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SEPARATION OF PERILLYL ALCOHOL FROM KOREAN ORANGE PEEL BY SOLVENT EXTRACTION AND CHROMATOGRAPHY

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Abstract – Perillyl alcohol, abundant mainly in oranges, has chemotherapeutic activity against carcinogenesis. The peel of Korean oranges was extracted by methanol, and the extract was partitioned by methanol-extract/water/chloroform, (20/5/30 vol%). To concentrate perillyl alcohol of the water-phase in the previous partition step, a glass column (2.5 i.d.×15 cm) with reversed-phase C_{18} packings (40-63 μm) was used. Finally, to obtain perillyl alcohol in a pure form, reversed-phase high-performance liquid chromatography (RP-HPLC) was applied. Mobile phases used were water, methanol, and acetonitrile. The flow rate of the mobile phase was 1 ml/min and UV wavelength was fixed at 205 nm. The resolution of perillyl alcohol from Korean orange peel was achieved on a μ-Bondapak C_{18} column (3.9×300 mm, 10 μm) and in-house chromatographic column packed with 15 μm C_{18} preparative packings. From the experimental results, the mobile phase composition was water/acetonitrile, (65/35 vol%) and the retention time of perillyl alcohol was 20.5 min in the analytical μ-Bondapak column. The effect of injection volumes was investigated in the preparative column.

Key words : Perillyl Alcohol, Korean Orange Peel, Solvent Extraction, Chromatography

INTRODUCTION

Plants have been recognized as major sources of an extremely wide range of organic chemicals. Many compounds of plant origin are used in food flavorings and colorings, and more importantly provide the basis for medicine [Glidewell, 1991]. Numerous epidemiological studies have revealed that high consumption of some plant products correlates with a reduction in cancer incidence [Burke et al., 1997].

The monoterpene, one of the simplest groups in natural plant products, is recognized to possess chemopreventive properties against mammary, liver, and lung carcinogenesis [Gelb et al., 1995; Haag et al., 1994]. The intake of diets containing fruits and vegetables, major sources of phytochemicals and micronutrients, may reduce the risk of developing cancer [Stark et al., 1995]. Monoterpenes are naturally occurring substances derived from orange peels, lavender, mints, and celery seeds [Reddy et al., 1997]. Perillyl alcohol, a monoterpenoid, is an hydroxylated product of d-limonene (p-mentha-1,8-diene) that is formed by the condensation of two isoprene molecules. Perillyl alcohol has chemotherapeutic activity against chemically induced rat mammary tumors with little toxicity to the host, which inhibits the proliferation of cultured human colon carcinoma cell. Moreover, perillyl alcohol is not only a potent breast anti-cancer agent but also an effective chemotherapeutic agent against advanced mammary tumors [Mills et al., 1995]. Monoterpenes are also used as a promising alternative to CFC cleaning solvents. Recently, such solvents as chlorinated hydrocarbons and chlorofluorocarbons (CFC), which are used for defluxing and precision cleaning, have been gradually replaced by naturally occurring products containing monoterpenes. The most frequently used compound is d-limonene [Nilsson et al., 1996]. The chemical is preferred mainly because of its environmentally less harmful properties [Nilsson et al., 1996].

Much effort has been made to commercialize the components in natural plants, especially by pharmaceutical companies. The most commonly used technique for biological samples is reversed-phase high performance liquid chromatography (RP-HPLC), which is normally done by n-octadecyl modified packings [Row and Larin, 1995a]. As the C_{18} are chemically bonded to the surface of tiny particles, these packings provide stability and reproducibility as well as selectivity [Row and Larin, 1995b]. The present work focused on the separation procedure of perillyl alcohol and d-limonene from the Korean orange [Row and Jung, 1997]. The peel of the Korean orange, disposed of as waste by home and food industry use, was extracted by solvents and separated by liquid chromatography. To obtain perillyl alcohol and d-limonene in a pure form efficiently, the pretreatment steps and the final chromatographic system need to be adequately configured, and this is the purpose of the work.

EXPERIMENTAL

1. Chemicals

The orange peel was purchased at a domestic market. The standard chemicals of perillyl alcohol and d-limonene were purchased from Aldrich Co. and Sigma Co., respectively. The concentration of perillyl alcohol and d-limonene dissolved in methanol was 10 mg/ml. The extra-pure grade solvents of

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hexane, methanol, ethanol, IPA, 1-propanol, chloroform, acetone, trichloroethylene, 1,1,1 trichloroethane, and methylene chloride were purchased from Dae Jung Chemicals & Metals Co., Ltd. (Korea). The water was distilled and deionized prior to use.

2. Analysis of Perillyl Alcohol and d-Limonene (GC)

The identification of peaks in the chromatogram was confirmed by the standard of perillyl alcohol and d-limonene. The extracts of Korean orange peel by solvents were analyzed with a Hewlett-Packard (HP) Model 5890 gas chromatograph (GC) equipped with a HP-5 (Crosslinked 5 % PH ME Siloxane, 30 m × 0.32 mm) capillary column and a flame ionization detector (FID). The oven temperature program was gradually raised from 50 °C to 80 °C by increases of 10 °C/min, followed from 80 °C to 250 °C by increases of 5 °C/min. The injector and detector temperatures were 250 °C and 300 °C, respectively. The flow rate of the carrier gas N_2 was 0.8 ml/min and the split ratio set at 1 : 50.

3. Extraction Step

Initially, perillyl alcohol and d-limonene from orange peel were extracted by solvents. The procedures were as follows. We weighed 100 g of dry Korean orange peel and chopped it into small pieces using a sharp knife. The chopped orange peel of 5 g was placed in a 500 ml triangle flask with 100 ml corresponding solvent. The temperature of the solvent was maintained at 20-60 °C. The orange peel with solvents was agitated over 3 hours in a stirrer. Then, the extracts were concentrated to 5 ml with a rotary evaporator (Resona technics, Switzerland).

4. Partition Step

The extract was mixed with water and chloroform, and the volume ratio of the extracts/water/chloroform was adjusted to experimentally determine the composition of solution in the partition step. After 30 min stirring, the two immiscible phases were collected with a funnel. Each phase was concentrated by rotary evaporator and the sample was analyzed by GC.

5. Open Column

To concentrate perillyl alcohol of the water-phase in the partition step, a glass column (2.5 i.d. × 15 cm) with reversed-phase C_{18} packings (40-63 μm, Merck, Germany) was used. The mobile phases were methanol, tetrahydrofuran, acetone, and acetonitrile by single or mixture state. The extracts, 2 ml, in the water-phase of the previous partition step were injected in the glass column. The effluent was collected every 10 min from the column outlet in ambient atmosphere. Each solution was concentrated to 1 ml for GC-analysis.

6. HPLC

The HPLC system was as follows : Waters Model 600 liquid chromatograph (Waters Associates, Milford, MA, U.S.A.) equipped with the Waters 600E Multisolvent Delivery System, a UV-visible tunable wavelength absorbance detector (Wasters 486), a U6K injector (2 ml sample loop). The data aquisition system was CHROMATE (V. 3.0, Interface Eng., Korea) installed in a PC. The mobile phases of water, methanol, and acetonitrile were experimented. The commercial analytical-column was μ Bondapak C_{18} (10 μm, 3.9 × 300 mm, Waters Co.) and preparative column (ODS, 15 μm, 3.9 × 300 mm) was in-house packed by vacuum pump. Flow rate of the mobile phase was fixed at 1.0 ml/min.

RESULTS AND DISCUSSION

To purify perillyl alcohol from the Korean orange, the steps developed in this work were extraction, partition, enrichment by an open glass column, and finally chromatographic separation.

1. Selection of Solvents for Perillyl Alcohol

The proper selection of solvent is very critical, because it depends on the efficiency and economics of the separation process. We used a wide range of polarity of solvents, hexane to methanol. The concentration of perillyl alcohol and d-limonene was experimentally measured, with the results summarized in Table 1. The two components were remarkably

Table 1. Concentration of perillyl alcohol and d-limonene in Korean orange peel with solvents

Experimental no.	Solvent	Concentration (μg/ml)	
		Perillyl alcohol	d-Limonene
1	Hexane	-	19.28
2	Methanol	0.72	12.11
3	Ethanol	0.24	2.86
4	IPA	0.33	17.59
5	1-Propanol	0.23	5.2
6	Chloroform	0.25	58.18
7	Acetone	0.26	86.73
8	Methylene chloride	-	204.35
9	Trichloroethylene	-	69.82
10	1,1,1 Trichloroethane	2.25	169.42

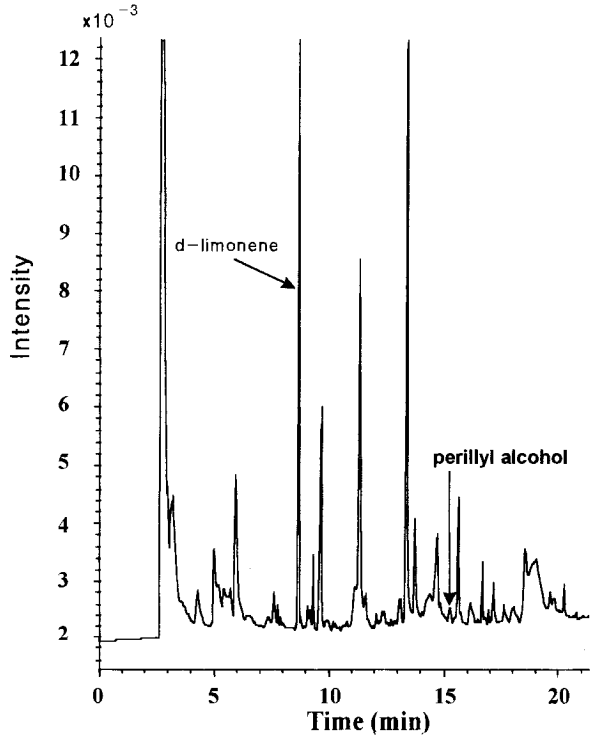

Fig. 1. Chromatogram of the extract by methanol on a HP-5 (30 m × 0.32 mm I.D.) capillary column.

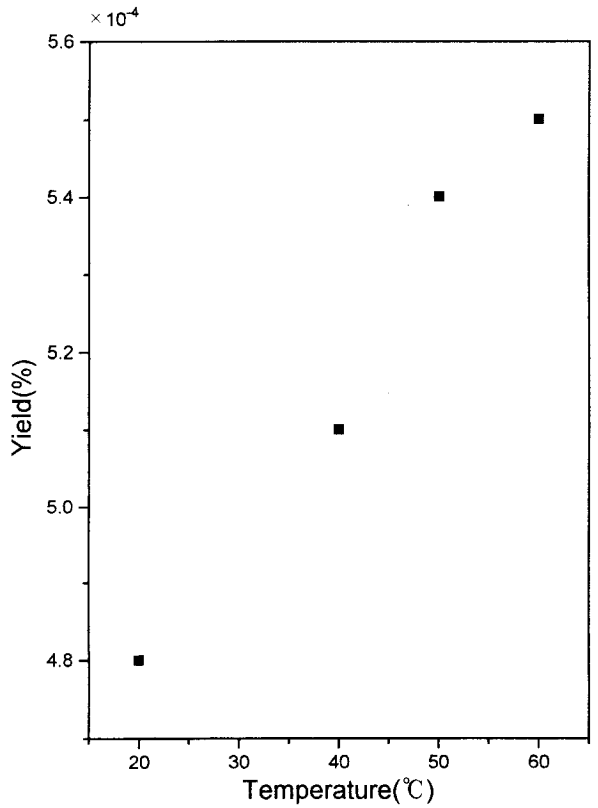

Fig. 2. Effect of methanol temperature on yield of perillyl alcohol.

dissolved in 1,1,1 trichloroethylene, but use of this solvent is restricted because of its ozone-depleting potential together with CFC 113. Methylene chloride had good solubility of d-limonene, but it did not dissolve perillyl alcohol well. The solubility of perillyl alcohol and d-limonene was greatly affected by different types of alcohols. For methanol, the GC-analysis of the extract is shown in Fig. 1. As listed in Table 1, the solubility of perillyl alcohol and d-limonene in methanol was superior to the other classes of alcohols, ethanol, IPA, and 1-propanol. The methanol cost was cheaper, but its toxicity was a important problem to solve prior to commercial use. Yield was defined by the percentage ratio of the weight of perillyl alcohol to the weight of dry Korean orange peel. Fig. 2 shows the effect of methanol temperature on yield of perillyl alcohol, which indicates that the yield increased with the temperature of the solvents.

2. Partition Step

Many components exist in the peel of a Korean orange. To remove the unnecessary components, the partition step was utilized as a pre-step to final chromatographic separation. In a funnel filled with chloroform and water, the nonpolar components in the methanol-extracted sample moved to chloroform, while the polar components to water. The concentrating effect can be enhanced by performing layer separation of the methanol-extracted sample between chloroform and water. The experimental results of different compositions of the extract, chloroform, and methanol are shown in Table 2. The concentrations of samples 1, 2 were 0.32 and 0.40 μg/ml, respectively. With a constant volume of extract, 20 ml, the volume ratio of chloroform and water was adjusted. It is desirable to decrease the amount of water for fast evaporation. From the GC-analysis of the top and bottom sample in the funnel, perillyl alcohol was dissolved in water, and d-limonene in chloroform. An adequate amount of methanol extracts should be added to be a miscible solution of chloroform and water. Perillyl alcohol was concentrated most satisfactorily in extract/water/chloroform (20/5/30 vol), where the amount of perillyl alcohol concentrated per that injected was 1.20. Perillyl alcohol was identified by the GC-analysis of the water phase in Fig. 3.

3. Enrichment by Glass Column

There are still a few useless components in the water phase containing perillyl alcohol. As a pretreatment step to the next chromatographic column, the sample was enriched by an open glass-column, where irregular shape reversed-phase C_{18} packings were filled. The sample passed down though the column by gravitational flow. The compositions of the mobile phase were varied as in Table 3. In addition to pure solvent, we experimented with binary and ternary mobile phases. When the mobile phase of pure water was used, the total elution time was very long. Perillyl alcohol strongly adsorbed on the C_{18} packing, so it was not eluted out within one hour. Similar trends were shown in the pure solvents of acetonitrile and acetone. Enrichment of perillyl alcohol by pure methanol was

Table 2. Concentration of perillyl alcohol in chloroform/water partition step

Sample /Run no.	Solvent (vol)			Concentration of perillyl alcohol (μg/ml)	Concentration effect based on the feed sample
	Methanol-extracts	Chloroform	Water		
Sample 1				0.32	1
Run #1	20	30	6	0.38	1.19
Run #2	20	40	10	0.33	1.03
Run #3	20	50	10	0.35	1.09
Run #4	20	40	5	0.34	1.06
Sample 2				0.40	1
Run #1	20	50	5	0.46	1.15
Run #2	20	40	5	0.47	1.18
Run #3	20	30	5	0.48	1.20
Run #4	20	20	5	no partition	
Run #5	20	10	5	no partition	

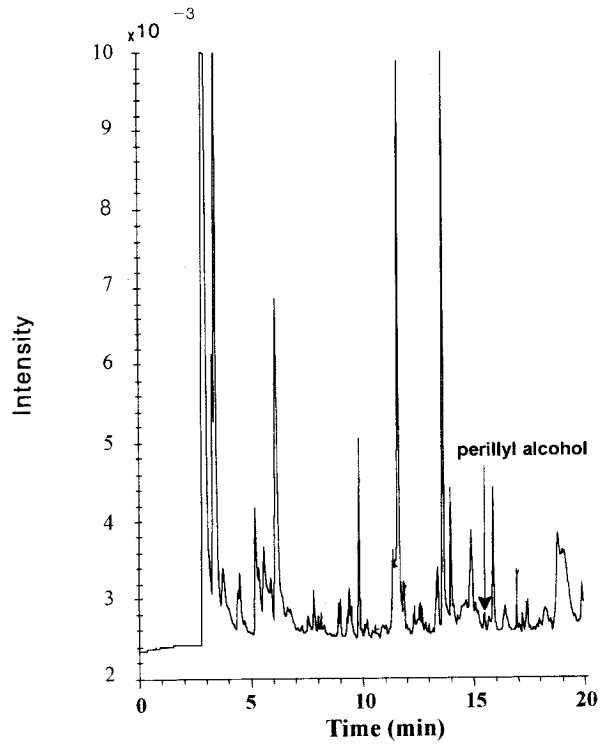

Fig. 3. Separation of perillyl alcohol in water-phase of the partition.

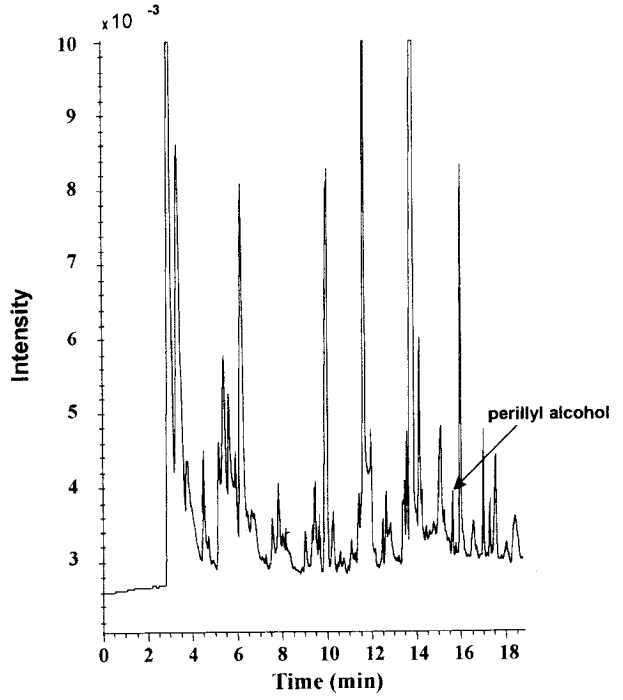

Fig. 4. Enrichment of perillyl alcohol by glass-column with methanol.

performed, and its experimental result is shown in Fig. 4. The pure solvent of methanol showed a concentrating effect, compared to the peak of perillyl alcohol in Fig. 3.

4. HPLC Separation

The Korean orange peel was extracted by methanol, and the extract was partitioned by chloroform/water, and further enriched by open glass-column. To further purify perillyl alcohol from the solution treated in the three steps mentioned above, final down-stream processing, RP-HPLC was used. Mobile phases were water, methanol, and acetonitrile. The two types of preparative (15 μm) as well as analytical (10 μm) packings were experimented at a constant flow rate of mobile phase, 1 ml/min. In a commercial analytical column (μ-Bondapak), the mobile phase composition was water/acetonitrile, 65/35 (vol%), and the injection volume was 10 μl. The two peaks of perillyl alcohol from a standard solution of 10 mg/ml (above) and the

Table 3. Types and composition of mobile phase used in open glass-column

Mobile phase (vol%)	Concentration of perillyl alcohol with time (μg/ml, min)						
	20	30	40	50	60	70	Sum
Acetonitrile	×	×	×	×	×	×	0
Acetone	×	×	×	×	×	×	0
Tetrahydrofuran	×	0.42	0.3	×	×	×	0.72
Methanol	×	0.45	1.19	0.75	0.32	×	2.71
Methanol/Tetrahydrofuran=70/30	×	0.33	0.58	0.83	0.66	×	2.4
Methanol/Tetrahydrofuran=50/50	×	0.62	0.8	0.39	0.34	×	2.15
Methanol/Tetrahydrofuran=30/70	×	0.75	0.56	0.44	×	×	1.75
Methanol/Acetonitrile=70/30	×	0.79	0.91	0.49	×	×	2.19
Methanol/Acetonitrile=50/50	×	0.71	0.35	×	×	×	1.06
Methanol/Acetonitrile=30/70	×	0.55	0.31	×	×	×	0.86
Methanol/Tetrahydrofuran/Acetone=20/40/40	×	0.34	0.65	0.54	×	×	1.53
Methanol/Tetrahydrofuran/Acetone=25/40/35	×	0.62	0.67	0.33	×	×	1.62
Methanol/Tetrahydrofuran/Acetone=30/40/30	×	0.75	0.86	0.33	×	×	1.94
Methanol/Tetrahydrofuran/Acetone=35/40/25	×	0.6	0.56	0.29	×	×	1.45
Methanol/Tetrahydrofuran/Acetone=30/45/25	×	0.92	0.64	0.41	0.25	×	2.22
Methanol/Tetrahydrofuran/Acetonitrile=20/40/40	×	0.24	0.53	0.38	×	×	1.15
Methanol/Acetonitrile/Acetone=20/40/40	×	0.56	0.44	×	×	×	1.0

(×: no detection)

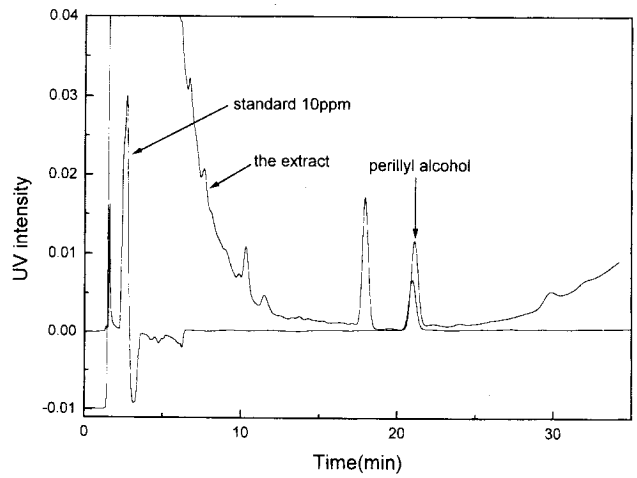

Fig. 5. Isocratic HPLC separation of Korean orange peel extracts.
(μ-Bondapak column, water/acetonitrile (65/35 vol%), injection vol: 10 μl, flow rate: 1 ml/min)

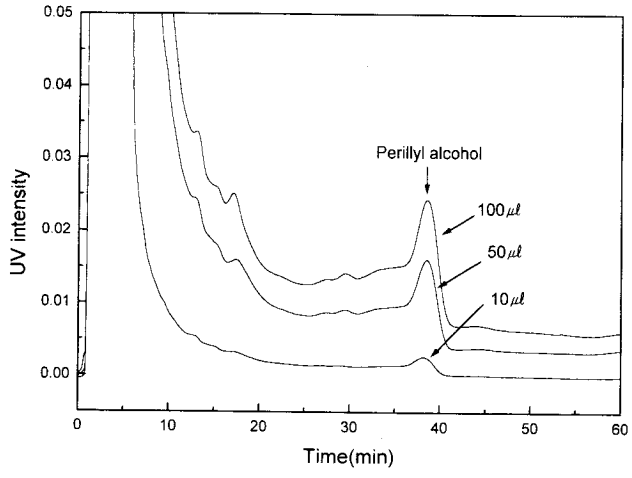

Fig. 6. Isocratic HPLC separation of Korean orange peel extracts.
(Preparative column, water/acetonitrile (65/35 vol%), injection vol: 10, 50, 100 μl, flow rate: 1 ml/min)

extract (below) are shown in Fig. 5. The retention time was 20.5 min, and unnecessary components in the peel were removed in the pretreatment steps. The in-house chromatographic column was packed by 15 μm preparative packings. At the same mobile phase as in the analytical column, the chromatogram is shown in Fig. 6 with different sample sizes of 10, 50, and 100 μl. Compared to the previous analytical column, the retention time of perillyl alcohol was increased to 38 min. Peak shapes changed to fronting as the injection volumes were larger.

CONCLUSION

For the separation of perillyl alcohol from Korean orange peel, the following configuration was systemized in this work: the extraction of perillyl alcohol by solvent, the partition step, the enrichment by glass column, and RP-HPLC. Among solvents, perillyl alcohol was well dissolved in methanol. To remove the unnecessary components from the methanol-extract, a partition step of chloroform and water was utilized. Then, perillyl alcohol was obtained by the water phase of extract/water/chloroform (20/5/30, vol%). The sample was further enriched by open glass-column, and pure methanol was used as the mobile phase. Finally, RP-HPLC was used to isolate perillyl alcohol from the solution. Two types of preparative (15 μm) as well as analytical (10 μm) packings were used at a constant flow rate of the mobile phase, 1 ml/min. In a μ-Bondapak analytical column, the retention time of resolved perillyl alcohol was 20.5 min in water/acetonitrile (65/35, vol%). The injection volume of sample was increased to 100 μl in the preparative chromatographic column.

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