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Synthesis of the flavour precursor, alliin, in garlic tissue cultures

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Abstract

The path of synthesis of alkyl cysteine sulphoxides, or flavour precursors, in the *Alliums* is still speculative. There are two proposed routes for alliin biosynthesis, one is from serine and allyl thiol while the other is from glutathione and an allyl source via γ glutamyl peptides. The routes have been investigated by exposing undifferentiated callus cultures of garlic and onion to potential pathway intermediates. After a period of incubation of 2 days the callus was extracted, and analysed for flavour precursors and related compounds by HPLC. Standards of alliin, isoallin and propiin were synthesised and their identity confirmed by HPLC and NMR. Putative intermediates selected included the amino acids serine and cysteine, as well as more complex intermediates such as allylthiol, allyl cysteine and glutathione. Both garlic and onion tissue cultures were able to synthesize alliin following incubation with allylthiol, and cysteine conjugates such as allyl cysteine. The ability of the tissue cultures to form alliin from intermediates was compatible with the proposed routes of synthesis of alliin.

Keywords: *Allium sativum*; *Alliaceae*; Garlic; Tissue cultures; Flavour precursor; Alliin

1. Introduction

The domestic *Alliums* (onion, garlic, chives and leek), contain high concentrations of organic sulphur compounds especially in the vegetative tissue of the swollen leaf bases and leaves (Granroth, 1970; Whitaker, 1976; Block, 1992). The sulphur is primarily as alkylcysteine sulphoxides and γ-glutamyl peptides which together make up over 70% of the total sulphur in garlic (Lawson, 1996). The alkyl cysteine sulphoxides, alliin, isoalliin, methiin (Table 1) and are degraded by alliinase enzymes on tissue disruption to release powerful smelling volatiles that give the characteristic odour and flavour to the domestic *Alliums*. In garlic the predominant flavour precursor is alliin with lower concentrations of isoalliin and methiin and trace amounts of propiin. In onion the major flavour precursor is isoalliin with smaller amounts of methiin and trace amounts of alliin and propiin. The relationship between the alkylcysteine sulphoxides and γ-glutamyl peptides is that γ-glutamyl peptides may be intermediates in the formation of the cysteine sulphoxides (Lancaster and Shaw, 1991).

In garlic the major site of synthesis is in the leaves (Granroth, 1970) but some synthesis is able to occur in the cloves. Two paths of synthesis of alliin have been proposed (Fig. 1) (Lawson, 1996). In one serine combines with an unknown allyl thiol source to give allyl cysteine which is oxidised to alliin, while in the other glutathione derived from cysteine combines with an allyl source to form allyl glutathione followed by successive loss of glycine and glutamate and finally oxidation of allyl cysteine to alliin. Evidence for the ability of plants to form *S*-allyl-L-cysteine from an allyl source and serine is

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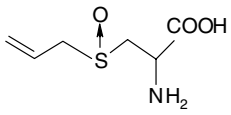
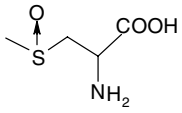
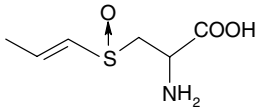
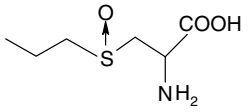
Table 1
Alk(en)yl cysteine sulphoxides (flavour precursors) in garlic and onion

Flavour precursor	Formula
S-allyl cysteine sulphoxide, alliin	
S-methyl cysteine sulphoxide, methiin	
trans S-1-propenyl cysteine sulphoxide, isoalliin	
S-propyl cysteine sulphoxide, propiin	

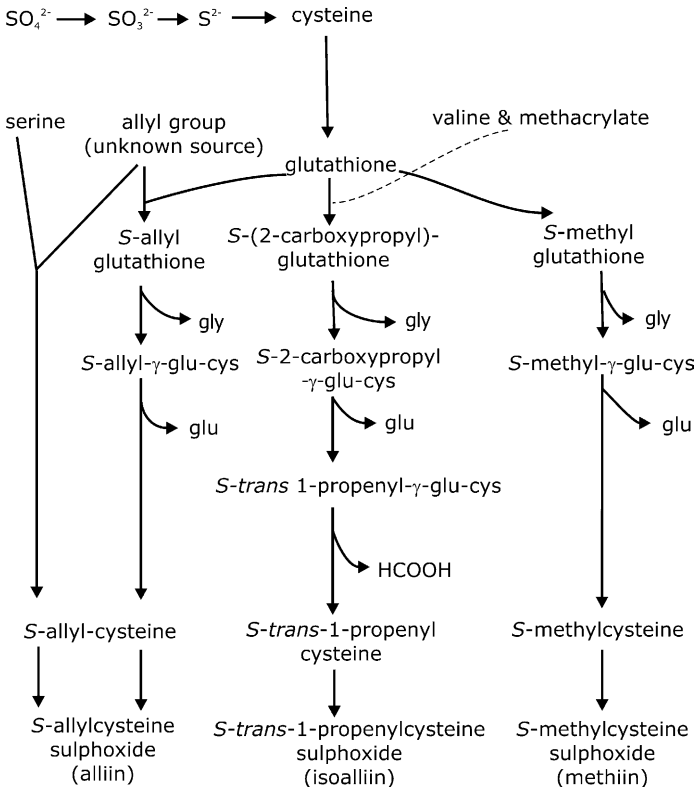


Fig. 1. Proposed paths of synthesis of alliin showing origins as serine allyl and glutathione (Lawson, 1996).

provided by the formation in vitro of *S*-allyl-L-cysteine from allylmercaptan and *O*-acetyl serine, in the presence of cysteine synthase extracted from *Spinacia oleracia* (Murakoshi et al., 1985). Ikegami et al. (1988) also showed that methyl thiol and allyl thiol could be converted to the cysteine conjugates, methyl cysteine and allyl cysteine by the enzyme β-cyano-L-alanine synthase from *Spinacia oleracia*. There are no reports of whether these reactions could occur *in planta*. Glutathione is thought to occupy a key role in the early stages of synthe-

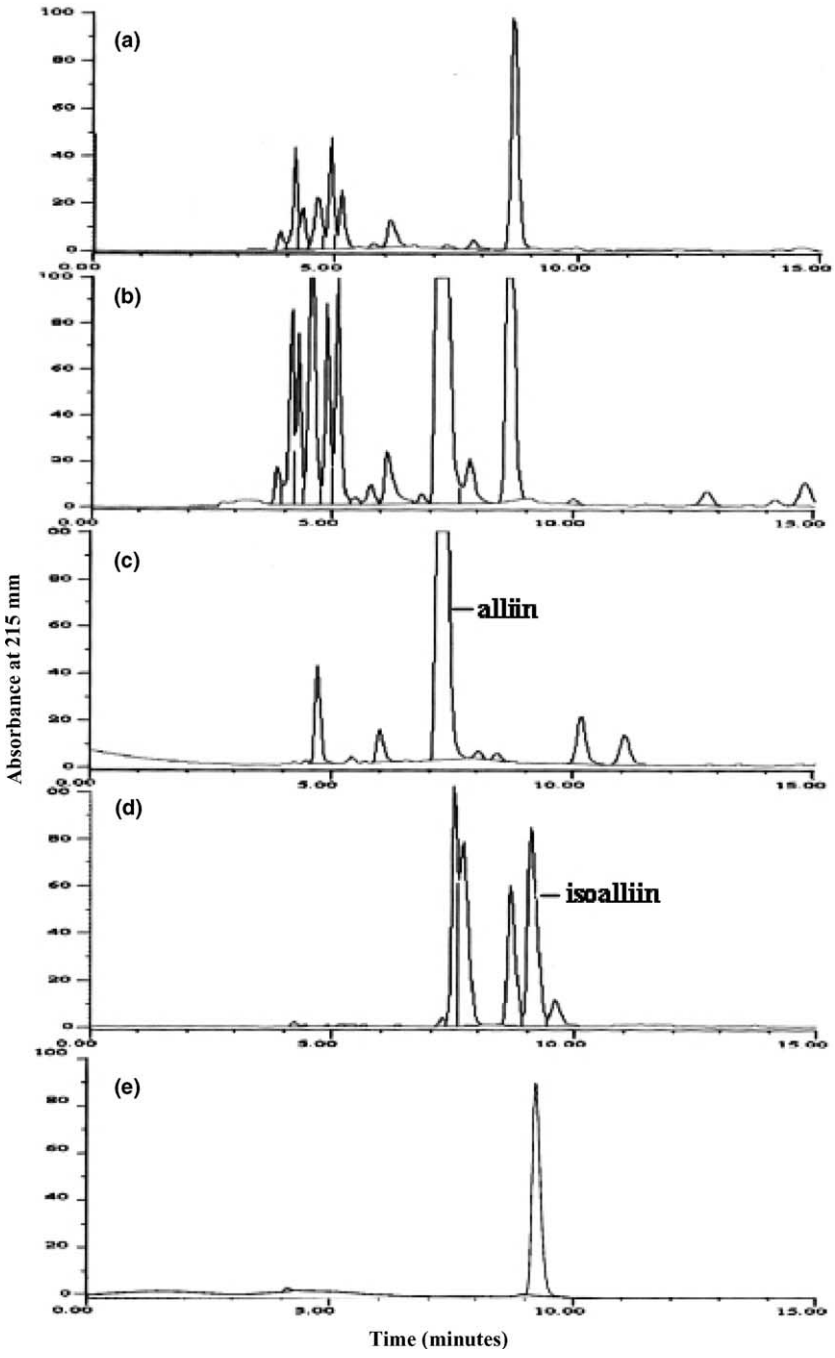


Fig. 2. HPLC traces comparing the profile of the extract from equivalent amounts of onion (a) garlic clove (b) and of synthesised flavour precursor standards, methiin, ethiin, alliin and propiin (c) synthetic alliin (d) and reaction mixture containing the isomer of isoalliin, *trans*(+)-*S*-propenyl-L-cysteine sulphoxide (e).

sis of the flavour precursors in both garlic and onion (Lancaster and Shaw, 1988). The final oxidation step from *S*-allyl-L-cysteine to alliin has been shown to occur in differentiating tissue cultures of garlic (Ohsumi et al., 1993).

Biosynthesis of the flavour precursors in *Alliums* has been investigated previously by exposing either tissue cultures, excised organs or intact plants to potential intermediates (Lancaster and Shaw, 1989; Lancaster et al., 1989; Turnbull et al., 1980). The advantage of using tissue cultures is that the impermeable cuticle on the leaf, root or cloves which prevents or reduces uptake is avoided. In addition because the tissue cells are intact the alliinase enzyme, which would normally cleave alkyl cysteine sulphoxides in damaged cells, is contained safely within the intact tissue. Cell suspensions, callus and differentiated tissue have been used successfully for studies of isoalliin synthesis in onion (Selby et al.,

1980; Turnbull et al., 1980) and alliin synthesis in garlic (Ohsumi et al., 1993).

The tissue chosen for an examination of the biosynthesis of alliin was garlic callus with the use of onion callus as a comparison.

2. Results and discussion

2.1. Separation of flavour precursors

A comparison of the flavour precursors in extracts from garlic cloves and onion bulbs and synthetic standards of the synthesised flavour precursors alliin and isoalliin is shown in Fig. 2(a)–(d). The HPLC of the onion had a single peak of isoalliin (Fig. 2(a)) whereas the garlic clove had a double peak of alliin and isoalliin (Fig. 2(b)), synthetic alliin on its own (Fig. 2(c)), a reaction mixture containing isoalliin and related compounds (Fig. 2(d)) and purified isoalliin obtained from this mixture (Fig. 2(e)).

2.2. Exposure of callus to potential intermediates for alliin and isoalliin synthesis

Portions of undifferentiated garlic callus originating from several clove apices were selected for uniformity

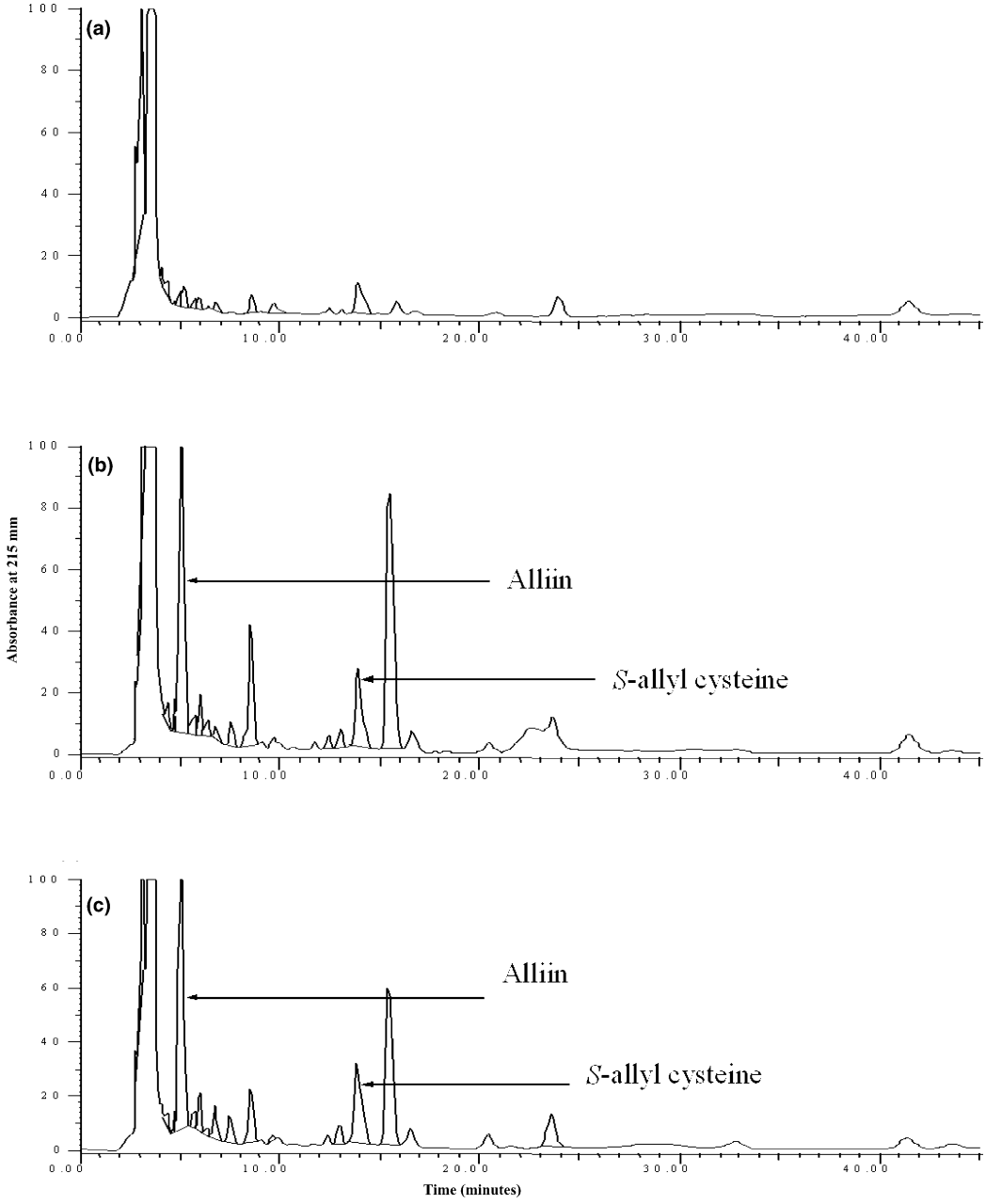


Fig. 3. HPLC traces comparing the profile of the extract from equivalent amounts of garlic callus alone (a) 2 days after incubation with 10 mM allyl cysteine (b), or 10 mM allyl thiol (c).

of growth rate and appearance. The callus as 0.5 g portions was transferred to Universal vials containing the maintenance medium to which had been added separately 10 mM cysteine, glutathione, serine, allyl cysteine or allyl thiol. The potential intermediates were sterilized by passage through 0.45 μm filters before adding to the autoclaved medium. Parallel cultures were incubated on maintenance medium alone. After 2 days the callus was extracted and the extracts separated by HPLC. Comparisons of HPLC profiles were made with the HPLC profiles of extracts taken from intact garlic clove, root and leaves and also retention times of the synthetic compounds.

There were no alliin or allyl cysteine peaks in the control callus which had been on maintenance medium alone for 2 days (Fig. 3(a)). After an incubation period of 2 days no alliin peaks were detected in the callus exposed to cysteine, glutathione, and serine (data not shown), whereas significant amounts of alliin was formed in the garlic callus following incubation with allyl cysteine and allyl thiol (Fig. 3(b) and (c)). In comparison to the incorporation of allyl cysteine and allyl thiol, the lack of response to serine and glutathione by the callus is difficult to understand. In a comparable study with onion callus the amino acid valine which is thought to be on the path of synthesis of isoalliin did not affect the production of isoalliin (Selby et al., 1980). Exposure of the callus to C14 valine however led to labelling in the isoalliin indicating that the pathway from valine to isoalliin was operating albeit slowly. Equally when onion callus was exposed to C14 serine or C14 cysteine there was significant uptake of the label into isoalliin and although the amount of amino acid that this represented was not assessed it was considered to be small. The amino acids serine and glutathione may be taken up by the garlic callus but could be restricted in their entry to the site of the secondary product pathway, i.e., that part of the pathway that leads from cysteine and glutathione to the synthesis of alliin and is specific for the *Alliums*, so that stimulation of alliin synthesis does not occur. In contrast allyl thiol and allyl cysteine may be incorporated into this pathway very readily and this leads to the production of alliin.

Where alliin was synthesised from the fed intermediates, allyl thiol and allyl cysteine, it is possible that the callus was able to metabolise these compounds via the serine–thiol or the glutathione–allyl pathway. Which pathway is operating in the callus would be determined by the presence and activity of the relevant enzymes. The initiation of the serine thiol pathway would be dependent on the presence of a cysteine synthase enzyme and the thiol glutathione pathway on a glutathione transferase enzyme. An enzyme capable of effecting the production of allyl cysteine from allyl thiol was detected in *Spinacia oleracea* by Murakoshi et al. (1985) and Ikegami et al. (1988). It is suggested that for garlic and onion a similar enzyme may be present which can make use of an organic thiol source.

Differentiation of the callus appears to unlock the inhibition to synthesis of the early stages in the path of synthesis of the flavour precursors since the differentiated garlic callus containing small roots showed the accumulation of both alliin and isoalliin. The positive effect of differentiation on the biosynthetic ability of onion and other tissue cultures is well known (Turnbull et al., 1981; Collin, 2001)) and probably relates to the appearance of complex cells and tissue types which are more metabolically competent.

2.3. Precursor feeding experiments using single source garlic callus

The response of the callus to incubation with allyl thiol and allyl cysteine was quite variable despite the selection for physical uniformity of the tissue culture portions at the start of the experiment. Although conditions of culture do favour uniformity, variation does arise in tissue cultures and can be a problem in quantifying a response (Bourgaud et al., 2001). The potential for variability was reduced by bulking the callus from one original callus source.

Potential intermediates, allyl thiol and allyl cysteine all at a concentration of 10 mM, were incorporated into the callus medium as before for cultures of garlic and onion callus both obtained from a single original source. The intermediates were chosen to see whether garlic and onion tissue were able to metabolize allyl thiol and allyl cysteine independently of substrate and plant source. Three replicates were prepared for each precursor and callus source. The tissue was sampled at 2 days. For the controls, no intermediates were added to the medium. The amounts of the allyl cysteine, and alliin produced by each callus was estimated by HPLC based on the response of standards (Table 2).

Following exposure of the garlic and onion callus to allyl thiol, small amounts of alliin were found in all samples and surprisingly in the untreated controls. The presence of allyl thiol caused no significant stimulus to the

Table 2
Accumulation of alliin and, allyl cysteine after exposing replicates of single clone callus of garlic(G) or onion (O) to allyl thiol (At), allyl cysteine (Ac) for 2 days of incubation

	GAt2	GAc2	Control
Garlic			
Alliin (mM)	0.026 ± 0.021	0.592 ± 0.123	0.020 ± 0.023
Allylcysteine (mM)	0.158 ± 0.131	0.936 ± 0.095	0.0 ± 0.0
	OnAt2	OnAc2	Control
Onion			
Alliin (mM)	0.113 ± 0.069	0.208 ± 0.038	0.126 ± 0.053
Allylcysteine (mM)	0.468 ± 0.257	2.182 ± 0.099	0.0 ± 0.0

concentration of alliin. The only treatment that showed a significant accumulation of alliin relative to the controls was the callus samples exposed to allyl cysteine, which might be expected as this intermediate is the closest to alliin in the proposed biosynthetic pathway. No allyl cysteine was detected in the controls but was present in the treated tissue. As allyl cysteine is regarded as an intermediate on the biosynthetic pathway its absence in the control tissue is not unexpected. The effect of feeding allyl cysteine to the callus was that there was a small increase in the alliin and a large accumulation of allyl cysteine. While the link between the allyl cysteine and alliin synthesis appears to be very active, the link between allyl thiol and allyl cysteine, and subsequently alliin appears to be weak.

The pattern of accumulation in response to the two intermediates is very similar in both sources of callus. This is surprising as alliin is not a major flavour precursor in the onion. The other finding is the fact that allyl thiol seems to be capable of making only a small contribution to allyl cysteine and alliin. This might bring into question the role of allyl thiol as a sole source for the synthesis of allyl cysteine and ultimately alliin. The alternative explanation is that the callus is a relatively inactive tissue so that while intermediates such as allyl cysteine may accumulate and be converted easily to alliin, allyl thiol accumulation may be redirected into alternative pathways rather than be converted to allyl cysteine via a limited enzyme complex.

The evidence for the allyl thiol functioning as an allyl source for the synthesis source of allin is provided by the precursor feeding data. It is not clear however whether the allyl thiol provides a pathway intermediate for the serine–thiol pathway or the allyl thiol–glutathione pathway. One approach for deciding on the importance of each is to identify the functional enzymes such as a specific cysteine synthase or glutathione transferase and their relative activities.

3. Experimental

3.1. Initiation of callus cultures of garlic and onion

Garlic cloves of variety Printanor (kindly provided by V. Chovelon, INRA, Avignon) were used as a source of excised shoot apices. These were isolated aseptically and the meristems transferred to Universal vials containing Murashige and Skoog inorganic salt medium, 3% sucrose, 0.1 mg l^{-1} naphthalene acetic acid (NAA), 0.5 mg l^{-1} 2iP (6-(γ-γ-dimethylallylamino purine) and 0.5% Phytogel (Barandiaran et al., 1999). The cultures were maintained at 25 °C on a 12 h light dark cycle with fluorescent lights of 400 μE m^{-2} s^{-1} photon flux density. After 3 months the small plantlets had formed roots which were excised and placed on a callus initiation medium containing Murashige and Skoog inorganic salt medium, 3.0 mg l^{-1} benzylamino purine, 0.5 mg l^{-1} 2,4-dichlorophenoxyacetic acid, 2.0 mg l^{-1} naphthalene acetic acid, 3% sucrose and 0.5% phytogel (Barandiaran et al., 1999). All chemicals were obtained from Sigma. The callus was maintained on the same medium with a monthly subculture. Differentiation was initiated by transferring the callus to the same medium but with the hormones omitted. Onion callus, variety Rijnsburger, was initiated and maintained as described by Dunstan and Short (1977).

3.2. Synthesis of flavour precursors and related compounds

Formation of the flavour precursor alliin required the synthesis of the respective cysteine conjugate. Allyl cysteine was made from cysteine and allyl bromide (Stoll and Seebeck, 1949) and alliin from the cysteine conjugate by the method of Stoll and Seebeck, 1949). The synthesis of *trans* isoalliin presented a problem. The early use of synthetic isoalliin as a marker (Lancaster and Shaw, 1989) did not take into account the fact that the chemical method of synthesis described by (Carson and Wong, 1963) yielded a mixture of *cis* and *trans* forms of isoalliin. It was important that the correct isomer (i.e., *trans*) was used as a marker to identify isoalliin in the garlic and onion. The mixture of the *cis* and *trans* isomers was separated by HPLC and *trans* isoalliin identified by NMR through a characteristic coupling constant of 15.4 Hz between the alkene protons. Full analytical details of NMR, MS and IR) for the alliin,isoalliin and allylcysteine are provided by Krest et al. (2000).The identity of these compounds in the current investigation was confirmed by NMR spectra as follows: Allyl-L-cysteine δ_H 300 MHz [D_2O] 2.95 (2H, *m*), 3.14 (2H, *d*, *J* 6.5), 3.83 (1H, *dd*, *J* 4.4, 7.4), 5.14 (2H, *m*, *J* 12), 5.79 (1H, *m*), (±)-Allyl-L-cysteine sulfoxide δ_H 300 MHz [D_2O] 3.13–3.46 (2H, *m*), 3.61(1H, *m*, *J* 6), 3.73 (1H, *m*), 4.18 (1H, *m*), 5.47 (1H, *m*, *J* 10, 17), 5.88 (1H, *m*) and (±)-(*trans*-1-propenyl)-L-cysteine sulfoxide δ_H 300 MHz [D_2O] 1.83 (3H, *dd*, *J* 6.8, 1.5), 3.28 (2H, *m*), 4.08 (1H, *m*), 6.35 (1H, *dq*, *J* 15.2, 1.5), 6.55 (1H, *dq*, *J* 6.8).

The *trans* form in the mixture was separated by preparative HPLC and the identity of the *trans* isoalliin confirmed by comparison with the HPLC profiles of onion and garlic extracts, where the major precursor were *trans* isoalliin and alliin, respectively. Stereochemistry of isoallin, formed following uptake of precursor-*S*-(2-carboxy-*n*-propyl) cysteine into onion seedlings and subsequent oxidative decarboxylation (Parry and Sood, 1989; Parry and Lii, 1991) and alliin following uptake of *S*-allyl cysteine into garlic callus and subsequent oxidation (Ohsumi, 1993),

showed that the isomers all had the L-(+)-configuration. This result confirmed that specific enzymatic reactions had occurred in contrast to a purely chemical process which would have produced a mixture of isomers. Since these earlier studies had confirmed that a specific enzyme reactions had occurred to convert the precursors into the final sulphoxides, it was assumed the same would be true for the garlic and onion callus used here.

3.3. Separation of flavour precursors

Flavour precursors were analysed by a modification of the method of Edwards et al. (1994). The hydroxylamine included in this method was removed from the extraction solution to reduce any possible secondary reactions with the *Allium* compounds. The activity of alliinase, previously inhibited by the presence of hydroxylamine, was minimised by the use of temperatures below −20 °C during the extraction. Passage of the extract through ion exchange columns as a purification step was also eliminated because of the considerable losses that occurred. This enabled smaller amounts of tissue (100 mg) to be analysed. The simple mobile phase of 0.03 M HCl was chosen as this allowed for the possibility of a direct MS analysis of the column filtrates in preference to the methods used by Mütsch-Eckner et al. (1992) and Krest et al. (2000) which employed a phosphate buffer in the mobile phase.

In the modified method, tissue was extracted by cutting it to approximate weight and immediately plunging it into liquid nitrogen. To each 100 mg of frozen material, was added 1 ml of extraction solution (methanol:chloroform:water, 12:5:3) which had been kept at −20 °C. The tissue was left to extract at −20 °C overnight. A volume of 1 ml of extractant was transferred to a centrifuge tube and to each 1 ml was added 0.55 ml of water and 0.45 ml of chloroform. The liquids were mixed and centrifuged at 6500*g* for 5 min. The upper phase was collected and frozen at −80 °C. After lyophilisation the solution was resuspended in 1 ml 0.03 M HCl for each 100 mg of starting material. The solution was filtered through a 0.45 μm Millipore filter (Whatman Laboratory Division, Springfield Mill, Maidstone, Kent) then 150 μl transferred to a 1.5 ml Eppendorf tube from which 50 μl was used directly for HPLC analysis.

All analyses were undertaken using a Gilson (Anachem) HPLC (234 Autoinjector, 307 Pump, 118 uv/vis Detector, 811b DynamicMixer) and a Phenomenex MAX-RP 80A reverse phase column. The mobile phase was 0.03 M HCl and this was passed through the column at 0.9 ml min^{-1} for 45 min. The column was washed after use with 70% acetonitrile.

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