Food & Function

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Journal:	*Food & Function*
Manuscript ID	FO-ART-05-2019-001053
Article Type:	Paper
Date Submitted by the Author:	17-May-2019
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Characterization of polymethoxyflavone demethylation during drying processes of citrus peels

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Abstract

Polymethoxyflavones (PMFs) are found almost exclusively in citrus peel and have attracted much attention due to their potential health benefits. Dried citrus peel is an important ingredient for applications in food and traditional Chinese medicine. However, the structural changes of PMFs during the drying processes of citrus peel remained unknown. In this study, for the first time we discovered that four major permethoxylated PMFs, i.e. sinensetin, nobiletin, heptamethoxyflavone and tangeretin underwent demethylation at 5-position on the A ring of their flavonoid structures to yield corresponding 5-demethylated PMFs during drying process of citrus peel. Our results further demonstrated that aforementioned PMF demethylation was through two mechanisms: acid hydrolysis and enzyme-mediated catalysis. PMF demethylation in citrus peel was systematically characterized during hot-air drying (HAD), vacuum-freeze drying (VFD) and sun drying (SD). The highest PMF demethylation was obtained in SD followed by HAD and VFD. This study provided solid scientific basis for rational control of PMF demethylation in citrus peel, which could facilitate the production of high-quality citrus peel and related products.

Keywords: citrus peels, drying process, polymethoxyflavones, demethylation, mechanism.

1 Introduction

Citrus is the fruit with the highest yield in the world. Besides being consumed fresh, the majority of citrus fruit is processed to yield juice and canned fruits along with the production of many citrus peel by-products.[1] There are also numerous citrus peel products in the market, such as citrus peel jam, candied citrus peel, and citrus peel tea, that are widely consumed as snack foods, cooking spices, etc. Among these products, dried citrus peel is the most popular food ingredient in many countries. Dried citrus peel is also an important ingredient in traditional Chinese medicine used for relieving indigestion and inflammatory syndromes such as bronchitis and asthma.[2,3]

There are many dehydration techniques that can be applied to drying citrus peel, such as sun drying, hot-air drying, vacuum-freeze drying, and so on. Different drying processes might have different effects on the chemical structures and biological activities of functional components of citrus peel.[4-9] It was found that total flavonoids content of air-dried, oven-dried, microwave-dried and freeze-dried yuzu peel was higher than those in fresh peel.[10] Similarly, freeze-drying was shown to increase the abundance of flavonoids in lemon peel.[11] In addition, the total content of flavonoids was found decreased at low drying temperature (≤ 80 °C), while increased at high temperature (90~100 °C) during oven-drying of citrus peel.[12] Furthermore, drying time also has important effects on flavonoids content in citrus peel during oven-drying.[4] The previous studies mainly focused on the effects of different drying processes on the overall flavonoid content, therefore, the specific chemical structural changes on the specific citrus flavonoids and their underlying mechanisms during drying of citrus peel remained unknown.

Polymethoxyflavones (PMFs) are a unique class of flavonoids with more than two methoxyl groups on their chemical skeletons and have been found exclusively exist in citrus peels.[13] PMFs have attracted growing interest in recent years due to their various biological activities, including anti-carcinogenic,[14], anti-inflammatory,[15] antioxidant,[16] antiviral,[3] and so on.[17] Demethylated polymethoxyflavones (Demethylated PMFs) are PMFs with hydroxyl groups that have replaced methoxyl groups on the structure. It has been reported that demethylated PMFs, mainly 5-demethylated PMFs, exhibit stronger biological activities than their corresponding permethoxylated counterpart compounds.[13,18-20] For example, the IC_{50} values of three major 5-demethylated PMFs (5-demethylnobiletin, 5-demethylhexamethoxyflavone and 5-demethyltangeretin) against colon cancer cells were approximately 2.1-fold, 3.1-fold and 6.6-fold lower than those of their permethoxylated counterparts nobiletin, heptamethoxyflavone and tangeretin, respectively.[18] Similarly, the inhibitory effects of 5-demethylnobiletin and 5-demethylhexamethoxyflavone on H1299 human lung cancer cells were 60% more potent than their permethoxylated counterparts, i.e. nobiletin and hexamethoxyflavone.[21] It is important to understand the mechanism of PMF demethylation.

Studies have demonstrated that demethylation of PMFs could occur via *in-vivo* metabolism,[22,23] and chemical reactions.[18,20,24] For example, nobiletin, tangeretin and other flavonoids could be demethylated by cytochrome P450s in liver microsome at enzyme, cellular, and animal levels.[22,24-27] In addition, an acidic condition was found conducive to the transformation of PMFs to 5-demethylated PMFs chemically.[13,14,20,24] Based on these previous findings, we hypothesized that PMFs could undergo demethylation during the drying processes of citrus peel due to the acidic condition and presence of enzymes in the citrus peel.

To test this hypothesis, the objective of this study was to determine the effects of different drying processes on PMF demethylation in citrus peel and the underlying mechanism. The findings from this study is potentially useful for the rational utilization of citrus and citrus products as sources of demethylated PMFs for health promotion.

2 Materials and Methods

2.1 Chemicals and regents

Sinensetin (compound **1**), nobiletin (compound **2**), heptanmethphoxyflavones (compound **3**) and tangeretin (compound **4**) were purchased from Sigma Co., Ltd. (Shanghai, China). HPLC-grade methanol, dichloromethane, ethyl acetate, formic acid and acetonitrile were bought from Fisher Scientific (Shanghai, China). Dimethyl sulfoxide (DMSO) and ethylene diamine tetraacetic acid (EDTA) were purchased from Sigma-Aldrich (Shanghai, China). Hydrochloric acid, sodium bicarbonate and sucrose were obtained from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Silica gel (100–200, 200–300 mesh) was purchased from Shanghai Titan Scientific Co., Ltd. (Shanghai, China). Tris (hydroxymethyl) aminomethane (99.85%) and dithiothreitol (DTT) were purchased from Acros Organics (Shanghai, China) and Thermo Scientific (Shanghai, China), respectively. Ultrapure water used in hydrochloric acid solution and mobile phase was prepared using a Milli-Q system (Millipore, Bedford, USA).

2.2 Preparation of citrus peels samples

Fresh *hybrid citrus* (*Citrus sinensis L. Osbeck* × *Citrus unshiu Marc.* × *Citrus reticulata Blanco*) and *valencia orange* (*Citrus sinensis L. Osbeck*) fruits were grown in Wanzhou district (Chongqing, China) and each citrus variety was collected from the same plantation in February 2017. The average weights of citrus fruits were 175.0± 2.5 and 160.0± 2.0 g for *hybrid citrus* and *valencia orange*, respectively. The fruits were first cleaned and wiped dry, then cut into eight pieces and peeled carefully by hand. The citrus peel were powdered under liquid nitrogen using a Cryogenic Sample Crusher (CKL-100, Beijing sanyoulianchuang Instrument Co., Beijing, China) for 3 min, and whisked for 30 s to make sure the samples were uniformly mixed. Moisture contents are the quantity of water contained in a material. The initial moisture contents of *hybrid citrus* peel and *valencia orange* peel powder were measured by Moisture Analyzer (MJ33, Mettler-Toledo, Ohio, USA), which were 3.25± 0.10 and 2.57± 0.15 g water/g dry basis, respectively. The soluble solids of *hybrid citrus* peel and *valencia orange* peel were measured using a digital refractometer (DR102, TO YOU OPTICAL Instrument Co., Shandong, China), which were 3.96± 0.24% and 3.62± 0.20%, respectively. The pH values of fresh *hybrid citrus* peel and *valencia orange* peel were measured by a Mettler Toledo pH meter (Fe20-K, Shanghai, China), which were 2.80 ± 0.14 and 3.32 ± 0.09, respectively.

2.3 Drying processes and conditions

Citrus peels were dried by 3 different drying methods, i.e., hot-air drying (HAD), vacuum-freeze drying (VFD), and sun drying (SD). The continuous weight loss was recorded by an electronic balance with the precision of 0.1 mg. All individually drying processes were carried out in triplicates. The description of each drying process is described in detail below.

HAD: It was performed using an oven (GZX-9240MBE, Shanghai Boxun Co., Ltd., Shanghai, China) at the different temperatures of 40, 50, 60 and 70 ^{o}C with a fixed air velocity of 2.1 m/s. Three grams of citrus peel were put on a round sample tray with diameter of 90 mm, which was placed in the middle of the oven. Both the distances from the tray to top heater and bottom heater were 20 cm. The samples were weighed after being dried for 1, 2, 4, 8, 16, 32 and 64 h.

VFD: It was carried out in a vacuum freeze dryer (D2F6090, Shanghai Jinghong Laboratory Instrument Co., Ltd., Shanghai, China) with a fixed vacuum degree of 3×10^{-3} MPa at -50 ^{o}C. Three grams of citrus peel were put on a round sample tray with a diameter of 90 mm, which was placed in the drying chamber with the same distance from the tray to top heater and bottom heater of 15 cm. The samples were weighed after being dried for 1, 2, 4, 8, 16, 32 and 64 h.

SD: Three grams of citrus peel powder was laid on a round sample tray with a diameter of 90 mm and exposed to the sunlight from 9 a.m. to 4 p.m at ambient temperature in the range of 25± 5 ^{o}C with relative humidity within 30%–40%. The samples were placed in the dark room for the remaining time, which was not included in the drying time. Samples were dried by SD for 1, 2, 4, 8, 16 and 32 d, and then weighed.

As the moisture ratio (MR) was investigated to evaluate the effect of different drying methods, MR at each time during drying was evaluated as below:

$$MR(\%) = \frac{Wt - Wd}{Wf - Wd} \times 100\%$$

where W_d is the weight of dry sample, W_f is the weight of fresh sample, and W_t is the weight of sample at t time.

2.4 PMF extraction in citrus peel

The extraction of PMFs from fresh or dried citrus peel was performed according to our previous reports with some modification[28]. Two-step extraction with low-polar solvent (ethyl acetate) and polar solvent (distilled water) was carried out to extract the PMFs effectively. 3g of the fresh and the corresponding dried *hybrid citrus* peel powder samples were extracted with 20 mL ethyl acetate in a 50-mL tapered plastic centrifuge tube, and broken at 10000 rpm/min for 15 seconds and two times with a high-speed blender (ULTRA-TURRAX T25 digital, IKA, Germany). The ethyl acetate extraction was collected through suction filtration with a Buchner filter, and 20 mL distilled water was added. The water layer was extracted twice with equal volume of ethyl acetate. Finally, the combined 60 mL of ethyl acetate extracts were evaporated with a Rotary Evaporator (RE-2000B, Shanghai Yarong Co., Ltd., Shanghai, China), and dissolved in methanol for HPLC analysis.

2.5 Identification of demethylated PMFs by LC-MS/MS analysis

Identification of demethylated PMFs in the fresh and dried citrus peel was completed with a LC-MS/MS system (Agilent, Santa Clara, CA, USA). Separations were accomplished on an Agilent Eclipse XDB-C18 column (100 mm× 2.1 mm i.d., 3.5 μm) and the column temperature was 35 °C. The UV absorption wavelength was set as 326 nm, the injection volume was 10 μL and flow rate maintained 1 mL/min. The mobile phase system was comprised of 0.1% (v/v) formic acid in ultrapure water as mobile phase A and acetonitrile as mobile phase B for the LC separation. An optimal gradient program was applied in the elution: 0-5 min, 30% B; 5-10 min, 30%-40% B; 10-15 min, 40% B; 15-22 min, 40%-50% B; 22-30 min, 50%-65% B; 30-35 min, 65%-80% B; 35-40 min, 80% B. MS/MS analysis was implemented on a high resolution mass spectrometer (Agilent G6545 Q-TOF) with an electrospray ionization (ESI) source in positive ionization mode. Before entering the MS system, the column eluate was split to 0.4 mL/min. The parameters of the source were set as follows: nebulizer gas pressure 45.00 psi; dry gas flow 6.00 L/min; electrospray voltage 4000 V; capillary temperature 360 °C; target mass m/z 400; scan range m/z 100−1000; Fragmentor 150.0 V. Nitrogen was used as damping and sheath gas (> 99.99%). The data processing was performed on Agilent MassHunter workstation.

2.6 Synthesis of demethylated PMFs standards

5-demethylsinensetin (compound **5**), 5-demethylnobiletin (compound **6**), 5-demethylhexamethoxyflavones (compound **7**) and 5-demethyltangeretin (compound **8**) were chemically synthesized from compounds **1**, **2**, **3** and **4**, respectively, according to our previous study.[20] Taking compound **5** as an example, it was directly obtained through acid hydrolysis (reflux in 3 M HCl/MeOH for 72 h) from compound **1**, next it was separated from the reaction mixture by silica gel column with the eluent of dichloromethane/methanol (100: 1), and further purified using preparative thin layer chromatography (PTLC) with silica gel plates (GF254, Yantai, China). Compounds **6**, **7** and **8** were chemically synthesized from compounds **2**, **3** and **4** with the same method, respectively. The HPLC profiles of the synthetic 5-demethylated PMFs standards showed that their purities were up to 98%, and their chemical structures were identified by LC-MS/MS and ^{1}H NMR data according to previous reports.[13,14]

2.7 The simulation of acidolysis of PMFs

In order to demonstrate that PMF could undergo 5-demethylation via an acid hydrolysis mechanism in the citrus peel, we simulated the chemical environment of this reaction by using the following chemical methods. Each 3 mg PMF standard (compounds **1**-**4**) was dissolved in methanol, and the pH value was adjusted to 3.0 with diluted hydrochloric acid (the pH value of citrus peels was about 3.0), in which the total volume of the solution was 10 mL. After refluxed for 64 h under 90 ^oC and naturally cooled down to room temperature, the pH value was adjusted to 7.0 with saturated $NaHCO_3$ aqueous solution. The mixture was then extracted three times with equal volumes of ethyl acetate, and the combined 30 mL of ethyl acetate extracts were dried under vacuum and re-dissolved with methanol (30 mL) for HPLC analysis to detect whether PMF demethylation occurred.

2.8 The simulation of enzymatic demethylation of PMFs

Biological method was implemented to simulate enzymes in citrus fruit that catalyze the demethylation reaction of PMFs. Extraction of enzyme was carried out by differential centrifugation according to previous report with some modification.[29] Specifically, 2.5 g *hybrid citrus* peel with essential oil squeezed out were ground to a fine powder by a chilled mortar and pestle after liquid nitrogen refrigeration. 20 mL of ice-cold extraction buffer (250 mM sucrose, 50 mM pH 7.5 Tris, 1 mM DTT and 1 mM EDTA) were mixed with the *hybrid citrus* peel powder in a 50 ml tube. Then, the supernatant was collected after high-speed shearing (40 S) with a high-speed blender at 8000 rpm/min. The homogenate was filtered through three layers of Miracloth, and then centrifuged at high-speed (10000 rpm/min, 40 min) at 4 ^{o}C. Finally, after ultracentrifugation (50000 rpm/min, 90 min) of the supernatant at 4 ^{o}C, the microsomal pellet was obtained, and 1 mL of the ice-cold extraction buffer were used to re-suspend the precipitated enzyme. 3 mg of each PMF standard (compounds **1**-**4**) was dissolved with DMSO, and mixed with 1 ml of the fresh enzyme buffer solution, in which the content of DMSO was less than 0.5% (v/v). After incubation at 37 ^{o}C for 12 h, the mixture was extracted three times with equal volumes of ethyl acetate. The combined ethyl acetate extracts were dried under vacuum and re-dissolved with methanol (20 mL) for HPLC analysis to detect whether PMF demethylation occurred.

2.9 Quantification of PMFs & demethylated PMFs, and calculation of PMF demethylation ratio

Quantitative analysis of PMFs and demethylated PMFs in fresh and dried citrus peel was completed by HPLC with the method mentioned above. The demethylation ratio of PMFs was calculated as follows.

$$\text{PMF demethylation ratio (\%)} = \frac{C_{(5\text{-demethylated PMF})}}{C_{(PMF)} + C_{(5\text{-demethylated PMF})}} \times 100\%$$

where $C_{(5\text{-demethylated PMF})}$ is the concentration of 5-demethylated PMF in sample, and $C_{(PMF)}$ is the concentration of PMF in sample. The demethylation ratio of PMFs could accurately reflect the content of demethylated PMFs in dried citrus peel samples. With this method, the PMF demethylation at different drying time and temperature in HAD, and other drying methods were measured to analyze the content changes of demethylated PMFs in different drying procedures.

2.10 Data analysis

The results of the drying experiments were reported as means and standard deviations based on dry basis, which were calculated by Origin 8.0 (OriginLab Inc., Northampton, MA, USA). The data were subjected to the analysis of variance (ANOVA), and the significance of the difference between means was determined by Duncan's multiple range test ($p < 0.05$) using SPSS 22.0 (IBM SPSS Inc., Chicago, IL, USA). All individually extracted samples were analyzed in triplicates.

3 Results and discussion

3.1 Identification of 5-demethylated PMFs generated in citrus peel during drying processes

Drying is an important method of processing for citrus peel. Previous studies have reported the change of total flavonoid content in citrus peel during drying processes.[9] In this study, the effects of different drying methods and conditions on PMF structures and contents were determined by LC-MS/MS. As shown in HPLC profiles (**Fig. 1a**), the contents of compounds **1-4** in citrus peel decreased, while compounds **5-8** increased after hot-air drying for 4 h at 60 °C. In order to elucidate the conversion relationship between those compounds, MS/MS was used to determine the chemical structures. Based on the core flavone structure, PMFs are more likely to lose methyl radicals ($CH_3^•$) in their structures and yield the basic fragment peaks, which included the major characteristic fragments of $[M+H-n\times15]^+$ and those losing CH_4 (16), H_2O (18), CO (28), CH_4+CH3$^•$ (31), $H_2O+CH_3^•$ (33), $CO+CH_3^•$ (43), CO_2 (44), $CO+H_2O$ (46), 4CH3$^•$ (60) or $CO+H_2O+CH_3^•$ (61) to form diagnostic fragments.[30-32] As shown in **Fig. 1b**, the LC-MS/MS spectra of compounds **1-8** demonstrated the characteristic peaks of individual PMFs. For the permethoxylated PMFs, taking compound **1** as an example, combination with element matching, the quasi-molecular ($[M+H]^+$) ion at m/z 373.1209 was identified as $C_{20}H_{20}O_7$. Compared to the basic flavone structure, this compound was speculated as a pentamethoxyflavone. Prominent ions at m/z 343.0811 $[M+H-2CH_3]^+$ and m/z 357.09582 $[M+H-CH_3]^+$ by loss of 30 Da ($2CH_3$) and 15 Da (CH_3) from the precursor ion $[M+H]^+$, respectively, were identified as the characteristic fragment ions of PMFs. In addition, there were relatively low abundance ions that were the products of the fragmentation pathways of retro-Diels−Alder (RDA) cleavage from the 1,4-position of the C-ring. The fragment ions at m/z 181.0119 $[^{1,3}A^+-2CH_3]$ and m/z 163.0751 $[^{1,3}B^+]$ indicated that there were three methoxyl substituents on ring A and two on ring B. By comparing the retention time and UV absorption, MS/MS spectrum library and ^1H NMR data,[32-34] compound **1** was identified as sinensetin. Similarly, compounds **2-4** were identified as nobiletin, heptamethoxyflavone and tangeretin, respectively (**Table 1**). For the 5-demethylated PMFs, taking compound **5** as an example, its quasi-molecular ion ($[M+H]^+$) at m/z 359.1053 indicated the molecular formula as $C_{19}H_{18}O_7$ by element matching. The similar fragmentation profile with compound **1** indicated compound **5** was a sinensetin derivative. Compared with compound **1**, only a 14 Da (CH_2) was lost in its molecular structure, which suggested that demethylation might be involved. The fragmentation pathways of RDA produced relatively low abundance ions at m/z 167.0174 [$^{1,3}A^+{-}2CH_3$] and m/z 163.0751 [$^{1,3}B^+$], which suggested that the demethylation occurred on A-ring of compound **1**. Combined with the retention time and UV absorption, compound **5** was identified as 5-demethylsinensetin.[32-34] Following the same pattern (**Table 1**), compounds **6-8** were identified as 5-demethylnobiletin, 5-demethylheptamethoxyflavone and 5-demethyltangeretin, respectively. They are the corresponding 5-demethylated products of compounds **2-4**, respectively.

In order to further identify and quantify PMFs and demethylated-PMFs, compounds **5-8** were synthesized as reference standards according to our previous report (**Fig. 2a**).[20] It has been reported that the neighboring participation effect could conduce to the demethylation of PMFs on the 5-position of the A-ring under acidic condition.[24] A proton from hydrochloric acid could be coordinated with the two oxygen atoms from 4-carbonyl atom and 5-methoxy group, respectively, forming a stable six element ring in structure; Thus, it was more easily broken down between

5-oxygen and its connected methyl group to form 5-hydroxyl. HPLC analysis of the synthesized demethylated-PMFs and standards mixture. And they were further confirmed through LC-MS analysis. All of the compounds **1-8** could be separated completely through HPLC with the retention time of 14.8, 18.3, 20.8, 22.9, 22.4, 26.0, 28.0, and 29.7 min, respectively. The identification of demethylated-PMFs in dried citrus peels were confirmed by comparing their retention time with that of synthesized standards through HPLC analysis (**Fig. 2b**). In addition, the standard curves for compounds **1-8** were plotted as follows: y=20.2x-34.6 (r^2=0.9976), y=13.5x-14.2 (r^2=0.9993), y=11.3x-5.3 (r^2=0.9993), y=16.4x+3.3 (r^2=0.9993), y=12.9x-8.8 (r^2=0.9989), y=8.8x-1.9 (r^2=0.9989), y=9.0x-2.1 (r^2=0.9991), y=17.0x-9.9 (r^2=0.9993), respectively. The linearity ranges for compounds **1** and **2** were 0.1 to 200 μM, and the others (compounds **3-8**) were 0.1 to 100 μM. Based on these positive linear relationships between the response value and concentration, quantification of the compounds were successfully conducted. Generally speaking, the content of PMFs **1-4** significantly decreased, while 5-demethylated PMFs **5-8** significantly increased after drying, which demonstrated the conversion of permethoxylated PMFs to 5-demethylated PMFs in citrus peel during the drying process. The effects of different drying methods and conditions on PMF demethylation were further investigated in the following sections.

3.2 Two mechanisms of PMF demethylation during drying of citrus peel

According to our measurement, pH values of fresh *hybrid citrus* peel and *valencia orange* were 2.80 ± 0.14 and 3.32 ± 0.09, respectively. Our previous studies have provided evidence for potential acidolysis of PMFs to 5-demethylated PMFs by stomach acid *in vivo*.[34] In addition, acid hydrolysis could also efficiently facilitate the demethylation of PMFs on the 5-position of the A-ring via chemical reaction, e.g. 3M hydrochloric acid.[20] Therefore, we hypothesized that in citrus peel, especially during the drying process, the acidic environment promoted the PMF demethylation. In order to verify this hypothesis, the acidic tissue condition of citrus peel was simulated in an *in-vitro* chemical reaction (pH= 3.0). As shown in **Fig. 3**, no demethylated-PMF was produced after 64 h at 30 ^{o}C in the solution of 4 permethoxylated PMFs standards (**1-4**) without addition of acid. Interestingly, after reflux at 90 ^{o}C for 64 h with pH of 3, 5-demethylated PMFs (compounds **5-8**) can be formed; and under this simulated acidolysis condition, the demethylation ratios of compounds **1-4** were 1.10%, 0.23%, 0.10% and 0.85%, respectively. The neighboring participation effect was the main reason for the easier conversion of PMFs to their 5-demethylated PMF counterparts under acidic condition.[24] Similarly, stomach acid *in vivo* could also induce the PMF demethylation on 5-position.[34] Therefore, an acidic environment could promote PMF demethylation, which suggests that acid hydrolysis is one of the mechanisms of PMF demethylation. This mechanism could be used to interpret the effect of acidolysis on the PMF demethylation ratios during drying process of citrus peels; from this point of view, lowering pH was beneficial to PMF demethylation in dried citrus peel.

Meanwhile, there are many enzymes in the genus Citrus, which can catalyze a series of reactions of the compounds in citrus. Thus, we hypothesized that enzyme catalysis might be another mechanism for PMF demethylation during citrus peel drying. To test this hypothesis, extraction of enzymes from fresh citrus peel and co-incubation of the enzymes and PMFs were carried out, and HPLC was also used to determine the extent to which PMF demethylation took place. As shown in **Fig. 3**, no demethylated-PMFs produced in the PMF solution without addition of enzymes from citrus peel; whereas, four 5-demethylated PMFs were produced in the PMF solution after treatment with enzyme from citrus peel. The observed demethylation ratios of compounds **1**-**4** were 1.40%, 0.31%, 0.15% and 0.60%, respectively. These results indicate the catalysis of enzyme could lead to the transformation of permethoxylated PMFs (**1-4**) to 5-demethylated PMFs (**5-8**) during citrus peel drying. Hence, enzymatic catalysis was confirmed to be another potential mechanism for the PMF demethylation that occurred in citrus peel. It was reported that cytochrome P450s, the main metabolic enzymes *in vivo*, have been found to catalyze flavonoid demethylation.[22,35] And this kind of enzymes also existed in citrus cells.[36] Moreover, for the simulation of enzymatic demethylation of PMFs, extraction of enzyme from citrus peel was carried out by following the procedure for extracting cytochrome P450 enzyme from plant according to previous report.[29] Therefore, we hypothesized that the enzyme that catalyzes the demethylation of PMFs on the 5-position of the A-ring during the drying processes of citrus peel might be cytochrome P450 enzymes. However, more definite and direct evidence need to be explored to prove the crude enzymes isolated from citrus peel is really the P450 enzymes. Different from human P450 enzymes, which mainly catalyzes the demethylation on 3'-position and 4'-position,[22,23] our results indicate that the potential enzymes in citrus peel might favor demethylation of C5. These findings also help understand PMF demethylation during the citrus peel drying process. During drying processing, the acidic tissue environment and enzyme exist simultaneously in citrus peel, which would promote the conversion from permethoxylated PMFs to 5-demethylated PMFs. The understanding of the mechanism of PMF demethylation could help better control the PMF demethylation in citrus peel during food processing.

3.3 Effects of different HAD drying conditions on PMF demethylation

Hybrid citrus peels were dried by HAD for 32 h at 40, 50, 60 and 70 ^{o}C, respectively. Overall, drying time and temperature had significant influence on the moisture ratio of *hybrid citrus* peel and PMF demethylation (**Fig. 4**). The demethylation ratios of the 4 PMFs (**1-4**) shared the same trend, in which the ratio increased rapidly first, then leveled out gradually with a slight decline and finally increasing slowly with the extension of drying time at each temperature. It should be noted that there was a significant drop in demethylated-PMF content during the ascent, which was a critical point during prolonged drying. In the initial period of drying, the moisture ratio in the sample was higher (**Fig. 4a**) and the rapid increase of the PMF demethylation ratio appeared to be associated with the combined effects of enzymes and acid in citrus peel, and the enzyme might be the main contributing factor impacting demethylation. As drying went on, the decrease of moisture ratio in samples might lead to the decrease of enzyme activity resulting in the significant decrease of the PMF demethylation reaction rate. In addition, flavonoids could also be oxidized and decomposed by other enzymes like oxidase during drying.[11] After reaching the lowest moisture ratio, catalysis of the enzymes disappeared and the effect of pH became the predominant factor. The decrease of moisture ratio also led to lower pH value in the peels, which accelerated the demethylation; therefore the demethylation ratios increased slowly along with drying time after reaching the lowest moisture ratio. Although the trends of PMF demethylation for the 4 PMFs were similar, the demethylation ratios of compound **1** was the highest, followed by **2**, **3** and **4** (**Fig. 4b**, **4c**, **4d**, and **4e**).

Our results showed that the demethylation ratios of PMFs varied at different temperature. And there were slight differences in demethylation between these PMFs with HAD by different temperature. Taking compound **1** as an example, after drying for 8 h, the demethylation ratio varied from 11.17 ± 0.09% at 60 °C to 9.31 ± 0.10% at 70 °C (**Fig. 4b**). The significant drop in the PMF demethylation ratios existed at all temperatures; the higher the heating temperature was, the earlier this point appeared. At 40 °C, the drop point of compound **1** was at 4 h, while the critical time point advanced to 2 h when the drying temperature was 60 °C. The moisture ratio decreased faster when the temperature rose from 40 °C to 60 °C, which was in accordance with the drop point of demethylation. Meanwhile, it is noteworthy that the temperature for the highest demethylation ratio of different PMFs also varied. In general, higher temperature was more conducive to demethylation. For example, the demethylation ratio of compound **1** reached its maximum at 60 °C while for compound **3** it was at 70 °C; It has been reported that the activity of many enzymes in fruits and vegetables could be improved at relatively high temperatures, for example, the optimum temperature at which peroxidase reacted with guaiacol was 60 ^{o}C.[37] Similar to these enzymes, the optimum temperature of enzymes in citrus was between 60 and 70 ^{o}C, and a higher drying temperature led to a more acidic condition that was conducive to PMF demethylation.[20] Whereas there were some exceptions for some PMFs, and the highest demethylation ratio of compound **2** was at 40 ^{o}C. The reason might be that the demethylation of different PMFs might be dominated by different enzymes, for which the optimum temperature is different. Moreover, as an endothermic reaction, PMF demethylation might adsorb the heat energy provided by relatively high temperature, resulting in the acceleration of the 5-demethylated PMF formation process. Flavonoids are mainly deposited in vacuoles within the cellular structure of citrus peel,[9,38] and as drying temperature increased, the cellular structure was gradually destroyed, which led to the PMF release, which increased the contact between PMFs and enzymes and in turn resulted in accumulation of 5-demethylated PMFs. However, the demethylation ratio of compound **1** was reduced significantly at 70 °C, which suggests that high temperature might lead to degradation of the 5-demethylated PMFs. During HAD processing of *valencia orange* peel, a similar trend was observed for PMF demethylation (**Fig. S1**).

3.4 Effects of different drying methods on PMF demethylation

Citrus peel can be traditionally processed into dried products which are widely used as healthy medical herbs and food ingredients. Herein, we investigated the effects of three prevailing conventional dehydration techniques (HAD, VFD and SD) for citrus peel drying on PMF demethylation. For the *hybrid citrus* peels, the moisture ratio of the peels and demethylation ratios of the 4 PMFs dried by VFD and SD processes were shown in **Fig. S2**. The moisture ratio of citrus peel during the two processes decreased rapidly over time (**Fig. S2a** and **S2b**). The demethylation ratio trends of the 4 PMFs during VFD and SD were roughly similar to those in the HAD process. Specifically, the demethylation ratio increased rapidly first to reach a relatively high level, then declined slightly, and finally increase slowly during the remaining drying time (**Fig. S2c** and **Fig. S2d**). The demethylation ratio of compound **1** was also the highest, followed by **3**, **2** and **4**. Generally, the four major PMFs in the samples shared the same demethylation trend in the different drying methods. The highest demethylation ratio was found in SD samples followed by HAD and VFD samples, which were all significantly higher than that of the fresh samples (**Fig. 5a)**. For example, in the three drying processes, the demethylation ratio of compound **2** ranged from 6.56 ± 0.29% to 7.46 ± 0.10%, which was higher than that of the fresh sample (4.79 ± 0.02%). Herein, the results showed that the drying processes could facilitate demethylation effectively and different drying technologies produced variable demethylation ratios. As for SD samples, the content of 5-demethylated PMFs were the highest after 16 days of sun-drying. Both acidic tissue environment and enzymes exist simultaneously in citrus peel contribute to for the conversion from permethoxylated PMFs to 5-demethylated PMFs during SD process. Notably, the enzymes effect might be the most significant factor. Because the time extension of SD at mild temperature was conducive to the contact of PMFs with oxygen and enzyme, which gave more chance to produce 5-demethylated PMFs. This led to sufficient accumulation of 5-demethylated PMFs in SD samples. However, SD is often time-consuming and the most vulnerable to contamination by a variety of debris such as dust, sand and litter, as well as exposure to bacteria, parasites, birds and insects. Another limitation of SD is the changeable and unpredictable weather that can also restrict its application. In practice, the quality difference of the citrus peel dried by SD was remarkable, and also this drying method could not meet the demand of wholesale industrialization. The lowest amounts of 5-demethylated PMFs were found in the VFD samples, which indicates that a lower amount of oxygen might restrict the production of the 5-demethylated PMFs and a lower temperature might inhibit the enzyme in citrus peel. Notably, frozen water formed inside the cell[39] might be another factor that could not be ignored, as it might significantly limit PMF demethylation. Taking these findings into consideration, HAD appears to be a practical and economical method to dry citrus peels, for the production of 5-demethylated PMFs. As for *valencia orange* peels, the trends of the moisture ratio and PMF demethylation ratios during VFD and SD processes (shown in **Fig. S3**) were consistent with the results obtained during the HAD process. As shown in **Fig. 5b**, for different drying methods, compounds **1**, **2** and **4** in *valencia orange* peels shared the same demethylation trend, and the highest demethylation ratio was observed in SD samples followed by HAD, VFD and the fresh samples. These results of *valencia orange* peels further confirmed the trend of PMF demethylation during different drying processes.

4 Conclusion

In summary, this study for the first time discovered demethylation reaction of permethoxylated PMFs to produce corresponding 5-demethylated PMFs in citrus peel during different drying processes. The PMF demethylation was simulated under chemical and biological conditions, which revealed the two demethylation mechanisms (acid hydrolysis and enzymatic catalysis) in citrus peel. And cytochrome P450 enzymes might be the enzyme that catalyzes the demethylation of PMFs on the 5-position of the A-ring during the drying processes. However, more definite and direct evidence need to be explored. The influence of different drying processes on the PMFs demethylation was also systematically investigated, and the dominant demethylation mechanism depended on the moisture ratio of the citrus peel. HAD was the most appropriate choice for drying citrus peel in a large scale to obtain high content of 5-demethylated PMFs as compared with VFD and SD. In addition, the optimal HAD operating conditions were determined to be at 60 ^{o}C for 4 h. The results obtained in this study provided valuable scientific basis for the rational control of PMF demethylation in citrus peel, as well as for the production of high quality citrus peel and related products.

Conflicts of interest

The authors declare no competing financial interest.

Acknowledgements

We would like to acknowledge the financial support of the National Natural Science Foundation of China (Nos. 31401581 and 31428017), Collaborative Innovation of Chinese Academy of Agricultural Sciences

(CAAS-XTCX201605-1~6), a Special Fund for Supervision of Quality and Safety of Agricultural Products (Risk Assessment) from the Chinese Ministry of Agriculture and Rural Affairs (GJFP 2018011), and fund from United States Department of Agriculture. We would also acknowledge the support of Elite Youth Program (Chinese Academy of Agricultural Sciences).

Supplementary Material

Supplementary data for the moisture ratio and demethylation ratios of 4 PMFs in *hybrid citrus* peels and *valencia orange* peels during HAD, VFD and SD processes.

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Figure captions

Fig. 1 HPLC profiles (a) and MS/MS spectra (b) of flavonoids extraction in *hybrid citrus* peels before and after hot-air drying for 4 h at 60 ^{o}C.

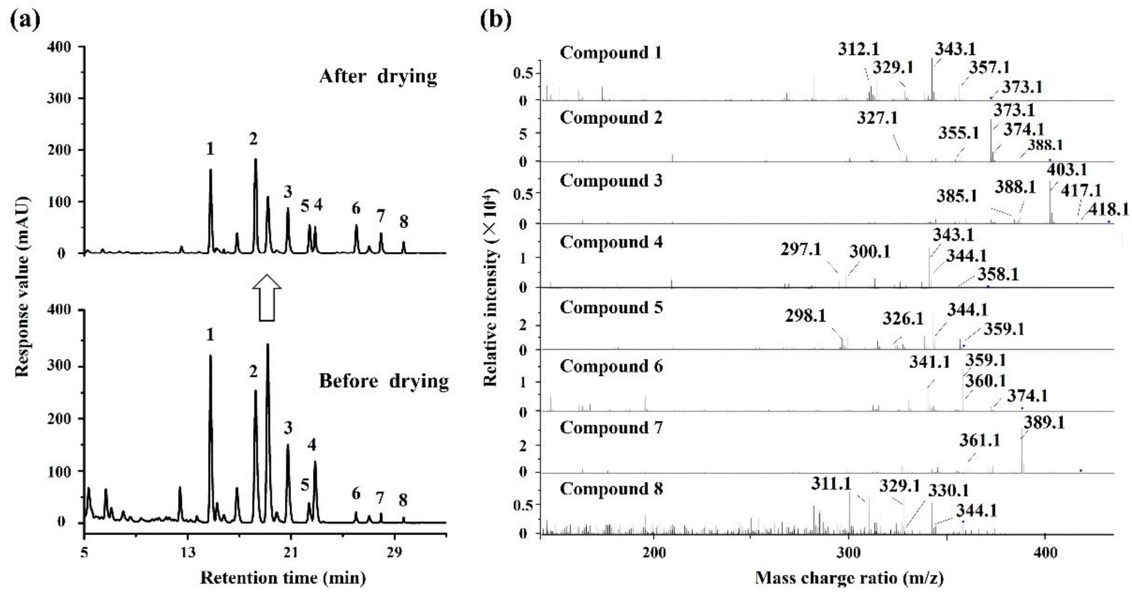
Fig. 2 Synthetic schemes of 5-demethylated PMF standards (a), and their HPLC validation (b). (Compounds **1**-**8** are sinensetin, nobiletin, heptamethoxyflavone, tangeretin, 5-demethylsinensetin, 5-demethylnobiletin, 5-demethylhexamethoxyflavone, and 5-demethyltangeretin, respectively).

Fig. 3 HPLC profiles of PMF standards, PMF solution after placed at 30 ^{o}C for 64 hours, acid hydrolysis of PMFs, and enzyme treatment of PMFs (a), and their enlarged views (b).

Fig. 4 The moisture ratio (a), and the demethylation ratios of sinensetin (b), nobiletin (c), heptamethoxyflavone (d) and tangeretin (e) in *hybrid citrus* peels during HAD process.

Fig. 5 Effects of different drying methods (F, fresh sample; HAD, hot air drying at 60 ^{o}C for 4 h; VFD, vacuum freeze drying for 2 h; SD, sun drying for 16 d) on the demethylation ratios of PMFs (sinensetin, nobiletin, heptamethoxyflavone and tangeretin) in *hybrid citrus* peels (a) and *valencia orange* peels (b). Means ± standard deviations and different letters presented the demethylation ratio data of PMFs and significant difference between PMFs with $P < 0.05$, respectively.

Table 1 LC-MS/MS characterization of compounds **1-8** in the *hybrid citrus* peels after drying.



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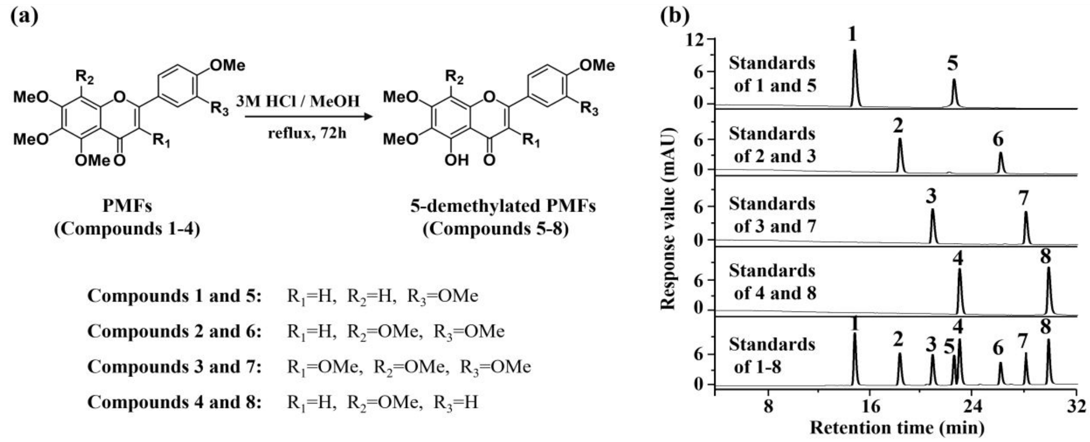

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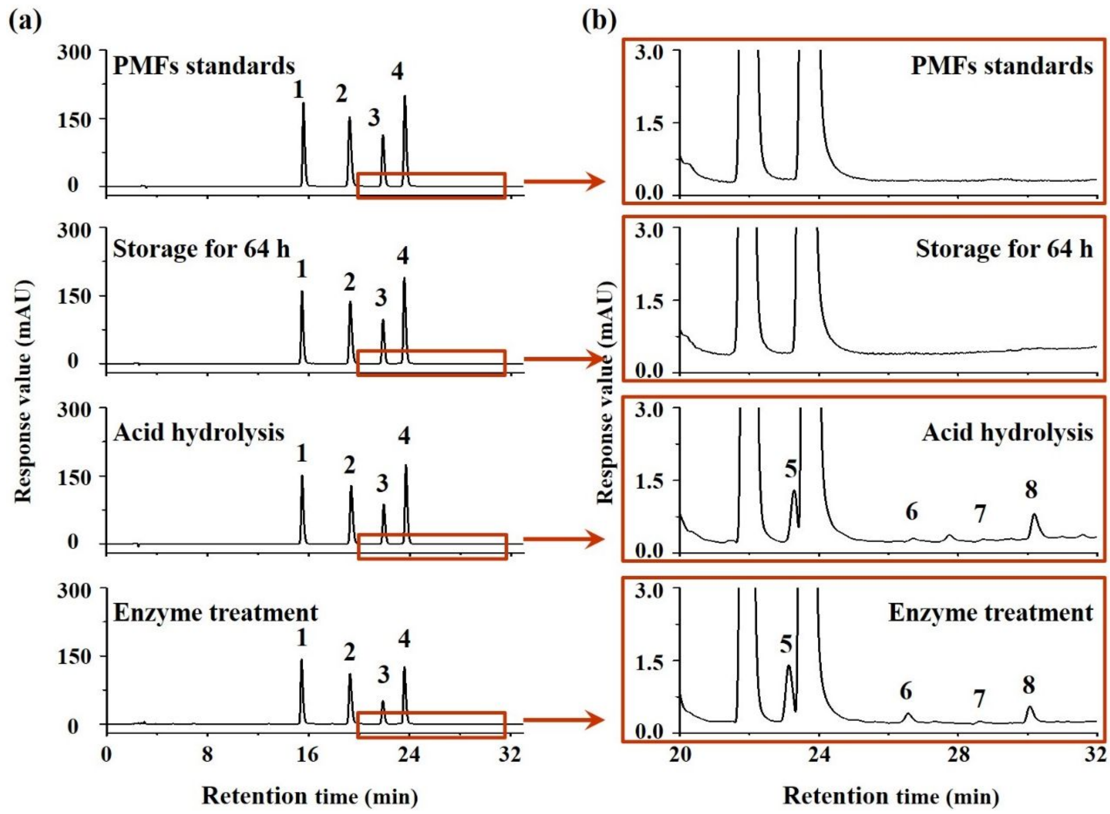

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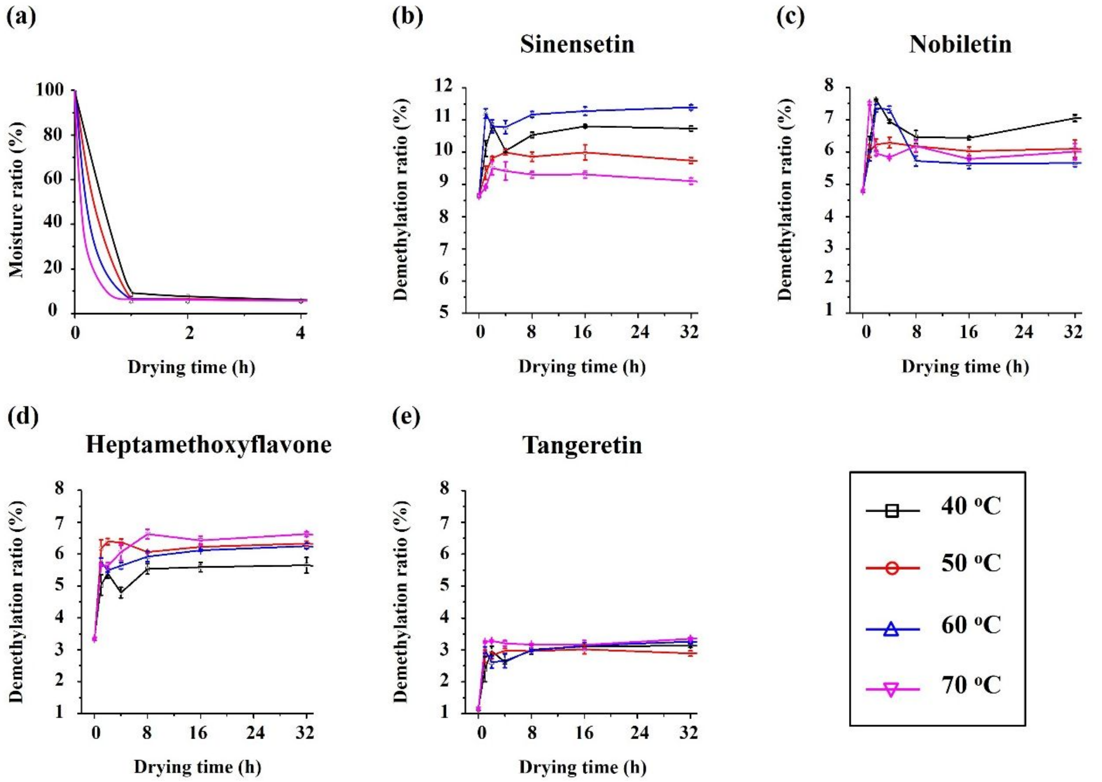

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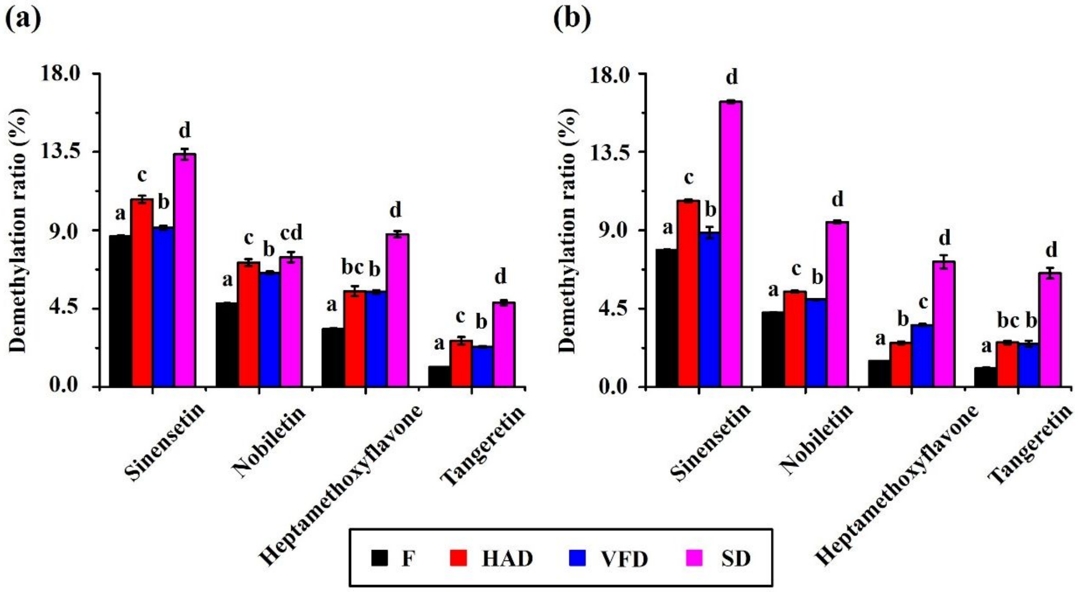

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Table 1 LC-MS/MS characterization of compounds **1-8** in the *hybrid citrus* peels after drying.

No	t_R/min	Formula	$[M+H]^+$	Assignment	MS^E ion fragments		Abundance/%
1	14.8	$C_{20}H_{20}O_7$	373.1	sinensetin	358.1	$[M+H-CH_3]^+$	7
					357.1	$[M+H-CH_4]^+$	36
					343.1	$[M+H-2CH_3]^+$	100
					329.1	$[M+H-CH_4-H_2O]^+$	22
					312.1	$[M+H-CH_3-CO-H_2O]^+$	32
2	18.3	$C_{21}H_{22}O_8$	403.1	nobiletin	388.1	$[M+H-CH_3]^+$	1
					374.1	$[M+H-CH_3-CH_2]^+$	20
					373.1	$[M+H-2CH_3]^+$	100
					355.1	$[M+H-2CH_3-H_2O]^+$	6
					327.1	$[M+H-CH_3-CO-H_2O]^+$	16
3	20.8	$C_{22}H_{24}O_9$	433.1	heptamethoxyflavone	418.1	$[M+H-CH_3]^+$	2
					417.1	$[M+H-CH_4]^+$	4
					403.1	$[M+H-2CH_3]^+$	100
					388.1	$[M+H-3CH_3]^+$	15
					385.1	$[M+H-2CH_3-H_2O]^+$	8
4	22.9	$C_{20}H_{20}O_7$	373.1	tangeretin	358.1	$[M+H-CH_3]^+$	4
					344.1	$[M+H-CH_3-CH_2]^+$	20
					343.1	$[M+H-2CH_3]^+$	100
					300.1	$[M+H-3CH_3-CO]^+$	32
					297.1	$[M+H-2CH_3-CO-H_2O]^+$	19
5	22.4	$C_{19}H_{18}O_7$	359.1	5-demethyl-sinensetin	344.1	$[M+H-CH_3]^+$	25
					326.1	$[M+H-CH_3-H_2O]^+$	4
					298.1	$[M+H-CH_3-CO-H_2O]^+$	13
					211.0	$^{1,2}A^+$	14
					163.1	$^{1,3}B^+$	2
6	26.0	$C_{20}H_{20}O_8$	389.1	5-demethyl-nobiletin	374.1	$[M+H-CH_3]^+$	2
					360.1	$[M+H-CH_3-CH_2]^+$	26
					359.1	$[M+H-2CH_3]^+$	100
					341.1	$[M+H-2CH_3-CO]^+$	40
					197.0	$[^{1,3}A^+-2CH_3]^+$	34
7	28.0	$C_{21}H_{22}O_9$	419.1	5-demethyl-hexamethoxy flavone	389.1	$[M+H-2CH_3]^+$	100
					361.1	$[M+H-2CH_3-CO]^+$	21
					227.1	$^{1,3}A^+$	2
					197.0	$[^{1,3}A^+-2CH_3]^+$	3
8	29.7	$C_{19}H_{18}O_7$	359.1	5-demethyl-tangeretin	344.1	$[M+H-CH_3]^+$	23
					330.1	$[M+H-CH_3-CH_2]^+$	3
					329.1	$[M+H-2CH_3]^+$	88
					311.1	$[M+H-2CH_3-H_2O]^+$	83
					197.0	$[^{1,3}A^+-2CH_3]^+$	45