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Jesús G. Díaz, Laura Chacón Cruz, David Díaz Díaz

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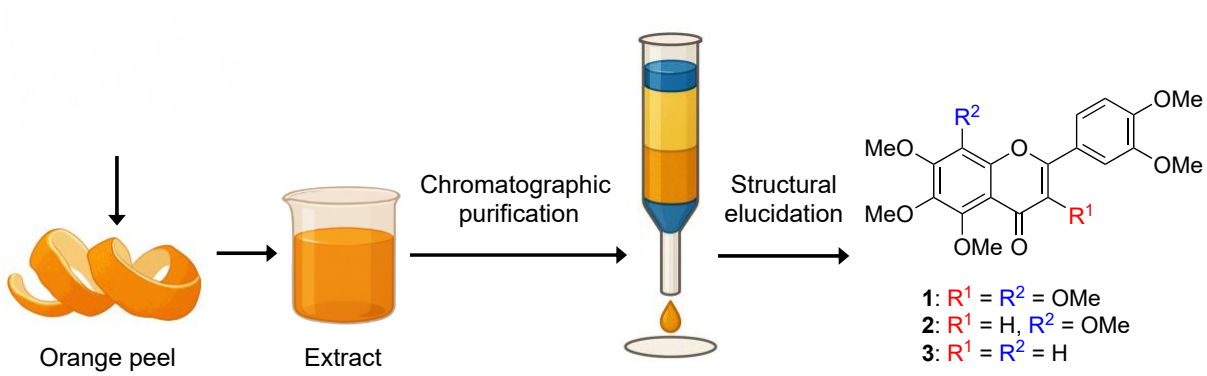
From waste to value: Isolation and structural elucidation of polymethoxylated flavones from orange (*Citrus sinensis*) peel

Jesús G. Díaz,[a,b]* Laura Chacón Cruz,[a] David Díaz Díaz[a,b]*

[a] *Instituto Universitario de Bio-Orgánica Antonio González (IUBO-AG), Universidad de La Laguna, Avda. Astrofísico Francisco Sánchez 2, La Laguna 38206, Spain.*
[b] *Departamento de Química Orgánica, Universidad de La Laguna, Avda. Astrofísico Francisco Sánchez 3, La Laguna 38206, Spain.*
**Corresponding author: Dr. David Díaz Díaz, Instituto Universitario de Bio-Orgánica Antonio González (IUBO-AG), Universidad de La Laguna, Avda. Astrofísico Francisco Sánchez 2, La Laguna 38206, Spain. Tel.: +34 922318584. E-mail address: ddiazdiaz@ull.es (D.D.D.).*

Graphical Abstract



Abstract: This study explores the valorization of orange (*Citrus sinensis*) peel waste as a source of bioactive polymethoxylated flavones (PMFs) with nutraceutical potential. A dichloromethane extract from fresh orange peel (including both albedo and flavedo) was subjected to sequential chromatographic separation, yielding three known compounds: 3,5,6,7,8,3′,4′-heptamethoxyflavone (**1**), nobiletin (**2**), and sinensetin (**3**). Structural elucidation was achieved through 1D and 2D nuclear magnetic resonance (NMR) techniques, supported by high-resolution mass spectrometry. While these compounds are already known, their recovery from citrus waste underscores the potential of agri-food by-products as valuable sources of health-promoting natural products. The findings contribute to sustainable waste valorization and support the development of high-value ingredients for pharmaceutical and nutraceutical applications.

Keywords: *Citrus sinensis, orange peel, polymethoxylated flavones, nobiletin, sinensetin, NMR spectroscopy.*

INTRODUCTION

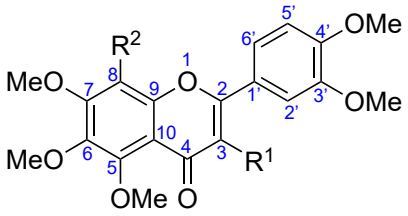
Plants remain an essential source of pharmacologically active compounds, and citrus fruits, particularly sweet oranges (*Citrus sinensis*), stand out for their nutritional and therapeutic relevance.[1,2] Traditionally used to alleviate ailments such as bronchitis, hypertension, and digestive disorders, *C. sinensis* has long been appreciated not only for its vitamin C content and antioxidant properties, but also for its diverse secondary metabolites.[3,4]

The global citrus industry, especially in countries such as Spain, Brazil, and the United States, generates vast quantities of peel waste through juice processing.[5] A substantial fraction of this biomass is diverted to low-value uses like animal feed or composting,[6] despite the fact that orange peels, particularly the flavedo and albedo regions, are rich in bioactive phenolic compounds,[7] including polymethoxylated flavones (PMFs). These compounds, characterized by multiple methoxy substituents on the flavone backbone, display enhanced lipophilicity, membrane permeability, and metabolic stability compared to their hydroxylated counterparts.[8]

PMFs such as nobiletin and sinensetin have been associated with a broad spectrum of biological activities,[9] including antioxidant,[10,11,12] anti-inflammatory,[12,13] antidiabetic,[12,14,15] neuroprotective,[12,16,17] and anticancer[12,18,19] effects. Their mechanisms of action involve the modulation of key cellular pathways (e.g., MAPK, PI3K/Akt), inhibition of reactive oxygen species, and suppression of pro-inflammatory mediators.[20,21] Owing to these properties, PMFs are gaining attention as nutraceuticals, bioactive compounds derived from food sources with preventive or therapeutic benefits.[22]

The recovery of such flavonoids from agro-industrial by-products aligns with the principles of green chemistry and the circular economy, offering a sustainable route to high-value applications in health-related sectors.[23] Notably, products like Daflon® 500 mg, based on flavonoids such as diosmin and hesperidin from citrus peels, exemplify the pharmacological relevance of these compounds.[24]

In this context, the present study investigates the extraction, isolation, and structural elucidation of three major PMFs (Figure 1) from *Citrus sinensis* peel waste using chromatographic and spectroscopic techniques. Although the isolated compounds are known, their targeted recovery from underutilized biomass underscores the relevance of revisiting familiar botanical sources in the pursuit of sustainable natural product valorization.



1: $R^1 = R^2 = OMe$
2: $R^1 = H$, $R^2 = OMe$
3: $R^1 = R^2 = H$

Figure 1. Chemical structures of the three PMFs **1**–**3** isolated and described in this work.

EXPERIMENTAL SECTION

General

All the solvents were distilled before use. Thin-layer chromatography (TLC) was carried out on silica gel plates (20 × 20 cm, SIL G-60, 0.20 mm thick) from Macherey-Nagel. Various solvent mixtures were used for elution in order to achieve optimal polarity for the desired separation. Visualization was performed by fluorescence under UV light at 254 nm and/or 360 nm. Spots were developed by spraying with oleum (i.e., mixture of CH_3COOH:H_2SO_4:H_2O in a ratio 8:0.4:1.6), followed by heating. Size-exclusion column chromatography was performed on Sephadex® LH-20 Pharmacia (ref. 17-0090-01). The resin was preconditioned by soaking in methanol for at least 12 h. HPLC separations were carried out on a JASCO Pu-980 series pumping system equipped with a JASCO UV-975 detector and a semipreparative Waters Kromasil Si 5 µm (10 × 250 mm) column using hexane–EtOAc gradients as the eluent. Melting points were measured on a Reichert Thermovar apparatus without correction. Nuclear magnetic resonance (NMR) spectra (^1H and ^{13}C) were obtained via Bruker Advance II 500 or Bruker Advance III 600 spectrometers in $CDCl_3$ and C_6D_6, with residual solvent signals serving as internal references, (δ_H 7.26; δ_C 76.7), (δ_H 7.16; δ_C 128.39), respectively. The pulse conditions for 1D and 2D (HSQC, HMBC) NMR were applied as previously reported.[25] Chemical shifts (δ) and coupling constants (*J*), are expressed in parts per million (ppm) and hertz (Hz), respectively. High-resolution mass spectra (HRMS) were recorded on a Waters LCT Premier XE instrument (ESI-TOF). Mass spectra, 1D and 2D NMR spectra of all compounds are provided in the Supplementary Material.

Material

Fresh sweet oranges (*Citrus sinensis* var. *Navel Lane Late*), sourced from Castellón, Valencia, Spain, were purchased in La Laguna, Spain. Peels (1.5 kg) were separated, chopped, and macerated in EtOH/H_2O (95:5) for 7 days at room temperature.

After filtration and evaporation under reduced pressure, the crude extract (185 g) with brown gummy appearance was suspended in water and partitioned successively with CH_2Cl_2 (×3) and *n*-BuOH (×3). The CH_2Cl_2 fraction (7.1 g), rich in lipophilic flavonoids as evidenced by TLC analysis (Figure S1), was selected for purification.

Purification

An aliquot (1.85 g) of the dichloromethane extract was fractionated by column chromatography using a 50 cm × 8 cm Sephadex® LH-20 using MeOH:CH_2Cl_2:hexane (1:1:2) as mobile phase. A total of 25 fractions of 50 mL each were collected, analyzed by TLC, and grouped by similar UV profiles. An intense yellow coloration of the spots in the TLC after development with oleum is indicative of the presence of aromatic compounds with conjugated systems, such as flavone and flavonol derivatives. The most active fraction (fraction 3, 560 mg) was subjected to a medium-pressure column chromatography on silica gel (SiO_2, 35–75 mesh, Art. 7748) using hexane–EtOAc gradients. A total of 23 fractions of 100 mL each were collected. Based on their behavior in TLC, these fractions were regrouped into subfractions 11–14 (88 mg), 16–17 (13.5 mg), and 22–23 (8.5 mg), which were subjected to semipreparative HPLC (Kromasil, hexane–EtOAc 30:70) to afford pure compounds.

RESULTS AND DISCUSSION

The chromatographic analysis of the dichloromethane extract from orange peel revealed three major polymethoxylated flavones. TLC analysis indicated the presence of UV-active compounds with retention factors, NMR and HRMS spectroscopic data consistent with known PMFs: 3,5,6,7,8,3′,4′-heptamethoxyflavone (**1**), nobiletin (**2**) and sinensetin (**3**). Structural elucidation of the compounds was based on the following approaches:

(i) *Analysis of carbon chemical shifts of methoxy-substituted carbons.* It is well established[13] that the resonances of aromatic methoxy groups bonded to di-*ortho*-substituted carbons appear significantly downfield (ca. 60 ppm) compared to those attached to carbons with one or no *ortho* substituents (ca. 55 ppm). This behavior serves as a valuable diagnostic tool for the structural analysis of PMFs. Notably, methoxy groups at position C-3 of the quasi-aromatic heterocyclic C-ring of flavones are also considered di-*ortho*-substituted.

(ii) *Solvent-induced shift method (C_6D_6 vs. $CDCl_3$).*[26] This technique allows discrimination between ^{1}H NMR signals of aromatic methoxy groups located *ortho* to aromatic protons and those that are not. Methoxy groups in *ortho* proximity to aromatic protons exhibit marked upfield shifts in spectra recorded in deuterated benzene. In our case, the methoxy groups at positions C′-3 and C′-4 displayed considerably greater upfield shifts than the remaining methoxy groups.

(iii) *2D HSQC and HMBC spectroscopy*. These techniques enable the identification of heteronuclear correlations over one, two, and three bonds, respectively. This approach allowed for the complete assignment of all proton and carbon signals (Table 1 and Table 2).

Table 1 ^{1}H RMN data (600 MHz) of FPMs **1**, **2** and **3** in $CDCl_3$ and C_6D_6*

H	1	1*	2	3	3*
H-3			6.63 s	6.63 s	6.33 s
H-8				6.81 s	6.42 s
H-2’	7.80 (d, 2.0)	7.83 (d, 2.0)	7.42 (d, 2.0)	7.33 (d, 2.0)	7.15‡
H-5’	7.00 (d, 8.5)	6.60 (d, 8.5)	6.99 (d, 8.5)	6.97 (d, 8.5)	6.52 (d, 8.5)
H-6’	7.84 (dd, 8.5, 2.0)	7.93 (dd, 8.5, 2.0)	7.57 (dd, 8.5, 2.0)	7.52 (dd, 8.5, 2.0)	7.35 (dd, 8.5, 2.0)
OMe 3	3.94 (s)	3.87 (s)			
OMe 5	4.00 (s)	4.03 (s)	3.95 (3H, s)	3.97 (3H, s)	4.13 (3H, s)
OMe 6	3.09 (s)	3.69 (s)	4.02 (3H, s)	3.93 (3H, s)	3.97 (3H, s)
OMe 7	4.09 (s)	3.78 (s)	4.10 (3H, s)	4.00 (3H, s)	3.18 (3H, s)
OMe 8	3.98 (s)	3.75 (s)	3.95 (3H, s)		
OMe 3’	3.97 (s)	3.55 (s)	3.97 (3H, s)	3.99 (3H, s)	3.38 (3H, s)
OMe 4’	3.97 (s)	3.38 (s)	3.96 (3H, s)	4.00 (3H, s)	3.31 (3H, s)

‡ masked by the benzene signal.

Table 2 ^{13}C RMN data (125 MHz) of FPMs **1**, **2** and **3** in $CDCl_3$ and C_6D_6*

C	1	1*	2	3	3*
2	153.2	153.1	161.2	162.3	161.3
3	140.9	141.8	107.1	107.4	108.4
4	174.0	173.5	177.5	177.2	176.6
5	148.4	149.4	148.6	151.8	154.0
6	138.0	138.8	138.2	140.3	141.6
7	151.5	151.3	151.6	157.6	158.2
8	144.0	144.8	144.3	96.3	96.9
9	146.9	147.5	147.9	154.4	155.2
10	115.3	116.7	115.1	112.9	114.3
1’	123.6	124.6	124.2	124.2	125.2

2’	111.38	112.4	108.8	108.7	110.0
3’	148.9	150.2	149.5	149.3	150.6
4’	151.3	152.3	152.1	152.6	153.0
5’	111.17	112.1	111.5	119.6	112.1
6’	122.5	122.5	119.8	111.2	120.0
OMe 3	60.0	59.9			
OMe 5	62.1	62.7	62.03*	62.1	62.7
OMe 6	62.5	61.8	62.2	61.5	62.0
OMe 7	61.8	61.7	61.6	56.08	55.8
OMe 8	62.0	61.9	62.05*		
OMe 3’	56.0	55.9	56.1•	56.2	56.0
OMe 4’	56.2	55.7	56.2•	56.1	55.8

* Interchangeable with each other

• Interchangeable with each other

All 1D and 2D NMR spectra are provided in the Supplementary Material, while the corresponding characterization data are detailed below.

Compound 1: 3,5,6,7,8,3′,4′-Heptamethoxyflavone

Subfractions 11–14 showed a single compound by TLC analysis under UV light (R_f = 0.70, hexane–EtOAc 1:1). After concentration and solvent removal under reduced pressure, it yielded a white needle-like solid with a melting point of 118–121 °C (hexane–EtOAc). This compound exhibited a molecular formula of $C_{22}H_{24}O_9$, as determined by high-resolution electrospray ionization mass spectrometry (HRESIMS), showing a molecular ion at m/z 433.1502 $[M+H]^+$ (100%), consistent with the calculated value for $C_{22}H_{25}O_9$ (m/z 433.1499). Together with its ^{13}C NMR data (Table 2), this indicates eleven degrees of unsaturation.

The ^1H NMR spectrum in $CDCl_3$ revealed resonances corresponding to a 1,3,4-trisubstituted benzene ring (δ_H 7.84, 1H, dd, J = 8.5 and 2.1 Hz; δ_H 7.81, 1H, d, J = 2.1 Hz; δ_H 7.00, 1H, d, J = 8.5 Hz), along with seven singlets attributable to aromatic methoxy groups at δ_H: 4.09, 4.00, 3.976, 3.970 (×2), 3.94, and 3.89 (each 3H, s). These data are consistent with a PMF structure, with the A-ring fully substituted by methoxy groups. Additionally, the pattern of substitution on the B-ring was determined based on the chemical shifts and coupling constants. The ^1H NMR spectrum of compound **1** revealed an ABX system (H′-2, H′-5, and H′-6), supporting substitution by two methoxy groups at C′-3 and C′-4 on the B-ring.

The full substitution pattern matched that of 3,5,6,7,8,3′,4′-heptamethoxyflavone (**1**) (Figure 2), a compound previously reported to exhibit significant biological activity, including neuroprotective effects against neuronal cell death in mice,[27,28] and antiproliferative activity against various types of human tumor cell lines.[29]

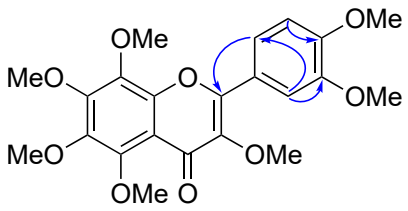


Figure 2. Chemical structure and main HMBC correlations for 3,5,6,7,8,3′,4′-heptamethoxyflavone (**1**).

Compound 2: Nobiletin

Subfractions 16–17 (R_f = 0.38, hexane–EtOAc 1:1) yielded a white solid after crystallization in hexane–EtOAc, with a melting point of 120–122 °C. The high-resolution mass spectrum showed a molecular ion at m/z 403.1395 $[M+H]^+$ (100%), consistent with the calculated value for $C_{21}H_{23}O_8$ (m/z 403.1393).

The ^1H NMR spectrum of the above solid in $CDCl_3$ was very similar to that of the previously discussed compound. It exhibited signals corresponding to a 1,3,4-trisubstituted benzene ring (δ_H 7.57, 1H, dd, J = 8.5 and 2.1 Hz; δ_H 7.42, 1H, d, J = 2.1 Hz; δ_H 6.99, 1H, d, J = 8.5 Hz), along with six singlets for aromatic methoxy groups at δ_H: 3.95, 4.02, 4.10, 3.95, 3.97, and 3.96 (each 3H, s). An additional olefinic proton signal at δ_H 6.63 (s) was assigned to H-3. The HMBC correlation between the signal at δ_H 6.63 (s) and δ_C 177.5 (C-4), 161.2 (C-2), and 115.1 (C-10) confirmed the assignment of H-3.

The above data and HMBC correlations matched the reported values for 5,6,7,8,3′,4′-hexamethoxyflavone (nobiletin) (**2**) (Figure 3),[30] a citrus PMF with antitumor, anti-inflammatory, and cognitive-enhancing effects.[31,32]

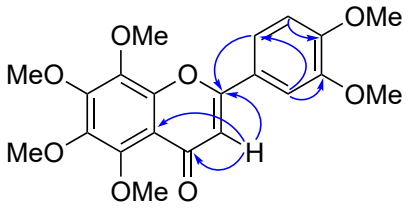


Figure 3. Chemical structure and main HMBC correlations for nobiletin (**2**).

Compound 3: Sinensetin

Subfractions 22–23 (R_f = 0.29, hexane–EtOAc 1:1) yielded a white amorphous solid with a melting point of 150–152 °C. The high-resolution mass spectrum showed a molecular ion at m/z 372.1239 $[M]^+$ (100%), consistent with the calculated value for $C_{20}H_{20}O_7$ (m/z 372.1209).

The ^{1}H NMR spectrum in $CDCl_3$ closely resembled that of compound **2**, differing mainly in the absence of one methoxy group and the appearance of a singlet aromatic proton at δ_H 6.81 (s). A significant solvent-induced shift was observed for one of the aromatic methoxy groups, moving from δ_H 4.00 (3H, s) in $CDCl_3$ to δ_H 3.18 (3H, s) in C_6D_6 ($\Delta\delta$ = 0.82), suggesting that the proton is located at C-5 or C-8 of the A-ring. HMBC correlations between the signal at δ_H 6.42 (s) (H-8) and δ_C 141.58 (C-6), 158.24 (C-7), 155.19 (C-9), and 114.33 (C-10) confirmed the position of the new aromatic proton at C-8.

The above data and the observed HMBC correlations were consistent with 5,6,7,3′,4′-pentamethoxyflavone (sinensetin) (**3**) (Figure 4),[30] a PMF known for exhibiting a wide range of pharmacological properties, including anti-inflammatory, anticancer, antioxidant, antithrombotic, antibacterial, vasorelaxant, anti-dementia, and antitrypanosomal activities, with minimal toxicity,[33] vasorelaxant and antithrombotic activities.[10,34]

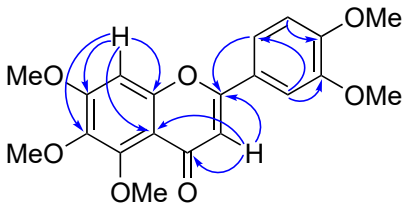


Figure 4. Chemical structure and main HMBC correlations for sinensetin (**3**).

CONCLUSIONS

It is widely recognized that natural products have long served as a fundamental source of pharmaceuticals. Today, the continued search for therapeutic agents with improved efficacy and reduced side effects has renewed interest in bioactive compounds from natural sources. In this context, the present study reinforces the potential of citrus by-products, particularly the peel of *Citrus sinensis*, as a promising therapeutic resource, owing to their high content of methoxylated flavonoids with well-documented biological activities.

Although the compounds isolated in this study, i.e., 3,5,6,7,8,3′,4′-heptamethoxyflavone (**1**), nobiletin (**2**), and sinensetin (**3**), are all known structures, the research remains highly valuable. The expectation of discovering new molecules was not fulfilled; however, the ability to recover bioactive flavones in significant yields from an abundant and underutilized agri-food residue demonstrates the relevance of this work. In particular, it highlights the importance of reporting and discussing such outcomes, which contribute to a more complete and realistic understanding of natural product exploration and resource valorization.

The applied methodology, combining chromatographic purification, 1D/2D NMR, and high-resolution mass spectrometry, proved efficient and reliable for the structural elucidation of these compounds. This valorization strategy not only adds value to citrus processing by-products but also promotes a more sustainable and circular model in line with the principles of the green economy. Moreover, it opens new economic, scientific, and commercial opportunities for a more competitive and innovation-driven citrus industry, one firmly committed to sustainability.

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