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Efficient and scalable method in isolation of polymethoxyflavones from orange peel extract by supercritical fluid chromatography

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Abstract

Polymethoxyflavones (PMFs) from citrus genus are of particular interest because of their broad spectrum of biological activities, such as anti-inflammatory, anti-carcinogenic, and anti-atherogenic properties. Recently, the exploration into the beneficial health properties of PMFs in citrus fruits has dramatically increased. However, the supply of pure PMFs in the *in vivo* study is a limiting factor due to the difficulties in large-scale isolation of the interested PMFs. Therefore, the development of an efficient and a scalable separation method of PMFs is necessary and significant. In this paper, we report a newly developed method for efficient and relatively large-scale isolation of four PMFs from sweet orange (*Citrus sinensis*) peel by employing supercritical chromatography (SFC): nobiletin, tangeretin, 3,5,6,7,8,3′,4′-heptamethoxyflavone and 5,6,7,4′-tetramethoxyflavone.

Keywords: Sweet orange peel (*Citrus sinensis)*; Polymethoxyflavones; Isolation and characterization; Supercritical fluid chromatography

1. Introduction

There are many research reports about polymethoxyflavones (PMFs), mainly about their biological activities, including anti-inflammatory, anti-carcinogenic, and anti-atherogenic properties [1–6]. Majority of the activity studies of PMFs were performed *in vitro*, which consumes only milligram amount of materials. As the supply of large quantities of pure PMFs remains problematic, the *in vivo* study of PMFs has been limited to the use of a mixture of extracts from citrus plants. Hence, the investigation of pharmacokinetic properties and the bioavailability of a single compound of PMFs has been rarely performed. Although some PMFs are commercially available, the cost is too high to perform efficacy studies. For instance, 3,5,6,7,8,3′,4′-heptamethoxyflavone have been reported to exhibit potent anti-tumor activity and to be a chemopreventive agent against nitric oxide carcinogenesis [7,8]. However, more thorough *in vitro* investigation and efficacy study of 3,5,6,7,8,3′,4′-heptamethoxyflavone has not been initiated, mainly because of its limited availability and high cost ($300/mg). An efficacy study in animals of lower species (mice, rats, etc.) may easily consume grams of 3,5,6,7,8,3′,4′-heptamethoxyflavone at a price of $300,000/g and kilogram quantities ($300 million/kg) in animals of higher species (dogs, monkeys) and in clinical trials. Therefore, it is necessary and urgent to develop an efficient method for large-scale separation of PMFs, to dramatically reduce the cost of pure PMF and to remove the bottle neck in the discovery of PMF as a novel nutraceutical series.

Other studies of PMF properties have been focused on the analysis and identification of individual PMFs, using various analytical methods such as gas chromatography (GC) [9], gas chromatography–mass spectrometry (MS) [10], high-performance liquid chromatography (HPLC) [11–14], HPLC-MS/nuclear magnetic resonance (NMR) [15–17], and supercritical fluid chromatography (SFC) [18,19]. However, these methods are pure analytical methods for the purpose of individual PMF identification. The purification and isolation of PMFs have been untouched until recently, a separation method of PMFs using high-speed counter-current chromatography being reported [20]. Although this method was able to isolate some PMFs in multi-milligram quantities, it is laborious and time-consuming,

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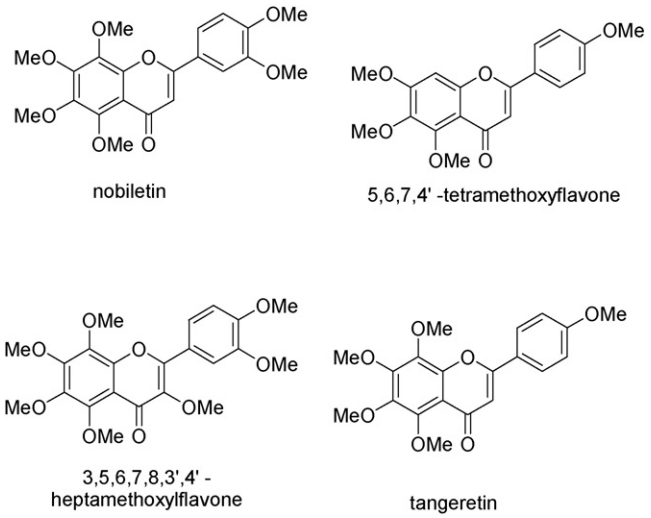

Fig. 1. Structures of four polymethoxyflavones isolated from sweet orange peel using SFC and chiral separation technology.

which in turn limited its scalability and application in larger scale separations.

During the course of isolation and biological activity studies of polymethoxyflavones from sweet orange (*Citrus sinenesis*) peel, by screening various separation methods, such as normal phase chromatography (silica gel, diol, cyano and amine columns, etc.), C18 reverse phase HPLC, chiral HPLC and SFC separation techniques, we developed an efficient and scalable SFC method for the large-scale separation of four common PMFs. With a potential application to be a common technology in large-scale isolation, this SFC technology has many advantages over the other separation methods in cost effectiveness, time efficiency and fully automation. This is the first reported SFC application in preparative separation of PMFs. It is of significance because it has not only provided an efficient and large-scale preparation of PMFs, but also explored a new application of the SFC technology. The four PMFs isolated in this study are nobiletin, tangeretin, 3,5,6,7,8,3′,4′-heptamethoxyflavone and 5,6,7,4′-tetramethoxyflavone (Fig. 1).

2. Experimental

2.1. Materials

Sweet orange peel extract (OPE) was obtained from Florida Flavors Company, Lakeland, Florida, USA. Pre-packed silica gel (60 Å, 32–63 μm) columns (330 g) for normal phase chromatography were purchased from Teledyne Isco, Inc. (Lincoln, NE). Octadecyl (C_{18}) derivatized silica gel reversed phase analytical (4.6 mm × 50 mm, 5 μm) and preparative (30 mm × 75 mm, 10 μm, ODS-A) columns for high-performance liquid chromatography was purchased from YMC Inc. (Kyoto, Japan). Another semi-preparative C18 reverse phase column, Xterra OBD™ (19 mm × 100 mm, μm) was purchased from Waters Corporation, Milford, MA (USA). DAICEL AD SFC chiral column (30 mm × 250 mm, 5 μm) purchased from Daicel Chemical Industry (Japan) was used on preparative SFC.

2.2. Flash column system

An automated flash chromatography system (Model Foxy 200, sg100, Teledyne Isco, Inc., Lincoln, NE) equipped with a pre-packed silica gel (particle size 35–60 μm) flash column from Teledyne Isco, Inc. (Lincoln, NE) was used. The mobile phase for normal phase flash column consisted of ethyl acetate and hexanes in varying proportions and the flow rate was 96 mL/min. The eluent was monitored with a single channel UV detector at a wavelength of 254 nm.

2.3. HPLC system

An automated high-performance liquid chromatograph from Gilson Inc. (Middleton, WI) was used for preparative purpose. This semi-preparative HPLC system was equipped with two pumps (322 HPLC pump with H2 pump heads), an UV–vis diode array detector (155) and an automated injection system (215 liquid handler with syringe pump and 819 injection module). The mobile phase for the HPLC system was 35% acetonitrile and 65% water (isocratic method) with a flow rate set at 20 mL/min. The eluent was detected with dual UV wavelength at 326 and 254 nm.

2.4. NMR instrument

NMR spectra were recorded on a Varian 300 Spectrometer (Varian Inc., Palo Alto, CA). With TMS serving as internal standard, ^{1}H NMR was recorded at 300 MHz and ^{13}C NMR at 75 MHz.

2.5. Liquid chromatography (LC)–electron spray ionization mass spectrometry (ESI-MS)

An HPLC-MS system was composed of an auto-sampler injector, an HP1090 HPLC system, with an UV–vis diode array detector (190–500 nm), an ELSD (Evaporative Light Scattering Detector) and an ESI-MS detector from Micromass VG Platform II mass analyzer (Micromass, Milford, MA). ESI-MS conditions were as follows: acquisition mode, ESI-positive; mass scan range, 100–800 amu; scan rate, 0.4 s; cone voltage, 25 V; source temperature, 150 °C; and probe temperature, 550 °C. Analytical HPLC conditions on HPLC-MS: column, Chromegabond WR C_{18}, 3 μm, 120 Å; 30 mm × 3.2 mm; injection volume, 5 μL; flow rate, 2 mL/min; and run time, 3 min. Mobile phase consisted of acetonitrile and H_2O with 0.05% TFA, typical gradient of 10–90% acetonitrile and the gradient varied.

2.6. Preparative supercritical fluid chromatography

Preparative SFC was performed on Berger MultiGram II Supercritical Fluid Chromatography system (Model SD-1) from Mettler-Toledo AutoChem Berger Instruments, Newark, DE, USA. The system consisted of an automatic liquid injection system with a DAICEL AD chiral column, 5 mL loop used to make injections and a thermal control module (TCM) used to control column temperature. Chromatographic conditions are as fol-

lows: SFC separations were performed at a temperature of 30 °C, a flow rate of 70 mL/min, and CO_2 pressure of 100 bar. Knauer variable wavelength UV detector (supplied by Mettler-Toledo) with high pressure flow cell was used for SFC detection. Detection in SFC was performed by measurement of UV absorbance at 220 nm.

2.7. General separation procedures from crude sweet orange peel extract

The crude OPE mixture (100 g) was dissolved in a mixture of methylene chloride (12 mL) and hexanes (8 mL) and loaded onto a 330 g pre-conditioned silica gel flash column. The elution was started with 10% ethyl acetate and 90% hexanes and modified linearly to 80% ethyl acetate and 20% hexanes over a period of 35 min. Then the isocratic mobile phase (80% ethyl acetate–20% hexanes) was applied for another 10 min (total run of 45 min). The fractions that had UV absorbance at 254 nm were analyzed by LC–ESI-MS and TLC. The fractions were combined into six groups (Groups I–VI) as shown in Fig. 2, according to their retention time and molecular weight obtained from LC/MS analysis. Further separation of two groups, Group IV, tangeretin group mainly containing a mixture of tangeretin and 3,5,6,7,8,3′,4′-heptamethoxyflavone and Group V (nobiletin group), a mixture of nobiletin, 5,6,7,4′-tetramethoxyflavone and other minor components, was performed by reverse phase HPLC and SFC.

2.8. Separation procedures of polymethoxyflavones by reverse phase HPLC

The fractions at each group (Group I to Group VI in Fig. 2) were combined and concentrated. A small amount of residues of Groups IV (tangeretin group) and V (nobiletin group) was dissolved in acetonitrile, respectively. The dissolved solution was loaded onto a C_{18} reverse phase HPLC system. A gradient method was used from 25% acetonitrile–75% water to 60% acetonitrile–40% water for 20 min with a flow rate of 20 mL/min. The monitoring UV absorbance was set at 280 nm. The fractions were analyzed by LC–ESI-MS. Both the pure compounds and mixtures were collected. The pure fractions by LC–ESI-MS were combined and concentrated or lyophilized to dryness and the dried compounds were analyzed by MS, UV and NMR.

2.9. Separation procedures of polymethoxyflavones by SFC

A portion of the Group V (nobiletin group, Fig. 2) residue (132 mg) was dissolved in methylene chloride (2 mL) and loaded onto the SFC system. The mobile phase consists of 45% methanol (0.25% diethylamine) and 55% liquid CO_2. Nobiletin (67 mg) and 5,6,7,4′-tetramethoxyflavone (52 mg) were collected. 2.01 gram of Group IV (tangeretin group, Fig. 2) was dissolved in methylene chloride (12 mL) and hexanes (8 mL). For each purification cycle, the injection volume was 0.8 mL (80 mg) and the runtime was 6.5 min. There were a total of 25 injections and the total runtime was 163 min. The mobile phase consists of 50% methanol (0.25% diethylamine) and 50% liquid CO_2. Tangeretin (0.72 g) and 3,5,6,7,8,3′,4′-heptamethoxyflavone (0.73 g) were collected.

3. Results and discussion

In an attempted separation of PMFs, several methods have been employed in preparative scale isolation of pure PMFs from orange peel extract. The most commonly used methods including silica gel normal phase chromatography and C_{18} reverse phase HPLC encountered difficulty. Recently, by using high speed counter-current chromatography, five PMFs have been isolated from tangerine peel in milligram quantities [20]. Chiral column based preparative HPLC has been reported to efficiently isolate gram scale quantity of nobiletin [21]. Although further investigation of large-scale separation of other PMFs using Chiral HPLC is still in process in our laboratory, the application of SFC technology in the separation of PMFs has been conducted. By using SFC technology, an efficient and scalable separation method was developed and a total

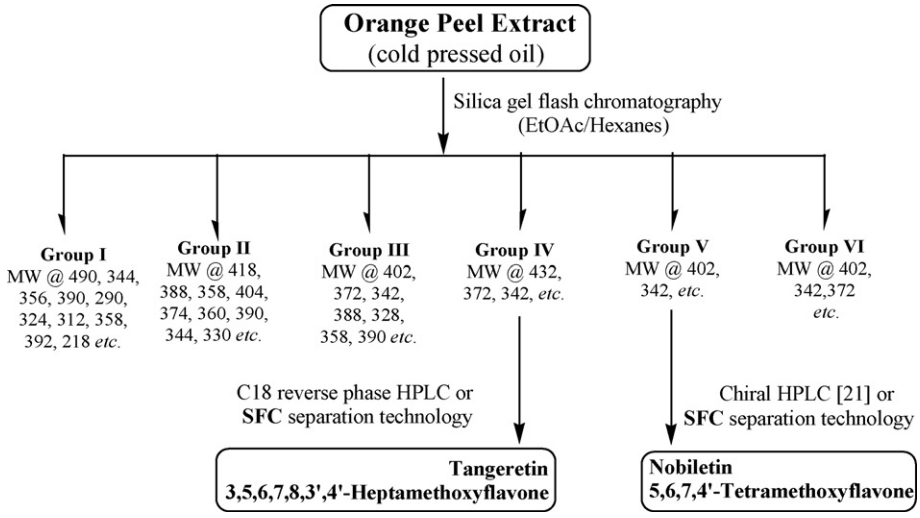

Fig. 2. Flow chart of PMF separation from OPE.

of four polymethoxyflavones were isolated from the orange peel extract in a relatively large scale. These four compounds are nobiletin, tangeretin, 3,5,6,7,8,3′,4′-heptamethoxyflavone and 5,6,7,4′-tetramethoxyflavone (Fig. 1). The MS and NMR data of the four isolated PMFs are in agreement with the literature values.

3.1. Normal phase chromatography separation

In the normal phase separation with silica gel chromatography, various solvent systems like hexanes-ethyl acetate, hexanes-dichloromethane and hexanes-isopropanol and different gradient methods were examined. With a larger column (330 g of silica gel column) and a small sample amount (less than five hundred milligrams of crude OPE), the isocratic method of ethyl acetate (45%) and hexanes (55%) gave the best result in terms of separation. The other solvent system that gave reasonable separation was 20% of isopropanol and 80% of hexanes. However, any attempt to isolate a pure PMF like 3,5,6,7,8,3′,4′-heptamethoxyflavone, tangeretin, nobiletin or other single PMF in multi-milligram scale with previously optimized analytical separation conditions (silica gel, diol, cyano and amine flash chromatography) either failed or many purification cycles had to be repeated. Also, these separation methods were very laborious and time consuming with high consumption of organic solvents and normal phase materials. Therefore, it is not practical to use conventional chromatographic (normal and reverse phases) methodology to meet the required large quantities for efficacy and metabolism studies.

Although silica gel chromatography demonstrates poor efficiency in isolating any single compound directly from orange peel extract, it is worthwhile to mention that the silica gel flash chromatography is the most convenient method for the process of removing residual wax and non-PMF oil and dividing the dewaxed OPE into different subgroups, allowing for further investigational studies (Fig. 2).

3.2. Separation on C_{18} reverse phase HPLC system

The study of PMF purification was also performed with reverse phase HPLC with a C_{18} stationary phase material. For example, Group V was loaded onto the reverse phase HPLC according to our reported procedure [21]. Nobiletin and 5,6,7,4′-tetramethoxyflavone can be separated when only 10 mg or less of Group V was loaded onto a semi-prep HPLC system (column size 30 mm × 75 mm). Previous research also concluded that these two PMFs could not be separated from higher loading material as a result of screening various solvent systems and optimising the flow rate. In the HPLC separation of Group IV whose components are mainly tangeretin and 3,5,6,7,8,3′,4′-heptamethoxyflavone, the optimum separation conditions were established to obtain a maximum amount of 15 mg of pure 3,5,6,7,8,3′,4′-heptamethoxyflavone during each cycle of purification with an Xterra C_{18} OBD™ reverse phase column from Waters Corporation (Milford, MA). This HPLC method is superior than the reported high-speed counter-current chromatography in terms of isolating pure 3,5,6,7,8,3′,4′-heptamethoxyflavone and tangeretin, as it is much faster and more cost effective [20]. However, many purification cycles must be repeated without increasing the column size should a larger amount of a single pure PMF, such as 3,5,6,7,8,3′,4′-heptamethoxyflavone, be requested for *in vivo* activity studies.

3.3. Separation on chiral HPLC column

As previously reported, the Whelk-O 1 stationary phase has a unique feature in terms of its binding with polymethoxyflavones [21]. Therefore, a very efficient large-scale separation of nobiletin and 5,6,7,4′-tetramethoxyflavone has been achieved from a crude mixture of the two compounds as the dominant components. The mobile phase was optimized to be 35% absolute ethanol and 65% hexane. Using this method, gram scale separation of pure nobiletin and 5,6,7,4′-tetramethoxyflavone was obtained in a single purification cycle. Therefore, this method has many advantages over silica gel chromatography and reverse phase HPLC in terms of separation efficiency and robust scalability. However, using this method, the separation efficiency was dropped when there is large percentage of 5,6,7,4′-tetramethoxyflavone in the mixture. The other disadvantages include the cost of the preparative Whelk-O 1 chiral column ($25,000), consumption of large volume of solvent and lack of full automation that are limiting factors to its widespread application.

3.4. Separation using supercritical fluid chromatography technology

In our investigation, we screened various columns that were used on normal phase and reverse phase HPLC. Both chiral and non-chiral columns were examined on SFC. It was found that the DAICEL AD chiral column gave the best separation results for the polymethoxylated flavones. The stationary phase of DAICEL AD chiral column is packed with amylose tris (3,5-dimethylphenyl carbamate). The amylose is a linear polymer, which comprises D-glucose residues connected through α-1,4-glucosidic bonds, while the cellulose is formed with 1,4-β-linkage. As discussed in the literature, the chiral discrimination is believed to be the stereoselective inclusion of enantiomers in the helically chiral cavity of amylose/cellulose and other interactions including hydrogen bond, dipole–dipole and π–π interaction with the phenyl rings on the derivatives. Although PMFs differ by one or more methoxy groups and are not regioisomers or enantiomers, chiral stationary phases were used and found to have a better separation than non-chiral columns, which may be because of the greater number of molecular interactions, such as multiple hydrogen bonds between the methoxy groups of PMF and the hydroxyl groups of cellulose, π–π interactions between the aromatic rings of PMF and the derivatized phenyl rings on the stationary phase.

Group V (Fig. 2) mainly comprises nobiletin and 5,6,7,4′-tetramethoxyflavone. Although the two PMFs differ by two methoxy groups, they have similar chromatographic behavior in both normal phase and reverse phase HPLC [21]. It has been demonstrated that these two compounds can be separated by

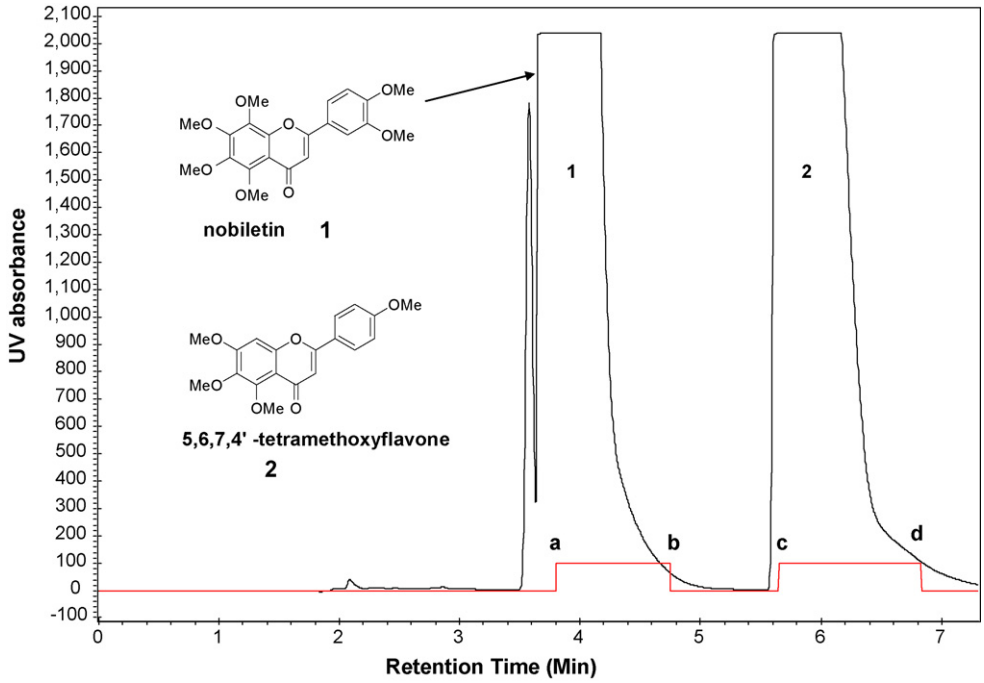

Fig. 3. SFC separation of Group V (nobiletin group). (Peak 1, nobiletin; peak 2, 5,6,7,4′-tetramethoxyflavone; SFC conditions, mobile phase 45% methanol (containg 0.25% diethylamine) and 55% liquid CO_2, flow rate, 70 mL/min; temperature, 30 °C; CO_2 pressure, 100 bar; UV absorbance, 220 nm; column, DAICEL AD chiral column, 30 × 250 mm, 10 μm; a and c, start collection point; b and d, stop collection point).

a chiral HPLC system in a large scale [21]. In this paper, we report that the two PMF compounds can also be clearly separated on an SFC system equipped with a chiral column in a much shorter time than that of the chiral HPLC system. Upon loading 132 mg of Group V onto the SFC system, 67 mg nobiletin and 52 mg of 5,6,7,4′-tetramethoxyflavone were obtained purely in less than 8 min. The mobile phase was 45% methanol (0.25% diethylamine) and 55% supercritical CO_2 (Fig. 3).

As the isocratic mobile phase allows repeated injections (stack injection) without concerning column cleaning, this in turn enables the application of many repeated purification cycles and a huge time saving without sacrificing separation efficiency. An example of stacking injection was provided in the separation of Group IV (tangeretin group, Fig. 2).

In the separation of Group IV (Fig. 2), 2.01 g of residue, mainly containing tangeretin and 3,5,6,7,8,3′,4′-heptamethoxyflavone after silica gel flash chromatography, was dissolved in methylene chloride (12 mL) and hexanes (8 mL). An isocratic method with a mobile phase consisting of 50% methanol (0.25% diethylamine) and 50% supercritical CO_2 was adapted in the separation. A stack injection was applied. For each purification cycle, the injection volume was 0.8 mL (80 mg) and the runtime was 8 min (Fig. 4). The injection and fraction collection were fully automated. In the stack injection method, injection of the next sample occurred at 6.5 min. There were a total of 25 injections and a total runtime of 163 min. Tangeretin (0.72 g) 3,5,6,7,8,3′,4′-heptamethoxyflavone (0.73 g) were collected. Fig. 5 shows the stack injection diagram. Therefore, in less than 3 h, 25 SFC purification cycles were performed automatically and more than 700 mg of pure tangeretin and pure 3,5,6,7,8,3′,4′-heptamethoxyflavone were obtained. Thus, this technology provides a significant solution for the scalable and economical supply of pure PMFs for *in vitro* and *in vivo* studies. The research of separating each of the PMF components of orange peel extract by SFC technology is in progress.

The isolation results of PMF using SFC and chiral HPLC were summarized in Table 1. In the table, we can see that the significant advantage of using SFC is the short time per purification cycle. With the current SFC system, Daicel AD SFC chiral column and a flow rate at 70 mL/min, it takes only 7 min for one purification cycle, which is significantly shorter than that of using chiral HPLC method (35 min run time in a comparable column size). A large amount separation of PMF can be achieved by using stack injection or a larger SFC purification system being able to deliver CO_2 and a modifier at a faster flow rate and using a larger column. The separation quality and capacity of SFC showed that it is the preferred choice over other preparative methods in isolation of PMF from OPE. The SFC uses liquid CO_2 and organic solvents as modifiers, such as methanol, isopropanol in PMF isolation, as the mobile phase. Liquid CO_2 is evaporated immediately and naturally when it is eluted from the column, as the liquid CO_2 is too volatile to exist in a liquid form at an ambient temperature and a normal atmosphere. Hence, the fraction volume from SFC is much less compared with the conventional chromatography. Consequently, it takes shorter time and less energy to evaporate the solvent in the collected fractions. In addition to its high separation efficiency and volatility, liquid CO_2 is much more inexpensive than other regular organic solvents. Therefore, the separation efficiency, scalability, tremendous time and cost saving in purification and

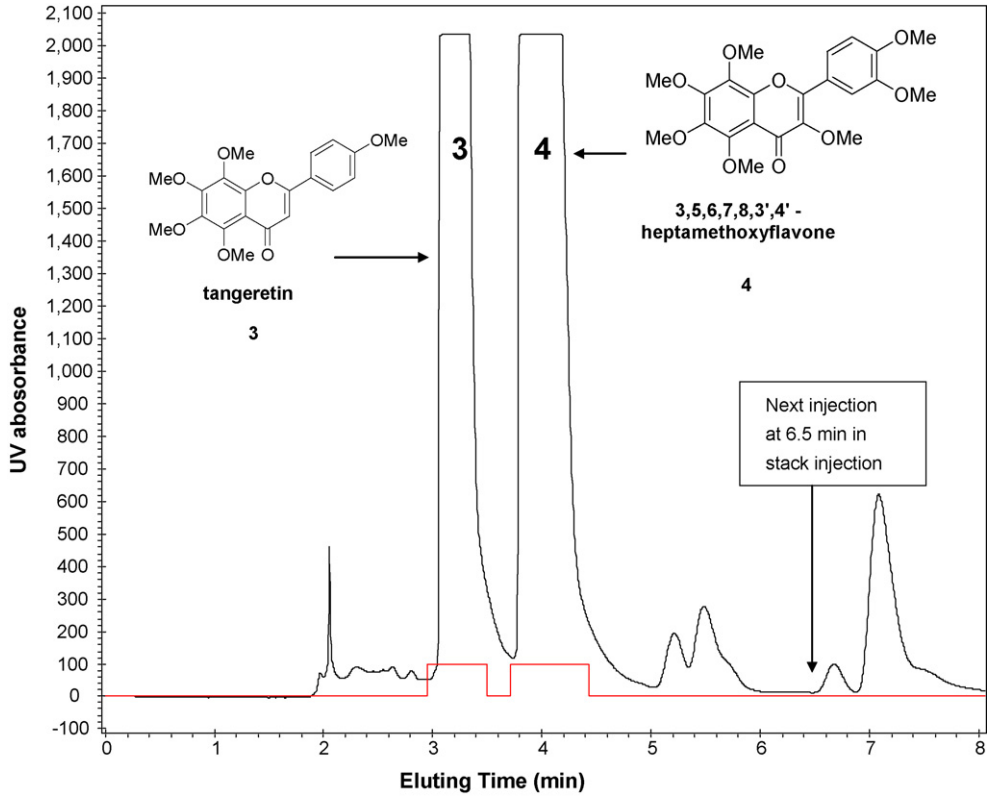

Fig. 4. SFC separation of Group IV (tangeretin group). (Peak 3, tangeretin; peak 4, 3,5,6,7,8,3′,4′-heptamethoxyflavone; SFC conditions, mobile phase 50% methanol (containing 0.25% diethylamine) and 50% liquid CO_2, flow rate, 70 mL/min; temperature, 30 °C; CO_2 pressure, 100 bar; UV absorbance, 220 nm; column, DAICEL AD chiral column, 30 mm × 250 mm, 10 μm).

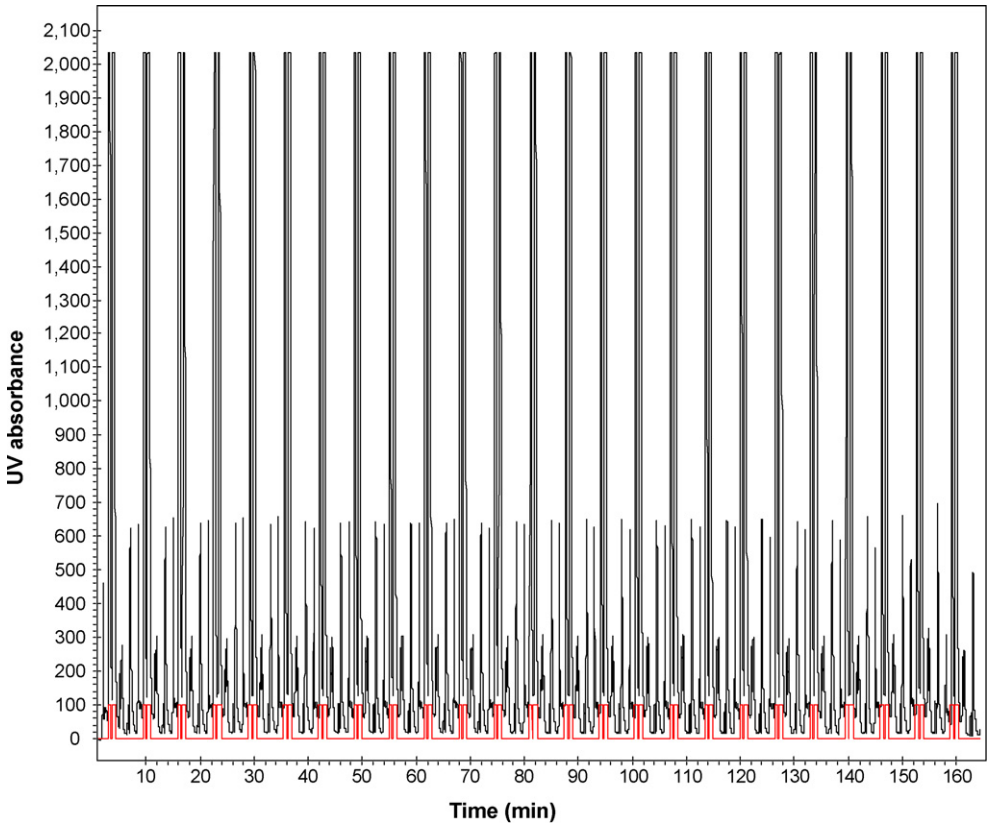

Fig. 5. Stack injection of SFC separation of Group V. (Total of 25 continuous injections, 80 mg of crude V per injection, other conditions in Fig. 4).

Table 1
Summary of PMF isolation by SFC and chiral HPLC

Method	Supercritical fluid chromatography (SFC)			Chiral HPLC [21]
Column	Daicel AD SFC, 30 × 250 mm, 5 μm			Welk-O 1 (R,R), 450 g
Solvent	Methanol			Ethanol/hexanes
Group in Fig. 2	Group IV	Group V		Group V[a]
Amount loaded	2.01 g[b]	132 mg[c]		3.54 g
Isolated PMF	Tangeretin	3,5,6,7,8,3′,4′-Heptamethoxy-flavone	5,6,7,8,4′-Tetramethoxy-flavone	Nobiletin	5,6,7,4′-Tetramethoxy-flavone
Isolated amount	0.72 g	0.73 g	67 mg	52 mg	2.48 g	0.85 g
Purity by HPLC-UV-ELSD[d]-MS	100	100	100	100	>99	>99
Time per purification cycle (min)	6.5		7		45	
Capability of stack injection	Yes		Yes		Possible	

Notes: a different batch of OPE; b total injection amount in 25 purification cycles; c amount in one injection; d evaporative light scattering detector (ELSD).

solvent evaporation make the SFC the most attractive separation method in large-scale isolation of PMF and a myriad of other chiral and non-chiral compounds.

4. Conclusions

The separation of PMFs from orange peel extracts was attempted under normal phase liquid chromatography and reverse phase HPLC as well as supercritical fluid chromatography, with non-chiral as well as chiral stationary phases. Owing to the unique physical properties of supercritical CO_2, SFC technology has been found to have the best separation efficiency, especially under chiral mode. More advantageous findings in SFC technology are the capability of short time purification cycles and fully automated stack injection, which provides ideal conditions for scalability of large quantity separation. Data from this research have shown that SFC technology has dramatic advantages over other separation methods and is well suited for providing large amounts of PMFs from orange peel extract.

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