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LC−MS^n Methods for Saccharide Characterization of Monoglycosyl Flavonoids Using Postcolumn Manganese Complexation

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A simple tandem mass spectrometry method for differentiating isomeric monoglycosyl flavonols, flavones, and flavanones using manganese complexation is reported. Dissociation of the [Mn(II) (L) (L − H)]$^+$ and [Mn(II) (L)$_2$ (L − H)]$^+$ species provides unique fragment ions that allow the identification of the saccharide moiety as glucose, galactose, arabinose, or xylose. The glycosylation site of the flavonoid can also be determined by the fragmentation pathways of the Mn complexes. The Mn complexation method was adapted for on-line liquid chromatography−mass spectrometry analysis and tested using flavonoid extracts from Fuji apples (*Malus domestica* Borkh. cv. Fuji) and red onions (*Allium cepa* L.). Using fragmentation data obtained from collisional activated dissociation of the deprotonated flavonoid glycosides and their Mn complexes, the major flavonoid species in these extracts were identified.

Flavonoids are polyphenolic secondary metabolites that are produced by nearly all green plants. Studies have linked human intake of flavonoids with concrete health benefits. For example, the consumption of flavonoid-rich foods has been shown to be inversely correlated with the incidence of heart disease and cardiovascular ailments.[1,2] Flavonoids have also been shown to have antioxidant, antiproliferative, antibiotic, and antiinflammatory properties.[3,4] Several thousand flavonoids have been discovered, sharing a similar $C_6-C_3-C_6$ skeleton, but differing in the number and location of substituents such as hydroxyl, methoxyl, and saccharide groups. Small differences in flavonoid structure may have a large effect on the biological activity of the molecule.[5−7] The positions and identities of saccharide moieties also affect bioactivity.[8−10] The precise identification of individual flavonoids and their derivatives is thus a crucial step in the investigation of their chemopreventive properties.

Mass spectrometry (MS), both alone and in combination with high-performance liquid chromatography (HPLC), has long been an important tool in the study of flavonoids. The use of mass spectrometry for flavonoid identification and structural analysis has been reviewed by Stobiecki[11] and more recently by Cuyckens and Claeys.[12] Tandem mass spectrometry of protonated and deprotonated flavonoid aglycons can often be used to positively identify these molecules;[13−17] and in the case of flavonoid glycosides, information can be obtained about the number of saccharide moieties and their molecular weights.[18−20] In a few specific cases, the fragmentation patterns of protonated or deprotonated flavonoid glycosides can yield information about the position and identity of the saccharide moieties.[21−25] But, in general, mass spectrometry has not provided complete structural information for the flavonoid glycosides, requiring the use of additional analytical techniques such as nuclear magnetic resonance (NMR) spectroscopy.[26−28]

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Unlike mass spectrometry, these auxiliary techniques are usually not readily compatible with HPLC; therefore, isolation, purification, and individual analysis of each flavonoid species are required. Due to the difficulty in characterizing flavonoid glycosides, some researchers opt to remove the saccharide moieties via hydrolysis prior to analysis and thus identify only the aglycon portion of molecules in their extracts and samples.[29−34] Because the saccharide moieties play an important though not well-understood role in determining bioactivity, such an approach results in the loss of critical information.

Only one mass spectrometric method for identifying isomeric monosaccharide moieties (i.e., glucose vs galactose) of flavonoid glycosides has been published,[35] but the method requires isolation and an overnight derivatization of the analytes prior to analysis. Similarly, little has been published about the determination of the glycosylation sites of monoglycosyl flavonoids by MS, though we have recently developed a tandem mass spectrometry method based on Mg complexation for identifying compounds glycosylated at five of the most commonly encountered sites of conjugation.[36] In this work, we report the use of tandem mass spectrometry to both differentiate isomeric saccharide moieties and determine the glycosylation site of 18 monoglycosyl flavonols, flavones, and flavanones (Figure 1) by examining the fragmentation patterns of flavonoid glycoside/manganese(II) complexes. Our group has previously shown that performing tandem mass spectrometry on flavonoid glycoside/metal complexes can provide more structural information than the more conventional analysis of protonated and deprotonated species.[36−41] Here we successfully apply a metal complexation strategy to on-line liquid chromatography−mass spectrometry (LC−MS) analysis of extracts from the peel of Fuji apples and the flesh of red onions via postcolumn addition of $MnCl_2$. Used in combination with information obtained in the negative ion mode, more complete structural information on the flavonoid glycosides is obtained than is possible without metal complexation.

EXPERIMENTAL SECTION

Materials. Quercetin-3-*O*-glucoside, quercetin-3-*O*-galactoside (hyperoside), syringetin-3-*O*-glucoside, syringetin-3-*O*-galactoside, quercetin-4′-*O*-glucoside (spiraeoside), naringenin-7-*O*-glucoside (prunin), and luteolin-6-C-glucoside (homoorientin) were purchased from Extrasynthèse (Genay, France). Kaempferol-3-*O*-glucoside (astragalin), isorhamnetin-3-*O*-glucoside, luteolin-4′-*O*-glucoside, apigenin-7-*O*-glucoside, luteolin-7-*O*-glucoside, apigenin-6-C-glucoside (isovitexin), apigenin-8-C-glucoside (vitexin), luteolin-8-C-glucoside (orientin), and quercetin were purchased from Indofine (Somerville, NJ). Quercetin-3-*O*-arabinofuranoside (avicularin), quercetin-3-*O*-xyloside (reynoutrin), and quercetin-7-*O*-glucoside were purchased from Apin (Abingdon, U.K.). Manganese(II) chloride was purchased from Aldrich (Milwaukee, WI). All materials were used without further purification.

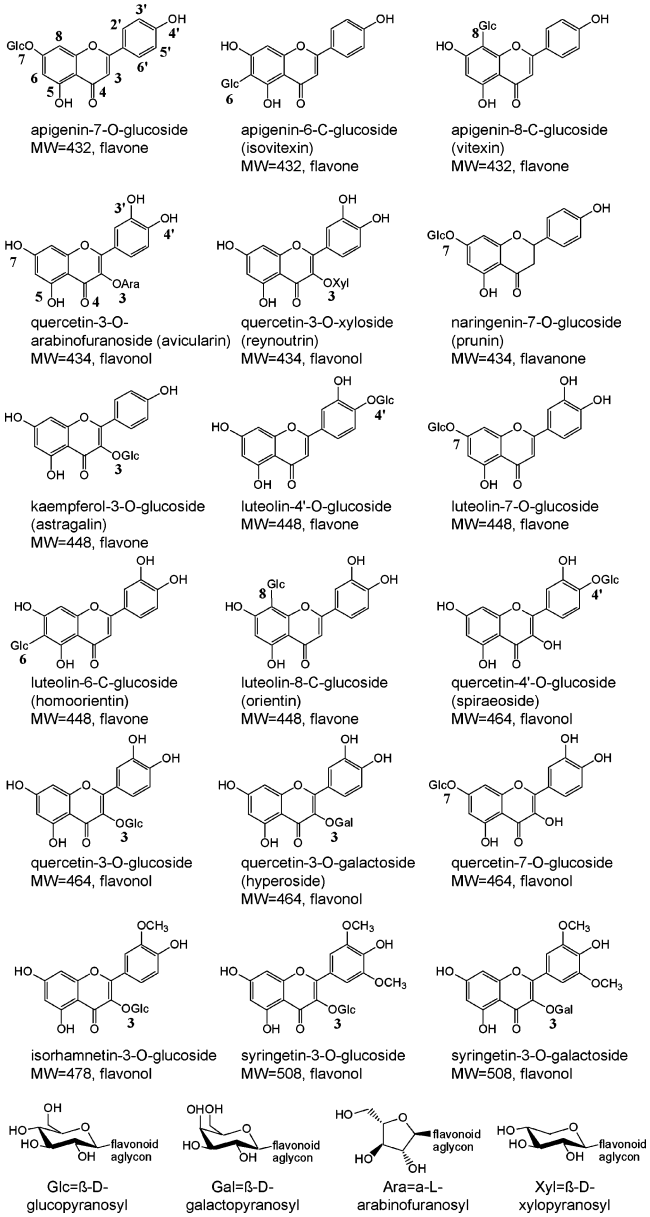

Figure 1. Structures of the flavonoid glycosides included in this study. The standard numbering scheme for the flavonoid carbons is shown.

Direct Infusion MS. All experiments were performed using an LCQ Duo quadrupole ion trap mass spectrometer (Thermo-Finnigan, San Jose, CA) with an electrospray ionization (ESI) source. For direct infusion experiments, the sample introduction

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rate was 5 μL/min and the ion injection time was 10 ms for full scans and 50 ms for collisional activated dissociation (CAD) experiments. A total of 100 microscans were averaged for each spectrum. The metal complexes were analyzed in positive ion mode using the following spray conditions: spray voltage, +5 kV; sheath gas flow rate, 5 arbitrary units; no auxiliary gas; heated capillary temperature, 200 °C; capillary voltage, +20 V; tube lens offset, +20 V. Analyte solutions were made in methanol, with the flavonoid standard and metal salt each added at 1.0×10^{-5} M. The ion abundances from the CAD experiments are reported relative to the most abundant ion in the spectrum, which is designated as 100%. When collecting CAD data, the collision energy was increased until the parent ion was reduced to 5−10% abundance. Collision energies are reported as a percentage of the maximum 5 V_{p-p}, normalized for the m/z of the parent.[42] Isolation windows of 4−6 m/z were used because many of the metal complexes require a wide window in order to yield a stable fragmentation spectrum. It has been our observation that many types of noncovalent complexes require wider than average windows for effective isolation.

Flavonoid Extraction from Apple Peel. Organic Fuji apples (*Malus domestica* Borkh. cv. Fuji) were purchased from a local supermarket and refrigerated until used. The extraction procedure was adapted from one described by Dick et al.[43] One apple was washed and peeled, and the peel (~15 g) was immersed in liquid nitrogen before being chopped in a blender. The finely chopped apple peel was added to a flask containing 100 mL of methanol. The flask was heated to 50 °C and stirred for 30 min. The contents of the flask were then filtered through a fritted glass funnel, and the extract solvent was passed through 0.2-μm PTFE syringe filters (Fisher, Pittsburgh, PA). This cleanup procedure proved ineffective as a solid precipitate formed upon refrigeration of the sample. The methanol was then evaporated with nitrogen, and the extract was redissolved in 8 mL of water with 0.33% formic acid (mobile phase A). A C18 SepPak (Waters, Milford, MA) was used to purify and concentrate the flavonoid species in the extract. The cartridge was conditioned with 10 mL of acetonitrile with 0.33% formic acid (mobile phase B) followed by 8 mL of mobile phase A. The extract was loaded and washed with 5 mL of 98:2 A/B. The flavonoid species were eluted with 2 mL of 67:33 A/B. The sample was refrigerated until analyzed and was injected onto the HPLC column without dilution.

Flavonoid Extraction from Onions. Red onions (*Allium cepa* L., unknown cultivar) were purchased from a local supermarket and refrigerated until used. The extraction procedure was adapted from one described by Marotti and Piccaglia.[29] An onion was peeled, sliced, and then immersed in liquid nitrogen before being chopped in a blender. Approximately 20 g of the chopped onion was added to a flask containing 50 mL of 50:42:8 methanol/water/acetic acid. The flask was covered and refrigerated for 48 h. The extraction mixture was then filtered through a fritted glass funnel, and the solvent was evaporated with nitrogen. The extract was redissolved in 15 mL of mobile phase A. A solid-phase extraction procedure similar to the one described for the apple peel extract was used to purify and concentrate the onion extract. The sample was refrigerated until analysis and was diluted 10-fold with methanol prior to injection onto the HPLC column.

LC−MS Conditions. Chromatography was performed using a Waters Alliance 2690 HPLC system. The column was a Waters Symmetry C18, 2.1 × 50 mm, 3.5-μm particle size, with a guard column. The flow rate was 0.3 mL/min, and an injection volume of 10 μL was used for all experiments. For the apple peel extract, the two-component mobile-phase system was held isocratically at 12% B for 26 min and then was increased to 98% B over 2 min before a return to initial conditions and reequilibration for 7 min. For the onion extract, the gradient began at 10% B, increased to 25% B over 10 min, and then to 95% B over 1.5 min before a return to initial conditions and reequilibration for 8.5 min. An ultraviolet (UV) detector monitored the column effluent at 280 nm. The column effluent was directed into the LCQ mass spectrometer without splitting. For negative ion mode experiments, the following MS conditions were used: spray voltage, −4.5 kV; sheath gas, 20 units; auxiliary gas, 5 units; heated capillary temperature, 200 °C; capillary voltage, −45 V; tube lens offset, −45 V. When flavonoid glycoside/Mn complexes were analyzed, 500 μM $MnCl_2$ dissolved in methanol was added at 20 μL/min via a mixing tee between the UV detector and the mass spectrometer. In these cases, the positive ion mode was employed, using the same spray conditions with the following exceptions: spray voltage, +4.5 kV; capillary voltage, +44 V; tube lens offset, +35 V.

RESULTS AND DISCUSSION

Direct Infusion Experiments. The first goal of this project was to develop a simple MS method for differentiating isomeric monoglycosyl flavonoids that differ only in the identity of the saccharide moiety (glucose vs galactose and arabinose vs xylose). Some of the flavonoid glycosides employed in this part of the experiment are diastereomeric pairs, since glucose and galactose differ only in their stereochemistry (Figure 1). CAD of protonated, deprotonated, and sodium-cationized species does not provide a sufficient means for differentiating these compounds. In a previous publication, we showed that the fragments afforded by CAD of flavonoid glycoside/Mg complexes were dependent on the identity of the saccharide moiety of the flavonoid glycoside.[36] A wide variety of metals, including Mg, Ca, Sr, Ba, Mn(II), Fe(II), Co(II), Ni(II), Cu(II), Zn(II), K, Ag, and Al was evaluated for use in differentiating the monoglycosyl flavonoids in the present study. ESI-MS analysis of solutions containing a flavonoid glycoside and a metal(II) salt results in detection of 1:1 complexes, $[M(II)\ (L - H)]^+$, and 2:1 complexes, $[M(II)\ (L)\ (L - H)]^+$. The ion abundance from these metal complexes is typically on the same order as the protonated flavonoid glycoside, $(L + H)^+$, with the 2:1 complex generally more intense than the 1:1 complex. The fragmentation patterns of both the 1:1 and 2:1 flavonoid glycoside/metal complexes were evaluated, and MS^3 fragmentation was performed on key diagnostic fragment ions. Some degree of isomer differentiation was possible using the MS/MS spectra of several of the 1:1 complexes; however the fragmentation patterns were always very complicated and subject to postdissociation solvent adduction, and in many cases, differentiation was based only on *relative intensities* of fragment ions rather than on the presence or absence of *unique diagnostic* fragment ions. In contrast, the MS/MS spectra of the 2:1 complexes were much

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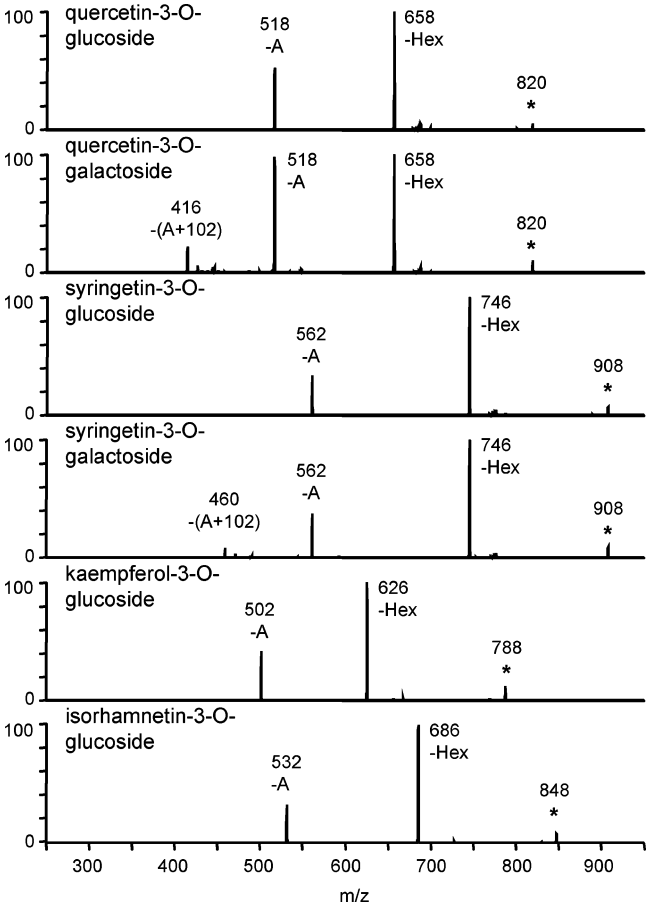

Figure 2. MS^3 spectra of $[Mn(II)\ (L)\ (L - H)]^+$ complexes of 3-*O*-hexosides following the loss of one hexose moiety. The parent ion is denoted by an asterisk (*). Fragment ions are labeled as follows: −Hex (loss of a hexose moiety); −A (loss of an aglycon portion). The CAD energy was 22−23% in all cases.

simpler but usually did not provide sufficient differentiation of isomers.

The best differentiation of the glucoside/galactoside pairs comes from MS^3 experiments using the Mn(II) complexes. Several species are observed in the full scans (Supporting Information, Figure S-1), including $(L + H)^+$ and $[Mn(II)\ (L - H)]^+$, but typically the most abundant species under the working conditions is the 2:1 complex, $[Mn(II)\ (L)\ (L - H)]^+$. When the 2:1 complexes of the 3-*O*-hexosides are subjected to CAD, the only fragment ion is the result of the loss of one hexose moiety, −162 u (data not shown). However, performing a second stage of CAD on this key primary fragment ion leads to a clear differentiation of the 3-*O*-glucosides and -galactosides (Figure 2). All of the complexes exhibit losses of the second hexose moiety and of one aglycon unit, but the two flavonoid galactoside complexes display the additional loss of an aglycon plus 102 u, providing a means to distinguish these compounds from the corresponding flavonoid glucosides. The loss of 102 u from saccharide/metal complexes was reported by Gaucher and Leary in a study to differentiate hexose sugars using a Zn(II)−dien complex.[44] They performed isotopic labeling studies indicating that sugar carbons 3−6 were lost. Based on this information, they proposed a mechanism for this fragmentation, which we have

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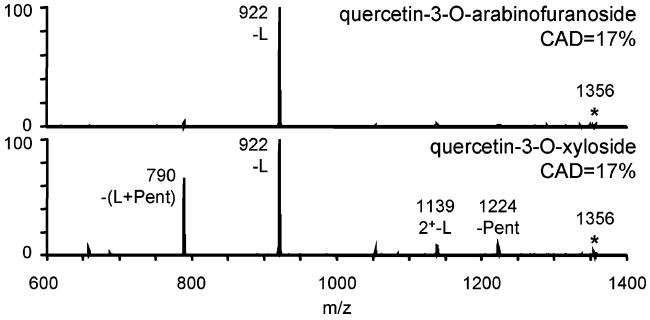


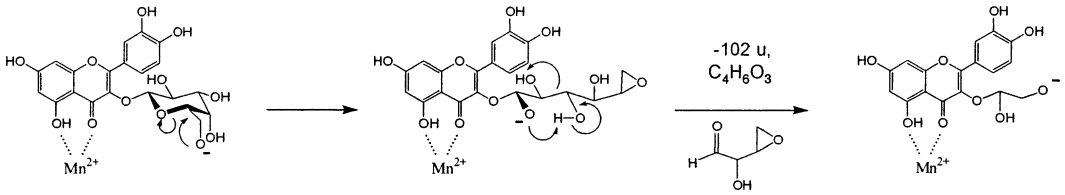
Figure 3. MS/MS spectra of $[Mn(II)\ (L)_2\ (L - H)]^+$ complexes of 3-*O*-pentosides. The parent ion is labeled with an asterisk (*). Fragment ions are labeled as follows: −L (loss of a flavonoid glycoside); −Pent (loss of a pentose moiety); 2^+−L ($[2\ Mn(II)\ (L)_4\ (L - H)_2]^{2+} - L$).

adapted for the present case (Scheme 1). Starting with the 2:1 complex, one galactose moiety is lost in the first stage of dissociation, followed by the aglycon in the second stage, in effect leaving behind a 1:1 complex. The additional loss of 102 u is attributed to a rearrangement/fragmentation of the remaining galactose moiety, resulting in the elimination of $C_4H_6O_3$. The flavonoid glucoside complexes do not display this fragmentation pathway even up to 50% CAD energy, presumably because of conformational differences that disfavor this process.

None of the 2:1 flavonoid glycoside/metal complexes provides a satisfactory differentiation of quercetin-3-*O*-arabinofuranoside and quercetin-3-*O*-xyloside. Instead, differentiation is achieved based on CAD of the 3:1 Mn complexes, $[Mn(II)\ (L)_2\ (L - H)]^+$ (Figure 3). Below 20% CAD energy, the arabinoside complex gives only one signficant product ion stemming from the loss of one flavonoid glycoside. However, the xyloside complex also yields an abundant fragment ion corresponding to the loss of a flavonoid glycoside plus a pentose moiety (*m/z* 790), in addition to some lower abundance fragments. Using a CAD energy around 17% provides the best differentiation of the two isomers.

In addition to allowing the confident identification of the saccharide moiety, the Mn(II) complexes also provide a means to determine glycosylation sites of flavonoid hexosides in a manner similar to that reported previously by using magnesium complexation.[36] When MS/MS is performed on the $[Mn(II)\ (L)\ (L - H)]^+$ complexes, the resulting fragmentation patterns are diagnostic for the five most common glycosylation sites: attachment to the flavonoid aglycon through an oxygen atom at position 3, 4′, or 7 or through a carbon atom at position 6 or 8. Table 1 summarizes the fragment ions obtained from each complex and shows how these data can be used to determine the glycosylation site. The loss of a hexose residue, −162 u, is indicative of O-glycosylation; the complexes of the C-glycosides do not display this loss. The 3-*O*-hexoside complexes yield no other significant fragmentation products, but the 4′-*O*-hexoside complexes also display the loss of two hexose moieties, the loss of an aglycon unit, and the loss of an intact flavonoid glycoside. The amount of CAD energy required to fragment the 4′-*O*-hexoside complexes is also higher than that required for the 3-*O*-hexoside complexes. The 7-*O*-glucoside complexes are the only ones to display both the loss of a hexose residue and a 0,2 cross-ring saccharide cleavage (−120 u). Further confirmation of the 7-*O*-glucosides can obtained by MS^3 using the ion that results from the loss of one hexose

Scheme 1. Proposed Mechanism for the Loss of 102 u from Mn Complexes of Flavonoid Galactosides (Hyperoside Shown as Example)[a]



[a] Adapted from ref 44.

Table 1. Summary of Fragment Ions from [Mn(II) (L) (L − H)]$^+$ Complexes of Flavonoid Hexosides (*N* = 2 or 3)[a,b]

glycosylation site	MW	flavonoid glycoside	av CAD energy (%)	-Hex[c]	−2 Hex	−A	−L	2^+ −L	−120	−(2 × 120)	−90	$-H_2O$	−2 H_2O	−3 H_2O	−66	−96	-(90 + H_2O)	-(120 + H_2O)
3-O	448	kaempferol-3-Glc	18.8	100	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	464	quercetin-3-Glc	17.8	100	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	464	quercetin-3-Gal	18.3	100	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	478	isorhamnetin-3-Glc	17.9	100	–	–	–	–	–	–	–	–	–	–		–	–	–
	508	syringetin-3-Glc	17.6	100	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	508	syringetin-3-Gal	18.0	100	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4′-O	448	luteolin-4′-Glc	25.9	100	10	7	8	4	–	–	–	–	–	–	–	–	–	–
	464	quercetin-4′-Glc	22.4	100	5	8	5	–	–	–	–	–	–	–	–	–	–	–
7-O	432	apigenin-7-Glc	23.9	100	8	2	–	–	14	–	–	2	–	–	–	–	–	–
	434	naringenin-7-Glc	20.7	100	15	3	–	5	6	–	–	–	–	–	–	–	–	–
	448	luteolin-7-Glc	24.3	100	10	2	2	–	13	–	–	2	–	–	–	–	–	–
	464	quercetin-7-Glc	22.2	100	12	2	2	–	11	–	–	3	–	–	–	–	–	–
6-C	432	apigenin-6-Glc	23.0	–	–	–	3	–	100	5	4	40	4	3	3	2	2	15
	448	luteolin-6-Glc	22.6	–	–	–	3	–	100	5	4	40	4	3	3	2	2	14
8-C	432	apigenin-8-Glc	20.3	–	–	–	–	–	100	6	6	3	–	–	–	–	–	–
	448	luteolin-8-Glc	19.9	–	–	–	–	–	100	6	7	2	–	–	–	–	–	–

[a] The CAD energy was chosen such that the parent ion was reduced to 5−10% relative abundance. [b] The abundance of each fragment is reported as a percentage relative to the most abundant ion in the spectrum. Fragments below 2% abundance are not reported. [c] Fragments are abbreviated as follows: −Hex, loss of 1 hexose moiety; −2 Hex, loss of two hexose moieties; −A, loss of one aglycon portion; −L, loss of one flavonoid glycoside molecule; 2^+−L, loss of one flavonoid glucoside molecule from [2 Mn(II) $(L)_4$ $(L - H)_2]^{2+}$; −120, 0,2 cross-ring saccharide cleavage; −90, 0,3 cross-ring saccharide cleavage; −66, 2,3 cross-ring saccharide cleavage with loss of two water molecules; −96, 0,4 cross-ring saccharide cleavage with loss of two water molecules.

moiety from the 2:1 complex (Supporting Information, Figure S-2). C-Glucosylation is indicated by the 0,2 cross-ring cleavage product[25] as the most prominent fragment ion in the CAD spectrum. Useful diagnostic ions for differentiating the 6-C- and the 8-C-glucosides include a very abundant dehydration product and the combined loss of 120 u and a water molecule (both characteristic of 6-C-glucosides). The MS/MS spectra of the 2:1 Mn complexes involving flavonoid glycosides with molecular weight 448 (spanning five sites of glycosylation) are provided in the Supporting Information, Figure S-3. Because the dissociation patterns are dependent primarily on the glycosylation site of the flavonoid rather than on the nature of the aglycon portion of the molecule, it is likely that a similar analysis of the Mn(II) complexes of unknown flavonoid monohexosides would yield information regarding the position of the saccharide moiety.

Manganese complexation is superior to the previously described method using magnesium complexation for several reasons. First, the magnesium complexes [Mg(II) (L) (L − H)]$^+$ suffer from an isobaric overlap with a sodium adduct, [Na $L_2]^+$, that may complicate spectral interpretation. The Mn(II) complexes do not have this problem. Second, manganese is monoisotopic, leading to a potentially simpler spectrum and greater concentration of the molecular ion intensity into a single peak. Finally, Mn(II) provides the ability both to determine glycosylation sites and to identify the saccharide moiety (in the case of 3-*O*-glycosides), whereas Mg is not reliable for the latter application.

LC−MS Analysis of Fuji Apple Peel Extract. To prove the simplicity of the metal complexation approach and its applicability to on-line chromatographic analysis, we attempted to identify the flavonoid species in food extracts, specifically the peel of Fuji apples and the flesh of red onions. Apples were a special target of interest because they are known to contain several monoglycosyl flavonoid isomers that differ only by the identity of the saccharide moieties and thus make an excellent test subject for the newly developed methods. The extracts were examined using both negative ion mode and positive ion mode (for the Mn complexes) because complementary information was obtained from each type of experiment. The extracts were prepared as described in the Experimental Section.

The UV chromatogram of the Fuji apple peel extract (Figure 4) shows eight major flavonoid components, labeled **1**−**8**. LC−MS^n analysis in the negative ion mode provides the molecular weights of the flavonoid glycosides (full scan mode) and the aglycon portions (MS/MS mode), as well as the number and weight of the saccharide moieties for each compound (MS/MS mode). For example, a loss of 162 u indicates an *O*-hexoside, a loss of 146 u indicates an *O*-deoxyhexoside, and a loss of 132 u indicates an *O*-pentoside.[12] Losses associated with cross-ring saccharide cleavages, such as 120 or 90 u, are indicative of C-glycosylation.[25] Upon sequential stages of fragmentation (MS^n), the saccharide moieties can be enumerated and some of their possible identities can be eliminated based on molecular weight.

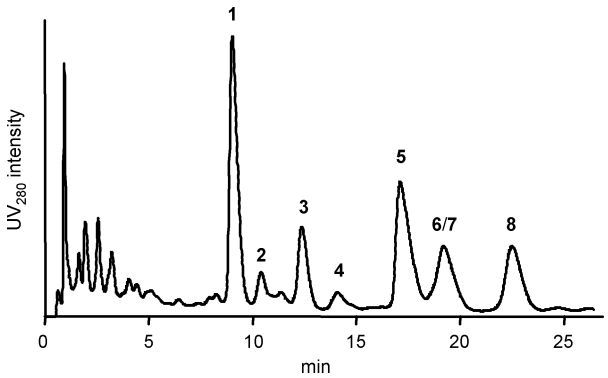


Figure 4. UV chromatogram of the Fuji apple peel extract, 280 nm. Flavonoid components are numbered.

Scheme 2. Summary of Structural Data from the Flavonoid Components in the Fuji Apple Peel Extract, Using Negative Ion Mode

1: 463 $\xrightarrow{-162}$ 301 (aglycon)

2: 463 $\xrightarrow{-162}$ 301 (aglycon)

3: 433 $\xrightarrow{-132}$ 301 (aglycon)

4: 433 $\xrightarrow{-132}$ 301 (aglycon)

5: 433 $\xrightarrow{-132}$ 301 (aglycon)

6: 433 $\xrightarrow{-132}$ 301 (aglycon)

7: 447 $\xrightarrow{-146}$ 301 (aglycon)

8: 567 $\xrightarrow{-(132+162)}$ 273 (aglycon)

Following the loss of all of the saccharide moieties, the weight of the aglycon portion is obtained, and the aglycon may be identified by comparing the fragmentation pattern with standards or by elucidating its structure based on well-known dissociation pathways of the flavonoid aglycons.[12−16] The process of determining this information for the eight major flavonoid species observed in the Fuji apple peel extract is summarized in Scheme 2. Compounds **1**−**7** all lose a single saccharide moiety, leaving behind a deprotonated aglycon with mass 301. Further fragmentation of m/z 301 identifies the aglycon as quercetin based on comparison to published spectra[14] and to the CAD spectrum of a commercial standard. Thus, **1** and **2** are quercetin *O*-hexosides, **3**−**6** are quercetin *O*-pentosides, and **7** is a quercetin *O*-deoxyhexoside. **8** was later determined to be a member of the chalcone family, a minor flavonoid class that is outside the scope of the current study.

Negative ion mode LC−MS^n provides useful structural information but does not reveal the nature of the saccharide moieties. At this point one would typically need to isolate each flavonoid glycoside and undertake NMR characterization to identify the saccharide moieties, perhaps using micro NMR coils with volumes less than 1 μL to reduce sample consumption. Another approach would be to inject standards to check for matching retention times (although many flavonoid glycosides are not commercially available). In this study, LC−MS^n with postcolumn Mn(II) complexation is used to probe the identities and locations of the saccharides. LC−MS/MS on the 2:1 Mn(II) complexes of **1** and **2** yields a single fragment stemming from the loss of one hexose

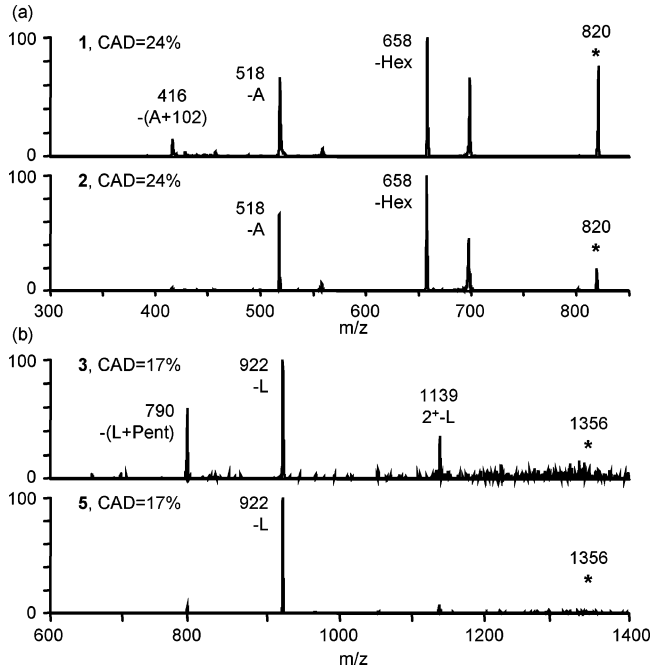


Figure 5. (a) MS^3 and (b) MS/MS spectra of Mn complexes involving components of the Fuji apple peel extract. The parent ion is denoted by an asterisk (*). Fragment ions are labeled as follows: −Hex (loss of a hexose moiety); −A (loss of an aglycon portion); −L (loss of a flavonoid glycoside); −(L+Pent) (loss of a flavonoid glycoside and a pentose moiety)' 2^+−L ($[2\ Mn(II)\ (L)_4\ (L-H)_2]^{2+}$ − L). Some of the additional peaks in the spectra are due to post-CAD adduction of acetonitrile (+41 u).

residue (Supporting Information, Figure S-4). This is the hallmark of 3-O-glycosylation. A second stage of CAD undertaken on this fragment ion results in spectra that differentiate the two analytes (Figure 5a). The diagnostic loss of one aglycon unit plus 102 u is observed only for **1**. Therefore, **1** is determined to be a galactoside and **2** is identified as a glucoside. Upon integration of all of these data, **1** and **2** are identified as quercetin 3-*O*-galactoside and quercetin-3-*O*-glucoside, respectively. This is in agreement with the observation that flavonoid galactosides generally elute before flavonoid glucosides.[45−47] This same LC−MS and postcolumn Mn complexation technique has also been used recently to identify several flavonol 3-*O*-hexosides in an extract from a species in the *Asteraceae* plant family.[48]

Of the quercetin pentosides, only **3** and **5** gave Mn(II) complexes of sufficient abundance for analysis. To determine the identity of the saccharides, LC−MS/MS was performed on the 3:1 complex, $[Mn(II)\ (L)_2\ (L-H)]^+$ (Figure 5b). The CAD spectra of these complexes identify **3** as a xyloside and **5** as an arabinofuranoside. Fragmentation of **7** is not necessary to identify this analyte as a quercetin *O*-rhamnoside because rhamnose is the only deoxyhexose known to form natural flavonoid conjugates.[12,35]

Retention time matching with commercial standards confirms the identities of **1** and **2**. The retention times of **3**, **5**, and **7** match those of quercetin-3-*O*-xyloside, quercetin-3-*O*-arabinofuranoside,

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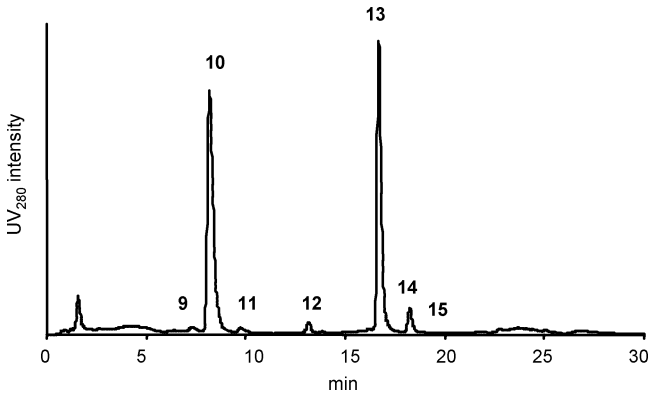

Figure 6. UV chromatogram of the red onion extract, 280 nm. Flavonoid components are numbered.

Scheme 3. Summary of Structural Data from the Flavonoid Components in the Red Onion Extract, Using Negative Ion Mode

9: 625 $\xrightarrow{-162}$ 463 $\xrightarrow{-162}$ 301 (aglycon)

10: 625 $\xrightarrow{-162}$ 463 $\xrightarrow{-162}$ 301 (aglycon)

11: 639 $\xrightarrow{-162}$ 477 $\xrightarrow{-162}$ 315 (aglycon)

12: 463 $\xrightarrow{-162}$ 301 (aglycon)

13: 463 $\xrightarrow{-162}$ 301 (aglycon)

14: 477 $\xrightarrow{-162}$ 315 (aglycon)

15: 301 (aglycon)

and quercetin-3-*O*-rhamnoside, respectively. The retention order of these five compounds agrees with other published studies of apple flavonoids.[43,47,49−51] The Mn complexation method provided no additional information on compounds **4**, **6**, and **8**. Using as a guide a particularly detailed study of apple waste components by Sánchez-Rabaneda et al.,[49] we believe that **4** is quercetin-3-*O*-arabinopyranoside and **8** is phloretin-2′-*O*-xyloglucoside (mistakenly labeled phloretin-2-*O*-xyloglucoside in the reference). From the same source,[49] some of the early-eluting peaks are thought to be various cinnamic acid derivatives. Like us, Sánchez-Rabaneda et al. observed an unidentified quercetin *O*-pentoside (**6**) coeluting with quercetin-3-*O*-rhamnoside. We speculate that **6** may be an apiose derivative of quercetin.[50]

LC−MS Analysis of Red Onion Extract. There are at least seven different flavonoid species present in the onion extract, labeled **9**−**15** in order of elution (Figure 6). As with the apple extract, negative ion mode LC−MS^n is used to determine the weights of the aglycon portions and the number and types of glycosyl groups. These data are summarized in Scheme 3. The deprotonated aglycon with mass 301 (**9**, **10**, **12**, **13**, **15**) is determined to be quercetin based on comparison of the fragmentation pattern to published spectra[14] and to the CAD spectrum acquired from a commercial standard. **15** is further confirmed to be quercetin based on comparison of the retention time with a commercial standard. The deprotonated aglycon with m/z 315 (**11**, **14**) is similarly determined to be isorhamnetin based on comparison of the fragmentation pattern with published spectra[47] and with a commercial standard. **9**−**11** all contain two hexose moieties while **12**−**14** have one each. This agrees with the observation that additional saccharide moieties increase the polarity of a flavonoid glycoside, thus leading to faster elution in reversed-phase chromatography.[45]

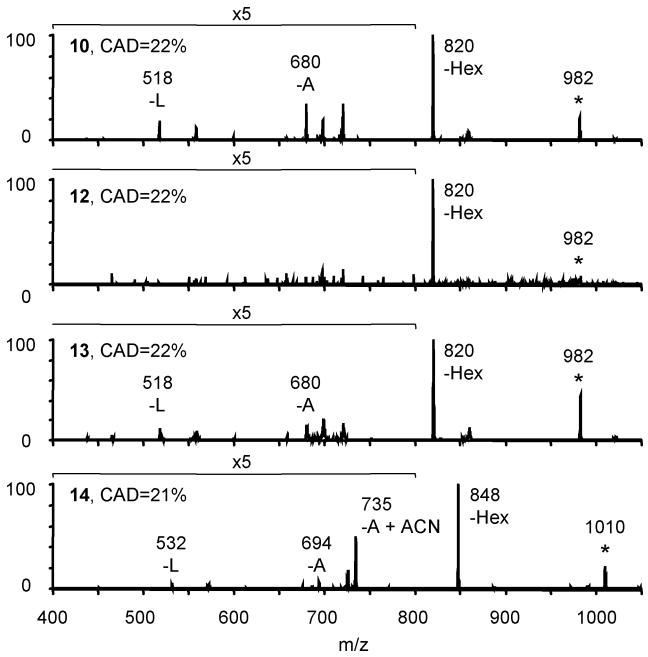

Figure 7. CAD spectra of Mn complexes involving components of the red onion extract. The parent ion is denoted by an asterisk (*). Fragment ions are labeled as follows: −Hex (loss of a hexose moiety); −A (loss of an aglycon portion); −L (loss of one flavonoid glycoside molecule). Many of the unlabeled peaks are post-CAD adducts of acetonitrile (+41 u) or water (+18 u).

To reveal the identities, locations, and configurations of the saccharide moieties of these analytes, postcolumn Mn complexation was performed. **9** and **11** do not form sufficient quantities of the complexes due to their low abundance, and **15** (a flavonoid aglycon, quercetin) is fully characterized in the negative ion mode, so only **10** and **12**−**14** were studied using Mn complexation. The MS/MS spectra of the 2:1 complexes (Figure 7) are compared to Table 1 in order to determine the glycosylation sites of these compounds. The lack of any cross-ring saccharide cleavage fragments suggests that all four are 3-*O*- or 4′-*O*-glycosides. Fragments indicative of 4′-*O*-glycosides include the loss of one flavonoid glycoside molecule and the loss of an aglycon portion, whereas 3-*O*-glycoside complexes generally display only one major loss of a saccharide. **13** clearly shows these additional fragments, while they do not appear for its isomer **12**; therefore, **12** is suggested to be a 3-*O*-glycoside while **13** is assigned as a 4′-*O*-glycoside. **14** also shows these diagnostic fragment ions, so it is identified as a 4′-*O*-glycoside of isorhamnetin. **10** is a special case: diglycosyl flavonoids are generally outside the scope of this study, but **10** nonetheless forms complexes of the type [Mn(II) (L) (L − H)]$^+$ at m/z 1306. Performing sequential stages of fragmentation on this complex yields m/z 982 after the loss of two hexose moieties, presumably one from each flavonoid glyco-

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side molecule. At this point, what remains is effectively a 2:1 monoglycosyl flavonoid complex, so the position of the remaining hexoses can be studied using the same techniques as discussed earlier. The fragments obtained from *m/z* 982 match the ones from **13**; thus, it is concluded that at least one of the hexose moieties of **10** is at the 4′-O position.

The spectral quality is somewhat worse for these LC−MS experiments compared to the direct infusion experiments. Considering that the diagnostic ions in Figure 7 are of fairly low abundance, supporting information regarding the glycosylation site is desirable. Additional evidence is obtained through a judicious choice of CAD energy. According to Table 1, the CAD energy required to reduce the parent ion of the 3-*O*-glycoside complexes to 5−10% overall abundance is always below 19%, while for the 4′-*O*-glycoside complexes it is above 22%. If a collision energy between these values is chosen, the survival rate of the parent ion can be used as a useful piece of confirmational evidence. When a CAD energy of 21−22% is used, a significant amount of the parent ions survive for **10**, **13**, and **14**, which is consistent with their assignments as 4′-*O*-glycosides. The parent ion of **12** does not survive, supporting its identification as a 3-*O*-glycoside. Additionally, knowledge about the typical elution order of flavonoid glycosides can be used.[45,52] Our observation has been that 3-*O*-glycosides generally elute earlier than 4′-*O*-glycosides. This is consistent with the assignment of **12** and **13** as 3-*O*- and 4′-*O*-glycosides of quercetin, respectively.

Finally, a second stage of fragmentation can determine the identity of the hexose moiety of **12**. When the ion at *m/z* 820 is activated, the fragment ion corresponding to the loss of the quercetin aglycon plus 102 u is missing (Supporting Information, Figure S-5). Comparing this result to Figure 2, it is clear that the hexose cannot be galactose and is therefore assigned as glucose. **10**, **13**, and **14** also lack the diagnostic ion for galactose, but this evidence is inconclusive because no standards are available to determine whether the Mn complexes of 4′-*O*-galactosides can be differentiated from 4′-*O*-glucosides in the same manner as at the 3-O-position.

In summary, the peak assignments based solely on these LC−MS methods are as follows: **9**, quercetin dihexoside; **10**, quercetin dihexoside (with at least one of the hexoses located at the 4′ position); **11**, isorhamnetin dihexoside; **12**, quercetin-3-*O*-glucoside; **13**, quercetin-4′-*O*-hexoside (glucoside); **14**, isorhamnetin-4′-*O*-hexoside (glucoside); **15**, quercetin. Standards of quercetin-3-*O*-glucoside and quercetin have the same retention times as **12** and **15**, respectively, confirming those identifications. A quercetin-4′-*O*-glucoside standard has the same retention time as **13**. No standard for isorhamnetin 4′-*O*-glucoside was available, but isorhamnetin-3-*O*-glucoside does not match the retention time of **14**. The accuracy of these assignments is assessed by comparison with several previously published studies of onion flavonoid components.[29,53−55] Based on the literature, we believe that we have correctly identified **12**−**15** using the LC−MS method with Mn complexation. **10** is almost certainly quercetin-3,4′-di-*O*-glucoside,[53] and **9** may be quercetin-7,4′-di-*O*-glucoside.[55] **11** may be isorhamnetin-3,4′-di-*O*-glucoside, which has been reported as a minor flavonoid glycoside in onions.[56]

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CONCLUSIONS

Performing CAD on complexes of the form $[Mn(II)\ (L)\ (L-H)]^+$ and $[Mn(II)\ (L)_2\ (L-H)]^+$, where L is a monoglycosyl flavonoid, provides information on the glycosylation site and saccharide identity of these compounds based on unique and consistent fragmentation patterns. This method of identifying flavonoid glycosides was applied to the on-line LC−MS analysis of a Fuji apple peel extract and a red onion extract. Combined with information obtained from the deprotonated analytes, this method allowed the identification of several of the flavonoid glycosides in the extracts. Supporting evidence was obtained by using retention time comparison with commercial standards and knowledge of the elution order associated with various structural features. The identifications were in agreement with the literature. Although complete identifications could not be made in all cases, significantly more structural information was obtained than was previously possible using conventional LC−MS techniques. Similar results may be possible using a triple quadrupole mass spectrometer. Furthermore, metal complexation has been shown to be a promising approach to mass spectrometric differentiation of flavonoid glycosides even when standards are not available. As more flavonoid glycosides become commercially available, we hope that further correlations between structure and dissociation behavior will be found such that full characterization of these compounds will be possible using simple LC−MS techniques. This would be a significant advantage over the isolation and purification of each compound followed by NMR analysis that is currently the standard method for characterizing the saccharide moieties of flavonoid glycosides.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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