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Efficient, High-Yield Purification of Pulegone from the Oriental Herb, *Schizonepeta tenuifolia* Briquet and Demonstration of Supramolecule Formation by Cyclodextrins

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Pulegone is an organic compound obtained from the essential oils of a variety of plants. Biomaterial screening of various herbs has demonstrated that large amounts of pulegone are present in *Schizonepeta tenuifolia* Briquet. To purify this useful volatile compound for nano-biomaterials, we prepared extracts from 220 g of *Schizonepeta tenuifolia* Briquet with chloroform at 55 ºC for 12 h and concentrated the extracts under reduced pressure. We obtained a total of 9.48 g, which was then solubilized with hexane and subjected to silica gel column chromatography and eluted with hexane/chloroform solvents. The resulting pulegone was more than 99 % pure, based on estimates using multiple spectroscopic analyses, including APCI-MS, gas chromatography, GC-MS and UV-visible absorbance. Supramolecule encapsulation of purified pulegone with cyclodextrins was shown by the fluorescence spectrum. These supramolecules have the ability to permeate hydrophilic biological membranes and might offer important advantages in various applications for cosmetic, pharmaceutical and food-flavour industries.

Key Words: Pulegone, *Schizonepeta tenuifolia* Briquet, Purification, Cyclodextrins, Supramolecules.

INTRODUCTION

Schizonepeta tenuifolia Briquet, the dried aerial part of Jingjie, is one of the more popular oriental medicinal herbs and has a number of useful biological properties. It is used in the treatment of common cold with headache, fever, allergic dermatitis, pruritus and eczema. The carbonized herb also exhibits hemostatic properties and has thus been recommended as a remedy to stop bleeding[1].

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Pulegone (Fig. 1) is a monoterpene ketone present in essential oils from many mint species, including *Hedeoma pulegoides* and *Mentha pulegium*, both of which are referred to as pennyroyal[2,3]. Pennyroyal oil has been used as a flavouring agent in foods and beverages, a fragrant constituent and a flea repellent[4]. Pulegone has also been reported to exhibit bioactivity, showing antinociceptive, antibacterial, fumigant and acaricidal properties[5-8]. Pulegone has been afforded generally recognized as safe (GRAS) status by the United States food and drug administration[9,10] and is therefore widely used in flavouring agents, perfumery and aromatherapy. Because of its chemical properties as a terpene, pulegone has been extensively used to enhance the transdermal permeability of a number of drug molecules, including 5-fluorouracil, propranolol hydrochloride, indomethacin, ketoprofen and tamoxifen[11,12]. A number of major components and structures, such as glucosides (schizonepetosides), volatile oils (menthone and pulegone) and flavonoids (diosmetin, hesperetin and luteolin) have been isolated and identified in *Schizonepeta tenuifolia* Briquet[13-15]. Although the content of pulegone in the *Schizonepeta tenuifolia* Briquet spike is reportedly between 0.5 and 4.1 %[16], there have been no reports describing the purification of pulegone from this source.

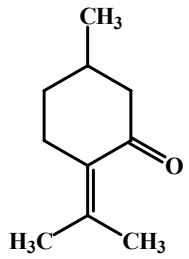

Fig. 1 Chemical structure of pulegone.

Pulegone is insoluble in hydrophilic solvents[2]. To increase the solubility of pulegone in hydrophilic environments, a hydrophilic group can be artificially introduced into pulegone through organic chemical reactions. However, these procedures are costly and time consuming. They may also alter the physical and chemical properties of pulegone, leading to unfavourable changes in its efficacy and stability. Therefore, it is very important that procedures used to solubilize pulegone do not alter its physico-chemical properties and thereby limit its practical use. One promising method to increase pulegone solubility in water is to encapsulate it with a host molecule, such as cyclodextrin, cucurbiturils, or calixarenes. Cyclodextrins have a hydrophilic exterior due to the presence of hydroxyl side-groups on the edges of the rings and an apolar cavity that provides a hydrophobic matrix. Consequently, cyclodextrins are able to encapsulate a variety of hydrophobic target molecules in an aqueous environment[17-19]. Using ^{1}H NMR, IR and UV-visible spectrometry and thermogravimetric analysis, we recently demonstrated the formation of supramolecules between pulegone and water-soluble macrocyclic cyclodextrin host compounds[20]. To further

our research goals, we required a means to prepare large amounts of high-purity pulegone. Although pulegone is commercially available, it is not clear what methods are used to prepare it. To the best of our knowledge, commercially available pulegone is mainly prepared through organic chemical synthesis[21] or by extraction from penny-royal oil of mixed mint species[22]. Because our ultimate goal in these studies is to use materialize pulegone as a nano-biomaterial, it is crucial that we know the exact origin and derivation of pulegone[23].

Here we report a simple procedure for purifying pulegone from *Schizonepeta tenuifolia* Briquet, well-defined origins and demonstrate its purity using APCI-mass, gas chromatography (GC), GC-mass and UV-visible absorbance spectroscopic methods. In fluorescence spectroscopic studies, we also provide evidence of supra-molecular encapsulation by cyclodextrins.

EXPERIMENTAL

All solvents and reagents were analytical or HPLC grade. Standards for menthone, pulegone, limonene, vanillin and β- and γ-cyclodextrins were purchased from Sigma-Aldrich (St. Louse, MO, USA). Chloroform, hexane and ethanol were purchased from Duksan Corp., (Ansan, Korea). Plant material (*Schizonepeta tenuifolia* Briquet) was obtained from commercial sources in Chungnam area (Kumsan, Korea). Thin layer chromatography (TLC) plates were purchased from Merck (Whitehouse Station, NJ, USA).

Preparation of *Schizonepeta tenuifolia* Briquet extract and silica gel column chromatography: *Schizonepeta tenuifolia* Briquet (220 g) was chopped, crushed and ground using a mixer (Hanil, HMF-1000) and then mixed with 4.5 L of chloroform at 55 ºC. After stirring for 12 h, the extracts were filtered using a glass filter and then concentrated under reduced pressure at 50 ºC using a lyophilizer. A total of 9.48 g of powder was obtained from 220 g of *Schizonepeta tenuifolia* Briquet for a yield of 4.31 %. Crude *Schizonepeta tenuifolia* Briquet extract (2.8 g) was loaded onto an open column (180 × 30 mm) containing activated C18 silica gel (Fluka; 230-400 mesh) and eluted step-wise with hexane: chloroform (10:0 to 5:5 v/v). The purification steps are shown schematically in Fig. 2.

Gas chromatography of crude *Schizonepeta tenuifolia* Briquet extract: The analyses of volatile compounds were carried out on a Shimadzu GC-2010 gas chromatograph equipped with a flame ionization detector (FID) and a fused-silica DB-5 column (Agilent; 30 m × 0.32 mm i.d.; film thickness, 0.25 μm). The carrier gas was helium, which was adjusted to a flow rate of 1.5 mL/min. The program used was 5 min at 60 ºC (isothermal), then a gradient from 60 to 200 ºC at a rate of 3 ºC/min, followed by a 30 ºC/min ramp up to 320 ºC and an isothermal hold at 320 ºC for 5 min. The injection port and the detector temperature were 260 and 280 ºC, respectively. *Schizonepeta tenuifolia* Briquet crude extracts and the purified pulegone fraction were injected a ratio of 1:40 in a total volume of 1 μL using the split sampling technique. The per cent composition of the target compound was determined from GC peak areas.

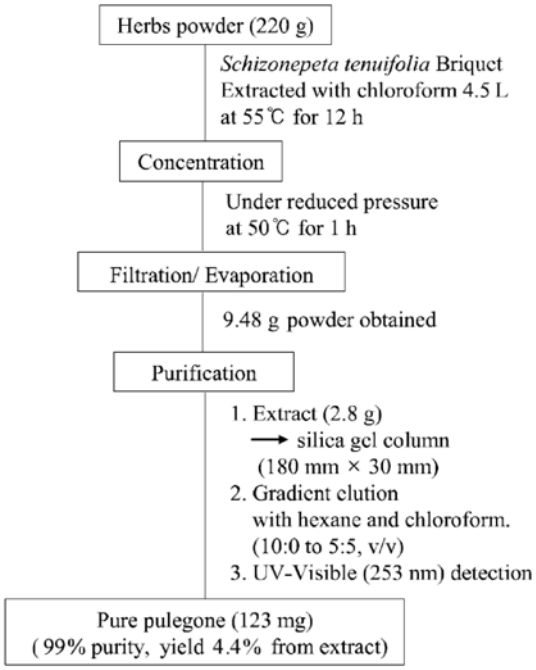

Fig. 2. Flowchart of procedures to purify pulegone from *Schizonepeta tenuifolia* Briquet

Pulegone assay after silica gel column chromatography: Fractions from silica gel column chromatography were monitored for purity of pulegone. Fractions (3 µL) were spotted onto silica gel 60 TLC plates (aluminum backed), eluted with hexane: chloroform (1:1, v/v) and then detected with vanillin/sulfuric acid at 100 ºC for 5 min. Fractions from silica gel column chromatography and standard solutions were confirmed by spectroscopy. UV-visible absorbance was detected using an Agilent 8453 UV-visible spectrophotometer. Mass spectrometry was conducted using a mass ZQ analyzer (Waters) in scan mode with positive ion mode on. The APCI parameters were as follows: corona-discharge current, 0.5 mA; capillary temperature, 150 ºC and vapourizer temperature, 350 ºC.

Gas chromatography-mass spectrometry of purified pulegone: Gas chromatography-mass spectrometry analysis was performed on a GCMS-QP2010 (Shimadzu) instrument. A DB-5MS column (60 m × 0.32 mm i.d.; stationary phase thickness, 0.25 µm) was used with the following temperature program: an initial temperature of 40 ºC for 5 min, then a gradient from 40 to 200 ºC at 4 ºC/min, followed by a gradient to 280 ºC at 20 ºC/min. Helium was used as the carrier gas at a flow rate of 1.5 mL/min. The interface temperature was 280 ºC. The MS operated in electron impact (EI) ionization mode at 70 eV and a temperature of 200 ºC. Total ion current (TIC) chromatograms were recorded in a mass range of 35-350 amu. The components were identified by comparing the mass spectra obtained with the database of Wiley 7, Nist 27 and Nist 147.

Assay of supramolecule formation by fluorescence spectroscopy: Pulegone (15.2 mg; 0.1 mmol) was added directly to a 10-mL aqueous solution of 1 mM β- or γ-cyclodextrins to yield a final pulegone concentration of 10 mM (*i.e.*, 10-times higher than that of each cyclodextrin). The solutions were stirred for 1 h at room temperature and the solvent was removed under reduced pressure. The residue was thoroughly washed with diethyl ether to eliminate uncomplexed pulegone and then dried under vacuum at room temperature. The fluorescence spectrum was measured using a Shimadzu RF-5301 PC spectrofluorophotometer.

RESULTS AND DISCUSSION

Extraction of pulegone from *Schizonepeta tenuifolia* Briquet: Pulegone was extracted and purified as presented in Fig. 2. After extraction with chloroform at 55 ºC for 12 h, the crude extract of *Schizonepeta tenuifolia* Briquet was assayed for pulegone by TLC (Fig. 3), which is a rapid and simple method for monitoring pulegone during purification steps. We obtained 9.48 g of lyophilized powder from 220 g of *Schizonepeta tenuifolia* Briquet. The precise concentration of pulegone in crude extracts of the *Schizonepeta tenuifolia* Briquet was determined by GC and the result is shown in Fig. 4. GC data also showed that limonene and menthone were present together with pulegone in the crude extracts (Fig. 4B). The calculated extracted yield was 17.2 % by GC chromatogram, which is a higher content than that previously reported[16]. This difference may reflect differences in the plant cultivation environments and the portions of the herb used to prepared extracts.

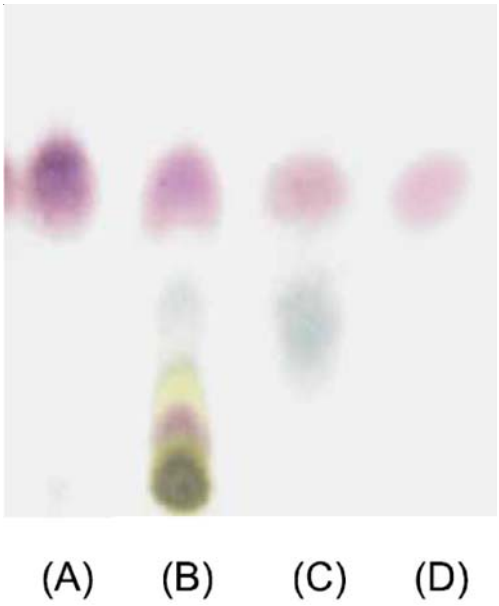

Fig. 3. TLC analysis of each purification step. A, authentic pulegone; B, *Schizonepeta tenuifolia* Briquet extract; C, partially purified fraction; D, purified pulegone

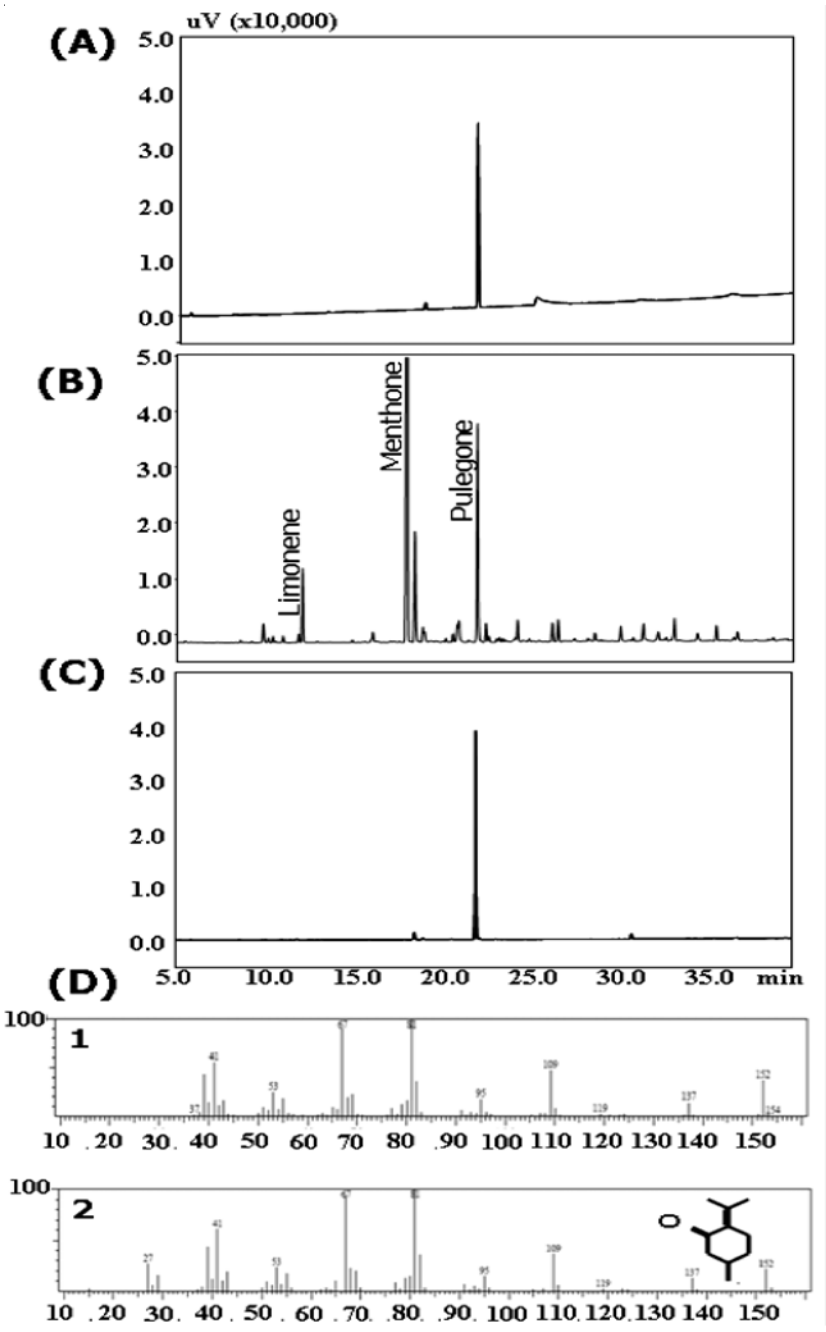

Fig. 4. GC spectra of authentic pulegone (A), *Schizonepeta tenuifolia* Briquet extract (B), purified pulegone (C) and GC-MS fragmentation patterns (D) of authentic pulegone (D, 1) and purified pulegone (D, 2). Note that the GC chromatogram also showed that limonene and menthone were major constituents of *Schizonepeta tenuifolia* Briquet extracts (B)

Purification of pulegone from *Schizonepeta tenuifolia* Briquet extract: One third of the crude powder (2.8 g) was loaded onto the silica gel column and chromatographed using hexane and chloroform as mobile phases (Fig. 2). The elution was performed stepwise and a total of 681 mL fractions were collected as follows: fractions 1-10, 100 % hexane elution; fractions 16-23, 80 % hexane/20 % chloroform elution; fractions 26-34, 60 % hexane/40 % chloroform elution; fractions 36-49, 55 % hexane/45 % chloroform elution; and fractions 51-68, 50 % hexane/50 % chloroform elution. Pure pulegone was obtained between fractions 37 and 59, as confirmed by TLC and GC analysis. The collected fractions exhibited a single, clear spot on TLC plates (Fig. 3D). The GC chromatogram of authentic pulegone showed that the peak corresponded to a retention time of 22 min (Fig. 4A). The purified pulegone also exhibited a single peak by GC analysis with the same retention time as that of authentic pulegone (Fig. 4C). We obtained a total of 123 mg pure pulegone from 2.8 g crude extract for a yield of 4.4 %, which is almost 10 times higher than previously reported values[16]. Moreover, the purity of present purified pulegone as calculated from GC chromatograms exceeded 99 % (Fig. 4C), which is higher than that of pulegone purchased from Aldrich (98 % pure).

Characterization of purified pulegone: Purified pulegone was further characterized by UV-visible spectroscopy, which is an important tool for verifying purity and by mass spectroscopy. The UV-visible spectra of purified pulegone (Fig. 5B) and authentic pulegone (Fig. 5A) obtained in 52 % hexane were nearly identical, with both exhibiting absorption maxima at 253 nm.

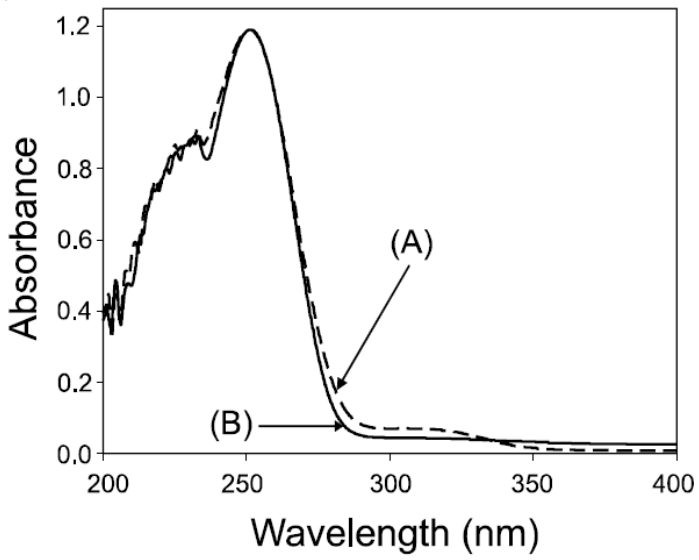

Fig. 5. UV-Visible absorption spectra of authentic pulegone (A) and purified pulegone (B)

Fig. 6 shows the APCI-MS spectra of authentic pulegone (Fig. 6A) and purified pulegone (Fig. 6B). The protonated molecular ion $[M-H]^+$ was observed as a base peak at m/z 153, whereas the pulegone fragment $[M-(CH_3)_3]^+$ was observed at m/z 107. The MS data for both preparations were very similar to each other, although there were unidentified peaks at m/z 119, 133, 147 in authentic pulegone and m/z 113, 115 in present purified pulegone.

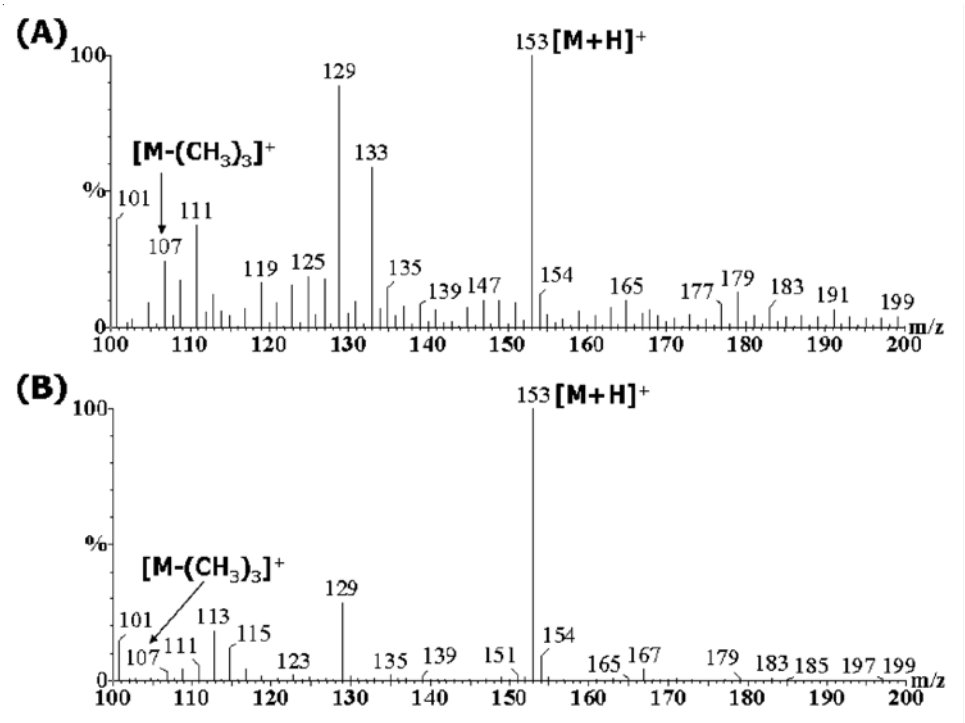

Fig. 6. MS spectra of authentic pulegone (A) and purified pulegone (B). The protonated ion $[M-H]^+$ was observed as a base peak at m/z 153 and the purified pulegone fragments $[M-(CH_3)_3]^+$ (arrow) was observed at m/z 107

Purified pulegone analysis by GC-MS: GC-MS has been a powerful tool in the study of essential oils from medicinal plants. One advantage of GC-MS is its ability to identify all constituents of sample in a single run. Using GC-MS to confirm the purity and mass identification of purified pulegone, we found that pulegone had been purified to high levels, as indicated by the TLC chromatogram. Purified pulegone exhibits a characteristic pattern of ion fragments that can be used for identification (Fig. 4D2). Using the NIST library as an established reference data (Fig. 4D1), we confirmed that the purified compound was pulegone.

Assay of supramolecule formation by fluorescence spectroscopy: Recently, using ^{1}H NMR, IR and UV-visible spectral studies and thermogravimetric analysis, we reported the supramolecular encapsulation of pulegone by β- and γ-cyclodextrin[20].

We now report an additional, simple fluorescence spectral method to assay for the formation of supramolecules. Since pulegone is not water soluble and free β- and γ-cyclodextrins exhibit no emission peak (excitation at 253 nm) in the vicinity of 510 nm due to the absence of appropriate fluorophores (Fig. 7, dotted line), the appearance of fluorescence bands in an aqueous solution of cyclodextrins must reflect the formation of complexes containing pulegone. The appearance of the characteristic pulegone fluorescence emission spectra (λ_{ex} = 253 nm, λ_{em} = 509 nm) in an aqueous solution confirms encapsulation of pulegone into β-cyclodextrin (Fig. 7A) and γ-cyclodextrin (Fig. 7B). The reported UV-visible spectrum of γ-cyclodextrin supramolecule is 1.34-fold larger than that of β-cyclodextrin supramolecule[20]. The new fluorescence emission spectrum attributable to pulegone encapsulation by γ-cyclodextrin (Fig. 7B) is 1.38 fold larger than that due to β-cyclodextrin encapsulation (Fig. 7A). Based on the results of previous studies that the pulegone molecule is encapsulated by both β- and γ-cyclodextrin with a 1:1 stoichiometry[20], these differences in spectroscopic characteristics should be arise from the differences in absorption coefficients between β- and γ-cyclodextrin supramolecules.

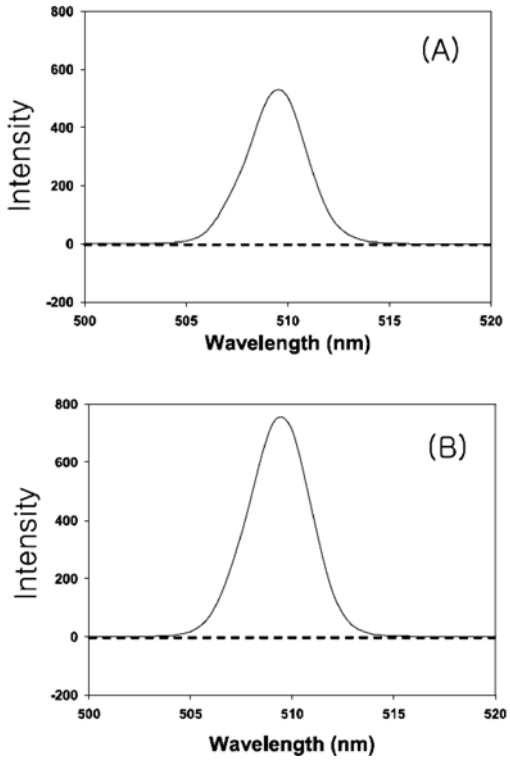

Fig. 7. Fluorescence emission spectra in water of (A) pulegone encapsulated by β-cyclodextrin (solid line) and β-cyclodextrin alone (dashed line); (B) pulegone encapsulated by γ-cyclodextrin (solid line) and γ-cyclodextrin alone (dashed line)

Conclusion

Here we report the development of a simple method to isolate, identify and purify pulegone from *Schizonepeta tenuifolia* Briquet. Pulegone is a useful volatile phytochemical that has been widely used in flavouring agents, perfumery and aromatherapy. It is also a terpene, which endows it with properties that have been exploited to enhance the transdermal permeability of a number of drug molecules. Although many reports have demonstrated the presence of pulegone in various plants or described the micro-level preparation and organic synthesis of pulegone, there are no reports on the purification of pulegone from *Schizonepeta tenuifolia* Briquet, single defined origin. Using silica gel chromatography, we were able to purify pulegone simply and with high efficiency. The purity and yield of pooled pulegone fractions were confirmed by APCI mass, GC, GC-mass and UV-visible absorbance spectroscopic methods. Present methods for purifying and identifying pulegone are conventional, but very powerful and rapid, producing high yields of high purity pulegone within two days. We also established a method for detecting the formation of supramolecules between purified pulegone and the host compounds, β- and γ-cyclodextrin, using fluorescence emission spectrum analysis.

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