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A strategy of EIC-MS coupled with diagnostic product ions analysis for efficient discovery of new hydroxylated polymethoxyflavonoid glycosides from the leaves of *Murraya paniculata* L. using HPLC-DAD-MS/MS

Jia-Yu Zhang,[ab] Jian-Qiu Lu,[a] Fang Wang,[b] Guang-shang Cao,[c] Peng-Fei Tu*[d] and Yan-Jiang Qiao*[b]

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Hydroxylated polymethoxyflavonoid glycosides (OH-PMFGs) are a rare kind of highly methoxylated flavone glycosides, which demonstrate many health-promoting bioactivities. Here we aim to identify OH-PMFGs from the leaves of *Murraya paniculata* using EIC (extracted ion chromatogram)-MS coupled with diagnostic product ions (DPIs) analytical method. Respective DPIs for glycosides of polymethoxyflavones, polymethoxyflavanones and polymethoxychalcones were obtained from the fragmentation pathways of eleven PMF (polymethoxylated flavonoid) standards. A sensitive HPLC-DAD-ESI-MS/MS was established for simultaneous qualitative and quantitative determination of the main OH-PMFGs in the extract. 54 OH-PMFGs including 49 flavone glycosides and 5 flavanone or chalcone glycosides were screened and identified from the leaves of *M. paniculata*. Meanwhile, the contents of three main OH-PMFGs in the extract were determined by HPLC-UV. It was the first systematic report of the presence of rare OH-PMFGs in the plants of the genus *Murraya*. The results indicated that the methodology developed could be employed as an effective technique for structural characterization of OH-PMFGs in complex extracts of TCMs (traditional Chinese medicines).

Introduction

Nowadays, traditional Chinese medicines (TCMs) have gained increasing popularity worldwide owing to the prevalence of chronic and systematic diseases and limitations of western medicines.[1,2] Therefore, comprehensive analytical methods for characterization of the chemical constituents and quality evaluation of a complex chemical system are urgently required to address the inherent holistic nature of TCMs. In recent years, HPLC-ESI-MS/MS has become a very powerful approach for the rapid identification of chemical constituents in botanic extracts and crude material of TCMs.[3–8] However, HPLC-ESI-MS/MS has been faced with some new challenges in information processing, making it a quite difficult and tedious task to deal with the extremely large information data. Therefore, strategies for efficient mass spectra analysis are needed for rapid characterization of the naturally occurring substances in TCMs.[9–12] In this paper, we have developed a universal strategy of EIC-MS coupled with diagnostic product ions (DPIs) analysis for rapid structural identification. Owing to the complexity and similarity of the constituents in TCMs, many trace components were too weak to be screened and identified. So the EIC-MS (extracted ion chromatogram) method by m/z was employed to identify the components especially the micro constituents in TCMs. Meanwhile, compounds in TCMs could usually be structurally classified into several families with the same carbon skeletons or substructures, from which the same diagnostic product ions (DPIs) could be determined by the tandem mass spectrometry. Therefore, DPIs have been adopted to verify the EIC-MS peaks that were picked out by EIC-MS method from the complex TCMs system.

Flavonoids are one of the most widespread groups of polyphenolic C6–C3–C6 secondary plant metabolites. Many TCMs contain substantial quantities of dietary flavonoids that show great potential as cancer chemopreventive agents in cell culture studies.[13,14] However, owning to their low bioavailability as result of conjugative metabolism, this does not translate well into *in vivo* activity.[15] Polymethoxylated flavonoids (PMFs), the flavonoid subclass in which all or almost hydroxyls are capped by methylation, have high oral bioavailability.[16–22] Meanwhile, hydroxylated polymethoxyflavonoids have drawn more and

[a]*Center of Scientific Experiment, Beijing University of Chinese Medicine, Beijing 100029, China*

[b]*School of Chinese Pharmacy, Beijing University of Chinese Medicine, Beijing 100029, China. E-mail: yanjiangqiao@sina.com; Tel: +86-10-84738660*

[c]*The Affiliated Hospital of Shandong University of Traditional Chinese Medicine, Jinan 250011, China*

[d]*Modern Research Center of Traditional Chinese Medicines, Beijing University of Chinese Medicine, Beijing 100029, China. E-mail: pengfeitu@vip. 163.com; Tel: +86-10-82802750*

more attention recently because accumulating evidence has suggested that OH-PMFs have much stronger health-promoting biological activities compared with their permethoxylated counterparts.[23–25] Hydroxylated polymethoxyflavonoid glycosides (OH-PMFGs), which are rare compounds in the botanic extracts might have much stronger health-promoting biological activities.[26,27] So far as we are aware, flavonoid glycosides could induce the bacteria in lower digestive tract to produce corresponding enzymes, which hydrolyze glycosides into aglycones to facilitate their absorption so as to strengthen the effect for diseases.[28]

Murraya paniculata (L.) Jack is an important TCM that shows many strong bioactivities, such as febrifuge, astringent, toothache remedy, and stimulant.[29,30] PMFs were considered to be the representative constituents of this plant.[31,32] Therefore, we previously established an HPLC-DAD-MS/MS method to screen and identify seventy PMFs in *M. paniculata*, and eight OH-PMFGs were first reported from the genus *Murraya*.[33] According to the analytical results, three OH-PMFGs (**PG-1**, **PG-2** and **PG-3**) have been obtained from *M. paniculata* by repeated silica gel column chromatography and semi-preparative HPLC, two of which were reported as new compounds.[34] Although the study provided a significant clue for the phytochemical research on *M. paniculata* and the plants of genus *Murraya*, it was not adequate for systematic report of OH-PMFGs. A number of OH-PMFGs in its extract are still unclear, most of which are very possibly new compounds.

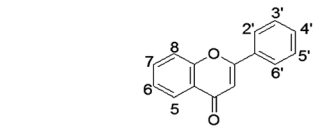
For selective phytochemical identification and further targeted isolation of OH-PMFGs in *M. paniculata* and genus *Murraya*, a methodology of EIC-MS coupled with DPIs analysis for characterization of OH-PMFGs in the leaves of *M. paniculata* using HPLC-DAD-ESI-MS/MS is described in this paper.

Experimental

Chemicals and materials

Eleven PMF reference compounds, including 5,4′-dihydroxy-6,3′-dimethoxyflavone-7-*O*-β-D-glucopyranoside (**PG-1**), 5,3′-dihydroxy-6,7,4′- trimethoxyflavone-8-*O*-β-D-glucopyranoside (**PG-2**), 5-hydroxy-6,7,3′,4′- tetramethoxyflavone-8-*O*-β-D-glucopyranoside (**PG-3**), 5,7,3′,4′-tetramethoxyflavone (**P-1**), 5,7,8,3′,4′-pentamethoxyflavone (**P-2**), 5,3′-dihydroxy-7,8,4′,5′-tetramethoxyflavone (**P-3**), 5-hydroxy-6,7,3′,4′,5′-pentamethoxyflavone (**P-4**), 5,6,7,3′,4′-pentamethoxyflavanone (**P-5**), 5,7,3′,4′,5′-pentamethoxy-flavanone (**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 previously extracted, isolated and identified from *M. paniculata* in our laboratory based on Fig. 1.[34–37] Their purities were determined to be no less than 98% 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). Ultra-pure water was produced by a Milli-Q purification system (18.2 Ω cm^{-1} at 25 °C) (Millipore, Bedford, MA, USA).

Material of *M. paniculata* was purchased from China Resources Sanjiu Medical & Pharmaceutical Co., Ltd (Shenzhen City, China). The material was authenticated by Professor Pengfei Tu. The voucher specimen was deposited at the Center of Scientific Experiment, Beijing University of Chinese Medicine, Beijing, China.



PMFs	-OH[a]	-OCH_3[a]	-O-Glucoside[a]
PG-1	5, 4'	6, 3'	7
PG-2	5, 3'	6, 7, 4'	8
PG-3	5	6, 7, 3', 4'	8
P-1	-	5, 7, 3', 4'	-
P-2	-	5, 7, 8, 3', 4', 5'	-
P-3	5, 3'	7, 8, 4', 5'	-
P-4	5	6, 7, 3', 4', 5'	-

[a]: The positions of the substituent groups

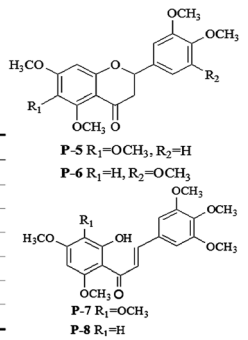


Fig. 1 Structures of eleven PMF standards isolated from *Murraya paniculata*.

Instrumentation

An HPLC-DAD-ESI-MS/MS instrument (Agilent Technologies, Santa Clara, CA, USA) equipped with a binary pump, an autosampler, a photodiode array detector, a column temperature controller and an MSD Trap XCT Plus Mass spectrometer, was used to analyze the OH-PMFGs in the leaves of *M. paniculata*.

Analytical methods

Sample preparation. Powdered dried leaves of *M. paniculata* were weighed accurately (0.5 g) and placed into a 50 mL flask containing 25 mL of methanol/water (70 : 30, v/v). The mixture was then extracted in an ultrasonic bath (Eima Ultrasonics Corp., Germany) at room temperature for 0.5 hour, and the same solvent was added to compensate for the lost weight during the extraction. 10 mL of the filtrate was evaporated and suspended in 2 mL 70% methanol, which was filtered through a 0.22 μm membrane for analysis.

Liquid chromatographic conditions. The sample injection volume was set at 15 μL as commonly used for the HPLC method. The column used was Agilent Zorbax Eclipse Plus C_{18} column (250 × 4.6 mm, 5 μm), and the column temperature was set at 25 °C. The mobile phase for the LC separation was composed of formic acid aqueous solution (0.1% v/v, solvent A) and acetonitrile (solvent B). The elution conditions were applied with a linear gradient as follows: 0–10 min, 10–21% B; 10–60 min, 21% B; 60–81 min, 21–36% B; 81–90 min, 36–100% B. The flow rate was at 1.0 mL min^{-1}, and peaks were detected at 330 nm.

Mass spectrometry. The chromatographic column eluate was split to 0.25 mL min^{-1} which was directed to a trap mass spectrometer through ESI interface. ESI tandem MS analysis was performed in positive ionization mode with the important parameters settings as follows: nebulizer gas pressure of 35.00 psi; drying gas (nitrogen) flow rate of 11.00 mL min^{-1}; electrospray voltage of 3500 V; capillary temperature of 350 °C; capillary exit voltage of 121.0 V; skimmer voltage of 40.0 V; target mass of *m*/*z* 500; scan range of *m*/*z* 100–900; AutoMS operation

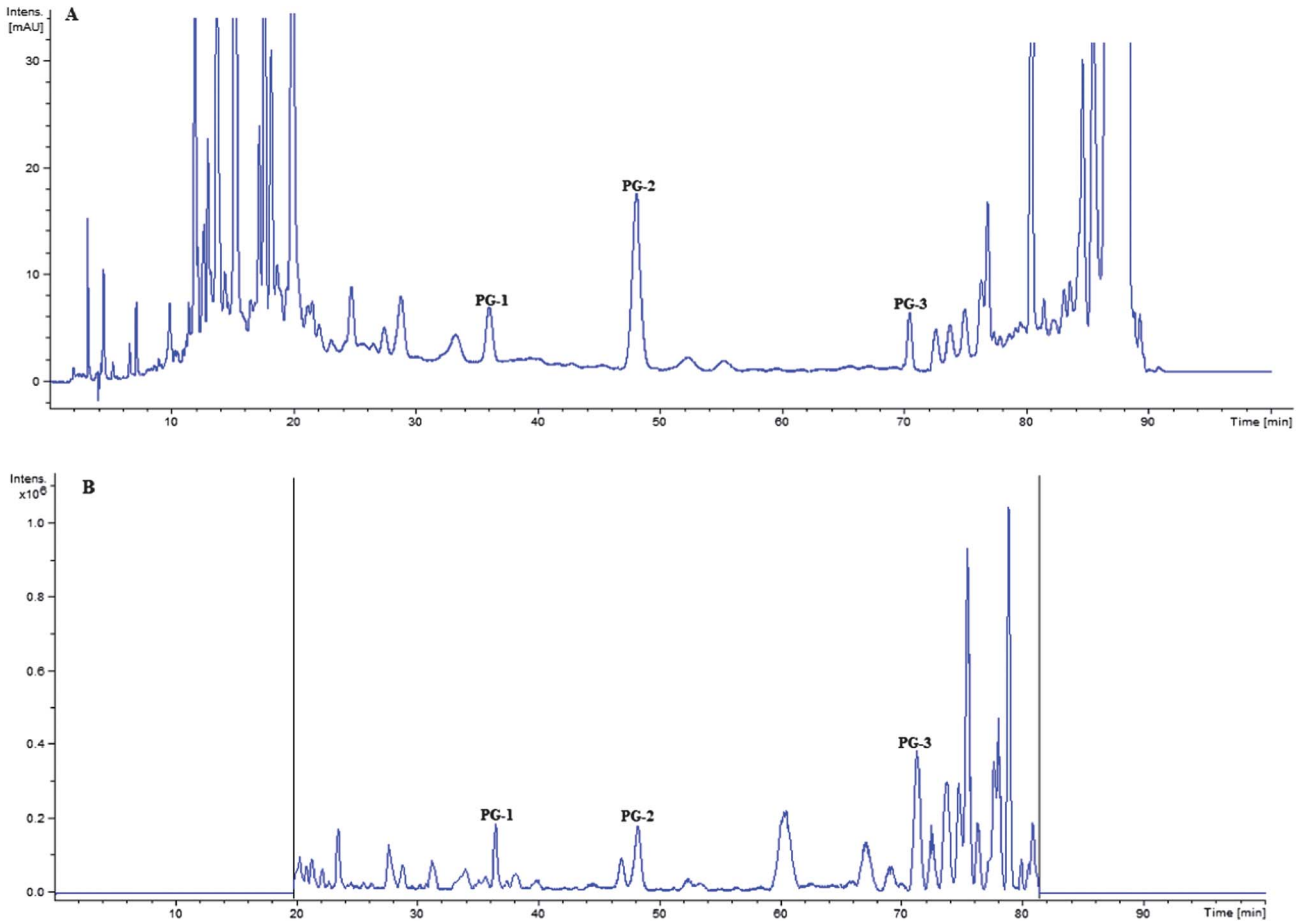


Fig. 2 HPLC-DAD-MS/MS analysis of the OH-PMFGs in extract of *Murraya paniculata*. (A) HPLC-DAD chromatogram of the extract at 330 nm; (B) the ESI-MS base peak chromatogram (BPC) of the extract in positive mode.

mode; collision energy of 1 eV; "SmartFrag" mode (to provide programming energy over a wide range in order to achieve as many product ions as possible), Start Ampl of 30%, End Ampl of 200%. A data-dependent program was used in the HPLC-ESI-MS^n analysis so that the protonated molecules could be isolated for further MS^n analysis. Nitrogen (>99.99%) and He (>99.99%) were used as sheath and damping gas, respectively. Data was acquired and processed through an Agilent 6300 Series Trap Control workstation (version 6.1).

EIC-MS coupled with DPIs analysis method. Owing to the universal presence of the homeomorphic compounds and the difference of their contents, many constituents especially the minor peaks in the TIC spectra are usually hard to see clearly. The EIC-MS method has provided a shortcut to display every peak attributed to the identical mass charge ratio. Meanwhile, the EIC-MS results also contain some peaks that are irrelevant to the targeted compounds. Therefore, it is necessary to develop a straightforward method to distinguish the targeted compounds from the irrelevant ones.

The constituents in TCMs are typically sorted into several classes on the basis of their carbon skeletons or substructures. It is easily understood that the compounds with same carbon skeletons will undergo similar fragmentation pathways in Collision-Induced Dissociation (CID) mode and thus generate similar DPIs from the common carbon skeletons. Therefore, a series of DPIs representing a certain parent nucleus or substitution groups can be used as the standards to judge the EIC-MS peaks into the corresponding chemical family.

The critical step is to determine the DPIs that are valuable in screening and deducing nontargeted compounds of the same class. Eleven representative PMF standards were subsequently analyzed by ESI-IT-MS/MS to obtain the common DPIs for OH-PMFGs. Once the carbon skeletons were determined by the recognition of DPIs, the chemical groups of a certain compound could be easily deduced from the quasi-molecular ions and the corresponding MS/MS product ions. Then the structurally characterized DPIs could be used as a useful screening standard for rapidly locating the candidates containing such a substitution group and/or substructure.

Quantitative method. Chromatographic conditions used here were the same as that described above. The reference compounds, **PG-1**, **PG-2** and **PG-3**, were accurately weighed, dissolved in methanol and diluted with methanol to an appropriate concentration. Calibration curves were plotted by the peak area *versus* six appropriate amounts injected in triplicate of each analyte. The limits of detection (LOD) and quantification (LOQ) were determined on the basis of signal-to-noise (*S*/*N*) ratio of 3 and 10, respectively. The intra-day and inter-day

Table 1 Characterizations of three OH-PMFGs standards in *Murraya paniculata* by CID-MS/MS

Peak	$[M+H]^+$ (m/z)	MS^2 (m/z)			MS^3 (m/z)			MS^4 (m/z)		
		P-ion[a] (%)	Loss[b]	DPIs	P-ion[a] (%)	Loss[b]	DPIs	P-ion[a] (%)	Loss[b]	DPIs
PG-1	493	331[e] (100)	162	$[M+H-Glc]^+$	316[e] (100)	15	$[331-CH_3^{\cdot}]^+$	301 (100)	15	$[316-CH_3^{\cdot}]^+$
								273 (19.4)	43	$[316-CH_3^{\cdot}\text{-}CO]^+$
								285 (6.9)	31	$[316-CH_3^{\cdot}-CH_4]^+$
PG-2	523	361[e] (100)	162	$[M+H-Glc]^+$	346[e] (100)	15	$[361-CH_3^{\cdot}]^+$	328 (100)	18	$[346-H_2O]^+$
					328 (46.6)	33	$[361-CH_3^{\cdot}\text{-}H_2O]^+$	331 (15.5)	15	$[346-CH_3^{\cdot}]^+$
					331 (7.6)	30	$[361-2CH_3^{\cdot}]^+$	300 (6.9)	46	$[346-CO-H_2O]^+$
PG-3	537	375[e] (100)	162	$[M+H-Glc]^+$	360[e] (100)	15	$[375-CH_3^{\cdot}]^+$	342 (100)	18	$[360-H_2O]^+$
					342 (49.2)	33	$[375-CH_3^{\cdot}-H_2O]^+$	345 (15.7)	15	$[360-CH_3^{\cdot}]^+$
					345 (10.3)	30	$[375-2CH_3^{\cdot}]^+$	314 (6.9)	46	$[360-CO-H_2O]^+$
P-1	343	328[e] (100)	15	$[M+H-CH_3^{\cdot}]^+$	299[e] (100)	29	$[328-HCO^{\cdot}]^+$	284 (100)	15	$[299-CH_3^{\cdot}]^+$
		327 (53.3)	16	$[M+H-CH_4]^+$	312 (67.1)	16	$[328-CH_4]^+$	270 (47.3)	29	$[299-HCO^{\cdot}]^+$
		299 (5.1)	44	$[M+H-CO_2]^+$				255 (24.6)	44	$[299-CO_2]^+$
P-2	403	373[e] (100)	30	$[M+H-2CH_3^{\cdot}]^+$	345[e] (100)	28	$[373-CO]^+$	315 (100)	30	$[345-2CH_3^{\cdot}]^+$
		388 (72.4)	15	$[M+H-CH_3^{\cdot}]^+$	358 (25.5)	15	$[373-CH_3^{\cdot}]^+$	284 (86.5)	61	$[345-CO-H_2O-CH_3^{\cdot}]^+$
					344 (10.1)	29	$[373-HCO^{\cdot}]^+$	312 (76.2)	33	$[345-CH_3^{\cdot}-H_2O]^+$
P-3	375	360[e] (100)	15	$[M+H-CH_3^{\cdot}]^+$	345[e] (100)	15	$[360-CH_3^{\cdot}]^+$	327 (100)	18	$[345-H_2O]^+$
		345 (90.4)	30	$[M+H-2CH_3^{\cdot}]^+$	327 (31.3)	33	$[360-CH_3^{\cdot}-H_2O]^+$	314 (26.0)	31	$[345-CH_3^{\cdot}-CH_4]^+$
		346 (19.0)	29	$[M+H-HCO^{\cdot}]^+$	342 (14.1)	18	$[360-H_2O]^+$	317 (20.4)	28	$[345-CO]^+$
P-4	389	356[e](100)	33	$[M+H\text{-}H_2O-CH_3^{\cdot}]^+$	328[e] (100)	28	$[356-CO]^+$	295 (100)	33	$[328-CH_3^{\cdot}-H_2O]^+$
		328 (67.9)	61	$[M+H\text{–}CO\text{–}H_2O-CH_3^{\cdot}]^+$	295 (6.9)	61	$[356-CO-H_2O-CH_3^{\cdot}]^+$	313 (29.6)	15	$[328-CH_3^{\cdot}]^+$
		374 (35.6)	15	$[M+H-CH_3^{\cdot}]^+$				267 (27.7)	61	$[328-CO-H_2O-CH_3^{\cdot}]^+$
P-5	375	211[e] (100)	RDA	$^{1,3}A^{+c}$	196[e] (100)	15	$[211-CH_3^{\cdot}]^+$	178 (100)	18	$[196-H_2O]^+$
		191 (37.1)	RDA	$^{1,4}B^{+c}$	178 (34.7)	33	$[211-CH_3^{\cdot}-H_2O]^+$	150 (51.5)	46	$[196-CO-H_2O]^+$
		357 (16.6)	18	H_2O	150 (19.2)	61	$[211-CO-H_2O-CH_3^{\cdot}]^+$	168 (5.0)	28	$[196-CO]^+$
					183 (15.5)	28	$[211-CO]^+$			
P-6	375	221[e] (100)	RDA	$^{1,4}B^{+c}$	193[e] (100)	28	$[221-CO]^+$	175 (100)	18	$[193-H_2O]^+$
		181 (24.1)	RDA	$^{1,3}A^{+c}$	190 (61.9)	31	$[221-CH_3^{\cdot}-CH_4]^+$	163 (35.1)	30	$[193-2CH_3^{\cdot}]^+$
					191 (40.0)	30	$[221-2CH_3^{\cdot}]^+$	178 (34.3)	15	$[193-CH_3^{\cdot}]^+$
					206 (30.5)	15	$[221-CH_3^{\cdot}]^+$	149 (8.4)	44	$[193-CO_2]^+$
P-7	405	221[e] (100)	RDA	$^{x}B^{+d}$	193 (100)	28	$[221-CO]^+$	175 (100)	18	$[193-H_2O]^+$
		387 (31.6)	18	$[M+H-H_2O]^+$	190 (51.5)	31	$[221-CH_3^{\cdot}-CH_4]^+$	178 (37.0)	15	$[193-CH_3^{\cdot}]^+$
		211 (28.3)	RDA	$^{y}A^{+d}$	191 (43.4)	30	$[221-2CH_3^{\cdot}]^+$	163 (28.3)	30	$[193-2CH_3^{\cdot}]^+$
					206 (31.6)	15	$[221-CH_3^{\cdot}]^+$	147 (15.7)	46	$[193-CO-H_2O]^+$
P-8	375	221[e] (100)	RDA	$^{x}B^{+d}$	193[e] (100)	28	$[221-CO]^+$	175 (100)	18	$[193-H_2O]^+$
		181 (30.0)	RDA	$^{y}A^{+d}$	190 (61.9)	31	$[221-CH_3^{\cdot}-CH_4]^+$	163 (33.2)	30	$[193-2CH_3^{\cdot}]^+$
					191 (40.0)	30	$[221-2CH_3^{\cdot}]^+$	178 (31.3)	15	$[193-CH_3^{\cdot}]^+$
					206 (22.3)	15	$[221-CH_3^{\cdot}]^+$	147 (17.3)	46	$[193-CO-H_2O]^+$

[a] P-ion (%), the product ions and the relative intensity. [b] Loss, Da. [c] The fragment ions from the RDA cleavage on the C-ring of flavanones. [d] The fragment ions from the RDA cleavage from the C-ring of chalcones. [e] Precursor-ion for next stage MS.

variations, which were chosen to determine the precision of the developed method, were investigated by determining the 3 analytes in six replicates during a single day (0, 2, 4, 8, 12, 16 h) and by duplicating the experiments during three consecutive days (0, 2, 8, 12, 24, 36 h). Variations of the peak area were taken as the measures of precision and expressed as percentage relative standard deviations (RSD). To assure the repeatability, six different solutions prepared from the sample were assessed. Recovery test was used to evaluate the accuracy of this method. Recoveries of the quantified constituents were determined using the sample, whose respective chemical contents had been predetermined by repeatability test above. The mixed standard solute was spiked at a close concentration with the sample (**PG-1** 0.224 μg mL^{-1}, **PG-2** 0.486 μg mL^{-1}, **PG-3** 0.182 μg mL^{-1}). 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. Variability was expressed by RSD (%).

Results and discussion

Separation of the analytes

In order to obtain satisfactory extraction efficiency for all of the OH-PMFGs, extraction conditions, including extraction method (ultrasonication, refluxing and standing overnight), extraction solvent (50%, 70% and 100% methanol), extraction solvent

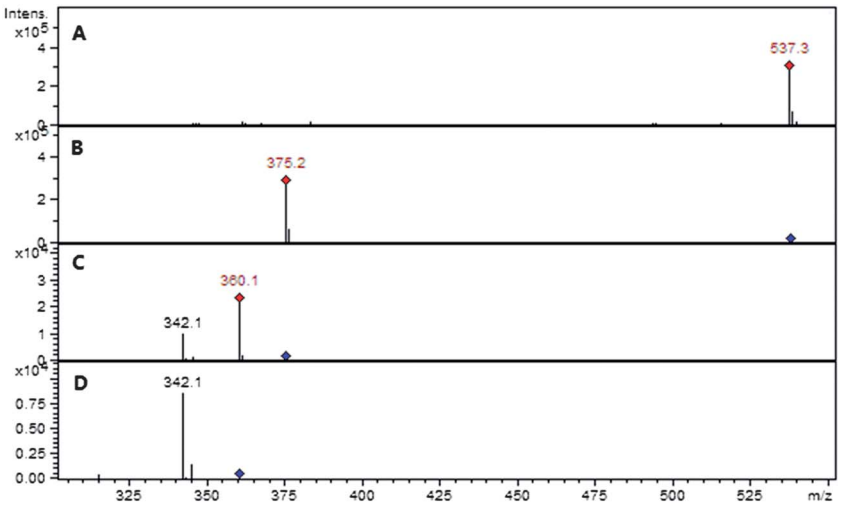


Fig. 3 MS^n spectra of **PG-3**: (A) MS spectrum; (B) MS^2 spectrum (precursor-ion was *m*/*z* 537); (C) MS^3 spectrum (precursor-ion was *m*/*z* 375); (D) MS^4 spectrum (precursor-ion was *m*/*z* 360).

volume (10, 25 and 50 mL), and extraction time (20, 30 and 40 min) were assessed on the basis of single-factor experiments. Meanwhile, the different HPLC parameters including mobile phases (methanol/water and acetonitrile/water), category of RP-ODS columns (Agilent Zorbax extended C_{18} column, 250 × 4.6 mm, 5 μm; Agilent Zorbax Eclipse Plus C_{18} column, 250 × 4.6 mm, 5 μm, and Waters Symmetry Shield C_{18} column, 250 × 4.6 mm, 5 μm), column temperature (20, 25 and 30 °C) and flow rate (0.8, 1.0 and 1.2 mL min^{-1}) were examined. The peaks were detected at 330 nm to display as many OH-PMFGs as possible (Fig. 2).

Optimization of ESI-MS/MS conditions

In the study, all factors related to MS performance including ionization mode, nebulizer gas pressure, electrospray voltage and collision energy have been evaluated. The results indicated ESI in positive ion mode was more sensitive to OH-PMFGs than that in negative ion mode. Preliminary test showed that OH-PMFGs distributed between 20 and 80 min of the TIC spectrum. In order to avoid unnecessary contamination to the mass spectrometer and to improve the signal intensity of OH-PMFGs, the chromatographic column eluates of 0–20 min, and 81–100 min were diverted to waste instead of to source. The major OH-PMFGs were detected (Fig. 2), and most exhibited quasi-molecular ions $[M + H]^+$ and product ions with rich structural information in the positive mode of CID-MS/MS.

DPIs determinations and fragmentation patterns analysis for OH-PMFGs

To clarify structures of the OH-PMFGs in the leaves of *M. paniculata*, eleven PMF standards were analyzed by HPLC-DAD-ESI-MS/MS techniques. According to their chemical structures of

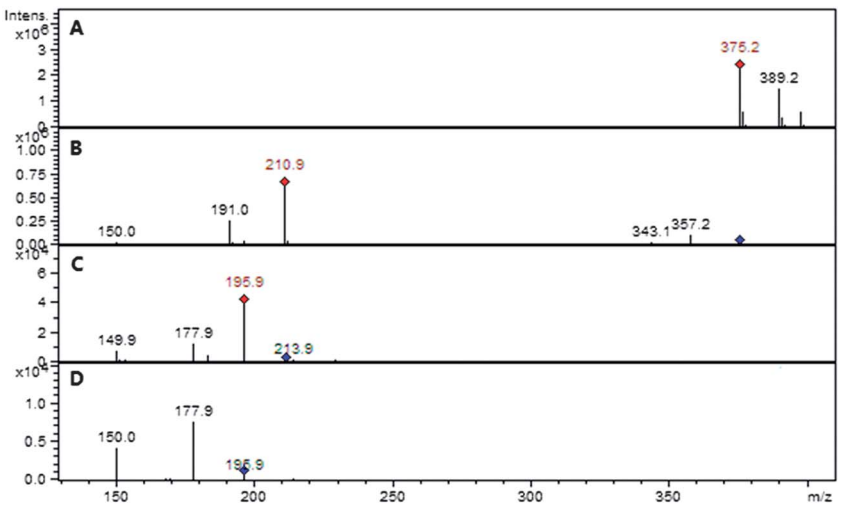


Fig. 4 MS^n spectra of **P-6**: (A) MS spectrum; (B) MS^2 spectrum (precursor-ion was *m*/*z* 375); (C) MS^3 spectrum (precursor-ion was *m*/*z* 211); (D) MS^4 spectrum (precursor-ion was *m*/*z* 196).

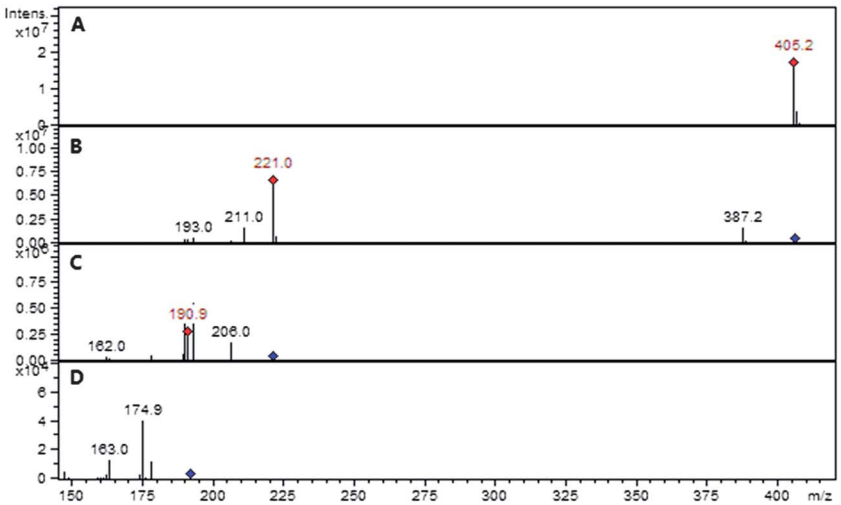


Fig. 5 MS^n spectra of **P-7**: (A) MS spectrum; (B) MS^2 spectrum (precursor-ion was *m/z* 405); (C) MS^3 spectrum (precursor-ion was *m/z* 221); (D) MS^4 spectrum (precursor-ion was *m/z* 191).

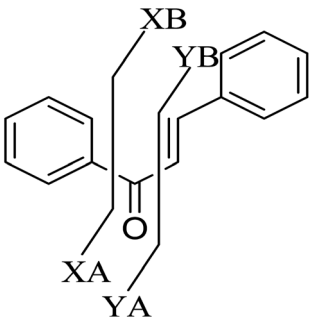


Fig. 6 Proposed MS fragmentation pathway for chalcone derivatives.

aglycones and dominant fragmentation pathways, the OH-PMFGs could be classified into three groups including glycosides of hydroxylated polymethoxyflavones, polymethoxyflavanones and polymethoxychalcones. All of the OH-PMFG and PMF standards exhibited $[M + H]^+$ ions of sufficient intensity that could be subsequently isolated automatically and subjected to CID-MS/MS analysis (Table 1). The DPIs from the proposed fragmentation patterns were helpful to clarify the structural identification of OH-PMFGs in the leaves of *M. paniculata*. The nomenclature commonly used for mass products of flavonoids was adopted in this work.[38]

DPIs determinations for hydroxylated polymethoxylated flavone glycosides

Seven PMF standards, including three polymethoxylated flavone glycosides and four polymethoxylated flavones, were subsequently analyzed first in the CID-MS/MS experiment. By comparison of the product ion spectra of the OH-PMFG standards (Fig. 3), some characteristic dissociation pathways could be summarized for further characterization of the other polymethoxylated flavones. First, all of the $[M + H]^+$ ions could lose one glucose moiety in the MS/MS spectra and formed the base peaks of $[aglycone + H]^+$ ($[M + H - 162]^+$). Second, all of the $[aglycone + H]^+$ ions could lose one to four methyl radicals ($CH_3^{\cdot}$) in their MS/MS spectra, and formed the base peaks of $[M + H - 15]^+$, $[M + H - 30]^+$, $[M + H - 45]^+$ and $[M + H - 60]^+$. Third, the other dissociation pathways of $[aglycone + H]^+$ by loss of 16 (CH_4), 18 (H_2O), 28 (CO), 31 ($CH_3^{\cdot} + CH_4$), 33 ($H_2O + CH_3^{\cdot}$), 43 ($CH_3^{\cdot} + CO$) and 46 ($H_2O + CO$) were detected

Table 2 Chemical formula and mass of all possible polymethoxylated flavone glycosides

Substituents	OH	2OH	3OH	4OH	5OH	6OH
OCH_3	$C_{22}H_{22}O_9$ 430	$C_{22}H_{22}O_{10}$ 446	$C_{22}H_{22}O_{11}$ 462	$C_{22}H_{22}O_{12}$ 478	$C_{22}H_{22}O_{13}$ 494	$C_{22}H_{22}O_{14}$ 510
$2OCH_3$	$C_{23}H_{24}O_{10}$ 460	$C_{23}H_{24}O_{11}$ 476	$C_{23}H_{24}O_{12}$ 492	$C_{23}H_{24}O_{13}$ 508	$C_{23}H_{24}O_{14}$ 524	
$3OCH_3$	$C_{24}H_{26}O_{11}$ 490	$C_{24}H_{26}O_{12}$ 506	$C_{24}H_{26}O_{13}$ 522	$C_{24}H_{26}O_{14}$ 538		
$4OCH_3$	$C_{25}H_{28}O_{12}$ 520	$C_{25}H_{28}O_{13}$ 536	$C_{25}H_{28}O_{14}$ 552			
$5OCH_3$	$C_{26}H_{30}O_{13}$ 550	$C_{26}H_{30}O_{14}$ 566				
$6OCH_3$	$C_{27}H_{32}O_{14}$ 580					

Table 3 Chemical formula and mass of all possible polymethoxylated flavanone or chalcone glycosides

Substituents	OH	2OH	3OH	4OH	5OH	6OH
OCH_3	$C_{22}H_{24}O_9$ 432	$C_{22}H_{24}O_{10}$ 448	$C_{22}H_{24}O_{11}$ 464	$C_{22}H_{24}O_{12}$ 480	$C_{22}H_{24}O_{13}$ 496	$C_{22}H_{24}O_{14}$ 512
$2OCH_3$	$C_{23}H_{26}O_{10}$ 462	$C_{23}H_{26}O_{11}$ 478	$C_{23}H_{26}O_{12}$ 494	$C_{23}H_{26}O_{13}$ 510	$C_{23}H_{26}O_{14}$ 526	
$3OCH_3$	$C_{24}H_{28}O_{11}$ 492	$C_{24}H_{28}O_{12}$ 508	$C_{24}H_{28}O_{13}$ 524	$C_{24}H_{28}O_{14}$ 540		
$4OCH_3$	$C_{25}H_{30}O_{12}$ 522	$C_{25}H_{30}O_{13}$ 538	$C_{25}H_{30}O_{14}$ 554			
$5OCH_3$	$C_{26}H_{32}O_{13}$ 552	$C_{26}H_{32}O_{14}$ 568				
$6OCH_3$	$C_{27}H_{34}O_{14}$ 582					

as diagnostic products in their MS^3 and MS^4 spectra, which were in accordance with the fragmentation pathways of PMF standards (**P1–P4**). These main product ions mentioned above could also form the ESI-MS^n DPIs of hydroxylated polymethoxyflavone glycosides, which could be used to rapidly screen out them from the complex TCM extracts.

DPIs determinations for glycosides of hydroxylated polymethoxyflavones, flavanones and chalcones

Unfortunately, there have been no hydroxylated polymethoxyflavanone and polymethoxychalcone glycosides isolated from *M. paniculata* until now due to their low abundance in the medical plant. However, all of the fragmentation pathways of polymethoxylated flavanones and chalcones that we have ever isolated from *M. paniculata* provide some clues for their respective glycosides,[33] since flavonoid-*O*-glycosides usually prefer to eliminate glucose moiety to form $[aglycone + H]^+$ ions rather than perform other fragmentation reactions in the first step of the fragmentation reaction.

In the CID-MS/MS experiment, the fragmentation pathways of two polymethoxylated flavanones derivatives (**P-5** and **P-6**) were similar to each other. For example, **P-6** gave the $[M + H]^+$ ion at *m*/*z* 375 in ESI-MS spectrum, which further generated a prominent ion at *m*/*z* 221 as base peak in MS/MS spectrum (Fig. 4). It could be deduced that the dominating fragmentation pathway was Retro–Diels–Alder (RDA) cleavage from the 1,4-position of C-ring.[33] Meanwhile, the minor ion at *m*/*z* 181 was also detected, owing to the RDA fragmentation from the 1,3-position of C-ring. The loss of 15 ($CH_3^{\cdot}$), 28 (CO), 30 ($2CH_3^{\cdot}$) and 31 ($CH_3^{\cdot} + CH_4$) from the base peak at *m*/*z* 221, could also generate a series of DPIs for polymethoxylated flavanone glycosides in the CID-MS/MS spectra. This kind of fragmentation pathway that the $[M + H]^+$ ions underwent RDA reaction prior to the neutral loss of $CH_3^{\cdot}$, H_2O, CO, *etc.*, was strikingly different from ordinary flavanones. Therefore, the particular pathways and DPIs could be adopted as a shortcut to distinguish polymethoxylated flavanone glycosides from ordinary flavones glycosides rapidly.

Compounds **P-7** and **P-8**, two polymethoxylated chalcone standards, were also analyzed by the CID-MS/MS method. Their dissociation pathways of MS spectra were the same as each other. Taking **P-7** for example (Fig. 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 could also be simultaneously detected in MS/MS spectrum first (Fig. 6). The fragmentation pathway was highly similar to what happened to polymethoxylated 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.[39,40] Meanwhile, the product ions detected from the loss of 15 ($CH_3^{\cdot}$), 16 (CH_4), 18 (H_2O), 28 (CO), 30 ($2CH_3^{\cdot}$) and 31 ($CH_4 + CH_3^{\cdot}$) could be also adopted as DPIs for polymethoxylated chalcones. Thus, according to their fragmentation pathways, it was easy to tell the difference between glycosides of polymethoxychalcones and flavones, but difficult to distinguish them from glycosides of polymethoxyflavanones. Meanwhile, the abundances of both the polymethoxyflavanone glycosides and polymethoxychalcone glycosides were too low to obtain online UV absorption spectra, so they were identified together in the end.

EIC-MS coupled with DPIs analysis method for rapid identification of OH-PMFGs in *M. paniculata*

Until now three kinds of OH-PMFGs, *i.e.* glycosides of polymethoxylated flavones, flavanones and chalcones have been detected from these medical plants. Owing to the common phenomenon of substitution isomerism and the great differences of contents in raw materials, it is extremely difficult to discover all of them in the ESI-MS spectrum. Some EIC-MS peaks were too weak to be seen clearly in the TIC spectrum. Moreover, the retention times of some EIC-MS peaks were so similar that they could not be identified simultaneously in TIC spectrum. However, PMFs have regularity in elemental composition as they have the basic aglycone structures with maximum seven substituents such as methoxyl group (OCH_3) and/or hydroxyl group (OH) on their A, B and C rings. The molecular weights of basic

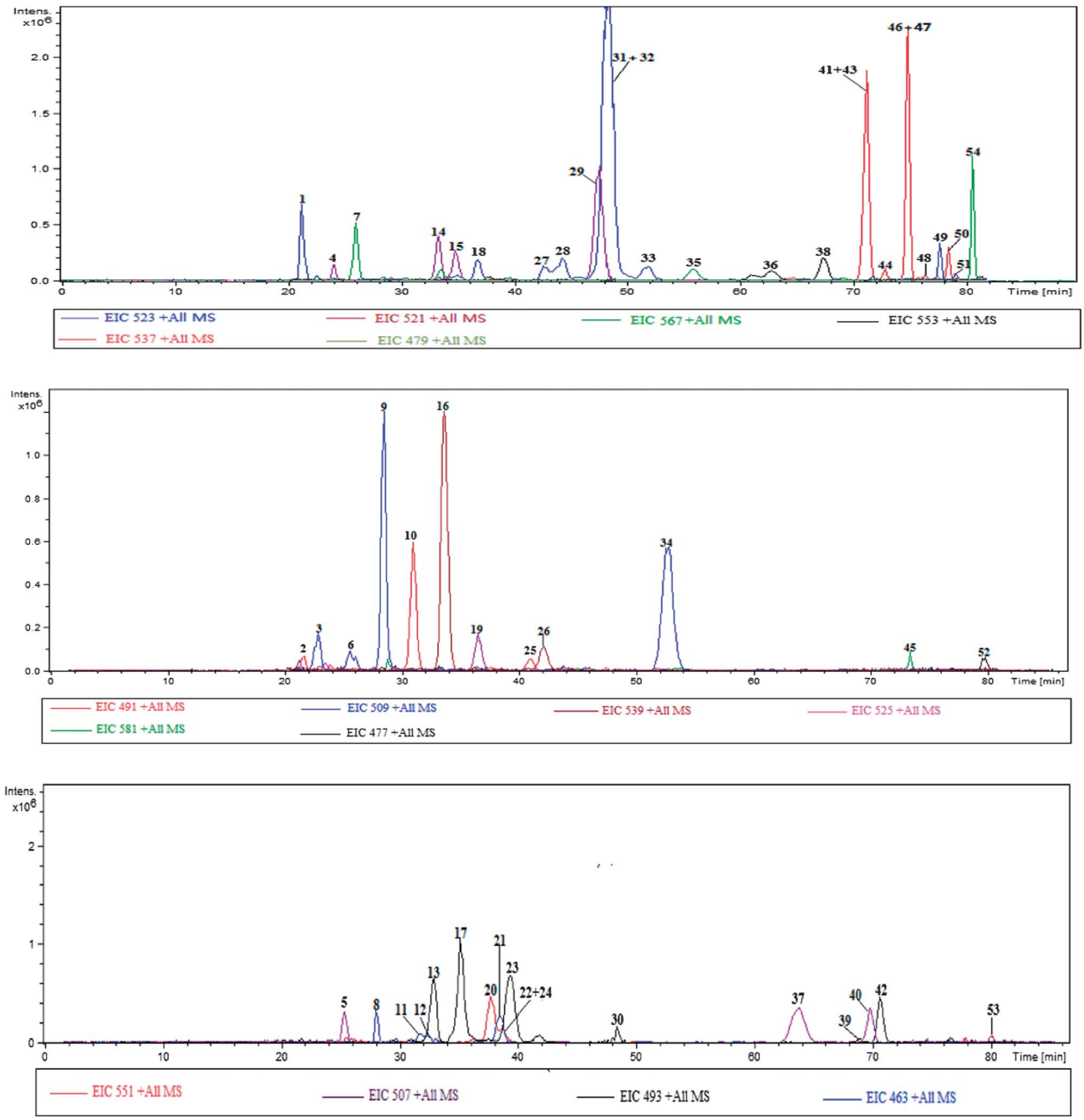


Fig. 7 The EIC-MS peaks of all possible OH-PMFGs in *Murraya paniculata*.

structures of aglycones are 222, 224 and 224 u for flavones, flavanones and chalcones, respectively, which are increased by 30 or 16 when a methoxyl or hydroxyl was attached. Meanwhile, all of their $[M + H]^+$ ions detected readily eliminated 162 Da to produce the corresponding $[aglycone + H]^+$ ions as base peak in MS/MS spectra, all involved were monosaccharide glycosides of PMFs. Based on the numbers and the types of the substituent groups, the chemical formula and mass of every possible OH-PMFGs isomer can be designated in advance (shown in Table 2 and 3). Thus, the EIC-MS method adopted in our study was confirmed to be one kind of powerful weapon to screen the preliminarily ingredients in highly complex extracts of TCMs (shown in Fig. 7 and Table 4).

The DPIs deduced above were used as markers for screening and classifying OH-PMFGs detected into known categories. After screening the molecular weights and DPIs analysis, 54 compounds including 49 flavone glycosides and 5 flavanone glycosides or chalcone glycosides were all verified as OH-PMFGs, among which three compounds were unambiguously identified by comparison with reference substances (shown in Table 5).

Table 4 Characterization of OH-PMFGs in *Murraya paniculata* by HPLC-DAD-ESI-MS/MS

No.	t_R[a]	$[M + H]^+$ (m/z)	MS^2 (m/z) P-ion[b] (%, loss)	MS^3 (m/z) P-ion[b] (%, loss)	MS^4 (m/z) P-ion[b] (%, loss)
1	21.54	523	361[c] (100, 162)	211[c] (100, RDA), 177 (15.3, RDA)	196 (100, 15), 178 (10.4, 33), 183 (8.4, 28)
2	21.85	491	329[c] (100, 162)	314 (100, 15), 299 (39.8, 30)	—[e]
3	23.09	509	347[c] (100 162)	193[c] (100, RDA), 329 (83.8, 18), 181 (31.5, RDA)	160 (100, 33), 178 (49.6, 15)
4	24.12	521	359[c] (100, 162)	344 (100, 15), 329 (81.8, 30), 331 (3.6, 28)	—[e]
5	25.51	507	345[c] (100, 162)	330 (100, 15)	—[e]
6	26.04	509	347[c] (100, 162)	332 (100, 15), 287 (32.5, 60)	—[e]
7	26.24	479	317[c] (100, 162)	302[c] (100, 15)	274 (100, 28)
8	27.90	463	301[c] (100 162)	283 (100, 18)	—[e]
9	28.68	509	347[c] (100, 162)	332[c] (100, 15)	304 (100, 28), 317 (27.9, 15)
10	31.11	491	329[c] (100, 162)	314[c] (100, 15)	285 (100, 29), 286 (69.6, 28)
11	32.11	463	301[c] (100, 162)	270 (100, 31), 273 (78.4, 28), 286 (77.8, 15)	—[e]
12	32.50	463	301[c] (100, 162)	286 (100, 15)	—[e]
13	32.91	493	331[c] (100, 162)	181 (100, RDA), 177 (30.3, RDA)	—[e]
14	33.64	521	359[c] (100, 162)	344 (100, 15), 298 (84.5, 61), 329 (23.9, 30)	—[e]
15	33.92	521	359[c] (100, 162)	298 (100, 61), 344 (38.1, 15), 314 (36.9, 45)	—[e]
16	34.24	539	377[c] (100, 162)	362[c] (100, 15), 344 (6.3, 33)	229 (100, RDA), 347 (92.3, 15), 344 (91.4, 18)
17[d]	35.86	493	331[c] (100, 162)	316 (100, 15)	301 (100, 15), 273 (18.7), 285 (7.2)
18	36.70	523	361[c] (100, 162)	346 (100, 15), 331 (69.5, 32), 328 (53.3, 33), 332 (30.6, 29)	—[e]
19	36.81	525	363[c] (100, 162)	345 (100, 18), 331 (46.6, 32), 348 (37.3, 15), 333 (23.9, 30)	—[e]
20	37.90	551	389[c] (100, 162)	328 (100, 61), 345 (26.1, 44), 359 (26.0, 30), 374 (24.0, 15)	—[e]
21	38.12	463	301[c] (100, 162)	286 (100, 15)	—[e]
22	38.41	551	389[c] (100, 162)	359[c] (100, 30), 328 (33.0, 61), 374 (31.5, 15)	267 (100, 61), 21 1(79.9, RDA), 297 (71.5, 31)
23	39.41	493	331[c] (100, 162)	316 (100, 15)	—[e]
24	39.98	551	389[c] (100, 162)	374[c] (100, 15), 359 (60.1, 30), 373 (58.2, 16)	359 (100, 15), 343 (24.9, 31), 345 (23.0, 29)
25	41.12	491	329[c] (100, 162)	314 (100, 15), 300 (20.4, 29)	—[e]
26	42.51	539	377[c] (100, 162)	362 (100, 15), 344 (26.9, 33)	—[e]
27	43.12	523	361[c] (100, 162)	346 (100, 15), 331 (43.0, 30), 328 (32.0, 33)	—[e]
28	44.80	523	361[c] (100, 162)	346 (100, 15), 328 (11.0, 33)	—[e]
29	47.51	521	359[c] (100, 162)	344[c] (100, 15), 343 (58.3, 16)	298 (100, 46), 315 (28.3, 29), 280 (10.1, 64)
30	48.10	493	331[c] (100, 162)	316 (100, 15), 315 (43.9, 16)	—[e]
31[d]	48.62	523	361[c] (100, 162)	346[c](100, 15), 328 (47.5, 33), 331 (9.6, 30)	328 (100, 18), 331 (15.5, 15), 300 (7.2, 46)
32	49.57	523	361[c] (100, 162)	346[c] (100, 15), 328 (24.5, 33), 331 (12.0, 30)	328 (100, 18), 298 (40.0, 48)
33	52.23	523	361[c] (100, 162)	346[c] (100, 15)	331 (100, 15)
34	53.28	509	347[c] (100, 162)	332 (100, 15)	—[e]
35	56.81	479	317[c] (100, 162)	302 (100, 15)	—[e]
36	62.10	553	391[c] (100, 162)	376[c](100, 15), 358 (38.9, 33), 361 (15.2, 30)	358 (100, 18), 342 (61.8, 34), 361 (35.1, 15), 331 (16.9, 45)
37	63.88	507	345[c] (162, 100)	330[c] (100, 15), 312 (83.5, 33)	284 (100, 28)
38	67.01	553	391[c] (100, 162)	376 (100,15), 358 (55.8, 33), 361 (19.5, 30)	—[e]
39	68.71	493	331[c] (100, 162)	316 (100, 15)	—[e]
40	70.05	507	345[c] (100, 162)	330 (100, 15), 312 (92.2, 33), 327 (43.4, 18)	—[e]
41[d]	70.66	537	375[c] (100, 162)	360[c] (100, 15), 342 (50.4, 33), 345 (10.7)	342 (100, 18), 345 (15.4, 30), 314 (7.8, 46)
42	71.28	493	331[c] (100, 162)	316[c] (100, 15)	288 (100, 28)
43	71.61	537	375[c] (100, 162)	360[c] (100, 15), 342 (48.3, 33), 345 (9.7, 30)	342 (100, 18), 225 (11.9, RDA)
44	72.81	537	375[c] (100, 162)	360 (100, 15), 342 (45.4, 33), 346 (12.7, 29)	—[e]
45	73.40	581	419[c] (100, 162)	389[c] (100, 30), 404 (37.1, 15), 358 (15.2, 61)	356 (100, 33), 361 (82.8, 28), 299 (71.3, 90), 346 (53.9, 43)
46	74.83	537	375[c] (100, 162)	360[c] (100, 15), 359 (29.9, 16), 342 (28.6, 33)	342 (100, 18), 314 (86.9, 36)
47	75.18	537	375[c] (100, 162)	360[c] (100, 15), 342 (19.9, 33), 314 (10.9, 61)	342 (100, 18), 183 (29.3, RDA), 178 (29.1, RDA)
48	76.25	537	375[c] (100, 162)	360 (100, 15), 345 (19.5, 30), 342 (14.6, 33)	—[e]
49	77.60	523	361[c] (100, 162)	197 (100, RDA), 191 (28.8, RDA), 343 (19.7, 18)	—[e]

Table 4 (*Contd.*)

No.	t_R[a]	$[M + H]^+$ (m/z)	MS^2 (m/z) P-ion[b] (%, loss)	MS^3 (m/z) P-ion[b] (%, loss)	MS^4 (m/z) P-ion[b] (%, loss)
50	78.21	537	375[c] (100, 162)	360 (100, 15), 314 (30.7, 61)	—[e]
51	79.11	523	361[c] (100, 162)	211[c](100, RDA)	177 (100, 34), 196 (50.9, 15)
52	79.66	477	315[c] (100, 162)	300 (100, 15)	—[e]
53	80.00	507	375[c] (100, 162)	360 (100, 15)	—[e]
54	80.46	567	405[c] (100, 162)	390[c] (100, 15), 375 (85.3, 30), 389 (61.0, 16), 357 (32.1, 48)	375 (100, 15), 359 (91.0, 31), 344 (60.2, 46), 341 (54.9, 49)

[a] t_R, retention time. [b] P-ion (%, loss), the product ions, the relative intensity and the loss (Da). [c] Precursor-ion for next stage MS. [d] Compounds identified by comparison with reference standards. [e] Too low to be detected.

Determination of three OH-PMFGs in the leaves of *M. paniculata*

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 thrice for linearity. As shown in Table 6, good linearity and high sensitivity under the optimal chromatographic conditions were obtained with correlation coefficients more than 0.998 and relative low LOD (0.57–0.71 ng) and LOQ (1.87–2.43 ng).

As demonstrated in Table 7, the results of precision and accuracy showed good reproducibility for quantification of three OH-PMFGs with intra- and inter-day variation less than 0.31 and 1.51%, respectively. The RSDs (relative standard deviations) of the repeatability experiments were less than 1.04% for all analytes. The overall recoveries of the three investigated compounds ranged from 97.26 to 99.37% with RSD from 0.71 to 1.97%.

Base on the above results, the developed method is precise, accurate and sensitive enough for the quantitative determination of the three main OH-PMFGs in the leaves of *M. paniculata*.

The developed method was subsequently applied to the simultaneous determination of three bioactive markers in the leaves of *M. paniculata*. The sample was analyzed three times and the mean contents of OH-PMFGs were calculated as follows: 10.147 μg g^{-1} of **PG-1**, 23.791 μg g^{-1} of **PG-2** and 9.014 μg g^{-1} of **PG-3**.

Table 5 The MWs and structural identification of all possible OH-PMFGs detected in *Murraya paniculata*

Peaks	Amounts	OH-PMFGs	No. of $-OCH_3$	No. of –OH	Molecular weight
8, 11, 12, 21	4	Trihydroxy-monomethoxyflavone-hexose	1	3	462
52	1	Dihydroxy-dimethoxyflavone-hexose	2	2	476
7, 35	2	Tetrahydroxy-monomethoxyflavone-hexose	1	4	478
2, 10, 25	3	Monohydroxy-trimethoxyflavone-hexose	3	1	490
13	1	Monohydroxy-trimethoxyflavanone-hexose *or* Monohydroxy-trimethoxychalcone-hexose	3	1	492
17, 23, 30, 39, 42	5	Trihydroxy-dimethoxyflavone-hexose	2	3	492
5, 37, 40, 53	4	Dihydroxy-trimethoxyflavone-hexose	3	2	506
3	1	Dihydroxy-trimethoxyflavanone-hexose *or* Dihydroxy-trimethoxymethoxychalcone-hexose	3	2	508
6, 9, 34	3	Tetrahydroxy-dimethoxyflavone-hexose	2	4	508
4, 14, 15, 29	4	Monohydroxy-tetramethoxyflavone-hexose	4	1	520
18, 27, 28, 31–33	6	Trihydroxy-trimethoxyflavone-hexose	3	3	522
1, 49, 51	3	Monohydroxy-tetramethoxyflavanone-hexose *or* Monohydroxy-tetramethoxychalcone-hexose	4	1	522
19	1	Pentahydroxy-dimethoxyflavone-hexose	2	5	524
41, 43, 44, 46–48, 50	7	Dihydroxy-tetramethoxyflavone-hexose	4	2	536
16, 26	2	Tetrahydroxy-trimethoxyflavone-hexose	3	4	538
20, 22, 24	3	Monohydroxy-pentamethoxyflavone-hexose	5	1	550
36, 38	2	Trihydroxy-tetramethoxyflavone-hexose	4	3	552
54	1	Dihydroxy-pentamethoxyflavone-hexose	5	2	566
45	1	Monohydroxy-hexamethoxyflavone-hexose	6	1	580

Table 6 Calibration curves, linearity, LOD and LOQ for three OH-PMFGs

Compounds	t_R[a] (min)	Regression equation[b]	r	Linear range (ng)	LOD (ng)	LOQ (ng)
PG-1	35.86	$Y = 4129.3X - 17.381$	0.9999	14.2–284	0.68	2.32
PG-2	48.62	$Y = 3716.5X - 17.314$	1.0000	20.8–416	0.57	1.87
PG-3	70.68	$Y = 3151.9X - 10.769$	0.9999	8.3–166	0.71	2.43

[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

Compounds	Intra-day RSD (%, $n = 6$)	Inter-day RSD (%, $n = 6$)	Repeatability RSD (%, $n = 6$)	Recovery (%, $n = 6$)	Recovery RSD (%, $n = 6$)
PG-1	0.31	0.89	0.59	99.37	0.71
PG-2	0.45	0.76	0.72	98.49	1.42
PG-3	1.15	1.51	1.04	97.26	1.97

Conclusions

In the present study, a sensitive HPLC-DAD-ESI-MS/MS method was established for simultaneous qualitative and quantitative determination of the OH-PMFGs present in the leaves of *M. paniculata*. Owing to regularities of OH-PMFGs in elemental composition, the EIC-MS method by molecular weights was employed to screen the homeomorphic OH-PMFGs from the extract. Meanwhile, the respective DPIs deduced from the fragmentation pathways of glycosides of hydroxylated polymethoxyflavones, flavanones and chalcones could be adopted as the standards for further analysis and verification the OH-PMFGs in the extract. Finally, 54 OH-PMFGs were preliminarily identified. This is the first systematic report of the presence of OH-PMFGs in genus *Murraya*, most of which were probably new compounds. Meanwhile, the contents of three main OH-PMFGs in the leaves of *M. paniculata* were determined by HPLC-UV. All of the results indicated that the established methodology of EIC-MS coupled with DPIs analysis could be employed as a rapid, effective technique to screen and identify OH-PMFGs from a complex extract of TCMs. The study also provided a significant clue for phytochemical research into *M. paniculata* and the plants of genus *Murraya*. It is possible for the methodology to be extended to the fields of elucidating compounds from other organic matter mixtures such as substances analysis in vegetables, water quality analysis, natural organic matter analysis in soil, pesticide multiresidue analysis in food, and so on, in view of the fact that the compounds contained in such matrix can also be classified into families based on the common carbon skeletons.

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