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Long term stability of organic selenium species in aqueous solutions

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Abstract Long term stability of organic selenium compounds (selenocystine, selenomethionine, trimethylselenonium ion) has been studied over a one year period for 2 analyte concentrations: 25 and 150 µg/L Se, at pH 4.5 in the dark, under different storage conditions: temperature of –20°C, 4°C, 20°C, 40°C; in Pyrex, Teflon, or polyethylene containers; in an aqueous matrix or in the presence of a chromatographic counter ion (pentyl sulfonate at 10^{-4} mol/L concentration). Light effects have also been tested. The stability of the selenium species was monitored by HPLC-ICP/MS.

Storage conditions can drastically alter the stability of organic selenium species. Organoselenium compounds were shown to be stable in the dark over a one year period in an aqueous matrix at pH 4.5 in Pyrex containers at both 4°C and 20°C. Pyrex vials exposed to natural sunlight at room temperature resulted in a steady decrease of the selenoamino acid concentration. Teflon containers caused losses of less than 25% at both 4° C and 20° C in the dark. However, polyethylene vials presented, at all temperatures tested, a rapid decrease of the $TMSe^+$ concentration. The stability of the Se species studied did not show significant differences between 4° C and 20° C in any container material used. Storage of solutions at 40° C led to slight differences between the Pyrex and Teflon containers. However, polyethylene presented a drastic decrease of the three species over time at this higher temperature. Solutions frozen at –20° C in polyethylene vials did not stabilize the $TMSe^+$ signal. Finally, concentrations and matrices of the samples did not significantly affect the stability of the species.

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Introduction

Selenium can be highly toxic for animals and human beings at high concentration levels, but it provides beneficial effects at appropriate concentrations [1, 2] depending on the chemical form in which it is present. As a result, a wide variety of analytical techniques for speciation has been developed during the last decade.

Analytical quality assurance requires reference samples, e.g., for calibration purposes, intercomparison, or method validation (certified reference materials). A major prerequisite is that samples must remain stable over time. Several studies have, therefore, been conducted by the SM&T programme (formerly BCR) to check the stability of chemical species in solutions [3–6] and certified reference materials [7]. A series of stability studies on model samples of inorganic selenium species has been performed which demonstrated that aqueous solutions were stable for 1 year leading to the production of two certified reference materials (CRMs) [8].

Among organic selenium species, the most frequently quoted in environmental and biological studies are volatile methylated selenium, selenocystine, selenocysteine, selenomethionine, and the trimethylselenonium ion [9]. This paper presents and discusses the long term stability of selenocystine (SeCys), selenomethionine (SeMet), and trimethylselenonium ion ($TMSe^+$) in simple aqueous solutions over a one year period. Selenocysteine was not considered in the present work due to the lack of commercially available calibrants. Determination of selenium species were performed by HPLC-ICP/MS. The general problems likely to be encountered in such a project are: interconversion of species, adsorption on the container walls, thermal degradation, loss by volatilization, photo decomposition, microbiological degradation, and environmental contamination, etc. The main factors which can generate any of the above changes in the samples are [10]: a) analyte chemical formulation, b) analyte concentration, c) physico-chemical conditions of solution storage (pH, presence of complexing or/and oxydizing agents, dis-

solved gases e.g. oxygen, suspended matter load, and the occurrence of micro-organisms), d) storage material properties (chemical composition of the container, cleaning procedures), and e) external factors such as temperature, light, etc.

Following other stability tests applied to inorganic selenium [4, 11], different storage conditions and physico-chemical parameters have been selected: polyethylene, Teflon and Pyrex storage containers; four temperatures (–20° C, 4° C, 20° C, and 40° C); aqueous matrix with and without a complexing agent, two Se concentrations (25 and 150 µg/L); and sunlight.

This study is part of a broader project on Se-speciation initiated by the SM&T Programme in 1992 with regard to general quality assurance. Extensive knowledge of species stability is essential to promote future candidate reference materials.

Experimental

Reagents

Pure water was obtained from a Milli-Q system (Millipore Corp. Bedford USA). The methanol and sodium pentyl sulfonate used as HPLC organic mobile phase were purchased from Prolabo and Sigma, respectively, and were of highest purity grade. Hydrochloric acid used to adjust pH of the solutions and the mobile phase was obtained from Merck (Suprapur grade). Stock standards of selenium were prepared by dissolving the appropriate amount of the solid compound (seleno-DL-cystine and seleno-DL-methionine from Sigma Chemicals 99%) in Milli Q water acidified with HCl to pH = 2 to facilitate dissolution, especially for SeCys. The trimethylselenonium chloride calibrant was obtained from University Complutense of Madrid, where it was synthesized following the procedure of Palmer et al. [12]. Working solutions were prepared daily by dilution of the stock calibrant solutions.

Containers

The storage materials classically used by laboratories [HDPE (high density polyethylene), Teflon PTFE (polytetrafluoroethylene), and Pyrex] were evaluated for the storage stability tests. Before being used the 125 mL containers were flushed and rinsed with tap water, then soaked in 2% RBS-50 detergent solution in hot water for 2 h and thereafter abundantly rinsed in tap water. They were then immersed in a 10% HNO_3 bath for 48 h, rinsed several times with Milli Q water, filled with Milli-Q water, and sealed in plastic bags until use.

Design of the storage experiment

Two different concentrations (25 and 150 µg/L Se) corresponding to Se levels typically found in environmental samples have been tested [13] at four temperature levels (–20°C, 4°C usually considered to represent normal storage conditions, 20°C for room temperature, and 40°C as extreme temperature storage). The effect of the addition of ion-pairing pentyl sulfonate was investigated to evaluate the possible risks of adsorption or species inter-conversion due to its complexing capacity.

pH 4.5 for storage was chosen to compromise between the necessity to study the possible degradation of organo-selenium compounds and the necessity to minimize the development of micro-organisms, and to facilitate chromatographic separation. All different storage conditions selected are summarized in Table 1.

Initial solutions containing the 3 selenium species for storage were made up in 5 L batches for the two different concentrations. The ion-pairing agent (sodium pentyl sulfonate), when used, was at 10^{-4} mol/L. All solutions were adjusted to pH = 4.5 with hydrochloric acid. Sample homogeneity was achieved by stirring the solution with a magnetic agitator for 2 h prior to bottling. Dissolved oxygen was removed by bubbling the stock 5 L solution with He for 2 h as recommended by Cobo et al. [4].

The stock solutions were split into 125 mL polyethylene, Teflon and Pyrex containers and kept in the dark at –20°C, 4°C, 20°C, and 40°C, and in sunlight at 20°C. For the 40°C temperature conditions, the vials were stored in the dark in an oven. The 20°C conditions were provided by a cupboard in a thermostated clean room. The 4°C conditions were obtained by a refrigerator. A deep freeze set at –20°C kept the polyethylene vials frozen. The storage temperature was checked periodically. The storage flasks were filled to the sealing cup to minimize oxygen contamination.

Determination of selenium species were made after 1 day, 15 days, and 1, 2, 3, 6, 8, 10 and 12 months.

A total of six vials were stored at each experimental condition (temperature, container material, matrix, species concentration, sunlight). For each analytical procedure, one to two bottles were randomly opened in each lot and subsampled immediately. The vials were then returned to storage until the next analytical determination. The pH of 4.5 was randomly checked during the whole experiment and was found to be stable over time.

Apparatus

Organic selenium species determinations were performed by HPLC-ICP/MS. The HPLC pump was a Titanium Perkin-Elmer Series 410 Bio. The six-port Rheodyne valve used for the sample injection was also made of titanium. The injection volume of the

Table 1 Experimental design of the stability study on organic selenium compounds

Temperature	Stock material	Matrix	Conc[a] (µg/L Se)	Series[b]	
–20°C	Polyethylene	H_2O, Ion-pairing	25, 150	4	Dark
+4°C	Polyethylene	H_2O, Ion-pairing	25, 150	4	Dark
	Pyrex			4	
	Teflon			4	
+20°C	Polyethylene	H_2O, Ion-pairing	25, 150	4	Dark
	Pyrex			4	
	Teflon			4	
+40°C	Polyethylene	H_2O, Ion-pairing	25, 150	4	Dark
	Pyrex			4	
	Teflon			4	
+20°C	Pyrex	H_2O	25, 150	2	Light

[a] Conc: concentration of the species as Se content.
[b] 6 vials per series
Solutions have been stored at pH = 4.5

sample loop was 100 µL. The chromatographic column selected for separation was a reversed phase Hamilton PRP1. The mobile phase used was a methanol/water mixture (2/98) with 10^{-4} mol/L sodium pentyl sulfonate at pH = 4.5.

The interface between the chromatographic column and the spray chamber of the ICP/MS was a PEEK tube. The nebulization chamber was fitted with the standard cross flow nebulizer and a Ryton spray chamber. A SCIEX Perkin-Elmer ELAN 5000 was used as detector. These analytical conditions have been optimized and are described elsewhere [14].

Analytical procedure

Stock calibrant solutions of each compound were initially prepared separately at 100 mg/L Se. They were kept in the dark at 4°C. Working solutions used for calibration (25 and 150 µg/L Se) were prepared on the day of analysis by dilution of the stock solutions. Calibration curves were performed prior to each set of analyses. Drift of the instrumental conditions was corrected by calibration of the instrument after every 8 samples. All samples of one concentration level were run during the same day.

Statistical evaluation

The effect of the different parameters selected for storage was evaluated by statistical treatment [15]. Stability can be assessed by calculating the coefficient $R_x = X_x/X_{ref}$, where X_x is the mean value of measurements made at the different storage conditions, and X_{ref} is the mean value taken as reference (obtained following the preparation of solutions).

The uncertainty (U_x) was obtained from the RSD for each set of analyses (X_x) according to the following equation:

$$U_x = [(RSD)_x^2 + (RSD)_{ref}^2]^{1/2} R_x/100$$

Table 2 Homogeneity tests of the initial solutions

	25 µg/L Se water	25 µg/L Se ion-pairing	150 µg/L Se water	150 µg/L Se ion-pairing
SeCys	24.1 ± 0.5	23.8 ± 2.0	149.7 ± 16.0	143.4 ± 15.0
SeMet	24.0 ± 1.0	23.6 ± 1.8	144.1 ± 4.0	142.4 ± 17.4
$TMSe^+$	25.0 ± 1.4	24.9 ± 2.0	154.2 ± 5.0	152.8 ± 11.6

Under ideal conditions of stability, R_x should equal 1. However, there are always random variations related to uncertainties in the measurement procedure. The overall stability can be evaluated by plotting R_x versus time. Ideal storage conditions should display values ranging between $R_x + U_x$ and $R_x - U_x$.

Results and discussion

Homogeneity of the samples prior to storage

In order to test the homogeneity of the different sample series three different bottles taken randomly from each series were analyzed to determine X_{ref}. This procedure also allowed direct quality control of the solutions. The mean concentration and the standard deviation of each solution series is given in Table 2. An analysis of variance (F-test) was applied to test the series homogeneity [15], and the experimental F-value found was lower than the theoretical value for a 95% estimated confidence interval implying that the initial series solutions were homogeneous.

A typical chromatogram obtained at the beginning of the storage experiment (t = 0) of one of the initial solutions containing the three organic selenium compounds is presented in Fig. 1.

Effect of the container material

The effect of the container composition (polyethylene, Teflon and Pyrex) was evaluated for different storage temperatures (–20° C, 4° C, 20° C, and 40° C). The polyethylene containers induced severe losses of the trimethylselenonium ion after only two weeks of storage. The losses are illustrated in Fig. 2 for three different temperatures. For 20° C and 40° C (Figs. 2b and 2c), the loss of $TMSe^+$ ranges between 80% and 90% after the first 15 days. At 4° C (Fig. 2a) the $TMSe^+$ decreases regularly with time.

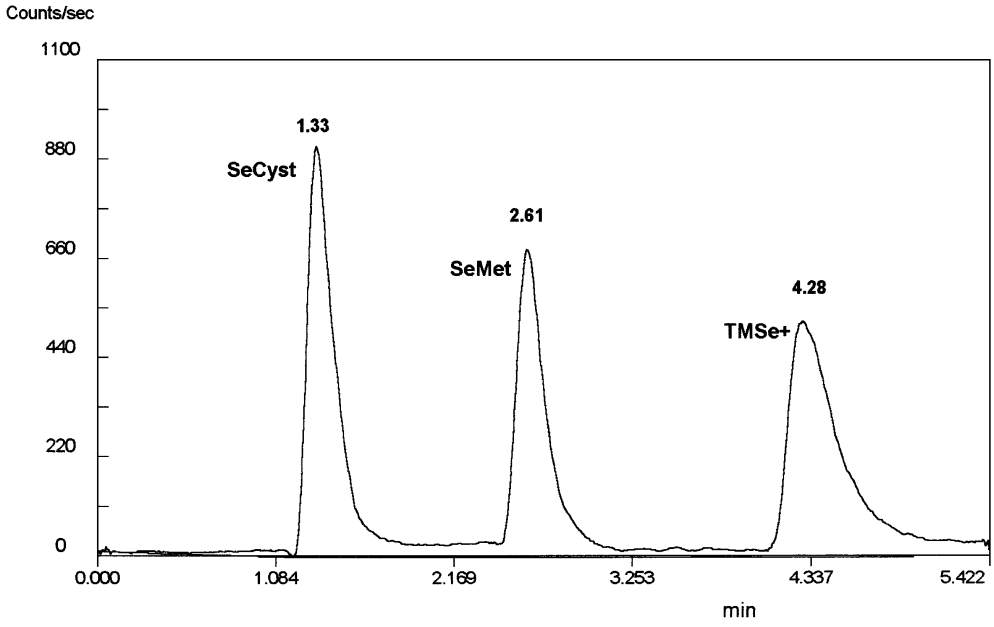

Fig. 1 Chromatogram of the organic Se compounds obtained by HPLC-ICP/MS at the beginning of the stability study (t = 0). Chromatographic conditions: Hamilton PRP1 column; water + methanol (98 + 2) $+10^{-4}$ mol/L sodium pentyl sulfonate; pH = 4.5. Concentration of each species: 25 µg/L Se

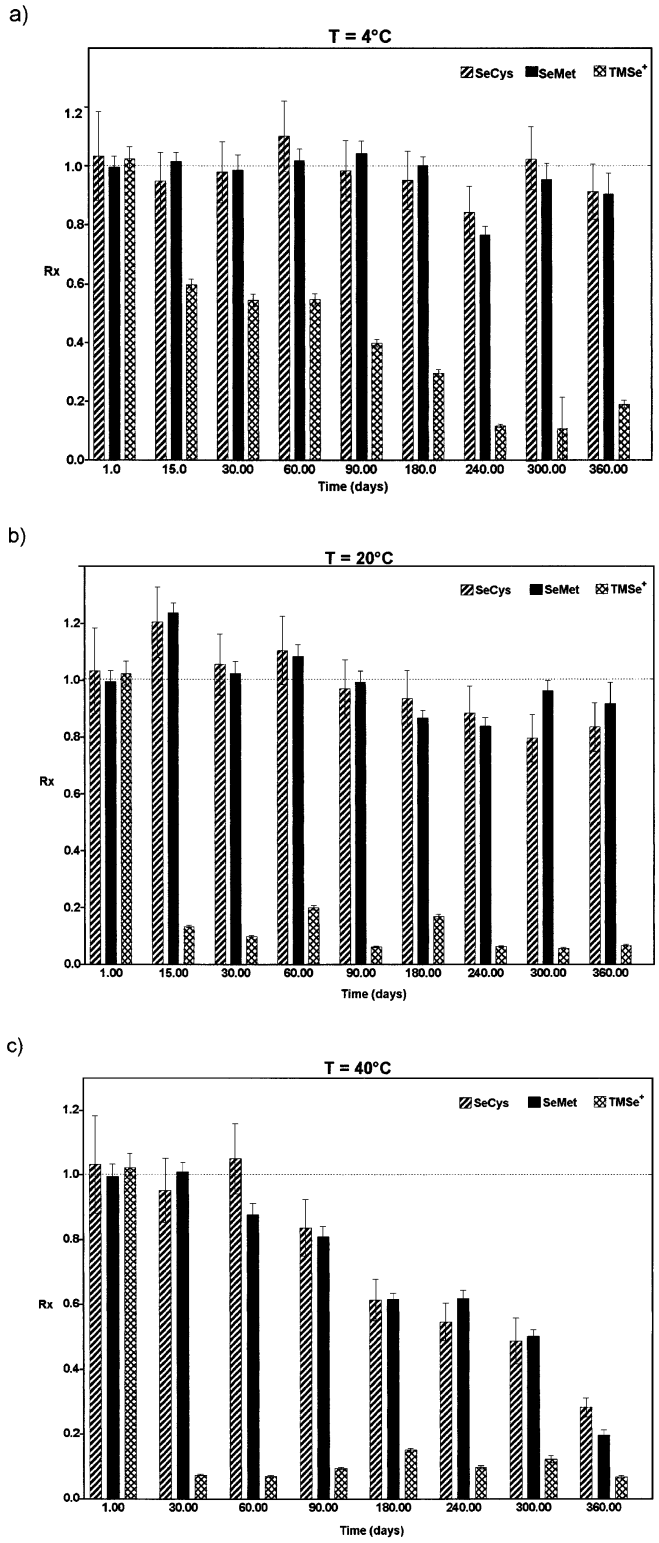

Fig. 2a–c Stability of the organic Se species in polyethylene containers: **a**) 4°C ; **b**) 20°C; **c**) 40°C. Concentration of species 150 µg/L Se; aqueous matrix

This phenomenon can be related to the possible adsorption of $TMSe^+$ on the container walls. The different adsorption rate observed at 4° C compared to 20° C and

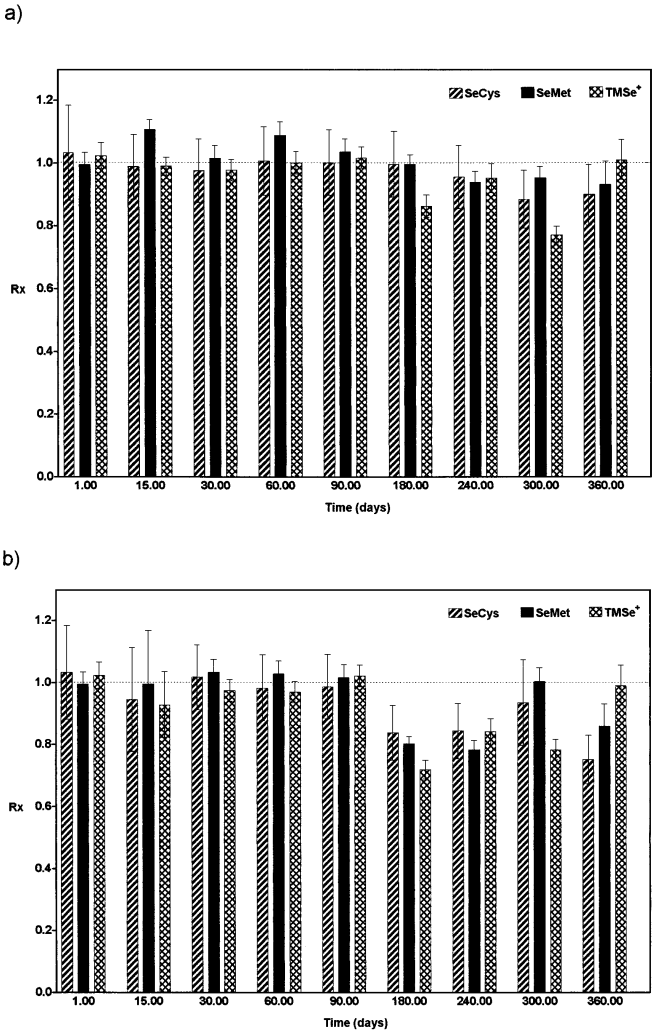

Fig. 3a, b Stability of the organic selenium species at 20°C in **a** Pyrex containers and **b**) Teflon containers. Concentration of the species 150 µg/L Se; aqueous matrix

40° C would suggest a reduction in the adsorption rates of the $TMSe^+$ cations at lower temperatures. This behavior is only apparent in the polyethylene containers.

The hypothesis of a transformation of the species has been discarded because of the absence of unknown peaks in the chromatogram and because HPLC confirmed the loss of $TMSe^+$. Additionally, the stability of SeMet and SeCys over the whole year eliminated the supposition of an interconversion among the species.

The other container materials provided better stability conditions for the 3 organic selenium species. Pyrex appears to be slightly superior to Teflon at 20° C (Fig. 3). Excellent stability is observed over the first 3 months under all conditions. A slight decrease of stability is then detected, being larger for the Teflon containers. The increased variability of response recorded after 6 months is unclear, but could be related to the ageing of the chromatographic column or to a partially reversible adsorptive behavior of the species. In conclusion, Pyrex appears to be the preferred storage material under any circumstances.

Effect of temperature

The effect of temperature is much less pronounced than that of the container material. Independent of the matrix used, no significant differences in the stability for all the species studied were seen at either 4° C and 20° C in Pyrex or Teflon containers (Fig. 3). In addition, Pyrex containers did not cause significant differences for the 3 temperatures investigated (4° C, 20° C, 40° C) or for the 3 selenium species.

Contrary to what is commonly accepted, samples kept frozen at –20°C in the polyethylene vials displayed a rapid loss of $TMSe^+$ similarly to the other temperature conditions (4° C, 20° C) along with a variable and slight decrease of selenoamino acid (Fig. 4). The rapid loss of $TMSe^+$ could be caused by rapid adsorption during the warming up of the sample before the measurements. Adsorption of the selenoamino acid could also occur during the melting of the sample. In neither case was chromatographic evidence of species interconversion found. This result is unexpected, as previous stability studies dealing with inorganic selenium species (SeO_4^{2-} and SeO_3^{2-}) at –20° C in polyethylene vials displayed excellent stability and they were used as reference solutions for the statistical treatment (X_{ref}) [4].

Results obtained at 40° C are very stable with little variation in the Pyrex and Teflon containers in water. Variabilities are slightly higher than those obtained at 20°C. However, at this temperature the most drastic changes of the analyte concentrations were observed in polyethylene (Fig. 2c). In this case a rapid loss of the $TMSe^+$ is recorded within less than 30 days. Both selenoamino acids also show a steady decrease after 30 days. After 12 months, only 20% of SeMet and 30% of SeCys can be recovered. These larger losses were confirmed by monitoring the total Se content.

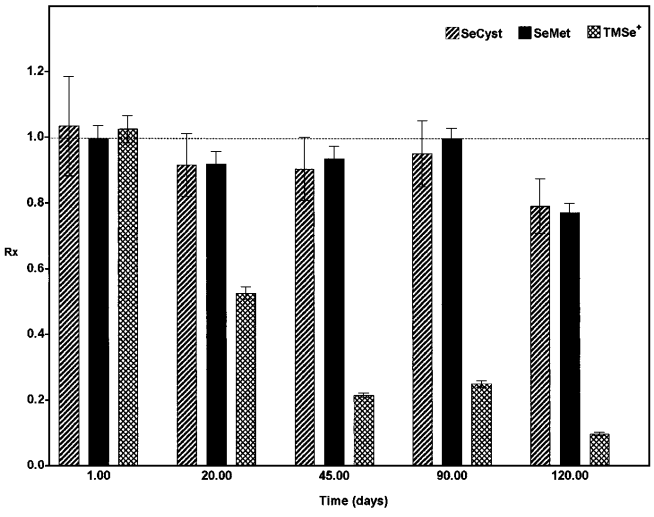

Fig. 4 Stability of organic Se compounds at –20°C in polyethylene containers. Concentration of the species 150 µg/L Se; aqueous matrix

Several hypotheses can explain the changes observed. The first explanation is related to adsorption phenomena on polyethylene walls. Whereas for these temperatures, the results obtained with Pyrex suggest that no surface interaction takes place, the results with polyethylene are very different for all selenium species. The loss of the selenoamino acids could be related to chemical interactions between the analytes and the stabilizers or catalyst agents used for the polymerization of the polyethylene. These agents are classically added to provide a good resistance of the polyethylene chains with respect to light, temperature, and oxidizing agents. These additives can either be metallic or organometallic compounds or metal oxides. They are grafted onto the ends of the polymeric chain and hence are likely to interact (adsorption, degradation) with our analytes. This effect could be enhanced by the modification of the polyethylene surface resulting from our acidic cleaning procedure.

Another explanation is the potential growth of algae or bacteria which is favored at these temperatures (37–40°C). The choice of pH = 4.5 cannot completely rule out the growth of some resistant micro-organisms. This biological activity could then turn the selenoamino acids into volatile compounds, such as dimethylselenium (DMSe) and generate a loss via volatilization which can be confirmed by monitoring the total Se content present in the solution. However, this loss must then be limited to the polyethylene vial series. Polyethylene under these temperature conditions is then supposed to favor bacterial development on the wall of the container.

Effect of light

The effect of light was only studied in two series of solutions, stored at 20°C in Pyrex containers in Milli Q water matrix, at both concentrations (25 and 150 µg/L Se). Two vials of each series were stored under light exposure and were used for the whole experiment. Repeated subsampling led to an increased introduction of oxygen compared to other series tested.

Figure 5 shows significant changes to the chromatographic records. In comparison to Fig. 1, some selenium species eluted in the void volume. These species could possibly be assigned to inorganic selenium species (SeO_3^{2-}, SeO_4^{2-}, SeO_2...) which are not retained under our chromatographic conditions. The appearance of these species can be caused by degradation of the organic selenium species to inorganic or other organic forms. The amino acids were more affected by this degradation compared to $TMSe^+$.

Figure 6 presents the evolution of the concentrations recorded after storage of the solution in the presence of light at 25 µg/L Se. $TMSe^+$ is the least affected with total loss after one year of around 20%. Selenoamino acids present a more pronounced decrease. SeCys concentration diminishes steadily until almost complete disappearance after 12 months. SeMet also showed significant losses of around 60% after 12 months. Several effects may account

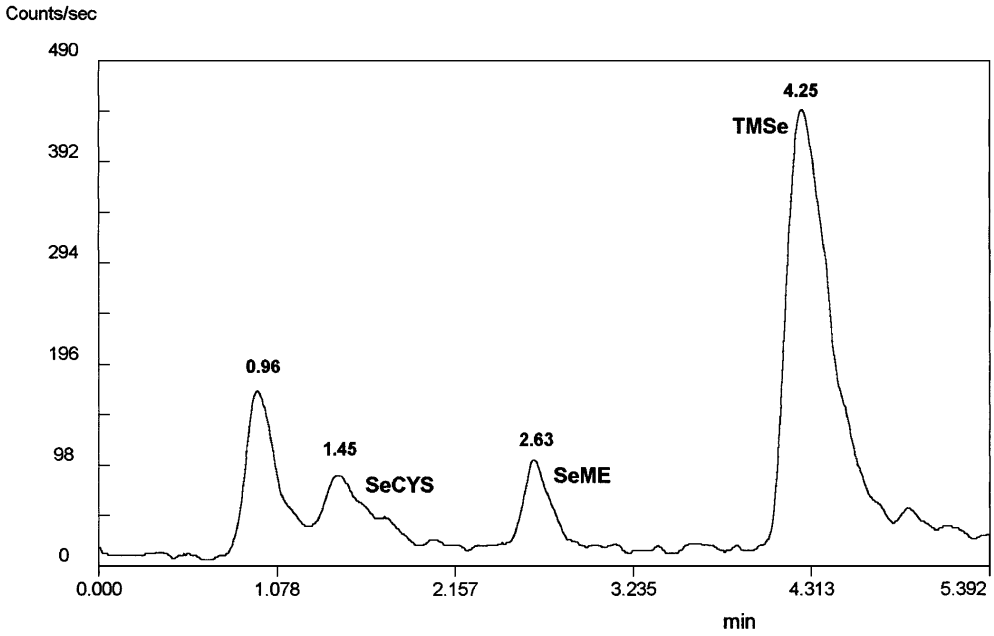

Fig. 5 Chromatogram profile of a sample after 12 months of storage in Pyrex containers at 20°C under sunlight conditions. Concentration of the species 25 µg/L Se; aqueous matrix

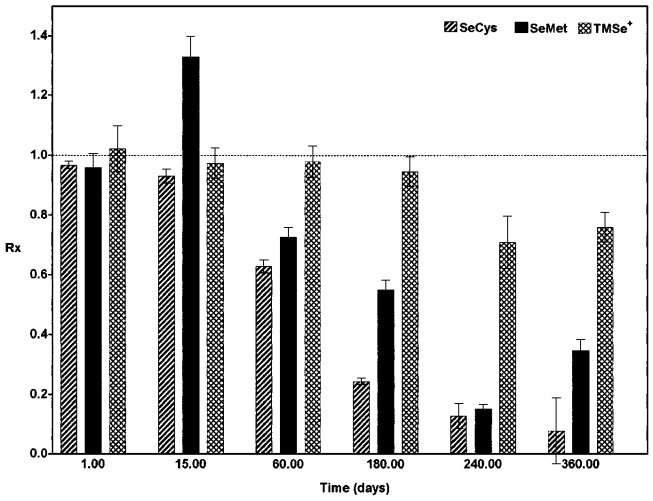

Fig. 6 Stability of the organic Se compounds stored in Pyrex containers at 20°C under sunlight conditions. Concentration of Se species 25 µg/L Se; aqueous matrix

for the degradation. Under light, the presence of oxygen introduced by repeated subsampling could have generated oxidation reactions resulting in photolytic decomposition. The oxidation products remain in solution and are unknown (inorganic ions, other amino acids, or selenoproteins, etc.). This hypothesis is supported by the fact that the total Se content has not decreased in these solutions exposed to light.

Effect of the ion-pairing agent

The effects generated by the addition of sodium pentyl sulfonate to the matrix in large excess to the organoselenium compounds (e.g. 100 times the 150 µg/L Se concentration) was investigated. Samples were stored in this matrix for one year under the same conditions (temperature, container material, analyte concentration) as those prepared in Milli Q water.

The objective of this addition was to evaluate potential stabilization by this agent via weak complexation of the analytes in solution to prevent adsorption losses or species interconversion. However, the same overall stability trends were observed as in the water matrices.

Figure 7 shows the results obtained at 20°C for two materials, Pyrex and Teflon. Results were in agreement with those obtained under the same conditions without addition of the ion-pairing agent to the matrix (Fig. 3). However, a higher RSD was noted, which may be due to less stable analytical conditions induced by the presence of ion-pairing matrices during the overall analytical protocol. In conclusion, the addtion of this agent as stabilizer is not recommended.

Effect of the analyte concentration

The two different concentration levels (25 and 150 µg/L Se) were selected with respect to analytical capabilities of the method used [14]. General results indicate that the concentration levels selected did not affect the overall stability trends.

Overall results are presented in Fig. 8 using the “Box and Whiskers” format for the 2 concentrations. The main differences between the two plots are related to SeMet. Dispersion around the median is significantly larger for studies performed at low concentration in the aqueous matrix (Fig. 8a). This discrepancy is not observed for SeCys or $TMSe^+$.

This larger dispersion with respect to SeMet can be related to some chromatographic retention problems. Partial sorption/retention of SeMet onto the chromatographic columns may have occurred. Random desorption of the

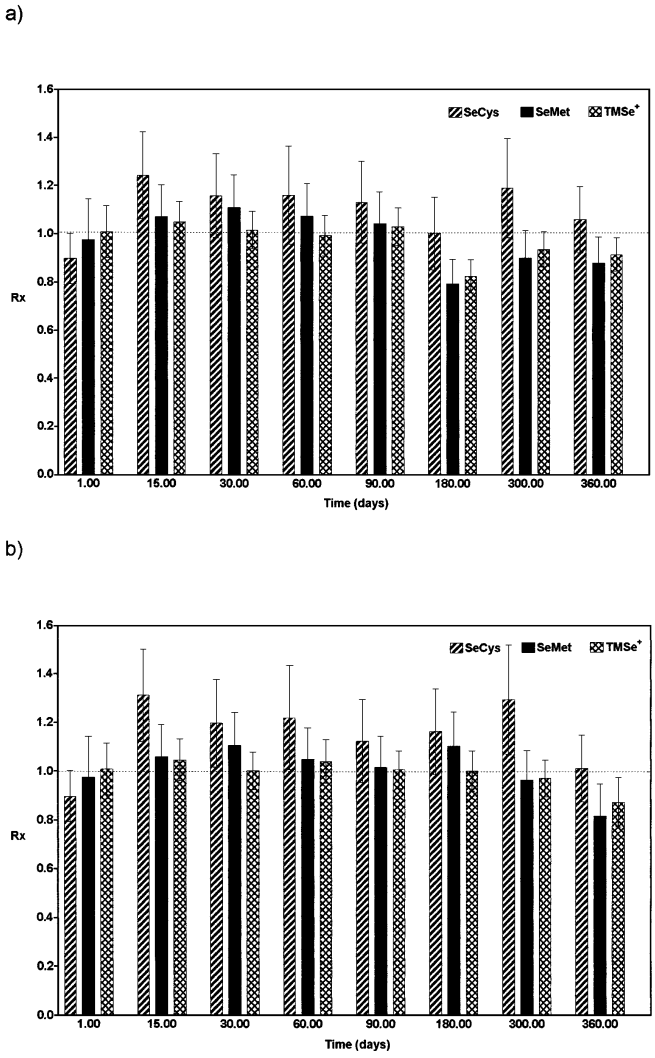

Fig. 7a, b Stability of organic Se compounds at 20°C in an aqueous matrix containing an ion pairing agent (pentyl sulfonate at 10^{-4} mol/L), and stored in **a**) Pyrex containers, and **b**) Teflon containers; concentration of the species 150 µg/L Se

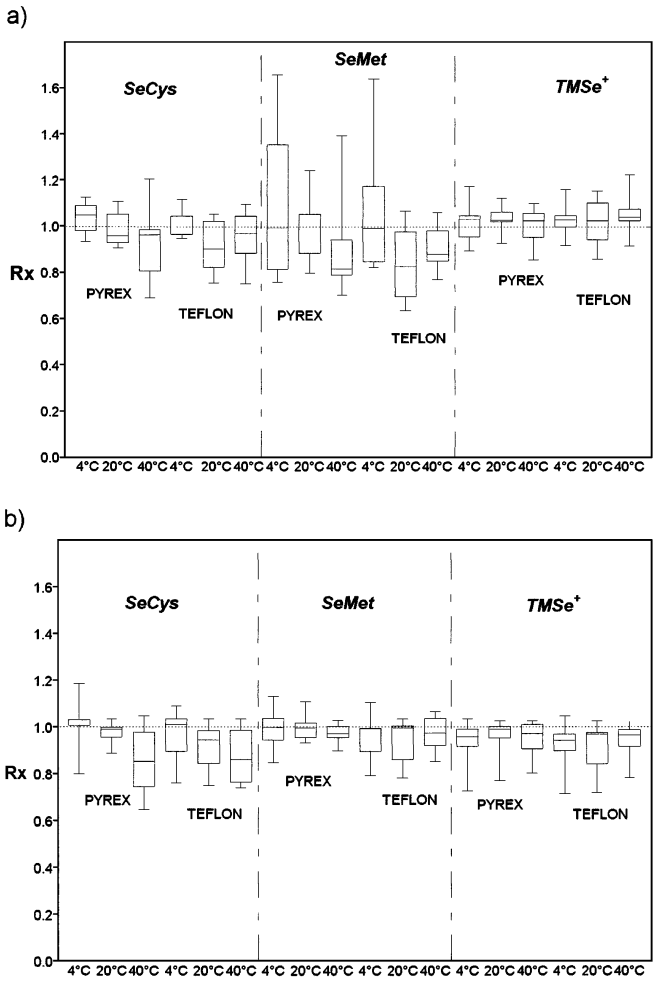

Fig. 8a, b Optimal conditions of stability (Pyrex and Teflon containers) for two different concentrations: **a**) 25 µg/L Se; **b**) 150 µg/L Se, aqueous matrix

SeMet during the next chromatographic run could contribute to higher concentration levels. This effect would be most obvious for the lower concentration level as can be seen in Fig. 8. However, this partial sorption/retention of SeMet by the chromatographic column is still an unknown phenomenon and must be justified in more specific studies. Ageing of the chromatographic column could also contribute to increased tailing of SeMet profiles resulting in an increased dispersion within the analytical data sets.

Conclusion

General results obtained in these stability studies for organoselenium species show that very different responses were obtained for different storage conditions. The container material is the most influential parameter. The temperature influence is much less pronounced. Best results for stability over one full year were obtained with Pyrex containers at 4°C and 20°C in the dark. Rapid loss of $TMSe^+$ (within less than 2 weeks) was observed under any conditions in polyethylene vials. Samples stored in Pyrex vials and exposed to natural sunlight conditions presented also showed a progressive degradation of mainly the seleno-amino acid species. This degradation has been assigned to the combination of light and the presence of oxygen introduced in the subsampling process. In order to prevent such a phenomenon, oxygen could be eliminated by bubbling an inert gas through the solutions after each subsampling. The addition of a stabilizing agent (pentyl sulfonate) to the solution resulted in worse repeatability and had no influence on the stability of the species, and is therefore, not recommended. Finally, the concentration of the analytes showed little influence on their stability at the 25–150 µg/L level.

A missing capability was the simultaneous determination of organic and inorganic Se species in order to identify the species formed during the storage period, which should be a goal of a future study.

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