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Characterization of Polymethoxylated Flavonoids (PMFs) in the Peels of 'Shatangju' Mandarin (*Citrus reticulata* Blanco) by Online High-Performance Liquid Chromatography Coupled to Photodiode Array Detection and Electrospray Tandem Mass Spectrometry

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ABSTRACT: A sensitive HPLC-DAD-ESI-MS/MS method was established to screen and identify the polymethoxylated flavonoids (PMFs) in the peels of 'Shatangju' mandarin (*Citrus reticulata* Blanco). Eight PMF standards, including four polymethoxylated flavones, two polymethoxylated flavanones, and two polymethoxylated chalcones, were first to be analyzed in positive mode by CID-MS/MS. On the basis of the ESI-MS^n characteristics of PMFs and the results of EIC-MS/MS experiment, 32 PMFs including 24 flavones and 8 flavanones or chalcones were screened from the complex extract of the peels of 'Shatangju' mandarin. Among them, 10 PMFs were hydroxylated polymethoxyflavonoids (OH-PMFs), and the rest were all permethoxylated PMFs. This was the first systematic report of the presence of PMFs in the peels of 'Shatangju' mandarin, especially for polymethoxylated flavanones and chalcones. Meanwhile, the contents of the three main PMFs and total flavonoids in the peels of 'Shatangju' were determined by HPLC and UV spectrophotometry, respectively. The results indicated that the developed analytical method could be employed as an effective technique for the characterization of PMFs.

KEYWORDS: *HPLC-DAD-ESI-MS/MS, polymethoxylated flavonoids (PMFs), hydroxylated polymethoxyflavonoids (OH-PMFs), characterization, 'Shatangju' mandarin (Citrus reticulata Blanco)*

INTRODUCTION

Dietary flavonoids and other polyphenols show great potential as cancer chemopreventive agents in cell culture studies.[1,2] However, because of their low bioavailability as a result of conjugative metabolism, this does not translate well into in vivo activity.[3] However, polymethoxylated flavonoids (PMFs), the flavonoid subclass in which all or almost hydroxyls are capped by methylation, have high oral bioavailability, displaying antiallergic, antioxidant, antibacterial, antiproliferative, anti-inflammatory, and anticancer activities,[4−10] which have aroused the interest of the food, nutraceutical, and pharmaceutical industries for the use of these compounds as specialty ingredients. Meanwhile, hydroxylated polymethoxyflavonoids (OH-PMFs), which are less abundant PMFs in comparison with permethoxylated PMFs,[11] have drawn more and more attention recently, because accumulating evidence has suggested that OH-PMFs have much stronger health-promoting biological activities compared with their permethoxylated counterparts. For example, 5-hydroxy polymethoxyflavones exhibited greater potencies in anticarcinogenic and anti-inflammatory effects.[12−14]

As PMFs are widely distributed in the *Citrus* genus with wide dynamic range, it is of great importance to screen out and identify PMFs, especially OH-PMFs in such samples, which can give a wide outlook on the applications of PMFs. Early reported methods for analysis of PMFs were based on high-performance liquid chromatography (HPLC) separation coupled with ultraviolet (UV) detection.[15,16] However, some constituents could not be detected owing to low abundance, coelution, and the high background of HPLC. Therefore, high-resolution chromatographic methods coupled to highly sensitive and selective detectors are needed. Mass spectrometry, especially coupled to a soft ionizationsource such as electrospray ionization (ESI), has turned the possibility of coupling with the HPLC instrument into a reality and provided rich information including molecular mass and structural information online. Recently, HPLC-ESI-MS and HPLC-ESI-MS/MS have become very powerful approaches for the rapid identification of constituents in botanic extracts.[17−22]

'Shatangju' mandarin (*Citrus reticulata* Blanco) (STJ) is a kind of citrus fruit belonging to *Citrus* speciesl its peels have been used as Pericarpium Citri Reticulatae (Chen-Pi in Chinese) in traditional herbal medicine. Although many previous studies on PMFs from different *Citrus* species (e.g., *Citrus aurantium*, *Citrus sinensis*) and *Citrus* juices have been reported, the composition of PMFs can be significantly different among *Citrus* species.[23−27] To the best of our knowledge, there have been few systematic studies on the identification of characteristic flavoring compounds (PMFs et al.) in the peels of STJ until now. Therefore, in the purpose of selective phytochemical screening and structural characterization of PMFs in the peels of STJ, a developed HPLC-DAD-ESI-MS/MS method was adopted to investigate the fragment patterns of eight PMFs and its application in the identification of PMF compounds from botanic extracts.

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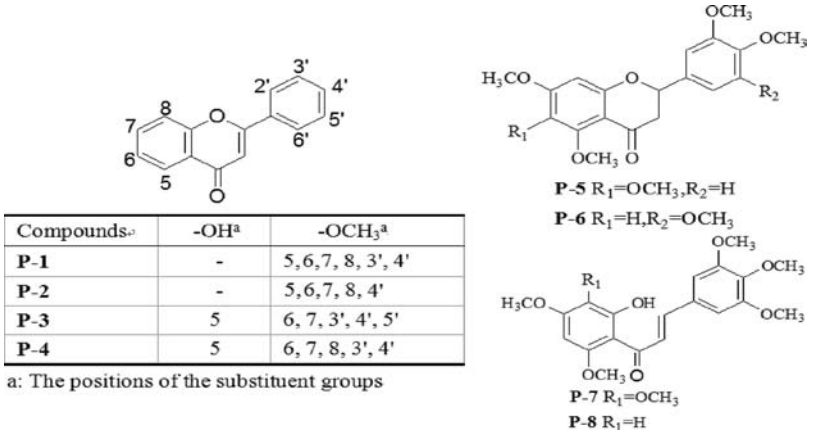

Compounds	-OH[a]	-OCH_3[a]
P-1	-	5, 6, 7, 8, 3', 4'
P-2	-	5, 6, 7, 8, 4'
P-3	5	6, 7, 3', 4', 5'
P-4	5	6, 7, 8, 3', 4'

a: The positions of the substituent groups

Figure 1. Chemical structures of PMFs reference standards **P1**−**8**.

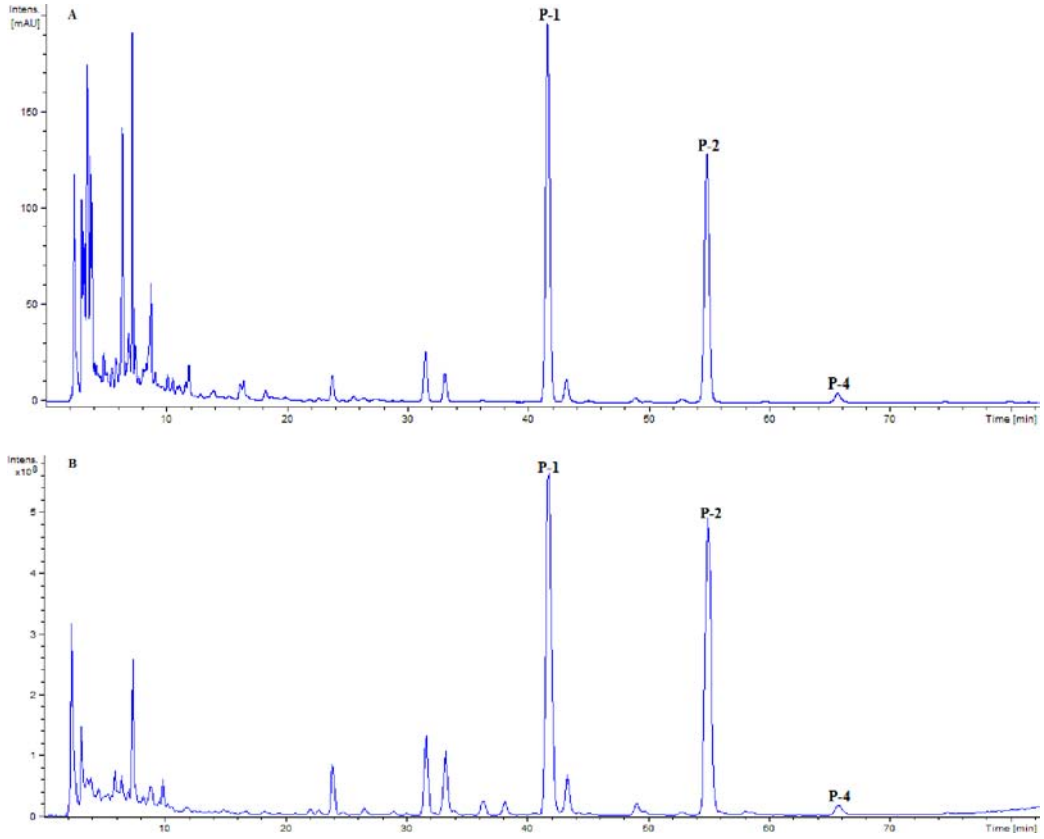

Figure 2. HPLC-DAD-MS/MS analysis of PMFs in the peels of 'Shatangju' mandarin (*Citrus reticulata* Blanco): (A) HPLC-DAD chromatogram at 330 nm; (B) ESI-MS total ion chromatogram (TIC) in positive mode.

MATERIALS AND METHODS

Samples. Fruits were collected at random from trees in Tongzhou County, Beijing City, China, in October 2011. The sample consisted of 20 fruits and was freeze-dried. The peels were divided from the freeze-dried fruits and deposited in a cool and dry place prior to analysis. It was authenticated by Professor Yan-Jiang Qiao. A voucher specimen was deposited at the Center of Scientific Experiment, Beijing University of Chinese Medicine, Beijing, China.

Chemicals. Eight PMF reference compounds, including 5,6,7,8,3′,4′ -hexamethoxyflavone (**P-1**), 5,6,7,8,4′-pentamethoxyflavone (**P-2**), 5-hydroxy-6,7,3′,4′,5′-pentamethoxyflavone (**P-3**), 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone (**P-4**), 5,6,7,3′,4′-pentamethoxyflavanone (**P-5**), 5,7,3′,4′,5′-pentamethoxyflavanone (**P-6**), 6′-hydroxy-3,4,5,2′,4′,5′-hexamethoxychalcone (**P-7**), and 6′-hydroxy-3,4,5,2′,5′-pentamethoxychalcone (**P-8**), were purchased from Xiantong Times (China) and identified in our laboratory for qualitative and quantitative analysis (shown in Figure 1). Their purities were determined to be no less than 95% by HPLC-UV. HPLC grade acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Formic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized water used throughout the experiment was purified by a Milli-Q Gradient A 10 System (Millipore, Billerica, MA, USA). The 0.22 μm membranes were purchased from Xinjinghua Co. (Shanghai, China).

Sample Preparation. Powdered dried peels of STJ were dried at 40 °C in the oven for 2 h before analysis. The sample was weighed accurately (0.3 g) and placed into a 50 mL flask containing 25 mL of methanol/water (70:30, v/v), and then the mixture was extracted in an ultrasonic bath (Eima Ultrasonics Corp., Germany) at room temperature for 0.5 h. The methanol solution was filtered through a

Table 1. Characterizations of Eight PMF Standards by CID-MS/MS

		$MS^2(m/z)$			$MS^3(m/z)$		
compd	$[M + H]^+$ (m/z)	P-ion[a] (%)	loss[b]	radical loss	P-ion[a] (%)	loss[b]	radical loss
P-1	403	373* (100)	30	$2CH_3^•$	327 (100)	46	$CO + H_2O$
		388 (64.6)	15	$CH_3^•$	358 (54.8)	15	$CH_3^•$
		342 (12.8)	61	$CO + H_2O + CH_3^•$	345 (10.1)	28	CO
P-2	373	358* (100)	15	$CH_3^•$	343 (100)	15	$CH_3^•$
		343 (63.5)	30	$2CH_3^•$	312 (16.9)	46	$CO + H_2O$
		312 (11.9)	61	$CO + H_2O + CH_3^•$	297 (4.4)	61	$CO + H_2O + CH_3^•$
P-3	389	356* (100)	33	$H_2O + CH_3^•$	328 (100)	28	CO
		328 (67.9)	61	$CO+H_2O + CH_3^•$	295 (6.9)	61	$CO + H_2O + CH_3^•$
		374 (35.6)	15	$CH_3^•$			
P-4	389	359* (100)	30	$2CH_3^•$	341[a] (100)	18	H_2O
		341 (43.6)	43	$CH_3^•$ + CO	328 (62.8)	31	$CH_4 + CH_3^•$
		374 (38.6)	15	$CH_3^•$	344 (19.3)	15	$CH_3^•$
		356 (23.3)	33	$H_2O + CH_3^•$	331 (18.4)	28	CO
P-5	375	211* (100)	RDA	$^{1,3}A^{+c}$	196 (100)	15	$CH_3^•$
		191 (37.1)	RDA	$^{1,4}B^{+c}$	178 (34.7)	33	$H_2O + CH_3^•$
		357 (16.6)	18	H_2O	150 (19.2)	61	$CO + H_2O + CH_3^•$
					183 (15.5)	28	CO
P-6	375	221* (100)	RDA	$^{1,4}B^{+c}$	193 (100)	28	CO
		181 (24.1)	RDA	$^{1,3}A^{+c}$	190 (61.9)	31	$CH_4 + CH_3^•$
					191 (40.0)	30	$2CH_3^•$
					206 (30.5)	15	$CH_3^•$
P-7	405	221* (100)	RDA	$^{x}B^{+d}$	193 (100)	28	CO
		387 (31.6)	18	H_2O	190 (51.5)	31	$CH_4 + CH_3^•$
		211 (28.3)	RDA	$^{y}A^{+d}$	191 (43.4)	30	$2CH_3^•$
					206 (31.6)	15	$CH_3^•$
P-8	375	221* (100)	RDA	$^{x}B^{+d}$	193 (100)	28	CO
		181 (30.0)	RDA	$^{y}A^{+d}$	190 (61.9)	31	$CH_4 + CH_3^•$
					191 (40.0)	30	$2CH_3^•$
					206 (22.3)	15	$CH_3^•$

[a]P-ion (%), the product ions and the relative intensity. *, precursor ion for next stage MS. [b]Loss, Da. $^{c}{}^{1,3}A^+$, $^{1,4}B^+$ stand for the fragment ions from the RDA cleavage from 1,3-position or 1,4-position on the C-ring of flavanones. $^{d}{}^{y}A^+$, $^{x}B^+$ stand for the fragment ions from the RDA cleavage from the C-ring of chalcones.

0.22 μm membrane before injection to the HPLC-MS system for analysis.

HPLC-DAD-ESI-MS/MS Analysis. The Agilent 1100 series HPLC-MS system (Agilent Technologies, Santa Clara, CA, USA) used in the experiment was equipped with a binary pump, an autosampler, a photodiode array detector, a column temperature controller, and an MSD Trap XCT Plus Mass spectrometer. Separations were carried out using an Agilent Zorbax Extended C_{18} column (250 × 4.6 mm i.d., 5 μm) with the oven temperature maintained at 25 °C. Formic acid aqueous solution (0.1% v/v, solvent A) and acetonitrile (solvent B) were used as mobile phase for the LC separation. The elution conditions were applied with a linear gradient as follows: 0−5 min, 20−28% B; 5−70 min, 28−42% B; 70−90 min, 42−64% B; 90−95 min, 64−100% B. The DAD acquisition wavelength was set in the range of 200−400 nm. The flow rate was at 1.0 mL/min, and peaks were detected at 330 nm. After passing through the flow cell of the DAD, the column eluate was split to 0.25 mL/min, which was directed to a trap mass spectrometer through an electrospray ionization (ESI) interface. ESI-MS was performed in positive ionization mode with source settings as follows: nebulizer gas pressure of 35.00 psi; dry gas flow rate of 11.00 L/min; electrospray voltage of the ion source of 3500 V; capillary temperature of 350 °C; compound stability of 50%; trap drive level of 100%; target mass of m/z 400; scan range of m/z 100−700; AutoMS(n) operation mode; collision energy of 1 V; SmartFrag Start Ampl of 30%, and SmartFrag End Ampl of 200%. A data-dependent program was used in the HPLC-ESI-MS^n analysis so that the protonated or deprotonated ions could be selected for further MS^n analysis. Nitrogen (>99.99%) and He (>99.99%) were used as sheath and damping gas, respectively. An Agilent 6300 Series Trap Control workstation (version 6.1) was used for the data processing.

For quantitative analysis, different concentrations of standards were analyzed by an Agilent 1100 series HPLC. Chromatographic conditions were the same as described above.

Method Validation. The reference compounds, including **P-1**, **P-2**, and **P-4**, were accurately weighed, dissolved in methanol, and diluted with methanol to an appropriate concentration. The solutions were brought to room temperature and filtered through a 0.22 μm membrane, and an aliquot of 10 μL was injected into HPLC for analysis. Calibration curves were plotted by the peak area versus at least six appropriate concentrations in triplicate of each analyte. The limits of detection (LOD) and quantification (LOQ) were determined on the basis of signal-to-noise (S/N) ratios of 3 and 10, respectively.

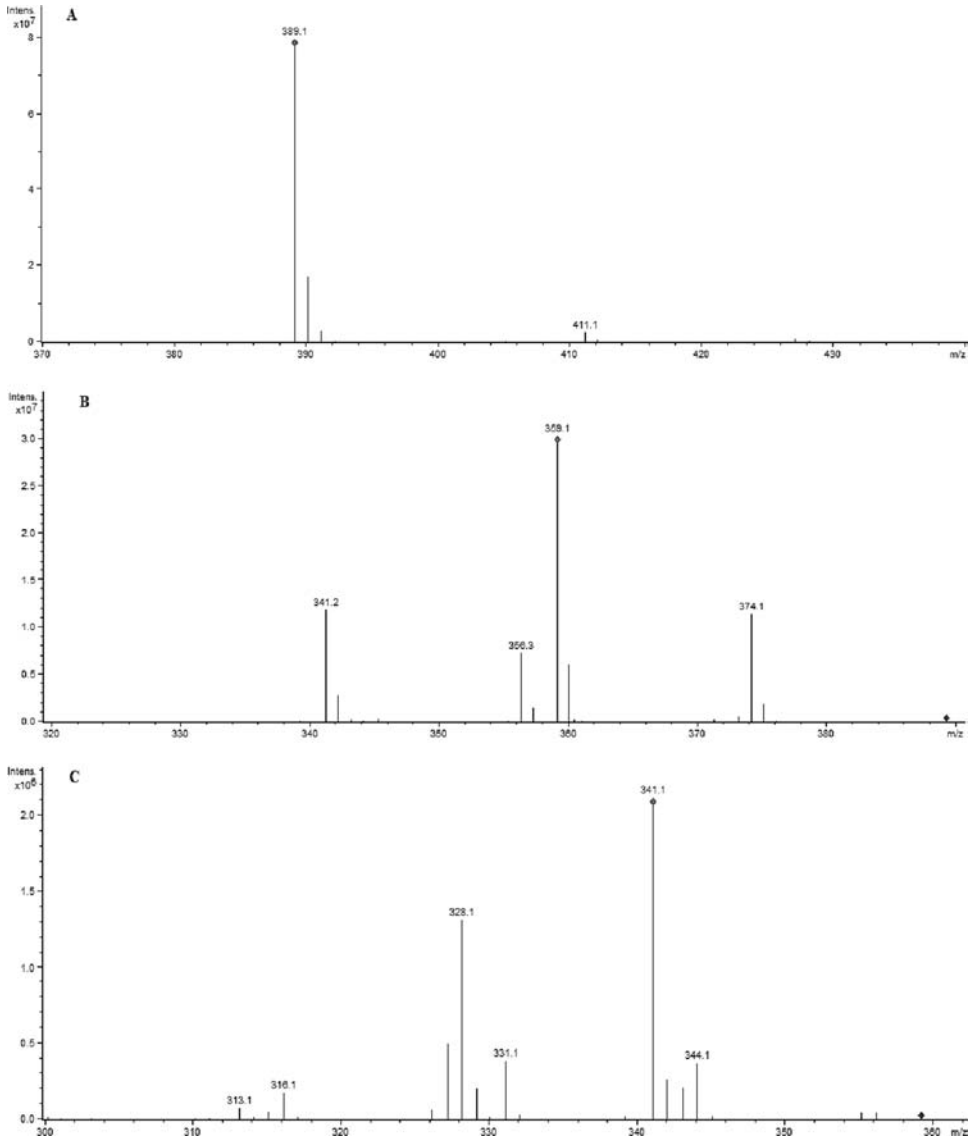

Figure 3. MS^n spectra of **P-4**: (A) MS spectrum; (B) MS^2 spectrum (precursor ion was m/z 389); (C) MS^3 spectrum (precursor ion was m/z 359).

The intra- and interday precision assays were performed by analyzing calibration samples during a single day and on three consecutive days, respectively. To ensure the repeatability, six different working solutions prepared from the same sample were assessed. Recoveries of the quantified constituents were determined using the sample for which respective chemical contents had been predetermined. Each standard solute was spiked at a close concentration with the sample. Then, recoveries were calculated on the basis of the difference between the total amount determined in the spiked samples and the amount observed in the nonspiked samples.

Determination of Total Flavonoids Content. The total flavonoids of the sample were determined by UV spectrophotometry at a wavelength of 510 nm after the extraction reacted with coloring agents.[28] Rutin was used as a standard, and results were expressed as milligrams of flavonoids equivalent per gram of dry sample.

RESULTS AND DISCUSSION

Optimization of HPLC Conditions. To obtain satisfactory extraction efficiency for all of the PMFs, extraction conditions, including extraction methods (ultrasonication, refluxing, and standing overnight), extraction solvents (30, 50, 70, and 100% methanol), and extraction time (20, 40, and 60 min) were assessed on the basis of single-factor experiments.[29] The best extraction efficiency was obtained by ultrasonication extraction with 70% ethanol for 60 min. Meanwhile, the different HPLC parameters including mobile phases (methanol/water and acetonitrile/water), the concentration of formic acid in water (0.05, 0.1, and 0.3%), category of RP-ODS columns (Agilent Zorbax Extended C_{18} column, 250 × 4.6 mm i.d., 5 μm; Agilent Zorbax Eclipse Plus C_{18}, 250 × 4.6 mm i.d., 5 μm; and Waters Symmetry Shield C_{18} column, 250 × 4.6 mm i.d., 5 μm), column temperature (20, 25, and 30 °C), and flow rate (0.8, 1.0, and 1.2 mL/min) were examined. The addition of formic acid was advantageous to obtain the best resolution of adjacent peaks during chromatographic separation (shown in Figure 2).

Optimization of ESI-MS/MS Conditions. To achieve optimum conditions to identify as many PMFs in the peels of STJ as possible, all factors related to MS performance including ionization mode, nebulizer gas pressure, electrospray voltage of the ion source, and collision energy have been evaluated. The results showed that ESI in positive ion mode was more sensitive to PMFs than ESI in negative ion mode. The major constituents were well detected (shown in Figure 2), and most of the investigated compounds exhibited quasi-molecular

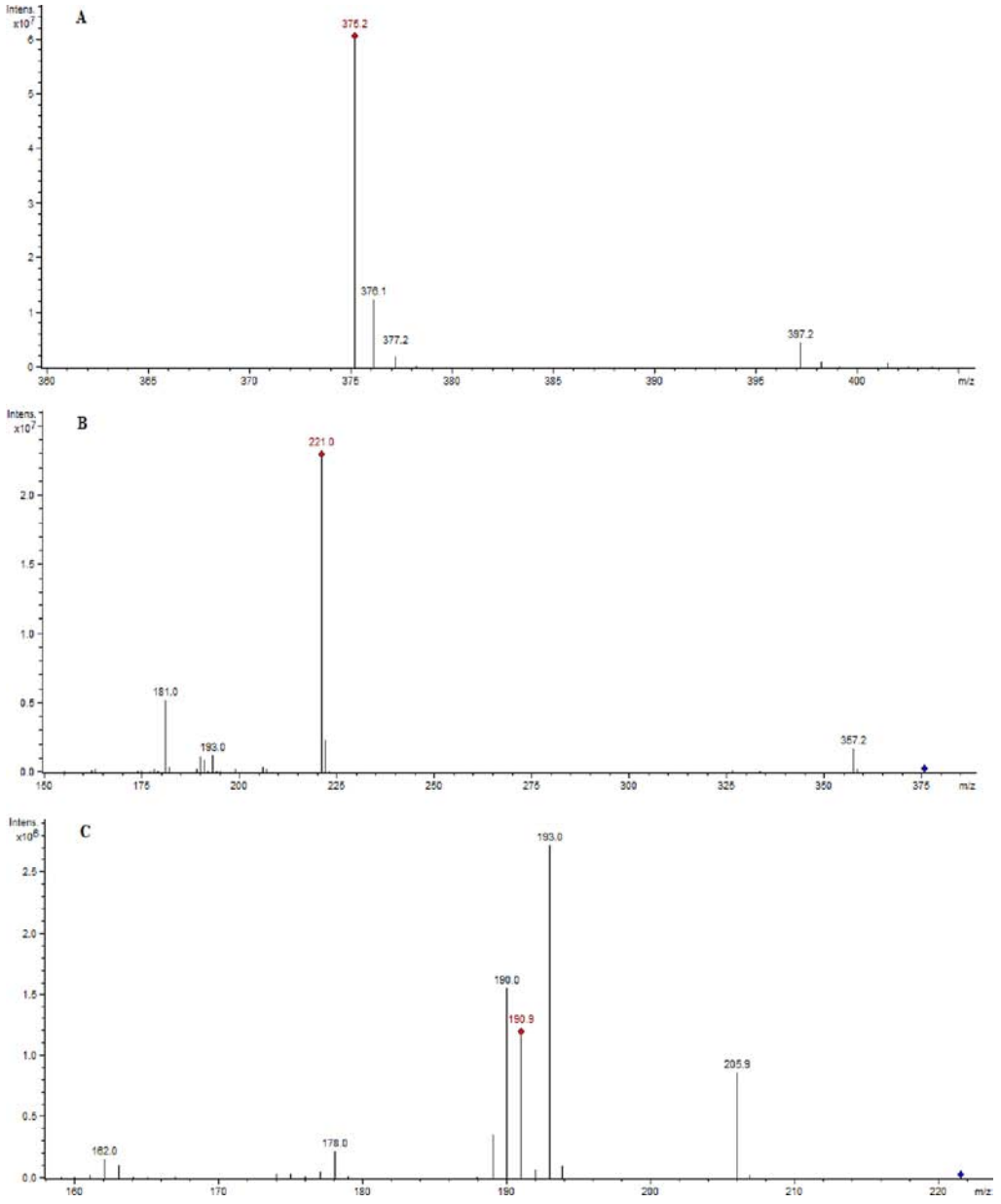

Figure 4. MS^n spectra of **P-6**: (A) MS spectrum; (B) MS^2 spectrum (precursor ion was m/z 375); (C) MS^3 spectrum (precursor ion was m/z 221).

ions $[M + H]^+$ and product ions with rich structural information in the positive mode of CID-MS/MS.

HPLC-DAD-MS/MS Analysis of Authentic Compounds. To identify structures of the constituents in the peels of STJ, eight reference compounds were analyzed by HPLC-DAD-ESI-MS/MS techniques. According to their chemical structures, UV absorption maxima, and dominant fragmentation pathways, the authentic compounds could be classified into three groups including polymethoxylated flavones, flavanones, and chalcones. In their full scan mass spectra, most of PMF standards exhibited $[M + H]^+$ ions of sufficient abundance that could be subsequently isolated automatically and subjected to CID-MS/MS analysis (shown in Table 1). The proposed fragmentation patterns were helpful to clarify the structural identification of constituents in the peels of STJ. The nomenclature commonly used for mass fragments of flavonoids was adopted in this work.[30]

Four polymethoxylated flavone standards were subsequently analyzed first in the CID-MS/MS experiment. By comparison of the product ion spectra of the standards (shown in Figure 3), some characterized dissociation pathways could be summarized for further characterization of the other polymethoxylated flavones. First, all of their $[M + H]^+$ ions of standards could lose one to four methyl radicals ($CH_3^\bullet$) in their MS/MS spectra and formed the base peaks of $[M + H -- 15]^+$, $[M + H - 30]^+$, $[M + H - 45]^+$, or $[M + H - 60]^+$. This fragmentation pathway can be taken as the major diagnostic characteristic for polymethoxylated flavones. Second, the other dissociation pathways by loss of 16 (CH_4), 18 (H_2O), 28 (CO), 31 ($CH_4 + CH_3^\bullet$), 33 ($H_2O + CH_3^\bullet$), 43 ($CO + CH_3^\bullet$), 46 ($CO + H_2O$), 60 ($4CH_3^\bullet$), and 61 ($CO + H_2O + CH_3^\bullet$) were also frequently detected as diagnostic fragments in their MS/MS and MS/MS/MS spectra. These main product ions mentioned above could form the characteristic ESI-MS^n "fingerprint" of PMFs, which could be used to screen out the polymethoxylated

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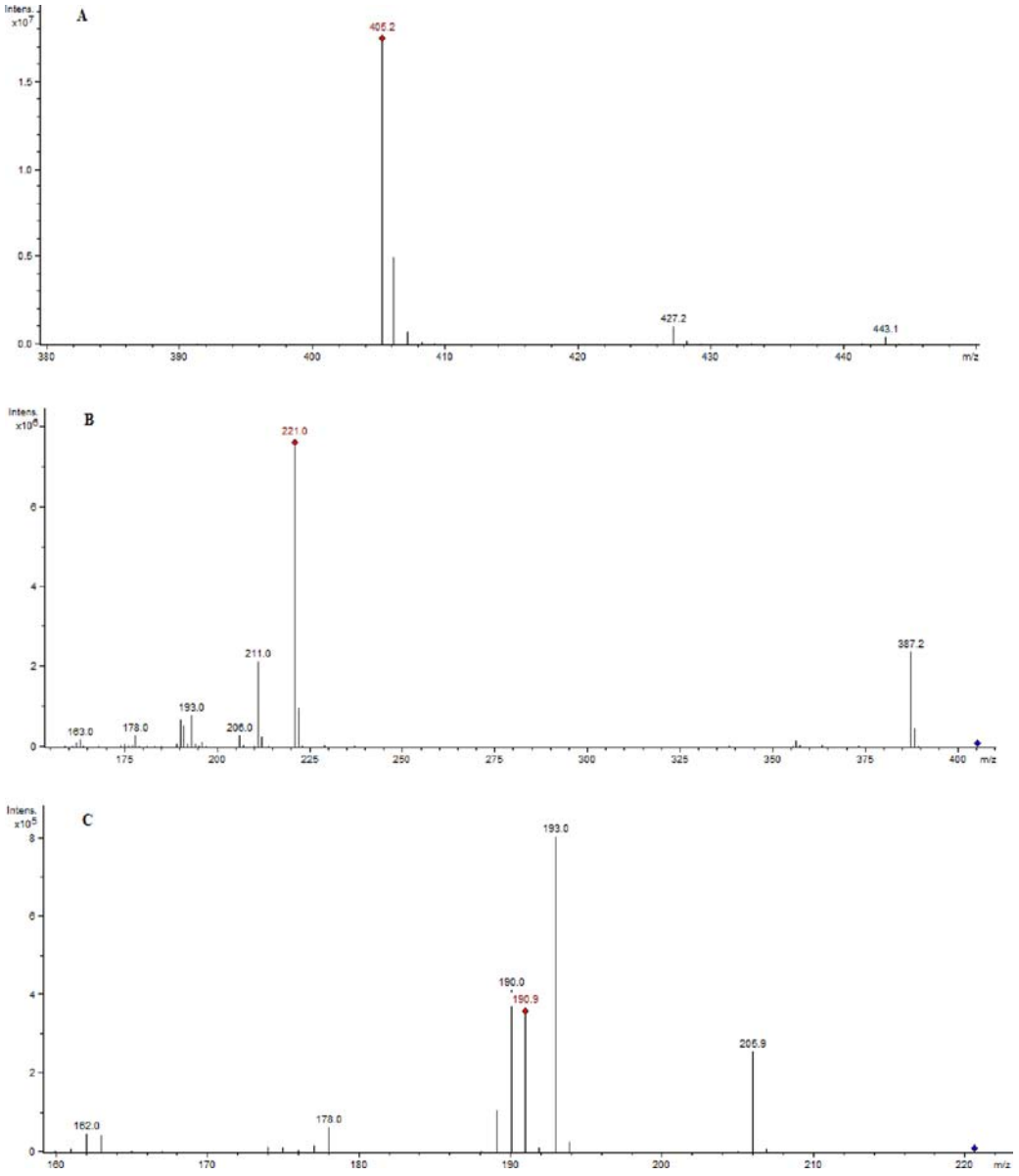

Figure 5. MS^n spectra of **P-7**: (A) MS spectrum; (B) MS^2 spectrum (precursor ion was m/z 405); (C) MS^3 spectrum (precursor ion was m/z 221).

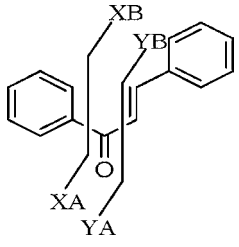

Figure 6. Proposed MS fragmentation pathway for chalcone derivatives.

flavones from the complex botanic extracts rapidly. Among them, some diagnostic fragments such as 18, 28, and 44 detected in the product ion spectra were frequently reported in the characterization of ordinary flavonoids, too.[31]

In CID-MS/MS experiment, the fragmentation pathways of two polymethoxylated flavanone derivatives (**P-5** and **P-6**) were similar to each other. **P-6**, for example, gave the $[M + H]^+$ ion at m/z 375, which further generated the prominent ion at m/z 221 as base peak in the MS/MS spectrum (shown in Figure 4). It could be deduced after careful analysis that its dominating fragmentation pathway was RDA cleavage from the 1,4-position of the C-ring. Meanwhile, the minor ion at m/z 181 was also detected, owing to the RDA fragmentation from the 1,3-position of the C-ring. The loss of 15 ($CH_3^{\bullet}$), 28 (CO), 30 ($2CH_3^{\bullet}$), and 31 ($CH_3^{\bullet}$ + CH_4) from the base peaks at m/z 221 could be also detected as minor fragmentation ions in the CID-MS/MS spectra. This kind of fragmentation pathway revealed that the $[M + H]^+$ ion underwent RDA reaction prior to the neutral loss of $CH_3^{\bullet}$, H_2O, CO, etc., and was noticeably different from ordinary flavanones. Therefore, it could be adopted as a shortcut to rapidly distinguish polymethoxylated flavanones from ordinary flavones.

Compounds **P-7** and **P-8**, two polymethoxylated chalcone standards, were analyzed by the CID-MS/MS method, too. Their dissociation pathways of MS spectra were similar to each other. In **P-7**, for example (shown in Figure 5), the RDA cleavage at bond X to yield the base peak ion $^{X}B^{+}$ at m/z 221 and at bond Y to yield the minor ion $^{Y}A^{+}$ at m/z 211 (shown in

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Table 2. Chemical Formulas and Masses of All Possible Polymethoxylated Flavones

substituent		OH	2OH	3OH	4OH	5OH
$2OCH_3$	$C_{17}H_{14}O_4$ 282	$C_{17}H_{14}O_5$ 298	$C_{17}H_{14}O_6$ 314	$C_{17}H_{14}O_7$ 330	$C_{17}H_{14}O_8$ 346	$C_{17}H_{14}O_9$ 362
$3OCH_3$	$C_{18}H_{16}O_5$ 312	$C_{18}H_{16}O_6$ 328	$C_{18}H_{16}O_7$ 344	$C_{18}H_{16}O_8$ 360	$C_{18}H_{16}O_9$ 376	
$4OCH_3$	$C_{19}H_{18}O_6$ 342	$C_{19}H_{18}O_7$ 358	$C_{19}H_{18}O_8$ 374	$C_{19}H_{18}O_9$ 390		
$5OCH_3$	$C_{20}H_{20}O_7$ 372	$C_{20}H_{20}O_8$ 388	$C_{20}H_{20}O_9$ 404			
$6OCH_3$	$C_{21}H_{22}O_8$ 402	$C_{21}H_{22}O_9$ 418				
$7OCH_3$	$C_{22}H_{24}O_9$ 432					

Table 3. Chemical Formulas and Masses of All Possible Polymethoxylated Flavanones or Chalcones

substituent		OH	2OH	3OH	4OH	5OH
$2OCH_3$	$C_{17}H_{16}O_4$ 284	$C_{17}H_{16}O_5$ 300	$C_{17}H_{16}O_6$ 316	$C_{17}H_{16}O_7$ 332	$C_{17}H_{16}O_8$ 348	$C_{17}H_{16}O_9$ 364
$3OCH_3$	$C_{18}H_{18}O_5$ 314	$C_{18}H_{18}O_6$ 330	$C_{18}H_{18}O_7$ 346	$C_{18}H_{18}O_8$ 362	$C_{18}H_{18}O_9$ 378	
$4OCH_3$	$C_{19}H_{20}O_6$ 344	$C_{19}H_{20}O_7$ 360	$C_{19}H_{20}O_8$ 376	$C_{19}H_{20}O_9$ 392		
$5OCH_3$	$C_{20}H_{22}O_7$ 374	$C_{20}H_{22}O_8$ 390	$C_{20}H_{22}O_9$ 406			
$6OCH_3$	$C_{21}H_{24}O_8$ 404	$C_{21}H_{24}O_9$ 420				
$7OCH_3$	$C_{22}H_{26}O_9$ 434					

Figure 6) could also be simultaneously detected in the MS/MS spectrum first. The fragmentation pathway was highly similar to what happened to flavanones. This is reasonable because cyclization of 6′-hydroxychalcones to flavanones has been reported in a number of studies demonstrating an intramolecular equilibrium being present between a flavanone-type and a chalcone-type molecular ion.[32,33] At the same time, the loss of 15 ($CH_3^{\bullet}$), 16 (CH_4), 18 (H_2O), 28 (CO), 30 ($2CH_3^{\bullet}$), and 31 ($CH_4 + CH_3^{\bullet}$) could be also detected. Thus, according to their fragmentation pathways, it was easy to tell the difference between polymethoxylated chalcones and flavones but difficult to distinguish them from polymethoxylated flavanones. However, the differences of UV spectra between polymethoxylated chalcones and polymethoxylated flavanones provided a shortcut to classify them, because the maximum absorption of chalcones usually ranged from 330 to 370 nm, whereas flavanones maintained at about 320 nm.

HPLC-DAD-MS/MS Analysis of the PMFs in the Peels of STJ. Three kinds of PMFs, that is, polymethoxylated flavones, flavanones, and chalcones, have been detected from the natural drugs. Owing to the common phenomenon of substitution isomerism and the great differences of contents in raw materials, it is great difficult to distinguish them from each other. However, PMFs have regularity in elemental composition as they have the basic aglycone structure with maximum seven substituents such as methoxyl group (OCH_3) and/or hydroxyl group (OH) on their A-, B-, and C-rings. The molecular masses of basic structures of aglycone are 222, 224, and 224 Da for flavones, flavanones, and chalcones, respectively, which are increased by 30 or 16 Da when a methoxyl or hydroxyl was attached. On the basis of the numbers and types of substituent groups, the chemical formula and mass of every possible PMF isomer can be designated in advance (shown in Tables 2 and 3). Because of the complexity and similarity of the components in the peels of STJ, the EIC-MS (extracted ion chromatogram) method was employed to analyze the PMFs in the peels of STJ (shown in Figure 7 and Table 4).

In the study, the abundances of both the flavanone and chalcone were too low to obtain online UV absorption spectra, so it was difficult to distinguish between them. Therefore, they were identified together.

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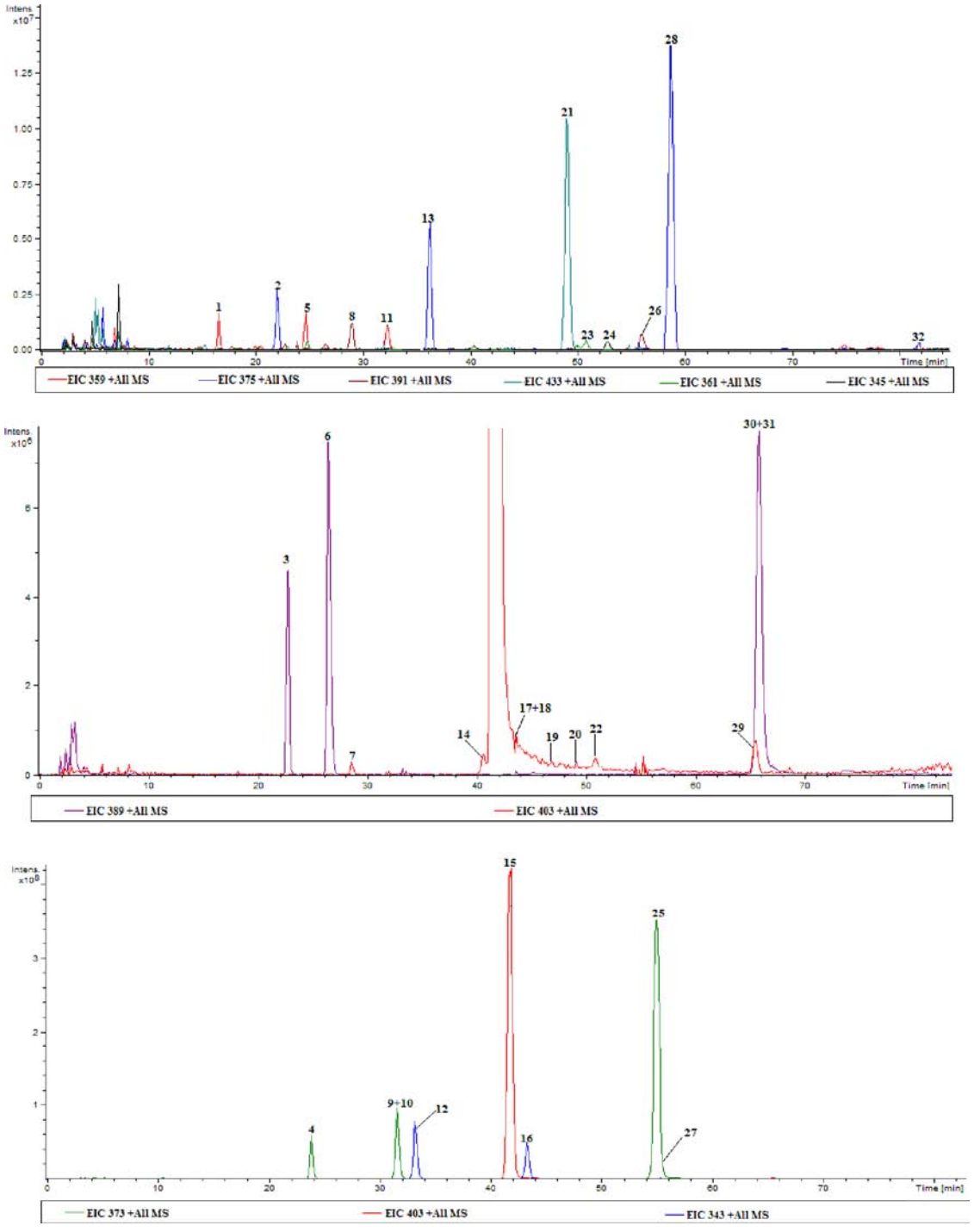

Figure 7. EIC-MS peaks of all possible PMFs in the peels of 'Shatangju' mandarin (*Citrus reticulata* Blanco).

After screening the molecular masses with the EIC-MS method, 32 PMFs including 24 polymethoxylated flavones and 8 flavanones or chalcones were tentatively identified (shown in Table 5) from the peels of STJ. Among them, 10 PMFs were OH-PMFs, whereas the rest were all permethoxylated PMFs. Some EIC-MS peaks were too weak to be seen clearly in the total ion chromatogram (TIC) spectra. Meanwhile, the retention times of some EIC-MS peaks were so similar that they could not be identified simultaneously in TIC spectra, either. Thus, the EIC-MS method adopted in our study was confirmed to be one kind of powerful weapon to screen the ingredients preliminarily in highly complex botanic extracts.

Verification with the Diagnostic Characteristic of PMFs. By EIC-MS/MS, all of the candidates for PMFs were preliminarily identified from the peels of STJ. However, further verification with the diagnostic characteristic of PMF standards was still needed to be performed. The $[M + H]^+$ ions of polymethoxylated flavones all eliminated masses of 15, 30, and 60 as the base peak for MS/MS spectra, except compounds **9**, **10**, and **16**, all of which yielded major $[M - n \times CH_3^{\bullet}]^+$ ions in their mass spectra, too. Meanwhile, other diagnostic fragment losses of 16 (CH_4), 18 (H_2O), 28 (CO), 29 ($HCO^{\bullet}$), 31 ($CH_4 + CH_3^{\bullet}$), 33 ($H_2O + CH_3^{\bullet}$), 43 ($CO + CH_3^{\bullet}$), 44 (CO_2), 46 ($H_2O + CO$), 60 ($4CH_3^{\bullet}$), and 61 ($CO + H_2O + CH_3^{\bullet}$) could

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Table 4. Characterization of PMFs in the Peels of 'Shatangju' Mandarin (*Citrus reticulata* Blanco) by HPLC-DAD-ESI-MS/MS

no.	t_R[s] (min)	$[M + H]^+$ (m/z)	MS^2(m/z) P-ion (%, loss)[b]	MS^3(m/z) P-ion (%, loss)[b]	MS^4(m/z) P-ion (%, loss)[b]
1	16.61	359	344* (100, 15), 326 (75.6, 33), 298 (5.2, 61)	326* (100, 18), 298 (11.4, 46)	298 (100, 28)
2	22.03	375	211*(100, RDA), 191 (46.3, RDA), 357 (18.7, 18)	196* (100, 15), 178 (25.1, 33), 150 (19.2, 61), 183 (14.9, 28)	178 (100, 18), 150 (85.7, 46), 168 (15.8, 28)
3	22.76	389	374* (100, 15), 359 (69.1, 30), 356 (10.1, 33)	359* (100, 15), 341 (33.4, 33), 356 (9.5, 18)	344 (100, 15), 341 (88.1, 18), 343 (53.1, 16), 316 (47.3, 43), 331 (40.1, 28)
4	23.77	373	343* (100, 30), 358 (70.6, 15)	315* (100, 28)	272 (100, 43), 153 (43.8, RDA), 163 (37.7, RDA), 300 (12.6, 15)
5	24.79	359	344* (100, 15), 329 (47.1, 30), 298 (9.7, 61)	329* (100, 15), 298 (18.5, 46), 283 (8.0, 61), 311 (6.5, 33)	283 (100, 46), 311 (44.1, 18), 314 (42.2, 15), 301 (32.7, 28), 286 (29.8, 43)
6	26.41	389	359* (100, 30), 374 (82.3, 15), 328 (17.2, 61)	313* (100, 46), 344 (68.2, 15), 341 (40.7, 18), 331 (32.2, 28), 316 (26.6, 43)	285 (100, 28), 298 (61.3, 15), 283 (46.6, 30), 284 (41.6, 29)
7	28.50	403	373* (100, 30), 388 (41.7, 15), 370 (41.2, 33), 342 (27.2, 31), 387 (14.8, 16)	345* (100, 28), 358 (38.3, 15), 330 (29.9, 33), 344 (11.3, 29)	330 (100, 15)
8	29.09	391	241* (100, RDA), 226 (15.9, RDA), 373 (8.8, 18)	226* (100, 15), 211 (11.8, 30), 208 (10.0, 33)	211 (100, 15), 208 (59.5, 18), 180 (40.2, 46), 183 (30.1, 43)
9	31.21	373	312* (100, 61), 358 (57.7, 15), 329 (28.3, 44), 343 (18.6, 30), 357 (15.8, 16), 340 (14.4, 33)	151 (100, RDA), 297 (74.6, 15), 15, 269 (44.8, 43), 296 (36.5, 16)268 (23.4, 44), 281 (16.3, 31), 284 (11.9,2 8)	–[c]
10	32.32	373	312* (100, 61), 358 (79.8, 15), 329 (29.5, 44), 343 (29.1, 30), 340 (22.8, 3 3), 357 (21.6, 16)	297 (100, 15), 151 (94.3, RDA), 296 (24.0, 16)	–
11	32.38	359	344* (100, 15), 329 (31.1, 30)	329* (100, 15), 311 (28.8, 33), 326 (11.6, 18)	311 (100, 18) , 286 (42.1, 43), 301 (33.6, 28)
12	33.09	343	313* (100, 30), 328 (79.8, 15)	285* (100, 28), 243 (9.2, 60)	242 (100, 43), 267 (86.1, 18), 153 (58.8, RDA)
13	36.32	375	211* (100, RDA), 191 (5.8, RDA)	196* (100, 15), 168 (32.1, 43), 167 (6.6, 44)	168 (100, 28), 122 (23.6, 84), 150 (21.1, 46)
14	40.65	403	373* (100, 30), 388 (69.3, 15), 342 (14.7, 31), 355 (8.1, 18)	327* (100, 46), 358 (63.0, 15), 355 (27.6, 18)	281 (100, 46), 312 (94.9, 15), 299 (91.8, 28)
15	42.87	403	373* (100, 30), 388 (64.7, 15), 342 (13.9, 61)	327* (100, 46), 358 (59.2,1 5), 345 (30.2,28), 355 (29.0, 18), 330 (22.7, 43)	312 (100, 15), 283 (62.9, 44), 299 (58.2, 28), 284 (51.7, 43)
16	43.01	343	282* (100, 61), 328 (70.0, 15), 299 (31.5, 44), 310 (18.7, 33), 313 (17.8, 30)	254 (100, 28), 267 (29.6, 15), 251 (28.2, 31), 239 (13.0, 43)	–
17	43.98	403	373* (100, 30), 388 (60.8, 15), 342 (14.0, 61)	327* (100, 46), 358 (47.7, 15), 355 (29.2, 18), 345 (24.5, 28), 330 (23.1, 43)	299 (100, 28), 312 (97.2, 15), 269 (62.2, 58)
18	44.56	403	373* (100, 30), 388 (64.9, 15), 342 (16.7, 61)	327* (100, 46), 358 (50.1, 15), 345 (30.7, 28), 330 (29.5, 43), 355 (27.0, 18)	297 (100, 30)
19	46.81	403	373* (100, 30), 388 (64.9, 15), 342 (13.0, 61)	327* (100, 46), 358 (53.8, 15), 355 (27.0, 18), 345 (23.1, 28), 330 (20.7, 43)	299 (100, 28), 297 (72.3, 30), 271 (45.8, 56), 312 (44.2, 15)
20	47.99	403	373* (100, 30), 388 (62.6, 15), 342 (16.1, 61)	327* (100, 46), 358 (49.9, 15), 355 (44.9, 18), 345 (28.4, 28), 330 (23.4, 28)	297 (100, 30), 312 (75.9, 15)
21	49.30	433	403* (100, 30), 418 (56.9, 15), 417 (18.4, 16)	388* (100, 15), 373 (77.5, 30), 387 (64.0, 16), 360 (49.6, 43), 385 (45.8, 18), 375 (43.7, 28)	342 (100, 46), 329 (53.5, 58), 370 (25.9, 18), 301 (25.0, 87), 357 (22.2, 31)
22	50.37	403	373* (100, 30), 388 (64.9, 15), 342 (15.3, 61)	327* (100, 46), 358 (53.3, 15), 345 (28.6, 28), 330 (25.6, 43), 355 (20.1, 18)	201 (100, RDA), 312 (95.9, 15), 269 (57.1, 58)
23	50.89	361	197* (100 RDA), 191 (29.8, RDA)	182* (100, 15), 136 (38.1, 61), 164 (15.8, 33)	164 (100, 18), 136 (72.0, 46)
24	52.51	345	211* (100, RDA), 196 (2.9, RDA)	196* (100, 15), 168 (28.2, 43), 167 (13.2, 44), 150 (10.5, 61)	168 (100, 28), 167 (69.6, 29), 121 (61.1, 75)
25	55.17	373	358* (100, 15), 343 (62.9, 30), 312 (11.2, 61)	343* (100, 15), 312 (17.3, 46), 325 (5.9, 33), 297 (5.5, 61)	328 (100, 15), 325 (48.7, 18), 300 (32.0, 43), 315 (29.6, 28)
26	55.96	391	227* (100, RDA), 253 (2.0, RDA)	212* (100, 15), 166 (11.0, 61)	179 (100, 33), 194 (66.2, 18), 166 (22.8, 46)
27	56.77	373	358* (100, 15), 343 (59.6, 30), 312 (13.3, 61)	343* (100, 15), 312 (11.4, 46), 297 (8.0, 61)	297 (100, 46), 328 (42.3, 15), 300 (41.2, 43), 325 (22.5, 18), 315 (13.5, 28)
28	58.64	375	241* (100, RDA), 359 (7.6, 16)	226* (100, 15), 208 (19.9, 33), 225 (12.7, 16), 211 (6.9, 30)	211 (100, 15), 165 (53.3, 61)
29	65.32	403	373* (100, 30), 388 (82.5, 15), 355 (19.0, 48)	355* (100, 18), 358 (68.9, 15), 345 (27.8, 28), 355 (27.2, 18)	327 (100, 18), 319 (40.7, 46), 340 (40.3, 15)307 (31.7, 48), 337 (23.6, 18), 326 (15.0, 33)
30	66.55	389	359* (100, 30), 341 (42.2, 48), 374 (39.4, 15), 356 (22.9, 33), 328 (19.9, 61)	341* (100, 18), 328 (61.0, 31), 344 (20.0, 15), 331 (18.3, 28),343(13.5, 32) 9 (11.7, 30)	313 (100, 28), 155 (48.9, RDA), 151 (48.2, RDA), 326 (43.2, 15)
31	67.01	389	359* (100, 30), 341 (47.7, 48), 374 (47.1, 15), 356 (31.9, 33), 328 (17.7, 61)	343 (100, 16), 341 (99.6, 18), 331 (82.6, 28), 344 (65.9, 15), 316 (30.6, 43), 315 (19.1, 44)	–
32	81.66	375	211* (100, RDA), 191 (6.7, RDA)	196* (100, 15), 168 (14.7, 43)	178 (100, 18), 150 (59.5, 46), 168 (48.4, 28)

[s] t_R, retention time. [b] P-ion (%, loss), product ions, relative intensity, and loss (Da). *, precursor ion for next stage MS. [c] –, too low to be detected.

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Table 5. Structural Identification of All Possible PMFs Detected in Peels of 'Shatangju' Mandarin (*Citrus reticulata* Blanco)

peaks	amount	PMFs	no. of $-OCH_3$	no. of $-OH$	mol mass
1, 5, 11	3	monohydroxytetramethoxyflavone	4	1	358
2, 13, 28, 32	4	pentamethoxyflavanone or pentamethoxychalcone	5	0	374
3, 6, 30, 31	4	monohydroxypentamethoxyflavone	5	1	388
4, 9, 10, 25, 27	5	pentamethoxyflavone	5	0	372
7, 14, 15, 17−20, 22, 29	9	hexamethoxyflavone	6	0	402
8, 26	2	monohydroxypentamethoxyflavanone **or** monohydroxypentamethoxychalcone	5	1	390
12, 16	2	tetramethoxyflavone	4	0	342
21	1	heptamethoxyflavone	7	0	432
23	1	monohydroxytetramethoxyflavanone **or** monohydroxytetramethoxychalcone	4	1	360
24	1	tetramethoxyflavanone **or** tetramethoxychalcone	4	0	344

Table 6. Calibration Curves, Linearity, LOD, and LOQ for Three PMFs

compd	t_R[a] (min)	regression eq[b]	r	linear range (μg)	LOD (ng)	LOQ (ng)
P-1	42.84	$Y = 3707.3X - 12.673$	0.9999	92.8−1856	0.37	1.25
P-2	55.19	$Y = 4256.9X - 18.360$	0.9999	92.4−1828	0.44	1.43
P-4	66.51	$Y = 3320.6X - 11.747$	0.9999	11.8−236	0.45	1.51

[a] t_R, retention time. [b] Y, value of peak area; X, value of amount injected (ng).

Table 7. Validation Results of the Analytical Method Using the Extract Solution

compd	intraday RSD (%, $n = 6$)	interday RSD (%, $n = 6$)	repeatability RSD (%, $n = 6$)	recovery (%, $n = 6$)	recovery RSD (%, $n = 6$)
P-1	0.25	0.33	0.45	98.94	0.74
P-2	0.24	0.33	0.29	97.62	0.92
P-4	0.75	1.22	0.93	101.21	1.41

be also detected. For all of the $[M + H]^+$ ions of polymethoxylated flavanones and chalcones, RDA fragmentation always happened as the major dissociation pathway prior to the neutral loss of the diagnostic fragments mentioned for polymethoxylated flavones. The results were well in accord with the fragmentation pathways deduced from the reference standards. Therefore, 32 compounds including 24 flavones and 8 flavanones or chalcones were all verified as PMFs.

Validation of the Analytical Method. The validation of the proposed chromatographic method was assessed by several analytical parameters. For determination of the bioactive markers, a calibration curve for each marker was constructed and tested three times for linearity. As shown in Table 6, good linearity and high sensitivity under the optimal chromatographic conditions were obtained with correlation coefficients >0.999 and relatively low LOD (0.37−0.45 ng) and LOQ (1.25−1.51 ng).

As demonstrated in Table 7, the results of precision and accuracy showed good reproducibility for quantification of three PMFs with intra- and interday variations of less than 0.75 and 1.22%, respectively. The relative standard deviations (RSDs) of the repeatability experiments were <0.93% for all analytes. The overall recoveries of the three investigated compounds ranged from 97.62 to 101.21% with RSDs from 0.74 to 1.41%.

On the basis of the above results, the developed method is precise, accurate, and sensitive enough for the quantitative determination of the three main PMFs in the peels of STJ.

Determination of Three PMFs and Total Flavonoids in the Peels of STJ. The developed method was subsequently applied to the simultaneous determination of three bioactive markers in the peels of STJ. The sample was analyzed three times, and the mean contents of PMFs were calculated as follows: 5.347 mg/g of **P-1**, 3.608 mg/g of **P-2**, and 0.241 mg/g of **P-4**. The mean contents of total flavonoids that had been determined three times by UV spectrophotometry method were 15.56 mg/g. Therefore, the results indicated that the contents of the PMFs play a vital role in the quality evaluation of raw material.

In conclusion, a sensitive HPLC-DAD-ESI-MS/MS method was established that could be used to simultaneously identify and screen the PMFs in the peels of STJ. Eight PMF standards including four flavones, two flavanones, and two chalcones were analyzed by CID-MS/MS first to obtain the respective characteristics of fragmentation pathways, which could be adopted as the basis for further analysis of the PMFs in the extract. Owing to regularities of PMFs in elemental composition, the EIC-MS method by molecular masses was employed to screen the homeomorphic PMFs from the extract. In the end, 32 PMFs including 10 OH-PMFs and 22 permethoxylated PMFs were identified preliminarily. This was the first systematic report of the presence of PMFs in the peels of STJ, especially for polymethoxylated flavanones and chalcones, most of which were probably new compounds. Meanwhile, the contents of three main PMFs and total flavonoids in the peels of STJ were determined by HPLC and UV spectrophotometry, respectively. All of the results indicated that the developed HPLC-DAD-ESI-MS/MS method could be employed as an effective technique to characterize PMFs from botanic extracts.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

HPLC-DAD-ESI-MS/MS, high-performance liquid chromatography coupled to photodiode array detection and electrospray tandem mass spectrometry; PMFs, polymethoxylated flavo-

noids; OH-PMFs, hydroxylated polymethoxyflavonoids; STJ, 'Shatangju' mandarin (*Citrus reticulata* Blanco).

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