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Identification of Sinensetin Metabolites in Rat Urine by an Isotope-Labeling Method and Ultrahigh-Performance Liquid Chromatography−Electrospray Ionization Mass Spectrometry

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ABSTRACT: Sinensetin (SIN), one of the major polymethoxyflavones (PMFs) contained mainly in the citrus peels, has been reported to possess various bioactivities, including antifungal, antimutagenic, anticancer, and anti-inflammatory activities. Although the biotransformation of SIN in fungi and insects has been reported, the information about the metabolism of SIN in mammals is still unclear. In this study, formation of SIN metabolites in rats was investigated. Four isotope-labeled SINs ([4′-D_3]SIN, [3′-D_3]SIN, [5-D_3]SIN, and [6-D_3]SIN) were synthesized and administered to rat. The urine samples were collected and main metabolites were monitored by ultrahigh-performance liquid chromatography−electrospray ionization mass spectrometry. The administered compound and four SIN metabolites were detected in rat urine. These metabolites were identified as 4′-hydroxy-5,6,7,3′-tetramethoxyflavone, 5-hydroxy-6,7,3′,4′-tetramethoxyflavone, 6-hydroxy-5,7,3′,4′-tetramethoxyflavone, and 7-hydroxy-5,6,3′,4′-tetramethoxyflavone sulfate.

KEYWORDS: *sinensetin, urinary metabolites, isotope-labeling, polymethoxyflavones, electrospray ionization mass spectrometry*

INTRODUCTION

Citrus fruits are very popular worldwide with various applications. Although generally eaten fresh, citrus juice is one of the major beverage products in the food industry, essential oils obtained from peels are widely used in foods and cosmetics, and the highly contented ascorbic acid is used for prevention of scurvy. They are also important ingredients in the kitchen. Recently, many studies indicated that the health benefits of citrus fruits are more than prevention of scurvy. They have been proved to possess a wide range of biological activities, including anticarcinogenic, anti-inflammatory, antimutagenic, antiviral, and antithrombogenic properties, and these bioactivities may be contributed by the flavonoids contained.[1−6] Among the flavonoids identified in citrus, polymethoxyflavones (PMFs) are of particular interest. Citrus PMFs exist mainly in peels and may have inhibitory activity against fungi that cause plant disease;[7] additionally, many studies also indicated that PMFs may have a broad spectrum of health-promoting properties in humans.[8,9]

The biotransformation of bioactive compounds in the body has a great impact on their biological effects. There are evidence that PMFs may undergo biotransformation in vivo and produce metabolites with various bioactivities,[10−13] in that the metabolism of PMFs is of importance. The major metabolic pathway of PMFs is demethylation catalyzed by cytochrome P-450 (CYP), mainly on the B-ring;[14,15] therefore, the major metabolites of PMFs are corresponding hydroxylated PMFs, and some of them are positional isomers.[16] Although many of PMFs have been extensively studied during the past 2 decades for their biological properties, information on their metabolic profiles is still rare. One of the major challenges in the metabolism study of PMFs is the structure identification of metabolites, because many of the metabolites generated are positional isomers with the same molecular weights. Identification of these metabolites usually requires confirmation with synthetic standards.

Sinensetin (5,6,7,3′,4′-pentamethoxyflavone; SIN) is one of the major PMFs identified in citrus fruits. Its bioactivities include antimutagenic,[13] antiproliferative,[17] and anti-inflammatory activities.[18,19] The biotransformation of sinensetin by *Aspergillus niger* and the larvae of *Spodoptera litura* had been reported,[13,20] but its metabolism in mammals is not yet clear.

The isotope-labeling method is proven as an effective method for the qualitative identification of PMF metabolites.[21] In this study, we extended the application of this method to study the formation of sinensetin metabolites. Four deteruated methoxy isomers of sinensetin, [4′-D_3]SIN, [3′-D_3]SIN, [5-D_3]SIN, and [6-D_3]SIN were synthesized and administered to rats. The metabolites in urine were monitored and identified by ultrahigh-performance liquid chromatography−electrospray ionization mass spectrometry (UPLC−ESI-MS). Our future goal will be to synthesize the identified metabolites as standards and to quantitatively measure the distribution of sinensetin and its metabolites in the serum and organs of rats orally administered with sinensetin.

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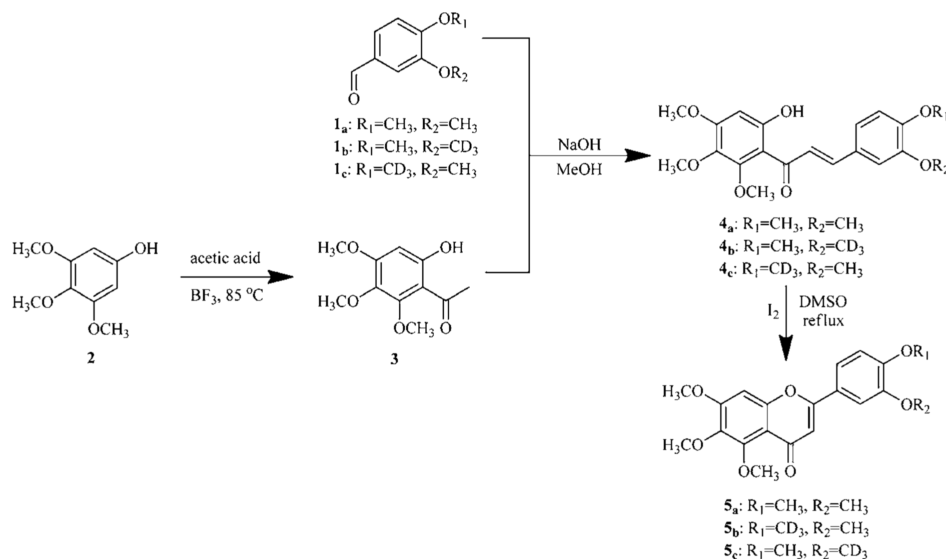

Figure 1. SIN (5_a), [4′-D_3]SIN (5_b), and [3′-D_3]SIN (5_c) syntheses.

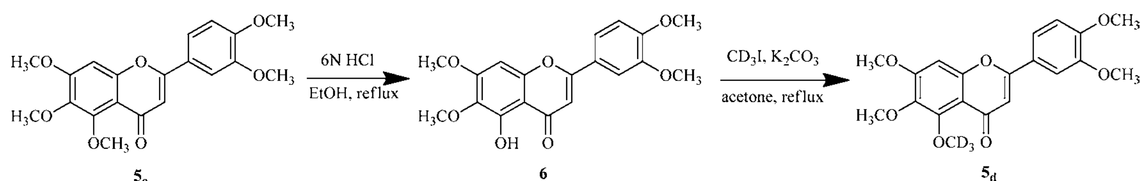

Figure 2. [5-D_3]SIN (5_d) synthesis.

■ MATERIALS AND METHODS

Chemicals. All solvents and chemicals were used without further purification unless otherwise stated. 3,4,5-Trimethoxyphenol was purchased from Alfa Aesar (Heysham, UK), and 3,4-dimethoxybenzaldehyde, 3-hydroxy-4-methoxybenzaldehyde, 2,6-dimethoxy-1,4-benzenediol, 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).

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

GC–MS. Gas chromatography–mass spectrometry (GC–MS) spectra were obtained with an Agilent (Agilent Technologies, Wilmington, DE) 5973 mass spectrometer coupled to a 6890 gas chromatography. A fused silica capillary column (SPB-5, 30 m × 0.25 mm i.d. × 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 temperature was 150 °C. Mass spectra were obtained by EI at 70 eV.

UPLC–ESI-MS. A Waters Acquity UPLC was connected to a TQD triple quadrupole mass spectrometer (Waters, Manchester, U.K.) equipped with an electrospray ionization source. The chromatographic separation was carried out using a Kinetex C18 column (2.6 μm, 2.1 × 100 mm) (Phenomenex, Torrance, CA), operated at 40 °C with a flow rate of 0.2 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 a 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; desolvation temperature, 350 °C.

Animals. Eight 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 four groups with two rats in each group and fasting for 16 h before tube-feeding with 50 mg/kg bw D_3-SINs dissolved in saline solution. Animals in each group were administrated with one of four D_3-SINs (5_b, 5_c, 5_d, or 5_e) individually. Urine from each rat was collected in an 8 h period.

Metabolite Extraction. 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 down under nitrogen stream at 40 °C and reconstituted with 200 μL of reconstitution solvent (MeOH:water = 7:3).

Synthesis. 1-(2-Hydroxy-4,5,6-trimethoxyphenyl)ethanone (**3**) was prepared by heating 3,4,5-trimethoxyphenol (**2**, 12.0 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, ethyl acetate/hexanes 1:6) to give 8.4 g of off-white solids. GC–MS: m/z 211 (100), and m/z 226 (85). 3,4-Dimethoxy[3-D_3]benzaldehyde (1_b) and 3,4-dimethoxy[4-D_3]-benzaldehyde (1_c) were prepared by reacting 3-hydroxy-4-methoxybenzaldehyde and 4-hydroxy-3-methoxybenzaldehyde with iodomethane-d_3 in acetone.[21]

SIN (5_a), [4′-D_3]SIN (5_b), and [3′-D_3]SIN (5_c) were prepared by coupling 1-(2-hydroxy-4,5,6-trimethoxyphenyl)ethanone (**3**, 3 g, 13 mmol) with corresponding benzaldehyde (1_a, 1_b, 1_c; 3.0 g, 18 mmol) to yield chalcone, (4_a, 4_b, 4_c), followed by cyclization and oxidation with 0.05 equiv of I_2 in DMSO to give SIN (5_a), [4′-D_3]SIN (5_b), and [3′-D_3]SIN (5_c) (Figure 1). SIN (5_a): ^{1}H NMR δ = 3.88 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 3.95 (6H, s, OCH_3), 6.54 (1H, s, H-8), 6.75 (1H, s, H-3), 6.92 (1H, d, J = 8.5 Hz, H-2′), 7.26 (1H, d, J = 2.1 Hz, H-5′), 7.45 (1H, dd, J = 2.1, 8.5 Hz, H-6′); ESI-MS $[M + H]^+$ = 373. [4′-D_3]SIN (5_b): ^{1}H NMR δ = 3.88 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 3.95 (6H, s, OCH_3), 6.54 (1H, s, H-8), 6.76 (1H, s, H-3), 6.92

(1H, d, J = 8.5 Hz, H-2′), 7.28 (1H, d, J = 2.1 Hz, H-5′), 7.45 (1H, dd, J = 2.1, 8.5 Hz, H-6′); ESI-MS $[M + H]^+$ = 376. [3′-D_3]SIN ($\mathbf{5_c}$): ^{1}H NMR δ = 3.88 (3H, s, OCH_3), 3.91 (3H, s, OCH_3), 3.95 (6H, s, OCH_3), 6.54 (1H, s, H-8), 6.75 (1H, s, H-3), 6.92 (1H, d, J = 8.5 Hz, H-2′), 7.27 (1H, d, J = 2.1 Hz, H-5′), 7.45(1H, dd, J = 2.1, 8.5 Hz, H-6′); ESI-MS $[M + H]^+$ = 376.

The preparation of [5-D_3]SIN ($\mathbf{5_d}$) was via the methylation of 5-hydroxy-6,7,3′,4′-tetramethoxyflavone (**6**), which was generated by selected demethylation of SIN ($\mathbf{5_a}$), as shown in Figure 2. 5-Demethyl-SIN (**6**): ^{1}H NMR δ = 3.93 (3H, s, OCH_3), 3.97 (3H, s, OCH_3), 3.98 (3H, s, OCH_3), 3.99 (3H, s, OCH_3), 6.54 (1H, s, H-8), 6.58 (1H, s, H-3), 6.97 (1H, d, J = 8.5 Hz, H-2′), 7.33 (1H, d, J = 2.1 Hz, H-5′), 7.51 (1H, dd, J = 2.1, 8.5 Hz, H-6′), 12.76 (1H, s, OH). [5-D_3]SIN ($\mathbf{5_d}$): ^{1}H NMR δ = 3.88 (3H, s, OCH_3), 3.92 (3H, s, OCH_3), 3.94 (3H, s, OCH_3), 3.96 (3H, s, OCH_3), 6.56 (1H, s, H-8), 6.76 (1H, s, H-3), 6.93 (1H, d, J = 8.5 Hz, H-2′), 7.29 (1H, d, J = 2.1 Hz, H-5′), 7.45 (1H, dd, J = 2.1, 8.5 Hz, H-6′); ESI-MS $[M + H]^+$ = 376.

Figure 3 shows the synthesis of [6-D_3]SIN ($\mathbf{5_e}$). 1-(3,6-Dihydroxy-2,4-dimethoxyphenyl)ethanone (**8**) was prepared by heating 2,6-

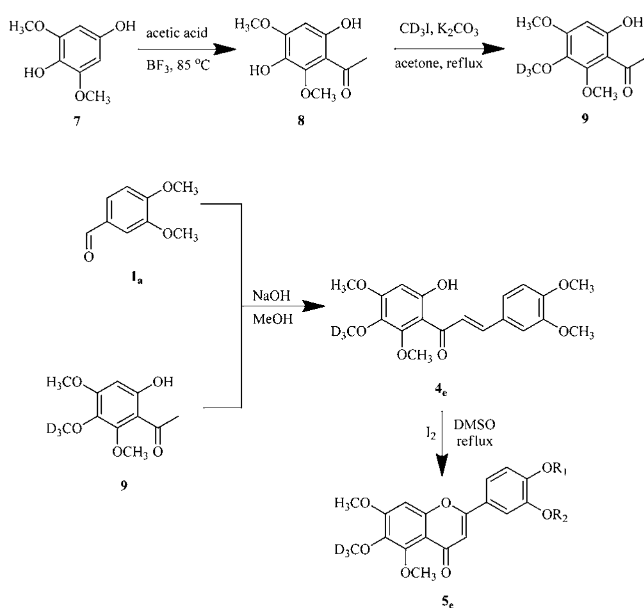

Figure 3. [6-D_3]SIN ($\mathbf{5_e}$) synthesis.

dimethoxy-1,4-benzenediol (**7**, 2.0 g, 12 mmol) with boron trifluoride diethyl etherate (3.5 g, 25 mmol) and acetic acid (10 mL) at 85 °C for 3 h. The crude product was purified by column chromatography (silica gel, ethyl acetate/hexanes 1:4) to give 1.5 g of compound **8**: GC−MS m/z 212 (100). 1-(2-Hydroxy-4,5,6-trimethoxyphenyl)[5-D_3]ethanone (**9**) was prepared by refluxing compound **8** (1.4 g, 7 mmol) with iodomethane-d_3 (1 mL) and 1.0 g of anhydrous potassium carbonate (7 mmol) in 15 mL of acetone overnight. The crude product was purified by column chromatography (silica gel, ethyl acetate/hexanes 1:6) to give 1.1 g of off-white solid: GC−MS m/z 229 (100). [6-D_3]SIN ($\mathbf{5_e}$): ^{1}H NMR δ = 3.93 (3H, s, OCH_3), 3.95 (3H, s, OCH_3), 3.96 (6H, s, OCH_3), 6.56 (1H, s, H-8), 6.77 (1H, s, H-3), 6.94 (1H, d, J = 8.5 Hz, H-2′), 7.29 (1H, d, J = 2.1 Hz, H-5′), 7.48 (1H, dd, J = 2.1, 8.5 Hz, H-6′); ESI-MS $[M + H]^+$ = 376.

■ RESULTS AND DISCUSSION

The molecular weight of SIN ($\mathbf{5_a}$) with formula $C_{20}H_{20}O_7$ is 372. The molecular weights of four deuterium-labeled SIN compounds ($\mathbf{5_b}$, $\mathbf{5_c}$, $\mathbf{5_d}$, and $\mathbf{5_e}$) administered to rats with formula $C_{20}D_3H_{17}O_7$ are 375 (Figure 4). If the molecular weight of demethyl-SIN detected in urine of rats has molecular weight of 358, it is suggestive of a mono-demethyl-SIN with a formula $C_{19}H_{18}O_7$; the demethylation occurs at the deuterated methoxy group. If the molecular weight of demethyl-SIN detected is 361, it is suggestive of a mono-demethyl-SIN with a formula $C_{19}D_3H_{15}O_7$; the demethylation occurs at any one of four undeuterated methoxy groups.

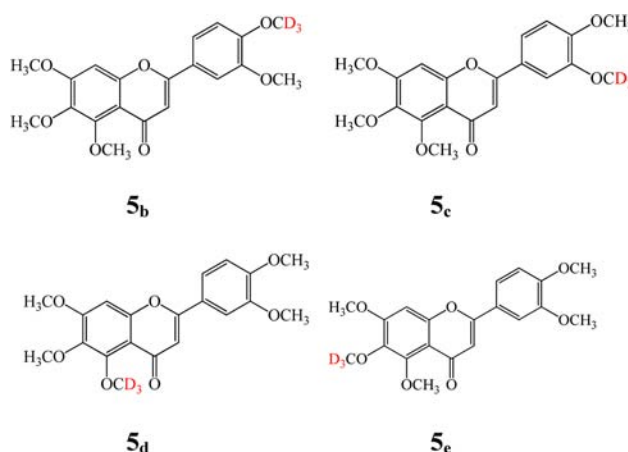

Figure 4. Structures of compounds administered.

SIN possesses five methoxy groups. In order to identify the positions of demethylation, four SINs with one deuterated methoxy group at different positions have to be synthesized. The main strategy for the preparation of most deuterated SINs is via their corresponding chalcones, followed by cyclization and oxidation. Chalcones were prepared by aldol condensation between an acetophenone and a benzaldehyde. Acetophenones serve as the building blocks for the A-ring, and benzaldehydes for the B-ring of chalcones and flavones, the final products. By coupling different building blocks for A- and B-rings, SINs with deuterated methoxy group at different positions could be secured.

[4′-D_3]SIN and [3′-D_3]SIN were prepared by using 3,4-dimethoxy[3-D_3]benzaldehyde ($\mathbf{1_b}$) and 3,4-dimethoxy[4-D_3]-benzaldehyde ($\mathbf{1_c}$) as the building blocks for the B-ring. [6-D_3]SIN was prepared by using 1-(2-hydroxy-4,5,6-trimethoxyphenyl)[5-D_3]ethanone (**9**) as the building block for the A-ring. 1-(2-Hydroxy-4,5,6-trimethoxyphenyl)[5-D_3]-ethanone (**9**) was synthesized by selected methylation of 1-(3,6-dihydroxy-2,4-dimethoxyphenyl)ethanone (**8**) with iodomethane-d_3.

The preparation of [5-D_3]SIN is more straightforward, just via the methylation of 5-hydroxy-6,7,3′,4′-tetramethoxyflavone (**6**), which was prepared by acid hydrolysis of SIN, with iodomethane-d_3.

The isolated metabolites from urine of rats administered [4′-D_3]SIN ($\mathbf{5_b}$) were analyzed by UPLC−ESI-MS. Figure 5 shows the m/z 359, 362, and 376 ion chromatograms extracted from the total ion chromatogram. [4′-D_3]SIN exhibited a protonated molecule $[M + H]^+$ at m/z 376. Four major metabolites, named **M-1**, **M-2**, **M-3**, and **M-4**, were detected. Metabolite **M-1** coeluting with **M-3** at 4.93 min and exhibited a protonated molecule $[M + H]^+$ at m/z 359. It is generated from the demethylation of compound $\mathbf{5_b}$ at the B-ring 4′-position having a deuterated methoxy group; thus, **M-1** is identified as 4′-hydroxy-5,6,7,3′-tetramethoxyflavone. Metabolite **M-2** and **M-3**, with retention times 5.02 and 4.93, both exhibited protonated molecules $[M + H]^+$ at m/z 362. It indicates that both **M-2** and **M-3** are mono-demethyl-SIN structures with a deuterated methoxy group at the B-ring 4′-position retained.

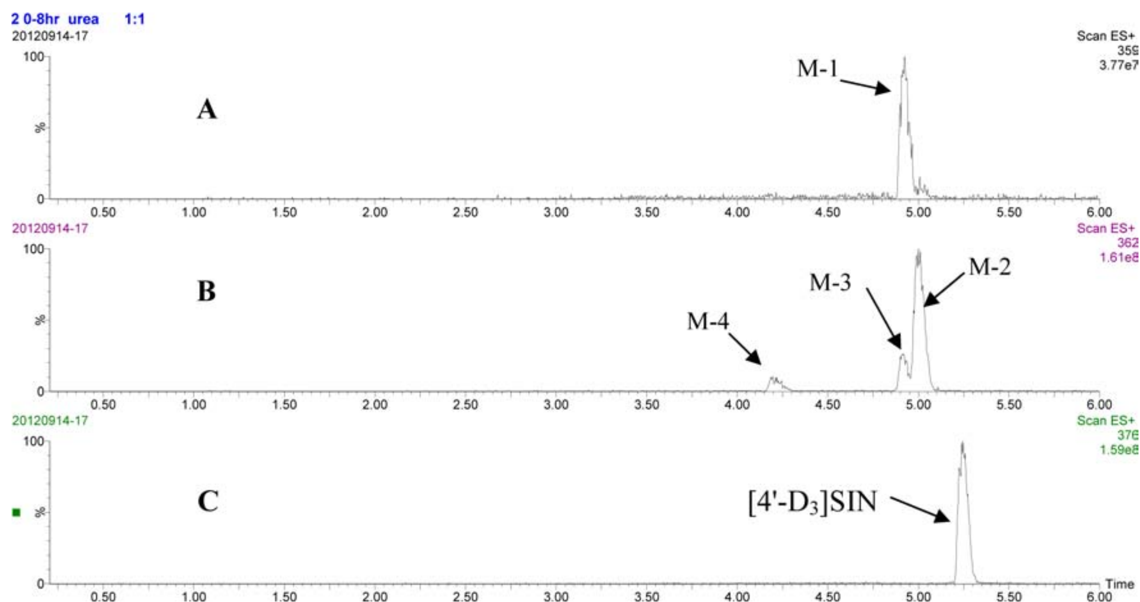

Figure 5. UPLC−ESI-MS analysis of urine of rats administered [4′-D_3]SIN. Ion chromatogram of (A) m/z 359, (B) m/z 362, and (C) m/z 376.

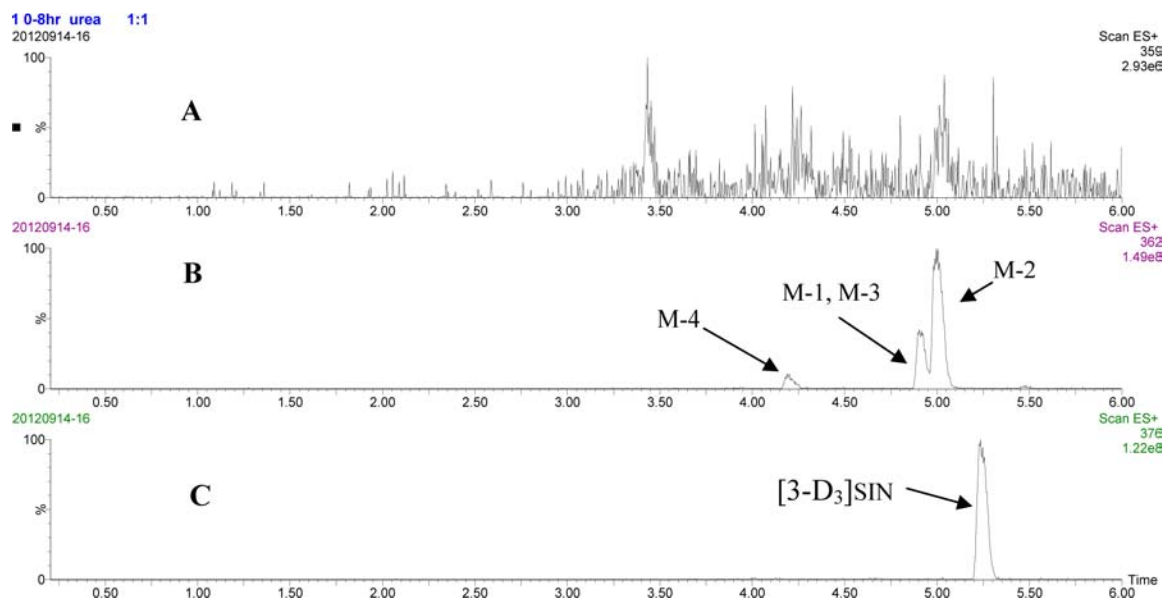

Figure 6. UPLC−ESI-MS analysis of urine of rats administered [3′-D_3]SIN. Ion chromatogram of (A) m/z 359, (B) m/z 362, and (C) m/z 376.

The urine of rats administered [3′-D_3]SIN (**5**$_c$) was also collected and analyzed by UPLC−ESI-MS (Figure 6). [3′-D_3]SIN (**5**$_c$) also exhibited a $[M + H]^+$ at m/z 376, but no metabolite with a protonated molecule $[M + H]^+$ at m/z 359 was detected. It indicates that no 3′-hydroxy-5,6,7,4′-tetramethoxyflavone was produced. It is interesting to note that the cytochrome P450 in rat has demethylation selectivity between the 3′-methoxy group and 4′-demethoxy group on the B-ring of sinensetin. It is also interesting to note that nobiletin, which has one more methoxy group on the A-ring compared to sinensetin, has both 3′-demethylnobiletin and 4′-demethylnobiletin as major metabolites.[22]

Figure 7 shows the results of rats administered [5-D_3]SIN (**5**$_d$). Metabolite **M-2**, eluting at 5.02 min, exhibited a protonated molecule $[M + H]^+$ at m/z 359. It is generated from the demethylation of compound **5**$_d$ at the A-ring 5-position having a deuterated methoxy group; therefore, **M-2** is identified as 5-hydroxy-6,7,3′,4′-tetramethoxyflavone.

Metabolite **M-3**, eluting at 4.93 min, exhibited a protonated molecule $[M + H]^+$ at m/z 359 when rats were administered [6-D_3]SIN (**5**$_e$) (Figure 8). It is generated from the demethylation of compound **5**$_d$ at the A-ring 5-position having a deuterated methoxy group; therefore, **M-3** is identified as 6-hydroxy-5,7,3′,4′-tetramethoxyflavone.

When any one of four deuterated SINs was administered, metabolite **M-4** with retention time 4.18 min always exhibited a protonated molecule $[M + H]^+$ at m/z 442 and a neutral loss of 80 amu. The neutral loss of 80 amu is suggestive a loss of sulfate from a mono-demethyl-SIN sulfated metabolite, and the mono-demethyl-SIN is 7-hydroxy-5,6,3′,4′-tetramethoxyflavone; therefore, **M-4** is identified as 7-hydroxy-5,6,3′,4′-tetramethoxyflavone sulfate (Table 1).

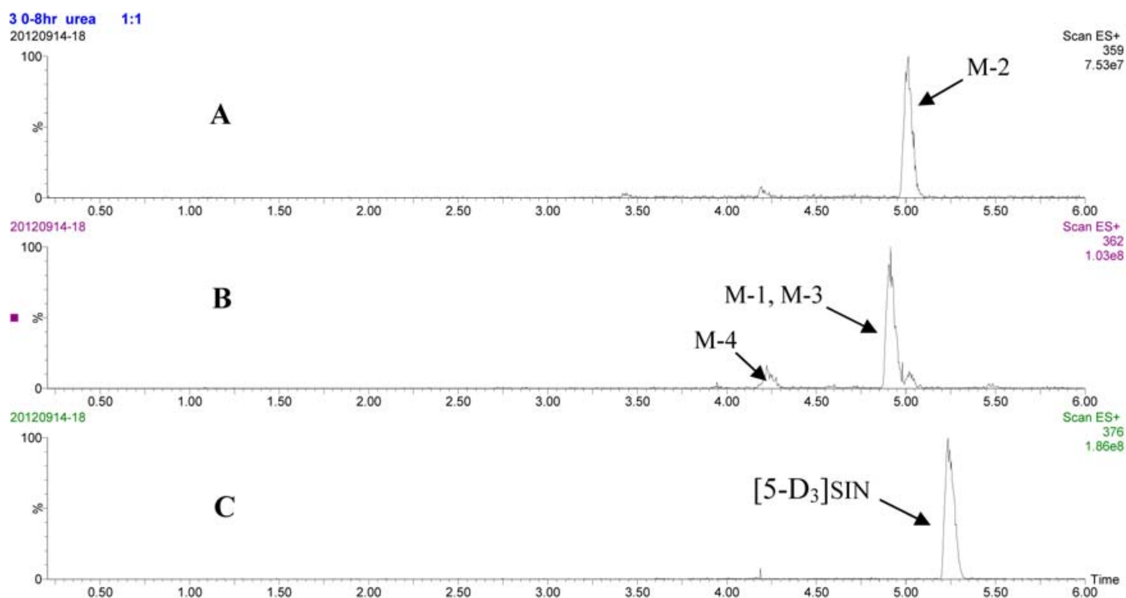

Figure 7. UPLC–ESI-MS analysis of urine of rats administered [5-D_3]SIN. Ion chromatogram of (A) *m*/*z* 359, (B) *m*/*z* 362, and (C) *m*/*z* 376.

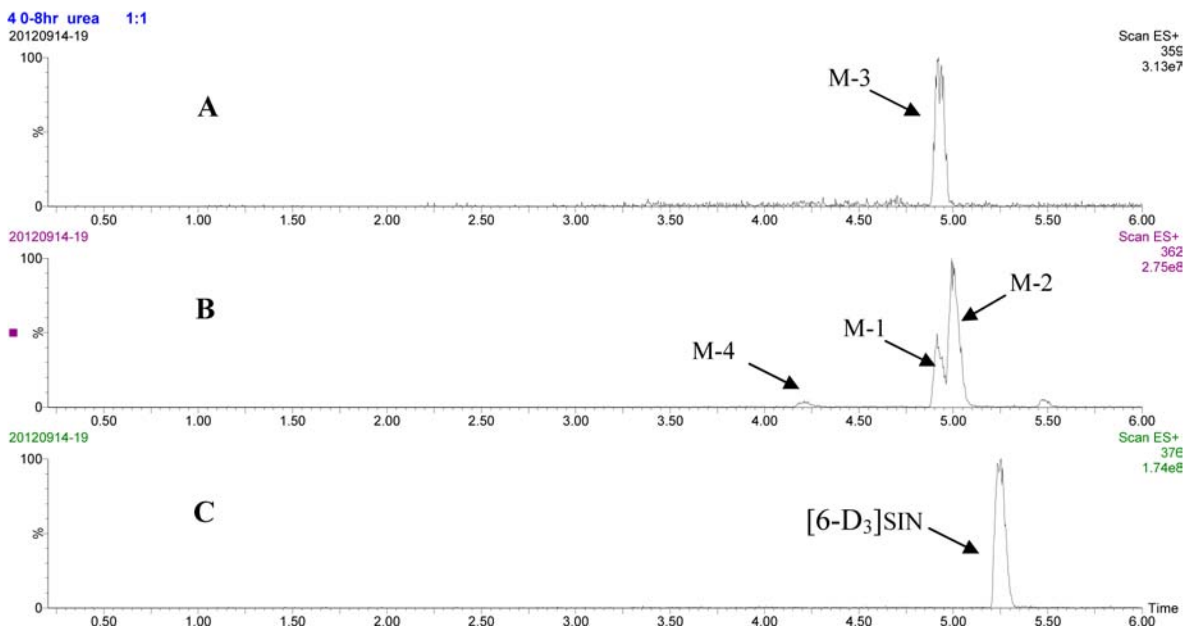

Figure 8. UPLC–ESI-MS analysis of urine of rats administered [6-D_3]SIN. Ion chromatogram of (A) *m*/*z* 359, (B) *m*/*z* 362, and (C) *m*/*z* 376.

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

	m/*z* of $[M + H]^+$			
compds identified	[4′-D_3]SIN	[3′-D_3]SIN	[5-D_3]SIN	[6-D_3]SIN
[D_3]SINs	376	376	376	376
M-1	359	362	362	362
M-2	362	362	359	362
M-3	362	362	362	359
M-4	442	442	442	442

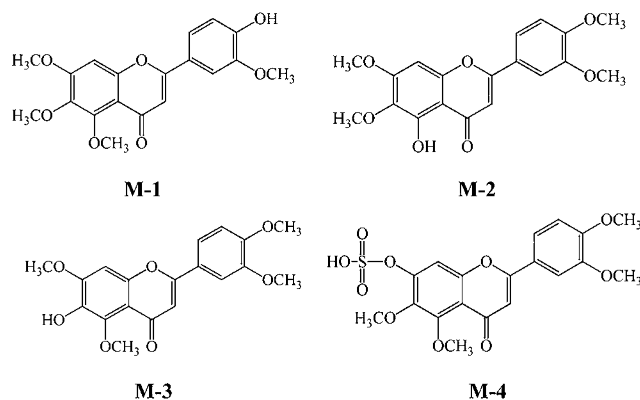

Figure 9. Sinesetin metabolites identified in rat urine.

In this study, the isotope-labeling method was successfully applied to the identification of sinensetin metabolites, and four sinensetin metabolites in rat urine were identified by UPLC–ESI-MS (Figure 9). The major metabolic pathway of sinensetin

in rat is demethylation, which mainly occurs at all positions but the B-ring 3′-position. Earlier, Okuno and Miyazawa reported that sinensetin was metabolized to 4′-hydroxy-5,6,7,3′-tetramethoxyflavone by *A. niger*.[13] They also reported that sinensetin was metabolized to 4′-hydroxy-5,6,7,3′-tetramethoxyflavone, 6-hydroxy-5,7,3′,4′-tetramethoxyflavone, and 5,7,3′,4′-tetramethoxyflavone-6-*O*-β-$_{D}$-glucoside by the larvae of *S. litura*.[20] For the B-ring, the 4′-position is likely more favorable for demethylation then other positions; therefore, 4′-hydroxy-5,6,7,3′-tetramethoxyflavone is one of the major metabolites in many species. It is worth mentioning that 4′-hydroxy-5,6,7,3′-tetramethoxyflavone showed better antimutagenic activity than sinensetin.[13]

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

CYP, cytochrome P-450; GC−MS, gas chromatography−mass spectrometry; HPLC−MS, high-performance liquid chromatography−mass spectrometry; NMR, nuclear magnetic resonance; PMF, polymethoxyflavones; SIN, sinensetin; UPLC−ESI-MS, ultrahigh-performance liquid chromatography−electrospray ionization mass spectrometry

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