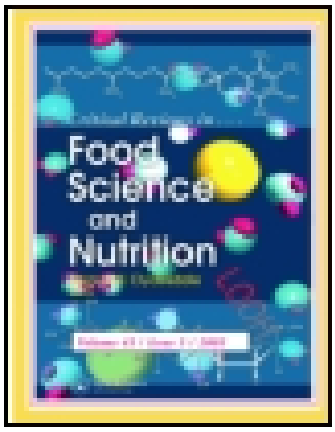

Critical Reviews in Food Science and Nutrition
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/bfsn20

Analyzing B-Vitamins in Human Milk: Methodological Approaches

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Accepted author version posted online: 15 Jan 2015.

To cite this article: Daniela Hampel & Lindsay H. Allen (2015): Analyzing B-Vitamins in Human Milk: Methodological Approaches, Critical Reviews in Food Science and Nutrition, DOI: 10.1080/10408398.2013.783550

To link to this article: http://dx.doi.org/10.1080/10408398.2013.783550

Analyzing B-Vitamins in Human Milk: Methodological Approaches

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ABSTRACT

According to the World Health Organization (WHO) infants should be exclusively breastfed for the first six months of life. However, there is insufficient information about the concentration of nutrients in human milk. For some nutrients, including B-vitamins, maternal intake affects their concentration in human milk but the extent to which inadequate maternal diets affect milk B-vitamin content is poorly documented. Little is known about infant requirements for B-vitamins; recommendations are generally set as Adequate Intakes (AI) calculated based on the mean volume of milk (0.78L/day) consumed by infants exclusively fed with human milk from well-nourished mothers during the first six months, and the concentration of each vitamin in milk based on reported values. Methods used for analyzing B-vitamins, commonly microbiological, radioisotope dilution or more recently chromatographic, coupled with UV, fluorometric and MS detection, have rarely been validated for the complex human milk matrix. Thus the validity, accuracy and sensitivity of analytical methods is important for understanding infant requirements

for these nutrients, the maternal intakes needed to support adequate concentrations in breast milk. This review summarizes current knowledge on methods used for analyzing the B-vitamins thiamin, riboflavin, niacin, vitamin B-6 and pantothenic acid, vitamin B-12, folate, biotin, and choline in human milk, their chemical and physical properties, the different forms and changes in concentration during lactation, and the effects of deficiency on the infant.

KEYWORDS

Human milk, infant nutrient requirements, B-vitamin deficiency, microbiological assays, chromatographic analyses

INTRODUCTION

According to the World Health Organization (WHO) infants should be exclusively breastfed for the first 6 months of life rather than the 4-6 months previously recommended. This advice was based substantially on findings that exclusive breastfeeding has protective effects against gastrointestinal infections (WHO 2001). A breastfed infantôs nutritional status, and especially B-vitamin status (Allen 2012) therefore relies on the amounts of the vitamins in breast milk, which are highly influenced by maternal dietary intake. Poor maternal status or inadequate intake of B-vitamins during lactation is usually reflected in lower breast milk concentrations and can subsequently cause infant deficiency.

Lack of reliable data reflecting the infantôs response to different levels of nutrient intake inhibits the setting of Estimated Average Requirements (EARs) and therefore the determination of Recommended Dietary Allowances (RDAs). Thus, the infantsôrecommended intakes of B-vitamins (and other nutrients) are generally set as Adequate Intakes (AI), calculated as the mean intakes by full-term infants exclusively fed with human milk from well-nourished mothers during the first six months (I.O.M, 1998). This calculation is based on the mean concentration of the vitamins from 2 - 6 months of lactation using consensus values from several reported studies, and an average milk intake of 0.78L/day (I.O.M. 1998). Depending on the vitamin in question the studies used for setting the AIs include as few as 22 women (thiamin) and up to 111 women (vitamin B-12) and were conducted between 1951 and 1997 (I.O.M. 1998). Thus, the current AIs are based on data from outdated studies with a low number of subjects, indicating a potential need for re-evaluation. Additional problems with existing data include inconsistent timing and

methods of milk collection, different stages of lactation, and lack of information about the effects of any maternal supplements.

Methods used for analysis of B-vitamins are commonly microbiological, or in more recent years, chromatographic coupled with UV, fluorometric or MS detection (Sakurai, et al. 2005, Shi, et al. 2011, Hampel, et al. 2012). Other techniques include radioisotope dilution assays, animal studies and voltammetry (McCosh, et al. 1931, Thomas, et al. 1979, Mikheeva, et al. 2009). However, these approaches are known to be suboptimal regarding time, sample volumes and cost, and are not necessarily validated for analysis of B-vitamins in the complex human milk matrix (Tamura and Picciano 2006b). In some cases the results obtained with different methods were not in agreement (Shane, et al. 1980, Roughead and McCormick 1990). Therefore, evaluating the suitability of methods for the task on hand is crucial.

In this review, different methodological approaches for analyzing the B-vitamins thiamin, riboflavin, niacin, vitamin B-6 and pantothenic acid, vitamin B-12, folate, biotin, and choline in human milk are discussed including the potential advantages and disadvantages of each method. Relevant physical and chemical properties of each of the vitamins are described to assist in optimizing sample preparation or detection. Moreover, information on the different forms in milk and their change in concentration during lactation are described as well as their effects of deficiency on the infant.

CURRENT KNOWLEDGE

Thiamin (Vitamin B1)

Thiamin (3-(4ô-amino-2ô-methylpyrimidinyl-5ô-ylmethyl)-5-(2-hydroxyethyl)-4-methyl-thiazole) was first isolated and structurally characterized in 1926. This water-soluble vitamin (S_W: 100g/100mL) exists naturally as free thiamin and phosphorylated as thiamin monophosphate (TMP) and thiamin pyrophosphate (TPP). Vitamin B1 is very susceptible to heat and to neutral and alkaline pH. Mild acidic conditions (pH 2 ï 4) are preferred for storage in solution, but degradation still occurs when exposed to heat. The spectral properties of thiamin vary with pH; thiamin hydrochloride, a form of vitamin B1 used for food fortification and pharmaceuticals, shows a single maximum at 246nm at pH 2.9. Between pH 5 and 7, two absorption maxima are observed at 234nm and 264nm, representing the pyrimidine and thiazole rings. Above pH 8, thiamin and its phosphate esters can be oxidized quantitatively to thiochrome and thiochrome phosphate esters respectively. Unlike the substrate, the biologically inactive oxidation products are a strongly fluorescent (Ex ə: 375nm, Em ə: 432-435nm), a quality which has been widely used for quantitative analysis of vitamin B1 in various matrices since the 1930s (Ellefson 1985, Ball 1994, Eitenmiller and Landen 1999). TPP is essential for normal carbohydrate, nucleic acid, and amino acid metabolism, e.g. decarboxylation of α-keto acids, the transketolase reaction in the pentose phosphate pathway and synthesis of the primary neurotransmitter acetylcholine. Thiamin phosphates are also involved in nerve impulse transmission (Rindi 1982, Gibson 1990, Gerrits, et al. 1997, Eitenmiller and Landen 1999). Beriberi is the classical syndrome of thiamin deficiency, but cardiovascular, muscular, nervous and gastrointestinal systems are also affected (Gibson 1990). Thiamin concentrations in human milk depend on maternal status but maternal supplementation results in only a limited increase (Pratt and Hamil 1951, Nail, et al. 1980, Thomas, et al. 1980, Lönnerdal 1986). During pregnancy its deficiency can cause problems such

as alterations in glucose tolerance and intrauterine growth, increasing the risk of low birth-weight in the offspring (Baker, et al. 2000, Ortega, et al. 2004) and it is also thought to be a possible cause of sudden infant death syndrome (Bernshaw 1991). Thiamin nutritional status is usually measured by transketolase activity in erythrocytes which reflects the adequacy of body stores (Gibson 1990).

Free thiamin and thiamin monophosphate are the main forms of vitamin B1 in human milk with increasing concentrations as lactation proceeds (Emmett and Rogers 1997, Picciano 1998, Stuetz, et al. 2011). Maternal supplementation of thiamin is not reflected in milk concentrations but in increased urinary excretion, limiting the amount that can be transferred into milk (Slater and Rial 1942, Lönnerdal 1986). Methods for analysis include the classic thiochrome reaction, and microbiological and HPLC methods. However, the first quantitative measurement of ñvitamin Bò (and also riboflavin) was carried out by measuring the growth of young rats whose diet was supplemented with human milk as the only vitamin B source (McCosh, et al. 1931, Donelson 1934). By feeding various amounts of pooled and individual breast milk to young albino rats, the so-called ñunit-growthò was determined as well as the influence of more adequate vitamin intake (Bourquin and Sherman 1931, Chase and Sherman 1931). By nature, this approach offers only a measurement of physiologically available thiamin. Besides being a very time consuming and expensive process, the results vary considerably. Furthermore, certain bacteria in the intestinal tract are able to synthesize thiamin and provide it to the animal, which adds to the complexity and inaccuracy of this biological assay (Ellefson 1985).

Thiamin quantitation by microbiological assays was first developed using *Lactobacillus fermenti*. However, the assay is compromised by the bacteriaôs susceptibility to both stimulatory and inhibitory matrix constituents such as sugars, reducing agents and calcium. Other organisms tested include *S. cerevisiae*, *O. malhamensis* and *L. viridescens* (ATCC 12706). The latter provides results comparable to quantification obtained by the thiochrome assay and is commonly accepted for food analysis. The extraction procedures for microbiological assays generally follow the thiochrome analysis procedures such as extraction under acidic conditions (0.33 M H_2SO_4) and heating prior to inoculation (Ford, et al. 1983). Microbiological assays require enzymatic hydrolysis of the phosphate esters due to differential growth response to TMP, TDP and TPP (Deibel, et al. 1957, Banhidi 1958, Ford, et al. 1983, Eitenmiller and Landen 1999, Fernández-Muiño, et al. 2008).

Since its development (Barger, et al. 1935), the thiochrome method has been widely used for thiamin analysis in biological matrices. For analyzing free and total thiamin in human milk samples were subjected to acidic hydrolysis (0.1N H_2SO_4) and heat followed by incubation overnight with (total thiamin) or without (free thiamin) clarase at pH 4.5. The vitamin was isolated by column chromatography prior to oxidation with potassium ferrocyanide yielding thiochrome. After extraction with isobutanol the samples were ready for analysis with a photofluorometer (Roderuck, et al. 1945). Although thiamin-phosphates are also converted under these conditions, the resulting thiochrome phosphates are not soluble in isobutanol and are therefore undetectable (Kinnersley and Peters 1938) allowing differentiation between free thiamin and total thiamin in the sample as described. Use of the thiochrome method for human milk has been reported at intervals over the last 60 years (Pearson, et al. 1946, Solov'eva and

Khrustaleva 1954, Ortega, et al. 2004). On the other hand, Hoshi (1956) described a colorimetric analysis using diazotized p-aminoacetophenone to form a colored precipitate under acidic conditions. However heavy metal salts, potassium ferricyanide, cysteine, iodine and other compounds interfere with the analysis resulting in artificially lower thiamin values (Prebluda and McCollum 1939, Sealock and Goodland 1944).

In the last 30 years, HPLC methods have become a commonly used technique for thiamin analysis in human milk still imbedding the well-known thiochrome reaction followed by fluorescence detection (Wielders and Mink 1983, Böhm, et al. 1997, McGready, et al. 2001, Sakurai, et al. 2005); however, HPLC-UV analysis has also been reported for human milk (Shi, et al. 2011). Using the thiochrome approach, HPLC methodology offers pre- or post-column derivatization of thiamin and its phosphate esters. While earlier reports used a post-column modification of the vitamin (Wielders and Mink 1983, Böhm, et al. 1997), pre-column derivatization of thiamin analysis in human milk has been described for the last ten years (McGready, et al. 2001, Stuetz, et al. 2011, Stuetz, et al. 2012). Recently Stuetz *et al.* (2011, 2012) analyzed thiamin and its monophosphate in human milk based on pre-column derivatization, which can be conducted fully automated. The analysis is then carried out by reversed-phase liquid chromatography coupled with fluorescence detection. Most recently we have developed an UPLC-MS/MS method for human milk samples analyzing thiamin simultaneously with other B-vitamins, specifically vitamin B2, niacin and pyridoxal after removal of proteins and non-polar constituents (Hampel, et al. 2012). Since pre- or post-column derivatization has been rendered obsolete when using this method and multiple vitamins are analyzed within one analytical run, more information is available in less time and with lower

cost. To account for matrix effects influencing analyte recovery stable isotope internal standards were used for quantitation (Hampel et al. 2012).

A similar approach has been described for the simultaneous determination of thiamin, riboflavin, nicotinamide, nicotinic acid, pantothenic acid, pyridoxal, and pyridoxine using LC/MS-TOF in nutritional yeast powder as a model food matrix (Hälvin et al., 2013). A comparison between various enzyme and acid treatments was conducted to optimize the release of the B-vitamins from their co-enzymatic forms. While thiamin and riboflavin both could be liberated using enzyme preparations such as takadiastase, α-amylase, β-glucosidase and acid phosphatase, acid treatment appeared to be ineffective but then again supported the release of pyridioxal, which was only marginally liberated by the enzymes (Hälvin et al., 2013). Neither nicotinamide nor pantothenic acid could be lilberated under any tested conditions, illustrating the complexity and difficulties of simultaneous analysis of multiple analytes present in free and bound forms.

Riboflavin (Vitamin B2)

Riboflavin (7,8-dimethyl-10-ribityl-isoalloxazine) was originally isolated from milk whey simultaneously with thiamin in 1879 and given the name lactochrome or vitamin G (Powers 2003, Buehler 2011). The coenzymes flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), the most important biologically active forms of riboflavin, were characterized between 1934 and 1938. While relatively stable under heat and acidic conditions, vitamin B2 is readily degraded above pH 7.0 and by UV- and visible light. Both coenzymes are easily converted to riboflavin below pH 5.0. Unlike FAD and FMN ($S_{W(Na\ salt)}$: 30 - 67g/$L_{25°C}$)

riboflavinôs yellow-orange crystals are poorly soluble in water (S_W å 0.1g/$L_{25°C}$). In aqueous solution, riboflavin shows absorption-maxima at 223, 226, 373 and 445nm, but flavins also possess a strong native fluorescence (Ex ə: 400 to 500, Em ə: 520 to 530). On an equimolar basis, riboflavin and FMN fluoresce similarly, while FAD shows only 10-20% of the intensity. However, when flavins are bound to proteins a dramatic quenching of fluorescence is observed (Maggiorelli 1958, Shah 1985, Russell and Vanderslice 1992, Ball 1994, Eitenmiller and Landen 1999, Zielenkiewicz, et al. 2010). Humans require riboflavin for DNA repair, energy production, fatty acid and amino acid synthesis, folic acid activation, the formation of the free radical scavenger glutathione, and the conversion of tryptophan to niacin in the liver. Since this vitamin is poorly stored and not synthesized by vertebrates it must be constantly supplied by diet (e.g. meat, milk and dairy products). Therefore, maternal riboflavin deficiency rapidly results in low milk concentrations of the vitamin and biochemical signs of depletion occur within only a few days of dietary deprivation (Bates, et al. 1982b, Powers 2003). Biochemical methods, such as the analysis of the FAD-dependent erythrocyte glutathione reductase (EGR), are essential for confirming clinical and subclinical cases of aribоflavinosis. Riboflavin deficiency usually occurs along with other water-soluble vitamin deficiencies and causes growth retardation, anemia, skin lesions or degenerative changes in the nervous system (Gibson 1990, Van Herwaarden, et al. 2007, Buehler 2011).

FAD and riboflavin are the prevalent forms of vitamin B2 in human milk (Roughead and McCormick 1990, Sakurai, et al. 2005) with an average concentration of 0.35mg/L in well-nourished women (Allen 2005). Other flavins present include 10-hydroxy-ethylflavin (2ï 10%; formed during the anaerobic bacterial degradation of riboflavin) and traces of 10-formyl-

methylflavin, 7α-hydroxyriboflavin, 8α-hydroxy-riboflavin (both products of riboflavin catabolism) and FMN (Roughead and McCormick 1990, Sakurai, et al. 2005). Throughout lactation the vitamin B2 concentration in human milk remains constant (Emmett and Rogers 1997). Quantitative methods for analysis of vitamin B2 in human milk include animal assays, and microbiological and spectroscopic (UV, fluorescence) techniques (McCosh, et al. 1931, Donelson 1934, Thomas, et al. 1980, Ford, et al. 1983, Ronnholm 1986, Toyosaki, et al. 1986, Roughead and McCormick 1990). As already mentioned, the first quantitative measurement of ñvitamin Gò combined with thiamin was described by Donelson and Macy (1934) and McCosh *et al.* (1931) (McCosh, et al. 1931, Donelson 1934) based on the growth of young rats, which used to be the original standard for other methods (Shah 1985). However, the results of this assay tend to be overestimates due to additional amounts of the vitamin in the food. Such animal studies are time consuming, expensive and least accurate, but they best reflect riboflavin bioavailability (Cosner and Schuck 1948, Shah 1985).

Most microbiological assays for riboflavin have been performed with *Lactobacillus rhamnosis* (formerly *L. casei*) ATCC 7469 and were first described for milk from different animal species in the 1940s (Williams, et al. 1942, Pearson, et al. 1946). Sample preparations consist of acidic hydrolysis, protein precipitation and neutralization prior to incubation with the growth medium. Riboflavin-dependent cell growth is used for indirect quantification by turbidimetry. However, common matrix constituents such as starch, protein degradation products or free fatty acids affect the growth of the culture. Furthermore, the response to FMN or FAD differs from the growth promotion by riboflavin; therefore all methods based on microbiological analysis are for total riboflavin (Bates, et al. 1985, Eitenmiller and Landen 1999).

Fluorometric techniques take advantage of the fact that riboflavin undergoes different routes of degradation depending on the pH. In acidic and neutral conditions the loss of the ribityl side chain results in lumichrome (6,7-dimethyl alloxazine) formation, while under alkaline pH irradiation generates lumiflavin (6,7,9-trimethylisoalloxazine) by cleaving the deoxy-end carbon of the ribityl side chain. The latter is an important analytical tool for flavin detection due to the significantly stronger fluorescence of lumiflavin compared to the native riboflavin. Even though the specificity can be enhanced by additional preparation steps (e.g. chloroform extraction of lumiflavin), the actual reaction does not proceed quantitatively and depends strongly on the experimental conditions and instrumental set-up (Strohecker and Henning 1965, Bates, et al. 1982a, Woodcock, et al. 1982, Eitenmiller and Landen 1999).

In the last 20 years HPLC methods have been introduced for riboflavin analysis in human milk utilizing the spectral properties of vitamin B2 for detection. In 1945, Roderuck and Coryell described the analysis of ñfreeò and ñboundò riboflavin in human milk by acidic hydrolysis with and without subsequent enzymatic digestion. The samples were purified by column chromatography and analyzed by fluorescence reading with appropriate filters (Roderuck and Coryell 1945), a method which was modified from riboflavin analysis in food products (Conner and Straub 1941). Due to the degradation of FAD and FMN into riboflavin below pH 5.0, the so-called ñfree riboflavinò in this study not only refers to actual freely available riboflavin but also to converted FAD and FMN, while the ñboundò fraction, analyzed after enzymatic degradation, also captures riboflavin previously bound by other constituents in the matrix (Watts, et al. 1948). Flavin analysis in human milk by HPLC and fluorescence detection described by Roughead and McCormick (1990) follows the same analytical principle. The

sample preparation, adapted from a goat and cowôs milk work-up procedure (Owen and West 1971), includes protein precipitation with trichloro-acetic acid (TCA) followed by phenol extraction of the flavins. Adding water to the phenol phase and back-extracting the organic solvent with diethyl-ether results in an aqueous sample ready for analysis by RP-HPLC. The authors pointed out that the values obtained have to be corrected for the internal quenching in FAD caused by the formation of an intramolecular complex between the isoalloxazine ring and the adenine moieties, which might not have been considered in earlier investigations and be a possible explanation for the discrepancy in their findings (McCormick 1968, Roughead and McCormick 1990). In contrast, studies utilizing an enzymatic treatment for a quantitative conversion of FAD to riboflavin in human milk prior to fluorometric analysis show comparable results (Rettenmaier and Vuilleumier 1983, Ronnholm 1986, Roughead and McCormick 1990). HPLC coupled with fluorescence detection has been described for flavin analysis in human milk for the last two decades and has been used recently (Sakurai, et al. 2005, Van Herwaarden, et al. 2007, Israel-Ballard, et al. 2008). However, riboflavin and FAD can also be analyzed simultaneously with thiamin, niacin and pyridoxal using UPLC-MS/MS; thus both prevalent forms can be analyzed directly in their native forms allowing distinction between the free vitamin and its coenzyme (Hampel, et al. 2012).

In 2009 Mikheeva *et al.* developed a rapid riboflavin analysis in breast milk by voltammetry (VA). It is less labor-intense and does not require large amounts of reagents and expensive equipment, compared to the previously discussed methods (Mikheeva, et al. 2009). This procedure is based on the oxidizability of riboflavin at a glassy-carbon (GC) indicator electrode. The samples are subjected to mild acidic hydrolysis (0.1N HCl), followed by an

ammonium-sulfate protein precipitation sediment removal prior to analysis. For quantitation purposes, the anodic differential voltammogram is recorded. However, the complexity of the pH dependence of the potential of vitamin B2 has to be considered, given that pH influences both the rate of the electrode process and its mechanism (Mikheeva, et al. 2009).

Niacin

Niacin refers to nicotinic acid (pyridine-3-carboxylic acid) and nicotinamide (pyridine-3-carboxylic acid amide) and was recognized as a curative factor for pellagra in 1937 (Spies, et al. 1938). Symptoms of marginal deficiency include insomnia, loss of appetite, weakness, soreness of mouth and tongue and indigestion. Dietary intake of the vitamin can be established from the ratio of urinary excretion of Nô-methylnicotinamide (NMN) and Nô-methyl-2-pyridone-5-carboxamide (2-pyridone). However the niacin metabolites only reflect niacin status so potential depletion might not be recognized (Gibson 1990). Humans produce nicotinic acid *in vivo* in the liver utilizing 60mg of the amino acid tryptophan to produce 1mg of nicotinic acid, a process which can be inhibited by excess leucine in the diet (Heidelberger, et al. 1949, Gibson 1990, Ball 1997, Eitenmiller and Landen 1999). Rich sources of niacin include oil seeds, legumes, lean meat and cereals. The latter contain niacin bound as niacytin, of which only very little is biologically available after hydrolysis by gastric acid (Gibson 1990). Niacytin can be degraded by soaking cereals in calcium hydroxide to release the niacin, for example in the process called nixtamalization used in some Latin American countries. Both forms are interconvertable; the free acid is readily converted into nicotinamide, which serves as precursor for the synthesis of nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate

(NADP). These coenzymes are important co-factors in oxidation-reduction reactions such as electron carriers for intracellular respiration, acting as a co-dehydrogenase involved in the oxidation of fuel molecules (NAD) or as a hydrogen donor in reductive processes for biosynthesis of fatty acids (NADP) (Eitenmiller and De Souza 1985, Eitenmiller and Landen 1999). Both free forms are stable in aqueous solution and not very susceptible to heat or pH. The amide is significantly more water-soluble (S_{Wamide} = 100g/100mL, S_{Wacid} = 1.67g/100mL) and hydrolyzes easily in acidic or basic conditions converting to the free acid (Eitenmiller and De Souza 1985). The coenzymes NAD and NADP show two UV maxima at 260nm (nicotinamide ring) and 340nm (adenine ring) as well as fluorescent qualities (Em λ = 470nm, when excited at 260 or 340nm). Free niacin however lacks fluorescence and shows relatively low specificity for UV detection. Therefore, the spectral properties are suboptimal for quantitation purposes and require time consuming purification and isolation procedures to remove interferences. Nonetheless, due to its stability, acidic or alkaline hydrolysis as well as heat can be used to isolate niacin from coenzyme structures and destroy sample matrices (Eitenmiller and Landen 1999).

Nicotinamide, NAD and NADP have been reported as forms of niacin in human milk (Greer 2001, Sakurai, et al. 2005). However, there has been little interest regarding the content of this vitamin in human milk, and quantitation has usually occurred in the context of multi-vitamin studies (Coryell, et al. 1945, Ford, et al. 1983, Sakurai, et al. 2005, Shamsia 2009, Shi, et al. 2011). Its concentration increases with the duration of lactation from colostrum to mature milk (Emmett and Rogers 1997) but niacin deficiency remains challenging in dietary depleted areas where corn and other cereals are major dietary staples. Maternal supplementation with this

vitamin is reflected in the milk content and increases the amount available to the infant (Pratt and Hamil 1951, Lönnerdal 1986, Eitenmiller and Landen 1999). Methods routinely used for niacin analysis include chemical (colorimetric) and microbiological procedures, though other analytical techniques have become available in more recent years.

The basis of the colorimetric analysis is the König reaction (Pelletier and Campbell 1959), in which free niacin and cyanogen bromide form a pyridinium intermediate. By undergoing rearrangements the newly formed derivatives couple with aromatic amines to form colored polymethine products, which absorb at a VIS-maximum of 436nm. The analysis is susceptible to interferences by pyridine derivatives under certain conditions (e.g. trigonelline under alkaline pH) (Sarett, et al. 1940); therefore the choice of temperature, pH, and aromatic amine as a coupling-agent for maximum color development are critical factors for quantitation and need to be controlled (Pelletier and Campbell 1959, Strohecker and Henning 1965, Eitenmiller and De Souza 1985, Eitenmiller and Landen 1999). Although applied to niacin analysis in various matrices, this method does not appear to have been employed for human milk studies.

Most niacin analyses in human milk have been conducted using microbiological assays. Among others, *Lactobacillus plantarum*, *L. mesenteroides* and *Tetrahymena thermophila* have been used for niacin analysis in general. *L. plantarum* shows an equimolar response to nicotinic acid, nicotinamide and NAD offering a reliable analysis of total niacin in biological matrices, while *L. mesenteroides* only responds to nicotinic acid, and *Tetrahymena thermophila* equally but not additively responds to both free niacin forms (Eitenmiller and De Souza 1985, Eitenmiller and Landen 1999). With regard to human milk, *L. arabinosus* has been reported

many times to be the microorganism of choice (Coryell, et al. 1945, Macy 1949, Pratt and Hamil 1951, Ford, et al. 1983). Coryell *et al.* (1945) were the first group to analyze niacin in human milk using *L. arabinosus*, which had been introduced as a suitable microorganism by Snell and Wright (1941) and modified by Krehl *et al.* (1943) and subsequently used by other groups. The samples were autoclaved and hydrolyzed under acidic conditions (0.1N HCl) followed by protein precipitation at pH 4.6 and diluted before the basal medium was added. After inoculating with the bacterium the nicotinic acid concentration was determined by turbidimetry (Coryell, et al. 1945). Due to higher sensitivity and specificity, the microbiological method offers better detection limits for samples with low amounts of niacin than the colorimetric analysis. However, the assay is susceptible to growth-stimulating or growth-depressing interferences causing errors during the analysis (Eitenmiller and De Souza 1985, Eitenmiller and Landen 1999).

Only recently have HPLC methods been applied for niacin analysis in human milk. Sakurai *et al.* (2005) described the analysis of unbound nicotinamide in breast milk by HPLC using a UV detector at 260nm for quantitation. The proteins in the sample were precipitated with acetonitrile and removed by centrifugation prior to analysis, a procedure also employed by Shi *et al.* (2011). Protein precipitation was carried out using a precipitation solution consisting of zinc acetate, polyhydrated phosphotungstic acid and glacial acetic acid, a method adapted from the analysis of water-soluble vitamins in supplementary foods (Zafra-Gómez, et al. 2006). HPLC coupled to a diode array detector (DAD) using the method of the Association of Official Analytical Chemists has also been applied to human milk samples (AOAC, 1990, (Shamsia 2009). Using UPLC-MS/MS nicotinamide can be analyzed simultaneously with thiamin, riboflavin, FAD and pyridoxal after protein and fat-removal (Hampel, et al. 2012).

Vitamin B6

Vitamin B6 (2-methyl-3-hydroxy-5-hydroxy methyl pyridine derivatives) is the collective term for the biologically active equivalent and metabolically interconvertible pyridoxine (PN), pyridoxal (PL), and pyridoxamine (PM). In 1934, Gyorgy introduced the term vitamin B6 to differentiate between this factor and other hypothetical growth factors. Pyridoxine was the first B6 vitamer to be structurally identified in 1938 followed by pyridoxal and pyridoxamine in the 1940s. All vitamers exist in their phosphorylated forms i.e. pyridoxal-5ô-phosphate (PLP), pyridoxine-5ô-phosphate (PNP) and pyridoxamine-5ô-phosphate (PMP). The latter two can be oxidized to the metabolically active form, PLP, which serves as a co-factor in numerous amino acid transformations such as transamination, decarboxylation or racemization by forming a Schiff base (Gyorgy 1934, Gyorgy and Eckardt 1939, Snell 1944, Polansky, et al. 1985, Eitenmiller and Landen 1999, Leklem 2001). PN is the most stable form of vitamin B6, followed by PL and PM. All vitamers are quite stable under acidic conditions but degrade greatly above neutral pH. All forms are light sensitive even under acidic conditions; near-UV and UV radiations cause especially significant degradation of the vitamers. The spectral properties are pH- and compound-dependent; all vitamers including their 5ô-phosphates show a maximum absorption at 290nm when dissolved in 0.1N HCl, while at neutral pH the UV maxima of PL and PLP shift to 390nm. The remaining vitamers maximally absorb at 253 and 325nm under the given conditions. The non-phosphorylated vitamers as well as PNP and PMP possess a strong fluorescence (Ex ə: 310 ï 365nm; Em ə: 365 ï 430nm) from slightly acidic to strongly alkaline pH while PLP only shows weak fluorescent properties since the phosphate ester of the 5-position

of the pyridine ring prohibits the hemiacetal formation necessary for a strong fluorescence (Bridges, et al. 1966, Saidi and Warthesen 1983, Eitenmiller and Landen 1999, Ubbink 2000).

General symptoms of B6 deficiency include weakness, sleeplessness, appetite or growth depression and various dermatologic disorders. Clinical status tests include the analysis of plasma PLP and urinary measurements of PL and 4-pyridoxic acid (4-PA). Furthermore, PLP is also involved as a coenzyme in the kynurenine stage of niacin formation from tryptophan; therefore, B6 deficiency increases urinary excretion of kynurenine and 3-hydroxykynurenine, an effect also resulting from oral loading of tryptophan, known as the tryptophan load test. The latter combined with the plasma B6 test currently provides excellent biochemical confirmation of vitamin B6 status. The vitamers are found in all foods of both animal and plant origin such as meats, cereal grains, vegetables or nuts with extremely variable bioavailability (Polansky, et al. 1985, Eitenmiller and Landen 1999). Vitamin B6 is a crucial dietary factor during the early stages of central nervous system development in early infancy and the already limited pool of this vitamin in adult humans rapidly depletes with deficient intake. However, vitamin B6 intake of lactating women appears to influence the concentration of the vitamin in their milk and is a strong predictor of infant vitamin B6 status (West and Kirksey 1976, Morrison and Driskell 1985, Gibson 1990, Kang-Yoon, et al. 1992).

PL has been reported as the principal form of vitamin B6 in human milk, with possible contributions of PLP (7-64%), PMP, PN and PM (mostly traces) (Vanderslice, et al. 1983, Hamaker, et al. 1985, Morrison and Driskell 1985). Its concentration increases throughout lactation from colostrum to mature milk but decreases with lactation beyond 7 months (Karra, et al. 1986, Emmett and Rogers 1997). While maternal vitamin B6 status has been correlated with

milk concentrations, supplementation with this vitamin seems not to be reflected in the milk (Thomas, et al. 1980, Prentice, et al. 1983, Styslinger and Kirksey 1985, Lönnerdal 1986). Microbiological assays and LC based methods have been established as the common techniques for vitamin B6 analysis. However, one of the first quantitative estimations of vitamin B6 carried out by Gyorgy (1936) utilized an animal assay to determine the volume of the so-called ñPetersô eluateò (equivalent to 1 ñrat-day doseò) which has a curative effect against dermatitis in rats (Gyorgy 1936).

The most common organism used for vitamin B6 analysis in biological samples by microbiological assay is *Saccharomyces uvarum* (ATCC 9080) and this has been used for human milk analysis (West and Kirksey 1976, Roepke and Kirksey 1979, Thomas, et al. 1980, Sneed, et al. 1981, Borschel, et al. 1986, Karra, et al. 1986, Eitenmiller and Landen 1999, Leklem 2001). Besides possible inhibition of growth due to high salt concentrations, different growth responses for PN, PL and PM add to the assayôs complexity. Therefore, after hydrolyzing the samples the vitamers are separated chromatographically prior to addition to the yeast basal medium allowing the determination of each vitamer individually (Parrish, et al. 1955, Parrish, et al. 1956, Storvick, et al. 1964, Thiele and Brin 1966, Polansky 1981, Eitenmiller and Landen 1999, Fernández-Muiño, et al. 2008). Other microorganisms such as *Kloeckera brevis* and *Lactobacillus casei* have also been described for vitamin B6 analysis in human milk (Barton-Wright 1962, Ford, et al. 1983, Wilson and Davis 1984). Since *Lactobacillus casei* only responds to pyridoxal, other forms of this vitamin have to be converted by hydrolysis with glycolic acid at 100°C for 15min (PM) or with manganese dioxide at room temperature (PN) (Wilson and Davis 1984). In any

case, the extensive sample preparation and intricacies of microbiological assays have led to the development of chromatographic methods for vitamin B6 analysis.

HPLC methods introduced for human milk analysis generally describe a chromatographic separation coupled with fluorometric detection. Vanderslice *et al.* (1983) treated their samples with sulfosalicylic acid as an extraction agent followed by further clean-up procedures. The B6 vitamers were separated by anion exchange chromatography prior to detection. Results obtained by HPLC analysis were in good agreement with the microbiological assay (Vanderslice, et al. 1983). Further methods described include (RP)-ion pair HPLC separation (Morrison and Driskell 1985) or a simple RP-HPLC separation, with subsequent post-column bisulfate derivatization to enhance PLP-detection. Samples were diluted in mobile phase buffer and proteins were precipitation with TCA. After centrifugation, the supernatant was collected and washed with diethyl ether to remove remaining TCA and fats prior to analysis (Hamaker, et al. 1985). The results obtained with the analytical method were in good agreement with the microbiological assay and also comparable to values obtained with the ion-exchange HPLC methods. RP-HPLC has continued to be described as a suitable method for vitamin B6 analysis in more recent years introducing a post-column photochemical conversion of PLP for improved detection (Gatti and Gioia 2005). However, UPLC-MS/MS allows the analysis of pyridoxal simultaneously with thiamin, riboflavin, FAD and niacin after protein and fat-removal directly without need for derivatization (Hampel, et al. 2012).

Cobalamin (Vitamin B12)

Vitamin B12 is the collective term for cobalt-containing corrinoids, which were first isolated in 1948 (Rickes, et al. 1948, Smith 1948) and structurally elucidated by Hodgkin and coworkers (Hodgkin, et al. 1956, Hodgkin, et al. 1962, White 1962, Brink-Shoemaker, et al. 1964). The molecule is composed of a central cobalt atom coordinated by a nearly planar corrin ring. Its axial coordination sites are occupied by dimethylbenzimidazole and various other groups such as cyano-, hydroxo-, nitrito- and sulfite-functions, a bonding that can be reversibly broken and reformed (Chin 1984, Eitenmiller and Landen 1999). The prevalent form of vitamin B12 in biological materials is coenzyme B12, in which the axial group is either occupied by a methyl group or an adenine nucleoside connected by a cobalt-carbon (Barker, et al. 1960, Lenhert and Hodgkin 1961, Lenhert 1968, I.o.M. 1998). These coenzymes are involved in the folate-dependent methylation of homocysteine to methionine and the conversion of methylmalonyl-coenzyme A to succinyl-coenzyme A (Gibson 1990, I.o.M. 1998). Animal source foods such as meats, sea food, eggs and dairy products represent the main source of B12, while plant derived foods usually provide none of this vitamin (Eitenmiller and Landen 1999, Allen 2009).

Being the most stable vitamer, cyanocobalamin (CNCbl) is used in vitamin preparations, supplements and medical or fortified foods. CNCbl is soluble in water (1g/80mL at 25°C), showing absorption maxima at 278, 361, and 551 nm. Further possible dissolving agents include alcohols, phenols and other polar solvents with a hydroxyl group. Its red crystals are stable when protected from light; they do not melt but decompose above 200°C. CNCbl is most stable between pH 4.0 and 4.5 but will be inactivated under strong alkaline or acidic conditions as well as by UV, strong visible light and oxidizing agents (Herbert 1996, Eitenmiller and Landen 1999, Lindemaus 2000, Beck 2001).

Since B12 is bound to proteins in food, the vitamin needs to be released by enzymatic and acidic hydrolysis in the stomach prior to absorption. Further steps include binding to salivary proteins (R-binders) and to the intrinsic factor secreted by gastric parietal cells prior to absorption in the ileum. B12 is either complexed with a delivery protein transcobalamin (TCII) or with the storage protein (haptocorrin, HC). B12 deficiency is generally caused by inadequate dietary intake, failure to secrete the gastric intrinsic factor necessary for B12 absorption (pernicious anemia, an autoimmune condition), or achlorhydria. Since vitamin B12 - as well as folate - is involved in DNA synthesis, B12 deficiency can result in megaloblastic anemia. Other symptoms include neurological disorders such as neuropathy or spinal cord dysfunction. General symptoms of pernicious anemia include glossitis, weakness, loss of appetite and weight, taste and smell, memory impairment, mild depression and hallucinations (Gibson 1990, Wardinsky, et al. 1995, Eitenmiller and Landen 1999, Allen 2009).

Even though biologically active (cobalamin) and inactive (cobinamide) forms of vitamin B12 are found in plasma, serum, bile and liver, only the biologically active cobalamins are selectively transported into milk (Kolhouse, et al. 1978, Adjalla, et al. 1993, Adjalla, et al. 1994). Compared to serum, human milk contains about 100-fold higher amounts of HC, mostly in the form of apoHC, which potentially interferes with B12 quantification (Sandberg, et al. 1981, Lildballe, et al. 2009). Methylcobalamin (MeCbl) was reported as being the dominant form of the B12 vitamers in human milk followed by 5ô-deoxyadenosylcobalamin (AdoCbl) and small amounts of hydroxocobalamin (OHCbl) and CNCbl, all bound to haptocorrin (Craft, et al. 1971, Sandberg, et al. 1981, Adjalla, et al. 1994). Throughout lactation vitamin B12 decreases in

concentration and low maternal intake is reflected in low milk concentrations (Lönnerdal 1986, Trugo and Sardinha 1994, Emmett and Rogers 1997, Deegan, et al. 2012).

Early approaches for analysis of B12 in human milk were carried out by microbiological assays using *Euglena gracilis* as the test organism (Jadhav, et al. 1962, Jathar, et al. 1970, Craft, et al. 1971, Sandberg, et al. 1981) based on a method developed for body fluids (Ross 1952) but adding enzymatic digestion with papain to release the B12 from binding to haptocorrin. Further steps include the conversion of the different forms into CNCbl by sodium cyanide prior to analysis. Alternatively, *Lactobacillus leichmanii* (NCIB 8118) has also been used to assay B12 microbiologically, and proteins were precipitated by heat in the presence of acetate buffer (Samson and McClelland 1980). However, microbiological assays using Lactobacilli can be influenced by desoxyribonucleosides such as thymidine and other compounds, which have been found to replace B12 as a growth factor resulting in a lower specificity and an overestimate of B12 concentrations (Shive, et al. 1951, Ross 1952).

Alternatively, the radioisotope dilution assay (RIDA) first described by Lau *et al.* (1965) for serum B12 has also been applied for human milk analysis (Areekul, et al. 1977, Thomas, et al. 1979, Thomas, et al. 1980, Van Zoeren-Grobben, et al. 1987, McPhee, et al. 1988, Donangelo, et al. 1989, Specker, et al. 1990, Adjalla, et al. 1994, Trugo and Sardinha 1994, Casterline, et al. 1997, Patel and Lovelady 1998). The method is based on competitive binding of endogenous vitamin B12 and added radioactive B12 to limited binding sites on intrinsic factor. Therefore, the amount of bound radioactive B12 is inversely correlated to the amount of endogenous B12 (Lau, et al. 1965, Chin 1985). While it has been mentioned that modified versions of the radioisotope dilution procedure using commercially available radio immunoassay

kits are not validated for human milk, it has also been stated that this approach for vitamin B12 quantitation in human milk is acceptable (Areekul, et al. 1977, Sneed, et al. 1981). However, several different commercially available radio assay kits have been used for analysis, and none of them has been developed for the human milk matrix. Modifications to the method include a hot extraction of the sample with ethanol or digestions using papain prior to the radioassay (Specker, et al. 1990, Adjalla, et al. 1994, Keizer, et al. 1995). Analysis of human milk from a mother of a diagnosed B12-deficient infant using a competitive protein binding assay resulted in very low B12 concentrations compared to values obtained with the same method from well-nourished lactating women (McPhee, et al. 1988). Since no validation was carried out prior to application, the accuracy of the values remains uncertain.

Lildballe *et al.* (2009) proposed the removal of apo-haptocorrin (apoHC) by a cobinamide-sepharose affinity column prior to B12 analysis due to the fact that the large amounts of the free form of haptocorrin in human milk may interfere with the analysis. Analyzing untreated samples with high amounts of apoHC using different commercially available immunoassay systems coupled with chemiluminescence detection resulted in spuriously high (Centaur analyzer, Siemens) or low (Architect i2000 analyzer, Abbott; Cobas 6000 E immunoassay system, Roche Diagnostics) concentrations of B12, while concentrations of apoHC below 10nM appeared not to influence the analysis (Lildballe, et al. 2009). Competitive protein binding coupled with chemiluminescence detection with and without apoHC removal prior to analysis appears to be the method of choice for B12 analysis in human milk in recent years (Israel-Ballard, et al. 2008, Honzik, et al. 2010, Deegan, et al. 2012).

Folate

Folate refers to the large group of heterocyclic compounds based on the pteroic acid structure conjugated with one (folic acid) or more L-glutamates (polyglutamyl folate) linked to the γ-carboxyl of the amino acid, all exhibiting the biological activity of folic acid (pteroylglutamic acid). Folate deficiency was first recognized in 1931 and this anti-anemia factor was designated ñvitamin Mò (Day, et al. 1938). In 1946, folic acid was identified and synthesized using techniques previously used to study other pteridines, such as butterfly pigments. Even though folic acid is not found in nature, this slightly water-soluble form is used for food fortification and pharmaceuticals and must be reduced to dihydro- or the active co-enzyme form tetrahydro-folate (THF). As a co-enzyme, folate is involved in the transfer of single-carbon atom groups such as formyl- (CHO), methyl- (CH3) or formimino-functions (CH=NH) in the metabolism of amino acids and also in purine and pyrimidine synthesis in nucleic acid formation (Gibson 1990, Eitenmiller and Landen 1999, Brody and Shane 2001).

Folates are labile to changes in pH, oxidizing agents, heat and light exposure. They show characteristic UV absorption spectra generally with three absorbance maxima. The p-aminobenzoyl-glutamic acid (PABG) moiety influences the UV (UV_{max} 270-280nm) as well as the fluorescence properties of folates while reduced pterins without PABG do not possess native fluorescence. While the reduced forms show excitation and emission maxima at λ_{Ex} 300-320 nm and λ_{Em} 360-425 nm, folate itself fluoresces at maxima of λ_{Ex} 360-380 nm and λ_{Em} 450-460 nm respectively. Fluorescence intensity is also significantly influenced by pH and buffer composition (Uyeda and Rabinowitz 1963, Ball 1994, Bates and Prentice 1994, Eitenmiller and Landen 1999, Fernández-Muiño, et al. 2008).

Signs of marginal deficiency include tiredness, irritability, and decreased appetite. Megaloblastic anemia indicates severe folate deficiency, which has been reported in pregnant and lactating women in developing countries where dietary intakes may not meet their high requirements. Moreover, low folate levels during the periconceptional period combined with genetic susceptibility can cause neural tube defects (NTD) in infants and may be deleterious to fetal growth and normal development of the fetal nervous system. The main forms of folate in foods are the polyglutamates found in liver, yeast, leafy vegetables or nuts (Donangelo, et al. 1989, Gibson 1990, Shaw, et al. 1995, Eitenmiller and Landen 1999).

In human milk, folates are quantitatively bound to whey binding proteins and predominantly present as pteroylpolyglutamates and as N-5 methyltetrahydrofolate (N-5-MeTHF) with a minor contribution of reduced folacin derivatives (Smith, et al. 1985, Brown, et al. 1986, Selhub 1989, O'Connor, et al. 1991, Tamura and Picciano 2006a). Recent experiments confirmed the presence of folic acid, *p*-aminobenzoylglutatmate (*p*ABGA) and its acetamide derivative a-*p*ABGA in the picomolar-range in breast milk (Álvarez-Sánchez, et al. 2010). The folate content of human milk increases throughout lactation; however, a correlation between maternal intake and milk folate has not been found (Ramasastri 1965, Cooperman, et al. 1982, Smith, et al. 1983, Lönnerdal 1986, Greer 2001, Tamura and Picciano 2006a).

The commonly accepted method for folate quantitation in the past has been a microbiological assay using *Lactobacillus casei* ATTC 7469, since this bacterium responds to all forms of folate (O'Connor, et al. 1997). However, other microorganisms (*Streptococcus faecalis*, *Pediococcus cerevisiae*) have been suggested for folate measurement due to differential responses to different folate derivatives, and were tested initially on serum samples (Herbert

1961, Tamura, et al. 1972). Ramasastri (1965) suggested autoclaving the samples in the presence of ascorbic acid under neutral pH conditions to prevent oxidation of the analytes. Adjusting the pH to 4.5 resulted in clear filtrates before the pH was then adjusted to pH 6.8 prior to analysis by the microbiological assay. Using the different bacteria for analysis (*L. casei*, *S. faecalis*, *P. cerevisiae*) different growth responses were observed, presenting the first differential analysis of folate forms in human milk. Various studies have been conducted using *L. casei* and/or the other stated microorganisms, with and without folate conjugase treatment (to convert folate polyglutamates into the monoglutamate form) for total and free folate content respectively, or to differentiate between the different forms of folate in breast milk (Metz, et al. 1968, Jathar, et al. 1970, Tamura, et al. 1980, Butte and Calloway 1981, Sneed, et al. 1981, Cooperman, et al. 1982, Ek 1983, Ford, et al. 1983, Smith, et al. 1983, Eitenmiller, et al. 1984, Bank, et al. 1985, Smith, et al. 1985, Udipi, et al. 1985, Brown, et al. 1986, Udipi, et al. 1987, Swiatlo, et al. 1990, Keizer, et al. 1995). Lim et al. (1988) suggested a tri-enzyme treatment of the milk samples with α-amylase and protease in addition to the folate conjugase prior to the microbiological assay resulting in increased values for the vitamin, an approach used in subsequent studies (Lim, et al. 1998, Mackey and Picciano 1999, Villalpando, et al. 2003, Hyun and Tamura 2005, Khambalia, et al. 2006, Han, et al. 2010).

Chromatographic methods have been used rarely for folate analysis. Sakurai et al. (2005) described a HPLC method using fluorometric detection, an approach described by Allfrey and coworkers in 1949 (Allfrey, et al. 1949, Sakurai, et al. 2005). However, this method appears to not be suitable since it was developed for analyzing the synthetic form of the vitamin, usually not present in biological samples. Furthermore, reduced folates were not protected by reducing

agents during the sample preparation and the described permanganate oxidation to cleave the C9-N10 bond is incomplete when a sample contains other folates such as 5-MeTHF and 10-formylTHF, the main folates found in biological samples. Therefore, results obtained most likely underestimate the actual folate content (Tamura and Picciano 2006b). Shi et al. (2011) also referred to a LC-fluorometric detection technique, described for the analysis of folic acid in vitamin-enriched (dairy) milk and infant nutrition products (Zafra-Gómez, et al. 2006). Therefore, the pteroylpolyglutamates and N-5 methyltetrahydrofolate present in human milk might not be reflected in the results. Recently, a new fully automated on-line SPE-HILIC-MS/MS has been introduced for determining folic acid and its catabolites *p*ABGA and the acetamide derivative a-*p*ABGA in human biofluids. Using only the whey fraction, the samples are subjected to solid-phase extraction (SPE) using ion exchange mode. Elution of the analytes was carried out with the mobile phase allowing the sample to be directly loaded onto the hydrophilic interaction liquid chromatography (HILIC)-system for analysis (Álvarez-Sánchez, et al. 2010). A high-throughput method for quantifying total folate by derivatization of the folates to *p*ABA (para-aminobenzoic acid) prior to analysis by LC-MS/MS using 96-well plates has also been reported (Owens et al. (2007). Even though the analysis is described for whole blood, this approach might be adaptable for breast milk analysis in the future.

Alternatively, competitive radio- and chemiluminescence assays have also been described for folate anlaysis (Van Zoeren-Grobben, et al. 1987, Donangelo, et al. 1989, Israel-Ballard, et al. 2008) mostly using commercially available assay kits. However, a comparison between several radioassays and the microbiologiocal technique revealed a potential overestimate of the free folacin content by radioassay when polyglutatmate forms of 5-MeTHF are present in the

sample (Shane, et al. 1980). Additionally, kits available for folate analysis are commonly validated for serum, plasma or red blood cells and not tested for human milk.

Pantothenic Acid

Pantothenic acid (d(+)-N-(2,4-dihydroxy-3,3-dimethyl-1-oxobutyl)-β-alanine) consists of pantoic acid coupled to β-alanine by an amide linkage. The optical activity derives from the hydroxylated carbon; however, only the d(+)-enantomers are biologically active and present in nature. This compound was first isolated and identified as a growth factor from yeast in 1933 with wide distribution in biological tissues (Williams, et al. 1933). Later, this growth factor was related to the chick antidermatitis factor, establishing the status of pantothenic acid as a vitamin for animals (Jukes 1939, Woolley, et al. 1939). Unlike many vitamins, pantothenic acid is not very susceptible to oxidative conditions or light exposure. Since this vitamin does not contain a chromophore, only weak absorption occurs below 210 nm due to the carbonyl function. Thermal stability is best at pH 5-7. Pantothenic acid is highly hygroscopic; therefore, the calcium salt or the alcohol pantothenol are commonly used for food fortification and pharmaceutical products (Eitenmiller and Landen 1999, Plesofsky 2001).

Being a component of coenzyme A (CoA) and acyl carrier protein (ACP), pantothenic acid is vital to many enzymatic processes and the fatty acid synthase complex and is involved in the regulation of alcohols, amines, and amino acids, and the oxidation of pyruvate by the pyruvate hydroxygenase complex to form acetyl-CoA. Pantothenic acid is essential to all forms of life and is readily available in good quantities in foods such as liver, kidney, yeast, egg yolk and broccoli. Deficiency of this vitamin may result in headaches, fatigue, insomnia, intestinal

disturbance and paresthesia (abnormal sensation) in hands and feet (Wyse, et al. 1985, Eitenmiller and Landen 1999, Plesofsky 2001).

In breast milk, pantothenic acid is mostly available in its free form (85-90%) while only a minor proportion is some bound form. Despite the importance of this vitamin in lipid metabolism, pantothenic acid is not entrapped by the large milk fat globules nor does it occur in significant amounts in the lipid fraction (Song, et al. 1984). Its concentrations in milk increase throughout lactation and appear to correlate with maternal intake (Lönnerdal 1986, Emmett and Rogers 1997). The majority of analyses carried out in human milk have been done by microbiological assays. Coryell et al. (1945) described a procedure using *Lactobacillus casei* no. 1769; the diluted samples were autoclaved, adjusted to pH 4.6 and boiled to precipitate the casein. After filtration, the basal medium was added and the sample autoclaved again prior to inoculating. The growth was measured by turbidimetry. Enzymatic treatment using mylase P or clarase did not seem to affect the assay. However *L. arabinosus* was also tested for suitability and showed a better growth response and reproducibility to pantothenic acid. Besides the mentioned microorganisms, *Lactobacillus plantarum* has also been employed for pantothenic acid analysis in human milk. Additional double enzyme treatment prior to sample preparation has been used to distinguish between free and total pantothenic acid. Enzymes applied include takadiastase/papain, intestinal phosphatase/pigeon liver extract or amylase/papain (Pearson, et al. 1946, Pratt and Hamil 1951, Ford, et al. 1983, Friend, et al. 1983, Goldsmith, et al. 1983, Sakurai, et al. 2005).

Alternatively, pantothenic acid has been quantified by radioimmunoassay (RIA) as described by Song et al. (1984). Samples were incubated at 37°C with bovine intestinal alkaline

phosphatase and pantetheinase. Additional testing of deproteinization methods revealed incomplete removal of the proteins by thermal treatment such as boiling or autoclaving compared to chemical treatment or dialysis prior to analysis by RIA as described for blood and other tissues (Wyse, et al. 1979). D-pantothenic acid is conjugated with bovine serum albumin via a bromoacetyl derivative of the vitamin for which the antibody was raised in rabbits. The sample and radiolabeled sodium D-pantothenate were then added to the antiserum, and incubated for 5min at room temperature before precipitation of the antibody-bound pantothenic acid by ammonium sulfate. The radioactivity of the precipitate was used for quantification (Wyse, et al. 1979).

Even though pantothenic acid does not possess a strong UV-absorption, HPLC coupled to UV-detection at 195 nm has been described for pantothenic acid analysis in supplemented foods such as vitamin-enriched milk and infant nutrition products and has been adapted for human milk. Samples were subjected to protein precipitation using aqueous acetic acid solution containing zinc and wolframium salts and filtered prior to analysis (Zafra-Gómez, et al. 2006, Shi, et al. 2011). Given the lack of chromophores in the molecule, this detection technique still allowed quantitation of 0.5 mg/kg (ppm).

Biotin

Biotin (*cis*-hexahydro-2-oxo-1H-thieno [3,4-d] imidazole-4-pentanoic acid) was found to be a requirement for mammals by Boas in 1927. So-called ñegg white injuryò was observed in rats fed raw egg white, while a curative effect was achieved by feeding them raw liver. In 1936, biotin was isolated and crystallized from egg yolk by Koegel and Tonnis, and Snell showed the

glycoprotein, avidin, functions as the biotin antagonist responsible for the toxicity of raw egg white in 1941. The structure was elucidated in 1942 with synthesis in 1943 (Boas 1927, Eitenmiller and Landen 1999, Mock 2001, Ball 2006). The bicyclic structure consists of an ureido ring fused to a tetrahydrothiophene ring with a valeric acid side chain. Of the eight known stereoisomeres only d(+)-biotin exhibits biological activity and is found in nature. In solution, biotin is quite stable in a pH range of 4 ï 9. This vitamin is insoluble in organic solvent and only sparingly water-soluble (20mg/100mL). The carbonyl group reveals only weak UV-absorption at ∂_{max} 204 nm. Both UV-absorbance and fluorescence can be used for detection but the vitamin needs to be derivatized in order to achieve reasonable sensitivity (Eitenmiller and Landen 1999).

Biotin functions as a covalently bound prosthetic group in four carboxylase enzymes that play key roles in gluconeogenesis, fatty acid biosynthesis, amino acid metabolism, and odd-chain fatty acid catabolism (Ball 2006). Symptoms of inadequate biotin levels include anorexia, nausea, vomiting, glossitis, pallor, mental depression, hair loss (alopecia), dry scaly dermatitis and an increase in serum cholesterol and bile pigments. Lack of biotinidase due to an inborn metabolic defect causes infant deficiency. This enzyme cleaves biotinyl-lysine bonds in biocytin resulting in inefficient release of protein bound biotin in the gastrointestinal tract if not available, less salvage of biotin at the cellular level and through increased renal loss. Organ meats, egg yolks and milk are good food sources of biotin (Eitenmiller and Landen 1999, Fernández-Muiño, et al. 2008).

In human milk, more than 95% of biotin is present in the skimmed milk fraction. Less than 3% is reversibly bound to macromolecules and less than 5% is covalently bound to macromolecules (Mock, et al. 1997). Forms found in early and transitional human milk include

biotin and its metabolites bisnorbiotin (~ 50%) and biotin sulfoxide (~ 10%); however, with maturation post partum the biotin concentration increases while bisnorbiotin and biotin sulfoxide only account for about 25% and 8% respectively. Biotin concentration remains fairly constant within one feeding but can vary substantially over 24h (Mock, et al. 1992a, Mock, et al. 1992b, Eitenmiller and Landen 1999, Mock 2001).

Microbiological assays for the determination of biotin have been described since the 1940s (Coryell, et al. 1945, Goldsmith, et al. 1982, Ford, et al. 1983, Friend, et al. 1983, Goldsmith, et al. 1983, Sakurai, et al. 2005). *Lactobacillus arabinosus* and *lactobacillus plantarum* are the common microorganisms used for the assay. Testing different conditions for hydrolysis such as hydrochloric acid or enzymatic treatment (papain, clarase, mylase P) did not produce a significant increase in biotin concentration compared to non-treated samples; however, enzymes used in the study contained large amounts of endogenous biotin compared to the milk sample causing inconclusive results (Coryell, et al. 1945). Moreover, growth-stimulating compounds such as oleic and aspartic acid can interfere with the determination resulting in an overestimate of the results (Hudson, et al. 1984).

Even though biotin itself does not contain an adequate chromophore for UV-detection, Hudson and coworkers developed a HPLC method using UV detection at 230 nm analyzing biotin in multivitamin products in the working range of 75 ï 300 ε g/mL. However, due to the limited number of mobile phase solvents with an appropriate UV cutoff and dissolved oxygen interference the analysis remains challenging. The latter can be resolved by degassing the solvents e.g. via sonication under vacuum. Besides limitations due to low UV-absorption, co-

elution with riboflavin and pyridoxine complicates quantification of biotin and this technique appears not to be applicable for the more complex human milk matrix.

Alternatively, Mock and DuBois developed a sequential solid phase assay using ^{125}I-labeled avidin for biotin analysis in physiologic fluids, which has also been applied to human milk (Mock and DuBois 1986, Mock, et al. 1992a, Mock, et al. 1992b, Mock, et al. 1997). Earlier avidin-binding assays using ^{14}C-caboxybiotin lack sensitivity for measuring physiologic concentrations of biotin due to the low specific activity of the radio labeled compound (Mock and DuBois 1986). A known amount of ^{125}I-labeled avidin is incubated with varying amounts of biotin in buffer (standard curve) and with several dilutions of the unknown samples. An aliquot is then transferred to microtiter plates previously coated with biotin linked to albumin, which will occupy the remaining avidin binding sites. The wells are washed after incubation and counted individually. Increasing amounts of biotin in the standards or samples results in fewer counts bound to the plate (Mock and DuBois 1986).

Choline

Choline (*N*-trimethylethanolamine) was discovered by Strecker in 1862 and chemically synthesized in 1866. Its biosynthetic pathway was first described by duVigneaud in 1941 (Canty and Zeisel 1994, Zeisel and Holmes-McNary 2001). The colorless viscid liquid is soluble in water and in solvents such as methanol, ethanol or acetonitrile. Diluted aqueous solutions of choline are fairly stable compared to concentrated solutions, particularly when heated to 100°C. Being strongly alkaline, choline forms salts with organic and inorganic acids, which are stable in

aqueous solutions. UV and fluorometric properties have been used for detection after derivatization of choline or its metabolites (Venugopal 1985).

As a dietary component, choline is important for the structural integrity of cell membranes, methyl metabolism, cholinergic neurotransmission, transmembrane signaling and lipid and cholesterol transport and metabolism and is needed in relatively large quantities compared to the other vitamins. Choline is a precursor of the neurotransmitter acetylcholine, and betaine. Lack of choline causes cell death by apoptosis and can lead to fatty liver or liver, muscle, and DNA damage as well as in alterations in lymphocyte gene expression (Venugopal 1985, Koc, et al. 2002, Holm, et al. 2003, Zeisel and Caudill 2010). Since this vitamin is involved in the conversion of homocysteine to methionine, requirements for dietary choline must be considered in relation to methionine and folate metabolism (Zeisel 2006). Moreover, choline plays a critical role in fetal development, particularly in the brain as its availability appears to have an effect on neural tube closure and cognition. Therefore, low choline intake during pregnancy is associated with an increased risk of birth defects in the fetus. During pregnancy this nutrient is provided to the fetus across the placenta, and is secreted into human milk from the maternal circulation (Ilcol, et al. 2005, Caudill 2010, Fischer, et al. 2010).

Choline is commonly available in foods as free choline, choline esters or betaine. Good food sources include egg yolks, organ meats, spinach, nuts or wheat germ (Canty and Zeisel 1994, Zeisel and Caudill 2010). In human milk, forms of choline include free choline and its metabolites phosphocholine (PC) and glycerophosphocholine (GPC) as main contributors as well as lipophilic phosphatidylcholine (lecithin) and sphingomyelin (SM). Its concentration doubles 6

ï 7 days after birth, mostly due to increasing amounts of phosphocholine and glycerophosphocholine (Holmes-McNary, et al. 1996, Holmes, et al. 2000).

One of the first approaches for analyzing choline in human milk was described by Zeisel and coworkers adapting a radioenzymatic assay introduced for choline and acetylcholine in brain. Samples were precipitated with chloroform/methanol, and the pellet washed and resuspended in chloroform/water prior to analysis. 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 (Reid, et al. 1971, Haubrich and Reid 1974, Haubrich, et al. 1975, Zeisel, et al. 1982, Zeisel, et al. 1986).

In the 1990s ^{1}H-NMR was introduced for choline analysis by Holmes and coworkers. Water-soluble choline, PC and GPC were extracted with perchloric acid and neutralized with potassium hydroxide. To distinguish the metabolites from other components possessing similar chemical shift values such as carnitine, samples were run at pH 2 and 7. Field locking, quantitation and shift referencing was achieved by adding deuterated trimethylsilylpropionate. Fat-soluble metabolites were extracted using chloroform, dried and redissolved in deuterated chloroform/water containing TMS and 1,3,5-trichlorobenzene as chemical shift reference and quantitation standard respectively (Holmes, et al. 1996, Holmes, et al. 1998, Holmes, et al. 2000).

HPLC as well as GC-MS has been employed for choline analysis in human milk since the mid 1990s; more emphasis on these analytical applications has been given within the last decade (Holmes-McNary, et al. 1996, Koc, et al. 2002, Sakurai, et al. 2005, Fischer, et al. 2010). Holmes-McNary and coworkers analyzed chlorine and its metabolite present in human milk

using a GC-MS method described for tissues such as plasma, serum or liver (Pomfret, et al. 1989, Zeisel and DaCosta 1990). Samples were partitioned into an organic and aqueous phase; while the aqueous metabolites were purified via HPLC, the fat-soluble metabolites were isolated using thin-layer chromatograohy (TLC) prior to hydrolysis and derivatization into volatile propionyl ester. After removing the N-methylgroup, the sample is ready for GC-MS analysis. SM analysis was adapted from a spectrophotometric method described for plasma. Samples were digested in concentrated perchloric acid at 200°C and phosphorus was quantified after reaction with molybdate, spectrometrically at 825nm (Svanborg, et al. 1961). However, this laborious chromatographic approach requires different types of instrumentation for purifying each metabolite separately and their analysis and SM has to be analyzed separately, amounting to a very complex analysis. An alternative approach used HPLC analysis after hydrolysis of the sample and electrochemical detection after enzymatic reaction with choline oxidase on an immobilized enzyme column (Sakurai, et al. 2005).

Koc and co-workers introduced liquid chromatography/electrospray ionization-isotope dilution mass spectrometry (LC/ESI-IDMS) analyzing betaine, choline, acetylcholine, cytidine diphosphocholine, PC, GPC, lecithin and SM simultaneously in liver, plasma and various foods, a method also used in recent years for human milk. Samples are partitioned into organic and aqueous phases using methanol and chloroform and analyzed directly without the need for isolation and derivatization (Koc, et al. 2002, Fischer, et al. 2010). Other HPLC methods have been described for choline analysis, using different detection methods such as fluorometric detection or continuous-flow fast atom bombardment mass spectrometry (Ricny, et al. 1992, Ishimaru, et al. 1993). However, none of these methods have been used for human milk analysis.

CONCLUSIONS:

Multiple methods have been applied for analyzing B-vitamins in human milk samples. Commonly used methods include microbiological assays and chromatographic techniques. However, the form of the vitamin present in human milk dictates the choice of methodological approach for analysis (Table 1). Chromatographic methods are the most recent approaches for multiple B-vitamins such as thiamin, riboflavin, niacin, vitamin B6 and choline. These vitamins are usually not bound to proteins and can be analyzed individually or simultaneously, and even as different forms (i.e. riboflavin and its coenzymatic form FAD). Microbiological assays appear to be the analysis of choice for folate and panthothenic acid, although for different reasons. Pantothenic acid is mostly present in its unbound form, but the lack of chromophores in the molecule complicates the detection after chromatographic separation, whereas folate is bound to multiple enzymes and chromatographic approaches for quantitation have been shown to be inaccurate. Competitive protein binding assays using radiolabel or chemiluminescence for detection are the common approaches for analyzing biotin and vitamin B12. From this review it is evident that many methods have been used for the analysis of B vitamins in human milk. A substantial number do not give accurate information, and this review should be helpful in enabling users to better interpret the validity of published values in the literature.

ACKNOWLEDGMENTS:

USDA is an equal opportunity employer and provider.

FUNDING

The authors would like to acknowledge funds by the Melinda and Bill Gates Foundation (OPP53107) provided by the University of North Carolina (Linda S. Adair and Margaret E. Bentley) and intramural USDA-ARS Project #5306-51000-003-00D.

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Table 1: Summary of the most useful, valid methods that can be applied today, in the opinion of these authors

Vitamin	Methodology	References
Thiamin	UPLC-MS/MS,	Hampel et al. 2012,
	HPLC-FLD	Stuetz et al. 2011
Riboflavin	UPLC-MS/MS,	Hampel et al. 2012,
	HPLC-FLD	Roughead and McCormick 1990
Niacin	UPLC-MS/MS	Hampel et al. 2012
Vitamin B6	UPLC-MS/MS,	Hampel et al. 2012,
	RPLC-FLD	Hamaker et al. 1985
Cobalamin	Competitive Protein Binding - Chemiluminescence	Lildballe et al. 2009
Folate	Microbiological Assay	Lim et al. 1988
Pantothenic Acid	Microbiological Assay	Coryell et al. 1945,
	Radioimmuno Assay	Song et al. 1984
Biotin	Competitive Protein Binding - Radiodetection	Mock and DuBois 1986
Choline	LC/ESI-IDMS	Koc et al. 2002
	GC-MS / HPLC-Radiodetection	Promfret et al. 1989

Supplementary Table 2A: Summary of thiamin concentrations in human milk as reported in the literature

Supplementary Table 2B: Summary of riboflavin concentrations human milk as reported in the literature

Supplementary Table 2C: Summary of niacin concentrations human milk as reported in the literature

Supplementary Table 2D: Summary of vitamin B6 concentrations human milk as reported in the literature

Supplementary Table 2E: Summary of cobalamin concentrations human milk as reported in the literature

Supplementary Table 2F: Summary of folate concentrations human milk as reported in the literature

Supplementary Table 2G: Summary of pantothenic acid concentrations human milk as reported in the literature

Supplementary Table 2H: Summary of biotin concentrations in human milk as reported in the literature

Supplementary Table 2I: Summary of choline concentrations in human milk as reported in the literature

