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ORIGINAL RESEARCH ARTICLE

Theoretical Determination of the pK_a Values of Betalamic Acid Related to the Free Radical Scavenger Capacity: Comparison Between Empirical and Quantum Chemical Methods

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Abstract Health benefits of dietary phytochemicals have been suggested in recent years. Among 1000s of different compounds, Betalains, which occur in vegetables of the *Cariophyllalae* order (cactus pear fruits and red beet), have been considered because of reducing power and potential to affect redox-modulated cellular processes. The antioxidant power of Betalains is strictly due to the dissociation rate of the acid moieties present in all the molecules of this family of phytochemicals. Experimentally, only the pK_a -values of betanin were determined. Recently, it was evidenced it was evidenced as the acid dissociation, at different environmental pHs, affects on its electron-donating capacity, and further on its free radical scavenging power. The identical correlation was studied on another Betalains family compound, Betalamic Acid. Experimental evidences showed that the free radical scavenging capacity of this compound drastically decreases at pH $>$ 5, but pK_a values were experimentally not measured. With the aim to justify the Betalamic Acid behavior as free radical scavenger, in this paper we tried to predict in silico the pK_a values by means different approaches. Starting from the known experimental pK_as of acid compounds, both phytochemicals and small organic, two empirical approaches and quantum–mechanical calculation were compared to give reliable prediction of the pK_as of Betalamic Acid. Results by means these computational approaches are consistent with the experimental evidences. As shown herein, in silico, the totally dissociated species, at the experimental pH $>$ 5 in solution, is predominant, exploiting the higher electron-donating capability (HOMO energy). Therefore, the computational estimated pK_a values of Betalamic Acid resulted very reliable.

Keywords Betalamic Acid · Antioxidants · pK_a prediction · Empirical methods · DFT calculation

1 Introduction

The man daily ingests, with diet, about five hundred grams of chemical compounds, the majority of which are components of plants or vegetables in general. Besides the well-known protein, fat, carbohydrates and essential micronutrients such as vitamins and minerals, the plant world provides phenols, terpenes, terpenoids, alkaloids, purines, pyrimidines, nucleic acids, steroids and so on, exercising potent biological activities [1]. These components are referred to generically as phytochemicals and include 1000s of compounds belonging to various chemical classes and botanical families also extremely different, responsible for the color and the organoleptic properties of many plants and fruits. Plants synthesize these compounds to protect themselves against infection and damage caused by microorganisms, insects, or others. The contents of these substances in plants can be affected by many factors, such as season, regional differences and methods of cultivation and conservation (light, humidity, temperature, time). Epidemiological studies have shown that diets rich in plant-based foods help to prevent many diseases such as cardiovascular, metabolic, neurodegenerative and

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inflammatory [2–4]. Among the 1000s of different phenolic compounds, terpenoids, and sulfur-containing compounds, capable of reducing power and interfering in the redox processes modulated by cells [5–7], the Betalains, present in the plant kingdom, especially in *Chenopodiaceae* plants belonging to the *Caryophyllales* order, such as the prickly pear (Opuntia genus) and red beet (*Beta vulgaris*) as the main dietary sources, are compounds with high antioxidant (radical scavengers) capability of preventing the oxidation of biological molecules mediated by oxygen and free radicals and therefore can protect against diseases related to oxidative stress. Therefore, consumers can benefit from the consumption of products rich in Betalains. In order to have the favorable effects, the Betalains, taken through food, must be absorbed in adequate amounts, transported in the bloodstream and distributed to tissues. Many studies demonstrated that after dietary intake of the fruit of the prickly pear [8] and beetroot juice [9, 10], indicaxanthin and betanin (Fig. 1) plasma levels are in the order micromolar (which is a fairly high value compared to other phytochemicals) [11–13].

Because of the benefits that Betalains exert on human health, it is important to study all the parameters that characterize the properties of these compounds and the potential effects on the human organism. The antioxidant power of Betalains is closely related to, at different pH, the dissociation rate of the acid moieties present in all the molecules of the family. Experimentally, for betanin only two over three pK_a values are known and in this regard a recent study by Gliszczynska-Swiglo et al. [14] showed that the acid dissociation rate at different pH levels affects the ability of betanin as electron donor and therefore on its ability of "free radical scavenger." Tesoriere et al. [15] also published a study in which the pK_a values of the acid moiety of indicaxanthin were calculated in order to assess the influence on bioavailability. However, in a recent paper Gandía-Herrero et al. [16] have dealt with the effect of the protonation states of Betalamic Acid on its ability to free radical scavenger. Evaluating the free radical scavenging capacity of this phytochemical compound on solution of stable colored radical ABTS°+ [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)], the researchers observed that, at pH > 5.5 it was associated the species with a notable increase of the free radical scavenging activity. Only basal scavenging activity was observed at pH < 5.5 but they did not define any pKa values experimentally. Given the need to determine the pK_a values of this compound to better focus properties and benefits and the potential uses, in this paper is reported in silico prediction of pK_a values of the Betalamic Acid, for which there are no, at our knowledge, experimental measures.

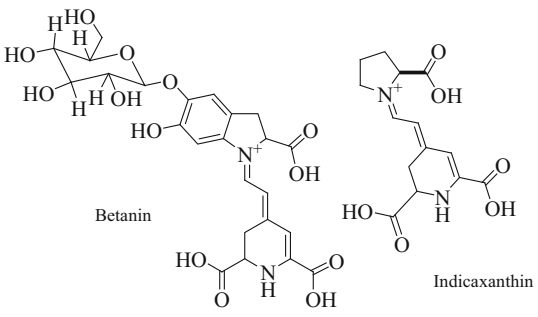

Fig. 1 Indicaxanthin and betanin

2 In Silico pK_a Determination

To date, pK_a prediction for organic substances can be performed with approaches which could be divided into two major groups: empirical methods and quantum chemical methods. The empirical methods can be further divided into three groups: (1) linear free energy relationships (LFER) methods utilizing the empirical relations of Hammett and Taft, (2) quantitative structure–property relationships (QSPR), methods correlating calculated structural descriptors with pK_a values, and (3) methods searching of similar structures in database of molecules with known measured pK_a values [17]. Main strength of the empirical methods is their high speed, while quantum chemical methods are supposed to have higher accuracy because they are based on, or closer to, first principles when calculating quantum mechanical descriptors. However, these approaches are much more time-consuming than empirical ones. In our paper, we compared experimental versus predicted pK_as of a series of acid phytochemicals by means of three different methods, two empirical and one quantum chemical with the aim to obtain the most reliable pK_a values for Betalamic Acid.

3 Materials and Methods

3.1 Experimental Data

The experimental pK_as values (at room temperature) used in this study are taken from the literature [17–30]. In reference papers, the experimental information related to each pK_a values is given. Structures are reported in Fig. 2.

3.2 Marvin Method

The Marvin version 5.0.6.1 (ChemAxon) method [31, 32] considers empirically calculated physicochemical parameters (mainly Mulliken partial charges) that are obtained from ionization-site-specific regression equations. It uses

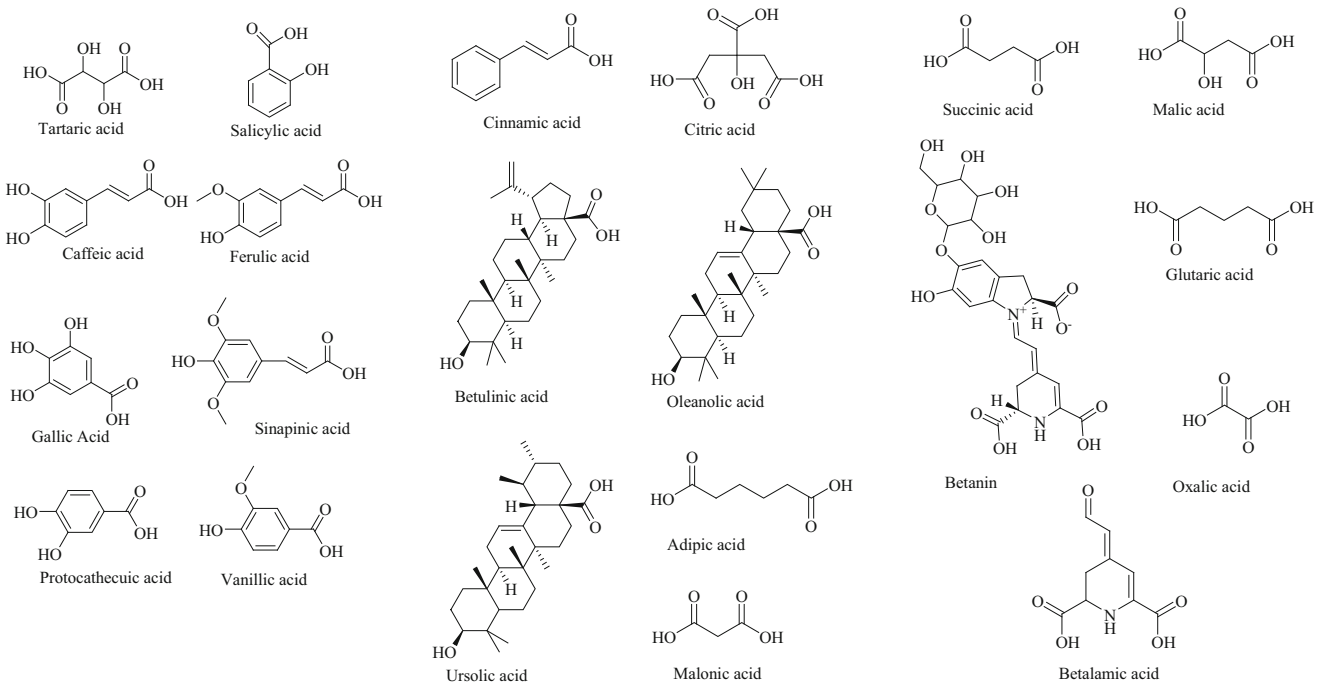

Fig. 2 Structures of the phytochemical object of this study

three types of calculated parameters (intramolecular interactions, partial charges and polarizability) to establish the micro-ionization constants pK_a of monoprotic molecules [32]:

$$\mathrm{p}K_a = a \times Q + b \times P + c \times S + d \times S + d \quad (1)$$

where Q and P are, respectively, the partial charge and the polarizability increments, S is the sum of the structure-specific (steric strain or/and hydrogen bond) increments; a, b, c and d are regression coefficients specific to the ionization site.

All of these pK_a increments are calculated from ionization-site-specific regression equations. Then, the ratio of microspecies is calculated to give calculated pK_a values to the atoms of the molecule object of study. At the end, macro-pK_a values are obtained from the theoretical relations that hold between macro- and micro-pK_a values. When a molecule bears more than one ionizable atom (multiprotic compound), it is important to make a distinction between micro- and macro-acid dissociation constants. The micro-acid dissociation constant is obtained from the equilibrium concentration of the conjugated acid–base pairs. The macro-acid dissociation constant is obtained from the global mass and charge conservation law.

3.3 Epik Method

The Epik [33, 34] method endorses the combination of two closely related linear free energy approaches based upon the Hammett equation for aromatic molecules and the Taft equation for aliphatic molecules to predict the pK_a values of organic acids and bases. Hammett and Taft equations are intended to predict microscopic pK_a values.

3.4 Jaguar Method

Jaguar [35, 36] uses a combination of correlated ab initio quantum chemistry, a self-consistent reaction field (SCRF) continuum treatment of solvation and empirical corrections to repair deficiencies in both the ab initio and continuum solvation models. Jaguar calculates the pK_a values using the thermodynamic cycle as shown in Fig. 3 and Eq. (2):

$$\mathrm{p}K_a = \frac{1}{2.3RT} D \quad (2)$$

where D is the free energy change involved in the step D, and it is calculated from the free energy changes in the other cycle steps:

$$D = A + C - B.$$

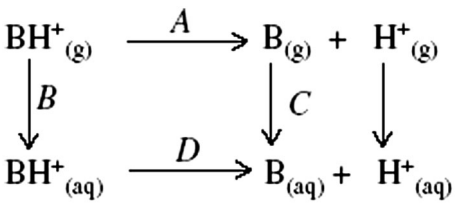

Fig. 3 Thermodinamic cycle used by Jaguar for pK_a calculation

As the calculated results partly depend on the conformation of the target molecule, first a conformational search was performed with MacroModel [37]. Schrodinger recommends using the lowest energy conformers found for further pK_a calculations. Jaguar calculates microscopic (atomic) pK_a values, but not macroscopic (experimental) pK_a values. If two or more microscopic pK_a values lie within one pK_a unit of each other, the macroscopic pK_a values can noticeably diverge from the corresponding microscopic values. Therefore, with the aim to get the macroscopic pK_a values for a multiprotic molecule, Schrodinger suggests to run 2^n states (n being the number of close pK_a values in a multiprotic molecule) and then to track the titration curve. In the case of two equivalent sites for protonation and deprotonation, the need for a statistical correction factor arises from the increased entropy of the appropriate species. A correction of $+0.60$ ($\log 2^2$) or -0.60 was added manually to the result obtained from running the pK_a prediction module on the basis of whether the calculated molecule has two equivalent acid sites, because Jaguar does not automatically recognize equivalent sites [38]. Calculations were run with the QM method DFT B3LYP/6-31G** level of theory. Free energy of solvation in water was computed by single-point calculation of the gas-phase-optimized geometry using the Poisson–Boltzmann solvation model (PB).

4 Data Analysis

To compare the predicted values versus experimental ones, a graphical analysis of pK_a results was first performed. Then statistical tests to compare the three prediction methods (Marvin, Epik, and Jaguar) are performed.

4.1 Indices of Performances of models

The bias factor (B_f) and the accuracy factor (A_f) are two indices of performance that allow to compare the goodness-of-fit of challenging models [39, 40].

The bias factor gives a clue of the average deviation between the model predictions and the average deviation between the model predictions and observed results; it is defined according to Eq. (3):

$$B_f = 10^{\frac{\sum \log \frac{pK_a^{cal}}{pK_a^{exp}}}{n}} \tag{3}$$

where pK_a^{cal} is the predicted pK_a value, pK_a^{exp} is the experimental pK_a value and n is the number of observations.

A bias factor of 1 signifies perfect agreement between observed and predicted pK_a values. Because over- and under-predictions may cancel out, the bias factor provides no indication of the range of the deviation between predictions and observations. A bias factor higher (resp. lower) than 1 indicates that the model predicts, on average, pK_a values higher (resp. lower) than experimental ones [39, 40].

The accuracy factor (A_f) seeks to provide an estimate of the average deviation between prediction and observation, and is defined according to Eq. (4):

$$A_f = 10^{\frac{\sum \left|\frac{pK_a^{cal}}{pK_a^{exp}}\right|}{n}} \tag{4}$$

We calculated the RMSE of the predicted pK_a values for each method according to Eq. (5):

$$\mathrm{RMSE} = \sqrt{\frac{\sum_{i=1}^{n} (\Delta pK_a)^2}{n}} \tag{5}$$

Then, a normalization of the residuals (noticing that 95 % of these points must be located between -2 and $+2$) is performed on the predicted values using the following equations:

$$\text{Normalized residual} = \frac{\Delta pK_a - \overline{\Delta pK_a}}{\sigma_{\Delta pK_a}} \tag{6}$$

where $\Delta pK_a = pK_a^{calc} - pK_a^{exp}$ is the error on predicted value; ΔpK_a and $\overline{\Delta pK_a}$ are the average and the standard deviation of the prediction errors on pK_a values, respectively, and are given by:

$$\overline{\Delta pK_a} = \frac{\sum_{i=1}^{n} \Delta pK_a}{n} \tag{7}$$

$$\sigma_{\Delta pK_a} = \frac{\sum_{i=1}^{n} \left(\Delta pK_a - \overline{\Delta pK_a}\right)^2}{n} \tag{8}$$

5 Results and Discussion

The aim of this work was to in silico predict the pK_a of Betalamic Acid for which there are no experimental measures. To argue this claim, a dataset of acid compounds which bear at least one carboxylic moiety and with known pK_a values was submitted to three different computational approaches, spacing from empirical to quantum mechanics methods. Comparison of the three different methods allowed to define the best reliable one to predict the unknown pK_a values of Betalamic Acid related to free radical scavenger activity and electronic properties.

5.1 Execution Speed

Programs execution was very fast for software based on empirical methods (Marvin and Epik). The pK_a prediction of Jaguar was very time-consuming, and it was strongly

dependent on the size and flexibility of the molecule. Calculation run on a HP pro-desk PC with Intel® Core™ i7-4770 CPU @ 3.40 GHz consumed from few minutes to several hours, in particular for betanin, Oleanolic, Betulinic and Ursolic acids. Jaguar predicted all the pK_a values for large and multiprotic substances, opposite of conclusion of Nicklaus and Liao in their benchmark study [38] who considered Jaguar as "a not practical solution for large, flexible and multiprotic compounds" due to the fact that this program failed to predict some pK_a values. For all of the compounds of our dataset, pK_as were predicted by the three different programs.

5.2 Prediction Results, Bias Factor and Accuracy Factor

The prediction results for the 30 pK_a protonation sites are shown in Table 1, and predicted versus experimental values are plotted in Fig. 4.

A linear fit of predicted pK_as data enables to establish the coefficient of determination (R^2) of each competed model. As results, we got, respectively, $R^2 = 0.87$ for Marvin, $R^2 = 0.75$ for Epik and $R^2 = 0.64$ for Jaguar. These results are consistent with the prediction capability observed in previous work [38] in which, notwithstanding Jaguar performs, in theory, the more accurate calculation, resulted as the worst predictor. However, it is very difficult to compare the three prediction methods from this graph, as there are a lot of points for which one method gives better predicted pK_a values compared to the others. The calculated values of the bias and accuracy factor are shown in Table 2.

The bias values for Marvin and Jaguar are negative, showing underestimation of the pK_as values. For Marvin, only the pK_a value for the carboxylic function C17 of betanin was calculated higher than the experimental value; however, this value actually has a margin of error as known in the literature [41]. For Jaguar, underestimations are

Table 1 Experimental and calculated pK_a values

S. no.	Compound		Exp. pK_a	Marvin	Epik	Jaguar/DFT
1	Tartaric acid	COOH	2.98	2.72	3.11	2.9
2		COOH	4.34	4.79	3.8	4.1
3	Salicylic acid	COOH	2.97	2.79	2.96	2.8
4	Caffeic acid	COOH	4.38	3.64	4.68	3.9
5	Ferulic acid	COOH	4.5	3.77	4.63	3.9
6	Gallic acid	COOH	4	3.94	4.7	3.7
7	Sinapinic acid	COOH	4.47	3.61	4.58	4.1
8	Protocatechuic acid	COOH	4.35	4.16	4.7	3.6
9	Vanillic acid	COOH	4.42	4.16	4.59	3.7
10	Cinnamic acid	COOH	4.5	4.51	4.45	3.6
11	Citric acid	COOH(3)	3.14	3.05	3.08	3
12		COOH(1)	4.77	4.67	3.87	3.5
13		COOH(5)	6.39	5.39	4.69	4.1
14	Betulinic acid	COOH	5.5	4.75	5.12	4.9
15	Oleanolic acid	COOH	5.11	4.74	5.17	4.8
16	Ursolic acid	COOH	5.29	4.74	5.18	4.7
17	Adipic acid	COOH	4.43	3.92	4.48	3.9
18		COOH	5.41	4.7	5.23	5.1
19	Malonic acid	COOH	2.83	2.43	3.1	2.3
20		COOH	5.69	5.92	5.4	3.5
21	Succinic acid	COOH	4.16	3.55	4.23	3.6
22		COOH	5.61	5.69	5.14	4.8
23	Malic acid	COOH(1)	3.4	3.2	3.74	2.9
24		COOH(4)	5.11	5.13	4.47	3.2
25	Glutaric acid	COOH	4.31	3.76	4.26	3.9
26		COOH	5.41	4.56	5.29	5.1
27	Oxalic acid	COOH	1.25	1.36	2.07	1.2
28		COOH	4.14	4.11	5.37	2.4
29	Betanin	COOH	3.3	3.12	2.96	4
30		COOH conjugated	3.3	3.79	5.19	2.9

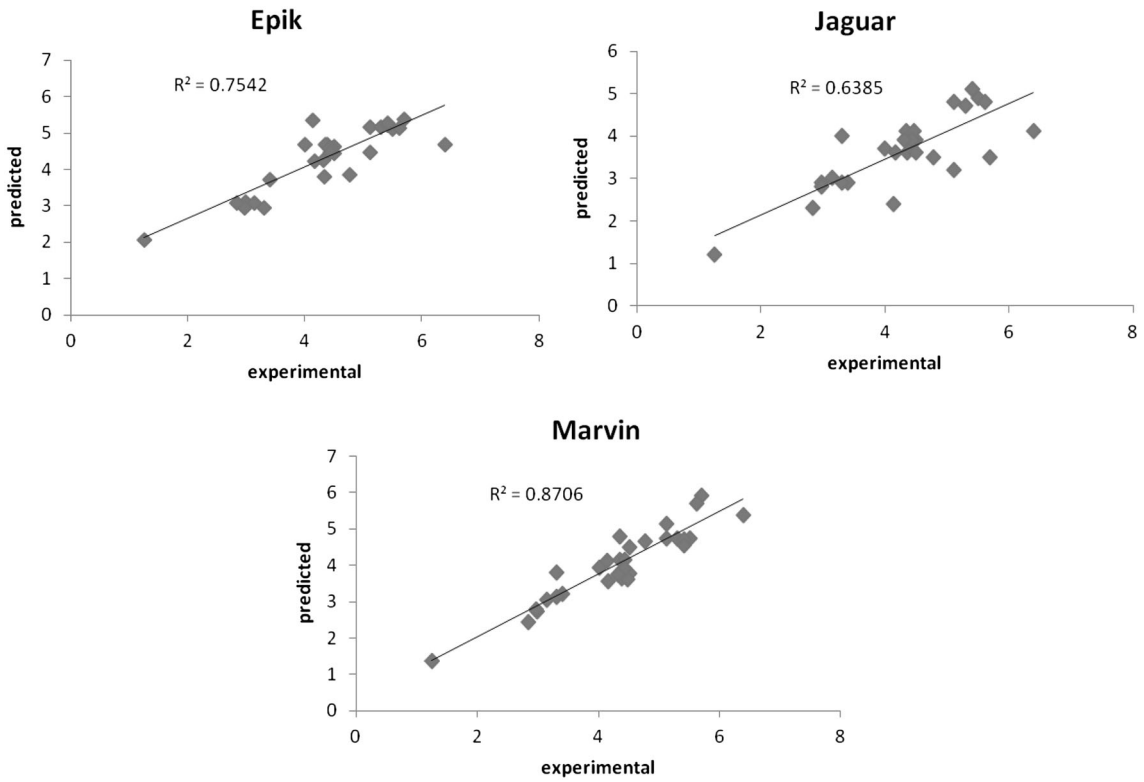

Fig. 4 Plots of predicted vs experimental pK_a values

Table 2 Bias, accuracy factor, and RMSE

	Bias	Accuracy	RMSE
Marvin	−0.67	0.93	0.48
Epik	0.21	1.04	0.63
Jaguar/DFT	−1.58	1.71	0.91

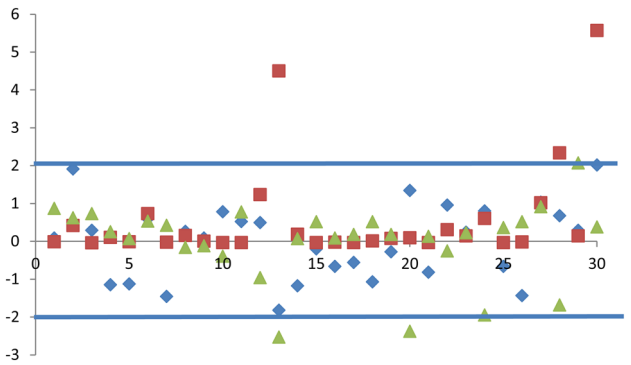

Fig. 5 Normalized residual plot, *diamond* Marvin, *square* Epik, *triangle* Jaguar

really higher when pK_{a2} and pK_{a3} were calculated for citric, malonic, malic and oxalic. In particular, the pK_{a3} of citric acid which has a $\Delta pK_a = -2.29$ while the pK_a values for betanin were predicted with opposite values respects the experimental ones. For Epik, the majority of predictions are underestimated, but the Bias values are positive probably because the pK_a of C17 of betanin was predicted with a $\Delta pK_a = 1.89$ which represents the worst prediction for Epik. The accuracy factor was really near to 1 for Marvin and Epik, while the value is 1.70 for Jaguar which means this method produces less accurate predictions. The RMSE of the overall predicted pK_a values for each method compared to experimental results was, respectively, 0.48 for Marvin, 0.63 for Epik and 0.91 for Jaguar. These results are in good agreement with literature reports [38, 42], confirming Marvin and Epik as the best predictors between the three methods exploited. Plotting the normalized residuals (that should ideally be equal to zero) of the three prediction methods, it is possible to evidence that for Marvin only for value #30 the residual is just higher than 2.00, for Epik three prediction are over 2.00 (pK_{a3} for citric acid, pK_{a2} for oxalic acid, and pK_a C17 for betanin), but the same show the lowest spread between −2 and +2. For Jaguar, two values are lower than −2.00 (pK_{a2} malonic acid and pK_{a3} citric acid) and one value is higher than +2.00 (pK_a c15 betanin) (Fig. 5).

All the three methods seem to be less accurate when predicting the second and the third dissociation constant for multiprotic acid. On the basis of these statistical parameters, Marvin and Epik resulted the most reliable methods to

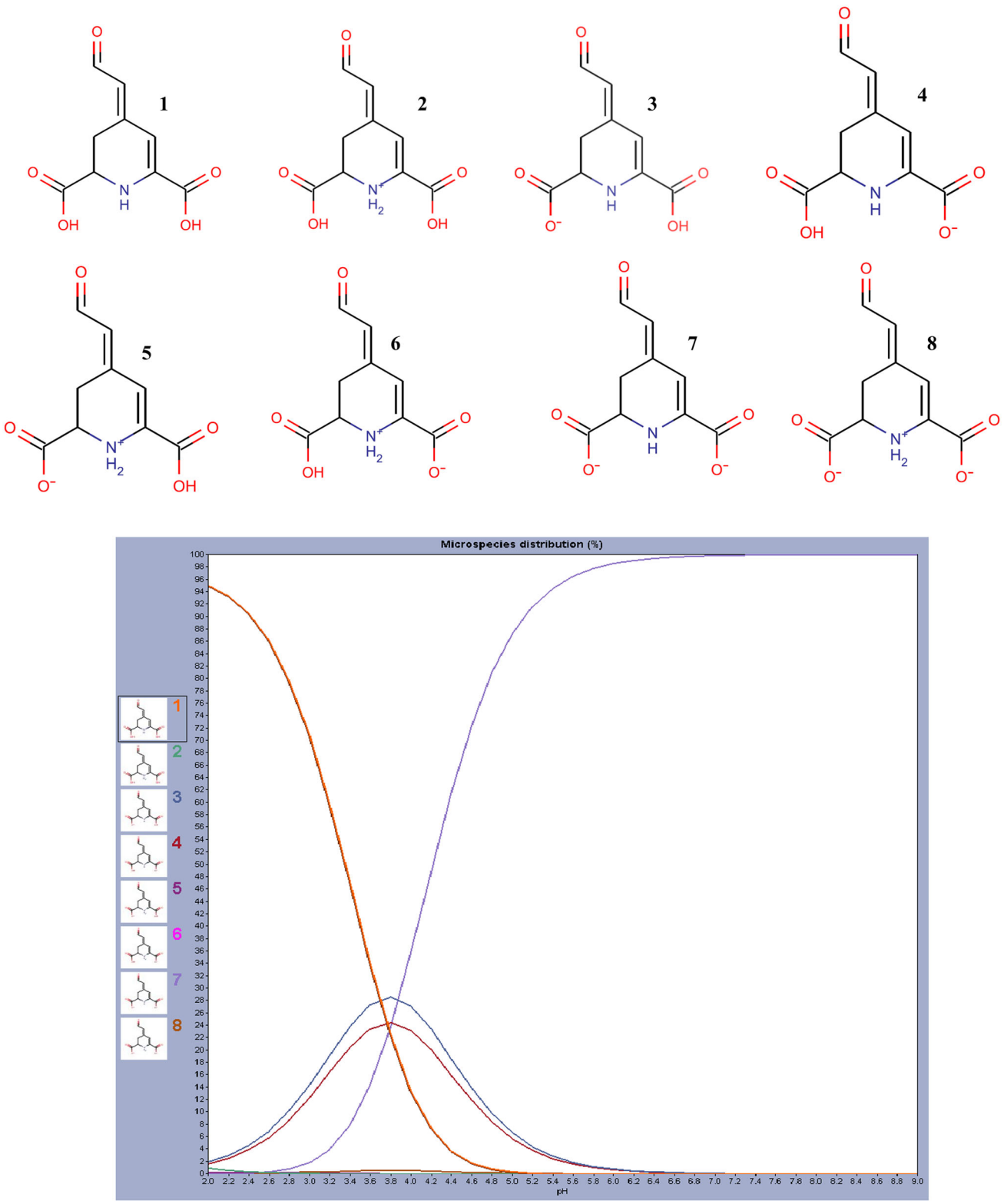

Fig. 6 Identified microspecies of betalamic acid and microspecies distribution related to environmental $2 < \text{pH} < 9$

predict the unknown pK_a, in particular multiprotic acid, values for Betalamic Acid also by virtue of the prediction of betanin pK_as which structure is strictly related to Betalamic Acid. As stated, Jaguar made reverse prediction for the known pK_as of betanin, determining the conjugated C17 carboxylic function more acid than the C15,

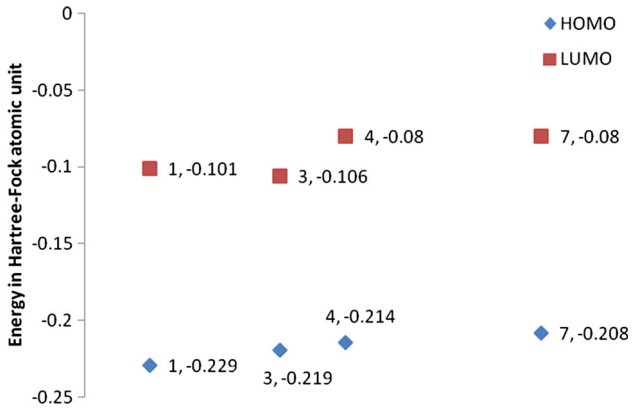

Fig. 7 HOMO and LUMO energies of dissociated and undissociated forms of Betalamic Acid

unexpectedly. For these reasons, we considered the prediction of Marvin and Epik as the most significant. A particular challenge for pK_a predictions methods is compounds that contain multiple titratable groups in which corresponding pK_as are similar in magnitude. This case drastically complicates the titrations, and experimental values are an average of several simultaneous protonation "events." This ambiguity also complicates pK_a prediction, as the models are generally trained to predict the microscopic pK_as of isolated centers [42]. Marvin has in their algorithm facilities which estimate the distribution of species among potential microstates and then estimate the apparent pK_as resulting from that distribution. Probably for these reasons, Marvin gives the better accurate prediction than the other methods. Marvin predicts C7 conjugated carboxylic function p$K_a = 4.16$, and C10 p$K_a = 3.43$, Epik p$K_a = 5.18$ and 2.62, respectively. As presumable, the conjugated acid was predicted to have a higher pK_a value in both methods. Analyzing microspecies present in solution in a pH range from 2 to 9, eight microspecies of the Betalamic Acid were identified as shown in Fig. 6.

Three of these microspecies have neutral total charge, one with total charge +1, three with total charge −1 and one with total charge −2. The distribution of the microspecies along the considered pH range showed that dissociated C10 is always in major percentage than the dissociated C7, but above all pK_a values of the two carboxylic moieties are very close to each other. At pH > 5, the completely dissociated microspecies is prevalent. Other microspecies (2, 5, 6, 8) in which the cyclic nitrogen is protonated are in negligible percentage with respect to others. The pK_a prediction of Betalamic Acid obtained by empirical methods Marvin and Epik is really consistent with results of Gandía-Herrero et al. [16]. As observed in silico, at such pH values, the di-anionic microspecies predominant in solution, which, exploiting the considerable electron-donating ability, is able to reduce in exhaustive manner the initial concentration of the radical colorant. These observations were further confirmed by the energetic calculation of HOMO and LUMO for the undissociated and dissociated predominant microspecies of Betalamic Acid. In fact, these calculations showed that the totally dissociated microspecies (7) present in major percentage at pH > 5.5 has the higher value of HOMO energy and is the best electron-donating one over the all possible microspecies (Fig. 7). For these reasons, in silico calculated pK_a values of Betalamic Acid in the range of 2.82–3.43, and 4.16–5.18, respectively, for the C10 and the C7 result consistent with experimental evidence.

6 Conclusion

The antioxidant power of Betalains is strictly due to the dissociation rate of the acid moieties present in all the molecules of this family of phytochemicals; in particular, a Betalain family compound Betalamic Acid showed potent free radical scavenging activity, but pK_a values were experimentally not measured. In this paper, we tried to in silico predict the Betalamic Acid pK_a values of by means different computational approaches. Starting from the known experimental pK_as of acid compounds, both phytochemicals and small organic, two empirical approaches and quantum mechanical DFT calculation were compared to give reliable prediction of the pK_as of Betalamic Acid. Obtained results by means of these computational approaches are consistent (pK_a values in the range of 2.82–3.43, and 4.16–5.18, respectively, for the C10 and the C7) with the experimental evidences of Gandía-Herrero et al. [16], which showed that the free radical scavenging capacity drastically decreases at pH > 5. In fact, as shown herein, in silico, at the experimental pH > 5 in solution, the totally dissociated species is predominant, exploiting the high electron-donating capability (HOMO energy). Therefore, the computationally calculated pK_a values of Betalamic Acid resulted very reliable and could be really useful for further applications.

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