JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Simultaneous Analysis of Six Polymethoxyflavones and Six 5-Hydroxy-polymethoxyflavones by High Performance Liquid Chromatography Combined with Linear Ion Trap Mass Spectrometry

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Supporting Information

ABSTRACT: Polymethoxyflavones (PMFs) and monohydroxylated polymethoxyflavones (OH-PMFs) exist exclusively in the citrus genus, particularly in citrus peels. Currently, due to the broad application of PMFs and OH-PMFs in nutraceuticals, pharmaceuticals, and functional foods, their identification and quantification will be of great significance and the first criteria to meet. We have developed a validated method with high performance liquid chromatography coupled with linear ion trap mass spectrometry. The method was fully validated in linearity, precision, accuracy, and recovery. Six PMFs and their monohydroxyl counterparts, six 5-OH-PMFs, were simultaneous analyzed within 20 min for the first time. The LOD (limit of detection) and LOQ (limit of quantitation) were calculated as 0.02−0.23 and 0.05−0.76 μg/mL, respectively. The method was performed on the samples of acid treated citrus peel extracts. The citrus peel extracts with high content of PMFs and 5-OH PMFs may provide reliable and economical resources in biological activity studies and development of health beneficial products.

KEYWORDS: *polymethoxyflavones, liquid chromatography, linear ion trap mass spectrometry, citrus peel extract*

INTRODUCTION

Flavonoids are widely distributed among fruits, vegetables and grains. As a major class of polyphenolic compounds, flavonoids possess various health-beneficial properties due to their strong antioxidant and radical scavenging activity. Polymethoxyflavones (PMFs) and hydroxylated polymethoxyflavones (OH-PMFs) constitute multiple methoxyl groups among flavonoids and are almost exclusively found in *Citrus* species.[1,2] The peel residue is considered a byproduct and hence is the primary waste from the citrus juice manufacturing industry. Therefore, their applications have drawn much attention recently as food additives of natural origin or nutraceutical products.

The PMFs have been found to have health-related properties. Many biological studies have focused on two of the most abundant PMFs in citrus peels: nobiletin and tangeretin. Evidence of the protective activities of PMFs in vitro and in vivo include anti-inflammatory,[3−6] anticarcinogenic,[7−10] anti-atherosclerosis activities,[11−13] and substantial hypolipidemic response.[13−15] Moreover, the same spectrum of biological activities such as anti-inflammatory[16,17] and anticarcinogenic activities[18−22] were also found in OH-PMFs, the mono- and didemethylated PMFs that were identified as mammalian metabolites.[23−25] More and more studies have revealed that OH-PMFs are significantly more bioactive than PMFs.[21,26] The changes in hydrophobicity, ligand binding property, permeability to biological membranes, and metabolic pathway may contribute to these outcomes. Hence, it is of critical importance both in the qualification and quantification of PMFs and OH-PMFs.

Many chromatographic and spectroscopic techniques have been applied on isolation, characterization, and quantification of PMFs and OH-PMFs in citrus fruits.[1,27−31] Because of the close proximity among PMFs and their hydroxylated counterparts, OH-PMFs, the baseline separation has not been accomplished when many compounds exist in a sample using a single liquid chromatographic analysis. Although a few validated methods had been reported separately for the quantification analysis of PMFs[32] or OH-PMFs[33,34] in citrus peel extracts (CPEs) using the HPLC technique, simultaneous analysis of both PMFs and OH-PMFs has not been reported.

Here, we report a validated quantification method that can simultaneously analyze six PMFs and six OH-PMFs in one single analysis. It is an LC-MS analysis method in which a liquid chromatograph coupled with a linear ion trap mass spectrometer (LC/MS) using electrospray ionization (ESI) source and collision dissociation (CID) MS^n is adopted to validate the fingerprints of PMFs/OH-PMFs and the quantification method. With this rapid analytical method, we further performed validation with standard compounds and a few acid treated CPEs, which laid the foundation for screening and structural characterization of PMFs and OH-PMFs in CPEs either naturally occurring or via the acid treatment.

MATERIALS AND METHODS

Chemicals and Solvents. Six PMFs (Figure S1 of the Supporting Information): 5,6,7,4′-tetramethoxyflavone (TetraMF), tangeretin, sinensetin, nobiletin, 3,5,6,7,3′,4′-hexamethoxyflavone (HexaMF), 3,5,6,7,8,3′,4′-heptamethoxyflavone (HeptaMF), and six 5-OH-PMFs (Figure S1 of the Supporting Information): 5-hydroxy-6,7,4′-

Received: September 9, 2012
Revised: November 21, 2012
Accepted: November 26, 2012
Published: November 26, 2012

trimethoxyflavone (5-OHtriMF), 5-hydroxy-6,7,8,4′-tetramethoxyflavone (5-OHtangeretin), 5-hydroxy-6,7,3′,4′-tetramethoxyflavone (5-OHsinensetin), 5-hydroxy-3,6,7,3′,4′-pentamethoxyflavone (5-OHpentaMF), 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone (5-OHnobiletin), and 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (5-OHhexaMF) were isolated from cold-pressed sweet orange peel extract. Their purity is more than 99% by LC/MS. The structures were characterized by ^{1}H and ^{13}C NMR and high resolution MS.[2] Daidzein was purchased from Indofine (Hillsborough, NJ, USA). HPLC-grade acetonitrile, tetrahydrofuran, and water (Merk, Darmstadt, Germany) were used. Analytical grade ethyl acetate, hexanes, and sodium hydroxide were purchased from Merck (Darmstadt, Germany). Hydrogen chloride (above 35%) was purchased from Union Chemical Works LTD (Hsin-Chu, Taiwan). Analytical-grade 100% ethanol was used as a sample solvent and was purchased from J. T. Baker (Phillipsburg, NJ, USA). One commercially available orange peel extract (OPE), which contained all PMFs/5-OH PMFs in the study (WellGen, North Brunswick, NJ, USA), was analyzed for the sample recovery experiment.

Citrus Materials and Ethyl Acetate Extract Sample Preparation. Four species of citrus fruits, ponkan (*Citrus reticulata* Blanco), liucheng (*C. sinensis* (L.) Osbeck), murcott (*C. reticulata* × *C. sinensis*), and tonkan (*C. tankan* Hayata) were purchased from a local market (Chia-Yi, Taiwan) between October 2009 and February 2010. The fruits were washed and hand peeled into edible pulp and inedible peel portions. The peels were dried under a warm stream in the oven at 40 °C for three days and then ground into fine powders. One hundred grams of each dried citrus peel powder was weighted and placed in a glass flask. Afterward, the citrus peels were extracted by the sequence of 500 mL of hexanes and 500 mL of ethyl acetate for 24 h under vigorous shaking. The extracts were filtered through filter paper, and only ethyl acetate supernatants were evaporated under vacuum using a rotary evaporator. After concentration, the ethyl acetate extracts were lyophilized for further demethylation reaction.

OH-PMF Sample Preparations. Hydroxylated PMFs were prepared according to the method of Li et al. with modification.[22] Briefly, 2 g of citrus ethyl acetate extract obtained previously was dissolved in absolute alcohol, and to the solution was added 6 M aqueous hydrochloric acid. The solution was heated and reflux for 5 h. After the reaction was cooled, the solution was titrated to neutral by sodium hydroxide, and the ethanol was removed in vacuo. Ethyl acetate and water were added, and the organic layer was collected. The aqueous layer was extracted with ethyl acetate twice. After concentration, the residues were lyophilized to give a light yellow solid of hydroxylated PMFs. Prior to MS analyses, each of the extracted solids was dissolved in absolute alcohol with the proper amount of internal standard. The samples were stored at −18 °C prior to sample analysis.

Standard Preparation. A stock solution of 1000 μg/mL of each PMF and 5-OH PMF standards was prepared in ethanol with a proper amount of daidzein as the internal standard (IS). Spiking solutions were prepared by mixing stock solutions of each analyzed compound and diluting in ethanol to create concentrations of 0.5, 1, 10, 25, 50, 75, or 100 μg/mL.

Assay Validation. The method was validated according to ICH (International Conference on Harmonisation) guidelines on specificity, sensitivity, precision, and recovery.[35] The limit of detection (LOD) is defined as the lowest amount of analyte that can be detected above baseline noise, typically S/N = 3. The analyte response at the limit of quantitation (LOQ) is defined as the lowest amount of analyte, which can be quantitated above the baseline noise level, typically S/N at least equal to 10. To evaluate the assay specificity, mixed standard compounds in solution were tested to demonstrate the capability of elution and identification of specific components. Intraday precision was performed by analysis of the QC samples of PMF and 5-OH-PMF standards at three concentrations (2, 20, and 40 μg/mL, n = 3) in the same day. Interday precision (n = 3) was determined by repeated analysis of the same QC samples over 3 consecutive days. Precision was determined by coefficient variation (CV %) of each measured concentration. The deviation of the mean from the true value served as the determination of accuracy (RE %). Recovery was determined by standard addition procedures in OPE samples; three different concentrations were used to represent the low, medium, and high concentrations. Each sample was analyzed in replications of three.

HPLC Analysis. The HPLC chromatographic conditions were modified from the previous published PMF method with slight modification as follows:[32] column, Ascentis RP-Amide C_{16} column (150 × 4.6 mm i.d., 3 μm particle size, Supelco, Bellefonte, PA, USA); binary gradient elution was used with a mobile phase composed of solvent A (water at 0.15% acetic acid) and solvent B (acetonitrile). The elution program is described in Table S1 of the Supporting Information for PMF derivatives analysis. The flow rate was 0.6 mL/min, and the column temperature was 35 °C. The injection volume was 10 μL for each sample.

LC-MS^n Analysis. A Thermo LC-ESI-MS system equipped with a Surveyor MS pump, a Surveyor autosampler, and an LCQ linear ion trap mass detector (Thermo Finnigan, San Jose, CA, USA) incorporated with an electrospray ionization (ESI) interface was used. The acquisition parameters were set as follows: the positive ion polarity mode was set for the ESI ion source with the voltage on the ESI interface maintained 4.0 kV; nitrogen gas was used in sheath gas at a flow rate of 65 arbitrary (arb) units and auxiliary gas at a flow rate of 10 arb units; capillary temperature, 300 °C; capillary voltage, 33 V; tube lens offset voltage, 95 V; and mass range, from m/z 120 to 600. The characteristic spectra information was obtained by tandem mass spectrometry (MS^n) through collision-induced dissociation (CID) with a relative collision energy setting of 35%. The CID for each standard solution was varied and optimized through direction infusion analysis. The selected reaction monitoring (SRM) or consecutive reaction monitoring (CRM) scan type was applied to the specific compound. The paired ions which were monitored and optimized CID are listed in Table 1. Both HPLC and MS systems were controlled by Xcalibur 2.06 software.

Table 1. Selective Reaction Monitoring (SRM) and Consecutive Reaction Monitoring (CRM) Transitions and Collision Induced Dissociation (CID) Energy for Six PMFs and Six 5-OHPMFs[a]

PMFs/5-OHPMFs	MS $[M + H]^+$ (m/z)	MS^2 product ion (m/z)	CID (%)
sinensetin	373	312	29
HexaMF	403	373	29
nobiletin	403	388	26
TetraMF	343	282	25
HeptaMF	433	418	41
tangeretin	373	358	28
5-OHsinensetin	359	344	28
5-OHpentaMF	389	356	18
5-OHnobiletin	389	341	20
5-OHhexaMF	419	389	24
5-OHtriMF	329	314	26
5-OHtangeretin	359	344	28

[a]MS^3 CRM transition (m/z 356→310, CID energy 17%) for 5-OHpentaMF.

RESULTS AND DISCUSSION

LC-ESI/MS^n Conditions. The aim of this study was to establish a valid qualitative and quantitative method for PMFs and OH-PMFs in a single HPLC run. Many studies had focused on the cinnamic acids or flavonoids in citrus peels. In order to reduce routine instrument cleaning, such as atmospheric pressure ionization source and ion transfer capillary, and effectively avoid the possible interference in citrus samples and acquire good chromatographic peak shape,

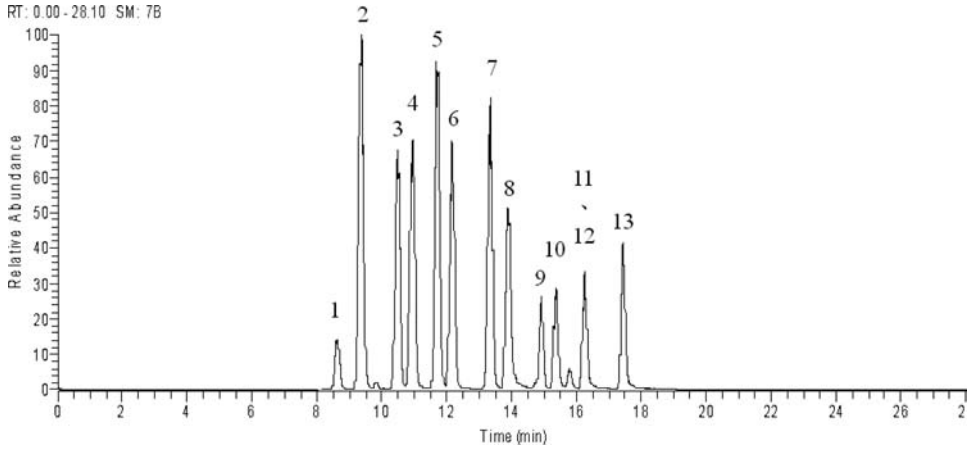

Figure 1. LC-MS chromatogram of internal standard, six PMF, and six 5-OHPMF standards in positive ionization mode. Peak 1, daidzein; peak 2, sinensetin; peak 3, HexaMF; peak 4, nobiletin; peak 5, TetraMF; peak 6, heptaMF; peak 7, tangeretin; peak 8, 5-OHsinensetin; peak 9, 5-OHpentaMF; peak 10, 5-OHnobiletin; peaks 11 and 12, 5-OHtriMF and 5-OHhexaMF, peak 13, 5-OHtangeretin.

the special operation has been added to the divert valve as described in Table S1 of the Supporting Information. The valve was connected after the HPLC column. The sample eluted from the column could divert the LC flow between the MS detector and waste based on the divert valve's configuration.

By following the previous reports, a polar embedded C16 phase provided excellent peak shape and efficiency in PMF and 5-OH PMF analyses. With mass spectrometry, several scan types can be operated depending on the various purposes of analyses. In general, the full scan provides a full mass spectrum of each analyte. The full scan for all the compounds in this study is shown in Figure 1. There were six selected PMFs and six 5-OH PMFs in 20 min. For each standard compound, the molecular ion $[M + H]^+$ was easily detected and confirmed under positive mode as shown in Table 1. Because of the PMF structural characteristics, their MS^n product-ions were considered as diagnostic fingerprints. Upon the first step of CID, the protonated PMFs dissociate predominantly by a loss of 61, 30, 15, 33, 48, 28, and 44, corresponding to the loss of ($CO+H_2O+CH_3^{\bullet}$), ($2CH_3^{\bullet}$or MeO), ($CH_3^{\bullet}$), ($H_2O+CH_3^{\bullet}$), ($2CH_3^{\bullet}+H_2O$), (CO) and (CO_2), respectively (data not included).[1,27] In order to obtain the maximum product ions, different voltages were optimized and applied to individual compounds as shown in Table 1. Under SRM, due to monitoring the pairs of ions, the specificity of the target compound identification was higher. Furthermore, SRM can improve the detection limit and allow for the very rapid analysis of trace components in a complex mixture. Although the coelution of 5-OHtriMF and 5-OHhexaMF was observed under full mass scanning, under SRM, 5-OHtriMF and 5-OHhexaMF were well determined as in Figure 2. Since all PMF derivatives have similar structures, polymethoxyl groups, much attention has to be paid to those compounds with the same molecular formula. The reasons are that their retentions are close and that the retention times for 5-OHpentaMF and 5-OHnobiletin were 15.06 and 15.50 min, respectively (Figure 2). They have the same molecular formula with molecular weight at 388.37. If only the SRM scans were applied on individual compounds, their high similarity in MS^2 spectra may introduce confusion both in qualitative and quantitative analyses. Therefore, the elution condition we applied is a consecutive reaction monitoring (CRM; $n = 3$ scan power), in which a multistep reaction path was monitored for 5-OHpentaMF. Under the circumstances, all distinguished peaks represent the distinct compounds shown in Figure 2.

The elution order was internal standard, daidzein, followed by PMFs and 5-OH-PMFs. Except for 5-OHtriMF and 5-OHhexaMF (peaks 11 and 12), with this optimized method, the column was able to fully resolve 11 compounds under a single-stage full scan type (internal standard was included). On the basis of this chromatography, it was easy to demonstrate that 5-OH PMFs were less polar than PMFs. The demethylation in the 5-position of PMFs provided the hydroxyl group to form the intramolecular hydrogen bond with the carbonyl oxygen at the 4-position of the flavone skeleton resulting in the higher hydrophobic property of the 5-demethylated PMF molecules.

Method Validation. Upon the development of instrument methodology, the linearity of the detector response was evaluated based on the calibration graphs of each compound. All PMFs had been quantified using internal calibration method. The HPLC/MS^n calibration curves were obtained by diluting 5 independent stock solutions over the concentration range as shown in Table S2 of the Supporting Information. Regression analysis was used to assess the linearity of the analytical method. In the regression equation $y = ax + b$, x is the concentration of standard solution (μg/mL), y the relative peak area, a the slope of the line, and the b the intercept of the straight line with the y-axis. The resulting correlation coefficients (r^2) were greater than 0.99 for all 12 standards, indicating that the relationship between relative peak area and the concentration of PMFs and OH-PMFs in the analyzed concentration was well established.

In determining the values of LOD and LOQ, at least seven replicates of ethanol solvent spiked with PMFs and OH-PMFs at the level close to the detection limit were performed. The results showed that the obtained LOD and LOQ values were between 0.02−0.23 and 0.05−0.76 μg/mL, respectively (Table S2 of the Supporting Information), which were lower than the values obtained in previous measurements that were only based on UV light absorption.

Intraday precision was evaluated by analyzing three independent ethanol solutions with spiked standard compounds at three different concentration levels (2, 20, and 40 μg/mL) during the same day, under the same experimental conditions. Interday precision was measured at the same three

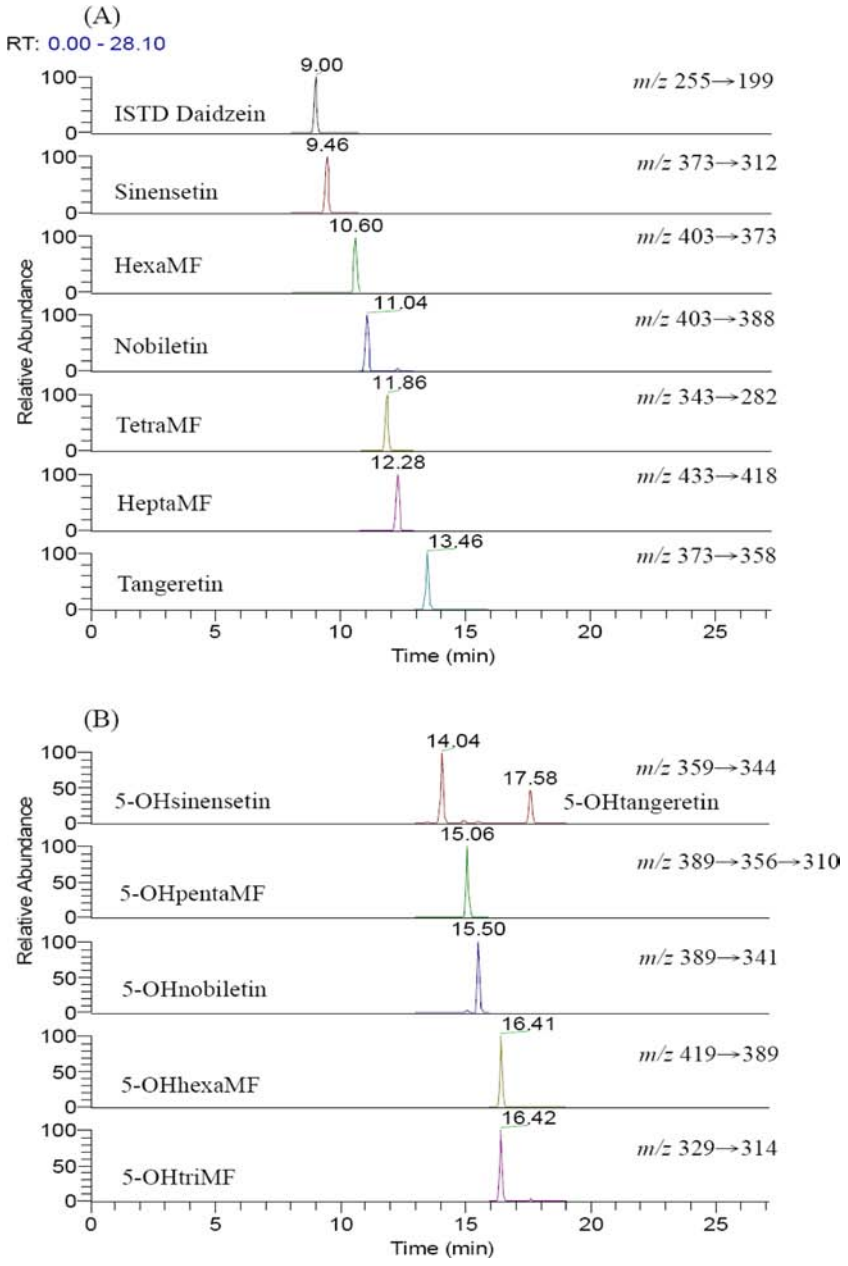

Figure 2. Selective reaction monitoring (SRM) and consecutive reaction monitoring (CRM) chromatograms and retention times for (A) internal standard (daidzein), six PMF, and (B) six 5-OHPMF standards.

Table 2. LC-MS^n Analysis of PMFs and OH-PMFs in Four Citrus Peel Ethyl Acetate Extracts[a]

	PMFs weight percentage (%)						
local name	total	sinensetin	HexaMF	nobiletin	TetraMF	HeptaMF	tangeretin
Ponkan	17.39 ± 0.52			8.37 ± 0.36			9.02 ± 0.38
Liucheng	6.55 ± 0.09	1.02 ± 0.02	0.55 ± 0.02	3.05 ± 0.04	0.50 ± 0.06	1.13 ± 0.05	0.30 ± 0.01
Murcott	19.38 ± 0.43	0.53 ± 0.00		12.28 ± 0.26		1.61 ± 0.10	4.96 ± 0.33
Tonkan	19.64 ± 0.38	0.53 ± 0.04		12.73 ± 0.27		3.23 ± 0.21	3.15 ± 0.14
	OH-PMFs weight percentage (%)						
	total	5-OHsinensetin	5-OHpentaMF	5-OHnobiletin	5-OHtriMF	5-OHhexaMF	5-OHtangeretin
Ponkan	30.00 ± 0.27	0.15 ± 0.03		21.33 ± 0.17	0.82 ± 0.02		7.70 ± 0.20
Liucheng	10.95 ± 0.24	1.82 ± 0.09	0.40 ± 0.03	5.61 ± 0.19	2.40 ± 0.10	0.34 ± 0.02	0.38 ± 0.01
Murcott	19.15 ± 0.31	0.48 ± 0.07		14.43 ± 0.22	0.55 ± 0.18	0.38 ± 0.01	3.30 ± 0.08
Tonkan	21.24 ± 0.25	1.13 ± 0.07		15.84 ± 0.23	0.11 ± 0.00	1.50 ± 0.06	2.66 ± 0.07

[a]The values are expressed as the mean ± SD of triplicate tests. The blank cells are the PMFs and OH-PMF below the quantification limit.

different concentrations in three different days. The intraday and interday CV % values of the observed concentrations were in the range of 1.3−9.9 and 0.9−12.5%, respectively, as shown in Tables S3 and S4 of the Supporting Information. The accuracy of this method was ascertained by spiking three known concentrations of standard PMFs and OH-PMFs. Their intraday and interday RE % values were in the range of −12.3−12.9 and −7.8−11.4%, respectively, as shown in Tables S3 and S4 of the Supporting Information. In the recovery study, the standard addition for all standard PMFs and OH-PMFs with three different concentrations in the OPE sample was performed. The high recovery is shown in Tables S5 and S6 of the Supporting Information. The results showed that the CV % and RE % were both less than 15%. Hence, the verified results from above-mentioned experiments indicated that the method and system were adequate to perform the desired analyses.

With strong acid treatment, the PMFs can be transformed into 5-OH-PMFs in the citrus peel extracts, which contained high amounts of PMFs. In this study, Liucheng citrus peels only contain 6.6% (weight percentage %) PMFs (Table 2). Other citruses, such as Ponkan, Murcott, and Tonkan, contain 17.4−19.6% PMFs. However, OH-PMFs reached 30.0% in Ponkan citrus extracts after 5 h and with 3 N hydrochloride treatment. The high yield of 5-OHnobiletin and 5-OHtangeretin was obtained under this treatment.

In the present study, the results showed that a reliable instrumental method and conditions were developed and optimized for simultaneous and rapid determination of six PMFs and six OH-PMFs. The obtained data of PMFs and OH-PMFs in terms of weight percentage have showed that the compositions of various citrus fruits in the same year vary a lot. With the acid treatment of certain citrus species, the desired combination amount of PFMs/OH-PMFs can be the further applied successfully in both in vitro and in vivo experiments.

■ ASSOCIATED CONTENT

Supporting Information

LC program for chromatographic separation for six PMFs and six 5-OHPMFs; linearity of the calibration curve and sensitivity data for six PMF standards and six 5-OHPMF standards; precision and accuracy of intraday and interday for six PMF standards and six 5-OHPMF standards; recovery data of six PMF standards and six 5-OHPMF standards; and chemical structures of the six polymethoxyflavones and the six 5-hydroxyl polymethoxyflavones. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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