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Comparison of different chloroformates for the derivatisation of seleno amino acids for gas chromatographic analysis

C. Haberhauer-Troyer [a,*], G. Álvarez-Llamas [b], E. Zitting [c], P. Rodríguez-González [b], E. Rosenberg [a], A. Sanz-Medel [b]

[a] *Analytical Chemistry Division, Institute of Chemical Technology and Analytics, Vienna University of Technology, Getreidemarkt 9/164 AC, 1060 Vienna, Austria*
[b] *Department of Physical and Analytical Chemistry, Faculty of Chemistry, Julián Clavería 8, 33006 Oviedo, Spain*
[c] *Department of Chemical Technology, Helsinki University of Technology, P.O. Box 6100, FIN-02015 Hut, Helsinki, Finland*

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Abstract

Three chloroformate reagents, ethyl chloroformate (ECF), methyl chloroformate (MCF) and menthyl chloroformate (MenCF), have been used for the derivatisation of seleno amino acids and their performance was compared. Chromatographic parameters and the inertness of the different instrumental configurations used (gas chromatography–atomic emission detection (GC–AED), and GC–MS) were shown to have a significant influence on the detection of various seleno amino acids (selenomethione, selenoethione and selenocysteine) and some sulphur-containing amino acids (methionine, cysteine, cystine and methylcysteine) which were included in the experiments for comparison. Methyl chloroformate was the preferred derivatisation reagent, since it generally performed best in terms of derivatisation yield and reproducibility and also showed less significant conditioning effects than ethyl chloroformate. Methyl and ethyl chloroformate derivatives of selenomethionine, selenoethionine, cysteine and methionine were detectable, while the detection of the menthyl chloroformate derivatives of selenocystine and cystine was not reproducible. Overall efficiencies for the determination of selenomethionine and selenoethionine from aqueous extracts ranged from 40 to 100% for methyl chloroformate, over 30–75% for ethyl chloroformate to 15–70% for menthyl chloroformate for different series measured over a period of months. The relative standard deviation of the method for the methyl and menthyl chloroformate derivatisation ranged from 7 to 13% without internal standard and was improved to 2% for the determination of selenomethionine using selenoethionine as internal standard. This indicates that, despite the limited reproducibility of the method, its repeatability is good enough to allow accurate determination of seleno amino acids, which was also demonstrated by the analysis of selenium supplementation tablets for human diet that contained selenomethionine.

Keywords: Derivatisation, GC; Selenium; Amino acids; Seleno amino acids; Chloroformates

1. Introduction

Selenium, like sulphur, exists in the environment in several oxidation states and in a variety of inorganic and organic compounds like selenite, selenate,

* Corresponding author. Fax: +43-1-58801/15199.
E-mail address: ctroyer@mail.zserv.tuwien.ac.at (C. Haberhauer-Troyer).

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dimethyl selenide, dimethyl diselenide (DMDSe), dimethyl selenone, selenomethionine, selenocysteine, trimethylselenonium and selenoproteins. Knowledge of the different chemical forms and their environmental distribution and physiological levels is important because of the dependence of the bioavailability and toxicity of selenium on the species in which it is present [1]. Selenium is now well known as an essential element for biological systems. Selenocysteine, which has been called the 21st amino acid essential for ribosome-directed protein synthesis, is present at the active sites of 5′-deiodinase, selenoprotein P and glutathione peroxidase, an enzyme that protects cell membranes by removing hydrogen peroxide and other free peroxides [2]. The fact that the range between the necessary selenium intake and its toxic dose is very narrow and the increasing knowledge of the metabolism and biological effects of trace elements have led to the need not only to determine the total levels of selenium in body tissues and fluids, but also to measure quantitatively its different chemical forms [3–8].

Capillary electrophoresis [9,10] and high resolution liquid chromatography coupled to ICP–MS or atomic fluorescence spectrometry [11,12] are some of the techniques applied to the speciation of selenium-containing compounds. Gas chromatography (GC) as separation technique has the advantages of high resolution, speed of analyses and low instrumental costs and has therefore also been in use for selenium speciation. Volatile selenium compounds like dimethyl selenide and dimethyl diselenide can be analysed directly [13], while the non-volatile seleno amino acids require a previous derivatisation step in order to achieve a quantitative conversion of the analytes into volatile compounds. Some reviews have been published dealing with advances in derivatisation techniques for GC [14,15]. *N*(*O*,*S*)-acyl alkyl esters have been widely used as amino and Se-amino acid derivatives [16–20]. These derivatives are formed in a two-step procedure: the esterification of the carboxylic acid group with an alcohol and the subsequent acylation of the amino group with an anhydride. Alkyl chloroformates have also been used as derivatisation reagents for amino acids resulting in *N*(*O*,*S*)-alkyloxycarbonyl-alkyl esters in a single one-step reaction. This derivatisation method offers the additional advantage of minimal sample handling, the use of an aqueous reaction medium and inexpensive reagents, together with the speed of derivatisation, as the volatile derivatives are formed within a few seconds at room temperature. Derivatisation with ethyl chloroformate (ECF), based on the procedure proposed by Hušek [21], has been used successfully for seleno amino acids by several authors [2,22–28]. However, some authors report on problems with the ECF derivatisation and prefer the time consuming esterification/acylation procedure; nevertheless, problems reported like extensive formation of by-products [18] and low suitability at low concentrations [20] seem not to have been encountered by some other authors applying the ECF derivatisation. In fact, Cai et al. [22] reported on unsuccessful attempts to volatilise selenocystine with ECF, while David et al. [26] and Janák et al. [25] have reported on unproblematic selenocystine determinations after derivatisation with ECF.

Derivatisation with isobutylchloroformate in combination with solid phase microextraction was recently applied by Vonderheide et al. [29] for selenomethionine, selenoethionine and selenocystine. Derivatisation with methyl chloroformate (MCF) [21] and menthyl chloroformate (MenCF) [30], proposed for S-amino acids, have not yet been used for seleno amino acids. Derivatisation with optically pure chiral MenCF offers the additional advantage of forming diastereomers with the amino acid enantiomers which can theoretically be separated even with non-chiral column coatings.

The conflicting conclusions in the literature, with regard to the applicability of chloroformates as derivatisation reagents for seleno amino acids, have prompted us to carry out a more detailed study on analytical limitations of the rapid and simple chloroformate derivatisation method. The reagents methyl and menthyl chloroformate were studied for the first time as derivatisation reagents for seleno amino acids and were compared with ethyl chloroformate. The applicability of the proposed methods has been demonstrated by the analysis of Se-methionine in selenium supplementation tablets for human diet.

Exploiting the capability of gas chromatography–atomic emission detection (GC–AED) for highly sensitive and selective detection of S- and Se-containing amino acids (which in many biological systems show similar behaviour), both elements were measured,

although the main aim of this work was the GC determination of seleno amino acids.

2. Experimental

2.1. Reagents

Selenomethionine (purity >99%), selenoethionine (>99.5%) and selenocystine (>98%) were purchased from Sigma. Methionine (≥99%), cysteine (≥97%), cystine (≥99%), methyl chloroformate (≥99%), (−)-menthyl chloroformate (99% enantiomeric excess) and pyridine (>99%), di-*tert*-butyl sulfide (DtBS), dimethyl diselenide and diphenyl selenide (DPhSe) were purchased from Aldrich (Steinheim, Germany), *S*-methyl-L-cysteine (≥99%), ethyl chloroformate (≥98%) and chloroform (≥99.8%) from Fluka (Buchs, Switzerland). Methanol (p.a.), ethanol (p.a.), hexane (p.a.) and acetonitrile (>99.9%) were purchased from Riedel-de Haën (Seelze, Germany), dimethyl disulfide (DMDS, ≥99%) from Merck (Darmstadt, Germany) and methyl propyl sulfide (MPS, ≥99%) from Lancaster (Mühlheim, Germany).

2.2. Preparation of standards

Stock solutions of the amino acids (approximately 1 g/l) and all further dilutions were prepared in 0.1 M HCl.

2.3. Preparation of calibration solutions

Due to the substance independent response of the AED the instrument can be calibrated with any suitable compound containing the respective heteroelement. For this purpose, a stock solution of DPhSe, DMDSe, DMDS, DtBS and MPS and further dilutions of these compounds were prepared in hexane.

2.4. Derivatisation procedures

Derivatisation parameters were optimised, using the sulphur amino acids, based on the procedure proposed by Hušek for the ECF and MCF derivatisation [21] and the procedure proposed by Domergue et al. for MenCF [30].

2.4.1. Derivatisation with ECF

The conditions proposed by Hušek [21] for the ECF derivatisation were followed. Forty microliters of the amino acid solution in 0.1 M HCl is diluted with 400 μl water–ethanol–pyridine (60:32:8 by volume) in a microreaction vessel (1 ml volume, Supelco, Bellefonte, PA, USA). Forty microliters ECF is added and the mixture is shaken until the evolution of carbon dioxide ends and for additional 20 s. 400 μl chloroform containing 1% ECF is added. The derivatives are extracted by shaking for 20 s. All dosages are controlled gravimetrically. The organic phase is then transferred to a GC autosampler vial with a pasteur pipette. The mechanism of the derivatisation reaction has been described in detail elsewhere [31].

2.4.2. Derivatisation with MCF

The addition of acetonitrile to the reaction medium of the MCF derivatisation as proposed by Hušek [21] was dropped, since it resulted in a decrease of the derivatisation efficiency of methionine, while cysteine was unaffected. Derivatisation is therefore carried out like the ECF derivatisation, but methanol and MCF are used instead of ethanol and ECF.

2.4.3. Derivatisation with MenCF

The procedure proposed by Domergue et al. [30] for the MenCF derivatisation of amino acids could be shortened, since derivatisation seems to take place instantaneously. The amount of alcohol in the reaction medium was reduced to 30%, since higher amounts of alcohol decreased the recovery of methylcysteine, while methionine was unaffected. Derivatisation is carried out like the ECF derivatisation. As solvent, 400 μl water–methanol–pyridine (60:30:10 by volume) is used. Forty microliters MenCF is added for derivatisation and extraction is done with 400 μl chloroform. The mechanism of the derivatisation reaction has been described in detail elsewhere [30].

The MCF, ECF and MenCF derivatised analytes were stable in tightly closed vials for at least 1 week at −20 °C when the chloroform layer was separated from the aqueous phase (storage stability of cystine and selenocystine was not investigated, since these compounds could not be detected).

2.5. Extraction of selenium tablets

Three Selamin tablets (Richter Pharma, Wels, Austria) containing selenium as L-selenomethionine (100 μg Se per tablet) are crushed, homogenised with 1 ml 2 M HCl and diluted with 10 ml of distilled water. Then, 500 μl of the selenoethionine stock solution (1 μg Se/μl) is added as internal standard and the mixture is extracted four times with 10 ml of chloroform. Finally, the aqueous phase is collected and centrifuged for 15 min with a Hettix Rotofix II centrifuge to remove the starch also contained in the tablet. Aliquots of the aqueous phase are then derivatised as described previously.

2.6. Instrumentation

Gas chromatographic analysis was carried out with different HP 5890 II and 6890 gas chromatographs (Hewlett-Packard, now Agilent Technologies, Palo Alto, CA, USA), equipped with split/splitless as well as direct on-column injection (OCI) ports. Three different detection systems were assayed: a flame ionisation detector (FID), a mass spectrometer (MS, HP 5989A MS Engine, electron impact ionisation) and an atomic emission detector (AED, HP 5921A). All analyses were carried out with freshly silanised liners (used for a maximum of 200 injections).

The AED was operated with 2.1 bar O_2, 0.7 bar H_2 and 20 ml/min He (purity >99.9996%) total flow for the detection of sulphur at 181 nm [32]. Optimum experimental conditions for Se detection at 196 nm were found to be 3 bar H_2, 40 ml/min He total flow and no oxygen reagent gas. No compromise conditions for the simultaneous detection of sulphur and selenium could be found, since addition of oxygen even in small amounts led to a strong decrease of the selenium response, while sulphur could not be analysed without oxygen due to strong peak tailing, leading to peak widths of several minutes at the base. Carbon was detected at 193 nm, but the signal was only followed for diagnostic reasons. The transferline temperature was 290 °C and the cavity temperature 300 °C.

2.7. Optimisation of chromatographic parameters

Inlet temperatures between 200 and 300 °C and column flows of 2.5–4.5 ml/min were investigated. A mixture of DL-methionine, DL-cysteine and DL-cystine (0.25 mg/ml each) was used for the ECF and MCF derivatisations. For the derivatisation with MenCF a mixture of DL-methionine and *S*-methyl-L-cysteine was used (the cysteine derivative showed decomposition products when analysed by GC–MS and the cystine derivative could not be detected even with on-column injection). For the seleno amino acids study a mixture of selenomethionine and selenoethionine (0.33 mg/ml each) was also used.

Chromatographic separations were achieved on an HP Ultra 2 capillary column (25 m, 0.32 mm i.d., 0.53 μm film coating; temperature program for ECF and MCF: 150 °C (2 min), with 5 °C/min to 175 °C, with 30 °C/min to 300 °C, 1 min hold; temperature program for MenCF: 170 °C (2 min), with 5 °C/min to 195 °C, with 30 °C/min to 300 °C, 2 min hold) and a MN GC-Optima 1701 (30 m, 0.25 mm i.d., 0.25 μm film thickness, Macherey-Nagel, Düren, Germany).

Also other columns tested (HP 624 (30 m, 0.32 mm i.d., 1.8 μm), HP FFAP (30 m, 0.25 mm i.d., 0.25 μm)) for the separation of the diastereomeric *N*-menthyloxycarbonyl methyl ester derivatives proved to be not successful for baseline separation of the sought diastereomers.

3. Results and discussion

3.1. Identification and detection of the volatile derivatives

3.1.1. Mass spectrometry studies

All seleno amino acid derivatives under scrutiny, with the exception of the selenocystine ones, could be identified with GC–MS by the detection of their molecular ion, as well as of their typical fragment ions (the fragmentation of *N*-ethoxycarbonyl ethyl esters of amino acids is explained in detail by Huang et al. [33]). Selenocystine derivatives could not be identified with GC–MS even at high concentration levels and varying chromatographic conditions. In contrast to this, all three methionine derivatives and the ECF and MCF derivatives of cysteine could be identified and confirmed by GC–MS. However, cysteine derivatised with MenCF showed three peaks of very low abundance in the total ion chromatogram, even when a derivatised stock solution was injected. Thus,

Table 1
Detectability of the different seleno and sulphur amino acids with different experimental set-ups after derivatisation with MCF, ECF and MenCF

Compound	MS			AED		
	MCF	ECF	MenCF	MCF	ECF	MenCF
SeMet	+	+	+	+	+	+
SeEt	+	+	+	+	+	+
SeCys	−	−	−	0[a]	0[a]	0[a]
Met	+	+	+	+	+	+
Cysteine	+	+	−[a]	+	+	n.i.
Cystine	0	0	−	0	0	0[a]
MeCys	n.i.	n.i.	+	n.i.	n.i.	+

(+): detectable; (−): not detectable; 0: detection not reproducible; n.i.: not investigated.
[a] Decomposition products detected.

derivatisation of the SH-group seems to be crucial, since MenCF derivatisation of methylcysteine resulted in a pronounced single peak easily identified by GC–MS, as *N*-menthyloxycarbonyl methyl ester derivative of methylcysteine (Domergue et al. [30] also found that free cysteine, derivatised with MenCF, was not stable enough to be analyzed). While the ECF and MCF derivative of cystine could be identified in a first measurement series, this result was not repeatable and later experiments did not show any peaks even of derivatised stock solutions. Also, the MenCF derivative of cystine could never be detected (see Table 1).

3.1.2. AED studies

All methionine, selenomethionine and selenoethionine derivatives, as well as the ECF and MCF derivative of cysteine and the MenCF derivative of methylcysteine, showed single repeatable peaks at the S/Se channel at retention times corresponding to those observed by GC–MS measurements (splitless or on-column injection). Since the detection of cystine and selenocystine turned out to give irreproducible results this derivatisation was more thoroughly studied: three different measurement series with different stock solutions of the two amino acids, different reagent bottles and discharge tubes were analysed over a period of several months between measurement series. In all those series different final column, transfer line and cavity temperatures were studied to investigate possible thermal decomposition. Only one of the series with on-column injection showed small peaks for the ECF and MCF derivatives of cystine at the sulphur channel. Also, in only one series with split/splitless injection the ECF and MCF derivatives of cystine showed single S-containing peaks at the corresponding retention times. Peak areas (PAs) increased with decreasing final column, transfer line and cavity temperatures, giving best results at the lowest temperatures investigated (210–220 °C) indicating thermal decomposition of the analytes. The MenCF derivative of cystine as well as all selenocystine derivatives could not be detected, but several very broad S/Se-containing peaks, whose intensity decreased with increasing temperatures.

The observed detectability of the different derivatives with MS and AED is summarised in Table 1.

Cai et al. [22] also report on unsuccessful attempts to volatilise selenocystine with ECF and subsequent GC–AED analysis, leading to a small long-retention time GC peak on the Se-trace, thought to be elemental selenium. These results are in contrast to those of David et al. [26], who reported unproblematic detection of cystine and selenocystine derivatised with ECF by GC–AED.

From our results, it can be concluded that chloroformate derivatives of cystine and selenocystine are decomposed to varying degrees depending on the different chromatographic systems. This decomposition reaction seems to be not only dependent on temperature, but to a significant degree, also on the inertness of the system. This could explain why the results of measurements carried out even with the same instrument can be largely differing and why there is no general consensus on the analytical applicability of the chloroformate derivatisation to seleno amino acids in the literature.

3.2. Optimisation of GC parameters (detection with FID)

The optimisation of the inlet temperature and the column flow for split/splitless injection showed that low inlet temperatures and high column flows led to improved sensitivities. This is a further indication of the limited thermal stability of the derivatised sulphur and seleno amino acids.

An inlet temperature of 200–220 °C in the splitless mode and of 240–260 °C in the split mode (for the

sulphur amino acids) were used for all analyses, along with 4.5 ml/min helium column flow in both modes. Even with the optimised inlet conditions and freshly silanised liners, up to 20 injections of derivatised sulphur or seleno amino acids were necessary in order to achieve a stable GC–AED response. An increase of peak area at the beginning of each sequence, independent of the type of detector used, was observed over the whole concentration range investigated, reaching up to 50% when low concentrations of amino acids (30–50 pg Se) were injected. This increase was most pronounced for ECF. Consequently, our set-up had to be conditioned before running the actual analysis or, alternatively, internal standards (e.g. selenoethionine) had to be used for compensation.

3.3. Derivatisation and extraction efficiency, overall efficiency

To study the derivatisation and extraction efficiencies of the amino acids, an aliquot of the aqueous amino acid solution was derivatised with the three investigated chloroformates and extracted as described in Section 2. The remaining aqueous solutions were derivatised and then extracted for a second time. The analysis of both extracts by GC–AED revealed that more than 93% of the amino acids are derivatised and extracted in a single one-step derivatisation and extraction. This result agrees with the results of Váquez Peláez et al. [18], who report that only about 10% of the initial Se concentration remained in the aqueous phase after the derivatisation/extraction procedure.

In addition to data on derivatisation and extraction efficiency, the overall efficiency (E_{ov}) also indicates potential losses in the GC system during the transfer of the analytes from the injector to the detector. For the determination of the overall efficiency atomic emission detection was chosen, as the response of this detector is compound independent, and so it ideally shows no dependence on the structure of the actual Se or S compound. Assuming this condition, calibration with any substance containing the element of interest is possible [34,35], provided that the transfer of the calibration compounds through the column and into the detector is quantitative (or at least has the same value for all investigated compounds). For this reason compounds of different volatility, including methyl propyl sulphide, dimethyl disulphide, di-*tert*-butyl sulphide, diphenyl selenide and dimethyl diselenide were used as calibrants for GC–AED measurements. Experimental conditions had been previously optimised for the sulphur compounds [36] and were confirmed for the selenium compounds demonstrating a total transfer of the calibrants onto the column.

First, the AED was calibrated with calibrant solutions, whose concentrations were selected to match the peak areas of the derivatised amino acids. The sensitivity for S or Se for each compound, *i*, and an average response was calculated.

The sensitivity, *S*, was defined as:

$$S_{S/Se,i} = \frac{PA_i}{m_{S/Se,i}}$$

where $S_{S/Se,i}$ is the sensitivity for compound *i* from the calibration solution (PA/(pg S or Se)); PA_i the peak area for compound *i* of the calibration solution; $m_{S/Se,i}$ the mass of sulphur/selenium in compound *i* injected in 1 μl of the calibration solution (pg).

The sensitivity of the different calibration compounds is averaged and the overall efficiency can then be calculated for each derivatised amino acid under scrutiny according to the following equation:

$$E_{ov} = \frac{PA_{aa} \times 100}{m_{aa} \times S_{S/Se,av}}$$

where E_{ov} is the overall efficiency (%); PA_{aa} the peak area of the derivatised amino acid; m_{aa} the mass of amino acid (pg S or Se) injected in 1 μl of chloroform assuming 100% derivatisation and extraction efficiency (calculated from the gravimetrically controlled addition of the standard and the solvent); $S_{S/Se,av}$ the averaged sensitivity (PA/(pg S or Se))

3.4. Figures of merit

The figures of merit for the derivatisation of selenomethionine and selenoethionine with MCF and MenCF are presented in Table 2. The overall efficiency was determined for both splitless and on-column injection. It was found to be independent of the concentration of the amino acid solution derivatised and did not decrease even at the lowest concentration level investigated. This finding is in contrast to the statement of Guo and Wu [20], that derivatisation with chloroformates is not suitable for lower concentration

Table 2
Figures of merit for the derivatisation of selenomethionine and selenoethionine by MCF, ECF and MenCF

	Limit of detection (μg/l)		Relative standard deviation of the method (%)		Overall efficiency (%)[a]
	Without I.S.[b]	With I.S.[c]	Without I.S.[b]	With I.S.[c]	
MCF					
SeMet	270	160	7	2	40–100
SeEt	260	–	8	–	40–100
MenCF					
SeMet	160	60	13	1	15–70
SeEt	110	–	13	–	15–70
ECF					
SeMet	n.i.	n.i.	n.i.	n.i.	30–75
SeEt	n.i.	n.i.	n.i.	n.i.	30–75

n.i.: not investigated.
[a] In splitless injection.
[b] Calculation based on absolute values (peak areas).
[c] Calculation based on selenoethionine as internal standard.

levels when only a limited amount of material is available. For splitless injection overall efficiency ranged from 30 to 75% for ECF, from 40 to 100% for MCF and from 15 to 70% for MenCF. The repeatability of the overall efficiency calculated within a sequence ranged between 2 and 15% relative standard deviation (R.S.D.). The reproducibility of the overall efficiency, calculated for different series measured over a period of several months, yielded %R.S.D. values of about 40% for ECF, 30% for MCF and 60% for MenCF. Hence, overall efficiency is not only highly variable but also significantly lower than the derivatisation and extraction efficiency, which indicates irreproducible losses in the GC system. Derivatisation with ECF showed slightly lower overall efficiencies and required more extensive conditioning than MCF and was therefore not further investigated. Although the optically pure (−)-MenCF also shows lower overall efficiencies, it appeared still attractive for chiral derivatisation. However, no separation of the diasteromers better than 70% valley could be achieved with the non-chiral column coatings investigated (see Section 2). On-column injection significantly increased overall efficiency of all investigated MenCF derivatives in all test series, which indicates that splitless injection is partially responsible for the losses of the MenCF derivatives in the GC system. For the ECF and MCF derivatives, no significant reduction in overall efficiency due to splitless injection compared to OCI could be demonstrated, but this may rather be caused by the strong variations in overall efficiencies. An indication of possible decomposition reactions in the split/splitless injector is the occasional formation of black deposits in the liner after approximately 300 injections of derivatised amino acid standards in chloroform. The optimisation of the inlet temperature for splitless injection also showed a distinct sensitivity of the derivatives to increased temperatures. Besides sample introduction there seem to be other crucial steps, which are mainly responsible for the losses in the GC system, since even with on-column injection overall efficiency is highly variable and ranges from 40 to 100%. Decomposition reactions are also possible on the column during transfer of the analytes into the detector. From these results it can be concluded that not only the cystine and selenocystine derivatives are prone to decomposition reactions in the analytical system, but also all other investigated amino acids, although to a lesser extent.

Limits of detection of the entire procedure (derivatisation, extraction and GC–AED analysis with splitless injection) were determined according to the calibration method and using standards in the range of 0.4 to 4 μg/ml as Se (concentration of the initial amino acid solution). They are given in Table 2. Detection limits of selenomethionine can be improved significantly by the use of selenoethionine as internal standard due to repeatability improvements (see comparative relative standard deviations in Table 2). Detection limits observed were better for MenCF than for MCF, which can mainly be attributed to the much narrower

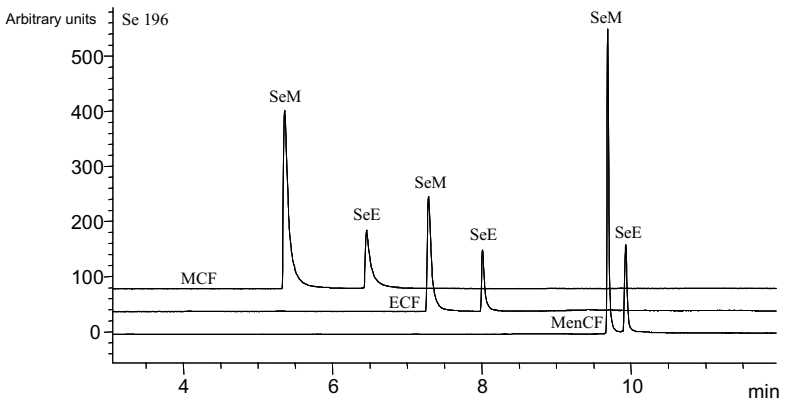

Fig. 1. Se-specific GC–AED-chromatogram of selenomethionine (SeM) after extraction from supplementation tablets and derivatisation by MCF, ECF, or MenCF, respectively (from top to bottom). Selenethionine (SeE) has been added as internal standard. GC and detector conditions are given in Section 2.

peak shape of the MenCF derivatives in comparison to the ECF and MCF derivatives (see Fig. 1). The use of a more polar column (e.g. MN 1701 or equivalent) drastically improves the peak shape of the ECF and MCF derivatives and would therefore improve the detection limits for these derivatives. The calibration graphs were linear in the range investigated (0.4–20 μg Se/ml).

No blanks or significant by-products were observed for any of the three derivatisation techniques. This is in contrast to reports on the extensive formation of by-products, particularly the occurrence of a methyl derivative when ECF is used for derivatisation [18]. In that case, a contamination of the solvent may have been responsible for that observation.

3.5. Analysis of selenomethionine-containing tablets

Selenomethionine-containing tablets, as used for Se supplementation to human diet, were extracted and derivatised according to the procedure described in Section 2 and analysed by GC–AED with splitless injection (chromatograms shown in Fig. 1). Two independent measurement series were made. For both series three extractions were carried out and each extract was derivatised three times with each of the derivatisation reagents investigated. Results are given in Table 3. For ECF and MCF recoveries were not significantly different from 100%, while for MenCF recovery was significantly lower than 100%, although selenoethionine was used as internal standard. The cause of this underestimation of selenomethionine from the tablets is not clear, since selenoethionine is a structurally very similar internal standard and should compensate not only for instrumental instabilities but also for differences in overall efficiency.

Table 3
Recoveries of the different chloroformate derivatives of selenomethionine from supplementation tablets ($c = 100$ μg Se per tablet as L-SeMet), determined in two independent series ($n = 3$)

Recovery (%)	ECF	MCF	MenCF
First series	98 ± 5	98 ± 2	81 ± 7
Second series	101 ± 4	101 ± 2	93 ± 4

4. Conclusion

Derivatisation of seleno and sulphur amino acids with alkyl chloroformates for gas chromatographic analysis shows advantages over other common multi-step derivatisation techniques like minimal sample handling, the use of an aqueous reaction medium and inexpensive reagents, together with the speed of derivatisation. Extraction and derivatisation efficiency is higher than 90% also for low concentration levels and the derivatised compounds can be stored for at least up to 1 week. However, the detection of the cystine and selenocystine derivatives by AED and MS is

not reproducible indicating decomposition reactions. This finding is supported by the fact, that all investigated derivatives show a distinct temperature sensitivity, which is evident from the optimisation of the inlet temperature and measurement series at different final column, inlet and detector temperatures. However, decomposition reactions seem to be not only dependent on temperature, but to a significant degree also on the inertness of the system. The use of the most inert materials available, e.g. Siltek-deactivated liners [37] is thus highly recommendable.

Besides sample introduction there seems to be other crucial steps, also responsible for analyte losses. The decomposition reactions are likely to cause the conditioning effects observed at the beginning of each measurement series. The losses in the GC system also lead to poor reproducibility, although the overall efficiency is remarkably constant within one series, indicating good repeatability. Overall efficiency is in most measurement series significantly lower than the more than 90% expected from the extraction and derivatisation efficiency.

Derivatisation with chiral MenCF did not allow the separation of the diastereomers on non-chiral column coatings. Furthermore, MenCF derivatisation seems to give rise to a significant negative bias (underestimation of the actual Se-methionine contents in supplementation tablets) when compared to the results of MCF and ECF derivatisation.

In brief, our results contribute to explain contradictory conclusions regarding chloroformate derivatisation for seleno amino acid GC analysis: while GC analysis of amino acids after alkyl chlorofomate derivatisation is repeatable within a series, they are highly irreproducible on a longer timescale with the extent of the losses being dependent on the state of the instrument in use. In any case, the %R.S.D. of the overall method using a suitable internal standard (e.g. selenoethionine for selenomethionine) is remarkably good (1–2%) allowing for excellent quantitative results, as temporal variations and the influence of instrument conditions are largely compensated.

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