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<table>
<thead>
<tr>
<th>Journal:</th>
<th>Analytical and Bioanalytical Chemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID:</td>
<td>ABC-02055-2013.R1</td>
</tr>
<tr>
<td>Type of Paper:</td>
<td>Note</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>n/a</td>
</tr>
<tr>
<td>Complete List of Authors:</td>
<td>García-Sevillano, Miguel Ángel; Universidad de Huelva, Dpto. Química y Ciencias de los Materiales, Facultad de Ciencias Experimentales García-Barrera, Tamara; University of Huelva, Química y Ciencia de Los Materiales Gomez Ariza, José Luis; Universidad de Huelva, Dpto. Química y Ciencias de los Materiales, Facultad de Ciencias Experimentales;</td>
</tr>
<tr>
<td>Keywords:</td>
<td>Mass spectrometry / ICP-MS, HPLC, Speciation, Metals / Heavy metals</td>
</tr>
</tbody>
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Simultaneous speciation of selenoproteins and selenometabolites in plasma and serum by dual size exclusion-affinity chromatography with online isotope dilution inductively coupled plasma mass spectrometry

M.A. GARCÍA-SEVILLANO*ab, T. GARCÍA-BARRERA*ab, and J.L. GÓMEZ-ARIZA*ab

*Department of Chemistry and Materials Science, Faculty of Experimental Science, University of Huelva. Campus de El Carmen, 21007 Huelva, SPAIN
†Department of Excellence International ceiA3. University of Huelva. SPAIN
‡Research Center of Health and Environment (CYSMA). University of Huelva. Campus de El Carmen. 21007 Huelva. SPAIN
*E-mail: ariza@uhu.es; tamara@dqmcm.uhu.es

A method for the simultaneous speciation of selenoproteins and selenometabolites in mouse plasma has been developed based on in series two-dimensional size exclusion and affinity high performance liquid chromatography (2D/SE-AF-1HPLC), using two columns of each type, and hyphenation to inductively coupled plasma-(quadrupole) mass spectrometry (ICP-QMS). The method allows the quantitative determination of selenoprotein P (SeP), extracellular glutathione peroxidase (eGPx), selenoalbumin (SeAlb) and selenometabolites in mouse plasma using species-unspecific isotope dilution (SUID). The 2D chromatographic separation is proposed to remove typical spectral interferences in plasma from chloride and bromide on 75Se (67Ar+3Cl) and 77Se (67Br+3I). In addition, the approach increases chromatographic resolution allowing the separation of eGPx from Se metabolites of low molecular mass. The method is robust, reliable and fast with a typical chromatographic runtime less than 20 min. Precision in terms of relative standard deviation (n=5) is in the order of 4% and detection limits are in the range of 0.2 to 1.0 ng Se g⁻¹. Method accuracy for determination of total protein-bound to Se was assessed by analyzing human serum reference material (BCR-637) certified for total Se content, and latterly applied to mouse plasma (Mus musculus). In summary, a reliable speciation method for the analysis of eGPx, selenometabolites, SeP and SeAlb in plasma/serum samples is proposed for the first time applicable to the evaluation of Se status in human in clinical studies and other mammals for environmental or toxicological assessment.

Keywords: selenoproteins, isotopic dilution analysis, inductively coupled plasma-mass spectrometry, Mus musculus, selenium speciation.
**Introduction**

Selenium (Se) is now recognized as an essential element in mammals, and the importance of its biochemistry has been widely reported. The exact mechanism used by Se to play its essential role in human health is still unclear. However, it has been checked that most biological functions attributed to Se are mediated by selenoproteins [1-2] such as extracellular glutathione peroxidase (eGPx) and selenoprotein P (SeP), which contain selenocysteine specifically incorporated at their active sites, versus selenoalbumin (SeAlb), not considered as a selenoprotein, because the element is not specifically incorporated into the albumin moiety as selenomethionine (SeMet) [3-4]. SeP is a major selenoprotein in mammalian plasma, and its concentration is a good indicator of Se status in humans [3-4], while eGPx activity in human serum is a useful parameter of oxidative stress in clinical studies [5-8]. On the other hand, SeAlb is assumed to be transported to the liver for new synthesis of SeP that is then released into the bloodstream [9]. Although the antioxidant activity of purified SeP has been demonstrated, as well as the reduction of phospholipid hydroperoxides in an eGPx-like manner, its biological action mechanisms is still unclear [10]. In addition, selenometabolites (selenium aminoacids and inorganic forms of selenium) also play important physiological roles in plasma, although the information about their presence in this fluid is scarce and not convincingly documented. For these reasons, a method for the simultaneous determination of SeP, eGPx, SeAlb and low molecular mass selenium species free of interferences and with high resolution, sensibility and precision is mandatory.

In this sense, the use of inductively coupled plasma mass spectrometry (ICP-MS) for selenium analysis is very suitable due to its trace analysis capability, low detection limits, isotope ratios measurements, tolerance to matrix and large linearity range [11-13]. On the other hand, species-unspecific isotope dilution mode (SUID) is especially useful in speciation either when the structure and composition of analysed species is not exactly known or when the corresponding isotopically labelled compound is not commercially available [13]. Several chromatographic methods have been proposed in the literature for the separation of selenoproteins in mammalian plasma or serum, based on anion exchange chromatography (AEC) [14-16], size exclusion chromatography (SEC) [17] and affinity chromatography (AFC) [14]. However, SEC cannot provide precise quantitative results for selenoproteins analysis because of low chromatographic resolution, which causes overlapping between peaks corresponding to high abundance selenium containing proteins [17, 18-19]. Alternatively, a number of methods have introduced a heparin-Sepharose column prior to SEC chromatographic separation in order to improve the resolution by retention
of SeP, but the time of analysis increases to 60 minutes [20]. On the other hand, AEC provides good
recoveries of analytes but chromatographic resolution is not satisfactory either [14]. Finally, when AFC is
used, the weakly-retained eGPx together to other non-target matrix Se-components are not resolved and they
are quantified together [14, 21-22]. Moreover, it should be remarked the high concentration of Cl− and Br− in
biological fluids, such as plasma and serum (∼3.5 g L⁻¹ and ∼3.5 mg L⁻¹, respectively) [23], which coelute
with eGPx and Se metabolites in the previously cited procedures [21-22] and represent a serious
interference, due to formation of polyatomic species ⁴₀Ar⁺ and ⁷⁹Br⁺¹¹⁺ isobaric with ⁸²Se isotope in the
ICP-MS device. For this reason, hydrogen is usually recommended as reaction gas in an octopole reaction
system (ORS) for accurate quantification of selenium, especially in human serum samples, which contains
high levels of bromide [14-15]. Most recently, multidimensional approaches based on AEC has been
coupled on line to AFC for the analysis of selenium containing proteins, alleviating the spectral interferences
of Cl− and Br− by using ICP-ORS-MS [21-22].

In this work, a method for the quantification of selenium-tagged proteins and selenometabolites has
been developed in plasma from laboratory mice (Mus musculus), using species-unspecific isotope dilution
(SUID)-ICP-ORS-qMS online coupled to a 2D/SE-AF-HPLC instrumental arrangement, which includes two
columns of both SE and AF for selenium species separation. Using this chromatographic arrangement
spectral interference produced by bromide and chloride are removed with a total chromatographic runtime
less than 20 min. Consequently, a reliable speciation method for the analysis of eGPx, selenometabolites,
SeP and SeAib in plasma/serum samples is proposed for the first time applicable to the evaluation of Se
status serum/plasma that can be applied in human in clinical studies. The analytical approach was validated
using a human serum reference material (BCR-637) certified for total Se content.

Experimental

Reagents and materials

All reagents used for sample preparation were of the highest available purity. Phenylmethanesulfonyl fluoride (PMSF) and tris(2-carboxyethyl)phosphine hydrochloride (TCEP)
(BioUltra grade, >98%) were obtained from Sigma Aldrich (Steinheim, Germany). Hydrogen used as
reaction gas in SEC-AF-ICP-ORC-MS system was of high-purity grade (>99.999%).
The human serum certified reference material (CRM) BCR-637 was purchased from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium). Standard solutions containing 1000 mg L\(^{-1}\) of Se and 1000 mg L\(^{-1}\) of Br, both stabilized in 5\% (v/v) Suprapur nitric acid were purchased from Merck (Darmstadt, Germany). Enriched \(^{75}\)Se was obtained from Cambridge Isotope Laboratories (Andover, MA, USA) as elemental powder and it was dissolved in the minimum volume of nitric acid (Suprapur grade) and diluted to the appropriate volume with ultrapure water. The concentration of this solution was established by reverse isotope dilution analysis as described elsewhere [14].

**Instrumentation**

A microwave oven (CEM Matthews, NC, USA, model MARS) was used for the mineralization of plasma and serum samples. Selenium trace levels and selenium-linked biomolecules were analyzed with an inductively coupled plasma mass spectrometer Agilent 7500ce (Agilent Technologies, Tokyo, Japan) equipped with an octopole collision/reaction cell. Chromatographic separations were performed using a Model 1100 HPLC pump with as delivery system with an UV detector (Agilent, Wilmington, DE, USA).

ICP-MS measurement conditions are shown in table 1.

The complete resolution of the selenium species was carried out by two 5 ml HiTrap® Desalting Columns (GE Healthcare, Uppsala, Sweden), in series connected to two affinity columns, with stationary phases of heparin-sepharose (HEP-HP) and blue-sepharose (BLU-HP), both purchased from GE Healthcare, Uppsala, Sweden.

**Procedures**

**Animals and sample preparation**

*Mus musculus* (inbred BALB/c strain) mice were obtained from Charles River Laboratory (Spain). Ten mice of 7 weeks of age were fed ad libitum with feed conventional pellets. The animals were allowed to acclimate for 5 days with free access to food and water under controlled conditions (temperature (25-30°С) and a 12 h light-dark cycle) prior to start the exposure experiment. All mice were sacrificed the seventh day from the beginning of the experience. Mice were individually anesthetized by isoflurane inhalation and exsanguinated by cardiac puncture. Plasma collection was carried out by centrifugation (4000rpm, 30 min, 4°С) after addition of heparin (ANTICLOTT) as an anticoagulant for separation into plasma and red blood cells (RBCs).

In addition, 10 mg of 100 mM of PMSF and 100mM of TCEP mixture were added as proteases inhibitor and
redactant agent, respectively [24]. To avoid changes in selenium species, the samples were directly injected into the column without dilution. Mice were handled according to the norms stipulated by the European Community. The investigation was performed after approval by the Ethical Committee of the University of Huelva (Spain).

**Total Se determination by isotopic dilution analysis ICP-ORS-MS**

For the analysis of serum reference material and mice plasma, sample amounts of 0.2000 g were weighed directly into 5-ml microwave polytetrafluoroethylene (PTFE) vessels Xpress (CEM Matthews, NC, USA). An appropriate amount of the $^{75}$Se spike and 800 μL of a mixture containing nitric acid and hydrogen peroxide (4:1 v/v) were added. After 10 min, the PTFE vessels were closed and introduced into the microwave oven. The mineralization was carried out at 400 W from room temperature ramped to 160°C for 15 min and hold for 40 min at this last temperature. After that, the solutions were made up to 10 g with ultrapure water and the total selenium concentration was analyzed by isotopic dilution analysis (IDA) and ICP-ORS-MS. All the analyses were performed using three replicates.

**Selenoproteins speciation by two-dimensional size exclusion-affinity chromatography hyphenated to species-unspecific isotope dilution ICP-MS (2D/SE-AF-HPLC-SUID-ICP-ORS-MS)**

Separation of the analytes was performed by in series stacking of two 5 ml HiTrap® Desalting Columns in series connected with a dual affinity column arrangement comprising a 1 ml heparin-sepharose column (HEP-HP) and a 1 ml blue-sepharose column (BLU-HP) interconnected by a six-way switching column valve (Fig. 1). The HiTrap column is based on size-exclusion principle, and the combination of two columns increases the resolution of the chromatographic separation. On the other hand, the HEP-HP column is able to retain selectively SeP whereas BLU-HP column retains both SeP and SeAlb [15, 25-26].

The 2D/SE-AF-HPLC-SUID-ICP-ORS-MS coupling was performed by connecting the outlet of the chromatographic unit with the T piece of Micromist nebulizer inlet of ICP-MS by means of a 30cm PEEK tubing (0.6 i.d. mm). Post column isotope dilution analysis was performed by the direct introduction of $^{75}$Se via a T connector into the ICP-ORS-MS. Speciation of selenium in mouse plasma and human serum was...
carried out using the operating conditions summarized in Table 1. Ammonium acetate was used as mobile phase with a gradient from 0.05 mol L\(^{-1}\) (pH 7.4) to 1.5 mol L\(^{-1}\) (pH 7.4), at a flow rate of 1.5 ml min\(^{-1}\), using an injection volume of 100 \(\mu\)L. Plasma and serum samples aliquots were injected into the column switching method passing the mobile phase A through the system (Position A, Fig. 1). Then, selenium containing proteins (eGPx, SeAlb and SeP) are separated by SE (HiTrap columns) from both low molecular weight selenium species and bromide and chloride interferences. Then, SeP is retained in the HEP-HP column and SeAlb in the BLUE-HP one. Since, eGPx, selenometabolites and Br+Cl interferences were not retained in affinity columns they were eluted resolved in three well differentiate peaks. After this step, mobile phase B was pumped through the system for the elution of SeP (Position B, Fig. 1). Finally, the valve was switched on again to the initial position for the elution of SeAlb (Position A, Fig. 1). Finally, mobile phase A was passed through the system for equilibration during 10 minutes prior to the next injection.

In order to verify the absence of other selenium species, the eGPx fraction separated by 2D/SE-AF-HPLC-SUID-ICP-ORS-MS was analyzed by AEC-HPLC-ICP-ORS-MS using the same operational conditions previously described by Garcia-Sevillano et al. [24]. The results obtained are in concordance with those previously published by P. Jitaru et al [15] in the GPx fraction purified by RP-HPLC-ICP-MS, but in our case the peak of bromide interference was not observe (data not shown), due to the exclusion caused by SEC separation (Fig. 3). The quantification of selenium containing proteins and selenium-metabolites in the different chromatographic peaks was carried out by post-column specie-unspecific isotopic dilution analysis as described by C. Sariego-Muñiz et al., 2001 [27].

Results and discussion

**Optimization of the analysis of selenium containing proteins and selenium metabolites by 2D/SE-AF-HPLC-SUID-ICP-ORS-MS**

During the chromatographic separation, the isotopes \(^{74}\text{Se},\ {^{76}\text{Se},}\ {^{77}\text{Se},}\ {^{78}\text{Se},}\ {^{80}\text{Se},}\ {^{82}\text{Se},}\ {^{79}\text{Br},}\ {^{81}\text{Br}}\) and \(^{81}\text{Kr}\) \(^{82}\text{Se}^1\text{H}^+\) were simultaneously monitored online by ICP-ORS-qMS to ensure the absence of isobaric interference from Br (Fig. 2b and 2c). The use of two small SE chromatographic columns arranged in series (HiTrap Desalting Column) allows a good resolution in the speciation of eGPx regarding to
selenometabolites, with retention times of 4 and 7 min, respectively, while spectral interference caused by Br presents in plasma is overcome, since Br appears in the peak at 9 min (Fig. 2a). This fact is confirmed when comparing Fig. 2a and 2c, in which a high intensity peak with retention time at 9 min can be related to Br by monitoring $^{75}$Se ($^{79}$Br$^{4}$H$^{-}$), Fig. 2a, and $^{79}$Br and $^{81}$Br, Fig. 2c. However, this peak is absent when $^{77}$Se, $^{75}$Se and $^{79}$Se are monitored (Fig. 2b). On the other hand, the important interference caused by Cl on the signal of $^{77}$Se (ion $^{37}$Cl$^{6}$Ar$^{-}$), is eliminated by using hydrogen in the reaction cell. This fact is confirmed by the accordance of natural isotopic abundance of $^{75}$Se and $^{77}$Se (49.6% and 7.63%, respectively) with the corresponding isotopic abundance in the chromatogram, and the absence of peaks of $^{76}$Se, $^{75}$Se, and $^{79}$Se at 9 min (Fig. 2b). After conversion of selenium isotopes intensity and polyatomic interferences to mass flow chromatogram for the quantification of selenium species in plasma and serum samples, using mathematical equation corrections [27], the peak about 9 minutes (BrH$^{-}$) is not present when isotope $^{79}$Se is used for quantification of selenium species (Fig. 3).

The speciation method has been validated using a CRM of human serum (BCR-637) certified for total Se content (Se$_{r}$ 81±7 ng mL$^{-1}$). This material was additionally spiked with 50 ng mL$^{-1}$ of inorganic selenium (sodium selenate) to evaluate the recovery and precision of the proposed method for inorganic selenium. The concentration of different selenium species obtained in the BCR-637 are in concordance with previous results published by Jitaru et al. [28] using AE-SPE prior to AF-HPLC-ICP-MS. However, selenoaminoacids (selenomethionine (SeMet) and selenocysteine (SeCys)) are not retained by AE-SPE and they elutes in the void volume besides eGPx [21]. This drawback is overcome by the use of a double SE unit previous to the AF arrangement, proposed in this study, due to absence of interactions of Se-species with SE stationary phase. The results obtained are show in table 2. In addition, total Se concentration calculated by IDA-ICP-ORS-MS which agrees with the sum of Se content in the previous species. The methodological improvement provided by the double SEC unit in the chromatographic resolution is clearly demonstrated, considering that the approach removes typical interferences from Cl and Br in plasma/serum samples and avoids the usual interactions of selenoproteins with other chromatographic components prior to the affinity chromatographic unit, which causes selenium species losses.

**Application of selenium speciation method to mouse plasma.**

**Evaluation of the analytical performance**
Quantification of selenium containing proteins and low molecular weight selenium species has been performed in mouse plasma using the optimized speciation method. Repeatability in terms of relative standard deviation (n=5), detection limits, selenium species concentration (eGPx, SeP, SeAlb, low molecular mass selenium species) in mice plasma, and total Se concentration evaluated by IDA-ICP-ORS-MS are show in table 2. These results confirm method reliability by the good accordance of the sum of selenium concentration in the different selenoproteins and total Se obtained by IDA-ICP-ORS-MS after acid digestion.

Conclusions

This study shows the reliability of the 2D/SE-AF-HPLC-SUID-ICP-ORS-MS approach for complete simultaneous speciation analysis of selenium in human serum and mouse plasma including selenoproteins and selenometabolites. The chromatographic method and the ICP-ORS-MS system remove typical spectral interferences from serum/plasma samples such as Cl and Br and increases the chromatographic resolution by the in series introduction of a double SE unit allowing the separation of eGPx from selenometabolites of low molecular mass, as well as from non-target matrix components that limit accurate quantification of these species. Subsequently online dual affinity chromatographic arrangement separates SeP and SeAlb. Repeatability in terms of relative standard deviation (n=5) is 4% for eGPx, 5% for seleno-metabolites, 3% for SeP, 5% for SeAlb and detection limits are in the range 0.2 to 1.0 ng Se g⁻1. The method is time-efficient and reliable, using post-column species-unspecific isotopic dilution analysis, and improves other methods based on off-line sample preparation by SPE. In summary, a reliable speciation method for the analysis of eGPx, selenometabolites, SeP and SeAlb in plasma/serum samples is proposed for the first time applicable to the evaluation of Se status in human in clinical studies and other mammals in environmental or toxicological assessment.

Acknowledgements

This work was supported by the projects CTM2012-38720-C03-01 from Ministerio de Economía y Competitividad (Spain), and P008 FQM-3554 and P009-FQM-4659 from Consejería de Innovación, Ciencia y Empresa (Junta de Andalucía-Spain). Miguel Angel García Sevillano thanks the Ministerio de Educación for a predoctoral scholarship. We want to thank to the group of Prof. Sanz-Medel at the
References


FIGURES

FIGURE 1. Schematic diagram of 2D/SE-AF-HPLC-SUID-ICP-ORS-MS arrangement for selenium containing proteins speciation in plasma and serum samples.
FIGURE 2. (A) Typical $^{75}$Se chromatogram obtained from mice plasma samples using the 2D/SE-AF-HPLC-SUID-ICP-ORS-MS coupling showing the interference of $^{80}$Br-H$^+$ (m/z 80) at 9 minutes. (B) Monitoring of $^{75}$Se, $^{77}$Se and $^{79}$Se isotopes, showing the absence of the Br$^+$ interference. (C) Monitoring of $^{79}$Br and $^{81}$Br isotope.

FIGURE 3. Mass flow chromatogram of $^{78}$Se/$^{80}$Se isotope ratios in plasma mouse using 2D/SE-AF-HPLC-SUID-ICP-ORS-MS.

Table 1. Operating conditions of 2D/SE-AF-HPLC-SUID-ICP-ORS-MS

<table>
<thead>
<tr>
<th>ICP-MS conditions</th>
<th>Value</th>
</tr>
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<tr>
<td>Forward power</td>
<td>1500 W</td>
</tr>
<tr>
<td>Plasma gas flow rate</td>
<td>15 L min$^{-1}$</td>
</tr>
<tr>
<td>Auxiliary gas flow rate</td>
<td>1 L min$^{-1}$</td>
</tr>
<tr>
<td>Carrier gas flow rate</td>
<td>0.15 L min$^{-1}$</td>
</tr>
<tr>
<td>Sampling depth</td>
<td>7 mm</td>
</tr>
<tr>
<td>Sampling and skimmer cones</td>
<td>Ni</td>
</tr>
<tr>
<td>H$_2$ flow</td>
<td>4 mL min$^{-1}$</td>
</tr>
</tbody>
</table>
Nebuliser: Micromist (Glass Expansion)
Torch: Shield (with long life platinum shield plate)
Qsub: -18 V
Qp: -16 V
Points per peak: 1
Integration time: 0.3 per isotope
Replicates: 1
Isotopes monitored: \(^{70}\text{Se}, ^{72}\text{Se}, ^{77}\text{Se}, ^{78}\text{Se}, ^{79}\text{Se}, ^{82}\text{Se}, ^{79}\text{Br}, ^{81}\text{Br}\) and \(^{87}\text{Kr}\)
Dead time detector: 47 ns

**Chromatographic conditions of 2D/SE-AF-HPLC-SUID-ICP-ORS-MS**

- Sample loop: 100 µL
- Flow rate: 1.3 ml min\(^{-1}\)
- Mobile phase A: 0.05 M ammonium acetate pHi 7.4
- Mobile phase B: 1.5 M ammonium acetate pHi 7.4
- Gradient: 6-7 min 100% A, 6-18 min 100% B, 18-20 min 100% A
- Valve position: 1-10 min Injet, 10-17 min Load, 17-20 min Injet

---

**Table 2. Quantification of selenium species in human serum (BCR-637) and BCR-637 spiked samples (50 ng of Se g\(^{-1}\); as sodium selenate). Quantification of selenium species in plasma from *Mus musculus* mice**

<table>
<thead>
<tr>
<th>Selenium Species</th>
<th>Human serum BCR-637 (µg Se g(^{-1}))</th>
<th>SD</th>
<th>Human serum BCR-637 (µg Se g(^{-1}))</th>
<th>SD</th>
<th>Human serum BCR-637 Spiked (50 ng g(^{-1}))</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>eGIPx</td>
<td>15 ± 4</td>
<td>11 ± 1</td>
<td>11 ± 1</td>
<td>11 ± 1</td>
<td>11 ± 1</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>SeP</td>
<td>60 ± 7</td>
<td>52 ± 2</td>
<td>53 ± 2</td>
<td>53 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SeAlb</td>
<td>13 ± 4</td>
<td>17 ± 2</td>
<td>18 ± 1</td>
<td>18 ± 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Se metabolites</td>
<td>-----</td>
<td>&lt;LOD</td>
<td>51 ± 2</td>
<td>51 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sum of species</td>
<td>79 ± 3</td>
<td>80 ± 2</td>
<td>133 ± 2</td>
<td>133 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TOTAL Se(^{b})</td>
<td>-----</td>
<td>82 ± 1</td>
<td>131 ± 1</td>
<td>131 ± 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Certified value</td>
<td>87 ± 9</td>
<td>81 ± 7</td>
<td>-----</td>
<td>-----</td>
<td></td>
<td></td>
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</tbody>
</table>

**Plasma from *Mus musculus* mice**

<table>
<thead>
<tr>
<th>Selenium Species</th>
<th>Mean (µg g(^{-1}) as Se)</th>
<th>Relative standard deviation (RSD, %)</th>
<th>Detection limits (LD, ng g(^{-1}) of Se)</th>
</tr>
</thead>
<tbody>
<tr>
<td>eGIPx</td>
<td>10</td>
<td>4</td>
<td>0.2</td>
</tr>
<tr>
<td>SeP</td>
<td>221</td>
<td>3</td>
<td>0.7</td>
</tr>
<tr>
<td>SeAlb</td>
<td>25</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>Se metabolites</td>
<td>21</td>
<td>5</td>
<td>0.4</td>
</tr>
<tr>
<td>Sum of species</td>
<td>277</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>TOTAL Se(^{b})</td>
<td>281</td>
<td>6</td>
<td>0.1</td>
</tr>
</tbody>
</table>

---

\(^{a}\) Values reported by P. Jitaru et al, 2010 [28]

\(^{b}\) Quantification of total selenium by IDA-ICP-ORS-MS.
Dear Editor,

Please find enclosed the amended version of the manuscript (ID ABC-02055-2013) “Simultaneous speciation of selenoproteins and selenometabolites in mouse plasma by dual size exclusion-affinity chromatography with online isotope dilution inductively coupled plasma mass spectrometry” by M.A. García-Sevillano, T. García-Barrera*, J.L. Gómez-Ariza* to be published in Analytical and Bioanalytical Chemistry. All the corrections have been marked in red in the new version of the manuscript and a detailed point-by-point response to each comment raised in the review is given below. All the comments have been taken into account and the new version of the manuscript has been considerably improved.

Sincerely yours,

José Luis Gómez Ariza
Manuscript No. ABC-02055-2013

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Corresponding author: Dr. José Luis Gomez Ariza

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****Please consider the following comments.

Editor Comments:

The work carried out is practical and useful and so this Note is of interest to ABC speciation readers. However the real goal of the work is doubtful and must be better clarified (control of the spectral interferences or actual quantification of the Se-compounds in mouse plasma). Moreover, the referee has made other important comments and suggestions to improve the quality of this Note that should be dealt with adequately in the Major revision of the MS required before publication can be recommended.

Along with the revised text, as customary in ABC, an itemised list of responses to all the points raised by the referees is required as well.

Referee Comments:

This note describes a 2D chromatographic coupling (SE and AF columns) which helps the separation of Br- and Cl-spectral interferences from Se-compounds, and therefore allows their determination by ICP-MS without spectral interferences derived from the presence of bromine within the sample. However, major changes and clarifications should be addressed.

The introduction is not clear enough. The first two paragraphs are a little bit confusing. It is difficult to clearly understand which are the Se-compounds of interest and, the relation among them. As well, more analytical information on the behaviour of the studied Se-compounds is need, instead of the biochemical one included. I miss a short description of the spectral interferences caused by Br and Cl to further understand the importance of the results obtained. Do both interferents (Br and Cl) have the same importance on Se-compounds determination?

Under your suggestion, the introduction has been rewritten in the new version of the manuscript.

What is the real goal of the manuscript? The control of the spectral interferences or the quantification of the Se-compounds in mouse plasma?

The aim of the present work is the development of a method for the simultaneous quantification of selenium containing proteins and low molecular mass selenium species in plasma and serum with high sensibility, precision, high resolution and free of interferences occasioned by the high content of bromide and chloride in these matrix. In summary, a reliable speciation method for the analysis of eGPx, selenometabolites, SeP and SeAlb in plasma/serum samples is proposed for the first time, which is applicable to the evaluation of Se status in human in clinical studies and other mammals in
environmental and toxicological assessment. In addition, the title has been changed to reflect the true contribution of this work.

The quality of the figure must be improved. The sub-figures A, B, C, and D are too small. Also, I consider that these sub-figures should be presented in, at least, 2 independent figures.

Following your recommendation, figures have been modified in the new version of the manuscript

☐ pp 5: Indicate how the different studied Se-compounds were identified after the chromatographic separation.

We have checked the eGPx fraction in order to verify the absence of other selenium species, the eGPx fraction separated by 2D/SE-AF-HPLC-SUID-ICP-ORS-MS was analyzed by AEI-ICP-ICP-ORS-MS using the same operational conditions previously described by M.A. García-Sevillano et al., 2012 [24]. Our results are in concordance with a previously published by P. Jitaru et al., 2010 [15] in the GPx fraction purified with RP-HPLC-ICP-MS, but in our case a peak of bromide interference was not observe, excluded in this work during SEC separation (Fig. 4). This observation has been included in the new version under your suggestion. The identity of SeP and SeAlb, retained by heparin sepharose and BLUE-sepharose, respectively, are well established in literature. Finally, bromide and chloride interferences and selenometabolites were confirmed by spike experiments of the sample.

☐ pp 6 / line 15: The retention times mentioned within the text do not agree with the one observed in the figures. Please check.

It has been modified in the new version

☐ A table including, for instance, the results obtained for the BCR-673 and the mouse samples is missing.

It has been modified in the new version

Editorial Office Comments:

Before submitting your revised paper, we ask that you kindly check that:
1. the manuscript is preceded by 3 to 6 keywords (not required for Feature Articles)
2. the Abstract captures the main points of the paper (an Abstract is not required for Feature Articles)
3. all figures are submitted in clearly reproducible photographs or diagrams
4. references are cited according to the journal’s guidelines for authors
5. axes in graphics are labelled with initial capital letters (e.g. Time)
6. if you have submitted Electronic Supplementary Material, please refer to it in the article, for example " (see Electronic Supplementary Material Fig. Sxxx or Table Sxx ) "
7. the email address of the corresponding author is included on the front page of the paper
8. all your revisions are clearly marked in the electronic version of the manuscript
9. in your cover letter all Editor and Reviewer change requests and your responses are listed in an itemized fashion.
10. As a new feature, online abstracts of published articles may now include an accompanying graphic that will be visible to all readers of the online edition.
Therefore, if your manuscript has an abstract, we ask you to kindly submit an attractive, eye-catching image (maybe from your manuscript or from a presentation of your research) for consideration for the graphical online abstract. This image should be colored and must be submitted in a separate file, preferably in one of the following formats: jpg, tiff, bmp, doc, ppt. Please label the figure ‘Online Abstract Figure’. The file designation is ‘figure’. Please make sure that you are the owner of the copyright or have the permission to reprint the image.

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Response letter:
We also kindly request that you prepare a response to the Editor and reviewers: Please respond in an itemized fashion to each comment made by the Editor and the reviewer(s) and also document any changes you made to the original manuscript. Kindly copy this response into the space provided. In order to expedite the editorial processing of the revised manuscript, please be as specific as possible.

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Prof. J.L. Gómez-Ariza
Universidad de Huelva
Departamento de Química y Ciencia de los Materiales
Facultad de Ciencias Experimentales
Campus de El Carmen
21007-Huelva
Spain
TF:  +34 959 219968
FAX:  +34 959 219942