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Aggregation State and pK_a Values of (*E*)-Resveratrol As Determined by Fluorescence Spectroscopy and UV−Visible Absorption

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Whether or not (*E*)-resveratrol can be used as a functional ingredient in foods depends on its structure at different concentrations and pH values. For this reason, the aggregation state of (*E*)-resveratrol at acidic and basic pH is investigated here for the first time. The data show that (*E*)-resveratrol forms aggregates above a certain critical concentration, and this concentration is lower at acid (12.5 μM at pH 5.5) than at basic pH values (37 μM at pH 10.5). Moreover, although several papers have focused on the effect of the protonation state of (*E*)-resveratrol on its biological activity, different data concerning the acidic dissociation constants of this natural antioxidant have been reported in the literature. The present paper shows a way of determining the acidic dissociation constants (pK_{a1} = 8.8, pK_{a2} = 9.8, pK_{a3} = 11.4) of (*E*)-resveratrol in aqueous medium using both absorbance and fluorescence spectroscopy. Furthermore, the spectroscopic study of (*E*)-resveratrol under different ionization states corresponding to the deprotonation of the hydroxyl groups present in the molecule shows significant differences among the absorbance, excitation, and emission spectra of (*E*)-resveratrol that have not previously been reported.

KEYWORDS: Resveratrol; acidic dissociation constants; fluorescence; aggregation state

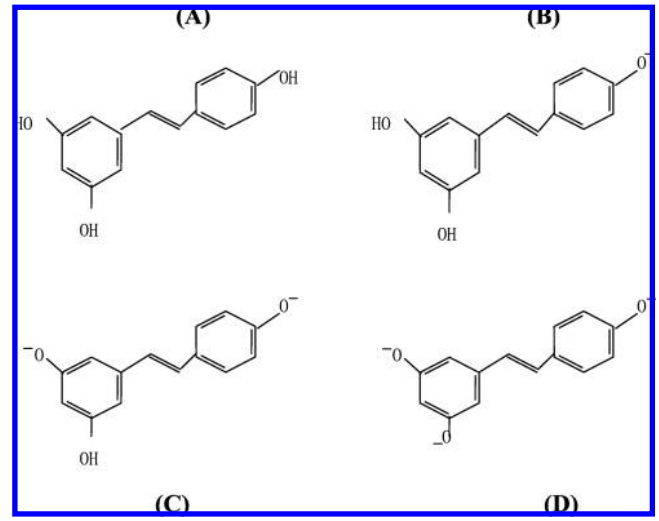
INTRODUCTION

(*E*)-Resveratrol (3,5,4′-trihydroxystilbene, **Scheme 1A**) is a phytoalexin with a stilbene structure found in several genera and families of plants (*1*). Moreover, this natural antioxidant can be found in foods such as grapes, wine, peanuts and derivatives, cranberries, chocolate, cocoa, and tomato (*2–4*).

In recent years, (*E*)-resveratrol has shown several beneficial biological effects on human health (*5*), and there is growing interest in its use as a functional ingredient in foods as a fortifier or nutraceutical compound (*6*). However, problems related with its poor solubility in aqueous media, its poor bioavailability, and its propensity to oxidize and the paucity of information concerning conformational changes in its structure resulting from variations in pH and aggregation state variations have meant that no food has been enriched with this important antioxidant compound (*7–9*).

Regarding its solubility, (*E*)-resveratrol is soluble in ethanol, DMSO, and other organic solvents, whereas different authors have put its solubility in water at <1 mg/mL, limiting its use as a fortifier in the food industry. No research has focused on the behavior of (*E*)-resveratrol in aqueous solutions at concentrations >1 mg/mL, that is, approaching the aggregation state of this antioxidant. In solution, some molecules exhibit an aggregation behavior that, depending on the pH, may lead to the formation of true micelles or merely to a dispersed oil phase (*10*). The onset of aggregation can be estimated by different techniques, and a critical concentration, at which a change in the physicochemical state of the molecule is produced, can be determined. Several works published by our group have shown that the aggregation state of different molecules may play an important role in their enzymatic or physiological activity (*10, 11*). Furthermore, some researchers have demonstrated that the aggregation state of some compounds can influence the same

Scheme 1. Protonated and Deprotonated Forms of (*E*)-Resveratrol



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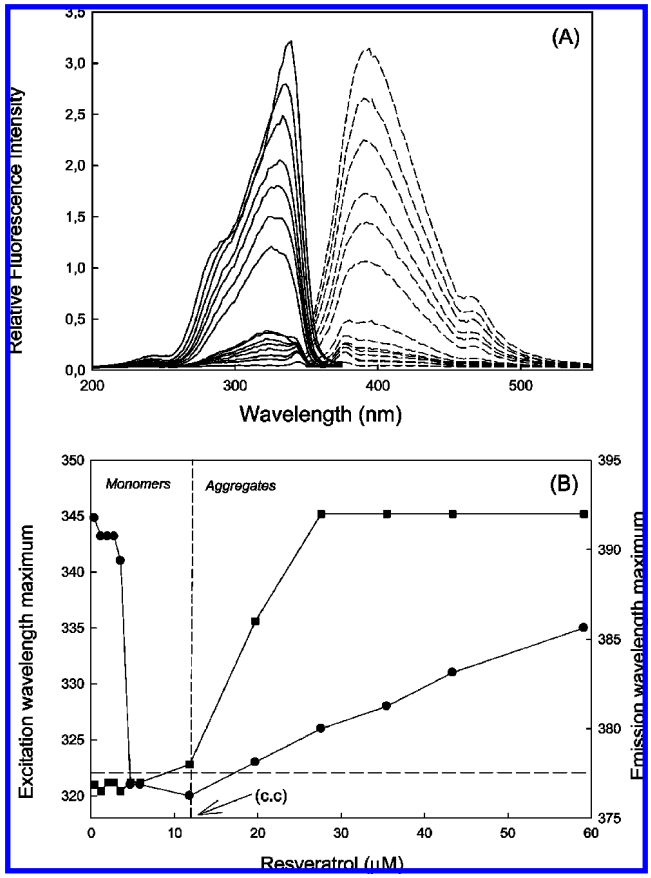

Figure 1. (**A**) Fluorescence spectra (—, excitation; - - -, emission) (λ_{exc} = 334 nm; λ_{emi} = 389 nm) for (*E*)-resveratrol in aqueous solution at increasing concentrations (0.5, 1.5, 2.5, 3.5, 4.2, 5.5, 7.0, 12.5, 23.0, 32.0, 41.5, 50.5, 69.0, 92.5, 140 μM) at pH 5.5. (**B**) Effect of concentration on the maximum excitation wavelength (●) and emission wavelength (■) of (*E*)-resveratrol in aqueous solution.

effects on health that have been attributed to resveratrol. These include anticancer (*1*), cardioprotection (*12*), antioxidant (*13*), antiviral (*14*), cytogenetic (*5*), estrogenic (*15*), and antiinflammatory (*16*) activities and the inhibition of platelet aggregation (*17*).

However, in the research into (*E*)-resveratrol, most studies thus far have focused on demonstrating its biological activities, obtaining evidence for its potent biological effects in clinical studies while searching for synergistic effects of (*E*)-resveratrol with other diet/beverage components, but no paper has considered the aggregation state of this natural antioxidant.

On the other hand, it has been found that the biological activity of (*E*)-resveratrol and its analogues depends to a significant extent on structural determinants (*18*), such as (i) the number and position of hydroxyl groups, (ii) intramolecular hydrogen bonding, (iii) stereoisomery, and (iv) double bond.

Many researchers have studied the role of hydroxyl groups on the properties of (*E*)-resveratrol and found that a 4′-hydroxy group is essential to (*E*)-resveratrol activities such as cytogenetic, radical scavenging, antioxidant, or antiproliferative (*19–23*).

However, the stability of *p*-4-hydroxyl in (*E*)-resveratrol is weak because it is more acidic than the other two *m*-hydroxyl groups (*24*). Moreover, Cao et al. (*25*) indicated that it is easier to abstract 4-H than 3-H and 5-H. These authors showed that the 4-hydroxyl group of (*E*)-resveratrol is more reactive than those at the the 3- and 5-positions because the resonance effects of the 4-radical are more stable than those of the 5-radical. For these reasons, the protection and stabilization of the 4-hydroxyl group of (*E*)-resveratrol are essential to the biological activities of this natural antioxidant. A knowledge of the protonation state of (*E*)-resveratrol is fundamental for understanding its biological activities because when the pH of the medium is higher than the first pK_a, the biological activity of (*E*)-resveratrol will be strongly reduced. This means that is necessary to determine the acidic dissociation constants (pK_{a1}, pK_{a2}, pK_{a3}) of (*E*)-resveratrol to know the protonation state of this compound at each pH. However, different data have been published on the (*E*)-resveratrol pK_a values. Some authors (*26*) calculated pK_{a1} = 8.2 and pK_{a2} = 9.7, compared with pK_{a1} = 9.49 calculated electrophoretically (*27*) or the reported values of pK_{a1} = 8.1, pK_{a2} = 9.9 and pK_{a3} = 10.5 calculated photometrically in aqueous medium by extrapolation of experimental values in different hydromethanolic media (*28*). Such contradictory results about the ionizations corresponding to the deprotonation of the hydroxyl groups present in the molecule led us to undertake the precise determination of the three (*E*)-resveratrol pK_a values.

In this work we have studied the aggregation state of (*E*)-resveratrol at acidic and basic pH values, determining the (*E*)-resveratrol concentration value at which this stilbene changes from its monomeric to aggregate form. The results clearly show that the aggregation mode of (*E*)-resveratrol is affected by the pH. Moreover, bearing in mind the importance of the ionization state of (*E*)-resveratrol in determining several beneficial biological effects of this compound on human health, and due to the contradictory results existing in the literature about the three (*E*)-resveratrol acidic dissociations constants, another aim of this work was to determine the correct pK_a of this stilbene in aqueous solution. For this reason, an absorption and fluorescence study of (*E*)-resveratrol was carried out; these spectroscopic studies revealed absorption, excitation, and emission bands not previously observed.

MATERIALS AND METHODS

Materials. Biochemicals were purchased from Fluka (Madrid, Spain). (*E*)-Resveratrol was from Sigma-Aldrich (Steinhelm, Germany) and was used without further purification.

Stock Standard Solution of (E)-Resveratrol. An accurately weighed 0.1 g standard sample of (*E*)-resveratrol was dissolved in ethanol, transferred into a 100 mL standard flask, diluted to the mark with ethanol, and mixed well. The solution was stored at 4 °C. The stock standard solution was diluted to 30 μg mL^{-1} with 0.1 M sodium phosphate before being used.

(*E*)-Resveratrol. is sensitive to the light, and the irradiation of solutions of the analyte induces the formation of (*Z*)-resveratrol; if the irradiation is intense, it leads to the formation of a highly fluorescent compound. Because of this, the samples were stored in darkness to protect the solutions against the light.

To minimize the risk of a basic hydrolysis, the measurements carried out to calculate the acidic dissociation constants of (*E*)-resveratrol were determined only 30 s after the preparation of the sample.

Equipment and Experimental Procedure. *Fluorescence and Absorbance Spectroscopy.* A PiStar-180 spectrometer (Applied Photophysics Ltd.) equipped with a xenon lamp source and quartz cell was used to perform all absorbance and fluorescence measurements. Excitation and emission bandwidths were both set at 2 nm. For both absorbance and fluorescence spectroscopy, excitation and emission spectra were recorded at 25 °C. To avoid self-absorbing (inner filter) effects, 2 mm quartz cells were used.

pH Assays. The pH was varied by the addition of small volumes (on the order of microliters) of aqueous hydrochloric acid or sodium hydroxide solutions in the 0.01−1.0 M concentration range, in the presence of 0.1 M sodium phosphate.

RESULTS AND DISCUSSION

Although there are many papers on the structure and solubility of (*E*)-resveratrol (*8, 24, 29–31*), the aggregation state of this compound has not been studied to date. As indicated in the Introduction, the aggregation state of compounds is strongly dependent on the pH. Although the majority of the papers published about (*E*)-resveratrol have been made at the "physiological" pH region between 4.5 and 7.5, in the literature we can find some papers that study the oxidation of (*E*)-resveratrol by some enzymes, that is, lipoxygenase, at basic pH values, including pH values above 9.5 (*32–34*). For this reason, we studied the dependence of the aggregation state of (*E*)-resveratrol on the concentration at two different pH values: 5.5 and 10.5, ascertaining the possible appearance of aggregates at increasing concentrations by measuring the fluorescence intrinsic to this antioxidant.

Study of Aggregation State of (E)-Resveratrol at Acid pH. **Figure 1A** illustrates the dependence of the relative intensity of both emission and excitation on (*E*)-resveratrol concentration at pH 5.5. As can be observed, the emission and excitation spectra differ greatly at high and low (*E*)-resveratrol concentrations, showing bathochromic displacement of the maximum emission and excitation wavelengths and a nonlinear dependence of the relative intensity of fluorescence on the (*E*)-resveratrol concentration. With regard to fluorescence excitation, at the lowest (*E*)-resveratrol concentration tested (0.46 μM), a single maximum excitation band at 345 nm was observed. However, when the (*E*)-resveratrol concentration was increased to 12.5 μM, this band suffered a bathochromic reduction of its maximum wavelength of 25 nm, showing a new principal band at 320 nm (**Figure 1B**) (solid circles). When the (*E*)-resveratrol concentration was increased from 12.5 to 60 μM, there was a sharp increase in the maximum excitation wavelength (**Figure 1B**).

A possible explanation for the dependence of (*E*)-resveratrol fluorescence on the concentration is that this molecule forms aggregates above a certain critical concentration. Following the method described by our group previously to determine the critical micelle concentration (cmc) of several compounds (*10*), we were able to determine graphically the critical concentration of (*E*)-resveratrol as the cross-point between the straight lines that define the trend of the maximum wavelength and the maximum relative excitation intensity in the pre- and postaggregation regions (12.5 μM at pH 5.5) (**Figure 1B**). This determination makes it possible to establish the conditions in which (*E*)-resveratrol is molecularly dispersed and does not form aggregates. The results were in good agreement with those presented previously by our group (*10, 11*), where different fatty acids formed aggregates that were detected by measuring the fluorescence of a probe such as diphenylhexatriene (the quantum yield of which increased when surrounded by an apolar environment) such as that of the aggregate core above a certain critical concentration.

To corroborate these results, the dependence of the maximum emission wavelength of (*E*)-resveratrol on the concentration is also shown in **Figure 1B**. As can be seen, at concentrations lower than 12.5 μM, (*E*)-resveratrol showed a constant maximum emission wavelength at about 376 nm. However, when the concentration was increased above 12.5 μM, a bathochromic displacement to 393 nm was observed (solid squares). This result confirms that 12.5 μM is the highest (*E*)-resveratrol concentration value at which this potent antioxidant is molecularly dispersed and does not form aggregates.

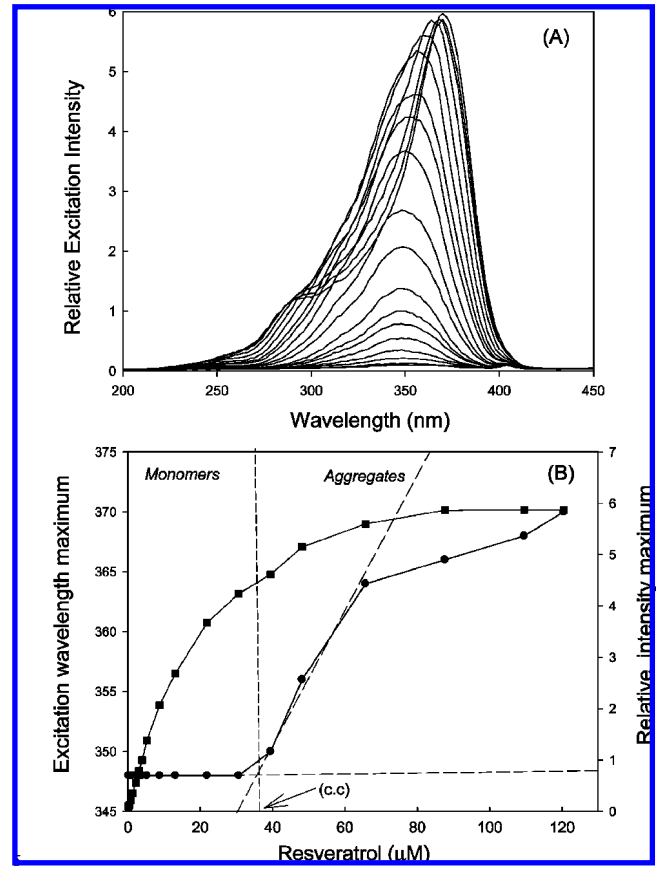

Figure 2. (**A**) Excitation spectra for (*E*)-resveratrol in aqueous solutions at increasing concentrations (0−120 μM) at pH 10.5 (λ_{emi} = 486 nm). (**B**) Effect of (*E*)-resveratrol concentration on the maximum excitation wavelength (●) and maximum excitation relative intensity (■) of (*E*)-resveratrol in aqueous solutions at increasing concentrations (0−120 μM) at pH 10.5.

Study of the Aggregation State of (E)-Resveratrol at Basic pH. Besides the fact that the aggregation state of several compounds is clearly in relation to their protonation state, the strong dependence of the structure of (*E*)-resveratrol on the pH shown in **Scheme 1** makes it necessary to study the aggregation state of (*E*)-resveratrol at basic pH values. Moreover, one of the main goals of this paper is to study the aggregation state of (*E*)-resveratrol to understand the kinetic behavior of this molecule as substrate or inhibitor of some enzymes present in foods. In fact, we can find in the literature several works that study the inhibition of oxidative and antioxidative enzymes by (*E*)-resveratrol (i.e., lipoxygenase, polyphenol oxidases, peroxidase, catalase, and 1-aminocyclopropane-1-carboxilic acid oxidase) (*32, 34*). In these papers, high concentrations of (*E*)-resveratrol from 0 to 500 μM were used to inhibit the enzymatic activities. For this reason, we have used a wide concentration range of (*E*)-resveratrol in this study.

As shown in **Figure 2A**, the excitation spectrum of (*E*)-resveratrol is clearly dependent on the concentration of this compound. However, although the bathochromic displacement of the maximum wavelength is of a magnitude similar to that observed at pH 5.5 (about 25 nm), (*E*)-resveratrol forms aggregates at a critical concentration above that calculated at acid pH. **Figure 2B** shows that, at pH 10.5, (*E*)-resveratrol forms aggregates at concentrations higher than 37 μM, which is more than twice the concentration determined for pH 5.5.

Finally, the relative fluorescence intensity of (*E*)-resveratrol is also dependent on concentration. However, although intensity

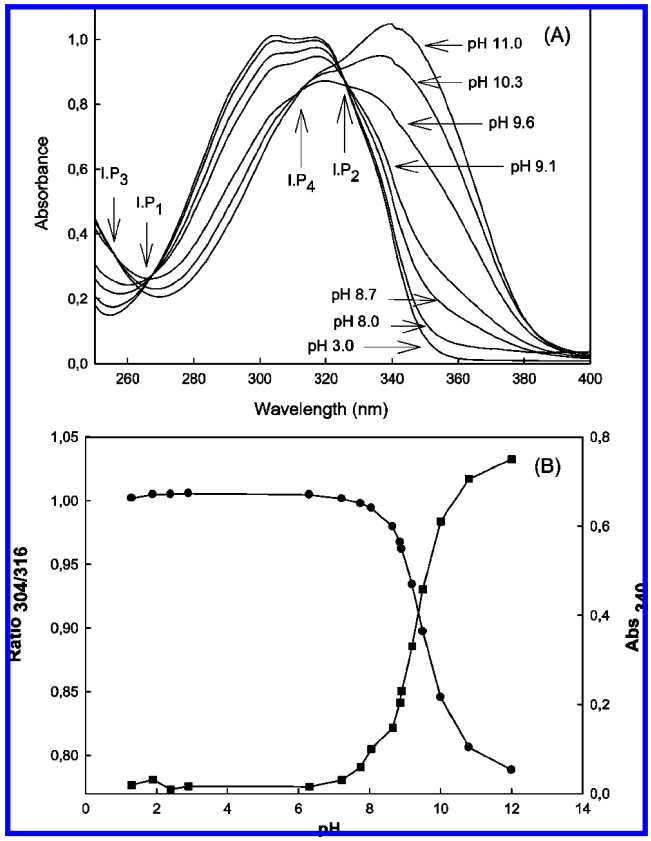

Figure 3. (**A**) Absorption spectra of (*E*)-resveratrol at 140 μM in aqueous solution at increasing pH values: 3.0, 8.0, 8.7, 9.1, 9.6, 10.3, and 11.0. (**B**) Effect of pH on the absorbance ratio 304/316 nm (●) and on the absorbance at 340 nm (■). Each point is the mean of three replicates.

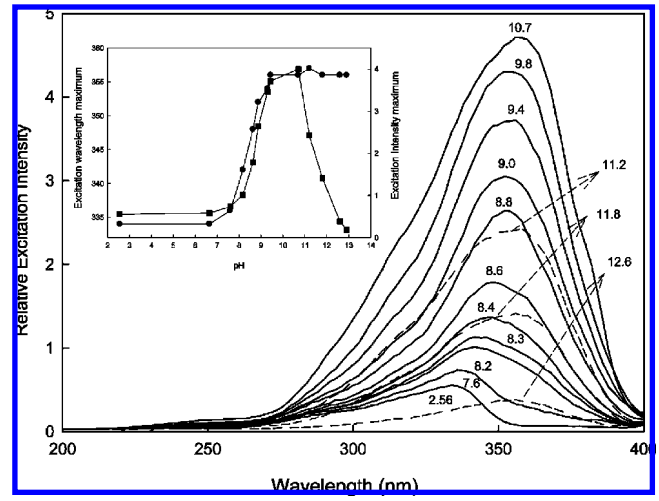

Figure 4. Excitation spectra of (*E*)-resveratrol at 140 μM in aqueous solution at different pH values (λ_{emi} = 440 nm). Each curve has been labeled with the corresponding pH value. (Inset) Effect of pH on the maximum excitation wavelength of (*E*)-resveratrol at 140 μM (●) and on the maximum relative excitation intensity (■). Each point is the mean of three replicates.

increased with the concentration of (*E*)-resveratrol, as was expected, this intensity became saturated in the presence of (*E*)-resveratrol aggregates.

Absorbance Study of (E)-Resveratrol. To calculate the acidic dissociation constants (pK_{a1}, pK_{a2}, and pK_{a3}) of (*E*)-resveratrol, both UV−vis absorption and fluorescence spectroscopy techniques were used.

The absorption spectrum of (*E*)-resveratrol in aqueous solutions at different pH values can be observed in **Figure 3A**. The results obtained show that, at pH values between 3.0 and 9.1, a single absorption band was observed around 300 nm with a bandwidth of 20 nm and two small maxima centered at 304 and 316 nm. However, the relationship between these two maxima bands changed as the pH increased. As can be seen in **Figure 3B**, the ratio between the absorbance determined at 304 and that observed at 316 increased sharply when the pH was >6.5. Moreover, in the pH region between 3.0 and 9.1, two isosbestic points at 270 and 330 nm were determined (IP_1 and IP_2). These results indicate that species A and B illustrated in **Scheme 1**, corresponding to the totally protonated structure of (*E*)-resveratrol (**A**) and the only deprotonated hydroxyl group in the structure of (*E*)-resveratrol (**B**), predominate in this pH zone.

As the pH shifted to higher values, the absorption intensity at 304 and 316 nm was reduced, and that near the 340 nm band was increased (**Figure 3B**). In this pH region (between pH 9.6 and 11.0), another two new isosbestic points at 255 and 315 nm (IP_3 and IP_4) appeared, indicating that species **B** and **C** shown in **Scheme 1** are predominant. Moreover, in **Figure 3B** it can be observed that the absorbance at 340 nm increased strongly when the pH of the medium was >6.5, just at the pH value where the ratio between the absorbance at 304 and 316 nm significantly decreased.

Although these bathochromic changes in the (*E*)-resveratrol UV−vis spectrum in acid, neutral, and basic media show clearly the dependence of the maximum wavelength of (*E*)-resveratrol on the pH, this method is not adequate to determine the three acidic dissociation constants of (*E*)-resveratrol because no method is known that relates the concentration of the absorbent species with the wavelength. For this reason, the next step in our investigation was to determine the three pK_a values of (*E*)-resveratrol through another spectroscopic technique: fluorescence spectroscopy.

Fluorescence Study of (E)-Resveratrol. The effect of pH on the relative excitation and emission fluorescence intensity of (*E*)-resveratrol has been tested, and comparative tests at various pH values showed that both the relative intensity and the maximum wavelength of (*E*)-resveratrol changed with pH.

A study of the excitation spectra of (*E*)-resveratrol at acid and neutral pH values showed a single excitation band at around 335 nm (**Figure 4**). However, the maximum excitation wavelength shifted to 355 nm when the pH was increased to the basic zone (**Figure 4**, inset) (solid circles). Moreover, as can be seen in **Figure 4** (inset), the relative excitation intensity of (*E*)-resveratrol reached its maximum at pH 10.8 and then decreased sharply (solid squares). These results are in good agreement with those published by Galeano-Díaz et al. (*26*). However, the emission fluorescence behavior of this stilbene determined in our study has not been published previously. At acid and neutral pH values (**Figure 5A**,) the antioxidant presents a principal emission wavelength at around 385 nm, followed by a shoulder at 445 nm and another lower intensity emission wavelength at 475 nm. However, at pH 9.0 the relative emission intensities of the three wavelengths are very similar (**Figure 5B**); at pH 10.2 the emission band at around 475 nm presents the highest relative emission intensity, followed by the band at 445 nm, and, finally, the lowest intensity belongs to the emission wavelength around 385 nm (**Figure 5C**). In summary, the emission wavelength that showed the highest relative intensity at both acid and neutral pH values presented the lowest relative emission intensity at basic pH values and vice-versa. However,

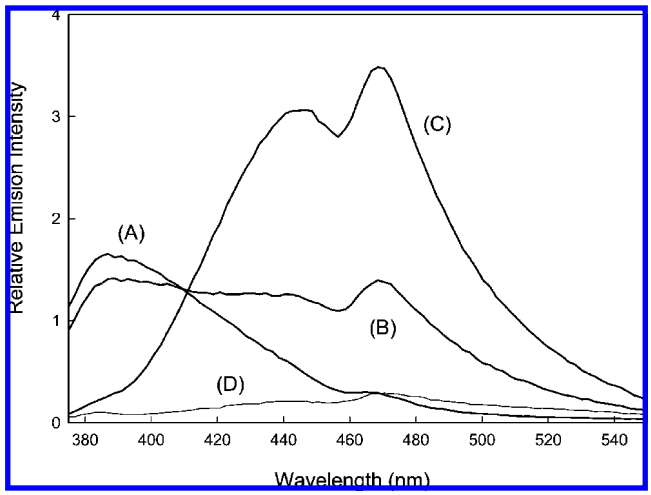

Figure 5. Emission spectra of (*E*)-resveratrol at 140 μM in aqueous solution at different pH values: (**A**) pH 2.5; (**B**) pH 9.0; (**C**) pH 10.2; (**D**) pH 12.5. (λ_{exc} = 356 nm.)

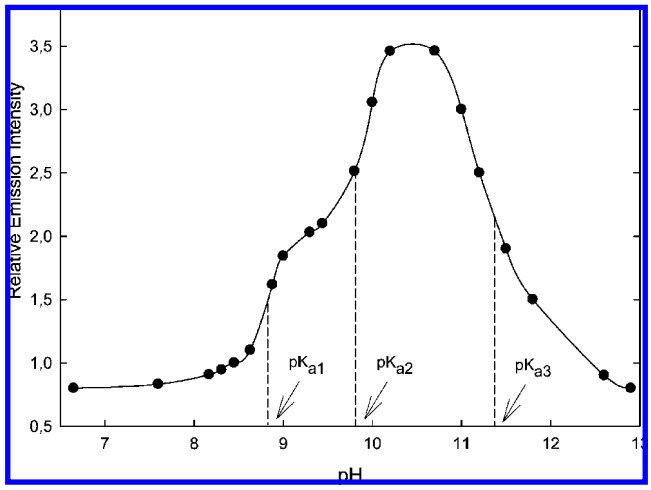

Figure 6. Effect of pH on the relative emission intensity of (*E*)-resveratrol at 140 μM (λ_{exc} = 356 nm; λ_{emi} = 440 nm). Each point is the mean of three replicates.

at pH values >10.5, a drastic change in the emission spectrum of (*E*)-resveratrol was registered with a strong decay in the relative emission intensity (**Figure 5D**).

As will be observed in the next section, these differences in the emission bands could be due to the fact that each of the four protonation states of (*E*)-resveratrol (**Scheme 1**) presents a characteristic emission band.

Determination of pK_{a1}, pK_{a2}, and pK_{a3} of (E)-Resveratrol. As can be seen in **Figure 6**, when the pH of the reaction medium is increasing, the relative emission intensity (λ_{exc} = 356 nm; λ_{emi} = 440 nm) of (*E*)-resveratrol shifts. However, the increase is not linear with the pH. Indeed, its behavior is similar to that observed for the well-known sigmoid titration curves used to determine the pK_a of several compounds.

The data presented suggest the existence of three successive equilibria in the pH 8.2−12.8 range. The first ionization predominates in the pH 8.2−9.3 range, the second one in the pH 9.5−10.8 range, and the third one in the pH 10.8−12.8 range. Thus, we calculated pK_{a1} = 8.8, pK_{a2} = 9.8, and pK_{a3} = 11.4. These ionizations correspond to the deprotonation of the three hydroxyl groups present in (*E*)-resveratrol. The first pK_a (pK_{a1} = 8.8) is associated with the deprotonation of 4-OH because the abstraction of 4-H is easier than that of 3-H and 5-H. The second pK_a (pK_{a2} = 9.8) indicates the deprotonation of 3-OH or 5-OH (the 3- and 5-positions have the same structures because the molecule is symmetric). The third pK_a (pK_{a2} = 11.4) indicates the deprotonation of 5-OH or 3-OH. Due to the high (*E*)-resveratrol concentrations used to determine pK_{a1}, pK_{a2}, and pK_{a3}, these can be considered as apparent pK_a values.

The value calculated for the first pK_a is quite different from those determined by Galeano-Díaz et al. (*26*) (pK_{a1} = 8.2), Takagai et al. (*28*) (pK_{a2} = 8.1), and Cao et al. (*27*) (pK_{a1} = 9.49). However, the second pK_a determined (pK_{a2} = 9.8) is very close to that determined by Galeano-Díaz et al. (*26*) (pK_{a1} = 9.7) and Takagai et al. (*28*) (pK_{a2} = 9.9). Moreover, except in the case of Takagai et al. (*28*) (pK_{a3} = 10.5), these researchers did not calculate the third pK_a of this antioxidant.

The three acidic dissociation constants calculated on the basis of the variation on the fluorescence of emission signals with the pH results confirm the hypothesis formulated previously about the fluorescence emission bands of (*E*)-resveratrol. When the pH value of the reaction medium is lower than the pK_{a1} (8.8), (*E*)-resveratrol shows a characteristic emission spectrum (**Figure 5A**) with a principal emission band at λ = 385 nm corresponding to the noncharged form of (*E*)-resveratrol (**Scheme 1A**). Moreover, when the pH of the medium is situated between pK_{a1} and pK_{a2}, three emission bands with similar intensities at 385, 445, and 475 nm, which belong to **Scheme 1B**, are presented (**Figure 5B**). When the pH value is increased to the region between the second and third pK_a values, the emission spectrum of (*E*)-resveratrol presents a main band at 475 nm (**Figure 5C**) due to the deprotonation of two hydroxyl groups (**Scheme 1C**). Finally, at pH values higher than the third pK_a (11.4), the spectrum observed in **Figure 5D** corresponds to the fully deprotonated form of (*E*)-resveratrol (**Scheme 1D**).

Conclusions. In recent years, interest in the study of antioxidant compounds, including (*E*)-resveratrol has grown. However, although many researchers have studied the positive effects of (*E*)-resveratrol on human health, few studies have focused on the important variations of the structure of this compound when both the (*E*)-resveratrol concentration and the pH of the reaction medium are modified. The aggregation state of (*E*)-resveratrol, which has not previously been studied, shows that this antioxidant compound forms aggregates above a certain critical concentration, which is dependent on the pH of the reaction medium. Moreover, although several studies have proposed the protection and stabilization of the 4-hydroxyl group of (*E*)-resveratrol as an essential step to maintaining the biological activities of this phytoalexin, published data on its dissociation constants are very different. The present paper aims to clarify the differences existing in the literature about the protonation state of (*E*)-resveratrol, and the three pK_a values of this compound have been determined using a spectrophotometric titration based on measurements of absorption, emission, and excitation. The data presented in this work concerning both the protonation and aggregation state of (*E*)-resveratrol will permit new investigation into the biological activities of this antioxidant at different pH values and in different states of aggregation. Finally, knowledge of the behavior of (*E*)-resveratrol at different pH values and substrate concentrations is of great importance for the food industry because of the growing interest in developing foods enriched with this natural antioxidant, among others.

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