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Determination of the Deprotonation Constants of Seleno-DL-cystine and Seleno-DL-methionine and Implication to their Separation by HPLC

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We have determined the deprotonation constants (pK_a) of seleno-DL-cystine and seleno-DL-methionine together with those of DL-cystine and DL-methionine for comparison, by potentiometric measurements. In the case of seleno-DL-cystine, the difference between the pK_a values for the two amino groups was found to be only slightly lower than that observed for DL-cystine itself. In contrast, the difference between the two pK_a values for the carboxylic groups was found to be much smaller for seleno-DL-cystine than for DL-cystine. In both seleno-amino-acids, the zwitterionic species appear to be dominant in the pH range between 4 and 7, while positively charged protonated species are found to be present at pH values lower than 4. Based on a knowledge of the ionic species distributions as a function of pH, we have proposed an interpretation for the chromatographic separation of selenocystine and selenomethionine by HPLC.

Keywords: seleno-DL-cystine; seleno-DL-methionine; pK_a; HPLC

INTRODUCTION

Selenium plays an important role in the environment, both as an essential nutrient and as a toxic substance,[1,2] depending on its concentration levels and the type of species present.

Among naturally occurring seleno species, selenoamino-acids play an essential role in the selenium biogeochemical cycle. These compounds are generally separated by liquid chromatography but the literature is still scarce.[1,3–6] This lack of information is mainly related to the high sensitivity required. The coupling between HPLC (high-performance liquid chromatography) and ICP–MS (inductively coupled plasma/mass spectrometry) appears to be an appropriate method as it provides both a good separation for these species and high sensitivity to detect them at natural concentration levels, which are usually very low ($\mu g\ l^{-1}$–$ng\ l^{-1}$). Moreover, owing to the possibility of distinguishing between isotopic species, the ICP–MS technique offers good selectivity by allowing for the detection of possible interferences.

In order to improve our knowledge of the chemical behaviour of organoselenium species, there is still a need for the determination of several chemical properties. In particular, the acid–base equilibria of these organic compounds which contain selenium atoms instead of sulphur atoms has not yet been studied extensively,[7–9] and for many naturally occurring seleno species the deprotonation constants remain unknown. This is the case for selenoamino-acids such as selenocystine and selenomethionine. Potentiometric titration combined with a computer program performing linear and non-linear regression has been shown to be a convenient tool for determination of deprotonation constants and elucidation of the species distribution over a wide range of pH.[10–14]

In this work, we have used this method to determine the deprotonation constants of seleno-DL-cystine and seleno-DL-methionine in aqueous solutions. The determination of the known depronation constants of the analogous sulphur

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amino-acids[10,13,15,16] has also been performed for comparison and to verify the accuracy of the method used. The pK_a values we determined for the selenoamino-acids and the equilibrium distribution of the species as a function of the pH appear to be of interest for interpreting the chromatographic behaviour of these compounds.

EXPERIMENTAL

Reagents

All reagents and solvents were of the highest-purity grade. DL-methionine, DL-cystine, seleno-DL-methionine, and seleno-DL-cystine were reagent-grade chemicals (>99% purity) purchased from Sigma Chemicals. They were used without further purification. Potassium chloride used as supporting electrolyte was a reagent-grade chemical from Merck. Carbonate-free solutions of 0.1000 mol l^{-1} KOH were prepared from Dilut-it ampoules (J. T. Baker) and were standardized by titration with potassium acid phthalate purchased from Merck. Reagents used for chromatographic separations were of analytical-purity grade.

Potentiometric equilibrium measurements

Potentiometric studies of seleno-DL-cystine, seleno-DL-methionine, DL-cystine and DL-methionine were carried out in a thermostated room with a pH-meter/ionometer (Orion model 720A) with a resolution of 0.001 pH units fitted with blue glass and Argenthal reference electrodes (Ingold). It was calibrated with standard HCl, 10^{-2} mol l^{-1} [μ=0.100 mol l^{-1} (KCl)] and CO_2-free KOH solutions to give a reading of the pH values after correction of the Nernstian slope. This calibration permits us to determine experimentally and directly the difference between the observed and the calculated pH values.[17] The pH reproducibility is <0.002. The temperature of the solution was maintained at 25 °C with a Cryothermostat Ministat Bioblock, and the ionic strength (μ) was adjusted to 0.100 mol l^{-1} by the addition of an appropriate amount of KCl, which takes into account the presence of HCl (10^{-2} mol l^{-1}) used to solubilize the analytes.

Samples of about 0.10 mmol of DL-methionine and DL-cystine or 0.05 mmol of seleno-DL-methionine and seleno-DL-cystine were diluted with 50 ml of Milli-Q (Millipore) water previously boiled to remove dissolved CO_2. Some HCl was added to the ligand solutions to ensure a total solubilization. A sealed thermostatic vessel with gas inlet and outlet for nitrogen bubbling was used for the whole experiment. The nitrogen was purified by flow through a solution of KOH and pyrogallol. All the solutions were titrated with 0.09906 mol l^{-1} standard CO_2-free KOH and a burette titration (Schott Geräte Model T80/10). This study was performed in the pH range from 2 to 12.

Computations

The proton association constants of seleno-DL-methionine, seleno-DL-cystine, DL-methionine and DL-cystine were calculated with the aid of the interactive program BEST.[17,18] The dissociation constant of water at 25 °C (pK_w) was taken from Ref. 17. With the exception of the unknown deprotonation constants to be determined, all the other constants were kept fixed during refinement.

The input data for the BEST program consist of millimoles of each component, the initial estimates for the equilibrium constants of each species thought to be formed from the solution components, and the experimentally determined curves of pH vs base added. At each increment of base added, the program sets mass-balance equations for all species present and solves for the concentration of hydrogen ions, which is compared with the experimental value.

The calculation starts with a set of known and unknown (estimated) overall deprotonation constants of all possible equilibrium reactions in the system and computes [H^+] at the equilibrium for each value of base added. The initial estimate of the unknown constants is made on the basis of the slope variations in the experimental pH curve. The chosen values are taken equal to a pH value slightly inferior to the pH for which a change in the slope is noticed. The fitting process consists of two stages:

(1) The minimization of the weighted sum of squares of the deviations between the observed and the calculated pH values expressed by Eqn [1]:[17]

$$U = \sum w(\mathrm{p}H_{\mathrm{obs}} - \mathrm{p}H_{\mathrm{calc}})^2 \quad [1]$$

where $w = 1/(\mathrm{p}H_{i+1} - \mathrm{p}H_{i-1})^2$ is a weighting factor which serves to lessen the influence of less accurate pH values in the steeply sloped region of the pH profile on the calculations:

(2) the adjustment of the unknown stability constants and repetition of the calculations until no further minimization of U can be obtained, thus providing the final calculated β values. The standard deviation in pH units is given by the value of σ_{fit} expressed by Eqn [2]:

$$\sigma_{\mathrm{fit}} = (U/N)^{1/2} \quad [2]$$

where $N = \sum w$.

The species distribution curves were calculated with the aid of the program SPECIES, which requires the output file of the program BEST, and which produces an output file which is composed of the molar percentage of all the species processed by the program BEST as a function of pH. Further details of the method of calculation are given in Ref. 17.

Chromatographic measurements

The HPLC pump used for the separation of selenoamino-acids was a Perkin-Elmer Series 410 Bio, made of titanium to avoid the risk of reagent or analyte decomposition. A reversed-phase chromatographic column (Hamilton PRP1) was used for the separation of organo-selenium compounds. The column outlet was directly connected to the spray chamber of the ICP–MS. A Sciex Perkin–Elmer ICP mass spectrometer (Elan 5000) was used as detector. The experimental conditions we have retained throughout this work are reported in Table 1. More details of the system used are given in a forthcoming publication.[19]

The mobile phase consisted of a mixture of water/methanol containing pentanesulphonic acid as an ion-pairing agent. The pH was adjusted to the desired value by addition of hydrocholoric acid.

RESULTS AND DISCUSSION

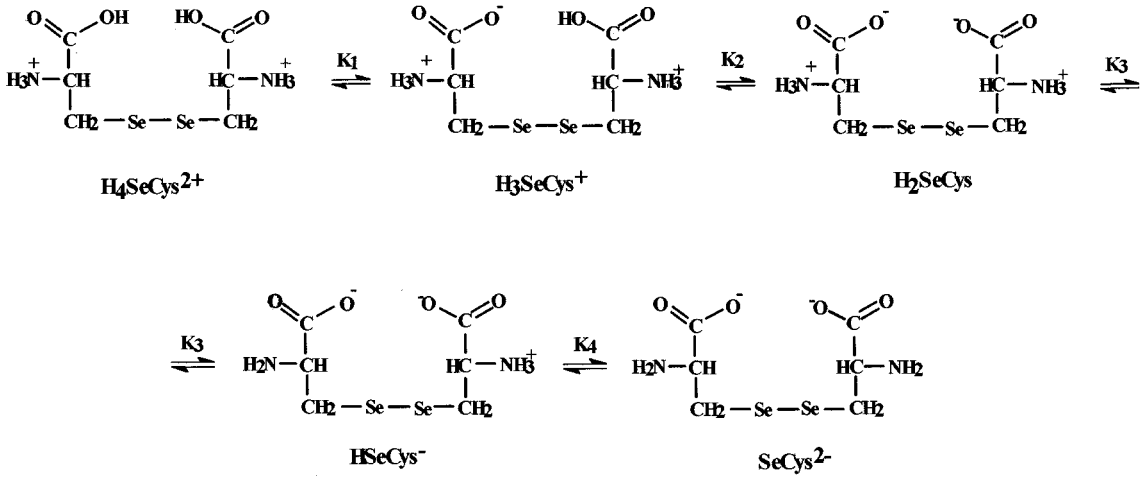
Deprotonation constants for seleno-DL-cystine

The equilibrium reactions between the four possible protonated and non-protonated species of seleno-DL-cystine are listed in scheme 1. They are arranged in order of occurrence, going from low to high pH with their equilibrium constants (deprotonation constants) ranging from K_1 to K_4.

The experimental titration curve is represented in Fig. 1. It displays one long buffer zone from $a = 0$ to $a = 10$ (where a = mol of base per mol of ligand). In this range, the added base is consumed both for the neutralization of the excess of HCl (10^{-2} mol l^{-1}) initially added to solubilize the ligand, and also for the deprotonation of the carboxylic groups. This explains why the curve

Table 1. Experimental conditions retained throughout this work for the separation by HPLC and ICP/MS detection of selenoamino-acids

Chromatographic conditions	Stationary phase	PRP1
	Mobile phase	Water/methanol (98:2) + 10^{-4} mol l^{-1} $C_5H_{11}SO^{3-}$
	pH	4.5
	Pump flow rate	1 ml min^{-1}
	Sample volume	100 μl
Interface	PEEK connection	0.5 mm i.d.
ICP–MS conditions	Ar flow rate:	
	Plasma	15 l min^{-1}
	Auxiliary	0.8 l min^{-1}
	Nebulization	1.0 l min^{-1}
	Power supply	
	Acquisition parameters	
	Dwell time	250 s
	No. of readings	650
	Analysis time	300 s
	Isotopes selected	82_{Se}



Scheme 1

begins at a pH around 2. According to results reported for similar amino-acids,[16] neutralization of the two carboxylic groups is expected to take place in this pH range or even at lower pH values and, as a consequence, it cannot be observed distinctly on the curve. Nevertheless the titration curve profile in the low pH range is sensitive enough to the presence of the equilibrium reactions involving the protonation of the carboxylic groups to allow the determination of the corresponding pK_a values by the parametrization process. Two weak buffer zones can be noticed at pH values between 8.0 and 9.0 (a=11 and 12 respectively). They probably correspond to the sequential neutralization of the two ammonium groups.

The deprotonation constants determined from this curve as reported in Table 2. Their values are in good agreement with the values found in the literature for other amino-acids.[16] As for DL-cystine, two different pK_a values were found for the deprotonation of the carboxylic groups of DL-selenocystine. These values were obtained from the parametrization process, which takes into account the initial presence of HCl to fit the curve in the range where the neutralization of the carboxylic groups occurs.

The relative distribution of the involved species as a function of pH is shown in Fig. 2. In this

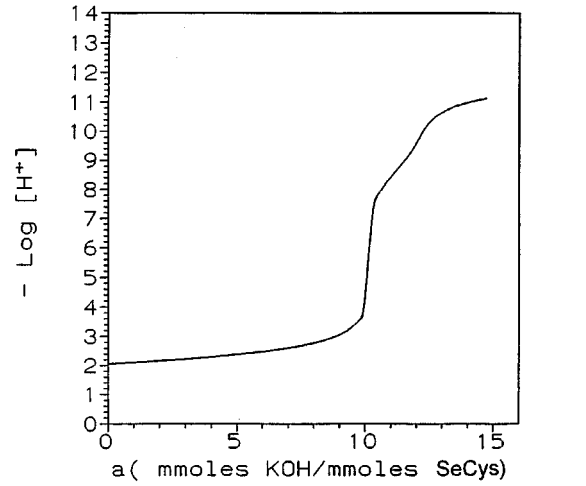


Figure 1 Potentiometric pH profile for a solution containing 9.40×10^{-4} mol l^{-1} seleno-DL-cystine (a=mol of base added per mol of seleno-DL-cystine, t=25.0 °C; μ=0.100 mol l^{-1} KCl).

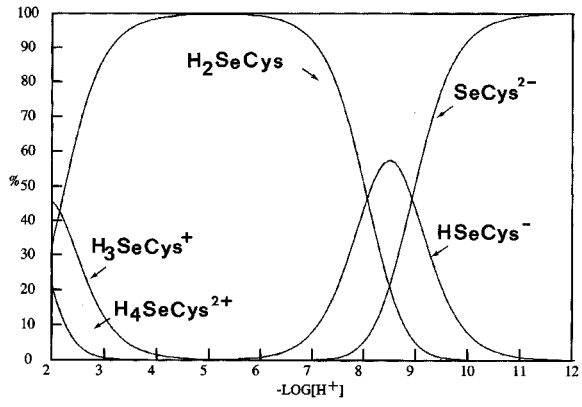


Figure 2 Species distribution curves for a solution containing 9.40×10^{-4} mol l^{-1} seleno-DL-cystine. $SeCys^{2-}$ is the completely deprotonated form of ligand and $HSeCys^{-}$, H_2SeCys, H_3SeCys^{+} and H_4SeCys^{2+} are the mono-, di-, tri- and tetra-protonated forms of the ligand.

Table 2. Logs of deprotonation constants of seleno-DL-cystine, DL-cystine, seleno-DL-methionine and DL-methionine ($t = 25.0$ °C; $\mu = 0.100$ mol l^{-1})

Amino-acid	$\log\left(\frac{[H_4L]}{[H_3L][H^+]}\right)$	$\log\left(\frac{[H_3L]}{[H_2L][H^+]}\right)$	$\log\left(\frac{[H_2L]}{[HL][H^+]}\right)$	$\log\left(\frac{[HL]}{[L][H^+]}\right)$
DL-Selenocystine[e]	1.68 ± 0.02 2.4[c]	2.15 ± 0.01	8.07 ± 0.01 8.9[c]	8.94 ± 0.02
DL-Cystine[f]	1.51 ± 0.03 1.50 ± 0.01[b]	2.79 ± 0.02 2.05 ± 0.05[b]	8.25 ± 0.02 8.03[a]	8.97 ± 0.02 8.80[a]
DL-Selenomethionine[g]	—	—	2.19 ± 0.02 2.6[c]	9.05 ± 0.01 8.9[c]
DL-Methionine[h]	—	—	2.23 ± 0.02 2.16 ± 0.03[d]	9.08 ± 0.02 9.08 ± 0.04[d]

[a]Ref. 16 ($t = 20$ °C; $\mu = 0.1$ mol l^{-1}). [b]Ref. 16 ($t = 37$ °C; $\mu = 0.15$ mol l^{-1}. [c]Ref. 6. [d]Ref. 16 ($t = 25$ °C; $\mu = 0.1$ mol l^{-1}). [e]σ-fit = 0.00342. [f]σ-fit = 0.00619. [g]σ-fit = 0.00971. [h]σ-fit = 0.00775.

figure, the totally deprotonated form ($SeCys^{2-}$) arises at pH 7.2 and its abundance increases with the pH, reaching 99.9% at pH 12. The monoprotonated species ($HseCys^-$) exists only in the pH range between 6.0 and 11.5 with a maximum percentage of 57.5% at pH 8.5. The diprotonated species (H_2SeCys), which has a zwitterionic character, is the major species formed in the pH range from 2.0 to 8.0. It amounts to 99.7% at pH 4.8 and is largely dominant over all other species in the pH range 4.0–7.0. The tri- (H_3SeCys^+) and tetra-protonated (H_4SeCys^{2+}) species occur at low pH (<4.0). At pH 2.0, while the triprotonated species constitutes 45.8%, the tetraprotonated species amounts to only half of this value (21.8%). Above pH 3.0 it can be considered to be non-existent.

The intercept of the distribution curves for two species interchanging a proton corresponds to the pK_a value for the equilibrium reaction. Accordingly, as derived from the parametrization of the titration curve, the pK_a value for the second deprotonation of the carboxylic group is shown in Fig. 2 at pH 2.15 where the two curves corresponding to the H_3SeCys^+ and H_2SeCys species intercept. The deprotonation of the first carboxylic group is found from the parametrization to start below pH 2.0 and it cannot be made evident in this figure.

At pH 8.5, where the monodeprotonated amino group species ($HSeCys^-$) reaches 60%, the completely deprotonated species ($SeCys^{2-}$) and the zwitterionic (H_2SeCys) species exist both with a 20% abundance.

For comparison, a potentiometric titration of DL–cystine was also performed. The deprotonation constants determined are shown in Table 2. The very small σ_{fit} values computed between the experimental and the calculated pH curves allow us to consider with confidence the results obtained for both DL-cystine and seleno-DL-cystine.[17] Moreover, compared with the values found in the literature, our values appear to be in good agreement.[10,16] The largest discrepancy is observed in the case of the deprotonation of the H_3L species of DL-cystine and can be assigned to the different experimental conditions used. The differences in the pK_a values between DL-cystine and its seleno analogue are small, except for the deprotonation of the H_3L species. Nevertheless, we notice a general tendency for the pK_a values of seleno-DL-cystine to be lower than those of DL-cystine. This observation may result from differences in intramolecular interactions between selenium and sulphur, or from differences in molecular conformations.

The influence of intramolecular interactions on pK_a values and particularly of interactions between functional groups attached respectively to the two halves of the molecules (Scheme 1) is shown by the different values of pK_a determined by the same type of functional groups.[7] Interestingly, we note that the replacement of sulphur atoms by selenium appears to affect differently the pK_a of the homologue caboxyl and amino groups. While the difference in the pK_a values of the two amino groups increases only slightly, a considerable decrease for the difference in the

pK_a of the carboxylic groups is observed.

Such a result indicates that, in seleno-DL-cystine, the protonated carboxylic group (H_3SeCys^+ species) undergoes weaker electrostatic interaction from the negative charge located on its homologue group than in DL-cystine. From crystallographic data[20,21] it has been shown that the tetraprotonated and the zwitterionic form of DL-cystine have a quite different geometry (Fig. 3). Moreover, in solution, it has been suggested[22,23] that each enantiomer molecule may exist as a distribution of different conformers. Accordingly it is likely that the change in pK_a values in going from DL-cystine to seleno-DL-cystine is due in a large part to changes in the geometry (or mean geometry) of the species involved.

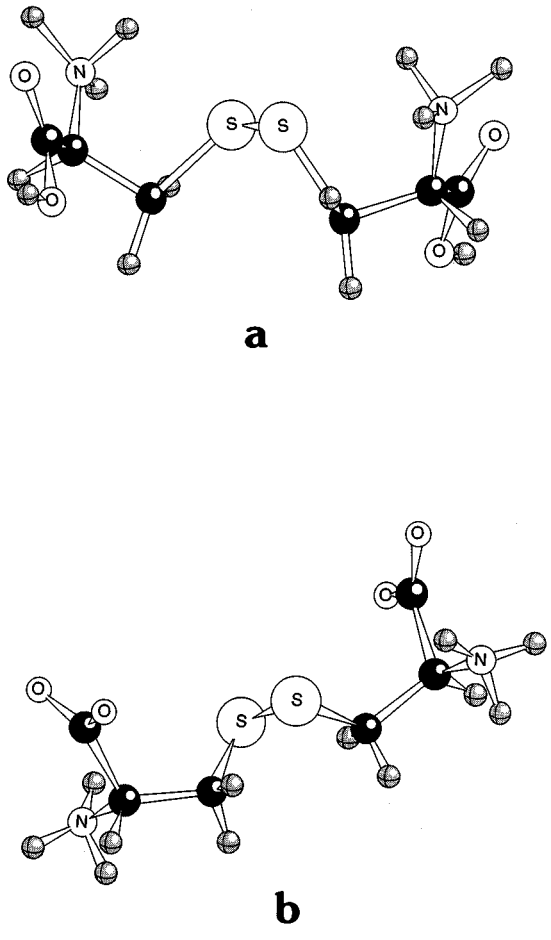


Figure 3 Perspective views drawn from crystallographic data of the cystine molecule in two different forms: (a) totally protonated form (chloride derivative) from Ref. 20; (b) neutral form (zwitterionic) from Ref. 21.

Deprotonation constants of seleno-DL-methionine

The two equilibria which operate in the case of seleno-DL-methionine are described in Scheme 2.

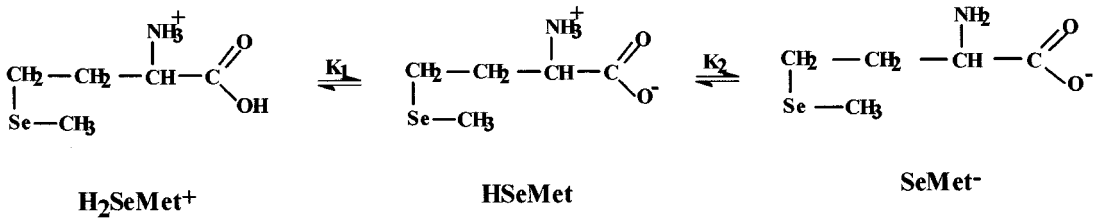
The titration curve plotted in Fig. 4 shows a buffer zone for a value of $a = 2$ and for pH around 9.0. It corresponds to the neutralization of a proton released by the amino group. As for seleno-DL-cystine, the carboxylic group theoretically has a pK_a value close to 2.0, but because of the presence of HCl used for solubilization, we could not make measurements at pH values lower than 3 and the corresponding buffer zone cannot be seen on this curve. Nevertheless, the deprotonation constants could be determined by parametrization. They are given in Table 2.

Similarly to seleno-DL-cystine, Fig. 5 shows that the completely deprotonated species ($SeMet^-$) occurs at high pH starting at pH 7.0 up to pH 12.0, where it is 99.9% formed. The zwitterionic species (HSeMet) exists over a wide range of pH, from 2.0 to 11.5, and between pH 4 and 7 it is practically the only species present in solution. The completely protonated form of seleno-DL-methionine (H_2SeMet^+) occurs only for pH values lower than 5.0. At pH 2.0, it is 60.6% formed.

For comparison, we have also determined the deprotonation constants of DL-methionine. The values we found are in good agreement with those reported in the literature.[10,13,16] These values are shown in Table 2. We observe that the pK_a values for the seleno-DL-methionine are only slightly lower than for DL-methionine. This is an indication that in both DL-methionine and seleno-DL-methionine molecules, the interactions of sulphur or selenium atoms with the carboxylic and amino groups influence only slightly the deprotonation constants. Such a result may be assigned to the fact that the presence of two methylene groups between the sulphur or selenium atom and the functional groups minimizes intramolecular interactions (Scheme 2).[7]

Chromatographic separation of seleno-DL-cystine and seleno-DL-methionine

Following the Jiang and Houk[5] procedure for the separation of the sulphur amino-acids, cysteine and methionine, we have applied a reversed phase chromatography method based on the



Scheme 2

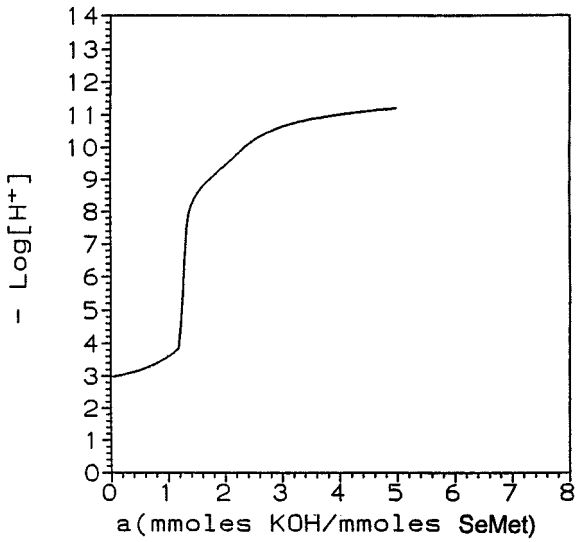


Figure 4 Potentiometric pH profile for the solution containing 9.95×10^{-3} mol l^{-1} seleno-DL-methionine (a = mol of base added per mol of seleno-DL-methionine, t = 25.0 °C; μ = 0.100 mol l^{-1} KCl).

formation of ion-pairs. A similar approach to the separation of this type of compounds has also been attempted by other authors.[6]

The chromatogram in Fig. 6 has been obtained with a mobile phase which consists of a water/

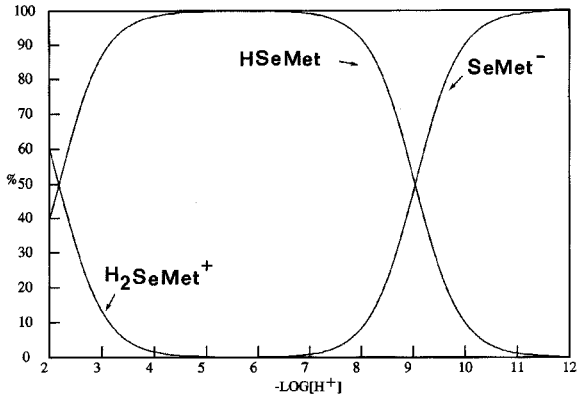


Figure 5 Species distribution curves for a solution containing 9.95×10^{-3} mol l^{-1} seleno-DL-methionine. $SeMet^-$ is the completely deprotonated form of the ligand; HSeMet and H_2SeMet^+ are the mono- and the di-protonated forms of the ligand.

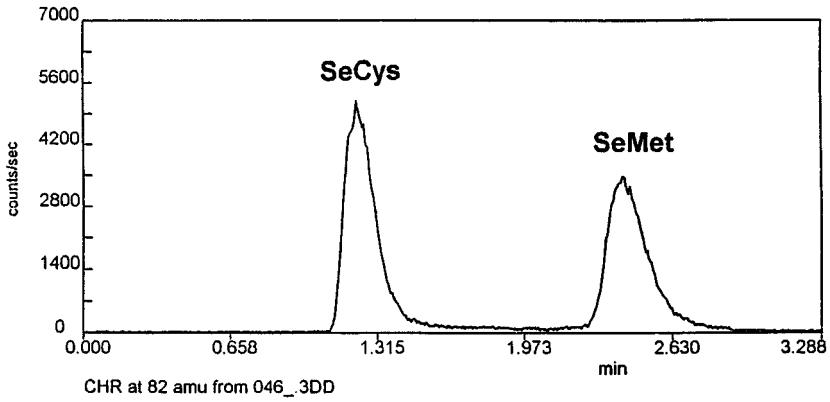


Figure 6 Chromatogram of seleno-DL-cystine and seleno-DL-methionine obtained by HPLC under optimized experimental conditions. Stationary phase:reverse-phase Hamilton PRP1. Concentration of the analytes:100 μg l^{-1}. Mobile phase: water/methanol (98:2) + 10^{-5} mol l^{-1} pentanesulphonic acid. pH:3.5.

methanol (98:2) mixture containing 10^{-5} mol l^{-1} of pentanesulphonic acid as ion-pairing agent and at a pH of 3.5. The concentration of each compound in the solution to be tested was 100 $\mu g\, l^{-1}$. These optimal conditions were retained after optimization of several parameters such as pH, mobile phase composition, eluent polarity, ionic strength, liquid flow rate, etc.[19] These values lead to the narrowest peaks with low peak tailing and to the best separation between the two peaks, thus offering a good sensitivity for detecting these molecules.

The ionic strength of the mobile phase was adjusted by addition of the ion-pairing pentanesulphonate. This molecule contains a hydrophobic aliphatic chain which would have a strong affinity with the apolar stationary phase, and a sulphonic group (SO_3^-) which would be orientated towards the analytes passing through the column. In favourable pH conditions the ammonium groups (positively charged) of the selenoamino-acids may be thought to interact with the negatively charged sulphonic groups. According to our titration results, such conditions are expected to occur for pH values lower than 4, where positively charged species are present for the formation of ion-pairs with sulphonic groups (Figs 2 and 5). In practice, the optimal pH condition for chromatographic separation was established to be at pH values between 3.5 and 4. These values correspond in fact to a compromise between the required low pH conditions as given by the titration results and the lowest pH value which may be used safely with the column for long-term stability and reproducibility of the stationary phase. Thus, they are consistent with the predominance of an ion-pair formation mechanism for the retention of SeCys and SeMet. However, in addition to this mechanism, other partition mechanisms based on molecular size or dipolar interactions between the highly polar mobile phase and the stationary phase may perhaps operate.

Over the pH range explored (3–7), we observed a smaller retention time for SeCys than for SeMe. Figure 7 shows the variation of the capacity factor k' (defined as $k' = (T_R - T_o)/T_o$, where T_R is the retention time of each compound and T_o is the column dead time) versus pH. The column dead time T_o corresponds to the retention time measured for species like SeO_3^{2-} and AsO_4^{3-}, which are not retained in the column because of their anionic character. The largest difference in k' values between the two species

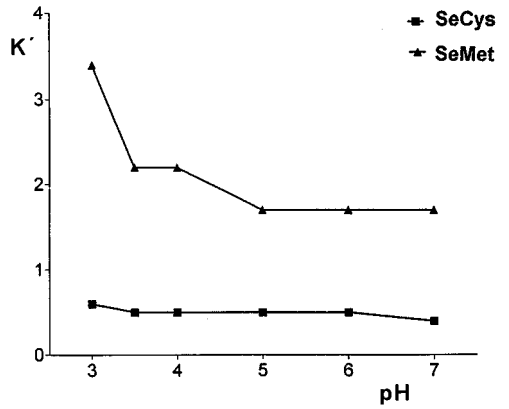


Figure 7 Influence of the pH on the retention of the two selenoamino-acids (SeCys and SeMet) separated by HPLC/ICP–MS. This retention is expressed as k' (capacity factor) $= T_R - T_o/T_o$, where T_R is the retention time of each compound and T_o is the column dead time.

was obtained at pH 3, which is the lowest pH value we used for performing the separation. This difference appears to result essentially from an increase of the value of k' for SeMet in going to low pH values, starting from about pH 5.0.

This increasing difference in k' values between SeCys and SeMet in going to low pH could be explained by the increase in the concentration of the monocationic forms (H_3SeCys^+ and H_2SeMet^+) which are in equilibrium with the zwitterionic forms (Figs 2 and 5). At low pH, whereas in the case of SeMet the monocationic forms contains a single positive charge, in the case of SeCys it contains two positive charges but also one negative charge. Because of the presence of this negative charge we may infer that the electrostatic interaction of these forms with SO_3^- pendant groups is less favoured than that with the monocationic forms of SeMet and is even comparable with that of the zwitterionic form. Such an interpretation would be consistent with the fact that, contrary to SeMet, the value of k' for SeCys is observed to be insensitive to the pH in the range 3–7 (Fig. 7).

In the pH range 5–7, the differences in the k' values are almost constant. In this pH range both compounds exist essentially as zwitterions. The lower retention time observed for SeCys, whatever the pH, indicates that the SeCys molecule is only weakly retained by the stationary phase. This could be caused by the presence of equilibrium between conformers, by polarity effects or by size exclusion mechanisms which

depend on the shape and the mass of the analyzed molecules.

CONCLUSIONS

Characterization of the species present in aqueous solutions of seleno-DL-cystine, seleno-DL-methionine, DL-cystine and DL-methionine has been achieved by potentiometric studies. Four deprotonation constants were determined for seleno-DL-cystine and DL-cystine, two for the carboxylic groups and two for the amino groups. Deprotonation constants for, respectively, the carboxylic group and for the amino group of seleno-DL-methionine and DL-methionine, were also determined and compared.

In both selenoamino-acids, the zwitterionic species appear to be the dominant species in the pH range between 4 and 7.

In the case of seleno-DL-cystine, the difference between the pK_a values for the two amino groups were found to be only slightly smaller than that observed for DL-cystine itself. In contrast, the difference between the two pK_a values for the carboxylic groups was found to be much smaller for seleno-DL-cystine than for DL-cystine. From this result, it has been inferred that in the case of seleno-DL-cystine, the protonated carboxylic group (H_3SeCys^- species) undergoes a weaker electrostatic interaction from the negative charge located on its homologue group than in DL-cystine itself, probably because of differences in their molecular structures.

The species distribution determined as a function of the pH accounts satisfactorily to the fact that the best separation was obtained in the pH range below pH 4, where the zwitterionic forms are in equilibrium with the carboxyl monoprotonated forms.

According to these results, the large difference at low pH in the retention times between seleno-DL-cystine and seleno-DL-methionine appears essentially to rely upon the fact that the diprotonated form of seleno-DL-methionine carries a single positive charge, thus ensuring an optimum interaction with the ion-pairing agent which is on line with a long retention time. In contrast, the triprotonated form of seleno-DL-cystine carries two positive charges but also one negative charge which may be supposed to weaken the interaction with the ion-pairing agent, thus inducing a short retention time.

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