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Gas-Phase Hydrogen/Deuterium Exchange and Conformations of Deprotonated Flavonoids and Gas-Phase Acidities of Flavonoids

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Abstract: Gas-phase hydrogen/deuterium (H/D) exchange was used to probe the conformations, gas-phase acidities, and sites of deprotonation of isomeric flavonoid aglycons and glycosides. The flavonoids in each isomer series were differentiated on the basis of their relative rate constants and total numbers of exchanges. For example, flavonoids that possess neohesperidose-type disaccharides may undergo faster and far more extensive exchange than isomeric rutinoside flavonoids. The structural factors that promote or prevent H/D exchange were identified and correlated with collisionally activated dissociation (CAD) patterns and/or molecular modeling data (both high-level ab initio acidity calculations and conformational analysis with molecular dynamics (MD) simulations), thus providing a framework for the use of H/D exchange reactions in the structural elucidation of new flavonoids.

1. Introduction

Flavonoids are antioxidants that hold promise for preventing age-related diseases, including heart disease and cancer. Because of their well-known health benefits and chemopreventive properties,[1−3] flavonoids have become a popular target of biological, biochemical, and analytical studies. Thousands of different flavonoids have been identified in plants, and although most have a common phenyl-benzopyrone skeleton (Figure 1), they differ with respect to the positions of hydroxyl and methoxy groups; the location, number, and identities of saccharides involved in glycosylation; and the intersaccharide linkage in the case of polysaccharides.[4] Structural characterization and isomer differentiation of the thousands of flavonoids is essential for identifying and quantifying individual flavonoids in food sources and biological samples and in understanding their metabolism, bioavailability, and structure−activity relationships. Mass spectrometry offers an excellent way to characterize the structures of flavonoids in a systematic fashion with good sensitivity.[5−20] Collisionally activated dissociation (CAD) has been commonly used to structurally characterize the different flavonoids in the negative mode[5−12] or in the positive mode by protonation[8−9,13−17] or by metal complexation[11,18−20] in conjunction with electrospray ionization (ESI) or fast atom bombardment (FAB).

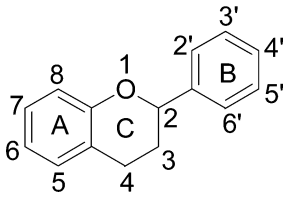

Figure 1. General structure of flavonoids.

One of the greatest analytical challenges posed by the flavonoids is that many of them are isomeric, differing only in the arrangement of the hydroxyl groups or the position of the glycosides. MS/MS/MS methods offer some merit for isomer differentiation of flavonoids,[11,19] but the need for other auxiliary mass spectrometric methods, both for distinction of isomers and for providing complementary information about structural

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features that influence the dissociation behavior of isomers, is apparent.

Hydrogen/deuterium (H/D) exchange offers a rather unique way to probe the structures of gas-phase ions, and it is becoming a widespread tool for structural elucidation and isomer differentiation.[21−34] In general, H/D exchange reactions provide a sensitive way for exploring subtle structural or conformational differences in gas-phase ions on the basis of either the rates or the average extent of deuterium incorporation. The rates and extent of H/D exchange correlate with several key factors of the compounds of interest: the gas-phase basicity or gas-phase acidity, the location of the charge site, and the conformation of the resulting ion in the gas phase. In some cases, H/D exchange proves to be more useful than conventional CAD methods for isomer differentiation because it does not involve energization of ions, meaning that isomers are less likely to rearrange or intraconvert during analysis. In addition, H/D exchange reactions can be used to correlate similarities in reaction behavior with common structural features, thus providing a road map for further studies involving compounds with unknown structures. Especially interesting cases are those that involve isomers that have both several different potential ionization sites and conformations, such as phenolic compounds such as the flavonoids.

Flavonoids are good candidates for H/D exchange because of their multiple hydroxyl groups (providing labile hydrogens and sites of deprotonation) and resonance structures through three conjugated ring systems. There has been only one published study on the H/D exchange of protonated flavonoid aglycons in the gas phase, which was performed via chemical ionization (CI) with D_2O.[35] Under the CI conditions, not only the hydrogens of the hydroxyl groups but also the aromatic hydrogens *ortho* and *para* to the hydroxyl groups underwent exchange easily.

As a complement to conventional CAD methods for evaluating the flavonoids, we have investigated the possibility of structural characterization and isomer differentiation of flavonoids (both aglycons and glycosides) by H/D exchange reactions of deprotonated flavonoids or their metal complexes in a quadrupole ion trap mass spectrometer (QIT-MS). H/D exchange was employed specifically to probe the small structural differences of the flavonoids (Figure 2), in addition to investigating the conformational effects of the flavonoids that promote or prevent extensive H/D exchange. The rates of H/D exchange were correlated with the CAD fragmentation patterns. Molecular modeling means were also performed to further understand the mechanisms of H/D exchange, using both ab initio calculations and molecular dynamics (MD) simulations.

2. Experimental Section

2.1. Chemical Reagents. Acacetin, daidzein, hesperetin, hesperidin, naringenin, naringin, neohesperidin, rutin (Figure 2), and sucrose were purchased from Sigma (St. Louis, MO). Apigenin, apigenin-7-*O*-glucoside, astragalin (kaempferol-3-*O*-glucoside), 3,7-dihydroxyflavone, isorhoifolin, isovitexin (apigenin-6-*C*-glucoside), kaempferol, luteolin, luteolin-4′-*O*-glucoside, luteolin-7-*O*-glucoside, narirutin, quercetin, quercitrin, orientin (luteolin-8-*C*-glucoside), rhoifolin, and vitexin (apigenin-8-*C*-glucoside), (Figure 2) were purchased from Indofine (Somerville, NJ). Daidzin, fortunellin, linarin, and luteolin-7,3′-*O*-diglucoside (Figure 2) were purchased from Extrasynthese (Genay, France). 2,2′-Bipyridine and $CoBr_2$ were purchased from Aldrich (Milwaukee, WI). D_2O, CD_3OD, and ND_3 were purchased from Cambridge Isotopes (Andover, MA). All the above compounds were used without further purification.

All the stock solutions (10^{-4} to 10^{-2} M) of the above flavonoids and sucrose as well as the metal complexation reagents were prepared in HPLC grade methanol and then diluted in methanol to obtain working solutions ($1-4 \times 10^{-5}$ M).

2.2. H/D Exchange and CAD.

2.2.1. H/D Exchange. The instrument used for H/D exchange was a Hitachi 3DQ quadrupole ion trap (QIT) (model: M-8000 LC/3DQMS) equipped with an electrospray ionization (ESI) source (Japan). The nitrogen sheath gas was set between 2 and 3 kg/cm^2, and the helium damping gas was set at 3 kg/cm^2. The flow rate of the flavonoid solutions ($\sim 4 \times 10^{-5}$ M) was kept at 15 μL/min with a makeup flow of methanol at 150 μL/min. The temperature settings were as follows: assistant gas heater 200 °C, desolvator 200 °C, and aperture 1 190 °C. The ESI probe, drift, and focus voltages were optimized as necessary. The photomultiplier detector voltage was set at 600 V. All the flavonoids were detected either as deprotonated molecules or as their metal complexes. The pseudo-molecular ion cluster was allowed to undergo gas-phase H/D exchange. The isomers within each series of flavonoids were run back to back under very similar if not identical conditions and repeated at least once on another day.

Deuterium oxide (D_2O), deuterated ammonia (ND_3), or deuterated methanol (CD_3OD) was used as an exchange reagent and introduced to the ion trap through a home-built leak valve setup. Briefly, a precision leak valve assembly and an ionization gauge were mounted on a standard conflat flange tee fitting (1.5-in. tube diameter) over a custom inlet to the vacuum chamber of the 3DQ ion trap. In this arrangement the exchange gas was admitted to the vacuum chamber independently of the helium buffer gas (the base pressure was $\sim 5 \times 10^{-5}$ Torr read from the ion gauge). The extent of exchange was examined by monitoring the relative abundance of the precursor pseudo-molecular ion and its deuterated species while the pressure of the exchange reagent (up to 5×10^{-4} Torr) and/or the reaction time (up to 10 s) allowed for exchange was varied. Kinetic plots and H/D exchange constants were obtained after data fitting using KinFit.[36] All the data points were normalized before fitting, and the same set of differential equations as defined by KinFit[36] was used for fitting. Exchange factors (at 1 s) were calculated as the sum of the peak heights of all deuterium-exchanged ions divided by the peak height of the unexchanged precursor ion.[28] Isotope correction was performed before any calculation or data fitting.

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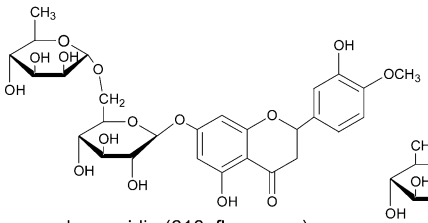
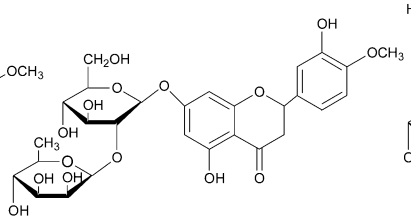

hesperidin (610, flavanone)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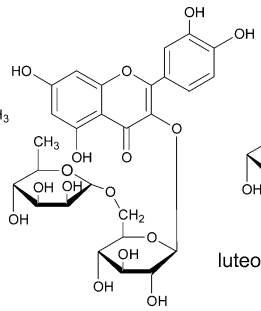
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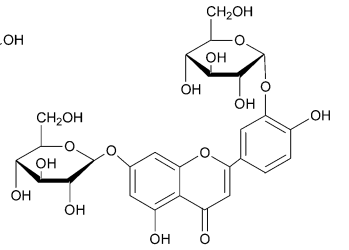
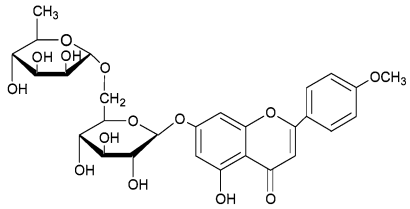

luteolin-7,3'-O-diglucoside (610, flavone)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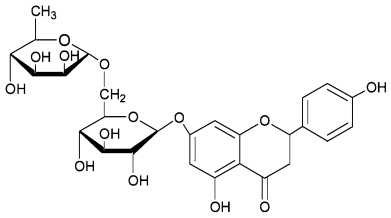
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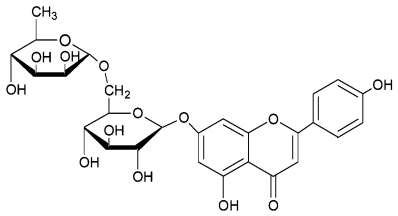
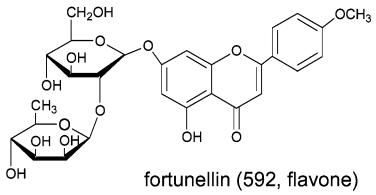

isorhoifolin (578, flavone)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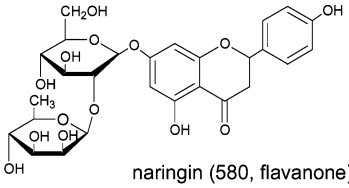
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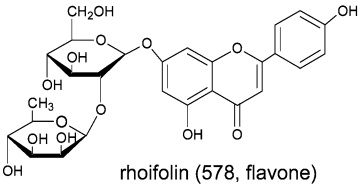
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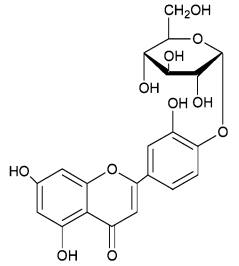
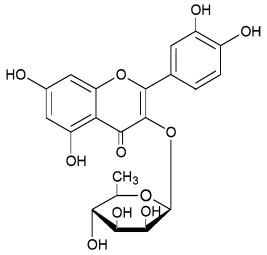

luteolin-4'-O-glucoside (448, flavone)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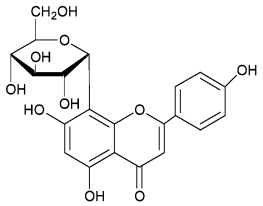
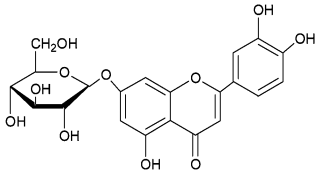
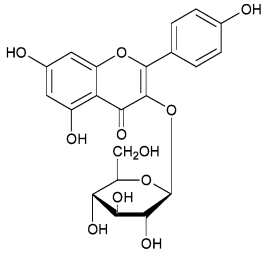
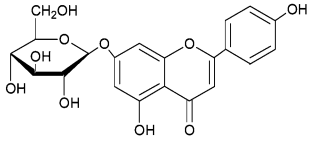
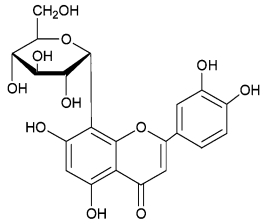
(448, flavonol)

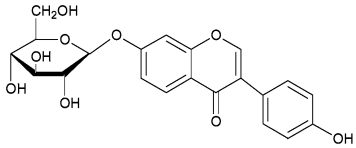

vitexin (apigenin-8-C-glucoside)
(432, flavone)

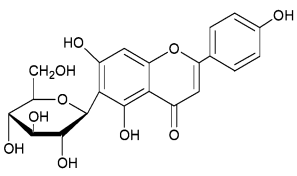

luteolin-7-O-glucoside (448, flavone)


astragalin (kaempferol-3-O-glucoside)
(448, flavonol)


apigenin-7-O-glucoside (432, flavone)


orientin (luteolin-8-C-glucoside)
(448, flavone)


daidzin (416, isoflavone)


isovitexin (apigenin-6-C-glucoside)
(432, flavone)

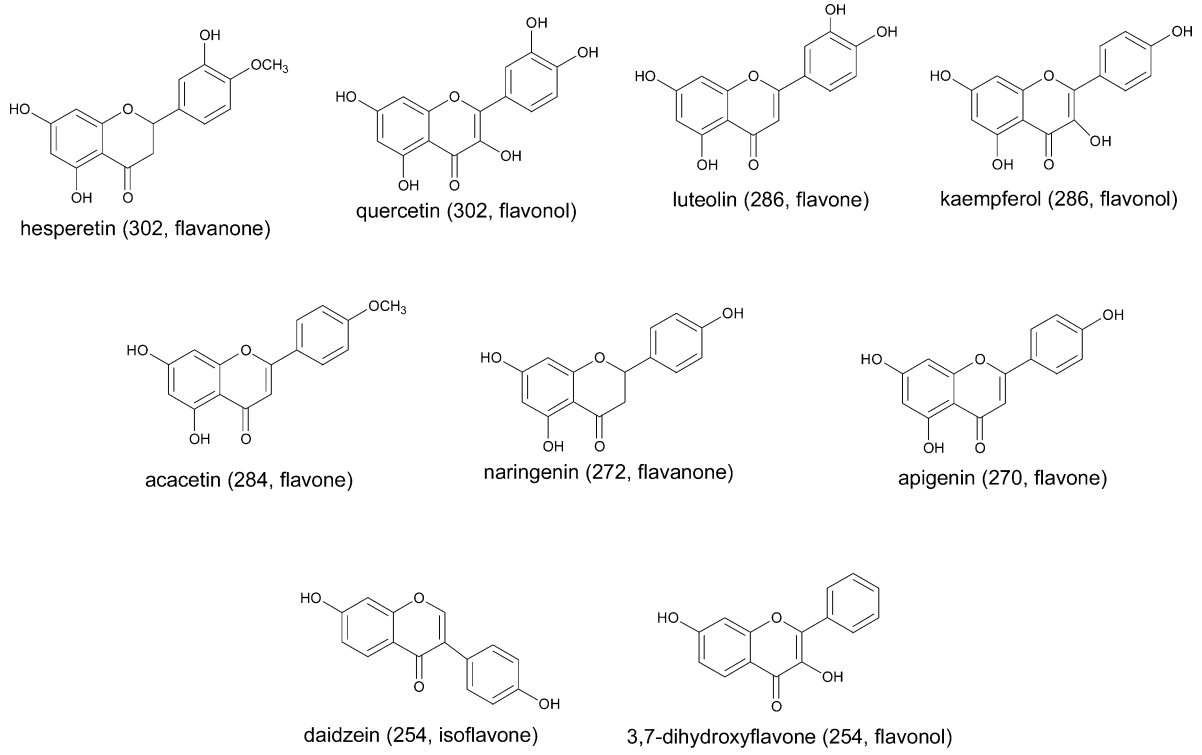


Figure 2. Flavonoid structures (molecular weight, class of flavonoid) in order of decreasing molecular weight.

2.2.2. CAD. A Thermo Finnigan LCQ Duo quadrupole ion trap instrument equipped with an ESI source (San Jose, CA) was used for all the CAD studies. The flow rate of the flavonoid solutions ($\sim 1 \times 10^{-5}$ M) was set at 5 μL/min. The heated capillary temperature was kept at 200 °C. The ESI spray voltage was set at −4.5 kV. The other instrumental parameters were tuned to optimize the relative abundance of the ions of interest. The deprotonated parent molecule of each flavonoid disaccharide was isolated and allowed to undergo CAD. The CAD energy was varied such that the relative abundance of the surviving parent ion was about 5−10%. Each rutinose/neohesperidose isomeric pair was run under the same experimental conditions on the same day for reliable comparison.

2.3. Molecular Modeling. High-level ab initio calculations of flavonoid acidities and long-period MD simulations of selected flavonoids were performed on the IBM pSeries 690 supercomputer of NCSA (www.ncsa.uiuc.edu).

2.3.1. Acidity Calculations. The Gaussian 03 software package[37] was used for the acidity calculations. The structures of selected flavonoids, saccharides, and three exchange reagent molecules (water, methanol, and ammonia) and their anions were optimized at the HF/6-31G(d) level. Zero-point energies (ZPE) were calculated at the same level and then scaled by a factor of 0.9135[38] to obtain the corrected ZPE. Single-point energies (E_{elec}) were calculated at three different levels, HF/6-31G(d), HF/6-311+G(d,p), and MP2/6-311+G(d,p), to gain insight into the impact of different models and different basis sets on the calculated acidities. B3LYP/6-311+G(d,p) was also used to calculate the single-point energies of water, methanol, and ammonia. The total energy of a species is approximately the sum of its single-point energy and corrected ZPE: $E = E_{elec} + \text{ZPE (corrected)}$. The energy difference between an anion (A^-) and its neutral species (HA) is the calculated acidity: $\Delta H_{acidity} = \Delta E_{elec} + \Delta\text{ZPE (corrected)}$, where $\Delta H_{acidity} = H_{A^-} - H_{HA}$, $\Delta E_{elec} = E_{A^-} - E_{HA}$, and $\Delta\text{ZPE} = \text{ZPE}_{A^-} - \text{ZPE}_{HA}$ (corrected).

2.3.2. MD Simulations. To study the conformations of deprotonated flavonoids in the gas-phase surrounded by a few D_2O molecules, MD simulations were carried out for selected flavonoids (naringin, rhoifolin, luteolin-7,3′-diglucoside, fortunellin, and linarin) using the AMBER 7.0 suite of programs.[39] To balance the computational efficiency and accuracy, each flavonoid having several potential deprotonation sites was cut into two (or three for luteolin-7,3′-*O*-diglucoside) residues, an aglycon and a saccharide, to calculate the restrained electrostatic potential charges (RESP) as suggested in the AMBER package. The initial structure of a deprotonated flavonoid was built by splicing the two residues together, and then 14−18 (depending on the size of the flavonoid) D_2O molecules were randomly placed around the molecule. The exchange reagent molecules were restricted within 16 Å of the mass center of the deprotonated flavonoid during the MD simulations. The molecular mechanical force-field parameters of these flavonoids were obtained from a general AMBER force field developed by Wang et al.[40,41] For each deprotonated flavonoid, the MD simulations were carried out at 300 K with a time step of 2.0 fs. After system equilibration

(37) Frisch, M. J.; Trucks, G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Montgomery, J. A., Jr.; Vreven, T.; Kudin, K. N.; Burant, J. C.; Millam, J. M.; Iyengar, S. S.; Tomasi, J.; Barone, V.; Mennucci, B.; Cossi, M.; Scalmani, G.; Rega, N.; Petersson, G. A.; Nakatsuji, H.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Klene, M.; Li, X.; Knox, J. E.; Hratchian, H. P.; Cross, J. B.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Ayala, P. Y.; Morokuma, K.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Zakrzewski, V. G.; Dapprich, S.; Daniels, A. D.; Strain, M. C.; Farkas, O.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Ortiz, J. V.; Cui, Q.; Baboul, A. G.; Clifford, S.; Cioslowski, J.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Challacombe, M.; Gill, P. M. W.; Johnson, B.; Chen, W.; Wong, M. W.; Gonzalez, C.; Pople, J. A. *Gaussian 03*; Gaussian, Inc.: Pittsburgh, PA, 2003.
(38) Foresman, J. B.; Frisch, Æ. *Exploring Chemistry with Electronic Structure Methods*; Gaussian, Inc.: Pittsburgh, PA, 1996; Chapter 4.
(39) Case, D. A.; Pearlman, D. A.; Caldwell, J. W.; Cheatham, T. E., III; Wang, J.; Ross, W. S.; Simmerling, C. L.; Darden, T. A.; Merz, K. M.; Stanton, R. V.; Cheng, A. L.; Vincent, J. J.; Crowley M.; Tsui, V.; Gohlke, H.; Radmer, R. J.; Duan, Y.; Pitera, J.; Massova, I.; Seibel, G. L.; Singh, U. C.; Weiner, P. K.; Kollman, P. A. *AMBER7*; University of California: San Francisco, CA, 2002.
(40) Wang, J.; Cieplak, P.; Kollman, P. A. *J. Comput. Chem.* **2000**, *21*, 1049.
(41) Wang, J.; Wolf, R.; Caldwell, J.; Kollman, P.; Case, D. *J. Comput. Chem.*, in press.

for 2 ns, 5000 snapshots were collected (2 ps per snapshot) for postprocessing analysis.

3. Results and Discussion

Hydrogen/deuterium exchange reactions of a series of deprotonated or metal-cationized flavonoids (including 18 glycosides and nine aglycons) and the model disaccharide, sucrose, were monitored. To assist in explanation of the observed H/D exchange behaviors of the flavonoid isomers or structurally similar flavonoids, molecular modeling was performed on selected flavonoids and the exchange reagents. For gas-phase H/D exchange reactions to occur between a weak exchange reagent (with low acidity, such as D_2O in this work) and a compound of interest with relatively high acidity, a relay mechanism has been proven to occur,[23] in which hydrogen bonds form between the exchange reagent and the deprotonation site as well as with neighboring acidic hydrogen(s) of the compound, and the hydrogen-bonded intermediate then facilitates the exchange process. Therefore, some knowledge about the deprotonation site of a compound of interest is critical to explain its H/D exchange behavior. High level ab initio acidity calculations were first carried out to predict the possible deprotonation sites. MD simulations were then undertaken to gain insight into the conformations of the resulting deprotonated flavonoids. CAD was also performed on selected deprotonated flavonoid disaccharides to probe correlations, if any, between the H/D exchange behavior and dissociation behavior.

3.1. Gas-Phase Acidities. Because the thermodynamics and kinetics of the H/D exchange reactions are directly related to the relative acidities of the compounds involved in the reactions, knowledge about the gas-phase acidities of the various hydroxyl groups of the flavonoids is important for rationalizing the observed trends in H/D exchange. Thus, extensive ab initio calculations were undertaken to estimate the acidities of the flavonoid constituents.

3.1.1. Model Selection. The compounds listed in Table 1 were chosen as models for estimating the gas-phase acidities. Ammonia, water, and methanol were also selected as model compounds to allow comparison of the ab initio results to known experimental acidities (404, 391, and 382 kcal/mol for ammonia, water, and methanol, respectively).[42] For simple small molecules, HF/6-31G(d) and HF/6-311+G(d,p) calculations provided the same relative acidity order (ammonia < water < methanol) as obtained by MP2/6-311+G(d,p), but the two HF treatments underestimated the absolute acidities[42] because of their lack of utilization of electron correlation, the energy contributions arising from electrons interacting with one another, which is important for anions. Though the acidity values were closer to the experimental values[42] when a larger basis set was used and with addition of diffuse functions and polarization functions for hydrogen (HF/6-311+G(d,p) vs HF/6-31G(d)), electron correlation had to be considered to obtain excellent agreement between the calculated and experimentally derived acidities for even such small molecules. B3LYP is a correlated model (based on the density functional theory, DFT) that is often used when a balance between available computation resources and accuracy must be sought. To test the accuracy of such a DFT-based model, single-point energies were also calculated at the B3LYP/6-311+G(d,p) level using the HF/6-31G(d)-optimized structures for water, methanol, and ammonia (Table 1). Though the B3LYP results were better than the HF models, the B3LYP model could not compete with the MP2 model for accuracy. Therefore, no further calculations were undertaken with the DFT-based model B3LYP. For larger and more complex molecules such as saccharides and flavonoids, the three ab initio models (HF- and MP2-based) were generally in good agreement on the relative orders of acidities, but some divergence occurred (e.g., quercetin). Since the MP2/6-311+G(d,p) model is the most reliable based on the results for the small molecules, results obtained from this model are used exclusively to compare the relative acidities of the different deprotonation sites for the flavonoids and to explain the experimental results.

3.1.2. Acidities of Flavonoids and Saccharides. Because of the large size of the flavonoid glycosides, direct calculation of the acidities of the various hydroxyl groups located on the aglycon and the saccharide portions was computationally impractical. As a compromise, the acidities of the aglycons and saccharides were calculated separately. Gas-phase acidities were calculated for nine flavonoid aglycons that possessed from two to five acidic hydroxyl groups, and the values are summarized in Table 1. In general, the most acidic groups are the 7-OH, and 4′-OH or 3′-OH, and the least acidic is the 5-OH group. For acacetin and 3,7-dihydroxyflavone, two aglycons that do not have a 4′-OH group, the 7-OH is most acidic. The 4′-OH is the most acidic group for quercetin and apigenin, whereas the 3′-OH or 7-OH is the most acidic site for luteolin or kaempferol, respectively. For all the above cases, the charge at either the 4′- or 7-position may be stabilized through resonance between the B and C or the A and C rings as shown in Schemes 1 and 2 for kaempferol and 3,7-dihydroxyflavone, respectively. When two adjacent hydroxyl groups exist on the B ring (i.e., quercetin and luteolin), intramolecular hydrogen bonding is another major stabilization factor for the anions (Scheme 3, structure A). For naringenin and daidzein, the charge at the 7-position may be stabilized through resonance between the A and C rings (similar to the processes shown in Schemes 1 and 2), while the charge at the 3′- or 4′-position will be restricted on the B ring due to the single bond (between C2 and C3) of nesperetin or naringenin or the unusual B ring position of daidzein. It is thus reasonable to expect that the 7-OH is the most acidic group for these three aglycons. It is notable that the flavanones naringenin and hesperetin have two possible configurations (R and S), each as a result of the single bond between C2 and C3. Since the S configuration of naringenin is more stable (its energy is 2.5 kcal/mol lower as calculated at the HF/6-31G(d) level), only the S configuration is considered for the flavanones. For all the flavonoids studied, the 5-OH group (if present) is always the least acidic, which is not surprising since it is likely that a hydrogen bond between the 5-OH and 4-keto oxygen exists in the neutral form of the aglycons, thus making removal of this proton energetically more unfavorable. As can be seen from the above ab initio predictions, charge stabilization through resonance and hydrogen bonding are the two major determining factors for the deprotonation process and resulting acidities.

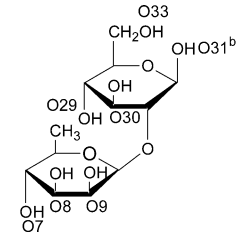
Gas-phase acidities were calculated for two disaccharides, each having numerous hydroxyl groups that represent possible deprotonation sites. The hydroxyl groups of 1,6-rhamnose-glucose (rutinose) and 1,2-rhamnose-glucose (neohesperidose)

(42) Gas-phase acidity values from NIST Standard Reference Database Number 69. http://webbook.nist.gov/chemistry/ (released March, 2003).

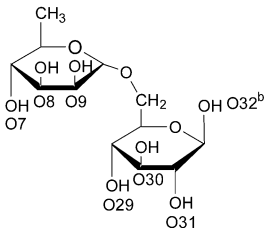
Table 1. Gas Phase Acidities of Selected Flavonoids, Saccharides, and H/D Exchange Reagents

compound	charge site[a]	acidity (kcal/mol) HF/6-31G(d)	HF/6-311+G(d,p)	MP2/6-311+G(d,p)
ammonia		434.2	412.5	403.7 (402.2[c])
water		421.0	398.3	389.8 (387.5[c])
methanol	CH_3O^-	397.8	389.1	382.0 (378.0[c])
quercetin	3	348.9	346.5	336.3
	5	349.1	346.1	340.8
	7	336.4	333.6	327.8
	3′	358.1	353.0	339.8
	4′	335.1	332.0	321.8
hesperetin (S)	5	353.4	349.6	342.7
	7	341.4	338.4	328.1
	3′	349.2	345.0	334.8
kaempferol	3	350.4	347.8	336.3
	5	349.0	345.9	340.3
	7	335.8	333.0	327.3
	4′	340.9	337.1	328.9
luteolin	5	355.2	351.0	345.3
	7	339.2	335.6	329.3
	3′	339.9	336.3	323.8
	4′	342.0	339.0	331.0
acacetin	5	357.2	353.1	347.0
	7	341.3	337.8	331.1
naringenin (S)	5	354.9	350.9	343.9
	7	339.2	335.8	328.8
	4′	350.2	346.0	337.5
apigenin	5	356.6	352.4	346.2
	7	340.6	336.9	330.3
	4′	336.7	333.6	327.0
3,7-dihydroxyflavone	3	353.0	349.9	340.1
	7	336.4	333.4	327.7
daidzein	7	339.9	336.5	329.7
	4′	355.2	351.0	342.0
1,2-rhamnose-glucose	O7	355.8	353.0	339.7
	O8	351.2	349.5	335.6
	O9	354.7	352.8	338.7
	O29	359.8	356.3	343.7
	O30	352.5	350.8	336.9
	O31[b]	355.2	352.6	337.9
	O33	374.9	368.8	357.2
1,6-rhamnose-glucose	O7	368.9	364.6	352.5
	O8	364.0	360.7	348.1
	O9	367.6	364.1	351.1
	O29	361.5	357.3	345.3
	O30	358.9	355.5	341.6
	O31	369.2	365.1	350.5
	O32[b]	358.9	355.5	339.8
glucose	O7	365.5	360.8	349.1
	O8	363.8	359.7	347.2
	O9	387.2	380.6	369.2
	O10[b]	359.2	356.5	341.6
	O12	381.9	375.1	363.8

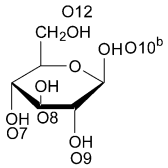
[a] The charge sites of the saccharides are the following:



1,2-rhamnose-glucose



1,6-rhamnose-glucose

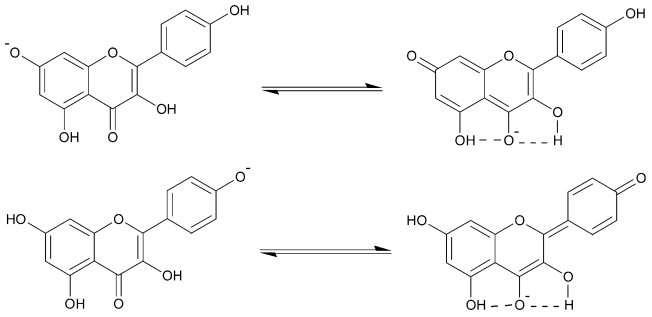


glucose

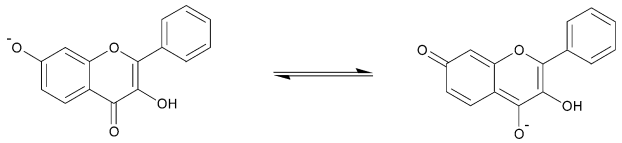
[b] Linked to aglycons in flavonoid glycosides. [c] Value calculated at the B3LYP/6-311+G(d,p) density functional theory level.

have different calculated acidities, with the hydroxyl groups of 1,2-rhamnose-glucose more acidic on average than those of 1,6-rhamnose-glucose (Table 1). The acidity difference is related to the differences in the intersaccharide linkage (1−6 vs 1−2), the bond(s) linking the two saccharides ($-CH_2-O-$ vs $-O-$), and rotational flexibility (1,6-rhamnose-glucose is more flexible because of the $-CH_2-O-$ linkage). These three factors cause differences in both the spatial distance between the two saccharide moieties and the extent of the intramolecular hydrogen bonding network. These factors ultimately make 1,2-

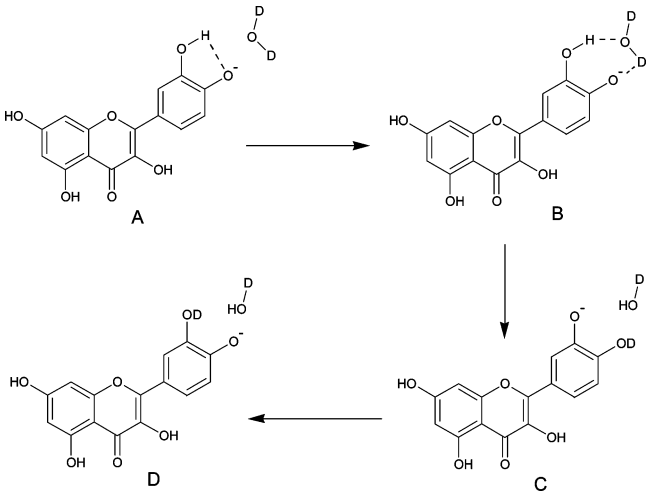
Scheme 1. Charge Migration of Deprotonated Kaempferol from an Oxygen at the 7- or 4′-Position to the 4-Position through Resonance



Scheme 2. Charge Mobility of Deprotonated 3,7-Dihydroxyflavone through Resonance



Scheme 3. Proposed Mechanism of H/D Exchange of Deprotonated Flavonoids with Adjacent Hydroxyl Groups on the B Ring as Illustrated for Quercetin



rhamnose-glucose substantially more acidic, presumably via better solvation of the incipient negative charge via hydrogen bonding from proximate hydroxyl groups. Note that the flavonoid glycosides having the 1−6 intersaccharide linkage are generally referred to as rutinosides, while those with the 1−2 linkage are neohesperidosides. In general, for the rutinose-type flavonoids, the most acidic site of an aglycon (unless the aglycon has only one hydroxyl group and the sole hydroxyl group is located at the 5- position) is thus predicted to be more acidic than the most acidic site of the disaccharide. Thus, it is expected that for a flavonoid rutinoside, deprotonation will occur on the aglycon. For neohesperidose-type flavonoids, the acidities of a few of the hydroxyl groups on the saccharide portions are predicted to be similar to the acidities of the most acidic hydroxyl groups of the aglycon portions. Therefore, deprotonation of a flavonoid neohesperidoside may occur competitively on either the disaccharide or the aglycon portion and is especially dependent on whether the aglycon possesses a highly acidic 3′-OH, 4′-OH, or 7-OH. This predicted difference in the favored sites of deprotonation for the rutinose versus neohesperidose-type flavonoids is anticipated to have an impact on the H/D exchange behavior. It should be noted that Fernandez and co-workers calculated gas-phase acidity values for a few flavonoid aglycons at the B3LYP/6-31G(d,p) level.[43] It would be interesting to compare their acidity results with our values, but it is not possible because their data is not listed in the only available abstract.[43]

3.2. H/D Exchange. Our experimental strategy involved monitoring the kinetics and extent of H/D exchange reactions for a variety of isomeric flavonoids whose structures varied based on the type of intersaccharide linkage, the type of saccharide (disaccharide: neohesperidose or rutinose, or monosaccharide: glucose or rhamnose), the presence of a single or double bond between C2 and C3, and the positions of the hydroxyl groups (Figure 2). The rate constants and extent of the H/D exchange reactions obtained by KinFit modeling[36] and exchange factor calculations[28] are interpreted by the favored sites of deprotonation, the mobility of the charge sites, and the conformations of the flavonoids and their ability to adopt resonance structures. This represents a different and complementary structural perspective than that obtained by CAD. Six pairs or series of isomeric flavonoid glycosides and the corresponding aglycons, listed in Figure 2, were used in this study.

First, the influence of the type of exchange reagent (ND_3, CD_3OD, D_2O) on the rates of H/D exchange of deprotonated flavonoids was investigated using deprotonated neohesperidin and rutin as model flavonoids to select the most effective exchange reagent for the remainder of the study. While lower rates and lower numbers of exchange were observed when ND_3 was used as the exchange reagent (e.g., one partial exchange for neohesperidin with $K1 = 0.01$), very similar rates and numbers of exchanges were observed for both D_2O and CD_3OD (e.g., up to four exchanges for neohesperidin with K values larger than 0.17). Since ND_3 is the least acidic among the three exchange reagents (gas-phase acidity values: NH_3 404, H_2O 391, and CH_3OH 382 kcal/mol[42]), the much lower exchange reactivity of ND_3 is expected. Though differing by 9 kcal/mol in their acidity values (based on the nondeuterated species), CD_3OD and D_2O have similar H/D exchange reactivities with deprotonated neohesperidin and rutin. This similarity may be related to the possibility of multiple concerted exchanges for D_2O compared to the sequential exchange of CD_3OD[23] and also to the similar differences in acidities of these two reagents relative to the acidities of the flavonoids. D_2O was used exclusively for the rest of the experiments because of its high reactivity with the deprotonated flavonoids.

An example of the H/D exchange kinetic data is shown in Figure 3 for deprotonated neohesperidin acquired at a nominal D_2O pressure of 5×10^{-4} Torr. As the reaction time is extended, the relative abundance of the initial nondeuterated precursor ion, D(0), decreases while those of deuterated ions, D(1), D(2), D(3), and D(4), increase as expected. Kinetic plots similar to the one shown in Figure 3 were used to determine the relative rate constants that are reported in Table 2 for the deprotonated flavonoid glycosides and in Table 3 for the deprotonated flavonoid aglycons. Exchange factors are also shown in Tables

(43) Martins, H.; Leal, J. P.; Lopes, V. H. C.; Cordeiro, N. D. S.; Fernandez, M. T. *IMSC*, Edinburgh, 2003.

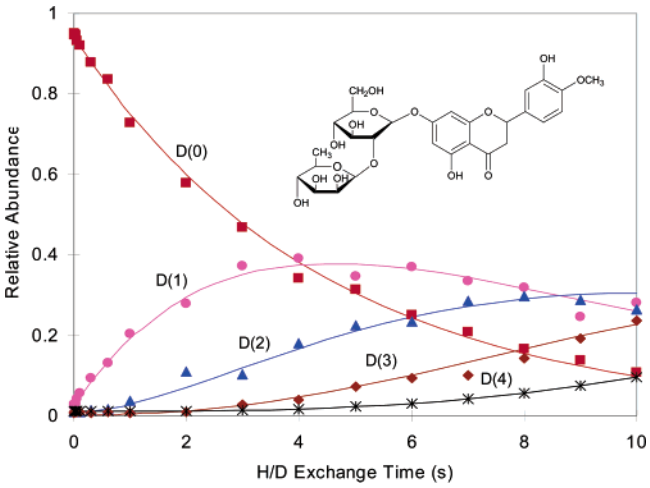


Figure 3. Kinetic plot of the H/D exchange reactions of deprotonated neohesperidin fitted with KinFit.[36] D(0) represents the initial parent ion, and D(1), D(2), D(3), and D(4) represent ions incorporating from one to four deuteriums.

2 and 3 to facilitate the comparison of the relative efficiencies of exchange for different flavonoid isomers.

3.2.1. H/D Exchange of Deprotonated Flavonoid Glycosides.

MW 610 Flavonoids. The extent and rates of exchange vary among the flavonoids in each isomeric series (Table 2). For example, hesperidin and neohesperidin contain the same aglycon (hesperetin, a flavanone) and the same disaccharide rhamnose-glucose at the 7-position. The only difference is the intersaccharide linkage: 1−6 for hesperidin (a rutinose) and 1−2 for neohesperidin (a neohesperidose). However, this small structural difference in the intersaccharide linkage causes significant differences in the observed kinetics and extent of H/D exchange (Table 2). Deprotonated hesperidin exchanges five of its seven labile hydrogens while deprotonated neohesperidin exchanges only four. The rate constants obtained for deprotonated hesperidin are also larger than those of deprotonated neohesperidin. The change in the rates and extent of H/D exchange for the rutinose versus neohesperidose-type flavanones is explained by their difference in conformations (see section 3.3.3).

Rutin is also a rutinose-type glycoside like hesperidin but it has a different aglycon (quercetin, a flavonol), and its glycosylation site is at the 3-position instead of the 7-position. On the basis of the acidity calculations presented earlier, it is expected that rutin deprotonates at the 4′-OH position. Deprotonated rutin exchanges five of its nine labile hydrogens with much higher rates than observed for either deprotonated hesperidin or neohesperidin, and this striking difference in the H/D exchange behavior may be attributed to both the presence of adjacent hydroxyl groups on the B ring and the glycosylation position at the 3-position (instead of the 7-position). Rutin is the only flavonoid disaccharide studied that has adjacent hydroxyl groups on the B ring. The presence of adjacent hydroxyl groups facilitates fast exchange at the hydroxyl group next to the deprotonated 4′-hydroxyl group (see section 3.2.2). The importance of adjacent hydroxyl groups on fast H/D exchange is more apparent when the exchange behaviors of the aglycons are discussed in detail (see section 3.2.2). The large number of exchanges of deprotonated rutin (five out of nine labile hydrogens) indicates that some exchanges must happen on the disaccharide, which may be a result of one or both of the following possibilities: the disaccharide is spatially close to the deprotonation site (4′-position) and/or the negative charge migrates from the 4′-position to the 4-position (through resonance), thus the charge becomes close to the disaccharide (3-position).

Luteolin-7,3′-*O*-diglucoside is a flavone isobaric to the above three flavonoids in which its two glucoses are attached to two different rings (A and B). It undergoes very extensive and rapid H/D exchange with rate constants that are higher than observed for the two flavanone glycosides described above. The fact that the hydroxyl groups on both glucoses undergo exchange (eight out of a total of nine labile hydrogens) suggests that the two glucoses may be spatially close to each other, and at least one of them is initially close to the deprotonation site (deprotonation is likely to occur at the 4′-position; see section 3.1.2). The conformation of deprotonated luteolin-7,3′-*O*-diglucoside is discussed later (see section 3.3.2).

MW 592 Isomers. Linarin and fortunellin are two isomers that share the same aglycon (acacetin, a flavone) and the same glycosylation site (7-position). They differ only in their disaccharide linkage (rutinose vs neohesperidose), just like hesperidin and neohesperidin, respectively. Surprisingly, deprotonated linarin only undergoes one slow exchange while deprotonated fortunellin exchanges all of its six labile hydrogens at much higher rates (Table 2). Since these two isomers do not have any hydroxyl groups on the B or C rings, the only deprotonation site on the aglycon is the 5-OH on the A ring. All the other possible deprotonation sites are solely located on the disaccharide moieties. The very extensive and rapid exchange of deprotonated fortunellin points out the possibility of deprotonation on the disaccharide (as also suggested by our ab initio calculations; see section 3.1.2), where the resulting charge could be stabilized by an extended hydrogen-bonding network and the charge is close to the labile hydrogens. The deprotonation site of linarin is less clear. Though most of the hydroxyl groups of its disaccharide (a rutinose) are less acidic than the 5-OH, one such hydroxyl group is more acidic than the 5-OH based on our acidity calculations (Table 1). As discussed later (see section 3.3.3), deprotonation of linarin is likely to occur on the disaccharide as well. The dramatic variation in the H/D exchange reactivity of these isomers indicates that the conformations of the rutinoses and neohesperidoses might be quite different, which is also suggested by the CAD results (see section 3.4 for the correlation between H/D exchange and CAD). MD simulations were performed on these two isomers to rationalize this hypothesis (see section 3.3.3).

MW 580 Isomers. Narirutin and naringin are the rutinose and neohesperidose forms of naringenin (a flavanone), again similar to hesperidin and neohesperidin, respectively, with the exception that each possesses a highly acidic 4′-OH group. Both deprotonated narirutin and naringin undergo extensive exchange (seven or eight exchanges) (Table 2), but deprotonated naringin exhibits faster exchange than deprotonated narirutin. The first deuterium incorporation is not necessarily the fastest reaction (e.g., $K1 < K2 < K3$ for deprotonated naringin). This kind of unusual profile is observed for both the reactions of deprotonated narirutin and naringin as well as for deprotonated luteolin-7,3′-*O*-diglucoside and orientin (luteolin-8-*C*-glucoside) (Table 2), which may be an indication of multiple (instead of sequential)

Table 2. Rate Constants for H/D Exchange of Deprotonated Flavonoid Glycosides Reacting with D_2O

MW	flavonoid[a]	exchange factor at 1 s[b]	*K* values[c]							
			*K*1	*K*2	*K*3	*K*4	*K*5	*K*6	*K*7	*K*8
610	hesperidin (7)	0.847	0.303	0.308	0.290	0.219	0.133			
	neohesperidin (7)	0.527	0.266	0.220	0.195	0.171				
	rutin (9)	∞	5.67	4.411	3.039	1.657	0.173			
	luteolin-7,3′-*O*-diglucoside (9)	0.976	0.413	0.887	0.886	0.841	0.793	0.664	0.423	0.200
592	linarin (6)	0.111	0.0212							
	fortunellin (6)	17.5	large	large	large	0.476	1.208	0.799		
580	narirutin (7)	0.981	0.401	0.779	0.730	0.664	0.609	0.511	0.400	0.279
	naringin (7)	0.993	0.143	1.97	3.63	2.80	1.57	0.710	0.083	
578	isorhoifolin (7)	0.090	0.052							
	rhoifolin (7)	0.140	0.082							
448	luteolin-4′-*O*-glucoside (6)	<0.05	0.015							
	luteolin-7-*O*-glucoside (6)	∞	11.5							
	orientin (7)	2.55	0.033	1.50	1.48	0.280	0.060			
	quercitrin (6)	∞	large	4.21	0.140					
	astragalin (6)	<0.05	<0.001							
432	vitexin (6)	<0.05	0.010							
	apigenin-7-*O*-glucoside (5)	<0.05	<0.001							
	isovitexin (6)	<0.05	0.016							
416	daidzin (6)	<0.05	<0.001							

[a] The number of exchangeable hydrogens is shown in parentheses. Only those hydrogens attached to oxygens are counted. [b] Exchange factors (at 1 s) were calculated as defined in the Experimental Section as well as in the literature[28] after isotope correction. [c] Exchange constants were obtained from KinFit[36] fitting after isotope correction. Only deuterated species that have more than 5% relative abundance (after isotope correction) were considered for data fitting. When D(1) was less than 5%, *K*1 was still calculated. For very fast exchanges (fortunellin and quercitrin), the first few *K* values could not be obtained because of the very low abundances of the species involved.

Table 3. Rate Constants for H/D Exchange of Deprotonated Flavonoid Aglycons Reacting with D_2O

MW	flavonoid[a]	exchange factor at 1 s[b]	*K*1[c]
302	hesperetin (2)	1.84	0.742
	quercetin (4)	9.99	2.30
286	luteolin (3)	∞	10.8
	kaempferol (3)	<0.05	0.002
284	acacetin (1)	<0.05	0.002
272	naringenin (2)	0.05	0.016
270	apigenin (2)	<0.05	<0.001
254	daidzein (1)	<0.05	<0.001
	3,7-dihydroxyflavone (1)	0.27	0.172

[a] The number of exchangeable hydrogens is shown in parentheses. Only those hydrogens attached to oxygens are counted. [b] Exchange factors (at 1 s) were calculated as defined in the Experimental Section as well as in the literature[28] after isotope correction. [c] Exchange constants were obtained from KinFit[36] fitting after isotope correction.

exchanges.[23] Since D_2O is the exchange reagent used, multiple exchanges are possible, but the specific exchange sites are impossible to determine. The difference in H/D exchange rates of these two flavanone isomers likely reflect differences in their acidities and conformations, especially as revealed by the distance between the initial charge site (deprotonation occurs most likely at the very acidic 4′-OH position or at the disaccharide (naringin only) as supported by our ab initio results; section 3.1.2) and labile hydrogen(s) of the disaccharide.

MW 578 Isomers. Isorhoifolin and rhoifolin are the flavone analogues of the flavanones narirutin and naringin, respectively. These two isomers share the same aglycon (apigenin, a flavone), and they differ from narirutin and naringin only by a double bond instead of a single bond between C2 and C3. Both deprotonated isorhoifolin and rhoifolin undergo only one exchange, and both of these flavonoids, based on the *K* values, are much less reactive with D_2O than narirutin and naringin (Table 2). The bond character between C2 and C3 is found to significantly affect the relative acidities of the hydroxyl groups at the 4′-positions of the corresponding aglycons: 327 kcal/mol for apigenin (a flavone) versus 337 kcal/mol for naringenin (a flavanone) (see Table 1), a change resulting from the possibility of charge stabilization through resonance for apigenin but not for naringenin. The conformational flexibility of the flavonoid glycosides also seems to be an important factor. The single bond between C2 and C3 of narirutin and naringin allows free rotation of the B ring relative to the plane defined by the A ring (C ring is not exactly on the same plane). For flavones such as isorhoifolin and rhoifolin, the double bond between C2 and C3 allows extended conjugation among the three rings; thus, the B ring is likely to be coplanar or close to be coplanar with the A and C rings (the corresponding torsion angle is less than 20° after structural optimization at the HF/6-31G(d) level). The conformational difference (B ring relative to the plane defined by the A ring) results in a difference in the distance between the deprotonation site and labile hydrogen(s), which is a critical factor for H/D exchange reactivity. The conformations of deprotonated rhoifolin and naringin are shown in section 3.3.1, from which the conformational differences are apparent.

MW 448 Flavonoids. Modest structural differences also cause a large impact on the H/D exchange of five flavonoid monosaccharides, as shown in Figure 4 and Table 2. The monosaccharide isomers include luteolin-4′-*O*-glucoside and luteolin-7-*O*-glucoside, two O-bonded glucosides of luteolin (a flavone); orientin (luteolin-8-*C*-glucoside), a C-bonded glucoside; and quercitrin (quercetin-3-*O*-rhamnoside) and astragalin (kaempferol-3-*O*-glucoside), two 3-O-bonded glycosides. Each of the five monosaccharides is distinguished by the total number of exchanges and the rate constants for exchange: one slow exchange for deprotonated luteolin-4′-*O*-glucoside, one very fast exchange for deprotonated luteolin-7-*O*-glucoside, five moderately fast exchanges for deprotonated orientin, three very fast exchanges for deprotonated quercitrin, and almost no exchange for deprotonated astragalin (see typical H/D exchange spectra shown in Figure 4). The fact that deprotonated luteolin-7-*O*-glucoside, orientin, and quercitrin undergo H/D exchange much faster than deprotonated luteolin-4′-*O*-glucoside and astragalin clearly shows the importance of two adjacent hydroxyl groups

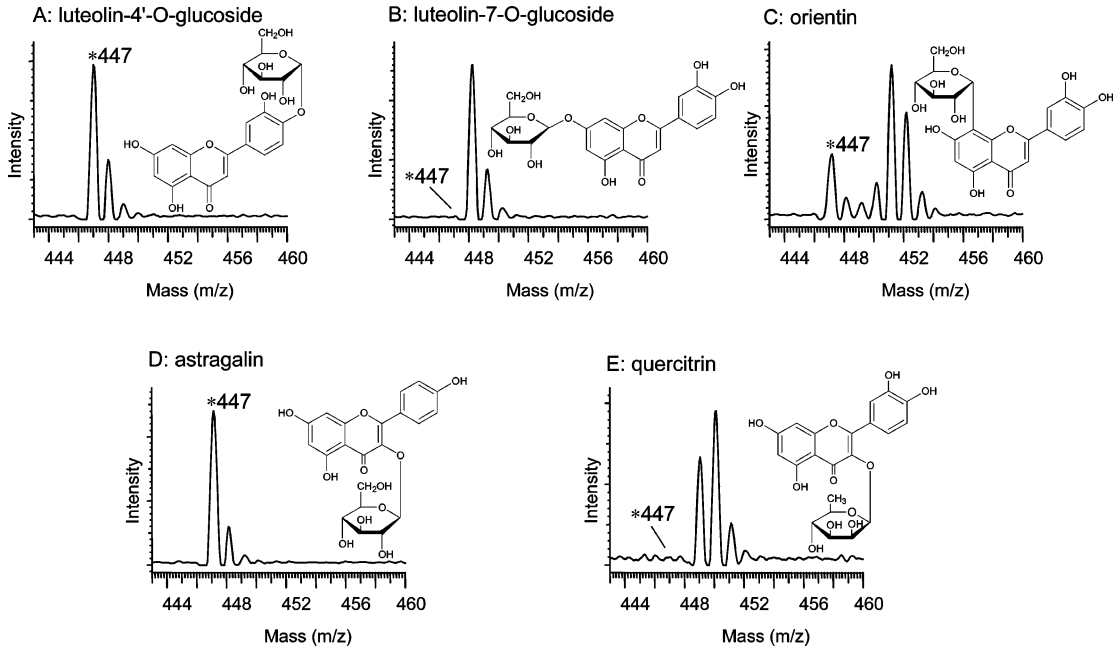


Figure 4. H/D exchange of five deprotonated flavonoid monosaccharides after 10 s.

on the B ring for fast H/D exchange (see section 3.2.2, Scheme 3). The extent of deuterium incorporation is attributed to the spatial arrangement of the saccharide relative to the deprotonation site as discussed earlier for deprotonated rutin. The attachment of the glucose residue at the 8-C or 3-O positions facilitates interaction of the hydroxyl groups of the sugar with the site of deprotonation, thus promoting H/D exchange. It is surprising that deprotonated astragalin does not undergo facile exchange despite its structural similarity to quercitrin. The rather unusual behavior of astragalin is believed to be a result of the saturation of the 4-keto oxygen with two hydrogen bonds (formed with the 5-OH and a hydroxyl group of the saccharide) after charge migration (Scheme 1). It is reasonable to assume that D_2O is less likely to form a stable ion−molecule complex with the charged 4-keto oxygen when the oxygen is already saturated with two hydrogen bonds.

MW 432 Isomers. Vitexin, isovitexin, and apigenin-7-*O*-glucoside are three monosaccharide derivatives of apigenin (flavone) that are glycosylated at the 8-C-, 6-C-, and 7-O-position, respectively. These three isomers undergo at most one exchange, and the exchange process is very slow. This H/D exchange behavior suggests that the deprotonation site is isolated from the other active hydrogens. The generally low extent of H/D exchange for these monosaccharide flavonoids (except the ones with adjacent hydroxyl groups), especially in comparison to the extensive H/D exchange noted earlier for many of the disaccharide flavonoids, is consistent with the rationalization that the longer, more flexible disaccharide moieties may interact with remote parts of the flavonoids, thus allowing migration of the charge site and consequently interaction and exchange with D_2O.

Daidzin. Daidzin is another monosaccharide with a great distance between its sole hydroxyl group on the B ring (the predicted deprotonation site based on the ab initio calculations discussed in section 3.1.2) and its hydroxyl groups on the glucose (the only other labile hydrogens) (Figure 2). It was chosen as a model compound to confirm the impact of the distance between the labile hydrogens and the charge site on H/D exchange of deprotonated ions. The fact that deprotonated daidzin does not exchange to any observable extent suggests that proximity of a labile hydrogen to a charge site is required for H/D exchange of the deprotonated flavonoids, as expected based on the collection of results thus far. Similarly, it has been demonstrated by the Beauchamp group[23] that the site of protonation should be close to the labile hydrogen(s) to allow H/D exchange to occur for protonated peptides when D_2O is the exchange reagent.

Comparison of Structurally Similar but Nonisomeric Flavonoids. The pattern of H/D exchange reactivity that emerges from the examination of the isomeric series discussed above clearly reflects the impact of the site of deprotonation and the possibility of charge migration, two factors that are directly related to the specific structures of each flavonoid. Flavonoids that are not isomeric but differ by one structural feature give further insight into the factors that mediate H/D exchange. For example, the only difference between naringin and rhoifolin (or narirutin and isorhoifolin) is a single versus double bond between C2 and C3 (Figure 2). This seemingly modest structural difference leads to dramatic differences in their H/D exchange behavior as shown in Table 2, with deprotonated naringin undergoing fast, extensive exchange whereas deprotonated rhoifolin undergoes only one, rather slow exchange. The 4′-OH of rhoifolin is estimated to be about 10 kcal/mol more acidic than the 4′-OH of naringin calculated at the MP2/6-311+G(d,p) level (Table 1). The result is that the deprotonation site of rhoifolin is strongly favored at the 4′-OH position, while that of naringin may be either at the 4′-OH position or on the disaccharide or a distribution of both. Deprotonation of the 4′-OH position of rhoifolin leaves the charge site far from the active hydrogens of the disaccharide moiety. If deprotonation of naringin occurs on the disaccharide, extensive H/D exchange along the disaccharide moiety is expected. If deprotonation of naringin occurs at the 4′-OH, then the flexibility of the B ring still allows some interaction of the disaccharide with the charge

site, a feature that is not possible for the more rigid rhoifolin molecule (see section 3.3.1).

Fortunellin differs from rhoifolin only by the functional group at the 4′-position: methoxy vs hydroxyl. The fact that deprotonated fortunellin is much more reactive with D_2O than deprotonated rhoifolin indicates that the nature of the 4′-functional group is important for the H/D exchange reactivity. As discussed above, the most likely deprotonation site of rhoifolin (or similar flavonoid disaccharides with a free hydroxyl group at the 4′-position) is at the 4′-position, which is far away from the disaccharide. The result is slow and limited exchange of deprotonated rhoifolin. On the other hand, the methoxy group at the 4′-position of fortunellin ensures that it must deprotonate at a site other than the 4′-position (i.e., the 5-OH position or the disaccharide), which results in fast, extensive exchange because the charge site is close to the disaccharide to facilitate exchange with numerous labile hydrogens.

It can also be seen in Table 2 that the deprotonated neohesperidoses (neohesperidin, fortunellin, naringin, and rhoifolin) in general exchange faster than their rutinose isomers (hesperidin, linarin, narirutin, isorhoifolin). This contrasting behavior suggests that the conformations of the disaccharides and/or the acidities of the hydroxyl groups of the disaccharides may differ in a significant way for the rutinose versus neohesperidose-type flavonoids. As shown in the sections 3.1.2 and 3.3.3, both the acidities and conformational flexibilities differ between the neohesperidose and rutinose isomeric pairs, thus explaining many of the notable differences in the H/D exchange behavior of the flavonoid isomers in the gas phase.

3.2.2. H/D Exchange of Deprotonated Flavonoid Aglycons. The H/D exchange reactions of all the aglycons were also studied (Figure 2 and Table 3). In direct comparison to their glycosides, the deprotonated aglycons are less reactive and only undergo up to one exchange, usually with a low rate constant ($K1 < 1.0$). The results are reasonable since, unlike the glycosides, the aglycons do not generally have multiple hydroxyl groups that provide labile hydrogens and alternative charge sites that are close to each other, a feature which is required by the relay mechanism of H/D exchange.[23] The exceptions are quercetin and luteolin, which have adjacent 3′,4′-dihydroxyl groups that ensure fast exchange of one labile hydrogen ($K1 > 1.0$).

When two adjacent hydroxyl groups exist on the B ring, one is easily deprotonated (i.e., the 4′-OH for quercetin as suggested by our ab initio calculations described in section 3.1.2) since the resulting negative charge may be stabilized through delocalization by resonance and through formation of an intramolecular hydrogen bond with the hydrogen of the second hydroxyl group. When D_2O is introduced to the ion trap, the deprotonated flavonoid may then form a relatively stable complex with D_2O through formation of two hydrogen bonds (instead of one for the deprotonated flavonoid itself) and a seven-membered ring system. The resulting complex is likely to be an intermediate that facilitates H/D exchange. The incorporated deuterium could then migrate to the neighboring oxygen to release the original deprotonation site (Scheme 3), which completes the reaction process.

3.2.3. H/D Exchange of Deprotonated Sucrose. Sucrose is a simple 1,1-glucose-fructose disaccharide that is similar to the rhamnose-glucose disaccharides in the flavonoids. The 1−1 intersaccharide linkage (−O−) in sucrose is more similar to the 1−2 linkage (−O−) than to the 1−6 linkage ($-CH_2-O-$) in the rhamnose-glucose disaccharides. Thus, it is reasonable to assume that the H/D exchange behaviors of deprotonated sucrose and flavonoid neohesperidoses (with a 1−2 linkage) are similar when deprotonation occurs on the disaccharides. Deprotonated sucrose exchanges all of its seven labile hydrogens within 1 s (data not shown), which is faster than any of the deprotonated flavonoid disaccharides. The flavonoids are in general more acidic than simple sugars. As a result, the acidity difference between a simple sugar and D_2O is smaller than that of a flavonoid and D_2O, and thus H/D exchange is faster for a disaccharide when not coupled to the aglycon. It is thus clear that the relative acidities of the exchange reagent and analyte play a critical role on the kinetics of H/D exchange. Simple model compounds provide only a limited and often misguided perspective for predicting the overall H/D exchange of complex, multifunctional molecules.

3.2.4. H/D Exchange of Metal Complexes of Rutin and Sucrose. In the positive mode, most flavonoids do not always protonate efficiently but instead frequently form sodium complexes. Though protonated or sodiated flavonoids have some merit for analytical characterization, the signal may be low compared to deprotonation unless the sample is desalted or analyzed with LC/MS. For detection of flavonoids in the positive ESI mode, complexation of the flavonoids with transition metals and auxiliary ligands is an alternative to protonation that provides excellent detection limits and highly diagnostic CAD patterns.[18−20] H/D exchange reactions were examined for these two types of metal complexes of rutin: $(\text{rutin} + \text{Na})^+$ and $[\text{Co}^{\text{II}}(\text{rutin} - \text{H})\ \text{bipy}]^+$, as well as the sodium complex of sucrose: $(\text{sucrose} + \text{Na})^+$. No exchange was observed for any of the above metal complexes (data not shown), which is not unexpected since the charge site of such a complex is located on the metal and is immobilized compared to the deprotonated flavonoids, whose charge may migrate through resonance to a position close to labile hydrogens. The crown ether complexes of diamines and glycine oligomers,[26] the platinum complexes of diethylenetriamine,[27] as well as the alkali metal complexes of polyamines[28] have also been shown to undergo very slow (if any) H/D exchange, while their protonated counterparts undergo fast H/D exchange. Our studies of the metal/flavonoid complexes underscore that immobilization of the charge site greatly reduces the H/D exchange reactivity of gas-phase ions.

3.3. Gas-Phase Conformations of Deprotonated Flavonoids. As suggested by the H/D exchange results, the conformations of neohesperidose/rutinose isomeric pairs or structurally similar flavonoids may be different, and this factor could contribute significantly to the different H/D exchange rates and CAD patterns (see section 3.4) observed for some of the similar flavonoids. To gain insight into the conformations in the gas phase, MD simulations were performed on selected flavonoid glycosides including rhoifolin, naringin, luteolin-7,3′-*O*-diglucoside, fortunellin, and linarin.

3.3.1. Conformations of Deprotonated Rhoifolin and Naringin. MD simulations were undertaken for deprotonated rhoifolin and naringin because these similar flavonoids (one a flavanone, the other a flavone) have dramatically different H/D exchange behaviors. The conformations of deprotonated rhoifolin (deprotonated at the 4′-position) do not change very much

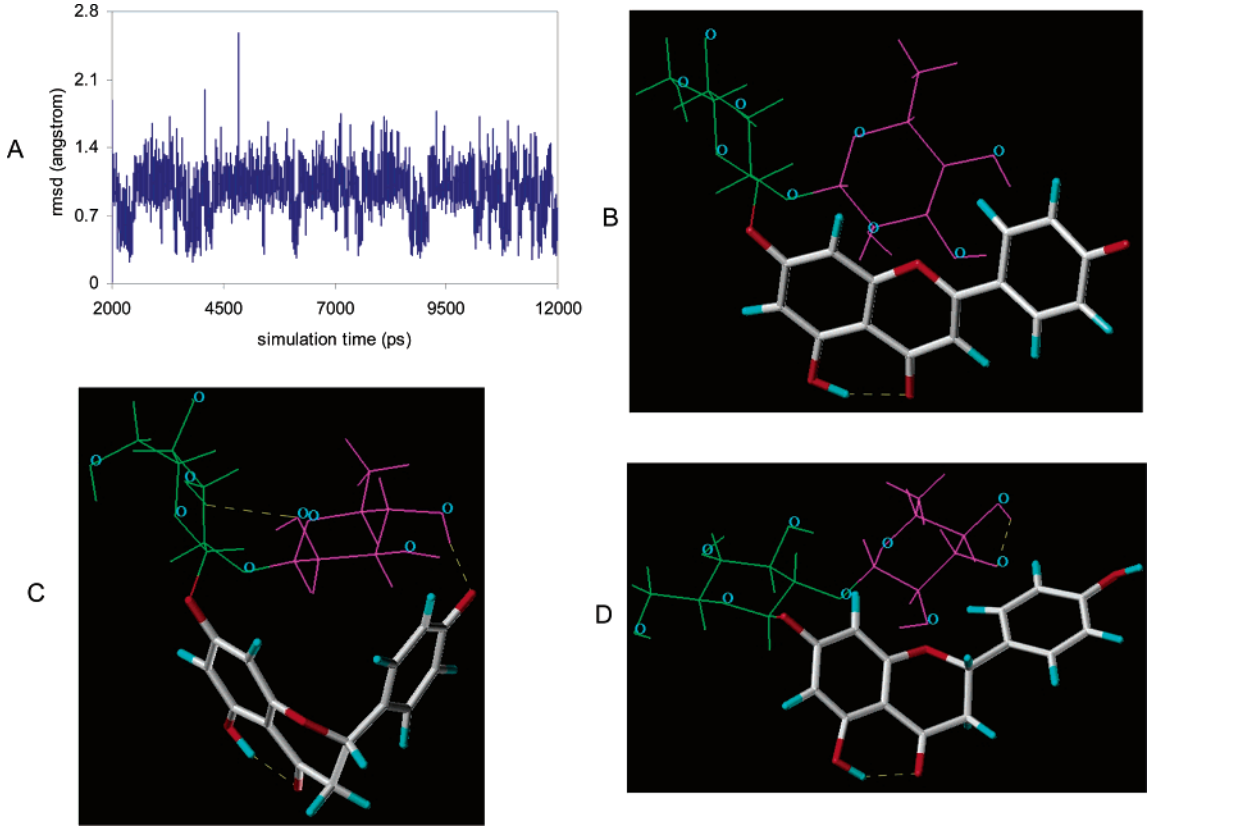


Figure 5. Root-mean-square deviations (Å) and representative snapshots of deprotonated rhoifolin and naringin. A and B: rmsd (A) and a conformation of rhoifolin deprotonated on the aglycon (4′-position) (B). C and D: conformations of naringin deprotonated on the aglycon (4′- position) (C) and disaccharide (O8) (D).

over time as indicated by its small root-mean-square deviation (rmsd) (Figure 5A). One of the conformational snapshots is shown in Figure 5B as an example. The disaccharide is quite far away from the deprotonation site, a situation which does not facilitate deuterium incorporation into the disaccharide. The single slow exchange observed should occur at the 5-position after the negative charge migrates to the 4-keto oxygen through resonance. Though only differing from rhoifolin by the bond characteristics between C2 and C3 (single instead of double), deprotonated naringin has very different conformations (Figure 5C,D) and undergoes very fast and extensive H/D exchange. Naringin could deprotonate on its aglycon (at the 4′-position just like rhoifolin) or on its disaccharide because of the similar calculated acidities of the hydroxyl groups (Table 1). There should be no question that naringin will undergo extensive and fast deuterium incorporation when deprotonation occurs on the disaccharide (Figure 5D) based on the H/D exchange behaviors of other deprotonated flavonoid neohesperidoses and deprotonated sucrose, a neohesperidose-like disaccharide, as discussed in sections 3.2.1 and 3.2.3. When deprotonation occurs at the 4′-position of the aglycon, the disaccharide and the B ring of naringin can orient themselves close to each other such that a natural hydrogen bond forms between one hydroxyl group of the disaccharide and the 4′-O carrying the negative charge (Figure 5C), thus allowing D_2O to interact with the initial charge site and to allow migration of the charge site to the disaccharide, which ensures fast exchange on the disaccharide.

3.3.2. Conformations of Deprotonated Luteolin-7,3′-*O*-diglucoside. Deprotonated luteolin-7,3′-*O*-diglucoside was modeled because it is the only flavonoid with two monosaccharides at separate positions. Conformation also plays an important role in the H/D exchange reactivity of deprotonated luteolin-7,3′-*O*-diglucoside. The favored deprotonation site of this flavone is the 4′-position, as shown in section 3.1.2. Likely due to the large flexibility of the two separate glucose residues, two distinct conformation regions are identified from the MD simulations (Figure 6A). Two representative snapshots are shown in Figure 6B,C. For both cases, natural hydrogen bonds are formed both between the two glucose residues and between the glucose linked to the aglycon at the 3′-position and the 4′-oxygen carrying the negative charge. Apparently it is this network of hydrogen bonds that allows shuttling of the charge site from the 4′-oxygen to the glucose residues during the reaction with D_2O, resulting in efficient H/D exchange. The surprising hydrogen bond(s) formed between the two glucose residues of deprotonated luteolin-7,3′-*O*-glucoside indicates that the two glucoses are spatially very close and demonstrates that the structure shown in Figure 2 gives a poor depiction of the conformation and spatial arrangement of the sugars relative to the aglycon.

3.3.3. Conformations of Deprotonated Linarin and Fortunellin. To confirm our hypothesis about the deprotonation sites and to probe the conformational differences of the neohesperidose/rutinose isomeric pairs, conformations of both acacetin glycosides (linarin and fortunellin) with two different deprotonation sites each were studied by MD simulations. Deprotonated linarin, a rutinose-type flavone, undergoes only one slow exchange. Deprotonated fortunellin, a neohesperidose-type flavone, undergoes very fast, multiple exchanges. Because of the low acidity of the 5-OH of acacetin, the two acacetin

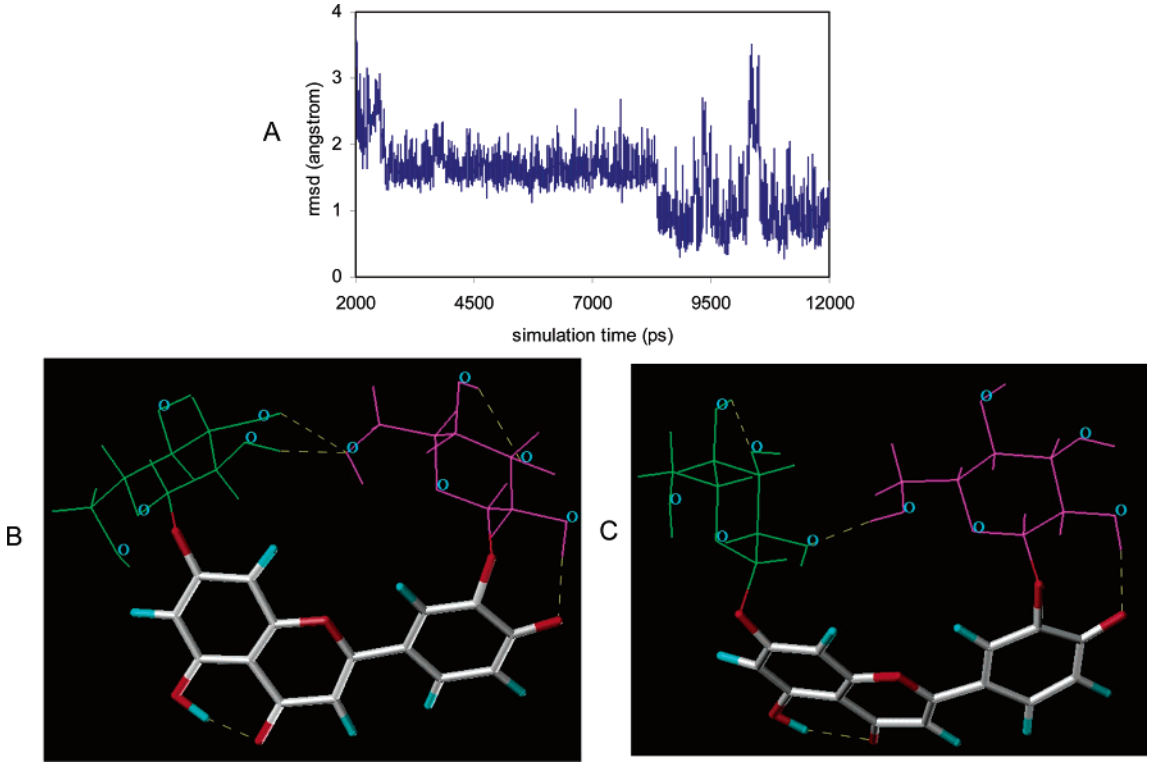


Figure 6. Root-mean-square deviations (Å) and representative snapshots of deprotonated luteolin-7,3′-*O*-diglucoside. (A) rmsd. (B and C) Snapshots that represent two conformation regions.

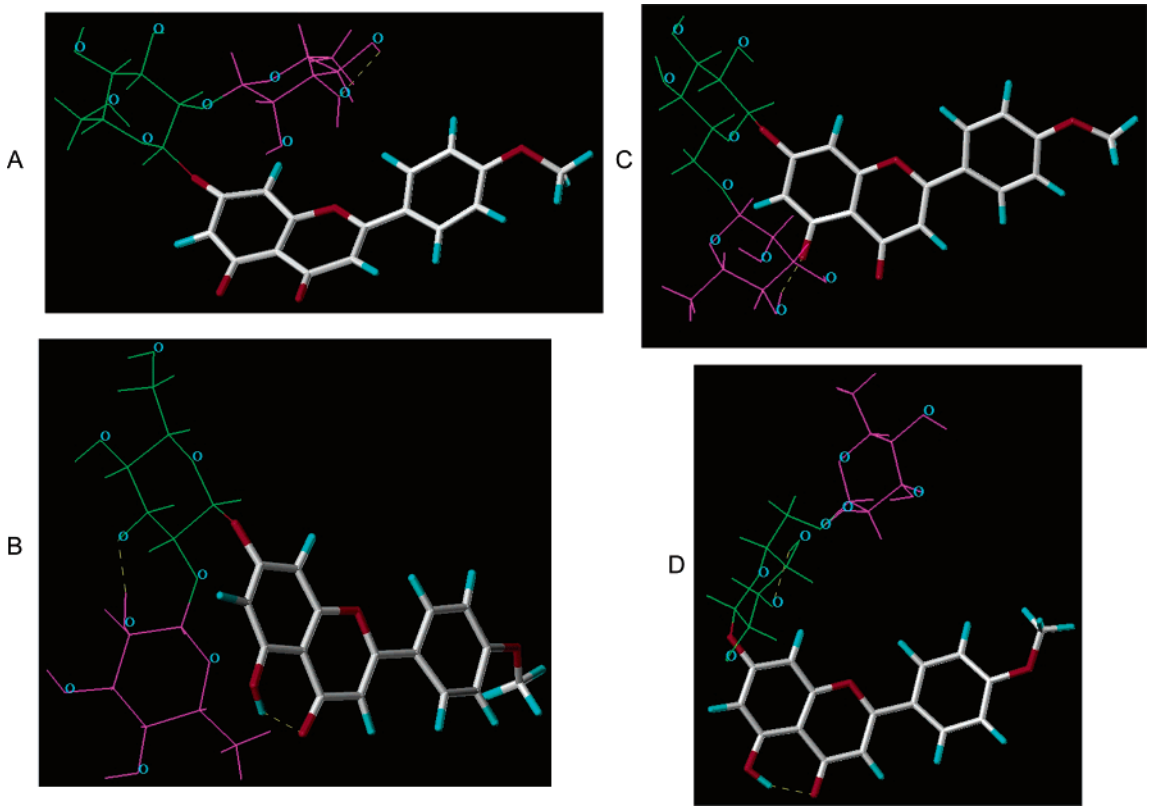


Figure 7. Representative snapshots of deprotonated fortunellin and linarin. A and B: fortunellin deprotonated on the aglycon (5-position) (A) and disaccharide (O30) (B). C and D: linarin deprotonated on the aglycon (5-position) (C) and disaccharide (O30) (D).

glycoside isomers are likely to deprotonate on the disaccharide moieties. The representative conformation snapshots are shown in Figure 7A,B for deprotonated fortunellin and in Figure 7C,D for deprotonated linarin. It is interesting that it is the disaccharide of fortunellin that is closer to the aglycon if both flavone glycosides deprotonate on the disaccharide portions (Figure

Table 4. CAD of Deprotonated Flavonoid Rutinoses and Neohesperidoses

				CAD product ions (%)[a]								
				P-disaccharide	others							
MW	flavonoid	CAD energy (%)	parent P	P-308 Y_0^-	P-120 $^{0,2}X_0^-$	P-146 Y_1^-	P-162 Y*	P-164 Z_1^-	P-206 $^{1,3}X_0^-$	P-266 $^{0,2}X_0Y_1^-$	P-266-18 $^{0,2}X_0Y_1^-$–W	others
610	hesperidin	23.0	609	301 (100)								
	neohesperidin	29.0	609	301 (64)	489 (11)		447 (4)		403 (5)	343 (12)	325 (4)	
592	linarin	17.5	591	283 (100)								
	fortunellin	22.0	591	283 (85)	471 (11)					325 (4)		
580	narirutin	22.5	579	271 (100)								
	naringin	26.0	579	271 (17)	459 (61)		417 (4)		373 (4)	313 (9)		235 (5)
578	isorhoifolin	24.0	577	269 (100)								
	rhoifolin	30.5	577	269 (81)	457 (4)	431 (4)		413 (5)	371 (2)	311 (4)		

[a] The nomenclatures proposed in the literature[8,44] are adopted to denote the CAD product ions. X ions stem from cross-ring cleavages, and Y ions indicate loss of sugars.

7B,D), but it is linarin that has more interactions between its disaccharide and aglycon if deprotonation occurs on the aglycon (Figure 7A,C).

The dramatic difference in the H/D exchange behavior of deprotonated fortunellin and linarin is rationalized on the basis of the difference in the proximity of the charge site and the labile hydrogens. Deprotonated linarin (charge on the disaccharide) is only able to form intraglucose hydrogen bond(s), thus restricting its deuterium incorporation to one of the disaccharide moieties (glucose). In addition, the acidity difference between the charge site and other hydroxyl groups of the disaccharide is very large (compared to that of deprotonated fortunellin), which causes difficulty in charge transfer during its H/D exchange. On the other hand, deprotonated fortunellin (charge on the disaccharide) forms an intersaccharide hydrogen bond that makes exchange on both glucose and rhamnose moieties possible. Because of the similar acidities of several hydroxyl groups, deprotonation at other sites of the disaccharide may also be possible for fortunellin, and the corresponding conformations may be even more favorable for deuterium incorporation than the one shown in Figure 7B.

3.4. CAD of Deprotonated Flavonoid Disaccharides. The standard technique for evaluating structural differences of gas-phase ions is CAD. CAD was thus used to characterize the deprotonated flavonoid disaccharides. The CAD product ions were assigned in accordance with previously proposed nomenclatures.[8,44] The types of fragmentation pathways, their relative abundances, and the level of CAD energy needed to convert ~90% of the parent ions to fragment ions are shown in Table 4.

It can be seen in Table 4 that the only significant fragmentation route for all of the deprotonated rutinoses (hesperidin, linarin, narirutin, and isorhoifolin) is the loss of the disaccharide residue, while all the deprotonated neohesperidoses (neohesperidin, fortunellin, naringin, and rhoifolin) have at least one cross-ring cleavage product ($^{0,2}X_0^-$, $^{1,3}X_0^-$, and $^{0,2}X_0Y_1^-$, etc.), including the loss of the rhamnose residue, besides the loss of the disaccharide. The CAD energies required for fragmentation are significantly different for the isomeric pairs. Deprotonated neohesperidose-type flavonoids require more CAD energy than their rutinose isomers for the same extent of dissociation. Correspondingly, deprotonated neohesperidoses generally (neohesperidin and hesperidin are the only exceptions) undergo H/D exchange much faster and to a greater extent than their rutinose isomers, which is a result of closer distance between the labile hydrogens and the charge site. It is clear that there are some correlations between the CAD and H/D exchange results that suggest that some of the same features (charge site, conformation) govern the two types of behaviors in the gas phase.

4. Conclusions

H/D exchange is sensitive to subtle structural differences within the flavonoid isomers. Structural characteristics that promote exchange include the presence of 3′,4′-dihydroxyl groups, the proximity between the charge site and labile hydrogens, the existence of a single instead of a double bond between C2 and C3, and a 1−2 intersaccharide linkage (neohesperidose) for the disaccharides. The factors that inhibit exchange are an overly large difference in acidity between the exchangeable sites and the reagent, the presence of a 3-hydroxyl group, as well as a 1−6 intersaccharide linkage for the disaccharides. Monosaccharide and aglycon flavonoids are much less reactive toward H/D exchange than the disaccharide flavonoids.

The high level ab initio calculations convey the relative acidities of the different deprotonation sites of selected flavonoids and confirm our hypothesis about the two major determining factors for acidities: charge delocalization through resonance and stabilization through hydrogen bonding. A disaccharide with a 1−2 intersaccharide linkage is generally more acidic than that with a 1,6-disaccharide linkage. Thus, neohesperidoses are more acidic than their rutinose isomers if deprotonation occurs on the disaccharides (instead of the aglycons). Different, dramatic in some cases, gas-phase conformations of similar flavonoids are identified using MD simulations, which can be used to explain the different H/D exchange behaviors observed. The gas-phase conformations are found to play an important role in the extent and rates of H/D exchange of flavonoid glycosides.

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