



Interactions of tetracyclines with ovalbumin, the main allergen protein from egg white: Spectroscopic and electrophoretic studies

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ARTICLE INFO

Article history:

Received 22 December 2016

Received in revised form 8 February 2017

Accepted 12 April 2017

Available online 17 April 2017

Keywords:

Ovalbumin

Egg white

Interaction

Tetracyclines

Spectroscopic techniques

Electrophoresis

ABSTRACT

The interactions of tetracycline (TC), oxytetracycline (OTC) and chlortetracycline (CTC) with ovalbumin (OVA), the main allergen protein of egg white, were investigated by molecular spectroscopy and electrophoresis at three pH conditions (1.5, 4.6 and 7.4). Molecular and synchronous fluorescence, UV-vis spectroscopy, electrophoresis and ¹H NMR were used to study the interaction process. Tetracyclines interact with ovalbumin fluorescence by a static quenching mechanism with non-fluorescent complex formation changing the native protein structure. The binding constant (K_b) ranged from 2.11×10^4 to $58.4 \times 10^4 \text{ L mol}^{-1}$, and corresponding thermodynamic parameters were measured at different temperatures and pH values. The binding process was spontaneous ($\Delta G < 0$), and the magnitude of the interaction increased in the following order: TC < CTC < OTC. Hydrogen, electrostatic, and Van der Waals forces played a major role in stabilizing the complex. The distances between the donor (protein) and receptors (TC, OTC and CTC) were determined by FRET and varied of 2.95–3.52 nm. Fe(III) and Zn(II) ions increase the affinities of TC and CTC for OVA, while OTC did not suffer a significant influence by the competitor metallic species evaluated.

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1. Introduction

There are about 40 different proteins in egg white, which are source of amino acids and nitrogen that are required for growth, maintenance, and the general well-being of humans [1]. However, the egg is one of the foods whose allergenicity is most altered by cooking or processing, and egg whites contain proteins with considerably higher allergenic potential [2]. Ovalbumin (OVA) is the major protein from egg white, representing 54% (m/m) of the total protein content, which is comprised of a complex mixture of proteins such as ovotransferrin, ovomucoid, and lysozyme [3]. The chicken egg is one of the most consumed foods in the world because it is inexpensive and an ingredient with a high nutritional value [4]. The industrial importance of ovalbumin is related to its functional properties, in this case its gelation, foaming, and emulsifying potential, among others, which are useful in preparing a variety of food

types [5]. Moreover, ovalbumin is used in immunological studies as a carrier protein in vaccines and a model protein in egg sensitivity (allergy) tests [6].

Tetracycline antibiotics are often used as feed additives for food-producing animals because they are antimicrobial agents that exhibit activity against a large range of Gram (–) and Gram (+) bacteria. Due to their broad activity spectrum, low toxicity, and low cost, tetracyclines are widely used as veterinary drugs for the prevention and treatment of farm animals as well as used as additives to promote animal growth [7]. Among the most used tetracyclines for livestock intended for human consumption are tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC) and doxycycline (DC) [8].

The continuous and widespread use of tetracyclines in food-producing animals may result in the presence of their residues in food derivatives. Human exposure to these residues may cause adverse effects from allergic reactions to cancer. Furthermore, exposure to tetracycline residues can produce resistant microorganisms and debilitate the therapeutic action of these drugs [9].

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There are few studies related to the interaction between organic and inorganic species with OVA when compared to studies with human serum albumin (HSA) and bovine serum albumin (BSA). In one such study, Lu et al. studied the interaction between OVA and quercetin using spectroscopic techniques and molecular modelling simulations under physiological conditions [10]. The results suggested that the binding constant value was on the order of 10^4 L mol^{-1} and the main forces involved in the interaction were hydrophobic and hydrogen bonding.

Wang et al. evaluated the interaction of alkaloids present in coffee (caffeine, theophylline, and diprophylline) with OVA using spectroscopic techniques in physiological conditions [11]. The results showed that binding constant values were on the order of 10^4 L mol^{-1} , the distance between the protein and alkaloids varied from 2.20 to 2.25 nm and the main forces involved in the interaction were Van der Waals and hydrogen bonds. Shen et al. evaluated the interaction between OVA and lysozymes with green tea polyphenols simulating gastrointestinal digestion [12]. Additionally, Ognjenovi et al. conducted studies about the interaction between epigallo catechin-3-gallate and OVA. In both studies, the interaction results indicate binding constant values of approximately 10^4 L mol^{-1} [13].

The majority of studies evaluating interactions with OVA are associated with compounds present in foods. However, considering the inadequate practices of veterinary medicine use, the ovalbumin (food protein) could be an indirect contaminant to the consumer since it is widely distributed in different forms. In this context, there is no description of interaction studies of this food protein with veterinary drugs and investigations into the manner by which this process can toxicologically change these species. Thus, the objective of this work was to assess the interactions between OVA and tetracyclines (TC, OTC and CTC) to investigate the most important aspects governing this process *in vitro* at different pH values using spectroscopic techniques and electrophoresis.

2. Materials and methods

2.1. Chemicals and solutions

The protein ovalbumin (*albumin from chicken egg white*) and the antibiotics tetracycline (TC), oxytetracycline (OTC) and chlortetracycline (CTC) employed in the interaction studies were purchased from the Sigma-Aldrich Chemical Company (USA). The ovalbumin stock solution ($6 \mu\text{M}$) was prepared in a 100 mM solution of HCl (pH adjusted to 1.5) containing 100 mM NaCl, and then, the working solutions were prepared by appropriate dilution. Similarly, stock protein solutions were prepared in 50 mM acetic acid/acetate buffer (pH = 4.6) and 50 mM Tris buffer (pH = 7.4), with both containing 100 mM NaCl. The stock ovalbumin *in natura* (without purification) solutions were prepared from solubilizing the egg white aliquot directly in 50 mM Tris buffer (pH = 7.4) containing NaCl. The ovalbumin concentration (commercial and *in natura* form) was determined by the application of Beer's law based on the absorbance signal at 280 nm and molar extinction coefficient ($30957 \text{ M}^{-1} \text{ cm}^{-1}$) after appropriate dilution of the solution [14]. Tetracycline (TC, OTC and CTC) stock solutions were prepared by directly dissolving the solid in ultrapure water at 1.0 mM, and they were stored in amber bottles at 4 °C.

2.2. Fluorescence spectroscopy

Molecular fluorescence measurements were performed on Shimadzu spectrofluorimeter (model 5301PC, Japan) using quartz cuvettes with a 10 mm optical path, height of 45 mm, width of 12.5 mm and depth of 12.5 mm with four polished windows and

3.5 mL of chamber volume. The OVA ($2.0 \mu\text{M}$) fluorescence emission spectra in the absence and presence of tetracyclines ($2\text{--}80 \mu\text{M}$) were recorded from 250 to 450 nm employing $\lambda_{\text{ex}} = 280 \text{ nm}$. The excitation and emission slits were 3 and 5 nm, respectively. All fluorescence assays were carried out in the following three different pH conditions: 1.5, 4.6 and 7.4.

Synchronous fluorescence spectra of the OVA in the absence and presence of different amounts of tetracyclines were obtained by variation in simultaneous excitation and emission monochromators. The difference in the excitation wavelength ($\Delta\lambda = \lambda_{\text{em}} - \lambda_{\text{ex}}$) was individually fixed at $\Delta\lambda = 15 \text{ nm}$ and $\Delta\lambda = 60 \text{ nm}$, and thus, the spectrum shows only the spectroscopic behaviour of tyrosine and tryptophan residues, respectively [15].

To assess the protein-ligand interaction and to calculate the critical distance at which the energy transfer occurs from tryptophan residues of OVA ($2 \mu\text{M}$) to tetracyclines (TC, OTC and CTC all at $2 \mu\text{M}$), Förster resonance energy transfer (FRET) studies were performed [16].

2.3. Molecular absorption spectroscopy (UV-vis)

The molecular absorption measurements were performed in a Micronal scanning spectrophotometer (AJX-6100PC model, Brazil) with a double-beam equipped with quartz cuvettes having a 10 mm optical path, height of 45 mm, width of 12.5 mm and depth of 12.5 mm with two polished windows and 3.5 mL of chamber volume. The absorption spectra of complexes of ovalbumin and tetracyclines were recorded in the range of 220–450 nm.

2.4. Polyacrylamide gel electrophoresis (SDS-PAGE and native-PAGE)

The electrophoretic assays (SDS-PAGE and native-PAGE) were performed employing 10% (m/m) polyacrylamide gel. The pH values of the samples were fixed at 7.4, while the concentrations of OVA and the tetracyclines were $18 \mu\text{M}$ and 9 mM , respectively. Samples were analyzed under reducing and non-reducing conditions at a constant voltage (85 V) using the Mini Protean® system (Tetra Cell, Bio Rad). In the SDS-PAGE, the reducing agent 2-mercaptoethanol (5%, v/v) and sodium dodecyl sulphate (SDS, 2%, m/v) were added into the system. Gel without denaturing (native-PAGE) was performed in the absence of SDS and reducing agent. Protein molecular weight markers (Jena Bioscience) were employed in the experiments.

2.5. Protein–ligand interactions by ^1H NMR

In this study, the interactions between the TC and OVA were evaluated by profiling the ^1H NMR spectrum and observing variations in the chemical shifts (δ , ppm) of TC in the absence and presence of protein. For this purpose, we used 1.4 mg of OVA and 1 mM tetracycline in 600 μL of phosphate buffer (10 mM, pH = 7.4) with the addition of 10 μL of sodium trimethylsilylpropionate (TMSP) at 2.50 mM (internal standard). All solutions were prepared with deuterated water (Tedia, D_2O , 99.9%). A Bruker 400 NMR spectrometer ($B_0 = 9.4 \text{ T}$) operating at 400.35 MHz for ^1H and equipped with an indirect detection probe of 5 mm was used.

2.6. Statistical analysis

All experiments were performed in triplicate ($n = 3$) to ensure accuracy and reliability of the results. In the quantitative evaluations, the results were presented considering the mean value and the respective standard deviation (SD) of three independent experiments. The linear regression analyses for Eqs. (1)–(3) were

performed at a 95% confidence interval using the software Origin-Pro 9 (OriginLab Corporation).

3. Results and discussion

The interaction studies between OVA and TC, OTC and CTC were performed at different pH conditions. The pH values were selected to simulate the biological environment of the protein in the egg (pH = 7.4), at the isoelectric point of the protein (pH = 4.6) and finally, in stomach conditions (pH = 1.5).

3.1. Kinetic study

The effect of time on the interaction process between OVA and TC (compound selected as a model for the presentation of the results) was evaluated at pH values of 1.5, 4.6 and 7.4. The fluorescence ratio (F_0/F) between the protein intrinsic fluorescence ($\lambda_{em} = 335$ nm) in the absence (F_0) and presence of TC (F) was used as the analytical parameter. In this study, the interaction between OVA and TC occurred immediately regardless of the pH. Therefore, the kinetic interaction was favourable and showed good stability for the time investigated (60 min).

3.2. Evaluation of the mechanism of interaction and the binding constant

To evaluate the interactions between tetracyclines (TC, OTC and CTC) and OVA, spectrofluorometric titration in the stationary state based on the protein intrinsic fluorescence was explored [17]. The fluorescence spectral profile of OVA titrated with TC is shown in Fig. 1a. Similar profiles were obtained for OTC and CTC (Fig. S1a and S1b, supplementary material).

Ovalbumin has three tryptophan residues (Trp160, Trp194 and Trp275). Thus, if the protein structure changed due to interaction, these residues might stay more accessible. The structure of OVA indicating the tryptophan residues is shown in Fig. S1c (supplementary material). The ovalbumin presented an intense and broad band centred at 335 nm when excited at 280 nm, and the addition of tetracyclines to the system led to a decreased intensity and emission band shift from 335 to 331 nm. The reduction in the intensity of the signal and maximum wavelength shift was assigned to conformational changes in the protein [18].

As proven, the interactions of TC, OTC, and CTC with OVA resulted in a reduction of the intrinsic fluorescence of the protein. To examine the mechanism of interaction between tetracyclines and OVA, the Stern-Volmer equation was applied as follows:

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

where F_0 and F are the fluorescence intensities in the absence and presence of the ligand, respectively, K_q is the bimolecular diffusion quenching rate constant ($2.0 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}$) and τ_0 is the average lifetime (10^{-8} s). $[Q]$ is the ligand concentration (quencher) and K_{SV} is the Stern–Volmer quenching constant [19]. The binding constant was calculated according to Eq. (2):

$$\log \frac{F_0 - F}{F} = n \log K_b + n \log \left(\frac{1}{[Q] - \frac{(F_0 - F)[P]_T}{F_0}} \right) \quad (2)$$

where K_b is the binding constant, n is the number of binding sites and $[P]_T$ is the total protein concentration. Fig. 1b and c show linearization of Eqs. (1) and (2), respectively, to calculate the binding parameters K_{SV} , K_b and n [20]. Thus, the effects of the pH value (1.5, 4.6 and 7.4) and temperature (22, 30 and 38 °C) on the interactions of the three tetracyclines with OVA were evaluated. The results of this study are presented in Table S1 (supplementary material).

Dynamic quenching is indicated when K_{SV} values increase with the temperature since higher temperatures lead to a larger diffusion coefficient and therefore a greater number of collisions between the fluorophore (protein) and quencher (tetracyclines). When the constant values decrease with increasing temperature, it indicates static quenching since higher temperatures affect the stability of the fluorophore-quencher complex or, in this particular case, the protein-ligand complex. According to the results (Table S1), the values of the quenching constant (K_{SV} and K_q) decreased with increasing temperatures independent of the ligand and pH. Thus, the interaction process among the three tetracyclines and OVA occurred predominantly through the static quenching mechanism based on non-fluorescent complex formation [21].

According to Shu et al., when the K_q is less than $2.0 \times 10^{10} \text{ L mol}^{-1} \text{ s}^{-1}$ dynamic quenching occurs, while higher K_q values are indicative of a static quenching mechanism [22]. The K_q values obtained in this study (Table S1) varied from 1.37 to $5.69 \times 10^{12} \text{ L mol}^{-1} \text{ s}^{-1}$, and thus, the interaction processes of TC, OTC, and CTC with OVA occurred through static quenching. Finally, the K_{SV} values decreased with increasing pH values. This fact can be related to the exposure of the major tryptophan residues to a more acidic medium, leading to less folding in the protein structure causing greater interaction [14].

Once the interaction mechanism was defined using molecular fluorescence, it was necessary to evaluate the degree of interaction among TC, OTC, and CTC with OVA. The affinity of a particular ligand with OVA is a relevant parameter for the protein–ligand interaction study, and it can be expressed by binding constant (K_b) values and the number of sites occupied (n) by the ligands in the protein structure.

Tetracyclines have different chemical functional groups in their structure, and depending on the pH of the solution, they could be in a protonated or deprotonated form. Therefore, the global charge of the tetracycline will differ depending on the pH, and thus, the interaction with the protein may differ depending on the hydrogen ion concentration of the solution. Based on the three different pK_a values of TC, it could exist in solution in up to four different species with various degrees of protonation depending on the pH as follows: H_3TC^+ , H_2TC , HTC^- and TC^{2-} . The species distribution as a function of the pH is shown in Fig. S2 (supplementary material). Each of these species shows acid-base behaviour and different global charges. Therefore, in studies about the interaction between a ligand and a macromolecule, it is important to know the acid-base aspects of the ligand derived from a weak acid and its particular charge at a particular pH value. Thus, the fully associated TC form (H_3TC^+) will be predominant at $pH < 3$ with a positive charge. The neutral form (H_2TC) will be predominant when $3.5 < pH < 6.5$, while the anionic form (HTC^-) will be predominant in the pH range of 8–9. Finally, the dianionic specie (TC^{2-}) will be predominant for $pH > 10$. The same logic can be used for the OTC and CTC as a function of pH with small variations due to pK_a differences. Therefore, the interaction of a tetracycline with the protein depends on its protonated form.

For the three pH values assessed (1.5, 4.6 and 7.4), the magnitude of the interaction (K_b) increased in the following order: $TC < CTC < OTC$ (Table 1). The OTC was the tetracycline that interacted most efficiently with OVA, whereas TC showed the lowest degree of interaction with the protein. This fact may be associated with an additional hydroxyl group in the OTC structure, facilitating the formation of an additional hydrogen bond compared with TC and CTC. The binding constants to tetracyclines decrease with pH, and the temperature is increasing, as expected due to the static quenching mechanism [23]. At $pH < 2$, the α -helix structure of OVA is changed to a rich β -sheet structure according to Kang et al. [24]. Therefore, the amino acids become more accessible to the ligands favouring the interaction process and leading to an increased K_b .

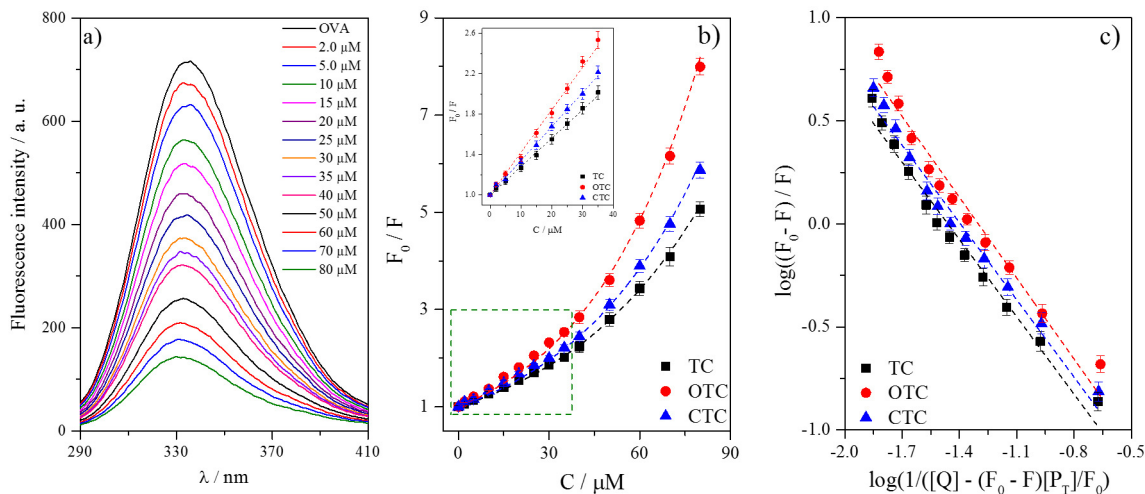


Fig. 1. a) Emission spectral profile of OVA (2 μM) at different concentrations of TC at a pH of 7.4 and 30 °C. b) Stern-Volmer quenching plot (linear range used for inset graph). c) Double logarithmic curve for calculation of the binding constant. Data represent mean ± SD of three experiments (n = 3).

Table 1
Binding and thermodynamics parameters of the interaction of OVA with tetracyclines at different pH and temperature conditions. Data represent mean ± SD of three experiments (n = 3).

Ligand	pH	Temperature (°C)	Binding constant			Thermodynamic parameters			
			$K_b (\times 10^4 \text{ L mol}^{-1})$	n	r	$\Delta G (\text{kJ mol}^{-1})$	$\Delta H (\text{kJ mol}^{-1})$	$\Delta S (\text{J mol}^{-1})$	Preferential interaction
TC	1.5	22	25.8 ± 0.3	1.10 ± 0.11	0.9876	−30.59	−40.24	−32.71	Hydrogen bond Van der Waals forces
		30	16.9 ± 0.6	1.06 ± 0.02	0.9885	−30.32			
		38	11.1 ± 0.2	1.29 ± 0.06	0.9864	−30.06			
	4.6	22	4.68 ± 0.11	1.14 ± 0.06	0.9964	−26.30	−23.85	+ 8.31	Electrostatic
		30	3.53 ± 0.16	1.12 ± 0.07	0.9945	−26.36			
		38	2.84 ± 0.10	1.02 ± 0.05	0.9956	−26.43			
	7.4	22	3.16 ± 0.19	1.13 ± 0.12	0.9912	−25.47	−19.22	+ 21.20	Electrostatic
		30	2.74 ± 0.23	1.14 ± 0.13	0.9878	−25.64			
		38	2.11 ± 0.21	1.16 ± 0.14	0.9854	−25.81			
OTC	1.5	22	58.4 ± 0.5	1.23 ± 0.09	0.9920	−32.63	−36.24	−12.22	Hydrogen bond Van der Waals forces
		30	41.7 ± 0.3	1.25 ± 0.11	0.9898	−32.53			
		38	27.3 ± 0.2	1.26 ± 0.10	0.9894	−32.44			
	4.6	22	5.85 ± 0.30	1.07 ± 0.05	0.9954	−26.92	−16.84	+ 34.17	Electrostatic
		30	4.89 ± 0.33	1.11 ± 0.04	0.9964	−27.19			
		38	4.11 ± 0.24	1.15 ± 0.05	0.9938	−27.46			
	7.4	22	5.32 ± 0.09	0.95 ± 0.04	0.9956	−26.56	−14.15	+ 42.07	Electrostatic
		30	4.26 ± 0.11	0.97 ± 0.05	0.9952	−26.90			
		38	3.96 ± 0.07	1.01 ± 0.04	0.9970	−27.23			
CTC	1.5	22	32.4 ± 0.4	1.26 ± 0.14	0.9985	−31.31	−46.83	−52.60	Hydrogen bond Van der Waals forces
		30	24.3 ± 0.5	1.18 ± 0.13	0.9991	−30.89			
		38	12.1 ± 0.3	1.15 ± 0.10	0.9986	−30.47			
	4.6	22	5.08 ± 0.28	1.16 ± 0.04	0.9980	−26.61	−14.94	+ 39.58	Electrostatic
		30	4.59 ± 0.21	1.15 ± 0.03	0.9987	−26.93			
		38	3.71 ± 0.22	1.25 ± 0.07	0.9957	−27.25			
	7.4	22	3.71 ± 0.08	1.02 ± 0.02	0.9987	−25.78	−19.83	+ 20.18	Electrostatic
		30	2.89 ± 0.11	1.01 ± 0.02	0.9983	−25.94			
		38	2.45 ± 0.13	1.05 ± 0.01	0.9994	−26.10			

under this condition. At pH values of 4.6, which is the isoelectric point (pI) of OVA, the protein is neutrally charged [25], and the tetracyclines are also in their neutral forms. In this case, the interaction process was less favoured at a pH of 1.5, yet it was more effective than at a pH of 7.4. Finally, at a pH of 7.4, both OVA and tetracyclines have a negative charge, and thus, the interaction was less favoured possibly due to some repulsion between the protein and ligand.

After evaluation of the binding constants, the binding site number (n) values were calculated, where it was observed that for TC, OTC and CTC, the values range from 0.95 to 1.29, indicating that the interactions between these molecules and macromolecule occur in a ratio of 1:1.

Tetracycline was selected as a model to evaluate the binding constant (K_b) against OVA *in natura* (directly from egg white without purification) at a pH of 7.4 (30 °C). The K_b was $6.61(\pm 0.09) \times 10^5 \text{ L mol}^{-1}$ and $n = 1.48$ ($r = 0.9991$). From these results, the binding constants for the protein *in natura* were greater than for the evaluated OVA, probably due to the presence of other proteins in the medium when compared with commercial and purified OVA. This result shows that the presence of antibiotics in food can be a route of contamination to humans, and they can possibly be associated with toxic and allergic effects of certain foods or derivatives.

3.3. Thermodynamic parameters

The study of the fluorescence interaction process at different temperatures allowed calculation of the thermodynamic parameters involved in the interaction. According to Wang et al. [11], from the thermodynamic parameters, it is possible to infer the nature of the forces involved in the protein-ligand interaction and the spontaneity of the process. Thermodynamic parameters (ΔH and ΔS) shown in Table 1 were calculated according to the linearization of the Van't Hoff equation, whereas the heat of reaction does not change with temperature [26]:

$$\ln K_b = -\frac{\Delta H}{R} \cdot \left[\frac{1}{T} \right] + \frac{\Delta S}{R} \quad (3)$$

where T is the temperature in Kelvin (K) and R is the ideal gas constant ($8.314462 \text{ J K}^{-1} \text{ mol}^{-1}$). The Gibbs free energy was calculated by Eq. (4):

$$\Delta G = \Delta H - T\Delta S \quad (4)$$

Considering the negative values of ΔG obtained for TC, OTC and CTC, the evaluated interaction process was thermodynamically spontaneous. The main relationships amongst the forces involved in the interaction process and the enthalpy and entropy are as follows: $\Delta H > 0$ and $\Delta S > 0$ (hydrophobic forces); $\Delta H < 0$ and $\Delta S > 0$ (electrostatic forces) and $\Delta H < 0$ and $\Delta S < 0$ (hydrogen bonding and Van der Waals forces) [27]. Regarding the thermodynamics parameters, ΔH values were negative indicating exothermic interaction processes for all of the tetracyclines.

The ability of a protein to bind selectively and with high affinity to a ligand depends on the noncovalent formation of some weak bonds such as hydrogen bonds, electrostatic interactions, and Van der Waals interactions in addition to hydrophobic interactions [28]. Thus, at a pH of 1.5, the protein and tetracyclines are positively charged so the electrostatic interactions are less effective, with hydrogen bonds and Van der Waals forces being predominant. Regarding experiments at pH values of 4.6 and 7.4, the electrostatic interaction was prevalent due to the presence of groups in the ligand and protein with negative and positive charges. Once the ligand-protein interaction is established, the water molecules associated with the protein (or solvent) are displaced. Thus, the large number of solvent molecules is responsible for the positive change in the system entropy during the interaction [29].

3.4. Synchronous fluorescence

To observe possible changes in the polarity of the OVA microenvironment related to tyrosine and tryptophan residues in the presence of different tetracyclines, studies by synchronized fluorescence were performed [30]. These results also show which tetracyclines changed the OVA microenvironment more significantly. The Stern-Volmer constant (K_{SV}) based on $\Delta\lambda = 15 \text{ nm}$ (tyrosine) and 60 nm (tryptophan) was the parameter to assess interactions at different pH values and with various tetracyclines. The results for TC in the different pH conditions are shown in Fig. S3 (supplementary material). A similar spectral profile was obtained for OTC and CTC in the interaction process. A summary of all synchronous fluorescence results is available in the supplementary material (Table S2, supplementary material).

According to the results (Fig. S3), it was observed that there was no maximum emission shift at pH values of 1.5 and 4.6 for the tyrosine residue, regardless of the tetracycline evaluated against the OVA. However, at a pH of 7.4, a maximum protein emission shift (1–4 nm) occurred for all of the evaluated tetracyclines (Table S2). This behaviour was possibly due to that at lower pH values, the protein structure was not organized as at the normal egg pH. Therefore, the interactions with the evaluated tetracyclines did

not influence the exposure of tyrosine residues (pH = 1.5 and 4.6). Since the amino acids residues were probably already exposed to the solvent (water), which is an opposite situation with regards to the conformation of OVA at a pH of 7.4. Moreover, the tyrosine residues are less sensitive to changes in the polarity of the medium in comparison to tryptophan [31].

Tryptophan residues are more sensitive to alterations in the solution (increasing or decreasing the polarity) and were affected at all pH values, regardless of tetracycline. Maximum emission shifts of the protein were observed from 1 to 2 nm (Table S2). These results show that the evaluated tetracyclines altered the OVA structure to an unfolded conformation when compared with the native structure. Therefore, the tyrosine (pH = 7.4) and tryptophan (all pH values) residues of OVA became exposed to a more polar microenvironment after interaction with tetracyclines.

Finally, according to Table S2, based on the K_{SV} constants among the assessed tetracyclines, OTC presented the highest values. This result indicates that OTC alters the OVA conformation more effectively than other tetracyclines, confirming the greater K_b values. This fact may be associated with the additional hydroxyl group (compared to TC and CTC), which increases the possibility of hydrogen bond formation and hence the degree of interaction with the protein leading to changes in its structure.

3.5. Tridimensional fluorescence (3D)

The conformational changes in the OVA secondary structure were characterized by tridimensional fluorescence spectral comparison in the absence and presence of tetracyclines simulating the pH of eggs (pH 7.4). The results of the evaluation with TC are given in Fig. 2. The spectral profiles for the evaluations with OTC and CTC are available in the supplementary material (Fig. S4).

The 3D fluorescence graphics of free OVA (Fig. 2a) and after the addition of TC (Fig. 2b) presented three peaks (1, 2 and 3). Peak 1 corresponds to Rayleigh scattering, which is characterized by radiation re-emission ($\lambda_{ex} = \lambda_{em}$) by the solvent in which the compound is dissolved, and a small fraction of the absorbed radiation is scattered in all directions at the same wavelength [19]. Peak 2 corresponds to the emission of tryptophan and tyrosine residues, and peak 3 is the emission of the polypeptide backbone of the OVA [32]. After the addition of TC, the fluorescence intensities of peaks 2 and 3 were reduced by approximately 60 and 78%, respectively. According to Zhang et al., when the ligand and protein are in the same chemical system and a change in the excitation wavelength (λ_{ex}) or emission wavelength (λ_{em}) related to the fluorescence peaks is observed, it indicates that the protein structure was altered when interacting with the ligand [33].

Similar spectral profiles of 3D fluorescence graphics for OTC and CTC in the presence of OVA (Fig. S4b and S4c) were obtained when compared with TC (Fig. 2b) due to a significant fluorescence reduction in peaks 2 and 3, indicating interactions of OTC and CTC with the protein. Tables S3 and S4 (supplementary material) present a compilation of the results of the 3D fluorescence studies of OVA in the presence of TC, OTC, and CTC at a pH of 7.4. The results indicate that the addition of tetracyclines to the OVA system led to a decrease in the fluorescence intensity of peaks 2 and 3 of the protein. When OTC was added, peaks 2 and 3 had intensity reductions of 65.7 and 71.9%, respectively, while for CTC, reductions of 69.5 and 76.5%, were observed, respectively.

The decrease of peak 2 indicated changes in the tyrosine and tryptophan microenvironments, and the reduction of peak 3 shows the modification of the native structure of the protein. This behaviour was possibly due to alterations in the polypeptide protein chain, thus changing the microenvironments of the amino acids and the original degree of folding [11]. Finally, based on the synchronous and 3D fluorescence, it became evident that there was

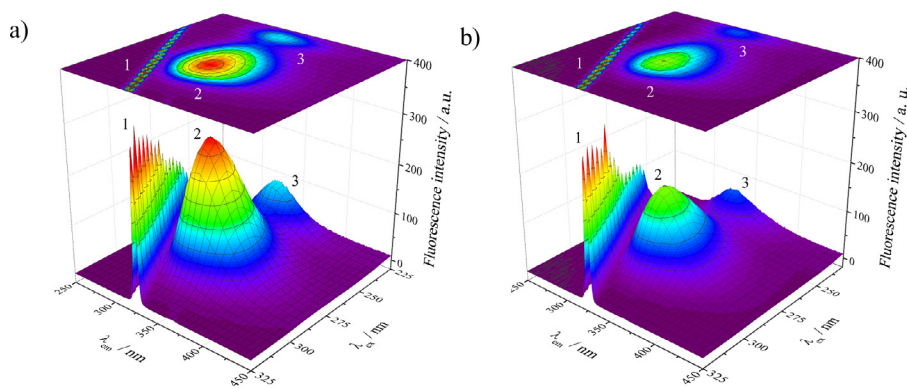


Fig. 2. Tridimensional fluorescence spectra of (a) OVA and (b) the OVA-TC complex at a pH of 7.4. The protein and ligand are at 2 and 20 μ M, respectively.

a change in the native OVA structure, which can range from the digestibility of the protein to justifying the appearance of allergies [2].

3.6. Förster resonance energy transfer (FRET)

The FRET studies were conducted to determine the distance of the energy transfer that occurs upon the interaction of OVA tryptophan (donor) with TC, OTC and CTC (receptors). The distance (r_0) between the donor and receptor can be calculated using Eq. (5):

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r_0^6} \quad (5)$$

where F_0 and F are the fluorescence intensities of the donor in the absence and presence of the ligand, respectively. E is the energy fraction transferred from the donor to the receptor, r_0 is the distance between the ligand and the donor, and R_0 is the Förster critical distance when 50% of the donor emission energy is transferred to the receptor. The critical distance (R_0) is described as follows:

$$R_0^6 = \frac{8.8 \times 10^{-12} k^2 \Phi J}{N^4} \quad (6)$$

where k^2 is the dipole orientation of the donor and receptor ($k^2 = 2/3$), N is the medium refractive index ($N = 1.36$), Φ is the fluorescence quantum yield of the protein ($\Phi = 0.118$), and J represents the overlap area of the normalized emission spectrum of the donor about the absorption spectrum of the receptor. The overlap area (J) can be calculated by the following equation:

$$J = \frac{\sum F(\lambda) \varepsilon(\lambda) \lambda^4 \Delta \lambda}{\sum F(\lambda) \Delta \lambda} \quad (7)$$

where $F(\lambda)$ is the fluorescence of the donor and $\varepsilon(\lambda)$ is the molar absorption coefficient of the receptor, which can be obtained by Beer's law [34].

The area of overlap of the fluorescence spectrum of free OVA and the molecular absorption of TC, OTC and CTC at three pH values (1.5, 4.6 and 7.4) was evaluated, and the spectral overlap chart between OVA and tetracyclines is shown in Fig. S5. Table S5 (supplementary material) displays the calculated parameters related to the FRET process.

According to the results obtained (Table S5), the transferred energy (E) ranged from 9 to 21%, indicating less than 50% of the energy was transferred, which implies that $R_0 < r_0$. The values of the distances of r_0 to TC, OTC and CTC ranged from 2.95 to 3.52 nm. Thus, the r_0 critical distance between tetracyclines and the tryptophan residue of the OVA was less than 8–10 nm [22]. These results indicated that non-radiative energy transfer among the evaluated tetracyclines and ovalbumin was influenced by variation in the pH. The obtained results, represented as the binding constant

(K_b), number of binding sites (n) and distance between the ligand and donor (r_0), were in agreement with the literature works, as described in Table S6 (supplementary material).

3.7. Metal ion competition

The influence of some competing ions including Ca(II), Mg(II), Zn(II), Cu(II) and Fe(III) on the interaction of OVA with tetracyclines was evaluated only at the food pH (pH = 7.4). In this process, the following possibilities can occur: i) the species compete for the same site as the ligand in the protein; ii) the formation of competitor-tetracycline complexes occurs, and these complexes interact less or more with the protein and iii) both processes occur simultaneously. These cations were selected because of their presence in the food (egg). The ratio of the binding constant in the presence of competitor (K_b') and absence (K_b) was used as a parameter for comparison. Thus, when $K_b'/K_b > 1$, complex formation is favoured, and oppositely, when $K_b'/K_b < 1$, protein and tetracycline complex formation is disfavoured. The results are shown in Fig. 3.

Based on the results (Fig. 3), the addition of the ions Ca(II), Zn(II), Cu(II) and Fe(III) increased the K_b values between TC and OVA. In contrast, the addition of Mg(II) ions decreased the binding constant. For OTC, the addition of Ca(II), Mg(II), Zn(II), Cu(II) and Fe(III) ions reduced the K_b values compared to complex formation in the absence of metal ions. For CTC, the addition of Ca(II), Mg(II) and Cu(II) ions led to a decrease in the value of the constant. However, the addition of Zn(II) and Fe(III) ions resulted in an increase of the K_b value. When the addition of an ion increases the binding constant, this may imply that the presence of this species altered the protein structure making it more favourable to interact with the ligand. When the opposite occurs, there is competition between the ion and the ligand [35].

3.8. UV-vis evaluation

To evaluate the effect of the pH on the interaction of OVA with tetracyclines, experiments by UV-vis were also performed, and these results are shown in Table S7 and Fig. S6 (supplementary material).

The tetracyclines have two chromophore regions; the first is located in ring A, and the second in the BCD rings. These areas present three UV absorption bands at 365, 270 and 217 nm, respectively. The π - π^* transition about the tricarbonyl ring (system A) contributes only to the absorption band at 270 nm, while the π - π^* transition of the BCD chromophore contributes to all of the absorption bands [36].

The OVA absorption is due to tryptophan and tyrosine residues, and these amino acids preferably present π - π^* transitions. When OVA is added to the medium containing the tetracyclines, an

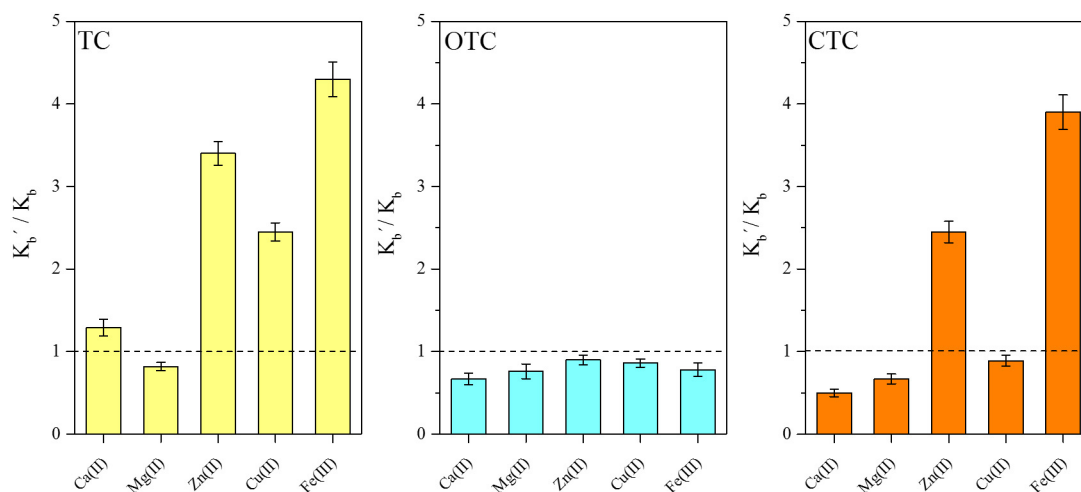


Fig. 3. Influence of metal ion competitors on the interaction process of OVA and tetracyclines. Protein and competitors 2 μ M; tetracyclines 2–80 μ M at a pH of 7.4 and 30 °C. Data represent mean $\pm$ SD of three experiments ($n = 3$).

increase in the absorption bands at 277 and 374 nm occurs due to the formation of the protein-ligand complex, wherein the pH of the medium may lead to a disadvantage for complex formation.

Changes in the protein absorption spectrum can be observed after the addition of the ligand, with the absorbance increasing and the maximum absorption shifting. When there is a progressive increase in the absorbance following the addition of a ligand, it is an indication of protein-ligand complex formation [37]. For this purpose, the signal from the complex formed should be different (greater or minor) than the sum of the signals of the free protein and ligand. If the sum of the signals of free species equals that of the mixture, it would be only an additive effect, without evidence of supramolecular complex formation. Fig. S6 shows that with increasing pH, a decrease in the difference between the complex spectra and the spectra of the free tetracyclines occurred.

Table S7 presents the quantitative evaluation of variation in the absorbance bands shown in Fig. S6. As noted, the difference calculated by $\Delta A = A_{\text{complex}} - (A_{\text{OVA}} + A_{\text{tetracycline}})$ was never zero, indicating that in all cases, complex formation occurred between the protein and ligand. This profile remained according to the two wavelengths monitored. The wavelength 277 nm was selected for the original protein band and the wavelength of approximately 374 nm (with variations of ± 5 nm depending on the evaluated tetracycline) was selected for the ligands. In both cases, the observed profiles were similar, as shown in Table 3. The variation was directly related to conformational changes of the protein and the tetracycline charge distribution due to the pH of the medium.

The increase of the pH led to a decrease in the absorbance variation (ΔA), and therefore, the formation of the complex was disfavoured (for all tetracyclines). This fact corroborates with the values of constants calculated by fluorescence, where the greatest constants were obtained at a pH of 1.5, and the constants were minor when the pH was 7.4.

3.9. Electrophoresis

To evaluate the electrophoretic migration effect of the commercial and *in natura* (from egg white) protein in the absence and presence of tetracyclines, studies employing polyacrylamide gel electrophoresis (native-PAGE and SDS-PAGE) were performed. In this evaluation, the effect of the addition of tetracyclines (9.0 mM, 500 times excess) was evaluated in relation to OVA (commercial and *in natura*) at 18 μ M (pH = 7.4). The results are presented in Fig. 4.

Based on the results obtained, the native-PAGE (Fig. 4a) showed three bands, and SDS-PAGE (Fig. 4b) showed only two bands. The changes in the OVA after the TC and CTC additions include protein band enlargement as well as an increase in the electrophoretic mobility. The addition of OTC caused band enlargement too, but it reduced the electrophoretic mobility of the protein.

According to Sun and Pan, when there are protein-ligand interactions, the electrophoretic mobility is altered, and migration occurs in a different way compared to that of the protein in the absence of ligand [38]. Based on the results obtained, there was a higher electrophoretic migration when TC and CTC were added to the commercial and *in natura* ovalbumin. This behaviour was possibly due to that the protein become more anionic when interacting with TC and CTC [39]. On the other hand, the addition of OTC led to a reduction in the electrophoretic migration for both sets of conditions.

The additions of TC and CTC led to increases in the electrophoretic migrations of both proteins (commercial and *in natura*) due to surface interactions changing the overall charge of OVA. The results of electrophoresis confirm the results of molecular fluorescence and UV-vis studies as well as confirming the interaction between the tetracyclines and OVA.

3.10. Evaluation of the interaction by ^1H NMR

For this study, TC was selected as a model and the chemical shift (δ) was one of the parameters selected to monitor the effect of interaction with the protein by the nuclear magnetic resonance of hydrogen (^1H NMR) at a pH of 7.4 (normal egg pH). Sodium trimethylsilylpropionate (TMSP) was used as an internal reference standard. The results of this study are presented in Fig. 5 and Table 2.

The ^1H NMR spectra of TC in the absence and presence of OVA with all of the hydrogens assigned are shown in Fig. 5. In the presence of OVA, the TC signals were observed to be enlarged, and the chemical shifts varied for all of the hydrogens. These changes were caused by the intermolecular interaction between the TC and OVA. Since the chemical shift is a parameter determined by the local magnetic field and is associated with the electrons that surround the nucleus, often this field opposes the external field applied. The shifts of the TC signals in the presence of OVA suggested that the effective magnetic field of the TC decreased after interaction with the protein [40]. The variation in the chemical shifts ($\Delta\delta$) for TC hydrogen atoms in the absence and presence of OVA were calculated and verified that $\Delta\delta$ varied from 0.01 to 0.02 (Table 2),

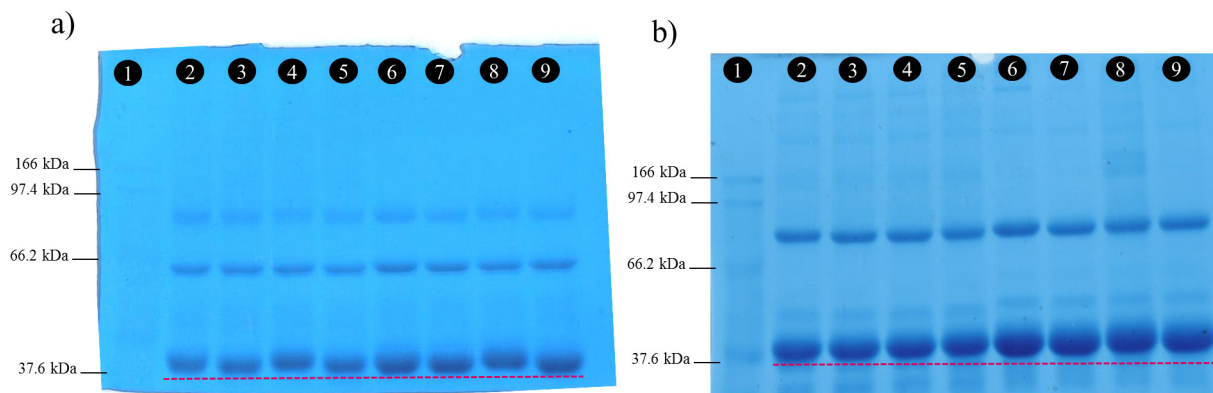


Fig. 4. a) Native-PAGE and b) SDS-PAGE. Planes: 1) protein molecular weight, 2) OVA, 3) OVA + TC, 4) OVA + OTC, 5) OVA + CTC, 6) OVA *in natura*, 7) OVA *in natura* + TC, 8) OVA *in natura* + OTC and 9) OVA *in natura* + CTC. Ovalbumin (commercial and *in natura*) and tetracyclines were at a pH of 7.4.

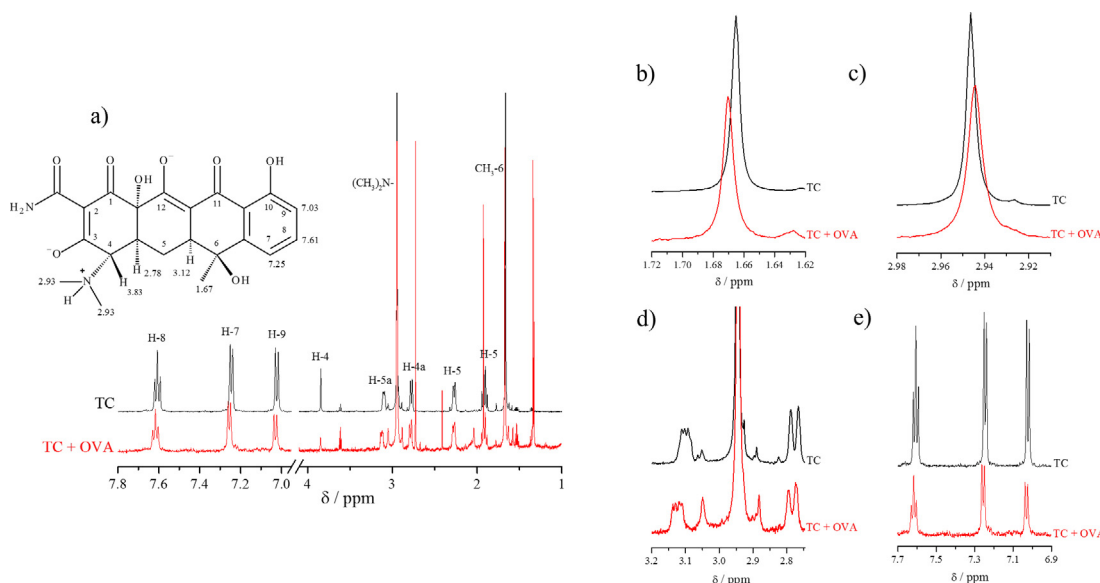


Fig. 5. a) ^1H NMR spectra (400 MHz) of TC in the absence and presence of OVA at a pH of 7.40. Expansion of different regions (b, c, and d) of the TC and TC-OVA spectra.

Table 2

Chemical shift (δ) variation and longitudinal relaxation time (T_1) for TC in the absence and presence of OVA at a pH of 7.4.

Hydrogen	δ , ppm (mult., J (Hz))		$\Delta\delta$	T_1 (s)	
	TC	TC + OVA		TC	TC + OVA
H-8	7.61 (t) 8.4 Hz	7.62 (t) 9.2 Hz	0.01	1.45	1.52
H-7	7.24 (d) 7.6 Hz	7.25 (d) 8.31 Hz	0.01	0.86	0.84
H-9	7.02 (d) 9.3 Hz	7.03 (d) 9.1 Hz	0.01	2.60	2.32
H-4	3.85 (s)	3.84 (sl)	0.01	0.63	0.78
H-5a	3.10 (dd)	3.12 (dd)	0.02	0.58	0.52
(CH_3) ₂ N-	2.95 (s)	2.94 (s)	0.01	0.83	0.72
H-4a	2.77 (dd)	2.78 (dd)	0.01	0.56	0.54
H-5	2.27; 1.91 (d)	2.27; 1.91	0.00	0.69	0.59
CH_3 -6	1.66 (s)	1.67 (s)	0.01	0.27	0.26

indicating that all of the TC hydrogen atoms were shifted when interacting with the OVA.

To evaluate which preferential region of the TC is interacting with the OVA, longitudinal relaxation time studies (T_1) were accomplished. The values of T_1 to all of the TC hydrogen atoms in the absence and presence of OVA were calculated (Table 2). From the results obtained, the T_1 values for TC hydrogens H-8 and H-4 increased in the presence of OVA, while those for H-7, H-9, H-5, H-5a, -N(CH_3)₂, H-4a and CH_3 -6 decreased after the addition of protein. In this manner, the TC region where the hydrogen atoms had

a reduction in the longitudinal relaxation time preferentially interacted with OVA [41]. Due to macromolecules relaxing quickly due to their high molecular weights, the ligand hydrogens are closer to the ovalbumin and have their relaxation time decreased.

4. Conclusions

This work showed that the tetracyclines evaluated bind to OVA with a high affinity, demonstrating the potential for food contamination and enabling problems related to allergies. The magnitude

of the interaction process followed the order TC < CTC < OTC, and static quenching was the preferential mechanism. The thermodynamic parameters indicate that the predominant binding forces are hydrogen bonds, Van der Waals forces and electrostatic interactions depending on the pH value of the medium. Through 3D and synchronous fluorescence, changes in the native protein structure induced due to interaction process with tetracyclines were verified. By FRET, the intermolecular distances between OVA and tetracyclines were found to range from 2.95 to 3.52 nm. Competition studies showed that Fe(III) and Zn(II) ions increase the affinities of TC and CTC for protein, while OTC did not suffer a significant influence by the competitor metallic species assessed. From the electrophoresis studies exploring SDS-PAGE and native-PAGE, it was observed that TC and CTC changed the migration profile of the OVA band more significantly. Finally, through ^1H NMR studies employing chemical shift (δ) and T_1 values, it was possible to verify the preferential region of interaction between TC and OVA.

Compliance with ethics requirements

This research does not contain any studies with human or animal subjects.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgements

M.D.A. Dantas wishes to thank CAPES for a Master of Science scholarship. The authors are grateful for the financial support from the CNPq (Grant Number 472145-2012-0) and FAPEAL (Alagoas, Brazil).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ijbiomac.2017.04.052>.

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Supplementary material – FIGURES

Interactions of tetracyclines with ovalbumin, the main allergen protein from egg white: spectroscopic and electrophoretic studies

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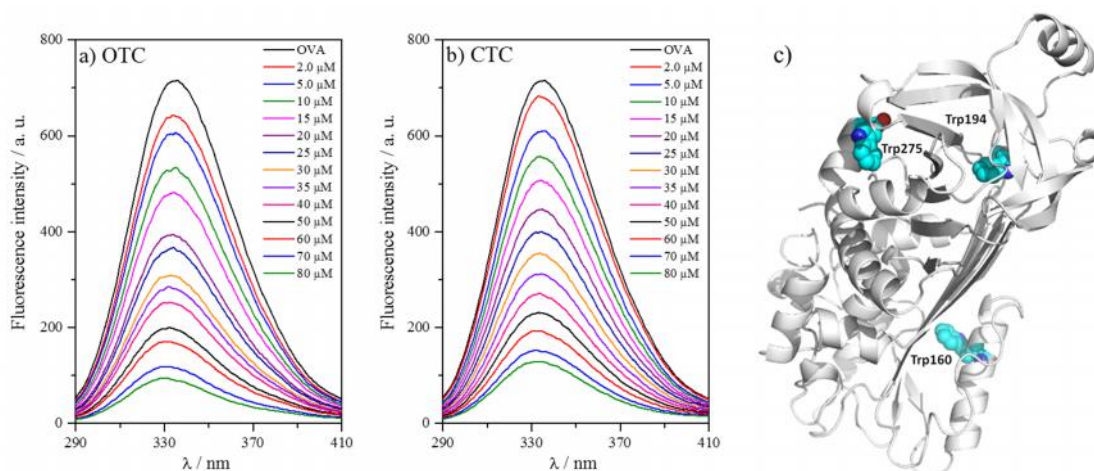


Fig. S1. Emission spectral profile of OVA (2 μM) at different concentrations of a) OTC and b) CTC at a pH of 7.4 and 30°C. c) Tridimensional OVA structure highlighting the tryptophan residues. The protein model was based on 1OVA obtained from the Protein Data Bank (PDB) and

R.E. Stein, A.G. Leslie, J.T. Finch, R.W. Carrell, Crystal structure of uncleaved ovalbumin at 1.95 Å resolution. J. Mol. Biol. 221 (1991) 941-959.

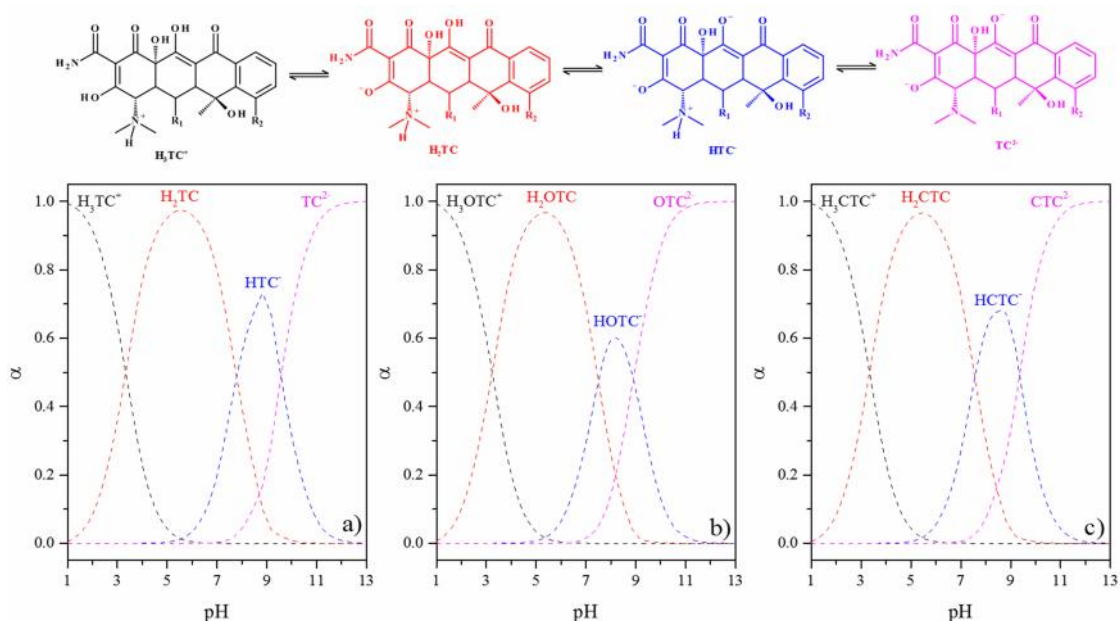


Fig. S2. The distribution of tetracycline species according to variation in the pH: a) TC (R₁ = H, R₂ = H), b) OTC (R₁ = OH, R₂ = H) and c) CTC (R₁ = H, R₂ = Cl). TC (pK_{a1} = 3.32/pK_{a2} = 7.72).

7.78/ $pK_{a3} = 9.58$), OTC ($pK_{a1} = 3.22/pK_{a2} = 7.46/pK_{a3} = 8.94$) and CTC ($pK_{a1} = 3.33/pK_{a2} = 7.55/pK_{a3} = 9.33$).

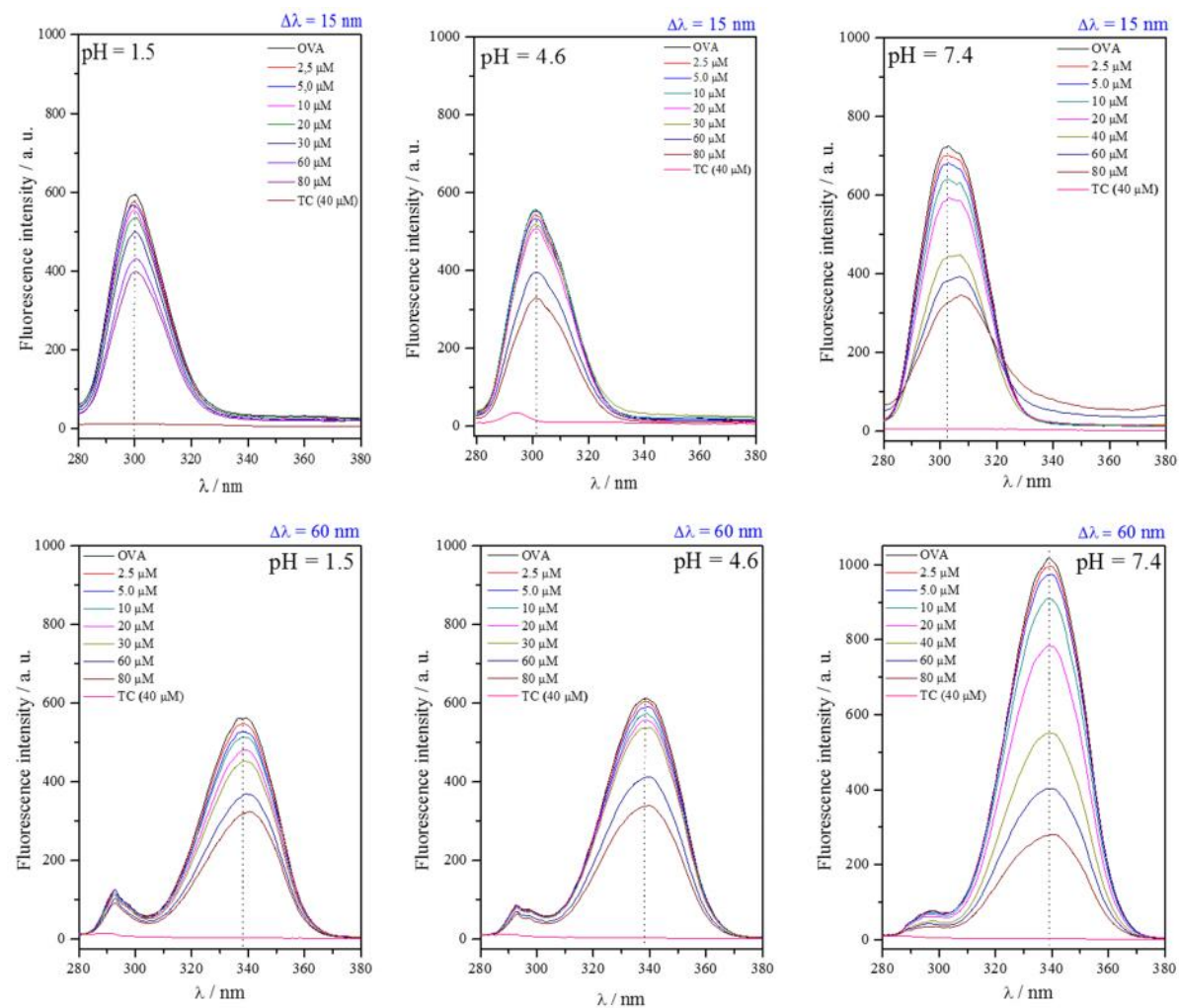


Fig. S3. Synchronous fluorescence spectra of OVA (2 μM) upon the addition of increasing concentrations of TC (2.5–80 μM) at various pH values.

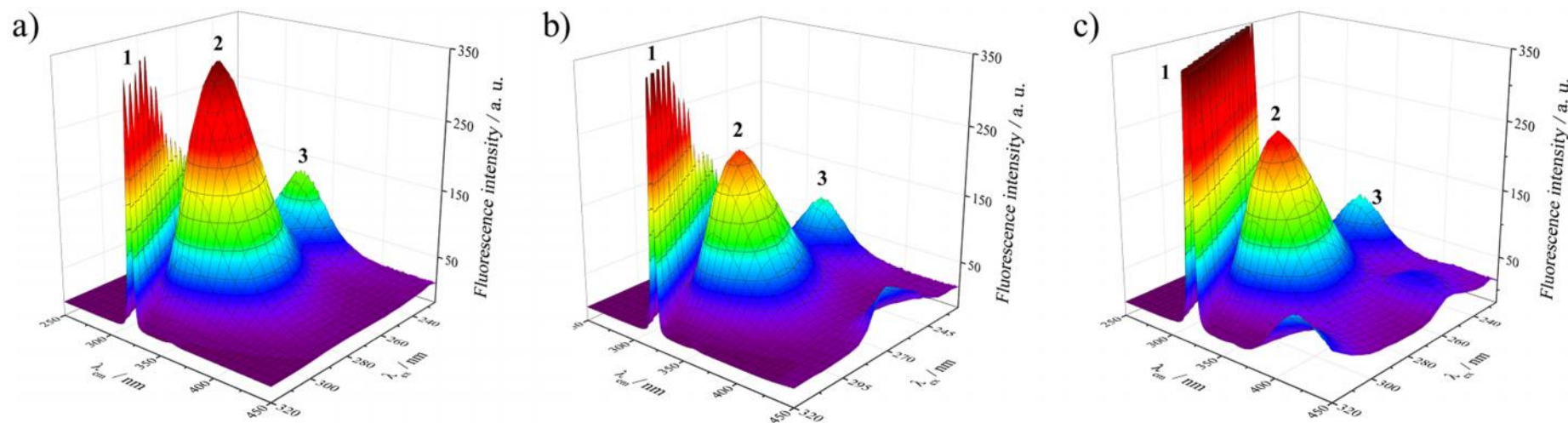


Fig. S4. Tridimensional fluorescence spectra of (a) OVA, (b) OVA-OTC complex and (c) OVA-CTC complex. The protein and ligand concentrations are 2 and 20 μM , and a pH of 7.4 was used.

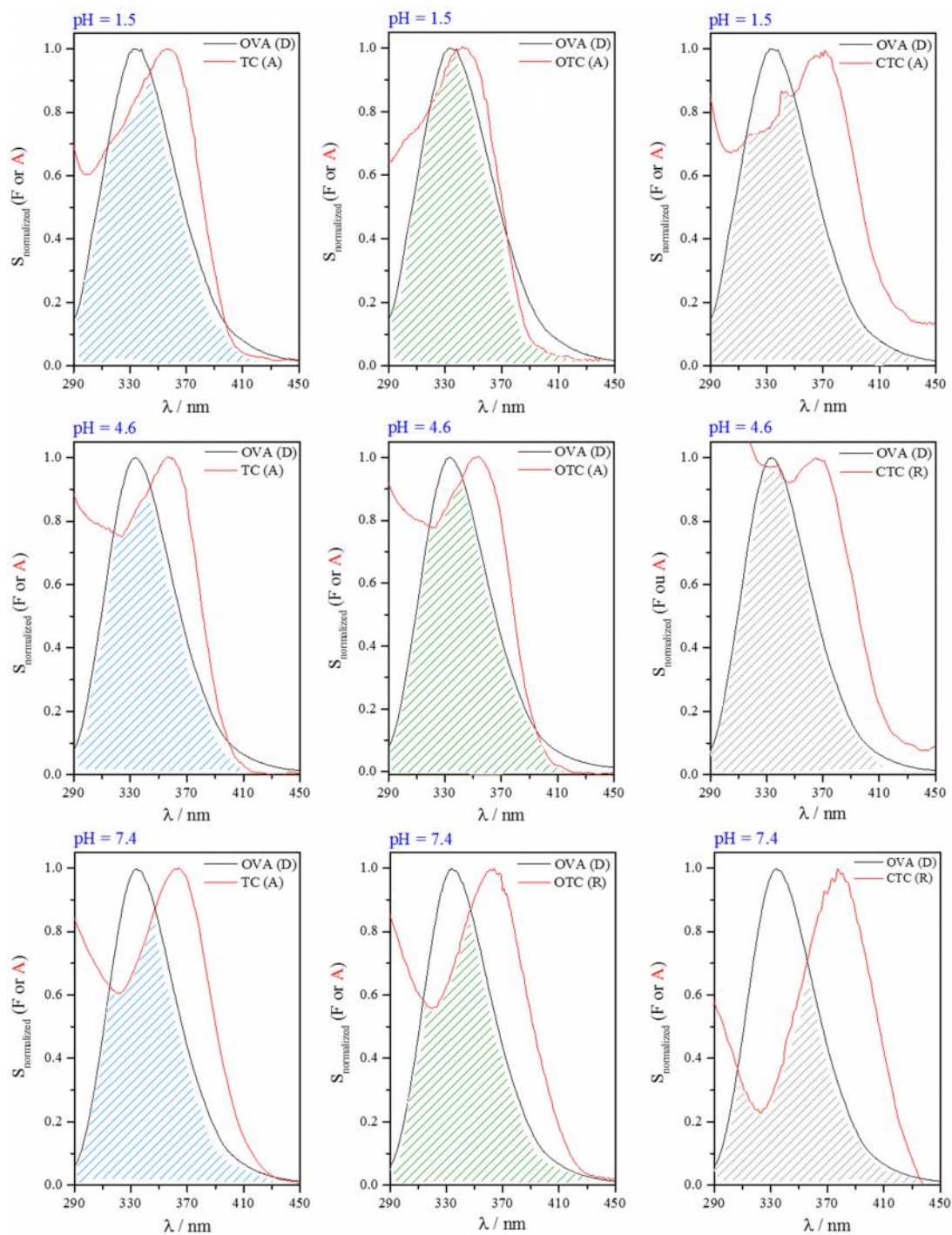


Fig. S5. Normalized overlapped spectra of the OVA (donor, D) fluorescence spectrum and tetracycline (receptor-R) absorption spectrum in different pH conditions. The fluorescence and absorbance signals (S) were normalized. The protein and ligands are at 2 μM .

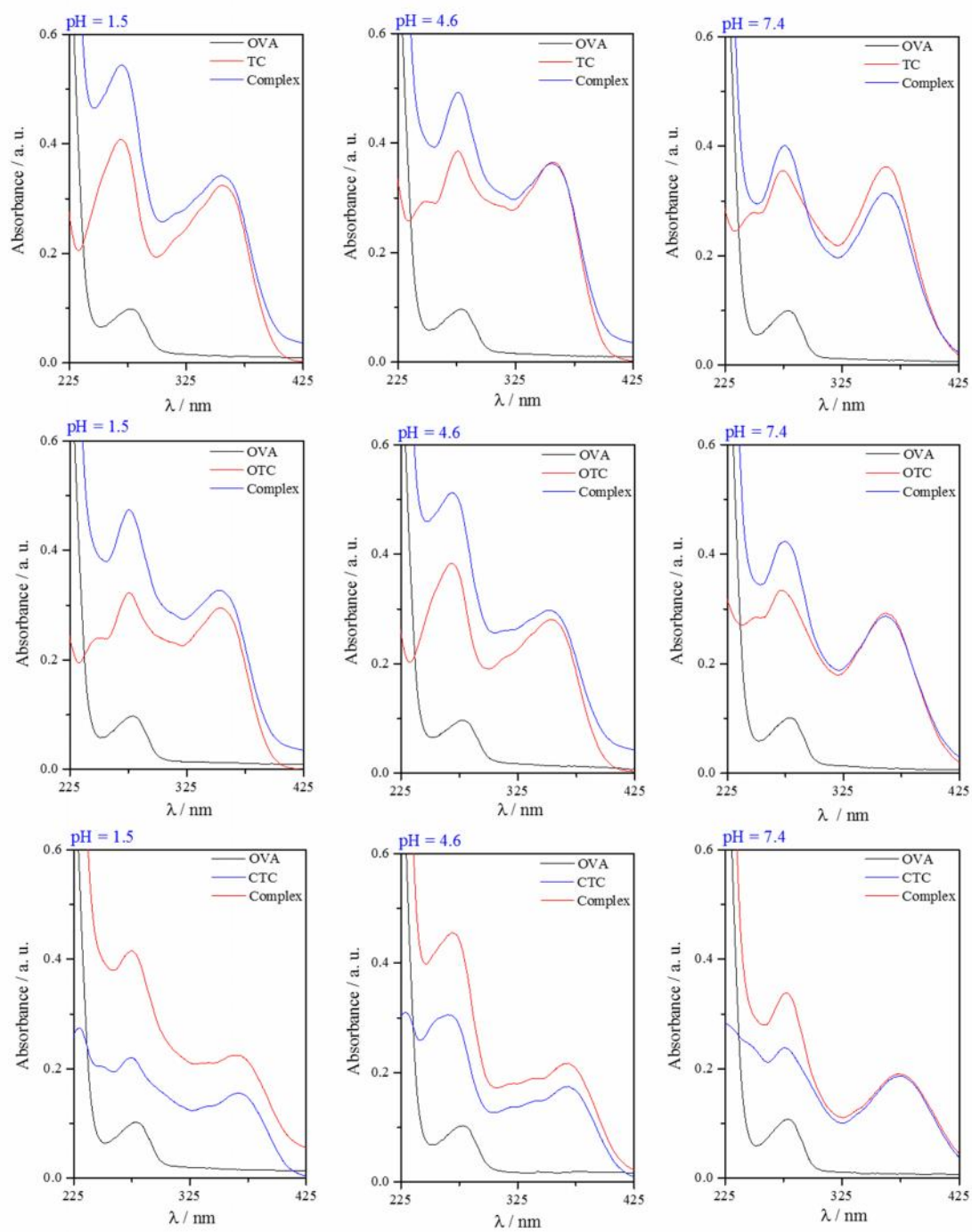


Fig. S6. UV-vis spectral profile of OVA (1 μM) in the absence and presence of tetracyclines (16 μM) in different pH conditions.

Supplementary material – TABLES

Interactions of tetracyclines with ovalbumin, the main allergen protein from egg white: spectroscopic and electrophoretic studies

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Table S1. Stern-Volmer constant parameters for TC, OTC and CTC at different pH values and temperature conditions. Data represent mean $\pm$ SD of three experiments ($n = 3$).

Ligand	pH	Temperature (°C)	Stern-Volmer constant		
			K_{SV} ($\times 10^4 \text{ L mol}^{-1}$)	r	K_q ($\times 10^{12} \text{ L mol}^{-1} \text{ s}^{-1}$)
TC	1.5	22	5.69 ± 0.02	0.9995	5.69
		30	5.41 ± 0.04	0.9997	5.41
		38	5.10 ± 0.05	0.9895	5.10
	4.6	22	4.85 ± 0.31	0.9987	4.85
		30	4.36 ± 0.10	0.9988	4.36
		38	3.93 ± 0.15	0.9995	3.93
	7.4	22	1.76 ± 0.05	0.9969	1.76
		30	1.56 ± 0.03	0.9975	1.56
		38	1.34 ± 0.09	0.9964	1.34
OTC	1.5	22	3.51 ± 0.06	0.9925	3.51
		30	3.47 ± 0.02	0.9920	3.47
		38	3.40 ± 0.03	0.9910	3.40
	4.6	22	2.45 ± 0.12	0.9945	2.45
		30	2.33 ± 0.09	0.9944	2.33
		38	2.16 ± 0.06	0.9951	2.16
	7.4	22	2.26 ± 0.10	0.9939	2.26
		30	2.12 ± 0.11	0.9935	2.12
		38	2.04 ± 0.06	0.9936	2.04
CTC	1.5	22	2.03 ± 0.08	0.9928	2.03
		30	1.93 ± 0.03	0.9930	1.93
		38	1.87 ± 0.02	0.9920	1.87
	4.6	22	1.96 ± 0.05	0.9934	1.96
		30	1.83 ± 0.02	0.9956	1.83
		38	1.71 ± 0.01	0.9948	1.71
	7.4	22	1.78 ± 0.07	0.9982	1.78
		30	1.59 ± 0.13	0.9984	1.59
		38	1.37 ± 0.04	0.9992	1.37

Table S2. Synchronous fluorescence interaction parameters for TC, OTC and CTC with OVA at different pH values. Data represent mean $\pm$ SD of three experiments ($n = 3$).

pH	$\Delta\lambda$ (nm)	TC			OTC			CTC		
		K_{SV} ($\times 10^3$ L mol $^{-1}$)	r	Variation* (nm)	K_{SV} ($\times 10^3$ L mol $^{-1}$)	r	Variation* (nm)	K_{SV} ($\times 10^3$ L mol $^{-1}$)	r	Variation* (nm)
1.5	15	6.09 ± 0.16	0.9978	0	9.63 ± 0.40	0.9956	0	7.07 ± 0.41	0.9920	0
	60	9.23 ± 0.27	0.9973	3	17.8 ± 0.9	0.9951	3	6.52 ± 0.31	0.9958	2
4.6	15	6.53 ± 0.47	0.9922	0	7.58 ± 0.12	0.9778	0	5.88 ± 0.46	0.9943	0
	60	4.65 ± 0.28	0.9929	2	9.61 ± 0.33	0.9970	2	6.63 ± 0.73	0.9945	2
7.4	15	11.6 ± 0.6	0.9977	4	13.0 ± 0.6	0.9966	4	8.40 ± 0.46	0.9970	1
	60	12.1 ± 0.9	0.9948	2	17.1 ± 0.6	0.9934	2	8.09 ± 0.59	0.9912	2

*concerning the maximum displacement of tryptophan or tyrosine residues.

Table S3. Tridimensional fluorescence parameters of OVA and the OVA-TC complex at a pH of 7.4. The protein and ligands are at 2 and 20 μ M, respectively.

Peak	OVA			OVA-TC		
	Position ($\lambda_{ex}/\lambda_{em}$)	Stokes shift (nm) ¹	F ²	Position ($\lambda_{ex}/\lambda_{em}$)	Stokes shift (nm)	F
1	$\lambda_{ex} = \lambda_{em}$	0	332	$\lambda_{ex} = \lambda_{em}$	0	292
2	282/337	55	265 (100%)	281/338	57	158 (59.6%)
3	237/338	101	95 (100%)	237/335	98	74 (77.9%)

¹Stokes shift is the $\Delta\lambda$ (nm) = $\lambda_{em} - \lambda_{ex}$; ²F = fluorescence intensity.

Table S4. Tridimensional fluorescence parameters of OVA, OVA-OTC complex and OVA-CTC complex at a pH of 7.4. The protein and ligands are at 2 and 20 μ M, respectively.

Peak	OVA			OVA-OTC			OVA-CTC		
	Position ($\lambda_{ex}/\lambda_{em}$)	Stokes shift (nm) ¹	F ²	Position ($\lambda_{ex}/\lambda_{em}$)	Stokes shift (nm)	F	Position ($\lambda_{ex}/\lambda_{em}$)	Stokes shift (nm)	F
1	$\lambda_{ex} = \lambda_{em}$	0	356	$\lambda_{ex} = \lambda_{em}$	0	402	$\lambda_{ex} = \lambda_{em}$	0	1008
2	282/337	55	341 (100%)	282/334	52	224 (65.7%)	281/337	56	237 (69.5%)
3	237/338	101	132 (100%)	237/335	98	95 (71.9%)	234/332	98	101 (76.5%)

¹Stokes shift is the $\Delta\lambda$ (nm) = $\lambda_{em} - \lambda_{ex}$; ²F = fluorescence intensity.

Table S5. FRET parameters of the interactions of tetracyclines and OVA under different pH conditions.

Ligand	pH	J ($10^{-15} \text{ cm}^3 \text{ L mol}^{-1}$)	E (%)	R_0 (nm)	r_0 (nm)
TC	1.5	8.90	0.13	2.38	3.26
	4.6	7.17	0.17	2.29	2.98
	7.4	8.53	0.16	2.36	3.11
OTC	1.5	9.58	0.10	2.41	3.47
	4.6	7.72	0.19	2.32	2.95
	7.4	8.71	0.14	2.37	3.20
CTC	1.5	9.34	0.09	2.39	3.52
	4.6	8.93	0.21	2.38	2.96
	7.4	6.40	0.15	2.25	3.00

Table S6. Comparison of the binding parameters for tetracyclines and OVA with similar works in the literature.

Condition	pH	Protein	pI	Trp residues	T (°C)	Ligand	$K_b (10^4 \text{ L mol}^{-1})$	n	$r_0 \text{ (nm)}$	Reference
10 mM Tris	8.0	BSA	4.9	2	25	TC OTC CTC	460 320 80	3.6 2.1 2.9	-	[1]
50 mM Tris and 100 mM NaCl	7.4	HSA	4.7	1	37	TC OTC CTC	5.59 5.76 5.40	1.20 1.19 1.10	2.25 2.16 2.28	[2]
		BSA	4.9	2		TC OTC CTC	6.21 7.05 5.74	1.08 1.06 1.05	2.28 2.18 2.30	
200 mM NaH_2PO_4 and Na_2HPO_4	7.6	HSA	4.7	1	35	TC	0.31	0.97	4.98	[3]
50 mM Tris	7.5	HSA BSA Lysozyme	4.7 4.9 9.3	1 2 6	35 35 35	TC	4.20 5.22 5.56	1.01 1.43 1.17	3.35 3.46 3.08	[4]
100 mM Tris and 100 mM NaCl	7.4	OVA	4.6	3	37	Quercetin	31.5	-	5.71	[5]
100 mM Tris and 80 mM NaCl	7.4	OVA	4.6	3	24	Diprophylline Caffeine Theophylline	1.67 1.08 0.44	1.09 1.06 0.97	2.23 2.25 2.20	[6]
50 mM Tris and 10 mM NaCl	7.4	OVA	4.6	3	30	TC OTC CTC	2.74 4.26 2.89	1.14 0.97 1.01	3.11 3.20 3.00	This work

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Table S7. UV-vis parameters of OVA (1 μM) in the absence and presence of evaluated tetracyclines (16 μM).

Ligand	λ (nm)	System	Absorbance (A)		
			pH = 1.5	pH = 4.6	pH = 7.4
TC	277	$A_{\text{OVA}} + A_{\text{TC}}$	0.498	0.480	0.451
		A_{complex}	0.544	0.493	0.401
		ΔA	+0.046	+0.013	-0.050
	374	$A_{\text{OVA}} + A_{\text{TC}}$	0.336	0.378	0.371
		A_{complex}	0.342	0.362	0.315
		ΔA	+0.006	-0.016	-0.056
OTC	277	$A_{\text{OVA}} + A_{\text{OTC}}$	0.419	0.470	0.430
		A_{complex}	0.474	0.512	0.423
		ΔA	+0.055	+0.042	-0.007
	374	$A_{\text{OVA}} + A_{\text{OTC}}$	0.307	0.293	0.301
		A_{complex}	0.327	0.297	0.287
		ΔA	+0.020	+0.004	-0.014
CTC	277	$A_{\text{OVA}} + A_{\text{CTC}}$	0.319	0.398	0.345
		A_{complex}	0.415	0.454	0.339
		ΔA	+0.096	+0.056	-0.006
	374	$A_{\text{OVA}} + A_{\text{CTC}}$	0.171	0.192	0.196
		A_{complex}	0.225	0.216	0.189
		ΔA	+0.054	+0.024	-0.007

$$\Delta A = A_{\text{complex}} - (A_{\text{OVA}} + A_{\text{tetracycline}})$$