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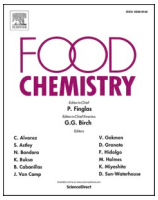

Seeing the smell of garlic: Detection of gas phase volatiles from crushed garlic (*Allium sativum*), onion (*Allium cepa*), ramsons (*Allium ursinum*) and human garlic breath using SESI-Orbitrap MS

Hendrik G. Mengers [a], Christina Schier [b], Martin Zimmermann [a], Martin C. H. Gruhlke [b], Eric Block [c], Lars M. Blank [a,*], Alan J. Slusarenko [b,*]

[a] Institute of Applied Microbiology – iAMB, Aachener Biology and Biotechnology – ABBt, RWTH Aachen University, Aachen, Germany
[b] Department of Plant Physiology, RWTH Aachen University, Aachen, Germany
[c] Department of Chemistry, University at Albany, SUNY, Albany, NY 12222, USA

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ABSTRACT

Allicin is the main flavour component of crushed raw garlic. This plant defence molecule has strong antibiotic properties. While measurements in the liquid phase using LC-MS are established, accessing reactive organosulfur compounds in the gas phase is still a challenge due to heat-degradation in the gas chromatograph. Using a gentle secondary electrospray ionisation coupled Orbitrap mass spectrometry procedure (SESI-Orbitrap MS), we measured gas phase concentrations of allicin evaporating from a pure solution. Despite the mild conditions, two quantitatively major allicin-derived breakdown products were found. The SESI-Orbitrap MS technique was used to follow the known chemistry of alliin, isoallin and methiin conversion in garlic, onion and ramsons. Allicin and its metabolites were also measured over two hours in human breath after garlic consumption. These results demonstrate the utility of SESI-Orbitrap MS for analysis of sulfur-containing volatiles from plants in the genus *Allium* and potentially for capturing volatilomes of foodstuffs in general.

1. Introduction

Over the past two decades, the analysis of volatile organic compounds (VOCs) has become increasingly important. VOCs are typically small, low molecular mass compounds with low boiling points and therefore high vapour pressures (Koppmann, 2007). They are emitted into the environment by a variety of anthropogenic and biogenic processes. Their biological functions are almost limitless, ranging from cell-to-cell to interspecies communication. For humans, volatiles are of particular interest in "food analysis" because of their contribution to odour and flavour (Ebert, Halbfeld, Blank & Keine Angabe, 2017). The entirety of volatile substances released into the gas phase by an organism is referred to as its volatilome (Tejero Rioseras et al., 2017).

Garlic (*Allium sativum* L.), which has been used as a food and medicinal plant for thousands of years, is a source of numerous volatile organic sulfur-containing compounds (VOSCs). Garlic VOSCs are responsible for the characteristic flavour of garlic, resulting in a distinct odour and taste that is much valued and used in many cuisines (Block, 2017). Furthermore, VOSCs can have antimicrobial activity (Block, 2010) and function as defence molecules against microbial pathogens and herbivores.

The major contributor to the smell of freshly crushed garlic is allicin (diallyl thiosulfinate, IUPAC *S*-prop-2-en-1-yl prop-2-ene-1-sulfinothioate). Allicin was first identified as an antibacterial molecule by Cavallito in 1944 (Cavallito & Bailey, 1944). The reactive compound allicin does not occur free in intact garlic tissue, but rather is formed after cellular damage (see Fig. 1a). The cytoplasmic non-protein amino acid alliin (*S*-allyl-L-cysteine sulfoxide) is cleaved after cell damage by the vacuolar enzyme alliinase (E.C. 4.4.1.4). This reaction leads to the production of dehydroalanine and 2-propenesulfenic acid. Two molecules of 2-propenesulfenic acid spontaneously condense to form one molecule of allicin. Notably, although this biochemical reaction sequence was long postulated, 2-propenesulfenic acid formation as a precursor for allicin was first confirmed by Direct Analysis in Real Time (DART)-MS in 2010 (Block, Dane, Thomas, & Cody, 2010) following up on earlier LC-MS studies (Calvey et al., 1994a; Calvey et al., 1994b).

Allicin is a reactive sulfur species (RSS) and causes oxidative stress in cells (Gruhlke & Slusarenko, 2012, see Tab. S1-3). It reacts with low

* Corresponding authors.
E-mail addresses: lars.blank@rwth-aachen.de (L.M. Blank), alan.slusarenko@bio3.rwth-aachen.de (A.J. Slusarenko).

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molecular weight thiols such as glutathione (GSH) and, in Gram-positive bacteria, the GSH functional analogue bacillithiol (BSH). Both GSH and BSH are required for the maintenance of cellular redox homeostasis in their respective hosts. Other targets for allicin *S*-thioallylation are accessible cysteine thiols in proteins (Müller et al., 2016; Chi et al., 2019, see Tab. S1-4). Allicin's antimicrobial effects against multiple pathogens occur either by direct contact in solution, where it has an efficiency comparable to many commercially available antibiotics (Borlinghaus, Albrecht, Gruhlke, Nwachukwu, & Slusarenko, 2014), or via the vapour phase (Borlinghaus et al., 2021; Reiter et al., 2020). In particular, its activity against human lung pathogenic bacteria, including multidrug-resistant (MDR) strains (Reiter et al., 2017), and the associated potential treatment of infectious diseases via the pulmonary route, make allicin a pharmacologically interesting substance (Reiter et al., 2020).

It is interesting to consider what is known about the characteristic differences in VOC production between the *Allium* spp. we investigated. Although onions, garlic and ramsons all belong to the genus *Allium* and all have alliinase activity, onions (*Allium cepa* L.) do not synthesise alliin, but rather its isomer isoalliin (*trans*-(+)-*S*-(1-propenyl)-L-cysteine sulfoxide) (Block, 2010) (Fig. 1). Like alliin, isoalliin is stable and odourless, and also makes up about 10% of the alk(en)yl cysteine sulfoxides present in garlic. After tissue injury it is converted by alliinase into 1-propenesulfenic acid and dehydroalanine (see Fig. 1c). The lachrymatory factor synthase (LFS) enzyme in onion, which is absent from garlic and ramsons, converts 1-propenesulfenic acid to propanethial *S*-oxide, the onion lachrymatory factor (LF). Some of the 1-propenesulfenic acid also spontaneously condenses to bis(1-propenyl) thiosulfinate, which then undergoes rearrangement to various zwiebelanes. The LF is volatile and is responsible for the eye irritation and the tears which result while chopping onions (Imai et al., 2002). In this way, the sulfenic acids derived from the respective alk(en)yl cysteine sulfoxides, coupled with the LFS activity in onion, determine the distinctively different VOSC chemistry between these two *Allium* species. In the related *Allium ursinum* (ramsons), methiin (methyl cysteine sulfoxide), alliin and isoalliin are the major alliinase substrates present. In all three *Allium* species variable amounts of propiin (propyl cysteine sulfoxide) may also be present. The sulfoxides undergo cleavage by alliinases giving the

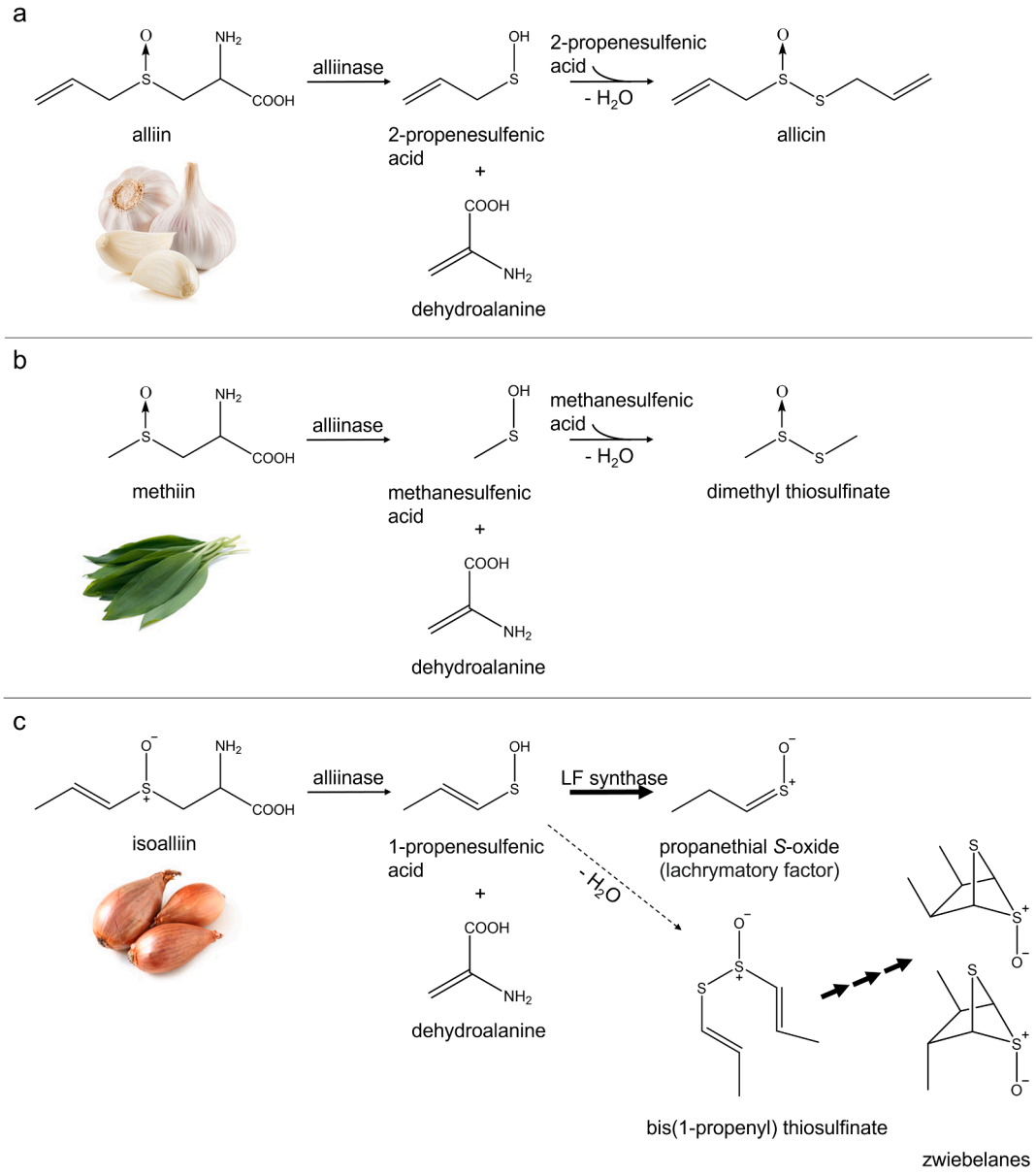

Fig. 1. Comparison of the conversion of (a) alliin from garlic, (b) methiin from ramsons, and (c) isoalliin from onion, by the alliinase enzymes. The pathways shown take place when plant tissue is injured, as the vacuolar alliinases then interact with their cytosolic substrates (see text for details).

corresponding sulfenic acids which condense in a statistical manner giving dimethyl thiosulfinate, isomeric allyl methyl thiosulfinates, allicin and other indistinguishable $C_6H_{10}S_2O$ molecules as the VOSC products (Block, 2010)(Fig. 1b). These compounds are also present in other *Allium* species. A good overview is given by Block et al. (Block, 2010).

Because of the rich flavour and potential uses for garlic, its sulfur compounds have been extensively studied. Initially, garlic volatiles were measured using gas chromatography (GC, liquid-injection or headspace), but this technique poses problems since allicin extensively degrades during GC (Lawson, Wood, & Hughes, 1991). Previously, the exploration of the underlying sulfur chemistry was performed by high-performance liquid chromatography (HPLC) (Lawson et al., 1991) or supercritical fluid chromatography (SFC) (Calvey et al., 1994a; Calvey et al., 1994b). Because of the characteristically flavourful odours of different alliums, non-destructive methods were sought for directly and rapidly observing the evolution and reactions of sulfur compounds in the gas phase. The authentic volatilome is measured directly from the gas phase above a sample and must be differentiated from treatments which might affect the release of molecules from the sample. For example, methods such as Direct Analysis in Real Time-Mass Spectrometry (DART-MS), electrospray ionisation (ESI) based techniques like desorption ESI (DESI), extractive ESI (EESI) or internal EESI (iEESI) bombard a solid sample with an ion-stream, thus ionising and volatilising compounds at the surface (see Tab S1-1 for references). Gas phase DART (Li, 2012) and Real-Time Proton-Transfer Reaction-Mass Spectrometry (PTR-MS) (Joyce, Eady, Silcock, Perry, & van Klink, 2013) are methods to observe the true volatilome, but until now, to the best of our knowledge, have only been used for onion samples. Atmospheric pressure photoionization (APPI) MS also detects the true volatilome and was applied to crushed garlic samples and garlic breath, but without the second-scale resolution the other methods offer (Zhou et al., 2017). Garlic breath was also observed by selected-ion flow-tube (SIFT) MS, but without attention to allicin (Munch & Barringer, 2014) (see Tab. S1-2). The work reported here is a progression from these earlier studies.

Secondary electrospray ionisation high-resolution Orbitrap mass spectrometry (SESI-Orbitrap MS) offers the possibility of measuring the true volatilome in a time-resolved manner on a scale measured in seconds (Gaugg et al., 2016), but it has not yet been applied to the measurement of *Allium* VOSCs. In this study, we use SESI-Orbitrap MS to analyse garlic, ramsons and onion VOSCs time-resolved in the gas-phase. The atmospheric pressure ionisation (API) source device that we use generates an electrospray through which the analyte gas stream is then passed. The electrospray consists of charged water clusters, containing a single positive or negative charge, depending on the setting. The charged water clusters collide with the gas-phase molecules and transfer their charge, although the exact mechanism is yet to be fully resolved (Barrios-Collado, Vidal-de-Miguel, & Martinez-Lozano Sinues, 2016). Since only the analyte gas stream is subject to ionisation, only molecules which are already in the gas phase are detectable. This system does not rely on analyte separation, so the time between molecules entering the SESI and reaching the high vacuum in the Orbitrap is only about 2 *sec*, reducing degradation by fragmentation and bimolecular processes. Also, the ionisation is very soft and can be achieved at atmospheric pressure and low temperature (Gaugg et al., 2016). SESI-Orbitrap MS allows real-time analysis because the need for chromatographic separation is bypassed, and full-MS scans can be recorded every 0.3 s. Because no sample treatment is required, any possible sampling bias is avoided. Moreover, in comparison to most GC–MS methods, low temperatures and a soft ionisation method are used. That being said, these advantages come at the cost of analyte resolution and the lack of molecule-specific retention times, allowing identification of unknown compounds only by their molecular formula by high-resolution (HR) MS, augmented by MS/MS methods identifying characteristic fragmentation pathways.

The aim of this study was to address the difficulties of characterizing heat-labile sulfur molecules from various *Allium* spp. in the gas phase in a non-degradative manner. SESI-Orbitrap MS was used in this work to analyse the gas phase over a solution of synthesized allicin and to distinguish between the volatilomes of garlic, ramsons, and onions. Our analysis of VOSCs in exhaled breath after garlic consumption is a novel use of the SESI technique.

2. Material and methods

2.1. Chemicals

Allicin synthesis was performed slightly modified according to Albrecht, Leontiev, Jacob, and Slusarenko (2017). Diallyl-disulfide was oxidised with H_2O_2 and formic acid as a catalyst. Instead of chromatographic separation, hexane and dichloromethane extraction was used (Albrecht et al., 2017). Fresh garlic (*Allium sativum* L.) and onion (shallot, *Allium cepa*) were purchased at a local supermarket and ramsons leaves (*Allium ursinum*) were freshly picked from local woodland and all used the same day.

2.2. SESI-Orbitrap mass spectrometry

A Super-SESI unit (Fossiliontech, Madrid, Spain) was coupled to a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). For all measurements, mechanically sharpened nano-electrospray emitters Sharp Singularity (Fossiliontech) were used. If not otherwise mentioned, the temperature at the SESI intake line, the SESI core, and the Orbitrap intake capillary were set to 60 °C. The scan range was set to 50–500 *m/z* with a resolution of 70,000 and with 10 microscans, resulting in a speed of approximately 0.4 Hz (for more details, see S2.1). The method used for MS/MS experiments is given in Tab. S2-1. The lock-masses used are depicted in Tab. S2-2. External mass calibration was performed regularly, and the mass shift of the machine was usually between 2 and 3 ppm (for the calculation, see S3.1).

Measurements with synthetic allicin were performed at 30 °C, 60 °C, 100 °C, and 130 °C. For these experiments, after measuring the background laboratory air, a microreaction tube with 1 mL of a 50 mM aqueous solution of synthetic allicin was placed 1 cm before the intake line. The lab temperature was 21 °C and the surface area of liquid available for evaporation was approx. 0.6 cm^2. For the measurement of garlic cloves, ramsons or onions, a glass funnel was installed in front of the SESI allowing a consistent distance. After measuring the background, a whole garlic clove, an equivalent large part of an onion or two ramsons leaves were crushed inside the funnel using a garlic press (depicted in Fig. S2-1). For all measurements, a background of laboratory air was measured for at least 1 min after which the sample was measured for at least 2 min. For human breath analysis, a mouthpiece was fitted to the SESI intake line and the aux gas flow rate was set to 2 arbitrary units (a.u.).

2.3. Data treatment and analysis

The raw files were converted to a user-accessible Excel format with the intensity over time profile for every feature using the open-source software MZmine2 (Pluskal, Castillo, Villar-Briones, & Oresic, 2010). The following data processing steps were carried out: scan-to-scan filtering with a Savitzky-Golay filter (5 datapoints), mass detection (noise cut-off 10,000), and ADAP-chromatogram builder (minimum group size 30 scans, minimum intensity 1E04). The Excel files were post-processed with a self-written Python script, which sorted the measured features into *noise* and *sample derived* based on their relative intensity compared to the background. For analysis of MS/MS experiments, averaged MS/MS spectra were used, exported from FreeStyle (Thermo Fisher Scientific, Waltham, MA, USA). Compound identification was performed by matching HR-MS *m/z* values against the METLIN (Smith et al., 2005) database or a molecular formula calculator (Patiny & Borel, 2013) (for more details, see S2.3).

3. Results and discussion

3.1. Measurement of synthetic allicin at different SESI temperatures.

In most GC measurements, allicin degrades in the heated injection port or during the chromatography and is therefore not detectable. This problem can be circumvented by using a SESI-Orbitrap MS system. We showed that varying the temperature at the intake line, ionizer core and intake capillary had a huge impact on the composition and intensity of measured volatiles. Allicin has a molecular formula of $C_6H_{10}OS_2$ with an $[M + H]^+$ of *m/z* 163.0246. The SESI-Orbitrap MS apparatus used has a resolution of 70,000 and usually works within an error range of 3 ppm. A feature with the same exact mass as protonated allicin was clearly visible in all tests, but not in the background, therefore this feature was assumed to be non-degraded allicin. Furthermore, a small amount of allicin dimer ($[2\ M + H]^+$ of *m/z* 325.0419) was found. This is not a covalently bonded molecule, but a so-called proton-bound dimer typically seen under electrospray MS conditions (Dryahina, Polášek, Smith, & Španěl, 2021). Additionally, among the most intensive features were the $[M + Na]^+$ (*m/z* 185.0065) and $[M + NH_4]^+$ (*m/z* 180.0511) species of allicin as well as $[M + H]^+$ isotopes of allicin with ^{13}C (*m/z* 164.0279) or ^{34}S (*m/z* 165.0204) incorporated (see S3.2). The temperatures of the intake line, ionizer core and intake capillary are known to have considerable, sometimes concurring, or antagonising effects. For example, higher temperatures influence the electrospray formation and ionisation capacity (Barrios-Collado et al., 2016), but heat-labile molecules are more strongly degraded at higher temperatures. A further complication is that the degradation of a highly ionisable species, in view of charge competition between different molecules, can result in other species being observed at apparently higher intensities. These effects cause difficulties when comparing results over several temperatures. Nevertheless, as shown in Fig. 2a, the intensity of detected allicin decreased with increasing temperature of the SESI unit. The highest intensity was observed at 30 °C with 6.8×10^8 molecules per scan.

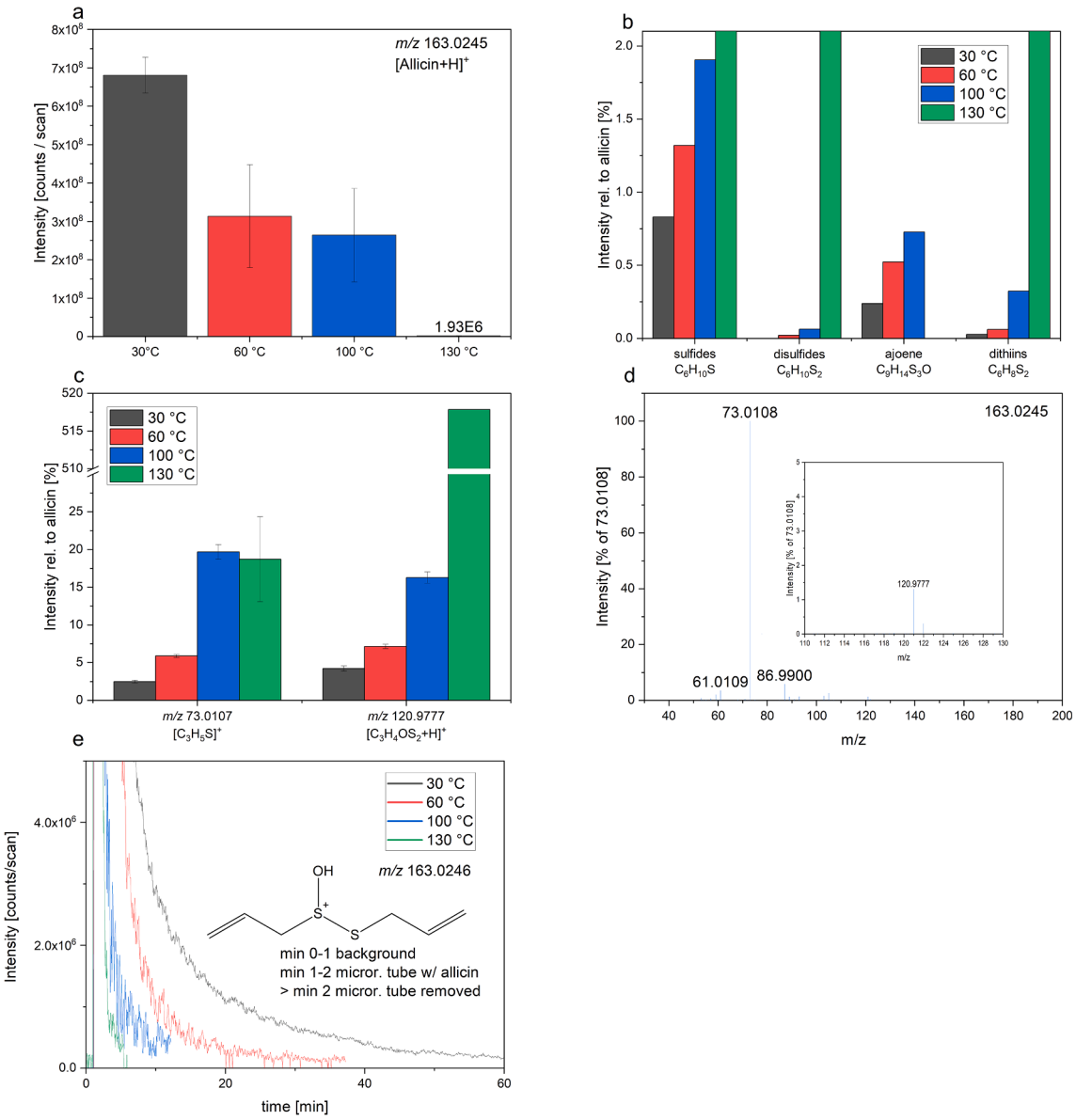

Fig. 2. (a) Intensity of allicin in the gas phase over an open microreaction tube at different temperatures of the SESI unit in a SESI-Orbitrap MS system. (b) Relative intensity of products of heat-induced reactions of allicin, cut off at 2 % relative intensity for clarity (c) Relative intensity of the most intensive fragments or protonated molecules smaller than allicin. (d) MS/MS spectrum of $^1H^{12}C^{32}S$ isotopomer of protonated allicin (*m/z* 163.0245). (e) The time-resolved allicin signal after placing an open microreaction tube with synthetic allicin solution in front of the machine for 1 min with subtraction of the 0–1 min background; the tube is removed after 2 min. The experiments for a, b, and c were performed n = 3; c 130 °C for *m/z* 120.9777 is shown without error bars for clarity.

Considering the scan rate of 3.7 Hz (1 scan consists of 10 microscans) and the intake volume of approximately 0.8 L min^{-1}, the gas phase concentration of the measured allicin was at least 0.3 nmol m^{-3}. In negative controls with an empty microreaction tube, only one single unidentifiable molecular species was observed and that was only consistently present in all three replicates of the 100 °C run. This result suggests that volatiles potentially emitted from the reaction vessel did not interfere with the measurements.

Although the SESI-Orbitrap MS detected protonated allicin molecules, finding fragments and degradation products of this reactive, labile molecule was not surprising. Several pathways to these chemical species are possible. Heat-induced unimolecular (or in solution, bimolecular) reactions of allicin can yield neutral molecules, which are later protonated in the SESI to $[M + H]^+$ species. These can include transformation to sulfides, disulfides, polysulfides, ajoene, dithiins (Lawson & Hunsaker, 2018), and smaller degradation products such as thioacrolein (in Fig. 3, the source of the two dithiins) and 2-propenesulfenic acid. Protonated allicin could fragment to 2-propenesulfenic acid and the thioallyl cation, $CH_2 = CHCH_2S^+$ (*m/z* 73.0107). Finally, in the gas phase, protonated allicin could lose propylene (C_3H_6) giving acylic/cyclic/bicylic *m/z* 121 $C_3H_5OS_2$ species **A-D**, corresponding to protonated forms of 5*H*-1,2-dithiole 1-oxide. Features with the corresponding masses for the protonated sulfide $C_6H_{10}S$ (115.0576 *m/z*), disulfide $C_6H_{10}S_2$ (147.0297 *m/z*), ajoene ($C_9H_{14}S_3O$, 235.0280 *m/z*), and dithiins ($C_6H_8S_2$, 145.0140 *m/z*) were found. The amounts of these compounds relative to the intensity of allicin for the different temperatures are shown in Fig. 2b. The intensity for all these molecules increased with the SESI-temperature.

The two most abundant species at all tested temperatures, besides the allicin variants, are *m/z* 73.0107 and *m/z* 120.9777. Because these two species might arise either via thermal degradation and protonation in the first case, or fragmentation following protonation in both cases, their exact nature remains ambiguous unless how they arise can be clarified. As seen in Fig. 2c, their abundance increased similarly with increasing temperature up to 100 °C. At 130 °C, the intensity of *m/z* 73.0107 did not increase further, whereas *m/z* 120.9777 became five times more intense than allicin. This suggests that the *m/z* 73.0107 is most likely the ionisation-induced fragment $CH_2 = CHCH_2S^+$ rather than the $[M + H]^+$ of thioacrolein, which is known to be thermally stable. However, given the early temperature dependence of the *m/z* 73 peak, it is possible that some thioacrolein is still formed at lower temperatures and protonated in competition with ionisation-induced fragmentation (Block, 2010). The *m/z* 120.9777 species is more likely the $[M + H]^+$ of a thermal degradation product. To further clarify this point, MS/MS spectra of allicin were recorded (see Fig. 2d). While *m/z* 73.0108 is the major fragmentation product, only minor amounts *m/z* 120.9777 were observed. MS/MS spectra of the ^{13}C and ^{34}S isotopes of allicin gave similar results (see S3.3). This further points towards *m/z* 73.0107 being an ionisation-induced fragment and the latter being primarily a thermal degradation product, with a small fraction derived from fragmentation. The molecular formula calculator from ChemCalc.org identified *m/z* 73.0108 as $[C_3H_5S]^+$ with an error of −0.72 ppm, and *m/z* 120.9777 as $[C_3H_4OS_2 + H]^+$ with an error of −0.55 ppm; $[C_3H_5S]^+$ is commonly described as a CID-fragment of not only allicin, but other allyl sulfides, disulfides and polysulfides (Calvey, Roach, & Block, 1994b; Zhou et al., 2017).

Several papers have previously reported *m/z* 120.9777, as the predominant fragment of allicin under ESI conditions, formed by loss of propylene, C_3H_6, and documented by MS/MS in most cases (Chen, Wortmann, Zhang, & Zenobi, 2007; Ferary & Auger, 1996; Wang, Song, Hang, Zhang, & Chen, 2009; Zhou et al., 2008; Zhou et al., 2017). At the same time this peak is absent in several other MS studies using chemical ionization conditions where protonated intact molecules are seen rather than fragments (Block et al., 2010; Calvey et al., 1994a). Various structures have been proposed for the $C_3H_5OS_2$ species (Chen et al., 2007; Wang et al., 2009; Zhou et al., 2008; Zhou et al., 2017). As shown in Fig. 3, we suggest that following the loss of C_3H_6 from protonated allicin in the cited literature cases, the charge-delocalised allylSSO$^+$ ion **A** undergoes intramolecular cyclisation via ion **B** giving bicyclic oxysulfonium ion **C**, which should be the most stable protonated form of the $C_3H_4OS_2$ neutral compound 5*H*-1,2-dithiole 1-oxide (favoured isomer with the divalent sulfur in conjugation with the C=C bond). 5*H*-1,2-Dithiole 1-oxide is currently unknown but should be isolable. Based on our results, we suggest that depending on the type of spectrometer

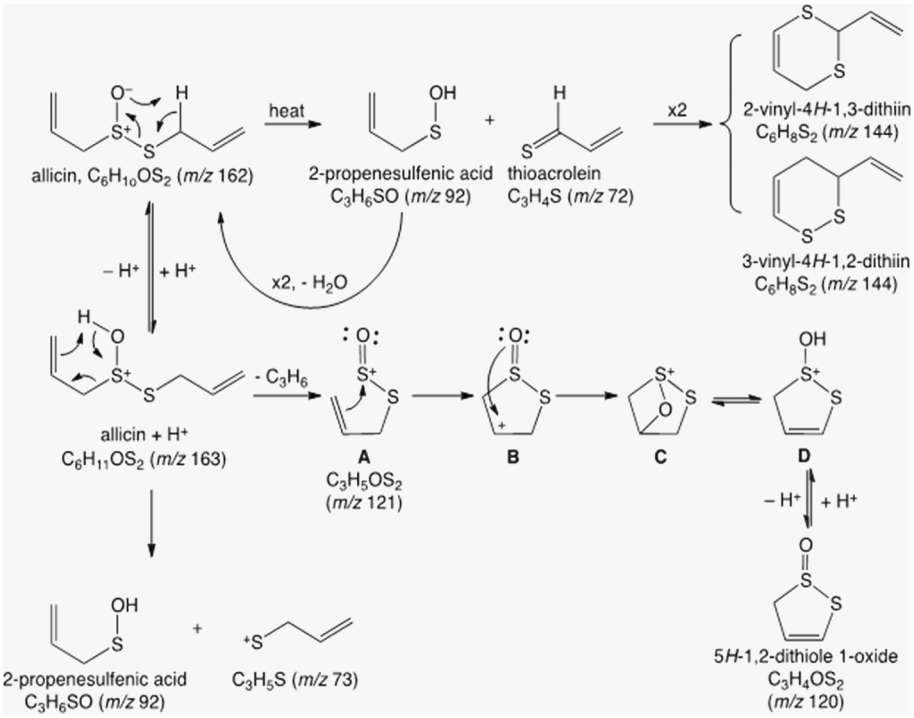

Fig. 3. Proposed degradation and fragmentation pathways of allicin.

employed, $C_3H_5OS_2$ can directly result from ionisation-induced fragmentation as well as protonation/heat-induced gas phase decomposition, or both.

For all the considered molecules, the measurements at 130 °C yielded the highest amounts of products and at 30 °C the lowest amounts relative to allicin. Of the temperatures tested, this suggested 30 °C to be the most suitable for allicin measurements in the gas phase, because it caused the least amount of degradation. However, sulfur compounds tend to adhere to surfaces (Herrera, Ysinga, & Jenkins, 2019; Slavin et al., 2012), and in order to start a new measurement run, all signals needed to be below a certain threshold, i.e., the machine should be cleared of all traces of the preceding sample. Therefore, in addition to measuring the intensities of allicin, the time between measurements has to be characterised. Therefore, after 1 min of background measurement, an open microreaction tube with allicin solution was placed 1 cm in front of the intake line for 1 min, and measurements continued for up to 70 min after removing the tube. Fig. 2e depicts the intensity of the allicin signal, shown only to approximately 1/3 peak height (5×10^6 molecules/scan) for better visibility. The same degree of signal decay takes 50 min at 30 °C, 20 min at 60 °C and only 10 min at 100 °C (for more details, see S3.4). Because of the impractically long times needed to clear the machine between runs at the lowest operating temperature, 60 °C was selected as the operating temperature for the short 2 *sec* period in the SESI unit before allicin reached the high vacuum in the Orbitrap and 15–20 min were allowed between runs for signal decay.

3.2. The volatiles of fresh garlic, onion and ramsons

Fresh garlic cloves, similarly sized onion pieces, or ramsons leaves were crushed in the funnel in front of the SESI-Orbitrap in triplicate experiments with the SESI temperature set to 60 °C.

The intention was to assess the suitability of the SESI-Orbitrap method to distinguish between the volatilomes of the three alliums relying on their already known chemistry (Fig. 1) and gain new knowledge on the kinetics of VOSC production. Thus, we monitored the production of the sulfenic acid precursor molecules and the expected major thiosulfinate products with time. For garlic the precursor is $[M + H]^+$ of 2-propenesulfenic acid ($[C_3H_6OS + H]^+$, *m/z* 91.0212) and the $[M + H]^+$ of allicin ($[C_6H_{10}OS_2 + H]^+$, *m/z* 163.0245) is the end product. For all elements, stable isotopes occur at distinct masses and abundancies. Thus, the incorporation of, e.g., ^{13}C into biogenic molecules reflects this natural isotope abundance. Therefore, in addition to the main molecular (parent) peak, predictable isotope peaks should be visible in the MS spectra. Therefore, to strengthen the identification of a particular molecular species, a search was made for daughter peaks at the appropriate masses and with the expected intensities. In this way, ^{13}C and ^{34}S isotope peaks were investigated to verify the number of C or S atoms, respectively. As shown in S3.6, the SESI-Orbitrap MS set up used can distinguish, for example, between the mass differences between ^{13}C and ^{12}C (+1.0033 *m/z*) or ^{2}H and ^{1}H (+1.0063 *m/z*). For the garlic samples, isotope analysis was performed for allicin and for 2-propenesulfenic acid. Fig. 4 depicts the intensity of the isotope peaks relative to the mother peak and the intensity expected based on the assigned number of C or S atoms and the natural abundance of ^{13}C and ^{34}S. While the intensities for ^{13}C are minimally lower than expected, the intensities for ^{34}S are slightly higher. Overall, the expected and measured intensities are in good agreement, thus confirming the assigned molecular formulae.

For garlic, allicin was by far the most abundant molecule with nearly 1.6×10^9 molecules/scan. The next most intense molecular species were the allicin dimer, with 32 % of the abundance of allicin, and $C_3H_4OS_2$ with 11 %. In this complex biological sample, it is impossible to distinguish if this is the above discussed thermal degradation product of allicin or an independently generated molecule (for more details, see S3.5). Only 13 of the postulated chemical species had at least 2 % of the intensity of allicin, whereby two are alternative ionisation products, one

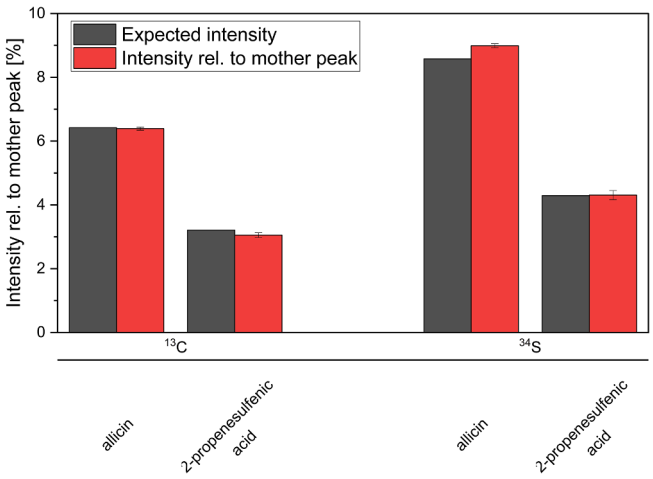

Fig. 4. Intensity of the ^{13}C or the ^{34}S isotope peak relative to the intensity of the mother peak for verification of the molecular formula of allicin and its precursor 2-propenesulfenic acid. All measurements are n = 3.

is the allicin dimer and four are isotope peaks of allicin, highlighting again allicin's importance as the quantitatively major early product making up garlic smell.

A considerable advantage of the SESI-Orbitrap MS system over most MS methods is that it allows kinetics to be followed by using the possibility to measure in a time resolved manner with the scanning speed of the mass spectrometer, in this case 0.4 Hz. As described above, the known VOSC chemistry of injured garlic begins with the production of 2-propenesulfenic acid from alliin upon cell damage. Therefore, 2-propenesulfenic acid should be visible before allicin but decline rapidly as the condensation to allicin is fast (Fig. 1a). As seen in Fig. 5a and b, a feature with *m/z* 91.0212 $[C_3H_6OS + H]^+$ is visible before *m/z* 163.0245 $[C_6H_{10}OS_2 + H]^+$, but at much lower concentrations. Additionally, the intensity of 2-propenesulfenic acid decreases after roughly 20 s, most likely because it condenses to allicin and the precursor alliin becomes depleted. In contrast, because onions produce mainly isoalliin instead of alliin, 1-propenesulfenic acid is produced and rapidly converted by the LFS enzyme into propanethial *S*-oxide (LF), both with the same molecular formula C_3H_6OS and therefore not distinguishable in this machine. However, a small amount of the 1-propenesulfenic acid spontaneously condenses into bis(1-propenyl) thiosulfinate which than rearranges into zwibelanes, once more having the same molecular formula $C_6H_{10}OS_2$ as allicin (Fig. 1c). Again the kinetics and absolute amounts shown in Fig. 5c and d match this chemistry as *m/z* 91.0212 $[C_3H_6OS + H]^+$ is visible earlier and in much higher abundances than the C6 compounds. Further, no decrease in the intensity of C_3H_6OS was observed, as this is here no intermediate as for allicin, but the end-product.

Ramsons also produce alliin, which results in kinetics similar to that seen for garlic, but in addition they also have considerable amounts of isoalliin. However, because ramsons do not have the LFS, only 1-propenesulfenic acid and zwiebelanes form, which are again not distinguishable from 2-propenesulfenic acid and allicin, respectively, in our instrument. In contrast to the other two alliums, ramsons also produce high amounts of methiin (*S*-methyl cysteine *S*-oxide), present in greater amounts than alliin and isoalliin. When acted upon by alliinase, this produces not only methanesulfenic acid ($[CH_4OS + H]^+$ *m/z* 65.0056) and dimethyl thiosulfinate ($[C_2H_6OS_2 + H]^+$ *m/z* 110.9933), but also allicin and the isomeric allyl methyl thiosulfinate ($[C_4H_8OS_2 + H]^+$ *m/z* 137.0089), in accordance with previous studies of ramsons volatiles (Sobolewska, Podolak, & Makowska-Wąs, 2015). Heterocondensation giving isomeric allyl methyl thiosulfinates is statistically more likely than homocondensation giving allicin and dimethyl thiosulfinate, as is observed. The predicted chemistry was observed, as shown in Fig. 5e and Fig. 4f. The intensity of dimethyl thiosulfinate relative to that of

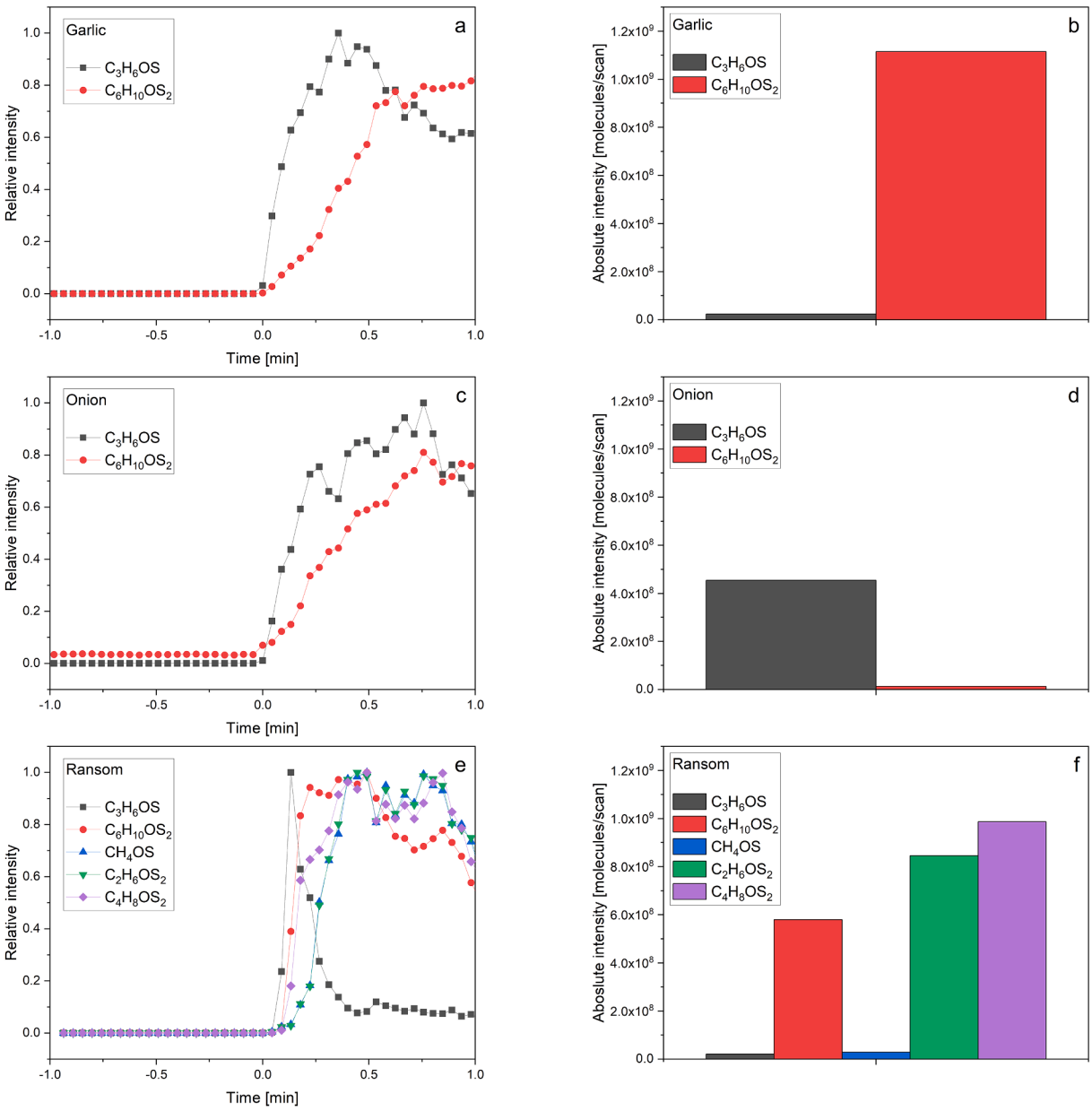

Fig. 5. (a), (c), and (e) kinetics and (b), (d), and (f) show the absolute intensities of selected VOSCs at 0.5 min for garlic (a), (b), onion (c), (d), and ramsons (e), (f). All species were found as $[M + H]^+$. The kinetics show each molecular species normalised to its own maximum and the first appearance of a VOSCs was set to zero.

allicin in Fig. 5f may be exaggerated due to the greater volatility of the former since HPLC analysis of ramsons extracts shows lower levels of dimethyl thiosulfinate relative to allicin (Sobolewska et al., 2015). As C_3H_6OS is visible before CH_4OS, the conversion of alliin/isoalliin to the corresponding sulfenic acids, and subsequently to the corresponding thiosulfinates, seems to be faster than the conversion of methiin. In contrast to the other alliums the 1- or 2-propenesulfenic acid signal quickly deteriorates. This could be due to a lower pool of alliin or to a higher reaction speed of the 2-propenesulfenic acids as more condensation partners are available. Thus, using the SESI-Orbitrap MS procedure it was possible to discriminate between garlic, onion and ramsons based on the intensity of their respective C6 and C3 VOSCs. Allicin was shown to adhere to the instrument to some extent (see Fig. 2e). This could potentially quench allicin signals in the beginning and exaggerate the signal at the end of the measurement. Future kinetic studies should try to elucidate this.

3.3. *Allicin detected in exhaled human breath*

One of the potential applications of allicin might be in treating human lung infections. Consuming garlic or allicin orally is generally considered unlikely to achieve therapeutically appropriate levels systemically in the body (Reiter et al., 2017). This is because allicin is rapidly hydrolysed to 2-propenethiol and is titrated out by reacting with glutathione and other thiols in cells and in the bloodstream. Allicin metabolites such as allyl methyl sulfide (AMS, C_4H_8S) are rapidly formed and have been detected by HPLC as a major contributor to the unpleasant smell of garlic breath (Lawson & Hunsaker, 2018). Other studies using GC–MS have shown AMS and other metabolites in the urine and breath after consumption of garlic or ramsons (*Allium ursinum*) (Scheffler, Sharapa, Amar, & Buettner, 2018) Against this background, the measurement of allicin after garlic consumption in human breath is of considerable interest. SESI-Orbitrap MS is a method that was originally developed for analysis of biomarkers in breath (Nowak et al., 2021).

In the present study, as a control prior to allicin consumption, one deep exhalation per minute into the machine was performed by a 26-year-old male subject. After 15 exhalations, one clove of raw garlic was crushed and consumed by the subject with a minimum amount of bread and approximately 50 mL of water. Subsequently, 4 measurement sets with 15 exhalations, with a 15 min break between each set, were performed. Exhaled breath from the lungs passes through the buccal cavity. Therefore, any VOSCs adhering to the mucosal surface in the mouth area, and swept out by exhalation, will also be detected. Fig. 6a shows the absolute intensity of allicin compared to the known lung biomarker 2-octenal (Singh et al., 2019). Allicin, with an initial maximum intensity of 4.7×10^6 molecules/scan, was clearly visible after, but not before, garlic consumption. Because of the limited ionisation capacity of the SESI, the presence of such well ionisable species in high concentration quenches all other signals, which explains the observed decreasing intensity of the 2-octenal signal. In Fig. 6b a logarithmic scale was used to show the maximum allicin intensity per exhalation over 63 exhalation events, taking 130 min. It can be seen that the intensity of allicin declines rapidly as it is either swept out of the buccal cavity or metabolised. Assuming an exponential decay, regression analysis with the smoothed data yielded a half-life for allicin of approximately 12 min in exhaled breath after eating raw garlic (for the calculations, see S3.7).

W. Zhou et al. (2017) analysed garlic breath using an APPI-MS system, but with a less sensitive and lower resolution detector than our SESI-Orbitrap MS and did not report finding allicin in human breath. This is perhaps not surprising given that the subject gargled with 100 mL of water before the breath was analysed. Further, the exhaled breath only after garlic consumption contained a myriad of other components (see Supplementary 2) like *m/z* 75.0264 $[C_3H_6S + H]^+$, which matches the molecular formula of 2-propenethiol, or *m/z* 89.0422 $[C_4H_8S + H]^+$, which matches the molecular formula of AMS, which are commonly described as principle components of garlic breath (Munch & Barringer, 2014; Zhou et al., 2017). The C_4H_8S signal intensity decreased in the first 60 min, but then increased again (Fig. 6d). A feature with the molecular formula C_4H_8S was also produced by crushed garlic (see Supplementary 2), and presumably the early, decreasing signal was from garlic VOSCs trapped in the buccal cavity, whereas the later, increasing signal may have come from AMS derived from allicin metabolism and exhaled through the lungs. This interpretation is supported by the results of a further experiment, in which garlic was simply chewed and spat out (i.e. not swallowed). No AMS increase was visible after 60 min, only a continual decrease in signal up to 120 min (Fig. 6c). In future studies breath analysis could be enhanced by using machinery to standardize exhalation, e.g. EXHALION (Fossiliontech, Madrid, Spain), which is specifically designed to work with the Super-SESI.

4. Conclusions

Allicin is the major active component in crushed garlic, but its reactivity and heat instability cause challenges in the analysis of gas phase samples. Volatile compounds are often analysed by GC–MS where temperatures >100 °C are commonly used. These conditions do not necessarily reveal the natural volatilome because compounds not volatile at lower temperatures might be volatilised at higher operating temperatures but with artefact production likely with heat-labile compounds, such as allicin.

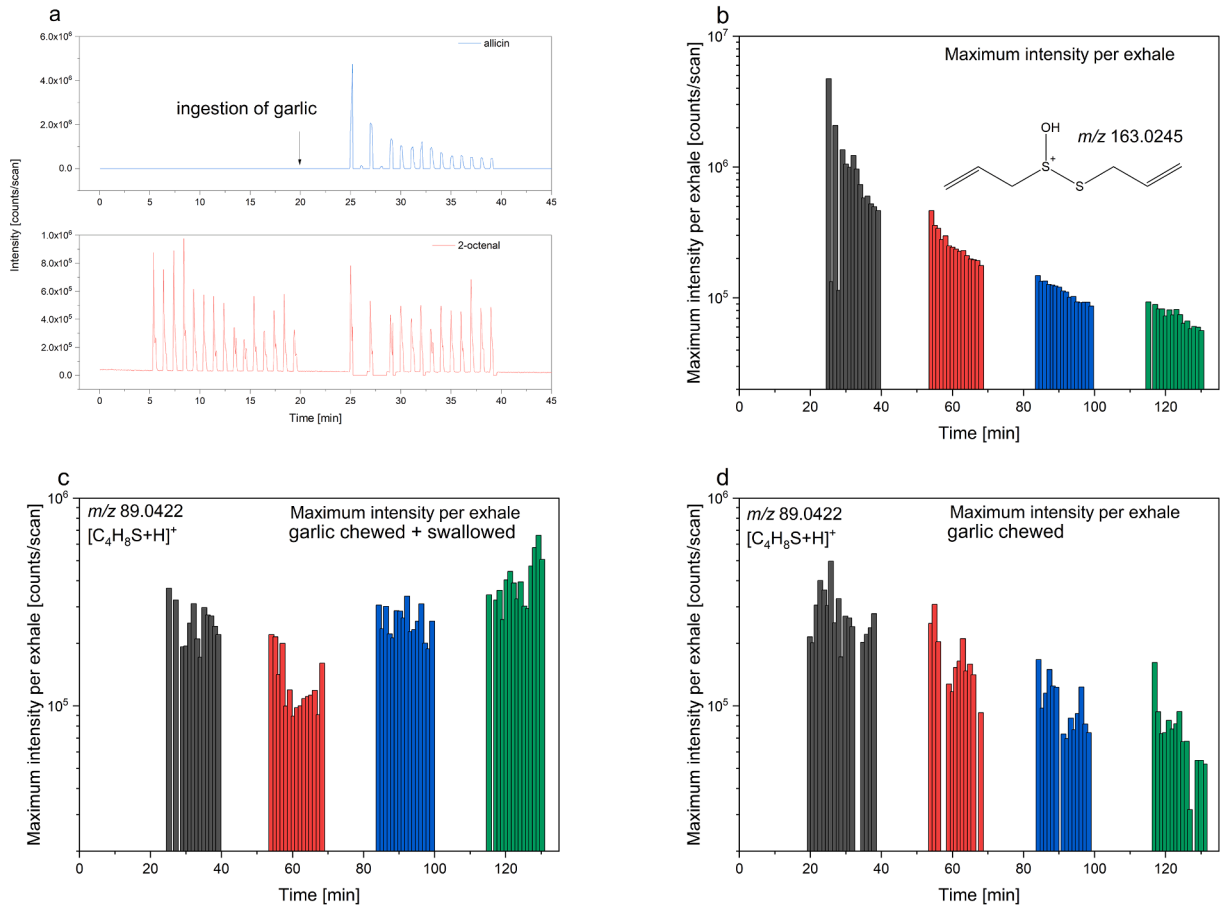

Fig. 6. Human breath SESI-Orbitrap MS analysis showing: (a) The intensities of allicin and a known human lung metabolite 2-octenal before (min 0–15) and after consumption of a raw garlic clove (min 20–40) in human breath, (b) the maximum intensity per exhale of allicin in human breath after the consumption of raw garlic, (c) the intensity of C_4H_8S in human breath after chewing and swallowing (c) and just chewing (d) raw garlic.

With the use of novel SESI-Orbitrap MS techniques, we were able to measure intact allicin from ambient air. We showcased this by measuring a solution of synthetic allicin. The major breakdown products of allicin (or protonated allicin) were the thermal degradation product $[C_3H_4OS_2 + H]^+$, which we propose to be 5*H*-1,2-dithiole 1-oxide, and $[C_3H_5S]^+$ which is most likely a fragment generated by the ionisation procedure. Furthermore, the kinetics of VOSCs arising from freshly crushed garlic, onion, and ramsons were recorded every 3 s. In garlic, the allicin precursor 2-propenesulfenic acid showed a sharper increase and a maximum concentration roughly 20 s after the start, while allicin continuously increased in intensity over time. In onion, no decrease in C_3H_6OS was detectable since this molecular formula is shared by both the precursor 1-propenesulfenic acid and the lachrymatory factor. In ransoms, the 2-propenesulfenic acid again is visible before all alliin and methiin derived products, but only for a very short period. Because allicin is a potential candidate for treating lung infections via the pulmonary route, human breath analysis after garlic consumption, measuring the intensity of allicin over multiple orders of magnitude, was carried out.

Moreover, the potential of SESI-Orbitrap MS measurements, showcased by the experiments presented here, could be applied, with only minor changes in the experimental design, to other flavour and fragrance compounds. This is true not only for reactive sulfur compounds, but also for very many volatile compounds in foods and fragrances.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2022.133804.

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