SPECIAL GUEST EDITOR SECTION

Current Methods for the Analysis of Selected Novel Nutrients in Infant Formulas and Adult Nutritionals

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Infant formula is designed to provide the human infant with a sole source of nutrition and it is intended to imitate breast milk. In recent years, advances in the science of infant nutrition have led to an increasing number of novel ingredients that are supplemented into infant formula. As the list of these nutritionally important nutrients is lengthy, this review summarizes contemporary analytical methods that have been applied to a representative selection (lutein, carnitine, choline, nucleotides, inositol, taurine, sialic acid, gangliosides, triacylglycerides, oligosaccharides, α-lactalbumin, and lactoferrin).

Despite evolutionary adaptation to the species specificity of mammalian milk, there is abundant evidence for the use of unmodified domesticated animal milk in human infant feeding as far back as 2000 B.C. Fast forward to the modern era, and since the early to mid-20th century, the continual humanization of primarily bovine milk has been the fundamental intent that supports the manufacture of modern infant formulas that are designed to provide the human infant with a sole source of nutrition that more closely resembles breast milk. Indeed, such initiatives have also been adopted where other sources, such as caprine milk and soy, have been exploited. Other articles in this *Special Guest Editor Section* discuss the significant compositional differences between bovine milk and human milk and the evolution of industry processing and formulation advances to address this complex issue.

International legislation mandates a very wide range of nutritional components (>30) that must be declared as within accepted limits to ensure compliance with regulatory bodies across borders, and many of these components with respect to analytical considerations have been discussed elsewhere in this *Special Guest Editor Section*. Additionally, many other ingredients that are considered nutritionally important in the humanization of infant formula are increasingly used during manufacture to enhance the status of infant formula within the medical community.

As the list of all potential ingredients is lengthy, we have limited this review of analytical methods to a representative selection that has been applied to the analysis of nutrients that are commonly used but that do not fall easily within convenient or obvious classifications.

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Lutein

Lutein belongs to the xanthophyll family of oxygenated carotenoids and is naturally present in many foods, particularly vegetables and fruits, as the all-E (all-*trans*) isomer. Lutein is a dihydroxy derivative of α-carotene and its molecular structure underlies its importance in protecting against oxidative and blue-light damage in the retina (1–3). Of the carotenoids, only lutein and its structural isomer zeaxanthin are specifically accumulated in neonatal macular retinal epithelium tissue within the eye. Research has confirmed further that lutein is the predominant carotenoid in human brain tissue, with implications for cognitive neural development (4).

As the carotenoid composition of mammalian milk is significantly influenced by diet, bovine milk is dominated by β-carotene and lutein, whereas, in contrast, human milk contains a qualitatively wider range of carotenoids. The supplementation of bovine-milk–based pediatric formulas with carotenoids is increasingly being practiced, although not yet routinely. Although the evidence for efficacy in infant visual development is not unequivocal, lutein has been progressively added into infant formula to levels that are equivalent to those found in human milk, thereby facilitating elevated neonatal plasma lutein concentrations that are comparable with those found in breast-fed infants (5–9).

Analytical methods for carotenoids in foods have been reviewed previously (10–15). In samples with high fat content, such as milk and infant formula, alkaline or enzymatic hydrolysis techniques have commonly been applied to remove potentially interfering lipid, to improve recovery, and to convert xanthophyll esters in advance of solvent extraction (8, 10, 11, 16–18).

Although reversed-phase HPLC (RPLC) and ultra-HPLC (UHPLC) platforms with either C_{18} or C_{30} column chemistries are most commonly used, normal-phase separations have also been reported because of superior retention of polar xanthophylls and enhanced resolution of carotenoid isomers (13, 14, 19, 20). Because of subtle differences in the UV-visible absorbance spectra of various carotenoids, photodiode array (PDA) detection is considered essential for identification. More recently, mass spectrometry (MS) detection methods have been used increasingly to identify constituent carotenoids further (13, 15, 16, 21, 22). Nonetheless, in view of the potential presence of both multiple structural and *cis-trans* isomeric forms in foods, UHPLC using an as-yet commercially unavailable UHPLC C_{30} carotenoid column, coupled to both PDA detection and

high-resolution MS, has been proposed for the unequivocal analysis of all carotenoids, including lutein (23).

Methods for the analysis of lutein in infant formula to support label claims have been reported as using RPLC following, most commonly but not exclusively, saponification and solvent extraction techniques (8, 24–26). The Stakeholder Panel on Infant Formula and Adult Nutritionals (SPIFAN) membership supported multianalyte testing of lutein with other carotenoids to include α-carotene, β-carotene, and lycopene. A *Standard Method Performance Requirement* (SMPR®) that mandates both chromatographic resolution of lutein and zeaxanthin and the differentiation of *cis*- and *trans*-lutein isomers has been defined. Candidate methods for potential AOAC *Official Method* status are currently under review for their potential to achieve official dispute resolution method status.

Carnitine

L-Carnitine is a quaternary ammonium compound that is biosynthesized from the amino acids lysine and methionine and is considered a conditionally essential nutrient, with dietary supplementation being particularly important during infant development. The key functional role of carnitine is the transport of fatty acids across the mitochondrial membrane where they undergo β-oxidation for energy production (27). Carnitine is found in milk as free carnitine and short-, medium-, and long-chain acyl carnitine. However, as acid-insoluble long-chain acyl carnitines are a minor component, total carnitine quantitation is usually restricted to the sum of free carnitine and short- and medium-chain acyl carnitine forms (28).

Strategies for sample preparation depend on which carnitine forms are the targets of the analysis. For the purpose of routine product release, analysis of infant formula is often limited to analysis of supplemented and endogenous free carnitine. Sample preparation for the analysis of free carnitine is straightforward, with protein removal being achieved by the use of acid (29, 30) or centrifugal ultrafiltration (31).

As carnitine lacks a chromophore, the analysis of carnitine in infant formula in the past has commonly been performed by an enzymatic assay (30). The method is based on the carnitine acetyltransferase-initiated acetylation of carnitine and the subsequent stoichiometric release of coenzyme A, which reacts with dithio-nitrobenzoate to form the chromophore detected at 412 nm. To detect total carnitine, different saponification strategies allow soluble and insoluble esters to be determined (28). This technique has been automated to speed up the routine analysis of infant formulas using flow injection analysis (FIA; 32).

The proliferation of LC-MS for routine analysis has led to the development of less labor-intensive methods for the detection and quantitation of carnitine. A microwave-assisted acid hydrolysis technique was developed for the analysis of free carnitine and total choline in four food-based certified reference materials including infant formula (33). A simplified technique for the analysis of free carnitine by dilution and filtering makes use of the high degree of selectivity and sensitivity of modern LC-MS/MS instruments (34).

Sample preparation for total carnitine typically consists of acid precipitation of protein followed by alkaline hydrolysis to release short- to medium-chain acyl carnitines (28, 30, 35), although direct saponification without previous protein removal is described in AOAC First Action Method **2014.17** (36).

Under reversed-phase conditions, chromatographic separation of carnitine is challenging because of its ionic nature. Ion pair (IP) chromatography using heptafluorobutyric acid has been used to retain carnitine on C_8 (34) and C_{18} (29) stationary phases. Alternative column chemistries can offer significant advantages for the retention of polar and ionic analytes without the need for IP reagents and hydrophilic interaction liquid chromatography (HILIC) columns (36), and mixed-mode reversed-phase and ion-exchange columns (33, 35) have been used. Mixed-mode columns allow for a high degree of selectivity by modification of mobile-phase pH, ionic strength, and organic solvent, which may be beneficial, particularly in the analyses for which MS/MS is not used. Electrospray ionization (ESI) in positive mode with either MS (29, 33) or MS/MS (34–36) has been applied to the quantitative analysis of carnitine.

Choline

Choline is a low molecular mass, quaternary amine, zwitterionic compound that is considered a conditionally essential nutrient, especially for the human infant dependent on a single nutritional source. In the human, choline is the precursor for the neurotransmitter acetylcholine, which is an important structural and signaling component of cell membrane phospholipids and, via betaine, functions as a significant source of labile methyl groups in intermediate metabolism. Choline plays a critical role during fetal neural tube development, stem cell proliferation, and apoptosis, with significant consequences for brain structure and lifelong function. Choline deficiency can manifest as fatty liver and hemorrhagic kidney necrosis, with prolonged deficiency leading to potential hepatic, renal, pancreatic, memory, and growth disorders (37, 38).

Choline is present in a wide range of foods as free choline and in various metabolically interrelated esterified forms including phosphocholine, phosphatidylcholine, glycerophosphocholine, sphingomyelin, and acetylcholine (39). Although choline is biosynthesized endogenously in the human body, current guidelines recommend an exogenous dietary intake for infants and adults to maintain health (40). Choline is actively transported from maternal blood to milk, and in recognition of its nutritional importance, pediatric formulas are increasingly supplemented with either the free salt or choline bound in the form of lecithin (approximately 25% w/w phosphatidylcholine) to enable infants to maintain serum levels that are equivalent to those of breast-fed counterparts.

Analytical strategies for the estimation of the total choline content of a food require the determination of either the aggregate of free and individual esterified forms or free choline following acid and/or enzymatic hydrolysis of choline esters, and they have been comprehensively reviewed (41). Early end-point analytical strategies based on gravimetric determination of the reineckate complex, spectrophotometry of the enneaiodide complex, microbial methods, and physiological methods are no longer used. The analysis of choline and choline esters in foods has been described using enzymatic–colorimetry (42, 43), biosensor (44, 45), FIA-electron capture detection (46, 47), NMR (48), GC-MS (49), and capillary electrophoresis (CE)–indirect UV (50) methods. More recently, methods based on HPLC-MS (29, 33, 51–53), HPLC–fluorescence (54, 55),

and high-performance ion chromatography (HPIC)–suppressed conductivity (56) have proliferated. HPLC-based methods using HILIC columns have demonstrated enhanced capability for the simultaneous separation of individual choline species that differ widely in hydrophobicity, and the commercial availability of isotope-labeled standards will facilitate the application of isotope-dilution MS methods (41).

The AOAC INTERNATIONAL SPIFAN initiative has adopted two procedures, based on HPLC-MS/MS (57) and HPIC–conductivity (58), as Official First Action for application to infant formulas and adult nutritionals, with the latter currently being subject to a multilaboratory study for possible adoption as a dispute resolution Final Action method.

Nucleotides

Nucleotides perform critical roles in cellular function as components of coenzymes, as building blocks of nucleic acids, and as key intermediaries of the transfer of metabolic energy. Numerous studies have reported the physiological benefits of nucleotide addition to infant formula (59), although supplementation remains controversial as unequivocal clinical evidence is lacking (60). However, infant formula products are currently considered safe when supplemented to levels that are equivalent to the free nucleotide concentrations in human milk (61).

Methods for the analysis of nucleotides in infant formula have been reviewed previously (62). In recent years, advances in column and instrument technology have led to the development of new methods that offer fast, reliable, and accurate analyses of nucleotides in infant formulas.

Various approaches have been used to remove protein material before chromatographic analysis. Protein precipitation with acids, such as perchloric (63), formic (64), hydrochloric (65), trichloroacetic (66), and acetic (67, 68), followed by neutralization, filtering, or centrifugation steps, has been used. Acid precipitation offers the advantage of a simple, rapid, and inexpensive sample preparation, although interfering artifacts that can complicate the chromatographic analysis may be introduced. Strong anion exchange solid phase extraction (SPE) (69) and physical removal by ultracentrifugation (70–72) generally produce cleaner extracts for analysis although they can add significantly to the cost per sample.

A variety of chromatographic techniques have been applied to separate nucleotides in infant formula extracts. Because of the anionic structure of nucleotides, ion-exchange chromatography (IEC) is a suitable technique for the separation of nucleotides with predictable retention behavior because the interactions between the negatively charged analyte and the positively charged stationary phase are predominantly electrostatic. Anion-exchange chromatographic analysis of infant formula extracts using quaternary amine stationary phases (66, 70) and a dendrimer stationary phase (65) has been reported, although the use of phosphate-containing mobile phases precludes its use in LC-MS analyses. In the past, IP-RPLC was the most prevalent technique for the analysis of nucleotides in infant formulas (62); however, in recent years only one study has reported the use of IP-RPLC to separate nucleotides in infant formula extracts (63). Advances in column technologies have led to the availability of more options for the retention of highly polar compounds without the need for IP reagents. This is of particular advantage in MS methods in which IP reagents either might be incompatible or might significantly complicate spectra. RPLC and HILIC methods have been developed to analyze nucleotides in infant formula, with sufficient retention and resolution demonstrated (64, 68, 69, 71). HILIC is particularly advantageous when coupled to MS detectors, in that the high organic content of the mobile phase enhances spraying and desolvation, thereby increasing signal intensity; also, there is less need for gradient elution, thereby reducing the impact of variances in the composition of the mobile phase on ion suppression.

CE has been used to separate nucleotides in infant formula (67, 72). Because of their inherent negative charge, CE readily analyzes nucleotides, and such methods are generally considered faster and cheaper than comparable LC methods, because inexpensive buffer salts rather than organic solvents are used.

Although LC detection using UV is still common (63, 65–70), MS and MS/MS with ESI in both positive (64, 71, 73) and negative (72) modes have increasingly been adopted. The use of MS/MS with isotope-labeled internal standards provides for a highly selective and accurate assay.

A method for the analysis of nucleotides in infant formula has been studied collaboratively (74) as part of the SPIFAN process to validate reference methods in infant formulas, and it is now an AOAC Final Action method (75, 76).

Inositol

Inositol is a cyclohexitol sugar that can exist in nine possible stereoisomeric forms, although only myo-inositol has confirmed biochemical significance in eukaryotic cells as the structural basis of several secondary messengers, including multiple inositol phosphates, phosphatidylinositol, and phosphatidylinositol phosphate lipids (77–79). In humans, myo-inositol was initially classified as a B-group vitamin, as a dietary source was originally considered essential. However, it is now regarded as a conditionally essential nutrient, given that most mammals, including humans, are capable of its in vivo synthesis from glucose-6-phosphate (79).

Although present as free myo-inositol, the compound is more commonly found in foods with various degrees of phosphorylation. For example, inositol hexaphosphate (phytate), which is present in many plant-based foods, is considered to be unavailable in humans and has historically been described as an antinutrient because of its binding of niacin and dietary minerals with consequent potential deficiency symptoms. More recently, however, it has been demonstrated that phytate may indeed contribute to human health, and its prevalence in mammalian neural tissue has also been confirmed, although its function within the central nervous system remains poorly understood (78, 80). Whereas phytate is not yet generally considered as a nutritional source of myo-inositol for humans, other lower phosphates such as inositol 1,4,5-trisphosphate probably are, as evidenced by their common occurrence in blood (81). Further, as the inositol bound within phosphatidylinositol is readily available to humans because of phospholipase activity within the digestive tract, analytical methodology for foods should ideally include contributions from both phosphatidylinositol and inositol phosphates (n = 0–3), but should possibly exclude phytate.

Milk is a significant source of myo-inositol, with human milk containing higher levels than bovine milk, and myo-inositol is

therefore added to infant formulas to ensure against potential deficiency during early neonatal development (82). Although the Codex Alimentarius Commission legislates a minimum of 1 mg/100 kJ for infant formulas, it does not specify whether inositol phosphates, including or excluding phytate, or phosphatidylinositol molecular species are to be included.

The analysis of free myo-inositol in foods and biological tissues has generally been facilitated following a simple protein precipitation step in advance of end-point analysis. Methods targeting the aggregate of free and bound forms have used acidic or alkaline hydrolysis followed by traditional microbiological assay (83), enzymatic assay (84), GC subsequent to previous derivatization (85–88), HPLC or high-performance anion-exchange chromatography (HPAEC) techniques using UV, evaporative light-scattering detection (ELSD), or pulsed amperometric detection (PAD) (82, 89–94), and HPLC or UHPLC coupled with MS (95–97). A review has summarized the techniques that are applicable for the analysis of inositol and related compounds, albeit specific to soybean tissues (98).

A GC method for the compliance analysis of the total myo-inositol content in milk, infant formula, and adult nutritional products, using acid hydrolysis and conversion to the volatile trialkylsilyl derivative, has been reported (88). The AOAC INTERNATIONAL SPIFAN initiative has adopted two procedures, both based on acid hydrolysis followed by HPAEC-PAD, as Official First Action (99, 100) for application to infant formulas and adult nutritionals. The methods prescribe equivalent HPAEC-PAD conditions and, although both yield comparable results when applied to the analysis of free myo-inositol, there are differences when applied to bound analyte because of fundamental differences in sample preparation. AOAC **2012.12** (100) adopts an exhaustive acid hydrolysis to recover total myo-inositol before end-point analysis, whereas AOAC **2011.18** (99) aggregates free and phosphatidyl-bound myo-inositol, which are estimated in two separate determinations. The latter method has been adopted as an AOAC Final Action method following a multilaboratory study.

Taurine

Taurine, 2-aminoethanesulfonic acid, is prudently added to infant formulations as part of the widespread strategy to match infant formula with human breast milk, and to adult energy drinks, perceivably to improve brain cognition. As it is involved in many biochemical reactions, a deficiency of taurine can have serious consequences for individuals who are unable to process sufficient in vivo concentrations (101, 102). Sometimes referred to as an amino acid, it is not found within protein structures and it is easily extracted from milk, milk powders, and infant formulations (103). As it is a small molecule without a chromophore, detection of the extracted taurine requires derivatization or electrochemistry in the same way as for many other amino acids or carboxylic acids.

Traditional amino acid profiles were obtained using ion-exchange columns to separate underivatized amino acids, followed by postcolumn ninhydrin reaction in discrete amino acid analyzers (104, 105). Similarly, AOAC **993.13** (106) describes a method for free amino acids in premixes using ion-exchange separation and postcolumn ninhydrin detection (107). Such devices are still useful, but they have largely been replaced by HPLC and UHPLC techniques that are less expensive and more versatile.

Following deproteinization of liquid or reconstituted powder with Carrez solutions, AOAC **997.05** (108) describes the analysis of taurine in dairy products using a precolumn dansyl chloride derivatization reaction and reversed-phase HPLC with UV detection (254 nm) or fluorescence detection (ex: 330 nm, em: 530 nm), a method that has been widely used (109). Biondi et al. (110) also used dansyl chloride derivatization to detect taurine in formulations, but used heat to accelerate the reaction.

Other derivatization chemistries in precolumn format, such as 4-fluoro-7-nitrobenzofurazan (111) and 2,4-dinitrofluorobenzene (112), have been used for taurine detection in milk and beverages. Taurine in cheese has also been separated from many other free amino acids as phenylisocyanate derivatives (113). The authors also describe many other derivatization reagents that are available for the precolumn or postcolumn determination of amino acids, many of which are potentially suitable for the detection of taurine. In offline mode using manual derivatization, slow-reacting derivatives are preferred, thereby improving method performance. The increasing availability of injector automation has provided new focus to instrumental precolumn derivatization with *o*-phthalaldehyde, a technique that has been successfully used with UV detection for infant formula and breast milk (114), with fluorescence detection for biological fluids (115), and electrochemically for tissues (116). If AOAC **997.05** (108) is to be replaced as the preferred SPIFAN method for taurine, then an automated *o*-phthalaldehyde reaction provides a potentially good option.

A viable alternative to precolumn derivatization is the use of cation IEC with PAD, in which taurine is detected electrochemically in its native state. This technology has been demonstrated successfully for the quantitation of taurine in dietary supplements and urine (117), various mammalian milks (118), and a wide variety of foods (119).

Despite the advantages demonstrated by LC-MS for detecting taurine in supplemented beverages (120) and the need for ultimate sensitivity and selectivity for taurine and its metabolites (121), routine quality assessments of taurine in infant formulas are probably adequately provided by existing reversed-phase HPLC or IEC technologies. This situation would change for multicomponent testing or in matrices in which taurine is not easily discriminated, such as protein hydrolysates.

Sialic Acid

Sialic acid is the trivial name for a family of acylated derivatives of a 9-carbon carboxylated monosaccharide, with the most common forms being Neu5Ac (*N*-acetylneuraminic acid) and Neu5Gc (*N*-glycolylneuraminic acid). Human milk contains predominantly Neu5Ac bound to free oligosaccharides, whereas bovine milk contains both Neu5Ac and significant quantities of Neu5Gc bound to glycoproteins. Although the nutritional importance of sialic acid is not yet well understood, it might be a conditionally essential nutrient and may play a role in brain development (122–124).

The analysis of sialic acids in dairy products has been reviewed (122). Infant formula samples are typically reconstituted in water; however, pretreatment by removal of the fat layer post-centrifugation and protein precipitation with trichloroacetic acid (10% w/v) has also been reported (125).

Acid hydrolysis using sulfuric acid is typically performed with sample digestion at 80°C for 1–2 h (125–130).

Sample cleanup using SPE anion-exchange resins (Dowex 1X8) has typically been used (127–129), although more recently, the Dionex OnGuard II A SPE cartridge has been reported as simplifying the analysis and reducing the test time (126).

Spectrophotometric methods using resorcinol (129) or thiobarbituric acid (125) have been used for the analysis of total sialic acid in infant formula. Because of its laborious nature, poor precision, poor selectivity, and the limitation to measuring total sialic acid, this technique has been superseded by chromatographic methods. RPLC methods with fluorescence detection of the 1,2-diamino-4,5-methylenedioxybenzene derivatives of Neu5Ac and Neu5Gc have been applied to the analysis of infant formulas (127, 128). A comparison of a UHPLC version of this method and an HPAEC-PAD method illustrated that both methods were sensitive for the analysis of Neu5Ac and Neu5Gc in infant formula (126). Of the two methods, UHPLC with fluorescence detection was more sensitive, although it required a derivatization step, whereas HPAEC-PAD was the faster method. An HILIC-MS/MS method for the determination of Neu5Ac and Neu5Gc in infant formula (130) was developed. The use of MS/MS analysis of Neu5Ac and Neu5Gc ions afforded similar selectivity to that afforded by fluorescence without the need for derivatization, thereby simplifying the analysis and reducing the analysis time. However, as isotope-labeled standards were not available, calibration was achieved by the use of matrix-matched standards, which can complicate quantitation.

Gangliosides

Gangliosides are a group of glycosphingolipids comprised of a hydrophilic oligosaccharide chain with one or more sialic acid moieties and a ceramide group consisting of sphingosine and fatty acids with a variety of chain lengths and degrees of saturation (122, 131). The ganglioside classes of GD3 and GM3 account for >80% of the total ganglioside content in bovine milk (132), but comprise approximately 50% of the total ganglioside content in human milk (133).

The extraction of gangliosides from dairy products is complex because of the high fat content and their localization in the milk-fat globule membrane. Typically the extraction of gangliosides from infant formulas is based on modifications to the extraction method described by Svennerholm and Fredman (134), whereby the gangliosides are extracted twice from the sample matrix, the supernatants are pooled, and the gangliosides are separated from other lipids by phase partitioning with water (131, 132, 135). A Folch extraction with methanol: chloroform: 0.01M KCl (132) or SPE (131, 135) has been used to purify the crude lipid extracts further.

Total gangliosides can be quantitated in purified extracted lipid by sialic acid determination, and this technique has been used in the determination of gangliosides in infant formulas (133). LC-MS affords a high degree of selectivity, sensitivity, and robustness, and can be applied to the routine analysis of analytes in difficult matrices, and such methods for the determination of gangliosides in milk and infant formulas have been reported (131, 132). An RPLC separation of GD3 and GM3 was performed with ESI in negative mode, with compensation for suppression effects, necessitating quantitation by multilevel standard addition via the autosampler, adding a significant burden to the test cost and the total analysis time (131). A reduction in analysis time was achieved with the development and validation of a HILIC-MS method for the analysis of GM3 and GD3. No matrix effects were observed with this method, allowing for external standardization and thereby simplifying and speeding the analysis (132). Further enhancements to ganglioside analysis have been achieved, with the publication of a UHPLC-MS/MS method for the analysis of GD3 and GM3 in which the run time was reduced to 8 min and quantitation was achieved by an internal standard using GM1 (135).

Triacylglycerides

The esters formed between glycerol and fatty acids produce a vast array of potential mono-, di-, and triacylglycerides that have been studied well because of their contribution to energy production, body insulation, and as a source of specific polyunsaturated fatty acids (136, 137).

Triacylglycerides, the predominant neutral lipid compounds, have three regiospecific positions for esterification (described as sn-1, sn-2, and sn-3), with the central sn-2 position being optically active in either the S or R conformation. The carbon number (CN) in a triacylglyceride is a convenient method of categorization; thus tripalmitolein with three palmitic acids is CN48, also described as PPP. The positions of the fatty acids in triacylglycerides have assumed greater importance in infant nutrition because pancreatic lipase removes sn-1 and sn-3 fatty acids more readily and leaves the sn-2 fatty acid as a monoacylglyceride, which is subsequently readily absorbed and is available for in vivo energy production (138). Additionally, free long-chain fatty acids from the sn-1 and sn-3 positions are reported to form calcium soaps, depleting the availability of this essential bone mineral (139).

Animal (140, 141) and human (142) studies have shown a faster absorption rate of certain stereospecific triacylglyceride isomers with a focus on the prevalent CN52 triacylglycerides. 1,3-Dioleoyl-2-palmitoylglycerol (OPO), created by the transesterification of palm oil (143), is incorporated into modern infant formulations such that the fatty acid profiles and the sn-2 compositions become more closely aligned with those in human milk (144–146). However, there is insufficient evidence to establish a cause-and-effect relationship between the consumption of sn-2 palmitate and an increase in calcium absorption, and further nutritional studies are suggested (147).

As an ingredient of infant formula, a method of differentiating OPO from the endogenous milk-fat triacylglycerides, particularly other CN52 triacylglycerides, is desirable. AOAC **986.19** (148) uses gas chromatography with flame-ionization detection (GC-FID) with packed column technology to separate intact triacylglycerides, but it is unlikely to include sufficient discrimination for OPO quality-control purposes. An International Standards Organization (ISO) method for the determination of the triacylglyceride composition of fats and oils (149) uses GC-FID separation on a 25 m phenylmethyl-polysiloxane capillary column. The underivatized triacylglycerides elute in order of increasing CN and, for similar CNs, triacylglycerides with greater unsaturation are retained longer. Similarly, an ISO method for the determination of milk-fat purity (150) separated groups of triacylglycerides, although neither method specifically targeted OPO detection.

Straarup et al. (151) used multidimensional chromatography to study extensively the triacylglyceride composition of infant formulations, and they compared sn-2 data with those for human milk. Thin-layer chromatography (TLC) separated the extracted fats before GC-FID, therefore providing consistently reliable data. Similarly, Haddad et al. (146) used TLC to separate lipid classes, but improved selectivity of minor compounds using dual-column HPLC with fraction collection of the peaks. Each fraction was examined for fatty acid and triacylglyceride composition using GC-FID and LC-MS to identify the individual compounds. The most abundant compound was a dioleoyl-palmitoyl isomer OOP, CN52:2 (the *:2* signifies the total number of double bonds), although the stereochemistry was not proven, with other CN52 compounds following in order CN52:4 (PLL/PoOL/POLn)>CN52:3 (POL/PoOO)=CN52:1 (PSO)>CN52:1 (PSS)>CN52:2 (PSL/PoSO)>CN52:0 (PSS)=CN52:5 (PoLL) (L=linoleic acid, Ln=linolenic acid, O=oleic acid, *P*=palmitic acid, Po=palmitoleic acid, S=stearic acid), illustrating the difficulty of quantitative testing of intact triacylglycerides. LC-refractive index detection (RID) has demonstrated reliability for triacylglyceride separations in vegetable oils, as illustrated in AOAC **993.24** (152). Under nonaqueous conditions with a C_{18} column, the triacylglycerides were eluted with increasing effective carbon number (ECN), a calculation that included a reduction in CN by unsaturated double bonds. ECN was described in the method as $ECN = CN - (2.60 \times O_n) - (2.35 \times L_n) - (2.17 \times Ln_n)$, where O_n, L_n, and Ln_n are the number of double bonds of oleic, linoleic, and linolenic acids, respectively, in the triacylglyceride. Using this equation, as OPO has an ECN of 46.8, it should elute just after triolein (OOO, ECN 46.2). Although it has not been demonstrated, this method has the potential to quantitate OPO in infant formulations.

The ELSD detector allows gradient elution with consequently enhanced triacylglyceride separation (153), a procedure that was optimized to study the natural composition of human milk (154). Similar to AOAC **993.24**, quantitation was by area normalization assuming equal detector response, with the identification of unknown triacylglycerides by the comparison of selectivity factors (log α) relative to triolein and by LC-MS with atmospheric pressure chemical ionization. The results were comparable to those of Haddad et al. (146), with the most abundant triacylglycerides being dioleoyl-palmitoyl (CN52:2, 27%) and oleoyl-palmitoyl-linolenoyl (CN52:3, 17%), but acknowledging the variability between samples that is caused by external factors such as dietary habit.

Other studies using MS detectors (155–157) for plant oils suggest that this technique has future potential for targeted OPO determinations. The need to detect a single analyte with known structure and in a well-established matrix, coupled with available standards, simplifies the analytical challenges (158). Future official methods are likely to be derivatives of nonaqueous argentation LC, permitting greater triacylglyceride separation by exploiting Ag^+–double-bond interactions, followed by detection with ELSD, Charged Aerosol Detector, or MS (159).

Oligosaccharides

Carbohydrates are vital components in metabolism, as individual monosaccharides, as polymers of various chain lengths, or in combination with proteins (glycoproteins), lipids (glycolipids), and many other biochemicals. Oligosaccharides are typically 3–9 sugars in length, comprising one or several simple sugars bonded through their hydroxyl groups. Thus gluco-oligosaccharides are homopolymers of glucose with α(1→4) or α(1→6) *O*-glycosidic links from maltotriose upward, whereas hetero-oligosaccharides contain ≥2 sugars such as pectic-oligosaccharides derived from fruits. Some oligosaccharides, notably trisaccharides such as raffinose, exist in nature although less predominantly than the long-chain polysaccharides found throughout the plant kingdom. The enzymatic hydrolysis of the polysaccharides can be controlled commercially to produce a substantial array of oligosaccharides for human dietary use (160, 161).

The principal purpose of supplementary oligosaccharides is to improve intestinal health, which in turn can enhance physical and immunological well-being. Such oligosaccharides, called prebiotics, survive the digestion process and are fermented in the colon, promoting the growth of beneficial bacteria such as *Bifidobacterium* and *Lactobacillus*. Those most commonly used for food fortification are fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS), both being water soluble and easily incorporated into liquids or powders. The addition of FOS or GOS at low-percentage concentrations to later-stage infant formula is becoming increasingly common.

There are two general analytical approaches to detecting FOS, one targeting the intact oligomers and the other hydrolyzing the glycoside bonds with subsequent measurement of liberated monosaccharides. Neither presents an easy challenge in infant formulations and adult nutritionals because of the presence of other carbohydrates at much higher concentration. Thus FOS, with its linked β(2→1) fructose (Fru) and terminal glucose (Glu) monomers, abbreviated Fru_n and $Glu–Fru_n$ (*n* typically 1–7), can be difficult to measure in products with high sucrose or maltodextrin levels (162). Methods for fructans are described in AOAC **997.08** (163) and AOAC **999.03** (164), based on HPAEC-PAD (165) and colorimetry, respectively (166). Both measure the net increase in fructose after enzymatic hydrolysis, correcting for non-FOS contributions. An enzymatic–spectrophotometric approach has been reported for inulin/FOS in a wide range of foods, including dairy products at 1% FOS, and this approach has been compared with results using AOAC **997.08**, demonstrating the usefulness of such methods without expensive equipment (167). AACC method 32-31.01 (168) is similar to AOAC **997.08**, using HPAEC-PAD to detect fructan oligomers before and after selective hydrolysis with separate amyloglucosidase and fructanase hydrolyses.

GOS is manufactured by *trans*-galactosylation of lactose with various bacterial enzymes, creating many variants of raw materials, some of which will contain free glucose and galactose (Gal) (169), making quality control even more complex. The testing of GOS is more complex than that of FOS because of its diverse linear β(1→3), β(1→4), and β(1→6) galactose chains, usually with terminal glucose molecules ($Glu–Gal_n$). In products with a significant lactose background, the analytical challenge is increased because this β(1→4) *O*-linked disaccharide will dominate the liberation of monosaccharides after hydrolysis. AOAC **2001.02** (170) describes a method for raw materials; it hydrolyzes oligomers with β-galactosidase and compensates for residual lactose by HPAEC-PAD (171), and it is similar to AACC method 32-33.01 (172).

HPAEC with integrated PAD and optimized wave-forms will probably play a significant role in future AOAC internationally harmonized methods for oligosaccharides, albeit challenged for sensitivity and selectivity in infant formulations. Thus AOAC **997.08**/AACC 32-31.03 for FOS and AOAC **2001.02**/AACC 32-33.01 for GOS can potentially be updated with respect to sample preparation and chromatography to improve their reliability at low concentrations. For methods involving oligomer hydrolysis, the average degree of polymerization needs to be determined to calculate the contribution of water at each linkage.

The detection of intact oligomers provides a viable alternative method to enzymatic hydrolysis but relies on standard materials being available for each component. In the case of FOS, the major compounds are known, namely 1-kestose (Glu–Fru_2), nystose (Glu–Fru_3), and 1-fructofuranosyl-nystose (Glu–Fru_4), and are commercially available for investigational use (173, 174). The future availability of β(2→6)-bonded neoFOS isomers will also be helpful. In the absence of suitable standards, response factors need to be determined by interpolation or by involving oligomer profiles of the relevant FOS raw material (175). The GC-FID profiles of volatile oxime trimethylsilyl derivatives selectively separated Glu–Fru_n and Fru_n in the presence of malto-, isomalto-, and galacto-oligosaccharides, and this method is a viable candidate for the routine analysis of infant formula, despite the need for previous derivatization.

The situation regarding GOS is more difficult, but Austin et al. (176) tackled this using a Dionex IC system equipped with a CarboPac PA 100 column to measure GOS in infant formulations. Calibration was achieved using two common marker peaks found in the sample and the raw ingredient material. Additionally, fluorescent derivatization with 2-aminobenzamide provided a promising quantitative method for GOS in infant formulations, the molecular mass of each oligomer being decided by flow splitting to a mass spectrometer.

MS detectors have been very successful in oligomer identification based on fragmentation patterns (177–179) and they lend themselves to the routine testing of infant formulations in the future.

α-Lactalbumin

α-Lactalbumin is a single chain, Ca^{2+}-binding protein comprising 123 amino acids, no free thiols, and four disulfide bonds, has a molecular mass of approximately 14 kDa, and is present in all mammalian milks. It is the second most abundant whey protein in bovine milk and the dominant whey protein in human milk, with 74% sequence homology between these species, and when bound to Ca^{2+}, it is relatively heat stable compared with other whey proteins (180, 181).

α-Lactalbumin is a critical component of the lactose synthase system within the mammary gland, and its structural similarity to lysozyme suggests an evolutionary relationship (180, 182). Aggregated forms of human apo-α-lactalbumin have been reported to be apoptotic to certain tumor cells, which is speculated to be protective to the suckling neonate (183).

α-Lactalbumin is of high nutritional value to the neonate, and it is a source of the essential amino acids tryptophan and cysteine. Based on the accepted nutritional suitability of human milk, infant formulas derived from bovine milk are increasingly humanized by adding bovine whey protein fractions enriched with α-lactalbumin to provide a plasma amino acid profile that is comparable with that of breast-fed infants (184). Research suggests that α-lactalbumin may also be physiologically active by moderating gut microflora, mineral absorption, and immune function (181).

The increasing trend toward the supplementation of infant formulas with α-lactalbumin, and its potential as a marker of heat treatment, have stimulated the need for reliable concentration assays for its determination at endogenous levels in milk, at supplemental levels in infant formula, and at pharmaceutical levels in milk protein isolates. Many analytical techniques are available for the determination of α-lactalbumin, either alone or simultaneously with other whey proteins, with a range of electrophoretic and LC strategies commonly described and more recently incorporating MS detection (185–193).

In view of their inherent specificity for native protein conformation and their sensitivity, immunological methods offer an alternative quantitative approach, and they have also found application in studies of the denaturation of α-lactalbumin and species adulteration of milk-based products. Radial immunodiffusion, nephelometric immunoassay, immunoelectrophoresis, and enzyme-linked immunosorbent assay (ELISA) techniques have commonly been described for the determination of α-lactalbumin in milk, with surface plasmon resonance (SPR) based biosensor methods being reported more recently (193–203). A notable attribute of biospecific biosensor immunoassays is the inherent sensitivity of SPR detection, facilitating direct sample dilution in buffer in advance of automated analysis.

Lactoferrin

Lactoferrin is an approximately 80 kDa, basic (pI: approximately 9.0), iron-binding, bilobal secretory transport sialylated glycoprotein of known amino acid sequence, and is a member of the transferrin family. It is characterized by the carbonate-anion-dependent, high-affinity (K_D approximately 10^{-20} M) (204), and pH-reversible binding of two Fe^{3+} ions per molecule yielding a pink complex (λ_{max}: 470 nm) (205, 206). Although lactoferrin is present in milk as a result of in situ synthesis within the mammary gland, it is also present in several other exocrine fluids. The fact that milk shares several antimicrobial components, including lactoferrin, with other glandular secretions might be evidence of functions that predate the nutritional role of lactogenesis (207). In addition to its antimicrobial activity, lactoferrin, and its proteolysis-generated peptide lactoferricin, might function as a prebiotic in intestinal iron uptake and regulation, immune response, growth-factor activity, and antioxidant activity (205, 208, 209). The lactoferrin content is species dependent, with significantly higher levels in human milk and colostrum than in the bovine equivalent (210). Based on the accepted nutritional suitability of human milk, infant formulas derived from bovine milk are increasingly humanized by their supplementation with fractionated lactoferrin isolated from bovine milk or whey (recombinant human lactoferrin has also been advocated) (211, 212), despite a 69% primary sequence homology with human lactoferrin and a poorly characterized understanding of its absorption in the infant gastrointestinal tract.

The increasing commercial interest in exploiting the therapeutic value of lactoferrin has stimulated the need for reliable concentration assays for its determination at

endogenous levels in milk and colostrum, at supplemental levels in infant formulas, and at pharmaceutical levels in milk protein isolates. Analytical chromatographic techniques for bovine whey proteins have been reviewed (187, 213), with LC, UHPLC-MS/MS, and electrophoretic techniques for the quantitation of lactoferrin in milk, whey, and infant formulas being reported (214–219).

A review of the immunological techniques that are available to estimate the concentration of lactoferrin concluded that immunodiffusion techniques have inherently low sensitivity and have generally been superseded by the more sensitive ELISA techniques (220). More recently, enzymatic and nephelometric immunoassays for lactoferrin in milk have been reported (221–226). Conventional immunoassay techniques have been further enhanced through the development of immunosensor platforms exploiting electrochemical transduction techniques, which have been applied to the determination of lactoferrin in mammalian milk (227, 228). Alternative real-time, label-free optical biosensor techniques based on SPR transduction have also been reported for the quantitation of lactoferrin in bovine milk and infant formula milk (194, 229). Such automated biosensor immunoaffinity technologies exploit the specific and reversible interaction between antibody and protein antigen, and they are versatile, robust, and capable of producing rapid and reliable data for the quantitative analysis of lactoferrin, and other proteins, in complex food matrices with minimal sample preparation.

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