A Novel HPLC Column Switching Method Coupled to ICP-MS/QTOF for the First Determination of Selenoprotein P (SELENOP) in Human Breast Milk

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ABSTRACT

In this work, we describe for the first time the presence of selenoprotein P in human breast milk (colostrum). To this end, a novel analytical method has been developed based on a two-dimensional column switching system, which consisted of three size exclusion columns and one affinity column coupled to inductively coupled plasma mass spectrometry (ICP-MS). The method combines the accurate quantification of selenoproteins and selenometabolites by species unspecific isotopic dilution ICP-MS, with the unequivocal identification by quadrupole time of flight mass spectrometry. Several selenopeptides, which contain the amino acid selenocysteine (U, SeCys), were identified after tryptic digestion followed by their separation. The results reveal that the relative selenium concentration in colostrum follows the order: glutathione peroxidase (GPX)~selenoprotein P (SELENOP)> selenocystamine (SeCA)>other selenometabolites (SeMB), in contrast with previously published papers (GPX>SeCA>SeC>SeMet). A mean concentration of 20.1±1.0 ng Se g-1 as SELENOP (1.45 µg SELENOP/g) was determined in colustrum (31% of total selenium).

Keywords

Breast milk, selenoprotein P, Inductively coupled plasma mass spectrometry, Two-dimensional column switching.

1. Introduction

The extraordinary beneficial roles of both selenium and human breast milk have been extensively investigated. Human breast milk contains stem cells whose function is not fully understood today, but they can be later differentiated into neurons(Sani et al., 2017). In addition, although apparently breast milk was thought to be sterile, there is nothing further from trusty. The origin of milk microbiota is unknown, but the hypotheses point to the mammary gland, the flora of the breast skin and a “gut-breast microbiota axis” where intestinal bacteria are transferred from mother breast to the newborn(Rautava, 2016). Nowadays, it is known that selenium is transferred to the fetus through placenta(Burk et al., 2013), but also through breast milk, like stem cells and microbiota.

Among other key roles, selenium is an essential element, antioxidant and anticancer, acts against free radicals and inhibits the toxicity of some xenobiotics(Arias-Borrego et al., 2019) . Moreover, dietary selenium affects gut microbiota which, in turn, influence the expression of selenoproteins and selenium status(Kasaikina et al., 2011a). During pregnancy, deficient levels of selenium have been associated with spontaneous abortion, preeclampsia, gestational diabetes, preterm birth and intrauterine growth restriction among other obstetric complications(Pieczyńska & Grajeta, 2015; Rayman, Bode, & Redman, 2003; Rayman, Wijnen, Vader, Kooistra, & Pop, 2011) . However, an overdose of selenium has also deleterious effects(Rayman, 2012).

In human serum, the total concentration of selenium is about 90 ng g^{-1} and the relative abundance of the selenospecies follows the order: selenoprotein P (SELENOP)>glutathione peroxidase (GPX)~selenoalbumin (SeAlb)>selenometabolites (SeMB, selenium species <1500 Da)(García-Sevillano, García-Barrera, & Gómez-Ariza, 2014). Given the importance of selenium during pregnancy and its known health benefits, the concentration of the different species in human breast milk has been extensively investigated and reviewed, especially SeMB(Dorea, 2002). The total concentration of selenium in breast milk, in a study of 30 mother-child couples was 68.6 ± 7.8 μg L^{-1} (Özdemir et al., 2008), very similar than that found in maternal serum and 1.7 times higher than that corresponding to cord se-

rum, but the concentration varies along lactation and under many other factors. Human selenoproteome comprises 25 selenoproteins, the majority of them without a clear function defined today (Arias-Borrego et al., 2019), but in human breast milk only glutathione peroxidase (GPX) has been identified. It has been reviewed that the relative concentrations of the selenospecies in human breast milk are: GPX (4-32% total selenium)>selenocystamine(SeCA)>selenocystine(SeC)>seleno-methionine (SeMet)(Dorea, 2002). Experiments in mice deficient in SELENOP demonstrated by means of behavioral tests that the offspring exhibit defects in growth and motor coordination as well as reduced activity of GPX in kidney(Schweizer, Michaelis, Köhrle, & Schomburg, 2004). Another study also carried out in mice, perform an in situ hybridization of the lactating mammary gland showing that SELENOP mRNA is present, but GPX3 is not, suggesting that the sources of these proteins are breast gland and plasma, respectively (Hill, Motley, Winfrey, & Burk, 2014).The authors also detect SELENOP and GPX in mouse milk by SDS-PAGE autoradiography and suggest that SELENOP genotype accounts for the most of selenium in mouse milk since total selenium in milk decrease 27 % in knock out mice (Hill et al., 2014).

The presence of a metal or metalloid into a molecule allows determining the last by inductively coupled plasma mass spectrometry (ICP-MS) using the metal as a tag (heteroatom-targeted proteomics) (Sanz-Medel, Montes-Bayón, Bettmer, Luisa Fernández-Sanchez, & Ruiz Encinar, 2012). The main advantages are that the metal/metalloid (*e.g.* selenium) can be detected at very low detection limits with a large linearity range and in complex samples, due to its high tolerance to matrix(García-Barrera et al., 2012; Hughes et al., 2018). On the other hand, quantification can be performed by species specific/unspecific isotopic dilution analysis (IDA), with important advantages (Rodríguez-González, Marchante-Gayón, García Alonso, & Sanz-Medel, 2005). The species unspecific isotopic dilution analysis (SUID) is especially recommended for un-targeted analysis or when the isotopically labelled specie is not commercially available (Rodríguez-González et al., 2005). One drawback is the presence of polyatomic or isobaric interferences in the plasma (*e.g.* $^{40}Ar^{2+}$ and $^{79}Br^{1}H^{+}$ on ^{80}Se signal), but they can be overcome using an ICP-MS equipped with a reaction/collision cell(Hinojosa Reyes, Marchante-Gayón,

78 García Alonso, & Sanz-Medel, 2003; Jitaru, Goenaga-Infante, Vaslin-Reimann, & Fisicaro, 2010) or
79 with a triple quadrupole time of flight (Borovička et al., 2019).

80 Herein we develop a new analytical method based on a two dimensional column switching system cou-
81 pled to ICP-MS, which separates selenoproteins and selenometabolites from human breast milk combin-
82 ing size exclusion chromatography and affinity chromatography (2D-SEC-SECxSEC-AF). Absolute
83 quantification of selenometabolites and selenoproteins was accurately carried out by SUID and
84 SELENOP were identified by quadrupole time of flight mass spectrometry (QTOF), after tryptic diges-
85 tion and separation of peptides by ultra-high performance liquid chromatography (UHPLC). This ana-
86 lytical method allows for the first time the identification of SELENOP in human breast milk.

87 2. Materials and methods

88 2.1. Reagents, solutions, and materials.

89 Water (18.2 MΩ cm) was purified with a Milli-Q® Direct 8 system (Millipore, Watford, UK). Enriched
90 ^{74}Se was obtained from Cambridge Isotope Laboratories (Andover, MA, USA) as elemental powder and
91 it was dissolved in the minimum volume of nitric acid (Suprapur grade) and diluted with ultrapure wa-
92 ter. Ammonium acetate used for the mobile phases was purchased from Sigma Aldrich, (Steinheim,
93 Germany). The standards selenomethionine (SeMet), selenocysteine (SeCys), selenocystamine (SeCA),
94 and selenomethylselenocysteine (SeMetSeCys) were purchased from Sigma–Aldrich (Steinheim, Ger-
95 many). The BCR-637 human serum certified reference material (CRM) was purchased from the Institute
96 for Reference Materials and Measurements (IRMM, Geel, Belgium).

97 Dithiotreithol, urea and iodoacetamide were purchased from Bio-Rad (Madrid, Spain) while Trypsin
98 (EC 3.4.21.4) TPCK and NH4HCO3 were obtained from Sigma-Aldrich (Steinheim, Germany).

99 2.2. Instrumentation

100 The extraction of selenometabolites and selenospecies from human breast milk samples was performed
101 using an ultracentrifuge model Beckman coulter optima L-90 K (Beckman, Palo Alto, CA, USA).

102 Chromatographic separation of selenometabolites and selenopeptides was performed using an HPLC
103 model 1260 Infinity Quaternary LC (Agilent Technologies, Tokyo, Japan). The column switching was
104 arranged by stacking of two 5 mL HiTrap® Desalting columns (SEC, bed size: 16 mm × 25 mm, col-
105 umn ID: 16 mm) (GE Healthcare, Uppsala, Sweden) connected by means of a six-way valve to another
106 5 mL HiTrap® Desalting column followed by a 1 mL heparin-sepharose column (HEP-HP, bed size: 7 ×
107 25 mm, column ID: 7 mm), both purchased from GE Healthcare, Uppsala, Sweden.

108 This column switching system was connected to the ICP-MS system using a T shape connector directly
109 introduced into the Micromist nebulizer inlet (Glass Expansion,Switzerland) using a 30 cm PEEK tub-
110 ing (0.6 mm i.d.).

111 The accurate quantification of selenium species was carried out by SUID by the on-line introduction of
112 ^{74}Se after the chromatographic separation and before the ICP-MS. The determination of selenium was
113 carried out in a triple quadrupole ICP-MS model 8800 Triple Quad (Agilent Technologies, Tokyo, Ja-
114 pan), using oxygen of high-purity grade (>99.999%), and pure hydrogen gas (>95%) as reaction gases.
115 Selenium was determined using a mixture of H_2 (2mL min^{-1}) and O_2 (40%). Instrumental conditions
116 were optimized using a tuning aqueous solution containing Tl, Y, Co and Li at 1 μg L^{-1}. Nickel skim-
117 mer and sampling cones were employed (sampling depth 10 mm). For instrumental conditions, see Sup-
118 porting Information Table S1.

119 Peptides were separated and identified by coupling a UHPLC system model Agilent 1290 Infinity to a
120 6550 iFunnel QTOF mass spectrometer (Agilent Technologies, Tokyo, Japan). A volume of 10 μl of the
121 tryptic digest was injected into a Zorbax RRHD Rapid resolution C18 (50 mmx2.1, 18 μm).

122 **2.3. Sample collection**

123 Five human breast milk samples (colostrum) were taken at the Department of Obstetrics and Gynecolo-
124 gy, Hospital de Riotinto in Huelva, Spain, during 2015. Samples were taken from the pregnant women
125 within 48 h after the delivery. As a proof of concept, five pregnant women (age >18 years) who gave
126 birth at term and without any maternal or neonatal risk were recruited for this study. Women with dis-

127 eases, multiple gestation and perinatal complications such as obstructed labour, low Apgar score (below
128 5) or suspicion of infant pathology were excluded from the study. This study was approved by the Local
129 Ethics Committee and a written informed consent was obtained from all the participants.

130 **2.4. Sample preparation**

131 Sample preparation consisted of the ultracentrifugation of human breast milk to remove
132 the caseins micelles and fats from whey using a previously proposed method (De La Flor St Remy,
133 Sánchez, Sastre, & Sanz-Medel, 2004). The ultracentrifugation of samples was carried out at 31,000 g
134 during 90 min at 4ºC (cut-off 105 nm). To this end, the ultracentrifugation method allowed separating 3
135 phases from human breast milk (caseins micelles in the pellet, fats in the upper phase and whey in the
136 intermediate phase). Then, the supernatant with the whey was filtered through Iso-Disc poly-(vinylidene
137 difluoride) (PVDF) filters (20-mm diameter, 0.45-µm pore size) to avoid column clogging and over-
138 loading. Finally, 100 µL of whey was injected into the columns switching system 2D-SEC-SECxSEC-
139 AF to determine the selenocompounds. On the other hand, 100 µL certified serum reference material
140 (BCR-637) was treated with the same sample procedure and injected into the 2D-SEC-SECxSEC-AF
141 system to validate the analysis.

142 **2.5. Analytical procedure for speciation of selenoproteins and selenometabolites from human**
143 **breast milk by 2D-SEC-SECxSEC-AF-ICP-MS**

144 As above described, the extraction of selenometabolites and selenospecies from human breast milk
145 samples was performed by ultracentrifugation using a previously proposed method (De La Flor St Remy
146 et al., 2004). Whey milk samples were filtered through through Iso-Disc poly-(vinylidene difluoride)
147 (PVDF) filters (20-mm diameter, 0.45-µm pore size) to avoid column clogging and overloading. A vol-
148 ume of 100 µL of whey milk was injected into the HPLC-ICP-MS. Serum CRM (100 µL) were directly
149 injected into the HPLC loop after filtration with the above described filters. A chromatographic gradient
150 was used as follows: Mobile phase A (0-12 min): 50 mM ammonium acetate, Mobile phase B (12-35

151 min): 1.5 M ammonium acetate. The column switching valve was programmed at position A: 0 min,
152 position B: 5.4 min, position A: 12 min. The flow rate was 1.3 mL min^{-1}.

153 The accurate quantification of selenium species was carried out by SUID by the on-line introduction of
154 ^{74}Se after the chromatographic separation and before the ICP-MS.

155 **2.6. Isolation of SELENOP from human breast milk**

156 The superimposed chromatograms of serum reference material and human breast milk give a suspected
157 peak of SELENOP in the last at the same retention time than in the former sample. The suspected
158 SELENOP peak was isolated from the 2D-SEC-SECxSEC-AF system as described in Section 2.5. To
159 this end, 100 µL of 10 undiluted aliquots were injected into the 2D-SEC-SECxSEC-AF-ICP-QQQ-MS
160 system using the operational conditions for ICP-MS and the chromatographic conditions described in
161 sections 2.2 and 2.5, respectively. SELENOP fractions, which elutes at 24.6 min, were collected in 50
162 mL Falcon tubes. For this purpose, the peek tube that connects the end of the column to the nebulizer of
163 ICP-QQQ-MS was disconnected and introduced in the Falcon tube at the retention time of the suspected
164 SELENOP. Subsequently, SELENOP fraction was pre-concentrated using a freeze-drier and stored at -
165 80ºC until analysis. Tryptic digestion and purification of peptides for the identification of SELENOP are
166 described in section 2.7.

167 **2.7. Tryptic digestion and purification of peptides.**

168 Tryptic digestion was carried out by adding to the lyophilized fraction (100 mg) a solution containing 8
169 M urea and 50 mM NH_4HCO_3 (200 µL, pH 8.3) and 5 µL of dithiothreitol (DTT, 180 mM). After incu-
170 bation (1h, 37 ºC) iodoacetamide was added (5µL, 400 mM) and incubated again (1 h, darkness). Later,
171 it was re-dissolved in NH_4HCO_3 (800µL, 50 mM) and trypsin (4µL, 0.1 mg mL^{-1}) and incubated at
172 room temperature during 12 h. The reaction was then stopped by addition of 100 µL of trifluoroacetic
173 acid (0.1% v/v). This procedure was adapted from a previously published one (Kinter & Sherman,
174 2005). After tryptic digestion, the peptides were desalted, purified and preconcentrated using ZipTips
175 C18 (Millipore, USA).

176 **2.8. Standardless identification of SELENOP in human breast milk after peptides separation by**

177 **UPLC-QTOF**

178 A volume of 10 µl of the tryptic digest was injected into a Zorbax RRHD Rapid resolution C18 (50

179 mmx2.1, 18 µm). Peptides were separated using mobile phase A (0.1% formic acid in water) and B

180 (90% acetonitrile in water with 0.1% formic acid) with the following gradient: 0 min 3% B, 10 min 35%

181 B, 12 min 90% B, 14 min 90% B, 15 min 3% B, at a flow-rate of 0.5 mL min^{-1}. The QTOF mass spec-

182 trometer operated in positive ion mode using the following parameters: drying gas flow 14 L min^{-1},

183 temperature 250ºC, sheath gas temperature 250ºC, sheath gas flow 11 L min^{-1}, nebulizer gas 35 psi, ca-

184 pillary voltage 3,500 V, MS range (m/z) 300-1700, MS^2 range 50-1,700, MS scan range 8 spec-

185 tra/second and MS^2 scan rate 3 spectra/second. LC/MS data were analysed using the Agilent Mas-

186 sHunter Qualitative Analysis Software B.06. Peptide sequence of SELENOP was obtained from

187 UNIPROT database (http://www.uniprot.org/; SEPP1_HUMAN; Uniprot accession no. P49908) and

188 compared with the experimental data using the Agilent MassHunter Bioconfirm Software B.06.

189 **3. Results and Discussion**

190 **3.1. Optimization of the chromatographic separation of selenometabolites and selenoproteins**

191 The separation in the HPLC was based on a two-dimensional column switching method, which consist

192 of three size exclusion columns (SEC) and one affinity column (AFC, Heparine-sepharose column). The

193 2D-SEC-SECxSEC-AF-ICP-MS arrangement is shown in Figure 1. Initially, the valve is in position A,

194 then low buffer concentration mobile phase (eluent A) carries the sample through whole system (three

195 size exclusion columns and the affinity column) eluting GPX3 because it is not retained in the AFC col-

196 umn (Heras, Palomo, & Madrid, 2011; Jitaru, Cozzi, Gambaro, Cescon, & Barbante, 2008; Jitaru et al.,

197 2010, 2009), while SELENOP is retained in the AF column due to its high affinity for heparin. At 5.4

198 minutes, the valve changed to position B to isolate the retained SELENOP in the AFC column, while all

199 the selenometabolites elute from the first two SEC columns giving two separated peaks, one for SeCA

200 and another one for all other SeMB. Then, after 12 minutes, the mobile phase increased the buffer con-

201 centration and the valve switched to position B for the elution of SELENOP. Finally, SELENOP elutes
202 from the AFC column and after that, the buffer concentration of the mobile phase is restored to 50 mM
203 to achieve the initial conditions. It is noteworthy that SeCA has a different behavior because it elutes
204 from the two first SEC columns after SeMB in a different peak, but it could be retained in the AFC due
205 to its affinity for the heparin contained in the stationary phase, like SELENOP. Certainly, at the first
206 stages of the optimization of the chromatographic separation of selenospecies from human breast milk,
207 the most important pitfall was the separation of SeCA and SELENOP, because they both have affinity
208 for the heparin-sepharose column. The separation was achieved isolating SELENOP in the AFC column
209 by switching the valve for the elution of SeCA from the first two SEC columns directly to the ICP-MS
210 (Position B). The separation of GPX and SeMB was also difficult, but it was possible by coupling two
211 SEC columns which increases the resolution. The third SEC column, located in the loop of the valve
212 was used to enhance peak shape of SELENOP, but it was not critical for the separation, which is not the
213 case of the first two SEC columns, responsible of the separation of GPX, SeMB and SeCA.

214 This method has been optimized from a previous one developed for the determination of selenometabo-
215 lites and selenoproteins in human serum (García-Sevillano, García-Barrera, & Gómez-Ariza, 2013),
216 which allowed for the first time the separation of SeMB and GPX, as well as SELENOP and SeAlb, but
217 it was not applicable to human breast milk due to the presence of SeCA, which co-elutes with
218 SELENOP under those conditions. Due to the *a priori* absence of SeAlb in human breast milk, the sec-
219 ond AFC column (blue-sepharose column) used for the analysis of human serum (García-Sevillano et
220 al., 2013) was unnecessary in this novel method for human breast milk. Figure 2 shows the superim-
221 posed mass flow chromatograms obtained from human breast milk, standards and human serum certi-
222 fied reference material with this novel method. Under these conditions, selenoalbumin co-elutes with
223 GPX in human serum, but the separation of these proteins can be achieved with the previously pub-
224 lished method for human serum (García-Sevillano et al., 2013). In human breast milk, several selenome-
225 tabolites coelute in the same peak, except SeCA. This novel analytical method demonstrates that the

true players in the mother-offspring transfer of selenium through human breast milk follows the order: GPX3~SELENOP>SeCA>SeMB in contrast with previously published papers, which reported: GPX>SeCA>SeC>SeMet. The chromatographic separation of selenium containing species using SEC-SEC-ICP-MS (without AF and column switching) and the proposed 2D-SEC-SECxSEC-AF-ICP-MS are shown in Figures S1 and S2, respectively.

3.2. Comparison with other similar methods

The separation of selenometabolites and selenoproteins can be performed combining different orthogonal chromatographic systems before the ICP-MS detection. In this sense, selenoproteins have been separated from human plasma by anion exchange chromatography (Hinojosa Reyes et al., 2003; Palacios, Ruiz Encinar, Schaumlöffel, & Lobinski, 2006; Xu, Yang, & Wang, 2008) (AEC), size exclusion chromatography (SEC) (Jitaru et al., 2010) and affinity chromatography (Hinojosa Reyes et al., 2003) (AFC). However, the separation of eGPX, SELENOP, SeAlb and SeMB in human serum was attained by coupling two SEC columns to two AFC columns before the ICP-MS using a column switching valve (García-Sevillano et al., 2013). More recently, isotopically enriched selenopeptides and species specific isotopic dilution combined with HPLC coupled to ICP-MS/MS, have been proposed for the absolute quantification of SELENOP in human plasma (Deitrich et al., 2016). However, the arrangement of the columns and the developed column switching method allows separating for the first time in human breast milk GPX, SeCA, Selenometabolites and SELENOP.

3.3. Mass spectrometric identification of SELENOP in human breast milk

The unequivocally identification of selenoprotein P was carried out for the first time in human breast milk, after a tryptic digestion of the selenium containing fraction followed by the separation of peptides in an ultra-high performance liquid chromatography (UHPLC) coupled to quadrupole time of flight. First, the suspected SELENOP peak was isolated from the 2D-SEC-SECxSEC-AF system after the injection of 10 whey milk samples obtained from human breast milk. Figure 3 shows the SELENOP sequence obtained from the analyzed tryptic digest as well as two of the identified tryptic selenopeptides

251 (where U is SeCys) that matches with predicted masses (highlighted in green). The selenium isotopic
252 distribution of one of the successfully identified SELENOP peptides is also shown. The score of this
253 identification was 80%.

254 In the human bloodstream, SELENOP is the best biomarker of selenium status(Jitaru et al., 2010). This
255 selenoprotein is produced in the liver and transported to peripheral having pivotal roles in homeostasis
256 and transport of selenium (Hill et al., 2012). SELENOP is also located in the endothelium, where it
257 bounds to heparin and carbohydrates and acts as an antioxidant (Burk, Hill, & Motley, 2003), defends
258 human astrocytes and endothelial cells from oxidative damage (Steinbrenner, Alili, Bilgic, Sies, &
259 Brenneisen, 2006), shields plasma proteins against oxidation and nitration (Arteel et al., 1998)and re-
260 duces phospholipid hydroperoxides. Moreover, this protein and its receptor apolipoprotein E receptor 2
261 (apoER2) favors selenium retention in brain against other organs. In fact, SELENOP has the same re-
262 ceptor than ApoE, whose polymorphisms represent a genetic risk of Altheimer disease (Muñoz-
263 Gutiérrez, Pierlé, Schneider, Baszler, & Stanton, 2016). SELENOP affects hyperphosphorylated tau ag-
264 gregation and Aβ and possess signaling functions though neuronal ApoER2 (Solovyev et al., 2018).
265 Then, could SELENOP hold the key to the newborn neurodevelopment attributed to human breast milk?
266 On the other hand, if selenium alters gut microbiota(Kasaikina et al., 2011b), is SELENOP closing the
267 gap around the described "gut-breast microbiota axis" (Rautava, 2016). Thus, what composition should
268 formula milk has? And what should nutraceutical and functional food have? Thus, the bioaccessibility
269 of SELENOP after by *in vitro* gastrointestinal digestion should be assessed in further works.

270 As a proof of concept, the analytical method was applied for the accurate quantification of selenometab-
271 olites and selenoproteins in five samples of human breast milk (Table 1). As can be seen in Table 1, the
272 mean concentration of selenium (ng Se g^{-1}) follows the order:
273 GPX3(24.0±3.4)~SELENOP(20.1±1.0)>SeCA(11.6±0.6)>SeMB(9.4±1.5). All these concentrations are
274 referred as ng of selenium per gram of sample, which in turn implies a mean concentration of
275 SELENOP of 1.45 μg of protein per gram of sample (assuming that SELENOP present 10 atoms of Se

276 in its structure). Thus, GPX accounts for the highest content of selenium in human breast milk (37%),
277 very close to previously reported data (4-32%) (Dorea, 2002), but for the first time reported, followed
278 by SELENOP (31%), SeCA (18%) and other SeMB (14%). It is possible that the assigned GPX peak,
279 which elutes in the void volume, contains other(s) non-retained selenocompoud(s), and should be fur-
280 ther investigated. In fact, when human breast milk was analyzed with the column switching method
281 firstly developed for human serum (García-Sevillano et al., 2013), a peak was detected at the retention
282 time of SeAlb, but the presence of this selenoprotein in human breast milk has not been confirmed by
283 organic mass spectrometry. The mean selenium concentrations (μg Se L^{-1}±σ) determined in our labora-
284 tory in 83 healthy mother and cord sera at the time of birth were, respectively: SELENOP(42.5±9.5 vs
285 28.1±7.7)>SeAlb(11.6±3.6 vs 14.1±4.3)~GPX3(11.2±3.7 vs 10.5±3.5)>SeMB(4.3±2.9 vs 4.0±2.4) and
286 total selenium 68.9±15.23 vs 56.1±14.6 (Santos et al., 2017). Thus, in mother serum SELENOP ac-
287 counts for the highest content of selenium (61%), followed by SeAlb (17%), GPX3 (16%) and SeMB
288 (6%). It is noteworthy that selenium concentration as SELENOP decrease to 50% and SeAlb increase to
289 25%, in cord serum against mother serum. This difference could be related with the immature child sys-
290 tem, which can not produce enough SELENOP. Moreover, the results reported in this work could lead to
291 the hypothesis that the remaining SELENOP required for the newborn should be transferred by human
292 breast milk. Several authors reported that SELENOP is quite an unstable protein (since it's so redox-
293 active) and it decomposes quite easily to form Se (VI) for instance (Solovyev, Berthele, & Michalke,
294 2013). Thus, differences found between the current study and the previous ones can be related, at least
295 partially to this fact.

296 Further studies, should be performed into a higher number of samples to establish more accurate per-
297 centages and mean values for human breast milk.

298 4. Conclusions

299 A novel analytical method has been developed for the separation of selenometabolites and selenopro-
300 teins from human breast milk. The two dimensional coupling of three size exclusion columns and one

affinity column using a column switching valve allows separating SELENOP from SeCA, which present affinity for the heparin-sepharose stationary phase of the AFC. The method allows the absolute quantification of GPX3, SELENOP, SeCA and SeMB by SUID-ICP-MS, and for the first time the identification of SELENOP in human breast milk demonstrating that it is implicated in the mother-offspring transfer of selenium, along with other selenospecies: GPX3~SELENOP>SeCA>SeMB. The results reported in this paper are in contrast with previously published papers, which reported: GPX>SeCA>SeC>SeMet. The development of this method enables the accurate and absolute quantification in human breast milk samples of SELENOP, a new biomarker of the mother-offspring transfer of selenium, related with cancer and Alzheimer´s disease (Arias-Borrego et al., 2019). The results open possibilities to new investigations related with the precise mechanisms underlying the mother-offspring transfer of selenium depending on the chemical specie, the role of selenoprotein P in human breast milk and the development of new nutraceutical and functional food (e.g. formula milk) enriched in selenium.

Appendix A. Supplementary material

Experimental conditions for ICP-MS meassurements (file type, i.e., PDF).

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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467 **FIGURES**

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469 Figure 1. 2D-SEC-SECxSEC-AF-ICP-MS Column switching method for the separation of GPX, SeMB,
470 SeCA and SELENOP in human breast milk.

471 Figure 2. 2D-SEC-SEC/SEC-AF-ICP-MS superimposed chromatograms obtained from human breast
472 milk (HBM, red line), human serum certified reference material (CRM, blue line) and standard solution
473 of selenometabolites (SeMB, green line) at 15 µg Se L^{-1} each: selenomethionine (SeMet), inorganic se-
474 lenium (iSe), selenocysteine (SeCys) and selenomethylselenocysteine (SeMetSeCys). Selenocystamine
475 (SeCA) was also added to the standard solution at 15 µg Se L^{-1} and elutes in a separated peak from
476 SeMB.

477 Figure 3. ESI-MS obtained from two peptides after the tryptic digestion of the suspected SELENOP
478 isolated by 2D-SEC-SECxSEC-AF. Identified tryptic peptides that matches with theoretical sequences
479 are highlighted in green. A. Mass spectrum of the SELENOP peptide EGYSNISYIVVNHQGISSRLK.
480 The inset shows one of the double charged peptide successfully identified. B. Mass spectrum of the
481 SELENOP selenopeptide NQAKKUEUPSNC. The inset shows the typical natural Se isotopic abun-
482 dance. U=selenocysteine (SeCys).

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494 **Table 1.** Selenium concentration (ng Se g-1) in n=5 human breast milk samples: glutathione peroxidase (GPX3),
495 Selenoprotein P (SELENOP), selenocystamine (SeCA) and other selenometabolites (SeMB).

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Sample	*GPX3	SeMB	SeCA	SELENOP	Total Se
1	24.2	9.8	11.6	19.9	65.5
2	25.2	8.0	10.8	21.1	65.1
3	18.3	7.6	12.4	18.5	56.8
4	25.0	11.1	11.9	20.2	68.3
5	27.5	10.4	11.1	20.8	69.7
Mean±σ	24.0±3.4	9.4±1.5	11.6±0.6	20.1±1.0	65.1±5.0
% of total Se	37	14	18	31	--

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504 *GPX3 and non-retained species
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