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High-performance ion-pair chromatography method for simultaneous analysis of alliin, deoxyalliin, allicin and dipeptide precursors in garlic products using multiple mass spectrometry and UV detection☆

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Abstract

The quality of garlic and garlic products is usually related to their alliin content and allicin release potential. Until now no analytical method was able to quantify simultaneously allicin, its direct precursor alliin (*S*-allyl-L-cysteine sulfoxide), SAC (*S*-allyl-L-cysteine) as well as various dipeptides that apparently serve as storage compounds in garlic. It is well known that all these intermediates are involved in the allicin biosynthetic pathway. A simple and rapid HPLC method suitable for routine analysis was developed using eluents containing an ion-pairing reagent. Particularly, heptanesulfonate as ion-pairing reagent guarantees a sufficient separation between alliin and the more retained dipeptides at very low pH. Allicin was eluted after 18 min on a 150×3 mm column. Synthetic reference compounds were characterized by the same chromatographic method using a diode-array UV detector and an ion trap mass spectrometer (electrospray ionization) in the multiple MS mode. In routine analysis of garlic bulbs, powders and other products, the diode-array detector is sufficient for a relevant quantification. Our method has been used in studies to improve the quality of garlic and its derived products.

Keywords: Garlic; Food analysis; Ion-pairing reagents; Peptides; Alliin; Deoxyalliin; Allicin; Organosulfur compounds

1. Introduction

Garlic is considered as a medicinal plant and especially one of the best disease-preventive food against some forms of cancer and cardiovascular disorders. Its beneficial widespread effect on health is attributed to sulphur compounds [1] and particularly to thiosulfinates.

When the clove of garlic is cut or crushed, the enzyme alliinase is released from its compartment

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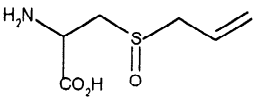
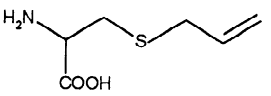
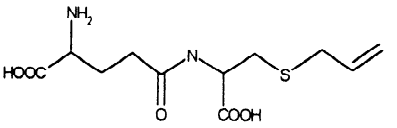
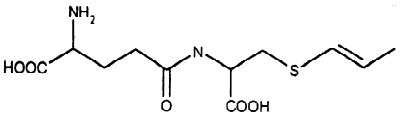
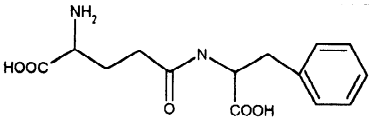
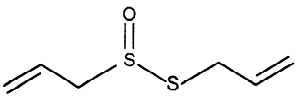
and transforms *S*-allyl-L-cysteine sulfoxide or alliin (**1**) into diallyl thiosulfinate or allicin (**6**), the characteristic compound of garlic flavor. This very unstable thiosulfinate is rapidly degraded to related organosulfur compounds like (poly)sulfides, vinyldithiines or ajoene [2]. In fact alliin (**1**) like others *S*-alk(en)yl-L-cysteine sulfoxides comes from γ-glutamyl-*S*-alk(en)yl-L-cysteines (GLUACs) via *S*-allyl-L-cysteine (SAC) or deoxyalliin (**2**) (see Table 1 for compound identities). A general scheme of thiosulfinates formation from GLUACs in *Allium* spp. is shown in Fig. 1.

To evaluate quality of garlic and garlic products, it is important to consider all the precursors and the biological active substances. Less is known about GLUACs only that they appear to provide a reserve for *S*-alk(en)yl-L-cysteine sulfoxides [3] particularly alliin in garlic, where GLUPeCs (**4**) the major precursor of onion is also present and seems to interfere with allyl compounds biosynthesis.

Until now, no analytical method is able to quantify simultaneously all sulfur compounds (**1**–**4**) implicated in biosynthesis of allicin, allicin itself and its degradation compounds.

Among these methods previously described in this field (Table 2), GC is scarcely used [5,9] and RP-

Table 1
Name and chemical structure of analysed compounds

Compound	Name	Abbreviation	Chemical structure
1	*S*-Allyl-L-cysteine sulfoxide	Alliin	
2	*S*-Allyl-L-cysteine, deoxyalliin,	SAC	
3	γ-Glutamyl-*S*-allyl-L-cysteine	GLUAlCs	
4	γ-Glutamyl-*S*-(*trans*-1-propenyl)-L-cysteine	GLUPeCs	
5	γ-Glutamyl phenylalanine	GLUPheAla	
6	Diallyl thiosulfinate	Allicin	

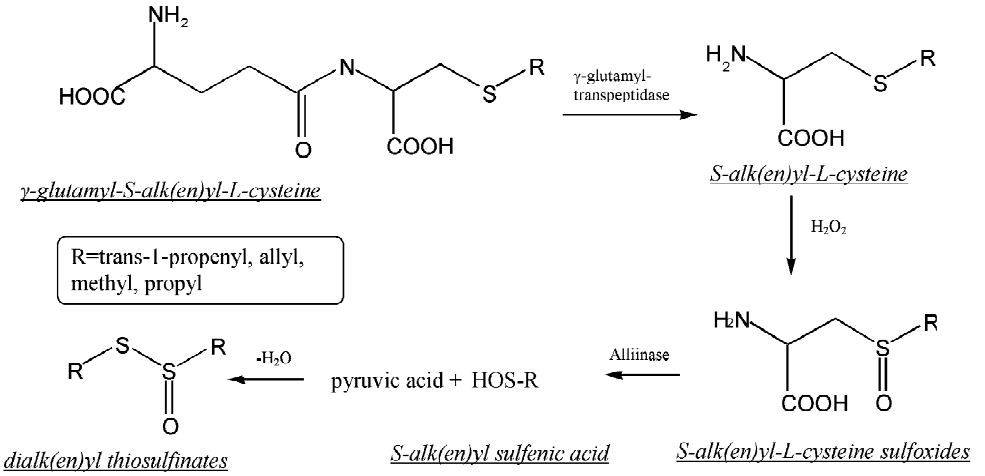


Fig. 1. Biosynthesis of dialk(en)yl thiosulfinates from γ-glutamyl-*S*-alk(en)yl-L-cysteine via *S*-alk(en)yl-L-cysteine sulfoxides in *Allium* spp.

HPLC with UV detection is the most frequently used. Most of them allow determination of alliin [4,6,9,10,12,13] or allicin [5,8,11] alone. Only one reports the quantification of GLUACs (**3**,**4**) with alliin (**1**) [7].

When the sensitivity is not sufficient, fluorimetric detection [4,6,7] or pre-column derivatization procedures [6,7,9,10,12,13] are preferred. When no reference compounds are available, UV [3,8], electrochemical detection [6] or coupling to mass spectrometry [8] are used.

For instance, in our laboratory we used RP-HPLC–UV with the phenylisothiocyanate pre-column derivatization method Pico-Tag [13].

Our purpose, in the present study, is to develop a method able to quantify in the same run compounds (**1**)–(**6**) in garlic and garlic products.

Due to the ionic nature of these compounds at low pH, the best way to separate sufficiently these substances is to evaluate a reversed-phase high-performance ion-pair chromatography method [14].

2. Experimental

2.1. Plant materials and chemicals

2.1.1. Chemicals

Alliin (**1**) was synthesized by alkylation of L-cysteine with allyl bromide and then by oxidation of the resulting deoxyalliin (**2**) with hydrogen peroxide according to the procedure of Iberl et al. [12].

Allicin (**6**) was synthesized by oxidation of diallyl disulfide with peracetic acid according to the procedure of Iberl et al. [12], too.

The synthesis of γ-glutamyl-*S*-allyl-L-cysteine (**3**) consists in coupling (**2**) with the γ-carboxy-activated glutamic acid prepared by treating *N*-Boc-L-glutamic (where Boc=*tert.*-butoxycarbonyl) acid α-*tert.*-butyl ester with *N*-hydroxysuccinimide and 1,3-dicyclohexylcarbodiimide. This reaction was done in the presence of $NaHCO_3$ followed by solvent removal and deprotection with trifluoroacetic acid (TFA). More details about this procedure are described by Lawson et al. [3]. Crude product was purified using preparative HPLC and the TFA salts were removed using ion-exchange chromatography.

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.1.2. Plant materials, cultivation and sampling

2.1.2.1. Field trial

Garlic bulbs were produced at Crest in France. Certified seeds material variety Printanor were supplied by National Institute of Agronomy Research of Avignon in France. Sulfur fertilisation was provided by dehydrated $CaSO_4$ (50% SO_4) applied before

Table 2
List of published chromatographic methods used for garlic analysis

Chromatographic technique/detection	Column type/wavelengths or ΔE/elution mode	Remark	Compounds tested	Ref.
HPLC–UV	RP-C_{18}/220 nm/isocratic	–	(**3**)+(**4**)+(**6**)	[3]
HPLC–UV	RPTMS/210 nm/isocratic	–	(**1**)	[4]
HPIPC–FL	RP-C_{18}/405–480 nm/isocratic	Ion-pairing reagent: tetra-*n*-butylammonium bromine	(**1**)	
GC–MS	Porous-layer open tubular/–/–	–	(**6**)	[5]
HPLC–UV	RP-C_{18}/337 nm/SMGE and isocratic	Pre-column derivatization: OPA–*tert*.-BuSH	(**1**)	[6]
HPLC–ED	RP-C_{18}/750 mV/SMGE and isocratic			
HPLC–FL	RP-C_{18}/230–420 nm/SMGE and isocratic			
HPLC–DAD	RP/260 and 337 nm/gradient and isocratic	Pre-column derivatization: OPA–*tert*.-BuSH	(**1**)+(**3**)+(**4**)	[7]
HPLC–FL	RP/230–420 nm/gradient and isocratic			
HPLC–DAD	RP-C_{18}/240 nm/isocratic	–	(**6**)	[8]
HPLC–MS	RP-C_{18}/–/isocratic			
GC–FID	Wall-coated open tubular/–/–	Pre-column derivatization: ECF–NaI+acetyl chloride	(**1**)	[9]
HPLC–UV	RP-C_{18}/335 nm/gradient	Pre-column derivatization: OPA–2-methylpropanethiol	(**1**)	[10]
HPLC–DAD	RP-C_{18}/254 nm/isocratic	Post-column photochemical derivatization	(**6**)	[11]
HPLC–ECD	RP-C_{18}/1.7 V/isocratic			
HPLC–UV	RP-C_{18}/337 nm/isocratic	Pre-column derivatization: OPA–*tert*.-BuSH	(**1**)	[12]
HPLC–UV	RP-C_{18}/254 nm/isocratic	–	(**6**)	
HPLC–DAD	RP-C_{18}/254 nm/gradient	Pre-column derivatization: PITC	(**1**)	[13]

(**1**): alliin; (**3**): GLUAlCs; (**4**): GLUPeCs; (**6**): allicin (see Table 1); OPA: *o*-phthaldialdehyde; PITC: phenylisothiocyanate; DAD: diode array detection; FL: fluorimetric detection; RP: reversed-phase; ED: electrochemical detection; ECF: ethyl chloroformate; IP: ion-pair; SMGE: selective multisolvent gradient elution; FID: flame ionisation detection; –: no data.

bulb formation. Four levels of SO_4 were experimented: 0, 100, 200 and 400 kg/ha (1 ha= 10 000 m^2). The treatments were designed in a bloc-randomized experiment with four replicates of 100 plants each.

The garlic bulbs were harvested when considered as mature (above 30° Brix of the juice). They were naturally air-dried and cured when completely dry (3–4 weeks later). Bulbs were then weighted and shipped to Dijon, France, for the powder processing.

2.1.2.2. Powdering

The bulbs were mechanically peeled in a four-step process which consisted in heating (3–5 h at 50 °C), cracking, blowing and fine cutting with high pressure air blow. Slicing produced 0.5 mm thick pieces of garlic that were then dehydrated in the following hours. The dehydrating process started at 70 °C the first 2 h, followed by 65 °C overnight and 60 °C with 10% air renewal for the last 2 h. Samples were dehydrated separately to control the evolution of the

dry matter content. When stabilized, the dry matter content was registered and the dry slices were cooled before to be powered in a mill (<25 μm particles).

2.1.2.3. Garlic dry powder extraction under alliinase inhibiting condition

First, 1 g of garlic dry powder was extracted at room temperature using 10 ml methanol–water (80:20, v/v)+0.05% formic acid (pH <3). An aliquot was diluted five times and filtrated (0.2 μm). Then, 10 μl were injected.

2.1.2.4. Garlic dry powder extraction under alliinase activating condition

First, 1 g of garlic dry powder was extracted at room temperature using 10 ml water (pH 6–8). An aliquot is diluted five times and filtrated (0.2 μm). Then, 10 μl were injected.

2.2. Instrumentation and methods

2.2.1. HPLC–UV analysis

Focussing method and routine analysis of garlic extracts were performed with a Waters 616 pump and a DAD 996 diode-array detector (Waters, Milford, MA, USA). Compounds were separated on a 150×3 mm I.D., 3 μm particle Hypurity Elite C_{18} column Thermo Quest, at 38 °C (Thermo Hypersil, Keystone, Bellefonte, PA, USA) and a UV detector operated at 208 nm. The column flow-rate was 0.4 ml/min. The mobile phase consisted of: (A) 20 m*M* sodium dihydrogenphosphate+10 m*M* heptanesulfonic acid, pH 2.1 (adjusted with orthophosphoric acid 85%); and (B) acetonitrile–20 m*M* sodium dihydrogenphosphate+10 m*M* heptanesulfonic acid, pH 2.1 (50:50, v/v). The gradient program used is listed in Table 3. Data acquisition is performed using Millennium software from Waters.

Table 3
Gradient elution program for HPLC analysis

Eluant	Time (min)						
	0	5	25	26	28	30	40
A	100	70	46	0	0	100	100
B	0	30	54	100	100	0	0

A: 20 m*M* sodium dihydrogenphosphate+10 m*M* heptanesulfonic acid, pH 2.1 (adjusted with orthophosphoric acid 85%); B: acetonitrile–20 m*M* sodium dihydrogenphosphate+10 m*M* heptanesulfonic acid, pH 2.1 (50:50, v/v).

2.2.2. Multiple mass spectrometric (S^n) analysis

Synthesis of dipeptides (**3**), (**4**) and (**5**) was validated using a Thermo Finnigan LCQ quadripole ion trap mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with an electrospray ionization (ESI) source. $[M+H]^+$ ions were formed using ESI in positive mode.The sample dissolved in ammonium formate buffer at pH 3 was introduced to the ESI source by infusion at a flow-rate of 10 μl/min using a Harvard syringe pump drive (Harvard Apparatus, Holliston, MA, USA). A potential of +5.0 kV, a sheath gas flow-rate at 30% and auxiliary gas flow-rate at 50% were employed. The heat and voltage of capillary were, respectively, maintained at 250 °C and 23 V. The $[M+H]^+$ ion intensity was optimised by adjustment of the tube lens offset.

3. Results and discussion

3.1. Profiles

The choice of heptanesulfonate, associated with a very acid condition (pH 2.1) and a suitable fast gradient leads to good profiles with a sufficient resolution for each interesting compound. This method shows actually drastic differences between sampling conditions: when alliinase is inhibited, alliin (**1**) is the major peak (Fig. 2), whereas it disappears with alliinase activity in favor of allicin (**6**) (Fig. 3).

Specific ions were obtained from all the synthetic compounds up to MS^4 (Table 4) which allow to differentiate easily isomers like (**3**) and (**4**). In fact, onion characteristic dipeptide (**4**) was—surprisingly—very abundant in our garlic samples.

3.2. Linearity, detection limit of the HPLC method on synthetic reference compounds

We tested the linearity for compounds (**1**)–(**3**) in the range of 0.2–30 nmol and the coefficients of

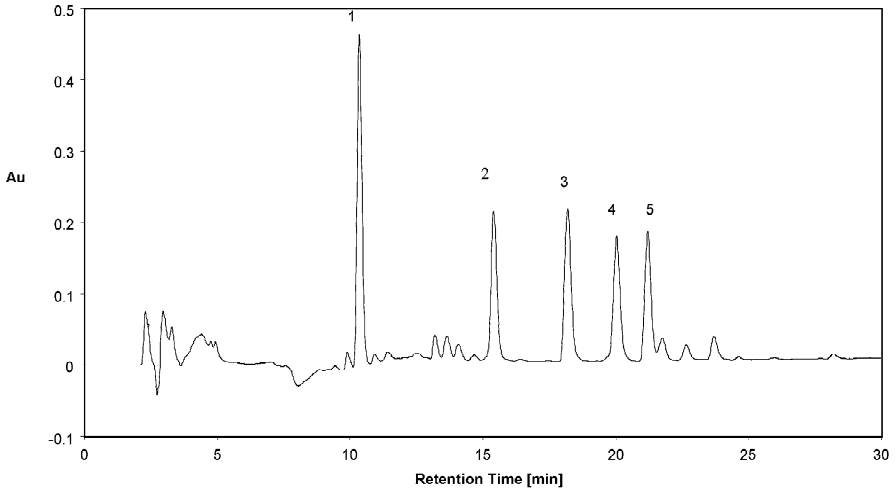


Fig. 2. Chromatogram of garlic powder under alliinase inhibiting condition. See Table 1 for the identity of compounds.

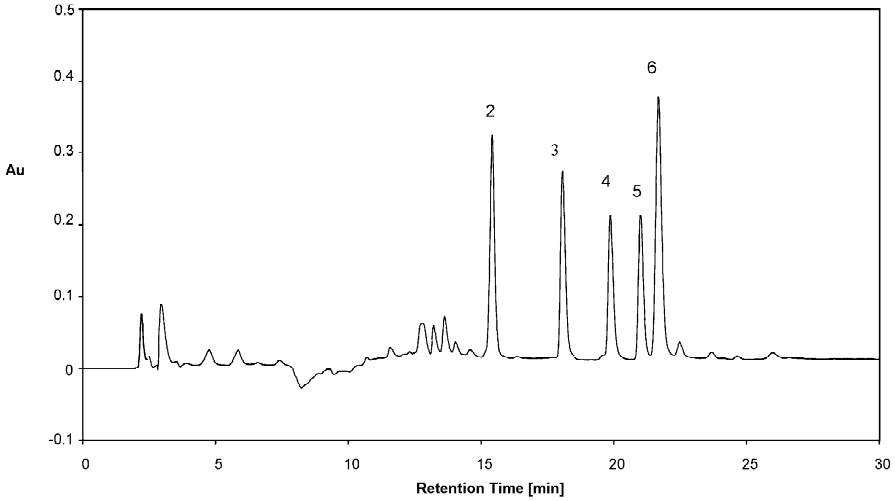


Fig. 3. Chromatogram of garlic powder under alliinase activating condition. See Table 1 for the identity of compounds.

Table 4
HPLC–MS^n of the dipeptides

Compound	*m*/*z* fragments			
	MS^1 (M+H)	MS^2	MS^3	MS^4
3	291	162, 145, 291	145	73
4	291	162, 170, 291, 145	145, 116, 162	55, 99, 73
5	295	166, 278, 120	120, 166, 149	–

See Table 1 for the identity of compounds.

correlation (R^2) are 0.9976, 0.9957 and 0.9944, respectively.

The detection limits for (**2**), (**3**) and (**6**) with this method were about 0.1 and 0.5 nmol for (**1**) which is sufficient for the majority of garlic samples. However, this method is much less sensitive than one using a derivatization procedure like the Pico Tag method [13].

3.3. Effect of sulfur fertilisation on alliin concentration in garlic

Alliin in garlic Printanor powders with four sulfur fertilisation levels (0, 100, 200 and 400 kg/ha) was analysed. Fig. 4 shows results in mg/g dry powder. Concentration of alliin increases significantly with sulfur fertilisation level but as the level 400 seems to have no more effect on alliin concentration than the level 200, we can suppose there is a maximum sulfur level, which can be incorporated in garlic.

3.4. Discussion

This new HPLC method without derivatization allows simultaneous quantification of alliin, SAC, allicin and dipeptides. It requires no particular sample preparation and the run time is rather short (30 min). The sensitivity is weaker compared to a pre-column derivatization method, but sufficient enough for the majority of all thinable application in the field. Furthermore, the response for each compound is strictly linear.

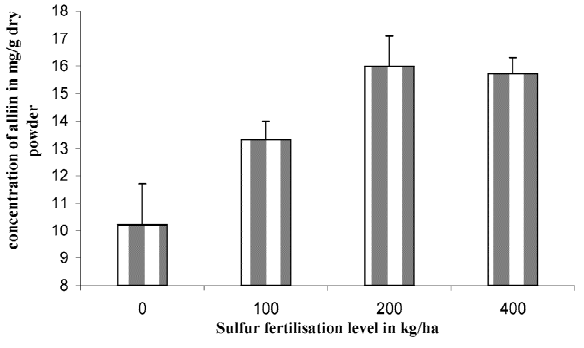


Fig. 4. Diagram of alliin concentration in garlic powder with four sulfur fertilisation levels.

4. Conclusions

This analytical method opens a fast and convenient approach to the quality and therapeutic value of garlic and garlic products by screening all the compounds of interest. Furthermore, this method can be useful to compare varieties of garlic and its hybrids with other *Allium* spp.

We envisage to adapt this method to analyse various *Allium* spp. containing three other *S*-alk(en)yl-L-cysteine sulfoxides in addition to alliin, related dipeptides, thiosulfinates, zwiebelanes and their degradation compounds. Particularly in *Allium cepa*, it will be interesting to compare all the available shallot varieties.

Acknowledgements

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References

[1] H. Harunobu, B.L. Petesh, H. Matsumara, S. Kasuga, J. Nutr. Suppl. 131 (2001) 955S.
[2] E. Block, Sci. Am. 252 (1985) 114.
[3] D.L. Lawson, Z.J. Wang, G.B. Hugues, J. Nat. Prod. 54 (1991) 436.
[4] E. Mochizuki, A. Nakayama, Y. Kitado, K. Saito, H. Nakazawa, S. Suzuki, M. Fujita, J. Chromatogr. 455 (1989) 271.
[5] K. Saito, M. Horie, Y. Hoshino, N. Nose, E. Mochizuki, H. Nakazawa, M. Fujita, J. Assoc. Anal. Chem. 72 (1989) 917.
[6] S.J. Ziegler, O. Sticher, Planta Med. 55 (1989) 372.
[7] M. Mütsch-Eckner, O. Sticher, J. Chromatogr. 625 (1992) 183.
[8] S. Ferary, E. Thibout, J. Auger, Rapid Commun. Mass Spectrom. 10 (1996) 1327.
[9] R. Kubec, M. Svobodovà, J. Velìsek, J. Chromatogr. A 862 (1999) 85.
[10] I. Krest, J. Glodek, M. Keusgen, J. Agric. Food Chem. 48 (2000) 3753.
[11] P. Bocchini, C. Andalò, R. Pozzi, G.C. Galletti, A. Antonelli, Anal. Chim. Acta 441 (2001) 37.
[12] B. Iberl, G. Winkler, B. Müller, K. Knobloch, Planta Med. 56 (1990) 320.
[13] J. Auger, F. Mellouki, A. Vannereau, J. Boscher, L. Cosson, N. Mandon, Chromatographia 36 (1993) 347.
[14] A. Fialaire, R. Postaire, R. prognon, D. Peradeau, J. Liq. Chromatogr 16 (1993) 3003.