 with hydrochloric acid. Topp et al. (61) extracted fat from samples with acetone–dichloromethane (9:1, v/v), discarded the supernatant, and extracted

nucleosides from the sediment with 70% (w/v) ethanol. Proteins were then removed by addition of acetone, and the supernatant was concentrated by rotary evaporator before analysis.

The preferred sample extraction technique depends on the aim of the analysis. First, it is necessary to eliminate endogenous enzyme activity and then 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 neutralization is advocated before extract storage. In analyses where the total nucleotide content is required, elimination of enzyme activity without protein precipitation is needed for total recovery of protein-bound analytes.

Extract Fractionation

Further purification of protein-free extracts before analysis has often been recommended, and the early use of charcoal adsorption has been reported (31, 62). However, charcoal has variable adsorption characteristics, and more selective means of purifying extracts have been preferred in recent studies.

Phenylboronate affinity chromatography.—

The use of a phenylboronate-modified affinity gel to improve the chromatographic selectivity of nucleosides in urine has been described (63, 64). The affinity gel contains an immobilized phenylboronic acid functionality capable of binding *cis*-diols, such as those found on the 2- and 3-C of the ribose moiety of nucleosides. The affinity ligand is immobilized via its m-aminophenyl derivative to various gel supports. Under alkaline conditions, nucleosides are selectively retained as boronate complexes before elution with dilute acid.

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% (61), and TPAN, with recoveries of 76–104% (36). Furthermore, this phenylboronate gel was found 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 solution (55).

Reversed-phase chromatography.—

In the analysis of hypoallergenic infant formulas containing partially hydrolyzed proteins, chromatographic analysis is more complicated because of the co-elution of peptides under conditions that are suitable for the separation of nucleotide monophosphates. A solid-phase extraction (SPE) cleanup procedure before chromatography was evaluated, and initial results obtained with a Chromabond C_{18}ec column showed only partial recovery of cytidine, guanosine, and adenosine, whereas uridine was not retained on the column (55).

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 nucleotide mono- and diphosphates and nucleotide diphosphate sugars (24, 25, 31, 54, 57). Formate was subsequently removed by freeze-drying (24, 25, 54), by cation exchange (57) or by charcoal treatment (31).

More recently, a strong anion-exchange (SAE) 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% (55). 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 SAE columns from different manufacturers and established that two Bakerbond quaternary amine columns in series were optimal, with repeatability relative standard deviation (RSD) values of 0.8–2.7%, and recovery of individual nucleotides ranging from 93 to 113%.

Analytical Liquid Chromatography

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

A growing understanding of the role that nucleotides play in nutrition, coupled with rapid advances in the development of liquid chromatography (LC), has led to extensive application of this technique for the analysis of nucleos(t)ides. Before the availability of high-performance liquid chromatographic (HPLC) systems, final analysis of nucleotides obtained from crude extracts was performed by paper chromatography or paper electrophoresis, following a second low-pressure chromatographic separation (24, 25, 31, 54, 57). However, HPLC has now superseded other forms of chromatography applied to the determination of nucleos(t)ides.

Three main modes of LC are used in the analysis of nucleos(t)ides: ion-exchange chromatography (IEC), reversed-phase liquid chromatography (RPLC), and ion-pair reversed-phase liquid chromatography (IP-RPLC).

Ion-exchange chromatography.—

IEC 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 behavior of nucleotides under IEC 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 (53).

Separation of nucleotide mono-, di-, and triphosphates of adenosine, guanosine, inosine, xanthosine, cytidine, uridine, and thymidine was achieved with an SAE column (Partisil 10-SAX) and an acidic phosphate buffer gradient (Figure 2; 65). This method was also applied in the analysis of nucleotide mono- and diphosphates in human milk (23). Isocratic elution was used for the analysis of human milk by a similar approach, and good separation of nucleotide monophosphates was achieved (56).

Reversed-phase liquid chromatography.—

With the development of robust stationary phases based on porous silica and flexibility in mobile phase optimization, RPLC, with or without the addition of ion-pair reagents, has become the method of choice for the analysis of nucleos(t)ides in milks.

The separation of nucleotides by RPLC is somewhat limited with conventional C_{18} columns because of inherently poor interaction of the highly polar analytes with the non-polar C_{18} phase under the required conditions of low organic modifier content, resulting in poor retention and resolution. However, by increasing the ionic strength and reducing the pH through the addition of acidic phosphate buffer, nucleotides are adequately retained and resolved, with the order of elution typically correlated with hydrophobicity. Organic modifiers such as methanol or acetonitrile added to phosphate buffer can facilitate improved resolution (52). 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 (66–68). 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 µBondapak C_{18} column with isocratic elution of a 0.01 M ammonium phosphate mobile phase adjusted to pH 6.0 (60). Human milk and infant formulas were analyzed using a µBondapak C_{18} column with a phosphate buffer–methanol–water linear gradient. Detection of the nucleotide monophosphates, nucleosides, and nucleobases was possible, although baseline resolution was not always achieved, and a second protocol was necessary to separate CMP from orotic acid (47). Nucleosides and methylated nucleosides in human milk were quantitated with ternary elution gradient of 0.01 M ammonium phosphate buffer–methanol–acetonitrile (61).

Recently, Gill and Indyk (28) developed a method for the simultaneous analysis of nucleotide monophosphates and corresponding nucleosides in human and bovine milks, skim milk powders, and infant formulas using RPLC (Figure 3). 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 with the compliance-critical nucleotides.

Ion-pair reversed-phase liquid chromatography.—

IP-RPLC has become the prevalent technique for the analysis of nucleotides in milk and pediatric products in recent years. The ionic nature of the phosphate ester facilitates strong interactions with cationic ion-pair reagents at the appropriate pH, thereby enhancing nucleotide retention and resolution. 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.

Spherisorb C_{18} column with tetrabutylammonium hydrogen sulfate (TBAHS) as ion-pair reagent and gradient elution was used for the analysis of dairy products (Figure 4; 29, 69). Perrin et al. (55) described a method based on isocratic elution with a mobile phase incorporating tetrabutylammonium dihydrogen phosphate as ion-pair reagent, where two Nucleosil 120-C_{18} columns in series were required for adequate resolution. Sugawara et al. (26) used a Capcellpak C_{18} column with TBAHS for the analysis of nucleotide mono-, di-, and triphosphates in human milk. 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.

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Automated dual column system.—

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 utilization of an *m*-aminophenylboronic acid substituted gel and column switching, online dual column cleanup and analysis of nucleosides in protein-free extracts was achieved (70).

Further development of this technique allowed for the analysis of proteinaceous material such as milk (59, 71). With a novel bonded-phase material prepared by immobilization of phenylboronic acid to a size exclusion gel support, two different modes of separation based on size exclusion and affinity were simultaneously exploited and applied to the analysis of nucleosides in human and bovine milks (2, 27). Martin and Schlimme (72) reported the use of Ca^{2+} and Mg^{2+} ions (50 mmol/L) to mask the negative charge from the nucleotide phosphate group in the simultaneous analysis of nucleotides and nucleosides. The recovery of AMP was acceptable (86–97%), but the recoveries of CMP, GMP, and UMP were much lower and further method optimization is required. Without the incorporation of these cations, nucleotides remained unbound to the pre-column.

Peak identification.—

Pyrimidines and purines readily absorb light in the UV range between 240 and 270 nm. However, because the chromatographic pattern of milk extracts is frequently complex, characterization of putative peaks by co-chromatography with detection at a single wavelength is generally insufficient for unambiguous identification.

The ratio of the absorbances at 254 and 280 nm, co-elution with authentic standards and enzymatic conversion were used for confirmation of peak identity of nucleic acid metabolites in bovine milk (60). Characteristic peak shifting, or quenching, due to pre-chromatographic chemical or enzymatic treatments can assist in the identification of nucleos(t)ides. After a tentative classification of a chromatographic peak, either a substrate-specific enzyme or a reagent known to selectively modify the target analyte is used, such that the peak disappears with the possible appearance of an additional peak in the subsequent chromatogram. Thus, pre-chromatographic modifications by enzymatic (e.g., adenosine deaminase, purine nucleoside phosphorylase) and chemical (e.g., periodate oxidation, Dimroth rearrangement, glyoxal modification) treatments have been used in the identification of nucleosides (2, 27).

In recent years, photodiode array (PDA) detectors have been increasingly used to detect and identify of nucleos(t)ides in milk (28, 29, 47, 55, 69). 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 maximized and chromatographic interferences may be minimized.

In general, the dominant strategy used for nucleos(t)ides analysis in milks and pediatric formulas has been protein removal by acid precipitation, followed by HPLC-UV analysis of the crude or fractionated extract. However, the field of clinical chemistry has generated numerous methods for the analysis of nucleos(t)ides by using more recently developed techniques such as capillary electrophoresis (73), matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS; 74), and LC-MS (75). Such techniques offer a high level of sensitivity and will be increasingly applied to the analysis of milk-based nucleos(t)ides in the future.

Enzymatic Analysis

An enzymatic assay for the determination of individual nucleotide monophosphates and total nucleotides was developed by Hernández and Sánchez-Medina (54) based on the method of Keppler (76). The method was applied to the analysis of cow, goat, sheep (24), and human milks (25). Nucleotide monophosphates 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 catalyzed decrease of NADH at 340 nm (AMP, CMP + UMP, GMP), whereas UMP was determined by enzymatic conversion to UDP-glucose. The recovery of AMP, CMP, GMP, and UMP was estimated at 96% with an RSD between determinations of < 4%, comparing favorably to an ion-exchange technique (54). Determination of UDP-glucose in milk extracts was performed by a modification of the method of Keppler and Decker (77), whereby an increase in absorption at 340 nm (due to the stoichiometric reduction of NAD^+ catalyzed by UDP-glucose dehydrogenase) was measured. UDP-galactose was determined by conversion to UDP-glucose catalyzed by UDP-glucose-hexose-1-phosphate uridylyltransferase in the presence of glucose-1-phosphate. Free nucleotide monophosphates 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 milk (54).

Although 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 nucleotide and nucleoside species. In the TPAN analysis of human milks, a number of enzymes have been used to characterize 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 with phosphatase. In this manner, contributions from polymeric nucleotides, monomeric nucleotides, nucleosides, and nucleotide adducts to TPAN were separately estimated (36, 78). The recovery of nucleotides ranged from 76%

for guanosine to 104% for cytidine, with an RSD of 2.0% for cytidine, guanosine, and adenosine, and 3.6% for uridine (36).

Adenosine 5′-triphosphate (ATP) in bovine milk was measured enzymatically using the luciferase-ATP reaction, with light detection by scintillation counter (79). Luciferase catalyzes the oxidative decarboxylation of D-luciferin and, when ATP is the limiting reagent, the photon count is proportional to the ATP present.

Radioimmunoassay

The cyclic nucleotides adenosine 3′,5′-cyclic monophosphate (cAMP) and guanosine 3′,5′-cyclic monophosphate (cGMP) in milk were determined using a radioimmunoassay technique. This assay is based upon competitive binding between the cyclic nucleotide and an isotopically labeled derivative for a specific cyclic nucleotide antibody (58, 80).

Microbiological Assay

Larson and Hegarty (81) described a microbiological assay for the determination of orotic acid pyrimidine nucleotides in ruminant milks. This method is of limited applicability because only pyrimidine nucleotides are measured and they are not individually differentiated.

Conclusions

The analysis of nucleos(t)ide content in mammalian milks and infant formulas may be required to satisfy a variety of purposes, including food safety, nutritional database information, regulatory compliance, quality control, quality assurance, and clinical studies. The different functions of academic, commercial, and regulatory laboratories will therefore influence method selection, and each of the analytical techniques available has attributes that suggest their use, depending on the intended purpose of the analysis.

Over the past decade, HPLC has become the dominant technique for the analysis of nucleotides, nucleosides, and nucleobases in milks and milk products. 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. However, despite the abundance 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 in this area difficult to standardize. Therefore, there is a clear need for an HPLC-based reference method to measure intact nucleotides. It is probable

that in the near future, a method based on LC-MS^n will be developed to support the more frequently used HPLC-UV methods currently in use.

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Table 1. Free nucleotide 5′-monophosphate ranges in mature human milk (µmol/dL)[a]

AMP[b]	CMP	GMP	IMP	UMP	Reference
0.3	3.3	0.2	—[c]	0.4	(31)
1.5–2.6	1.8–2.6	nd[d]–0.3	—	0.7–1.3	(25)[e]
0.4–0.5	1.0–1.6	0.3–0.5	0.6–0.8	1.0–1.7	(23)[f]
nd–0.4	0.3–4.3	nd–0.1	nd–0.1	nd–0.3	(26)
0.2–1.9	4.1–10.6	0–0.6	nd	0.5–2.1	(47)[g]
nd	nd–1.3	nd	nd	0.2–0.5	(28)

[a] Collated results for milks > 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] — = Not reported

[d] nd = Not detected

[e] Adapted from results at 15 days, 1 month, and 3 months post-partum

[f] Adapted from results reported as mg/dL at 4, 8, and 12 weeks post-partum

[g] Adapted from range of results reported as µmol/L at 3–24 weeks post-partum

Table 2. Free nucleotide 5′-monophosphate ranges in mature bovine milk (µmol/dL)[a]

AMP[b]	CMP	GMP	IMP	UMP	Reference
nd[c]	0.9	nd	—[d]	nd	(31)
nd–0.4	0.9–2.7	nd	—	nd	(30)
1.8–2.9	1.2–4.9	nd	—	nd	(24)[e]
2.0–2.8	1.9–3.3	nd	—	nd	(24)[f]
—	0.3	0.2	—	—	(57)[g]
Trace	3.0	nd	nd	nd	(69)[h]
0.1	1.0	nd	0	0.1	(26)
nd	0.2–0.3	nd	nd	nd	(28)

[a] Collated results for milks > 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] nd = Not detected

[d] — = Not reported

[e] Ion-exchange chromatography

[f] Enzymatic analysis

[g] Adapted from results reported as µmol/L

[h] Adapted from results reported as mg/dL

Table 3. Summary of methods for analysis of nucleos(t)ides in milks and infant formulasa

Analytes	Sample	Preparation of crude extract	Extract cleanup	Analysis	Ref.
Nucleotide 5'-monophosphates, nucleotide diphosphate sugars	Cow and goat milk	10% v/v TCA, removed with diethyl ether	Ion-exchange chromatography	Paper chromatography	(57)
Nucleotide 5'-monophosphates, nucleotide diphosphates, nucleotide diphosphate sugars	Cow and human milk	2M PCA, neutralized with 2M potassium hydroxide	Ion-exchange chromatography	Ion-exchange chromatography, paper chromatography	(31)
Pyrimidine nucleotides	Cow and sheep milk	0.1M acetate buffer, adjusted to pH 7.0 with sodium hydroxide	–	MBA	(81)
Adenosine 5'-triphosphate	Cow milk	Adjusted to 2% TCA, neutralized to pH 7.4 with 2M sodium hydroxide	–	Enzymatic assay	(79)
Cyclic nucleotides	Human milk	10–20% TCA, neutralized with calcium carbonate	–	RIA	(58)
Nucleotide 5'-monophosphates, nucleotide diphosphates, nucleotide diphosphate sugars	Cow, goat, sheep, and human milk	1M PCA, neutralized to pH 6.5–7.0 with 5M potassium hydroxide	Ion-exchange chromatography	Paper chromatography	(24, 25)
Nucleotide 5'-monophosphates, nucleotide diphosphate sugars	Cow, goat, sheep, and human milk	1M PCA, neutralized to pH 7.5–8.0 with 0.2M triethanolamine–0.16M potassium carbonate	–	Enzymatic analysis	(24, 25)
Nucleotide 5'-monophosphates, nucleotide 5'-diphosphates	Human milk	0.6M PCA, neutralized to pH 6–7 with 3M potassium hydroxide	–	Ion-exchange HPLC	(23)
Nucleotide 5'-monophosphates, nucleosides, nucleobases	Cow milk	Addition of sodium dodecyl sulfate to 1%	–	RPLC	(60)
Nucleosides, modified nucleosides	Cow, goat, and human milk	pH adjusted to 3.4–4 with formic acid	Phenylboronate affinity and size exclusion chromatography	RPLC	(2, 27, 59)

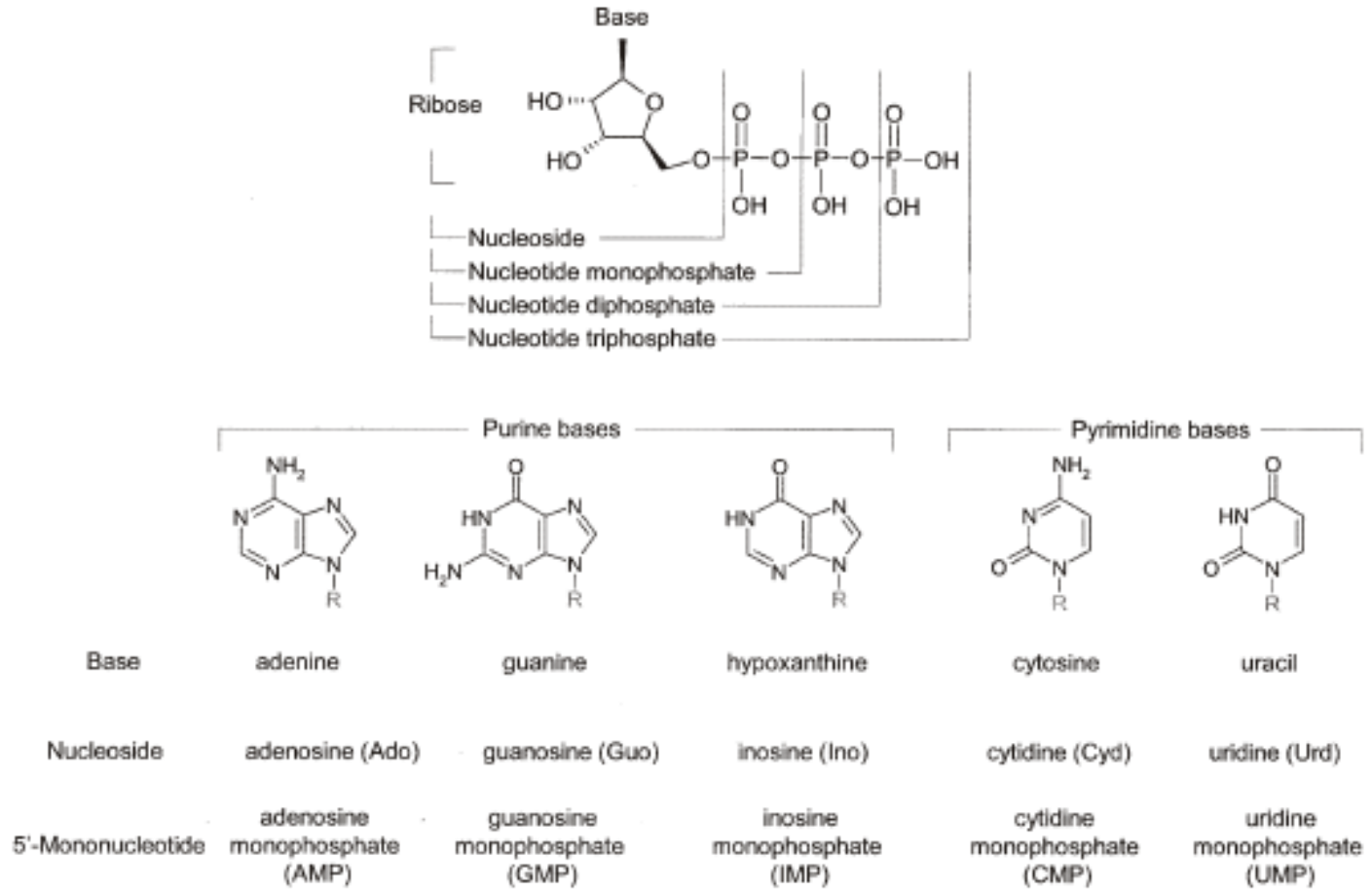

Figure 1. Structural relationship between nucleotides, nucleosides, and nucleobases

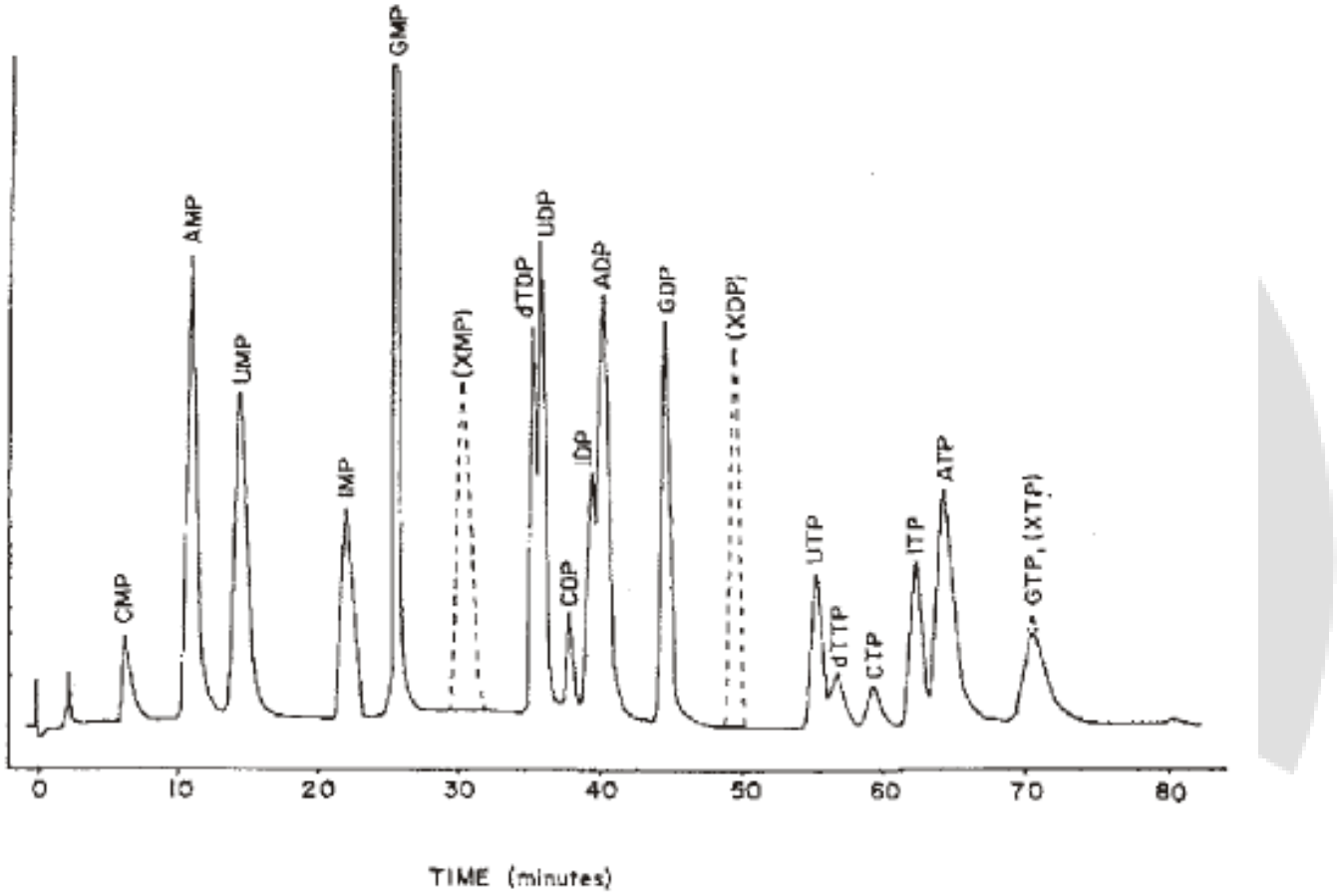

Figure 2. Ion-exchange chromatographic separation of mono-, di-, and triphosphate nucleotides of adenine, guanine, hypoxanthine, xanthine, cytosine, uracil, and thymine (from ref. 65 with permission from Elsevier)

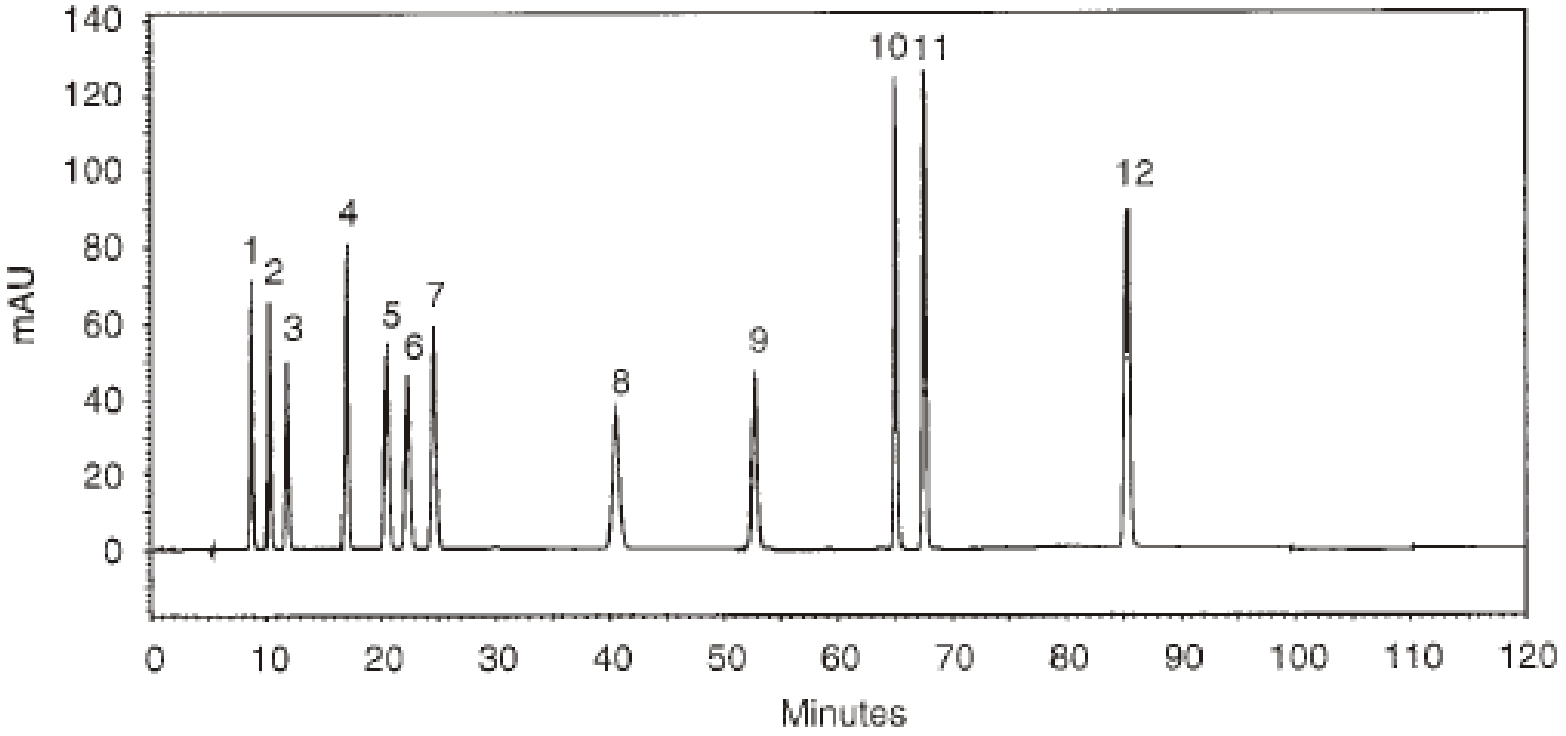

Figure 2. Reversed-phase chromatographic separation of a standard mixture of (1) cytidine 5′-monophosphate, (2) orotic acid, (3) uridine 5′-monophosphate, (4) uric acid, (5) guanosine 5′-monophosphate, (6) inosine 5′-monophosphate, (7) cytidine, (8) uridine, (9) adenosine 5′-monophosphate, (10) inosine, (11) guanosine, and (12) adenosine (from ref. 28 with permission from Elsevier)

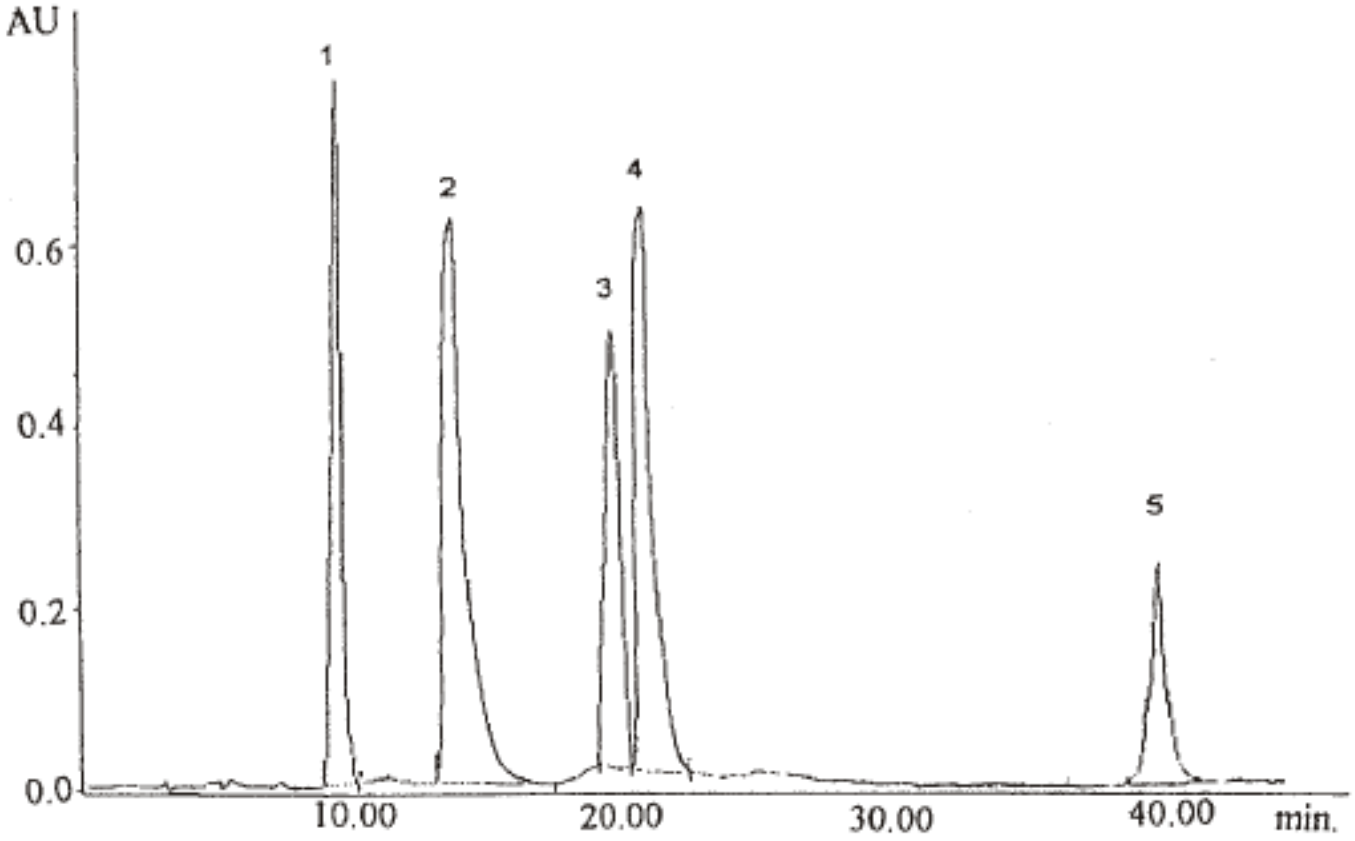

Figure 3. Ion-pair reversed-phase chromatographic separation of 5′-nucleotides: (1) cytidine 5′-monophosphate, (2) uridine 5′-monophosphate, (3) guanosine 5′-monophosphate, (4) inosine 5′-monophosphate, and (5) adenosine 5′-monophosphate (from ref. 69 with permission from Elsevier)