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Gas chromatographic determination of *S*-alk(en)ylcysteine sulfoxides

Roman Kubec, Markéta Svobodová, Jan Velíšek*

Department of Food Chemistry and Analysis, Institute of Chemical Technology (ICT), Technická 1905, 166 28 Prague, Czech Republic

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Abstract

A new GC method for determination of *S*-alk(en)ylcysteine sulfoxides, important secondary metabolites occurring in many plant genera, has been developed. The method is based on isolation of the amino acid fraction by ion-exchange chromatography followed by derivatization with ethyl chloroformate at ambient temperature and reduction of derivatized *S*-alk(en)ylcysteine sulfoxides by sodium iodide. The main advantages of the new method are its high sensitivity, excellent resolution capability, accuracy and reliability, as well as the possibility to identify unknown compounds by means of GC–MS. The content of alliin and other *S*-alk(en)ylcysteine sulfoxides was determined in nine different samples of garlic (*Allium sativum* L.) originating from the Czech Republic, France, and China. The total content of *S*-alk(en)ylcysteine sulfoxide pool ranged between 0.53 and 1.3% fresh weight, with *S*-allylcysteine sulfoxide (alliin) being predominant. A novel *S*-alkylcysteine derivative, *S*-ethylcysteine sulfoxide (ethiin), not previously reported to occur in *Allium* species, was found in some of the samples examined. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: *S*-Alk(en)ylcysteine sulfoxide

1. Introduction

Numerous methods for determination of *S*-alk(en)ylcysteine sulfoxides, important aroma precursors of *Allium* and *Brassica* species, have been developed. These methods, ranging from the simple testing to the use of complex physicochemical methods, can be divided into the direct and indirect ones. The former allow determination of the content of *S*-alk(en)ylcysteine sulfoxides before their enzymatic splitting, whereas the latter are mostly based on determination of various products arising after enzymatic conversion of the precursors (thiosulfinates, thiopropanal sulfoxide, pyruvic acid, ammonia, or disulfides and vinyldithiins) [1].

With regard to thermal instability of *S*-alk(en)ylcysteine sulfoxides, a leading role among the direct methods plays HPLC. Using a sample derivatization with *ortho*-phthaldialdehyde (OPA) and 2-mercaptoethanol, Gustine detected *S*-methylcysteine sulfoxide (MCSO, methiin) in some cruciferous plants [2]. Ziegler and Sticher [3] used an OPA/*tert*.-butylthiol sample derivatization procedure to detect methiin and quantify alliin in garlic. So far, many other studies using the OPA-derivatization have been published in the literature and this technique has become one of the most applied to precise determination of *S*-alk(en)ylcysteine sulfoxides [4–9]. Alternative methods to preparing the OPA derivatives involve a sample derivatization with 9-fluorenylmethyl chloroformate (FMOC) [10] or dansyl chloride [11]. The preparation of FMOC- or dansyl-amino acid derivatives yields adducts with a

*Corresponding author. Fax: +420-2-311-9990.
E-mail address: jan.velisek@vscht.cz (J. Velíšek)

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better stability and avoids the use of the noxious thiol reagent.

Although HPLC generally allows a facile, reproducible, and accurate determination of *S*-alk(en)ylcysteine sulfoxides, its resolving power is the most limiting factor for identification of trace quantities of the minor alliin analogues. In addition, a retention time variation can lead to possible misidentification of peaks, compounds having a minor UV absorption may be overlooked, and finally, present limitations of LC–MS instrumentation do not often allow identification of unknown peaks.

From this point of view, a more promising technique seems to be gas chromatography with its excellent resolution capability and sensitivity. Unfortunately, the extremely thermolabile sulfoxide group of alliin and other *S*-alk(en)ylcysteine sulfoxides seriously complicates the applicability of this technique. No wonder that only two studies have so far been published. Gas chromatographic determination (GC–FPD) of alliin in garlic and garlic-based products was first elaborated by Saito et al. in 1988 [12]. In this method, alliin was derivatized with trifluoroacetic acid anhydride (TFAA) following GC analysis by using a short packed column (3 mm×1.5 m). The high instability of the TFA-derivative (80% decomposed after the exposure to direct sunlight for 15 min) and a poor column resolution seem to be the most important drawbacks limiting the broader use of this method. The second method, developed by Hayashi et al., is based on the trimethylsilylation of alliin [13]. However, neither method affords a satisfactory peak resolution and sensitivity.

Therefore, the main aim of the study presented herein was to develop a new method allowing a highly sensitive and reproducible determination of *S*-alk(en)ylcysteine sulfoxides (including minor derivatives) by means of GC.

2. Experimental

2.1. Reagents and materials

Ethyl chloroformate (ECF) and methyl chloroformate (MCF) were obtained from Fluka Chemie AG (Buchs, Switzerland). Solvent grade acetonitrile and methanol, phthaldialdehyde, *tert*.-butyl mercaptan (2-methyl-2-propanthiol), and dichloromethane were obtained from Merck KGaA (Darmstadt, Germany).

All other chemicals used were of analytical grade and of the highest available purity. These were purchased mostly from Lachema (Brno, CZ). Acetyl chloride and pyridine were freshly distilled prior to use. Distilled and deionized water was used throughout this study.

All the samples of garlic were obtained from Mr. Pavel Havránek (Department of Botany, the University of Palacký, Olomouc, CZ). These were harvested in July 1998 and stored in the refrigerator prior to analysis (February and March 1999). Normal doses of common nitrate fertilizers were applied during their growth. The garlic originating from France and China was purchased in a local market in March 1999.

2.2. Reference compounds

S-Alk(en)yl-L-cysteines were synthesized by alkylation of L-cysteine with the appropriate alk(en)yl halides according to the slightly modified procedure of Stoll and Seebeck [14]. (*Z*)-*S*-(1-Propenyl)-L-cysteine was synthesized by base-catalyzed isomerization of *S*-allyl-L-cysteine with potassium *tert*.-butoxide according to the procedure of Carson and Boggs [15].

(±)-*S*-Alk(en)yl-L-cysteine sulfoxides were prepared by oxidation of the corresponding *S*-alk(en)yl-L-cysteines with hydrogen peroxide followed the procedure of Yu et al. [16].

2.3. Apparatus and methods

A Hewlett-Packard 5890 chromatograph (Palo Alto, CA) equipped with a flame ionization detector (FID) and an HP-5 or HP-INNOWax fused-silica capillary column (30 m×0.25 mm I.D.; film thickness of 0.25 μm; Hewlett-Packard) was used. The sample (1 μl) was injected using a split ratio of 1:10. The operating conditions were employed as follows: injector and detector temperatures of 180°C and 250°C, respectively; a nitrogen carrier gas flow rate of 2 ml/min; the temperature program raised linearly from 180°C to 220°C at 2 C°/min and held at the final temperature for 15 min.

Mass spectra were collected by using a Hewlett-Packard G1800A chromatograph. The operating conditions were the same as described above for GC–FID analyses, with the exception of a helium carrier gas (99.99%) at a flow rate of 0.6 ml/min. Mass spectra were obtained by EI ionization at 70 eV over the range of 15–425 mass units. The ion source temperature was maintained at 250°C.

HPLC analyses were performed by using a binary pump system (ConstaMetric® 3500 and ConstaMetric® 3200) equipped with an automatic sample injector (AS 100, 20-μl loop) and employing a variable UV wavelength detector, SpectroMonitor® 3200 (Thermo Instrument System, Inc., Riviera Beach, FL). A reversed-phase column Nova Pak® C_{18} (250×4.6 mm×4 μm) with a guard column Waters Guard-Pak® C_{18} (Waters, Milford, MA) was used for separation. Amino acids were detected as the *ortho*-phtaldialdehyde (OPA) derivatives at 337 nm using the gradient profile as follows: a constant flow rate of 0.8 ml/min; solvent A, 0.5 *M* sodium phosphate buffer (pH 6.5) diluted 1:9 (v/v) with water; solvent B, methanol; 0 min, 90% A; 10 min, 50% A; 20 min, 10% A; 25 min, 10% A; 35 min, 90% A.

2.4. Isolation of amino acids

About 10 g of carefully peeled garlic cloves were steeped overnight into cold methanol to allow penetration of methanol into the cellular tissue. The plant material was then cut in small pieces and after addition of *S*-butylcysteine sulfoxide (BCSO, 20 mg/ml) homogenized by using a high speed tissue homogenizer. The homogenate was then extracted with 2×100 ml of boiling methanol. The combined methanolic extracts were reduced to approximately 15 ml at 40°C and adjusted to 25 ml by addition of 3% HCl. A precipitate usually appeared on the acidification. The extract was filtered and a volume of 3 ml was passed through a column (1×5 cm) of a cation-exchange resin (Dowex 50 WX 4, H^+ form, 50–100 mesh), which was pretreated with 10 ml of 3% HCl. The column was then treated with 10 ml of 3% HCl and 20 ml of deionized water to remove interfering non-cationic substances present in the extract. These fractions were discarded. The amino acids were eluted from the column with 50 ml of 1 *M* ammonium hydroxide. The eluate was evaporated to dryness by using a rotary flash evaporator (at 40–50°C). The residue obtained (usually white or yellowish) was used for both GC and HPLC analyses.

2.5. Derivatization procedures

2.5.1. OPA derivatization procedure

The amino acid residue was dissolved in 1 ml of water and an aliquot of 0.1 ml was mixed with 0.9 ml of the OPA derivatization reagent. The vials were allowed to stand for 30 min and the isoindole derivatives formed were analyzed by means of HPLC. The OPA reagent used for the pre-column derivatization of amino acids was prepared as follows: 140 mg of *ortho*-phthaldialdehyde (OPA) were dissolved in 5 ml of methanol. After addition of 100 μl of *tert*.-butylthiol, the solution was adjusted to 50 ml with 0.05 *M* sodium phosphate buffer (pH 9.5).

2.5.2. ECF derivatization and reduction procedure

A slightly modified derivatization procedure reported by Hušek was followed [17–19]. The evaporated amino acid residue (usually 20–30 mg in total) was dissolved in 0.3 ml of a mixture of ethanol–water–pyridine (32:60:8 v/v/v) and 100 μl of ethyl chloroformate (ECF) were added to form the corresponding *N*-ethoxycarbonyl ethyl esters (foaming usually occurred due to carbon dioxide evolution). The derivatization was completed within a few seconds after mixing and brief shaking. The *S*-alk(en)ylcysteine sulfoxides present in the sample were then reduced by addition of 0.2 ml sodium iodide aqueous solution (1 g/ml) and 50 μl of acetyl chloride. The reaction mixture was allowed to stand at room temperature for 24 h, and then the liberated iodine was removed by addition of a few crystals of stannous chloride. The amino acids derivatives were extracted with 0.4 ml of dichloromethane and an aliquot of the organic phase (mostly the upper layer) was analyzed by means of GC.

2.6. Quantitative determination

Quantification of *S*-alk(en)ylcysteine sulfoxides was done relative to the internal standard, *S*-butylcysteine sulfoxide (BCSO), added prior to

sample homogenization. Due to unavailability of the standard samples of isoalliin (*S*-1-propenylcysteine sulfoxide, PeCSO), its response factor to the FID was assumed to be the same as for alliin. At least two individual sample extracts were analyzed. Duplicate or triplicate analyses of each extract were done.

3. Results and discussion

All preliminary attempts to analyze *S*-alk(en)ylcysteine sulfoxides by GC immediately after derivatization failed. Despite many derivatization methods tried (e.g. silylation, acetylation followed by esterification, methylation), all GC analyses were unsuccessful. Two different capillary columns (HP-INNOWax and HP-5), various temperature programs and injector temperatures (120–250°C) were tested. Nevertheless, under all the conditions studied, a substantial decomposition of *S*-alk(en)ylcysteine sulfoxides was observed, leading to multiple peaks in the chromatogram.

Similar problems have been described in connection with GC determination of the glucosinolates having a sulfoxide moiety in the side chain (glucoiberin, glucoraphanin, glucoallysin) [20]. Apparently, these problems are due to the presence of the highly polarized and extremely labile sulfoxide group. Thus, the best way (if not the only) to analyze *S*-alk(en)ylcysteine sulfoxides by GC seems to be the removal of this group prior to the injection.

3.1. The choice of the reducing agent

Deoxygenation of sulfoxides to the corresponding sulfides was the subject of an extensive research in the 1970s. A survey of the literature indicates that sulfoxides can be reduced under various conditions, for example with hydrogen halides, trivalent phosphorus compounds, hydrides, low valent metal ions, sulfhydryl compounds, or by activation with an acyl reagent [21]. However, needless to emphasize, just a few methods can accomplish the reduction rapidly, in high yields, and under mild conditions (preferably in water).

Our initial experiments to reduce alliin by stannous chloride, sodium dithionite ($Na_2S_2O_4$), sodium disulfite ($Na_2S_2O_5$), and thioacetic acid revealed that the reduction does not proceed quantitatively enough at temperatures below 60°C. Fortunately, it was found that sodium iodide is able to reduce alliin at room temperature quite rapidly. The reduction is strongly accelerated by addition of a few drops of acetyl chloride. Similar method has been described by Roepstorff et al. who reduced methionin sulfoxide by potassium iodide [22]. The only drawback of this reduction procedure is the production of iodine which can subsequently complicate the analysis (e.g. it substantially increases the detection limits). Therefore, the iodine formed should be removed by addition of stannous chloride prior to the injection.

3.2. The choice of the derivatization agent

The second important point was the choice of an appropriate derivatization agent. Regarding the thermal instability of *S*-alk(en)ylcysteine sulfoxides, the requirements on the derivatization agent were as follows:

- an ability to derivatize both amino and carboxy groups, best in an aqueous medium, producing stable volatile derivatives amenable to GC separation,
- an instaneous or very rapid reaction proceeding at room temperature or under very mild conditions (at temperatures lower than 80°C),
- a simple sample handling with preferably one reagent only.

It is clear that only a few derivatization agents can fully satisfy all of the above requirements. Fortunately, a few years ago, a new class of derivatization agents was described in the literature, namely alkyl chloroformates [17–19]. The applicability of methyl (MCF) and ethyl chloroformate (ECF) was tested using a mixture of twelve *S*-alk(en)ylcysteines. It was found that both reagents yield stable derivatives which are excellently separable by GC. The ethyl derivatives, however, exhibit a better detector response (Fig. 1).

3.3. The choice of an internal standard

An internal standard was added to the sample before homogenization to reduce potential errors occurring during extraction, sample clean-up, as well as during the derivatization and reduction procedure.

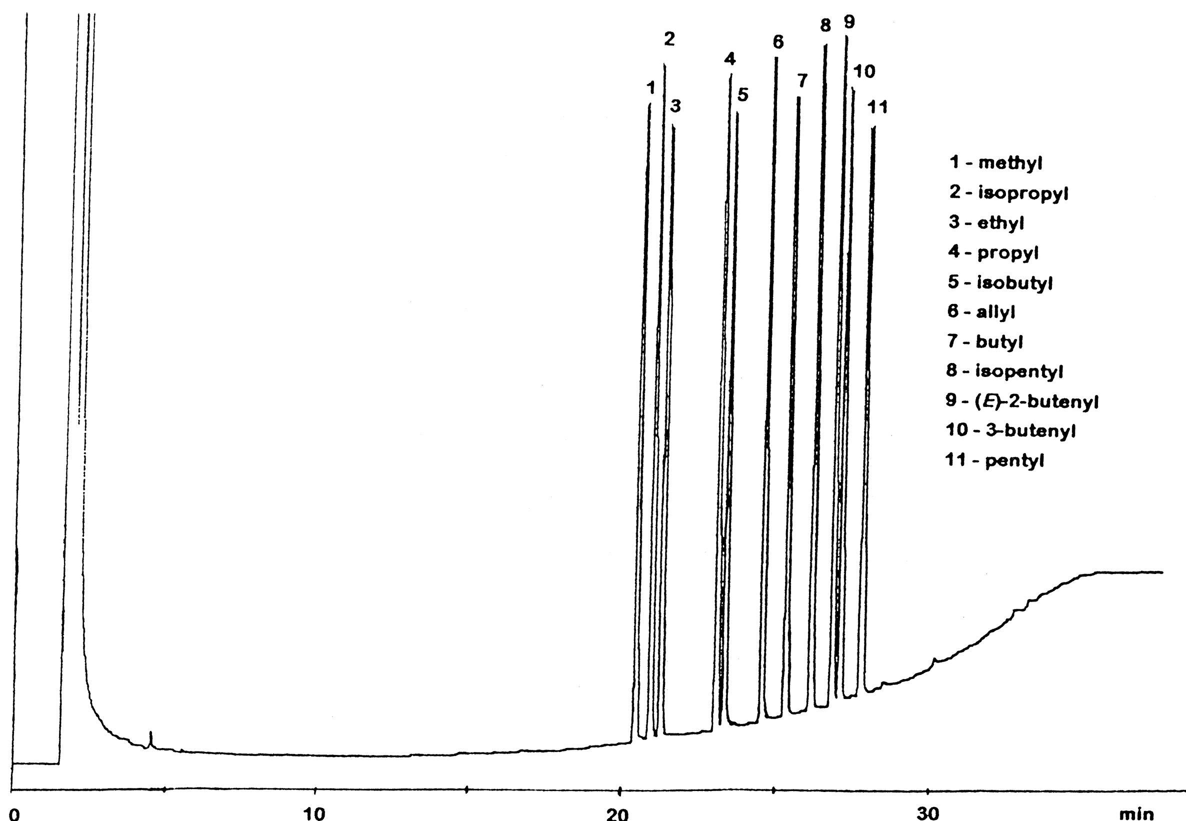


Fig. 1. Separation of a mixture of S-alk(en)ylcysteines after derivatization with ethyl chloroformate (ECF).

S-Butylcysteine sulfoxide (BCSO) was chosen as the internal standard for a number of reasons:

- its thermal stability, solubility, isoelectric point, and chemical behavior are very similar to those of the analytes,
- it can serve as a substrate for alliinase, thus possible losses of the analytes due to incomplete alliinase inactivation are corrected,
- its retention time does not coincide with any other peaks usually found in the extracts,

S-butylcysteine sulfoxide yields a sharp, well-defined peak with a very similar detector response to the analytes, it is not commercially available, but it can be easily prepared in a high yield and purity.

3.4. Testing of the method

In this study, the stability of derivatized S-allylcysteine was tested by making replicate injections over 2 h periods. No decrease in the peak areas was found for 24 h.

The reproducibility of the derivatization and reduction steps, which are critical to the overall precision of the analytical method, was checked by duplicate injections of replicately derivatized standards. Calibration curves for S-methyl-, S-ethyl-, S-propyl-, and S-allylcysteine sulfoxides (MCSO, ECSO, PCSO, and ACSO, respectively) were computed using solutions prepared from the synthesized amino acids. Eight-point calibration curves were established by at least duplicate injections where the correlation coefficient values for each of the curves was $r \geq 0.986$.

It has also been speculated that various propyl-containing volatiles found in some studies might arise by the reduction (hydrogenation) of allylic or 1-propenyl groups at high injector/column temperatures [1]. However, in this study, no conversion of

S-allylcysteine or (*Z*)-*S*-(1-propenyl)cysteine into *S*-propylcysteine was observed at the injector and column temperatures used.

Also, the elution profiles of *S*-alk(en)ylcysteine sulfoxides were studied in detail to ensure that all the analytes are retained on the ion-exchanger and then completely eluted from the column. It was found that under the conditions employed all the *S*-alk(en)ylcysteine sulfoxides are retained in the column and then completely eluted with the first 25 ml of 1 *M* ammonia (Fig. 2). All the *S*-alk(en)ylcysteine sulfoxides present in the extracts as well as *S*-butylcysteine sulfoxide (the internal standard) have almost identical elution profiles.

3.5. Comparison of the GC method with HPLC determination

Despite many attempts to optimize HPLC conditions (mobile phase composition, isocratic or gradient elution), the peak separation achieved by HPLC/OPA was unsatisfactorily. As can be seen in the representative chromatogram, alliin and especially methiin co-eluted with some other components present in the extracts making the precise comparison of both analytical methods impossible. Therefore, these methods were compared only using a model mixture consisting of MCSO, ACSO and BCSO as the internal standard. The results obtained are given in Table 1.

Table 1
Comparison of the GC method with the HPLC/OPA determination

Analyte	Conc. (mg/ml)	Determined by	
		GC	HPLC/OPA
MCSO	0.740	0.763 (3.1%)[a]	0.711 (3.9%)
ACSO	0.493	0.492 (0.2%)	0.505 (2.4%)

[a] Mean value of three determination. Relative difference between the mean and the actual value in parentheses.

3.6. Determination of alliin and other S-alk(en)ylcysteine sulfoxides in garlic

Typical GC and HPLC chromatograms of an extract of garlic (*A. sativum* L. *cv.* Záhorský) are shown in Fig. 3. As can be seen, the resolution capability of both methods is incomparable.

Seven different cultivars of garlic grown in the Czech Republic and two samples of garlic originating from China and France were examined. Even though most samples were grown under identical climatic conditions (excluding those originating from France and China), the total content of *S*-alk(en)ylcysteine sulfoxides varied considerably between 0.53 and 1.3% fresh weight (Table 2). On the

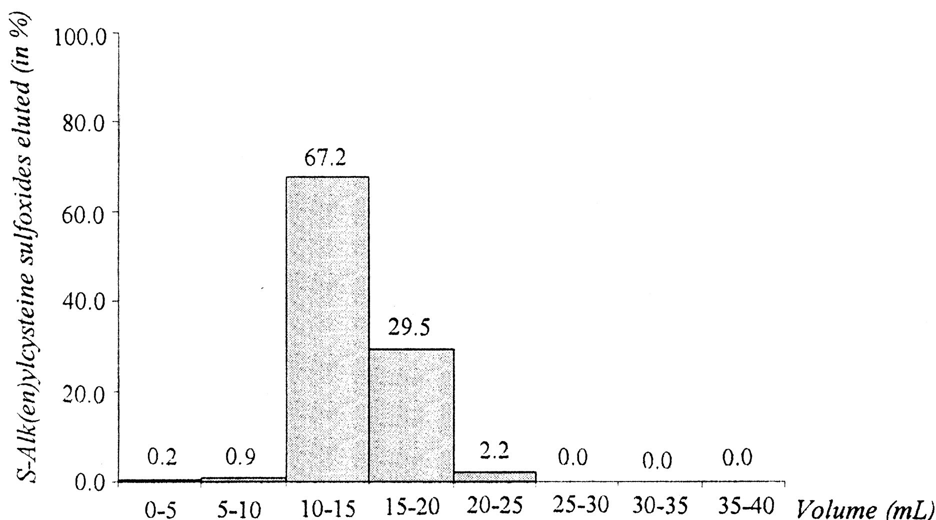
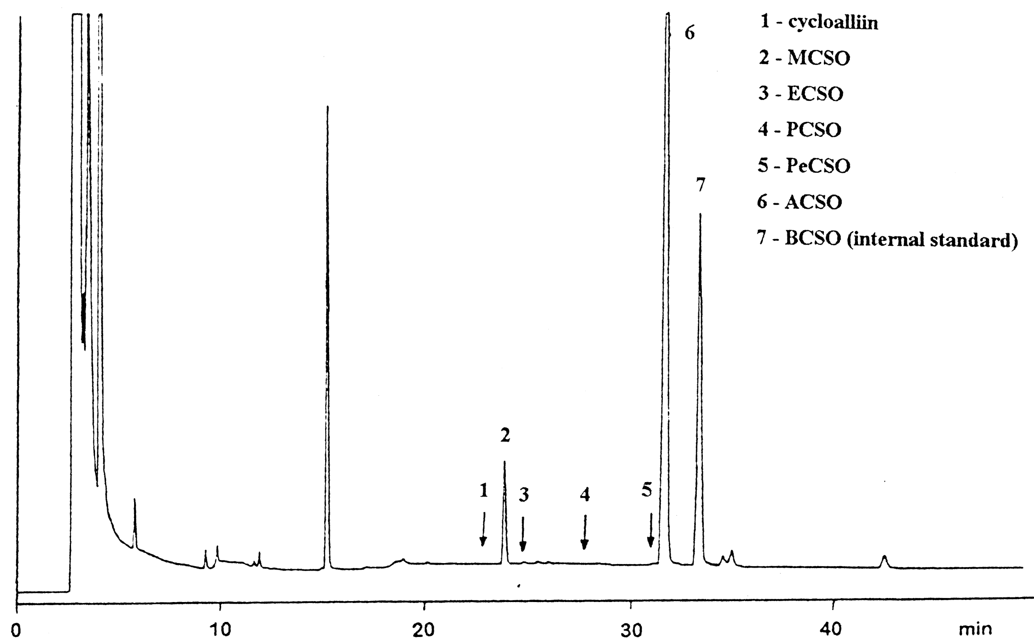


Fig. 2. Elution profile of *S*-alk(en)ylcysteine sulfoxides present in an extract from *Allium sativum* L. *cv.* Záhorský.



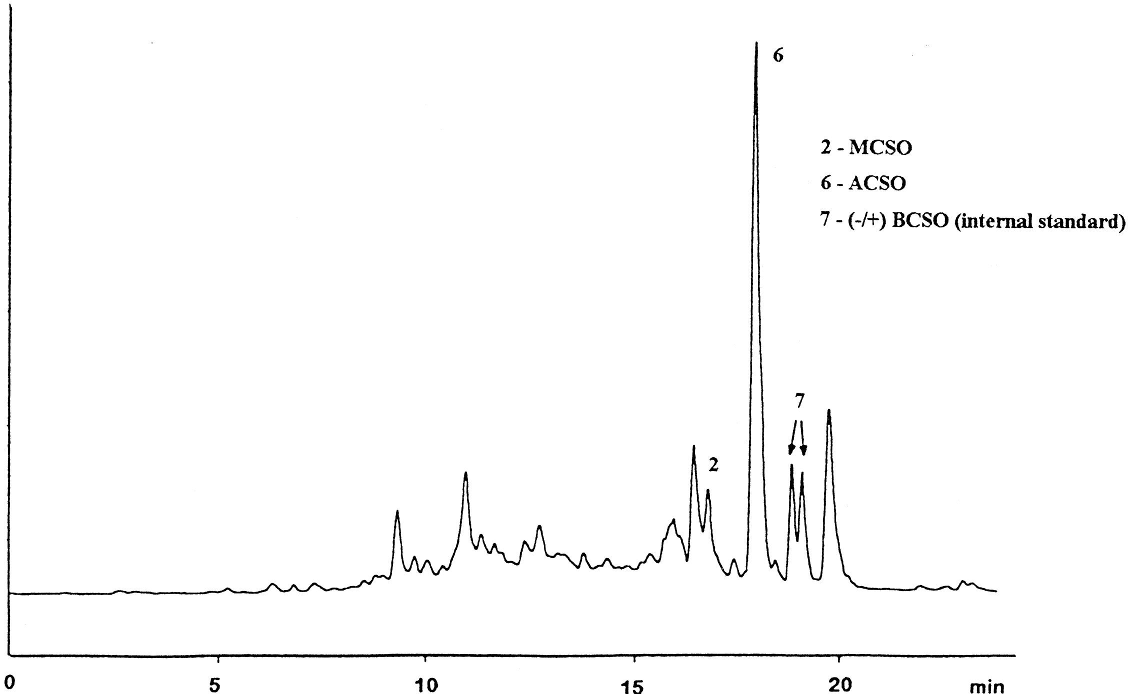


Fig. 3. GC (above) and HPLC (below) chromatograms of an extract from *Allium sativum* L. *cv*. Záhorský.

Table 2
The content of *S*-alk(en)ylcysteine sulfoxides in garlic (*Allium sativum* L.)

Sample analyzed	Content of *S*-alk(en)ylcysteine sulfoxides (in mg/100 g fw)					
	MCSO	ECSO	PCSO	ACSO	PeCSO	Total
A. sativum L. *cv.* Záhorský	57±3	tr[a]	n.d.[b]	847±29	tr	903±27
A. sativum L. *cv.* Tantal	65±3	tr	n.d.	632±41	tr	697±42
A. sativum L. *cv.* OL 1450	78±10	n.d.	n.d.	1140±106	tr	1218±116
A. sativum L. *cv.* Thermidrôme	82±5	tr	n.d.	905±47	tr	986±49
A. sativum L. *cv.* Benátčan	126±7	tr	tr	1049±37	tr	1175±43
A. sativum L. *cv.* Tristan	54±1	n.d.	n.d.	851±15	tr	904±14
A. sativum L. *cv.* Bianco Veneto	81±11	n.d.	n.d.	939±14	tr	1024±10
A. sativum L. (China)	50±1	tr	n.d.	481±16	tr	531±24
A. sativum L. (France)	122±13	n.d.	tr	1077±35	tr	1199±22

[b] n.d. – not detected (<0.1 mg/100 g fw).
[a] tr-traces (<0.2 mg/100 g fw).

other hand, the relative proportions of MCSO/ACSO were nearly identical in all the samples analyzed, varying in a very close range of (6–11)/(89–94). Consistent with other reports, isoalliin (PeCSO) was present only as a minor derivative and *S*-propylcysteine sulfoxide (PCSO) was even absent in most the samples. In general, the values determined are in good agreement with those reported in the literature [1,3–5,7,11,12,23].

As can be determined from Table 2, the coefficients of variation range between 2.0–13.1% and 1.4–9.3% with the mean of 6.7% and 4.4% for MCSO and ACSO, respectively. This variation in the content determined can be attributable primarily to sample-to-sample variation due to nonuniform distribution of the analytes in the plant tissue.

Isoalliin is known to cyclize readily under alkaline conditions, affording 3-carboxy-5-methyl-1,4-thiazane sulfoxide (cycloalliin), as shown in Fig. 4 [24]. In this study, almost total conversion of isoalliin into cycloalliin was observed during the analysis. This can be most likely due to the alkaline treatment during the ion-exchange chromatography or during the derivatization step. Unfortunately, all attempts to eliminate this conversion were unsuccessful. Therefore, two peaks belonging to the *R*/*S* isomers of reduced cycloallin were typically observed in the chromatogram along with a minor peak of non-cyclized isoalliin (the total content of PeCSO was expressed as the sum of the content of isoalliin and cycloalliin). Mass spectra of derivatized isoalliin and cycloalliin are shown in Fig. 5.

A novel *S*-alkylcysteine derivative, *S*-ethylcysteine sulfoxide (ECSO, ethiin), not previously reported to occur in *Allium* species (except the doubtful finding of Hörhammer et al. [25]), was found in most the samples examined as a minor component. However, respecting its very low content in the tissue, it does not appear to contribute significantly to the overall aroma of garlic. None of the other *S*-alk(en)ylcysteine derivatives (e.g. isopropyl, butyl, or pentyl) were detected, limiting possible levels of

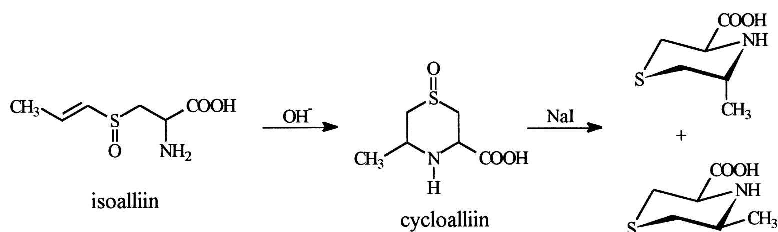


Fig. 4. Cyclization and reduction of isoalliin, *S*-(1-propenyl)cysteine sulfoxide.

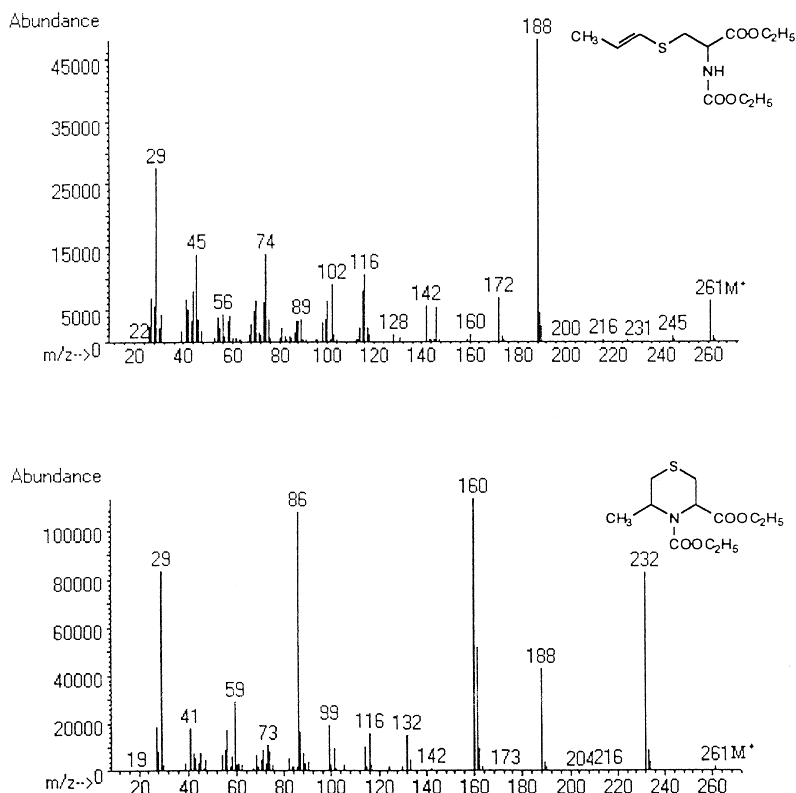


Fig. 5. Mass spectra of derivatized isoalliin (above) and cycloalliin (below).

each of these components to≤1 ppm in fresh weight (to detect *S*-butylcysteine sulfoxide no internal standard was added to the sample).

4. Conclusions

On the basis of the results obtained it can be stated that the main advantages of the new method described herein are its outstanding sensitivity, excellent resolution capability, accuracy and reliability, as well as the possibility to identify unknown compounds by means of GC–MS. The limit of detection can be estimated to be about 0.0001% (1 ppm) of an individual amino acid in the fresh tissue. Thus, these limits are far better than e.g. those reported for the HPLC method of Thomas and Parkin (~ 0.001%) [23]. Of course, when needed, enrichment of analytes can be easily achieved by a simple method modification (higher aliquot volumes introduced on the ion-exchanger, a splitless injection, etc.). The sensitivity of the method can be also substantially enhanced using more selective detectors (FPD, AED, or MS in the SIM mode).

On the other hand, sample preparation (homogenization, extraction, clean-up, and derivatization) takes about 90 min. The most time-consuming step, the reduction of sulfoxides, proceeds quite rapidly. Only for practical reasons, the procedure was continued 24 h in our experiments. However, it was found that the 24 h period can be shorten to about 2 h. Likewise, the time needed for the sample homogenization and extraction might be significantly shorten by freeze-drying and pulverizing of the sample prior to extraction. Time requirements are a serious drawback for use in routine analysis and the application may, therefore, be limited to more complex or special problems. Anyhow, a high accuracy, repeatability, and sensitivity were of greater importance than the ability to handle large numbers of samples.

The second limitation is the inability of the method to resolve between *S*-alk(en)ylcysteines and their sulfoxides. However, in most cases this inability does not represent any limiting factor, regarding to negligible contents of the reduced forms in plants [26].

Some other possible applications of the method can be found. For example, the method can be successfully used for the quantitation of alliin and its analogues in various food and pharmaceutical garlic-based products, as a somewhat better alternative to the dominantly used HPLC determination. Furthermore, the method can be applied in labeling experiments studying metabolic pathways, since the isotope-labeled compounds can be easily identified by means of GC–MS (unlike common HPLC methods). Also, the use of the method in the analysis of selenium-containing alliin analogues seems to be very promising (in connection with atomic emission spectral detection) [27].

Acknowledgements

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