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Flavonoid Deactivation of Excited State Flavins: Reaction Monitoring by Mass Spectrometry

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Supporting Information

ABSTRACT: Flavin mononucleotide (FMN, as a B_2 vitamin model) was shown to induce dimerization of flavonoids (flavanone, apigenin, naringenin, eriodictyol, taxifolin, catechin, kaempferol, luteolin, quercetin, rutin, and seven smaller model phenols studied) as the major photoreaction, when aqueous solutions were exposed to visible light using a new, real-time electrospray ionization mass-spectrometric (ESI-MS) technique supported by LC−MS and MS^2 analysis. Electrophilic intermediates such as transient radical cations, *o*-quinones, and *p*-quinone methide were proposed to be involved in the coupling process. The C_3-OH in flavon-3-ols gave rise to atypical compounds such as a depside or a dioxane-linked dimer. Flavonoid dimers, formed in vegetal extracts added to food during storage in light and for which structures are proprosed based on MS and MS^2, may affect colloidal stability, color, astringency, and antioxidative capacity.

KEYWORDS: *antioxidants, flavonoids, flavins, mass spectrometry, photooxidation*

INTRODUCTION

Oxidative instability in foods leads to formation of oxidation products that change flavor perception and eventually affect product quality. Although exact mechanisms often remain elusive, onset of oxidation is assumed to depend on various factors, including enzyme activity, the presence of transition metals, partial oxygen pressure, and temperature.[1] Also light exposure triggers oxidation, a pathway that is particularly effective in the presence of strongly absorbing sensitizer molecules such as riboflavin, an essential nutrient (vitamin B_2) that prevails in beverages and dairy products.[2−4] Its light-harvesting potential is ascribed to the presence of an isoalloxazine moiety, which accounts for absorption of the blue part of the visible spectrum (bands at 375 and 445 nm).[5] The high molar absorptivity ($>10^4$ M^{-1} cm^{-1}) is typical of a $\pi-\pi^*$ transition, generating the singlet-excited state (^{1}RF*) upon excitation.[6] Subsequent intersystem crossing yields triplet-excited riboflavin (^{3}RF*), which efficiently transfers energy excess to molecular oxygen with production of singlet oxygen (type II mechanism). Alternatively, ^{3}RF* acts as powerful oxidant (E ∼ +1.7 V)[7] in a type I mechanism, degrading suitable substrates in a radical process.[6] Both flavin-mediated mechanisms (i.e., type I and type II) occur in foods,[8−16] the importance of which is levered by increasing use of transparent packaging materials. Beer, wine, and dairy products are particularly sensitive, as thresholds below ppm level have been reported for perception of their light-induced off-flavors.[17−20] Still, despite the risk of severe shelf life reduction and potential consumer rejection, foods and beverages are exposed to intense illumination during retail. In response to this, novel pathways are explored to mitigate photodegradation, one of which includes addition of sacrificial naturally occurring flavonoids to quench intermediate excited states. Fast electron or hydrogen transfer to the reactive flavin intermediates produces resonance-stabilized radicals which are expected to break the radical chain,[21,22] but the fate of these flavonoid species remains largely elusive.

To complete reaction mechanisms of flavin-mediated photooxidations, the screening of a series of flavonoid reactions with a fast and readily implementable methodology was aimed at. Spectrophotometry was considered as a swift and responsive tool for time-resolved evaluation of flavonoid oxidations, but application is hampered by the limited structural information provided. A superior tool for real-time analysis, involving irradiation of the reaction mixture with simultaneous infusion in the electrospray ionization source of a mass spectrometer (real-time ESI-MS), was therefore elaborated and implemented for studying photodegradation of flavonoids. In this new approach, primary photoproducts, which are essential for mechanistic interpretation, were instantly monitored and could thus be discriminated from interfering secondary products. Structures were tentatively proposed from the corresponding molecular ions and later confirmed by chromatographic separation combined with tandem mass spectrometry (MS^2) and by comparison with products from benchmark oxidation methods. The combination of (time-resolved) mass spectrometric data thus obtained with earlier insights in photooxidation kinetics and in radical intermediates[21] is a unique approach that undoubtedly contributes to sound understanding of the chemistry underlying the flavonoid deactivation of excited flavin states. In view of possible use of flavonoids in food, these insights are particularly valuable and possibly lead to the use of transparent packaging without compromising food quality.

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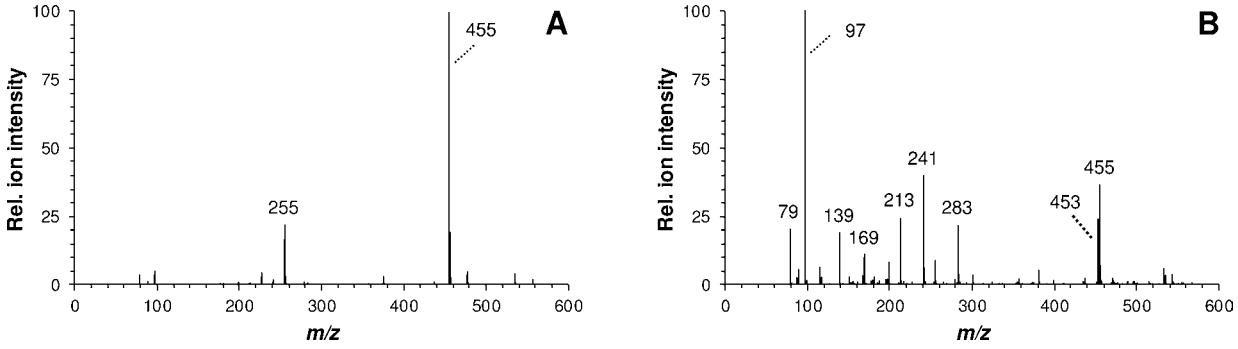


Figure 1. Real-time ESI-MS spectra of a FMN solution, 2 min (A) and 12 min (B) after exposure to visible light was initiated.

EXPERIMENTAL SECTION

Chemicals. Substrates (flavonoids and model compounds) were supplied by Acros (Beerse, Belgium) and by Aldrich (St.Louis, MO, USA), while the 2,2-diphenyl-1-picrylhydrazyl radical and the sodium salt of flavin mononucleotide were purchased from Aldrich. Acetonitrile (Lab-Scan, Dublin, Ireland) was of spectrophotometric grade. Aqueous solutions were prepared using a Milli-Q purification device (Millipore, Bedford, MA, USA) (R = 18 MΩ cm).

Photoreaction Screening by ESI-MS. In order to screen reactions for primary, nonvolatile photoreaction products, a method for real-time product analysis on exposure to visible light was used. To avoid precipitation of buffer molecules in the ionization source, reaction mixtures were prepared in a water−acetonitrile mixture (1:1, v/v). Besides enhancing solubility of flavonoid compounds, acetonitrile improved spray characteristics with increased instrument sensitivity as result. The sodium salt of flavin mononucleotide (FMN) was preferred (over riboflavin) as light-absorbing species due to its higher solubility in water. Final concentrations in the photoreactions were 0.75 mM flavonoid and 0.3 mM FMN. After purging with nitrogen, the mixture was loaded in a transparent syringe and exposed to visible light (commercial halogen lamp, 20 W) with concurrent continuous flow injection in the electrospray ionization source (Z-spray) of a quadrupole time-of-flight (Q-ToF) hybrid mass spectrometer (Waters, Manchester, U.K.) (see Supporting Information for details). The following settings were applied: source and desolvation temperatures were set to 80 and 120 °C, respectively; desolvation gas, 450 L h^{-1}; nebulizer gas, 20 L h^{-1}; capillary voltage, 2000 V; cone voltage, 33 V; extractor voltage, 1 V; ion energy, 1.8 V; collision energy, 4 V. Mass spectra were acquired over a mass range from 50 to 1500 Da, in the negative ion mode.

By using a time-of-flight mass spectrometer with high sensitivity in wide-range scan mode, formation of photooxidation products was readily deduced from appearance of new ions in the time-resolved mass spectra. Subsequent extraction of selected ions from the total ion chromatogram (TIC) gave the desired formation traces. Continuous flow injection in the electrospray ionization source occurred at a rate of 20 μL min^{-1} and was maintained by a Harvard syringe pump, equipped with a 0.25 mL Hamilton glass syringe. Although irradiation time in such a setup was limited by syringe size, a small diameter was preferred because of the homogeneous irradiation of the reaction mixture. On the other hand, slow pumping speed accounted for a dead volume of approximately 2.5 min between syringe and electrospray source.

LC−MS and MS^2. Identical mixtures as prepared for ESI-MS were prepared for batch irradiation experiments (using a 20 W halogen lamp as excitation source). After purging with nitrogen, mixtures were loaded in transparent vials (5 mL) and exposed to irradiation. At varying exposure time, samples were analyzed by liquid chromatography with mass spectrometric detection. Injection volume was 3 μL, and separation was performed on an analytical C_{18} column (Alltech Altima, Grace, Deerfield, IL; 4.6 × 250 mm, 5 μm, kept at 20 °C) at a flow rate of 0.7 mL min^{-1}. Gradient elution was optimized using a solvent system consisting of water (solvent A) and a methanol−acetonitrile mixture (1:1, v/v; solvent B) each with 0.025% HCOOH added. The following gradient profile with satisfying separation was applied: 0−3 min at 15% B, raise to 24% B (3−8 min), stay for 3 min (8−11 min), raise to 34% B (11−18 min), raise to 44% B (18−28 min), raise to 81% B (28−36 min), raise to 95% B (36−42 min), stay for 8 min (42−50 min), return to 15% B (50−57 min), and stay for 5 min (57−62 min). Mass spectrometer settings were as follows: source and desolvation temperatures were set to 80 and 120 °C, respectively; desolvation gas, 450 L h^{-1}; nebulizer gas, 20 L h^{-1}; capillary voltage, 2500 V; cone voltage, 33 V; extractor voltage, 1 V; ion energy, 1.8 V; collision energy, 4 V. Mass spectra were acquired over a mass range from 50 to 1000 Da, in the negative ion mode. Relevant samples were further analyzed by tandem mass spectrometry (MS^2). Appropriate mass functions (ToF product ion), with collision energies set between 20 and 40 eV, were programmed for fragmentation of selected compounds from LC−MS analyses.

DPPH Benchmark Reaction. Oxidation of relevant flavonoids by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical was carried out in order to compare reaction products with those from flavin-mediated oxidations. Therefore, 250 μL of DPPH solution (1 mM in acetonitrile) was added to 750 μL of relevant flavonoid solution (1 mM in aqueous acetonitrile 1:1, v/v) and the mixture was left reacting for 60 min prior to analysis using the above-mentioned methods. Reaction mixtures were compared with blank reactions, in which the DPPH solution was replaced by 250 μL of acetonitrile.

RESULTS AND DISCUSSION

Real-Time ESI-MS Reaction Monitoring. Visible light irradiation of a mixture of (poly)phenolic compounds and flavin mononucleotide (FMN) in aqueous acetonitrile (1:1, v/v) was previously shown to produce relatively long-lived radical intermediates.[21] Since fate of these species was unknown, fast real-time ESI-MS (see Supporting Information for method details) was applied for initial screening of product formation. Negative ion mode was selected because the negatively charged phosphate moiety in FMN induced a strong response in aqueous acetonitrile mixtures. Thus, irradiation of the solution containing only FMN produced a series of ions that were attributed to light-induced autoreaction products (Figure 1). The most characteristic molecular ions ($[M-H^+]^-$) thus formed were m/z 241 and m/z 283, corresponding to formation of lumichrome and formylmethylflavin, respectively.[23,24] Concurrent rise of m/z 213 (Figure 1) was ascribed to release of the ribitylphosphate chain, but also other disintegration paths, possibly initiated by radical cleavage reactions, gave phosphate-containing fragments (m/z 169, m/z 139, and the characteristic m/z 97, which refers to the phosphate moiety itself). Besides fragmentation, the appearance of m/z 453 pointed to oxidation with ketone formation in the ribityl chain, as previously reported for flavin irradiation experiments.[23] All of these different FMN degradation pathways were most likely initiated by triplet−triplet

Scheme 1. Mechanism of FMN Ground State Quenching (A) and Flavonoid Deactivation (B) of Triplet-Excited FMN

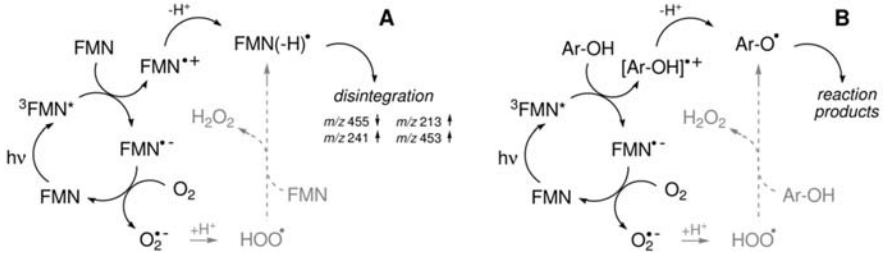
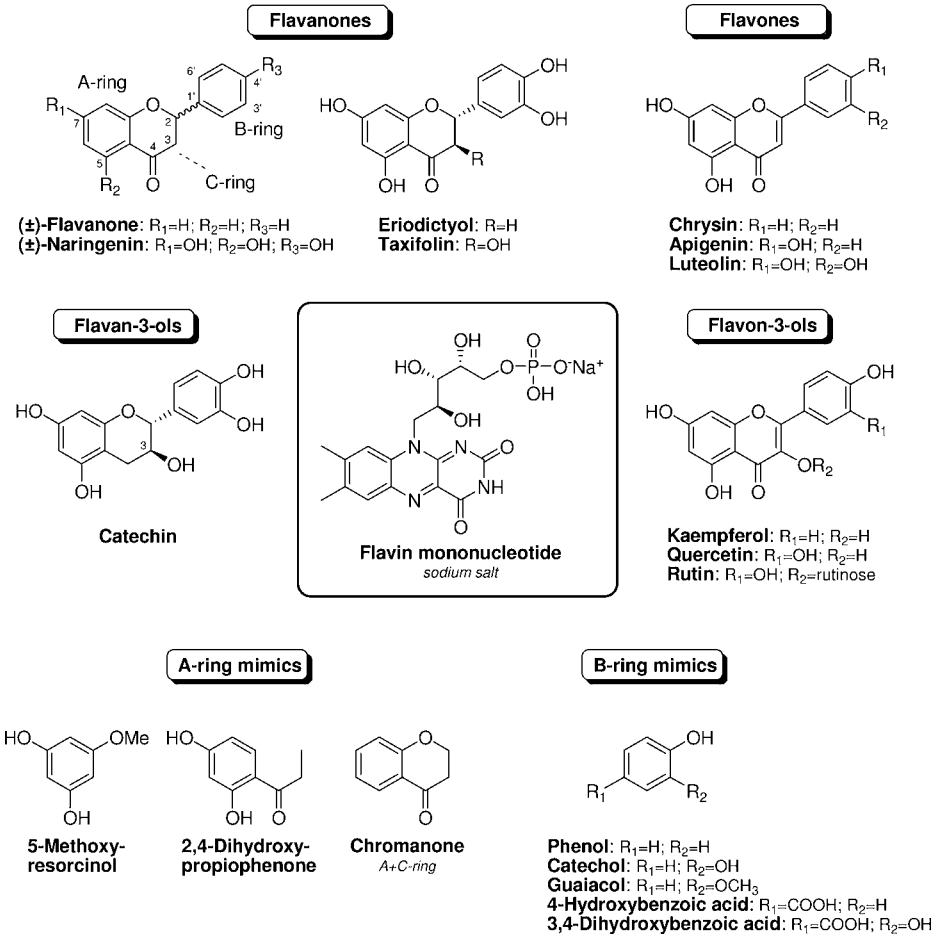


Chart 1. Structures of FMN, Flavonoids (Including Flavones, Flavon-3-ols, Flavan-3-ols, and Flavanones) and Phenolic Model Compounds



annihilation or triplet-ground state quenching,[25] the latter mechanism which is depicted in Scheme 1A. Electron transfer from FMN to the oxidizing ^{3}FMN* gives the reduced flavin radical (FMN$^{\bullet-}$), which is readily oxidized by residual oxygen with regeneration of the incipient flavin and concomitant formation of superoxide ($O_2^{\bullet-}$).[26] The fact that both this reactive oxygen species and a highly reactive flavin radical cation (FMN$^{\bullet+}$) were previously characterized in identical photoreaction mixtures[27] is in agreement with the proposed mechanism.

Photooxidation of (Poly)phenols. Addition of chromanone or flavanone (for structures, see Chart 1) did not affect FMN degradation traces, but the presence of phenolic compounds considerably changed the reaction path. Decay of m/z 455 (FMN ion) and the rise of m/z 241 (lumichrome ion) were inhibited (Figure 2), most likely because the pathway involving the flavin triplet−triplet or triplet−ground state interactions was cut off by fast electron transfer from the antioxidant ($k \sim 10^9$ L mol^{-1} s^{-1})[21] with reduction of ^{3}FMN* (Scheme 1B). As mentioned, FMN$^{\bullet-}$ is regenerated to FMN by residual oxygen, and it is likely that the resulting superoxide or the more reactive hydroperoxyl radical (HOO$^{\bullet}$) contributes to the observed degradation of flavin and substrate. These pathways (Scheme 1B) account for consumption of naringenin and apigenin during irradiation, with emerging ions (m/z 541 and m/z 537, respectively) referring to dimer formation (Figure 2D). Similar observations were made when a set of model phenolic substrates were investigated, for example with intense dimerization (m/z 185) in the case of phenol (Table 1). Minor hydroxylation (m/z 109) favored a disproportionation mechanism (with intermediate phenoxonium as electrophile)[21] rather than formal radical coupling. No oxidation products were

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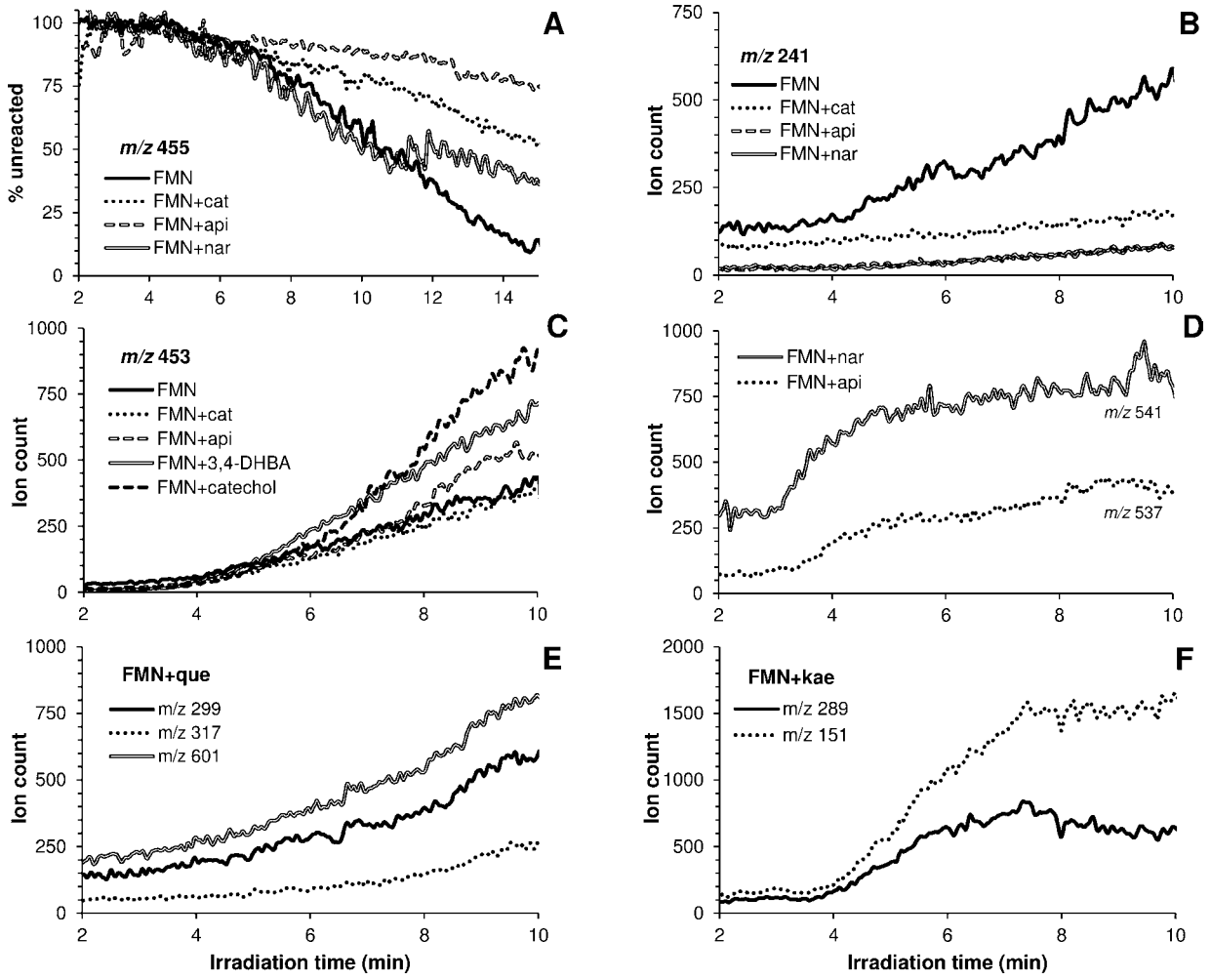

Figure 2. Extracted ion chromatograms from a real-time ESI-MS experiment in which a FMN solution was irradiated in the absence (FMN) or in the presence of selected flavonoids (FMN + substrate) (cat: catechin, api: apigenin, nar: naringenin, 3,4-DHBA: 3,4-dihydroxybenzoic acid). (A) Relative decay of FMN (m/z 455). (B) Formation of lumichrome (m/z 241). (C) Formation of keto-FMN (m/z 453). (D) Formation of dimers from naringenin and apigenin. (E) Formation of dimer (m/z 601), quinone (m/z 299), and benzofuranone (m/z 317) from quercetin (que). (F) Formation of a depside (m/z 289) and its major fragment (m/z 151) from kaempferol (kae).

detected for 4-hydroxybenzoic acid, suggesting either a pivotal role for the *para*-position in dimerization or a highly stabilizing effect from the carboxylate moiety on the phenoxyl radical. The absence of oxidation products contrasts with the observation of a dimeric species upon irradiation of 3,4-dihydroxybenzoic acid, which carries a catechol moiety that is generally considered beneficial for antioxidative activity. Catechol however gave only minor dimer formation, but the m/z 453 ion trace (corresponding to ketone formation in FMN) was particularly intense (Figure 2C) and possibly pointed to interference of secondary oxidation pathways. This peculiar behavior was suppressed by methylation of one of the hydroxyls, as in guaiacol, with mainly dimerization (m/z 245) being observed.

Catechin (m/z 289), a compound with a nonconjugated catechol B-ring, was readily converted during irradiation. The ion trace at m/z 287 was ascribed to oxidation to the *o*-quinone (or *p*-quinone methide) product, while also formation of dimers (m/z 577) and further oxidized derivatives thereof (m/z 575) was readily detected. Next to dimers, there were a couple of reaction products (m/z 303 and m/z 321) that probably resulted from oxygenation of the quinone (net result +14 Da) with subsequent water addition (net result +32 Da). Luteolin, which also bears a catechol moiety, failed to produce the corresponding *o*-quinone (no m/z 283), and dimerization (m/z 569) was poor. The moderate rise of m/z 583 suggested formation of a dimeric compound, which possibly came from cross-coupling with minor oxygenated luteolin derivatives. The introduction of a hydroxyl function at C_3, as in quercetin, however, changed reactivity as now both quinone (m/z 299) and dimer (m/z 601) traces could be extracted from the total ion chromatogram (Figure 2E). Moreover, addition of water appeared to be involved, furnishing a previously characterized 3,4-flavanedione or, after isomerization, benzofuranone compound (m/z 317).[28,29] These reactions were inhibited in rutin, most likely because the rutinoside moiety blocked the pivotal C_3-OH from participating in the oxidation mechanism. Due to the importance of this moiety, early events in the photooxidation of kaempferol were expected to be similar to those for quercetin, but, instead of a benzofuranone compound, the major product was tentatively identified as a depside (m/z 289). Dimers or a *p*-quinone methide were not detected.

It is generally accepted that reactivity of flavonoids (except for flavon-3-ols) originates in the phenolic function(s) of the B-ring moiety. Indeed chrysin, which is similar to apigenin but lacks the $C_{4'}$-OH, failed to produce dimer compounds on irradiation. Furthermore, reactions of A-ring models like 5-

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Table 1. Product Ions Observed by ESI-MS Monitoring (in Negative Ion Mode) of Flavin-Mediated Photooxidations

		reaction product ions			
substrate	$[M-H^+]^-$	$[M-H^+]^-$	Δ (Da)[a]	MIC[b]	TA[c]
phenol	93	185	92	++	dimer
		109	16	±	*p*-hydroquinone
		123	30	±	oxygenated phenol
		277	184	±	trimer
		369	276	−	tetramer
catechol	109	215	106	−	dimer(−2H)
guaiacol	123	245	122	++	dimer
		367	244	±	trimer
4-HBA[d]	137	X[e]			
3,4-DHBA[f]	153	303	150	±	dimer(−2H)
		259	106	±	dimer(−2H, $-CO_2$)
5-MR[g]	139	277	138	±	dimer
		429	290	±	NI[h]
2,4-DHP[i]	165	329	164	−	dimer
chromanone	147	X[e]			
flavanone	223	X[e]			
apigenin	269	537	268	±	dimer
		285	16	−	hydroxylated apigenin
naringenin	271	541	270	+	dimer
		287	16	±	hydroxylated naringenin
catechin	289	287	−2	±	*o*-quinone
		303	14	±	oxygenated quinone
		321	32	±	oxygenated (2×) catechin
		577	288	±	dimer
		575	286	±	dimer(−2H)
kaempferol	285	151	−134	++	depside fragment
		289	4	+	depside
		169	−116	+	depside fragment
		179	−106	±	hydrolyzed compound
		301	16	±	benzofuranone
luteolin	285	583	298	±	oxygenated dimer
		569	284	−	dimer
		299	14	−	oxygenated luteolin
quercetin	301	601	300	±	dimer
		299	−2	±	*o*-quinone or *p*-quinone methide
		317	16	±	benzofuranone
rutin	609	X[e]			

[a]Difference (Da) with substrate mass. [b]MIC: maximum ion count observed in the 2.5−10 min irradiation window; 100 < − < 200 < ± < 500 < + < 1000 < ++. [c]TA: tentative product assignment. [d]4-Hydroxybenzoic acid. [e]No product ions observed. [f]3,4-Dihydroxybenzoic acid. [g]5-Methoxyresorcinol. [h]Not identified. [i]2,4-Dihydroxypropiophenone.

methoxyresorcinol (as model for the A-ring in flavan-3-ols) showed less dimerization than phenol, but it was mainly the carbonyl substituent (as in 2,4-dihydroxypropiophenone, the A-ring model for other flavonoids in this study) that inhibited the A-ring from contributing to product formation.

Validation of ESI-MS by LC−MS. Results from direct infusion of a reaction mixture should be interpreted with care, as the observer may be misled through unforeseen in-source phenomena such as oxidation reactions, ion suppression, or ionic adduct or cluster formation. Thus, to validate real-time ESI-MS analysis as a useful technique for monitoring photooxidations, formation of dimers and other reaction products was investigated by chromatographic analyses coupled to mass spectrometric detection (LC−MS). For this purpose, identical mixtures as used for ESI-MS were shortly exposed to irradiation (≤1 h) to avoid excessive formation of secondary photoproducts. Longer reaction time led to extensive degradation of substrate (particularly in the case of flavon-3-ols) which severely hampered mechanistic interpretations (see Supporting Information).

Photooxidation of Flavanones and Flavones. Initial LC−MS analysis of simple model systems revealed that the dimer from phenol prevailed as two major isomers which, due to coinciding ion traces, could not be discriminated by ESI-MS (Table 2). Moreover, the possibility of combining LC−MS with collision-induced fragmentation of analytes (MS^2) was favorable in view of collecting additional structural information. Accordingly, phenol dimers were found to release only small fragments (−28 Da, CO loss; −18 Da, water loss) at highest collision energy, suggesting dimer formation occurred via strong, irreversible C−C bonding. Naringenin, a flavanone compound with a phenol B-ring, produced two major dimers as well (Figure 3), but fragmentation patterns were significantly more complex (Table 2). However, the presence of two hydroxylated naringenin compounds (both *m*/*z* 287) in the photoreaction mixture appeared to be very helpful in elaborating a general reaction mechanism (Scheme 2). After initial electron transfer from naringenin with subsequent proton loss, resulting radical **i** underwent disproportionation with formation of a quinone methide (**ii**). Such species are highly electrophilic,[30,31] and, particularly in the presence of protic solvent molecules or mild nucleophiles, only transient lifetimes were expected. Resonance stabilization (**ii** ↔ **iii**) was in agreement with formation of two hydroxylated compounds (2-hydroxynaringenin and 2′-hydroxynaringenin), which were neatly characterized (using detailed guidelines for interpretation of flavonoid mass spectra)[32,33] by C-ring fragmentation in MS^2 analyses (dashed reaction arrows in Scheme 2). A similar mechanism was expected in the formation of dimers, with naringenin substituting for water as nucleophile. Most likely, the less hindered **ii** was attacked by one of the phenolic hydroxyls in a Michael-type reaction, thus accounting for more hydrophobic products (as concluded from longer retention times) with respect to naringenin.

Apigenin, having an additional C_2-C_3 unsaturation with respect to naringenin, was photooxidized with formation of two dimers as well (Figure 3). MS^2 patterns of these compounds (both *m*/*z* 537) mainly showed intense, intact monomer fragments (*m*/*z* 269, Table 2), which points to dimerization via reversible ether (−C−O−C−) connection. The consumption of a hydroxyl function in this process was indeed in agreement with the longer retention time of the products compared to the parent apigenin. Still, structures of the two dimers must have differed significantly as only the first compound (at 38.1 min) further disintegrated in the collision cell and produced other fragments next to the abundant apigenin monomer ion. In this respect, the loss of a phenol moiety (transition 537 > 443, corresponding to the breaking of the $C_2-C_{1'}$ bond) was only feasible if conjugation between the B and C-ring was lost, as for example due to dimerization involving addition to the C_2-C_3 unsaturation. The second dimer (39.5 min) was much more stable and retro-addition as sole fragmentation mechanism made structure elucidation highly speculative. But, despite the limited information, it was concluded that formation of a C_3-

Table 2. Details of LC−MS and MS² Analyses of Reaction Products from Flavin-Mediated Photooxidations

				reaction products				
substrate	$[M-H^+]^-$	t_R[a]	tI[b]	$[M-H^+]^-$	t_R[a]	PI[c]	CE[d]	fragment ions[e]
phenol	93	X[f]	60	185	36.6	∞	40	157 (100), 185 (72)
				185	38.4	∞	40	167 (100), 185 (59)
				277	35.6	∞	40	277 (100)
				277	38.1	∞	40	277 (100), 259 (61)
				277	40.0	∞	40	259 (100), 277 (68)
				277	41.0	∞	40	259 (100), 277 (98)
				277	42.4	∞	40	200 (100), 170 (67), 277 (63)
catechol	109	16.4	40	223	19.5	2	30	135 (100), 109 (90), 179 (31), 223 (8)
guaiacol	123	X[f]	60	245	38.7	∞	40	230 (100), 215 (52), 187 (15)
naringenin	271	36.5	20	287	28.1	7	30	177 (100), 108 (42), 287 (39), 150 (15)
				287	33.9	3	30	151 (100), 135 (42), 287 (41), 107 (7)
				541	39.5	20	40	389 (100), 151 (82), 415 (66), 237 (22), 177 (20), 541 (14), 263 (9)
				541	40.6	14	40	363 (100), 125 (56), 541 (54), 177 (53), 317 (48), 151 (44), 415 (19), 449 (11)
apigenin	269	37.3	20	285	35.3	3		not measured
				537	38.1	9	40	269 (100), 443 (10), 537 (7), 151 (5)
				537	39.5	6	40	537 (100), 269 (66)
luteolin	285	35.4	60	569	34.1	25	40	569 (100), 332 (78), 325 (69), 391 (47), 459 (31)
				583	35.8	<1		not measured
				585	37.5	<1		not measured
				629[g]	35.0	5		not measured
				629[g]	35.5	2		not measured
				629[g]	38.0	7		not measured
catechin	289	13.1	20	575	21.9	4	35	575 (100), 394 (57), 413 (29), 449 (26), 243 (15) 287 (16), 229 (13), 137 (12)
				575	25.8	18	40	575 (100), 394 (52), 287 (39), 229 (33), 271 (28), 137 (27)
kaempferol	285	37.4	20	301	24.1	8	30	273 (100), 301 (51), 207 (42), 245 (23), 257 (18)
				289	31.8	43	20	151 (100), 289 (39), 169 (9)
				569	39.0	35	40	311 (100), 151 (95), 283 (25), 330 (22), 417 (22), 449 (19), 257 (19), 569 (15)
				179[g]	19.1	55	30	151 (100), 107 (28), 179 (19), 63 (17), 83 (15)
				333[g]	26.7	99	30	315 (100), 333 (55), 283 (15)
				347[g]	33.8	32	30	315 (100), 347 (98), 283 (15)
quercetin	301	35.2	20	317	18.5	4	20	317 (100), 191 (11), 163 (9), 207 (9)
				169	25.6	6	30	151 (100), 125 (88), 169 (85), 83 (76), 57 (33)
				299	36.2	18	40	299 (100), 271 (53), 194 (24), 151 (18)
				601	38.6	52	30	299 (100), 601 (11)
				901	39.5	16	30	299 (100), 901 (24)
				349[g]	22.7	114	30	331 (100), 299 (48), 349 (32), 179 (27), 151 (18), 271 (9)
				331[g]	28.9	25	30	331 (100), 299 (80), 179 (69), 151 (40), 271 (29)
				649[g]	35.5	62	20	649 (100), 299 (31)
				663[g]	37.4	16	20	663 (100), 299 (42)
eriodictyol	287	34.2	20	301	35.3	3	30	301 (100), 151 (76), 125 (43), 186 (18), 177 (16), 257 (11), 239 (10)

[a]Retention time (minutes). [b]Irradiation time (minutes). [c]PI: relative peak intensity, calculated as the ratio of product peak area vs peak area of the remaining substrate (∞ indicates no substrate compound was retrieved after irradiation). [d]CE: collision energy (eV). [e]m/z value of fragments (relative ion intensity in mass spectrum). [f]Substrates most likely eluted without retention or response was silenced by ion suppression. [g]Products only observed if irradiation was carried out in the presence of methanol.

$C_{3'}$-linked or a C_3-C_3-linked dimer, as reported for reaction with alkaline potassium ferricyanide,[34] was highly unlikely.

Despite the similarities with apigenin, the presence of a catechol B-ring in luteolin is expected to affect reactivity. Such a catechol moiety was found important in two-electron processes, e.g., in the deactivation of ferrylmyoglobin[35] or in bulk electrolysis.[28] One-electron mechanisms such as enzymatic or free radical (AIBN) oxidations remarkably failed to produce detectable reaction products.[29,36] As for the photooxidation reaction, a one-electron process as well, the weak ion trace (m/z 569) in ESI-MS corroborated the poor product formation, which is possibly a consequence of long-lived, resonance-stabilized radical (or radical anion) formation.[21] Still, chromatographic analysis (Figure 3) indicated the presence of a single dimer that, according to real-time data, must be resulting from a secondary pathway.

Photooxidation of Flavan-3-ols. Catechin dimers, such as procyanidins, are widespread and have been identified in various foods including fruits and chocolate.[37−39] Their structures are often complex with multiple bonds between the catechin monomers, giving compounds that are typically catalogued as A-type procyanidins. B-type analogues are less complex and basically consist of monolinked catechin units. These compounds were detected in the photoreaction mixture (m/z 577), but prevalence was only minor compared to further oxidized dimers (m/z 575). Interestingly, identical products

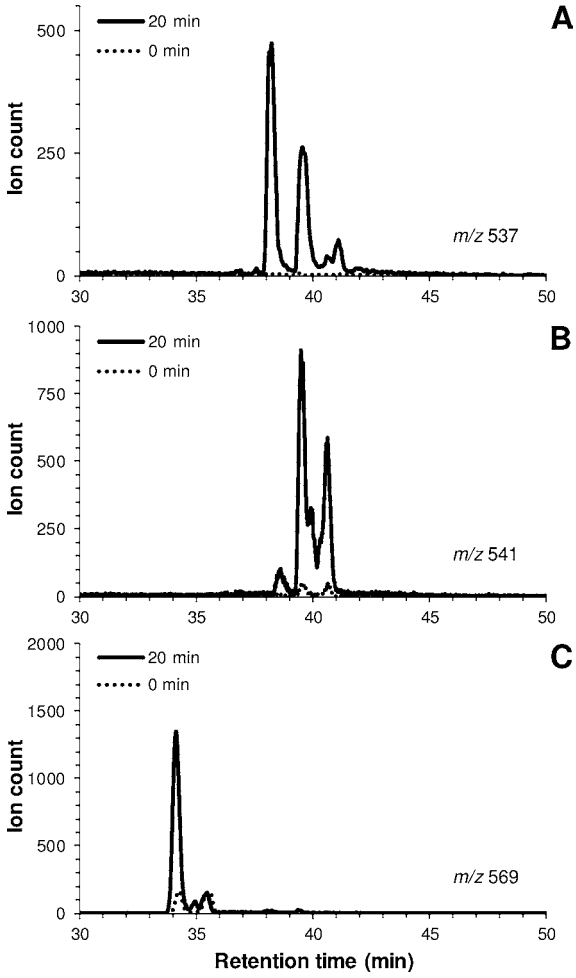
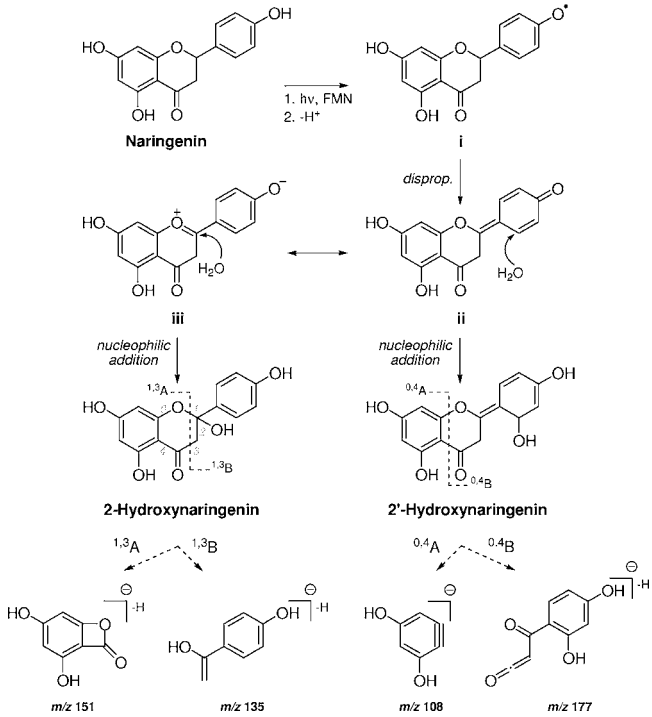


Figure 3. LC–MS extracted ion chromatograms showing dimer compounds from apigenin (A), naringenin (B), and luteolin (C) before (0 min) and after (20 min) irradiation in the presence of FMN.

(matching retention times and *m/z* values) were found when oxidation was carried out with the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) as oxidant. Products from this benchmark reaction were described in the literature,[40,41] allowing comparison of reported structures with experimental MS^2 data from the flavin-mediated oxidation. The most prevalent compound (retention time 25.8 min), producing a rich fragmentation pattern, was thus identified as dehydrodicatechin A (Scheme 3). Particularly ion *m/z* 394 was characteristic since the $A^{0,4}$ cleavage indicated that the A-ring of at least one of the substrates was involved in dimerization. Previous investigation of radical intermediates showed that oxidation was initiated at the B-ring catechol with formation of radical **iv** (or radical anion, depending on reaction pH).[21] Subsequent disproportionation (or a consecutive one-electron oxidation) was found to give the corresponding *o*-quinone (*m/z* 287), which was sufficiently stable to be detected in ESI-MS. Following Michael-type addition via the A-ring of a second catechin yields a B-type dimer which is prone to secondary oxidations due to the presence of a labile hydrogen (benzylic and bisallylic position, see Scheme 3). The involvement of the A-ring as nucleophile was not observed with naringenin as substrate and was most likely attributed to the absence of an electron-withdrawing carbonyl moiety. Indeed, taking basicity as a measure for nucleophilicity, the difference in pK_a value between the C_7-OH

Scheme 2. Mechanism of Naringenin Photooxidation with Formation of Hydroxylated Derivatives[a]



[a]Dashed lines indicate cleavage sites in MS^2 that give formation of a series of fragment ions (dashed arrows refer to collision-induced fragmentation reactions).

in catechin and in taxifolin (9.4 vs 6.7, respectively) strongly supported this assumption.[42−44]

Photooxidation of Flavon-3-ols: Role of the C_3-OH. The presence of a C_3-OH moiety in flavon-3-ols has been predicted by theoretical studies to significantly alter reactivity with respect to the corresponding flavones. Particularly equilibrium with the C_3-keto tautomer was presumed responsible for this shift since its C_2-H is exceedingly labile (bond dissociation energy of 64.3 kcal mol^{-1} vs 74.6 kcal mol^{-1} for the $C_{4'}$-hydroxyl in the B-ring).[45] Such values predict ready participation in radical reactions, giving **viii** and **ix** upon hydrogen abstraction from kaempferol and quercetin, respectively (Scheme 4). As such, quercetin was reported to be easily oxidized in one-electron processes (including enzymatic oxidation and DPPH and AIBN free radical oxidations),[29,36,46,47] thus showing very different behavior than the corresponding flavone luteolin (vide supra). Flavin-mediated photooxidation gave one major species, a dimer (*m/z* 601), that was considerably more hydrophobic than quercetin (retention time 38.6 min vs 35.2 min) and that was identical to the product from a benchmark free radical oxidation (DPPH) (see Supporting Information). MS^2 spectra, recorded at low collision energy, were dominated by retro-Diels–Alder fragmentation (Scheme 4; Table 2) and were thus in support of a dioxane linkage. Such a product has been identified before and was suggested to result from Diels–Alder cycloaddition,[29] from radical coupling[48] or from disproportionation of two quercetin radicals with subsequent ionic coupling.[36] The intervention of a flavilium-like intermediate (**xv** or its resonance **xiii**, Scheme 4) in the latter mechanism was particularly interesting, since it corroborates formation of (mixed) methanol adducts via nucleophilic solvent addition to **xiii** and **xvii** if irradiations

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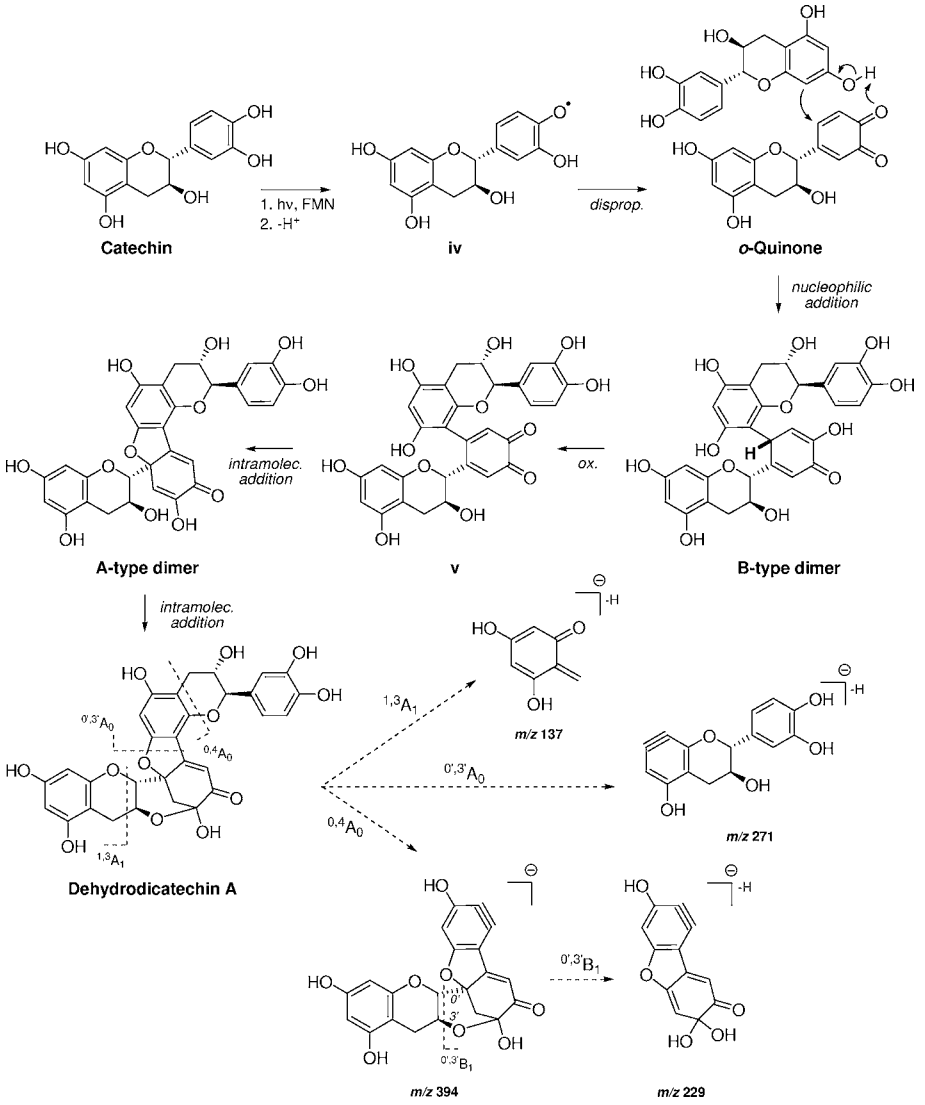
Scheme 3. Mechanism of Catechin Photooxidation with Formation of an A-Type Procyanidin[a]



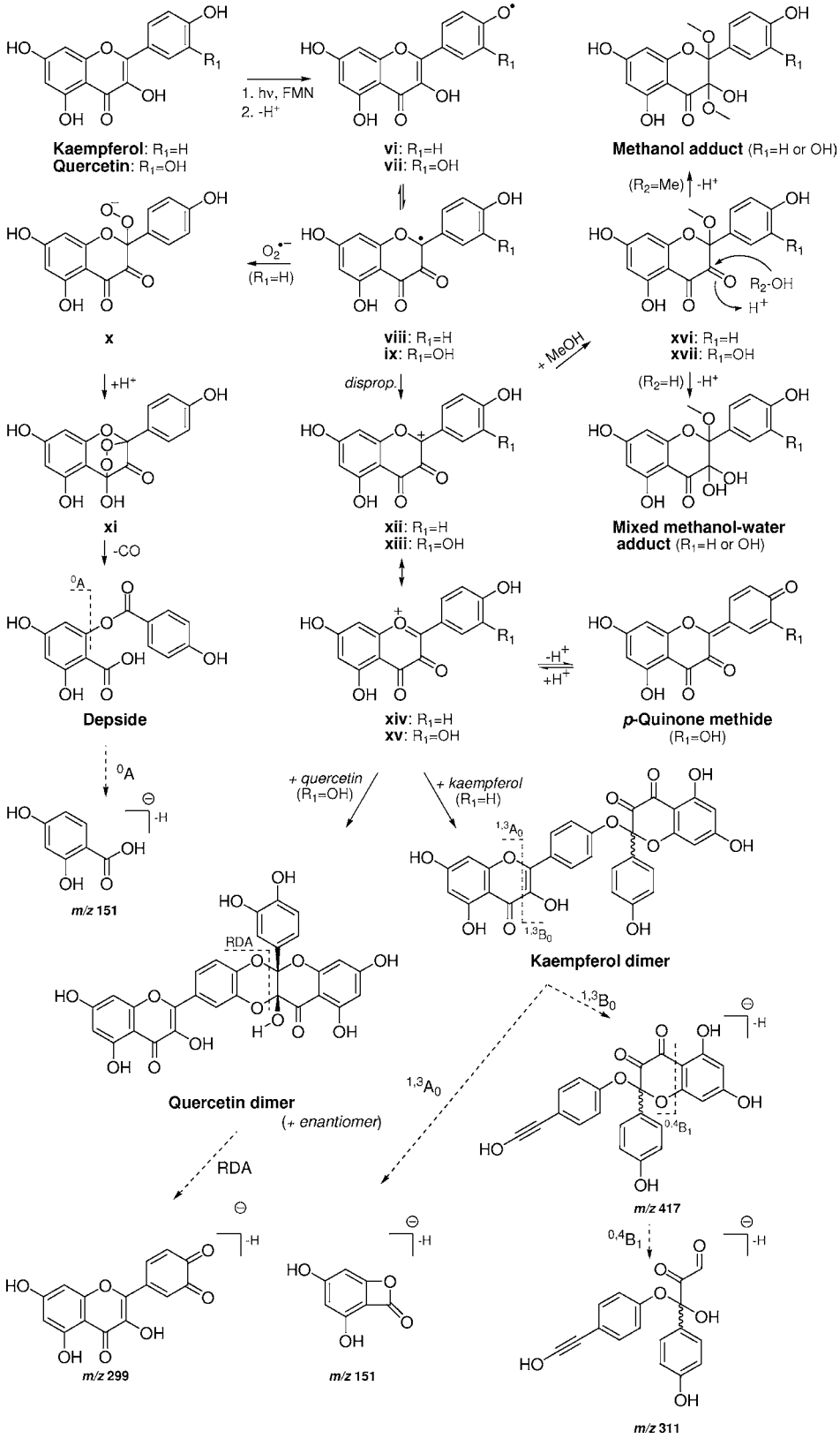
[a]Dashed lines indicate cleavage sites in MS^2 that give formation of a series of fragment ions (dashed arrows refer to collision-induced fragmentation reactions).

were carried out in aqueous acetonitrile/methanol mixtures. Such adducts were not formed with luteolin or rutin (a quercetin glycoside which has the C_3-OH protected by a rutinoside sugar moiety), but were readily observed if kaempferol was the substrate (Figure 4). This supports the pivotal role of C_3-OH oxidation and, consequently, the interference of electrophilic intermediates **xiii** and **xv**, in quercetin product formation. Accordingly, dioxane linking in the dimerization process was suggested to occur via O-addition of the catechol (or catecholate from disproportionation) to these electrophiles, followed by second O-addition to the neighboring ketone at C_3.

When focusing on kaempferol, it is clear that the lack of *o*-dihydroxy pattern formally refutes formation of a dioxane link. Dimerization (m/z 569) was still observed, albeit in competition with depside formation. MS^2 of the latter showed cleavage of the ester link (with loss of a 4-hydroxybenzoic acid fragment) with intense formation of a 2,4-dihydroxybenzoate ion (transition 289 > 151, see Scheme 4). This fragment was also found to dominate the ESI-MS chromatogram (Figure 2) and was accordingly associated with *in-source* fragmentation. Although the depside formation mechanism was not fully elucidated, the difference in carbon atoms with kaempferol (14 vs 15) was previously explained by loss of CO.[49] Interestingly, interference of superoxide was suggested,[50] a reactive oxygen species that was abundantly formed during irradiation of flavin solutions.[27] Combination of this information with insights in flavon-3-ol behavior leads to following mechanism. The C_2-centered kaempferol radical (**viii**, Scheme 4), a pivotal intermediate formed during light exposure in the presence of flavins, recombines with superoxide ($O_2^{\bullet-}$) to give peroxyl anion **x**. After intramolecular addition, the resulting kaempferol endoperoxide (**xi**) decomposes with release of CO and eventually forms the depside. The feasibility of this mechanism was supported by the absence of depside formation in the reference DPPH oxidation, as this mechanism does not involve superoxide generation.

Sacrificial Use in Food Systems: A Perspective. A final consideration is attributed to practical use of naturally occurring flavonoids (e.g., under the form of a vegetal extract) as

Scheme 4. Mechanism of Flavon-3-ol Photooxidations with Formation of a Depside (Only for Kaempferol), Dimers, and Solvent Adducts[a]



[a]Dashed lines indicate cleavage sites in MS^2 that give formation of a series of fragment ions (dashed arrows refer to collision-induced fragmentation reactions; RDA refers to retro-Diels–Alder fragmentation).

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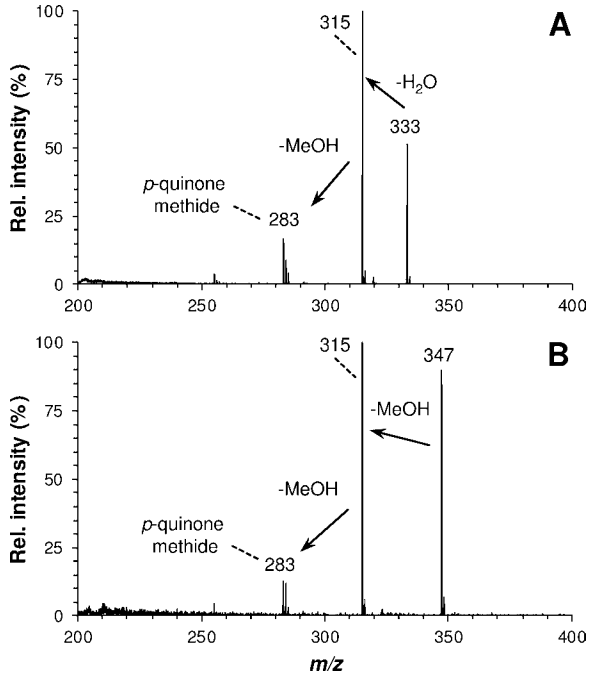

Figure 4. MS^2 spectra (recorded at collision energy of 30 eV) of the kaempferol mixed methanol−water adduct (A) and double methanol adduct (B), as formed from irradiation in the presence of FMN and methanol.

sacrificial compounds in the protection of food matrices against light-induced degradation. Our efforts in this field recently showed that addition of green tea extract (rich in flavan-3-ols) was effective in protecting soft cheese against light-induced lipid oxidation,[51] thus exemplifying the possibility to counter quality regression during retail under strong illumination. The use of vegetal flavonoid extracts as natural conservation agents nicely fits the trend toward sensible additive use, but, obviously, care should be taken about flavor impact and, most importantly, safety of spent products. In this respect, various flavonoid dimers naturally occur in frequently consumed fruits, vegetables, or derived products, thus risk for human health is not of concern. On the contrary, some of these compounds have been associated with a series of health beneficial properties, including among others anticancer and antibacterial activity.[52−54] Moreover, most of these dimers still have significant antioxidative power because of the remaining free phenolic functions.[55,56] This also implies that potential tannin action can affect colloidal stability or that progressive oxidation can alter product color. As for flavor perception, however, flavonoid dimers are expected to be less astringent than their monomers and, when present at low concentrations, possibly contribute to pleasant mouthfeel.

In conclusion, real-time ESI-MS analysis was a useful tool for fast and direct probing of photoreactions, particularly when screening of primary photoproducts was the objective. Using this methodology, strong evidence was provided for the fact that flavins, under the influence of visible light, degraded flavonoids. Tentative identification via ESI-MS, in combination with more elaborate LC−MS (and MS^2) analyses, showed that structures of resulting products strongly depended on the presence of specific functionalities. The presence of a B-ring phenol was essential for dimerization, but a B-ring catechol showed ambiguous behavior. Isolated catechols, like in catechin, were found relatively reactive, and dimeric photoproducts were readily observed. On the other hand, conjugated catechols (as in luteolin) were less prone to photooxidation, and only minor primary photoproduct generation was monitored in real-time analysis. The C_3-OH in flavon-3-ols was found more reactive, most likely due to its facile hydrogen donation and its ability to generate transient cations from radical disproportionation. These electrophilic intermediates were proposed to be pivotal in the formation of atypical photoreaction products such as a depside or a dioxane-linked dimer. A free C_3-OH, i.e., without C_2−C_3 unsaturation, was relatively inert toward photooxidation but could still participate in product formation via intramolecular addition.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Real-time ESI MS method details, as well as additional data from flavonoid photodegradation (after prolonged irradiation) and radical (DPPH) reactions. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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