Distribution of *S*-Alk(en)ylcysteine Sulfoxides in Some *Allium* Species. Identification of a New Flavor Precursor: *S*-Ethylcysteine Sulfoxide (Ethiin)

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The content of *S*-alk(en)ylcysteine sulfoxides, important nonvolatile flavor precursors, was determined in 15 different *Allium* species by means of gas chromatography. The method employed is based on derivatization of *S*-alk(en)ylcysteine sulfoxides with ethyl chloroformate followed by their reduction with sodium iodide. The total content of *S*-alk(en)ylcysteine sulfoxides varied considerably in the wide range between 0.02 and 1.3% fresh weight. Not only the total content but also relative proportions of individual derivatives varied to a great extent. A novel *S*-alkylcysteine derivative, *S*-ethylcysteine sulfoxide (ethiin), not previously reported to occur in *Allium* species, was found in most of the samples examined in trace amounts. None of the other *S*-alk(en)ylcysteine sulfoxides, for example, isopropyl, (*Z*)-1-propenyl, butyl, or pentyl, were detected in any of the samples analyzed, limiting possible levels of each of these components to ≤1 ppm in fresh weight.

Keywords: *S-alk(en)ylcysteine sulfoxides; alliin; methiin; propiin; ethiin; isoalliin; flavor precursor; Allium; ethyl chloroformate*

INTRODUCTION

Garlic, onion, leek, and other members of the genus *Allium* (Alliaceae) typically contain 1−5% dry weight of nonprotein sulfur amino acids. So far, four isomers have definitely been identified in *Allium* species, namely, *S*-allyl-, *S*-propyl-, *S*-methyl-, and (*E*)-*S*-(1-propenyl)-cysteine sulfoxides (trivially named alliin, propiin, methiin, and isoalliin, respectively) (Whitaker, 1976; Lancaster and Boland, 1990; Block, 1992). On the other hand, the presence of *S*-ethyl-, *S*-butyl-, *S*-isopropyl-, and *S*-pentylcysteine sulfoxides in *Allium* species has been suggested but never confirmed. Bernhard (1970) has proposed, from gas chromatographic analyses, that thioethyl compounds (and by implication the *S*-ethylcysteine sulfoxide precursor) may be present in certain wild species of onion. Likewise, Boelens et al. (1971) found isopropyl propyl disulfide and trisulfide among onion volatiles. Moreover, the report of significant amounts of thiopentyl compounds among chive volatiles (methyl pentyl disulfide and pentyl hydrodisulfide) raises the possibility of the occurrence of *S*-pentylcysteine sulfoxide (Hashimoto et al., 1983; Kameoka and Hashimoto, 1983). At present, nothing is known about the origin of such volatiles carrying different substituents. They may, of course, originate from precursors of an entirely different type. There has also been a suggestion that both *S*-ethyl and *S*-butylcysteine sulfoxides are present in garlic (Hörhammer et al., 1968). Such a possibility seems to be much greater in light of the recently published results of Prince et al. (1997), who fed ethanethiol to onion. Their study clearly showed that a metabolic pathway exists in onion leading to the formation of *S*-ethylcysteine sulfoxide (and probably the other derivatives as well). Therefore, it would be quite interesting to re-examine these findings by modern separation techniques.

A variety of methods for the determination of *S*-alk(en)ylcysteine sulfoxides have been published in the literature (Whitaker, 1976; Lancaster and Boland, 1990). These 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 estimation of various products arising after enzymatic conversion of the precursors (thiosulfinates, thiopropanal sulfoxide, pyruvic acid, ammonia, or disulfides and vinyldithiins).

With regard to the thermal instability of *S*-alk(en)-ylcysteine sulfoxides (Kubec, 1999), HPLC plays a dominant role among the direct methods, due to its very mild separation conditions (Ziegler and Sticher, 1989; Marks et al., 1992; Edwards et al., 1994; Thomas and Parkin, 1994; Nakamura et al., 1996; Mochizuki et al., 1997; Yoo and Pike, 1998). Although HPLC generally allows a facile, reproducible, and accurate determination of *S*-alk(en)ylcysteine sulfoxides, its resolving power and sensitivity are the most limiting factors for identification of trace quantities of minor alliin analogues. In addition, a retention time variation can lead to possible misidentification of peaks, and present limitations of LC/MS instrumentation often do not allow unknown peaks to be identified.

Recently, we have developed a new method allowing a highly sensitive determination of *S*-alk(en)ylcysteine sulfoxides by means of GC (Kubec et al., 1999). The method is based on isolation of an amino acid fraction by ion-exchange chromatography followed by derivatization with ethyl chloroformate (ECF) at ambient temperature and reduction of derivatized *S*-alk(en)ylcysteine sulfoxides by sodium iodide (Figure 1). Therefore,

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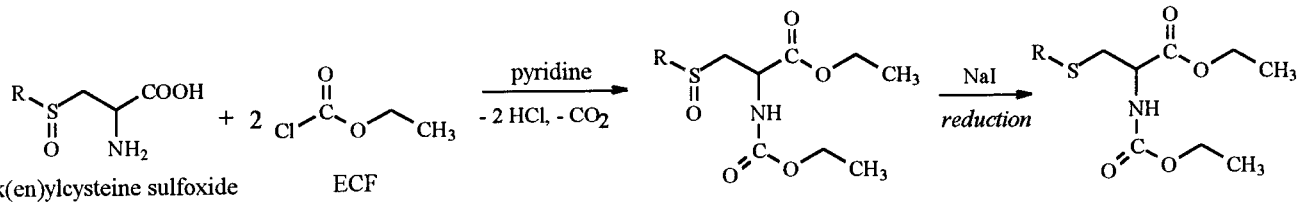

Figure 1. Derivatization and reduction of *S*-alk(en)ylcysteine sulfoxides with ECF.

the main aim of the presented study was focused on the determination of *S*-alk(en)ylcysteine sulfoxide profiles in individual *Allium* species. The emphasis was put on identification of some new derivatives, especially those mentioned above, that is, *S*-ethyl-, *S*-isopropyl-, *S*-butyl-, and *S*-pentylcysteine sulfoxides.

EXPERIMENTAL PROCEDURES

Caution: Pyridine, acetyl chloride, and ECF are harmful and irritating agents and should be manipulated only in a good hood.

Chemicals. ECF and methyl chloroformate (MCF) were obtained from Fluka Chemie AG (Buchs, Switzerland). Solvent grade dichloromethane was purchased from Merck KGaA (Darmstadt, Germany).

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

Plant Material. All of the samples examined were obtained from Mr. Pavel Havránek (Department of Botany, The University of Palacký, Olomouc, Czech Republic). Shallot and *Allium longicuspis* Rgl. were harvested in July 1998 and stored in the refrigerator prior to analysis (February 1999). The leaves of wild garlic (*Allium ursinum* L.) were picked in a forest (April 1999; Grygov near Olomouc, Czech Republic) and immediately analyzed. The fresh leaves of the other *Allium* species examined were picked and immediately analyzed in April 1999. Except for *A. ursinum* all of the plants were grown under identical climatic conditions. Normal doses of common nitrate fertilizers were applied during their growth.

Synthesis of 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 (1948). (*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 (1966).

Fourteen *S*-alk(en)ylcysteines were prepared in total. These were as follows: *S*-methyl-, *S*-ethyl-, *S*-propyl-, *S*-isopropyl-, *S*-butyl-, *S*-isobutyl-, *S*-allyl-, (*Z*)-*S*-(1-propenyl)-, *S*-(2-butenyl)-, *S*-(3-butenyl)-, *S*-pentyl-, *S*-isopentyl-, *S*-prenyl-, and *S*-(methylthiomethyl)cysteines.

(±)-*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. (1994).

The authenticity of the amino acids synthesized was proved by ^{1}H, ^{13}C, and IR spectroscopy. Their purity (>99%) was checked by HPLC, TLC, and GC. Some details (yields, melting points, and NMR and IR data) can be found elsewhere (Kubec, 1999).

Isolation of Amino Acids. About 10 g of carefully peeled garlic cloves or 20 g of shallot bulbs were steeped in cold methanol (4 °C) overnight to allow penetration of methanol into the cellular tissue. When leaves were analyzed, ~40 g was usually weighed out. 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 twice with 100 mL of boiling methanol. The combined methanolic extracts were reduced under vacuum to ~15 mL at 40 °C and adjusted to 25 mL by addition of 3% HCl. A precipitate usually appeared on acidification. The extract was filtered, and a volume of 3 mL was passed through a column (5 × 1 cm) of a cation-exchange resin (Dowex 50WX4, 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 noncationic 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 then derivatized as described below.

ECF Derivatization and Reduction Procedure (Figure 1). The derivatization procedure reported by Hušek (1991a,b) was followed with some modifications. 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 ECF was added at ambient temperature 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 the addition of 0.2 mL of aqueous sodium iodide 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 the addition of a few crystals of stannous chloride. The amino acid 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.

GC-FID Analysis. 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 = 0.25 μm; Hewlett-Packard) was used. The sample (1 μL) was injected using a split ratio of 1:10. The operating conditions employed were as follows: injector and detector temperatures of 180 and 250 °C, respectively; a nitrogen carrier gas flow rate of 2 mL/min; a temperature program that rose linearly from 180 to 220 °C at 2 C°/min and held at the final temperature for 15 min.

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 [(*E*)-S-(1-propenyl)cysteine sulfoxide] and cycloalliin (3-carboxy-5-methyl-1,4-thiazane sulfoxide), their response factors to the FID were assumed to be the same as for alliin. Two individual sample extracts were usually analyzed. Duplicate or triplicate analyses of each extract were done.

GC-MS Analysis. 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 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.

RESULTS AND DISCUSSION

Fifteen different *Allium* species were analyzed in total. Along with garlic (*A. sativum* L.), which was

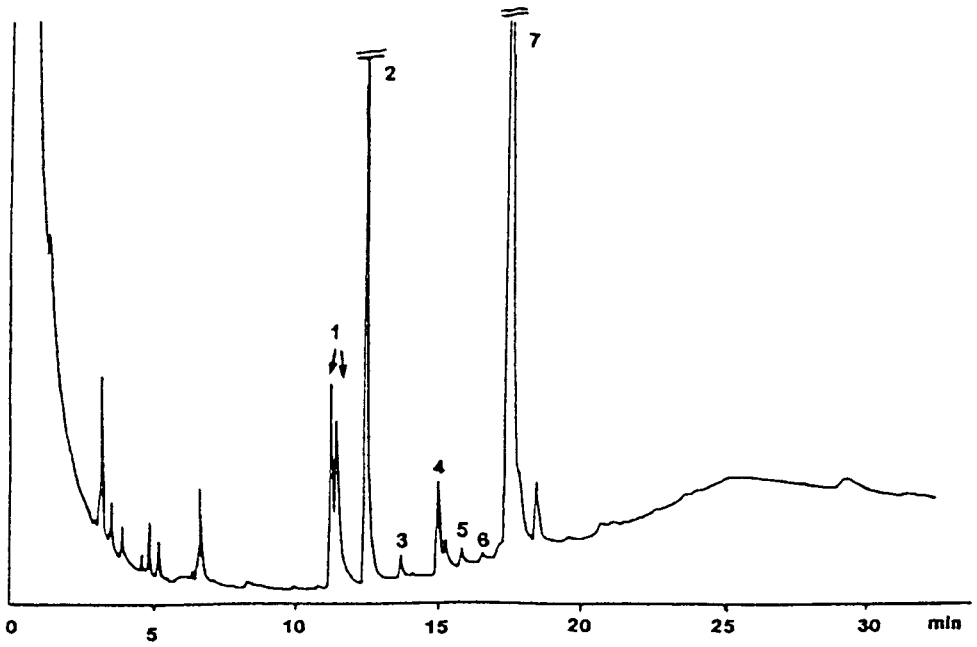

Figure 2. GC chromatogram of an extract from *A. nutans* L. (1, cyloalliin, two peaks; 2, MCSO, *S*-methylcysteine sulfoxide; 3, ECSO, *S*-ethylcysteine sulfoxide; 4, PCSO, *S*-propylcysteine sulfoxide; 5, PeCSO, *S*-1-propenylcysteine sulfoxide; 6, ACSO, *S*-allylcysteine sulfoxide; 7, BCSO, *S*-butylcysteine sulfoxide, internal standard).

Table 1. Content of *S*-Alk(en)ylcysteine Sulfoxides in the *Allium* Species Analyzed

			content of *S*-alk(en)ylcysteine sulfoxides[a] (mg/100 g of fw)					
vegetable analyzed	common name	tissue	MCSO	ECSO	PCSO	ACSO	PeCSO	total
A. aflatunense B. Fedt.		leaves	49.7	1.8	nd[b]	tr[c]	tr	51.5
A. altaicum Pall.		leaves	4.3	0.8	3.0	0.6	12.4	21.1
A. altyncolicum Fries.		leaves	35.2	1.6	9.6	1.1	33.3	80.8
A. ampeloprasum L.	elephant garlic	leaves	97.0	2.6	2.8	207.7	tr	310.1
A. ascalonicum auct.[d]	shallot	bulbs	41.4	2.9	17.7	1.1	92.7	155.8
A. ascalonicum auct.[e]	shallot	bulbs	21.7	1.9	3.4	2.6	19.7	49.3
A. cepa × *A. pskemense*[f]		bulbs	34.8	5.2	6.3	5.2	50.3	101.8
A. chinense G. Don	rakkyo	leaves	199.8	1.3	tr	13.5	tr	214.6
A. fistulosum L.	scallion	leaves	5.6	0.7	1.8	tr	13.1	21.2
A. longicuspis Rgl.[g]		cloves	104.7	tr	tr	1184	tr	1288
A. longicuspis Rgl.[h]		cloves	107.3	tr	nd	924.1	tr	1031
A. nutans L.		leaves	20.5	0.9	3.8	0.6	20.7	46.5
A. ochotense		leaves	816.8	5.3	3.3	34.7	tr	860.1
A. porrum L.	leek	stem	4.0	tr	tr	tr	17.6	21.6
A. schoenoprasum L.	chive	leaves	32.2	0.5	6.6	2.1	31.0	72.4
A. ursinum L.	wild garlic	leaves	60.0	0.4	1.2	40.3	tr	101.9
A. victorialis L.	caucas	leaves	524.7	2.3	0.3	81.4	nd	608.7

[a] Abbreviations: MCSO, *S*-methylcysteine sulfoxide, methiin; ECSO, *S*-ethylcysteine sulfoxide, ethiin; PCSO, *S*-propylcysteine sulfoxide, propiin; ACSO, *S*-allylcysteine sulfoxide, alliin; PeCSO, *S*-1-propenylcysteine sulfoxide, isoalliin. [b] nd, not detected (<0.1 mg/100 g of fw). [c] tr, traces (<0.2 mg/100 g of fw). [d] Convariety Griselle. [e] Convariety Sibirskyj Žoltyj. [f] Hybrid between *A. cepa* L. and *A. pskemense* B. Fedt. [g] Convariety Brussel. [h] Convariety Iscul.

analyzed in the previous study (Kubec et al., 1999), these represent the most important species used worldwide for cooking, including Chinese and Japanese cuisines. A typical GC chromatogram of an extract of *Allium nutans* L. is shown in Figure 2.

Even though all species (except *A. ursinum*) were grown under identical conditions (soil composition, climate, and fertilization), the total content of *S*-alk(en)ylcysteine sulfoxides varied considerably within the wide range of 0.02−1.3% fresh weight, as shown in Table 1. Generally, the values determined are consistent with those already reported in the literature (Thomas and Parkin, 1994; Nakamura et al., 1996; Mochizuki et al., 1997; Yoo and Pike, 1998). Some discrepancies in the data may result partly due to varietal differences and partly due to the method of analysis.

As already mentioned in the previous study (Kubec et al., 1999), almost total conversion of isoalliin [(*E*)-S-(1-propenyl)cysteine sulfoxide] into cycloalliin (3-carboxy-5-methyl-1,4-thiazane sulfoxide) occurred during the isolation and/or reduction step. Due to unavailability of the standard samples of these two amino acids, their response factors to the FID were assumed to be the same as for alliin. This may have affected the accuracy of the data presented herein.

A novel *S*-alkylcysteine derivative, *S*-ethylcysteine sulfoxide (ECSO), not previously reported to occur in *Allium* species [except the doubtful finding of Hörhammer et al. (1968)], was found in most of the samples examined as a minor component. This was named ethiin, in a close analogy to alliin. It generally accounted for <4% of the *S*-alk(en)ylcysteine sulfoxide pool (Table 2), and thus it probably does not contribute significantly to the aroma formation of common *Allium* species. None of the other *S*-alk(en)ylcysteine derivatives, for example, *S*-isopropyl, (*Z*)-*S*-(1-propenyl), *S*-butyl, or *S*-pentyl,

Table 2. Relative Proportions of *S*-Alk(en)ylcysteine Sulfoxides in *Allium* Species Found in This and Other Studies

		relative proportions[a] (%)					
vegetable analyzed	tissue	MCSO	ECSO	PCSO	ACSO	PeCSO	ref
A. aflatunense B. Fedt.	leaves	97	3	nd[b]	nd	tr[c]	
	bulbs	94		6			*d*
A. altaicum Pall.	leaves	20	4	14	3	59	
A. altyncolicum Fries.	leaves	44	2	12	1	41	
A. ampeloprasum L.	leaves	31	1	1	67	tr	
	cloves	17			63	20	*e*
	cloves	31−34			61−67	2−5	*f*
A. ascalonicum auct.	bulbs	27	2	11	1	59	
	bulbs	44	4	7	5	40	
	bulbs	5				96	*e*
	bulbs	32		59		9	*d*
	bulbs	22		45		33	*f*
A. cepa × *A. pskemense*	bulbs	34	5	6	5	49	
A. chinense G. Don	leaves	93	1	tr	6	tr	
	bulbs	43		35		22	*d*
A. fistulosum L.	leaves	26	3	8	tr	62	
	leaves	10				90	*e*
	bulbs	24		65		10	*d*
	whole plant	17		53		30	*f*
A. longicuspis Rgl.	cloves	8−10	tr	tr	90−92	tr	
A. nutans L.	leaves	44	2	8	1	45	
A. ochotense	leaves	95	1	<1	4	tr	
A. porrum L.	stem	19	tr	tr	tr	81	
	stem	8				92	*e*
	stem	27				73	*g*
	stem	29		62		8	*d*
	stem	27		42		31	*f*
A. sativum L.	cloves	6−11	tr	tr	89−94	tr	*h*
	cloves	5			84	11	*e*
	cloves	17			83	tr	*g*
	cloves	13		2	85		*d*
	cloves	2−24			74−95	2−11	*f*
A. schoenoprasum L.	leaves	44	1	9	3	43	
	leaves	48			6	46	*e*
	bulbs	29		64		7	*d*
	leaves	9		72		19	*f*
A. ursinum L.	leaves	59	<1	1	40	tr	
	leaves	67			33		*i*
	cloves	52			48		*i*
	cloves	32		5	64		*d*
	cloves	45			54	1	*f*
A. victorialis L.		86	<1	<1	13	nd	

[a] Abbreviations: MCSO, *S*-methylcysteine sulfoxide, methiin; ECSO, *S*-ethylcysteine sulfoxide, ethiin; PCSO, *S*-propylcysteine sulfoxide, propiin; ACSO, *S*-allylcysteine sulfoxide, alliin; PeCSO, *S*-1-propenylcysteine sulfoxide, isoalliin. [b] nd, not detected (<0.1 mg/100 g of fw). [c] tr, traces [<0.1% of the total *S*-alk(en)ylcysteine sulfoxide pool]. [d] Freeman and Whenham (1975). [e] Yoo and Pike (1998). [f] Block (1992). [g] Thomas and Parkin (1994). [h] Kubec et al. (1999). [i] Sendl and Wagner (1991).

were detected in any of the samples analyzed, limiting possible levels of each of these components to ≤1 ppm in fresh weight (to detect *S*-butylcysteine sulfoxide, no internal standard was added to the sample).

To ensure that the detection of *S*-ethylcysteine sulfoxide was not just an artifact of analysis, the derivatization of cysteine with ECF was studied in more detail. However, no production of *O,S*-diethyl *N*-(ethoxycarbonyl)cysteine due to the hypothetical decarboxylation of the cysteine derivative was observed at the injector/column temperatures used in this study (Figure 3). Furthermore, ethiin was detected in the samples even when methyl chloroformate (MCF) was used as the derivatization agent instead of ECF, indicating that ethiin is indeed present in the tissue. The mass spectrum of derivatized and reduced ethiin, *O,S*-diethyl *N*-(ethoxycarbonyl)cysteine, is shown in Figure 4.

Both the total content and relative proportions of each of the *S*-alk(en)ylcysteine sulfoxides varied to a great extent (Table 2). The proportions determined by other investigators are also given for comparison.

Some interesting conclusions can be drawn from the above results. Almost all of the species typically contain all five *S*-alk(en)ylcysteine sulfoxides, with either *S*-methyl-, *S*-allyl-, or *S*-(1-propenyl) derivatives being predominant. In all samples methiin accounts for at least 10% of the *S*-alk(en)ylcysteine sulfoxide pool. In *A. aflatunense*, *A. chinense*, *A. ochotense*, and *A. victorialis* it totally predominates. Conversely, no species contains considerable amounts of both alliin and isoalliin. Propiin was found only in small to moderate amounts in some samples.

As with *A. longicuspis* Rgl., the garlic's wild ancestor, the ratios of MCSO/ACSO were almost identical in both

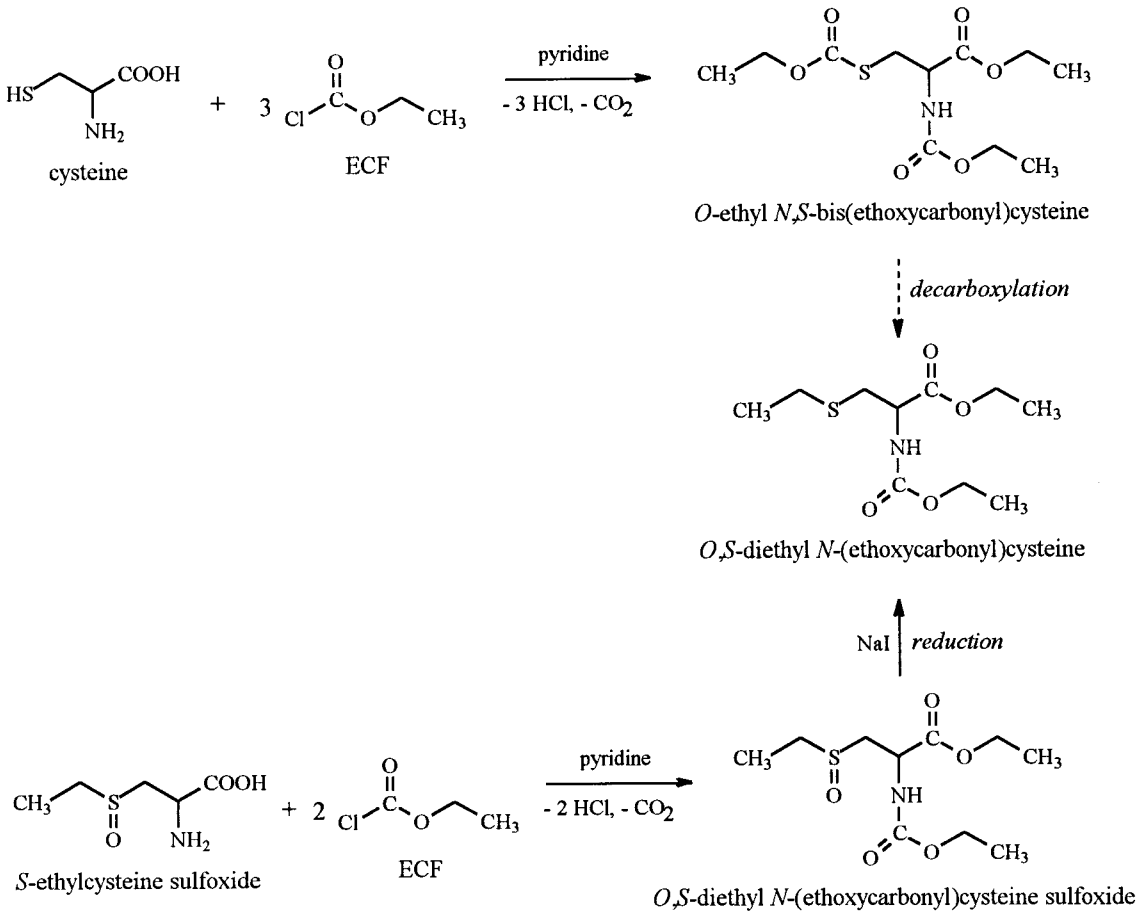

Figure 3. Derivatization of cysteine and *S*-ethylcysteine sulfoxide with ECF.

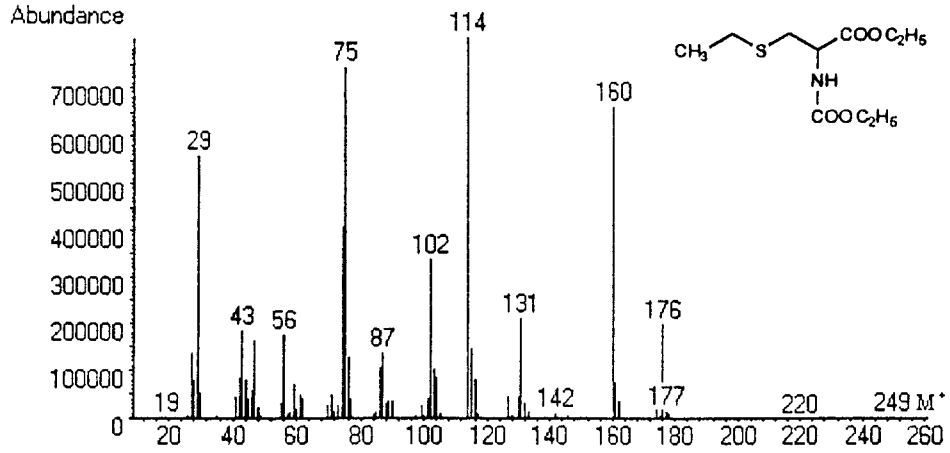

Figure 4. Mass spectrum of derivatized and reduced *S*-ethylcysteine sulfoxide (ethiin).

of the samples examined, varying in a very close range of (8−10)/(90−92). Garlic (*A. sativum* L.) showed a very similar MCSO/ACSO ratio of (6−11)/(89−94), supporting the close genetical relationship between these two species (Kubec et al., 1999).

It must be kept in mind that the total amount of the flavor precursors will vary with variety, cultural and climatic conditions (soil location and its composition, temperature, irrigation, sulfur and nitrogen nutrition), maturity, harvest date, postharvest handling, etc. Furthermore, these factors can significantly influence also relative proportions of individual *S*-alk(en)ylcysteine sulfoxides (Lancaster and Boland, 1990; Block, 1992). Therefore, increased attention must be paid to the choice of the plant material, particularly in the case of precise subgeneric taxonomic studies. Cultivation and storage of the studied material under identical conditions seems to be the best approach.

ABBREVIATIONS USED

GC, gas chromatography; FID, flame ionization detector; MS, mass spectrometry; MCSO, *S*-methylcysteine sulfoxide, methiin; ECSO, *S*-ethylcysteine sulfoxide, ethiin; PCSO, *S*-propylcysteine sulfoxide, propiin; ACSO, *S*-allylcysteine sulfoxide, alliin; PeCSO, *S*-1-propenylcysteine sulfoxide, isoalliin; BCSO, *S*-butylcysteine sulfoxide, internal standard; ECF, ethyl chloroformate; MCF, methyl chloroformate.

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