Journal of Chromatography A, 1216 (2009) 6957–6963

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Chromatographic methods for determination of *S*-substituted cysteine derivatives—A comparative study

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ARTICLE INFO

Article history:
Received 29 June 2009
Received in revised form 10 August 2009
Accepted 13 August 2009
Available online 18 August 2009

Keywords:
Allium
Garlic
Onion
FMOC
OPA
Dansyl
MEKC
Alliin
Isoalliin
Methiin
Glutamyl dipeptide

ABSTRACT

A novel HPLC method for determination of a wide variety of *S*-substituted cysteine derivatives in *Allium* species has been developed and validated. This method allows simultaneous separation and quantification of *S*-alk(en)ylcysteine *S*-oxides, γ-glutamyl-*S*-alk(en)ylcysteines and γ-glutamyl-*S*-alk(en)ylcysteine *S*-oxides in a single run. The procedure is based on extraction of these amino acids and dipeptides by methanol, their derivatization by dansyl chloride and subsequent separation by reversed phase HPLC. The main advantages of the new method are simplicity, excellent stability of derivatives, high sensitivity, specificity and the ability to simultaneously analyze the whole range of *S*-substituted cysteine derivatives. This method was critically compared with other chromatographic procedures used for quantification of *S*-substituted cysteine derivatives, namely with two other HPLC methods (derivatization by *o*-phthaldialdehyde/*tert*-butylthiol and fluorenylmethyl chloroformate), and with determination by gas chromatography or capillary electrophoresis. Major advantages and drawbacks of these analytical procedures are discussed. Employing these various chromatographic methods, the content and relative proportions of individual *S*-substituted cysteine derivatives were determined in four most frequently consumed alliaceous vegetables (garlic, onion, shallot, and leek).

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1. Introduction

S-Substituted cysteine derivatives are important secondary metabolites occurring in many families of plants, mushrooms and algae. The most important group of these sulfur-containing compounds are *S*-alk(en)ylcysteine *S*-oxides which are the precursors of an extraordinary variety of sensory-active and health-beneficial compounds of *Allium* vegetables (e.g. garlic, onion, leek and chive etc.). Five *S*-alk(en)ylcysteine *S*-oxides are typically present in commonly consumed alliaceous plants, namely *S*-methyl-, *S*-allyl-, (*E*)-*S*-(1-propenyl)-, *S*-propyl- and *S*-ethylcysteine *S*-oxides (methiin, alliin, isoalliin, propiin, and ethiin, respectively, **1a**–**5a**) (Fig. 1), with propiin and ethiin usually occurring only at trace levels [1]. Two other cysteine derivatives, *S*-butyl- and *S*-(3-pyrrolyl)cysteine *S*-oxides, were isolated from ornamental *Allium* species [2,3] and *S*-(3-pentenyl)cysteine *S*-oxide was recently found in a locally-grown variety of onion *Allium cepa* var. *tropeana* [4]. A quite distinct cysteine derivative occurring in alliaceous plants is 3-carboxy-5-methyl-1,4-thiazane *S*-oxide (cycloalliin, **6**). Although this unusual secondary amino acid was originally considered to be only an artifact formed by cyclization of isoalliin (**3a**) during isolation, it was recently proved to be a component naturally present in *Allium* species [5].

Typically, *S*-alk(en)ylcysteine *S*-oxides are accompanied by substantial amounts of the corresponding γ-glutamyl dipeptides (**1b**–**4b** and **1c**–**4c**) in the plant tissue (Fig. 1). It has been shown that there exists equilibrium between the free cysteine derivatives and their dipeptide forms, with their ratio depending mainly on the growth stage and storage conditions [5–9]. The glutamyl dipeptides are not cleaved by alliinase and thus they do not actively participate in the enzymatic formation of *Allium* flavor. However, they have recently started to receive much more attention due to their significant health-promoting properties [10,11].

Quantitative determination of *S*-substituted cysteine derivatives in *Allium* species is apparently of great importance for both food and pharmaceutical industry. Numerous methods for quantitation of *S*-alk(en)ylcysteine *S*-oxides (**1a**–**5a**) have been developed. These methods, ranging from simple semi-quantitative screening to employing modern chromatographic techniques, can be classified as either indirect or direct. The former ones are based on determination of various products (e.g. thiosulfinates, pyruvate or ammonia) arising after enzymatic conversion of **1a**–**5a**. On the other hand, direct methods allow determination of *S*-alk(en)ylcysteine *S*-oxides before their enzymatic decomposition.

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doi:10.1016/j.chroma.2009.08.032

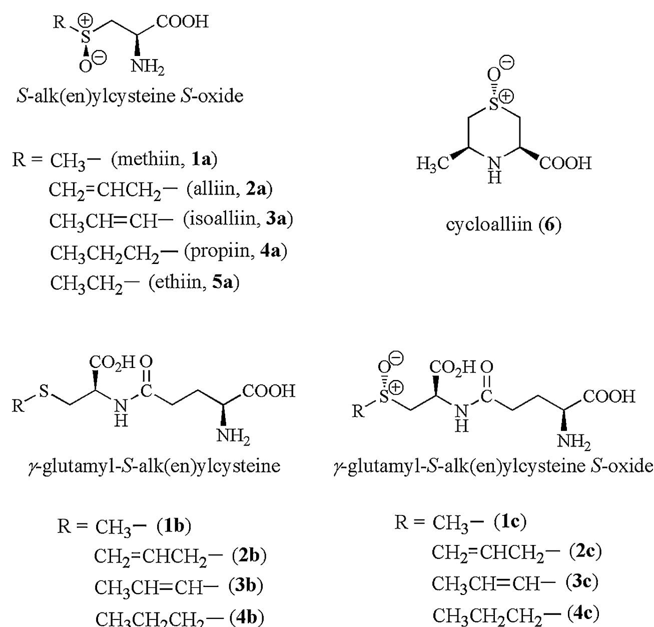

Fig. 1. Major *S*-substituted cysteine derivatives present in common *Allium* species.

A leading role among the direct methods plays HPLC determination after pre-column derivatization, with *o*-phthaldialdehyde (OPA)/*tert*-butylthiol having been the most commonly used derivatization reagent [12–15]. Much less frequently, fluorenylmethyl chloroformate (FMOC) [16], dansyl chloride (Dns-Cl) [17] or phenyl isothiocyanate (PITC) [18,19] have been applied as pre-column derivatization reagents in combination with HPLC separation. Alternatively, *S*-alk(en)ylcysteine *S*-oxides can be reliably quantified by gas-chromatography (GC) [1,19–22] or by capillary electrophoresis (CE) [23,24].

On the other hand, much less attention has been paid to the analysis of the γ-glutamyl dipeptide forms (**1b**–**4b** and **1c**–**4c**). This situation has been mostly due to commercial unavailability of these compounds and the necessity of their laborious synthesis or isolation. Only a few reports on their quantitative determination have thus far been published, with HPLC being the most commonly employed method [13,19,25–27]. However, these methods do not usually allow simultaneous determination of the whole range of *S*-substituted cysteine derivatives (**1a**–**5a**, **1b**–**4b**, **1c**–**4c** and **6**) in a single run.

The main aim of this study was to critically evaluate various chromatographic procedures used for quantification of **1a**–**5a** and to develop a simple HPLC method allowing simultaneous determination of the whole range of *S*-substituted cysteine derivatives in a single run. Such a method would be suitable not only for analysis of fresh vegetables but also for testing various products made of garlic and onion (e.g. garlic-derived dietary supplements, spices etc.). It could also be a powerful tool for evaluation of changes in the profile of *S*-substituted cysteine derivatives during growth, storage and processing of *Allium* species.

2. Experimental

2.1. Reagents and materials

Chemicals were obtained from the Sigma–Aldrich group (St. Louis, MO, USA) and Spolana (Neratovice, Czech Republic), HPLC-grade methanol and acetonitrile were purchased from LabScan (Dublin, Ireland). Garlic (*Allium sativum* L., China), yellow onion (*A. cepa* L., Netherlands), leek (*A. ampeloprasum* var. *porrum* L., Netherlands) and shallot (*A. ascalonicum* auct., Netherlands) were purchased from a local market in June 2008. The white onion (Spain) used for isolation of isoalliin (**3a**) and γ-glutamyl-*S*-(*E*)-(1-propenyl)cysteine *S*-oxide (**3c**) was purchased from a local market in September 2006.

2.2. Reference compounds

S-Substituted cysteines (*S*-methyl-, *S*-ethyl-, *S*-propyl-, *S*-allyl- and *S*-isobutyl-L-cysteines) and diastereomeric mixtures of the corresponding *S*-alk(en)yl-L-cysteine *S*-oxides were synthesized by methods described in [20]. The naturally-occurring (S_S,R_C)-diastereomers of alliin (**2a**) and propiin (**4a**) were obtained by repeated recrystallizations from aqueous acetone or ethanol, respectively. Isoalliin (**3a**) and γ-glutamyl-*S*-(*E*)-(1-propenyl)cysteine *S*-oxide (**3c**) were isolated from white onion [27,28]. The γ-glutamyl dipeptides (**1b**–**4b**, **1c**–**2c**, **4c**) were synthesized from the corresponding *S*-alk(en)ylcysteines according to [29] and the naturally-occurring (S_S,R_{C3},S_{C5})-diastereomer of cycloalliin (**6**) was prepared according to Sakai and Yoneda [30]. The identity and purity (≥98%) of the reference compounds were checked by ^{1}H and ^{13}C NMR, HPLC and TLC.

2.3. Apparatus and methods

HPLC separations were performed on a Dynamax SD-210 binary pump system (Varian, Palo Alto, CA, USA), employing a Varian PDA 335 detector and a C-18 reverse phase column (Rainin Microsorb-MV 100 Å, 250 mm × 4.6 mm, 5 μm). Samples were injected manually using a 20 μl injection loop. The chromatographic conditions were as follows: (A) Dns-derivatives: 50 mM sodium acetate buffer (pH 5.0, solvent A) and methanol (solvent B) were used as the mobile phase, with a flow rate of 0.9 mL min^{-1} and the gradient A/B 70/30 (0 min), 60/40 (in 35 min), 25/75 (in 60 min), 25/75 (in 65 min) and 70/30 (in 70 min), detection wavelength of 250 nm; (B) FMOC-derivatives: 50 mM sodium acetate buffer (pH 4.2, solvent A) and methanol (solvent B), flow rate of 0.9 mL min^{-1} and the gradient A/B 65/35 (0 min), 35/65 (in 40 min), 35/65 (in 45 min) and 65/35 (in 50 min), detection wavelength of 265 nm; (C) OPA-derivatives: 50 mM KH_2PO_4 buffer (pH 6.5, solvent A) and methanol (solvent B), flow rate of 0.9 mL min^{-1} and the gradient A/B 75/25 (0 min), 25/75 (in 50 min), 25/75 (in 55 min) and 75/25 (in 60 min), detection wavelength of 337 nm.

CE analyses were carried out on a fully automated system Spectraphoresis 2000, equipped with a UV–vis scanning detector (Thermo Separation Products, Fremont, CA, USA). Separations were performed using a fused-silica capillary (70 cm × 75 μm I.D., Supelco; the effective length to the detector was 67 cm). Injections were achieved by application of vacuum for 2 s. The detection wavelength was set at 265 nm. The separation buffer (pH 9.2) consisted of 20 mM sodium tetraborate, 20 mM sodium dodecyl sulfate (SDS) and 10% (v/v) methanol. The applied voltage of +20 kV resulted in an electrophoretic current of 30 μA. The temperature around the capillary was maintained constant at 25 °C [23].

GC analyses were conducted on a Varian 3800 chromatograph (Varian, Palo Alto, CA, USA), equipped with both a flame ionization detector (FID) and a Varian 4000 MS detector. Samples (1 μL) were injected using a split ratio of 1:10 on an HP-5MS fused silica capillary column (30 m × 0.25 mm I.D.; film thickness 0.25 μm; Agilent Technologies, Santa Clara, CA, USA). The operating conditions employed were as follows: injector and detector temperatures of 180 and 250 °C, respectively; a helium carrier gas flow rate of 1.3 mL min^{-1}; a temperature linear gradient from 130 (3 min hold) to 220 °C at 2 °C min^{-1} was applied [1,20].

2.4. Isolation and derivatization procedures

The amino acids and γ-glutamyl dipeptides were extracted from fresh vegetables by 90% aqueous methanol containing 10 mM HCl [25]. Typically, about 10 g of carefully peeled garlic cloves were homogenized in 150 ml of acidified methanol by using a tissue homogenizer. The homogenate was allowed to gently boil under reflux for 5 min, filtered and repeatedly extracted with another 150 ml portion of boiling methanol. The combined methanolic extracts were concentrated at reduced pressure (at 40 °C) to approximately 10–15 ml and adjusted to 25 ml by 20 mM borate buffer (pH 9.2). This extract was stored in the refrigerator (−18 °C) until derivatization.

Dansyl derivatives were prepared by mixing 100 μl of the sample extract with 250 μl of the Dns-Cl reagent (10 mM dansyl chloride in acetonitrile) and 0.65 ml of 20 mM borate buffer (pH 9.2). The mixture was briefly shaken, allowed to stand at room temperature for 15 min, filtered through a 0.45 μm nylon filter and analyzed by HPLC (gradient A).

FMOC derivatives were obtained by mixing 100 μl of the sample extract with 250 μl of the FMOC reagent (10 mM fluorenylmethyl chloroformate in acetonitrile) and 0.65 ml of 20 mM borate buffer (pH 9.2). The mixture was briefly shaken, allowed to stand at room temperature for 5 min and extracted by 1 ml of pentane to remove the excess of FMOC. After clearing the layers, the aqueous (the lower) one was filtered through a 0.45 μm nylon filter and analyzed by HPLC (gradient B) or CE.

OPA derivatives were prepared by mixing 100 μl of the sample extract with 900 μl of the OPA reagent. The mixture was briefly shaken, allowed to stand at room temperature for 15 min, filtered through a 0.45 μm nylon filter and analyzed by HPLC (gradient C). The OPA reagent was prepared by dissolving 140 mg of *o*-phthaldiadehyde in 5 ml of methanol. After addition of 100 μl of *tert*-butylthiol, the solution was adjusted to 50 ml with 50 mM KH_2PO_4 buffer (pH 9.5).

For GC determination, the extract was acidified by 1% HCl to pH 2.5–3.0 and an aliquot of 3 ml was passed through a column (1 cm × 5 cm) of a cation-exchange resin (Dowex 50WX4, H^+ form, 50–100 mesh). After washing the column with 10 ml of 1% HCl and 20 ml of H_2O, the amino acids were eluted with 50 ml of 1 M ammonium hydroxide. The eluate was evaporated to dryness (at 40 °C) and the residue obtained was dissolved in 0.3 ml of a mixture of ethanol/H_2O/pyridine (32/60/8, v/v/v). Ethyl chloroformate (ECF, 100 μl) was added to the mixture (evolution of CO_2 was usually observed). After brief shaking, 0.2 ml of aqueous sodium iodide (1.0 g ml^{-1}) and 50 μl of acetyl chloride were added. The reaction mixture was allowed to stand overnight at room temperature and the iodine formed was removed by addition of a few crystals of $SnCl_2$. The ECF derivatives were extracted by CH_2Cl_2 (0.4 ml) and analyzed by GC [20].

Identification of the amino acids and dipeptides was achieved by matching retention times of components in sample extracts with those of authentic standards and/or spiking sample extracts with the standards. The quantification was done relative to the internal standard of *S*-isobutylcysteine *S*-oxide (*i*-BCSO, 20 mg ml^{-1}) which was added to the samples before homogenization. Five-point calibration curves for both amino acids and dipeptides were generated using solutions prepared from the synthesized/isolated standards. For the assessment of the linearity of detector response, solutions of the following concentrations of derivatives were tested in triplicate: 0.01, 0.1, 0.5, 1.0 and 2.0 mM. The extraction efficiency and recovery of the analytes were determined in triplicate by adding a mixture of **1a**–**5a**, **1b**–**4b** and **1c**–**4c** at two different concentrations to garlic before homogenization. To allow better comparison of the tested methods, only a single extract was prepared from each *Allium* species and subjected to the various derivatization procedures. All extracts were analyzed at least in triplicate.

2.5. Testing of derivatives stability

When testing the stability of FMOC, OPA and Dns-tagged alliin, 1 ml of an alliin solution (5 mM in 20 mM borate buffer, pH 9.2) was mixed with 1 ml of α-naphthylacetic acid (5 mM in acetonitrile). The resulting mixture was derivatized as described above. The individual derivatized samples were stored in capped 2 ml clear glass vials at laboratory temperature without any protection from light. The samples were repeatedly analyzed in triplicate using the HPLC methods described above. The quantification was done relative to the peak area of α-naphthylacetic acid.

3. Results and discussion

In the first stage, we examined the applicability of various pre-column derivatization reagents for HPLC analysis of *S*-alk(en)ylcysteine *S*-oxides (**1a**–**5a**), γ-glutamyl-*S*-alk(en)ylcysteines (**1b**–**4b**), γ-glutamyl-*S*-alk(en)ylcysteine *S*-oxides (**1c**–**4c**) and cycloalliin (**6**). The following three reagents, *o*-phthaldialdehyde (OPA)/*tert*-butylthiol, fluorenylmethyl chloroformate (FMOC) and dansyl chloride (Dns-Cl), were chosen for several reasons. These reagents have already been successfully applied to HPLC analysis of **1a**–**5a**, with OPA/*tert*-butylthiol having been by far most frequently used. All three reagents are known to readily form derivatives with amino acids and dipeptides, with the reactions proceeding in aqueous solutions and at ambient temperature. The derivatives formed exhibit very high extinction coefficients, allowing sensitive and specific detection. Finally, the derivatization protocols do not involve any time-consuming clean-up steps, which enables high sample throughputs in routine applications.

3.1. Optimization of the HPLC separation

We have modified previously published HPLC procedures for separation of **1a**–**5a** [12–17] to achieve satisfactory separation of the complete range of *S*-substituted cysteine derivatives. Compared with the original separation conditions, we used shallower gradients which resulted in longer analysis times (50–70 min). In return, much better separation of the compounds of interest was typically achieved and a mixture of standards **1a**–**5a**, **1b**–**4b** and **1c**–**4c** was satisfactorily separated by all three optimized HPLC methods.

Standard samples of synthetically prepared *S*-alk(en)yl-L-cysteine *S*-oxides are always mixtures of two diastereomers which differ in the absolute configuration of the sulfoxide group (pure isomers can be obtained by time- and material-consuming fractional recrystallization). The individual diastereomers were found to be easily separable from each other using all three derivatization procedures. In all three cases, it was found that the naturally-occurring (+)-diastereomers of **1a**–**5a** elute from a C-18 HPLC column later than their levorotary counterparts. Interestingly, the migration order of the diastereomers of **1a**–**5a** is opposite during the CE/FMOC separation, with the naturally-occurring (+)-isomers migrating faster [23].

It is worth mentioning that the derivatives with a three-carbon side chain (alliin **2a**, isoalliin **3a**, and propiin **4a**) exhibited quite different elution patterns when analyzed by the tested methods. Whereas the elution pattern is **2a**–**3a**–**4a** during the HPLC/FMOC and HPLC/OPA separation, the dansyl-tagged derivatives elute in the order of **3a**–**2a**–**4a** (i.e. Dns-isoalliin elutes faster than Dns-alliin). This observation is in contrast with the study of Yoo and Pike [16] who reported a different elution sequence of **2a**–**3a**–**4a** for

Table 1
The stability of various alliin derivatives at room temperature (relative%).

Derivative	Days of storage				
	0	1	2	7	14
OPA/*tert*-butyl	100	89.9	67.8	33.5	10.7
FMOC	100	98.7	96.0	85.8	76.9
Dns	100	99.8	99.3	98.3	97.2

Dns-derivatives. It should be mentioned that no significant differences were observed between separations performed using C-18 and C-8 columns. For comparison, an elution order of **2a**–**4a**–**3a** was observed during the GC separation (using a nonpolar HP-5 column) and a migration order of **2a**–**3a**–**4a** during the CE/FMOC determination [23].

Unlike the OPA/*tert*-butyl reagent, both FMOC and Dns-Cl are capable of reacting with secondary amino acids. For example, the latter two compounds readily produced stable derivatives with proline under the experimental conditions used in this study. Thus, we also employed FMOC and Dns-Cl in the analysis of cycloalliin (**6**). It was found, however, that neither of these bulky reagents reacted with this amino acid, perhaps due to steric hindrance of the secondary amine group of **6**. None of the three derivatization procedures examined in this study therefore permits HPLC analysis of cycloalliin.

3.2. The stability of derivatives and derivatization reagent

All three derivatization reagents examined (OPA/*tert*-butylthiol, FMOC and Dns-Cl) were found to be stable upon storage in the refrigerator (4–5 °C) for at least 2 weeks, as no statistically significant differences were observed in derivatization of a standard sample of alliin (**2a**) during this period. Although the OPA reagent turned slightly opaque within 3 days of storage, it still retained its derivatization ability.

The stability of FMOC-, Dns- and OPA-tagged alliin upon storage at room temperature was evaluated by repeated injections over a 2-week period (Table 1). Whereas the Dns and FMOC-derivatives showed excellent stability, the OPA/*tert*-butylthiol-derivatized sample of alliin decomposed quite rapidly. Similar observations of the profound instability of OPA/*tert*-butylthiol-derivatized propiin (**4a**) were reported by Thomas and Parkin [17]. In our hands, *Allium* extracts turned opaque within 15–30 min after mixing with the OPA/*tert*-butylthiol reagent and a vividly colored (usually green) precipitate occasionally formed in the vials upon standing for several hours. To protect the column, the OPA-derivatized *Allium* samples had to be filtered immediately before injection, making automated analysis of multiple samples very complicated. On the other hand, large sets of FMOC- or Dns-tagged *Allium* samples could be prepared in advance, placed in an autosampler and analyzed within several hours without compromising the accuracy of results.

3.3. Sensitivity and accuracy

Detector responses were found to be linear ($r^2 \geq 0.98$) in the range of 0.01–2.0 mM for all OPA/*tert*-butylthiol-, FMOC-, and Dns-derivatives. The limits of detection (LOD) (calculated as a signal-to-noise ratio of 3) were found to be approximately 1.1, 2.0 and 1.5 pmol for OPA/*tert*-butylthiol-, FMOC-, and Dns-tagged alliin, respectively. These LOD values are similar to those determined by Fürst et al. for most protein amino acids [31]. For comparison, the LOD of 0.3 pmol was reported for alliin determination by micellar electrokinetic capillary electrophoresis (MEKC) after derivatization by FMOC [23]. The HPLC detection limits correspond to alliin content in garlic of approximately 0.3 $\mu g\, g^{-1}$ fresh weight. As *S*-substituted cysteine derivatives are typically present in substantially higher concentrations (>100 $\mu g\, g^{-1}$ fresh weight) in *Allium* plants, the sensitivity of all tested methods is more than sufficient for most purposes. If needed, the protocols can be easily modified to achieve even higher sensitivity of determination.

The extraction efficiency and recovery of the analytes were determined by adding two different concentrations of each organosulfur compound (**1a**–**5a**, **1b**–**4b** and **1c**–**4c**) to garlic before homogenization. The validated extraction procedure of Ichikawa et al. [25] was used, employing acidified 90% methanol as the solvent. In accordance with their observations, recoveries of 91–103% were typically determined by all three derivatization methods, with relative standard deviation (RSD) values lower than 7.9%.

3.4. Choice of an internal standard

A great deal of attention was paid to finding a suitable internal standard which would reduce potential errors occurring during extraction, derivatization and injection steps. In our previous studies [1,20,21], we used *S*-butylcysteine *S*-oxide as an internal standard. However, this amino acid was subsequently found to occur abundantly in *Allium siculum* and at trace levels in onion [2]. Therefore, we decided to use *S*-isobutylcysteine *S*-oxide (*i*-BCSO) which was chosen for a number of reasons. Most importantly, this amino acid has not been reported to occur in *Allium* species. Although *i*-BCSO is not commercially available, it can be easily prepared in high yield and purity. Its thermal stability, solubility and chemical behavior are very similar to those of **1a**–**5a**. *i*-BCSO can also serve as a substrate for alliinase, thus possible enzymatic cleavage of **1a**–**5a** during homogenization and extraction steps is partially corrected. Furthermore, the two peaks of the individual *i*-BCSO diastereomers do not usually coincide with any other compounds found in the extracts. If one of them accidentally co-elutes with some other extract component, the quantification can still be performed relative to the area of the second *i*-BCSO diastereomer.

3.5. Determination of *S*-alk(en)ylcysteine *S*-oxides in *Allium* species

Four different *Allium* species, representing the most commonly consumed alliaceous vegetables worldwide, were analyzed (garlic, onion, shallot, and leek). The content of **1a**–**5a** in these species was simultaneously examined by the three optimized HPLC methods, along with the previously developed GC and CE procedures [20,23]. Typical HPLC and GC chromatograms and a CE electropherogram of an extract from garlic are shown in Fig. 2. The total content of *S*-alk(en)ylcysteine *S*-oxides (**1a**–**5a**) varied considerably within a wide range of 0.49–13.1 $mg\, g^{-1}$ fresh weight (Table 2). Whereas alliin (**2a**) was found in substantial amounts only in garlic, isoalliin (**3a**) was the major *S*-alk(en)ylcysteine *S*-oxide occurring in onion, shallot, and leek. Methiin (**1a**) was present in all four species as a second most abundant derivative. Quite interestingly, alliin (**2a**) was found to occur at trace levels also in shallot, along with the predominant isoalliin (**3a**). On the other hand, propiin (**4a**) and ethiin (**5a**) were not detected in substantial amounts in any of the samples analyzed. In general, the values found in the present study are consistent with data reported previously [1,5,12–20].

3.6. Determination of γ-glutamyl dipeptides in *Allium* species

Unfortunately, the optimized HPLC/OPA/*tert*-butylthiol and HPLC/FMOC methods did not permit a satisfactory separation of dipeptides **1b**/**c**–**4b**/**c** in real *Allium* extracts, as significant peak overlapping was often observed. Usually one or two dipeptide peaks co-eluted with other components present in the extracts. We did not succeed in further optimizing the methods (by changing the mobile phase composition or buffer pH) to satisfactorily improve

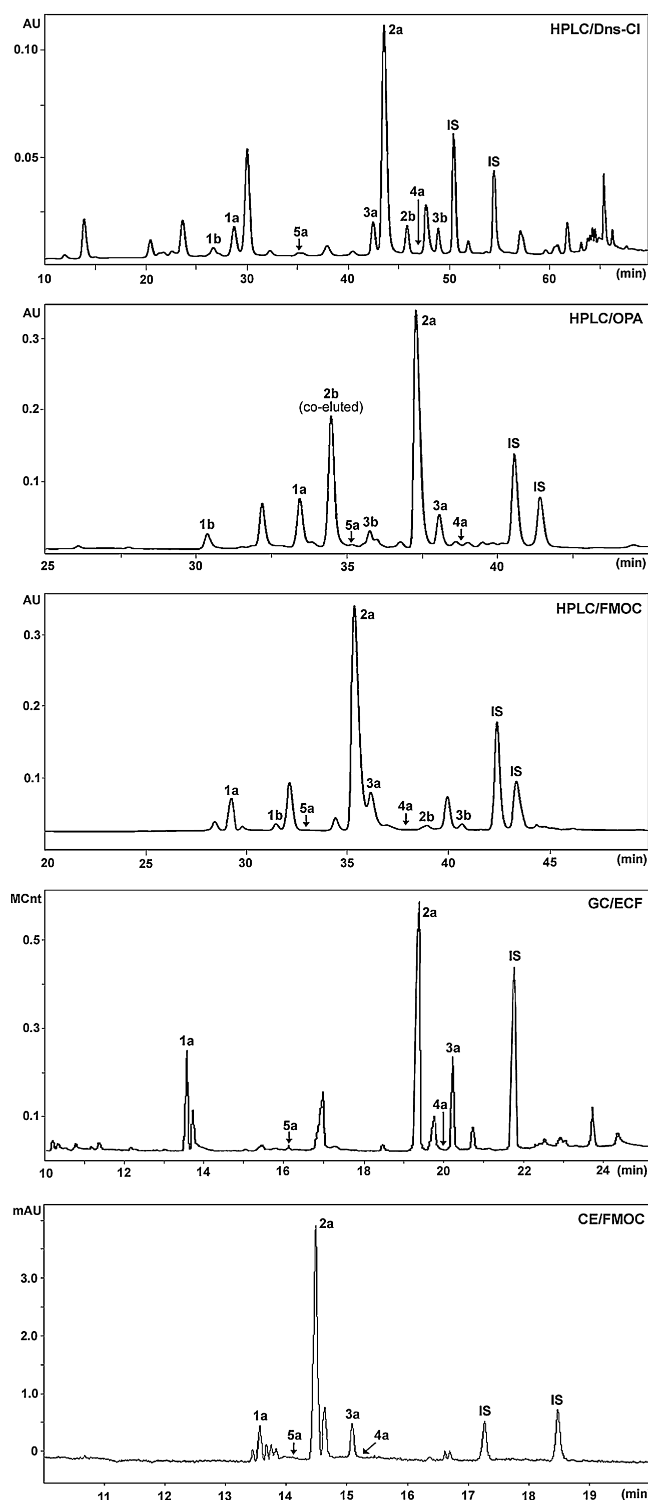

Fig. 2. Separation of *S*-substituted cysteine derivatives in an extract from garlic by various chromatographic procedures.

the separation of **1b**/**c**–**4b**/**c** from the interfering compounds. The dipeptide content was therefore determined only by the Dns-Cl method which proved to offer superior separation of all compounds of interest also in real *Allium* extracts.

As shown in Table 3, γ-glutamyl dipeptides (**1b**/**c**–**4b**/**c**) were present in the samples analyzed in considerably lower quantities compared with the corresponding *S*-alk(en)ylcysteine *S*-oxides (**1a**–**4a**). γ-Glutamyl-*S*-alk(en)ylcysteines (**1b**–**4b**) occurred quite abundantly only in garlic, whereas their content in onion, shallot and leek was very limited. On the contrary, the latter three *Allium* species contained γ-glutamyl-*S*-(*E*)-(1-propenyl)cysteine *S*-oxide (**3c**) as the most abundant dipeptide. In accordance with previous reports [7,8,25], no oxidized dipeptide forms (i.e. **1c**–**4c**) were detected in garlic, limiting their possible levels to <0.01 mg g^{-1} fresh weight. Our results are generally in a good agreement with the data reported previously (Table 3). It should be kept in mind, however, that the content of these dipeptides can fluctuate dramatically during the growth cycle and subsequent storage. For example, Matsuura et al. [7] reported a more than six-fold increase in the content of **2b** during an 8-week-period of garlic growth.

3.7. Comparison of the various chromatographic methods

All tested methods showed quite satisfactory reproducibility of determination (expressed as relative standard deviations, RSD). The RSD values were as follows: 3.5–5.2% (mean 4.4%), 5.5–6.5% (mean 5.9%), 4.7–9.0% (mean 6.6%), 1.6–8.1% (mean 5.4%) and 3.4–6.1% (mean 4.8%) for HPLC/Dns-Cl, HPLC/FMOC, HPLC/OPA, CE/FMOC and GC/ECF determinations, respectively. As can be seen in Table 2, no significant differences were observed among the values determined by the five chromatographic procedures tested in this study. Relative standard deviations of the various determinations varied between 4.1% and 8.2%, with a mean of 5.7%. All five methods also exhibited relatively good reproducibility of retention/migration times, with the GC determination showing best characteristics. Intraday RSD of retention/migration times of **1a**–**3a** observed were as follows: 0.1–0.3% (HPLC/Dns-Cl), 0.1–0.3% (HPLC/FMOC), 0.1–0.2% (HPLC/OPA), 0.1–0.4% (CE/FMOC) and <0.1% (GC/ECF). The RSD of retention/migration times of day-to-day runs typically varied between 0.1% and 1.2%, with a mean of 0.4%.

Although each of the three HPLC methods investigated permits reliable and sensitive quantitative determination of *S*-alk(en)ylcysteine *S*-oxides (**1a**–**5a**), the Dns-Cl procedure also allows superior separation of the γ-glutamyl dipeptide forms (**1b**/**c**–**4b**/**c**). Using this method, *S*-alk(en)ylcysteine *S*-oxides (**1a**–**5a**), γ-glutamyl-*S*-alk(en)ylcysteines (**1b**–**4b**), and γ-glutamyl-*S*-alk(en)ylcysteine *S*-oxides (**1c**–**4c**) can be separated and quantified in a single run. Thanks to its simplicity and excellent stability of the derivatives, the HPLC/Dns-Cl method is also suitable for routine automated analysis of multiple samples. On the other hand, the widely used OPA/*tert*-butylthiol derivatization procedure does not offer any significant advantage compared to the Dns-Cl method described in this study. The OPA-tagged derivatives are considerably less stable, making automated analysis of multiple samples quite problematic. Furthermore, the OPA derivatization reagent consists of the noxiously smelling thiol, thus the use of a well ventilated hood is absolutely necessary for sample preparation and handling. A summary of major analytical features of the different methods is given in Table 4.

Another chromatographic method of choice for quantitative analysis of **1a**–**5a** can be the recently reported determination by capillary electrophoresis after derivatization with FMOC [23]. This method is comparatively sensitive and specific as the HPLC procedures. Main advantages of this rapid method are simplicity, high specificity, sensitivity and very low running costs, making it suitable for routine analysis of large numbers of samples. One run takes only 20 min compared with a typical 40–70 min HPLC or GC analysis. On the other hand, migration time variations (resulting in possible peak misidentifications) belong to its most significant drawbacks, if the capillary is not rinsed and equilibrated properly (Table 4).

S-Alk(en)ylcysteine *S*-oxides can also be reliably analyzed by gas chromatography [20]. Unlike the HPLC and CE determination, the GC method is significantly more elaborative. In return, it offers

Table 2
The content of *S*-alk(en)ylcysteine *S*-oxides in *Allium* species (in mg g^{-1} fresh weight) as determined by various analytical methods.

Species	*S*-Alk(en)ylcysteine *S*-oxide						
	1a	**2a**	**3a**	**4a**	**5a**	Total	Method
Garlic	1.37 ± 0.06	10.12 ± 0.34	1.20 ± 0.05	n.d.[a]	tr[b]	12.7 ± 0.4	HPLC/Dns-Cl
	1.24 ± 0.08	10.68 ± 0.49	1.14 ± 0.07	n.d.	n.d.	13.1 ± 0.7	HPLC/FMOC
	1.31 ± 0.07	9.51 ± 0.54	1.22 ± 0.08	n.d.	tr	12.0 ± 0.6	HPLC/OPA
	1.19 ± 0.07	9.62 ± 0.37	1.02 ± 0.07	n.d.	tr	11.8 ± 0.6	GC/ECF
	1.29 ± 0.05	9.97 ± 0.17	1.05 ± 0.05	n.d.	n.d.	12.3 ± 0.2	CE/FMOC
Onion	0.12 ± 0.01	n.d.	0.44 ± 0.02	tr	tr	0.56 ± 0.03	HPLC/Dns-Cl
	0.10 ± 0.01	n.d.	0.42 ± 0.02	tr	n.d.	0.52 ± 0.03	HPLC/FMOC
	0.13 ± 0.01	n.d.	0.47 ± 0.03	tr	tr	0.60 ± 0.04	HPLC/OPA
	0.09 ± 0.01	n.d.	0.40 ± 0.01	tr	tr	0.49 ± 0.02	GC/ECF
	0.10 ± 0.01	n.d.	0.49 ± 0.03	n.d.	n.d.	0.59 ± 0.04	CE/FMOC
Shallot	0.20 ± 0.01	0.05 ± 0.01	0.98 ± 0.06	tr	n.d.	1.23 ± 0.11	HPLC/Dns-Cl
	0.22 ± 0.01	tr	1.07 ± 0.06	tr	n.d.	1.29 ± 0.08	HPLC/FMOC
	0.24 ± 0.02	0.03 ± 0.01	1.12 ± 0.10	tr	n.d.	1.40 ± 0.13	HPLC/OPA
	0.19 ± 0.01	0.04 ± 0.01	1.01 ± 0.07	tr	tr	1.25 ± 0.12	GC/ECF
	0.25 ± 0.03	tr	1.10 ± 0.09	n.d.	n.d.	1.35 ± 0.11	CE/FMOC
Leek	0.18 ± 0.02	n.d.	1.27 ± 0.05	tr	tr	1.44 ± 0.07	HPLC/Dns-Cl
	0.22 ± 0.02	n.d.	1.32 ± 0.07	tr	tr	1.54 ± 0.10	HPLC/FMOC
	0.17 ± 0.02	n.d.	1.19 ± 0.04	tr	tr	1.36 ± 0.09	HPLC/OPA
	0.20 ± 0.01	n.d.	1.21 ± 0.05	tr	tr	1.41 ± 0.09	GC/ECF
	0.19 ± 0.02	n.d.	1.35 ± 0.06	tr	n.d.	1.53 ± 0.08	CE/FMOC

[a] n.d., not detected.
[b] tr, traces (<0.02 mg g^{-1} fresh weight).

excellent separation capability and sensitivity. In combination with a MS detector, it is an indispensable tool when looking for new *S*-substituted cysteine derivatives. Unfortunately, the GC procedure does not permit analysis of γ-glutamyl dipeptides, due to their unsufficient volatility (Table 4).

There should also be mentioned some other chromatographic procedures developed for quantitative determination of *S*-substituted cysteine derivatives (including the γ-glutamyl dipeptides). The method of Ichikawa et al. [25] is based on HPLC separation of **1a**–**4a** and **1b**–**4b** without pre-column derivatization. However, this procedure is not capable of simultaneous separation of the complete range of *S*-substituted cysteine derivatives in a single run and separate injections on two different columns (aminopropyl and C-18) are required to quantify **1a**/**b**–**4a**/**b**. No information has also been given about the applicability of this method for the analysis of species other than garlic. On the other hand, this method is, at present, the only reliable chromatographic procedure allowing quantitative determination of cycloalliin (**6**).

Another HPLC method for separation and quantitation of *S*-substituted cysteine derivatives is based on pre-column derivatization by phenylisothiocyanate (PITC) [19]. Although this method permits simultaneous separation of both *S*-alk(en)ylcysteine *S*-oxides (**1a**–**4a**) and several γ-glutamyl dipeptides, the procedure involves time-consuming extract fractionation on an ion-exchanger and a laborious derivatization step, limiting its applicability in routine analyses of large sample sets. Furthermore, Fürst et al. also observed a considerable shortening of the lifetime of the column used for separation of PITC-tagged amino acids [31].

Finally, Arnault et al. [26] reported an HPLC method for simultaneous quantification of alliin (**2a**) and two major γ-glutamyl dipeptides (**2b** and **3b**) present in garlic and garlic-derived products. The method utilizes sodium heptanesulfonate as an ion-pairing reagent and it also permits quantification of a few other sulfur secondary metabolites (allicin, *S*-allylcysteine). However, determination of other important cysteine derivatives, most notably methiin (**1a**) and isoalliin (**3a**), was not performed by this

Table 3
The content of γ-glutamyl-*S*-alk(en)ylcysteine dipeptides in *Allium* species (in mg g^{-1} fresh weight) as determined by the HPLC/Dns-Cl method.

Species	γ-Glutamyl-*S*-alk(en)ylcysteine dipeptide					
	1b	**2b**	**3b**	**3c**	Total	Ref.
Garlic	0.48 ± 0.05	0.96 ± 0.07	0.73 ± 0.04	n.d.[a]	2.18 ± 0.16	[b]
	n.q.[c]	0.19–5.77	0.33–3.30	n.q.	0.52–9.1	[7]
	0.5–1.3	4.0–6.9	4.1–8.0	n.d.	9.4–15.4	[8]
Onion	0.08 ± 0.01	n.d.	tr[d]	1.07 ± 0.06	1.15 ± 0.07	[b]
	n.q.	n.q.	n.q.	1.49–2.92	1.49–2.92	[6]
	n.d.	n.d.	0.08	0.53	0.61	[8]
	n.q.	n.q.	n.q.	0.04–2.33	0.04–2.33	[19]
	n.q.	n.q.	n.q.	1.24–2.18	1.24–2.18	[27]
Shallot	0.02 ± 0.01	n.d.	tr	0.20 ± 0.02	0.22 ± 0.02	[b]
	n.d.	n.d.	0.18	0.85	1.03	[8]
Leek	0.08 ± 0.01	n.d.	tr	0.23 ± 0.02	0.31 ± 0.02	[b]
	n.d.	n.d.	0.02	n.d.	0.02	[8]

[a] n.d., not detected.
[b] Present study.
[c] n.q., not quantified.
[d] tr, traces (<0.02 mg g^{-1} fresh weight).

Table 4
Comparison of various chromatographic methods for determination of *S*-substituted cysteine derivatives.

	Method				
	HPLC/Dns-Cl	HPLC/OPA/*t*-BuSH	HPLC/FMOC	GC/ECF	CE/FMOC
Limit of detection[a]	1.5 pmol	1.1 pmol	2.0 pmol	N.D.[b]	0.3 pmol
Retention/migration times reproducibility	0.1–0.3%	0.1–0.2%	0.1–0.3%	<0.1%	0.1–0.4%
Stability of derivatives	Excellent	Fair	Very good	Very good	Very good
Reagent stability	Very good	Very good	Very good	Very good	Very good
Elution order of diastereomers	(−)/(+)	(−)/(+)	(−)/(+)	N.A.[c]	(+)/(−)
Elution/migration pattern	**3a**/**2a**/**4a**	**2a**/**3a**/**4a**	**2a**/**3a**/**4a**	**2a**/**4a**/**3a**[d], **4a**/**3a**/**2a**[e]	**2a**/**3a**/**4a**
Determination of dipeptides	Yes	Not whole range	Not whole range	No	No
Advantages	Simplicity; excellent derivative stability	Simplicity	Simplicity; good derivative stability	Excellent separation capability; convenient peak verification by GC/MS	Simple and cheap; short analysis times (20 min)
Major drawbacks	Long analysis times (70 min)	Limited derivative stability; noxiously smelling reagent	Peak broadening and overlapping	Elaborative sample preparation	Need for regular capillary rinsing; possible peak misidentification

[a] Limit of detection (LOD) of alliin.
[b] N.D., not determined.
[c] N.A., not applicable (the chiral sulfoxide group is reduced during derivatization step).
[d] Elution order on an HP-5 GC column (non-polar).
[e] Elution order on an HP-INNOWax GC column (polar), Ref. [20].

method. Also, the applicability of this procedure for analysis of other *Allium* species (e.g. onion or leek) has not been demonstrated yet.

4. Conclusions

In conclusion, we believe that the newly developed HPLC/Dns-Cl method could become a generally used procedure for analysis of various *S*-substituted cysteine derivatives in *Allium* species, as it offers several advantages over the most commonly used OPA/*tert*-butylthiol procedure as well as other HPLC methods. It is a simple but powerful tool for analysis of the whole range of *S*-substituted cysteine derivatives not only in fresh vegetables but also in various *Allium*-derived products (e.g. garlic-based food supplements, spices etc.). The applicability of this method can also be extended to determination of methiin (**1a**) and γ-glutamyl-*S*-methylcysteine (**1b**) in cruciferous plants.

Acknowledgements

Financial support provided by the Ministry of Education of the Czech Republic (grants OC123 – COST 924 and MSM6007665806) is greatly appreciated. The authors also thank Dr. Jan Schraml (Institute of Chemical Processes, Czech Academy of Sciences, Prague, Czech Republic) for conducting NMR analyses and Dr. Martin L. Shaw (Institute for Crop and Food Research, Christchurch, New Zealand) and Wakunaga Pharmaceutical Company (Hiroshima, Japan) for providing samples of γ-glutamyl-*S*-(*E*)-(1-propenyl)cysteine *S*-oxide and cycloalliin, respectively.

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