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Separation of Perillyl Alcohol from the Peel of Citrus Unshiu by Supercritical CO_2 and Preparative High-Performance Liquid Chromatography

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Abstract−To separate perillyl alcohol (POH), a potential anti-cancer agent, the peel of citrus unshiu was extracted by supercritical CO_2 extraction (SCE) system at 50 °C, 200 bar and 6 kg CO_2/hr/kg sample. The extracts were partitioned by acetonitrile/hexane (90/10, vol%). POH was eluted in the acetonitrile phase. An open-tubular chromatography with silica gel (40-63 μm) was used to purify POH from the acetonitrile phase. Mobile phase was hexane/ethyl acetate (90/10, vol%). To obtain POH in a pure form, finally preparative high-performance liquid chromatography was applied. The collection of POH from citrus unshiu peel was achieved on a laboratory-prepared chromatographic column (300×3.9 mm) packed with 15 μm C_{18} preparative packings. The composition of mobile phase was water/acetonitrile (50/50, vol%). The flow rate of the mobile phase was 1 ml/min and UV wavelength was fixed at 205 nm. It was found that the total yield of POH was 1.6×10^{-3} (wt%) as the dry powder of citrus unshiu peel.

Key words: Perillyl Alcohol, Citrus Unshiu Peel, Supercritical Fluid Extraction, Preparative HPLC

INTRODUCTION

Perillyl alcohol (POH; NSC-641066) is a cyclic monoterpene, which is a hydroxylated derivative of *d*-limonene, occurring in numerous species of plants, including mints, lavendar, perilla, citrus, and cranberries. Monoterpenes such as limonene and POH have the potential to both prevent and treat a variety of cancers including breast cancer [Gould, 1995; Kelloff, 1996]. POH is also used as a fragrance in perfumes, soaps, detergents, lotions, and creams.

Citrus peel oils, which are usually produced by cold pressing of the citrus fruits, consist of hundreds of compounds. These compounds can be grouped into the hydrocarbon terpenes and oxygenated terpenes. The former should be removed to stabilize the product, because they are unstable to heat, light and oxygen, rapidly decompose, and produce undesirable compounds [Brandani et al., 1990; Temelli, 1988]. Some of the terpenes are removed from cold-pressed oils to concentrate the flavor compounds into a more soluble and stable product. Vacuum distillation, steam distillation, solvents extraction, and desorption are the processes for concentrating cold-pressed oils. The disadvantages of all these process are low yield and formation of degradation product.

Supercritical fluid extraction (SFE), which uses predominantly supercritical carbon dioxide as an extraction medium, appears to be an alternative process, because it offers the advantages of low operating temperature and no solvent residue [Ernesto, 1997; Masaki, 1995]. The diffusivity of supercritical fluid is one to two orders of magnitude higher than those of other liquids, which permits rapid mass transfer and it can thus reduce the overall time required for the separation. It has been suggested that supercritical fluids can be used as a powerful alternative to organic solvents for the extraction of natural products, improving the purification process by achieving a selective extraction of the desired sample components through careful control of the extraction conditions. Thus, SFE can be seen as a very attractive alternative procedure to classical extraction methods.

The POH, which is a naturally occurring substance in citrus unshiu peel, had been isolated by solvent extraction and chromatography [Jung and Row, 1998a, b]. POH in citrus unshiu peel was extracted by means of the supercritical fluid extraction technique [Lee et al., 1999, 2000a, b, 2001]. The effects of the operating condition of supercritical fluid extraction on the yield of POH were presented [Lee et al., 2000a]. The experimental variables used to vary the extraction conditions were temperature and pressure of the supercritical fluid as well as the flow rate of CO_2. 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].

Over 600,000 tons of the citrus unshiu have been produced annually, but there is no special application of its peel. The peel of the citrus unshiu, wasted by homes and the food industry after its use, was extracted by SFE and separated by liquid chromatography. Pretreatment steps and preparative high-performance liquid chromatography (prep HPLC) were used to obtain POH in a pure form. The present work was focused on developing the separation procedure of POH from citrus unshiu peel.

EXPERIMENTAL

1. Chemicals

Commercial grade carbon dioxide (99.95%) was used as a sup-

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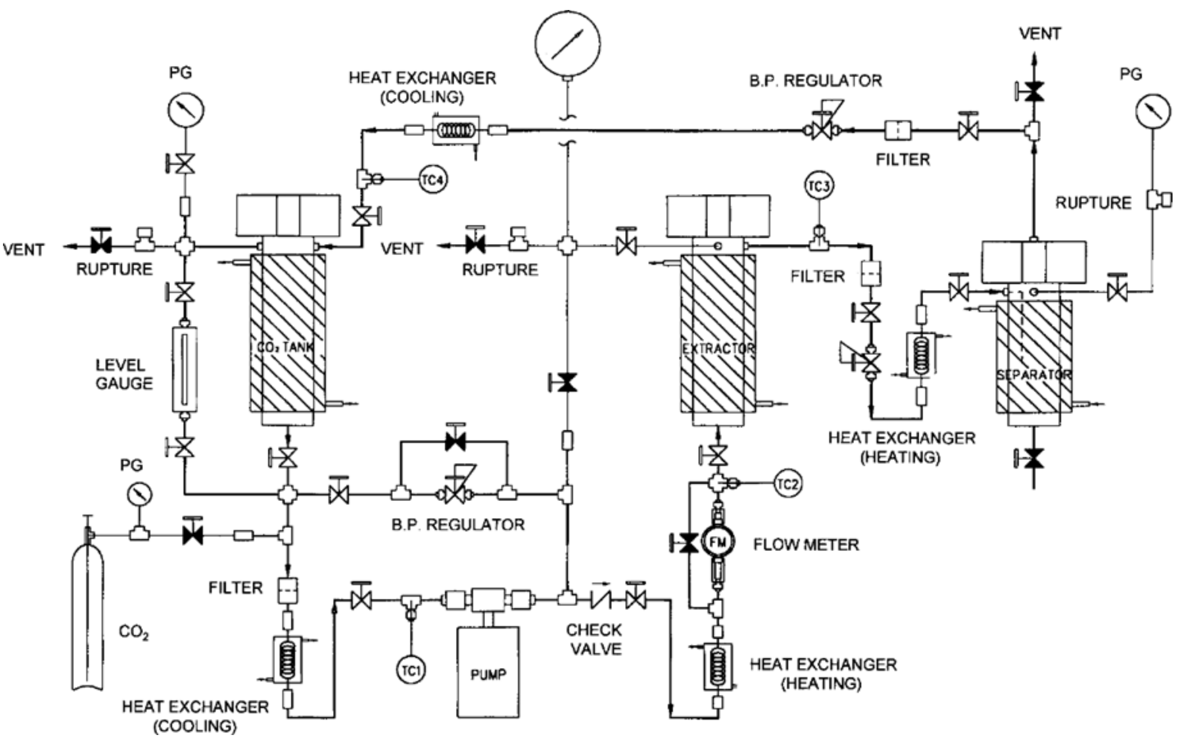

Fig. 1. Schematic diagram of SFE system used in this study.

ercritical fluid. The citrus unshiu peel was purchased at a domestic market in Korea. The standard chemicals of (*R*)-(+)-perillyl alcohol was purchased from Aldrich Co. The extra-pure grade solvents of hexane, methanol, and acetonitrile were purchased from Dae Jung Chemicals & Metals Co., Ltd. (Korea).

2. Extraction by Supercritical Fluid CO_2

The pilot plant used in this work mainly consisted of an extraction vessel (258 mmOD×870 mmH, 10 L), a separator (258 mmOD×545 mmH), 4 set heat exchanger and a CO_2 recycle storage tank (258 mmOD×870 mmH) as shown in Fig. 1. The dry powder of peel (0.3-1 mm, particle size) was put into a cylindrical extraction vessel. CO_2 from the cylinder was delivered by a pump (Milroyal pump, MCP13D10B1M) into the extraction vessel. The pressure in the extractor was controlled by a backpressure regulator. The extracts dissolved in the supercritical solvent were separated from the CO_2 by pressure reduction and collected in the separator. The separator was equipped with a cooling jacket at room temperature. The extraction pressure and the temperature were 200 bar and 50 °C, respectively, and the mass flow rate of CO_2 was set at 6 kg CO_2/hr/kg sample. The extraction was performed for 14 hr.

3. Analysis of Perillyl Alcohol by Gas Chromatography (GC)

The extract of citrus unshiu peel was dissolved in acetone and analyzed by a Hewlett-Packard (HP) Model 5890 gas chromatography (GC) equipped with an HP-5 (crosslinked 5% PH ME Siloxane, 30 m×0.32 mm) capillary column and a flame ionization detector. The oven temperature was gradually raised from 50 °C to 80 °C at a rate 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 1 ml/min and the split ratio was at 1 : 50. A calibration graph for POH was constructed by plotting the peak area against concentration. The relationship between peak area and concentration of POH was linear in the range of 10-1,000 g/ml [Lee et al., 2001]. The identification of peaks in chromatogram was confirmed by the standard POH.

4. Open-tubular Chromatography (OTC)

The extract after partition was injected into the open-tubular chromatograph and the effluents were collected from the column outlet. The effluent was monitored by thin layer chromatograph (TLC) detected by a 254 nm UV lamp. Normal phase and reversed phase of open-tubular chromatography (30 cm×2.5 I.D.) were used. In normal phase, OTC was filled with silica 60 (40-63 μm, Merck) and mobile phase was hexane/ethyl acetate (90/10, vol%). TLC was performed by using pre-coated plates with silica gel and eluted by hexane/ethyl acetate (75/25, vol%). In reversed phase, OTC was filled with octadecylsilica (40-63 μm, YMC) and mobile phase was methanol/water (40/60, vol%). TLC was performed by using glass plates covered with a 0.25 mm layer of C_{18} (RP-18F_{254s}, Merck) and eluted by chloroform.

5. Preparative HPLC

HPLC system was as follows: Waters Model 616 liquid chromatograph (Waters Associates, Milford, MA, U.S.A.) equipped with the Waters 600 s Multisolvent Delivery System, a UV-visible dual λ absorbance detector (Waters 2487), a Rheodyne injector (2 ml sample loop). The data acquisition system was Millennium 32 (V. 3.05, Waters, U.S.A) installed in a PC. The mobile phases of water, methanol, and acetonitrile were tested. The preparative column (ODS, 15 μm, 3.9×300 mm) was in-house packed by vacuum pump. Flow rate of mobile phase was 1.0 ml/min and UV wavelength was 205 nm.

RESULTS AND DISCUSSION

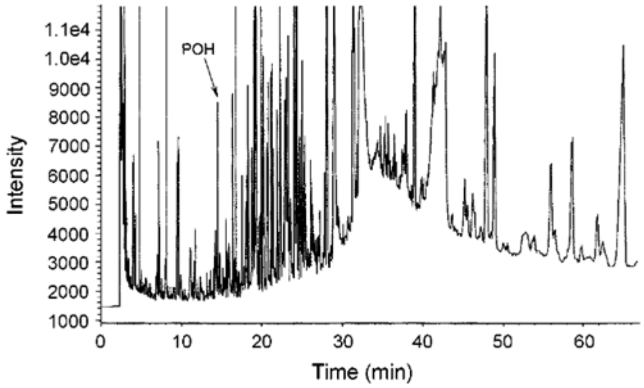

Fig. 2. Supercritical fluid extraction of POH from the extract.

The step to purify POH from citrus unshiu peel was the extraction by supercritical fluid CO_2, followed by partition, the purification by an open tubular column, and finally the preparative chromatographic separation.

1. Extraction by Supercritical Fluid CO_2

A system of supercritical fluid extraction (SFE) used in this work is shown in Fig. 1. The extraction yield was 2.5 (wt%) and the concentration of POH was 2.8×10^{-3} (wt%) as dry basis, which indicated that SFE was approximately 30 times more efficient than the solvent-extraction method [Lee et al., 1999, 2000a]. The percentage yield was expressed as the mass of effluents to the total dry powder of citrus unshiu peel loaded in the extraction vessel. The yield was obtained by a linear calibration graph for POH over the concentration range 10-1,000 μg/ml [Lee et al., 2001a]. The extract obtained by SFE was diluted with acetone to be analyzed by GC as shown in Fig. 2. 10 g of the extracts was dissolved in 15 ml of acetone and the injection volume of the sample was 1 μl. The retention time of POH was 15.6 min and the peak of POH was identified [Lee et al., 2000a]. High solvating power of supercritical fluid caused the complexity of the chromatogram. Quite a few of oil and wax components were followed by POH. Before 30 min, oil components were eluted while wax components later.

2. Partition

Many unnecessary components existed in the extract from SFE. To remove impurities, the partition step was utilized as a pre-step to open tubular chromatography. The extract was added in the solvent of acetonitrile/hexane (80/20, vol%) to purify POH. 10 g of the sample was partitioned with 120 ml of the acetonitrile phase and 30 ml of the hexane phase. The each phase was separated and evaporated. The samples in the acetonitrile and the hexane phases were analyzed by GC, and its results are shown in Fig. 3 and Fig. 4, respectively. 1 g of sample was diluted with 1.5 ml of acetone. The injection volume of the acetonitrile and hexane phases was 1 μl. The retention time of POH was 15.6 min. The selectivity of POH could be adjusted by the composition of acetonitrile/hexane. The composition of the solvents was chosen to relieve the interfering compounds in SFE without loss of POH. In the acetonitrile phase, larger amount of POH was present. However, in hexane phase, negligible amount of POH was detected, which allowed POH to be isolated from the impurities present in a citrus unshiu peel.

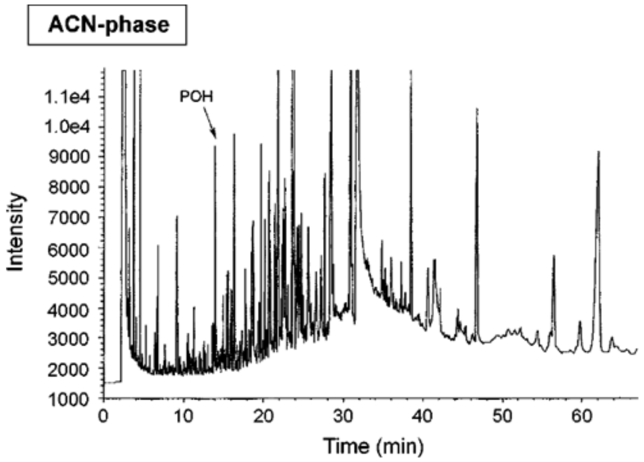

Fig. 3. Solvent extraction of POH in acetonitrile-phase.

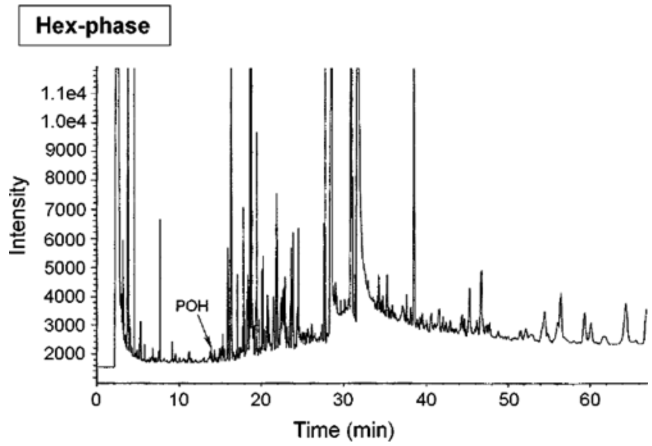

Fig. 4. Solvent extraction of POH in hexane-phase.

3. Open Tubular Chromatography (OTC)

As a pretreatment step to the chromatographic column, the acetonitrile phase was further purified by open tubular chromatography. Both reversed-phase C_{18} and silica gel were filled to the chromatographic column to find out the adsorption character of POH and chromatographic interferents. The sample passed down through the column by gravitational flow. During the separation, the effluents were checked by TLC and monitored with UV detection at 254 nm. The effluents were analyzed by GC; the chromatograms for normal-phase

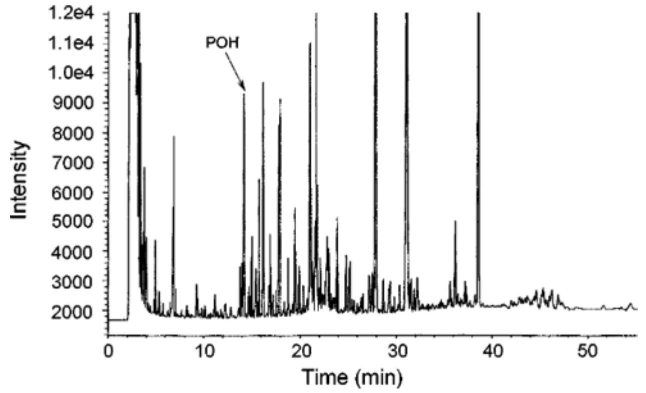

Fig. 5. Separation of POH from the effluent by open tubular chromatography with silica gel.

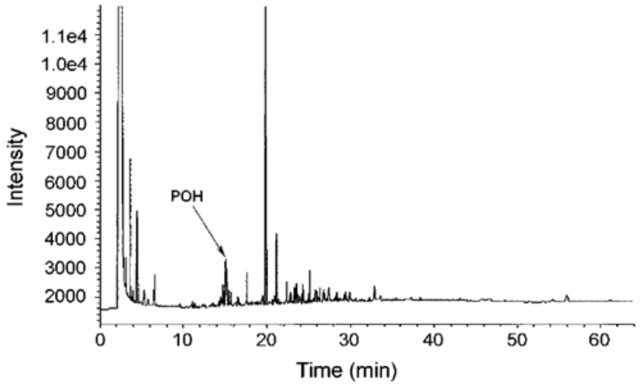

Fig. 6. Separation of POH from the effluent by open tubular chromatography with C_{18}.

and reversed-phase packings are shown in Figs. 5 and 6, respectively. Although OTC with reversed-phase was more selective for POH, the total elution time was long and the yield was low. Considering the efficiency and economics, OTC with normal-phase packings was selected as a pretreatment step for separating POH with preparative HPLC.

4. Preparative HPLC

The peel of citrus unshiu was extracted by SFE, and the extract was partitioned by acetonitrile/hexane, and purified by OTC. To purify POH treated in the three steps mentioned above, finally the preparative HPLC was used. The mobile phase was water/acetonitrile (50/50, vol%) and the injection volume was 0.2 ml. POH, separated by preparative HPLC, was collected at the end of chromatographic column several times. The effluent was concentrated by evaporation. 0.1 g of the concentrated sample was diluted with 6 ml of methanol and injected 5 μl to chromatographic column to confirm the purity of POH. As shown in Fig. 7, unnecessary components in the citrus unshiu peel were removed. The retention time of POH was 16.03 min. Preparative HPLC was successfully utilized to obtain POH.

CONCLUSIONS

Naturally, a very small amount of POH was contained in the citrus unshiu peel. To extract POH efficiently with a better yield, unnecessary components should be removed by subsequent separation steps. For the separation of POH from citrus unshiu peel, the following configuration was proposed in this work: the extraction of POH by SFE, the partition step, the purification by open tubular chromatography, and preparative LC. The qualitative and quantitative analyses of effluents have been used successfully by GC-FID system. The extract from the powder of citrus unshiu peel was obtained by supercritical CO_2 at the operating conditions of 50 °C, 200 bar, and 6 CO_2 kg/hr/kg sample. To remove the unnecessary components from the extracts by SFE, the partition of acetonitrile/hexane (80/20, vol%) was utilized. POH was obtained by the acetonitrile phase, which was further purified by open tubular chromatography. From an economical aspect, normal phase OTC, which is filled with silica gel, was preferred and the mobile phase was hexane/ethyl acetate (90/10, vol%). Finally to obtain POH with high purity, preparative LC was utilized. The injection volume was 0.2 ml and the mobile phase was water/acetonitrile (50/50, vol%) at a fixed flow rate of 1 ml/min. The extraction yield was 2.5 (wt%) and the concentration of POH was 2.8×10^{-3} (wt%) as dry basis. The total yield of POH was 1.6×10^{-3} (%, wt) as the dry powder of citrus unshiu peel. These procedures might be extended to the separation of POH from citrus unshiu peel on a production scale.

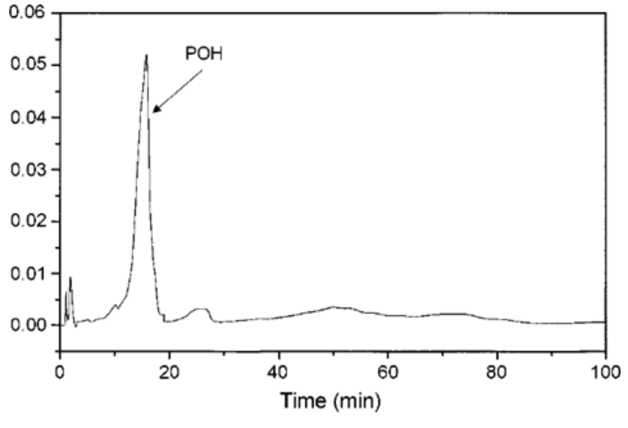

Fig. 7. Chromatogram of purified POH by preparative HPLC.

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