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Influence of saliva on individual in-mouth aroma release from raw cabbage (*Brassica oleracea var. capitata f. rubra L.*) and links to perception

Damian Frank*, Udayasika Piyasiri, Nicholas Archer, Jenifer Jenifer, Ingrid Appelqvist

CSIRO, 11 Julius Ave, North Ryde, NSW 2113, Australia

* Corresponding author.

E-mail address: damian.frank@csiro.au (D. Frank).

Abstract

Raw or minimally processed vegetables are popular for health reasons and for their unique textural and flavor attributes. While many aroma volatiles are produced *in situ* when plant tissues are mechanically disrupted, enzymes expressed in bacteria in oral microbiota such as cysteine-β-lyase (EC 4.4.1.13) may also contribute to aroma formation in-mouth during consumption. Interactions between raw cabbage and fresh human saliva (n = 21) were measured *ex vivo* by real-time monitoring of sulfur volatile production by proton transfer reaction mass spectrometry (PTR-MS). Inter-individual differences in the concentration of sulfur volatiles from the breakdown of *S*-methyl-L-cysteine sulfoxide (SMCSO) in fresh cabbage by saliva were characterized and a 10-fold difference in the extent of sulfur volatile production was measured across individuals. The overall intensity and garlic odor of raw cabbage was positively correlated with the concentration of sulfur volatiles after incubation with fresh human saliva. A buildup of SMSCO-derived sulfur volatiles *in vivo*, over twenty repeated

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mouthfuls was demonstrated, indicating that these reactions can affect sensory perception within the timescale of eating. These findings show the perceived odor experienced when eating cabbage differs, thus resulting in a unique flavor experience between individuals.

Keyword: Food science

1. Introduction

Many consumers seek out the unique texture and flavor of raw or minimally processed plant foods [1]. Raw or minimally processed salads or slaws are popular due to greater retention of some vitamins and nutrients compared to thermally processed equivalents and for their characteristic "fresh" flavor profile [2]. Complex enzyme-induced reactions rapidly generate odor volatiles when raw plant tissues are mechanically broken down during mastication. For example, lipoxygenases present in plant tissue produce a range of C-6 alcohols and aldehydes, associated with "green" flavors [3, 4]. Some volatiles may be present in the form of non-volatile glycosides, requiring glucosidase enzyme activity from α-amylase present in saliva, for release and perception [5]. Sulfur containing glucosinolates are well-characterized in brassica vegetables, constituting only a minor component (0.1−0.6% dry weight) [6]. Myrosinase (thioglucoside) enzyme activity present in plant tissue is essential to convert glucosinolates into their bioactive and volatile isothiocyanate form [6]. While glucosinolates are well-known in brassicas, the presence of *S*-alkyl-L-cysteine conjugates is less familiar, although the latter constitute up to 1−2% of dry weight [7]. *S*-methyl-L-cysteine sulfoxide (SMCSO, PubChem CID: 182092), is a non-volatile amino acid abundant in many brassica vegetables [7, 8]. The breakdown of SMCSO requires the activity of cysteine-*S*-conjugate-beta-lyase (CBL) enzyme (EC 4.4.1.13), naturally present in plant tissues [9, 10]. Various CBL-subtypes have also been characterized in human tissue extracts and play an important role in liver detoxification pathways [11, 12]. Considerable CBL activity is present within anaerobic bacteria naturally present in the oral cavity and saliva, such as *Fusobacterium nucleatum,* which contributes directly to the breakdown of L-cysteine-S-conjugates [8]. Cystathionine β-lyase enzyme (EC 4.4.1.8), present in *Veillonela spp.* bacteria in saliva also has CBL activity [13].

CBL catalyzes the cleavage of C−S bonds of L-cysteine-*S*-conjugates in the presence of pyridoxal-5-phosphate co-factor (P5P), to liberate methanesulfenic acid, ammonia and pyruvate (Fig. 1) [7]. Methanesulfenic acid is unstable and spontaneously undergoes disproportionation to generate the volatile compound methanethiol (MT, PubChem CID: 140171) which then dimerizes to form the odor active volatiles dimethyl disulfide (DMDS, PubChem CID: 12232) and dimethyl trisulfide (DMTS, PubChem CID: 19310) [14, 15]. SMSCO and its non-volatile decomposition products (*S*-methyl methanethiosulfinate (PubChem CID: 95200), *S*-methyl

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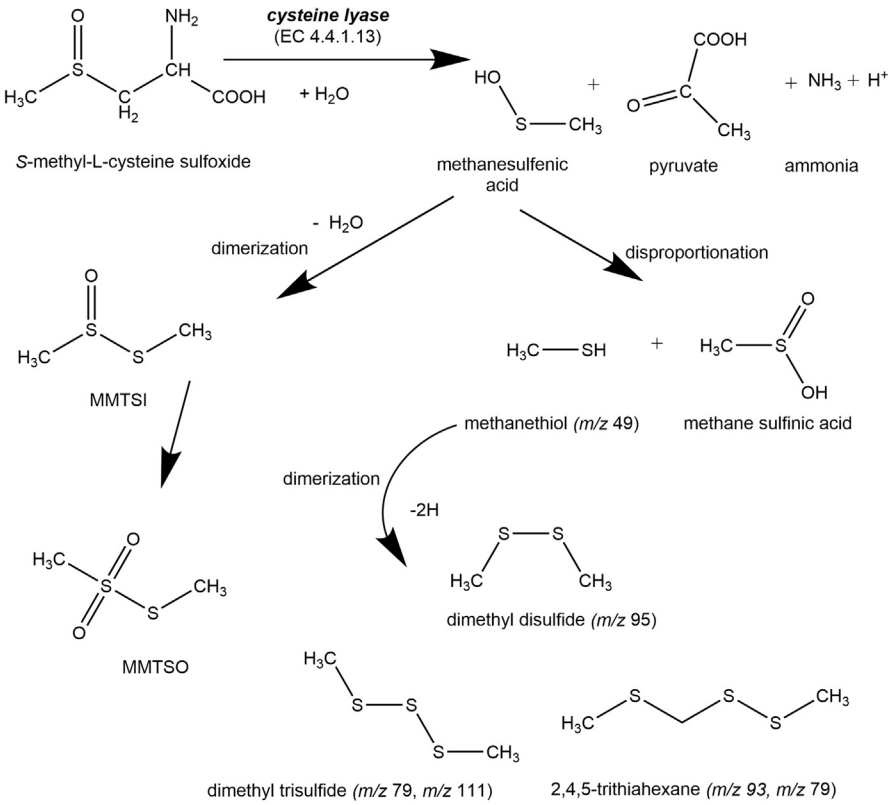

Fig. 1. Diagram showing breakdown products of *S*-methyl-L-cysteine sulphoxide through the actions of cysteine lyase enzyme. Modified and adapted from (Edmands, Gooderham, Holmes, & Mitchell, 2013). MMTSO = *S*-methyl methanethiosulfonate, MMTSI = *S*-methyl methanethiosulfinate. Ion fragments (*m/z*) corresponding to volatiles measured by proton transfer reaction mass spectrometry denoted.

methanethiosulfonate (PubChem CID: 18064)) exhibit anti-microbial, anti-carcinogenic and other physiological effects [7, 12, 16, 17].

Differences in the composition of the human oral microbiome have been characterized, with most sites in the oral cavity having up to 20 to 30 different predominant species and the number of predominant species ranging from 34 to 72 between individuals. Species from genera *Gemella, Granulicatella, Streptococcus,* and *Veillonella* are common in the human oral microbiome [18]. We hypothesized that differences in the composition of individual oral microbiota would lead to individual differences in CBL activity, and hence, the degree of breakdown of SMSCO and the amount of sulfur volatile production in the mouth. This study characterized inter-individual differences in the extent of in-mouth sulfur volatile generation from plant material (raw cabbage) and subsequent aroma development using an *ex vivo* saliva monitoring technique and sensory evaluation. Build-up of sulfur volatiles in the mouth over repeated mouthfuls was also demonstrated in an *in vivo* experiment.

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2. Materials and methods

2.1. Chemicals and reagents

Volatile reference standards were purchased from Sigma-Aldrich (Castle Hill, Australia); dimethyl disulfide, dimethyl trisulfide, hexanal, (*E*)-2-hexenal, 1-hexanol, allyl isothiocyanate (2-propenyl isothiocyanate) and 4-methyl-1-pentanol. 2,3,5-trithiahexane (PubChem CID: 93236) was supplied by Penta Manufacturing Corporation (Livingston, NJ, USA) and *S*-methyl-L-cysteine-sulfoxide was purchased from Cayman Chemicals (Sapphire Bioscience, Beaconsfield, NSW, Australia).

2.2. Ethics and saliva collection

Approval to collect and use human saliva in the *ex vivo* PTR-MS experiments was obtained from CSIRO low- risk ethics committee (LR-02-2016-F). Twenty one healthy subjects, 13 female (45 $\pm$ 12 years) and 8 male (42 $\pm$ 12 years) participated in the study and experiments were conducted one subject at a time over two separate two week periods. Saliva was collected between 9:00 and 11:00 hr. Subjects were instructed to have their usual breakfast and to brush their teeth using their normal dental care product and regime. Subjects were asked to refrain from using mouthwash and to stop eating and drinking (with the exception of water) one hour before collection. All subjects provided written informed consent before participation. Subjects were instructed to rinse their mouth twice with room temperature water (Pureau®, Noble Beverages, St Marys, Australia). After 5 minutes, subjects were asked to chew on a piece of 4 $\times$ 4 cm^2 wax Parafilm® (Bemis, Oshkosh, WI, USA). Stimulated saliva ($\sim$30 mL) was collected into 50 mL centrifuge tubes. During collection and handling ($\sim$5$-$10 min), saliva was kept on ice. Half of the fresh saliva ($\sim$15 mL) was deactivated by microwaving the loosely closed plastic tube in a beaker of water using defrost mode (Sharp R-230F, 800 W), until the beaker water was visibly boiling ($\sim$10 s). The sample was removed and cooled and the microwaving process was repeated twice. Deactivated saliva was cooled to room temperature before using. Small loses in volume were corrected by addition of protein free artificial saliva buffer [19]. Using the *ex vivo* volatile method described below (Section 2.6), we demonstrated that the microwave conditions were sufficient to completely inhibit production of sulfur volatiles associated with CBL activity (data not shown). Deactivated saliva samples were required as controls for each subject, because mucin and amylase protein content varies considerably between individuals and both are known to affect volatile release [20, 21]. Prior to performing experiments, saliva samples were incubated for (15 min) to reach a temperature of 37 °C in a temperature controlled incubator (Sanyo, Japan).

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2.3. Preparation of cabbage for experiments

The amount and distribution of gluscosinolates and SMSCO in brassica vegetables varies widely according cultivar and growing conditions [6]. To obtain consistent material for use across experiments, a homogeneous batch of cabbage powder was prepared. Fresh whole red cabbages (*Brassica oleracea var. capitata f. rubra, L.*) ~2 kg, were purchased from a local supermarket. After washing and rinsing with Milli-Q water, the outer leaves were removed and discarded. The cabbages were cut into quarters and processed a quarter at a time. For the *ex vivo* assay, roughly chopped cabbage pieces (~1 cm^2) were transferred into liquid nitrogen (Linde Australia) and blended in a stainless steel vessel into a fine powder until the whole cabbage was processed. The cabbage powder was pooled, mixed and distributed into 20 separate plastic storage tubes (50 mL) sealed and stored frozen at −80 °C until later use. For the *in vivo* study, roughly fresh cut fresh cabbage pieces (2 cm^2) were weighed into plastic cups (4 g). Cooked cabbage was prepared by steaming for 5 minutes, cooling and cutting into ~2 cm^2 pieces.

2.4. Quantitative measurement of SMSCO in cabbage

SMCSO was dissolved in acidified 70% methanol solution (formic acid 0.1 %) and a series of concentrations were used to construct an external calibration curve between 0.1 and 2 mg/mL. Raw and cooked cabbage was macerated in 70% acidified methanol (70%) using an Ultra-Turrax (T 25) followed by centrifugation. Samples were analyzed using a Dionex Ultimate-3000 liquid chromatograph coupled with triple quadrupole mass spectrometer (TSQ-Quantiva, Thermo Scientific, USA). The chromatographic separation was performed on an Intrada amino acid column (Imtakt Corporation, Japan) (3 mm × 150 mm) and the column oven was kept at 35 °C. Calibration standard solutions and extracts were injected by autosampler (2 μL injection volume). The mobile phases were 100 mM ammonium nitrate (A) and 0.1% formic acid in acetonitrile (B). The flow rate was 600 μL/min and the gradient program began at 14% B (3 min), then ramped to 100% B at 10 min and held for 1.5 min and then ramped to 14% B at 12.5 min and held for 2.5 min. The water content of raw cabbage was taken as 92% to calculate the SMSCO content on a dry weight basis [22]. The mass spectrometer was operated in negative electrospray ionization mode at a spray voltage of −2500 V and capillary temperature of 420 °C. The SMCSO precursor ion (*m/z* 150) and the following product ions (*m/z* 48, 63 & 86) with the corresponding collision energies (34.93, 10.25 & 10.25 V) were used for identification and quantification.

2.5. Solid phase microextraction (SPME) and gas chromatography-mass spectrometry

Frozen cabbage powder (1 g) was transferred quantitatively into headspace vials (20 mL) and 20 μL of 4-methylpentanol internal standard (40 μg/mL) and 1 mL of Milli-

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Q water (37 °C) were added. Immediately after collection, either fresh or deactivated saliva (2 mL) was immediately added and vials were sealed with a gas-tight Teflon® seal. Samples were incubated at 37 °C for 30 min. After incubation saturated calcium chloride solution (1 mL) was injected into the vials through the septum using a stainless steel cannula (24-gauge). To evaluate the effect of saliva on volatile development, replicate samples of cabbage incubated with fresh (n = 2) and deactivated (n = 2) saliva samples across the subjects (n = 10, 40 samples total) were measured. Headspace vials were placed into the auto sampler for the GC-MS analysis (AOC-5000 Plus, Shimadzu, Kyoto, Japan). The headspace was extracted using solid phase microextraction (SPME) (Carboxen/divinylbenzene/polydimethylsiloxane, Stableflex™ (Supelco, USA), 50/30 μm, 23 gauge) fibers at 37 °C (30 min) with sample agitation and analyzed by gas chromatography-mass spectrometry (Shimadzu 2010 GC-MS). The SPME fiber was desorbed at 240 °C (splitless) for 5 min. Separation was achieved using a Zebron wax capillary column (0.25 mm × 30 m × 0.50 μm film, Phenomenex, Lane Cove, Australia). The GC oven was programmed from 100 °C (held 1 min) to 250 °C at 10 °C/min (held 3 min.). The MS was set to electron ionization (EI) mode to scan between 45-250 mass/charge ratio (*m/z*). Volatiles were initially identified through electron impact mass spectral matches in the National Institute of Standards and Technology (NIST) database (Version 2.0, United States of America, 2002) and in most cases identification was confirmed using reference standards and matching retention times. SPME volatile integrated area data were calculated using the LabSolutions® software (Shimadzu).

2.6. *Ex vivo* saliva PTR-MS protocol

Volatiles were measured using high-sensitivity quadrupole mode PTR-MS (Ionicon Analytic GmbH, Innsbruck, Austria). The inlet flow rate was set to 100 mL/m. The temperatures of inlet tube and reaction chamber were 70 °C and 80 °C, respectively. The drift tube voltage was 600 V and the pressure was 2.19 mbar. Frozen cabbage powder (4 g) was weighed into a sealed Schott bottle with a Teflon stir bar and thawed to room temperature (60 minutes). Immediately prior to experiments, 10 mL of protein-free artificial saliva buffer (37 °C) and was added to the vessel and connected to the PTR-MS via a Luer lock connection as described previously [23]. Fresh and deactivated saliva were kept on ice until placing in an incubator (37 °C) for 15 m prior to the experiment. The time between saliva collection and PTR-MS experiments was no more than 15 min. During piloting experiments, the headspace volatiles were measured in scan mode from *m/z* 40−150. A number of major ions increased after either macerating fresh cabbage samples (without addition of saliva) or after addition of fresh saliva to cabbage. Pure reference volatile standards (~5 μg/L) in water were used to determine the PTR-MS fragmentation patterns for key volatile compounds identified by GC-MS (Table 1). Most of the volatiles had common ions. For example, the main fragment for DMDS was the

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Table 1. Details of the main volatiles present in cabbage headspace, associated odor character, quantitative ion (*m/z*) monitored by gas chromatography-mass spectrometry (GC-MS) and main ions (*m/z*, %) measured for reference compounds by proton transfer reaction mass spectrometry (PTR-MS), odor thresholds in water and mean concentrations (n = 10 subjects) in cabbage powder incubated with either deactivated (Deact) or fresh saliva measured by GC-MS. P value for comparison of deactivated and fresh saliva.

Volatile	Odor character	GC/MS *m/z*	Main PTR-MS ions *m/z* (%)	Odor threshold µg/L	Deact µg/kg	Fresh µg/kg	P value
Methanethiol (MT)	*Sulfurous, putrid*	47	49 (100%)	0.02[a]	0.47	0.48	ns
dimethyl sulfide (DMS)	*Asparagus, cooked*	62	63 (100%), 65 (5%)	1.0[a]	3.0	2.5	ns
dimethyl disulfide (DMDS)	*Cabbage-like*	94	95 (100%), 79 (49%), 97 (12%)	7.6[a]	320	2160	<0.001
dimethyl trisulfide (DMTS)	*Cabbage-like*	126	79 (100%), 81 (36%), 93 (32%), 61 (14%), 127 (12%)	0.01[a]	3410	4680	0.004
(*E*)-2-hexenal	*Marzipan, green*	69	57 (100%), 99 (24%), 81 (22%)	316[a]	340	370	ns
hexanal	*crushed leaves*	56	55 (100%), 83 (61%), 101 (3.2%)	4.5[a]	800	540	ns
1-hexanol	*Fatty, green*	56	41 (100%), 43 (94%), 57 (36%), 85 (33%)	2500[a]	30	120	<0.001
allyl-isothiocyanate	*Mustard, pungent*	99	41 (100%), 100 (55%)	375[a]	1220	1060	ns
2,3,5-trithiahexane (TTH)	*Onion, penetrating*	61	93 (100%), 61 (40%)	0.8[b]	60	460	<0.001

a (Belitz, Grosch, & Schieberle, 2009), b (Spadone, Matthey-Doret, & Blank, 2006).

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$M+H^+$ ion, *m/z* 95 (100%), however it also produced a significant amount of *m/z* 79 ($(CH_3)S_2^+$, 49%), which was the most abundant ion for DMTS. Although no reference standard was available for methanethiol (MT), the fragment at *m/z* 49 (100%) was assigned to this molecule consistent with previous reports [24]. It should be noted that other non-characterized volatile species present in individual saliva samples may have also contributed to some of the PTR-MS ions monitored. A selected ion monitoring method was programmed such that a full acquisition cycle of ions of interest (Table 1) was completed every 4 s; *m/z* 49, *m/z* 51, *m/z* 57, *m/z* 59 (acetone), *m/z* 61, *m/z* 63, *m/z* 65, *m/z* 79, *m/z* 81, *m/z* 83, *m/z* 85, *m/z* 93, *m/z* 95, *m/z* 97, *m/z* 99, *m/z* 100 and *m/z* 127. For the *ex vivo* saliva measurements, cabbage powder solutions were scanned for 50 cycles (200 s) to reach a steady state baseline, before introduction of either fresh (8 mL) or deactivated (8 mL) saliva through a syringe via a cannula into the Schott bottle vessel [23]. Samples were then scanned for a further 100 cycles (400 s). The area under the curve (AUC) for the first 10 cycles (40 s), the first 20 cycles (80 s) and the full 100 cycles (400 s)) was calculated using Excel® (Microsoft). The AUC values for deactivated saliva samples were also measured. Two replicates for fresh and deactivated saliva were measured for 10 subjects. Further fresh saliva *ex vivo* samples for an additional 11 subjects were measured in duplicate, so that data for a total of 21 subjects were available to understand potential relationships between the degree of individual volatile production after incubation with fresh saliva and sensory quality.

2.7. Consecutive mouthful in vivo volatile release experiment

The purpose of the *in vivo* experiment was to test whether significant buildup of SMSCO-derived sulfur volatiles occurred within the timescale of a typical eating event, e.g. over 20 consecutive mouthfuls (24 s each) over a total consumption period of 480 s (8 min) period. Cooked cabbage (5 min steaming) was used as a control sample to confirm that there was no significant generation of sulfur volatiles from thermally processed material. Room temperature roughly chopped raw or cooked cabbage samples (~4 g) were weighed into individual plastic cups (n = 20). An animated visual guide was programmed (Adobe Flash®) to coordinate the breath cycles and intake of twenty consecutive mouthfuls of either fresh or cooked cabbage [25]. A plastic disposable cannula was firmly placed in the subject's nostrils by tightening the plastic tubes at the back of the subject's head. The inlet of the PTR-MS was connected via peek tubing to the cannula and volatiles were measured in multiple ion monitoring mode as described in the previous section. The subject was asked to follow the animation on the computer screen for the consumption protocol. Initially they breathed for 5 cycles and then placed the cabbage sample in their mouth and chewed for 2–3 times prior to swallowing (24 s period). After another 5 breathe cycles another 19 consecutive cabbage samples were consumed according to the strict protocol. For the *in vivo* measurements a flow

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rate of 400 mL/min was used. To increase sensitivity, only *m/z* 49, *m/z* 79, *m/z* 95, *m/z* 111 and *m/z* 127 were measured (1 scan/s). Replicate fresh (n = 6) and cooked (n = 6) samples were consumed by one trained assessor according to the strict eating and breathing paradigm to ensure good temporal alignment of data. The area under the concentration curve (AUC) from the first to tenth mouthful (AUC-1-10) and from the eleventh to twentieth mouthful (AUC-11-20) were calculated and used in statistical comparisons.

2.8. Triangle testing and sensory evaluation

Within 30 min of completion of the *ex vivo* PTR-MS measurements, a volume (2 mL) of remaining samples from the deactivated and fresh saliva treatments were transferred into individual plastic cups and closed with a lid. Duplicate series of either; two deactivated saliva and one fresh saliva (BBA), or two fresh saliva and one deactivated saliva (AAB) samples were labelled with a random 3-digit code and presented to individual subjects (n = 21) as an alternative forced choice test (3-AFC) where subjects were required to choose the sample that differed from the other two presented in randomized order (a total of 4 assessments). Subjects were blindfolded in comfortable seated position. A technician removed the sample lids one at a time and held each of the three samples below the subject's nose and asked the subject to guess which sample was different. The total number of correct guesses for the four separate tests was calculated (0−4). Subjects were then asked to rate whether the following attributes were stronger or weaker in the fresh saliva sample compared to the deactivated saliva sample on a 100-mm line scale; green-odor, garlic-odor and overall odor intensity. The midpoint on the scale represented the same intensity or no difference. The left hand anchor was labeled weaker and the right hand anchor labelled stronger. The average intensity of each attribute was calculated for the fresh saliva samples and used to compare to volatile profiles.

2.9. Data processing and analysis

Volatile concentration (μg/L) was calculated using the PTR-MS software. PTR-MS data files were imported into Excel® (Microsoft Corporation). The area under the concentration curve (AUC) was calculated for the first 10 cycles (AUC-10, 40 s) and the first 20 cycles (AUC-20, 80 s) and for 200 complete cycles (AUC-200, 800 s). Replicate volatile data from GC-MS and PTR-MS experiments (n = 10 subjects) were subjected to Multivariate Analysis of Variance (MANOVA) using saliva (fresh, deactivated) and subject as fixed main factors. To understand relationships between various parameters, Pearson's correlation coefficients and associated two-sided *p*-values and correlation plots were generated using the standard procedures available in GenStat® (16th Edition, VSN International, Hemel Hempstead, UK).

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The one sample binomial test (Genstat) was used to analyze the data from the triangle tests. Principal components analysis was performed using the standard procedure in Genstat after normalizing data (1/standard deviation).

3. Results and discussion

3.1. Solid phase microextraction measurement of cabbage headspace volatiles

The SPME headspace profiles of fresh macerated cabbage (without addition of either fresh or deactivated saliva) was dominated by dimethyl trisulfide (DMTS) and dimethyl disulfide (DMDS) consistent with previous studies (Table 1) [26, 27]. Only trace amounts of methanethiol (MT) were measured by the SPME method (Table 1). 2,3,5-trithiahexane (TTH, or methyl methylthiomethyl disulfide) was also identified as a major volatile component in the raw cabbage headspace, previously reported only in cooked brassica vegetables [27, 28]. The low olfactory threshold concentrations and the measured concentration of MT, DMTS and TTH indicated that these sulfur volatiles had high odor impact relative to the other volatiles present in cabbage [28, 29]. In the presence of deactivated saliva, a background level of SMSCO-derived sulfur volatiles was measured, indicating a contribution of endogenous plant CBL enzyme activity. Allyl-isothiocyanate (2-propenyl isothiocyanate) was the major glucosinolate breakdown product present in the cabbage headspace, as expected [6, 26]. Typical C-6 volatiles generated from lipoxygenase pathways were also major components; hexanal, 1-hexanol and (*E*)-2-hexenal [3]. After incubation with fresh saliva, the concentration of DMDS, DMTS were significantly higher ($p < 0.001$), indicating that CBL activity also was present in human saliva (Table 1). No differences were measured for (*E*)-2-hexenal or hexanal. A significantly higher amount of 1-hexanol was measured after treatment with fresh saliva. The significantly higher concentration of 1-hexanol in fresh saliva may have indicated the presence of hexyl β-D-glucoside (not measured) in cabbage and release of 1-hexanol due to the activity of salivary α-amylase [5, 30, 31, 32]. Despite the higher amount of 1-hexanol in the fresh saliva, the concentration was still below the olfactory threshold and was considered unlikely to affect the sensory properties. The reason for the lower concentration of hexanal in the fresh saliva may have been due to a change in the confirmation of the denatured saliva proteins, leading to greater binding. The heat denatured saliva proteins in the deactivated saliva may have interacted with this volatile differently [5, 31]. In summary, the SPME GC-MS data demonstrated significant increases in key odor-active sulfur volatiles, typically generated from the breakdown of SMSCO through CBL enzyme activity [7, 8]. Few differences were measured for volatiles produced through lipoxygenase pathways, for example (*E*)-2-hexenal and hexanal.

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3.2. Ex-vivo and saliva measurement using PTR-MS

Typical PTR-MS volatile profiles for *ex vivo* fresh saliva experiments are shown for the most concentrated volatile ions for two individuals; a relative high (Subject 18) and low (Subject 3) producer of sulfur volatiles (Fig. 2). After the addition of saliva at (cycle 50) there was an almost immediate increase in the amount of MT (*m/z* 49), which was by far the most abundant sulfur volatile measured. After a short lag period a clear increase in *m/z* 95 (DMDS) and then *m/z* 79 (DMDS and DMTS) were measured. Significant increases in *m/z* 111, *m/z* 93 and *m/z* 127 were measured after a longer induction period. In previous studies, there has been speculation that DMDS and DMTS may form spontaneously when MT comes in contact with heated or metallic surfaces such as the injector inlet of a gas chromatograph and hence may be heat induced artifacts [33]. The PTR-MS data did not support this, confirming recent findings from other groups [24].

The ion *m/z* 93 was the main PTR-MS fragment from TTH and also a major ion from DMTS. The *m/z* 127 ion corresponded to the $M+H^+$ ion for DMTS. The ion *m/z* 111 increased in all samples after the addition of fresh saliva, although *m/z* 111 ion was not present in the reference PTR-MS spectra for either DMTS or TTH. In contrast,

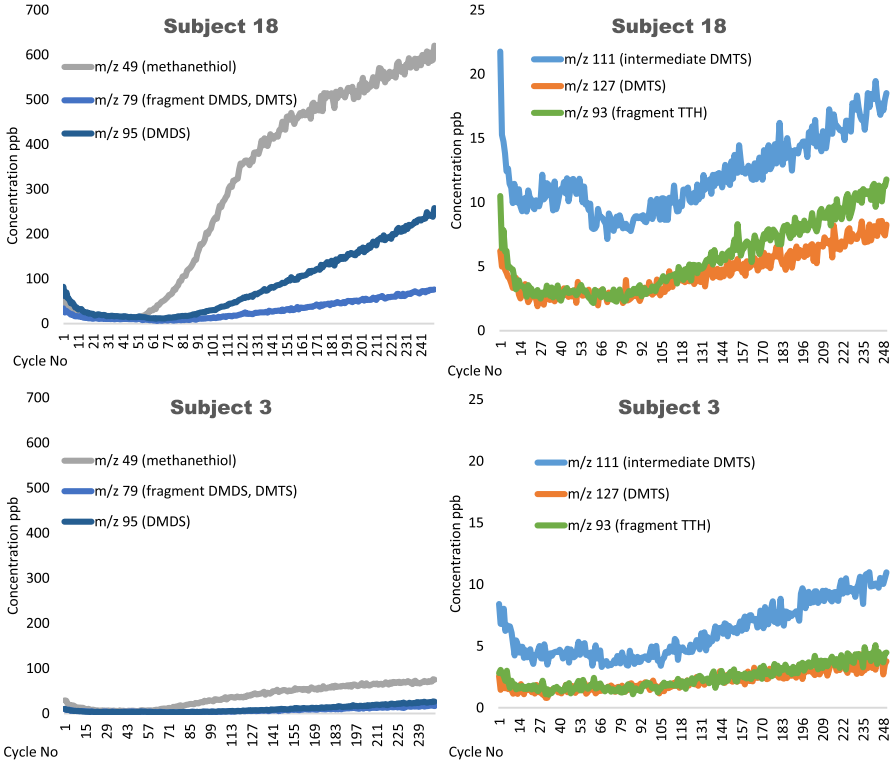

Fig. 2. Representative real time *ex vivo* saliva PTR-MS profiles from two human subjects, showing increases in main volatile ions after addition of fresh saliva at 50 cycles. Methanethiol (*m/z* 49), fragment from dimethyl trisulfide and dimethyl disulfide (*m/z* 79), dimethyl disulfide (*m/z* 95), 2,3,5-trithiahexane and dimethyl trisulfide (*m/z* 93), unidentified ion (*m/z* 111) and dimethyl trisulfide (*m/z* 127).

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the electron impact mass spectrum of DMTS has a prominent ion at *m/z* 111 (16.2%) (National Center for Biotechnology Information. PubChem Compound Database; CID = 19310, https://pubchem.ncbi.nlm.nih.gov/compound/19310 (accessed Dec 11, 2018)) likely corresponding to the positive ion fragment $(CH_3)S_3^+$. The presence of ion *m/z* 111 in *ex vivo* and *in vivo* PTR-MS data indicate that this may be an unstable chemical intermediate in the formation of DMTS from MT and DMDS. Addition of deactivated saliva did not result in the increase over time of any of the ions associated with the SMSCO-derived volatiles. The concentration of ions corresponding to other cabbage volatiles, for example (*E*)-2-hexenal (*m/z* 99), and 1-hexanol (*m/z* 57) decreased at similar rates over time after addition of both fresh and deactivated saliva *ex vivo*, indicating that these volatiles were not significantly increased by salivary enzymes. This was in contrast to the GC-MS result for 1-hexanol, in which a higher amount was measured in fresh saliva.

The AUC after 10 (40 s), 20 (80 s) and 200 (800 s) cycles for selected monitored ions for fresh and deactivated saliva for ten subjects were measured (Table 2). Significant differences ($p < 0.05$) were measured for most ions between fresh and deactivated saliva and also between individuals at each time point (Table 2). MT (*m/z* 49) was significantly higher in all samples with fresh saliva ($p < 0.001$). There were also clear differences in *m/z* 49 between individuals at each time point. For example, there was an almost 10-fold difference between the concentration of MT between subject 1 and 6 for AUC-200. Large differences in the concentration of *m/z* 51 in fresh saliva between individuals was also measured. The fragment *m/z* 51 was consistent with the ^{34}S- isotope of methanethiol which has a natural abundance of around 4% [34]. Increases in the concentration of *m/z* 79, *m/z* 93, *m/z* 95 and *m/z* 111 in fresh saliva were measured after a period of time. Significantly higher *m/z* 127 was only measured after 200 cycles (800 s). The rapid initial formation of *m/z* 49 and *m/z* 95, indicated that the formation of MT and DMDS was under enzymatic control (CBL), whereas the formation of *m/z* 79 (DMDS, DMTS), *m/z* 93 (DMTS, MMTMDS), *m/z* 111 and *m/z* 127 (DMTS) was slower and appeared to be determined by chemical addition reactions. No consistent or large increases between fresh and deactivated saliva for *m/z* 57 (1-hexanol and other volatiles) or *m/z* 99 ((*E*)-2-hexenal) were measured (in contrast to *m/z* 49 and *m/z* 95) indicating that salivary enzymes did not significantly affect their generation. Enzymes (lipoxygenases) present mainly in plant tissues (not saliva) were expected to be responsible for the generation of these volatiles, hence these findings were not surprising.

3.3. Relationship between *ex vivo* saliva data and odor attributes

Significant differences ($p < 0.001$) were measured between individuals at AUC-200 (800 s) for *m/z* 49, *m/z* 51, *m/z* 79, *m/z* 93, *m/z* 95, *m/z* 111 and *m/z* 127 (Table 2). The data also clearly showed differences between individuals in the rate of production of

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Table 2. Mean (n = 2) area under the concentration curve (AUC) data for volatile ions (*m/z*) after 10 cycles (AUC-10), 20 cycles (AUC-20) and 200 cycles (AUC-200) for 10 subjects for deactivated (deact) and fresh saliva measured by proton transfer reaction mass spectrometry. P values and standard errors of determination (SED) given for the effects of saliva (deact, fresh) and subject. *m/z* 49 (methanethiol); *m/z* 51 (^{34}S-methanethiol), *m/z* 57 (1-hexanol), *m/z* 79 (dimethyl dilsufide, dimethyl trisulfide), *m/z* 93 (2,3,5-trithiahexane, dimethyl trisulfide), *m/z* 95 (dimethyl disulfide), *m/z* 99 (E)-2-hexenal, *m/z* 111 ($CH_3S_3^+$) and *m/z* 127 (dimethyl trisulfide).

AUC-10

Ion	Treatment	1	2	3	4	5	6	7	8	9	10	Saliva	Subject	
m/z 49	Deact	96	89	141	72	122	66	84	76	261	112	<0.001	<0.001	P value
	Fresh	895	397	214	89	239	86	247	144	476	169	113	26	SED
m/z 51	Deact	51	58	40	36	47	43	38	45	58	53	<0.001	<0.001	P value
	Fresh	82	71	49	35	59	46	51	50	72	51	2.1	4.4	SED
m/z 57	Deact	555	784	537	636	725	477	498	639	483	810	0.534	<0.001	P value
	Fresh	601	732	519	508	757	479	539	571	561	695	29	61	SED
m/z 79	Deact	134	257	106	108	177	107	154	127	142	271	0.007	<0.001	P value
	Fresh	199	327	146	132	208	148	210	164	192	266	14	30	SED
m/z 93	Deact	33	57	24	28	35	21	34	25	29	56	0.017	<0.001	P value
	Fresh	43	71	31	30	42	29	46	32	40	54	3	6	SED
m/z 95	Deact	53	96	49	53	77	52	59	53	71	88	0.029	0.001	P value
	Fresh	102	121	58	59	83	62	83	58	82	88	6.4	13.4	SED
m/z 99	Deact	159	193	159	218	192	132	129	201	117	194	0.028	0.008	P value
	Fresh	152	165	135	155	187	122	125	156	139	155	9	19	SED
m/z 111	Deact	122	213	78	108	142	98	108	112	83	197	0.006	<0.001	P value
	Fresh	157	245	110	126	154	125	145	145	130	181	8.9	18.8	SED
m/z 127	Deact	19	44	15	15	26	15	21	20	19	37	0.1	<0.001	P value
	Fresh	23	46	18	15	29	20	28	23	28	33	1.9	4	SED

(*continued on next page*)

Table 2. (*Continued*)

AUC 20

Ion	Treatment	1	2	3	4	5	6	7	8	9	10	Saliva	Subject	
m/z 49	Deact	203	178	290	127	247	120	154	143	514	221	<0.001	<0.001	P value
	Fresh	4280	2349	878	344	1135	266	1217	692	2012	758	67	141	SED
m/z 51	Deact	142	152	86	74	96	83	93	93	114	113	<0.001	<0.001	P value
	Fresh	300	255	125	87	169	97	135	122	197	131	6.5	14	SED
m/z 57	Deact	1002	1486	967	1128	1324	854	917	1152	854	1492	0.535	<0.001	P value
	Fresh	1105	1341	935	941	1392	846	977	1032	1006	1278	51	109	SED
m/z 79	Deact	267	493	201	190	331	191	279	231	261	509	<0.001	<0.001	P value
	Fresh	539	726	283	248	415	258	406	306	394	503	26	56	SED
m/z 93	Deact	65	111	48	51	67	38	63	46	55	106	0.016	<0.001	P value
	Fresh	97	140	58	57	79	50	84	58	74	100	6	12	SED
m/z 95	Deact	103	183	88	89	138	87	107	92	132	160	<0.001	<0.001	P value
	Fresh	422	441	140	117	213	109	199	124	236	196	12	25	SED
m/z 99	Deact	289	354	287	387	352	240	238	364	208	357	0.034	0.003	P value
	Fresh	280	305	242	288	344	218	228	283	250	288	16	33	SED
m/z 111	Deact	232	406	144	195	266	180	197	206	153	368	0.006	<0.001	P value
	Fresh	314	467	200	237	288	221	265	263	238	335	17	35	SED
m/z 127	Deact	38	89	28	29	50	28	37	36	35	69	0.087	<0.001	P value
	Fresh	51	90	34	29	54	35	51	42	52	61	3.4	7.2	SED

(*continued on next page*)

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Table 2. (*Continued*)

AUC-200

Ion	Treatment	1	2	3	4	5	6	7	8	9	10	Saliva	Subject	
m/z 49	Deact	845	810	1421	646	1137	533	826	523	2543	968	<0.001	<0.001	P value
	Fresh	51635	34423	15491	13769	17212	5164	17212	8606	34423	15491	1165	2457	SED
m/z 51	Deact	2264	2230	970	856	744	631	1349	699	878	1171	<0.001	<0.001	P value
	Fresh	4282	4142	1886	1375	2348	957	1610	1387	2512	1702	96	202	SED
m/z 57	Deact	4197	6496	4312	5116	5789	3540	4016	4687	3616	6376	0.601	<0.001	P value
	Fresh	4849	5744	4117	4501	6091	3535	4215	4164	4194	5557	223	471	SED
m/z 79	Deact	1675	2812	1216	1269	1775	1017	1550	1027	1433	2651	<0.001	<0.001	P value
	Fresh	9538	8614	2018	3518	1020	3555	1575	4761	2418	8614	345	727	SED
m/z 93	Deact	411	659	294	270	373	211	364	280	317	571	<0.001	<0.001	P value
	Fresh	723	566	163	271	89	241	114	302	73	566	51	107	SED
m/z 95	Deact	578	911	460	425	630	369	512	392	708	727	<0.001	<0.001	P value
	Fresh	11728	11481	3333	5556	864	5679	2099	6667	4198	11481	431	908	SED
m/z 99	Deact	1208	1499	1254	1614	1552	1017	1043	1577	893	1525	0.066	<0.001	P value
	Fresh	1244	1351	1064	1244	1523	927	998	1270	1056	1269	65	138	SED
m/z 111	Deact	1329	2216	792	1083	1396	932	1059	960	830	1847	<0.001	<0.001	P value
	Fresh	2333	2717	1172	1405	1671	1088	1450	1277	1346	1764	95	200	SED
m/z 127	Deact	224	505	163	191	264	151	208	153	190	353	<0.001	<0.001	P value
	Fresh	589	700	272	282	414	203	365	195	395	394	24	51	SED

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Table 3. Ex vivo mean (n = 2) fresh saliva data for 21 subjects. Evaluation of odor attributes (garlic, green, intensity) on a 100 mm line scale, number of correct guesses 3-alternative forced choice (3-AFC, triangle test, n = 4), mean area under concentration curve (AUC) data after 200 cycles for ions (*m/z*) corresponding to sulfur volatiles measured by proton transfer reaction mass spectrometry and total sum of volatiles (AUC total). *m/z* 49 (methanethiol); *m/z* 51 (^{34}S-methanethiol), *m/z* 79 ($CH_3S_2^+$, dimethyl disulfide, dimethyl trisulfide), *m/z* 93 (2,3,5-trithiahexane, dimethyl trisulfide), *m/z* 95 (dimethyl disulfide), *m/z* 111 ($CH_3S_3^+$) and *m/z* 127 (dimethyl trisulfide).

Subject No	Garlic odor	Green odor	Odor intensity	Correct (3-AFC)	*m/z* 49	*m/z* 51	*m/z* 79	*m/z* 93	*m/z* 95	*m/z* 111	*m/z* 127	Total AUC
1	55	50	54	3	51635	4282	9538	723	11728	2333	365	80604
2	81	41	74	4	34423	4142	8614	566	11481	2717	194	62138
3	67	34	66	4	15491	1886	2018	163	3333	1172	91	24154
4	50	33	50	3	13769	1375	3518	271	5556	1405	149	26043
5	43	51	28	0	17212	2348	1020	89	864	1671	52	23256
6	61	51	62	3	5164	957	3555	241	5679	1088	157	16840
7	58	50	56	3	17212	1610	1575	114	2099	1450	42	24102
8	51	47	48	3	8606	1387	4761	302	6667	1277	205	23205
9	55	38	49	3	34423	2512	2418	73	4198	1346	41	45011
10	97	20	95	4	15491	1702	8614	566	11481	1764	194	39811
11	96	20	95	4	20929	9884	3138	942	5468	2133	709	43204
12	69	26	65	3	22114	6225	4893	1305	8671	3337	1077	47621
13	64	34	65	4	29494	6239	5082	1410	6719	4983	1186	55112
14	65	28	76	3	21354	7173	4446	1080	9337	3899	1291	48580
15	68	22	68	4	47100	9557	6842	1328	15126	3743	983	84679
16	68	41	51	3	9056	5419	1665	530	2253	1317	480	20722
17	58	38	42	2	17657	7774	3227	951	5460	2089	727	37885
18	57	43	69	2	74994	11410	6755	1244	20514	2480	984	118380
19	51	37	36	2	17258	6771	2306	671	4201	1982	561	33749
20	74	20	72	2	11964	15006	6027	1641	7477	5969	1372	49457
21	77	24	76	2	52431	6603	4306	1026	8088	2252	784	75490

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Table 4. Correlation plot (n = 21) showing correlation coefficient and associated *p* values (* = p < 0.05; **p < 0.01, *** = p < 0.001) for *m/z* 49 (methanethiol); *m/z* 51 (^{34}S-methanethiol), *m/z* 79 (dimethyl dilsufide, dimethyl trisulfide), *m/z* 93 (2,3,5-trithiahexane, dimethyl trisulfide), *m/z* 95 (dimethyl disulfide), *m/z* 111 ($CH_3S_3^+$), *m/z* 127 (dimethyl trisulfide), sum of total volatiles (Total AUC) and odor attributes (garlic, green, intensity) measured on a 100 mm line scale.

methanethiol (*m/z* 49)	-										
^{34}S-methanethiol (*m/z* 51)	0.37	-									
fragment DMDS, DMTS (*m/z* 79)	0.49 *	0.23	-								
fragment TTH, DMTS (*m/z* 93)	0.38	0.87 ***	0.45 *	-							
dimethyl disulfide (*m/z* 95)	0.75 ***	0.43 *	0.81 ***	0.55 **	-						
fragment DMTS (*m/z* 111)	0.19	0.73 ***	0.4	0.86 ***	0.35	-					
dimethyl trisulfide (*m/z* 127)	0.28	0.85 ***	0.26	0.96 ***	0.42	0.85 ***	-				
Total AUC	0.95 ***	0.57 **	0.65 **	0.62 **	0.87 ***	0.42	0.50 ***	-			
Garlic odor	0.005	0.28	0.38	0.34	0.24	0.22	0.23	0.15	-		
Green odor	-0.041	-0.52 **	-0.22	-0.60 **	-0.24	-0.51 *	-0.57 **	-0.22	-0.70 ***	-	
Odor intensity	0.19	0.3	0.49 *	0.42	0.46 *	0.32	0.34	0.34	0.90 ***	-0.67 ***	-
	***m/z* 49**	***m/z* 51**	***m/z* 79**	***m/z* 93**	***m/z* 95**	***m/z* 111**	***m/z* 127**	**Total AUC**	**garlic**	**green**	**intensity**

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the measured sulfur volatiles at different times during the oral processing of cabbage (10, 20 and 200 cycles). The varying concentration of sulfur volatiles in *ex vivo* saliva were expected to result in differences in sensory perception.

To determine whether differences in sensory perception, could be perceived triangle tests (3-AFC) were completed on cabbage incubated with fresh and deactivated saliva. Overall, there were 84 separate triangle tests performed (21 subjects, 4 triangle tests). A total of 61 tests were correct, significantly higher than chance ($p < 0.001$), indicating that sensory differences between deactivated and fresh saliva samples were able to be perceived by most assessors (Table 3). Individual AUC-200 sulfur volatiles (*m/z* 49, *m/z* 79, *m/z* 93, *m/z* 95, *m/z* 111 and *m/z* 127) across individuals (n = 21) were significantly ($p < 0.05$) positively correlated with each other (Table 4). MT (*m/z* 49) and DMDS (*m/z* 95) were strongly correlated ($r = 0.76$, $p < 0.001$). The odor intensity and garlic odor character were significantly correlated to each other ($r = 0.89$, $p < 0.001$). Although the intensity and garlic attributes were positively correlated with most volatiles, the strongest relationships were with higher

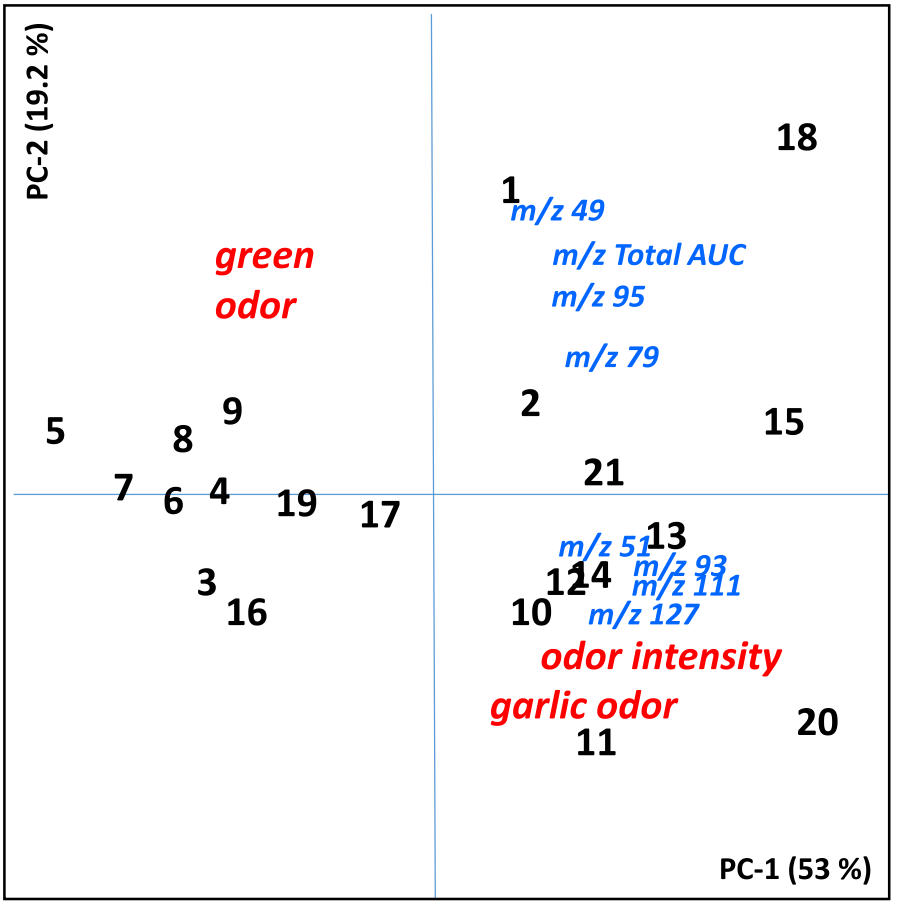

Fig. 3. PCA plot for PT-RMS volatile production from cabbage incubated with fresh saliva and perceived sensory measures (green, garlic and intensity) for the 21 study participants (1−21).

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mass sulfur compounds; e.g.; *m/z* 79 (DMTS), *m/z* 93 (DMTS, TTH) and *m/z* 95 (DMDS). Green character decreased significantly as the intensity and garlic odor increased ($r = -0.70$, $p < 0.001$). As no clear differences between deactivated and fresh saliva in background green volatile ions (e.g.; *m/z* 57, *m/z* 99) were measured by PTR-MS, masking of the green odor by the sulfur volatiles was indicated. Differences between the sulfur volatile composition and sensory attributes of *ex vivo* saliva samples for all subjects (n = 21) are summarized in a principal component bi-plot (Fig. 3). Low producers of sulfur volatiles (left hand side) generally reported greater green character than the garlic or odor intensity in *ex vivo* saliva samples. High sulfur volatile producers (right hand side) reported higher odor intensity and garlic odor character, particularly associated with *m/z* 93 and *m/z* 111, which are key ions from DMTS and TTH. Although based on only a small number of subjects (n = 21), these data suggest increased SMCSO derived sulfur volatile production was positively related to the perceived intensity and garlic odor and negatively associated with green odor character in *ex vivo* extracts.

3.4. *In vivo* consecutive mouthful experiment

The average *in vivo* release for the last ten mouthfuls (AUC-11-20) compared to the first ten mouthfuls (AUC-1-10) for raw and cooked cabbage are shown in Fig. 4. It should be emphasized, that the cabbage samples were swallowed and that the volatiles measured were mainly due to residual cabbage juice and pieces present on the

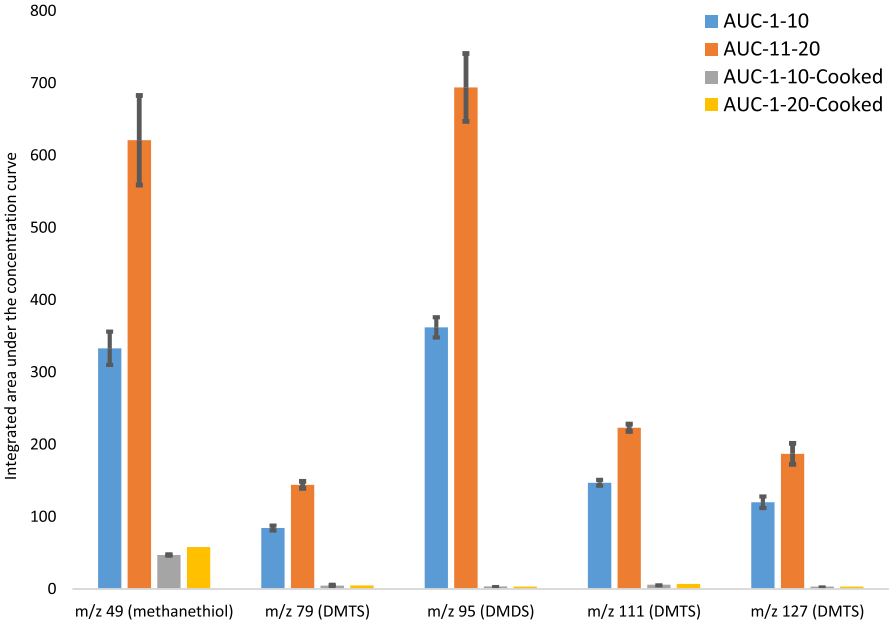

Fig. 4. Mean (n = 6) area under curve for 1−10 mouthfuls (AUC-1-10) and AUC for 11−20 mouthfuls (AUC-11-20) of raw and cooked cabbage (AUC-1-10-Cooked, AUC-11-20-cooked), for methanethiol (*m/z* 49), dimethyl disulfide (*m/z* 95) and dimethyl trisulfide (*m/z* 79, *m/z* 127, bottom) and unidentified ion (*m/z* 111).

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surfaces of the oral cavity in contact with saliva. All sulfur volatiles increased in the raw cabbage over the twenty mouthfuls and were significantly higher ($p < 0.001$) for the latter ten mouthfuls. There was no significant increase or build up over time of any volatile in the cooked cabbage samples. These data demonstrated that SMSCO-derived sulfur volatiles were generated within the mouth during the typical time-scales of an eating event (8 min period). The amount of SMSCO in the fresh cabbage powder was estimated to be 932 mg/100g and 875 mg/100 g after steaming for 5 minutes. In a previous study, cysteine *S*-methyl−sulfoxide conjugates were mostly retained (85.7%) in *Allium* vegetables after steaming for 4 minutes, but were substantially lost with longer cooking times (<15 minutes) [35]. The enzyme co-factor pyridoxal-5′-phosphate (P5P, vitamin B-6) and associated pyridoxal kinase enzyme activity are required for CBL activity [12]. We speculate that P5P was not available in the cooked cabbage samples, preventing CBL activity.

4. Conclusions

An *ex vivo* PTR-MS method was developed for real-time measurement of SMSCO breakdown in red cabbage by CBL activity in bacteria present in saliva and quantitative differences between individuals were characterized for the first time. Significant buildup of SMSCO-derived volatiles *in vivo* over an eight minute repeated mouthful eating session was also demonstrated. Relationships between the degree of volatile production and the perception of raw cabbage aroma in *ex vivo* saliva samples was determined using human subjects (n = 21). This study clearly showed for the first time in raw plant tissue (cabbage) almost instantaneous production of MT, before the formation DMDS, DMTS and TTH. The PTR-MS data from *ex vivo* experiments showed that there was up to a 10-fold difference in the concentration of MT between the lowest and highest producing individuals, affecting sensory properties. The rates of formation of higher molecular weight sulfur volatiles also differed widely between individuals. The presence of sulfur volatiles generated in mouth from SMSCO by bacterial enzymes may be part of the unique flavor experience of eating raw or minimally processed cabbage and other brassica vegetables. In contrast, there was little evidence that the breakdown of aroma glycosides by salivary α-amylase enzyme played any role in the sensory differences of the *ex vivo* cabbage samples.

Apart from differences in individual aroma release in the oral cavity and perception as shown in this study, the breakdown of SMSCO during the oral phase of digestion may have wider implications for individual digestion, the gut microbiome and health [11, 12]. Future research using a larger cohort will be required to confirm the results described in this study and better ascertain whether the degree of in mouth volatile production from SMSCO is related to brassica vegetable liking and/or consumption frequency. Better characterization of the variation in different bacterial species in

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individual oral microflora, the level of CBL enzyme produced and the degree of sulfur volatile production may be warranted in future investigations. Additionally, the effects of bacterial enzymes resulting in differences in odor release and flavor perception should be further explored to better understand whether this is a common feature of other plant foods.

Declarations

Author contribution statement

Damian Frank: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Udayasika Piyasiri: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Nicholas Archer, Ingrid Appelqvist: Conceived and designed the experiments; Analyzed and interpreted the data.

Jenifer Jenifer: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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