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Reversed-phase high-performance liquid chromatography of S-alk(en)yl-L-cysteine derivatives in *Allium sativum* including the determination of (+)-S-allyl-L-cysteine sulphoxide, γ-L-glutamyl-S-allyl-L-cysteine and γ-L-glutamyl-S-(*trans*-1-propenyl)-L-cysteine

M. Mütsch-Eckner and O. Sticher

Department of Pharmacy, Swiss Federal Institute of Technology (ETH) Zurich, CH-8092 Zürich (Switzerland)

B. Meier

Zeller AG, CH-8590 Romanshorn (Switzerland)

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ABSTRACT

The separation of six S-alk(en)yl-L-cysteine sulphoxides and γ-L-glutamyl-S-alk(en)yl-L-cysteines as genuine constituents of *Allium sativum* L. is reported. After automated precolumn derivatization with *o*-phthaldialdehyde–*tert.*-butanethiol the reaction products, sulphur-substituted isoindole derivatives, were analysed by reversed-phase high-performance liquid chromatography (RP-HPLC) followed by UV detection at 337 and 260 nm or fluorescence detection (excitation wavelength 230 nm, emission wavelength 420 nm). The method described allowed the qualitative and quantitative determination of the characteristic genuine polar garlic components in a single run. The accuracy and precision of the assay method, including external calibration, were evaluated. To validate the system the two main γ-glutamyl peptides, γ-L-glutamyl-S-allyl-L-cysteine and γ-L-glutamyl-S-(*trans*-1-propenyl)-L-cysteine, were determined using two different chromatographic procedures: they were determined as isoindole derivatives with UV detection as described above and by RP-HPLC with UV detection at 210 nm without previous derivatization. The method can be applied to the standardization of garlic and garlic preparations. Several garlic bulb samples were investigated and the total amount of the three main compounds was found to vary by a factor of about 2.5.

INTRODUCTION

Sulphur containing L-cysteine derivatives have been reported to be characteristic, genuine constituents of various *Allium* species [1,2]. The S-alk(en)yl-L-cysteine sulphoxides, especially (+)-S-allyl-L-cysteine sulphoxide (alliin), are precursors of a variety of more lipophilic products derived from enzymatic conversion of, *e.g.* alliin to allicin by the alliinase after cell rupture and further transformation to ajoenes, vinyldithiins or sulphides [3]. Most of the previous chromatographic analyses tended to concentrate on the latter compounds [4–12], because they are considered to be associated with the biological activity of garlic [13,14]. In this case the cysteine sulphoxides act as prodrugs. Less is known about the biological activity of the γ-glutamyl peptides [15] or the pharmacokinetic properties of all the cysteine derivatives mentioned.

The determination of alliin was presented for the

Correspondence to: Professor Dr. O. Sticher, Department of Pharmacy, Swiss Federal Institute of Technology (ETH) Zurich, CH-8092 Zürich, Switzerland.

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first time by Ziegler and Sticher [16] and Mochizuki *et al.* [17]. After precolumn derivatization, alliin was analyzed as isoindole and a fluorophore derivative, respectively. Recently Lawson *et al.* [18] reported the separation of the γ-glutamyl peptides by reversed-phase high-performance liquid chromatography (RP-HPLC) and their determination after UV detection at 220 nm. Lancaster and Kelly [19] developed a rather complicated method using a combination of electrophoresis, two-dimensional thin-layer chromatography and densitometric detection for the quantitative analysis of garlic extracts. Kappenberg and Glasl [20] described an assay method using thin-layer chromatography and detected alliin after derivatization with ninhydrin.

The aim of this work was to develop an RP-HPLC method for the selective qualitative analysis of cysteine derivatives in garlic and a validated chromatographic procedure for the determination of the main γ-glutamyl peptides, γ-L-glutamyl-S-allyl-L-cysteine (GLUACS) and γ-L-glutamyl-S-(*trans*-1-propenyl)-L-cysteine (GLUPRENCS), and (+)-S-allyl-L-cysteine sulphoxide [alliin, (+)-ACSO] in garlic in a single run.

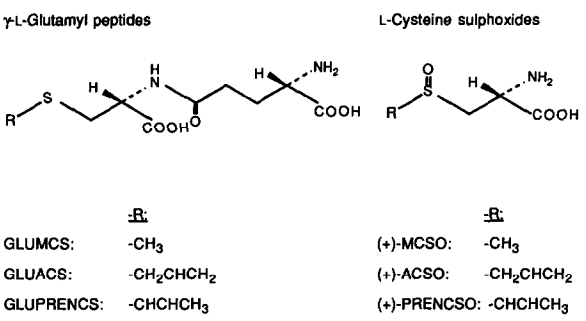
EXPERIMENTAL

Plant material

All of the garlic bulb samples were purchased in 1991 at local markets in Zürich (Switzerland). They were cultivated either in France, Switzerland or Spain.

Reference compounds

S-Alk(en)yl-L-cysteine sulphoxide derivatives, (+)-S-allyl-L-cysteine sulphoxide [alliin, (+)-ACSO] and (±)-S-methyl-L-cysteine sulphoxide [(±)-MCSO] were synthesized; details are given in ref. 21. Additionally, alliin was isolated from garlic leaves [22]. (+)-S-(*trans*-1-Propenyl)-L-cysteine sulphoxide [(+)-PRENCSO] and the two γ-glutamyl peptides, γ-L-glutamyl-S-allyl-L-cysteine (GLUACS) and γ-L-glutamyl-S-(*trans*-1-propenyl)-L-cysteine (GLUPRENCS), were isolated as monoammonium salts from garlic bulbs, details of the isolation procedure and the analytical data have been described in ref. 22. γ-L-Glutamyl-S-methyl-L-cysteine (GLUMCS) was isolated from chive seeds [18] and was a gift from Dr. L.D. Lawson (Murdock



	R:
GLUMCS:	$-CH_3$
GLUACS:	$-CH_2CHCH_2$
GLUPRENCS:	$-CHCHCH_3$

	R:
(+)-MCSO:	$-CH_3$
(+)-ACSO:	$-CH_2CHCH_2$
(+)-PRENCSO:	$-CHCHCH_3$

Healthcare, Springville, UT, USA). The purity of the reference compounds was checked by means of reversed-phase HPLC and on normal-phase thin-layer chromatography.

Solvents

Methanol, acetonitrile, tetrahydrofuran and 1,4-dioxane were of HPLC quality (Romil Chemicals, Shepshed, UK). Water was obtained using a NANOpure Cartridge system (Skan, Basle-Allschwil, Switzerland). All other reagents employed for the preparation of buffer solutions or derivatization procedures were of analytical-reagent grade and were obtained from Merck (Darmstadt, Germany) or Fluka (Buchs, Switzerland). Aqueous buffer solutions needed for the mobile phases were prepared with sodium dihydrogenphosphate dihydrate, adjusted to the desired pH value and passed through a 0.45 μm membrane filter.

Instrumentation

HPLC analyses were performed using a Hewlett-Packard instrument (Model 79994A analytical workstation, Model 1090M liquid chromatograph, Model 1040 diode-array detector, Model 1046A fluorescence detector). UV detection was performed at 337 nm and 260 nm; for fluorescence detection the excitation wavelength (λ_{ex}) was set at 230 nm and emission was recorded at wavelength (λ_{em}) 420 nm.

Chromatographic conditions

The analytical column for method 1 (100 x 4.6 mm I.D.) was packed with Spherisorb ODS II, 3 μm (Phase Separations, Queensferry, UK); for method 2 the column (250 x 4 mm I.D.) was filled with Lichrosorb RP-18, 5 μm (Merck). The column temperature was maintained at 25°C.

For method 1 the mobile phase consisted of sol-

vent A [tetrahydrofuran–1,4-dioxane–acetonitrile–0.045 *M* aqueous phosphate buffer (pH 7.10) (1.6:6.0:13.1:79.3)] and solvent B [tetrahydrofuran–1,4-dioxane–acetonitrile–0.045 *M* aqueous phosphate buffer (pH 7.10) (3.4:12.9:28.1:55.6)] with the following gradient 0 to 5 min, 100% A; 10 min, 90% A; 15 min, 65% A; 20 min, 60% A. The isocratic analyses of method 2 were performed with methanol–0.015 *M* aqueous phosphate buffer (pH 3.2) (14:86) as the eluent. The flow-rate was maintained at 1.0 ml/min for method 1 and 1.1 ml/min for method 2.

Sample preparation

About 0.8–1.0 g (accurately weighed) of freeze-dried and pulverized plant material was extracted for 5 min with 50.0 ml of methanol–water (50:50) containing 0.05% of formic acid using a Polytron (Kinematica, Kriens, Switzerland). After filtration, 5.0 ml of the filtrate were passed through a Bond Elut C_{18} cartridge. To ensure complete elution, an additional volume of about 4.5 ml of the extraction solvent was passed through. The solution obtained was adjusted to a final volume of 10.0 ml in a volumetric flask. From each specimen three samples were prepared. According to method 2, 15 μl were injected into the chromatographic system.

Derivatization

The reagent used for precolumn derivatization of the cysteine derivatives was prepared as follows: 81 mg of *o*-phthaldialdehyde and 60 μl of *tert.*-butanethiol were dissolved in 5 ml of methanol and diluted to 20.0 ml in a volumetric flask with 0.07 *M* aqueous borate buffer (pH 9.60). The reagent was stored at room temperature and replaced every week. The automated precolumn derivatization was performed at ambient temperature by mixing 9 μl of the reagent with 3 μl of the extract sample for four times. After waiting for 5 min the derivatized sample was injected into the chromatographic system.

Standard solutions

The reference compounds, (+)-S-allyl-L-cysteine sulphoxide [alliin, (+)-ACSO], γ-L-glutamyl-S-allyl-L-cysteine (GLUACS) and γ-L-glutamyl-S-(*trans*-1-propenyl)-L-cysteine (GLUPRENCS) were dissolved in methanol–water (50:50) containing 0.05% of formic acid. The solutions were stored at low temperature for not more than 1 day except for the solutions of alliin, which were stable at room temperature for several days.

Calibration was performed using the external standard method and calculating the peak areas. For the determination of the linearity, eight-point calibration lines were obtained with concentrations of the standard solutions between about 0.030 and 0.400 mg/ml. Each calibration point was measured three times. The correlation coefficient (r^2) was $\geqslant$ 0.997.

RESULTS AND DISCUSSION

Extraction and sample preparation

Freeze-drying and pulverizing the plant material guaranteed a homogeneous sample for the subsequent extraction. The relative standard deviation of the constituents determined was thereby slightly reduced with respect to the freshly extracted specimen. Extraction in an acidic methanolic–aqueous medium using a Polytron was rapid to perform, protected the extract components from enzymatic activity (*e.g.* by alliinase) and therefore favoured the stability of the extracts; a decrease in the peak areas could not be measured for at least 5 h. The stability of extracts is often a crucial problem in the automation of the chromatographic procedure. Extraction with a methanolic–aqueous solution of O-(carboxymethyl)hydroxylamine, reported to be a specific inhibitor of alliinase [23], did not result in a higher yield of either alliin or GLUACS and GLU-

TABLE I

RESULTS OF RECOVERY EXPERIMENTS

For conditions, see Experimental.

Compound	Recovery (%)[a]	
	Method 1 (337 nm)	Method 2 (210 nm)
GLUACS	99.2 (1.7)	98.9 (1.3)
GLUPRENCS	98.1 (2.1)	98.4 (1.4)
(+)-ACSO	99.5 (2.6)	–[b]

[a] Mean (n = 3) with relative standard deviation (%) in parentheses.

[b] Not determined.

PRENCS. The recovery of all three compounds added to the extraction medium was not less than 98% (n = 3, Table I), confirming not only their quantitative extraction but also their complete elution from the reversed-phase sample clean-up cartridges.

Precolumn derivatization

Improved detection possibilities of primary amines, especially of amino acids and smaller peptides, could be achieved by their reaction with *o*-phthaldialdehyde and *tert.*-butanethiol in an alkaline medium to give 1-butylthio-N-substituted isoindole derivatives [21]. In the investigation presented, the precolumn derivatization was automated and the conditions of the derivatization were optimized: the concentrations of the reagents were adjusted to guarantee a complete reaction on the one hand and to avoid precipitation during derivatization on the other. Owing to its buffer capacity especially at more basic pH values, borate buffer was preferred to phosphate buffer. The reaction time of the cysteine derivatives at ambient temperature was varied between 2 and 15 min and a waiting time of 5 min was found to be adequate. The reproducibility of the automated derivatization step was checked by injection of replicate derivatized standards and extracts. Relative standard deviations for the peak areas of reference compounds were 0.53% (GLUACS), 0.49% (GLUPRENCS) and 0.60% [(+)-ACSO)], (n = 4, detection at 337 nm). The linearity of the reaction system and of the detector employed was controlled by derivatizing alliin,

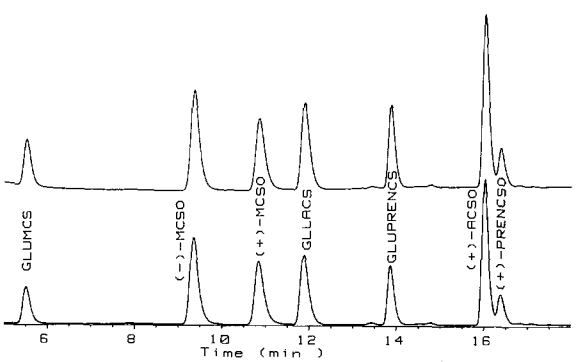


Fig. 1. Chromatographic separation of the derivatized reference compounds (method 1) with UV detection at 260 nm (top) and 337 nm (bottom). For conditions, see Experimental.

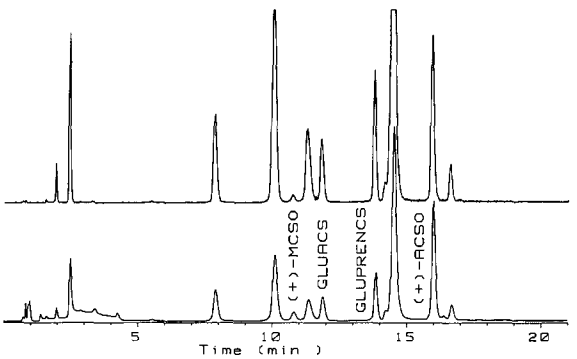


Fig. 2. Typical elution profile of a derivatized garlic bulb extract (method 1) with fluorescence detection (top) and UV detection at 337 nm (bottom). For conditions, see Experimental. Further main peaks eluted were mainly identified as ubiquitous amino acids or γ-glutamyl peptides of ubiquitous amino acids (see text).

GLUACS and GLUPRENCS in amounts ranging from about 0.15 to 1.5 μg.

Qualitative analysis

The separation of the derivatized extract components was performed using a reversed-phase C_{18} stationary phase. Owing to the different chemical properties of the compounds to be separated, an efficient and selective procedure for the optimization of the mobile phase was required. Satisfactory resolution was achieved by means of a four solvent system applying the PRISMA model [24]. Performing a gradient elution, the solvent strength of the mobile phase was increased as a factor of time. Figs. 1 and 2 show chromatograms of the separation of the derivatized reference compounds and a derivatized extract sample. To obtain highly reproducible results the column temperature was maintained at 25°C.

There is great versatility for the selective and sensitive detection of the 1-butylthio-N-substituted isoindole derivatives. The UV characteristics are fixed primarily by the chromophoric system of the isoindole moiety. It shows a UV maximum at 337 nm and a shoulder at 260 nm. In addition to specific UV detection, more sensitive electrochemical or fluorescence detectors can be employed. Ziegler and Sticher [25] investigated the advantages and disadvantages of these detection techniques and also described results of in-series coupling experiments with two detectors (*e.g.* fluorescence and electro-

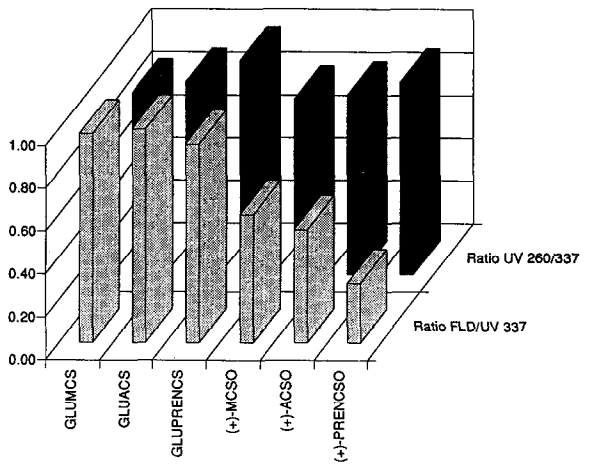


Fig. 3. Graphical representation of detector ratios of derivatized cysteine derivatives. Results were obtained by calculating ratios of integrated peak areas. Ratios were normalized to a value of 1.0 for the largest value calculated. UV = ultraviolet detection; FLD = fluorescence detection.

chemical). In this work, UV detection at two different wavelengths (260 and 337 nm) was combined with fluorescence detection. Optimum detector settings which were consistent with the results of Ziegler and Sticher [25] for (+)-ACSO were λ_{ex} 230 nm and λ_{em} 420 nm. The baseline disturbance in the front region of the chromatogram obtained with UV detection was due to the excess of derivatization reagent and did not interfere with the separation or determination of the cysteine derivatives.

Within the chromatographic system described not only the isomeric pairs GLUACS–GLUPRENCS and (+)-ACSO–(+)-PRENCSO, could be separated, but also epimeric mixtures of the synthesized sulphoxides [*e.g.* (±)-MCSO, see Fig. 1]. The identification and peak purity control of the plant constituents was established by comparing their retention times and the ratios of the signals measured at two different wavelengths or by in-series coupling of UV and fluorescence detection with the corresponding values for the reference substances (Fig. 3). Both detector ratios determined for the γ-glutamyl peptides and the cysteine sulphoxides differed significantly; compared with the cysteine sulphoxides the γ-glutamyl peptides showed increased fluorescence activities and higher UV absorbance values at 260 nm.

The fingerprint chromatogram (Fig. 2) of the garlic bulb samples examined exhibited similar elution profiles for all extracts, whereas the relative concentrations of the eluted substances varied. Under standard chromatographic conditions (+)-S-methyl-L-cysteine sulphoxide [(+)-MCSO], (+)-S-allyl-L-cysteine sulphoxide [alliin, (+)-ACSO], γ-L-glutamyl-S-allyl-L-cysteine (GLUACS) and γ-L-glutamyl-S-(*trans*-1-propenyl)-L-cysteine (GLUPRENCS) were detected in all of the garlic bulb samples analysed. γ-L-Glutamyl-S-methyl-L-cysteine (GLUMCS) could be identified in two garlic bulb samples as a minor compound. (+)-S-(*trans*-1-Propenyl)-L-cysteine sulphoxide [(+)-PRENCSO] occurred in very small amounts. In the extract samples the compound could not be detected by fluorescence detection owing to its low fluorescence activity when compared with UV detection. Further peaks eluted were mainly identified as ubiquitous amino acids (*e.g.* aspartic acid, glutamic acid, glutamine, arginine, alanine; other chromatographic conditions [22]) or γ-glutamyl dipeptides (γ-glutamylmethionine and γ-glutamylphenylalanine [22]). They showed no interference with the S-alk(en)ylcysteine derivatives.

Determination

The relevant major compounds of garlic bulb extracts, (+)-S-allyl-L-cysteine sulphoxide [alliin, (+)-ACSO], γ-L-glutamyl-S-allyl-L-cysteine (GLUACS) and γ-L-glutamyl-S-(*trans*-1-propenyl)-L-cysteine (GLUPRENCS), were determined. To validate the reproducibility and precision of the method described above (method 1) the determination of the γ-glutamyl peptides in garlic bulb samples was performed by an additional chromatographic procedure (method 2). Applying the more specific and sensitive method 1 the cysteine derivatives were separated as their isoindole derivatives and the detection limit of (+)-ACSO was found to be in the picomole range (detection at 337 nm) [25]. Method 2 (see experimental) allowed the isocratic determination of the γ-glutamyl peptides avoiding any chemical reaction. Both systems were calibrated using the pure compounds, (+)-ACSO, GLUACS and GLUPRENCS, as external standards and the linearity of the determination was ensured by regression analysis (eight-point measurements, $r^2 \geqslant 0.997$).

Using method 2, the two γ-glutamyl peptides

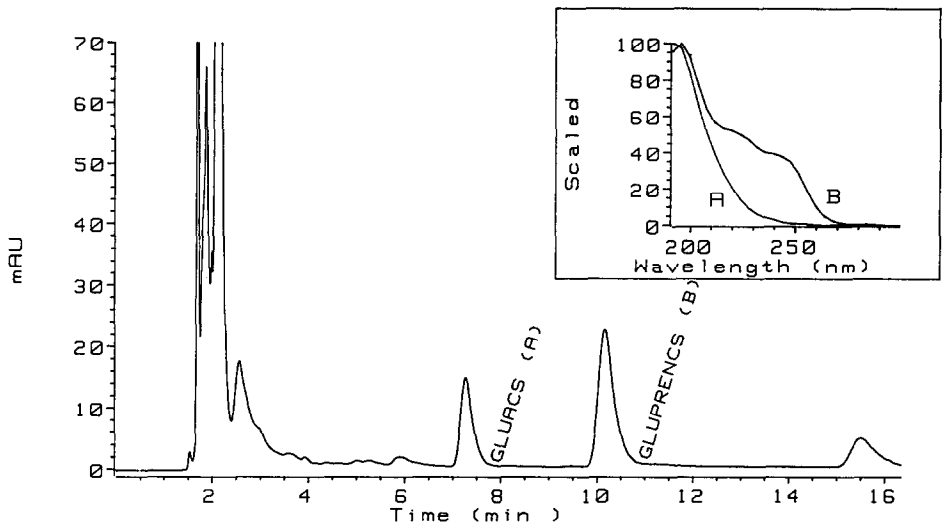


Fig. 4. Chromatogram of a garlic bulb extract according to method 2 with UV detection at 210 nm and on-line recorded UV spectra of the two γ-glutamyl peptides. For conditions, see Experimental.

were separated in an isocratic run within less than 12 min using RP-HPLC. Fig. 4 shows the chromatogram of the separation of a garlic bulb sample. The acidic buffered mobile phase decreased the fronting of the γ-glutamyl peptide peaks due to ionized carboxyl groups. In the chromatographic system described, alliin eluted in the front region of the chromatogram and overlapped with ubiquitous amino

TABLE II

RESULTS OF ALLIIN AND γ-GLUTAMYL PEPTIDE ASSAYS OF SELECTED GARLIC BULB SAMPLES

For conditions, see Experimental.

Sample No.	Origin	Compound	Content (% dry weight)[a]		
			Method 1		Method 2 (210 nm)
			260 nm	337 nm	
I	Switzerland	GLUACS	0.36 (1.38)	0.36 (1.60)	0.35 (1.02)
		GLUPRENCS	0.63 (2.76)	0.63 (2.47)	0.63 (0.87)
		(+)-ACSO	0.98 (2.70)	1.00 (2.31)	–[b]
II	Spain	GLUACS	0.99 (1.23)	0.99 (1.75)	1.02 (0.56)
		GLUPRENCS	0.63 (1.70)	0.63 (1.59)	0.65 (0.87)
		(+)-ACSO	2.62 (2.29)	2.70 (2.43)	–[b]
III	France	GLUACS	1.08 (0.93)	1.09 (0.88)	1.06 (1.58)
		GLUPRENCS	0.59 (2.88)	0.59 (1.18)	0.58 (1.03)
		(+)-ACSO	1.41 (2.51)	1.45 (2.79)	–[b]
IV	Switzerland	GLUACS	1.77 (2.07)	1.78 (1.32)	1.77 (0.86)
		GLUPRENCS	1.44 (1.30)	1.44 (1.60)	1.43 (0.75)
		(+)-ACSO	2.52 (2.06)	2.58 (1.36)	–[b]

[a] Mean (n = 3) with relative standard deviation (%) in parentheses.
[b] Not determined.

acids. Owing to the lack of a chromophoric system, UV detection was performed at 210 nm. Peak identity and homogeneity were established by comparing the retention times and on-line recorded UV spectra of the reference and extract compounds. The detection limit of method 2 at a signal-to-noise ratio of *ca.* 2 was at 20–30 ng of injected γ-glutamyl peptide.

In order to obtain some information on the precision and accuracy of both methods, the recoveries of the reference substances added to the garlic bulb samples prior to extraction were determined. The results indicated complete recovery and suggested that there is no loss of compounds during sample clean-up (Table I).

Yields of sample III (Table II), determined six times applying both methods 1 and 2, revealed no significant difference between the two methods by means of the two-tailed Student's *t*-test. Results of the analyses of several garlic extract samples are summarized in Table II. The relative standard deviations did not exceed 3.0% for GLUACS, GLUPRENCS and (+)-ACSO ($n=3$). Comparable results of the quantitative analyses based on the mean values and the standard deviations were obtained by performing UV detection at either 260 or 337 nm.

The results presented in Table II revealed striking variable contents of all three compounds determined, probably owing to the origin, variety, cultivation, harvesting and storage conditions. Assuming a water content of about 65%, total values of all three compounds constituted up to 2.0% of the fresh material. Varying contents are in agreement with the results of Lawson *et al.* [18] for the γ-glutamyl peptides and Ziegler *et al.* [26] and Iberl *et al.* [27] for alliin. The alliin content was always higher than that of either of the γ-glutamyl peptides. The ratio of the content of the two γ-glutamyl peptides was not constant between the samples tested, although there is a tendency towards higher levels of GLUACS which could be explained with an accelerated transformation of GLUPRENCS during storage [18].

Conclusions

The chromatographic analysis of polar garlic bulb extracts revealed a characteristic pattern of genuine amino acids and dipeptides. (+)-S-Allyl-L-cysteine sulphoxide (alliin), γ-L-glutamyl-S-allyl-L-cysteine and γ-L-glutamyl-S-(*trans*-1-propenyl)-L-cysteine were found to be the major cysteine derivatives of garlic bulbs. They are not only the main compounds in garlic under the conditions described but as sulphur-containing constituents also relevant for *Allium* species in general.

The assay method presented emphasized the occurrence of two main groups of cysteine derivatives in garlic: the sulphoxides and the γ-glutamyl peptides. Biosynthetic investigations confirmed the latter to be precursors of the cysteine sulphoxide derivatives [28]. Their qualitative and quantitative determination including fingerprint profiles, provided some information for quality control of the raw material and of pharmaceutical garlic preparations and should also be suitable for investigations of the pattern of cysteine derivatives in various *Allium* species.

In addition, the three cysteine derivatives are prodrugs and could therefore act as marker compounds for the pharmacologically active transformation products. Their yield in garlic samples provides concise information about the proportion of the various known transformation products to be formed, *e.g.*, after enzymatic conversion.

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