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Removal of Toxic Aqueous Essential Oils from *Chamaecyparis obtusa* by Liquid-Liquid Extraction and Solid-phase Extraction

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A method was developed to remove five toxic aqueous essential oils (α-terpinene, γ-terpinene, linalool, α-terpineol and α-terpinyl acetate) from *Chamaecyparis obtusa* by liquid-liquid extraction and solid-phase extraction with gas chromatography analysis. The best liquid-liquid extraction conditions were as follows: extraction solvent, 1-octanol and an essential oil:1-octanol ratio of 7:3. The best solid-phase extraction conditions were as follows: sorbent, C_{18}; and washing and elution step solvents of 2 mL 1-octanol and 1 mL water, respectively. GC-FID was used to detect the five toxic aqueous essential oils. This method was simple and could remove the toxic aqueous compounds from natural product oils.

Key Words: Liquid-liquid extraction, Solid-phase extraction, Toxic aqueous, Essential oils, *Chamaecyparis obtusa*.

INTRODUCTION

Chamaecyparis obtusa is also called Japanese cypress in Japan and Taiwan. The species of *Chamaecyparis* become well known in South Korea from the 1990s[1,2]. The essential oils extracted from the leaves of the *C. obtusa* tree are used generally as a functional additive with good fragrance in soaps, toothpastes and cosmetics[3]. The essential oils from *C. obtusa* have a range of biological activities, such as antibacterial, antifungal, anti-mite, anti-termite and acaricidal *etc*.[4]. Fig. 1 shows the structures of five target compounds, such as α-terpinene, γ-terpinene, linalool, α-terpineol and α-terpinyl acetate. Of the essential oils, α-terpinene (1-isopropyl-4-methyl-1,3-cyclohexadiene) is found in a wide range of valuable and aromatic plants. Plants and essential oils including α-terpinene are used extensively in traditional medicine and cosmetics. On the other hand, α-terpinene exhibits embryofoeto toxicity[5]. Other target compounds, such as γ-terpinene, linalool, α-terpineol and α-terpinyl acetate, have some bioactivities, such as antimicrobial, anticonvulsant *etc*.[6-8]. These compounds have some toxic compounds and there are many studies on the essential oil bioactivities and evaluated toxicity[9,10]. Liquid-liquid extraction is a common sample preparation method in an analytical process. This method has several advantages, such as increased selectivity by separating the analyte from the mixture and concentrating the analyte from a large sample volume[11]. On the other hand, liquid-liquid extraction has some problems, such as time consuming, expensive and requiring large amounts of toxic organic solvents[12].

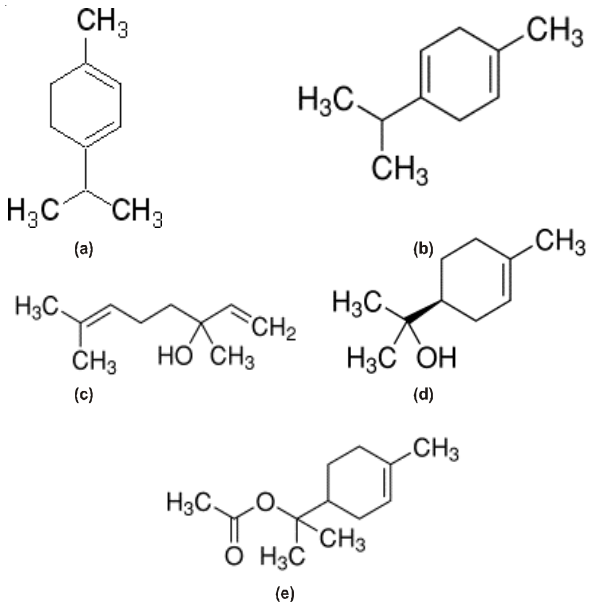

Fig. 1. Structures of (a) α-terpinene, (b) γ-terpinene, (c) linalool, (d) α-terpineol and (e) α-terpinyl acetate

Solid-phase extraction is used extensively as a sample preparation method for the purification of target compounds

and for applications, such as the removal of toxic or active constituents. Solid-phase extraction become well known in the early 1970s because it minimizes the weakness of liquid-liquid extraction[13]. A normal solid-phase extraction cartridge is composed of a short column (open syringe barrel) packed with a sorbent[14]. Solid-phase extraction is founded on the distinct transfer of compounds absorbed and desorbed during eluted between the sorbent material and mobile phase. Retention is related to the hydrophobic, polar, ion exchange interactions between the target compounds and the surface of the sorbent[15].

In this study, solid-phase extraction separation was performed using liquid-liquid extraction to separate the five toxic aqueous essential oils from *Chamaecyparis obtusa*. The efficiencies of the different solvents were obtained.

EXPERIMENTAL

Chamaecyparis obtusa was obtained from Jangseong (Jeollanam-do, Korea). The α-terpinene, γ-terpinene, linalool, α-terpineol and α-terpinyl acetate were supplied by Sigma-Aldrich (Milwaukee, WI, USA). Cyclohexanol, 1-octanol, dodecanol, oleyl alcohol, methanol and ethanol were obtained from Duksan Pure Chemical Co. Ltd. (Ansan, Korea). All chemicals and reagents were of HPLC grade and distilled water was filtered using a vacuum pump (Division of Millipore, Waters, USA) and filter (HA 0.45, Division of Millipore, USA) prior to use.

Chromatography conditions: Chromatography was performed using a Younglin 6100 GC system and Younglin flame ionization detector (Younlin Co. Ltd., Korea). Data analysis was performed using Autochro-3000 software. The GC consisted of a flame ionization detector (FID) and DB-1701 capillary column, 30 × 0.23 mm × 1.00 μm I.D. The injector and detector temperatures were 280 ºC and 300 ºC, respectively. The oven temperature program used was as follows: 5 min at 50 ºC, 10 min at 100 ºC and 1 min at 250 ºC, then held at that temperature for 5 min. Nitrogen, hydrogen and air were used as the carrier gases.

Preparation of standard solution and sample solution: A stock solution of the five target compounds at 100 μg/mL was prepared in methanol. *Chamaecyparis obtusa* was powdered and 20 g of the resulting powder was weighed and extracted with 200 mL water for 24 h by hydro distillation at 100 ºC. The extracts were then combined. After centrifugation and filtration, the extracts were collected and stored at room temperature.

Liquid-liquid extraction procedure: Different extraction solvents (cyclohexanol, 1-octanol, dodecanol and oleyl alcohol) were used to determine which could extract the highest amount of the target compounds. After extraction, different ratios of essential oil aqueous solvent/organic solvent (1:9, 3:7, 5:5, 7:3 and 9:1) were examined to determine which could remove the large amount of target compound. For this process, the extract was collected in a 5 mL vial and stored for injection.

Solid-phase extraction procedure: Commercial solid-phase extraction polypropylene cartridges (diameter 0.9 cm, 3 mL) with 200 mg of C_{18} sorbent were purchased from Alltech (Deerfield, IL, USA). 2.0 mL of the essential oils from *C. obtusa* were loaded into the solid-phase extraction cartridge, washed with 1 mL of 1-octanol and then eluted with 2 mL of water, ethanol and methanol, sequentially.

Linearity and reproducibility: A standard solution containing α-terpinene, γ-terpinene, linalool, α-terpineol and α-terpinyl acetate were diluted (10, 20, 50, 70 and 100 ng/mL) in methanol. As a result, linear regression equations (Y = a + b) of the five compounds were obtained within the concentration range studied. X and Y represent the peak areas and concentrations of the analyte, respectively. Table-1 lists the results of regression analyses along with the correlation coefficients (r^2). The high correlation coefficients ($r^2 > 0.9990$) indicated good linearity between their peak areas (X) and examined the compound concentrations (Y, ng/mL) in a relatively wide concentration range. Assays of the repeatability, which were calculated as the standard deviations (SD), were performed. A SD < 5.0 ng/g showed acceptable precision and accuracy.

TABLE-1
REGRESSION EQUATIONS OF TARGET COMPOUNDS
(Y: CONCENTRATION, x: PEAK AREA)

Target compounds	Regression equation	r^2
α-Terpinene	Y = 2025.2x + 76981	0.9995
γ-Terpinene	Y = 2172.0x + 88594	0.9998
Linalool	Y = 1608.5x + 63568	0.9990
α-Terpineol	Y = 1691.3x + 70293	0.9998
α-Terpinly acetate	Y=166.8x + 58681	0.9992

RESULTS AND DISCUSSION

Effect of liquid-liquid extraction: The selected extraction solvent is an important process for increasing the extraction efficiency. The target compounds can be dissolved in alcohol (methanol, ethanol *etc.*) but cannot be used owing to their solubility in water. Liquid-liquid extraction extracted the target compounds from a water solution into an extraction solvent immiscible in water. The driving force of liquid-liquid extraction is the distribution coefficients of the target compounds between the extraction solvent and water solution and transport across the liquid-liquid interface arises by diffusion[16]. In this study, four different extraction solvents, cyclohexanol, 1-octanol, dodecanol and oleyl alcohol, were examined. Table-2 lists the respective amount remaining after extraction in the water phase by the different solvents. Cyclohexanol has a cyclic structure and 1-octanol, dodecanol and oleyl alcohol are linear with different lengths of the carbon chain. Generally, dodecanol and oleyl alcohol were used as surfactants but in this experiment, dodecanol and oleyl alcohol with long carbon chain lengths were used to extract the target compounds, so they can remove a small amount of the target compound in essential oil. 1-Octanol can remove many target compounds from essential oil and retain a small amount of the target compounds on the sorbent. Therefore, 1-octanol was selected as the optimal extraction solvent. After selected extraction solvent, the amounts of five target compounds extracted using different 1-octanol ratios (essential oil: 1-octanol, 1:9, 3:7, 5:5, 7:3 and 9:1) were investigated. The results are listed in Table-3. Fig. 2 shows that at a higher essential oil:1-octanol ratio, more target

TABLE-2
REMAINED AMOUNT AFTER LIQUID-LIQUID EXTRACTION IN WATER PHASE BY DIFFERENT SOLVENTS

Solvent	Remained amount (ng/g, n = 3)				
	α-Terpinene	γ-Terpinene	Linalool	α-Terpineol	α-Terpinyl acetate
Essential oil	902.32	1848.51	412.93	209.66	3387.79
Cyclohexanol	24.00 ± 0.5	10.06 ± 0.6	31.57 ± 0.5	33.31 ± 0.4	172.11 ± 1.1
1-octanol	3.44 ± 0.2	10.87 ± 1.1	17.30 ± 0.9	11.49 ± 0.1	11.13 ± 0.8
Dodecanol	8.34 ± 0.4	22.95 ± 0.8	7.55 ± 0.5	3.42 ± 0.7	6.31 ± 0.8
Oleyl alcohol	40.21 ± 0.2	47.59 ± 1.2	31.39 ± 1.5	24.39 ± 0.6	221.13 ± 0.7

TABLE-3
REMAINED AMOUNT IN THE WATER PHASE AFTER LLE BY DIFFERENT ESSENTIAL OIL:1-OCTANOL RATIOS

Essential oil: octanol ratio	Remained amount (ng/g, n=3)				
	α-Terpinene	γ-Terpinene	Linalool	α-Terpineol	α-Terpinyl acetate
Essential oil	902.32	1848.51	412.93	209.66	3387.79
1:9	26.83 ± 1.1	87.00 ± 0.2	183.27 ± 1.9	136.13 ± 6.4	18.20 ± 0.8
3:7	17.85 ± 0.3	40.66 ± 0.7	50.44 ± 0.4	46.50 ± 0.6	16.69 ± 1.1
5:5	14.48 ± 0.7	24.28 ± 1.2	31.25 ± 2.9	11.49 ± 0.5	11.14 ± 1.1
7:3	3.45 ± 1.1	10.89 ± 0.8	17.30 ± 0.4	11.00 ± 0.6	3.52 ± 1.1
9:1	10.58 ± 0.5	31.21 ± 0.6	24.65 ± 1.0	37.70 ± 0.3	5.35 ± 1.3

TABLE-4
REMAINED AMOUNT OF THE FIVE TARGET COMPOUNDS WITH DIFFERENT SOLVENTS IN ELUTION STEP. (C_{18} CARTRIDGE)

Elution solvent	Remained amount (ng/g, n=3)				
	α-Terpinene	γ-Terpinene	Linalool	α-Terpineol	α-Terpinyl acetate
Essential oil	902.32	1848.51	412.93	209.66	3387.79
Water	-	-	-	6.75 ± 1.8	2.77 ± 1.8
Ethanol	23.55 ± 2.0	6.51 ± 0.8	177.85 ± 10.0	110.79 ± 5.0	-
Methanol	27.51 ± 1.8	62.88 ± 2.0	458.22 ± 12.7	89.30 ± 2.0	-

- = not detected

compounds remained in the essential oil. The five toxic aqueous compounds could be removed from the essential oils at an essential oil : 1-octanol ratio of 7:3.

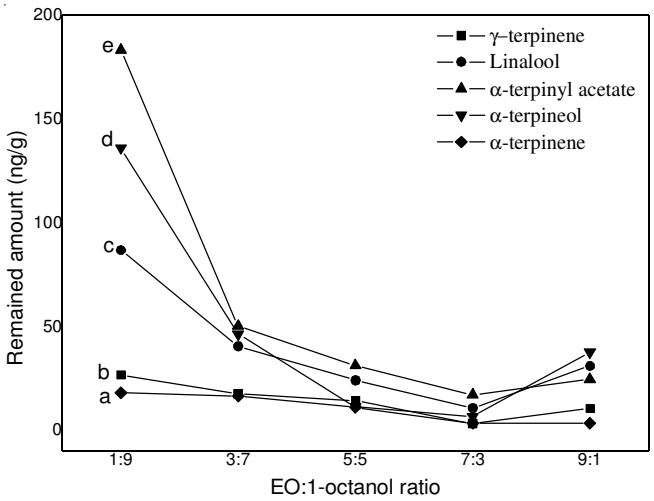

Fig. 2. Effect of essential oil: 1-octanol ratio of liquid-liquid extraction. (a) α-terpinene, (b) γ-terpinene, (c) linalool, (d) α-terpineol and (e) α-terpinyl acetate

Effect of solid-phase extraction: The selected washing solvent and elution solvent is important in solid-phase extraction. According to the result of the liquid-liquid extraction process, 1-octanol was selected as the washing solvent because it is the best solvent to remove the target compounds from essential oils. To determine the optimal volume of washing solvent, the essential oil and 1-octanol ratio to be applied in the liquid-liquid extraction process was found to be 7:3. select because this ratio remove very well target compounds in essential oils. 1 mL of 1-octanol was used as the washing condition. Table-4 lists the amounts of five target compounds extracted by the different elution solvents. Methanol and ethanol could not be used elute solvent, because the target compounds are soluble in alcohol. Many other compounds were also eluted from the C_{18} sorbent but water did not elute the aqueous toxic components from the C_{18} sorbent. Therefore, water was used as the elute solvent. α-Terpinene, γ-terpinene and linalool were not detected but α-terpineol and α-terpinyl acetate were detected. On the other hand, the amounts remaining were very small. The driving force of solid-phase extraction is the intermolecular forces between the target compounds, activity range on the surface of the sorbent and liquid phase[17]. Equilibrium developed between the liquid and solid phases (or liquid and liquid)[18]. Fig. 3 shows the solid-phase extraction process after most of the five toxic aqueous target compounds had been removed. Fig. 4 compares the liquid-liquid extraction and solid-phase extraction methods. As a result, solid-phase extraction was more effective method in removing the aqueous toxic compounds from the *Chamaecyparis obtusa* essential oils than liquid-liquid extraction.

Effect of removal toxic aqueous: Terpinene-4-ol is a major compound in *C. obtusa* essential oils. Terpinene-4-ol has bioactivities, such as anti-candida, antifungal and anti-inflammation *etc.*[19], but no toxic effects. Table-5 lists the related peak area of the target compounds and terpinene-4-ol

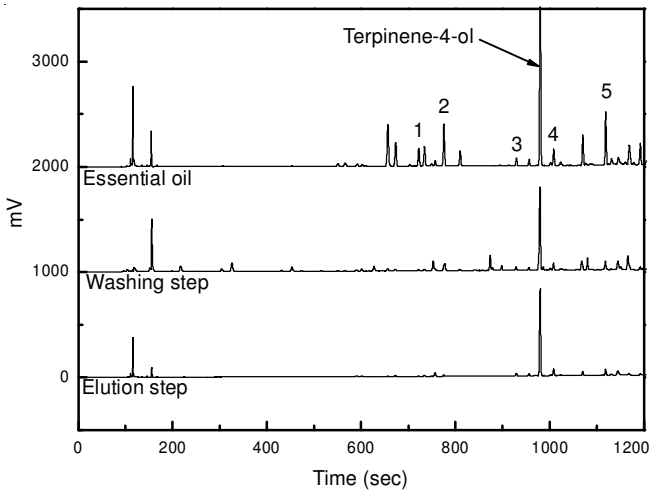

Fig. 3. Chromatogram of extracted essential oil, washing and elution step of SPE; (Column: DB-1701, 30 × 0.23 mm × 1 μm i.d, detector: FID, carrier gas: H_2, N_2, air, Injector temp.: 280 ºC, Oven temp.: 250 ºC, Detector temp.: 300 ºC, injection vol.: 2 μL, SPE condition: washing solvent: 1-octanol, 1: α-terpinene, 2: γ-terpinene, 3: Linalool, 4: α-terpineol, 5: α-terpinyl acetate)

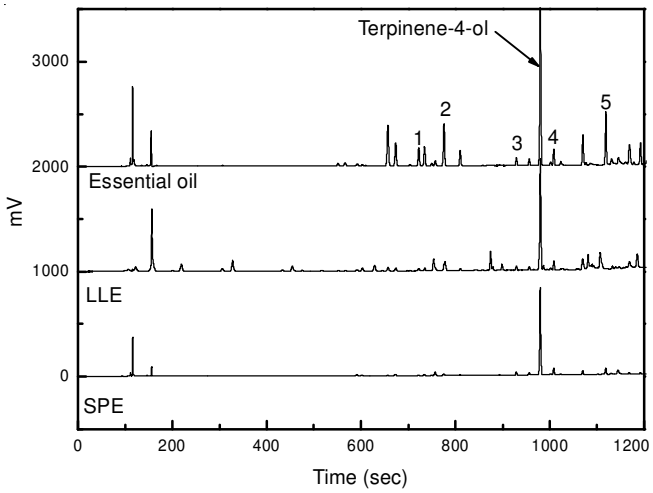

Fig. 4. Comparison of the chromatograms of essential oils after SPE and LLE; (Column: DB-1701, 30 × 0.23 mm × 1 μm i.d, Detector: FID, carrier gas: H_2, N_2, air, Injector temp.: 280 ºC, Oven temp.: 250 ºC, Detector temp.: 300 ºC, injection vol.: 2 μL, SPE condition: washing solvent: 1-octanol, 1: α-terpinene, 2: γ-terpinene, 3: Linalool, 4: α-terpineol, 5: α-terpinyl acetate)

TABLE-5
RELATED PEAK AREAS OF REMAINED MAJOR AND TARGET COMPOUNDS AFTER LLE AND SPE

Compounds	Related peak areas (%)		
	Essential oil	LLE	SPE
α-Terpinene	2.73	0.25	0.22
γ-Terpinene	6.17	1.73	0.08
Linalool	2.26	1.49	1.06
α-Terpineol	3.76	3.32	2.19
α-Terpinyl acetate	7.31	3.30	2.79
Terpinene-4-ol	24.92	42.47	43.06

after liquid-liquid extraction and solid-phase extraction. As the target compounds had been removed by liquid-liquid extraction and solid-phase extraction, the related peak had decreased from 6.17 to 1.73 % (for liquid-liquid extraction) and 0.08 % (for solid-phase extraction). On the other hand, the related peak area of terpinene-4-ol increased from 24.92 to 42.47 % (for liquid-liquid extraction) and 43.06 % (for solid-phase extraction). In solid-phase extraction, the target compounds are extracted from the solid and liquid because these compounds have greater affinity for the solid phase than for the sample (retention or adsorption step). The compounds remained on the solid phase and were removed after elution step with an organic solvent with greater affinity for the target compounds (elution or desorption step). Accordingly, the liquid-liquid extraction and solid-phase extraction methods can purify the useful compounds and remove the toxic compounds. Therefore, five toxic aqueous compounds were removed and the bioactive compounds remained in the essential oils after liquid-liquid extraction and solid-phase extraction.

Conclusion

Liquid-liquid extraction and solid-phase extraction methods were used to remove five toxic aqueous compounds in *Chamaecyparis obtusa* essential oil. After the solid-phase extraction process, the toxic aqueous essential oils were removed. The optimal conditions of liquid-liquid extraction were an extraction solvent of 1-octanol at an essential oil: 1-octanol ratio of 7:3. Under this condition, the amounts of the five toxic aqueous compounds removed, α-terpinene, γ-terpinene, linalool, α-terpineol and α-terpinyl acetate, were 3.45, 10.89, 17.30, 11.00 and 16.69 ng/g, respectively. The optimal conditions of solid-phase extraction were as follows: C_{18} sorbent, a 2 mL loading of the essential oil, 2 mL of 1-octanol as the washing solvent and 1 mL water as the elution solvent. Under these conditions, of the five toxic aqueous compounds, α-terpinene, γ-terpinene and linalool were not detected and only small amounts of α-terpineol (6.75 ng/g) and α-terpinyl acetate (2.77 ng/g) were detected. The low deviation error demonstrated the method to be a viable alternative tool for further studies. When the five toxic aqueous compounds were removed from the essential oils, the related peak area of terpinene-4-ol was increased from 24.92 to 42.47 % (for liquid-liquid extraction) and 43.06 % (for solid-phase extraction).

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