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Determination of Aroma Components in *Pinus densiflora* (Pine Needles) Studied by Using Different Extraction Methods

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추출방법에 따른 솔잎의 휘발성 성분 조성 비교

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Abstract

The efficiency of six different extraction methods for the analysis of aroma components from pine needle (*P. densiflora*) was compared by gas chromatography-mass selective detector(GC-MSD). The six methods were dynamic headspace (DHS), reduced pressure headspace (RPHS), solid-phase microextraction (SPME), simultaneous distillation-extraction (SDE), supercritical fluid extraction (SFE) and pyrolysis distillation extraction (PDE). A total of 65 compounds were identified by using the six different extraction methods. These compounds are classified into six categories in terms of chemical functionality: 25 hydrocarbons, 16 alcohols, 9 carbonyls, 6 esters, 7 acids, and 2 ethers. The aroma compounds having low boiling point were more abundant in DHS, RPHS, and SPME extracts. On the other hand, the aroma compounds having high boiling point were more abundants in SDE, SFE and PDE extracts. The acid compounds were extracted by heat-based extraction methods such as SDE, SFE, PDE, but not by DHS, RPHS and SPME, which used neither solvent nor heat. The oxygenated terpens, hexanal, hexanol, and hexadienal were more abundant in DHS and RPHS extracts, compared with the other methods.

Key words: extraction methods, aroma components, pine needle, extraction condition, GC-MS

Introduction

Pinus densiflora is one of the most important forest species in the South Korean region. For centuries throughout eastern Asia, various parts of the pine tree, such as pine needles, pine cones, and pine pollen have been widely used for promoting health as folk medicine or as food[1]. The essential oil of *Pinus densiflora* (pine needles), which is obtained by steam distillation, is used extensively as fragrance and flavor components in medicine, detergents, beverages, candy, baked goods, and perfumes[2,3]. Volatile aroma components of *P. densiflora* have been investigated by several researchers using gas chromatography-mass spectrometry (GC-MS). Lee *et al.*[4,5] analyzed the volatile components of *P. densiflora* needles, grown in Korea, using a purge and trap headspace appa-

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ratus, and using double-shot pyrolyser. Also, Yu *et al.* analyzed the differences in the extraction of volatile components of *P. densiflora*, grown in Korea using SDE and SPME[6]. In addition, Jo *et al.*[7] and Kim *et al.*[8] analyzed the volatiles from twigs, needles and sprouts of *P. densiflora*, grown in Korea.

Various analytical methods can be used for the analysis of the aroma components from *P. densiflora* pine needles. It is important that researchers have the choice of a suitable extraction method to obtain an extract that contains all the aroma compounds contained in the original pine needles without alteration. Schlich *et al.*[9] recommended the validation of extract representativeness by preliminary sensory testing before analysis by gas chromatography-olfactometry (GC-O) and gas chromatography-mass spectrometry (GC-MS). Fischer *et al.*[10] have clearly demonstrated that the composition of aroma extracts is dependent on the isolation procedures employed. The fidelity between the aroma of the starting material and that of the isolated extract provides the basis for judging analytical techniques. Hence, the conditions employed should be as mild as possible to avoid oxidation, thermal degradation, and chemical change in the sample.

Traditionally, a number of techniques have been employed including solvent extraction, SDE, and headspace trapping. Solvent extraction followed by concentraction under nitrogen or in a rotary evaporator was one of the earliest methods used to recover the organic components from plants[11]. SDE was first used by Likens and Nickerson[12] and later applied for the analysis of essential oil from plants. The headspace is a very popular method for analyzing volatile components in plants as well as in other products such as airborne and VOCs in water[13]. SPME has been applied prevalently in the analysis of aroma components[14,15]. It has become the method of choice in aroma analysis offering solvent-free, rapid sampling with low cost, ease of operation and sensitivity[16]. SFE is a recent development in which the extraction can be fine-tuned by controlling the solvating power of the extraction by optimizing extraction temperature and pressure[17]. PDE was attempted for analysis of the aroma components by Lee *et al.*[5] in the most recent. In this PDE technique, the aroma components are distillated by pyrolysis and then injected at GC along with a carrier gas.

The purpose of this study was to investigate the difference of aroma components obtained by 6 kinds of extraction methods: dynamic headspace(DHS), reduced pressure headspace(RPHS), SPME, SDE, SFE, and PDE.

Materials and Methods

1. Materials

P. densiflora (pine needles) were collected from mountains near Daejeon, South Korea in July 2004. Pine-needle samples collected were stored in solvent-cleaned glass jars with aluminium foil-lined lids, and were refrigerated at 2℃ in the laboratory until required for analysis. Pine needles were cut to 2 mm lengths immediately before use. All organic solvents were of analytical grade and were purchased from Sigma.

2. Isolation Methods of Volatile Components

1) Dynamic Headspace (DHS)

The purge and trap equipment for the isolation of aroma components from pine needle was manufactured in our laboratory. The purging vessel was a 600 mL glass column of 50 cm length and 4 cm inner diameter provided with a glass frit for solid sample. A pine-needle (20 g) was put in the purging vessel and connected to nitrogen gas (flow rate: 50 mL/min). Before its first use, Tenax GR (1 g) was put in the trap and preconditioned for 3 h at 350℃ in a stream of nitrogen. The aroma components were adsorbed in the Tenax trap (Tenax GR, 60-80 mesh) for 2 h at room temperature.

The Tenax GR was put in the sample tube of JHS-100A (JAI, Japan), directly connected to a gas chromatography system. The aroma components were desorbed from Tenax GR by heating at 250℃ for 10 min and then transferred by a carrier gas stream (He) to an adsorption tube, cooled to −40℃ by liquid nitrogen. Injection into the GC was performed by curie-point heating (350℃, 10 sec).

2) Reduced Pressure Headspace (RPHS)

Pine needle (20 g) were placed in a 1 L round-bottom flask connected to the RPHS apparatus. Which was manufactured in our laboratory. Aroma components were extracted for 2 h at room temperature with reduced pressure (100 mmHg) of a 1 L sample flask. The aroma components were condensed in two cold traps cooled with

liquid nitrogen for extracting. The condensed aroma components were dissolved by diethyl ether (10 mL). The moisture including in the solvent part was excluded with anhydrous sodium sulfate. The solvent part including aroma components was concentrated to 1 mL in nitrogen gas.

3) Solid-phase Microextraction (SPME)

After placing pine needle (2 g) into the 50 mL vial, the vial was sealed using a Teflon-faced septum cap. The SPME fiber (Supelco, USA) was fitted into the sealed vial, whence it was exposed and its height was adjusted in the headspace above pine needles. Then, the vial was placed in a heating block and the extraction commenced (see below for condition).

In these experiments, a 100 μm polydimethylsiloxane (PDMS) was used as a SPME fiber, and it was conditioned by fitting it into the GC injector at 250℃ for 30 min prior to the extraction experiments. The SPME fiber was exposed to the headspace above the sample for 20 min at 50℃ in a heating block. After adsorption, the SPME fiber was removed from the sample vial and inserted into the injection port of GC for 2 min at 230℃ in order to transfer the volatile components into the GC-MS.

4) Simultaneous Distillation Extraction (SDE)

Pine needles (20 g) and distilled water (500 mL) were placed in a 2 L round-bottom flask. Diethyl ether (30 mL) and pentane (30 mL) were placed in a 100 mL round-bottom flask, and the two flasks were connected to the modified Likens-Nickerson micro SDE apparatus[13]. The extraction was performed for 2 h, during which time chilled water was circulated through the cold finger condenser. The fractions in the solvent flask were dried with anhydrous sodium sulfate, filtered and then concentrated by blowing with nitrogen.

5) Supercritical Fluid Extraction (SFE)

Supercritical fluid extraction was carried out using CH-8307 (Nova, Switzerland). In this study, extraction was performed by filling 40 mL volume extraction vessel with 20 g of pine needle. The pine needle was then extracted with supercritical CO_2 under 200 atm pressure and 50℃ temperature for 2 h. A Duraflow manual variable restrictor was used in the SFE system to collect the extracted components. The supercritical CO_2 flow rate through the Duraflow restrictor was 1.0 mL/min. To dissolve the extracted components in the restrictor, 20 mL of diethyl ether was poured in the restrictor and concentrated to 3 mL with nitrogen at room temperature. The extracted components were stored in sealed vials at 2℃ until evaluation.

6) Pyrolysis Distillation Extraction (PDE)

Volatile aroma components were extracted by using a curie point-pyrolyzer JHS-3 (JAI, Japan), which was connected directly to the injector of the GC. Helium (high purity, 99.99%) was used both as the GC carrier gas and as the inert atmosphere for thermal desorption. Pine needle (1 mg) was placed into the pyrofoil, which was then put in the quartz tube and was introduced into the injector port of GC. In order to evaporate the moisture before commencing the thermal desorption, the system was purged for a short time (30 s) with the carrier gas. After purging, the quartz tube was heated, whence the volatile components were transferred from the quartz tube to GC-MS without significant loss. Experiments were carried out for 0.1 min (heating time) at temperature of 160℃.

7) Gas Chromatography-Mass Selective Detector (GC/MSD)

GC/MSD was performed with Agilent-6890/5973N. The injector and interface parts were both operated at 250 ℃. An INNOWAX fused silica capillary column (60 m × 0.25 mm; film thickness 0.25 μm) was used for the chromatography. Samples were injected in the split mode with a splitting ratio of 1:50. Helium was used as the carrier gas with a flow rate of 0.5 mL/min. The oven temperature of GC was programmed from 50℃ to 220℃ by increasing at 3℃/min. Ion source energy of 70 eV and electron multiplier of 1,450 V were utilized. Identification of volatile compounds was achieved by comparison of the GC retention time and mass spectra with those, when available, of the pure standard compounds. All mass spectra were compared with those of the data system library of the GC/MSD (wiley7n.l.).

Results and Discussion

A summary of the data obtained by the six methods evaluated is presented in Table 1. This table gives com-

Table 1. Comparison on aroma components obtained from different extraction methods

Peak No.	RI[a]	Components	Peak area (%)					
			DHS[b]	RPHS[c]	SPME[d]	SDE[e]	SFE[f]	PDE[g]
1	978	Ethyl acetate	0.03	-[h]	-	-	-	-
2	1001	Ethanol	1.06	2.07	-	-	-	0.12
3	1014	2-Ethyl furan	0.08	0.03	-	-	-	-
4	1046	Tricyclene	0.49	0.69	0.38	0.78	-	0.79
5	1055	α-Pinene	15.66	20.07	6.27	12.37	2.71	24.61
6	1083	Camphene	2.42	1.79	0.75	2.41	0.34	1.77
7	1095	Hexanal	1.17	0.20	0.01	0.02	-	0.07
8	1116	β-Pinene	5.41	4.10	1.47	3.50	0.65	2.42
9	1129	Sabinene	0.73	0.62	0.39	0.18	0.03	0.30
10	1170	β-Myrcene	15.63	12.18	4.75	5.93	1.17	5.52
11	1183	α-Terpinene	0.58	0.10	0.01	0.15	-	-
12	1207	Limonene	4.47	4.88	1.88	1.85	0.42	2.29
13	1219	β-Phellandrene	15.29	25.65	9.97	7.08	1.78	15.14
14	1233	2-Hexenal	2.54	2.46	-	0.30	-	1.17
15	1255	γ-Terpinene	0.39	0.14	0.05	0.42	0.02	0.03
16	1282	p-Cymene	0.45	0.06	0.02	0.03	-	-
17	1292	α-Terpinolene	6.63	2.18	0.80	4.07	1.16	0.80
18	1330	2-Pentenol	0.29	0.14	-	0.07	-	-
19	1361	Hexanol	1.09	0.22	0.01	0.14	0.01	0.09
20	1380	*trans*-3-Hexenol	0.21	0.13	-	-	-	-
21	1395	*cis*-3-Hexenol	9.31	3.02	0.03	2.01	0.32	0.38
22	1414	*trans*-2-Hexenol	0.29	0.06	-	0.03	-	-
23	1422	2,4-Hexadienal	0.09	0.10	-	-	-	-
24	1469	α-Cubebene	0.09	0.08	0.39	0.13	-	0.09
25	1485	α-Longipinene	0.07	0.09	0.16	-	-	-
26	1486	Acetic acid	-	-	-	-	0.46	0.32
27	1490	Furfural	-	0.04	-	-	-	-
28	1495	Bicycloelemene	0.09	0.05	1.08	0.55	0.50	-
29	1508	α-Copaene	0.13	0.18	0.82	0.20	0.21	0.29
30	1544	Camphor	0.49	0.22	0.13	0.08	0.20	0.11
31	1553	Benzaldehyde	0.20	0.12	0.11	0.16	0.12	0.18
32	1565	β-Cubebene	0.07	0.10	0.33	0.08	0.46	-
33	1590	Junipene	0.40	0.37	5.51	0.38	0.46	0.01
34	1599	Bornyl acetate	0.72	1.03	2.33	2.11	0.56	1.41

Table 1. Continued

Peak No	RI[a]	Components	Peak area(%)					
			DHS[b]	RPHS[c]	SPME[d]	SDE[e]	SFE[f]	PDE[g]
35	1605	β-Elemene	0.21	0.34	2.98	1.20	1.23	0.64
36	1611	Thymyl methyl ether	0.34	1.07	1.88	0.59	0.46	0.49
37	1617	β-Caryophyllene	3.62	5.30	17.15	8.07	9.05	7.86
38	1627	Aromadendrene	0.07	0.07	0.41	0.12	-	-
39	1692	α-Caryophyllene	0.51	0.06	2.98	1.72	1.73	1.13
40	1708	α-Amorphene	0.16	0.29	2.01	0.64	0.59	0.70
41	1723	α-Terpineol	-	-	-	1.04	0.34	-
42	1728	Borneol	0.10	0.06	0.23	0.22	0.81	0.07
43	1734	Germacrene D	1.25	5.29	14.47	7.41	8.63	11.14
44	1745	α-Muurolene	0.06	0.28	1.90	1.04	0.43	0.58
45	1759	Bicyclogermacrene	0.20	0.70	2.59	1.34	1.55	1.44
46	1780	□-Cardinene	0.21	0.52	4.32	2.80	1.90	1.35
47	1783	γ-Cardinene	0.16	0.28	2.37	0.61	0.94	0.91
48	1839	Hexanoic acid	-	-	-	0.06	-	-
49	1880	Benzyl acetone	0.14	-	-	0.13	-	-
50	1962	3-Hexenoic acid	-	-	-	0.30	2.23	0.01
51	2003	Caryophyllene oxide	-	-	-	0.41	0.28	0.39
52	2007	4-Phenyl-2-butanol	-	-	-	0.30	0.57	-
53	2028	Methyl eugenol	-	-	-	0.21	0.14	-
54	2055	Nerolidol	-	-	-	0.38	0.18	-
55	2066	1,6-Germacradien-5-ol	-	-	-	0.65	2.00	0.46
56	2088	3-Phenyl propenal	-	-	-	-	-	0.01
57	2102	Octanoic acid	-	-	-	-	-	1.83
58	2141	Spathulenol	-	-	-	0.66	0.48	0.53
59	2146	3-Hexenyl benzoate	-	-	-	0.73	0.13	0.02
60	2205	α-Cadinol	-	-	-	2.74	1.75	0.51
61	2462	Benzoic acid	-	-	-	0.22	7.08	0.01
62	2495	Lauric acid	-	-	-	0.23	1.18	-
63	2595	Thunbergol	-	-	-	2.13	7.88	1.13
64	2650	Hexenyl cinnamate	-	-	-	1.54	0.51	-
65	2686	Benzyl benzoate	-	-	-	0.07	-	-

[a] Retention indices on an innowax column relative to C_6~C_{26} n-alkanes, [b] DHS : Dynamic Headspace method,
[c] RPHS : Reduced Pressure Headspace method, [d] SPME : Solid Phase Microextraction method,
[e] SDE : Simultaneous Distillation Extraction method, [f] SFE : Supercritical Fluid Extraction method,
[g] PDE : Pyrolysis Distillation Extraction method, [h] (−) : Not detected.

parative information of the compounds isolated by each technique. A total of 65 compounds were identified by using the six different extraction methods. These compounds are classified into six categories in terms of chemical functionality: 25 hydrocarbons, 16 alcohols, 9 carbonyls, 6 esters, 7 acids and 2 ethers. Among these compounds, the major components were α-pinene (2.7~24.6%), β-myrcene (1.2~15.6%), β-pinene (0.7~5.4 %), β-phellandrene (3.6~17.2%), and germacrene D (1.3 ~ 14.5%). These findings were some difference to those of Roussis *et al.*[18], who reported that the major components of volatile compounds from five pine species grown in Greece were α-pinene (5.1~52.2%), β-myrcene (1.3~8.8%), β-pinene (1.1~45.7%), β-phellandrene (0.3 ~ 13.8%), and germacrene D (0.1~19.1%). Also, the analysis of Woo *et al.*[19] using a SFE device on Korean pine twigs (*P. densiflora*) found that α-pinene (5.3~11.7%), β-myrcene (11.5-17.3%), β-pinene (10.8~18.5 %), Limonene (32.6~43.4%), and germacrene D (5.6~11.3%). Thus, it can be seen that these variations are due to differences not only in extraction methods, but also to differences in the organic compounds composition of pine needles and twigs of the same species. Eakin has reported that compounds that give rise to flavouring properties described as herb, spicy, and citrus are not terpenoid hydrocarbons but are oxygenated terpens, such as terpene alcohols or terpene esters[20]. This study verified the presence of the oxygenated terpene substrates 2-hexenal, 2-pentenol, *trans*-2-hexenol, *trans*-3-hexenol, and *cis*-3-hexenol.

As shown in Table 1, the aroma compounds identified by the six different extraction methods were divided into two categories. The aroma compounds having low boiling point and low molecular masses were more abundant in DHS, RPHS, and SPME extracts, compared with the other methods. On the other hand, the aroma compounds having high boiling point and high molecular mass were more abundant in SDE, SFE and PDE extracts. These results bear some resemblance to those reported by Valero[21] in his analysis of aroma compounds of cheese by using analytical techniques such as DHS and SDE. DHS is assumed to be the most effective method for the analysis of aroma compounds with low boiling point [22,23]. In addition, aroma component that have low boiling point such as ethyl acetate was identified in the DHS method,

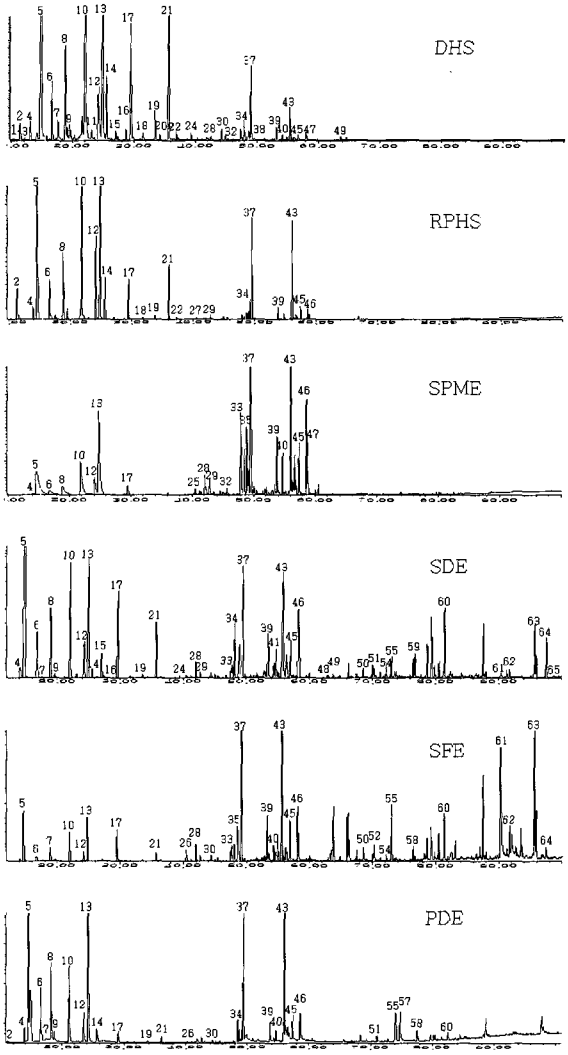

Fig. 1. Total ion chromatograms of aroma components obtained from different extraction methods.

but this component was not detected in the RPHS and SPME methods.

The compounds with high boiling point such as 3-hexenoic acid, methyl eugenol, nerolidol, spathulenol, α-cardinol, benzoic acid, thunbergol and hexenyl cinnamate, were found by SDE, SFE and PDE methods, but not by DHS, RPHS or SPME methods. For analyzing high boiling point compounds, the SDE is assumed to be the most effective method.

The oxygenated terpenes, such as hexanal, hexenal, hexanol, hexenol, and hexadienal, which are formed by oxidation of unsaturated fatty acids, were more abundant in DHS and RPHS extracts, compared with the other methods.

Among the oxygenated terpenes, *cis*-3-hexenol constituted 9.3% in DHS, whereas this component was present at levels from 0.1% to 0.4% in SPME, SFE and PDE. Meanwhile, *trans*-3-hexenol and 2,4-hexadienal were identified by DHS and RPHD only.

Thus, the headspace extraction methods are assumed to be the most effective to extract alcohol and carbonyl compounds from pine needle. The acid compounds, such as acetic acid, octanoic acid, benzoic acid and lauric acid, were extracted by heat-based extraction methods such as SDE, SFE and PDE, but not by DHS, RPHS and SPME, which used neither solvent nor heat.

The PDE method is deemed among the six methods as the most straightforward technique. This method is not as effective as SDE in terms of extraction capability but useful for the analysis of many samples for a short period of time given its simple process.

From all these results, it can be concluded that the SDE technique is the most effective for the analysis of high boiling point aroma compounds extracted from pine needle whereas the DHS method is the most effective for the analysis of low boiling point compounds. Furthermore, the PDE method is deemed to be suitable for the analysis of many samples in a short time because of its fast and simple extraction process.

Conclusion

The aroma compounds of pine needle (*P. densiflora*) were extracted by DHS, RPHS, SPME, SDE, SFE and PDE techniques. The extracted aroma compounds were compared by gas chromatography-mass spectrometry (GC-MS).

A total of 65 compounds were identified by using the six different extraction methods. These compounds are classified into six categories in terms of chemical functionality: 25 hydrocarbons, 16 alcohols, 9 carbonyls, 6 esters, 7 acids, 2 ethers. Among these compounds, the major components were α-pinene (2.7～24.6%), β- myrcene (1.2～15.6%), β-pinene (0.7～5.4%), β-phellandrene (3.6～17.2%), and germacrene D (1.3～14.5%). The SDE technique was found to be the most effective for the analysis of high boiling point aroma compounds extracted from pine needle while the DHS method was the most effective for the analysis of low boiling point compounds. The PDE method was found to be suitable for the analysis of many samples in a short time because of its fast and simple extraction process.

요 약

식물체의 향기성분을 분리하는데 주로 사용되는 headspace(DHS, RPHS)법, SPME법, SDE법, SFE법과 최근에 개발된 PDE법으로 솔잎의 휘발성 성분을 추출하였다. 추출된 성분을 GC/MS를 이용하여 총 65종의 휘발성 성분을 확인하였다. 확인된 성분은 terpene류 25종, alcohols류 16종, carbonyls류 9종, esters류 6종, acids류 7종, esters 화합물이 2종인 것으로 나타났다.

Headspace(DHS, RPHS) 법과 SPME 법에서는 휘발성이 강한 ethyl acetate, 2-ethyl furan, β-myrcene 등과 같은 저비점 휘발성 성분들이 많이 추출되었다. 반면에, 고비점 휘발성 성분들인 nerolidol, spathulenol, benzoic acid 등은 휘발 성분 추출시 열이 가해지는 SDE 법, SFE 법, PDE 법 등에서는 확인되었으나 headspace 법과 SPME 법에서는 확인되지 않았다. 이들 6가지 추출방법에서 저비점 휘발성 성분 분석에는 headspace (DHS) 법이 가장 좋은 것으로 나타났으며, 고비점 휘발성 성분 분석에는 SDE 법이 가장 적합한 것으로 나타났다.

최근에 개발된 PDE법은 SDE법에 비해 추출효율면에서 약간 떨어지나 전처리 방법이 간단하므로 짧은 시간에 많은 시료를 분석할 때 가장 적합한 방법인 것으로 판단된다.

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