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ORIGINAL ARTICLE

Modeling the acid–base properties of glutathione in different ionic media, with particular reference to natural waters and biological fluids

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Abstract The acid–base properties of γ-L-glutamyl-L-cysteinyl-glycine (glutathione, GSH) were determined by potentiometry (ISE-H$^+$, glass electrode) in pure $NaI_{(aq)}$ and in $NaCl_{(aq)}/MgCl_{2(aq)}$, and $NaCl_{(aq)}/CaCl_{2(aq)}$ mixtures, at $T = 298.15$ K and different ionic strengths (up to $I_c \sim 5.0$ mol L^{-1}). In addition, the activity coefficients of glutathione were also determined by the distribution method at the same temperature in various ionic media ($LiCl_{(aq)}$, $NaCl_{(aq)}$, $KCl_{(aq)}$, $CsCl_{(aq)}$, $MgCl_{2(aq)}$, $CaCl_{2(aq)}$, $NaI_{(aq)}$). The results obtained were also used to calculate the Specific ion Interaction Theory (SIT) and Pitzer coefficients for the dependence on medium and ionic strength of glutathione species, as well as the formation constants of weak $Mg_jH_i(GSH)^{(i+2j-3)}$ and $Ca_jH_i(GSH)^{(i+2j-3)}$ complexes. Direct calorimetric titrations were also carried out in pure $NaCl_{(aq)}$ and in $NaCl_{(aq)}/CaCl_{2(aq)}$ mixtures at different ionic strengths ($0.25 \le I_c$/mol L$^{-1} \le 5.0$) in order to determine the enthalpy changes for the protonation and complex formation equilibria in these media at $T = 298.15$ K. Results obtained are useful for the definition of glutathione speciation in any aqueous media containing the main cations of natural waters and biological fluids, such as Na$^+$, K$^+$, Mg^{2+}, and Ca^{2+}. Finally, this kind of systematic studies, where a series of ionic media (e.g., all alkali metal chlorides) is taken into account in the determination of various thermodynamic parameters, is useful for the definition of some trends in the thermodynamic behavior of glutathione in aqueous solution.

Keywords Glutathione · Protonation · Activity coefficients · Distribution coefficients · Thermodynamic parameters · SIT and Pitzer models

Abbreviations

c_x	Analytical concentration, in the molar scale, of the component "x"
m_x	Analytical concentration, in the molal scale, of the component "x"
I	Ionic strength
K_i^H	ith protonation constant
K_i^{H0}	ith protonation constant at infinite dilution
γ_x	Activity coefficient of species "x" in the molal scale
y_x	Activity coefficient of species "x" in the molar scale
K_D	2-Methyl-1-propanol/aqueous salt solution distribution ratio
K_D^0	2-Methyl-1-propanol/pure water distribution ratio
k	Setschenow coefficient

Introduction

The importance of glutathione (γ-L-glutamyl-L-cysteinyl-glycine, GSH) and its derivatives (oxidized glutathione, phytochelatins, etc.) from a biological and environmental point of view is very well attested by the extensive number of studies and publications dealing with these ligands. New functions, properties, and applications are being regularly discovered and reported in literature (see, e.g., some recent books and reviews (Tew and Townsend 2011;

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Mohammadirad and Abdollahi 2011; Jan et al. 2011; Foyer and Noctor 2011; Yadav 2010; Markovic et al. 2010; Kuo and Chen 2010; Kulinsky and Kolesnichenko 2010; Yuan and Kaplowitz 2009; Szalai et al. 2009; Perricone et al. 2009; Pallardò et al. 2009; Martin and Teismann 2009; Marì et al. 2009; Fraternale et al. 2009; Franco and Cidlowski 2009; Forman et al. 2009; Masella and Mazza 2009)), and any attempts of their description in this manuscript would result in an incomplete and reductive report. Nevertheless, it is important to underline here that all the main properties of glutathione and its derivatives are strictly dependent on their chemical behavior in aqueous solution, since biological fluids, natural and waste waters, and soil solutions are "aqueous media". Above all, the acid–base properties affect both (1) the binding ability of glutathione toward several metal cations and other ligands (e.g., (Noszal and Szakacs 2003; Dorcak and Krezel 2003; Wang et al. 2009; Mah and Jalilehvand 2008)) and (2) its redox behavior (e.g., (Madej and Wardman 2007; Gough and Lees 2005)). This is one of the main reasons that induced us to undertake a systematic study on the dependence on medium and ionic strength of glutathione acid–base behavior. In fact, the most of work done over the years on the protonation and complex formation of glutathione (and its derivatives) does not report any modeling of this dependence, though it is well established (Buffle 1988; Grenthe and Puigdomenech 1997; Millero 2001) that modeling studies are of fundamental importance to get information on the chemical behavior of a molecule in aqueous systems of very variable composition, as in the case of several natural waters and biological fluids where glutathione plays key roles.

Therefore, the acid–base properties of glutathione were determined in this work by potentiometry in pure $NaI_{(aq)}$ and in $NaCl_{(aq)}/MgCl_{2(aq)}$, and $NaCl_{(aq)}/CaCl_{2(aq)}$ mixtures, at $T = 298.15$ K and different ionic strengths (up to $I_c \sim 5.0$ mol L^{-1}). In addition, the activity coefficients of glutathione were also determined by the distribution method at the same temperature in various ionic media ($LiCl_{(aq)}$, $NaCl_{(aq)}$, $KCl_{(aq)}$, $CsCl_{(aq)}$, $MgCl_{2(aq)}$, $CaCl_{2(aq)}$, $NaI_{(aq)}$). Results obtained in this and other work (Crea et al. 2007) were also used to calculate the specific ion interaction theory (SIT) (Biederman 1975, 1986; Grenthe and Puigdomenech 1997) and Pitzer (Millero 1982, 2001; Pitzer 1973, 1991) coefficients for the dependence on medium and ionic strength of glutathione species, as well as the formation constants of weak $Mg_jH_i(GSH)^{(i+2j-3)}$ and $Ca_jH_i(GSH)^{(i+2j-3)}$ complexes. Finally, direct calorimetric titrations were also carried out in pure $NaCl_{(aq)}$ and in $NaCl_{(aq)}/CaCl_{2(aq)}$ mixtures at different ionic strengths ($0.25 \leq I_c/\text{mol L}^{-1} \leq 5.0$) in order to determine the enthalpy changes for the protonation and complex formation equilibria in these media at $T = 298.15$ K.

Experimental section

Symbols used

A list of the most common symbols used in the manuscript is reported in the abbreviations section. Symbols not frequently used are defined directly in the text. In general, "c" or "m" subscripts in various symbols refer to the molar (mol L^{-1}) or molal (mol $kg^{-1}[H_2O]$) concentration scales, respectively.

Chemicals

Glutathione was used without further purification and its purity, checked alkalimetrically, was found to be >99%. Alkali and alkaline earth metal chlorides and sodium iodide aqueous solutions were prepared by weighing pure salts dried in an oven at $T = 383.15$ K. Magnesium and calcium chlorides stock solutions were previously standardized against EDTA standard solutions (Flaschka 1959). Hydrochloric acid and sodium hydroxide solutions were prepared by diluting concentrated ampoules and were standardized against sodium carbonate and potassium hydrogen phthalate, respectively, previously dried in an oven at $T = 383.15$ K for 2 h. Hydroxide solutions were preserved from atmospheric CO_2 by means of soda lime traps. All solutions were prepared with analytical grade water ($R = 18$ MΩ cm^{-1}) using grade A glassware. All chemicals were purchased from Sigma Aldrich (Italy).

Apparatus and procedure for potentiometric measurements

Potentiometric measurements were carried out (at $T = 298.15 \pm 0.1$ K in thermostatted cells) by two operators using two different setups in order to minimize systematic errors and to check the repeatability of the systems. The first setup consisted of a Model 713 Metrohm potentiometer, equipped with a half-cell glass electrode (Ross type 8101, from Thermo-Orion) and a double-junction reference electrode (type 900200, from Thermo-Orion), and a Model 765 Metrohm motorized burette. The apparatus was connected to a PC, and automatic titrations were performed using a suitable homemade computer program to control titrant delivery, data acquisition and to check for emf stability. The second setup consisted of a Metrohm model 809 Titrando apparatus controlled by Metrohm TiAMO 1.2 software equipped with combination glass electrode (Ross type 8102, from Thermo-Orion). Estimated precision was ±0.15 mV and ±0.003 mL for the emf and titrant volume readings, respectively, and was the same for both setups. All the potentiometric titrations were carried out under magnetic stirring and bubbling purified presaturated N_2

through the solution in order to exclude O_2 and CO_2. Titrand solutions were prepared by adding different amounts of glutathione ($2.0 \leq c_{GSH}/\text{mmol L}^{-1} \leq 5.0$), hydrochloric acid ($3.0 \leq c_H/\text{mmol L}^{-1} \leq 7.5$), and ionic medium in order to obtain pre-established ionic strength values ($0 < I_c/\text{mol L}^{-1} \leq 4.0$ for measurements in $NaI_{(aq)}$; $1.0 \leq I_c/\text{mol L}^{-1} \leq 5.0$ for measurements in $NaCl_{(aq)}/MgCl_{2(aq)}$ and $NaCl_{(aq)}/CaCl_{2(aq)}$ mixtures, using different $c_{Na}{:}c_{Mg}$ and $c_{Na}{:}c_{Ca}$ ratios). Potentiometric measurements were carried out by titrating 25 or 50 mL of the titrand solutions with standard NaOH solutions up to pH ~10.5. For each experiment, independent titrations of strong acid solutions with standard base were carried out under the same medium and ionic strength conditions as the systems to be investigated, with the aim of determining the electrode potential (E^0) and the acidic junction potential ($E_j = j_a\,[H^+]$). In this way, the pH scale used was the total scale, $\text{pH} \equiv -\log_{10}[H^+]$, where $[H^+]$ is the free proton concentration. The reliability of the calibration in the alkaline pH range was checked by calculating the appropriate pK_w values. For each titration, 80–100 data points were collected, and the equilibrium state during titrations was checked by adopting some usual precautions (Braibanti et al. 1987). These included checking the time required to reach equilibrium and performing back titrations.

Procedure for distribution measurements

Distribution measurements were carried out by mixing, in a separatory funnel, 25 mL of 2-methyl-1-propanol (isobutanol) with 25 mL of an aqueous solution containing known amounts of glutathione ($2.0 \leq c_{GSH}/\text{mmol L}^{-1} \leq 10.0$) and the desired salt (LiCl, NaCl, KCl, CsCl, $MgCl_2$, $CaCl_2$, NaI) in order to obtain pre-established ionic strength values ($0.5 \leq I_c/\text{mol L}^{-1} \leq 5.0$). The mixtures were then shaken for at least 4 h in a thermostatted room at $T = 298.15$ K and, after the complete separation of the two immiscible phases (4–5 h), potentiometric titrations on the aqueous solutions were performed as described above in order to calculate the glutathione concentrations. The glutathione concentrations in the organic phase were calculated by difference between the total initial glutathione concentration and its concentration in the aqueous phase after equilibrium. Some random checks were also carried out by titrating the organic phase after its dilution with an aqueous solution in the ratio 1:5 (organic phase/aqueous solution). The results obtained by the different procedures were in each case in good agreement.

Apparatus and procedure for calorimetric measurements

Calorimetric measurements were carried out at $T = 298.150 \pm 0.001$ K by means of a Tronac (model 450) isoperibolic titration calorimeter coupled with a Keithley 196 system Dmm digital multimeter. The apparatus was connected to a PC, and automatic titrations were performed using a suitable computer program to control calorimetric data acquisition. Measurements were performed by titrating with hydrochloric acid 50.0 mL of a solution containing different amounts of glutathione ($2.0 \leq c_{GSH}/\text{mmol L}^{-1} \leq 5.0$), sodium hydroxide (to fully deprotonate GSH), and the supporting electrolyte (NaCl or NaCl/$CaCl_2$ mixtures at different ionic strengths, $0.25 \leq I_c/\text{mol L}^{-1} \leq 5.0$). The titrant was delivered by a 2.5 mL capacity model 1002TLL Hamilton syringe with a precision of ±0.001 mL. For each experimental condition at least three measurements were performed. The precision of the calorimetric apparatus was $Q \pm 0.008$ J and was checked by titrating a THAM [tris(hydroxymethyl)aminomethane] buffer solution with HCl (the heat of protonation resulted $\Delta H = -47.53 \pm 0.06\ \text{kJ mol}^{-1}$). The enthalpy of dilution was measured before each experiment under the same experimental conditions as for the calorimetric measurements. The enthalpy changes for the ionization of water used in the calculations were taken from De Stefano et al. (2001).

Calculations

The non-linear least squares computer program ESAB2M (De Stefano et al. 1987) was used for the refinement of all the parameters of the acid–base titration (E^0, pK_w, liquid junction potential coefficient j_a, analytical concentration of reagents). The BSTAC (De Stefano et al. 1993) and STACO (De Stefano et al. 1996) computer programs were used for the calculation of the protonation and complex formation constants. Both programs can deal with measurements at different ionic strengths. The LIANA (De Stefano et al. 1997) computer program was used to fit the potentiometric and calorimetric data through the equations reported in the following sections. The calorimetric data were analyzed by the ES5CMI (De Robertis et al. 1986a) computer program. The ES4ECI (De Stefano et al. 1993) program was used to draw the speciation diagrams and to calculate the species formation percentages. Details on models used for the dependence on ionic strength are given in the following sections. Formation constants, concentrations and ionic strengths are expressed in the molar (c, mol L^{-1}) or molal (m, $\text{mol kg}^{-1}[H_2O]$) concentration scales. Molar to molal conversions were made using appropriate density values.

If not differently specified, protonation equilibria are expressed as

$$H_{i-1}(GSH)^{(i-4)} + H^+ = H_i(GSH)^{(i-3)} \quad K_i^H \tag{1}$$

$\log K_i^H = pK_{a(5-i)}$, with $1 \leq i \leq 4$. Formation constants of

$Mg_jH_i(GSH)^{(i+2j-3)}$ and $Ca_jH_i(GSH)^{(i+2j-3)}$ species are expressed as ($M^{2+} = Mg^{2+}$ or Ca^{2+})

$$jM^{2+} + H_i(GSH)^{(i-3)} = M_jH_i(GSH)^{(i+2j-3)} \quad K_{ij} \tag{2a}$$

$$jM^{2+} + iH^+ + GSH^{3-} = M_jH_i(GSH)^{(i+2j-3)} \quad \beta_{ij} \tag{2b}$$

The analysis of experimental data

Activity and Setschenow coefficients from distribution measurements

The chemical potential (μ) of a component (x) in a given phase may be expressed as

$$\mu_{(x)} = \mu^0_{(x)} + RT\ln a_{(x)} = \mu^0_{(x)} + RT\ln\left(m_{(x)}\gamma_{(x)}\right) \tag{3}$$

where $\mu^0_{(x)}$ is the standard chemical potential and $a_{(x)}$ the activity. When the component is in thermodynamic equilibrium between two phases, its chemical potential is the same in both phases. As a consequence, for the distribution of glutathione between an aqueous (aq) salt solution and an organic (org) solvent, it is

$$\mu^0_{(aq)} + RT\ln\left(m_{(aq)}\gamma_{(aq)}\right) = \mu^0_{(org)} + RT\ln\left(m_{(org)}\gamma_{(org)}\right) \tag{4}$$

This expression may be rearranged to

$$\exp\left(\frac{\mu^0_{(aq)} - \mu^0_{(org)}}{RT}\right) = \frac{m_{(org)}\gamma_{(org)}}{m_{(aq)}\gamma_{(aq)}} = K_D\frac{\gamma_{(org)}}{\gamma_{(aq)}} \tag{5}$$

If no supporting electrolyte is added to the aqueous solution, Eq. 5 becomes

$$\exp\left(\frac{\mu^0_{(aq)} - \mu^0_{(org)}}{RT}\right) = \frac{m_{(org)}}{m_{(aq)}} = K^0_D \tag{6}$$

From the combination of these two equations, it is

$$\frac{\gamma_{(aq)}}{\gamma_{(org)}} = \frac{K_D}{K^0_D} \tag{7}$$

In the case that the concentration of the component in the organic phase is sufficiently low, its activity coefficient $\gamma_{(org)}$ may be approximated to 1, so that Eq. 7 may be written as

$$\gamma_{(aq)} = \frac{K_D}{K^0_D} \tag{8}$$

This expression is also valid (with the opportune symbols) if the molar (mol L^{-1}) concentration scale is used. From this point, the (aq) subscript will be omitted for simplicity for quantities referred to aqueous solutions.

Equation 8 may be clearly exploited to determine the activity coefficient and the salt effect (*salting-in* and *salting-out*) of a neutral species from distribution measurements carried out in pure water and in salt solutions at different ionic strengths. In the case of glutathione, the concentration of the neutral species ($H_3(GSH)$) may be obtained from the total ligand concentration (c_L) by applying the correct mass balance equation

$$c_L = [GSH^{3-}] + [H(GSH)^{2-}] + [H_2(GSH)^-] + [H_3(GSH)] + [(H_4GSH)^+] \tag{9}$$

By indicating the concentration of the neutral species $[H_3(GSH)]$ as c_0, Eq. 9 becomes

$$c_L = c_0\left(1 + \frac{1}{K^H_3[H^+]} + \frac{1}{K^H_2K^H_3[H^+]^2} + \frac{1}{K^H_1K^H_2K^H_3[H^+]^3} + K^H_4[H^+]\right) \tag{10}$$

The combination of Eq. 8 (in both the molal or molar concentration scales) with the simplest forms of the Setschenow (1889) equation for the expression of the activity coefficient of a neutral species (γ_N or y_N) as a function of the concentration of the supporting electrolyte (m_{MX} or c_{MX}), i.e.,

$$y_N = k_c c_{MX} \tag{11a}$$

$$\gamma_N = k_m m_{MX} \tag{11b}$$

makes the determination of Setschenow coefficients possible from distribution measurements:

$$\log K_{cD} = \log K^0_D + k_c c_{MX} \tag{12a}$$

$$\log K_{mD} = \log K^0_D + k_m m_{MX} \tag{12b}$$

In general, k_c and k_m are true values, but they may be also dependent on the concentration of the supporting electrolyte, as in the cases reported here. According to previous papers (Bretti et al. 2007, 2008), k_m and k_c were expressed here as

$$k_c = \left(k_{c\infty} + \frac{k_{c0} - k_{c\infty}}{c_{MX} + 1}\right) \tag{13a}$$

$$k_m = \left(k_{m\infty} + \frac{k_{m0} - k_{m\infty}}{m_{MX} + 1}\right) \tag{13b}$$

Extended Debye–Hückel (EDH) and Specific ion Interaction Theory (SIT) approaches

The dependence on medium and ionic strength of the protonation and complex formation constants of glutathione was taken into account by an EDH type equation and by the SIT (Biederman 1975, 1986; Grenthe and Puigdomenech 1997) model:

$$\log K^H_i = \log K^{H0}_i - z^*DH + L_iI \tag{14}$$

where

$$z^* = \Sigma(\text{charges})^2_{\text{reactants}} - \Sigma(\text{charges})^2_{\text{products}}$$

and where

$$\text{DH} = AI^{1/2}\left(1 + 1.5I^{1/2}\right)^{-1}$$

is the Debye–Hückel term, with $A = 0.510$ at $T = 298.15$ K in water. Depending on the concentration scale used,

$L_i \equiv C_i$ in the molar scale (EDH equation)

or

$L_i \equiv \Delta\varepsilon_i$ in the molal scale (SIT equation).

Both C_i and $\Delta\varepsilon_i$ are generally true constants, but several studies (e.g., Bretti et al. 2006, 2007, 2008; Crea et al. 2007; De Stefano et al. 2006; Cigala et al. 2010) demonstrated that they may be better expressed as a function of the ionic strength:

$$C_i = c_{\infty i} + (c_{0i} - c_{\infty i})(I_c + 1)^{-1} \tag{15a}$$

$$\Delta\varepsilon_i = \Delta\varepsilon_{\infty i} + (\Delta\varepsilon_{0i} - \Delta\varepsilon_{\infty i})(I_m + 1)^{-1} \tag{15b}$$

An extensive discussion on the applications of both the EDH and SIT models to protonation data and activity coefficients may be found, e.g., in (De Stefano et al. 2006; Bretti et al. 2007; Crea et al. 2007) and references therein. Below, the expressions of $\Delta\varepsilon_i$ coefficients are reported, just as an example, for glutathione protonation constants (Eq. 1) determined using a generic MX salt as ionic medium:

$$\Delta\varepsilon_1 = \varepsilon\left(\text{GSH}^{3-}, \text{M}^{z+}\right) + \varepsilon(\text{X}^{z-}, \text{H}^+) - \varepsilon\left(\text{HGSH}^{2-}, \text{M}^{z+}\right) \tag{16}$$

$$\Delta\varepsilon_2 = \varepsilon\left(\text{HGSH}^{2-}, \text{M}^{z+}\right) + \varepsilon(\text{X}^{z-}, \text{H}^+) - \varepsilon(\text{H}_2\text{GSH}^-, \text{M}^{z+}) \tag{17}$$

$$\Delta\varepsilon_3 = \varepsilon(\text{H}_2\text{GSH}^-, \text{M}^{z+}) + \varepsilon(\text{X}^{z-}, \text{H}^+) - k_m \tag{18}$$

$$\Delta\varepsilon_4 = k_m + \varepsilon(\text{X}^{z-}, \text{H}^+) - \varepsilon(\text{H}_4\text{GSH}^+, \text{X}^{z-}) \tag{19}$$

Pitzer model

Pitzer equations were also used to model the dependence of the stability constants of glutathione species on ionic strength, both in their simplified and classical forms (the historical aspects and the theoretical basis one may refer, for example, to Pitzer (1973, 1991) and Millero (1982, 2001), while references (De Stefano et al. 2006; Bretti et al. 2007; Crea et al. 2007) may be useful for their application to protonation data). The most directly applicable and widely used forms for the expression of activity coefficients of single ions, for a cation M^{z+} or an anion X^{z-}, are reported below:

$$\begin{aligned}\ln\gamma_{\text{M}} = {} & z_+^2 f^\gamma + 2\Sigma_{\text{a}} m_{\text{a}}(B_{\text{Ma}} + EC_{\text{Ma}}) \\ & + \Sigma_{\text{a}}\Sigma_{\text{c}} m_{\text{c}} m_{\text{a}}\left(z_+^2 B'_{\text{ca}} + z_+ C_{\text{ca}}\right) \\ & + \Sigma_{\text{c}} m_{\text{c}}(2\Theta_{\text{Mc}} + \Sigma_{\text{a}} m_{\text{a}} \Psi_{\text{Mca}}) + \Sigma_{\text{a}}\Sigma_{\text{a}'} m_{\text{a}} m_{\text{a}'} \Psi_{\text{Maa}'}\end{aligned} \tag{20}$$

$$\begin{aligned}\ln\gamma_{\text{X}} = {} & z_-^2 f^\gamma + 2\Sigma_{\text{c}} m_{\text{c}}(B_{\text{Xc}} + EC_{\text{Xc}}) \\ & + \Sigma_{\text{a}}\Sigma_{\text{c}} m_{\text{c}} m_{\text{a}}\left(z_-^2 B'_{\text{ca}} + z_- C_{\text{ca}}\right) \\ & + \Sigma_{\text{a}} m_{\text{a}}(2\Theta_{\text{Xa}} + \Sigma_{\text{c}} m_{\text{c}} \Psi_{\text{Xac}}) + \Sigma_{\text{c}}\Sigma_{\text{c}'} m_{\text{c}} m_{\text{c}'} \Psi_{\text{Xcc}'}\end{aligned} \tag{21}$$

and for neutral species

$$\ln\gamma^0_{\text{MX}} = 2\lambda I \tag{22}$$

E is the equivalent molality ($E = ½\ \Sigma_i m_i |z_i|$), and

$$f^\gamma = -A_\Phi\left[I^{1/2}\left(1 + 1.2I^{1/2}\right)^{-1} + (2/1.2)\ln\left(1 + 1.2I^{1/2}\right)\right] \tag{23}$$

where A_Φ represents the Debye–Hückel term that, in Pitzer equations, is $A_\Phi = 0.3915$ at $T = 298.15$ K.

$$B_{\text{MX}} = \beta^{(0)}_{\text{MX}} + \beta^{(1)}_{\text{MX}} f\left(\alpha_1 I^{1/2}\right) + \beta^{(2)}_{\text{MX}} f\left(\alpha_2 I^{1/2}\right) \tag{24}$$

$$B'_{\text{MX}} = \left[\beta^{(1)}_{\text{MX}} f'\left(\alpha_1 I^{1/2}\right) + \beta^{(2)}_{\text{MX}} f'\left(\alpha_2 I^{1/2}\right)\right]/I \tag{25}$$

$$C_{\text{MX}} = C^{\Phi}_{\text{MX}}/\left(2|z_{\text{M}} z_{\text{X}}|^{1/2}\right) \tag{26}$$

with

$$f(\text{x}) = 2[1 - (1 + x)\exp(-x)]/x^2 \tag{27}$$

and

$$f'(x) = -2\left[1 - (1 + x + x^2/2)\exp(-x)\right]/x^2 \tag{28}$$

Generally, $\alpha_1 = 2.0$ and $\alpha_2 = 0.0$ [(kg mol^{-1})$^{1/2}$] for all kind of electrolytes, except for 2–2 salts where $\alpha_1 = 1.4$ and $\alpha_2 = 12$, though several examples are reported in literature where different α_1 and α_2 terms were used (see, e.g., (Pitzer 1991)). $\beta^{(0)}$, $\beta^{(1)}$, $\beta^{(2)}$, and C^ϕ represent Pitzer interaction parameters between two ions of opposite signs and are "specific" for each ion pair. Θ represents the specific interaction parameters ($+\,+$ or $-\,-$) between two ions of the same sign, Ψ represents triple interaction parameters ($+\,-\,+$, $-\,+\,-$) between two similarly charged ions and an ion of opposite charge. Finally, λ is the interaction parameter for neutral species with the ions in the solution and is related to the molal Setschenow coefficient by the equation (for a 1:1 electrolyte):

$$\lambda = (\ln 10 k_m)/2 \tag{29}$$

Pitzer model can also be used in some simplified forms, especially when one or more parameters are not known or

Table 1 Protonation constants of glutathione in $\mathrm{NaI_{(aq)}}$ at different ionic strengths, at $T = 298.15$ K

I_c[a]	I_m[b]	$\log K^{\mathrm{H}}_{c1}$	$\log K^{\mathrm{H}}_{m1}$	$\log K^{\mathrm{H}}_{c2}$	$\log K^{\mathrm{H}}_{m2}$	$\log K_{c3}\mathrm{H}$	$\log K_{\mathrm{m}3}\mathrm{H}$	$\log K^{\mathrm{H}}_{c4}$	$\log K^{\mathrm{H}}_{m4}$
0.100	0.101	9.549 ± 0.001	9.546	8.692 ± 0.002	8.689	3.508 ± 0.003	3.505	2.117 ± 0.005	2.114
0.476	0.486	9.401 ± 0.002	9.392	8.617 ± 0.002	8.608	3.473 ± 0.004	3.464	2.199 ± 0.006	2.190
0.951	0.988	9.424 ± 0.002	9.407	8.699 ± 0.002	8.682	3.563 ± 0.003	3.546	2.260 ± 0.003	2.243
2.840	3.191	9.983 ± 0.009	9.932	9.230 ± 0.011	9.179	4.000 ± 0.012	3.949	2.780 ± 0.014	2.729
3.741	4.381	10.360 ± 0.010	10.291	9.530 ± 0.011	9.461	4.270 ± 0.012	4.201	3.110 ± 0.013	3.041

K^{H}_i refer to equilibrium: $\mathrm{H}_{i-1}(\mathrm{GSH})^{(i-4)} + \mathrm{H}^+ = \mathrm{H}_i(\mathrm{GSH})^{(i-3)}$, ±3 standard deviation

[a] In mol $\mathrm{L^{-1}}$

[b] In mol $\mathrm{kg^{-1}}$ ($\mathrm{H_2O}$)

when the high co-linearity between different parameters results in "unreliable" values. In one of these forms, Pitzer equations for a generic stability constant (both protonation or complex formation) in a simple MX ionic medium can be written as follows:

$$\log K_{mi} = \log K^0_{mi} + \left[z^* f^\gamma + 2p_{1i} I_m + p_{2i} I_m^2 + p_{3i}\left(2I_m f\left(2I_m^{1/2}\right)\right) + 1/2z^*\left(2I_m f'\left(2I_m^{1/2}\right)\right)\beta^{(1)}_{MX}\right]/\ln 10 \qquad (30)$$

where p_{1i} represents the summation (with sign) of all the classical Pitzer coefficients dependent on I_m, for all species involved in the formation equilibrium (i.e., $\beta^{(0)}$, Θ, λ); p_{2i} takes into account the coefficients dependent on I_m^2 (i.e., C^Φ, Ψ), and p_{3i} all $\beta^{(1)}$. For example, in the case of the first protonation constant of glutathione in $\mathrm{NaI_{(aq)}}$, as $\log K^{\mathrm{H}}_{m1}$, it is

$$p_{11} = \beta^{(0)}_{\mathrm{HI}} + \beta^{(0)}_{\mathrm{Na(GSH)}} - \beta^{(0)}_{\mathrm{NaH(GSH)}} + \Theta_{\mathrm{HNa}} \qquad (31a)$$

$$p_{21} = \mathrm{C}^\Phi_{\mathrm{HI}} + \mathrm{C}^\Phi_{\mathrm{Na(GSH)}}/\sqrt{3} - \mathrm{C}^\Phi_{\mathrm{NaH(GSH)}}/\sqrt{2} + \mathrm{C}^\Phi_{\mathrm{NaI}} + \Psi_{\mathrm{HNaI}} \qquad (31b)$$

$$p_{31} = \beta^{(1)}_{\mathrm{HI}} + \beta^{(1)}_{\mathrm{Na(GSH)}} - \beta^{(1)}_{\mathrm{NaH(GSH)}}. \qquad (31c)$$

Results and discussion

Glutathione protonation constants in pure $\mathrm{NaI_{(aq)}}$ and in $\mathrm{NaCl_{(aq)}/MgCl_{2(aq)}}$, and $\mathrm{NaCl_{(aq)}/CaCl_{2(aq)}}$ mixtures, at different ionic strengths

The protonation constants of glutathione were determined in this work in pure $\mathrm{NaI_{(aq)}}$ and in $\mathrm{NaCl_{(aq)}/MgCl_{2(aq)}}$, and $\mathrm{NaCl_{(aq)}/CaCl_{2(aq)}}$ mixtures, at $T = 298.15$ K and different ionic strengths (up to $I_c \sim 5.0$ mol $\mathrm{L^{-1}}$). Since magnesium and calcium, together with sodium and potassium, are among the most important cations in natural waters and biological fluids (Millero 2001), the results here reported could be helpful for a deeper comprehension of several natural processes involving glutathione. On the contrary, sodium iodide was selected as supporting electrolyte mainly for two practical reasons. First, the use of a different supporting electrolyte than chloride salts would be of interest for the estimation of the effect of the anion on the acid–base behavior of glutathione, especially in the acidic pH range where the formation of the positively charged $\mathrm{H_4(GSH)^+}$ occurs. The second main reason is that protonation constants obtained in $\mathrm{NaI_{(aq)}}$ would allow the calculation of both the Pitzer and SIT coefficients among various H_i $(\mathrm{GSH})^{(i-3)}$ species and iodide. As a consequence, it could be eventually possible to calculate the same coefficients for the interactions of these species with $\mathrm{(C_2H_5)_4N^+}$, which was used in form of iodide salt as supporting electrolyte in a previous study (Crea et al. 2007).

Glutathione protonation constants determined in $\mathrm{NaI_{(aq)}}$ are reported in Table 1 in both molar and molal concentration scales. For a fast comparison, some selected values previously obtained in $\mathrm{NaCl_{(aq)}}$ (Crea et al. 2007) are also reported in Table 2. The analysis of these tables evidences that the apparent protonation constants in sodium iodide are generally higher than in sodium chloride, for all four glutathione protonation steps. This is better shown in Fig. 1 where, for example, $\log K^{\mathrm{H}}_{\mathrm{c}1}$values in both $\mathrm{NaCl_{(aq)}}$ and $\mathrm{NaI_{(aq)}}$ are reported versus the square root of ionic strength (in the molar concentration scale; curves in Figure represent the dependence on ionic strength by the EDH model, as discussed in the next sections).

The protonation constants of glutathione in $\mathrm{NaCl_{(aq)}/MgCl_{2(aq)}}$ and $\mathrm{NaCl_{(aq)}/CaCl_{2(aq)}}$ mixtures were determined varying both the ionic strength and the medium composition (i.e., the $\mathrm{Mg^{2+}}$ or $\mathrm{Ca^{2+}}$ concentration). Corresponding values are reported in Tables 3 and 4, respectively, and clearly show that, at given ionic strength, the higher the concentration of alkaline earth metal cation, the lower the apparent protonation constant. This trend is better observed in Fig. 2 where, as an example, $\log K^{\mathrm{H}}_{c2}$ are reported at different ionic strengths versus the $\mathrm{Ca^{2+}}$ concentration. Looking at protonation data of Tables 3 and 4, it is also interesting to remark that, within the experimental error and the slight different experimental conditions, the acid base properties of glutathione in the presence of $\mathrm{Mg^{2+}}$ or

Table 2 Protonation constants of glutathione in $NaCl_{(aq)}$ (from Crea et al. 2007) at different ionic strengths, at $T = 298.15$ K

I_c[a]	I_m[b]	$\log K^H_{c1}$	$\log K^H_{m1}$	$\log K^H_{c2}$	$\log K^H_{m2}$	$\log K^H_{c3}$	$\log K^H_{m3}$	$\log K^H_{c4}$	$\log K^H_{m4}$
0.104	0.105	9.498	9.496	8.701	8.699	3.557	3.555	2.188	2.186
0.468	0.473	9.298	9.293	8.553	8.548	3.461	3.456	2.173	2.168
0.926	0.945	9.302	9.293	8.582	8.573	3.491	3.482	2.241	2.232
1.836	1.908	9.430	9.413	8.705	8.688	3.580	3.563	2.333	2.316
3.050	3.256	9.694	9.666	8.930	8.902	3.751	3.723	2.489	2.461
3.596	3.889	9.827	9.793	9.058	9.024	3.807	3.773	2.466	2.432
4.490	4.966	10.081	10.037	9.230	9.186	3.930	3.886	2.574	2.530

K^H_i refer to equilibrium: $H_{i-1}(GSH)^{(i-4)} + H^+ = H_i(GSH)^{(i-3)}$

[a] In mol L^{-1}

[b] In mol kg^{-1} (H_2O)

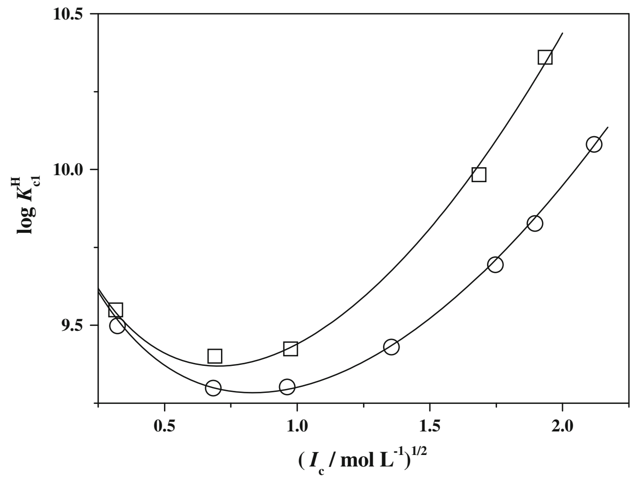

Fig. 1 Dependence of the first protonation constant (as $\log K^H_{c1}$) of GSH versus the square root of ionic strength (in mol L^{-1}), at $T = 298.15$ K. *open squares* $NaI_{(aq)}$, *open circles* $NaCl_{(aq)}$ (Crea et al. 2007)

Ca^{2+} are similar for all the protonation steps. This results in a similar distribution of the protonated glutathione species in both $NaCl_{(aq)}/MgCl_{2(aq)}$, and $NaCl_{(aq)}/CaCl_{2(aq)}$ mixtures, independently of the nature of the alkaline earth metal cation, as shown in Fig. 3.

Distribution measurements and Setschenow coefficients of glutathione

Distribution measurements were carried out following the above-described procedure. Initially, the possible isobutanol/water distribution of various salts was checked at different ionic strengths, observing that their solubility in the organic phase is always much lower than 0.1% up to $I_c = 3.0$ mol L^{-1}, and less than 0.3% at $I = 5.0$ mol L^{-1}. The values of the isobutanol/water distribution ratio of the $H_3(GSH)$ species, experimentally determined in various ionic media at different salt concentrations in both the molar and molal scales, are reported in Tables 5 and 6, respectively, and are shown in Fig. 4. The curves reported in this Figure represent the dependence of $\log K_{cD}$ on the salt concentration, according to Eqs. 12a, b. In fact, the fitting of the data reported in Tables 5 and 6 to Eqs. 12a, b and 13a, b allowed the calculation of the Setschenow coefficients of glutathione in different ionic media (reported in Table 7 in both molar and molal concentration scales), as well as its isobutanol/pure water distribution ratio (±standard deviation):

$$\log K^0_D = -1.344 \pm 0.002$$

Figure 4 gives an immediate picture of the effect of various salts on the distribution of glutathione (and, as a consequence, on the activity coefficient), indicating marked salting-in and salting-out effects, depending on the nature of the salt. For example, within the alkali metal chlorides, a different trend is observed for Li^+ and Na^+ with respect to K^+ and Cs^+. This is mainly due to the different size of the ions of the supporting electrolyte, which determine the different salt effect. In fact, though several exceptions are known, it has been demonstrated since the 50s (Bergen and Long 1956) that the salt effect varies regularly with the dimensions of ions: a salting-in effect is generally observed for large ions, while smaller ones are usually responsible of the salting-out. As reported in previous sections, Setschenow coefficients reported in Table 7 are also useful to calculate the activity coefficients of neutral $H_3(GSH)$ in different conditions by means of Eq. 11a, b.

Dependence on medium and ionic strength by EDH and SIT approaches

The dependence of the protonation constants of glutathione in $NaI_{(aq)}$ was modeled according to the EDH and SIT equations, and corresponding parameters are reported in Table 8 (simplified Pitzer parameters are also shown in the same Table). Concerning the apparent protonation constants obtained in $NaCl_{(aq)}/MgCl_{2(aq)}$ and $NaCl_{(aq)}/CaCl_{2(aq)}$

Table 3 Protonation constants of glutathione in $MgCl_{2(aq)}/NaCl_{(aq)}$ mixtures at different ionic strengths, at $T = 298.15$ K

I_c [a]	I_m [b]	c_{Mg} [a]	m_{Mg} [b]	log K^H_{c1}	log K^H_{m1}	log K^H_{c2}	log K^H_{m2}	log K^H_{c3}	log K^H_{m3}	log K^H_{c4}	log K^H_{m4}
0.993	1.018	0.051	0.052	9.414 ± 0.003	9.403	8.422 ± 0.005	8.411	3.465 ± 0.008	3.454	2.273 ± 0.014	2.262
1.009	1.038	0.103	0.106	9.400 ± 0.006	9.388	8.369 ± 0.005	8.357	3.463 ± 0.006	3.451	2.297 ± 0.012	2.285
1.012	1.043	0.204	0.210	9.332 ± 0.026	9.319	8.235 ± 0.022	8.222	3.414 ± 0.027	3.401	2.215 ± 0.038	2.202
2.994	3.214	0.098	0.105	9.734 ± 0.012	9.703	8.727 ± 0.011	8.696	3.723 ± 0.016	3.692	2.586 ± 0.021	2.555
2.988	3.227	0.196	0.212	9.650 ± 0.017	9.617	8.651 ± 0.017	8.618	3.701 ± 0.025	3.668	2.661 ± 0.030	2.628
3.048	3.328	0.416	0.454	9.385 ± 0.016	9.347	8.484 ± 0.012	8.446	3.541 ± 0.021	3.503	2.387 ± 0.027	2.349
5.004	5.647	0.098	0.111	10.340 ± 0.079	10.288	9.355 ± 0.065	9.303	4.070 ± 0.064	4.018	2.921 ± 0.064	2.869
5.003	5.680	0.196	0.223	10.377 ± 0.045	10.322	9.202 ± 0.039	9.147	4.004 ± 0.038	3.949	2.843 ± 0.042	2.788
5.003	5.748	0.416	0.478	9.860 ± 0.092	9.800	8.984 ± 0.079	8.924	3.953 ± 0.080	3.893	2.805 ± 0.087	2.745

K_i^H refer to equilibrium: $H_{i-1}(GSH)^{(i-4)} + H^+ = H_i(GSH)^{(i-3)}$, ±3 standard deviation

[a] In mol L^{-1}

[b] In mol kg^{-1} (H_2O)

Table 4 Protonation constants of glutathione in $CaCl_{2(aq)}/NaCl_{(aq)}$ mixtures at different ionic strengths, at $T = 298.15$ K

I_c [a]	I_m [b]	c_{Ca} [a]	m_{Ca} [b]	log K^H_{c1}	log K^H_{m1}	log K^H_{c2}	log K^H_{m2}	log K^H_{c3}	log K^H_{m3}	log K^H_{c4}	log K^H_{m4}
0.993	1.019	0.051	0.052	9.294 ± 0.009	9.283	8.515 ± 0.009	8.504	3.418 ± 0.013	3.407	2.066 ± 0.021	2.055
0.983	1.012	0.101	0.104	9.229 ± 0.002	9.216	8.491 ± 0.003	8.478	3.404 ± 0.006	3.391	2.101 ± 0.009	2.088
1.000	1.033	0.200	0.207	9.123 ± 0.006	9.109	8.388 ± 0.006	8.374	3.351 ± 0.009	3.337	2.027 ± 0.011	2.013
1.998	2.103	0.106	0.112	9.405 ± 0.003	9.383	8.587 ± 0.003	8.565	3.519 ± 0.005	3.497	2.255 ± 0.010	2.233
1.960	2.074	0.200	0.212	9.403 ± 0.006	9.378	8.487 ± 0.010	8.462	3.466 ± 0.017	3.441	2.254 ± 0.024	2.229
2.002	2.130	0.294	0.313	9.305 ± 0.010	9.278	8.457 ± 0.011	8.430	3.434 ± 0.015	3.407	2.125 ± 0.021	2.098
2.998	3.226	0.106	0.114	9.599 ± 0.006	9.567	8.847 ± 0.010	8.815	3.661 ± 0.013	3.629	2.370 ± 0.020	2.338
3.000	3.252	0.200	0.217	9.558 ± 0.007	9.523	8.790 ± 0.008	8.755	3.624 ± 0.012	3.589	2.272 ± 0.025	2.237
3.000	3.289	0.400	0.439	9.456 ± 0.010	9.416	8.691 ± 0.014	8.651	3.514 ± 0.020	3.474	2.251 ± 0.030	2.211
4.018	4.430	0.106	0.117	9.938 ± 0.007	9.896	9.106 ± 0.013	9.064	3.882 ± 0.023	3.840	2.604 ± 0.032	2.562
4.000	4.441	0.200	0.222	9.859 ± 0.010	9.814	9.025 ± 0.016	8.980	3.813 ± 0.025	3.768	2.510 ± 0.033	2.465
3.998	4.519	0.506	0.571	9.688 ± 0.028	9.634	8.874 ± 0.041	8.820	3.596 ± 0.061	3.542	2.791 ± 0.069	2.737
5.014	5.673	0.106	0.120	10.565 ± 0.020	10.511	9.374 ± 0.021	9.320	4.115 ± 0.029	4.061	2.930 ± 0.037	2.876
5.001	5.698	0.200	0.228	10.454 ± 0.030	10.397	9.213 ± 0.052	9.156	4.014 ± 0.078	3.957	2.810 ± 0.101	2.753
5.001	5.817	0.505	0.587	9.423 ± 0.056	9.357	9.174 ± 0.038	9.108	3.978 ± 0.061	3.912	3.161 ± 0.071	3.095

K_i^H refer to equilibrium: $H_{i-1}(GSH)^{(i-4)} + H^+ = H_i(GSH)^{(i-3)}$, ±3 standard deviation

[a] In mol L^{-1}

[b] In mol kg^{-1} (H_2O)

mixtures, the calculation of both EDH and simplified SIT parameters was not performed. The reason for this choice is mainly due to the fact that, for a correct data interpretation, two sets of parameters would be necessary to model the dependence on ionic strength in these mixtures: a term dependent on the sodium concentration, and one dependent on the alkaline earth metal cation. Such a procedure is possible, but the characteristics of simplicity and direct applicability of both the EDH and simplified SIT models would be lost. Moreover, the availability of the Setschenow coefficients of neutral $H_3(GSH)$ determined in various ionic media by distribution measurements makes the calculation of the classical SIT coefficients possible for various glutathione species. Nonlinear least square calculations gave the results reported in Table 9 for all the media investigated in this and previous work (Crea et al. 2007), together with the SIT coefficients for H^+–Cl^- and H^+–I^- interactions, taken from (Bretti et al. 2006). This Table shows some interesting results and needs to be carefully analyzed and commented. As can be noted, two sets of coefficients are reported for the interaction of glutathione species with Na^+, while several different coefficients are tabled for the same interaction of positively charged $H_4(GSH)^+$ species with chloride. During preliminary calculations, data in both $NaCl_{(aq)}$ and $NaI_{(aq)}$ were analyzed simultaneously by LIANA program in order to "constrain"

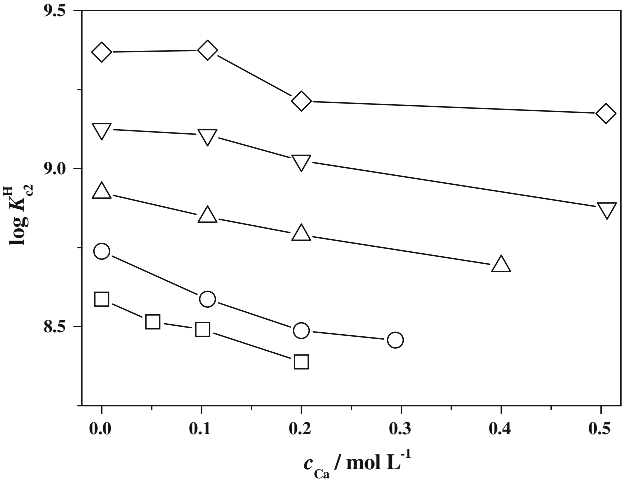

Fig. 2 Dependence of the second protonation constant (as log K^{H}_{c2}) of GSH versus c_{Ca} (in mol L^{-1}), in $NaCl_{(aq)}$/$CaCl_{2(aq)}$ mixtures at different ionic strengths and $T = 298.15$ K. *open squares* $I_c \sim 1.0$ mol L^{-1}, *open circles* = $I_c \sim 2.0$ mol L^{-1}, *open triangles* $I_c \sim 3.0$ mol L^{-1}, *open inverted triangle* $I_c \sim 4.0$ mol L^{-1}, *open diamonds* $I_c \sim 5.0$ mol L^{-1}

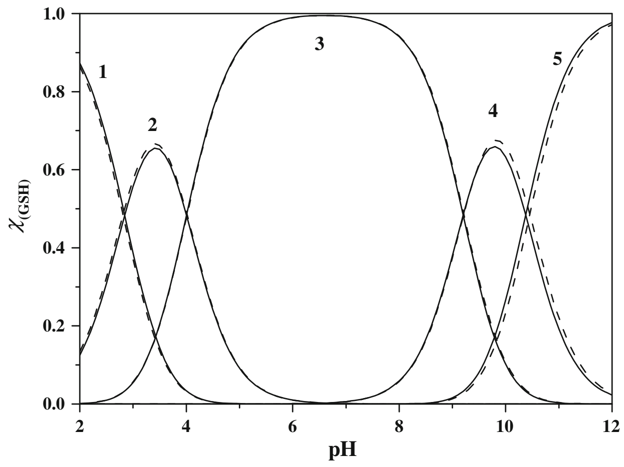

Fig. 3 Speciation diagrams of glutathione versus pH in $NaCl_{(aq)}$/$MgCl_{2(aq)}$ (*solid lines*) and $NaCl_{(aq)}$/$CaCl_{2(aq)}$ (*dashed lines*) mixtures at $T = 298.15$ K and $I = 5.0$ mol L^{-1}. $c_{GSH} = 0.005$ mol L^{-1}, $c_{Mg} = c_{Ca} = 0.200$ mol L^{-1}. *1* $H_4(GSH)^+$, *2* $H_3(GSH)^0$, *3* $H_2(GSH)^-$, *4* $H(GSH)^{2-}$, *5* $(GSH)^{3-}$

the coefficients for $(GSH)^{3-}$–Na^+, $H(GSH)^{2-}$–Na^+ and $H_2(GSH)^-$–Na^+ interactions to the same value in both ionic media as recommended by the SIT theory. The same procedure was adopted for the log K^{H}_{m4} values in all the alkali metal chloride media where the Setschenow coefficients of neutral $H_3(GSH)$ were available (i.e., $LiCl_{(aq)}$, $NaCl_{(aq)}$, $KCl_{(aq)}$, $CsCl_{(aq)}$) in order to refine a single set of SIT coefficients for the Cl^-–$H_4(GSH)^+$ species (Eq. 19). In both cases unreliable values of interaction coefficients were obtained during refinements, associated with very high systematic errors. This behavior is not infrequent in such calculations and may be usually ascribed to the fact that SIT model, in its original formulation, does not take into account other kinds of interactions than those between ions of opposite sign. On the contrary, for ligands like glutathione containing different kinds of functional groups, the possibility of further interactions is common. Analogous considerations should be done in the case of the SIT coefficients reported in Table 9 for the interactions of glutathione species with Mg^{2+} and Ca^{2+}, where very high errors are observed.

Dependence on medium and ionic strength by Pitzer approach

Difficulties occurred during SIT calculations suggested to follow different approaches in the experimental data analysis. The dependence on medium and ionic strength of glutathione protonation constants was therefore modeled by the Pitzer equations. The simplified Pitzer parameters for log K^{H}_{mi} values in $NaI_{(aq)}$ were already reported in Table 8. Concerning data in $NaCl_{(aq)}$/$MgCl_{2(aq)}$ and $NaCl_{(aq)}$/$CaCl_{2(aq)}$ mixtures, considerations and problems arose during SIT calculations suggested to bypass this step for these systems, proceeding directly to the determination of the classical Pitzer parameters. In fact, though the formulation of Pitzer equations in their classical form may appear quite complex, it was previously pointed out that the original formulation of Pitzer model explicitly takes into account the possibility of further interactions by different terms (specifically same sign and triple interaction terms), dependent on both the cations and/or anions of supporting electrolytes. In the case, for example, of the dependence of the log K^{H}_{m4} values on ionic strength in the alkali metal chloride media, good results (with a mean deviation on the whole fit m.d. = 0.023) were obtained by considering the possibility of same sign (Θ) interactions for the $H_4(GSH)^+$ species with Li^+, Na^+, K^+ (not for Cs^+), and a triple interaction for Li^+ only (ψ, $H_4(GSH)^+$–Li^+–Cl^-). The same considerations hold for the protonation constants in $NaCl_{(aq)}$ and $NaI_{(aq)}$. When refined simultaneously, a reasonably good fit of data (m.d. = 0.008) was obtained by considering the presence of same sign and triple interactions with iodide. Refined Pitzer parameters for the interactions of glutathione species with alkali metal cations and chloride or iodide are summarized in Table 10. Known parameters used to calculate these values were taken from refs. Pitzer (1991) and Millero (1982). Values related to glutathione interactions in pure $NaCl_{(aq)}$ were then fixed and used to calculate the Pitzer parameters related to $H_i(GSH)^{(i-3)}$–Mg^{2+} and $H_i(GSH)^{(i-3)}$–Ca^{2+} interactions from the apparent protonation constants in $NaCl_{(aq)}$/$MgCl_{2(aq)}$ and $NaCl_{(aq)}$/$CaCl_{2(aq)}$ mixtures, respectively. Several attempts were made, in which the refinement of different combinations of $\beta^{(0)}$, $\beta^{(1)}$, $\beta^{(2)}$, C^{Φ}, Θ, and ψ parameters was tested. Unfortunately,

Table 5 Isobutanol/water distribution ratios of neutral glutathione (H_3GSH) in different aqueous media and salt concentrations, at $T = 298.15$ K

c_s [a]	log K_{cD}	$LiCl_{(aq)}$		$NaCl_{(aq)}$		$KCl_{(aq)}$		$CsCl_{(aq)}$		$MgCl_{2(aq)}$		$CaCl_{2(aq)}$		$NaI_{(aq)}$	
		c_s [a]	log K_{cD}	c_s [a]	logK_{cD}	c_s [a]	log K_{cD}	c_s [a]	log K_{cD}	c_s [a]	log K_{cD}	c_s [a]	log K_{cD}	c_s [a]	log K_{cD}
0	−1.339	0.505	−1.204	0.504	−1.242	0.506	−1.377	1.008	−1.474	0.172	−1.199	0.168	−1.257	1.012	−1.025
0	−1.338	0.505	−1.202	0.504	−1.241	0.506	−1.375	1.008	−1.474	0.172	−1.199	0.168	−1.257	1.012	−1.025
0	−1.373	1.010	−1.155	0.504	−1.246	1.011	−1.406	2.001	−1.585	0.172	−1.199	0.168	−1.255	2.007	−0.939
0	−1.373	1.010	−1.155	0.504	−1.246	1.011	−1.406	2.001	−1.589	0.172	−1.199	0.168	−1.255	2.007	−0.937
0	−1.381	1.010	−1.155	0.504	−1.241	1.011	−1.408	2.801	−1.673	0.344	−1.131	0.337	−1.206	2.810	−0.903
0	−1.347	1.010	−1.155	0.504	−1.241	1.011	−1.408	2.801	−1.677	0.344	−1.132	0.337	−1.205	2.810	−0.903
0	−1.345	3.031	−1.066	1.008	−1.231	2.998	−1.520	1.012	−1.475	0.344	−1.132	0.337	−1.204	1.001	−1.010
		3.031	−1.065	1.008	−1.232	2.998	−1.522	1.012	−1.475	0.344	−1.131	0.337	−1.203	1.001	−1.010
		4.659	−1.052	1.008	−1.216	2.998	−1.524	2.007	−1.589	1.011	−1.077	0.893	−1.147	2.000	−0.934
		4.659	−1.053	1.008	−1.218	2.998	−1.519	2.007	−1.589	1.011	−1.077	0.893	−1.147	2.000	−0.933
				1.008	−1.237			2.810	−1.673	1.011	−1.078	0.893	−1.146	2.800	−0.900
				1.008	−1.219			2.810	−1.673	1.011	−1.078	0.893	−1.147	2.800	−0.900
				2.988	−1.201										
				2.988	−1.203										
				2.988	−1.194										
				2.988	−1.193										
				2.988	−1.173										
				2.988	−1.193										
				2.988	−1.171										
				5.008	−1.217										
				5.008	−1.217										
				5.008	−1.215										
				5.008	−1.215										

[a] Salt concentration in mol L^{-1}

unexpected, unreliable results were obtained in each case. The reasons of the failure of these refinements may be found in problems frequently occurring during Pitzer modeling: high correlation and co-linearity between different parameters, and the risk of over-parameterization during fitting procedures. For example, high correlation coefficients are generally observed between $\beta^{(0)}$ and Θ, or C^{Φ} and ψ terms, so that Θ and ψ are usually neglected. Nevertheless, same sign and triple interactions (represented by these coefficients) may be sometimes fundamental, as in the case of polyfunctional ligands like glutathione. For example, when considering the monoprotonated $H(GSH)^{2-}$ species, where the first proton is bound to the amino group (Crea et al. 2007), the interaction with Cl^- must be considered, and this is accounted by Θ in Pitzer formalism. As another example, while ammonium cation of GSH interacts with chloride, carboxylate–Mg^{2+} or Ca^{2+} interactions take place simultaneously, i.e., triple $H(GSH)^{2-}$–M^{2+}–Cl^- interactions must be taken into account, as well as other (not less important) $- + +$ interactions like $H(GSH)^{2-}$–M^{2+}–Na^+. Of course, similar considerations hold for other glutathione species.

Formation of $Mg_jH_i(GSH)^{(i+2j-3)}$ and $Ca_jH_i(GSH)^{(i+2j-3)}$ species

In order to bypass these problems, alternative approaches must be followed. Both SIT and Pitzer models explain the lowering of the apparent protonation constants in $NaCl_{(aq)}$/$MgCl_{2(aq)}$ and $NaCl_{(aq)}$/$CaCl_{2(aq)}$ mixtures with respect to pure $NaCl_{(aq)}$ by the variation of the activity coefficients of ions involved in the protonation equilibria (i.e., proton and glutathione in its free and protonated forms). Alternatively, this lowering can be interpreted in terms of ion pair formation between glutathione species and Mg^{2+} or Ca^{2+}. Though the accurate determination of the stability of weak complexes is always quite difficult (Daniele et al. 2008), this approach is probably one of the most useful when non-reliable parameters can be obtained from classical theories for the modeling of the dependence of activity coefficients on ionic strength, as happened in the case of both SIT and Pitzer approaches applied to glutathione protonation constants in $NaCl_{(aq)}$/$MgCl_{2(aq)}$ and $NaCl_{(aq)}$/$CaCl_{2(aq)}$ mixtures. Potentiometric measurements in these media, at different ionic strengths, were therefore reanalyzed by both

Table 6 Isobutanol/water distribution ratios of neutral glutathione (H_3GSH) in different aqueous media and salt concentrations, at $T = 298.15$ K

m_s [a]	$\log K_{m\mathrm{D}}$	$\mathrm{LiCl_{(aq)}}$		$\mathrm{NaCl_{(aq)}}$		$\mathrm{KCl_{(aq)}}$		$\mathrm{CsCl_{(aq)}}$		$\mathrm{MgCl_{2(aq)}}$		$\mathrm{CaCl_{2(aq)}}$		$\mathrm{NaI_{(aq)}}$	
		m_s [a]	$\log K_{m\mathrm{D}}$	m_s [a]	$\log K_{m\mathrm{D}}$	m_s [a]	$\log K_{m\mathrm{D}}$	m_s [a]	$\log K_{m\mathrm{D}}$	m_s [a]	$\log K_{m\mathrm{D}}$	m_s [a]	$\log K_{m\mathrm{D}}$	m_s [a]	$\log K_{m\mathrm{D}}$
0	−1.340	0.512	−1.210	0.510	−1.247	0.515	−1.384	1.056	−1.494	0.173	−1.202	0.169	−1.260	1.054	−1.043
0	−1.339	0.512	−1.208	0.510	−1.246	0.515	−1.382	1.056	−1.494	0.173	−1.202	0.169	−1.260	1.054	−1.043
0	−1.374	1.034	−1.165	0.510	−1.251	1.043	−1.420	2.198	−1.626	0.173	−1.202	0.169	−1.258	2.176	−0.974
0	−1.374	1.034	−1.165	0.510	−1.251	1.043	−1.420	2.198	−1.630	0.173	−1.202	0.169	−1.258	2.176	−0.972
0	−1.382	1.034	−1.165	0.510	−1.246	1.043	−1.422	3.202	−1.731	0.347	−1.135	0.341	−1.211	3.153	−0.953
0	−1.348	1.034	−1.165	0.510	−1.246	1.043	−1.422	3.202	−1.735	0.347	−1.136	0.341	−1.210	3.153	−0.953
0	−1.346	3.236	−1.094	1.030	−1.240	3.304	−1.562	1.060	−1.495	0.347	−1.136	0.341	−1.209	1.042	−1.028
		3.236	−1.093	1.030	−1.241	3.304	−1.564	1.060	−1.495	0.347	−1.135	0.341	−1.208	1.042	−1.028
		5.157	−1.096	1.030	−1.225	3.304	−1.566	2.205	−1.630	1.036	−1.088	0.916	−1.158	2.168	−0.969
		5.157	−1.097	1.030	−1.227	3.304	−1.561	2.205	−1.630	1.036	−1.088	0.916	−1.158	2.168	−0.968
				1.030	−1.246			3.214	−1.731	1.036	−1.089	0.916	−1.157	3.141	−0.950
				1.030	−1.228			3.214	−1.731	1.036	−1.089	0.916	−1.158	3.141	−0.950
				3.185	−1.229										
				3.185	−1.231										
				3.185	−1.222										
				3.185	−1.221										
				3.185	−1.201										
				3.185	−1.221										
				3.185	−1.199										
				5.616	−1.267										
				5.616	−1.267										
				5.616	−1.265										
				5.616	−1.265										

[a] Salt concentration in mol kg^{-1} [H_2O]

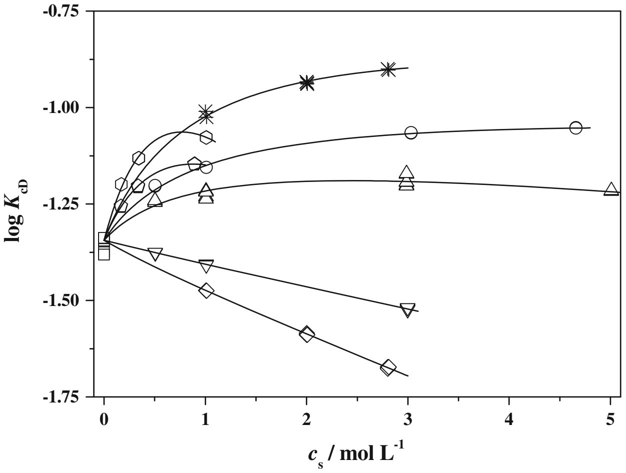

Fig. 4 Dependence of the isobutanol/water distribution ratio (as log $K_{c\mathrm{D}}$) of neutral glutathione (H_3GSH) versus c_s (salt concentration in mol L^{-1}), at $T = 298.15$ K. *open squares* pure water, *open circles* $\mathrm{LiCl_{(aq)}}$, *open triangles* $\mathrm{NaCl_{(aq)}}$, *open inverted triangles* $\mathrm{KCl_{(aq)}}$, *open diamonds* $\mathrm{CsCl_{(aq)}}$, *open hexagons* = $\mathrm{MgCl_{2(aq)}}$, *open pentagons* = $\mathrm{CaCl_{2(aq)}}$, *stars* = $\mathrm{NaI_{(aq)}}$

STACO and BSTAC computer programs following this approach. Once the protonation constants of glutathione were fixed in the programs to their values in pure $\mathrm{NaCl_{(aq)}}$, together with their ionic strength dependence parameters in this medium, it has been possible to determine the formation constants of several $M_jH_i(GSH)^{(i+2j-3)}$ species ($M^{2+} = Mg^{2+}$ or Ca^{2+}) at various ionic strengths. The stability constants of $Mg_jH_i(GSH)^{(i+2j-3)}$ and $Ca_jH_i(GSH)^{(i+2j-3)}$ species at infinite dilution are reported in Table 11 together with the corresponding parameters for their dependence on ionic strength (by the EDH and simplified SIT models, Eqs. 14 and 15a). The results in this Table confirm, in some way, what was already stated in previous paragraphs about the similar behavior shown by glutathione toward Mg^{2+} and Ca^{2+}. In both cases, the formation of the three mononuclear $M(GSH)^-$, $MH(GSH)$, and $MH_2(GSH)^+$ and the dinuclear $M_2(GSH)^+$ species was observed, with a very similar stability for both cations. Only in the acidic pH range, Ca^{2+} forms the triprotonated $CaH_3(GSH)^{2+}$ species, not observed for Mg^{2+}. The importance of these $Ca_jH_i(GSH)^{(i+2j-3)}$ species may be better appreciated, for example, looking at Fig. 5, where the speciation diagram of glutathione in a $\mathrm{NaCl_{(aq)}}$/$\mathrm{CaCl_{2(aq)}}$ mixture is reported, considering their formation. In the conditions of the diagram (i.e., $I = 1.0$ mol L^{-1}, $c_{GSH} = 0.001$ mol L^{-1}, $c_{Ca} = 0.200$ mol L^{-1}), Ca^{2+}/GSH ion pairs are present in the whole pH range in appreciable

Table 7 Setschenow parameters (Setschenow 1889) of glutathione in different ionic media in the molar (mol L^{-1}) and molal (mol kg^{-1} [H_2O]) concentration scales

Salt	$k_{c\infty}$	k_{c0}	m.d.[a]
LiCl	−0.009 ± 0.001[b]	0.395 ± 0.006[b]	0.005
NaCl	−0.026 ± 0.001	0.281 ± 0.005	0.010
KCl	−0.057 ± 0.001	−0.067 ± 0.006	0.001
CsCl	−0.105 ± 0.002	−0.154 ± 0.006	0.001
$MgCl_2$	−0.478 ± 0.017	1.013 ± 0.019	0.005
$CaCl_2$	−0.254 ± 0.012	0.645 ± 0.014	0.001
NaI	−0.023 ± 0.003	0.664 ± 0.009	0.004

Salt	$k_{m\infty}$	k_{m0}	m.d.[a]
LiCl	−0.017 ± 0.001[b]	0.382 ± 0.004[b]	0.005
NaCl	−0.031 ± 0.001	0.266 ± 0.005	0.011
KCl	−0.061 ± 0.001	−0.084 ± 0.005	0.001
CsCl	−0.101 ± 0.002	−0.187 ± 0.006	0.002
$MgCl_2$	−0.474 ± 0.016	0.994 ± 0.019	0.004
$CaCl_2$	−0.255 ± 0.012	0.624 ± 0.014	0.001
NaI	−0.036 ± 0.003	0.631 ± 0.008	0.005

[a] Mean deviation of the fit

[b] ±Standard deviation

Table 8 Parameters for the dependence of protonation constants of glutathione on ionic strength in $NaI_{(aq)}$, according to EDH, simplified SIT and Pitzer models

Parameter	$\log K_1^H$	$\log K_2^H$	$\log K_3^H$	$\log K_4^H$
EDH				
$\log {}^{T}K^{H}$ [a]	10.135	9.077	3.713	2.124
z^*	6	4	2	0
c_∞	0.411 ± 0.015[b]	0.348 ± 0.006[b]	0.306 ± 0.012[b]	0.245 ± 0.009[b,c]
c_0	0.647 ± 0.028	0.548 ± 0.016	0.196 ± 0.024	
SIT				
$\log {}^{T}K^{H}$ [a]	10.134	9.075	3.712	2.123
z^*	6	4	2	0
$\Delta\varepsilon_\infty$	0.318 ± 0.009[b]	0.265 ± 0.005[b]	0.228 ± 0.008[b]	0.199 ± 0.006[b,d]
$\Delta\varepsilon_0$	0.691 ± 0.027	0.548 ± 0.013	0.232 ± 0.021	
Pitzer				
$\log {}^{T}K^{H}$ [a]	10.134	9.075	3.712	2.123
z^*	6	4	2	0
p_1	0.587 ± 0.022	0.541 ± 0.020	0.377 ± 0.027	0.215 ± 0.026
p_2	−0.012 ± 0.006	−0.037 ± 0.006	−0.017 ± 0.010	0.025 ± 0.008
p_3	2.81 ± 0.09	1.74 ± 0.07	0.57 ± 0.12	−0.26 ± 0.11

K_i^H refer to equilibrium: $H_{i-1}(GSH)^{(i-4)} + H^+ = H_i(GSH)^{(i-3)}$, Eqs. 14 and 15a, in mol L^{-1}, Eqs. 14 and 15b, in mol kg^{-1} (H_2O), Eqs. 30 and 31, in mol kg^{-1} (H_2O)

[a] From (Crea et al. 2007)

[b] ±3 standard deviation

[c] C_4 from Eq. 14

[d] $\Delta\varepsilon_4$ from Eq. 14

amounts and, in some cases, they reach formation percentages higher than 50%, as observed in the case of $CaH_2(GSH)^+$, the main species at the typical pH of the most important natural fluids (e.g., pH ~7.4 for blood plasma, pH ~8.1 for seawater).

Formation enthalpy and entropy changes

The experimental conditions for the calorimetric measurements were already described in the experimental section. The enthalpy changes calculated by the ES5CMI computer

Table 9 SIT coefficients of glutathione species in various aqueous media, at $T = 298.15$ K

X^{z-}	$M^{z'+}$	ε_{∞}	ε_0
Cl^-	H^+	0.136[a]	0.0848[a]
I^-	H^+	0.173[a]	0.204[a]
$(GSH)^{3-}$	Li^+	0.145 ± 0.005[b]	1.064 ± 0.022[b]
$(GSH)^{3-}$	Na^+ [c]	0.125 ± 0.003	1.217 ± 0.009
$(GSH)^{3-}$	Na^+ [d]	0.257 ± 0.009	1.526 ± 0.030
$(GSH)^{3-}$	K^+	0.109 ± 0.004	0.879 ± 0.006
$(GSH)^{3-}$	Cs^+	−0.029 ± 0.007	0.800 ± 0.019
$(GSH)^{3-}$	Mg^{2+}	−1.11 ± 0.24	1.30 ± 0.36
$(GSH)^{3-}$	Ca^{2+}	−0.19 ± 0.13	−1.04 ± 0.45
$H(GSH)^{2-}$	Li^+	0.094 ± 0.004	0.711 ± 0.017
$H(GSH)^{2-}$	Na^+ [c]	0.008 ± 0.002	0.786 ± 0.007
$H(GSH)^{2-}$	Na^+ [d]	0.112 ± 0.005	1.039 ± 0.019
$H(GSH)^{2-}$	K^+	0.016 ± 0.003	0.291 ± 0.006
$H(GSH)^{2-}$	Cs^+	−0.057 ± 0.006	0.173 ± 0.017
$H(GSH)^{2-}$	Mg^{2+}	−0.49 ± 0.17	−1.59 ± 0.28
$H(GSH)^{2-}$	Ca^{2+}	−0.27 ± 0.11	−1.34 ± 0.39
$H_2(GSH)^-$	Li^+	−0.019 ± 0.003	0.406 ± 0.012
$H_2(GSH)^-$	Na^+ [c]	−0.047 ± 0.001	0.426 ± 0.005
$H_2(GSH)^-$	Na^+ [d]	0.020 ± 0.004	0.659 ± 0.015
$H_2(GSH)^-$	K^+	−0.047 ± 0.002	−0.040 ± 0.004
$H_2(GSH)^-$	Cs^+	−0.084 ± 0.004	−0.032 ± 0.011
$H_2(GSH)^-$	Mg^{2+}	−0.43 ± 0.11	0.66 ± 0.18
$H_2(GSH)^-$	Ca^{2+}	−0.46 ± 0.08	−0.11 ± 0.24
Cl^-	$H_4(GSH)^+$ [e]	0.001 ± 0.004	0.479 ± 0.007
Cl^-	$H_4(GSH)^+$ [c]	0.044 ± 0.003	0.182 ± 0.006
Cl^-	$H_4(GSH)^+$ [f]	−0.031 ± 0.004	−0.128 ± 0.008
Cl^-	$H_4(GSH)^+$ [g]	−0.011 ± 0.009	−0.205 ± 0.037
Cl^-	$H_4(GSH)^+$ [h]	−0.03 ± 0.02	0.18 ± 0.04
Cl^-	$H_4(GSH)^+$ [i]	−0.10 ± 0.02	0.70 ± 0.04
I^-	$H_4(GSH)^+$ [d]	−0.121 ± 0.007	0.845 ± 0.024

[a] From (Bretti et al. 2006)

[b] ±3 standard deviation

[c] In $NaCl_{(aq)}$

[d] In $NaI_{(aq)}$

[e] In $LiCl_{(aq)}$

[f] In $KCl_{(aq)}$

[g] In $CsCl_{(aq)}$

[h] In $MgCl_{2(aq)}$

[i] In $CaCl_{2(aq)}$

program for the protonation of glutathione in $NaCl_{(aq)}$ at different ionic strengths are reported in Table 12 together with the corresponding entropy values. As observed, protonation equilibria of glutathione are moderately exothermic (except for the fourth step at low ionic strength). By taking into account the contribution of both ΔH and $T\Delta S$ to ΔG, it is also evident that protonation is an enthalpy-driven process, where $T\Delta S$ values for the binding of a proton is approximately constant ($T\Delta S \sim 14 \pm 2$ kJ mol^{-1}), independently of both the ionic strength and the protonation step. The protonation enthalpy changes of Table 12 were then fixed in the data analysis of calorimetric measurements performed in $NaCl_{(aq)}/CaCl_{2(aq)}$ mixtures in order to determine the thermodynamic formation parameters of $Ca_jH_i(GSH)^{(i+2j-3)}$ species. In the experimental conditions of these measurements, the enthalpy changes relative to the formation of the $CaH_3(GSH)^{2+}$ and $Ca_2(GSH)^+$ species were not determined; the others are reported in Table 13 at different ionic strengths, together with corresponding $T\Delta S$ and ΔG. The analysis of these data clearly evidences that the binding of one Ca^{2+} ion to the free or protonated glutathione is an endothermic process. Moreover, the contribution of $T\Delta S$ to this process is generally higher than corresponding ΔH, indicating that entropy changes associated with complexation are much more important than in the case of glutathione protonation.

Both protonation and complex formation enthalpies reported in Tables 12 and 13 proved fairly dependent on ionic strength, as shown, for example, for ΔH_1^H in Fig. 6. Curve in the same Figure represents the modeling on this dependence by the SIT approach, according to the equation (De Stefano et al. 2004):

$$\Delta H_{ij} = \Delta H_{ij}^0 - z^* \mathrm{DH}' + \Delta\varepsilon' I \qquad (32)$$

Table 10 Pitzer parameters of glutathione species in various aqueous media, at $T = 298.15$ K

Species	M^+	X'^-	$\beta^{(0)}$	$\beta^{(1)}$	C^{Φ}	Θ	ψ
$(GSH)^{3-}$	Li^+		0.7075 ± 0.045[a]	3.943 ± 0.175[a]	−0.1726 ± 0.020[a]	–	–
$(GSH)^{3-}$	Na^+	I^-	0.5324 ± 0.015	4.522 ± 0.052	−0.1111 ± 0.007	0.292 ± 0.010[a]	−0.0593 ± 0.005[a]
$(GSH)^{3-}$	K^+		0.6173 ± 0.013	3.734 ± 0.038	−0.0974 ± 0.010	–	–
$(GSH)^{3-}$	Cs^+		0.4633 ± 0.004	3.704 ± 0.013	0.0067 ± 0.002	–	–
$H(GSH)^{2-}$	Li^+		0.4077 ± 0.040	1.890 ± 0.154	−0.0889 ± 0.014	–	–
$H(GSH)^{2-}$	Na^+	I^-	0.2054 ± 0.013	2.407 ± 0.045	−0.0549 ± 0.005	0.225 ± 0.009	−0.0458 ± 0.005
$H(GSH)^{2-}$	K^+		0.2124 ± 0.013	1.564 ± 0.037	−0.0114 ± 0.008	–	–
$H(GSH)^{2-}$	Cs^+		0.2491 ± 0.003	1.147 ± 0.010	−0.0102 ± 0.001	–	–
$H_2(GSH)^-$	Li^+		0.0862 ± 0.026	0.843 ± 0.101	−0.0268 ± 0.007	–	–
$H_2(GSH)^-$	Na^+	I^-	−0.0053 ± 0.009	1.082 ± 0.032	−0.0126 ± 0.003	0.171 ± 0.008	−0.0342 ± 0.004
$H_2(GSH)^-$	K^+		0.0278 ± 0.008	0.162 ± 0.023	−0.0110 ± 0.003	–	–
$H_2(GSH)^-$	Cs^+		0.0275 ± 0.002	0.171 ± 0.007	–	–	–
$H_4(GSH)^+$	Li^+	Cl^-	−0.3458 ± 0.040	0.7525 ± 0.094	0.0436 ± 0.015	0.527 ± 0.028	−0.076 ± 0.013
$H_4(GSH)^+$	Na^+	Cl^-	−0.3458 ± 0.040	0.7525 ± 0.094	0.0436 ± 0.015	0.420 ± 0.024	−0.035 ± 0.011
$H_4(GSH)^+$	K^+	Cl^-	−0.3458 ± 0.040	0.7525 ± 0.094	0.0436 ± 0.015	0.177 ± 0.012	–
$H_4(GSH)^+$	Cs^+	Cl^-	−0.3458 ± 0.040	0.7525 ± 0.094	0.0436 ± 0.015	–	–

[a] ±3 standard deviation

Table 11 Stability constants of $Mg_jH_i(GSH)^{(i+2j-3)}$ and $Ca_jH_i(GSH)^{(i+2j-3)}$ species at $T = 298.15$ K and infinite dilution, and EDH and SIT parameters for their dependence on ionic strength in $NaCl_{(aq)}$

i	j	$\log \beta^0_{ij}$	z^*	c_∞	c_0	$\Delta\varepsilon_\infty$	$\Delta\varepsilon_0$
$Mg_jH_i(GSH)^{(i+2j-3)}$							
0	1	3.17 ± 0.06[a]	12	0.071 ± 0.006[a]	0.078 ± 0.009[a]	0.057 ± 0.003[a]	0.098 ± 0.006[a]
1	1	12.85 ± 0.03	14	0.387 ± 0.003	0.390 ± 0.006	0.317 ± 0.002	0.454 ± 0.008
2	1	20.45 ± 0.04	14	0.540 ± 0.008	0.550 ± 0.002	0.438 ± 0.003	0.633 ± 0.013
0	2	4.34 ± 0.09	16	0.127 ± 0.012	0.145 ± 0.008	0.098 ± 0.009	0.165 ± 0.004
$Ca_jH_i(GSH)^{(i+2j-3)}$							
0	1	3.04 ± 0.03	12	0.298 ± 0.004	0.409 ± 0.009	0.249 ± 0.004	0.469 ± 0.013
1	1	12.75 ± 0.02	14	0.444 ± 0.006	0.560 ± 0.012	0.365 ± 0.005	0.633 ± 0.016
2	1	20.92 ± 0.02	14	0.587 ± 0.006	0.706 ± 0.008	0.478 ± 0.005	0.797 ± 0.012
3	1	23.77 ± 0.02	12	0.582 ± 0.008	0.716 ± 0.012	0.463 ± 0.006	0.798 ± 0.012
0	2	4.88 ± 0.04	16	0.170 ± 0.007	0.309 ± 0.015	0.134 ± 0.009	0.338 ± 0.015

β_{ij} refer to equilibrium: $jM^{2+} + iH^+ + GSH^{3-} = M_jH_i(GSH)^{(i+2j-3)}$, Eqs. 14 and 15a, b

[a] ±3 standard deviation

where

$$\mathrm{DH}' = A' I^{1/2}\left(1 + 1.5 I^{1/2}\right)^{-1}$$

with

$$A' = RT^2 \ln 10(\partial A/\partial T) = 1.5$$

at $T = 298.15$ K, and

$$\Delta\varepsilon' = RT^2 \ln 10(\partial \Delta\varepsilon/\partial T)$$

Refined enthalpy changes at infinite dilution (ΔH^0_{ij}) and derivative SIT interaction coefficients ($\Delta\varepsilon'$) for both the protonation constants of glutathione and the formation constants of $Ca_jH_i(GSH)^{(i+2j-3)}$ species are shown in Table 14.

The values of the enthalpy changes reported in Tables 12 and 13 can be also used for the calculation of the corresponding stability constants at other temperatures than $T = 298.15$ K, by applying the van't Hoff equation (neglecting ΔC_p), which can be written as

$$\log K_{ijT} = \log K_{ij\theta} + \Delta H_{ij}(1/298.15 - 1/T)/2.303R \qquad (33)$$

where log K_{ijT} is the stability constant at a given temperature (in Kelvin), log $K_{ij\theta}$ is the corresponding value at $T = 298.15$ K and with $R = 8.314472(15)$ J K^{-1} mol^{-1} when ΔH_{ij} is expressed in J mol^{-1}. Both Eqs. 32 and 33,

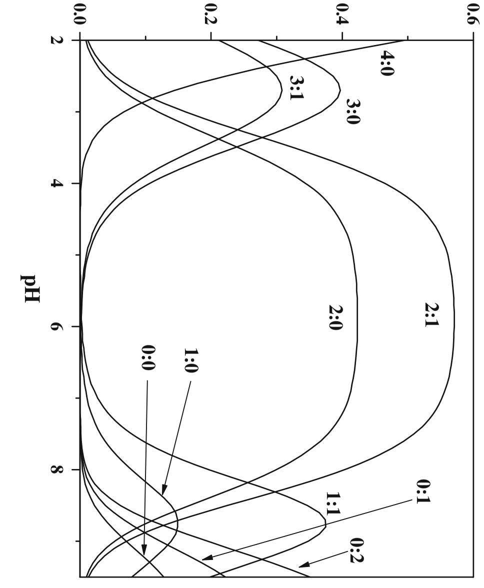

Fig. 5 Speciation diagrams of glutathione versus pH in $NaCl_{(aq)}$/$CaCl_{2(aq)}$ mixtures at $T = 298.15$ K and $I = 1.0$ mol L^{-1}, considering the formation of Ca^{2+} ion pairs. $c_{GSH} = 0.001$ mol L^{-1}, $c_{Ca} = 0.200$ mol L^{-1} *i*:*j* indexes in figure refer to $Ca_jH_i(GSH)^{(i+2j-3)}$ species

together with the equations for the dependence of the stability constants on ionic strength, are very useful for the definition of glutathione speciation in several natural fluids where this ligand and its calcium complexes may play key roles. As an example, the log K_{ij} values relative to both the protonation constants of glutathione and the stability constants of $Ca_jH_i(GSH)^{(i+2j-3)}$ species were calculated in similar ionic strength and temperature conditions of blood plasma and seawater, i.e., at $T = 310.15$ K and $I = 0.15$ mol L^{-1}, and at $T = 288.15$ K and $I = 0.7$ mol L^{-1}, respectively, and are reported in Table 15.

Literature comparisons

A detailed literature analysis on glutathione protonation in various aqueous media was already preformed in another paper (Crea et al. 2007) by this group. Successively, to our knowledge only few papers, published from 2007 to present time, reported new protonation constant values for this ligand (Ferretti et al. 2007; Leverrier et al. 2007; Enyedy et al. 2008; Yang et al. 2008; Wang et al. 2009). Most of these new data were obtained in $KCl_{(aq)}$ or in buffer solutions, and will not be discussed in detail here, since protonation constants in this medium were the subject of another contribution (Crea et al. 2007). Nevertheless, it is possible to assert briefly that there is a generally good agreement between new data and those previously published. During the literature analysis, no data were found on the most of results reported in this work: (1) the apparent protonation constants in $NaI_{(aq)}$ and in $NaCl_{(aq)}$/$MgCl_{2(aq)}$, and $NaCl_{(aq)}$/$CaCl_{2(aq)}$ mixtures at different ionic strengths; (2) the EDH, SIT, and Pitzer parameters for their dependence on medium and ionic strength and for

Table 12 Thermodynamic parameters for glutathione protonation in $NaCl_{(aq)}$, at $T = 298.15$ K

I_c/mol L^{-1}	ΔH_1^{H} [a]	ΔG_1^{H}	$T\Delta S_1^{H}$	ΔH_2^{H} [a]	ΔG_2^{H}	$T\Delta S_2^{H}$	ΔH_3^{H} [a]	ΔG_3^{H}	$T\Delta S_3^{H}$	ΔH_4^{H} [a]	ΔG_4^{H}	$T\Delta S_4^{H}$
0.25	−37.0	−53.45	16.5	−68.8 (−31.8)[b]	−102.45 (−49.00)[b]	33.7 (17.2)[b]	−72.6 (−3.8)[b]	−122.25 (−19.80)[b]	49.7 (16.0)[b]	−67.5 (5.1)[b]	−134.51 (−12.26)[b]	67.0 (17.3)[b]
1.00	−38.4	−52.65	14.3	−71.3 (−32.9)	−101.08 (−48.43)	29.8 (15.5)	−76.6 (−5.3)	−120.48 (−19.40)	43.9 (14.1)	−73.8 (2.8)	−132.46 (−11.98)	58.7 (14.8)
2.00	−40.4	−53.62	13.2	−74.7 (−34.3)	−102.62 (−49.00)	27.9 (14.7)	−81.0 (−6.3)	−122.70 (−20.08)	41.7 (13.8)	−83.0 (−2.0)	−135.60 (−12.90)	52.6 (10.9)
3.00	−42.4	−54.76	12.4	−78.0 (−35.6)	−105.25 (−50.49)	27.2 (14.8)	−85.4 (−7.4)	−126.13 (−20.88)	40.7 (13.5)	−92.1 (−6.7)	−139.65 (−13.52)	47.5 (6.8)
5.00	−45.2	−60.24	15.0	−82.9 (−37.7)	−113.75 (−53.51)	30.8 (15.8)	−91.8 (−8.9)	−137.19 (−23.44)	45.4 (14.6)	−96.5 (−4.7)	−153.91 (−16.72)	57.4 (12.0)

ΔX_i^{H} refer to equilibrium: $iH^+ + GSH^{3-} = H_i(GSH)^{(i-3)}$, in kJ mol^{-1}

[a] ±0.3–0.9 standard deviation

[b] Values in parenthesis refer to equilibrium: $H_{i-1}(GSH)^{(i-4)} + H^+ = H_i(GSH)^{(i-3)}$

Table 13 Thermodynamic parameters of $Ca_jH_i(GSH)^{(i+2j-3)}$ species in $NaCl_{(aq)}$, at $T = 298.15$ K

I_c/mol L^{-1}	ΔH_{01}^{a}	ΔG_{01}	$T\Delta S_{01}$	ΔH_{11}^{a}	ΔG_{11}	$T\Delta S_{11}$	ΔH_{21}^{a}	ΔG_{21}	$T\Delta S_{21}$
Overall equilibrium[b]									
1.00	45.2	−5.42	50.6	−0.1	−59.33	59.3	−22.7	−106.73	84.0
2.00	39.8	−5.36	45.1	−3.1	−59.78	56.7	−29.5	−108.04	78.6
3.00	23.9	−6.10	30.0	−15.4	−61.21	45.8	−40.1	−110.27	70.2
5.00	−8.6	−8.44	−0.1	−51.7	−5.03	13.3	−91.5	−115.74	24.3
Stepwise equilibrium[c]									
1.00	45.2	−5.42	50.6	38.3	−6.68	45.0	48.6	−5.65	54.2
2.00	39.8	−5.36	45.1	37.3	−6.16	43.5	45.2	−5.42	50.7
3.00	23.9	−6.10	30.0	27.0	−6.45	33.4	37.9	−5.02	43.0
5.00	−8.6	−8.44	−0.1	−6.5	−4.79	−1.7	−8.6	−1.99	−6.5

In kJ mol^{-1}

[a] ±0.6–0.9 standard deviation

[b] $\Delta X_{ij}{}^{\cdot}$ refer to equilibrium: $jCa^{2+} + iH^{+} + GSH^{3-} = Ca_jH_i(GSH)^{(i+2j-3)}$

[c] ΔX_{ij} refer to equilibrium: $jCa^{2+} + H_i(GSH)^{(i-3)} = Ca_jH_i(GSH)^{(i+2j-3)}$

the calculation of activity coefficients; (3) the distribution data; (4) the Setschenow coefficients; and (5) the parameters for the dependence on ionic strength of protonation and complex formation enthalpy changes are reported here for the first time. On the contrary, few old papers were found on the protonation enthalpies of glutathione (Vander Jagt et al. 1972; Corrie and Williams 1976), on the stability of $Mg_jH_i(GSH)^{(i+2j-3)}$ (Singh et al. 2001) and $Ca_jH_i(GSH)^{(i+2j-3)}$ (Singh et al. 2001; Schubert 1954; Touche and Williams 1976) species and on their formation enthalpy changes (Singh et al. 2001).

In their paper Vander Jagt et al. (1972) measured, by direct calorimetric titrations, the enthalpy changes for the protonation of glutathione at $T = 298.15$ K in dilute solutions, without ionic medium. Values obtained by these authors for the stepwise protonation equilibria (Eq. 1) are $\Delta H_1^{H} = -35.0$ kJ mol^{-1}, $\Delta H_2^{H} = -31.7$ kJ mol^{-1}, $\Delta H_3^{H} = -0.6$ kJ mol^{-1}, and $\Delta H_4^{H} = -1.8$ kJ mol^{-1}. Corresponding data at infinite dilution obtained in this work are, within the experimental errors, in good agreement: $\Delta H_1^{H} = -33.9$ kJ mol^{-1}, $\Delta H_2^{H} = -29.8$ kJ mol^{-1}, $\Delta H_3^{H} = -2.9$ kJ mol^{-1}, and $\Delta H_4^{H} = 5.3$ kJ mol^{-1}. The same good agreement is also observed between the protonation enthalpy changes determined in this work at $T = 298.15$ K and $I = 3.0$ mol L^{-1}. and the corresponding values reported by Corrie and Williams (1976) in the same conditions (but in $NaClO_{4(aq)}$). These authors obtained, for the stepwise protonation equilibria (Eq. 1) $\Delta H_1^{H} = -37.1$ kJ mol^{-1}, $\Delta H_2^{H} = -35.1$ kJ mol^{-1}, $\Delta H_3^{H} = -1.8$ kJ mol^{-1}, and $\Delta H_4^{H} = -4.6$ kJ mol^{-1}, against $\Delta H_I^{H} = -42.4$ kJ mol^{-1}, $\Delta H_2^{H} = -35.6$ kJ mol^{-1}, $\Delta H_3^{H} = -7.4$ kJ mol^{-1}, and $\Delta H_4^{H} = -6.7$ kJ mol^{-1} of this work.

Concerning the stability of $Ca_jH_i(GSH)^{(i+2j-3)}$ species, Schubert (1954) reported for the first time some data on the interactions among Ca^{2+} and glutathione, investigated by the ion exchange technique under physiological conditions, approximately. This author, however, only reports a value of $\log K_f = 0$, where $\log K_f$ is defined as the formation quotient of the complex, i.e.,

$$\log K_f = \left(\left(K_d^0/K_d\right) - 1\right)/A$$

where K_d^0 and K_d are the distribution coefficients of Ca^{2+} between the resin and the solution phases in the absence and presence of glutathione (A), respectively. Some values of stability constants of the $Ca_jH_i(GSH)^{(i+2j-3)}$ species were reported for the first time by Touche and Williams (Touche and Williams 1976). These authors obtained, at $I = 0.15$ mol L^{-1} in $NaClO_{4(aq)}$ and $T = 303.15$ K, the following overall formation constants (Eq. 2b): $\log \beta_{01} = 3.84$, $\log \beta_{11} = 12.89$, $\log \beta_{21} = 20.68$, $\log \beta_{-11} = -6.46$, relative to the $Ca(GSH)^{-}$, $CaH(GSH)$, $CaH_2(GSH)^{+}$, and $Ca(OH)(GSH)^{2-}$ species, respectively. The agreement between these values and those calculated in this work in the same conditions (Table 15) is only moderately satisfactory. Probably, one of the main reasons for the discrepancies between values here reported and those by Touche and Williams (1976) is the different speciation scheme proposed. In fact, in their experimental conditions, these authors neither observed the formation of the triprotonated $CaH_3(GSH)^{2+}$ species, nor that of the dinuclear $Ca_2(GSH)^{+}$. On the contrary, they proposed the $Ca(OH)(GSH)^{2-}$ species, not observed in this work.

A different mention is necessary in the case of the paper by Singh et al. (2001). These authors report results on the evaluation of various thermodynamic parameters of several bivalent metal complexes of glutathione, including Mg^{2+} and Ca^{2+}. The whole work is abundant and well designed to get information on the effect of ionic strength,

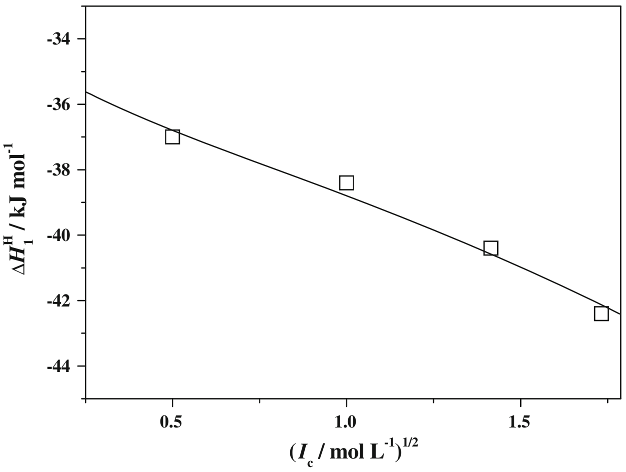

Fig. 6 Dependence of the enthalpy changes of the first protonation step of GSH versus the square root of ionic strength (in mol L^{-1}), at $T = 298.15$ K, in $NaCl_{(aq)}$

Table 14 Enthalpy changes for the protonation of glutathione ($j = 0$) and the formation of $Ca_jH_i(GSH)^{(i+2j-3)}$ species at $T = 298.15$ K and infinite dilution, SIT parameters for their dependence on ionic strength in $NaCl_{(aq)}$

i	j	ΔH^0_{ij} [a]	z^*	$\Delta\varepsilon'$ [a]
1	0	−33.9 ± 0.5	6	−1.3 ± 0.1
2	0	−63.7 ± 0.6	10	−2.3 ± 0.1
3	0	−66.6 ± 0.4	12	−3.2 ± 0.1
4	0	−61.3 ± 0.9	12	−5.5 ± 0.4
0	1	71.6 ± 2.4	12	−13.9 ± 1.9
1	1	29.1 ± 2.2	14	−13.4 ± 2.2
2	1	12.6 ± 2.7	14	−17.8 ± 2.6

ΔH^0_{ij} refer to equilibrium: $j\mathrm{Ca}^{2+} + i\mathrm{H}^{+} + \mathrm{GSH}^{3-} = \mathrm{Ca}_j\mathrm{H}_i(\mathrm{GSH})^{(i+2j-3)}$, in kJ mol^{-1}, Eq. 32

[a] ±3 standard deviation

temperature, and solvent on the interaction between GSH and divalent metal cations in a wide pH range. Several results are reported for each investigated metal/ligand systems at (1) one temperature ($T = 298.15$ K) and different ionic strengths ($0.05 \le I_c$/mol L^{-1} ≤ 0.2 in $NaClO_{4(aq)}$); (2) one ionic strength ($I_c = 0.1$ mol L^{-1}) and different temperatures ($288.15 \le T$/K ≤ 318.15); and (3) one temperature and ionic strength ($T = 298.15$ K and $I_c = 0.1$ mol L^{-1}), and different solvent mixtures, i.e., water/methanol (90:10, 80:20 and 70:30 w:w), water/ethanol (80:20), and water/dimethylformamide (80:20) mixtures. Data at different ionic strengths and temperatures allowed the authors to extrapolate the results at infinite dilution and to calculate the entropy and enthalpy changes for various metal/ligand interactions. Unfortunately, these results can be hardly compared with those reported in this work, since the experimental data obtained by Singh et al. from the potentiometric titrations in the above-cited conditions were used to determine only one log K value (and corresponding ΔH and ΔS) for each investigated Mg^{2+}/GSH system and condition, so that these formation parameters are "apparent", as originally intended by Schwarzenbach (Schwarzenbach 1957).

Final considerations

This paper reports the results of an extension of a previous study on the determination of some thermodynamic parameters for the protonation complex formation and activity coefficients of reduced glutathione in different conditions. These results have been discussed and commented in detail along the manuscript. Here it is interesting to pay attention on some other aspects. Several studies (e.g., (Foti et al. 1997; Bretti et al. 2007)) demonstrated that, at least for the simplest systems, different models for the dependence of various thermodynamic parameters

Table 15 Stability constants for the protonation of glutathione ($j = 0$) and the formation of $Ca_jH_i(GSH)^{(i+2j-3)}$ species in $NaCl_{(aq)}$ at different temperatures and ionic strengths

i	j	log β_{ij}			
		$I = 0.15$ mol L^{-1}		$I = 0.7$ mol L^{-1}	
		$T = 298.15$ K	$T = 303.15$ K	$T = 298.15$ K	$T = 288.15$ K
1	0	9.45	9.35	9.28	9.51
2	0	8.64	17.89	8.56	18.27
3	0	3.50	21.38	3.47	21.77
4	0	2.14	23.53	2.19	23.94
0	1	1.60	1.79	1.02	0.69
1	1	11.08	11.15	10.46	10.39
2	1	19.27	19.29	18.73	18.78

log β_{ij} refer to equilibrium: $j\mathrm{Ca}^{2+} + i\mathrm{H}^{+} + \mathrm{GSH}^{3-} = \mathrm{Ca}_j\mathrm{H}_i(\mathrm{GSH})^{(i+2j-3)}$

(mainly stability constants, but enthalpy and entropy changes too) on medium and ionic strength are generally equivalent. As a consequence, the choice of a model rather than another sprang often from personal reasons, and rarely other routes are explored by various investigators. The case of glutathione reported in this paper is a clear example of a system where the use of the tested models (EDH, SIT, Pitzer, and ion pair formation) is not indifferent and where complementary information can be obtained. For example, when the advantage of the simplicity must be privileged, EDH and/or SIT models should be preferred: SIT coefficients of Table 9 are useful to model with sufficient accuracy the acid–base behavior of glutathione in aqueous media containing a single supporting electrolyte. On the contrary, the acid–base behavior of this ligand in multi-electrolyte aqueous solutions could be better described by the Pitzer model. Moreover, both SIT and Pitzer coefficients can be also exploited to calculate the coefficients of glutathione species in other aqueous media such as, for example, $RbCl_{(aq)}$, $(CH_3)_4NCl_{(aq)}$, and $(C_2H_5)_4NI_{(aq)}$, whereas the protonation constants are already available in literature (Crea et al. 2007). However, when quite strong interactions occur or when unreliable parameters are obtained by these models, the application of the ion pair formation model is the simplest and safest solution.

A last consideration is related to the fact that a complete series of thermodynamic data in all (except Fr^+, of course) the alkali metal chlorides is given in this work. Once again, it is important to stress that the results obtained in this paper are useful for the definition of glutathione speciation in any aqueous media containing the main cations of natural waters and biological fluids, such as Na^+, K^+, Mg^{2+}, and Ca^{2+} and may be helpful for the comprehension of several properties and mechanisms involving this fundamental ligand. In addition, it is worth mentioning that this kind of systematic studies, where a complete series of cations is taken into account, is not so frequent, though this investigation would allow the definition of some trends in the thermodynamic behavior of various ligands in aqueous solution. This group already performed similar studies for the protonation and alkali metal complex formation of simple (Capone et al. 1986; Daniele et al. 1982; De Robertis et al. 1985) and complex (Cucinotta et al. 1981; De Robertis et al. 1986b; De Stefano et al. 2003; Crea et al. 2008) ligands, and some empirical relationships have been proposed. For example, the alkali metal complexes of ATP can be modeled as a function of the crystal radii of the complexing alkali metal cation. In the case of glutathione, similar trends may be also observed for most of the thermodynamic parameters determined in this work (e.g., protonation constants, Pitzer coefficients, SIT parameters, etc.). Nevertheless, though some empirical relationships have been found during data analysis, they have not been proposed here, because of their quite complex mathematical formulation. Probably, simple one or two-parameter equations are not reliable in the case of glutathione, due to the different nature of functional groups involved in its protonation and complex formation.

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