Journal of Pharmaceutical and Biomedical Analysis 49 (2009) 207–213

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Characterization of polymethoxylated flavones in *Fructus aurantii* by off-line two-dimensional liquid chromatography/electrospray ionization-ion trap mass spectrometry

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ARTICLE INFO

Article history:
Received 17 March 2008
Accepted 20 October 2008
Available online 11 November 2008

Keywords:
2D-LC
ESI-ITMS
Fructus aurantii
Matrix effects
Polymethoxylated flavones

ABSTRACT

Off-line two-dimensional reversed-phase liquid chromatography (2D-RPLC) coupled to electrospray ionization-ion trap mass spectrometry (ESI-ITMS) was operated in positive mode (PI) to characterize polymethoxylated flavonoids (PMFs) in botanical sample. The fragments of $[M+H-n\times15]^+$ produced by loss of one or more methyl group from the protonated molecules, as well as $[M+H-14]^+$, $[M+H-29]^+$, $[M+H-33]^+$, $[M+H-43]^+$, $[M+H-46]^+$ and $[M+H-61]^+$ fragments formed the multiple MS (MS^n) "fingerprint" of PMFs. 42 target compounds were tentatively identified from the extract of *Fructus aurantii* (*F. aurantii*) based on this "fingerprint". Experimental outcomes indicated that the application of 2D separation method can reduce the matrix suppression of analytes caused by the coelution with interferential components and the column overloading of interferential components. 42 versus 23 target compounds were detected through 2D versus 1D method, which confirm the superiority of 2D coupled to MS in elimination of matrix effects.

1. Introduction

Herbal medicines are greatly complex mixtures, containing usually hundreds of chemically different constituents ranging in concentration from mg/g to pg/g. The analysis and identification of constituents in herbal medicines is becoming one of the hotspots and difficulties for current analytical chemistry. The routine procedure of identifying the components in herbal medicine is normally divided into three steps: detection → isolation → identification. Detection is the essential step, which reveals the existence and distribution of constituents in samples analyzed and offers information for further isolation and identification. Chromatography is one of the analytical instruments used mostly in this step and the signs for existence of constituents are chromatographic peaks. However, some constituents maybe have no "peaks" during analysis by chromatography for three main reasons: coelution, low abundance and high background. To overcome such problems, high-resolution chromatographic methods coupled to highly sensitive and selective detectors are needed. Satisfying such analytical requirements with respect to the characteristic of herbal medicines, LC–MS (MS/MS) is maybe the optimal choice.

In our former study, a LC/atmospheric pressure chemical ionization (APCI)-MS/MS method was employed to analyze PMFs in *F. aurantii* and 29 target compounds were tentatively identified [1]. However, 1D-LC employed in the study has limited separation ability, which could not eliminate effectively the MS signal suppression resulting from matrix effects. Therefore, some traces of PMFs maybe were missed in the analysis. Matrix effects refer to the phenomenon that the "soft ionization" source MS signal of analytes is suppressed or enhanced (suppressed mostly) by the molecules coeluting [2,3]. The matrix effects are believed to result from the competition between analytes and matrix components in access to the droplet surface for gas phase emission during ionization. To eliminate the matrix effects in LC–MS analysis, off-line and on-line 2D separation method were reported recently [4–6].

Multiple dimensional (MD) chromatography is a powerful separation method, which refer to a technique having more than one step of separation is applied to a sample, each step being considered an independent separation dimension [7]. Though the selectivity of the multiple separation mode is not completely orthogonal and the achievable peak capacity (PC) is lower than expected, but the method still has huge potential to increase PC and provide a possibility for total separation of complex mixtures [8]. MD chromatography may be either on-line or off-line, referring to whether subsequent dimensions are directly coupled to the previous one, or whether "manual" intervention is required to transfer analytes.

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doi:10.1016/j.jpba.2008.10.044

On-line comprehensive MD separation has a higher throughput and can be automated without sample loss, but direct coupling is technically challenging. Meanwhile, the analysis speed of the second dimension should be fast enough to guarantee completing the analysis before the next sample injection [9,10]. The separation ability is unavoidably sacrificed when the analysis of second dimension is carried out in short separation time. Off-line MD separation can be achieved with easily coupling and the second dimension can obtain high separation ability in long analysis time, whereas the method is time consuming along with sample loss [11,12]. In these years, MD chromatography has been widely used to separate biomolecules [9,12], polymer [13], herbal medicines [14–16] and other complex mixtures [17,18] due to its powerful separation ability.

In this paper, an off-line 2D-LC/ESI-ITMS method was developed for separation and identification of PMFs in extract of *F. auranti*. Off-line method was employed for its high separation ability and easy implementation. The effect of liquid chromatographic separation on matrix-related signal suppression in ESI-ITMS was investigated by comparing the results of analysis through 2D versus 1D separation methods.

2. Experimental

2.1. Standards and reagents

PMFs standards, sinensetin (SIN, 3′,4′,5,6,7-pentamethoxyflavone, MW 372) was purchased from Meryer Co. (Sweden); tangeretin (TAN, 4′,5,6,7, 8-pentamethoxyflavone, MW 372) was purchased from Xian-tong-shi-dai Co. (China). The following agents were in HPLC grade: acetonitrile purchased from Merck Co. (Germany); ethanol, methanol and ethyl acetate were purchased from Yu-wang Co. (China). Reverse osmosis Milli-Q water (18.2 MΩ) (Millipore, USA) was used for all solutions and dilutions.

2.2. Instrumentation

Agilent 1100 Series LC/MSD Trap XCT (Agilent, USA) with a photodiode array detector (DAD) monitoring at 326 nm was used in this study. Eluent: (A) water, (B) acetonitrile. The linear gradient was listed below and the column temperature was 30 °C. The three analytical columns used in this study were listed below: Hypersil ODS2 C18 (Elite, China), 250 mm × 4.6 mm, 5 μm; ZORBAX RX-C8 (Agilent, USA), 250 mm × 4.6 mm, 5 μm; ZORBAX SB-CN (Agilent, USA), 250 mm × 4.6 mm, 5 μm.

ESI mass spectra were acquired in PI mode. Nitrogen was used as the nebulizing gas at 35 P.S.I and as drying gas at a flow rate of 10 L/min and at a temperature of 350 °C. Ions were obtained in the range of *m/z* 250–450. MS^n spectra was obtained by auto-MS^3 mode (the ion of base peak is selected as precursor ion for next stage MS automatically), the fragmentation amplitude (FA) was 1.5 V (SmartFrag: 30–200%) and the MS^n isolation width was 4.0 *m/z*. 0.25 mL/min mobile phase was entered MS from outlet of DAD via diffluence.

2.3. Plant material

F. aurantii were collected from Kai county, Chongqing City, China. The herb was authenticated by Institute of Medication, Xiyuan hospital of China Academy of Traditional Chinese Medicine. The procedures of extraction were as follow: 100 kg herb was grounded into powder and decocted in 1000 L water at 100 °C for 120 min. Then the residue was collected and re-decocted in 1000 L water at 100 °C for 90 min. The decoction in both times were collected and dried by spray drying. Then 1.5 kg residue was dissolved in 15 L water–ethanol (30:70, v/v). After stirred continuously for 0.5 h, the solution was stored at room temperature for 12 h. The mixture was filtered using φ7 cm qualitative filter paper and the filtrate was dried with a rotary evaporator at 60 °C. The residue was dissolved in 1 L water and extracted twice by 5.25 and 2.25 L ethyl acetate, respectively. The organic layers in both times were collected, combined, and dried with a rotary evaporator at 60 °C. 10 g residue was dissolved in 100 mL acetonitrile and filtered through 0.45 μm filters. This solution was separated through Purification Factory system (Waters, USA). The fraction of PMFs (FP) (see Fig. 1) was collected and dried with a rotary evaporator at 60 °C. The residue was dissolved in acetonitrile and filtered through 0.22 μm membranes before LC analysis.

3. Results and discussion

3.1. The 2D-LC separation of the FP

In order to choose the suitable chromatography modes in fractionation of FP and followed analysis by LC–ESI/ITMS, the separations of FP on C18, C8, and CN columns were performed, and the acquired chromatograms were shown in Fig. 2. Though the *F. aurantii* extract was separated by Purification Factory system and only FP (see Fig. 1) was collected for analysis, the components were still very complex (see Fig. 2). Different pattern of chromatograms were observed due to different chemical selectivity on these three columns, separation of a solute on C18, C8, and CN. Comparing with the experimental results given in Fig. 2, the C18 and C8 column

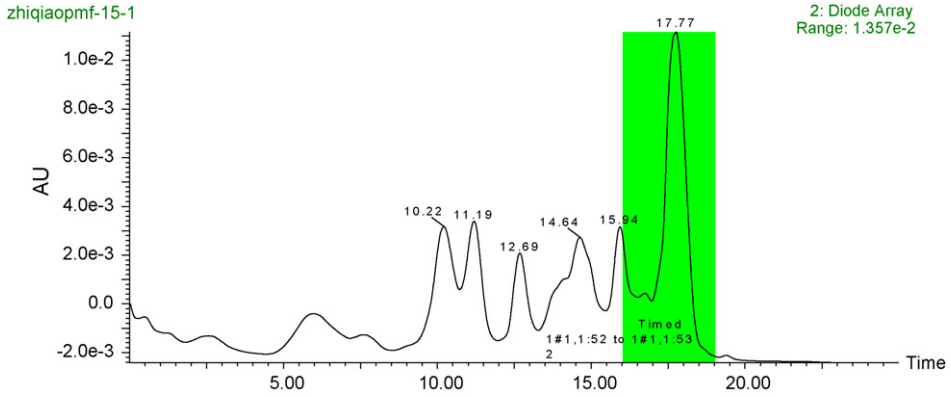

Fig. 1. Chromatogram of the *F. aurantii* extract on Purification Factory system. Experimental conditions: column: XTerra™ C18 (Waters, USA), 100 mm × 19 mm, 5 μm; eluent: (A) water, (B) methanol; linear gradient: 0–5 min, 30% B; 5–15 min, 30–70% B; 15–16 min, 70–95% B; 16–25 min, 95% B; flow rate: 16.37 mL/min; column temperature: 30 °C; injection volume: 0.15 mL; sample concentration: 100 mg/mL. FP was collected between 16 and 19 min.

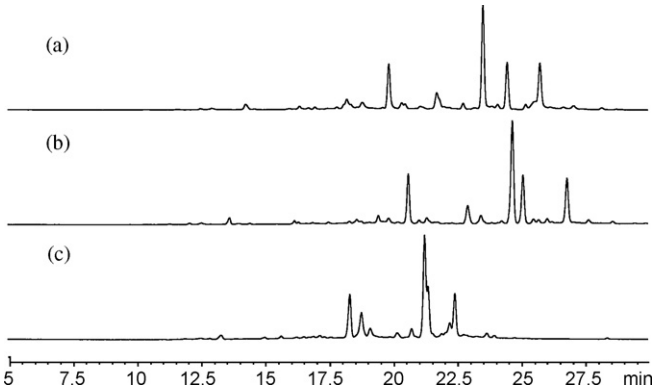

Fig. 2. Chromatograms of FP on C18 (a), C8 (b) and CN (c) column. Experimental conditions: eluent: (A) water, (B) acetonitrile; linear gradient: 0–30 min, 20–90% B; flow-rate: 1 mL/min; injection volume: 20 μL; sample concentration: 0.1 mg/mL.

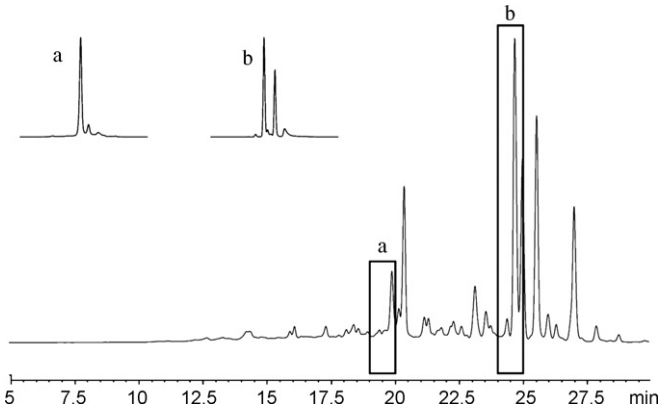

Fig. 3. Chromatogram for fractionalizing of FP on C8 column. Experimental conditions: eluent: (A) water, (B) acetonitrile; linear gradient: 0–30 min, 20–90% B; flow-rate: 1 mL/min; injection volume: 20 μL; sample concentration: 10 mg/mL. a: the second-dimensional chromatogram on C18 of fraction 10 (RT = 19–20 min); b: the second-dimensional chromatogram on C18 of fraction 15 (RT = 24–25 min). Experimental conditions of the second-dimension: eluent: (A) water, (B) acetonitrile; linear gradient: 0–30 min, 20–90% B; flow-rate: 1 mL/min; injection volume: 20 μL.

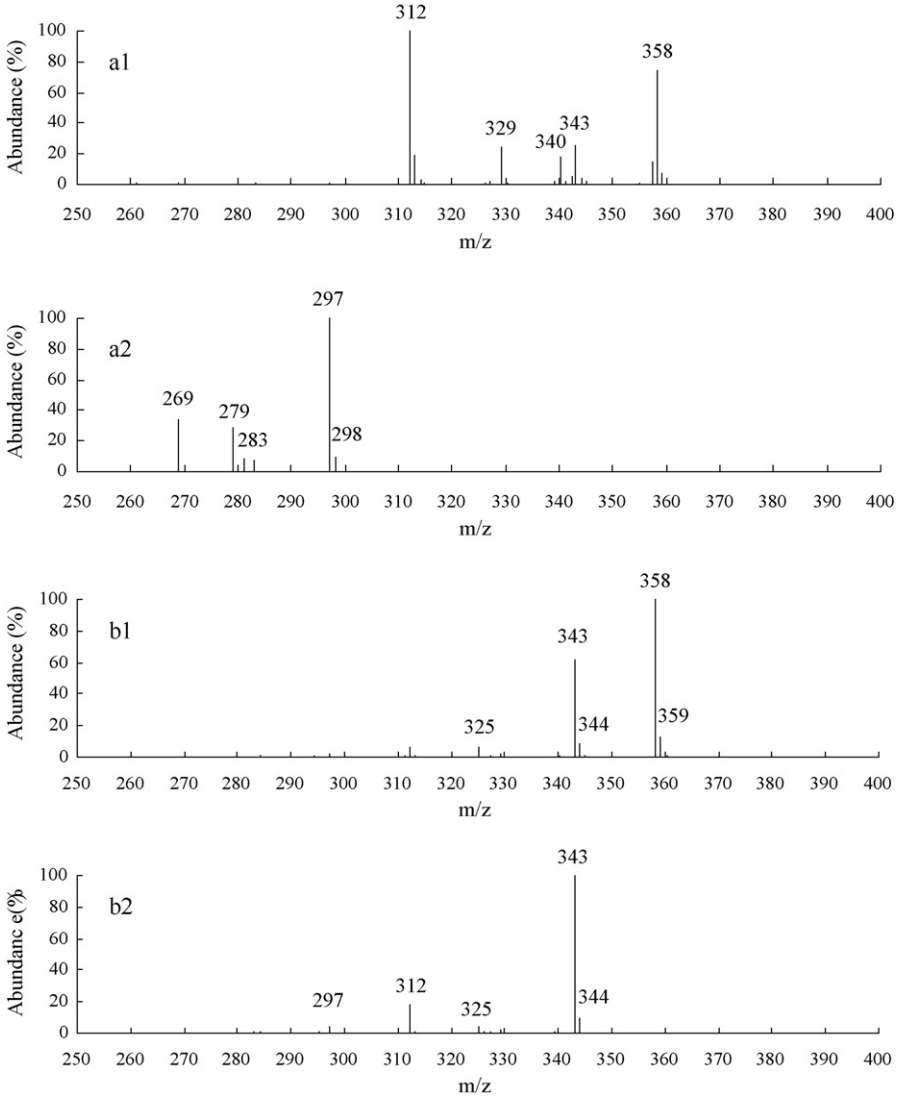

Fig. 4. MS^n spectra of SIN and TAN: (a1) MS^2 spectrum of SIN (precursor-ion was m/z 373($[M+H]^+$)); (a2) MS^3 spectrum of SIN (precursor-ion was m/z 312 ($[M+H-61]^+$)); (b1) MS^2 spectrum of TAN (precursor-ion was m/z 373 ($[M+H]^+$)); (b2) MS^3 spectrum of TAN (precursor-ion was m/z 358 ($[M+H-15]^+$)).

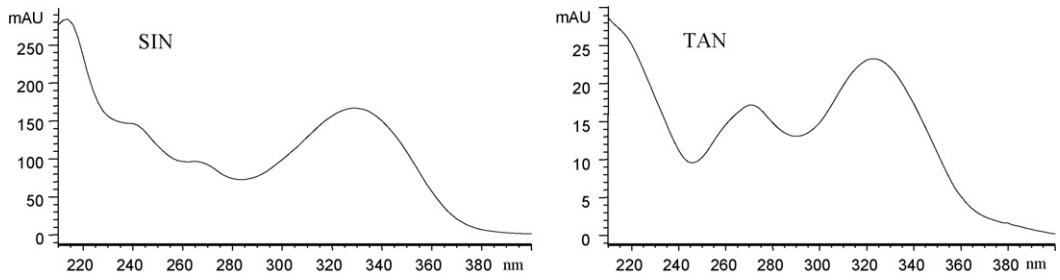

Fig. 5. The UV spectra of SIN and TAN.

show better resolution and higher efficiency compared with the CN column. It was reported that the C8/C18 system demonstrated the greatest efficiency in elimination matrix effects for most compounds [4,5]. Therefore, the C8 and C18 column were chosen for further separation of FP as the first- and second-dimension, respectively.

The chromatogram for fractionalizing of the FP on C8 was illustrated in Fig. 3. It could be seen all the components in the extract were eluted completely from the column in 30 min. So, the eluate from the C8 column was collected every 1 min manually from retention time (RT) 11 to 30 min (PMFs distributing in this RT range), and a total number of 20 fractions were collected for further treatment and analysis. In order to increase the concentrations of components in the collected fractions and enhance their detection sensitivity in the following analysis by LC–ESI/ITMS, each of the 20 fractions was dried with nitrogen evaporator and the residues were diluted in 0.2 mL acetonitrile for further analysis. All the collected and concentrated fractions were injected into C18 column for further separation under the same elution gradient as first-dimension. Passing through the procedure including collection, evaporation, resolution, and injection into second-dimensional column, the amount of 1/40 analytes as initial injection should be transferred into MS detector if no sample loss occurred.

3.2. MS^n "fingerprint" and UV spectra of PMFs obtained from standards SIN and TAN

For characterization PMFs in FP, the diagnostic MS^n "fingerprint" and UV spectra were obtained firstly from two commercially available PMF standards, TAN and SIN. The FA was 1.5 V in MS^n analysis of the standards, which was selected through optimized experiments. Ions were obtained in the range of m/z 250–450, which was selected because low m/z fragments were rarely detected [1]. Our former study indicated some diagnostic fragments such as $[M+H-n\times15]^+$, $[M+H-29]^+$, $[M+H-33]^+$, $[M+H-43]^+$, $[M+H-46]^+$ and $[M+H-61]^+$ formed the diagnostic MS^n "fingerprint" of PMFs by APCI-ITMS [1]. Though ESI source was employed in this study, similar MS fragment patterns were expected. Meanwhile, the UV spectra of flavonoids shows characteristic "double-peaks" phenomenon and the absorption maxima near 330 nm for PMFs [19], which can be used as another diagnostic marker.

Fig. 4 shows the MS^n product-ions spectra of protonated TAN and SIN, in which some diagnostic fragments were observed. The protonated flavones TAN and SIN dissociated predominantly via loss of one or two methyl radical(s) (15 or 30 Da) and formed the fragments $[M+H-15]^+$ or $[M+H-30]^+$, which was the most distinct characteristic for PMFs [1,20]. Other diagnostic fragments include product ions corresponding to the loss of 14, 29, 33, 43, 46 and 61 from precursors. These fragmentations were observed previously by APCI or ESI-MS analysis of PMFs [1,21]. Fragments due to neutral loss of 18, 28 and 44 were observed too, which were reported previously in characterization of flavonoids by MS [21]. Through MS^n experiments of the two standards, the diagnostic fragmentations for PMFs were acquired, which formed the "fingerprint" for further characterization of target compounds in highly complex mixtures. Fig. 5 shows UV spectra of TAN and SIN, which demonstrate characteristic "double-peaks" phenomenon and the absorption maxima were near 330 nm. The main fragment ions and UV information of SIN and TAN were summarized in Table 1.

3.3. EIC-MS method to screen out potential PMFs in FP

Through standards SIN and TAN, the diagnostic MS^n "fingerprint" and characteristic UV spectra of PMFs had been obtained. Next, the candidates for PMFs in FP should be screened out by some methods for further verification by these characters. PMFs have the basic aglycone structure and differ in the position and number of methoxyl groups (OCH_3) and/or hydroxyl groups (OH) on the A, B and C rings of the aglycone. The molecular weight (MW) of basic structure of aglycone is 222 Da (see Fig. 6), which is increased by 30 or 16 when a methoxyl or hydroxyl group added. Through this regularity, the MWs of all possible structure of PMFs can be designed in advance. After screening these MWs with EIC-MS method by LC–MS, all possible PMFs compounds existing in FP could be screened out.

3.4. MS^n "fingerprint" and UV spectra to characterize PMFs

Nearly 100 candidates for PMFs were screened out through the EIC-MS method by MWs, therefore further verification with their MS^n information and UV spectra were still needed. Among those candidates, 42 compounds were tentatively identified as PMFs (see Table 2) by their MS^n information and UV spectra. These compounds distributed in 12 fractions from fraction 6 to fraction 17. Though some target compounds distribute synchronously in two consecutive fractions (cross distribution), but the fractions with high-concentration target compounds were selected to exhibit (see Fig. 7).

The main MS^n information of 42 compounds was summarized in Table 3. In addition, the specific UV information of eight com-

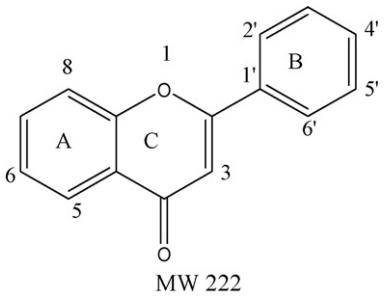

Fig. 6. The basic structure of flavone aglycones.

Table 1
MS^n data of protonated SIN and TAN.

Comp	RT (min)	FA (V)	$[M+H]^+$	MS/MS			MS/MS/MS			UV spectra (absorption maxima)	
				P-ion (%)	Loss (Da)	L-R	P-ion (%)	Loss (Da)	L-R	Band I (nm)	Band II (nm)
SIN	14.66	1.5	373	312※(100)	61	$CO+H_2O+CH_3^•$	297 (100)	15	$CH_3^•$	266	330
				358 (74.6)	15	$CH_3^•$	269 (34.1)	43	$CO+CH_3^•$		
				343 (25.9)	30	$2CH_3^•$	279 (28.7)	33	$H_2O+CH_3^•$		
				329 (24.0)	44	CO_2	298 (9.5)	14	$CH_2^{••}$		
				340 (18.6)	33	$H_2O+CH_3^•$	283 (7.4)	29	$HCO^•$		
TAN	16.85	1.5	373	358※(100)	15	$CH_3^•$	343 (100)	15	$CH_3^•$	266	326
				343 (61.7)	30	$2CH_3^•$	312 (17.6)	46	$CO+H_2O$		
				359 (13.1)	14	$CH_2^{••}$	344 (9.9)	14	$CH_2^{••}$		
				344 (9.0)	29	$HCO^•$	297 (4.2)	61	$CO+H_2O+CH_3^•$		
				325 (6.8)	48	$2CH_3^•+H_2O$	325 (4.2)	33	$H_2O+CH_3^•$		

$[M+H]^+$ represents the *m*/*z* of the protonated flavone. P-ion (%) represents the production-ion and the relative abundance. The production-ions tag with "※" represent precursor-ion for next stage MS. L-R represents the radical loss.

Table 2
The MW and structural identification of all PMFs detected in FP by 2D LC–MS method.

No.	Amounts	Structural identification	OCH_3	OH	MW
1–3	3	Monohydroxy-trimethoxyflavone	3	1	328
4–5	2	Trimehydroxy-trimethoxyflavone	3	3	360
6–7	2	Tetramethoxyflavone	4	0	342
8–12	5	Monohydroxy-tetramethoxyflavone	4	1	358
13–15	3	Dihydroxy-tetramethoxyflavone	4	2	374
16–18	3	Trihydroxy-tetramethoxyflavone	4	3	390
19–22	4	Pentamethoxyflavone	5	0	372
23–28	6	Monohydroxy-pentamethoxyflavone	5	1	388
29–32	4	Dihydroxy-pentamethoxyflavone	5	2	404
33–36	4	Hexamethoxyflavone	6	0	402
27–40	4	Monohydroxy-hexamethoxyflavone	6	1	418
41–42	2	Heptamethoxyflavone	7	0	432

pounds was obtained too. The MS^n information of the 42 target compounds had three distinct characteristic which accord with the "fingerprint" of PMFs obtained by SIN and TAN (see Table 3). Firstly, except compound 7 (*m*/*z* 343), 8 (*m*/*z* 359), 21 (*m*/*z* 373) and 27 (*m*/*z* 389), the predominant ions in MS^2 spectra were fragments corresponding to the loss of one or two methyl radicals ($CH_3^•$) from precursors. Secondly, the main product-ions of the compounds in MS^n spectra were those diagnostic fragments which correspond to the loss of 14, 29, 33, 43, 46, and 61 from precursors. Thirdly, besides those diagnostic fragments for PMFs, other main product-ions were fragments corresponding to the neutral loss of 18, 28 and 44 from precursors. Meanwhile, the UV spectra obtained for compound 7 (*m*/*z* 343), 19 (*m*/*z* 373), 21 (*m*/*z* 373), 22 (*m*/*z* 373), 26 (*m*/*z* 389), 28 (*m*/*z* 389), 36 (*m*/*z* 403), and 41 (*m*/*z* 433) show the same characteristic as standard TAN and SIN, which further validated the identification by their MS^n "fingerprint". In these compounds, compound 21 (*m*/*z* 373) and 22 (*m*/*z* 373) were identified as SIN and TAN respectively, since they have the identical RTs, UV spectra and MS^n information with the standards. Other compounds were characterized as PMFs by their MS^n information and UV spectra, without confirmation of the exact position of the substituent groups.

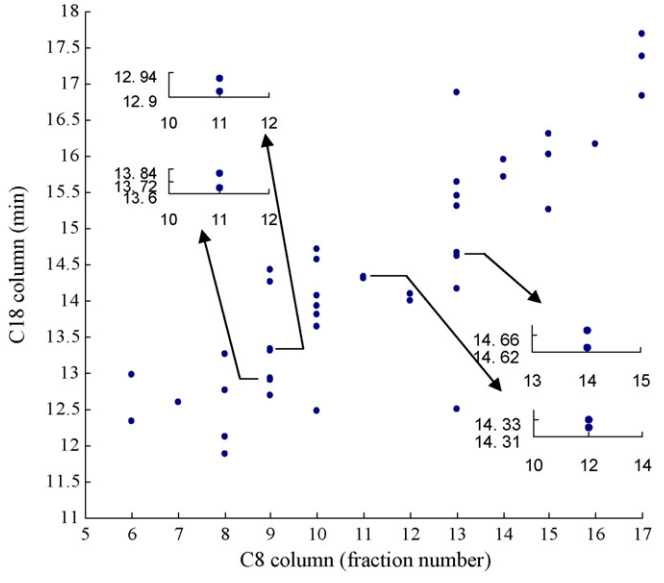

Fig. 7. 2D chromatographic RTs of PMFs in FP (each point represents a target compound). Experimental conditions of the second-dimension: eluent: (A) water, (B) acetonitrile; linear gradient: 0–30 min, 20–90% B; flow-rate: 1 mL/min; injection volume: 20 μL. In this figure, each point represents a target compound. As some points are too closer to discern in the figure, we enlarge which as insets indicated by arrow.

3.5. 2D versus 1D methods

In order to prove that 2D separation can eliminate matrix effects in MS application, the analysis of FP using 1D method without previous fractionation on the C8 column was performed as described above. To enhance separation efficiency of LC under gradient conditions, the gradient slope was decreased. In this situation, 23 target compounds were detected (data not shown). Compared to 2D method, nearly a half of target compounds were missed in 1D analysis due to matrix-related signal suppression, though the amount of each component into MS was nearly same.

The main causes for signal suppression in LC–MS system were believed to be the coelution of analytes with matrix components then eluted into the ESI source simultaneously. 2D method has high ability of chromatographic separation to reduce the coelution and remove the matrix effects to a certain extent. However,

Table 3
MS^n data and UV information of protonated PMFs in FP.

No.	RT (min)	Fraction (No.)	$[M+H]^+$	MS/MS-loss (Da)%	MS/MS/MS-loss (Da) %	UV spectra (absorption maxima)	
						Band I (nm)	Band II (nm)
1	11.87	8	329	30※(100),15(77.4),44(5.2),29(5.2),14(3.8)	28(100),16(10.6),27(4.3),30(2.5)		
2	12.77	8	329	15※(100),61(94.7),44(33.7),30(27.6),16(17.3)	46(100),15(26.1),29(19.6),18(14.5),45(6.2)		
3	12.47	10	329	15※(100),14(9.9),30(6.2),29(2.1),31(0.7)	15(100),46(58.1),18(49.8),28(8.8),29(2.9)		
4	13.27	8	361	15※(100),33(81.4),18(17.8),17(15.9),19(14.4)	18(100),90(27.6),48(19.9),61(12.7),31(7.6)		
5	14.44	9	361	15※(100),60(27.8),18(26.1),46(21.7),30(17.7)	46(100),31(93.8),17(65.9),59(61.6),48(59.8)		
6	15.26	15	343	30※(100),15(58.4),14(7.2),29(7.0),44(2.2)	28(100),27(20.4),56(10.5),30(3.9),42(3.4)		
7	16.02	15	343	61※(100),15(70.0),44(27.8),30(16.1),16(15.9)	15(100),31(37.8),28(13.0),29(7.5)	268	320
8	12.13	8	359	19※(100),30(68.6),63(65.1),15(51.0),18(18.2)	18(100),44(86.2),28(80.2),64(78.5),90(74.2)		
9	12.93	9	359	15※(100),61(59.3),33(33.4),30(16.9),44(13.7)	46(100),29(28.2),18(19.1),45(15.2),28(3.6)		
10	13.65	10	359	15※(100),30(56.0),61(11.1),14(9.2),29(6.8)	15(100),46(22.5),14(11.9),61(5.7),33(5.6)		
11	14.09	12	359	15※(100),60(20.7),33(19.7),14(19.0)30(15.2)	18(100),46(35.2),33(25.7),15(21.2),30(21.0)		
12	14.63	13	359	15※(100),30(31.1),48(9.0),14(8.2),33(6.8)	15(100)33(36.0),18(19.5),14(7.20,46(3.3)		
13	12.34	6	375	15※(100),14(13.1),30(8.9),19(5.4),16(3.2)	15(100),33(21.6),21(15.8),71(13.5),14(12.8)		
14	14.57	10	375	15※(100),33(54.5),30(35.4),14(13.2),29(10.4)	18(100),15(86.7),48(14.1),65(8.5),28(7.9)		
15	15.64	13	375	15※(100),30(86.2),33(44.3),48(33.6),61(18.7)	15(100),33(32.5),18(28.8),46(14.3),18(6.2)		
16	12.97	6	391	15※(100),30(56.2),14(22.5),33(12.8),29(11.8)	15(100),18(36.0),33(18.7),14(16.5),17(9.3)		
17	14.08	10	391	30※(100),15(76.5),31(55.5),17(54.9),29(27.9)	18(100),17(28.9),28(13.2),15(8.7),21(8.2)		
18	15.46	13	391	30※(100),15(71.9),31(49.7),29(31.4),17(30.1)	28(100),27(16.0),43(4.0),30(3.6),21(2.8)		
19	13.99	12	373	30※(100),15(69.4),29(19.7),14(7.9),44(3.5)	28(100),27(3.6),19(3.5),77(3.3),43(3.0)	270	340
20	12.51	13	373	15※(100),46(60.7),43(37.5),28(19.5),30(15.2)	30(100),57(90.7),14(52.5),27(13.5),16(10.7)		
21	14.67	13	373	61※(100),15(57.1),44(30.1),33(20.9),30(20.5)	15(100),43(60.5),16(49.1),61(27.4),44(25.4)	266	330
22	16.84	17	373	15※(100),30(63.7),14(13.3),29(9.9),61(8.4)	15(100),46(17.6),14(13.8),33(6.5),45(3.1)	266	326
23	12.69	9	389	15※(100),30(81.0),29(28.5),14(27.1),32(14.9)	15(100),46(37.5),18(29.2),14(13.4),70(34.1)		
24	12.91	9	389	15※(100),14(29.3),32(12.4),30(10.7),31(6.1)	18(100),15(83.1),14(21.1),17(20.4),32(13.5)		
25	13.31	9	389	15※(100),29(36.6),14.(25.2),28(12.9),27(5.0)	15(100),14(24.6),17(9.6),32(5.3),31(3.9)		
26	13.93	10	389	15※(100),30(96.6),29(30.7),14(27.0),60(7.0)	15(100),14(13.6),45(6.8),33(2.8),45(2.6)	272	336
27	15.95	14	389	33※(100),15(33.6),32(23.4),30(8.7),14(8.0)	28(100),43(77.6),15(71.4),46(67.4),29(19.5)		
28	17.69	17	389	30※(100),15(56.4),32(29.3),29(27.4),47(21.3)	18(100),31(76.7),28(19.4),17(16.0),32(15.7)	284	342
29	12.60	7	405	30※(100),15(96.9),29(36.1),14(24.0),28(6.6)	18(100),15(29.4),28(11.4),17(5.9),47(4.6)		
30	14.27	9	405	15※(100),30(35.9),14(21.7),29(13.4),28(3.6)	15(100),14(14.2),32(8.5),17(5.0),31(3.5)		
31	14.72	10	405	15※(100),30(45.7),29(21.3),14(20.5),32(5.9)	15(100),17(18.2),14(11.1),32(1.0),31(0.3)		
32	17.38	17	405	30※(100),15(55.5),29(26.6),14(12.5),32(11.5)	28(100),18(13.9),27(13.0),17(2.1),30(1.8)		
33	14.33	11	403	30※(100),15(46.0),32(29.0),29(26.1),31(13.0)	31(100),46(44.6),28(39.1),30(25.4),16912.1)		
34	14.17	13	403	30※(100),29(29.7),15(29.2),28(15.2),14(7.9)	26(100),15(53.7),16(39.1),30(36.7),27(25.3)		
35	15.30	13	403	30※(100),16(68.9),33(42.9),15(31.7),29(25.6)			
36	15.72	14	403	30※(100),15(88.3),29(38.6),14(22.7),28(6.7)	46(100),15(59.7),28(35.4),18(31.4),43(24.1)	272	334
37	13.33	9	419	30※(100),15(94.9),29(21.9),14(16.1),48(16.0)	43(100),28(77.9),18(61.5),15(53.5),33(41.5)		
38	13.80	10	419	15※(100),29(78.0),28(26.0),14(22.7),30(2.6)	15(100),14(13.4),16(1.9),32(1.8),31(1.3)		
39	14.32	11	419	15※(100),30(92.5),29(24.2),14(23.5),31(4.7)	15(100),14(20.8),30(3.7),18(3.5),32(3.3)		
40	16.88	13	419	15※(100),30(81.7),29(21.5),14(19.9),33(14.3)	15(100),18(28.5),14(20.0),16(3.9),33(3.6)		
41	16.30	15	433	15※(100),29(34.6),14(22.7),28(6.9),14(3.9)	15(100),14(13.5),30(4.6),32(3.6),18(3.1)	270	328
42	16.16	16	433	30※(100),15(87.7),29(37.4),14(29.6),28(11.7)	15(100),30(59.0),28(46.1),18(38.8),14(23.1)		

$[M+H]^+$ represents the protonated molecule. Loss (Da) (%) represents the lossing radicals and its relative abundance. The production-ion tag with "※" represents precursor-ion for next stage MS.

the 2D-LC system based on C8/C18 mode in this study had limited orthogonality. As illuminated in Fig. 3, the second-dimensional chromatograms of fraction 10 and 15 have limited resolution. In this situation, the removing of the matrix effects was affected not only by high ability of separation, but also by significant reduction of column overloading of matrix components [4,5].

4. Conclusions

2D-LC/ESI-ITMS was performed in three steps to identify PMFs in complex matrix. Firstly, the diagnostic MS^n "fingerprint" and UV spectra of PMFs were acquired through analysis of standards SIN and TAN. Secondly, the candidates for PMFs were screened out by their MWs through EIC method. Thirdly, the MS^n information and UV spectra of candidates were summarized for theirs further verification. Totally 42 PMFs in FP were tentatively identified through this procedure.

In addition, the effect of LC separation on matrix-related signal suppression was investigated in analysis of complex mixture by MS or MS/MS. Coelution of interferential components with analytes and column overloading of interferential components are believed to be the main causes for signal suppression. 2D separation method is a highly effective and efficient approach to reduce MS signal suppression effects by elimination of the coelution and the column overloading. In comparison, 42 versus 23 target compounds were detected through 2D versus 1D method, which confirmed the ability of 2D method in elimination of matrix effects.

Acknowledgement

This work was supported by "The Knowledge Innovation Program of DICP, CAS (K2006A4)".

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