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Title page

Preference for *O*-demethylation reactions in the oxidation of 2´-, 3´-, and 4´-methoxyflavones by human cytochrome P450 enzymes

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Abbreviations used,

P450 or CYP, cytochrome P450; b_5, cytochrome b_5; DLPC, L-α-1,2 dilauoryl-*sn*-glycero-3-phosphocholine; 4´MeF, 4´-methoxyflavone; 3´MeF, 3´-methoxyflavone 2´MeF, 2´-methoxyflavone; 4´OHF, 4´-hydroxyflavone; 3´OHF, 3´-hydroxyflavone; 2´OHF, 2´-hydroxyflavone; 6OHF, 6-hydroxyflavone; 3´4´diOHF, 3´,4´-dihydroxyflavone; 57diOHF, and 5,7-dihydroxyflavone (chrysin).

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Abstract

1. 2´-, 3´-, and 4´-Methoxyflavones (MeFs) were incubated with nine forms of recombinant human cytochrome P450 (P450 or CYP) enzymes in the presence of an NADPH-generating system and the products formed were analyzed with LC-MS/MS methods.

2. CYP1B1.1 and 1B1.3 were highly active in demethylating 4´MeF to form 4´-hydroxyflavone (rate of 5.0 nmol/min/nmol P450) and further to 3´,4´-dihydroxyflavone (rates of 2.1 and 0.66 nmol/min/nmol P450, respectively). 3´MeF was found to be oxidized by P450s to *m/z* 239 (M-14) products (presumably 3´-hydroxyflavone) and then to 3´,4´-dihydroxyflavone. P450s also catalyzed oxidation of 2´MeF to *m/z* 239 (M-14) and *m/z* 255 (M-14, M-14 +16) products, presumably mono- and di-hydroxylated products, respectively.

3. At least two types of ring oxidation products having *m/z* 269 fragments were formed, although at slower rates than the formation of mono- and di-hydroxylated products, on incubation of these MeFs with P450s; one type was products oxidized at the C-ring, having *m/z* 121 fragments, and the other one was the products oxidized at the A-ring (having *m/z* 137 fragments).

4. Molecular docking analysis indicated the preference of interaction of *O*-methoxy moiety of methoxyflavones in the active site of CYP1A2.

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5. These results suggest that 2´-, 3´-, and 4´-methoxyflavones are principally demethylated by human P450s to form mono- and di-hydroxyflavones and that direct oxidation occurs in these MeFs to form mono-hydroxylated products, oxidized at the A- or B-ring of MeF.

Introduction

Many plant flavonoids, such as flavones, flavanones, flavonols, isoflavones, catechins, and anthocyanins, have been suggested to have various biological effects, e.g. anti-cancer, anti-oxidative and anti-inflammatory activities in experimental animal model and epidemiological studies, preventing cancer, heart diseases, bone loss, and other diseases (Arct and Pytkowska, 2008; Kale et al., 2008, Walle et al., 2007; Zhang et al., 2005; Hostetler et al., 2017). These activities vary with the number and substitution positions of hydroxyl and/or methoxy groups in the flavonoid molecules (Breinholt et al., 2002, Hodek et al., 2002; Kim et al., 2005; Walle et al., 2007; Zhang et al., 2005). Androutsopoulos and associates have reported that CYP Family 1 enzymes in human breast cancer MDA-MB-468 cell lines play important roles in the activation of some methoxylated flavonoids (e.g., eupatorin (3´,5-dihydroxy-4´,6,7-trimethoxyflavone), diosmetin (3´,5,7-trihydroxy-4´-methylflavone), and nobiletin (3´,4´,5,6,7,8-hexamethoxyflavone)), to active metabolites with antitumor effects and to the suppression of metastasis in human cancer cells

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(Androutsopoulos, 2008; 2009a; 2009b; Surichan 2012). Interestingly, these methoxylated flavonoids are found to be bioactivated by CYP1 enzymes to singly-demethylated products (Surichan et al., 2018a; 2018b). We have previously shown that various flavonoid derivatives strongly inhibit human Family 1 P450 enzymes, particularly CYP1B1.3, and that 2´-, 3´-, and 4´-methoxyflavones (2´MeF, 3´MeF, and 4´MeF) are more potent in inhibiting CYP1B1.3 than flavone (Shimada et al., 2009; 2010; 2013). In addition, the introduction of 4´-methoxy or 3´,4´-dimethoxy moieties to 5,7-dihydroxyflavone (57diOHF, chrysin) has been shown to cause significant increases in inhibiting CYP1B1.3 activities (Shimada et al., 2010). It is of interest in this connection to note the results of Fatokun et al. (2013; 2014) who showed that 4´MeF and 3´,4´-dimethoxyflavone can effectively prevent decreases in cell viability of Hela and SH-SY5Y cells caused by *N*-methyl-*N*´-nitro-*N*-nitrosoguanine, which induces parthanatos, a form of programmed cell death that is distinct from other cell death processes such as necrosis and apoptosis. These methoxyflavones, but not other three hydroxy-methoxyflavones and eleven other methoxyflavones examined, have been shown to reduce the synthesis and accumulation of poly ADP-ribose and thus protected cortical neurons against cell death by the toxic nitrosamine (Fatokun et al., 2013; 2014).

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In this study, we examined how 2´MeF, 3´MeF, and 4´MeF—three model mono-methoxyflavones whose levels and concentrations in plants and environments are not known at present—are metabolized by human P450 enzymes, analyzing product formation using LC-MS/MS. Human P450 enzymes used in this study include recombinant CYP1A1, 1A2, 1B1.1, 1B1.3, 2A6, 2A13, 2B6, 2C9, and 3A4. Molecular docking analysis of the interaction of these flavonoids using CYP1A2 as a model is also reported.

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Materials and methods

Chemicals

Flavone, 2´-, 3´-, and 4´-methoxyflavoneflavone (2´MeF, 3´MeF, and 4´MeF, Figure 1), and 5,7-dihydroxyflavone (57diOHF) were kindly donated by Dr. Maryam K. Foroozesh of Xavier University of Louisiana (New Orleans, LA, USA). 4´-Hydroxyflavone (4´OHF), 3´,4´-dihydroxyflavone (3´4´diOHF), and 5,7-dihydroxyflavone (chrysin, 57diOHF) were purchased from Tokyo Kasei Co. (Tokyo), Sigma-Aldrich (St. Louis, MO, USA), and Wako Pure Chemicals (Osaka, Japan). Other chemicals and reagents were obtained from sources described

previously or were of the highest quality commercially available (Kakimoto et al, 2019; Nagayoshi et al., 2019a; 2019b).

Enzymes

Purified preparations of wild type of human CYP1A1, CYP1A2, CYP1B1.1, CYP1B1.3, CYP2A6, CYP2A13, CYP2C9, and CYP3A4 expressed *in Escherichia coli* were obtained by the methods described previously (Sandhu et al., 1993; 1994; Parikh 1997; Shimada et al., 2018; Han et al., 2012; Kim et al., 2018). Recombinant *E. coli* membranes expressing both CYP2B6 and NADPH-P450 reductase were prepared by the method as described previously (Han et al., 2012; Kim et al., 2018). NADPH-P450 reductase and cytochrome b_5 (b_5) were purified from membranes of recombinant *E. coli* by the methods as described elsewhere (Shen et al., 1989; Guengerich 2014).

Oxidation of 2´MeF, 3´MeF, and 4´MeF by recombinant human P450 enzymes

The oxidation of 2´MeF, 3´MeF, and 4´MeF by P450 enzymes was determined by the methods described previously (Kakimoto et al, 2019; Nagayoshi et al., 2019a; 2019b). Briefly, reconstituted monooxygenase systems consisting of each purified P450 (50 pmol), NADPH-P450 reductase (100 pmol), b_5 (100 pmol, in the cases of CYP2A6, 2A13, 2C9, and 3A4 enzymes), and

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L-α-1,2 dilaouryl-*sn*-glycero-3-phosphocholine (DLPC) (50 μg) were incubated (0.25 mL of total volume) with 60 μM 2´MeF, 3´MeF, or 4´MeF at 37 °C for 20 min, following a pre-incubation of 1 min before adding an NADPH-generating system (0.5 mM $NADP^+$, 5 mM glucose 6-phosphate, and 0.5 unit of yeast glucose 6-phosphate dehydrogenase/mL). CYP2B6 membranes in *E. coli* that express both CYP2B6 and NADPH-P450 reductase were also incubated with these MeFs (with 50 pmol P450). Each reaction was terminated by the addition of 0.5 mL of ice-cold CH_3CN. The mixture was mixed vigorously (with a vortex device) and centrifuged at 10,000 × *g* for 5 min, and an aliquot of the upper CH_3CN layer was injected and analyzed with LC-MS/MS as described (Kakimoto et al, 2019; Nagayoshi et al., 2019a; 2019b).

LC-MS/MS analyses were performed using an HPLC system (ACQUITY UPLC I-Class system; Waters, Milford, MA) coupled to a tandem quadruple mass spectrometer (XevoTQ-S; Waters) by the methods as described previously (Kakimoto et al, 2019; Nagayoshi et al., 2019a; 2019b). MS/MS analysis was performed in the positive electrospray ionization mode with a capillary voltage of 3000 V and cone voltage of 30 V as described previously (Kakimoto et al, 2019; Nagayoshi et al., 2019a; 2019b).

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Other assays

P450 and protein contents were determined by the methods described previously (Omura and Sato, 1964; Brown et al., 1989).

Docking simulations of 2´MeF, 3´MeF, and 4´MeF with CYP1A2

The one available crystal structure of CYP1A2 (Protein Data Bank 2H14) was used in this study (Sansen et al., 2007). The chemical structures of 2´MeF, 3´MeF, and 4´MeF were taken from PubChem (an open chemistry database at the National Institutes of Health) and were optimized in MOE software (ver. 2018.0101, Computing Group, Montreal, Canada). Simulations were carried out in the MOE by the methods as described previously (Nagayoshi et al., 2019a). Ligand-interaction energies (*U* values) were obtained by use of the program ASEdock in MOE.

Statistical analysis

Kinetic parameters were determined by non-linear regression analysis (mean ± standard error) employing the Michaelis-Menten equation ($v = V_{max} \times [S]/(K_m + [S])$) or the allosteric Hill equation model ($v = V_{max} \times [S]^n/(S^n_{50} + [S]^n)$) using the program Kaleida-Graph (Synergy Software, Reading, PA, USA) or GraphPad Prism (GraphPad, La Jolla, CA, USA).

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Results

Oxidation of 2´MeF, 3´MeF, and 4´MeF by human P450 enzymes

2´MeF, 3´MeF, and 4´MeF were incubated with human P450s and product formation was detected by LC-MS/MS analysis (Figures 2 and 3). We tentatively termed these 4´MeF products 4´Me239a (*m/z* 239 product), 4´Me255a (*m/z* 255 product), and 4´Me269a, b, c, d, and e (*m/z* 269 products) (Figure 2A-2F) and this tentative naming scheme was also used for 2´MeF (Figure 2G-2L) and 3´MeF (Figure 3). The *m/z* 239 and *m/z* 255 products are suggested to be mono- and di-hydroxylated flavones, respectively, and the *m/z* 269 products are mono-hydroxylated methoxy-flavones (*vide infra*).

3´MeF and 4´MeF were first examined for product formation with CYP1A2, 1B1.1, and 1B1.3 (Figure 2). The upper three figures (2A, 2B, and 2C for 4´MeF and 2G, 2H, and 2I for 3´MeF) showed formation of *m/z* 239 and *m/z* 255 products. Standard 4´OHF and 3´4´diOHF were available and used to confirm that 4´Me239a was 4´OHF and 4´Me255a was 3´4´diOHF, respectively (Figure 2A, 2B, and 2C). The 3´Me239a product was 3´OHF; the retention time was similar to that of 4´OHF and the LC-MS/MS product ion spectrum of 3´Me239a was very similar

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to that of 4´OHF (*vide infra*, Figure 4). We also showed that 3´Me255a was 3´4´diOHF by comparing retention times and product ion spectra (Figure 2G, 2H, and 2I).

The lower parts of Figures 2D, 2E, and 2F (4´MeF^{o} and 2J, 2K, and 2L (3´MeF) showed products having an *m/z* 269 ion, which indicated the formation of ring oxidation of 4´MeF and 3´MeF, respectively, by these P450 enzymes. Because the peak intensities of the *m/z* 269 products were lower than those of *m/z* 239 and 255 products, the highest peak levels (vertical axis) in Figure 2D, 2E, 2F, 2J, 2K, and 2L used were 2×10^5, while those in Figure 2A, 2B, 2C, 2G, 2H, and 2I were 2×10^7. The results showed that human P450s oxidized 4´MeF to at least five *m/z* 269 products, namely 4´Me269a, 4´Me269b, 4´Me269c, 4´Me269d, and 4´Me269e, although the peak intensities of these products were very low compared with those of the *m/z* 239 and *m/z* 255 products (Figure 2). 3´MeF was also found to be oxidized by these P450s to produce the *m/z* 269 products (mainly 3´Me269a); the peak intensities of 3´Me269a by CYP1A2, 1B1.1, 1B13 were about 19-, 6-and 16-fold lower, respectively, than those of 3´Me239a (Figure 2).

Oxidation of 2´MeF by P450s was also determined using nine human P450s, CYP1A1, 1A2, 1B1.1, 1B1.3, 2A6, 2A13, 2B6, 2C9, and 3A4 (Figure 3). We measured the formation of the *m/z* 239, 255, and 269 products, and the results of peak intensities (vertical axis used) were the same as 2×10^6 at the highest value (Figure 3). As expected, the *m/z* 239 product, namely

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2´Me239a that was suggested to be 2´OHF (*vide infra*), was formed in these P450s at higher rates with CYP1B1.1, 1B1.3, 2A13, and 2B6 (Figure 3C1, 3D1, 3F1, and 3G1), followed by CYP1A1, 1A2, and 2A6 (Figure 3A1, 3B1, and 3E1). Formation of the *m/z* 239 product was not as high with CYP2C9 and 3A4 (Figure 3H1 and I1). Interestingly, formation of the *m/z* 255 products was low with 2´MeF as compared with 3´MeF and 4´MeF with these nine human P450s. CYP1A2 was found to produce two products, 2´Me255a and 2´Me255b (Figure 3B2), and the former product was also formed with CYP1A1, 1B1.1, 1B1.3, and 2B6 (Figure 3A2. 3C2, 3D2, and 3G2). In contrast to the 2´Me255 products, four *m/z* 269 products were formed by oxidation of 2´MeF with P450s at relatively high rates and, among these products determined, 2´Me269b was the major one with CYP1A1, 1B1.1, 1B1.3, 2A6, and 2A13, whereas CYP2B6 and 2C9 produced 2´Me269a (Figure 3G3 and 3H3). We also found that both CYP1B1.1 and 1B1.3 were more active in producing 2´Me269c than other P450s examined (Figure 3C3 and 3D3).

Activities of oxidation of 2´MeF, 3´MeF, and 4´MeF to mono- and di-hydroxylated products by nine forms of human P450s.

Because 4´OHF and 3´4´diOHF were available in this study, we calculated specific activities of formation of these products (Figures 4B, 4C, and 4F). Formation of other products was compared using intensities of LC-MS/MS peak heights, although it is not known whether the relative

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intensities of the minor peaks are completely accurate indicators of the yields of products (Figures 2E, 2H, and 2I and Figure 3).

CYP1B1.1 and 1B1.3 were highly active in catalyzing the oxidation of 4´MeF to 4´OHF at a rate of 5 min^{-1} and to form 3´4´diOHF at rates of 2.1 and 0.66 min^{-1}, respectively (Figure 4). 4´MeF was also oxidized by CYP1A1, 1A2, and 2A13 to form 4´OHF at rates of 1.1, 1., and 2.1 min^{-1}, respectively, and to form 3´4´diOHF at rates of 0.36, 1.1, and 1.74 min^{-1}, respectively (Figure 4). 3´MeF was oxidized by CYP1A2 to form 3´Me239a (possible 3´OHF), as well as by CYP2A13, 1A1, 1B1.1, and 1B1.3 at lower rates (Figure 4C). CYP1B1.1 oxidized 3´MeF to form 3´4´diOHF at a rate of 3.3 min^{-1}, higher than the activity when 4´MeF was used as a substrate (Figure 4D and 4B).

In a case of 2´MeF, CYP1B1.1, 1B1.3, and 2B6 were highly active in forming 2´Me239a (possibly 2´OHF), followed by CYP2A13, 2A6, 1A1, and 1A2 (Figure 4). CYP1A2, 1B1.1, 1B1.3, 2B6, and 1A1 oxidized 2´MeF to form 2´Me255a at very low levels, and other P450s did not show detectable activities for this reaction (Figures 3 and 4).

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Product ion spectra of *m/z* 239, 255, and 269 products obtained in the oxidation of three MeFs with P450 enzymes

The above results showed that various oxidative products were formed during oxidation of 2´MeF, 3´MeF, and 4´MeF with human P450s. We analyzed fragment patterns of these products, namely, the *m/z* 239 (Figure 6), *m/z* 255 (Figure 7), and *m/z* 269 products (Figure 8) as well as the parent compounds 4´MeF, 3´MeF, and 2´MeF (Figure 5) by analyzing their product ion spectra using LC-MS/MS.

4´MeF and 3´MeF were similar in their fragmentation patterns, which had *m/z* 238, 210, 132, and 121 ions as well as an *m/z* 253 ion (Figure 5A and 5B). The spectrum of 2´MeF was somewhat different from others; the 2´MeF contained *m/z* 221, 120, and 105 ions as well as fragments of *m/z* 253, 238, 210, 132, and 121 ions (Figure 5C).

The spectrum of products for 4´Me239a (Figure 6B) was very similar to that of standard 4´OHF (Figure 6A), which showed a parent *m/z* 239 ion and several fragment ions of *m/z* 65, 92.7, 118.7, 120.7, 120.9, and 144.7, indicating that 4´OHF was formed by *O*-demethylation of 4´MeF by CYP1A2. Interestingly, the fragmentation patterns of 3´Me239a and 2´Me239a were very similar to that of 4´OHF, suggesting that both 2´MeF and 3´MeF were *O*-demethylated to form

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2´OHF and 3´OHF, respectively, by mechanisms similar to that for 4´MeF oxidation by P450s (Figure 6C and 6D). The spectrum of 6OHF, a reference standard, was dissimilar to those of 4´OHF and the products 4´Me239a, 3´Me239a, and 2´Me239a; the former spectra showed the oxidation at A-ring, with *m/z* 136.9 (Figure 6E).

We also analyzed the product ion spectra of the *m/z* 255 products formed from 4´MeF, 3´MeF, and 2´MeF incubated with P450s and detected four *m/z* 255 products, namely 4´Me255a, 3´Me255a, 2´Me255a, and 2´Me255b, all formed through oxidation by P450s (Figure 7). The spectrum of 4´Me255a (Figure 7b) was very similar to that of 3´4´diOHF (Figure 7A), and the other products 3´Me255a, 2´Me255a, and 2´Me255b showed very similar patterns to those of 3´4´diOHF and 4´Me255a, suggesting that these products were oxidized at the B-ring of 2´OHF (Figures 7C, 7D, and 7E). The product ion spectrum of 57diOHF, di-oxidized at the A-ring, was very different from those of 3´4´diOHF and the other products obtained in this study (Figure 7F).

As described above, we identified five *m/z* 269 products (4´Me269a, b, c, d, and e) from 4´MeF, two *m/z* 269 products (3´Me269a and b) from 3´MeF, and four *m/z* 269 products (2´Me269a, b, c, and d) from 2´MeF formed on incubation with P450s (Figures 2 and 3). We tentatively classified these into two types of these products, one oxidized in the A-ring of MeFs and the other in the B-ring of MeFs (Figure 8). For example, 4´Me269c, 3´Me269b, 2´Me269b,

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and 2´Me269d showed fragment ions of *m/z* 137 ion as well as *m/z* 226, 254, and 269 ions, indicating that oxidation occurred at the A-ring of these MeFs by P450s. In contrast, 4´Me269a and 2´Me269c showed different fragment profiles, which did not have the *m/z* 137 ion but instead contained an *m/z* 121 ion, suggesting that these products were oxidized at the B-ring (Figures 8G-8H). In this study, we also observed other *m/z* 269 products, such as 4´Me269a, 4´Me269d, and others, which had *m/z* 227, 254, and 269 ions but did not show an *m/z* 121 or 137 ion for the molecule (Figure 8I and 8J).

Molecular docking analysis of interaction of 2´MeF, 3´MeF, and 4´MeF with CYP1A2

Interaction of MeFs with the active site of CYP1A2 was examined using molecular docking analysis as described in Experimental Procedures (Figure 9). The results showed that methoxy moieties of 2´MeF, 3´MeF, and 3´MeF were interfaced to the active sites of CYP1A2 and that the distances between the methoxy groups of 2´MeF, 3´MeF, and 3´MeF and the iron center of CYP1A2 were calculated to be 4.50, 4.40, and 4.61 Å, respectively (Figure 8). The ligand-interaction energies (*U* values) of interaction of 2´MeF, 3´MeF, and 3´MeF with active site of CYP1A2 were –47.3, -51.6, and -30.9, respectively (Figure 9). Our molecular docking analysis also showed that 2´MeF interacted with the CYP1A2 protein at the C3 position as well as the 2´-methoxy moiety, and this former interaction was not seen with 4´MeF or 3MeF (Figure 9).

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Discussion

Methoxylated flavonoids, which are distributed widely in plants, particularly in the citrus fruits, have been reported to show increased metabolic stability and membrane transport in animals and possibly in humans, thus enhancing the effectiveness in their biological activities (Walle, 2007; Walle et al., 2007; Koirala et al., 2016; Wen et al., 2017). Oxidative metabolism of methoxylated flavonoids by human P450 enzymes has been reported by several investigators (Breinholt et al., 2002; Walle and Walle, 2007; Cheng et al., 2011). Androutsopoulos and his associates reported that several methoxylated flavonoids—e.g., hesperidin, nobiletin, tamarixetin, tangeretin, and diosmetin— are demethylated by human P450 enzymes, particularly CYP Family 1 enzymes, to active metabolites that inhibit proliferation of human cancer cells (Androutsopoulos et al., (2008; 2009a; 2009b; 2009c; 2010; 2013; Surichan et al., 2012; 2018a; 2018b).

In the present study, we found that three methoxylated flavones, 2´MeF, 3´MeF, and 4´MeF, were *O*-demethylated to form mono-hydroxylated and further di-hydroxylated products by nine forms of recombinant human P450 enzymes and that ring oxidation also occurred, although at much slower rates than the *O*-demethylation reactions. We found that CYP1B1.1 and 1B1.3 were highly active in catalyzing the oxidation of 4´MeF to form 4´OHF (4.9 min^{-1}) and then to produce 3´4´diOHF at 2.1 and 0.66 min^{-1}, respectively, and that CYP1A1, 1A2, and 2A13 oxidized 4MeF

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to 4´OHF and 3´4´diOHF at significant levels. The above P450 enzymes tended to have similar, but nevertheless different, catalytic properties in oxidizing 3´MeF and 2´MeF to the *m/z* 239 products (possibly 3´OHF and 2´OHF, respectively) and the *m/z* 255 products (3´4´diOHF and unidentified products, respectively) (Figures 2 and 3). Other P450s including CYP2A6, 2B6, 2C9, and 3A4 were found to be less active in oxidizing 2´MeF, 3´MeF, and 4´MeF, except that CYP2B6 efficiently catalyzed the oxidation of 2´MeF to the *m/z* 239 products; the activities were almost similar to those catalyzed by CYP1B1.1 and 1B1.3 (Figure 4E).

In this study, the ring oxidation of 2´MeF, 3´MeF, and 4´MeF by P450s was identified by determining the *m/z* 269 products by LC-MS/MS. Although the peak intensities of the *m/z* 269 products were found to be usually low as compared with those of the *m/z* 239 and *m/z* 255 products in 2´MeF, 3´MeF, and 4´MeF, except that the intensities of 2´´Me269b were higher than those of 2´Me255a in CYP1A1, 1B1.1, 1B1.3, 2A6, and 2A13 (Figures 2 and 3). Fragmentation of the *m/z* 269 products clearly indicated these were the ring oxidation products, having fragments *m/z* 269, 254, and 226. We found that there were at least two types of *m/z* 269 products; one was the products oxidized at A-ring of the MeFs and the other was at B-ring of MeFs (Figure 8). However, the nature of the other *m/z* 269 products was not characterized with regard to which positions were oxidized in this study.

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Our molecular docking analysis supported the preference of interaction of methoxy moieties of 2´MeF, 3´MeF, and 4´MeF with active site of CYP1A2, thus resulting in the initial formation of mono-hydroxyflavones and then di-hydroxyflavones after the demethylation reaction. The distances between the methoxy groups of these 2´MeF, 3´MeF, and 4´MeF and the iron center of CYP1A2 were very similar, but the *U* values of interaction of 3´MeF and 4´MeF were found to be smaller than that of 2´MeF. Docking analysis also showed that there was a possible interaction (7.3 Å distance) between C3 position in C-ring of 2´MeF and active site of CYP1A2, and such an interaction was not seen with 3´MeF or 4´MeF (Figure 9). The formation of 2´Me255a (*m/z* 255 product) was very low as compared with 2´Me239a (possibly 2´OHF), but in cases of 3´MeF and 4´MeF the formation of 3´4´diOHF (the *m/z* 255 product) was not so low compared with 4´Me239a (4´OHF) and 3´Me239a (possibly 3´OHF). It is not known, however, whether such differences in molecular interaction between the three MeFs with active site of CYP1A2 affect the catalytic differences in oxidation of these MeFs.

In conclusion, our present results showed that three mono-MeFs, 2´MeF, 3´MeF, and 4´MeF, were preferentially *O*-demethylated to form mono-hydroxyflavones and then di-hydroxyflavones by human P450 enzymes. Molecular docking analysis indicated the preference of interaction of the *O*-methoxy moiety of methoxyflavones with active site of CYP1A2. Of the

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nine forms of P450s used in this study, CYP1A1, 1A2, 1B1.1, 1B1.3, and 2A13 were found to have important roles in all of the reactions studied, and CYP2B6 and 2A6 showed significant roles in the *O*-demethylation of 2´MeF. CYP2C9 and 3A4 had relatively minor roles in these reactions. Although the formation of ring oxidation reactions occurred in these MeFs (to form products oxidized in the A- and/or B-ring), the levels of product formation were much lower than for the mono- and di-hydroxyflavones formed. The results provide useful information on the metabolism of methoxylated flavones by human P450 enzymes.

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Declaration of interest Statement

The authors declare no conflict of interest associated with this manuscript.

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Figure legends

Figure 1. Structures of 4´MeF, 3´MeF, and 2MeF.

Figure 2. Oxidation of 4´Mef (A-F) and 3´MeF (G-L) by CYP1A2 (A, D, G, and J), CYP1B1.1 (B, E, H, and K), and CYP1B1.3 (C, F, I, and L). The upper three figures (A, B, and C) and (G, H, and I) show formation of the *m/z* 239 and 255 products obtained on incubation of 4´MeF and 3´MeF, respectively, with individual P450s. The lower three figures (D, E, and F) and (J, K, and L) show the formation of the *m/z* 269 products obtained on incubation of 4´MeF and 3´MeF, respectively, with individual P450s. The maximum peak intensities for the detection of the *m/z* 239 and 255 products used were 2×10^7 (A-C and G-I) and that for the *m/z* 269 products was 2×10^5 (D-F and J-L). These products were tentatively named as shown in the figures. N: uncharacterized (non-specific) peaks that were also detected in the absence of an NADPH-generating system.

Figure 3. Oxidation of 2´MeF by CYP1A1 (A1-A3), 1A2 (B1-B3), 1B1.1 (C1-C3), 1B1.3 (D1-D3), 2A6 (E1-E3), 2A13 (F1-F3), 2B6 (G1-G3), 2C9 (H1-H3), and 3A4 (I1-I3). Product formation was examined using the ions *m/z* 239 (A1-I1), *m/z* 255 (A2-I4), and

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m/z 269 (A3-I3) in LC-MS/MS analysis. The peak intensities used were at the same sensitivity in these figures.

Figure 4. Formation of hydroxylated products of 4´MeF (A, B, and C), 3´MeF ‘D, E, and F), and 2´MeF (G, H, and I) on incubation with nine forms of human P450 enzymes. Because standard 4´OHF and 3´4´diOHF were available, product formation was calculated as specific activity based on P450 content. In other cases, the extents of product formation of 3´Me239a (E, putatively 3´OHF), 2´Me239a (H, putatively 2´OHF), and 2´Me255a (I, putatively diOHF) were expressed as peak intensities. All values were means ± SD or SE from two or three determinations.

Figure 5. Product ion spectra of substrates 4´MeF (A), 3´MeF (B), and 2´MeF (C) used in this study. Proposed fragments are included.

Figure 6. Product ion spectra of the *m/z* 239 products of 4´MeF (B), 3´MeF (C), and 2´MeF (D) incubated with human CYP1A2. Standard 4´OHF (A) and 6OHF (E) are also shown. Proposed fragments are included.

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Figure 7. Product ion spectra of the *m/z* 255 products of 4´Me255a (C), 3´Me255a (E), 2´Me255a (D), and 2´Me255b (F) incubated with human CYP1A2. Standard 3´4´diOHF (A) and 57diOHF (E) are shown. Proposed fragments are included.

Figure. 8. Product ion spectra of *m/z* 269 products of 4´MeF, 3´MeF, and 2´MeF incubated with human P450 enzymes. P450s are shown at the right of the figure. Proposed fragments are included.

Figure 9. Molecular docking analysis of the interaction of 2´MeF (A), 3´MeF (B), and 4´MeF (C) with CYP1A2. The distances between the methoxy groups of MeFs and the iron center of CYP1A2 is shown in red and the ligand-interaction energy (*U*) is also included in the figure.

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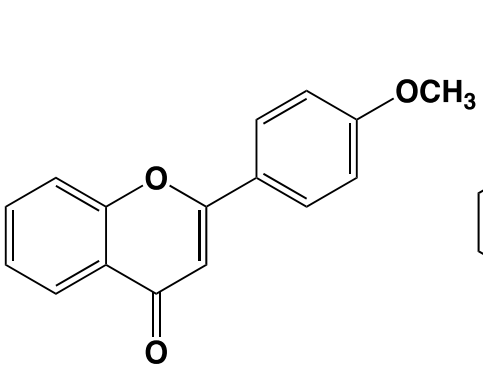
Figure 1



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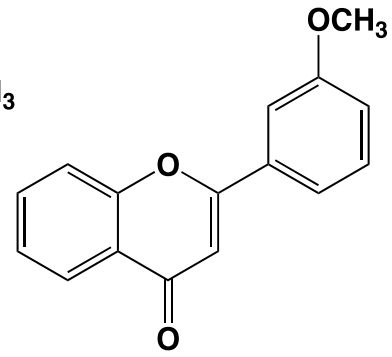

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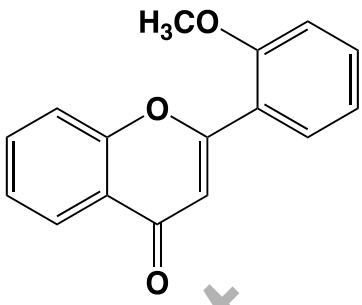

2'Methoxyflavone
(2'MeF)

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Figure 2

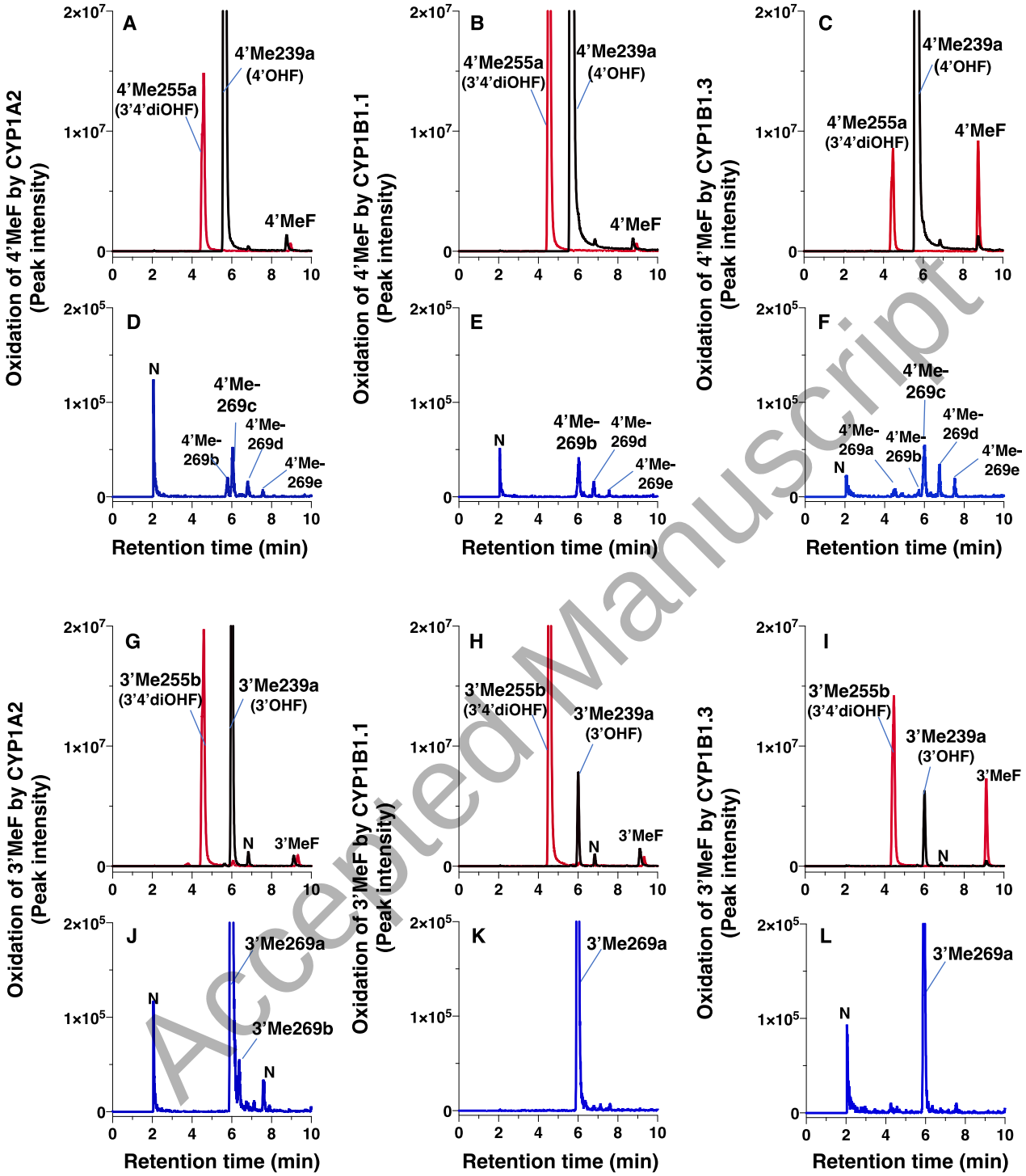

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Figure 3

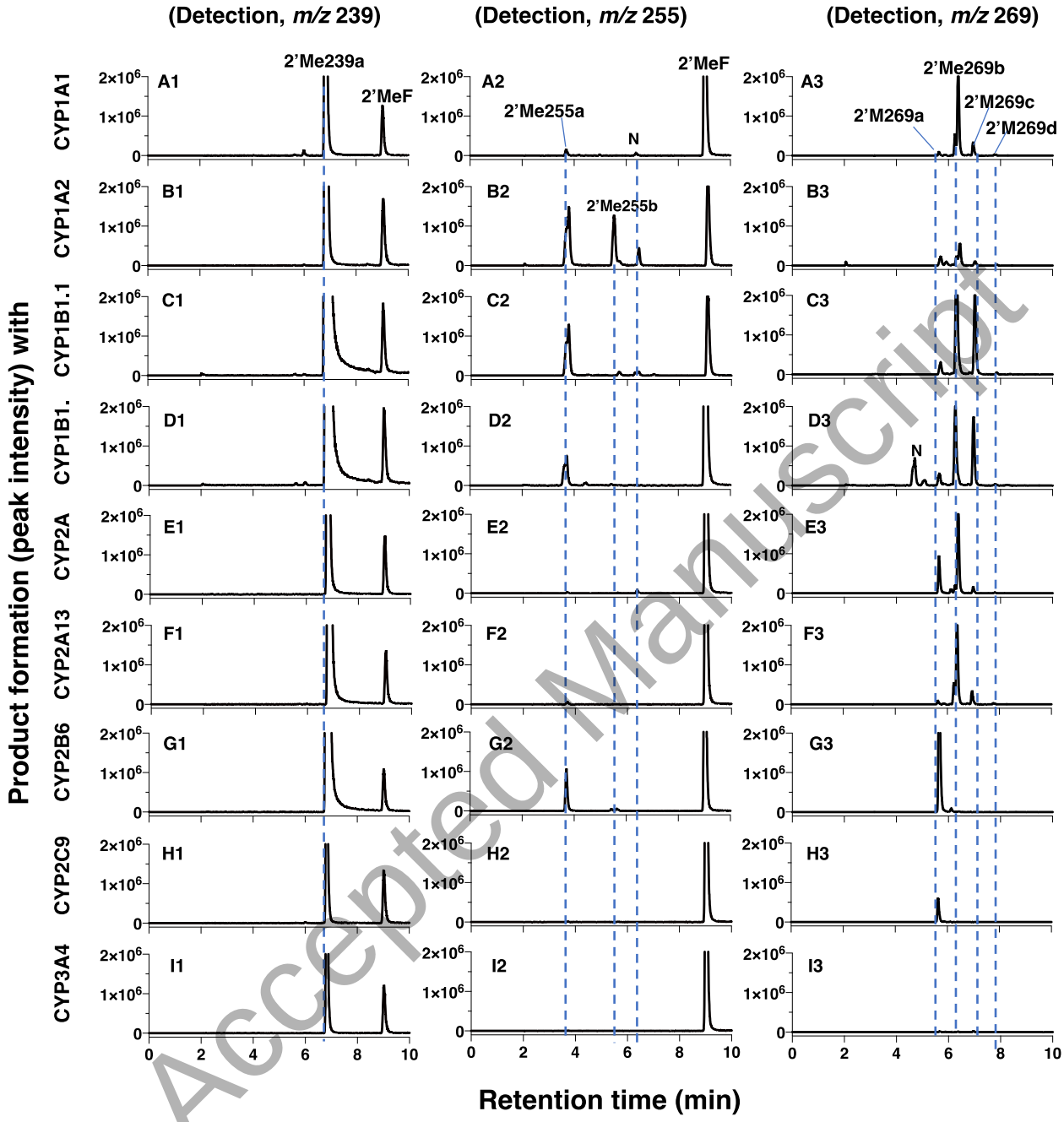

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Figure 4

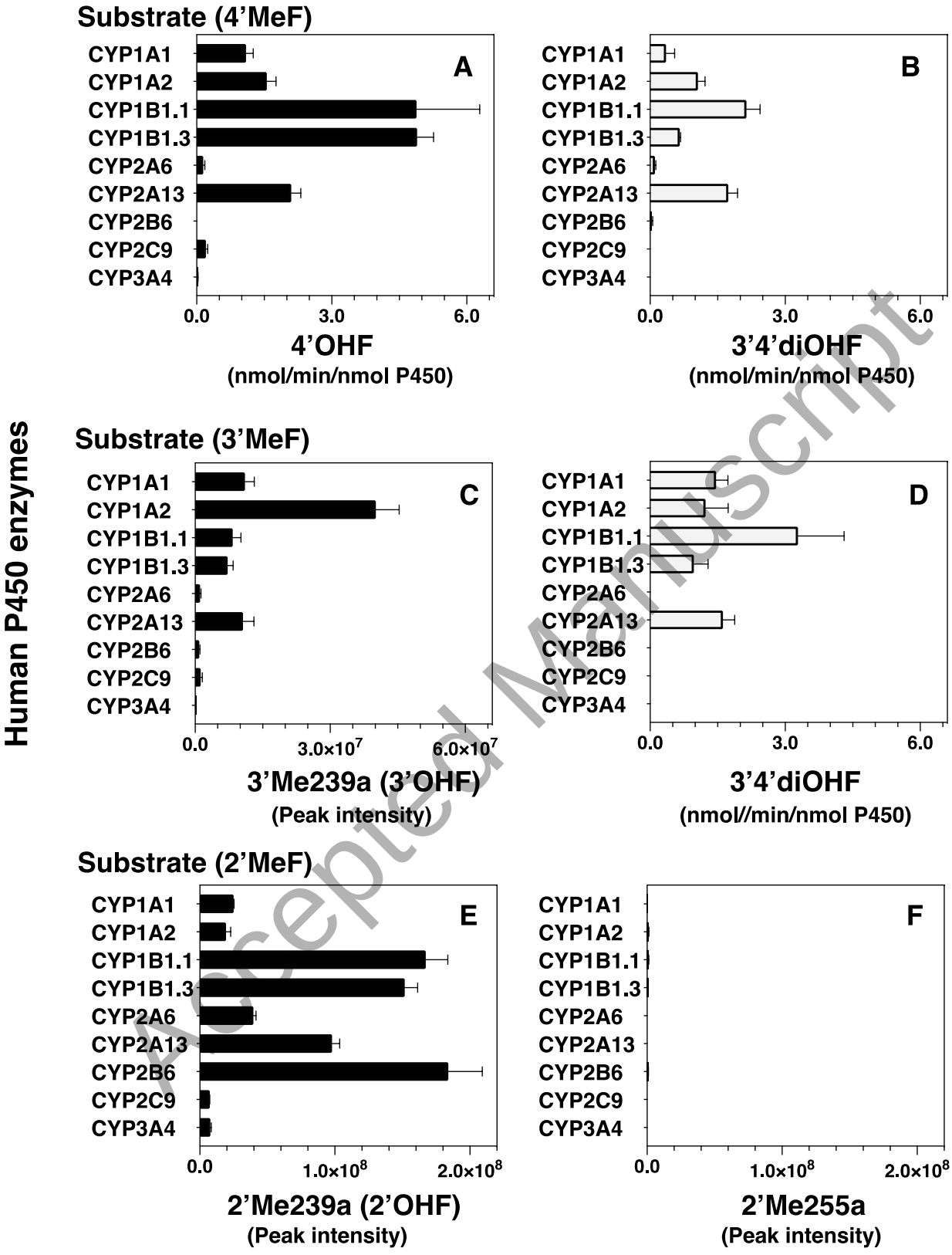

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Figure 5

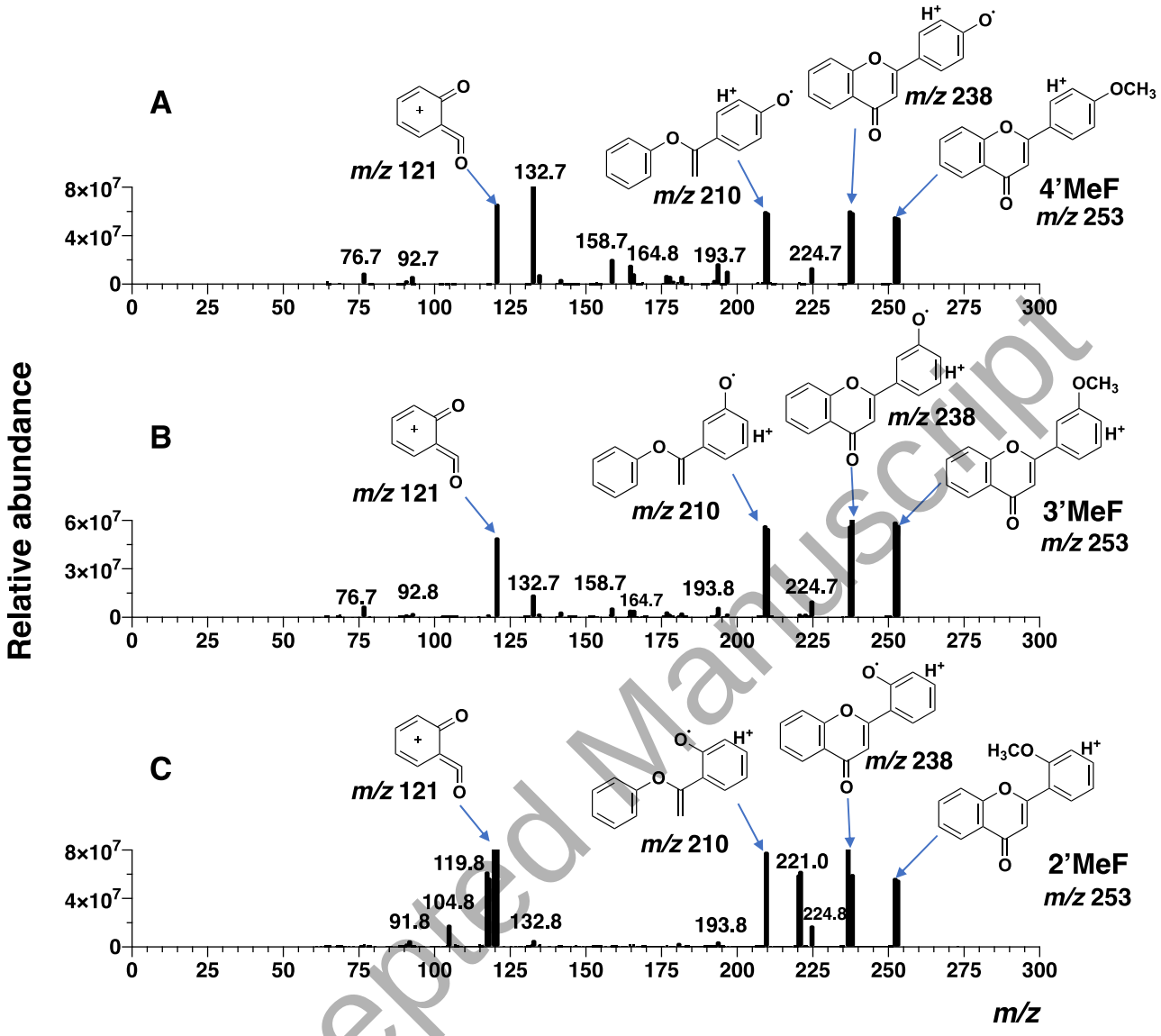

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Figure 6

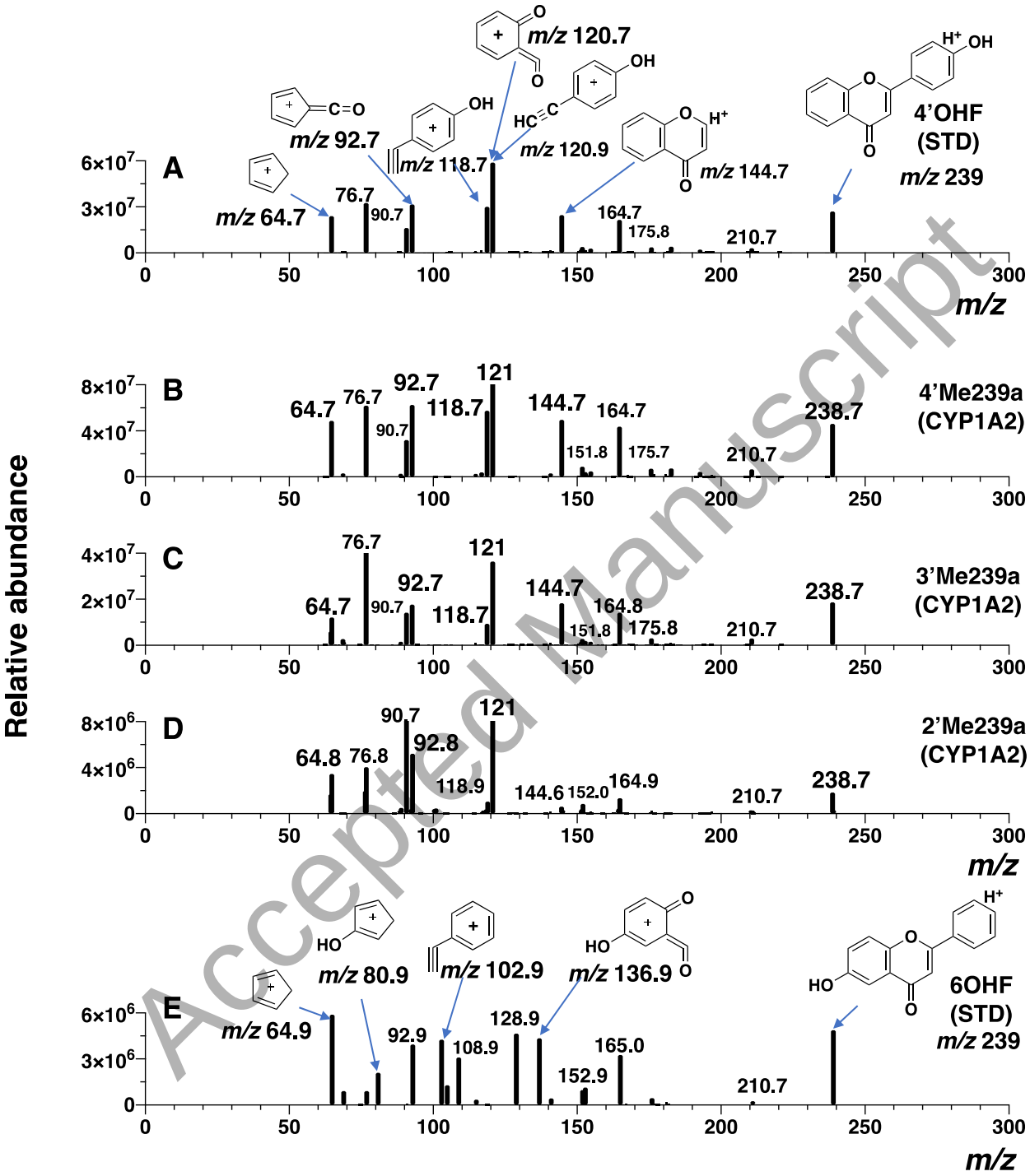

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Figure 7

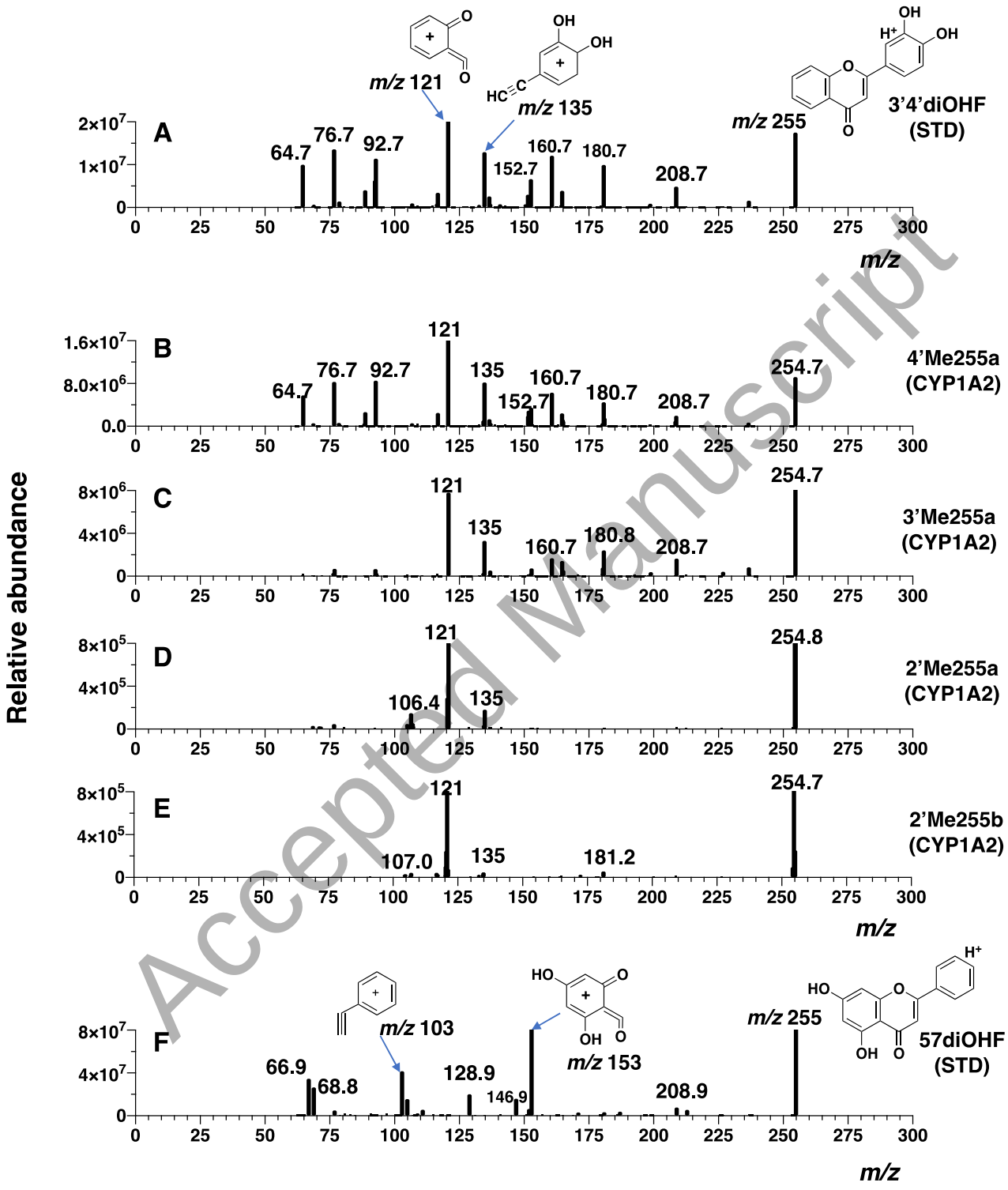

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Figure 8

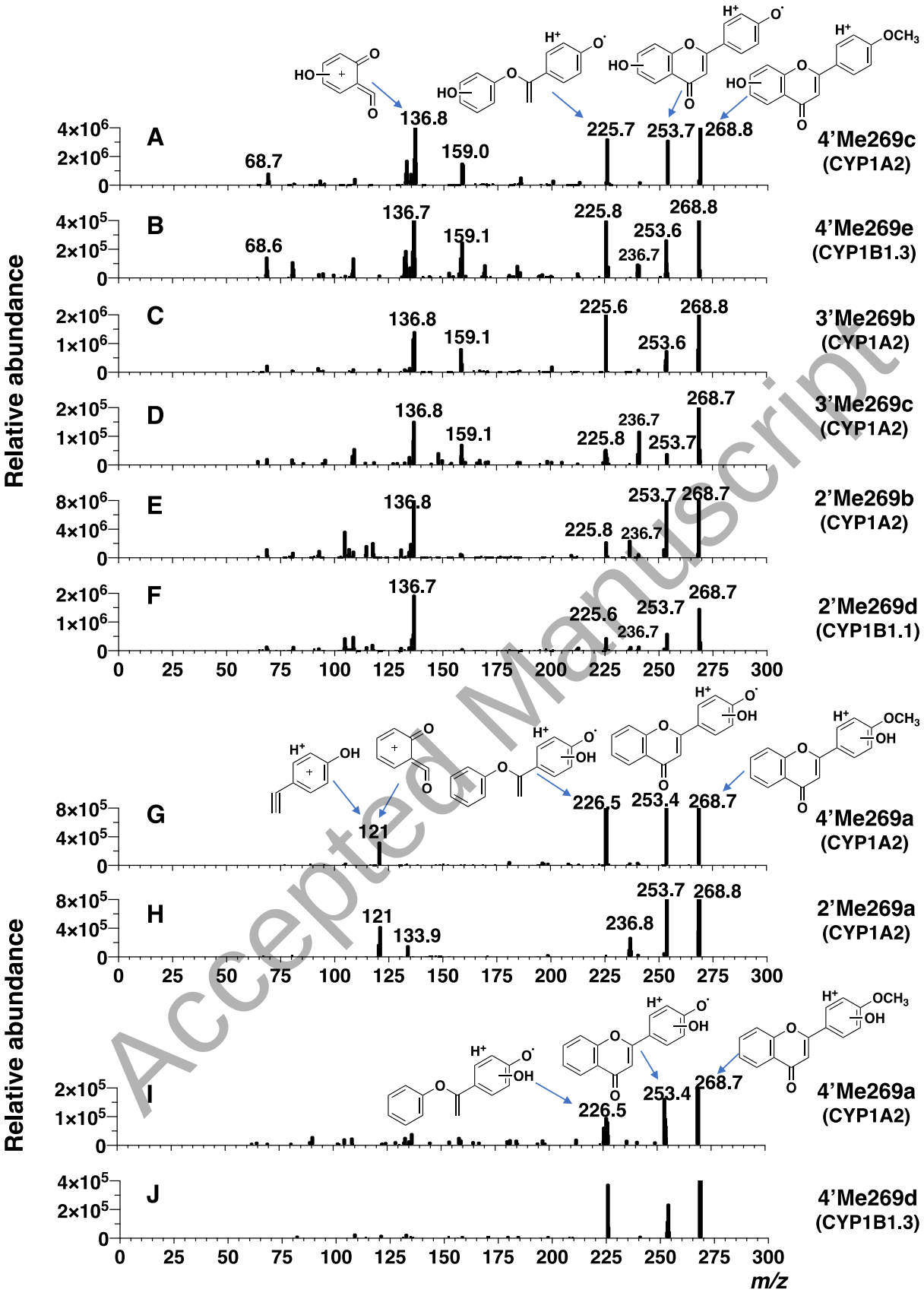

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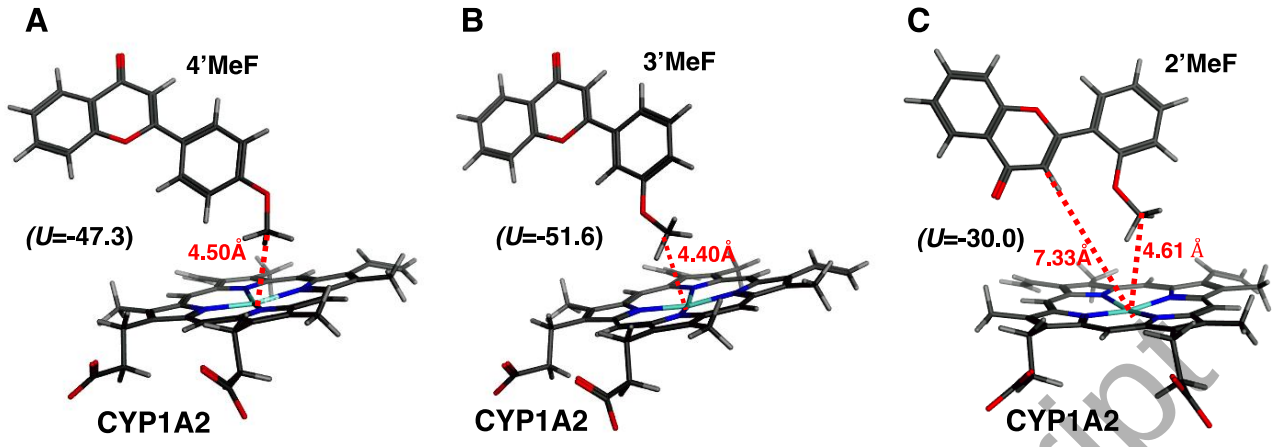
Figure 9



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