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Short communication

Extraction and purification of perillyl alcohol from Korean orange peel by reversed-phase high-performance liquid chromatography

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Abstract

To obtain perillyl alcohol, a potential anti-cancer agent, the peel of Korean orange was extracted by methanol or ethanol, and the extracts were partitioned by water–chloroform or hexane, respectively. Open-tubular chromatography (15×2.5 cm I.D.) with reversed-phase C_{18} packings (40~63 μm) was used to concentrate perillyl alcohol from the extracts. Finally to separate perillyl alcohol in a pure form, reversed-phase high-performance liquid chromatography was applied. Mobile phases used were water, methanol, ethanol and acetonitrile. The resolution of perillyl alcohol from Korean orange peel was achieved on a μBondapak C_{18} column (300×3.9 mm, 10 μm) and on a laboratory-prepared chromatographic column packed with 15 μm C_{18} preparative packings.

Keywords: Fruits; Perillyl alcohol; Terpenoids

1. 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 [1]. Numerous epidemiological studies have revealed that high consumption of some plant products correlate with a reduction in cancer incidence [2].

The monoterpene is one of the simplest groups in natural plant products, and it is recognized as possessing chemopreventive properties against mammary, liver and lung carcinogenesis [3,4]. The intake of diets containing fruits and vegetables, major sources of phytochemicals and micronutrients, may reduce the risk of developing cancer [5]. Monoterpenes are naturally occurring substances derived from orange peels, lavender, mints and celery seeds [6]. Perillyl alcohol, a monoterpenoid, is an hydroxylated product of *d*-limonene (*p*-mentha-1,8-diene) which 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 cells. Moreover, perillyl alcohol is not only a potent breast anti-cancer agent but also an effective chemotherapeutic agent against advanced mammary tumors [7,8]. The monoterpenes are also used as a promising alternative to chlorofluorocarbon (CFC) cleaning solvents. Recently, chlorinated hydrocarbons and CFCs used for defluxing and precision cleaning, have been gradually replaced by naturally occurring products containing monoterpenes. The most frequently used compound is *d*-limonene. The chemical is preferred mainly because of its environmentally less harmful properties [9].

Many efforts have been made to commercialize

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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 [10]. As the C_{18} is chemically bonded to the surface of tiny particles, these packings provide stability and reproducibility as well as selectivity [11]. The present work focused on the separation procedure of perillyl alcohol and *d*-limonene from Korean orange. The peel of the Korean orange, wasted by home and food industry after its use, was extracted by solvents and separated by liquid chromatography. To efficiently obtain perillyl alcohol and *d*-limonene in a pure form, the pretreatment steps and the final chromatographic system needed to be adequately configured, and this was the purpose of the work.

2. Experimental

2.1. Chemicals

The orange peel was purchased at a domestic market in South Korea. The perillyl alcohol and *d*-limonene standards were purchased from Aldrich and Sigma, respectively. The concentration of perillyl alcohol and *d*-limonene dissolved in methanol was 10 mg/ml. The extra-pure grade solvents of hexane, methanol, ethanol, 2-propanol (IPA), 1-propanol, chloroform, acetone, trichloroethylene, 1,1,1-trichloroethane and methylene chloride were purchased from Dae Jung Chemicals and Metals (Korea). Water was distilled and deionized prior to use.

2.2. Analysis of perillyl alcohol and d-limonene (GC)

The identification of peaks in chromatogram was confirmed by the standards of perillyl alcohol and *d*-limonene. The extracts of Korean orange peel by solvents were analyzed using a Hewlett-Packard (HP) Model 5890 gas chromatography (GC) system equipped with a HP-5 (crosslinked 5% PH ME siloxane, 30 m×0.32 mm) capillary column and a flame ionization detection (FID) system. 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.

2.3. Extraction step

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

2.4. Partition step

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

2.5. Open-tubular chromatography

To concentrate perillyl alcohol of the water phase in the partition step, open-tubular chromatography (15 cm×2.5 cm I.D.) 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 into the open-tubular chromatograph. The effluents were collected every 10 min from the column outlet in an ambient atmosphere. Each solution was concentrated to 1 ml for GC analysis.

2.6. HPLC

The HPLC system was as follows: Waters Model 600 liquid chromatography (Waters Associates, Milford, MA, USA) equipped with a Waters 600E Multisolvent Delivery System, a UV–visible tunable-wavelength absorbance detector (Wasters 486), a U6K injector (2-ml sample loop). The data acquisition system was CHROMATE (V. 3.0, Interface Eng., Korea) installed in a personal computer. The commercial analytical-column was a μBondapak C_{18} (10 μm, 300×3.9 mm, Waters) and preparative column (ODS, 15 μm, 300×3.9 mm) were packed by pump in the laboratory. Mobile phases in RP-HPLC were water, methanol and acetonitrile. Flow-rate of mobile phase was fixed at 1.0 ml/min.

3. Results and discussion

To purify perillyl alcohol from Korean orange, the steps developed in this work were composed of the extraction step, the partition step, the enrichment by open-tubular chromatography, and finally the chromatographic separation.

3.1. 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 concentrations of perillyl alcohol and *d*-limonene were experimentally measured with the various kinds of solvents. The two components were remarkably dissolved in 1,1,1-trichloroethylene, but this solvent's use is restricted because of its ozone-depleting potential together with CFC 113. Methylene chloride had good solubility for *d*-limonene, but it did not dissolve perillyl alcohol well. The solubility of perillyl alcohol and *d*-limonene were greatly affected by different types of alcohols. For the solvent of methanol, the GC analysis of the extract is shown in Fig. 1. The solubility of perillyl alcohol and *d*-limonene in methanol was superior to the other classes of alcohols, ethanol, IPA and 1-propanol. Cost of methanol was cheaper, but its toxicity was an important problem to solve prior to a commercial.

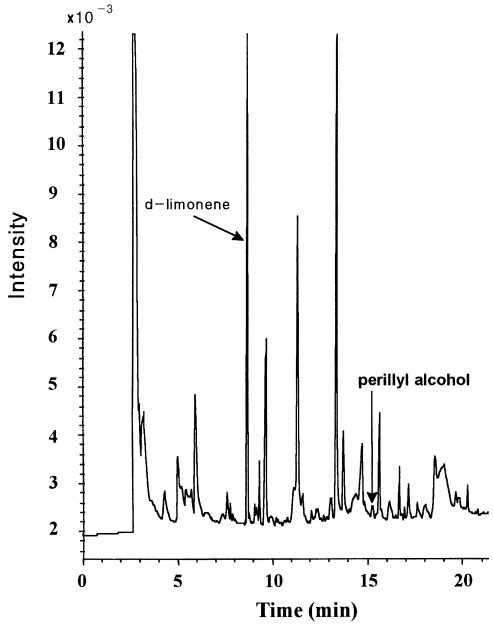

Fig. 1. GC chromatogram of the extract by methanol on a HP-5 capillary column.

Yield was defined by the percentage ratio of the mass of perillyl alcohol to the mass of dry Korean orange peel.

3.2. Partition step

Many components exist in the peel of 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 non-polar components in the methanol-extracted sample moved to chloroform, while the polar components moved to water. The concentrating effect can be enhanced by performing layer separation of the methanol-extracted sample between chloroform and water. With the 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 analysis of the top and bottom sample in the funnel, perillyl alcohol was dissolved in water, and *d*-limonene in chloroform. Chloroform and water were not immiscible with smaller amounts of chloroform or water. Perillyl alcohol was concentrated most satisfactorily in extract–water–chloroform

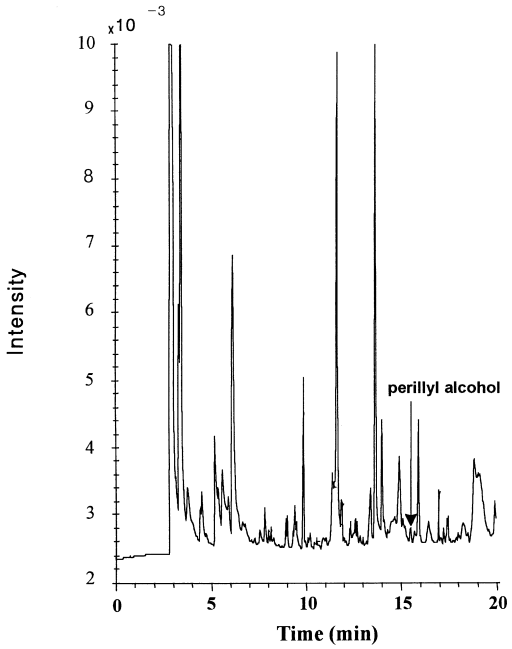

Fig. 2. Separation of perillyl alcohol in the water phase of the partition.

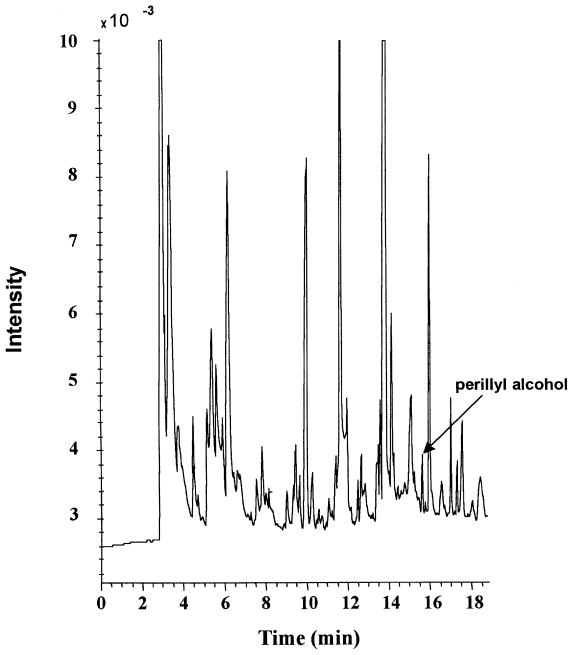

Fig. 3. Enrichment of perillyl alcohol by open-tubular chromatography with methanol.

(20:5:30, v/v). Perillyl alcohol was identified by the GC analysis of the water phase in Fig. 2.

3.3. Enrichment by open-tubular chromatography

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 open-tubular chromatography, where rough reversed-phase C_{18} packings were filled. The sample passed down though the column by a gravitational flow. In addition to pure solvent, the binary and ternary mobile phases were studied. 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 1 h. Similar trends were shown in the pure solvents of acetonitrile and acetone. Enrichment of perillyl alcohol by pure methanol was performed, and its experimental result is shown in Fig. 3. The pure solvent of methanol showed the concentrating effect, compared to the peak of perillyl alcohol in Fig. 2.

3.4. HPLC separation

The peel of Korean orange was extracted by methanol, the extracts partitioned by chloroform–water, and further enriched by open-tubular chromatography. To purify further perillyl alcohol from the solution treated in the three steps mentioned above, the final downstream 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 the constant flow-rate of mobile phase, 1.0 ml/min. In a commercial analytical column (μBondapak), the mobile phase composition was water–acetonitrile (65:35, v/v), and the injection volume was 10 μl. The retention time of perillyl alcohol was 20.5 min, and unnecessary components in the peel was removed in the pretreatment steps.

Instead of methanol, previously used as a solvent for extraction, ethanol was studied to extract perillyl alcohol. The ethanol extracts were mixed with hexane to remove unnecessary components. The resulting extracts were further purified by open-tubular chromatography with a mobile phase of pure

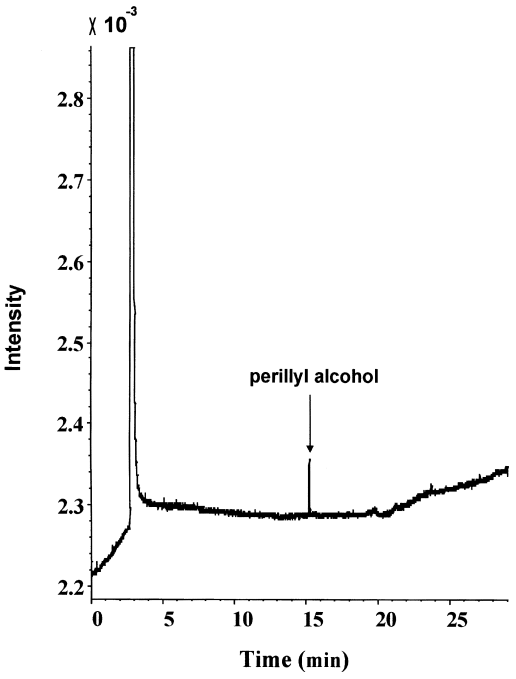

Fig. 4. GC chromatogram of purified perillyl alcohol collected by HPLC.

ethanol in a similar way to methanol. The effluents from the open-tubular chromatography were separated by the analytical μBondapak column. The injection volume of the sample was 10 μl, and mobile phase was water–ethanol (55:45, v/v) at 0.5 ml/min. The resulting perillyl alcohol was collected and analyzed by GC as showed in Fig. 4. Almost pure perillyl alcohol was separated. In this work, 28.1 μg of perillyl alcohol per 1 g of dry peel of Korean orange was obtained. In the preparative column, the injection volume was increased to 100 μl. The retention time of perillyl alcohol was 49 min, and it did change with sample sizes. Isolation of an (*R*)- and (*S*)-mixture of perillyl alcohol was not performed in the present work.

4. Conclusions

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 open-tubular chromatography and RP-HPLC. Among the various solvents used, perillyl alcohol was well dissolved in methanol and ethanol. The partition step of chloroform and water was utilized for the methanol extract, while hexane was used in the partition step for the ethanol extract. The samples were further enriched by open-tubular chromatography, and the pure methanol or ethanol was used as a mobile phase. Finally, to purify perillyl alcohol from the mixture, RP-HPLC was used. In the μBondapak analytical column, the retention times of a resolved perillyl alcohol from the methanol and ethanol extract were 20.5 and 37.0 min in water–acetonitrile (65:35, v/v) and water–ethanol (55:45, v/v), respectively. The injection volumes of sample were increased to 50~100 μl in the preparative chromatographic column (15 μm packings).

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