2102 *J. Agric. Food Chem.* **2008,** *56,* 2102–2111

JOURNAL OF
AGRICULTURAL AND
FOOD CHEMISTRY

Impact of Different Green Manures on the Content of *S*-Alk(en)yl-L-cysteine Sulfoxides and L-Ascorbic Acid in Leek (*Allium porrum*)

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This field study investigated the impact of various fertilization strategies with red clover (*Trifolium pratense* L.) green manure on the levels of *S*-alk(en)yl-L-cysteine sulfoxides (ACSO) and L-ascorbic acid in leek. Two of the 12 treatments were controls, one without fertilizers and the other with a commercial mineral fertilizer. The remaining 10 treatments were different forms and quantities of green manure prepared from red clover. One treatment consisted of direct incorporation into soil of the preceding red clover crop. The other 9 treatments comprised three types of red clover green manure [anaerobically digested red clover biomass (biodigestate), composted red clover, fresh red clover as mulch] applied at three different doses. Yield was increased only at the highest dose of compost and the highest dose of mulch. High doses of green manure decreased dry matter content in leek. The fertilizer treatments increased the nitrogen uptake and the nitrogen content of leek. Sulfur uptake and sulfur levels were increased only by the mineral fertilizer and by the compost. Nonfertilized leek contained 20.4 ± 5.8 g/kg of dry weight (dw) ACSOs as determined by LC-MS/MS and 1.57 ± 0.01 g/kg of dw ascorbic acid as determined by HPLC. The ACSOs were to 92–96% isoalliin, the rest being methiin. Alliin was identified in only 1 of 72 samples. The ACSO level was increased by 37% by the mineral fertilizer. Whereas direct incorporation of red clover, mulch, and red clover biodigestate had no influence on the ACSO level, the highest dose of compost increased the ACSO level by 55%. Ascorbic acid levels were not influenced by the mineral treatment. Green manures increased ascorbic acid levels only on a dry weight basis. A high correlation between the content of sulfur and ACSO indicated that delivering capacity of sulfur from the manure to the plant strongly affected the ASCO content of the leek. In conclusion, the composted green manure was the most useful organic fertilizer in this study and reached at least the efficiency of the mineral fertilizer.

KEYWORDS: ACSO; *S*-alk(en)yl-L-cysteine sulfoxides; *Allium porrum*; ascorbic acid; green manure; leek; mineral fertilizer; organic farming

INTRODUCTION

The genus *Allium* includes several hundred species, of which a few, such as garlic (*A. sativum*), wild garlic (*A. ursinum*), great-headed (elephant) garlic (*A. ampeloprasum* L. var. *holmense*), common onion (*A. cepa* L.), scallion (*A. fistulosum*), shallot chives (*A. schoenoprasum* L.), Chinese chives (*A. tuberosum* L.), and leek (*A. porrum*), are important food plants and are also used as drugs in folk medicine (for their antimicrobial, lipid lowering, cardiovascular, hypocholesteraemic, antitrombotic, hypoglycemic, and antitumorigenic activities) (*1*). 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 (*S*-alk(en)ylcysteine sufoxides; ACSOs). It has been estimated that around three-fourths of the sulfur in plants of the *Allium* family occurs in the form of ACSOs or the storage form γ-glutamyl-ACSOs (*2*). It is the ACSOs in these plants that are the precursors both to flavors and to the lachrymatory factor and other bioactive molecules (*1, 3*).

As availability of sulfur is important for onion plants (*4–6*), they are normally grown on soils with sufficient sulfur to support plant growth and flavor precursor accumulation (*7*). However, sulfur deficiency might become a reality for *Allium* crops in

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10.1021/jf071710s CCC: $40.75 © 2008 American Chemical Society
Published on Web 02/26/2008

the future in analogy with what has already happened worldwide for *Brassica* crops due to the use of sulfur-free fertilizers and efficient reduction of environmental sulfur pollution in industrial countries (*8*). Other minerals the availability of which could influence onion growth and quality include nitrogen (*9–11*).

The ACSO content of *Allium* crops is influenced by genetic (cultivar) and environmental factors (*12–16*). Such environmental factors include sulfur fertilization (*11, 13, 17, 18*), nitrogen fertilization (*11, 19*), soil properties and climate (*20*), and storage conditions of the harvested bulbs (*19*). Flavors and bioactive compounds are formed when ACSOs are cleaved by the endogenous enzymes allinase and lachrymatory factor synthase, respectively (*3*). Under normal conditions, in intact cells, ACSO degradation is fairly limited but it increases on cell destruction. The resulting aroma profiles are species-specific and depend on the precursor profile in the plant.

Six different ACSOs have been reported in alliums (*7, 21, 22*). They are *S*-methylcysteine sulfoxide (methiin), *S*-propylcysteine sulfoxide (propiin), *trans*-*S*-(1-propenyl)cysteine sulfoxide (isoalliin), *S*-(2-propenyl)cysteine sulfoxide (alliin), *S*-ethylcysteine sulfoxide (ethiin), and *S*-butylcysteine sulfoxide (butiin). Usually, not all of these compounds are found in each species. For example, alliin, which is the precursor for the characteristic garlic flavor, occurs in garlic and a few less common onion species only. The common onion, *Allium cepa*, instead, as the major ACSO, contains isoalliin, which is a precursor for the lachrymatory factor. Leek (*Allium porrum* L.), which has a mild onion flavor, contains a few of these ACSOs. Leaves and the long blanched stem of leek are usually eaten cooked, but leek can also be cut into thin slices and used in salads.

L-Ascorbic acid (ascorbic acid) is a water-soluble antioxidant of great importance for the living organism. The vitamin readily scavenges reactive oxygen species and reactive nitrogen species, in addition to singlet oxygen and hypochlorite. It also regenerates other antioxidants, such as vitamin E and glutathione. Plants and almost all animal species synthesize their own ascorbic acid, but humans cannot, and require 50–60 mg of this vitamin in the diet daily (*23*). This requirement can be satisfied by the consumption of fruits and vegetables.

This study formed part of the project "The Ecology of the Growing System—Green Manure as a Multifunctional Tool in Vegetable Production" and investigated the impact of various fertilization strategies using different types of red clover-based green manures on the levels of ascorbic acid and the ACSO flavor precursors as indicators of quality in leek. The agricultural aspects of the project, including mineral nutrient efficiency and availability, and soil microbial interactions are addressed elsewhere (*24–26*).

MATERIALS AND METHODS

Leek [*A. porrum* L., syn. *A. ampeloprasum porrum* ((L.) J. Gray)], cultivar Hilari, was grown in a moderately fertile (2% soil organic matter) loam with high silt content (approximately 65%) and a pH of 6.1 at Krusenberg, 60 km north of Stockholm, Sweden, in 2004.

Experimental Design. The experiment used a randomized complete block design including 12 different treatments and 4 replicates per treatment (**Table 1**). Two of the treatments were controls, one without fertilizers and the other with commercial mineral fertilizers. The remaining 10 treatments were different forms and quantities of green manure prepared from red clover (*Trifolium pratense* L. cv. Viva). One treatment consisted of direct incorporation of the preceding red clover crop into the soil. The other nine treatments comprised three types of red clover green manure [anaerobically digested red clover biomass (biodigestate), composted red clover, fresh red clover as mulch] applied at three different doses (I, II, III).

Table 1. Amount of Nutrients Supplied in the Different Treatments in the Form of Different Types of Green Manure or as Mineral Fertilizer

treatment	fertilization in relation to direct incorporation	dose (ton/ha)	C (kg/ha)	N (kg/ha)	S (kg/ha)
unfertilized		0	0	0	0
mineral fertilizer[a]	equivalent N and S dose		0	190	21
direct incorporation	reference treatment	58	3600	217	17
shoots			2341	153	8
stubble and roots			1260	65	9
biodigestate I	equivalent available N dose	45	826	119	10
biodigestate II	equivalent N dose	78	1431	206	16
biodigestate III	equivalent C dose	187	3432	493	38
compost I	equivalent N dose	15	1724	208	21
compost II	equivalent C dose	30	3448	416	42
compost III	equivalent available N dose	60	6896	832	84
mulch I	equivalent N and C dose	60	3649	224	11
mulch II	equivalent available N dose	110	6690	411	20
mulch III	area 4 times	229	13927	855	42

[a] Nitrate of lime 1336 kg/ha + PKS 7–25–3.8, 567 kg/ha.

For the control that received mineral fertilizer, nitrogen (N) and sulfur (S) doses were adjusted to give the same amounts of these minerals as was supplied by red clover in the direct incorporation treatment. Phosphorus (P) and potassium (K) were supplied in relation to the sulfur dose of the PKS fertilizer. The green manure doses were chosen in such a way that one experimental group per treatment contributed a total nitrogen dose equivalent to that of the direct incorporation treatment. Another dose was chosen to give the same amount of plant available nitrogen as that delivered by direct incorporation. The various treatments with green manure giving "equivalent available nitrogen dose" were calculated from analytical determinations of the amount of total nitrogen in the manures. In the calculation, template values for nitrogen availability in different organic green manures were used (35% for direct incorporation, 60% for biodigestate, 9% for compost, and 20% for mulch) to obtain information on the amount of nitrogen that may be available to the crop during the growing season (*23*). The third dose of the compost and biodigestate contained the same amount of carbon as that delivered by direct incorporation of red clover into soil. The third mulch dose, however, was chosen to represent a practically appropriate mulch application (*24*).

Production of Green Manures. The methods used for composting and production of biodigestate have been described elsewhere (*24*). Mulching material was harvested from a neighboring red clover field directly outside the experimental plot on the same day as the mulch treatments were applied.

Preceding Crops and Pretreatments. Before sowing of the preceding crop, the entire experimental field was fertilized with beef cattle slurry at a rate corresponding to 20 tonnes/ha. The preceding crops in the plots with the direct incorporation treatment were red clover (seed rate = 18–20 kg/ha) insown in barley (*Hordeum vulgare* L. cv. Cecilia, seed rate = 170 kg/ha), the latter cut at anthesis. Plots with other treatments used barley (seed rate = 200 kg/ha) grown to full ripeness alone as the preceding crop. The clover was incorporated into the soil on June 1 using a tractor-powered rotavator. All other pretreatments were autumn-plowed.

Treatments during the Field Study. The field study started with the planting of 8-week-old leek seedlings on June 15. Each experimental plot comprised 12.6 m^2 (3.5 m × 3.6 m) with leeks planted in seven rows (24 leeks per row) at 15 cm from each other and with 50 cm between rows. This design resulted in 168 leek plants per plot.

Half of the nitrogen [604 kg/ha nitrate of lime (15.5% N)] and PKS (535 kg/ha; 7% P−25% K−3.8% S) used in the mineral fertilizer control treatment was supplied as a starting dose (June 14) and the other half as a top dressing after 8 weeks (August 10). The two lowest doses of biodigestate were split into two applications (June 14, August 10), and the highest dose was split into four applications (June 14, July 14, August 10, and September 9). The fresh mulch, obtained from a neighboring field with a pure stand of red clover, was spread on the soil between the rows of leeks 2 weeks after planting (July 1). The compost was supplied as a single application in conjunction with planting (June 14). All fertilizers/green manures that were applied on June 14 were rotavated into the soil. Applications of biodigestate on other dates were hoed into the soil between the rows of leek. The experiment was irrigated at planting and when required by a 10 mm water deficit according to historical rainfall data of the locality and the general requirements of leek crops. The field trial was kept weed-free by hand clearing. The experiment was terminated on October 5.

Sampling of Green Manures. To determine the amounts of green manure to be supplied in the different treatments, samples of the different green manures were taken for chemical analysis shortly before they were to be applied in the field.

The green manure samples were analyzed for dry matter content, total carbon, and mineral nutrients. These chemical analyses showed that the biodigestate contained 57% mineral nitrogen in the form of NH_4-N, whereas the compost contained 7% mineral nitrogen, nearly all (6%) in the form of NO_3-N. These nitrogen doses could be compared with the 95% NO_3-N supplied by the mineral fertilizer. The amounts of nutrients (carbon, nitrogen, and sulfur) supplied in the form of different types of green manure and as mineral fertilizer are summarized in **Table 1**.

Sampling of Leek. The leeks were harvested after 16 weeks (October 5). Twenty plants from one row were pulled up with the roots intact and divided into two groups of 10 leeks each. Roots were trimmed directly in the field to a few millimeters in length. After the fresh leeks had been cleaned, one group was weighed (fresh weight), chopped into small pieces, and ground before analysis of dry matter content.

Five of the other 10 leeks were directly packed in paper bags/boxes and sent by air to the Institute of Chemical Technology in Prague, where they were analyzed for L-ascorbic acid and *S*-alk(en)ylcysteine sulfoxide contents. During storage and transport, samples were kept at 4–6 °C. Most samples were analyzed on the day after their arrival in Prague, but isolated samples were analyzed after 2–3 days of storage in the refrigerator.

The outer leaves and lower part of the leek plant (with the roots) were removed, and the remaining edible part was used for sample extraction. Each leek was divided into fourths along its length, and one-fourth from each leek was cut into smaller pieces, not more than 2 cm in length.

Chemical Analysis of Manure and Leek Constituents. *Chemicals.* Standards of methiin, alliin, and propiin were purchased from Prof. Velíšek, Prague, who had synthesized methiin and propiin according to the method of Theodoropulos (*27*) and alliin (>99% pure) according to the method of Stoll and Seebeck (*28*). Isoalliin was identified on the basis of mass spectra and quantified as alliin. L-Ascorbic acid (>99.9% pure) was purchased from PENTA, Czech Republic. The internal standard in the analysis for the ACSOs, DL-norleucine (>99.0% pure), was obtained from Fluka (USA). Stock solutions of the ACSO standards at 1 mg/mL were prepared in water and stored in the freezer at −20 °C until analysis. New standards of ascorbic acid were prepared for each set of analyses. Of other analytical chemicals, the alliinase inhibitor *O*-(carboxymethyl)hydroxylamine hemihydrochloride (>97% pure) was purchased from Fluka (Switzerland).

Analysis of Red Clover and Manure. Fresh red clover, dried red clover, and the various green manures (biodigestate, compost, and mulch) sampled at the time of spreading were analyzed for dry matter content and content of the minerals carbon, nitrogen (NO_3-N, NH_4-N), sulfur, potassium, phosphorus, magnesium, iron, copper, and zinc. Dry matter content was determined after drying in the oven at 105 °C. The carbon analyses were performed with a Leco analyzer (CN 2000) in accordance with the method of Dumas (*29*), whereas nitrogen was analyzed by the Kjeldahl method after pretreatment of the sample with salicylic acid and thiosulfate (*30*). Analyses of the other mineral nutrients were carried out using inductively coupled plasma emission spectrometry (ICP) after wet digestion with nitric acid (HNO_3). Ten milliliters of concentrated HNO_3 was added to 1 g of a dry ground sample in a 50 mL Kjeltec tube and kept at room temperature overnight. The sample was boiled for 6 h (1 h at 60 °C, 1 h at 100 °C, and the final 4 h at 125 °C), 5 mL of HNO_3 being added after 4 h of boiling. After hydrolysis, samples were diluted with distilled water to a total volume of 50 mL, filtered, and analyzed by ICP using a Perkin-Elmer Optima 3000 DV.

Table 2. MS Detector Setting for *S*-Alk(en)yl-L-cysteine Sulfoxide Determination

	methiin	alliin; isoalliin	propiin	norleucine
transition monitored (*m/z*)	152.0→88.0	178.0→88.0	180.0→88.0	132.0→86.0
isolation width (*m/z*)	1.0	1.0	1.0	1.0
capillary voltage (V)	38.5	2.5	6.5	26.0
discharge current (*μ*A)	5.0	0.5	0.5	5.0
activation amplitude (%)	20.0	20.0	20.0	30.0
activation Q	0.25	0.25	0.25	0.25
activation time (ms)	30.0	30.0	30.0	30.0

Analysis of Leek. In the case of ACSO analysis, a 25–35 g subsample of leek was mixed with 60 mL of *O*-(carboxymethyl) hydroxylamine hemihydrochloride (OCMHA; 1.1 g/L) and 2 mL of norleucine (5 g/L) and homogenized for 1 min. OCMHA was added to inhibit allinase activity. The subsamples for L-ascorbic acid analysis were directly homogenized for 1 min in 60 mL of 3% $(HPO_3)_n$. The homogenates were then filtered (Büchner funnel) and made up to 100 mL with 3% $(HPO_3)_n$. A smaller quantity of each semipurified homogenate was filtered through a 5 *μ*m microfilter (Rotilabo, PTFE, Karlsruhe, Germany) prior to analysis.

S-Alk(en)ylcysteine sulfoxides in leek extracts were determined in one run together with internal standard (DL-norleucine) determined by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) using an Agilent HP 1100 liquid chromatograph (Hewlett-Packard) with a mass spectrometry detector, Finnigan LCQ Deca (Finnigan) operated in selected reaction monitoring (SRM) mode. The analytical conditions of the liquid chromatography were a HyPurity Aquastar (250 mm × 4 mm; 5 *μ*m) (Thermo) column, with 2% acetic acid as mobile phase, having a flow rate of 0.8 mL/min at a column temperature of 20 °C. The injection volume was 1 *μ*L. The mass spectrometry conditions made use of an APCI interface with MS-MS detection mode, a positive ionization mode, a sheet gas flow rate of 90 arb, an aux gas flow rate of 10 arb, a capillary temperature of 155 °C, and a vaporizer temperature of 480 °C.

Table 2 summarizes the MS detector setting for ACSO determinations. For the identification of individual ACSOs in analyzed samples, retention time and selective transition *m/z* was used. For quantification of individual compounds from peak areas, external calibration based on ACSO matrix standards was used. The blank matrix was a leek extract not supplied with the inhibitor OCMHA until 1 h after extraction, but before analytical standards were added to the extract. The equations of the quadratic regression of calibration curves and correlation coefficients for individual ACSOs were as follows: $y = 284.47x^2 + 2 \times 10^6x - 2 \times 10^7$ for methinn with $R^2 = 0.9985$; $y = 754.38x^2 - 49822x + 628706$ for propiin with $R^2 = 1$; $y = 113.62x^2 + 126417x - 3 \times 10^6$ for alliin with $R^2 = 0.9928$; and $y = 98.219x^2 + 10^6x - 2 \times 10^7$ for norleucine with $R^2 = 0.9968$. The LC-MS/MS method used had limits of detection (LOD) of 10 mg/kg for methiin, 20 mg/kg for alliin and isoalliin, and 40 mg/kg for propiin. The relative standard

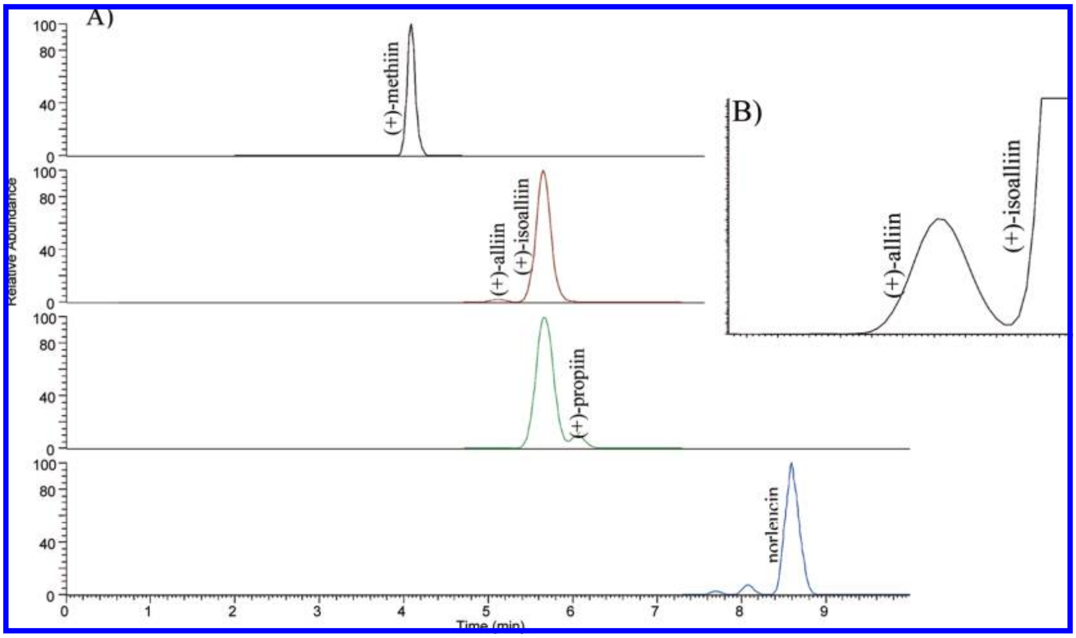

Figure 1. (**A**) LC-MS/MS chromatogram of a leek sample fertilized with biodigestate II; (**B**) zoom of the chromatogram in the region of traces of alliin.

Table 3. Yield (Fresh Weight), Dry Matter Content, and Average Amount (Grams per Kilogram of Dry Weight ± SD) of *S*-Alk(en)ylcysteine Sulfoxides (ACSOs) and L-Ascorbic Acid in Full-Grown Leek[a]

treatment	yield (ton/ha)	dry matter (%)	methiin[b] (g/kg)	propiin[b] (g/kg)	isoalliin[b] (g/kg)	alliin[b] (g/kg)	total amount of ACSOs (g/kg)	L-ascorbic acid (g/kg)
unfertized	44.1 ± 4.0 c	10.69 ± 0.40 a	1.3 ± 1.3 a	<0.04	19.1 ± 4.6 cd	<0.02	20.4 ± 5.8 cd	1.57 ± 0.010 cd
mineral fertilizer	45.8 ± 7.8 c	9.89 ± 0.77 abc	1.5 ± 0.5 a	<0.04	26.5 ± 2.8 ab	<0.02	28.0 ± 2.8 ab	1.74 ± 0.013 abcd
direct incorporation	47.5 ± 8.9 c	10.40 ± 0.69 a	1.5 ± 0.7 a	<0.04	17.6 ± 2.6 d	<0.02	19.2 ± 3.2 d	1.46 ± 0.009 d
biodigestate I	42.6 ± 5.1 c	10.30 ± 0.62 ab	1.6 ± 1.0 a	<0.04	21.6 ± 2.7 bcd	<0.02	23.2 ± 3.6 bcd	1.89 ± 0.027 abcd
biodigestate II	47.3 ± 3.8 c	10.03 ± 0.56 ab	1.1 ± 0.8 a	<0.04	21.0 ± 1.7 cd	<0.02	22.1 ± 1.5 cd	2.03 ± 0.028 abc
biodigestate III	45.5 ± 2.8 c	9.50 ± 0.66 bcd	1.5 ± 0.5 a	<0.04	23.5 ± 5.3 bc	<0.02	24.9 ± 5.1 bc	2.12 ± 0.036 abc
compost I	48.1 ± 3.5 bc	10.61 ± 0.42 a	1.7 ± 1.0 a	<0.04	20.2 ± 5.1 cd	<0.02	21.9 ± 6.1 cd	1.64 ± 0.029 bcd
compost II	44.9 ± 6.1 c	9.94 ± 0.80 ab	1.7 ± 0.8 a	<0.04	23.5 ± 3.8 bc	<0.02	25.2 ± 3.8 bc	2.23 ± 0.096 a
compost III	56.7 ± 3.1 ab	9.12 ± 0.82 cd	1.3 ± 0.3 a	<0.04	30.4 ± 6.8 a	<0.02	31.7 ± 6.7 a	2.28 ± 0.038 a
mulch I	48.3 ± 3.1 bc	9.55 ± 0.59 bcd	1.8 ± 1.6 a	<0.04	20.2 ± 2.4 cd	0.02[c]	22.0 ± 3.6 cd	1.86 ± 0.043 abcd
mulch II	50.1 ± 8.1 abc	8.87 ± 0.70 d	1.1 ± 0.5 a	<0.04	20.9 ± 2.7 cd	<0.02	22.0 ± 2.9 cd	2.15 ± 0.047 ab
mulch III	58.7 ± 1.0 a	9.00 ± 0.45 d	0.6 ± 0.5 a	<0.04	20.3 ± 1.5 cd	<0.02	20.9 ± 2.0 cd	2.00 ± 0.036 abcd

[a] Values bearing different letters are significantly different according to a *t* test (LSD) ($p < 0.05$). [b] Methiin, (+)-*S*-methyl-L-cysteine sulfoxide (MCSO); propiin, (+)-*S*-propyl-L-cysteine sulfoxide (PCSO); isoalliin, *trans*-(+)-*S*-(1-propenyl)-L-cysteine sulfoxide (PESCO); alliin, *S*-(2-propenyl)-L-cysteine sulfoxide (ACSO). [c] One of four samples contained 0.09 g of alliin/kg of fresh weight.

deviations (RSD) for methiin, alliin, isoalliin, and propiin were 4.0, 3.5, 3.5, and 3.6%, respectively. The first two were obtained from our leek experiment, the second two, due to the very low levels in leek, from garlic (alliin) and chive (propiin) samples, respectively.

L-Ascorbic acid content was determined separately using high-performance liquid chromatography (HPLC) with UV detection. Due to the limited stability of the analyte, analysis was carried out immediately after sample extraction. At analysis, a 5 μL test sample was injected into an Agilent HP 1100 liquid chromatograph with a diode array detector (DAD) at 251 nm. Separation was carried out on a LiChroCART column (250 mm × 4.6 mm; 5 μm), Lichrospher 100 RP-18, with a precolumn, LiChroCART (4 × 4 mm; 5 μm), and Lichrospher 100 RP-18 (Merck, Germany). The mobile phase was 5% methanol acidified with H_3PO_4 (pH 3). Flow rate was 0.8 mL/min and column temperature 35 °C (**Figure 1**).

Retention time and DAD spectra were used for identification of ascorbic acid in analyzed samples. External calibration was used for quantification, the equation of the linear regression of the calibration curve being $y = 14.697x - 5.177$, the correlation coefficient being $R^2 = 0.9988$; the response was linear within the range of 0.00025–0.6 mg/mL. The LOD of the analytical method was 1 mg/kg of fresh weight. The average recovery in three test samples was 89.7%, with a RSD of 8.6%.

Statistical Analyses. Factorial analysis of variance was performed, using the PROC glm-model in SAS. The model used data obtained from harvested leek to determine the effects of the various green manuring strategies on the quantity of the compounds analyzed (df = 47). A multivariate analysis of variance (MANOVA, df = 47) was performed to check for correlations between studied parameters.

RESULTS

The only green manuring strategies that influenced leek yield at 16 weeks of growth (harvest October 5) were the highest dose of compost and the highest dose of mulch, which both increased the yield (**Table 3**). There was no indication of sulfur or nitrogen deficiency in the harvested crop (**Table 4**). Compared with leek produced without fertilizer, the dry matter content of leek decreased with increasing nitrogen dose, giving

Table 4. Carbon, Nitrogen, and Sulfur Contents (in Dry Weight) in 16-Week-Old Leek Grown in Soils Given Different Forms of Organic Fertilizers[a]

treatment	C (g/kg of dw)	N (g/kg of dw)	S (g/kg of dw)
unfertilized	434.6 ab	18.7 f	3.36 de
mineral fertilizer	433.4 abc	27.5 a	4.79 a
direct incorporation	436.8 a	20.9 def	3.09 e
biodigestate I	436.9 a	21.9 cde	3.21 de
biodigestate II	432.5 abcd	22.5 cd	3.22 de
biodigestate III	427.8 d	27.2 a	3.70 cd
compost I	429.8 bcd	19.8 ef	3.28 de
compost II	429.2 cd	23.6 bc	4.05 bc
compost III	428.7 cd	25.9 ab	4.28 b
mulch I	432.0 abcd	20.6 def	3.01 e
mulch II	429.5 bcd	24.4 bc	3.39 de
mulch III	431.4 bcd	27.8 a	2.91 e
LSD	5.1	2.2	0.49

[a] Values bearing different letters are significantly different according to a *t* test (LSD) ($p < 0.05$).

a significant decrease in dry matter content at high doses of green manure (**Tables 1** and **3**). This was particularly evident for higher doses of mulch.

Levels of *S*-Alk(en)ylcysteine Sulfoxides in Leek. The influence of type and amount of green manure applied to the leek crop on its content of ACSOs (alliin, isoalliin, methiin, and propiin) and L-ascorbic acid when harvested full-grown are shown in **Table 3**. The nonfertilized leeks contained 20.4 ± 5.8 g of ACSOs/kg of dry weight. The mineral fertilizer resulted in a significant 37% increase in total amount of ACSOs, whereas direct incorporation of the previous red clover crop had no influence on the ACSO content of leeks. Of the three surface-applied green manures, the mulch and the red clover biodigestate had no influence on the ACSO level, whereas ACSO content increased with increasing dose of compost. The highest dose of compost resulted in the highest ACSO levels observed after any of the manuring strategies tested in this study, the increase (55%) being slightly higher than that produced by the mineral fertilizer. A significantly increased ACSO level in leek given mineral fertilizer or the highest dose of compost was evident also when the data were expressed on a fresh weight basis (**Figure 2A**).

A more detailed study of the various ACSOs showed that 92–96% of the total amount of ACSOs in full-grown (16-week) leek was isoalliin. Nearly all of the remaining ACSO was methiin (4–8% of the total ACSO content). Alliin levels were frequently below the LOD (0.02 g/kg of fw). In some samples traces below the limit of quantification (LOQ; 0.06 g/kg of fw) were found, and in 1 of the 72 samples quantifiable amounts were detected (0.09 g/kg of fw). Traces of propiin between the LOD (0.04 g/kg of fw) and LOQ (0.12 g/kg of fw) were found in some samples, but levels were never high enough to be quantified. Because of the dominance of isoalliin, the influence of the various green manuring treatments on this ACSO paralleled the influence of the various manures on the total amount of ACSOs. It was noted that the content of methiin in leeks receiving the highest mulch dose was reduced to 3% of the total ACSO content, resulting in an increased isoalliin/methiin ratio (data not shown).

Levels of L-Ascorbic Acid in Leek. In control plots, leek contained on average 1.57 ± 0.01 g of ascorbic acid/kg of dry weight, with a fairly modest variation in ascorbic acid level between samples (**Table 3**). The high standard deviation in compost II was due to one of the four samples containing much higher levels of ascorbic acid than all other samples. Outlier analysis did not allow this sample to be excluded. The mineral fertilizer had no influence on ascorbic acid level. In relation to unfertilized leek, composts II and III had a significantly higher ascorbic acid level. Compared with direct incorporated red clover, high levels of processed green manure (biodigestate and compost) resulted in increased ascorbic acid content when measured on a dry weight basis. However, the latter differences in ascorbic acid level at high green manure levels were observed only for compost II when the amount was expressed on a fresh weight basis (**Figure 2B**). This difference was probably due to a lower dry matter content in leek treated with higher green manure levels (**Table 3**), attested by a significant negative correlation ($r = -0.466$; $p = 0.0009$) between dry matter content and ascorbic acid content on a dry weight basis (**Figure 3B**).

Interaction between Mineral Status of Leek and Its Content of ACSOs and Ascorbic Acid. The three doses of each form of green manure supplied different amounts of carbon, nitrogen, and sulfur to the leek crop (**Table 1**) and resulted in dose-dependent increases in the nitrogen content of the leek (**Table 4**). The total sulfur content of leek increased with increasing dose of biodigestate and compost, but the increase was significant only for compost. The different doses of mulch had no influence on the sulfur content of leek.

In comparison to unfertilized control, leek that had been given a mineral fertilizer showed increased contents of nitrogen and sulfur. Green manure treatments that resulted in total nitrogen doses similar to that of the mineral fertilizer (direct incorporation, biodigestate II, compost I, and mulch I) had no influence on the carbon, nitrogen, and sulfur contents of the leek compared with unfertilized leek except for biodigestate II, which increased the nitrogen content.

Figure 3 shows the correlation between total ACSOs and ascorbic acid content of leek and the total content of carbon (**Figure 3A**,**B**), nitrogen (**Figure 3C**,**D**), and sulfur (**Figure 3E**,**F**). There was a strong correlation between the sulfur and total ACSO content in leek, whereas a weaker correlation was found for the nitrogen and ascorbic acid contents of leek, the nitrogen and ACSO contents of leek, and the carbon and ascorbic acid contents of leek. The level of none of these compounds or minerals showed any significant correlation with yield (data not shown).

As shown in **Figure 4A**, all green manuring strategies improved total uptake of nitrogen per hectare (nitrogen yield) in full-grown leeks, but the effect was statistically significant only for the mineral fertilizer and the highest dose of the three green manures (biodigestate III, compost III, and mulch III). The green manures had a more modest influence on the total uptake of sulfur per hectare (**Figure 4B**). It was only the mineral fertilizer and the highest dose of compost that significantly increased sulfur yield compared with the control.

DISCUSSION

There are very few data in the literature on ACSO levels in alliums produced in controlled field studies. Most data are from products purchased in the market or harvested from experimental greenhouse studies. In the latter, onions and garlic were frequently grown in sand and given sulfur and nitrogen in the form of a nutrient solution. This experimental situation thus differs significantly from that of field studies. In our case, leeks

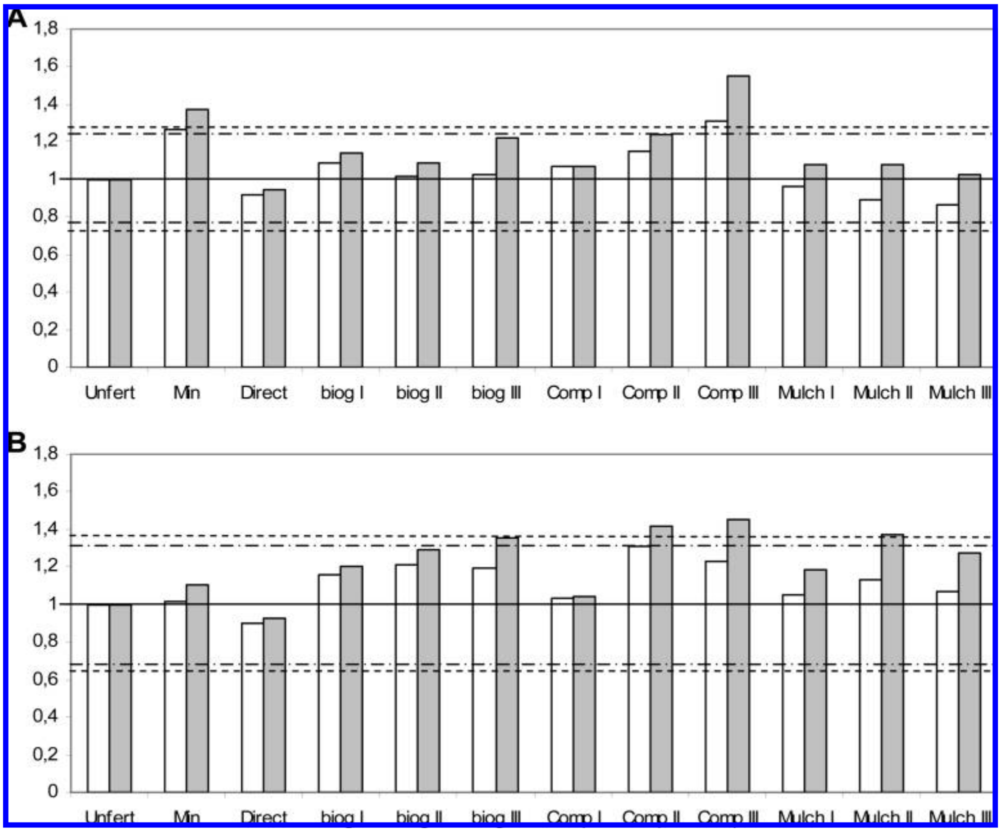

Figure 2. Relative amounts of ACSO (**A**) and ascorbic acid (**B**) in leek under various applied fertilization regimens as compared to amounts in unfertilized (Unfert) leek. Open columns shows amounts expressed per kilogram of fresh weight and shaded columns amounts per kilogram of dry weight. The broken lines show the 1 ± LSD level for fresh weight (alternating dot-dashed line) and dry weight (dashed line) in the unfertilized leek. Bars that are higher than the upper levels are significantly different from unfertilized at the 5% level: Unfert, unfertilized; Min, mineral fertilizer; Direct, direct incorporation; biog, biodigestate; Comp, compost.

were grown in the field under normal agricultural conditions, using various forms and doses of green manures with well-defined sulfur and nitrogen composition. The field-grown leek crop therefore characterizes typical products available for consumers.

ACSO Content of Field-Grown Leek. To our knowledge, this study is the first time an LC-MS/MS method has been used for determination of ACSOs in *Allium* species. The successful quantification of the individual ACSOs required the inclusion of an efficient inhibitor of *Allium* allinase in the extraction fluid (*31*). Without this inhibitor [*O*-(carboxymethyl)hydroxylamine hemihydrochloride], the ACSOs would quickly have been degraded, primarily to thiosulfinates, which upon mild heating or even at room temperature decompose by disproportionation to form symmetrical and mixed mono-, di-, and trisulfides, as well as sulfur dioxide (*32*). It is these degradation products that are responsible for the typical aroma and flavor of onions (*33–35*).

Three of the eight published studies devoted to this class of sulfur constituents have deduced the ACSO composition of leek from gas chromatographic or HPLC data on the degradation products and drew the wrong conclusion that propiin is the major ACSO in leek (*36–38*). A fourth study found substantial amounts of propiin, around one-third of the total amount of ACSOs (*35*), but these investigators pointed out that the detection of propiin in leek is most likely an artifact caused during extraction and analysis of the ACSOs (*34, 35*), confirming the suspicions of other investigators (*38–40*).

We found leek to contain isoalliin and methiin and, possibly, traces of alliin. Our findings are supported by the results of three other research teams, whose data are presented in **Table 5** (*21, 40, 41*). In contrast to our study, which analyzed leeks grown with various forms of organic manure, all earlier studies analyzed conventionally grown leeks. The data show that ripe leek contains between 1 and 2.8 g of total ACSO/kg of fresh weight. This means that leeks contain less ACSOs than most other types of onions, as amounts between 0.2 and 117.5 g/kg of fresh weight have been reported for various other *Allium* species (*41, 42*). We found that isoalliin predominated, constituting 92–96% of the total ACSOs, the rest being methiin, an ACSO that has been found in all *Allium* species analyzed. Other investigators have made similar observations, but reported less pronounced dominance of isoalliin (**Table 5**). Quantifiable levels of alliin were found in only 1 of 72 samples in our study, whereas propiin occurred only in traces between the limits of detection (40 mg/kg of fw) and quantification in some samples. Although we did not analyze for ethiin in any of the test samples, we analyzed for this compound in leeks purchased at the local market in Prague, using an analytical method without standard but with improved detection limits. We found no ethiin in this purchased sample, nor did we find any selenium analogues to ACSOs (the S in ACSOs being substituted by Se), but in this case our detection limit was rather poor because we had no standard and the selenium/sulfur ratio is unfavorable in leek.

Green Manure Effects on Leek Yield and Quality. The composition and concentration of plant secondary metabolites were determined by the genetic predisposition, but they are also very much influenced by environmental factors. Fertilization strategy during cultivation is one of these environmental factors,

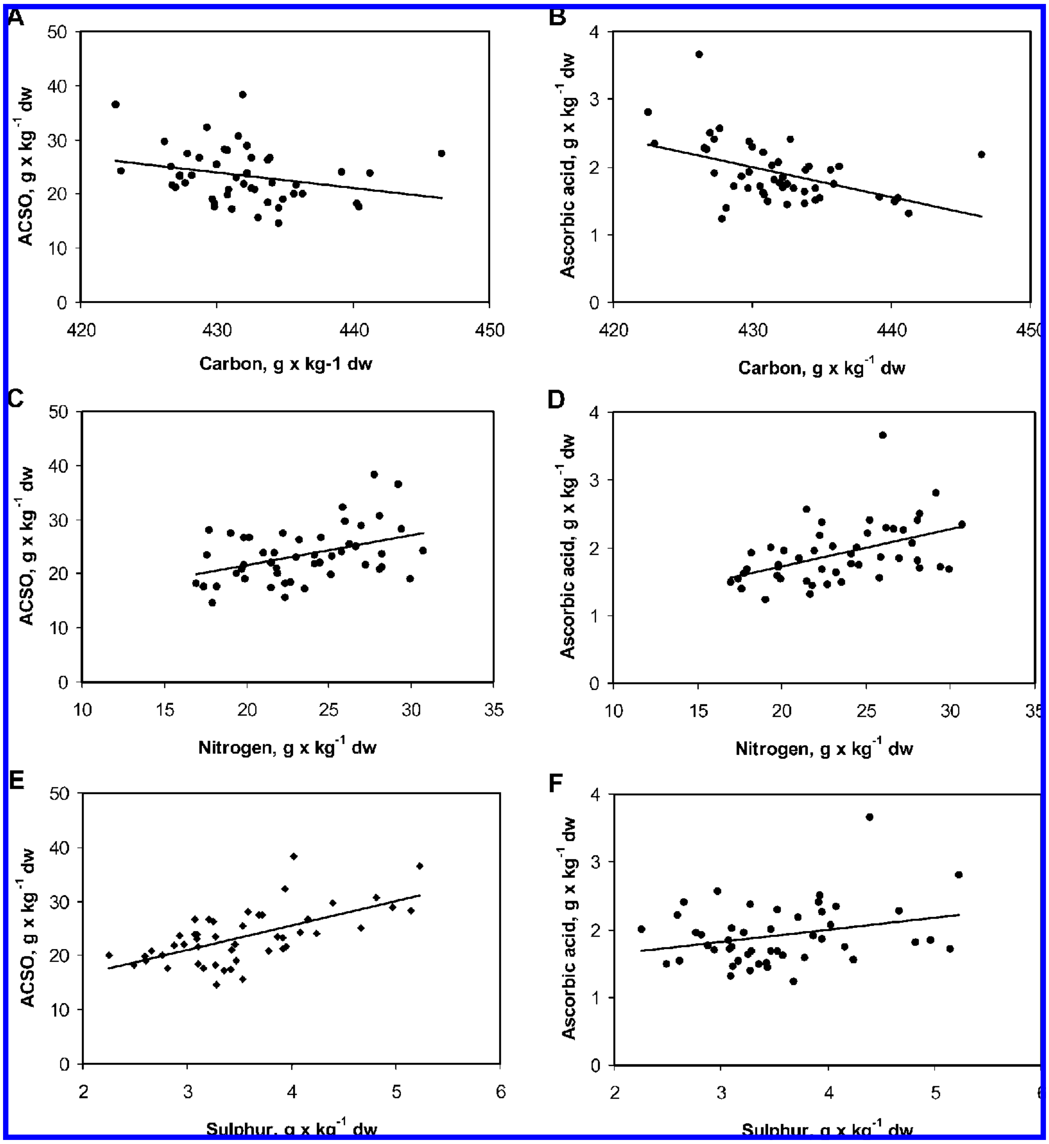

Figure 3. Correlation between the content of carbon (**A**, **B**), nitrogen (**C**, **D**), or sulfur (**E**, **F**) and the content of ASCOs (**A**, **C**, **E**) or L-ascorbic acid (**B**, **D**, **F**) in full-grown leek treated with different fertilizers. Correlation coefficients: (**A**) $r = 0.2674$ ($p = 0.0661$); (**B**) $r = 0.4584$ ($p = 0.0010$); (**C**) $r = 0.4196$ ($p = 0.0030$); (**D**) $r = 0.4611$ ($p = 0.0010$); (**E**) $r = 0.6264$ ($p = 0.0001$); (**F**) $r = 0.2821$ ($p = 0.0520$).

being important not only for yield but also for the quality as determined by the quantity and/or structure of plant components. In the present study, yield increased with dose of green manure, presumably as a consequence of the increased uptake of nitrogen (**Figure 4A**). The exception was biodigestate, which showed no dose response on yield. This could be due to loss of ammonium by emission during the season, as well as a high content of fatty acids influencing growth (*44*). However, mineral nitrogen fertilization did not affect the leek yield. It has been reported that drought stress during cultivation depresses plant growth, nitrogen uptake, and nitrogen uptake efficiency in leek (*45*) and that good water supply increases the influence of nitrogen on production of onion bulbs (*46*). In the present study, the weather was dry during a large part of the growing season, which might have influenced leek growth. As the mulch was able to keep the humidity in soil, this green manure treatment was least influenced by the drought conditions and produced the highest leek yield of all the green manures tested. However, mulch also resulted in the lowest dry matter content.

The various green manure treatments influenced L-ascorbic acid content less than they did the ACSO content. When carbon content increased, a slight reduction in ascorbic acid was noted (**Figure 3B**). The low level of ascorbic acid in mineral-fertilized leeks may have been due to the late application, with half of the nitrogen fertilizer applied 8 weeks after planting. This late supply may have given the leek crop a nitrogen boost, leading to strong growth and a decrease in ascorbic acid content compared with leek given green manure. In general, high N mineral fertilization is known to stimulate N uptake and reduce the content of ascorbic acid (*47*). In our study, this was not observed after the green manure treatments, indicating a more even uptake of N corresponding to the crop N requirements. Due to the decreased dry matter content in green manure-treated leek, the differences in the content of ascorbic acid between mineral fertilized or unfertilized leek and above-ground green manure fertilized leek were more pronounced when the content was expressed on a dry weight basis than on a fresh weight basis (**Figure 2B**).

In sulfur-requiring crops, sulfur is commonly bound in a set of sulfur-containing compounds. Because of the majority of sulfur being present in specific compounds, there is frequently a good correlation between sulfur content in *Allium* plants and the level of these constituents (*2, 6, 17*). Our data confirm that

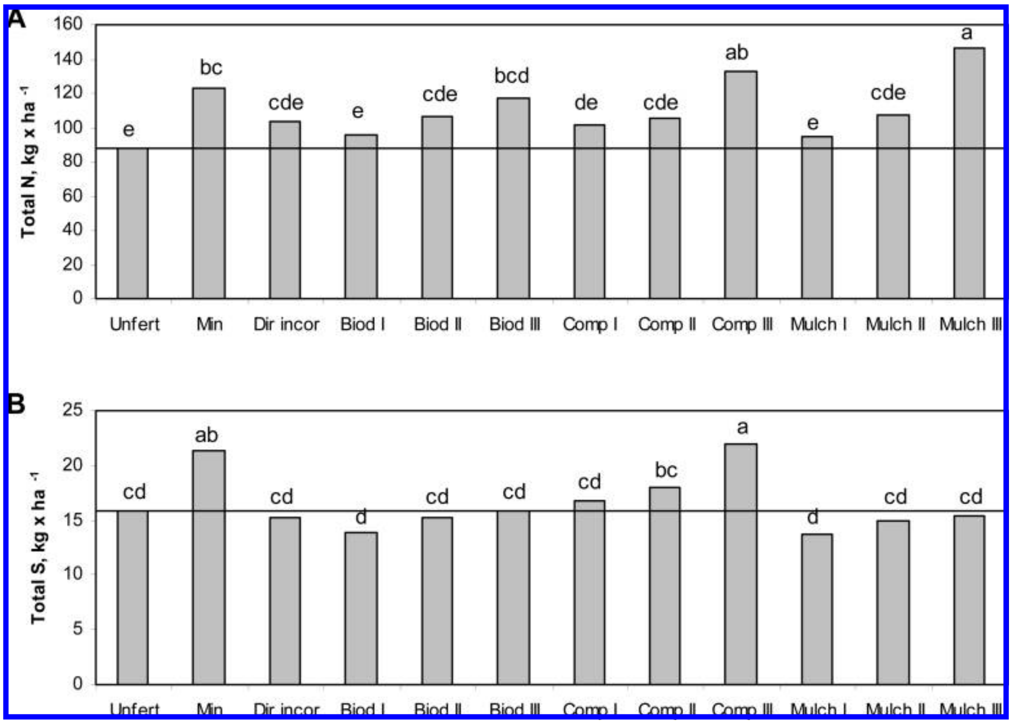

Figure 4. Total yield (kilograms per hectare) of nitrogen (**A**) and sulfur (**B**) in full-grown leek supplied with various fertilizers. The horizontal line represents nitrogen and sulfur yield of control unfertilized leek. Unfert, unfertilized; Min, mineral fertilizer; Dir incor, directed incorporation; Biod, biodigestate; Comp, compost.

Table 5. Amount and Distribution of Isoalliin, Methiin, Propiin, and Alliin in Leek (*Allium porrum*)

ref	isoalliin (g/kg of fresh wt)	methiin (g/kg of fresh wt)	propiin (g/kg of fresh wt)	alliin (g/kg of fresh wt)
present study, organic leek	1.82–2.75 (92–96%)	0.06–0.18 (4–8%)	nd[d]	traces
present study, conventional leek	2.61 (95%)	0.15 (5%)	nd	nd
Thomas and Parkin, 1994 (HPLC)[a]	0.75 (73%)	0.28 (27%)		nd
Yoo and Pike, 1998 (HPLC)[b]	2.28 (92%)	0.19 (8%)	nd	nd
Kubec et al., 2000 (GC)[c]	0.18 (81%)	0.04 (19%)	traces	traces

[a] Leek bulbs. [b] *A. ampeloprasum*, leaf. [c] *A. porrum*, stem; found traces of *S*-ethyl-L-cysteine sulfoxide (ethiin). [d] Not detected.

this is also the situation in leek (**Figure 3E**). ACSO levels in leek were better correlated to the sulfur content than to the nitrogen content. This has also been observed in studies on other alliums (*2*). One reason for nitrogen being less well correlated to ACSO level than sulfur could be that nitrogen more strongly stimulates methiin synthesis than isoalliin synthesis (*11, 48*). As isoalliin predominates in leek, a change in methiin level will not have any strong influence on the ACSO level compared with a change in isoalliin level (**Table 3**). Hence, the availability of sulfur may be the main factor affecting ACSO content in leek. Although the humidity was high in the mulched soil, the uptake of sulfur was poor (**Figure 4B**), probably due to a high C/S ratio in the mulch manure (**Table 1**), as a high C/S ratio results in a strong immobilization of sulfur (*49*). As a consequence, the leeks from these treatments contained low levels of sulfur, resulting in a low content of ACSOs (**Table 3**, **Figure 2A**). The compost and mineral fertilizer resulted in the highest sulfur uptake and the highest ACSO content (**Table 3**, **Figure 2A**).

In conclusion, a high supply of compost resulted in the highest levels of ACSO and ascorbic acid in leek. This was the consequence of a better sulfur delivery from compost to leek than from the other fertilizers. In the harvested crop the treatments with unprocessed green manure (direct incorporation and mulch) and with biodigestate were less efficient at delivering sulfur to the leek crop. As compost delivered sulfur favorably and to some extent maintained soil humidity, this form of green manure seemed to be most useful of the organic fertilizers studied.

ABBREVIATIONS USED

ACSO, *S*-alk(en)yl-L-cysteine sulfoxide; METHIIN, (+)-*S*-methyl-L-cysteine sulfoxide; PROPIIN, (+)-*S*-propyl-L-cysteine sulfoxide; ISOALLIN, *trans*-(+)-*S*-(1-propenyl)-L-cysteine sulfoxide; ALLIN, *S*-(2-propenyl)-L-cysteine sulfoxide; BUTIIN, *S*-butylcysteine sulfoxide; OCMHA, *O*-(carboxymethyl)hydroxylamine hemihydrochloride; RSD, relative standard deviation.

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Received for review June 11, 2007. Revised manuscript received December 4, 2007. Accepted January 8, 2008. We thank the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning, the Swedish Farmers' Foundation for Agricultural Research, the National Food Administration in Sweden, and the Ministry of Education, Youth and Sports, Czech Republic (MSM 6046137305 and COST OC924) for financial support.

JF071710S