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Influence of Cultivar and Harvest Time on the Amounts of Isoalliin and Methiin in Leek (*Allium ampeloprasum* var. *porrum*)

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Supporting Information

ABSTRACT: Extracts of 31 leek cultivars were analyzed using liquid chromatography−tandem mass spectrometry (HPLC-MS/MS) to determine the distribution of the two most abundant *S*-alk(en)yl-L-cysteine sulfoxides (ACSOs) in leek, that is, isoalliin and methiin. The isoalliin concentration of the white shaft and green leaves of the 31 leek cultivars varied from 15 to 53 mg/g dry weight (dw) and from 9 to 45 mg/g dw, respectively, whereas the methiin concentration varied from 3 to 16 mg/g dw and from 1 to 10 mg/g dw, respectively. Leek cultivar and tissue had an effect on the ACSO amounts. Cultivars Artico and Apollo F1 rated highest for the mean isoalliin and methiin concentration, respectively. In general, the whole leek plant of the winter leek cultivars contained a significantly higher ACSO amount than the summer and autumn cultivars. To determine whether this difference was attributed to the cultivar background or time of harvest, ACSOs were also quantitated in nine leek hybrids at four different stages during the next growth season. The amounts of ACSO changed significantly during the growth season, indicating the importance of harvest at specific time moments, although there was still an effect of cultivar on the ACSO amounts.

KEYWORDS: *leek, isoalliin, methiin, HPLC-MS/MS, genetic diversity, harvest time*

INTRODUCTION

Leek (*Allium ampeloprasum* var. *porrum*) is a monocotyledonous plant of the Alliaceae family (genus *Allium*). Indonesia is the largest producer of leeks in the world followed by Turkey, France, Belgium, China, and Poland.[1] Leek is one of the most important field vegetable crops in Belgium, accounting for 16% of the production value, and is grown for its thickened cylindrical stem made up of long leaf bases.[2] This biennial crop is grown as a short-lived annual in commercial cropping.[3] The first leek cultivars were landraces and were highly variable in agronomic and morphological traits. During the second half of the 20th century, numerous new cultivars were developed. These cultivars were maintained by open pollination after selection for specific traits, including winter-hardiness, long shafts in winter varieties, erectness of the leaves, dark leaf color, disease resistance, and uniformity.[4] Nowadays, F1 hybrids are gaining popularity among growers, due to their higher yields and improved uniformity compared with open-pollinated cultivars.[5] Selection and breeding efforts have resulted in many types of leek cultivars, each specifically adapted for growth in a specific part of the leek production season and with typical properties concerning growth rate, leaf color, frost resistance, shaft length, and harvest time.[6] In Belgium, summer leek types are harvested from June until September, autumn leek from September until January, and winter leek from December until March. As a result, leeks are supplied to the market year-round.

The *Allium* genus comprises approximately 700 species, including economically important vegetables and flowering ornamentals as well as wild species from Europe, Asia, and North America.[7] *Allium* species including onion (*Allium cepa* L.), garlic (*Allium sativum* L.), leek (*A. ampeloprasum* var. *porrum*), chives (*Allium schoenoprasum* L.), and bunching or Welsh onion (*Allium fistulosum* L.) are extensively used for food flavoring.[8] These vegetables have been recognized as rich sources of secondary metabolites, including the organosulfur compounds, which are responsible for the characteristic taste, aroma, and lachrymatory effects of the *Allium* species. This group of molecules has been considered to be the main factor responsible for the claimed health-promoting effects of these species, including anticancer, antimutagenic, antiplatelet, antihyperglycemic, antidiabetic, and antioxidant effects.[9−16] All *Allium* species produce sulfur-containing compounds. Up to a few percent of the dry weight may be nonprotein sulfur amino acids, better known as *S*-alk(en)yl-L-cysteine sulfoxides (ACSOs). These amino acids are enzymatically formed by

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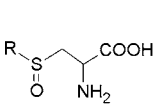
hydrolysis of the corresponding γ-glutamyl-*S*-alk(en)ylcysteine storage dipeptides.[17] It has been estimated that approximately 75% of the sulfur in *Allium* species occurs as ACSOs or the storage form γ-glutamyl-ACSOs. These nonvolatile, odorless ACSOs are the precursors of flavors, odors, and the lachrymatory factor.[18−21] When the tissues of *Allium* species are damaged, characteristic flavor and aroma compounds are produced as a result of the cleaving of ACSOs by the endogenous enzymes alliinase and lachrymatory factor synthase.[22]

Ten ACSOs have been reported in *Allium* species (Figure 1) including *S*-methyl-L-cysteine sulfoxide (methiin, present in



R = CH_3	methiin
R = CH_2CH_3	ethiin
R = $CH_2CH_2CH_3$	propiin
R = $CH_2CH{=}CH_2$	alliin
R = $CH{=}CHCH_3$	isoalliin
R = $CH_2CH_2CH_2CH_3$	butiin
R = $CH_2CH_2CHCHCH_3$	*S*-(3-pentenyl)-L-cysteine sulfoxide
R = $CHCHCH_2CH_3$	homoisoalliin
R = CH_2SCH_3	marasmin
R = 	*S*-(2-pyrrolyl)-L-cysteine sulfoxide

Figure 1. Structure of the *S*-alk(en)yl-L-cysteine sulfoxides present in *Allium* species.

Allium species and some Brassicaceae), *S*-propyl-L-cysteine sulfoxide (propiin), *trans*-*S*-1-propenyl-L-cysteine sulfoxide (isoalliin; characteristic of onion), *S*-(2-propenyl)-L-cysteine sulfoxide (alliin; characteristic of garlic), *S*-ethyl-L-cysteine sulfoxide (ethiin), *S*-butyl-L-cysteine sulfoxide (butiin), *S*-(3-pentenyl)-L-cysteine sulfoxide (present in *A. cepa* var. *tropeana* seeds), *S*-(1-butenyl)-L-cysteine sulfoxide (homoisoalliin, present in *Allium siculum*), *S*-(methylthiomethyl)-L-cysteine sulfoxide (marasmin, present in *Allium stipitatum*), and *S*-(2-pyrrolyl)-L-cysteine sullfoxide (present in *Allium giganteum*).[23−28] The composition of these compounds is species-specific, but their concentrations can vary according to the environmental conditions or plant parts examined.[29,30] The degree of the respective contributions of these organosulfur compounds to flavor, bioactivity, and food discoloration depends on their alkyl moieties.[21]

Multiple papers describe the ACSO concentration in *Allium* species such as garlic,[31,32] onion,[33] and bunching onion.[34] Reports can be found on the composition and variability of ACSOs in leek, but the amount of data is scarce. Lundegardh et al.[35] investigated the impact of fertilization on the ACSO concentration (alliin, propiin, isoalliin, and methiin) in leek (cv. Hilari) using a HPLC-MS/MS system. Only isoalliin and methiin seemed to be present in significant amounts. Kubec and Dadakova[36] analyzed ACSOs in leek with the help of micellar electrokinetic capillary chromatography and found significant amounts of only isoalliin and methiin in the leek samples. Propiin and alliin were present in trace amounts. Yamazaki et al.[21] determined the distribution of 11 flavor precursors in seven *Allium* vegetables, including leek, and found only isoalliin, and methiin in significant amounts.[21] Moreover, few papers describe the quantitation of ACSOs as a function of the *Allium* cultivars.[32,37] In addition, limited information can be found with regard to changes in ACSO concentration in the leek plant during their growth season. In onion, the isoalliin concentration was remarkably lower when sold during summer than when sold during winter.[21] In garlic, it was observed that the ACSO concentration (alliin, methiin, and isoalliin) gradually decreased during the whole vegetation period (March until June) in the leaves and pseudostem, whereas the alliin and methiin concentrations in the bulbs of garlic initially decreased after planting but significantly increased in June and culminated before harvest.[37] On the other hand, isoalliin levels steadily decreased during the whole vegetation period in all parts of the plants. Coolong and Randle[33] reported a significantly higher total ACSO concentration in onions grown at high temperatures in comparison with ACSO concentration of onions grown at lower temperatures, indicating the effect of growth temperature on the ACSO concentration.

As information about the effect of leek cultivars on the ACSO composition is lacking, the aim of this study was to investigate the concentration of the two major ACSOs present in leek, that is, isoalliin and methiin, as a function of the cultivars of leek, harvested at their optimal condition according to their type (summer, autumn, winter). To clarify possible differences between cultivars in terms of harvest date, a selection of leek cultivars, representing summer, autumn, and winter types, was harvested at four time points from summer until winter.

This study can recommend that leek growers use specific cultivars, types, and practices to maximize the ACSO concentration. Moreover, the availability of data on ACSO levels can be considered as an important criterion for selection of genotypes from a genebank for use in crop improvement or other research-related or commercial activities.

MATERIALS AND METHODS

Plant Material. *Genetic Diversity.* Thirty-one leek (*A. ampeloprasum* var. *porrum*) cultivars (Table 1) were studied from a selection based on three criteria including (1) morphological type (pale green summer, dark green winter, intermediate autumn); (2) breeding strategy and multiplication scheme (hybrids, open-pollinated cultivars, farmer selections, and old landraces); and (3) seed company. The most recently developed cultivars, that is, the F1 hybrids included in this study, are a good representation of leek material commercially grown in the leek-producing areas.

Leek seeds were obtained from the collection of the Institute for Agricultural and Fisheries Research (ILVO). The 31 leek cultivars were sown at the same moment in triplicate (3 × 75 seeds) in April 2009 in an unheated greenhouse at ILVO. In June 2009, 3 × 15 plants of each cultivar were planted in the field (N 50° 58′ 86″, E 3° 46′ 56″) with each repetition in a different row. During the cultivar optimal harvest period, 3 × 10 plants (of the 3 × 15) of each cultivar were manually harvested. The summer leek types were harvested in the summer, the autumn types in the autumn, and the winter types in the winter, as performed in commercial leek production. Immediately after harvest, the leeks were washed, the roots and decayed leaves were removed, and the remainder was divided into two parts, that is, the lower white part (shaft) and the upper green part (leaves). With regard to the white shaft, the section starting from 1 cm of the basal plate until 2 cm under the transition between the shaft and green leaves was used as sample material. In addition, samples of the green leaves were taken starting 2 cm above the transition to 1 cm of the top of the leaves. The sections were then chopped into ∼1 cm^2 pieces. The 10 plants of each replication were pooled. The batches were stored at −80 °C prior to freeze-drying (CD-Energie, Eke, Belgium). The freeze-dried samples were milled to pass through a 1 mm sieve (Fritsch, Rotterdam, The Netherlands), and the dry powder obtained was stored in Falcon tubes at 4 °C until analysis. For each cultivar, three biological replicates containing the pooled material of 10 plants each and 2 technical replications were taken into account.

Table 1. Overview of the 31 Leek Cultivars Analyzed during Growing Season 2009−2010 and the 9 Cultivars (Indicated in Bold) Analyzed at Four Stages during Growing Season 2010−2011

commercial name	type	breeding category	commercial origin
Varna	summer	open pollinated	Royal Sluis
Albana	summer	open pollinated	Nunhems
Nelli	summer	open pollinated	Svalöf Weibull
Elefant	summer	old cultivar	IPK
Miracle F1	summer	F1 hybrid	Enza
Zeus F1	summer	F1 hybrid	S&G
Striker F1	summer	F1 hybrid	Bejo
Electra	autumn	open pollinated	Clause
Nebraska	autumn	old cultivar	Royal Sluis
Breugel F1	autumn	F1 hybrid	Rijkzwaan
Tadorna	autumn	old cultivar	Enza
Poribleu	autumn	open pollinated	Nickerson-Zwaan
Alcazar	autumn	open pollinated	Rijkzwaan
Belton F1	autumn	F1 hybrid	Nunhems
Pretan F1	autumn	F1 hybrid	Nickerson-Zwaan
Musselburgh	winter	breeder selection	D. T. Brown
Van Limbergen	winter	breeder selection	Sint Katelijne Waver
Buelens	winter	breeder selection	Onze Lieve Vrouw Waver
Coolidge F1	winter	F1 hybrid	Hortiplan
Apollo F1	winter	F1 hybrid	S&G
Artico	winter	old cultivar	IPK
Farinto	winter	open pollinated	Nunhems
Arkansas	winter	open pollinated	Royal Sluis
Gavia	winter	open pollinated	Enza
Toledo	winter	old cultivar	Thompson & Morgan
Uyterhoeven	winter	breeder selection	Onze Lieve Vrouw Waver
Engels	winter	breeder selection	Putte
Vervloet	winter	breeder selection	Sint Katelijne Waver
Harston F1	winter	F1 hybrid	Nunhems
Kenton F1	winter	F1 hybrid	Nunhems
Fahrenheit F1	winter	F1 hybrid	Royal Sluis

Harvest Time. In the next leek growth season (2010−2011), nine leek hybrids (Table 1, indicated in bold) were sown in triplicate in April 2010 in an unheated greenhouse. In June 2010, the nine leek cultivars were planted in the field (N 50° 58′ 53″, E 3° 46′ 43″) as described for the genetic diversity setup. In September 2010, November 2010, January 2011, and March 2011, three leek plants of each repetition in the field were harvested from each cultivar. The plants were pooled for each repetition and further processed as described for the analysis as a function of the genetic diversity. For each cultivar and each harvest time, three biological and two technical replications were taken into account.

Soil properties of the two leek parcels before planting are given in Table 2.

Reagents and Chemicals. Acetonitrile (MeCN, LC-MS) and formic acid (99%, LC-MS) were obtained from Biosolve B.V. (Valkenswaard, The Netherlands). Water was of HPLC grade (generated by a Milli-Q gradient purification system, Millipore, Bedford, MA). The alliin standard (purity ≥ 98%) was purchased from Sigma-Aldrich (Bornem, Belgium) and methiin from Enzo Life Sciences (Antwerp, Belgium). Because isoalliin was not available as a standard, isoalliin was identified on the basis of the mass spectra and quantitated as alliin.[35] As isoalliin and methiin are the most prevalent ACSOs in leek and standards of the other eight ACSOs are not readily commercially available, analysis focused on isoalliin and methiin only. *O*-(Carboxymethyl)hydroxylamine hemihydrochloride (OCMHA) was purchased from Sigma-Aldrich.

Table 2. Soil Properties of the Two ILVO Parcels before Planting

	season 2009−2010	season 2010−2011
total NO_3-N	89.0	101.7
total NH_4-N	19.1	13.8
pH	5.88	4.73
C (%)	0.84	0.85
P	20.2	16
K	9	5.3
Mg	15.3	9.9
Ca	92.8	67.3
Na	4.5	4

Sample Preparation. Extraction was performed as described by Lundegardh et al.[35] with some modifications. Briefly, a precisely weighed (100 mg) amount of freeze-dried leek powder was diluted with 10 mL of OCMHA (1.1 g/L). This mixture was shaken on a horizontal shaker for 10 min and centrifuged at 3946*g* during 10 min. Subsequently, the supernatant was 1:5 diluted by adding HPLC-H_2O containing 0.1% formic acid. This extract was filtered (Millex GV, Millipore, 0.22 μm) and used for the ACSO HPLC-MS/MS determination.

HPLC-MS/MS Method. *Instrumental Conditions.* ACSO levels were quantitated using a model 2695 Alliance LC system (Waters, Milford, MA, USA) interfaced to an MS equipment consisting of a Quattro LCZ (Waters) equipped with a Z-spray system.

Separation of the ACSOs was performed on a Hypurity Aquastar C_{18} column (2.1 × 150 mm) with 5 μm particle size protected with a C_{18} guard column (2.1 × 10 mm; 5 μm) (Thermo, Louvain-la-Neuve, Belgium). The HPLC eluent was water with 0.1% formic acid. The isocratic eluent flow was set at 0.15 mL/min and the injection volume at 20 μL. The column temperature was held at 20 °C for chromatographic separation. The LC effluent was connected to the interface via a divert valve to avoid clogging the cone of the mass spectrometer. The instrument operated in the selected reaction monitoring (SRM) mode with a dwell time of 0.50 s, an interchannel delay of 0.01 s, and an interscan delay of 0.10 s.

The MS system was controlled by version 4.1 of the MassLynx software (Waters, Zellik, Belgium).

Mass spectrometric characteristics such as cone voltage and collision energy were optimized (=tuned) by continuously infusing pure standards (1 μg/mL, 10 μL/min) into the mass spectrometer combined with a flow of 200 μL/min HPLC-H_2O + 0.1% formic acid using a T-piece. Standard stock solutions of alliin and methiin were prepared in water at a concentration of 1 mg/mL and stored at −18 °C. Tuning solutions of 1 μg/mL were obtained by diluting the working solution of 10 μg/mL in acetonitrile/water (50:50, v/v) containing 0.1% formic acid. For both compounds, ionization was performed in the electrospray (ES) positive mode. The precursor ion and the two product ions with the highest signal-to-noise (S/N) value and the highest peak intensity were selected for both analytes. The sum of both ions was used for quantitation. The detection parameters of alliin and methiin are listed in Table 3. Nitrogen was used as cone gas and desolvation gas at flow rates of 60 and 700 L/h, respectively. The source block and desolvation temperature were set at 120 and 300 °C, respectively. Collision gas pressure was 2.5×10^{-3} mbar.

For quantitation of individual compounds from peak areas, external calibration of alliin and methiin in a blank leek matrix was used (i.e., matrix-matched calibration curves). The blank matrix was a leek matrix (white shaft, cv. Krypton), which was left to stand during 24 h without the addition of OCMHA. Alliin and methiin were completely converted into breakdown products during these 24 h.[38] The same blank matrix (stored at −20 °C) was used for all analyses. Results were expressed as milligrams of isoalliin or methiin per gram dry weight (mg/g dw).

Method Validation. The analyte-dependent characteristics of the ACSO method concern specificity, linearity, possible matrix effects, apparent recovery (R_A), repeatability (RSD_r), intralaboratory reprodu-

Table 3. Mass Spectrometer Detector Settings for *S*-Alk(en)yl-L-cysteine Sulfoxide Determination

compd	ionization mode	precursor ion (m/z)	cone voltage (V)	product ions (m/z)	collision energy (eV)	retention time (min)
alliin	ES+	178.23	20	88.01/70.26	10/20	4.21
methiin	ES+	152.15	20	88.22/70.08	10/15	3.67

Table 4. Overview of the Percentage Apparent Recovery (R_A), Repeatability (RSD_r), and Interday Precision over 3 Days (RSD_R) at the Three Concentrations Used for Validation and Limits of Detection and Quantitation (LOD and LOQ) and Signal Suppression/Enhancement (SSE) for Alliin and Methiin

		low concn			medium concn			high concn					
	concn (mg/g)	R_A (%)	RSD_r (%)	RSD_R (%)	R_A (%)	RSD_r (%)	RSD_R (%)	R_A (%)	RSD_r (%)	RSD_R (%)	LOD (μg/g)	LOQ (μg/g)	SSE (%)
alliin	1.0−1.5−2.0	98	3	6	98	2	3	96	1	4	31	62	48
methiiin	1.0−1.5−2.0	108	4	11	108	2	3	109	2	3	22	44	13

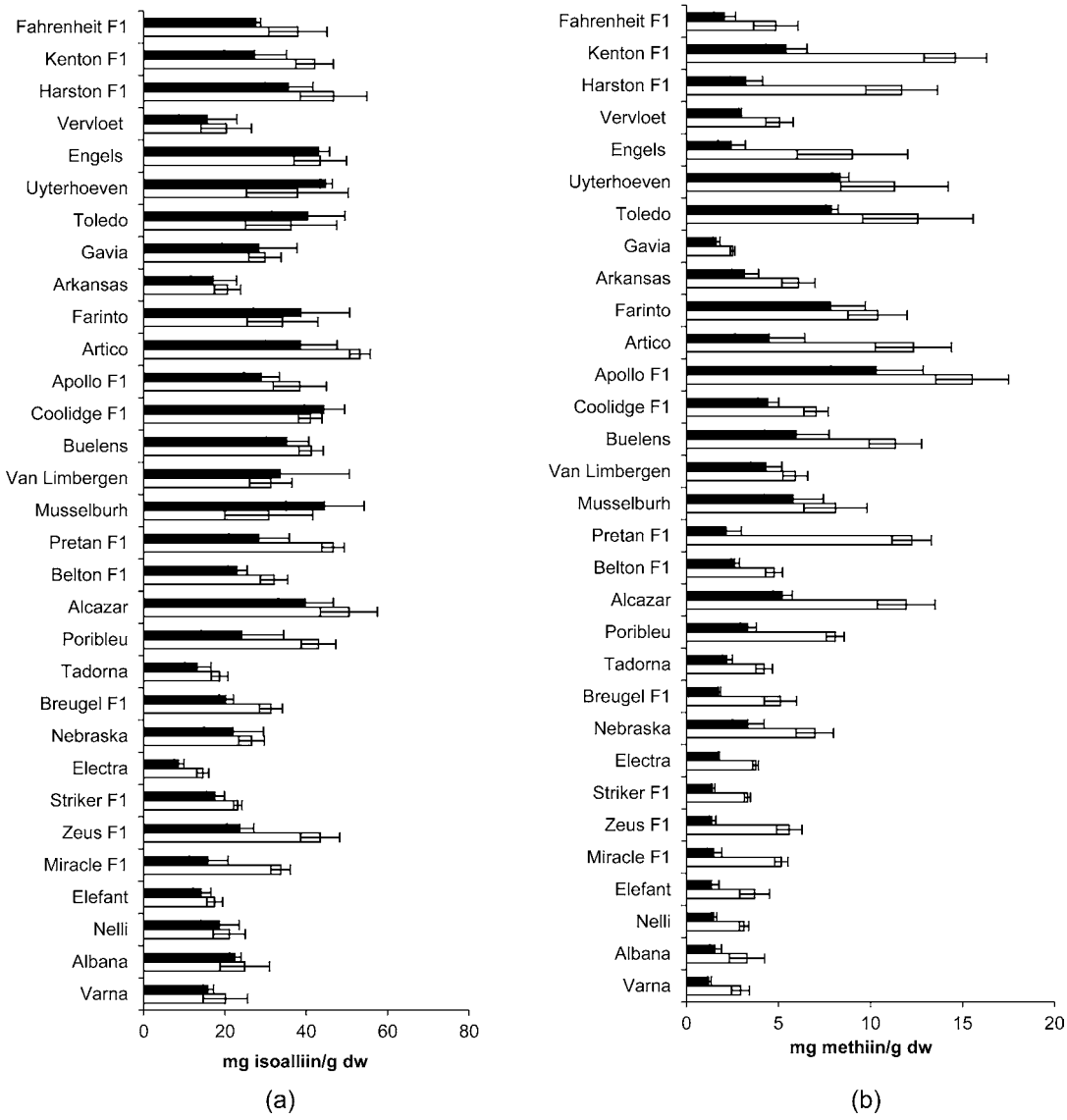


Figure 2. Isoalliin (a) and methiin (b) concentrations (mg/g dw) in the white shaft (white bars) and green leaves (black bars) of the analyzed leek cultivars.

cibility (RSD_R), and limits of detection (LOD) and quantitation (LOQ). These parameters were validated according to the guidelines of Commission Decision 2002/657/EC. A blank leek sample was used for spiking experiments. Specificity was checked by analyzing 1 μg/mL of each pure liquid standard separately and searching for signal interference among the various multiple selection monitorings (MRM). To evaluate linearity, possible matrix effects, R_A, and RSD_r, three series blank samples were spiked with pure ACSO standards. Each series included one specific spiking level in six replicates (Table 4). A matrix-matched calibration curve of at least five spiking concentrations was also taken into account. Linearity of the matrix-matched calibration curves was evaluated on the basis of graphical

interpretation of R^2 (≥0.99) and the F statistic (goodness-of-fit). A minimum of five data points for each calibration curve was used within the concentration range of 0−5 mg/g. R_A percentages at the three spiking levels were calculated by using the matrix-matched calibration curves for quantitation. Specifically, for each spiking level, the observed concentration levels were calculated by using the peak area and the matrix-matched calibration curve. Subsequently, the apparent recovery was expressed as a percentage by comparing these observed values to the actual spiked levels. The data obtained from these experiments conducted on a single day were used to study the intraday precision repeatability by calculating the relative standard deviation (RSD_r). For interday precision (RSD_R), these experiments were carried out on three separate days. For both compounds, the LOD and LOQ were calculated as 3 and 6 times the standard error of the intercept divided by the slope of the calibration curve, respectively. The calibration curves of the spiked extracts were used to determine possible matrix effects by comparing them to the corresponding calibration curves of the pure standards. These effects were expressed in terms of signal suppression/enhancement (SSE) and were calculated as follows: SSE (%) = 100 × $slope_{extract\text{-}matched\ standard}/slope_{pure\ standard}$.[39]

Statistical Analysis. The significance was set at $p < 0.05$. For evaluation of the linearity of the matrix-matched calibration curves, an F statistic (goodness-of-fit) using the Linest function was performed in Excel.

The data are presented as the mean ± standard deviation (SD) of six measurements. Analysis of variance (ANOVA) was performed using SPSS version 17 statistical program (SPSS Inc., Chicago, IL, USA) with "cultivar" and "plant tissue" as factors for the cultivar experiment and "cultivar" and "harvest time" for the harvest time experiment, taking their interaction into account. In case of significant differences, multiple comparison of means was established with the post hoc Sheffé test. For the harvest time experiment, repeated measurements were used.

A Pearson correlation test was used to determine the correlations between the ACSO levels of season 2009−2010 and season 2010−2011.

RESULTS AND DISCUSSION

Method Validation. Calibration curves of matrix-matched standards were used to evaluate linearity in terms of R^2 values and goodness-of-fit testing. For both compounds, linearity was found to be adequate; R^2 = 0.9920 (alliin) and R^2 = 0.9967 (methiin). The calibration curves of spiked extracts were used to determine SSE by comparing them to the corresponding calibration curves of the pure standards. The results shown in Table 4 indicate that both compounds seemed to be subject to signal suppression (SSE < 100%). The observed SSEs (in the range of 13−48%) emphasize the need to quantitate ACSOs in leek samples by means of matrix-matched calibration curves. These strong matrix effects could be also controlled by using an internal standard, if possible, an isotopically labeled ACSO compound.

Table 4 gives an overview of the overall percentage apparent recovery (R_A), repeatability (RSD_r), interday precision over 3 days (RSD_R), and LOD and LOQ (μg/g) for both compounds. The overall R_A was calculated as a mean of the three concentrations extracted and was within the range of 80−110% for both compounds, that is, the strictest limits set in Commission Decision 2002/657/EC. The repeatability and intralaboratory reproducibility were considered to be acceptable according to the guidelines stipulated in the performance criteria of Commission Decision 2002/657/EC. The HPLC-MS/MS method used had LODs of 31 μg/g for alliin and 22 μg/g for methiin and LOQs of 62 μg/g for alliin and 44 μg/g for methiin.

Genetic Diversity. The results of the isoalliin and methiin concentrations as a function of the cultivars of leek are shown in Figure 2, panels a and b, respectively. The cultivar and leek part had a significant effect on the isoalliin and methiin concentrations (Table 5). Moreover, there was a significant interaction between the cultivars analyzed and the part of the leek plant on the ACSO concentration.

Table 5. Analysis of Variance of Means Square for the Isoalliin and Methiin Concentrations of the 31 Leek Cultivars

	isoalliin[a]	methiin[a]
cultivar (A)	584.894*	50.381*
plant tissue (B)	1632.886*	601.975*
A × B	87.363*	7.942*
error	49.277	1.603

[a]*, significant at the 0.05 level.

The isoalliin concentration of the white shaft and green leaves of the 31 leek cultivars varied from 14.56 to 53.17 mg/g dw (mean = 33.30 mg/g dw) and from 8.73 to 44.90 mg/g dw (mean = 27.58 mg/g dw), respectively. For half of the leek cultivars, the isoalliin concentration was significantly higher in the white shaft than in the green leaves. The highest isoalliin concentration was obtained from the white shafts of the cultivars Artico, Alcazar, and Harston F1 and from the green leaves of the cultivars Uyterhoeven, Musselburg, and Coolidge F1. Among the 31 leek cultivars, the whole leek plant (55% white shaft and 45% green leaves) of the cultivar Artico rated the highest for the mean isoalliin concentration (46.69 mg isoalliin/g dw).

The methiin concentration of the white shaft and green leaves of the 31 leek cultivars varied from 2.93 to 15.52 mg methiin/g dw (mean = 7.50 mg/g dw) and from 1.24 to 10.34 mg methiin/g dw (mean = 3.65 mg/g dw). The white shaft of the 31 cultivars contained significantly higher amounts of methiin than the green leaves, except for cultivar Uytterhoeven. The highest methiin concentration was observed from the white shafts of the cultivars Apollo F1, Kenton F1, and Toledo and from the green leaves of the cultivars Apollo F1, Uyterhoeven, and Toledo. Among the 31 leek cultivars, the whole leek plant of the cultivar Apollo F1 rated highest for mean methiin concentration (13.19 mg methiin/g dw).

Lundegard et al.[35] reported ACSO concentrations in the edible leek plant (cv. Hilari) of approximately 23.0 mg isoalliin/g dw and 1.5 mg methiin/g dw. These values are in the range of our results, but less than the average amount of ACSOs quantitated in our study. These variations could be due to differences among cultivars, growing seasons, agricultural practices, and variations in the analytical method. Yamazaki et al.,[21] on the other hand, reported mean isoalliin and methiin concentrations of 37.4 and 7.7 mg/g dw, respectively, analyzing three leek pools from three regions (United States, Belgium, and Australia). These results are in the same range of our results.

In general, the white shaft of the summer, autumn, and winter leek types contained 30.11, 40.02, and 45.81 mg ACSOs (isoalliin + methiin)/g dw, respectively. The ACSO concentration of the white shafts of the winter cultivars was significantly higher than the concentration of shafts of the summer cultivars. Furthermore, the green leaves of the summer, autumn, and winter types had mean ACSO concentrations of

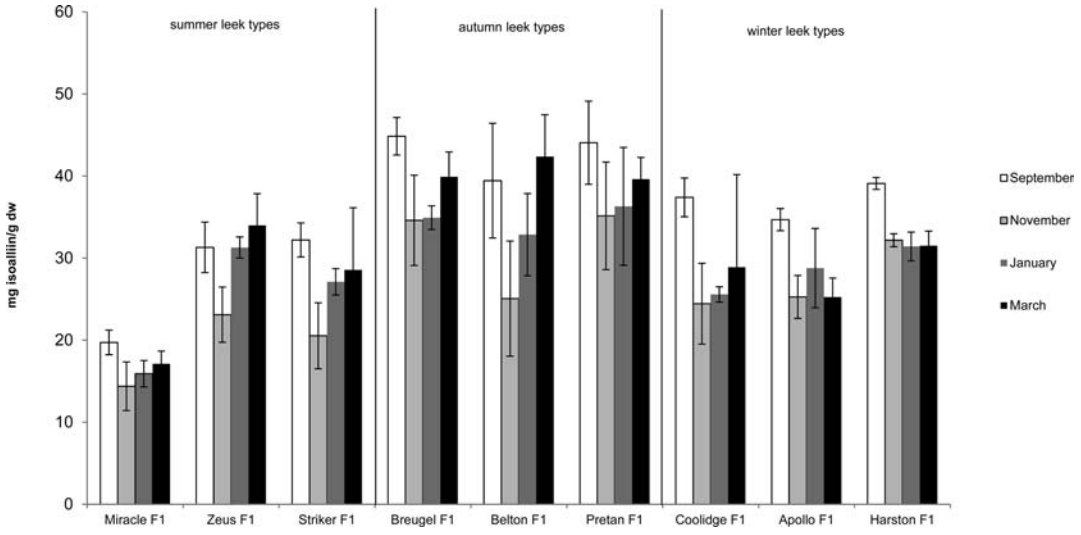


Figure 3. Isoalliin concentrations (mg/g dw) in the white shaft of nine leek cultivars harvested at four stages during their growth.

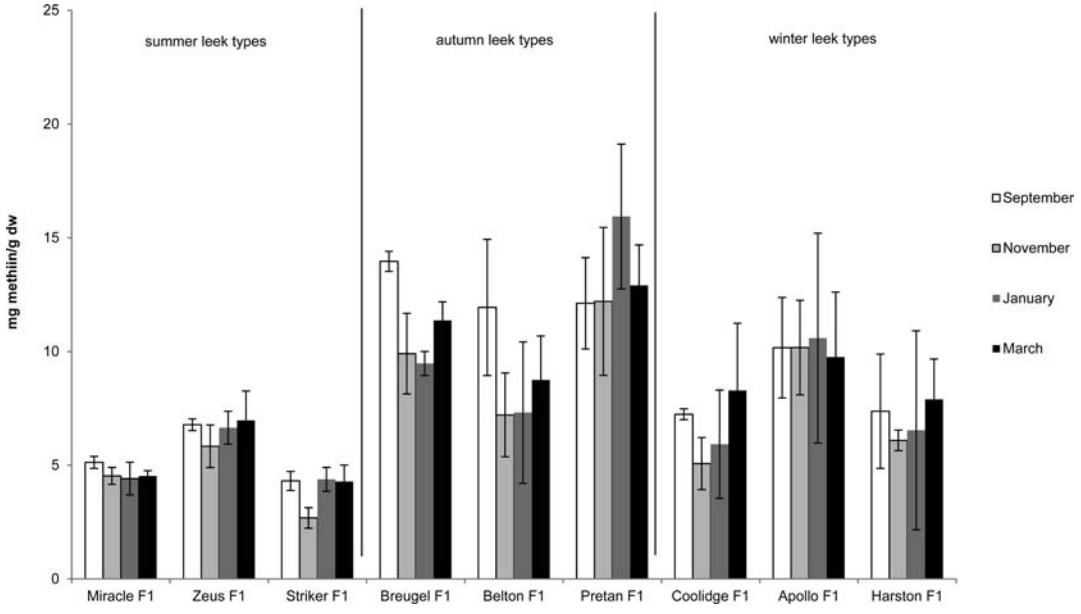


Figure 4. Methiin concentrations (mg/g dw) in the white shaft of nine leek cultivars harvested at four stages during their growth.

19.87, 25.35, and 39.15 mg/g dw, respectively. The green leaves of the winter cultivars contained a significantly higher ACSO concentration than the green leaves of the summer and autumn cultivars. In general, the whole leek plant of the winter cultivars contained a significantly higher amount of ACSOs. Similarly, the isoalliin concentration in onions sold during summer were remarkably lower than those sold during winter.[21] The increase of the ACSO concentration observed in the winter leek cultivars can be attributed to the role of these sulfur compounds in *Allium* plants, that is, defense against pests and predation, particularly in the overwintering bulb, and carbon, nitrogen, and sulfur storage and transport, which could result in the conversion of the corresponding γ-glutamyl dipeptides to sulfoxides, as observed in garlic.[32,40] Coley-Smith[41] hypothesized that plants obtaining the highest disease incidence among and within *Allium* species emit more organosulfur and/or different types of organosulfur compounds from their roots than the resistant plants. The different accumulation pattern of ACSOs between the summer, autumn, and winter leek types can also be explained by other environmental stress factors. Light radiation and water stress affect the biosynthesis of organosulfur compounds in onion.[42] In addition, the average growing temperature as well as the root zone temperature (RZT) could strongly affect the flavor composition of onion. However, an understanding of the influence of environmental factors and their interactions with agricultural practices in relation to ACSOs present in leek is still lacking.

The absolute concentration of these ACSOs is less important for their olfactory properties than their relative composition.[43] In this study, we found that isoalliin predominated in leek. It constituted 87% of the total ACSOs analyzed in the white shaft of the summer cultivars, 82% in the autumn cultivars and 80% in the winter cultivars. The same relative decreasing trend of isoalliin can be observed in the green leaves, 93% isoalliin in the summer cultivars, 88% in the autumn cultivars, and 87% in the winter cultivars. In the study of Lundegardh et al.[35] isoalliin constituted 92−96% of the ACSOs present in leek. In the study of Yamazaki et al.,[21] the molar ratio of isoalliin/methiin in the

edible portion of leek was 81:19 (%), whereas Yoo and Pike[30] found a ratio of 91.9:8.1 (%) in the leek leaves. Fritsch and Keusgen[44] reported a isoalliin/methiin ratio in leek of 79:13 (%) and also found 7% propiin. Yamazaki et al.[21] came to the conclusion that onion, Welsh onion, and leek generate similar flavors and result in an isoalliin/methiin/alliin ratio of 81−89:11−19:0 (%). On the basis of their research, Yoo and Pike[30] identified three distinctive *Allium* groups: the isoalliin, methiin, and alliin dominant groups. Leek belongs, along with onion, shallot, and bunching onion, to the isoalliin group. Species in this group contain no alliin or an undetectable amount of alliin. Garlic belongs to the alliin-dominant group, with an isoalliin/methiin/alliin ratio of 1:16:83 (%).[32]

Harvest Time. To determine whether the difference between the leek types (summer, autumn, winter) was attributed to the cultivar or time of harvest, ACSOs were also quantitated in nine leek hybrids at four different stages during the next growth season. The results of the isoalliin and methiin concentration in the white shaft during the growth season of nine leek cultivars are shown in Figures3 and 4, respectively. Cultivar and harvest time had a significant effect on the isoalliin and methiin concentrations (Table 6). Moreover, there was a significant interaction between the cultivars analyzed and the harvest time on the ACSO concentration.

Table 6. Analysis of Variance of Means Square for the Isoalliin and Methiin Concentrations of the White Shaft of Nine Leek Cultivars Harvested at Four Times

	isoalliin[a]	methiin[a]
cultivar (A)	1130.179*	221.883*
harvest time (B)	887.039*	28.601*
A × B	48.759*	9.342*
error	13.553	2.609

[a]*, significant at the 0.05 level.

The isoalliin concentration in the white shaft was significantly higher for all cultivars (except Pretan F1) when leek was harvested in September as compared with November harvest. For some of the cultivars (Miracle F1, Breugel F1, Apollo F1, and Harston F1), the isoalliin concentration was significantly higher when harvested in September in comparison with the other three harvest periods. For most of the cultivars, November harvest resulted in a significantly lower isoalliin concentration in the white shaft as compared with the plant material harvested at the other three harvest periods. The white shaft of the autumn cultivars contained in general the highest amount of isoalliin, irrespective of the time of harvest.

Next to the isoalliin concentration, also the methiin concentration in the white shaft was significantly higher for all cultivars (except for Zeus F1, Pretan F1, Apollo F1, and Harston F1) harvested in September in comparison with November. For some of the cultivars (Miracle F1, Breugel F1, and Belton F1), the methiin concentration was significantly higher when harvested in September in comparison with the other three harvest periods. November harvest resulted in a significantly lower methiin concentration in the white shaft of most of the cultivars in comparison with harvest at the other three periods. In general, the methiin concentration of the summer cultivars Miracle F1 and Striker F1 was significantly lower than those of the autumn and winter cultivars, irrespective of the date of harvest. The autumn cultivars contained the highest amount of methiin, followed by the winter cultivars.

In general, harvest in September 2010 resulted in the highest amount of ACSOs for each cultivar. As far as we know, no data have been published on the variation of ACSOs as related to growth stage in leek. However, in garlic, it was observed by Hornickova et al.[37] that the ACSO concentrations (alliin, methiin, and isoalliin) gradually decreased during the whole vegetation period (March until June) in the leaves and pseudostem, whereas the alliin and methiin concentrations in the bulbs of garlic initially decreased after planting but significantly increased in June and culminated before harvest. On the other hand, isoalliin levels steadily decreased during the whole vegetation period in all parts of the plants. Growth temperature can have an influence on the ACSO concentration. Like that, Coolong and Randle[33] reported a significantly higher total ACSO concentration in onions grown at high temperatures in comparison with ACSO concentration of onions grown at lower temperatures. In addition, it is documented that the average growing temperature as well as the RZT could strongly affect the flavor composition of onion. An RZT of 21 °C gave a higher ACSO concentration in onion in comparison with RZTs of either 12 or 34 °C.[45]

From the analysis of the nine leek cultivars as a function of harvest date, we can conclude that the autumn cultivars contain the highest amount of ACSO irrespective of the time of harvest. These results (season 2010−2011) do not support the results of the analysis as a function of the cultivars (season 2009−2010), where the winter cultivars contained the highest amount of ACSOs. Table 7 shows the Pearson correlation coefficients between the ACSO levels in the white shaft of seasons 2009−

Table 7. Pearson's Correlation Coefficients of the Isoalliin and Methiin Concentrations of the White Shaft of the Nine Cultivars of Seasons 2009−2010 and 2010−2011

	season 2009−2010	season 2010−2011 Sept	season 2010−2011 Nov[a]	season 2010−2011 Jan[a]	season 2010−2011 March[a]
season 2009−2010		0.25	0.44	0.28	0.12
		0.28	0.63	0.63	0.53
season 2010−2011 Sept			0.93*	0.90*	0.84*
			0.87*	0.74*	0.90*
season 2010−2011 Nov				0.87*	0.73*
				0.94*	0.93
season 2010−2011 Jan					0.91*
					0.90*

[a]*, significant correlation at $p < 0.05$.

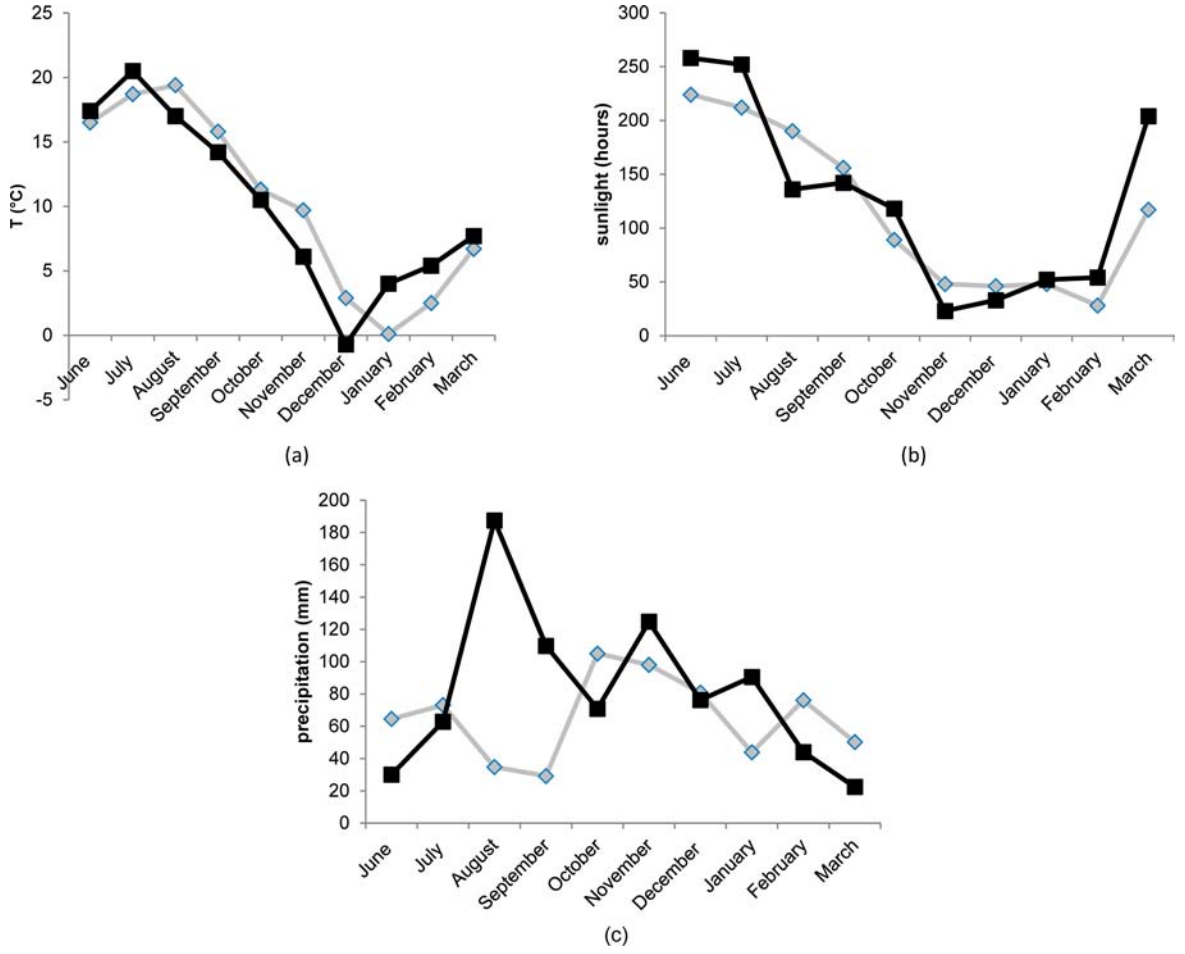


Figure 5. Average monthly temperature (a), hours of sunlight (b), and precipitation (c) for growing season 2009−2010 (gray curve) and 2010−2011 (black curve).

2010 and 2010−2011. The ACSO levels of the first growth period, although in the same range, were not correlated with the next growing season. However, the levels of season 2010−2011 at the four harvest dates were mutually correlated. As reported in the literature, the difference in ACSO concentration between the two seasons could be explained by environmental factors, including sulfur fertilization, nitrogen fertilization, and soil properties, which could be different for both leek growth locations.[11,19,42,46−48] Soil properties of the two leek fields before planting are given in Table 2. Sulfur analyses were not performed. The nitrate nitrogen was higher in season 2010−2011, whereas the other parameters were higher in season 2009−2010, which can have an influence on the ACSO amount. To eliminate these factors, the experiment should be repeated in the same locale. In addition, meteorological differences between the two growth seasons could also have contributed to the different results obtained. Figure 5 shows the differences in mean monthly temperature, sunlight hours, and precipitation for the region between the two growing seasons.[49] The mean monthly temperature in November and December was much lower in 2010−2011 in comparison with 2009−2010. This stress condition can be why the autumn cultivars of the second year contained more ACSOs. The results of the study of Hornickova et al.[32] indicate that the concentration of ACSOs in 52 garlic genotypes primarily depends on various genetic factors and postharvest storage conditions, whereas the climatic conditions (e.g., temperature, irrigation) during the growth influence their levels to a lesser extent. However, as already mentioned for the soil properties, further studies have to be performed to measure the effect of meteorological parameters in detail.

To cultivate leek with a high ACSO concentration, growers need to consider both growth conditions and genetic factors. Our results reveal that autumn and winter cultivars contain the highest ACSO concentrations, especially when harvested in September. Further studies will help to elucidate the combined effects of meteorological aspects, soil properties, genotype, harvest time, and production environment on ACSO concentration of leek and may lead to recommended practices to maximize the ACSO concentration.

■ ASSOCIATED CONTENT

Supporting Information

LC-MS/MS chromatogram of the extract of the white shaft of leek for the MRM of isoalliin (178.23 → 70.26, 88.01) and the MRM of methiin (152.15 → 70.08, 88.22). This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

R_A, apparent recovery; RSD_r, repeatability; RSD_R, reproducibility; LOD, limit of detection; LOQ, limit of quantitation; SSE, signal suppression/enhancement; HPLC-MS/MS, high-performance liquid chromatography−tandem mass spectrometry; S/N, signal-to-noise; ES, electrospray; MRM, multiple reaction monitoring; SRM, selection reaction monitoring; ACSO, *S*-alk(en)yl-L-cysteine sulfoxides; OCMHA, *O*-(carboxymethyl)-hydroxylamine hemihydrochloride; RZT, root zone temperature.

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