HPLC Comparison of Supercritical Fluid Extraction and Solvent Extraction of Coumarins from the Peel of *Citrus maxima* Fruit

Wen-Yuh Teng,¹ Chien-Chih Chen² and Ren-Shih Chung^{1*}

¹Department of Agricultural Chemistry, National Taiwan University, Taipei, Taiwan

²National Research Institute of Chinese Medicine, Taipei, Taiwan

The efficiency of carbon dioxide supercritical fluid extraction (SFE) of the biologically active compounds imperatorin, meranzin and meranzin hydrate from the fruit peel of *Citrus maxima* Merr. has been compared with that of solvent extraction with acetone. Under the best SFE conditions tested for the three coumarins, which involved extraction at 50°C and 27.6 MPa, the extractive efficiencies were 84, 76 and 18% for imperatorin, meranzin and meranzin hydrate, respectively. The presence of modifiers significantly affected the extraction efficiency: the highest extraction efficiency of the three coumarins was obtained with ethanol as modifier. Copyright © 2005 John Wiley & Sons, Ltd.

Keywords: Supercritical fluid extraction; modifiers; HPLC; imperatorin; meranzin; meranzin hydrate; *Citrus maxima*.

INTRODUCTION

Imperatorin [9-[(3-methyl-2-butenyl)-7H-furo[3, 2-9][1]benzopyran-7-one] (**1**), meranzin [8-(3,3-dimethyloxiranylmethyl)-7-methoxy-chromen-2-one] (**2**) and meranzin hydrate [8-(2,3-dihydroxy-3-methyl-butyl)-7-methoxy-chromen-2-one] (**3**) are coumarins isolated from the fruit peel of *C. maxima* (Rutaceae). Imperatorin exhibits a wide spectrum of biological effects (Yamahara *et al.*, 1985; Averbeck, 1989; Kumar *et al.*, 1994; Chen *et al.*, 1996; Kwon *et al.*, 1997; Chiou *et al.*, 2001; Colombain *et al.*, 2001; Liao, 2002), whilst **2** and **3** possess cytotoxic activities (Hitotsuyanagi *et al.*, 1996).

Together with coumarins, the fruit peel of *C. maxima* is rich in essential oil, and the specific isolation of the active compounds from peel by CC is tedious and time-consuming. Supercritical fluid extraction with carbon dioxide (SFE-CO₂) is an attractive alternative that offers several advantages, including speed of extraction, high mass transference, completeness of extraction, elimination of the analyte concentration step, cost savings, the absence of toxic solvent and simplicity of analysis (Williams, 1981; Brogle, 1982; Larson and King, 1986). The solvent strength of a supercritical fluid may be increased by increasing the density of SFE-CO₂ with appropriate modifiers (Hills and Hill, 1993; Sanagi and Ahmed, 1993; Hanson, 1994; Ashraf-Khorassani *et al.*, 1995).

The aim of the present study was: (a) to study the extraction efficiency of SFE-CO₂ for compounds **1–3** in peel of *C. maxima* (shaddock), and (b) to develop a method for the simultaneous quantitative determination of the three coumarins by the HPLC.

EXPERIMENTAL

Chemicals and reagents. Liquid CO₂ (with helium head) was purchased from the Chin Fun Gas Company (Taipei, Taiwan). Ethanol was purchased from the Taiwan Tobacco and Liquor Corporation (Taipei, Taiwan), whilst HPLC-grade acetone, acetonitrile and *n*-hexane were from Merck (Darmstadt, Germany).

Preparation of samples and standards. Fruit peel of *C. maxima* (shaddock) was dried at 45°C to constant weight and pulverised. Dried peel (2 kg) was extracted by SFE-CO₂ at 50°C and 27.6 MPa, and the extract (33.07 g) subjected to CC over silica gel. Successive elution with *n*-hexane:ethyl acetate mixtures (1:0, 15:1, 10:1, 5:1, 2:1, 1:1 and 0:1) gave 18 fractions. The fraction obtained with *n*-hexane:ethyl acetate at 10:1 was further subjected to CC over silica gel to yield **1** (1.43 g), **2** (1.02 g) and **3** (50 mg). The identities of these coumarins were confirmed by MS and NMR spectra, and their purities were determined as >98% using the HPLC method.

SFE-CO₂. Extractions were performed using a laboratory-constructed apparatus: the deliver system of the modifier consisted of an HPLC pump. An extraction cartridge was filled with 100 g of powdered dried fruit peel and extraction was carried out with a continuous flow of CO₂. Two temperature levels (40 and 50°C) combined with two pressure levels (27.6 and 34.5 MPa), yielding four treatments in all, were tested. Extraction was carried out for 0.5 h in static mode, following by 6 h in dynamic mode. The final extract was collected in a tube containing 50 mL of ethanol. In all extraction procedures, the CO₂ flow rate was maintained at 1 L/min and the restrictor heated was maintained at 65°C.

For extractions in the presence of modifier, 50 mL of *n*-hexane, acetone or ethanol were pumped into the

* Correspondence to: Ren-Shih Chung, Department of Agricultural Chemistry, National Taiwan University, Taipei, Taiwan.
E-mail: chungrs@ntu.edu.tw

extractive cartridge with the HPLC pump prior to static mode extraction. After 0.5 h of static extraction, the modifier was pumped into the CO₂ line and the flow rate set at 0.5 mL/min for 1 h; the remaining 5 h of dynamic extraction were performed with CO₂ alone as described above.

Conventional extraction. Powdered dried fruit peel (1 g) was extracted with acetone under reflux for 1 h, after which the extract was filtrated and the extraction process repeated three times. Finally the concentrated extracts were combined.

Preparation of samples for the quantitative determination of coumarins. (a) The SFE-CO₂ extract from dried fruit peel was dissolved in ethanol; (b) the residue (1 g) of dried fruit peel remaining after SFE-CO₂ extraction was extracted with acetone under reflux for 1 h and filtered; (c) the extract from the conventional extraction process was re-dissolved in acetone and diluted to 100 mL (as control).

An aliquot (1 µL) of sample was added to the same volume of 1% naphthalene (internal standard) before being subjected to HPLC. The coumarin content was calculated by means of respective calibration curves.

HPLC analysis. A Hewlett-Packard/Agilent (Palo Alto, CA, USA) Module 1100 LC system, equipped with a degasser, a pump and a UV detector, linked to a Rheodyne (Cotati, CA, USA) injection valve

with a 20 µL sample loop was used in this study. Determinations were carried out using a Merck ChromolithTM RP-18e column (100 × 4.6 mm i.d.) with a mobile phase consisting of acetonitrile (solvent A) and water containing 1% (v/v) acetic acid (solvent B). The gradient program commenced at 20:80 (A:B) for 5 min, followed by a linear gradient to 28:72 in 0.5 min, maintained at 28:72 for 6.5 min, followed by a linear gradient to 30:70 in 0.5 min, maintained at 30:70 for 7.5 min, followed by a linear gradient to 32:68 in 0.1 min, and maintained at 32:68 for 40 min. The flow rate was 2 mL/min, the injected volume was 20 µL, and the coumarins were detected at 315 nm.

RESULTS AND DISCUSSION

HPLC separation of coumarins

The HPLC separation of imperatorin (**1**), meranzin (**2**), meranzin hydrate (**3**) and the internal standard naphthalene is shown in Fig. 1; the retention times of the respective analytes were 51.2, 12.0, 5.3 and 33.8 min.

Calibration of quantitative method.

The regression equations for the coumarins were as follows:

Figure 1. HPLC profile of an acetone extract of the fruit peel of *Citrus maxima*. Key to peak identity: **a**, meranzin hydrate (**3**); **b**, meranzin (**2**); **c**, naphthalene (internal standard); **d**, imperatorin (**1**). (For chromatographic protocol see the Experimental section.)

- *imperatorin* (**1**) – $y = 9.0846x - 0.0013$ (where y is the ratio of the peak area of the coumarin to the internal standard and x is the coumarin concentration); $r = 0.9989$ ($n = 4$); concentration range 45–200 µg/mL;
- *meranzin* (**2**) – $y = 8.6642x - 0.0295$; $r = 0.9997$ ($n = 4$); concentration range 45–270 µg/mL;
- *meranzin hydrate* (**3**) – $y = 8.1897x + 0.0212$; $r = 1.0000$ ($n = 4$); concentration range 14–196 µg/mL.

In each case there was a good linear relationship between the peak area elicited by HPLC and the concentration of analyte, as demonstrated by regression coefficients that were not significantly different from 1 ($p < 0.05$).

Solvent extraction of coumarins

The amounts of **1**, **2** and **3** that could be extracted by acetone from the fruit peel of *C. maxima* were 1.34, 2.63 and 1.47 mg/g, respectively.

SFE extraction of coumarins

The extraction efficiency was the highest when the extraction temperature was 50°C and the pressure was 27.6 MPa (Table 1). However, portions of the solutes remained in the extraction pathways or became plugged in the restrictor and lowered the extraction efficiency. SFE-CO₂ extraction was able completely to remove imperatorin from the fruit peel of *C. maxima* since **1** was

not detected in the residues. In the case of **2**, the amount retained in the residue following SFE extraction was lowest under the conditions 50°C and 27.6 MPa.

The solubility of coumarins in CO₂ in the absence of modifier decreases as the polarity of the functional groups of the coumarins increases (Table 1). Choi *et al.* (1998) found that substitution of the functional group of a compound tended to reduce its solubility in the order methyl > methoxyl > hydroxyl. In the present study it was established that the extractive efficiency of SFE-CO₂ with the addition of a modifier, which alters the polarity of the CO₂, was higher than that determined in the absence of a modifier. The increase in extractive efficiency following the addition of ethanol was higher than with the other two modifiers tested and the extraction efficiency of **3** significantly increased (Table 1).

In conclusion, the results show that the addition of modifiers can improve extractive performance for the three coumarins. Ethanol proved to be the best of the modifiers tested. These solubility data are essential for the systematic application of SFE-CO₂ extraction of coumarins from plant sources.

Acknowledgements

The authors wish to express their gratitude to Dr. J. C. Ou, National Research Institute of Chinese Medicine, Taipei, Taiwan, for his valuable instruction of the SFE technique. The fruit peel from *C. maxima* (shaddock) used in these experiments was kindly provided by Dr. C. T. Chen, Hwa-Lien District Agricultural Improvement Station, Hwa-Lien, Taiwan.

Table 1. Extraction efficiency for coumarins 1–3 from the fruit peel of *Citrus maxima* by supercritical fluid extraction with carbon dioxide in the presence or absence of modifiers

SFE conditions	Modifier	Content (mg/g) ^a and extraction efficiency (%) ^b					
		Imperatorin (1)		Meranzin (2)		Meranzin hydrate (3)	
		SFE extract	Residue	SFE extract	Residue	SFE extract	Residue
27.6 MPa, 40°C	None	1.00 ^{def} (75)	n.d. ^c	1.71 ^d (65)	0.73 ^{deg}	0.15 ^f (10)	0.94 ^{de}
27.6 MPa, 50°C	None	1.12 ^{deg} (84)	n.d.	2.00 ^d (76)	0.71 ^{de}	0.27 ^f (18)	0.85 ^{de}
34.5 MPa, 40°C	None	0.92 ^{ef} (68)	n.d.	1.61 ^d (61)	1.12 ^g	0.27 ^f (18)	1.32 ^g
34.5 MPa, 50°C	None	0.84 ^f (63)	n.d.	1.94 ^d (74)	0.89 ^{dg}	0.18 ^f (12)	0.50 ^{ef}
27.6 MPa, 50°C	<i>n</i> -Hexane	1.18 ^{dg} (88)	n.d.	1.88 ^d (71)	0.58 ^{de}	0.52 ^e (35)	1.05 ^{dg}
27.6 MPa, 50°C	Acetone	1.09 ^{deg} (81)	n.d.	2.04 ^d (78)	0.68 ^{de}	0.65 ^d (44)	0.77 ^{ef}
27.6 MPa, 50°C	Ethanol	1.28 ^g (95)	n.d.	2.54 ^g (97)	0.48 ^e	1.20 ^g (81)	0.49 ^e

^a Data are expressed as the mean content. Values bearing the same lower case letters in the same column are not significantly different ($p < 0.05$) according to Duncan's multiple range tests ($n = 3$).

^b Extraction efficiency (shown in parentheses) is the ratio of coumarin extracted by SFE-CO₂ to that extracted by acetone (control) × 100%.

^c n.d. = none detected, below detection limit.

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