Available online at www.sciencedirect.com
ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 44 (2007) 63–69
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Identification of polymethoxylated flavones from green tangerine peel (*Pericarpium Citri Reticulatae Viride*) by chromatographic and spectroscopic techniques

Wang Dandan [a], Wang Jian [a], Huang Xuehui [b], Tu Ying [a], Ni Kunyi [a,*]

[a] *China Pharmaceutical University, Nanjing 210009, China*
[b] *Hangzhouhuadong Pharmaceutical Co. Ltd., Hangzhou 3100011, China*

Received 12 October 2006; received in revised form 21 January 2007; accepted 23 January 2007
Available online 3 February 2007

Abstract

Polymethoxylated flavones (PMFs) were extracted from *Pericarpium Citri Reticulatae Viride* using a procedure that obtained a consistent mixture of PMFs both in identity and proportion. The mixture consisted of isosinensetin (0.2%) (1), sinensetin (1.7%) (4), tetramethyl-*o*-isoscutellarein (0.3%) (5), nobiletin (40.5%) (6), tetramethyl-*o*-scutellarein (1.2%) (7), tangeretin (45.6%) (10), 5-demethylnobiletin (8.7%) (12), 5-demethyl tangeretin (0.8%) (14) and other flavonoids including heptamethoxyflavone (1.0%) (9), among which, compounds 1, 4, 5, 7 and 9 were identified based on their UV spectra, MS data and elution order described in the literature while compounds 6, 10, 12 and 14 were isolated and identified by UV, IR, MS, ^{1}H NMR, ^{13}C NMR and 2D NMR spectral studies. In addition, compound 14 was isolated and identified for the first time from *Citrus*.

Keywords: *Pericarpium Citri Reticulatae Viride*; Polymethoxylated flavones; Chromatographic and spectroscopic techniques

1. Introduction

A number of *Citrus* species have been recorded in the Chinese Pharmacopoeia as appropriate for medical use. The primary active biological constituents of *Citrus* species are flavonoids, a kind of sympathomimetic amine, synephrine, of which flavonoids have high content. Three types of flavonoids occur in *Citrus* species: flavanones, flavones and flavonols. Among these compounds, polymethoxylated flavones (PMFs) exhibit antimutagenic as well as antitumor properties and, therefore, may possess chemopreventive potential [1–3]. The composition of PMFs can be significantly different between different *Citrus* species [4,5].

Green tangerine peel (*Qing Pi* in Chinese) is the dried, immature fruit or immature fruit peel of *Citrus reticulata Blanco* [6]. In previous studies, determination of flavonone glycosides, synephrine, *N*-methyltyramine and volatile oil in *Pericarpium Citri Reticulatae Viride* was developed. Although many studies on PMFs from different *Citrus* species (e.g. *Citrus aurantium*, *Citrus sinensis*) and *Citrus* juices have been reported and most PMFs have been confirmed by UV, IR, MS, ^{1}H NMR and ^{13}C NMR [7–12], there is no systematic study on PMFs from *Pericarpium Citri Reticulatae Viride*.

The purpose of this work was to isolate and characterize PMFs in *Pericarpium Citri Reticulatae Viride* with some different chromatographic and spectroscopic techniques. Five compounds (compounds 1, 4, 5, 7 and 9) were identified based on their UV spectra, MS data and elution order described in the literature [10,12,13,15,17–19] while four other compounds (compounds 6, 10, 12 and 14) were isolated and identified by UV, IR, MS, ^{1}H NMR, ^{13}C NMR and 2D NMR spectral studies. Compound 14 was isolated and confirmed for the first time from *Citrus*.

2. Materials and methods

2.1. General procedure

Element analysis was conducted on an Eager 300 CHN element analyzer. UV spectra were performed on a SHIMADZU UV-2450 spectrometer. The UV diagnosis reagent was $AlCl_3$

* Corresponding author. Tel.: +86 25 83318799; fax: +86 25 83302827.
E-mail address: bailifenlan2005@126.com (K. Ni).

0731-7085/$ – see front matter
doi:10.1016/j.jpba.2007.01.048

ethanol solution. IR spectra were obtained with a Thermo-Nicolet AVATAR 360 infrared Fouriertransform spectometer. Nuclear magnetic resonance (NMR) spectra (δ, J in Hz) were recorded on a BRUKER 500 NMR spectrometer. Tetramethylsilane (TMS, Aldrich) was used as the internal reference (δ0.00) for ^{1}H NMR spectra measured in $CDCl_3$ (Dertero GmbH). This solvent was used for ^{13}C NMR spectra.

2.2. Isolation of the PMFs from Pericarpium Citri Reticulatae Viride

Five kilograms of dry powder of *Pericarpium Citri Reticulatae Viride* was refluxed with 75% (v/v) ethanol 25 l for 10 h. Then ethanol solution was concentrated at vacuum until the ethanol was eliminated. The concentrated solution was extracted with chloroform and the chloroform fraction was concentrated to dryness to give crude PMFs. Dissolve crude PMFs in chloroform-acetone (9:2, v/v), load the solution on a silica gel column (9 cm × 180 cm, I.D. silica gel 100–200 mesh), and eluted with chloroform:acetone (9:2, v/v), the colorless fraction was combined and concentrated to dryness to give fine PMFs (25 mg).

2.3. LC/PDA analysis

About 1 mg PMFs was dissolved in 10 ml of methanol (Merck) and 20 µl of the sample solution was injected.

An Agilent 1100 series HPLC was coupled with a photodiode-array detector (PDA) set at 333 nm. UV spectra were taken in the region of 200–400 nm. Chromatographic conditions were as follows: column, Agilent Extend C18 column (150 mm × 4.6 mm, I.D. 5 µm); eluent (A) H_2O, (B) acetonitrile (Merck). The gradient elution had the following profile: 0–20 min 68% H_2O; 20–40 min 68–50% H_2O; 40–50 min 50–20% H_2O. The flow rate was 1 ml/min. The H_2O used (HPLC grade) was purified on a Millipore (Simplicity 185) pure water system.

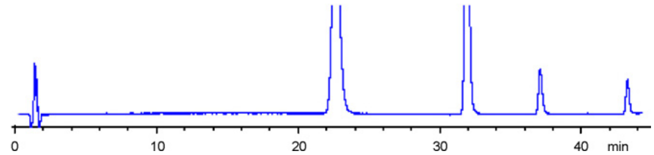


Fig. 1. Chromatogram of the four isolated compounds.

2.4. HPLC/MS/MS

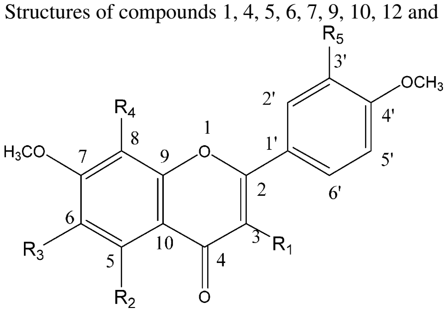
About 1 mg PMFs was dissolved in 10 ml of methanol (Merck) and 20 µl of the sample solution was injected.

LC/MS/MS experiments were performed on a system consisting of ion trap mass spectrometer with an ESI ionization interface (Bruke esquire 3000plus) and an Agilent 1100 HPLC system. ESI experiments were carried out in the positive mode. Mass range measured, m/z 50–1000 m/z; ion trap temperature 250 °C; EM 3.5 kV; drying N_2 10 ml/min; nebulizing N_2 30 psi.

2.5. Isolation and identification of four isolated Citrus flavonoids

The Preparative HPLC was performed with a Varian Prep Star equipped with SD2 pump and a ProStar UV/Vis detector (Model 320). An isocratic mobile phase comprised ethyl acetoacetate /cyclohexane (30:70, v/v) was used at a flow rate of 250 ml/min. The analytes were dissolved in chloroform and separated on a Prep HPLC column (L&L 4004-1L, 80 cm × 10 cm, I.D.) packed with silica gel (silica gel 200–300 mesh). The sample injection volume was 20 ml. Fractions 4–5, 7–10, 16–18 and 27–32 were combined, recrystallized and dried to give compound 6 (6.5 g), compound 10 (5.8 g), compound 12 (0.88 g) and

Table 1
Structures of compounds 1, 4, 5, 6, 7, 9, 10, 12 and 14



Peak no.	Compound	R1	R2	R3	R4	R5
1	Isosinensetin	H	OMe	H	OMe	OMe
4	Sinensetin	H	OMe	OMe	H	OMe
5	Tetramethyl-*o*-isoscutellarein	H	OMe	H	OMe	H
6	Nobiletin	H	OMe	OMe	OMe	OMe
7	Tetramethyl-*o*-scutellarein	H	OMe	OMe	H	H
9	Heptamethoxyflavone	OMe	OMe	OMe	OMe	OMe
10	Tangeretin	H	OMe	OMe	OMe	H
12	5-Demethylnobiletin	H	OH	OMe	OMe	OMe
14	5-Demethyl tangeretin	H	OH	OMe	OMe	H

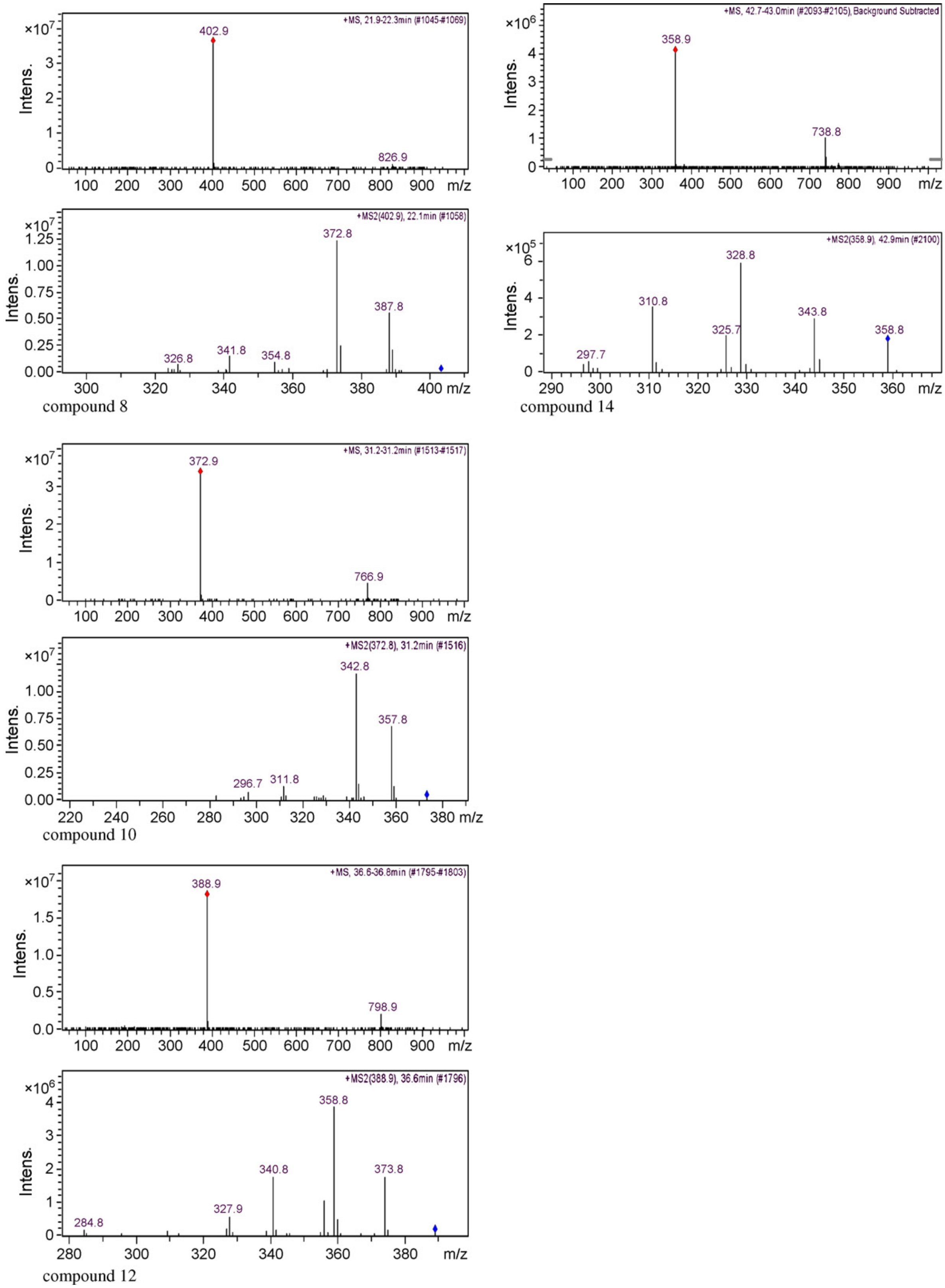


Fig. 2. MS/MS spectra of compound 8, 10, 12, 14.

compound 14 (0.066 g). Finally, the structures of compounds 6, 10, 12 and 14 were confirmed by UV, IR, MS, ^{1}H NMR, ^{13}C NMR and 2D NMR spectral studies. All reagents used were analytical grade.

Compound 6: Compound 6 was colorless needles; $C_{21}H_{22}O_8$; mp 137–138 °C; IR γ_{max} (KBr cm^{-1}): 2943.7, 2839.7, 1645.9, 1592.2, 1520.1, 1462.5, 1370.7, 1277.7, 1016.2, 840.7, 803.4; ^{1}H NMR (500 Hz, CDCl$_3$) δ: 7.61 (1H, dd, J = 8.5 and 1.7 Hz, H-6′), 7.45 (1H, d, J = 8.5 Hz, H-2′), 7.02 (1H, d, J = 8.5 Hz, H-5′), 6.74 (1H, s, H-3), 4.14, 4.05 (each 3H, s, OMe), 3.99 (12H, m, 4× OMe); ^{13}C NMR (500 MHz, CDCl$_3$) δ: 177.6 (C-4), 161.3 (C-2), 152.3 (C-4′), 151.7 (C-7), 149.7 (C-3′), 148.7 (C-5), 148.0 (C-9), 144.4 (C-6), 138.3 (C-8), 124.4 (C-1′), 119.9 (C-6′), 115.2 (C-10), 111.6 (C-5′), 109.0 (C-2′), 107.2 (C-3), 62.5, 62.2, 62.1, 61.9, 56.4, 56.3 (6× OMe). Compound 6 was identified as nobiletin [2-(3,4-dimethoxyphenyl)-5,6,7,8-tetramethoxy-4H-1-benzopyran-4-one] from these spectral data and physical properties [7,9,11,13,15,18,20].

Compound 10: Compound 10 was light yellow needles; $C_{20}H_{20}O_7$; mp 153–154 °C; IR γ_{max} (KBr cm^{-1}): 2947.0, 2842.2, 1650.7, 1608.1, 1586.7, 1513.5, 1462.6, 1362.1, 1264.8, 1110.7, 1074.4, 1016.3, 830.5; ^{1}H NMR (500 Hz, CDCl$_3$) δ: 7.88 (2H, d, J = 8.8, H-2′, 6′), 7.02 (2H, d, J = 8.5, H-3′, 5′), 6.62 (1H, s, H-3), 4.11, 4.03, 3.89 (each 3H, s, OMe), 3.96 (6H, s, 2 × OCH$_3$); ^{13}C NMR (500 MHz, CDCl$_3$) δ: 177.4 (C-4), 162.4 (C-4′), 161.3 (C-2), 151.4 (C-7), 148.4 (C-5), 147.8 (C-9), 144.1 (C-6), 138.1 (C-8), 127.8 (C-2′, 6′), 123.8 (C-1′), 114.9 (C-10), 114.6 (C-3′,5′), 106.7 (C-3), 62.3, 62.1, 61.9, 61.7, 55.6 (5× OMe). Compound 10 was identified as tangeretin [2-(4-methoxyphenyl)-5,6,7,8-tetramethoxy-4H-1-benzopyran-4-one] from these spectral data and physical properties [8,9,11,15,18,20].

Compound 12: Compound 12 was yellow needles; $C_{20}H_{20}O_8$; mp 144–145 °C; IR γ_{max} (KBr cm^{-1}): 2941.3, 2938.6, 1652.2, 1596.3, 1514.5, 1479.5, 1373.8, 173.3, 1144.7, 1022.5, 834.2, 809.8; ^{1}H NMR (500 Hz, CDCl$_3$) δ: 12.20 (1H, s, 5-OH), 7.60 (1H, dd, J = 8.5, 1.7, H-6′), 7.42 (1H, d, J = 1.7, H-2′), 7.00 (1H, d, J = 8.5, H-5′), 6.62 (1H, s, H-3), 4.12 (3H, s, OMe), 4.00 (12H, m, 4× OMe); ^{13}C NMR (500 MHz, CDCl$_3$) δ: 183.1 (C-4), 164.1 (C-2), 153.1 (C-4′), 152.6 (C-7), 149.7 (C-3′), 149.5 (C-5), 146.9 (C-9), 136.7 (C-6), 133.1 (C-8), 123.8 (C-1′), 120.3 (C-6′), 111.4 (C-5′), 108.9 (C-2′) 107.1 (C-10), 104.1 (C-3), 62.2, 61.9, 61.3, 56.3, 56.1 (5× OMe). Compound 12 was identified as 5-demethylnobiletin [5-hydroxy-2-(3,4-dimethoxyphenyl)-6,7,8-trimethoxy-4H-1-benzopyran-4-one] from these spectral data and physical properties[8,18].

Compound 14: Compound 14 was yellowish green needle; $C_{19}H_{18}O_7$; mp 176–177 °C; IR γ_{max} (KBr cm^{-1}): 2939.9, 2838.6, 1652.1, 1602.8, 1575.9, 1509.0, 1477.2, 1369.7, 1269.8,

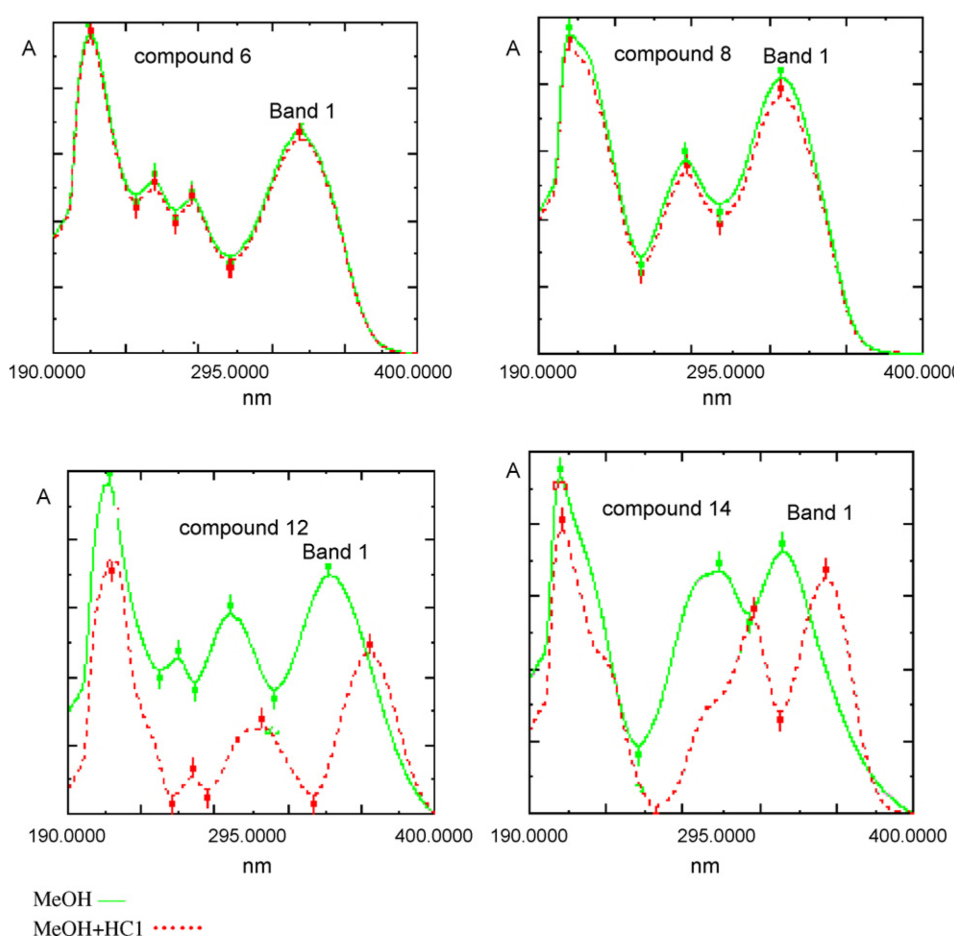


Fig. 3. UV spectra of compounds 6, 10, 12 and 14.

1253.6, 1068.6, 826.8; ^{1}H NMR (500 Hz, $CDCl_3$) δ: 12.35 (1H, s, 5-OH), 7.91 (2H, d, J = 8.8, H-2′,6′), 7.05 (2H, d, J = 8.8, H-3′, 5′), 6.61 (1H, s, H-3), 4.13, 3.99, 3.97, 3.91 (each 3H, s, OMe), 3.98 (6H, s, 2× OMe); ^{13}C NMR (500 MHz, $CDCl_3$) δ: 183.3 (C-4), 164.3 (C-4′), 163.0 (C-2), 153.2 (C-7), 149.8 (C-5), 146.0 (C-9), 136.8 (C-6), 133.2 (C-8), 128.3 (C-2′,6′), 123.7 (C-1′), 114.9 (C-3′, 5′), 107.2 (C-10), 104.0 (C-3), 62.4, 62.0, 61.4, 55.8 (4× OMe). Compound 14 was identified as 5-demethyltangeretin [5-hydroxy-2-(4-methoxyphenyl)-6,7,8-trimethoxy-4H-1-benzopyran-4-one] from these spectral data and physical properties [16].

3. Results

3.1. Identification of four isolated Citrus flavonoids

The chromatogram and mass spectra of compounds 6, 10, 12 and 14 are shown in Fig. 1 and Fig. 2, respectively. The structure of identified components is shown in Table 1.

In full scan mass spectra, compound 6, 10, 12 and 14 show intense molecular ions $[M + H]^+$ at m/z 403, 375, 373 and 359, respectively. In second-stage scan mass spectra, the fragmentation ions may result from the loss of $^\bullet CH_3$, H_2O, CH_3OH or CO. The mechanism of fragmentation pattern needs to be further studied.

For ^{13}H NMR spectra, spectra of 6 and 10 had no A-ring aromatic proton resonances, indicating methoxylation at C-5, 6, 7, 8. Spectra in the B-ring of compound 6 and 10 with ABX type aromatic proton signals showed a pattern of three protons. The size of the coupling constant (J = 1.7 and 8.5 Hz) is characteristic of *meta* and *ortho* coupling as found in 3′,4′-methoxylated flavonoids. Spectra 10 and 14 had a pair of two-proton, orth-coupled doublets arising from two pairs of degenerated protons (H-2′,6′ and H-3′,5′), showing the presence of an A_2B_2 pattern in the B-ring, typical of para-substituted benzene ring. For compounds 12 and 14, there is a signal at about 12 ppm in ^{1}H NMR, indicating a hydroxyl group in the structure.

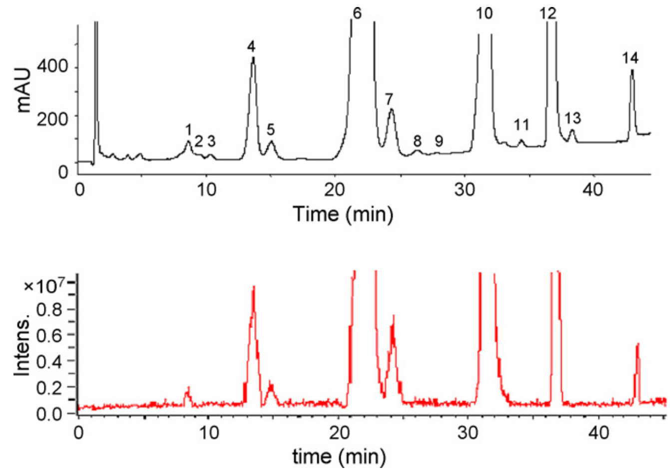


Fig. 4. Chromatogram and total ion current (TIC) spectra.

For further determination of the position of hydroxyl group for compounds 12 and 14, we conducted UV experiments with addition of UV diagnostic reagent $AlCl_3$/HCl as a tool. Results are shown in Fig. 3. For compounds 6 and 10 dissolved in methanol, their UV spectra were the same as those with addition of $AlCl_3$/HCl, which indicated there was no hydroxyl group at position 3 or/and 5; for compounds 12 and 14 dissolved in methanol, band in UV spectra with addition of $AlCl_3$/HCl had a 26 nm red shift compared with UV spectra without addition of $AlCl_3$/HCl, which indicated there was not only a hydroxyl group at position 5, but also a methoxyl group at position 7.

^{13}C NMR spectra assignments were given on the basis of DEPT (Distortionless Enhancement by Polarization Transfer), HMBC (Heteronuclear Multiple Bond Correlation) and HMQC (Heteronuclear Multiple Quantum Correlation) spectra.

In previous studies, compound 14 was isolated and identified from roots of *Ficus hirta* [16], but in our studies, we isolated and confirmed it for the first time from *Citrus*. Its biological properties are urgently needed for further studies.

Table 2
Peak assignment for analysis of PMFs

Peak no.	t_R (min)	$[M + H]^+$ (m/z)	MS/MS (m/z)	Identification
1	8.6	373	358, 343, 329, 312, 297	Isosinensetin
2	9.5	359	344, 329, 311, 298, 280	ND
3	10.2	389	374, 359, 341, 328, 313	ND
4	13.6	373	358, 343, 329, 312, 297	Sinensetin
5	15.1	343	328, 313, 285	Tetramethyl-*o*-isoscutellarein
6	22.1	403	388, 373, 355, 342, 327	Nobiletin
7	24.3	343	328, 313, 299, 282	Tetramethyl-*o*-scutellarein
8	26.2	375	360, 345, 327, 314	ND
9	27.8	433	403, 373, 343, 315, 284	Heptamethoxyflavone
10	31.5	373	358, 343, 312, 297	Tangeretin
11	33.0	296	166, 135	ND
12	36.7	389	374, 359, 356, 341, 328	5-Demethylnobiletin
13	38.3	329	314, 296, 268, 237	ND
14	43.0	359	344, 329, 326, 311, 298	5-Demethyl tangeretin

ND: not determined.

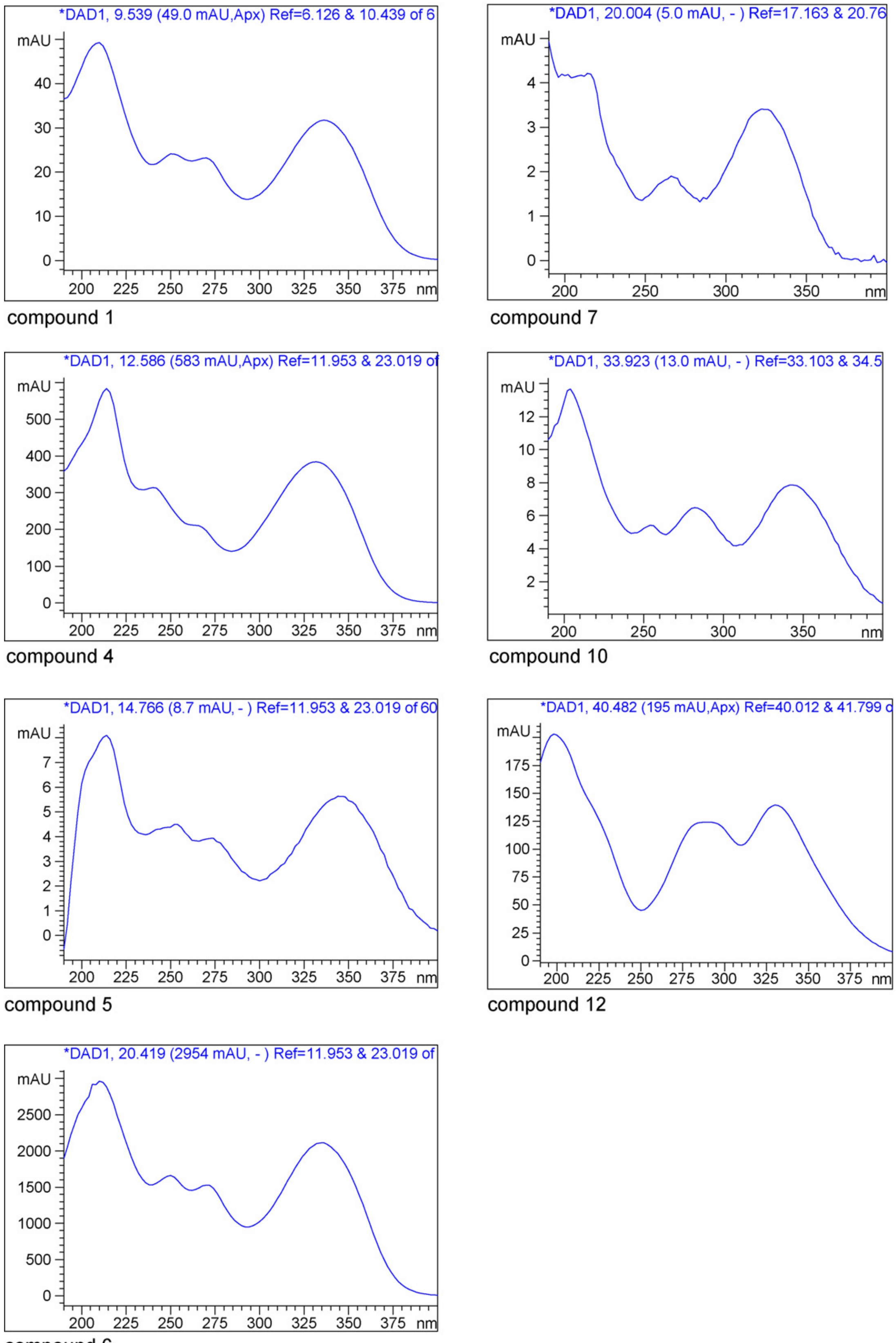


Fig. 5. UV spectra of compounds 1, 4, 5, 6, 7, 10, 12, 14.

3.2. Identification of PMFs from Pericarpium Citri Reticulatae Viride

Fig. 4 shows the chromatogram and total ion current (TIC) of LC/MS/MS experiment.

Compounds 1, 4, 5, 6, 7, 9 and 10 were indicated as tetra-, penta-, hexa-, and heptamethoxy-substituted flavones due to the protonated molecular ions $[M+\mathrm{H}]^+$ at m/z 343, 373, 403 and 433 in the positive mode. Compounds 2, 3, 12, 13 and 14 could be assigned as being monohydroxylated tri-, tetra- and pentamethoxyflavone due to protonated molecular ions $[M+\mathrm{H}]^+$ at m/z 329, 359 and 389. Fig. 5 shows UV spectra of compounds 1, 4, 5, 6, 7, 9, 10, 12 and 14. Compounds 1, 4, 5, 7 and 9 were identified based on their UV spectra, molecular ions, fragment ions and elution order described in the literature [10,12,13,15,17–19]. Compounds 6, 10, 12 and 14 are identified based on the comparison of their retention time, UV spectra, molecular ions and fragment ions with those of the isolated compounds and the literature data.

Compounds 2, 3, 8, 11 and 13 could be detected, but their UV spectra could not be achieved with HPLC coupled with a PDA. We tried to increase the injection volume to obtain their UV spectra, but these efforts had not been successful. Table 2 shows the peak assignment of PMFs.

References

[1] M.E. Bracke, E.A. Bruyneel, S.J. Vermeulen, K. Vennekens, V. Van Marck, M.M. Mareel, Food Technol. 48 (1994) 121–124.

[2] M. Miyazawa, Y. Okuno, M. Fukuyama, S. Nakamura, H. Kosaka, J. Agric. Food Chem. 47 (1999) 5244–5329.

[3] I. Yukiko, T. Yuko, J. Motoharu, Y. Masamichi, I. Chihiro, F. Hiroshi, M. Teruo, K. Masashi, T. Harukuni, N. Hoyoku, Cancer Lett. 163 (2001) 7–9.

[4] M. Mizuno, M. Iinuma, M. Ohara, T. Tanaka, M. Iwamasa, Chem. Pharm. Bull. 39 (1991) 945–949.

[5] K. Robards, X. Li, M. Antolovich, S. Boyd, J. Sci. Food Agric. 75 (1997) 87–101.

[6] Pharmacopoeia of PR China, Beijing, 2005.

[7] S. Qian, L. Chen, J. Chin. Med. Mater. 21 (1998) 301–302.

[8] X. Zhao, X. Ye, D. Zhu, S. Jiang, Chin. Tradit. Herbal Drugs 34 (2003) 11–13.

[9] X. Guo, T. Wang, M. Guo, Y. Chen, China J. Chin. Mater. Med. 25 (2000) 146–148.

[10] J.M. Sendra, S.L. Navarro, L. Izquierdo, J. Chromatogr. Sci. 26 (1998) 443–448.

[11] S. Zhang, B. Ma, D. Zheng, Q. Dai, J. Instrum. Anal. 23 (2004) 110–111.

[12] P. Mouly, E.M. Gaydou, A. Auffray, J. Chromatogr. A 800 (1998) 171–179.

[13] M. Miyazawa, Y. Okuno, M. Fukuyama, S. Nakamura, H. Kosaka, Agric. Food Chem. 47 (1999) 3269–5244.

[15] B. Weber, B. Hartmann, D. StÖckigt, K. Schreiber, M. Roloff, H.-J. Bertram, C. Schmidt, J. Agric. Food Chem. 54 (2006) 274–278.

[16] C. Li, P. Bu, D. Yue, Y. Sun, China J. Chin. Mater. Med. 31 (2006) 131–133.

[17] P. Dugo, L. Mondello, R. Stancanelli, G. Dugo, J. Pharm. Biomed. Anal. 24 (2000) 147–154.

[18] X. Wang, F. Li, H. Zhang, Y. Geng, J. Yuan, T. Jiang, J. Chromatogr. A 1090 (2005) 188–192.

[19] A.M. Pupin, M.-J. Dennis, M.C.F. Toledo, Food Chem. 63 (1998) 513–518.

[20] X. He, L. Lian, L. Lin, M.W. Bernart, J. Sci. Food Agric. 791 (1997) 127–134.