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SCIENTIFIC PAPER

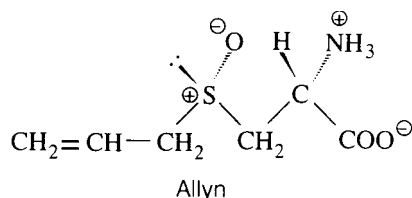
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## HPLC ANALYSIS OF THIOSULPHINATES IN AQUEOUS EXTRACTS FROM GARLIC (*Allium sativum* L.)

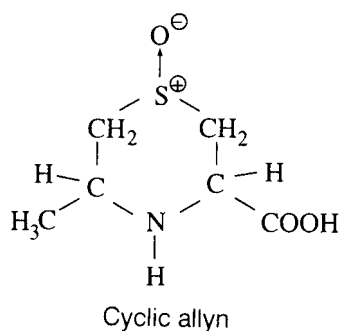
The qualitative and quantitative content of thiosulphinates in aqueous extracts of garlic powder, as well as from fresh garlic bulb (*Allium sativum* L.) was analysed. The stability of thiosulphinates in freshly prepared aqueous extract from garlic, aqueous extracts prepared from garlic powder stored 4 days at room temperature (293 K) as well as extract prepared from powder stored for 18 months at 278 K was also analyzed by use HPLC. Allicin (allyl thiosulphinate), allyl-methyl-/methyl-allyl-thiosulfinat and methyl-thiosulfinat are the most representative components in freshly prepared aqueous extracts from garlic bulbs and from garlic powder. The stability of these sulphinates is very low at room temperature. The highest stability was detected in the case of extract prepared from powder sample stored for 18 months at 273K. The content of allicin and other thiosulphinates in such extract is almost equal to the content of thiosulphinates in fresh garlic bulb.

Garlic contains a large number of bioactive organic compounds such as for example amino acids, ferments, hormones, vitamins, sterols, steroids, lipids, carbohydrates, antibiotics and many sulphur organic compounds [1,2-5]. Sulphur compounds are the most important compounds from the view point of pharmacology (alkyl-cistein derivatives, alkyl sulphides, alkyl disulphides, alkyl polysulphides, thiosulphinates, ajoens and vinyl ditiins) [6].

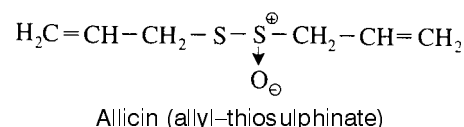
Stoll and Seebeck [7] isolated a mixture of different amino acids containing sulphur organic compounds and alkyl derivatives of sulphur organic compounds in which the content of allyl amino acids, diastereoisomers, (+)-S-allyl-L-cistein-sulphoxide was the highest:



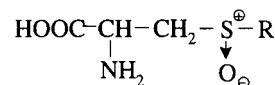
Cyclic allyl from garlic was isolated by Virtanen et al. [8]:



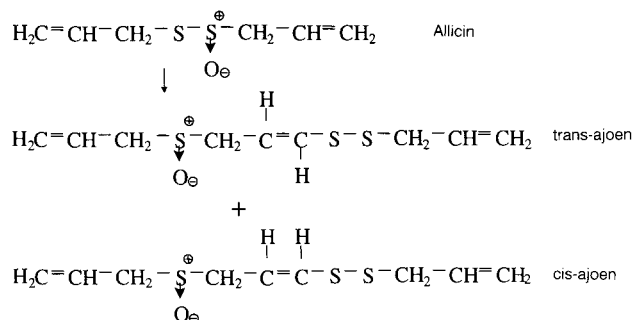
A series of S-alkyl-cistein-sulphoxides was identified in fresh garlic bulb by Hörhammer [3]: S-methyl-cistein-sulfoxide, S-ethyl-cistein-sulfoxide, S-methyl-cistein, S-propyl-cistein-sulfoxide and S-allyl-cistein (deoxyalliin). Allyl is a pharmacologically inactive compound and only under the influence of the enzyme *allinase* present in garlic bulb, the pharmacologically active compounds, allicine is formed. It is known nowadays that allicin is the most important compound for the characteristic flavor and pharmacological action of garlic, as well as for extracts obtained from garlic.



The hydrolytic reactions of many other compounds which are similar to allyl and alkyl-cistein-sulphoxide have been observed under the action of enzyme *allinase* [6]:



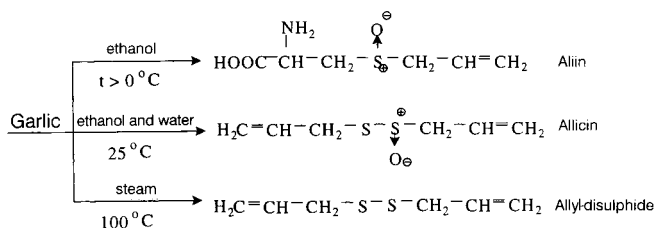
Ajoen is one of the compounds with high antiaterogenic activities, determined in fresh garlic bulb [9]. It can be synthesized from the allicin according to the scheme determined by Block [10]:



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The content of these pharmacologically active substances in garlic bulb, its tinctures or extracts depends on the origin, storage procedure of the different preparations of garlic bulbs, the procedure and manner of garlic aqueous extract preparation etc.

Alliin is a very unstable substance and can easily be transformed to ajoen *in vitro* under precisely determined conditions [11]. The synthesis of alliin and allyl-disulphides from allin could be presented by the following equation [11]:



$\gamma$ -Glutamyl-peptides were also found in the garlic bulb. Almost 15 compounds of such type were isolated [6]. These compounds from the garlic bulb are not identical to  $\gamma$ -glutamyl-peptides originating from the green part of the plants. It is usually considered that they represent reserve substances and are energy source for biochemical processes in a plant, as well as a factor of growth, so they appear in a plant immediately with the first green leaves of the plant [6].

Kominato detected organic sulphur compounds in garlic bulb which belong to the group of thioglycosides and scordinine [6]. They are different regarding some substituents in the thiazole ring of the nucleotide which are a constitutive part of scordinine. The main compound from this group is scordinine A, representing a complex of fructuronic acid, allyl-mercaptan and one peptide with glutamic acid as one of many other amino acids.

Many different methods have frequently been used for the quantitative and qualitative determination of sulphur compounds. They are based on spectrophotometry [12-16]; chromatography: thin layer chromatography (TLC) [12,16-18], gas chromatography (GC) [19-21], liquid chromatography under high pressure (HPLC) [5,17,22]; spectroscopic methods: mass spectrometry (MS) [23,24], nuclear magnetic resonances (NMR) [17,23] and a combination of GC-MS followed by computer identification (GC-MS-C) [19,21,24].

In the present paper a HPLC technique was used for the determination of the composition and thiosulphinate stability in aqueous extracts obtained by using fresh garlic bulb and previously prepared garlic powder. This method is very useful for the analysis of alliin (allyl-thiosulphinate), as well as for other alkyl- and alkenyl thiosulphinates, well known as unstable and degradable substances. Depending on the operating conditions (room temperature, chemical reactions with other constituents) they easily make different products of degradation. Moreover, in many cases, they are not of

interest from the pharmacological point of view because they are not active.

## EXPERIMENTAL PART

**Aqueous extract of fresh garlic bulb (A):** Granddad fresh garlic bulb (1g) was mixed with distilled water (10 cm<sup>3</sup>) and left for 10 min at room temperature (293 K) followed by moderate mixing from time to time. An extract was obtained by filtration under vacuum (glass filter G<sub>1</sub>), and then dissolved in a standard flask (10 cm<sup>3</sup>) with distilled water.

**Aqueous extract of garlic powder (B):** A measured amount of garlic powder (0.4 g) was mixed with distilled water (10 cm<sup>3</sup>) and left for 10 min at room temperature (293 K) followed by moderate mixing. An extract was obtained by filtration under vacuum (glass filter G<sub>1</sub>), and then dissolved in a standard flask (10 cm<sup>3</sup>) with distilled water.

## Liquid chromatography under high pressure (HPLC)

*Apparatus:* HPLC Spectra Physics SP 8100 XR

*Detector:* Spectra Physics SP 8440 UV/VIS

*Integrator:* Spectra Physics SP 4200; Degasser: Erma Erc-3520.

*Column:* Supelco LC-18, 250 x 4,6 mm, (10  $\mu$ m)

*Flow rate:* 1.0 cm<sup>3</sup>/min

*Mobil phase:* Methanol/water/ 50/50 v/v

*Detection:* UV Detector, 254 nm

*Operating parameters:* Pressure 93 bar, Temperature 291 K.

*Recording time:* 15 min

*Volume of injected solution (sample):* 50  $\mu$ l

Before the injection procedure was applied, all the sugars were precipitated from the solution (sample) using 50% methanol (ratio of sample/methanol, 1/10, v/v) and filtered on a membrane filter (d=0.45 mm).

## RESULTS AND DISCUSSION

The HPLC data obtained by using some standard thiosulphinates is shown in Figure 1 [17]. The results of HPLC analysis of the qualitative and quantitative content of thiosulphinates in a freshly prepared aqueous extract (fresh garlic bulb, and fresh garlic powder) are shown in Figure 2 and given in Table 1. The identification of thiosulphinates was performed by comparing the retention times with other data given recently in the literature (Figure 1) [17].

Both analyzed extracts, according to the results of HPLC analysis (Table 1 and Figures 1, 2a and 2b), indicated the same qualitative and very similar quantitative composition. The applied procedure of analysis enabled that six main components could be separated in the samples. Peaks in the chromatograms 2a and 2b with the retention times 3.57 and 3.56 min, respectively, correspond to methyl-thiosulphinate, which is the most present compound in the extracts (33.05% and 35.93%, for extracts A and B, respectively). Alliin

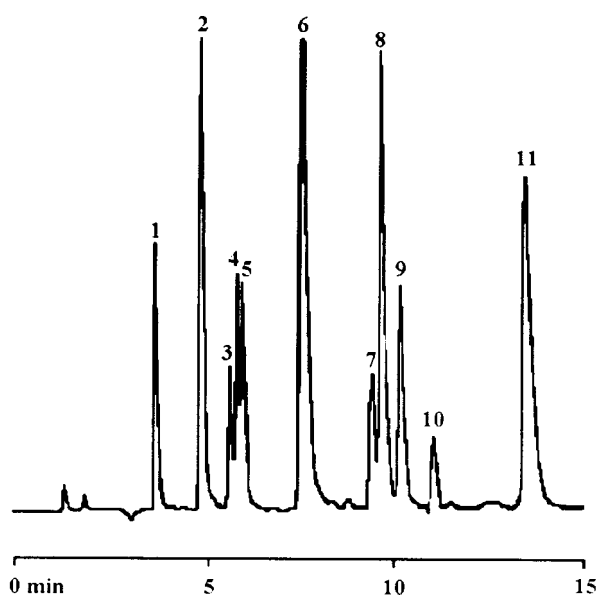


Figure 1. HPLC analysis of some standard thiosulphinates. Column: Supelco LC-18 250 x 4,6 mm; methanol/water 50/50, v/v; 1.0 cm<sup>3</sup>/min; UV detector, 254 nm: 1 methyl-, 2 allyl-methyl-/methyl-allyl-, 3 1-propenyl-allyl-, 4,5 propyl-methyl-/methyl-propyl- 6 allyl- (allicin), 7 allyl-1-propenyl-, 8 methyl-1-propenyl-/1-propenyl-methyl-, 9 propyl-allyl-, 10 1-propenyl-, 11 propyl-thiosulphinate.

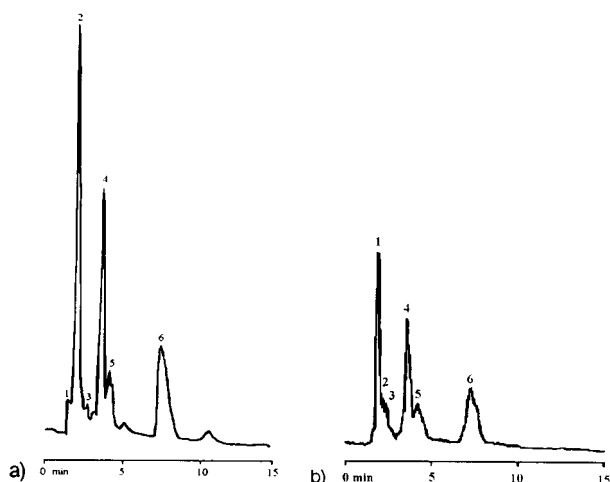


Figure 2. HPLC analysis of thiosulphinates in an aqueous extract of fresh garlic bulb (A), and in an aqueous extract of freshly prepared garlic powder (B). Column Supelco LC-18 250 x 4,6 mm; methanol/water 50/50 v/v; 1.0 cm<sup>3</sup>/min; UV detector: 254 nm

(allyl-thiosulphinate) was detected as a peak which appeared with the retention time 7.85 (extract A) and 7.8 min (extract B, Figures 2a and 2b). Its content (19.08%) is slightly lower in extract B compared to the content determined in extract A (20.37%). Peaks with the retention times 4.15 and 4.13 min were identified as allyl-methyl-/methyl-allyl-thiosulphinate, with much lower content compared to the allicin and methyl-thiosulphinate in both extracts. Moreover, data

Table 1. Literature data (HPLC analysis) of thiosulphinates [17] and HPLC data obtained from aqueous extracts A and B (experimental part)

| Sample                      | Peak | Ret.time (min) | Content* (%) | Thiosulphinates                      |
|-----------------------------|------|----------------|--------------|--------------------------------------|
| STANDARDS - THIOSULPHINATES | 1    | 3.55           | -            | Methyl-                              |
|                             | 2    | 4.75           | -            | Allyl-methyl-/methyl-allyl-          |
|                             | 3    | 5.65           | -            | 1-Propenyl-allyl-                    |
|                             | 4    | 5.70           | -            | Propyl-methyl-/methyl-propyl-        |
|                             | 5    | 5.80           | -            |                                      |
|                             | 6    | 7.70           | -            | Allyl- (allicin)                     |
|                             | 7    | 9.35           | -            | 1-Propenyl-                          |
|                             | 8    | 9.55           | -            | Methyl-1-propenyl/1-propenyl-methyl- |
|                             | 9    | 10.15          | -            | Propyl-allyl-                        |
|                             | 10   | 11.00          | -            | 1-propenyl-                          |
|                             | 11   | 13.85          | -            | Propyl-                              |
| EXTRACT "A"                 | 1    | 1.66           | 1.55         | Unidentified                         |
|                             | 2    | 1.88           | 32.32        | Unidentified                         |
|                             | 3    | 2.26           | 4.55         | Unidentified                         |
|                             | 4    | 3.57           | 33.05        | Methyl-                              |
|                             | 5    | 4.15           | 8.16         | Allyl-methyl-/methyl-allyl-          |
|                             | 6    | 7.85           | 20.37        | Allyl- (allicin)                     |
| EXTRACT "B"                 | 1    | 1.89           | 24.05        | Unidentified                         |
|                             | 2    | 2.10           | 3.97         | Unidentified                         |
|                             | 3    | 2.33           | 8.58         | Unidentified                         |
|                             | 4    | 3.56           | 35.95        | Methyl-                              |
|                             | 5    | 4.13           | 8.38         | Allyl-methyl-/methyl-allyl-          |
|                             | 6    | 7.80           | 19.08        | Allyl- (allicin)                     |

\*% of thiosulphinates in the total amount (area) of identified components.

which indicates that allicin, as the main pharmacological substance, is present almost in the same quantity in both extracts, show that garlic powder could be of more interest and importance for commercial production. Such a conclusion was derived knowing that allicin has greater stability in garlic powder than in the aqueous extract of garlic.

The stability of thiosulphinates in garlic powder and in an aqueous extract was analyzed by measuring changes of the thiosulphinate content in garlic powder, just after its preparation (Figure 1b) and in the aqueous extract of garlic powder stored at room temperature (293 K; Figure 3a) and in extract obtained from garlic powder stored for 18 months at 278 K (Figure 3b).

Some differences (qualitative and quantitative content) in the identified thiosulphinates and in some other (unidentified) compounds in garlic powder stored for 18 months at 278 K compared to fresh garlic powder were determined. Moreover, as the HPLC chromatogram of the aqueous sample (stored for 18 months at 278 K) indicates, 3 unidentified components (Figure 3b) and the

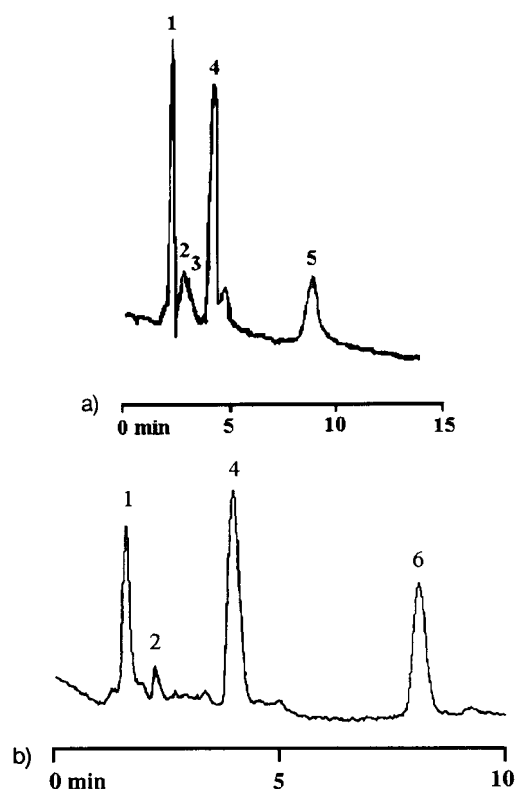


Figure 3. HPLC analysis of different thiosulphinates in an aqueous extract obtained from garlic powder stored for 4 days at 293 K (a) and in the same extract obtained from a garlic powder stored for 18 months at 278 K (b).

isomer allyl-methyl/methyl-allyl-thiosulphinate (peak 5, Figure 3b) were not detected. The area of peak 6, corresponding to allicin, increased as a result of the smaller number of identified compounds, indicating the existence of pharmacological activity of garlic powder. HPLC analysis of the freshly prepared aqueous extract and those stored for 4 days at room temperature (293 K) indicated instability of the aqueous extract. Namely, none of the thiosulphinates detected in extract A were not found in extract B stored for 4 days. Such a conclusion was supported by some newly registered compounds with retention times greater by 12% than the retention times of thiosulphinates and the presence of some other unidentified components.

## CONCLUSION

Allicin (allyl-thiosulphinate), allyl-methyl-/methyl-allyl-thiosulphinate and methyl-thiosulphinate are the most represented components in freshly prepared aqueous extracts from garlic bulb and from garlic powder. The stability of these thiosulphinates in the above extracts at room temperature (293 K) is very low. According to HPLC analysis, it was concluded that garlic powder has more valuable pharmacological activity compared to garlic bulb, as a result of the higher stabil-

Table 2. The results of HPLC analysis of thiosulphinates in an aqueous extract of garlic powder (extract and powder stored for different times)

| Sample  | Peak | R <sub>t</sub> (min) | Content* (%) | Identified thiosulphinates  |
|---|------|----------------------|--------------|-----------------------------|
| EXTRACT "B"   | 1.   | 1.89                 | 24.05        | Unidentified                |
|   | 2.   | 2.10                 | 3.97         | Unidentified                |
|   | 3.   | 2.33                 | 8.58         | Unidentified                |
|   | 4.   | 3.56                 | 35.95        | Methyl-                     |
|   | 5.   | 4.13                 | 8.38         | Allyl-methyl-/methyl-allyl- |
|   | 6.   | 7.80                 | 19.08        | Allyl- (allicin)            |
| EXTRACT "B" (stored for 4 days at 293 K)                                | 1.   | 2.05                 | 24.61        | Unidentified                |
|   | 2.   | 2.47                 | 5.12         | Unidentified                |
|   | 3.   | 2.53                 | 6.90         | Unidentified                |
|   | 4.   | 4.01                 | 44.06        | Unidentified                |
|   | 5.   | 9.29                 | 19.31        | Unidentified                |
| EXTRACT "B" (prepared from garlic powder stored at 278 K for 18 months) | 1.   | 1.80                 | 19.64        | Methyl-                     |
|   | 2.   | 2.20                 | 2.22         | Allyl-methyl/methyl-allyl-  |
|   | 3.   | ----                 | ----         | -----                       |
|   | 4.   | 3.58                 | 47.90        | Propyl-methyl-              |
|   | 5.   | ----                 | ----         | -----                       |
|   | 6.   | 7.90                 | 30.24        | Allyl-(allicin)             |

\*% of thiosulphinates in the total amount (area) of identified components.

ity of the thiosulphinates in powder. The highest stability of thiosulphinates was detected in garlic powder stored at 278 K. The content of allicin and other thiosulphinates in an aqueous extract of garlic powder stored for 18 months at 278 K was almost equal to those detected in aqueous extracts prepared from fresh garlic bulb.

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## IZVOD

### HPLC ANALIZA TIOSULFINATA U VODENIM EKSTRAKTIMA IZ SVEŽEG I PRAHA BELOG LUKA (*Allium sativum* L.)

(Naučni rad)

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Primenom tačne hromatografije pod visokim pritiskom (HPLC) analiziran je sastav tiosulfinata u vodenim ekstraktima sveže lukovice i praha belog luka. U vodenim ekstraktima svežeg belog luka i praha belog luka identifikovano je po tri tiosulfinata: alicin (alil-tiosulfinat), alil-metil/metil-alil- i metil-tiosulfinat. Kvantitativni sastav identifikovanih tiosulfinata takođe je u oba vodena ekstrakta približan. Stabilnost registrovanih tiosulfinata u vodenoj sredini je mala. Na ovo ukazuju rezultati HPLC analize gde su retenciona vremena pikova pomešana za oko 12% ka većim vrednostima. Potencijal alicina koji se oslobađa u vodenom ekstraktu iz praha belog luka koji je čuvan osamnaest meseci na +4°C je vrlo zadovoljavajući, tj. skoro odgovara sadržaju alicina u svežem belom luku. Na osnovu ovih rezultata zaključeno je da je farmakološka vrednost u potpunosti očuvana u prahu belog luka i da se on može koristiti za izradu komercijalnih fito preparata.

Key words: HPLC • Garlic • Alicin • Thiosulphinate • Retention time •  
Ključne reči: Tačna hromatografija pod visokim pritiskom • Beli luk • alicin • Tiosulfinati • Retenciona vreme •



Allyn

Cyclic allyn

Allicin

trans-ajoen

cis-ajoen

Aliin

Allicin

Ally-disulphide

**Garlic**

ethanol

ethanol and water

steam