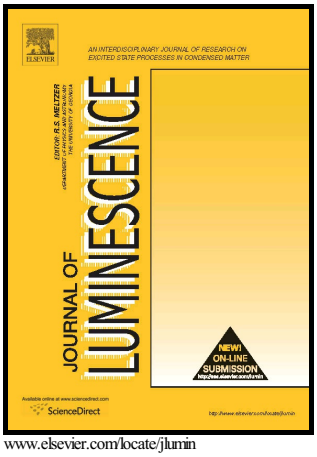

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Fluorescence spectroscopic evaluation of the interactions of quercetin, isorhamnetin, and quercetin-3'-sulfate with different albumins

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Abstract

Quercetin is one of the most commonly occurring flavonoids in nature. Although, quercetin and its metabolites express negligible fluorescence, the albumin-bound form of quercetin has a strong fluorescence property. Considering the structural variance of different albumins, we hypothesized that the fluorescence of albumin complexes of quercetin and its metabolites may vary significantly. Therefore, in this study the fluorescence enhancement of quercetin and some of its major metabolites in the presence of bovine (BSA), human (HSA), porcine (PSA), and rat serum albumins (RSA) were investigated by steady-state fluorescence spectroscopy in PBS buffer (pH 7.4). Among the tested quercetin metabolites, significant fluorescence signal was shown by albumin complexes of quercetin, isorhamnetin, and quercetin-3'-sulfate, while other metabolites (tamarixetin, quercetin-3-glucuronide, and isorhamnetin-3-glucuronide) expressed negligible fluorescence. BSA was the most potent enhancer of quercetin-3'-sulfate but it showed poor effects regarding other flavonoids. The strongest enhancement of isorhamnetin was caused by HSA, while it was less effective enhancer of quercetin and quercetin-3'-sulfate. PSA showed a strong fluorescence enhancement of quercetin and quercetin-3'-sulfate but it was poorly effective regarding isorhamnetin. RSA was the most potent enhancer of quercetin but it caused only a weak enhancement of isorhamnetin and quercetin-3'-sulfate. Large changes of the pH (such as pH 5.0 and pH 10.0) almost completely abolished the fluorescence signals of the complexes. Nevertheless, slight decrease (pH 7.0) reduced and slight increase (pH 7.8) generally enhanced the fluorescence of flavonoid-albumin complexes (only exceptions were quercetin-PSA and quercetin-RSA). Complex formations were also investigated by fluorescence quenching studies. Based on our results, the formations of quercetin-BSA, quercetin-HSA, isorhamnetin-BSA, isorhamnetin-HSA, isorhamnetin-PSA, and quercetin-3'-sulfate – HSA complexes followed 1:1 stoichiometry, while the presence of a secondary binding site of flavonoids was assumed regarding other

tested albumin complexes. Our study highlights that albumins can induce significantly different fluorescence enhancement of flavonoids, and even the stoichiometry of flavonoid-albumin complexes may differ.

Keywords: quercetin, isorhamnetin, quercetin-3'-sulfate, albumin, fluorescence spectroscopy

1. Introduction

Flavonoids are ubiquitous polyphenolic compounds in the plant kingdom. They appear in several fruits and vegetables, in numerous dietary supplements, herbal medicines, and in some medications [1,2]. One of the most frequently occurring flavonoids is quercetin which is an extensively studied molecule because of its complex mechanism of action and manifold pharmacological effects in the human organism [3-5]. Beyond that quercetin is part of the diet, many commercially available dietary supplements contain extremely high doses of quercetin [6]. After its oral consumption or administration, quercetin undergoes significant presystemic elimination [7-10]. During its biotransformation, sulfate and glucuronide conjugates as well as methylated metabolites of quercetin are formed [11,12]. In the human circulation, the parent compound quercetin represents only a few percent of the total quercetin, while quercetin-3'-sulfate (Q3'S) is the dominant circulating metabolite [12]. Furthermore, 3'- and 4'-methylation of quercetin by catechol-O-methyltransferase (COMT) enzyme result in the formation of isorhamnetin (IR; 3'-*O*-methylquercetin) and tamarixetin (4'-*O*-methylquercetin), respectively [8,13].

Albumin is the most abundant plasma protein in humans and animals. Albumin maintains the oncotic pressure of the blood, and it is able to form stable complexes with several compounds (including fatty acids, hormones, amino acids, bile acids, metals, drugs, natural compounds, and xenobiotics) which can strongly affect the distribution and elimination of these ligand molecules [14,15]. Therefore, albumin can significantly influence the pharmacokinetic properties of these substances [16-18]. Previous studies demonstrated that quercetin forms stable complexes with human serum albumin (HSA) and bovine serum albumin (BSA), occupying Sudlow's Site I (on subdomain IIA, which is the primary binding site of warfarin as well) [19-25]. However, a recent study suggest that flavonoid aglycones do not bind at warfarin high affinity site, but rather to different regions within the IIA subdomain of HSA

[26]. Investigation of quercetin-albumin interaction is an important issue because the complex formation may have both therapeutic advantages and unpleasant consequences. Based on previous studies, albumin nanoparticles can promote the stability of quercetin in the intestinal fluid [27], and albumin improves the positive effect of quercetin on survival of erythrocytes in visceral leishmaniasis [28]. On the other hand, the competitive interaction of quercetin with other Sudlow's Site I ligands can result in the displacement of drugs from albumin which may responsible for the disruption of drug therapy [24,29-32]. In addition, other investigations also highlighted the strong interaction of quercetin metabolites with HSA [6,33,34] which is very important because of the extensive biotransformation of quercetin in the body [4,7,12].

Albumin-ligand interactions are commonly investigated by fluorescence spectroscopy due to the quenching of the tryptophan residue(s) of albumins can be precisely followed with this technique. Besides quenching studies, some of the fluorescence investigations of quercetin-albumin interactions highlighted that the non-fluorescent (or poorly fluorescent) quercetin shows strong fluorescence in its albumin-bound form [20-22]. Despite their similarities, different albumin species show large variance regarding their interaction with ligand molecules [35,36]. Therefore, it is plausible to hypothesize that different albumins may cause significantly different fluorescence enhancement of quercetin or its metabolites.

In this study, the influence of BSA, HSA, porcine serum albumin (PSA), and rat serum albumin (RSA) on the fluorescence properties of quercetin and its metabolites (including IR, Q3'S, tamarixetin, quercetin-3-glucuronide, and isorhamnetin-3-glucuronide) was investigated. Since among the tested compounds, only quercetin, IR, and Q3'S (Fig. 1) gave significant fluorescence signals in the presence of albumins, complex formation of these flavonoids with the four different albumins were further investigated. During the high quercetin intake (through the consumption of dietary supplements and herbal medications), high concentrations of quercetin and its metabolites can reach the circulation. Therefore, the

deeper understanding of the flavonoid-albumin complex formations may give us important and pharmacologically relevant information. Furthermore, extrapolation of animal experiments to humans is commonly a controversial issue; therefore, similarities or differences between the binding abilities of flavonoid molecules to human and animal albumins are also important. In addition, the fluorescence enhancement of quercetin, IR, and Q3'S by albumins makes possible the fluorescence spectroscopic detection and investigation of these poorly fluorescent molecules.

2. Materials and methods

2.1. Reagents

All applied chemicals were of spectroscopic or analytical grade. Quercetin dihydrate (Q; product code: 1135S, purity: ⩾99% based on HPLC), isorhamnetin (IR; product code: 1120S, purity: ≥99%, HPLC), and tamarixetin (product code: 1140S, purity: ≥99%, HPLC) were from Extrasynthese. Human serum albumin (HSA; product code: A1653, purity: ≥96%, agarose gel electrophoresis), bovine serum albumin (BSA; product code: A2153, purity: ≥98%, agarose gel electrophoresis), porcine serum albumin (PSA; product code: A1830, purity: ≥98%, agarose gel electrophoresis), and rat serum albumin (RSA; product code: A6272, purity: ≥96%, agarose gel electrophoresis) were purchased from Sigma-Aldrich. Quercetin-3'-sulfate (Q3'S), quercetin-3-glucuronide, and isorhamnetin-3-glucuronide (purity of each conjugate: >95%, HPLC) were synthetized as described elsewhere [37]. 2000 μM stock solutions of flavonoids were prepared in spectroscopic grade dimethyl sulfoxide (Fluka); thereafter these solutions were diluted (in more steps) by the appropriate buffers (for example PBS) in order to produce the samples. Most of the measurements were carried out in PBS buffer (phosphate buffered saline, pH 7.4) in order to mimic extracellular physiological environment, because this buffer contains very similar concentrations of sodium, potassium,

and chloride ions which are typical of the extracellular space (including blood). To examine the influence of pH on the fluorescence enhancement of flavonoids by albumins, 0.05 M sodium acetate (pH 5.0), 0.05 M sodium-borate (pH 10.0), and PBS (pH 7.0 and 7.8) buffers were applied as well.

2.2. Spectroscopic measurements

Fluorescence spectra were recorded employing a Hitachi F-4500 fluorescence spectrophotometer (Tokyo, Japan). To test the influence of different albumins on the fluorescence signal of flavonoids, increasing albumin concentrations (0, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0, and 5.0 μM) were added to 2 μM flavonoid. Then fluorescence emission spectra were recorded using 445 nm (Q3'S) or 455 nm (quercetin and IR) as excitation wavelengths.
In order to investigate the complex formations of flavonoids with albumins, fluorescence quenching method was applied. During these experiments, increasing flavonoid concentrations (0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, and 6.0 μM) were added to 2 μM albumin. Fluorescence emission spectra of albumins were recorded using 295 nm as excitation wavelength. Quenching experiments were evaluated based on the graphical application of the Stern-Volmer equation:

$$\frac{I_0}{I} = 1 + K_{SV} * [Q] \qquad (1)$$

where I_0 and I are fluorescence emission intensities of albumin with and without flavonoids, respectively. K_{SV} is the Stern-Volmer quenching constant while *[Q]* is the concentration of the flavonoid. Thereafter, based on the quenching data, binding constants (*K*) were quantified using the graphical application of double logarithm Stern-Volmer equation as well [38,39]:

$$\lg \frac{(I_0 - I)}{I} = \lg K + n * \lg[Q] \quad (2)$$

where *n* is the number of binding sites on albumin.

Overall and stepwise binding constants were calculated by non-linear fitting the fluorescence emission data obtained for all the performed experiments (quenching of the albumin's fluorescence by flavonoids, and fluorescence enhancement of flavonoids by albumins) with the Hyperquad2006 program package [40,41]. To calculate the stability constants associated with the complex formation between albumins (*A*) and flavonoids (*F*), the following equations are implemented in the Hyperquad code:

$$pA + qF \leftrightarrow A_pF_q \qquad (3)$$

$$K_{pq} = \frac{[A_pF_q]}{[A]^p[F]^q} \qquad (4)$$

where *p* and *q* are the coefficients that indicate the stoichiometry associated with the possible equilibria in the solution. In Hyperquad2006 computer fitting program all equilibrium constants are defined as overall association/binding constants.

$$A + F \leftrightarrow AF \qquad \beta_1 = \frac{[AF]}{[A][F]} \qquad (5)$$

$$A + 2F \leftrightarrow AF_2 \qquad \beta_2 = \frac{[AF_2]}{[A][F]^2} \qquad (6)$$

$$A + qF \leftrightarrow AF_q \qquad \beta_q = \frac{[AF_q]}{[A][F]^q} \qquad (7)$$

The relationship between the overall association/binding constants and the stepwise association/binding constants calculated by the Hyperquad is the following.

$$\beta_1 = K_1; \beta_2 = K_1 \times K_2; \beta_q = K_1 \times K_2 ... \times K_q \qquad (8)$$

The stoichiometry and association/binding constants of the complexes were determined by the model associated with the lowest standard deviation.

3. Results and discussion

3.1. Fluorescence spectra of quercetin and quercetin metabolites in the presence of different albumins

First, the fluorescence excitation and emission spectra of 2 μM concentrations of quercetin and its metabolites – including isorhamnetin, quercertin-3'-sulfate, quercetin-3-glucuronide, isorhamnetin-3-glucuronide, and tamarixetin – were recorded in PBS buffer. Under the applied circumstances, the tested flavonoids showed no considerable fluorescence signals. Thereafter, increasing concentrations of BSA, HSA, PSA, and RSA (0-5 μM) were added to the standard 2 μM flavonoid concentrations. The presence of elevating albumin levels resulted in the significant fluorescence enhancement of quercetin, IR, and Q3'S (Fig. 2, Fig. 3, and Fig. S1). Albumins themselves did not show fluorescence signal under the applied conditions. We observed negligible fluorescence of quercetin-3-glucuronide, isorhamnetin-3-glucuronide, and tamarixetin, even in the presence of 5 μM albumin concentrations, regardless the albumin applied (data not shown). In our previous study, we demonstrated that tamarixetin binds with slightly lower (log$K$ = 5.19) while quercetin-3-glucuronide and isorhamnetin-3-glucuronide bind with much lower (log$K$ values were 4.73 and 4.55, respectively) affinities to HSA compared to the parent compound quercetin (log$K$ = 5.37) [6]. Despite their significant interaction with albumin, glucuronide metabolites and tamarixetin did not show strong fluorescence signal in their albumin-bound form.

In the presence of albumins, quercetin and IR showed fluorescence excitation maximum at 455 nm, while Q3'S expressed its maximal excitation intensity at 445 nm (quercetin and IR: λ_{em} = 525 nm; Q3'S: λ_{em} = 505 nm). Using these excitation wavelengths, emission spectra of flavonoid-albumin complexes were recorded (Fig. 3). Interestingly, the presence of different albumins led to quite different fluorescence emission intensities (Fig. 2). Regarding quercetin, complexation with BSA caused the weakest while complex formation with RSA resulted in the strongest fluorescence, and significant differences were observed in the presence of each

tested albumin (Fig. 2, left). Similar fluorescence signals of IR-albumin complexes were observed in the presence of BSA, PSA, and RSA; however, HSA showed much stronger effect compared to the other tested albumins (Fig. 2, middle). Furthermore, very strong increase of the fluorescence signal of Q3'S was observed in the presence of BSA, while HSA, PSA, and RSA had weaker effects showing distinct fluorescence intensities (Fig. 2, right). Q3'S-BSA complex showed the highest fluorescence among each tested flavonoid-albumin combination, strongly exceeding the fluorescence signals of both quercetin-RSA and IR-HSA complexes. Our results are consistent with previous studies which describes that quercetin in HSA-bound form expresses significant fluorescence at 525 nm [20-22]; however, the fluorescence of other quercetin-albumin complexes as well as the fluorescence enhancement of IR and Q3'S by albumins were not reported previously. Therefore, our results offer a good starting point to quantify quercetin, IR, and Q3'S (in fact, their albumin complexes) with fluorescence methods.

3.2. Effect of the pH on the fluorescence enhancement of quercetin, IR, and Q3'S by albumins

Our results highlighted that albumins are able to significantly improve the fluorescence properties of quercetin, IR, and Q3'S under extracellular physiological conditions. However, we were curious how the changes of the environmental pH can influence the fluorescence signals of these flavonoid-albumin complexes; therefore, only in this part of our experiments, measurements were carried out in non-physiological buffers as well. Fluorescence spectra of quercetin, IR, and Q3'S were recorded in sodium acetate (pH 5.0) and in sodium borate (pH 10.0) buffers. Under these circumstances, the fluorescence intensities of the flavonoid-albumin complexes were significantly decreased compared to their signals in PBS at pH 7.4 (such as Q3'S-BSA and Q3'S-HSA) or almost completely abolished (in each other case).

Thereafter, the influence of slight pH changes were tested in the same buffer as well. Fluorescence spectra of flavonoid-albumin complexes were recorded in PBS at pH 7.0, 7.4, and 7.8. Fig. 4 demonstrates that in most of the cases, slight decrease of the pH reduces while slight increase of the pH enhances the fluorescence of the tested flavonoid-albumin complexes. On the other hand, quercetin-PSA and quercetin-RSA complexes did not follow this principle because both the slight decrease and increase of the pH resulted in reduced fluorescence signals. Furthermore, our results also highlighted that the extent or magnitude of the fluorescence changes show strong differences. Altogether, we can state that the environmental pH significantly influences the fluorescence signal of the examined flavonoid-albumin complexes.

3.3. Fluorescence quenching studies

In order to get more information regarding the flavonoid-albumin complex formations, fluorescence quenching studies were performed. During this part of the study, increasing quercetin, IR, and Q3'S concentrations (0-6 μM) were added to 2 μM albumin. Fluorescence emission spectra of albumins in the absence and presence of flavonoids were recorded in PBS (pH 7.4) using 295 nm as excitation wavelength. As Fig. 5 demonstrates, increasing flavonoid concentrations resulted in the significant decrease of the fluorescence emission intensities at 334 nm. Flavonoids alone did not show fluorescence under the applied circumstances. Before the evaluation of fluorescence quenching studies, UV-VIS absorption spectra of quercetin, IR, and Q3'S were recorded; these data show negligible absorbance of flavonoids at 6 μM concentration (295 nm: A = 0.03-0.05; 344 nm: A = 0.03-0.06) suggesting their insignificant inner-filter effect. Fluorescence quenching of albumins by the tested flavonoids were evaluated using the graphical application of Stern-Volmer (SV) equation (Eq. 1). SV plots of flavonoid-albumin complexes are demonstrated in Fig. 6. SV plots of quercetin show good

linearity (R^2 = 0.990-0.999) regarding each tested albumin, suggestion the static quenching of albumins by quercetin and 1:1 stoichiometry of the complex formation. Linearity of IR-BSA, IR-HSA, and IR-PSA plots was excellent (R^2 = 0.996-0.999), and IR-RSA complex gave an acceptable fitting as well (R^2 = 0.989). While Q3'S-HSA complex had a relatively good linearity (R^2 = 0.990), SV plots of other Q3'S-albumin complexes lost their linearity in the presence of higher (4-6 μM) Q3'S concentrations (Fig. 6). This shape of the SV function may suggest that both static and dynamic components are involved as the quenching mechanism. On the other hand, relatively low flavonoid concentrations were applied and their absorbance is negligible at the wavelengths used. Another explanation of the poor linearity is the presence of more than one binding sites of some flavonoids in the proteins.

3.4. Quantitation of binding constants of flavonoid-albumin complexes

To determine the stabilities of flavonoid-albumin complexes as well as to test the hypothesis that some flavonoids occupy more binding sites on albumins, binding constants of flavonoid-albumin complexes were determined. During the quantitation of binding constants both the data regarding the fluorescence enhancement of flavonoids by albumins (Fig. 2) and data from quenching experiments (Fig. 5) were utilized.

Table 1 represents the binding constants and number of binding sites calculated from the quenching experiments employing the SV equation, double logarithm SV equation, and the Hyperquad2006 software (see details in the Materials and Methods section). SV equation suggested approximately 5.1-5.9 logK_{SV} values, while the double logarithm SV equation referred to significantly higher binding constants of some complexes. Based on double logarithm SV equation, logK_{SV} and log$K$ values were in a good agreement regarding quercetin-BSA, quercetin-HSA, IR-BSA, IR-HSA, IR-PSA, and Q3'S-HSA complexes ("group I"); however, other complexes including quercetin-PSA, quercetin-RSA, IR-RSA,

Q3'S-BSA, Q3'S-PSA, and Q3'S-RSA ("group II") showed much higher stabilities (log$K$ = 6.1-7.8). We also noticed that the latter group showed relatively higher n values (1.16-1.36) compared to group I (0.98-1.04). On the other hand, Hyperquad calculations of quenching data recommend similar magnitude of binding constants in each case (log$K$ = 5.2-6.1) than logK_{SV} data. Using the Hyperquad2006 software, binding constants were also calculated assuming 1:2 stoichiometry (two binding sites of ligands in one albumin molecule). In many cases, Hyperquad failed to interpret the 1:2 model (the only exception was IR-BSA complex among the members of group I), while 1:2 stoichiometry was compatible with every group II complexes.

As Table 2 represents, Hyperquad calculations of 1:1 and 1:2 models were performed using the data of the fluorescence enhancement of flavonoids by albumins as well. Using the 1:1 stoichiometry model, 5.2-6.2 log$K$ values were determined regarding most of the flavonoid-albumin complexes, with the exceptions of quercetin-RSA, Q3'S-BSA, Q3'S-HSA, Q3'S-PSA, and Q3'S-RSA (log$K$ = 6.4-7.3). On the other hand, the assumption of 1:2 stoichiometry resulted in much lower stabilities of the primary binding sites of these complexes (logK_1 = 5.2-5.8). Furthermore, with the exception of IR-RSA complex, 1:2 stoichiometry was compatible with each group II complex. Among group I complexes, only quercetin-BSA and Q3'S-HSA showed the possibility of 1:2 stoichiometry.

Considering the above listed observations, it is plausible to assume that group I members form 1:1 while group II members support formation of 1:2 complexes. HSA forms 1:1 and RSA forms typically 1:2 complexes with flavonoids tested. BSA and PSA can form 1:1 or 1:2 complexes depending on the ligand molecule. Binding constants of quercetin-, IR-, and Q3'S-albumin complexes (primary binding sites) are between 10^5 and 10^6 dm^3/mol (or in some cases slightly exceeding this range), which correlates well with previous studies regarding flavonoid-albumin interactions [42,43]. Stabilities of flavonoid-albumin complexes based on

the albumin species can show negligible, moderate, or high differences. Typically 0.1-0.5 differences of log*K* values were observed. Flavonoids commonly formed the most stable complexes with RSA.

4. Conclusions

In this study, the fluorescence enhancement of quercetin and its metabolites by different albumins (BSA, HSA, PSA, and RSA) was investigated using fluorescence spectroscopy. Our major observations and conclusions are the followings. (1) Among the tested compounds, fluorescence enhancement of quercetin, IR, and Q3'S was observed in the presence of albumins. (2) Depending on the albumin species, the extent of the fluorescence enhancement of flavonoids varied considerably. The strongest fluorescence enhancement of quercetin was observed in the presence of RSA then followed by PSA, HSA, and BSA (in this order). On the other hand, the presence of HSA resulted in the highest enhancement regarding IR, while RSA, PSA, and BSA were less effective enhancers of IR (these three IR-albumin complexes expressed similar fluorescence). Fluorescence enhancement of Q3'S-albumin complexes showed the following order: Q3'S-BSA > Q3'S-PSA > Q3'S-HSA > Q3'S-RSA. BSA was highly the most potent enhancer of Q3'S but it showed the lowest effects regarding quercetin and IR. HSA caused the strongest enhancement of IR while it was less effective enhancer of quercetin and Q3'S (despite the fluorescence of quercetin-HSA, IR-HSA, and Q3'S-HSA complexes were similar). PSA was a relatively strong enhancer of quercetin and Q3'S but it was poorly effective regarding IR. RSA was the most potent enhancer of quercetin, however, it was a weaker enhancer of IR and it was the least potent enhancer of Q3'S. (3) Fluorescence enhancement of quercetin, IR, and Q3'S by albumins were examined at non-physiological pH levels as well. At pH 5.0 and 10.0, the fluorescence of flavonoid-albumin complexes was almost completely abolished, while slight decrease (pH 7.0) reduced and slight increase (pH

7.8) generally enhanced the fluorescence of albumin complexes (only quercetin-PSA and quercetin-RSA showed weaker fluorescence at pH 7.8 than at pH 7.4). (4) Based on the fluorescence enhancement of flavonoids by albumins and quenching experiments, some of the formed complexes showed 1:1 stoichiometry (quercetin-BSA, quercetin-HSA, IR-BSA, IR-HSA, IR-PSA, and Q3'S-HSA), however, in other cases 1:2 stoichiometry was assumed (quercetin-PSA, quercetin-RSA, IR-RSA, Q3'S-BSA, Q3'S-PSA, and Q3'S-RSA) suggesting that some flavonoids may occupy a secondary binding site on albumins. (5) Despite these flavonoids express negligible fluorescence, the strong fluorescence enhancement of quercetin, IR, and Q3'S by albumins allows the fluorescence spectroscopic detection of these biologically important flavonoids, which may be a suitable tool for their fluorescence quantification. Furthermore, fluorescence detection of the described flavonoid-albumin complexes make possible to examine some of their molecular interactions by fluorescence spectroscopy.

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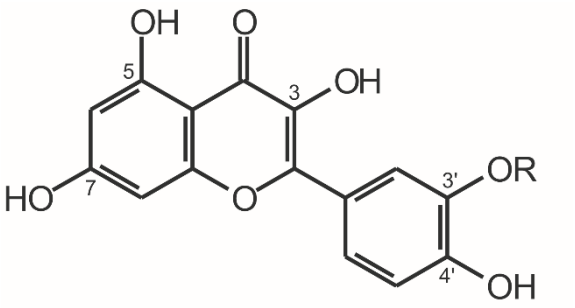
Figures:



	R
Quercetin (Q)	- H
Isorhamnetin (IR)	- CH_3
Quercetin-3'-sulfate (Q3'S)	- SO_3H

Fig. 1: Chemical structures of quercetin, isorhamnetin, and quercetin-3'-sulfate.

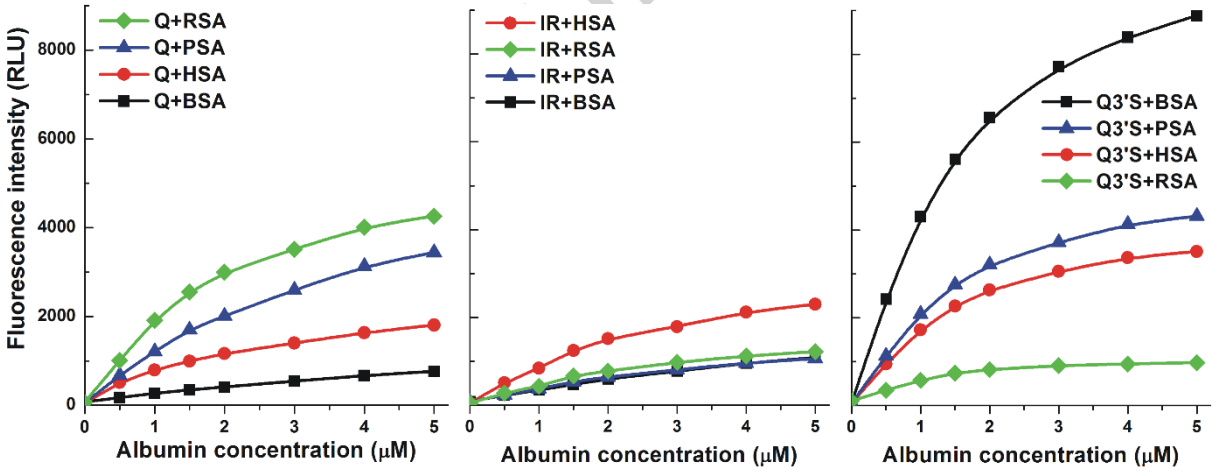


Fig. 2: Fluorescence emission intensities of 2 μM quercetin (Q; left), 2 μM isorhamnetin (IR; middle), and 2 μM quercetin-3'-sulfate (Q3'S; right) in PBS (pH 7.4) in the presence of increasing concentrations (0-5 μM) of BSA, HSA, PSA, and RSA (quercetin and IR: λ_{ex} = 455 nm, λ_{em} = 525 nm; Q3'S: λ_{ex} = 445 nm, λ_{em} = 505 nm).

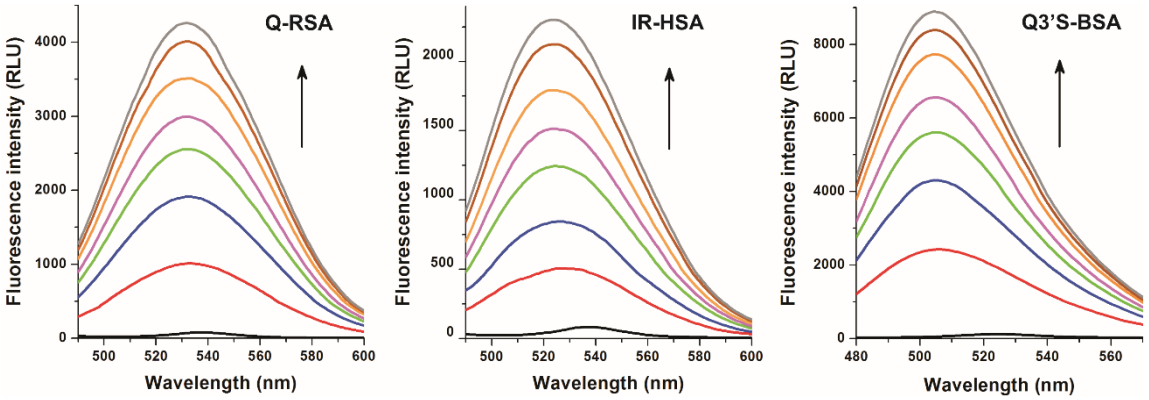


Fig. 3: Fluorescence emission spectra of 2 µM quercetin (Q; left), 2 µM isorhamnetin (IR; middle), and 2 µM quercetin-3'-sulfate (Q3'S; right) in the presence of increasing concentrations (0, 0.5, 1, 1.5, 2, 3, 4, and 5 µM) of RSA, HSA, and BSA, respectively (quercetin and IR: λ_{ex} = 455 nm; Q3'S: λ_{ex} = 445 nm; PBS, pH 7.4).

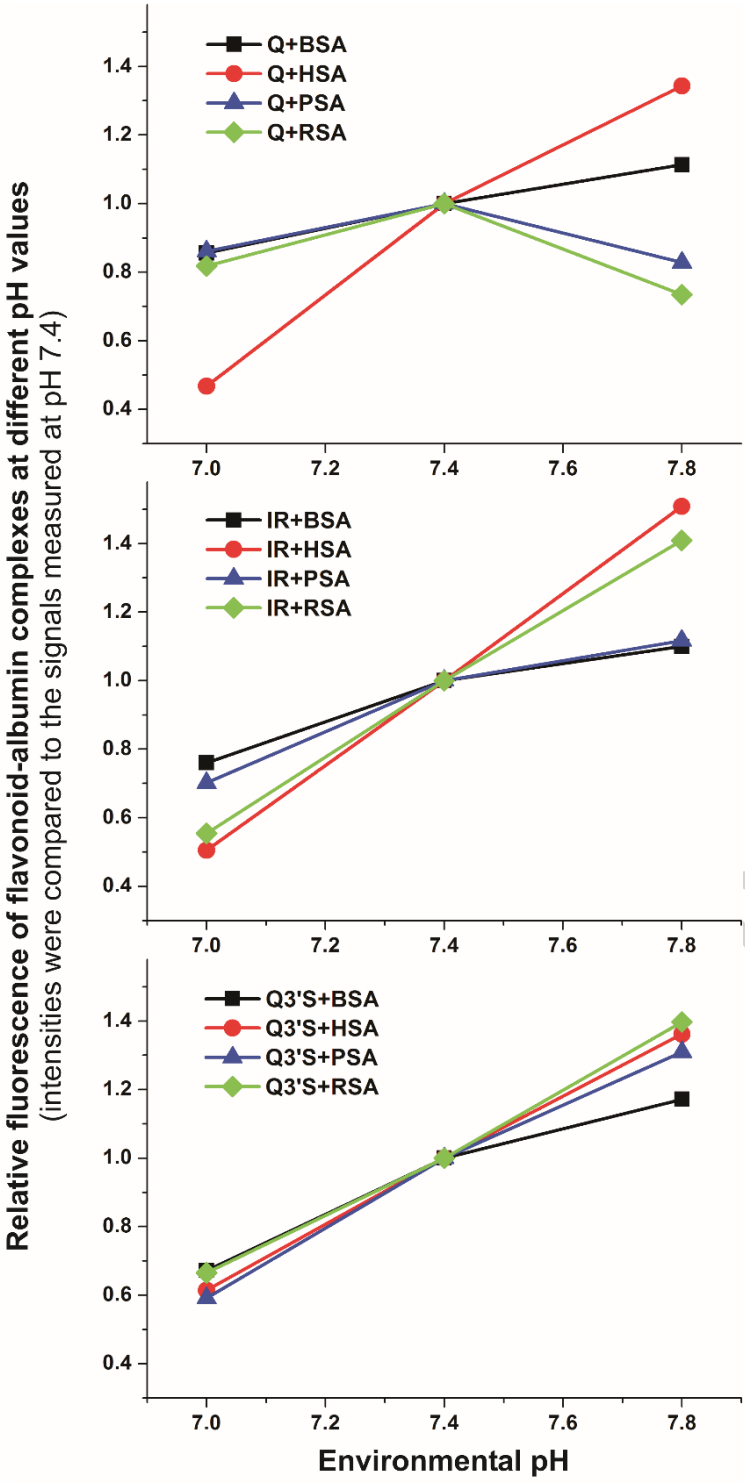


Fig. 4: Influence of the pH on the fluorescence of flavonoids-albumin complexes. Relative fluorescence emission of quercetin (Q; top), isorhamnetin (IR; middle), and quercetin-3'-sulfate (Q3'S; bottom) in the presence of increasing albumin concentrations (0-5 µM) in PBS at pH 7.0, 7.4, and 7.8 (quercetin and IR: λ_{ex} = 455 nm, λ_{em} = 525 nm; Q3'S: λ_{ex} = 445 nm, λ_{em} = 505 nm). Because the fluorescence emission signal of a flavonoid-albumin complex

determined at pH 7.0 and 7.8 was compared to the signal of the same complex recorded at pH 7.4, the relative intensities of complexes at pH 7.4 are always 1.0 which is the basis of the comparison.

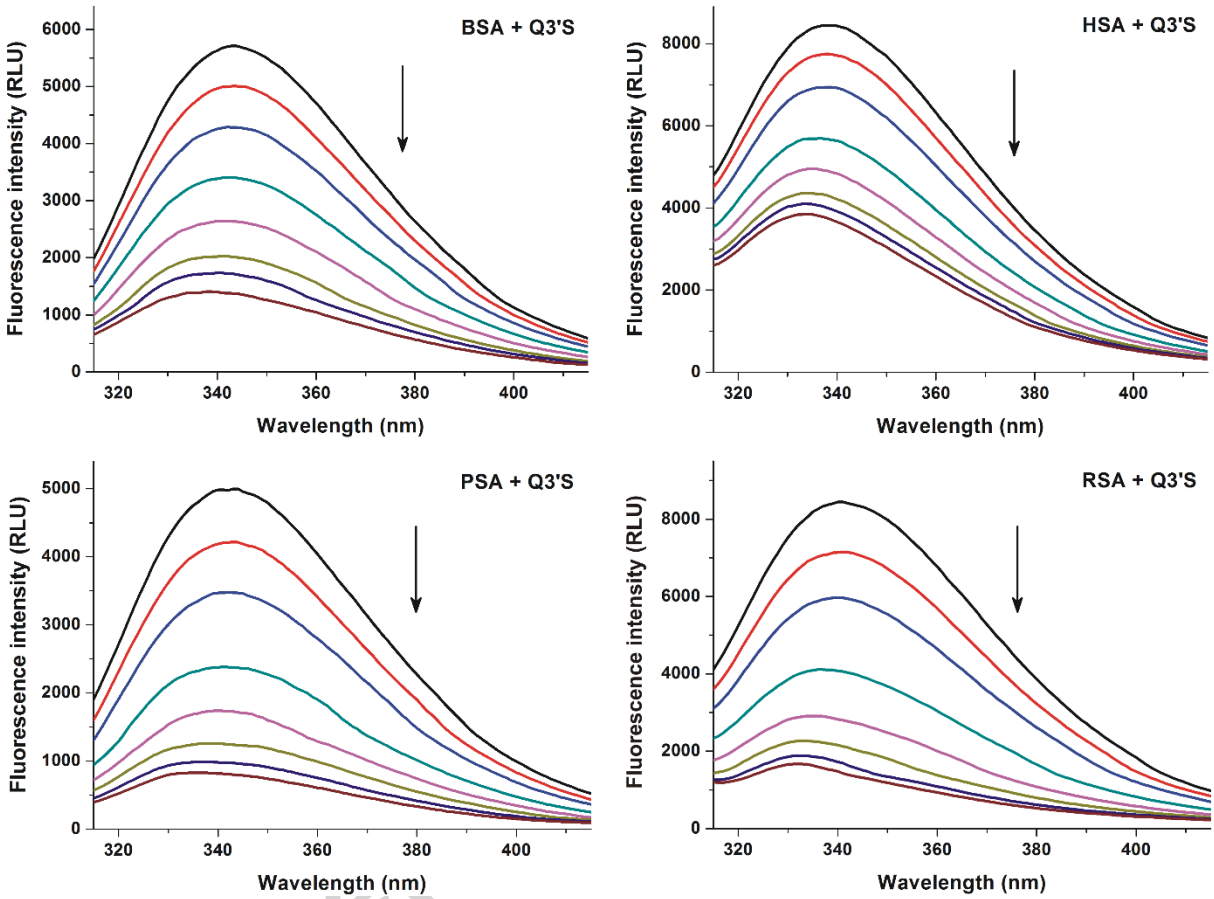
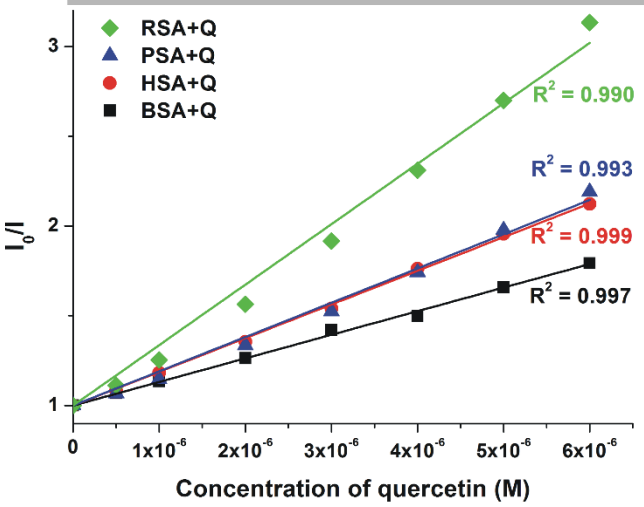
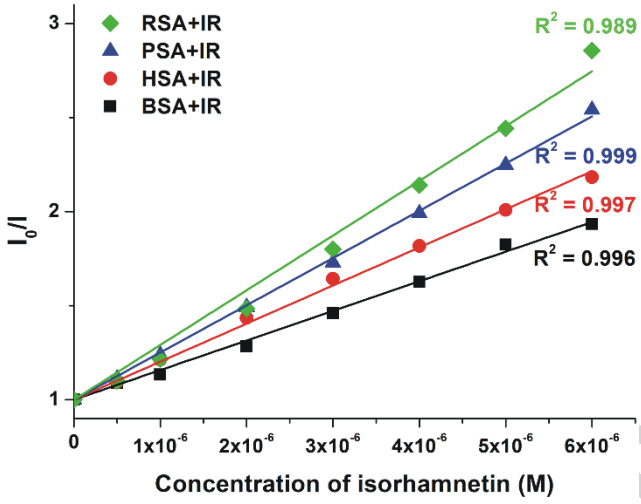


Fig. 5: Fluorescence quenching of albumins by quercetin-3'-sulfate (Q3'S). Emission spectra of BSA (left above), HSA (right above), PSA (left below), and RSA (right below) in the presence of increasing Q3'S concentrations (0, 0.5, 1, 2, 3, 4, 5, and 6 µM) in PBS (pH 7.4; λ_{ex} = 295 nm).





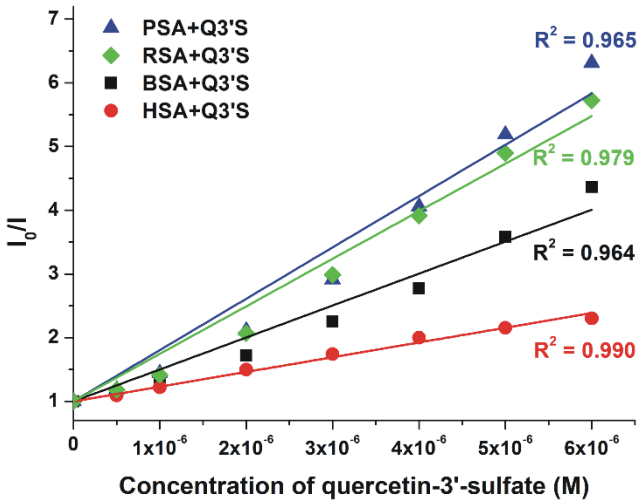


Fig. 6: Stern-Volmer plots of the fluorescence quenching of albumins (2 μM) by quercetin (Q), isorhamnetin (IR), and quercetin-3'-sulfate (Q3'S) in PBS (pH 7.4) (λ_{ex} = 295 nm, λ_{em} = 344 nm).

Tables:

Complex	Stern-Volmer 1:1	Double logarithm Stern-Volmer		Hyperquad 1:1	Hyperquad 1:2	
	logK_{SV} (R^2)	**log$K$ (R^2)**	***n***	**log$K$ (±SD)**	**logK_1 (±SD)**	**logK_2 (±SD)**
Q – BSA	5.14 (0.997)	5.00 (0.999)	0.98	5.25 (± 0.00)	-	-
Q – HSA	5.27 (0.999)	5.44 (0.999)	1.03	5.37 (± 0.00)	-	-
Q – PSA	5.28 (0.993)	6.13 (0.999)	1.16	5.36 (± 0.00)	5.32 (± 0.01)	4.66 (± 0.05)
Q – RSA	5.51 (0.990)	6.50 (0.999)	1.18	5.65 (± 0.00)	5.64 (± 0.01)	4.26 (± 0.22)
IR – BSA	5.19 (0.996)	5.39 (0.996)	1.02	5.29 (± 0.00)	5.26 (± 0.01)	4.34 (± 0.12)
IR – HSA	5.27 (0.997)	5.20 (0.999)	0.98	5.43 (± 0.01)	-	-
IR – PSA	5.40 (0.999)	5.63 (0.999)	1.04	5.52 (± 0.00)	-	-
IR – RSA	5.45 (0.989)	6.40 (0.999)	1.18	5.58 (± 0.00)	5.46 (± 0.01)	4.58 (± 0.13)
Q3'S – BSA	5.60 (0.964)	7.19 (0.999)	1.28	5.80 (± 0.00)	5.76 (± 0.01)	4.93 (± 0.07)
Q3'S – HSA	5.35 (0.990)	5.28 (0.996)	0.99	5.49 (± 0.01)	-	-
Q3'S – PSA	5.78 (0.965)	7.81 (0.999)	1.36	6.06 (± 0.00)	6.05 (± 0.00)	4.49 (± 0.10)
Q3'S – RSA	5.86 (0.979)	7.69 (0.999)	1.34	6.05 (± 0.01)	6.04 (± 0.01)	3.80 (± 0.10)

Table 1: Stern-Volmer quenching constants (K_{SV}), binding constants (K), and number of binding sites (n) of flavonoid-albumin complexes calculated from the data of fluorescence quenching experiments (see details in the Materials and Methods section).

	Hyperquad 1:1	Hyperquad 1:2	
	log*K* (±SD)	**logK_1 (±SD)**	**logK_2 (±SD)**
Q - BSA	5.33 (± 0.01)	5.32 (± 0.02)	5.26 (± 0.02)
Q - HSA	6.16 (± 0.05)	-	-
Q - PSA	5.72 (± 0.01)	5.75 (± 0.03)	4.83 (± 0.02)
Q - RSA	6.38 (± 0.03)	5.23 (± 0.04)	5.16 (± 0.02)
IR - BSA	5.26 (± 0.01)	-	-
IR - HSA	6.05 (± 0.03)	-	-
IR - PSA	5.70 (± 0.01)	-	-
IR - RSA	5.94 (± 0.02)	-	-
Q3'S - BSA	6.63 (± 0.04)	5.41 (± 0.06)	5.22 (± 0.02)
Q3'S - HSA	6.65 (± 0.04)	5.27 (± 0.02)	5.14 (± 0.12)
Q3'S - PSA	6.66 (± 0.04)	5.27 (± 0.02)	5.14 (± 0.14)
Q3'S - RSA	7.31 (± 0.11)	5.46 (± 0.02)	3.67 (± 0.11)

Table 2: Binding constants (*K*) of flavonoids-albumin complexes calculated from the data of the fluorescence enhancement of flavonoids by albumins (see details in the Materials and Methods section).