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Electrospray Mass Spectrometry and UV/Visible Spectrophotometry Studies of Aluminum(III)–Flavonoid Complexes

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Aluminum(III) complexes formed by the isoflavone biochanin-A and the flavones kaempeferol and quercetin in CH_3OH, CH_3CN, i-C_3H_7OH, and the mixed solvents CH_3CN/CH_3OH (1/1 v/v), CH_3CN/H_2O (1/1 v/v) CH_3CN/CH_2Cl_2 (1/1 v/v) and CH_3CN/i-C_3H_7OH (1/1 v/v), were investigated by means of electrospray mass spectrometry (ES-MS) and UV/visible absorbance spectrophotometry. Electrospray mass spectra of solutions that were indicated by their UV/visible spectra to have formed an Al-flavonoid complex displayed singly-charged ions corresponding in m/z to the 1:2, dimeric complex, viz., $[AlL_2]^+$, L = (flavonoid $- H^+)^-$. Lesser abundant singly-charged ions corresponding in m/z to $[AlR(L)]^+$, where R = H^-, HO^- or CH_3O^- were also sometimes observed. Formation of the 1:2 complex was favored in CH_3OH relative to all other solvent systems investigated. The relative amounts of complexed and free flavonoid in solution, as estimated by the appearance of the UV/visible spectra, correlated well with the abundance ratio of the 1:2, Al-flavonoid dimer to the protonated flavonoid (indicative of free flavonoid) observed in the ES mass spectrum of the same solution. © **1998 John Wiley & Sons, Ltd.**

KEYWORDS: flavonoids, electrospray mass spectrometry, UV/visible spectrophotometry, aluminum(III), complexes

INTRODUCTION

Bioflavonoids are an important group of plant phenolics, diverse in both chemical structure and suggested biological function.[1,2] Among a few of their important biological functions in animals are those of antioxidants, free radical scavengers, and metal chelators. In their role as antioxidants and free radical scavengers, for example, these compounds are thought to be important in preventing atherosclerotic plaque formation and in protecting against oxidative tissue damage related to neurodegeneration, aging, and the development of cancer.[2,3] As metal chelators, the bioflavonoids may play a role in both the bioavailability and toxicity of a variety of metals. For example, the complexation of Al(III) by various flavonoids has been suggested to reduce the overload of aluminum in the diet (such as that supplied by teas), a metal which has been implicated in Alzheimer's disease, among other maladies.[4]

From an analytical standpoint, this capability for metal complexation has been exploited both for identification of flavonoid structure[1] and for elemental determinations.[5,6] Flavonoids contain a highly conjugated aromatic system (see structures below), and therefore, exhibit intense, characteristic absorption or fluorescence spectra. Chelation with various metals causes spectral shifts that can be correlated with flavonoid structure. The UV/visible absorption spectra of flavonoids in the presence of Al(III) have been used to distinguish flavonoids that contain either a free 5- or 3-hydroxyl group. Flavonoids that do not contain these groups do not form complexes with Al(III) and therefore their spectra are unaltered.[1] Among those flavonoids that do complex Al(III), morin, quercetin and kaempferol have been used in the fluorometric determination of Al(III). In a neutral or acidic medium (typically ethanol), the characteristic fluorescence of the chelate provides detection levels for Al(III) as low as a few parts-per-billion.[5–7]

Despite the widespread use of these metal chelation reactions, debate continues regarding the stoichiometry of the Al(III)-flavonoid complex or complexes formed in solution. Experimentation has shown that a free 5- and or 3-hydroxyl group is necessary for complex formation, with metal binding apparently involving both this hydroxyl group and the 4-keto group. In various studies, employing several solvent systems, complexes of four different Al(III)-flavonoid stoichiometries have been proposed, viz., 1:1, 1:2, 1:3 and 2:3.[5–7]

To better understand Al(III)-flavonoid complexation, a method other than spectrophotometry and fluorescence is warranted. Traditional means to determine metal-ligand complex stoichiometry and structure include NMR and electrochemistry. ^{27}Al-NMR and potentiometric studies have been used, for example, to

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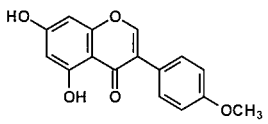
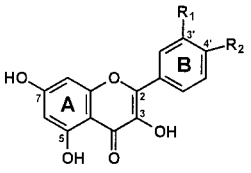
characterize the Al(III) chelates of several phenolic compounds found in a tea extract.[8] The emergence of electrospray mass spectrometry (ES-MS)[9] in recent years has greatly enhanced the application of mass spectrometry in this type of analysis. Colton and co-workers,[10] in particular, have been instrumental in demonstrating that the ions observed in the ES mass spectra of metal-ligand complexes, more often than not, reflect the ionic species present in the solution being electrosprayed. Therefore, ES-MS may be used, at a minimum, as a complement to NMR or any other means of metal-ligand characterization. Herein, our recent studies of the Al(III) complexes of the isoflavone biochanin-A (**1**, 5,7-dihydroxy-4′-methoxyisoflavone) and the flavones kaempferol (**2**, 3,5,7,4′-tetrahydroxyflavone) and quercetin (**3**, 3,5,7,3′,4′-pentahydroxyflavone) in a variety of solvents, using a combination of ES-MS and UV/visible spectrophotometry, are reported.



1: M_r = 284 Da



2: R_1 = H, R_2 = OH; M_r = 286 Da

3: R_1 = R_2 = OH; M_r = 302 Da

EXPERIMENTAL

The flavonoids and $Al(NO_3)_3 \cdot 9H_2O$ (99.997% w/w) were obtained commercially in the purest form available and used as received. Solvents were HPLC grade. Typically a stock flavonoid solution of 10 mM was prepared by dissolution in CH_3OH. Aliquots of this stock solution were further diluted with CH_3OH or another solvent, to obtain a final concentration of 100 μM. Solutions were spiked with Al^{3+} at the appropriate level (typically also 100 μM) by addition of a small volume from a stock $Al(NO_3)_3 \cdot 9H_2O$ solution (10 mM) prepared in CH_3OH or CH_3CN.

Perkin-Elmer SCIEX API165 and API365 mass spectrometers (Concord, Ontario, Canada), each equipped with a TurbolonSpray source, were used to acquire the ES-MS and ES-MS/MS (product ion spectra) data, respectively. Sample solutions were continuously infused via syringe pump at a flow rate of 5.0 μL/min. Solutions were pneumatic nebulized (N_2) without the use of the heated turbo gas. Typically, ES mass spectra were the sum of 10 scans acquired by scanning m/z 100–700 using a 0.1 m/z step size and 10 ms dwell time. Product ion spectra were acquired over a range of collision energies at a collision gas pressure, measured in the analyzer chamber, of 1.9×10^{-5} Torr (N_2). Product ion spectra discussed were the result of a single scan acquired by scanning Q3 over the m/z range of interest using a 0.1 m/z step size and 10 ms dwell time.

UV/visible spectra were obtained with a Shimadzu UV-2101PC scanning UV/visible spectrophotometer (Kyoto, Japan) using a 1 cm cell path and a scan speed of 200 nm/min (0.5 nm slit width).

RESULTS AND DISCUSSION

The UV/visible absorbance spectra of 100 μM solutions of biochanin-A (**1**), kaempferol (**2**) and quercetin (**3**) in CH_3OH, and CH_3OH containing 100 μM Al^{3+}, are displayed in Fig. 1. The UV/visible spectra of kaempferol and quercetin, are very similar. In CH_3OH alone, each spectrum shows an intense absorbance at ~370 nm (Band I) and at ~255–270 nm (Band II). With Al^{3+} added to the solution, Band I shifts to longer wavelength (Band Ia, ~430–440 nm) and also shows an inflection or lower intensity absorption to shorter wavelength (Band Ib, ~350–360 nm). Band II shifts slightly to longer wavelength (Band IIb) and shows a lower intensity absorbance at higher wavelength (Band IIa, ~305 nm). The spectrum of biochanin-A in CH_3OH shows an intense absorbance at 260 nm (Band II) and only a weak absorbance at 330 nm (Band I). In this and other isoflavones, the phenyl ring at the 3-position is not conjugated with the pyrone carbonyl group. As a result, Band I, which is associated with the conjugated lateral B ring, is either absent or considerably diminished in intensity.[1] Upon addition of Al^{3+}, the major absorbance (Band II) shifts slightly to a longer wave-

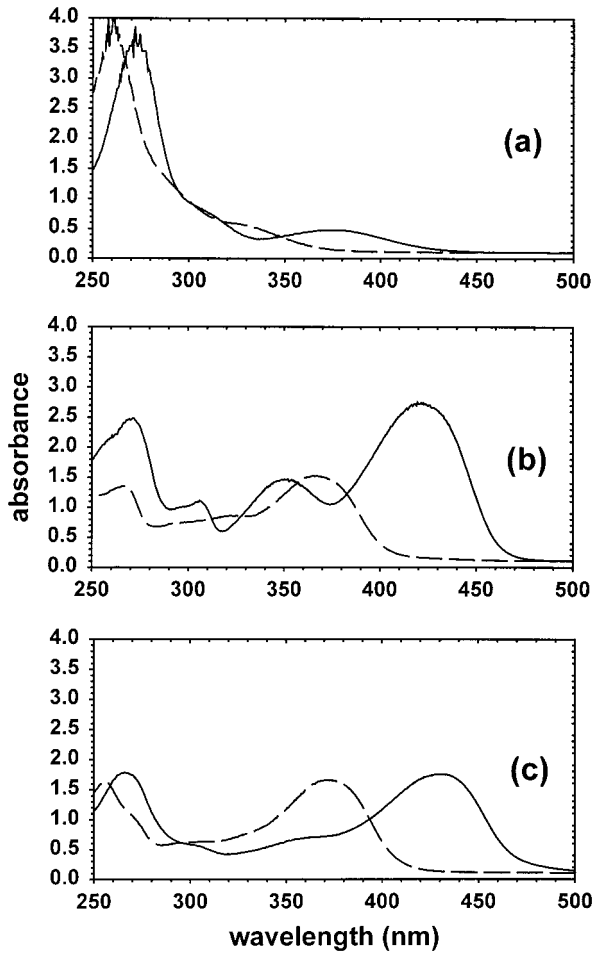


Figure 1. UV/Visible spectra of methanol solutions of (a) biochanin-A (**1**), (b) kaempferol (**2**) and (c) quercetin (**3**). Dashed curve is the spectrum recorded in methanol. Solid curve is the spectrum recorded in methanol containing 100 μM Al^{3+}. Concentration of the flavonoid in each case is 100 μM.

length. Band I also shifts to longer wavelength (~380 nm) and increases slightly in intensity.

The six UV/visible spectra in Fig. 1 are nominally the same as those reported in the literature for these molecules dissolved in ethanol and Al^{3+}-ethanol mixtures. Ethanol has traditionally been the solvent utilized for flavonoid structure determinations and for Al^{3+} determinations by means of Al(III)-flavonoid complex formation.[1,5,6] The spectral changes observed upon addition of Al^{3+} to solution have been attributed to formation of an Al(III)-flavonoid complex. There is some debate, however, over the stoichiometry of the actual complexes formed.[5–7]

The ES mass spectra obtained by spraying the solutions used in the acquisition of the UV/visible spectra presented in Fig. 1 are shown in Fig. 2. The mass-to-charge ratio of the base peak in all three spectra is consistent with the singly-charged 1:2, dimeric Al-flavonoid complex, viz., $[AlL_2]^+$, where $L = (1 - H^+)^-$ (m/z 593, Fig. 2a), $L = (2 - H^+)^-$ (m/z 597, Fig. 2b), or $L = (3 - H^+)^-$ (m/z 629, Fig. 2c). In the lower m/z range of the spectra, two relatively abundant peaks can be tentatively assigned as forms of the singly-charge 1:1, monomeric Al-flavonoid complex $[AlR(L)]^+$, where $R = H^-$ or HO^- in each case. These ions are observed at m/z 311 and 327 for **1**, m/z 313 and 329 for **2**, and m/z 329 and 345 for **3**. The abundance of these

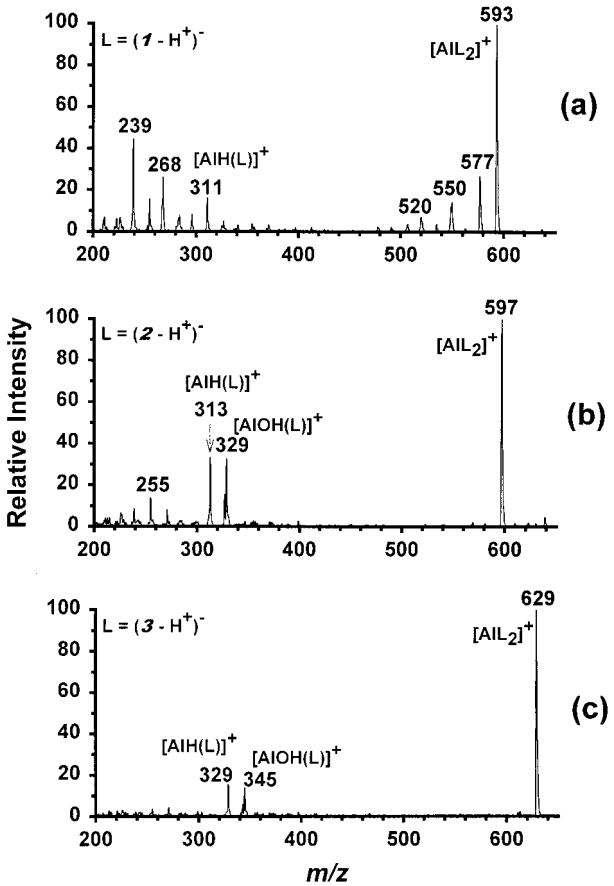


Figure 2. ES mass spectra recorded using the same solutions as those used to acquire the data in Fig. 1. (a) Biochainin-A (**1**), (b) kaempferol (**2**) and (c) quercetin (**3**). Solutions continuously infused at 5.0 μL/min.

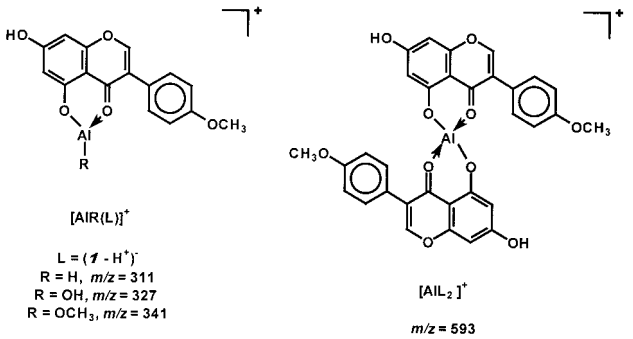


Figure 3. Proposed structures of the 1:1, and 1:2 Al-biochanin A (**1**) complexes.

two peaks in the biochanin-A spectrum is much reduced compared to the corresponding peaks in the spectra obtained for kaempferol and quercetin. This appears to be due to more facile fragmentation of these biochanin-A ions (as well as the dimer ions) under the given set of interface conditions (orifice 150 V). Under milder interface conditions (orifice 40 V), these biochanin ions are of slightly greater relative abundance. Also, an additional species corresponding in m/z to $[AlR(L)]^+$, where $R = CH_3O^-$, is observed. This species was also observed under similar interface conditions in the ES mass spectra of kaempferol and quercetin. In no case were ions corresponding to multiply-charged, Al-flavonoid complexes apparent in the ES mass spectra.

Possible structures of the 1:1 and 1:2 complexes of each of the flavonoids are shown in Figs 3, 4 and 5. The proposed structures are based on the observed m/z ratios of the ions in the ES mass spectra and on previous literature reports, which have concluded that Al^{3+} binding involves either a free 5- or 3-hydroxyl group and the 4-keto group.[1] In the case of kaempferol and quercetin, in particular, the opportunity exists to form several different dimer structures (Fig. 5). Literature reports indicate that binding of the Al^{3+} by the 3-hydroxy and 4-keto group is most favored. Therefore, one might speculate that structure B in Fig. 5, in which both flavonoid molecules bind to Al^{3+} through these

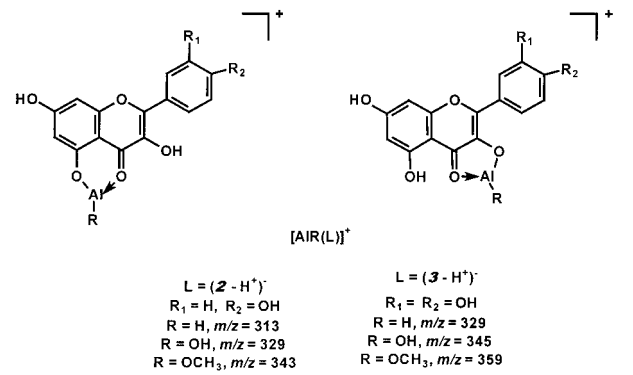


Figure 4. Proposed structures of the 1:1, Al complexes of kaempferol (**2**) and quercetin (**3**).

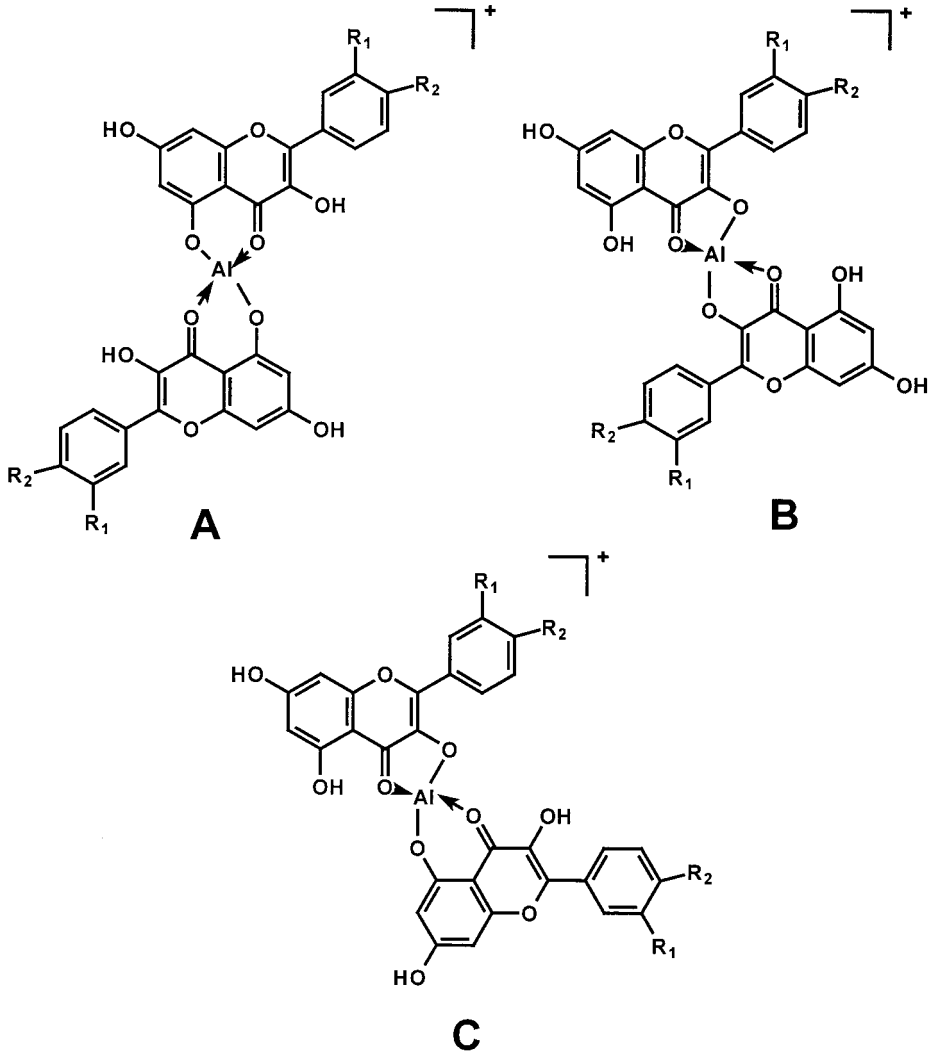


Figure 5. Proposed structures of the 1 : 2, Al complex of kaempferol (**2**) and quercetin (**3**).

positions, would be the favored structure. Of course, a number of other considerations, including steric factors, must influence the actual structure(s) adopted.

Without suitable standards for the dimeric complexes, determining the predominate structure or structures would be expected to be difficult by mass spectrometry alone. Actually sorting out these structural questions will require NMR, with tandem mass spectrometry possibly offering data that could support the NMR determined structure. Nonetheless, the base peak in the product ion spectrum of the 1 : 2, Al-complex of kaempferol (**2**) and quercetin (**3**) (data not shown) was consistent with the monomeric species $[AlOH(L)]^+$, observed at *m/z* 329 and *m/z* 345, respectively. A peak of much lower abundance was also observed in the respective MS/MS spectra consistent with $[AlH(L)]^+$ (*m/z* 313 and *m/z* 329, respectively). These fragments support the proposed dimer structures and indicate disruption of the Al-O bonding that links the individual flavones to be the predominant fragmentation channel. Furthermore, these data indicate that the ions in the ES mass spectra of these compounds that are assumed to be the 1 : 1 Al-flavonoid complex may arise in part from fragmentation of the 1 : 2 dimer complex in the atmospheric sampling interface. However, the ions corresponding to the 1 : 1 Al-flavonoid complex in the ES mass spectrum of biochanin-A were probably not formed via this route. The 1 : 2 dimer complex of bioachin-A was found by

MS/MS to fragment mainly via cleavage of the biochanin molecules rather than via disruption of the Al-O linkages (data not shown). No ions owing to the monomeric species were observed in the product ion spectra of this 1:2-dimer complex. While this is a very limited data set, the differing fragmentation of the dimers of biochanin-A, and kaempferol and quercetin suggests that one might be able to differentiate isoflavones from flavones on the basis of the gross features of the product ion spectra of the 1:2, Al-flavonoid complex. Apparently the isoflavone system is sufficiently fragile so that it fragments at a lower energy than that required to disrupt the Al-O binding required to split the dimer into monomeric pieces. Further experiments with flavone and isoflavone isomers are underway to support this initial observation.

If not formed via fragmentation of the dimer, the monomeric species observed in the ES mass spectra presented above might otherwise be formed either directly from ions present in the solution or possibly during desolvation of a multiply-charged 1:1 Al-flavonoid complex. For example, the ions observed at *m/z* 327 and 341 in the mass spectrum of biochanin-A might result from the following desolvation reactions involving the 1:1 complex solvated with H_2O (Eqn 1) and CH_3OH (Eqn 2), respectively ($L = (\mathbf{1} - H^+)^-$).

$$[Al(L)(H_2O)_2]^{2+} \rightarrow [AlOH(L)]^+ + H_3O^+ \quad (1)$$

$$[Al(L)(CH_3OH)_2]^{2+} \rightarrow [AlOCH_3(L)]^+ + [CH_3OH + H^+]^+ \quad (2)$$

At present we have no firm data to confirm their origin either way.

On the basis of the UV/visible spectra and corresponding ES mass spectra presented above, the shift in absorbance observed in the UV/visible spectra of the methanolic solutions of these flavonoids upon the addition of Al^{3+} appears to be indicative of the formation of predominantly the 1:2, dimeric Al-flavonoid complex. Additional evidence in support of this conclusion was obtained by spraying these solutions in negative ion mode. In this case only ions corresponding to the deprotonated flavonoids, formed by dissociation of an acidic phenolic group, were observed. We would expect it possible to observe under these conditions the deprotonated 1:3, AL-flavonoid complex if it had been formed in solution. However, such ions (e.g., $[AIL_3 - H^+]^-$, *m/z* 875 where $L = (\mathbf{1} - H^+)^-$) were not observed.

The UV/visible spectra in Fig. 6 and the ES mass spectra of the corresponding solutions shown in Fig. 7 illustrate the effect of the Al^{3+}:flavonoid concentration ratio on complex formation. The UV/visible spectra were recorded using a constant 100 μM concentration of quercetin with 1.0, 10 or 100 μM of Al^{3+} also in the solution. One notes that little if any complex was formed with only ~1.0 μM of Al^{3+} in the solution. No clear absorbance was noted at the wavelength (~440 nm) indicative of complexation. The spectra indicate that a significant amount of the complex was formed with 10 μM of Al^{3+} added. Nearly all of the quercetin appears complexed when 100 μm of Al^{3+} is added (disappearance of absorbance at 375 nm). When 500 μM is added, the nature of the absorbance at 440 nm changes, increasing slightly, but also shifting to longer wavelength with considerable broadening.

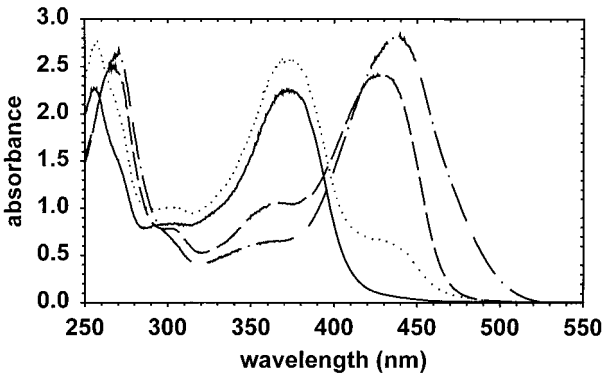


Figure 6. UV/Visible spectra of methanol solutions containing 100 μM quercetin (**3**) and differing concentrations of Al^{3+}. Solid curve = 1.0 μM Al^{3+}, dotted curve = 10 μM Al^{3+}, dashed curve = 100 μM Al^{3+}, dash-dot curve = 500 μM Al^{3+}.

The peaks observed in the ES mass spectra of these solutions reflect the same results. In Fig. 7(a), we see at low absolute abundance only ions corresponding to the protonated molecule, $[\mathbf{3} + H^+]^+$ (*m/z* 303), sodiated molecule $[\mathbf{3} + Na^+]^+$ (*m/z* 325), and a dimer that corresponds in mass to $[2(\mathbf{3}) + Na^+]^+$ (*m/z* 627). No ions assignable to an Al-flavonoid complex are observed. With 10 μM of Al^{3+} [Fig. 7(b)], the protonated and sodiated molecules are still observed (actually at slightly higher abundance), but not the $[2(\mathbf{3}) + Na^+]^+$ species. The base peak in this spectrum is the 1:2, Al-flavonoid dimer, $[AIL_2]^+$ (*m/z* 629, $L = (\mathbf{3} - H^+)^-$). Increasing the concentration of Al^{3+} to 100 μM yields the spectrum in Fig. 7(c) in which the 1:2, Al-quercetin dimer is by far the most abundant peak. The 1:1, Al-quercetin complexes (*m/z* 329 and 345) are also observed. Note that the absolute abundance of the flavonoid derived ions in this case are 10–100 greater than when the flavonoid is sprayed directly from methanol alone. Increasing the concentration of Al^{3+} further to 500 μM [Fig. 7(d)], yields a significantly more complex spectrum. Basically the signal intensity of the 1:2 complex is reduced by 99 percent. The absolute abundance of the 1:1 complexes are little changed from 7(b). However, the methoxy substituted 1:1 complex (*m/z* 359) is now observed as well as a number of other abundant ions not readily assigned as an Al-flavonoid complex. The UV/visible spectrum of this mixture (Fig. 6) also indicated a possible change in the nature of the species present in solution. It should be pointed out, however, that the high concentration of Al^{3+} might have affected the ES process, and therefore, the resulting ES mass spectrum.

We also investigated the effect of various solvents on the formation of the Al-flavonoid complexes. The UV/visible spectra of 100 μM quercetin/100 μM Al^{3+} in four different solvent systems are shown in Fig. 8. As before, in CH_3OH the maximum absorbance is noted at ~430–440 nm indicating formation of 1:2, Al-quercetin dimer. In both CH_3OH/CH_3CN (1/1 v/v) and *i*-CH_3H_7OH, the UV/visible spectrum indicates that the

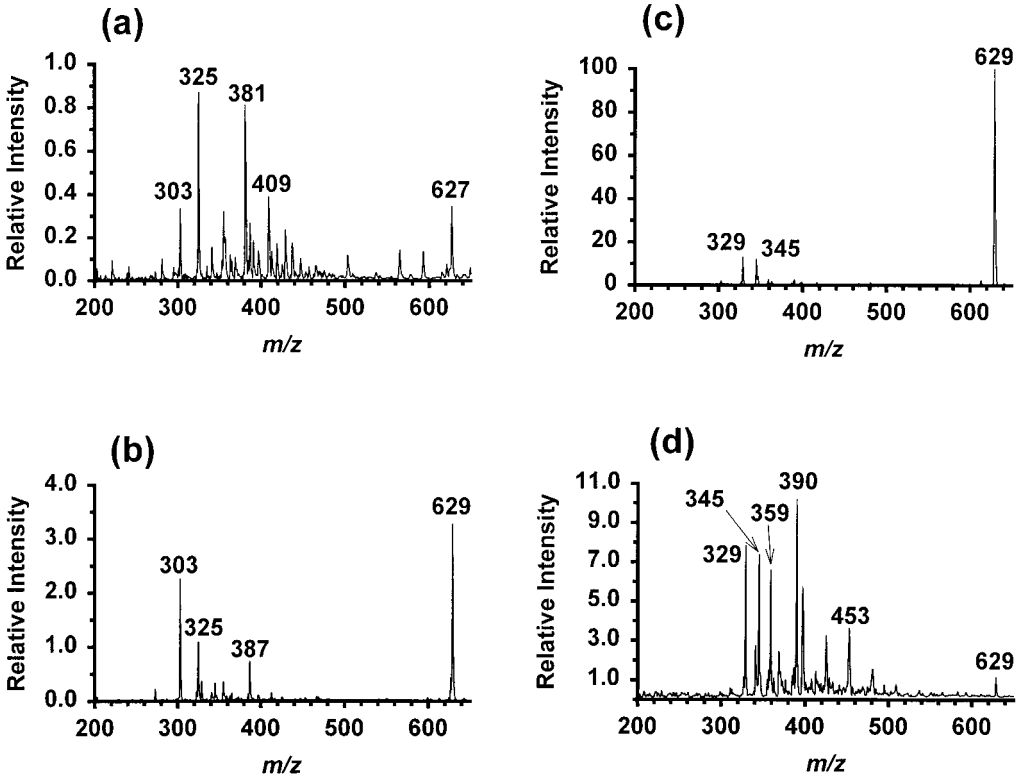


Figure 7. ES mass spectra recorded using the same solutions as those used to acquire the data in Fig. 6. (a) 100 μM quercetin (**3**)/1.0 μM Al^{3+}, (b) 100 μM quercetin (**3**)/10 μM Al^{3+}, (c) 100 μM quercetin (**3**)/100 μM Al^{3+} and (d) 100 μM quercetin (**3**)/500 μM Al^{3+}. Solutions continuously infused at 5.0 μL/min. All spectra normalized with respect to the base peak in (c).

solution contains a mixture (roughly 50% of each component assuming equal extinction coefficients, $abs_{430\ nm}/abs_{370\ nm} \approx 0.5$) of quercetin and the Al-quercetin dimer. In neat CH_3CN, the spectrum indicates that very little of the Al-quercetin complex is formed. Thus, one can conclude that the overall solvation effects in CH_3CN and the other solvents are such that Al^{3+} complexation is inhibited when compared with CH_3OH.

Once again, the ES mass spectra of these solutions reflect the UV/visible spectral observations. Figure 9 shows in bar graph form the relative abundance of the 1:2, Al-flavonoid dimer and the other major quercetin derived ion that was observed in the different spectra, i.e., the protonated molecule, $[\mathbf{3} + H^+]^+$. Note that in many cases the 1:1, Al-quercetin complexes were also present in the spectra, but at relatively low abundance

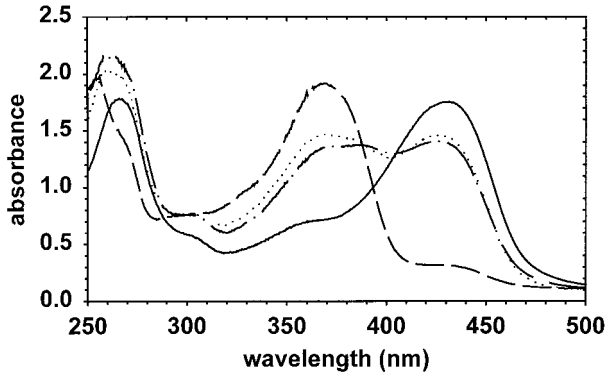


Figure 8. UV/Visible spectra of 100 μM quercetin (**3**)/100 μM Al^{3+} recorded in differing solvent systems. Solid curve = CH_3OH, dotted curve = CH_3OH/CH_3CN (1/1 v/v), dashed curve = CH_3CN and dash-dot curve = i-CH_3H_7OH.

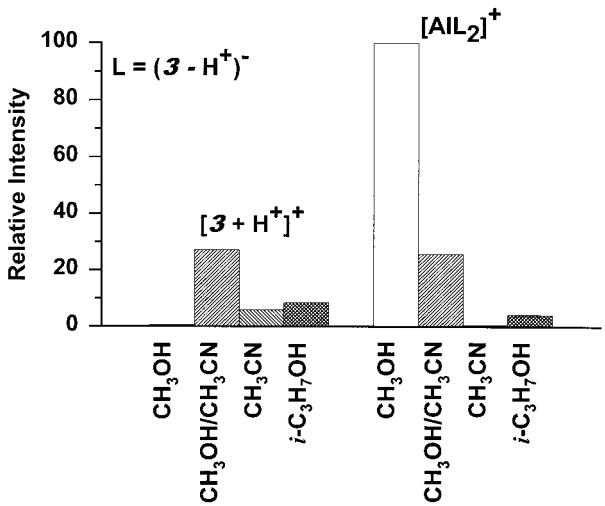


Figure 9. Normalized relative intensities for the major quercetin (**3**) derived ions, viz., the protonated molecule and the 1:2, Al-quercetin dimer, observed in the ES mass spectra recorded using the same solutions as those used to acquire the data in Figure 8. Data obtained by continuous infusion at 5 μL/min.

[e.g., see Fig. 7(c)]. The abundance of the ions are all normalized with respect to the absolute abundance recorded for the dimer in the spectrum obtained by spraying the CH_3OH solution. Comparison of the ES abundances of the dimer relative to the protonated molecule (representative of free quercetin in solution) for each solvent system shows a very good match with that expected on the basis of the UV/visible spectrum. The signal levels for the dimer obtained when spraying from i-CH_3H_7OH might be slightly less than expected. In fact, the overall signal level observed when spraying out of CH_3CN or i-C_3H_7OH are substantially lower compared to spraying from CH_3OH. Rather than a reflection of the actual ion abundance in solution, this observation may relate more to the nature of the solvent and its effect on the ES process and the liberation of the solution ions into the gas-phase.

A similar set of experiments, with some additional solvents systems, was carried out using kaempferol. The resulting UV/visible spectra and corresponding ES-MS results are shown in Figs 10 and 11, respectively. These results, as all of those above, demonstrate the general correspondence among the UV/visible spectra and ES mass spectra. These results also demonstrate that the degree of dimer formation is not only dependant on the solvent but on the flavonoid. In contrast to the results for quercetin [Figs (8) and (9)], we observed that 1:2, Al-flavonoid dimer formation was major for kaempferol in CH_3CN/CH_3OH (1/1 v/v). This data would imply that the binding of Al^{3+} by kaempferol is stronger than that of quercetin in this solvent system. The UV/visible spectrum of kaempferol in CH_3CN/H_2O (1/1 v/v), CH_3CN/CH_2Cl_2 (1/1 v/v) and CH_3CN/i-C_3H_7OH (1/1 v/v) indicates that a much smaller fraction of the dimer is formed. In fact, the ES-MS results show the protonated molecule to be of greater relative abundance than the dimer with these solvents. Note that the overall UV/visible signal is attenuated in CH_3CN/H_2O (1/1 v/v), but the fraction of dimer compared to free kaempferol (expressed as the ratio $abs_{430\ nm}/abs_{370\ nm}$) would be expected to be next highest compared to that observed for CH_3CN/CH_3OH (1/1 v/v). This was the ES-MS observation. The overall lower signal levels for

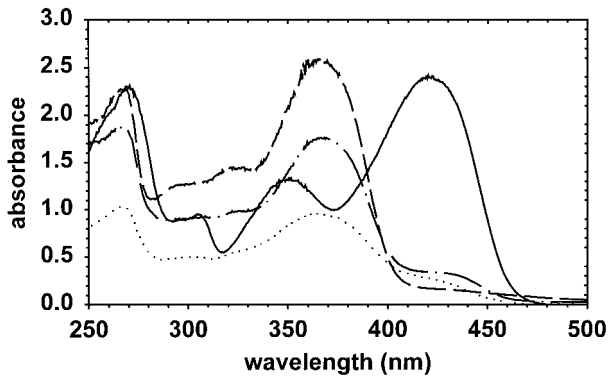


Figure 10. UV/Visible spectra of 100 μM kaempferol (**2**)/100 μM Al^{3+} recorded in differing solvent systems. Solid curve = CH_3CN/CH_3OH (1/1 v/v), dotted curve = CH_3CN/H_2O (1/1 v/v), dashed curve = CH_3CN/CH_2Cl_2 (1/1 v/v), and dash-dot curve = CH_3CN/i-CH_3H_7OH (1/1 v/v).

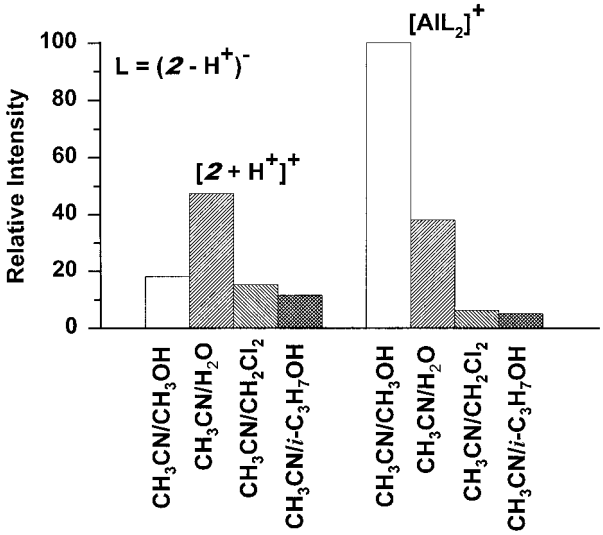


Figure 11. Normalized relative intensities for the major kaempferol (**2**) derived ions, viz., the protonated molecule and the 1:2, Al-kaempferol dimer, observed in the ES mass spectra recorded using the same solutions as those used to acquire the data in Fig. 10. Data obtained by continuous infusion at 5 μL/min.

CH_3CN/CH_2Cl_2 (1/1 v/v) and CH_3CN/i-C_3H_7OH (1/1 v/v) might again be the result of an ES ion generation phenomenon rather than a reflection of the absolute abundance of ionic components in solution.

CONCLUSIONS

Electrospray mass spectra of a variety of solvent systems indicated by UV/visible interrogation to contain Al-flavonoid complexes show the stoichiometry of the major complex formed to be 1:2. Both the ES mass spectra and UV/visible spectra demonstrated that the degree of complex formation was dependent on the Al^{3+}:flavonoid concentration ratio, on the solvent system employed, and on the flavonoid under study. In general CH_3OH favored formation of the 1:2 dimer, whereas dimer formation was suppressed in CH_3CN and all other solvents investigated. In all solvents, the 1:2 complex was predominant except when the solution concentration of Al^{3+} was several times that of the flavonoid. In these cases, ions corresponding in m/z to the monomeric 1:1 Al-flavonoid complexes tended to dominant the spectra.

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