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Enhanced Detection of Flavonoids by Metal Complexation and Electrospray Ionization Mass Spectrometry

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Metal complexation with the use of an auxiliary ligand is explored as an alternative to conventional protonation or deprotonation for analysis of a series of flavonoids by electrospray ionization mass spectrometry. Use of a neutral auxiliary ligand, 2,2′-bipyridine, results in formation of $[M^{II}(\text{flavonoid} - H)bpy]^+$, ternary complexes with intensities that are 2 orders of magnitude greater than the corresponding protonated flavonoids and up to 1.5 orders of magnitude greater than the deprotonated flavonoids, based on confirmation by collisionally activated dissociation patterns. The formation of ternary complexes with six divalent transition metals, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, and Fe^{2+} were compared. Cu^{2+} resulted in the most intense complexes and simplest mass spectra, while Co^{2+} gave the second most intense spectra and also produced two key products that could be useful for a selected ion monitoring strategy. Complexation with iron(III) bromide is also investigated to explore the feasibility of using triply charged metals.

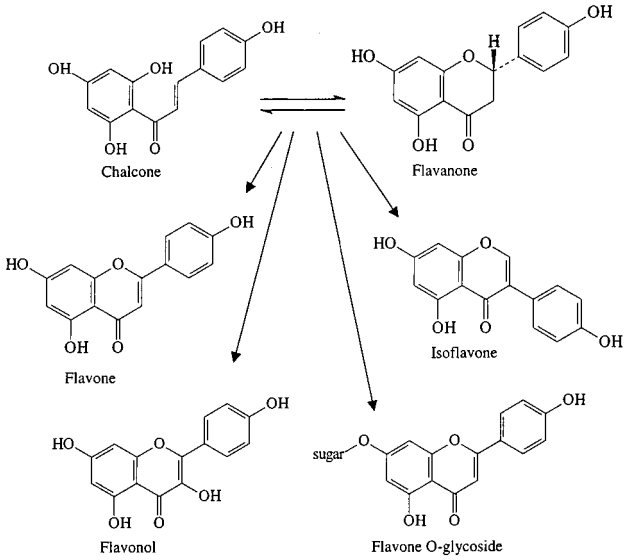

Figure 1. Common flavonoid structures.

Flavonoids, polyphenolic phytochemicals found in all plants except green algae and hornworts, are a ubiquitous part of the human diet. The basic flavonoid structure is the chalcone that can be modified biosynthetically to form structures identified as flavanones, flavones, isoflavones, and flavonols, among others (see Figure 1). Further modifications of the basic flavonoid structure in the plant or after consumption, such as methylation of the hydroxyl groups or glycosylation, frequently cause subtle chemical and biological changes.[1] For example, glycosylation increases the polarity of the flavonoid molecule and enhances storage in plant vacuoles but decreases antioxidant activity.

Flavonoids are of increasing interest due to their health benefits. They possess antioxidant activity, the ability to scavenge active oxygen species and act as metal chelators.[2−4] Their action as antioxidants has been implicated in lowered risk of atherosclerosis, in part due to their ability to prevent oxidation of low-density lipoproteins (LDLs).[5−7] Flavonoids also exhibit antitumor, antibacterial, and antiviral effects.[6,8−10] For example, a significant reduction in the frequency of tongue carcinomas of rats exposed to known carcinogens while being fed the flavonoids diosmin and hesperidin has been determined.[11] Other research has demonstrated the efficacy of flavonoids in preventing viral replication, specifically HIV[12] and the hepatitis B virus.[13] Epidemiological studies have shown an inverse association between coronary heart disease and flavonol and flavone consumption in humans.[6]

Study of the correlation between health benefits and flavonoid consumption depends on detailed analysis of foods and biological

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(1) Rice-Evans, C. A.; Packer, L. *Flavonoids in Health and Disease*; Marcel Dekker: New York, 1998.
(2) Morand, C.; Crespy, V.; Manach, C.; Besson, C.; Demigne, C.; Remesy, C. *Am. J. Physiol.* **1998**, *275*, R212−R219.
(3) Rice-Evans, C. A.; Miller, N. J. *Biochem. Soc. Trans.* **1996**, *24*, 790−795.
(4) Pratt, D. E. *ACS Symp. Ser.* **1992**, *No. 507*, 54−71.
(5) McAnlis, G. T.; McEneny, J.; Pearce, J.; Young, I. S. *Biochem. Soc. Trans.* **1997**, *25*, 142S.
(6) Wiseman, H. *Biochem. Soc. Trans.* **1996**, *24*, 795−800.
(7) Leake, D. S. In *Phytochemistry of Fruits and Vegetables*; Tomas-Barberan, F. A., Robins, R. J., Eds.; Clarendon Press: Oxford, 1997; pp 287−311.
(8) Hollman, P. C. H.; Hertog, M. G. L.; Katan, M. B. *Biochem. Soc. Trans.* **1996**, *24*, 785−789.
(9) Hertog, M. G. L.; Van Poppel, G.; Verhoeven, D. In *Phytochemistry of Fruit and Vegetables*; Tomas-Barberan, F. A., Robins, R. J., Eds.; Clarendon Press: Oxford, 1997; pp 313−329.
(10) Benavente-Garcia, O.; Castillo, J.; Marin, F. R.; Ortuno, A.; Del Rio, J. A. *J. Agric. Food Chem.* **1997**, *45*, 4505−4515.
(11) Tanaka, T.; Makita, H.; Ohnishi, M.; Mori, H.; Satoh, K.; Hara, A.; Sumida, T.; Fukutani, K.; Tanaka, T.; Ogawa, H. *Cancer Res.* **1997**, *57*, 246−252.
(12) Eich, E. *ACS Symp. Ser.* **1998**, *No. 691*, 83−96.
(13) Zembower, D. E.; Zhang, H. *J. Org. Chem.* **1998**, *63*, 9300−9305.

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fluids such as blood plasma and urine. Analytical methods to identify and quantitate flavonoids and their metabolites have lacked both the sensitivity and specificity to detect low levels in complex matrixes.[1] In addition, because the subgroups of flavonoids vary widely in structure, and because over 4000 different flavonoids have been identified, no single analytical method has been available for simultaneous analysis of all the flavonoids present in a particular food sample. Identification of flavonoids is key to understanding the structure/activity relationships.

Mass spectrometric methods such as desorption chemical ionization (DCI)[14] and fast atom bombardment (FAB),[14−16] as well as high-performance liquid chromatography−mass spectrometry (HPLC−MS)[17−23] and gas chromatography/ mass spectrometry (GC/MS),[24−28] have commonly been used for flavonoid analysis. For example, negative-ion fast atom bombardment mass spectrometry was used to identify flavonol glycosides in *Sedum telephium* juice.[29] HPLC−MS with electrospray ionization was used for identifying and quantifying plant flavonoids, such as quercetin, myricetin, and kaempferol, in edible berries.[30] These previous studies have highlighted some of the limitations of the mass spectrometric methods, including the inability to generate molecular ions for some of the diverse classes of flavonoids and ineffective isomer differentiation. The present work describes a versatile and sensitive method for detection of the flavonoids based on a novel metal complexation strategy.

The use of metal complexation to generate cationized species for mass spectrometric analysis has gained renewed popularity in recent years due to the ability to produce complexes by electrospray ionization (ESI).[31−40] Recent examples have focused on the metal complexation of peptides, polysaccharides, lipids, and proteins. Our group has carried out several studies involving metal complexation with a variety of organic ligands, such as crown ethers and quinolone antibiotics.[41−47] Only one previous study has used mass spectrometric analysis in an attempt to better understand the metal-chelating abilities of flavonoids. This work was undertaken by Van Berkel and Deng in which flavonoid complexation with Al^{3+} was studied.[48] Differences in the flavanone versus isoflavone complexation and dissociation were found, which the authors suggested could be used for structural differentiation.

The use of an auxiliary ligand to enhance metal coordination efficiencies is a promising way to expand the versatility of metal complexation strategies. Gatlin and his colleagues found that abundant complexes were formed between Cu(II) ions, amino acids or peptides, and 2,2′-bipyridine or 1,10-phenanthroline and that these complexes gave distinctive fragmentation patterns.[49−51] Alvarez et al. found that complexation with transition metal ions and various pharmaceutical compounds was possible with the addition of an auxiliary ligand, especially 2,2′-bipyridine.[41,42] In several cases, the abundance of the metal complex was significantly greater than that of the protonated antibiotic. Shen and Brodbelt showed that metal complexation with an auxiliary ligand could be incorporated into an effective postcolumn ionization method for HPLC−MS applications, as demonstrated for a group of quinolone antibiotics.[45] The success of this earlier work led us to evaluate the use of metal complexation in conjunction with auxiliary ligands for the ionization of flavonoids.

Our goal was to use transition metal complexation as an alternative to protonation and/or deprotonation for detection of flavonoids. As shown in this report, addition of 2,2′-bipyridine greatly improved the complexation efficiencies. Several transition metals were used to investigate the efficiencies of complex formation. In addition to using doubly charged transition metal salts of Co(II), Ni(II), Cu(II), Zn(II), Fe(II), and Mn(II), complexation with a triply charged metal ion, Fe(III), was investigated. With the triply charged transition metal salt, the auxiliary ligand proved unnecessary due to the higher charge of the metal ion which allowed the complex to retain a +1 charge despite the

(14) Domon, B.; Hostettmann, K. *Phytochemistry* **1985**, *24*, 575−580.
(15) Ma, Y. L.; Li, Q. M.; Van den Heuvel, H.; Claeys, M. *Rapid Commun. Mass Spectrom.* **1997**, *11*, 1357−1364.
(16) Price, K. R.; Rhodes, M. J. C. *J. Sci. Food Agric.* **1997**, *74*, 331−339.
(17) Krishnan, H. B. *Crop Sci.* **1998**, *38*, 1052−1056.
(18) Barnes, K. A.; Smith, R. A.; Williams, K.; Damant, A. P.; Shepherd, M. J. *Rapid Commun. Mass Spectrom.* **1998**, *12*, 130−138.
(19) Barnes, S.; Coward, L.; Kirk, M.; Sfakianos, J. *Proc. Soc. Exp. Biol. Med.* **1998**, *217*, 254−262.
(20) Chaves, N.; Rios, J. J.; Gutierrez, C.; Escudero, J. C.; Olias, J. M. *J. Chromatogr., A* **1998**, *799*, 111−115.
(21) Raffaelli, A.; Moneti, G.; Mercati, V.; Toja, E. *J. Chromatogr., A* **1997**, *777*, 223−231.
(22) Stevens, J. F.; Taylor, A. W.; Deinzer, M. L. *J. Chromatogr., A* **1999**, *832*, 97−107.
(23) Poon, G. K. *J. Chromatogr., A* **1998**, *794*, 63−74.
(24) Mazur, W.; Fotsis, T.; Wahala, K.; Ojala, S.; Salakka, A.; Aldercreutz, H. *Anal. Biochem.* **1996**, *233*, 169−180.
(25) Schulten, H.-R.; Simmleit, N.; Muller, R. *Anal. Chem.* **1989**, *61*, 221−227.
(26) Soleas, G. J.; Dam, J.; Carey, M.; Goldberg, D. M. *J. Agric. Food Chem.* **1997**, *45*, 3871−3880.
(27) Joannou, G. E.; Kelly, G. E.; Reeder, A. Y.; Waring, M.; Nelson, C. *J. Steroid Biochem. Mol. Biol.* **1995**, *54*, 167−184.
(28) Franski, R.; Bednarek, P.; Wojtaszek, P.; Stobiecki, M. *J. Mass Spectrom.* **1999**, *34*, 486-495.
(29) Mulinacci, N.; Vincieri, F. F.; Baldi, A.; Romani, A.; Favretto, D.; Traldi, P. *Rapid Commun. Mass Spectrom.* **1995**, *9*, 963−967.
(30) Hakkinen, S. H.; Karenlampi, S. O.; Heinonen, I. M.; Mykkanen, H. M.; Torronen, A. R. *J. Agric. Food Chem.* **1999**, *47*, 2274−2279.
(31) Olling, A.; Breimer, M. E.; Peltomaa, E.; Samuelsson, B. E.; Ghardashkhani, S. *Rapid Commun. Mass Spectrom.* **1998**, *12*, 637−645.
(32) Li, H.; Siu, K. W. M.; Guevremont, R.; Le Blanc, J. C. Y. *J. Am. Soc. Mass Spectrom.* **1997**, *8*, 781−792.
(33) Asam, M. R.; Glish, G. L. *J. Am. Soc. Mass Spectrom.* **1997**, *8*, 987−995.
(34) Sible, E. M.; Brimmer, S. P.; Leary, J. A. *J. Am. Soc. Mass Spectrom.* **1997**, *8*, 32−42.
(35) Veenstra, T. D.; Johnson, K. L.; Tomlinson, A. J.; Craig, T. A.; Kumar, R.; Naylor, S. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 8−14.
(36) Hsu, F.-F.; Bohrer, A.; Turk, J. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 516−526.
(37) Hu, P.; Loo, J. A. *J. Am. Chem. Soc.* **1995**, *117*, 11314−11319.
(38) Lavanant, H.; Hoppilliard, Y. *Eur. Mass Spectrom.* **1999**, *5*, 41−50.
(39) Chu, I. K.; Guo, X.; Lau, T.-C.; Siu, K. W. M. *Anal. Chem.* **1999**, *71*, 2364−2372.
(40) Konig, S.; Leary, J. A. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 1125−1134.
(41) Alvarez, E. J.; Vartanian, V. H.; Brodbelt, J. S. *Anal. Chem.* **1997**, *69*, 1147−1155.
(42) Alvarez, E. J.; Brodbelt, J. S. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 463−472.
(43) Blair, S. M.; Kempen, E. C.; Brodbelt, J. S. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 1049−1059.
(44) Hall, B. J.; Brodbelt, J. S. *J. Am. Soc. Mass Spectrom.* **1999**, *10*, 402−413.
(45) Shen, J.; Brodbelt, J. S. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 1381−1389.
(46) Vartanian, V. H.; Goolsby, B.; Brodbelt, J. S. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 1089−1098.
(47) Goolsby, B. J.; Brodbelt, J. S.; Adou, E.; Blanda, M. *Int. J. Mass Spectrom.* **1999**, *193*, 197−204.
(48) Deng, H.; Van Berkel, G. J. *J. Mass Spectrom.* **1998**, *33*, 1080−1087.
(49) Gatlin, C. L.; Turecek, F.; Vaisar, T. *J. Mass Spectrom.* **1995**, *30*, 1617−1627.
(50) Gatlin, C. L.; Turecek, F.; Vaisar, T. *J. Mass Spectrom.* **1995**, *30*, 1605−1616.
(51) Gatlin, C. L.; Rao, R. D.; Turecek, F.; Vaisar, T. *Anal. Chem.* **1996**, *68*, 263−270.

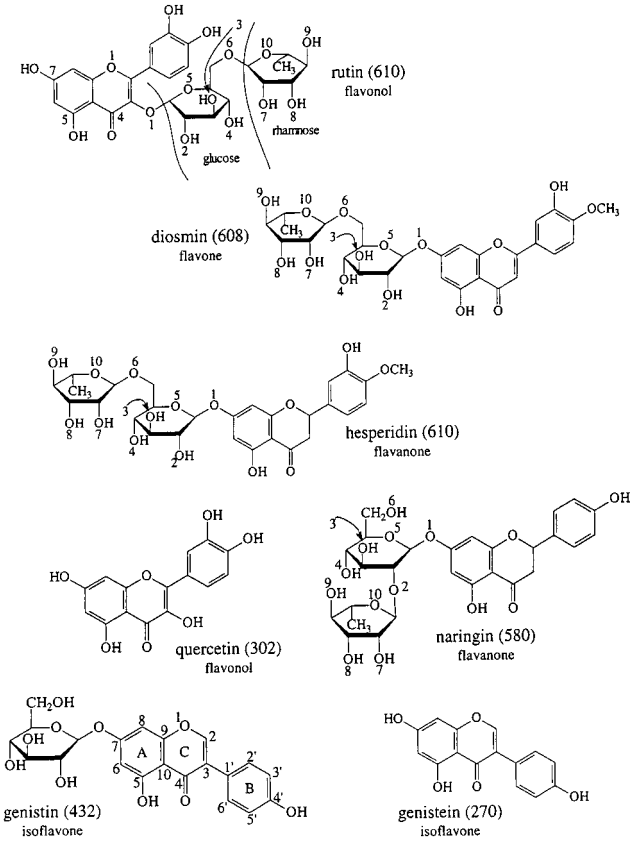

Figure 2. Structures of flavonoids used (molecular weight). Category of flavonoid is also given.

incorporation of two deprotonated flavonoids. Several flavonoid glycosides and two of their corresponding aglycons were investigated, including a flavone, flavonol, flavanone, and isoflavone in order to test the applicability of this method to a variety of structures (See Figure 2). The aim is to create complexes that exhibit significantly higher signal intensities than the protonated or deprotonated flavonoids to lower the detection limits of a wide variety of flavonoids in foods, bodily tissues, and fluids.

EXPERIMENTAL SECTION

All experiments were carried out on a Thermoquest LCQ Duo quadrupole ion trap mass spectrometer equipped with an electrospray interface. The flow rate of the electrospray solution was 5 μL/min, and the lens and octapole voltages, sheath gas flow rate, and capillary voltage were optimized for maximum abundance of the ions of interest. In solutions of the flavonoid without metal addition, the ion optimized was $[L - H]^-$ (negative mode) or $[L + H]^+$ (positive mode), in which L is the flavonoid. For solutions containing the transition metal salt and flavonoid, the $[M^{II}(L - H)]^+$ complex was optimized. Upon addition of the auxiliary ligand to the metal/flavonoid solution, the $[M^{II}(L - H)bpy]^+$ complex was optimized. For investigations involving triply charged metal ions, the complex $[M^{III}(L - H)_2]^+$ was optimized. The capillary temperature was kept at 200° C. The pressure, measured with the convectron gauge at the skimmer cone during electrospray experiments, was normally 0.9 Torr. The base pressure in the ion trap with helium added was typically 1.9×10^{-5} Torr, as measured with the ionization gauge. Full-scan spectra shown represent ion injection times of 1 ms and an average of 20 microscans.

Solutions were made from stock solutions of 1.0×10^{-4} M flavonoid/methanol, 1.0×10^{-3} M 2,2′-bipyridine/methanol and 1.0×10^{-2} M metal salts/methanol. For examination of the protonated flavonoids, a solution of 1.0×10^{-4} M flavonoid with 5% glacial acetic acid was used. When basic solutions were required for examination in the negative mode, solutions of 1.0×10^{-4} M flavonoid with 5% concentrated ammonium hydroxide were made. Solutions of a flavonoid and a transition metal salt were 1:1 flavonoid/metal. When 2,2′-bipyridine was added, the ratio of flavonoid to metal ion to 2,2′-bipyridine was 1:1:1. The molarity of all solutions sprayed, except those used in the limit of detection study, was 1.0×10^{-4} M. To examine the effect of nominal pH on complexation, solutions composed of the 1:1 flavonoid/metal or 1:1:1 flavonoid/metal/2,2′-bipyridine with 5% glacial acetic acid or 5% concentrated ammonium hydroxide were analyzed. The limit of detection of $[Cu^{II}(\text{naringin} - H)\ bpy]^+$ was determined by analysis of a solution of 5.0×10^{-11} M naringin/$CuBr_2$/2,2′-bipyridine in the ratio of 1:5:5 using an ion injection time of 10 s. Solutions with the same ratio of analytes but with higher concentrations were also analyzed. Solutions of 5.0×10^{-11}, 5.0×10^{-10}, 5.0×10^{-9}, and 5.0×10^{-8} M naringin with 5% ammonium hydroxide were analyzed under similar conditions to determine the detection limit of $[\text{naringin} - H^+]^-$

For computational modeling, PC Spartan Pro (Wavefunction, Irvine, CA) was used. Lowest energy conformers were determined by molecular modeling using MMFF94 force fields. The equilibrium geometries of the lowest energy conformers were optimized using semiempirical PM3 calculations. To ensure the validity of the final structures, the equilibrium geometries of several conformers were optimized.

The flavonoids hesperidin, rutin, naringin, genistin, and catechin were purchased from Sigma (St. Louis, MO). Diosmin, 3,7-dihydroxyflavone, 5,7-dihydroxyflavone, luteolin, 7,3′,4′-trihydroxyflavone, 7-hydroxyflavone, and 7,4′-dihydroxyflavone came from Indofine Chemical Co., Inc. (Somerville, NJ). 2,2′-Bipyridine, cobalt(II) bromide, nickel(II) bromide, copper(II) bromide, manganese(II) bromide, zinc(II) bromide, iron(II) bromide, and iron(III) bromide were purchased from Aldrich Chemical Co. (Milwaukee, WI). The HPLC grade methanol, glacial acetic acid, and ammonium hydroxide were purchased from EM Science (Gibbstown, NJ). All materials were used without further purification.

RESULTS AND DISCUSSION

Formation of the Protonated, Deprotonated, and Sodium-Cationized Flavonoids. Prior to investigation of transition metal complexation of the flavonoids, protonation and deprotonation were explored to establish benchmarks for the relative electrospray ionization efficiencies of the $[L + H]^+$ and $[L - H]^-$ ions. As shown in Table 1, most of the flavonoids investigated exhibited sodium complexation favored over protonation, even under acidic conditions. Figure 3A shows a typical ESI mass spectrum of the flavonoid naringin in which the base peak in acidic solution is identified as a dimer composed of two naringin molecules complexed to a sodium ion. The presence of sodium, a metal that is ubiquitous in the environment, makes the detection of the protonated flavonoids ineffective. For most flavonoid compounds, protonation is very inefficient.

Since the flavonoids are slightly acidic, the ESI-MS of the flavonoids were also examined in basic solutions using the

Table 1. ESI-MS of Flavonoids in Acidic, Basic, Metal, and Metal + 2,2′-Bipyridine Solutions (Relative Percentages ±5%)

	most intense ions (%)			
compound (MW)	in acidic solution	in basic solution	in Co^{2+} solution	in Co^{2+} solution with 2,2′-bipyridine
rutin (610)	$(L + H)^+$ (30) $[Na^I(L)]^+$ (25) $[Na^I(L)_2]^+$ (20) [aglycon + H]$^+$ (15) $(L -$ rhamnose$)^+$ (5)	$(L - H)^-$ (90) $[(L - H)L]^-$ (10)	$[Co^{II}(L - H)]^+$ (60) $[Co^{II}(L - H)L]^+$ (30) 974^+ (10)	$[Co^{II}(L - H)bpy]^+$ (60) $[Co^{II}(L - H)(bpy)_2]^+$ (40)
naringin (580)	$[Na^I(L)_2]^+$ (80) 798^+ (20)	$(L - H)^-$ (80) $[(L - H)L]^-$ (20)	$[Co^{II}(L - H)L]^+$ (75) $[Na^I(L)_2]^+$ (25)	$[Co^{II}(L - H)bpy]^+$ (40) $[Co^{II}(L - H)(bpy)_2]^+$ (60)
hesperidin (610)	$[Na^I(L)_2]^+$ (80) $(L + H)^+$ (10) $[Na^I(L)_3]^+$ (10)	$(L - H)^-$ (80) $[(L - H)L]^-$ (20)	$[Co^{II}(L - H)L]^+$ (45) $[Na^I(L)_2]^+$ (45) $(L + H)^+$ (10)	$[Co^{II}(L - H)bpy]^+$ (90) $[Co^{II}(L - H)L]^+$ (10)
diosmin (608)	925^+ (>95)	$(L - H)^-$ (90) $[(L - H)L]^-$ (10%)	$(L + H)^+$ (50) $[Co^{II}(L - H)^+$ (30%) $[Co^{II}(L - H)H_2O]^+$ (20%)	$[Co^{II}(L - H)bpy]^+$ (>95)
genistin (432)	$[Na^I(L)_2]^+$ (85) $(L + H)^+$ (15)	$[(L - H)L]^-$ (60) $(L - H)^-$ (40)	$[Na^I(L)_2]^+$ (40) $[Co^{II}(L - H)L]^+$ (30) $(L + H)^+$ (30)	$[Co^{II}(L - H)bpy]^+$ (95) $[Co^{II}(L - H)bpy - glucose]^+$ (5)
quercetin (302)	$(L + H)^+$ (> 95)	$(L - H)^-$ (50) $(L - H + 30)^-$ (40) $[(L - H)(L + 30)]^-$ (10)	$[Co^{II}(L - H^+)(CH_3OH)_2]^+$ (80) $(L + H)^+$ (10) $[Co^{II}(L - H)L]^+$ (10)	$[Co^{II}(L - H)bpy]^+$ (45) $[Co^{II}(L - H)(bpy)_2]^+$ (55)
genistein (270)	488^+ (45) 365^+ (35) 555^+ (20)	$(L - H)^-$ (95) $[L(L - H)]^-$ (5)	$[Co^{II}(L - H)]^+$ (>95)	$[Co^{II}(L - H)bpy]^+$ (90) $[Co^{II}(L - H)(bpy)_2]^+$ (10)

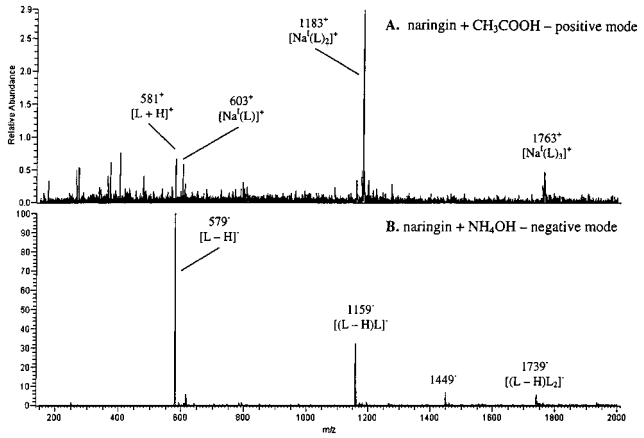

Figure 3. Comparison of ESI-MS of naringin in (A) positive mode (acidic solution) and (B) negative mode (basic solution). The vertical scales are directly comparable for this series of spectra, with maximum abundance being 100, and values less than 100 signifying proportionally lower abundances.

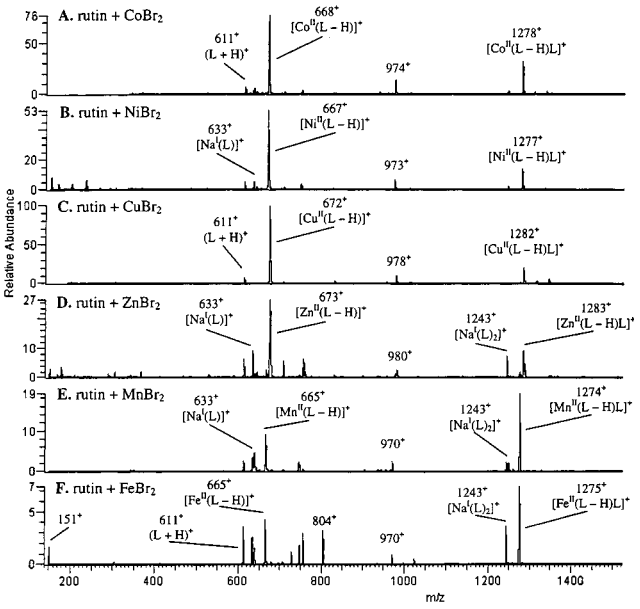

Figure 4. Spectra showing complexation of rutin with (A) cobalt(II), (B) nickel(II), (C) copper(II), (D) zinc(II), (E) manganese(II), and (F) iron(II). The vertical scales are directly comparable for this series of spectra, with maximum abundance being 100, and values less than 100 signifying proportionally lower abundances.

negative mode (See Table 1). As shown in Figure 3B, the prevalent ions were deprotonated flavonoids and dimers composed of a deprotonated flavonoid ion complexed to a neutral flavonoid molecule. Due to their acidity, flavonoids deprotonate easily, as illustrated by the superior signal intensity of the deprotonated flavonoid as compared to that of the protonated flavonoid.

Formation of Transition Metal Complexes with Doubly Charged Transition Metals. Complexation with transition metals was carried out with the goal of improving signal intensities. Flavonoids, with their multiple hydroxyl groups and the carbonyl group at carbon 4 of the C ring, have numerous sites for metal complexation. Initially, ESI-MS of solutions of the flavonoid and each of six transition metal salts, cobalt(II), nickel(II), copper(II), zinc(II), manganese(II), and iron(II) bromide, were examined. Cobalt, nickel, and copper produced consistently greater intensities of metal complexes than zinc, manganese, and iron with fewer clusters and adducts (see Figure 4). Optimization of the capillary temperature and sheath gas flow reduced the overall complexity of the spectra by eliminating ions due to clusters and adduct formation.

Of the flavonoid glycosides investigated, only rutin produced abundant complexes composed of a single deprotonated flavonoid and the doubly charged metal ion, $[M^{II}(L - H)]^+$. Ions observed

and their relative abundances are summarized in Table 1. For most of the flavonoid glycosides, the most abundant metal complexes were composed of one deprotonated flavonoid molecule, the transition metal ion, and an additional neutral flavonoid molecule, $[M^{II}(L - H)\ L]^+$. Two flavonoid glycosides, hesperidin and diosmin, produced minor $[M^{II}(L - H)]^+$ complexes, but the sodium-cationized and protonated complexes were much more abundant. Complexes with a second neutral flavonoid molecule were more abundant than those lacking the neutral flavonoid, presumably due to the preferred coordination numbers of the metal ions. Cobalt and nickel frequently favor octahedral coordination geometries, with coordination numbers of six, and copper often prefers square planar/tetragonal geometries (coordination number four) with oxygen donors.[52] Chelation by a flavonoid glycoside may involve several coordination sites, two on the flavonoid aglycon and others on the saccharide moiety, as suggested by molecular modeling experiments. Complexation of the metal ion with two flavonoids may involve two or more binding sites of one ligand with the second ligand providing the other necessary binding sites.

Due to the acidity of the flavonoid molecules, production of electroneutral complexes of the type $[M^{II}(L - H)_2]^0$ is possible. This type of complexation may explain why the flavonoid/transition metal solutions do not always give intense ESI mass spectra. To avoid production of these nondetectable complexes and to aid in simplification of spectra, addition of a neutral auxiliary ligand was explored.[41,42,49−51] On the basis of previous studies of metal complexation of antibiotics with an auxiliary ligand,[41,42] several ligands were initially investigated, 1,10-phenanthroline, 2,2′-bipyridine and 4,4′-bipyridine. 2,2′-Bipyridine was chosen on the basis of the intensity of the ternary complexes and the stability of the auxiliary ligand during dissociation. Upon ESI-MS of solutions containing 2,2′-bipyridine, a flavonoid and a transition metal salt, the $[M^{II}(L - H)\ bpy]^+$ complexes are the dominant complexes with a dramatic increase in signal as compared to the intensities of the corresponding protonated, deprotonated, and metal complexes (see Figure 5).

Addition of the auxiliary ligand proved to be a successful strategy for increasing the signal intensities of flavonoid−metal complexes for all the flavonoids investigated. Intensities of the key molecular ions often increased by as much as 2 orders of magnitude relative to the other ionization modes, and the spectra are generally cleaner with fewer types of products. The base peaks were identified as $[M^{II}(L - H)\ bpy]^+$ or $[M^{II}(L - H)\ (bpy)_2\ ]^+$, as summarized in Table 1. These complexes correspond to ternary complexes in which the auxiliary ligand serves as a chelating agent for the transition metal ion.[41,42,49−51] The deprotonated flavonoid molecule provides at least two other coordination sites for the metal ion. Previous studies[41,42,45] have shown the importance of the availability of an acidic site in the analyte of interest that can be deprotonated as well as the presence of an additional binding site to chelate the metal ion. The flavonoids investigated all have a carbonyl functionality close in proximity to one or more hydroxyl groups. The saccharide substituents do not cause sufficient steric hindrance to prohibit favorable coordination and are even involved in coordination, based on molecular modeling results.

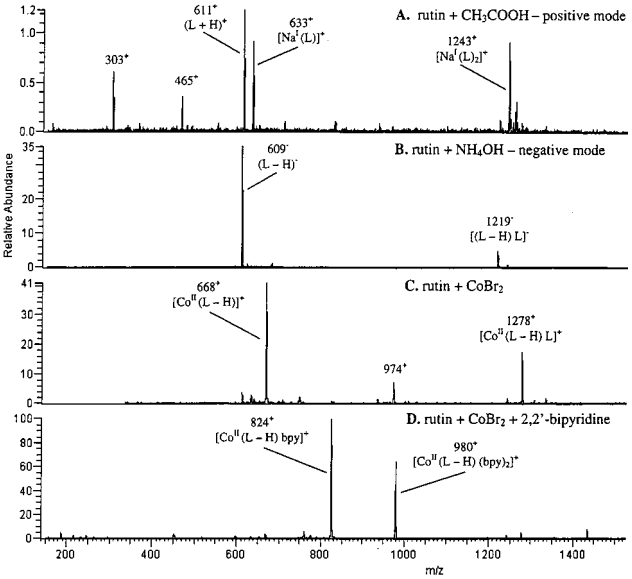

Figure 5. Effect of metal complexation and the auxiliary ligand, 2,2′-bipyridine, on signal intensity. The vertical scales are directly comparable for this series of spectra, with maximum abundance being 100, and values less than 100 signifying proportionally lower abundances.

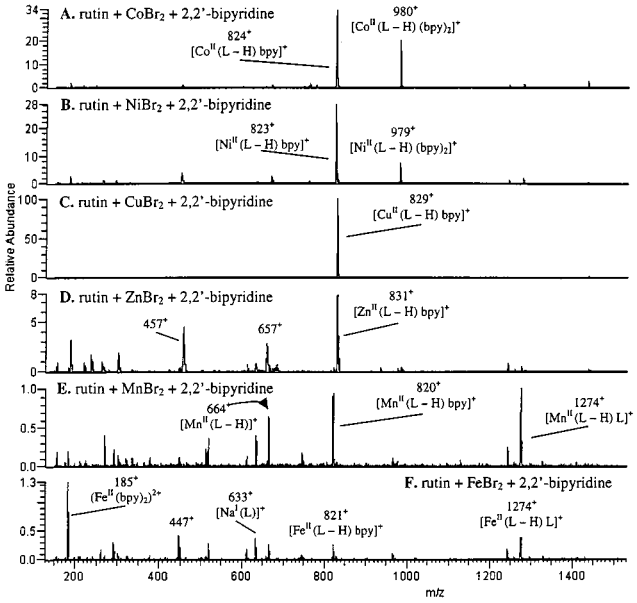

Figure 6. Effect of the transition metal ion on formation of ternary metal complexes with the auxiliary ligand, 2,2′-bipyridine (bipy). The vertical scales are directly comparable for this series of spectra, with maximum abundance being 100, and values less than 100 signifying proportionally lower abundances.

The type of metal ion also influences the favorable formation of ternary complexes, as illustrated in Figure 6. With cobalt, nickel, copper, manganese, and zinc, the base peak was identified as $[M^{II}(L - H)\ bpy]^+$. Iron(II) was the only metal ion that did not show significant complex formation with rutin upon addition of the auxiliary ligand. In addition to ternary complexes of the type $[M^{II}(L - H)\ bpy]^+$, complexes identified as bis-bipyridine species, $[M^{II}(L - H)\ (bpy)_2\ ]^+$, were also evident with the flavonoid glycosides rutin and naringin. This type of complex was never observed with copper. The Jahn−Teller distortion for Cu(II) results in stronger coordination of four sites with weaker coor-

(52) Martell, A. E.; Hancock, R. D. *Metal Complexes in Aqueous Solutions*; Plenum Press: New York, 1996.

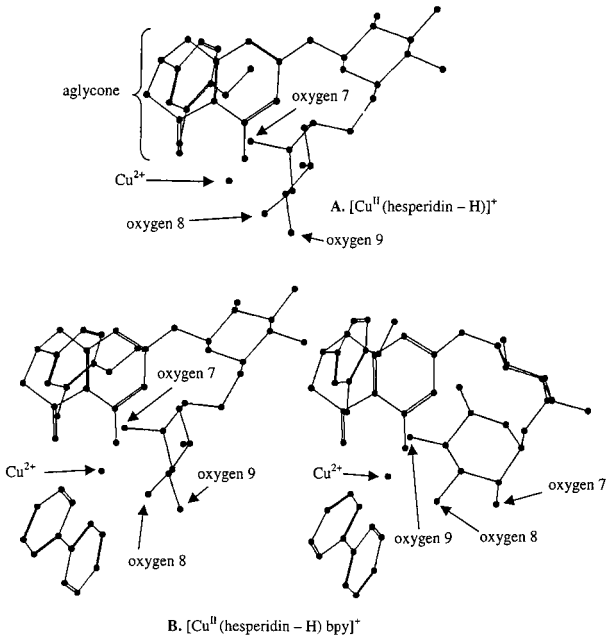

Figure 7. Models of (A) $[Cu^{II}(\text{hesperidin} - H)]^+$ and (B) $[Cu^{II}(\text{hesperidin} - H)\text{bpy}]^+$, reflecting the changes in distances to the oxygens of the disaccharides that occur when the auxiliary ligand is added. Hydrogens are not shown.

dination to the two axial sites.[52] In the absence of 2,2-bipyridine, coordination with four sites is evident in the $[Cu^{II}(L - H)]^+$ complex. In the presence of the auxiliary ligand, coordination to six sites occurs (see Figure 7 which is discussed later). Formation of bis-bipyridine complexes, $[Cu^{II}(L - H)\ (bpy)_2]^+$, is not favored, possibly due to the weak binding involving the two axial coordination sites. However, as the flavonoid glycoside has appropriate binding sites available in sterically favorable positions, coordination to the terminal sugar, rhamnose, occurs. As mentioned previously, cobalt and nickel prefer octahedral coordination and thus complexation with two 2,2′-bipyridine molecules is possible and favored, giving net coordination numbers of six. Neither diosmin, hesperidin, nor genistin showed evidence of formation of the bis-bipyridine complexes, $[M^{II}(L - H)\ (bpy)_2]^+$, perhaps related to the more favorable coordination between the terminal sugar and the metal ion.

The signal intensity of the copper complexes tended to be the greatest of all the metals (see Figure 6), in part due to lack of division of signal between mono- and bis-bipyridine complexes, as seen for cobalt and nickel. Signal reduction for zinc and manganese was attributed to overall poor complexation efficiency and the production of other analytically uninteresting ions, such as $[M^{II}(bpy)_2\ Br]^+$ and dehydration products, thus reducing the intensity of the base peak, $[M^{II}(L - H)\ bpy]^+$. Formation of the ternary complex was also affected by the affinity of the auxiliary ligand for the metal. For example, the base peak in the mass spectra of hesperidin with manganese(II), zinc(II), or iron(II) and 2,2′-bipyridine was $[M^{II}(L - H)\ L]^+$, indicating an apparent lower affinity of the auxiliary ligand for these metal ions or perhaps preferential coordination by the hydroxyl-rich saccharide moieties. These results are confirmed by the lower log K values of metal−2,2′-bipyridine complexes involving manganese, iron, and zinc (log K values 2.62−5.13)[53] relative to those involving cobalt, nickel, and copper (log K values 5.8, 7.0, and 6.3, respectively)[53] in aqueous solution (which likely parallel the trends in methanol). Complexes including the auxiliary ligand, $[M^{II}(L - H)\ bpy]^+$ were evident, but less abundant than $[M^{II}(L - H)\ L]^+$ when M^{II} = Mn(II), Fe(II), and Zn(II).

For successful formation of the ternary complexes, the binding strengths of the metal and auxiliary ligand must be similar to that of the metal and analyte. Although binding constants for the flavonoids have not been reported, binding constants of model compounds, i.e., oxygen-rich chelating agents, such as deprotonated 5-hydroxytetracycline, deprotonated 3-hydroxy-2-methyl-4-pyrone, and acetylacetonate, indicate a range of log K values of 5.4−5.5 for Co(II), 5.5−6.0 for Ni(II), and 7.6−8.25 for Cu(II) in aqueous solution.[53] Although the binding constants are for aqueous solutions, the trends in selectivity are probably similar in methanolic solutions. If the auxiliary ligand binds to the metal much more strongly than does the flavonoid, complexes composed of the metal ion and two or more auxiliary ligands will be dominant over those involving the deprotonated flavonoid. If coordination of the auxiliary ligand is relatively weak compared to the flavonoid, formation of electroneutral complexes, $[M^{II}(L - H)_2]^0$, will dominate, lowering the overall signal. The use of 2,2′-bipyridine with doubly charged transition metals for the complexation of flavonoids is successful in part due to the similarities of binding strengths of the deprotonated flavonoid and the auxiliary ligand.

To further investigate the optimal conditions for formation of metal complexes with an auxiliary ligand, the nominal pH of the solutions was varied. As expected based on the acidity of the flavonoids, lowering the nominal pH by addition of 5% acetic acid to the solution severely reduced the amount of complexation, due to the suppressed deprotonation of flavonoid molecules. Raising the pH by addition of 5% ammonium hydroxide also tended to decrease complexation, which is attributed to the increased deprotonation of the flavonoid molecules and favored formation of electroneutral complexes, $[M^{II}(L - H)_2]^0$. All further studies were carried out without manipulation of the pH, thus maintaining nominally neutral conditions in methanol.

To investigate the possibility of structural characterization of the flavonoids, collisional-activated dissociation (CAD) was performed, as shown in Figure 8 for the ternary complexes involving hesperidin. The dissociation patterns are easily assigned, uncluttered, and tunable based on the metal, providing complementary structural information to that of CAD of $[L - H]^-$. The CAD behavior will be explored in greater detail in another study.

Elucidation of Coordination Sites of Flavonoids. To elucidate the favored coordination sites of the flavonoids to the metal ions, a series of experiments were performed with flavonoid aglycons that have hydroxyl groups in differing positions, in conjunction with the presence or absence of the carbonyl moiety at carbon 4 on the C ring. The flavonoids used were 3,7-dihydroxyflavone, 5,7-dihydroxyflavone, luteolin, 7,3′,4′-trihydroxyflavone, 7,4′-dihydroxyflavone, 7-hydroxyflavone, catechin, and quercetin, all shown in Figure 9. Flavone, possessing no hydroxyl groups, does not form the $[M^{II}(L - H)\ bpy]^+$ complexes, presumably due to the lack of a suitable acidic hydrogen. The

(53) Smith, R. M.; Martell, A. E. *Critical Stability Constants*; Plenum Press: New York, 1975.

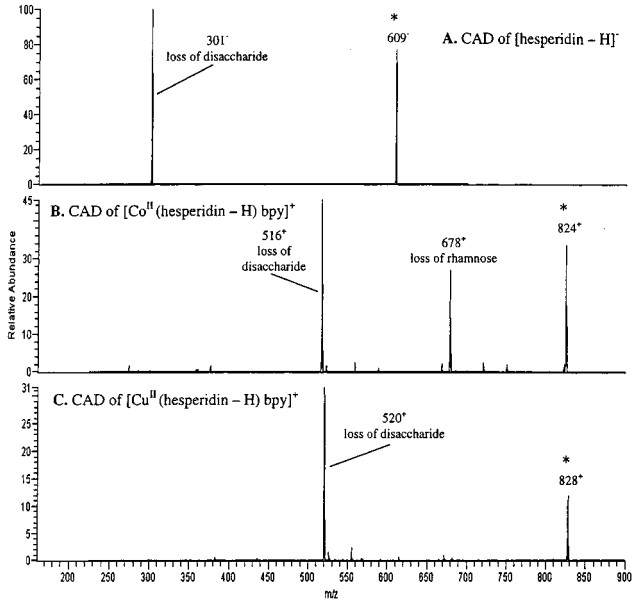

Figure 8. Dissociation patterns of deprotonated hesperidin, and ternary complexes of hesperidin with cobalt(II) or copper(II) and 2,2′-bipyridine in which the asterisk indicates the precursor.

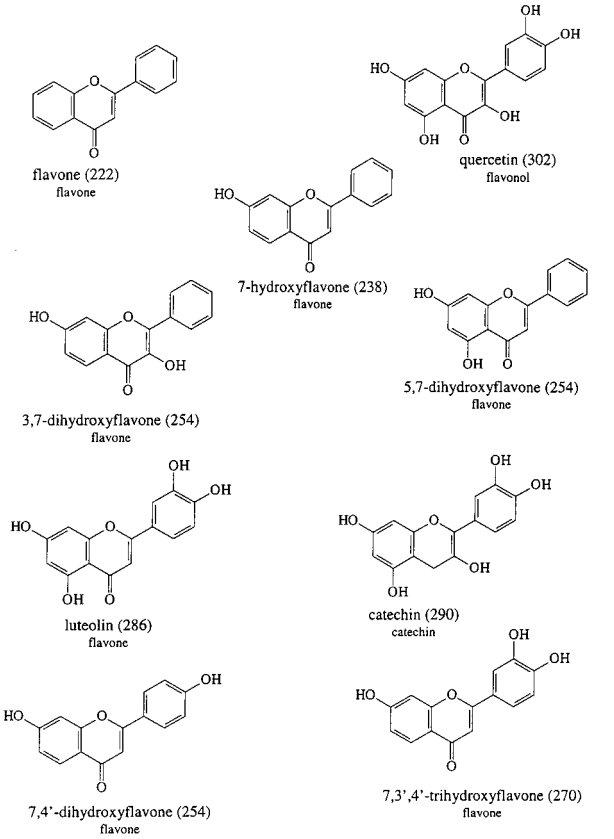

Figure 9. Flavonoids used in investigation of coordination sites of metal complexation (molecular weight). Category of flavonoid is also given.

four compounds that produce the lowest intensities of the metal−2,2′-bipyridine complexes are catechin, 7,3′,4′-trihydroxyflavone (Figure 10A), 7-hydroxyflavone, and 7,4′-dihydroxyflavone, each of which lack a pair of chelating oxygen atoms at the carbon 3 and carbon 4 or carbon 4 and carbon 5 positions. 5,7-Dihydroxyflavone, luteolin, and 3,7-dihydroxyflavone (Figure 10B) generated

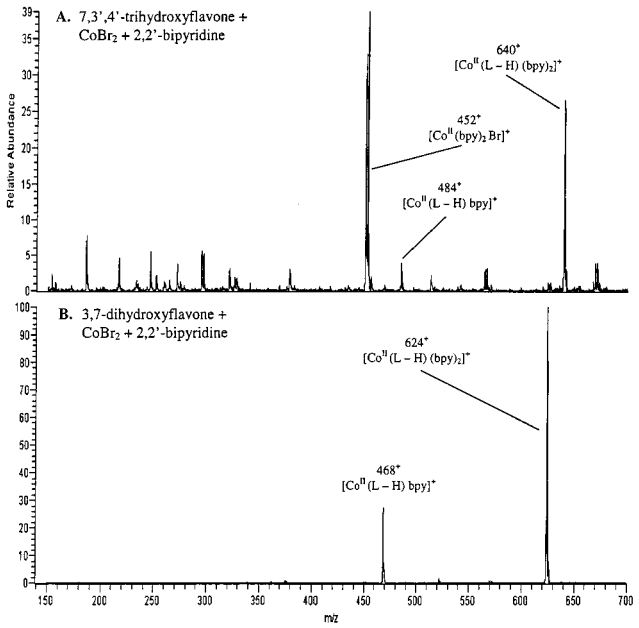

Figure 10. Comparison of ESI-MS of flavonoid model compounds with differing structural features that favor or discourage metal coordination. The vertical scales are directly comparable for this series of spectra, with maximum abundance being 100, and values less than 100 signifying proportionally lower abundances.

intense metal−2,2′-bipyridine complexes, and these three possess the favorable chelating oxygen atoms at the carbon 3 and carbon 4 or carbon 4 and carbon 5 positions. Quercetin is an interesting case because it possesses the important carbon 4 carbonyl in addition to hydroxyl groups at both the carbon 3 and carbon 5 positions. This compound also produces intense complexes, but less intense than 3,7-dihydroxyflavone and 5,7-dihydroxyflavone, which only have one of the two hydroxyl groups at the carbon 3 or carbon 5 positions. Apparently, the extra hydroxyl group exerts an electron-withdrawing effect that moderately destabilizes the chelation interaction to the metal ion. These results highlight the importance of the carbonyl and hydroxyl chelating pair in metal coordination, although complexes involving flavonoids lacking the carbonyl group were also evident to a lesser degree. The presence of at least one hydroxyl group appears to be essential for formation of the metal complexes.

The experimental results indicating preferential metal coordination between the deprotonated hydroxyl group at carbon 3 or carbon 5 and the carbon 4 carbonyl group parallel the dissociation constants measured spectrophotometrically for monohydroxyflavones.[54] Calculated pK_a values for the hydroxyl group of 3-hydroxyflavone and 5-hydroxyflavone were 9.6 and 11.56, respectively. The higher acidity of the proton at the carbon 3 position correlates well with its facile deprotonation and enhanced metal-binding ability.

Studies to determine the possible atoms of the saccharides of the flavonoid glycosides that may participate in metal coordination were undertaken by examination of solutions of individual monosaccharides and a metal salt. Four of the five flavonoid glycosides, rutin, naringin, hesperidin, and diosmin, are composed of a flavonoid aglycon attached to a disaccharide consisting of

(54) Wolfbeis, O. F.; Leiner, M.; Hochmuth, P.; Geiger, H. *Ber. Bunsen-Ges. Phys. Chem.* **1984**, *88*, 759−767.

glucose and rhamnose, with the terminal saccharide being rhamnose. As seen in Figure 2, differences between the four flavonoid glycosides involve the intersaccharide linkage and the saccharide−aglycon linkage. Rutin is a 3-*O*-rutinoside, naringin is a 7-*O*-neohesperidoside, and hesperidin and diosmin are both 7-*O*-rutinosides.

To encourage deprotonation and metal coordination, the monosaccharides were examined in basic solution with $CoBr_2$. On the basis of spectral intensities of the resulting $[Co^{II}(L - H)L]$ complexes, coordination to Co^{2+} is favored for glucose over rhamnose, probably due to the enhanced acidity of the methylene hydroxyl group. Of course, coordination with the flavonoids is also based on the proximity of the saccharide to the metal ion. Because rhamnose is the terminal saccharide, coordination between rhamnose and the metal ion may be favored over that involving glucose.

Molecular modeling was carried out to determine the most probable conformer of the ternary complexes. Due to the lack of availability of experimental parameters for cobalt and nickel, theoretical calculations were confined to copper. Using semiempirical calculations, conformer distributions for complexes of the type $[M^{II}(L - H)\ bpy]^+$ were performed. As expected based on the availability of binding sites on the saccharide, conformer distributions of the ternary complexes indicate coordination at several sites (see Figure 7). When molecular models of the ternary complexes were compared to molecular models of the metal complexes, $[Cu^{II}(L - H)]^+$, coordination of Cu(II) to the saccharides is altered somewhat to accommodate the auxiliary ligand. For example, molecular modeling results of the $[Cu^{II}$(hesperidin $- H)]^+$ complex indicate that the copper(II) ion is closest to oxygens 7 and 8 of the outer sugar, rhamnose, as seen in Figure 7. When 2,2′-bipyridine is added, the copper ion is closest in distance to oxygens 7 and 8 *or* 8 and 9 of rhamnose, permitting close coordination of the nitrogens of the auxiliary ligand. For each of the four flavonoids, the most favorable interactions (based on evaluation of the shortest metal−heteroatom distances in which the "shortest" distances are 2.0 ± 0.5 Å and the others range from 5 to 12.0 Å) involve the Cu^{2+} ion and the two oxygens from the aglycon portion (i.e., the deprotonated phenolic oxygen and the adjacent carbonyl oxygen), two oxygens involving the outer sugar, and the two nitrogens from 2,2′-bipyridine. On the basis of the different structures shown in Figure 7 for the binary versus ternary complexes, one would predict that these different complexes might dissociate in different ways. This interesting issue will be explored in a future report.

Limit of Detection of $[Cu^{II}$(naringin − H)bpy$]^{+\cdot}$. To determine the improvement in signal seen with the ternary metal complexes as compared to that of the deprotonated flavonoids, a limit of detection study was carried out. Solutions were prepared composed of 1:5:5 naringin/$CuBr_2$/2,2′-bipyridine beginning with a concentration of 1.0×10^{-11} M and followed by solutions of increasing naringin concentration with the same net ratio of flavonoid to metal salt and 2,2′ -bipyridine. Under optimum conditions, the limit of detection of the ternary complex, $[Cu^{II}$-(naringin − H)bpy$]^+$, based on the lowest naringin concentration at which known fragments were seen upon collisional-activated dissociation of the isolated complex, was determined to be 5.0×10^{-11} M, with a signal/noise level of greater than 3. On the same day using similar instrumental conditions, but tuned for the negative ionization mode and optimization of $[L - H]^-$, the limit of detection of [naringin − H]$^-$ was ∼1.5 orders of magnitude higher, also confirmed by its CAD pattern. These results illustrate the enhanced lower limit of detection possible with the ternary complexes as compared to the deprotonated flavonoid. Future reports will explore the quantitative application of metal complexation for flavonoid detection in biological matrixes.

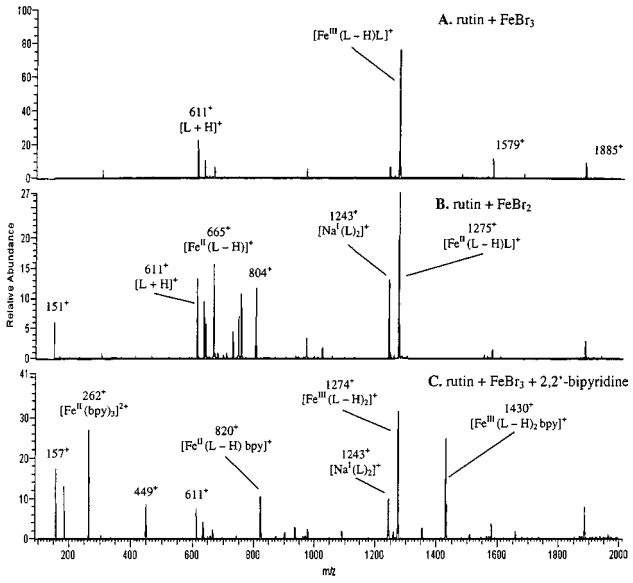

Figure 11. Comparison of rutin−metal complex formation with doubly and triply charged metal salts. The vertical scales are directly comparable for this series of spectra, with maximum abundance being 100, and values less than 100 signifying proportionally lower abundances.

Formation of Flavonoid−Metal Complexes with Triply Charged Metals. Since coordination of a flavonoid ion and the auxiliary ligand to a transition metal ion was successful with doubly charged metals, transition metals with +3 charge seemed natural candidates for flavonoid complexation, perhaps without the auxiliary ligand. Moreover, the formation of complexes involving two deprotonated flavonoids would lead to detectable singly charged complexes, $[Fe^{III}(L - H)_2]^+$. Iron(III) bromide was chosen as the metal salt since results could be directly compared with those of iron(II) bromide. As shown in Figure 11, spectra of flavonoids and iron(III) bromide were simpler and cleaner than those with iron(II) bromide. With every flavonoid investigated, complexes identified as $[Fe^{III}(L - H)_2]^+$ were identified as the base peaks. In solutions of iron(II), complexes identified as $[Fe^{II}(L - H)L]^+$ were usually evident upon visual inspection, but the spectra were cluttered with many other ions.

The auxiliary ligand, 2,2′-bipyridine, was added to the iron(III) solution to see if signal enhancement similar to that seen with doubly charged metal ions would occur. As shown in Figure 11C, complexes including the auxiliary ligand were identified, but not in the increased abundance seen with doubly charged metal ions. In general, use of triply charged metals offers another avenue for future studies with other analytes, but the ESI complexation efficiencies for the flavonoids were not superior to those obtained with the doubly charged metals.

CONCLUSION

Metal complexation of flavonoids with doubly charged transition metals has been investigated as an alternative to protonation and deprotonatation with resultant signal enhancement of the flavonoid−metal complexes. Addition of a transition metal salt to a flavonoid solution results in formation of $[M^{II}(L - H)]^+$ and/or $[M^{II}(L - H)L]^+$ complexes that are typically 1 order of magnitude more intense than the protonated flavonoids and up to 1 order of magnitude greater than the deprotonated flavonoids. The greatest signal enhancement occurs upon addition of an auxiliary ligand, 2,2′-bipyridine, to the flavonoid−metal solutions, thus preventing formation of electroneutral complexes and leading to at least a doubling of the ion intensities relative to those of the metal complexes. Of the various transition metals, Cu^{2+} produces the most intense $[M^{II}(L - H)]^+$ and $[M^{II}(L - H)bpy]^+$ complexes, presumably because the preferred coordination geometry of Cu^{2+} prohibits formation of several other types of complexes that are noted for the other transition metals, thus dividing the ion current into several different product channels. The limit of detection of the ternary complex $[Cu^{II}(naringin - H)\ bpy]^+$ has been measured as 1−1.5 orders of magnitude better than that of the deprotonated flavonoid, $[naringin - H]^-$, under optimal conditions.

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