JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Identification of 5,7,3′,4′-Tetramethoxyflavone Metabolites in Rat Urine by the Isotope-Labeling Method and Ultrahigh-Performance Liquid Chromatography−Electrospray Ionization−Mass Spectrometry

Wen-Chien Lu,[†] Jenn-Feng Sheen,[‡] Lucy Sun Hwang,*[,†] and Guor-Jien Wei*[,§]

[†]Graduate Institute of Food Science and Technology, National Taiwan University, Taipei 106, Taiwan

[‡]Department of Biotechnology, National Formosa University, Yunlin 632, Taiwan

[§]Department of Nutrition and Health Sciences, Kainan University, Taoyuan 33857, Taiwan

ABSTRACT: 5,7,3′,4′-Tetramethoxyflavone (TMF), one of the major polymethoxyflavones (PMFs) isolated from *Kaempferia parviflor*, has been reported possessing various bioactivities, including antifungal, antimalarial, antimycobacterial, and anti-inflammatory activities. Although several studies on the TMF have been reported, the information about the metabolism of TMF and the structures of TMF metabolites is still not yet clear. In this study, an isotope-labeling method was developed for the identification of TMF metabolites. Three isotope-labeled TMFs (5,7,3′,4′-tetramethoxy[3′-D_3]flavone, 5,7,3′,4′-tetramethoxy[4′-D_3]flavone, and 5,7,3′,4′-tetramethoxy[5,4′-D_6]flavone) were synthesized and administered to rats. The urine samples were collected, and the main metabolites were monitored by ultrahigh-performance liquid chromatography−electrospray ionization−mass spectrometry. Five TMF metabolites were unambiguously identified as 3′-hydroxy-5,7,4′-trimethoxyflavone, 7-hydroxy-5,3′,4′-trimethoxyflavone sulfate, 7-hydroxy-5,3′,4′-trimethoxyflavone, 4′-hydroxy-5,7,3′-trimethoxyflavone, and 5-hydroxy-7,3′,4′-trimethoxyflavone.

KEYWORDS: *5,7,3′,4′-Tetramethoxyflavone, metabolites, isotope labeling*

INTRODUCTION

5,7,3′,4′-Tetramethoxyflavone (TMF) is one of the major polymethoxyflavones (PMFs) identified in *Murraya paniculata*,[1] *Piper porphyrophyllum*,[2] and *Kaempferia parviflora*.[3] These plants have been widely used for the treatment of various illnesses, such as dysentery, gout, digestive disorders, gastric ulcer,[3,4] and allergy.[5] Besides, they are also reported possessing several bioactivities, including antimicrobial, anti-inflammatory,[6] anticholinesterase,[7] and anti-allergic activity.[8] Recently, many studies indicated that, among many compounds identified, TMF might be one of the main phytochemicals responsible for the bioactivities of these plants.[7,9−11]

PMFs are widely distributed in citrus plant. Demethylation is one of the major metabolic pathways of PMFs in animals,[12−14] in that the major metabolites of PMFs are hydroxylated PMFs. PMF metabolites have recently drawn much attention from researchers because some of them are with different or more potent bioactivities.[15−18] As a result, the identification of PMF metabolites is crucial to have a deeper understanding of biological activities of PMFs. Thus far, the metabolite identification relies mainly on high-performance liquid chromatography−mass spectrometry (HPLC−MS) and nuclear magnetic resonance (NMR). However, the identification of PMF metabolites faces a major obstacle; several metabolites are positional isomers with the same molecular weight, which are unable to be distinguished by HPLC−MS. It was also reported that some of these isomers are unable to be separated by the reverse-phase HPLC system,[19] which makes this task more difficult. Because only a very limited quantity of metabolites is available from animal urine or plasma, it will take a long time to collect enough metabolites for NMR analysis.

To the best of our knowledge, there is no report on *in vivo* metabolism of TMF. In this study, we report an isotope-labeling method for the identification of TMF metabolites. 5,7,3′,4′-Tetramethoxy[3′-D_3]flavone, 5,7,3′,4′-tetramethoxy-[4′-D_3]flavone, and 5,7,3′,4′-tetramethoxy[5,4′-D_6]flavone were synthesized and administered to rats. The main metabolites in urine were monitored and identified by ultrahigh-performance liquid chromatography−electrospray ionization−mass spectrometry (UPLC−ESI−MS).

MATERIALS AND METHODS

NMR Instrument. NMR spectra were obtained with a Bruker AVIII 500 MHz FT-NMR (Bruker, Rheinstetten, Germany) in $CDCl_3$ with tetramethylsilane as an internal standard.

Gas Chromatography−Mass Spectrometry (GC−MS). GC−MS spectra were obtained with an Agilent (Agilent Technologies, Wilmington, DE) 5973 mass spectrometer coupled to a 6890 gas chromatograph. A fused silica capillary column (SPB-5, 30 m × 0.25 mm inner diameter × 0.25 μm film thickness, Supelco, Bellefonte, PA) was employed. The injector temperature was 275 °C. The GC oven temperature was programmed as follows: 50 °C for 5 min, increased to 240 °C at a rate of 10 °C/min, and held at this final temperature for 5 min. The ion source temperature was 230 °C, and the analyzer

Received: May 10, 2012
Revised: July 18, 2012
Accepted: July 18, 2012
Published: July 19, 2012

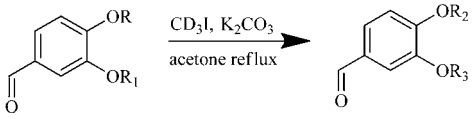
temperature was 150 °C. Mass spectra were obtained by electron impact (EI) at 70 eV.

UPLC–ESI–MS. A Waters Acquity UPLC was connected to a triple quadrupole mass spectrometer (Waters, Manchester, U.K.) equipped with an ESI source. The chromatographic separation was carried out using an Acquity BEH C18 column (Waters, MA), 1.7 μm, 2.1 × 50 mm, operated at 40 °C with a flow rate of 0.3 mL/min. The mobile phase consisted of methanol and water with 5 mM ammonium acetate. The elution program was 10% methanol for 0 min, raised to 100% methanol for 5–8 min, and reduced to 10% methanol for 9–13 min. The sample injection volume was 5 μL. The ESI was performed in positive ionization mode with parameters as follows: source temperature, 80 °C; capillary voltage, 3.2 kV; cone voltage, 30 V; desolvation gas, 700 L/h; and desolvation temperature, 350 °C.

Chemicals and Reagents. 3-Hydroxy-4-methoxybenzaldehyde, 3,5-dimethoxyphenol, boron trifluoride diethyl etherate, iodine, iodomethane-d_3, and vanillin were purchased from Sigma-Aldrich (St. Louis, MO). Acetic acid, acetone, dimethyl sulfoxide, and methanol were purchased from Tedia (Fairfield, OH). Anhydrous magnesium sulfate, potassium carbonate, and sodium hydroxide were purchased from Showa (Tokyo, Japan). All solvents and chemicals were used without further purification, unless otherwise stated.

Syntheses of Deuterium-Labeled TMFs. Deuterium-labeled TMFs were synthesized from their corresponding chalcones. It started with the preparation of 3,4-dimethoxy[3-D_3]benzaldehyde and 3,4-dimethoxy[4-D_3]benzaldehyde ($\mathbf{II_a}$ and $\mathbf{II_b}$), prepared by reacting 3-hydroxy-4-methoxybenzaldehyde ($\mathbf{I_a}$) and 4-hydroxy-3-methoxybenzaldehyde ($\mathbf{I_b}$) with iodomethane-d_3 in acetone[20] (Scheme 1). The mixture was stirred at room temperature for overnight, and then solvent was removed on a rotary evaporator. Water (30 mL) was added and extracted with CH_2Cl_2 (3 × 30 mL). The organic layers were combined, washed with brine, dried over anhydrous $MgSO_4$, and evaporated on a rotary evaporator to give $\mathbf{II_a}$ and $\mathbf{II_b}$ as off-white solids.

Scheme 1. 3,4-Dimethyl[3-D_3]benzaldehyde ($\mathbf{II_a}$) and 3,4-Dimethyl[4-D_3]benzaldehyde ($\mathbf{II_b}$) Syntheses



$\mathbf{I_a}$: R=CH_3, R_1=H
$\mathbf{I_b}$: R=H, R_1=CH_3

$\mathbf{II_a}$: R_2=CH_3, R_3=CD_3
$\mathbf{II_b}$: R_2=CD_3, R_3=CH_3

GC–MS spectra of both $\mathbf{II_a}$ and $\mathbf{II_b}$ showed a M^+ peak at m/z 169 (100%).

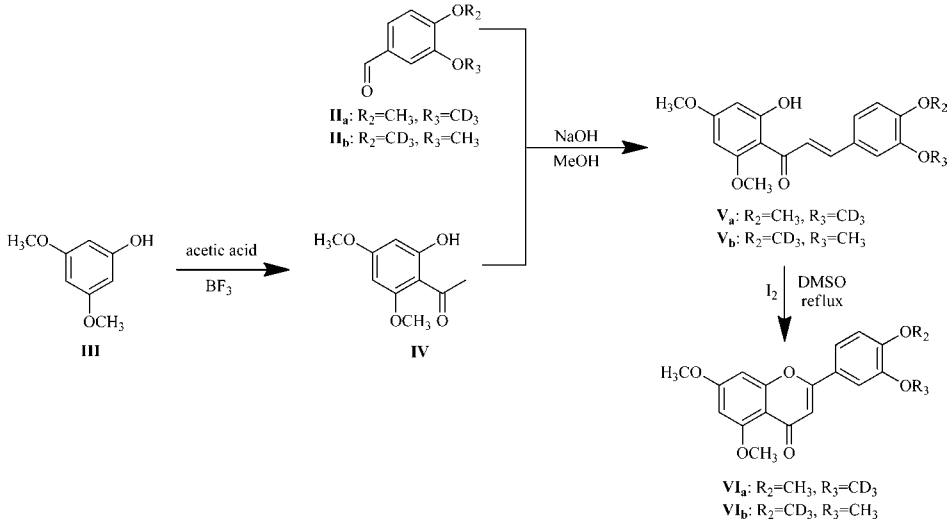
1-(2-Hydroxy-4,6-dimethoxyphenyl)ethanone (**IV**) was prepared by heating 3,5-dimethoxyphenol (**III**, 10.0 g, 65 mmol) with boron trifluoride diethyl etherate (25.5 g, 180 mmol) and acetic acid (50 mL) at 85 °C for 3 h. The crude product was purified by column chromatography (silica gel, 1:6 ethyl acetate/hexanes) to give 7.4 g of off-white solids. GC–MS spectrum of compound **IV** showed a M^+ peak at m/z 196 (100%).

Chalcones were prepared by aldol condensation of 1-(2-hydroxy-4,6-dimethoxyphenyl)ethanone (**IV**) with the corresponding benzaldehydes ($\mathbf{II_a}$ or $\mathbf{II_b}$). 1-(2-Hydroxy-4,6-dimethoxyphenyl)ethanone (**IV**, 2.1 g, 10 mmol) and 3,4-dimethoxy[D_3]benzaldehyde ($\mathbf{II_a}$ or $\mathbf{II_b}$, 2.0 g, 12 mmol) were dissolved in 40 mL of methanol. Sodium hydroxide (2.0 g, 50 mol) was added and stirred overnight. HCl (3 N) was then added until the solution was acidic. The yellow precipitate was filtered, washed with distilled water, and recrystallized from ethanol to give 2.6–2.8 g of chalcone ($\mathbf{V_a}$ or $\mathbf{V_b}$) as yellow crystals.

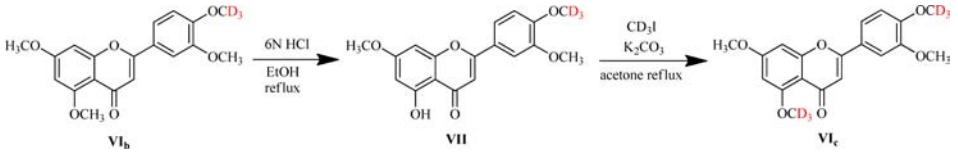
Yielded chalcone ($\mathbf{V_a}$ or $\mathbf{V_b}$) was then refluxed with 0.05 equiv of I_2 in dimethylsulfoxide (DMSO) for 30 min[21] (Scheme 2). After the mixture was cooled, it was poured into ice water saturated with $Na_2S_2O_3$ and extracted with ethyl acetate. The organic layers were combined, washed with brine, and dried over anhydrous $MgSO_4$. After evaporation of the solvent, the product was filtered through a plug of silica gel washed with ethyl acetate/hexanes (1:3, v/v). The solvent was removed on a rotary evaporator, and the crude product was crystallized from a mixture of ethyl acetate/hexanes (2:1, v/v) to give 1.2–1.4 g of 5,7,3′,4′-tetramethoxy[D_3]flavones ($\mathbf{VI_a}$ or $\mathbf{VI_b}$) as off-white solids. Structures were confirmed by ^{1}H NMR. $\mathbf{VI_a}$: δ = 3.89 (3H, s, OCH_3), 3.92 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 6.34 (1H, d, J = 2.3 Hz, H-6), 6.53 (1H, d, J = 2.3 Hz, H-8), 6.57 (1H, s, H-3), 6.92 (1H, d, J = 8.5 Hz, H-5′), 7.28 (1H, d, J = 2.1 Hz, H-2′), and 7.47 (1H, dd, J = 2.1 and 8.5 Hz, H-6′). $\mathbf{VI_b}$: δ = 3.89 (3H, s, OCH_3), 3.92 (3H, s, OCH_3), 3.93 (3H, s, OCH_3), 6.34 (1H, d, J = 2.3 Hz, H-6), 6.52 (1H, d, J = 2.3 Hz, H-8), 6.57 (1H, s, H-3), 6.92 (1H, d, J = 8.5 Hz, H-5′), 7.28 (1H, d, J = 2.1 Hz, H-2′), and 7.47 (1H, dd, J = 2.1 and 8.5 Hz, H-6′).

5,7,3′,4′-Tetramethoxy[5,4′-D_6]flavones ($\mathbf{VI_c}$) was synthesized via the methylation of 5-hydroxy-7,3′,4′-trimethoxy[4′-D_3]flavone (**VII**), which was generated by selected demethylation of 5,7,3′,4′-tetramethoxy[4′-D_3]flavones ($\mathbf{VI_b}$),[22] with iodomethane-d_3 in acetone (Scheme 3). Structures were confirmed by ^{1}H NMR. **VII**: δ = 3.88 (3H, s, OCH_3), 3.98 (3H, s, OCH_3), 6.35 (1H, d, J = 2.2 Hz, H-6), 6.48 (1H, d, J = 2.2 Hz, H-8), 6.57 (1H, s, H-3), 6.96 (1H, d, J = 8.4 Hz, H-5′), 7.32 (1H, d, J = 2.0 Hz, H-2′), 7.50 (1H, dd, J = 2.0 and 8.4 Hz, H-6′), and 12.78 (1H, s, 5-OH). $\mathbf{VI_c}$: δ = 3.89 (3H, s, OCH_3),

Scheme 2. 5,7,3′,4′-Tetramethoxy[3′-D_3]flavone ($\mathbf{VI_a}$) and 5,7,3′,4′-Tetramethoxy[4′-D_3]flavone ($\mathbf{VI_b}$) Syntheses



Scheme 3. 5,7,3′,4′-Tetramethoxy[5,4′-D_6]flavone (VI_c) Synthesis



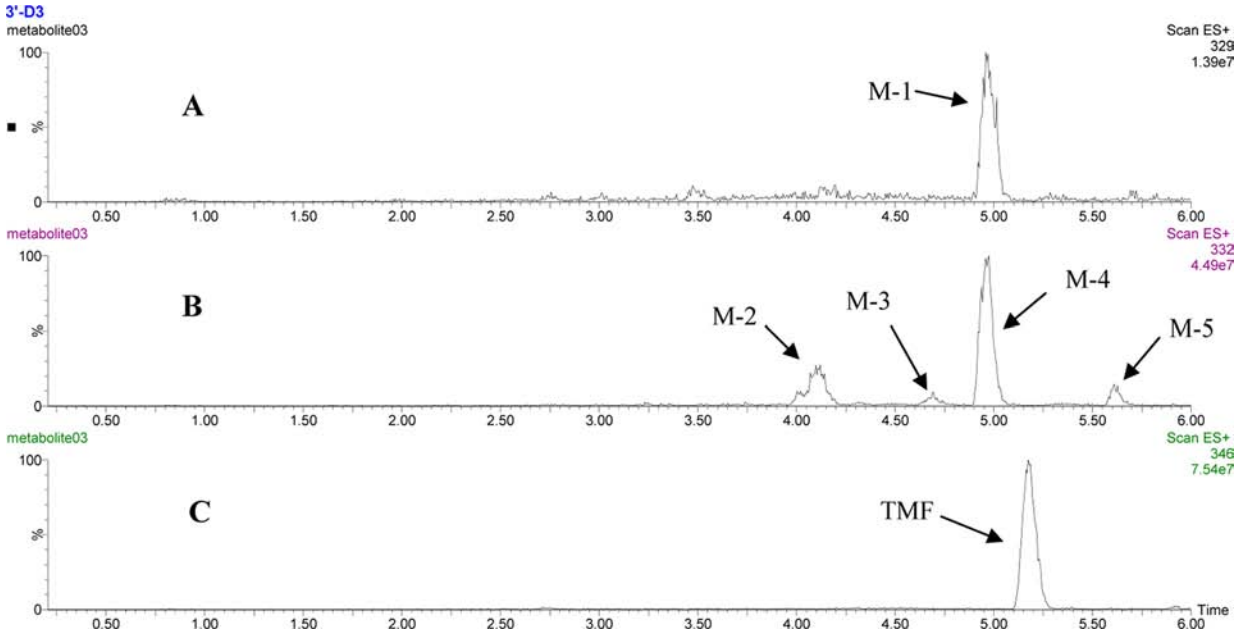


Figure 1. UPLC–MS analysis of the urine of rats administered 5,7,3′,4′-tetramethoxy[3′-D_3]flavone: (A) ion chromatogram of m/z 329, (B) ion chromatogram of m/z 332, and (C) ion chromatogram of m/z 346.

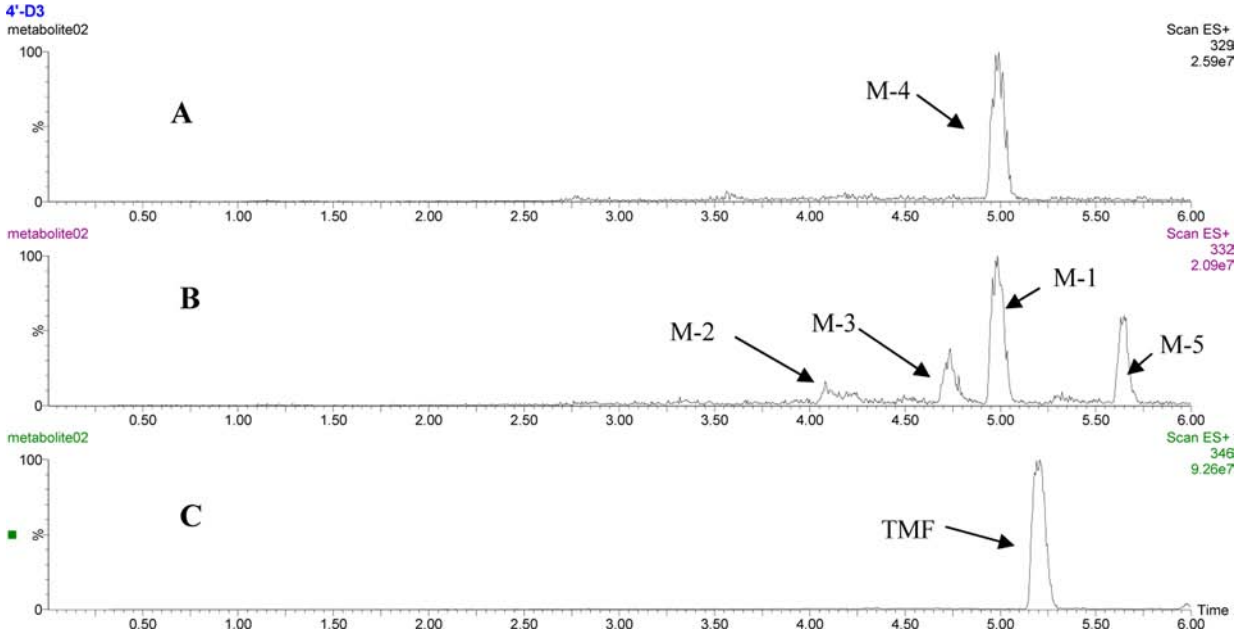


Figure 2. UPLC–MS analysis of the urine of rats administered 5,7,3′,4′-tetramethoxy[4′-D_3]flavone: (A) ion chromatogram of m/z 329, (B) ion chromatogram of m/z 332, and (C) ion chromatogram of m/z 346.

3.94 (3H, s, OCH_3), 6.34 (1H, d, J = 2.3 Hz, H-6), 6.53 (1H, d, J = 2.3 Hz, H-8), 6.57 (1H, s, H-3), 6.92 (1H, d, J = 8.5 Hz, H-5′), 7.29 (1H, d, J = 2.1 Hz, H-2′), and 7.47 (1H, dd, J = 2.1 and 8.5 Hz, H-6′).

Animals. Six 8-week-old male Sprague–Dawley rats weighing about 250–280 g were purchased from BioLASCO Co. (Taipei, Taiwan), housed in a temperature-controlled room at 24 ± 2 °C with a 12 h light–dark cycle and given *ad libitum* access to food and water. The rats were randomly grouped into three groups with two rats in each group and fasting for 16 h before tube-feeding with 50 mg of TMF/kg of body weight (bw) dissolved in saline solution. Animals in each group were administrated with one of three isotope-labeled

dx.doi.org/10.1021/jf302043a | *J. Agric. Food Chem.* 2012, 60, 8123–8128

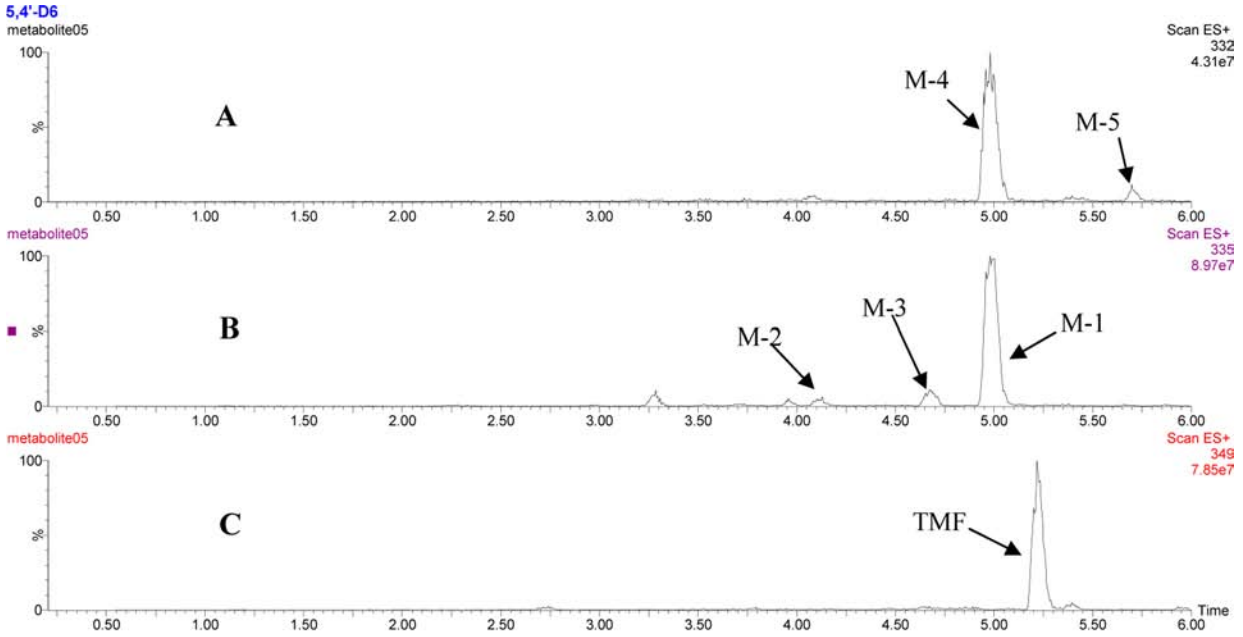


Figure 3. UPLC–MS analysis of the urine of rats administered 5,7,3′,4′-tetramethoxy[5,4′-D_6]flavone: (A) ion chromatogram of m/z 332, (B) ion chromatogram of m/z 335, and (C) ion chromatogram of m/z 349.

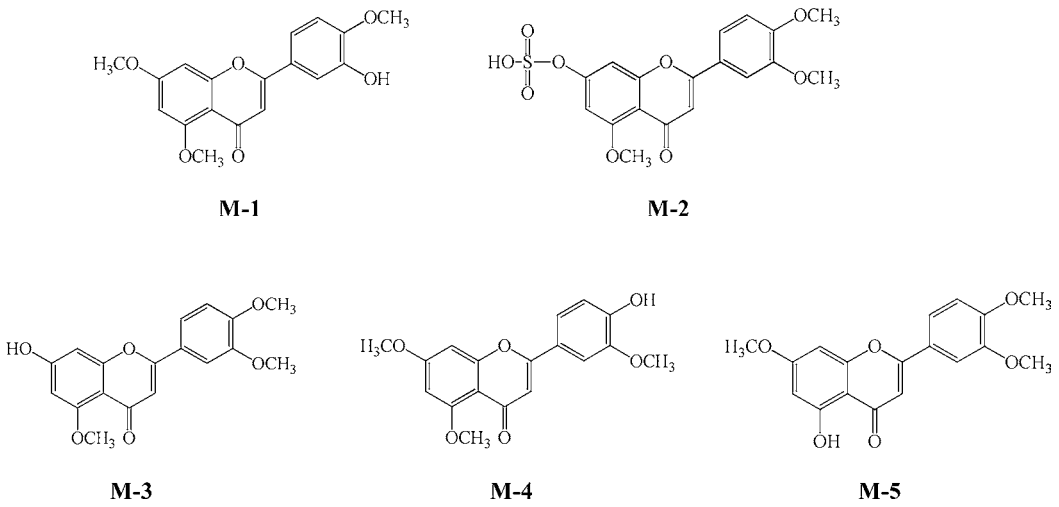


Figure 4. TMF metabolites identified in rat urine.

TMFs ($\mathbf{VI_a}$, $\mathbf{VI_b}$, and $\mathbf{VI_c}$) individually. Urine from each rat was collected in an 8 h period.

TMF Metabolite Isolation. The collected urine samples were freeze-dried and then dissolved in 5 mL of distilled water followed by ethyl acetate extraction (10 mL). The organic layer was transferred and centrifuged for approximately 10 min at 3000 rpm. The supernatant was dried under nitrogen stream at 40 °C and reconstituted with 200 μL of reconstitution solvent (MeOH/water = 7:3).

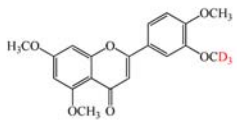
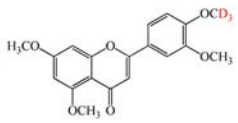
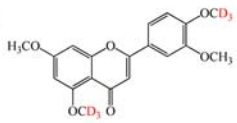
RESULTS

The isolated metabolites from the urine of rats administered 5,7,3′,4′-tetramethoxy[3′-D_3]flavone ($\mathbf{VI_a}$) were analyzed by UPLC–ESI–MS. Figure 1 shows the m/z 329, 332, and 346 ion chromatograms extracted from the total ion chromatogram. 5,7,3′,4′-Tetramethoxy[3′-D_3]flavone ($\mathbf{VI_a}$) exhibited a protonated molecule $[M + H]^+$ at m/z 346. Five major metabolites, named M-1, M-2, M-3, M-4, and M-5, were detected. Metabolite M-1 with a retention time of 4.96 min exhibited a protonated molecule $[M + H]^+$ at m/z 329. The mass difference between m/z 346 and 329 is 17, resulting from the demethylation of $\mathbf{VI_a}$ at the B-ring 3′ position having a deuterated methoxy group, and thus, M-1 was identified as 3′-hydroxy-5,7,4′-trimethoxyflavone. Metabolite M-2 with a retention time of 4.16 min exhibited a protonated molecule $[M + H]^+$ at m/z 412 and a neutral loss of 80 atomic mass units (amu), which suggested a loss of sulfate from a monohydroxy-trimethoxyflavone-sulfated metabolite. The position of the hydroxyl substituent could be at any but B-ring 3′ position. Metabolites M-3, M-4, and M-5 with retention times of 4.68, 4.96, and 5.62 min, respectively, all exhibited protonated molecules $[M + H]^+$ at m/z 332. The mass difference between m/z 346 and 332 is 14, which represented monohydroxy-trimethoxyflavone structures with a deuterated methoxy group at the 3′ position retained.

The urine of rats administered 5,7,3′,4′-tetramethoxy[4′-D_3]flavone ($\mathbf{VI_b}$) were also collected and analyzed by HPLC–

dx.doi.org/10.1021/jf302043a | J. Agric. Food Chem. 2012, 60, 8123–8128

Table 1. Protonated Molecules $[M + H]^+$ of TMFs and Metabolites Generated by Three Different Deuteriomethylated TMFs

	compd administrated		
			
	IV_a	IV_b	IV_c
compd identified	m/z of $[M+H]^+$		
TMF	346	346	349
M-1	329	332	335
M-2	412	412	415
M-3	332	332	335
M-4	332	329	332
M-5	332	332	332

ESI−MS (Figure 2). 5,7,3′,4′-Tetramethoxy[4′-D_3]flavone ($\mathbf{VI_b}$) also exhibited a $[M + H]^+$ at m/z 346. Metabolite M-4, coeluted with metabolite M-1, exhibited a protonated molecule $[M + H]^+$ at m/z 329. The mass difference between m/z 346 and 332 is 17, resulting from the demethylation of $\mathbf{VI_b}$ at the B-ring 4′ position having a deuterated methoxy group, and thus, M-4 was identified as 4′-hydroxy-5,7,3′-trimethoxyflavone.

To precisely locate the position of hydroxyl substituents on metabolite M-3 and M-5, the rats were administered with 5,7,3′,4′-tetramethoxy[5,4′-D_6]flavone ($\mathbf{VI_c}$) and then the urine was collected and analyzed in the same manner (Figure 3). 5,7,3′,4′-Tetramethoxy[5,4′-D_6]flavone ($\mathbf{VI_c}$) exhibited a protonated molecule $[M + H]^+$ at m/z 349, and metabolite M-5 exhibited a protonated molecule $[M + H]^+$ at m/z 332. The mass difference between m/z 349 and 335 is 17, which was generated by demethylation of $\mathbf{VI_c}$ at the A-ring 5 position or B-ring 4′ position, both having a deuterated methoxy group. The metabolite with demethylation at the B-ring 4′ position had been identified as M-4; therefore, M-5 was identified as 5-hydroxy-7,3′,4′-trimethoxyflavone.

In the same manner, metabolite M-3, exhibiting a protonated molecule $[M + H]^+$ at m/z 335, was identified as 7-hydroxy-5,3′,4′-trimethoxyflavone. The mass difference between $\mathbf{VI_c}$ and M-3 is 14, resulting from the demethylation of $\mathbf{VI_c}$ at either the A-ring 7 position or B-ring 3′ position. The metabolite with demethylation at the B-ring 3′ position had been identified as M-1; therefore, M-3 was identified as 7-hydroxy-5,3′,4′-trimethoxyflavone (Figure 4).

Metabolite M-2 exhibited a protonated molecule $[M + H]^+$ at m/z 415 and a neutral loss of 80 amu; it suggested that the aglycone was either 7-hydroxy-5,3′,4′-trimethoxyflavone or 3′-hydroxy-5,7,4′-trimethoxyflavone. As mentioned, when administered with 5,7,3′,4′-tetramethoxy[3′-D_3]flavone ($\mathbf{VI_a}$), M-2 exhibited a protonated molecule $[M + H]^+$ at m/z 412 and a neutral loss of 80 amu, in that the position of the hydroxyl substituent could be anywhere but the B-ring 3′ position. As a result, M-2 was identified as 7-hydroxy-5,3′,4′-trimethoxyflavone sulfate (Table 1).

DISCUSSION

Demethylation is the major phase I metabolic pathway of TMF, and therefore, the major metabolites generated were monohydroxy-trimethoxyflavones. Because all of these monohydroxy-trimethoxyflavones are positional isomers with the same molecular weights, it would be a very arduous task to identify these metabolites by liquid chromatography−mass spectrometry (LC−MS) without confirmation with synthetic standards. Our investigation revealed that some of these metabolites might not be separated by a reverse-phase liquid chromatography system, the most widely used separation system for metabolite identification. It makes the metabolite identification by other spectroscopic instruments, such as Fourier transform infrared (FTIR) spectroscopy or NMR, more difficult; not even confirmation with synthetic standards is able to identify all PMF metabolites. The isotope-labeling method that we developed is proven to be a very accurate and effective method for the PMF metabolite identification; it is able to unambiguously identify all metabolites by most LC−MS systems without confirmation with synthetic standards. We strongly believe that the reinvestigation of metabolism of other PMFs, such as tangeretin and nobiletin, is necessary.

AUTHOR INFORMATION

Corresponding Author

*Telephone: +886-2-23629984 (L.S.H.); +886-3-341-2500, ext. 2512 (G.-J.W.). Fax: +886-2-33664113 (L.S.H.); +886-3-270-5904 (G.-J.W.). E-mail: lshwang@ntu.edu.tw (L.S.H.); gwei@mail.knu.edu.tw (G.-J.W.).

Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

TMF, 5,7,3′,4′-tetramethoxyflavone; PMF, polymethoxyflavone; HPLC−MS, high-performance liquid chromatography−mass spectrometry; UPLC−ESI−MS, ultrahigh-performance liquid chromatography−electrospray ionization−mass spectrometry; NMR, nuclear magnetic resonance; GC−MS, gas chromatography−mass spectrometry

REFERENCES

(1) Kinoshita, T.; Firman, K. Myricetin 5,7,3′,4′,5′-pentamethyl ether and other methylated flavonoids from *Murraya paniculata*. *Phytochemistry* **1997**, *45*, 179−181.

(2) Rajudin, E.; Ahmad, F.; Sirat, H. M.; Arbain, D.; Aboul-Enein, H. Y. Chemical constituents from tiger's betel, *Piper porphyrophyllum* N.E.Br. (Fam. Piperaceae). *Nat. Prod. Res.* **2010**, *24*, 387−390.

(3) Yenjai, C.; Prasanphen, K.; Daodee, S.; Wongpanich, V.; Kittakoop, P. Bioactive flavonoids from *Kaempferia parviflora*. *Fitoterapia* **2004**, *75*, 89−92.

(4) Rujjanawate, C.; Kanjanapothi, D.; Amornlerdpison, D.; Pojanagaroon, S. Anti-gastric ulcer effect of *Kaempferia parviflora*. *J. Ethnopharmacol.* **2005**, *102*, 120−122.
(5) Tewtrakul, S.; Subhadhirasakul, S. Anti-allergic activity of some selected plants in the Zingiberaceae family. *J. Ethnopharmacol.* **2007**, *109*, 535−538.
(6) Ahmad, F.; Emrizal; Sirat, H. M.; Jamaludin, F.; Mustapha, N. M.; Ali, R. M.; Arbain, D.; Aboul-Enein, H. Y. Antimicrobial and anti-inflammatory activities of *Piper porphyrophyllum* (Fam. Piperaceae). *Arabian J. Chem.* **2011**, DOI: 10.1016/j.arabjc.2010.12.032.
(7) Sawasdee, P.; Sabphon, C.; Sitthiwongwanit, D.; Kokpol, U. Anticholinesterase activity of 7-methoxyflavones isolated from *Kaempferia parviflora*. *Phytother. Res.* **2009**, *23*, 1792−1794.
(8) Tewtrakul, S.; Subhadhirasakul, S.; Kummee, S. Anti-allergic activity of compounds from *Kaempferia parviflora*. *J. Ethnopharmacol.* **2008**, *116*, 191−193.
(9) Azuma, T.; Kayano, S.; Matsumura, Y.; Konishi, Y.; Tanaka, Y; Kikuzaki, H. Antimutagenic and α-glucosidase inhibitory effects of constituents from *Kaempferia parviflora*. *Food Chem.* **2011**, *125*, 471−475.
(10) Matsuda, H.; Morikawa, T.; Ando, S.; Toguchida, I.; Yoshikawa, M. Structural requirements of flavonoids for nitric oxide production inhibitory activity and mechanism of action. *Bioorg. Med. Chem.* **2003**, *11*, 1995−2000.
(11) Aboul-Enein, H. Y.; Kładna, A.; Kruk, I. Radical scavenging ability of some compounds isolated from *Piper cubeba* towards free radicals. *Luminescence* **2011**, *26*, 202−207.
(12) Nielsen, S. E.; Breinholt, V.; Cornett, C.; Dragsted, L. O. Biotransformation of the citrus flavone tangeretin in rats. Identification of metabolites with intact flavane nucleus. *Food Chem. Toxicol.* **2000**, *38*, 739−746.
(13) Manthey, J. A.; Cesar, T. B.; Jackson, E.; Mertens-Talcott, S. Pharmacokinetic study of nobiletin and tangeretin in rat serum by high-performance liquid chromatography−electrospray ionization−mass spectrometry. *J. Agric. Food Chem.* **2011**, *59*, 145−151.
(14) Li, S.; Wang, Z.; Sang, S.; Huang, M. T.; Ho, C. T. Identification of nobiletin metabolites in mouse urine. *Mol. Nutr. Food Res.* **2006**, *50*, 291−299.
(15) Matsuda, H.; Morikawa, T.; Ueda, K.; Managi, H.; Yoshikawa, M. Structural requirements of flavonoids for inhibition of antigen-induced degranulation, TNF-α and IL-4 production from RBL-2H3 cells. *Bioorg. Med. Chem.* **2002**, *10*, 3123−3128.
(16) Jiang, W.; Kou, J.; Zhang, Z.; Yu, B. The effects of twelve representative flavonoids on tissue factor expression in human monocytes: Structure−activity relationships. *Thromb. Res.* **2009**, *124*, 714−720.
(17) Li, S.; Sang, S.; Pan, M.-H.; Lai, C.-S.; Lo, C.-Y.; Yang, C. S.; Ho, C.-T. Anti-inflammatory property of the urinary metabolites of nobiletin in mouse. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 5177−5181.
(18) Li, S.; Pan, M.-H.; Lai, C.-S.; Lo, C.-Y.; Dushenkov, S.; Ho, C.-T. Isolation and syntheses of polymethoxyflavones and hydroxylated polymethoxyflavones as inhibitors of HL-60 cell lines. *Bioorg. Med. Chem.* **2007**, *15*, 3381−3389.
(19) Li, S.; Di, T.; Dushenkov, S.; Ho, C.-T. Polymethoxyflavones: Metabolite identification and pathway. In *Dietary Supplements*; Ho, C.-T., Simon, J. E., Shahidi, F., Shao, Y., Eds.; American Chemical Society (ACS): Washington, D.C., 2008; ACS Symposium Series, Vol. *987*, Chapter 15, pp 216−232.
(20) Vyas, G. N.; Shah, N. M. Quinacetophenone monomethyl ether. *Org. Synth.* **1963**, *4*, 836−838.
(21) Lokhande, P. D.; Sakate, S. S.; Taksande, K. N.; Navghare, B. Dimethylsulfoxide−iodine catalysed deprotection of 2′-allyloxychalcones: Synthesis of flavones. *Tetrahedron Lett.* **2005**, *46*, 1573−1574.
(22) Ho, C.-T.; Li, S.; Pan, M.-H.; Lo, C.-Y.; Dushenkov, S. Hydroxylated polymethoxyflavone compositions. U.S. Patent Application 20070275106 A1, Nov 29, 2007.