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New Factor Characterizing the In-Mouth Release of Odorants (Volatile Thiols): Compositional Changes in Odorants Exhaled from the Human Nose during Drinking

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The aim of this research was to investigate the relationship between the odorants in the model drink and the odorants reaching the olfactory epithelium using the Retronasal Flavor Impression Screening System (R-FISS). By application of the R-FISS to the odorants in the model drink, it was found that a methylthio ether [1-methoxy-3-methyl-3-(methylthio)butane] was detected with the original volatile thiol (4-methoxy-2-methyl-2-mercaptobutane) in the air exhaled through the nostrils via the nasal cavity after the model drink (including the original thiol) had been swallowed. In addition, this phenomenon was also observed in other volatile thiols (furfuryl mercaptan, ethyl 2-mercaptopropionate, 2-methyl-1-mercaptobutane, and 4-mercapto-4-methyl-2-pentanone). These compositional changes in thiols that were observed in the air exhaled through the nostrils could be affected by the chemical structure of each tested thiol and individual differences. These results pointed to a possibility that the odorants reaching the olfactory receptor via the throat during consumption of foods could not always retain their original chemical structures and compositions in foodstuffs. Therefore, the characteristic odor of volatile thiols might be perceived due to the stimuli of multiple compounds. To understand in detail flavor perception during the consumption of foods, not only the compositions or amounts of odorants in foodstuffs but also the compositional changes in odorants induced by biological reactions (reduction or methylation) need to be taken into consideration.

KEYWORDS: Flavor release; retronasal aroma; thiol; R-FISS; reduction; methylation

INTRODUCTION

For odorants in foodstuffs, the in-mouth release of odorants during the consumption of foods is also an important characteristic. Therefore, the characteristics of the in-mouth release of each odorant must be considered to understand in detail the characteristics of the odorants in foodstuffs. The perception of food aroma is the result of odorant/receptor interactions taking place in the olfactory receptors on the olfactory epithelium in the human nasal cavity. Therefore, sensing the aroma of food put into the human mouth requires that odorants in the food reach the olfactory epithelium via the throat. To understand the flavor perception during eating and drinking, it is important to determine the composition and amounts of the odorants that reach the olfactory epithelium (*1*, *2*). However, it is difficult to analyze the odorants reaching the olfactory epithelium. Therefore, to measure the odorants exhaled through the nostrils, several analytical techniques have been developed, such as nosespace analysis by APCI-MS (*3*), PTR-MS (*4*), EXOM (*5*), and R-FISS (*6*). These analytical techniques are based on the experimental results of Taylor et al., which show a better correlation between the intensity of the flavor perceptions during the consumption of foods and the amount of exhaled odorants through the nostrils via the nasal cavity than the amount of odorants included in the food themselves (*2*, *7*, *8*). By using these analytical techniques, the in-mouth release of odorants has been studied mainly from the physicochemical point of view. Many of these studies then demonstrated that the in-mouth release of odorants could be affected by the interaction of the food matrix with odorants and the chemical properties of the odorants.

Recent studies have indicated the possibility that the compositions of odorants reaching the olfactory epithelium during the consumption of foods do not always correspond to their original compositions in foodstuffs. Namely, odorants would be changed by human saliva (*9*, *10*), and nonvolatile cysteine-*S*-conjugates could provide the odorants (free thiols) in the human mouth (*11*). However, these results are focused on only the enzymatic or microbial reaction in human saliva, so that the relationship between the consumed odorants and the odorants reaching the olfactory epithelium (stimulating the olfactory receptor) appears not to be fully explained.

In this study, the Retronasal Flavor Impression Screening System (R-FISS) technique was selected to reveal the composition of odorants exhaled through the nostrils via the nasal cavity. The major advantage of R-FISS is the ability to improve the detection limit of low amounts of odorants and to determine the composition of the mixture consisting of a great number of odorants in one measurement. Therefore, the aim of this study was to investigate the relationship between the odorants in a

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Table 1. RSD of Peak Areas of 12 Odorants in Air Exhaled through the Nostrils via the Nasal Cavity

no.	compound	RSD[a] (%)
1	ethyl isobutyrate	8
2	3-hexanone	13
3	hexanal	5
4	butyl isobutyrate	4
5	1,8-cineole	14
6	2-octanone	12
7	octanal	4
8	isobutyl hexanoate	5
9	hexanol	4
10	decanal	10
11	octanol	2
12	decanol	12

[a] RSD was calculated using the peak areas from triplicate results obtained from identical panelists.

model drink and the compositions of odorants reaching the olfactory epithelium during drinking, focusing on the compositional changes in the odorants.

MATERIALS AND METHODS

Chemicals. The following odorants were obtained from the suppliers shown: 3-hexanone, ethyl isobutyrate, and ethyl 2-mercaptopropionate (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan); hexanal, octanal, octanol, and decanal (Kao Corp., Tokyo, Japan); butyl isobutyrate, isobutyl hexanoate, hexanol, decanol, and *p*-methylanisole (Inoue Perfumery Manufacturing Co., Ltd., Tokyo, Japan); 2-octanone and 2-methyl-1-mercaptobutane (Sigma-Aldrich Corp., Tokyo, Japan); 1,8-cineole (Nippon Terpene Chemicals, Inc., Kobe, Japan); (*Z*)-3-hexenol (Shin-Etsu Chemical Co., Ltd., Tokyo, Japan); furfuryl mercaptan (Oxford Chemicals, Ltd., Cleveland, U.K.). The following compounds were synthesized according to the literature procedures: 4-mercapto-4-methyl-2-pentanone (*12*) and 4-methoxy-2-methyl-2-mercaptobutane (*13*). Chemical purity was checked by gas chromatography−flame ionization detector (GC-FID) and gas chromatography−mass spectrometry (GC-MS).

On the other hand, methylthio ethers [furfuryl methyl sulfide, ethyl 2-(methylthio)propionate, 2-methyl-1-(methylthio)butane, 4-methylthio-4-methyl-2-pentanone, and 1-methoxy-3-methyl-3-(methylthio)butane] were synthesized as follows: approximately 4 g of thiol solution (1% in ethanol) was stirred with approximately 80 mg of iodomethane and 200 μL of a potassium hydroxide solution (2.0 mol/L). After stirring for 30 min at room temperature, 1 mL of a saturated ammonium chloride solution was added. Next, the reaction mixture was extracted with 0.5 mL of hexane. After a washing with a saturated sodium chloride solution, the organic layer was dried over anhydrous sodium sulfate and concentrated by a rotary evaporator. The products were analyzed by GC-MS, and each mass spectrum was confirmed.

Preparation of the Model Flavor. Twelve odorants given in **Table 1** were dissolved in ethanol (final concentration of each odorant was 0.5%). Also, 3-hexanone, ethyl isobutyrate, hexanal, hexanol, (*Z*)-3-hexenol, *p*-methylanisole, and five volatile thiols (furfurylmercaptan, ethyl 2-mercaptopropionate, 2-methyl-1-mercaptobutane, 4-mercapto-4-methyl-2-pentanone, and 4-methoxy-2-methyl-2-mercaptobutane) were independently dissolved in ethanol (final concentration of each odorant was 1%).

Preparation of the Model Drink. The model flavor was added at 0.1% to the syrup, which contains 10% granulated sugar and 0.1% citric acid in ion-exchanged water (final concentration of each odorant in the model drink was 5 or 10 ppm).

Trapping of In-Mouth Odorants Exhaled from the Human Nose via the Throat. To determine the in-mouth odorants exhaled from the human nose, after 30 mL of the model drink had been swallowed (a blank test was performed after only syrup had been swallowed), the air exhaled from the human nose was passed through a glass nosepiece fitted to the nose of each panelist (**Figure 1**). Ten breaths after the model drink had been swallowed were passed through a small glass column (6 cm × 5 mm i.d.) filled with Tenax TA (100 mg, 80/100 mesh, GL Science, Tokyo, Japan), which had been heated at 220 °C prior to the analysis. The end of the glass column was connected to a pump by a silicon tube, and during trapping of the air exhaled from the human nose, a suction of approximately 1 L/min was applied to the system. This sampling system allowed the panelists to exhale normally without the need to press the air through the Tenax column. After trapping, the water was removed from the Tenax TA with dry nitrogen (30 min, 100 mL/min). Three replicates of each experiment were performed with each panelist. These experiments were carried out at room temperature (25 ± 2 °C)

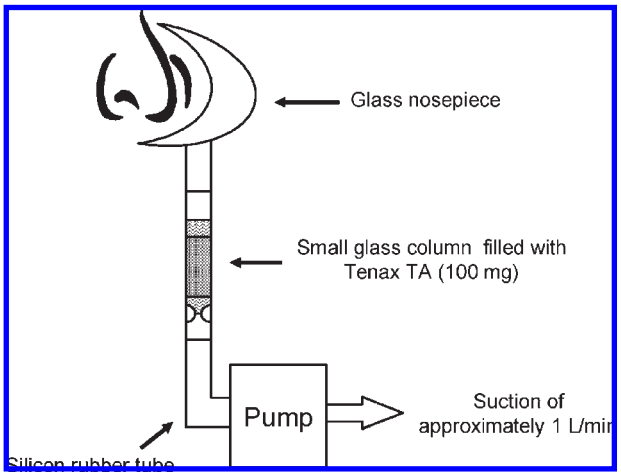

Figure 1. Schematic diagram of the trapping device for the odorants exhaled through the nostrils via the nasal cavity.

Dynamic Headspace (Purge-and-Trap) Analysis. A model drink was put into a glass flask. N_2 gas was then allowed to flow (at approximately 100 mL/min) through the model drink in the glass flask, and the odorants were trapped on Tenax TA, which had been heated at 220 °C prior to the analysis. After trapping, the water was removed from the Tenax TA with dry nitrogen (30 min, 100 mL/min).

GC-MS with Thermal Desorption. Thermal desorption of the trapped odorants on the Tenax TA was performed using a TDU thermal desorption system (Gerstel GmbH, Mulheim an der Ruhr, Germany) in combination with the ATEX option of an MPS2 autosampler (Gerstel GmbH) and a CIS-4 injector (Gerstel GmbH) for cryofocusing of the odorants prior to transfer onto the analytical column. The following sampling parameters were used: Thermal desorption was performed by programming the TDU from 20 to 220 °C (held for 3 min) at the rate of 12 °C/s and using the splitless mode. Cryofocusing was performed with liquid nitrogen at −150 °C. Injection was performed with a ramp of 12 °C/s from −150 to 220 °C (held for 3 min) and using the splitless mode. The odorants were analyzed by an Agilent 6890 N gas chromatograph coupled to an Agilent 5975 B series mass spectrometer (Agilent Technologies, Palo Alto, CA). The column was a 30 m × 0.25 mm i.d. DB-WAX fused silica capillary (J&W Scientific, Folsom, CA) with a film thickness of 0.25 μm. The column temperature was programmed from 30 °C (held for 3 min) to 210 °C at the rate of 5 °C/min. The flow rate of the helium carrier gas was 1 mL/min. The mass spectrometer was used with an ionization voltage of 70 eV (EI) and an ion source temperature of 150 °C. Also, the composition ratio of odorants (the tested thiol and the corresponding methylthio ether) was calculated from the peak area ratio of the total ion chromatogram.

Solvent Desorption from Tenax TA. The solvent desorption of trapped odorants on the Tenax TA was eluted with 5 mL of diethyl ether. The eluate was concentrated by N_2 gas flow.

Concentration of Odorants by the Column Adsorption Method. Thirty milliliters of the model drink was passed through the glass column (15 cm × 2 cm i.d.) filled with SP700 (5 mL, Mitsubishi Chemical, Tokyo, Japan). After the column had been washed with 50 mL of distilled water, the odorants on SP700 were eluted with 20 mL of dichloromethane. The eluate was concentrated by N_2 gas flow.

GC-MS with Liquid Injection. The concentrated odorants (the eluate from Tenax or SP700) were analyzed by an Agilent 6890 N gas chromatograph coupled to an Agilent 5973 series mass spectrometer (Agilent Technologies) and using the pulsed splitless mode (injection volume of

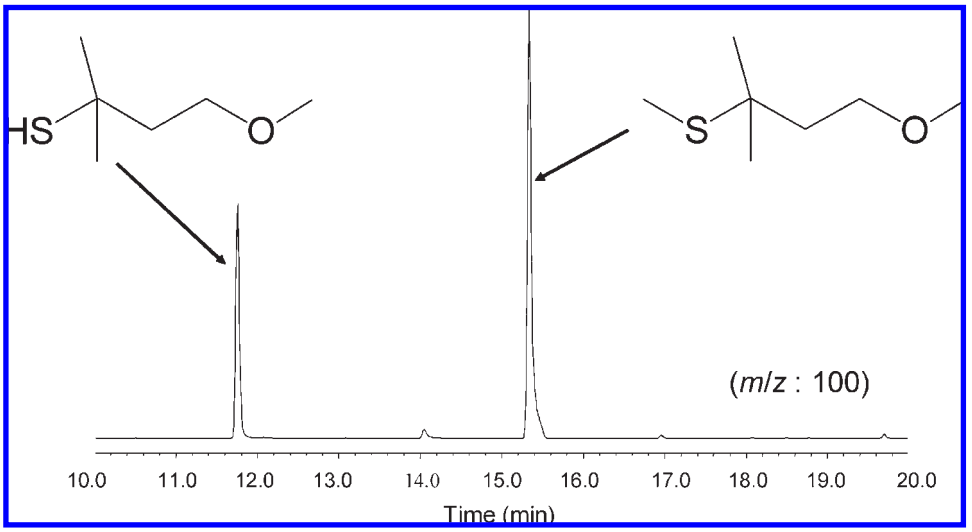

Figure 2. Ion chromatogram of the odorants exhaled through the nostrils after swallowing of the model drink. Concentration of 4-methoxy-2-methyl-2-mercaptobutane in the model drink was 10 ppm.

0.2 or 4 μL; inlet temperature of 250 °C; injection pulse pressure of 32.0 psi). The column was a 60 m × 0.25 mm i.d. DB-WAX fused silica capillary (J&W Scientific) with a film thickness of 0.25 μm. The column temperature was programmed from 40 to 210 °C at the rate of 5 °C/min. The flow rate of the helium carrier gas was 1 mL/min. The mass spectrometer was used with an ionization voltage of 70 eV (EI) and an ion source temperature of 150 °C.

RESULTS AND DISCUSSION

Application of R-FISS to the Odorants in the Model Drink. The concentrations of in-mouth odorants exhaled instantaneously from the human nose via the throat after a drink has been swallowed are extremely low, and many kinds of odorants are included in a real drink. The R-FISS technique is the modification to improve the detection limit based on the EXOM technique (*5*). In addition, R-FISS can achieve separation of the mixture consisting of a great number of odorants in one measurement as well as the EXOM (*5*). By application of R-FISS having these advantages, the reproducibility of peak areas of odorants exhaled through the nostrils was then examined after the model drink (including 12 mixed odorants; final concentration of each odorant in this model drink was 5 ppm) had been swallowed. As a result, the relative standard deviation [RSD (%) = SD 100/mean] of peak areas of the tested odorants showed good reproducibility of < 14% (**Table 1**) (*6*). This result indicated that the odorants exhaled from panelists would be reproducibly detected and that many odorants are certainly analyzed in one measurement, despite the facts that the odorants were exhaled in an instant through the nostrils via the nasal cavity and that their amounts were extremely low (*5*).

Compositional Changes in Odorants Exhaled through the Nostrils via the Nasal Cavity. To investigate the compositions of odorants between those in the model drink and the odorants exhaled from the human nose, the odorants exhaled through the nostrils after the model drink [independently including the following single odorants: 3-hexanone, ethyl isobutyrate, hexanal, hexanol, (*Z*)-3-hexenol, *p*-methylanisole, or 4-methoxy-2-methyl-2-mercaptobutane; final concentration of each odorant in this model drink was 10 ppm] had been swallowed were examined for one panelist using R-FISS. As a result, 1-methoxy-3-methyl-3-(methylthio)butane was detected with 4-methoxy-2-methyl-2-mercaptobutane in air exhaled through the nostrils after the model drink including 4-methoxy-2-methyl-2-mercaptobutane had been swallowed (**Figure 2**). Moreover, hexanol was detected with hexanal after the model drink including hexanal had been swallowed (**Figure 3**). That is, thiols were partially methylated to the corresponding methylthio ethers, and aldehydes were partially reduced to the corresponding alcohols. As a result, the compositions of those odorants exhaled through the nostrils were changed.

These compositional changes in odorants exhaled through the nostrils via the nasal cavity, especially volatile thiols, were examined with eight panelists (four males and four females). At first, the odorants exhaled through the nostrils were investigated after swallowing of the model drink (including 4-methoxy-2-methyl-2-mercaptobutane and 4-mercapto-4-methyl-2-pentanone; final concentration of each odorant in this model drink was 10 ppm). As a result, 1-methoxy-3-methyl-3-(methylthio)butane and 4-methylthio-4-methyl-2-pentanone were detected with their original thiols in air exhaled through the nostrils from all panelists.

To clarify that these compositional changes in odorants can be observed only in the air exhaled through the nostril via the nasal cavity, volatile thiols (4-methoxy-2-methyl-2-mercaptobutane and 4-mercapto-4-methyl-2-pentanone) at each stage of trapping odorants were analyzed by several analytical methods. At first, these thiols in the model drink were concentrated using the column adsorption method and analyzed by GC-MS. As a result, their corresponding methylthio ethers [1-methoxy-3-methyl-3-(methylthio)butane and 4-methylthio-4-methyl-2-pentanone] were not detected. These two thiols exhaled from the human nose after swallowing of this model drink were then trapped on Tenax TA and analyzed not only by thermal desorption but also by solvent desorption using diethyl ether. In both desorption techniques, their corresponding methylthio ethers were detected with the original tested thiols. On the other hand, after the headspace odorants of this model drink were trapped on Tenax TA using the dynamic headspace technique, they were analyzed by both thermal desorption and solvent desorption. However, their corresponding methylthio ethers were not detected by using either desorption technique. Thus, these results suggested that methylthio ethers were observed only in cases in which the odorants exhaled through the nostrils after swallowing of the model drink (including volatile thiols) were trapped. In other words, the compositional changes in volatile thiols appear to be common phenomena that occur in the oral or nasal cavity during the short period of time prior to the odorants being exhaled from the human nose via the throat.

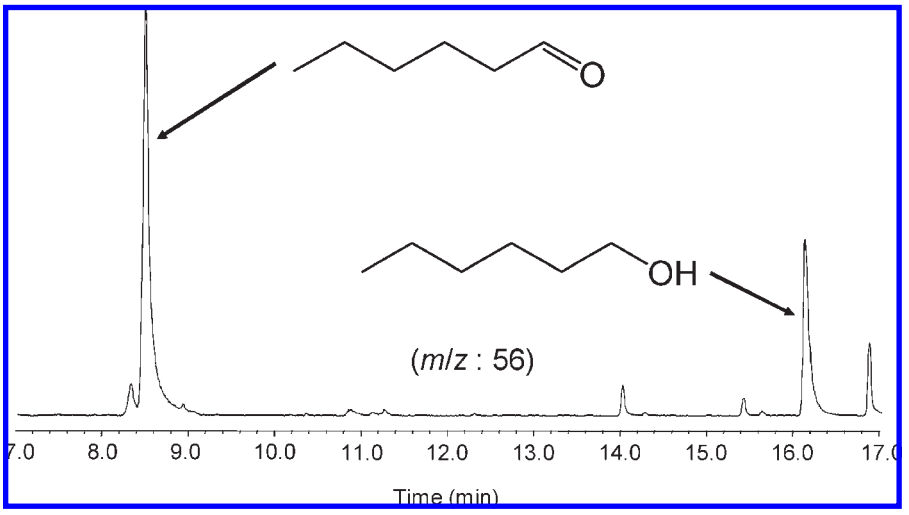

Figure 3. Ion chromatogram of the odorants exhaled through the nostrils after swallowing of the model drink. Concentration of hexanal in the model drink was 10 ppm.

As already stated, it has been previously indicated that enzymes in human saliva could change the odorants (*9*−*11*). However, after the model drink including thiols had been put in the mouth, the corresponding methylthio ethers were hardly observed in the drink expelled by the panelist. Therefore, given the present results showing that the compositional changes in odorants proceeded instantaneously, the enzymatic reactions in human saliva may be considered to have very little impact.

Methyl conjugation of thiols is a well-known reaction in mammals and microorganisms. Generally, thiol methylation in mammals is catalyzed by thiol methyltransferases that utilize *S*-adenosyl-L-methionine (AdoMet) (*14*). This enzyme has not yet been confirmed in the human nasal or oral cavity. However, numerous enzymes have been identified in the human nasal mucosa, for example, cytochrome P450, aldehyde dehydrogenases, UDP-glucuronosyl transferase, and glutathione *S*-transferases (*15*). Actually, it was already demonstrated that biotransformation of some kinds of odorants would occur in the human nasal cavity (*16*, *17*), and it was reported that odorants would be changed by enzymes in the mouse olfactory mucus (*18*). Therefore, there is a possibility that thiol methyltransferases could be present in the olfactory mucus and that they might be involved in methylations of volatile thiols. Also, AdoMet-dependent thiol methyltransferase activities are widespread in bacteria (*19*), and they are known to exist in the human oral and nasal cavity (*20*,*21*). Therefore, these bacteria might be involved in methylations of volatile thiols. Thus, in any case, this is the first study to show that volatile thiols consumed as an aqueous solution are partially methylated during the short period of time prior to the odorants being exhaled from the human nose via the throat. However, it will be one of the most important challenges to elucidate where the methylation occurs.

Influence of Chemical Structures and Individual Difference in the Methylation of Volatile Thiols. To determine whether the methylation would occur with other volatile thiols having different chemical structures, the odorants exhaled through the nostrils via the nasal cavity were examined by three panelists after the model drink (including furfuryl mercaptan, ethyl 2-mercaptopropionate, 2-methyl-1-mercaptobutane, 4-mercapto-4-methyl-2-pentanone, and 4-methoxy-2-methyl-2-mercaptobutane, separately; final concentration of each odorant in this model drink was 10 ppm) had been swallowed. As a result, despite the differences in chemical structures among these five kinds of thiols, all of their corresponding methylthio ethers [furfuryl methyl sulfide, ethyl 2-(methylthio)propionate, 2-methyl-1-(methylthio)butane, 4-methylthio-4-methyl-2-pentanone, and 1-methoxy-3-methyl-3-(methylthio)butane] were respectively detected with the original tested thiols in the air exhaled through the nostrils. The odor qualities of these methylthio ethers are different from those of their original thiols. Therefore, methylation during the consumption of food probably influences the flavor perception of thiols.

The compositional ratio of each thiol and its corresponding methylthio ether in air exhaled through the nostrils obtained from the experimental results of three panelists was then compared. As shown in **Figure 4**, the mean value of the compositional ratio differed considerably among five kinds of thiols. Moreover, the compositional ratio of ethyl 2-mercaptopropionate was highly individual, whereas that of 4-mercapto-4-methyl-2-pentanone was nearly the same between individuals. These results suggested that the compositional ratio of each thiol and its corresponding methylthio ether in air exhaled through the nostrils could be widely different between different kinds of thiols and between individuals. In addition, these ratios were slightly different between the concentrations of thiols in the model drink (data not shown). The previous study also indicated that concentrations of odorants would influence their decrease in human saliva (*10*). Therefore, the concentrations of thiols in the model drink can also influence their perception.

These differences seem to have some possible causes. At first, the chemical structure of each thiol seems to affect its compositional ratio in air exhaled through the nostrils via the nasal cavity. If an enzyme such as thiol methyltransferase could cause methylations of these thiols, the reaction must have more than a little substrate specificity. In fact, the high reactivity of furfuryl mercaptan is in good agreement with the previous study (*10*). However, to understand the substrate specificity in detail, it needs to be examined for more thiols. On the other hand, these individual differences might be caused by the difference in circumstances in the oral or nasal cavity between individuals (for example, the length of the pathway from the oral cavity to the nasal cavity via the throat, the amount of saliva, and the number of bacteria in the nasal or oral cavity, etc.).

These results pointed to a possibility that the odorants reaching the olfactory receptor via the throat during the consumption of foods could not always retain their original chemical structures and compositions in foodstuffs, so the characteristic odor of volatile thiols might be perceived due to the stimuli of multiple compounds. Therefore, to understand in detail flavor perception

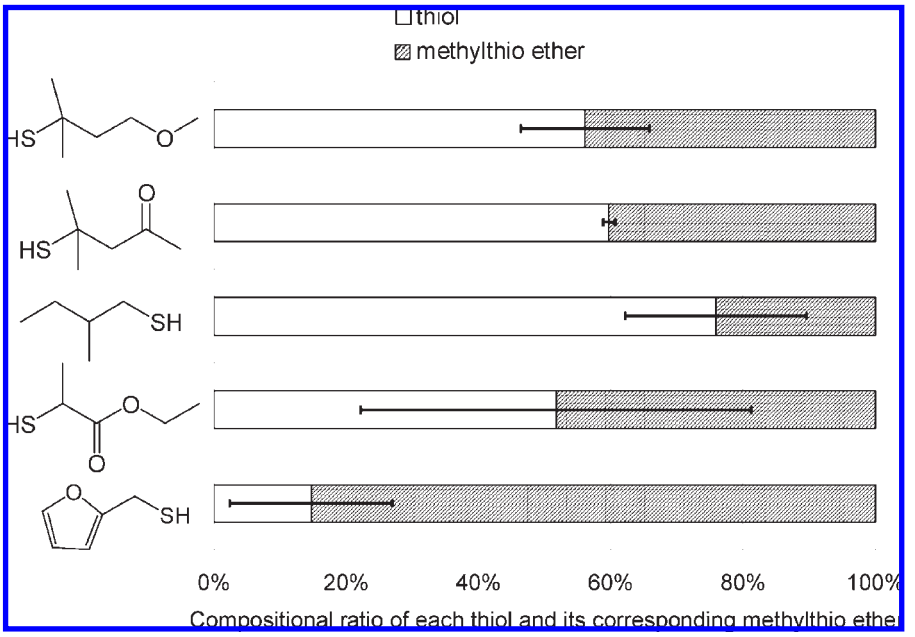

Figure 4. Compositional ratio of each thiol and its corresponding methylthio ether in air exhaled through the nostrils after swallowing of the model drink. Each compositional ratio was calculated from the peak area ratio of the total ion chromatogram. Concentration of each thiol in the model drink was 10 ppm. Values are the means of three panelists. Error bars show the standard deviations.

during the consumption of foods, not only the compositions or amounts of odorants in foodstuffs but also the compositional changes in odorants induced by biological reactions (reduction or methylation) need to be taken into consideration.

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