Influence of cation adduction on the separation characteristics of flavonoid diglycoside isomers using dual gate-ion mobility-quadrupole ion trap mass spectrometry

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An electrospray ionization-dual gate-ion mobility-quadrupole ion trap mass spectrometer was used to evaluate the separation characteristics of isomeric flavonoid diglycosides adducted with sodium, potassium, and silver. This instrumental configuration allows ions to be selectively accumulated within the ion trap on the basis of their gas phase conformation prior to mass analysis. For the metal cations examined, silver produced the most compact adducts with flavonoid diglycosides. Listed in order of increasing size, the trend of flavonoid diglycoside ion-neutral cross sections adducted with Na+, K+, and Ag+ was narirutin < naringin < hesperidin < neohesperidin < rutin. To examine the separation contribution of the carbohydrate group, hesperetin, the aglycone of hesperidin, and neohesperid were compared to quercetin, the aglycone of rutin. Separation of the flavonoid diglycosides indicated that quercetin-derived diglycosides drifted longer than their hesperetin-derived isomers. Combined with the observed collision assisted dissociation (CAD) data, these findings suggest that carbohydrate moiety plays a significant role in both the separation and metal chelating characteristics of flavonoid diglycosides. Copyright © 2006 John Wiley & Sons, Ltd.

KEYWORDS: IMS; QIT-MS; flavonoids; metal adduction; electrospray ionization

INTRODUCTION

Produced almost exclusively by the members of the plant kingdom, flavonoids represent a class of organic compounds that play vital roles in the regulation, development, and growth of plant tissues.1–3 Because of their significant contribution to the human diet (~1 g/day),4 antioxidant behavior,5,6 and chemopreventive properties,3,7,8 there is great interest in characterizing the chemistry of flavonoids in mammalian systems. At present, over 4000 flavonoid variants have been isolated and identified, each possessing a fifteen-carbon skeleton consisting of two phenyl rings (A- and B-rings) connected by a three carbon bridge (C-ring).5 The wide range of flavonoid functional activity is attributed to the structural complexity of flavonoids produced by modifying the carbon skeleton using hydroxyl, methoxy, methyl, and/or carbohydrate groups. In the case of carbohydrates, an addition to the flavonoid structure may occur directly through the carbon skeleton (C-linked) or through the hydroxyl groups (O-linked) and is further complicated by the stereochemical orientation of the carbohydrate rings. Given the aforementioned structural complexity, accurate and robust means of structure determination are needed to comprehensively assess the functional role of flavonoids in biological systems. Mass spectrometry (MS) is one such technique capable of providing detailed structural information regarding flavonoids while consuming a minimal amount of the sample. Earlier, examination of flavonoids and their glycosides was explored using electron impact combined with methylation prior to analysis.10 However, methylated side reactions involving flavonoid glycosides were observed, making this an undesirable approach.11 Field desorption,12 fast atom bombardment,13 and direct chemical ionization12 have also been used with varying degrees of success for the structural characterization of flavonoids. Unfortunately, these techniques too require extensive sample preparation prior to analysis. More recently, matrix assisted laser desorption ionization (MALDI) time-of-flight (TOF) MS combined with both in-source and postsource decay have been used to examine the structural features of flavonoids.14,15 Yet, difficulty assigning the binding position of the carbohydrate moieties has required additional analysis steps for structural identification. The advent of electrospray ionization (ESI)16 eased much of the difficulty in examining flavonoids, as this
technique accommodated the experimental conditions of liquid chromatography (LC) and the ionization needs of MS. Because ESI is a comparatively ‘soft’ ionization technique, a secondary means of producing fragment ions for structural assignment was necessary. Using ESI combined with MS experiments, a number of approaches have been developed to examine flavonoids isolated from natural systems.

Owing to their acidic nature, flavonoids and their glycosides produce an abundance of negative ions; however, the product ions resulting from low-energy fragmentation are limited in their ability to reveal structural composition. Analysis of protonated flavonol glycosides has been successful in yielding a broader range of product ions, but has been severely hindered by low signal intensities. Nevertheless, recent MS experiments using positive mode ESI combined with cation adduction have demonstrated the ability to produce a rich array of product ion spectra and structural information while maximizing signal intensity. 

Aluminum, alkaline earth, alkali, and transition metals have all been used to generate positive adducts of flavonoids and their glycosides. Often, an auxiliary ligand was used in conjunction with transition metals to enhance positive ion yield and generate useful fragmentation patterns. 

These methods of fragmenting flavonoid-metal adducts offer an attractive method to elucidate flavonoid structure; however, they often rely upon differential signal intensities to distinguish between isomeric variants. High-resolution ion mobility spectrometry (IMS), on the other hand, offers a unique post-ionization method of resolving gas phase isomers prior to mass analysis.

In the presence of a neutral drift gas, IMS separates gas phase ions on the basis of their respective drift times through a weak homogeneous electric field. In situations where the drift gas molecules are much smaller than the analyte ions, the observed ion mobility is proportional to the ion-neutral collision cross section, \( \Omega \), and charge, \( z \). When combined with MS, ion mobility provides a degree of specificity unattainable by MS alone. Ion mobility–mass spectrometry (IMMS) instruments have been used to derive conformational information for proteins, peptides, carbohydrates, and other biologically significant systems. However, until recently, reliable fragmentation data derived from mobility selected ions proved difficult to obtain. Combining an atmospheric pressure–dual gate-ion mobility spectrometer (DGT) with a quadrupole ion trap (QIT), it is possible to accumulate mobility selected ions prior to MS analysis. Using this instrumental configuration, we have investigated the separation characteristics of select isomeric flavonoid diglycosides adducted with sodium, potassium, and silver.

**EXPERIMENTAL**

**Chemicals and materials**

The flavonoid diglycosides hesperidin (M.W. 610.2), neohesperidin (M.W. 610.2), and rutin (M.W. 610.2) (Fig. 1) were obtained from Sigma-Aldrich (St Louis, MO) and used without further purification. Solutions of the individual flavonoid diglycosides (100 \( \mu l \)), silver nitrate (500 \( \mu l \)) (Sigma-Aldrich, St Louis, MO), and the chloride salts of potassium and sodium (100–500 \( \mu l \)) were prepared in a 90:10 mixture of methanol and water (J. T. Baker, Phillipsburg, NJ). Hesperetin (M.W. 302.3, Sigma-Aldrich, St Louis, MO) and quercetin (M.W. 302.3, Sigma-Aldrich, St Louis, MO), the respective aglycones of the isomeric flavonoid diglycoside series of mass 610, were prepared using the same conditions as the diglycosylated species. Naringin (Sigma-Aldrich, St Louis, MO) and narirutin (Indofine Chemical, Hillsborough, NJ), which possess a M.W. of 580.2 (Fig. 1), were also examined in the presence of silver, sodium, and potassium. Unless noted otherwise, a molar ratio of 2:1 was used for all of the metal-flavonoid diglycoside solutions, except in the case of silver where the ratio was increased to 5:1.

**Instrumentation**

**Electrospray source**

The electrical connection was applied to the sample solution through a zero–dead volume stainless steel union (Valco Instruments Co. Inc., Houston, TX) and was held 3 kV above the first ion mobility drift ring operated at 9.5 kV. Connected inline with the stainless steel union was a 13 cm fused silica, polyimide capillary (Polymerich Technologies, Phoenix, AZ). The electrospray capillary was held in position by a PTFE Teflon™ cylinder as shown in Fig. 2. Using a syringe pump, sample solutions were infused at a rate of 2 \( \mu l / min \).

**Dual gate-ion mobility-quadrupole ion trap mass spectrometer**

As the fundamental operating principles of dual gate-ion mobility coupled with QIT-MS have previously been described in detail, only a brief outline is presented. The stacked ring ion mobility tube constructed at Washington State University comprised alternating conductive stainless steel and insulating alumina rings. As shown in Fig. 2, differing sets of conducting drift rings were used throughout the IMS tube with the wider rings (6 × 47 mm, width and i.d., respectively) being employed for the desolvation region and the thinner conducting rings (4.25 × 47 mm, width and i.d., respectively) utilized for the drift region and ion trap interface. Electrical contact between the conducting stainless steel rings of the drift region was made through a series of 1 MΩ resistors (Caddock Electronics Inc, Roseburg, OR). By applying 9.5 kV to the ion mobility tube, a potential gradient of \( \sim 343 \) V/cm was created across the 24 cm long drift region. Two Bradbury–Nielsen ion gates separate the desolvation region and ion trap interface from the drift region. These two ion gates, shown in Fig. 2, consist of electrically isolated alternating sets of Alloy 46 wires (California Fine Wire Co., Grover Beach, CA) spaced 0.65 mm apart. Desolvation of electrosprayed ions was aided by a \( \sim 11/\min \) countercurrent flow of nitrogen gas held at 210°C. This desolvating drift gas was introduced by transmitting the gas through a hollow drift ring possessing radially distributed apertures directed toward the entrance of the drift tube. Throughout the described experiments, the ambient pressure in Fullman, WA, ranged from 690–710 Torr.
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Once through the second ion gate, ions were directed toward the heated transfer capillary (210°C) of an LCQ Deca (Thermo Electron, San Jose, CA) by an electric field equal to the drift region. The potential applied to the heated stainless steel capillary located at the end of the ion mobility system was varied to optimize transmission for selected ion populations. After determining the $m/z$ ratio of the parent ions, collisions were induced using an isolation window of 5 $m/z$. Collisionally activated dissociation (CAD) experiments were performed. Each displayed fragment ion spectrum was an average of 50 scans.

Figure 1. Selected flavonoid diglycosides examined using cation adduction and dual gate-atmospheric pressure ion mobility-quadrupole ion trap mass spectrometry.
The CAD energy used for fragmentation was varied to preserve 10–20% of the parent ion with respect to the most intense fragmentation peak.

**Instrumental modes of operation**

In addition to functioning as an ion transmission device, the ion mobility system may be operated in a dual gate scanning (DGS) or single mobility monitoring (SMM) mode. For both DGS and SMM experiments, the pulsing of the 1st ion gate was synchronized with the injection cycle of the QIT. The 2nd ion gate was synchronized in a similar fashion; however, its pulsing was delayed by a user specified quantity.

In the DGS experiment, the delay of the 2nd ion gate was sequentially increased to cover the range of desired drift times, while the ion gate pulse widths were held at 0.3 ms. To obtain a complete mobility spectrum, the ion transmission at each 2nd ion gate delay was recorded, with the final spectrum being a composite of the transmission at each step. Consequently, the incremental stepping function used to collect the double gate IMS spectra was slow compared to the traditional signal averaged IMS experiment using a single ion gate. To maximize efficiency, once a complete ion mobility scan was acquired, only a narrow range of drift times were examined for future SMM and DGS experiments.

Using the drift time(s) obtained from the DGS experiment, the SMM experiment, which employs a static 2nd gate drift region, the ion transmission at each 2nd ion gate delay was recorded, with the final spectrum being a composite of the transmission at each step. Consequently, the incremental stepping function used to collect the double gate IMS spectra was slow compared to the traditional signal averaged IMS experiment using a single ion gate. To maximize efficiency, once a complete ion mobility scan was acquired, only a narrow range of drift times were examined for future SMM and DGS experiments.

The Xcalibur suite of programs (Thermo Electron, San Jose, CA) was used to collect both m/z and IMS spectra produced by the DGS experiment. In addition to specifying the m/z range to be scanned along with the ion trap injection time, the Xcalibur program allowed the user to define the number of μ-scans or ion trap scan averages included for each data point. The number of ion mobility gate pulses accumulated during each QIT injection cycle was dependent upon the scan frequency of the IMS experiment and length of ion injection. Unless stated otherwise, each point within the presented ion mobility spectrum was the average of 750 ion gate pulses.

**Ion mobility calculations**

The reduced mobility values, \( K_0 \), reported were calculated using the following equation:

\[
K_0 = \frac{L^2}{t_d V} \times \frac{P}{760} \times \frac{273.15}{T} \tag{1}
\]

In the equation, the parameter \( L \) is the drift region length (24 cm), \( t_d \) is the drift time in seconds, \( V \) is the potential applied across the drift region (8240 V), \( T \) is the effective temperature of the system (483.2 K), and \( P \) is the pressure during the course of the experiment (690–710 Torr). The ion-neutral cross section, \( \Omega \), may also be derived from the ion mobility experiment.

\[
\Omega = \left( \frac{3}{16N} \right) \left( \frac{2\pi \mu kT}{\mu kT} \right)^{1/2} \left( \frac{z e t_d V}{L^2} \right) \tag{2}
\]

In addition to the parameters defined in the previous equation, this equation uses the number density of the drift gas, \( N \); the reduced mass, \( \mu \); Boltzmann’s constant, \( k \); the number of charges on the ion, \( z \); and the elementary charge, \( e \).

**RESULTS AND DISCUSSION**

When cation adduction is used as a strategy for the analysis of flavonoids in the positive mode, the valence state of the metal ion influences the abundance and ratio of the observed complex. For di- and trivalent cations, a 2:1 flavonoid-metal ratio is often observed. While effective for the production of positive flavonoid species, this method often requires multiple stages of MS\(^n\) analysis for structure elucidation and isomeric differentiation. Further, this approach may encounter difficulties if the 2:1 complex gets formed in the presence of isomeric flavonoids. To retain the flavonoid...
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glycoside complex in the positive mode but eliminate the occurrence of 2:1 flavonoid-metal ratios, auxiliary ligands have been used to coordinate the metal ion.\textsuperscript{23–28} In addition to producing structurally relevant product ions, the use of auxiliary ligands resulted in an increase in flavonoid glycoside signal intensity when compared to protonation or deprotonation. Unfortunately, however, this approach has been limited to flavonoid systems that possess a 4-keto group accompanied by at least one hydroxyl group located at the 3rd or 5th position of the fifteen-carbon skeleton.\textsuperscript{25} The silver adduction technique described by Zhang and Brodbelt is not limited in this respect and offers a more universal approach to the analysis of flavonoid–metal complexes in the gas phase.\textsuperscript{29}

\textbf{Silver-adducted flavonoid diglycosides}

Using silver as the coordinating metal, flavonoid diglycosides routinely produce the losses corresponding to the rhamnose residue (R, 146 Da), core aglycone (A), and disaccharide group (D, 308 Da). Further dissociation pathways are observed that include combinations of the above groups with and without the loss of water. Combined with the loss of the aglycone moiety, the removal of 64 Da is occasionally observed. These losses are believed to result from consecutive fragmentations that may include water and C\textsubscript{2}H\textsubscript{6}O or two water molecules and CO.\textsuperscript{29} Below the structure of the cation-adducted hesperidin, these fragmentation pathways are illustrated in Fig. 3. The product ion spectra shown in Fig. 3 were derived from the 1:1 flavonoid-metal complex.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{silver-adducted-flavonoid-diglycosides.png}
\caption{Structure and characteristic fragmentation pattern of silver-adducted flavonoid diglycosides. The structure of hesperidin is shown as a guide, with the product ion spectrum that originates from a mixture of silver-adducted isomeric flavonoid diglycosides (M.W. 610). The silver isotopes 107 (52\%) and 109 (48\%) give rise to the observed peak doublets. Fragmentation of the 717/719 parent species produced 7 primary peaks, with the three primary species being the loss of rhamnose (m/z: 571/573, \(\text{--R}\) (146 Da)), the aglycone moiety (m/z: 415/417, \(\text{--A}\)), and disaccharide group (m/z: 409/411, \(\text{--D}\) (308 Da)). In addition to the loss of water, product ions that result from multiple losses are observed. The loss of the aglycone and 64 Da is believed to occur through consecutive losses, with the 64 Da corresponding perhaps to water, followed by 46 Da (C\textsubscript{2}H\textsubscript{6}O or H\textsubscript{2}O and CO). The fragmentation diagram also shows the cross-ring cleavage observed for sodium-adducted flavonoid diglycosides.}
\end{figure}
(m/z 717/719) arising from a mixture of the 610 isomeric flavonoid diglycoside series. As not all flavonoid diglycosides produce the fragmentation pathways described above, a mixture was used to demonstrate the range of product ions possible. It should be noted that the peak doublets observed in Fig. 3 originate from the characteristic isotopes of silver, Ag<sup>107</sup> (52%) and Ag<sup>109</sup> (48%).

The union of DG-IM-QIT MS affords the opportunity to obtain detailed product ion spectra from mobility selected ions. The mobility separation and resulting product ion spectra for three isomeric flavonoid diglycosides adducted with silver are shown in Fig. 4. Using the DGS mode of operation, the respective reduced mobility values (Table 1) of the individual standards, hesperidin, neohesperidin, and rutin were determined as shown in Fig. 4, spectrum #2. The mixture separation of these three isomers along with the drift time’s windows used to selectively accumulate ions prior to fragmentation are shown in spectrum #1 of Fig. 4. Using the DGS mode of operation, the respective reduced mobility values (Table 1) of the individual standards, hesperidin, neohesperidin, and rutin were determined as shown in Fig. 4, spectrum #2. The mixture separation of these three isomers along with the drift time’s windows used to selectively accumulate ions prior to fragmentation are shown in spectrum #1 of Fig. 4. The product ion spectra of silver-adducted hesperidin (1a)) and neohesperidin (1b)), both m/z 717/719, resulted from the selective fragmentation of ions originating from drift time windows (a) and (b) respectively. Also shown in Fig. 4 is the fragmentation pattern of the rutin, silver complex (1c)), that was derived from drift window (c).

The two product ion spectra originating from hesperidin and neohesperidin, both 7-O-diglycosides, display virtually identical fragmentation patterns with predominant losses of rhamnose (R), the core aglycone (A), and the disaccharide group (D). The product ion spectrum of the rutin–silver complex, on the other hand, lacks the characteristic loss of the disaccharide group. Hence, the presence of the m/z 409/411 ion may be used as diagnostic marker when distinguishing between the 7-O-diglycosides and rutin; however, conclusive assignment of hesperidin and neohesperidin remains elusive without the aid of the preseparation that was provided by the ion mobility spectrometer. The product ions observed throughout the reported CAD experiments are summarized in Table 2.

The mobility characteristics of a second set of isomeric flavonoid diglycosides adducted with silver were determined using the methods described above. The reduced mobility values and their corresponding RSDs determined using the DG-IM-QIT MS technique for silver-adducted narirutin and naringin (M.W. 380) were 0.83 cm<sup>2</sup>/(V·s) ± 0.66% and 0.81 cm<sup>2</sup>/(V·s) ± 0.65%, respectively. As seen in Fig. 5, this difference was not large enough to allow for the baseline resolution of the two isomers. Nevertheless, the difference between these two isomers was sufficient for partial resolution. The mobility spectrum of the narirutin standard (Fig. 5) displays a pronounced shoulder, which indicates the presence of a gas phase conformer. An alternative conformation may arise from different modes of silver adduction and consequent charge location. The product ions derived from the SMM experiment of both narirutin and naringin are reported in Table 2. Both narirutin and naringin display the dominant loss of the rhamnose residue along with the aglycone group. Additional fragments are observed; however, these tend to be much less intense, reducing their utility as diagnostic ions.

### Table 1. Reduced mobilities and ion-neutral collision cross sections determined for select flavonoid diglycosides

<table>
<thead>
<tr>
<th>Flavonoid</th>
<th>Cation adduct</th>
<th>M.W.&lt;sup&gt;a&lt;/sup&gt;</th>
<th>K&lt;sub&gt;v&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
<th>K&lt;sub&gt;v&lt;/sub&gt; RSD&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Ω&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Ω&lt;sub&gt;RSD&lt;/sub&gt;&lt;sup&gt;d&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Hesperidin</td>
<td>Ag&lt;sup&gt;+&lt;/sup&gt;</td>
<td>717</td>
<td>aw 0.81</td>
<td>0.71</td>
<td>201.2</td>
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<td></td>
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<tr>
<td></td>
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<td>0.78</td>
<td>0.61</td>
<td>207.1</td>
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<td>0.77</td>
<td>0.61</td>
<td>210.1</td>
<td>0.6</td>
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<td>Neohesperidin</td>
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<td>0.65</td>
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<td></td>
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<td>0.78</td>
<td>0.64</td>
<td>208.7</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>K&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>0.80</td>
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<td>0.78</td>
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<td>Naringin</td>
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<td>0.64</td>
<td>208.4</td>
<td>0.7</td>
</tr>
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<td>Quercetin</td>
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<td>1.18</td>
<td>0.69</td>
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</table>

<sup>a</sup> The values reported are for the isotopic pair (107Ag/109Ag), though only the M.W. for the 107Ag isotope is reported in tabular form. The italicized values correspond to the second gas phase conformation of the given flavonoid diglycoside.

<sup>b</sup> The units of the reported reduced mobility, K<sub>v</sub>, are cm<sup>2</sup>/(V·s).

<sup>c</sup> The relative standard deviation (RSD) is expressed as a % of the reported value.

<sup>d</sup> Ion-neutral collision cross section measured in Å<sup>2</sup>.

### Sodium-adducted flavonoid diglycosides

To examine the effect of sodium adduction, experiments analogous to those conducted with silver were conducted using both DGS and SMM. Figure 6 illustrates the mixture separation of the M.W. 610 isomers adducted with sodium. While the mixture contained only three compounds, four distinct peaks were observed. Closer examination of the mobility spectrum acquired for the hesperidin standard (Fig. 6) shows two distinct conformers. When adducted to sodium, the narirutin standard also displays two pronounced conformations in the gas phase (Fig. 7). Unfortunately, the peak corresponding to the second conformer of narirutin significantly overlaps with the sodium-adducted naringin species. Despite the resolution of the primary sodium-adducted narirutin conformer, this overlap interferes with the SMM, making quantitative isomeric differentiation using this method difficult.

Compared to the silver-adducted species, the sodium complexes of both isomer sets displayed a broader range of CAD fragmentation pathways. In addition to the losses of the rhamnose, aglycone, and disaccharide groups, a prominent cross-ring cleavage pattern was observed. The 1<sup>3</sup>B fragment,
Table 2. Primary CAD product ions derived from cation-adduct flavonoid diglycosides

<table>
<thead>
<tr>
<th>Flavonoid</th>
<th>Cation</th>
<th>Parent m/z</th>
<th>Product ions derived from cation-adducted flavonoid diglycosidesa</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>−H₂O</td>
</tr>
<tr>
<td>Hesperidin</td>
<td>Ag</td>
<td>717 (17)</td>
<td>− 571 (100)</td>
</tr>
<tr>
<td></td>
<td>Na</td>
<td>633 (13)</td>
<td>615 (20) 487 (19)</td>
</tr>
<tr>
<td></td>
<td>K</td>
<td>639 (12)</td>
<td>− 503 (100)</td>
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<tr>
<td>Neohesperidin</td>
<td>Ag</td>
<td>717 (15)</td>
<td>− 571 (100)</td>
</tr>
<tr>
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<td>K</td>
<td>619 (19)</td>
<td>− 473 (100)</td>
</tr>
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</table>

a The m/z ratio of each ion species is reported along with its relative intensity in parentheses. Those ions possessing an intensity of 1–5% are indicated by a ‘*’, undetected ions are labeled with the character ‘−’.

b The values reported are for the isotopic pair (107Ag/109Ag), though only the M.W. for the 107Ag isotope is reported in tabular form.

c The 1–3 B neutral loss comprised the entire B-ring of the aglycone in addition to the 2 and 3 carbons of the C-ring (Refs 18 and 49).

d Possible explanations for the loss of 64 Da include consecutive losses involving the loss of water and a C₂H₆O group or/and additional water and CO.
Figure 4. Spectrum #1 illustrates the ion mobility mixture separation of the three isomers, hesperidin, neohesperidin, and rutin adducted with silver. Spectrum #2 shows the overlaid ion mobility spectra of the respective standards. Through the use of SMM, the ions contained in the drift time windows (a), (b), and (c) were fragmented to produce the spectra shown in 1(a), 1(b), and 1(c), respectively. Shown in bold text in 1(a) and 1(b), the presence of the ions 409 and 411 may be used to confirm the presence of either hesperidin or neohesperidin. However, the IMS separation prior to mass analysis is necessary to conclusively distinguish between the three isomers.

as shown in Fig. 3, consists of the B-ring combined with the 2 and 3 carbons of the aglycone C-ring (also referred to as the retro-Diels Alder pathway). Interestingly, the cross-ring cleavage was observed only for the 7-O-linked diglycosides and was always accompanied by an additional loss of the rhamnose residue. It should be noted that the CAD spectra acquired for secondary conformations did not yield fragmentation patterns different from the primary conformation.

Potassium-adducted flavonoid diglycosides

The behavior of potassium adducts for both sets of isomers was similar to that observed for sodium; however, their intensity and product ion spectra were dramatically decreased. The mobility spectra of the potassium complexes of narirutin and naringin are also shown in Fig. 7. The conformation of second narirutin-potassium adduct again overlaps with the naringin-potassium complex, making detailed CAD experiments difficult for mixtures. The ion mobility spectra of the potassium complexes of hesperidin, neohesperidin, and rutin are shown in Fig. 8. The mixture separation again displays four clearly resolved peaks, though the appearance of the fourth peak is not solely attributed to a second conformer of hesperidin. As the spectra of the individual flavonoid diglycoside standards shown in Fig. 8 indicate, neohesperidin also formed a second conformation when adducted with potassium. Only the loss of the rhamnose and diglycoside residues were observed for the product ion spectra of the potassium-adducted flavonoid diglycosides (Table 2). Because of their relatively small signal intensities, further identification of product ion peaks derived from potassium adducts proved difficult.

Flavonoid aglycones

Previous work by Brodbelt and coworkers suggests that the presence of the 4-keto and a neighboring hydroxyl group are necessary to form stable metal–flavonoid complexes. As they demonstrated, silver represents the exception to this trend and forms gas phase complexes with flavonoid aglycones. To further investigate the structural requirements of flavonoids required to bind metal species in the gas phase, the separation characteristics of the isomeric aglycones, hesperetin and quercetin, were examined. Hesperetin forms the core structure of hesperidin and neohesperidin, whereas quercetin serves as the core of rutin. Figure 9 shows the separation of quercetin and hesperetin as [M + Ag]⁺ ions. The most notable characteristic of this isomeric separation is the order of aglycone migration. As shown in Figs 4, 6, and 8, the drift times of the rutin complexes were significantly greater than those formed with hesperetin-derived diglycosides.
Influence of cation adduction on the ion mobility separation characteristics of flavonoid diglycoside isomers

Figure 5. Dual gate-ion mobility separation of the flavonoid diglycoside isomers (M.W. 580), narirutin and naringin adducted with silver. The peak profile of the narirutin displays a tailing shoulder, indicating the presence of a unresolved gas phase conformer.

This apparent reversal in migration order indicates that the carbohydrate group plays a dominant role in the separation characteristics of flavonoid diglycosides.

Though formed, the adducts of silver, sodium, and potassium with the aglycone species were found to be much less intense than that formed with the protonated aglycone. One possible explanation for the relative absence of metal–cation adducts may be the collisional cooling of ions through the ion mobility experiment. Compared to direct analysis of species formed at atmospheric pressure, ion mobility separations afford the benefit of collisional cooling prior to mass analysis. Such thermalization can minimize the observation of species that occupy less favorable transition states. No doubt, metal-aglycone species may be formed in the gas phase; however, given the effective temperature and collisional cooling of the ion mobility experiment, it is entirely possible that this species did not survive for mass analysis.

The absence of the carbohydrate group and its ability to stabilize the metal cation may provide an alternative explanation for the lack of cation-adducts of the aglycone species. This observation is further supported by the abundance and degree of flavonoid diglycoside product ions that are derived, and which contain an intact metal-carbohydrate residue (Table 2). The loss of the disaccharide residue for the examined flavonoid–metal complexes were of low intensity and in some cases absent. The removal of the rhamnose residue, on the other hand, was readily observed for all adducted flavonoid species. Combined with the aglycone separation data, the relative fragmentation patterns of the metal-adducted flavonoid diglycosides suggest that the carbohydrate group is often necessary to stabilize the metal ion in the gas phase – specifically the presence of a single monosaccharide residue located on the aglycone skeleton.

Deprotonated flavonoid diglycosides

In the negative mode, the separation characteristics of hesperidin, neohesperidin, and rutin are markedly different. Shown in Fig. 10 are the overlaid ion mobility spectra for the [M – H]− ions of the M.W. 610 isomer series. The deprotonated form of rutin displays a comparatively narrow peak width compared to the hesperetin-derived flavonoid diglycosides. In the case of the negatively charged hesperidin and neohesperidin, the peak profiles were overly broad and
The observed collision cross sections and separation order for hesperidin, neohesperidin, and rutin confirms assertions made by previous studies using computational methods and cobalt adduction. Briefly, Zhang, Brodbelt, and Wang modeled the helium accessible surface areas of the cobalt complexes with hesperidin, neohesperidin, and rutin. Compared to the metal complexes observed in this study, those formed with cobalt were found to contain two flavonoid diglycoside moieties for each metal ion. Using a custom-built genetic algorithm, Zhang, Brodbelt, and Wang found rutin to possess the largest helium accessible surface area and hesperidin the smallest. Though qualitative, this trend may be indicative of the relative binding affinities of the studied metals and flavonoid diglycosides.

In addition to the data derived from previous modeling studies, the differences in ionic radii and coordination geometries of the examined metals represent one potential explanation for ion-neutral cross section trends observed for the metal–flavonoid diglycoside complexes. This explanation, however, must be tempered with the knowledge that radii data currently available for ionic metal cations
Influence of cation adduction on the ion mobility separation characteristics of flavonoid diglycoside isomers

Figure 9. Separation of the protonated aglycone isomers, quercetin and hesperetin. Quercetin, the aglycone of rutin, displays a drift time of 1.22 cm²/(V·s), whereas hesperetin, the aglycone of hesperidin and neohesperidin, possesses a drift time of 1.18 cm²/(V·s). Compared to the separation order observed for the diglycosides isomers, the order of separation for the aglycone moieties are reversed. These data suggest that the linkage position of the disaccharide group plays a dominant role in the ion mobility separation process.

Figure 10. Overlaid negative mode ion mobility spectra of the deprotonated [M – H]⁻ forms of hesperidin, neohesperidin, and rutin. Compared to their cation-adducted forms, the deprotonated flavonoid diglycosides are not resolved in the ion mobility dimension. Multiple deprotonation sites may contribute to the abnormally broad peak profiles observed for the hesperetin-derived flavonoid diglycosides.

have been derived predominantly from solid molecular structures. Additionally, these ionic metal radii are highly dependent on the coordination geometry of the system and the corresponding anion. In light of these qualifications, silver has been found to possess ionic radii smaller than both sodium and potassium and the ability to adopt geometries with smaller coordination numbers. The range of sodium and potassium coordination geometries span from IV to XII with seven different configurations each. Regardless of the coordination number, potassium was found to have the largest ionic radius. Silver, on the other hand, was shown to occupy six different coordination geometries ranging from II to VIII including the square planar configuration. While experimental data regarding the coordination geometry and ionic radius of metal ions in the gas phase has yet to be convincingly furnished, the use of currently known radii data offer one potential explanation for the observed separation order of metal-adducted flavonoid diglycosides. Further elucidation of ion mobility separation trends may be provided by molecular modeling; however, numerous challenges exist until such goals are realized.

While molecular modeling has been used extensively to model ions in vacuum, to effectively model the IMS experiment and infer molecular structure, the interactions between the analyte ion and neutral drift gas must be considered. To simplify this task computationally, helium has often been used as the neutral drift gas. Experimental results obtained using larger drift gas molecules, such as nitrogen, have proven exceedingly difficult to interpret using existing computational methods as only a limited number of Leonard–Jones parameters are available for these drift gas molecules. When experimental resolution is paramount, as in the case of isomeric separations, drift gas molecules larger than helium and possessing smaller mean free paths, are often employed. These larger drift gas molecules afford a larger number of ion–neutral interactions over the course of the IMS experiment. As resolution of the flavonoid diglycosides examined in this study was achieved using nitrogen as a neutral drift gas, molecular modeling on this system was not performed.

CONCLUSIONS

Using a dual gate-ion mobility-quadrupole ion trap mass spectrometer, three sets of isobaric flavonoids were resolved from a mixture prior to mass analysis. Secondary gas phase conformations of the flavonoid diglycosides were observed when sodium and potassium were used as the complexing cation. In the case of hesperidin, neohesperidin, and rutin, these additional peaks were resolved from the primary flavonoid diglycoside species, whereas the secondary...
conformers of narirutin and naringin adducted with sodium or potassium were found to significantly overlap. Because the sodium and potassium adducts of narirutin and naringin were unresolved, CAD experiments for mixtures of these compounds were not possible. This was an unfortunate occurrence, as a larger array of CAD pathways were observed for sodium adducts. Further, because of the presence of additional conformations, quantification using sodium and potassium as adducts may prove difficult. Compared to silver and sodium addition, potassium complexes of the examined flavonoid diglycosides were of low intensity and yielded a very limited amount of product ions. Silver, in general, did not form secondary gas phase conformations and displayed the most compact flavonoid diglycoside species, followed by sodium, then potassium. Additionally, the silver complexes displayed an abundance of signal intensity and moderate levels of CAD pathways. The aglycones of the M.W. 610 isomer series, interestingly, displayed a separation order directly opposite to their diglycosylated forms. This information, combined with further examination of the fragment pathways of the flavonoid diglycosides, suggests that the carbohydrate group plays a large role in stabilizing the metal cation in the gas phase. Silver has demonstrated the ability to form robust complexes with flavonoid diglycosides that may provide a wide variety of CAD pathways. Combined with silver adduction, DC-IM-QIT MS (dual gate-ion mobility-quadrupole ion trap mass spectrometry) offers the ability to form robust complexes with flavonoid diglycosides, suggesting that the carbohydrate group plays a large role in stabilizing the metal cation in the gas phase. Silver has demonstrated the ability to form robust complexes with flavonoid diglycosides that may provide a wide variety of CAD pathways. Combined with silver adduction, DC-IM-QIT MS (dual gate-ion mobility-quadrupole ion trap mass spectrometry) offers a unique method to separate and conclusively identify isomeric flavonoid diglycosides in simple mixtures.

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REFERENCES


