FOOD COMPOSITION AND ADDITIVES

An HPLC Method for the Determination of Isoflavones and the Evaluation of Their Antioxidant Capacity in Both Homogeneous and Microheterogeneous Systems

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In this work, we developed an HPLC method to simultaneously quantify and hence evaluate the stability, distribution, and antioxidant capacity of six isoflavones: genistein, genistin, daidzein, daidzin, glycitin, and biochanin A. Isoflavones have been described as having an important estrogenic activity to treat menopausal symptoms and can reduce postmenopausal bone loss and also participate in the prevention of cardiovascular diseases. These beneficial properties are believed derived from their capacity to act as free-radical scavengers. Isoflavones are formulated in capsules and creams and also can be used as antioxidants in liposomes. HPLC separation was achieved on an Agilent Hypersil ODS C18 column. The mobile phase consisted of 0.02–0.2% orthophosphoric acid in water–acetonitrile with gradient elution. The diode array detector was operated at 260 nm. The hydrophobicity of isoflavones was determined through their distribution in octanol–buffer. These results allowed us to establish a relation between chemical structure, pKa, lipophilicity, and the characteristics of the dispersion medium. Photolysis of hydrogen peroxide was used to measure the $HO^{\bullet}$ scavenging capability of isoflavones. In liposomes, the order of reactivity of the studied compounds was genistein > biochanin A > genistin > daidzein > daidzin > glycitin.

Isoflavones are polyphenolic compounds widely distributed in plants, such as soybean or red clover, and, of course, are present in their products (1, 2). The principal isoflavones present in soy correspond to daidzein, genistein, and glycitein and their glycosidic derivatives daidzin, genistin, and glycitin. In red clover, the main isoflavones present are formononetin and biochanin A and their *O*-glucosides, ononin and sissotrin, respectively (3).

The chemical structure of isoflavones includes a C6–C3–C6 carbon framework, or more specifically, a phenylbenzopyran functionality (Figure 1). In Figure 1, the three rings are labeled A, B, and C, and the numbering starts from the oxygen of heterocyclic ring C. Isoflavones differ from flavonoids by the position of ring B, which is at C2 in flavonoids and at C3 in isoflavones (4).

Isoflavones have received great attention due to their estrogenic and antiestrogenic health-related benefits, such as the alleviation of symptoms of menopause and bone loss in postmenopausal women (5), reduction in the incidence of breast cancer (6), and prevention of cardiovascular diseases (7). Isoflavones are formulated in capsules and creams and also can be used as antioxidants in liposomes.

Some of the pharmacological properties of isoflavones are derived from their capacity to act as free-radical scavengers, such as the hydroxyl radical in homogeneous and microheterogeneous systems (8–10). In heterogeneous systems, the protective effect of phenolic compounds, particularly when incorporated into membranes, is mainly associated with their hydrophilicity, their degree of oligomerization, and the number of hydroxyl groups present in the whole molecule. In biological systems, many domains or compartments with different hydrophilicity and/or hydrophobicity can be found, so it can be expected that isoflavones having a wide range of water–lipid solubility would be unevenly distributed in these domains according to their chemical structure (11, 12).

A survey of the literature reveals the existence of several methods for the determination of isoflavones present in plants, foods, and pharmaceutical preparations (13–16). The techniques used include spectrophotometry (17), capillary electrophoresis (18), LC (19–21), LC coupled to MS (22), and GC coupled to MS (23); however, these methods lack the efficiency and robustness required for evaluating the stability and antioxidant capacity of isoflavones in microheterogeneus media. For this reason, in this work, a new HPLC method was developed and validated for the simultaneous determination of six isoflavones present in both homogeneous and microheterogeneous systems. The proposed method was applied to studies of stability, partition and/or distribution, and antioxidant capacity in solvents and lipid

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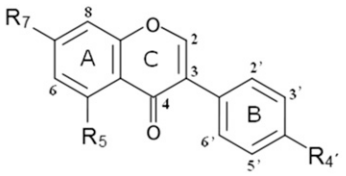

Isoflavone	Substituents			
	R-5	R-6	R-7	R-4′
genistein	OH	H	OH	OH
daidzein	H	H	OH	OH
genistin	OH	H	O-glucose	OH
daidzin	H	H	O-glucose	OH
glycitin	H	$O\text{-}CH_3$	O-glucose	$O\text{-}CH_3$
biochanin A	OH	H	OH	$O\text{-}CH_3$

Figure 1. Chemical structure of the isoflavones studied.

membranes as liposomes. All the parameters involved in the validation of the analytical methodology, including International Conference on Harmonization guidelines, were established for these compounds (24).

Experimental

Instrumentation

The HPLC system used was an Agilent Series 1100 (Agilent Technologies, Baden-Württemberg, Germany), equipped with a quaternary pump (Model No. G1311A), a degasser (Model No. G1379A), a thermostatized autosampler (Model No. G1329A), a thermostated column compartment (Model No. G1316A), and a photodiode array detector (PDAD; Model No. G1315B).

Reagents and Materials

Isoflavones daidzin (>99%), glycitin (>99%), genistin (>99.5%), genistein (>99%), daidzein (>99%; all from LC Laboratories, Woburn, MA), and biochanin A (>99%; Sigma, Steinheim, Germany) were used as received. Semisynthetic 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC; >99%) from Sigma (St. Louis, MO) was used without further purification.

HPLC grade solvents, such as methanol and acetonitrile, were purchased from Merck (Darmstadt, Germany). Dimethyl sulfoxide (DMSO) and orthophosphoric acid 85% (Merck) and hydrogen peroxide (H_2O_2; 30% solution; Merck, Billerica, MA) were reagent grade. Deionized water (typical 18.2 MΩ cm) was used throughout.

Preparation of stock standard solutions.—Stock solutions of each isoflavone were prepared by dissolving 3 mg corresponding compound in 5 mL methanol or DMSO to obtain a final concentration of 0.6 $mg \cdot mL^{-1}$. All working solutions were stirred in an ultrasonic bath for 10 min, light-protected, and maintained at 5°C.

Preparation of DPPC liposomes.—Small unilamellar vesicles were obtained by ultrasonication of DPPC suspensions (1–5 mM) in 0.1 M phosphate buffer pH 7.4 with a Cole Parmer Ultrasonic Homogenizer and stored at 5°C.

Isoflavones–DPPC liposomes.—Solutions of isoflavones incorporated into lipid membranes were prepared by addition of small volumes of a standard stock solution of isoflavone in DMSO to DPPC liposomes. The mixture was homogenized in a vortex shaker and heated in a bath at 43°C for 30 min. The solution was slowly cooled to 20°C and, if necessary, stored at 5°C.

Preparation of isoflavone-loaded liposome samples for HPLC analysis.—Samples were prepared by diluting 500 µL sample solution with 500 µL methanol in a 15 mL conical-bottomed disposable plastic tube. The mixture was shaken in a vortex mixer for 2–3 min, let stand for 10 min, and then centrifuged at 8000 rpm for 30 min. The supernatant was separated and centrifuged again under the same conditions and used as the sample for HPLC. The injection volume was 30 µL (25, 26).

Determination of Distribution and Partition Coefficients

To determine the distribution coefficients of isoflavone in the homogeneous media, 2.5 mL *n*-octanol and 2.5 mL buffer (pH 1, 3, 5, 7.4, and 9) were added to a flask and stirred vigorously for 24 h to allow the system to reach equilibrium. Both phases were mutually saturated with one another. Then, in duplicate, an aliquot of methanolic solutions of isoflavone (8 mM to ~3 $mg \cdot mL^{-1}$) was added to 2.5 mL *n*-octanol saturated with buffer and/or buffer saturated with *n*-octanol. After mixing and stirring for another 24 h, the biphasic mixture was centrifuged at 2000 rpm for 5 min at 25°C. The concentration of isoflavone in the organic–aqueous buffer phase was determined by HPLC–DAD analysis as described above.

For the determination of distribution coefficients of isoflavone in liposomes, an alternative UV-derivative spectrophotometric method was used. The instrument was a single-beam UV-Vis Agilent 8453 spectrophotometer (Agilent Technologies, Shanghai, China) equipped with 1 cm quartz cells connected to a computer loaded with UV-Vis ChemStation Software (Agilent Technologies). Samples were prepared by adding the appropriate amount of isoflavone solution in DMSO to the liposome suspension. The final sample volume, concentration of isoflavone, and percentage DMSO in the samples were 2.5 mL, 7×10^{-5} M, and 0.5%, respectively. Lipid concentrations varied from 0.25 mM (~0.18 mg/mL) to 2.5 mM (~1.8 µg/mL). Prior to measurement, samples were incubated for 30 min at 37°C. Zero-order spectra of isoflavones in buffer pH 7.4 and liposomes were obtained in the UV range of 200–500 nm at a scan speed of 1200 nm/min, data interval of 1.0 nm, and bandwidth of 2.0 nm. To eliminate residual background signal effects from the dispersed medium, derivative spectra, especially the second derivative, are frequently used (27–29). However, in this work, the best results were obtained using the first-derivative spectra (^1D) obtained by instrumental electronic differentiation (Savitzky–Golay algorithm on ChemStation Software). The changes produced in the first-derivative spectra of isoflavone incorporated at different concentrations of lipids were registered (Equation 1):

$$\frac{[L]}{\Delta D} = \left(\frac{1}{\Delta D_{max}}\right)[L] + \left(\frac{[W]}{K_p \Delta D_{max}}\right) \quad (1)$$

where [L] = molar lipid concentrations; $\Delta D = {}^1D - {}^1D_0$; ^1D = intensity of the first derivative of isoflavone solution in the presence of liposomes; $^1D_0$ = intensity of the first derivative of isoflavone in buffer pH 7.4; ΔD_{max} = extrapolated value of

intensity of the first derivative when 100% of isoflavone is bound to the lipid; and [W] = water concentration (55.5 mol/L).

Determination of Isoflavone Degradation Under Stress Conditions

Preparation of stock standard solutions for the degradation study.—A stock solution of each isoflavone was prepared in methanol to obtain a concentration of 0.1 mg/mL. The solutions were kept at 5°C.

Acid hydrolysis.—For the degradation study under acid conditions, a volume of 5 mL from the stock solution of each isoflavone was mixed with 10 mL of 0.1 N hydrochloric acid (HCl) in a 25 mL conical flask, heated in a water bath adjusted to 80°C for 3 h, cooled, and then neutralized with 0.1 N sodium hydroxide (NaOH). The solution was transferred quantitatively into a 25 mL volumetric flask, mixed well, filtered through a 0.22 μm Millex PVDF syringe filter (Millipore), and injected into the HPLC system using the chromatographic conditions stated.

Alkaline hydrolysis.—For the degradation study under basic conditions, a volume of 5 mL from the stock solution of each isoflavone was mixed with 10 mL of 0.1 N NaOH in a 25 mL conical flask, heated in a water bath adjusted to 80°C for 3 h, cooled, neutralized with 0.1 N HCl, and the procedure continued as described above for acidic hydrolysis.

Neutral hydrolysis.—For the degradation study under neutral conditions, a volume of 5 mL from the stock solution of each isoflavone was mixed with 10 mL water in a 25 mL conical flask, heated in a water bath adjusted to 80°C for 3 h, cooled, transferred quantitatively into a 25 mL volumetric flask, and the procedure continued as described above for acidic hydrolysis.

Photodegradation.—For the photostability study, a volume of 5 mL from the stock solution of each isoflavone was mixed with 10 mL methanol in a 25 mL conical flask, exposed to sunlight for 6 months, transferred quantitatively into a 25 mL volumetric flask, and diluted to volume with the same solvent.

Evaluation of Antioxidant Capacity: Time-Dependent Isoflavone Consumption by Reaction with the Hydroxyl Radical

Homogeneous media.—Aliquots of the stock isoflavone solution were mixed with 2.5 mL of 30% H_2O_2 solution to obtain a final concentration of 0.05 mM (~20 μg/mL) of each isoflavone. The mixture was homogenized in a vortex mixer. The solution was transferred to a 5 mL double-wall cell that was light-protected with black paint, but irradiation allowed through a centered window. Circulating water maintained the cell temperature at 20 ± 0.5°C. The irradiation of H_2O_2 was performed with a high-power mercury (Hg) UV lamp at 254 nm. Finally, the samples at different times were analyzed by HPLC–DAD.

Liposomes.—Aliquots of the isoflavone-loaded liposomes dispersed in 0.1 M phosphate buffer (pH 7.4) were mixed with 2.5 mL of 30% H_2O_2 solution to obtain a final concentration of 0.05 mM (~20 μg/mL). The mixture was homogenized in a vortex mixer. The solution was transferred to a 5 mL double-wall cell that was light-protected by black paint, but irradiation allowed through a centered window. Circulating water maintained the cell temperature at 20 ± 0.5°C. The irradiation of H_2O_2 was performed with a high-power Hg UV lamp at 254 nm. The samples were treated as described above and analyzed by HPLC–DAD.

Results and Discussion

Chromatographic Analysis

Columns from Agilent [Hypersil ODS C18 (4.6 × 250 mm, 5 μm particle size) and Eclipse Plus C8 (4.6 × 150 mm, 5 μm particle size)] and Merck [Chromolith Performance RP-18 monolithic (4.6 × 100 mm) and LiChrospher RP-select B (4.6 × 250 mm, 5 μm particle size)] from Agilent) were tested. Satisfactory separation results were obtained with the Agilent Hypersil ODS C18. Mobile phases of different composition (nature and volume fraction of the organic eluent modifier, effect of pH, and buffer concentration), isocratic or gradient elution at different temperatures (15–45°C), and flow rates (0.5–1.5 mL/min) were also tested. The best results were obtained using a mobile phase consisting of a combination of two solutions: phase A was 0.02% orthophosphoric acid solution–acetonitrile (35 + 65, v/v) and phase B was 0.2% orthophosphoric acid solution. The gradient started with an isocratic elution of mixture 25 + 75 (A + B, v/v) for 10 min, phase A solvent percentage linearly increased from 40 to 60% in 15 min, and, finally, from 20 to 30 min, phase B at 90%. The effect of flow rate on chromatographic resolution was performed in the range of 0.5–2.0 mL/min. Optimal conditions were obtained with 1.5 mL/min using the described gradient elution.

The asymmetry observed for all peaks increased with methanol presence, and the retention times increased when the mobile phase included a buffer with increasing pH from 5 to 7.

The column temperature was set at 20°C because the results obtained showed that temperature did not affect significantly the chromatographic efficiency.

The optimal detection condition was obtained using a PDAD set at 260 nm, corresponding to the average maximum absorption wavelength for the set of compounds analyzed. An inadequate reproducibility of peak area and poor linearity were achieved monitoring at 250 or 270 nm.

Under these conditions, the retention times (*see* Figure 2), for the studied compounds were (A) daidzin (6.5 min), (B) glycitin

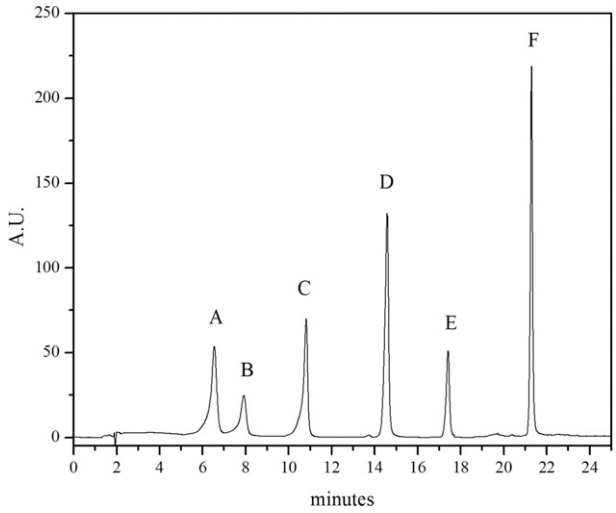

Figure 2. HPLC chromatogram of (A) daidzein, (B) glycitin, (C) genistin, (D) daidzin, (E) genistein, and (F) biochanin A in the methanol solutions.

Table 1. Chromatographic performance data of the method

Compound	Retention time, min	Retention factor, k′	Selectivity, α	Resolution between critical band pair
Daidzin	6.5	2.4	[a]	—[a]
Glycitin	7.9	3.2	1.3	1.8
Genistin	10.8	4.7	1.5	3.7
Daidzein	14.6	6.7	1.4	5.4
Genistein	17.4	8.2	1.2	6.2
Biochanin A	21.3	10.2	1.2	11.1

[a] — = Value not calculated because daidzin was used as the reference compound.

(7.9 min), (C) genistin (10.8 min), (D) daidzein (14.6 min), (E) genistein (17.4 min), and (F) biochanin A (21.3 min). Figure 2 represents the typical HPLC chromatogram obtained with these optimal conditions.

The chromatogram shows well-defined peaks for daidzein, genistein, and biochanin A. However, the peaks corresponding to glycosides (daidzin, glycitin, and genistin) show fronting due to the low affinity of these hydrophilic compounds for the C18 column. Chromatographic performance criteria—retention factor (k′), selectivity (α), and resolution—were calculated and are shown in Table 1. The retention factor (k′) was defined as $(t_R - t_0)/t_0$, where t_R = retention time of peak (min); and t_0 = void time (min). In the present method, the void time was 1.9 min. A retention factor in the range of $0.5 < k' < 20.0$ was desired to clearly separate the first peak from the void time and to avoid a higher retention time for the last compound. Selectivity (α) is the ratio of the retention factors of two peaks. A resolution of 2.0 or higher was desired for critical band pairs; glycosides daidzin and glycitin were the only pairs of compounds that did not meet this criterion.

Linearity and Range

The HPLC method was validated using the following performance criteria: linearity and linear range, sensitivity, intra-assay and interassay precision, accuracy, LOD, and LOQ.

Linearity, linear range, and sensitivity were established through a calibration curve obtained by the triplicate analysis of each isoflavone at nine concentration levels in methanol solutions. Linearity was demonstrated over the concentration range of 5–30 mg/L. The linear equation was obtained by least-squares linear regression analysis of the peak area of analyte standard in arbitrary units (AU) versus concentration (mg/L). The correlation coefficient of the linear standard curve ranged from 0.9991 (daidzin) to 0.9999 (genistein). Table 2 shows the slope ($AU \cdot M^{-1}$) of the calibration curves, representing the sensitivity of the method. Each point corresponds to three replicates.

Precision

The precision of the method was tested by measuring both intraday and interday reproducibility from the analysis of 10.00 mg/L of each isoflavone standard solution in methanol. The assays were performed using 10 solutions, and the solutions were analyzed in triplicate. Results of repeatability on 3 different days (mean, SD, and percentage RSD) are summarized in Table 3. The RSD values range between 0.76 and 1.93%. The criteria for the acceptability of the data included a precision of within ±15% RSD (30).

Accuracy

Accuracy studies were performed to determine the closeness between the true concentration and the experimental results. Samples of each isoflavone-loaded DPPC vesicle were prepared and treated as previously described.

Our experiments showed a recovery in the order of 75% for the isoflavones studied in liposomes. The recovery study was performed at three different concentration levels (0.014, 0.03, and 0.045 mM) of the target concentration. For each level, four preparations were tested (Table 3).

The results showed no significant difference between glycosides and their respective aglycones. For example, a recovery of 76.3% for genistin and 75.2% for genistein in DPPC liposomes were observed. Similar recoveries were obtained for all isoflavones studied.

Selectivity and Stability

Selectivity is the capacity of a method to accurately measure analyte response in the presence of potentially interfering sample components as degradation products or by studying the absence of any interference in the same chromatographic run. In this work, selectivity was evaluated by exposing isoflavone samples to the following stress conditions: acidic, alkaline, and neutral hydrolysis and photodegradation.

The results for acid and neutral hydrolysis showed no significant difference between the chromatogram obtained in the methanol solution and its degradation products. Under these conditions, the chromatograms only evidence the presence of unionized species. The isoflavones studied were stable to light

Table 2. Slopes of the response curve for each isoflavone in the standard solutions for the chromatographic method

Compounds	Sensitivity/$AU \cdot M^{-1}$ mean (RSD, %)
Daidzin	1.45×10^7 (2.8)
Glycitin	2.19×10^7 (1.2)
Genistin	3.72×10^7 (2.7)
Daidzein	2.40×10^7 (3.2)
Genistein	3.43×10^7 (1.5)
Biochanin A	4.12×10^7 (1.8)

Table 3. Summary of precision and accuracy

	Precision			Accuracy		
	Mean ± SD (RSD, %)			Mean (RSD, %)		
Compounds	Day 1	Day 2	Day 3	0.014 mM	0.030 mM	0.045 mM
Daidzin	9.87 ± 0.14 (1.42)	9.73 ± 0.14 (1.44)	9.85 ± 0.17 (1.73)	76.3 (0.67)	78.2 (0.65)	80.0 (0.65)
Glycitin	10.15 ± 0.14 (1.38)	10.11 ± 0.18 (1.78)	10.26 ± 0.11 (1.07)	72.8 (0.69)	72.9 (0.70)	77.1 (0.68)
Genistin	9.93 ± 0.18 (1.81)	10.01 ± 0.17 (1.70)	9.92 ± 0.14 (1.41)	75.0 (0.78)	76.2 (0.75)	76.3 (0.72)
Daidzein	10.27 ± 0.17 (1.66)	10.25 ± 0.13 (1.27)	10.27 ± 0.11 (1.07)	70.3 (1.30)	71.1 (1.17)	71.8 (1.15)
Genistein	10.52 ± 0.08 (0.76)	10.55 ± 0.10 (0.95)	10.55 ± 0.12 (1.14)	69.7 (1.35)	73.5 (1.30)	75.2 (1.25)
Biochanin A	9.85 ± 0.18 (1.83)	9.87 ± 0.19 (1.93)	9.82 ± 0.18 (1.83)	67.5 (1.59)	68.7 (1.47)	69.9 (1.45)

exposure for a period of 6 months. Furthermore, temperature did not influence the stability of the compounds at 20, 30, 40, 50, 60, and 70°C for 30 min each. Previous studies suggest that isoflavones like daidzein genistein, daidzin, glycitin, and genistin might become degraded as temperatures exceed 135°C (31–33).

The chromatogram of the alkaline solutions documented the disappearance of the peak corresponding to the isoflavone and the appearance of several degradation products. This effect was significantly greater in the case of the glycosides studied.

Evaluation of the Distribution of Isoflavones

In this work, the hydrophobicity of isoflavones was determined through the experimental measurement of the partition and/or distribution of these compounds in homogeneous media using an HPLC method and in micro-organized systems (liposomes) by a derivative spectrophotometric method. The results obtained allowed the establishment of a relation between the chemical structure, pKa, lipophilicity, and the characteristics of the dispersion medium.

At different pH values in buffered media (Table 4), the distribution of isoflavones was highly dependent on the position of the hydroxyl groups in the chemical structure and their pKa. In the acid medium (pH 1–5), aglycones (daidzein, genistein, and biochanin A) exhibited a marked hydrophobicity, with logD values greater than 2. However, due to the ionization of their hydroxyl groups, logD values decreased at physiological pH (7.4). Moreover, the effect of pH in the distribution of glycosides was unimportant.

The relationship between the partition or distribution coefficient and the retention factor obtained by HPLC under isocratic elution has been frequently used as a hydrophobicity predictor (34, 35). The retention times under isocratic conditions (100% mobile phase A) for daidzin, glycitin, genistin, daidzein, and genistein were 5.6, 5.9, 10.71, 31.5, and 83.6 min, respectively. Figure 3 shows the high correlation obtained for the plot of $\log k'$ versus logD [*n*-octanol–buffer (pH 3)] for the five isoflavones studied. Biochanin A was dropped from the study because, under these isocratic conditions, it presents a very long retention time (over 500 min).

DPPC liposomes prepared by sonication and dispersed in buffer pH 7.4 had an average size of 150 nm and ζ-potential of +0.9 mV. The hydrophobicity of isoflavones in liposomes was very similar. Polyphenols, such as isoflavones, promote adsorption on the surface of DPPC liposomes by electrostatic interaction of hydroxyl groups of isoflavones with the quaternary ammonium of DPPC. According to the results, the compound with the highest affinity for the liposomes was daidzin, whereas the lowest affinities corresponded to genistein and biochanin A. This can be explained by the fact that cationic DPPC would give higher affinity for compounds with greater capacity for ionization or have as many OH groups as glycosides.

Antioxidant Capacity

Several steady-state experiments allowed us to evaluate the applicability of this analytical method to the study of the kinetics of reaction between the hydroxyl radical and each isoflavone (in methanol and DPPC liposomes). Photolysis of H_2O_2 was used to measure the scavenging of hydroxyl radical by isoflavones (36, 37). The chemical reaction was evaluated following time-dependent isoflavone consumption by HPLC. This kinetics can be adjusted to a pseudo-first-order process according to Equation 2:

$$v = -\frac{d[\text{isoflav}]}{dt} = k \times [HO^{\bullet}][\text{isoflav}] = k_{obs}[\text{isoflav}] \qquad (2)$$

where k = chemical rate constant of the hydroxyl radical with isoflavone; $[HO^{\bullet}]$ = hydroxyl radical steady-state concentration; and k_{obs} = observed or experimental pseudo-first-order rate constant obtained from the steady-state experiments. Under strict control of experimental conditions, such as the concentration of H_2O_2, light intensity, temperature, and geometry of the cell, the hydroxyl radical concentration remained constant.

Table 4. Distribution of the isoflavones in the octanol–buffer and in the DPPC liposomes

	Distribution, logD					
	Octanol–buffer					
Compounds	pH 1	pH 3	pH 5	pH 7.4	pH 9	DPPC liposomes
Daidzin	0.42	0.29	0.28	0.26	0.14	4.89
Glycitin	0.57	0.40	0.33	0.29	0.09	4.77
Genistin	0.98	0.85	0.50	0.34	–0.50	4.56
Daidzein	2.65	2.03	1.95	1.61	0.47	4.40
Genistein	2.48	2.36	2.15	1.28	1.20	3.95
Biochanin A	2.78	2.63	2.38	1.47	0.69	4.08

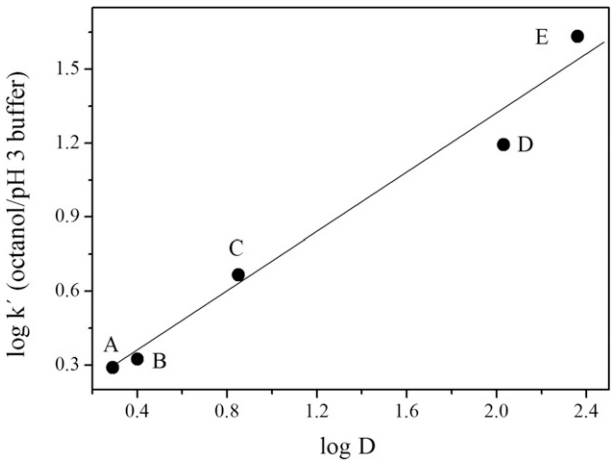

Figure 3. Plot of logk′ versus logD [*n*-octanol–buffer (pH 3)] of (A) daidzin, (B) glycitin, (C) genistin, (D) daidzein, and (E) genistein.

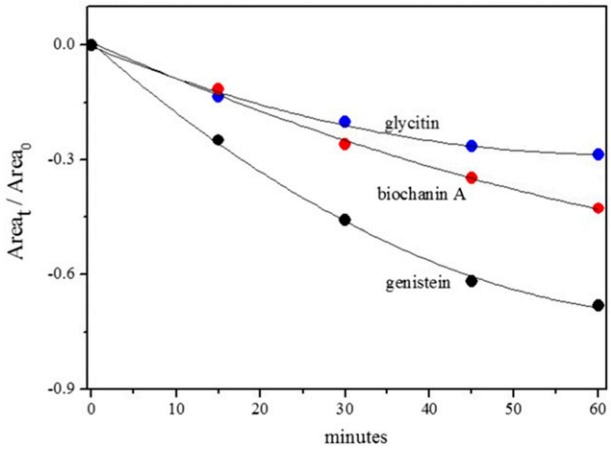

Figure 5. Plots of the ratio of area HPLC versus time for the reaction between glycitin, biochanin A, and genistein and the hydroxyl radical in methanol.

In methanol and DPPC liposomes, all isoflavones showed consumption, which depended on the structure of the isoflavone. A typical HPLC chromatogram for daidzein consumption at different times is given in Figure 4.

The experimental rate constants showed that genistein (k_{obs} of 0.01327 min^{-1}) was the most potent scavenger of the hydroxyl radical (Figure 5). The reactivity of daidzein and its glucoside daidzin was similar (k_{obs} of 0.0484 and 0.0486 min^{-1}, respectively; Figure 5). Thus, the antioxidant capacity was not affected when the hydroxyl group in C7 was replaced by glucose. Daidzein was less efficient than genistein due to the absence of a hydroxyl moiety at C5. The HPLC assay also showed glycitin was the less potent antioxidant (k_{obs} of 0.00471 min^{-1}), indicating that the loss of the hydroxyl group in the C6 position diminished notoriously the antioxidant capacity of the isoflavones. These results are similar to those reported in other studies using fluorescence and spectroscopic techniques (38, 39).

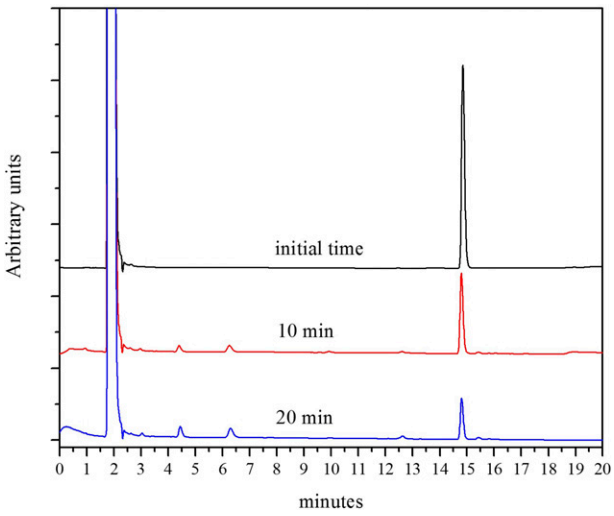

Figure 4. HPLC chromatogram of daidzein consumption in DPPC liposomes at 0, 10, and 20 min.

In DPPC liposomes, the order of reactivity was similar to methanol (genistein > biochanin A > genistin > daidzein > daidzin > glycitin). However, under the same experimental conditions, the consumption of all isoflavones was lower in the liposomes than in the homogeneous media. For example, in DPPC liposomes, the consumption of daidzein and glycitin was 60.2 and 48.4%, but in liposomes, the values decreased to 44.5 and 24.2%, respectively. This could be explained by the greater hydrophobicity of the isoflavones present in lipid media and also the reduction of hydroxyl radical access to isoflavone locations in the membrane.

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