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First approach of a methodological set-up for selenomethionine chiral speciation in breast and formula milk using high-performance liquid chromatography coupled to atomic fluorescence spectroscopy

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The chiral speciation of selenomethionine in breast and formula milk based on species separation by high-performance liquid chromatography followed by online microwave-assisted digestion and detection with hydride generation atomic fluorescence spectrometry (HPLC-MAD-HG-AFS) requires severe sample manipulation to avoid matrix influence. Sample clean-up for fat and protein elimination using centrifugation and ultrafiltration was optimized, and selenomethionine preconcentration based on cation exchange solid-phase extraction was studied and optimized. The resulting procedure is suitable for chiral selenium speciation in infant milk with detection limits of 3.1 and 3.5 ng ml^{-1} as Se for L-selenomethionine and D-selenomethionine, respectively. The time necessary for the analysis, about 90 min, including sample clean-up, analyte preconcentration and chromatographic separation, makes the approach suitable for routine analysis. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: chiral speciation; selenomethionine; atomic fluorescence; breast milk; formula milk; sample pretreatment

INTRODUCTION

Selenium is an essential nutrient of great importance for human health due to its being bound to antioxidant proteins involved in basic living organism functions, such as thyroid activity, reproduction capacity and defence against tumours and virus infections,[1] but the range between beneficial and toxic effects in human is narrow.[2] Moreover the toxicity of selenium is highly dependent on its chemical form.[3] Selenium enters the food chain through plants that take it up from the soil;[4] however, selenium intake from this source can be problematic due to the low abundance of selenium in food,[5] which does not reach human requirements for this element, ranging from 10 to 70 μg per day.[6,7] A more recent review from Rayman suggested a medium selenium intake of 75 μg per day for men and 60 μg per day for women to maximize the activity of the antioxidant selenoenzyme GPx in plasma.[8] For these reasons, introduction of selenium supplements into the diet can be necessary. Most selenium in the human body (less than 0.005% of the total body mass) is present as amino acids, such as selenomethionine and selenocysteine.[9,10] The first is synthesized by plants and the second is of animal origin.

Selenomethionine is more available and less toxic than inorganic selenium, and it is commonly used as a selenium source for human dietary supplements, usually as selenized yeast. However, selenoamino acids have chiral centers which can become D- and L-enantiomers, the latter being more easily assimilated into the body, although it can also be more toxic than the D-forms.[11] Therefore

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selenium chiral speciation has acquired great importance in evaluating the metabolic consequences of selenium supplements.[12–15]

Selenium intake has a basic importance during infant nutrition. Infancy is characterized by a high rate of synthesis of tissue cells, which requires high trace element inputs supplied not only by specific metal–biomolecule forms transferred via the mother's milk or by formula milk, but also from prenatal stores. This fact has special relevance for premature infants because of their lower stores of essential micronutrients.[16] In early infancy, breast milk or formula milk is the only dietary source of essential trace elements. The mother's milk provides a balanced supply of all micronutrients for the full-term infant. However, a number of elements, including selenium, are added to formula milk in chemical forms and concentrations different from those found in breast milk.[17,18] Total selenium presence in supplemented formula milk is similar to that in breast milk, ranging between 16 and 25 $\mu g\, l^{-1}$,[19] and some studies have been performed on selenium speciation in human milk,[20,21] but little data[22] has been reported about the presence of chiral selenium species in these matrices.

Several analytical approaches have been proposed for chiral speciation of selenium,[23] mainly based on the use of ICP-MS as a detector, coupled to liquid chromatography (HPLC-ICP-MS)[24–28] or gas chromatography (GC-ICP-MS),[29] although atomic fluorescent detection has recently been tested for this purpose (HPLC-MAD-HG-AFS).[22] An important question is the type of chiral stationary phase used to insure reliable enantiomer separations, and several alternatives have been proposed for this purpose.[23] Glycopeptide antibiotic columns (teicoplanin-Chirobiotic T), which contain ionic groups ($-NH_4^+$ and $-COO^-$), polar groups (hydroxyls) and non-polar carbon side chain moieties, enable enantiomeric separations with hydro-organic mobile phases. Generally, very low percentages of organic modifiers without buffers or salts are necessary for these columns, which is very convenient for the couplings involving chromatography and atomic detectors.[24] However, the relatively poor selectivity between amino acids and polypeptides that may be present in complex samples and the elevated cost of these columns, have motivated some alternative methods based on chiral derivatization and further separation by conventional reversed-phase chromatography.[27]

Although the use of the hyphenated system HPLC-MAD-HG-AFS has been previously reported for non-enantiomeric selenoamino acid speciation,[30,31] only one contribution, to our knowledge, has focused on the chiral speciation of DL-selenomethionine.[22] Generally, sample and species pretreatment is critical in the use of these instrumental approaches to transform the selenium species into chemical forms suitable for the AFS detector. The online decomposition of the organic part of these molecules and their later online generation of selenium hydride are common steps in selenium speciation with AFS. Selenium-enriched materials such as yeast, garlic and onion, have frequently been used as test samples to evaluate the reliability of proposed methods for this element.[24–27] In these cases sample pretreatment is relatively simple and it is generally based on enzymatic digestion or water extraction.[24,25,27] Some studies from Caruso *et al*.[26] have mimicked gastric enzymolysis using a mixture of pepsin, NaCl and HCl or alternatively treatment with nitric acid. Formula and breast milk are very complex matrices due to the high lipid content and the presence of proteins that make intensive clean-up necessary prior to the analytical speciation. In addition, the low concentration of selenium requires a species preconcentration step to reach the quantification limit of the AFS detector.

In the present study the optimization of an analytical approach for selenomethionine chiral speciation based on coupling HPLC-MAD-HG-AFS has been performed and special attention has been paid to the application of the method to breast and formula milk. Sample pretreatment based on ultrafiltration clean-up and solid-phase preconcentration to obtain a method suitable for these samples, based on the relatively cheap atomic fluorescence detector has been particularly considered. These features make the approach suitable for chiral selenomethionine speciation in routine analysis of human milk.

EXPERIMENTAL

Reagents and chemicals

Deionized water from a Milli-Q system (Millipore, Bedford, MA, USA) was used throughout. Glass and plastic ware were cleaned by soaking for one day in diluted (2 : 8) nitric acid and were rinsed with distilled water before use.

DL-Selenomethionine, L-selenomethionine and DL-seleno-ethionine were obtained from Sigma Chemical Co. (St Louis, MO, USA). Stock standard solutions containing 10–100 $\mu g\, l^{-1}$ of each compound in milli-Q water were stored in the dark at 4 °C. Working standard solutions were prepared daily by dilution.

Solutions of $NaBH_4$ (Panreac, Barcelona, Spain) were prepared freshly every day with concentrations of 1–4% (w/v), stabilized in 1% (w/v) NaOH (Merck) and filtered through a 0.45 µm cellulose acetate filter before use. Concentrated HBr, HNO_3 and $HClO_4$ (Merck, Darmstadt, Germany) and $KBrO_3$ (Panreac, Barcelona, Spain) and other chemicals were of analytical reagent grade or higher. Protease XIV (*Streptomyces griseus*) and lipase VII (*Candida rugosa*) obtained from Sigma (Gillingham, Dorset, UK) were used for sample treatment. Membrane filters of 0.45 mm were obtained from Millipore (Belford, MA, USA).

Formula milk was purchased at the pharmacy. Samples of human breast milk were generously provided by the Huelva

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Table 1. Principal components in the formula milk used in the study

Composition	Amount in 100 ml (reconstituted milk)
Fats	3.5 g
Proteins: lactalbumin and casein	0.85 g 0.56 g
Ca	50 mg
P	30 mg
Mg	6 mg
Fe	0.78 mg
K	65 mg
Na	20 mg
Cl	39 mg
Cu	50 μg
Mn	6.5 μg
Se	0.78 μg

Hospital, Spain. The composition of the formula milk is shown in Table 1.

Procedures and instrumentation

Elimination of fats and proteins

Breast or formula milk (3.5 g), the latter reconstituted in 25 ml of milli-Q water, were completely defatted following several successive steps: (i) centrifugation of the milk at 14 000 rpm for 30 min for fat elimination; (ii) casein precipitation by pH adjustment at 4.5 and centrifugation at 14 000 rpm for 30 min; and (iii) ultrafiltration of the extract using a stirred cell from Amicon mod 8050 (Millipore, Belford, MA, USA) of 50 ml provided with a PM 10, 44.5 mm diameter polyethersulphone (nominal molecular weight limit, 10 kDa) membrane (Millipore). The ultrafiltration was performed under nitrogen pressure (1.5 bar). The small selenomethionine molecules were separated from the proteins remaining in the extract after steps (i) and (ii).

Analyte preconcentration

The extract from the previous treatment (pH 4.5) was passed through 1 g of SCX (cation exchange solid-phase; Symta, Madrid, Spain) placed on a glass column (10 cm long and 1 cm i.d.). The column was previously conditioned with 10 ml of milli-Q water at 1 ml min^{-1} flow rate. The sample extract was passed at 1 ml min^{-1} through the column and the analytes eluted at the previous flow rate with 3 ml of 3 mol l^{-1} H_2SO_4. This operation was performed twice, and the final extract, neutralized with NH_3, injected in the chromatograph.

Chiral speciation of DL-selenomethionine

A 200 μl volume of sample was injected into the HPLC system, consisting of a quaternary pump (Jasco 1580-PU) equipped with a Rheodyne 7125 injector and a 200 μl loop (Rheodyne, Cotati, CA, USA). The column used was a 10 μm Chirobiotic T (250 × 4.6 mm i.d.; Advanced Separation Technologies Inc., NJ, USA). The separation of enantiomeric selenium forms was achieved using milli-Q water as mobile phase. The flow rate through the column was always 1 ml min^{-1}.

The online microwave-assisted digestion (MAD) of the selenium species eluted from the chromatograph was performed in a 3 m long PTFE loop placed inside a domestic microwave oven operated at 350 W. Two reagents were added online to complete species decomposition: (i) 15 mM $KBrO_3$ (flow rate 0.6 ml min^{-1}); and (ii) 47% HBr (flow rate 0.6 ml min^{-1}). A scheme of the instrumental coupling can be seen in Fig. 1. An ice-bath-cooled loop was placed after the microwave device for fast temperature reduction of the flow.

Hydride generation of selenium species previously separated by the chromatograph was performed by adding 1.2 ml min^{-1} of 1.5% (w/v) $NaBH_4$ in 1% (w/v) NaOH using a Gilson Minipulse-3 peristaltic pump (Gilson, Villiers, Le Bel, France). Volatile selenium hydride was separated from the liquid flow using a PSA Type A gas–liquid separator that introduced two argon flows, one to carry the hydrides to the separator (100 ml min^{-1}), and the other, at 200 ml min^{-1}, to transport them to the AFS system. Before detection, the argon stream was dried with a hygroscopic membrane drying tube (Perma Pure Inc, Toms River, NJ, USA). Air was used as

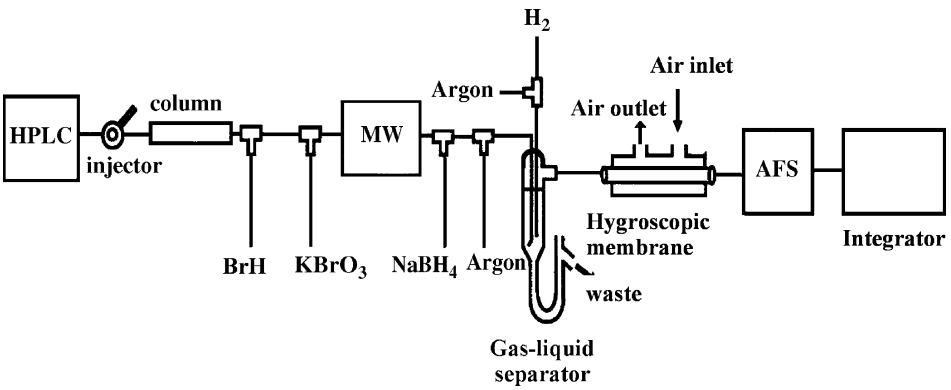

Figure 1. Scheme of HPLC-MAD-HG-AFS coupling. MW, microwave oven; AFS, atomic fluorescence detector.

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the drying gas at a flow rate of 3.0 l min^{-1}. A 60 ml min^{-1} hydrogen flow was added at the gas–liquid separator in order to maintain the argon–hydrogen diffusion flame.

Selenium was detected with an AFS detector (PSA Excalibur 10.33, PS Analytical, Orpington, Kent, UK) equipped with a selenium boosted-discharge hollow cathode lamp (Super lamp; Photron, Victoria, Australia). Measurements were performed at 196 nm. The analog signal output of the AFS detector was connected to a computer equipped with the Start chromatographic software (Varian, Palo Alto, CA, USA).

Total selenium analysis

The lyophilized or powdered milk samples (1 g, carefully weighted) were dissolved with an acid mixture (10 ml of HCl, 10 ml of HNO_3 and 5 ml of $HClO_4$) in PTFE open vessels using a sand bath for heating until the appearance of white fumes. The the treatment was repeated another time. Then the samples were evaporated almost to dryness, transferred to a volumetric flask, diluted with 1 ml of HNO_3 and diluted with water until a final volume of 5 ml. Total selenium determinations were performed using a flow injection device consisting of a microwave-assisted digestion unit followed by selenium hydride generation and detection with atomic fluorescence spectrometry [(FI)–MAD-HG-AFS], similar to Fig. 1 without the chromatographic separation. Samples were introduced with a manual valve.

A certified reference material (BCR 063) was used for analysis quality control. Recoveries obtained for the certified total selenium concentration, 129 ng g^{-1}, were of the order of 102%.

RESULTS AND DISCUSSION

Optimization of post-column selenium species treatment and AFS detection

Several on-line reactions had to be performed with the selenoamino acids eluted from the chromatographic systems to transform them in chemical forms suitable for the AFS detector. The use of a redox agent ($HBr/KBrO_3$ mixture) under the action of an energy source (namely microwave radiation) is usually the elected approach,[33–35] which converts all the selenium species into Se(IV) before the hydride generation step. Therefore, the method is suitable for complex molecules, especially selenoamino acids. The concentrations of the different solutions pumped with the peristaltic pump, $KBrO_3$, HBr and $NaBH_4$, were optimized earlier,[30] and results were confirmed for these chiral species. Different microwave powers were tested; an increase in the microwave power resulted in a significant increase in the signal, but also produced an increase in the baseline noise. A compromise value was obtained for 150 W.

Gas flows

Complete gas–liquid separation of selenium hydride is necessary to insure signal stability. Two argon flows are introduced into the gas–liquid separator. The main flow carries the hydride just from the separator to the detector, the auxiliary one, added after hydride generation, contributes to the fast transport of hydride to the separator, which reduces the baseline noise and improves the peaks resolution. A flow rate of 200 ml min^{-1} provided the optimum signal-to-noise (S/N) ratio, keeping the auxiliary argon flow at 100 ml min^{-1}. When the auxiliary flow had been optimized (keeping the main argon flow at 200 ml min^{-1}), the S/N ratio decreased sharply; therefore this auxiliary flow was kept at 100 ml min^{-1}.

Hydrogen significantly contributes to the background noise. Therefore, flow rates over 70 ml min^{-1} increased the S/N ratio, while flow rates lower than 50 ml min^{-1} resulted in poor net signal and increased the flame instability. Therefore, a flow of 60 ml min^{-1} was selected as optimum.

Figure 2 shows the chromatogram obtained for a racemic mixture of selenomethionine (100 μg l^{-1} as Se), when the optimized chiral HPLC-MAD-HG-AFS system was used under the conditions given in Table 2. Chiral separation was performed with good resolution with retention times of 5.7 and 7.5 min for L-selenomethionine and D-selenomethionine, respectively.

Analytical performance characteristics for chiral speciation of selenomethionine

The relative standard deviations of the peaks areas (RSD%) for five replicate injections of the same sample of a racemic mixture of DL-selenomethionine were found to be ±0.089 and ±2.47% for D-selenomethionine and L-selenomethionine, respectively, at a concentration of 50 μg l^{-1} as Se. The calibrations graphs (performed on milk whey) for the DL-selenomethionine racemates showed good linearity over the range of concentrations studied (10–200 μg l^{-1} as Se). The straight line calibration curve for L-selenomethionine

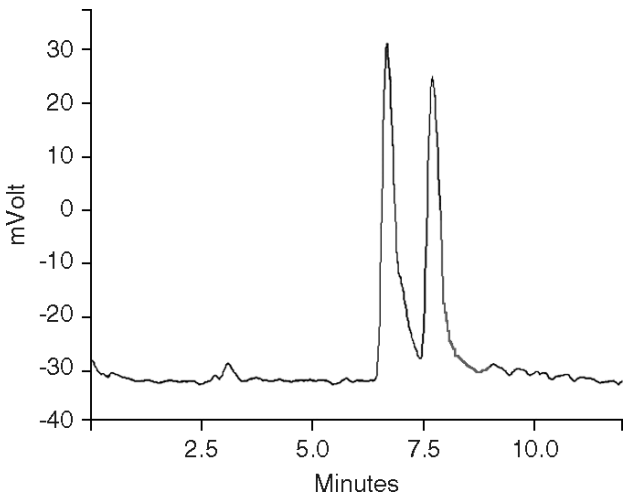

Figure 2. Chromatogram obtained for a racemic mixture of selenomethionine (100 μg l^{-1} as Se), using the coupling HPLC-MAD-HG-AFS.

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Table 2. Optimized operation conditions for the HPLC-MAD-HG-AFS system in the enantiomeric separation of DL-selenomethionine

Chromatographic conditions	
Column	Teicoplanin bonded phase (Chirobiotic T, 250 × 4.6 mm i.d., 10 μm particle size)
Mobile phase	Milli-Q water
Flow rate	1 ml min^{-1}
Temperature	Room temperature
Injection volume	200 μl
Sample decomposition hydride generation	
HBr	47% (w/v); 1 ml min^{-1}
$KBrO_3$	15 mM; 1 ml min^{-1}
$NaBH_4$	1.5% (w/v) in NaOH 1%; 1 ml min^{-1}
AFS detection	
Carrier gas	Ar (200 ml min^{-1})
Auxiliary gas	Ar (100 ml min^{-1})
H_2	60 ml min^{-1}
Lamp (BDHCL) intensity	20 and 25 mA

was described by the equation $y = 21963x - 24953$, with correlation coefficient $R = 0.9996$ and $y = 20370x - 12120$ ($R = 0.9997$) for the D-selenomethionine.

For the L-selenomethionine standard it was $y = 49259x - 7044$ ($R = 0.9998$), where y is the measured peak area and x the Se concentration in ppb or μg l^{-1}. The detection limits on milk whey samples for selenomethionine (evaluated as 3σ of the blank) were found to be 3.1 μg l^{-1} (as Se) for the L-enantiomer and of 3.5 μg l^{-1} for the D-enantiomer (in the racemic mixture). L-Selenomethionine standard showed a detection limit at 1.5 μg l^{-1}.

Analysis of selenomethionine in breast and formula milk

Sample clean-up

Milk is a complex food that contains fats and proteins which can dramatically affect metal speciation. In addition, chiral columns are critically affected by the sample matrix and other analytical steps such as sample preconcentration in the SCX column, and post-column hydride generation is also influenced by the matrix components. For these reasons, careful sample treatment is necessary to obtain an extract without fats and proteins suitable for the whole analytical approach.

The combined use of centrifugation and ultrafiltration was studied for this purpose. Further experiments involving the use of enzymatic hydrolysis (a mixture of protease and lipase)[36] did not provide successful results, because the elimination of fats and proteins in the milk was not complete.

Sample centrifugation and ultrafiltration

A clean-up approach combining centrifugation, pH adjustment and ultrafiltration was set up. One-dimensional optimization was used for this purpose. Several times and centrifugation rates were tested on milk samples without pH modification. The optimal conditions for fat elimination were obtained under centrifugation at 14 000 rpm during 30 min. For casein elimination we used the usual experimental conditions established in the literature that fix the pH at 4.5, promoting separation by centrifugation. However, less abundant proteins and some fat still remain in the resulting extract and a third step based on ultrafiltration was necessary for their complete elimination. A commercial ultrafiltration cell was used for this purpose, based on the application of a nitrogen flow under pressure to assist in the sample passing through a suitable pore size membrane and simultaneous sample stirring to avoid membrane clogging. The membrane nominal molecular weight limit (NMWL), 10 kDa, was selected for high molecular weight compound cut-offs such as fats and proteins; therefore, selenoamino acids are isolated in the resulting extract. Membranes with different NMWL were tested: 5, 10 and 30 kDa, but higher recoveries were reached with the 10 kDa membrane; in addition, several nitrogen pressures were also studied and optimum results were obtained for 1.5 bar.

Selenomethionine preconcentration from breast milk

The low concentration of selenium in breast milk, 7–10 ng ml^{-1}, which is usually distributed among different chemical species, makes the analysis of the extract resulting from the clean-up step using the HPLC-MAD-HG-AFS coupling difficult, because the quantification limits are not reached. Therefore, a preconcentration treatment is necessary to insure the speciation procedure reliability.

Solid-phase preconcentration has been tested for this purpose using cation and anion exchangers. The parameters controlling solid-phase extraction (SPE) of selenoamino acids were the pH and sample concentration, which affect the retention of selenium species, as well as the type, composition and volume of the eluents. The experiments were initially performed (three replicates) using spiked aqueous test samples with 625 μg of both D- and L-selenomethionine in 25 ml of milli-Q water.

The pH influence on the analyte retention was tested at three values: 2.0, 4.5, and 12.0, using H_2SO_4 and $NaOH/NH_3$ as pH reagents. The retention was quantitative for the SCX column at pH 4.5. The SAX adsorbent did not provide satisfactory results. Elution was performed using different solutions: (i) acids such as HNO_3 and H_2SO_4, at pH 2; (ii) water; and (iii) alkalis such as NH_3 and NaOH, at pH 12. The best recoveries were obtained with H_2SO_4 at pH 2 as eluent, with values of about 102%.

Selenomethione preconcentration from different spiked milk whey samples with concentrations ranging from 25

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to 5 ng ml^{-1} (enantiomers mixture) was tested. The results were quantitative with recoveries from 85 to 102%.

Breast and formula milk analysis

The content of L-selenomethionine in a pooled breast milk sample obtained from several mothers was evaluated in the resulting extract after sample pre-treatment, using the proposed method. Figure 3 shows the chromatograms obtained in the final extract, after sample clean-up and preconcentration. In breast milk [Fig. 3(a)] only one peak was observed, which matches the retention time of L-selenomethionine. This confirms that L-enantiomer is the form best assimilated by the body.[11]. However, the formula milk chromatogram [Fig. 3(b)] shows peaks corresponding to L- and D-selenomethionine, although concentration of the first is three-fold higher. Quantification of L-selenomethionine in breast milk was performed using standard addition calibration on the extract obtained after clean-up and preconcentration of the milk. A concentration of L-selenomethionine of 9.5 ± 0.1 μg l^{-1} (as Se) was obtained, which recovered about 71% of the total selenium present in the breast milk sample, 13.4 ± 0.3 μg l^{-1} (as Se), evaluated as described in the Experimental section. This suggests the presence of other selenium bioactive molecules in the milk, such as selenoproteins. In formula milk, L- and D-selenomethionine concentration in the extract obtained after treatment of samples reconstituted in water was 6.2 ± 0.1 and 2.2 ± 0.1 μg l^{-1} (as Se), respectively, for three replicates. In this case selenomethionine selenium species represented only the 64.2 and 23.1%, respectively, of the total selenium amount (9.5 ± 0.1 μg l^{-1}).

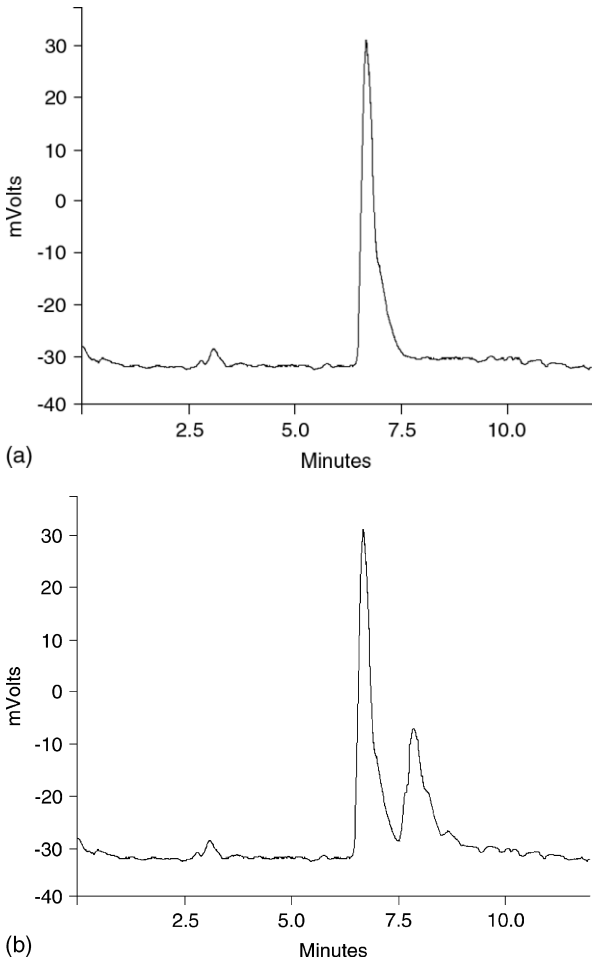

Figure 3. Chromatograms with the coupling HPLC-MAD-HG-AFS for: (a) breast milk and (b) formula milk.

CONCLUSIONS

The coupling HPLC-MAD-HG-AFS provides a good analytical approach for chiral speciation of selenomethionine in breast and formula milk, although careful sample clean-up and species preconcentration are necessary. The final procedure is sensitive and precise for selenomethionine enantiomers speciation in milk, and it is suitable for this amino acid chiral speciation in infant milk.

Comparison of HPLC-MAD-HG-AFS sensitivities for L-selenomethionine (3.1 μg L^{-1}, as Se) and D-selenomethionine (3.5 μg L^{-1}) with those corresponding to HPLC-ICP-MS, 0.8 μg L^{-1} as Se, for both chiral species,[24] confirms the higher sensitivity of ICP-MS detector if the Chirobiotic T chiral column is used. However, when HPLC-ICP-MS is applied to milk samples for selenomethionine chiral speciation, the recovery decreases[22] and the present approach provides more reliable results. Possibly, the discriminating action caused by the multiple post-column online treatments in the HPLC-MAD-HG-AFS coupling reduces the matrix influence. Finally, the best sensitivity for chiral speciation of selenomethionine was reached with the coupling GC-ICP-MS[29] using a L-valine-*tert*-butylamine (Chirasil-L-Val) column after with detection limits in the order of 0.1 μg l^{-1}; however, the selenoamino acids have to be analyzed as *N*-TFA-*O*-isopropyl-derivatives and the procedure has not been applied to milk samples.

The application of the HPLC-MAD-HG-AFS coupling to breast milk analysis revealed the presence of L-selenomethionine, representing about 70% of the total selenium content in the sample. In formula milk, some D-selenomethionine is also present. Results obtained confirmed the method's applicability to this type of sample.

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