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SUPPLEMENT

Micronutrients in Human Milk: Analytical Methods

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ABSTRACT

Exclusive breastfeeding is recommended by the WHO for the first 6 mo of life because human milk protects against gastrointestinal infections and supplies balanced and adequate nutrient contents to the infant. However, reliable data on micronutrient concentrations in human milk are sparse, especially because some micronutrients are affected by maternal diet. Microbiological and competitive protein-binding assays, nuclear magnetic resonance or inductively coupled plasma spectroscopy, and chromatographic analyses are among the methods that have been applied to human-milk micronutrient analysis. However, the validation or evaluation of analytical methods in terms of their suitability for the complex human-milk matrix has been commonly ignored in reports, even though the human-milk matrix differs vastly from blood, plasma, or urine matrixes. Thus, information on the validity, accuracy, and sensitivity of the methods is essential for the estimation of infant and maternal intake requirements to support and maintain adequate milk micronutrient concentrations for healthy infant growth and development. In this review, we summarize current knowledge on methods used for analyzing water- and fat-soluble vitamins as well as iron, copper, zinc, iodine, and selenium in human milk and their different forms in milk; the tools available for quality control and assurance; and guidance for preanalytical considerations. Finally, we recommend preferred methodologic approaches for analysis of specific milk micronutrients. *Adv Nutr* 2018;9:313S–331S.

Keywords: human milk, fat- and water-soluble vitamins, minerals, analytical methods, microbiological assay, competitive protein-binding immuno-assay (CPBA), inductively coupled plasma spectroscopy (ICP), liquid chromatography–mass spectrometry (LC-MS)

Introduction

The methods used for micronutrient analysis in human milk are commonly derived from methods developed for other matrixes, such as plasma or urine. Although neither plasma nor urine contains mentionable amounts of fat or sugars, these macronutrients constitute >10% of human milk by weight (1, 2), which will affect the physical and chemical behavior of the sample and requires adjustments in the sample preparation protocol. Micronutrients in human milk are commonly analyzed by using microbiological, colorimetric, and competitive protein-binding assays (CPBAs); GC and LC with the use of UV, fluorescence, or MS detection; atomic absorption spectroscopy (AAS); and inductively coupled plasma spectroscopy–atomic emission spectroscopy (ICP-AES) or ICP-MS. Additional techniques applied to human milk include animal studies and radioisotope dilution assays, or voltammetry. The latter approaches have been shown to be inferior to the newer techniques with regard to sample volume, costs, and time. Some reported methods are not suitable for analyzing micronutrients in human milk (3), or different methods for the same micronutrient analysis are not comparable (4–6). These concerns reiterate the importance of evaluating the suitability of methods chosen for analysis. In this review, we summarize the current knowledge of and evaluate the methodologic approaches reported for analyzing water- and fat-soluble vitamins, iron, copper, zinc, iodine, and selenium in human milk. In addition, we discuss the different forms of the micronutrients present in human milk.

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Abbreviations used: AA, ascorbic acid; AAS, atomic absorption spectroscopy; AES, atomic emission spectroscopy; AI, Adequate Intake; apoHC, apo-haptocorrin; ATCC, American Type Culture Collection; CPBA, competitive protein-binding assay; DHAA, dehydroascorbic acid; ECD, electrochemical detection; FLD, fluorescence detection; ICP, inductively coupled plasma spectroscopy; MS/MS, tandem MS; NMN, nicotinamide mononucleotide; NNA, neutron activation analysis; SRM, standard reference material; TMP, thiamin monophosphate; $1,15(OH)_2D$, 1,15-dihydroxyvitamin D; $24,25(OH)_2$, 24,25-dihydroxyvitamin D.

Current Status of Knowledge

Thiamin (vitamin B-1)

Thiamin in breast milk exists in its free form as well as in 2 of its phosphorylated forms: thiamin monophosphate (TMP) and thiamin pyrophosphate. Free thiamin and TMP are the main forms of vitamin B-1 in human milk (7, 8).

Analyses of thiamin in milk have been mostly conducted via the classic thiochrome reaction and microbiological and HPLC methods (9). Bacteria described for microbiological assays include *Lactobacillus fermenti*, *Saccharomyces cerevisiae*, *Ochromonas malhamensis*, and *Leptostylus viridescens* [American Type Culture Collection (ATCC) 12706]. Only *L. viridescens* provides results comparable to the thiochrome assay, whereas other bacteria are susceptible to matrix constituents (e.g., sugars, reducing agents, calcium). This type of analysis requires enzymatic hydrolysis of the phosphate esters due to differential growth response to thiamin, TMP, and thiamin pyrophosphate (10–14).

The thiochrome method has been widely used for thiamin analysis in biological matrixes. Free thiamin as well as its phosphate esters are derivatized with potassium ferrocyanide under alkaline conditions, yielding thiochrome. HPLC methods continue to use this well-known reaction via pre- or post-column derivatization of the thiamin vitamers, followed by fluorescence detection (8, 15–22); however, HPLC-UV analysis has also been reported for human milk (23). Free thiamin can also be quantified by ultraperformance LC–tandem MS (MS/MS) simultaneously with other B-vitamins, after removal of proteins and nonpolar constituents, without the need of derivatization (24, 25).

Thiamin values used as the basis for Adequate Intake (AI) estimates for infants aged 0–6 mo were obtained by using the thiochrome method (26–28). Even though this approach is less susceptible to matrix interferences than the microbiological assays, the more recent approaches that use chromatographic separation before fluorescence detection of the thiochrome derivatives is preferred due to the added reproducible separation of the analytes from the matrix and rapid accurate and stable quantitation of total thiamin in breast milk.

Riboflavin (vitamin B-2)

Riboflavin (7,8-dimethyl-10-ribityl-isoalloxazine) and its coenzymatic form FAD are the prevalent forms of vitamin B-2 in human milk. Other flavins present include 10-hydroxy-ethylflavin and traces of 10-formyl-methylflavin, 7α-hydroxy-riboflavin, 8α-hydroxy-riboflavin, and FMN (4, 18).

Quantitative analyses of vitamin B-2 in human milk include microbiological and spectroscopic (UV, fluorescence) methods (4, 12, 28–30). *Lactobacillus rhamnosis* (formerly *Lactobacillus casei*; ATCC 7469) has been the common choice for microbiological approaches, which include acidic hydrolysis, protein precipitation, and neutralization before incubation with the growth medium. However, matrix constituents (e.g., starch, protein degradation products, or FFAs) and different growth responses to the different forms of the vitamin deem this approach more susceptible to errors (13, 31).

Fluorometric techniques are based on the conversion of riboflavin to lumiflavin (6,7,9-trimethylisoalloxazine) under alkaline conditions, which possess significantly stronger fluorescence than does the native riboflavin. Although additional preparation steps can enhance the specificity, the actual reaction does not occur quantitatively and varies tremendously with experimental conditions and instrumental set-up (13, 32–34).

HPLC separation followed by fluorescence detection has emerged as a common technique for riboflavin analysis in human milk (18, 35, 36). However, values obtained need to be corrected for the internal quenching in FAD caused by the formation of an intramolecular complex, which might have been neglected in reports before 1990 (4, 37). Alternatively, FAD can be converted quantitatively into riboflavin by enzymatic treatment before fluorometric analysis (4, 29, 38). More recently, riboflavin and FAD analysis via ultraperformance LC-MS/MS has been described for the first time to our knowledge, enabling the analysis of the prevalent vitamin B-2 vitamers in their native forms (21, 24, 39).

Mikheeva et al. (40) described a rapid riboflavin analysis in breast milk by voltammetry. This approach takes advantage of the oxidizability of riboflavin at a glassy-carbon indicator electrode. The samples are subjected to acidic hydrolysis and protein precipitation before analysis. The riboflavin potential, however, varies considerably with pH, which also affects the rate of the electrode process and its mechanism, highlighting the intricacies of this method (40).

The AI value for infants aged 0–6 mo is based on riboflavin concentrations on human milk obtained by UV detection and fluorometric measurements after HPLC separation (4, 26). However, the direct analysis of riboflavin without the need of derivatization is preferred to avoid the intricacies attached to this mandatory additional sample preparation step.

Niacin (vitamin B-3)

Niacin refers to nicotinic acid (pyridine-3-carboxylic acid) and nicotinamide (pyridine-3-carboxylic acid amide). Nicotinamide and its coenzymatic forms nicotinamide mononucleotide (NMN), NAD, NAD(P), and nicotinamide riboside have been reported to be present in human milk (18, 41, 42).

Most niacin analyses in human milk have been conducted by using microbiological assays with the use of *Lactobacillus arabinosus* (12, 43–45). However, growth-stimulating or growth-depressing interferences might cause errors during the analysis (13, 46).

Current methodologic approaches for analyzing nicotinamide in human milk include HPLC coupled with UV, diode array detection, and MS/MS (18, 21, 23, 24, 47). Furthermore, ^{1}H-NMR has been used for measuring nicotinamide within a human-milk metabolome analysis in human milk (48), and a novel fluorometric enzyme-coupled assay has been reported for the analysis of nicotinamide

riboside, NMN, and NAD (42). Unfortunately, none of these techniques includes all forms of niacin for analysis.

The AI for niacin for infants aged 0–6 mo (26) is based on a single study by Ford et al. (12) that used a microbiological assay with the use of *L. arabinosus*, which continues to be a suitable choice for analysis. Alternatively, LC-MS/MS can be used for nicotinamide and nicotinic acid analysis, which will additionally provide information on other B-vitamin concentrations in the sample (24, 39), whereas the fluorometric enzyme-coupled assay offers quantitative data on nicotinamide riboside, NMN, and NAD (42).

Vitamin B-6

Vitamin B-6 (2-methyl-3-hydroxy-5-hydroxy methyl pyridine derivatives) refers to the biologically active equivalent and metabolically interconvertible pyridoxine, pyridoxal, and pyridoxamine and their phosphorylated forms. Pyridoxal represents the principal form of vitamin B-6 in human milk, with possible contributions of pyridoxal-5′-phosphate (7–64%), pyridoxamine-5′-phosphate, pyridoxine, and pyridoxamine (49–51).

Quantitative determination of vitamin B-6 is generally carried out by microbiological assays or LC-based methods. *Saccharomyces uvarum* (ATCC 9080) has been widely used for human-milk analysis (13, 28, 52–57). However, high salt amounts can suppress growth of the medium, and different growth responses for pyridoxine, pyridoxal, and pyridoxamine add to the assay's complexity. Hydrolyzing the samples followed by the chromatographic separation of the vitamers before addition to the yeast basal medium allows the determination of each vitamer individually (13, 14, 58–62). Other microorganisms such as *Kloeckera brevis* and *Lactobacillus casei* have also been described (12, 63, 64), but the extensive sample preparation and complexities of microbiological assays have led to the development of chromatographic methods for vitamin B-6 analysis (9).

HPLC coupled with fluorescence detection has emerged as a valid method for vitamin B-6 analysis in human milk. Sample analyses described include treatment with sulfosalicylic acid, bisulfate derivatization, photochemical conversion, or conversion into 4-pyridoxolactone (49–51, 65, 66). Results obtained with the analytical methods were in good agreement with the microbiological assay. The use of LC-MS/MS for the analysis of vitamin B-6 bypasses the mandatory derivatization for fluorescence detection and allows the direct analysis of the native form (21, 24, 39).

The vitamin B-6 AI for infants aged 0–6 mo is based on the mean concentration in milk of 19 well-nourished but unsupplemented mothers with intakes near the RDA (26, 52). Vitamin B-6 concentrations were analyzed by using microbiological assays. The recent developments of HPLC-based methods provide a more robust and rapid approach for modern vitamin B-6 analysis.

Cobalamin (vitamin B-12)

Vitamin B-12 is the collective term for cobalt-containing corrinoids. Only the biologically active cobalamins are selectively transported into human milk (67–69). Methylcobalamin represents the dominant form of vitamin B-12 in human milk followed by 5′-deoxyadenosylcobalamin and small amounts of hydroxocobalamin and cyanocobalamin, all bound to haptocorrin, which potentially interferes with vitamin B-12 analysis (69–72).

Early approaches for the analysis of vitamin B-12 in human milk were carried out by microbiological assays with the use of *Euglena gracilis* as the test organism (70, 71, 73, 74) utilizing enzymatic digestion with papain to release the vitamin B-12 from binding to haptocorrin and conversion of the different forms into cyanocobalamin. Alternatively, *Lactobacillus leichmanii* (National Collection of Industrial Bacteria 8118) has also been used to assay vitamin B-12 microbiologically (75), but interferences by deoxyribonucleosides, such as thymidine and other compounds, can result in an overestimate of vitamin B-12 concentrations (76, 77).

Radioisotope dilution assay, first described by Lau et al. (78) for serum vitamin B-12, has also been applied for human-milk analysis (28, 69, 78–88). This approach is based on competitive binding of endogenous vitamin B-12 and added radioactive vitamin B-12 to limited binding sites on intrinsic factor (78, 89), but no validation has been described for the use of human milk as matrix.

Competitive protein binding coupled with chemiluminescence detection appears to be the method of choice for vitamin B-12 analysis in human milk in current years (36, 90–92). Lildballe et al. (72) proposed the removal of apo-haptocorrin (apoHC) before vitamin B-12 analysis because the analysis of untreated samples with high amounts of apoHC resulted in artificially high or low vitamin B-12 concentrations, depending on the analyzer used; apoHC < 10 nmol/L appeared not to interfere with the analysis (72). The most recent report describes a competitive chemiluminescence enzyme immunoassay without the need of haptocorrin removal before sample preparation and analysis and lower detection limits for vitamin B-12 in milk (93), which was used for vitamin B-12 analysis in the most recent studies (21, 94–97).

Vitamin B-12 values used to set the AI for infants aged 0–6 mo were obtained from the milk of 9 well-nourished, unsupplemented Brazilian mothers analyzed via radioisotope dilution assay (26, 85). Given the lack of validation of these types of assays for human milk, the use of competitive chemiluminescence enzyme immunoassays as described above is the preferred method for vitamin B-12 analysis in human milk.

Folate (vitamin B-9)

Folate is the collective term for the large group of heterocyclic compounds that all possess the biological activity of folic acid (pteroylglutamic acid). Milk folate is covalently bound to whey-binding proteins and predominantly present as pteroylpolyglutamates and as N-5 methyltetrahydrofolate, with a minor contribution of reduced folacin derivatives (3, 98–101) and traces of folic acid, *p*-aminobenzoylglutatmate, and its acetamide derivative *p*-aminobenzoylglutatmate acetamide (102).

The commonly used method for folate analysis has been a microbiological assay with the use of *L. casei* ATCC 7469, because this bacterium responds to all forms of folate (103, 104). Other microorganisms used include *Streptococcus faecalis*, *Pediococcus cerevisiae*, and *Lactobacillus casei*. These bacteria possess differential responses to the different folate vitamers, allowing differential analysis of folate forms in human milk (12, 54, 74, 98, 105–118). More recent studies used α-amylase and protease in addition to the folate conjugase to aid in the liberation of the vitamin, which results in higher concentrations.

HPLC with fluorescence detection (FLD) has been used for folate in human milk (18); however, the method applied was not adjusted for the form of folate present in milk (3). Only recently, an LC-FLD method has been described that allows the analysis of the main forms of folate in milk (119).

Competitive protein-binding radio- and chemiluminescence assays have also been used for folate analysis in milk (36, 81, 84). However, these assays are not validated for the human-milk matrix and appear to overestimate free folacin in the presence of polyglutatmate forms of 5-methyltetrahydrofolate (5).

The folate AI for infants aged 0–6 mo has been estimated from several publications with the use of a microbiological assay for folate measurement (26, 99, 101, 120). This approach remains the method of choice for folate analysis in human milk; however, recent advances in the analytical field may offer a valid alternative in the future (119).

Pantothenic acid (vitamin B-5)

Pantothenic acid [d(+)-N-(2,4-dihydroxy-3,3-dimethyl-1-oxobutyl)-β-alanine] consists of pantoic acid bound to β-alanine. Approximately 85–90% of pantothenic acid in human milk is available in its free form. Although it is a key factor in lipid metabolism, this vitamin does not occur in the lipid fraction in substantial amounts (121).

Microbiological assays, RIA, and HPLC-UV analysis have been described for pantothenic acid analysis in human milk. Microbiological assays use microorganisms such as *L. casei*, *L. arabinosus*, and *Lactobacillus plantarum*; enzymatic treatments appeared not to increase pantothenic acid concentrations (12, 18, 43, 45, 122–124).

RIAs include incubation of the sample with bovine intestinal alkaline phosphatase and pantetheinase. However, complete protein removal was not achieved with commonly used techniques such as boiling or autoclaving (121, 125).

LC coupled with UV detection has been used for pantothenic acid analysis allowing the quantitation of 0.5 ppm, even though the vitamin lacks the necessary chromophores for strong UV absorption (23, 126). Recently, MS/MS detection has also been described for human-milk analysis, measuring several B-vitamins simultaneously (39). Alternatively, ^{1}H-NMR has been used in a human-milk metabolome study to quantify pantothenic acid along with nicotinamide and other metabolites such as sugars, amino acids, and energy metabolites (48).

The AI for pantothenic acid is based on a study in the United Kingdom that used a pooled sample from 96 women from 5 cities (26, 127). Unfortunately, no information about the methods used for analysis is available. Even though the majority of analyses have been conducted via microbiological assays, chromatographic separation followed by UV or MS/MS detection may be beneficial with regard to accuracy and reproducibility, sample volume, time, and costs.

Biotin (vitamin B-7)

Biotin (*cis*-hexahydro-2-oxo-1H-thieno [3,4-d]imidazole-4-pentanoic acid) in human milk accumulates to >95% in the skimmed-milk fraction. Less than 3% is reversibly bound and <5% is covalently bound to macromolecules (128). Forms found in early and transitional human milk include biotin and its metabolites bisnorbiotin (~50%) and biotin sulfoxide (~10%). Although biotin concentrations are fairly constant throughout lactation, the ratio of biotin to its metabolites shifts in favor of the actual vitamin (13, 129–131).

Biotin analyses in milk have been carried out by microbiological and sequential solid-phase assays. Microbiological approaches regularly use *L. arabinosus* and *L. plantarum* as test organisms (12, 18, 43, 123, 124, 132). Growth-stimulating compounds such as oleic and aspartic acid can interfere with the determination, resulting in overestimated concentrations (133).

Sequential solid-phase assays that use ^{125}I-labeled avidin have been suggested as an alternative technique for biotin in milk (128–130, 134). ^{125}I-labeled avidin is mixed with varying amounts of biotin (standard curve) and with several dilutions of the samples. The remaining avidin-binding sites will be bound to an immobilized biotin-albumin complex; its radioactivity is inversely related to the biotin concentration in the sample.

To our knowledge, no validation of a chromatographic method has been described for biotin analysis in human milk. UV detection has been used for multivitamin products (133), but due to the lack of chromophores in the biotin molecule this approach lacks the necessary sensitivity for biotin analysis in milk. However, LC-MS/MS has been mentioned to be a feasible approach for free biotin analysis in the human-milk matrix (24) and later described (39).

The biotin AI for infants aged 0–6 mo is based on values obtained from a few reports that used microbiological assays (26, 135–137). These types of assays are still commonly used. Future advances as indicated above are directing toward novel LC-MS/MS approaches for biotin analysis in human milk.

Choline

Forms of choline (*N*-trimethylethanolamine) in human milk include mainly free choline and its metabolites phosphocholine and glycerophosphocholine, with minor contributions of lipophilic phosphatidylcholine (lecithin) and sphingomyelin. Its concentration doubles 6–7 d after birth due to

increasing amounts of phosphocholine and glycerophosphocholine (138, 139).

Choline measurements in milk samples have been determined by using radioenzymatic assays, ^{1}H-NMR, and chromatographic techniques. The radioenzymatic assay is based on the conversion of choline to phosphorylcholine-^{32}P in the presence of choline kinase and ATP-γ-^{32}P and was used mainly in the 1970s (140–144).

^{1}H-NMR has been suggested for choline analysis. Water-soluble choline, phosphocholine, and glycerophosphocholine and the lipophilic metabolites were extracted before separate analysis of both fractions (48, 139, 145, 146). Both HPLC as well as GC-MS have been described for choline analysis in human milk. The GC approach uses laborious and complex sample preparation involving an array of equipment (18, 138, 147, 148). HPLC with electrochemical detection (ECD) can be applied after simple hydrolysis and enzymatic treatment (18, 149, 150). More recently, various LC-MS/MS methods have been introduced for choline analysis in human milk. Water- and fat-soluble forms of choline can be analyzed directly after a simple extraction step without further isolation or derivatization (147, 148, 151, 152).

The choline AI for infants aged 0–6 mo was based on 2 studies that used RIA and GC-MS analysis (26, 138, 143). However, given the possible radiation exposure and laborious sample preparation for the methods described, LC-MS/MS provides validated results with only minimal sample preparation without possible radiation exposure.

Vitamin C

Ascorbic acid (AA), as the principal form of vitamin C, and dehydroascorbic acid (DHAA) represent the biologically relevant forms of vitamin C in human milk (153, 154). Assays used for vitamin C quantitation in human milk include titration, colorimetric, and chromatographic techniques. Early approaches for AA measurement in human milk include AA oxidation to DHAA and titration with 2,6-dichlorophenolindophenol (155–157). However, other reducing substances present interfere with the accuracy of the method.

Colorimetric assays are mostly based on a method published for whole-blood and urine samples (158). After oxidizing AA, the DHAA is converted into its 2,4-dinitrophenylhydrazine derivative, which, under acidic conditions, forms a colored product for analysis (28, 54, 56, 80, 116, 154, 157, 159, 160). *O*-phenylenediamine fluorometry has also been described for human-milk analysis (161).

More recent approaches include HPLC with UV detection, FLD, or ECD (18, 36, 81, 124, 153, 154, 162, 163). The fluorometric approach is initiated by reducing AA to DHAA, which is converted into a quinolaxine derivative for analysis (81). Elaborative sample preparation for UV detection has also been described (18); however, the most recent approaches describe the reduction in DHAA by DTT to AA, which then will be analyzed after adding meta-phosphoric acid (154, 163).

A comparison of the chromatographic and the colorimetric approach showed that HPLC provided more satisfactory results, because the latter cannot determine total vitamin C content and results for AA were almost 40% lower (154). Moreover, the HPLC method uses less material and reagents and is simpler and less time consuming; thus, HPLC measurements of vitamin C in human milk should be carried out by using HPLC-UV.

The vitamin C AI for infants aged 0–6 mo is based on values mainly obtained by colorimetric assays (164). Given the described intricacies of that approach, HPLC methods should be used for vitamin C analysis in human milk.

Vitamin A

Forms of vitamin A in human milk include retinol, retinyl esters, and β-carotene (165, 166). Early approaches for measuring this fat-soluble vitamin in human milk describe colorimetric assays. Sample preparation includes protein precipitation with or without saponification for the removal of fatty constituents and extraction of the vitamin with petroleum ether. Treating the vitamin A–rich extract with antimony trichloride in chloroform results in a brilliant blue color (Carr-Price reaction), which is quantifiable via a photoelectric colorimeter (167–169). Alternatively, trifluoroacedic acid in chloroform can also be used as chromogenic solution for spectrophotometric analysis (166, 170).

Fluorometry has also been described for vitamin A analysis in human milk. Following the basic protocol of protein precipitation, saponification, and extraction, vitamin A concentrations are determined by using the fluorescent properties (31). The majority of vitamin A analyses, however, have been conducted by using HPLC coupled with UV, fluorescence, and MS detection (18, 36, 81, 171–211). Although protein precipitation has been carried out with ethanol or methanol, saponification has been described before or after the extraction procedure with the use of a range of nonpolar solvents such as hexane, petroleum ether, or diethyl ether. Potassium hydroxide has been commonly used for saponification; however, incubation time and temperature are not uniform, and enzymatic (lipase) hydrolysis may be used as a pretreatment to the saponification step to release retinol and carotenoids (179). Compounds used as internal standards include didehydroretinol acetate (189, 192), retinal (*o*-ethyl) oxime (207, 208), β-apo-8′ carotenal methyl oxime (181, 182), α-tocopherol acetate (171, 172), retinyl acetate (173), and β-apo-8′ carotenal (191). Didehydroretinol acetate can be added before saponification.

Recently, the iCheck FLUORO portable fluorometer (Bioanalyt GmbH) was introduced for rapid, quantitative analysis of vitamin A in milk, serum and plasma, or fortified foods. A comparison with the well-established HPLC-UV method showed that results obtained by this new technique highly correlated with the established method, but that values were greater with the use of HPLC, and the difference increased with increasing vitamin A concentrations (207).

Alternatively, LC-MS/MS has been described for vitamin A analysis following a similar sample preparation that includes saponification and hexane extraction (212).

The AI for vitamin A for infants aged 0–6 mo is based on values obtained from breast milk from 46 women by using colorimetric and HPLC methods (110, 171, 210, 211, 213). The latter has been the dominant technique for vitamin A analysis and allows chromatographic separation and rapid analysis of the different forms of vitamin A as well as separation from matrix constituents.

Vitamin D

Vitamin D in human milk is mostly present as vitamin D_2 (ergocalciferol) and vitamin D_3 (cholecalciferol), with contributions from their 25-hydroxy metabolites (214–216) and possibly 24,25-dihydroxyvitamin D and 1,15-dihydroxyvitamin D. These sterols are secreted into milk while bound to their plasma- or cytosol-binding proteins, but with time migrate into the lipid portion (215). A water-soluble form of vitamin D, D-3β-sulfate, has also been reported (217, 218), but has been shown to be biologically inactive and therefore has been discarded as a significant contributor to vitamin D activity in milk (216, 219).

A modified antimony chloride test has been described for vitamin D analysis in human milk (218); however, HPLC-UV and CPBA for better sensitivity for the minor vitamin D vitamers have been widely used. The samples undergo a stepwise purification process, including methanol precipitation, alkaline backwash for removal of interfering lipids, and preparative HPLC (215, 216, 219–225). Analytical HPLC-UV has also been applied after solid-phase extraction (18).

An isotope dilution LC-MS/MS method has been described for measuring vitamin D and its metabolites in human milk. Samples are purified by solid-phase extraction before analysis (226).

The vitamin D AI for infants aged 0–6 mo is not based on milk concentrations but on observations that a minimal intake of 2.5 μg/d most likely prevents rickets (227). This value was doubled to set the AI to account for the lack of vitamin D from sunlight exposure. However, the established HPLC and CPBA or LC-MS/MS methods should be applied when evaluating human-milk vitamin D status.

Vitamin E

Vitamin E refers to the 8 chemically related α-, β-, γ-, and δ-tocopherols and α-, β-, γ-, and δ-tocotrienols, which differ in structure and bioavailability (228, 229). α-Tocopherol is the only biologically active form of vitamin E; the other vitamers do not convert into the active form (230).

Early approaches for vitamin E analysis in human milk applied TLC and GC-MS. The lipid fraction is extracted by using ethanol, ethyl ether, and petroleum ether before saponification. After a purification step, the tocopherol fraction is used for TLC or GC-MS (231). Moreover, a colorimetric assay with the use of 2,2′-bipyridine-$FeCl_3$ has been described as being used parallel to TLC or paper chromatography (232, 233).

However, HPLC methods have been mainly used for vitamin E analysis in human milk, applying FLD, ECD, or UV detection. Sample preparation usually includes protein precipitation and extraction with the use of hexane. Analyses have been reported with and without saponification of the sample (163, 171, 172, 200, 202, 203, 206, 228–230, 234–249). However, saponification will convert α-tocopherol acetate into α-tocopherol; thus, α-tocopherol concentrations include the amounts of α-tocopherol acetate when the sample undergoes saponification (250). The use of LC–diode array detection–MS/MS has been reported for vitamin E analyses in milk from different animal species as well as human milk and can be considered a valid alternative for tocopherol analysis in human milk (212, 251).

The AI for vitamin E for infants aged 0–6 mo (164) is estimated from 5 studies that used HPLC for analyzing tocopherol content in human milk (171, 228, 236, 243, 252). HPLC coupled with fluorescence or UV detection is a well-studied and suitable technique for quantifying vitamin E in human milk; LC-MS/MS is a valid alternative.

Vitamin K

Vitamin K in human milk consists mainly of phylloquinone (vitamin K-1) and menaquinone-4 (vitamin K-2). Menaquinone-6 has been found in trace amounts (212, 253, 254).

The biological curative chicken test is one of the first methods described for vitamin K analysis (255). However, HPLC has superseded other techniques due to its superior sensitivity (254). Methods described include FLD, ECD, and UV detection. Generally, lipase treatment of the lipid extract is followed by a 2-step purification process that uses column chromatography and semi-prep HPLC before analysis (253, 254, 256–269). A 2 orders of magnitude higher sensitivity can be achieved when using FLD and ECD compared with UV detection; however, both require the conversion of the vitamin K vitamers into their reduced form for detection. This can be achieved chemically, electrochemically, photochemically, and online post–column solid-phase catalytic reduction by using zinc, platinum oxide, or platinum. The latter has been described as the easiest alternative for vitamin K reduction (254).

LC-MS/MS has been described for vitamin K analysis. Samples undergo lipase treatment, protein precipitation, and hexane extraction. After a silica cartridge clean-up step, the extract is ready for analysis (212).

The vitamin K AI for infants aged 0–6 mo is based on reports that used HPLC-FLD, HPLC-ECD, and UV detetction (213, 256–258, 260, 261). Given that ECD requires rigorous exclusion of oxygen, the reduction step used may be incomplete (254), and the lower sensitivity of UV detection, HPLC-FLD is the preferred method for vitamin K analysis. Alternatively, LC-MS/MS provides the needed sensitivity and no reducing agent for vitamin K analysis in human milk (212).

Iron

Iron in human milk is found in the lipid as well as in the low-molecular-weight compound fraction; only small amounts are bound to lactoferrin (270). Little is known about the mechanisms that regulate iron concentrations in human milk. It is transported by divalent metal transporter 1 through the basolateral membrane into aveoli and exported by ferroportin in the apical membrane (271).

Colorimetric techniques, such as the orthophenanthroline method, have been used for iron analysis in human milk as one of the first approaches (272, 273). In the more recent past, AAS has emerged as the method of choice for iron analysis. Sample preparation includes lyophilizing and ashing of the sample before acid (nitric acid, sulfuric acid) digestion; in addition, microwave digestion with the use of nitric acid and hydrogen peroxide have been described (270, 272, 274–302). Inductively coupled argon plasma spectrometry and ICP-MS have been proposed as a valid alternative for iron analysis in human milk (303–307). These approaches also require sample digestion by nitric acid or hydrogen peroxide.

The iron AI for infants aged 0–6 mo is based on 9 reports that mostly used AAS and inductively coupled argon plasma spectrometry for analysis (213, 275, 277, 281–284, 288, 289, 306). Both methods are suitable for iron analysis in human milk.

Copper

Copper is mostly found in the skim-milk fraction of human milk, but substantial amounts are also present in milk fat (308). Copper-binding proteins in milk include casein, serum albumin, and ceruplasmin (309, 310).

Early techniques for copper analysis in human milk include colorimetric assays, such as the diethyldithiocarbamate method (272, 311). A rapid wet digestion with the use of nitric, perchloric, and sulfuric acid is followed by deionization of interfering iron with citrate or pyrophosphate under alkaline conditions before analysis (311). However, AAS has been one of the main analytical techniques used for copper analysis in human milk over the past 30–40 y (272, 274, 275, 277–281, 283, 284, 286, 288–290, 293, 296–298, 302, 308, 312–322). Other techniques used for copper analyses include ICP-AES (303–306, 323, 324), ICP-MS (307, 325–329), or neutron activation analysis (NNA) (330).

The copper AI for infants aged 0–6 mo was established by the review of 16 reports (213). Methods used to determine copper concentrations in those reports include AAS, ICP-AES, and ICP-MS (275, 277, 279, 280, 284, 288, 289, 305, 306, 312, 315–317, 325, 327); both techniques are valid approaches for copper analysis in human milk.

Zinc

Like iron and copper, zinc can be found in both the whey and fat fractions of human milk (286). A substantial amount of zinc is associated with citrate, a low-molecular-weight binding ligand (272) as well as with casein and serum albumin as zinc-binding proteins (309).

Early approaches used colorimetric methods with the use of dithizone as a reagent (272, 331). However, AAS has emerged as one of the main techniques for zinc analysis in human milk (272, 274, 275, 277–280, 283–285, 288–290, 293, 295–300, 302, 308, 312–322, 329, 332–343). More recently, ICP-AES and ICP-MS have also been described for zinc analysis in milk (303, 304, 306, 323, 324, 326–329, 344).

The zinc AI for infants aged 0–6 mo is based on 12 reports that used AAS and ICP-AES or ICP-MS. All of the approaches are valid methods for analyzing zinc in human milk.

Iodine

More than 75% of the iodine content in human milk is present as ionic iodide (345–347). Iodine is concentrated by the lactating breast due to increased expression of the main iodine transporter during lactation. However, maternal intake also influences the iodine concentration in milk (348–351).

The main approach for analyzing iodine in breast milk has been a colorimetric measurement based on the Sandell-Kolthoff reaction, in which iodine catalyzes the reduction in cerium (IV) by arsenic (III) under acidic conditions. The sample undergoes an ashing process before analysis and can be measured by using an autoanalyzer (347, 349, 352–367). ICP-MS has been shown to provide comparable results to the colorimetric method without analytical bias between the 2 approaches (368–374). However, a recent study showed that ICP-MS should be the method of choice for analyzing breast-milk iodine concentrations due to its superior recovery and sensitivity when compared with the colorimetric Sandell-Kolthoff approach, indicating a previously unreported bias between the 2 methods (6).

Other analytical techniques for iodine analysis include neutron-activated analysis (301, 375), ion chromatography coupled with MS (376–378), and the use of an iodide-specific electrode (346, 379). The 2 latter approaches usually only provide results for iodide, not the total iodine content of breast milk.

The iodine AI for infants aged 0–6 mo is based on only a few reports that used the colorimetric approach (353, 366) or the iodide-specific electrode, capturing only the ionic iodide (346). On the basis of recent findings with regard to ICP-MS and the colorimetric assay (6), ICP-MS is the preferred methodologic approach for analyzing total iodine concentrations in human milk.

Selenium

The majority of selenium in human milk is bound to proteins, whereas only a minor faction is associated with the milk fat (380). Several analytical approaches have been used for selenium analysis: GC coupled with ECD (GC-ECD), a fluorometric method, AAS, NNA, and inductively coupled argon plasma spectrometry. GC-ECD analysis requires sample digestion and conversion of the various oxidation states of selenium into Se (IV) before derivatization with the use of 4-nitro-*o*-phenylenediamine and removal of interferences

with hydroxylamine sulfate, EDTA, and urea. The Se-derivative is extracted by toluene before analysis (380–386).

The fluorometric method includes the wet-ashing with the use of hydrogen chloride and perchloric acid ($HClO_4$) and derivatization by using 2,3-diaminonaphthalene and extraction into cyclohexane. Fluorescent interferences are removed by back-extraction of the selenium complex with concentrated nitric acid (324, 387–397).

Other methodologic approaches include hydride generation, flow-injection hydride, and electrothermal AAS (320, 322, 398–405); instrumental NNA (301, 330, 401, 406–408); ICP-MS and ICP-AES (326, 409, 410); and isotope dilution MS (411).

The selenium AI for infants aged 0–6 mo is based on 13 reports that used GC-ECD and NNA (164); however, not all of the reports used provide information about the methodologic approach for analysis. Compared with the methods used in the reports, AAS includes less sample handling and no radiation steps.

Quality assurance and method validation

Although external reference material is readily available for analysis in plasma or serum samples, there is, to our knowledge, no certified standard for analyzing micronutrients in human milk. The National Institute of Standards and Technology recently developed a fortified and nonfortified human-milk standard reference material (SRM) for organic contaminants such as polychlorinated biphenyl congeners or chlorinated pesticides. However, the respective certificates of analyses include some minerals such as copper, iron, or calcium and could be used for quality assurance of the listed minerals. Alternatively, National Institute of Standards and Technology SRM 1849a infant/adult nutritional formula has been analyzed for evaluation during method development for

TABLE 1 Preferred methods for vitamin and mineral analysis in human milk[1]

Vitamin	Forms reported in human milk	Methodology	References
Thiamin	Thiamin, TMP, TPP	LC-FLD, LC-MS/MS	(8, 22, 24)
Riboflavin	Riboflavin, FAD, 10-OH-ethylflavin, and traces of 10-formyl-methylflavin, 7α-OH-riboflavin, 8α-OH-riboflavin, and FMN	LC-MS/MS, LC-FLD	(4, 24)
Niacin	Nicotinamide, NAD, NADP, NR, NMN	LC-MS/MS, microbiological assay, fluorometric enzyme-coupled assay	(24, 42, 43)
Vitamin B-6	Pyridoxal, PLP, PN, PM	LC-MS/MS, LC-FLD	(24, 50)
Cobalamin	Methylcobalamin 5′-deoxyadenosylcobalamin, hydroxo-cobalamin, cyanocobalamin	CPBA–chemiluminescence	(72, 93)
Folate	Pteroylpolyglutamates, N-5 methyltetrahydrofolate folacin derivatives, folic acid, and *p*-aminobenzoylglutatmate and its acetamide derivative	Microbiological assay	(104, 120)
Pantothenic acid	Pantothenic acid	LC-MS/MS, microbiological assay	(25, 43)
Biotin	Biotin, bisnorbiotin, biotin sulfoxide	CPBA–radiodetection, LC-MS/MS	(25, 134)
Choline	Choline, phosphocholine, glycerophosphocholine, phosphatidylcholine, sphingomyelin	LC-MS, GC-MS/LC-radiodetection	(147, 415)
Vitamin C	Ascorbic acid, dehydroascorbic acid	LC-DAD	(154)
Vitamin A	Retinol, retinyl esters β-Carotene	LC-DAD, LC-MS/MS	(203, 212)
Vitamin D	Vitamins D_2 and D_3 25(OH)D_2, 25(OH)D_3 24,25$(OH)_2$D and 1,15$(OH)_2$D	CPBA/LC-DAD, LC-MS/MS	(220, 226)
Vitamin E	α-, β-, γ-, and δ-tocopherols α-, β-, γ-, and δ-tocotrienols	LC-FLD/DAD, LC-MS/MS	(203, 212)
Vitamin K	Phylloquinone, menaquinone-4	LC-FLD, LC-MS/MS	(212, 254)
Iron	Iron	ICAPS/ICP-MS AAS	(297, 306, 307)
Copper	Copper	ICAPS/ICP-MS, AAS	(297, 306, 307)
Zinc	Zinc	ICAP/ICP-MS, AAS	(297, 306, 307)
Iodine	Iodide, iodine	ICP-MS	(6)
Selenium	Selenium	AAS	(404)

[1] AAS, atomic absorption spectroscopy; CPBA, competitive protein-binding assay; DAD, diode array detector; FLD, fluorescence detection; ICAPS, inductively coupled argon plasma spectroscopy; ICP-MS, inductively coupled plasma–MS; MS/MS, tandem MS; NMN, nicotinamide mononucleotide; NR, nicotinamide riboside; PLP, pyridoxal-5′-phosphate; PM, pyridoxamine; PN, pyridoxine; TMP, thiamin monophosphate; TPP, thiamin pyrophosphate; LC; 1,15$(OH)_2$D, 1,15-dihydroxyvitamin D; 24,25$(OH)_2$D, 24,25-dihydroxyvitamin D; 25(OH)D_2, 25-hydroxyvitamin D_2; 25(OH)D_3, 25-hydroxyvitamin D_3.

vitamin analysis in human milk (24, 25, 412). However, in addition to the apparent matrix differences, the forms of vitamins present in the infant formula SRM differ considerably from those in milk (24); thus, its use for method validation for the human-milk matrix is very limited.

In lieu of a certified human-milk SRM, in-house pooled breast milk has been used for method validation and quality assurance (6, 8, 22, 24, 72, 93). Standard addition experiments should be used to validate the unknown concentrations of the micronutrients of interest to ensure accuracy and precision of the results (413). Without proper validation of the unknown concentration, the pooled milk samples may be used to evaluate precision but not accuracy.

Preanalytical considerations

Choosing a suitable protocol for human-milk sampling is as important as using an appropriate method for analysis. Although many studies have been devoted to milk micronutrient analyses, the variations in milk collection protocols are numerous. We found that the circadian variance was significant for fat- and water-soluble vitamin concentrations in milk from Bangladeshi mothers and that some vitamin concentrations differed on the basis of the collected aliquots within a feeding, but none of those differences were substantial (414). Maternal supplementation was reflected in breast-milk riboflavin and pyridoxal concentrations shortly after ingestion, showing the importance of the timing of sample collection when mothers consume supplements (414). Mock et al. (129) found significant differences in biotin concentrations between breasts for some study participants.

Some micronutrients such as minerals are generally stable and tolerate various storage conditions, but the use of trace element–free supplies is necessary to avoid cross-contamination. Vitamins, however, contain an array of different chemical and physical properties. Their light, temperature, and pH sensitivities (9) have to be considered when collecting milk samples. Collection under dim light in amber containers and sample storage at subambient temperatures (−70°C) are suggested to minimize potential analyte degradation. Nicotinamide, in particular, has been shown to be sensitive to storage and handling, showing some degradation within the analytical run and lower precision when samples were undergoing thaw-freeze cycles (also true for FAD) compared with other B-vitamins (24).

Thus, depending on the micronutrient of interest, sample collection and storage conditions should accommodate the specific needs of the micronutrient of interest to minimize analyte losses and cross-contamination. Maternal supplement consumption affects the milk concentrations of at least some milk vitamins and needs to be considered when scheduling the sample collection to ensure a representative sample collection.

Conclusions

A wide array of methodologic approaches have been described for analyzing micronutrients in human milk, including microbiological assays, chromatographic techniques, or ICP. The preferred method for analysis, however, is dependent on the micronutrient of interest and its (active) forms found in milk (**Table 1**). Although some micronutrients such as vitamin B-12 or folate are bound to milk proteins, others such as thiamin or riboflavin are found in their free as well as in their phosphorylated or coenzymatic forms. Nevertheless, several micronutrients can be analyzed simultaneously (e.g., vitamins A and E and carotenoids; iron, copper, and zinc; or multiple B-vitamins).

Although microbiological assays are the preferred choice for analyzing folate, niacin, and possibly pantothenic acid, chromatographic approaches have been adapted for the majority of the micronutrients discussed. Mineral analyses have evolved over time from the colorimetric approaches to more sophisticated techniques such as AAS or ICP-MS and ICP-AES, and vitamin B-12 is usually analyzed by using CPBAs. Nevertheless, a substantial number of methods used for micronutrient analysis in human milk fail to provide accurate and reliable data; moreover, conditions for sample collection and storage are equally important for the accurate determination of micronutrient concentrations in milk. The lack of certified human-milk standards can be overcome by validating an in-house pooled milk sample, preferably by standard addition experiments. The information available in this review should aid in the understanding and interpretation of the validity of values reported in the literature and in the selection of suitable methods for micronutrient analysis in human milk in future studies.

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