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# Feasibility study of ion-chromatography microwave assisted on-line species conversion hydride generation atomic absorption spectrometry for selenium speciation analysis of biological material

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A method for selenium speciation based on ion-chromatography (IC) microwave (MW) assisted on-line species conversion hydride generation (HG) atomic absorption spectrometry (AAS) is described. The conversion of selenium species into selenite was achieved by using hydrobromic acid and bromate. A potential interferent originating from the reagents was identified. Multivariate optimization of the IC-MW-HGAAS system was undertaken and the results show that the $HBr/BrO_3^-$ ratio is a very important parameter, which should be carefully controlled both to obtain a high conversion efficiency and to diminish the interferent. The feasibility of the IC-MW-HGAAS system for real samples is demonstrated by the application of selenium speciation in extracts of the certified reference material CRM 402. The system is shown to be sensitive to interferences but the detection capability is good, relative detection limits being 1.0–1.6 ng $g^{-1}$ (based on a 50 μL injection volume) for the species investigated: selenomethionine, selenite, selenate and selenocystine. Selenate was quantified in the CRM 402 extract at a level of $\approx$1.5 μg $g^{-1}$ corresponding to $\approx$22% of the total selenium content in the material. The method described is concluded to be operationally defined because of the extraction procedure used.

## Introduction

Selenium speciation in food and feed stuff is important because the uptake and excretion in humans and animals is dependent on the chemical form in which the selenium is present.[1] In samples of biological origin, selenium can be present as inorganic oxyanions: selenite and selenate and organic amino acids: selenomethionine, selenocysteine and selenocystine. There are certainly other selenium species existing in samples of biota, as for example shown to be the case in garlic.[2] It should also be noted that selenium is often incorporated in proteins, the enzyme glutathione peroxidase being one of the most studied.[3] Information is needed on the distribution and content of various selenium-containing compounds in food[4] and therefore there is a demand for speciation methods applicable to food samples. For an extensive overview of analytical techniques for selenium speciation, information can be found in comprehensive reviews.[5–8] The main trend in speciation is presently the use of inductively coupled plasma (ICP) mass spectrometry (MS) coupled with various modes of chromatography. However, a viable alternative is hydride generation (HG) atomic absorption spectrometry (AAS) which is a well-established and sensitive detection technique for selenium.[9] A number of reports on the coupling of this technique with chromatography for selenium speciation have already been published.[10–13] Prior to HG all selenium species must be converted to selenite, as this is the only species readily forming volatile $SeH_2$ when reacted with borohydride. It should however be noted that other selenium species than selenite were recently reported to form volatile selenium when reacted with sodium borohydride and therefore HG can not be considered to be fully selective for selenite.[14] For on-line conversion of selenium species to selenite, UV radiation[15] and microwave (MW) assisted chemical pretreatment techniques[10–13] have been utilized. The chemical reagents most frequently used combined with MW heating are $HBr–KBrO_3$[12,13] and $K_2S_2O_8$–NaOH/HCl.[10] Advantages of $K_2S_2O_8$–NaOH–HCl compared to $HBr–KBrO_3$ have been suggested to be a shorter residence time in the MW oven for quantitative conversion of species and no interference from the reagent in the hydride generation step.[16] Bromine is known to be a potential interferent in hydride generation and thus a plausible problem when applying the $HBr–KBrO_3$ reagent for species conversion.[17] On the other hand bromine has been shown to play a key role in the conversion of organoselenium compounds by the oxidative addition to divalent selenium compounds.[18] In this paper the effectiveness of the $HBr–KBrO_3$ reagent for conversion of selenium species to selenite was studied. Furthermore an interference originating from the reagent mixture was identified and reduced by careful optimization of influential parameters using multivariate methods.

Although the accurate and precise determination of selenium species in a solution can be difficult, the real challenge in speciation lies in the sample preparation steps. Criteria such as high and reproducible extraction efficiency and species preservation must be fulfilled. This paper presents a speciation method for selenium using IC-MW-HGAAS. A certified reference material CRM 402, white clover grown on selenium enriched soil, was used as a model sample. This sample is only certified for the total selenium content but results from selenium speciation analysis have previously been reported in the literature.[19–22] This makes a direct comparison of results possible. The application to real sample extracts also provides information about the feasibility of the IC-MW-HGAAS system for selenium speciation analysis.

## Experimental

### Reagents

All solutions were prepared with Milli-Q Plus 185 (Millipore, Millford, MA, USA) water resistivity (18.2 MΩcm). The 15 mmol $L^{-1}$ carbonate eluent for ion-chromatography was

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prepared by dissolving ammonium carbonate (food grade, Merck, Darmstadt, Germany) in water and adding methanol (gradient grade, Merck) 2% v/v. Ammonia (suprapur, Merck) was used to adjust the pH to 10. The 100 mmol $L^{-1}$ sodium hydroxide eluent was prepared using sodium hydroxide (suprapur, Merck) in water. All eluents were filtered through 0.22 μm hydrophilic filters (GVWP, Millipore). Stock standard selenium solutions in water were gravimetrically prepared from sodium selenite (pro analysi, Merck), sodium selenate (AnalaR, BDH, Poole, Dorset, UK), seleno-DL-methionine and seleno-DL-cystine (Sigma, St. Louis, MO, USA). For dissolving selenocystine, hydrochloric acid (suprapur, Merck) was used for adjusting the pH to 2. The stock standard solutions were checked with respect to total selenium concentration using electrothermal graphite furnace atomic absorption spectrometry (ETAAS) and a 1000 mg $L^{-1}$ of Se solution (Titrisol, Merck). Hydrobromic acid in water 48% m/m, purity 99.999 % with respect to metals (Aldrich, Milwaukee, WI, USA) was used as received. An appropriate amount of potassium bromate (A.C.S. reagent, Aldrich) was dissolved in water to obtain the desired concentration. Solutions of sodium borohydride (99%, Aldrich) were prepared daily by dissolving the salt in water together with 0.1% m/m of sodium hydroxide for stabilization.

## Instrumentation

The IC-MW-HGAAS system is depicted in Fig. 1. An inert GP-40 gradient pump (Dionex, Sunnyvale, CA, USA) was used to deliver the eluent in the flow-controlled mode. All wetted parts were made from PEEK or sapphire. All tubing used on the high-pressure side was PEEK tubing, and on the low pressure side, PTFE was used. Two different columns were used: PAX-100, 50 × 4 mm id and Ion Pac AS-10, 250 × 2 mm id both made from PEEK (Dionex). The two injection loops (50 μL, PEEK tubing) were connected to two electrically controlled inert valves (Cetac, Omaha, NE, USA) before and after the analytical column. The injection valve after the column was used for flow injection analysis. A peristaltic pump (Ismatec, Zurich, Switzerland) equipped with Tygon pump tubing (Ismatec) was used to deliver the reagents used for on-line reduction and oxidation of the separated selenium species. The effluent from the column was merged with the reagents using a mixing manifold (Perkin-Elmer, Norwalk, CT, USA). The solution was then passed through PTFE tubing (0.8 mm id) coiled and immersed into the cavity of a focused microwave oven Microdigest 301 (Prolabo, Paris, France). The gas evolution during microwave heating causes impaired precision due to an irregular change of the flow rate into the hydride generation unit. Therefore, a cooling device was made in-house consisting of the inner tube (PTFE, 0.8 mm id) where the effluent from the MW passed and an outer tubing (6 mm id) passing cooling water. After cooling, an additional peristaltic pump FIAS 400 (Perkin-Elmer) was incorporated to further stabilize the flow rate. The sodium borohydride solution was pumped using the same peristaltic pump and mixed with the analyte solution using a manifold. Helium gas N60 (Alphagaz, Air liquide, Liège, Belgium) was used as driving gas for the gas–liquid separator (Perkin-Elmer) which was equipped with a PTFE membrane for preventing liquid from being carried into the quartz cell. The quartz cell for MHS-10 (125 × 10 mm id) (Perkin-Elmer) was electrically heated to 900 °C and installed in a Perkin-Elmer 5000 AAS. An electrodeless discharge lamp for selenium was operated at 280 mA in continuous mode using a Perkin-Elmer System 2 power supply. The wavelength monitored was 196.0 nm and conventional $D_2$ background correction was applied. Data acquisition and evaluation was accomplished by using a custom-made interface and software (B. Radziuk, Frickingen, Germany). Other optimized operating conditions for the IC-MW-HGAAS system are given in Table 1. A flow injection set-up was used for initial experiments investigating the conversion rate of different selenium species into selenite. A FEP (fluorinated ethylene propylene) vessel (V = 20 mL) filled with the reagents and analytes was immersed into the MW cavity. Aliquots of sample from the FEP vessel was filled in a 500 μl injection loop by means of a peristaltic pump. A standard procedure for HG was then used to determine selenium assuming that only selenite gave rise to a response. For determinations of the total concentration of selenium a Perkin-Elmer 5100 ZL ETAAS was used. Samples (5 μL) were injected together with Pd modifier (5 μg) and $Mg(NO_3)_2$ modifier (3 μg). The following furnace program was used: drying 110 °C, 20 s; drying 130 °C, 35 s; ashing 1000 °C, 20 s; cool down 20 °C, 5 s, atomize, 1900 °C, 7 s and burn out, 2400 °C, 4 s.

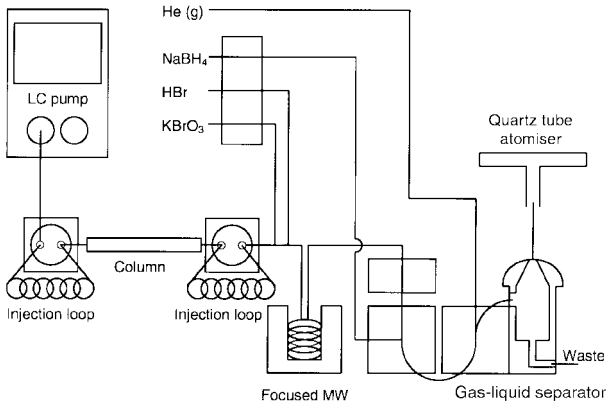


**Fig. 1** Instrumental set-up of the IC-MW-HGAAS system. For details see text.

## Extraction and cleanup

Extractions of selenium species from CRM 402, a white clover material (Institute for Reference Materials and Measurements, Geel, Belgium) was undertaken applying an extraction method described by Emteborg *et al.*[21] Approximately 0.5 g of CRM 402 was placed in a 100 mL conical glass flask, thereafter spikes were added where applicable, 10 mL of $H_2O$–methanol (1 + 1) with 4% ammonia was added and the flasks set in the ultrasonic bath Branson 8200 (Vel, Leuven, Belgium) for 30 min. The sample suspension was transferred to 30 mL FEP centrifuge tubes (Nalgene, Rochester, NY, USA) and centrifuged at 3500 rpm for 30 min using a Sigma Model 4-10 centrifuge (Bioblock Scientific, Paris, France). The supernatant was transferred to an acid washed plastic scintillation vessel. The extraction was repeated by adding 10 mL of the extraction solution. The combined supernatants were filtered using 0.45 μm cellulose acetate syringe filters (Alltech, Laarne, Belgium). From the filtrate a sub-sample was collected for determination of the total Se concentration by ETAAS. Further cleanup was achieved by passing the filtered samples twice through a C18 SPE column

**Table 1** Optimized operating conditions for the IC-MW-HGAAS system

| | |
|---|---|
| IC eluent flow rate[a] | 1.0 mL $min^{-1}$ |
| Injected sample volume | 50 μL |
| HBr (conc.) flow rate | 0.5 mL $min^{-1}$ |
| $KBrO_3$ (15 mmol $L^{-1}$) flow rate | 0.25 mL $min^{-1}$ |
| MW power | 20 W |
| Reaction coil length | 300 cm |
| Cooling coil length | 40 cm |
| $NaBH_4$ (0.5% m/m) flow rate | 1.0 mL $min^{-1}$ |
| He gas flow rate | 150 mL $min^{-1}$ |

[a] For the AS-10 microbore column an eluent flow rate of 0.3 mL $min^{-1}$ was used.

(Isolute™, IST, Mid-Glamorgan, UK). It should be noted that no reduction of the sample volume was undertaken as described by Emteborg *et al.*[21] Sub-samples were taken from the fully processed sample solutions for subsequent determination of total selenium concentration. The pellets were amended to an acid extraction by addition of 10 mL of a 10% (v/v) $HNO_3$ solution to each centrifuge tube. The tubes were closed and kept at room temperature for 24 h. Centrifugation as described above was undertaken and aliquots of the supernatant were withdrawn for determination of selenium.

### Multivariate optimization

The Modde 4.0 (Umetri, Umeå, Sweden) software was used for experimental design and multivariate analysis applied for screening and optimization of parameters influencing the analyte response from the IC-MW-HGAAS system.

## Results and discussion

### Rate of Se species conversion into selenite and reagent interference

The conversion rates of selenomethionine, selenate and selenocystine have been studied in microwave heated concentrated HBr medium using the FI set-up. As shown in Fig. 2a, selenomethionine and selenate are more resistant to conversion than selenocystine. But adding $KBrO_3$ to the hydrobromic acid leads to completely different results: an interferent is formed and a significant background absorbance was detected, suppressing the response for selenite (Fig. 2b) independently of the selenium species investigated. The interferent shows however a time dependence which can be seen in Fig. 2b. This observation is in accordance with that of D'Ulivo[17] who stated that interfering effects of an oxidizing agent in HGAAS is a kinetic problem and therefore every case must be evaluated in a specific manner. Furthermore, the interferent showed a strong correlation with the concentration of bromate for all four selenium species investigated. When the bromate concentration was decreased from 18 to 12 mmol $L^{-1}$ the time elapsed before the specific Se-signal appeared was reduced by 50%. Assuming the concentration of the interferent is temperature dependent, the time dependence of the interferent can be explained as a reflection of the increase in temperature with time. As the interferent disappears the analyte signal increases until a maximum is reached. These results demonstrate that the composition and total concentration of the pre-treatment reagent HBr–$BrO_3^-$ must be carefully optimized along with the time and temperature in order to avoid the formation of an interferent originating from the reagent itself. Most likely this interference is bromine or products from reactions with bromine although no direct evidence for this is presented here. Therefore hydrochloric acid was also tested, leading to a much slower conversion rate of selenomethionine (Fig. 2c). However, if hydrobromic acid was added the conversion rate was increased.

Utilizing the IC-MW-HGAAS system with the PAX-100 column after optimization, this difference between HCl and HBr becomes even more complex. In Fig. 3 two chromatograms are shown using HCl and HBr respectively mixed with $BrO_3^-$. The results presented above are confirmed as the responses for selenomethionine (peak A) and selenate (C) are significantly lower when applying HCl. Furthermore the depression of sensitivity caused by interference shown in Fig. 2b when HBr and $BrO_3^-$ are mixed can be seen as the sensitivity for selenite (B) and selenocystine (D) are higher when using HCl and thus avoiding the excessive formation of this interferent. Evidently there are two counteracting effects of the HBr/$BrO_3^-$ reagent, selenomethionine and selenate are converted into selenite more efficiently than if HCl is used, whereas the efficiency of selenium hydride formation or the atomization of the same is hindered by the use of this reagent.

### Flow rate stabilisation

In the IC-MW-HGAAS system, the direct introduction of the effluent from the MW into the HG unit results in inadequate repeatability. Excessive formation of gas bubbles caused a rather unstable flow of analyte into the HG unit. Cooling of the effluent from the MW gives improvement of repeatability. The final outcome was to use cooling combined with an additional peristaltic pumping of the effluent.

### Stability and purity of stock standard solutions

Each stock standard solution was controlled with respect to purity and concentration of total selenium. The concentration was checked against a Titrisol selenite solution using ETAAS. Standard solutions, approximately 100 ng $g^{-1}$ as Se, of each Se species was injected in the optimized IC-MW-HGAAS system using the PAX-100 column and the ammonium carbonate eluent. No detectable additional peaks were obtained and the concentrations determined agreed within ±5% with expected values. No breakdown of selenocystine could be observed when using HCl for dissolution, as reported in the literature.[21] This study was however conducted under the limited time period of one week. For further information on selenium species stability we refer to Muñoz Olivas.[23]

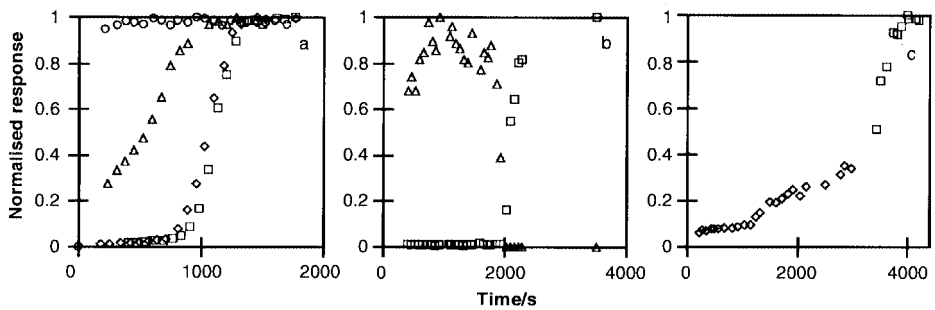


**Fig. 2** Transformation rates of Se species to $Se^{IV}$. All species were quantitatively converted within the time shown. MW was turned on 400 s after addition of the analyte in all experiments. The FI set-up was used for these measurements. (a) Selenite (○), selenomethionine (◇), selenate (□) and selenocystine (△) in 40% v/v HBr solution. (b) Specific (□) and background (△) absorbance obtained from a solution of selenite, 20 ng $mL^{-1}$ as Se, in 40% v/v HBr and 0.018 mol $L^{-1}$ $KBrO_3$, similar results were obtained for the other Se species considered here. (c) Selenomethionine in (◇) 40% v/v HCl solution; (□) 10% v/v HBr added.

### Optimization of the IC-MW-HGAAS system

The IC-MW-HGAAS system was optimized using the PAX-100 column and the ammonium carbonate eluent. Multilinear regression analysis (MLR) on a fractional factorial design was utilized for screening the effect of seven parameters on the response of selenomethionine, selenite, selenate and selenocystine. The parameters investigated were; HBr flow rate (HB), $KBrO_3$ flow rate (Br), reaction coil length (RC), microwave effect (MW), cooling coil length (Cc), $NaBH_4$ concentration (BH) and helium gas flow rate (He). It was not possible to achieve a statistically valid fit of all four responses using one model and therefore each response was fitted separately. This approach has no implication at this stage as the objective was to find the most influential parameters and there are no interactions between responses that are significant. Peak area measurements were used. After addition of some interactive terms, $R^2 \geq 0.92$, $Q^2 \geq 0.78$ were obtained for all four selenium species. From the results it could be seen that the only parameters showing significant influence were He, HB, Br and RC.

After reducing the number of variables to the four most influential, a central composite face centered (CCF) design was applied for optimization. The ranges for the variables were: He (150–200 mL $min^{-1}$), RC (200–400 cm), HB (0.5–1.0 mL $min^{-1}$) and Br (0.2–1.0 mL $min^{-1}$). Partial least square (PLS) analysis was used to fit the model to the experimental results. Total values for $R^2 = 0.93$ and $Q^2 = 0.77$ were obtained validating the projected model. With this approach it is now possible to optimize all the parameters with respect to all four responses using the same model.

The results from the optimization for the four species studied disclose two general response models, one for selenomethionine and selenate and another for selenocystine and selenite (results not shown). This can be explained by considering the stability of the species since the response from selenomethionine and selenate showed a significant dependence on reaction coil length or reaction time whereas the others did not. As can be seen in Fig. 4, all maximum responses are obtained at low flow rates of HBr although the effects on selenate and selenomethionine responses are less pronounced. This is more evident in Fig. 5 showing the actual effects of variables on the responses for selenomethionine (similar to selenate) and selenocystine (similar to selenite). The effects of HBr flow rate on the responses are most probably a result of impaired hydride generation efficiency or formation of an interferent. These effects are believed to be the same namely the formation of excessive amounts of bromine, which both consumes the borohydride and forms the background absorbing compound appearing in the atomizer. Selenocystine and selenite responses are significantly increased by higher flow rates of bromate when the HBr flow rate is kept low, the result is the opposite for selenomethionine and selenate. As the selenate must be reduced and the selenomethionine oxidized to obtain selenite, these results can not be simply explained by the redox properties of the reagents. Certainly the reaction time is longer for lower total flow rates which could be favorable for conversion of selenomethionine and selenate into selenite. It should however be noted here that the influence of microwave effect varied between 20–60 W was not significant for any of the responses. Therefore the conversion of selenate and selenomethionine seems to be very dependent on time. According to D'Ulivo *et al.*,[18] bromine must be present in excess of the organoselenium compound for transformation to be efficient. This prerequisite is however fulfilled under the conditions used here and therefore are the main effects of the bromate flow rate probably on the reaction time and the formation of interferences. There is no evidence for oxidation of the selenite formed as selenocystine and selenite show maximum response at high bromate flow rates.

The strong interaction between flow rates of bromate and hydrobromic acid supports the theory that the ratio of these reagents is very important for an efficient conversion of selenate and selenomethionine into selenite. It is evident from the results presented that compromised conditions must be applied for future experiments. All the optimized operating conditions are

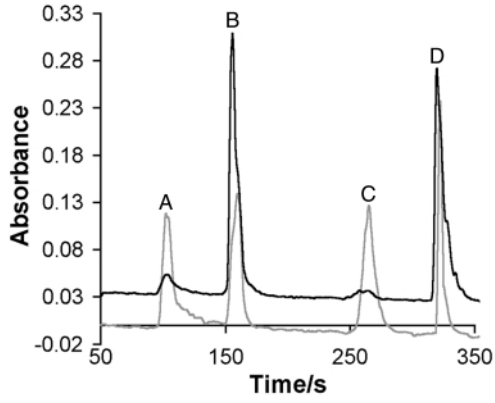


**Fig. 3** IC-MW-HGAAS chromatogram of a 50 μL mixed standard solution containing 100 ng $g^{-1}$ of selenomethionine (A), selenite (B), selenate (C) and selenocystine (D). Optimised conditions were used with $BrO_3^-$ and HBr (grey line) or HCl (black line) as pre-treatment reagents. For clarity the chromatogram obtained using HCl (black line) has been displaced by 0.03 absorbance units.

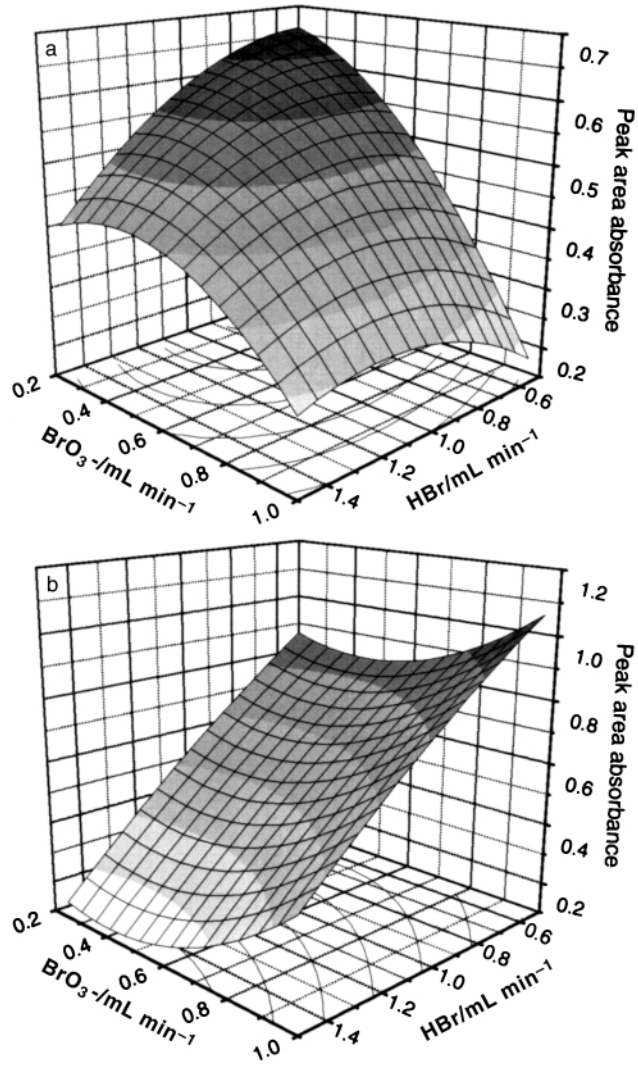


**Fig. 4** Response surface plots showing the peak area response dependency on $BrO_3^-$ and HBr flow rate for selenomethionine (a) and selenocystine (b). The model is based on the CCF designed experiments.

gathered in Table 1 together with other conditions optimized using univariate optimization.

## Calibration

In Table 2 figures of merit are given for the IC-MW-HGAAS system utilizing the PAX-100 50 × 4 mm id column. Detection limits were calculated based on the IUPAC recommended $s = 3\sigma$ criterion, standard deviations of the base-line noise were determined from six blank injections over a time interval similar to the base width of a typical chromatographic peak. Characteristic masses for the four selenium species were calculated from peak area absorbance values obtained at the 34 ng $mL^{-1}$ concentration level. All four selenium species were baseline resolved within 400 s. The standard deviation for the retention times for all species, selenocystine excluded, were 3.5 s or lower based on the calibration data. The elution time for an unretained analyte was calculated to be 84 s.

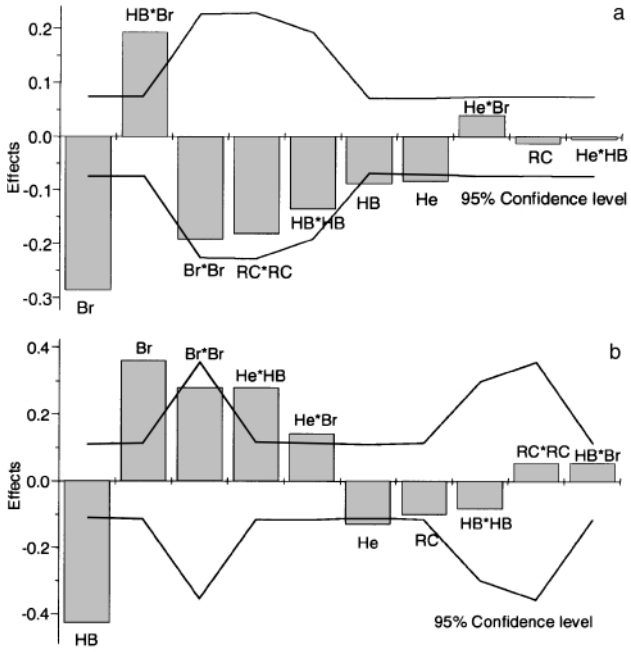


**Fig. 5** Diagrams showing the effects of the investigated factors and interactions between these factors in the CCF design. Effects on peak area responses are shown for selenomethionine (a) and selenocystine (b).

## Speciation of Se in CRM 402

**Extraction procedure.** For evaluation of the extraction and clean-up method, different procedures may be applied. Here the recovery of an added spike and the total extraction efficiency were used. Extractions were performed in triplicate and at two concentration levels of spiking. The spikes were mixtures of selenomethionine, selenite, selenate and selenocystine, adding ≈0.7 and ≈1.3 μg (low and high spike, respectively) of each species to ≈0.5 g of sample.

In Table 3 extraction efficiencies of total Se from the extract are shown. Compared to filtration, no significant losses of analytes during the clean up can be seen except for the high concentration spiking level. The extraction efficiency is generally higher for the spiked samples indicating that the incipient analytes are more difficult to extract than the spiked analytes. After the acid extraction procedure, approximately 80% of the total selenium content in the non-fortified samples was liberated. Comparison between the extraction efficiencies of selenium obtained by determination of total selenium after clean-up (Table 3, 2nd column) and by determination of each species, followed by summation of the different species (Table 4, right column), show good agreement for the original sample (≈45%). The extraction efficiencies for the spiked samples were somewhat lower using the speciation method compared to total selenium determinations. A likely explanation for this moderate difference would be the depression of the selenomethionine and selenite response in the speciation mode due to a co-eluting interferent. The very low recoveries obtained for selenocystine, see Table 4, (left column) suggest that this species is degenerated during the extraction procedure. It is however difficult to say where the product of selenocystine can occur in the chromatogram (Fig. 6) as no obvious unknown peaks appear. The recoveries of selenite are rather low (40–50%) and one possible explanation for this is the impaired chromatography, which makes the attempted quantification highly susceptible to bad accuracy[24] although the precision is reasonable. It should be noted here that the speciation results are based on the assumption that selenomethionine, selenite and

**Table 2** Figures of merit for the IC-MW-HGAAS system

| Species | Detection limit[a] ($3\sigma$ criterion)/ng $mL^{-1}$ | Characteristic mass, $m_0$/pg as Se | Linear calibrated conc. range/ng $mL^{-1}$ | $r^{2b}$ | Repeatability, peak area RSD (%) (n = 8)[c] | $t_R$/s |
|---|---|---|---|---|---|---|
| Selenomethionine | 1.4 | 15.5 | 7.9–97.1 | 0.996 | 4.4 | 104 |
| Selenite | 1.1 | 16.2 | 8.2–99.8 | 0.998 | 2.8 | 164 |
| Selenate | 1.6 | 15.2 | 8.2–100.3 | 0.996 | 4.6 | 284 |
| Selenocystine | 1.0 | 16.0 | 8.2–99.9 | 0.998 | 2.3 | - |

[a] Based on a 50 μL injection volume, see text for the definition of detection limit used. [b] A five point calibration curve ($n = 3$) was used. [c] Standards containing 34 ng $mL^{-1}$ of each selenium species were used to obtain these data.

**Table 3** Extraction efficiencies expressed as % of the total content of selenium in CRM 402 + spikes. All results obtained from determinations of selenium using ETAAS. The standard deviation, shown in parentheses, was calculated from all nine results obtained from each sample, *i.e.*, triplicate extractions and determinations

| | Extraction efficiency, (%) | | | |
|---|---|---|---|---|
| | After filtration | After clean-up[b] | After acid extraction | Total[c] |
| Sample | 45.9 (2.9) | 43.8 (1.9) | 33.7 (5.8) | 79.6 (6.5) |
| Sample + spike (2.7 μg)[a] | 64.0 (1.6) | 68.6 (1.5) | 28.0 (3.6) | 92.0 (3.9) |
| Sample + spike (5.2 μg)[a] | 73.1 (1.2) | 65.3 (4.7) | 24.8 (0.1) | 97.9 (1.2) |

[a] Total mass of selenium added to the sample. [b] Yield for sub-samples collected after clean-up on the C18 cartridge. [c] Calculated by summation of selenium masses determined after filtration and acid extraction.

**Table 4** Recoveries of the spiked selenium species from CRM 402, all extractions and determinations using the IC-MW-HGAAS system were performed in triplicate ($n$ = 3), uncertainty is expressed as one standard deviation of all nine measured values in each sample type, given in parentheses. Extraction efficiencies, expressed as % of total selenium content in the CRM 402, were calculated by summation of the determined concentrations of each selenium species. One standard deviation from the three extraction replicates of each sample type is given in parentheses

| | Recovery (%) | | | |
|---|---|---|---|---|
| | Spike 0.7 μg[a] | Spike 1.3 μg[a] | Extraction efficiency, (%) | |
| Selenomethionine | 92 (8) | 119 (11) | Sample | 45.7 (7.6) |
| Selenite | 41 (6) | 47 (7) | Sample + spike 0.7 μg | 51.7 (3.1) |
| Selenate | 92 (7) | 108 (9) | Sample + spike 1.3 μg | 61.3 (2.6) |
| Selenocystine | 4 (1) | 8 (1) | | |

[a] The samples (≈0.5 g) were spiked with ≈0.7 μg or ≈1.3 μg, as Se, of each selenium species determined.

selenocystine are not coeluting with any other selenium species. None of these species could be positively identified as discussed below.

**Species separation.** In Fig. 6 chromatograms of the extracts are shown. A large peak can be seen in the background signal at a retention time close to selenomethionine but also affecting selenite. This is certainly caused by ammonia present in the extracts, which probably also explains slight changes in retention times. The resulting lower resolution causes the peaks of selenomethionine (A) and selenite (B) to partly overlap.

To further control the identity of the selenium species separated, another system—AS-10 column and sodium hydroxide eluent—was used. Chromatograms are shown in Fig. 7 where (a) displays a separation of a standard solution. Selenocystine (C) shows a small peak compared to the other species. However, after selenate (D) has eluted, dilute $HNO_3$ was injected and two minor peaks were eluted. This shows that selenocystine probably is partly transformed during the chromatographic run. Chromatograms (b) and (c) show the unspiked

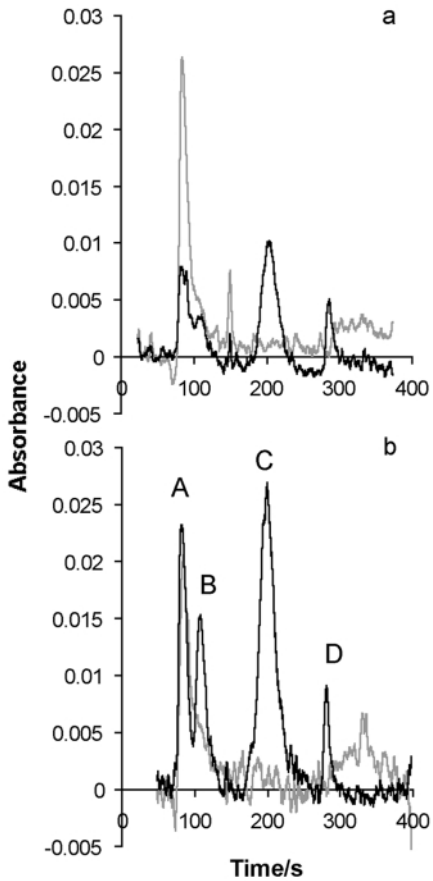


**Fig. 6** Chromatograms showing selenium specific absorbance (black line) and background absorbance (grey line) from extracts of CRM 402, see text for further details. Chromatogram (a) unspiked extract and (b) spiked extract with additions of 0.7 μg per species. The selenium species spiked to the sample were selenomethionine, selenite, selenate and selenocystine. The elution order shown in (b) was (A) selenomethionine, (B) selenite, (C) selenate and (D) selenocystine.

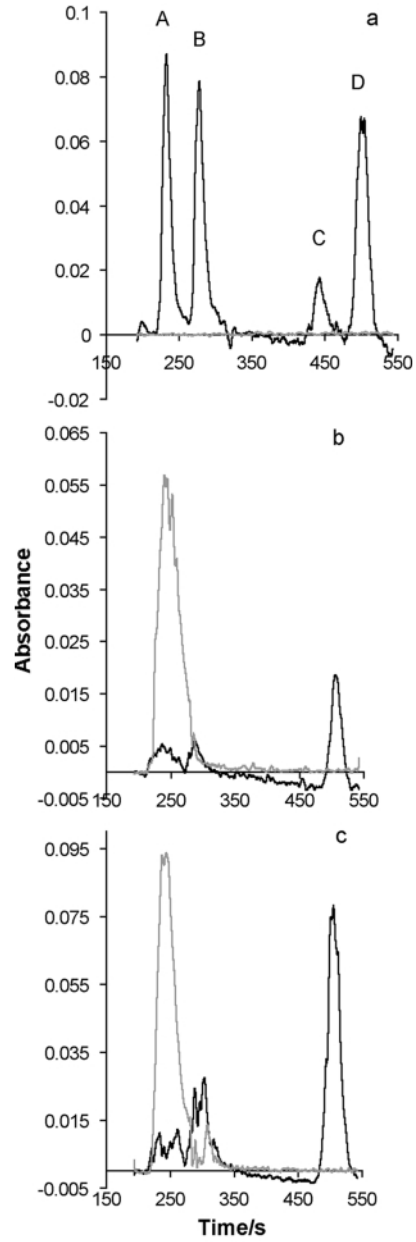


**Fig. 7** Chromatogram (a) shows the separation of a standard solution containing 67 ng mL$^{-1}$ of selenomethionine (A), selenite (B), selenocystine (C) and selenate (D) using the AS-10 column. Chromatograms (b) show the separation of the unspiked CRM 402 extract and (c) that of the spiked CRM 402 at the 1.3 μg per species spiking level. Black lines represent the selenium specific absorbance and the gray lines the background.

and spiked extracts, respectively. The extracts had been kept for 48 h in the refrigerator (+4 °C) prior to injection. This may have affected the results but anyway no trace of selenocystine can be discerned in these chromatograms: it may have degraded during the run since small peaks were eluted after injection of the acid similarly to what has been observed with the standard solution (see above). Furthermore signals for selenomethionine and selenite were severely impaired most likely caused by a coeluting interferent responsible for a high background absorbance peak. However it can not be ruled out that also these species have been affected by the storage of the extract.

From all these results it is obvious that the only species possible to positively identify and quantify in this sample, using this method, is selenate. Assuming a quantitative recovery for selenate (Table 4) the extractable amount of selenate in CRM 402 was determined to be $1.46 \pm 0.35$ μg $g^{-1}$ (95% confidence level) using the standard addition method. As the selenium content of CRM 402 is $6.70 \pm 0.25$ μg $g^{-1}$, it constitutes approximately 22% of the total selenium. The results obtained for the selenium speciation of CRM 402 are generally in accordance with Emteborg *et al*.[22] and Alsing Pedersen *et al*.[20] who also found selenate to be the only quantifiable species. It should also be noted that from the results obtained here (Fig. 7) selenomethionine is likely to be present in the extract, although further confirmation is needed. This was not the case in the other studies of this material.[20–22]

It must be emphasized that due to effects of the extraction procedure the speciation of selenium in refractory materials is operationally defined. This was recently shown by the use of different extraction methods applied to a yeast sample.[25] In the extraction method used here ammonia was used to liberate amino acid selenium species from protein binding sites. Ammonia may certainly also contribute to degradation of selenium compounds originally present in the sample. Further studies addressing these problems are currently being undertaken.

## Conclusions

The IC-MW-HGAAS system exhibits a relatively high complexity, which has been controlled by the use of multivariate methods for optimization. A potential interferent originating from the reagents used has been identified to most likely be bromine or products of reactions with bromine. Bromine is likely to have two counteracting effects on the responses from the IC-MW-HGAAS system, namely to increase the conversion rate of selenomethionine and eventually also selenate but also to decrease the hydride generation or atomization efficiency. The method shows low detection limits but is very sensitive to matrix interferences and therefore extensive clean up of extracts from real samples are necessary prior to determination. During extraction spiked analytes were revealed not to behave like the incipient analytes, which showed lower extraction efficiency. Selenate was quantified in the CRM 402 and the concentration was determined to represent about 22% of the total selenium content. The results from the speciation analysis of CRM 402 show good agreement with some of the previously published results in the same material. Further work is necessary to develop more efficient extraction methods for selenium speciation in biological materials.

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