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Short communication

Quantitative determination of *S*-alk(en)ylcysteine-*S*-oxides by micellar electrokinetic capillary chromatography

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ABSTRACT

A novel method for determination of *S*-alk(en)ylcysteine-*S*-oxides by capillary electrophoresis has been developed and validated. The method is based on extraction of these sulfur amino acids by methanol, their derivatization by fluorenylmethyl chloroformate and subsequent separation by micellar electrokinetic capillary chromatography. Main advantages of the new method are simplicity, sensitivity, high specificity and very low running costs, making it suitable for routine analysis of a large number of samples. Employing this method, the content of *S*-alk(en)ylcysteine-*S*-oxides was determined in 12 commonly consumed alliaceous and cruciferous vegetables (e.g. garlic, onion, leek, chive, cabbage, radish, cauliflower and broccoli). The total content of these amino acids in the *Allium* species evaluated varied between 0.59 and 12.3 mg g^{-1} fresh weight. Whereas alliin was found only in garlic, isoalliin was the major *S*-alk(en)ylcysteine-*S*-oxide in onion, leek, chive and shallot. On the other hand, the cruciferous species analyzed contained only methiin in the range of 0.06–2.45 mg g^{-1} fresh weight.

1. Introduction

S-Alk(en)ylcysteine-*S*-oxides are important secondary metabolites occurring in many families of plants, fungi and algae. These sulfur amino acids are precursors of an extraordinary variety of sensory-active and health-beneficial compounds of *Allium* and *Brassica* vegetables (e.g. garlic, onion, leek and cabbage, broccoli, kohlrabi, etc.). Six *S*-alk(en)ylcysteine-*S*-oxides have so far been found in alliaceous plants, namely *S*-methyl-, *S*-allyl-, (*E*)-*S*-(1-propenyl)-, *S*-propyl-, *S*-ethyl- and *S*-butylcysteine-*S*-oxides (methiin, alliin, isoalliin, propiin, ethiin and butiin, respectively, **1**–**6**) (Fig. 1) [1–9]. On the other hand, cruciferous plants typically contain only methiin (**1**) with traces of ethiin (**5**) [10–12]. Disruption of the plant tissue results in the release of a C-S lyase and subsequent enzymatic cleavage of *S*-alk(en)ylcysteine-*S*-oxides to form thiosulfinates [RS(O)SR′], the flavor principles of freshly comminuted *Allium* vegetables.

Numerous methods for quantitative determination of *S*-alk(en)ylcysteine-*S*-oxides (**1**–**6**) have been developed. A leading role among these methods plays HPLC determination after pre-column derivatization, with *ortho*-phthaldialdehyde (OPA)/*tert*-butylthiol being the most frequently used derivatization reagent [6]. Alternatively, *S*-alk(en)ylcysteine-*S*-oxides can be quantified by GC after derivatization with ethyl chloroformate and reduction of the thermolabile sulfoxide group by sodium iodide [1,2,10]. The GC method allows very sensitive determination in combination with the convenient possibility to verify the identity of analytes by mass-spectrometry. Surprisingly, only one method has thus far been published for determination of methiin (**1**) and alliin (**2**) by capillary electrophoresis (CE) [11]. However, no information was given regarding the applicability of this CE procedure to the analysis of other very important cysteine derivatives, namely isoalliin (**3**) and propiin (**4**). Quantification of isoalliin in both onion and garlic is of particular importance, as this amino acid is the key compound directly affecting the pungency of onion and the tendency of garlic to undesirable discoloration [13].

Thus, the main aim of this study was to develop a simple method allowing rapid, sensitive and reproducible determination of the whole range of *S*-alk(en)ylcysteine-*S*-oxides by means of capillary electrophoresis. Such a method would be suitable not only for analysis of fresh vegetables but also for evaluation of various products made of garlic and onion (e.g. garlic-based supplements, spices,

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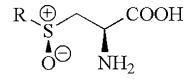

S-alk(en)ylcysteine-*S*-oxide

R = CH_3— (methiin, **1**)
CH_2=$CHCH_2$— (alliin, **2**)
CH_3CH=CH— (isoalliin, **3**)
$CH_3CH_2CH_2$— (propiin, **4**)
CH_3CH_2— (ethiin, **5**)
$CH_3CH_2CH_2CH_2$— (butiin, **6**)

Fig. 1. *S*-Alk(en)ylcysteine-*S*-oxides in *Allium* species.

etc.). The emphasis was put on the simplicity of isolation, clean-up and derivatization steps to ensure low financial costs per sample and general applicability of the method.

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 solvents (methanol and acetonitrile) were purchased from LabScan (Dublin, Ireland). Garlic (*Allium sativum* L., China), yellow onion (*Allium cepa* L., Netherlands), chive (*Allium schoenoprasum* L., Czech Republic), leek (*Allium ampeloprasum* var. *porrum* L., Netherlands), shallot (*Allium ascalonicum* auct., Netherlands), cabbage (*Brassica oleracea* L. convar. *capitata* var. *alba*, Czech Republic), Pekingese cabbage (*Brassica pekinensis* Lour., Czech Republic), broccoli (*B. oleracea* DC. var. *asparagoides*, Spain), kohlrabi (*B. oleracea* L. var. *gongylodes*, Czech Republic), cauliflower (*B. oleracea* L. var. *botrytis*, Italy), radish (*Raphanus sativus* L. var. *radicula*, Czech Republic) and white radish (*R. sativus* DC. subsp. *niger* var. *albus*, Italy) were purchased from a local market in June 2008.

2.2. Reference compounds

S-Substituted cysteines (*S*-methyl-, *S*-ethyl-, *S*-propyl-, *S*-allyl-, *S*-butyl- 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 [1]. The naturally occurring (S_S,R_C)-diastereomers of alliin (ACSO, **2**) and propiin (PCSO, **4**) were obtained by repeated recrystallizations from aqueous acetone or ethanol, respectively. Isoalliin (PeCSO, **3**) was isolated from white onion according to the procedure of Carson et al. [14]. 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

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 as 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) MeOH. 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.

2.4. Isolation and derivatization procedure

The amino acids were extracted from fresh vegetables by 90% aqueous methanol containing 10 mM HCl [1–3]. 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 reduced (at 40 °C) to approximately 10–15 ml and adjusted to 25 ml by 20 mM borate buffer (pH 9.2). The extract was filtered with a 0.45-μm nylon filter and an aliquot of 100 μl was mixed with 150 μl of fluorenylmethyl chloroformate (FMOC-Cl; 10 mM in MeCN) and 0.75 ml of the borate buffer. The mixture was briefly shaken, allowed to stand at room temperature for 5 min and extracted by 1 ml of pentane. After clearing the layers, the aqueous (the lower) one was analyzed.

The quantification was done relative to the internal standard of *S*-isobutylcysteine-*S*-oxide (*i*-BCSO, 20 mg ml^{-1}) which was added prior to sample homogenization. All samples were analyzed in triplicate. Calibration curves for the analytes were generated using solutions prepared from the synthesized/isolated standards.

3. Results and discussion

3.1. Method development

Initially, we attempted to adopt the recently published CE procedure of Horie and Yamashita [11]. They developed a capillary-electrophoretic method that is based on indirect detection of methiin (**1**) and alliin (**2**). Using this method, they determined the content of these two amino acids in garlic and a few other *Allium* and *Brassica* vegetables. After a few slight modifications of the original procedure, we achieved quite good separation of a mixture of the standards. However, this method proved to offer absolutely unsatisfactory results for analyzing real sample extracts. The capillary deteriorated rapidly after a few injections, resulting in loosing the separation capability, even if the capillary was rinsed thoroughly after every run. Moreover, significant migration time variations and peak overlapping were observed, rendering identification and quantification of individual peaks extremely unreliable.

Therefore, we turned our attention to developing a completely novel method. We decided to test the applicability of the FMOC-Cl derivatization procedure for CE analysis of compounds **1**–**6**. FMOC-Cl is known to readily form stable derivatives with compounds possessing primary and secondary amino groups in virtually quantitative yield [15]. The reaction proceeds in aqueous solutions within minutes with no time-consuming clean-up procedure required. Moreover, the derivatives exhibit very high extinction coefficients, allowing their sensitive and specific detection. Thus, it is not surprising that FMOC-Cl belongs to the most frequently used derivatization reagents for analysis of amino acids in combination with HPLC or CE. In fact, FMOC-Cl was already successfully employed for analysis of *S*-alk(en)ylcysteine-*S*-oxides in *Allium* plants by HPLC [4].

When looking for optimal separation conditions, we tested various concentrations of sodium dodecyl sulfate (SDS, 0–30 mM) and MeOH (0–15%) in the running borate buffer. The FMOC-tagged derivatives were found to be optimally resolved in a system consisting of 20 mM borate buffer (pH 9.2), 20 mM SDS and 10% MeOH. Under these conditions, all compounds of interest were satisfactorily resolved within 20 min. The individual diastereomers of synthetically prepared *S*-alk(en)yl-L-cysteine-*S*-oxides were easily separable, with the naturally occurring (+)-isomers migrating