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Radiolysis of Kaempferol in Water/Methanol Mixtures. Evaluation of Antioxidant Activity of Kaempferol and Products Formed

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Oxidative reaction between hydroxymethyl radical ($\bullet CH_2OH$) and kaempferol, in methanol and methanol/water mixtures, was studied by γ-radiolysis using a ^{60}Co source. Radiolysis was performed with concentrations and doses ranging from 5×10^{-5} M to 5×10^{-3} M and from 0.5 kGy to 14 kGy, respectively. Kaempferol degradation was followed by HPLC. Results showed that $\bullet CH_2OH$ reacts with kaempferol at the 3-OH group and produces two depsides (**K1** and **K2**) and other products including **K3**. **K1**, **K2,** and **K3** were identified by NMR, LC-MS, and HRMS. The kaempferol degradation pathway leading to the **K1**, **K2,** and **K3** formation is proposed. It was observed that the more water concentration in the irradiation medium increases, the more **K2** concentration increases. Comprehension of food preservation is not clear because many phenomena occurring during irradiation are not established. Radiolysis of kaempferol in water/methanol mixtures helps to elucidate the phenomenon and it is possible that during the treatment of nutriments by γ-irradiation, a series of products such as depside **K2** could be formed. Antioxidant properties of kaempferol radiolysis products were evaluated according to their capacity to decrease the EPR DPPH (1,1-diphenyl-2-picrylhydrazil) signal and to inhibit superoxide radicals formed by the enzyme reaction "xanthine + xanthine oxidase".

KEYWORDS: Kaempferol; gamma irradiation; depsides; antioxidant activity

INTRODUCTION

Flavonols, belonging to the flavonoid natural compounds, have biological activities that may be beneficial to the health (*1*−*3*). Flavonols are present in fruits and vegetables (*4*, *5*). They have been a subject of increasing research concerning their antioxidant capacity including radical scavenging, metal chelation, and enzyme inhibition. Structure−activity relationships to scavenge some reactive species including superoxide, hydroxyl, and peroxyl radicals have been proposed repeatedly (*6*−*9*). Quercetin and kaempferol are the most efficient radical scavengers.

At the present time, comprehension of food preservation is a major problem since certain phenomena occurring during irradiation are not totally established. Some studies have observed that the levels of polyphenols, including flavonoids, decline when plant foods undergo γ irradiation treatment (*10*−*12*). It was also observed that γ irradiation can cause a change of coloration of some irradiated food stuffs (*12*, *13*). Recently, we have demonstrated that radiolysis of quercetin in methanol solution forms {2-[(3′,4′-dihydroxybenzoyl)oxy]-4,6-dihydroxyphenyl}(oxo) methyl acetate (a depside) (*14*). To understand flavonoid radiolysis and then preservation of foods by γ-irradiation, we have studied the radiolysis of kaempferol and have evaluated the antioxidant activity of radiolysis products.

MATERIALS AND METHODS

Chemicals and Reagents. HPLC Methanol grade (99.8%) was purchased from SdS (Peypin, France), acetic acid from Merck (Darmstadt, Germany), and kaempferol from Sigma (St. Louis, MO).

Irradiation Treatment. Different solutions of kaempferol (5×10^{-5} M, 10^{-4} M, 5×10^{-4} M, 5×10^{-3} M) dissolved in methanol were prepared and were irradiated in aliquots of 1 mL with different doses (0.5−20 kGy) at a dose rate of 800 Gy/h in the ^{60}Co source carrier type Oris experimental irradiator.

HPLC Analyses. For each analysis of pure kaempferol (5×10^{-5} M, 10^{-4} M, 5×10^{-4} M, 5×10^{-3} M), as control, and irradiated kaempferol, 20 μL were injected into the analytical HPLC system, a Waters model equipped with a 600 model pump, a variable wavelength photodiode array detector (PDA 996) and a 600 model controller. The column used was a 250 × 4.6 mm i.d.,10 μm, μBondapak C18 cartridge (Waters). The mobile phase consisted of 100% methanol (A) and 1% aqueous acetic acid (B). Analyses were performed using a linear gradient from 20% A to 80% A during 40 min at 1 mL/min.

Isolation Procedure Using Preparative HPLC. Purification was performed by semipreparative HPLC using a 100 × 25 mm i.d.,10 μm, μBondapak C18 cartridge (Waters) and the same solvent system as above. The gradient was 20−80% A at 5 mL/min during 60 min. Solutions were evaporated at 45 °C under vacuum.

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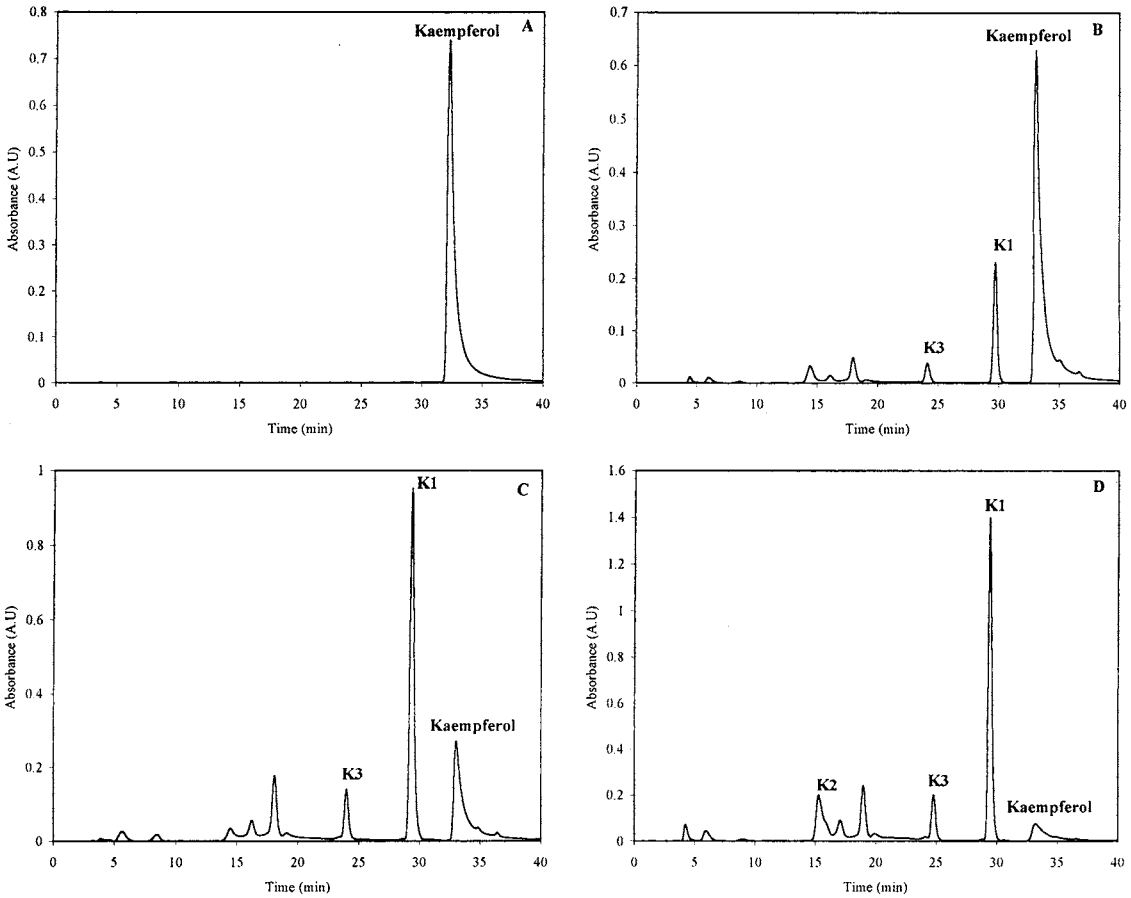

Figure 1. HPLC chromatograms, recorded at 280 nm, of A. kaempferol and irradiated kaempferol (5×10^{-3} M) at different doses: B, 2 kGy; C, 8 kGy; and D, 14 kGy.

Identification of Products K1, K2, and K3. The identity of these radiolysis products was achieved by NMR, ES-MS, and FAB-LSIMS. ^{1}H NMR and ^{13}C NMR spectra were measured in CD_3OD at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR on a Bruker DPX Avance Spectrometer using tetramethylsilane as internal standard. Complete proton and carbon assignments were based on 1D (^{1}H standard, ^{13}C *J*mod) and 2D (^{1}H-^{1}H correlation spectroscopy (COSY), ^{1}H-^{13}C heteronuclear multiple quantum coherence (HMQC), and ^{1}H-^{13}C heteronuclear multiple bond correlation (HMBC, Bruker pulse program for acquisition: inv4gslplrnd)) NMR experiments. ES-MS analysis was performed on a Waters Alliance system equipped with a Waters electrospray interface. The source was operated in the negative and positive ion modes (ES^-) and (ES^+) with a 40 V cone voltage. The FAB-LSIMS mass spectroscopy was performed at "Centre Régional de Mesures Physiques de l'Ouest" (Rennes, France), using metanitrobenzylic alcohol (mNBA) matrix. The determination of molecular formula was based on the observation of ion peaks corresponding to $[M - H]^-$, $[M + H]^+$, and $[M + Na]^+$.

DPPH Scavenging Test. Antioxidant activity was assessed on the basis of the scavenging activity of stable 1,1-diphenyl-2-picrylhydrazil (DPPH, from Sigma) free radical using EPR spectroscopy (*15*). Reaction mixtures contained 100 μL test samples and 100 μL DPPH ethanolic solution (5×10^{-4} M). EPR spectra were obtained with a Bruker ESP300E spectrometer using microsampling pipets at a room temperature under the following conditions: frequency modulation, 100 kHz; amplitude modulation, 0.197 mT; scanning field, 349.7 mT; receiver gain, 1.25 10^5; sweep time, 11 s; microwave power, 4 mW; microwave frequency, 9.78 GHz. All spectra were recorded in 3 min after homogenization by agitation. Percent inhibition was calculated using the double integral of the signal and eq 1

$$\text{percent inhibition} = \frac{\text{ref} - \text{test}}{\text{ref} - \text{bg}} \times 100 \qquad (1)$$

where ref is the reference signal (DPPH + methanol), test is the test signal, and bg is the background signal. The data were the mean of four measurements.

Superoxide Radical Scavenging Test. Oxidation of lucigenin by superoxide radicals was assayed by the chemiluminescence (CL) response of lucigenin (*7*). Superoxide radicals ($O_2^{\bullet -}$) were generated by the action of 20 μL xanthine oxidase (0.5 U) on 60 μL xanthine (2 mM) in a reaction mixture containing 400 μL phosphate buffer (pH 7.4), 50 μL lucigenin (1 mM), and 5 μL test reagent (methanol as reference signal). The mixture was quickly vortexed, and CL was measured in a luminometer analyzer (Lumat LB 9507—Berthold) at room temperature for 60 s using 75 × 12 mm tubes. Percent inhibition was calculated using formula 1. Data were the mean of four measurements.

RESULTS AND DISCUSSION

***γ* Irradiation of Kaempferol in Methanol.** Radiolysis of kaempferol in methanol solution was performed with different kaempferol concentrations irradiated at doses ranging from 0.5 to 20 kGy. Chromatograms obtained for kaempferol (5×10^{-3} M) irradiated at 2, 8, and 14 kGy are given in **Figure 1**. These HPLC data show a decrease in concentration of kaempferol (retention time of 34 min) between 2 and 14 kGy. A dose of 6 kGy decomposes 50% of kaempferol (5×10^{-3} M) and 14 kGy is required for a complete degradation. For low concentrations ($<10^{-4}$ M), 3 kGy is sufficient to cause total degradation of kaempferol. Similar results were obtained for quercetin radiolysis (*14*). **Figure 1B**, 1C, and 1D revealed that degradation of kaempferol led to the formation of several compounds and their concentrations increase when those of kaempferol decrease. **K1** (eluted at 30 min) was detected as the major product formed during radiolysis. Other products were formed in low yields. A

Table 1. Radiation Chemical Yields of Kaempferol, **K1**, **K2,** and **K3** versus the Initial Concentration of Kaempferol and versus the Different Percentage of Water/ Methanol Mixtures Utilized as System Solvent in the Radiolysis of Kaempferol

$G(\mu mol/J)$	kaempferol concentration (M) water/methanol mixtures	5×10^{-5}	1×10^{-4}	5×10^{-4}	5×10^{-3}
G(-kaempferol)	0/100%	0.0416	0.0679	0.1660	0.4070
	10/90%	0.0369	0.0611	0.1490	0.3540
	15/85%	0.0312	0.0490	0.1310	0.3100
	20/80%	0.0270	0.0428	0.1080	0.2530
	25/75%	0.0229	0.0370	0.0923	0.2120
G(K1)	0/100%	0.0314	0.0420	0.1550	0.3060
	10/90%	0.0220	0.0304	0.1120	0.2170
	15/85%	0.0144	0.0193	0.0690	0.1490
	20/80%	0.0094	0.0130	0.0465	0.0920
	25/75%	0.0063	0.0084	0.0323	0.0640
G(K2)	0/100%	0.0017	0.0028	0.0105	0.0291
	10/90%	0.0020	0.0032	0.0131	0.0340
	15/85%	0.0022	0.0036	0.0146	0.0372
	20/80%	0.0024	0.0038	0.0159	0.0401
	25/75%	0.0026	0.0041	0.0197	0.0447
G(K3)	0/100%	0.0054	0.0081	0.0303	0.0469
	10/90%	0.0046	0.0068	0.0288	0.0397
	15/85%	0.0038	0.0056	0.0220	0.0317
	20/80%	0.0029	0.0045	0.0171	0.0260
	25/75%	0.0021	0.0032	0.0119	0.0188

similar result was also observed for quercetin radiolysis (*14*). **K1** and two minor radiolysis products named **K2** (retention time of 16 min) and **K3** (retention time of 25 min) were separated, from a 5×10^{-3} M kaempferol methanol solution (30 mg) irradiated at 14 kGy, by repeated reversed-phase semipreparative HPLC and gave 14.5 mg (**K1**), 6.2 mg (**K2**), and 4.9 mg (**K3**). During the evaporation step, we observed that **K1** was transformed into compound **K2**. Concentrations of **K1**, **K2**, and **K3** were calculated by the use of calibration curves performed with purified materials. Radiation chemical yields of radiolysis products were calculated from the slope of linear concentration-dose equations and with the formula (2) (*16*)

$$G(\mu mol/J) = \frac{10^6}{0.79}\frac{\Delta M}{D} \qquad (2)$$

where D is the dose (Gy), ΔM the concentration (M) of the molecules formed, and it is well known that G is the radiation chemical yield, which corresponds to the number of molecules liberated per 100 eV of absorbed energy.

$G(-K)$, $G(K1)$, $G(K2)$, and $G(K3)$ are summarized in **Table 1**. The radiation chemical yield of each compound increases with initial concentration of kaempferol and seemed to saturate for high concentrations ($>10^{-3}$ M) (data not shown). This agreement with a classic radiolytic process where the solute reacts with the radiolytic species because of solvent, until all species are consumed.

***γ* Irradiation of Kaempferol in Water/Methanol Mixtures.** Kaempferol in different mixtures of water/methanol (10/90, 15/85, 20/80, 25/75) was irradiated. **Figure 2** reports HPLC chromatograms of kaempferol (5×10^{-3} M) irradiated at 14 kGy versus the different percentage of water/methanol. Compared with the result obtained for radiolysis of kaempferol in methanol (**Figure 1D**), the chromatograms are quite identical, indicating that in the presence of water, **K1**, **K2**, and **K3** are also formed. Radiation chemical yields of **K1**, **K2**, **K3**, and **K** were calculated and summarized in **Table 1**. We observed that kaempferol consumption and **K1** and **K3** formation decreases when methanol percentage decreases. **K2** radiation chemical yield increases with the water percentage. This indicates that the radiolysis of water led to the formation of some radical species which favor the **K2** formation.

UV/Visible Spectra of K1, K2, and K3. The UV spectra of these compounds exhibited two major bands in the 222−227 nm range, and in the 270−280 nm range (**Figure 3**). Compared with the kaempferol spectrum, we concluded that both the characteristic absorption bands at 265 nm and at 365 nm (attributed to A−C and B−C ring conjugation, respectively) disappeared. This indicated that the C-ring of kaempferol was destroyed. A similar result was established for quercetin (*14*).

Identification of K1, K2, and K3. These three radiolysis products, formed during the radiolysis of kaempferol, were identified by ^{1}H, ^{13}C, COSY, HMQC, and HMBC NMR and by negative and positive ES mass data, and their molecular formula were determined by HRMS.

The observed signals from the **K2** ^{1}H NMR spectrum (**Table 2**) corresponded to six aromatic protons: two meta coupled ($J = 2.2$ Hz) at 6.27 ppm and at 6.21 ppm, and four ortho coupled ($J = 8.8$ Hz) at 7.96 ppm (2H) and at 6.92 ppm (2H). These data indicated the presence of an asymmetrical A ring and a symmetrical B ring, tetra- and disubstituted, respectively. The positions of all protons were deduced from ^{1}H-^{1}H COSY spectrum of the proton−proton coupling connectivities.

The ^{13}C *J*mod spectrum of **K2** gave fifteen carbons signals (**Table 2**): six tertiary carbons at δ 133.9 ppm (C-2′/C-6′), 116.6 ppm (C-3′/C-5′), 105.0 ppm (C-5), and 101.7 ppm (C-3) and nine quaternary carbons. The latter were identified as three carbonyl carbons at δ 188.6 ppm (C-7), 165.7 ppm (C-8), and 165.9 ppm (C-7′) representing ketone, acid, and ester groups, respectively; four O-linked aromatic carbons at δ 168.2 ppm (C-4), 167.7 ppm (C-2), 164.8 ppm (C-4′), and 155.3 ppm (C-6) and two C-linked aromatic carbons at δ 120.5 ppm (C-1′) and 105.7 ppm (C-1). The positions of the six tertiary carbons were assigned from the ^{1}H-^{13}C HMQC experiments (**Table 2**): the carbons at 133.9, 116.6, 105.0, and 101.7 ppm correlated with protons at 7.96, 6.92, 6.21, and 6.27 ppm, respectively. These data confirmed the asymmetrical and the symmetrical structures of A and B rings. The contributions of chemical shifts to the different O-linked and C-linked aromatic carbons were deduced from the carbon−proton long-range couplings obtained from the HMBC experiments (**Table 2**).

In the ROESY and the NOESY spectra of **K2**, no correlation was observed between H3 and H6′ or H2′. This indicates that the stable conformation is obtained for a torsion angle between the A and B rings which implicates a high distance (≥4−5 Å) between the cited protons. However, the connection between the two rings was established from the HMBC spectrum. First, the ^3J correlation between C-7′ (165.9 ppm) and the protons H2′/H6′ (7.96 ppm), together with the chemical shift of C-1′ (120.5 ppm) which corresponds to a C-linked carbon, demonstrates the C1′−C7′ linkage. Second, C-7′ (165.9 ppm) was identified as an ester group and C-2 (167.7 ppm) as a non-hydroxylated O-linked carbon because of the A ring structure. This led us to establish the connection between A and B rings through the C7′−*O*−C2 linkage.

The absence of three bond couplings between any protons and the C-7 (188.6 ppm) and C-8 (165.7 ppm) carbons, together with the presence of the quaternary C-linked carbon (105.7 ppm, C-1), led us to propose the C1−C7−C8 linkage. Finally, **K2** was identified as {2-[(4′-hydroxybenzoyl)oxy]-4,6-dihydroxyphenyl}(oxo) acetic acid (**Figure 4**).

ES-MS analysis of **K2** suggested 318 as a molecular weight. The high-resolution mode of the FAB-MS revealed that the

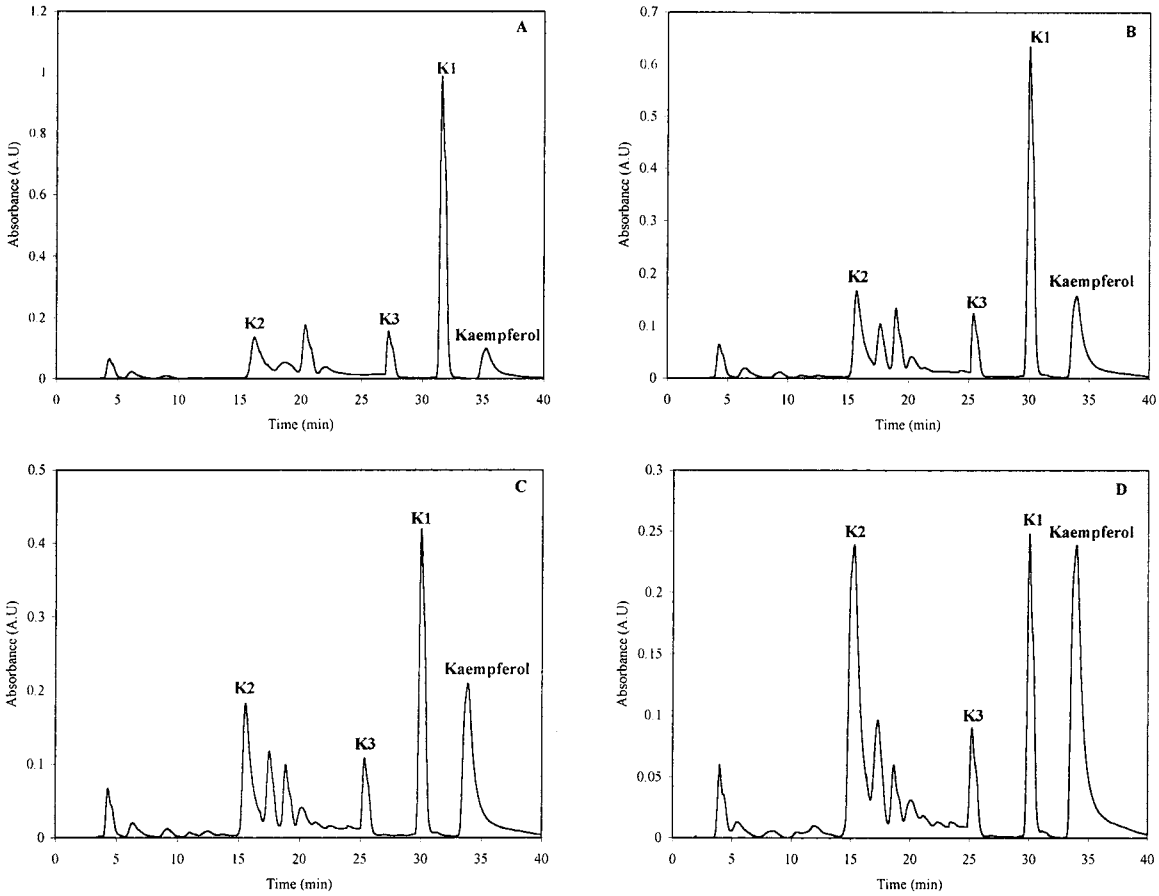

Figure 2. HPLC chromatograms, recorded at 280 nm, of irradiated kaempferol methanol/water solutions (5×10^{-3} M) at dose of 14 kGy. A, 90/10; B, 85/15; C, 80/ 20; and D, 75/25.

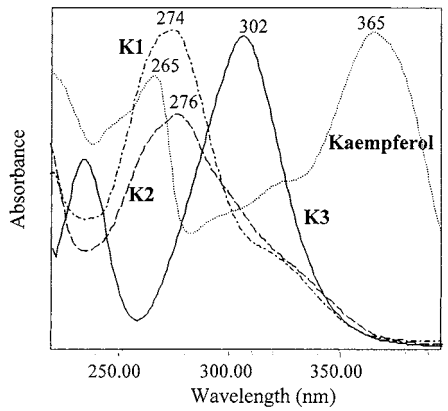

Figure 3. UV spectra of products **K1**, **K2, K3,** and kaempferol.

molecular formula of **K2** was $C_{15}H_{10}O_8$ (calculated for $C_{15}H_{10}O_8Na$, 341.02734; found, 341.02730) corresponding to the structure obtained from the NMR analysis. In addition, the positive ES-MS spectrum displayed other ion peaks at *m*/*z* 121 and 199 corresponding to the fragment C7′O linked to the B ring and to the residue of K2 (+2H), respectively (**Figure 4**). This confirms the connection between the A and B rings through C2−*O*−C7′.

The ^{1}H NMR and ^{13}C spectra (**Table 2**) of **K1** were very similar to those of **K2**, and it appeared that they belonged to the same analogous series, differing only by the presence of a methoxyl group represented by a carbon at 53.3 ppm and by a three-proton singlet (3.46 ppm). The chemical structure of **K1** was determined by procedures similar to those used for identification of **K2**. The methoxyl position was assigned because of a ^3J correlation observed between the methoxyl protons (3.46 ppm) and the C-8 carbon (165.4 ppm). Consequently, **K1** was identified as {2-[(4′-hydroxybenzoyl)oxy]-4,6-dihydroxyphenyl}(oxo) methyl acetate (**Figure 4**).

The ES-MS and the high-resolution mode of the FAB-MS demonstrated that the molecular formula of **K1** was $C_{16}H_{12}O_8$ (calculated for $C_{16}H_{13}O_8$ and $C_{16}H_{12}O_8Na$, 333.0610 and 355.0429; found, 333.0607 and 355.0428, respectively) confirming the structure obtained from the NMR analysis.

The ^{1}H spectrum of **K3** consisted of three signals. Two, corresponding to four aromatic protons, detected at 7.84 ppm (H2/H6) and 6.88 ppm (H3/H5), were ortho coupled ($J = 8.8$ Hz), and one signal at 3.93 ppm revealed the presence of a methoxyl group. The ^{13}C spectrum consisted of four tertiary carbons at δ 133.8 ppm (C-2/C-6) and 116.9 ppm (C-3/C-5), three quaternary signals at δ 125.2 ppm (C-1), 165.8 ppm (C-4), and 166.5 ppm (C-8), a carbonyl group at δ 186.5 ppm (C-7) and one signal at 53.0 ppm, confirming the presence of a methoxyl group. The positions of the tertiary and the quaternary carbons were identified by the HMQC and HMBC spectra, respectively, and the position of OCH_3 was deduced according to the ^3J correlation between the methoxyl protons (3.93 ppm) and the C-8 carbon. Finally, **K3** was identified as [4-hydroxyphenyl](oxo) methyl acetate (**Figure 4**) or methyl 4-hydroxyphenylglyoxylate (*17*). Mass spectroscopy analyses demonstrated that the molecular weight of **K3** is 180 corresponding to the molecular formula $C_9H_8O_4$ and confirming the structure obtained by NMR analyses.

Formation of K1 and K3. To understand the radiolysis of solutes, two effects are proposed, direct and indirect. The first type concerns direct interactions between molecules in solution

Table 2. ^{1}H (400 MHz); ^{13}C (100 MHz) NMR Data and Correlations Observed in COSY, HMQC, and HMBC Spectra of Products **K1** and **K2** in CD_3OD/TMS (δ ppm; J Hz)

position	^{1}H	COSY	^{13}C J_{mod}/ HMQC	HMBC
		Product **K1**		
1			105.5	
2			167.5	
3	6.26 *d* (2.2)	H-5	101.6	C-5; C-1
4			168.0	
5	6.19 *d* (2.2)	H-3	104.8	C-1; C-3
6			155.4	
7			188.6	
8			165.4[a]	
8-OCH3	3.46 s		53.3	C-8
1′			120.9	
2′	7.99 *d* (8.7)	H-3′	133.9	C-4′; $C_{6'/2'}$; C-7′
3′	6.88 *d* (8.7)	H-2′	116.5	C-1′; $C_{5'/3'}$
4′			164.8	
5′	6.88 *d* (8.7)	H-6′	116.5	C-1′; $C_{3'/5'}$
6′	7.99 *d* (8.7)	H-5′	133.9	C-4′; $C_{2'/6'}$; C-7′
7′			165.9[a]	
		Product **K2**		
1			105.7	
2			167.7	
3	6.27 *d* (2.2)	H-5	101.7	C-5; C-1
4			168.2	
5	6.21 *d* (2.2)	H-3	105.0	C-1; C-3
6			155.3	
7			188.6	
8			165.7[a]	
1′			120.5	
2′	7.96 *d* (8.8)	H-3′	133.9	C-4′; $C_{6'/2'}$; C-7′
3′	6.92 *d* (8.8)	H-2′	116.6	C-1′; $C_{5'/3'}$
4′			164.8	
5′	6.92 *d* (8.8)	H-6′	116.6	C-1′; $C_{3'/5'}$
6′	7.96 *d* (8.8)	H-5′	133.9	C-4′; $C_{2'/6'}$; C-7′
7′			165.9[a]	

[a] Assignments may be interchangeable.

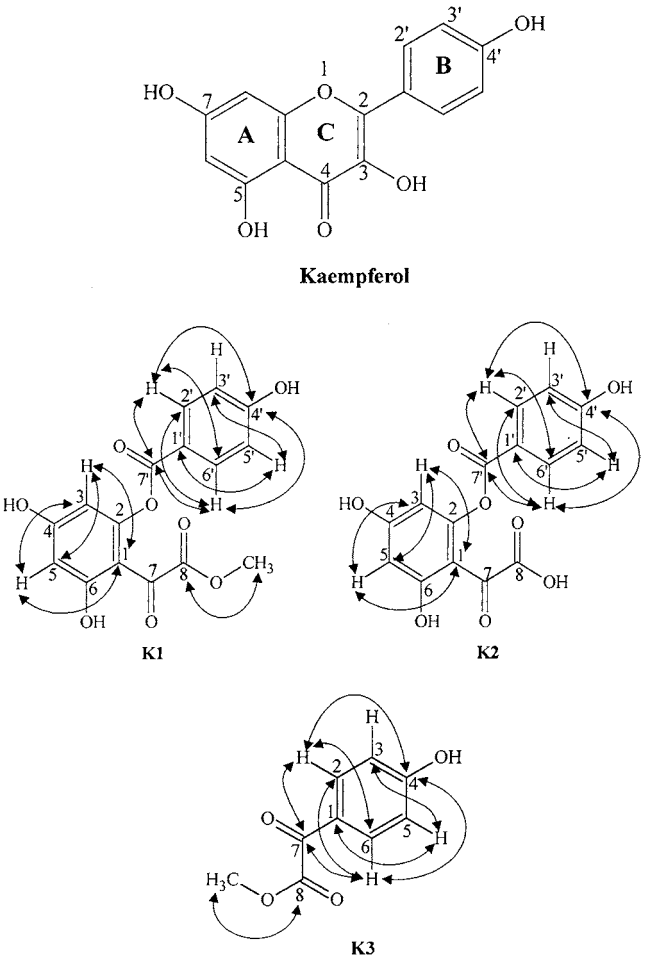

Figure 4. Selected ^{1}H–^{13}C long-range correlations (^3J) (↔) observed in HMBC spectra of products **K1**, **K2,** and **K3**.

and γ-rays. This leads to excitation and ionization of molecules. In fact, direct effects could occur when using solutions with high concentrations or with large molecules such as polymers or DNA. In our study, we worked with concentrations of kaempferol lower than 5×10^{-3} M, so direct effects could only be considered as a minor contribution. The second effect is responsible for chemical degradation of molecules. Indeed, the reactive species formed during the radiolysis of solvent react with the solute and lead to the formation of other radiolysis products. In our investigation, methanol and water were utilized as solvents. Radiolysis of these two solvents has been well established for many years (*6*, *18*). It has been shown that 50 ns after the beginning of irradiation, several reactive species including solvated electron (e_S^-), H_2, $H^\bullet$, $CH_3O^\bullet$, $^\bullet CH_2OH$, $(CH_2OH)_2$, H_2CO, H^+ such as $CH_3OH_2^+$ are formed from methanol, and (e_S^-), H_2, $H^\bullet$, $HO^\bullet$ from water. For low concentrations ($<5 \times 10^{-3}$ M), these radical species react with the solute and lead to its degradation. The strong potential of electron transfer of the cited species implicates that the solute degradation is attributed to redox reactions, including stereospecific oxidation, hydroxylation, and scission of molecules.

It has been reported (*6*, *8*, *19*) that antioxidant potential of kaempferol is attributed to the presence of hydroxyl substituents in positions 3 and 4′ which permit redox transfers and to the π electron delocalization between the B ring, the 2,3-double bond, and the 4-keto group which is responsible for stabilization of flavonoxy radicals produced after oxidative reactions. Therefore, kaempferol has potential for redox reactions occurring during radiolysis. This kaempferol chemical degradation led to the formation of depsides **K1** and **K2**. Flavonols transformation to depsides was observed, more than 30 years ago, by Gottlieb and co-workers (*20*). They demonstrated that aerial oxidation of quercetin leads to saturation of the 2,3-double bond, scission of C-ring, and consequently formation of depside. To understand the radiolysis process as well as the radical reactions occurring in the irradiated medium, we propose hypothetical degradation mechanism for kaempferol (**Figure 5**). We have observed that the consumption of kaempferol decreased with the presence of water in the irradiated medium. Radiolysis of both solvents methanol and water leads to the formation of $^\bullet H$. We conclude that the degradation of kaempferol is probably not attributed to $^\bullet H$ radicals and propose that the first step of the degradation pathway comprises the abstraction of the hydrogen atom of the 3-OH group, by the $^\bullet CH_2OH$ ($^\bullet OCH_3$) radical, leading to the flavonoxy radical ($K^\bullet$) and to the methanol formation. The second step is the formation of a keto group in position 3 by mesomeric effect. The formed radical (K11) reacts with methanol and leads to formation of an intermediate product K12. Another $^\bullet CH_2OH$ ($^\bullet OCH_3$) radical could react with K12 yielding the K13 radical. The formation of keto group in position 2 of radical K13 requires scission of the C-ring. Two possible pathways are proposed: (1) Scission of the C2−C3 bond and consequently formation of an intermediate radical K14 which could react with the methanol to give the depside **K1** and (2) scission of the C2−O1 bond (**Figure 4**) to give the radical K15 followed by the scission of the C3−C4 bond and then formation of the radical K16. This latter reacts with methanol yielding the compound **K3**. **K1** was detected as the major product formed

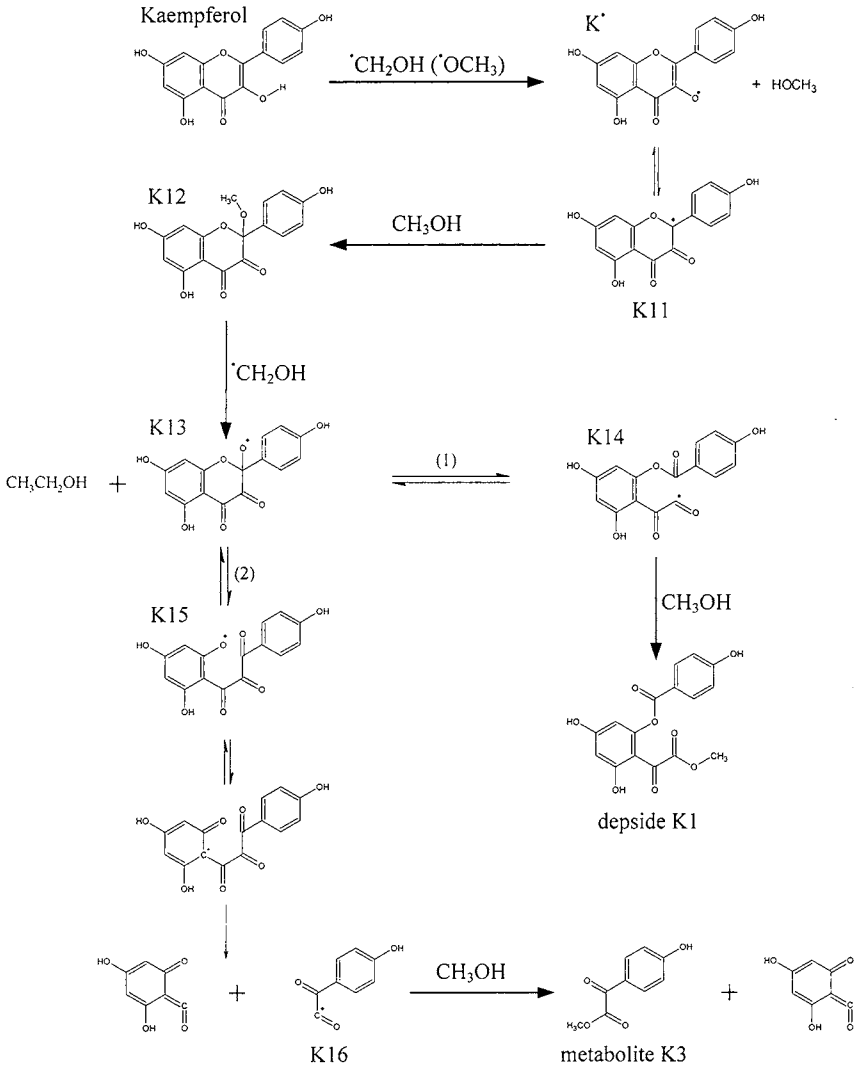

Figure 5. Proposed kaempferol-degradation mechanism yielding to the **K1** and **K3** formation.

during radiolysis. This result demonstrated that cleavage of K13 at the C2−C3 bond is more probable than that at the C2−O1 bond.

Kaempferol and quercetin are flavonols distinguished only by hydroxylation of position 3′ for quercetin. The mechanism leading to the formation of **K1** and the major radiolysis product of quercetin (*14*) do not implicate the B ring. Hence, we conclude that 3′ and 4′ hydroxylation do not influence formation of the major radiolysis products obtained by irradiation of both quercetin and kaempferol. However, it is possible that these groups react with radicals of the irradiated medium to form minor radiolytic compounds. It is well known that oxidation of the B ring leads to flavonoxy radicals and quinone forms. Presence of $^{\bullet}OCH_3$ radicals in the irradiated medium can also lead to methoxylation of the B ring.

Formation of K2. K2 was detected as a minor metabolite in the irradiated kempferol solution, and it was also formed during evaporation of **K1,** out of the irradiated medium. Indeed, the eluate containing **K1** was collected with approximately 70% methanol and 30% water. During the evaporation phase, water seemed to hydrolyze the methoxyl group of **K1** and forms the product **K2** through an reverse esterification reaction. In presence of methanol, we observed esterification of **K2** and the following chemical equilibrium is established:

$$K2 + CH_3OH \rightleftharpoons K1 + H_2O$$

The same result has been observed for the quercetin radiolysis products (*14*).

Concerning the **K2** formation in the irradiated medium, attacking of **K1** by the radiolytic species could be suggested, but this proposition is not confirmed because we have not observed any product after irradiation of **K1**. Therefore, we postulate that the **K2** formation in the irradiated medium is attributed to the presence of low concentrations of $^{\bullet}OH$ radicals which probably react with the K14 radical (**Figure 5**) to form **K2**. This hypothesis is confirmed because during kaempferol radiolysis in water/methanol mixtures, the more water concentration increases, the more **K2** concentration increases.

In conclusion, we observed that the presence of the OH group in position 3 was the key for the γ-degradation of kaempferol in methanol solution. This was confirmed because no transformation was observed on the flavones, apigenin and luteolin, analogous to the flavonols kaempferol and quercetin, respectively, when we studied radiolysis in the same experimental conditions used for kaempferol.

Flavonoid metabolism and absorption has been the subject of investigation and certain studies reported the role of intestinal microflora (*8*, *21*). Several phenolic compounds and aromatic acids have been observed which were formed by detachment of the A ring from the flavonoid molecule and by opening of the heterocyclic C-ring. For example, Winter and co-workers (*22*) reported that four *Clostridium strains*, recovered from

Table 3. IC_{50} Values of Kaempferol as Control, **K1**, **K2**, and **K3** According to the Inhibition of Superoxide Anion and the Reduction of EPR DPPH Signal

	superoxide anion		DPPH radical	
	IC_{50} (μM)	SD	IC_{50} (mM)	SD
kaempferol	1.18	1.22%	0.19	0.79%
K1	18.12	2.45%	3.12	1.50%
K2	5.37	1.60%	2.76	1.31%
K3	83.91	2.14%	13.10	1.42%

human feces, synthesize enzymes capable of cleaving the C-ring of some flavonoids such as quercetin, kaempferol, and naringenin at the C3−C-4 bond. This type of C-ring opening was observed for the anaerobic degradation of flavonoids by *Eubacterium ramulus* (*23*). Opening the pyrone ring was also found during the degradation of quercetin by methanogenic *Consortia* (*24*).

Our study has shown that γ-irradiation allows the C-ring cleavage at C2−C3 double bond to form the depsides **K1** and **K2** and at the C2−O1 bond giving **K3** via oxidative reactions similar to those occurring in microorganisms. For example, oxygenation of flavonols obtained by copper (II) complexes reproduced the in vivo quercetin and kaempferol transformation to depsides. This reaction is catalyzed by dioxygenase quercetinase (*25*). Recently, it has been demonstrated that in the presence of free radicals such as 2,2,6,6-tetramethyl-1-piperidinyloxyl (TEMPO) and 2,6-di-*tert*-butyl-a-(3,5-di-*tert*-butyl-4-oxo-2,5-cyclohexadien-1-ylidene)-*p*-totylox yl (galvinoxyl), flavonols undergo catalytic oxygenation to the corresponding depsides with concomitant CO release (*26*). Compared with our study, the loss of CO was not observed in the **K1** and **K2** formation mechanism, but the $^{\bullet}CH_2OH$ radicals play the same role as that of TEMPO and galvinoxyl because all these radicals begin the flavonol degradation process at the 3-OH group.

Antioxidant Capacity of Kaempferol Radiolysis Products. We observed that γ-irradiation transformed kaempferol to a major depside **K1** and to other minor radiolysis products, among which we identified **K2** and **K3**. We have discussed the depside formation in microorganisms but no published work has dealt with the antioxidant activity of these products. We isolated **K1**, **K2**, and **K3** from kaempferol methanol solution (5×10^{-3} M) irradiated at 14 kGy, and we evaluated their antioxidant ability to scavenge DPPH and superoxide radicals using the EPR and CL, respectively. IC_{50} values were calculated by linear regression and are listed in **Table 3**. The highest superoxide and DPPH scavenging activities were observed for kaempferol, followed by depsides **K2** and **K1**. The metabolite **K3** possessed the lowest scavenging capacity.

Flavonoid structure radical-scavenging abilities relationships have been reported repeatedly (*6*−*9*, *27*). More specifically, three criteria for radical-scavenging capacity are suggested (*19*). First, presence of the catechol group in B ring, which has better electron-donating properties. Second, the 2,3-double bond conjugated with the 4-oxo group, which is responsible for electron delocalization. Third, presence of a 3-hydroxyl group in the heterocyclic ring. Therefore, the antioxidant activity of kaempferol was attributed to the 4′, 3, 5, and 7-OH groups and to the C2−C3 double bond. 4′, 5, and 7-OH remain during the kaempferol transformation to **K1** and **K2**. The H-donor capacity of these three groups (4′-OH, 4-OH, and 6-OH in **Figure 4**) can explain the antioxidant activity of **K1** and **K2.** π Electron delocalization can exist from the A ring to the 8-keto group and from the B ring to the 7′-keto group. Additionally, the superoxide scavenging activity of **K2** is 6 times as high as **K1**. This demonstrates the importance of the 8-OH group in **K2**.

Fruits, vegetables, and beverages are rich sources of flavonols. The irradiation process has been reported as a potential treatment for extending the shelf life of foods (*28*−*30*). γ-irradiation of *Citrus clementina* increases the synthesis of total phenolic compounds such as phenolic acids and flavonoids (*31*) but little is known concerning the degradation behavior of these products when foods undergo irradiation treatment. Low solubility of most flavonoids in neutral aqueous media prevented us from performing the radiolysis of kaempferol in water. Therefore, we have chosen methanol as solvent. Although this is not the exact medium of flavonoids in fruits and there are not the same radicals constituting the irradiated medium during the preservation treatment, we have also studied kaempferol radiolysis in different water/methanol mixtures and have demonstrated that formation of depside **K2** increased in the presence of water. It is possible that during the preservation process of foods by γ-irradiation, a series of products such as depside **K2** could be formed.

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