

# UHPLC-(ESI)QTOF MS/MS Profiling of Quercetin Metabolites in Human Plasma Postconsumption of Applesauce Enriched with Apple Peel and Onion

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**ABSTRACT:** An ultrahigh pressure liquid chromatography accurate mass quadrupole time-of-flight mass spectrometry with electrospray ionization (UHPLC-(ESI)QTOF MS/MS) method was developed for measuring individual quercetin metabolites in human plasma with high sensitivity and high selectivity. Quercetin (3,3',4',5,7-pentahydroxyflavone) occurs as glycosides in foods. The composition of glycosides is species and cultivar specific. In humans, quercetin undergoes extensive biotransformation, resulting in a range of metabolites. The bioactivity of quercetin metabolites will depend on the type and position of the conjugates. Herein, individual quercetin metabolites (i.e., sulfate, glucuronide or methyl conjugates) were identified by accurate mass MS in human plasma (females = 8 and males = 8) over 24 h after consumption of applesauce enriched with either micronized apple peel (AP) or onion powder (OP). The AP and OP contained ~180 μmol of quercetin glycosides. The relative amounts of quercetin metabolites were quantified in plasma. The complement of identified quercetin metabolites was similar after consumption of AP and OP. Primary metabolites included the following: quercetin sulfate, quercetin glucuronide, and quercetin diglucuronide. A quercetin glutathione adduct was identified in negative ion mode but not apparent in positive ion mode. The pharmacokinetic parameters for  $AUC_{0-24\text{ h}}$  and  $C_{\max}$  were significantly different for AP and OP. For example, consumption of the AP resulted in  $C_{\max}$  of quercetin sulfate, 4.6 ng/mL; quercetin glucuronide, 15.5 ng/mL; quercetin diglucuronide, 9.3 ng/mL; quercetin glucuronide sulfate, 1.3 ng/mL; methyl quercetin glucuronide, 7.5 ng/mL; and methyl quercetin diglucuronide, 3.6 ng/mL, whereas the OP resulted in  $C_{\max}$  of quercetin sulfate, 37.3 ng/mL; quercetin glucuronide, 212.8 ng/mL; quercetin diglucuronide, 168.8 ng/mL; quercetin glucuronide sulfate, 43.0 ng/mL; methyl quercetin glucuronide, 90.1 ng/mL; methyl quercetin diglucuronide, 65.4 ng/mL. Gender-related differences in the  $AUC_{0-24\text{ h}}$  for quercetin sulfate and quercetin sulfate glucuronide metabolites were also observed.

**KEYWORDS:** Apple, onion, quercetin, metabolite, pharmacokinetics, gender, LC-(ESI)MS/MS, accurate mass, UHPLC-(ESI)QTOF MS/MS

## INTRODUCTION

Among flavonoids, quercetin is of great interest, as it has antioxidative, anticarcinogenic, and anti-inflammatory effects.<sup>1</sup> Major dietary sources of quercetin include onions (28.4–48.6 mg/100 g) and apples (i.e., peel, 2.1–7.2 mg/100 g).<sup>1</sup> Most flavonoids are stored in plant cell vacuoles as the more soluble glycoside. The dominant type(s) of quercetin glycoside vary among species and cultivars. In apples, quercetin is present as a mixture of 3-O-galactoside, 3-O-glucoside, 3-O-rhamnoside, and 3-O-rutinoside, whereas it occurs primarily as the 4'-O-glucoside and 3,4'-O-diglucoside in onions.<sup>2,3</sup>

The glycoside compositions of a food can have important biological consequences. For example, the pharmacokinetics and bioavailability of flavonoids from different food sources can vary based upon the glycoside composition of the food.<sup>2,4,5</sup> Hollman et al. (1997) demonstrated that the bioavailabilities of quercetin from apples and the rutinoside were both 30% relative to that of fried onions.<sup>5</sup> Graefe et al. (2001) demonstrated that the pharmacokinetics and bioavailability of a purified quercetin 4'-O-glucoside was similar to consuming onion dominating in the 4'-O-glucoside,<sup>6</sup> again suggesting that the glycoside influences bioavailability to a greater extent than

the food matrix. More recently, Lee et al. (2012) demonstrated that the consumption of applesauce containing onions as a source of quercetin (~180 μmol of quercetin glycosides) led to 3-fold greater absorption of quercetin as compared with applesauce containing apple peel as a source of quercetin (~180 μmol of quercetin glycosides) in healthy humans.<sup>2</sup> The absorption of quercetin glycosides from the gastrointestinal tract involves deglycosylation by luminal lactase phlorizin hydrolase (EC 3.2.1.108; LPH) and/or cleavage within the enterocyte by β-glucosidases (EC 3.2.1.21).<sup>7,8</sup> The affinity of these enzymes for the various quercetin glycosides varies<sup>9</sup> and is therefore likely to affect their absorption and, thus, bioavailability.

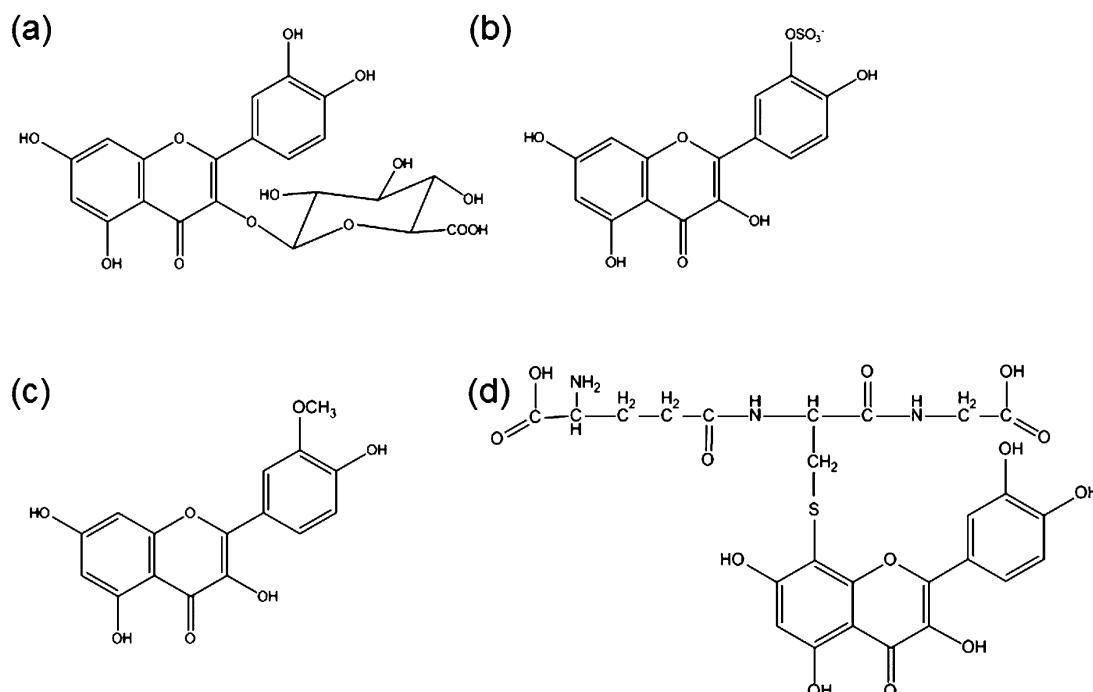
Biotransformation of quercetin appears to occur primarily in the enterocyte during absorption and also in the liver prior to systemic circulation,<sup>10</sup> and mixed conjugates are common.<sup>11,12</sup> Chemical structures of selected quercetin metabolites (i.e.,

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**Figure 1.** Chemical structures of selected quercetin metabolites, including (a) quercetin 3-O-glucuronide, (b) quercetin 3'-O-sulfate, (c) isorhamnetin, and (d) quercetin glutathione conjugate.

quercetin glucuronide, quercetin sulfate, isorhamnetin, and quercetin glutathione conjugate) are given in Figure 1. The primary enzymes involved in the biotransformation of quercetin include UDP glucuronyl transferase (EC 2.4.1.17; UGT), sulfotransferase (EC 2.8.2; SULT), and catechol O-methyl transferase (EC 2.1.1.6; COMT). The distribution of UGT and SULT enzyme systems varies throughout the gastrointestinal tract; therefore, the site of absorption of quercetin may influence the resulting metabolite profiles. In addition, colonic glucosidases may cleave the flavonoid ring, generating ring fission products such as hydroxyl phenylacetic acids.<sup>13</sup> The generation and biological fate of hydroxyl phenylacetic acids from flavonoids is not well understood.<sup>14</sup> However, in a recent study, several potential glutathione-related metabolites of quercetin were identified in human urine as mercapturic acids of common hydroxyphenylacetic acids.<sup>15</sup> Quercetin is a biologically active catechol, and one-electron reductions give rise to an *o*-quinone, followed by the isomerization of the *o*-quinone to its *p*-quinone methide isomer (a-b). Formation of *o*-quercetin quinone and quinone methide type metabolites is of interest in the context of genotoxicity and carcinogenicity. Several in vitro studies employing the quercetin aglycone report mutagenic activity<sup>16–19</sup> and the covalent binding of quercetin to cellular protein and DNA.<sup>20</sup> In vitro studies of the aglycone indicate that 6-glutathionyl-quercetin and 8-glutathionyl-quercetin adducts of the quinone intermediates can occur.<sup>21,22</sup> The toxicological risk associated with quercetin as a pro-oxidant in humans may be low due to the extensive metabolism of the catechol group of quercetin.<sup>23</sup> Additionally, it is difficult to extrapolate data from in vitro models employing the aglycone and relate it to humans consuming quercetin glycosides in the diet. Nonetheless, the metabolism of quercetin via glutathionylation is of toxicological interest in terms of the formation of quercetin quinones.

Considerable in vitro evidence indicates that quercetin metabolites have biological activity and that the biological

activity of these metabolites depends on the type and position of the conjugate group on the quercetin aglycone.<sup>24,25</sup> For example, the in vitro lag time of LDL oxidation was increased by quercetin-3-glucuronide significantly more than by quercetin aglycone, but it was not affected by equivalent concentrations of quercetin-3'-sulfate or isorhamnetin-3-glucuronide.<sup>25</sup> Xanthine oxidase was inhibited by quercetin glucuronides in the order 4' -> 3' -> 7 -> 3.-<sup>24</sup> Quercetin 4'-glucuronide inhibited xanthine oxidase at low micromolar concentration, as did quercetin aglycone, whereas the inhibitory constant ( $K_i$ ) of quercetin 3-sulfate was 300-fold higher than that of quercetin-4'-glucuronide.<sup>24</sup> These studies indicate that it is necessary to investigate qualitatively and quantitatively the range of quercetin metabolites in plasma after the consumption of defined glycoside combinations in order to understand how specific foods rich in different quercetin glycosides will influence health and biological pathways.

LC-(ESI)MS methods with quadrupole instruments in full scan mode have been used to successfully identify metabolites in plasma, but the sensitivity of these techniques does not allow for monitoring individual metabolites in plasma, beyond 6 h.<sup>11,12,26</sup> Typical LC-(ESI)MS/MS quantitation of quercetin metabolites has relied on the enzymatic cleavage of the glucuronide or sulfate conjugate and concomitant measurement of the quercetin aglycone and isorhamnetin (methyl-quercetin aglycone). Although this approach is useful in establishing pharmacokinetics, a great deal of information is lost regarding circulating metabolites.

Quadrupole MS/MS is typically used for targeted analysis and not the identification of unknowns. Elucidation of metabolite structure is highly facilitated by accurate mass MS/MS. This is especially true with complex mixtures of flavonoid metabolites, as commercial standards are not available. High-resolution MS such as quadrupole time-of-flight analysis (QTOF MS) provides the high sensitivity and high selectivity needed to distinguish among isobaric ions and

provide the molecular formula to aid in the identification of nontargeted and unknown compounds. Also, accurate mass analysis of product ions in MS/MS mode facilitates structural elucidation for identification. Combined with ultrahigh pressure liquid chromatography (UHPLC), high resolution MS generates information rapidly and efficiently.

In a previous study, we demonstrated that the bioavailability of an equivalent amount of total quercetin ( $\sim 180 \mu\text{mol}$  of quercetin glycosides) in healthy humans consumed from micronized onions was significantly higher than that consumed from micronized apple peels.<sup>2</sup> Holman et al. (1997) had shown this previously in ileostomy patients.<sup>5</sup> Possibilities for differential absorption include the following: different affinities of  $\beta$ -glycosidases or LPH for the various glycosides;<sup>27</sup> food matrix components influencing the absorption of quercetin glycoside;<sup>6</sup> or differences in the location of absorption of different glycosides in the gut. Any of these possibilities may affect both metabolism and the range of circulating metabolites.

The current study compares the pharmacokinetics of quercetin metabolites over 24 h in 16 participants (8 males and 8 females) postconsumption of a quercetin-free applesauce enriched with either micronized apple peel (AP) or onion (OP) using UHPLC-(ESI)QTOF MS. Gender differences in the pharmacokinetics of quercetin metabolites were also investigated.

## MATERIALS AND METHODS

**Chemicals and Reagents.** Quercetin 4'-O-glucoside was purchased from Extrasynthese (Genay, France). L-(+)-Ascorbic acid was obtained from ACROS Organics (Geel, Belgium). HPLC-grade methanol, 88% doubly distilled formic acid, and LC/MS grade acetonitrile were obtained from Fisher Scientific (Fair Lawn, NJ, USA), GFS Chemicals (Columbus, OH, USA), and Burdick and Jackson (Muskegon, MI, USA), respectively.

**Study Design.** The study protocol was approved by the Institutional Review Board of the University of California, Davis, and written informed consent was obtained from all participants. A randomized crossover study of 16 healthy volunteers (8 male and 8 female) was performed. The mean age was  $23.3 \pm 2.8$  year, and the mean body mass index was  $21.5 \pm 2.0 \text{ kg/m}^2$ .<sup>2</sup> The participants completed all study treatments, acted as their own controls, and entered the study in random order. The participants followed a low-flavonoid (quercetin-free) diet for 2 days prior to the study (washout period) and during the treatment day. After an overnight fast, either apple peel powder-enriched applesauce (AP) or onion powder-enriched applesauce (OP) was provided as breakfast. The dried apple peel or onion powders (85 g of apple peel or 47.5 g of onion), accounting for  $\sim 180 \mu\text{mol}$  of total quercetin glycosides, were mixed fresh daily with 100 g of applesauce and one cup of water. The quercetin glycoside content of these foods was determined by HPLC-(ESI) MS/MS as reported previously.<sup>2</sup> On the treatment day, a quercetin-free lunch and a snack were provided at 4 and 8 h, respectively, after consumption of each treatment. Blood samples were collected before (baseline) consumption of the foods and 0.5, 1, 2, 4, 6, 8, and 24 h after consumption. Blood samples were centrifuged, and plasma was separated from blood cells. Aliquots of plasma were stored at  $-80^\circ\text{C}$  until analysis.

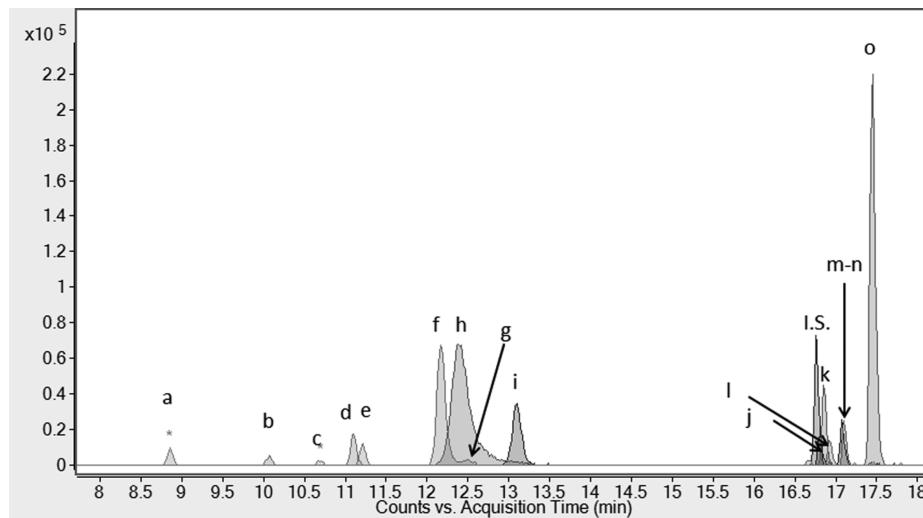
**Extraction of Quercetin Metabolites in Plasma.** The extraction method was modified from the method of Mullen et al.<sup>12,28</sup> Recovery was measured using quercetin 4'-O-glucoside as an internal standard (IS) added to baseline plasma (at final

concentrations of 10 and  $1000 \text{ ng/mL}$  plasma). Mullen reported recoveries of 85–87% using an acetonitrile extraction followed by a methanol extraction. This method was modified herein by adding ascorbic acid to plasma prior to extraction and employing methanol for both extractions. Briefly, a  $500 \mu\text{L}$  aliquot of plasma was mixed with  $50 \mu\text{L}$  of 0.1 M ascorbic acid, 0.1 ng of quercetin 4'-O-glucoside, and 1.5 mL of methanol. Quercetin 4'-O-glucoside was used as an IS because, in previous studies, it was not found in plasma postconsumption of quercetin glycosides or quercetin glycoside-rich foods.<sup>29,30</sup> The mixture was vortexed for 30 s every 2 min for 5 times and centrifuged at  $4000\text{g}$  at  $4^\circ\text{C}$  for 10 min. The supernatant was collected, and the pellet was extracted again as described above. The supernatants were combined and evaporated in vacuo, and reconstituted in  $250 \mu\text{L}$  of initial mobile phase (0.1% formic acid in 95% water and 5% acetonitrile). The extract was centrifuged at  $4^\circ\text{C}$  for 10 min and filtered through a  $0.2 \mu\text{m}$  filter prior to LC-(ESI)-MS/MS analysis. Measured recoveries ranged from 87 to 108%. All samples were analyzed in duplicate.

**UHPLC-(ESI)QTOF MS/MS Analysis.** Quercetin metabolite analysis was performed on an Agilent 1290 Infinity ultrahigh pressure liquid chromatography system coupled to a 6530 accurate mass quadrupole time-of-flight mass spectrometer (UHPLC-(ESI)QTOF MS/MS) with electrospray ionization (ESI) via Jet Stream Technology (Agilent Technologies, Santa Clara, CA, USA). The UHPLC was equipped with a binary pump with an integrated vacuum degasser (G4220A), an autosampler (G4226A) with a thermostat (G1330B), and a thermostated column compartment (G1316C). Quercetin metabolites were separated with a Poroshell C<sub>18</sub> column (2.1  $\times$  100, 2.7  $\mu\text{m}$ , Agilent Technologies) with a C<sub>18</sub> guard column (2.1 mm  $\times$  5 mm, 1.8  $\mu\text{m}$ , Optimize Technologies, Inc.) maintained at  $30^\circ\text{C}$ . The mobile phase consisted of a linear gradient of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) as follows: 5–10% B, 0–5 min; 10–12% B, 5–8 min; 12–15% B, 8–10 min; 15% B, 10–15 min; 15–55% B, 15–18 min; 55–90% B, 18–20 min. The column was re-equilibrated between injections for 4 min with the initial mobile phase. The flow rate was 0.4 mL/min, and the injection volume was  $5 \mu\text{L}$ .

To identify all possible quercetin metabolites, total ion spectra were collected over a mass range of  $m/z$  100–1000 in both negative and positive modes at an acquisition rate of 1.0 spectra/s. The drying gas temperatures and flow rate were  $225^\circ\text{C}$  and 8.0 L/min, respectively. The sheath gas temperature and flow rate were  $300^\circ\text{C}$  and 10.0 L/min, respectively. The nebulizer gas pressure, skimmer voltage, octopole RF, and fragmentor voltage were 45 psi, 65 V, 750 V, and 125 V, respectively. The capillary voltage was 2.5 kV (negative) or 3.5 kV (positive). Continuous internal calibration was performed during analysis to achieve the desired mass accuracy of recorded ions with ions of  $m/z$  of 119.0363 (proton abstracted purine) and 966.0007 (formate adduct of protonated hexakis (1H, 1H, 3H-tetrafluoropropoxy)phosphazine or HP-921) in negative mode and with ions of  $m/z$  of 121.0509 (protonated purine) and 922.0098 (HP-921) in positive mode. The peaks of eluents corresponding to possible quercetin metabolites were further investigated with negative UHPLC-(ESI)QTOF MS/MS by applying the optimum collision energy for different analytes.

Relative quantification of each quercetin metabolite was performed in the QTOF in the MS<sup>1</sup> mode (QTOF) using



**Figure 2.** Representative UHPLC-(ESI)QTOF MS extracted-ion chromatograms of the quercetin metabolites in human plasma 2 h after the consumption of OP at negative ion mode. Peaks: a, methyl quercetin diglucuronide; b, quercetin diglucuronide; c, methyl quercetin diglucuronide; d, quercetin diglucuronide; e, methyl quercetin diglucuronide; f, quercetin diglucuronide; g, quercetin diglucuronide; h, quercetin glucuronide sulfate; i, quercetin 3-O-glucuronide; j, quercetin 3'-O-glucuronide; k, methyl quercetin 3-O-glucuronide; l, quercetin glutathione; m, quercetin glucuronide; n, methyl quercetin glucuronide; o, quercetin 3-O-sulfate.

**Table 1. Tentative Assignments of Quercetin (Q) Metabolites in Plasma ( $n = 16$ ) Postconsumption of Applesauce with Added Apple Peel or Onion as a Source of Quercetin Glycosides<sup>a</sup>**

peak	RT (min)	possible compds	type of mol ion	predicted $m/z$	obsd $m/z$	error (ppm)
a	8.86	methyl Q <sup>a</sup> diglucuronide	[M - H] <sup>-</sup>	667.1152	667.1146	0.9
b	10.07	Q diglucuronide	[M - H] <sup>-</sup>	653.0996	653.0987	1.4
c	10.70	methyl Q diglucuronide	[M - H] <sup>-</sup>	667.1152	667.1136	2.4
d	11.10	Q diglucuronide	[M - H] <sup>-</sup>	653.0996	653.0986	1.5
e	11.22	methyl Q diglucuronide	[M - H] <sup>-</sup>	667.1152	667.1147	0.6
f	12.18	Q diglucuronide	[M - H] <sup>-</sup>	653.0996	653.0989	1.1
g	12.32	Q diglucuronide	[M - H] <sup>-</sup>	653.0996	653.0989	1.1
h	13.38	Q glucuronide sulfate	[M - H] <sup>-</sup>	557.0243	557.0240	0.5
i	13.10	Q 3-glucuronide	[M - H] <sup>-</sup>	477.0675	477.0671	0.8
j	16.80	Q 3'-glucuronide	[M - H] <sup>-</sup>	477.0675	477.0673	0.4
k	16.85	methyl Q 3-glucuronide	[M - H] <sup>-</sup>	491.0831	491.0832	-0.2
l	16.90	Q glutathione	[M + HCOO] <sup>-</sup>	651.1012	651.0998	2.2
m	17.07	Q glucuronide	[M - H] <sup>-</sup>	477.0675	477.0672	0.6
n	17.10	methyl Q glucuronide	[M - H] <sup>-</sup>	491.0831	491.0831	0.0
o	17.45	Q 3-sulfate	[M - H] <sup>-</sup>	380.9922	380.9925	-0.8

<sup>a</sup>Q, quercetin. <sup>a</sup>Assignments are based upon negative mode ESI and accurate mass QTOF MS/MS data.

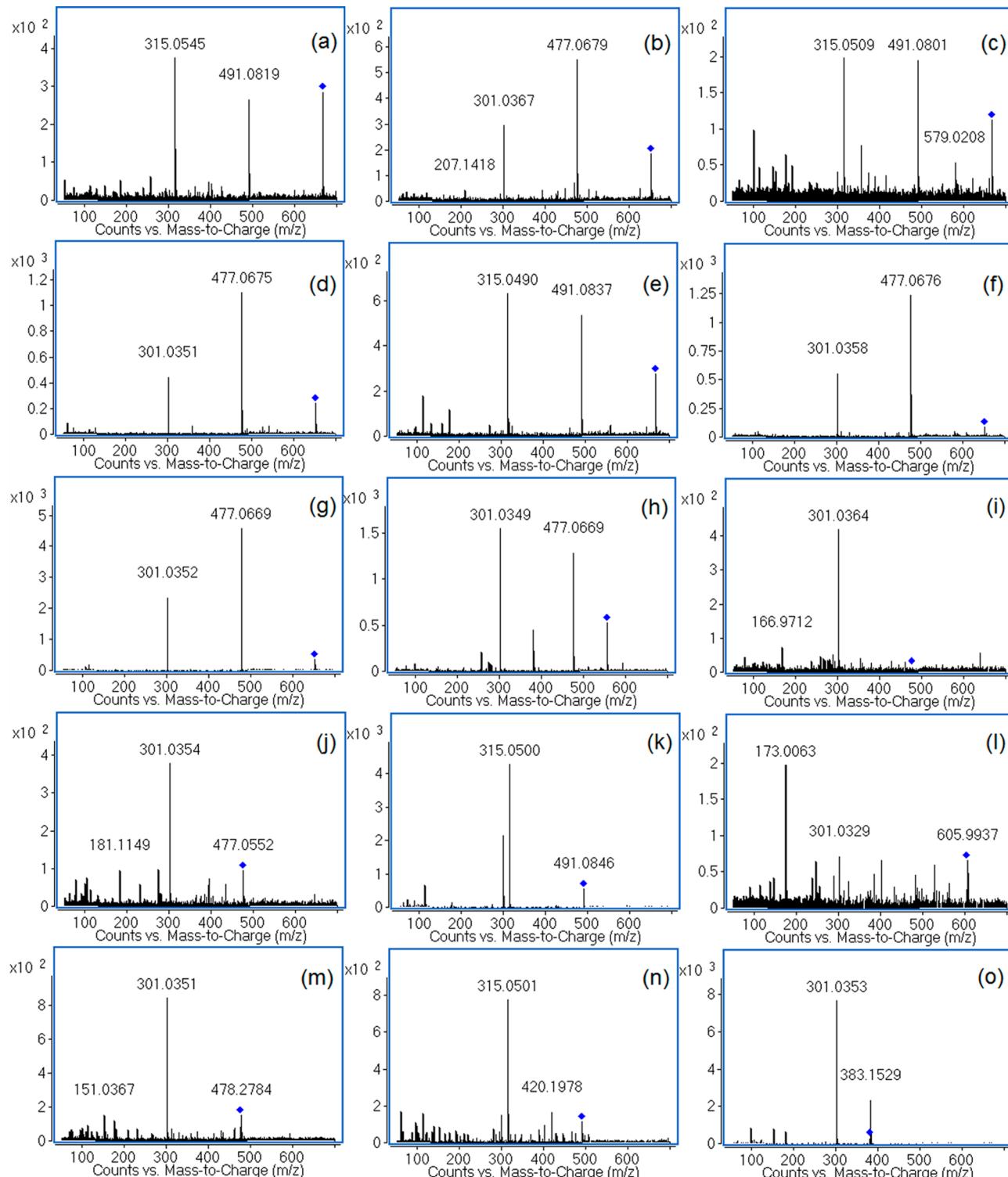
quercetin 4'-O-glucoside as an IS. Calibration was achieved using the standard addition method of spiking known amounts of stock standard to pooled plasma. The standards were extracted by the same method as used for samples. The limit of IS detection was 1 ng/mL plasma, and the linear dynamic range spanned 3 orders of magnitude. The quality control samples were prepared at the concentrations 20 and 100 ng IS/mL plasma.

**Data Analysis.** An accurate mass database of all potential flavonoid metabolites was built using a personal compound database library (PCDL) manager (Agilent Technologies). This library included quercetin metabolites previously reported in the literature and based upon calculated masses of possible metabolites obtained from known biotransformation pathways. The exact mass for each possible metabolite was calculated based on metabolite formula. This database was imported into MassHunter Qualitative Analysis Software (Agilent Technologies) and used to search the MS datafile obtained for each

plasma sample. Samples were analyzed in the QTOF in MS<sup>1</sup> mode. Potential quercetin-related metabolites in plasma were identified based on comparison of accurate mass, abundance of the isotopes, and isotope spacing to the calculated theoretical masses and abundances (performed automatically in Mass-Hunter Qualitative Analysis). Tandem MS (targeted MS<sup>2</sup>) was subsequently used for each quercetin metabolite identified by database matching.

**Pharmacokinetics.** Pharmacokinetic variables measured included peak plasma quercetin concentration ( $C_{\max}$ ), time to reach  $C_{\max}$  ( $t_{\max}$ ), and area under the plasma concentration–time curve from 0 to 24 h ( $AUC_{0-24\text{ h}}$ ).  $AUC_{0-24\text{ h}}$  was calculated using the trapezoidal rule. The calculation was performed using PK Function add-ins (Allergan, Irvine, CA) for Microsoft Excel (Microsoft Corp., Redmond, WA).

**Statistical Analysis.** Statistical analysis was performed using IBM SPSS statistics software (v. 20.0, SPSS, Inc., Chicago, IL). Significant differences in plasma pharmacokinetic

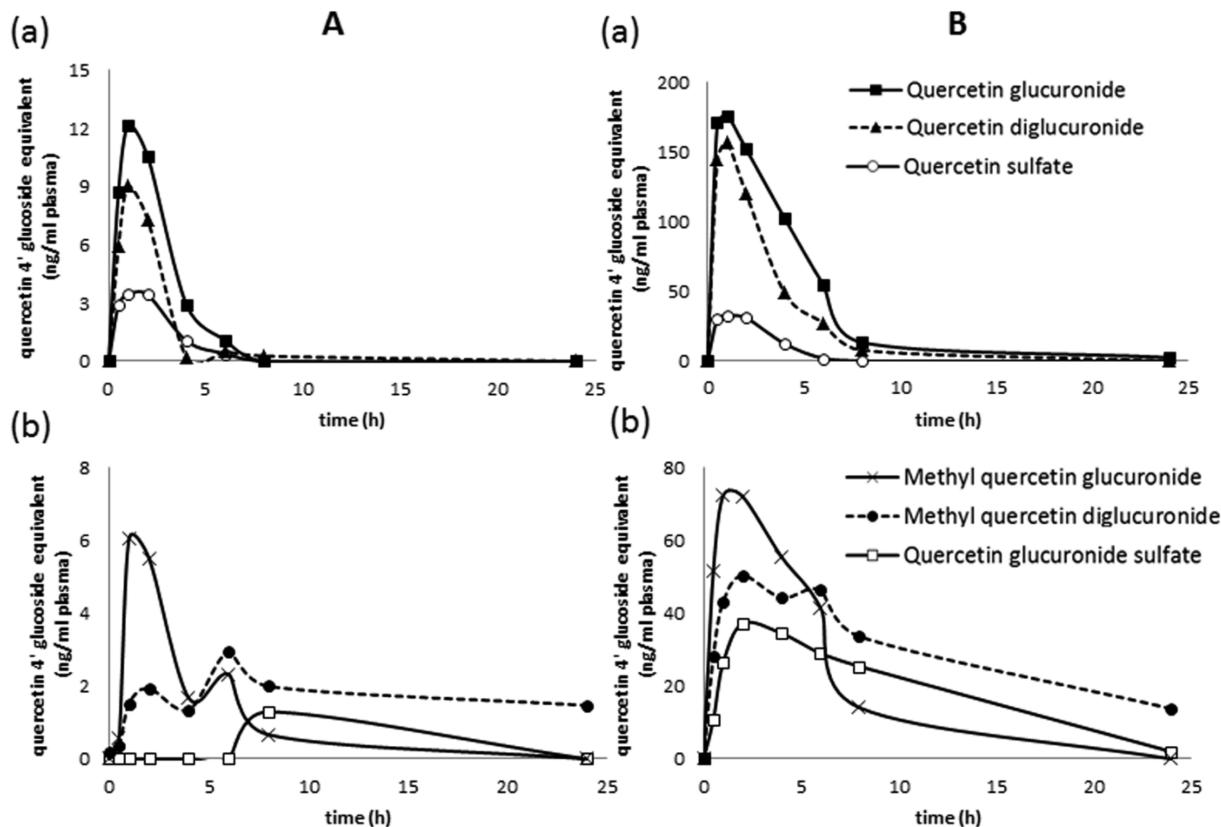


**Figure 3.** UHPLC-(ESI)QTOF MS spectra of chromatographic peaks obtained from human plasma extract. A blue diamond indicates the pseudomolecular ion of precursor ions. (a) Peak 1, methyl quercetin diglucuronide; (b) peak 2, quercetin diglucuronide; (c) peak 3, methyl quercetin diglucuronide; (d) peak 4, quercetin diglucuronide; (e) peak 5, methyl quercetin diglucuronide; (f) peak 6, quercetin diglucuronide; (g) peak 7, quercetin diglucuronide; (h) peak 8, quercetin glucuronide sulfate; (i) peak 9, quercetin 3-O-glucuronide; (j) peak 10, quercetin 3'-O-glucuronide; (k) peak 11, methyl quercetin 3-O-glucuronide; (l) peak 12, quercetin glutathione; (m) peak 13, quercetin glucuronide; (n) peak 14, methyl quercetin glucuronide; (o) peak 15, quercetin 3-O-sulfate.

parameters among food treatments were determined using a paired *t*-test at  $P < 0.05$ . Gender differences for plasma pharmacokinetic parameters were evaluated with an independent *t*-test at  $P < 0.05$ .

## RESULTS AND DISCUSSION

The quercetin glycosides composition in standardized AP and OP were characterized and quantified by LC-(ESI)-MS/MS as described previously.<sup>2</sup> Each treatment in the present study



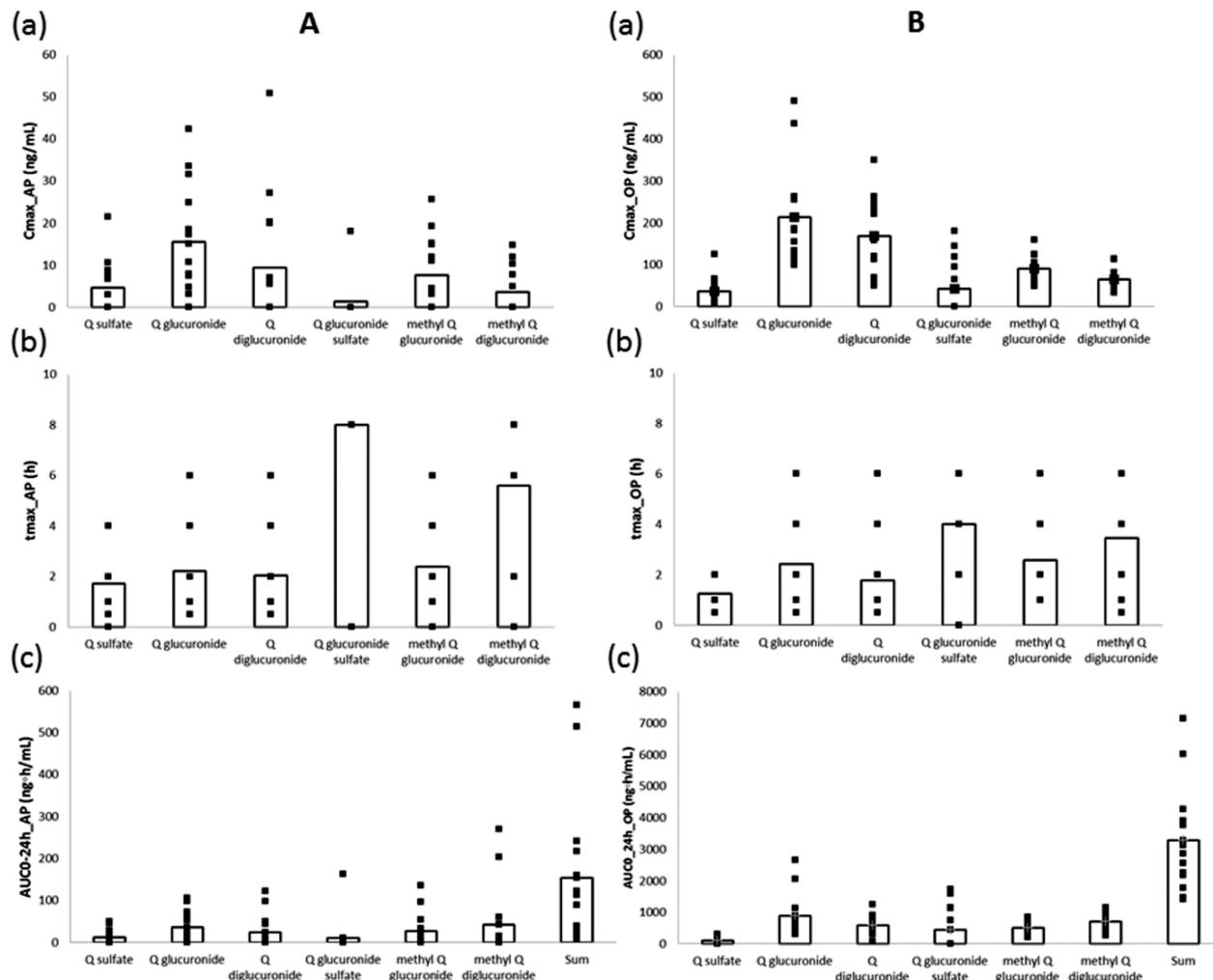
**Figure 4.** Individual quercetin metabolite concentration–time profiles after oral consumption of applesauce fortified with  $\sim 180 \mu\text{mol}$  quercetin glycosides from either (A) apple peel or (B) onion. (a) Concentration of quercetin sulfate, quercetin glucuronide, and quercetin diglucuronide; (b) concentration of methyl quercetin glucuronide and methyl quercetin diglucuronide, and quercetin glucuronide sulfate [y-axes for apple peel, 0–15 ng (IS equivalent)/mL plasma; y-axes for onion, 0–200 ng (IS equivalent)/mL plasma].

delivered  $\sim 180 \mu\text{mol}$  of total quercetin glycosides (100 mg of total quercetin glycosides). In OP, quercetin was present primarily as the 4'-O-glucoside ( $44.9 \pm 1.3 \text{ mg}$ ;  $96.7 \pm 2.8 \mu\text{mol}$ ) and 3,4'-O-diglucoside ( $48.7 \pm 0.8 \text{ mg}$ ;  $77.7 \pm 1.3 \mu\text{mol}$ ), whereas it occurred as a mixture of O-arabinopyranose ( $17.6 \pm 0.1 \text{ mg}$ ;  $40.5 \pm 0.2 \mu\text{mol}$ ), 3-O-galactoside ( $16.8 \pm 0.2 \text{ mg}$ ;  $36.2 \pm 0.4 \mu\text{mol}$ ), 3-O-glucoside ( $16.1 \pm 0.1 \text{ mg}$ ;  $34.7 \pm 0.2 \mu\text{mol}$ ), and 3-O-rhamnoside ( $30.6 \pm 2.4 \text{ mg}$ ;  $68.2 \pm 5.4 \mu\text{mol}$ ) in the AP.

UHPLC-(ESI)QTOF MS was used for identification and relative quantification of the range of quercetin metabolites in the plasma samples. A representative extracted ion chromatogram of the quercetin metabolites identified in human plasma 2 h after the consumption of OP is given in Figure 2. The peaks with  $m/z$  values corresponding to quercetin metabolites based upon calculated masses were further identified by QTOF MS/MS. Quercetin has a calculated monoisotopic mass of 302.0427 Da. All quercetin-related metabolites produce a fragment ion at  $m/z$  301.0354 corresponding to quercetin without the conjugate [ $M - \text{conjugate}$ ] $^-$ . The exception is methylated quercetin-related metabolites, which produce fragment ions at  $m/z$  315.0510 corresponding to the isorhamnetin ion without the conjugate [ $M - \text{conjugate}$ ] $^-$ . For example, the QTOF MS/MS spectra of quercetin glucuronide demonstrate characteristic fragment ions at  $m/z$  of 301.0354, 178.9986, and 151.0037 (corresponding to quercetin fragmentation) and at  $m/z$  113.0244 for the glucuronide moiety.

Using this approach, fifteen quercetin metabolites, including glucuronide, sulfate, methyl, glutathione, and mixed conjugates

were identified in plasma after the consumption of the AP or OP (Table 1). The differences between predicted and observed  $m/z$  values were less than 2.5 ppm. Peaks 1, 3, and 5 (Figure 2) were identified as methyl quercetin diglucuronide isomers. These peaks produced a strong pseudomolecular ion at  $m/z$  667.1152 [ $M - H$ ] $^-$  and fragment ions at  $m/z$  491.0831 [ $M - H - 176.0326$ ] $^-$  and  $m/z$  315.0510 [ $M - H - 176.0326 - 176.0326$ ] $^-$ , corresponding to the loss of two molecules of glucuronide (Figure 3a, c, and e). Four quercetin diglucuronides (peaks 2, 4, 6, and 7) were identified by monitoring a strong pseudomolecular ion at  $m/z$  at 653.0996 [ $M - H$ ] $^-$ . Fragment ions, resulting from the loss of two glucuronide moieties, were observed at  $m/z$  477.0675 [ $M - H - 176.0326$ ] $^-$  and  $m/z$  301.0354 [ $M - H - 176.0326 - 176.0326$ ] $^-$  (Figure 3b, d, f, and g). A quercetin glucuronide sulfate (peak 8) gave a pseudomolecular ion at  $m/z$  557.0243. The MS/MS spectrum featured characteristic ions of  $m/z$  477.0675 and 301.0354, derived from the loss of sulfate and glucuronide, respectively (Figure 3h). Peaks 9, 10, and 13 were identified as quercetin 3-O-glucuronide, quercetin 3'-O-glucuronide, and quercetin glucuronide, respectively, by monitoring ions corresponding to the [ $M - H$ ] $^-$  at  $m/z$  477.0675. The precursors produced fragment ions at  $m/z$  301.0354 [ $M - H - 176.0326$ ] $^-$ , corresponding to loss of a glucuronide (Figure 3i, j, and m). The position of the glucuronide group of peaks 9 and 10 was identified by comparing the HPLC retention time and MS/MS with authentic quercetin metabolite standards. Two peaks (11 and 14) had an [ $M - H$ ] $^-$  at  $m/z$  491.0831 and a predominant



**Figure 5.** Plasma pharmacokinetic parameters for quercetin (Q) metabolites postconsumption of applesauce enriched with either (A) apple peel or (B) onion ( $n = 16$ ). (a)  $C_{\max}$  peak plasma quercetin concentration [y-axes for apple peel, 0–60 ng (IS equivalent)/mL plasma; y-axes for onion, 0–600 ng/mL plasma]; (b)  $t_{\max}$  time to reach  $C_{\max}$ ; (c)  $AUC_{0-24\text{ h}}$  area under the plasma concentration–time curve from 0 to 24 h [y-axes for apple peel, 0–600 ng·h/mL plasma; y-axes for onion, 0–8000 ng·h/mL plasma].

fragment ion at  $m/z$  315.0510 [ $M - H - 176.0326$ ] $^-$ , corresponding to methylated quercetin glucuronides (Figure 3k and n). The identification of peak 11 as methyl quercetin 3-O-glucuronide was confirmed using an authentic standard. Quercetin glutathione was identified in plasma in negative ionization mode (peak 12) by observing ions at  $m/z$  651.1012 [ $M + \text{HCOO}$ ] $^-$  and at  $m/z$  605.0957 [ $M - H$ ] $^-$ , and a fragment ion at  $m/z$  301.0354 [ $M - H - 304.0609$ ] $^-$  (quercetin) (Figure 3l). This peak was not visible in positive ion mode. Comparisons with an authentic standard indicated that peak 15 corresponded to quercetin 3-sulfate. The MS/MS spectra demonstrated an ion at  $m/z$  301.0354 [ $M - H - 79.9574$ ] $^-$  corresponding to the loss of a sulfate group (Figure 3o).

To date, a range of quercetin metabolites have been described in biological fluids<sup>31–33</sup> with the identification relying primarily on enzymatic hydrolysis of the conjugating moiety. In studies of rats fed quercetin 4'-O-glucoside, 10 quercetin metabolites were identified in plasma, whereas 17 metabolites were identified in the intestine.<sup>34</sup> Twenty-one flavonol-related metabolites were identified in the urine of humans that had consumed lightly sautéed onions; however, plasma metabolites were not identified in this study.<sup>11</sup> More recently, five quercetin

metabolites (i.e. quercetin-3-sulfate, quercetin-3-glucuronide, isorhamnetin-3-glucuronide, a quercetin diglucuronide, and a quercetin glucuronide sulfate) were detected in human plasma in quantifiable amounts for up to 6 h after ingestion of onions.<sup>12</sup> Trace levels of six additional quercetin metabolites were also observed in this study.<sup>12</sup> There are several analytical challenges associated with quantifying individual quercetin metabolites in plasma, including a lack of commercially available standards and, importantly, instrument sensitivity.

Quercetin metabolite levels decrease rapidly in plasma over the first 6 h and present analytical challenges in terms of monitoring individual metabolites in plasma. Previously, Mullen et al.<sup>12</sup> was able to monitor five quercetin metabolites in plasma for 6 h after the consumption of onions.<sup>12</sup> No detectable quercetin metabolites were present in these samples at 24 h. Although Mullen et al.<sup>12</sup> provided a far more detailed description of the fate of quercetin metabolites than previous studies; data were limited by the detection limits of the methodology available. In the present study, it was possible to obtain more detailed insight into the metabolic fate of quercetin glucosides compared to previous studies for several reasons. First, modifications in extraction method resulted in an improvement in measured recoveries of 87–108%, but more

**Table 2.** Plasma Pharmacokinetic Parameters for Quercetin (Q) Metabolites Postconsumption of Applesauce Enriched with Either Apple Peel Powder (AP) or Onion Powder (OP) ( $n = 16$ )<sup>a</sup>

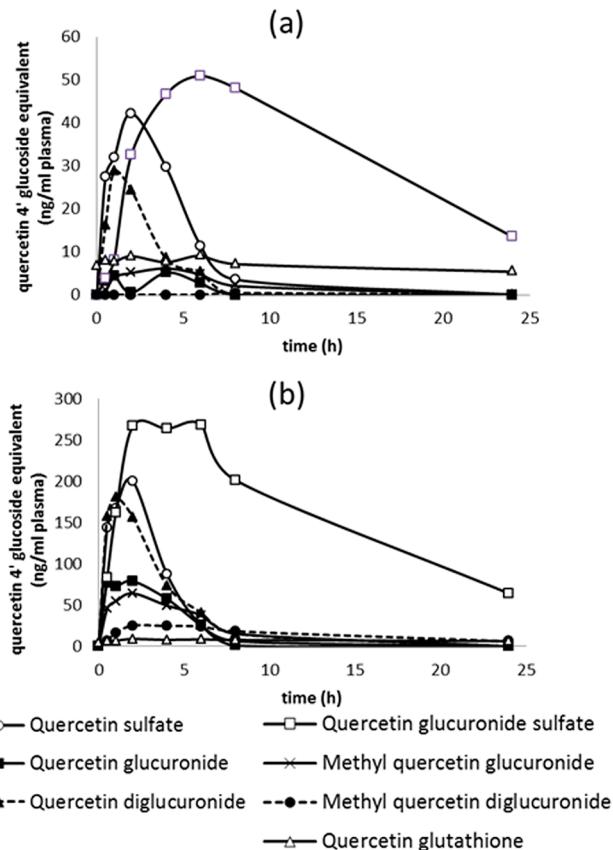
		$C_{\max}$ (ng/mL)	$t_{\max}$ (h)	$AUC_{0-24\text{ h}}$ (ng·h/mL)
AP				
Q <sup>b</sup> sulfate		4.6 ± 6.3	1.7 ± 1.2	12.0 ± 17.3
Q glucuronide		15.5 ± 13.3	2.2 ± 1.6	37.3 ± 36.9
Q diglucuronide		9.3 ± 15.2	2.0 ± 1.6	24.4 ± 40.6
Q glucuronide sulfate		1.3 ± 4.8	8.0 ± 0.0	11.6 ± 43.6
methyl Q glucuronide		7.5 ± 8.7	2.4 ± 1.8	27.2 ± 41.8
methyl Q diglucuronide		3.6 ± 5.4	5.6 ± 2.2	42.6 ± 85.8
Sum				155.1 ± 179.7
OP				
Q sulfate		37.3 ± 31.2 <sup>d</sup>	1.3 ± 0.7	113.9 ± 86.7 <sup>d</sup>
Q glucuronide		212.8 ± 120.1 <sup>d</sup>	2.4 ± 1.9	897.9 ± 656.3 <sup>d</sup>
Q diglucuronide		168.8 ± 93.1 <sup>d</sup>	1.8 ± 1.6	591.0 ± 293.5 <sup>d</sup>
Q glucuronide sulfate		43.0 ± 64.7 <sup>c</sup>	4.0 ± 1.4	453.6 ± 668.4 <sup>c</sup>
methyl Q glucuronide		90.1 ± 31.0 <sup>d</sup>	2.6 ± 2.0	509.6 ± 204.3 <sup>d</sup>
methyl Q diglucuronide		65.4 ± 25.6 <sup>d</sup>	3.5 ± 2.2	717.2 ± 284.0 <sup>d</sup>
Sum				3283.1 ± 1665.3 <sup>d</sup>

<sup>a</sup>Values are mean ± SD.  $C_{\max}$ , peak plasma quercetin concentration;  $t_{\max}$ , time to reach  $C_{\max}$ ;  $AUC_{0-24\text{ h}}$ , area under the plasma concentration–time curve from 0 to 24 h. <sup>b</sup>Q, quercetin. <sup>c</sup>Significantly different between treatments (AP and OP) at  $P < 0.05$ . <sup>d</sup>Significantly different between treatments (AP and OP) at  $P < 0.001$ .

importantly, the UHPLC-(ESI)QTOF MS/MS method used herein provided a significant improvement in both the sensitivity and selectivity, as compared with previous HPLC-(ESI)MS/MS and HPLC (ESI) ion trap methods. The improved sensitivity of the QTOF MS method enable the kinetic monitoring of individual quercetin metabolites over 24 h, extending the kinetic studies of Muelen et al.<sup>12</sup>

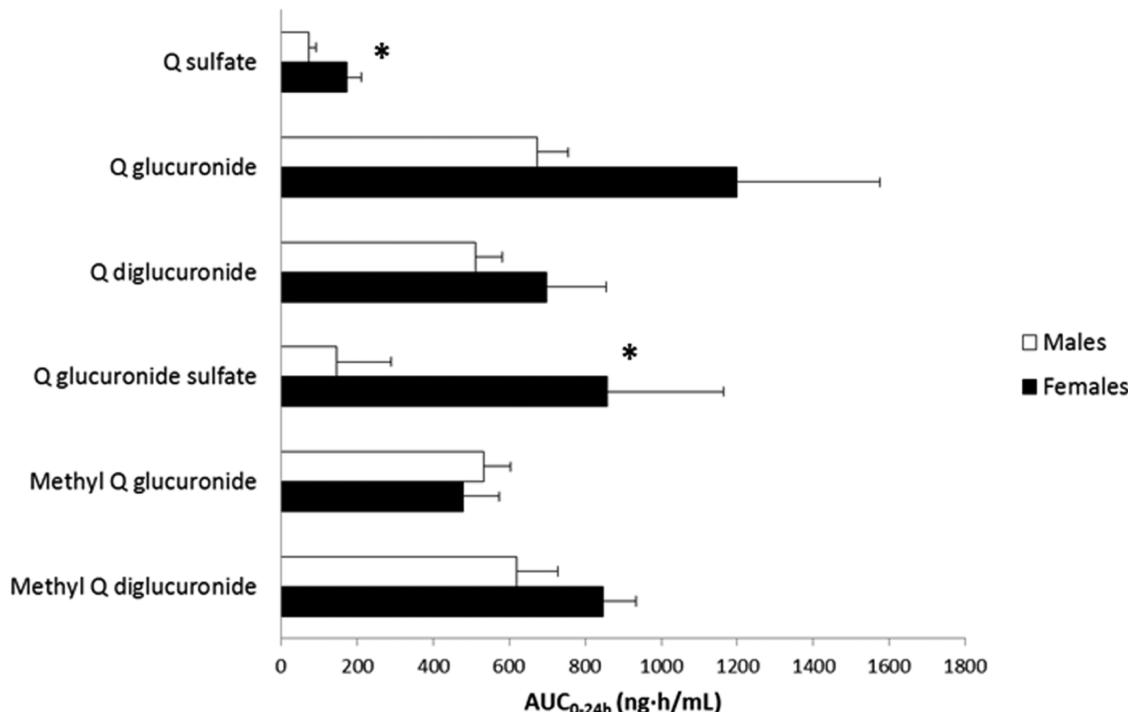
To simplify data presentation, the fifteen identified quercetin metabolites were grouped into seven categories by combining isomers. These categories include the following: quercetin sulfate, quercetin glucuronide, quercetin diglucuronide, quercetin glucuronide sulfate, quercetin glutathione, methyl quercetin glucuronide, and methyl quercetin diglucuronide metabolites. Quantitation was performed for each group relative to the quercetin 4'-O-glucoside (IS).

The time course of the individual quercetin metabolites identified in plasma by positive mode ESI was followed for 24 h after consumption of the AP and OP as shown in Figure 4. The metabolites identified were similar following consumption of either the AP or OP; however, the plasma concentrations of the individual quercetin metabolites were significantly higher after the consumption of OP compared to consumption of AP. After the consumption of either AP or OP, the major quercetin metabolites identified included the following: quercetin glucuronide, quercetin diglucuronide, and methyl quercetin glucuronide. The range of metabolites identified herein is



**Figure 6.** Individual quercetin metabolite concentration–time profiles after consumption of applesauce fortified with ~180 μmol of quercetin glycosides from either (a) apple peel or (b) onion: quercetin sulfate, quercetin glucuronide, quercetin diglucuronide, quercetin glucuronide sulfate, quercetin glutathione, methyl quercetin glucuronide, and methyl quercetin diglucuronide in ESI negative [y-axes for apple peel, 0–60 ng (IS equivalent)/mL plasma; y-axes for onion, 0–300 ng (IS equivalent)/mL plasma].

similar to metabolites reported in previous studies following the consumption of onions by humans and rats.<sup>12,35</sup> It is likely that the range of quercetin metabolites will be similar after the consumption of most foods rich in quercetin and that the overall levels of the metabolites will be dependent upon the glycoside composition of the food. Herein, the quercetin diglucuronide concentration was lower than the quercetin glucuronide concentration over the 24 h period following either AP or OP consumption. Twenty four hours after the consumption of OP, the methyl quercetin diglucuronide concentration (13.8 ± 9.2 ng/mL) was significantly higher than that at 0 h (before consumption; 0.2 ± 0.7 ng/mL) ( $P < 0.001$ ). Interestingly, an obvious three-phase sequential phase absorption phase was noted for the methyl quercetin glucuronide (after consumption of OP) and methyl quercetin diglucuronide (after consumption of AP and OP). In a previous study, three-sequential quercetin absorption phases were found in 40% of the study participants after consuming the OP.<sup>2</sup> This second absorption phase associated with the methyl metabolites may reflect enterohepatic recirculation. Methylation and conjugation with glucuronic acid would result in metabolites with sufficient molecular weight for biliary excretion (491 Da). This observation may also potentially be explained by the liberation of quercetin glycosides by colonic bacteria, methylation in the gut, and delayed absorption.



**Figure 7.** Gender differences in the plasma pharmacokinetics of quercetin metabolites postconsumption of applesauce enriched with onion powder (OP). AUC<sub>0-24 h</sub>, area under the plasma concentration–time curve from 0 to 24 h; \*, significantly different between genders at  $P < 0.05$ ; Q, quercetin. Values are mean  $\pm$  SE.

The pharmacokinetic parameters between OP and AP were significantly different (Figure 5; Table 2). The pharmacokinetic parameters were calculated based on positive mode ESI. Consumption of OP led to significantly greater concentrations of metabolites in plasma ( $C_{\max}$ ) as compared with the AP ( $P < 0.05$ ). The total absorption of quercetin glycosides as determined from the area under the plasma concentration–time curve was significantly higher in the OP (AUC<sub>0-24 h</sub> =  $3283.1 \pm 1665.3$  ng·h/mL) as compared with the AP (AUC<sub>0-24 h</sub> =  $155.1 \pm 179.7$  ng·h/mL) ( $P < 0.05$ ). The enzymatic hydrolysis of the glycosidic moiety is required for intestinal absorption.<sup>7,30</sup> Our data suggests that the different affinities of intestinal glycosylase enzymes for the various quercetin glycosides may affect the absorption of quercetin and thus the range and concentration of metabolites circulating in plasma. Apple peel contains the flavonoid phlorizin (phloretin glucoside) at levels between 80 and 420 mg/kg depending upon variety.<sup>36</sup> Phlorizin is hydrolyzed to phloretin by LPH in the brush border of the small intestines.<sup>37</sup> Therefore, another possible explanation for the lower absorption from apples is through the competitive inhibition of LPH by phlorizin.

The time to reach the  $t_{\max}$  of all quercetin metabolites was not significantly different between the AP and OP treatment groups, with the exception of methyl quercetin diglucuronide, which was slightly higher postconsumption of AP compared to that of OP, although it was not significantly different between the two treatments (calculated  $P = 0.053$ ). This is the first report of the  $t_{\max}$  for methyl quercetin diglucuronide. The later  $t_{\max}$  observed for glucuronide sulfate (4.0–8.0 h) and methyl quercetin diglucuronide (3.5–5.6 h) indicated that these metabolites formed more slowly than quercetin glucuronide and quercetin sulfate ( $t_{\max}$  of 1.3–2.6 h). This result suggests that when the absorption of quercetin glycosides is from the lower intestine (slower), mixed conjugates may predominate.

The  $t_{\max}$  of quercetin sulfate ( $t_{\max}$  of 1.3–1.7 h) was shorter than  $t_{\max}$  of quercetin glucuronide ( $t_{\max}$  of 2.2–2.4 h). In general, SULTs are considered low capacity–high specificity enzymes that are efficient at low substrate concentrations, whereas UGTs are high capacity–low specificity enzymes that are more efficient when SULTs are saturated.<sup>38</sup> The difference in the  $t_{\max}$  between these two metabolites may reflect saturation of the SULTs.

The average  $t_{\max}$  values of methyl quercetin glucuronide ( $5.2 \pm 1.1$  h) and methyl quercetin diglucuronide ( $6.0 \pm 0.0$  h) were shorter in participants displaying three-sequential quercetin absorption phases as compared to participants with two-sequential quercetin absorption phases ( $t_{\max}$  of methyl quercetin glucuronide  $1.4 \pm 0.5$  h and  $t_{\max}$  of methyl quercetin diglucuronide  $2.1 \pm 1.2$  h).

Because authentic standards are not currently commercially available for most metabolites and most are not chemically stable, differences in the ionization efficiency of each individual quercetin metabolite could not be evaluated and corrected for absolute quantification. Herein we observed that the polarity used for ESI had a significant effect on the ionization efficiency of individual quercetin metabolites. For example, the predominant metabolites in positive mode ESI were quercetin glucuronide and quercetin diglucuronide (Figure 4) whereas quercetin glucuronide sulfate was the predominant metabolite in negative mode ESI (Figure 6). Mullen et al. (2006) reported that the predominant quercetin metabolites in human plasma after ingestion of onions were the quercetin-3'-sulfate, quercetin-3-glucuronide, and to a lesser extent quercetin glucuronide sulfate, isorhamnetin-3-glucuronide, and a quercetin diglucuronide.<sup>12</sup> In this study, negative mode ionization was used and authentic standards were only available for the quercetin-3-sulfate, quercetin-3-glucuronide, quercetin-3'-glucuronide, and methyl quercetin-3-glucuronide. Interestingly, a

potential quercetin glutathione (GSH) adduct was detected in negative ESI mode at  $m/z$  651.0998 but not in positive ESI mode. Although accurate mass data was obtained, and identification confirmed through QTOF MS/MS (Figure 3*l*), confirmation of this adduct remains to be achieved with an authentic standard. GSH is known to react easily with quinoid compounds, and this reaction would be an expected detoxification pathway for quercetin. This putative Q-GSH conjugate was present at T = O and persisted in the 5–7 ng/mL range throughout the 24-h sampling period. Previous investigations with chemical systems have shown that quercetin is capable of reaction with GSH to generate mono- and diglutathionyl adducts.<sup>22,39–41</sup>

To our knowledge, this is the first report of a glutathione conjugate of quercetin detected in human plasma. The fact that the ionization mode of the instrument greatly influences the ionization of individual metabolites greatly limits quantitative interpretation of the data. Until authentic standards are available for the metabolites, the choice of analytical method used will influence the observed results and should be carefully considered in future studies and when interpreting reports in the literature.

The AUC<sub>0–24 h</sub> for quercetin metabolites based upon gender is given in Figure 7. In general, the AUC<sub>0–24 h</sub> for females ( $4,258.5 \pm 2,066.5$ ) was greater than the AUC<sub>0–24 h</sub> for males ( $2,551.6 \pm 820.5$ ) but not significantly (calculated  $P = 0.053$ ) for all metabolites, with the exceptions of quercetin sulfate and quercetin glucuronide sulfate, which were significantly greater in females than in males ( $P < 0.05$ ). This was observed for both the OP and AP treatment groups. This data suggest that increasing the number of participants may reveal significant gender differences in the metabolism of quercetin.

In summary, the complement of quercetin metabolites identified in plasma is similar following consumption of AP and OP; however, the AUC<sub>0–24 h</sub> for all quercetin metabolites was greater after the consumption of OP than after consumption of AP. The major quercetin metabolites identified included the following: quercetin sulfate, quercetin glucuronide, and quercetin diglucuronide regardless of treatment. Quercetin glutathione was identified in plasma in negative ionization mode after consumption of onions but not in positive mode. Gender-related differences were observed in the values for AUC<sub>0–24 h</sub> (ng·h/mL) of quercetin sulfate and quercetin glucuronide sulfate; levels were significantly greater in females than in males postconsumption of OP ( $P < 0.05$ ).

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### Notes

The authors declare no competing financial interest.

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## ABBREVIATIONS USED

AP, apple peel powder enriched applesauce; OP, onion powder enriched applesauce; ESI, electrospray ionization; UHPLC, ultra high pressure liquid chromatography; QTOF, quadrupole time-of-flight; IS, internal standard

## REFERENCES

- (1) Erlund, I. Review of the flavonoids quercetin, hesperetin, and naringenin. Dietary sources, bioactivities, bioavailability, and epidemiology. *Nutr. Res.* **2004**, *24*, 851–874.
- (2) Lee, J.; Mitchell, A. E. Pharmacokinetics of quercetin absorption from apples and onions in healthy humans. *J. Agric. Food Chem.* **2012**, *60*, 3874–3881.
- (3) Lee, J.; Mitchell, A. E. Quercetin and isorhamnetin glycosides in onion (*Allium cepa* L.): varietal comparison, physical distribution, coproduct evaluation, and long-term storage stability. *J. Agric. Food Chem.* **2011**, *59*, 857–863.
- (4) Nielsen, I. L. F.; Chee, W. S. S.; Poulsen, L.; Offord-Cavin, E.; Rasmussen, S. E.; Frederiksen, H.; Enslen, M.; Barron, D.; Horcajada, M. N.; Williamson, G. Bioavailability is improved by enzymatic modification of the citrus flavonoid hesperidin in humans: a randomized, double-blind, crossover trial. *J. Nutr.* **2006**, *136*, 404–408.
- (5) Hollman, P. C.; van Trijp, J. M.; Buysman, M. N.; van der Gaag, M. S.; Mengelers, M. J.; de Vries, J. H.; Katan, M. B. Relative bioavailability of the antioxidant flavonoid quercetin from various foods in man. *FEBS Lett.* **1997**, *418*, 152–156.
- (6) Graef, E. U.; Wittig, J.; Mueller, S.; Riethling, A. K.; Uehleke, B.; Drewelow, B.; Pforte, H.; Jacobasch, G.; Derendorf, H.; Veit, M. Pharmacokinetics and bioavailability of quercetin glycosides in humans. *J. Clin. Pharmacol.* **2001**, *41*, 492–499.
- (7) Day, A. J.; Gee, J. M.; DuPont, M. S.; Johnson, I. T.; Williamson, G. Absorption of quercetin-3-glucoside and quercetin-4'-glucoside in the rat small intestine: the role of lactase phlorizin hydrolase and the sodium-dependent glucose transporter. *Biochem. Pharmacol.* **2003**, *65*, 1199–1206.
- (8) Sesink, A. L.; Arts, I. C.; Faassen-Peters, M.; Hollman, P. C. Intestinal uptake of quercetin-3-glucoside in rats involves hydrolysis by lactase phlorizin hydrolase. *J. Nutr.* **2003**, *133*, 773–776.
- (9) Arts, I. C.; Sesink, A. L.; Faassen-Peters, M.; Hollman, P. C. The type of sugar moiety is a major determinant of the small intestinal uptake and subsequent biliary excretion of dietary quercetin glycosides. *Br. J. Nutr.* **2004**, *91*, 841–847.
- (10) Boersma, M. G.; van der Woude, H.; Bogaards, J.; Boeren, S.; Vervoort, J.; Crubben, N. H.; van Iersel, M. L.; van Bladeren, P. J.; Rietjens, I. M. Regioselectivity of phase II metabolism of luteolin and quercetin by UDP-glucuronosyl transferases. *Chem. Res. Toxicol.* **2002**, *15*, 662–670.
- (11) Hong, Y.-J.; Mitchell, A. E. Metabolic profiling of flavonol metabolites in human urine by liquid chromatography and tandem mass spectrometry. *J. Agric. Food Chem.* **2004**, *52*, 6794–6801.
- (12) Mullen, W.; Edwards, C. A.; Crozier, A. Absorption, excretion and metabolite profiling of methyl-, glucuronyl-, glucosyl- and sulpho-conjugates of quercetin in human plasma and urine after ingestion of onions. *Br. J. Nutr.* **2006**, *96*, 107–116.
- (13) Mullen, W.; Rouanet, J. M.; Auger, C.; Teissedre, P. L.; Caldwell, S. T.; Hartley, R. C.; Lean, M. E.; Edwards, C. A.; Crozier, A. Bioavailability of [2-(14)C]quercetin-4'-glucoside in rats. *J. Agric. Food Chem.* **2008**, *56*, 12127–12137.
- (14) Li, C.; Lee, M. J.; Sheng, S.; Meng, X.; Prabhu, S.; Winnik, B.; Huang, B.; Chung, J. Y.; Yan, S.; Ho, C. T.; Yang, C. S. Structural identification of two metabolites of catechins and their kinetics in

- human urine and blood after tea ingestion. *Chem. Res. Toxicol.* **2000**, *13*, 177–184.
- (15) Hong, Y. J.; Mitchell, A. E. Identification of glutathione-related quercetin metabolites in humans. *Chem. Res. Toxicol.* **2006**, *19*, 1525–1532.
- (16) Bjeldanes, L. F.; Chang, G. W. Mutagenic activity of quercetin and related compounds. *Science* **1977**, *197*, 577–578.
- (17) Nakayasu, M.; Sakamoto, H.; Terada, M.; Nagao, M.; Sugimura, T. Mutagenicity of quercetin in Chinese hamster lung cells in culture. *Mutat. Res.* **1986**, *174*, 79–83.
- (18) Nagao, M.; Morita, N.; Yahagi, T.; Shimizu, M.; Kuroyanagi, M.; Fukuoka, M.; Yoshihira, K.; Natori, S.; Fujino, T.; Sugimura, T. Mutagenicities of 61 flavonoids and 11 related compounds. *Environ. Mutagen.* **1981**, *3*, 401–419.
- (19) van der Hoeven, J. C.; Bruggeman, I. M.; Debets, F. M. Genotoxicity of quercetin in cultured mammalian cells. *Mutat. Res.* **1984**, *136*, 9–21.
- (20) Walle, T.; Vincent, T. S.; Walle, U. K. Evidence of covalent binding of the dietary flavonoid quercetin to DNA and protein in human intestinal and hepatic cells. *Biochem. Pharmacol.* **2003**, *65*, 1603–1610.
- (21) Awad, H. M.; Boersma, M. G.; Vervoort, J.; Rietjens, I. M. Peroxidase-catalyzed formation of quercetin quinone methide-glutathione adducts. *Arch. Biochem. Biophys.* **2000**, *378*, 224–233.
- (22) Boersma, M. G.; Vervoort, J.; Szymusiak, H.; Lemanska, K.; Tyrakowska, B.; Cenas, N.; Segura-Aguilar, J.; Rietjens, I. M. Regioslectivity and reversibility of the glutathione conjugation of quercetin quinone methide. *Chem. Res. Toxicol.* **2000**, *13*, 185–191.
- (23) Zhu, B. T.; Ezell, E. L.; Liehr, J. G. Catechol-O-methyltransferase-catalyzed rapid O-methylation of mutagenic flavonoids. Metabolic inactivation as a possible reason for their lack of carcinogenicity in vivo. *J. Biol. Chem.* **1994**, *269*, 292–299.
- (24) Day, A. J.; Bao, Y.; Morgan, M. R.; Williamson, G. Conjugation position of quercetin glucuronides and effect on biological activity. *Free Radical Biol. Med.* **2000**, *29*, 1234–1243.
- (25) Janisch, K. M.; Williamson, G.; Needs, P.; Plumb, G. W. Properties of quercetin conjugates: modulation of LDL oxidation and binding to human serum albumin. *Free Radical Res.* **2004**, *38*, 877–884.
- (26) Wang, L.; Morris, M. E. Liquid chromatography-tandem mass spectroscopy assay for quercetin and conjugated quercetin metabolites in human plasma and urine. *J. Chromatogr., B: Anal. Technol. Biomed. Life Sci.* **2005**, *821*, 194–201.
- (27) Boyer, J.; Liu, R. Apple phytochemicals and their health benefits. *Nutr. J.* **2004**, *3*, 5–19.
- (28) Mullen, W.; Boitier, A.; Stewart, A. J.; Crozier, A. Flavonoid metabolites in human plasma and urine after the consumption of red onions: analysis by liquid chromatography with photodiode array and full scan tandem mass spectrometric detection. *J. Chromatogr., A* **2004**, *1058*, 163–168.
- (29) Wittig, J.; Herderich, M.; Graefe, E. U.; Veit, M. Identification of quercetin glucuronides in human plasma by high-performance liquid chromatography-tandem mass spectrometry. *J. Chromatogr., B: Biomed. Sci. Appl.* **2001**, *753*, 237–243.
- (30) Sesink, A. L.; O'Leary, K. A.; Hollman, P. C. Quercetin glucuronides but not glucosides are present in human plasma after consumption of quercetin-3-glucoside or quercetin-4'-glucoside. *J. Nutr.* **2001**, *131*, 1938–1941.
- (31) Erlund, I.; Alftan, G.; Siren, H.; Ariniemi, K.; Aro, A. Validated method for the quantitation of quercetin from human plasma using high-performance liquid chromatography with electrochemical detection. *J. Chromatogr., B: Biomed. Sci. Appl.* **1999**, *727*, 179–189.
- (32) Moon, Y. J.; Wang, L.; DiCenzo, R.; Morris, M. E. Quercetin pharmacokinetics in humans. *Biopharm. Drug Dispos.* **2008**, *29*, 205–217.
- (33) Manach, C.; Morand, C.; Crespy, V.; Demigne, C.; Texier, O.; Regerat, F.; Remesy, C. Quercetin is recovered in human plasma as conjugated derivatives which retain antioxidant properties. *FEBS Lett.* **1998**, *426*, 331–336.
- (34) Mullen, W.; Graf, B. A.; Caldwell, S. T.; Hartley, R. C.; Duthie, G. G.; Edwards, C. A.; Lean, M. E.; Crozier, A. Determination of flavonol metabolites in plasma and tissues of rats by HPLC-radioisotope counting and tandem mass spectrometry following oral ingestion of [2-(14)C]quercetin-4'-glucoside. *J. Agric. Food Chem.* **2002**, *50*, 6902–6909.
- (35) Graf, B. A.; Mullen, W.; Caldwell, S. T.; Hartley, R. C.; Duthie, G. G.; Lean, M. E.; Crozier, A.; Edwards, C. A. Disposition and metabolism of [2-(14)C]quercetin-4'-glucoside in rats. *Drug Metab. Dispos.* **2005**, *33*, 1036–1043.
- (36) Escarpa, A.; Gonzalez, M. C. High-performance liquid chromatography with diode-array detection for the determination of phenolic compounds in peel and pulp from different apple varieties. *J. Chromatogr., A* **1998**, *823*, 331–337.
- (37) Malathi, P.; Crane, R. K. Phlorizin hydrolase: a beta-glucosidase of hamster intestinal brush border membrane. *Biochim. Biophys. Acta* **1969**, *173*, 245–256.
- (38) Mulder, J. G. Competition between conjugations for the same substrate. In *Conjugation Reactions in Drug Metabolism*; Mulder, J. G., Ed.; Taylor & Francis: London, 1990; pp 39–48.
- (39) Awad, H. M.; Boersma, M. G.; Boeren, S.; van der Woude, H.; van Zanden, J.; van Bladeren, P. J.; Vervoort, J.; Rietjens, I. M. Identification of o-quinone/quinone methide metabolites of quercetin in a cellular in vitro system. *FEBS Lett.* **2002**, *520*, 30–34.
- (40) Awad, H. M.; Boersma, M. G.; Boeren, S.; van Bladeren, P. J.; Vervoort, J.; Rietjens, I. M. The regioselectivity of glutathione adduct formation with flavonoid quinone/quinone methides is pH-dependent. *Chem. Res. Toxicol.* **2002**, *15*, 343–351.
- (41) Galati, G.; Moridani, M. Y.; Chan, T. S.; O'Brien, P. J. Peroxidative metabolism of apigenin and naringenin versus luteolin and quercetin: glutathione oxidation and conjugation. *Free Radical Biol. Med.* **2001**, *30*, 370–382.