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Chemical Characterization of *Allium ursinum* L. Depending on Harvesting Time

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Sulfur-containing compounds of ramson (*Allium ursinum* L.) are responsible for its traditional use in terms of culinary and medicinal purposes. Leaves and bulbs were investigated for their contents of cysteine sulfoxides (volatile precursors) as well as volatile compounds released from minced plant material. Plants were analyzed during the whole vegetation period, focused on the months from March to June. Additionally, within the dormancy period bulbs were analyzed again and alliinase activity was determined. The pattern of volatile compounds was analyzed both by SPME/GC-MS and by SDE/GC-MS. Compared to each other, SDE exhibited a wider spectrum of detectable volatile compounds. The quality and quantity of volatiles significantly depended on the time of harvest. The highest amounts of volatile precursors can be gained in March and April, shortly before flowering time (up to 0.4% of total cysteine sulfoxides). The main cysteine sulfoxides were alliin and isoalliin. It has been found that alliinase of *A. ursinum* exhibited properties similar to those of alliinase of garlic (*Allium sativum* L.), but differing in terms of substrate specificity.

KEYWORDS: ***Allium*; alliin; ramson; alliinase; cysteine sulfoxide; diallyl disulfide**

INTRODUCTION

Leaves of ramson (also named wild garlic or bear's garlic, *Allium ursinum* L., Alliaceae, subgenus *Amerallium* Traub), a wild-growing *Allium* species in the forests of Europe and northern Asia, are wildly used in traditional medicine and as a spice. Consequently, attempts are currently undertaken to cultivate *A. ursinum*. The herbaceous plants grow up to a height of 50 cm and feature pseudoumbels with white flowers as well as elongated bulbs not exceeding 6 cm in size. Ramson bears trichotomic capsules with black seed as fruits (*1*).

Similar to garlic (*Allium sativum* L.), the best-known representative of the genus *Allium*, ramson also contains high amounts of cysteine sulfoxides (*2*, *3*) as well as the enzyme alliinase (EC 4.4.1.4) (*4*). Volatile sulfur-containing compounds—being formed by the reaction of alliinase and cysteine sulfoxides in disrupted plant material—create the characteristic flavor of *Allium* species and are suggested to have various therapeutic effects. By this reaction, the thiosulfinate allicin is enzymatically formed from the cysteine sulfoxide alliin. *A. sativum*, for example, is commonly used as an herbal medicine for lowering the blood lipid level. Also, antibiotic, antioxidative, antidiabetic, and cancer-preventing properties of *Allium* have been reported (*5*−*7*). The cysteine sulfoxides themselves as well as their degradation products, especially those derived from *S*-2-propenyl-L-cysteine sulfoxide (alliin), are believed to be responsible for these effects. Consequently, high amounts of cysteine sulfoxides have a direct impact on the quality of ramsom as a medicinal plant and as a spice. Up to now, ramson was only rarely subject to in vitro investigations, although plant extracts showed effects similar to those shown by garlic (*5*).

The basic chemistry of ramson and also investigations concerning alliinase were reviewed by Sendl (*1*). However, no detailed investigation on both bulbs and leaves was published until now. To gain more information about the sulfur compounds of ramson, the study presented here is focused on the changes of the cysteine sulfoxide contents and patterns in different organs of *A. ursinum* during the vegetation period. The sulfur-containing degradation products should be investigated simultaneously. Moreover, the enzyme alliinase should be characterized with respect to its activity properties.

MATERIALS AND METHODS

Reagents. Unless otherwise specified, all chemicals were purchased from the firms Merck (Darmstadt, Germany) or Fluka (Sigma-Aldrich Chemie) (Steinheim, Germany). Acetonitrile of HPLC grade was obtained from SDS (Peypin, France). Reference substances of the used cysteine sulfoxides were synthesized following the procedures described

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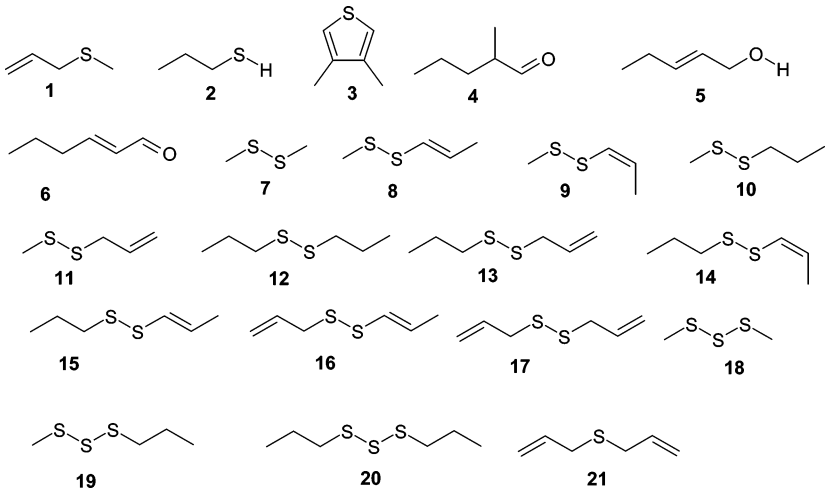

Figure 1. Chemical structures of typical compounds of *Allium* detected by GC-MS: **1**, methyl 2-propenyl sulfide (AlMeS); **2**, propylthiol (PrSH); **3**: dimethylthiophene (Me_2Thio); **4**, 2-methylpentanal (MePent); **5**, hex-3-en-1-ol; **6**, 2-hexenal; **7**, dimethyl disulfide (Me_2S_2); **8**, (*E*)-methyl 1-propenyl disulfide [(*E*)-$PeMS_2$]; **9**, (*Z*)-methyl 1-propenyl disulfide [(*Z*)-$PeMS_2$]; **10**, methyl propyl disulfide ($MePrS_2$); **11**, methyl 2-propenyl disulfide ($MeAlS_2$); **12**, dipropyl disulfide (Pr_2S_2); **13**, 2-propenyl propyl disulfide ($AlPrS_2$); **14**, (*Z*)-1-propenyl propyl disulfide [(*Z*)-$PePrS_2$]; **15**, (*E*)-1-propenyl propyl disulfide [(*E*)-$PePrS_2$]; **16**, (*E*)-1-propenyl 2-propenyl disulfide [(*E*)-$PeAlS_2$]; **17**, di-2-propenyl disulfide (Al_2S_2); **18**, dimethyl trisulfide (Me_2S_3); **19**, methyl propyl trisulfide ($MePrS_3$); **20**, dipropyl trisulfide (Pr_2S_3); **21**, di-2-propenyl sulfide (Al_2S).

by Theodoropoulus (*8*), Stoll and Seebeck (*9*), and Iberl et al. (*10*) as well as Freeman and Huang (*11*). Their analytical data were published by Krest et al. (*2*). Isoalliin was gained from a macerate of *Allium cepa* L.

Authentic reference samples for gas chromatography (GC) and mass spectrometry (MS) were obtained from Oxford Chemicals, Harlepool, U.K. (allyl mercaptan, methyl propyl sulfide, diallyl sulfide, dipropyl sulfide, allyl methyl sulfide, allyl propyl sulfide, allyl propyl disulfide, allyl methyl disulfide, diallyl disulfide, diallyl trisulfide, dimethyl trisulfide, allyl propyl trisulfide, allyl methyl trisulfide), Symrise GmbH & Co. KG, Holzminden, Germany (dipropyl disulfide, dipropyl trisulfide), and Merck (dimethyl disulfide). The (*E*) and (*Z*) isomers of methyl-1-propenyl disulfide were distinguished according to the published data of Brodnitz et al. (*12*) as well as Kallio and Salorinne (*13*).

Plant Material. The samples (whole plants of *A. ursinum*) were collected in 2001 and 2002 in the area of Quedlinburg (Germany). Individual plants of the same population were analyzed by HPLC according to their contents of cysteine sulfoxides as well as by GC-MS due to their amount and pattern of volatile constituents throughout the vegetation period, focused on the months from March to June 2001. Besides the leaves, various other parts of the plants (bulb, storage leaf of the bulb and inner part of the bulb, fruit, and leaf stalk) were characterized as well in mid-June. Harvesting dates were March 21, 2001 (week 0; at least one green, fully developed leaf), April 3, 2001 (week 2), April 17, 2001 (week 4), May 2, 2001 (week 6), May 15, 2001 (week 8), May 29, 2001 (week 10), and June 12, 2001 (week 12; leaves turned yellow afterward). In terms of the bulb, the authors differentiate between the "storage leaf" and the remaining "inner part of the bulb". After the growing plant has exhausted it, the storage leaf disappears. Its remainder dissolves in the soil. After that, only the remaining bulb was analyzed. Additionally, in September, within the dormancy period of the plants, the bulb was analyzed again. The sample of June was also used for the investigation of the enzyme alliinase.

Methods. *The cysteine sulfoxides* of an average of two to three plants were analyzed by HPLC-UV after deactivation of the alliinase by boiling in methanol for 10 min, extraction in methanol/water (1:1), and precolumn derivatization with a reagent containing *o*-phthaldialdehyde and 2-methylpropanethiol following the method of Ziegler and Sticher (*14*). HPLC analysis was performed with a Shimadzu LC-4A chromatograph combined with a Chromatopac C-R3A and a UV detector set at a wavelength of 335 nm. The stationary phase was a Spherimarge 80 ODS 2 RP column (particle size, 5 μm; 250 mm $\times$ 4 mm). The mobile phase consisted of acetonitrile and phosphate buffer (0.05 M, pH 6.5) with a gradient profile. The method is described in detail by Krest et al. (*2*).

The pattern of volatile compounds was determined by solid-phase microextraction (SPME) headspace GC-MS as published in Keusgen et al. (*15*) and Storsberg et al. (*16*).

Approximately 1−2 g of the freshly minced sample (chopping was performed using a Waring blender, model 36BL28, Waring Products, New Hartford, CT, or an ordinary kitchen knife) was incubated in distilled water and transferred to a poly(dimethylsiloxane)-coated fiber. After thermal desorption, the sample was analyzed by gas chromatography and detected by mass spectrometry. The analyzing equipment consisted of a Hewlett-Packard gas chromatograph HP 5890 series II with an HP Innowax fused silica capillary column (0.5 μm bonded PEG phase, 60 m $\times$ 0.25 mm i.d. coupled to a Hewlett-Packard MSD model HP 5972). Operational parameters are described in detail by Keusgen et al. (*15*). The detected compounds are presented in **Figure 1**. Generally, the results are related to the total oil value of the sample. The oil value was determined by simultaneous distillation/extraction (SDE) following the method described by Likens and Nickerson (*17*) using 30 g of minced sample. After the addition of 0.32 mg of 6-methylhept-5-en-2-one as an internal standard, the pentane extract was analyzed according to the GC method used for the investigation of the profile of volatile compounds. The oil value was calculated from the sum of all peak areas.

Also, the extraction procedure and the characterization of *alliinase* are published in ref *2*. The obtained crude protein extract was analyzed on the basis of a method published by Schwimmer and Mazelis (*18*). K_m and V_{max} values were derived from a Lineweaver−Burk plot. Substrate specificities toward *rac*-(±)-alliin, (+)- and (−)-alliin, (+)-isoalliin, (+)-methiin, *rac*-ethiin [(±)-*S*-ethyl-L-cysteine sulfoxide], (+)-propiin, and *rac*-butiin [(±)-S-butyl-L-cysteine sulfoxide] as well as temperature and pH optima were determined.

RESULTS

Total Cysteine Sulfoxides. Analysis of plant material was started after *A. ursinum* showed at least one fully developed, freshly green leaf (March 21). In each investigated plant organ of *A. ursinum*, the highest amounts of total cysteine sulfoxides were detected in the middle of June (**Figure 2**). During the vegetation period, a significant decrease of the cysteine sulfoxide content in the leaves from 0.42% at the beginning of the study in March (week 0) to <0.1% in June (week 12) was observed. For this sample collected in June, additionally fruits and stalks

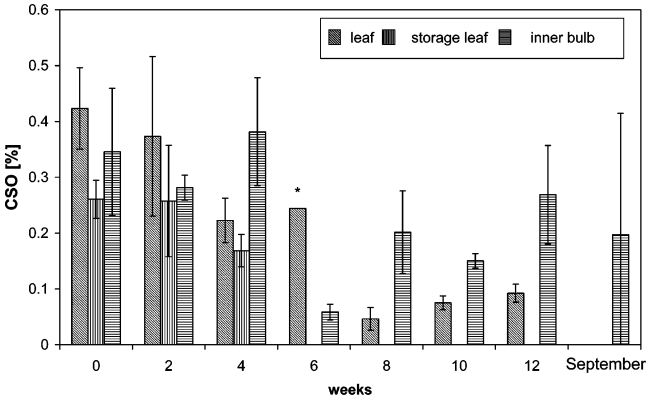

Figure 2. Change of the total cysteine sulfoxide (CSO) content in several parts of the plant depending on harvesting date. The content is related to the fresh weight of sample. Harvesting dates were March 21, 2001 (week 0), April 3, 2001 (week 2), April 17, 2001 (week 4), May 2, 2001 (week 6), May 15, 2001 (week 8), May 29, 2001 (week 10), and June 12, 2001 (week 12). * Because of the low number of green leaf samples (most leaves were yellow), error bars are not given at week 6.

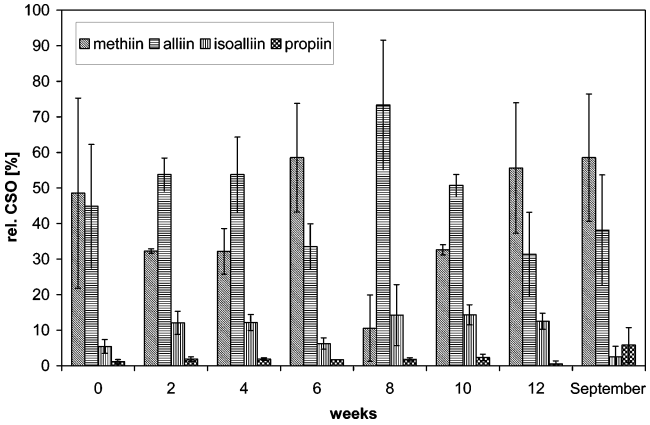

Figure 3. Change of the cysteine sulfoxide (CSO) pattern in the bulb of *A. ursinum* depending on harvesting time.

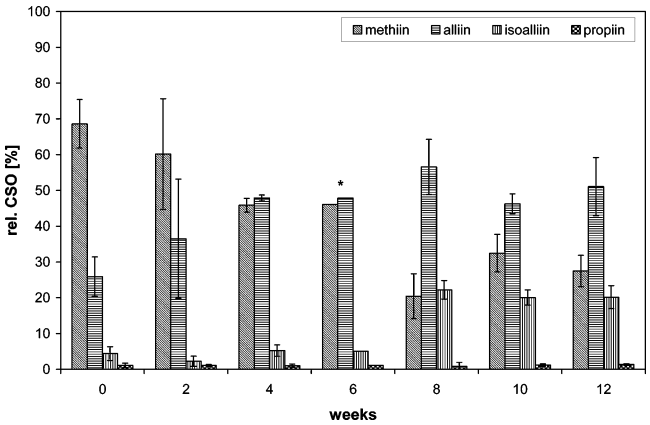

Figure 4. Change of the cysteine sulfoxide (CSO) pattern in the leaves of *A. ursinum* depending on harvesting time. * Because of the low number of green samples (most leaves were yellow), error bars are not given at week 6.

of leaves were analyzed (data not given in **Figure 2**). The content in the fruits (0.25% of fresh weighed sample) was as high as the content in the bulbs. Interestingly, leaf stalks showed a concentration of 0.15% total cysteine sulfoxides, which is significantly higher as the average of the whole leaf at the same time (0.09%). In the bulbs, we did not find a parallel development but first a reduction of the analyzed compounds from 0.35% in week 0 to 0.06% at the beginning of May (week 6) followed by an increase to 0.27% in week 12. The following September, the results of the investigated bulb showed an unexpected high standard deviation. This made it necessary to perform a repetition of the analysis with two bulbs of the same population in September of the following year. The content depicted is the average value of the results of all four investigated bulbs. It is similar to the cysteine sulfoxide content in June (0.21%).

Up to week 4 (mid-April), we also investigated the outer, fleshy storage leaf of the bulbs. After that date, they came off the inner part of the bulb and degraded. Therefore, they could not be harvested any more. The storage leaves showed fewer cysteine sulfoxides than the inner parts of the bulbs themselves analyzed at the same time. The reduction of the cysteine sulfoxides could be observed here as well.

Pattern of Cysteine Sulfoxides. Moreover, the pattern of the four investigated cysteine sulfoxides, alliin, isoalliin, methiin, and propiin, differed in dependence on the plant organ and time of harvest. *A. ursinum* is a methiin/alliin-type *Allium* species, exhibiting high amounts of these compounds but only traces of isoalliin and propiin (*1*−*3*). **Figure 3** shows the development of the cysteine sulfoxide pattern in the bulbs of *A. ursinum* during the vegetation period. In the beginning of the investigation, the bulb contained as much methiin as alliin. In the following weeks, alliin got the major compound of the cysteine sulfoxide fraction, whereas the content of methiin decreased to 15% (±9%; alliin, 73 ± 18%) in week 8 related to the content of total cysteine sulfoxides. After week 8, a rise of methiin led to a relationship of cysteine sulfoxides, which was similar to the situation at the beginning of the investigation in March (week 0). The relative content of isoalliin was ∼10%, and the relative concentration of propiin was always below 5%. In the sample out of the dormancy period we detected a pattern comparable to that of the bulb from mid-June. In the leaves, the development of the pattern during the vegetation period exhibited a great variance as shown in **Figure 4**. On the one hand, the content of methiin was reduced significantly from March to June. On the other hand, alliin increased from 26% in week 0 to >50% of the total cysteine sulfoxides in week 12. Furthermore, the observed increase of isoalliin after flowering time was remarkable. For the analysis of leaves, only fresh green parts were used. However, leaf color turned to yellow and brown at the end of the flowering period. In week 6, only one intact leaf could be investigated for its cysteine sulfoxide content. For this reason, no standard deviations are given for this sample.

Oil Value. Volatile substances were mainly formed by the alliinase reaction (**Figure 1**). Therefore, plant material has to be minced before analysis. The relative yield of oil (sum of volatile substances) obtained from leaves of *A. ursinum* decreased significantly during the vegetation period and corresponded to the amounts of detected cysteine sulfoxides. The same development could be observed within the bulbs for the first 8 weeks. Beginning at week 10, yield of bulbs increased again. Two different mincing methods (using an ordinary kitchen knife and a Waring blender) were applied simultaneously to compare their suitabilities for sample preparation. It was found that the samples minced by the robot blender had significantly higher levels. Therefore, only the volatile compounds of those samples were recognized in this study.

Volatile Compounds. For the characterization of volatile compounds, two different analytical methods were applied. Besides the investigation by SPME-GC (**Figures 5** and **6**), results were also obtained by SDE and subsequent GC analysis.

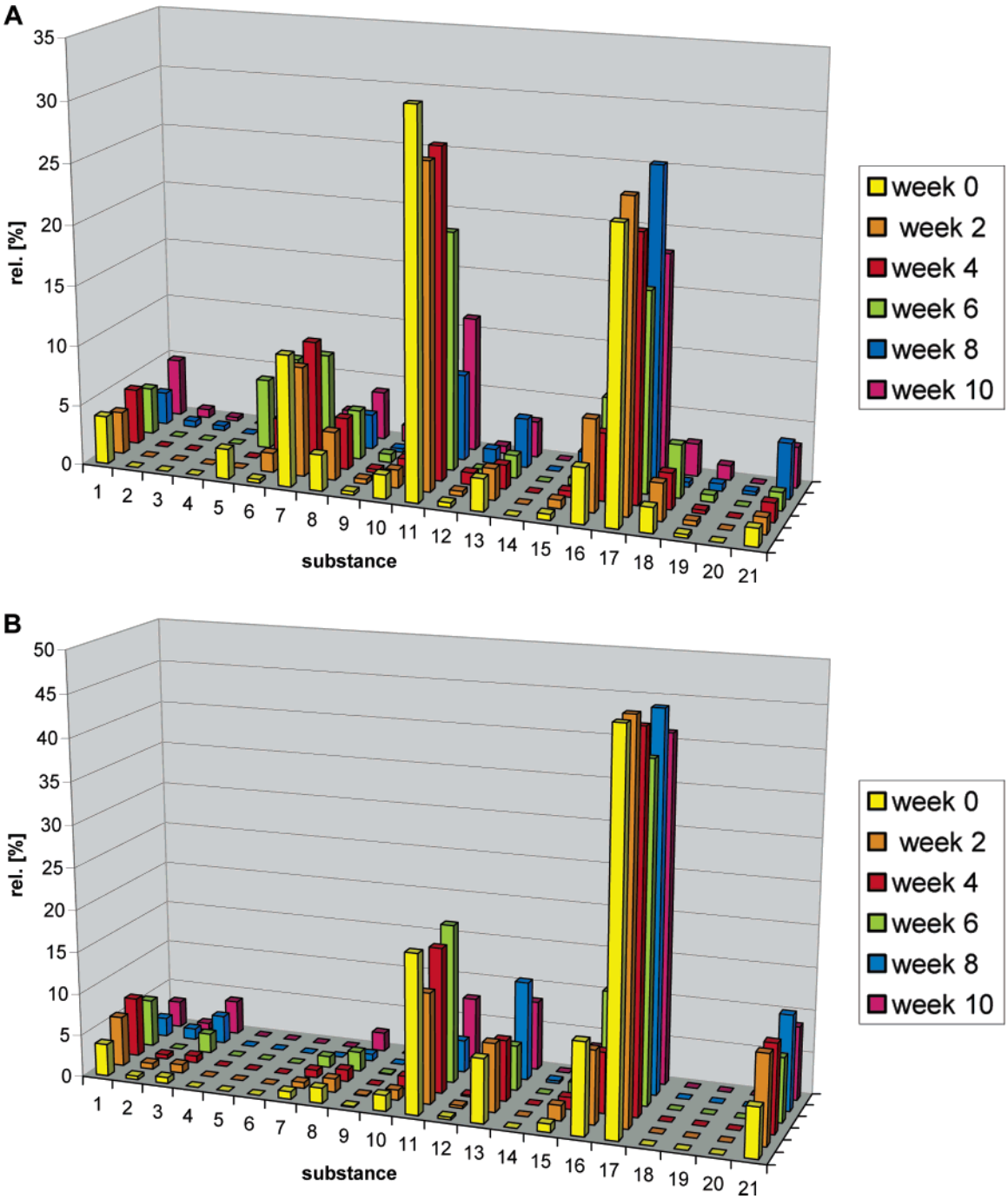

Figure 5. Change of the volatile compounds in leaves of *A. ursinum* depending on harvesting time: (**A**) SDE extraction; (**B**) SPME extraction. The numbering of compounds relates to **Figure 1**. The relative amounts refer to 100% = sum of all volatile compounds. Results are mean values for duplicate determinations.

The results of the SPME investigation showed diallyl disulfide as the major compound with a relative amount of ~50% in the leaves (**Figure 5**) and nearly 80% in the bulbs (**Figure 6**). On the other hand, the SPME method did not supply information about several volatile compounds such as 2-hexenal or hex-3-en-1-ol, whereas the SDE cleanup method yielded also traces of them. Dimethyl disulfide and dimethyl trisulfide, the two major compounds within the profile obtained by SDE-GC measurements (up to 11% in the leaves), were detected in significantly lower amounts when headspace SPME-GC analysis was applied; dimethyl trisulfide was not detectable in all samples. Allyl methyl sulfide, allyl methyl disulfide, diallyl sulfide, and (*E*)-allyl 1-propenyl disulfide were found by both methods as major compounds in the volatile fraction. SDE-GC detected more allyl methyl disulfide (up to 30%) than SPME-GC (<20%). It was found that neither propyl and (*Z*)-2-propenyl sulfides nor non-sulfur volatiles such as 2-hexenal contributed much to the profile of volatile compounds of *A. ursinum*.

In the leaves, significant decreases of allyl methyl disulfide and dimethyl disulfide could be observed during the span of investigation. Vice versa, the relative content of (*E*)-allyl 1-propenyl disulfide increased. The allyl sulfides were found to show comparatively constant amounts. In the bulbs, only small amounts of the methyl sulfides (dimethyl disulfide, allyl methyl disulfide, dimethyl trisulfide) were detected during the flowering period (week 8), but these substances increased to original amounts by September. Generally, the changes of the

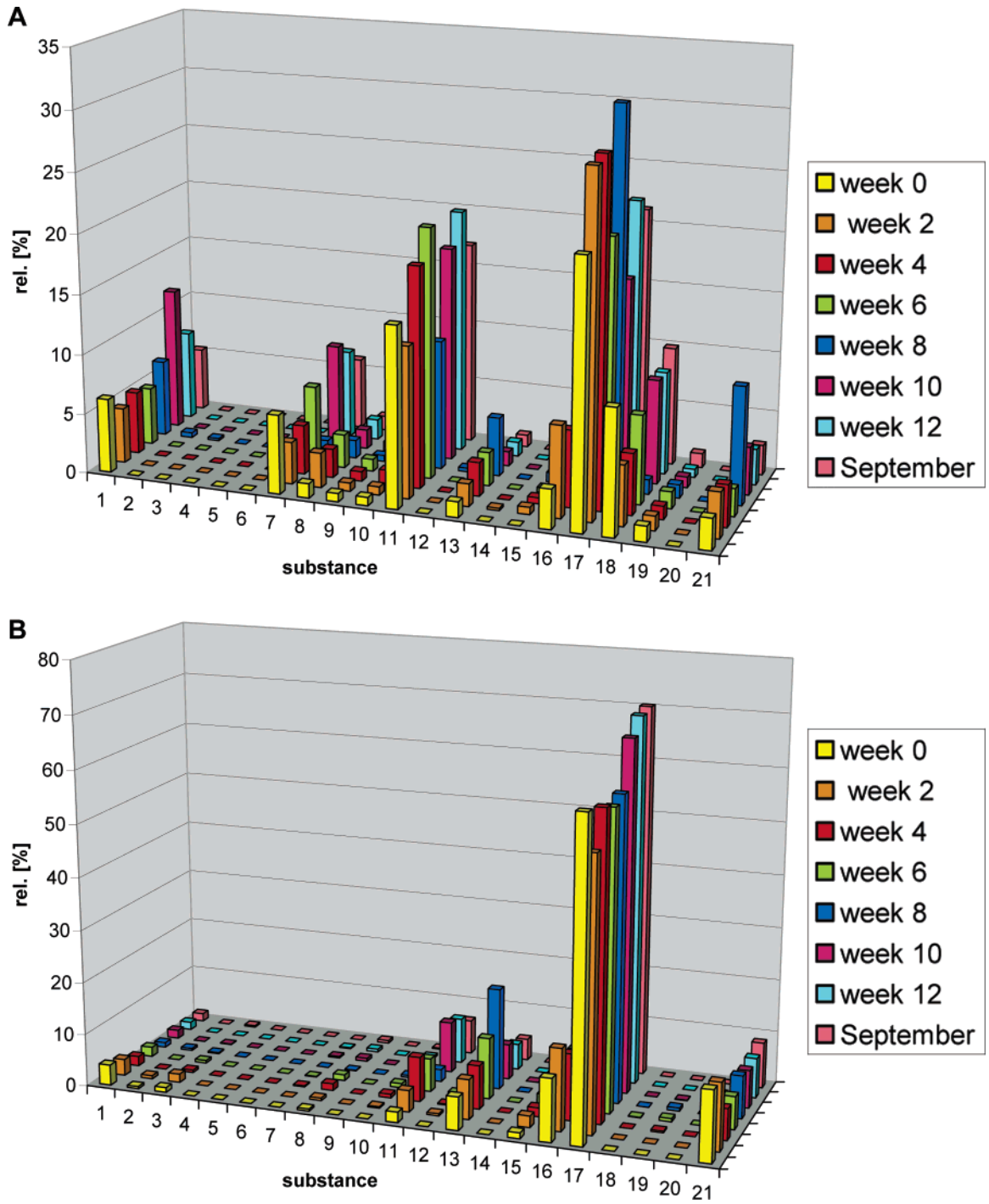

Figure 6. Change of the volatile compounds in bulbs of *A. ursinum* depending on harvesting time: (**A**) SDE extraction; (**B**) SPME extraction. The numbering of compounds relates to **Figure 1**. The relative amounts refer to 100% = sum of all volatile compounds. Results are mean values for duplicate determinations.

pattern of volatile compounds in leaves and bulbs corresponded very well to the results of cysteine sulfoxide investigation.

Alliinase. The results of the investigation of a protein extract of *A. ursinum* toward alliinase activity are shown in **Table 1**. V_{max} is related to the specific activity toward alliin. We found an enzymatic activity of 11 μmol min^{-1} mg^{-1}. Half of this activity was reached at 1.2 mM alliin (K_m value). The alliinase of *A. ursinum* showed highest activity at a pH of 7.5 and at a temperature optimum of 35 °C. Alliinase activity toward (+)-alliin [128% relative to (±)-alliin] was found as high as the activity toward (+)-isoalliin (125%) and significantly higher than those to the other compounds. Lowest activity was found for methiin (27%) and butiin (31%). The activity toward (−)-alliin (57%) was significantly lower than that toward its (+)-homologue.

DISCUSSION

Cysteine Sulfoxides and Volatile Compounds. Qualitative and quantitative results of both investigations mostly corresponded to each other. It has been found that the date of harvest has great impact on the quantity as well as the composition of volatile components. The content of total sulfuric compounds mainly depended on the content of methyl compounds, which varied significantly during the investigated span

Table 1. Kinetic Characteristics of Crude Alliinase Preparations Obtained from *A. ursinum* and *A. sativum*[a]

	A. ursinum	*A. sativum*
V_{max} (μmol min^{-1} mg^{-1})	11	10
K_m (mM)	1.2	1.3
pH optimum	7.5	7.0
temp optimum (°C)	35	40
substrate specificity (%)		
rac-(±)-alliin	100	100
(+)-alliin	128	122
(−)-alliin	57	20
isoalliin	125	123
methiin	27	7
ethiin	58	24
propiin	35	8
butiin	31	13

[a] Values of *A. sativum*, as published in ref *1*, were determined with exactly the same method, the same reagents, and devices. To calculate the relative substrate specificity, the activity toward racemate of alliin was set to 100%

of time but did not contribute considerably to the pharmacological impact. Nevertheless, the content of alliin decreased during the vegetation period. Therefore, as a result of this study the best time for harvest would be during March and April. Interestingly, garlic (*A. sativum* L.) shows an opposite behavior by which alliin increased during ontogenesis (*19*, *20*).

Two biogenetic pathways have been discussed in the literature (*21*, *22*). Neither theory included transformation reactions, for instance, alliin or isoalliin from methiin. Changes in the cysteine sulfoxide pattern are therefore not caused by interactions between the volatile precursors. Cysteine sulfoxides such as alliin and isoalliin, which are the two best investigated substances, may be synthesized during the vegetation period. Thus, decreasing and increasing contents may be explained by N-terminal substitution reactions with (−)-*N*-(1′-deoxy)-1′-β-D-fructopyranose as described by Mütsch-Eckner (*24*) or γ-glutamylpeptides (*23*). Cysteine sulfoxides bound this way cannot be detected by the applied methods. Because the biogenetic pathways are not directly coupled to each other (see above), the ratio between individual cysteine sulfoxides may vary in a broad range, also demonstrated by this investigation. Diallyl disulfide, the major compound in the volatile pattern during the vegetation period, is thought to be responsible for the inhibition of cholesterol synthesis (*25*). Therefore, further studies concerning the health-promoting effects of the medicinal plant *A. ursinum* are highly advisable. A significant maximum for this compound in leaves could not be determined in this investigation. However, it can be assumed that fresh, young leaves are favorable.

The different results gained by SDE-GC and SPME-GC show the great influence of sample preparation techniques on the development of volatiles in *Allium* species. Generally, SPME yielded significantly lower amounts compared to SDE. Furthermore, both solvent polarity and temperature have an effect on the pattern of volatile compounds (*17*). For this reason the use of pentane extraction during the SDE procedure might not simulate exactly the natural pattern of volatile substances produced in an aqueous medium. On the other hand, SPME discriminates against the adsorption of specific volatiles present in the headspace of ramson samples. Applying both methods simultaneously may therefore be preferable for improved investigations of volatile compounds in various *Allium* species.

Alliinase. The specific activity was nearly identical to the specific activity of alliinase from *A. sativum* and comparable to those of various wild relatives (*2*). It must be taken into account that the enzyme was not purified to homogeneity but reflects the situation in minced plant material. This is of great practical importance, because formation of sulfur-containing, volatile compounds strictly depends on an active enzyme. The K_m describes the affinity of an enzyme toward a substrate by representing the substrate concentration at 50% of maximum velocity. The K_m value of the obtained extract is as high as that of *A. sativum*. This implies that alliinase of ramson might be as active as that of garlic.

Moreover, alliinase of *A. ursinum* is less specific to alliin than alliinase of *A. sativum*, but shows higher relative activity toward the cleavage of other cysteine sulfoxides. Remarkable are the activities toward methiin and isoalliin. Interestingly, these two substrates, which naturally occur in ramson, were not tested in previous investigations (*4*). Furthermore, a pH maximum was described at pH 6.0 for the isolated enzyme, which is not in accordance with our findings (pH 7.5). A reason for these differences might be that the alliinase naturally occurs as a lectin complex (*26*). During extraction and purification, no high salt concentrations or pH shifts were used. Therefore, native protein complexes were not destroyed. It can be assumed that kinetic properties of this complex might be different from those of the separated enzyme.

ACKNOWLEDGMENT

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