Enantiomeric Analysis of Flavor Compounds by Multiple Headspace Solid-Phase Microextraction Gas Chromatography-Mass Spectrometry

Koichi SAITO*, Yukino OSHIRO, Osamu SAKATA, Rie ITO

Department of Analytical Chemistry, Faculty of Pharmaceutical Sciences, Hoshi University, 2-4-41, Ebara, Shinagawa-ku, Tokyo 142-8501, Japan

Abstract
A quality assessment method for eight commercially available optically active flavor compounds: menthol, menthyl acetate, perillaldehyde, 1,8-cineole, α-pinene, limonene, neomenthol, and neomenthyl acetate, was developed for commercial foods. The simultaneous determination of the eight flavor compounds and the optical purity test for the complete enantiomeric separation of each flavor compound were achieved by gas chromatography-mass spectrometry (GC/MS) equipped with a tandem capillary column consisting of a fused silica column (DB-17MS) coupled with a cyclodextrin chiral column (β-DEX). The extraction of the flavor compounds from the food samples was carried out by a multiple headspace (MHS) solid-phase microextraction (SPME) technique in order to improve the quantitativeness of SPME. Optimization of MHS-SPME using a mathematical technique with repeated extraction yielded the total peak area of each flavor compound and excellent recoveries from the food samples in three repeated measurements. Of the twelve commercial samples subjected to the optical purity test, some were found to have undesirable enantiomers that are not designated as flavor compounds by the Food Hygiene Law of Japan. The results show the efficiency of the developed method, and suggest a need to conduct further optical purity tests for the quality assessment of flavor compounds in commercial foods.

Keywords: Flavor compound; Enantiomeric analysis; Multiple headspace solid-phase microextraction; GC/MS

1. Introduction
Flavor compounds used as food additives are often derived from natural products, and thus there is less concern about their safety compared to other food additives, such as artificial preservatives and sweeteners. Moreover, international regulations for flavor compounds have not yet been drawn up. In recent years, there have introduced the Positive List System for flavor compounds in the EU. In Japan as well, the importance of quality assurance of flavor compounds has been increasingly recognized. However, there is one problem regarding quality assurance, that is, the purity of flavor compounds is not known. As an example, impurities generated in the process of synthesizing and purifying flavor compounds cause flavor deterioration and produce an offensive odor [1].

To ensure food hygiene, it is imperative to measure the purity of flavor compounds. However, the Food Hygiene Law of Japan does not require indication of the content and purity of flavor compounds on food labels. Because of this, the content and purity of flavor compounds present in commercial foods is unclear. To determine accurately the content of flavor compounds, the direct analysis of foods is warranted. However, flavor compounds are generally volatile, and a decrease in sensitivity for the target compounds sometimes occurs due to the so-called matrix effect of food samples. For this reason, a pretreatment procedure, such as sample cleanup, is necessary. However, cumbersome and tedious cleanup creates another problem in that the amount of flavor compound necessary for measurement cannot be recovered on account of its high volatility [2].

Conventionally, the purge and trap method [3], the liquid-liquid extraction method [4], and the solid-phase extraction method [5] are used for the pretreatment of flavor compounds. However, these methods have several problems...
and deficiencies, such as sensitivity, reproducibility, and cost performance [6]. In order to overcome these problems, a headspace solid-phase microextraction (HS-SPME) method was introduced in the early 90s by Zhang and Pawliszyn [7]. The HS-SPME method is widely applied in food and environmental analyses because it is a simple, rapid, solvent-free sample preparation technique that realizes sampling, cleanup, and concentration in one step. The HS-SPME method has been applied to volatile constituents in foods and flavor compounds and so on [8-11].

On the other hand, there are also reports that chiral analysis is necessary for the quality evaluation of flavor compounds [12,13]. This is because the effect of flavor enantiomers on fragrance cannot be ignored, that is, fragrance differs depending on the enantiomer, and the threshold for the perception of fragrance also differs depending on the enantiomer [14]. For instance, d-limonene ((R)-enantiomer) is used as a food additive because of its orange flavor, whereas l-limonene ((S)-enantiomer) smells of fresh pine needles. Generally, the aroma of the (R)-form is more intense than that of the (S)-form [15]. Therefore, the influence of an enantiomer of a flavor compound cannot be ignored. Such regulatory authorities as the U.S. Food and Drug Administration require pharmaceutical manufacturers to specify the enantiomeric purity of all optically active compounds prior to marketing, as well as to provide information on the activity and/or toxicity of both enantiomers and racemates [16]. Therefore, it is important to study the enantiomeric resolution of chiral flavor compounds, and to develop precise methods for the determination of enantiomeric purities up to enantiomeric excess (ee) for reference standards.

In our previous work [17], we developed an evaluation method of reagent purity and ee for standard compounds, and reported the usefulness of the method. In the previous work, a quality assessment of foods containing flavor compounds was performed by analyzing the amount of flavor compounds and the enantiomeric purity of flavor compounds that are added to commercial foods.

Among the flavor compounds designated by the Ministry of Health, Labor and Welfare as optically active compounds, eight compounds, namely, menthol, methyl acetate, perillaldehyde, 1,8-cineole, α-pinene, limonene (α-pinene and limonene are impurities in 1,8-cineole), neomethyl (a structural isomer of menthol), and neomenthol acetate (a structural isomer of menthyl acetate), were selected as substances to be measured (Fig. 1). We attempted to construct an enantiomeric purity evaluation method for flavor compounds in commercial foods using the SPME-GC/MS method.

The conventional HS-SPME method has the following drawbacks: it is susceptible to disturbance by sample matrices and has inferior quantitativeness, even if it is a HS extraction or a liquid-phase extraction method. Because quantification is indispensable for the evaluation of the optical purity of flavor compounds, in the present study, quantitative accuracy was improved by measuring the same sample vial many times with the HS method, and the so-called multiple headspace (MHS)-SPME-GC/MS method [18-20] was examined.

Fig. 1. Chemical structures of eight flavor compounds.

2. Principle of MHS-SPME

MHS-SPME is a stepwise HS method that is useful for the quantitative analyses of volatile and semivolatile compounds in complex liquid samples. It can be used as an alternative to other quantification methods to avoid possible matrix effects [18-20]. If carried out until exhaustive extraction in a single extraction as a conventional HS-SPME, the various compounds derived from matrices may also be accumulated and affect the phase equilibrium, which resulted in matrix effect. MHS-SPME in a non-equilibrium condition enables estimation of the total peak area corresponding to the complete extraction of the analyte, by performing several consecutive extractions from the same sample. Therefore, the MHS-SPME has a potential to minimize possible matrix effects. The principle of MHS-SPME is as follows: The analyte total peak area (A_t) corresponding to the cumulative extraction yield after multiple extractions at a specific time is the sum of the peak areas obtained from individual extractions when the extractions are exhaustive. The outline is shown in Fig. 2.

The amount of analyte extracted by the fiber is proportional to the initial amount, and the peak area decays exponentially with the number of extractions. The total peak area can be estimated by performing three or four successive extractions by HS-SPME.

The peak area (A_i) measured by the HS-SPME, which is based on the amount extracted by the fiber before equilibration (the amount that adsorbed to the i-th fiber) can...
be approximated by the following equation: \( A_1 \) is the peak area obtained after the first extraction, and \( \beta \) is a constant calculated from the slope of the regression line in Fig. 2.

\[
A_i = A_1 \beta^{i-1} \quad \ldots \quad (Eq. 1)
\]

The \( A_T \) obtained by successively repeating this extraction operation is shown in Eq. 2.

\[
A_T = \sum_{i=1}^{N} A_i = \frac{A_1}{1 - \beta} \quad \ldots \quad (Eq. 2)
\]

Here, when Eq. 1 is converted into a logarithmic expression, linear function Eq. 3 with \( \ln A_i \) and \( (i - 1) \) as variables is obtained.

\[
\ln A_i = (i - 1) \ln \beta + \ln A_1 \quad \ldots \quad (Eq. 3)
\]

When three or more measurements with the MHS-SPME method are conducted and \( \ln A_i \) is plotted on the Y axis and \( (i - 1) \), on the X axis, \( \beta \) is calculated from the slope of the regression line in Fig. 2, and \( A_1 \) is determined from the intercept. Next, substituting each value into Eq. 2 makes it possible to calculate the \( A_T \). In this way, the total peak area corresponding to complete extraction can be calculated through a mathematical equation when the extraction is not exhaustive.

![Fig. 2. Calculation of total peak area measured by MHS-SPME GC/MS. The same sample is measured sequentially three times. (A) is an overlay chromatogram of each extraction. (B) is a regression line of logarithm of peak area versus extraction times obtained from (A).](image)

MHS-SPME can be used in a non-equilibrium condition, the matrix effect can be avoided, and calibration can be easily performed using aqueous solutions. In this case, the distribution constants of the analyte between the fiber and the sample and between the HS and the sample, and the volumes of the three phases, namely, sample, HS, and fiber, must be constant. The other parameters that influence SPME (sample agitation, exposure time, etc.) must also be constant in each extraction.

### 3. Experimental

#### 3.1. Materials and reagents

Menthol, methyl acetate, neomenthol, neomenthyl acetate, perillaldehyde, 1,8-cineole, α-pinene, and limonene, available commercially in enantiomer and/or racemate forms, were selected for this study. The chemical standards of these enantiomer and racemate forms were purchased from Sigma-Aldrich Japan K.K. (Tokyo, Japan), Fluka Chemie GmbH (Buchs, Switzerland), Kanto Chemical Co., Inc. (Tokyo, Japan), FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan), and Tokyo Chemical Industry Co., Ltd (Tokyo, Japan). Most of the organic solvents, including n-hexane, acetone, isopropanol, and ethanol, were of pesticide analysis grade and obtained from Kanto Chemical or FUJIFILM Wako Pure Chemical Corporation. Water was purified with a Milli-Q Gradient A10 system equipped with an EDS-PAK® polisher (Merck Ltd., Tokyo, Japan). All other chemicals were of special grade.

#### 3.2. Preparation of standard solution

Stock solutions at 1 mg/mL were prepared in acetone for all flavor compounds tested in the present study. Working standard solutions for method validation, such as GC calibration and recovery study, were prepared by diluting the stock solution with acetone to the appropriate concentrations.

#### 3.3. Apparatus and operating conditions

The GC/MS instrument used was an Agilent GC 5980 Series II Plus gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a Model 5971 mass spectrometer (Agilent Technologies). The column was a tandem combination capillary column of a DB-17MS (30 m × 0.25 mm i.d., 0.25 μm film thickness; Agilent Technologies) and a β-DEX 120 (30 m × 0.25 mm i.d., 0.25 μm film thickness; Supelco, Bellefonte, PA, USA). Helium was used as the carrier gas at the flow rate of 1.4 mL/min. The GC oven program was as follows: 50°C (5 min) heated to 100°C at 2°C/min, and held for 2 min, then heated to 150°C at 2°C/min, and finally raised to 210°C at 10°C/min. The injector temperature was 250°C, and the interface temperature was 280°C. The measurement was made in the splitless injection mode accompanied by MHS-SPME.

The MS was operated in the SCAN or the selected ion monitoring (SIM) mode with electron impact ionization at the ionization energy of 70 eV; the ion source temperature
was 250°C, and the quadrupole temperature was 150°C. Quantitative analysis was performed in the SIM mode using each target ion and one reference ion of m/z 93 (92) for α-pinene, m/z 68 (93) for limonene, m/z 81 (71) for 1,8-cineole, m/z 71 (81) for neomenthol, m/z 71 (81) for menthol, m/z 95 (81) for neomenthol acetate, m/z 95 (81) for menthol acetate, and m/z 68 (79) for perillaldehyde. The number in parentheses is the m/z of the reference ion used for confirmation. Target and reference ion abundances were determined by injecting standards under the same chromatographic conditions.

3.4. Operating conditions for MHS-SPME

For each MHS-SPME analysis, 5 mL of sample solution was introduced into a 20 mL HS vial, and 1 g of sodium chloride (NaCl) was added. The vial was tightly sealed with a polytetrafluoroethylene (PTFE) septum. The vial containing the sample solution was set in the MHS-SPME system, and autosampling was carried out by using a COMBI-PAL (CTC Analytics AG, Switzerland). The operating conditions were as follows: fiber: polydimethylsiloxane (PDMS, film thickness 100 μm), pre-incubation time: 1 min, incubation temperature: 40°C, extraction time: 10 min, agitator speed: 250 rpm, and thermal desorption time at the GC injection port: 10 min. The extraction operation was performed three consecutive times per sample. The MHS-SPME procedure employed to analyze the calibration standards was the same as that described above.

3.5. Preparation of sample solution for foods

As solid and liquid food samples, we purchased commercially available jams, candy balls, and beverages imported to Japan. The sample solutions for candy balls and jams were prepared as follows: approximately 1 to 10 mg thereof was precisely weighed after crushing, and dissolved in 10 mL of water. Thereafter, 2 g of NaCl was added to the solution. The sample solutions for beverages were prepared as follows: a beverage was diluted 10 to 100 times with pure water, and NaCl was added to the concentration of 20%.

4. Results and discussion

4.1. Optimization of enantiomeric separation by using GC chiral column

In our previous work [17], we developed a test method that can calculate the optical purity of flavor compounds: menthyl acetate, 1,8-cineole, perillaldehyde, borneol, menthol, α-pinene, and limonene, which are designated by the Ministry of Health, Labor and Welfare as compounds having enantiomers among the flavor compounds described in the designated food additives. In the present study, we added neomenthol and neomenthyethyl acetate, which are the structural isomers of menthol, and menthyl acetate, respectively, to the substances to be measured, and examined the conditions under which chiral separation could be performed. When we employed the chiral column β-DEX 120 alone used in the previous work [17], mutual separation of the peaks of menthyl acetate and menthol and menthyl acetate and neomenthol was insufficient. Although the GC/MS measurement in the SIM mode had high selectivity, because menthol and neomenthol, and menthyl acetate and neomenthyl acetate are structural isomers, the ions (m/z) measured by MS were the same. As a result, mutual separation and quantification of the above-mentioned flavor compounds were difficult even in the SIM mode. Then, we examined the mutual separation by using the so-called tandem column, which is a combination of a chiral column and a non-polar, slightly polar or moderately polar fused silica column, the separation mode of which differs from that of the chiral column.

![Fig. 3](image-url)
The fused silica columns were non-polar type CP-Sil 5 CB (Agilent Technologies), slightly polar type SLB-5ms (Sigma-Aldrich Japan), and moderately polar types CP-Sil 24 CB (Agilent Technologies) and DB-17ms (Agilent Technologies). Their sizes were unified into 30 m length, 0.25 mm internal diameter, and 0.25 μm film thickness. Moreover, the influence of the difference in order of connection, namely, fused silica column-chiral column and chiral column-fused silica column, was also examined. As a result, by adopting a tandem column in which DB-17MS and β-DEX 120 are connected in series and in that order, mutual separation and chiral separation of almost all the flavor compounds were accomplished (Fig. 3).

4.2. Optimization of MHS-SPME

By optimizing the conditions for MHS-SPME, we had expected that the amount of extracted target compound would be increased, interfering substances would be removed, reproducibility would be improved, and extraction time would be shortened. Factors influencing MHS-SPME are extraction time, partition coefficient, mobility of the target compound, and sample solubility in the aqueous phase. The type of fiber is essential to improve the partition coefficient. Agitation, the volume ratio of aqueous phase to gas phase, and temperature are factors affecting the mobility of the target compound. Furthermore, the types and concentrations of coexisting salts and pH are factors affecting sample solubility. Therefore, the optimization of the extraction conditions for each item was carried out.

4.2.1. Selection of fiber

The influence of the type of fiber on the analytic extraction by MHS-SPME was studied using PDMS, polyacrylate (PA), PDMS/divinylbenzene (DVB), carboxen (CAR)/PDMS, and CAR/DVB, all of which are commonly used for SPME. To a 10 mL vial for SPME, 500 μL of a mixed solution of the eight kinds of flavor standards dissolved in acetone and 4.5 mL of 20% aqueous NaCl solution were added, and the vial was sealed with a dedicated septum. Using this 10 ng/mL mixed solution of flavor standards, the type of fiber was examined according to section 3.4. Fiber having a high extraction rate would be easily influenced by the matrix, so that β in the above equation (Eq. 3) shown as 0.4 < β < 0.95 was selected as the index [18]. As a result, the highest extraction rate was obtained when PDMS/DVB was used, and the percentage area of the target compound to be measured other than limonene was almost 100%, but β was less than 0.4 for all the flavor compounds. In contrast, when PA was used, the optimum condition of 0.4 < β < 0.95 was achieved for all the flavor compounds, but reproducibility was poor. On the other hand, when PDMS (100 μm) was used, only menthyl acetate had a β value of around 0.35, and the optimum condition of 0.4 < β < 0.95 was achieved for the other flavor compounds. Therefore, PDMS (100 μm) was employed as the optimum fiber type.

4.2.2. Effect of agitation

Agitation influences microextraction because it can accelerate the transfer of analytes from the sample matrix to the fiber coating. An experimental system similar to section 4.2.1 was adopted to examine the influence of agitation on the recovery rate. Agitation of the sample was performed at 250 rpm using an autosampler equipped with a temperature-controlled vial agitator tray. At that time, PDMS (100 μm) was used as the fiber, and agitation was performed for 10 min with an autosampler (Combi-PAL LHS-7). Because the recovery rate (approximately 1.1 to 1.7 times) was improved by agitation, we decided to perform agitation for all the eight types of flavor compounds.

4.2.3. Volume ratio of aqueous phase to gas phase

As the volume of the gas phase decreases with the increase of the sample volume (aqueous phase) in an HS vial, the effect of changing the volume of the gas phase on HS-SPME was examined. An experimental system similar to the above was adopted, and the peak area of each flavor compound obtained by the HS-SPME method was examined in the sample volume range of 1 to 5 mL in the 20 mL SPME vial. As a result, the peak area of all the eight flavor compounds except neomenthol showed a tendency from 1.1 to 1.4 times with the increase of the aqueous phase volume from 3 mL to 5 mL. Neomenthol reached maximum value at 3 mL and a slightly decreasing trend with a further increase of aqueous phase volume. However, it showed a value of 70% even at 5 mL. Therefore, in the present study, 5 mL of liquid phase was employed.

4.2.4. Effects of salt and pH

The types of salts (NaCl, KCl, and CaCl₂) to be added to the liquid phase and their concentrations were investigated for their salting-out effects. First, to investigate the types of salts, three types of salts were prepared as 5% aqueous solutions, respectively, and the relative peak areas of the eight flavor compounds according to the type of salt were compared. No significant difference was observed among the three salts, and NaCl was used. Accordingly, the optimum concentration of NaCl added was examined. Five, 10, 15, and 20% aqueous solutions of NaCl were prepared. Using an experimental system similar to the above, the relative peak areas of the eight flavor compounds were compared at various NaCl concentrations. α-Pinene, limonene, menthyl acetate, and neomenthyl acetate (Group A) showed 65% or larger peak areas even at a low NaCl concentration (Fig. 4). On the other hand, in the case of 1.8-cineole, menthol, neomenthol, and perillaldehyde...
(Group B), a significant dependence of the salting-out effect on NaCl concentration was observed, and the peak areas were largest when NaCl concentration was 20% (Fig. 4).

![Fig. 4.](image)

In light of the structural formulas of the eight flavor compounds, Group A (α-pinene, limonene, menthyl acetate, and neomenthyl acetate), for which the salting-out effect was not apparent, have no polar functional groups. On the other hand, such polar functional groups as ether oxygen, carbonyl group, and hydroxy group are present in Group B (1,8-cineole, menthol, neomenthol, and perillaldehyde), in which the salting-out effect was remarkable. From this, it was presumed that salting out effectively accelerates the equilibrium state from the aqueous phase to the gas phase for compounds with high polarity. It was inferred that these results are theoretically based on the octanol-water partition coefficient (log$P_{ow}$), that is, the eight flavor compounds in Fig. 4 are categorized into two groups on the basis of log$P_{ow}$. Those in the range of 3.6 to 4.86 are in the Group A, and those in the range of 1.55 to 3.19 are in the Group B. It was revealed that most of the profile patterns in Fig. 4 are related to log$P_{ow}$. As for the optimization of the added NaCl concentration, the Group A showed the largest peak area at approximately 15%. On the other hand, the Group B showed the largest peak area at approximately 20%. Therefore, their largest common divisor was approximately 20%.

4.2.5. Extraction time and extraction temperature

The relative peak areas at the extraction times of 5, 10, 20, 30, 60, and 100 min were compared to clarify the optimum extraction time. The time-dependent extraction profiles in Fig. 5-(A) indicated that 10 min was sufficient for all analytes except α-pinene and limonene. Although α-pinene and limonene showed maximum values at 5 min and a decreasing trend thereafter, the relative peak areas were 70% or larger even after 10 min. Therefore, the optimal extraction time was set at 10 min, which applies to all analytes.

Next, the optimum extraction temperature was examined. In a similar experimental system, the relative peak areas at the extraction temperatures of 40, 50, 60, and 70°C were compared, while keeping the extraction time at 10 min. Most of the substances to be measured showed maxima at the extraction temperature of 40°C; thus, the optimal extraction temperature was set at 40°C (Fig. 5-(B)).

![Fig. 5.](image)
4.3. Method validation

Experiments to create a calibration curve and recovery tests of the eight flavor compounds were performed to validate the analysis method. The limit of quantitation (LOQ) ranged from 0.01 to 1 ng/mL, and the calibration curve showed good linearity with a correlation coefficient \( r \) of > 0.995 from LOQ to 100 ng/mL (Table 1).

In the recovery tests, commercially available candy balls and beverages were spiked with standard flavor compounds at a low concentration (10 ng/g) and a high concentration (50 ng/g), respectively. Then, recovery tests were carried out with three replicates for each concentration. The average recoveries for candy balls spiked with a low concentration of the standard flavor compounds were in the range of 84.1 to 96.1%, and the relative standard deviations (RSDs) ranged from 2.0 to 12.1%. Those for candy balls spiked with a high concentration of the standard flavor compounds ranged from 81.6 to 103.9%, and RSDs were from 0.3 to 14.5% (Table 2).

On the other hand, the average recoveries for beverages spiked with a low concentration of the standard flavor compounds ranged from 86.6 to 119.2%, and RSDs were from 1.5 to 14.1%, and those for beverages spiked with a high concentration of the standard flavor compounds ranged from 82.3 to 106.5%, and RSDs were from 3.7 to 14.3% (Table 3).

Table 1. Validation of proposed MHS-SPME GC/MS method for flavor compound analysis.

| Flavor compounds | Enantiomer | Linear range (ng/mL) | Correlation coefficient \( r \) |
|------------------|------------|----------------------|-------------------------------|
| α-Pinene         | \( l \)    | 1 – 100              | 0.997                         |
|                  | \( d \)    | 1 – 100              | 0.996                         |
| Limonene         | \( l \)    | 0.5 – 100            | 0.999                         |
|                  | \( d \)    | 0.5 – 100            | 0.999                         |
| 1,8-Cineole      | \( l \)    | 1 – 100              | 0.999                         |
| Neomenthol       | \( l \)    | 1 – 100              | 0.998                         |
|                  | \( d \)    | 1 – 100              | 0.998                         |
| Menthol          | \( l \)    | 1 – 100              | 0.998                         |
| Neomenthyl acetate | \( l \) | 0.01 – 100          | 0.996                         |
| Menthyl acetate  | \( d \)    | 0.01 – 100           | 0.999                         |
| Perillaldehyde   | \( l \)    | 1 – 100              | 0.996                         |
|                 | \( d \)    | 1 – 100              | 0.995                         |

Table 2. Recoveries of flavor compounds from spiked food sample (candy ball).

| Flavor compounds | Spiked amount (ng/g) | Average recovery (ng/g) | RSD (%) | Spiked amount (ng/g) | Average recovery (ng/g) | RSD (%) |
|------------------|----------------------|-------------------------|---------|----------------------|-------------------------|---------|
| α-Pinene         | \( l \)              | 10                      | 96.1    | 12.1                 | 50                      | 101.5   | 9.3    |
|                  | \( d \)              | 10                      | 86.7    | 7.5                  | 50                      | 97.6    | 12.1   |
| Limonene         | \( l \)              | 10                      | 93.9    | 7.9                  | 50                      | 100.3   | 7.8    |
|                  | \( d \)              | 10                      | 86.1    | 8.3                  | 50                      | 99.1    | 7.9    |
| 1,8-Cineole      |                      | 10                      | 93.8    | 5.9                  | 50                      | 94.7    | 12.6   |
| Neomenthol       | \( l \)              | 10                      | 86.1    | 2.0                  | 50                      | 101.7   | 12.1   |
|                  | \( d \)              | 10                      | 84.2    | 10.1                 | 50                      | 95.7    | 9.0    |
| Menthol          | \( l \)              | 10                      | 84.1    | 5.6                  | 50                      | 92.0    | 14.5   |
|                  | \( d \)              | 10                      | 89.0    | 3.8                  | 50                      | 97.0    | 8.7    |
| Neomenthyl acetate | \( l \)           | 10                      | 93.0    | 6.0                  | 50                      | 98.0    | 1.1    |
| Menthyl acetate  | \( d \)              | 10                      | 94.6    | 7.1                  | 50                      | 96.7    | 1.8    |
| Perillaldehyde   | \( l \)              | 10                      | 84.5    | 13.3                 | 50                      | 81.6    | 2.1    |
|                 | \( d \)              | 10                      | 94.0    | 2.8                  | 50                      | 99.7    | 3.9    |

Data were based on three replicate analyses.
with a shiso odor; it is extracted from shiso, a kind of Labiatae plant, and the leaves are used as a food additive to impart a shiso flavor. However, because the detected d-form is not designated as a flavor compound by the Food Hygiene Law of Japan, we considered that this candy ball has food hygiene problem.

In the case of beverages, menthol was detected from liquid sample No. 9 (Brazil; cashew) and liquid sample No. 10 (Brazil; passion fruit). In the latter (No. 10), the d-form was not detected, but in the former (No. 9), d-menthol and l-menthol were detected in almost equal amounts, which revealed a racemic mixture. We considered that chemically synthesized menthol was added.

In addition, menthol and menthyl acetate were detected in both liquid sample No. 6 (China; sarsaparilla) and liquid sample No. 7 (Taiwan: guava juice). In sample No. 7, those two flavor compounds were present in l-forms, and the d-forms, which are not designated as a flavor compound by the Food Hygiene Law of Japan, were found in trace amounts. On the other hand, in sample No. 6, both d-form and l-form were detected, and the d-form of menthol was present in an amount that was approximately twice as large as the l-form, whereas the d-form of menthol was approximately 2.7 times more abundant than the l-form. From the results, menthol and menthyl acetate were detected in almost equal amounts. However, from the other samples analyzed in this study, we suspected artificial causes as well, because such cases do

### Table 3. Recoveries of flavor compounds from spiked food sample (beverage).

| Flavor compounds | Spiked amount (ng/mL) | Average recovery (ng/mL) | RSD (%) | Spiked amount (ng/mL) | Average recovery (ng/mL) | RSD (%) |
|------------------|-----------------------|--------------------------|---------|-----------------------|--------------------------|---------|
| α-Pinene         | l                     | 10                       | 115.4   | 50                    | 86.1                     | 9.7     |
|                  | d                     | 10                       | 116.5   | 50                    | 84.8                     | 12.0    |
| Limonene         | l                     | 10                       | 103.5   | 50                    | 82.6                     | 13.7    |
|                  | d                     | 10                       | 105.1   | 50                    | 82.8                     | 7.1     |
| 1,8-Cineole      | l                     | 10                       | 110.9   | 50                    | 84.3                     | 13.0    |
|                  | d                     | 10                       | 107.9   | 50                    | 106.5                    | 9.2     |
| Neomenthol       | l                     | 10                       | 104.9   | 50                    | 103.8                    | 10.3    |
|                  | d                     | 10                       | 107.9   | 50                    | 106.5                    | 9.2     |
| Menthol          | l                     | 10                       | 101.4   | 50                    | 93.7                     | 11.5    |
|                  | d                     | 10                       | 87.5    | 50                    | 95.7                     | 14.3    |
| Neomenthyl acetate | l                  | 10                       | 102.0   | 50                    | 89.1                     | 6.9     |
|                  | d                     | 10                       | 101.0   | 50                    | 87.9                     | 7.3     |
| Menthy l acetate | l                     | 10                       | 100.3   | 50                    | 88.7                     | 6.1     |
|                  | d                     | 10                       | 86.6    | 50                    | 82.3                     | 3.7     |
| Perillaldehyde   | l                     | 10                       | 106.3   | 50                    | 87.6                     | 4.9     |
|                  | d                     | 10                       | 119.2   | 50                    | 93.9                     | 4.0     |

Data were based on three replicate analyses.

### 4.4. Application to real samples and quality assessment

Five solid samples (four candy balls and one jam) and seven liquid samples (seven beverages) were analyzed using the proposed MHS-SPME method. Limonene, 1,8-cineole, menthol, menthyl acetate, and perillaldehyde were detected (Table 4). In the case of solid sample No. 1 (jam; made in Korea), the product label of which claims to have yuzu flavor, limonene was detected at a relatively high concentration, and both d- and l-enantiomers were detected in almost equal amounts, indicating a racemic mixture. Therefore, we considered that the limonene flavor added to this food was not from natural yuzu, but from a chemically synthesized product.

Menthol was detected in solid sample No. 2 (Korean ginseng candy balls made in Korea) and solid sample No. 3 (strawberry- and banana-flavored candy balls made in Canada). Most of the menthols detected in solid sample No. 3 were the l-form, and only a few were the d-form. On the other hand, menthol detected in solid sample No. 2 was presumed to be a racemic mixture because the d-form and the l-form were detected in almost equal amounts. Thus, we considered that chemically synthesized menthol and not natural menthol was added, as in the case of solid sample No. 1.

Solid sample No. 5 (made in China; Siraitia grosvenorii) was also presumed to be a racemic mixture because the d- and l-forms of perillaldehyde were detected in almost equal amounts. It was thought that chemically synthesized perillaldehyde was added. Perillaldehyde is an essential oil with a shiso odor; it is extracted from shiso, a kind of Labiatae plant, and the leaves are used as a food additive to impart a shiso flavor.
not occur. Because the d-form was detected in relatively large amounts, the remaining chemicals that were removed as pure l-form from the chemically synthesized racemic mixture by fractional crystallization, etc. might have been used as additives. Alternatively, we presumed that the asymmetric synthesis is insufficient and low optical purity enantiomers may have been used as additives. In any case, further detailed investigations are necessary.

Based on these results, the developed method could measure the optical purity of flavor compounds used as food additives, and would be useful in the quality evaluation and quality control of commercial foods.

| Flavor compounds | No.1 | No.2 | No.3 | No.4 | No.5 | No.6 | No.7 | No.8 | No.9 | No.10 | No.11 | No.12 |
|------------------|------|------|------|------|------|------|------|------|------|-------|-------|-------|
| α-Pinene         | d    | l    | l    | l    | l    | l    | l    | l    | l    | l     | l     | l     |
| Limonene         | l    | d    | 15.8 | 15.2 | l    | d    | l    | d    | l    | d     | d     | d     |
| 1,8-Cineole      |      |      |      |      |      |      |      |      |      |      |      | 230.1 |
| Neomenthol       | l    | d    |      |      |      |      |      |      |      |      |      | 120.5 |
| Menthol          | l    | d    | 5.0  | 0.02 | —    | —    | —    | —    | —    | —     | —     | 145.2 |
| Neomenthol acetate | l  | d    |      |      |      |      |      |      |      |      |      | 58.2  |
| Methyl acetate   |      |      |      |      |      |      |      |      |      |      |      | —     |
| 1,8-Cineole      |      |      |      |      |      |      |      |      |      |      | —     | —     |
| Linalool         |      |      |      |      |      |      |      |      |      |      |      | —     |

Sample types and concentration units: No. 1: jam (µg/g), Nos. 2–5: candy balls (µg/g), Nos. 6–12: beverage (µg/mL). Country of origin: Nos. 1 and 2: South Korea, Nos. 3 and 4: Canada, Nos. 5 and 6: China, Nos. 7 and 8: Taiwan, Nos. 9 and 10: Brazil, No. 11: Vietnam, No. 12: Philippines. —: not detected (flavor compound concentration is lower than LOD). Tr: trace (flavor compound concentration is ≥ LOD and < LOQ).

5. Conclusions
The chiral separation of multiple flavor compounds by GC is not sufficient with only one type of chiral column. In the present study, we examined a tandem column with different separation modes. As a result, the chiral separation of eight flavor compounds was achieved with a tandem column composed of a DB-17MS column and a β-DEX 120 column. In addition, optimizing the extraction conditions in the MHS-SPME method, which is the pre-treatment step, for application to the eight flavor compounds resulted in good recoveries (81.6 to 119.2%) and quantitative performance.

The application of this method to commercial foods, such as candy balls, jam, and beverages, enabled us to infer whether the added flavor compound was a natural flavor compound or an artificial one, based on the enantiomeric ratio of the detected flavor compound. Enantiomers that are not designated in the Food Hygiene Law on Japan were detected in some samples, suggesting the possibility that low-quality synthetic chemical products were added as flavor compounds.

In addition, because the method developed in the present study could be easily automated, it could be used to determine flavor compounds in both solid and liquid foods with high accuracy and precision, and could be a practical method for evaluating the optical purity of flavor compounds used as food additives.

References
[1] Okamura, H. Tech. J. Food Chem. & Chemicals 2007, 65-72.
[2] Mani, V.; Woolley, C. FFI J. Jpn. 1995, 163, 94-103.
[3] Campillo, N.; Aguinaga, N.; Viñas, P.; López-Garcia, I.; Hernández-Córdoba, M. J. Chromatogr. A 2004, 1061, 85-91.
[4] Buser, H. R.; Zanier, C.; Tanner, H. J. Agric. Food Chem. 1982, 30, 359-362.
[5] López, R.; Aznar, M.; Cacho, J.; Ferreira, V. J. Chromatogr. A 2002, 966, 167-177.
[6] Groning, M.; Hakkarainen, M. J. Chromatogr. A 2004, 1052, 61-68.
[7] Zhang, Z.; Pawliszyn, J. Anal. Chem. 1993, 65,
1843-1852.

[8] Steffen, A.; Pawliszyn, J. J. Agric. Food Chem. 1996, 44, 2187-2193.

[9] Yang, X.; Peppard, T. J. Agric. Food Chem. 1994, 42, 1925-1930.

[10] Elmore, J. S.; Erbahadir, M. A.; Mottram, D. S. J. Agric. Food Chem. 1997, 45, 2638-2641.

[11] Jia, M.; Zhang, Q. H.; Min, D. B. J. Agric. Food Chem. 1998, 46, 2744-2747.

[12] Caja, M. M.; Blanch, G. P.; Herraiz, M.; Ruiz Del Castillo, M. L. J. Chromatogr. A 2004, 1054, 81-85.

[13] Flores, G.; Ruiz Del Castillo, M. L.; Blanch, G. P.; Herraiz, M. Food Chem. 2006, 96, 334-339.

[14] Ravid, U.; Putievsky, E.; Katzir, I.; Ikan, R.; Weinstein, V. Flavor Frag. J. 1992, 7, 289-292.

[15] Mosandl, A.; Guenther, C. J. Agric. Food Chem. 1989, 37, 413-418.

[16] U.S. Food and Drug Administration, Chirality 1992, 4(5), 338-340.

[17] Saito, K.; Hosono, K.; Kitazawa, N.; Iwasaki, Y.; Ito, R.; Nakazawa, H. J. AOAC Int. 2011, 94(3), 923-930.

[18] Kolb, B. Chromatographia 1982, 15, 587-594.

[19] Kolb, B.; Pospisil, P.; Auer, M. J. Chromatogr. A 1981, 204, 371-376.

[20] Martinez-Uruñuela, A.; González-Sáiz, J. M.; Pizarro, C. J. Chromatogr. A 2005, 1089, 31-38.