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# Capillary electrophoretic methods for a clear identification of selenoamino acids in complex matrices such as human milk

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**Abstract**

Selenoamino acids from size-exclusionchromatographic fractions of human milk were identified and determined by capillary electrophoresis (CE). For this purpose, different CE methods were developed to separate these selenoamino acids from other molecules with similar molecular masses. Methods were introduced for the clear identification of the analytes. These methods were designed to allow the identification of selenoamino acids in spite of the presence of many molecules with similar mobilities. Further, they overcome identification problems caused by shifts of migration times (due to different ionic compositions) compared with standard solutions.

## 1. Introduction

The high separation potential of capillary electrophoresis (CE) covers a broad field of applications and supplements other separation methods such as HPLC. The benefits make CE techniques of interest in speciation investigations. There, the organic binding partners of metals have to be identified, e.g., by capillary zone electrophoresis (CZE) [1].

Selenium is an essential trace element for humans. Sufficient selenium supplementation can protect against several heart diseases and has been discussed in prevention against cancer [2,3]. Newborns show very low serum selenium levels, which decrease during the first year of life [3]. The bioavailability of an element (here Se) is dependent on its binding form (species) [1]. Therefore, a clear identification and determination of such species in human milk is necessary. Fig. 1 shows the general structure of a selenoamino acid.

When analysing body fluids or size-exclusion chromatographically (SEC) separated fractions, e.g., from human milk by CZE, even baseline-separated molecules usually show nearly identical migration times ($t_m$). Further, molecules of interest often are present in low concentrations, probably migrating close to highly concentrated substances.

Migration times are altered by changes in ionic strength of buffers and samples [4–7]. Migration times of low-concentration molecules are influenced by closely migrating high-concentration compounds. Therefore, comparing standard $t_m$ values with sample peak $t_m$ values can lead to erroneous identifications [6–8].

The adjustment of ionic strength by mixing samples with a small aliquot of CE buffer is not sufficient when analysing samples with very high

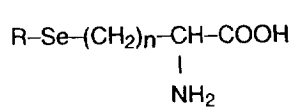


Fig. 1. General structure of a selenoamino acid. As an example, R = $CH_3$ and $n = 2$ for SM.

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ionic strength. Large additions of buffer are not possible owing to sample dilution, which may bring low-concentration molecules below the determination limit. Consequently, $t_m$ values in samples and standard solutions are different and a clear identification is no longer possible. Quantification may also be problematic owing to alterations in peak area for the same reasons.

Here, an easy and efficient method is described for identifying low-concentration amino acids in human milk despite $t_m$ alterations from sample to sample. Different levels of security in identification are introduced. Determination of the molecules of interest can easily be done in parallel.

## 2. Experimental

### 2.1. Sampling

Sampling of human milk was carried out as described by Schramel et al. [9]. The tubing of the manual pump and the sampling vessels were cleaned with nitric acid and doubly-distilled water to avoid the contamination that can occur with commercially available pumps.

### 2.2. Sample preparation

Pooled human milk (second to seventh day after delivery) was defatted and milk proteins were precipitated by centrifugation (25 840 *g*, 30 min, 8°C), as described elsewhere [10]. The supernatant was injected into the LC system for SEC fractionation.

### 2.3. SEC fractionation and fraction treatment

The SEC fractionation was carried out on a metal-free Econo-System (Bio-Rad, Munich, Germany). The mobile phase was doubly distilled water at a flow-rate of 3 ml/min. The chromatographic column (500 × 50 mm I.D.) was metal-free, filled with Toyo Pearl TSK HW 40 as the stationary phase and temperature controlled at 20°C. A UV detector monitored the separation at 232 nm.

*Fraction treatment*

Fractions were collected at 5-min intervals with a Model 100 fraction collector (Pharmacia). The fractions were frozen at −20°C and subsequently freeze-dried. The dry powder was resuspended with doubly-distilled water (500 μl) and used for the Se determinations (350 μl) and for different CE determinations (150 μl).

### 2.4. Capillary electrophoresis

A Biofocus 3000 capillary electrophoresis system (Bio-Rad) was used for the CE experiments. The capillary and carousel temperature was 20°C. Two CZE methods (A and B) were developed for each of GSH, Se-CM, SC and SM (Table 1).

A standard addition of the analytes improved analyte identification and quantification. Fig. 2 shows the flow chart of consecutive investigations. An increasing security in identification of the investigated compounds is achieved by the combination of different methods (SEC, CZE methods A + B) for identification, obtaining increasing levels of security (ISL = identification security level).

### 2.5. Se determination

Se determinations were carried out by inductively coupled plasma mass spectrometry with an instrument from Perkin-Elmer (Überlingen, Germany) coupled with a graphite furnace using the standard addition method for quantification. Electrothermal vaporization was chosen to overcome the well known polyatomic interferences.

### 2.6. Chemicals

TSK-gel (ToyoPearl HW 40 S) was obtained from Toso Haas (Stuttgart, Germany). GSH, selenocystamine (Se-CM), selenocystin (SC) selenomethionine (SM) were purchased from Sigma (Munich, Germany). The capillaries and borate and phosphate buffer (pH 2.5) were bought from Bio-Rad and $H_2PO_4^-$–$HPO_4^{2-}$, acetic acid and sodium acetate from Merck

Table 1
Parameters of the different CZE methods

| Analyte | Method | Capillary[a] (cm × μm I.D.) | Buffer | Polarity |
|---|---|---|---|---|
| GSH | A | 24 × 25 (c) | Borate buffer, pH 8,5 (Bio-Rad 148-5023) | −/+ |
| | B | 24 × 25 (c) | $H_2PO_4^-$–$HPO_4^{2-}$, 100 m*M*, pH 6.0 | −/+ |
| Se-CM | A | 50 × 50 | Borate buffer, pH 8.5 (Bio-Rad 148-5023) | +/− |
| | B | 50 × 50 | Na acetate, 100 m*M*, pH 5.5 | +/− |
| SC | A | 24 × 25 (c) | Phosphate buffer, 100 m*M*, pH 2.5 (Bio-Rad 148-5010) | +/− |
| | B | 24 × 25 (c) | Acetic acid, 1%, pH 1.9 | +/− |
| SM | A | 24 × 25 (c) | Phosphate buffer, 100 m*M*, pH 2.5 (Bio-Rad 148-5010) | +/− |
| | B | 24 × 25 (c) | Acetic acid, 1%, pH 1.9 | +/− |

Additional common parameters: injection by pressure, $1.03 \cdot 10^3$ Pa s; voltage, 10 kV; temperature, 20°C (sample carousels and capillary); wavelength, 200 nm; purge between runs, (1) water, 60 s, and (2) buffer, 90 s.
[a] (c) = coated.

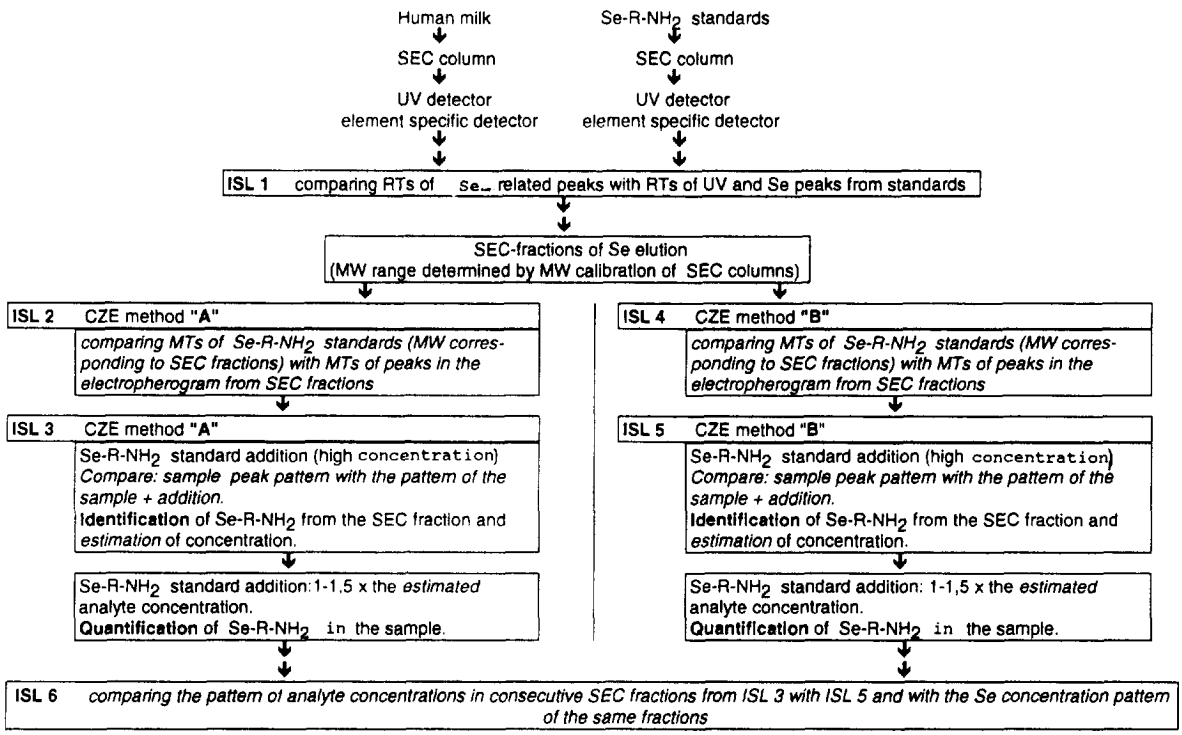


Fig. 2. Scheme of consecutive analytical steps for identification obtaining increasing levels of security. RT = Retention time; MT = migration time; MW = molecular mass.

(Darmstadt, Germany). The Se standards were purchased from Aldrich (Steinheim, Germany).

## 3. Results

The electropherogram of an individual SEC fraction showed good reproducibility for several replicates ($n = 5$), but a $t_m$ shift compared with standards was observed. This shift changed on analysing other fractions, owing to their different compositions. Therefore, the identification of an Se-R-$NH_2$ by comparing the standard CZE $t_m$ with the peak CZE $t_m$ in SEC fractions (ISL 2) was impossible.

Fig. 3 shows the analysis (methods A and B) of an SEC fraction containing GSH. A multitude of peaks migrated around the standard $t_m$ and a slight $t_m$ shift was obvious.

For Se-CM, SC and SM, no peaks were detected at the standard $t_m$ values in the SEC fractions where the elution of these molecules was expected from $M_r$ calibration of the SEC column (SM, Fig. 4d and e; Se-CM and SC, not shown). On the other hand, a peak with the SM standard CZE $t_m$ was seen in SEC fractions of higher molecular mass (Fig. 4a and b).

The addition of analyte standards to the samples (ISL 3) showed marked $t_m$ shifts in these samples. Now several sample peaks from different fractions could be correlated with analyte standards. These sample peaks "grew" with the standard additions, thus being identified by ISL 3 and ISL 5 procedures. Fig. 3b and c and 3e and f show the "growth" of the GSH peaks by methods A and B and Fig. 4e and f demonstrate the increasing peak height of SM.

The absence of the analytes in the resting SEC fractions was proved when a new peak appeared after a standard addition. As an example, Fig. 4b and c show the absence of SM in an SEC fraction where a sample peak simulates SM by an ISL 2 identification (Fig. 4a and b).

In some electropherograms, a hidden co-elution of another molecule was not totally excluded even by the ISL 3 procedure [e.g., Fig. 3b or for SC (not shown)]. Therefore, a second determination method was necessary for additional validation (ISL 4 and 5; e.g., Fig. 3d, e and f). The presence and the identity of the molecules were proved by the ISL 5 procedure in those fractions where they had already been determined by the ISL 3 procedure. Co-elutions of other molecules were now excluded.

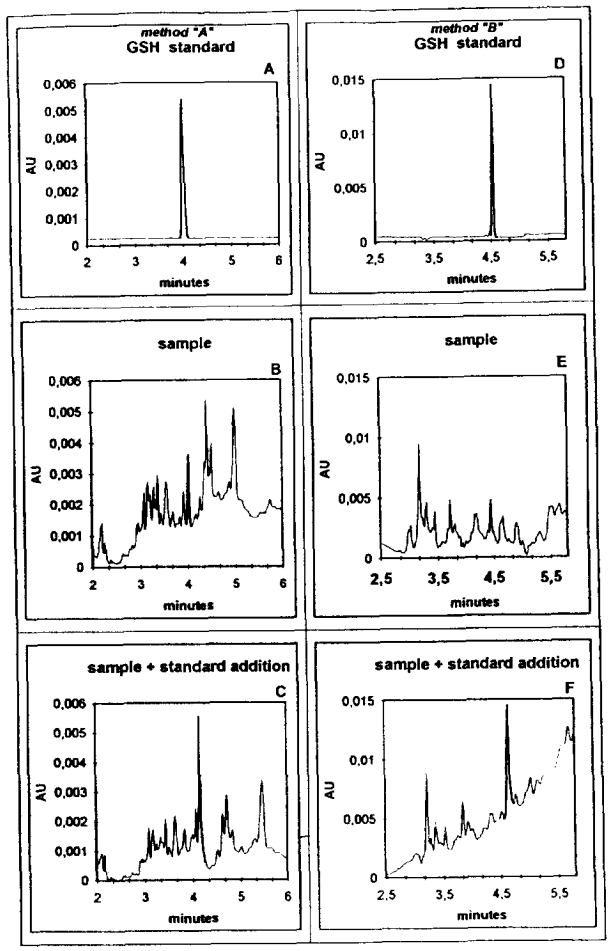


Fig. 3. Electropherograms of standards, samples and samples + standard addition for GSH by methods A and B. A clear identification by ISL 3 and ISL 5 procedures is shown.

The amounts of the analytes in consecutive SEC fractions (ISL 6) showed close conformity of the values determined by methods A and B (ISL 3 and 5). The concentration patterns were further comparable to those of Se in consecutive SEC fractions (Fig. 5). The total amount of Se-R-$NH_2$ in human milk in these experiments is shown in Table 2.

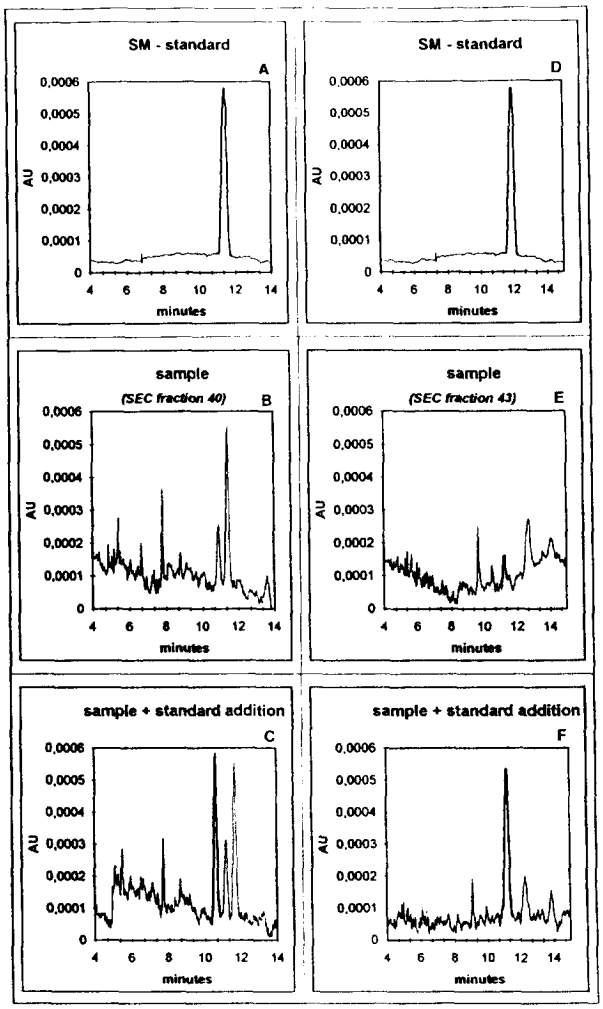


Fig. 4. (A) and (D) an SM standard is shown; (B) seemingly, SM is present in the sample according to ISL 2, but after addition of an SM standard (ISL 3) the lack of this analyte in the sample (SEC fraction 40) becomes obvious; (D) the SM standard; (E) seemingly, SM is not present in the sample according to ISL 2; (F) after addition of an SM standard (ISL 3), the presence of this analyte in the sample (SEC fraction 43) becomes obvious.

## 4. Discussion

The analytes were well separated from other molecules. Replicate electropherograms of one individual fraction were strongly reproducible with respect to $t_m$ and peak quantification.

The observed $t_m$ shift from a standard to a

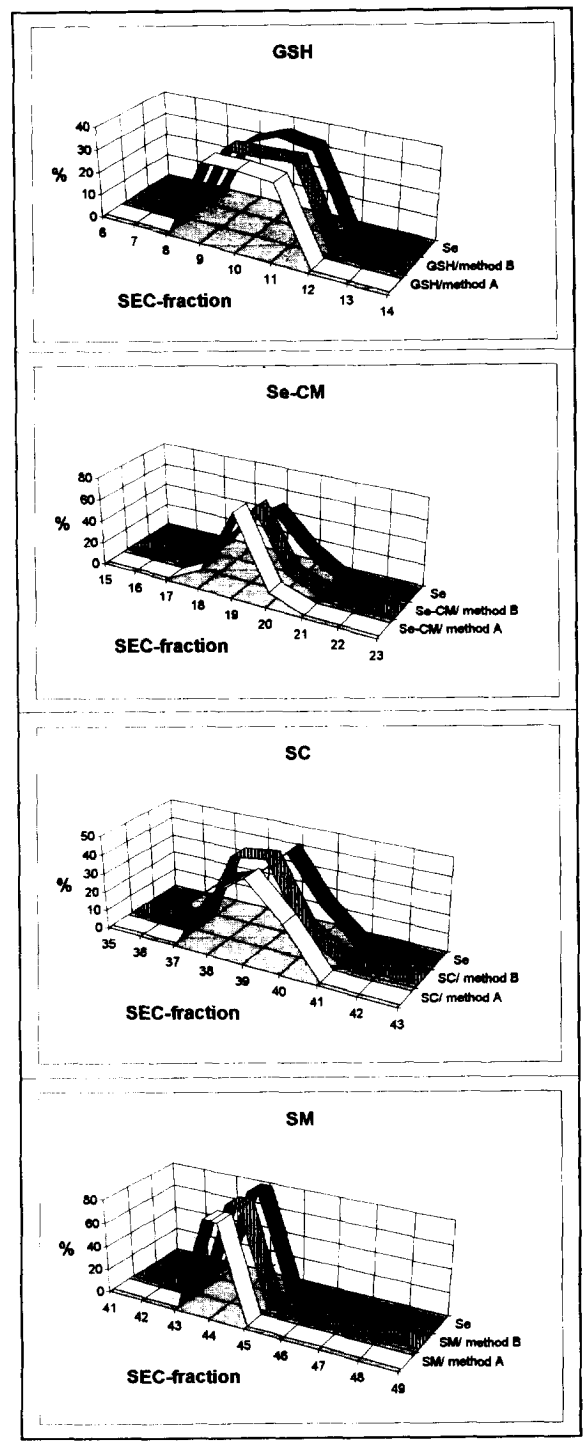


Fig. 5. Determination of Se and the four analytes in consecutive SEC fractions.

Table 2
Concentrations of analytes in human milk determined by different CZE methods

| Analyte | Concentration (mg/l) | | |
|---|---|---|---|
| | CZE method A | CZE method B | Average |
| GSH | 1.92 ± 0.20 | 1.95 ± 0.21 | 1.93 ± 0.19 |
| Se-CM | 1.99 ± 0.15 | 1.91 ± 0.20 | 1.95 ± 0.18 |
| SC | 3.96 ± 0.41 | 4.10 ± 0.25 | 4.02 ± 0.38 |
| SM | 1.08 ± 0.20 | 0.90 ± 0.20 | 1.03 ± 0.21 |

sample has been reported [5] and is explained by differences in ionic strength. The changes in $t_m$ shifts from consecutive SEC fractions were a logical consequence of the different compositions of these fractions. Taking this as a basis, identification of an analyte by the ISL 2 procedure can easily lead to a wrong identification in such complex samples. The ISL 3 procedure overcame these problems. The analytes were identified clearly (Fig. 3b and c). The ISL 3 procedure is therefore necessary for quality assurance.

A further necessary tool for quality assurance was the application of a second determination method B. Doubtful identifications and possibly wrong quantifications (referring to possible coelutions and closely migrating peaks) were now excluded by the ISL 5 procedure (Fig. 3e and f).

GSH was well separated from other molecules, but the multitude of closely migrating peaks called for the ISL 3 procedure. However, even after an ISL 3 identification, an accidental co-migration of another molecule was not totally excluded. Finally, the combination of ISL 3 and ISL 5 procedures provided absolute identification security. The amounts determined by the two methods were the same and the same concentration patterns in consecutive SEC fractions were observed (ISL 6), also indicating that there was no hidden peak.

A clear separation of Se-CM from other peaks [11] was provided. Identification and quantification were possible without interferences. Owing to the marked $t_m$ shifts, the ISL 3 and ISL 5 procedures were essential for identification and quality assurance.

Neither SC nor SM was identified by the ISL 2 procedure owing to the $t_m$ shifts (SM, Fig. 4d and e). Again, the ISL 3 and ISL 5 procedures helped to identify these molecules clearly in those SEC fractions where they should be expected. The necessity for the ISL 3 combined with the ISL 5 procedure for quality assurance is demonstrated impressively in Fig. 4a–c. There, SM seemed to appear at the standard $t_m$. Only the ISL 3 and ISL 5 procedures demonstrated clearly a marked $t_m$ shift and the absence of the analyte in this SEC fraction.

For each analyte, the total amounts and the concentration patterns in consecutive SEC fractions were in accordance for both methods (Table 2). The selenium patterns agreed with the analyte patterns. Both findings strengthen further the identification security.

In the literature, the identification problems described here often seem to play a minor role. Shifts in $t_m$ can often be observed (e.g., [12,13]), but usually no identification problems are referred to. This may be due to the investigation of model or standard solutions. However, when analysing complex, changing matrices, only standard additions with at least two methods (ISL 3 and ISL 5) guarantee sufficient identification and quality assurance.

This aspect can be found in other articles also. Deyl and Struzinsky [4] stated that there is a "large step from separations of a model solution to a real sample" and Gurley et al. [5] suggested a standard addition analogous to the ISL 3 procedure. However, even there, possible co-migration of other molecules (suspected by the authors themselves) could not be totally excluded.

A "normalization" as suggested by several groups [6–8] is not practicable for the samples analysed here. The $t_m$ and peak areas were referred to an internal standard, which is not present in the original sample. However, the prediction of an analyte $t_m$ in consecutive SEC fractions can hardly be done, because the $t_m$ shifts vary from fraction to fraction.

The four analytes investigated here have been

partly described in the literature: GSH is well known in breast milk [14] and GSH with covalently bound Se has been described [15]. SC and SM have the same pathways as their sulfur analogues and close metabolic relationships between each of the analytes are known [15–17].

## 5. Conclusion

Selenium supplementation in human milk is provided by four Se ligands, which can be clearly identified and quantified by the ISL 3 and ISL 5 procedures. These identification procedures overcome the problems resulting from $t_m$ shifts and from problems known from other identification techniques such as "normalization". They are necessary for quality assurance and will be the basis for identifications of selenium binding partners in future Se speciation investigations.

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