Available online at www.sciencedirect.com
ScienceDirect
Journal of Chromatography B, 846 (2007) 291–297
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

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

Shiming Li [a,*], Ted Lambros [a], Zhenyu Wang [a], Robert Goodnow [a], Chi-Tang Ho [b]

[a] *Department of Drug Discovery, Hoffmann-La Roche Inc., Nutley, NJ 07110, USA*
[b] *Department of Food Science, Rutgers University, 65 Dudley Road, New Brunswick, NJ 08901-8520, USA*

Received 8 March 2006; accepted 8 September 2006
Available online 10 October 2006

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,

* Corresponding author. Tel.: +1 973 235 4615; fax: +1 973 235 6084.
E-mail address: shiming.li@roche.com (S. Li).

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.09.010

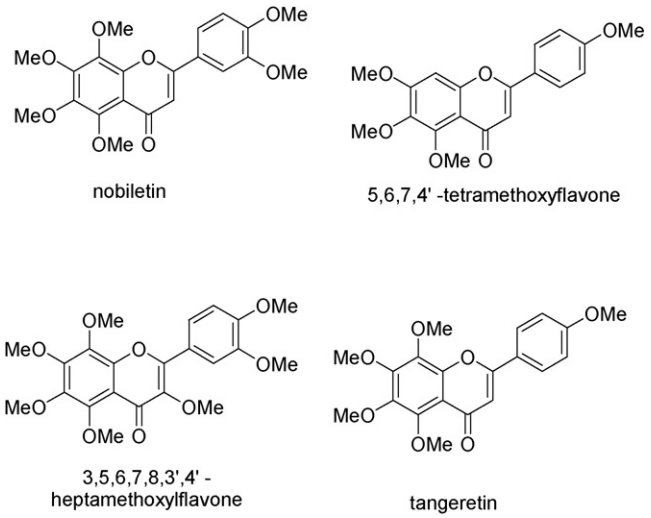

Fig. 1. Structures of four polymethoxyflavones isolated from sweet orange peel using SFC and chiral separation technology.

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-