Identification of Fragrances from Chestnut Blossom by Gas Chromatography-Ion Trap Mass Spectrometry

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In far-eastern Asia including Korea, Japan, and China, chestnuts are an important source of food. The scientific name for the Korean chestnut tree that widely distributed in Korean peninsular is *Castanea crenata var dulcis* Siebold et Zuccarini (Fagaceae) or *Castanea kusakuri*. The Japanese chestnut is classified as *Castanea crenata*, the Chinese chestnut as *Castanea mollissima* or *Castanea bungeana* Blume, the European chestnut as *Castanea sativa*, the American chestnut as *Castanea dentata* and some American species the chinquapin as *Castanea pumila*. It was commonly found on mountains, hills, and slopes in gravelly or rocky, well-drained glacial soils. The flowers of the Korean chestnut appeared in June, producing a spectacular display of creamy-yellow blossoms and were strongly scented. The flowers were similar to those of the Japanese chestnut. Chestnut honey was produced in this season. Interestingly, it has been believed that chestnut blossoms emit strong sperm-like scents for attracting mate. The edible nuts ripened and dropped from September. The bark and wood were rich in tannic acid which provided tannin for use in the tanning of leather. To date, however, chemical composition of fragrances emanated from chestnut blossom has never been studied.

The main purpose of this study is to characterize the fragrance composition emanated from chestnut blossom. The screening of new compounds and combination formulas for cosmetics, aroma therapy and many other applications is the final goal. In this study, solid-phase trapping-solvent extraction (SPTE) was used to collect fragrances emitted from chestnut blossom. Then, fragrant compounds were analysed by GC-MS^n with electron impact (EI) ionization and methane chemical ionization (CI) modes for the cross-checking. For the first time, fragrance constituents of chestnut blossom have been reported here.

Experimental Section

The freshly picked flower samples of chestnut (*Castanea crenata var dulcis* Siebold et Zuccarini) were collected at Mt. Soraksan, Mt. Sooraksan, and Mt. Yoomyungsan in June, 2000. Samples were analyzed immediately after arrival. Ethylvinyl bezene divinyl benzene copolymer (Porapak Q, 149-125 μm: 50-80 mesh) was purchased from Supleco (Bellefonte, PA, USA). Special precaution is required prior to use Porapak Q, because it contains *m-*, *o-*, *p-* isomers of diethylbenzene as impurities. Before use, Porapak Q particles were pre-rinsed with organic solvent in order to remove impurities. All fragrance standards were of analytical grade (purity, 99.9%) and were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Tokyo Kasei (Nihonbashi, Tokyo, Japan). All organic solvents of chromatographic grade were purchased from Sigma-Aldrich.

Fragrance compounds were collected from the chestnut blossom by using a SPTE apparatus as described in our previous reports.[1-4] About 15 g of chestnut flower samples were filled in a clean, dry barrel of the hypodermic glass syringe (50 mL, 3 cm I.D. $\times$ 14 cm long) with its plunger and needle removed. And then, two syringe barrels were fitted together with a polytetrafluoroethylene (PTFE, Teflon) spacer gasket and held by a joint clip. A Pasteur pipet (0.565 cm I.D. $\times$ 15 cm long) was used as a trap-housing which was packed with Porapak Q adsorbent (500 mg) and glass wool plugs. The inlet of the Pasteur pipet was attached to the luer taper tip of the barrel containing the flower cut. An oil-free electric vacuum pump (Vacuubrand GMBH, Wertheim, Germany, diaphragm ME2 model, 2.4 m^3/h) and a PTFE valve restrictor were connected with Tygon tubing to the outlet end of the trap via glass-manifold. A purified nitrogen gas (purity, 99.99%) flow at *ca.* 400 mL/min was passed into a couple of barrels and out through the adsorbent trap under reduced pressure. The collection was continued for 3 h at ambient temperature. After a run, the trap was then removed and the trapped fragrance compounds were eluted by two extractions with 2 mL of petroleum ether in portions to the new syringe to which the trap was attached and forcing the solvent through with the syringe plunger. Aliquots were analysed by GC-MS.

GC-MS was performed on a Trace GC 2000 with GC-Q Plus ion trap MS^n (Thermoquest-Finnigan, Austin, TX, USA) gas chromatograph-mass spectrometer with Xcalibur software system. The columns were fused silica capillary columns coated with different stationary phase: a 5% phenyl poly(dimethylsiloxane) column (SPB-5, Supelco, 60 m $\times$ 0.25 mm $\times$ 0.25 μm film thickness) and a polyethylene glycol (Supelcowax-10, 30 m $\times$ 0.32 mm $\times$ 0.25 μm) column. The oven temperature program for SPB-5 column was 50 ^{o}C(3 min)-4 ^{o}C/min-200 ^{o}C-8 ^{o}C/min-240 ^{o}C(5 min)-50 ^{o}C/

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min-290 °C (5 min). Injector temperature and transfer line temperature were 250 °C and 275 °C, respectively. The oven temperature program for Supelcowax-10 column was 40 °C(5 min)-4 °C/min-150 °C-8 °C/min-240 °C; injector, 230 °C; transfer line, 230 °C; all other conditions were the same as those of a SPB-5 column. Carrier gas (He, 99.9995%) was adjusted to a flow of 1.0 mL/min, the sample volume injected was 1 μL. The split ratio was 1 : 30. The electron impact (EI) ionization mass spectrometer was operated as follows: ionization voltage, 70 eV; ion source temperature, 200 °C. Excitation voltage for MS^2 mode was 1 V. Methane (99.5% purity) reagent gas pressure for chemical ionization (CI) was 70 mtorr, ion source temperature was 150 °C.

All the compounds were identified by comparison of their Kovats retention indices (*I*) and mass spectral information with those of authentic substances used as references. Kovats retention indices were determined by using a solution containing the homologous series of normal alkanes (C_8-C_{20}).

Results and Discussion

Figure 1 shows the total ion chromatograms (TIC) of fragrances collected by SPTE from chestnut blossom. Figure 1A is a TIC separated by using a SPB-5 column, B is a TIC by a Supelcowax column. A total of eleven compounds were detected. The quantitative determination was carried out using authentic standard. The amount of each analyte in chestnut sample was calculated according to the following equation:

$$W_{analyte} = (A_{analyte} \times C_{ST} \times V_{TOTAL})/(A_{ST} \times W_{sample})$$

where $W_{analyte}$ (mg/g) is the weight of analyte in chestnut blossom sample, $A_{analyte}$ is the peak area of analyte, C_{ST} is the concentration (ng/μL) of authentic standard solution injected, V_{TOTAL} is the total volume (1,000 μL) extracted, A_{ST} is the peak area of authentic standard, and W_{sample} is the weight (15 g) of chestnut blossom sample obtained, respectively. It can be seen that acetophenone (24.5 mg/g ± 2.5% RSD),

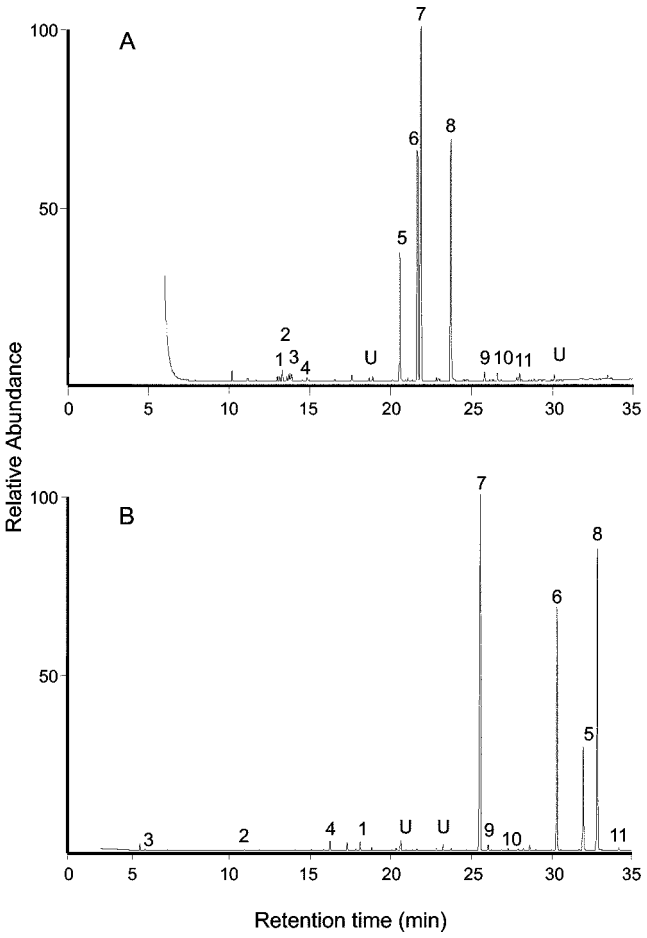

Figure 1. Total ion chromatograms of fragrances in chestnut blossom analysed by solid phase trapping-solvent extraction and gas chromatography ion trap mass spectrometry with electron impact ionisation using (A) 5% phenyl poly(dimethylsiloxane) column (SPB-5, Supelco, 60 m × 0.25 mm × 0.25 μm film thickness) and (B) polyethylene glycol (Supelcowax-10, 30 m × 0.32 mm × 0.25 μm) column. Peak numbers corresponding to the numbers indicated in Table 1. For analytical conditions, see experimental section.

Table 1. The retention times (t_R), retention factors (k) and retention inces (I) on 5% phenylpoly(dimethylsiloxane) column and polyethylene glycol column

Peak No.	Compound	Non-polar column: 5% Phenylpoly(dimethylsiloxane) Supelco SPB-5, 60 m × 0.25 mm × 0.25 μm		Polar column: Polyethylene glycol Supelcowax-10, 30 m × 0.25 mm × 0.25 μm	
		t_R	I	t_R	I
1	2-Methylbutyric acid	13.00	844	18.83	1417
2	2-Hexyn-1-ol	13.13	847	10.98	1201
3	Ethylbenzene	13.57	859	4.90	1019
4	Allyl vinyl ether	13.75	864	16.26	1344
5	Benzyl alcohol	20.87	1043	31.92	1839
6	α-Methyl benzyl alcohol	21.99	1072	30.30	1801
7	Acetophenone	22.18	1076	25.57	1591
8	2-Phenethyl alcohol	24.05	1126	32.80	1860
9	Ethyl benzoate	25.80	1174	26.03	1613
10	Azulene	26.58	1195	27.88	1736
11	Phenethyl acetate	28.87	1258	34.13	1892

benzyl alcohol (6.1 mg/g ± 3.2% RSD), 2-phenethyl alcohol (10.2 mg/g ± 0.6% RSD) and α-methyl benzyl alcohol (0.2 mg/g ± 12.5% RSD) were the abundant constituents. On the other hand, 2-methyl butyric acid, 2-hexyn-1-ol, ethyl benzene, allyl vinyl ether, ethyl benzoate, azulene, and phenethyl acetate were also detected as trace constituents. In all

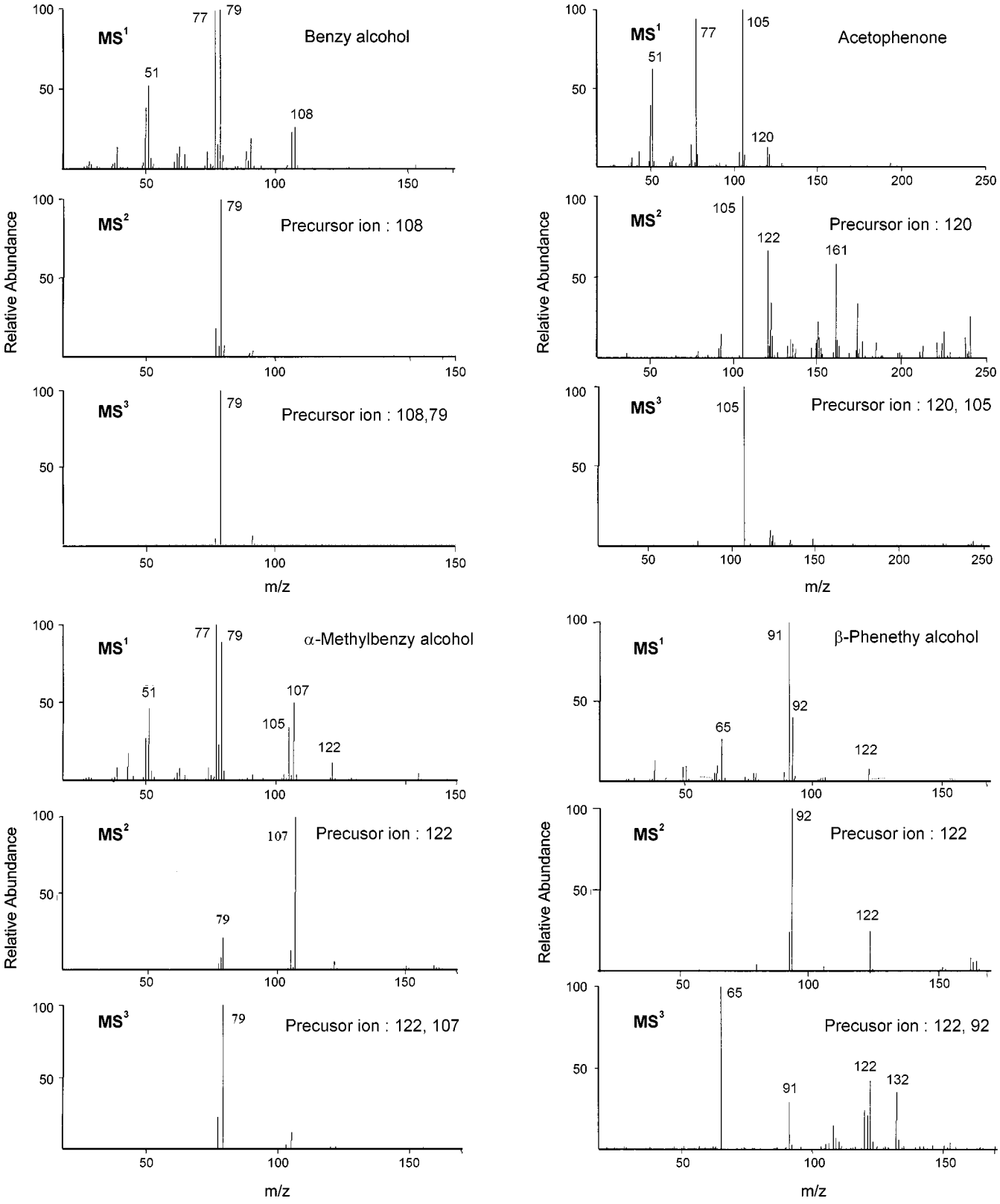

Figure 2. GC-EI-MS^n product ion spectra for benzyl alcohol, α-methylbenzyl alcohol, aceto-phenone and β-phenethyl alcohol in chestnut blossom.

Table 2. Characteristic methane CI mass spectral ions for benzyl alcohol, α-methylbenzyl alcohol, acetophenone, and 2-phenyl ethyl alcohol identified from chestnut blossom

Reagent ion m/z (%, species)	Analytes	M_r	Base peak m/z (100%)	Characteristic mass spectral ion (CI-MS) m/z (relative abundance)
29 (100, $C_2H_5^+$)	Benzyl alcohol	108	91	79 (27.7), 53 (10.6), 92 (8.0), 65 (5.3), 107 (3.2), 108 (1.1)
19 (73, H_3O^+)	α-Methylbenzyl alcohol	122	105	79 (23.8), 91 (14.4), 106 (9.2), 53 (6.2), 107 (4.9), 122 (0.4)
41 (70, $C_3H_5^+$) 17 (9, CH_5^+)	Acetophenone	120	121	105 (31.3), 53 (23.9), 149 (18.8), 55 (18.3), 91 (15.6), 122 (8.7), 161 (2.8), 120 (1.8)
	2-Phenylethyl alcohol	122	105	91 (12.9), 79 (12.6), 103 (11.8), 106 (8.6), 53 (8.1), 133 (5.8), 145 (2.2), 122 (0.5)

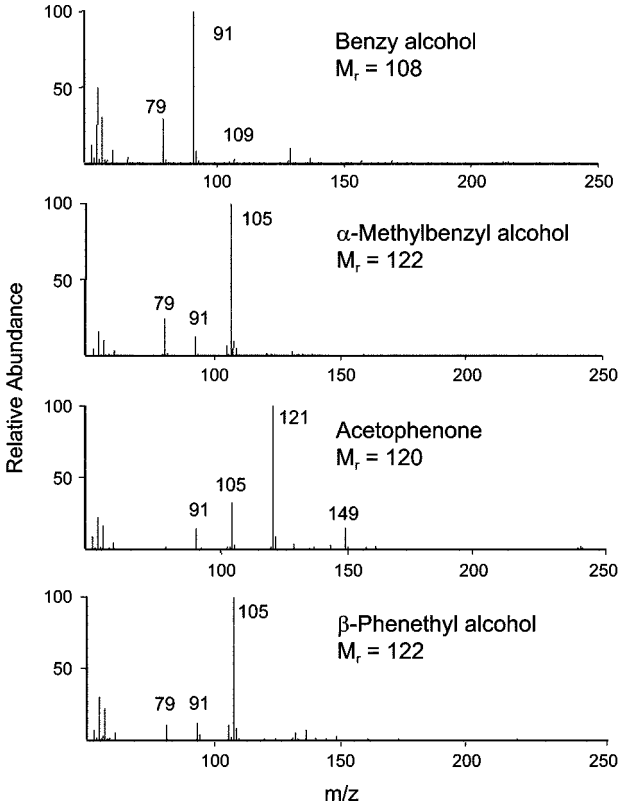

Figure 3. Methane CI-MS spectra for benzyl alcohol, α-methyl-benzyl alcohol, aceto-phenone and β-phenethyl alcohol in chestnut blossom.

samples of chestnut blossom collected from three different places, acetophenone, 2-phenethyl alcohol, α-methyl benzyl alcohol, and benzyl alcohols were found as predominant constituents.

Of these eleven constituents, four predominant compounds were positively identified by comparing their retention times as well as their EI and methane CI mass spectra, and by using reference standards. Table 1 lists Kovats retention indices (*I*) on a SPB-5 column and a Supelcowax column for reference standards mixture, in order of increasing retention time (t_R) on a SPB-5 column.

The mass spectra of above compounds were analyzed by GC-MS^n with EI ionization, as shown in Figure 2. In MS^2 mode, molecular ion was selected as a precursor ion. Molecular ion and base peak ion of MS^2 mode were selected as precursor ions of MS^3 mode. Simple base peak was observed at m/z 79 corresponding to $C_6H_7^+$ ion in both MS^2 and MS^3 modes for benzyl alcohol whereas many fragment ions in MS^1. Base peaks in EI-MS^2 spectra of α-methyl benzyl alcohol, acetophenone, and 2-phenethyl alcohol are observed at m/z 107 (M-CH_3), 105 (M-CH_3), 92 ($C_6H_5CH_3$), respectively. Base peaks in their EI-MS^3 spectra are observed at m/z 79, 105, 65, respectively. Each subsequent generation of product ions exhibits unique patterns that can be used for structural investigation. Mutiple MS (MS^n) experiments can provide the final piece of information key to identification of an unknown.

In addition, methane CI-MS spectra for benzyl alcohol, α-methyl benzyl alcohol, acetophenone, and 2-phenethyl alcohol were obtained, as shown in Figure 3 and Table 2. CI of acetophenone provides M+$C_2H_5^+$ ion at m/z 149 and abundant quasi-molecular ion (M+H). The corresponding CI spectra for benzyl alcohol, α-methyl benzyl alcohol, and 2-phenethyl alcohol are simpler and most of the ion signals are concentrated at m/z 91, 105, 105, respectively. CI spectra are complementary to EI spectra. MS^n and CI provides better results for structural elucidation.

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