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Polymethoxylated Flavone Extracts from Citrus Peels for Use in the Functional Food and Nutraceutical Industry

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Abstract Polymethoxylated flavones (PMFs) extracted from *Citrus sinensis* 'Jincheng' peel were characterized by chromatographic and spectroscopic techniques. Seven individual PMF were identified. 3, 3', 4', 5, 6, 7-hexamethoxyflavone (HEX), nobiletin (NOB), heptamethoxyflavone (HEP), 5-demethylnobiletin (DN), and tangeretin (TAN) were characterized through electrospray ionization mass spectrometry (ESI-MS) in positive mode of protonated molecular ions $[M+H]^+$, the diagnostic fragment ions, together with the UV-Vis spectra and high performance liquid chromatography (HPLC) elution order from literature data. Sinensetin (SIN) and tetramethyl-*O*-scutellarein (SCU) were isolated and identified through their MS, ^{1}H nuclear magnetic resonance (NMR) and UV-Vis spectral studies. The levels of PMFs in peels from different cultivars of citrus fruits grown in China were determined for the first time. The results showed that *C. aurantium* 'Bitter orange' peel was the most promising variety for HEP. *C. sinensis* peel was a good source for SIN and SCU.

Keywords: citrus, peel, polymethoxylated flavone, spectrometry, chromatography

Introduction

With the large amount of citrus being processed into juice, a considerable by-product has evolved to utilize the residual peels, membranes, seeds, and other compounds. The peel of citrus fruits is considered an important source of polymethoxylated flavones (PMFs) of higher concentration than in other plants (1). The composition of PMFs can be significantly different between different citrus cultivars with the peels being a rich source of flavones with PMFs as major constituents (2,3). Ortuno *et al*. (4) reported PMFs content in different tissues of tangelo Nova mature fruits. The result showed that PMFs level in peels (71.7 mg/100 g d.w.) was significant higher than whole fruit (15.5 mg/100 g d.w.), and was not detected in the pulp. Mouly *et al*. (5) investigated PMFs level in citrus juice. Tangor juice showed PMFs content of 13.8 mg/L, but Florida orange and mixture of orange-tangor juices contained less PMFs than tangor juice. Thus the peel can be a significant source of PMFs in citrus crops. PMFs are of particular interest due to their documented broad spectrum of biological activity with nobiletin (NOB) and tangeretin (TAN) the most studied PMFs. TAN and NOB seem to be cytotoxic towards cancer cells, which are believed to be potential anti-tumor promoting agents (6). NOB can inhibit the proliferation of human prostate cancer cells, as well as the skin, breast, and colon carcinoma cell lines (7). NOB is also known to decrease both the rate of erythrocyte aggregation and blood cell sedimentation in humans, and TAN was shown to inhibit the development of HL-60 cells, which are implicated in leukemia (8). NOB and TAN have anti-viral and antimicrobial capacity, which confer a certain degree of resistance against microbial infections in citrus (4,9-11). 5-Demethylnobiletin (DN) shows strong inhibitory activities against proliferation and also induces apoptosis of HL-60 cell lines (12). Heptamethoxyflavone (HEP) may inhibit lipopolysaccharide (LPS)-induced monocyte expression of tumor necrosis factor macrophage inflammatory protein production (13). HEP was also shown to prevent the proliferation of different human cancer cells and induced HL-60 differentiation (14,15).

Due to the importance of flavonoids of citrus fruits and products as contributors of beneficial health effects, the identification or structural determination of such bioactive compounds plays an important role in many areas of science. PMFs from *Citrus* cultivars (e.g. *Citrus aurantium*, *Citrus sinensis*) and citrus juices have been confirmed by ultraviolet (UV), infrared (IR), mass spectrometry (MS), ^{1}H nuclear magnetic resonance (NMR), and ^{13}C NMR (5, 16-21). *Citrus* species have been cultivated in China for at least 1,700 years and the current citrus acreage and production have made China the world leader. However, there are very few studies in China about citrus PMFs among locally grown cultivars and varieties. *Jinchen* is a native sweet crop of *C. sinensis* Osbeck, which originated from Sichuan province and has been widely planted in the middle & upper regions of the Yangtze River. It possesses important economic value to the citrus industry in China, but considerable residual organic compounds resulting from processing (5,000 kton/year), presents an adverse environmental impact. Residue peels from citrus juice production are a source of pectin, essences, pectin, limonoids, and flavonoids.

The purpose of this study was to isolate and characterize PMFs in *C. sinensis* 'Jincheng' peel by different chromatographic and spectroscopic techniques. The levels of PMFs in peel from different cultivars of citrus fruits

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grown in the middle & upper regions of the Yangtze River in China were also compared.

Materials and Methods

Plant materials The fresh citrus peel samples were collected in November 2008 from the Wangchunhua Citrus Valley Co., Ltd., Songzi, Hubei. There were 9 *Citrus* varieties: *C. sinensis* Osberk ('Jincheng', 'Hamlin', 'Blood orange'), *C. unshiu* Marc ('Owari satuma', 'Miyagawa Wase', 'Guoqing No.1'), *C. paradisi* Macf ('Red grapefruit', 'White grapefruit'), and *C. aurantium* Linn ('Bitter orange').

Chemicals Standard compounds of nobiletin (NOB, 3', 4', 5, 6, 7, 8-hetramethoxyflavone), tangeretin (TAN, 4', 5, 6, 7, 8-pentamethoxyflavone), and baicaletin (BAI, 5, 6, 7-trihydroxyflavone) dissolved in methanol were purchased from Shanxi Huike Botanical Development Co., Ltd. (Xi'an, China). Methanol and acetonitrile (HPLC grade) were purchased from Fisher Chemical Co. (Waltham, MA, USA). Cellulase was purchased from Shanghai Dongfeng Biochemical Technology Co., Ltd. (Shanghai, China). Ultrapure water (Milli-Q) was used. High performance liquid chromatography (HPLC) and semipreparative HPLC (Waters, Milford, MA, USA). Liquid chromatography (LC)-MS (Agilent, Santa Clara, CA, USA). Other chemicals were (analytical grade) from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

Extraction and purification of PMFs Citrus peels were oven-dried to constant weight at 40°C and milled into a powder (particle size 0.425 mm). Approximately 100 g of each powder was extracted exhaustively by means of enzymatic hydrolysis using 95% ethanol (1,500 mL) and 5% cellulase (15 μ/mg; μ/mg is the number of enzyme units/mL divided by the concentration of protein in mg/mL) at 60°C for 2.5 hr. The ethanol extracts were concentrated and treated with diethyl ether (200 mL×3) as long as appreciable components were extracted. The combined diethyl ether extracts were washed with 0.4% NaOH solution until the aqueous fractions were colorless. The diethyl ether layers were collected, concentrated, and freeze-dried to yield PMFs mixture.

HPLC analysis Quantitative analysis was performed on a Waters HPLC coupled with a photodiode-array (PDA) detector set at 330 nm. About 1 mg of PMFs from each variety was dissolved in 10 mL of methanol and filtered through a 0.45-μm nylon membrane before analysis. A Hypersil ODS C18 (250×4.6 mm) (Elite, Dalian, China) column was used and the mobile phase consisted of H_2O-acetic acid (100:2, v/v) (A) and acetonitrile (B). The gradient profile was 50% (B) in 25 min. The column was equilibrated for 15 min prior to each analysis. The flow rate was 0.6 mL/min and the injection volume was 20 μL. The UV spectra were taken in the region of 200-400 nm. The quantities of NOB and TAN were determined from the area calculated by the integrator using the response factor of the corresponding standards. The other PMFs were determined using a semi-quantitative method by adding a known weight of flavone (BAI) as internal standard.

LC-MS identification LC-MS identifications of PMFs were performed on a system consisting of ion trap mass spectrometer with an electrospray ionization (ESI) interface and LC system (Agilent 1100 series LC/MSD Trap). ESI experiments were carried out in the positive mode. Separation was performed using a ZORBAX SB-C18 5 μm (150×2.1 mm) (Agilent) column. The column was equilibrated for 7 min prior to each analysis. Five μL of samples and standards solution (NOB and TAN mixture) were injected and separated under the same HPLC gradient program mentioned above. Flow rate was 0.25 mL/min and injection volumn was 10 μL. Drying N_2 was heated to 150°C and introduced to the capillary region at a flow rate of 8 mL/min. The pressure of nebulizing N_2 was set at 30 psi. The capillary temperature was kept at 250°C and the mass range measured was 100-1,00 *m/z*.

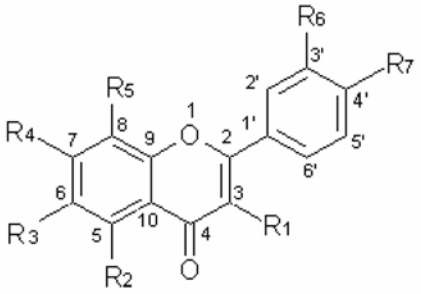
Isolation and NMR analysis The semipreparative HPLC (Waters 600E-2487) was performed with a UV-Vis detector set at 330 nm. The PMFs mixture of *C. sinensis* 'Jincheng' peel was dissolved in acetonitrile and separated with a Phenomsil Prep-ODS C18 (250×10 mm) (Tianjin, China) column. Elution was carried out using 45% acetonitrile in 1.5% acetic acid. The column temperature was 35°C and the injection volume 200 μL. The flow rate was fixed at 5 mL/min. Fractions were collected according to the corresponding retention times of peaks. The fractions corresponding to compounds 1 and 4 were concentrated, cooled to recrystallize for analysis.

Data statistics All experiments were performed at least 3 times and the statistic analysis was carried out by Origin 7.0. The quantitative values were expressed as mean± standard deviation (SD).

Results and Discussion

Identification of PMFs from *C. sinensis* 'Jincheng' peels LC-MS was used to determine the identity and proportions of the individual PMFs. With the advent of atmospheric pressure ionization techniques, ESI and atmospheric pressure chemical ionization (APCI), MS has become a powerful analysis tool in phytochemistry due to its sensitivity, rapidity, and low levels of sample consumption.

The structural identification of individual PMFs compound 1-7 are shown in Fig. 1 and Table 1 through the spectroscopic analyses with MS and ^{1}H NMR through comparison with reported literature. The data corresponded well with literature studies (18-23). The literature data is from analysis of citrus oil, juice, and peels. Citrus peels contain higher levels of PMFs than juice and pulp. ESI-MS was operated in positive mode to characterize PMFs through their specific radical cations by collision-induced dissociation (CID). The chromatogram and mass spectra of PMFs are shown in Fig. 2 and 3, respectively. The flavones were transferred to the mass spectrometer by positive ESI, resulting in the formation of protonated flavones. The positive ESI mass spectra showed only the molecular ions, so the molecular weights of these flavones were confirmed. The structures were characterized by CID to generate the



Sinensetin: R_1=R_5=H, R_2=R_3=R_4=R_6=R_7=OMe
Nobiletin: R_1= H, R_2=R_3=R_4= R_5=R_6=R_7=OMe
Tangeretin: R_1=R_6= H, R_2=R_3=R_4= R_5 =R_7=OMe
Tetramethyl-o-scutellarein: R_1=R_3=R_6=H, R_2=R_4=R_5=R_7=OMe
Heptamethoxyflavone: R_1=R_2=R_3=R_4= R_5=R_6=R_7=OMe
Hexamethoxyflavone: R_5=H, R_1=R_2=R_3=R_4=R_6=R_7=OMe
5-Demethylnobiletin: R_1=H, R_2=OH, R_3=R_4=R_5=R_6=R_7=OMe

Fig. 1. Chemical structure of polymethoxylated flavones (PMFs).

fragmentation patterns of the protonated flavones, as summarized in Table 1 and Fig. 3. Upon the stage of collisional activated dissociation, the protonated flavones dissociate predominantly via loss of a methyl radical ($\bullet CH_3$) and form the radical cation $[M+H-15]^+$ as base peak (24). This type of fragmentation pathway is highly characteristic of methoxylated flavones but is insufficient for total structural characterization. At the same time, other main fragments, corresponding to the loss molecular weights of 30 ($2CH_3\bullet$), 28 (CO), 33 ($H_2O+CH_3\bullet$), 46 ($CO+H_2O$), 48 ($2CH_3\bullet+H_2O$), 44 (CO_2), and 61 ($CO+H_2O+CH_3\bullet$), from the protonated molecule were also observed. As reported by Zhou *et al.* (19), the fragments of $[M+H-n\times15]^+$ produced by loss of one or more methyl groups from the protonated molecule, as well as $[M+H-28]^+$, $[M+H-33]^+$, $[M+H-43]^+$, $[M+H-46]^+$, and $[M+H-61]^+$ fragment ions were diagnostic for the polymethoxylated species. The mechanism of fragmentation pattern needs to be further studied. In full scan mass spectra, compound 1 and 7, 2 and 3 expressed the identical molecular ions $[M+H]^+$ at *m/z* 373 and 403, which is representative of pentamethoxyflavone and hexamethoxyflavone, respectively. Because of the identical molecular weights, a mixed solution of the 2 standards (TAN and NOB) was injected into the LC-MS as reference, and peak 7 was found to be consistent with that of TAN, and peak 3 with NOB.

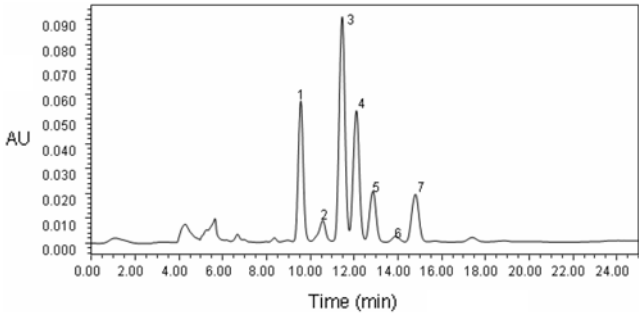


Fig. 2. HPLC profile of PMFs from *C. sinensis* 'Jincheng' peel. 1, SIN; 2, HEX; 3, NOB; 4, SCU; 5, HEP; 6, DN; 7, TAN.

In addition, methoxylation patterns of compound 1 and 4 were determined from their ^{1}H NMR spectra (Table 2). Spectra of compound 1 and 4 had 1 A-ring aromatic proton resonance, suggesting 3 methoxylation substitution at A-ring. Spectra of the B-ring of compound 1 indicated a pattern of 3 protons because of the ABX type aromatic proton signals. The size of the coupling constant (J=2.0 and 6.4 Hz) is characteristic of *meta* and *ortho* coupling as found in 3', 4' -methoxyflavone. Compound 4 had a pair of 2-protons, showing the presence of an A_2B_2 pattern in the B-ring of the spectra. It is typical of *para*-substituted benzene ring, *ortho*-coupled doublets arising from 2 pairs of degenerated protons (H-2', 6' and H-3', 5'). ^{1}H NMR spectra for compound 1 and 4 showed 5 and 4 methoxyl signals, respectively. The location of methoxyl groups for compound 1 and 4 confirmed the absence of any other hydrogen bearing substitutions. Thus, compound 1 and 4 were identified as sinensetin (SIN) and tetramethyl-*O*-scutellarein (SCU) according to their MS, ^{1}H NMR, and UV-Vis spectra. This is consistent with comparison with reported data (25-27). Compound 2, 5, and 6 were characterized as 3, 3', 4', 5, 6, 7-hexamethoxyflavone (HEX), HEP, and DN through comparison to the MS and UV-Vis spectra with reported data (4,18,19,23).

PMFs analysis of different cultivars There are few literature studies that have determined the levels of PMFs in citrus peels. This study represents the first time that PMFs have been characterized in detail in different citrus cultivars grown in China, among which *C. sinensis* 'Jincheng' and *C. unshiu* 'Guoqing NO.1' are native varieties in China. Table 3 shows the levels of PMFs found in the mature fruit of different cultivars according to their response factors by HPLC analysis. *C. aurantium* 'Bitter

Table 1. The molecular weights and structural identification of PMFs

Peak no.	t_R (min)	$[M+H]^+$ (*m/z*)	MS/MS (*m/z*)	UV Peak (λ/nm)	Identification
1	9.56	373	358, 343, 329, 327, 312	239, 271, 331	SIN
2	10.57	403	387, 373, 355, 339, 327	247, 336	HEX
3	11.45	403	388, 373, 357, 327	248, 271, 331	NOB
4	12.10	343	327, 313, 297, 282	266, 320	SCU
5	12.86	433	418, 403, 385	254, ~[1)]270, 342	HEP
6	13.92	389	374, 359, 356, 341, 327	253, 273, 345	DN
7	14.80	373	358, 343, 327, 312	232, 271, 327	TAN

[1)]Shoulder peak.

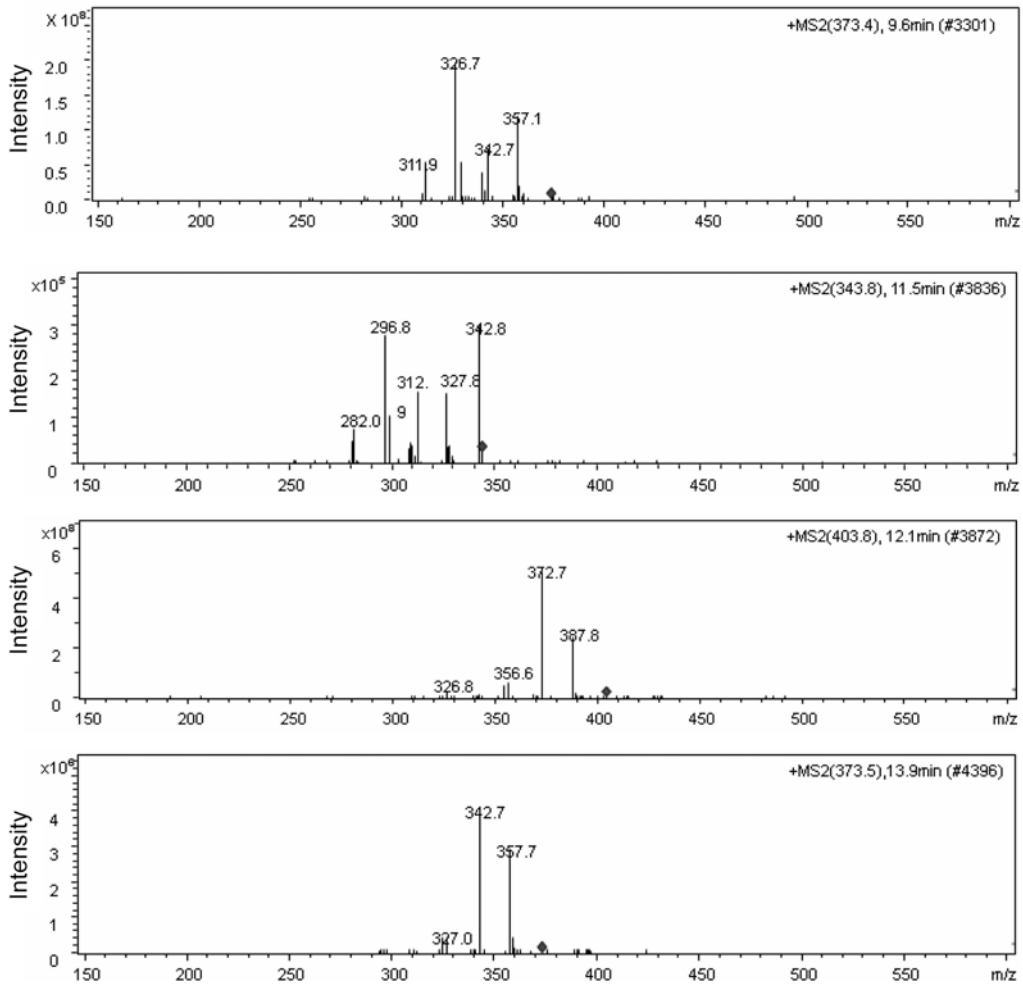


Fig. 3. MS/MS spectra of compound 1, 3, 4, 7.

orange', though not a commercial fruit in China due to its sourness, in examined cultivars, was shown to be a good source for NOB and TAN. The highest levels of NOB were detected in *C. aurantium* 'Bitter orange' (63.81±0.09 mg/100 g d.w.), followed by *C. unshiu* 'Owari satuma' (40.18±0.15 mg/100 g d.w.), *C. sinensis* of 'Blood orange' (29.02±0.18 mg/100 g d.w'), 'Hamlin' (24.94±0.22 mg/100 g d.w.), and 'Jincheng' (20.56±0.18 mg/100 g d.w.), respectively. The lowest level of NOB occurred in *C. paradisi* grapefruit (8.7±0.20 mg/100 g d.w.). The levels of TAN in these tested cultivars were similar to NOB. Among the cultivars examined, the 3 varieties of *C. unshiu* were the richest in HEP. 'Owari satuma' showed the highest level of HEP (16.77±0.12 mg/100 g d.w.), followed by levels of 25.33±0.14 mg/100 g d.w. in 'Miyagawa Wase' and 21.88±0.13 mg/100 g d.w. in 'Guoqing NO.1'. *C. sinensis* contained PMFs with 7 different structures, with SCU the dominant PMF (12.63-22.93 mg/100 g d.w.). 'Blood orange' variety showed the highest level of SCU (22.93±0.28 mg/100 g d.w.), followed by 'Hamlin' (15.33±0.15 mg/100 g d.w.) and 'Jincheng' (12.63±0.18 mg/100 g d.w.), respectively. Moreover, SIN was detected at a much higher level in *C. sinensis* (11.07-18.49 mg/100 g d.w.) than *C. aurantium* 'Bitter orange' (1.62±0.05 mg/100 g d.w.) and was not detected in the other cultivars, which was consistent with reported data from Lu *et al.* (20) and Ortuno *et al.* (28). HEX and DN were detected in *C. sinensis*, but the levels were low. Similarly, Ortuno *et al.* (28) also reported that *C. sinensis* was a good source for PMFs compared to grapefruit in terms of composition and concentration of PMFs. The results in this study are in

Table 2. ^{1}H NMR chemical shifts (δH in $CDCl_3$) of compound 1 and 4

H	SIN[1)]	SCU[1)]
3	6.61 s	6.76
8	6.80 s	6.82
2'	7.33 d (1.9)	7.84 d
5'	6.97 d (8.4)	7.03 d
6'	7.51 dd (2.0, 6.4)	7.87 d
3'		7.01 d (8.8)
5 (OCH_3)	4.01 s	3.99 s
6 (OCH_3)	3.93 s	3.93 s
7 (OCH_3)	3.99 s	3.89 s
3' (OCH_3)	4.00 s	
4' (OCH_3)	3.97 s	3.89 s

[1)]Chemical shift values are in ppm and *J* values in parentheses (Hz). s, singlets; d, doublets.

Table 3. Levels of individual PMFs in peels of *Citrus* cultivars grown in China

Scientific name	Local name	NOB	TAN	HEP	SCU	SIN	HEX	DN
C. sinensis Osberk	Jinchen	20.56±0.18[1)]	9.82±0.25	5.20±0.14	12.63±0.18	11.07±0.11	2.31±0.16	0.67±0.04
C. sinensis Osberk	Hamlin	24.94±0.22	11.22±0.21	5.74±0.13	15.33±0.15	13.04±0.35	2.20±0.09	0.99±0.15
C. sinensis Osberk	Blood orange	29.02±0.18	13.19±0.05	7.89±0.10	22.93±0.28	18.49±0.14	3.75±0.10	1.17±0.12
C. aurantium Linn	Bitter orange	63.81±0.09	63.69±0.08	16.77±0.12	5.60±0.10	1.62±0.05		
C. paradisi Macf	Red grapefruit	8.90±0.11	8.23±0.07	3.96±0.09				
C. paradisi Macf	White grapefruit	8.56±0.11	7.92±0.13	3.69±0.13	0.78±0.18			
C. unshiu Marc	Owari satuma	40.18±0.15	24.30±0.13	48.69±0.38	2.20±0.16			
C. unshiu Marc	Miyagawa Wase	17.83±0.08	13.73±0.11	25.33±0.14	1.03±0.12			
C. unshiu Marc	Guoqing NO.1	11.78±0.16	9.75±0.16	21.88±0.13	0.56±0.08			

[1)]Data represent mean±SD (n=3) (mg/100 g d.w.).

agreement with the reported data obtained for other *Citrus* cultivars, in which PMFs levels were dependent on the cultivar (20,23,28,29).

As secondary metabolites, composition and concentration data of citrus PMFs can provide important characteristics of each *Citrus* cultivar, which can be employed to standardize citrus quality and provide a valuable fingerprint of different cultivars. Taking into consideration the potential for industrial and pharmacological applications, the results outlined show *C. aurantium* 'Bitter orange' to be the most valuable citrus variety for isolating NOB and TAN, while *C. unshiu* 'Owari satuma' is the most appropriate variety for HEP. *C. sinensis* contains 7 individual PMFs, with significant levels of SIN and SCU. HEX and DN were only detected in *C. sinensis*, but the levels were low.

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