ELSEVIER

Journal of Chromatography A, 807 (1998) 71–80

JOURNAL OF CHROMATOGRAPHY A

Application of capillary zone electrophoresis–inductively coupled plasma mass spectrometry and capillary isoelectric focusing–inductively coupled plasma mass spectrometry for selenium speciation

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Abstract

The high resolution potential of capillary electrophoresis (CE) makes CE techniques valuable for separations of selenium species, both, organic and inorganic. Such effective separations of charged species are possible using the CZE mode (Z = zone). Very powerful separations can be achieved by capillary isoelectric focusing, when analysing molecules showing different isoelectric points (p*I*) values, such as organic Se species. Inductively coupled plasma (ICP) MS is an element-specific multi-element detector, providing extremely low detection limits. The combination of CE with ICP-MS promises a powerful tool for metal speciation. Therefore, an online hyphenation of CE [using capillary zone electrophoresis (CZE) or capillary isoelectric focusing (cITP) mode] with ICP-MS, which was developed earlier, was modified and applied to selenium speciation. Separation was differentiated from the detection step during hyphenation. This resulted in short separation times (10 min) and a subsequent detection step lasting 100 s. Firstly, a CZE method was applied, providing a separation of six Se species of interest in one run [Se(IV), Se(VI), selenium carrying glutathione (GSSeSG), selenomethionine (SeM), selenocystine (SeC), selenocystamine (SeCM)]. This CZE method used an alkaline background electrolyte (Na_2CO_3/NaOH). The Se species were separated sufficiently from each other. Detection limits were calculated as 10 or 20 μg Se l^{-1} for inorganic Se species and 30–50 μg Se l^{-1} for organic Se species. The method was applied to standard mixtures and body fluids like human milk and serum. Secondly, a cIEF separation (pH range 2–10) was employed for organic Se species only in parallel. Detection limits were around 10–30 μg Se l^{-1}. The method was applied to standard mixtures and body fluids like human milk and serum. © 1998 Elsevier Science B.V.

Keywords: Human milk; Capillary electrophoresis–mass spectrometry; Isoelectric focusing; Selenium; Organoselenium compounds

1. Introduction

Selenium, both a toxic and an essential element, plays an important role in environmental analysis as well as in health studies. Excess Se intake can cause toxic reactions in living organisms [1]. Therefore, inorganic selenium compounds, mostly of anthropogenic origin and emitted into the environment [2], are of significant interest in environmental studies. On the other hand, selenium is an essential trace element for humans. Sufficient selenium supplementation can protect against several heart diseases and is discussed in cancer prevention [3,4]. Detoxificational effects of Se are proven and are described for several heavy metals [3,5]. Se deficiency can cause many diseases like haemolysis [6], muscular

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0021-9673/98/$19.00
PII: S0021-9673(97)01231-4

dystrophy [4,7,8], cystic fibrosis, congestive cardiomyopathy, Kashin–Becks disease, legionnaires disease, multiple sclerosis, myotonic dystrophy, neural ceroid lipofusicinosis and rheumatic arthritis [8]. Furthermore, thyroid metabolism seems to be impaired, because many deiodinases are Se-proteins [9].

Selenium is of particular importance for pregnant women, as the foetus receives a lot of Se during intra-uterine growth [8]. Se deficiency is most critical for the brain and growth of infants [9].

Biological and toxicological effects of Se are strongly dependent on its chemical form, specifically on its chemical 'species' [10–12]. Therefore, there is an increasing interest in the differentiation of selenium species (inorganic and organic).

The main analytical problems in Se species determination were recognised as a lack of efficient separation and poor sensitivity of determination [12]. Chromatographic separation techniques were already widely employed [13]. But for all separation procedures in speciation the most important condition is the minimisation of possible influences on existing binding forms [14,15]. This is a principle problem in speciation analysis. Chromatographic separations provide interactions of species with stationary and mobile phases, probably resulting in the destruction of complexes [15,16]. Thus, carrierless CE has principle advantages compared to liquid chromatography [15]. Several articles therefore were applying CE to Se-speciation problems [11,17,18]. There, analytical solutions were given to only some of the Se species, we were interested in our current studies. These species are selenite [Se(IV)], selenate [Se(VI)], selenocystamine (SeCM), selenocystine (SeC), selenomethionine (SeM) and selenium carrying glutathione (GSSeSG; oxidized form) [11,19]. In a former work [20] a capillary zone electrophoresis (CZE) method was developed to separate the six Se species of interest because former methods, described in literature, did not match all our requirements:

Ref. [11] analysed only organic Se species in different CZE runs without element selective detection. Refs. [18,21] included only inorganic Se species, but both were already using an online coupling of CE to inductively coupled plasma (ICP) MS. Ref. [17] used only conventional CE for only four Se species.

Preliminary investigations [20] were still in the developing stage and were focusing on an optimisation of the CZE method and the coupling to ICP-MS.

Now, in this subsequent work the method has been perfected and applied to aqueous solutions as well as to 'real' samples like human milk or human serum.

In such matrices however, predominantly organic Se species are present and are important for essentiality. Therefore, a completely different separation technique [capillary isoelectric focusing (cIEF)] was developed in parallel in this work, aiming to discover isoelectric point (p*I*) properties of organic species. IEF is known for its extremely high resolving power [22,23]. A chosen pH gradient (e.g. pH 2–10) is spread along the total capillary length, resulting in high resolution. cIEF has already been used in quality control experiments for speciation [14]. A major advantage is the increase of sample volume by filling nearly the whole capillary with sample, thus decreasing concentration detection limits. The two-step procedure, used for ICP–MS coupling is derived from detection steps of cIEF [22,24–26] and predestines this separation method for hyphenation.

In this work, cIEF interfaced to ICP-MS was firstly applied to single standards and standard mixtures of the organic Se species of interest. Subsequently, the method was applied to real samples like human milk or human serum in parallel to the CZE method.

2. Experimental

2.1. Sample preparation

2.1.1. Standards

Se(IV) and Se(VI) stock solutions as well as SeCM, SeC and SeM stock solutions (final concentration each: 0.5 g $Se\,l^{-1}$) were prepared by dissolving K_2SeO_3, $Na_2SeO_4 \cdot 10H_2O$, selenocystamine·2HCl or seleno-DL-cystine in Milli-Q water. Seleno-DL-methionine was dissolved in 20 m*M* KOH as it is nearly insoluble in pure water. GSSeSG was prepared according to the reaction given in [27] and already used in [20]:

$$\text{Selenite} + 4\,\text{GSH} \rightarrow \text{GSSeSG} + \text{GSSG}$$

where GSH = glutathione (reduced form) and GSSG = glutathione (oxidized form).

• CZE mode

When using CZE mode for interfacing CE with ICP–MS, a 10 m*M* Na_2CO_3 (adjusted to pH 11.5 by KOH) electrolyte was used for dilution to final concentrations. The concentrations were 150 μg (Se) l^{-1} for standard mixtures (also for species identifications by standard addition), or 0, 50, 100, 500, 1000 (each μg l^{-1}) for calibration curves [20]. Calibration curves were performed to check the linear detection range and to get an estimation of concentrations in 'real' samples. Quantitations were performed by standard addition procedure (adding one-, two- and three-fold of estimated Se species amount to the sample).

• IEF mode

For cIEF coupling, standard solutions of organic Se species were diluted to a concentration of 300 μg (Se) l^{-1} with Milli-Q water and an addition of 'Ampholytes' (Bio–Rad, final concentration 2%) for single standards and standard mixtures. The ampholytes provided a pH gradient within the capillary after application of voltage. Se species then were moving to their individual isoelectric points, thus being separated from each other.

Detection limits for both methods (CZE, cIEF) were calculated according to IUPAC recommendations (3σ criterion [28]).

2.1.2. Real samples

Sampling of the human milk was carried out as described in [29]. After sampling, the human milk samples were pooled (different volunteer women, healthy, seventh to fourteenth day after delivery) and frozen immediately for storage at −20°C. The processing of human milk followed the descriptions in Ref. [11]: Pooled human milk was defatted, and milk proteins were precipitated by centrifugation (25 840×*g*, 30 min, 8°C). The supernatant was used for CE investigations. One ml aliquots of the human milk supernatant were preconcentrated by freeze drying (sample temperature controlled at 0°C) and resuspending in 100 μL of 10 m*M* Na_2CO_3 (CZE mode) or Milli-Q water with 2% ampholytes (cIEF mode).

Sampling of blood and the preparation of serum was performed analogous to the method in Ref. [30]: Blood samples were kept standing for 30 min after sampling from healthy volunteers and subsequently centrifuged (1 250×*g*, 20 min). Serum resulted as the supernatant and aliquots were frozen immediately at −20°C for storage. For speciation experiments serum aliquots were thawed and diluted 1: 1.5 (v:v) with 10 m*M* Na_2CO_3 (CZE mode) or Milli-Q water with ampholytes (final ampholyte concentration = 2%, cIEF mode).

2.2. Capillary electrophoresis

A Biofocus 3000 capillary electrophoresis system (Bio–Rad, Munich, Germany) was used as the CE device. The temperature was set to 20°C for sample carousel (air cooling) and capillary (liquid cooling) during all experiments.

The CZE method used Na_2CO_3 (10 m*M*, adjusted to pH 11.5 by KOH) as the electrolyte and an uncoated capillary (150 cm×50 μm I.D.). Before each run, the capillary was purged with Milli-Q water (150 s, 8 bar) and electrolyte (150 s, 8 bar). Sample injection was carried out by pressure (8 bar, 10 s). The applied voltage was set to +18 kV, resulting in an electrophoretic mobility of anions towards the injection point, but also in a faster endosmotic flow (EOF) towards the detector. Separation was stopped after 10 min. Detection was carried out by pressing Milli-Q H_2O into the capillary (130 s, 8 bar) and forcing separated molecule bands to the nebuliser ('detection step'). Previous experiments [21] showed that it takes 120 s at 8 bar to move molecules from the capillary inlet to the ICP–MS system). This two-step procedure was successfully employed already in extensive CE–ICP–MS experiments for Se and Pt speciation [20,21,31] and was derived from cIEF (without coupling to an element selective detector) [22,24–26].

The cIEF method used 100 m*M* NaOH as the catholyte at capillary inlet and 100 m*M* H_3PO_4 as the anolyte at the capillary outlet/nebuliser [22,23]. The capillary (120 cm×50 μm I.D.) was coated, being a necessary prerequisite for cIEF [23]. Before each run, the capillary was purged with Milli-Q water (150 s, 8 bar) and 2% ampholyte solution. Sample injection was carried out by pressure (8 bar, 60 s). The applied voltage was set to −18 kV, resulting in a pH gradient from pH 10 at the inlet (cathode) to pH 2 at the nebuliser (anode). Focusing

was stopped after 7 min. Mobilisation was carried out by pressing Milli-Q water into the capillary (130 s) and forcing separated molecule bands to the nebuliser in analogy to the CZE-detection step [21].

2.2.1. Hyphenation and nebulisation

For temperature control of the capillary, a 'user assembled cartridge' (Bio–Rad) was used. This cartridge leads the coolant coaxially around the capillary up to the nebuliser (and back again into cooling circuit). Temperature control of the total capillary was necessary for high reproducibility and precision [31–33].

The modified Meinhard nebuliser was home-made and especially designed for the requirements of the CE–ICP-MS interface [21]. Special care was focused on an exact and optimised positioning of the capillary end by turning a nut and on a reliable closing of the CE electrical circuit during nebulisation. A coaxial sheath flow around the CE capillary [10 m*M* Na_2CO_3 (CZE) or anolyte (cIEF)] showed a flow-rate of 10 $\mu l\,h^{-1}$ and provided the electrical connection from the capillary outlet to the outlet electrode in a buffer reservoir at the end of the nebuliser [21]. The newly developed spray chamber was specially designed considering the nebulisation characteristics of the CE–ICP-MS nebuliser: An additional Ar/H_2-gas stream was directed to the torch of ICP-MS and was 'coating' the inner surface of the spray chamber. This avoided an aerosol condensation at the walls of the chamber and the aerosol was transported completely into the plasma [20].

2.3. Quality control: Estimation of a possible suction driven flow by nebulisation gas stream and species stability

A liquid flow through the capillary, produced by a suction from the nebulisation gas stream would be very undesirable during the separation or focusing. The performance of CE separations, especially isoelectric focusing, could be decreased drastically. For estimation of this possible flow, experiments were carried out in analogy to the experiments in Refs. [21,33].

According to Ref. [31], several experiments were carried out to investigate, whether species were destroyed during separation or new species were generated. Newly generated species, migrating faster than the original ones, could reach the ICP-MS during the separation step. Therefore, ICP-MS was monitoring also the separation step.

For elucidating systematic signals, probably produced by the total device (e.g. 'start peaks', peaks when switching from separation mode to detection mode), runs without sample injection were carried out and monitored by ICP-MS during separation and detection steps [21,31].

2.4. ICP-MS

The ICP-MS system (ELAN 5000, Sciex, Canada) was used in the graphic mode with the homemade nebuliser fitting into the new, homemade spray chamber. The very small amount of aerosol coming from the CE capillary is transported more efficiently to the Ar plasma. Se was determined at $m/z=78$ and 77 incessantly for monitoring possibly occurring interferences (altering the signal ratio of $^{78}Se/^{77}Se$). Instrumental parameters were derived from [34] with following modifications:

RF power: 1200 W; nebuliser gas: Ar 0.85 $l\,min^{-1}$; auxiliary gas for spray chamber: Ar/H_2 0.2 $l\,min^{-1}$. The total gas flow (nebuliser gas + auxiliary gas) was 1.05 $l\,min^{-1}$, being again in the optimal range already found in former investigations [21,33]. Dwell time: 50 ms.

2.5. Chemicals

The uncoated capillary was from Laser 2000 (Wessling, Germany) and the coated one from Bio–Rad. Ampholytes, anolyte (H_3PO_4) and catholyte (NaOH) were purchased from Bio–Rad, whereas acetic acid and Na_2CO_3 were obtained from Merck (Darmstadt, Germany). The Se species standards (selenite, selenate, SeCM, SeC, SeM) and GSH were purchased from Sigma (Munich, Germany). Argon and Ar/H_2 were bought from Messer–Griesheim (Munich, Germany).

3. Results

Firstly, the quality control investigations were performed concerning a suction flow and species

stability during separation and detection. These experiments proved that no suction driven flow was altering the separation during nebulisation. Further, Se species were neither impaired by the CZE methods nor by cIEF separation: No Se compounds were seen during the separation step at ICP-MS nor in the inlet vials. There, Se could have been monitored when Se species alteration or degradation would have occurred.

Secondly, the CZE method was applied on single standard solutions for determination of detection limits and calibration graphs. The detection limits (3σ criterion according IUPAC recommendations) were determined in the low ppb range for all Se species: inorganic Se species at 10–20 μg (Se) l^{-1} and organic Se species at 30–50 μg (Se) l^{-1}. Linear calibration graphs (Five points, see Section 2) were carried out for the Se species and resulted in regression coefficients ranging from 0.997 (SeM) to 0.999 [Se (VI)]. Migration times (time of appearance during the detection step) were reproducible for single standard runs ($n=5$), but were shifted when performing mixtures. However, when keeping species concentrations below 300 μg l^{-1}, cross interferences between standards remained negligible. Se species still were clearly separated. As an example, Fig. 1 shows the performance of inorganic Se species (upper) and of organic ones (lower), monitored by ICP–MS. The high resolution of the method is demonstrated as well as good peak shapes. Peak widths are ca. 3.5 s for each species. Detection times in mixed samples were determined at 6 s (SeCM), 13 s [Se(VI)], 20 s (SeC), 26 s [Se(IV)], 33 s (SeM), 45 s (GSSeSG).

The next step for elucidating the capabilities of the CZE method, was the application of real samples. For this purpose, both, a preconcentrated low-molecular-mass fraction of human milk and a serum sample were used. In primary experiments, the total Se amount of the human milk fraction was determined (15.8±0.9 μg Se l^{-1}), resulting in 158 μg Se l^{-1} after ten-fold preconcentration.

Fig. 2 shows an electropherogram of the preconcentrated human milk fraction. Three peaks are detected at 7 s, 20 s and 47 s. An identification of the Se species was achieved, when analysing the fraction after standard additions of Se species (not shown). These electropherograms pointed to SeCM (peak at 7 s), SeC (peak at 20 s) and GSSeSG (peak at 47 s) as

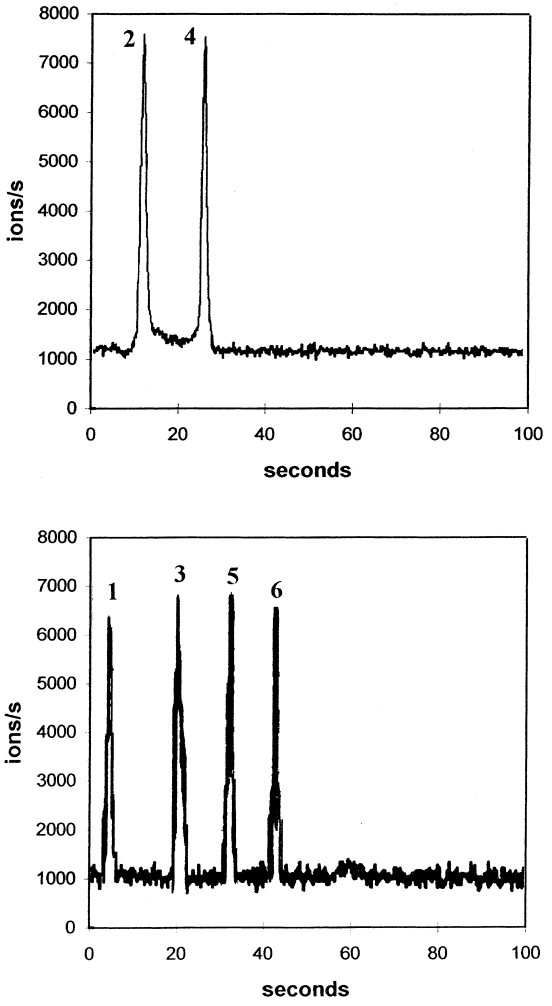

Fig. 1. Separation of inorganic (upper) and organic (lower) Se species, each 150 μg Se l^{-1}. Only the ^{77}Se isotope is shown, as the ^{78}Se isotope is interfered by polyatomic interferences more frequently. This results in 'pseudo-Se-signals' and decreases the signal-to-noise ratio. The species are monitored at (1) 6 s (SeCM), (2) 13 s (Se (VI), (3) 20 s (SeC), (4) 26 s (Se (IV), (5) 33 s (SeM) and (6) 45 s (GSSeSG).

Se species in this fraction. The addition of SeM was generating a new peak at 33 s, thus not being identified in the original sample.

Unfortunately, the variation of peak areas was considerably high in consecutive runs ($n=4$, mean area±60%, without standard addition), as the Se concentrations per peak/species were at/close to detection limits. Thus, quantification was hardly performed at such low concentrations and in a rough matrix like preconcentrated human milk. But an

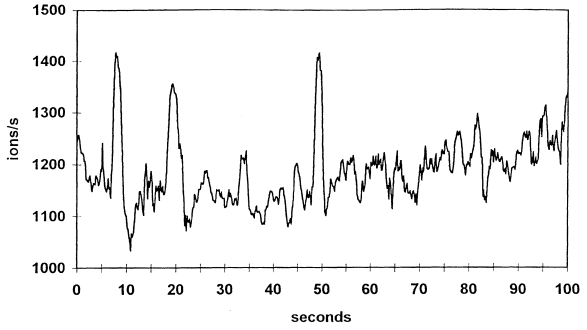

Fig. 2. A CZE–ICP-MS electropherogram is shown (only $m/z=77$ is shown for the same reasons given in legend Fig. 1) from a human milk fraction after ten-fold preconcentration. The found Se species are still close to detection limit. Identification and quantification of species was done by standard additions (GSSeSG, SeCM, SeC). The results fitted satifactorily with earlier findings (see text and Table 1, [36]).

estimation of Se concentrations per peak still was possible when comparing peak areas with calibration curves of corresponding Se species or after standard addition procedure (one-, two- and three-fold addition of estimated value). The concentrations for peaks 1–3 were ca. 50 $\mu g\,l^{-1}$, 36 $\mu g\,l^{-1}$ and 45 $\mu g\,l^{-1}$ in the preconcentrated sample (or calculated for the original sample without preconcentration: 5.0 $\mu g\,l^{-1}$, 3.6 $\mu g\,l^{-1}$ and 4.5 $\mu g\,l^{-1}$). When calculating mass balances, the sum of estimated Se species concentrations (ca. 131 $\mu g\,l^{-1}$) was compared to the concentration of the whole preconcentrated milk fraction (158 $\mu g\,l^{-1}$), resulting in a estimated recovery of around 83%.

Human serum was taken as another ‘real sample’. Fig. 3 demonstrates an electropherogram of human serum ($n=4$ replicates). Several Se species are resolved, the most prominent e.g. appearing at 5 s, 11 s, 14 s, 23 s, 33 s, 51 s, 55 s, 107 s and 119 s. When performing standard addition procedure for identifications, several peaks were increasing in height: the peak at 11 s after SeC addition, the peak at 14 after Se (IV) addition and the peak at 33 s after GSSeSG addition. This demonstrates that detection times of these species are markedly shifted in serum! The addition of other Se species created new peaks, thus, not being identified in the original sample. The identification of further Se peaks in the electropherogram still remains to be done. As the serum experiments are still in a preliminary state and most

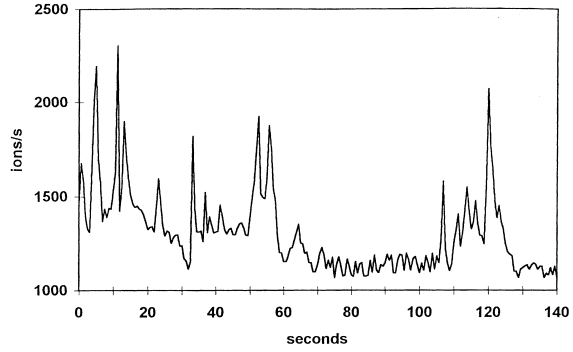

Fig. 3. A CZE–ICP-MS electropherogram from a human serum is shown (only $m/z=77$ is shown for the same reasons given in legend Fig. 1). Many peaks are seen, of which only some are identified up to now (see text).

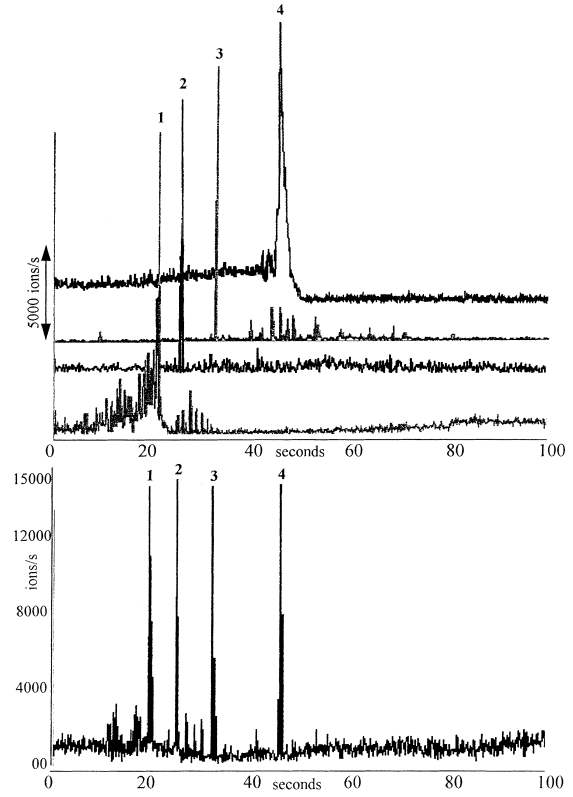

Fig. 4. Application of single standards (upper) or a standard mixture (lower) is demonstrated (only $m/z=77$ is shown for the same reasons given in legend Fig. 1), using the cIEF–ICP-MS method. Species concentrations: 300 $\mu g\,l^{-1}$ each. Detection times: (1) SeM: 21 s, (2) GSSeSG: 26 s, (3) SeCM: 32 s, (4) SeC: 45 s. Polyatomic interferences are seen also in the ^{77}Se-trace, mainly in the SeM and SeCM electropherograms as small minor/side peaks.

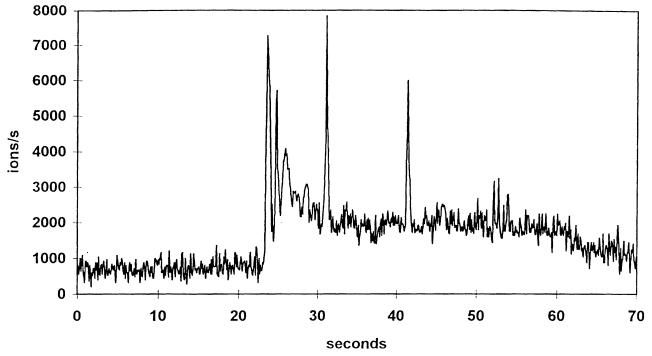

Fig. 5. A cIEF–ICP-MS electropherogram is shown (only $m/z=77$ is shown for the same reasons given in legend Fig. 1) from a human milk fraction after preconcentration. The found Se species are still not too far from detection limit. Identification and quantification of species (GSSeSG, SeCM, SeC) was performed by standard addition procedure. The results fitted well with the CZE–ICP-MS method and earlier findings (see text and Table 1, [36]). At least two unidentified species are seen additionally (e.g. at 26 s, 27 s).

of the peaks remained unidentified, quantitative considerations were postponed.

As a third step, the cIEF–ICP-MS experiments were performed. Here, only organic Se species were investigated. Single standard runs firstly were used to elucidate detection times during detection step as well as determination of detection limits (3σ criterion according IUPAC recommendations).

Fig. 4 shows cIEF electropherograms (ICP-MS) of single Se species (upper) and of a mixture (lower). The species were monitored at 21 s (SeM), 26 s (GSSeSG), 32 s (SeCM) and 45 s (SeC) with peak widths of 1.5 s (SeM, GSSeSG, SeCM) or 3.5 s (SeC) in single applications or ca. 1.5–2 s in mixtures. Detection limits were found in the range 10–30 μg $Se\,l^{-1}$, thus being slightly decreased compared to the CZE method (30–50 μg $Se\,l^{-1}$).

The next step was the application of the method to real samples. Again the ten-fold preconcentrated low-molecular-mass fraction of human milk (158 μg $Se\,l^{-1}$) and a serum sample were chosen.

Fig. 5 shows the mobilisation of the preconcentrated human milk fraction after 7 min focusing. Five peaks were detected at 25 s, 26 s, 27 s, 32 s and 43 s. An identification of the Se species by standard additions of Se species pointed to GSSeSG (25 s), SeCM (32 s) and SeC (43 s). SeM was not seen in the sample, but at least two minor peaks (26 s and 27 s) remained unidentified. Quantitation resulted in concentrations (±S.D.) of 63±6 μg $Se\,l^{-1}$ (GSSeSG), 53±6 μg $Se\,l^{-1}$ (SeCM) and 38±8 μg $Se\,l^{-1}$ (SeC) for identified peaks and ca. 47 μg $Se\,l^{-1}$ as a sum for peaks at 26 s and 27 s. This resulted in a mass balance of 127±18% compared to the total Se amount.

The next application was performed with human serum as another ‘real sample’. Fig. 6 demonstrates mobilisation of human serum ($n=3$ replicates) after 7 min focusing. Several Se species are seen, e.g. appearing between 11 s and 23 s or at 27 s, around 35 s and 40 s or at 49 s and 56 s. Identifications by standard additions pointed to GSSeSG at 27 s and SeC at 49 s. This indicated an alteration of the pH

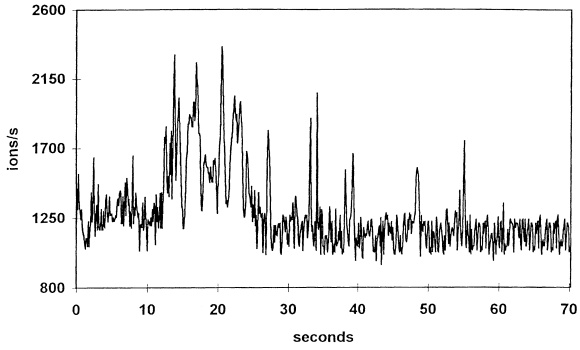

Fig. 6. A cIEF–ICP-MS electropherogram is shown (only $m/z=$ 77 is shown for the same reasons given in legend Fig. 1) from human serum. A multitude of peaks is seen, of which only some are identified up to now (see text). Due to the low total Se concentration (70 μg $Se\,l^{-1}$) being distributed to the high number of Se species, single species Se concentrations are low and at detection limits.

gradient in the capillary according to the very high buffering capacity of serum and the relatively low concentration of ampholytes in this sample. The identification of further Se peaks in the electropherogram still remains to be done. For the same reasons, as above, quantitation was postponed to future experiments.

4. Discussion

The speciation of Se is of growing interest, due to its toxic (excess concentrations) and essential (physiological concentrations) properties [2–4,12]. An important analytical prerequisite is an efficient separation [12] as possible with less interaction with stationary phases [15]. Therefore, the development of CE methods is of great importance, especially when using element selective detection systems [17]. The presented work demonstrates (a) a CZE separation method coupled to ICP-MS for six Se species (inorganic/organic) and (b) the application of the cIEF method coupled to ICP-MS for organic Se species, both methods applied to aqueous solutions and real samples with rough matrices.

The on-line coupling of CE to ICP-MS was set up analogous to previous investigations [20,21,31]. An important modification was brought by the new spray chamber, which was stabilising signal intensity especially at low concentrations and (thus) decreasing detection limits.

Quality control investigations were performed analogous to earlier hyphenation experiments [21,31,33] and lead to analogous satisfactory results: No interfering suction flow was seen and Se species stability was guaranteed during the analytical procedures. The lack of a suction flow was of specially importance for cIEF coupling experiments.

Two isotopes (^{77}Se, ^{78}Se) were monitored and signal ratio of both calculated for peaks (^{77}Se: ^{78}Se = 1: 3.1). This was carried out for elucidating signal alterations by polyatomic interferences. Both isotopes are known for being interfered e.g. by Ar–K, Ar–Cl or Ar–Ca and Ar–Ar [35]. When a isotope ratio of 3.1 is seen such interferences are excluded, whereas different ratios indicate an interference resulting in ‘pseudo Se signals’.

The performance of the CZE-hyphenation was first checked and compared with results obtained at the end of the developing stage [20]. The detection limits and detection times of the Se species were again reproduced and the signal stability and resolution were slightly improved compared to those in [20]. As a next step, the application to complex, real matrices was possible. These matrices (human serum and a human milk fraction) were taken out of other speciation projects, thus being in part, already characterised, and were used as a basis for method validation. The total Se content of the used human milk was determined previously [36] (15.8 μg Se l^{-1}) with single species concentrations given in Table 1. The concentrations here were below concentration detection limits for these Se species. Therefore, the sample had to be preconcentrated. A major difficulty for analysing that preconcentrated sample was its high viscosity, resulting in inhomogeneity problems and problems for reproducible injections. These problems, as well as the concen-

Table 1
Comparison of Se species concentrations in a human milk fraction

Se species	c_{Se} (SEC–ICP-MS [36])[a]	c_{Se} (CZE–ICP-MS)[a]	c_{Se} (cIEF–ICP-MS)[a]
GSSeSG	7.1±0.4	4.5	6.3±0.6
SeCM	4.2±0.8	5,0	5.3±0.6
SeC	3.5±0.3	3.6	3.8±0.8
SeM	1.0±0.2	n.d.	n.d.
Other	n.d.	n.d.	4.7
Total Se	15.8±0.9	13.1	20.1±0.8

This fraction was characterised earlier [36]. Results of both hyphenation techniques are compared with these earlier results for validation.
[a] The values are given in μg Se l^{-1} ± S.D., (n.d. = not detected). No S.D. values are given for CZE–ICP-MS and ‘other’ species (cIEF–ICP-MS), as these values are at the detection limits of these species (CZE–ICP-MS) or not identified (cIEF–ICP-MS). Therefore, these concentrations have to be considered as ‘estimated’ (according to IUPAC, see Section 3).

trations still being at/close to detection limits may be the reason for some species concentrations being slightly different to formerly determined values and to cIEF determinations (c.f. Table 1). On the other hand it is striking that three of four Se species in the human milk fraction were found and inorganic Se species again were not detected. This corresponds to earlier findings [36]. The fourth Se species (SeM) was five times below its detection limit even after preconcentration [36].

According to Gauss' law, the analytical error at the detection limit must be at least 60%. Taking this into consideration, the results were quite satisfactorily matching earlier findings. The estimated mass balance of ca. 83% was acceptable, as the found species were close to their detection limits. Thus, the reliability of the method was satisfactory even in a rough matrix.

The method was also applicable for serum and demonstrated a high resolution. At least ten different species were monitored. This high number of Se compounds in human serum and tissue is known in literature. The Se species are participating in widespread Se-metabolism, having many intermediate Se molecules [27,37]. The identification of these Se species still remains to be done, e.g. by parallel runs of CE-electrospray (ESI) MS. Quantifications of species principally are only useful when carried out in correspondence to species-specific calibrations [38] and therefore are only possible after identification. Thus, this point was postponed to future investigations.

The newly developed cIEF–ICP-MS hyphenation is still in the development stage. However, the results are promising. In general, cIEF is well known for its high resolution power [22,23,25,26]. The resolution is very efficient as shown (e.g. in Fig. 4) and can be further increased, when choosing a smaller pH range, but using the same or increased capillary length. For the investigations shown in this work, a pH range of 2–10 was built up in a 120 cm capillary: The capillary was filled with 2% ampholyte solution (ca. 25% of total capillary length) and sample/2% ampholyte solution (ca. 75% of total capillary length). By filling the capillary totally with sample or using longer capillaries in future investigations, the total sample intake will be further increased in parallel to a decrease of relative detection limits.

However, some alterations are seen in electropherograms of SeM and SeCM, coming up as increased noise (SM: 0–20 s) or small side peaks (SeM: around 30 s and SeCM: 40–50 s). The noise (SeM) was proven to be an impurity of the inorganic Se species remainder, drifting at the front of the pH gradient after focusing. Side peaks (SeM and SeCM) were recognised as polyatomic interferences by 'wrong' isotope ratios.

The application to human milk resulted in clear, highly resolved peaks, of which three were identified and quantified, but minor peaks remained unidentified. Again, SeM was not found (although it was determined in earlier investigations), as its concentration was below detection limit even after preconcentration. But quantification of known species fitted well with earlier size-exclusion chromatography (SEC)–ICP-MS determinations and mostly also with CZE–ICP-MS results (c.f. Table 1).

When looking at the serum electropherogram, two findings are striking: (a) the high number of Se species and (b) the signals being close to noise. The multitude of Se species found corresponds principally with CZE findings and literature data [27,37]. Besides, the increased separation of cIEF gave reason for further resolution of Se species. The total Se content of serum (ca. 70 μg Se l^{-1}) is distributed to many individual Se species. The concentrations of these species are obviously around their individual detection limits, resulting in signals close to noise.

Summarising, both coupling methods were very sensitive and highly resolving. The CZE–ICP-MS method demonstrated its advantage in analysing six Se species, including two inorganic ones. The cIEF–ICP-MS technique principally has decreased detection limits due to higher sample intake and an increased resolution.

Future investigations are planned for more routine applications and for parallel runs of CZE–ESI-MS/cIEF–ESI-MS applications.

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