JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 10705–10709 10705
DOI:10.1021/jf102035a

Interaction between Vine Pesticides and Bovine Serum Albumin Studied by Nuclear Spin Relaxation Data

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Pesticides are chemicals usually used in agriculture to prevent possible diseases to crops, such as grapes, caused by parasites. Even if most of the pesticides are degraded during the wine process, residual levels remain in the final product. The most commonly used pesticides in vine belong to several classes of chemical compounds; among them, triazoles and anilinopyrimidines have been commercially used since the 1970s and 1990s, respectively. In this work, we investigated the interaction between three of the most used fungicides belonging to the chemical classes mentioned above (myclobutanil, triadimenol, and pyrimethanil) and bovine serum albumin (BSA) by nuclear spin relaxation analysis. We found that all of the pesticides were able to form a complex with BSA; nevertheless, there were strong differences in their affinity toward the plasma protein. The nuclear magnetic resonance approach used on the basis of the analysis of selective relaxation rate enhancements of pesticide protons in the presence of BSA allowed for the calculation of the affinity indexes and the equilibrium constants of the three systems. Myclobutanil showed the highest affinity toward BSA, whereas triadimenol gave the weakest interaction with the protein. The differences in the capacity of the three pesticides to bind to albumin highlighted the existence of different binding strengths on the protein. These results indicate that myclobutanil and triadimenol, despite their structure similarity, may have very different residence times in the plasma and rates of clearance.

KEYWORDS: Pesticides; relaxation rate; affinity index; serum albumin

INTRODUCTION

Pesticides are chemical compounds used in agriculture with the aim to avoid diseases originated by insect plagues, fungi, or other pests to crops, such as grapes. The most common parasites of vine in the Mediterranean countries are *Lobesia botrana* (the grape moth), *Plasmopora viticola* (downy mildew), *Uncinula necator* (powdery mildew), and *Botrytis cinerea* (gray mold) (*1*, *2*).

An amount of pesticide remains deposited on the grapes and decreases gradually, until the grapes are harvested. Residues are found in the final products, such as wine (*3*), vinegar, etc., and their presence in the end products depends upon several factors, such as the technological processes, the concentration factor of the fruit (*4*), and the chemical stability of the molecules (*5*).

The pesticides mainly used against parasite attacks belong to the chemical classes of acylanilines, triazoles, and dicarboximides and have been commercially used since the 1970s.

The main effect of the protracted use of these fungicides is resistance and a strong decrease of their efficacy. Only in the 1990s, new fungicides belonging to new chemical classes (strobilurines, anilinopyrimidines, dinitroanilines, and phenylpyrroles) that showed good activity became available (*2*).

Fungicides belonging to the triazole family, such as myclobutanil and triadimenol, were reported to exhibit a range of toxicological properties in mammalian species (*6*). Among the "new generation" pesticides, pyrimethanil, an anilinopyrimidine used to control gray mold (*7*), was shown to cause an increase of liver metabolism and thyroid tumors in rats (*8*).

Cabras et al. (*2*) reported that pyrimethanil residues on grapes remained constant up to harvest and that it was present in wine at the same concentration as on the fruit. Athanasopoulos et al. (*9*) studied the decomposition of myclobutanil and triadimefon in grapes, finding pseudo-first-order degradation kinetics.

The presence of fungicides in the fruit (i.e., grapes) and in the final products (i.e., wine and vinegar), even at very low concentrations (*1*, *10*, *11*), may represent a possible risk for human health. It is known that the interaction of pesticides with blood components is able to affect their distribution and biomacromolecules functioning (*12*). For this reason, the investigation of their binding to plasmatic proteins represents a very important tool to obtain pharmacological information.

Albumin is the most important plasma protein, having a key role in the physiological transport of xenobiotic compounds often through the formation of noncovalent complexes (*13*), thus influencing the circulation and elimination of exogenous compounds (lowering the rate of clearance and increasing the plasma half-life) and affecting the characteristics of their physiological effects (*14*).

In this work, we investigated the interaction between bovine serum albumin (BSA) and three of the most used fungicides

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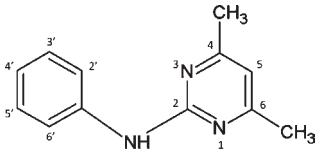
Published on Web 09/21/2010

(myclobutanil, triadimenol, and pyrimethanil) by nuclear spin relaxation analysis. The used approach on the basis of the analysis of proton selective relaxation rates of the pesticides in the presence of the protein allowed for the calculation of the "affinity index" as well as the thermodynamic equilibrium constants for the formation of the complexes. Myclobutanil showed the strongest interaction with BSA with respect to pyrimethanil and triadimenol, suggesting a longer residence time in the plasma flux and a capacity to diffuse to body tissues.

MATERIALS AND METHODS

Materials. Pyrimethanil (4,6-dimethyl-*N*-phenylpyrimidin-2-amine) (**1**), myclobutanil (2-(4-chlorophenyl)-2-(1*H*-1,2,4-triazol-1-ylmethyl)-hexanenitrile) (**2**), and BSA (molecular mass of 66 200 Da) were purchased from Sigma Chemical Co. (Seelze, Germany). Triadimenol (1-(4-chlorophenoxy)-3,3-dimethyl-1-(1*H*-1,2,4-triazol-1-yl)butan-2-ol) (**3**) was purchased from Supelco, Bellefonte, PA. All of the compounds were used without any further purification.



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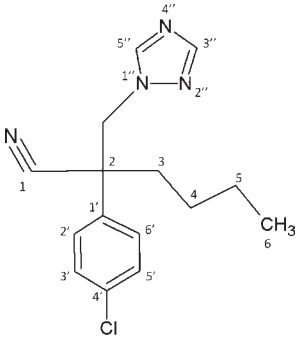

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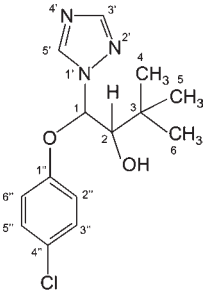

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NMR Measurements. All of the ^{1}H spectra were performed on a Bruker AMX 400. The solutions for the NMR experiments were obtained by dissolving the appropriate amounts of pesticides and protein in DMSO-d_6/D_2O (3:2). The solvent mixture was required because of the low solubility of fungicides in D_2O. In all of the experiments, their concentration was 2×10^{-2} mol dm^{-3}.

The spin−lattice relaxation rates were measured using the $(180°-\tau-90°-t)_n$ sequence. The τ values used for the selective and non-selective experiments were 0.01, 0.02, 0.04, 0.06, 0.08, 0.1, 0.2, 0.4, 0.8, 1, 1.4, 2, 3, 4, 5, 8, and 20 s, respectively, and the delay time t was 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) (*15*). The free induction decay (FID) was processed using an exponential window function, with line broadening of 1 Hz. The selective and non-selective spin−lattice relaxation rates refer to the H-2′,6′ of pyrimethanil, H-2″,6″ of triadimenol, and H-2′,6′ of myclobutanil. Because, in general, the recovery of proton longitudinal magnetization after a 180° pulse is not a single exponential, because of 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 of the spectra were processed using the Bruker Software XWINNMR, version 2.5, on Silicon Graphics, O_2 equipped with a RISC R5000 processor, working under the IRIX 6.3 operating system.

Calculation of the Affinity Index. The explicit forms of non-selective R_1^{NS} and selective R_1^{SE} proton spin−lattice relaxation rates are (*16*−*21*)

$$R_1^{NS} = \frac{1}{10}\frac{\gamma_H{}^4\hbar^2}{r_{ij}{}^6}\left[\frac{3\tau_c}{1+\omega_H{}^2\tau_c{}^2}+\frac{12\tau_c}{1+4\omega_H{}^2\tau_c{}^2}\right] \quad (1)$$

$$R_1^{SE} = \frac{1}{10}\frac{\gamma_H{}^4\hbar^2}{r_{ij}{}^6}\left[\frac{3\tau_c}{1+\omega_H{}^2\tau_c{}^2}+\frac{6\tau_c}{1+4\omega_H{}^2\tau_c{}^2}+\tau_c\right] \quad (2)$$

where $\hbar$ is reduced Plank's constant, γ_H and ω_H are the proton magnetogyric ratio and Larmor frequency, respectively, r_{ij} is the internuclear distance, and τ_c is the effective correlation time, which modulates the $i-j$ magnetic interaction.

As can be seen from eqs 1 and 2, the values of R_1^{SE} and R_1^{NS} depend upon ligand dynamics to different extents: $R_1^{NS} > R_1^{SE}$ in the fast molecular motion regime, typical of the free ligand ($\omega_0\tau_c \ll 1$), and $R_1^{SE} > R_1^{NS}$ when the ligand dynamics are restricted because of the binding to a macromolecule ($\omega_0\tau_c \gg 1$).

The ligand NMR parameter most affected by drastic changes in the correlation time τ_c is R_1^{SE}, which is expressed by the following equation:

$$R_{1obs}^{SE} = \chi_F R_{1F}^{SE} + \chi_B R_{1B}^{SE} \quad (3)$$

where R_{1obs}^{SE} is the experimentally determined selective relaxation rate and R_{1F}^{SE} and χ_F and R_{1B}^{SE} and χ_B are the selective spin−lattice relaxation rates and the ligand fractions of the free and bound environments, respectively.

If we consider ligand−macromolecule equilibrium

$$M + L \leftrightarrows ML \quad (4)$$

and assuming $[L] \gg [M_0]$, we obtain

$$\Delta R_1^{SE} = \frac{KR_{1B}^{SE}}{1+K[L]}[M_0] \quad (5)$$

The plot ΔR_1^{SE} ($\Delta R_1^{SE} = R_{1obs}^{SE} - R_{1F}^{SE}$) versus macromolecule concentration ($[M_0]$) would have a straight line, with slope (*22*)

$$[A]_L^T = \left(\frac{KR_{1B}}{1+K[L]}\right) \quad (6)$$

where K is the thermodynamic equilibrium constant. $[A]_L^T$ was defined as the "affinity index" (dm^3 mol^{-1} s^{-1}) (*23*), which is a constant if the temperature and ligand concentration are specified, as suggested by the L and T sub- and superscripts in the affinity index symbol.

Because there can be differences in the dynamics of different portions of the molecule, which can affect proton selective relaxation rates, R_1^{SE} values need to be normalized to the relaxation rate of the free ligand. This will remove the effects of different correlation times and different proton densities and isolate the effects of restricted motions because of the interaction of the ligand with the macromolecule, leading to a normalized affinity index (*24*)

$$\Delta R_{1N}^{SE} = \frac{KR_{1B}^{SE}[M_0]}{(1+K[L])R_{1F}^{SE}} \quad (7)$$

The dependence of the normalized relaxation rate enhancements ΔR_{1N}^{SE} on the macromolecule concentration $[M_0]$ is represented by a straight line

passing through the origin of the axes with slope

$$[A_I^N]_L^T = \frac{KR_{1B}^{SE}}{(1+K[L])R_{1F}^{SE}} \quad (8)$$

$[A_I^N]_L^T$ is still a constant at fixed temperature and ligand concentration and is defined as the "normalized affinity index" ($dm^3\ mol^{-1}$).

Calculation of the Binding Constants of Pesticide−BSA Complexes. Equation 5 may be rewritten as

$$\frac{1}{\Delta R_I^{SE}} = \frac{(1+K[L])}{KR_{1B}^{SE}[M_0]} \quad (9)$$

and

$$\frac{1}{\Delta R_I^{SE}} = \frac{1}{KR_{1B}^{SE}[M_0]} + \frac{[L]}{R_{1B}^{SE}[M_0]} \quad (10)$$

Plotting $1/\Delta R_I^{SE}$ in relation to the ligand concentration [L], we should observe a linear behavior in which the slope and the intercept of the straight line are given by

$$S = \frac{1}{R_{1B}^{SE}[M_0]} \quad (11)$$

and

$$I = \frac{1}{KR_{1B}^{SE}[M_0]} \quad (12)$$

Because the concentration of the macromolecule is known and the relaxation rate of the free ligand can be directly measured, the relaxation rate of the bound ligand R_{1B}^{SE} can be calculated using eq 11. This value can be used to obtain the value of the equilibrium constant K from eq 12.

RESULTS AND DISCUSSION

Panels **a**, **b**, and **c** of **Figure 1** report the low field portion of the proton spectrum of pyrimethanil, myclobutanil, and triadimenol, respectively, with resonance assignments, which have been carried out performing double-quantum filtered correlation spectroscopy (DQF-COSY) and nuclear Overhauser effect spectrometry (NOESY) spectra (data not shown).

To evaluate the existence of pesticide−BSA interactions, proton spin−lattice relaxation rates of pyrimethanil, myclobutanil, and triadimenol solutions in the absence and presence of BSA have been measured. The experimental results are reported in **Table 1**. Data show that the presence of the protein did not affect R_{1N}^{NS}; nevertheless, an increase in R_{1N}^{SE} with an increasing BSA concentration within the three systems was observed. It can be noted that BSA influenced selective relaxation rate values to different extents depending upon the fungicide within the system under study. This result suggests the existence of binding processes between the three pesticides and BSA and, thus, the formation of pyrimethanil−BSA, myclobutanil−BSA, and triadimenol−BSA complexes. As reported in the Material and Methods, R_{1N}^{SE} enhancements (ΔR_{1N}^{SE}), caused by the presence of molecular interactions, can be quantified in relation to the protein concentration. ΔR_{1N}^{SE} dependence of the BSA content is always linear in this kind of system, and the slope of the straight line provides a useful index, the affinity index, $[A_I^N]_L^T$, which is related to the strength of the overall interaction processes (specific and non-specific binding) occurring between the pesticides and the protein. The plot reported in **Figure 2** shows the experimental values of ΔR_{1N}^{SE} versus the BSA concentration together with data fitting and affinity index calculation for the three systems under investigation. The results indicate that myclobutanil had the highest affinity for BSA ($[A_I^N]_L^T = 73676\ mol^{-1}\ dm^3$), whereas pyrimethanil and triadimenol showed lower affinity for the protein, giving affinity indexes of 33458 and 20813 $mol^{-1}\ dm^3$, respectively. In particular, the affinity of myclobutanil for BSA was about 3.5 times that of triadimenol.

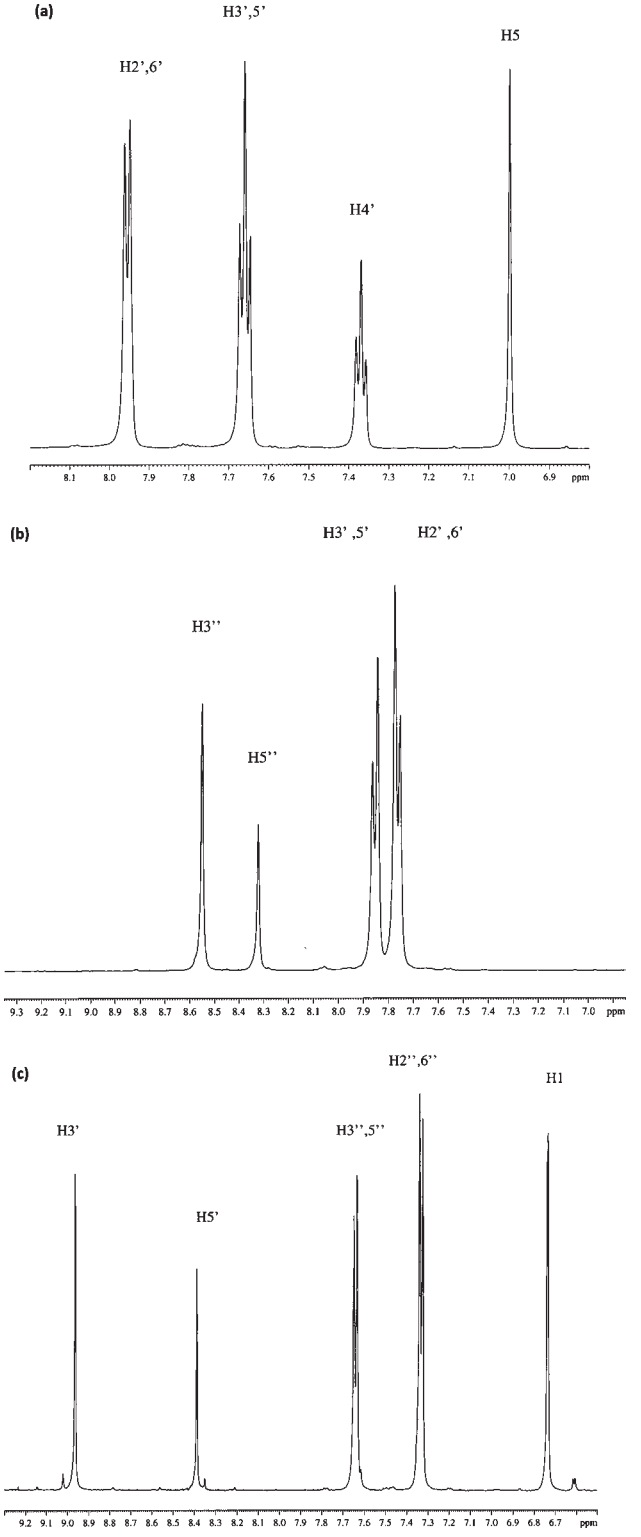

Figure 1. Low field portion of the proton spectrum at 400 MHz of (a) pyrimethanil, (b) myclobutanil, and (c) triadimenol with resonance assignments.

The thermodynamics of the interaction processes can be deeply investigated by calculating the values of the equilibrium constants that describe the formation of pesticide−BSA complexes.

As reported in the Material and Methods, the contribution to the proton selective relaxation rate because of the fraction of

Table 1. Experimental Values of R_{1N}^{NS} and R_{1N}^{SE} of the Three Pesticides in the Absence and Presence of Increasing Amounts of BSA, Normalized to the Relaxation Rates of the "Free" Fungicides[a]

		pyrimethanil				myclobutanil				triadimenol			
BSA concentration (mg/mL)	BSA concentration (mol dm^{-3})	R_{1N}^{NS} (s^{-1})	SD (%)	R_{1N}^{SE} (s^{-1})	SD (%)	R_{1N}^{NS} (s^{-1})	SD (%)	R_{1N}^{SE} (s^{-1})	SD (%)	R_{1N}^{NS} (s^{-1})	SD (%)	R_{1N}^{SE} (s^{-1})	SD (%)
0	0	1.00	11	1.00	1	1.00	1	1.00	1	1.00	1	1.00	1
1	1.45×10^{-5}	1.26	7	1.56	1	1.29	1	1.76	2	1.00	2	1.78	2
3	4.35×10^{-5}	1.22	4	2.91	2	1.30	2	4.30	3	0.88	1	2.52	4
4	5.80×10^{-5}	1.26	5	3.38	2	1.33	2	6.72	9	1.00	1	2.22	3
5	7.25×10^{-5}	1.39	4	3.79	2	1.40	2	7.17	10	1.00	2	2.72	3
8	1.16×10^{-4}	1.26	7	4.55	3	1.40	3	8.96	2	1.07	2	3.17	4

[a] Pyrimethanil, R_1^{NS} = 0.50 s^{-1} and R_1^{SE} = 0.40 s^{-1}; myclobutanil, R_1^{NS} = 1.05 s^{-1} and R_1^{SE} = 0.93 s^{-1}; triadimenol, R_1^{NS} = 0.99 s^{-1} and R_1^{SE} = 0.75 s^{-1}.

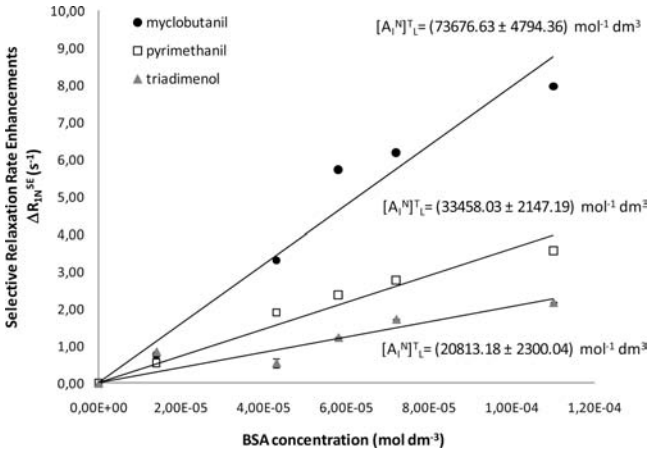

Figure 2. Experimental values of ΔR_{1N}^{SE} versus the BSA concentration together with data fitting and affinity index calculation.

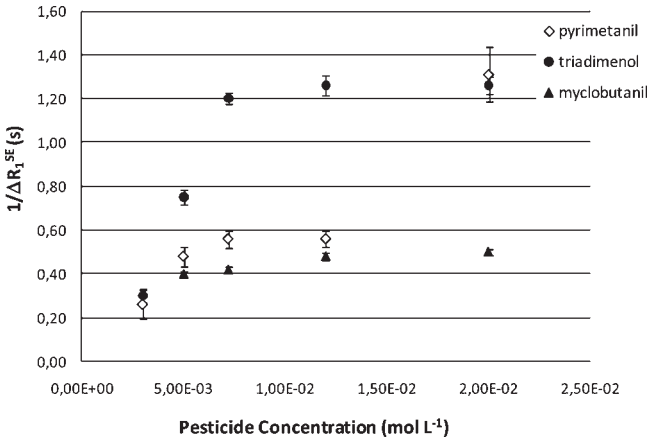

Figure 3. Experimental values of $1/\Delta R_1^{SE}$ in relation to the pyrimethanil, myclobutanil, and triadimenol concentrations ranging from 3×10^{-3} to 2×10^{-2} mol dm^{-3}.

pesticide bound to the protein may also be dependent upon the pesticide concentration. In particular, the reciprocal of relaxation rate enhancements measured varying pesticide amounts at a fixed BSA concentration, which typically shows a linear trend in relation to the pesticide concentration, can be used to calculate the thermodynamic equilibrium constant (K) as well as the relaxation rate of the bound ligand. **Figure 3** shows the experimental values of $1/\Delta R_1^{SE}$ in relation to the pyrimethanil, myclobutanil, and triadimenol concentrations ranging from 3×10^{-3} to 2×10^{-2} mol dm^{-3}. Data clearly showed the existence of binding site saturation processes occurring at pesticide concentrations higher than 1.2×10^{-2} mol dm^{-3}. The evidence that saturation of

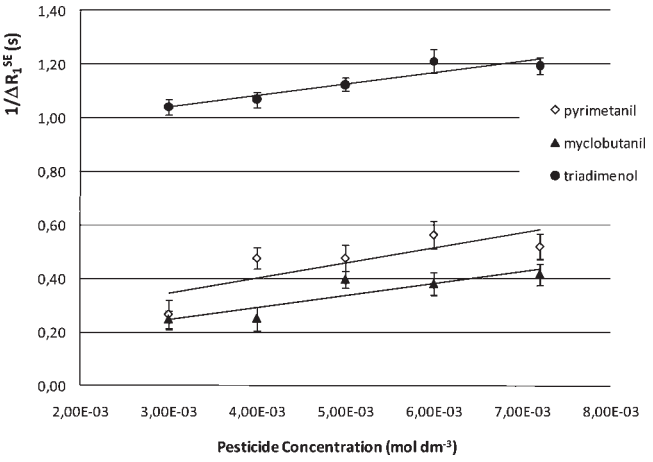

Figure 4. Experimental values of $1/\Delta R_1^{SE}$ versus the pyrimethanil, myclobutanil, and triadimenol concentrations ranging from 7.2×10^{-3} to 3.0×10^{-3} mol dm^{-3}.

BSA binding sites occurred at the same concentration for all of the tested pesticides, which, however, showed differences in the calculated affinity indexes, deserves some comments. Motion restriction of the pesticides at the protein interface because of the complex formation occurs independently of the nature of the binding site (i.e., specific or non-specific interaction). For this reason, the affinity index calculated from the proton relaxation rate enhancement caused by the change in the molecular dynamics of the ligand (i.e., from fast reorientational motion to slow motion) represents the "global" affinity of a ligand toward a macromolecule, providing an evaluation of the overall recognition process occurring through noncovalent binding. The fact that the three pesticides were able to saturate all of the binding sites on BSA at the same concentration but, at the same time, showing strong differences in the $[A_1^N]_L^T$ may suggest the existence of specific strong interaction processes involving mainly myclobutanil. It is known, in fact, that the carrier properties of BSA derive from the presence of two major and structurally selective binding sites (sites I and II), located in three homologous domains (*25*). The binding affinity of site I is essentially due to hydrophobic interactions, while site II involves either hydrophobic, hydrogen bonding, or electrostatic interactions (*26*). However, because both sites contain mainly hydrophobic residues, pesticide binding may be strongly stabilized by hydrophobic interactions. Moreover, BSA usually interacts with small ligands mainly through a single high-affinity binding site and several nonspecific binding sites (*27*). Thus, the differences in the affinity indexes of the three systems under investigation highlighted a more specific interaction of myclobutanil with BSA, with respect to pyrimethanil and triadimenol. As reported above, the

equilibrium constant for the complex formation can be calculated in the range of concentrations in which a linear dependence of $1/\Delta R_1^{SE}$ versus the pesticide concentration exists. For this reason, experiments in the range of the pesticide concentration in the linear regime were performed. In particular, concentrations ranged from 7.2×10^{-3} to 3.0×10^{-3} mol dm^{-3}. **Figure 4** reports $1/\Delta R_1^{SE}$ versus the pyrimethanil, myclobutanil, and triadimenol concentrations. Using eqs 10−12, R_{1B}^{SE} and K values were calculated. R_{1B}^{SE} values were 393.91 ± 0.01 s^{-1} (pyrimethanil), 517.45 ± 0.01 s^{-1} (triadimenol), and 493.88 ± 0.01 s^{-1} (myclobutanil). Equilibrium constant values for pesticide−BSA complexes were 494.43 ± 0.54 mol^{-1} dm^3 (myclobutanil), 315.37 ± 0.76 mol^{-1} dm^3 (pyrimethanil), and 46.73 ± 0.77 mol^{-1} dm^3 (triadimenol). The data highlighted that myclobutanil−BSA was the most stable complex and confirmed the weakness of the pyrimethanil−BSA interaction process, in agreement with affinity index results.

In this work, we investigated the interaction between three pesticides (myclobutanil and tradimenol, belonging to the triazole family, used since the 1970s, and pyrimethanil, belonging to the so-called "new generation" pesticide, used since the 1990s) and BSA. Interaction processes occurred for all of the tested pesticides, suggesting that myclobutanil, tradimenol, and pyrimethanil were able to form molecular complexes with BSA. Our approach, on the basis of nuclear spin relaxation data analysis, allowed for the calculation of the affinity index for the three systems. This index, calculated from the selective spin−lattice relaxation rate enhancements of pesticide protons caused by the presence of interactions occurring at the protein interface, is related to the strength of the recognition processes and collects the contributions of all of the protein binding sites. Moreover, nuclear spin relaxation data allowed for the calculation of the thermodynamic equilibrium constants for pesticide−BSA complex formation. Myclobutanil showed the highest affinity for BSA, whereas tradimenol gave the weakest interaction with the protein. The differences in the ability of the three fungicides to interact with BSA indicated a differentiation in their plasma residence time and availability and their capacity to diffuse from the circulatory system to body tissues.

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Received for review June 3, 2010. Revised manuscript received August 31, 2010. Accepted September 8, 2010. The authors thank the University of Siena for financial support.