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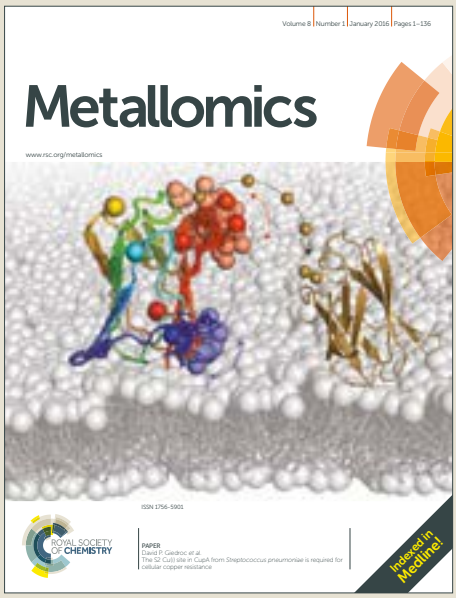

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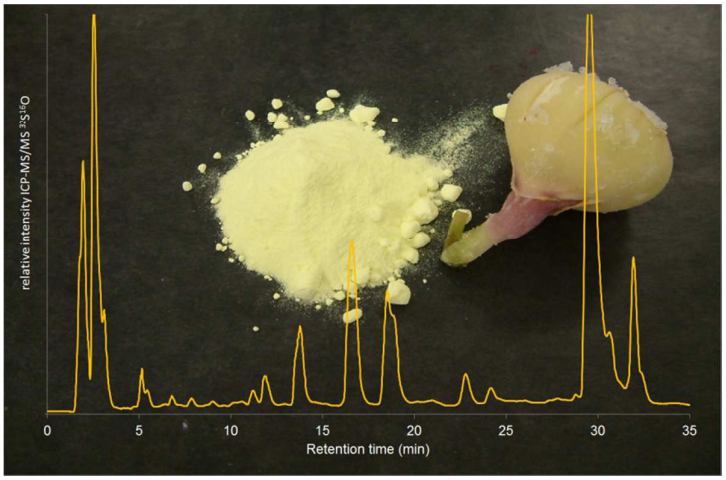
Comprehensive non-target analysis identifies 54 sulphur containing compounds in garlic.



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Sulphur fertilization influences the sulphur species composition in *Allium sativum:* sulphomics using HPLC-ICPMS/MS-ESI-MS/MS

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Abstract

Garlic (*A. sativum*) contains a large number of small sulphur (S)-containing metabolites, which are important for its taste and smell and vary with *A. sativum* variety and growth conditions.

This study was designed to investigate the influence of different sulphur-fertilization regimes on the low molecular weight S-species by attempting the first sulphur mass balance in *A. sativum* roots and bulbs using HPLC-ICPMS/MS-ESI-MS/MS.

Species unspecific quantification of acid soluble S-containing metabolites was achieved using HPLC-ICP-MS/MS. For identification of the compounds high resolution ESI-MS (Orbitrap LTQ and q-TOF) was used.

The plants contained up to 54 separated sulphur-containing compounds, which constitute about 80 % of the total sulphur present in *A. sativum*. Roots and bulbs of *A.sativum* contained the same compounds, but not necessarily the same amounts and proportions. The S-containing metabolites in the roots reacted more sensitive to manipulations of sulphur fertilization than those compounds in the bulbs. In addition to known compounds (eg. γ-glutamyl-S-1-propenylcysteine) we were able to identify and partially quantify 31 compounds. Three as yet undescribed S-containing compounds were also identified and quantified for the first time. Putative structures were assigned to the oxidised forms of S-1-propenylmercaptoglutathione, S-2-propenylmercaptoglutathione, S-allyl/propenyl-containing PC-2 and 2-amino-3-[(2-carboxypropyl)sulfanyl]propanoic acid.

The parallel use of ICP-MS/MS as sulphur-specific detector and ESI-MS as molecular detector simplifies the identification and quantification of sulphur containing metabolites without species specific standards. This non-target analysis approach enables a mass balance approach and identifies the occurrence of so far unidentified organosulphur compounds. The experiments showed that the sulphur-fertilization regime does not influence sulphur-speciation, but the concentration of some S-containing compounds in roots is depending on the sulphur fertilization.

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Significance to Metallomics

Sulphur is not a metal, but the similarity to Se which is featured in the journal should make an S-based study eligible. The multitude of S-containing metabolites in allium is difficult to quantify using traditional methods. We developed a species independent quantification method coupled with simultaneous identification using HPLC-ICPMS/ESI-MS to give a holistic (sulphomic) view on the acid soluble low molecular weight S-metabolites. This is in the spirit of non-metal non target speciation analysis as laid out recently.[1]

Introduction

A wide variety of *Allium sativum* (garlic) is cultivated worldwide for use as spice in the kitchen and for their medical properties.[2] Like all alliums garlic contains a variety of volatile and non-volatile sulphur (S) containing metabolites, which are mainly responsible for its typical smell and taste. Several sulphur containing phytochemicals present in *A. sativum* show at least *in vitro* medical properties, namely alliin and allicin,[3] supporting the use of garlic in traditional medicine. Suggestions of health benefits resulting from consumption of garlic range from reducing the risk of coronary heart disease to anti-cancer properties.[4,5] Allicin, the major volatile S-species produced by crushing garlic and first identified in 1944 by Cavallito, was shown to have significant bacteriostatic activity *in vitro*.[2] Clinical trials have, however, so far failed to show conclusive evidence for significant health benefits.[6]

Research in sulphur-containing compounds (S-containing compounds) of garlic focuses predominantly on alliin, allicin and some of their major derivatives. The presence of some di- and tripeptides of the γ-glutamyl cysteinyl family containing an S-allyl or S-propenyl moiety is known. The best known are γ-glutamyl-S-allyl-L-cysteine (GSAC), γ-glutamyl-S-1-propenyl-L-cysteine (GSPC) and γ-L-glutamyl-S-methyl-L-cysteine (GSMC). [5,7,8] Whether they are precursors and / or sulphur storage peptides, especially during favourable growing conditions, for the eventual formation of alliin and allicin, is one example of what is not known in the biosynthesis of S-containing molecules.

The main reasons for this lack of knowledge are the use of unspecific analytical techniques for the determination of non-volatile S-containing compounds; mainly HPLC-UV with quantification & identification of compounds by synthetic standards is used. Rarely ESI-MS is used for identification of S-containing compounds in garlic. Quantification is always done using synthesised standard compounds.[8,7] When ESI-MS is used for identification of unknown S-containing compounds high accuracy not only with regard to *m/z*, but also the isotopic pattern is required. The latter is important, since the sulphur isotopic pattern is not very different from the isotopic pattern contributed by carbon, oxygen, nitrogen and hydrogen present in the

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compound. ^{32}S constitutes about 95 % of the present sulphur with ^{33}S contributing about 0.8 %, ^{34}S 4.2% and ^{36}S 0.02%. Sulphur has also a small mass-deficiency, a compound of similar composition (m: 305 g mol^{-1}) containing one sulphur atom is about 0.0905 g mol^{-1} lighter than a compound containing only C,N,O and H. Both mass defect and isotope pattern shift can be applied to identify unknown compounds when using high-resolution accurate MS instruments.[9] But a non-target analysis with mass balance approach is not possible using molecular mass spectrometry.

The aim of this feasibility study was to test firstly whether the parallel use of ICP-MS/MS and ESI-MS is advantageous for quantification and identification of S-containing compounds in roots and bulbs of *A. sativum* without the use of species specific standards. Secondly we applied this approach to study the influence of the levels of sulphur fertilization on the generation of the different S-containing compounds and calculated a complete sulphur mass balance (sulphomics).

Material and methods

Chemicals and Standards

MilliQ water (18 MΩ cm, Millipore UK) was used throughout for the preparation of every solution except growing of *A. sativum*. Other chemicals (hydrogen peroxide, concentrated nitric acid, cysteine, formic acid) were of at least p.a. quality (all from Sigma, UK), methanol was of HPLC-grade (Sigma, UK). The sulphur standard for total sulphur determination and the germanium solution used as continuous internal standard were from High Purity Standards (USA). As certified reference material (CRM) for total S determination RM 8415 (whole egg powder, NIST, Gaithersburg, USA) and Seronorm urine blank (Sero, Norway) were used. No CRM for sulphur-speciation was available.

Plants

A. sativum (single bulb garlic of Chinese origin) was bought in a shop in Aberdeen, UK and grown hydroponically for 6 weeks at 19 +/- 1ºC with ambient lighting. Each bulb was individually grown in a plastic beaker. The plants were fertilized using Hoagland's solution (20 % strength) with the sulphur levels adapted by either replacing the sulphur containing salts from Hoagland-solution by chloride containing salts or by adding increased levels of magnesium sulphate (see details ESI). The solutions were replaced 3 times per week. Sulphur levels at which the plants were grown for 6 weeks were 0.1, 0.5 and 2 mM sulphate, 20 bulbs were grown on each level and 4 or 5 plants randomly selected for sampling in two separate (8 weeks difference in planting), but otherwise identical experiments. Dry weight (d.w.) of plant parts was determined by freeze drying. Roots contained 9.9 ± 0.5 % dry matter and bulbs 24 ± 2.7 %.

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Sample preparation

The plants were harvested after 6 weeks. Their roots rinsed with deionised water and blotted dry. Roots and shoot were separated from the bulb. Only freshly formed (during the 6 weeks of the experiment) bulb tissue was used. Roots and bulb were separately grounded to fine powder using liquid nitrogen as soon as separated. Material intended for species determination was kept frozen with liquid nitrogen until extraction with 1 % (v/v) formic acid in water in an ice bath (~ 1ºC) for 15 min (1 g plant / 4 mL extraction solution). The extract was centrifuged and the supernatant was used immediately for speciation analysis. Formic acid was used to suppress allinase (EC 4.4.1.4) activity which is irreversibly inhibited at pH below 3.5, as described by Ichikawa *et al*.[7]

For total sulphur determination the plant material and reference materials (100 ± 0.1 mg) was digested using 1 mL nitric acid and 2 mL hydrogen peroxide in a microwave oven (Mars5, CEM) for 30 min at 95ºC in unpressurised vessels. The digests were diluted with water to 50 g before sulphur determination. Extracts prepared for speciation analysis were diluted 1 to 50 with 1 % nitric acid for determination of total extracted sulphur.

Instrumentation

Species separation

An Agilent 1100 HPLC or a 1290 HPLC system with cooled autosampler was used for separation. The extract was separated using an Agilent Eclipse C18 column (4.6 * 150 mm) with a linear water methanol gradient (both 0.1 % v/v formic acid) in 20 min to 20 % methanol and held for 10 min. The flow rate was 1 mL min^{-1}, after the column the flow was split 1:3 with 1 part introduced into the ICP-MS/MS and the rest into the ESI-MS (QuickSplit Post-column Flow splitter, Analytical Scientific Instruments, USA). The sample volume was 0.1 mL and the column oven set to 40ºC.

Quantification and S-specific detection using ICP-MS/MS

An 8800 Agilent ICP-MS/MS was used for all measurements in MS/MS mode using oxygen (30 %; ~ 0.3 mL min^{-1} O_2) and hydrogen (1.1 mL min^{-1} H_2) as reaction gases. The energy discrimination was set to -7 mV with a wait time of 2 ms, all other parameters were optimised daily as required. For total sulphur determination and determination of sulphur in the extracts the instrument was used with nickel cones in standard set-up. Due to the high concentration, only sulphur isotopes 33 and 34 with mass shift of 16 were measured on *m/z* 49 and *m/z* 50 (Q1: *m/z* 33 and 34, $^{33}S^+$ and $^{34}S^+$, Q2: *m/z* 49 or 50, $^{33}S^{16}O^+$ and $^{34}S^{16}O^+$). Germanium was used as continuous internal standard added online and measure on mass (*m/z* 72).

For sulphur speciation analysis the instrument was used in organic mode with Pt-cones, micro-PFA nebulizer and the addition of 6 % oxygen/argon (20:80) to the nebulizer gas. Q1 was set to *m/z* 32 or 34 while Q2 was set in the mass shift mode to *m/z* 48 ($^{32}S^{16}O^+$) and 50 ($^{34}S^{16}O^+$). Continuous internal standard (germanium) was

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added via a T-piece before the nebulizer and measured on mass (*m/z* 72). The influence of the methanol gradient on signal intensity was corrected for as described in Amayo *et al.*[10] Standards used for quantification were prepared fresh every day from cysteine in 1 % (v/v) formic acid. For peak integration PeakFit (Jandel Scientific) was used. The program was used with Method I Residuals and the integration model used was EMG + GMG, baseline setting was 0.1 % linear. Peak parameters varied during integration were residuals, width and shape. Starting peaks were set manually at the signal maximums. The results of five chromatograms for experiments 1, respectively 2 were integrated repeatedly at 3 different days (independently), the peak areas for individual peaks varied on average by 3 to 5 %. An example of the results is given in Fig. S32.

Identification of S-species using ESI-MS/MS

An Orbitrap Discovery (Thermo Scientific) was used for the identification of the eluting compounds, when the instrument was coupled to the HPLC in parallel to the ICP-MS/MS as described in more detail elsewhere.[11] The instrument was used in positive mode with 4.5 kV source voltage at 30.000 resolution in MS-mode and a scan range from 100-1500. One MSMS was measured after each MS-spectrum when it was triggered (minimum 10000 counts) at a resolution of 7500 in CID mode (activation Q: 0.25, normalised collision energy: 35, isolation width: 1 *m/z*, activation time: 30, wideband activation). Additionally experiments were run on an Agilent 6200 series TOF/6500 series Q-TOF instrument using the same HPLC conditions and similar ESI-conditions with a scan rate of 1.5 Hz, scan range from 100 - 1000, variable CID energy, 3.5 kV source voltage, fragmentor 175 V (± 200 %) and reference masses (121.05087 and 922.00979) enabled. The instruments were optimised as required. For identification / confirmation of fragmentation patterns MetFusion[12] was used with ChemSpider[13] as database. The molecular formulas were accepted as correct when Δppm was less than 3 ppm of the theoretical *m/z*.

Statistical analysis

All significant levels were tested using SigmaPlot 13.0 One Way ANOVA. Errors are always given as standard deviation of nine biological replicates if not mentioned otherwise. Mintab 17 was used as a platform for chemometric calculations: a) unsupervised principal component analysis (PCA) and cluster analysis was employed for the 22 identified and quantified low molecular weight S-containing metabolites including sulphate from all fertilization stages.

Results and discussion

Single bulb garlic used in these experiments is an *A. sativum* variety with significantly lower pungency than “normal” multiple clove forming *A. sativum* varieties. The term single bulb garlic was for simplicity shortened to garlic throughout the paper.

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Total sulphur in garlic roots and freshly formed bulb-tissue

Contamination of all liquids from environmentally present gaseous S-containing compounds is always a risk, leading to elevated sulphur background levels. All solutions were therefore not stored any longer than necessary and standards were prepared on the same day as the samples and stored under identical conditions. To reduce the risk of sulphur contamination, due to the presence of sulphur in the chemicals used for digestion including the water, the dilution factor of the plant digests was kept relative small and the sulphur concentration in the standards relative high (up to 0.6 mmol kg^{-1}). To reduce the amount of ions hitting the detector sulphur was determined via ^{16}O mass shift on ^{33}S and ^{34}S. In RM8415 with a certified value of (5100 ± 500) mg S kg^{-1} (4713 ± 100) mg S kg^{-1} (n = 4) was determined, Seronorm urine (blank) (658 ± 70) mg S kg^{-1} (certified: 545 ± 70, n = 3). The recovery of the reference materials was between 92 and 121 %. The limit of determination was between 10 and 20 µmol kg^{-1} (0.3 – 0.6 mg kg^{-1}) sample (n = 5).

Total sulphur concentration in roots was strongly depending on sulphur fertilization ($p < 0.01$). Plants fertilised with 0.1 mM sulphate for 6 weeks containing significantly less sulphur than plants fertilised with either 0.5 or 2 mM (Table 1). From the growth behaviour of garlic it can be estimated that at least 0.5 mM bio-available sulphur are required for optimal growth of the roots (details not shown). The sulphur content in roots increased linearly over the three tested sulphur levels (r^2: 0.91). In contrast to the roots there was no significant difference in the sulphur concentration of the newly formed bulb material ($p = 0.236$) (Table 1), but the sulphur content of the individual bulbs varied significantly within the groups. Montano *et al*.[8] also found a high variability of specific S-containing compounds in cloves of the same bulb and between bulbs (up to 36% depending on *A. sativum* variety) without determining the total sulphur content. The same variability is likely to occur for total sulphur since the majority of S is present as small acid extractable species. The amount of newly formed bulb tissue did not seem to be influenced by the availability of sulphur from the fertilizer within 6 weeks of growth.

A large proportion of sulphur could be extracted (80.8 ± 14.8) % (n = 54) using 1 % formic acid as solvent independent of the amount of sulphur fertilization (Table 1). This indicates that the majority of S-containing compounds present in garlic are small acid soluble S-containing compounds. About 20 % of the sulphur is not extractable by 1 % (v/v) formic acid and might be present as protein-bound sulphur.

Comparing the S-concentration found here with literature values showed that garlic grown to maturity in some field trials contained significantly more sulphur.[15] Sulphur content seems to be highly variable and depending on the availability of sulphate in soil and on *A. sativum* variety. In a field trial applying different Se and humic acid concentrations sulphur concentration between 0.3 and 0.5 % d.w. were found in bulbs (96 - 158 mmol S kg^{-1} d.w., the sulphur concentration in soil was not

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specified).[14] These values are similar to the ones found in the hydroponically grown bulbs here. In contrast bulbs of *A. sativum* L. var. Thermidrome grown under different S and N regimes in Germany contained roughly 10 to 20 times more sulphur at growth stages 2 and 3 depending on fertilization (these stages are comparable with our harvested plants).[15]

Garlic roots are normally not studied, the ones studied here contained between 2 and 3.5 times as much S than the bulb. The reason is currently unknown.

Table 1 amount of sulphur determined in root and bulb of garlic plants exposed to variable amounts of sulphate for 6 weeks in mmol S kg^{-1} d.w. (mean ± standard deviation, n per group = 9).

	0.1 mM S	0.5 mM S	2 mM S
Root total	202 ± 88[a,b]	279 ± 71[a,c]	466 ± 59[b,c]
Root extractable (% extraction efficiency)	164 ± 71[a] (81 %)	211 ± 45[b] (76 %)	353 ± 50[a,b] (76 %)
Root sum chromatogram[#] (% column recovery)	138 ± 53[b] (84 %)	172 ± 89[c] (82 %)	383 ± 86[b,c] (108 %)
Bulb total	135 ± 25	130 ± 33	133 ± 40
Bulb extractable (% extraction efficiency)	116 ± 42 (86 %)	110 ± 32 (85 %)	116 ± 48 (87 %)
Bulb sum chromatogram[#] (% column recovery)	97.7 ± 41 (84 %)	90.3 ± 35 (82 %)	100 ± 46 (86 %)

[a,b,c]: statistically significant difference between groups by One Way ANOVA $p < 0.001$
[#] sum chromatogram: sum of all individually integrated peaks

Sulphur-speciation analysis (Sulphomics)

For speciation analysis sulphur was measured on *m/z* 48 ($^{32}S^{16}O^{+}$) to increase the sensitivity of the ICP-MS/MS, since only a quarter of the injected sample was infused into the instrument (the rest being directed to the ESI-MS). Chromatograms of garlic root and bulb extract separated under the chosen conditions contained around 54 separated S-containing peaks, some peaks contained several compounds. The sulphur content in each chromatographic peak was quantified using external calibration with correction for the carbon-effect as described by Amayo *et al.*[10] The concentration of the detected S-containing compounds ranged from 0.01 to 100 mmol S kg^{-1} d.w. per chromatographic peak as determined by ICP-MS/MS. The l.o.d. was about 0.005 mmol S kg^{-1} d.w. The inter-plant variability was very high (depending on compound between 10 and 160 % RSD were found, n = 9) and only

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with the statistical power of using 9 replicates, differences on the compound level became significant between the fertilization stages.

The results of the quantification by ICP-MS/MS were compared with literature values determined by HPLC-UV. These values do not necessarily compare, since HPLC-UV results may not be free of interfering compounds co-eluting with the compound in question. In none of the publications the possibility of interfering compounds in real samples was studied, although due to the complexity of the matrix and the number of S-containing compounds (and others) present in extracts co-eluting UV-active compounds cannot be excluded. These interfering compounds will influence the quantification results using HPLC-UV with external calibration and also with standard addition. The comparison of concentrations between the quantification using HPLC-UV and HPLC-ICP-MS/MS can therefore only be used as a guide.

Set prerequisites for the identification as S-containing compound:

(i) Signal in the sulphur trace of the ICP-MS/MS
(ii) Manual mining of the ESI-MS data at retention time of signal
(iii) Extraction of EIC of potential *m/z* within less than 2 ppm error of the theoretical *m/z*
(iv) The shape of the chromatographic peak of the extracted mass charge ratio with the shape of the sulphur peak from the ICP-MS/MS trace
(v) The potential elemental composition (Δppm < 3 ppm) and
(vi) The MS^2 data (when available).

In this way the identification of the major compounds was unambiguous but the identification of isomer/diastereomer would need confirmation from e.g. NMR. An example is shown in Fig. 1, the Figures for other compounds can be found in the electronic supplement (Fig. S1 to S27), while Fig. 2 illustrates the complexity of the data mining showing the EIC for 9 different S-containing compounds in one chromatogram. In Fig. S30 & S31 examples of sulphur traces for 3 root and their corresponding bulb extracts are shown. Table 2 contains a summary of all identified compounds. The identification of minor S-containing compounds was still difficult, especially since S-containing compounds proved to be very reactive under ESI-MS conditions (eg. Fig. S8). Compounds containing the moiety $–S(O)C_3H_5$ were especially reactive. Depending on compound this led to in-source fragmentation, in-source oxidation/reduction or multimer formation impeding the identification of the compound. The majority of secondary sulphur metabolites in *A. sativum* are relatives of cysteine or γ-glutamyl-cysteine often containing an additional sulphur moiety (related to alliin). Methionine containing compounds were a minority. A number of these compounds were present in different isoforms and / or diastereomers.

The major S-containing compounds in garlic were hydrophilic compounds (including sulphate) and the alliin-variants (cyclo-alliin, methiin, alliin, isoalliin and propiin), together containing between 11 and 26 % of the total sulphur in root (Table S1) and

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20 % in bulb. The sulphate, alliin, isoalliin and propiin content increased significantly between low and high ($p < 0.05$) or medium and high exposure groups ($p < 0.05$) in roots, but not in bulb (Table S1). The inter-plant variability in bulb material was higher than in roots, which may contribute to the fact that no influence of sulphur fertilization in bulb for any compound was found which confirmed the finding by Montano and co-workers. [8]

Major S-containing compounds cited in the literature and quantified

Alliin and its isomers are the precursors for allicin and other thiosulfinates, which are the major compounds responsible for the typical garlic taste and smell. Their quantification therefore is of major interest to producers of garlic containing food and food supplements. Determination by HPLC-UV (the standard method used in the literature) results in highly variable alliin (59 and 298 mmol alliin kg^{-1} d.w.) content in bulbs. [8,15,16] The variation of this dominant species results most likely from different garlic varieties and culture conditions. The content also varied with age of the plant.[15] The garlic grown in our experiment is a very mild variety and this also showed in the alliin content, which was significantly lower than literature values both in bulb (21 mmol alliin kg^{-1} d.w., Table S1) and root. The alliin content in root was depending on the level of S-fertilization (between 14 and 79 mmol alliin kg^{-1} d.w.). The isoalliin content in bulbs (4 mmol kg^{-1} d.w., Table S1) was within the range mentioned in the literature.[7,8] Cycloalliin eluting very early on was not cleanly separated from other S-containing compounds(Table S13).

The published concentrations of the γ-glutamyl relatives of aliin and isoalliin (γ-glutamyl-S-allyl-cysteine (GSAC) and γ-glutamyl-S-1-propenyl-cysteine (GSPC)) (structures Table S4) range from 7.5 to 224 mmol kg^{-1} d.w. [7,8,16] for GSAC and for GSPC from 27 to 312 mmol kg^{-1} d.w.[7,8,16] The content of both compounds determined here via sulphur was significantly lower (Table S4). The content of GSPC was higher than that of GSAC both in bulb and root. Roots contained slightly more GSAC than bulb, but less GSPC than bulbs. GSPC content in root was depending on S-fertilization. The oxidised isomers of GSAC (GSAC(O)) and GSPC (GSPC(O)) eluted significantly earlier than the parent compounds. Both compounds have been reported as being present in garlic by Yamazaki *et al*.[17] They were quantified by Hughes *et al.*[18] studying changes in GSAC(O) and GSPC(O) in garlic bulbs and cloves due to storage conditions with concentrations found for GSAC(O) between 6.2 and 68 mmol kg^{-1} and between 27 and 202 mmol kg^{-1} for GSPC(O) with no indication of whether it was determined on a dry or fresh weight basis.[18] In our study the amount of GSPC(O) in bulbs was lower than that of GSAC(O) in contrast to Hughes findings (Table S5). Roots contained similar amounts of both compounds at the low and medium fertilization level, but significantly more GSAC(O) than GSPC(O) at the high level (with a very high inter-plant variability in all cases. In all groups the concentrations of the oxidised forms in roots were by more than a factor of 10 higher than the reduced forms GSAC and GSPC (Table S4 and S5).

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The content of the methiin relative γ-glutamyl-S-methyl-cysteine (GSMC) showed a sulphur-fertilization dependency in roots (up to ca. 1.7 mmol kg^{-1} d.w., Table S6), whereas the content in bulbs was about 0.08mmol GSMC kg^{-1} d.w. (Table S6), low when compared to published values. Literature values for GSMC for garlic range between 0.38 and 144 mmol kg^{-1} d.w.[7,8]

Reduced glutathione (MS^2 by Orbitrap, but not q-TOF) co-eluted with propiin and methionine showing a very small signal in ES-MS. Oxidised glutathione (GSSG) eluted without co-elution of any other S-containing compound. Its concentration was not influenced by the S-fertilization regime. Bulbs contained about 0.09 mmol kg^{-1} d.w. GSSG (equiv. 0.18 mmol reduced GSH) and roots between 0.17 and 0.27 mmol GSSG kg^{-1} d.w. (equiv. 0.35 to 0.55 mmol kg^{-1} d.w. reduced GSH) (Table S7). Since the signal for reduced GSH also contained co-eluting propiin and methionine (~ 0.9 mM S kg^{-1} d.w. in bulb and between 1.1 and 3.2 mmol S kg^{-1} d.w. in root), no quantification of reduced GSH was possible. However, the values for GSSG compared well with the 0.6 to 1.9 mmol kg^{-1} d.w. as total GSH in bulbs after reduction as reported by Bloem *et al*.[15] The fact that GSSG is the more dominant form of GSH is slightly unusual, but not an artefact of sample preparation since dissolving reduced GSH in 1 % formic acid does not lead to GSSG formation (data not shown). Therefore GSSG must be naturally the more dominant of the two forms.

Compounds so far not quantified in the literature

The content of propiin, the co-eluting methionine and reduced glutathione increased in roots with S-fertilization (1.2 to 3.2 mmol sulphur kg^{-1} d.w.), whereas bulbs contained on average 1 mmol S kg^{-1} d.w. (Table S13) at this retention time. None of the compounds was as yet quantified in garlic. Assuming similar sensitivity in ESI-MS for all three compounds (likely due to their similarity) propiin was the dominant compound.

The S-allyl-cysteine (SAC, deoxyalliin) content in bulbs was slightly lower than in roots and did not depend on the S-fertiliser regime (Table S3), possibly due to high inter-plant variability. SAC identification by extracted ion-chromatogram was difficult, since a whole host of other compounds showed in-source fragments at *m/z* 162.0583 among these were S-1-propenylmercaptoglutathione, S-2-propenylmercaptoglutathione and S-propylmercaptoglutathione, GSMC, GSAC, GSPC and especially C393, which gave the most intense signal in the extracted ion-chromatogram of *m/z* 162.0583. The signal was identified by a combination of not identifying any other S-containing compound at that retention time which might plausibly give an in-source fragment of *m/z* 162.0583 and estimation of possible retention times under the separation conditions used here with the retention time determined by Yamazaki as described by Block.[17]

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S-1-propenylmercaptoglutathione and S-2-propenylmercaptoglutathione were described as S-containing compounds in garlic first by Nakabayashi *et al.*[9] but not quantified. Both compounds are relatives of glutathione containing an additional C_3H_5S-group bound to the –SH-group of glutathione. Bulbs contained more S-1-propenylmercaptoglutathione than S-2-propenylmercaptoglutathione. In roots the concentration of S-2-propenylmercaptoglutathione was higher than that of S-1-propenylmercaptoglutathione (Table S8). Neither compound showed the least dependency on S-fertilization levels.

Phytochelatin (PC-2, γ-Glu-Cys-γ-Glu-Cys-Gly) a pentapeptide often occurring in plants was present in root and bulb in its intramolecular oxidised form, showing a similar behaviour to GSH. The reduced form was not found. PC-2 co-eluted with GSAC(O) compound 1a and was therefore not individually quantified, but its concentration in both root and bulb as estimated from the amount of eluting sulphur was relative low (Table S11).

Newly identified S-containing compounds in garlic

In Fig. 3 the proposed structures for the newly identified compounds in garlic are summarised. The extracted ion chromatogram of $[M+H]^+$ 265.0843 (GSMC) showed one strong signal (GSMC) and several smaller signals of the same *m/z*, some of which were in-source fragmentation products. One new compound with $[M+H]^+$ 265.0843 was identified from its fragmentation pattern as γ-glutamyl-homocysteine (γ-Glu-HCy). The concentration of γ-Glu-HCy in root was S-fertilization rate independent with about 0.25 mmol kg^{-1} d.w., bulbs contained about 0.07 mmol kg^{-1} d.w. (Table S6, Fig. S16).

S-propylmercaptoglutathione ($C_{13}H_{23}N_3O_6S_2$), theoretical *m/z* 381.1028, not yet mentioned in the literature was identified by ESI-MS/MS, eluting shortly after S-2-propenylmercaptoglutathione. It is co-eluting with one of the S-allyl/propenyl-PC2 isomers. The amount of sulphur eluting at the retention time was low (Fig. S21). S-1-propenylmercaptglutathione and S-2-propenylmercaptoglutathione both can occur, at least theoretically, in their oxidised forms similar to GSAC and GSPC. In the case of S-1-propenylmercaptglutathione and S-2-propenylmercaptoglutathione 4 different isomers may occur, since the SO-group can be formed by either of the sulphur atoms of molecule (Table S9). These compounds should elute somewhere between GSAC(O)/GSPC(O) and their reduced parent-compounds when they behave similar to GSAC and GSPC. The EIC-trace showed both in root and bulb the presence of 4 compounds at $[M+H]^+$ 396.0894 eluting at about the expected retention times. For three of the four compounds ESI-MS/MS spectra were measured. Compounds 1a and b showed more pronounced fragments at *m/z* 131.0459 ($C_4H_7N_2O_3$) and *m/z* 263.01 ($C_8H_{11}N_2O_4S_2$) (Fig. S22 and S23 a and b). Compound 2a showed a more pronounced fragment at *m/z* 120.0126 ($C_3H_6NO_2S$)

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(Fig. S23c). The last of the mentioned fragments may indicate that for this compound the HS-group of GSH was oxidised. No further identification with regard to the influence of double bond position and oxygen position on retention time and therefore compound characterisation was possible from the ESI-MS/MS data. Compound 1a co-eluted with oxidised PC-2 (Table S13), whereas compounds 1b, 2a and 2b eluted without any co-eluting S-containing compounds. Bulbs contained lower concentrations of the three quantifiable forms than roots (Table S10). Their γ-glutamyl-cysteine counterparts ($C_{11}H_{18}N_2O_5S_2$ at $[M+H]^+$ 323.0729 and $C_{11}H_{18}N_2O_6S_2$ at $[M+H]^+$ 339.0679) were not detectable.

The, as yet undescribed, S-allyl/propenyl-containing PC-2 (C611) was present in both root and bulb in at least four different isomeric forms (Fig. S25), indicating that the S-allyl/propenyl-group can be bound to either SH-group of PC2. The sulphur amount eluting at its retention time was higher than that estimated for PC2 especially in bulbs (Table S13).

Among the precursor molecules described as part of the biosynthetic pathway by Block and co-workers[2] one of the first steps during the synthesis of alliin and its relatives is the addition of 2-methylprop-2-enoic acid ($C_4H_6O_2$) to either γ-Glu-Cys or GSH. The products of these two reaction should give molecules with $[M+H]^+$ of 337.1064 (2-amino-5-({1-carboxy-2-[(2-carboxypropyl)sulfanyl]ethyl}amino)-5-oxopentanoic acid, C336) and $[M+H]^+$ of 394.1279 (2-amino-5-({1-[(carboxymethyl)amino]-3-[(2-carboxypropyl)sulfanyl]-1-oxopropan-2-yl}amino)-5-oxopentanoic acid, C393). Not mentioned in the published biosynthetic pathway is the cysteine-derivative at $[M+H]^+$ 208.0638 (2-amino-3-[(2-carboxypropyl)sulfanyl]propanoic acid, C207). None of these was yet mentioned anywhere else in the literature. C207 and C393 showed strong signals in both ICP-MS/MS and ESI-MS in both bulb and root, whereas C336 was not detectable except as in-source fragment of C393. C207 is the more dominant precursor in root. The amount of both compounds was depending on the amount of S-fertilization rate in roots but not bulbs (Table S12). Concentrations of C207 and C393 in root were significantly higher for the highest S-fertilization rate (2 mM S) than in the low and medium exposure groups (Table S12).

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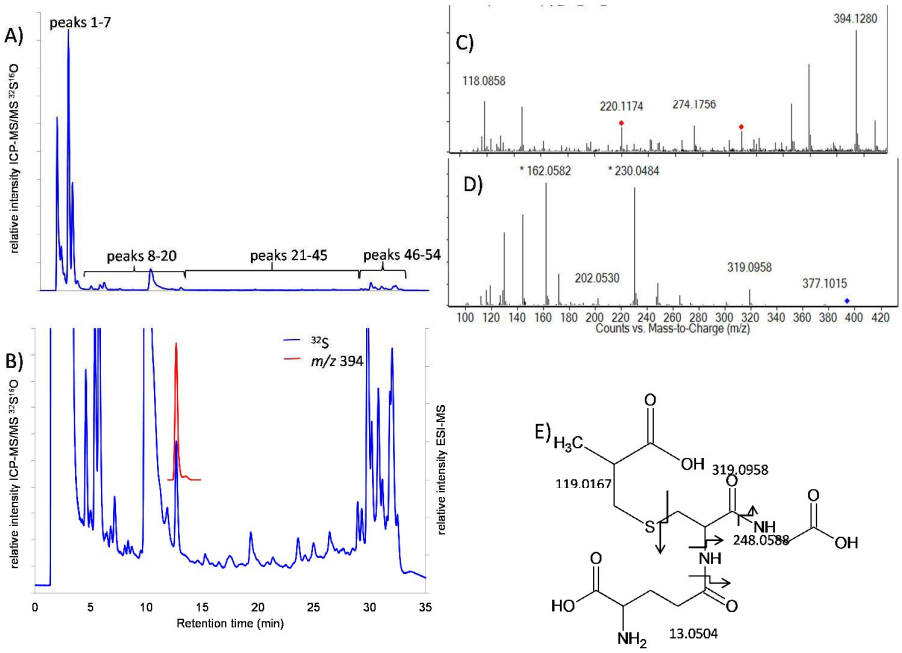

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461 **Fig. 1** panel A) ^{32}S-trace from ICP-MS/MS; panel B) details of ^{32}S-trace and
462 extracted ion chromatogram of m/z 394.1280 of root extract exposed to 2 mM S;
463 panel C) ESI-MS-spectrum of compound; panel D) ESI-MS^2-spectrum of compound;
464 panel E) proposed structure and main fragmentation sites; for HPLC-ICP-MS/ESI-
465 MS conditions please see Instrumentation; details of all other compounds can be
466 found in the electronic supplement.

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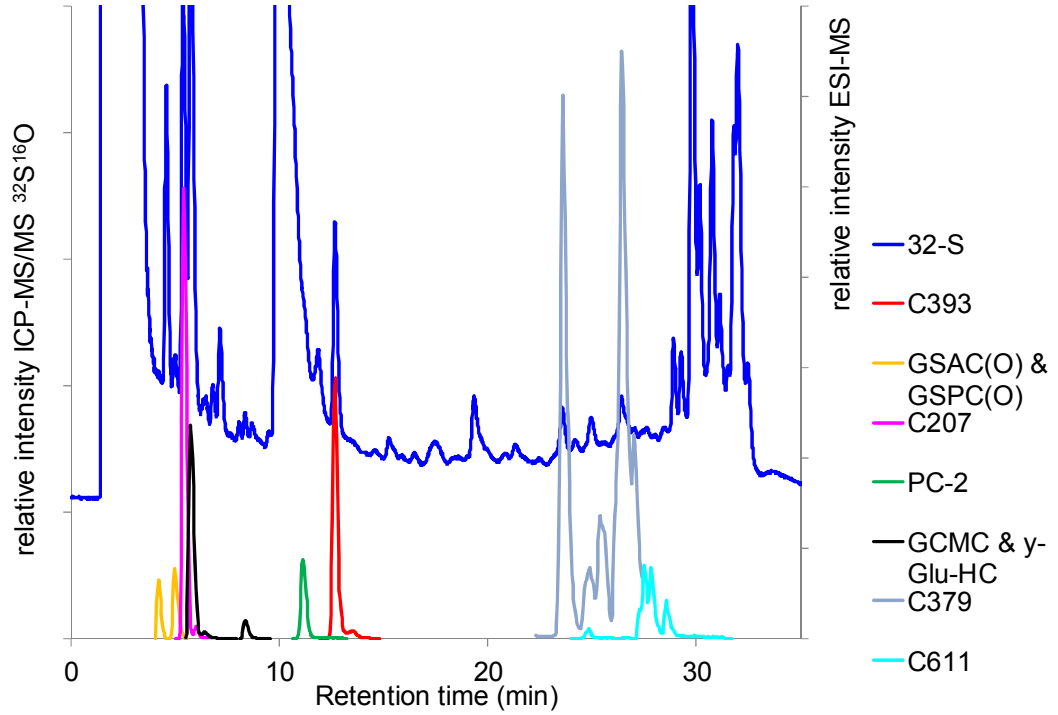

Fig. 2 ^{32}S-trace from ICP-MS/MS and extracted ion chromatograms of 9 sulphur containing compounds of root extract exposed to 2 mM S; for HPLC-ICP-MS/ESI-MS conditions please see Instrumentation; detailed Figures for all compounds can be found in the electronic supplement.

Table 2 overview of S-containing compounds identified in garlic.

Compound name	Molecular weight / $[M+H]^+$	Co-elution	ID by MS/MS	Quantified in literature
Known compounds in garlic				
alliin	178.0532		y	y
Isoaliin	178.0532		y	y
cycloalliin	178.0532	y	y	y
methiin	152.0376	y		y
propiin	180.0689		y	
methionine	150.0583	y	y	
methylcysteine	136.0427	y		
γ-glutamyl-S-allyl-cysteine	291.1009		y	y
γ-glutamyl-S-1-propenyl-cysteine	291.1009		y	y
γ-glutamyl-S-2-propenylcysteine sulfoxide	307.0958		y	y
γ-glutamyl-S-1-propenylcysteine sulfoxide	307.0958		y	y
γ-glutamyl-S-methyl-cysteine	265.0853		y	y
Glutathione	308.0911	y	y	y

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Oxidised glutathione	613.1592		y	y
S-allyl-cysteine	162.0583		y	
S-1-propenylmercaptoglutathione	380.0944		Y	
S-2-propenylmercaptoglutathione	380.0944		y	
Newly identified compounds in garlic				
Phytochelatin 2	538.1272	y	y	
C264 (putative: γ-Glu-HCy)	265.0853		y	
S-propylmercaptoglutathione	382.1105	y	y	
Oxidised forms of S-1-propenylmercaptoglutathione and S-2-propenylmercaptoglutathione	396.0894	partly	y	
C611 (putative: S-allyl/propenyl-containing PC-2)	612.1462	partly	y	
C207 (putative: 2-amino-3-[(2-carboxypropyl)sulfanyl]propanoic acid)	208.0638		y	
C336 (putative: 2-amino-5-({1-carboxy-2-[(2-carboxypropyl)sulfanyl]ethyl}amino)-5-oxopentanoic acid)	337.1064			
C393 (putative: 2-amino-5-({1-[(carboxymethyl)amino]-3-[(2-carboxypropyl)sulfanyl]-1-oxopropan-2-yl}amino)-5-oxopentanoic acid)	394.1279		y	

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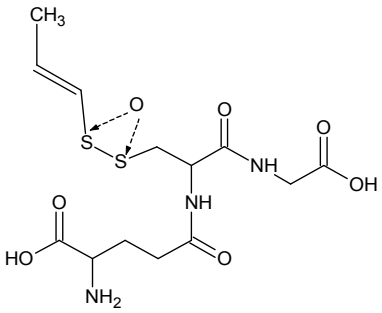
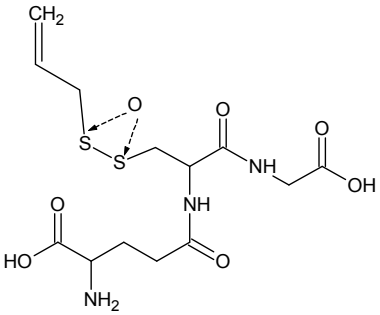
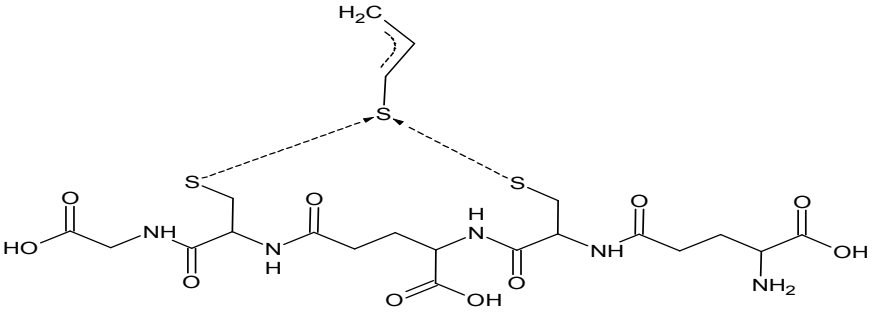
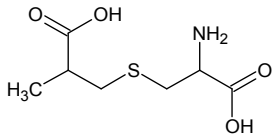


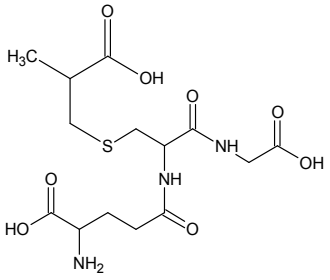


Oxidised S-1- and S-2-propenylmercaptoglutathione

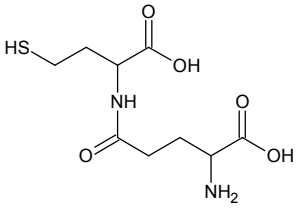


S-allyl/propenyl-containing PC-2

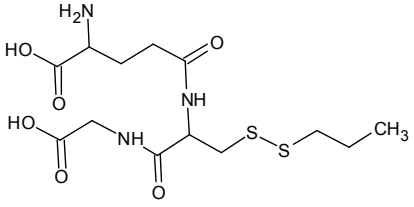




Precursor molecules for S-alk(en)ylcysteine sulfoxides



γ-Glu-HC



S-propylmercaptoglutathione

Fig. 3 **P**roposed structures for newly in garlic identified compounds.

Sulphate

Inorganic sulphate was eluting from the column in the dead volume. Although by far the main component, possible co-elution of unknown small sulphur containing

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compounds cannot be entirely be excluded. Its concentration in roots was depending on the amount of sulphate in the fertilizer, whereas sulphate in bulbs did not show any dependency on the fertilizer (Table 3, Fig. 4).

Table 3 Mass balance: Sulphate, chromatographically separated unknown organic S- containing compounds (unk S_{org}) and chromatographically separated identified organic S-containing compounds (known S_{org}) in mmol S kg^{-1} d.w. (mean ± standard deviation, n = 9 per group), in brackets as average % of total sulphur.

	Sulphate	unk S_{org}	known S_{org}
Root (0.1 mM S)	26 ± 23[a,c] (13 %)	78 ± 46 (39 %)	34 ± 14[d] (17 %)
Root (0.5 mM S)	63 ± 42[a,b] (23 %)	62 ± 53 (22 %)	45 ± 21[e] (16 %)
Root (2.0 mM S)	151 ± 40[b,c] (32 %)	92 ± 46 (20 %)	140 ± 50[d,e] (30 %)
Bulb (0.1 - 2 mM S)	7.7 ± 4.9 (5.9 %)	57 ± 40 (43 %)	32 ± 8.8 (24 %)

[b, c]: statistically significant difference between groups by One-way ANOVA $p < 0.001$
[a]: statistically significant difference between groups by One-way ANOVA $p < 0.05$
[d, e]: statistically significant difference between groups by One-way ANOVA $p < 0.01$

Overall it is apparent that at lower fertilization rate, the amount of sulphate was lower and concentration of the identified organosulphur compounds increased, while the proportion of unidentified S-species decreased (Table 3).

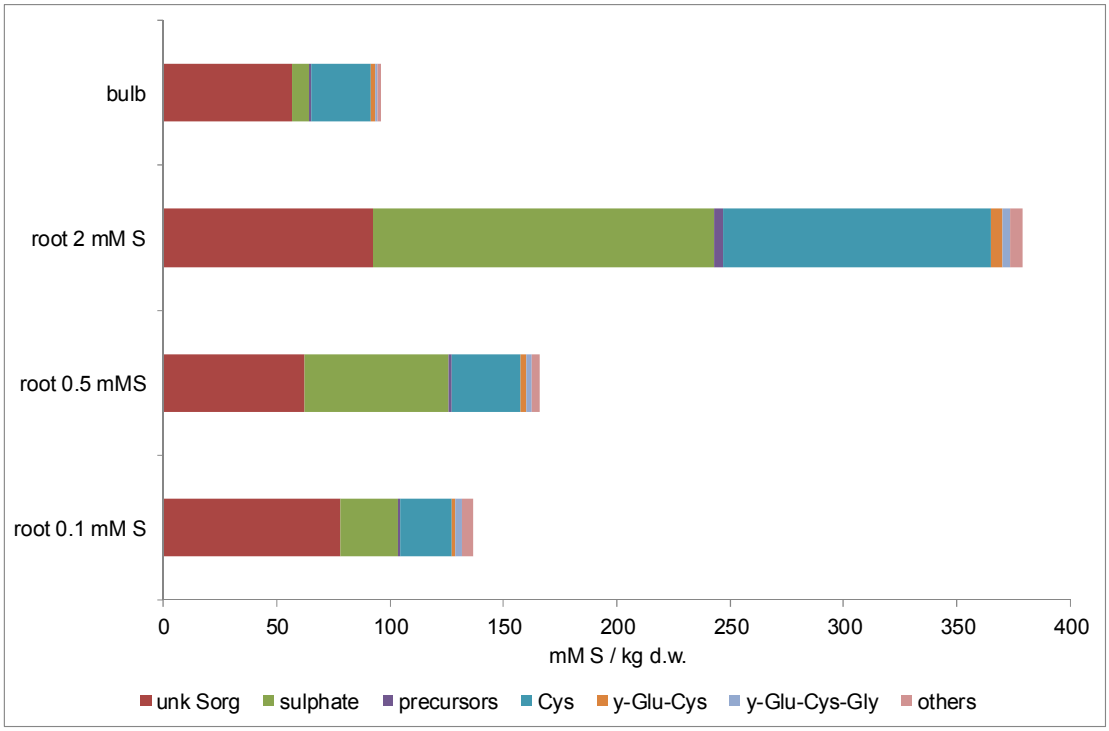

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Fig. 4 A list of average concentrations (mmol S kg^{-1}) of all S-species in garlic roots growing under a different S-fertilization regime. Unk Sorg= unknown organic S-containing compounds, precursors = sum of C207 and C393, Cys = sum of methiin, alliin, isoalliin, propiin and SAC, γ-Glu-Cys = sum of GSAC, GSPC, GSAC(O) and GSPC(O), γ-Glu-Cys-Gly = sum of reduced and oxidised propenylmercapto-glutathions, others = sum of all other compounds mentioned in the text.

Comparing root and bulb sulphur patterns showed that both plant parts were similar in their speciation (Fig. S30 & S31) though not necessarily in concentration of the individual compounds. The experiments also showed that inter-plant variability was very high with regard to concentrations of S-containing compounds. The experiments showed that manipulation of sulphur fertilization rates under hydroponic growth conditions did not significantly influence the sulphur content in bulbs . In contrast to bulbs the amount of certain S-containing compounds in roots was strongly depending on sulphur availability as shown not only by one-way ANOVA, but also by PCA-analysis (Fig. 5, Fig. S28 and S29). The main difference was between the group exposed to 2 mM sulphur while the 0.1 and 0.5 mM S-fertilized plants were similar. The excess of S in the hydroponic solution created a significantly different species distribution of the low molecular weight sulphur species. From the loading plot (Fig. S28) it was apparent that the difference were due to the following sulphur species: C393, GSAC(O), propiin+GSH+methionin. A smaller influence was exerted by S-allyl/propenyl PC-2, but not by alliin and isoalliin as was originally expected.

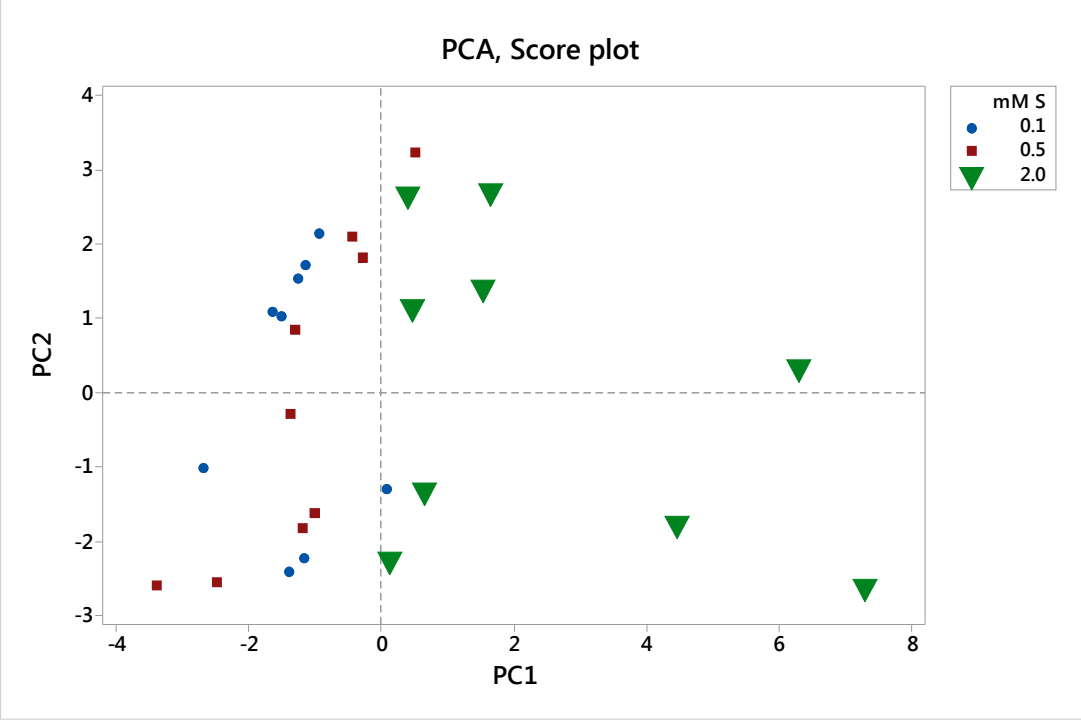

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Fig. 5 Principal component analysis of all individual plants exposed to different S-fertilization regime show significant differences in their low molecular weight S-containing species.

Summary

This has been the first attempted global S-speciation in a plant. Having the ICP-MS/MS available for a non-targeted analysis made it possible to determine a full sulphur mass balance and identified gaps in our knowledge about the multitude of S-containing compounds in *A. sativum*.

Using elemental and molecular detectors in parallel (HPLC-ICPMS/MS-ESI-MS/MS) allowed us to identify the molecular composition and assign at least tentative structures for between 16 and 30 % of the total sulphur. Only a limited number of the compounds had been identified and quantified in the literature before due to different factors chiefly among them availability of standards, which are necessary when UV is used for quantification. Among the multitude of identified compounds five not yet elsewhere described as occurring in garlic were found and quantified for the first time and for a further four the first concentration estimates could be determined. Having used a non-targeted S-analysis a further 20 to 43 % of the total sulphur was identified as organosulphur compounds but so far no structures and or molecular compositions were deducible.

The use of ICP-MS/MS in contrast to the use of UV-detection allowed the quantification of all S-containing compounds independent of whether they were chromatographically resolved or not without requiring the chromatographic separation of every UV-absorbing compound (at the chosen wavelength) and without the requirement of species-specific standards. Nevertheless quantification of compounds on the basis of their molecular weight is still limited in the case of not fully resolved chromatographic peaks. Hence, chromatography needs to be improved if single so far non-resolved compounds need to be quantified in a target analysis.

The study also showed, that despite garlic being a well-studied system, there are still unknown S-containing compounds present and the use of high resolution ESI-MS/MS is absolutely required for at least tentative identification of compounds. The amount of some of these newly identified compounds was strongly influenced by S-fertilization.

The separation of *A. sativum* extracts and detection of its sulphur metabolites showed also that the chromatographic separation needs to be improved in order to quantify the myriad of S-containing compounds. This sulfomics study by using a non-target analysis HPLC-ICPMS/MS/ESI-MS demonstrated that all S-containing

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compounds can potentially be detected in plants and it is therefore a useful tool when environmental influences on plants are going to be studied.

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