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# Regiospecificity of Human UDP-glucuronosyltransferase Isoforms in Chalcone and Flavanone Glucuronidation Determined by Metal Complexation and Tandem Mass Spectrometry

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## Abstract

The glucuronidation of a series of chalcones (2'-hydroxychalcone, 2',4'-dihydroxychalcone, 3,2'-dihydroxychalcone, 4,2'-dihydroxychalcone, and cardamonin) and their corresponding cyclized flavanones (7-hydroxyflavanone, 3'-hydroxyflavanone, 4'-hydroxyflavanone, and alpinetin) by nine human UDP-glucuronosyltransferase (UGT) 1A enzymes was evaluated. A post-column metal complexation LC-MS/MS strategy was used successfully to produce characteristic mass spectrometric product ions that were utilized in combination with elution order trends to identify chalcone and flavanone monoglucuronides unambiguously, thus allowing determination of the regioselectivities of the UGT1A isoforms. The presence of hydroxy groups on the A or B-ring had a significant effect on the glucuronide product yield and the site where glucuronidation occurred. For example, for reaction with UGT1A9, formation of the 2'-O-glucuronide was increased for dihydroxychalcones with A-ring hydroxy substituents. In contrast, although UGT1A8 reacted with 3,2'-dihydroxychalcone and 4,2'-dihydroxychalcone to yield 2'-O-glucuronide products, the presence of a B-ring hydroxy group at the 4' position on cardamonin and 2',4'-dihydroxychalcone quenched the reaction at the OH-2' position. Moreover, the A-ring OH-4 group promoted glucuronidation at the 2' position for the reaction of 4,2'-dihydroxychalcone with UGT1A1 and 1A3. For UGT1A7, hydroxy group substituents on the chalcone A-ring also promoted cyclization and formation of the corresponding flavanone glucuronide.

Chalcones are a subclass of flavonoids that have been shown to exhibit promising biological activities for chemotherapeutic applications, including potent antioxidant,[1] antiangiogenic,[2] and anti-inflammatory[3,4] properties.[5] Although considered to be minor flavonoids due to their typical low concentrations within plants,[6] chalcones play an important role as precursors and intermediates in all flavonoid synthetic pathways.[7] Chalcones are often characterized as"open-chain flavonoids" because of their structure in which a three-carbon αβ unsaturated ketone connects the two aromatic rings (see Figure 1). This unsaturated bond is the source of many of the biological properties of the chalcones, since nucleophilic species react readily with it.[1] Compared to other flavonoids, chalcone derivatives can be synthesized with relative ease, with the addition of new functional groups leading to changes in bioactivity.[8,9]

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**Supporting Information**
CID product ions for the metal/flavonoid complexes in this study.

Like other flavonoids, chalcones are xenobiotics that undergo metabolism in the human body upon ingestion. Metabolism converts flavonoids to more hydrophilic forms through methylation, glucuronidation, and sulfonation to allow excretion of these exogenous compounds from the body through the urine or bile. Glucuronidation, in particular, is one of the most important metabolic pathways in humans and other vertebrates.[10] Presystemic metabolism of flavonoids occurs primarily in the small intestine and liver[11] where uridine diphosphate (UDP) glucuronosyltransferase enzymes are responsible for catalyzing the addition of the glucuronyl moiety from endogenous uridine 5'-diphosphoglucuronic acid (UDPGA) to nucleophilic functional groups, leading to glucuronidation at these sites.[12] In the case of flavonoids, this biotransformation process most often occurs at hydroxy groups, producing flavonoid O-glucuronides. Interestingly, although the metabolism process generally decreases the bioactivity of xenobiotics, several flavonoid glucuronides have shown increased antioxidant activity and biological uptake compared to their unconjugated parent aglycones.[13]

Liquid chromatography (LC) paired with mass spectrometry (MS) has become the preferred analytical technique for the separation and identification of flavonoid glucuronides.[14,15] After separation of the flavonoid metabolite(s) from the parent aglycone and other components within the sample matrix by LC, tandem MS (MS/MS) or high-resolution MS measurements are then used for structural elucidation.[15] In addition to considerable gains in sensitivity and selectivity compared to other methods, LC-MS also allows for direct detection of metabolites, a distinct advantage over enzyme-based or derivatization methods.[16,17] Even with the impressive analytical metrics of LC-MS, however, distinguishing isomeric glucuronides and/or determining the site of glucuronidation remains a considerable analytical challenge.[15] Since the position of glucuronidation may strongly influence the bioactivity of flavonoid metabolites,[11,18] the development of techniques for the unambiguous identification of flavonoid glucuronides is particularly important. For example, pairing LC-MS with a UV shift method has allowed for identification of the glucuronidation site of model flavonoids, particularly flavones and flavonols.[19–22] Our group explored pairing metal complexation strategies with collision-induced dissociation (CID) MS/MS to characterize isomeric flavonoid glycosides,[23–26] and has extended this strategy to the structural identification of glucuronides enzymatically synthesized from a range of common flavonoids.[27–29] Using this technique, flavonoid glucuronides are complexed with a metal and an auxiliary organic ligand in solution prior to transfer to the gas phase by electrospray ionization and characterization by collision-induced dissociation, thus affording an integrated approach that provides unique fragmentation patterns for each isomeric glucuronide and allows for their unambiguous structural identification.

In the current study, previous work is expanded upon using this metal complexation MS/MS strategy to investigate the glucuronidation of a series of 2'-hydroxychalcones (2'-hydroxychalcone, 2',4'-dihydroxychalcone, 3,2'-dihydroxychalcone, 4,2'-dihydroxychalcone, and cardamonin) by nine UPD-glucuronosyltransferase (UGT) 1A enzymes. UGT1A enzymes (1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9 and 1A10) were chosen for this investigation because they have been shown previously to possess greater reactivity than the UGT2B enzymes in catalyzing the glucuronidation of flavonoids.[12,29] These particular chalcones were selected for their biological relevance, and also because the OH-2' group in general has been identified as being important in enhancing the biological properties of chalcones, especially anti-inflammatory and antibacterial activities.[30,31] In addition to altering the biological activity of the molecule, the presence of the OH-2' group allows for cyclization of chalcones to flavanones through the light-induced pathway depicted in Figure 1.[32] As a result, 2'-hydroxychalcones interconvert readily to their corresponding flavanones. Therefore, as part of this study, the glucuronidation of four flavanones (7-hydroxyflavanone, 3'-hydroxyflavanone, 4'-hydroxyflavanone, and alpinetin) was also investigated, with the

flavanones corresponding to the cyclized versions of the 2'-hydroxychalcones examined. Despite the recent interest in chalcones as novel pharmaceutical targets, with the exception of isoliquiritigenin,[33–36] there have been few studies to date focusing on the metabolism of these compounds. The present study therefore provides benchmark data on the regioselectivity of UGT1A enzymes in the glucuronidation of 2'-hydroxychalcones as well as a translatable analytical strategy for the identification of isomeric chalcone glucuronides, providing a foundation for future studies of chalcone metabolism.

## RESULTS AND DISCUSSION

In order to determine the regioselectivity of UGT1A enzymes in the glucuronidation of the selected 2'-hydroxychalcones, it was necessary to develop a protocol to identify unambiguously the chalcone glucuronide products as well as the unconjugated chalcone aglycone within each enzyme reaction mixture. Based on previous work,[27–29] reaction mixtures were first analyzed using LC-ESIMS to identify the chalcone and glucuronide products based on their respective molecular ions. For example, Figures 2A and 2B present typical extracted ion chromatograms for the reaction of 2',4'-dihydroxychalcone with UGT1A10. Each total ion chromatogram was first searched for the protonated chalcone aglycone, $[M + H]^+$ (*m/z* 241 for the dihydroxychalcones), as shown in Figure 2A. Total ion chromatograms were also searched for the monoglucuronidated chalcone, $[M + gluc + H]^+$ (240 + 176 + 1 = *m/z* 417 for the dihydroxychalcones), as shown in Figure 2B. Finally, total ion chromatograms were searched for diglucuronidated chalcones $[M + 2gluc + H]^+$ (240 + 176 + 176 + 1 = *m/z* 593 for the dihydroxychalcones). However, a diglucuronidated species was observed only for a single UGT reaction (cardamonin with UGT1A1) and it was present only in trace amounts.

Further inspection of Figure 2A shows the presence of multiple species within the enzyme reaction mixture that correspond to ions of *m/z* 241 with two major and one minor chromatographic peaks observed. Two of these peaks, at 27 and 33 min, had mass spectra generally consistent with the expected 2',4'-dihydroxychalcone, primarily *m/z* 241, while the other was identified as a monoglucuronidated species, $[M + gluc + H]^+$, *m/z* 417, with a mass spectrum containing a fragment for the corresponding aglycone (i.e., the spontaneous loss of the glucuronide moiety during ionization to re-generate a precursor-like product). Due to the ability of chalcones to cyclize readily in solution, it was investigated whether the additional eluting species (*m/z* 241) present in the UGT reaction mixture may be due to formation of the flavanone aglycone. Chromatographic analysis was used to determine the retention times of 7-hydroxyflavanone, the cyclized form of 2',4'-dihydroxychalcone, and the corresponding chalcone. Based on those results (see Table 1), the peak at 27 min was assigned as 7-hydroxyflavanone and the peak at 33 min as 2',4'-dihydroxychalcone. In Figure 2B, one major chromatographic peak was observed at 21.5 min with a minor peak at 29 min, both with mass spectra corresponding to protonated glucuronides, $[M + gluc + H]^+$. However, although LC-ESIMS allowed the detection of monoglucuronides in the enzyme reaction mixture based on the masses of the products, this method alone could not be used to identify the site of glucuronidation, thus requiring the metal complexation-MS/MS method described in the next section.

In order to distinguish isomeric monoglucuronides, a post-column metal complexation strategy followed by CID of the resulting metal complexes was utilized.[27–29] Metal complexes of the flavonoid glucuronide (FG) and an auxiliary ligand (Aux) of the form $[M^{n+}(FG - H)(Aux)_2]^+$ that readily assemble in solution and can be transferred to the gas phase by ESI were subjected to CID. Survey experiments indicated that using the auxiliary ligand 4,7-dimethyl-1,10-phenanthroline along with $Co^{2+}$ yielded glucuronide metal complexes that provided the most diagnostic fragment ions upon CID. However,

cardamonin, a methoxy-substituted chalcone, and its corresponding flavanone, alpinetin. did not yield sufficient quantities of the $[Co^{2+}(FG - H)(Aux)_2]^+$ complex for CID analysis. Therefore, the single auxiliary ligand metal complex $[Co^{2+}(FG - H)(Aux)]^+$ was used for the CID analysis of these two compounds. The CID fragmentation patterns of the $[Co^{2+}(FG - H)(Aux)_2]^+$ and $[Co^{2+}(FG - H)(Aux)]^+$ complexes, explicitly summarized in Table 2, provide the crucial molecular fingerprints for differentiation of the chalcone and flavanone glucuronides.

## Analysis of Single Product Reactions: Flavanones and 2'-Hydroxychalcone

Initial studies confirmed that the UGT reaction mixtures for all of the dihydroxychalcones showed the presence of the parent chalcone, the corresponding flavanone, and if glucuronidation occurred, up to three monoglucuronidated species. Glucuronidation most likely occurs at the nucleophilic -OH groups, so dihydroxychalcones should have only two probable glucuronidation sites while their corresponding flavanones have one, all resulting in species with the same molecular weight. Owing to the complexity of these mixtures, initial metal complexation studies focused on the analysis of compounds expected to form only a single monoglucuronidated product: 7-hydroxyflavanone, 3'-hydroxyflavanone, 4'-hydroxyflavanone, and 2'-hydroxychalcone. The three flavanones were selected because they correspond to the cyclized versions of the dihydroxychalcones in this study, with an ultimate goal of identifying glucuronidated flavanones based on CID fragmentation patterns and chromatographic retention times in the chalcone reaction mixtures. 2'-Hydroxychalcone was chosen because the cyclized version of this molecule, flavanone, contains no hydroxy groups and is therefore not expected to form any glucuronidated products. Furthermore, each of the dihydroxychalcones in this study contains a 2'-hydroxy group, so determining the CID fragmentation patterns for metal complexes of the 2'-O-glucuronide would further aid in distinguishing isomeric glucuronides in more complicated chalcone reaction mixtures.

Example CID spectra of the $[Co^{2+}(FG - H)(Aux)_2]^+$ metal complexes of these glucuronidated compounds are presented in Figure 3. As previous studies of other flavonoids have shown,[27–29,37] the position of glucuronidation has a strong influence on the CID fragmentation patterns observed. For example, in Figure 3A, a single product, a 7-O-glucuronide, is observed for the reaction of 7-hydroxyflavanone with UGT1A1. The CID mass spectrum of this glucuronide metal complex shows a high abundance loss of glucuronic acid (−GlcA, −176 Da) with lower abundance losses of the flavanone aglycone (−Agl, −240 Da) and the auxiliary ligand combined with the aglycone or glucuronic acid. In general, the same product ions were observed upon CID of the 7-O-glucuronide metal complexes produced from all the UGT enzyme reactions studied (Table S1, Supporting Information). In a CID study of metal complexes of 7-O-glucuronides produced from common flavonoids (e.g., quercetin, luteolin), Davis and Brodbelt[27] observed similar diagnostic ions including losses of GlcA and the auxiliary ligand, both individually and concurrently, as well as loss of the aglycone, which they determined was a diagnostic fragment for glucuronidation at the OH-7 position.

CID of the metal complex formed with the 3'-O-glucuronide in Figure 3B showed loss of the auxiliary ligand (−Aux, −208 Da) as the most abundant product ion as well as losses of the aglycone, glucuronic acid, and a combined loss of the aglycone and auxiliary ligand. These same CID product ions were also observed for 3'-O-glucuronide metal complexes produced from other UGT enzyme reactions (Table S2, Supporting Information). Previous work has shown that loss of the auxiliary ligand is a characteristic fragment ion for CID of B-ring flavanone glucuronide metal complexes.[27] For 4'-O-glucuronide metal complexes, as shown in Figure 3C, loss of GlcA and concurrent loss of the flavanone aglycone and auxiliary ligand (−(Agl + Aux), −442 Da) were the most prominent CID fragment ions. In addition, CID of 4'-O-glucuronide metal complexes showed the loss of auxiliary ligand, flavanone

aglycone, and concurrent loss of GlcA and the auxiliary ligand, with similar product ions observed for all UGT reactions studied (Table S3, Supporting Information). Finally, CID of the 2'-O-glucuronide metal complex, as shown in Figure 3D, resulted in a single fragment ion from the concurrent loss of GlcA and the auxiliary ligand (−(GlcA + Aux), −384 Da). The same characteristic CID fragment ion was observed for 2'-O-glucuronide metal complexes produced from all UGT reactions (Table S4, Supporting Information).

A summary of the product ions observed for CID of the metal complexes of these flavanone and chalcone glucuronides is shown in Table 2. Diagnostic CID fragment ions were noted for each glucuronidation position including characteristic high abundance losses for the 7-O-glucuronide (−GlcA), 3'-O-glucuronide (−Aux), 4'-O-glucuronide (−(Agl + Aux)), and 2'-O-glucuronide (−(GlcA + Aux)).

## Flavanone Glucuronides and 2'-O-Glucuronide

As expected, 7-hydroxyflavanone, 3'-hydroxyflavanone, 4'-hydroxyflavanone, and 2'-hydroxychalcone all formed a single monoglucuronidated product when reacted with UGT1A enzymes, with two exceptions. UGT1A4 showed no reactivity toward these flavonoids and produced no glucuronidated species. This result is not surprising since UGT1A4 is primarily involved in the preferential glucuronidation of tertiary amines[12] rather than hydroxy moieties, and has been previously noted to have no reactivity with flavonoids,[12,29] flavones,[38] and the chalcone isoliquiritigenin.[33] Likewise, the reaction of 4'-hydroxyflavanone with UGT1A7 and UGT1A9 produced no glucuronidated products.

Glucuronide product distributions were estimated for each UGT enzyme reaction and are presented in Figure 4. For the reaction of 7-hydroxyflavanone with UGT1A enzymes (Figure 4A), the 7-O-glucuronide was produced in the highest yield by UGT1A3 and 1A9 and in the lowest by UGT1A7. UGT1A1, 1A8, and 1A9 all exhibited the highest glucuronide product formation for the reaction of 3'-hydroxyflavanone with UGT1A isoforms (Figure 4B), while UGT1A3, 1A7 and 1A10 had the lowest. For the reaction of 4'-hydroxyflavanone with UGT1A enzymes (Figure 4C), 1A8 yielded the greatest glucuronidated product formation while 1A3 had the lowest. In general, 7-hydroxyflavanone, which has a hydroxy group on the flavanone A-ring, produced higher yields of monoglucuronidated product with a greater number of UGT isoforms than the flavanones with B-ring hydroxy substituents, 3'-hydroxyflavanone, and 4'-hydroxyflavanone. Finally, for the reaction of UGT1A enzymes with 2'-hydroxychalcone (Figure 4D), 1A9 produced the greatest amount of glucuronidated product while 1A3 produced the least.

## Analysis of Multiple Product Reactions: Dihydroxychalcones

Figure 5A presents a typical CID total ion chromatogram (*m/z* 890) of the glucuronide metal complexes formed from the reaction of 2',4'-dihydroxychalcone with UGT1A9. Although 2',4'-dihydroxychalcone is expected to form at most two monoglucuronidated products based on the number of hydroxy groups in the molecule, the CID total ion chromatogram shows the presence of three monoglucuronidated species. Based on the presence of 7-hydroxyflavanone within the chalcone reaction mixture, it was investigated whether the third product is formed from glucuronidation of the cyclized chalcone. Chromatographic retention time studies showed that for all chalcones in this study, the corresponding flavanone aglycone always eluted *prior* to the chalcone aglycone, as shown in Table 1. For example, for the reaction of 2',4'-dihydroxychalcone with UGT1A9, the flavanone aglycone eluted at 22 min while the chalcone eluted at 28 min. Glucuronidation increases the hydrophilicity of the molecules, so the resulting glucuronides are expected to elute *before* their parent aglycones.[39] Therefore, the monoglucuronide in Figure 5A, eluting after the

flavanone aglycone at 24 min, corresponds to a chalcone glucuronide. The monoglucuronides that elute at 17 and 19 min, prior to either of the aglycones, may be either flavanone or chalcone glucuronides. In Figure 5B, the CID mass spectrum of the earliest eluting peak at 17 min shows loss of GlcA as the highest abundance product ion along with losses of the aglycone (−Agl) and concomitant losses of the auxiliary ligand with both glucuronic acid [−(GlcA + Aux)] and the aglycone [−(Agl + Aux)]. As shown in Table 2, this CID fragmentation pattern is characteristic of the metal complex of a 7-O-glucuronide and the peak at 17 min is therefore assigned as the monoglucuronide of 7-hydroxyflavanone. The CID mass spectrum of the chromatographic peak at 19 min is presented in Figure 5C and shows the combined loss of GlcA and the auxiliary ligand [−(GlcA + Aux)] as the highest abundance product ion, allowing it to be assigned as a 2'-O-glucuronide (see Table 2). For the last-eluting peak at 24 min, the CID mass spectrum (Figure 5D) showed loss of the auxiliary ligand (−Aux) as the highest abundance ion along with loss of GlcA and combined losses of the auxiliary ligand with both GlcA and the aglycone. Based on elution order, this monoglucuronide must be formed from the chalcone and can therefore be assigned as the 4'-O-glucuronide.

This same strategy of utilizing characteristic CID fragmentation patterns for the metal glucuronide complexes in combination with elution order trends was used to identify unambiguously all monoglucuronides produced for the reactions of 2'4'-dihydroxychalcone, 3,2'-dihydroxychalcone, and 4,2'-dihydroxychalcone with the UGT1A enzymes studied (Tables S5 – S7, Supporting Information). These results showed two general trends: (1) for all dihydroxychalcones studied, the flavanone monoglucuronide elutes before any of the chalcone glucuronides; and (2) when comparing monoglucuronides formed from a single dihydroxychalcone, the 2'-O-glucuronide always elutes earliest.

## Glucuronides of Dihydroxychalcones

After identifying the individual monoglucuronides for reactions of the dihydroxychalcones with UGT1A enzymes, glucuronide product distributions were calculated and are presented in Figure 6. For all of the dihydroxychalcones studied, reaction with the UGT1A4 isoform did not produce any glucuronidated products. However, each of the dihydroxychalcones showed unique isoform-dependent variations in both the number of monoglucuronides produced as well as the site where glucuronidation occurred. For example, product distributions for 2',4'-dihydroxychalcone with UGT1A enzymes are shown in Figure 6A. UGT1A8 exhibited relatively low reactivity with 2',4'-dihydroxychalcone, with only trace amounts of glucuronidated products formed. The reactions of 2',4'-dihydroxychalcone with UGT1A1, 1A7, and 1A10 formed only a single monoglucuronide in greater than trace amounts: the 4'-O-glucuronide with UGT1A1 and 1A10 and the 2'-O-glucuronide with UGT1A7. Both UGT1A6 and 1A9 produced two chalcone monoglucuronides, the 4'-O-glucuronide and the 2'-O-glucuronide, with UGT1A9 producing the highest overall yield of glucuronidated products of all reactions of 2',4'-dihydroxychalcone with UGT1A isoforms. UGT1A3 was the only isoform to produce the monoglucuronidated flavanone, a 7-O-glucuronide, in greater than trace amounts and also formed a 4'-O-glucuronide.

For the reactions of 3,2'-dihydroxychalcone with UGT1A enzymes (Figure 6B), UGT1A6 was found to have low reactivity and produced only trace amounts of glucuronidated product. Reaction of 3,2'-dihydroxychalcone with UGT1A10 produced a single monoglucuronide, a 2'-O-glucuronide, while UGT1A1, 1A3, and 1A7 all formed two products, the monoglucuronidated flavanone, 3'-O-glucuronide, as well as the chalcone glucuronide, 3-O-glucuronide, with UGT1A7 producing the largest yield of glucuronides of the isoforms studied. UGT1A9 also produced two monoglucuronidated products upon reaction with 3,2'-dihydroxychalcone: the 3'-O-glucuronide (flavanone) and the 2'-O-glucuronide. When UGT1A8 reacted with 3,2'-dihydroxychalcone, three monoglucuronides

were formed in greater than trace amounts: the 3'-O-glucuronide (flavanone), the 2'-O-glucuronide, and the 3-O-glucuronide.

For the reactions of 4,2'-dihydroxychalcone with UGT1A enzymes (Figure 6C), UGT1A6, 1A7, 1A8, 1A9 and 1A10 produced only a single monoglucuronide in greater than trace amounts. UGT1A6, 1A8, and 1A9 formed the 2'-O-glucuronide, UGT1A7 produced only the glucuronidated flavanone, 4'-O-glucuronide, while UGT1A10 formed a 4-O-glucuronide. Reaction of 4,2'-dihydroxychalcone with UGT1A3 produced two monoglucuronidated products, the 2'-O-glucuronide and the 4-O-glucuronide, while UGT1A1 formed three glucuronidated products in greater than trace amounts, the 4'-O-glucuronide (flavanone), the 4-O-glucuronide, and the 2'-O-glucuronide.

## Analysis of a Methoxy-Substituted Dihydroxychalcone: Cardamonin

As shown in Figure 1, cardamonin is identical to 2',4'-dihydroxychalcone but with a methoxy group added to the chalcone B-ring at the 6' position. Like the other dihydroxychalcones in this study, reaction of cardamonin with UGT1A enzymes generated complex mixtures containing multiple products. Therefore, initial metal complexation studies focused on the analysis of the flavanone, alpinetin, the cyclized form of cardamonin, which has one free hydroxy group at the 7 position and is therefore expected to form only a single well-defined monoglucuronidated product. Reaction of alpinetin with UGT1A enzymes followed by CID of the resulting metal/ligand complexes (Table S8, Supporting Information) showed loss of the aglycone (−Agl, −270 Da) as the most abundant fragment ion with smaller losses of glucuronic acid (−GlcA), concomitant loss of GlcA and the auxiliary ligand [−(GlcA + Aux)], and loss of an aglycone fragment (−Agl fragment, −134 Da), as summarized in Table 2.

Chromatographic retention time analysis showed that, like other chalcones in this study, alpinetin eluted prior to cardamonin within the UGT reaction mixtures (see Table 1). A similar strategy using characteristic CID fragmentation patterns for the metal glucuronide complexes in combination with elution order trends could therefore be used to identify glucuronide products for the reaction of cardamonin with UGT1A enzymes (Table S9, Supporting Information).

## Cardamonin and Alpinetin Glucuronides

Following identification of the individual glucuronides for the reactions of alpinetin and cardamonin with UGT1A enzymes, glucuronide product distributions were determined and are presented in Figure 7. For alpinetin, UGT1A4, 1A6 and 1A7 did not catalyze the formation of any glucuronidated products. For the other UGT1A isoforms, reaction with alpinetin led to the formation of a single monoglucuronidated product as expected, with UGT1A3 and 1A9 forming the highest yields of the monoglucuronide, 7-O-glucuronide-5-methoxyflavanone.

Like other chalcones and flavanones in this study, UGT1A4 produced no glucuronidated products upon reaction with cardamonin and UGT1A6 and 1A10 formed only trace amounts of monoglucuronide products. UGT1A1, 1A3, 1A7 and 1A8 produced only a single monoglucuronide, the 4'-O-glucuronide, in greater than trace amounts when reacted with cardamonin while UGT1A9 produced two monoglucuronide products, the 4'-O-glucuronide and the 2'-O-glucuronide. None of the UGT1A isoforms produced the flavanone glucuronide, 7-O-glucuronide-5-methoxyflavanone, in greater than trace amounts upon reaction with cardamonin. Cardamonin was the only dihydroxychalcone in this study to produce a diglucuronide, and it was found in trace amounts after reaction with UGT1A1.

### Reactivity Trends for Flavanone Glucuronides

The flavanones in this study had only one likely glucuronidation site but the yield of monoglucuronide product was highly dependent on both the UGT1A isoform and flavanone structure. The UGT1A9 isoform promoted the highest yield of monoglucuronide product for both 7-hydroxyflavanone and 3'-hydroxyflavanone and the second highest glucuronide product yield for alpinetin. However, UGT1A9 produced no glucuronidated product upon reaction with 4'-hydroxyflavanone. Chen et al. similarly noted that when the UGT1A9 isoform reacted with quercetin, it strongly promoted glucuronidation at the OH-7 site but was much less effective at producing glucuronides at the OH-3' and OH-4' positions.[40] Although Robotham and Brodbelt found that the trihydroxyflavanone naringenin never glucuronidated at the OH-4' position when reacted with UGT1A enzymes,[29] it was observed in the present study that several UGT isoforms promoted the formation of the 4'-O-glucuronide, particularly UGT1A8, which produced the monoglucuronide in high yield. UGT1A3 strongly promoted glucuronidation at the OH-7 position of the flavanone A-ring in 7-hydroxyflavanone and alpinetin, but led to much lower glucuronide product yields for flavanones with B-ring hydroxy groups such as 3'-hydroxyflavanone and 4'-hydroxyflavanone. Similarly, Brodbelt and Robotham also found that UGT1A3 showed a preference for the production of 7-O-glucuronides for the flavonols, quercetin and isorhamnetin.[29] While UGT1A1 catalyzed the formation of glucuronide products in relatively high yields for all of the flavanones studied, in general, glucuronide product formation was less for the flavanones with a B-ring hydroxy group compared to those with an A-ring hydroxy moiety.

Comparing 7-hydroxyflavanone and alpinetin – which only differ in structure by the methoxy group at the 5 position on the flavanone A-ring – both compounds had similar trends in reactivity, with UGT1A1, 1A3 and 1A9 catalyzing the largest yields of glucuronide product. However, addition of the methoxy group decreased the overall amount of monoglucuronide formed for all UGT1A isoforms studied.

### Isoform- and Structure-Dependent Selectivity Trends for Chalcone Glucuronides

The UGT1A enzymes displayed unique regioselectivity for the site of glucuronidation and this selectivity was affected strongly by structural differences among the dihydroxychalcones in this study. However, several of the UGT1A isoforms showed poor reactivity toward the chalcones including UGT1A4, 1A6, and 1A10. Although UGT1A4 and 1A6 have been reported previously to have minimal reactivity with other flavonoids,[12,20,29,37] UGT1A10 was noted to promote glucuronidation strongly in a trihydroxychalcone, isoliquiritigenin,[33] suggesting that chalcone structure impacts the reactivity of the 1A10 isoform.

UGT1A9 promoted glucuronidation at the OH-2' position, and formation of the 2'-O-glucuronide was enhanced for dihydroxychalcones with hydroxy substituents on the A-ring. In fact, UGT1A9 was the only isoform to glucuronidate cardamonin at the OH-2' position. These results are not surprising since the UGT1A9 isoform was found to produce the highest yields of the 2'-O-glucuronide product when reacted with 2'-hydroxychalcone. The effect of hydroxy group substituent location on the formation of the 2'-O-glucuronide was even more pronounced for reactions of the dihydroxychalcones with the UGT1A8 isoform. For example, although UGT1A8 reacted with 3,2'-dihydroxychalcone and 4,2'-dihydroxychalcone to form 2'-O-glucuronides, the presence of the B-ring hydroxy group at the 4' position in both cardamonin and 2',4'-dihydroxychalcone rendered the OH-2' position inactive to glucuronidation with the 1A8 isoform. Furthermore, the A-ring OH-4 group was found to promote glucuronidation at the 2' position for the reaction of 4,2'-dihydroxychalcone with UGT1A1 and 1A3. No other dihydroxychalcones in this study

formed a 2'-O-glucuronide upon reaction with UGT1A1 and 1A3, although reaction of these isoforms with 2'-hydroxychalcone led to a glucuronide product, although in low yields. In their study of isoliquiritigenin, a chalcone with an A-ring hydroxy group at the 4 position as well as OH-2' and OH-4' substituents, Guo et al. similarly found that UGT1A1 strongly promoted formation of the 2'-O-glucuronide.[33]

For UGT1A7, hydroxy substituents on the chalcone A-ring enhanced the overall yield of glucuronide product and especially promoted formation of the corresponding flavanone glucuronide. For example, UGT1A7 had relatively low reactivity with 2',4'-dihydroxychalcone and cardamonin, but it strongly catalyzed glucuronidation of the dihydroxychalcones with A-ring hydroxy substituents, promoting chalcone cyclization to the flavanone and subsequent glucuronidation to form the flavanone glucuronide in large yields, particularly for 4,2'-dihydroxychalcone. This is a particularly interesting result since the reaction of 4'-hydroxyflavanone with UGT1A7 failed to yield any glucuronidated product. Although chalcones by themselves are able to cyclize upon exposure to light,[32] little evidence was found for spontaneous cyclization in the chalcone stock solutions on hand. In fact, based on LC-UV and LC-MS analysis, only traces (<1%) of the cyclic flavanones were detected, suggesting that cyclization is more prevalent in the presence of some UGT1A enzymes.

Addition of a methoxy group to the chalcone B-ring at the 6' position activated glucuronidation at the OH-4' site for the reaction of cardamonin with UGT1A1, 1A8 and 1A9, giving high 4'-O-glucuronide product yields compared to 2',4'-dihydroxychalcone. The B-ring methoxy group on cardamonin also generally decreased the reactivity of the OH-2' position to glucuronidation. For example, a 2'-O-glucuronide was formed during the reaction of 2',4'-dihydroxychalcone with UGT1A6 and 1A7, but was only found in trace amounts upon reaction of these isoforms with cardamonin. In addition, although the reaction of cardamonin with UGT1A9 yielded a 2'-O-glucuronide product, the overall yield was substantially reduced in comparison with 2',4'-dihydroxychalcone. While the B-ring methoxy group enhanced glucuronidation upon reaction with some UGT isoforms, it decreased the reactivity with others. Although UGT1A6 and 1A10 produced relatively low glucuronide yields with 2',4'-dihydroxychalcone, these isoenzymes catalyzed only trace amounts of glucuronide product upon reaction with cardamonin. Finally, addition of the B-ring methoxy group promoted formation of trace amounts of diglucuronide upon reaction of cardamonin with UGT1A1, the only chalcone/enzyme reaction in which a diglucuronide was observed.

In conclusion, a metal complexation MS/MS strategy was successfully used to evaluate the glucuronidation of a series of 2'-hydroxychalcones and their corresponding flavanones by nine UGT1A enzymes. Metal complexes of the form $[Co^{2+}(FG - H)(Aux)_2]^+$ were used for analysis of hydroxyflavanones, 2'-hydroxychalcone, and dihydroxychalcones, while $[Co^{2+}(FG - H)(Aux)]^+$ complexes were used for cardamonin and alpinetin. CID of these metal glucuronide complexes yielded characteristic fragmentation patterns that were used in combination with elution order trends to identify unambiguously monoglucuronides produced for all UGT reactions. Selectivity of the UGT1A isoforms was affected strongly by structural differences among the reactants, and for the 2'-hydroxychalcones, in particular, the presence of additional hydroxy groups on the A or B-ring as well as B-ring methoxy substitution was found to have a substantial impact on the overall yield of glucuronide product and the site where glucuronidation occurred. For some UGT1A enzymes such as 1A7, hydroxy substituents on the chalcone A-ring also promoted cyclization and formation of the corresponding flavanone glucuronide. This study provides important benchmark data on the regioselectivity of UGT1A enzymes in the glucuronidation of 2'-hydroxychalcones and their corresponding flavanones. In addition, it shows the benefits of using a metal

complexation LC-MS/MS strategy for the unambiguous identification of isomeric monoglucuronides in complex reaction mixtures. Because metal complexation MS methods have been previously used to identify flavonoid glucuronides in urine[27,41] and plasma,[27] this research is also a first step toward analyzing chalcone glucuronides within biological fluids.

## EXPERIMENTAL SECTION

### General Experimental Procedures

HPLC separation of chalcones and flavanones from their corresponding glucuronides was accomplished primarily on a Shimadzu LC-20AT chromatograph with photodiode array detection. For several reaction mixtures, a combined Waters Alliance 2695 (Milford, MA) and Hitachi L-7000 (Hitachi High Technologies America, Pleasonton, CA) HPLC system with a photomultiplier tube detector was used. The gradient elution method for all analyses used a Waters Symmetry $C_{18}$ column (2.1 mm × 50 mm, 3.5 μm particle size) and guard column with 0.1% formic acid in water (mobile phase A) and methanol (mobile phase B). The solvent program was adapted from Guo et al.[33] as follows: 0 – 25 min, 20 to 70% B; 25 – 30 min, 70 to 90% B; 30 – 32 min, hold at 90% B; 32 – 34 min, return to 20% B; 34 – 44 min, hold at 20% B to equilibrate the column prior to the next analysis. The injection size was 50 μL and the flow rate was 0.3 mL/min.

Mass spectrometric (MS) detection was accomplished by directing the column effluent from the HPLC into a Thermo Electron LCQ Duo quadrupole ion trap mass spectrometer with electrospray ionization. All analyses were completed in the positive ESI mode using a spray voltage of 4.1 kV, a heated capillary temperature of 200 °C, and with the automatic gain control set to $5 \times 10^7$ ions with a maximum injection time of 500 ms and 5 microscans averaging. Other parameters such as auxiliary gas (nitrogen; 5 – 15 arbitrary units), sheath gas (nitrogen; 15 – 25 arbitrary units), capillary voltage (17 – 46 V), octapole offsets and tube lens offset were optimized for individual compounds.

All reagents and solvents were purchased from commercial sources and used without further purification. Most analytical standards including the dihydroxychalcones (2',4'-dihydroxychalcone, 98%; 3,2'-dihydroxychalcone, 98%; 4,2'-dihydroxychalcone, 98%), 2'-hydroxychalcone (98%), and hydroxyflavanones (7-hydroxyflavanone, 99+%; 3'-hydroxyflavanone, 98%; 4'-hydroxyflavanone, 97%) were purchased from Indofine Chemical Company, Inc. Alpinetin (secondary standard) was obtained from ChromaDex and cardamonin (>98%, HPLC grade) was purchased from Sigma-Aldrich Co.

### Preparation of Chalcone and Flavanone Glucuronides

All chalcone and flavanone glucuronides were prepared using a procedure developed by Robotham and Brodbelt[29] as based on a previously published method.[28] Briefly, human nonhepatic UGT supersomes (1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, and 1A10 at 5.0 mg/mL in 0.1 M Tris-HCl buffer, pH 7.5) were purchased from BD Biosciences and stored at −80 °C until use. Chalcone and flavanone stock solutions were prepared in methanol at concentrations of 10 mM and stored at 4 °C. All chalcone solutions were protected from light during preparation, use, storage and analysis to minimize photodegradation. Aqueous UDPGA (65 μL; 2 mM) was added to potassium phosphate buffer (378.75 μL; 20 mM, pH 7.0) and the chalcone or flavanone stock solution in methanol (6.25 μL; 10 mM). Methanol increases flavonoid solubility within the UGT reaction mixtures[29,37] and previous work has shown that low methanol concentrations (< 2%) have no effect on UGT enzyme activities.[42] The resulting solution was vortexed then transferred into a 25 μL aliquot of the appropriate UGT enzyme. After vortexing again, the enzyme mixture was placed in a block heater at 37 °C for approximately 15 h, a sufficient incubation time to reach a final equilibrium concentration of reactants and glucuronide products. The reaction was stopped by the

addition of 1.5 mL of acetone, the mixture was vortexed, centrifuged at 16,000 × *g*, then the supernatant was removed and placed in a Thermo Electron Savant DNA 120 SpeedVac Concentrator for 100 min on low heat. The resulting solution (~ 0.5 mL) was stored at 4 °C until analysis.

### Analysis of UGT Reaction Mixtures

Each enzyme reaction mixture was analyzed first using mass spectrometric detection to identify the chalcone or flavanone reactant and glucuronide products based on their respective molecular ions. A second analysis using post-column metal derivatization was then completed to further identify the glucuronide products. Metal/auxiliary ligand complexes were formed with chalcone or flavanone glucuronides by delivery of a methanolic solution of 10 μM $CoCl_2$/4,7-dimethyl-1,10-phenanthroline (dmphen, 208 g/mol) to the column effluent via a tee placed after the column and prior to the mass spectrometer. The $Co^{2+}$/dmphen solution was delivered at a rate of 20 μL/min using a syringe pump. Collision induced dissociation analysis used an isolation width of 5 Da[43] with 35% normalized collision energy to fragment the resulting metal complexes.

### Determination of Glucuronide Product Distributions

During a chromatographic analysis using the Shimadzu system, UV spectra were recorded for each analyte over a narrow range using the photodiode array detector. Integrated peak areas were then determined for each chalcone or flavanone reactant as well as the glucuronide product(s) based on the extracted chromatogram at 360 nm. For the Waters/Hitachi system, the detection wavelength was set at 360 nm and integrated peak areas were measured. Since HPLC retention times are important for identifying chalcone and flavanone aglycones in the UGT reaction mixtures, each chalcone and flavanone standard was also injected individually and retention times were determined using identical chromatographic conditions.

For each UGT reaction mixture, the relative glucuronide product distribution was estimated by dividing the peak area for each glucuronide by the sum of the peak areas for the chalcone or flavanone reactant and all glucuronide products for a given reaction. Although the molar absorptivities of the flavanones are typically lower than those of the chalcones, relative product distributions were determined to allow facile comparison of reactivity trends. Furthermore, because UV molar absorptivities do not correlate linearly with electrospray ionization (ESI) efficiencies, all quantitative measurements of product distributions were based on UV absorbance rather than ESI ion abundance. Relative standard deviations were calculated for replicate measurements and due to peak area deviations that occurred, all product distributions were rounded and reported to the nearest 5%.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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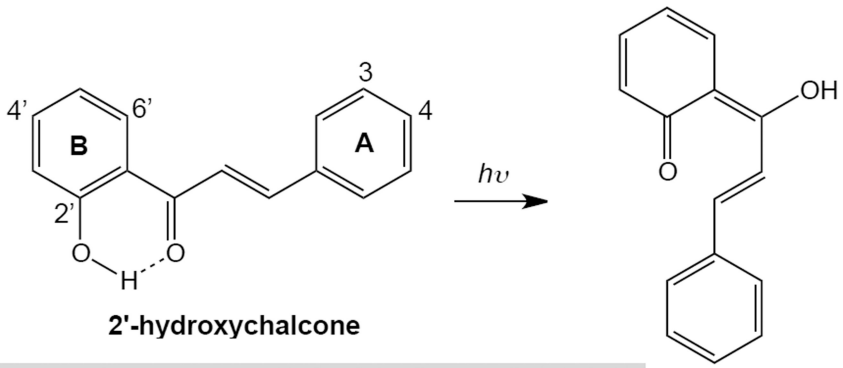
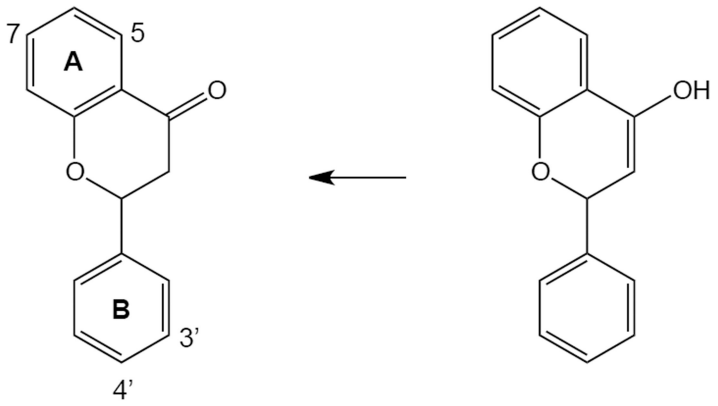



**2'-hydroxychalcone**

| Name | MW (g/mol) | -OH | -OCH$_3$ |
|---|---|---|---|
| 2'-hydroxychalcone | 224 | 2' | |
| 2',4'-dihydroxychalcone[1] | 240 | 2', 4' | |
| 3,2'-dihydroxychalcone[2] | 240 | 2', 3 | |
| 4,2'-dihydroxychalcone[3] | 240 | 2', 4 | |
| cardamonin[4] | 270 | 2',4' | 6' |

[1] This chalcone may cyclize to give 7-hydroxyflavanone.
[2] This chalcone may cyclize to give 3'-hydroxyflavanone.
[3] This chalcone may cyclize to give 4'-hydroxyflavanone.
[4] This chalcone may cyclize to give alpinetin.




**flavanone**

| Name | MW (g/mol) | -OH | -OCH$_3$ |
|---|---|---|---|
| 7-hydroxyflavanone | 240 | 7 | |
| 3'-hydroxyflavanone | 240 | 3' | |
| 4'-hydroxyflavanone | 240 | 4' | |
| alpinetin | 270 | 7 | 5 |

**Figure 1.**
Mechanism for the light-induced cyclization of chalcone to flavanone (adapted from Matsushima[32]) and structures of the chalcones and flavanones used in this study.

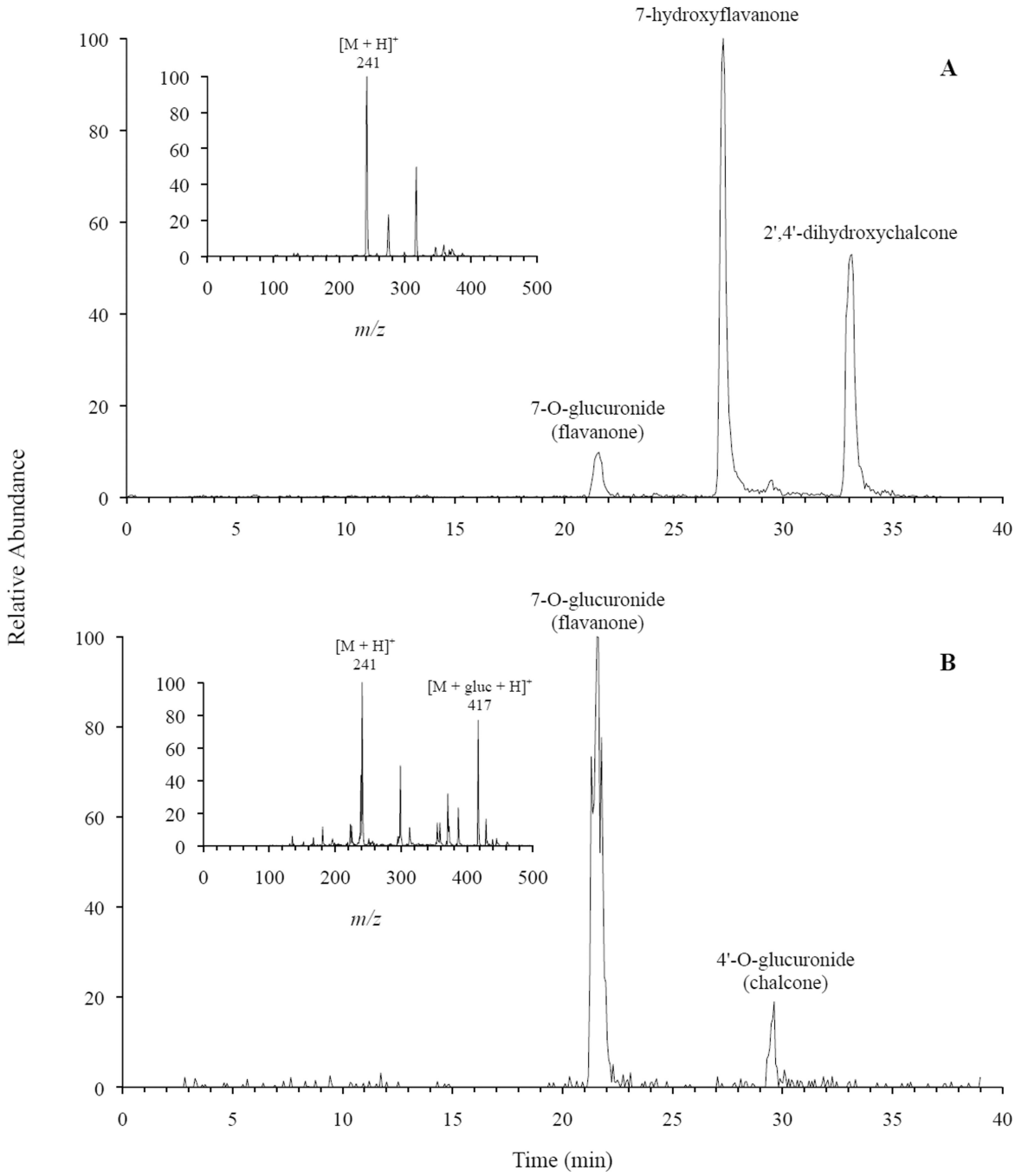



**Figure 2.**

Reaction of 2',4'-dihydroxychalcone with UGT1A10: (A) Selected ion chromatogram of *m/z* 241 for the protonated species, $[M + H]^+$; inset is the mass spectrum for the chromatographic peak at 33 min, (B) Selected ion chromatogram of *m/z* 417 for the monoglucuronidated species, $[M + gluc + H]^+$; inset is the mass spectrum for the chromatographic peak at 21.5 min.

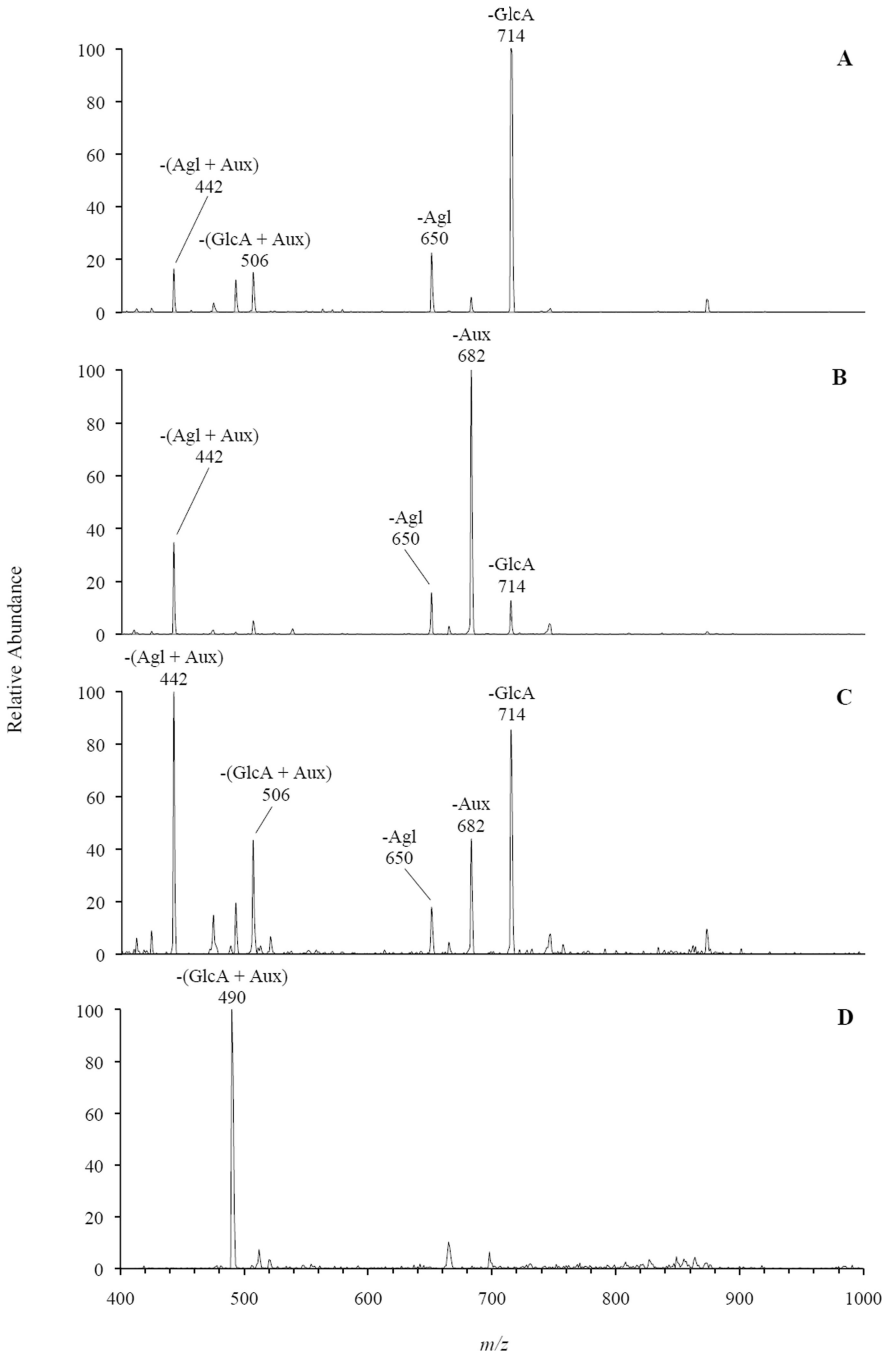



**Figure 3.**

CID mass spectra of $[Co^{2+}(FG - H)(Aux)_2]^+$ complexes formed from the reaction of UGT1A1 with: (A) 7-hydroxyflavanone (CID of *m/z* 890), (B) 3'-hydroxyflavanone (CID of *m/z* 890), (C) 4'-hydroxyflavanone (CID of *m/z* 890), (D) 2'-hydroxychalcone (CID of *m/z* 874). −Agl (loss of flavonoid aglycone); −Aux (loss of auxiliary ligand); -GlcA (loss of glucuronic acid moiety); $-H_2O$ (loss of water).

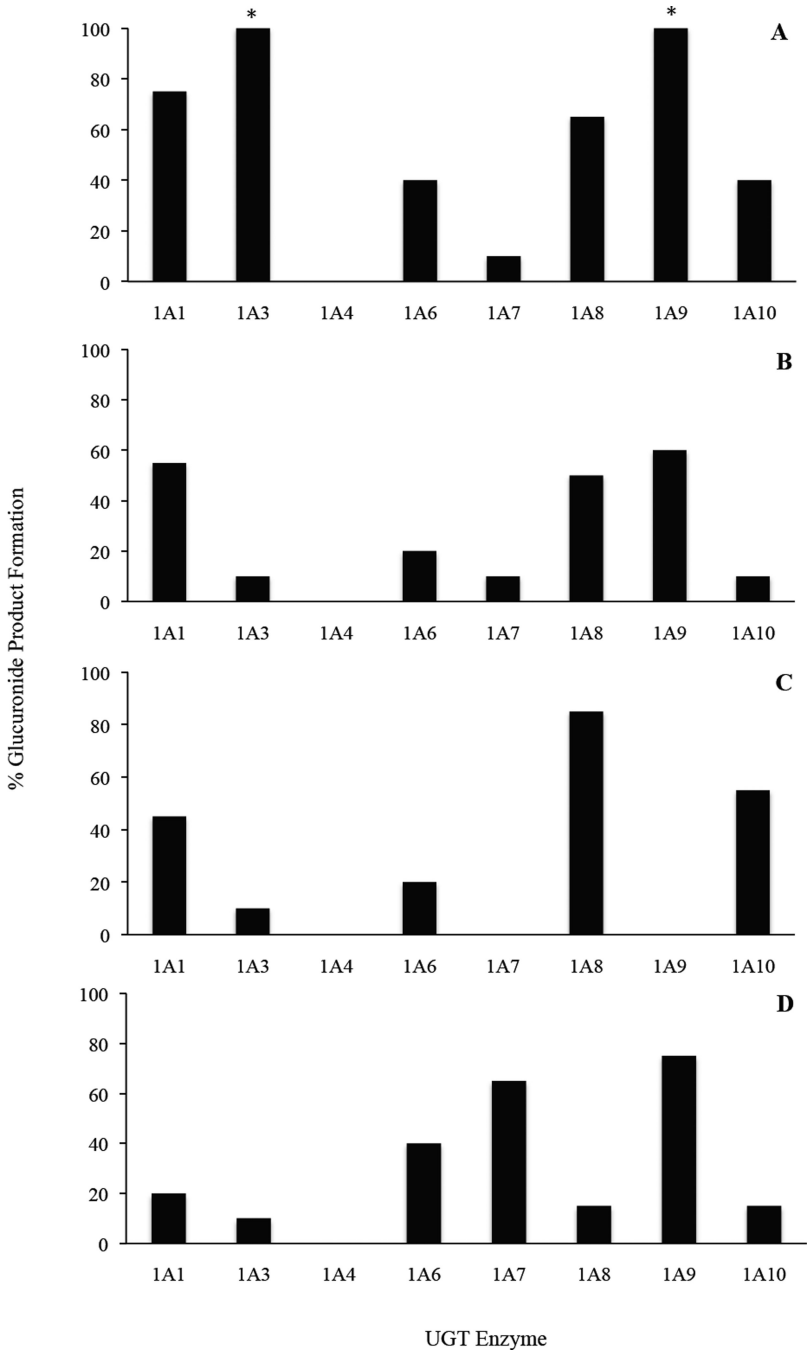



**Figure 4.**

Relative percent glucuronide product formation for the reactions of UGT enzymes with: (A) 7-hydroxyflavanone, (B) 3'-hydroxyflavanone, (C) 4'-hydroxyflavanone, (D) 2'-hydroxychalcone. A * indicates that less than 5% of the flavonoid aglycone remains. Glucuronide product distributions were estimated using LC-UV chromatograms by dividing the glucuronide peak area by the sum of the peak areas for the unreacted aglycone and glucuronide product for a given reaction. Relative standard deviations were generally less than 10% for replicate measurements.

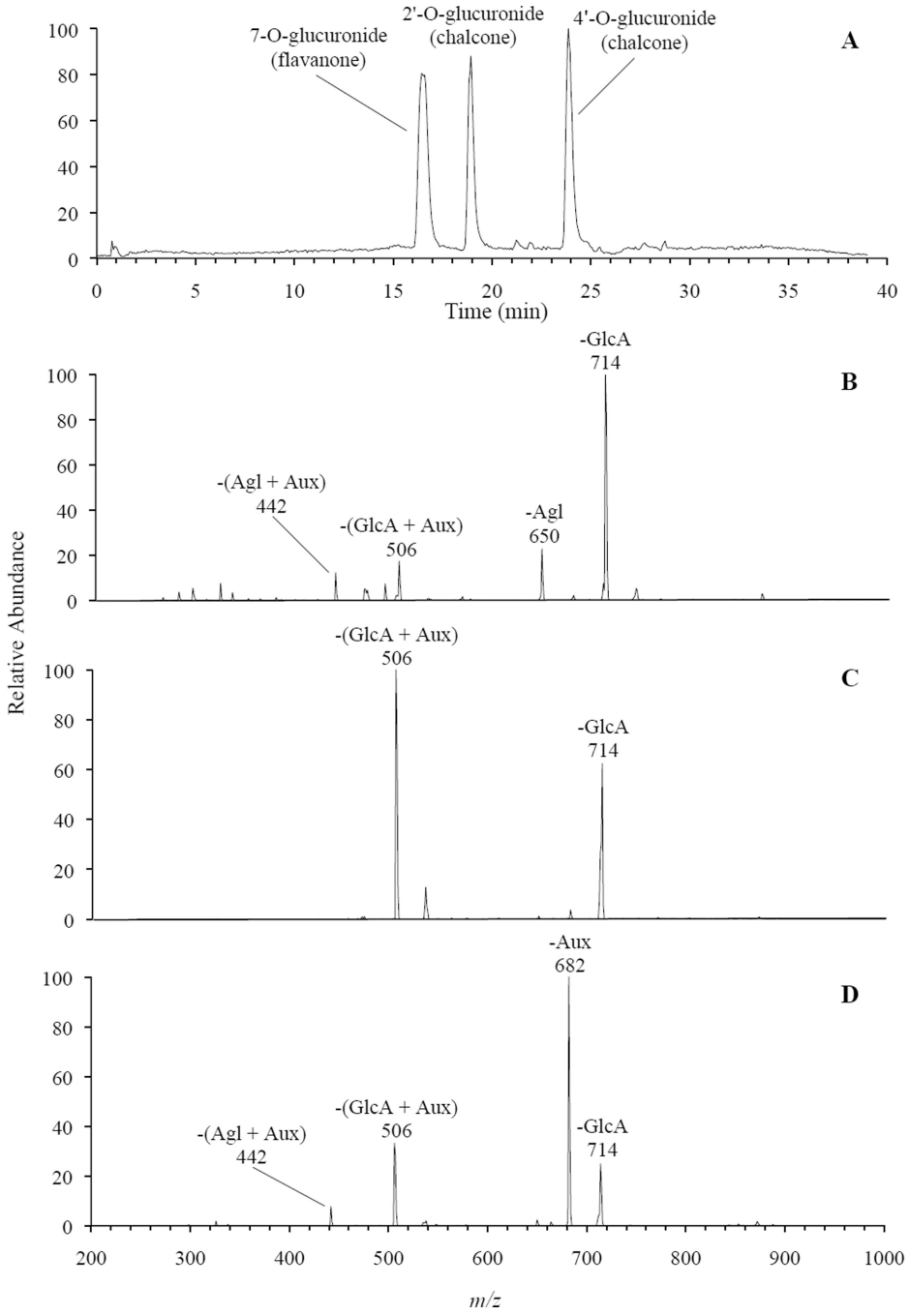



**Figure 5.**

Reaction of 2',4'-dihydroxychalcone with UGT1A9: (A) total ion chromatogram for CID of the glucuronide metal complex of *m/z* 890, (B) CID mass spectrum of the peak at 16.4 min, (C) CID mass spectrum of the peak at 18.9 min, (D) CID mass spectrum of the peak at 23.9 min. −Agl (loss of flavonoid aglycone); −Aux (loss of auxiliary ligand); −GlcA (loss of glucuronic acid moiety).

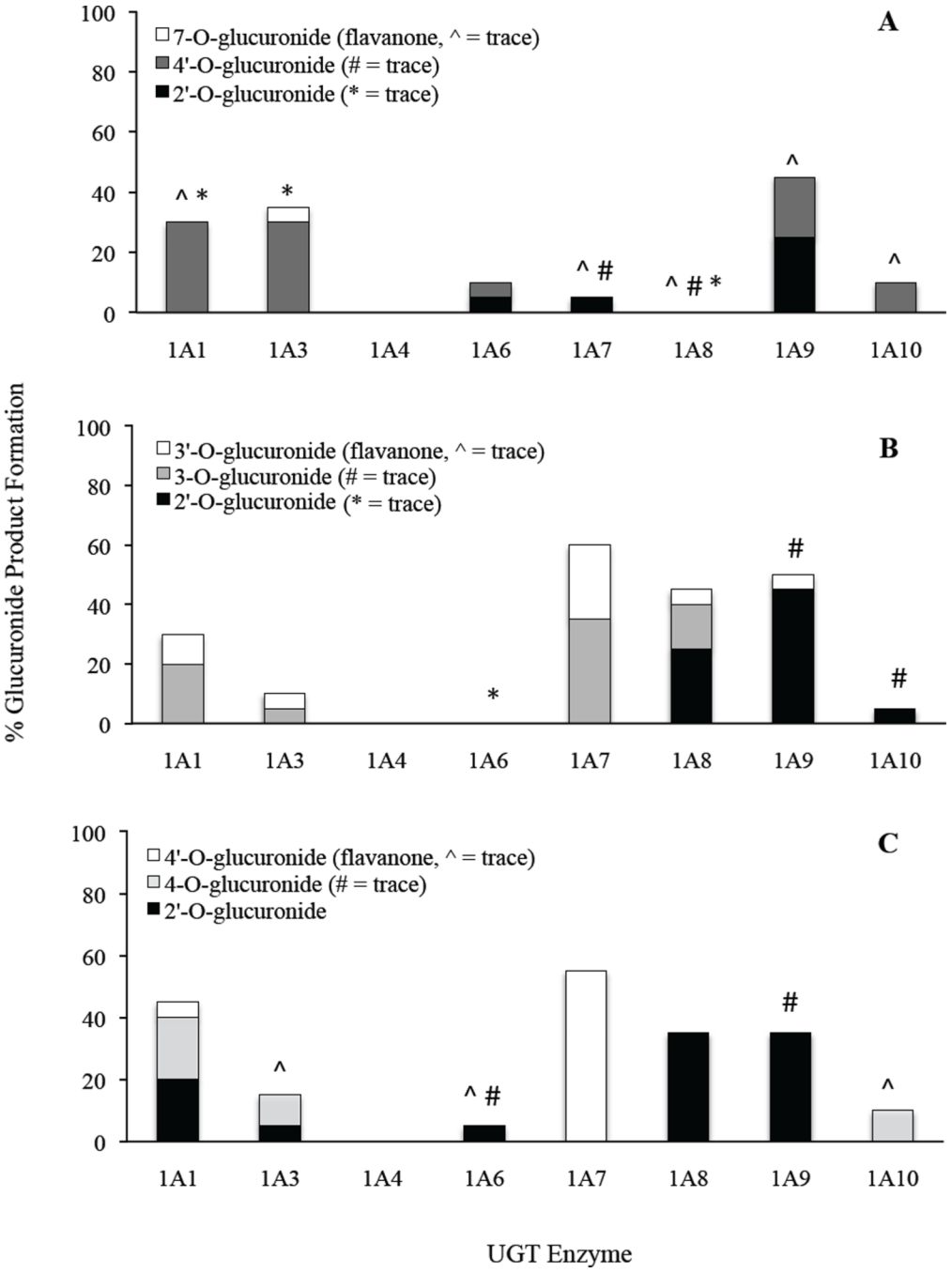



**Figure 6.**

Relative percent glucuronide product formation for the reactions of UGT enzymes with: (A) 2',4'-dihydroxychalcone, (B) 3,2'-dihydroxychalcone, (C) 4,2'-dihydroxychalcone. Glucuronide product distributions were estimated using LC-UV chromatograms by dividing the individual glucuronide peak area by the sum of the peak areas for the unreacted aglycone and all glucuronide products for a given reaction. Relative standard deviations were generally less than 10% for replicate measurements.

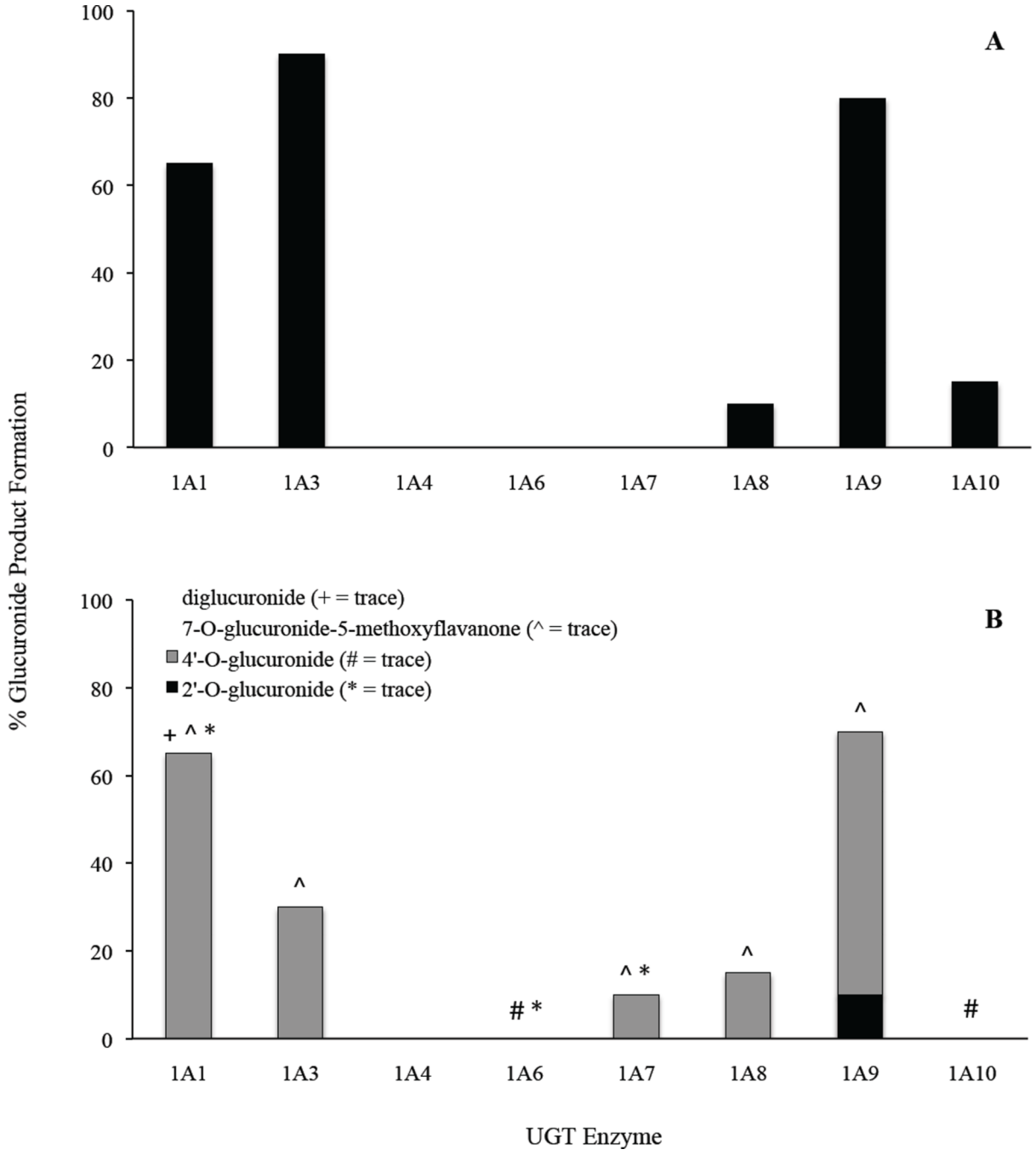



**Figure 7.**

Relative percent glucuronide product formation for the reactions of UGT enzymes with: (A) alpinetin, (B) cardamonin. Glucuronide product distributions were estimated using LC-UV chromatograms by dividing the individual glucuronide peak area by the sum of the peak areas for the unreacted aglycone and all glucuronide products for a given reaction. Relative standard deviations were generally less than 10% for replicate measurements.

## Table 1

Average Chromatographic Retention Times ($t_R$) for Selected Flavanone and Chalcone Aglycones[a]

| flavanone | $t_R$ (min) | chalcone (min) | $t_R$ (min) |
|---|---|---|---|
| 7-hydroxyflavanone | 26.3 | 2',4'-dihydroxychalcone | 32.9 |
| 3'-hydroxyflavanone | 26.2 | 3,2'-dihydroxychalcone | 32.3 |
| 4'-hydroxyflavanone | 25.7 | 4,2'-dihydroxychalcone | 32.4 |
| flavanone | 31.1 | 2'-hydroxychalcone | 36.6 |
| alpinetin | 24.9 | cardamonin | 34.0 |

[a]Values were determined by averaging the retention times for the respective flavanone or chalcone in all UGT1A reactions analyzed using the Shimadzu HPLC system.

**Table 2**

Characteristic Product Ions and Retention Times ($t_R$) Observed upon CID of $[Co^{2+}(FG - H)(Aux)_2]^+$ and $[Co^{2+}(FG - H)(Aux)]^+$ Complexes

| glucuronide, complex mass (*m/z*) | $t_R$ (min) | CID base peak(s) and corresponding mass loss (Da) | other minor fragment ions[a] and corresponding mass loss (Da) |
|---|---|---|---|
| 7-O-glucuronide (flavanone), 890[b] | 20.4 | −GlcA, −176 | −Agl, −240; −(GlcA + Aux), −384 −(Agl + Aux), −448 |
| 3'-O-glucuronide (flavanone), 890[b] | 21.5 | −Aux, −208 | −GlcA, −176; −Agl, −240 −(Agl + Aux), −448 |
| 4'-O-glucuronide (flavanone), 890[b] | 20.8 | −GlcA, −176 −(Agl + Aux), −448 | $-H_2O$, −18; −Aux, −208; −Agl, −240 −(GlcA + Aux), −384 |
| 7-O-glucuronide-5-methoxyflavanone, 712[c] | 20.1 | −Agl, −270 | −GlcA, −176; −Agl fragment, −134 −(GlcA + Aux), −384 |
| 2'-O-glucuronide (chalcone), 874[b] | 24.2 | −(GlcA + Aux), −384 | −Aux, −208 |
| 4'-O-glucuronide (chalcone), 890[b] | 28.6 | −Aux, −208 | −GlcA, −176; −(GlcA + Aux), − 384 −(Agl + Aux), −448 |
| 3-O-glucuronide (chalcone), 890[b] | 27.5 | −Aux, −208 | −(GlcA + Aux), −384 −(Agl + Aux), −448 |
| 4-O-glucuronide (chalcone), 890[b] | 27.9 | −Aux, −208 | −(GlcA + Aux), −384 |

[a] Listed in order of decreasing *m/z* value.

[b] $[Co^{2+}(FG - H)(Aux)_2]^+$ complex.

[c] $[Co^{2+}(FG - H)(Aux)]^+$ complex.