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Accurate measurement of the essential micronutrients methionine, homocysteine, vitamins B₆, B₁₂, B₉ and their metabolites in plasma, brain and maternal milk of mice using LC/MS ion trap analysis



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ABSTRACT

Methionine, homocysteine, vitamins B₆, B₁₂, B₉, and their metabolites are crucial co-factors and substrates for many basic biological pathways including one-carbon metabolism, and they are particularly important for brain function and development and epigenetic mechanisms. These are essential nutrients that cannot be synthesized endogenously and thus need to be taken in via diet. A novel method was developed that enables simultaneous assessment of the exact concentrations of these essential micronutrients in various matrices, including maternal milk, plasma, and brain of neonatal mice. The protocol for analysis of these components in the various matrices consists of a cleanup step (i.e. lipid extraction followed by protein precipitation) combined with a liquid chromatography mass spectrometry (LC/MS) ion trap method with high sensitivity and selectivity (SRM mode). This novel method enables the measurement of these essential nutrients with good recoveries (69–117%), and high intra-day (<10%) and high intra-day precision (defined as <15% for compounds with an isotopologue and <20% for compounds without an isotopologue as internal standard) in plasma, maternal milk, and brain of mice at low and high levels. In addition, lower limits of quantitation (LOQ) were determined for the various matrices in the range for methionine (700–2000 nmol/L), homocysteine (280–460 nmol/L), vitamins B₆ (5–230 nmol/L), B₁₂ (7–11 nmol/L), B₉ (20–30 nmol/L). Degradation of vitamins and oxidation of homocysteine is limited to a minimum, and only small sample volumes (30 µL plasma, 20 mg brain and maternal milk) are needed for simultaneous measurement. This method can help to understand how these nutrients are transferred from mother to offspring via maternal milk, as well as how these nutrients are absorbed by the offspring and eventually taken up in various tissues amongst the brain in preclinical and clinical research settings. Therefore the method can help to explore critical periods in lactating mothers and developing offspring.

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1. Introduction

Micronutrient status is important for many biological processes, and it is essential for healthy development. Methionine, homocysteine, vitamins B₆, B₁₂, B₉ (folic acid), and their metabolites belong to a group of essential micronutrients that are particularly relevant for brain development [1]. Biochemically, these nutrients act as substrates and cofactors for enzymes in the one-carbon metabolism

[2], as illustrated in Fig. 1. Adequate dietary intake of these nutrients is important throughout life as most mammals (including mice and humans) cannot synthesize them endogenously. In periods of high nutritional demands (e.g., pregnancy and lactation), deficiencies of these nutrients during embryonic development have been strongly associated with neural-tube defects [4,5]. Fortification with folic acid reduces the incidence of such developmental neurological anomalies [6] and is currently strongly recommended during gestation [7].

The postnatal developmental period is critical to life. During lactation, the nutritional composition of the maternal milk is the main determinant of micronutrient status in the offspring. Therefore, there has been an increasing interest in the possibility of

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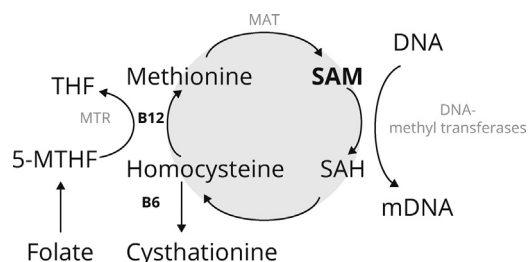


Fig. 1. Diagram of the one-carbon metabolism pathway and its implication in DNA methylation. Different dietary compounds (folate, vitamin B12, vitamin B6) act as cofactors in the enzymatic reactions in cycle.

Abbreviations: 5-MTHF: 5-methyltetra hydrofolate; MAT: methionine adenosyl-transferase; MTR: 5-methyltetrahydrofolate- homocysteine methyl transferase; mDNA: methylated DNA; SAH: s-adenosylhomocysteine; SAM: s-adenosyl methionine; THF: tetra-hydrofolate. Methionine, homocysteine, vitamins B₆, B₁₂, B₉ and their metabolites were investigated in this study in different matrices (maternal milk, plasma, and brain) from the same individual.

supplementing these micronutrients in various pathological conditions. For example, even though breastfeeding is advised under all circumstances, under certain pathological conditions (e.g., pre-term delivery or postpartum depression), the quality of maternal milk might be compromised; however, little is known yet about the exact nutrient levels in the mother's milk under healthy conditions and how these levels might be altered by stress or pathological conditions. In addition, there is gap in knowledge in how these nutrients are being distributed in the plasma of the offspring and how they are eventually transferred to the brain as a major target organ. This knowledge is important for our understanding of how the composition of maternal milk and its supplementation can influence brain structure and function and how it can modify disease susceptibility.

Until now, techniques were unable to simultaneously assess and quantify all of these essential nutrients in the same sample, especially when these samples had a very small volume (as is often the case in preclinical studies when using animal models). Therefore, we have developed a rapid and easy purification method in combination with LC/MS iontrap analysis that enables measurements of these essential nutrients within a single run. The simultaneous measurement of the different components (amino acids, dietary methyl-donors, metabolites, and co-factors) is important as these micronutrients interact and can affect each other's levels [8,9]. However, simultaneous measurement is also very challenging; although all nutrients are involved in the same biochemical pathway, they greatly differ in terms of chemical properties such as molecular weight, polarity, and acid dissociation constants. Thus, the extraction and clean-up step should be kept generic in order to determine this broad range of compounds in a single run. Another difficulty is the presence of some vitamins at trace levels in the samples and their high sensitivity to light, air, and heat, which requires specific conditions during sample preparation. In particular, vitamins B₆, vitamin B₁₂, folic acid, and 5-methyltetrahydrofolate (5-MTHF) are sensitive for degradation processes [10–12]. Thus conditions such as addition of antioxidants [13–15] should be chosen carefully to prevent degradation of vitamins. Although several antioxidants are available, an additional reagent was needed to prevent oxidation of homocysteine to homocystine. Dithioerythritol (DTE), for instance, has the properties to enhance stability for the specific B vitamins and to reduce homocysteine oxidation.

For the separation of water-soluble vitamins and amino acids several liquid chromatography applications that employ reversed-phase LC [11,12,16,17] and ion pair reagents have been reported [18–22]. Although most of the water-soluble vitamins can be retained on a reversed phase analytical column [11,16], the amino acids have hardly any retention on these columns. As a

consequence, reversed-phase separations without using an ion pair reagent is not suitable since amino acids cannot be separated. Therefore, in this study a reversed-phase LC method using ion-pair reagents was applied to be able to separate the wide variety of micronutrients into a single run.

In order to gain insight into how these nutrients are transferred from mother to offspring via maternal milk and to understand how these nutrients are being absorbed by the offspring and eventually taken up in the brain, the three matrices (milk, plasma, and brain) were collected from each individual. Whereas the method presented here was standardized for mice, the protocol can easily be adapted for other types of samples (including human), and it can be used for a broad range of possible applications in preclinical as well as clinical research settings to determine levels of these essential nutrients in healthy as well as pathological conditions or after nutritional intervention.

2. Materials and methods

2.1. Standards and chemicals

Amino acid standards of L-methionine and D,L-homocysteine and the B vitamins pyridoxal (B6), pyridoxine (B6), pyridoxamine (B6), pyridoxal-5-phosphate (B6), folic acid (B9), 5-methyltetrahydrofolate (5-MTHF), and cyanocobalamin (B12) were purchased from Sigma-Aldrich (Milford, NH, USA).

[¹³C₁₁]folic acid, [D₄]homocysteine, and [U-¹³C₅-¹⁵N]methionine were purchased from Cambridge Isotope Laboratories (Woburn, MA, USA). [D₃]Pyridoxamine and [D₂]pyridoxine were purchased from Sigma-Aldrich (Milford, NH, USA) and used as internal standards.

Methanol and formic acid (all HPLC grade or higher) were purchased from Biosolve (Valkenswaard, The Netherlands) and ultrapure water was obtained from a Milli-Q purifier (Millipore, Eschborn, Germany). Heptafluorobutyric acid (HFBA) and DTE were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands); trichloroacetic acid (TCA) and hydrochloric acid (HCl) were purchased from Merck (Darmstadt, Germany).

2.2. Sample collection

All experimental animal procedures were conducted under national law and European Union directives on animal experiments and were approved by the animal welfare committee of the University of Amsterdam. On postnatal day 9, C57Bl/6J mouse pups were rapidly removed from their cage, sexed, weighted, and sacrificed by rapid decapitation. Trunk blood was collected in ice-cold EDTA-coated tubes (Sarstedt, Etten-leur, The Netherlands) and placed on ice and centrifuged at 15,871 × g for 15 min; afterwards, the plasma was stored at –20 °C. Brains were quickly isolated, and hippocampi of both hemispheres were dissected in ice-cold saline. The brain tissue was put in an Eppendorf tube, immediately frozen on dry-ice, and stored on –80 °C until further use. Pup stomach milk (which is quite solid) was collected from the stomach via an incision in the stomach wall. The milk was immediately frozen on dry-ice and stored at –80 °C until further use. When all the pups were sacrificed, the dam was weighted and sacrificed by rapid decapitation. Dam blood plasma was collected in the same way as pup plasma (see above).

2.3. Standard preparation

Standard solutions of 2.5 mmol/L were prepared for methionine, homocysteine, pyridoxine, pyridoxal, and pyridoxamine; 1.0 mmol/L solution was prepared for pyridoxal-5-phosphate; and solutions of 0.5 mmol/L were prepared for vitamin B₁₂, vitamin

B9, and 5-methyltetrahydrofolate and the isotopologues were prepared in equal concentrations as the standards. Methionine was prepared in 0.1 M HCl and stored at -20°C ; the other standards were prepared in a mixture of water and DTE and they were stored at -80°C to prevent degradation of some compounds. Since some of the compounds are unstable, repeated freezing–thawing cycles of the standards should be avoided.

An internal standard solution for the labeled amino acids and B vitamins was prepared freshly in a mixture of purified water and DTE. Standard solutions for the construction of the calibration curve were freshly prepared as well. Calibration curves were constructed at adequate levels for methionine (0.1–40 $\mu\text{mol/L}$), homocysteine (40–3500 nmol/L), pyridoxal (20–3200 nmol/L), pyridoxine (0.7–130 nmol/L), pyridoxamine (1.3–250 nmol/L), pyridoxal-5-phosphate (0.7–500 nmol/L), vitamin B9 (3–550 nmol/L), 5-MTHF (3–650 nmol/L), and vitamin B12 (1–200 nmol/L).

2.4. Sample preparation

2.4.1. Plasma

To 30 μL of plasma, 20 μL of internal standard solution, 40 μL of 100 mM DTE, 80 μL of water, and 200 μL of hexane were added. The sample was mixed and allowed to react for 15 min. After centrifugation (5 min, $10,000 \times g$), the hexane layer was removed and 50 μL of 10% TCA was added for protein precipitation. Then after mixing and centrifugation (5 min, $10,000 \times g$), the supernatant was diluted with 20 μL of 0.1% HFBA solution, and filtered (0.22 μm filter regenerated cellulose, Grace, Deerfield, USA) and finally transferred to a micro autosampler vial.

2.4.2. Brain

To 20 mg of brain tissue, 20 μL of internal standard solution, 40 μL of 100 mM DTE, 80 μL of water, and 200 μL of hexane were added. The brain sample was homogenized and allowed to react for 15 min. After centrifugation (5 min, $10,000 \times g$), the water layer was transferred to a new container and precipitated with 50 μL of 10% TCA. Then after mixing and centrifugation (5 min, $10,000 \times g$), the supernatant was diluted with 20 μL of 0.1% HFBA solution, and filtered (0.22 μm filter regenerated cellulose, Grace, Deerfield, USA) and finally transferred to a micro autosampler vial.

2.4.3. Milk

To 20 mg of milk, 20 μL of internal standard solution, 40 μL of 100 mM DTE, 80 μL water, and 200 μL of hexane were added. The milk sample was homogenized and allowed to react for 15 min. After centrifugation (5 min, $10,000 \times g$), the water layer was transferred to a new container and precipitated with 50 μL of 10% TCA. Then after mixing and centrifugation (5 min, $10,000 \times g$), the supernatant was diluted with 20 μL of 0.1% HFBA solution, and filtered (0.22 μm filter regenerated cellulose, Grace, Deerfield, USA) and finally transferred to a micro autosampler vial.

2.5. Liquid chromatographic conditions

Ultra-high performance liquid chromatography (UHPLC) was used to separate the samples. The UHPLC Thermo Scientific Accela system (Thermo Fisher Scientific, San Jose, CA, USA) was equipped with a degasser, an autosampler with a cooled sample tray, a column oven, and a quaternary pump. Compound elution was performed at a stable temperature of 35°C using a Waters Acquity BEH C18 column (100 \times 2.1 mm i.d., 1.7 μm particle size) (Waters, Milford, MA, USA). The mobile phases consisted of 0.02% HFBA and 0.4 mM DTE (A) and methanol with 0.1% formic acid (B). Ultra-pure, LC/MS-quality water was used to eliminate excessive background signals and to prevent the formation of sodium or potassium adducts. A step-wise gradient starting at 10% B was employed at

a flow-rate of 0.2 mL/min. From 2 to 6 min, the percentage of B was linearly increased to 15%. Between 6 and 12 min, the gradient was linearly increased to 50% B and finally it was increased to 100% B in 1 min with a final hold for 3 min. The total run-to-run time (including equilibration prior to injection of the next sample) was 20 min. The injection volume was 20 μL and each sample was analyzed in duplicate.

2.6. Mass spectrometer instrument settings

The effluent of the UHPLC Thermo Scientific Accela system was directly passed to a Linear Ion Trap Velos Pro mass spectrometer (LTQ Velos ProTM) equipped with a heated electrospray interface (Thermo Fisher Scientific, San Jose, CA, USA) operating in the positive and negative mode. The spray voltage of the ion source was set at 3.0 kV, and the S-Lens RF Level was set at 68%. The sheath gas was 60 arbitrary units (abu), the auxiliary gas was 20 abu, and the sweep gas was 0 abu. The capillary temperature was set at 300°C . For collision induced dissociation (CID), ultra-pure detector helium was used and ion abundance was monitored using the selected reaction monitoring mode with a collision energy of 35 eV. In Table 1 an overview is given of the SRM transitions that were used for each compound.

2.7. Method validation

The method was validated for the linearity, limits of quantitation, intra-day precision, inter-day precision, recovery, and matrix effects. The upper and lower limits of quantitation (LOQ) were determined and defined by the domain of linearity of the concentration curves, which were measured at six different days and 10 concentration levels. While matrix-matched calibration curves are the best methods to account for possible ion suppression effects, blank matrices of plasma, maternal milk, and brain of mice were not available in this study. Since various artificial matrices were also not blank for all the compounds of interest, the calibration curves were prepared in an aqueous solvent of water/TCA/DTE/HFBA that had the same composition as plasma, maternal milk, and brain after sample preparation. To take into account possible matrix interferences from the samples, matrix effects were evaluated by post-column infusion of internal standards and the injection of an internal standard free matrix to reveal possible ion suppression. The precision was determined by analyzing pools of plasma, brain, and milk samples that were spiked at relevant levels for each matrix, see Table 3 for an indication of the concentration range of the control samples. The intra-day and inter-day precisions were evaluated by analyzing the same pools of plasma, brain, and milk samples in triplicate on six different days. Recovery was determined by the analysis of plasma, brain, and milk samples that were spiked with known amounts of analytes before and after sample purification. Since some of the compounds are known to degrade in solution, a stability test of the sample extracts stored at $4-8^{\circ}$ and $<-18^{\circ}\text{C}$ was performed. Finally, the performance characteristics were evaluated for each compound by using EP Evaluator (Data Innovations, LLC, South Burlington, VT, USA).

3. Results and discussion

3.1. Sample preparation

In preliminary pilot experiments, the samples were prepared based on a method using a protein precipitation step with acetonitrile. Although this method was suitable for maternal milk, it caused problems for plasma and brain samples after repeated injections of sample extracts (e.g., severe peak tailing). It also resulted in the formation of an unidentified gel in the organic layer after protein

Table 1
Optimized MS parameters and SRM transitions for each compound and isotope labelled internal standard.

Compound	Parent ion (<i>m/z</i>)	Quantifier ion (<i>m/z</i>)	Qualifier ion (<i>m/z</i>)	Internal standard	Polarity
Homocysteine	140.1	118	90	[D ₄]Homocysteine	Positive
Methionine	150.1	133	104	[U- ¹³ C ₅ - ¹⁵ N]Methionine	Positive
Pyridoxal	168.1	150	n/a	[D ₂]Pyridoxine	Positive
Pyridoxal-5P	248.1	150	n/a	[D ₄]Homocysteine	Positive
Pyridoxine	170.1	152	n/a	[D ₂]Pyridoxine	Positive
Pyridoxamine	168.1	150	n/a	[D ₃]Pyridoxamine	Positive
Folic acid (B9)	440.2	311	422	[¹³ C ₁₁]Folic acid	Negative
5-MTHF	460.2	313	331	[¹³ C ₁₁]Folic acid	Positive
Vitamin B12	678.3	997.6	997.6	[¹³ C ₁₁]Folic acid	Positive

Table 2
Precision of the method determined by measurement of control samples of, respectively, plasma, brain, and maternal milk at low and high concentration levels.

Compound	Concentration range (nmol/L)	Intra-assay precision low level (<i>n</i> = 18) (%) ^a	Intra-assay precision high level (<i>n</i> = 18) (%) ^a	Inter-day precision low level (<i>n</i> = 6) (%) ^a	Inter-day precision high level (<i>n</i> = 6) (%) ^a
Homocysteine	900–17000	5.8/4.5/3.6	6.6/8.2/6.2	11.5/13.2/12.7	11.6/12.6/12.2
Methionine	2000–132,000	4.8/5.0/3.6	5.3/6.1/6.0	10.8/11.3/12.9	11.1/12.4/10.6
Pyridoxal	250–2500	4.1/5.4/4.7	4.0/5.4/6.6	12.7/14.6/11.0	11.2/8.2/8.7
Pyridoxal-5P	100–3400	8.5/5.8/4.9	5.6/9.8/7.5	19.1/11.0/11.5	15.0/12.5/11.2
Pyridoxine	20–500	4.8/N.D./4.2	5.2/N.D./6.4	8.6/6.1/7.9	7.9/11.1/7.1
Pyridoxamine	60–600	7.0/6.3/5.9	7.0/6.0/6.2	8.3/13.9/11.6	7.0/11.0/6.5
Folic acid (B9)	90–875	8.9/6.4/6.5	5.1/8.8/6.5	10.8/12.8/14.5	8.3/14.7/7.8
5-MTHF	130–1100	6.7/6.7/7.9	6.2/10.6/8.3	18.3/19.9/31.2	13.2/17.1/25.6
Vitamin B12	135–1100	8.0/5.3/4.7	7.0/9.8/7.0	12.2/14.1/18.7	10.6/15.8/11.4

^a Precision in order of plasma, brain and maternal milk method.

precipitation of the plasma from mouse pups (but not in the plasma of adult mice). This phenomenon caused peak tailing and deterioration of the column. Based on the results found by Hůšek [23], a TCA protein precipitation step was introduced for plasma. As a result, gel formation did not appear, and no peak tailing and column deterioration were observed. Therefore, the TCA precipitation step was added to the sample preparation method for brain and maternal milk samples. To ensure that all lipids were removed as well, a hexane extraction step was introduced before the protein precipitation step. Since some of the B vitamins are unstable and sensitive to degradation by exposure to light or oxygen, addition of antioxidants were tested to prevent degradation during sample preparation. It was found that DTE, which is also used for reduction of homocystine to homocysteine, had good degradation-preventing properties without interfering with the measurements. Therefore, a high concentration of DTE was added to prevent the degradation of the unstable compounds during the sample preparation, and to increase the stability of the sample extract. This did not result in any severe ion suppression effects observed in the mass spectrometer.

3.2. Liquid chromatography optimization

Since the compounds of interest in this study possess a broad range of physical properties, the LC method had to be suitable for the separation of a wide variety of compounds in terms of

molecular weight, polarity, and pKa. Van der Ham et al. [22] used an UHPLC method that had the capability of separating polar compounds such as methionine, homocysteine, and B6 vitamins as well as the more lipophilic compounds vitamin B9 and B12 in a single run. Since UHPLC separations obtains narrower and higher peaks resulting in better signal-to-noise ratio's, this technique was used as well in our study instead of high pressure LC separations. When using ion trap mass spectrometry, it is essential that compounds are baseline separated from interfering signals to prevent loss of sensitivity. Simultaneous loading of the ion trap with analytes and matrix suppresses the compound signal due to the limited capacity of the ion trap. Therefore, UHPLC separations also benefit in a higher peak capacity per time unit, which is particularly desirable for compound analysis in complex samples such as plasma and brain. Based on the method of van der Ham et al. [22], the amount of HFBA added to the aqueous mobile phase was increased to 0.02% to improve separation between the amino acids and some of the B6 vitamins. Addition of higher amounts of HFBA resulted in peak broadening of vitamin B12. Addition of formic acid or acetic acid increased ion-suppression effects and was only added to the organic mobile phase for that reason. In addition, methanol was used as organic solvent instead of acetonitrile due to the increased sensitivity of most of the compounds in mass spectrometric analysis. Although van der Ham et al. [22] obtained good compound separation using a Waters Acquity HSS T3 analytical column, in

Table 3
Results of the upper and lower LOQ (*n* = 6 days) for the various matrices, the linearity of the calibration curves (*n* = 6), and the recovery of the sample preparation methods.

Compound	Lower LOQ (nmol/L) ^a	Upper LOQ (nmol/L) ^a	Linearity (<i>R</i> ²)	Recovery (%) ^b
Homocysteine	280/460	189 ^E 3/310 ^E 3	0.9995	133/131/124
Methionine	700/2000	525 ^E 3/860 ^E 3	0.9998	107/113/100
Pyridoxal	140/230	125 ^E 3/20 ^E 3	0.9982	71/93/100
Pyridoxal-5P	5/8	2200/3500	0.9989	33/92/95
Pyridoxine	5/8	2200/3500	0.9996	116/98/97
Pyridoxamine	10/16	6900/10 ^E 3	0.9995	117/79/98
Folic acid (B9)	20/30	15 ^E 3/24 ^E 3	0.9997	84/92/101
5-MTHF	20/30	600/940	0.9907	83/97/82
Vitamin B12	7/11	5000/8000	0.9995	96/69/87

^a Lower and upper LOQ in plasma and secondly both brain and maternal milk.

^b Recovery in order of plasma, brain and maternal milk method.

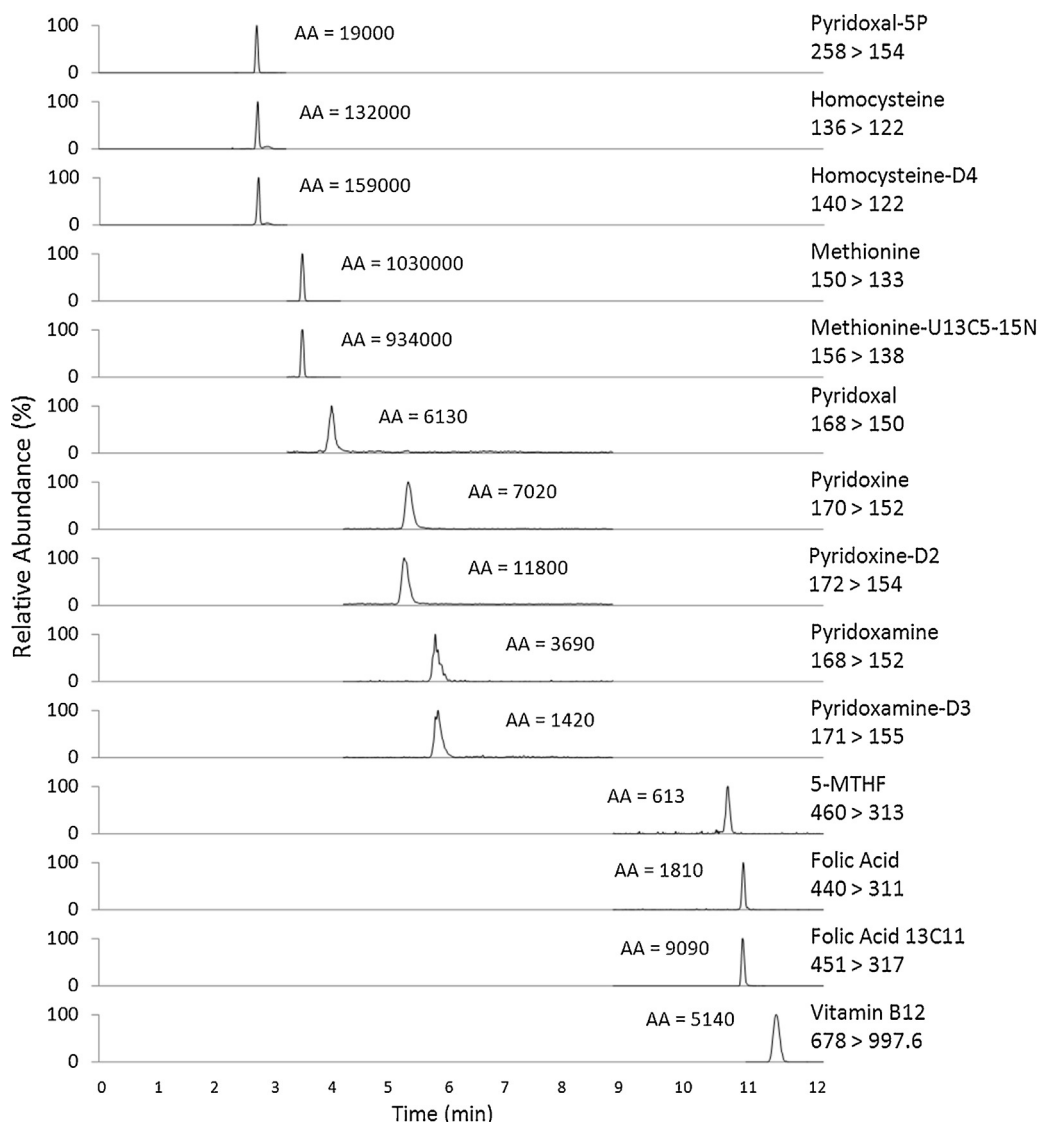


Fig. 2. Representative ion chromatogram of the specific B-vitamins and amino acids obtained from a plasma control sample at low level that was prepared according to the developed purification method. The absolute abundance (AA) and SRM transitions are presented in each ion chromatogram.

this study the use of this column resulted in peak tailing for some compounds (e.g., pyridoxal-5P and 5-MTHF). Instead, the Waters Acquity BEH C18 analytical column gave good results for the separation of these wide variety of compounds, as demonstrated in Fig. 2. Non-specific binding to PEEK tubing and stainless steel parts was observed for 5-MTHF. Therefore, all tubing was replaced by inert glass lined peek (Peeksil); this prevented most non-specific binding of 5-MTHF. Addition of DTE to the mobile phase decreased this binding of 5-MTHF to a minimum.

3.3. Mass spectrometry

Optimal MS ion source conditions for each individual compound were obtained by introduction of the compounds mixed with mobile phase using a PEEK T-piece to mimic the chromatographic conditions. Most of the compounds had the highest sensitivity in the positive ion mode, except folic acid which had the highest sensitivity in the negative mode. To achieve maximum sensitivity and accuracy, the ion trap MS was operating in the selected reaction monitoring (SRM) mode for compound detection, which is a scanning mode with high selectivity. All compounds could be sensitively measured using these selected transitions. For the measurement of

Table 4
Stability of the sample extracts at different temperatures over time.

Compound	Storage at 4–8 °C (days)	Storage at ≤18 °C (days)
Homocysteine	14	14
Methionine	14	14
Pyridoxal	5	5
Pyridoxal-5P	2	2
Pyridoxine	5	5
Pyridoxamine	2	2
Folic acid (B9)	14	14
5-MTHF	2	2
Vitamin B12	2	2

vitamin B12 using a triple quad instrument, the most abundant daughter fragment ion was found to be m/z 147 in SRM mode [16]; however, when vitamin B12 was measured with iontrap analysis, this fragment could not be used due to the so called “1/3 rule”, that is, that the smallest viewable product mass is about 1/3 of the original precursor mass. As a consequence, a fragment with a lower abundance had to be selected; this resulted in a lower sensitivity of vitamin B12 compared to the other measured compounds.

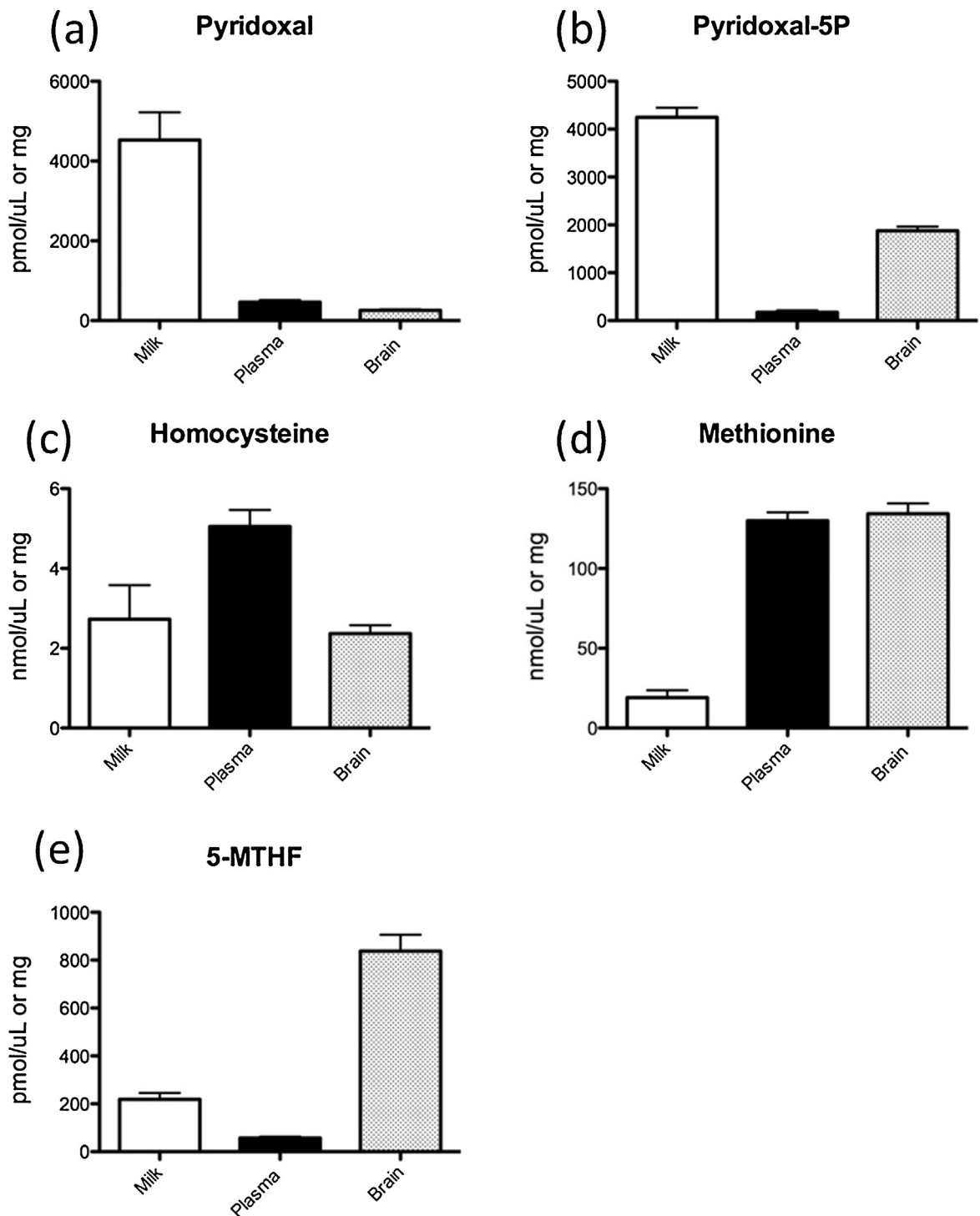


Fig. 3. Mean concentrations (\pm SEM, $n = 8-9$ individuals) of pyridoxal (a), pyridoxal-5P (b), homocysteine (c), methionine (d), and 5-MTHF (e) in stomach milk (concentration per mg), plasma (concentration per μ L) and brain (concentration per mg).

3.4. Method validation

The method was validated for the upper and lower limits of quantitation (LOQ), which were determined by the domain of linearity using the upper and lower part of the calibration curve in aqueous solvent. The linear range for each compound was determined by EP Evaluator (Data Innovations, LLC, South Burlington, VT, USA) for methionine (0.1–75 μ mol/L), homocysteine (0.04–27 μ mol/L), pyridoxal (20–1750 nmol/L), pyridoxine

(0.7–280 nmol/L), pyridoxamine (1.3–990 nmol/L), pyridoxal-5-phosphate (0.7–280 nmol/L), vitamin B9 (2.8–2100 nmol/L), 5-MTHF (3–85 nmol/L), and vitamin B12 (1–720 nmol/L). In addition, the linearity expressed in R^2 was >0.99 for all the calibration curves. The upper and lower LOQ's for the various compounds in plasma, brain, and maternal milk were derived from the linear range and are presented in Table 3. To take into account possible matrix interferences from the samples, matrix effects were evaluated by post-column infusion of internal standards and the

injection of an internal standard free matrix to reveal ion suppression. Post-column infusion revealed no matrix effects for most of the compounds; only pyridoxal-5P and homocysteine had matrix interferences. However, this can be corrected by using an internal standard of homocysteine, which has nearly the same retention as pyridoxal-5P. Since no labeled internal standards were available in this study for 5-MTHF and vitamin B12, the labeled internal standard of vitamin B9 was used to correct for variations in the sample preparation. Similarly, the labeled internal standard for pyridoxine was used to correct for pyridoxal, and homocysteine-D₄ was used to correct for pyridoxal-5P. Furthermore, the sample preparation methods had good recoveries for almost all compounds (69–117%), as presented in Table 3. Although the recovery's of pyridoxal-5P in plasma (33%) and homocysteine in all the matrices (130%) were deviant, the values were reproducible. The precision of the method was evaluated by analyzing pools of plasma, maternal milk, and brain samples spiked at relevant levels. The intra-assay precision was better than 10% for each of the compounds, and the inter-day precision was better than 15% for all the compounds with labeled internal standards. Although a few compounds without a labeled counterpart as an internal standard had a lower inter-day precision in some cases (<20%), it was still acceptable for this study compared to the biological variation of the samples. The inter-day precision of 5-MTHF was low in maternal milk (around 30%); this can probably be improved by using a labeled counterpart of 5-MTHF as an internal standard. An overview of the method validation results is presented in Tables 2 and 3. Since some of the vitamins are unstable, the stability of the sample extracts were determined as well. As viewed in Table 4, some vitamins are already degraded after 48 h of storage. Therefore the sample extracts should be analyzed directly after preparation, and a limited number of samples should be prepared. In case of inevitable sample storage, the sample extracts should be stored at –80 °C.

3.5. Application of the method

This technique enables us, for the first time, to measure exact levels of essential nutrition components in maternal milk as well as offspring's blood plasma and brain to gain further insight into their transfer from one matrix to the other. Levels of 5-MTHF, pyridoxal-5P, pyridoxal, homocysteine, and methionine levels could be determined in all three matrices, and concentrations were found as depicted in Fig. 3. Other nutrients (e.g., folic acid) were detected in milk ($167,4 \pm 8,1$ pmol/gram milk $n = 16$) but not in plasma or brain, indicating degradation of the component to metabolite. Analyzing the nutrient levels in the different matrices obtained from the same individual enabled the analysis of the relation between levels in milk, plasma, and brain. There were no significant correlations between nutrient levels in stomach milk and nutrient levels in plasma. However, there was a positive Pearson's correlation (two-tailed) between levels in plasma and levels in brain tissue for the nutrients homocysteine ($r(29) = 0.49$ $P = 0.005$), methionine ($r(29) = 0.67$ $P < 0.001$), and 5-MTHF ($r(13) = 0.594$ $P = 0.02$), (e.g., high nutrient levels in plasma correspond with high levels of these nutrients in brain). The simultaneous assessment of all the essential micronutrients involved in the one-carbon metabolism in the various matrices can be used in future research to study the transfer and uptake of these nutrients via maternal milk to offspring's plasma and eventually uptake in the offspring's brain. This knowledge is important for our understanding of how maternal milk composition exerts its effects on the brain and disease susceptibility and can be used to study the effects of maternal nutritional supplementation on nutritional status of the offspring.

4. Conclusions

A novel procedure for the measurement of the essential micronutrients methionine, homocysteine, vitamins B₆, B₁₂, B₉ (folic acid), and their metabolites in plasma, brain, and maternal milk samples in combination with LC/MS ion trap analysis has been demonstrated. This method is capable of measuring a wide variety of compounds in terms of molecular weight, polarity, and acid dissociation constants in a single LC/MS run. Despite the presence of some compounds at trace levels and the sensitivity to light, air, and heat, we were able to develop a rapid and simple method for measurement of small-sample amounts of various matrices of mice pups. The concentration of the specific micronutrients could be determined with high inter-day precisions (<15%) in the samples at low and high concentration levels. In general, the compounds without an isotopologue as internal standard had a lower inter-day precision (<20%). However, a low intra-day precision was observed in maternal milk for 5-MTHF, which should be improved by using a labeled internal standard of 5-MTHF. Furthermore, the sample preparation methods had good recoveries (69–117%) for most of the compounds, and only ion-suppression effects were observed for homocysteine and pyridoxal-5P by post-column infusion experiments. The sample extracts were stable for 48 h at 4 °C and should be kept at –80 °C (ultra-freezer) for longer storage. In conclusion, this novel method provides a rapid and easy procedure for the preparation of plasma, maternal milk, and brain of mice for the measurement of the concentration of specific micronutrients using an LC/MS system with high sensitivity and selectivity.

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