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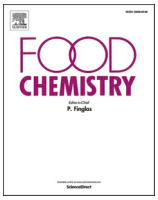

Characterization of free and bound polymethoxyflavones in the dried peel of *Citrus reticulata* "Chachi" through fingerprint RDA ions and reversed-phase column retention by UPLC-Q-TOF-MS/MS

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ABSTRACT

Polymethoxyflavones (PMFs) from citrus peel, including permethoxylated PMFs and hydroxylated PMFs (OH-PMFs), have attracted much attention due to their potential strong biological activities. However, characterization of PMFs through LC-MS analysis was challenged due to numerous substituent positions in flavone. In this study, twelve PMF standards were analyzed by UPLC-QTOF-MS/MS to present fingerprint retro-Diels-Alder (RDA) ions ($[^{0,2}B]^+$, ^{1,3} A and ^{1,3}B(C) associated ions). Based on UPLC-QTOF-MS/MS characteristics of PMFs, 29 PMFs, screened through extracted ion chromatograms in UPLC-QTOF-MS analysis, were identified in free and bound extracts from dried peel of *Citrus reticulata* "Chachi" through fingerprint RDA ions and reversed-phase column retention in UPLC-QTOF-MS/MS experiment. It was found that permethoxylated PMFs and 5-OH PMFs existed mainly in free form; while di/trihydroxy PMFs existed mainly in bound form in dried peel of *Citrus reticulata* "Chachi". The present study is expected to provide new analytical strategy in characterizing PMFs in PMFs metabolites and citrus.

1. Introduction

Citrus are one of the world's major fruit crops that are produced in many countries, and are widely consumed in the world due to the flavorful and pro-health properties. Except for the large consumption as fresh fruits, citrus fruit has been mainly manufactured as juice in food industry, along with the production of many citrus peel by-products. Among these products, dried citrus peel has been commonly utilized as culinary seasoning, and also used as herbal medicine for treating cough, inflammatory respiratory diseases, et al. (Chinese Pharmacopoeia Committee, 2020).

Flavonoids are the main bioactive compounds in dried citrus peel, mainly including flavonoid glycosides and polymethoxyflavones (PMFs) (Yu et al., 2018; Yu et al., 2018). Despite much more content of flavonoid glycosides than PMFs in dried citrus peel, PMFs were reported to better reflect the biological activity of dried citrus peel (Chen et al., 2017; Zeng et al., 2018). Besides, the two largest PMFs (tangeretin and nobiletin) were first listed in the Chinese Pharmacopoeia (2020 edition) as the index components of dried peel of *Citrus reticulata* "Chachi" from Xinhui County (Guangdong province, China), which has always been well-regarded to have superior quality than dried peel derived from other cultivars (Fu et al., 2017; Liu et al., 2013).

PMFs can be divided into permethoxylated PMFs and hydroxylated PMFs (OH-PMFs), among which the former is more abundant than the latter in citrus peel (Li et al., 2006). Thus, current research on PMFs in dried citrus peel mainly focused on permethoxylated PMFs, including tangeretin, nobiletin, sinensetin, and 3,5,6,7,8,3′,4′-heptamethoxyflavone, while research on OH-PMFs was mainly limited in 5-demethylnobiletin.(Duan et al., 2017; Yin et al., 2019; Zheng et al., 2020) However, more and more research showed that OH-PMFs exhibited more significant antifungal and anti-inflammatory activities than permethoxylated PMFs (Li et al., 2007; Liu et al., 2012), and dihydroxy PMFs showed stronger anti-tumor and anti-inflammatory activity than monohydroxy PMFs (Guo et al., 2018; Song et al., 2020). Besides, it was reported that PMFs could remove one, two, or three methyl groups from methoxy group at C-3′, C-4′, C-5, C-6, or C-7 to form corresponding

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mononhydroxy, dihydroxy, or trihydroxy PMFs through cytochrome enzyme P450 metabolism in vivo, with the metabolites OH-PMFs being identified through NMR-validated synthesized standard (Shakour et al., 2020; Zheng et al., 2015). And permethoxylated PMFs in citrus peel can be demethylated at C-5 to form corresponding 5-OH PMFs through acid hydrolysis and enzyme-mediated catalysis during drying process (Zhang et al., 2019). Additionally, many other OH-PMFs have also been reported in dried citrus peel through liquid chromatography-mass spectrometry (LC-MS) analysis, including monohydroxy-tri/tetra/pentamethoxyflavone, dihydroxy-di/tri/tetra/pentamethoxy flavone etc. (Zhang et al., 2020). However, characterization of these OH-PMFs through accurate mass value was challenged due to the presence of numerous substituent positions in A, B, C-ring of flavone.

Phenolics (including flavonoids, phenolic acids, etc) in plants generally occur in both free and bound form with the free form usually soluble in polar organic solvent, while the bound phenolics can link with macromolecules such as polysaccharides, protein and other substances via covalent bounds (Wang et al., 2020). It can be inferred that OH-PMFs might also exist in bound form. However, to the best of our knowledge, there have been few studies on the bound PMFs in dried citrus peel.

With the advantages of liquid separation and tandem mass spectrometry (MS/MS) fragmentation in structural characterization,(Yu et al., 2024) the objective of this study was to characterize free and bound PMFs in the dried peel of *Citrus reticulata* "Chachi" by considering both retention time and accurate mass of fragment ions in ultrahigh performance liquid chromatography-high resolution mass spectrometry (UPLC-HRMS). The UPLC-QTOF-MS and UPLC-QTOF-MS/MS analysis were adopted to investigate the retention time and fragment patterns of twelve commercially available PMFs standards with hydroxy or methoxy group at different substituent positions, so as to connect the structure of PMFs to the observed fragment ions and retention time. Then, the UPLC-QTOF-MS and UPLC-QTOF-MS/MS analysis were further applied in dried citrus peel to analyze free PMFs and bound PMFs isolated from different hydrolysis methods.

2. Materials and methods

2.1. Samples

Fresh *Citrus reticulata* "Chachi" were collected from Xinhui Distrcit, Jiangmen City, Guangdong Province in November 2020. After cleaning, the citrus peel was removed as three pieces through three knives and then subject to sun drying until there was no change on the weight of peel. Finally, the water content (wt/wt) of the dried citrus peel was at the level of 9 %. The dried citrus peel was stored at room temperature.

2.2. Chemicals

Twelve PMF reference compounds, including eupatilin (5,7-dihydroxy-6,3′,4′- trimethoxyflavone, P1), jaceosidin (5,7,4′-trihydroxy-6,3′-dimethoxyflavone, P2), nepetin (5,7,3′,4′-tetrahydroxy-6-methoxyflavone, P3), sinensetin (5,6,7,3′,4′-pentamethoxyflavone, P4), demethylsinensetin (5-hydroxy-6,7,3′,4′-pentamethoxyflavone, P5), eupatorin (5,3′-dihydroxy-6,7,4′-trimethoxyflavone, P6), tangeretin (5,6,7,8,4′-pentamethoxyflavone, P7), gardenin B (5-hydroxy-6,7,8,4′-tetramethoxyflavone, P8), lysionotin (5,7-dihydroxy-6,8,4′-triamethoxyflavone, P9), nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone, P10), demethylnobiletin (5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone, P11), 3,5,6,7,8,3′,4′-heptamethoxyflavone (P12) (>95 %) and LC-MS grade acetonitrile were purchased from ANPEL laboratory technologies Co., Ltd. (Shanghai, China). The water and formic acid used in mobile phase were purchased from Watson's Food & Beverage Co., Ltd. (Guangzhou, China) and Sigma-Aldrich (St. Louis, MO, USA), respectively. Cellulose (50 U/mg) was purchased from Yuanye biotechnology Co., Ltd. (Shanghai, China). NaOH and HCl were of analytical grade and purchased from ANPEL laboratory technologies Co., Ltd. (Shanghai, China). The 0.22 μm membranes were purchased from ANPEL laboratory technologies Co., Ltd. (Shanghai, China).

2.3. Sample preparation

Each PMF reference compound was dissolved separately in methanol to achieve a concentration of 100 ng/mL. The dried citrus peel was finely ground in high-speed universal grinder (FW100, Tianjin Tester Instrument Co., China).

Free PMFs extract from dried citrus peel was prepared according to the previous study(Zheng et al., 2020) with slight modification. Briefly, a certain amount of dried citrus peel powder (0.1 g) and 5 mL of methanol were added to a 15 mL centrifuge tube. After homogenization under a vortometer for 30 s, the mixture was then ultrasonically extracted at 50 °C for 30 min with ultrasonic power at 360 W. By cooling to room temperature, the mixture was then centrifuged at 6000 r/min for 10 min. Finally, the supernatant was collected as free extract. The residue was dried at 40 °C for further extraction of bound PMFs.

Bound PMFs extract from dried citrus peel was prepared by using three hydrolysis methods (acid/alkaline/enzyme hydrolysis) combined with ultrasonic extraction (Wang et al., 2019). Briefly, the dried residue was hydrolyzed in an ultrasonic bath with ultrasonic power of 360 W under following conditions: (1) 10 mL 4 M or 10 M HCl solution was mixed with residue for acid hydrolysis at 80 °C for 30 min, corresponding to bound PMFs extract by low and high acid hydrolysis; (2) 10 mL 4 M or 10 M NaOH solution was mixed with residue for alkaline hydrolysis at 80 °C for 30 min, corresponding to bound PMFs extract by low and high base hydrolysis; (3) 10 mL 1 % or 30 % (*w*/w, cellulase/residue) cellulase aqueous solution was mixed with residue for enzyme hydrolysis at 50 °C for 75 min, corresponding to bound PMFs by low and high enzyme hydrolysis. Subsequently, after adjusting pH to 4 using 12 M HCl, the mixture was extracted with 3 × 20 mL of ethyl acetate, being centrifuged at 4000 rpm for 3 × 10 min after stirring at 1000 rpm for 3 × 10 min. The pooled extraction was concentrated with a rotary evaporator at 40 °C and reconstituted in 2 mL of methanol.

Prior to UPLC-QTOF-MS and UPLC-QTOF-MS/MS analysis, the free or bound PMFs extract was diluted ten-fold with methanol and filtered through 0.22 μm micropore film. Each standard and each sample solution were prepared in triplicates.

2.4. Determination of total flavonoid content

The total flavonoid content of both free and bound extracts was determined using the method in literature (Zhong et al., 2022) with some modifications. A 1.5 mL of sample solution was mixed with 150 μL of 5 % $NaNO_2$ solution (*w*/*v*). After 5 min, 150 μL of 10 % $Al(NO_3)_3$ solution (w/v) was added and allowed to stand for 6 min, subsequently 2 mL of 1 M NaOH and 1.2 mL of 75 % ethanol (*v*/v) were added to the mixture. Then the mixture was allowed to incubate at room temperature for another 10 min. The absorbance of reaction solution was measured at 510 nm using a UV–Vis spectrophotometer (Evolution200, Thermo fisher scientific, USA). Rutin (0–200 μg/mL) was used as the standard to obtain the quantification calibration curve (R^2 = 0.9976). The results were expressed as mg of rutin equivalent per 1 g dried citrus peel (mg RE/g DW). Data were reported as means±SD for three replications.

2.5. UPLC-MS and UPLC-MS/MS analysis

UPLC-MS and UPLC-MS/MS analysis were performed on an UPLC system coupled to X500R QTOF mass detector, with an electrospray ionization (ESI) source (AB Sciex, USA). Standards and samples were separated on ACQUITY UPLC HSS T3 column (2.1 mm × 100 mm, 1.8 μm, Waters Corp., USA) and eluted at a flow rate of 0.4 mL/min with eluent A of 0.1 % formic acid and eluent B of acetonitrile. Gradient elution was employed as follows: 0 min, 5 % B; 3 min, 15 % B; 8 min, 25 % B; 9 min, 30 % B; 17 min, 45 % B; 19 min, 60 % B; 20 min, 90 % B; 22

min, 90 % B; 24 min, 5 % B. The injection volume was 1 μL and the column temperature was operated at 40 °C for all cases. The ESI was operated under positive polarity using the following MS and MS/MS parameters: curtain gas of 30 psi, nebulizer gas of 55 psi, auxiliary gas of 55 psi, source temperature of 600 °C, declustering voltage of 80 V and ion spray voltage of 5500 V. The ions were monitored in TOF-MS and TOF-MS/MS mode, corresponding to UPLC-MS and UPLC-MS/MS analysis. In TOF-MS mode, the scan range was set at *m/z* 10–800. In TOF-MS/MS mode, the ions corresponding to protonated PMFs ($[M + H]^+$) were monitored at *m/z* 299.09, 301.07, 313.11, 315.09, 329.10, 331.08, 343.12, 345.10, 347.08, 359.11, 361.09, 373.13, 375.11, 377.09, 389.12, 391.10, 403.14, 405.12, 419.13, 433.15, with collision energy at 35 V and scan range of 50–500.

The mass accuracy of protonated PMFs and their fragment ions were set to 10 ppm. Fragment ions were assigned according to the literatures (Lei et al., 2017; Zhang et al., 2012). Identification of individual free and bound PMFs in samples was carried out through characteristics of retention time and fragment ions of PMFs deduced from PMF reference compounds.

2.6. Method validation

The reference compounds, including P4, P5, P7, P8, P10, P11 and P12, were used as standards for quantitative analysis. The calibration curves were plotted by the peak area of ion $[M + H]^+$ versus different concentrations, and limits of detection (LOD) and quantification (LOQ) were determined on the basis of signal-to-noise (S/N) ratios of 3 and 10, respectively, with results shown in Table S1.

3. Results and discussion

3.1. UPLC-QTOF-MS and UPLC-QTOF-MS/MS analysis of authentic compounds

High-resolution mass spectrometry and tandem mass spectrometry have been a useful technique in structural characterization (Yu et al.,

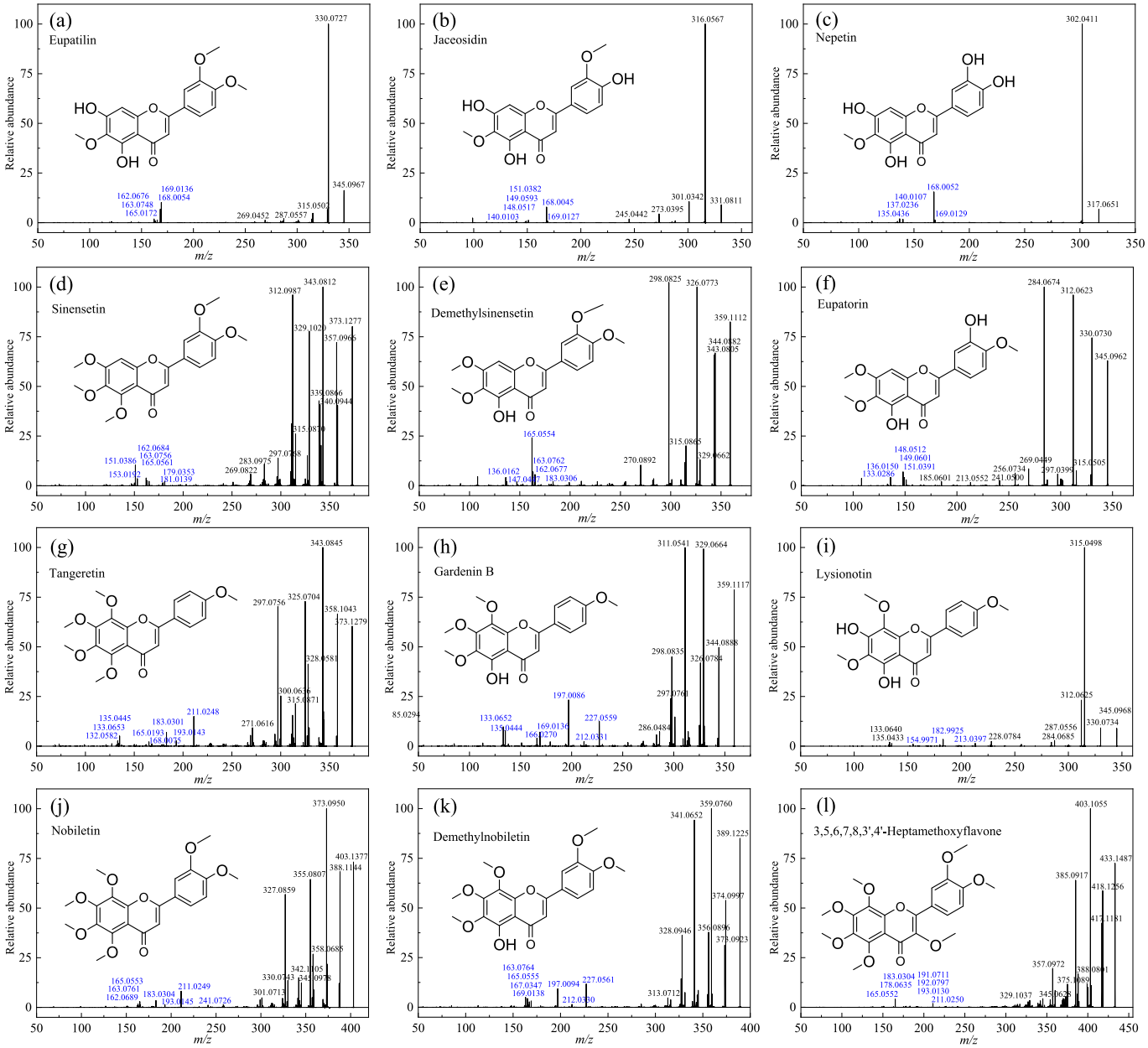

Fig. 1. MS/MS spectra of twelve PMF standards by QTOF-MS/MS.

2024; Yu et al., 2018) through accurate mass, isotopic pattern and fragmentation behaviour. In this study, to identify structures of free and bound PMFs in dried citrus peel, twelve PMFs standards were analyzed by UPLC-QTOF-MS and UPLC-QTOF-MS/MS analysis. Preliminary mass spectrometry analysis indicated that PMFs standards exhibited protonated ions ($[M + H]^+$) in TOF-MS spectrum, thus subsequently chosen as the precursor ion for further MS/MS analysis. Fig. 1&2, and Table S2 show the relative retention time, relative abundances of main fragment ions, as well as relative abundances of fingerprint fragment ions in range of *m/z* 50–240 for all PMFs standards. From Fig. 1&2, the following observations can be summarized for further characterization of other PMFs.

By using the standard compound tangeretin as a benchmark, the relative retention time (Table S2) revealed that, for PMFs with the same substituent positions (P1, P2, P3, P5 and P6; or P8 and P9), the more hydroxy groups there are, the stronger the polarity, thus the shorter the relative retention time in reversed-phase (RP) column. The exception was that 5-demethyl (5-OH) PMFs (P5, P8, P11) showed longer relative retention time than corresponding permethoxylated PMFs (P4, P7, P10), in accordance with literatures (Barberan et al., 1985; Li et al., 2006; Zheng et al., 2015). The explanation might be that the intramolecular hydrogen bond formed between 5-hydroxy and 4-carbonyl led to a decrease in polarity of PMFs (Goetz et al., 2014) and thus an increase in relative retention time in RP column.

Similar with EI-MS spectra of PMFs (Berahia et al., 1994; Kingston, 1971; Rizzi & Boeing, 1984), ESI-MS/MS spectra also showed two main dissociation pathways in PMFs (Wang et al., 2021; Wang & Zhang, 2009; Xing et al., 2017; Zhang et al., 2012) (Fig. 1&2, and Table S2). First, the protonated PMFs ($[M + H]^+$) could loss radical ($CH_3\cdot$, $CH_2\cdot$) or neutral molecules (CH_4, H_2O, CO), usually corresponding to the fragment ions with higher mass-charge ratio and higher abundance in MS/MS spectra. Second, the protonated PMFs could undergo retro-Diels-Alder (RDA) cleavage in 0,2- or 1,3-position of the C-ring (shown in Fig. 3), forming the ion $[^{0,2}B]^+$ and ^{1,3}A or ^{1,3}B(C) associated ions (^{1,3}B(C) refers to the

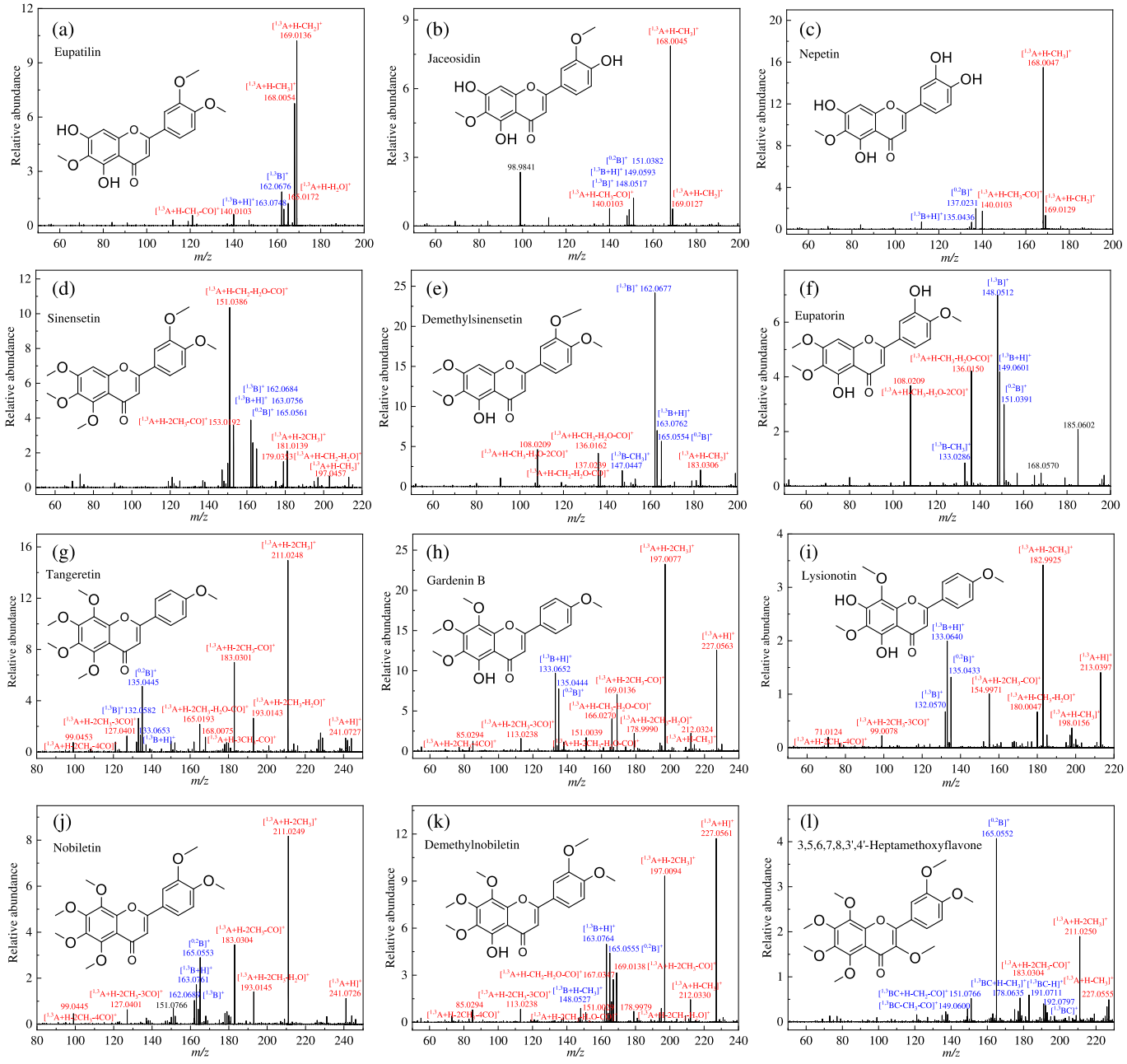

Fig. 2. Fingerprint fragment ions in range of *m/z* 50–240 for twelve PMF standards by QTOF-MS/MS.

structure containing B-ring with or without 3-substituent in C-ring), such as $[^{1,3}A + H\text{-}CH_3\cdot]^+$, $[^{1,3}A + H\text{-}H_2O]^+$, $[^{1,3}A + H\text{-}CH_3\cdot\text{-}CO]^+$, $[^{1,3}A + H\text{-}CH_3\cdot\text{-}H_2O\text{-}CO]^+$, $[^{1,3}B + H]^+$, $[^{1,3}B]^+$, $[^{1,3}B\text{-}CH_3\cdot]^+$, etc. Other ions such as $[^{1,3}BC\text{-}H]^+$, $[^{1,3}BC + H\text{-}CH_3\cdot]^+$, $[^{1,3}BC + H\text{-}CH_2\cdot\text{-}CO]^+$, and $[^{1,3}BC\text{-}CH_3\cdot\text{-}CO]^+$ ($^{1,3}BC$ refers to the structure containing B-ring and 3-substituent in C-ring), were observed in PMFs with 3-substituted C-ring (P12 in Fig. 2l). These RDA ions were of relative smaller intensity and could form the characteristic MS/MS fingerprint of PMFs, which could be used to identify substituents in A, B, C-ring in PMFs.

It was interesting that, with the exception of P5, the base peaks of $[M + H\text{-}CH_3\cdot]^+$ and $[M + H\text{-}2CH_3\cdot]^+$ were observed in PMFs with one methoxy and two or more methoxy groups in A-ring, respectively. It was reported that PMFs possessing 6-OCH_3 and/or 8-OCH_3 easily underwent demethylation to form stable quinoid ions (Kingston, 1971; Rizzi & Boeing, 1984), in conformity with low O—C bond dissociation enthalpy in 6-OCH_3 and 8-OCH_3 (Kleinová et al., 2024). Additionally, the base peaks of $[M + H\text{-}CH_3\cdot]^+$ (P1, P2, P3), $[M + H\text{-}2CH_3\cdot]^+$ (P4, P7, P8, P9, P10, P11, P12), $[M + H\text{-}CH_3\cdot\text{-}H_2O]^+$ (P6), or $[M + H\text{-}CH_3\cdot\text{-}H_2O\text{-}CO]^+$ (P4, P5, P6) were in accordance with the observed highest $^{1,3}A$ associated ion, that is $[^{1,3}A + H\text{-}CH_3\cdot]^+$ (or $[^{1,3}A + H\text{-}CH_2\cdot]^+$), $[^{1,3}A + H\text{-}2CH_3\cdot]^+$, $[^{1,3}A + H\text{-}CH_3\cdot\text{-}H_2O\text{-}CO]^+$ (or $[^{1,3}A + H\text{-}CH_2\cdot\text{-}H_2O\text{-}CO]^+$). The above phenomenon suggested that the protonated PMFs underwent RDA fragmentation prior to the radical/neutral loss, with the radical/neutral loss ($CH_3\cdot$, $CH_2\cdot$, H_2O, CO) mostly from A-ring, in consistent with previous reports on EI-MS of PMFs (Berahia et al., 1994; Kingston, 1971; Rizzi & Boeing, 1984). Besides, the most observed B-ring associated RDA ion were $[^{0,2}B]^+$, $[^{1,3}B + H]^+$, $[^{1,3}B]^+$, with negligible radical/neutral loss in $^{1,3}B$ associated ions, in conformity to the previous conclusion.

Moreover, as shown in Fig. 1(a, b, c, i), the radical/neutral loss ions from aforementioned first dissociation pathway were of less intensity and abundance in compounds (P1, P2, P3, P9) as being compared with other compounds. According to the structures of 12 reference compounds, it can be easily found again that the radical/neutral loss ions were closely related with substituents in A-ring, and the presence of ortho-methoxy substituents in A-ring was favorable to the radical/neutral loss from protonated PMFs. In addition, the presence of ortho-methoxy substituents in A-ring or B-ring was necessary for CH_4 loss through the absence of CH_4 loss in Fig. 1(b, c, i) (P2, P3, P9). The correlation between neutral loss of CH_4 and ortho-methoxy groups at B-ring was also reported previously (Wang & Zhang, 2009).

3.2. UPLC-QTOF-MS analysis of free and bound PMFs in dried peel of Citrus reticulata "Chachi"

The molecular weight of PMFs can be calculated by adding n × CH_2O (n, the number of methoxyl groups) and m × O (m, the number of hydroxyl groups) to the basic structure of flavone ($C_{15}H_{10}O_2$). Thus, the chemical formula and accurate mass of every possible PMFs in protonated form can be obtained as shown in Table S3. By screening the accurate masses in Table S3 with extracted ion chromatograms (EIC) from TOF-MS based total ion chromatogram spectra, 29 PMFs including 12 permethoxylated PMFs and 17 hydroxyl PMFs were detected in dried peel of *Citrus reticulata* "Chachi" as shown in Table S4, from which it can be found that high acid hydrolysis was the best method to obtain bound PMFs in citrus peel with larger number (13 PMFs) and higher content (shown in peak area) of PMFs than alkaline (8 PMFs) and enzyme (1 PMFs) hydrolysis. Additionally, there were 21 PMFs detected in free extract and 13 PMFs in bound extract. And five PMFs were detected in both free and bound extract, including four monohydroxy PMFs and one dihydroxy PMFs. Besides, 12 permethoxylated PMFs existed only in free extract, and bound extract only contained hydroxylated PMFs (five monohydroxy PMFs, four dihydroxy PMFs and four trihydroxy PMFs), in conformity with the attachment of bound PMFs via hydroxyl group with macromolecules (Wang et al., 2020). Moreover, mainly PMFs with five (12 PMFs) or six substituents (7 PMFs), and some little PMFs with three (1 PMFs), four (5 PMFs) and seven substituents (4 PMFs) were detected in dried peel of *Citrus reticulata* "Chachi".

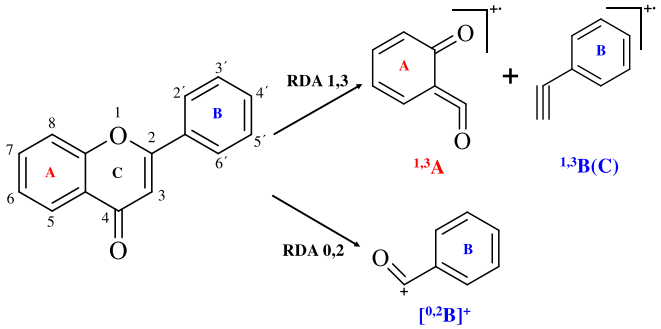

Fig. 3. RDA cleavage in 0,2- or 1,3-position of the C-ring for PMFs.

3.3. UPLC-QTOF-MS/MS analysis of free and bound PMFs in dried peel of Citrus reticulata "Chachi"

After screening the molecular masses through EIC-MS method, MS/MS analysis were performed on the detected protonated PMFs for further characterization of free and bound PMFs in citrus peel. The results were well in accordance with the dissociation pathways deduced from reference compounds. The radical/neutral loss and RDA cleavage were observed in all detected PMFs in dried citrus peel. The major radical/neutral loss ions of detected PMFs were designated in Table S5 through accurate mass. It can be found that, except for the radical/neutral loss of $CH_3\cdot$, $CH_2\cdot$, CH_4, H_2O, CO observed in reference compounds, the loss of HCO· could also be detected in PMFs, in agreement with the literature (Zhang et al., 2012).

Based on the numbers and types of substituent groups in A-, B-, and C-ring of PMFs, the chemical formula and accurate mass of all possible RDA ions can be obtained and shown in Table S6 in advance. It should be noted from Table S6 and Table S2 that the same charge-mass ratio integer can come from different RDA ions, such as *m/z* 165.0182 and *m/z* 165.0546 with the same integer 165, thus the accurate mass was necessary in characterizing PMFs. According to the accurate mass of RDA ions shown in Table S6, the major RDA ions ($^{1,3}A$ or $^{1,3}B(C)$ associated ions, and $[^{0,2}B]^+$) of detected PMFs in citrus peel are also designated in Table S5, through which the substituents in A-, B-, and C-ring of detected PMFs were obtained (shown in Table 1).

By comparing RDA ions and relative retention time between detected PMFs (Table S5) and reference compounds (Table S2), the compounds 8, 9, 11, 13, 21, 23, 26 in Table S5 were identified as sinensetin (P4), tangeretin (P7), demethylsinensetin (P5), gardenin B (P8), nobiletin (P10), demethylnobiletin (P11), and heptamethoxyflavone (P12), respectively. It was interesting that, different from other hydroxylated PMFs, 5-demethyl PMFs showed larger content in free extract than bound extract according to Table S4, which could be used along with relative retention time to distinguish 5-demethyl PMFs and other hydroxylated PMFs.

According to the distribution of substituents in Table 1, as well as the comparison between the neutral/radical loss and $^{1,3}A$ associated ions in Table S5 with those of reference compounds (Table S2), the compounds 4 and 6 could be tentatively identified as 5,6,7,4′-tetramethoxyflavone (similar with sinensetin (P4, 8) in A-ring) and 5-OH-6,7,4′-trimethoxyflavone (similar with demethylsinensetin (P5, 11) in A-ring), respectively; and the compound 12 might be from 3′ or 4′-demethylation from sinensetin (P4, 8). The compounds 5,6,7,4′-tetramethoxyflavone and 5-OH-6,7,4′-trimethoxyflavone were also isolated in sweet orange peel and characterized by MS, UV and NMR techniques in the previous report (Li et al., 2006). In addition, the compounds 19 and 20 both possessing 5,6,7,8-tetermethoxy in A-ring were different from nobiletin (P10, 21) in substituent positions of two methoxy groups. In B-ring of PMFs, 4′-methoxy or 3′,4′-dimethoxy were mostly observed (Li et al., 2006; Uckoo

Table 1
Structural identification of free and bound PMFs detected in dried peel of *Citrus reticulata* "Chachi" through UPLC-MS/MS analysis.

No.	PMFs	Relative t_R	Substituent in A ring	Substituent in B ring	Substituent in C ring	Characteristic RDA ions (*m/z*)
1	trimethoxyflavone	0.94	$2OCH_3$	OCH_3	–	181.0501, 135.0455, 133.0647
2	tetramethoxyflavone	0.86	$2OCH_3$	$2OCH_3$	–	167.0342, 165.0565
3	tetramethoxyflavone	0.89	$3OCH_3$	OCH_3	–	181.0128, 153.0181, 135.0440
4	tetramethoxyflavone	0.96	$3OCH_3$	OCH_3	–	181.0140, 150.0311, 135.0449, 133.0652
5	monohydroxytrimethoxyflavone	0.70	$2OCH_3 + OH$	OCH_3	–	182.0205, 164.0108, 136.0165, 135.0446, 108.0213
6	monohydroxytrimethoxyflavone	1.02	$2OCH_3 + OH$	OCH_3	–	136.0164, 135.0447, 133.0654
7	pentamethoxyflavone	0.81	$3OCH_3$	$2OCH_3$	–	181.0136, 165.0565, 153.0185
8	pentamethoxyflavone (P4)	0.92	$3OCH_3$	$2OCH_3$	–	181.0136, 165.0552, 162.0673, 151.0393
9	pentamethoxyflavone (P7)	1.00	$4OCH_3$	OCH_3	–	211.0243, 183.0299, 165.0198, 135.0452, 127.0405
10	monohydroxytetramethoxyflavone	0.80	$2OCH_3 + OH$	$2OCH_3$	–	183.0290, 165.0557, 163.0750, 162.0703
11	monohydroxytetramethoxyflavone (P5)	1.00	$2OCH_3 + OH$	$2OCH_3$	–	183.0290, 165.0551, 162.0675, 148.0526, 136.0155
12	monohydroxytetramethoxyflavone	1.01	$3OCH_3$	$OCH_3 + OH$	–	181.0126, 149.0597
13	monohydroxytetramethoxyflavone (P8)	1.04	$3OCH_3 + OH$	OCH_3	–	227.0554, 197.0088, 169.0133, 135.0441, 133.0647
14	dihydroxytrimethoxyflavone	0.91	$2OCH_3 + OH$	$OCH_3 + OH$	–	151.0376, 148.0517, 136.0153, 108.0207
15	dihydroxytrimethoxyflavone	0.94	$3OCH_3 + OH$	OH	–	227.0554, 197.0088, 169.0133, 121.0299
16	trihydroxydimethoxyflavone	0.68	$OCH_3 + 2OH$	$OCH_3 + OH$	–	168.0050, 151.0390,149.0606
17	trihydroxydimethoxyflavone	0.75	$OCH_3 + 2OH$	OCH_3	OH	147.0441, 135.0441
18	trihydroxydimethoxyflavone	0.76	$OCH_3 + 2OH$	$OCH_3 + OH$	–	168.0053, 151.0400
19	hexamethoxyflavone	0.86	$4OCH_3$	$2OCH_3$	–	165.0541
20	hexamethoxyflavone	0.94	$4OCH_3$	$2OCH_3$	–	165.0540
21	hexamethoxyflavone (P10)	0.96	$4OCH_3$	$2OCH_3$	–	241.0727, 211.0245, 183.0300, 165.0558, 127.0403
22	hexamethoxyflavone	1.02	$4OCH_3$	OCH_3	OCH_3	211.0238, 161.0597, 135.0442, 121.0657
23	monohydroxypentamethoxyflavone (P11)	1.02	$3OCH_3 + OH$	$2OCH_3$	–	227.0554, 197.0081, 165.0551, 163.0756
24	dihydroxytetramethoxyflavone	0.97	$3OCH_3 + OH$	$OCH_3 + OH$	–	197.0073, 151.0370, 149.0613
25	trihydroxytrimethoxyflavone	0.76	$2OCH_3 + 2OH$	$OCH_3 + OH$	–	213.0402, 182.9924, 151.0400, 149.0586
26	heptamethoxyflavone (P12)	0.99	$4OCH_3$	$2OCH_3$	OCH_3	211.0241, 191.0710, 165.0551, 151.0763, 149.0597
27	monohydroxyhexamethoxyflavone	1.00	$3OCH_3 + OH$	$2OCH_3$	OCH_3	227.0556, 165.0550, 151.0785, 149.0597
28	monohydroxyhexamethoxyflavone	1.03	$3OCH_3 + OH$	$2OCH_3$	OCH_3	227.0553, 165.0549, 151.0774
29	trihydroxytetramethoxyflavone	0.96	$3OCH_3 + OH$	$OCH_3 + OH$	OH	227.0544, 151.0397, 149.0236

Relative t_R, the relative retention time of compounds by using the retention time of tangeretin as a benchmark. "——" means no substituent in C-3 at C-ring.

et al., 2012). PMFs with B ring of 2′,5′-dimethoxy (3,6,7,8,2′,5′-hexamethoxyflavone) was also isolated from Valencia orange peel and identified using NMR technique (Lee et al., 2023).

By considering the retention time and free/bound content in Table S4, as well as distribution of substituents in Table 1, the compounds 5 and 10 could not be 5-OH PMF since they showed shorter retention time than corresponding permethoxylated PMFs, and larger content in bound extract than free extract; while the compound 28 could be tentatively identified as 5-OH-3,6,7,8,3′,4′-hexamethoxyflavone through longer retention time than heptamethoxyflavone (P12, 26), as well as larger content in free extract than bound extract. The compound 27 was different from 28 in position of hydroxy in A-ring and might be 7-OH-3,5,6,8,3′,4′-hexamethoxyflavone since C-7 was another commonly observed demethylation site in A ring (Uckoo et al., 2012; Wang et al., 2021; Burapan et al., 2017; Zeng et al., 2017; J. Zheng et al., 2015). Besides, 5-OH-3,6,7,8,3′,4′-hexamethoxyflavone and 7-OH-3,5,6,8,3′,4′-hexamethoxyflavone were also isolated in citrus previously (Uckoo et al., 2012).

Additionally, according to the distribution of substituents in Table 1, as well as reported PMFs isolated from citrus (Uckoo et al., 2012), the compound 3 might be 5,7,8,4′-tetramethoxyflavone; the compound 7 might be 5,7,8,3′,4′-pentamethoxyflavone, being different from sinensetin (P4, 8) in three methoxy positions in A-ring; and the compound 22 could be tentatively identified as 3,5,6,7,8,4′-hexamethoxyflavone. The compound 17 was characterized to contain 3-OH and one methoxy in B-ring. The 3-OH PMFs were also commonly observed in citrus (Li et al., 2006; Liu et al., 2012; Uckoo et al., 2012). The compounds 14 and 15 might be from twice demethylation from sinensetin (P4, 8) and tangeretin (P7, 9), respectively; and the compounds 16 and 18 might be from triple demethylation from compound 7 or 8. The compounds 24 and 25 were probably from twice and triple demethylation from nobiletin (P10, 21), conforming to the aforementioned phenomenon in relative retention time that PMFs with larger number of hydroxy groups showed shorter relative retention time. The metabolites of nobiletin in mouse urine were reported to contain OH-PMFs with demethylation at 4′-, 3′,4′-, 3′-, 5,3′-, 5,4′-, and 5,3′,4′-position (Zheng et al., 2015). The compound 29 was probably from triple demethylation from heptamethoxyflavone (P12, 26). It was reported that PMF demethylation during metabolism generally followed the order C-7 > C-4′ ≈ C-3′ > C-5 > C-3 (Burapan et al., 2017).

Overall, just like permethoxylated PMFs, 5-OH PMFs (6,11,13,23,28) mainly existed in free extract; while di/trihydroxy PMFs detected were mainly in bound extract, and probably mainly from demethylation from the main and most studied permethoxylated PMFs, such as sinensetin (P4, 8), tangeretin (P7, 9), nobiletin (P10, 21) and heptamethoxyflavone (P12, 26). Moreover, larger content was favorable to the observation of abundant RDA ions.

3.4. Determination of seven PMFs and total flavonoids in free and bound extract in dried peel of Citrus reticulata "Chachi"

The total flavonoids determined by UV spectrometry (Table S7) also indicated that free extract showed much larger flavonoids than bound extract in dried citrus peel, and high acid hydrolysis was the best method to obtain bound flavonoids. Following the identification of seven PMFs through reference compounds, the contents of these seven PMFs in free and bound extract (high acid hydrolysis) were determined (shown in Table 2) according to Table S1. It could be found from Table 2 that PMFs were mainly present in free form and of negligible content in bound form in dried citrus peel. Additionally, permethoxylated PMFs showed much larger content than corresponding 5-OH PMFs, in accordance with the previous reports (Fu et al., 2017; Li et al., 2006). Besides, the most abundant permethoxylated PMFs (tangeretin and nobiletin) were in agreement with the characteristic indexes of dried peel of *Citrus reticulata* "Chachi" in Chinese Pharmacopoeia (2020 edition).

4. Conclusion

In sum, the protonated PMFs could undergo radical/neutral loss and RDA cleavage in 0,2- or 1,3-position of the C-ring in QTOF-MS/MS

Table 2
Contents of seven PMFs in free and bound extracts in dried peel of *Citrus reticulata* "Chachi" (mg/g dry sample).

No.	PMFs	Free content	Bound content
8	pentamethoxyflavone (sinensetin)	0.065 ± 0.009	nd
9	pentamethoxyflavone (tangeretin)	0.69 ± 0.05	nd
11	monohydroxytetramethoxyflavone (demethylsinensetin)	0.0017 ± 0.0002	0.0002 ± 0.0001
13	monohydroxytetramethoxyflavone (demethyltangeretin)	0.0047 ± 0.0012	0.0005 ± 0.0001
21	hexamethoxyflavone (nobiletin)	1.12 ± 0.08	nd
23	monohydroxypentamethoxyflavone (demethylnobiletin)	0.058 ± 0.003	nd
26	heptamethoxyflavone	0.12 ± 0.01	nd

analysis. The radical/neutral loss ions were closely related with substituents in A-ring, while RDA ions formed the characteristic MS/MS fingerprint of PMFs. The combination of fingerprint RDA ions and RP column retention was further used to identify substituents in A, B, C-ring in detected 21 free PMFs and 13 bound PMFs isolated from dried peels of *Citrus reticulata* "Chachi". It was found that high acid hydrolysis was the best method to obtain bound PMFs in dried citrus peels of *Citrus reticulata* "Chachi"; permethoxylated PMFs were only detected in free form, 5-OH PMFs mainly existed in free form, while di/trihydroxy PMFs were mainly in bound form. The presented strategy could be employed as an effective method to characterize PMFs from other botanics and metabolites.

CRediT authorship contribution statement

Xiangying Yu: Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Difan Li:** Methodology, Investigation. **Yahui Yu:** Writing – review & editing. **Longqing Li:** Writing – review & editing. **Mingyu Jin:** Writing – review & editing. **Jing-Kun Yan:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2025.142831.

Data availability

Data will be made available on request.

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