# Hydroxylated Polymethoxyflavones and Methylated Flavonoids in Sweet Orange (*Citrus sinensis*) Peel

SHIMING LI, CHIH-YU LO, AND CHI-TANG HO*

Department of Food Science, Rutgers University, 65 Dudley Road, New Brunswick, New Jersey 08901-8520

Polymethoxyflavones (PMFs) from citrus genus have been of particular interest because of their broad spectrum of biological activities, including antiinflammatory, anticarcinogenic, and antiatherogenic properties. There have been increasing interests in the exploration of health beneficial properties of PMFs in citrus fruits. Therefore, the isolation and characterization of PMFs from sweet orange (*Citrus sinensis*) peel will lead to new applications of the byproducts from orange juice processes and other orange consumption in nutraceutical and pharmaceutical products. In our study, eight hydroxylated PMFs, six PMFs, one polymethoxyflavanone, one hydroxylated polymethoxyflavanone, and two hydroxylated polymethoxychalcones were isolated from sweet orange peel and their structures were elucidated by various MS, UV, and different NMR techniques. Some of the hydroxylated PMFs and chalcones are newly isolated from sweet orange peel.

**KEYWORDS: Sweet orange peel (*Citrus sinensis*); hydroxylated polymethoxyflavones; polymethoxyflavonoids; chalcones; isolation and characterization**

## INTRODUCTION

Citrus production worldwide in selected major producing countries in 2003−2004 was 73.1 million metric tons (161 billion lbs). Total citrus production in the United States was 14.85 million metric tons (33 billion lbs) (National Agricultural Statistics Service). Around 34% of these products were used for juice production (*1*), yielding approximately 44% (4−5 billion lbs in the United States) of peels as byproducts. In some regions of the world, orange peel has been a traditional medicine for relieving stomach upset, skin inflammation, muscle pain, and ringworm infections. Its major constituents include flavonoids, mainly polymethoxylated flavonoids; terpenoids, such as limonene and linalool; and other volatile oils. Traditionally, orange peels were processed to obtain the volatile and nonvolatile fractions for some applications in foods, drugs, and cosmetics. However, the application of these extracted chemicals is limited because the overall demand for these compounds is insignificant.

Citrus flavonoids, especially polymethoxyflavones (PMFs), have been of particular interest because many of these flavonoids exhibit a broad spectrum of biological activity, including antiinflammatory, anticarcinogenic, antiviral, antioxidant, antithrombogenic, and antiatherogenic properties (*2*−*13*). PMFs exist almost exclusively in the citrus genus, particularly in the peel of sweet oranges (*Citrus sinensis*) and mandarin oranges (*Citrus reticulata*). So far, there are more than 20 polymethoxylated flavonoids being isolated and identified from different tissues of citrus plants. The types and contents of PMFs vary among different varieties of citrus species (*1*, *2*). Thus, the types and concentrations of PMF may serve taxonomic purposes in botanical and agricultural sciences.

Polymethoxyflavonoids have been shown to block adhesion molecule biosynthesis by cytokine-induced endothelial cells, to block activation-induced degranulation of neutrophils and mast cells, to inhibit expression of tumor necrosis factor-α (TNFα), to reduce the invasiveness of tumors in animal models, to induce the differentiation of myeloid leukemic cells, to suppress proliferation while promoting apoptosis, to reduce lymphocyte proliferation and platelet aggregation, and to suppress ethanol-induced gastric hemorrhagic lesions while promoting chloride secretion by human colonic epithelial cells etc. (*4*, *12*, *13*). Because of the hydrophobic nature of methoxy groups as compared to hydroxyl groups, PMFs are more lipophilic as compared to polyhydroxylated flavonoids, such as quercetin, luteolin, and narigenin, etc. Therefore, the PMFs may have higher permeability through the small intestine and are readily absorbed into the blood circulation system of the human body (*14*, *15*).

The isolation and biological activities of polymethoxyflavonoids and their related compounds or derivatives have been of particular interest. During the course of our biological activity studies of polymethoxyflavonoids from sweet orange (*C. sinensis*) peel (cold-pressed oil), eight hydroxylated PMFs, six PMFs, one polymethoxyflavanone, one hydroxylated polymethoxyflavanone, and two hydroxylated polymethoxychalcones were isolated (**Figure 1**) and their structures were elucidated by liquid chromatography−electrospray ionization−mass spectrometry (LC-ESI-MS), electron impact (EI)-MS, high-resolution mass spectrometry (HRMS), UV, and NMR [$^{1}$H, $^{13}$C, and two-

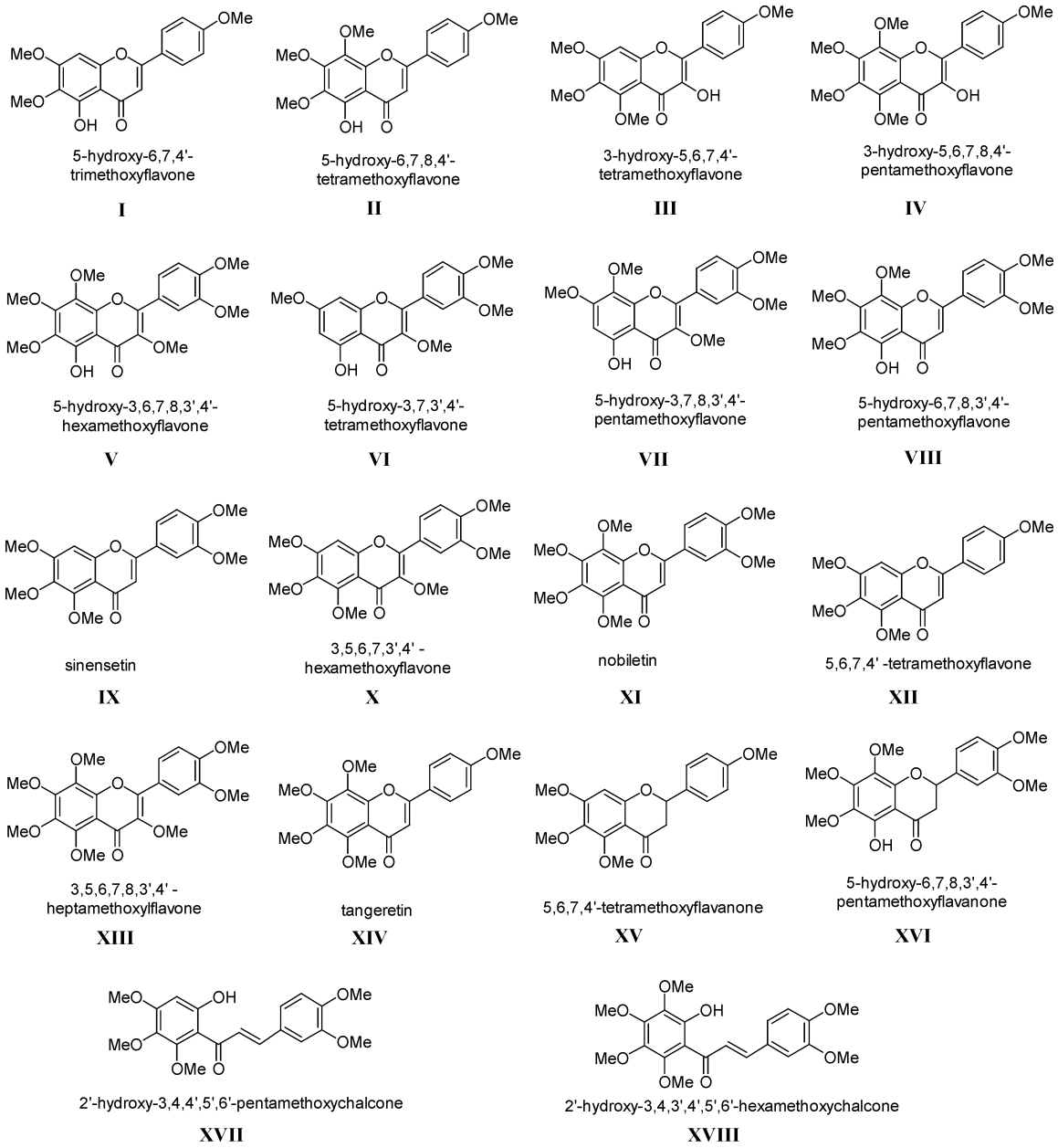



**Figure 1.** Hydroxylated PMFs, PMFs, polymethoxyflavanones, and polymethoxychalcones isolated from sweet orange peel.

dimensional (2D)] and were found to be compatible with available known structures of the same compounds. This paper reports the isolation and characterization of these compounds existing in sweet orange peel.

## MATERIALS AND METHODS

**Materials.** Sweet orange peel extract (OPE, from cold-pressed orange peel oil) was obtained from Florida Flavors Co. (Lakeland, FL). Prepacked silica gel (60 Å, 32−63 $\mu$m) columns, whose sizes were 4, 12, 40, 80, 120, and 330 g, for normal phase chromatography and octadecyl ($C_{18}$) derivatized silica gel (60 Å) for reversed phase flash chromatography were purchased from Teledyne Isco, Inc. (Lincoln, NE). Octadecyl ($C_{18}$) derivatized silica gel (60 Å) reversed phase analytical and preparative columns for high-performance liquid chromatography (HPLC) were purchased from YMC Inc. (Kyoto, Japan). Chiral analytical and preparative columns of Regis Welk-O 1 (R,R) were purchased from Regis Technologies, Inc. (Morton Grove, IL).

**Flash Column System.** An automated flash chromatography system (model Foxy 200, sg100, Isco Inc.) equipped with a prepacked silica gel (particle size 35−60 $\mu$m) flash column from Teledyne Isco Inc. was used. The mobile phase for normal phase flash column consisted of either ethyl acetate and hexanes or 2-propanol and hexanes in varying proportions, and the flow rate was varied between 40 and 100 mL/min depending on the column size. The eluent was monitored with a single channel UV detector at a wavelength of 254 nm.

**HPLC System.** An HPLC equipped with a pump (Waters Delta Prep 4000 delivery pump, Milford, MA), UV−vis detector (Waters 486 tunable absorbance detector), and an injector (Waters U6K injector) was used. A Regis Welk-O 1 (R,R) 450 g column (Regis Technologies, Inc.) was used for the HPLC system. The mobile phase for the HPLC system was 35% absolute ethanol and 65% hexanes with a flow rate set at 90 mL/min. The eluent was detected with a UV wavelength at 326 nm.

**NMR Instrument.** NMR spectra were recorded on a Varian 300 and Varian 500 Spectrometer (Varian, Inc., Palo Alto, CA). With tetramethylsilane serving as the internal standard, $^{1}$H NMR was recorded at 300 and 500 MHz; $^{13}$C NMR was recorded at 75 and 125 MHz.

**Mass Spectrometer.** EI-MS spectra were obtained on a MicroMass AutoSpec HF (Micromass, Beverly, MA). MS conditions: mass scan range, 100−1500 amu; 250 °C (EI-MS).

**LC-ESI-MS.** An HPLC-MS system was composed of an autosampler injector (Switzerland), an HP1090 system controller, with a variable UV wavelength 190−500 nm) detector, an evaporizing laser-

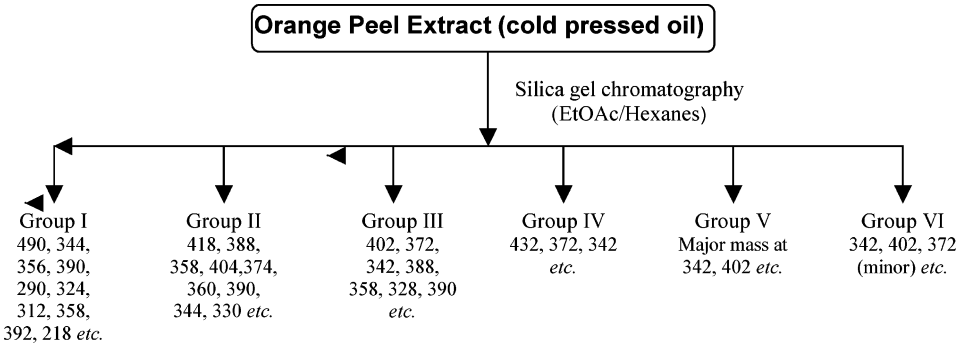



**Figure 2.** Detected molecular weight of compounds from OPE.

scattered deposition detector, and an ESI-MS detector from Micromass VG Platform II mass analyzer (Micromass). ESI-MS conditions were as follows: acquisition mode, ESI positive; mass scan range, 100−800 amu; scan rate, 0.4 s; cone voltage, 25 V; source temperature, 150 °C; and probe temperature, 550 °C. Analytical HPLC conditions on HPLC-MS: column, Chromeabond WR $C_{18}$, 3 $\mu$m, 120 Å; length and OD, 30 mm × 3.2 mm; injection volume, 15 $\mu$L; flow rate, 2 mL/min; and run time, 3 min. The mobile phase consisted of acetonitrile and $H_2O$ with 0.05% trifluoroacetic acid and a typical gradient of 10−90% acetonitrile, and the gradient varied.

**General Separation Procedures from Crude Sweet OPE (Cold-Pressed Oil).** The commercial OPE mixture from Florida Flavors Co. (10 g) was dissolved in a mixture of methylene chloride (2 mL) and hexanes (2 mL) and loaded onto a 120 g preconditioned silica gel flash column. The gradient was started with 10% ethyl acetate and 90% hexanes and went to 40% ethyl acetate and 60% hexanes within 35 min. Then, the isocratic mobile phase (40% ethyl acetate−60% hexanes) was applied for another 15 min (total run of 50 min). The fractions that had UV absorbance at 254 nm were analyzed by LC-ESI-MS, and the fractions were combined into six groups according to their molecular weight obtained from LC-MS analysis (**Figure 2**). Further separation of each group was done using reversed phase HPLC and chiral separation technology.

**Separation Procedures of PMFs, Hydroxylated PMFs, Polymethoxyflavanones, and Polymethoxychalcones by Reversed Phase HPLC and Chiral Column HPLC.** The fractions that contain hydroxylated PMFs characterized by LC-ESI-MS were concentrated, and the residue was dissolved in acetonitrile and water. The dissolved solution was loaded onto a $C_{18}$ reverse phase HPLC system. A gradient method was used from 25% acetonitrile−75% water to 60% acetonitrile−40% water in 25 min with a flow rate of 20 mL/min. The fractions were analyzed by LC-ESI-MS. Both the pure compounds and the mixtures were collected. The pure fractions by LC-MS were combined and concentrated or lyophilized to dryness. The dried compounds were analyzed by MS, UV, and NMR. For instance, tangeretin (**XIV**) and 3,5,6,7,8,3′,4′-heptamethoxylflavone (**XIII**) in group IV (**Figure 2**) were separated in small scale by this reversed phase HPLC. The mixtures containing two or more compounds by LC-MS were concentrated under reduced pressure. The resulted residue was dissolved in a minimum amount of methylene chloride. The solution was then loaded onto an HPLC system equipped with the Welk-O 1 (R,R) Regis column. The mobile phase was composed of 35% absolute ethanol and 65% hexanes. The monitoring UV absorbance was set at 280 nm. A detailed procedure of this technique was described previously (*7*). Pure PMFs, hydroxylated PMFs, polymethoxyflavanones, and polymethoxychalcones were eluted, and the fractions were collected and concentrated, respectively. MS, UV, and NMR were taken for these pure compounds.

## RESULTS AND DISCUSSION

Using silica gel normal phase chromatography and $C_{18}$ reverse phase HPLC techniques as well as chiral HPLC separation technology, a total of eight hydroxylated PMFs, six PMFs, two polymethoxyflavanones, and two polymethoxychalcones (**Figure 1**) were isolated from the OPE obtained from Florida Flavors Co. These compounds are identified as 5-hydroxy-6,7,4′-trimethoxyflavone (**I**), 5-demethyltangeretin (5-hydroxy-6,7,8,4′-tetramethoxyflavone, **II**), 3-hydroxy-5,6,7,4′-tetramethoxyflavone (**III**), 3-hydroxytangeretin (3-hydroxy-5,6,7,8,4′-pentamethoxyflavone, **IV**), 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (**V**), 5-hydroxy-3,7,3′,4′-tetramethoxyflavone (**VI**), 5-hydroxy-3,7,8,3′,4′-pentamethoxyflavone (**VII**), 5-demethylnobiletin (5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone, **VIII**), sinensetin (5,6,7,3′,4′-pentamethoxyflavone, **IX**), 3-methoxysinensetin (3,5,6,7,3′,4′-hexamethoxyflavone, **X**), nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone, **XI**), 5,6,7,4′-tetramethoxyflavone (**XII**), 3-methoxynobiletin (3,5,6,7,8,3′,4′-heptamethoxyflavone, **XIII**), tangeretin (5,6,7,8,4′-pentamethoxyflavone, **XIV**), 5,6,7,4′-tetramethoxyflavanone (**XV**), 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavanone (**XVI**), 2′-hydroxy-3,4,4′,5′,6′-pentamethoxychalcone (**XVII**), and 2′-hydroxy-3,4,3′,4′,5′,6′-hexamethoxychalcone (**XVIII**). All of these isolated compounds were characterized by MS, UV, and NMR ($^1$H, $^{13}$C, and 2D NMR when necessary). Some analytical data of these compounds including UV, low-resolution MS (both EI and ESI), and HRMS are listed in **Table 1**.

**Separation of PMFs, Hydroxylated PMFs, and (Hydroxylated) Polymethoxyflavanones.** The separation started with silica gel chromatography. Various solvent systems were screened in the initial separation, and no solvent system was found to be capable of separating all or most of the components of cold-pressed OPE. However, the gradient elution solvent system, ethyl acetate and hexanes, was used in the coarse separation from crude OPE. Thus, the OPEs were divided into six groups (**Figure 2**) based on the behavior of individual compounds on the silica gel column. The molecular mass of each group was obtained by analyzing the results of HPLC-ESI-MS of each group. **Figure 2** shows the molecular mass of the containing compounds from group I to group VI. Each group was composed of several compounds that have similar behaviors on normal phase chromatography. Further separation of each compound within each group would be laborious and difficult due to the poor resolution on silica gel flash column.

Reversed phase HPLC was employed for screening each group of compounds resulting from a normal phase flash column. Although in some cases there was some differentiation between the components within a group such as in group IV, the overall separation result was poor. Therefore, it is unfeasible to isolate each PMF compound on preparative reversed phase HPLC, despite good separations of PMF compounds being obtained from reversed phase analytical HPLC by many research groups.

Ultimately, clear separation results in preparative scale were obtained from chiral HPLC separation technology. Previously, we reported the efficient and large scale isolation of nobiletin using an HPLC system equipped with the Welk-O 1 (R,R) Regis

**Table 1.** Analytical Data of Isolated Polymethoxylated Compounds from Sweet Orange Peel

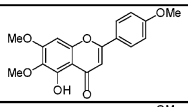
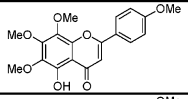
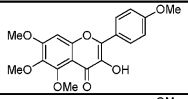
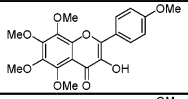
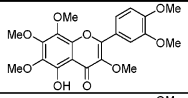
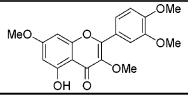
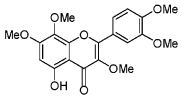
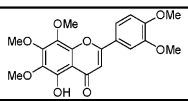
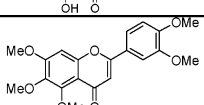
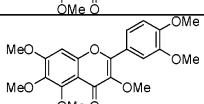
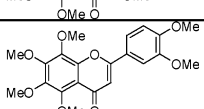
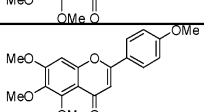
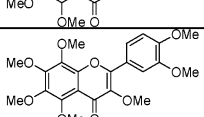
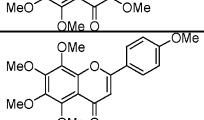
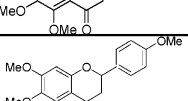
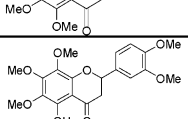
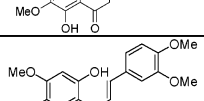
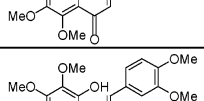
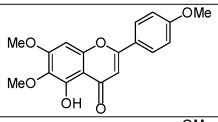
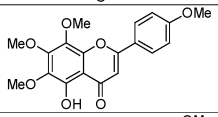
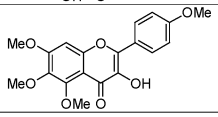
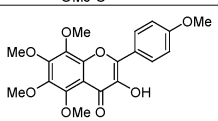
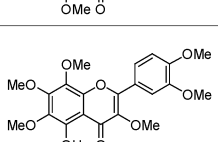
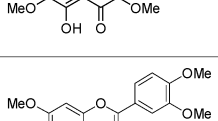
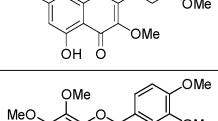
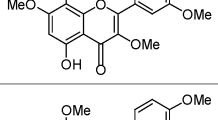
| | Structure | ESI-MS m/z [$MH^+$] | EI-MS | | HRMS [$MH^+$] | | UV Acetonitrile-$H_2O$ (nm) |
|---|---|---|---|---|---|---|---|
| | | | $M^+$ | Frag-ments | Observed | Calculated | |
| I |  | 329 | | | | | 274.5<br>330.5 |
| II |  | 359 | | | | | 288.5<br>328.5 |
| III |  | 359 | | | | | 258.5<br>350.5 |
| IV |  | 389 | | | | | 262.5<br>354.5 |
| V |  | 419 | | | | | 258.5<br>278.5<br>346.5 |
| VI |  | 359 | 358 | 345<br>315 | 359.1123 | 359.1126 | 252.5<br>352.5 |
| VII |  | 389 | 388 | 373<br>358<br>345<br>330 | 389.1228 | 389.1231 | 256.5<br>272.5<br>362.5 |
| VIII |  | 389 | | | | | 280.5<br>342.5 |
| IX |  | 373 | | | | | 238.5<br>330.5 |
| X |  | 403 | | | | | 250.5<br>334.5 |
| XI |  | 403 | | | | | 248.5<br>334.5 |
| XII |  | 343 | | | | | 264.5<br>322.5 |
| XIII |  | 433 | | | | | 252.5<br>342.5 |
| XIV |  | 373 | | | | | 270.5<br>322.5 |
| XV |  | 345 | 344 | 210<br>195<br>167 | 345.1330 | 345.1333 | 226.5<br>276.5 |
| XVI |  | 391 | | | | | 286.5 |
| XVII |  | 375 | 374 | 210<br>195<br>167 | 375.1436 | 375.1439 | 374.5 |
| XVIII |  | 405 | 404 | 240<br>225<br>197<br>182 | 405.1541 | 405.1544 | 352.5<br>366.5 |

**Table 2.** $^1$H NMR of Hydroxylated PMFs in DMSO-$d_6$

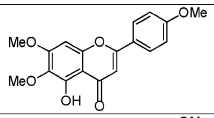
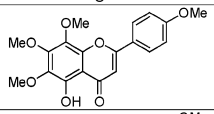
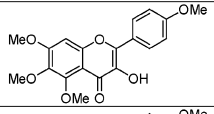
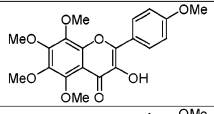
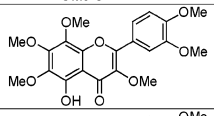
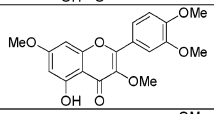
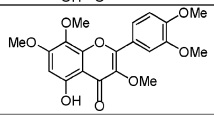
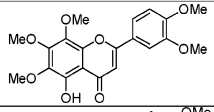
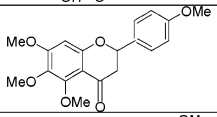
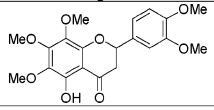
| | Structure | -O<u>H</u> | Ar-<u>H</u> | -OCH$_2$-<u>H</u> |
|---|---|---|---|---|
| **I** |  | 12.95 (s, 1H) | 8.078 (d, J= 9 Hz, 2H)<br>7.129 (d, J= 9 Hz, 2H)<br>6.965 (s, 1H)<br>6.945 (s, 1H) | 3.939 (s, 3H)<br>3.872 (s, 3H)<br>3.744 (s, 3H) |
| **II** |  | 12.75 (s, 1H) | 8.046 (d, J=9 Hz, 2H)<br>7.157 (d, J=9 Hz, 2H)<br>6.954 (s, 1H) | 4.026 (s, 3H)<br>3.920 (s, 3H)<br>3.866 (s, 3H)<br>3.821 (s, 3H) |
| **III** |  | 9.103 (s, 1H) | 8.172 (dd, J=9 Hz, 2 Hz, 2H)<br>7.182 (s, 1H)<br>7.125 (dd, J=9 Hz, 2 Hz, 2H) | 3.954 (s, 3H)<br>3.856 (s, 3H)<br>3.851 (s, 3H)<br>3.783 (s, 3H) |
| **IV** |  | 9.241 (s, 1H) | 8.156 (d, J= 9 Hz, 2H)<br>8.158 (d, J= 9 Hz, 2H) | 4.033 (s, 3H)<br>3.963 (s, 3H)<br>3.856 (s, 3H)<br>3.853 (s, 3H)<br>3.834 (s, 3H) |
| **V** |  | 12.40 (s, 1H) | 7.721 (dd, J=9 Hz, 2 Hz, 1H)<br>7.647 (d, 2 Hz, 1H)<br>7.151 (d, J=9 Hz, 1H) | 4.018 (s, 3H)<br>3.898 (s, 3H)<br>3.861 (s, 3H)<br>3.843 (s, 3H)<br>3.828 (s, 3H)<br>3.816 (s, 3H) |
| **VI** |  | 12.62 (s, 1H) | 7.735 (dd, J=9 Hz, 2 Hz, 1H)<br>7.665 (d, J=2 Hz, 1H)<br>7.172 (d, J=9 Hz, 1H)<br>6.804 (d, J=2 Hz, 1H)<br>6.393 (d, J=2 Hz, 1H) | 3.879 (s, 3H)<br>3.872 (s, 3H)<br>3.866 (s, 3H)<br>3.830 (s, 3H) |
| **VII** |  | 12.42 (s, 1H) | 7.188 (dd, J=9 Hz, 2 Hz, 1H)<br>7.665 (d, J=2 Hz, 1H)<br>7.191 (d, J=9 Hz, 1H)<br>6.578 (s, 1H) | 3.914 (s, 3H)<br>3.871 (s, 3H)<br>3.851 (s, 3H)<br>3.835 (s, 3H)<br>3.829 (s, 3H) |
| **VIII** |  | 12.72 (s, 1H) | 7.633 (dd, J=9 Hz, 2 Hz, 1H)<br>7.512 (d, J=2 Hz, 1H)<br>7.110 (d, J=9 Hz, 1H)<br>6.989 (s, 1H) | 4.022 (s, 3H)<br>3.919 (s, 3H)<br>3.863 (s, 3H)<br>3.846 (s, 3H)<br>3.819 (s, 3H) |

column (*7*). This technique was applied to each group of compounds resulted from silica gel chromatography. For example, nobiletin and 5,6,7,4′-tetramethoxyflavone in group VI were clearly separated from each other on the chiral column. The detailed procedure of this technology was published previously (*7*).

From 10 g of crude OPE (cold-pressed oil), the isolated yield for PMFs was high and some were in gram quantities: sinensetin (**IX**, 0.23 g), 3-methoxysinensetin (3,5,6,7,3′,4′-hexamethoxyflavone, **X**, 0.17 g), nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone, **XI**, 1.12 g), 5,6,7,4′-tetramethoxyflavone (**XII**, 0.41 g), 3-methoxynobiletin (3,5,6,7,8,3′,4′-heptamethoxylflavone, **XIII**, 1.48 g), and tangeretin (5,6,7,8,4′-pentamethoxylflavone, **XIV**, 0.86 g). For other compounds, the isolated yields also from 10 g of crude OPE (cold-pressed oil) were as follows: 0.7 mg of 5-hydroxy-6,7,4′-trimethoxyflavone (**I**), 2.9 mg of 5-demethyltangeretin (**II**), 5.9 mg of 3-hydroxy-5,6,7,4′-tetramethoxyflavone (**III**), 19.8 mg of 3-hydroxy-5,6,7,8,4′-pentamethoxyflavone (**IV**), 87.5 mg of 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (**V**), 12.6 mg of 5-hydroxy-3,7,3′,4′-tetramethoxyflavone (**VI**), 26.4 mg of 5-hydroxy-3,7,8,3′,4′-pentamethoxyflavone (**VII**), 44.1 mg of 5-demethylnobiletin (5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone, **VIII**), 0.9 mg of 5,6,7,4′-tetramethoxyflavanone (**XV**), 0.6 mg of 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavanone (**XVI**), 8.8 mg of 2′-hydroxy-3,4,4′,5′,6′-pentamethoxychalcone (**XVII**), and 1.2 mg of 2′-hydroxy-3,4,3′,4′,5′,6′-hexamethoxychalcone (**XVIII**). The isolated yield of each compound can have some variations for different batches and commercial sources of OPE.

**Hydroxylated PMFs.** Compounds **I**−**VIII** in **Figure 1** and **Table 1** are hydroxylated PMFs. These compounds were previously reported. Compound **I**, also called salvigenin, was previously isolated from *Achillea depressa* (*16*), *Marrubium velutinum*, and *Marrubium cylleneum* (*17*). Compound **II**, known as gardenin B, 5-demethyltangeretin, or 5-desmethyltangeretin, was isolated from Scrophulariaceae (*Limnophila geoffrayi*), which is used as an antidote for detoxification of poison (*18*); from *Limnophila aromatica*, a medicinal herb in Southeast Asia (*19*); and from the leaf surface of the genus *Oscimum* (*20*). Also, it was reported that 5-demethyltangeretin is one of the flavone components as a taste modifier. It can enhance refreshing flavor and deliciousness, reduce saltiness and the flavor associated with acetic acid, and inhibit unpleasant lasting of sweetness (*21*). 5-Hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (**V**), one of the most abundant hydroxylated PMFs in the batch of OPE being studied, was first isolated from Valencia orange (*C. sinensis*) by Tatum and Berry (*22*), then from leaves of *Polanysia trachysperma* (*23*), and recently from *Melicope borbonica* and *Melicope obscura* (Rutaceae, *24*). It was also identified from OPE by Ghai et al. using atmospheric pressure

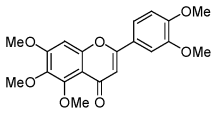
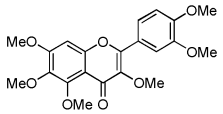
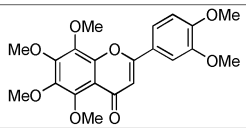
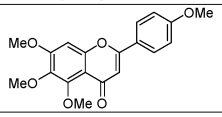
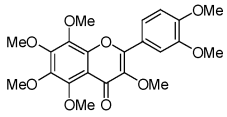
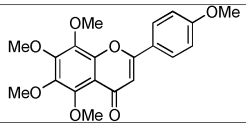
**Table 3.** $^{13}C$ NMR of Hydroxylated PMFs in DMSO-$d_6$

| | Structure | $C_2$ | $C_3$ | $C_4$ | $C_5$ | $C_6$ | $C_7$ | $C_8$ | $C_9$ | $C_{10}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| I |  | 163.60 | 103.34 | 182.26 | 152.04 | 131.90 | 158.67 | 91.64 | 152.66 | 105.12 |
| II |  | 163.87 | 103.41 | 182.70 | 148.65 | 135.96 | 152.63 | 132.75 | 145.35 | 106.32 |
| III |  | 142.64 | 137.62 | 170.99 | 151.02 | 139.29 | 157.51 | 96.92 | 152.99 | 109.96 |
| IV |  | 142.90 | 137.75 | 171.23 | 146.95 | 143.07 | 150.70 | 137.43 | 146.15 | 112.28 |
| V |  | 155.60 | 138.06 | 178.66 | 148.47 | 135.49 | 152.46 | 132.44 | 144.42 | 106.78 |
| VI |  | 155.53 | 138.31 | 178.13 | 160.92 | 97.88 | 165.23 | 92.52 | 156.37 | 105.27 |
| VII |  | 155.19 | 138.00 | 178.34 | 156.38 | 95.79 | 158.24 | 128.77 | 147.84 | 104.49 |
| VIII |  | 163.48 | 103.45 | 182.54 | 148.51 | 135.75 | 152.43 | 132.51 | 145.16 | 106.16 |
| XV[a] |  | 78.96 | 45.25 | 189.29 | 156.67 | 137.53 | 160.08 | 97.49 | 160.02 | 109.36 |
| XVI |  | 78.65 | 42.11 | 198.23 | 150.82 | 133.35 | 154.87 | 132.77 | 148.72 | 103.93 |

| | $C_{1'}$ | $C_{2'}$ | $C_{3'}$ | $C_{4'}$ | $C_{5'}$ | $C_{6'}$ | OMe | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | $C_3$ | $C_5$ | $C_6$ | $C_7$ | $C_8$ | $C_{3'}$ | $C_{4'}$ |
| I | 122.72 | 128.34 | 114.59 | 162.41 | 114.59 | 128.34 | | | 60.62 | 56.46 | | | 55.59 |
| II | 122.81 | 128.43 | 114.87 | 162.65 | 114.87 | 128.43 | | | 60.67 | 62.00 | 61.59 | | 55.69 |
| III | 123.48 | 128.83 | 114.00 | 160.14 | 114.00 | 128.83 | | 61.90 | 61.05 | 56.46 | | | 55.33 |
| IV | 123.54 | 128.78 | 114.20 | 160.27 | 114.20 | 128.78 | | 61.76 | 61.38 | 61.95 | 61.55 | | 55.30 |
| V | 122.11 | 110.90 | 148.09 | 151.43 | 111.78 | 122.01 | 59.75 | | 60.58 | 61.79 | 61.49 | 55.42 | 55.69 |
| VI | 122.10 | 111.60 | 148.49 | 151.35 | 111.26 | 122.07 | 59.83 | | | 56.16 | | 55.68 | 55.69 |
| VII | 122.22 | 111.71 | 148.44 | 151.32 | 110.88 | 121.88 | 59.74 | | | 56.51 | 60.98 | 55.34 | 55.65 |
| VIII | 122.64 | 111.67 | 148.94 | 152.27 | 109.03 | 119.89 | | | 60.48 | 61.74 | 61.42 | 55.67 | 55.61 |
| XV | 131.53 | 128.79 | 114.54 | 154.10 | 114.54 | 128.79 | | 61.94 | 61.50 | 56.92 | | 55.88 | |
| XVI | 130.85 | 110.46 | 149.07 | 150.36 | 111.60 | 119.10 | | | 60.64 | 61.23 | 60.99 | 55.56 | 55.56 |

[a] NMR solvent is $CDCl_3$.

**Table 4.** $^1$H NMR of PMFs Isolated from Sweet Orange Peel in $CDCl_3$

| | Name | Structure | Ar-H | -$OCH_2$-H |
|---|---|---|---|---|
| **IX** | Sinensetin |  | 6.60 (s, 1H, H3)<br>6.80 (s, 1H, H8)<br>6.97 (d, J=9 Hz, 1H, H5′)<br>7.33 (d, J=2 Hz, 1H, H2′)<br>7.52 (dd, J=9 Hz, 2 Hz, 1H, H6′) | 3.93 (s, 3H)<br>3.97 (s, 3H)<br>3.98 (s, 3H)<br>3.99 (s, 6H) |
| **X** | 3,5,6,7,3′,4′-hexamethoxyflavone |  | 6.75 (s, 1H, H8)<br>6.99 (d, J=9 Hz, 1H, H5′)<br>7.70 (d, J=2 Hz, 1H, H2′)<br>7.71 (dd, J=9 Hz, 2 Hz, 1H, H6′) | 3.87 (s, 3H)<br>3.92 (s, 3H)<br>3.97 (s, 3H)<br>3.98 (s, 6H)<br>4.01 (s, 3H) |
| **XI** | Nobiletin |  | 6.63 (s, 1H, H3)<br>7.00 (d, J=9 Hz, 1H, H5′)<br>7.42 (d, J=2 Hz, 1H, H2′)<br>7.57 (dd, J=9 Hz, 2 Hz, 1H, H6′) | 3.95s (6H)<br>3.97s (3H)<br>3.98s (3H)<br>4.03s (3H)<br>4.11s (3H) |
| **XII** | 5,6,7,4′-tetramethoxyflavone |  | 6.59 (s, 1H, H3)<br>6.80 (s, 1H, H8)<br>7.01 (d, J=9 Hz, 2H, H3',5′)<br>7.83 (d, J=9 Hz, 2H, H2',6′) | 3.89s (3H)<br>3.92s (3H)<br>3.98s (3H)<br>3.99s (3H) |
| **XIII** | 3,5,6,7, 8,3′,4′-heptamethoxyflavone |  | 7.01 (d, J=9 Hz, 1H, H5′)<br>7.80 (d, J=2 Hz, 1H, H2′)<br>7.84 (dd, J=9 Hz, 2 Hz, 1H, H6′) | 3.89s (3H)<br>3.95s (3H)<br>3.97s (9H)<br>4.00s (3H)<br>4.10s (3H) |
| **XIV** | Tangeretin |  | 6.61 (s, 1H, H3)<br>7.02 (d, J=9 Hz, 2H, H3',5′)<br>7.87 (d, J=9 Hz, 2H, H2′, 6′) | 3.89s (3H)<br>3.95s (6H)<br>4.02s (3H)<br>4.10s (3H) |

chemical ionization and EI-MS methodology in 2001 (*25*). 5-Hydroxy-3,7,3′,4′-tetramethoxyflavone (**VI**), also called retusin or 3,3′,4′,7-tetramethylquercetin, was isolated from *Pogostemon cablin* (Blanco) for antifungal activity screening (*26*) and from the aerial parts of *Ballota undulate* (Lamiaceae, *27*). 5-Hydroxy-3,7,8,3′,4′-pentamethoxyflavone (**VII**) was previously isolated from Valencia orange (*22*) and from mandarin orange peel (*28*). 5-Demethylnobiletin, or 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavone (**VIII**), was isolated from many species including Lamiaceae (*29*); *Stachys aegyptiaca*; peels of *Citrus changshan-huyou*, mandarin oranges, *Citrus tankan Hayata*, and leaves of *C. reticulata* cultivar; and orange peel oil (*30*).

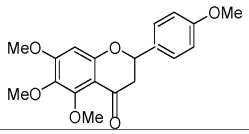
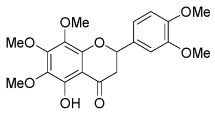
Among the eight hydroxylated PMFs, the hydroxyl group is either at the 5-position (compounds **I**, **II**, and **V**−**VIII**) or at the 3-position (**III** and **IV**). It is an interesting observation that the hydrophilic affinity of 5-hydroxylated PMF compounds is less than their corresponding 5-methoxyflavones judging from their elution order from the $C_{18}$ reverse phase HPLC system and from normal phase chromatography. For example, 5-demethylnobiletin is less polar than nobiletin, i.e., 5-demethylnobiletin is more retentive on reversed phase HPLC. As to the behavior of hydroxylated PMFs and PMFs on silica gel normal phase chromatography, the 5-hydroxylated PMFs were eluted earlier than their PMF counterparts. This is because there is a six member ring intramolecular hydrogen bond formation between the hydrogen of 5-OH group and the oxygen of the 4-carbonyl group. As a result, there are increased interactions between the 5-hydroxylated PMFs and the C18 alkyl chain (reversed phase HPLC columns) and deceased interactions between the 5-demethylated PMFs and the silinol groups on the silica gel column. Further evidence is the profound downfield movement ($\delta = 12-14$ ppm) of the 5-OH proton of a flavonoid on proton NMR spectroscopy (**Table 2**). The downfield proton resonance around 12.5 ppm on $^1$H NMR is characteristic and diagnostically signaling a proton at the 5-position of the flavonoid skeleton structure (*31*). For compounds **III**−**VII**, the lack of a resonance at 6.6 ppm in $^1$H NMR indicated that there is a substitute at the 3-position. In the $^1$H NMR, the $A_2B_2$ pattern (compounds **I**−**IV**) and ABX pattern (compounds **V**−**VIII**) of B-ring evidenced the existence of 1′,4′-disubstituted and 1′,3′,4′-trisubstituted flavones. (*8*). Horrie et al. had a detailed discussion about the behavior of the $^{13}$C NMR for PMFs and hydroxylated PMFs (*32*). The elucidated structures in this study were compatible with published data.

**PMFs.** Six PMFs have been isolated from sweet OPE. Structures and analytical data of these isolated PMFs are illustrated in **Figure 1** and **Table 1** (compounds **IX**−**XIV**), respectively. All six PMFs are known compounds and have been previously isolated from different citrus plants. The structures of six PMFs were elucidated from their analytical data and proton NMR (**Table 4**) and by comparison of these data with literature values (*8*, *32*, *33*).

**Polymethoxyflavanone and Hydroxylated Polymethoxyflavanone.** Previously, 5,6,7,4′-tetramethoxyflavanone (**XV**) was isolated from *Chromolaena arnottiana* (*34*) and from *Polygonum nepalense* (*35*). 5-Hydroxy-6,7,8,3′,4′-pentamethoxyflavanone (**XVI**) was first isolated from the peels of *Citrus mitis* Blanco (*36*). Manthey and Buslig also identified a few polymethoxyflavanones (and/or polymethoxylated chalcones) from orange oil residue by UV, HPLC, and MS (*37*). The structures of the two isolated flavanones are also shown in **Figure 1**, the analytical data are listed in **Table 1**, and $^1$H NMR and $^{13}$C NMR data are in **Tables 5** and **3**, respectively.

The $^1$H NMR spectrum of **XV** has two distinct pairs ($A_2B_2$ pattern) of ortho aromatic protons at 7.38 and 6.95 ppm with a coupling constant of 9 Hz, and they are the B-ring protons ($H_{2',6'}$ and $H_{3',5'}$). The low resonance (6.34 ppm) singlet aromatic proton indicates the proton at the 8-position. Four methoxy groups are

**Table 5.** $^1$H NMR of Polymethoxyflavanones Isolated from Sweet Orange Peel in $CDCl_3$

| | Name | Structure | OH | Ar-H | Aliphatic Hs | -$OCH_2$-H |
|---|---|---|---|---|---|---|
| **XV** | 5,6,7,4'-Tetramethoxy-flavanone |  | - | 7.385 (d, J=9 Hz, 2H)<br>6.951 (d, J=9 Hz, 2H)<br>6.336 (s, 1H) | 5.344 (dd, J=13.5 Hz, 3 Hz, 1H, H2)<br>3.023 (dd, J=16.5 Hz,, 13.5 Hz, 1H, H3a)<br>2.753 (dd, J=16.5 Hz,, 3 Hz, 1H, H3e) | 3.942 (s, 3H)<br>3.868 (s, 3H)<br>3.834 (s, 3H)<br>3.825 (s, 3H) |
| **XVI** | 5-Hydroxy-6,7,8,3',4'-pentamethoxy-flavanone |  | 11.76 | 7.006 (d, J=2.5 Hz, 1H)<br>6.988 (dd, J=9Hz, 2.5 Hz, 1H)<br>6.897 (d, J=9 Hz, 1H) | 5.398 (dd, J=13 Hz, 3 Hz, 1H, H2)<br>3.089 (dd, J=17 Hz, 13 Hz, 1H, H3a)<br>2.907 (dd, J=17 Hz, 3 Hz, 1H, H3e) | 4.088 (s, 3H)<br>3.906 (s, 6H)<br>3.859 (s, 3H)<br>3.797 (s, 3H) |

displayed at 3.94, 3.88, 3.83, and 3.82 ppm on $^1$H NMR and 61.94, 61.50, 50.92, and 55.88 ppm on $^{13}$C NMR. The protons on the saturated $C_2-C_3$ bond display an ABX system at 5.34 (dd, $J$ = 13.5 Hz, 3 Hz, 1H, $H_2$), 3.02 (dd, $J$ = 16.5 Hz, 13.5 Hz, 1H, $H_{3a}$), and 2.75 (dd, $J$ = 16.5 Hz, 3 Hz, 1H, $H_{3e}$). The resonance at 189.29 ppm on the $^{13}$C NMR further confirmed this flavanone skeleton (*8*, *34*, *35*). Further evidence of $C_2-C_3$ being a single bond is the short wavelength (226.5 and 276.5 nm in acetonitrile and water, **Table 1**) in its UV spectrum. The lack of UV absorption larger than 300 nm indicates the absence of conjugation between A- and C-rings. The EI-MS spectrum of **XV** showed $M^+$ at *m*/*z* 344. More abundant peaks at 210 (90%), 195 (100%, base peak), and 167 were attributed to retro Diels−Alder fragmentation. The dominant retro Diels−Alder fragmentation pattern of **XV** on EI-MS confirmed the existence of a $C_2-C_3$ single bond and is in agreement with the established fragmentation pattern of flavanones (*8*). **Figure 3** shows the fragmentation pathway of 5,6,7,4′-tetramethoxyflavanone.

Compound **XVI** has the same flavanone skelton structure as compound **XV**, 5,6,7,4′-tetramethoxyflavanone. The ABX system on the $^1$H NMR identified its $H_2$ at 5.4 ppm, $H_{3a}$ (axial proton) at 3.1 ppm, and $H_{3e}$ (equatorial proton) at 2.9 ppm. The signal at 198 ppm on $^{13}$C NMR spectrum confirmed that the carbonyl group is attached to a saturated carbon atom—flavanone (*38*). The diagnostic resonance at 11.8 ppm on the $^1$H NMR spectrum indicates the existence of a 5-OH group. Five methoxy groups are apparently seen from both $^1$H NMR (3.78−4.10 ppm) and $^{13}$C NMR (55.6−61.2 ppm). There are a pair of ortho aromatic protons, $H_{6'}$ (6.98 ppm) and $H_{5'}$ (6.89 ppm), with a large coupling constant of 9 Hz. The coupling between meta protons, $H_{2'}$ (7.01 ppm) and $H_{6'}$ (6.98 ppm), is very small (2.5 Hz). Therefore, it is determined that there are two methoxy groups on the B-ring and three methoxy groups on the 6−8-positions. (*8*). Thus, the structure of **XVI** is determined as 5-hydroxy-6,7,8,3′,4′-pentamethoxyflavanone. The short wavelength (286.5 nm in acetonitrile and water) of **XVI** on the UV spectrum also indicates the lack of conjugation between A- and C-rings, a characteristic feature of flavanones.

**Polymethoxychalcones.** 2′-Hydroxy-3,4,4′,5′,6′-pentamethoxychalcone (**XVII**) was isolated from *Chromolaena odorata*, a

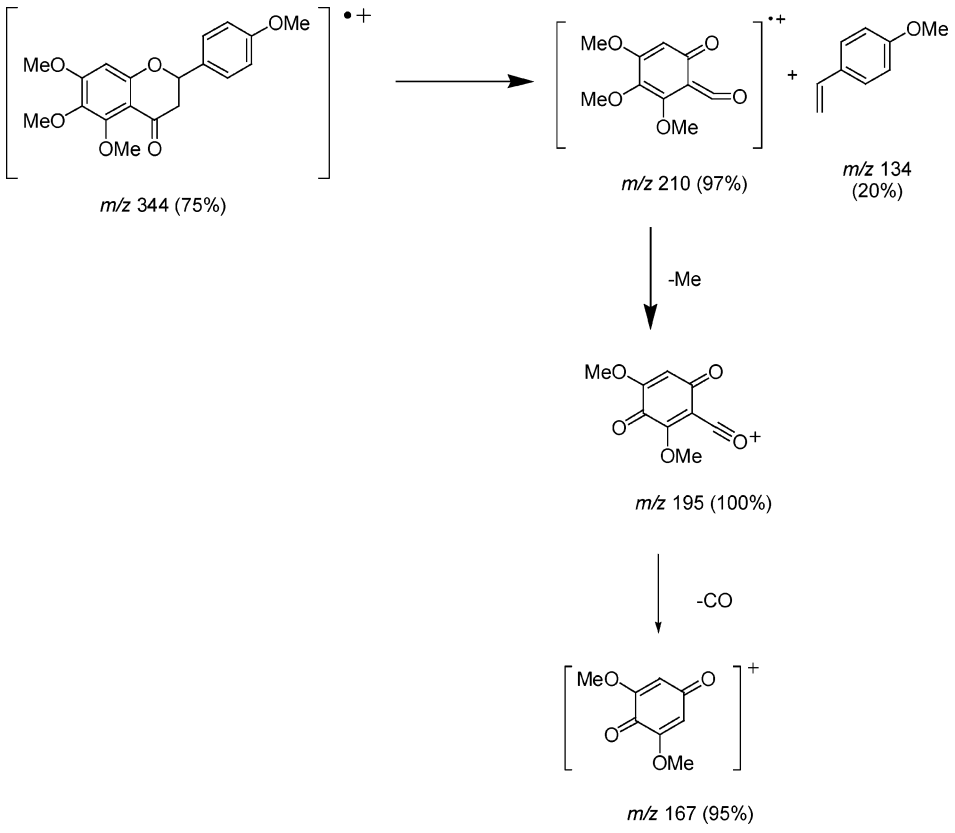
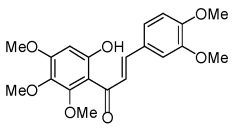
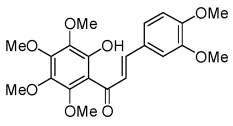



**Figure 3.** Fragmentation pathway of 5,6,7,4′-tetramethoxyflavanone.

**Table 6.** $^1$H NMR (DMSO-$d_6$) Data of Polymethoxychalcones

| | Name | Structure | OH | Ar-H | -$OCH_2$-H |
|---|---|---|---|---|---|
| **XVII** | 2′-Hydroxy-3,4,4′5′6′-pentamethoxychalcone |  | 12.38 (12.02[a]) | 7.566 (d, J=16 Hz, 1H)<br>7.451 (d, J=16 Hz, 1H)<br>7.315 (d, J=2 Hz, 1H)<br>7.287 (dd, J=8 Hz, 2 Hz, 1H)<br>7.024 (d, J=8 Hz, 1H)<br>6.383 (s, 1H) | 3.845 (s, 6H)<br>3.829 (s, 3H)<br>3.817 (s, 3H)<br>3.708 (s, 3H) |
| **XVIII** | 2′-Hydroxy-3,4,3′,4′5′6′-hexamethoxychalcone |  | 10.04 (13.24[a]) | 7.398 (d, J=16 Hz, 1H)<br>7.342 (d, J=3 Hz, 1H)<br>7.266(dd, J=9 Hz, 3 Hz, 1H)<br>7.159 (d, J=16 Hz, 1H)<br>6.996 (d, J=9 Hz, 1H) | 3.926 (s, 3H)<br>3.814 (s, 3H)<br>3.805 (s, 3H)<br>3.773 (s, 3H)<br>3.750 (s, 3H)<br>3.736 (s, 3H) |

[a] $^1$H NMR data in $CDCl_3$.

**Table 7.** $^{13}$C NMR Data of Isolated Polymethoxychalcones in DMSO-$d_6$

| | C=O | aromatic and alkene carbon | OMe |
|---|---|---|---|
| **XVII** | 192.56 | 157.77, 157.52, 153.01, 151.17, 148.99, 143.88, 134.60, 127.39, 124.95, 122.85, 111.74, 110.75, 110.60, 96.40 | 61.47, 60.62, 55.99, 55.59, 55.52 |
| **XVIII** | 192.86 | 151.24, 149.02, 148.95, 147.06, 145.73, 144.76, 138.55, 137.18, 127.15, 125.88, 123.23, 116.72, 111.62, 110.68 | 61.51, 61.04, 60.93, 60.86, 55.57 |

perennial shrubby Compositae native to tropical Central and South America (*39*), and 2′-hydroxy-3,4,3′,4′,5′,6′-hexamethoxychalcone (**XVIII**) was isolated from the bark of *Macaranga peltata Muell*. (*40*). The structures, analytical data, and $^1$H NMR of the two polymethoxychalcones are illustrated in **Figure 1** and **Tables 1** and **6**, respectively.

The EI-MS spectrum of **XVII** (**Figure 1**) showed $M^+$ at *m*/*z* 374 (92%). The abundant peaks at 210 (95%), 195 (100%, base peak), and 167 (84%) were attributed to the cleavage of the multiple conjugated system, reminiscent of pseudo-retro-Diels−Alder fragmentation, an almost identical fragmentation pattern with its flavanone counterpart. The dominant fragmentation pattern of **XVII** on EI-MS provides significant information that the basic skeleton has a chalcone skeleton. The UV absorbance of **XVII** at 374.5 nm in acetonitrile and water indicates the existence of a multiple conjugated system, different from the flavanone skeleton structure. The diagnostic resonance of 192.56 ppm in $^{13}$C NMR provides evidence of a chalcone carbonyl carbon, whereas the resonance of the carbonyl carbon in flavones appears at the region between 170 and 178 ppm. Moreover, the NMR spectra also show the existence of an unsubstituted carbon at the 8-position signaled by the resonance of 96.4 ppm on the $^{13}$C NMR and 6.39 ppm (singlet, 1H) on the $^1$H NMR. Five methoxy groups are characteristic both on $^1$H NMR (3.85 ppm, 6H; 3.83 ppm, 3H; 3.82 ppm, 3H; and 3.71 ppm, 3H) and on $^{13}$C NMR spectra (61.47, 60.62, 55.99, 55.59, and 55.52 ppm). The AB doublet pattern depicts the existence of two alkene protons (7.57 and 7.45 ppm) with a large coupling constant on the $^1$H NMR. The typical ABC system on the aromatic proton NMR as mentioned early in this section indicates that the substitution pattern on the A-ring is 3,4-disubstitution. Therefore, on the basis of these analytical data, the structure of **XVII** is determined as 2′-hydroxy-3,4,4′,5′,6′-pentamethoxychalcone.

The spectrum analysis of **XVIII** also applies to **XVII** due to the similarity of these two compound structures. The EI-MS spectrum of **XVIII** showed $M^+$ at *m*/*z* 404 (52%) and its fragments at 240 (100%, base peak), 225 (88%), 197 (64%), and 182 (58%), which follows the exact same fragmentation pattern of **XVII**. The structure of **XVIII** is determined as 2′-hydroxy-3,4,3′,4′,5′,6′-hexamethoxychalcone being based on the following data analysis: the multiple conjugated system indication from UV absorbance of **XVIII** at 352.5 and 366.5 nm in acetonitrile and water, the existence of characteristic carbonyl resonance of basic chalcone structure at 192.86 ppm in $^{13}$C NMR, a trans alkene system characterized by the AB doublet pattern of two alkene protons (7.38 and 7.16 ppm) with a large coupling constant ($J$ = 16 Hz) on the $^1$H NMR, the 3,4-disubstitution on the A-ring evidenced by typical ABC type aromatic protons on proton NMR, and six methoxy groups characterized on $^1$H and $^{13}$C NMR spectra (*2*).

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