Interaction of Quercetin and Its Conjugate Quercetin 3-*O*-*β*-D-Glucopyranoside with Albumin as Determined by NMR Relaxation Data

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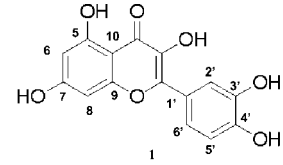
NMR methodology has been developed in order to study phytochemical−macromolecular receptor interactions. This approach is based on the analysis of proton selective spin–lattice relaxation rate enhancements of the ligand in the presence of the macromolecule, to calculate an affinity index, $[A]_L^T$, related to the strength of the interaction process. This index has been modified by normalization to the relaxation rate of the free ligand, in order to take into account the effects of motional anisotropies and different proton densities. The normalized affinity index, $[A_N]_L^T$, isolates the contribution due to a decrease in the ligand dynamics caused by the binding with the protein. This methodology has been applied to the interaction between two flavonoids (quercetin, **1**, and quercetin 3-*O*-*β*-D-glucopyranoside, **2**) and bovine serum albumin (BSA). The calculated values of the affinity indexes and thermodynamic equilibrium constants suggested a much stronger capacity of **1** to interact with BSA when compared with its glucosylated derivative, **2**.

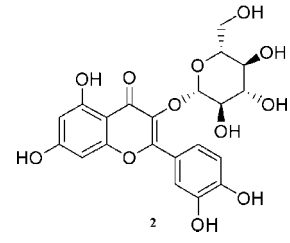
Phenolic compounds and organic acids may play a crucial role in the protection against various diseases, due to their antioxidant potential.[1] Antioxidants are of great interest because they may help to protect the organism against reactive oxygen species (ROS). It is known that they are able to avoid free radical mediated lipid peroxidation of low-density lipoproteins (LDL), which is responsible for cell aging and chronic diseases such as atherosclerosis.[2–4] In fact, the oxidation induced by ROS can result in cell membrane disintegration, membrane protein damage, and DNA mutation and can be the first step toward the development of many diseases, such as cancer, liver injury, and cardiovascular disease.[5] In order to more deeply understand the mechanisms of natural compound biological and biochemical activities, the study of their interactions with possible biological targets such as nucleic acids,[6] enzymes,[7] and other proteins may become crucial. At the cellular level, phenols are able to bind to numerous proteins,[8] inducing enzyme inhibition and the modulation of biological effects mediated by these receptors.[9]

There is a wide range of experimental and theoretical approaches to study recognition processes between ligands and receptors.[10–14] Nuclear magnetic resonance has been used widely for the characterization of ligand–receptor complexes,[15–19] due to the advantage of noninvasivity and nonalteration of the normal biofunctionality of the biomolecules under investigation as well as the large number of spectroscopic parameters that can be measured and analyzed (chemical shift,[20] relaxation rates and line width,[21] NOE[22]), together with NMR methods such as pulsed gradient diffusion.[23] In particular, relaxation rate techniques, such as measurements of proton spin–lattice selective relaxation rates, represent a powerful tool to investigate the binding affinity of the ligand toward the receptor as well as the dynamic properties of ligand−protein complexes.[15,24]

In this paper, we have used a development of a NMR methodology, based on the quantitative analysis of proton spin–lattice selective relaxation rate enhancements (ΔR_1^{SE}) of the ligand induced by the formation of the complex with the protein. These contributions, arising from the fraction of the bound ligand, have allowed the calculation of the *"affinity index"*, $[A]_L^T$, a parameter related to the strength of all nonspecific and/or specific interactions occurring within the system.[25,26] Since motional anisotropies and different spin densities at ligand proton sites may affect the observed selective relaxation rates, the affinity index has been normalized to the relaxation rate of the free ligand. The new calculated parameter, $[A_N]_L^T$, the normalized affinity index, appears to be totally independent from the intrinsic relaxation properties of any proton nuclei and can be proposed as a more suited parameter to compare the recognition processes between a protein and different ligands.[27] Furthermore, the analysis of $1/(\Delta R_1^{SE})$ in relation to ligand concentration gave the complex equilibrium constant K and the relaxation rate of the ligand bound to the protein.

In the present work we have applied this approach to the investigation of the interaction between natural compounds such as quercetin (**1**) and quercetin 3-*O*-*β*-D-glucopyranoside (**2**) and bovine serum albumin (BSA). Albumin, in being one of the most abundant carrier proteins, plays a fundamental role in the transport of endogenous and exogenous ligands present in the plasma.[28] In fact, both the distribution and the metabolism of many bioactive compounds in humans are related to their affinity toward albumin.[29] Thus, investigations on phenol−albumin recognition processes may play a key role in order to study important properties of these natural compounds such as their bioavailability, toxicology, and antioxidant capacity, which are affected by interaction processes.[30]





Quercetin (**1**) is regularly consumed by humans, as it is the major flavonoid found in the diet.[31] A number of beneficial effects of **1** on human health have been known for some time.[32,33] This flavonoid is reported to decrease capillary fragility, to protect against

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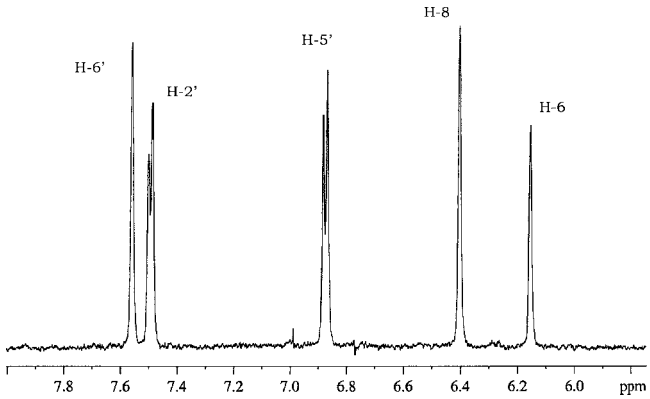

Figure 1. Aromatic portion of the ^{1}H NMR spectrum of quercetin (**1**, 2×10^{-2} mol L^{-1} at 298 K), with the assignment of the signals.

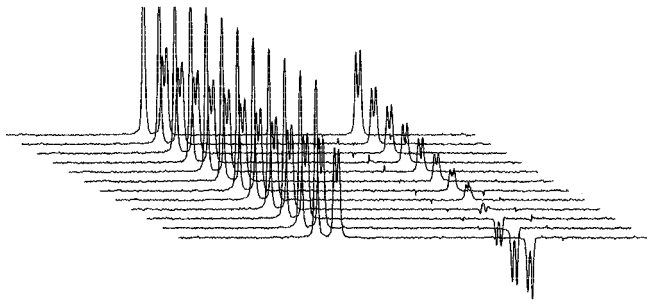

Figure 2. Partially relaxed spectra of the selective inversion of H-5′ proton of quercetin (**1**). The chemical shift axes range from 7.8 to 6.6 ppm.

Table 1. R_1^{SE} and R_1^{NS} Values Calculated for the H-5′ Proton of Quercetin (**1**, 2×10^{-2} mol L^{-1}) and 3-*O*-*β*-D-glucopyranoside (**2**, 2×10^{-2} mol L^{-1}) in the Presence of Variable Albumin Concentrations at 298 K

albumin concentration (mg mL^{-1})	albumin concentration (mol L^{-1})	**1**		**2**	
		R_1^{SE} (s^{-1}) H-5′	R_1^{NS} (s^{-1}) H-5′	R_1^{SE} (s^{-1}) H-5′	R_1^{NS} (s^{-1}) H-5′
0	0	0.40	0.58	0.47	0.65
1	1.51×10^{-5}	1.10	0.56	0.54	0.58
3	2.98×10^{-5}	4.16	0.50	0.65	0.60
4	6.04×10^{-5}	5.87	0.52	0.75	0.55
5	7.46×10^{-5}	6.11	0.49	0.83	0.63

diabetic cataracts, to possess antiviral and antiallergenic activities, to inhibit platelet aggregation and the oxidation of low-density lipoproteins, and to act as an anti-inflammatory agent.[34] Quercetin is found in the plasma also in the conjugate form, quercetin 3-*O*-*β*-D-glucopyranoside (**2**), which cannot be absorbed and is hydrolyzed by intestinal enzymes and colon microflora.[35]

Results and Discussion

Figure 1 shows the aromatic region of the proton NMR spectrum of quercetin (**1**), including the H-5′ proton used for the selective and nonselective relaxation rate measurements.

The NMR parameters able to give information about the existence of interaction processes between the ligand and the protein are the nonselective and the selective proton relaxation rates, measured in the absence and presence of albumin. Figure 2 shows the spectra with the selective inversion of the H-5′ proton used for the calculation of R_1^{SE} (see Supporting Information).

Table 1 reports the values of R_1^{SE} and R_1^{NS} of the H-5′ proton of quercetin (**1**) and quercetin 3-*O*-*β*-D-glucopyranoside (**2**) in relation to albumin concentration. The results show that for all the observed

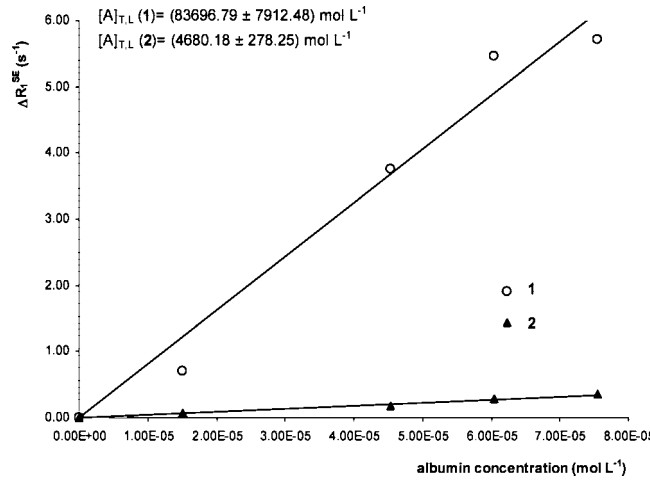

Figure 3. Linear regression analysis of the H-5′ selective relaxation enhancement, ΔR_1^{SE}, as a function of albumin concentration of a solution of (a) quercetin (**1**) and (b) quercetin 3-*O*-*β*-D-glucopyranoside (**2**, 2×10^{-2} mol L^{-1} at 298 K). The value of the affinity indexes $[A]_L^T$ is also reported with the corresponding error.

protons, in the absence of BSA, $R_1^{NS} > R_1^{SE}$, while increasing protein concentration R_1^{SE} becomes greater than R_1^{NS}. The selective relaxation rate enhancements reveal the existence of a large contribution from the bound ligand fraction to the observed relaxation rate, which suggests the presence of an interaction between **1** and **2** and BSA. Nevertheless, it can be noted that the selective relaxation rate enhancements of the quercetin protons were much greater than those related to its conjugate, **2**. The presence of relatively high concentrations of protein in solution may cause an increase in the viscosity of the system, which can cause a lowering in the ligand dynamics. The results of this phenomenon may lead to an increase of R_1^{SE} even if the interaction processes did not take place. In some papers published by the authors of this work[26,27] it has been demonstrated that the concentrations of BSA used in this study did not change the viscosity of the system, and therefore the observed R_1^{SE} enhancements can be directly related to the formation of the ligand−macromolecule complex.

In order to evaluate the strength of the binding process, the affinity indexes $[A]_L^T$ for **1**−albumin and **2**−albumin systems were calculated from the slope of the straight line describing the dependence of proton selective relaxation rate enhancements on protein concentration. Figure 3 shows the plot of ΔR_1^{SE} vs BSA concentration for the H-5′ proton of **1** and **2** together with the calculated affinity indexes for each compound. In order to remove the effects due to motional anisotropies in the ligand molecule that may affect the spin–lattice relaxation rates, $[A]_L^T$ was normalized to the selective spin–lattice relaxation rate of the free ligand, and the so-called "*normalized affinity index*" $[A^N]_L{}^T$ was calculated. Figure 4 shows the effect of the normalization on $[A]_L^T$. The values of the normalized affinity indexes were found to be 209 242 mol^{-1} L for quercetin (**1**) and 9957 mol^{-1} L for quercetin 3-*O*-*β*-D-glucopyranoside (**2**). These results indicate that **1** has a stronger affinity for BSA compared with **2**. This behavior is in agreement with other studies concerning the investigation of the interaction between BSA and rutin (a glucoside of quercetin, containing the disaccharide rutinose).[29] It is clear, in fact, that the structure of the polyphenols plays a key role in affecting their recognition processes with proteins.[36] In particular, flavonoids interact mainly via hydrophobic interactions, which are stronger for **1**, being an aglycon, with respect to its conjugate **2**.

In order to calculate the values of the relaxation rates of bound ligand and the complexation equilibrium constant, the selective relaxation rates of quercetin (**1**) and 3-*O*-*β*-D-glucopyranoside (**2**) were measured at different concentrations in the presence of a

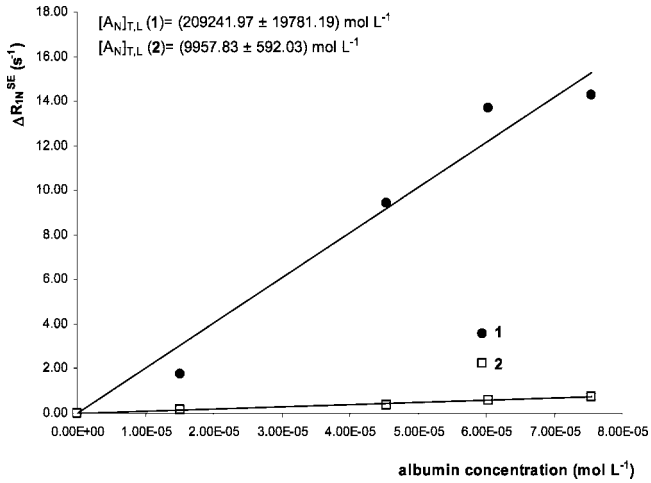

Figure 4. Linear regression analysis of the H-5′ selective relaxation enhancement, ΔR_{1N}^{SE}, as a function of albumin concentration of a solution of (a) quercetin (**1**) and (b) quercetin 3-*O*-*β*-D-glucopyranoside (**2**, 2×10^{-2} mol L^{-1} at 298 K) as a function of albumin concentration. The value of the normalized affinity indexes $[A_N]_L^T$ is also reported with the corresponding error.

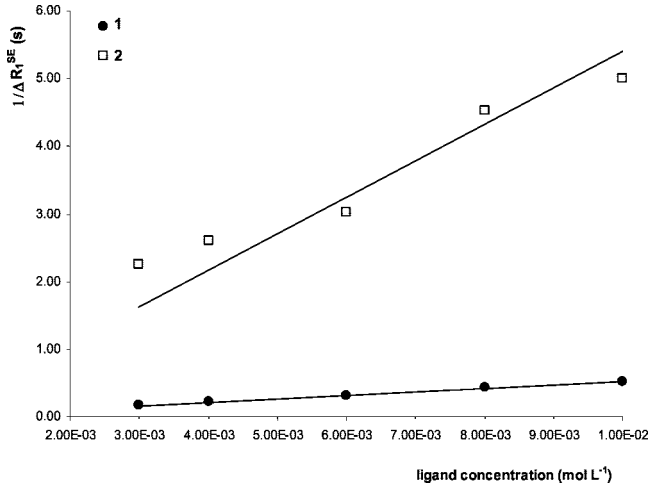

Figure 5. Calculated values of $1/\Delta R_1^{SE}$ of quercetin (**1**) and quercetin 3-*O*-*β*-D-glucopyranoside (**2**) measured at variable concentrations. Both the values of K and R_{1b}^{SE} were calculated from the slope and the intercept of the straight line using the equations $S = 1/(R_{1b}^{SE}[M_0])$ and $I = 1/(KR_{1b}^{SE}[M_0])$.

constant amount of albumin. Figure 5 shows the calculated values of $1/\Delta R_1^{SE}$ of the phytochemicals measured at different concentrations. Using the values of the calculated slopes and applying eq 11 (Supporting Information), R_{1b}^{SE} was calculated as 434.63 ± 1.63 s^{-1} for **1** and 53.20 ± 0.45 s^{-1} for **2**. These results allowed the calculation of the equilibrium constants associated with the complex formation, which were found to be 3077.25 ± 104.22 mol^{-1} L for **1** and 456.33 ± 21.32 mol^{-1} L for **2**. These results are in agreement with those found for the affinity index calculations, confirming that the strength of the interactions follows the order **1**−BSA > **2**−BSA.

In conclusion, the calculated values of the affinity indexes and the binding constants, K, for the **1**−BSA and **2**−BSA systems indicate that the binding affinity was strongest for quercetin (**1**); in particular, **1** showed a value of $[A_N]_L^T$ about 20 times larger than its derivative. The proposed approach, using a fast and simple methodology to evaluate the strength of the interaction between bioactive small molecules and macromolecules such as proteins, may represent a useful tool for natural compound−protein recognition screening.

This study provides further insights into the complex behavior of quercetin (**1**), a major dietary flavonoid, compared to its glucosylated form, **2**. In particular all the covalent and noncovalent binding sites were shown to be highly selective for quercetin.

Experimental Section

General Experimental Procedures. All the ^{1}H spectra were obtained on a Bruker 600 DRX spectrometer operating at 600.13 MHz.

Materials. Quercetin, 3,5,7,3′,4′-pentahydroxyflavone (**1**), quercetin 3-*O*-*β*-D-glucopyranoside (**2**), and bovine serum albumin (molecular mass 66200 Da) were purchased from Sigma Chemical Co. and used without any further purification.

NMR Measurements. The solutions for the NMR experiments were obtained by dissolving the appropriate amounts of ligand and protein in DMSO-d_6−D_2O (2:3). The solvent mixture was required due to the low solubility of **1** and **2** in D_2O. In all the experiments the ligand concentration was 2×10^{-2} mol L^{-1}.

The spin–lattice relaxation rates were measured using the $(180°-\tau-90°-t)_n$ sequence. The τ values used for the selective and nonselective experiments were 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4, 0.8, 1, 1.5, 2, 3, 4, 5, 7, and 20 s, respectively, and the delay time t in this case is 20 s. The 180° selective inversion of the proton spin population was obtained by a selective soft Gaussian perturbation pulse (width: 60 ms, power: 120 dB).[37] The FID was processed using an exponential window function with line broadening of 1 Hz. All the selective and nonselective spin–lattice relaxation rates refer to the H-5′ of **1** and **2**. Since in general the recovery of proton longitudinal magnetization after a 180° pulse is not a single exponential, due to the sum of different relaxation terms, the selective spin–lattice relaxation rates were calculated using the initial slope approximation and subsequent three-parameter exponential regression analysis of the longitudinal recovery curves. The maximum experimental error in the relaxation rate measurements was 5%. The affinity index was calculated by linear regression analysis of the experimental data.

All the spectra were processed using the Bruker Software XWINNMR, version 2.5, on a Silicon Graphics O_2 equipped with a RISC R5000 processor, working under the IRIX 6.3 operating system.

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Supporting Information Available: The theory of the methodology to study ligand−macromolecule interactions adopted in this work. This material is available free of charge via the Internet at http://pubs.acs.org.

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