Studies on Factors Affecting the Flavor Release from Foods: Development of New Approaches to Estimate the Flavor Release Characteristics

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2017

Contents

List of Abbreviations

ACK	Acesulfame Potassium
AdoMet	S-adenosyl-L-methionine
AEDA	Aroma Extract Dilution Analysis
ANOVA	Analysis of Variance
APCI-MS	Atmospheric Pressure Chemical Ionization-Mass Spectrometry
ASP	Aspartame
ATEX	Automated TDU-liner Exchange
CAR	Carboxen
CIS	Cooled Injection System
DVB	Divinylbenzene
EI	Electron Ionization
EXOM	Exhaled Odorant Measurement
FD-factor	Flavor Dilution Factor
FFT	2-Furfurylthiol (Furfuryl Mercaptan)
FID	Flame Ionization Detector
FMS	Furfuryl Methyl Sulfide
GC	Gas Chromatography
GC-MS	Gas Chromatography-Mass Spectrometry
GC-O	Gas Chromatography-Olfactometry
HS-SPME	Headspace-Solid Phase Microextraction
IS	Internal Standard
MPS	Multi-Purpose Sampler
MS	Mass Spectrometer
PDMS	Polydimethylsiloxane
PTR-MS	Proton Transfer Reaction-Mass Spectrometry
QDA	Quantitative Description Analysis
RAS	Retronasal Aroma Simulator

RF	Response Factor
R-FISS	Retronasal Flavor Impression Screening System
RI	Retention Index
RSD	Relative Standard Deviation
SAFE	Solvent-assisted Flavor Evaporation
SD	Standard Deviation
SIM	Selected Ion Monitoring
SIM-SCAN	Selected Ion Monitoring and Scan
SPE	Solid Phase Extraction
SPME	Solid Phase Microextraction
TDU	Thermal Desorption Unit
TI	Time-intensity

General Introduction

Decisive Factors for Food Palatability

The palatability of foods is perceived through the five physical senses comprehensively. Sensory factors, such as flavor (aroma and taste), temperature, appearance (color and shape), mouthfeel (texture, viscosity, etc.), sound, external environment (atmosphere, room temperature and humidity), etc., are combined in the brain and finally recognized as the palatability of foods (**Table 0.1**) [1]. Aroma and taste are, especially, the most important factors determining the flavor perception of foods.

Flavor is considered to be a multi-modal sense resulting from multiple stimuli elicited by the aroma and taste compounds released from foods. Taste is perceived when the taste compounds in foods stimulate the gustatory receptors on the tongue. The taste sense basically consists of five tastes, i.e., sweet, salty, sour, bitter, and umami. Although pungency and astringency are not exactly the taste sense, these senses are also included as the taste sense in a wide meaning because their sensory information is transmitted to the brain and integrated with the taste sensory information. Aroma is perceived when the odorants released from foods stimulate the olfactory receptor on the olfactory epithelium located in the nasal cavity. In general, foods contain hundreds of aroma compounds. The aroma perception is an extremely complex sense resulting from multiple stimuli elicited by a number of aroma compounds. Aroma with such a complexity rather than taste seems to be responsible for the diverse flavors of foods, thus we can say that aroma plays a crucial role in the acceptability and palatability of foods.

Table 0.1 Factors for food palatability.

Factor	Sense		
Aroma	Smell	Aroma	Flavor
Taste	Taste	Sweet	Flavor
		Sour	Flavor
		Salty	Flavor
		Bitter	Flavor
		Umami	Flavor
	(Pain)	Pungency	Flavor
	(Touch)	Astringency	Flavor
Texture (Mouthfeel)	Touch	Hardness	
		Viscosity	
Temperature	Temperature	Temperature	
Appearance	Sight	Color	
		Shape	
Sound	Hearing	Sound	
External environment		Atmosphere	
		Temperature	
		Humidity	
Eating experience		Eating habits	
		Experience	
In vivo environment		Hunger	
		Physical conditions	

Characteristics of Aroma Components in Food and Flavor Release

As already described, food aroma plays an important role in determining the flavor perception and palatability of foods. It is a major and essential challenge in the flavor

science field to explore unique odorants having a favorable quality and to clarify the characteristics of these odorants. AEDA [2] introduced by Grosch et al. is one of the analysis techniques using GC-O, in which an aroma concentrate is analyzed several times by GC after the step-by-step dilution, and the separated aroma compounds by the GC are individually sniffed by a human nose. The contribution of each aroma component to the flavor perception is evaluated based on the dilution ratio of the aroma concentrate (FD-factor), which reflects the detection limit of the odorants. AEDA is a technique combining the instrumental analysis using GC and the sensory analysis, and we can obtain information about the aroma quality of each odorant as well as its contribution to the overall aroma of foods using this technique. This technique can also be used to clarify the effects of materials and processing conditions on the behavior of the aroma compounds, and to consider the mechanism whereby the aroma characteristics change. Such usefulness causes a situation that AEDA has been widely used to estimate the importance of each aroma component.

On the other hand, flavor release from foods (the diffusion behavior or persistence of odorants during eating and drinking) has been acknowledged as an important factor

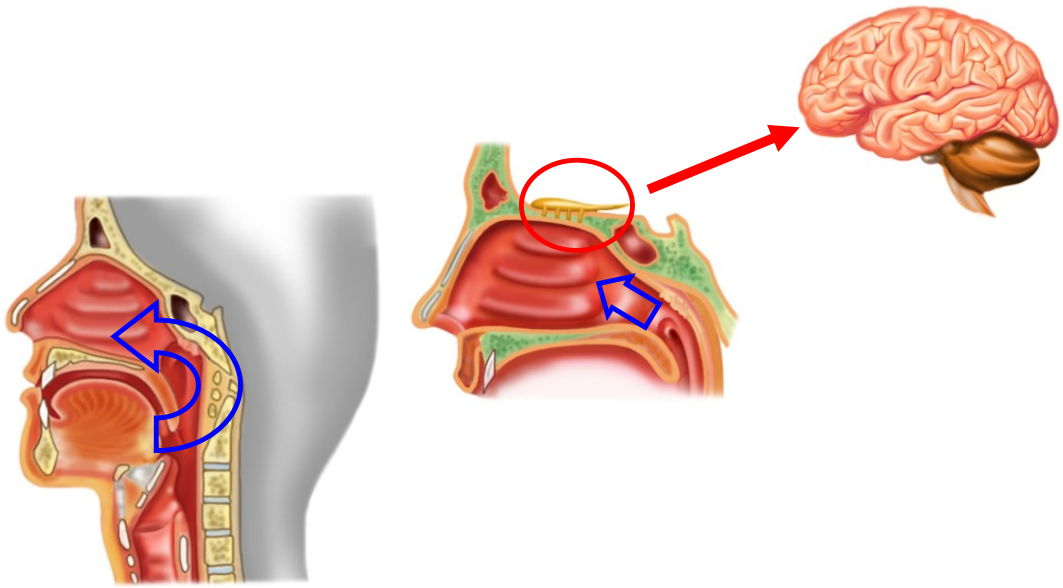


Figure 0.1 Flavor release from foods during eating and drinking.

determining food quality. The aroma perception of foods is divided into 2 types. One is the orthonasal aroma. In this case, odorants released from foods stimulate the olfactory receptors after directly passing through the nostrils. Another is the retronasal aroma. In this case, odorants released from foods in the oral cavity stimulate the olfactory receptors after reaching the nasal cavity via the throat. Perceived food aroma during eating and drinking is mainly the retronasal aroma (**Figure 0.1**). The composition and amount of aroma compounds in the retronasal aroma are closely related to our flavor perceptions during eating and drinking. Therefore, the behavior of odorants in the retronasal aroma, that is, the flavor release from foods during eating and drinking, should be clarified in order to understand the characteristics of the food aromas to produce high-quality foods having a pleasant flavor.

Analysis of Flavor Release -Method and Current Problems-

As already described, the perception of aroma is based on the phenomenon that the odorants released from foods stimulate the olfactory receptors on the olfactory epithelium located in the nasal cavity. When we feel food aroma during eating and drinking, odorants in the foods reach the olfactory epithelium via the throat. Therefore, in order to understand the aroma perception during eating and drinking, we should know the composition and amounts of odorants actually reaching the olfactory epithelium via the throat [3, 4]. It is, however, very difficult to analyze the odorants directly reaching the olfactory epithelium. An alternative way is to analyze the retronasal aroma, that is, odorants released from foods in the mouth and exhaled from the human nose. This methodology is rationalized by the result that the perceived flavor intensity has a better correlation with the aroma contents in the retronasal aroma than those originally present in foods as reported by Prof. Taylor and his coworkers [4-6]. Several analytical techniques have already been developed to analyze the retronasal aroma. There are three major techniques, and their features are described as follows (**Table 0.2**).

The first one is the MS Nose™, in which the odorants exhaled from the human nose during eating and drinking are directly introduced to an MS instrument. The odorants are ionized at atmospheric pressure and mainly detected as a protonated molecule ($[M + H]^+$). This technique has been widely used to examine the flavor release because it can monitor the in-mouth odorants in real time and simultaneously measure the temporal changes in the amounts of the in-mouth odorants while sensorially evaluating the flavor intensity. The odorants having the same molecular weights, however, cannot be identified because the odorants are introduced to the MS instrument without separation. Therefore, it is difficult to determine a number of odorants in one analysis. Although the MS Nose™ normally utilizes APCI-MS [5, 7-9] as a detector, another real-time analysis technique which utilizes PTR-MS [10-12] as a detector has been developed. PTR-MS also detects a protonated molecule and has the same problems as the APCI-MS.

The second one is EXOM [13], in which odorants released from foods in the mouth and exhaled from the human nose during eating and drinking are trapped on an adsorption resin with aspiration by a pump. After the trapped odorants are desorbed from the resin by an organic solvent, the odorants are concentrated and analyzed using a GC-MS instrument. This technique has the ability to identify and determine a number of odorants in the retronasal aroma in one analysis because the odorants are separated and detected by a GC-MS instrument. It is, however, hard to detect low amounts of odorants because the odorants trapped on the resin were desorbed and diluted by an organic solvent resulting in a dilute solution of the odorants.

The third one is RAS [14], in which the odorants in a sample are concentrated on an SPME fiber in the head space of a glass flask heated at approximately 37°C while the sample is stirred. The concentrated odorants are analyzed by a GC-MS instrument using the HS-SPME method. In addition to the good repeatability of the analysis results, the detection limit is expected to be improved because this technique utilizes a human mouth mimicked by a glass flask and easily increases the aroma contents in the sample. A previous study reported that the analysis results obtained by the RAS have a good correlation with the flavor release in the human mouth [15]. The mimicked human mouth is, however, just a model device and complete reproduction of the human mouth

Table 0.2 Advantages and problems of analysis techniques for flavor release.

	Advantages	Problems
MS Nose™ (APCI-MS)	Real-time analysis	Unable to separate isomers (Difficult to determine many odorants in one analysis)
EXOM	Ability to determine many odorants in one analysis	Low sensitivity (Hard to detect low amounts of odorants)
RAS	Good reproducibility	Not real mouth cavity (Correlation with flavor perception is poorly understood)

is extremely difficult because the device cannot replicate the saliva circulation and the complicated mastication process of the human mouth due to its structural problem.

In addition to these techniques, Beauchamp et al. [16] and Fransnelli et al. [17] also developed other analytical techniques that can analyze the odorants actually passing through the nasopharynx and reaching the olfactory epithelium in order to understand the flavor release in more detail. All the previously described existing techniques are mainly targeting model foods containing a limited number of odorants at relatively high concentrations. Although a lot of knowledge has been accumulated by applying these techniques with respect to the relationship between the odorant contents in the retronasal aroma and their perceived flavor intensity during eating and drinking, such knowledge cannot satisfactorily explain the favor release behavior of real foods. That is, in these studies, it has not been completely considered that the actual foods include various aroma components, and they reach the nasal cavity interacting with each other to cause the perception of flavor. Because of such technical problems, we cannot say that the existing techniques are currently capable of analyzing and understanding the flavor release from actual foods containing a number of aroma components at various concentrations.

Recently, food varieties are increasing year after year according to the diversification of diets. Many types of foods are being newly developed one after another. Aroma is required to give pleasant flavors to these newly-developed foods. Moreover, the aroma should have excellent flavor release characteristics optimized for each type of food. Therefore, it is essential to understand the flavor release characteristics of odorants in foods in order to develop high quality foods having pleasant flavors. However, few studies are available that have investigated in detail the flavor release from actual foods containing various aroma components. Thus, the relationship between the perceived flavor and the basic knowledge of aroma compounds (i.e., composition and amount) in the retronasal aroma during eating and drinking still remain unclear.

Purpose of This Study

As described above, flavor release from foods has been acknowledged as an important factor determining the quality of foods. Knowledge about the flavor release characteristics is expected to significantly contribute to the production of high quality foods having a pleasant flavor. It is, however, difficult to examine the flavor release from foods containing a number of odorants by the existing analysis techniques of the retronasal aroma. The flavor release characteristics of foods composed of various components still remain unclear. Therefore, in order to understand in more detail the flavor release characteristics, a new technique is required to analyze the retronasal aroma during the consumption of actual foods containing a number of odorants and estimate the flavor release characteristics of the odorants.

In order to obtain the fundamental knowledge about the flavor release from foods, a new analytical technique of the retronasal aroma applicable to actual foods was first developed. Based on the advantages and problems of the existing techniques, the following 3 points should be considered when developing the new analytical technique. First, in order to examine the perceived food aroma during eating and drinking, the

in-mouth odorants exhaled from the human nose should be analyzed. Second, in order to apply the technique to actual foods containing a number of aroma components, use of a GC is required to separate the aroma components. Finally, since the aroma contents in the retronasal aroma are presumed to be extremely low, the in-mouth odorants should be injected into the GC-MS without any loss to improve the detection limit. Based on these 3 points, R-FISS was developed as a new analytical technique of the retronasal aroma. The objective of this study is to investigate in detail the flavor release characteristics of odorants during the eating and drinking of two types of foods, chewing gum and beverages, using this new analytical technique.

The flavor release characteristics of the odorants in chewing gum are discussed in Chapter 1. In general, aroma plays a more important role in determining the quality of chewing gum compared to the case of other foods. The aroma of chewing gum is strongly required to have excellent in-mouth release characteristics, that is, the immediate flavor perception and the persistence of flavor during chewing, in addition to an excellent odor quality and the pleasant intensity. For the development of high-quality chewing gum products having a pleasant flavor, the formulation or the design considering the in-mouth release characteristics of odorants in chewing gums during chewing is necessary. However, no study has been available that investigates the flavor release from chewing gum containing a number of aroma components. This situation requires the accumulation of the fundamental knowledge about the flavor release characteristics of various odorants in the chewing gum. Therefore, in Chapter 1, the in-mouth release characteristics of a number of odorants exhaled from the human nose during chewing were investigated in detail by applying R-FISS to chewing gum products. Furthermore, the relationship between these characteristics of odorants and their physicochemical properties was demonstrated.

The flavor of chewing gum is perceived when odorants released from the chewing gum during chewing reach the nasal cavity via the throat. There are a few studies that have investigated in detail the odorant behavior in each step (i.e., mastication, dilution with saliva, mass transfer from the oral cavity to the nasal cavity, etc.) of flavor release during chewing. The influence of these behaviors on the flavor release characteristics of

odorants in chewing gum still remains unclear. In order to understand in more detail the flavor release from chewing gum, the odorant behavior during each step of chewing should be revealed. Therefore, in the latter part of Chapter 1, the elution characteristics of odorants in chewing gums were investigated using a chewing apparatus [18], focusing on the elution step of odorants from chewing gum into saliva during chewing.

Flavor release from beverages and their characteristics are described in Chapter 2. Beverages are made from various ingredients depending on the type of beverages. The main role of aroma in beverages is to impart a desirable or pleasant flavor to the beverages. The appropriate flavor release, especially in the mouth, is also indispensable. Therefore, it is worthwhile to research the effects of the ingredient compositions in the beverages on the flavor release and flavor perception in order to improve the quality of the beverage products.

Most studies on flavor release are conducted based on the assumption that the odorants released from foods in the oral cavity reach the nasal cavity via the throat without any change in their chemical structures. Recent studies have, however, indicated the possibility that the compositions of odorants reaching the olfactory epithelium during eating and drinking do not always correspond to their original compositions in the foods. The impact of these compositional changes on the flavor perception cannot be ignored. Actually, previous studies have reported that odorants are changed by human saliva [19, 20], and nonvolatile compounds can be changed to volatile compounds in the human mouth [21]. However, there have been few studies that compare the composition of odorants in the original foods and that of odorants reaching the olfactory epithelium (retronasal aroma). The compositional changes of odorants in the mouth during eating and drinking should significantly affect the flavor release and the flavor perception of foods. Therefore, in Chapter 2, the relationship between the odorants in beverages and the odorants in the retronasal aroma was investigated, especially focusing on the compositional changes in the odorants, by applying R-FISS to the model beverages.

The composition of the base components besides the aroma components should have a significant impact on the flavor perception of beverages. Coffee is often enjoyed

with milk or a creamer. In general, adding milk or a creamer to coffee significantly affects the aroma perceptions during drinking. Recently, the use of low-calorie sweeteners instead of sugar is highly required for making low-calorie beverages to meet the growing consumer demand for healthier foods. It was previously demonstrated that the intensities and quality of the perceived flavor of beverages were changed according to the types of sweeteners used in the beverage bases [22-24]. However, there have been few studies about the effects of the beverage compositions, especially the composition of the base components on the flavor release and flavor quality. Therefore, it is an important challenge to reveal the influence of the beverage compositions on the retronasal aroma in order to understand the relationship between the composition of the base components and flavor perception. In this chapter, the influence of milk and sweeteners on the flavor release and flavor perceptions was also investigated by comparing the amounts of the odorants in the retoronasal aroma during drinking coffee beverages and other model beverages.

Chapter 1

Studies on Development of New Approaches to Estimate the Flavor Release from Chewing Gum

1.1 A New Approach to Estimate the In-mouth Release Characteristics of Odorants in Chewing Gum

1.1.1 Introduction

In general, aroma plays a more important role in determining the quality of chewing gum compared to the case of other foods. The aroma of chewing gum is strongly required to have excellent in-mouth release characteristics, that is, the immediate flavor perception and the persistence of flavor during chewing, in addition to an excellent odor quality and an appropriate intensity. For the development of high-quality chewing gum products having a pleasant flavor, the formulation or the design considering the in-mouth release characteristics of odorants in chewing gums during chewing is necessary. However, the in-mouth release characteristics such as the immediate flavor perception and the persistence of flavor during chewing still remain unclear. Therefore, the aims of this study were to develop a new approach to estimate the in-mouth release characteristics of odorants in chewing gum during chewing and to apply the approach to the prediction of those in-mouth release characteristics.

1.1.2 Materials and Methods

Materials. Commercial and model chewing gums were tested. The commercial chewing gum was purchased from a local market. The model chewing gum was prepared by

kneading a mixture of about 60 odorants (see **Table 1.2**; final concentrations of the odorants were each ca. 30 ppm dissolved in glyceryl triacetate) or a single odorant (final concentration of ethyl propionate or menthyl acetate was 400 ppm dissolved in glyceryl triacetate), powdered sugar (64 g), and 85%-sugar syrup (13 g), with a gum base (23 g).

Trapping of In-mouth Odorants Exhaled through the Nostrils. Panelists placed the chewing gum (the weights of the commercial chewing gums were approximately 2.5 g and those of the model chewing gums were approximately 2 g) into their mouth cavities and chewed at the rate of 100 chews per minute using a metronome. In order to determine the in-mouth odorants, the breath exhaled through the nostrils was passed through a glass nosepiece fitted to the nose of each panelist (**Figure 1.1**). The breaths during chewing for 1 min or 10 min were passed through a small glass column (6 cm × 5 mm i.d.) filled with 100 mg of Tenax TA (80/100 mesh, GL Science, Tokyo, Japan), which had been heated at 220°C for 2 hours prior to the analysis. The end of the glass column was connected to a pump by a silicon tube, and a suction of approximately 1 L/min was applied to the system during trapping of the air exhaled through the nostrils. This sampling system allowed the panelists to normally exhale without the need to press air through the Tenax column. After trapping of the in-mouth odorants, the water was removed from the Tenax TA by flowing dry nitrogen (30 min, 100 mL/min). Three replicates of each experiment were performed by each panelist. These experiments were carried out at room temperature (25 ± 2°C).

GC-MS. 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 and a CIS4 injector (Gerstel GmbH) according to the following parameters. Thermal desorption was performed by programming the TDU from 20°C to 220°C (held for 3 min) at the rate of 12°C/s in the split mode (the split ratio was 30:1). Cryofocusing was performed with liquid nitrogen at -150°C. Injection was performed with the ramp of 12°C/s from -150°C to 220°C (held for 3 min) in the split mode (the split ratio was 5:1). The odorants were analyzed by an Agilent 6890N GC with an Agilent 5975B series MS (Agilent Technologies, Palo Alto, CA, USA). The column was a 30 m × 0.25 mm i.d.

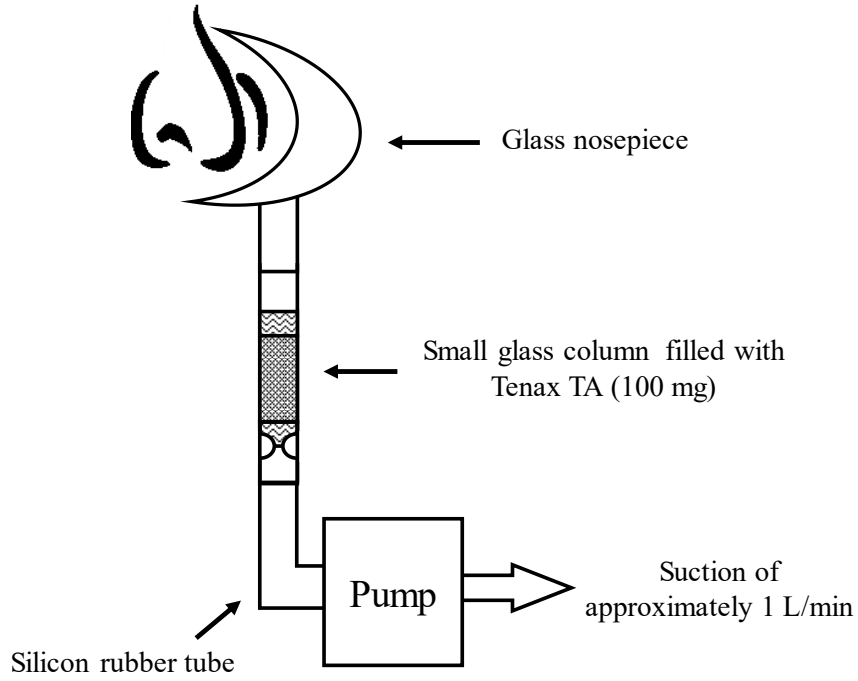


Figure 1.1 Schematic diagram of the trapping device for the odorants exhaled through the nostrils via the nasal cavity.

DB-WAX fused silica capillary (J & W Scientific, Folsom, CA, USA) 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 MS was used with an ionization voltage of 70 eV (EI) and operated in the SCAN mode. The ion source and quadrupole temperature were set at 230°C and 150°C, respectively. The peak area ratio of each odorant exhaled through the nostrils was calculated by dividing the peak area of the extracted ion for 10 min by that for 1 min.

Sensory Evaluation. Five panelists (two females and three males) were trained for the TI analysis of the flavor sensation of chewing gums during chewing. They were asked to rate the perceived flavor intensity with time while chewing the gums (100 chews/min). The flavor intensity was scored on a scale of 1 (weak)–10 (strong). Ten results per sample were obtained from the five panelists (each panelist evaluated a model chewing gum containing a single odorant twice). The scores were normalized as a percentage of the maximum score per sample, and then the ten normalized scores were averaged.

1.1.3 Results and Discussion

Estimation of the In-mouth Release Characteristics of Odorants in Chewing Gum. It seems to be quite difficult to analyze the in-mouth release from chewing gum because the air exhaled from the human nose contains a number of odorants and their amounts are extremely low. Therefore, in order to analyze the in-mouth odorants exhaled from the human nose during consumption of actual foods such as chewing gum, the in-mouth odorants need to be concentrated and separated by GC. A technique with combination of the trapping of odorants in the gas phase on an adsorption resin such as Tenax and a GC-MS instrument equipped with a thermal desorption and cryofocusing injection system have been used for the dynamic headspace analysis. This technique has the ability to improve the detection limits of odorants and to determine the composition of the mixture containing a significant number of odorants in one analysis. By applying

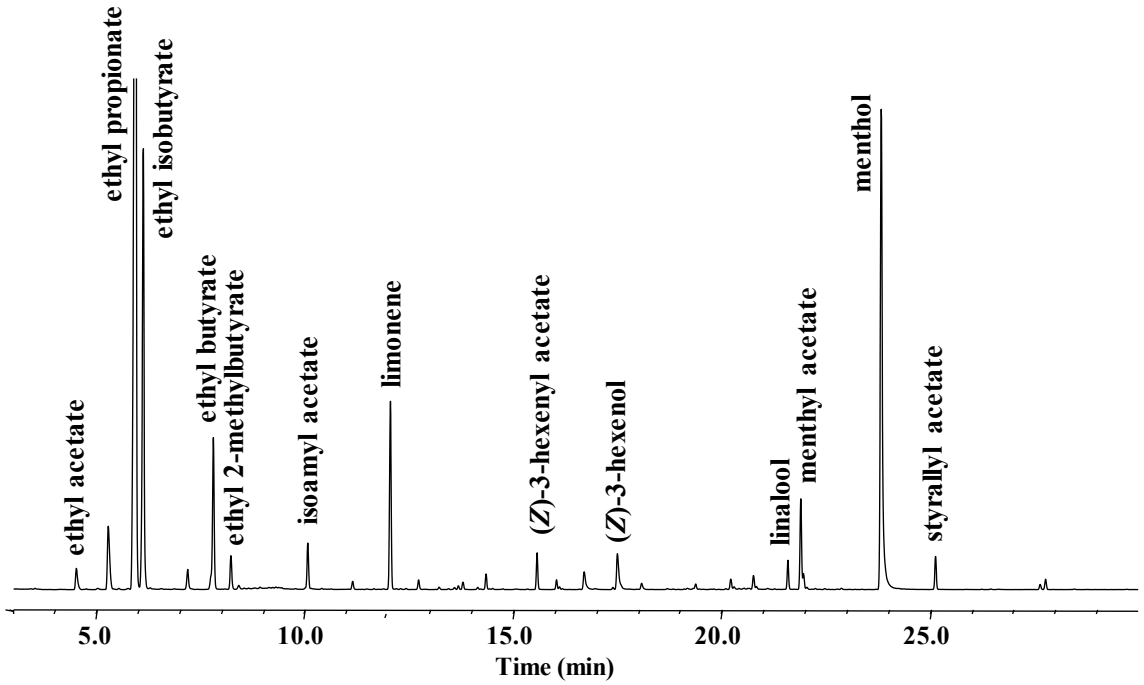


Figure 1.2 Typical gas chromatogram of the exhaled in-mouth odorants from the human nose (commercial chewing gum).

this analytical technique to the in-mouth odorants in commercial chewing gum, various concentrations of odorants could be successfully separated by optimizing the TDU-CIS4 injection system and GC conditions (**Figure 1.2**).

The reproducibility of the peak areas of odorants in commercial chewing gum was examined by six panelists (**Table 1.1**). The RSD (%) of the peak areas of the main

Table 1.1 Comparison of the RSD (%) of the peak area and peak area ratio of the main exhaled odorants in the commercial chewing gum.

		RSD (%)[a]		
		Peak Area[b]		Peak Area Ratio[c]
No	Compound	Same Panelist[d]	Between Different Panelists	Between Different Panelists
1	ethyl acetate	17	51	23
2	ethyl propionate	8	84	19
3	ethyl isobutyrate	8	85	15
4	ethyl butyrate	9	92	30
5	ethyl 2-methylbutyrate	8	88	15
6	isoamyl acetate	9	78	24
7	limonene	9	89	24
8	(*Z*)-3-hexenyl acetate	7	93	24
9	(*Z*)-3-hexenol	12	99	32
10	linalool	12	82	18
11	menthyl acetate	10	91	28
12	menthol	6	67	22
13	styrallyl acetate	18	91	25

[a] Each RSD (%) was calculated based on the value from six panelists.

[b] Peak area was the mean value of the triplicate results for 10 min.

[c] Peak area ratio of each compound was calculated by comparing the peak area for 10min to the peak area for 1 min (10 min/1 min).

[d] RSD (%) was the mean value from six panelists.

odorants exhaled from the human nose trapped for 10 min from the beginning of chewing by each panelist showed good reproducibility of ≤ 18% (RSD (%) = SD × 100/mean). However, the average peak areas among the six panelists showed high RSD values (51 < RSD (%) < 99). On the other hand, the RSD (%) of the peak area ratios calculated by dividing the peak area of each in-mouth odorant trapped for 10 min by that trapped for 1 min were significantly lower than those of the average peak areas among the six panelists. Namely, there were small individual differences in the peak area ratios regardless of the significant individual differences in the peak area values of the odorants exhaled from the human nose. These findings indicated the possibility that the in-mouth release characteristics of odorants in chewing gum would be estimated by comparing the peak area ratios because the peak area ratio appeared to indicate the in-mouth release kinetics of each odorant. In preliminary experiments with this sampling system, the peak area of the odorants in chewing gum exhaled from the human nose during chewing increased in proportion to the length of time from the beginning of chewing (the range from 1 min to 10 min). Therefore, 1 min and 10 min were selected as the trapping times in order to indicate more definitely the difference in the peak area ratios of the in-mouth odorants. It was demonstrated that each odorant had the specific peak area ratio by comparing the peak area ratios of the odorants (**Figure 1.3**). Since the peak area ratios of the odorants seem to correspond to their in-mouth release kinetics during chewing, the peak area ratio was assumed to indicate the in-mouth release characteristics of odorants in the chewing gum. Based on these results, ethyl propionate showing a low peak area ratio and menthyl acetate showing a high peak area ratio were expected to have an immediate flavor perception and a longer persistence of flavor perception, respectively.

In order to examine the relationship between the difference in the peak area ratios and the in-mouth release kinetics of the odorants in chewing gum, the flavor perception of ethyl propionate and menthyl acetate in the model chewing gum was compared by sensory evaluation using the TI measurement. The TI curves of those 2 odorants produced by plotting the normalized flavor intensities showed similar patterns among 5 panelists despite the individual differences in the perceived flavor intensities. **Figure 1.4**

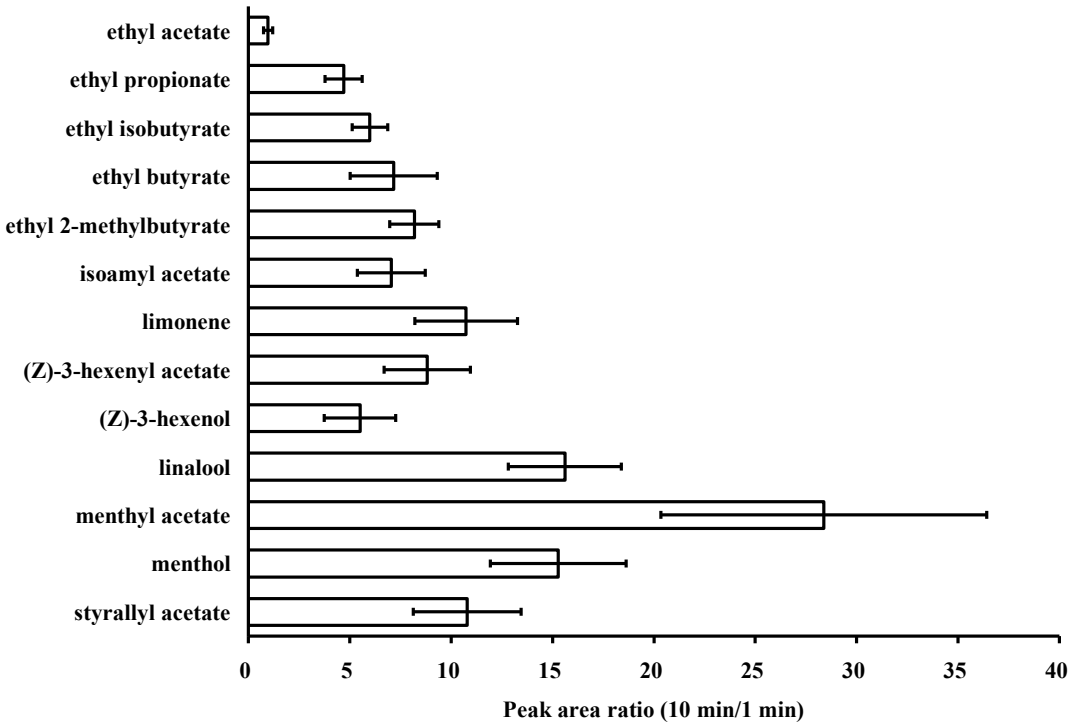


Figure 1.3 Average peak area ratios, which were calculated by dividing the peak area for 10 min by the peak area for 1 min, of the exhaled odorants in commercial chewing gum. Each average ratio was the mean value of triplicate results from six panelists.

shows the change in the flavor intensities of ethyl propionate and menthyl acetate in the model chewing gum. As a result, their TI curves were quite different. The flavor intensity of ethyl propionate decreased in a short time. In contrast to that, the flavor intensity of menthyl acetate lasted for a long time. These results indicated that the peak area ratios obtained by R-FISS agreed well with the results of the sensory TI analyses, and the technique can be used to estimate the in-mouth release characteristics of the odorants in chewing gum.

Prediction of the In-mouth Release Characteristic of Odorants in Chewing Gum. It was reported that the hydrophobicity/hydrophilicity (*Log P*) and vapor pressure (*Log ρL*) of each aroma compound are the most important factors on the persistence of their flavor perception, which means the in-mouth release characteristics of the compounds, during consumption of an aqueous solution containing those volatile compounds [25]. In

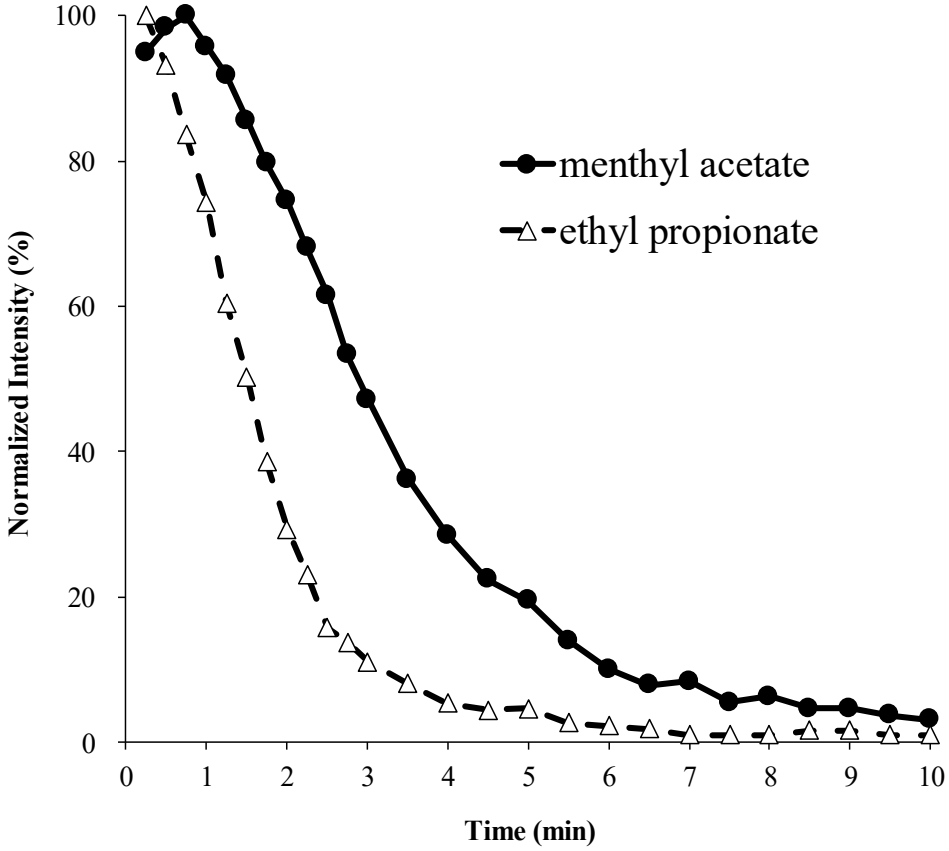


Figure 1.4 Simultaneous time-intensity curves of ethyl propionate and menthyl acetate in the model chewing gum. Ten results per sample obtained from five panelists (two replicates of each chewing gum per panelist), and the scores of each perceived intensity have been normalized for easy comparison.

addition, a previous study also suggested that the interaction between polarity and boiling point of each compound plays a role in the rate and time of flavor release from mint-flavored sweets [26]. In general, the vapor pressure (boiling point) and hydrophobicity/hydrophilicity (polarity) are also major factors on the retention and separation of each compound in GC analyses [27, 28]. For instance, the vapor pressure or the interaction between polarity and vapor pressure of compounds are regarded as the major factors affecting their retention on apolar GC columns such as DB-1 or polar GC columns such as DB-WAX, respectively. Based on these factors, it can be presumed that

Table 1.2 Peak area ratios[a] of the tested odorants in the model chewing gum.

No.	RI DB-Wax	RI DB-1	ΔI[c]	Compound	Extracted ion[b] (m/z)	Peak area ratio (10 min/1min)	No.	RI DB-Wax	RI DB-1	ΔI[c]	Compound	Extracted ion[b] (m/z)	Peak area ratio (10 min/1min)
1	890	593	297	ethyl acetate		1.4	30	1321	1079	242	propyl hexanoate	117	7.7
2	894	714	180	acetaldehyde dea		2.4	31	1316	888	428	2-heptanol		8.1
3	927	673	254	methyl isobutyrate		2.7	32	1356	1133	223	isobutyl hexanoate		8.1
4	956	690	266	ethyl propionate		3.6	33	1337	846	491	hexanol		6.4
5	965	735	230	ethyl isobutyrate		4.4	34	1371	1057	314	allyl hexanoate		8.0
6	990	661	329	2-pentanone		2.8	35	1385	836	549	(*Z*)-3-hexenol	67	6.1
7	962	850	112	isobutanal dea		3.4	36	1412	1074	338	methylthio hexanoate		7.8
8	971	705	266	methyl butyrate		3.6	37	1401	854	547	(*E*)-2-hexenol	82	6.2
9	1036	774	262	ethyl butyrate		4.9	38	1416	1177	239	butyl hexanoate	117	8.1
10	1058	760	298	3-hexanone		5.1	39	1437	1228	209	hexyl isovalerate		8.9
11	1055	837	218	ethyl 2-methylbutyrate		5.3	40	1453	1079	374	(*Z*)-6-nonenal	122	9.7
12	1074	758	316	butyl acetate		5.2	41	1448	972	476	1-octen-3-ol	57	10.3
13	1091	774	317	hexanal		4.8	42	1455	957	498	heptanol	70	7.6
14	1111	852	259	isoamyl acetate		5.8	43	1501	990	511	(*E*,*E*)-2,4-heptadienal	81	7.1
15	1136	875	261	ethyl valerate		6.0	44	1510	1089	421	2-nonanol		14.2
16	1149	934	215	butyl isobutyrate		6.6	45	1541	1280	261	ethyl nonanoate		9.1
17	1195	1010	185	1,4-cineole		5.5	46	1550	1084	466	linalool		15.4
18	1191	866	325	2-heptanone	114	6.1	47	1555	1052	503	octanol	84	13.1
19	1195	877	318	heptanal	96	5.8	48	1588	1110	478	fenchyl alcohol		11.7
20	1215	867	348	methylthio butyrate		5.7	49	1612	1371	241	hexyl hexanoate	117	9.4
21	1224	1027	197	1,8-cineole		4.5	50	1611	1373	238	octyl butyrate	89	9.5
22	1234	825	409	(*E*)-2-hexenal	98	5.2	51	1645	1171	474	menthol		10.6
23	1219	979	240	butyl butyrate	89	7.1	52	1724	1009	715	4-hexanolide	85	11.2
24	1241	871	370	(*Z*)-4-heptenal		5.1	53	1706	1183	523	α-terpineol		15.3
25	1264	928	336	ethyl amyl ketone		7.9	54	1842	1373	469	β-damascenone	121	12.3
26	1275	991	284	hexyl acetate		8.2	55	1854	1408	446	α-ionone	121	16.5
27	1294	959	335	2-octanone		8.3	56	1937	1215	722	4-octanolide	85	13.8
28	1299	980	319	octanal		6.3	57	1928	1104	824	2-phenylethyl alcohol	91	12.7
29	1320	983	337	(*Z*)-3-hexenyl acetate	82	7.7							

[a] Each peak area ratio was the mean value of triplicate results by one panelist.

[b] Each peak area ratio of the overlapped odorants on the gas chromatogram was determined by comparing the peak area of the extracted ions for 10 min to that for 1 min.

[c] ΔI is the difference of the RI values on the polar and apolar columns.

the peak area ratios of odorants, which correspond to their in-mouth release characteristics, obtained by R-FISS have a close relationship to the retention time or RI value in GC analyses. Furthermore, these RI values are expected to be used to predict the in-mouth release characteristics of odorants in chewing gum.

In order to reveal the relationship between the peak area ratio and RI value of each odorant, the peak area ratios of odorants having various functional groups (such as ethers, ketones, aldehydes, esters, and alcohols) were determined by R-FISS using the model chewing gum. **Table 1.2** shows the RI values on the polar and apolar GC column,

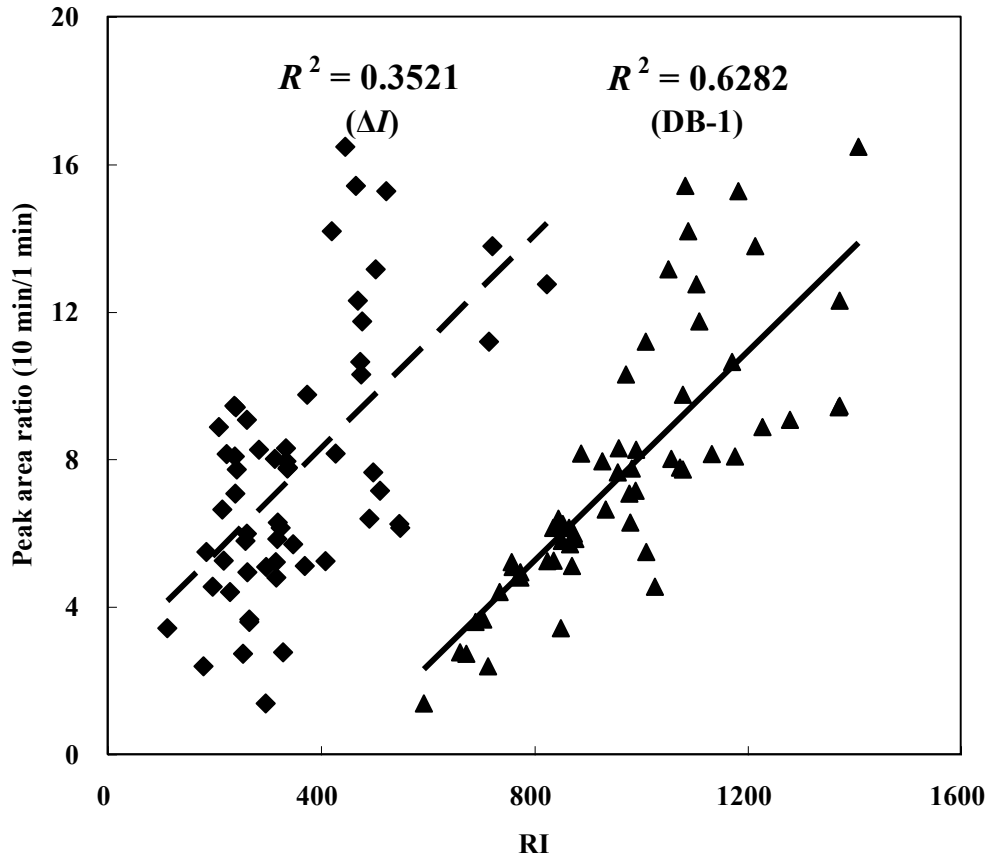


Figure 1.5 Relationship between the peak area ratio of odorants in the model chewing gum and its retention index (RI) values on the apolar stationary phase GC column (DB-1) and ΔI (ΔI is the difference in the RI values of the polar and apolar columns). Each ratio is the mean value of three replicates by one panelist.

the difference between those RI values (ΔI; the values correspond to the polarity of each odorant) and the peak area ratios of 57 odorants whose peak area ratios showed relatively good reproducibility. Each RI value corresponds to the vapor pressure, polarity, and interaction between polarity and vapor pressure of each compound, respectively. First, the relationships between the RI values on the apolar GC column or ΔI and the peak area ratios of the 57 odorants are shown in **Figure 1.5**. Those odorants in the model chewing gum showed a weak correlation between their peak area ratios and RI values on the apolar GC column ($R^2 = 0.6282$) or ΔI ($R^2 = 0.3521$). These results indicated that the vapor pressure and polarity of each odorant would play a role in their

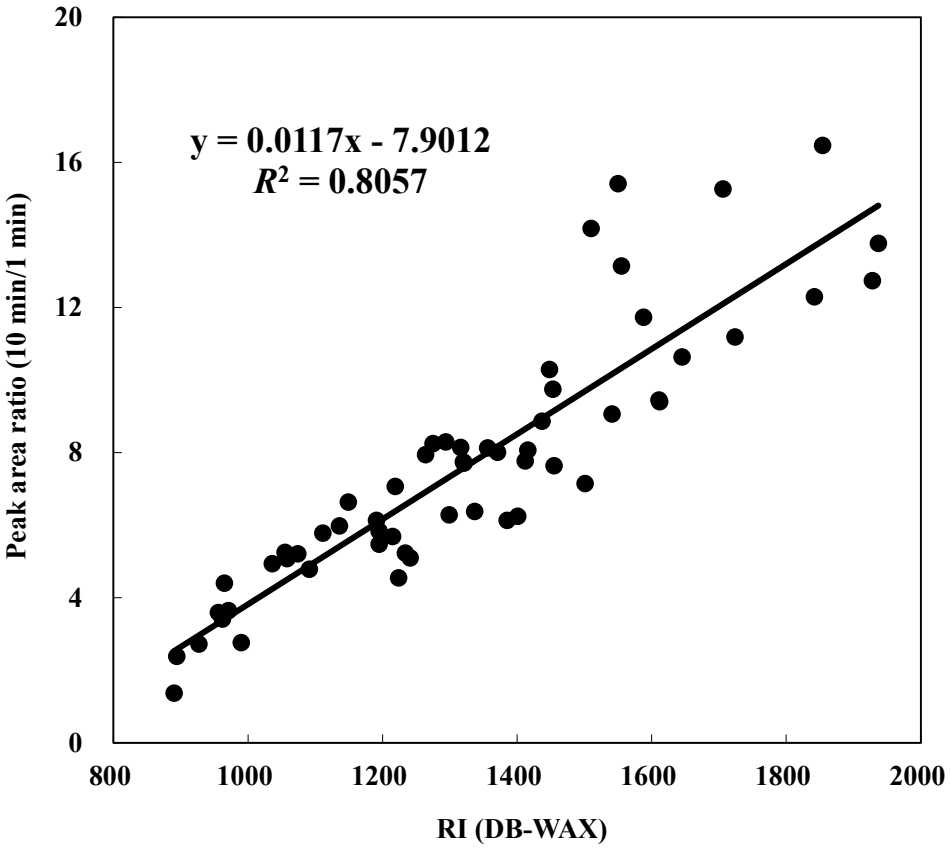


Figure 1.6 Relationship between the peak area ratio of odorants in the model chewing gum and its RI values on the polar stationary phase GC column (DB-WAX). Each ratio is the mean value of three replicates by one panelist.

in-mouth releases from chewing gum as was the case in the consumption of an aqueous solution containing volatile compounds. In addition, each coefficient of determination (R^2) suggested that the vapor pressure was a more important factor on the in-mouth release of odorants than the polarity. Furthermore, the relationship between the RI values on the polar GC column (the interaction between polarity and vapor pressure of compounds plays a role in their retention on the column) and the peak area ratios of the 57 odorants is shown in **Figure 1.6**. As a result, it was found that the peak area ratio of those odorants had a good correlation with their RI values on the polar GC column. The coefficient of determination was approximately 0.8. Therefore, the RI values on the polar GC column can be used to predict the in-mouth releases of various odorants in chewing gum.

These results suggested that the in-mouth release characteristics of odorants in chewing gum can be estimated by comparing their peak area ratios (10 min/1 min) obtained by R-FISS. Moreover, it was found that those in-mouth releases can be predicted from their RI values on the polar GC column (DB-WAX). This approach using R-FISS and RI values is relatively simple methodology. Thus, the approach is useful to reveal the in-mouth release characteristics of odorants in chewing gum. However, it is well known that the food composition has an effect on the release rate of odorants [29]. For instance, the fat content in food products is important on the flavor perception [7, 30]. There are many types of chewing gums (sugar or sugarless, stick or tablet, etc.), and it has been reported that the sucrose concentration in chewing gum and their form affect the perceived flavor intensity [31]. Furthermore, the functional groups of odorants used in this study were limited. Therefore, further investigations will be required to provide detailed information about the in-mouth release characteristics of the odorants in chewing gum such as the relationship between the chewing gum base and aroma release.

1.2 A New Approach to Estimate the Elution Characteristics of Odorants in Chewing Gum during Chewing

1.2.1 Introduction

The perception of flavor in chewing gum is a process that involves the release of odorants from the chewing gum and their transport via the retronasal route to the nasal cavity, where aroma perception takes place. The process of aroma perception involves many steps. First, odorants are released from the chewing gum and diluted with saliva during chewing. They are then volatilized into the headspace of the oral cavity and transported via the retronasal route to the nasal cavity, where they interact with the receptors in the olfactory epithelium. The receptors transform the sensory information into electrical signals. These signals are then transported to the brain and the aroma is perceived. Therefore, the in-mouth release characteristic of each odorant during chewing would be affected by each step in the process.

Recent studies have proposed some methodologies to determine the concentrations of odorants in the nose-space during food consumption by real-time mass spectrometry such as APCI-MS or PTR-MS [32, 33]. While these in vivo studies can provide insights into the in-mouth release characteristics of odorants and how flavor is perceived, these methods have limitations. One of the major limitations is the significant variability in the data. In addition, these methods cannot consider the individual effect of each step (mastication, dilution with saliva, mass transfer from the oral cavity to the nasal cavity, etc.) on the in-mouth release characteristics of odorants in chewing gum. Therefore, the in-mouth release characteristics of odorants in chewing gum such as the immediate flavor perception and their persistence still remain unclear.

In order to overcome these difficulties, a chewing simulator seemed to be one of the solutions. Substituting an artificial mouth for a human subject can provide a good reproducibility of mastication and quantitative data of odorants released from chewing gum. In this study, the in vitro chewing apparatus [18] was then used to investigate the elution characteristic of odorants in chewing gum during chewing, focusing on the

elution step of odorants from chewing gum into saliva. The chewing apparatus was mainly constructed to examine the release of pharmaceutical ingredients in chewing gum. The advantages of the apparatus are a good reproducibility of mastication and its easy operation to recover the test medium into which the tested ingredients were eluted. However, there are no previous studies that the chewing apparatus was applied to volatile ingredients, and it is unclear whether the apparatus can be applied to odorants in chewing gum or not. Therefore, the aims of this study were to develop a new approach using the chewing apparatus to estimate the elution characteristics of odorants from chewing gum into saliva during chewing and to apply the approach to the prediction of their elution ratios.

1.2.2 Materials and Methods

Preparation of the Model Flavors. Two different forms of flavors were used in the experiments; a powdered flavor and liquid flavors. The powdered flavor included 5 odorants (shown in **Table 1.3**; the concentration of each odorant in the powdered flavor was about 5%) and its emulsifier was gum arabic and starch hydrolysate. Liquid flavors were comprised a total of 43 odorants (shown in **Table 1.4**; the concentration of each odorant in the liquid flavor was 5%) dissolved in glyceryl triacetate.

Preparation of the Model Stick Gum and the Model Tablet Gum. Non-coated chewing gums were prepared as follows. Powdered sugar (64 g) and 85%-sugar syrup (13 g) softened in hot water were mixed with a gum base (23 g) and the mixture was kneaded for about 1 min. 0.5, 1.0 or 2.0 g of the powdered flavor was then added (the final concentration of each odorant in the chewing gum was about 250, 500 or 1000 ppm, respectively), and the mixture was kneaded adequately. The chewing gum was rolled and cut into about 1.8 g sticks. Meanwhile, 1 g of the liquid flavor was added to a mixed gum base (the final concentration of each odorant in the chewing gum was 500 ppm), and the mixture was kneaded adequately. The chewing gum was rolled and cut into about 1.0 g tablets.

Chewing Conditions for the Elution Tests. The following default settings of the chewing apparatus (DRT, ERWEKA GmbH, Heusenstamm, Germany) were used during chewing, unless specified otherwise: chew frequency: 40 strokes/min, distance between the chewing surfaces: 1.6 mm, twisting angle: 20°, and temperature of test cell: 37.0°C. A stick gum (folded in the middle) or a tablet gum was placed between the chewing surfaces. For all samples, 40 g of distilled water was used as the test medium. A sample volume of 0.5 mL was withdrawn from the test cell at different times; 2, 5, 10 min from the start of chewing. The chewing test was then stopped and the test medium was adequately stirred. The chewing test was restarted immediately after a sample was withdrawn each time. The total amount of the tested medium was recovered after a 20-min chewing run.

Concentration of Odorants by the SPE Method. Five microliters of an IS solution (2 μg/mL 2-octanol in ethanol) were added to 10 g of the tested medium recovered after a chewing run. The medium was passed through the glass column (15 cm × 2 cm i.d.) filled with 5 mL of SP700 resin (Mitsubishi Chemical Corporation, Tokyo, Japan), which was conditioned with distilled water before use, followed by washing with 50 mL of distilled water and eluting with 20 mL of dichloromethane. The eluate was dried with an excess amount of anhydrous sodium sulfate and then concentrated by rotary evaporation (35°C, 550 mmHg) to about 5 mL, followed by nitrogen stream evaporation to about 100 μL.

GC-MS with Liquid Injection. The concentrated odorants were analyzed by an Agilent 6890N GC with an Agilent 5973 series MS (Agilent Technologies) in the split mode (injection volume of 1 μL, inlet temperature of 250°C, split ratio of 30:1). 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°C to 230°C at a rate of 5°C/min. The flow rate of the helium carrier gas was 1 mL/min. The MS was used with an ionization voltage of 70 eV (EI) and operated in the SCAN mode. The ion source and quadrupole temperatures were set at 230°C and 150°C, respectively. Each odorant was identified by comparing its mass spectrum (quadrupole) and retention time to those of the reference compounds.

GC-FID with Liquid Injection. The aroma concentrates were analyzed by an Agilent 6850N GC with an FID (Agilent Technologies) in the split mode (injection volume of 1 μL, inlet temperature of 250°C, split ratio of 30:1). The column was a 30 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°C to 230°C at a rate of 5°C/min. The flow rate of the N_2 carrier gas was 0.7 mL/min. The odorants were determined by matching their retention times to those of the reference compounds and by comparing their elution order to those in the chromatograms analyzed by GC-MS. Quantitative data were obtained from the relative peak areas between the tested odorants and 2-octanol (IS material) in the chromatograms. These data were then converted to the concentrations in the tested chewing gum.

Concentration of Odorants by the HS-SPME Method. Five hundred microliters of the tested medium after a chewing run was placed in a 20-mL glass vial, followed by adding 10 μL of an IS solution (0.2 μg/mL 2-octanol in ethanol). The vial was then sealed with a silicone septum. An SPME fiber (Supelco, Bellefonte, PA, USA) coated with divinylbenzene-carboxen-polydimethylsiloxane (DVB/CAR/PDMS, 50/30 μm) was then inserted into the headspace of the pre-warmed (10 min at 60°C) sample vials for extraction (15 min at 60°C). During pre-warming and extraction, the sample vials were agitated at 500 rpm and 250 rpm, respectively. After extraction, the SPME fiber was removed and introduced into the injector port of GC-FID. These automated processes were achieved using a Combi PAL autosampler (CTC Analytics AG, Zwingen, Switzerland).

GC-FID with HS-SPME. The odorants concentrated on the SPME fiber were analyzed by an Agilent 6890N GC with an FID in the split mode (desorption time of 1 min, inlet temperature of 250°C, split ratio of 30:1). The column was a 30 m × 0.32 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°C to 230°C at a rate of 5°C/min. The flow rate of the helium carrier gas was 1 mL/min. The odorants were determined by matching their retention times to those of the reference compounds and by comparing their elution order to those in the chromatograms measured by GC-MS. Quantitative

data were obtained from the relative peak areas between the tested odorants and 2-octanol (IS material) in the chromatograms. These data were then converted to the concentrations in the tested chewing gum.

1.2.3 Results and Discussion

Application of the Chewing Apparatus to the Odorants in the Model Chewing Gum. It seems to be quite difficult to analyze the in-mouth releases of odorants in chewing gum, especially the process of elution from chewing gum into saliva, because the eluted odorants in saliva are swallowed and the absolute extraction of the remaining odorants in the chewing gum after chewing appears to have difficulty. Therefore, a model mouth can be used as a solution in order to examine the in-mouth elution characteristics of odorants from chewing gum into saliva during chewing. In the present study, the in vitro chewing apparatus was used to reveal the elution characteristics of odorants in chewing gum during chewing. As previously stated, the advantages of the chewing apparatus are the reproducible mastication and its easy operation to recover the tested medium (distilled water) into which the tested odorants were eluted. The reproducibility of the amounts of odorants eluted from the model stick gum (containing 5 odorants shown in **Table 1.3**; the final concentration of each odorant in the model stick gum was 500 ppm) after a 20-min chewing run was examined by using the apparatus. The odorants in the test medium were determined by GC-MS and their contents in the model stick gum were calculated from the results of GC-FID using the SPE method and the HS-SPME method. As a result, the RSD (%) of the amounts of eluted odorants in the tested medium showed good reproducibility of ≤ 8% in each analytical method (**Table 1.3**). This result demonstrated that the chewing apparatus can be applied to the elution test of odorants in chewing gum.

Development of a New Approach to Estimate the Elution Characteristics of Odorants in Chewing Gum. The elution characteristics of odorants in chewing gum during chewing could be characterized by the amounts of eluted odorants and their release curves (time-

Table 1.3 The average concentration (conc.)[a] and RSD (%)[b] of the eluted odorants from the model stick gum after a 20-min chewing run.

	SPE		HS-SPME	
Compound[c]	Average conc. (ppm)	RSD (%)	Average conc. (ppm)	RSD (%)
hexanal	34	6	9	6
isoamyl acetate	65	5	28	5
hexanol	167	6	35	7
menthol	126	2	142	8
hexanoic acid	180	3	3	7

[a] Each average concentration was the mean value of triplicate results.

[b] Each RSD (%) was calculated based on the value of triplicate results.

[c] The concentration of each compound in the model stick gum was 500 ppm.

amount curves). In order to reveal the elution characteristics, it is highly important to select an analytical method which has both a simplified operation and the capability of accurate quantitation since many data points are required to plot the release curves. In this study, the SPE method and the HS-SPME method were used to analyze the odorants in the tested medium after the chewing run. However, the quantitative data of odorants obtained by these 2 methods were quite different (**Table 1.3**). The accurate quantitation of odorants in the tested medium using HS-SPME was assumed to be difficult because the method does not analyze the odorants in the medium, but in the headspace. In contrast, the quantitative data of the odorants obtained with SPE are more reliable than those with HS-SPME. However, this method requires a multistep preparation process (absorption, desorption and condensation). Moreover, continuous measurement will be difficult because a large amount of tested medium (10 g of the initial 40 g) is required and it can influence the subsequent elution of the odorants. Thus, the SPE method cannot be applied to the present study since many data points are required. SPME liquid sampling was not used to analyze the odorants in tested medium for the same reason.

Although SPME liquid sampling might provide reliable data and a simplified operation, continuous measurement will be difficult because a large amount of the tested medium is required. Based on these advantages and disadvantages, both analytical methods are assumed to have difficulty in analyzing many samples accurately and easily when performed separately. Therefore, considering the possibility of combining the advantages of both methods, the quantitative data obtained from HS-SPME and SPE were compared.

In order to develop an analytical method that can determine the amounts of eluted odorants in the tested medium and their release curves, the relationship between the quantitative data of the eluted odorants at each chewing time (2, 5, 10 and 20 min from the beginning of the chewing test) obtained from SPE and HS-SPME with GC-FID was investigated using the chewing apparatus. These measurements were performed only once because the quantitative data for the eluted odorants showed good reproducibility (see **Table 1.3** and [18]). As a result, it was found that the quantitative data of the odorants in the model stick gum (containing 5 odorants shown in **Table 1.3**; the final concentration of each odorant in the model stick gum was 500 ppm) obtained from each method are in proportion (not including hexanoic acid because it could not be sufficiently detected by HS-SPME) (**Figure 1.7**). In addition, when the same chewing test using 2 other model stick gums (containing those 5 mixed odorants; the final concentration of each odorant in the model stick gums was 250 or 1000 ppm) was performed, the quantitative data obtained from each method were also in good proportion in each case (data not shown). Therefore, it would appear that the quantitative data obtained from HS-SPME could be easily converted to the data obtained from SPE, provided that the concentrations of odorants in chewing gum range from 250 ppm to 1000 ppm.

Based on these results, the elution characteristics of the odorants in the chewing gum will be measured according to the following steps. The tested medium at each chewing time (2, 5 and 10 min after the chewing runs) will be analyzed only by HS-SPME. Only the tested medium after a 20-min chewing run will then be analyzed by both SPE and HS-SPME. Because the quantitative data from HS-SPME were

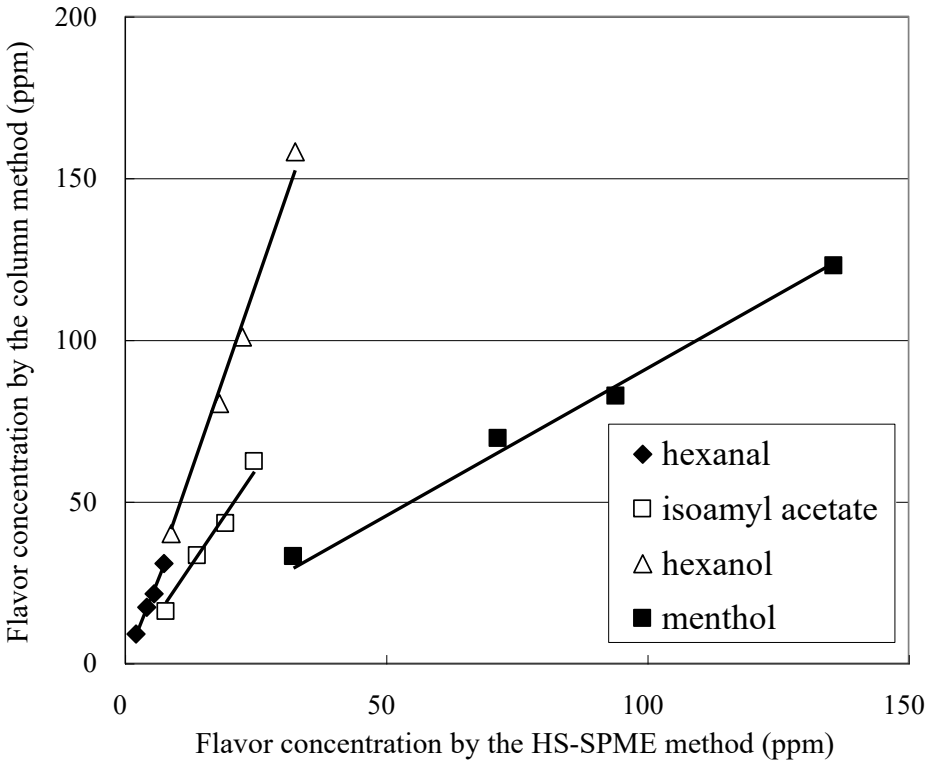


Figure 1.7 Relationship between the quantitative data for the odorants in the model stick gum obtained from SPE and HS-SPME. Each value is a single result (the measurement was performed only once) and the chewing times are 2, 5, 10, and 20 min corresponding to the order of increasing value. The concentration of each odorant in the model stick gum was 500 ppm.

proportional to those from SPE, the quantitative data obtained from HS-SPME was converted to that from SPE at each chewing time using the following equation:

$$C_n = X_n \times C_{20} / X_{20} \qquad \textbf{Eq.1}$$

In this equation, C_{20} and X_{20} are the quantitative data obtained from SPE and HS-SPME after the 20-min chewing run, respectively, and C_{20} / X_{20} is the coefficient to convert the quantitative data obtained from HS-SPME (X_n) to that obtained from SPE (C_n) after an n-min chewing run. The release curves of the odorants in the chewing gum determined by the new approach are shown in **Figure 1.8**. The elution ratios (amounts) of odorants eluted from the chewing gum at each chewing time would be compared

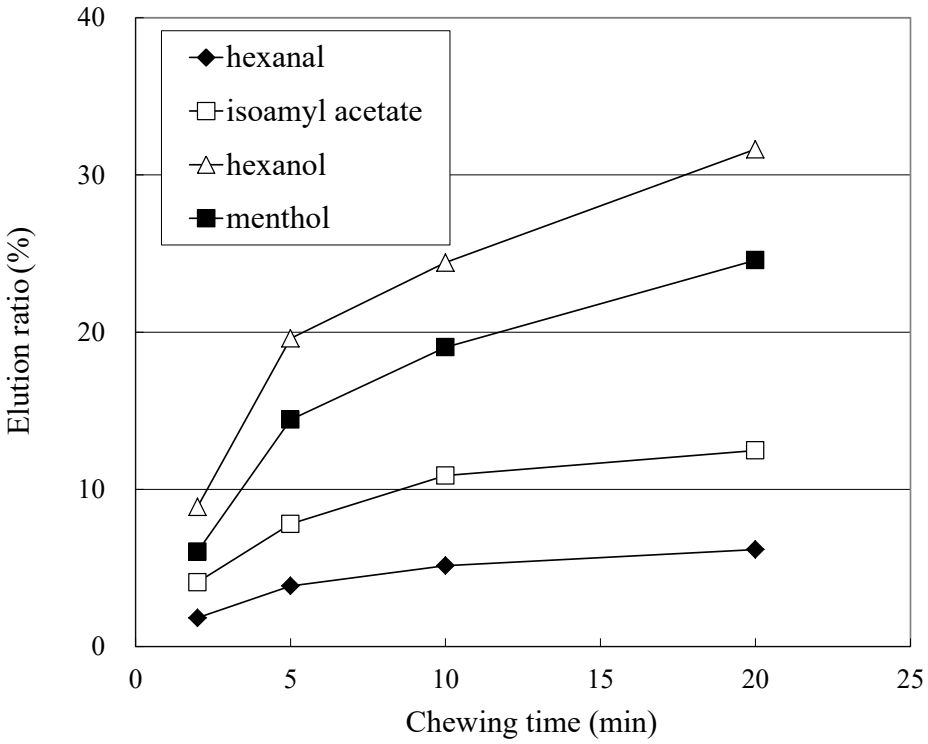


Figure 1.8 Release curves for the eluted odorants from the model stick gum into the tested medium. Each elution ratio was the percentage of the eluted odorants to the added odorants in the stick gum and was calculated from a single result (this measurement was performed only once). The concentration of each odorant in the model stick gum was 500 ppm.

using these release curves. Moreover, the elution rate can be compared by differentiating the approximation formula of these release curves. Therefore, the new approach using the chewing apparatus can be an effective method to estimate the elution characteristics (elution ratio and release curves) of various odorants (not including volatile acids) in chewing gum.

However, the release curves obtained from the approach mentioned above do not correspond to the common TI curves because the present release curves show the accumulated amounts of the eluted odorants during the chewing run while the sensory curves show the instantaneous sensory intensities of flavor perception at each chewing

time. If the perceived flavor intensity is proportional to the amounts of odorants instantaneously eluted at each chewing time, the curves showing the elution rate of the odorants at each chewing time might become similar to the TI curves. Moreover, the amounts of eluted odorants in the tested medium showed a tendency to decrease after a certain period of time in the present experimental results. It was also reported that the concentration of menthone was relatively steady during chewing in the nose-space release analyses [32, 34]. Therefore, the eluted odorants might reach equilibrium between the chewing gum base and the tested medium (distilled water) under the present experimental condition. In fact, the odorants eluted sufficiently when only the test medium was changed into a new test medium after the 20-min chewing run (data not shown). Furthermore, the approach in this study cannot consider the odorants that volatilized in the air during chewing. Recent studies have suggested that the amounts of volatilized odorants during chewing would also be high [35], and the release curves obtained from the present approach might reflect the influence of those volatilized odorants. These results suggested that the present approach using the chewing apparatus would also have limitations. Therefore, further examination will be required to clarify the relationship between the release curves of the eluted odorants and their sensory curves.

Prediction of the In-mouth Elution Ratio of Odorants in Chewing Gum. Elution ratios of odorants in chewing gum are supposed to be one of the most important factors determining their elution characteristics. The relationship between the elution ratios and physicochemical properties of odorants in the model chewing gum was investigated using the same approach. It appeared that the in-mouth elution of odorants from the chewing gum to saliva was the partition phenomenon between the hydrophobic chewing gum base and hydrophilic medium (saliva). Therefore, it can be presumed that the hydrophobicity/hydrophilicity (polarity) of each odorant would have an influence on the amounts of eluted odorants from the chewing gum. The polarity is also a major factor affecting the retention and separation of odorants in GC analyses [27, 28]. For example, the retention and separation of odorants on apolar GC columns (such as DB-1) or polar GC columns (such as DB-WAX) depend on their vapor pressure or the interaction

Table 1.4 Average elution ratios[a] of the tested odorants in the model chewing gum.

No.	RI			Compound	Elution ratio[c] (%)	No.	RI			Compound	Elution ratio[c] (%)
	DB-Wax	DB-1	ΔI[b]				DB-Wax	DB-1	ΔI[b]		
1	890	593	297	ethyl acetate	7	23	1337	846	491	hexanol	62
2	956	690	266	ethyl propionate	14	24	1347	804	543	ethyl lactate	33
3	965	735	230	ethyl isobutyrate	14	25	1356	1133	223	isobutyl hexanoate	2
4	977	606	371	2,3-butanedione	8	26	1385	836	549	(*Z*)-3-hexenol	71
5	990	661	329	2-pentanone	25	27	1401	854	547	(*E*)-2-hexenol	67
6	1036	774	262	ethyl butyrate	18	28	1402	1082	320	nonanal	4
7	1047	681	366	2,3-pentanedione	18	29	1412	1074	338	methyl thiohexanoate	4
8	1055	837	218	ethyl 2-methylbutyrate	14	30	1448	972	476	1-octen-3-ol	43
9	1058	760	298	3-hexanone	29	31	1453	1079	374	(*Z*)-6-nonenal	12
10	1091	774	317	hexanal	23	32	1518	932	586	benzaldehyde	62
11	1111	852	259	isoamyl acetate	16	33	1550	1084	466	linalool	35
12	1191	866	325	2-hetanone	31	34	1555	1052	503	octanol	32
13	1195	1010	185	1,4-cineole	15	35	1624	1098	526	3-(methylthio)propyl acetate	46
14	1215	867	348	methyl thiobutyrate	13	36	1645	1171	474	menthol	27
15	1224	1027	197	1,8-cineole	30	37	1667	1024	643	phenylacetaldehyde	31
16	1234	825	409	(*E*)-2-hexenal	39	38	1752	1252	500	decanol	5
17	1253	754	499	amyl alcohol	56	39	1758	1364	394	geranyl acetate	1
18	1264	928	336	2-octanone	21	40	1842	1373	469	β-damascenone	7
19	1275	991	284	hexyl acetate	9	41	1854	1408	446	α-ionone	4
20	1294	959	335	3-octanone	18	42	1893	1009	884	benzyl alcohol	102
21	1299	980	319	octanal	9	43	1928	1104	824	2-phenylethyl alcohol	98
22	1320	983	337	(*Z*)-3-hexenyl acetate	19						

[a] Each average elution ratio was the mean value of triplicate results.

[b] ΔI is the difference of the RI values of the polar and apolar columns.

[c] Each elution ratio was the percentage of the eluted odorants to the amount of mixed odorants in the model chewing gum.

between polarity and vapor pressure, respectively. Based on these factors, it can be presumed that the elution ratios (amounts) of the eluted odorants from the chewing gum have a close relation to the retention time or RI values in the GC analysis. In addition, these RI values can be used to predict the in-mouth elution characteristics of odorants in chewing gum.

In order to investigate the relationship between the elution ratio and RI values of odorants in the model chewing gum, the elution ratios of the odorants having various functional groups (ethers, ketones, aldehydes, esters and alcohols) after the 20-min chewing run were determined by SPE with GC-FID using the chewing apparatus. **Table 1.4** shows the elution ratios of 43 odorants, whose elution ratios showed relatively good reproducibility (RSD < 10%), in the model tablet gums (determined by GC-MS) after the 20-min chewing run. In addition, the table also shows the RI values on the polar and apolar GC columns and the difference between those RI values (ΔI). Each RI value can correspond to the vapor pressure, polarity, and interaction between polarity and vapor pressure of the compounds.

First, the relationship between the RI values on the polar or apolar GC column and the elution ratios of the 43 odorants are shown in **Figure 1.9** and **Figure 1.10**, respectively. The elution ratios of odorants in the model tablet gums have little correlation with both the RI values on the polar and apolar GC column. These results indicated that the elution ratios of odorants in chewing gum would hardly be related to their vapor pressure. The relationship between ΔI, which has a close relation to the polarity of compounds, and the elution ratios of odorants is shown in **Figure 1.11**. Consequently, a slightly strong correlation was observed between the elution ratio and ΔI of each odorant. This result suggested that the polarity of each odorant in the chewing gum had a significant impact on their elution ratios. The physicochemical commonality of the odorants, which deviated from the fitted line, was then investigated. It was found that the odorants whose molecular weight is greater than 150 tend to deviate appreciably from the fitted line. The elution ratios of such odorants were quite low regardless of their ΔI. These odorants appeared to scarcely elute from the model chewing gum due to their increased hydrophobicity since the influence of their

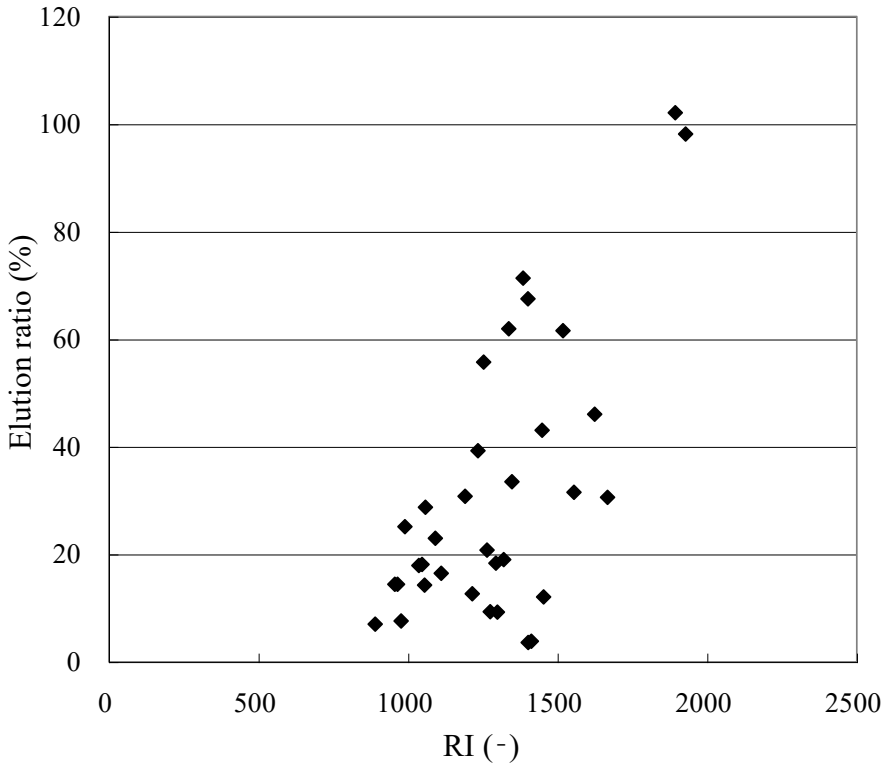


Figure 1.9 Relationship between the elution ratio for the odorants in the model tablet gums after a 20-min chewing and their RI values on the polar GC column (DB-WAX). Each value is the mean of triplicate results. The concentration of each odorant in the model tablet gums was 500 ppm.

functional groups on their hydrophobicity is relatively low compared to their molecular weight. The relationship between the ΔI and elution ratios of the selected odorants whose molecular weight is not greater than 150 is shown in **Figure 1.12**. The elution ratios of those odorants had a good correlation with their ΔI. The coefficient of determination (R^2) for the tested odorants (molecular weight $\leq$ 150) was nearly 0.8. Therefore, the relationship can be used to predict the elution ratios of various odorants in chewing gum using ΔI that has a close relation to the polarity of the compounds. This study is the first to show that the elution ratios of various odorants in chewing gum can be predicted by ΔI, which is one of their physicochemical properties, without the need to measure them.

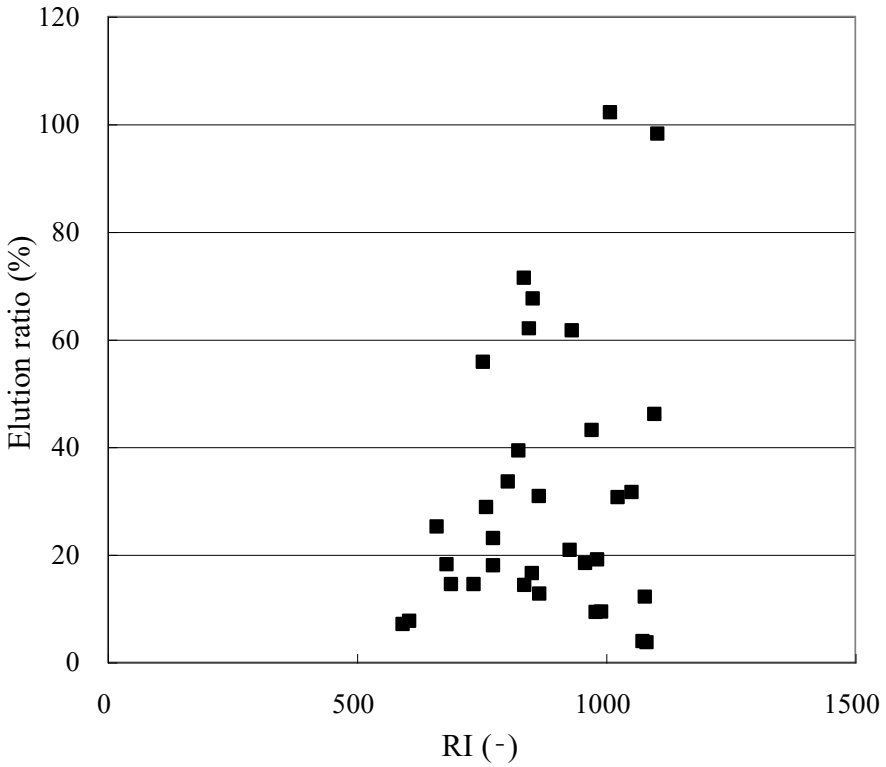


Figure 1.10 Relationship between the elution ratio for the odorants in the model tablet gums after a 20-minute chewing and their RI values on the apolar GC column (DB-1). Each value is the mean of triplicate results. The concentration of each odorant in the model tablet gums was 500 ppm.

Since distilled water was used as the test medium in the present study, the influence of saliva on the elution characteristics of odorants in chewing gum during chewing remains to be determined. It has already been reported that the addition of saliva has important effects on the in-mouth release and their release profiles [36]. Therefore, further investigation will be needed in order to understand the influence of saliva. Moreover, the number and type of functional groups of the tested odorants in the present study were limited. Therefore, it will be the future challenges to verify the precision of the predictive elution ratio from ΔI and to check the range of odorants to which this prediction approach can be applied. Furthermore, it will also be a future challenge to examine the relationship between the elution ratios and other physical parameter (dipole

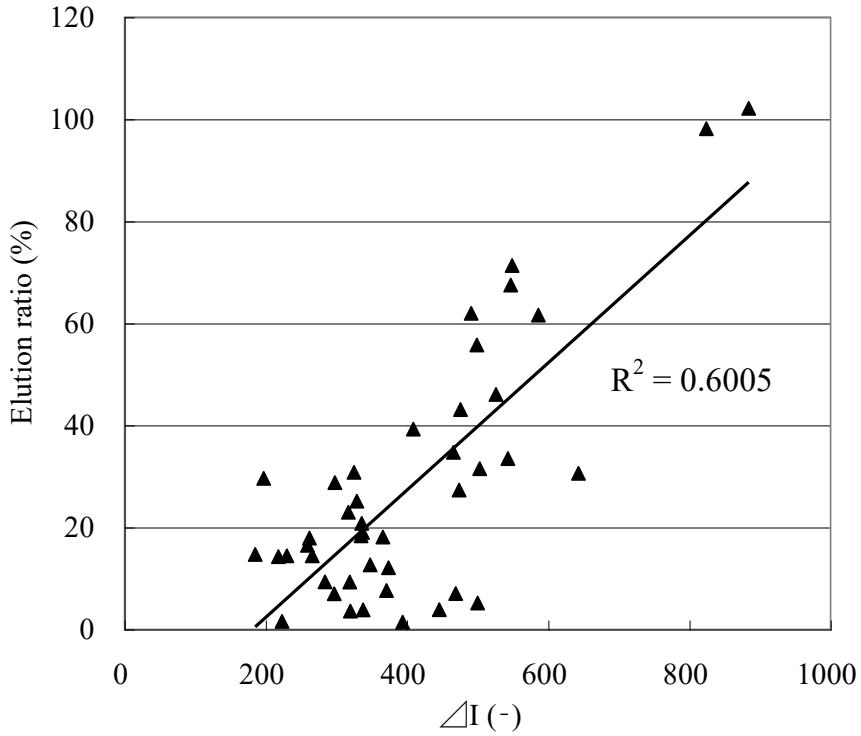


Figure 1.11 Relationship between the elution ratio for the odorants in the model tablet gums after a 20-min chewing run and ΔI (ΔI is the difference in the RI values of the polar and apolar GC columns). Each value is the mean of triplicate results. The concentration of each odorant in the model tablet gums was 500 ppm.

moment, etc.) of odorants. In addition, the composition of chewing gum could affect the elution ratios of odorants. In fact, previous studies have already reported that the composition of chewing gum (for example, the chewing gum bases, the carbohydrate and solvent) would affect the in-mouth release [37-39]. The composition of chewing gum is assumed to have some effects on the elution characteristics of odorants. Therefore, it will also be one of the future challenges to examine the impact of the composition of chewing gum on the elution ratios of odorants.

These results indicated that the elution characteristics (elution ratios and their release curves), which has been difficult to examine, of the odorants in chewing gum can be easily estimated by the approach with combination of SPE and HS-SPME using the chewing apparatus, and the approach can be used to predict the elution ratios of

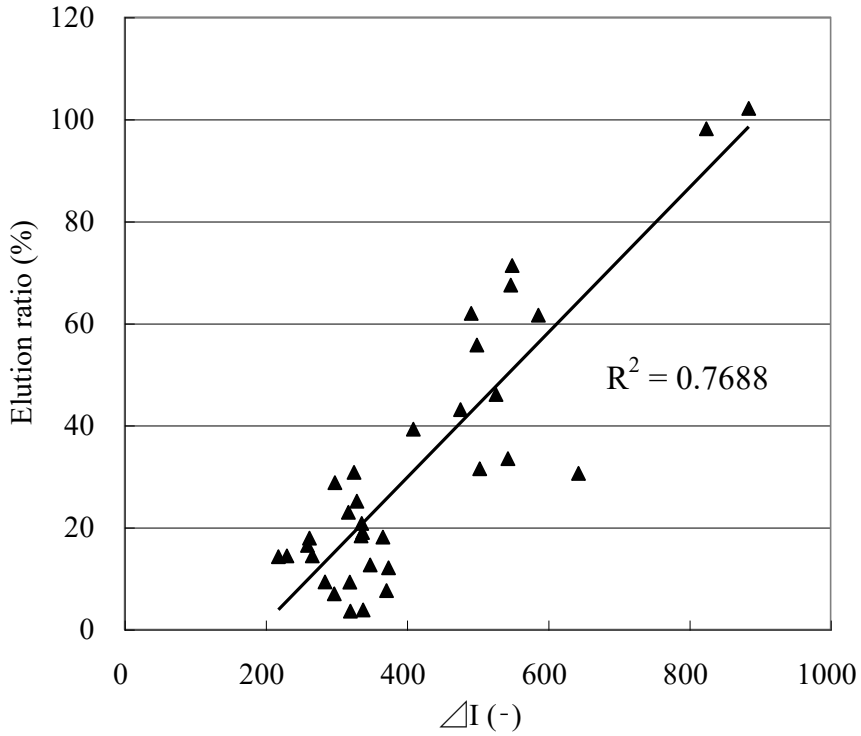


Figure 1.12 Relationship between the elution ratio for the odorants in the model tablet gums after a 20-min chewing run and ΔI (ΔI is the difference in the RI values of the polar and apolar GC columns). The odorants whose molecular weights are greater than 150 are not shown. Each value is the mean of triplicate results. The concentration of each odorant in the model tablet gums was 500 ppm.

odorants in chewing gum from their ΔI which is the difference between the RI values on the polar and apolar GC columns. This analytical technique and the prediction approach seem to be an effective methodology that can provide useful information in order to clarify the elution characteristics of odorants in chewing gum. Moreover, it was also suggested that hydrophobicity/hydrophilicity is the key factor determining the elution characteristics of odorants in chewing gum during chewing. In addition to these elution characteristics, by understanding the volatility characteristics of odorants and their mass transfer characteristics from the oral cavity to the nasal cavity, the overall flavor release from the chewing gum will be revealed in detail.

1.3 Conclusion

R-FISS was developed as a new technique to analyze the in-mouth odorants exhaled from the human nose during the consumption of foods. In this chapter, the in-mouth release characteristics of odorants in the chewing gum were estimated using R-FISS. The elution characteristics of odorants in chewing gum during chewing were also investigated using a chewing apparatus (DRT). The relationships between the in-mouth release characteristics or elution characteristics of the odorants in the chewing gum and their physicochemical properties were demonstrated.

The in-mouth release characteristics and elution characteristics of the odorants in the chewing gum significantly varied depending on the kinds of odorants. It was indicated that the in-mouth release characteristics of odorants would be predicted by their RI values on polar GC columns, and the elution characteristics would be predicted by their ΔI, which is the difference between the RI values on the polar and apolar GC columns and equivalent to the polarity of each odorant. These results indicated that the hydrophobicity/hydrophilicity and vapor pressure play an important role in determining the flavor release characteristics of odorants in chewing gum during chewing.

The flavor of chewing gum is perceived when odorants released from the chewing gum during chewing reach the nasal cavity via the throat. Therefore, the flavor release characteristics of odorants in chewing gum will be revealed in more detail by understanding the volatility characteristics of odorants in the mouth cavity and their mass transfer characteristics from the mouth cavity to the nasal cavity via the throat, in addition to the elution characteristics. Moreover, understanding the influence of the compositions of the base components in the chewing gum on the flavor release and revealing the flavor release characteristics of more odorants will be required. These fundamental findings about the flavor release will be useful to understand the flavor perception of chewing gum during chewing. Furthermore, they can contribute to the development of chewing gum products having an excellent flavor release and a pleasant flavor.

Chapter 2

Studies on Factors Affecting the Flavor Release and Flavor Perception of Beverages

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

2.1.1 Introduction

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 [19, 20], and nonvolatile cysteine-S-conjugates could provide the odorants (free thiols) in the human mouth [21]. However, these results are focused on only the enzymatic or microbial reaction in human saliva. The relationship between the consumed odorants and the odorants reaching the olfactory epithelium (stimulating the olfactory receptor) appears not to be fully explained. The aim of this study was then to investigate the relationship between the odorants in a model beverage and the compositions of odorants exhaled from the human nose during drinking using R-FISS, focusing on the compositional changes in the odorants.

2.1.2 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-methyl-1-mercaptobutane and 2-octanol (Sigma-Aldrich Japan, Tokyo, Japan); 1,8-cineole (Nippon Terpene Chemicals, Inc., Kobe, Japan); (*Z*)-3-hexenol (Shin-Etsu Chemical Co., Ltd., Tokyo, Japan); FFT (Oxford Chemicals, Ltd., Cleveland, U.K.). The following compounds were synthesized according to the literature procedures: 4-mercapto-4-methyl-2-pentanone [40] and 4-methoxy-2-methyl-2-mercaptobutane [41]. Chemical purity was checked by GC-FID and GC-MS.

Methylthio ethers [FMS, 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 shown in **Table 2.1** 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 (FFT, 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 through the Nostrils. To determine the in-mouth odorants exhaled through the nostrils, after 30mL of the model drink had been

swallowed, the air exhaled from the human nose was passed through a glass nosepiece fitted to the nose of each panelist (**Figure 1.1**). Ten breaths after the model drink had been swallowed were passed through a small glass column (6 cm × 5 mm i.d.) filled with 100 mg of Tenax TA (80/100 mesh, GL Science), which had been heated at 220°C for 2 hours prior to the analysis. The end of the glass column was connected to a pump by a silicon tube, and a suction of approximately 1 L/min was applied to the system during trapping of the air exhaled through the nostrils. This sampling system allowed the panelists to normally exhale without the need to press air through the Tenax column. After trapping of the in-mouth odorants, the water was removed from the Tenax TA by flowing dry nitrogen (30 min, 100 mL/min). Three replicates of each experiment were performed by each panelist. These experiments were carried out at room temperature (25 ± 2°C). A blank test was also performed under the same conditions using only syrup.

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 of the odorants, 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) in combination with the ATEX option of an MPS2 autosampler (Gerstel GmbH) and a CIS4 injector (Gerstel GmbH) according to the following parameters. Thermal desorption was performed by programming the TDU from 20°C to 220°C (held for 3 min) at the rate of 12°C/s in the splitless mode. Cryofocusing was performed with liquid nitrogen at -150°C. Injection was performed with the ramp of 12°C/s from -150°C to 220°C (held for 3 min) in the splitless mode. The odorants were analyzed by an Agilent 6890N GC with an Agilent 5975B series MS (Agilent Technologies). The column was a 30 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 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 MS was used with an ionization voltage of 70 eV (EI) and operated in the SCAN mode. The ion source and quadrupole temperatures were set at 230°C and 150°C, respectively. The composition ratio of odorants (the tested thiol and their corresponding methylthio ether) was calculated from the peak area ratio of the total ion chromatogram.

Solvent Desorption from Tenax TA. The solvent desorption of the trapped odorants on the Tenax TA was eluted with 5 mL of diethyl ether, followed by nitrogen stream evaporation to about 100 μL.

Concentration of Odorants by the SPE Method. Thirty milliliters of the model drink was passed through the glass column (15 cm × 2 cm i.d.) filled with 5 mL of SP700 resin (Mitsubishi Chemical Corporation), which was conditioned with distilled water before use, followed by washing with 50 mL of distilled water and eluting with 20 mL of dichloromethane. The eluate was dried with an excess amount of anhydrous sodium sulfate and then concentrated by rotary evaporation (35°C, 550 mmHg) to about 5 mL, followed by nitrogen stream evaporation to about 100 μL.

GC-MS with Liquid Injection. The concentrated odorants (the eluate from Tenax TA or SP700 resin) were analyzed by an Agilent 6890N GC with an Agilent 5973 series MS (Agilent Technologies) and using the pulsed splitless mode (injection volume of 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°C 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 operated in the SCAN mode. The ion source and quadrupole temperatures were set at 230°C and 150°C, respectively.

Identification of the Odorants. Each odorant was identified by comparing its Kovats GC RI and mass spectrum to those of the reference compounds.

2.1.3 Results and Discussion

Application of R-FISS to the Odorants in the Model Drink. The amounts of in-mouth odorants instantaneously exhaled from the human nose via the throat after a drink has been swallowed are extremely low, and many kinds of odorants are contained in an actual drink. The major advantages of the R-FISS technique are the ability to improve the detection limit of odorants by concentrating them on an adsorption resin and to determine the composition of the mixture including a significant number of odorants in one analysis. The reproducibility of peak areas of odorants exhaled through the nostrils was then examined using R-FISS having these advantages after the model drink (containing 12 mixed odorants; final concentration of each odorant in this model drink was 5 ppm) had been swallowed. As a result, the RSD [RSD (%) = SD × 100/mean] of peak areas of the tested odorants showed good reproducibility of ≤ 14% (**Table 2.1**). This result indicated that the odorants exhaled from panelists would be reproducibly detected and many odorants could be certainly analyzed in one analysis, despite the conditions that the odorants were exhaled in an instant through the nostrils via the throat and their amounts were extremely low [13].

Compositional Changes in Odorants Exhaled through the Nostrils via the Nasal Cavity. In order to investigate the difference in the compositions of odorants between those in the model drink and those in the air exhaled from the human nose, the odorants exhaled through the nostrils were examined for one panelist using R-FISS after the model drink [independently containing the following 7 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. As a result, 1-methoxy-3-methyl-3-(methylthio)butane was detected with 4-methoxy-2-methyl-2-mercaptobutane in the air exhaled through the nostrils after the model drink containing 4-methoxy-2-methyl-2-mercaptobutane had been swallowed (**Figure 2.1**).

Moreover, hexanol was detected with hexanal after the model drink containing hexanal had been swallowed (**Figure 2.2**). That is, thiols were partially methylated to

Table 2.1 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.

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 females and four males). At first, the odorants exhaled through the nostrils were investigated after swallowing of the model drink (containing 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 the air exhaled through the nostrils from all panelists.

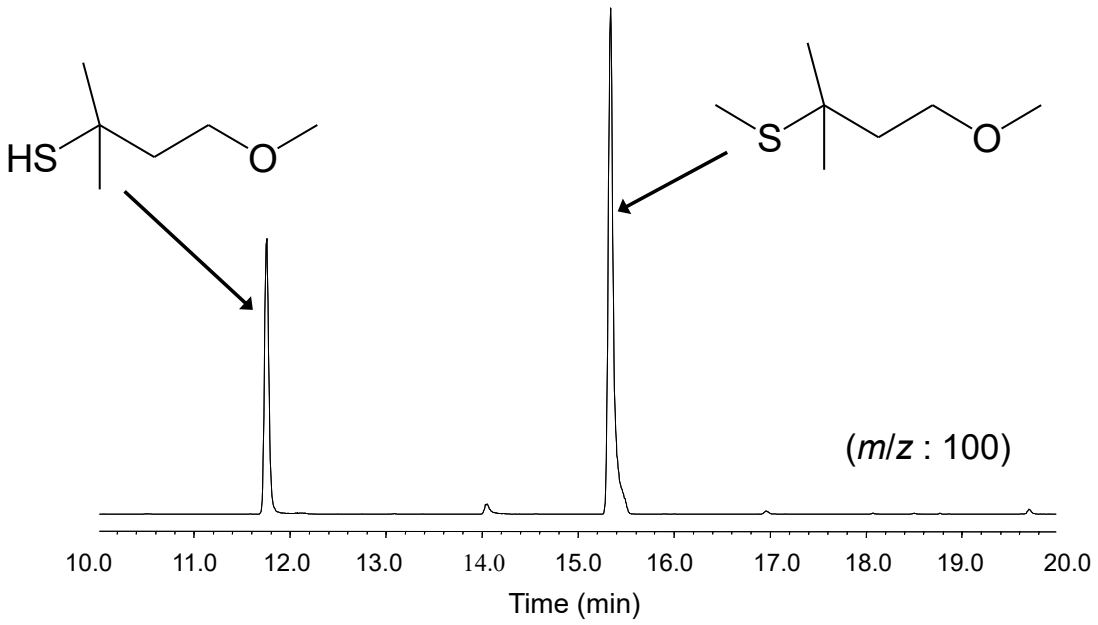


Figure 2.1 Ion chromatogram of the odorants exhaled through the nostrils after swallowing of the model drink. Concentration of 4-methoxy-2-methyl-2-mercaptobutane in the model drink was 10 ppm.

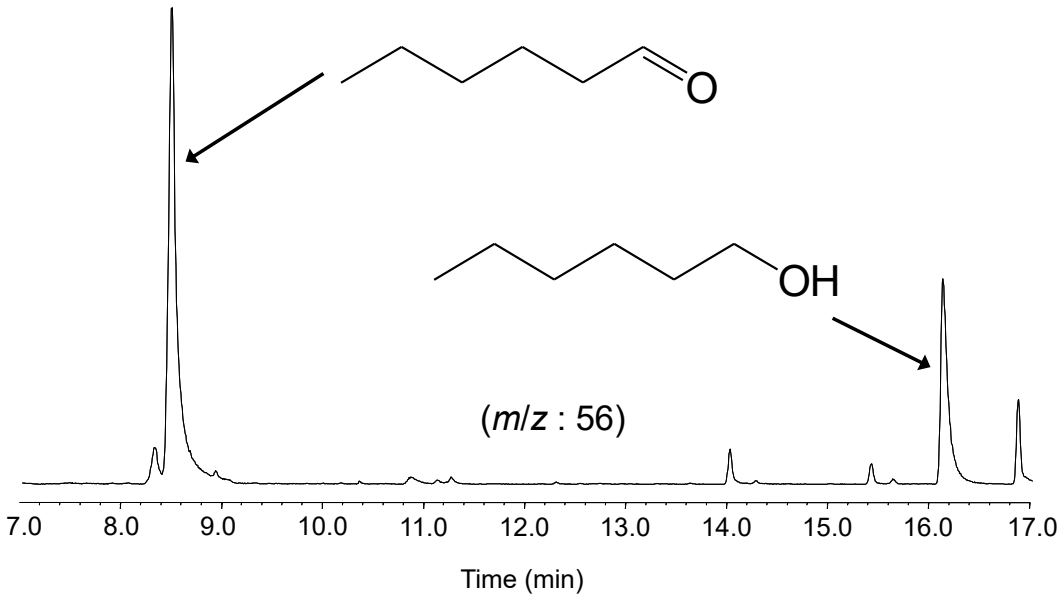


Figure 2.2 Ion chromatogram of the odorants exhaled through the nostrils after swallowing of the model drink. Concentration of hexanal in the model drink was 10 ppm.

In order 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 SPE 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 were then trapped on Tenax TA after swallowing of this model drink and analyzed not only by thermal desorption but also by solvent desorption using diethyl ether. Their corresponding methylthio ethers were detected with the original tested thiols in both desorption techniques. On the other hand, after the odorants in the headspace 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. Therefore, these results indicated that methylthio ethers were observed only in cases in which the odorants exhaled through the nostrils were trapped after swallowing of the model drink containing volatile thiols. That is, 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.

As already stated, it has been previously indicated that enzymes in human saliva could change the odorants [19-21]. However, after the model drink containing thiols had been put in the mouth, the corresponding methylthio ethers were hardly observed in the drink expelled by the panelist. Therefore, based on the present results that the compositional changes in odorants instantaneously proceeded, 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. In general, thiol methylation in mammals is catalyzed by thiol methyltransferases that utilize AdoMet [42]. 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 [43]. Actually, it was already demonstrated that biotransformation of some kinds of odorants would occur in the human nasal cavity [44, 45], and it was reported that odorants would be changed by enzymes in the mouse olfactory mucus [46]. 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 [47], and they are known to exist in the human oral and nasal cavity [48, 49]. 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. In order 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 (containing FFT, 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 [FMS, 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

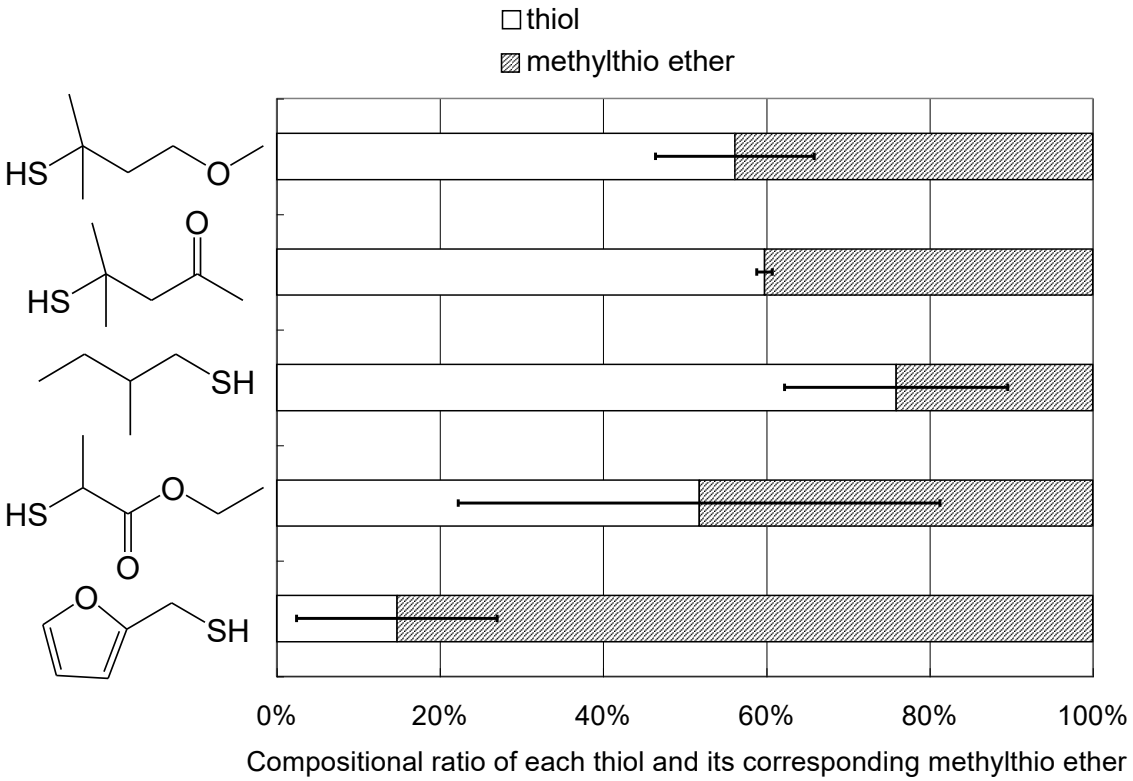


Figure 2.3 Compositional ratio of each thiol and its corresponding methylthio ether in air exhaled through the nostrils after swallowing of the model drink. Each compositional ratio was calculated from the peak area ratio of the total ion chromatogram. Concentration of each thiol in the model drink was 10 ppm. Values are the means of three panelists. Error bars show the standard deviations.

was then compared. As shown in **Figure 2.3**, the mean value of the compositional ratio considerably differed 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

[20]. 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 FFT is in good agreement with the previous study [20]. 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 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.

2.2 Influence of Milk on Aroma Release and Aroma Perception during Consumption of Coffee Beverages

2.2.1 Introduction

Coffee is widely appreciated for its characteristic aroma and taste, and is often enjoyed with milk or creamer. The purposes of adding these products to coffee are to develop a desirable color change, to reduce the bitter and sour tastes, and to reduce the astringency

of coffee. In general, adding milk or creamer to coffee significantly affects the aroma perceptions during drinking. Recent studies have demonstrated that the addition of milk products or milk components to coffee reduced the amounts of the aroma compounds released into the headspace of the coffee beverages [50, 51], likely altering the quality of the perceived aroma [50]. It is also well-known that the interaction between fat and odorants can affect the in-mouth release of odorants and their aroma perception [7, 52, 53]. The influence of fat on aroma release is highly related to the lipophilicity of the odorants. It has been reported that the aroma release of lipophilic compounds, which have high $K_{o/w}$ and *Log P* values, generally decreased with increasing fat content [53-56]. However, previous studies have not investigated in detail the influence of milk or creamer on the in-mouth release, especially the amounts of potent odorants in brewed coffee exhaled through the nostrils, during coffee consumption. Therefore, the aim of this study was to elucidate the influence of milk on the aroma release and aroma perception by investigating in detail the amounts of in-mouth odorants, including the potent odorants in brewed coffee, during the consumption of coffee using R-FISS.

2.2.2 Materials and Methods

Materials. Arabica coffee beans (Columbia Supremo) with a medium roasting degree were supplied by Unicafe, Inc. (Tokyo, Japan), and stored at -20°C until used. The roast degree of the beans was characterized by a color value of 20. The value was obtained by measuring coarsely milled coffee beans with a ZE2000 color-difference meter (Nippon Denshoku Industries Co., Ltd., Tokyo, Japan). Commercial milk (3.5% fat or more, sterilized at 130°C for 2 s; Meiji Co., Ltd., Tokyo, Japan) was purchased from a local market.

Chemicals. FMS was synthesized according to the procedure in the previous section (see 2.1.2 Materials and Methods). The following odorants were purchased from the suppliers shown: compounds (**Table 2.4**) 1-15, 19-22, 24-28, and 30-38 (Tokyo Chemical Industry Co., Ltd.); compounds 16-18, 23, 39, and FFT (Sigma-Aldrich

Japan); compound 29 (Wako Pure Chemical Industries Ltd., Osaka, Japan); 2-octanol (Nacalai Tesque, Inc., Kyoto, Japan).

Preparation of Coffee Brew. Five hundred grams of hot distilled water (ca. 90°C) were poured on 50 g of the ground Arabica coffee powder in a filter. The filtrate was immediately cooled in an ice water bath.

Preparation of Deodorized Coffee Brew. The coffee brew, which was prepared under the same conditions as described above, was lyophilized and the coffee powder was obtained (the solid content was 1.5%(w/w) of the coffee brew). To remove the remaining odorants in the coffee powder, the powder was distilled using the SAFE method [57] after being dissolved in 100 g of distilled water. The residue was then lyophilized again, and the deodorized coffee powder was obtained. It was then dissolved in distilled water (1.5 g/100 g). The residual odorants were confirmed by GC-MS using the SPE method [58]. FMS was completely removed, and almost all the FFT was also removed.

Quantitative Analysis of FMS in Coffee Brew. The aroma concentrate of the coffee brew was prepared by the SPE method. Fifty grams of the coffee brew were passed through a glass column (15 cm × 2 cm i.d.) filled with 5 mL of SP700 resin (Mitsubishi Chemical Corporation), which was conditioned with distilled water before use, followed by washing with 50 mL of distilled water and eluting with 20 mL of diethyl ether. Fifty microliters of an IS solution (10.8 mg/100 mL 2-octanol in dichloromethane) was added to the diethyl ether fraction for the quantitative analysis. The fraction was dried with an excess amount of anhydrous sodium sulfate and then concentrated by rotary evaporation (35°C, 550 mmHg) to about 5 mL, followed by nitrogen stream evaporation to about 100 μL. In order to determine the recovery rate of FMS using this method, the aroma concentrate of the deodorized coffee brew (100 ppb of FMS was added) was also prepared. These aroma concentrates were used as the samples for the GC-MS analysis (*a*).

Preparation of Coffee Beverages and Deodorized Coffee Beverages. The experimental design for the coffee beverages is shown in **Table 2.2**. FFT and FMS were dissolved in ethanol, and added at 0.1% to each coffee beverage so that the additive amounts of these

Table 2.2 Experiment design for coffee beverages, additive amount of FFT and FMS, and concentration of FMS in coffee beverages.

		Mixed amount (g)				Additive amount (ppb)		Concentration of FMS (ppb)	
Beverage	Beverage type	Coffee brew	Deodorized coffee brew	Distilled Water	Milk	FFT	FMS	from Coffee brew[a]	Sum[b]
1	Black coffee	100	-	100	-	-	-	22	22
2	Milk coffee	100	-	-	100	-	-	22	22
3	Deodorized black coffee	-	100	100	-	-	20[c]	-	20
4	Deodorized milk coffee	-	100	-	100	-	20[c]	-	20
5	FMS-added milk coffee	100	-	-	100	-	10[d]	22	32
6	Aroma-adjusted milk coffee	100	-	-	100	80[e]	10[d]	22	32

[a] calculated from the concentration of FMS in coffee brew and the dilution ratio with distilled water or milk.

[b] Sum of the concentration of FMS in each coffee beverage; Sum = additive amount of FMS + concentration of FMS derived from the coffee brew.

[c] determined by considering the concentration of FMS in coffee brew so that the concentration of FMS was about the same as in beverages 1 and 2.

[d] determined by considering the concentration of FMS in coffee brew and the different in-mouth release of FMS between beverages 3 and 4.

[e] determined so that the in-mouth release of FMS was about the same as in beverages 1 and 2.

compounds are the same as the concentrations described in **Table 2.2**. In order to prepare beverage 6, the amounts of FFT and FMS to be added to the milk coffee were determined according to the following procedure. The concentration of FMS in the coffee brew was determined to be 44 ppb by GC-MS using SPE and the recovery rate of FMS. The concentration of FMS in beverages 1 and 2 was estimated to be 22 ppb (**Table 2.2**) because the coffee brew was diluted with the same weight of distilled water or milk. In order to determine the in-mouth release of FMS derived from only FMS in beverages 1 and 2 during consumption of those coffee beverages, 20 ppb of FMS was then added to the deodorized coffee beverages so that the concentration of FMS in beverages 3 and 4 (**Table 2.2**) was about the same as in beverages 1 and 2. The in-mouth release of FMS during the consumption of beverage 4 was about two-thirds that of beverage 3. Therefore, based on the different in-mouth release of FMS between beverages 3 and 4, and the concentration of FMS in the milk coffee, the additive amount

of FMS to the milk coffee was determined to be 10 ppb so that the in-mouth release of FMS derived from only FMS in beverage 2 was about the same as that of beverage 1. Next, the relationship between the additive amounts of FFT to beverage 5 and the in-mouth release of FMS was investigated. The additive amount of FFT to the milk coffee was determined assuming that the different in-mouth release of FMS between beverages 1 and 5 (**Table 2.2**) resulted from the different in-mouth release of FFT in these coffee beverages. All of these beverages were kept at room temperature before use.

Semi-quantitative Analysis of In-mouth Odorants Exhaled through the Nostrils. In order to determine the in-mouth odorants, the breath exhaled through the nostrils was trapped according to the following procedure. After 30 mL of the coffee beverage at room temperature was placed in the mouth cavity, trained panelists paused for 1-2 seconds and then swallowed all of the liquid in one gulp. Ten breaths after the coffee beverage had been swallowed were passed through a small glass column (6 cm × 5 mm i.d.) filled with 100 mg of Tenax TA (80/100 mesh, GL Science), which had been heated at 220°C for 2 hours prior to the analysis. The end of the glass column was connected to a pump by a silicon tube, and a suction of approximately 1 L/min was applied to the system during trapping of the breath exhaled through the nostrils. The experiments lasted 1-3 min. This sampling system allowed the panelists to normally exhale without the need to press air through the Tenax column. After trapping of the in-mouth odorants, 5 μL of an IS solution (5 μg/mL 2-octanol in ethanol) was directly added to the Tenax column for the semi-quantitative analysis. The water and ethanol were then removed from the Tenax TA by flowing dry nitrogen (30 min, 100 mL/min). These experiments were carried out at room temperature (25 ± 2°C). The experiments using beverages 1 and 2 (**Table 2.2**) were performed by 4 trained panelists. The Tenax columns were used as the samples for the GC-MS analysis (*b*). A blank test was also performed under the same conditions using distilled water. In order to determine the additive amounts of FFT and FMS to the milk coffee, the experiments using beverages 1-6 (**Table 2.2**) were performed by a trained panelist. The Tenax columns were used as the samples for the GC-MS analysis (*c*).

Semi-quantitative Analysis of Thiols in Coffee Beverages. The aroma concentrate of the coffee beverage was prepared by SPE. Five microliters of a solution (0.5 w/v% FFT in ethanol) and 100 μL of an IS solution (105.1 mg/100 mL 2-phenoxyethanol in methanol) for the semi-quantitative analysis were added to 50 g of the coffee beverages (final concentration of FFT added to the coffee beverages was 500 ppb). The coffee beverages were passed through a glass column (15 cm × 2 cm i.d.) filled with 5 mL of SP700 resin (Mitsubishi Chemical Corporation), which was conditioned with distilled water before use, followed by washing with 50 mL of distilled water and eluting with 20 mL of diethyl ether. The diethyl ether fraction was dried with an excess amount of anhydrous sodium sulfate and then concentrated by rotary evaporation (35°C, 550 mmHg) to about 5 mL, followed by nitrogen stream evaporation to about 100 μL. These aroma concentrates were used as the samples for the GC-MS analysis (*d*).

GC-MS. (*a*) The aroma concentrates were analyzed by an Agilent 7890N GC with an Agilent 5975C series MS (Agilent Technologies) using the split mode (injection volume of 1 μL; inlet temperature of 250°C; split ratio 30:1). 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 80°C to 230°C at the rate of 3°C/min. The flow rate of the helium carrier gas was 1 mL/min. The MS was used with an ionization voltage of 70 eV (EI) and operated in the SIM mode. The ion source and quadrupole temperature were set at 230°C and 150°C, respectively. The selected ions of 45 and 81 were monitored for 2-octanol (IS material) and FMS, respectively. The content of FMS in the volatile fraction of the coffee brew was determined by the internal standard method using an RF. The RF of FMS to 2-octanol (IS material) was 1.5, and this RF was calculated from the ratio of the selected ion peak area of FMS to 2-octanol obtained by mass chromatography of a standard solution containing equal weights of FMS and 2-octanol. These selected ion peak areas were the mean values of triplicate results. Moreover, the concentration of FMS in the coffee brew was calculated from the content in the volatile fraction of the coffee brew and the recovery rate on the SPE method. (*b*) Thermal desorption of the trapped odorants on the Tenax TA was performed using a TDU thermal desorption system (Gerstel GmbH) in combination

with the ATEX option of an MPS2 autosampler (Gerstel GmbH) and a CIS4 injector (Gerstel GmbH) according to the following parameters. Thermal desorption was performed by programming the TDU from 20°C to 220°C (held for 3 min) at the rate of 12°C/s in the splitless mode. Cryofocusing was performed with liquid nitrogen at -150°C. Injection was performed with the ramp of 12°C/s from -150°C to 220°C (held for 3 min) in the splitless mode. The odorants were analyzed by an Agilent 6890N GC with an Agilent 5975B series MS (Agilent Technologies). The column was a 30 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 30°C (held for 3 min) to 120°C at the rate of 3°C/min, and then raised at the rate of 5°C/min to 230°C. The flow rate of the helium carrier gas was 1 mL/min. The MS was used with an ionization voltage of 70 eV (EI) and operated in the SIM-SCAN mode. The ion source and quadrupole temperatures were set at 230°C and 150°C, respectively. The selected ions listed in **Table 2.3** were monitored in the SIM mode for each potent odorant in coffee brew. The semi-quantitative amounts of in-mouth odorants were determined by the internal standard method from the total ion or the selected ion peak areas obtained by mass chromatography. The semi-quantitative amounts were calculated from the ratio of the total ion peak area of 2-octanol (IS material) and those of the in-mouth odorants obtained in the SCAN mode. Based on the ratio of the selected ion to the overall ions of the mass spectra for each reference compound, the total ion peak area of each compound was converted from the selected ion peak area obtained in the SIM mode; total ion peak area = selected ion peak area × conversion factor. The conversion factors are listed in **Table 2.3**. The response factor of each odorant to 2-octanol (IS material) was defined as 1. The odorants, whose peak areas were significantly larger than those in the blank test, were quantitated as in-mouth odorants. The results were then analyzed using a Student's t-test. (*c*) The conditions of the thermal desorption and GC-MS were the same as those in *b*. The results of beverages 1, 2, and 6 were then analyzed using a one-way ANOVA followed by Tukey's test. (*d*) The aroma concentrates were analyzed by an Agilent 7890B GC with an Agilent 5977A series MS (Agilent Technologies) using the split mode (injection volume of 1 μL; inlet temperature of 250°C; split ratio 30:1).

Table 2.3 Selected ions and conversion factors for mass chromatography in the SIM mode.

Odorants	Selected Ion (m/z)[a]		Conversion Factor[b]	Previous idendification[c]
	cal.	ref.		
3-methylbutanal	44	58	4.5	59
2,3-butanedione	86	43	5.0	59
2,3-pentanedione	100	43	6.1	59
ethyl 2-methylbutyrate	102	85	4.1	60
ethyl isovalerate	88	85	4.4	60
3-methyl-2-buten-1-thiol	102	41	7.3	59
2-methyl-3-furanthiol	114	85	3.5	59
2,3,5-trimethylpyrazine	122	42	2.2	59
2-isopropyl-3-methoxypyrazine	137	152	3.3	59, 60
2-methoxy-3,5-dimethylpyrazine	138	109	4.7	60
2-furfurylthiol (FFT)	81	114	2.3	59
methional	104	48	5.2	59, 60
2-ethyl-3,5-dimethylpyrazine	135	136	3.0	59
2,3-diethyl-5-methylpyrazine	150	135	6.5	59
(*Z*)-2-nonenal	41	70	7.7	60
2-isobutyl-3-methoxypyrazine	124	151	2.5	59, 60
3-mercapto-3-methylbutyl formate	69	102	3.1	59, 61
linalool	71	93	6.7	59, 60
(*E*)-2-nonenal	70	83	8.9	59, 60
3-mercapto-3-methylbutyl acetate	69	102	4.7	61
phenylacetaldehyde	91	120	2.0	59
isovaleric acid	60	87	2.0	59, 60
3-mercapto-3-methylbutanol	69	75	5.7	59
β-damascenone	121	190	5.3	59
3,4-dimethyl-1,2-cyclopentanedione	126	111	5.1	59
2-methoxyphenol	109	124	3.1	59
anisaldehyde	135	136	2.9	59
4-hydroxy-2,5-dimethyl-3(2*H*)-furanone	128	85	3.2	59
4-ethyl-2-methoxyphenol	137	152	2.4	59, 60
bis(2-methyl-3-furyl)disulfide	113	226	3.5	59
3-hydroxy-4,5-dimethyl-2(5*H*)-furanone	128	83	11.3	59, 60
2-methoxy-4-vinylphenol	150	135	3.5	59, 60
5-ethyl-3-hydroxy-4-methyl-2(5*H*)-furanone	97	142	4.8	59
vanillin	151	152	3.2	59, 60
furfuryl methyl sulfide[d]	81	128	2.8	-
2-octanol[e]	45	97	3.2	-

[a] cal.: quantifier ion, ref.: qualifier ion.

[b] The ratio of the selected ion (quantifier ion) to the overall ions of the mass spectra for each reference compound.

[c] See references.

[d] Selected as a target compound in the SIM mode to improve the qualitative accuracy.

[e] Internal standard material.

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 80°C to 230°C at the rate of 3°C/min. The flow rate of the helium carrier gas was 1 mL/min. The MS was used with an ionization voltage of 70 eV (EI) and operated in the SCAN mode. The ion source and quadrupole temperature were set at 230°C and 150°C, respectively. The semi-quantitative amounts were calculated from the ratio of the total ion peak area of 2-phenoxyethanol (IS material) and those of the thiols obtained in the SCAN mode. The RF of each odorant to 2-phenoxyethanol was defined as 1.

Identification of the Odorants. Each odorant was identified by comparing its Kovats GC RI and mass spectrum to those of the reference compounds.

Sensory Evaluation. Fifteen milliliters of the coffee beverages (beverages 1, 2, and 6 shown in **Table 2.2**) were placed in plastic cups, and these 3 samples were simultaneously presented to panelists in a dark room under red light. Ion exchanged water was provided for rinsing the mouth. The samples were evaluated by 31 panelists (17 females and 14 males) employed by Ogawa & Co., Ltd. All panelists had previously received extensive training in the descriptive sensory analysis of coffee beverages and had experience in the sensory profiling of various food samples. Milk coffee (beverage 2) was used as a control, and its intensity of the coffee-like aroma quality was defined as 4 for descriptive purposes. The panelists scored the intensity of the coffee-like aroma quality for the black coffee and aroma-adjusted milk coffee (beverages 1 and 6) using a seven-point scale from 1 (very weak) to 7 (very strong) compared to that for the milk coffee. The experiment was performed one time by each panelist. The sensory results were then analyzed using a one-way ANOVA followed by Tukey's test.

2.2.3 Results and Discussion

Influence of Milk on Aroma Release during Consumption of Black Coffee and Milk Coffee. In order to investigate the influence of milk on the aroma release, the in-mouth

release of odorants during the consumption of the black coffee and milk coffee (beverages 1 and 2 shown in **Table 2.2**) was compared using R-FISS. The SIM-SCAN mode was applied to a mass spectrometer in order to improve the detection limit of odorants. Thirty-five odorants, except for 2-octanol, listed in **Table 2.3** were selected as the target compounds in the SIM mode. These odorants except for FMS were previously identified as potent odorants in brewed coffee [59-61]. There is the possibility that their in-mouth release could have a significant impact on the aroma perception of coffee. As the result of in-mouth release analysis using R-FISS, thirty-nine odorants listed in **Table 2.4** could be detected and their amounts showed individual differences (**Table 2.4**). Previous studies have already indicated that the chemical structure of some odorants can be altered by the enzymatic activities of human saliva [19, 20]. Based on these results, the different in-mouth release among panelists might have resulted from differences in the oral cavity, volume and composition of the saliva, or swallowing activity. Thus, while there were individual differences in the in-mouth releases, the amounts of most in-mouth odorants did not significantly differ between the black coffee and milk coffee. However, the amounts of 3 odorants (No. 24, 32, and 36 in **Table 2.4**) exhaled through the nostrils during consumption of the milk coffee were significantly lower than those of the black coffee regardless of the panelists (**Table 2.4**).

Previous studies suggested the possibility that fat would have a greater impact on the aroma release than proteins [51, 62]. It was also reported that the influence of fat on the aroma release differed depending on the *Log P* values of the odorants [53-56]. In agreement with previous studies, milk significantly decreased the in-mouth releases of relatively lipophilic odorants whose *Log P* values are equal to or higher than 2 (No. 24, 32, and 36 in **Table 2.4**) in the present results. However, not all odorants whose *Log P* values are higher than 2 (No. 23, 24, 26, 28, 32, 36, 38, and 39 in **Table 2.4**) were significantly affected by the milk. These results indicated that the in-mouth releases of some odorants could be easily influenced by the milk, but those of other odorants could not be easily influenced even if those odorants have similar *Log P* values. Therefore, some factors other than the milk fat and the *Log P* values of the odorants might have an impact on the aroma release of the milk coffee.

Table 2.4 Semiquantitative amounts[a] and RSD (%)[b] of in-mouth odorants exhaled through the nostrils during the consumption of black coffee and milk coffee obtained by the SIM-SCAN mode.

No.	Compound	Odor threshold in water[c] (μg / L)	*Log P*[d]	Panelist 1						Panelist 2					
				Black Coffeee		Milk Coffee		Ratio[e] (Effect of Milk)	*p* value[f]	Black Coffeee		Milk Coffee		Ratio[e] (Effect of Milk)	*p* value[f]
				Amount (pg)	RSD (%)	Amount (pg)	RSD (%)			Amount (pg)	RSD (%)	Amount (pg)	RSD (%)		
1	2-methylbutanal[g]	1 - 6	1.23	1240	14	1080	19	0.87	n.s.	724	54	846	60	1.17	n.s.
2	3-methylbutanal[h]	0.2 - 2	1.25	955	30	903	14	0.95	n.s.	1100	27	785	35	0.71	n.s.
3	2,3-butanedione[h]	2.6 - 15	-1.34	1730	24	1740	18	1.01	n.s.	1020	14	902	10	0.88	n.s.
4	2,3-pentanedione[h]	20	-0.85	1670	22	1330	20	0.80	n.s.	1050	21	966	30	0.92	n.s.
5	1-methylpyrrole[g]	—	1.43	713	25	457	24	0.64	< 0.01	913	31	719	35	0.79	n.s.
6	pyridine[g]	7.9 - 2000	0.65	22300	20	19400	13	0.87	n.s.	11700	21	12700	19	1.09	n.s.
7	pyrazine[g]	50000 - 75000	-0.26	660	18	678	14	1.03	n.s.	335	37	383	13	1.14	n.s.
8	2-methylbutanol[g]	300 - 4150	1.26	84	30	61	13	0.73	n.s.	51	19	43	32	0.86	n.s.
9	isoamyl alcohol[g]	250 - 1005	1.26	173	41	126	25	0.73	n.s.	86	27	85	16	0.99	n.s.
10	furfuryl methyl ether[g]	—	1.14	285	23	211	21	0.74	< 0.05	327	25	279	26	0.85	n.s.
11	2-methyltetrahydrofuran-3-one[g]	—	-0.20	2470	18	2200	16	0.89	n.s.	998	22	963	16	0.97	n.s.
12	2-methylpyrazine[g]	60 - 105000	0.24	2990	24	2560	12	0.86	n.s.	1010	26	975	17	0.97	n.s.
13	2,5-dimethylpyrazine[g]	800 - 1800	1.03	746	25	632	13	0.85	n.s.	261	27	248	15	0.95	n.s.
14	2,6-dimethylpyrazine[g]	200 - 9000	1.03	653	30	563	14	0.86	n.s.	222	31	211	17	0.95	n.s.
15	2,3-dimethylpyrazine[g]	400 - 2500	1.03	258	26	209	12	0.81	n.s.	85	22	87	15	1.03	n.s.
16	2-methyl-2-cyclopenten-1-one[g]	—	1.26	114	16	100	12	0.88	n.s.	55	24	60	13	1.08	n.s.
17	2-ethyl-6-methylpyrazine[g]	40	1.53	853	24	717	17	0.84	n.s.	361	24	339	20	0.94	n.s.
18	2-ethyl-5-methylpyrazine[g]	16 - 100	1.53	559	23	478	15	0.85	n.s.	238	20	223	18	0.94	n.s.
19	2-ethyl-3-methylpyrazine[g]	130 - 500	1.53	194	21	163	14	0.84	n.s.	83	19	81	18	0.97	n.s.
20	2,3,5-trimethylpyrazine[h]	400 - 9000	1.58	99	28	80	18	0.81	n.s.	35	27	32	21	0.94	n.s.
21	furfural[g]	282 - 23000	0.83	4600	34	3360	19	0.73	n.s.	1380	27	1120	12	0.81	n.s.
22	acetoxy-2-propanone[g]	—	-0.19	707	35	574	14	0.81	n.s.	206	32	156	17	0.76	n.s.
23	2-ethyl-3,5-dimethylpyrazine[h]	0.04 - 1.0	2.07	50	30	40	18	0.81	n.s.	24	38	21	30	0.88	n.s.
24	furfuryl methyl sulfide[h]	—	2.00	197	24	103	30	0.52	< 0.001	277	36	132	25	0.48	< 0.01
25	2-acetylfuran[g]	10000	0.80	1760	22	1480	10	0.84	n.s.	769	34	675	14	0.88	n.s.
26	2,3-diethyl-5-methylpyrazine[h]	0.09 - 1.0	2.56	45	36	36	22	0.80	n.s.	25	21	20	28	0.79	n.s.
27	pyrrole[g]	20000 - 49600	0.75	1070	13	1010	14	0.94	n.s.	487	20	400	18	0.82	n.s.
28	2-isobutyl-3-methoxypyrazine[h]	0.002 - 0.016	2.86	14	39	5	23	0.38	< 0.01	15	92	9	60	0.58	n.s.
29	1-(2-furanyl)-2-propanone[g]	—	0.84	139	22	110	29	0.79	n.s.	97	16	73	24	0.76	< 0.05
30	furfuryl acetate[g]	—	1.09	1020	48	676	27	0.66	n.s.	528	23	411	28	0.78	n.s.
31	5-methylfurfural[g]	6000	1.38	1020	39	747	19	0.73	n.s.	374	25	311	12	0.83	n.s.
32	difurylmethane[g]	—	2.99	80	23	33	39	0.42	< 0.001	128	43	35	33	0.27	< 0.01
33	2-formyl-1-methylpyrrole[g]	37	1.18	506	28	402	10	0.79	n.s.	166	23	156	15	0.94	n.s.
34	2-acetyl-1-methylpyrrole[g]	—	1.11	174	26	133	11	0.76	n.s.	69	23	59	16	0.86	n.s.
35	furfuryl alcohol[g]	1000 - 2000	0.45	2060	34	1490	22	0.72	n.s.	875	27	762	26	0.87	n.s.
36	furfuryl pyrrole[g]	100	2.50	165	37	76	43	0.46	< 0.01	183	29	69	31	0.37	< 0.001
37	2-methoxyphenol[h]	3 - 21	1.34	109	16	118	34	1.08	n.s.	97	19	79	21	0.82	n.s.
38	4-ethyl-2-methoxyphenol[h]	20 - 50	2.38	19	18	21	28	1.08	n.s.	11	65	7	41	0.58	n.s.
39	2-methoxy-4-vinylphenol[h]	3 - 10	2.24	33	29	30	23	0.89	n.s.	15	35	10	58	0.64	n.s.

[a] Each amount is the mean value of seven-fold results obtained from each identical panelist. [b] RSD (%) is calculated using the amounts of the odorants from seven-fold results obtained from each identical panelist.

[c] Odor threshold values are obtained from Flavor-Base 9th Edition – the world's most extensive database for flavoring materials and food additives provided by Leffingwell & Associates.

[d] *Log P* values are obtained from Chemspider – open access online chemical database hosted by the Royal Society of Chemistry [63].

[e] Each ratio is calculated by dividing the amount of the in-mouth odorant during the consumption of milk coffee by that of black coffee.

[f] Student's t tests were performed.; n.s., not significant [g] detected in the SCAN mode. [h] detected in the SIM mode.

Table 2.4 Continued.

No.	Odorants	Odor threshold in water[c] (μg / L)	*Log P*[d]	Panelist 3						Panelist 4					
				Black Coffeee		Milk Coffee		Ratio[e] (Effect of Milk)	*p* value[f]	Black Coffeee		Milk Coffee		Ratio[e] (Effect of Milk)	*p* value[f]
				Amount (pg)	RSD (%)	Amount (pg)	RSD (%)			Amount (pg)	RSD (%)	Amount (pg)	RSD (%)		
1	2-methylbutanal[g]	1 - 6	1.23	113	28	120	35	1.06	n.s.	251	21	268	31	1.06	n.s.
2	3-methylbutanal[h]	0.2 - 2	1.25	155	17	166	32	1.07	n.s.	347	41	307	37	0.89	n.s.
3	2,3-butanedione[h]	2.6 - 15	-1.34	346	40	375	44	1.08	n.s.	796	38	879	11	1.10	n.s.
4	2,3-pentanedione[h]	20	-0.85	350	28	296	44	0.85	n.s.	786	50	738	32	0.94	n.s.
5	1-methylpyrrole[g]	—	1.43	147	45	79	31	0.54	< 0.05	458	26	286	47	0.63	< 0.05
6	pyridine[g]	7.9 - 2000	0.65	7570	24	6820	11	0.90	n.s.	19600	14	19200	25	0.98	n.s.
7	pyrazine[g]	50000 - 75000	-0.26	319	19	366	8	1.15	n.s.	678	43	570	29	0.84	n.s.
8	2-methylbutanol[g]	300 - 4150	1.26	22	41	22	28	0.98	n.s.	36	34	49	43	1.36	n.s.
9	isoamyl alcohol[g]	250 - 1005	1.26	37	23	43	35	1.16	n.s.	77	51	84	32	1.10	n.s.
10	furfuryl methyl ether[g]	—	1.14	77	53	43	30	0.56	n.s.	233	29	166	49	0.71	n.s.
11	2-methyltetrahydrofuran-3-one[g]	—	-0.20	974	21	880	11	0.90	n.s.	1880	23	1500	27	0.80	n.s.
12	2-methylpyrazine[g]	60 - 105000	0.24	1160	22	1130	8	0.97	n.s.	2290	31	2120	24	0.93	n.s.
13	2,5-dimethylpyrazine[g]	800 - 1800	1.03	294	26	282	6	0.96	n.s.	565	29	525	25	0.93	n.s.
14	2,6-dimethylpyrazine[g]	200 - 9000	1.03	249	35	224	21	0.90	n.s.	451	28	398	37	0.88	n.s.
15	2,3-dimethylpyrazine[g]	400 - 2500	1.03	104	22	101	9	0.97	n.s.	177	31	171	22	0.97	n.s.
16	2-methyl-2-cyclopenten-1-one[g]	—	1.26	50	13	49	12	0.96	n.s.	97	22	93	23	0.97	n.s.
17	2-ethyl-6-methylpyrazine[g]	40	1.53	303	26	270	9	0.89	n.s.	740	23	699	26	0.94	n.s.
18	2-ethyl-5-methylpyrazine[g]	16 - 100	1.53	206	25	184	8	0.89	n.s.	480	21	455	25	0.95	n.s.
19	2-ethyl-3-methylpyrazine[g]	130 - 500	1.53	70	14	67	12	0.96	n.s.	163	27	152	26	0.94	n.s.
20	2,3,5-trimethylpyrazine[h]	400 - 9000	1.58	41	28	39	14	0.95	n.s.	76	34	72	15	0.94	n.s.
21	furfural[g]	282 - 23000	0.83	1610	19	1510	15	0.94	n.s.	2110	40	1710	18	0.81	n.s.
22	acetoxy-2-propanone[g]	—	-0.19	294	17	280	9	0.95	n.s.	356	52	346	19	0.97	n.s.
23	2-ethyl-3,5-dimethylpyrazine[h]	0.04 - 1.0	2.07	20	27	17	21	0.88	n.s.	42	32	40	21	0.97	n.s.
24	furfuryl methyl sulfide[h]	—	2.00	62	68	21	31	0.34	< 0.05	145	35	74	34	0.51	< 0.01
25	2-acetylfuran[g]	10000	0.80	686	20	654	9	0.95	n.s.	1420	20	1320	21	0.93	n.s.
26	2,3-diethyl-5-methylpyrazine[h]	0.09 - 1.0	2.56	17	37	12	27	0.70	n.s.	42	29	37	30	0.87	n.s.
27	pyrrole[g]	20000 - 49600	0.75	264	51	143	30	0.54	n.s.	430	28	270	55	0.63	n.s.
28	2-isobutyl-3-methoxypyrazine[h]	0.002 - 0.016	2.86	3	28	1	27	0.48	< 0.01	10	17	6	26	0.63	< 0.01
29	1-(2-furanyl)-2-propanone[g]	—	0.84	67	65	36	30	0.53	n.s.	85	25	52	35	0.62	< 0.05
30	furfuryl acetate[g]	—	1.09	321	48	189	34	0.59	n.s.	339	30	249	39	0.74	n.s.
31	5-methylfurfural[g]	6000	1.38	424	23	373	13	0.88	n.s.	395	42	287	16	0.73	n.s.
32	difurylmethane[g]	—	2.99	27	77	4	54	0.15	< 0.05	48	37	18	42	0.37	< 0.01
33	2-formyl-1-methylpyrrole[g]	37	1.18	190	21	170	11	0.90	n.s.	344	33	296	22	0.86	n.s.
34	2-acetyl-1-methylpyrrole[g]	—	1.11	64	14	58	8	0.90	n.s.	129	28	109	22	0.84	n.s.
35	furfuryl alcohol[g]	1000 - 2000	0.45	1320	38	887	19	0.67	n.s.	961	20	955	38	0.99	n.s.
36	furfuryl pyrrole[g]	100	2.50	74	65	18	62	0.24	< 0.05	151	25	48	62	0.32	< 0.001
37	2-methoxyphenol[h]	3 - 21	1.34	99	20	94	16	0.94	n.s.	131	34	132	30	1.00	n.s.
38	4-ethyl-2-methoxyphenol[h]	20 - 50	2.38	10	54	7	36	0.72	n.s.	10	36	8	50	0.82	n.s.
39	2-methoxy-4-vinylphenol[h]	3 - 10	2.24	22	60	16	45	0.74	n.s.	19	52	12	51	0.62	n.s.

[a] Each amount is the mean value of seven-fold results obtained from each identical panelist. [b] RSD (%) is calculated using the amounts of the odorants from seven-fold results obtained from each identical panelist.

[c] Odor threshold values are obtained from Flavor-Base 9th Edition – the world's most extensive database for flavoring materials and food additives provided by Leffingwell & Associates.

[d] *Log P* values are obtained from Chemspider – open access online chemical database hosted by the Royal Society of Chemistry [63].

[e] Each ratio is calculated by dividing the amount of the in-mouth odorant during the consumption of milk coffee by that of black coffee.

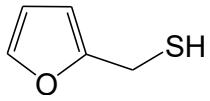
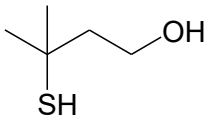
[f] Student's t tests were performed.; n.s., not significant [g] detected in the SCAN mode. [h] detected in the SIM mode.

FMS exhaled through the nostrils during the consumption of milk coffee could be derived from not only FMS but FFT, whose *Log P* value is 3.44 (obtained from Chemspider [63]), in the coffee beverages (see 2.1). Especially, it was also reported that nearly 90% of the FFT was methylated to FMS during a short period prior to the odorants being exhaled through the nostrils (**Figure 2.3**). Therefore, the different in-mouth release of FMS between the black coffee and milk coffee obtained from the present study would include the different in-mouth release of FFT. The interchanging of the sulfhydryl and disulfide groups within the protein or with external thiol groups are well-known reactions in protein chemistry [64]. Thiols would be lost by reacting with the sulfhydryl or disulfide groups on the protein. Therefore, the in-mouth release of FMS might be decreased by the addition of milk to the coffee because some of the FFT in milk coffee might be lost by these reactions with milk proteins, such as casein, and those FFTs could not be exhaled from the nostrils as FMS.

In order to examine the influence of milk on the contents of thiols in the milk coffee, the concentrations of thiols in the black coffee and milk coffee were compared using the SPE method. As a result, the concentration of FFT in the milk coffee was much lower than that in the black coffee (**Table 2.5**). This result indicated that the in-mouth release of FMS decreased by the addition of milk to the coffee because a large part of the FFT was lost by the reactions with the milk proteins, and the lost FFT could not be exhaled from the human nose as FMS. Furthermore, the decrease rate in the concentration of 3-mercapto-3-methyl-1-butanol in the milk coffee was quite different from that of FFT. These results also suggested the possibility the decrease rates in the contents of thiols in the milk coffee might significantly vary depending on their chemical structures. The present results strongly suggested the possibility that the addition of milk to the coffee could decrease the in-mouth release of FFT in addition to 3 odorants (No. 24, 32, 36 in **Table 2.4**).

Influence of FFT on Differences in Coffee-like Aroma Quality between Black Coffee and Milk Coffee. The different in-mouth release of FFT during consumption of coffee beverages may have a significant impact on the different flavor perception between black coffee and milk coffee based on the fact that FFT has an extremely low threshold

Table 2.5 Comparison of semi-quantitative concentrations of FFT and 3-mercapto-3-methyl-1-butanol in black coffee and milk coffee.

	Conc.[a] (ppb)		Ratio[b]
	Black Coffee	Milk Coffee	(Milk / Black)
	31	10	0.32
	136	118	0.87

[a] Semi-quantitative concentrations of thiols were calculated using the internal standard method with SPE.

[b] Each ratio was calculated by dividing the concentration of the thiol in milk coffee by that in black coffee, respectively.

and it is one of the most important potent odorants in brewed coffee. In order to investigate the influence of the in-mouth release of FFT on the different aroma perceptions of the black coffee and milk coffee, the aroma-adjusted milk coffee (beverage 6 shown in **Table 2.2**) was prepared by the addition of FFT and FMS to the milk coffee. The intensity of the coffee-like aroma quality of the aroma-adjusted milk coffee was then compared to those of the black coffee and milk coffee. The additive amounts of FFT and FMS to the milk coffee were determined to be 80 ppb and 10 ppb, respectively, based on the concentration of FMS in the coffee brew and the in-mouth release of FMS during consumption of the coffee beverages (see 2.2.2 Materials and Methods). The in-mouth release of FMS during the consumption of the aroma–adjusted milk coffee was then investigated. As a result, the amount of FMS exhaled through the nostrils was roughly the same as that of the black coffee (**Figure 2.4**).

The intensity of the coffee-like aroma quality during the consumption of the black coffee, milk coffee, and aroma-adjusted milk coffee was then examined by sensory evaluation. As a result, it was demonstrated that the coffee-like aroma quality of the milk coffee was perceived to be significantly weaker than that of the black coffee (**Figure 2.5**). Moreover, the coffee-like aroma quality of the aroma-adjusted milk coffee

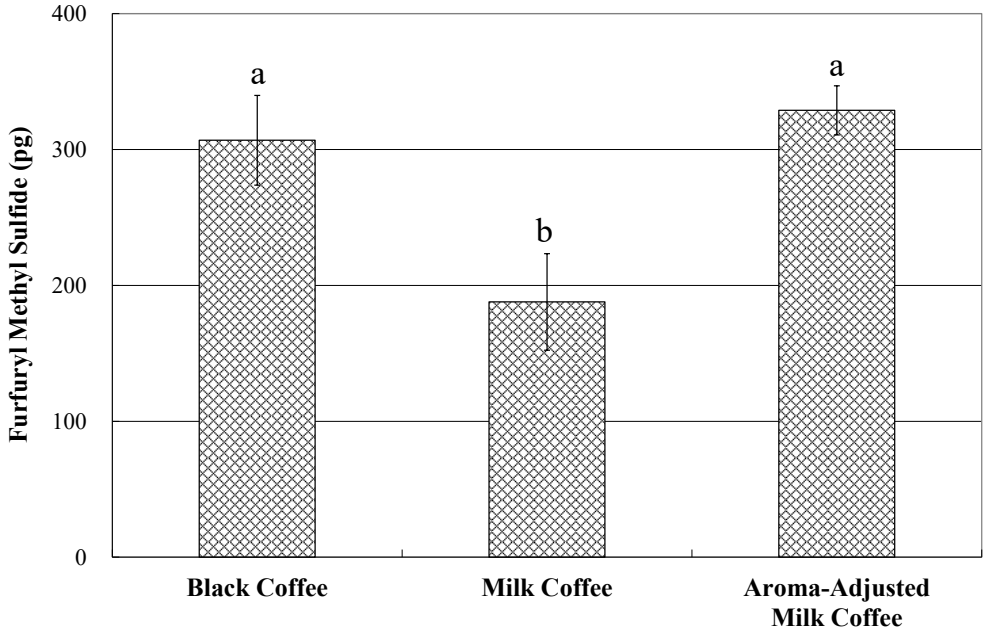


Figure 2.4 Semi-quantitative amounts of FMS exhaled through the nostrils during the consumption of black coffee, milk coffee, and aroma-adjusted milk coffee. Each amount of FMS is the mean value of triplicate results obtained from one panelist. Error bars show the standard deviations and the different letters indicate significant differences (Tukey's test, $p < 0.05$).

was perceived to be as strong as that of the black coffee (**Figure 2.5**).

These results suggested the possibility that the amount of FFT exhaled through the nostrils as FMS during consumption of the coffee is one of the factors that has a significant impact on the different intensity of the coffee-like aroma quality between the black coffee and milk coffee. The in-mouth release of some odorants (No. 5, 28, 29, 32, and 36 in **Table 2.4**) during the consumption of milk coffee was also significantly lower than that of the black coffee in more than one panelist. These odorants could be also presumed to have an impact on the different aroma perceptions between the black coffee and milk coffee. Moreover, the in-mouth releases of trace odorants which could not be detected by R-FISS in the present study, especially thiols that have a characteristic odor and react with milk proteins, are likely also affected by the milk. Their different in-mouth releases might have an impact on the different aroma perceptions between

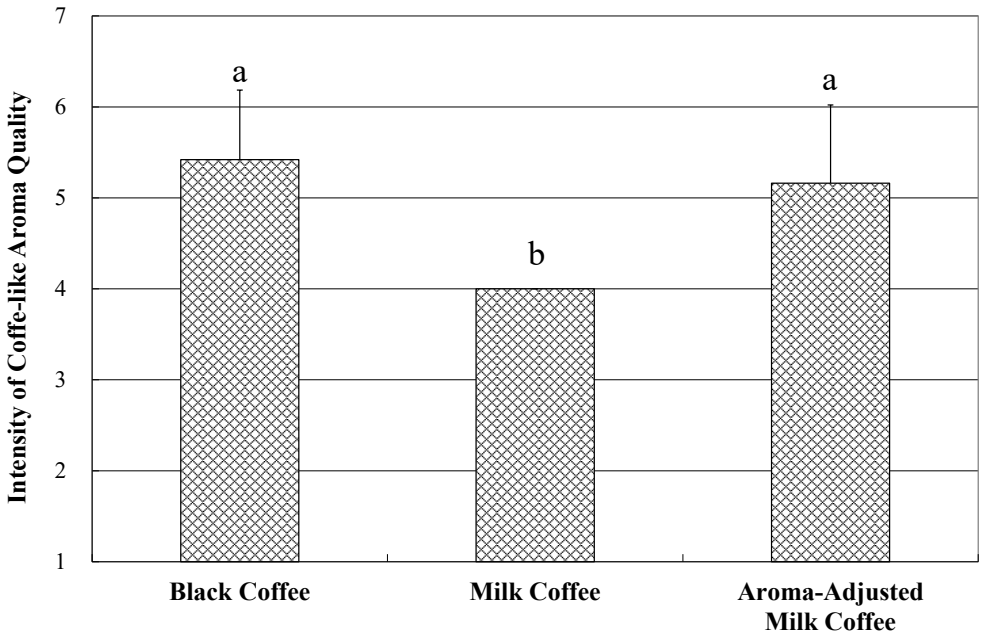


Figure 2.5 Intensity of coffee-like aroma quality during the consumption of black coffee, milk coffee (control, the intensity was defined as 4), and aroma-adjusted milk coffee. Each intensity is the mean value of 31 panelists using a seven-point scale from 1 to 7. Error bars show the standard deviations and the different letters indicate significant differences (Tukey's test, $p < 0.05$).

these coffee beverages. Furthermore, milk could have an influence on the release kinetics of the in-mouth odorants during the consumption of milk coffee. The different aroma composition of retronasal aroma caused by the different release kinetics might result in the different aroma perceptions between the black coffee and milk coffee.

The present results suggested the possibility that the significantly decreased intensity of the coffee-like aroma quality might result from the decreased aroma releases of a few odorants, including FFT, by the addition of milk to the coffee. Moreover, it was inferred that the different aroma release of thiols, especially FFT, would have a significant impact on the different aroma perceptions of the coffee-like aroma quality between the black coffee and milk coffee. An important future challenge will be to understand in more detail the relationship between the influence of milk on the aroma release of coffee and the perception of aromas.

2.3 Taste-aroma Interactions in Lemon-flavored Model Beverages: Influence of Sweeteners on Aroma Perception and In Vivo Aroma Release

2.3.1 Introduction

Flavor is considered to be a multi-modal sense resulting from multiple stimuli elicited by the aroma and taste compounds in foods, and is one of the most important characteristics determining the acceptance of foods by consumers. Recently, the increased consumer demand for healthier foods has resulted in a growing demand for low-calorie beverages with low-calorie sweeteners such as ACK, ASP and sucralose. These artificial sweeteners have been widely used as alternatives to sugar in various beverages. In general, flavor perceptions of beverages can vary by the types of sweeteners. It was previously demonstrated that the intensity of the perceived flavor and overall aroma of beverages differed based on the types of sweeteners used in the beverage bases [22-24]. Previous studies have also reported that the amounts of aroma compounds released into the headspace of the beverages and their behavior varied by the types of sweeteners or their concentration [65-70]. Moreover, some of the studies indicated the possibility that the changes in the aroma perception could result from differences in aroma release from the beverages [65, 66, 70]. These studies, however, investigated the influence of sweeteners on the in vitro aroma release from beverages using a model system such as a model mouth, etc., but the influence on the in vivo aroma release requires further explanation. Study of the influence of sweeteners on the in vivo aroma release from beverages would assist in understanding the relationship between beverage composition and the perception of aromas. Therefore, the aim of this study was to investigate the relationship between the influence of sweeteners on the in vivo aroma release from beverages and their aroma perception by comparing the amounts of the in-mouth odorants exhaled through the nostrils during the consumption of lemon-flavored model beverages containing different sweeteners.

2.3.2 Materials and Methods

Chemicals. The following chemicals were purchased from the suppliers shown: 11 odorants shown in **Table 2.6** (Tokyo Chemical Industry Co., Ltd.); 2-octanol and citric acid (Nacalai Tesque, Inc.); sucrose, ACK, ASP, sucralose (Wako Pure Chemical Industries, Ltd.). The chemical purity of 11 odorants shown in **Table 2.6** and 2-octanol was confirmed by GC-FID and GC-MS.

Preparation of the Model Lemon Flavor. The aroma composition of the model lemon

Table 2.6 Concentrations (Conc.), selected ions and response factors of odorants in the model lemon flavor for mass chromatography in the SIM mode.

Compound	Conc. in ethanol (% w/w)	Selected ion (m/z)[a]		Response factor[b]
		cal.	ref.	
α-pinene	0.20	93	91	1.3
camphene	0.10	121	93	3.0
myrcene	0.10	69	93	1.7
γ-terpinene	0.80	136	121	3.3
terpinolene	0.20	121	93	2.1
neral	0.40[c]	109	69	16.2
geranial		152	109	34.5
neryl acetate	0.40	136	154	51.8
geranyl acetate	0.20	93	136	16.1
nerol	0.20	69	93	13.4
geraniol	0.40	69	136	8.3

[a] cal.; quantifier ion, ref.; qualifier ion.

[b] calculated from the ratio of the selected ion peak area of each odorant to 2-octanol (IS material) obtained by mass chromatography of a standard solution containing equal weights of odorants and 2-octanol.

[c] Neral and geranial were mixed in the model lemon flavor as citral.

flavor is shown in **Table 2.6**. Eleven odorants were dissolved in 99.5% ethanol.

Preparation of the Lemon-flavored Model Beverages. The experimental design for the lemon-flavored model beverages is shown in **Table 2.7**. The model beverages were prepared so that the sweetness level of each model beverage containing the artificial sweeteners (beverages 2-4) was about the same as that of the beverage containing 4% sucrose (beverage 1). The equi-sweetness was determined by the sweetness power function for each sweetener based on experimental results from previous papers [71, 72]. Their sweetness levels were then confirmed by a sensory evaluation.

First, the artificial sweeteners and citric acid were completely dissolved in distilled water at room temperature. The model lemon flavor was then added to each solution and mixed thoroughly in a sealed glass flask. The model beverages were immediately used (within 2 hours at the most) without sterilization after addition of the flavor.

Sensory Evaluation of the Model Beverages by QDA. Fifty grams of the model beverages was weighed into plastic cups, and then each cup was immediately capped with a plastic cap. These 4 model beverages were evaluated by 10 panelists (5 males and 5 females) employed by Ogawa & Co., Ltd. All the panelists had previously received extensive training in the descriptive sensory analysis of citrus-flavored beverages and had experience in the sensory profiling of various food samples. First, the panelists generated 17 aroma attributes by comparing the aroma profiles of each model

Table 2.7 Experimental design for lemon-flavored model beverages.

Beverage number	Sucrose (% w/w)	Acesulfame K (ppm)	Aspartame (ppm)	Sucralose (ppm)	Citric acid (% w/w)	Lemon flavor (% v/v)
1	4.0	0	0	0	0.1	0.1
2	0	200[a]	0	0	0.1	0.1
3	0	0	200[a]	0	0.1	0.1
4	0	0	0	60[a]	0.1	0.1

[a] determined by the sweetness power function based on experimental results from previous papers so that the sweetness level of each model beverage was about the same as beverage 1.

beverage. These attributes were then ranked and clustered to determine the key attributes of the samples. Five key attributes were ultimately selected, and their definitions were confirmed by the panelists. Next, the 4 samples (beverages 1-4) were randomly presented to the panelists. Beverage 1 was also used as a control and was presented to panelists in addition to the 4 samples. The intensity of each attribute for the control was defined as 4. Ion exchanged water was provided for rinsing of the mouth. The panelists scored the intensity of each aroma quality for the 4 model beverages using a seven-point scale from 1 (very weak) to 7 (very strong) compared to that for the control. All samples were evaluated 3 times by each panelist. The sensory results were then analyzed using a one-way ANOVA followed by Tukey's test.

Trapping of In-mouth Odorants Exhaled through the Nostrils. In order to determine the in-mouth odorants, the breath exhaled through the nostrils was trapped according to the procedure described in a previous section (see 2.2.2 Materials and Methods). After 30 mL of the model beverage at room temperature was placed in the mouth cavity, the panelists paused for 1-2 seconds, and then swallowed all of the liquid in one gulp. Ten breaths after the beverage had been swallowed were passed through a small glass column (6 cm × 5 mm i.d.) filled with 100 mg of Tenax TA (80/100 mesh, GL Science), which had been heated at 220°C for 2 hours prior to the analysis. The end of the glass column was connected to a pump by a silicon tube, and a suction of approximately 1 L/min was applied to the system during trapping of the air exhaled through the nostrils. This sampling system allowed the panelists to normally exhale without the need to press air through the Tenax column. After trapping of the in-mouth odorants, five microliters of an IS solution (5 μg/mL 2-octanol in ethanol) was directly added to the Tenax column for the quantitative analysis. The water and ethanol were then removed from the Tenax TA by flowing dry nitrogen (30 min, 100 mL/min). These experiments were carried out at room temperature (25 ± 2°C). The experiments were performed by 4 trained panelists, and the Tenax columns were used as the samples for the GC-MS analysis. A blank test was also performed under the same conditions using distilled water.

GC-MS. Thermal desorption of the trapped odorants on the Tenax column was performed with a TDU thermal desorption system (Gerstel GmbH) in combination with

the ATEX option of an MPS2 autosampler and a CIS4 injector (Gerstel GmbH) according to the following parameters. Thermal desorption was performed by programming the TDU from 20°C to 280°C (held for 3 min) at the rate of 12°C/s in the splitless mode. Cryofocusing was performed with liquid nitrogen at -150°C. Injection was performed with the ramp of 12°C/s from -150°C to 260°C (held for 3 min) in the splitless mode. The odorants were analyzed by an Agilent 6890N GC with an Agilent 5975 B series MS (Agilent Technologies). The column was a 30 m × 0.25 mm i.d. DB-WAX fused silica capillary (J&W Scientific) with a film thickness of 0.25 μm. The oven temperature was programmed from 30°C (held for 3 min) to 120°C at the rate of 3°C/min, and then raised at the rate of 5°C/min to 230°C. The flow rate of the helium carrier gas was 1 mL/min. The mass spectrometer was used at an ionization voltage of 70 eV (EI) and operated in the SIM-SCAN mode. The ion source and quadrupole temperatures were set at 230°C and 150°C, respectively. The selected ions listed in **Table 2.6** and 45 for 2-octanol were monitored in the SIM mode. The quantitative amounts of the in-mouth odorants were determined by the internal standard method using an RF. The RFs of 11 odorants in the model lemon flavor to 2-octanol (IS material) are shown in **Table 2.6**, and these RFs were calculated from the ratio of the selected ion peak area of each odorant to 2-octanol obtained by mass chromatography of a standard solution containing equal weights of odorants and 2-octanol. These selected ion peak areas were the mean values of triplicate results. The odorants were then quantitated as in-mouth odorants. The results were then analyzed using a one-way ANOVA followed by Tukey's test.

Identification of the odorants. Each odorant was identified by comparing its Kovats GC RI and mass spectrum to those of the reference compounds.

2.3.3 Results and Discussion

Influence of Sweeteners on Aroma Perceptions during Consumption of the Lemon-flavored Model Beverages. In order to investigate the influence of the

sweeteners on aroma perceptions during consumption of the beverages, the aroma profiles of the lemon-flavored model beverages (**Table 2.7**, beverages 1-4), which had equi-sweet levels and the same aroma content, were compared by sensory evaluation using QDA. As a result, the intensity of the lemon-like aroma of beverage 2 was significantly weaker, while the intensity of the green aroma of beverage 4 and the intensity of the spicy aroma of beverages 3 and 4 were significantly stronger than the other beverages (**Figure 2.6**). It was previously reported that the intensity of the perceived flavor changed according to the types of sweeteners and their concentrations in the citrus-flavored model beverages [24]. The present results demonstrated that the aroma quality and flavor intensity of the beverages was perceived to differ due to the types of sweeteners.

These results suggested that differences in aroma perceptions according to the types

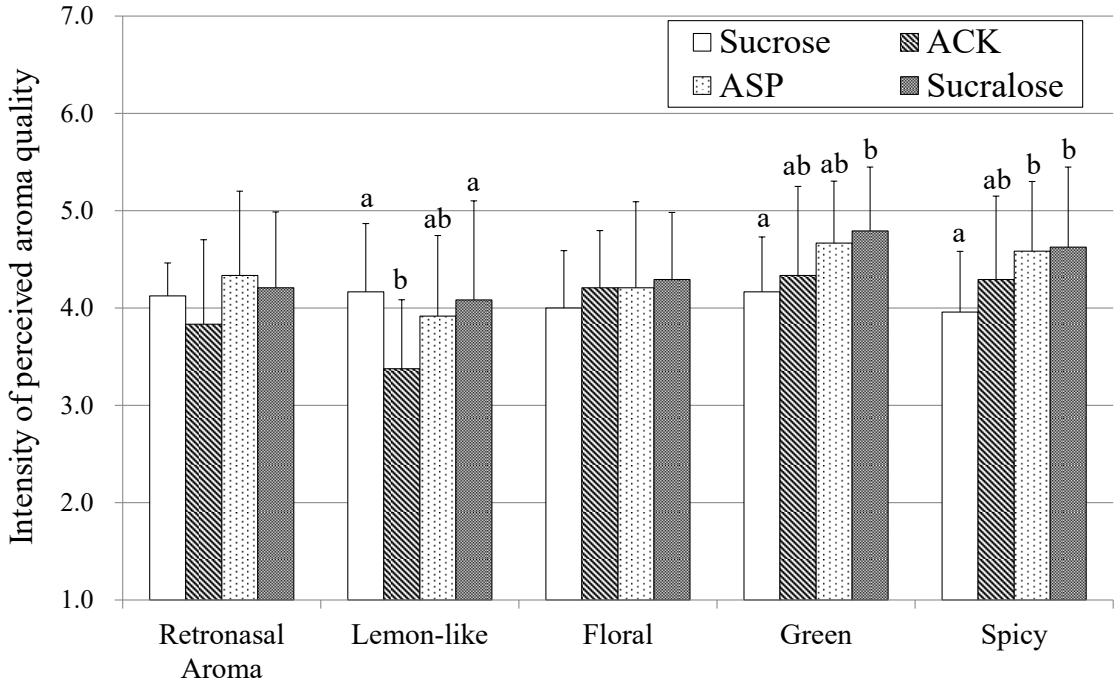


Figure 2.6 Intensity of perceived aroma quality during the consumption of the lemon-flavored model beverages. Each intensity is the mean value of 10 panelists using a seven-point scale from 1 to 7. Error bars show the standard deviations. The different letters indicate significant differences (Tukey's test, $p < 0.05$).

of sweeteners in the model beverages resulted from factors other than the sweetness level, sourness level and aroma content in the beverages, since, aside from the types of sweeteners and their concentrations, each model beverage consisted of the same ingredients and composition. As previously mentioned, it has been shown that the aroma release from beverages varied by the types of sweeteners and their concentrations [65-70], and differences in aroma release could affect the aroma perceptions of the beverages [65, 66, 70]. Therefore, it is possible that differences in aroma perceptions of the model beverages could be the result of changes in aroma release, due to the concentrations of the sweeteners or the physicochemical interactions between the ingredients in the beverage base and the odorants.

Influence of Sweeteners on the In Vivo Aroma Release during Consumption of the Model Beverages. In order to investigate in detail the influence of the sweeteners on the in vivo aroma release, the amounts of odorants exhaled through the nostrils during the consumption of the model beverages were compared by the 4 trained panelists using R-FISS. As a result, the amounts of in-mouth odorants showed individual differences (**Table 2.8**). Previous studies have already indicated that the chemical structure of some odorants can be changed by the enzymatic activities of human saliva in the mouth [19, 20]. Based on these results, differences in the in vivo aroma release among panelists might result from differences in the oral cavity, volume and composition of the saliva, or swallowing activity. Thus, the in vivo aroma release showed individual differences, but there were no in-mouth odorants that showed significant differences in their amounts common to the 4 panelists (**Table 2.8**). Therefore, it was found that the types of sweeteners or their concentrations likely had only a small impact on the in vivo aroma release during the consumption of the model beverages, whereas their aroma perceptions significantly differed in the sensory evaluation.

Although previous studies reported that the aroma release from beverages varied by the types of sweeteners and their concentrations [65-70], it is also possible that these changes are not noticeable within the range of the sweetener concentration generally used for beverages [65, 68]. Therefore, sweeteners might have a small impact on the in vivo aroma release due to the low concentrations of the sweeteners in the model

Table 2.8 Quantitative amounts[a] of in-mouth odorants exhaled through the nostrils during the consumption of the lemon-flavored model beverages.

Compound	Panelist 1				Panelist 2			
	Amount (ng)				Amount (ng)			
	Sucrose	ACK	ASP	Sucralose	Sucrose	ACK	ASP	Sucralose
α-pinene	17 ± 3.9	22 ± 8.9	13 ± 4.1	24 ± 6.6	32 ± 7.1	21 ± 3.5	28 ± 10.1	23 ± 5.7
camphene	11 ± 2.2	13 ± 5.2	8 ± 2.6	15 ± 4.1	18 ± 4.4	12 ± 2.0	15 ± 6.2	13 ± 3.3
myrcene	23 ± 4.2	24 ± 10.9	24 ± 4.6	25 ± 5.7	33 ± 7.5	34 ± 8.1	31 ± 8.6	27 ± 7.5
γ-terpinene	189 ± 33.6	237 ± 69.5	192 ± 33.2	208 ± 47.6	286 ± 77.7	276 ± 62.4	251 ± 72.3	234 ± 69.9
terpinolene	46 ± 9.5	60 ± 17.7	46 ± 8.8	47 ± 9.9	73 ± 22.3	57 ± 20.4	64 ± 20.5	43 ± 15.1
neral	20 ± 1.5	18 ± 1.9	17 ± 2.6	18 ± 1.8	16 ± 2.3 a	8 ± 1.9 b	12 ± 2.0 ab	10 ± 2.4 ab
geranial	21 ± 1.9	17 ± 1.8	18 ± 1.7	19 ± 2.3	16 ± 2.0 a	9 ± 1.6 b	13 ± 2.4 ab	10 ± 2.4 b
neryl acetate	81 ± 8.8	79 ± 8.8	66 ± 19.1	79 ± 12.8	144 ± 64.9	67 ± 47.0	105 ± 31.2	110 ± 37.6
geranyl acetate	21 ± 1.2	19 ± 1.8	16 ± 4.0	19 ± 2.3	35 ± 14.8	16 ± 10.5	26 ± 7.6	25 ± 9.0
nerol	46 ± 12.4	35 ± 8.5	30 ± 13.3	34 ± 8.2	29 ± 6.7	8 ± 4.4	23 ± 3.6	23 ± 4.6
geraniol	37 ± 9.0	39 ± 9.9	25 ± 5.6	37 ± 4.3	41 ± 5.8	16 ± 5.6	34 ± 5.5	30 ± 3.8

[a] Each amount is shown as the mean value ± standard deviation of four-fold results obtained from each identical panelist. Values followed by different letters are significantly different (Tukey's test, $p < 0.05$).

Table 2.8 Continued.

Compound	Panelist 3				Panelist 4			
	Amount (ng)				Amount (ng)			
	Sucrose	ACK	ASP	Sucralose	Sucrose	ACK	ASP	Sucralose
α-pinene	15 ± 3.6 ab	12 ± 1.7 a	20 ± 2.8 b	18 ± 4.7 ab	16 ± 3.3	15 ± 5.0	11 ± 4.6	13 ± 4.5
camphene	10 ± 2.3 ab	7 ± 1.1 a	12 ± 1.6 b	11 ± 3.0 b	9 ± 2.0	9 ± 2.7	7 ± 2.7	7 ± 2.8
myrcene	25 ± 3.0 a	28 ± 3.6 ab	37 ± 4.8 b	32 ± 8.6 ab	13 ± 3.9	12 ± 4.8	10 ± 4.1	10 ± 4.5
γ-terpinene	216 ± 34.1	241 ± 40.8	298 ± 51.3	251 ± 66.9	132 ± 27.8	120 ± 61.7	95 ± 38.1	97 ± 50.5
terpinolene	52 ± 7.8	66 ± 10.1	80 ± 18.6	60 ± 16.0	31 ± 5.9	28 ± 16.2	21 ± 9.7	21 ± 11.8
neral	21 ± 0.8	21 ± 5.0	16 ± 1.5	17 ± 1.5	7 ± 1.2 a	5 ± 0.5 ab	4 ± 0.9 b	6 ± 1.4 ab
geranial	21 ± 0.8	21 ± 4.1	18 ± 1.7	18 ± 1.6	7 ± 1.2	5 ± 0.3	5 ± 1.3	6 ± 1.1
neryl acetate	126 ± 24.1	149 ± 57.6	91 ± 37.0	108 ± 29.5	63 ± 20.5 a	30 ± 7.7 b	22 ± 10.1 b	32 ± 13.0 b
geranyl acetate	30 ± 5.2	35 ± 13.9	24 ± 7.5	30 ± 7.1	14 ± 4.3 a	7 ± 2.5 b	5 ± 2.0 b	8 ± 2.9 ab
nerol	38 ± 4.5	34 ± 9.0	27 ± 3.5	33 ± 5.4	23 ± 3.1 a	13 ± 2.3 b	11 ± 3.8 b	19 ± 7.3 ab
geraniol	35 ± 11.2	46 ± 10.9	30 ± 4.7	33 ± 4.2	24 ± 3.4 a	9 ± 1.4 b	10 ± 5.8 b	13 ± 5.7 b

[a] Each amount is shown as the mean value ± standard deviation of four-fold results obtained from each identical panelist. Values followed by different letters are significantly different (Tukey's test, $p < 0.05$).

beverages used in this study.

It was also reported that each sweetener has a different taste profile [72, 73]. ACK has a sweet taste, accompanied by a relatively stronger bitter taste than other sweeteners [72, 73]. ASP and sucralose have a relatively stronger sweet aftertaste than sucrose [73] in addition to the sweet taste quality that is similar to sucrose. Therefore, the bitter taste might have an inhibitory effect on the perceived lemon-like aroma, and the sweet aftertaste might have an enhancing effect on the perceived green/spicy aroma. Previous studies have already indicated the possibility that the multi-modal interactions between multiple stimuli elicited by the aroma and taste compounds (taste-aroma interactions) in foods could change their perceived aroma intensities [74-76]. Considering the present results, it is highly likely that the taste-aroma interactions caused by the aroma and taste compounds would have a significant impact on differences in the aroma perceptions of the beverages containing different sweeteners.

The present results suggested that differences in aroma perceptions according to the types of sweeteners in the model beverages would result from factors other than the amounts of in-mouth odorants exhaled from the human nose. Furthermore, it was inferred that the taste-aroma interactions could be one of the factors having a significant impact on the different aroma perceptions of the beverages containing different sweeteners. Sweeteners could, however, have an influence on the release kinetics of the in-mouth odorants during the consumption of the beverages, and consequently, change the composition of the retronasal aroma. Changes in the aroma composition due to the sweeteners may result in different aroma perceptions of the beverages. A future important challenge will be to understand the relationship between the taste compounds including sweeteners, in vivo aroma release, and their aroma perceptions resulting from multiple stimuli elicited by the aroma and taste compounds.

2.4 Conclusion

In this chapter, the influence of the compositions of the aroma and base components in beverages on the flavor release was investigated by applying R-FISS to beverages. The relationship between the different flavor release resulting from the influences and their flavor perception was revealed.

The different compositions of the aroma and base components would specifically change the chemical structures and the amounts of the in-mouth odorants exhaled through the nostrils during drinking beverages depending on the chemical structure of each odorant. It was indicated that these different flavor releases could result in different flavor perceptions. Meanwhile, milk and the types of sweeteners had a small impact on the flavor release of most odorants. These results also suggested that the differences in the aroma perceptions according to the compositions of the base components in beverages would result from factors other than the flavor release.

It was demonstrated that the chemical properties in addition to the physical properties of odorants have a significant impact on the flavor release from beverages. Therefore, in order to understand in detail the flavor perception of beverages, the possibility that the aroma might be perceived due to the stimuli induced by multiple compounds resulting from the compositional changes in the odorants during drinking beverages need to be taken into consideration. Moreover, it will be necessary to reveal the influence of the taste-aroma interactions caused by the aroma and taste compounds on the flavor perceptions in addition to the different flavor release according to the compositions of the base components. These findings will be useful to understand the relationship between the flavor release from beverages and their flavor perceptions. Furthermore, they can contribute to the development of beverage products having an appropriate flavor release for each composition of the base components and a pleasant flavor, and to the quality improvement of the beverage products.

Summary

Chapter 1

1.1 A New Approach to Estimate the In-mouth Release Characteristics of Odorants in Chewing Gum

R-FISS was developed as a new technique to analyze the in-mouth odorants exhaled from the human nose during the consumption of foods. By applying the R-FISS to chewing gum, the in-mouth release characteristics of odorants in chewing gum during chewing were indicated by the ratio of the peak area values of the in-mouth odorants for 10 minutes chewing to those for 1 minutes chewing. In addition, a good correlation coefficient was found between the peak area ratios of odorants in the model chewing gum obtained by R-FISS and their RI values on a polar stationary phase GC column (DB-WAX). Therefore, the in-mouth release characteristics of odorants in chewing gum can be predicted by the RI values on the DB-WAX column. Moreover, these results suggested that the vapor pressure and hydrophobicity/hydrophilicity are the key factors determining the in-mouth release of odorants in chewing gum.

1.2 A New Approach to Estimate the Elution Characteristics of Odorants in Chewing Gum during Chewing

A new approach was developed to estimate the elution characteristics of odorants from chewing gum into saliva during chewing using a chewing apparatus. The odorants eluted from the model chewing gum were analyzed by GC-FID using the SPE method and the HS-SPME method. By applying this approach to a model chewing gum, it was found that the quantitative data obtained from each analytical method are in proportion and that the measured value obtained from HS-SPME, which is easy to operate, could

convert the quantitative data obtained from the adsorptive column method. Therefore, it was demonstrated that the amounts of odorants eluted from chewing gum and their release curves could be easily determined by a combination of the two methods. In addition, it was recognized that there was a good overall regression coefficient between the elution ratio of the odorants in the model chewing gum after a 20-min chewing run and the difference between the RI values on the polar and apolar stationary phase of GC columns (ΔI). Therefore, ΔI is the most important factor for predicting the elution ratio of odorants in chewing gum during chewing.

Chapter 2

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

By applying 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 (containing the original thiol) had been swallowed. In addition, this phenomenon was also observed in other volatile thiols (FFT, 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.

2.2 Influence of Milk on Aroma Release and Aroma Perception during Consumption of Coffee Beverages

The amounts of in-mouth odorants exhaled through the nostrils during the consumption of black coffee and milk coffee were compared using R-FISS by 4 trained panelists. As a result, it was found that the amounts of most in-mouth odorants including the potent odorants in brewed coffee did not significantly differ between the black coffee and milk coffee. However, the amounts of FMS, difurylmethane, and furfuryl pyrrole exhaled through the nostrils during the consumption of milk coffee were significantly lower than those of the black coffee regardless of the panelist. It has been previously indicated that FMS could result from the methylation of FFT, one of the most important potent odorants in brewed coffee. Based on these results, FFT and FMS were added to milk coffee so that the amount of FMS exhaled through the nostrils was about the same as that of black coffee, resulting in improved intensity of the coffee-like aroma quality for the milk coffee. The present results suggested that the significantly decreased intensity of the coffee-like aroma quality might result from decreased aroma release of a few odorants, including FFT, by the addition of milk to the coffee. Moreover, it was inferred that the difference in aroma release of FFT would have an especially significant impact on the perception of coffee-like aroma quality between the black coffee and milk coffee.

2.3 Taste-aroma Interactions in Lemon-flavored Model Beverages: Influence of Sweeteners on Aroma Perception and In Vivo Aroma Release

Equi-sweet and lemon-flavored model beverages were prepared using sucrose, ACK, ASP and sucralose. The amounts of in-mouth odorants exhaled through the nostrils

during the consumption of the model beverages were compared using R-FISS by 4 trained panelists, and the relationship between the in vivo aroma release and their aroma perceptions were evaluated. As a result of the sensory evaluation by QDA, the lemon-like aroma of the beverage containing ACK was perceived significantly weaker, while the green aroma of the beverage containing sucralose and the spicy aroma of the beverages containing ASP and sucralose were perceived significantly stronger than the other beverages. In contrast, there were no in-mouth odorants which showed significant differences in their amounts common to the 4 panelists. Therefore, it was found that the sweeteners could have a small impact on the in vivo aroma release from the model beverages. These results suggested that the different aroma quality of the model beverages by the types of sweeteners would result from some factors other than the in vivo aroma release. Furthermore, it is highly likely that the taste-aroma interactions caused by the aroma and taste compounds would be one of the factors having a significant impact on the different aroma perceptions of the beverages containing the different sweeteners.

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List of Publications

Chapter 1

1.1 Kumazawa, K., Itobe, T., Nishimura, O., and Hamaguchi, T. (2008). A New Approach to Estimate the In-mouth Release Characteristics of Odorants in Chewing Gum. *Food Sci. Technol. Res.*, **14**, 269-276. Copyright © 2008, Japanese Society for Food Science and Technology

1.2 Itobe, T., Kumazawa, K., Inagaki, S., and Nishimura, O. (2012). A New Approach to Estimate the Elution Characteristics of Odorants in Chewing Gum during Chewing. *Food Sci. Technol. Res.*, **18**, 295-302. Copyright © 2012, Japanese Society for Food Science and Technology

Chapter 2

2.1 Itobe, T., Kumazawa, K., and Nishimura, O. (2009). New Factor Characterizing the In-Mouth Release of Odorants (Volatile Thiols): Compositional Changes in Odorants Exhaled from the Human Nose during Drinking. *J. Agric. Food Chem.*, **57**, 11297-11301. Copyright © 2009 American Chemical Society
http://pubs.acs.org/doi/abs/10.1021/jf902239g

2.2 Itobe, T., Nishimura, O., and Kumazawa, K. (2015). Influence of Milk on Aroma Release and Aroma Perception during Consumption of Coffee Beverages. *Food Sci. Technol. Res.*, **21**, 607-614. Copyright © 2015, Japanese Society for Food Science and Technology

2.3 Itobe, T., and Kumazawa, K. (2017). Taste-aroma Interactions in Lemon-flavored Model Beverages: Influence of Sweeteners on Aroma Perception and In Vivo Aroma Release. *Food Sci. Technol. Res.*, (in press).

List of Conference Presentations

Itobe, T., and Kumazawa, K. (2015). A New Approach to Investigate the Flavor Release from Different Beverage Compositions: Influence of Milk on Aroma Release during Consumption of Coffee Beverages. *The 2015 International Chemical Congress of Pacific Basin Societies (PACIFICHEM 2015)*: Honolulu, Hawaii, USA.

Acknowledgements

The author is deeply grateful to Dr. Yasuki Matsumura, Professor of Laboratory of Quality Analysis and Assessment, Graduate School of Agriculture, Kyoto University, for his continuous guidance, kind advices and many useful suggestions to prepare this manuscript.

The author would like to express his sincere gratitude to Dr. Kenji Kumazawa (Ogawa & Co., Ltd.) and Dr. Osamu Nishimura (Ogawa & Co., Ltd.) for their valuable discussion and advices, generous support and continuous encouragement throughout this study.

The author would acknowledge Mr. Yutaka Ogawa (Company President of Ogawa & Co., Ltd.), Mr. Keisuke Sakuda (Director of Ogawa & Co., Ltd.), Mr. Junya Tennojiya (Director of Ogawa & Co., Ltd.), and Mr. Wataru Kameda (Ex-Director of Ogawa & Co., Ltd.) for providing the opportunity to conduct this study. Furthermore, the author thanks all company members of Ogawa & Co., Ltd., especially staff of Analytical Laboratory for their useful discussion and continuous encouragement on this study.

Finally, the author sincerely thanks his wife, parents and children for their kind support and continuous encouragement.

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