UHPLC-ESI Accurate-Mass Q-TOF MS/MS Approaches for Characterizing the Metabolism and Bioavailability of Quercetin in Humans

Application Note

Food Quality and Pharmacology

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Abstract

The bioflavonoid quercetin occurs as a range of glycosides in foods, and the composition of these glycosides is species and cultivar specific. In humans, quercetin undergoes extensive phase II biotransformation, resulting in a complex mixture of metabolites. The bioactivity of the individual metabolites depends on the type and position of the conjugates formed during biotransformation. An Agilent 1290 UHPLC with an Agilent 6530 Accurate-Mass Q-TOF LC/MS was used to identify and measure the relative amounts of individual quercetin metabolites in human plasma, post consumption of applesauce enriched with either micronized apple peel (AP) or onion powder (OP). The AP and OP were standardized to 100 mg total quercetin glycosides. Metabolite identification was performed using a custom Agilent Personal Compound Database Library (PCDL) of quercetin-related products of phase II biotransformation and Agilent MassHunter Qualitative Analysis software. Q-TOF MS/MS fragmentation data and Agilent MassHunter Molecular Structure Correlator (MSC) software were used for structural confirmation of the identified metabolites.

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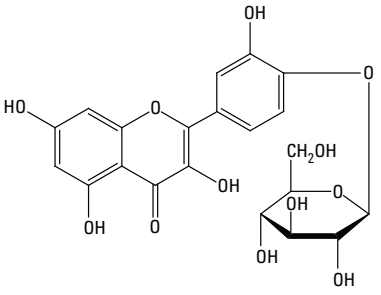
Introduction

A complementary report of this study of quercetin metabolism is published in the *Journal of Agriculture and Food Chemistry* [1]. Quercetin was chosen for study because epidemiological studies have shown that diets high in flavonoids may lower the risk of cardiovascular disease, cancer, and other age-related diseases [2,3,4]. As shown in Figure 1, quercetin occurs as glycosides in foods. The glycosidic form determines its bioavailability (absorption). The complement of glycosides present in a food is specific to the species and cultivar. The primary glycoside found in onions is quercetin 4'-*O*-gucloside. The primary form found in apples is the quercetin 3-*O*-galactoside.

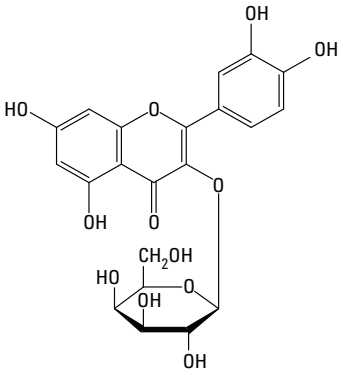
Quercetin glycosides are absorbed in the gut. The glycoside is cleaved by the action of β-glucosidase and lactase phlorizin hydrolase in the brush border membranes of the small intestine, and the aglycone is absorbed. The aglycone undergoes extensive metabolism by phase II biotransformation enzymes within the enterocytes. This results in a range of sulfated, glucuronidated, and methylated species. These metabolites are transported to the liver via the hepatic portal vein and undergo further phase II metabolism, prior to release into general circulation.

In vitro studies show that the activity of quercetin metabolites depends on the types and position of their conjugate groups. For example, Jansich *et al*. showed that the *in vitro* lag time of LDL oxidation was increased by quercetin-7-glucuronide more than quercetin aglycone, but was not affected by the same concentrations of quercetin-3'-sulfate or isorhamnetin-3-glucuronide [5]. Identifying the kinetic profiles of individual metabolites in the plasma is critical to understanding their mechanism of action and bioactivity [6].

Full-scan quadrupole ESI LC/MS methods have been used to identify metabolites in plasma successfully, but the sensitivity of these methods does not allow monitoring individual metabolites in plasma beyond six hours [7,8,9]. ESI LC/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, information regarding circulating metabolites is lost. Elucidation of metabolite structure in complex mixtures is greatly facilitated by accurate mass MS/MS. This is especially true for complex mixtures of flavonoid metabolites, such as quercetin, where commercial standards are not available. Accurate mass quadrupole time-of-flight (Q-TOF) MS methods provide the sensitivity and selectivity needed to distinguish among isobaric ions, and to provide a molecular formula to aid in the identification of non-targeted unknown compounds. Accurate mass analysis of product ions produced in the Q-TOF LC/MS mode facilitates structural elucidation, further aiding compound identification.



Q 4'-*O*-glucoside: primary form found in onions



Q 3-*O*-galactoside: primary form found in apples

Figure 1. Primary quercetin (Q) glycosides found in onions and apples.

This application note describes the tools used to explain the differences in quercetin bioavailability: UHPLC-ESI Accurate-Mass Q-TOF LC/MS analysis in combination with Agilent Personal Compound Database Library (PCDL) manager and Agilent MassHunter Qualitative Analysis software. Specifically, the authors investigated the quercetin metabolites present in plasma in response to two different foods (apple peel and onion powder) with the same total level of quercetin, but composed of different quercetin glycosides. The goal was to understand if the glycoside forms influence pharmacokinetic parameters (such as absorption and metabolism). Because standards are not available, semi-quantitative analysis was done by comparing the response of the metabolites to that of the reference standard.

Experimental

A detailed description of the experimental design and procedures can be found in the complementary report published in the Journal of Agriculture and Food Chemistry [1].

Study Design

The absorption and metabolism of quercetin glycosides in fortified applesauce was investigated using a randomized crossover feeding trial protocol approved by the Institutional Review Board of the University of California, Davis. The trial included 16 volunteers: eight female and eight male. 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 two days prior to the study and during the treatment day. After an overnight fast, either apple peel powder (AP) fortified applesauce or onion powder (OP) fortified applesauce was provided as breakfast. Both treatments delivered 100 mg total quercetin.

The quercetin glycoside composition of the fortified applesauce, shown in Table 1, was identified previously using triple quadrupole liquid chromatography mass spectrometry (LC/MS/MS) and quantified using high-pressure liquid chromatography (HPLC) [6]. In the applesauce fortified with AP, the primary forms of quercetin included 3-galactoside, 3-glucoside, and 3-rhamnoside. In the applesauce fortified with OP, quercetin 3,4′ diglucoside and quercetin 4′-glucoside predominated.

Subject blood samples were taken at 0, 0.5, 1, 2, 4, 6, 8, and 24 hours following consumption of the fortified applesauce. These samples were centrifuged, and the plasma was separated from blood cells.

Sample Preparation: Extraction of Quercetin Metabolites

The extraction method was modified from the method of Mullen *et al*. [8,10]. Recovery was measured using quercetin 4′-*O*-glucoside as a reference standard (RS) added to baseline plasma (at final concentrations of 10 and 1,000 ng/mL plasma). In previous studies, quercetin 4′-*O*-glucoside was not found in plasma after consumption of quercetin glycosides or quercetin glycoside-rich foods [11,12].

Table 1. Quercetin Glycoside Profile of Fortified Applesauce in mg

	100 g applesauce fortified with:	
Quercetin glycosides	**Apple peel powder**	**Onion powder**
quercetin 3,4′-*O*-diglucoside	0 mg	44.9 mg
quercetin 3-*O*–rutinoside (rutin)	6.5	0
quercetin 3-*O*-galactoside	16.8	0
quercetin 3-*O*-glucoside	16.1	4.9
quercetin -*O*-xyloside	9.3	0
quercetin -*O*-arabinofuranose	3.0	0
quercetin -*O*-arabinopyranose	17.6	0
quercetin 3-*O*-rhamnoside	30.6	0
quercetin 4′-*O*-glucoside	0	48.7
Total	99.9 ± 2.3 mg	98.5 ± 2.1 mg

Instrumentation

The extracted quercetin metabolites were analyzed using an Agilent 1290 Infinity Binary LC system coupled to an Agilent 6530 Accurate-Mass Q-TOF LC/MS system with Agilent Jet Stream technology for electrospray ionization. The UHPLC was equipped with a binary pump with an integrated vacuum degasser (G4220A), an autosampler (G4226A) with a thermostat (G1330B), and a thermostatted column compartment (G1316C). The UHPLC parameters are shown in Table 2.

To identify all possible quercetin metabolites, a Q-TOF MS^1 analysis of the extracted plasma samples was performed. Total ion spectra were collected over a mass range of *m/z* 100–1,000, in both negative and positive modes. The Q-TOF MS parameters are shown in Table 3.

To achieve the desired mass accuracy, continuous internal calibration was performed during analysis.

Relative quantification of each quercetin metabolite was performed using the Q-TOF MS^1 mode using quercetin 4′-*O*-glucoside as the RS. Calibration was achieved using the standard addition method of spiking known amounts of stock standard to pooled plasma. The limit of RS detection was 1 ng/mL plasma, and the linear dynamic range spanned three orders of magnitude. Peaks of eluents corresponding to possible quercetin metabolites were further investigated using Q-TOF in MS/MS negative ESI mode.

Table 2. UHPLC Parameters

Instrument	Agilent 1290 Infinity Binary LC
Mobile phases	A) 0.1% formic acid in water B) 0.1% formic acid in acetonitrile Initial 5% B
Gradient	Linear
	Time (min) %B 0–5 5–10 5–8 10–12 8–10 12–15 10–15 15 15–18 15–55 18–20 55–90
Flow rate	0.4 mL/min
Column	Agilent Poroshell EC18 UHPLC column 2.1 × 100 mm, 2.7 µm (p/n 695775-902) with an Agilent 2.1 mm × 5 mm, guard column (p/n 821725-911)
Post run time	4 minutes at initial mobile phase
Temperature	30 °C
Injection volume	5 µL

Table 3. Q-TOF LC/MS Parameters

Instrument	Agilent 6530 Accurate-Mass Q-TOF LC/MS
Ionization mode	Positive and negative electrospray with Agilent Jet Stream technology
Acquisition rate	1.0 spectra/s
Mass range	100–1,000 *m/z*
Drying gas temperature	225 °C
Drying gas flow rate	8.0 L/min
Sheath gas temperature	300 °C
Sheath gas flow rate	10.0 L/min
Nebulizer gas	45 psi
Skimmer voltage	65 V
Octopole RF	750 V
Fragmentor	125 V
Capillary	2.5 kV (negative mode) or 3.5 kV (positive mode).

Data Analysis

The quercetin metabolites were identified using the following steps:

1. A database of metabolites was created using metabolites previously reported, as well as calculated masses based upon known phase II biotransformation pathways. The exact masses for these potential quercetin metabolites were compiled into the PCDL Manager (Figure 2). This database was then imported into the MassHunter Qualitative Analysis software.
2. Data obtained from the Q-TOF MS[1] analysis were searched against the PCDL described in step 1 above, using the MassHunter Qualitative Analysis software. The resulting list of potential quercetin-related metabolites was identified based on their accurate mass, isotope abundance and isotope spacing. This step resulted in a list of possible compounds.
3. Structural confirmation of quercetin metabolites identified in step 2 was obtained by formula matching the Q-TOF MS/MS fragmentation data with the MassHunter MSC software.

Pharmacokinetics

Pharmacokinetic variables measured were 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 hours (AUC0–24 hours). Significant differences in plasma pharmacokinetic 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 $P < 0.05$.

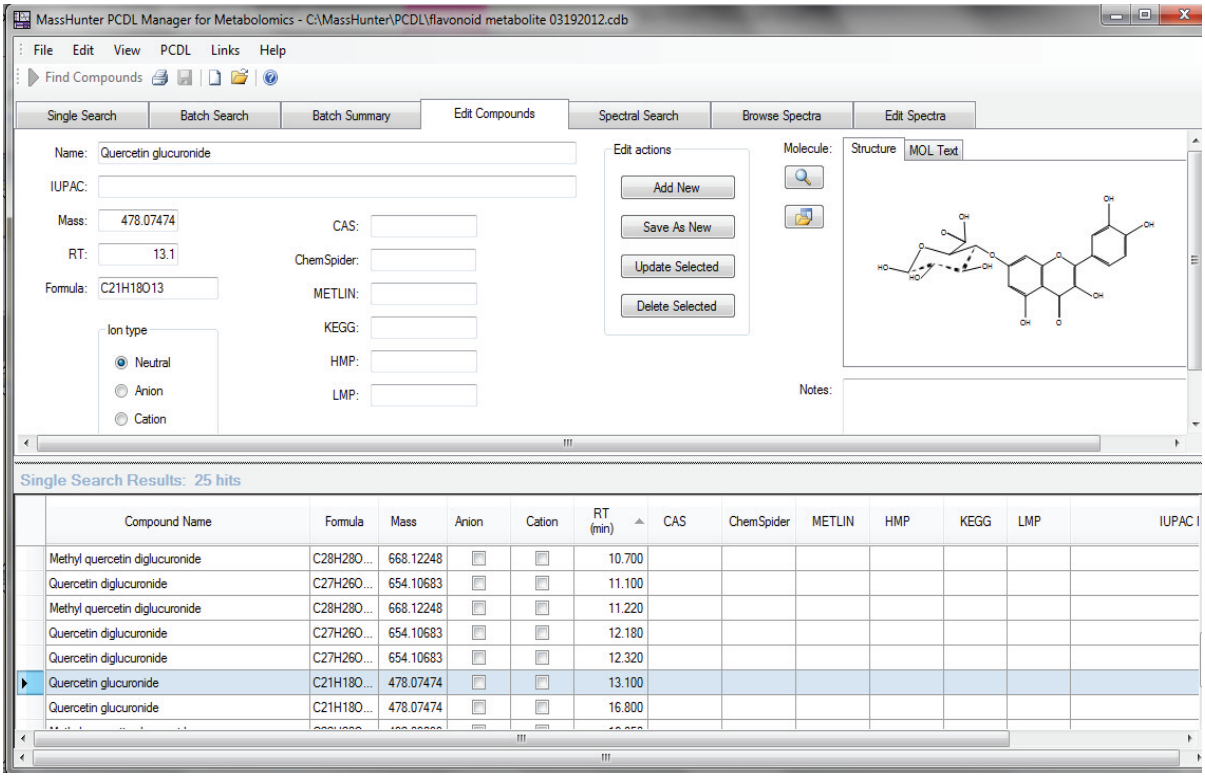


Figure 2. The MassHunter PCDL Manager was used to build and manage an accurate mass library of flavonoid metabolites. The software automatically calculates masses and formulas when compound structures and names are entered.

Results and Discussion

Identification and Confirmation of Quercetin Metabolites

Representative extracted ion chromatograms of the quercetin metabolites identified in the volunteers' plasma samples 2 hours after consumption of the fortified applesauce are shown in Figure 3. The identifications were made using a MassHunter Qualitative Analysis software search of the data against values imported from the PDCL library of known metabolites. Identifications were made based on accurate mass and isotopic fidelity.

Table 4 shows by peak number the list of the possible quercetin metabolites, along with their retention time, type of molecular ion, observed and predicted mass, and the mass error. The error between the observed and predicted *m/z* was consistently less than 2.5 ppm.

To obtain structural confirmation, a Q-TOF MS/MS analysis of the 15 identified possible quercetin metabolites was performed. Quercetin has a calculated monoisotopic mass of 302.0427 Da. All quercetin-related metabolites produce a negative ion fragment ion at *m/z* 301.0354 corresponding to quercetin aglycone (quercetin without the conjugate). The exception is methylated quercetin-related metabolites, which produce fragment ions at *m/z* 315.0510 corresponding to the methylated quercetin ion without the conjugate.

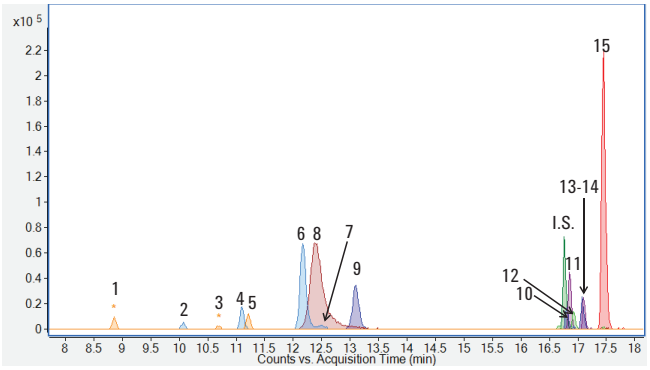


Figure 3. Extracted ion chromatograms of quercetin metabolites in plasma, two hours after consumption of the fortified applesauce. Identifications were made using MassHunter software and customized PDCL library.

Table 4. Possible Quercetin Metabolites by Peak Number with Retention Time, Type of Molecular Ion, Observed and Predicted Mass, and Mass Error

Peak	RT (min)	Possible compounds	Type of molecular ion	Predicted *m/z*	Observed *m/z*	Mass error (ppm)
1	8.86	Methyl Q diglucuronide	$[M-H]^-$	667.1152	667.1146	0.9
2	10.07	Q diglucuronide	$[M-H]^-$	653.0996	653.0987	1.4
3	10.70	Methyl Q diglucuronide	$[M-H]^-$	667.1152	667.1136	2.4
4	11.10	Q diglucuronide	$[M-H]^-$	653.0996	653.0986	1.5
5	11.22	Methyl Q diglucuronide	$[M-H]^-$	667.1152	667.1147	0.6
6	12.18	Q diglucuronide	$[M-H]^-$	653.0996	653.0989	1.1
7	12.32	Q diglucuronide	$[M-H]^-$	653.0996	653.0989	1.1
8	13.38	Q glucuronide sulfate	$[M-H]^-$	557.0243	557.0240	0.5
9	13.10	Q-3-glucuronide	$[M-H]^-$	477.0675	477.0671	0.8
10	16.80	Q-3'-glucuronide	$[M-H]^-$	477.0675	477.0673	0.4
11	16.85	Methyl Q 3 glucuronide	$[M-H]^-$	491.0831	491.0832	–0.2
12	16.90	Q glutathione	$[M+HCOO]^-$	651.1012	651.0998	2.2
13	17.07	Q glucuronide	$[M-H]^-$	477.0675	477.0672	0.6
14	17.10	Methyl Q glucuronide	$[M-H]^-$	491.0831	491.0831	0.0
15	17.45	Q-3-sulfate	$[M-H]^-$	380.9922	380.9925	–0.8

Figure 4 shows the Q-TOF MS/MS spectra of quercetin glucoronide and quercetin sulfate with the characteristic fragment ions at *m/z* of 301.0354 corresponding to the loss of a glucuronide from the molecular ion of 477.0673, and the loss of a sulfate group from the molecular ion a 380.9925. The *m/z* 113.0244 represents the glucuronide moiety.

Matching the observed MS/MS fragments with the proposed structure and its fragments provided additional confidence in the identifications made. MassHunter MSC software was used to obtain structural confirmation of the identified metabolites via formula matching. The MassHunter MSC software correlates each observed MS/MS fragment ion to the proposed structure using a systematic bond-breaking approach. Figure 5 provides an example of the MS/MS confirmation of quercetin glucoronide fragment ion with *m/z* 301.0354. Figure 6 shows the expected fragmentation of quercetin glucoronide provided by the MassHunter MSC software.

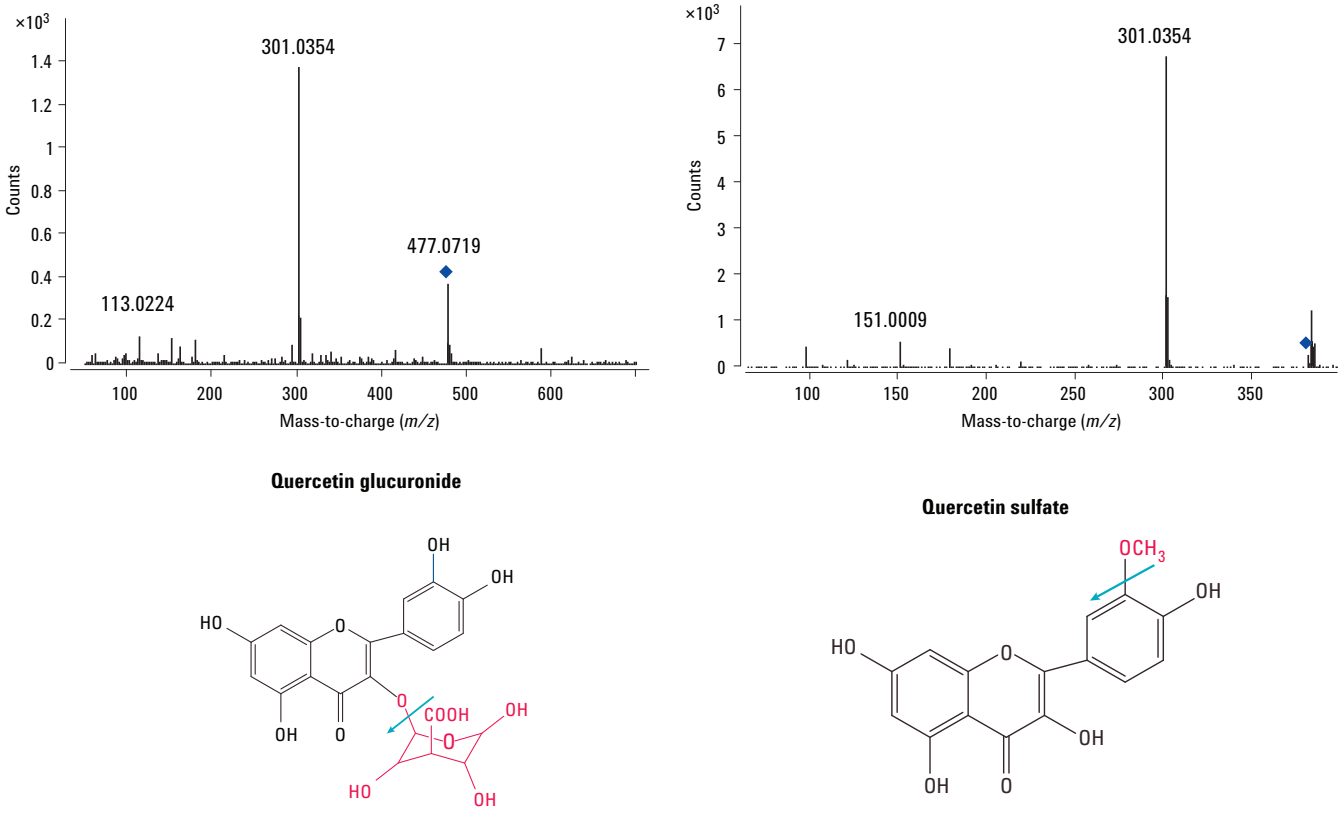


Figure 4. Q-TOF MS/MS spectra of quercetin glucoronide and quercetin sulfate with characteristic fragment ions.

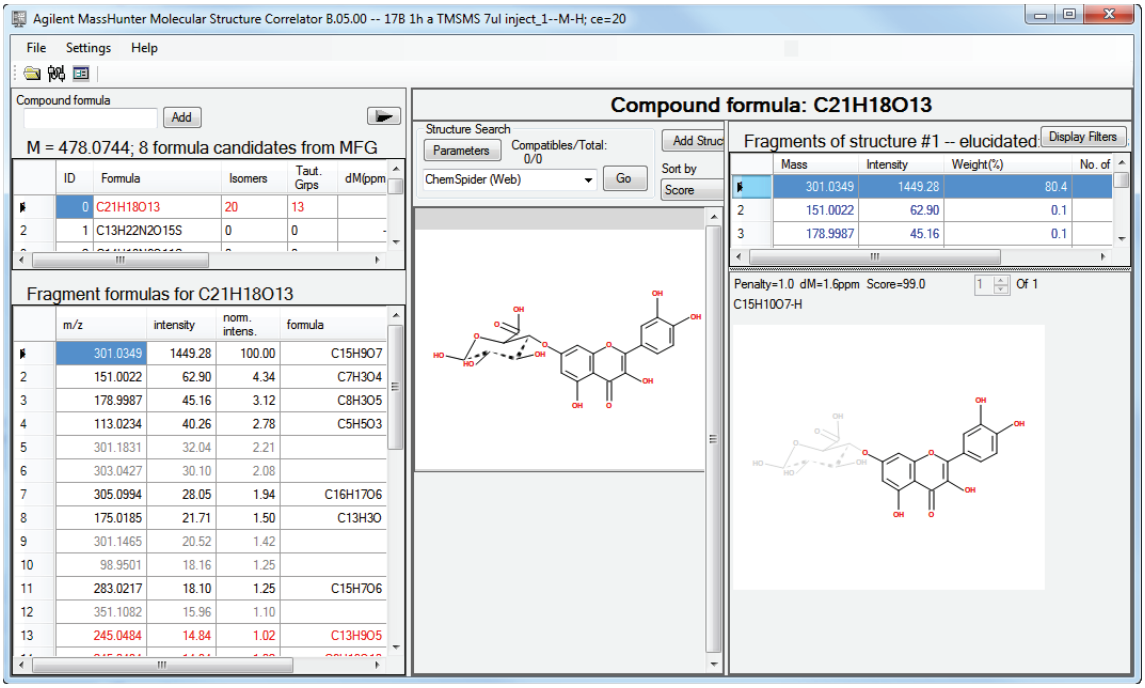


Figure 5. MS/MS confirmation of quercetin glucoronide fragment ion with m/z 301.0354 using MassHunter MSC software. The software displays the parent and fragment formulas, and presents the structures when a formula is highlighted.

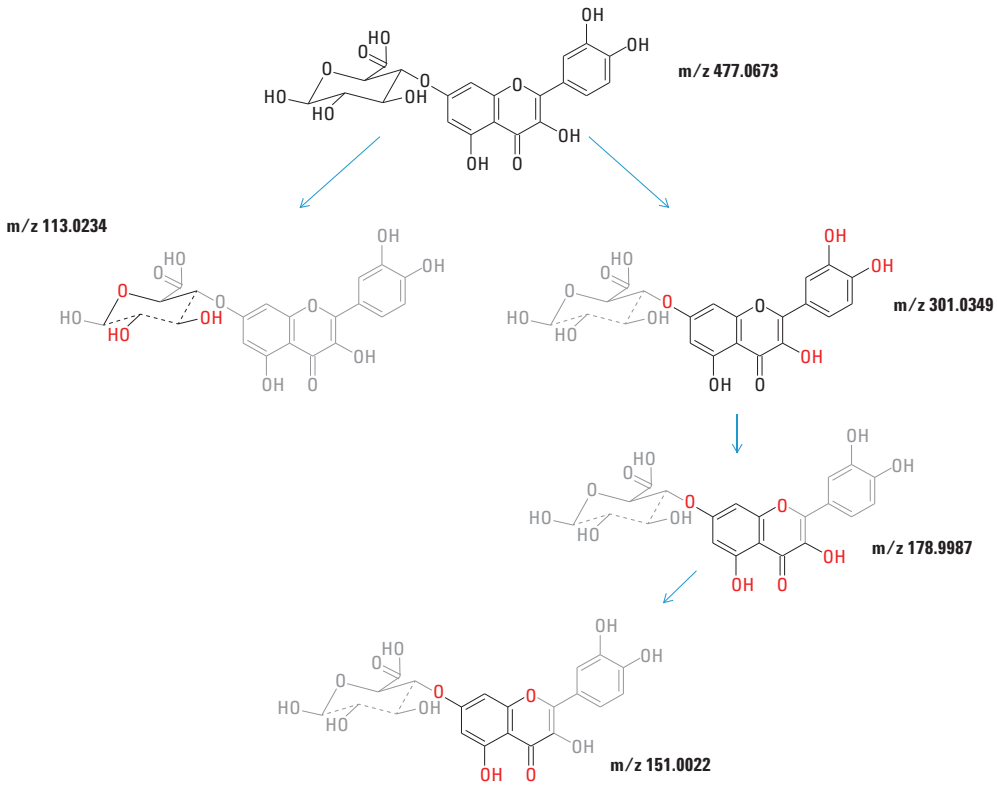


Figure 6. Expected fragmentation of quercetin glucoronide provided by MassHunter MSC software.

Pharmacokinetics

Pharmacokinetic parameters can easily be calculated for each metabolite given the sensitive detection the Q-TOF LC/MS allows over an extended time period (24 hours). In this application study, we found that quercetin metabolite profiles in plasma are essentially the same following consumption of either treatment; however, their bioavailability is different (Figure 7). The plasma concentrations of the individual quercetin metabolites were significantly higher after consumption of the OP fortified applesauce as compared to AP fortified applesauce. Because authentic standards were not used, the quantitative results shown in Figure 7 are relative, not absolute, values.

Conclusions

Accurate-Mass Q-TOF LC/MS enabled detection, identification, relative quantitation, and kinetic monitoring of quercetin metabolites in human plasma, with high sensitivity and selectivity. Similar quercetin metabolites were identified in the plasma of eight females and males over 24 hours after consumption of applesauce enriched with either micronized apple peel or onion powder containing 100 mg total quercetin.

Screening and identification were performed rapidly using a custom PCDL of flavonoid metabolites imported into MassHunter Qualitative Analysis software. Q-TOF MS/MS fragmentation data and MassHunter MSC software provided structural confirmation of the identified metabolites. The polarity of ESI impacted the ionization efficiency of the quercetin metabolites. For example, quercetin glutathione was identified in negative ESI mode but not in positive ESI mode.

The primary metabolites found included: quercetin sulfate, quercetin glucuronide, and quercetin diglucuronide, and were the same for both treatments. However, the pharmacokinetic parameters AUC0–24 h and Cmax were different for the two treatments. The plasma concentrations of the quercetin metabolites were significantly higher after consumption of the onion powder. Gender-related differences in the AUC0–24 h for quercetin sulfate and quercetin sulfate glucuronide metabolites were also found.

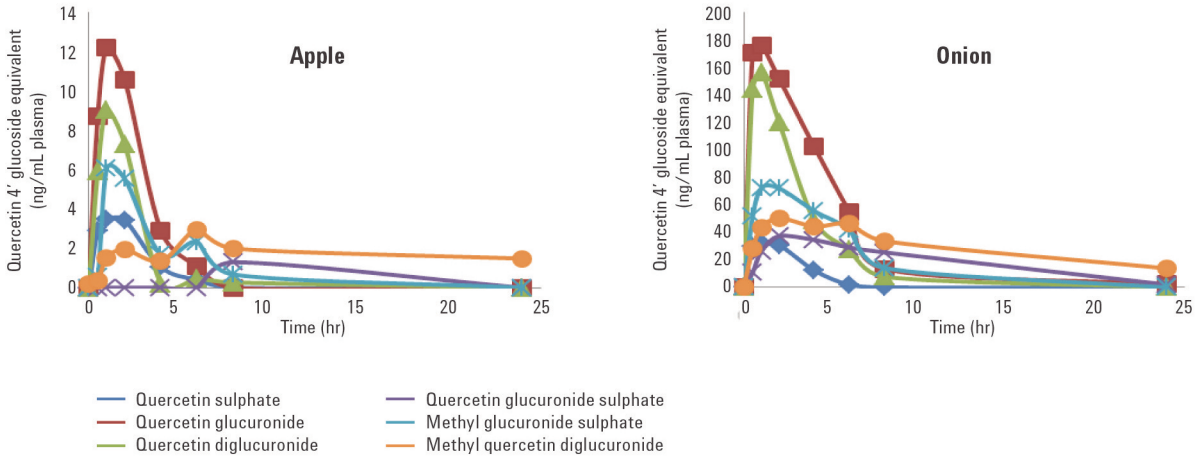


Figure 7. Quercetin metabolites found in plasma in positive electrospray ionization mode.

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