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Interaction of Flavonoids with Bovine Serum Albumin: A Fluorescence Quenching Study

ATHINA PAPADOPOULOU,† REBECCA J. GREEN,‡ AND RICHARD A. FRAZIER*,†

School of Food Biosciences, The University of Reading, P.O. Box 226, Whiteknights, Reading RG6 6AP, United Kingdom, and School of Chemistry, The University of Reading, P.O. Box 224, Whiteknights, Reading RG6 6AD, United Kingdom

The interaction between four flavonoids (catechin, epicatechin, rutin, and quercetin) and bovine serum albumin (BSA) was investigated using tryptophan fluorescence quenching. Quenching constants were determined using the Stern−Volmer equation to provide a measure of the binding affinity between the flavonoids and BSA. The binding affinity was strongest for quercetin and ranked in the order quercetin > rutin > epicatechin = catechin. The pH in the range of 5−7.4 does not affect significantly ($p < 0.05$) the association of rutin, epicatechin, and catechin with BSA, but quercetin exhibited a stronger affinity at pH 7.4 than at lower pH ($p < 0.05$). Quercetin has a total quenching effect on BSA tryptophan fluorescence at a molar ratio of 10:1 and rutin at approximately 25:1. However, epicatechin and catechin did not fully quench tryptophan fluorescence over the concentration range studied. Furthermore, the data suggested that the association between flavonoids and BSA did not change molecular conformation of BSA and that hydrogen bonding, ionic, and hydrophobic interaction are equally important driving forces for protein−flavonoid association.

KEYWORDS: Flavonoid; polyphenol; protein; albumin; tryptophan fluorescence quenching; spectroscopy

INTRODUCTION

Polyphenols are secondary plant metabolites and have received much attention because of their potential health benefits (*1*−*3*). Flavonoids are a major group of polyphenols and their structure is characterized by the diphenylpropane ($C_6C_3C_6$) skeleton. The family includes monomeric flavanoids, flavanones, anthocyanidins, flavones, and flavonols, and these compounds are found in almost every plant (*1*). Consumption of plants and plant products that are rich in flavonoids, such as cocoa, wine, tea, and berries, has been related with protective effects against cardiovascular disease and certain forms of cancer (*3*−*5*). They have been found to act as free-radical scavengers and have been widely studied for their antioxidant activity in vitro (*6*−*8*). However, the reality about their in vivo activity still remains uncertain, and questions concerning their absorption, metabolism, and bioavailability are still unanswered (*9*−*14*). Current knowledge suggests that factors such as protein binding may impair polyphenol absorption and bioavailability and even mask their antioxidant activity (*15*, *16*). Protein−polyphenol association is a well-known phenomenon; however, it is only relatively recently that any considerable information has been obtained in the area of how the structure of either the protein or the polyphenol may affect the interaction (*17*−*21*).

This paper investigates the association of flavonoids with bovine serum albumin (BSA); serum albumins are the major soluble protein constituents of the circulatory system and have many physiological functions (*22*). The most important property of this group of proteins is that they serve as a depot protein and as a transport protein for a variety of compounds. BSA has been one of the most extensively studied of this group of proteins, particularly because of its structural homology with human serum albumin (HSA). The BSA molecule is made up of three homologous domains (I, II, III) which are divided into nine loops (L1−L9) by 17 disulfide bonds. The loops in each domain are made up of a sequence of large−small−large loops forming a triplet. Each domain in turn is the product of two subdomains (IA, IB, etc.). X-ray crystallographic data (*23*) show that the albumin structure is predominantly α-helical (67%) with the remaining polypeptide occurring in turns and extended or flexible regions between subdomains with no β-sheets. BSA has two tryptophan residues that possess intrinsic fluorescence (*24*): Trp-134 in the first domain and Trp-212 in the second domain. Trp-212 is located within a hydrophobic binding pocket of the protein and Trp-134 is located on the surface of the molecule. Therefore, fluorescence quenching can be considered as a technique for measuring binding affinities.

Fluorescence quenching is the decrease of the quantum yield of fluorescence from a fluorophore induced by a variety of molecular interactions with quencher molecule. Fluorescence quenching can be dynamic, resulting from collisional encounters between the fluorophore and quencher, or static, resulting from the formation of a ground-state complex between the fluorophore and quencher (*25*). In both cases, molecular contact is required between the fluorophore and the quencher for fluorescence

* To whom correspondence should be addressed. Fax: +44 118 931 0080; telephone: +44 118 378 8709; e-mail: r.a.frazier@reading.ac.uk.
† School of Food Biosciences.
‡ School of Chemistry.

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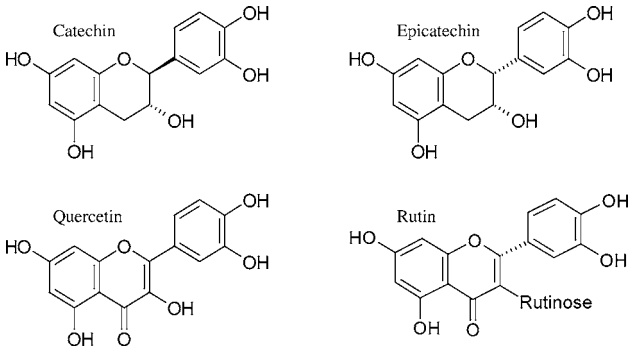

Figure 1. Flavonoid structures.

quenching to occur. Application of the fluorescence quenching technique can also reveal the accessibility of the fluorophores to quenchers.

There have been several studies on fluorescence quenching of proteins induced by drugs or other small molecules (*26*−*34*). In this paper, the quenching of the intrinsic tryptophan fluorescence of BSA has been used as a tool to study the interaction of four different flavonoids (catechin, epicatechin, rutin, and quercetin) with this transport protein in an attempt to characterize the chemical associations taking place.

MATERIALS AND METHODS

Reagents. BSA (≥99%), (+)-catechin (≥98%), (−)-epicatechin (≥98%), and dimethyl sulfoxide (DMSO) were purchased from Sigma (Poole, Dorset, U.K.). (+)-Rutin (quercetin-3-rutinoside, ≥97%) and quercetin (3,3′,4′,5,7-pentahydroxyflavone hydrate, ≥98%) were purchased from Lancaster Synthesis (Morecambe, Lancashire, U.K.). The chemical structures of the flavonoids are depicted in **Figure 1**. The buffers used were 0.1 M phosphate buffer at pH 6.8 and 7.4 and 0.05 M citrate buffer at pH 5.0 and 6.0. DMSO was used at a concentration of 10% v/v to solubilize the flavonoids.

Fluorescence Quenching Measurements. The fluorescence intensities were recorded with a Perkin-Elmer LS5 Luminescence Phosphorescence Spectrophotometer, using 2.5-nm excitation and 5-nm emission slit widths. To determine the linear concentration range for protein fluorescence, a series of BSA solutions with increasing concentrations (0−25 μM) were prepared in buffer. The maximum excitation wavelength (λ_{ex}) and maximum emission wavelength (λ_{em}) for BSA were 282 and 348 nm, respectively. The linear range of BSA fluorescence was between 0 and 5 μM. Therefore, 1.5 μM BSA was chosen as the concentration for fluorescence quenching experiments.

A dilution series of flavonoid solutions (0.01−0.5 mM) were prepared in a buffer:DMSO (9:1) solvent. For each data point, 0.25 mL of the appropriate flavonoid solution was added into 3 mL BSA solution, to give a final flavonoid concentration in the range 0.001−0.04 mM. The change in fluorescence emission intensity was measured within 1 min of adding flavonoid to the BSA. The addition of a constant volume of quencher to the protein solution avoided complications due to dilution effects within titration type experiments. Each measurement was repeated in triplicate and the mean and standard deviation were calculated. The fluorescence quenching data were plotted as relative fluorescence intensity (RFI = $F/F_0 \times 100$) against flavonoid concentration. For the calculation of quenching constants, the data were plotted as a Stern−Volmer plot of F_0/F against [Q] and the quenching constant was calculated by linear regression.

Catechin and epicatechin possess intrinsic fluorescence at the λ_{ex} of BSA (282 nm) and both display a corresponding λ_{em} at 318 nm. To eliminate the background effect on BSA fluorescence quenching values, the fluorescence emission intensities of both catechin and epicatechin were measured at 348 nm as a blank titration series (i.e., adding 0.25 mL of each flavonoid concentration into 3 mL buffer). The fluorescence values obtained were then subtracted from the fluorescence intensity values obtained for BSA quenching.

The emission spectra of BSA were also recorded, to allow observation of any changes in the BSA spectra because of the addition of the flavonoids at the same concentrations. For these experiments, a Perkin-Elmer LS50B Luminescence Spectrophotometer was used to determine emission spectra at an excitation wavelength of 282 nm.

To evaluate the quenching effect of DMSO, the effect of BSA dilution by buffer titration was evaluated and was compared to the effect of dilution with DMSO. It was observed that DMSO had the same affect on BSA fluorescence as the buffer dilution effect (data not shown). Thus, the effect of DMSO on the flavonoid interaction with BSA can be considered negligible in the amount used. Furthermore, no effect was observed on the BSA fluorescence emission spectrum with similar addition of DMSO (data not shown), which suggests no change in BSA conformation.

Principles of Fluorescence Quenching. Fluorescence quenching is described by the Stern−Volmer equation

$$\frac{F_0}{F} = 1 + k_q\tau_0[Q] = 1 + K_{SV}[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities before and after the addition of the quencher, respectively, k_q is the bimolecular quenching constant, τ_0 is the lifetime of the fluorophore in the absence of quencher, [Q] is the concentration of the quencher, and K_{SV} is the Stern−Volmer quenching constant. Hence, eq 1 was applied to determine K_{SV} by linear regression of a plot of F_0/F against [Q].

A linear Stern−Volmer plot is generally indicative of a single class of fluorophores, all equally accessible to the quencher. In many cases, the fluorophore can be quenched both by collision and by complex formation with the same quencher. When this is the case, the Stern−Volmer plot exhibits an upward curvature, concave toward the y-axis at high [Q], and F/F_0 is related to [Q] by the following modified form of the Stern−Volmer equation

$$\frac{F_0}{F} = (1 + K_D[Q])(1 + K_S[Q]) \quad (2)$$

where K_D and K_S are, respectively, the dynamic and static quenching constants. This modified form of the Stern−Volmer equation is second-order with respect to [Q], which accounts for the upward curvature observed at high [Q] when both static and dynamic quenching occur. There are further cases when quenching can occur because of specific binding interactions and in such cases the quenching appears to arise from static quenching. In these cases, the bimolecular quenching constant is calculated and compared to the maximum value possible for diffusion-limited quenching in water ($\sim 10^{10}$ M^{-1} s^{-1}). There have been several studies reporting BSA quenching due to specific interaction (*26*−*28*), and in these cases the quenching constant has been several magnitudes higher than the maximum value of diffusion-limited quenching in water.

Fourier Transform Infrared (FTIR) Spectroscopy. FTIR spectra were obtained using a ThermoNicolet Nexus FTIR Spectophotometer (Madison, WI). Samples were prepared using a deuterated buffer to prevent interference by water in the amide region of the protein spectra. The spectrometer was continuously purged with dry air to remove excess water vapor. The samples were placed in a liquid cell between two windows (CaF_2) separated with Mylar spacers (0.6 μm). The BSA concentration for FTIR spectroscopy was 0.25 mM. Spectra were obtained by adding 5 mM rutin or 10 mM epicatechin. FTIR spectra showed no effect on the BSA spectrum with the addition of DMSO.

Statistical Analysis. Statistically significant differences between quenching constants at different pH and for different flavonoids were determined using a two-way ANOVA with 95% confidence limits ($p < 0.05$).

RESULTS

Fluorescence Quenching. Figure 2 shows the raw data for quenching of BSA fluorescence at pH 7.4 by addition of rutin, quercetin, epicatechin, and catechin, respectively, plotted as RFI against flavonoid concentration. It was observed that titration

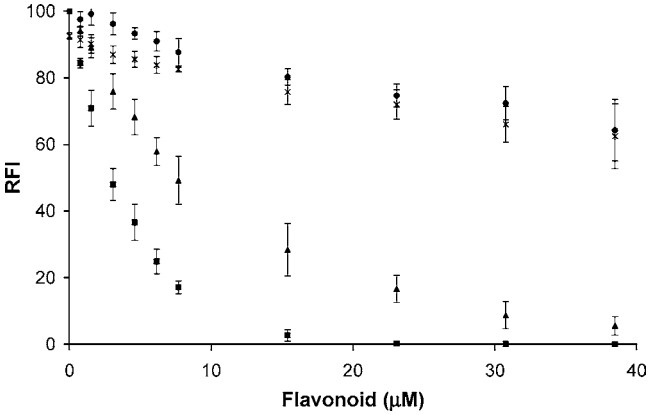

Figure 2. Tryptophan fluorescence quenching of BSA at pH 7.4, plotted as RFI ($F_0/F \times 100$) against flavonoid concentration for × catechin, ◆ epicatechin, ▲ rutin, ■ quercetin. Fluorescence emission intensity was recorded at $\lambda_{ex} = 282$ nm and $\lambda_{em} = 348$ nm. Data for epicatechin and catechin have been corrected for quencher fluorescence.

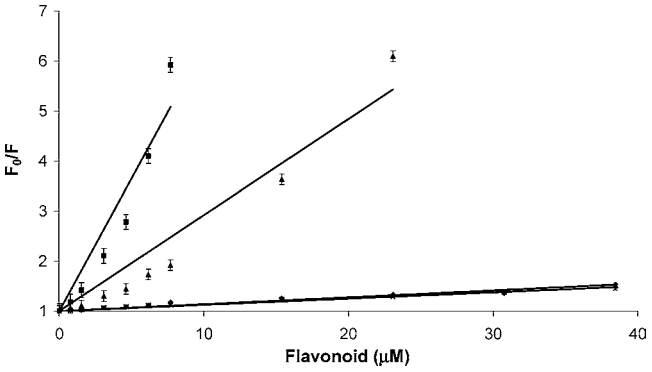

Figure 3. Stern–Volmer plot describing BSA tryptophan quenching at pH 7.4 caused by flavonoid association. × Catechin: $y = 12.5x + 1$, $R^2 = 0.97$; ◆ epicatechin: $y = 13.8x + 1$, $R^2 = 0.95$; ▲ rutin: $y = 192x + 1$, $R^2 = 0.94$; ■ quercetin: $y = 531x + 1$, $R^2 = 0.92$.

Table 1. Stern–Volmer Quenching Constants, K_{SV} ($\times 10^3$ M^{-1}), for the Interaction of Flavonoids with BSA at Varying pH[a]

flavonoid	pH 7.4	pH 6.8	pH 6.0	pH 5.0
quercetin	530 ± 72a	323 ± 9b	291 ± 3c	282 ± 20bc
rutin	192 ± 6a	166 ± 27a	153 ± 38a	198 ± 22a
epicatechin	13.8 ± 1.9a	17.3 ± 1.8a	12.1 ± 0.8a	13.3 ± 1.7a
catechin	12.5 ± 2.8ab	21.0 ± 1.8b	14.2 ± 1.0a	16.5 ± 3.4ab

[a] Different letters within rows denote significant differences ($p < 0.05$).

with both quercetin and rutin eventually leads to total quenching and that total quenching was achieved at a lower molar ratio of quercetin:BSA (10:1) than in the case of rutin (~25:1). Both epicatechin and catechin had a similar effect on BSA fluorescence; the RFI of BSA decreased slowly and almost linearly (**Figure 2**) but did not reach zero over the range of catechin/epicatechin concentrations studied.

The calculation of K_{SV} from Stern−Volmer plots (**Figure 3**) demonstrated that varying pH did not have a significant effect on fluorescence quenching by rutin, epicatechin, and catechin but did for quercetin between pH 6.8 and 7.4 ($p < 0.05$). **Table 1** summarizes the calculated K_{SV} for each flavonoid at each pH studied. As expected, quercetin had the highest quenching constant and the constants for catechin and epicatechin (which are isomers) were very similar (see **Figure 1**). Indeed, there was no statistically significant difference between the quenching

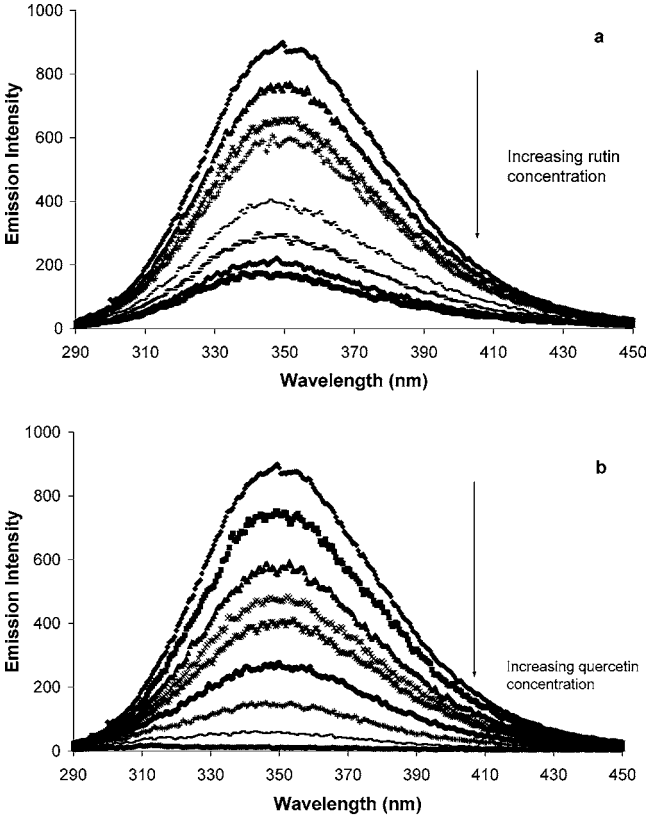

Figure 4. Emission spectra of 1.5 μM BSA at $\lambda_{ex} = 282$ nm showing the quenching effect of increasing concentrations of (**a**) rutin (1.5, 5, 8, 15, 23, 31, 38 μM) and (**b**) quercetin (0.8, 1.5, 3, 5, 6, 8, 15, 31 μM). Spectra were recorded at pH 7.4.

constants for catechin and epicatechin at any pH (**Table 1**, $p < 0.05$).

Effect of Flavonoids on BSA Spectra. Figure 4 shows the fluorescence emission spectra (at $\lambda_{ex} = 282$ nm) obtained for BSA at pH 7.4 with the addition of rutin and quercetin as quenchers. It was observed that a decrease in the fluorescence intensity was caused by quenching but that there was no significant λ_{em} shift with the addition of rutin and quercetin. The same trends were observed at other pH values studied.

In the titration with catechin and epicatechin (**Figure 5a**), there was a small λ_{em} shift to lower wavelengths (a blue shift from 348 to 320 nm) with increasing flavonoid concentration and an overall increase in fluorescence intensity was also observed. This was because both catechin and epicatechin are fluorescent at the λ_{ex} for BSA (282 nm). This was corrected by subtracting the spectra for epicatechin or catechin from quenching spectra, as described earlier, to yield the corrected data shown in **Figure 5b**, which displayed no significant λ_{em} shifts. To ascertain the validity of correcting the spectra in this way, spectra obtained by summing the individual spectra of epicatechin or catechin and BSA were compared with those obtained by subtracting epicatechin or catechin spectra from experimental BSA/epicatechin spectra. **Figure 5c** shows this comparison for epicatechin. When the epicatechin spectrum was added to the spectrum obtained for BSA, the resultant sum spectrum (**Figure 5c**, C) was blue shifted as observed for the corresponding BSA/epicatechin spectrum (**Figure 5c**, D). This suggests that the shift was an artifact introduced by the intrinsic fluorescence of epicatechin and was not due to changes in the structural conformation of BSA. Indeed, when the epicatechin spectrum was subtracted from the BSA/epicatechin spectrum, the resultant

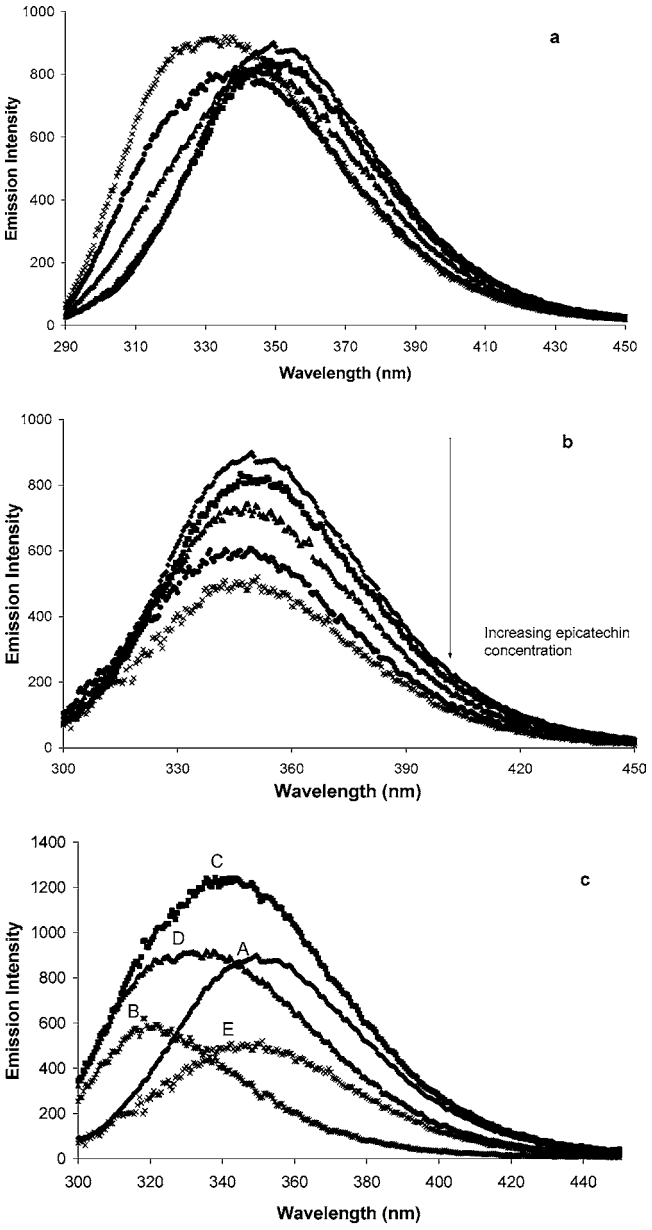

Figure 5. (**a**) Raw BSA emission spectra (λ_{ex} = 282 nm) obtained in the presence of increasing concentrations of epicatechin (0, 1.5, 8, 15, 31 μM) into 1.5 μM BSA. (**b**) Corrected BSA emission spectra with the subtraction of epicatechin emission spectra at the appropriate concentration and same λ_{ex}. (**c**) Manipulation of emission spectra: A, 1.5 μM BSA; B, 31 μM epicatechin; C, sum of 1.5 μM BSA and 31 μM epicatechin spectra (A+B); D, 1.5 μM BSA/31 μM epicatechin as recorded experimentally; E, subtraction of 31 μM epicatechin spectrum from BSA/epicatechin spectrum (D–B) to compensate for epicatechin fluorescence.

corrected spectrum no longer exhibited any significant blue shift (**Figure 5c**, E). In addition, the overall emission intensity was higher when two individual spectra were added (**Figure 5c**, C) than for the spectra obtained for BSA/epicatechin mixtures (**Figure 5c**, D). This suggests that quenching did occur.

To investigate further whether any structural change of BSA was associated with the interaction with flavonoids, FTIR spectra of BSA (0.25 mM) were recorded with and without the addition of flavonoid. No changes were observed in the spectra of the amide I and N−H residual amide II bands, which were present at 1653 and 1540 cm^{-1}, respectively (data not shown). This revealed that no changes to the secondary and tertiary structure of BSA had occurred because of the interaction with the flavonoids.

DISCUSSION

To interpret the data from fluorescence quenching studies, it is important to understand what kind of interaction takes place between the fluorophore and the quencher. As discussed earlier, the fluorescence quenching mechanism usually involves static or dynamic quenching. In this case, both dynamic and static quenching was involved, which was demonstrated by the fact that the Stern−Volmer plots deviated from linearity toward the *y*-axis at high flavonoid concentrations (**Figure 3**). Quenching can be caused also by the formation of a complex between the two compounds that does not fluoresce after returning from the exited state and that is due to a specific interaction (*27*). To verify that quenching is due to a specific interaction, it is essential to calculate the apparent bimolecular quenching constant. For BSA, the lifetime of the fluorophore is approximately 5 ns (*25*). The average quenching constant for epicatechin (having the lowest quenching effect) is 13.8×10^3 M^{-1}, thus $k_q^{app} = 2.76 \times 10^{12}$ M^{-1} s^{-1}. This value is 100-fold higher than the maximum value possible for diffusion-limited quenching in water ($\sim 10^{10}$ M^{-1} s^{-1}), which suggests that there was a specific interaction occurring between BSA and the flavonoids studied here.

BSA contains two tryptophan residues with intrinsic fluorescence; Trp-212 is located within a hydrophobic pocket of the protein and Trp-134 is located on the surface of the molecule. Furthermore, these residues are located in separate domains within the BSA molecule. This is significant because the quenching of tryptophan fluorescence does not show a preferential site of interaction, that is, the Stern−Volmer plot does not show deviation from linearity toward the *x*-axis at high flavonoid concentrations (*25*). On the contrary, after the linear part, the Stern−Volmer plots show deviation toward the *y*-axis at high flavonoid concentrations, which is an indication that both static and dynamic quenching occur together with complex formation as discussed earlier (*25*).

The observation that there was interaction occurring between BSA and flavonoids and that both tryptophan residues were quenched with similar affinities suggests two simultaneous modes of interaction: (i) the flavonoid molecules bind within the hydrophobic pockets of BSA and (ii) they surround the protein molecule. The latter mode of association agrees with earlier results where isothermal titration calorimetry was employed for the investigation of protein−tannin interactions (*19*). When considering the effect of flavonoids on the fluorescence spectra of BSA, there was no apparent λ_{em} shift. This suggests no other change in the immediate environment of the tryptophan residues except the fact that the flavonoids are situated at close proximity to the tryptophan residue for the quenching effect to occur. This means that the molecular conformation of the protein was not affected whatever the flavonoid mechanism of interaction.

The observation that the protein conformation was not affected with the addition of flavonoid was also demonstrated by FTIR spectroscopy. If there had been a change of BSA conformation, a shift in the peak of amide I band or disappearance of the peak corresponding to the N−H residual amide II band would have been observed, neither of which occurred. Johansson (*28*), who studied binding of the anaesthetic chloroform to BSA, observed that the ability of chloroform to quench intrinsic fluorescence was decreased markedly when loss of tertiary structural contacts in BSA was induced chemically. This

indicated that retention of protein conformation was crucial for ligand binding, which may also be the case for the flavonoids studied here. Another study on anaesthetic binding to HSA showed that occupancy of the tryptophan sites by the interacting ligands actually stabilized the native conformation of HSA, and it was suggested that the polarity of the ligand was an important driving force for the interaction (*30*). Previous studies on protein−polyphenol interactions (*35*−*37*) suggest that proteins form complexes with polyphenols that are stabilized by hydrophobic association and hydrogen bonding. Simon et al. (*38*) studied wine tannin−saliva protein complexes and observed that tannins complexed tightly to the peptide without modifying its secondary structure, which agrees with the results reported here.

The structure of the flavonoids appears to have some effect on the quenching process. Quercetin, as an aglycon, is more hydrophobic and demonstrates stronger affinity toward BSA compared to rutin at all pH ($p < 0.05$). Rutin is a glucoside of quercetin, incorporating the disaccharide rutinose. The presence of rutinose renders rutin less hydrophobic than quercetin and also more bulky. This latter fact may result in steric hindrance of its penetration of the hydrophobic pocket of Trp-212 and may generally affect the orientation of the rutin molecules in relation to the tryptophan residues. The difference of quercetin/rutin to epicatechin/catechin is the presence of a ketone functional group in the former two. This difference appears to have a significant effect on their binding affinity for BSA.

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