



Protonation equilibria of the tryptophan metabolite 8-hydroxyquinoline-2-carboxylic acid (8-HQA) and its precursors: A potentiometric and calorimetric comparative study

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ABSTRACT

The protonation constants of quinaldic acid (QA), 8-hydroxyquinoline (8-HQ) and 8-hydroxyquinoline-2-carboxylic acid (8-HQA) were determined potentiometrically in $\text{KCl}_{(\text{aq})}$ at $I = 0.2 \text{ mol dm}^{-3}$ at different temperatures ($288.15 \leq T/\text{K} \leq 318.15$). Their temperature dependence was modeled by the van't Hoff equation, which allowed the calculation of other thermodynamic parameters, such as ΔH^0 and ΔS^0 . Protonation enthalpy changes were also experimentally determined by isothermal titration calorimetry (ITC) at $T = 298.15 \text{ K}$ in the same medium and ionic strength conditions. From the obtained results, it emerged that all stepwise protonation reactions for the three ligands are exothermic, with protonation constants decreasing with increasing temperature. Then, thermodynamic protonation parameters obtained by both approaches were critically analyzed and compared, evidencing that protonation enthalpy changes obtained experimentally by direct calorimetry are more accurate than those derived by the van't Hoff equation. However, the latter approach proved useful to evidence possible variability of this thermodynamic parameter with temperature, thus allowing the eventual calculation of the corresponding ΔC_p . Furthermore, on the basis of both the analysis of the obtained parameters and the results of detailed 1D and 2D ^1H NMR studies, it was possible to unequivocally determine the protonation sequence of the different functional groups of 8-HQA (as well as QA and 8-HQ): from basic to acidic pH, the first group to undergo protonation is the phenolate, followed by the quinolinic nitrogen and, finally, by the carboxylate.

1. Introduction

Tryptophan (Trp) is an essential exogenous amino acid with a variety of physiological functions, playing a fundamental role in the immune, gastrointestinal nervous and the central nervous systems, with a significant impact on the intestinal microbiota. Nearly 95 % of Trp is metabolised via kynurenine pathway (Scheme S1), as it is the center of metabolism of peripheral Trp in most mammalian cells [1–3]. The corresponding metabolites belong to the group of 8-hydroxyquinoline (8-HQ) derivatives, explaining the increasing interest in quinolines

(and, specifically, in 8-HQ derivatives) in the last decades. Many examples of their anticancer, antimicrobial, antioxidant and anti-neurodegenerative activity can be found in the literature [1,2,4–6]. It is not surprising that, due to its rich and diverse properties, 8-HQ is also considered a privileged scaffold in medicinal chemistry. Interestingly, quinolinic carboxylic acid derivatives are widely distributed in nature, from plants to insects and bacteria. For example, 8-hydroxy-4-methoxyquinoline-2-carboxylic acid is a siderophore used for the efficient uptake of iron in *Pseudomonas fluorescens* [7] and 8-hydroxyquinoline-2-carboxylic acid (8-HQA) was recently reported as regulator for

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bacterial abundance and diversity in the midgut of *S. littoralis* larvae, mainly due to its iron chelation properties [8,9]. These results are consistent with the observed fact that their biological activity, as well as other properties, is mainly related to their sequestering ability towards biologically relevant metal cations as Fe^{3+} / Fe^{2+} , Cu^{2+} and even Zn^{2+} . In the last years, we studied the chemical speciation of different 8-HQA based systems, in particular Fe^{2+} and Fe^{3+} /8-HQA [9] and MoO_4^{2-} /8-HQA [10]. For this kind of studies, preliminary investigation of ligands [11–13] and cations acid-base properties [14–17] is essential. In our first investigation on 8-HQA [9], we determined its protonation constants in $\text{KCl}_{(\text{aq})}$ at $I = 0.2 \text{ mol dm}^{-3}$ (chosen due its similarity to blood plasma conditions, $I = 0.16 \text{ mol dm}^{-3}$) at $T = 298.15 \text{ K}$. Since real systems frequently occur at other temperatures, in this work we present the results of an investigation on the dependence of the acid-base properties of 8-HQA on temperature, performed by potentiometric (ISE- H^+ glass electrode) titrations in $\text{KCl}_{(\text{aq})}$ at $I = 0.2 \text{ mol dm}^{-3}$ and at different temperatures ($288.15 \leq T/\text{K} \leq 318.15$). This allowed the determination of the protonation constants of 8-HQA at different temperatures and, by means of the van't Hoff equation, the calculation of other thermodynamic parameters, such as ΔH^0 and ΔS^0 . Protonation enthalpy changes were also determined by isothermal titration calorimetry (ITC) at $T = 298.15 \text{ K}$ in the same medium and ionic strength conditions as in the potentiometric studies. Then, thermodynamic protonation parameters obtained by both approaches were critically analyzed and compared, to evidence possible statistically significant differences. Furthermore, despite the presence of three protonable groups in the 8-HQA structure (namely the quinolinic nitrogen, the carboxylate in position 2, and the hydroxylate in position 8), only two $\log K$ values were determined in our previous work [9], since the third protonation constant is quite low ($\log K_3 \ll 2$) and not relevant for speciation studies in most natural waters and biological fluids. Based on a literature analysis of similar systems, we hypothesized that the protonation sequence of 8-HQA starts with the phenolate followed by the quinolinic nitrogen, while the protonation of the carboxylate occurs only under very acidic conditions. As such, to get further insights and confirmations on our hypothesis, in this work we extended the above-mentioned studies to the two structural precursors of 8-HQA, namely the two bifunctional ligands quinaldic acid (QA) and 8-HQ (Fig. 1), which are 8-HQA analogues without the hydroxylate (QA) and the carboxylate (8-HQ) groups, respectively.

The thermodynamic protonation parameters and their comparison with those of 8-HQA were useful to derive additional information on the enthalpic and entropic contributions of the individual functional groups of these molecules, as well as further evidence for the correct protonation sequence. Finally, for unquestionable confirmation of this last aspect, a detailed 1D and 2D ^1H NMR study for all the three ligands was also performed.

2. Materials and methods

2.1. Chemicals

8-hydroxyquinoline-2-carboxylic acid (8-HQA, CAS: 1571–30–8), quinoline-2-carboxylic acid (quinaldic acid, QA, CAS: 93–10–7), quinolin-8-ol (8-hydroxyquinoline, 8-HQ, CAS: 148–24–3) solutions were prepared by weighing analytical pure compounds purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany) without further purification. Working solutions were prepared adding a minimum known amount of EtOH (POCH S.A., Gliwice, Poland) to promote initial ligands solubilization in water, never exceeding, in any case, 2 % v/v. Potassium chloride (KCl, CAS: 7447–40–7, Merck KGaA, Darmstadt, Germany) aqueous solutions were prepared by weighing the pure salt, previously dried in an oven at $T = 383.15 \text{ K}$ for at least 2 h. Potassium hydroxide (KOH, CAS: 1310–58–3) and hydrochloric acid (HCl, CAS: 7647–01–0) TitraFix™ were purchased from POCH S.A. (Gliwice, Poland) and the solutions were prepared by diluting the concentrated ampoules, and standardized against potassium hydrogen phthalate (CAS: 877–24–7, Standard Reference Material, Merck, Germany) and tris (hydroxymethyl)aminomethane (Tris, CAS: 77–86–1 ACS reagent, Sigma–Aldrich Chemie GmbH, Steinheim, Germany), respectively, previously dried in an oven at $T = 383.15 \text{ K}$ for at least 2 h. All solutions were prepared with ultrapure water ($R = 18 \text{ M}\Omega \text{ cm}^{-1}$) obtained by a Milli-Q water (Millipore Direct-Q 3, Merck) purifying system, using grade A glassware.

2.2. Potentiometric measurements

Potentiometric titrations were carried out by an Orion Star™ T900 Series Potentiometric Titrator (Thermo Scientific™, Waltham, MA, USA) equipped with an automatic burette and a combined ISE- H^+ glass

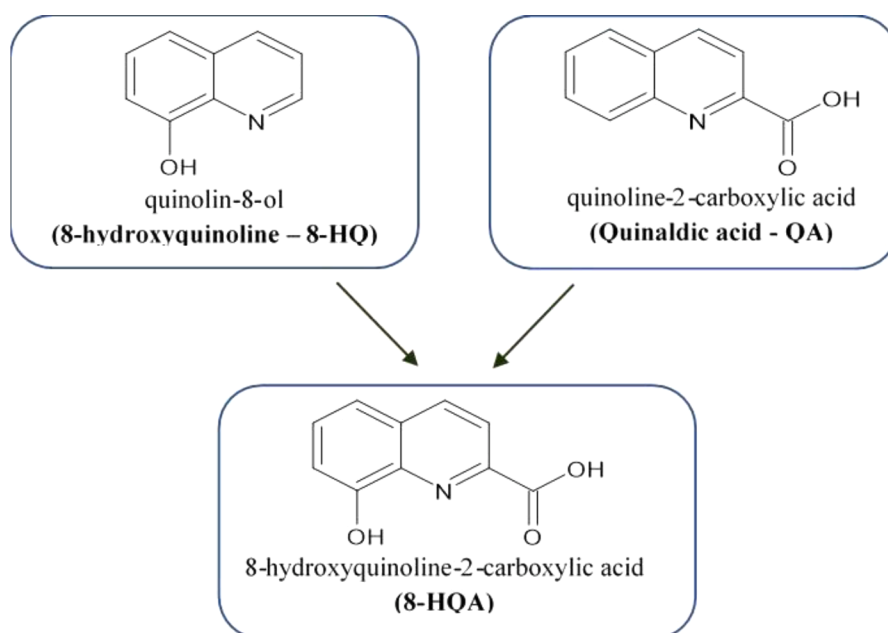


Fig. 1. Chemical structure of quinoline-2-carboxylic acid (QA), quinolin-8-ol (8-hydroxyquinoline, 8-HQ) and 8-hydroxyquinoline-2-carboxylic acid (8-HQA).

electrode Orion™ 8102BNUWP ROSS Ultra™ (Thermo Scientific). The estimated accuracy was ± 0.2 mV and 0.002 cm^3 for the electromotive force and titrant volume readings, respectively. The measurements were performed at different temperatures in the range $288.15 \leq T/K \leq 318.15$ and at the constant ionic strength of $I = 0.2 \text{ mol dm}^{-3}$ in $\text{KCl}_{(\text{aq})}$, in a double-walled, thermostatted glass cell, under magnetic stirring. Saturated argon was bubbled through the solution in order to exclude $\text{O}_{2(\text{g})}$ and $\text{CO}_{2(\text{g})}$ inside. Potentiometric measurements were carried out by titrating 25 cm^3 of the titrant solution with standard $\text{KOH}_{(\text{aq})}$ solution up to $\text{pH} \approx 10.5\text{--}11$. For each experiment, electrode calibrations were performed by independent titrations of $\text{HCl}_{(\text{aq})}$ with standard $\text{KOH}_{(\text{aq})}$ under the same experimental conditions (temperature, ionic medium and ionic strength) as the systems under investigation, in order to determine the standard electrode potential (E^0) and the acidic junction potential ($E_j = j_a [H^+]$). By this procedure, the pH scale is the free scale, $\text{pH} = -\log [H^+]$, where $[H^+]$ is the free proton concentration (not activity). The reliability of the calibration in the alkaline range was checked by calculating the ionic product of water ($\text{p}K_w$). For each titration, 80–100 data points were collected.

2.3. Isothermal titration calorimetry measurements

ITC experiments were carried out at $T = 298.15 \text{ K}$ in $\text{KCl}_{(\text{aq})}$ at $I = 0.2 \text{ mol dm}^{-3}$ with a nano-isothermal titration calorimeter Nano-ITC (TA Instruments, New Castle, USA), with an active cell volume of $980 \mu\text{L}$ and equipped with a $250 \mu\text{L}$ injection syringe. The reaction mixture in the sample cell was stirred at 250 rpm during the titration. Measurements were conducted in the overfilled mode so that liquid evaporation and the presence of the vapor phase can be neglected. Prior to the experiments, the calorimeter was chemically calibrated by a test HCl/TRIS reaction according to the procedure previously described [18]. The equipment was also double checked through an electrical calibration. In order to obtain the gross heat evolved or absorbed in the reaction, the power curves were integrated by the NanoAnalyze software (TA Instruments, New Castle, USA).

ITC measurements for the determination of the protonation enthalpy changes were performed by adding aqueous solutions of nitric acid (in $\text{KCl}_{(\text{aq})}$ 0.2 mol dm^{-3}) into the Trp-metabolites aqueous solutions (in $\text{KCl}_{(\text{aq})}$ 0.2 mol dm^{-3}). Preliminary solubility tests were carried out to define the optimal experimental conditions for the ITC titrations. A KOH solution was added to the cell solutions in order to have the ligands in the suitable deprotonated form. The concentration of HNO_3 (containing 0.2 mol dm^{-3} KCl), used as the titrant, ranged from 1 to 100 mmol dm^{-3} in order to explore different pH windows and optimize the formation of the expected species, in line with the findings from the potentiometric measurements. Details on the experimental conditions are summarized in Table 1.

Typically, 4 to 9 independent titrations were run for each system to collect an adequate number of points and obtain a satisfactory fit of the curve. In the case of QA an 8-HQA, to maximize the formation of each species according to the distribution diagrams, different pH windows were examined allowing an accurate determination of the ΔH^0 values

for each protonated species formed in solution (see Table 1). Nevertheless, data points collected at different pH windows were analyzed together using HypCal software [19] to obtain the final parameters. The heats of dilution were determined in separate blank experiments by titrating solutions of $\text{HNO}_{3(\text{aq})}$ (in $\text{KCl}_{(\text{aq})}$ at $I = 0.2 \text{ mol dm}^{-3}$) into a solution containing 0.2 mol dm^{-3} $\text{KCl}_{(\text{aq})}$ only (Fig. S1).

2.4. 1D and 2D ^1H NMR measurements

^1H NMR spectra were recorded using a Bruker Avance II 400 spectrometer. Solutions were prepared with a known ligand concentration ($c_L \approx 10^{-3} \text{ mol dm}^{-3}$) in $\text{KCl}_{(\text{aq})}$ at $I = 0.2 \text{ mol dm}^{-3}$ and at $T = 298.15 \text{ K}$, at different pH. Standard $\text{KOH}_{(\text{aq})}$ and $\text{HCl}_{(\text{aq})}$ solutions were used to adjust sample pH in the range $2 \leq \text{pH} \leq 11$, measured by a combined glass Orion™ 8103BNUWP ROSS Ultra™ semi-micro electrode, calibrated as above described. The solutions were transferred into 5 mm NMR tubes ($500 \mu\text{L}$) and a D_2O capillary was introduced in each NMR tube as external reference. It is worth mentioning that the use of a D_2O capillary avoids the preparation of the samples on a usual $\text{H}_2\text{O}/\text{D}_2\text{O}$ mixture. This allows the direct measurement of the pH of the sample without the need to evaluate the deuterium isotopic effect, which should be considered when D_2O is present in the solution, due to different binding affinities of protonating groups for D^+ vs H^+ [20–23]. Chemical shifts obtained at different pH were then refined to obtain the final $\log K$ values by the HypNMR program [24]. Structural assignments were made exploiting additional information obtained by gCOSY, NOESY and EASY-ROESY experiments. For this, a Varian 500 spectrometer (500 MHz) was used, and the samples were simply prepared by dissolving the investigated ligands in $\text{D}_2\text{O}/\text{EtOH}$ (9:1 v/v). Chemical shifts in the latter experiments are referenced to dioxane solvent ($\delta_{\text{H}} 3.70 \text{ ppm}$) as the internal standard [25,26].

3. Calculations

All the parameters of the potentiometric titrations (standard electrode potential (E^0), acidic junction potential coefficient (j_a), ionic product of water (K_w)) and the protonation constants of the studied ligands were determined by means of the BSTAC computer program [27]. For measurements performed at different temperatures, particular attention must be paid to the concentration values used in the experimental data analysis (e.g., components concentrations used as input in software for potentiometric data analysis). Changing the temperature of a solution prepared in the molar scale results in a change of volume and, thus, of concentration (and density, among others). As such, for a correct data analysis, all concentrations and volumes should be corrected for the new working temperature(s) using the appropriate density values, becoming the new input data used during calculations by dedicated software. Since, often, these changes are significant, neglecting this correction may turn into unreliable results.

PyES program [28] was used to draw the speciation diagrams and to calculate the species formation percentages.

Protonation constants in the manuscript are referred to the stepwise

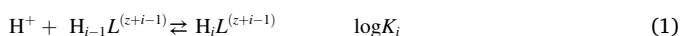
Table 1

Experimental conditions adopted for the ITC calorimetric measurements on QA, 8-HQ and 8-HQA at $T = 298.15 \text{ K}$ in $\text{KCl}_{(\text{aq})}$ at $I = 0.2 \text{ mol dm}^{-3}$.

Ligand	Equilibria	c_{burette}^a	c_{cell}^a	$c_{\text{burette}} / c_{\text{cell}}$	pH range
QA	$\text{H}^+ + \text{QA}^- \rightleftharpoons \text{H}(\text{QA})$	6.50	0.76–1.09	1.66–2.39	6.75–3.23
	$\text{H}^+ + \text{H}(\text{QA}) \rightleftharpoons \text{H}_2(\text{QA})^+$	100.47	1.02–1.03	27.23–27.49	4.02–1.79
8-HQ	$\text{H}^+ + 8\text{-HQ}^- \rightleftharpoons \text{H}(8\text{-HQ})$	1.00–1.05	0.08–0.10	2.79–2.82	9.29–4.66
	$\text{H}^+ + \text{H}(8\text{-HQ}) \rightleftharpoons \text{H}_2(8\text{-HQ})^+$				
8-HQA	$\text{H}^+ + 8\text{-HQA}^{2-} \rightleftharpoons \text{H}(8\text{-HQA})^-$	1.01	0.12–0.18	1.57–2.35	9.47–4.36
	$\text{H}^+ + \text{H}(8\text{-HQA})^- \rightleftharpoons \text{H}_2(8\text{-HQA})$	4.00	0.17–0.19	6.57–5.88	5.71–3.20
	$\text{H}^+ + \text{H}_2(8\text{-HQA}) \rightleftharpoons \text{H}_3(8\text{-HQA})^+$	39.99	0.17–0.19	65.66–58.75	4.13–2.12

^a Concentrations are expressed in mmol dm^{-3} ; HNO_3 is in the burette, whilst QA, 8-HQ or 8-HQA (containing the proper amount of base) are in the cell.

equilibria:



where L represents the fully deprotonated ligands of charge z (with sign) 8-HQA^{2-} , QA^- and 8-HQ^- , written in italic to distinguish from the generic ligand names (in their neutral form). Where not strictly necessary, charges are omitted for simplicity.

The protonation enthalpy changes (ΔH^0 , in kJ mol^{-1}) were calculated by fitting the protonation constants (expressed as $\ln K_i$, and converted to the molal concentration scale, mol kg^{-1} (H_2O)) using appropriate density values [29]) of the investigated ligands as a function of temperature (as $1/T$), according to the van't Hoff equation [30,31]:

$$\left(\frac{\partial \ln K}{\partial T}\right)_p = \frac{\Delta H^0}{RT^2} \quad (2)$$

In addition, using the well-known relationships between the thermodynamic quantities, the ΔG^0 can be calculated from Eq. (3).

$$\Delta G^0 = -RT \ln K \quad (3)$$

$T\Delta S^0$ can then be derived by Eq. (4):

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \quad (4)$$

The net heats of reaction were handled by HypCal [19], a software that allows the determination of the equilibrium constants and enthalpy changes by a non-linear least-squares minimization of the function:

$$U = \sum (Q_{obs.} - Q_{calc.})^2 \quad (5)$$

where $Q_{obs.}$ is the observed net heat for a given reaction step, while $Q_{calc.}$ is calculated as:

$$Q_{calc.} = - \sum \delta n \Delta H \quad (6)$$

where δn is the change in the number of moles of a reaction product and ΔH is the molar formation enthalpy of the reaction product. The sum is carried out over all the reaction steps; the squared residuals $(Q_{obs.} - Q_{calc.})^2$ are summed over all the titration points. The thermodynamic parameters were obtained by the simultaneous analysis of data obtained from different calorimetric titrations.

4. Results and discussion

4.1. Protonation constants at different temperatures

The study of the chemical speciation of 8-HQA in aqueous solutions seems to be a crucial aspect to access the insights about its action. However, considering the activity of the ligand in biological systems, studies carried out only at 298.15 K are not sufficient. For that purpose, we studied the effect of temperature on the chemical speciation of 8-HQA and its molecular precursors. In fact, this is particularly

important for natural waters, biological fluids and many other real aqueous systems, in which temperature can range from very few degrees (e.g., some natural waters) to relatively high values (e.g., thermal waters). Obviously, 310.15 K (i.e., 37 °C) is of chief importance in relation to human body, becoming a sort of “reference value” for speciation studies in biological systems. The protonation constants of QA, 8-HQ and 8-HQA, determined by potentiometric titrations at different temperatures, are shown in Table 2.

Information on the acid-base properties of the investigated ligands in a wide temperature range is scarce. Therefore, the only accurate comparison between our and literature protonation constants can be made with those published at $T = 298.15$ K. Values at $T = 298.15$ K are in excellent agreement with the literature data (QA [32]: $\log K_1 = 4.79$ and $\log K_2 = 1.9$ ($T = 298.15$ K in $\text{KNO}_3(\text{aq})$ 0.1 mol dm^{-3}); 8-HQ [32]: $\log K_1 = 9.63$ and $\log K_2 = 4.95$ ($T = 298.15$ K in $\text{KNO}_3(\text{aq})$ 0.1 mol dm^{-3}); 8-HQA [9]: $\log K_1 = 9.56$ and $\log K_2 = 3.96$ ($T = 298.15$ K in KCl_{aq} 0.2 mol dm^{-3})).

As can be noted, values of $\log K_2$ for QA and $\log K_3$ of 8-HQA are given in parenthesis in Table 2, and should be considered as indicative. Values determined by ISE- H^+ (glass electrode) potentiometric titrations must be “handled” with care, as the very acidic range in which they were determined highly affects their uncertainty (despite j_a was accounted in their calculations, thus increasing their accuracy). For the same reason, $\log K_3$ of 8-HQA was not determined in the previous work [9]. Since these constants are very useful for comparing the acid-base behavior of the tested ligands, they were determined in this work. However, it should be mentioned that their importance in the speciation of these ligands in natural systems is limited to a few cases (very acidic aqueous systems, such as some wastes), while it is less important in the case of the most common natural waters and biological fluids (whose pH is usually higher [33,34]). Standard deviations given in Table 2 for those constants are obtained from BSTAC program, used for data analysis. However, based on the authors experience, the first decimal digit can already be considered “uncertain”.

The analysis of the protonation constants for the three investigated ligands confirms the assumptions of the previous work [9] regarding the protonation sequence of 8-HQA. Its first protonation constants, with $\log K_1 > 9$, are very similar to $\log K_1$ of 8-HQ, which are related to the protonation of the hydroxylate group (not present in QA). Analogously, $\log K_3$ of 8-HQA are below 2 as in the case of $\log K_2$ of QA, and are related to the protonation of the carboxylate (not present in 8-HQ). As such, $\log K_2$ of 8-HQA and 8-HQ, as well as $\log K_1$ of QA, refer to the protonation of the quinolinic nitrogen, the only functional group present simultaneously in all three ligands. Differences between protonation constants for various ligands can be ascribed to the influence of the other functional groups present in the molecules. In particular, the presence of a third protonable group in the 8-HQA structure seems to have a more significant effect than the nature and position of a second group in the molecules: $\log K_2$ of 8-HQ (bearing the hydroxylate in position 8) are similar to $\log K_1$ of QA (with a carboxylate in position 2) and are higher

Table 2

Experimental protonation constants of QA, 8-HQ and 8-HQA, in $\text{KCl}_{\text{(aq)}}$ at $I = 0.2$ mol dm^{-3} and different temperatures.

Ligand	Species	$\log K_i^a$			
		288.15 K	298.15 K	310.15 K	318.15 K
QA	HL	4.820±0.001	4.750±0.001	4.679±0.001	4.636±0.001
	H ₂ L ^b	(0.93±0.01)	(1.06±0.01)	(1.27±0.01)	(1.33±0.01)
8-HQ	HL	9.808±0.001	9.639±0.002	9.470±0.002	9.385±0.002
	H ₂ L	5.160±0.001	4.991±0.002	4.789±0.002	4.679±0.001
8-HQA	HL	9.799±0.006	9.604±0.009	9.34±0.02	9.00±0.02
	H ₂ L	3.863±0.006	3.779±0.008	3.33±0.02	2.96±0.03
	H ₃ L ^b	(1.65±0.01)	(1.78±0.03)	(1.40±0.07)	(0.7±0.1)

^a $\log K_i$ refer to equilibrium: $H + H_{i-1}L = H_iL$, ± standard deviation.

^b values in parenthesis are tentative, see main text for discussion.

than $\log K_2$ of 8-HQA, where both the hydroxylate and the carboxylate are simultaneously present, and in the same positions of 8-HQ and QA, respectively. As such, for the protonation of the quinolinic nitrogen of the three molecules, the order is as follows:

$$\log K_2(8-HQ) \sim \log K_1(QA) > \log K_2(8-HQA)$$

The distribution diagrams of 8-HQA species as a function of pH at different temperatures are presented in Fig. 2. In the typical pH range of many biological fluids and natural waters ($6 < \text{pH} < 8$), 8-HQA almost exclusively occurs in its monoprotinated form HL^- , and the temperature effect is negligible. For the other species, since all distribution curves are slightly shifted towards lower pH with increasing temperature, significant differences are observed, especially at pH where their formation increases or decreases. For example, at $\text{pH} = 4.0$, 8-HQA is present as 17.6 % H_2L and 82.4 % HL^- at $T = 310.15 \text{ K}$, while it is 42.1 % H_2L and 57.7 % HL^- at $T = 288.15 \text{ K}$. As for the fully protonated species H_3L^+ , it starts to become significant ($> 10\%$) at very acidic pH values ($\text{pH} < 3$), while the fully deprotonated 8-HQA is only present under alkaline conditions at $\text{pH} > 8$.

4.2. Temperature dependence of the protonation constants: The van't Hoff method

For all the protonation constants reported in Table 2 (except $\log K_2$ of QA, but taking into account the abovementioned discussion about the uncertainties on these values), a fairly linear decreasing trend is observed with increasing temperature (see Fig. S2). The van't Hoff equation (Eq. (2)) gives the dependence of an equilibrium constant on temperature. Plotting the equilibrium constants (as natural logarithm, $\ln K$) as a function of $1/T$, if the corresponding enthalpy change is independent of temperature (i.e., $\frac{\partial \Delta H}{\partial T} = 0$, corresponding to $\Delta C_p = 0$), a linear trend is obtained, with a slope equal to $-\frac{\Delta H^0}{R}$. van't Hoff plots, presented in Fig. 3, were used to calculate the protonation enthalpy changes for all equilibria reported in Table 2.

The complete set of the protonation thermodynamic parameters (ΔG^0 , ΔH^0 and $T\Delta S^0$) is presented in Table 3 for the three studied ligands (the above discussion about protonation constants in parenthesis in Table 2 also holds for these thermodynamic parameters).

As expected, looking at the values in Table 3, the protonation enthalpy changes are negative (except for the second protonation of QA), indicating that all the stepwise protonation equilibria are exothermic. Interestingly, the presence of the three functional groups in the same molecule (8-HQA) results in an increase, with respect to the QA and 8-HQ, of the enthalpic contribution to the protonation, in particular of the quinolinic nitrogen.

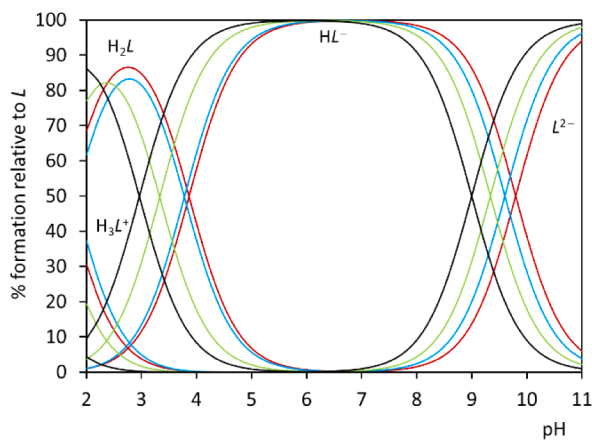


Fig. 2. Distribution diagram of 8-HQA species as a function of pH in $\text{KCl}_{(\text{aq})}$ at $I = 0.2 \text{ mol dm}^{-3}$ and $T = 288.15$ (red), 298.15 (blue), 310.15 (green) and 318.15 (black) K, $c_L = 10^{-3} \text{ mol dm}^{-3}$.

4.3. Thermodynamic parameters: ITC measurements

Isothermal titration calorimetry (ITC) allows for the accurate determination of the enthalpy changes of chemical equilibria, such as the protonation of the hydroxylate, quinolinic nitrogen and/or carboxylic groups of the ligands examined in this work, as the result of the direct measurement of heats involved in such reactions. As a general rule, the direct determination of a measurable quantity (enthalpy changes in this case) should (we would say must) always be preferred over its derivation from other measurable quantities (as carried out above by modeling the temperature dependence of the protonation constants): the reliability of the protonation enthalpy changes directly measured by ITC is, for sure, higher than that simply calculated by the van't Hoff equation. Furthermore, the thermodynamic details of the protonation equilibria do not promptly emerge by evaluating the stability constants alone and thus splitting of the standard Gibbs free energy term into its enthalpic and entropic components may allow one to unveil the driving forces of the processes occurring in solution [35], such as the interactions between the proton and the functional groups on the ligands, as well as desolvation upon protonation reactions.

The choice of the starting conditions (i.e., the protonation state of the ligand) and the proper pH window to be explored are of key relevance for accurately determining the ΔH^0 values for each protonated species formed in solution. The pH titration ranges were selected to maximize the formation of each species according to their distribution diagrams, and the resulting titration curves were treated simultaneously through the proper software. Typical calorimetric titrations for the protonation of 8-HQA are shown in Fig. 4, while the analogous curves for QA and 8-HQ are shown in Figs. S3 and S4. The thermodynamic parameters for the protonation of the hydroxylate, quinolinic nitrogen and/or carboxylate of the three ligands are listed in Table 4 and displayed in Fig. 5.

Noteworthy, the protonation of all the functional groups is an enthalpy and entropy favored process for all the systems under study. However, interesting insights can be envisaged when examining each single ligand. The first step for QA occurs with a favorable and almost comparable contribution of ΔH^0 and $T\Delta S^0$, while entropy slightly drives the protonation of the carboxylate moiety. The protonation of the hydroxylate in 8-HQ is driven by entropy with a quite large and favorable enthalpic contribution, while ΔH^0 is basically the only term that contributes to the Gibbs free energy in the reaction involving the nitrogen function. The values obtained for 8-HQ are nicely comparable with those already reported [36,37], although the latter parameters were obtained indirectly by measuring the temperature dependence of the equilibrium constants.

The proper experimental design enabled to obtain parameters for 8-HQA. It is observed that, as long as the acidity of the functional groups increases, the heat involved in the protonation process decreases, denoting that the reaction becomes less exothermic. The first protonation step is driven by enthalpy along with a favorable entropic contribution likely due to desolvation; the values and the forces are similar to those obtained for the $-\text{OH}$ group in 8-HQ. The protonation of the nitrogen unit is equally driven by both the entropic and enthalpic term, as observed in the case of the homologous group in QA. Parameters for the protonation of the carboxylate are again similar to those of QA, with very small amount of heat detected upon titration; the entropy term acts as the driving force which may be ascribed to desolvation and orientation disorder of the hydration water molecules upon COO^- protonation.

The thermodynamic parameters obtained by calorimetry for the different protonation steps of 8-HQA exhibit a trend and values which are consistent with those found for the homologous functional groups of QA and 8-HQ, as much as observed for the tendency of the protonation constants obtained by potentiometry in the same experimental conditions, thus supporting the assumptions on the assignment of the correct protonation sequence.

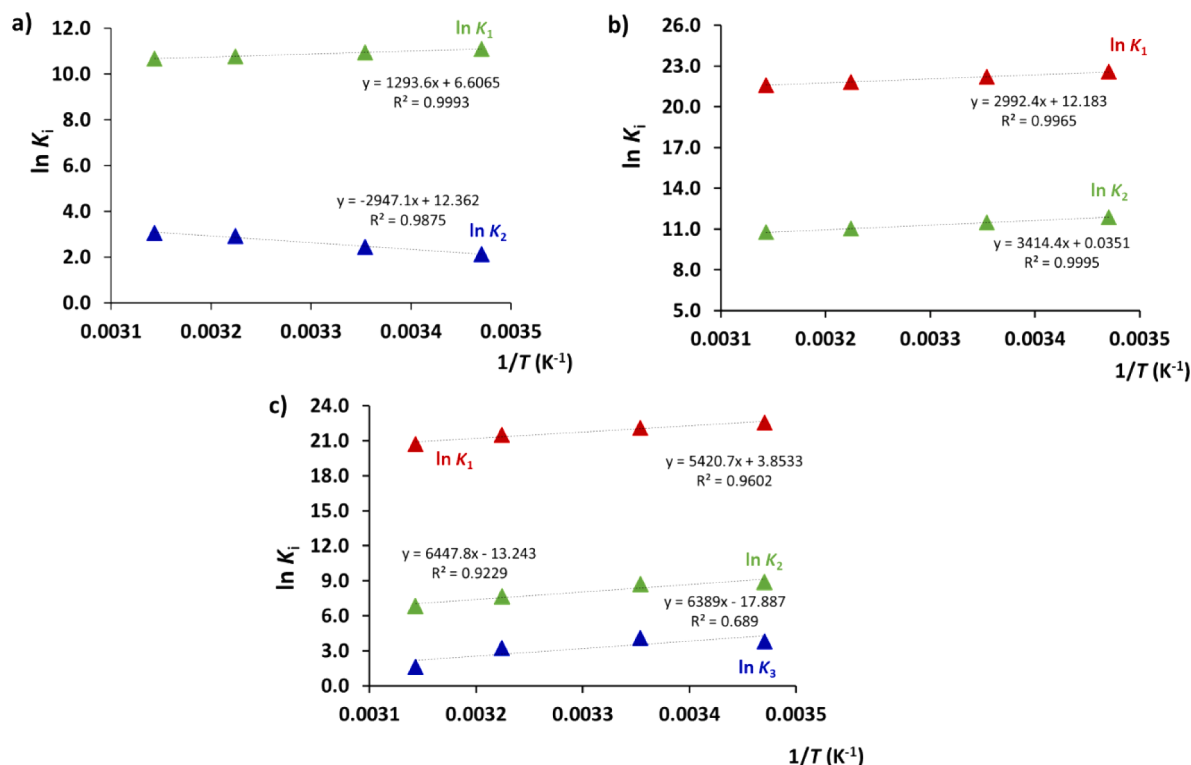


Fig. 3. van't Hoff plot of $\ln K_i$ versus $1/T$ (K^{-1}) for a) QA, b) 8-HQ and c) 8-HQA. Red symbols refer to the protonation of hydroxylate, green to the quinolinic nitrogen and blue to the carboxylate.

Table 3

Protonation thermodynamic parameters (ΔG^0 , ΔH^0 and $T\Delta S^0$) of QA, 8-HQ and 8-HQA obtained from the temperature dependence of the protonation constants (van't Hoff equation) in $KCl_{(aq)}$ at $I = 0.2 \text{ mol dm}^{-3}$ and $T = 298.15 \text{ K}$.

Ligand	Protonation equilibria	$\log K_i^a$	$\Delta G^0^{b,c}$	$\Delta H^0^{b,d}$	$T\Delta S^0^{b,e}$
QA	$H^+ + QA^- \rightleftharpoons H(QA)$	4.750	-27.11 ± 0.01	-10.8	16
	$H^+ + H(QA) \rightleftharpoons H_2(QA)^+$	$(1.06)^f$	$(-6.05 \pm 0.06)^f$	24.5	31
8-HQ	$H^+ + 8-HQ^- \rightleftharpoons H(8-HQ)$	9.639	-55.03 ± 0.01	-24.9	30
	$H^+ + H(8-HQ) \rightleftharpoons H_2(8-HQ)^+$	4.991	-28.49 ± 0.01	-28.4	0
8-HQA	$H^+ + 8-HQA^{2-} \rightleftharpoons H(8-HQA)^-$	9.604	-54.83 ± 0.05	-45.1	10
	$H^+ + H(8-HQA)^- \rightleftharpoons H_2(8-HQA)$	3.779	-21.57 ± 0.05	-53.6	-32
	$H^+ + H_2(8-HQA) \rightleftharpoons H_3(8-HQA)^+$	$(1.78)^f$	$(-10.2 \pm 0.2)^f$	-53.1	-43

^a $\log K_i$ refer to equilibrium: $H + H_i \cdot 1L = H_iL$.

^b in kJ mol^{-1} .

^c \pm standard deviation.

^d $\Delta H \pm 0.1 - 0.5 \text{ kJ mol}^{-1}$.

^e $T\Delta S \pm 1 - 2 \text{ kJ mol}^{-1}$.

^f values in parenthesis are tentative, see main text for discussion.

4.4. Thermodynamic parameters: Van't Hoff vs ITC

The comparison of Tables 3 and 4 clearly shows differences between protonation enthalpy changes (and, therefore, $T\Delta S^0$) obtained by van't Hoff and ITC. Comparisons, analogies and differences between the two approaches have been thoroughly discussed in literature [38,39]. Here we limit the discussion to some practical aspects and considerations, based on authors' experience and some statistical tools. As above mentioned, the direct determination of a measurable is preferable. In this work, this consideration holds when the goal is the accurate determination of the enthalpy changes. In fact, uncertainties associated to the thermodynamic parameters obtained by ITC are generally below 1 kJ mol^{-1} , and this level of accuracy is hardly achievable using the van't Hoff equation, which is a derived equation whose uncertainty on the refined parameter(s) must also consider the propagation of errors associated to the fitted stability constants. Reversely, when the aim of

research is the modeling of the dependence of equilibrium constants on temperature, obtaining these parameters from the fitting of these constants at different temperatures may give additional information. In fact, when an equilibrium constant is available at a given temperature together with the corresponding enthalpy change, the common practice is to calculate the same constant at another temperature by the application of the van't Hoff equation as shown in Eq. (2). However, as it is, Eq. (2) assumes that the dependence of $\ln K$ on T (as $1/T$) is linear throughout the considered T range (i.e., ΔH is constant and independent on T), while this is not always true. In such cases, especially if wide T ranges are considered, the dependence of $\ln K$ on T has a nonlinear trend, and the dependence of ΔH on T should also be taken into account as described, for example, by Clarke and Glew [40] (in some cases, the consideration of ΔC_p may ensure sufficient accuracy). Of course, though the determination of the dependence of ΔH on temperature (i.e., the determination of ΔC_p) by ITC is possible running experiments at

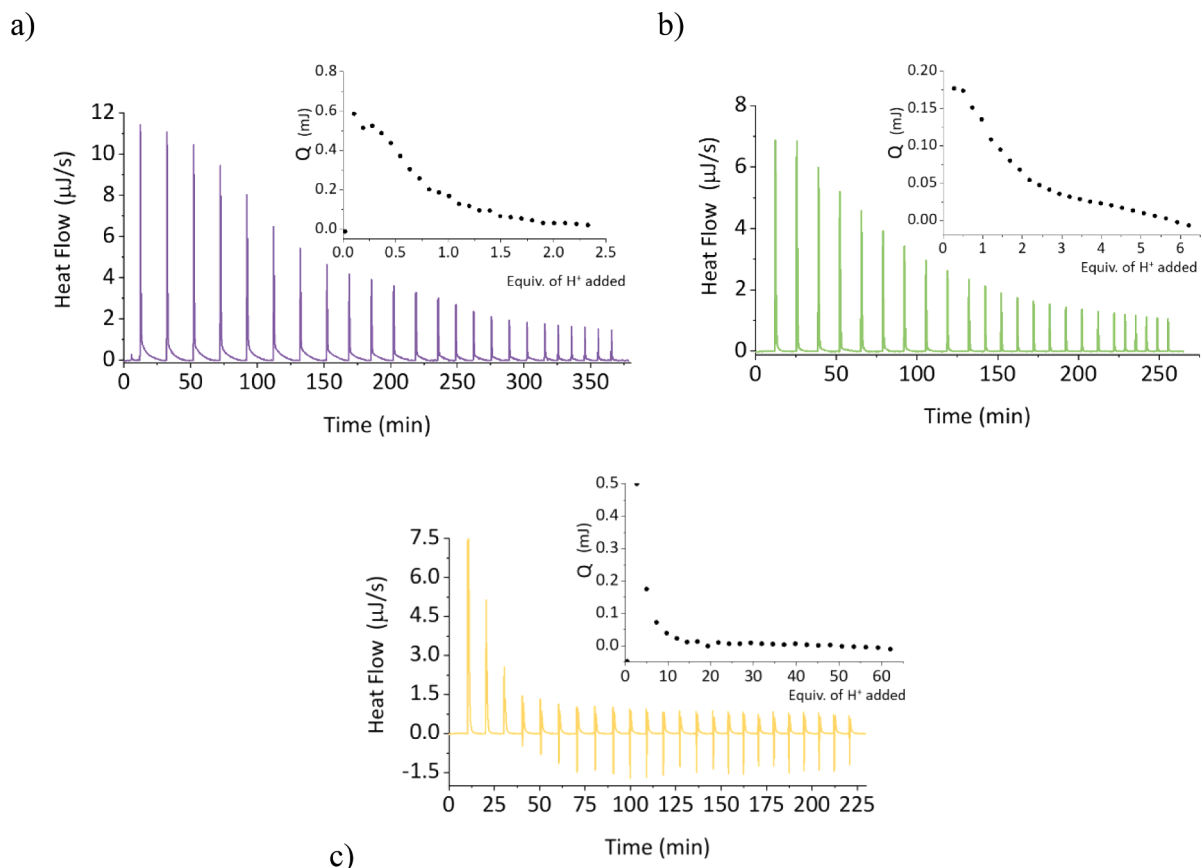


Fig. 4. Calorimetric titrations and corresponding net heat values of: a) HNO_3 $1.01 \text{ mmol dm}^{-3}$ into 8-HQA $0.12 \text{ mmol dm}^{-3}$ (pH range explored: 9.47 to 4.36); b) HNO_3 $4.00 \text{ mmol dm}^{-3}$ into 8-HQA $0.18 \text{ mmol dm}^{-3}$ (pH range explored: 5.71 to 3.20); c) HNO_3 $39.99 \text{ mmol dm}^{-3}$ into 8-HQA $0.18 \text{ mmol dm}^{-3}$ (pH range explored: 4.13 to 2.12), in $\text{KCl}_{(\text{aq})}$ at $I = 0.2 \text{ mol dm}^{-3}$ and $T = 298.15 \text{ K}$.

Table 4

Protonation thermodynamic parameters (ΔG^0 , ΔH^0 and $T\Delta S^0$) of QA, 8-HQ and 8-HQA obtained by ITC measurements in $\text{KCl}_{(\text{aq})}$ at $I = 0.2 \text{ mol dm}^{-3}$ and $T = 298.15 \text{ K}$.

Ligand	Protonation equilibria	$\log K_i^a$	ΔG^0 ^{b,c}	ΔH^0 ^{b,c}	$T\Delta S^0$ ^{b, d}
QA	$\text{H}^+ + \text{QA}^- \rightleftharpoons \text{H}(\text{QA})$	4.750	-27.11 ± 0.01	-14.1 ± 0.1	13
	$\text{H}^+ + \text{H}(\text{QA}) \rightleftharpoons \text{H}_2(\text{QA})^+$	(1.06)	$(-6.05 \pm 0.06)^e$	-2.0 ± 0.6	4
8-HQ	$\text{H}^+ + 8\text{-HQ}^- \rightleftharpoons \text{H}(8\text{-HQ})$	9.639	-55.03 ± 0.01	-22.4 ± 0.1	33
	$\text{H}^+ + \text{H}(8\text{-HQ}) \rightleftharpoons \text{H}_2(8\text{-HQ})^+$	4.991	-28.49 ± 0.01	-28.4 ± 0.3	0
8-HQA	$\text{H}^+ + 8\text{-HQA}^{2-} \rightleftharpoons \text{H}(8\text{-HQA})^-$	9.604	-54.83 ± 0.05	-29.2 ± 0.2	26
	$\text{H}^+ + \text{H}(8\text{-HQA})^- \rightleftharpoons \text{H}_2(8\text{-HQA})$	3.779	-21.57 ± 0.05	-11.1 ± 0.1	11
	$\text{H}^+ + \text{H}_2(8\text{-HQA}) \rightleftharpoons \text{H}_3(8\text{-HQA})^+$	(1.78)	$(-10.2 \pm 0.2)^e$	-3.3 ± 0.3	7

^a $\log K_i$ refer to equilibrium: $\text{H} + \text{H}_i\text{L} = \text{H}_i\text{L}$.

^b in kJ mol^{-1} .

^c \pm standard deviation.

^d $T\Delta S \pm 1 - 2 \text{ kJ mol}^{-1}$.

^e values in parenthesis are tentative, see main text for discussion.

different temperatures, it is not so frequent. The often-erroneous assumptions of the constancy of ΔH values in temperature ranges in which they effectively are not could be easily verified by the analysis of equilibrium constants determined at different temperatures. An example of such a behavior is evident by plotting the protonation constants of 8-HQA (Table 2) vs. temperature (Fig. S2), where a deviation from linearity is noticed compared to QA and 8-HQ. In such case, the fitting of the three protonation constants at different temperatures was performed by both neglecting and considering the ΔC_p parameter (*i.e.*, the classical van't Hoff Eq. (2) and the quadratic/Clarke and Glew equation [40]) and results were critically compared by means of the AIC (Akaike Information Criterion, see refs. and procedure in [41]) to look for statistically significant differences between the 1st (linear) and 2nd degree curves:

$$AIC = 2 \cdot k + n \cdot \ln \left(\frac{RSS}{n} \right) \quad (7)$$

where k is the number of independently adjusted parameters within the model, n is the number of datapoints used for the estimation, and RSS is the residual sum of squares, so that the lower the AIC the better the model performances are. In our case, $k = 2$ in the linear model (*i.e.*, the intercept and the slope, the latter including ΔH) and $k = 3$ in the second degree curve (if also the ΔC_p is considered). The results obtained for both models (Mod1 is the linear, Mod2 is the 2nd degree curve) for the three protonation constants of 8-HQA (Table S1) lead to the conclusion that Mod1 and Mod2 are significantly different. In practice, for those

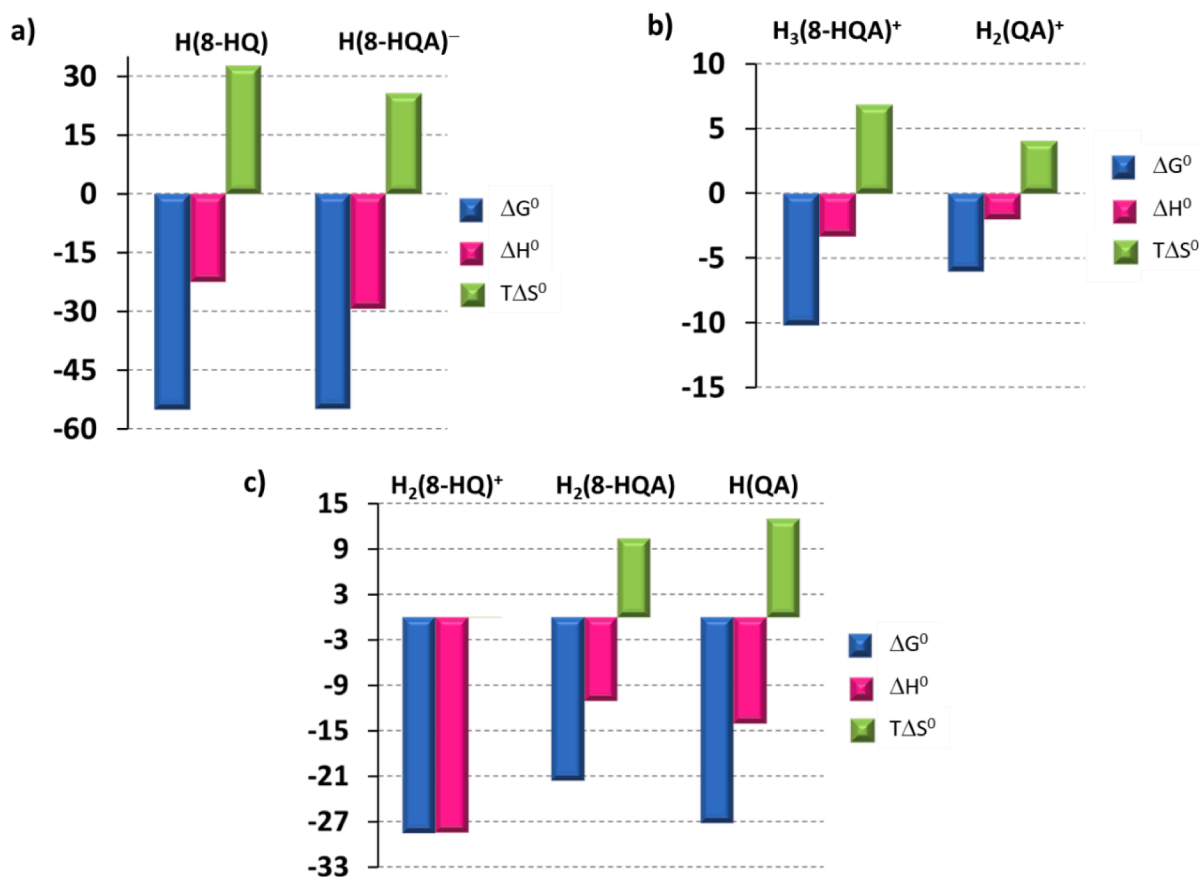


Fig. 5. Graphical representation of the protonation thermodynamic parameters (ΔG^0 , ΔH^0 and $T\Delta S^0$, in kJ mol^{-1}) obtained by ITC measurements in $\text{KCl}_{(\text{aq})}$ at $I = 0.2 \text{ mol dm}^{-3}$ and $T = 298.15 \text{ K}$ for the protonation reactions of 8-HQ, 8-HQA and QA: a) hydroxylate, b) carboxylate and c) quinolinic nitrogen.

equilibria, ΔH is not constant in the considered temperature range, so that considering ΔC_p would significantly improve the fitting (data in bold, Table S1), particularly for $\log K_3$.

Even after this discussion, the “issue” of differences between van’t Hoff (Table 3) and ITC (Table 4) results remains. We also tried to envisage if those differences are effectively (statistically) significant or not. To this aim, several *t*-tests (for average values) and *F*-tests (for uncertainties) were performed to check for significant differences between protonation enthalpy changes by ITC and van’t Hoff. The general formulation of the tests is as follows:

<i>F</i> -tests	<i>t</i> -tests
$H_0 : s(\Delta H)_{\text{ITC}}^2 = s(\Delta H)_{\text{van't Hoff}}^2$	$H_0 : \overline{\Delta H}_{\text{ITC}} = \overline{\Delta H}_{\text{van't Hoff}}$
$H_1 : s(\Delta H)_{\text{ITC}}^2 \neq s(\Delta H)_{\text{van't Hoff}}^2$	$H_1 : \overline{\Delta H}_{\text{ITC}} \neq \overline{\Delta H}_{\text{van't Hoff}}$

A scheme of the results obtained is given in Table S2. Rigorously, the uncertainty associated to values obtained by ITC and van’t Hoff for 8-HQA are not estimates of the same population (*F*-tests fail), so that *t*-tests have no strict statistical meaning. However, neglecting this last aspect, *t*-tests were performed, showing that ΔH_i^0 values for QA and ΔH_2^0 for 8-HQA obtained by van’t Hoff fitting are statistically different than those obtained by ITC, whereas the others are not.

4.5. 1D and 2D NMR study

In order to confirm the protonation sequence of 8-HQA and evaluating the analogies between the various precursors, as well as to gain a better insight into the effect of the presence of the different functional groups on the acid-base properties of 8-HQA, potentiometric and ITC measurements were complemented by a detailed NMR study. QA, 8-HQ

and 8-HQA were subjected to a series of 1D and 2D NMR (gCOSY, NOESY and EASY-ROESY) experiments. This investigation resulted in an unambiguous assignment of the resonances of the three compounds, fundamental for further analysis of their protonation sequence. Considering, for example, 8-HQA, the assignment of the peaks for its five protons was carried out by means of 2D NMR homonuclear correlation experiments, as well as NOESY through-space correlation experiments. At pH ~ 7 , at which the experiments were recorded, the 1D ¹H NMR spectrum features three doublets (all integrating for 1 nucleus) and a multiplet that accounts for 2 hydrogen atoms (Fig. 6a). The correlations observed in the gCOSY spectra, presented in Fig. 6c, indicate that the two resonances at lower fields (δ 8.56 and 8.05 ppm) are coupled, and so they belong to the carboxyl-pyridine ring spin-system, while the other two higher-field resonances account for the three hydrogen atoms in the phenolic ring. The complete assignment was obtained by a 2D ROESY spectrum (Fig. 6d), which showed a clear through-space cross-peak between the resonances at δ 8.56 ppm and part of the 7.45–7.56 ppm multiplet, indicating that these two resonances belong to the hydrogen atoms in the *peri* positions (H_c and H_d) of the molecule. The resonances of QA (Fig. S5) and 8-HQ (Fig. S6) were assigned similarly.

With the detailed assignments done, a clear analysis of the ¹H NMR titrations could be performed. For each compound, several ¹H NMR spectra were recorded at different pH values.

The graphical representation of the chemical shifts of the different 8-HQA protons, at various pH values is shown in Fig. 6b (the chemical shifts recorded in the same way for QA and 8-HQ are shown in Figs. S4 and S5). From Fig. 6b it is possible to observe that *i*) at acidic pH (ca. 1), the variation of chemical shifts goes in line with the deprotonation of the carboxylic group; *ii*) with the increase of pH (pH ≥ 3 –5), the significant change in the chemical shift evidences the quinolinic nitrogen deprotonation; *iii*) the variation of chemical shifts at pH ≈ 9 –10 results from

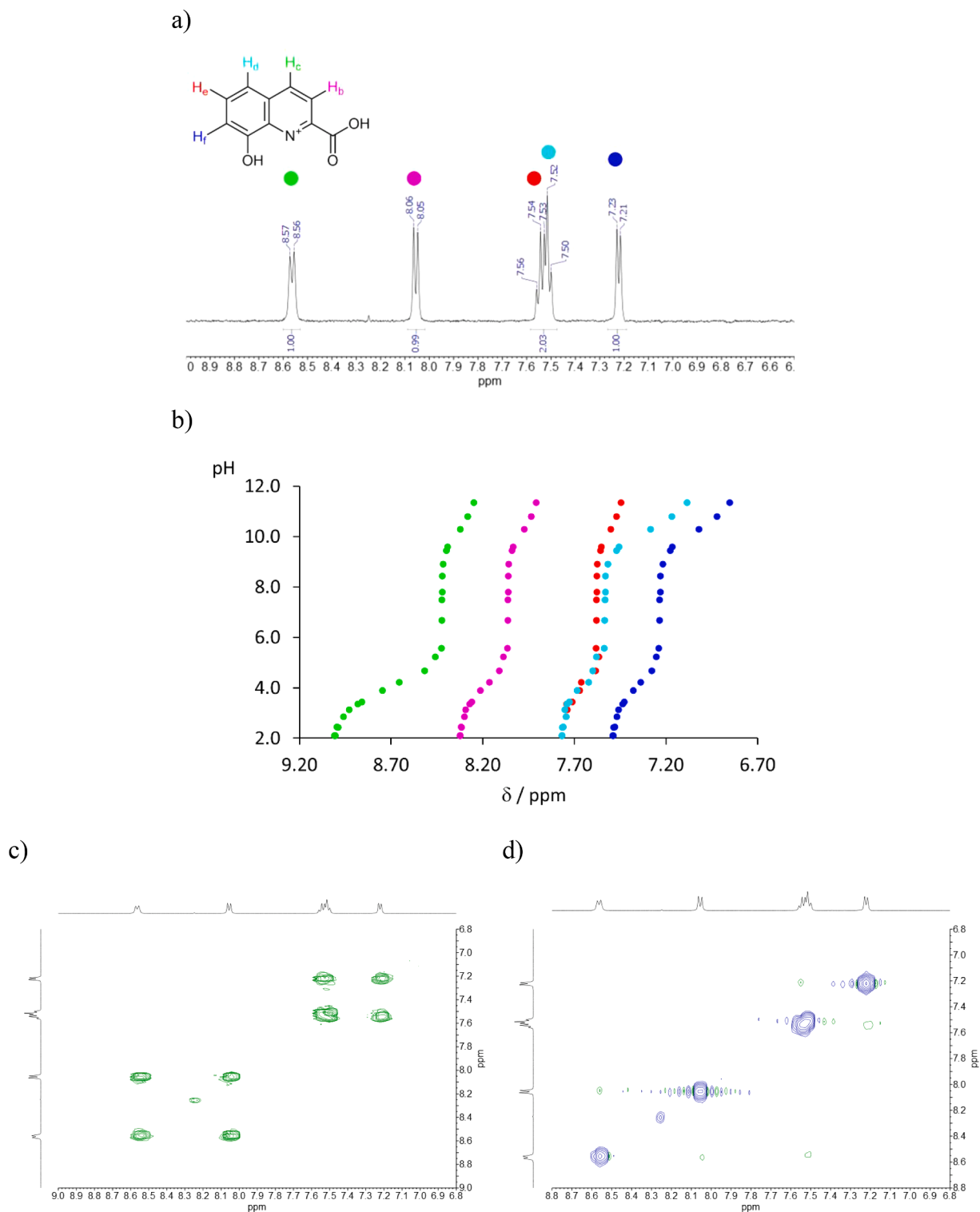


Fig. 6. (a) ^1H NMR spectrum (500 MHz, 298.15 K, $\text{D}_2\text{O}/\text{EtOH}$ 9:1 v/v), (b) graphical representation of the chemical shifts of each proton obtained from experimental spectra recorded at different pH values (400 MHz, 298.15 K, H_2O) (c) gCOSY spectrum and (d) EASY-ROESY spectrum (500 MHz, 298.15 K, $\text{D}_2\text{O}/\text{EtOH}$ 9:1 v/v) of 8-HQA.

the displacement of the hydrogen atom from the hydroxylic group, which unequivocally confirms the previous discussion on the protonation sequence. The obtained data were also fitted by HypNMR software [24] and the corresponding $\log K_i$ values and chemical shifts of each species (Table 5) were determined, resulting in full agreement with the values obtained by potentiometry ($\text{p}K_{-\text{COOH}} = 1.5 \pm 0.3$ for QA, $\text{p}K_{\text{N}} = 4.82 \pm 0.03$, 5.1 ± 0.1 and 4.0 ± 0.3 for QA, 8-HQ and 8-HQA,

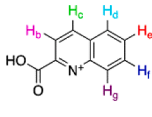
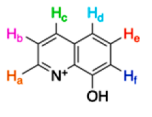
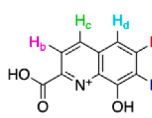
respectively; $\text{p}K_{-\text{OH}} = 9.91 \pm 0.09$ and 9.70 ± 0.03 for 8-HQ and 8-HQA).

5. Conclusions

The protonation constants of QA, 8-HQ and 8-HQA were determined by potentiometric titrations at different temperatures ($288.15 \leq T/\text{K} \leq$

Table 5

Calculated chemical shift (δ /ppm) of different (de)protonated species of QA, 8-HQ and 8-HQA, in $\text{KCl}_{(\text{aq})}$ at $I = 0.2 \text{ mol dm}^{-3}$ and $T = 298.15 \text{ K}$.

Ligand	Species	Nucleus (δ / ppm)						
		H _a	H _b	H _c	H _d	H _e	H _f	H _g
 QA	L^-	–	8.016	8.490	7.986	7.711	7.873	8.133
	HL	–	8.375	9.170	8.371	7.983	8.183	8.313
	H_2L^+	–	8.469	9.244	8.409	8.026	8.228	8.369
 8-HQ	L^-	8.262	7.435	8.694	6.966	7.445	7.185	–
	HL	8.393	7.598	8.814	7.241	7.521	7.528	–
	H_2L^+	9.087	8.044	9.007	7.519	7.793	7.790	–
 8-HQA	HL^-	–	7.999	8.359	7.352	7.541	7.086	–
	H_2L	–	8.068	8.426	7.547	7.575	7.239	–
	H_3L^+	–	8.323	9.006	7.776	7.768	7.491	–

318.15) in $\text{KCl}_{(\text{aq})}$ at $I = 0.2 \text{ mol dm}^{-3}$, and the van't Hoff equation was used to model the temperature dependence of the protonation constants, allowing the calculation of other thermodynamic parameters, such as ΔH^0 and ΔS^0 . Then, ITC measurements were also performed at $T = 298.15 \text{ K}$, in the same medium and ionic strength conditions, to determine the protonation enthalpy changes. Statistical analysis of obtained results showed that protonation enthalpy changes obtained by ITC are more accurate than those derived by the van't Hoff equation, though the latter proved useful to evidence possible variability of ΔH^0 with temperature. Thermodynamic parameters showed that the stepwise protonation reactions for the three ligands are exothermic. The analysis of the obtained parameters, together with 1D and 2D ^1H NMR studies, also confirmed the protonation sequence of the different functional groups of 8-HQA (and QA and 8-HQ as well). The first group to undergo protonation is the hydroxylate, followed by the quinolinic nitrogen and, finally, by the carboxylate.

Altogether, results obtained in this work contribute to give further insights on the acid-base properties of the three investigated ligands from both a thermodynamic and structural point of view, providing useful information for a thorough understanding of their solution behavior, with particular reference to their chemical speciation in biological fluids and other real aqueous systems.

CRedit authorship contribution statement

Anna Baryłka: Investigation, Writing – original draft. **Aneta Bagińska-Krakówka:** Investigation, Writing – original draft. **Lidia Zuccarello:** Investigation, Writing – original draft. **Francesca Mancuso:** Investigation, Writing – original draft. **Giuseppe Gattuso:** Data curation, Formal analysis, Methodology, Resources, Validation, Writing – review & editing. **Gabriele Lando:** Data curation, Formal analysis, Methodology, Validation. **Carmelo Sgarlata:** Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Validation, Writing – review & editing. **Concetta De Stefano:** Data curation, Resources, Writing – review & editing. **Beata Godlewska-Żyłkiewicz:** Funding acquisition, Project administration, Resources, Writing – review & editing. **Demetrio Milea:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing. **Sofia Gama:** Conceptualization, Data curation,

Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.tca.2023.179615](https://doi.org/10.1016/j.tca.2023.179615).

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