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Efficient determination of cysteine sulphoxides in *Allium* plants applying new biosensor and HPLC-MS² methods

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Summary

Cysteine sulphoxide (CSO) contents of 16 different accessions of garlic (*Allium sativum* L.) and 15 varieties of onion (*Allium cepa* L.) were measured using two different rapid analytical methods: a biosensoric approach and a newly developed HPLC-MS² technique. Both methods allow quantification of naturally occurring cysteine sulphoxides present in *Allium* plants without time-consuming sample pretreatment such as derivatisation of amino acid derivatives prior to HPLC-separation. It has been found that the amount of alliin, which is the predominant CSO occurring in garlic, varies between 0.2 and 2.2 g/100 g dry matter. Contrary to that, isoalliin representing the main CSO in onion has been detected in significantly lower amounts (0.3 to 1.25 g/100 g dry matter).

Introduction

Onion (*Allium cepa* L.) and garlic (*Allium sativum* L.) are economically the most important species of the genus *Allium*. Onion varieties (*Allium cepa*) can be found in many regions of Europe, Asia, North America and Africa. This species is one of the most important horticultural crops with an annual world-wide production of around 44 million tons (GRIFFITHS et al., 2002).

It is well-known that S-alk(en)yl-L-cysteine sulphoxides (CSO), which are mainly present in the bulb, act as flavour precursors generating the typical taste and odour in presence of the enzyme alliinase (*e.g.* dipropyl disulfide, diallyl disulfide, thiophene). Fig. 1 shows the biotransformation of sulphur compounds in *Allium* during processing of the plant raw material.

The alliinase reaction initiates the production of highly reactive sulphenic acids, which condense with each other to form thiosulphinates. These compounds are mainly responsible for the flavour of fresh onion or garlic. The thiosulphinates can disproportionate into thiosulphinoates and sulphides presenting the characteristic flavour of cooked onions. Steam-distilled oils obtained from various *Allium* species such as onion, garlic or leek are widely used for flavouring food and for production of food supplements. Several studies have been performed during recent years in order to evaluate potential *Allium* wild plants and hybrids with special regard to their aroma properties as well as pharmacological value (SCHULZ et al., 1998; SCHULZ et al., 2000; KEUSGEN et al., 2002; STORSBERG et al., 2004; SCHMITT et al., 2005). Besides sulphur compounds, also pyruvic acid and ammonia were formed in equimolar amounts, which can be used for a rapid biosensoric determination of cysteine sulphoxides (KREST and KEUSGEN, 2002; KREST and KEUSGEN, 1999). In the latter case, alliinase has to be immobilized with an ammonia-detecting device.

Further on, cepaenes (α-sulphinyl disulphides) were found in aged extracts of *Allium* species (RABINOWITCH and CURRAH, 2002) acting as potent inhibitors of platelet aggregation and different enzymes (*e.g.* cyclooxygenase, lipoxygenase) (BLOCK et al., 1997). The formation of particular compounds depends on different reaction conditions (*e.g.* temperature, solvent). In an ethanolic extract, the thiosulphinate allicin can react with itself (disproportionation) forming compounds with three sulphur atoms in the molecule (ajoens), which are especially known for their antithrombotic properties. The lachrymatory factor, built from 1-propenyl-sulphenic acid, can dimerize to bisulphines and cyclic zwiebelans.

As shown above, CSO play an important role in the biochemistry of *Allium* plants and significantly contribute to the health of men. Therefore, efficient analytical methods are required to determine the individual content of these compounds in plant material as well as to

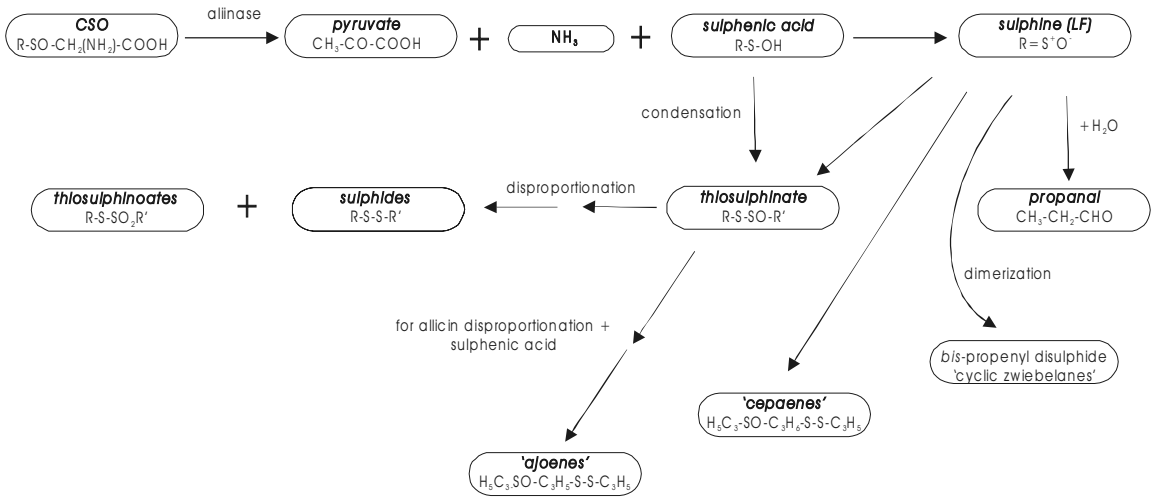

Fig. 1: Scheme presenting the formation of sulphur compounds starting from cysteine sulphoxides coming in contact with alliinase during processing of plant material. R = methyl, propyl, 2-propenyl and 1-propenyl; LF = lachrymatory factor (RABINOWITCH and CURRAH, 2002).

characterise herbal products obtained from *Allium* plant material. Several HPLC techniques for the determination of CSO, especially of alliin, have been described in the past (ZIEGLER and STICHER, 1989; IBERL et al., 1990; KEUSGEN, 1997) but all these analysis methods generally are time consuming because of extensive sample clean-up steps necessary to separate CSO from interfering matrix components. Therefore, two different sophisticated methods were applied to study the CSO content and distribution in various cultivars of *Allium cepa* as well as gene bank accessions of *Allium sativum*: a new biosensoric method based on immobilized alliinase (KREST and KEUSGEN, 2002) and a newly developed HPLC-MS^2 method. For the biosensoric analysis immobilized alliinase was used to initiate the degradation of CSO. Pyruvate and ammonia are by-products formed during this process. The amount of enzymatically formed ammonia is proportional to the level of cysteine sulphoxides and can be, therefore, used for an indirect quantification of these sulphur constituents in *Allium* plant material, if native alliinase was inactivated during extraction (methanol, heat).
The new HPLC-MS^2 method with selective ion monitoring (SIM) was developed to more rapidly screen extracts of various *Allium* species. The method was used for the analysis of the four main important CSO in *Allium* species: methiin, alliin, isoalliin and propiin. The special advantage of MS^2-detection should be used to detect the individual analyte molecule ions and their related fragments with high selectivity also in presence of other co-eluting non-sulphur compounds. Finally, we also will discuss the variation of CSO found in different cultivars/accessions of *A. cepa* and *A. sativum.*

Materials and methods

Reagents

Alliin was obtained from Carl Roth GmbH & Co KG (Karlsruhe, Germany). Methiin and propiin were prepared following the procedures described earlier (KEUSGEN, 1997; THEODOROPOULOS, 1959; STOLL and SEEBECK, 1951; FREEMAN et al., 1994; KOCH and KEUSGEN, 1998; KREST et al., 2000). Isoalliin, synthesized according to a new efficient procedure (NAMYSLO), was generously provided by the Institute of Organic Chemistry, Clausthal University of Technology, Germany. Alliinase was isolated, stabilized and purified according to methods developed by Krest and Keusgen (KREST and KEUSGEN, 2002; KREST and KEUSGEN, 1999).

Plant material

The investigated garlic plant material was obtained from the *Allium* collection of the Institute of Plant Genetics and Crop Plant Research in Gatersleben, Germany. The different onion varieties were cultivated by the „Agrargenossenschaft Calbe“, Calbe, Germany. The plants were grown under comparable conditions to reduce influences caused by environmental factors. The freshly harvested bulbs were directly cooled down (-80 °C) and subsequently freeze-dried in order to prevent losses of CSO caused by enzymatic reactions.

Sample preparation

HPLC-MS^2
About 200 mg of an exactly weighed freeze-dried sample were heated under reflux for 10 minutes in 20 mL of methanol. After that, the sample was crushed in a mortar and returned to the methanolic solution with the addition of 20 mL deionised water and heated again for 10 minutes. Then the extract was filtered and the residue was washed with 3x3 mL of methanol/water (50:50). The filtrates were evaporated to dryness under reduced pressure. For HPLC analysis the residue was redissolved in 2.5 mL of deionised water (THEODOROPOULOS, 1959). From each variety two samples were taken in order to get an overview about the variability of the CSO content within the analysed plant material.

Biosensor
For biosensor analyses the freeze-dried plant material (without green parts) was pulverised in a mortar. 200 mg of this material was transferred to a test tube containing 3 mL of methanol. This enzyme-inactivated solution was gently heated for five minutes in a heating block at 100 °C block temperature. Then the test tube was cooled down for three minutes and the sample was heated again for another 15 minutes after addition of 3 mL of deionised water. Finally, the mixture was transferred into a graduated flask (25.0 mL) and filled up with biosensor phosphate buffer (pH = 7). Prior to biosensor analysis, the sample was filtered and diluted with the buffer solution (1:20, v/v).

HPLC-MS^2 Analysis
The analyses were performed on an HP 1100 series HPLC system (Agilent) hyphenated with a Bruker Esquire 3000 mass spectrometer with electrospray ion source. A ZORBAX SB-C18 column (3 x 150 mm), particle size 3.5 µm, was used for the CSO separation.

Sample aliquots of 1-5 µL were injected into the analytical column and the CSO separation was performed using two solvents (A: deionised water, B: methanol). The following elution gradient was applied (flow rate = 0.5 mL/min).

Tab. 1: Gradient elution profile for HPLC separation of cysteine sulphoxides.

Step	Time (Min.)	% Eluent B (methanol)
1	0.00	1.0
2	2.00	1.0
3	7.00	6.0
4	7.10	1.0
5	9.00	1.0

The optimisation steps for detection of mass fragments were carried out in the flow injection mode using a scan range of 50-400 m/z for all analysed CSO. The quantification of CSO was performed on the basis of the extracted ion $[M+H]^+$ without further fragmentation within a given time window (Fig. 2).
The individual MS conditions were specially adapted to methiin, alliin, isoalliin and propiin. Accordingly the calibration equations were established for these CSO.

Flow-through analysis
The biosensoric method is based on the alliinase reaction, the enzyme was immobilized and placed inside a flow-through cartridge. Determination of free ammonia and enzymatically formed ammonia was determined by a flow-through-apparatus (MANA and SPOHN, 2000). Ammonia in equimolar amounts was obtained from cysteine sulphoxides by immobilized alliinase. The enzyme was isolated, stabilized and purified according to the methods already described earlier (KREST and KEUSGEN, 2002). The powdered garlic or onion sample was homogenized and a crude alliinase preparation was obtained by precipitation with ammonium sulphate or by ultra-

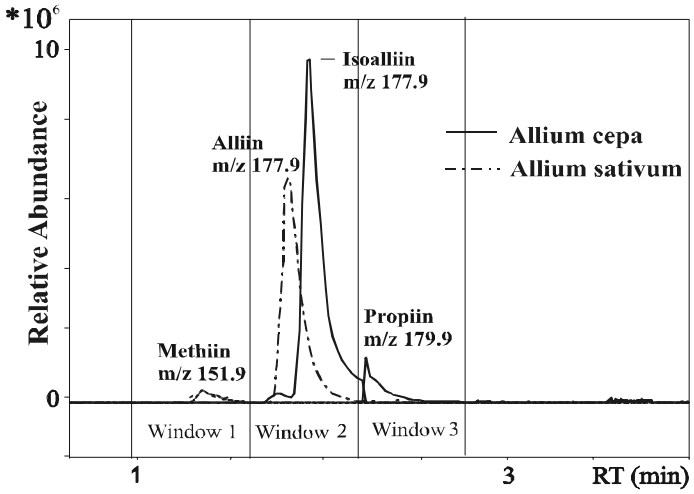

Fig. 2: The extracted ion chromatograms of CSO occurring in *Allium cepa* (black line) and *Allium sativum* (dotted line). Isolation of cysteine sulphoxide ions was performed using different time windows individually adapted to the masses of individual CSO

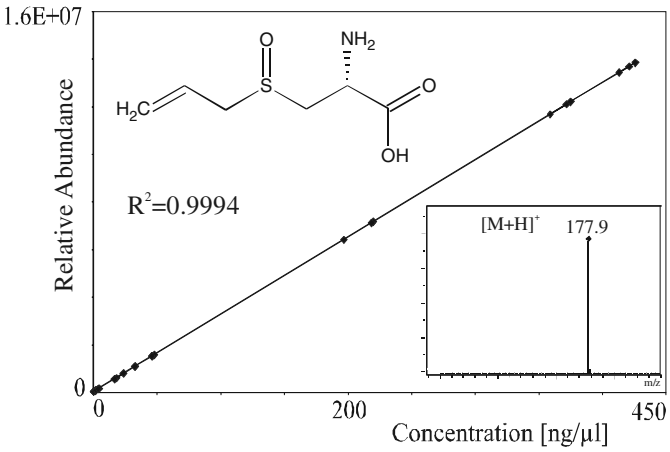

Fig. 3: HPLC-MS calibration curve obtained for alliin (based on the individual signal intensity of the protonated molecule ion at m/z 177.9) and MS spectrum of a pure alliin standard

filtration. Ultra-filtrated alliinase was used for standard experiments. Alliinase was stabilized with 10 % sucrose, 0.17 M NaCl and 25 mM pyridoxal-5'-phosphate (P-5'-P) dissolved in phosphate buffer (pH 7.0, 60 mM). Further purification was done by gel filtration followed by affinity chromatography. Immobilization of alliinase was performed on a ConA/agarose-carrier (Sigma, Ord.-No. C 7555). Briefly, the carrier was filled into a cartridge (60 µL) and rinsed with buffer. A solution containing alliinase was then pumped through this cartridge. Immobilized alliinase may be also renewed. Elution of the enzyme was performed with buffer containing mannose followed by rinsing with a glucose solution and buffer. The carrier may be newly loaded with alliinase as described above. For analysis of cysteine sulphoxides, the cartridge was operated with phosphate buffer (pH 7.0, 0.02 M containing 0.17 M NaCl). Analysis of cysteine sulphoxides by HPLC and the biosensor was repeated at least three times. Given values for cysteine sulphoxides were corrected by the amount of native ammonia before alliinase reaction.

Results and discussion

HPLC-MS^2 with SIM has been shown to be a suitable method to detect and to quantify naturally occurring CSO (methiin, alliin, isoalliin, propiin) in *A. cepa* and *A. sativum*. All four compounds could be individually detected. The newly developed method has been found to be very sensitive, robust, and reliable over a wide concentration range of the targeted compounds. The low detection limit allows to analyse CSO even in concentrations below 1 µg/mL sample. The linear range could be confirmed over three concentration decades. Sensitivity is sufficient even for the analysis of small *Allium* samples even in the order of a few mg.

The use of MS^2 detection has been proven to be particularly effective in analysing the non-volatile sulphur substances from *Allium* extracts, because other co-eluting *Allium* substances do not interfere with the significant key signals of the molecule ion $[M+H]^+$. The ESI interface and mass spectrometer parameter were optimised to obtain maximum sensitivity as displayed in Tab. 2 and Fig. 3. For calibration, pure CSO standards were used in different concentration ranges related to the authentic content which is usually measured in both *Allium* species. Within these considered ranges the individual calibration curves (Fig. 3) were found to be linear (Tab. 2).

The biosensoric method is also suitable for a simple and rapid determination of the total amount of CSO. Each analysis needs only less than five minutes. As shown in previous investigations with immobilized alliinase, results obtained by ammonium determination after alliinase digestion of CSO are comparable with those obtained by HPLC (KREST and KEUSGEN, 2002). The method is less sensitive than the HPLC-MS method but concentrations below 5 µg alliin/mL can be accurately determined. Samples described here in this work were diluted as least 20 times to reach the linear calibration range (about 2 µg to 100 ng/µL). So, at any rate the sensivity of the presented HPLC-MS method is high enough for quality control purposes.

Analysis of *Allium sativum*

The CSO concentration obtained for *Allium sativum* gene bank accessions are presented in Fig. 4. These data show the results of the biosensor and of the HPLC method as well.

Tab. 2: Characteristics of naturally occurring cysteine sulphoxides based on the individual molecule ions $[M+H]^+$

CSO	$[M+H]^+$ [m/z]	Fragment ion [m/z]	Retention time [min]	Precision R.S.D. (n=3)	Calibration range [ng/µL]	Calibration linearity (R^2)
methiin	151.8	88.2	1.5	0,17	0.1-20	0.9996
alliin	177.9	88.2	1.8	7.89	0.4-400	0.9994
isoalliin	177.9	88.2	2.0	6.83	0.4-400	0.9998
propiin	179.9	88.2	2.4	0.13	0.2-20	0.9996

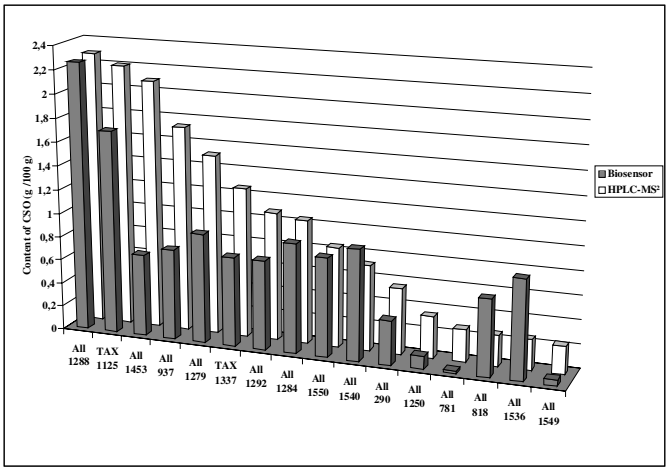

Fig. 4: Total CSO content in various garlic accessions analysed by biosensor and HPLC-MS² methods.

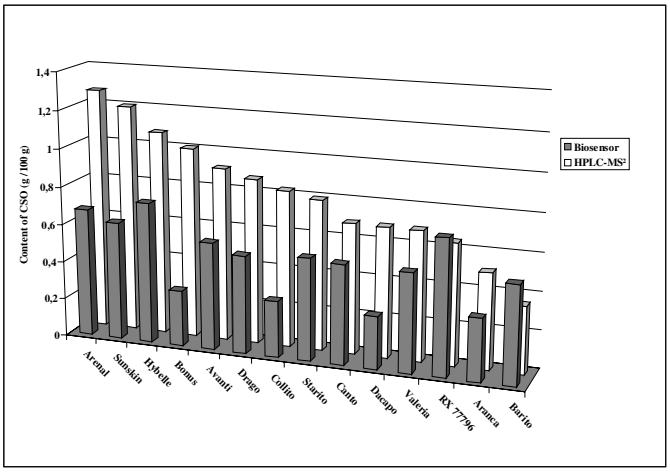

Fig. 5: Total CSO content in different *Allium cepa* varieties analysed by biosensor and HPLC-MS² methods.

We found a high variability regarding the CSO content within the measured *Allium sativum* accessions. Samples with the numbers TAX 1125 and ALL 1288 present highest content of total CSO (approx. 2.2 g/100 g dry matter) contrary to those plants with the numbers ALL 1549 or ALL 781 containing only comparatively low amounts of CSO of approx. 0.23 g/100 g dry matter. The mean difference between the two analysed samples A and B (for HPLC-MS²) was 0.23 g/100 g dry matter. The main CSO present in *A. sativum* is alliin, whereas methiin was only detected in traces (< 0.1 %). Mean standard deviation for the biosensor method was found to be 0.08 g/100 g dry matter.

The biosensor method reflected the same trend of CSO content of samples as described above for the HPLC-MS² method. However, there are some significant differences in detail. We assume that inhomogeneous sample material is the reason for this behaviour. To avoid losses of CSO before sample lyophilisation, cloves were only roughly cut. This causes some problems during lyophilisation. Some parts of the material could not be properly dried because the sample material got amber-like at its surface preventing water evaporation in the tissue under these zones. It can be assumed that this garlic material showed a dramatically reduced alliin content. Because of the limited amount of sample material, only small amounts of each accession could be analysed leading to a big influence of inhomogeneous sample material.

In order to truly compare both methods with each other, the individual ratio between the results obtained by the biosensor and those obtained by HPLC-MS was calculated. Because only very low amounts of CSO were detected in ALL 781, this sample was not included in the calculation. The average ratio was determined to be 0.97, demonstrating that both methods lead to nearly identical results and variations between the two series are highly randomized (as an effect of insufficient homogeneity of sample material).

Analysis of *Allium cepa*

The CSO contents determined by both techniques in 15 varieties of *A. cepa* are shown in Fig. 5. In agreement with the results reported in former studies (RABINOWITCH and CURRAH, 2002) it has been found that the content of total CSO is much lower than in the analysed *A. sativum* accessions (0.2 – 0.8 g/100 g dry matter).

Both series (Biosensor and HPLC) showed a rather good correlation. Especially those samples, which displayed a relatively low CSO concentration obtained by the biosensor (cultivars 'Dacapo', 'Collito', and 'Aranca') were also low by HPLC analysis. Interestingly, values obtained by the biosensor are lower than those obtained by the HPLC-MS² method.

These differences may be explained by the fact that CSO are not completely converted to ammonia by the alliinase immobilized in the biosensor. Currently, further studies are under investigation aiming to get more detailed information regarding the alliinase selectivity towards individual CSO in the near future.

In the case of *A. cepa*, sample preparation in terms of drying was much easier. Coarse cut pieces are rather thin and can be sufficiently freeze-dried. Therefore, onion sample material is much more homogenous than that obtained from garlic bulbs applying the same method. But nevertheless, because of the good correlation of both methods, the biosensor approach as well as HPLC-MS² seem to be suitable for the quality control of *A. cepa* material.

It could be demonstrated that methods described above are rather helpful for a time-saving and effective quality control of garlic and common onion. In case of common onion, freeze drying seems to be an appropriate method for sample preparation. Contrary to that, drying of garlic samples causes serious problems regarding sample homogeneity. In contrast to onion, garlic cloves consist of only one thick storage leaf covered with a rather thick skin, which makes freeze-drying more difficult. Cutting in smaller pieces probably leads to more homogenous sample material, but will definitely result in lower alliin amounts because of degradation of CSO at the cut surface. Further research studies are necessary to overcome these problems specially related to insufficient sample homogeneity.

Both devices used – biosensor and HPLC-MS² – are rather complex tools. However, the biosensor approach is much more inexpensive (a tenth of the price of an HPLC-MS²-equipment). Moreover, only the total amount of CSO can be gained by the biosensor approach. As favourable point of this device, it can be fully operated by aqueous solutions. Operating costs are, therefore, extremely low. On the other hand, the HPLC-MS² device is a rather complex tool, which provides full information about different CSO and can be also used for a broad range of further analytical tasks.

Therefore, this technique provides a very useful support to breeding projects as well as fast evaluation of *Allium* genebank accessions aiming at controlling the content of individual CSO. The HPLC-MS method presented in this study allows to perform an effective selection of single plants and to measure simultaneously the optimal distribution and amount of all naturally occurring CSO in one course of analysis. Furthermore, this method can be applied also for quality control purposes in order to check the level of valuable CSO in certain

Allium species (e.g. garlic, onion, ramson) used as raw materials for the production of various phytopharmaceutical preparations.

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