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Olfactory Perception of Cysteine−*S*-Conjugates from Fruits and Vegetables

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Volatile sulfur compounds have a low odor threshold, and their presence at microgram per kilogram levels in fruits and vegetables influences odor quality. Sensory analysis demonstrates that naturally occurring, odorless cysteine−*S*-conjugates such as *S*-(*R*/*S*)-3-(1-hexanol)-L-cysteine in wine, *S*-(1-propyl)-L-cysteine in onion, and *S*-((*R*/*S*)-2-heptyl)-L-cysteine in bell pepper are transformed into volatile thiols in the mouth by microflora. The time delay in smelling these volatile thiols was 20−30 s, and persistent perception of their odor occurred for 3 min. The cysteine−*S*-conjugates are transformed in free thiol by anaerobes. The mouth acts as a reactor, adding another dimension to odor perception, and saliva modulates flavors by trapping free thiols.

KEYWORDS: Mouth microflora; *Fusobacterium nucleatum*; saliva; cysteine−*S*-conjugates; sensory analysis

INTRODUCTION

In his book *The Taste of Wine*, the French enologist Emile Peynaud, considered the father of modern enology, described how the golden Sauvignon grape releases an aromatic fruity odor 20−30 s after being swallowed (*1*). Unknown precursors caused this so-called retroaromatic effect, which is a delayed perception of retronasal odor. The same retroaromatic effect can be observed with onion powder (from *Allium cepa* L. cultivars), bell pepper (*Capsicum annuum* L. cultivars), and other fruits and vegetables.

Tominaga et al. discovered that volatile sulfur compounds, such as the thiol (*R*/*S*)-3-sulfanylhexan-1-ol, are key odorants in Sauvignon white wine (*2*). They found that the cysteine−*S*-conjugate precursors of these sulfur compounds are derivatives of the glutathione metabolic pathway (*3*).

The interaction of saliva with sulfur compounds has been already demonstrated, but many questions remain (*4*). To study this retroaromatic effect in more detail, we selected three cysteine−*S*-conjugates **1**−**3** that occur naturally in common food products (**Figure 1**). The first conjugate selected was *S*-(*R*/*S*)-3-(1-hexanol)-L-cysteine **1**, which leads to the formation of (*R*/*S*)-3-sulfanylhexan-1-ol **4**. The second conjugate was *S*-propyl-L-cysteine **2**, present in *Allium* sp., which forms 1-propanethiol **5**. The third conjugate was *S*-(2-heptyl)-L-cysteine **3**, detected in our laboratories in bell pepper, a precursor of 2-heptanethiol **6** (*5*). These cysteine−*S*-conjugates were incubated in sterile or crude saliva or with *Fusobacterium nucleatum*, a mouth anaerobe, to follow the consumption of the precursor into volatile thiols.

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EXPERIMENTAL PROCEDURES

General. Commercially available reagents and solvents of adequate quality were used without further purification. 6-Acryloyl-2-dimethylaminonaphthalene (Acrylodan) was purchased from Molecular Probes, Inc., catalog no. A-433. An Acrodisc LC 13 mm syringe filter, 0.45 μm PVDF membrane, was from Pall Life Sciences. Mineral water was from Henniez (Switzerland).

UPLC-MS Analyses. Ultraperformance liquid chromatography (UPLC) was performed on a Waters Acquity system coupled to a Thermo Finnigan TSQ quantum. The separations and quantifications were performed on an Acquity BEH-C18 column (2.1 mm i.d. × 100 mm, 1.7 μm). The elution solvents were CH_3CN containing 0.1% of formic acid (solvent B) and water containing 0.1% of formic acid. The gradient profile was started at 8% B, held for 0.5 min, increased to 30% B in 8.4 min, and then increased to 90% B in 2.1 min. The flow rate was 0.3 mL/min. The mass spectrometer was a Thermo Finnigan TSQ quantum ultra-triple-quadrupole spectrometer, with electrospray ion source operated in positive mode (ESI^+). The spray voltage was 4.0 kV. The capillary temperature was 349 °C. The sheath gas was nitrogen at flow rate 60 (Finnigan arbitrary units). The auxiliary gas was also nitrogen at flow rate 5 (Finnigan arbitrary units). Analyses were performed in single ion monitoring (SIM) mode using $[M + 1]^+$ to measure *S*-(*R*/*S*)-3-(1-hexanol)-L-cysteine **1**:220.0.

Calibration Curves. Solutions were prepared from the pure synthesized compound in water containing 0.1% of formic acid. The internal standard was *S*-[1-(2-hydroxyethyl)-1-methylbutyl]-(L)-cysteinylglycine ($C_{12}H_{24}N_2O_4S$, MW 292.40). The linear calibration curve was established from solutions at 0.02, 0.10, 1.00, 5.00, and 10.00 mg/L ($R^2 = 0.9998$). External calibration was established with *S*-[1-(2-hydroxyethyl)butyl]-L-cysteine in SIM mode.

HPLC/Fluorometer. A Thermo Separation Product Spectra system P4000 quaternary pumps, with autosampler AS3000 and fluorometric detector FL2000 was used. Excitation was at 390 nm and emission at 500 nm. The separations were performed on a Zorbax SB-C18 column (3.0 mm i.d. × 150 mm, 3.5 μm particle size; Agilent, catalog no.

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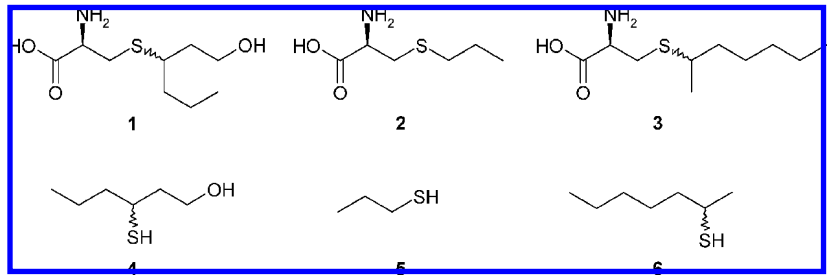

Figure 1. Transformation of naturally occurring *S*-(*R*/*S*)-3-(1-hexanol)-L-cysteine **1** in grapes, *S*-(1-propyl)-L-cysteine in **2** onion, and *S*-((*R*/*S*)-2-heptyl)-L-cysteine **3** in bell pepper into (*R*/*S*)-3-sulfanylhexan-1-ol **4**, 1-propanethiol **5**, and 2-heptanethiol **6**, by fermentation, plant enzymes, and by mouth microflora.

863954-302). The elution solvents were CH_3CN and water containing 0.02% of formic acid. The gradient profile was started at 30% CH_3CN, held for 0.5 min, and then increased to 100% CH_3CN in 10 min. The flow rate was 1 mL/min.

^{1}H and ^{13}C NMR Spectra. The NMR spectra were recorded on a Bruker Avance-500 spectrometer at 500.13 and 125.76 MHz, respectively. If not stated otherwise, the solvent was $CDCl_3$. δ values are in parts per million downfield from $(CH_3)_4Si$ (= 0 ppm). The cysteine−*S*-conjugates were measured in D_2O with sodium 3-(trimethylsilyl)-tetradeuteriopropionate as internal standard. The assignments by COSY, HSQC, and HMQC experiments were performed with standard Bruker software (XWINNMR 3.1).

Analysis of Flavors and Fragrances in Real-Time Monitoring (AFFIRM). Mouth and nose headspaces were measured with a Micromass Platform II equipped with an APCI source ($APCI^+$), corona discharge 4 kV heated transfer lines, and ion source at 105 °C. The gas flow rate was 600 L/h. The headspace samples were 50 mL/min.

Identification of *S*-((*R*/*S*)-2-Heptyl)-L-cysteine in *Capsicum annuum* L. Cultivar. Green bell peppers from a local supermarket (1500 g), in water (700 g), were minced with a KitchenAid (Krups chopper model KB720). The resulting emulsion was centrifuged at 4000 rpm for 45 min at 10 °C. The supernatant was lyophilized to give 68 g of solid. This solid (20 g) was diluted in water (100 g) and loaded on a Dowex (100 g, 50WX8, 200−400 mesh, conditioned with 0.1 M HCl) in a column (3 cm i.d.). The gradient elution was operated with portions of 100 mL at 0.3, 0.6, 0.9, 1.2, 1.5, 1.8 (fraction 6), 2.1, and 2.4 M (NH_4OH). The presence of *S*-((*R*/*S*)-2-heptyl)-L-cysteine **3** in fraction 6 was confirmed by UPLC-ESI in SIM M + 1 220.0 at the same retention time compared with the synthesized sample. The estimate of the concentration was based on the peak area obtained by SIM from fraction 6 and an external calibration established with the authentic sample: 0.057 (±0.002) mg/kg. The dosage was repeated on five different lots over different times of the year, and important variations were observed: 0.09−0.001 mg/kg.

Identification of *S*-(1-Propyl)-L-cysteine 2 in Commercial Onion Powder (Firmenich 2007-967746 Lot 3J005). Commercially used onion powders for snacks and food preparations are prepared from onions (*A. cepa* L. cultivar) that are cut in a few pieces and dehydrated in an industrial belt oven. This powder (20 g) was suspended in water (180 g) and an internal standard was added (I.S.): 0.05 mg of *S*-[1-(2-hydroxyethyl)-1-methylbutyl]-L-cysteinylglycine (*6*). The resulting suspension was centrifuged at 4000 rpm for 45 min at 10 °C. The supernatant (140 mL) was loaded on a Dowex column (3 cm i.d., 80 g, 50WX8, 200−400 mesh, conditioned with 0.1 M HCl). The column was rinsed with water (150 mL) and then eluted with a gradient operated with portions of 100 mL at 0.3, 0.6, 0.9, 1.2 (fraction 4), 1.5 (fraction 5), 1.8, 2.1, and 2.4 M (NH_4OH). *S*-(1-Propyl)-L-cysteine and the internal standard were detected in fractions 4 and 5. These fractions were pooled and lyophilized to give 600 mg of a brownish solid. A response factor was established after three consecutive injections of the internal standard and *S*-(1-propyl)-L-cysteine **2** in SIM mode (ions 164/293), which was 0.53 (±0.2), and then from the comparison of peak areas, the concentration of *S*-(1-propyl)-L-cysteine **2** was established at 1.9 mg/kg (±0.2 mg).

Preparation of *S*-((*R*/*S*)-2-Heptyl)-L-cysteine 3. To cysteine (6.05 g, 50 mmol), water (100 mL), and NaOH (12 g) was added (*R*/*S*)-2-bromoheptane (11.8 g, 66 mmol) with EtOH (50 mL). After 2 days with stirring, the solvent was evaporated under vacuum. The crude mixture was purified by HPLC on a LiChroprep RP-18, 40−63 μm (Merck, catalog no. 1.1390.0250) using a water−EtOH gradient starting at 0% EtOH and finishing with 100% EtOH. *S*-((*R*/*S*)-2-Heptyl)-L-cysteine **3** (mixture of two diastereoisomers) was obtained in a 7% yield (700 mg). UPLC/ESI^+ $[M + 1]^+$ 220. ^{1}H NMR (0.1 M NaOD): mixture of two diastereoisomers, 0.89 (t, J = 6.9 Hz, 6H), 1.28 (d, J = 6.8 Hz, 6H), 1.29−1.62 (16H), 2.67−2.77 (2H, −S−CH_2a*), 2.84−2.90 (2H, −S−CH−), 2.91−2.97 (2H, −S−CH_2b*), 3.36−3.40 (2H, −N−CH−) (*a and b = for "*abx*" system for both diastereoisomers). ^{13}C NMR (0.1 M NaOD): 183.6 (s, C1), 58.5 and 58.1 (d, C2), 43.1 and 42.6 (d, C4), 39.2 and 39.1 (t, C5), 39.1 and 38.4 (t, C3), 33.9 (2t, C-7), 28.7 (2t, C-6), 24.8 (2t, C-8), 23.6−25.5 (2q, $-CH_3$), 16.2 (2q, C-9).

***S*-(1-Propyl)-L-cysteine 2 and *S*-(*R*/*S*)-3-(1-Hexanol)-L-cysteine 1.** These were prepared as previously described (*7, 8*).

Dosage of the Free Thiol in the Presence of Saliva. The (*R*/*S*)-3-sulfanyl-hexan-1-ol **4** (2.5 μg/mL) was added to water (*Henniez Bleue* from Henniez Switzerland) (2 mL). A solution of Na_2HPO_4 (0.5 g, 0.2 M) was added followed by Acrylodan (0.1 mL, 0.1 mg/mL CH_3CN). The mixture was stirred on a vortex for 1 min and then stored for 45 min at 22 °C. This solution (0.5 mL) was added to formic acid 0.1% (0.5 mL) and injected on HPLC. The same operations were performed with 1.98 mL of water and 0.02 mL of sterile saliva (1%) and then repeated with 2, 3, 4, 5, 6, 7, 8, 9, and 10% of saliva.

Collection of Saliva. Whole saliva samples (10 mL) were collected from two male and two female adult volunteers in good oral and medical health. They were asked to spit out saliva directly into sterile polypropylene tubes 2 h after breakfast and after a thorough cleaning of their teeth with commercial toothpaste. The volunteers were nonsmokers and were undergoing no medical treatments at the time of the sampling. The experiment was repeated during four consecutive days so that enough materials could be gathered. Saliva samples were then pooled and centrifuged at 2880*g* for 5 min at 4 °C to remove excessive mucus and dead cells (crude saliva). Half of the crude saliva (80 mL) was centrifuged longer (2880*g*, 30 min, 4 °C), pasteurized at 60 °C for 1 h, and clarified by a final centrifugation (2880*g*, 30 min, 4 °C) (sterile saliva). Crude and sterile salivas were stored at −20 °C until used. The measured pH was 7.6 ± 0.1 (measured with a Mettler Toledo pH-meter) in crude and sterile salivas. The protein concentration was 0.59 ± 0.2 mg/mL (measured by the micromethod of Bradford with the Coomassie protein assay reagent) (*9*). A total count of 8×10^7 cfu/mL facultative and strict anaerobic bacteria was found in crude saliva samples after plating on Schaedler agar containing 5% sheep's blood in anaerobic conditions at 37 °C for 72 h. The anaerobic atmosphere was maintained by an anaerobe gas pack (Biomerieux) and checked by dipping an indicator strip into the jar. No bacteria were found in sterile saliva after anaerobic or aerobic incubation for 72 h at 37 °C.

Microbiological Culture and Growing Media. A bacterial solution of *Fusobacterium nucleatum* DSM 20482 (3.6×10^7 cfu/mL) was prepared in sterile, degassed saline solution (0.9% NaCl) from a 72 h anaerobic culture on a Schaedler agar plate (containing 5% sheep's blood). Anaerobic and aerobic oral bacterial solutions were prepared by incubating crude saliva samples on Schaedler agar (with 0.5% sheep's blood) in either aerobic or anaerobic conditions for 72 h at 37

°C. Bacterial counts were standardized at 5.6×10^7 and 7.0×10^7 cfu/mL for aerobic and anaerobic bacterial solutions, respectively. A Wilkins−Chalgren anaerobe liquid broth was used as rich liquid growing media for cultivating an anaerobic and facultative anaerobic strain (medium A). Minimal liquid growing media consisted of yeast extract (5 g/L), glucose (1 g/L), menadione (0.0005 g/L), and hemin (0.005 g/L) (medium B).

Bacterial Transformation of the Cysteine−*S*-Conjugates. Different bacterial solutions at 5×10^6 cfu consisting of *F. nucleatum* DSM 20482, anaerobic or aerobic oral cultivable bacteria (as described earlier), were incubated with 5 ppm of cysteine−*S*-conjugates diluted in 3 mL of either crude saliva, sterile saliva, or sterile physiological water (0.9% NaCl). Bacterial transformation at 37 °C was measured by analyzing the remaining cysteine−*S*-conjugate or thiol release in time. To measure cysteine−*S*-conjugate precursors, we took 0.5 mL of the reaction mix at various times and added it to 0.5 mL of formic acid solution (1%). The solution was filtered on Acrodisc, and 2 μL was injected on UPLC. The concentration of the precursor at $t = 0$ s corresponds to a precursor concentration of 2.5 mg/L or 100%.

Sensory Analysis. *Subjects.* Thirty trained panelists were from Firmenich S.A., Geneva.

Stimuli. Each solution tested was prepared by direct dilution of the product (sulfur compound or its cysteine conjugate) in mineral water. Dilutions were made in weight per volume. Tasting solutions of cysteine−*S*-conjugates were prepared from an odorless powder, corresponding to a neutral form.

Tasting Protocol for All Sensory Evaluations. Subjects received 30 mL samples in a cup (blind test). They were asked to take the entire sample in their mouths. They spit out the sample after 5 s and then answered the question.

Dose−Response Study. Seven different concentrations and a replicate for each free thiol were evaluated per session. Subjects rated the perceived retronasal odor on a linear scale from "not at all" to "very intense" (from 0 to 10). Student *t* tests were performed between the two replicated concentrations for each free thiol. No significant differences were perceived between the replicates at a confidence level of 5%.

Time−Intensity Study. For the time−intensity evaluation of free thiols and their conjugates, to avoid any fatigue or sensation overlap, only one sample was tasted per session. Subjects were asked to start the timer when they took the entire sample in their mouths. Each evaluation was rated on the same linear scale as for the dose−response study. Tests were repeated twice for the three pairs of products to demonstrate repeatability of sensory measurements. Student *t* tests were performed on each parameter (T_{begin}, I_{max}, T_{max}, I_{end}, and T_{end}) to compare data obtained from the cysteine−*S*-conjugate and its corresponding free thiol. The probability obtained for each of these tests indicates whether or not measurements were significantly different for the parameter under consideration. A significance level of 5% was taken.

Determination of the Retronasal Odor Threshold of (R/S)-3-Sulfanylhexan-1-ol 4 in Water. The three-alternative forced choice (3 AFC) method was performed with the same 30 trained panelists. The solutions were prepared from a daily preparation of 1 mg/L of the thiol diluted at the appropriate concentration with water. Eight concentrations were submitted: 4, 8, 16, 32, 64, 120, 250, and 500 ng/L. In one session, the eight 3 AFC tests, corresponding to the eight concentrations of the thiol, were presented in ascending order. Panelists had to determine for each test the odd sample among three, two water solutions and the sample with the thiol. The session was repeated twice. The panel threshold was calculated by using a geometric mean for the two sessions.

RESULTS AND DISCUSSION

We performed a sensory study, with 30 trained panelists, to evaluate the perceived retronasal odor intensity of the volatile sulfur compounds released from these cysteine−*S*-conjugates. To validate the sensory protocol, we established average perceived intensity as a function of the concentration of free thiols. The dose−response curves for the three thiols are similar (variance analysis 5%) (**Figure 2A**). A detection retronasal odor threshold for (*R*/*S*)-3-sulfanlyhexan-1-ol in water was determined to be 22 ng/L, which is comparable with published values of 1−60 ng/L (*10*).

Next, we evaluated the cysteine−*S*-conjugates and the free thiols in water and compared them by using a time−intensity method based on the same linear scale evaluation protocol and involving the same panelists. The 30 trained panelists were asked to take into their mouths a 1 mg/L solution of *S*-(*R*/*S*)-3-(1-hexanol)-L-cysteine **1** or, in another session, 0.01 mg/L of the corresponding free thiol, (*R*/*S*)-3-sulfanylhexan-1-ol **4**. The panelists noted the time when they started to perceive the sulfury retronasal odor, the time when they perceived its maximum intensity, and finally the time when it disappeared; if they still perceived an odor after 3 min, they estimated its intensity. The same study was performed with *S*-propyl-L-cysteine **2** at 20 mg/L and 0.01 mg/L of its corresponding free thiol **5**, as well as with *S*-((*R*/*S*)-2-heptyl)-L-cysteine **3** at 1 mg/L and 0.01 mg/L of its corresponding free thiol **6**. The concentrations used were about 10 times those that occur naturally to ensure that all panelists could perceive the sulfury odor well. The concentration in onion powder of *S*-propyl-L-cysteine **2** is 1.8 mg/kg (±0.2), and *S*-(2-heptyl)-L-cysteine **3**, in green bell pepper, was quantified at 0.09−0.001 mg/kg, the difference in concentrations reflects the seasonal variations and the differences in cultivated variety.

The (*R*/*S*)-3-sulfanylhexan-1-ol **4** was perceived immediately, but it took 12 (±2) s for participants to start to notice a retronasal odor for the corresponding cysteine−*S*-conjugate **1**. The maximum odor intensity for the free thiol was 16 (±5) s but shifted to 39 (±8) s for the cysteine−*S*-conjugate; in addition, the odor persisted for 100 (±12) s for the free thiol, whereas it lasted 163 (±9) s for the cysteine−*S*-conjugate (**Figure 2B**). This sensory analysis clearly demonstrates that the cysteine−*S*-conjugate was transformed into free thiol in the mouth. The same experiment was repeated with 1-propyl-*S*-cysteine **2**. The maximum odor intensity was perceived at 20 (±8) s for the free thiol **5** compared with 37 (±5) s for the conjugate; after 76 (±10) s, the odor of the free thiol was gone, but the odor could be perceived for up to 156 (±16) s for the conjugate (**Figure 2C**). Tasting of *S*-((*R*/*S*)-2-heptyl)-L-cysteine **3** gave results similar to those for the other cysteine−*S*-conjugates. Remarkably, bell pepper odor could still be detected after 3 min (**Figure 2D**) compared with 94 (±17) s for the free thiol **6**. All experiments were replicated and gave similar results (no significant differences at 95% according to the *t* test).

The only way for someone to smell an odor is to detect a volatile molecule when it reaches the olfactory epithelium in the nasal cavity. Therefore, the odor detected by the panelists in this study must result from the transformation of cysteine−*S*-conjugate into free thiol in the mouth. We wanted to know if it was possible to monitor the release of the free thiol. Our first approach was to use real-time analysis of the breath by using atmospheric pressure ionization mass spectrometry (MS). This technique consists of putting a piece of inert tubing linked directly to the mass spectrometer into the mouth or nose (*11, 12*). No signal was detected during real-time monitoring of the flavor release, even when 30 mL of 10 mg/L cysteine−*S*-conjugate **1** was taken into the mouth. The panelists were able to smell a strong odor, but the detection threshold of the instruments was too high to detect the thiol.

Many new techniques allow tracking of volatile thiols at the nanogram per liter level by gas chromatography coupled to mass spectrometry (GC-MS). The two approaches that we investigated were based on concentration by affinity chromatography with

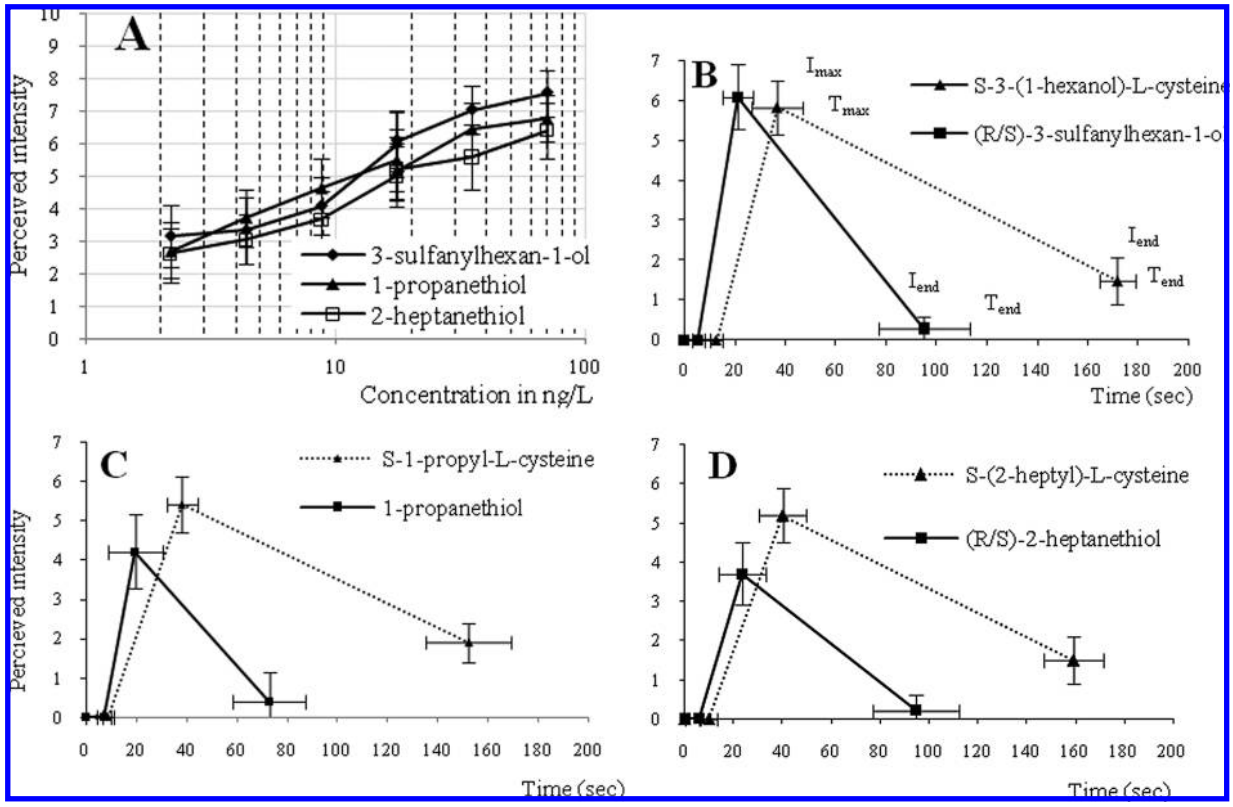

Figure 2. (**A**) Dose−response study for (*R*/*S*)-3-sulfanylhexan-1-ol, 1-propanethiol, and (*R*/*S*)-2-heptanethiol in water. Eight samples of one compound per session were tasted at seven different concentrations: 0, 2.2, 4.4, 8.8, 17, 35, and 70 ng/L (the sample at 17 ng/L was repeated). The subjects reported the perceived retronasal odor intensity for each sample. (**B**−**D**) Time−intensity study. The subjects reported the time of sulfur perception onset (T_{begin}), the maximum perceived sulfur intensity and its corresponding time (I_{max} and T_{max}), and the perceived intensity 3 min after they had sipped the sample (I_{end}) or the time when the sulfur intensity disappeared, if it was earlier than 3 min (T_{end}).

a mercury atom covalently linked to a resin (*13*) or by derivatization with pentafluorobenzyl bromide (*14*). Both approaches failed to show the presence of free thiols in saliva.

In medical studies of halitosis (breath malodor), highly volatile sulfur compounds such as hydrogen sulfide, methyl mercaptan, and dimethyl sulfide can be monitored by portable GC (OralChroma) (*15*) or by mouth air sampling and direct injection on a GC column. The compounds can then be detected by flame photometry (*16*). To avoid using concentrations and extractions, we developed a derivatization method for the free thiol with Acrylodan and monitored the concentration by high-performance liquid chromatography (HPLC) equipped with fluorometric detection. By using this method, we could detect free thiols in water at 0.001 mg/L, which is still less sensitive by a factor of about 100 compared with the human olfactory organ.

Next, we used this method to check the detection limit of the free thiol in saliva. Saliva is produced in the mouth by three major glands, the parotid, sublingual, and submandibular, and by other minor glands. Minor lingual saliva glands are crucial in taste perception because they irrigate taste buds. The role of saliva is to provide a coating on the mouth epithelium as a buffering system. Various enzymes are present: amylases, invertases, maltase, ureases, oxidases, lipases, phosphatases, and lysozymes (*17*). In addition, mucin glycoproteins lubricate oral surfaces, regulate the permeation of compounds to the taste buds, and modulate the colonization of oral bacteria. Human saliva is composed of about 98% water but is a dynamic system. The flow rate varies from 0.05 to 1 mL/min, and the protein concentrations vary from 0.6 to 1.2 mg/mL or from 230 to 250 mg/mL (*18*−*20*).

Saliva proteins interact strongly with free thiols. We found that the detection limit of (*R*/*S*)-3-sulfanylhexan-1-ol **4** in crude saliva was 60 (±10) mg/L, which is 3×10^6 above its odor threshold. To understand the importance of these protein−-thiol interactions, we diluted crude saliva at different concentrations in water. We observed that only the 10% solution of crude saliva quenched the detection of 1 mg/L **4**, whereas a 2% solution quenched the detection of 0.1 mg/L of this thiol. Sterile saliva quenched the detection of **4** less efficiently due to the loss of proteins during the clarification and pasteurization process (**Figure 3**). These analytical observations were confirmed by preparing a water solution in a cup containing 0.1 mg/L of (*R*/*S*)-3-sulfanylhexan-1-ol. When the participants sipped 20−30 mL of the free thiol into their mouths for 5 s, they perceived a strong odor. After spitting the solution out and smelling the cup again, the participants could no longer detect the odor in the cup, but the original solution was still described as having a strong sulfury odor, thereby confirming the interaction of saliva with free thiols.

Mouth microflora (*21*) and enzymes excreted by salivary glands (*22*) have been widely studied. Some investigators have observed a lag time in the release of malodorant molecules when cystine or oxidized glutathione was incubated with anaerobic Gram-negative bacteria (*22, 23*). *F. nucleatum*, a Gram-negative anaerobic rod, is associated with periodontal disease but is also found in healthy individuals. It can ferment carbohydrates and peptides and has been shown to be responsible for generating volatile organic sulfur compounds such as hydrogen sulfide and methyl mercaptan in human halitosis (*24*). Its concentration has been estimated to be 3.25 (±1.95) log cfu/mL in normal subjects (*25*). To demonstrate that saliva microflora have a role in the

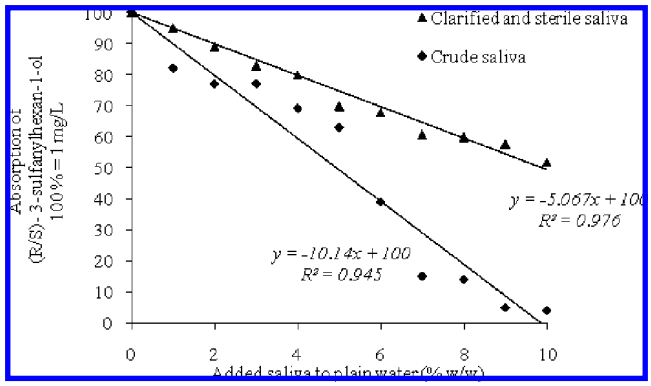

Figure 3. Interaction of free (*R*/*S*)-3-sulfanylhexan-1-ol (1 mg/L) with an increasing concentration of saliva in water. The intensity at 100% saliva represents the peak area of 1 mg/L of (*R*/*S*)-3-sulfanylhexan-1-ol in water. The quantification was performed by a high-performance liquid chromatography−fluorescent detection method after derivatization with Acrylodan.

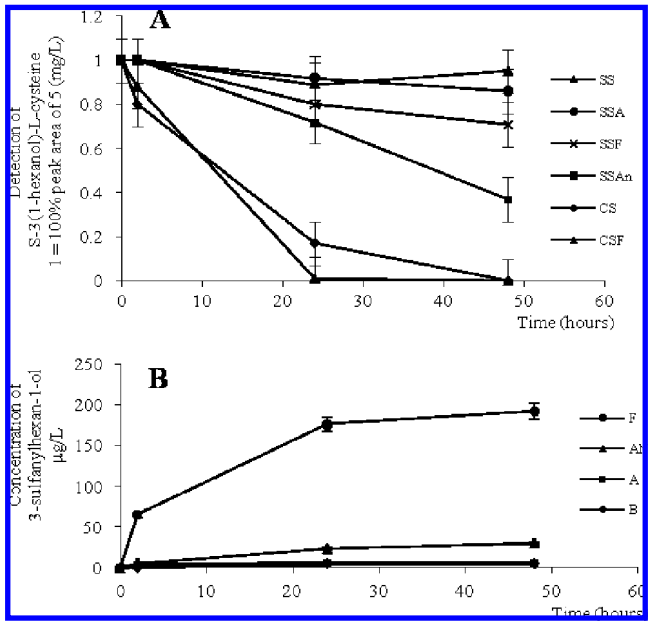

Figure 4. (**A**) Evolution of the concentration of *S*-3-(1-henanol)-L-cysteine in sterile saliva (SS), sterile saliva with aerobes added (SSA), sterile saliva with *Fusobacterium nucleatum* added (SSF), sterile saliva with anaerobes added (SSAn), crude saliva (CS), and crude saliva with *F. nucleatum* added (CSF), monitored by ultraperformance liquid chromatography−electrospray ionization (UPLC-ESI) in single ion monitoring mode. (**B**) Study of the formation of (*R*/*S*)-3-sulfanylhexan-1-ol in physiological water in the presence of *F. nucleatum* (F), anaerobes (An), aerobes (A), and blank (B), monitored by derivatization with Acrylodan and high-performance liquid chromatography−fluorometry detection.

transformation of cysteine−*S*-conjugates into odorant thiols, we added the precursor *S*-(*R*/*S*)-3-(1-hexanol)-L-cysteine **1** at a concentration of 5 mg/L to saliva and monitored the kinetics of the transformation by LC-MS in SIM mode. In crude saliva, 20% of the precursor disappeared after 2 h and 80% after 24 h at 37 °C. With the addition of *F. nucleatum*, the same result was obtained. In sterile saliva, however, <15% (±10%) of the precursor disappeared after 4 days of incubation at 37 °C. This difference clearly indicates that microorganisms transform cysteine−*S*-conjugates. When *F. nucleatum* was added to sterile saliva, 20% (±10%) of the precursor was consumed after 2 h in the same conditions. Compared with aerobes, which are inactive, the transformation by anaerobes was 60% (±10%) after 4 days (**Figure 4A**).

To verify whether the degradation of the precursor is correlated with the generation of (*R*/*S*)-3-sulfanylhexan-1-ol, we diluted 5 mg/L of its precursor in physiological water and incubated it with *F. nucleatum*. After 2 h at 37 °C, we observed the formation of 0.087 mg/L (±0.005) of (*R*/*S*)-3-sulfanylhexan-1-ol (4×10^3 above the odor threshold), which represented a conversion yield of 1.7%; after 1 day, the yield was 3.9%. This low yield is due to the decrease in the number of *F. nucleatum* in physiological water (**Figure 4B**). Indeed, the same experiment was repeated in a minimal growing medium containing peptones and yeast extracts, and after 3 days, 75% of the precursor was consumed and the observed yield of (*R*/*S*)-3-sulfanylhexan-1-ol was 40%. This evidence indicates that bacterial enzymes are responsible for the transformation of cysteine−*S*-conjugates into free thiols.

Cysteine−*S*-conjugates in various foods are transformed by mouth microflora enzymes into volatile odorant thiols. This transformation explains long-lasting sulfury odors in the mouth that give a second dimension to the flavor perception of food products. Saliva, however, is a strong modulator that absorbs free thiols. Whether this absorption results from physicochemical interactions, chemical transformations, or covalent linkage to glycoproteins remains unclear.

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