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A simplified method for inorganic selenium and selenoaminoacids speciation based on HPLC–TR–HG–AFS

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ABSTRACT

A simplified speciation method for the determination of selenite, selenate and three selenoaminoacids (selenocystine, selenomethylselenocysteine and selenomethionine) has being developed, based on the coupling of high performance liquid chromatography (HPLC), thermoreduction (TR), hydride generation (HG) and atomic fluorescence spectrometry (AFS). Most of the existing methods based on AFS detection employ a two step procedure to reduce selenate to selenite before HG: (i) Ultraviolet radiation followed by (ii) heating, to produce volatile hydrides of the selenium compounds. The proposed simplified method HPLC–TR–HG–AFS does not require ultraviolet radiation. Instead, KBr dissolved in a HCl solution is added during the heating step (thermoreduction), resulting in an effective hydride generation of the selenium species. Different variables (temperature, HCl and $NaBH_4$ concentrations) have been optimized, using both univariant and multivariant experimental designs. The proposed method is therefore less complex and allows limits of detection, reproducibility and repeatability values similar or better than the existing AFS detection methods described in the literature. A Certified Reference Material (SELM-1 with certified selenomethionine content) and a Se-enriched algae sample have been successfully analyzed with the proposed method. The results were also compared to an alternative technique (GC–MS) that provided similar results.

1. Introduction

Selenium is an essential element for higher organisms, although the range between deficiency and toxicity is narrow [1,2]. Selenium is found in biological samples in form of non-volatile inorganic selenium (selenite and selenate) and several organic selenium species (e.g., selenoaminoacids). In order to better understand the metabolism of selenium in organisms, analytical methods for the determination of selenium compounds in biological tissues must be readily available [3–5]. Therefore, speciation of selenium is of great importance in this respect, as this type of analysis gives information regarding the individual species. Speciation analysis allows to distinguish between non-covalently bound inorganic selenium, organic selenium species that are the result of the replacement of S by Se during supplementation (as it happens in selenomethionine), and other selenoaminoacids that participate in the genetically encoded incorporation of selenium into true selenoproteins [6–9].

Most methods for the speciation analysis of non-volatile selenium compounds are based on a separation technique, such as gas chromatography (GC) or high performance liquid chromatography (HPLC), which is coupled on-line to a suitable elemental or mass-selective detector. Certain approaches require the conversion of the analyte species into a form that is suitable for detection. If GC is the separation technique of choice, all selenoaminoacids must be derivatized (e.g., with chloroformates or cyanogen bromide) to volatile compounds prior to their detection either by mass spectrometry (MS) or inductively coupled plasma–mass spectrometry (ICP–MS) [10–14].

Liquid chromatography has the advantage that it can be employed both for the separation of inorganic selenium (selenite and selenate) and selenoaminoacids. The separation of the selenium species can be accomplished by using reversed-phase columns in combination with an ion-pairing agent [15–18], size-exclusion [19] or ion exchange columns [20–21]. HPLC has been coupled to MS [22] and ICP–MS [8,9,21,23]. Owing to its inherent selectivity and sensitivity, atomic fluorescence spectrometry (AFS), combined with hydride generation (HG), has gained in recent years a wide use as an element-specific detector [24].

A critical step in AFS is the necessity to convert the selenium compounds into Se(IV), in order to obtain afterwards the

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corresponding volatile hydride (SeH_2), a feature that is considered in detail in the existing literature. The early published approaches for this involve the use of either microwave radiation with a redox reagent (concentrated HBr/$KBrO_4$) [24] after the chromatographic separation to reduce Se(VI) and the selenoaminoacids, followed by HG and AFS detection. UV radiation [26] has also been employed as intermediate step to convert some selenoaminoacids into Se(IV). However, using only UV radiation as derivatization step does not allow the reduction of Se(VI) to Se(IV) [23]. Some authors have point in this sense that UV radiation has to be employed in combination with a reducing agent (e.g., potassium iodide) [27].

Nowadays, some authors have employed a commercially available device (Speciation Heated Coil) consisting of two Teflon reactions coils (a long one wrapped around a UV lamp, and a second short one wrapped around a heating block), in combination with concentrated HCl [18,28–31] for derivatization before hydride generation. Under these conditions, Se(VI) is effectively reduced to Se(IV), although the long reaction coils results in broaden peaks with some overlapping. Also, the need of a long resident time of the selenium species in the reaction coils requires low flow rates of the HPLC pump, ca. 0.5 ml min^{-1}, resulting in long retention times of ca. 35 min.

The present study considers the development of a simplified selenium speciation method (HPLC–TR–HG–AFS) that does not require the use of UV radiation for the decomposition of the selenoaminoacids and the reduction of Se(VI) to Se(IV). Instead, we propose a thermoreduction (TR) step before the hydride generation step, based on use of the short heated reaction coil of the Speciation Heated Coil device, and in the presence of KBr dissolved in concentrated HCl. This simplified method eliminates the use of dangerous UV radiation, reduces the length of the reaction coils, and allows faster flow rates of the chromatographic mobile phase. This results in shorter retention times in comparison with those methods described in the literature. The optimization of the experimental variables (temperature, HCl and $NaBH_4$ concentrations) was performed both by a univariant approach and multivariant experimental design. The proposed method was validated using a Certified Reference Material (SELM-1) and comparing the results of the certified value of the selenomethionine content with other analytical technique (GC–MS). This method has also been applied to an algae sample enriched with selenium.

2. Experimental

2.1. Reagents

All reagents were of analytical grade. Solutions were prepared with ultra pure Milli-Q water obtained from an Elix Advantage System (Millipore). The selenium compounds that were used in this study were: sodium selenite, sodium selenate, selenomethionine (SeMet), selenocystine (SeCyst) (Aldrich) and selenomethylselenocysteine (SeMetSeCys) (Flucka). Stock solutions of 1000 mg Se l^{-1} were prepared for selenite and selenate, and 500 mg Se l^{-1} (in 0.1 M HCl) for the three selenoaminoacids, and stored at 4 °C. Working solutions were prepared daily. The mobile phase for liquid chromatography was prepared with K_2HPO_4 and KH_2PO_4 (Merck). HCl 37% (v/v) (Albus) and $NaBH_4$ (Ridel de Hean) were employed for hydride generation. Protease and lipase for enzymatic hydrolysis of the samples, and KBr for thermoreduction were purchased from Sigma-Aldrich. Certified Reference Material SELM-1 (National Research Council of Canada), corresponding to yeast enriched with selenium, was employed for quality control.

2.2. Instrumentation

A schematic diagram of the instrumental coupling HPLC–TR–HG–AFS employed for selenium speciation is depicted in Fig. 1. The operating conditions are summarized in Table 1. The HPLC consisted of a Jasco PU-2080 Plus quaternary pump equipped with a Rheodyne 7125 injector and a 200 µl loop for sample introduction. The isocratic separation of the selenium compounds was accomplished by means of a strong anion exchange column (PRP-X100, Hamilton). The mobile phase employed for chromatographic separation was 80 mM potassium phosphate solution at pH 6.

The Speciation Heated Coil (PS Analytical) employed for thermoreduction allows performing two types of sample treatment, just changing the position of a two-way switching valve. In the first position of the valve, labelled as "UV+heating", the samples passes first through a 9 m long Teflon coil placed around a 9 W ultraviolet radiation source, and then through a 3 m Teflon tube wrapped around a heating block (maximum heating temperature of 200 °C). If the valve is switched to the second position, labelled as "Heating only", the sample passed only through the 3 m Teflon tube wrapped around the heating block. The internal diameter of the tubing is 0.5 mm. In this work, thermoreduction of the selenium compounds was achieved after chromatographic separation, with the valve of the Speciation Heated Coil placed in the "Heating only" position, at a temperature of 150 °C. The outlet of the chromatographic column was mixed with a reducing solution (5% KBr in 6 M HCl) and pumped into the Speciation Heated Coil with a peristaltic pump of the AFS instrument.

HG-AFS was performed employing an atomic fluorescence spectrometer (Millenium Excalibur, PS Analytical), equipped with two peristaltic pump, a glass gas-liquid separator, and a selenium boosted discharge hollow cathode lamp (Photron). Argon was added at the gas-liquid separator to carry the hydrides to the AFS detector. The flame of the detector was sustained by the hydrogen

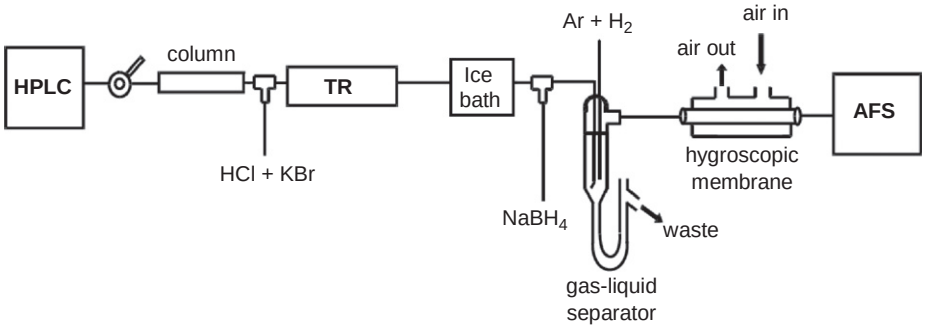

Fig. 1. Scheme of the instrumental coupling for selenium speciation based on high performance liquid chromatography–thermoreduction–hydride generation–atomic fluorescence spectrometry (HPLC–TR–HG–AFS).

Table 1
HPLC–TR–HG–AFS optimized conditions.

HPLC	
Column	Hamilton PRP × 100 (250 mm × 4.1 mm × 10 μm)
Mobile phase	80 mM KH_2PO_4/K_2HPO_4 buffer, pH 6.0
Flow rate	1.0 ml min^{-1}
Injection volume	200 μl
Thermoreduction (TR)	
Heating block temperature	150 °C
Reaction coil	3 m, i.d. 0.5 mm
Reductant	5% (w/v) KBr in 6 M HCl
Flow rate	1.0 ml min^{-1}
Hydride generation (HG)	
Reductant	1.2% (w/v) $NaBH_4$ in 0.4% (w/v) NaOH
Reductant flow rate	1.0 ml min^{-1}
Purging Ar gas flow rate	400 ml min^{-1}
Counter-current air flow rate	2 l min^{-1}
Atomic fluorescence spectrometry (AFS)	
Radiation source	Selenium super lamp (Photron)
Primary and boosted currents	25 mA (both)
Gain setting	100
Detection wavelengths	190–210 nm
H_2 flow rate	50 ml min^{-1}

gas generated from the decomposition of the excess of borohydride. Also, a 60 ml min^{-1} H_2 flow was added at the gas–liquid separator to help to support the AFS flame, as it has been previously described for speciation analysis with AFS detection [32].

For GC–MS analysis of selenomethionine, an Agilent 6890N Gas Chromatograph fitted with a HP-5MS fused silica column and a 5973 Mass Spectrometry detector was employed. The GC–MS was coupled to a Gerstel TDS 2 thermodesorption system equipped with an autosampler and a programmable temperature vaporization inlet. The temperature program of the GC and the operation conditions of the MS have been described elsewhere [33], as well as the derivatization of the analytes with chloroformate prior to the GC–MS analysis.

2.3. Algae sample enrichment with Se(IV)

For the selenium enrichment experiment, 1.2 l of a selenite solution was added to a 2 l Erlenmeyer flask, and inoculated with 300 ml of a stock algae culture (*Isochrysis galbana* clon T-Iso, 2×10^6 algae cell ml^{-1}) in the log growth phase. The concentration of selenite in the 1.5 l solution was 500 μg Se l^{-1}. The solution was cultured for 8 days under continuous illumination at 20.0 °C ± 1.0 °C. Afterwards, the algae were separated by centrifugation (15 min at 15,000 rpm), frozen and freeze-dried. The algae were stored at −20 °C until analysis.

2.4. Enzymatic hydrolysis

The biological samples considered in this study (an algae sample and Certified Reference Material SELM-1) underwent an enzymatic hydrolysis treatment to extract the selenium species prior to analysis. 0.05 g of freeze-dried sample were mixed with 15 mg of protease XIV-lipase (2:1) and 3 ml of water, and sonicated during 2 min at 50 w. After enzymatic hydrolysis, the sample was centrifuged for 10 min at 5000 rpm [33,34]. The supernatant was separated with a Pasteur pipette, weighted and analysed for selenium speciation.

2.5. Algae sample spiking procedure

The algae sample was spiked with the selenium species to evaluate the effect of enzymatic hydrolysis on the extraction efficiency. Sample spiking was performed before enzymatic hydrolysis. In each case, 0.05 g of sample was spiked with 0.3 μg (as Se) of SeCyst, SeMetSeCys, Se(IV) and Se(VI), and 0.6 μg (as Se) of SeMet. The volume of the spiking solution was 50 μl, containing each selenium species at a concentration of 6 mg Se l^{-1}, with a double concentration for SeMet (12 mg Se l^{-1}). A blank of the enzymatic hydrolysis was spiked following the same procedure.

2.6. Multivariant experimental design

The optimization of the experimental variables (HCl and $NaBH_4$ concentrations, and thermoreduction temperature) was performed by a Box–Behnken experimental design (CC Statistica, StatSoft Inc). The number of experiments (N) is defined by the equation $N=2k(k-1)+c_p$, where k represents the number of factors (the three experimental variables) and c_p is the number of replicates of the central point. The three experimental variables were evaluated at three levels. Therefore, this design involved 15 experiments, which were performed in random order.

3. Results and discussion

A classical approach based on univariant optimization was first considered for the variables involved in the HPLC–TR–HG–AFS coupling. The results were compared afterwards with those of an experimental design. In both cases, the experimental variables considered were the two ones involved in the hydride generation step (HCl and $NaBH_4$ concentration), the temperature of the thermoreduction step, and the addition of KBr to promote the reduction of Se(VI) to Se(IV). The concentration of KBr considered was always 5% (w/v), the maximum that can be dissolved in the HCl solution. Experiments with UV or thermoreduction were also considered, in order to compare results with the current methodology described in the literature.

3.1. Derivatization with HCl and $NaBH_4$

Experiments were performed injecting the analytes separately, and removing the HPLC column from the system, in order to obtain the best signal for each selenium compound when changing the value of each experimental variable while maintaining the others fixed. Individual solutions of each species (20 μg Se l^{-1}) were injected by triplicate and the peak areas were recorded.

Initially, the hydride generation step was evaluated, adding HCl 6 M and 1.5% $NaBH_4$. The results showed that under these conditions (without any additional derivatization step), only Se(IV) was effectively converted into the volatile hydride SeH_2. Small signals were obtained for the selenoaminoacids and selenate. Under these conditions, the addition of KBr to the HCl solution did not improved the signal of the analytes. These results are in accordance to the published experiments, indicating that an additional derivatization step is needed to obtain signal for those selenium analytes by AFS [28].

3.2. Derivatization with HCl, $NaBH_4$ and UV

A similar experience was then performed including an on-line ultraviolet derivatization step before hydride generation, as it has been described in the literature [25], using just the 9 m long Teflon loop wrapped around a low pressure Hg lamp of the

Speciation Heated Coil. The results in Fig. 2 correspond to experiments with and without addition of KBr to the 6 M HCl solution. It can be noticed that the use of UV allows to obtain signals for the three selenoaminoacids, always lower than the one of Se(IV). However, almost no signal was recorded for Se(VI). This pattern was not improved by addition of KBr. These results agreed with existing published articles, indicating that UV has little capacity to reduce selenate to selenite [28].

3.3. Derivatization with HCl, $NaBH_4$ and thermoreduction

An alternative new method that does not require the use of UV radiation was considered, using the Speciation Heated Coil with the valve in the "Heating only" position (see Section 2.2). Fig. 3 shows the results obtained at different heating temperatures: 90, 120 and 150 °C. No KBr was added to the HCl solution in this experiment. The increase of the temperature resulted in a small increment of the signal for the selenoaminoacids, but Se(VI) gave almost no signal, regardless the temperature (Fig. 3). The addition of KBr produced successful results, in combination with high temperature (Fig. 4). Se(IV), SeCyst and SeMetSeCyst gave signals of the same order of magnitude at 150 °C. In this case, similar signals were obtained for Se(VI) and SeMet.

Although the temperature of the heating device could be increased up to 200 °C, no higher temperature than 150 °C was considered at this stage, because of the great noise of the base line that difficulties the peak integration. In order to improve the signal quality, a cooling step was added before the hydride generation, as it has been considered in the literature by some authors [18]. It consisted of a 1 m Teflon coil (i.d. 0.5 mm) placed into an ice bath. This approach had several positive consequences: it reduced to one third the noise of the baseline, but also improved the net signal and the reproducibility of all the selenium species, as it can be seen in the last graph of Fig. 4.

3.4. Selenium speciation with HPLC–TR–HG–AFS and experimental design

The speciation of the selenium analytes was accomplished using a strong anion exchange column, under isocratic conditions.

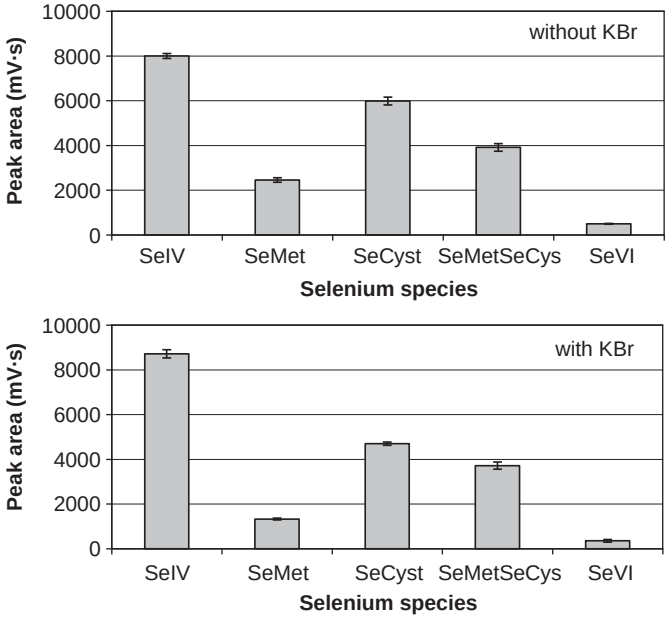

Fig. 2. Peak areas of Se(IV), SeMet, SeCyst, SeMetSeCys and Se(VI) with UV; (a) without KBr and (b) with KBr.

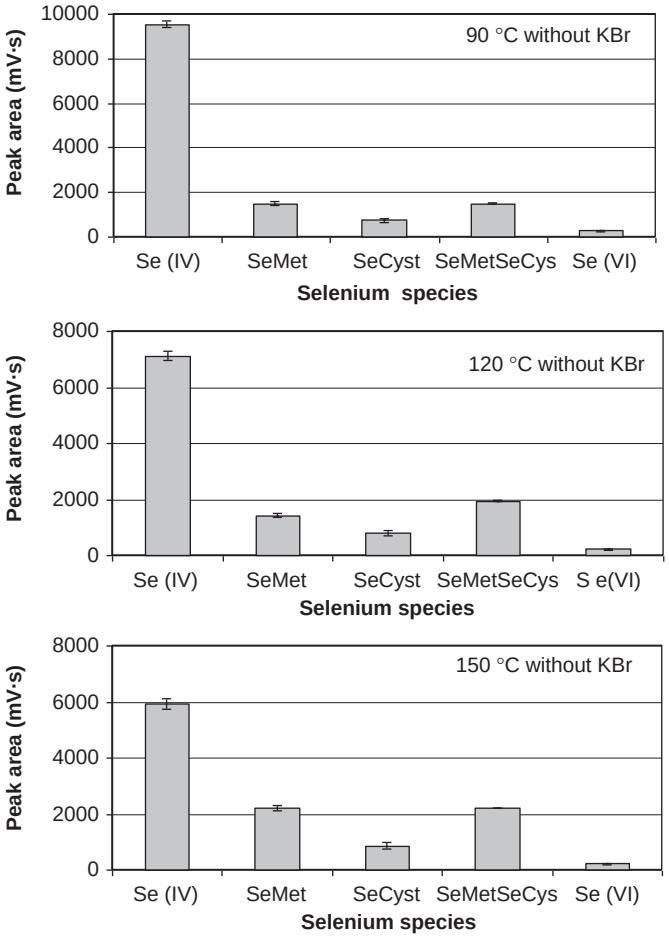

Fig. 3. Peak areas of Se(IV), SeMet, SeCyst, SeMetSeCys and Se(VI) with thermoreduction at different temperatures. No KBr was added to the HCl solution.

We avoided the use of a gradient program for the separation of the selenium species [26,27], as an equilibration time is required after each chromatographic separation. Initially, a separation under isocratic condition was tried with 40 mM phosphate buffer at pH 6, as it has been described in the literature [29]. Other mobile phases employed under isocratic condition, such as citrate [35], were not considered, as the peaks of some compounds (e.g., selenate) are almost twice wider than using phosphate buffer.

The elution order using the 40 mM phosphate buffer was SeCyst, SeMetSeCyst, Se(IV), SeMet and Se(VI). However, although the first four peaks elute in ca. 7 min, Se(VI) eluted after 30 min. Therefore, the phosphate buffer concentration was increased to 80 mM. This reduced significantly the retention time of Se(VI) to ca.18 min, without any significant change in the resolution of the other four peaks.

Some experiments were performed with and without the UV coil (in addition to the TR step) to support the idea that better signal are obtained without the UV reactor. The results indicated that the peak widths were about ca. a 10% narrower. Another advantage is the reduction of the elution time of all the analytes in ca. 1 min. Also, it improves the chromatographic resolution (Rs, defined as two times the difference of the retention times divided by the sum of the peaks width) between SeMet and Se(VI) from Rs=7.0 (with the UV coil) to Rs=6.0 (without the UV coil).

A Box–Behnken experimental design was also considered for the optimization of the variables of the HPLC–TR–HG–AFS coupling. Pareto Charts indicated that the signal of SeCyst has a strong correlation with the three considered variables: temperature, $NaBH_4$ and HCl concentrations. The signal of SeMetSeCys

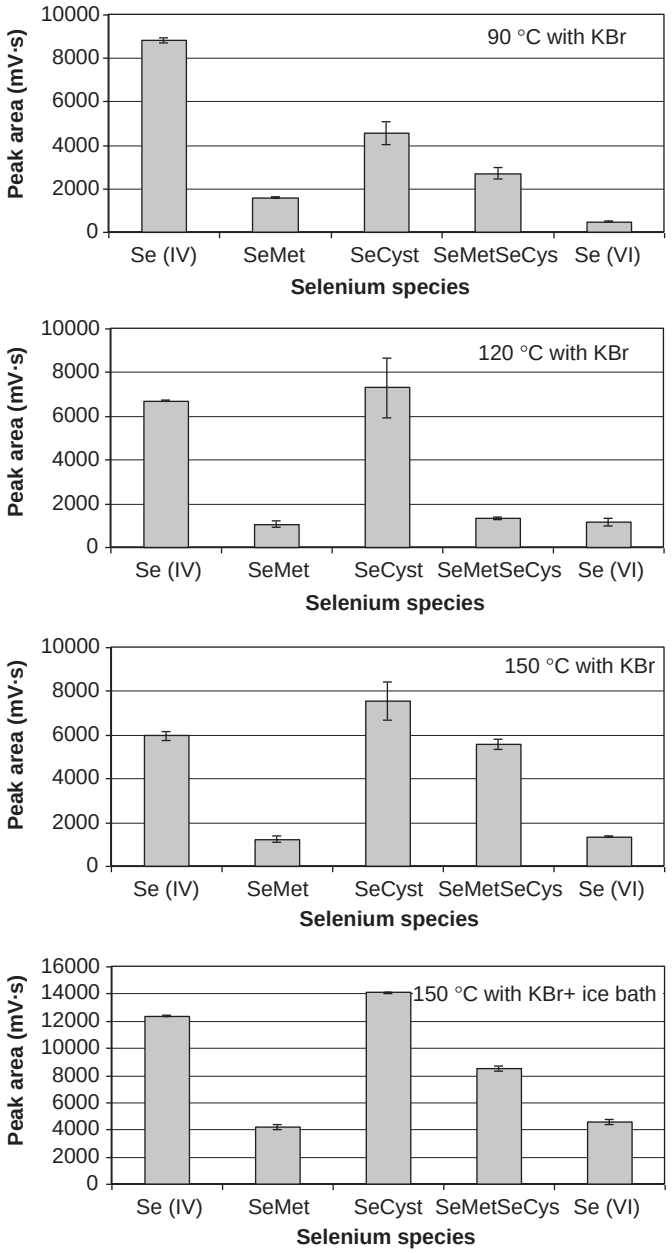

Fig. 4. Peak areas of Se(IV), SeMet, SeCyst, SeMetSeCys and Se(VI) with thermoreduction at different temperatures. KBr was added to the HCl solution. Results of the last graph include an ice bath prior to hydride generation.

depended on $NaBH_4$ and HCl concentrations, and the signal of Se(IV) depended on HCl concentration and temperature. For Se(VI), only HCl concentration showed a significant correlation. SeMet signal depended only on the temperature.

To determine the optimal level of variables, three-dimension surface plots were constructed. Fig. 5a shows the response surfaces for Se(VI), considering temperature and HCl concentration. Fig. 5b shows the response surface for SeCyst, considering $NaBH_4$ and HCl concentrations. The results obtained by the experimental design agreed with the previous univariant optimization, indicating that high HCl concentration (HCl 6 M), a medium $NaBH_4$ concentration of 1.2% (w/v), and a high temperature of 150 °C represented the best values for the experimental variables. A chromatogram of the selenium species under these conditions is depicted in Fig. 6.

Calibration curves were plotted for each compound, with concentrations 5–40 μg Se l^{-1} for SeCyst and Se(IV), 5–80 μg Se l^{-1} for SeMetSeCyst and Se(VI), and 10–80 μg Se l^{-1} for SeMet. Linear regression with R^2 values between 0.998 and 1.000 were obtained.

Limits of detection (LODs), reproducibility and repeatability were calculated for the proposed method based on thermoreduction (HPLC–TR–HG–AFS), and the values compared to the existing methods based on UV radiation (HPLC–UV–HG–AFS) are summarized in Table 2. LODs were calculated in two ways. In the first place, LODs were calculated with the data generated in the calibration graphs. LODs were defined as three times the estimated error ($S_{x/y}$), divided by the slope of the calibration graphs [36]. Second, LODs were estimated choosing the lowest concentration that can be measured with a signal to noise ratio of 3,

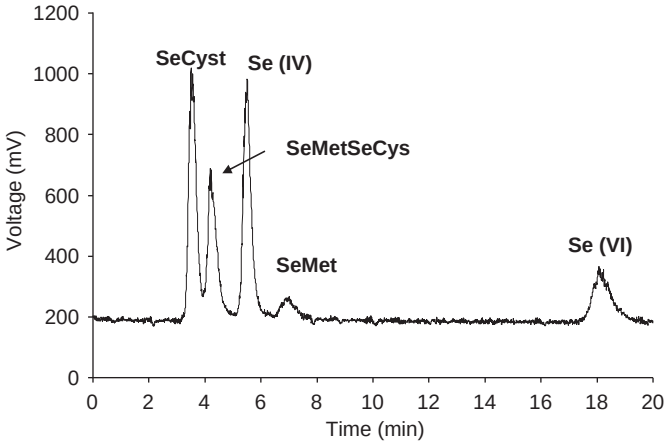

Fig. 6. HPLC–HG–TR–AFS chromatogram obtained with the optimized working condition for a standard solution containing each compound at a concentration of 40 μg Se l^{-1}.

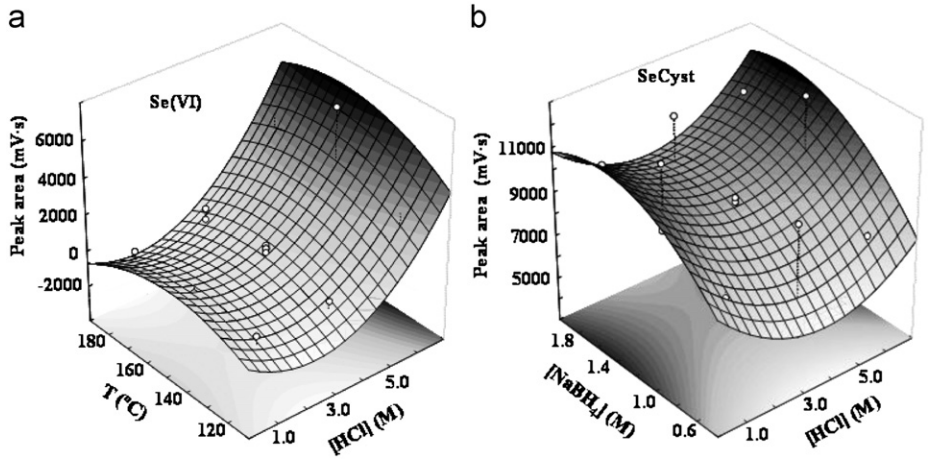

Fig. 5. Response surface plot showing the predicted values for the influence of: (a) $NaBH_4$ and HCl concentrations on the peak area of Se(VI), and (b) HCl concentration and temperature on the peak area of SeCyst.

Table 2
Limits of detection (LOD), reproducibility and repeatibility for selenium species obtained by the proposed method HPLC–TR–HG–AFS, compared to existing methods based on HPLC–UV–HG–AFS.

LOD ($\mu g\ Se\ l^{-1}$)						
Method of analysis	Se(IV)	Se(VI)	SeCyst	SeMetSeCys	SeMet	Ref.
HPLC–TR–HG–AFS[a]	1.0	3.3	0.4	2.8	4.6	This work
HPLC–TR–HG–AFS[b]	0.4	1.1	0.2	0.5	2.6	This work
HPLC–UV–HG–AFS	2.3	5.7	4.3	6.0	9.0	[29]
HPLC–UV–HG–AFS	1	–	5	3	5	[26]
HPLC–UV–HG–AFS	0.6	–	0.5	1.2	7.2	[40]
HPLC–UV–HG–AFS	0.6	14.5	0.9	–	5.9	[25]
Reproducibility (RSD, %)						
	Se(IV)	Se(VI)	SeCyst	SeMetSeCys	SeMet	Ref.
HPLC–TR–HG–AFS[c]	2.2	4.3	2.0	2.6	4.0	This work
HPLC–UV–HG–AFS	3.8	–	3.8	2.0	3.2	[26]
HPLC–UV–HG–AFS	1.5	–	0.5	1.8	5.6	[40]
HPLC–UV–HG–AFS	3.3	3.6	1.1	–	4.9	[25]
Repeatability (RSD, %)						
	Se(IV)	Se(VI)	SeCyst	SeMetSeCys	SeMet	Ref.
HPLC–TR–HG–AFS[d]	4.2	6.0	16.4	9.4	18.7	This work
HPLC–UV–HG–AFS	8	11	14	15	14	[29]

[a] LOD calculated as 3 times $s_{y/x}$ of the calibration graph, according to [36].
[b] LOD calculated as signal to noise ratio of 3, according to [37].
[c] Reproducibility calculated after three consecutive injections of a 10 $\mu g\ Se\ l^{-1}$ containing each seleniun species.
[d] Repeatability calculated after four non-consecutive injections in a 10 days period.

as defined by the ISO Norm 12530:1997 (*E*) [37]. In this second case, LODs were calculated from ten replicate analyses of an enzymatic hydrolysis blank.

LODs calculated considering the signal to noise ratio of the baseline were always lower than those obtained using the data of the calibration graphs, the latter found more in accordance with experimental data. In this work, the LODs obtained were similar or better than existing values, with the lowest one for SeCyst and the highest for SeMet. It is noteworthy that these LODs obtained with AFS detection are in the similar range than those reported by some authors employing ICP–MS detection (0.5–2 $\mu g\ Se\ kg^{-1}$) in [7], 0.7 $\mu g\ l^{-1}$ [38], although lower values can be obtained using ICP–MS equipped with a collision/reaction cell [39]. The reproducibility, calculated after three consecutive injections of a standard, ranged between 2.0–4.0%RSD, also similar to published values. Reproducibility was calculated after four analysis performed during ten non-consecutive days. The reproducibility of the results obtained by thermoreduction was also better or similar for all the selenium species compared to previous reported methods).

3.5. Method validation

A certified reference material, SELM-1 (Se-enriched yeast), with a certified amount of SeMet was analyzed with the proposed HPLC–TR–HG–AFS method, and the results were compared with an alternative method of analysis, gas chromatography-mass spectrometry (GC–MS). The sample was subjected to an enzymatic hydrolysis treatment before analysis. The results obtained by HPLC–TR–HG–AFS for SeMet (3283 ± 216 mg SeMet kg^{-1}) indicated that there were not significant differences compared to the certified value (3389 ± 173 mg SeMet kg^{-1}), the only selenium specie certified in this material. Similar results were obtained by GC–MS (3405 ± 187 mg SeMet kg^{-1}).

Also, the enzymatic hydrolysis of an algae sample (*Isochrysis galbana*) exposed to Se(IV) was analyzed with the proposed method. As selenite underwent biotransformation within the algae, other organic species were found in addition to Se(IV).

A spiking experiment was performed to evaluate the effect of enzymatic hydrolysis on the extraction efficiency of the selenium species of the algae sample. The sample was spiked before enzymatic hydrolysis, as described in the Experimental section. Also, a blank consisting of water spiked with the selenium species was also submitted to enzymatic hydrolysis. The recoveries of both experiments are summarised in Table 3. Good recoveries were obtained for all selenium species (89–101%) in the blank. However, spiking the sample before enzymatic hydrolysis resulted in the good recoveries for all species (90–100%), with the exception of Se(IV), with resulted in a recovery of 26%. This strong matrix effect obligated to its quantification by standard addition instead of external calibration.

Results in Table 4 show the selenium species concentration found in the algae sample, calculated both by external calibration and standard addition. SeMet was the main selenium specie found (3.4 mg Se kg^{-1}), followed by SeCyst (1.8 mg Se kg^{-1}) and SeMetSeCys (1.0 mg Se kg^{-1}). There were no significant difference between these values and those obtained by standard addition. The concentration of SeMet was also calculated by GC–MS, with a similar value of 3.6 mg Se kg^{-1}. The exception corresponded to Se(IV), that was not detected by external calibration, but found at a concentration of 0.2 mg Se kg^{-1} by standard addition, thus confirming the matrix effect on its quantification. Se(VI) was not detected in the algae sample.

Table 3
Percentage of recovery of selenium species of a blank and an algae sample spiked with selenium species before enzymatic hydrolysis. Results correspond to mean ± standard deviation ($n=2$). Analysis performed by HPLC–TR–HG–AFS.

	Se(IV)	Se(VI)	SeCyst	SeMetSeCyst	SeMet
Blank (recovery, %)	98 ± 1	96 ± 2	89 ± 6	101 ± 2	101 ± 1
Algae sample (recovery, %)	26 ± 1	94 ± 2	90 ± 3	100 ± 1	95 ± 1

Table 4
Concentration of selenium species (mg Se kg^{-1}) in a Se-enriched algae sample, determined by the proposed method (HPLC–TR–HG–AFS). Results correspond to mean ± standard deviation ($n=2$). SeMet determined also by GC–MS for comparison.

Sample	Method of analysis	Se(IV)	Se(VI)	SeCyst	SeMetSeCys	SeMet
Algae	HPLC–TR–HG–AFS	n.d.	n.d.	1.8 ± 0.4[a]	1.0 ± 0.2[a]	3.4 ± 0.4[a]
		0.2 ± 0.1[b]	n.d.	1.6 ± 0.4[b]	0.9 ± 0.3[b]	3.2 ± 0.4[b]
	GC–MS					3.6 ± 0.2

n.d. not detected.
[a] Calculated by external calibration.
[b] Calculated by standard addition.

4. Conclusions

The proposed method simplifies the existing methodology for selenium speciation based on AFS detection, and allows the determination of inorganic species (selenite and selenate) and several selenoaminoacids. With this approach, the UV step before heating, described in the scientific literature is not necessary. Instead, thermoreduction of Se(VI) to Se(IV) is achieved adding KBr to the HCl solution employed for hydride generation, heating at 150 °C. The limits of detection, reproducibility and repeatability values obtained by the proposed HPLC–TR–HG–AFS method are similar or better than those provided with UV.

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