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Simultaneous determination of four 5-hydroxy polymethoxyflavones by reversed-phase high performance liquid chromatography with electrochemical detection

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ABSTRACT

Accumulating evidence has suggested the potential health-promoting effects of 5-hydroxy polymethoxyflavones (5-OH-PMFs) naturally existing in citrus genus. However, research efforts are hampered by the lack of reliable and sensitive methods for their determination in plant materials and biological samples. Using reversed-phase high performance liquid chromatography (HPLC) equipped with electrochemical (EC) detection, we have developed a fast and highly sensitive method for quantification of four 5-OH-PMFs, namely 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone, 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone, 5-hydroxy-6,7,4′-trimethoxyflavone, and 5-hydroxy-6,7,8,4′-tetramethoxyflavone. The method was fully validated in terms of linearity, accuracy and precision. The limit of detection (LOD) was determined as being between 0.65 and 1.8 ng/mL (ppb), demonstrating an over 160 times higher sensitivity in comparison with the previously reported method using UV detection. The recovery rate of the method was between 96.17% and 110.82%, and the precision for the retention times and peak areas was all below 13%. The method was successfully used to quantify 5-OH-PMFs with a wide range of abundance in the citrus products and preparations, such as orange juice, citrus peel, and dried tangerine peel. The quantification method for 5-OH-PMFs developed herein could be useful for the nutritional and pharmacological studies of these compounds in future.

1. Introduction

Polymethoxyflavones (PMFs) are almost exclusively found in the citrus genus, particularly in the peels of sweet oranges (*Citrus sinensis*) and mandarin oranges (*Citrus reticulata*) [1]. Currently, more than 20 PMFs have been isolated and identified from different parts of citrus plants [1]. They exhibited a broad spectrum of biological activities, including anti-inflammatory [2,3], anti-carcinogenic [4,5], anti-atherogenic [6,7], antiviral and antioxidative [8,9] ones.

Hydroxylated polymethoxyflavones (OH-PMFs) are less abundant PMFs in comparison with permethoxylated PMFs in citrus peels [1]. OH-PMFs can be formed from their permethoxylated counterparts by auto-hydrolysis during long-term storage [10]. Recently, more attention has been focused on OH-PMFs, because accumulating evidence has suggested that OH-PMFs have much stronger health-promoting biological activities compared with their permethoxylated counterparts. For example, 5-hydroxy polymethoxyflavones (5-OH-PMFs) exhibited greater potencies in anti-carcinogenic and anti-inflammatory effects [10–12].

In order to facilitate research on biological activities of 5-OH-PMFs, a reliable and sensitive quantification method is needed. There are a few studies on the determination of permethoxylated PMFs by HPLC combined with mass spectrometry (MS) or ultraviolet (UV) detector [13,14]. Recently, Wang et al. developed a validated reversed-phase LC method with UV detector for quantitative analysis of permethoxylated PMFs in citrus peel extracts [15]. Based on this method, we have developed a quantification method for 5-OH-PMFs using HPLC with UV detection [16]. However, this method suffered from low sensitivity, which makes it impossible to detect trace amount of 5-OH-PMFs in certain citrus preparations and biological samples.

Electrochemical (EC) detection is a superior method for the quantification of phenolic compounds, in terms of sensitivity and selectivity [17]. In this report, we utilized EC detection to improve our previously reported method, and developed a simple, sensitive and rapid analytical method

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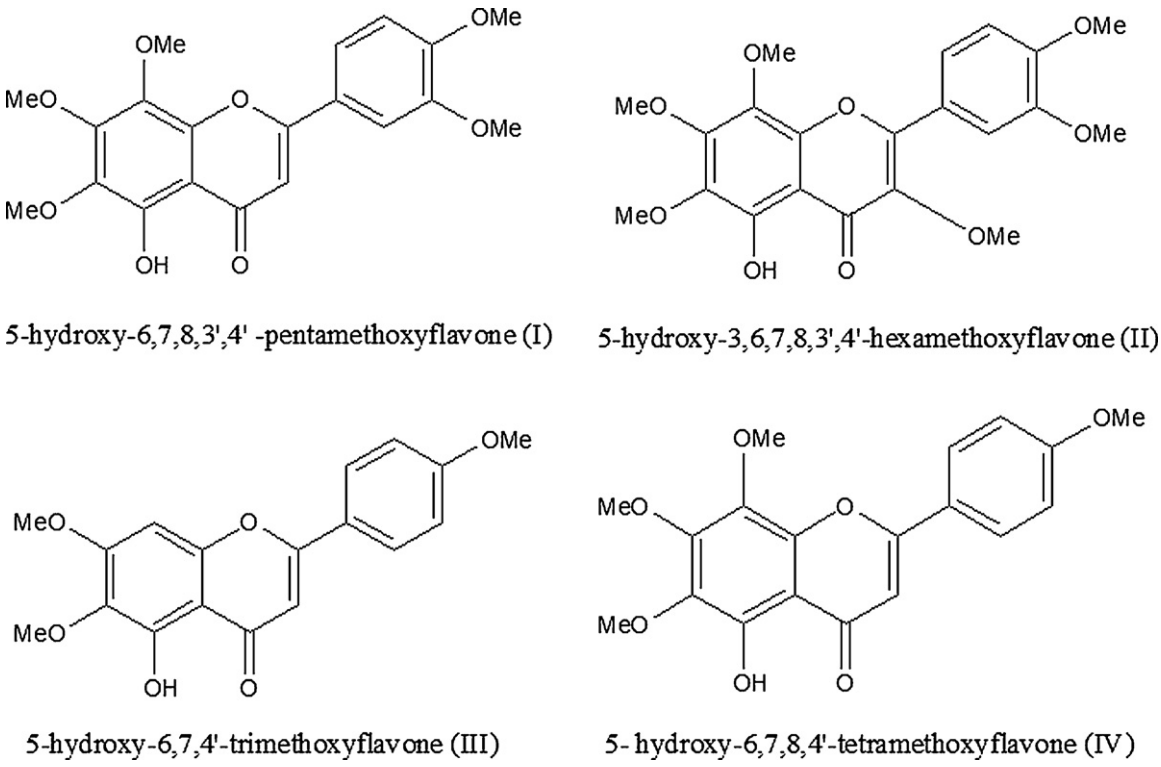

Fig. 1. Chemical structure and nomination of 5-OH-PMFs I-IV.

focusing on the analysis of four 5-OH-PMFs naturally existing in citrus, namely: 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone (I), 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (II), 5-hydroxy-6,7,4′-trimethoxyflavone (III) and 5-hydroxy-6,7,8,4′-tetramethoxyflavone (IV) (Fig. 1).

2. Materials and methods

2.1. Standards and reagents

Organic solvents, including methanol, acetonitrile (ACN), tetrahydrofuran (THF), trifluoroacetic acid (TFA), ethyl acetate, and hexanes were of HPLC grade and purchased from Fisher Scientific (Fairlawn, NJ, USA). Ammonium acetate was a product of EMD Chemicals Inc. (Gibbstwon, NJ, USA).

Four 5-OH-PMFs standards: 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone (I), 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (II), 5-hydroxy-6,7,4′-trimethoxyflavone (III) and 5-hydroxy-6,7,8,4′-tetramethoxyflavone (IV) were isolated from sweet orange (*C. sinensis*) peel extract and identified by MS, UV and NMR as described previously [1].

2.2. Instrumentation and chromatographic conditions

The CoulArray® HPLC system (Chelmsford, MA, USA) consisted of a binary solvent delivery system (model 584), an auto-sampler (model 542), a CoulArray® Multi-Channel EC detector (model 6210) and a UV detector (model 526) (Waters, Milford, MA, USA). Instrument control and data processing were performed with CoulArray 3.06 software. Ascentis RP-Amide reversed-phase HPLC column (15 cm × 4.6 mm id, 3 μm) (Sigma–Aldrich, MO, USA) was used. The mobile phase consisted of 50% water, 40% acetonitrile, 10% THF, 0.05% TFA and 50 mM ammonium acetate. Flow rate was 1.0 mL/min. The injection volume was 10 μL. Two EC detector cells (each contains four channels) were used, and the detecting potentials were set at 100, 200, 300, 400, 500, 600 and 700 mV. The last channel was connected to the UV detector. The wavelength of UV detector was 214, 280 and 326 nm. The temperature of auto-sampler was set to 4 °C. As a general practice, we included an injection of known standards with known concentrations for every five samples analyzed. If any noticeable decrease in sensitivity was observed for the known standards, a cleaning procedure was conducted according to the manufacturer's recommendation.

The pH of the mobile phase could affect the sensitivity of the EC detector on phenolic compounds [18]. Therefore, mobile phases with same composition but of different pH from 2 to 6.1 were tested to optimize the chromatographic condition. The pH of the mobile phase was adjusted using TFA.

2.3. Preparation of standards

The stock solution (10 mM) of each standard compound was prepared in DMSO and stored at 4 °C, and the final test solutions with concentrations ranging from 0.001 to 5 μM were prepared by diluting the stock solution with 20% methanol just before every experiment.

2.4. Method validation

Linearity, limit of quantification (LOQ) and limit of detection (LOD), recovery rate and precision of the current method were determined. The calibration curve was constructed using peak areas of 5-OH-PMFs standards and the known concentrations of standard solutions (μM). LOD and LOQ were calculated by determining signal-to-noise (S/N) ratio of the lowest measured concentrations and extrapolating to S/N values of 3 and 10, respectively [19]. Recovery rate was determined by the standard addition procedure in dried tangerine peel (P2); three different concentration levels (0.05, 0.5 and 5 μM) were used to represent the low, medium and high concentrations. Each sample was

analyzed in replication of three. Intraday and interday precision was determined for both retention time and peak area at low, medium and high concentrations similarly as in the recovery rate study.

2.5. Analyses of citrus samples

The quantification method developed herein was used to determine the abundance of 5-OH-PMFs in different citrus products and preparations, including three kinds of orange juice from different manufacturers (J1, J2 and J3) and two kinds of citrus peels with different aging periods (P1 and P2). 20 mL of orange juice was extracted with equal volume of ethyl acetate for three times. Ethyl acetate phase was pooled, evaporated to dryness, and dissolved in 10 mL of 75% methanol. The 75% methanol solution was washed three times with equal volume of hexanes. The methanol phase was then dried and redissolved in 20% methanol before the analysis by HPLC directly. P1 is freshly dried orange peel. P2 is the aged dry tangerine peel (used as traditional Chinese medicine). 20 g of P1 or P2 was incubated at 60 °C for over night to remove residue water, and then grinded to fine powder with a coffee bean grinder. The powder was extracted with methanol (20 mL/g dry powder) for five times at 85 °C (1 h each time). The extract was dried, dissolved in 75% methanol, washed with hexanes and analyzed using HPLC as described above.

3. Results and discussion

3.1. Optimization of the chromatographic conditions

Our purpose is to achieve baseline separation of four 5-OH-PMFs in a single HPLC run with relative short duration and less complex gradient profile. Based on our previous results, we chose to use Ascentis RP-Amide reversed-phase HPLC column (150 mm × 4.6 mm id, 3 μm), and water–ACN–THF as the mobile phase. After our test with different compositions of mobile phase, 40% of ACN and 10% THF in water was selected as isocratic mobile phase. This mobile phase resulted in a baseline separation of all four 5-OH-PMFs in 12 min with an isocratic composition.

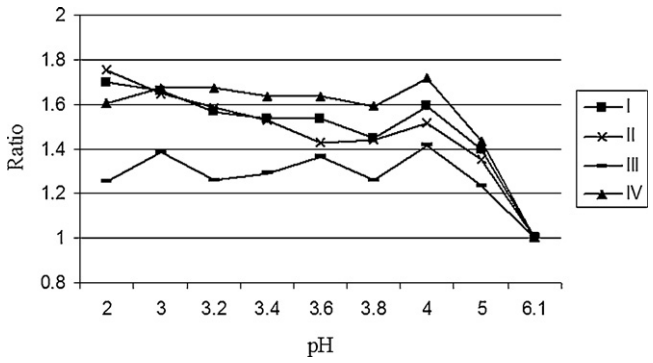

Fig. 2. The effect of pH of mobile phase on the sensitivity of EC detection of four 5-OH-PMFs. *X* axis is the pH value of mobile phase. *Y* axis is the ratio between the peak areas at certain pH and the peak area at pH 6.1. The peak areas of compound I, II and IV were determined from the signal at the channel 3 (300 mV). The peak areas of compound III were determined from the signal at the channel 4 (400 mV).

Ammonium acetate is one of the most commonly used buffer systems for EC detection [20,21]. EC detector is a sensitive detector whose sensitivity can be affected by the pH and ionic strength of the buffer systems in the mobile phase [18]. We compared the sensitivity of EC detector when a different ionic strength was used. It was found that that mobile phase with 50 mM ammonium acetate produced modestly higher sensitivity (about 10–15%) than the mobile phase with 20 mM ammonium acetate (data not shown). Further increase in ammonium acetate concentration did not improve sensitivity. Changes on the concentration of ammonium acetate did not affect retention times of any compounds tested. Therefore, we chose to use 50 mM ammonium acetate in the mobile phase. Next we tested the effects of pH of mobile phase with ammonium acetate (50 mM) as a buffer system on the sensitivity of the EC detector in terms of 5-OH-PMFs quantification. Initially, 0.05% TFA was added to mobile phase to improve the peak shape of 5-OH-PMFs, and the

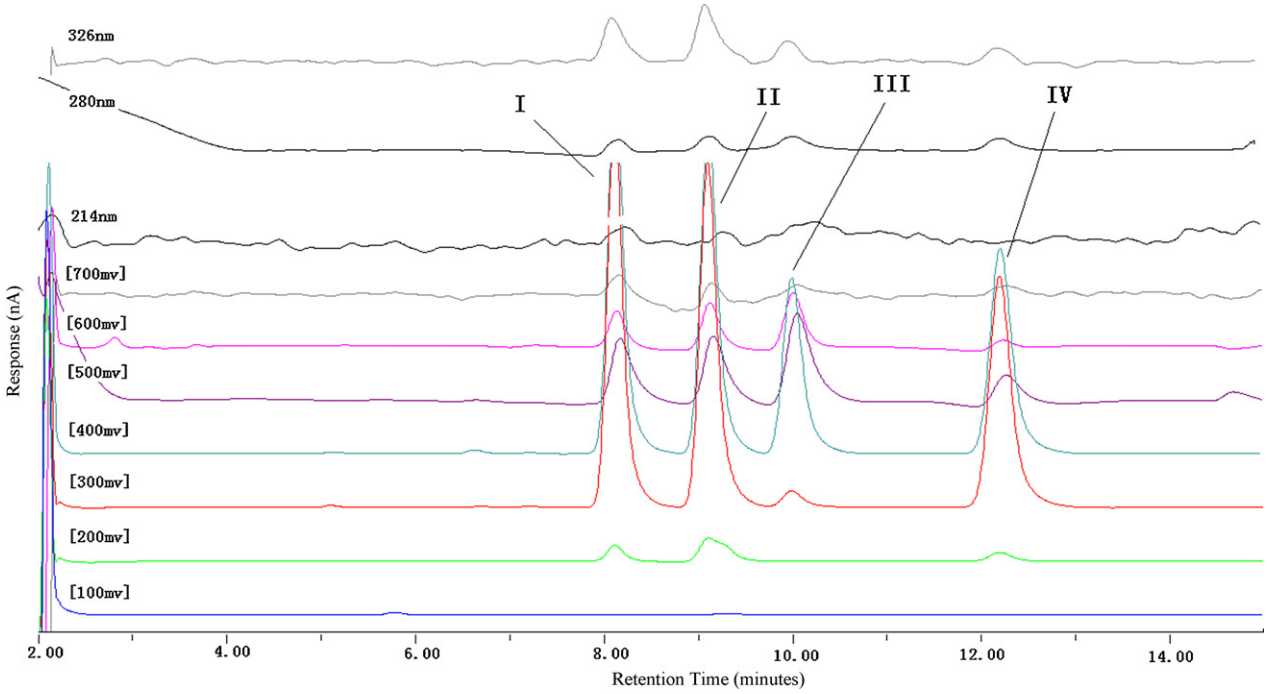

Fig. 3. Representative chromatograms of four 5-OH-PMFs. Experimental conditions: Ascentis RP-Amide column (15 cm × 4.6 mm id, 3 μm); Mobile phase (50% pure water, 40% acetonitrile, 10% THF, 0.05% TFA, 50 mM ammonium acetate, and pH 3–4); and flow rate at 1.0 mL/min. All concentrations of compounds I–IV were 0.2 μM.

Table 1
Linearity, LOD and LOQ of 5-OH-PMFs I–IV.

5-OH-PMFs	Concentration range (μM)	Linear regression equation	r^2	LOD (ng/mL)	LOQ (ng/mL)
I[a]	0.001–5	$y = 4287.6x + 71.121$	0.9990	0.65	2.1
II[a]	0.001–5	$y = 4429.3x + 78.716$	0.9989	0.85	2.8
III[b]	0.001–5	$y = 3148.8x + 74.848$	0.9992	0.95	3.3
IV[a]	0.001–5	$y = 4979.8x + 95.968$	0.9991	1.8	6.0

[a] Calculated using the potential of 300 mV.
[b] Calculated using the potential of 400 mV.

initial pH value is 6.1. Then pH of the mobile phase was adjusted from 6.1 to 2 by the addition of TFA. Using mobile phase with different pH, standard 5-OH-PMFs were analyzed by HPLC. The peak areas obtained from the initial mobile phase with pH of 6.1 were used as the standard, and peak areas obtained using the mobile phase with other pHs were normalized accordingly. As shown in Fig. 2, the sensitivity of EC detector for all four 5-OH-PMFs significantly increased, when the pH of mobile phase decreased from 6.1 to 4. The sensitivity for compounds III and IV was relatively stable while the pH of mobile phase was between 2 and 4. However, the sensitivity for compounds I and II showed a trend of increase when the pH of mobile phase decreased from 3.6 to 2. We prefer to use pH > 3.2 for HPLC analysis of all four 5-OH-PMFs because that baseline would become unstable if low pH (<3) was used for mobile phase, although low pH may render higher sensitivity for compounds I and II. At pH 4.0, there is a trend that sensitivity is slightly higher (less than 5%) than at pH 3.6. However, the pH of the mobile phase is not very stable when pH is adjusted to 4.0, which causes more variations among samples tested. Therefore, we chose pH 3.6 for all the analyses of 5-OH-PMFs.

Based on the results discussed above, the optimal conditions for mobile phase were as follows: 40% acetonitrile, 10% THF, 0.05% TFA, 50 mM ammonium acetate in water and pH 3.6 (adjusted with TFA). A typical chromatogram of four 5-OH-PMFs was shown in Fig. 3. Overall, this method provided baseline separation of four 5-OH-PMFs with slight structural differences, and a simple isocratic mobile phase was employed, which can eliminate potential unstable baseline caused by changing mobile phase composition during gradient elusion.

In order to determine suitable settings for potentials on different detecting channels of EC detector for different 5-OH-PMFs, stock solutions of each 5-OH-PMFs were prepared in 20% methanol in water at 2 μM, and then subjected to HPLC analysis with a serial of potentials set at 100, 200, 300, 400, 500, 600, and 700 mV on the seven channels of EC detector simultaneously. As shown in Fig. 3, all of these four 5-OH-PMFs could be oxidized when the electric voltage was higher than 200 mV. Compounds I, II and IV showed the maximum signals at 300 mV, while compound III had the maximum at 400 mV. These results suggested that under the sequentially increasing electric voltages, EC detector is the most sensitive to detect compounds I, II and IV at 300 mV, and compound III at 400 mV. Therefore, 300 mV was selected as the quantification voltage for compounds I, II and IV, and 400 mV was selected for compound III. In the same HPLC run, four 5-OH-PMFs were also detected by the UV detector with wavelength at 214, 280 and 326 nm (Fig. 3) [22,23]. Compared to the signals in EC channels, the UV signals of 5-OH-PMFs were much weaker, suggesting that the EC detector was much more sensitive than UV detector for the 5-OH-PMFs (see below for detailed discussion).

Table 2
Recovery.

5-OH-PMFs	Concentration (μM)	% Recovery	% RSD ($n = 3$)
I	0.05	110.82	6.46
	0.5	96.24	4.88
	5	99.81	6.72
II	0.05	108.82	12.25
	0.5	96.97	7.25
	5	98.80	10.43
III	0.05	106.30	8.00
	0.5	96.91	5.32
	5	100.78	10.65
IV	0.05	108.96	14.01
	0.5	96.17	3.82
	5	98.35	9.64

3.2. Method validation

The calibration curves of the four 5-OH-PMFs were constructed by plotting the peak areas against the corresponding concentrations of standard solutions prepared as described in Methods section. The concentration range was between 0.001 and 5 μM for all four 5-OH-PMFs. Regression analysis was used to assess the linearity of the analytical method. The regression equations and the correlation coefficients (r^2) were listed in Table 1. The correlation

Table 3
Precision data of four 5-OH-PMFs standards.

5-OH-PMFs	Concentration (μM)	Interday variation (%RSD) ($n = 3$)		Intraday variation (%RSD) ($n = 3$)	
		Retention time	Peak area	Retention time	Peak area
I	0.05	0.51	12.16	0.07	3.31
	0.5	0.56	7.13	0.06	1.19
	5	0.83	3.42	0	4.00
II	0.05	0.51	9.93	0.09	2.48
	0.5	0.55	3.63	0.05	2.30
	5	0.75	4.61	0	2.59
III	0.05	0.76	11.74	0.15	3.59
	0.5	0.79	9.67	0.05	0.72
	5	1.00	1.84	0.05	3.39
IV	0.05	0.84	5.38	0.07	3.94
	0.5	0.83	4.30	0	0.98
	5	1.02	9.56	0	3.55

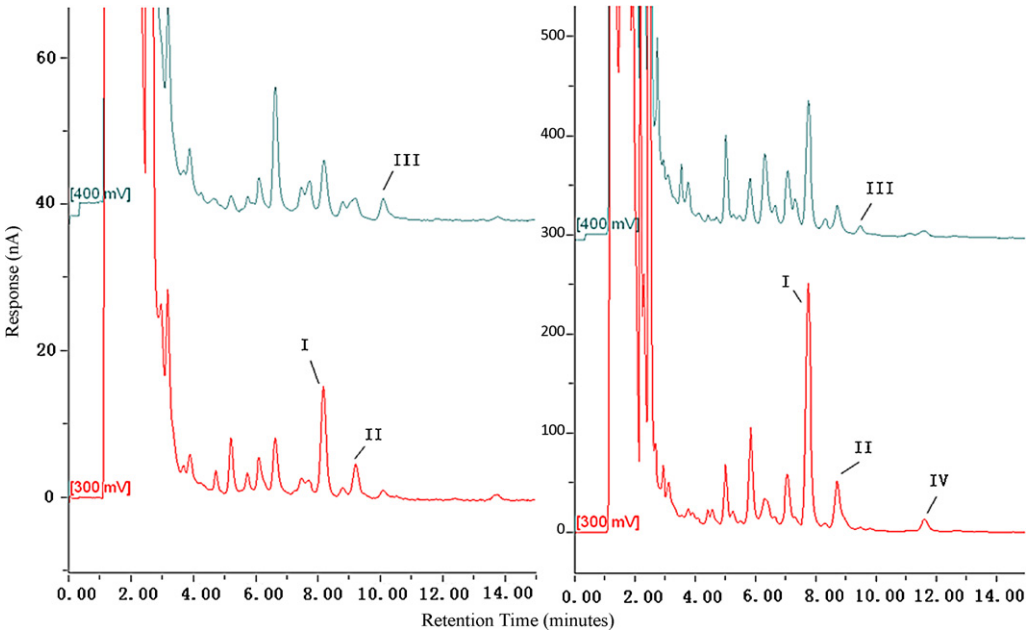

Fig. 4. Representative chromatograms for the determination of 5-OH-PMFs in different citrus products. (a) Orange juice (J1) (b) Citrus peel (P2).

coefficients (r^2) were around 0.999 for all four tested 5-OH-PMFs, suggesting a good linearity in a wide concentration range tested herein for all four 5-OH-PMFs.

Four 5-OH-PMFs showed noticeable difference in the LOD and LOQ. The LOD ranged between 0.65 and 1.8 ng/mL (ppb), and the LOQ ranged between 2.1 and 6 ng/mL (Table 1). Compared to our previous results by UV detection [16], the LOD and LOQ of 5-OH-PMFs using EC detector were about 160 times lower. These results demonstrated a superior sensitivity of EC detection to UV detection in terms of 5-OH-PMFs quantification by HPLC.

Recovery rate of the extraction and detection method was obtained by spiking known amount of 5-OH-PMFs in samples of dried tangerine peel (P2), preparing the samples following the extraction procedures described in Section 2.5, then analyzing the samples by HPLC. As shown in Table 2, the recovery rates were ranging from 96.17% to 110.82% with the RSD less than 15% for all four 5-OH-PMFs in 0.05, 0.5 and 5 μM that are representatives of the low, medium and high concentrations. We determined the intraday and interday variation of the HPLC method by analyzing same samples three times a day at different times and three times at 24 h interval, respectively. For intraday variation, the RSD of the retention time was between 0% and 0.09%, and the RSD of the peak area was less than 4.00% (Table 3). For the interday variation, the RSD of the retention time and the peak area was respectively in the range of 0.51–1.02% and 1.84–12.16% (Table 3). The results demonstrated that the method described herein has good reproducibility.

3.3. Analysis of 5-OH-PMFs in commercial citrus products and preparations

The abundance of 5-OH-PMFs (I–IV) in three brands of orange juice (J1, J2 and J3) and two kinds of citrus peel (P1 and P2) were determined by the HPLC method developed herein. The results were listed in Table 4. The representative chromatograms of J1 and P2 were showed in Fig. 4(a) and (b), respectively. The levels of 5-OH-PMFs in all three different orange juice were between 0.2 and 1.0 μM and similar to each other. Compounds I and III were more abundant than compound II. Compound IV was not detected in any orange juice. In two types of citrus peels tested (P1 and P2), the abundance of 5-OH-PMFs was much different from each other. The abundance of 5-OH-PMFs in P2 was about 4–15 times higher than in P1. P1 was freshly dried orange peel, while P2 was the aged dry tangerine peel that has been used as traditional Chinese medicine for centuries. P2 has been processed through many cycles of sun-drying, steaming, and storage. It is possible that the high levels of 5-OH-PMFs in P2 were generated by auto-hydrolysis of permethoxylated PMFs during the drying and steaming process. We and others have shown that 5-OH-PMFs had much stronger bioactivities than their corresponding permethoxylated PMFs [10–12]. Studies have demonstrated that aged dry tangerine peel exhibited various biological activities, including anti-shock, anti-arteriosclerosis, anti-carcinogenic, anti-thrombotic, etc. [24]. Some of these bioactivities may be associated with the relatively high abundance of the 5-OH-PMFs in aged dry tangerine peel.

Table 4
Levels of 5-OH-PMFs I–IV found in different citrus products.

Samples	I (μM)	II (μM)	III (μM)	IV (μM)
J1	0.98	0.295	0.585	NA
J2	0.752	0.258	0.842	NA
J3	0.705	0.222	0.713	NA
Samples	**I (ppm)**	**II (ppm)**	**III (ppm)**	**IV (ppm)**
P1	82.42	18.278	2.747	1.552
P2	343.972	66.154	33.081	23.055

4. Conclusion

Herein, we reported a simple, reliable and highly sensitive HPLC method for quantification of four different 5-OH-PMFs that have been associated with various biological activities. Our method provides a 160 times higher sensitivity compared to UV detection. The high sensitivity offered by our method is critically important to detect and quantify 5-OH-PMFs in biological samples such as blood and tissue homogenates where usually only trace amount of these compounds can be found. The method developed in this report is useful for future nutritional and pharmacological studies of 5-OH-PMFs in animals and humans.

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