Ca$^{2+}$ Complexation With Relevant Bioligands in Aqueous Solution: A Speciation Study With Implications for Biological Fluids

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A speciation study on the interaction between Ca$^{2+}$ and ligands of biological interest in aqueous solution is reported. The ligands under study are L-cysteine (Cys), D-penicillamine (PSH), reduced glutathione (GSH), and oxidized glutathione (GSSG). From the elaboration of the potentiometric experimental data the most likely speciation patterns obtained are characterized by only protonated species with a 1:1 metal to ligand ratio. In detail, two species, CaLH$_2$ and CaLH, for systems containing Cys, PSH, and GSH, and five species, CaLH$_5$, CaLH$_4$, CaLH$_3$, CaLH$_2$, and CaLH, for system containing GSSG, were observed. The potentiometric titrations were performed at different temperatures ($15 \leq t/°C \leq 37$, at $I = 0.15 \text{ mol L}^{-1}$). The enthalpy and entropy change values were calculated for all systems, and the dependence of the formation constants of the complex species on the temperature was evaluated. $^1$H NMR spectroscopy, MALDI mass spectrometry, and tandem mass spectrometry (MS/MS) investigations on Ca$^{2+}$-ligand solutions were also employed, confirming the interactions and underlying characteristic complexing behaviors of Cys, PSH, GSH, and GSSG toward Ca$^{2+}$. The results of the analysis of $^1$H NMR experimental data are in full agreement with potentiometric ones in terms of speciation models and stability constants of the species. MALDI mass spectrometry and tandem mass spectrometry (MS/MS) analyses confirm the formation of Ca$^{2+}$-L complex species and elucidate the mechanism of interaction. On the basis of speciation models, simulations of species formation under conditions of some biological fluids were reported. The sequestering ability of Cys, PSH, GSH, and GSSG toward Ca$^{2+}$ was evaluated under different conditions of pH and temperature and under physiological condition.

Keywords: Ca$^{2+}$, biological ligands, speciation in biological fluids, sequestration, potentiometry, $^1$H NMR spectroscopy, mass spectrometry, thermodynamic parameters

INTRODUCTION

Calcium is the fifth most important element in the human body. It is indispensable for life, for the regulation of metabolism and maintenance of structure (Peterlik and Stoepppler, 2004). It behaves like an intracellular “second messenger” in numerous processes, namely, neurotransmitter release, cellular proliferation and differentiation, and control of exocrine and endocrine secretions (Bringhurst and Potts., 1979; Broaoudus, 1993). In human body, about 99% of total calcium
(1.0–1.3 kg in adults) (Hluchan and Pomerantz, 2002) is found in the bones. The remaining part, 1%, is present in intra- and extracellular fluids. Free calcium concentration in the cell ranges between $10^{-6}$ and $10^{-8}$ mol kg$^{-1}$. It is about $10^{-3}$ mol kg$^{-1}$ in the sarcoplasm (Frausto da Silva and Williams, 2001a). The mean Ca$^{2+}$ concentration in the plasma
is 2.5 mmol L\(^{-1}\), of which about 50% is present as free ion; the remaining part is bound for 40% to plasma proteins and for 10% to citrate and phosphate. The rigid control of free calcium in the plasma is very crucial, as even small concentration changes can cause significant variations in the skeletal site, as well as intracellular free calcium, with harmful consequences for bone health (Peterlik and Stoeppler, 2004; Whedon, 1980). Calcium homeostasis is based on a dynamic equilibrium of its fluxes between three different body compartments, namely, extracellular fluid, intracellular one, and skeletal tissue. As regards the physiological role of calcium, it includes the control of many kinase reactions in metabolism, of dioxygen release in photosynthesis, and of dehydrogenases in oxidative phosphorylation (Frausto da Silva and Williams, 2001b). Ca\(^{2+}\) interacts preferably with oxygen donor groups. In the body fluids it can bind polymers, such as proteins, via carboxylate and phosphate sidechains. In the proteins, the main donor groups toward Ca\(^{2+}\) are represented by carboxylate and carbonyl centers (Frausto da Silva et al., 2001a).

Cys is one of the most important binding agents for metal cations in biological fluids (Laurie et al., 1979). Its concentration in normal human plasma is in the micromolar range (Bingham et al., 1960). The drug penicillamine, which has a very similar structure to Cys, was commonly employed in the treatment of Wilson’s disease (Walshe, 1956; Jones, 1991). GSH is a tripeptide consisting of the amino acids L-glutamic acid (Glu), Cys, and glycine (Gly). It exists in two forms: a reduced (GSH) and an oxidized one, i.e., dimer glutathione disulfide (GSSG) (Labib et al., 2016). GSH is ubiquitous antioxidant present in cells as well as in bacteria (Sies, 1999; Pompella et al., 2003; Kretzschmar et al., 2020; Meister and Anderson, 1983). In mammalian cells, concentrations greater than 12 mmol L\(^{-1}\) are reported (Dringen, 2000). Both GSH and its oxidized form, GSSG, are fundamental for the maintenance of the intracellular redox state (Shahid et al., 2020). They are considered biomarkers of oxidative stress in biological fluids as well as for the diagnosis of certain clinical disorders (Labib et al., 2016; Olmos Moya et al., 2017). The mechanism of antioxidant cellular defense in vivo is governed by GSH, oxidized continuously to disulfide glutathione (GSSG) (Davis and Hanumegowda, 2008). In healthy cells, the GSH form constitutes over 90% of glutathione (Labib et al., 2016). In addition to protecting cells from oxidative damage, GSH is involved in the complexation and transport reactions of metal ions (Olmos Moya et al., 2017). In blood, the normal values of GSH and GSSG are 3.8–5.5 and 0.2–0.5 μmol L\(^{-1}\), respectively (Labib et al., 2016).

Given all these aspects, reliable assessment of the speciation of biologically relevant ligands with Ca\(^{2+}\) is crucial to understand and to model the behavior of these systems. Ligands under study are reported in Figure 1. In this study, the experimental measurements were performed by different techniques: potentiometry, \(^1\)H NMR spectroscopy, MALDI mass spectrometry, and MS/MS. The potentiometric titrations were carried out at different temperatures, 15 ≤ t/°C ≤ 37 and I = 0.15 mol L\(^{-1}\) in NaCl. Some simulations of species formation under conditions of biological fluids were reported. The sequestering ability of all ligands understudy toward Ca\(^{2+}\) was evaluated under different conditions of pH and temperature.

### MATERIAL AND METHODS

#### Materials

The solutions containing calcium metal cation were obtained by weighing and dissolving the corresponding salt, calcium (II) chloride dihydrate (purity ≥99%, Fluka/Honeywell, Charlotte, North Carolina, US). Afterward, calcium solutions were standardized by titration with EDTA (Ethylenediaminetetraacetic acid disodium salt, BioUltra, ≥99%, Sigma-Aldrich/Merck, Darmstadt, Germany) standard solution. Ligand solutions were prepared by weighing and dissolving, without further purification, the following products: L-cysteine (purity ≥99.5%, Fluka/Honeywell, Charlotte, North Carolina, US), D-penicillamine (purity ≥97%, Alfa-Aesar/Thermo Fisher, Kandel, Germany), reduced glutathione (purity ≥98%, Alfa-Aesar, Thermo Fisher, Kandel, Germany), and oxidized glutathione (purity 98%, Sigma-Aldrich/Merck, Darmstadt, Germany). The purity of the ligands was checked by alkalimetric titration. It was found to be greater than 99%. Solutions of hydrochloric acid and sodium hydroxide were obtained by dilution of Fluka (Fluka/Honeywell, Charlotte, North Carolina, US) ampoules and afterward they were standardized with sodium carbonate (≥99.5%, Sigma-Aldrich/Merck, Darmstadt, Germany) and potassium biphthalate (≥99.5%, Sigma-Aldrich/Merck, Darmstadt, Germany), respectively. Both salts were previously dried in an oven at 110 °C. Solutions of sodium hydroxide were reprepared very frequently and were kept in bottles with soda lime traps. Solutions of sodium chloride were obtained by weighing the corresponding salt (puriss., Sigma-Aldrich/Merck, Darmstadt, Germany), previously dried in an oven at 110 °C. Distilled water (conductivity <0.1 μS cm\(^{-1}\)) and grade A glassware were employed for the preparation of all the solutions.

#### Potentiometric Apparatus and Procedure

Two distinct systems were employed for the potentiometric titrations. In detail, the systems consist in an identical configuration consisting in an automatic dispenser Metrohm Dosino 800, a Metrohm model 809 Titrando potentiometer, and a Metrohm LL-Unitrode WOC combined glass electrode. Each potentiometric system was connected to a PC and the experimental titration data were acquired by the Metrohm

| Technique | t/°C | C\(_{\text{Ca}}\)/mmol L\(^{-1}\) | C\(_{\text{Lig}}\)/mmol L\(^{-1}\) | M/L | pH range |
|-----------|------|------------------|------------------|-----|----------|
| Potentiometry | 15–37 | 1–5 | 2–6 | 0.33–2 | 2–10 |
| \(^1\)H NMR | 25 | 5–10 | 5–10 | 0.75–1.5 | 2–10.5 |
| \(^1\)H NMR | 25 | – | 10* | – | 2–10.5 |

*Protonation measurements for GSSG ligand.
TIAMO 2.2 software. It can control several parameters, such as e.m.f. stability, titrant delivery, and data acquisition. Estimated accuracy of this apparatus is ±0.15 mV and ±0.002 ml for e.m.f. and for readings of titrant volume, respectively.

Each titration consists in additions of volumes of NaOH standard to 25 ml of the solution containing Ca²⁺, ligand, and a supporting electrolyte (NaCl). Experimental details on potentiometric titrations are reported in Table 1. Glass jacket thermostated cells were employed for the measurements performed under different conditions of temperature (15 ≤ t/°C ≤ 37), by bubbling pure N₂ in order to avoid CO₂ and O₂ inside the solutions and under magnetic stirring. For each measurement, an independent titration of HCl with standard NaOH was performed to calculate the standard electrode potential E⁰ and the pKw value, under the same experimental ionic strength and temperature conditions.

NMR Apparatus and Procedure

The spectrometer employed for the collection of ¹H NMR spectra is a Varian 500 FT-NMR. 1,4-Dioxane was used as internal reference (δCH/CH₃ = 3.70 ppm); the chemical shifts are referred to tetramethylsilane (TMS). All the measurements were carried out in a 9:1 H₂O/D₂O solution at t = 25 °C. Presaturation technique was employed to suppress the water signal. Experimental details on ¹H NMR titrations are reported in Table 1.

Mass Spectrometric Apparatus and Procedure

A water solution of 2 equivalents of each ligand (Cys, PSH, GSH, GSSG) was added dropwise to 1 mmol of CaCl₂ dissolved in water with magnetic stirring for 2 h at room temperature. MALDI MS and MS/MS analyses were performed using a 5800 MALDI-TOF-TOF Analyzer (AB SCIEX) in reflection positive ion mode with a mass accuracy of 5 ppm. At least 5000 laser shots were typically accumulated with a laser pulse rate of 400 Hz and 1000 Hz in the MS and MS/MS mode, respectively. MS/MS experiments were performed using ambient air as collision gas with a medium mass accuracy of 5 ppm. After acquisition, spectra were processed using Data Explorer version 4.0. MALDI MS and MS/MS experimental conditions were optimized using sinapinic acid (SA, 5 mg/ml in H₂O/CH₃CN 40:60, v/v; with 0.1% TFA) as matrix for all ligands. The sample loading was performed by dried droplet method for all ligands, spotting 1 μL of sample/matrix premixed solution (1:5, v/v ratio).

Calculations

Experimental data of potentiometric titrations were processed using BSTAC and STACO programs. They allow for obtaining the best speciation model for each system under study, the formation constant values of the species, and the parameters of a titration (standard potential E⁰, analytical concentration of the reagents, and junction potential). The parameters for the dependence of complex formation constants on temperature were obtained by LIANA program. More details on software employed in the refinement of the experimental data are reported in De Stefano et al. (1997). For ¹H-NMR titrations, HypNMR software was employed to obtain protonation and formation constant values, as well as the individual chemical shift of each species, using the observed signals and assuming fast mutual exchange in the NMR time scale (Frassineti et al., 1995). HySS program was used to obtain the speciation diagrams and the formation percentages of the complex species (Alderighi et al., 1999).

RESULTS AND DISCUSSION

In the calculations, protonation constants of ligands understudy (Cardano et al., 2008; Crea et al., 2008; Cardano et al., 2013) and hydrolytic constant of Ca²⁺ were taken into account. They are reported in Supplementary Tables S1 and S2.

Potentiometric measurements were carried out under different conditions of temperature and metal-ligand ratios, to choose the most appropriate speciation model and to be able to refine the formation constants of the species in solution. The formation constants of Ca²⁺(M)-ligand(L) species are expressed as overall formation constants (K) and stepwise formation constants (Kᵢ). The reactions are the following (charges are omitted for simplicity):

\[ M + L + R = MLH \quad K_{MLH} \]  \hspace{1cm} (1)

\[ M + LH = MLH_i \quad K_{MLH_i} \]  \hspace{1cm} (2)

Within the speciation studies, the most reliable model for a metal-ligand system is chosen by taking into account several factors,
such as the simplicity of the model itself, the statistical parameters (standard and mean deviation on the fit), the variance ratio between the chosen model and others, and the formation percentages of the formed species (Filella, 2005).

**Speciation Profiles and Aqueous Behavior**

Formation constant values of Ca²⁺-Cys, PSH, GSH, GSSG species obtained via potentiometric measurements at different temperatures and $I = 0.15$ mol L⁻¹ were reported in Table 2. The speciation pattern for all the systems includes only 1:1 M:L species. Cys, PSH, and GSH show a very similar behavior with the same speciation model including only two significant species, namely, MLH₂ and MLH. For all three systems, the stability of complex species in terms of stepwise formation constants is between a minimum of 1.57 (MLH species for Ca²⁺-GSH system, $t = 25 \degree C$) and a maximum of 3.66 (MLH₂ species for Ca²⁺-PSH system, $t = 37 \degree C$). In Figure 2A the speciation diagram of Ca²⁺-Cys species is depicted at $I = 0.15$ mol L⁻¹ and $t = 15$, 37°C. Under physiological conditions ($t = 37 \degree C$, $I = 0.15$ mol L⁻¹), MLH₂ species is formed in the range 2 ≤ pH ≤ 9 and reaches a metal fraction of 0.4 in the range 3 ≤ pH ≤ 7. The main complex species in the range 8 ≤ pH ≤ 10 is MLH with a maximum metal fraction corresponding to 0.3 at pH = 9.5.

Formation constants of Ca²⁺-PSH species are quite higher with respect to Ca²⁺-Cys ones. For example, stepwise formation constant values at $t = 37 \degree C$ resulted between 3.58 and 3.66. The differences between stepwise formation constants of species formed by PSH and Cys with Ca²⁺, under physiological conditions, are $\Delta \log K = 1.3$ for both MLH₂ and MLH. The speciation diagram, represented in Figure 2B, refers to Ca²⁺-PSH system, under physiological conditions. MLH₂ species is formed in the wide interval 2 ≤ pH ≤ 8, reaching a maximum metal fraction of 0.4; MLH species is present in the range 8 ≤ pH ≤ 10 with a lower metal fraction (0.2).

For Ca²⁺-GSH system, stepwise formation constant values at $t = 37 \degree C$ are equal to 2.41 for both species. The differences between formation constants of Ca²⁺-PSH and -GSH species, under physiological conditions are $\Delta \log K = 1.2$ for both. The speciation diagram, depicted in Figure 2C, refers to Ca²⁺-GSH system, under physiological conditions. It shows that MLH is the main complex species in the wide interval 2 ≤ pH ≤ 9, with a maximum metal fraction of 0.4, and MLH predominates in the range 8.5 ≤ pH ≤ 10, with a metal fraction of 0.3.

A separate discussion must be made for the system containing GSSG. As expected from the presence of the numerous protonable groups on molecule, the speciation model is very rich in complex species, namely, MLH₅, MLH₄, MLH₃, MLH₂, and MLH. Their stability is comparable to the values found for the other ligands already discussed. As an example, at $t = 37 \degree C$ and $I = 0.15$ mol L⁻¹, logK values, referring to stepwise formation constants, range between 2.27 and 3.56 for the five species. Speciation profile for Ca²⁺-GSSG system is represented in Figure 2D. Under physiological conditions, the less significant species is MLH one, while the most significant species is MLH₂, which predominates in the pH range between 4.5 and 9 reaching metal fraction of 0.6. The most protonated species, MLH₅, MLH₄,
and MLH₃, predominant at pH < 4.5, reach maximum metal fractions equal to 0.65, 0.3, and 0.45, respectively. MLH species is significant only at pH > 9, with a metal fraction of 0.2.

**1H NMR Spectroscopy**

The interaction of ligands of biological interest with metal cations in aqueous solution had been already studied by our research group with several spectroscopic techniques, such as ¹H NMR (Cardiano et al., 2008; Cardiano et al., 2011; Cardiano et al., 2013; Cardiano et al., 2016), UV-Vis (Falcone et al., 2011; De Stefano et al., 2014), Mössbauer (Cardiano et al., 2006), and Raman (Cassone et al., 2019). In the literature, there are some recent papers that report ¹H NMR investigations on GSSH and PSH with metal cations other than Ca²⁺ (Sisombath et al., 2014; Kretzschmar et al., 2020). More in detail, Sisombath et al. report a complexation study on Pb²⁺ with PSH by ¹H NMR analysis, in D₂O solutions at pH = 9.6, at various M:L molar ratios. The data here reported are comparable with that reference and specifically it is possible to underline the same trend relative to the significant chemical shift of CH-2 (Δδ = 0.6 ppm) and of only one of the two -CH₃.

In this paper ¹H NMR spectra of Ca²⁺-Cys species, reported in Figure 3 at different pH values and t = 25 °C, show a chemical shift of the signals related to the proton in 2 and to the two protons in 3, indicated as H-2, H₃-3, and H₆-3, respectively for Cys. At pH < 8 there is a triplet for H-2 and a doublet (dd) for H₃-3 and H₆-3. At pH > 8, the complexity of the signals increases wherein a multiplet for H-2, a dd for H₃-3, and a dd for H₆-3 are shown. The chemical shift of the H-2 proton to the increase of pH is approximately 0.8 ppm upfield due to the increase in the negative charge for the deprotonation of the carboxyl group and subsequently of the thiol group. A similar trend is evident for protons in 3; in this case the chemical shift is about 0.3 ppm. Much more interesting is the splitting of the signals into two different dd, which can be interpreted with greater rigidity of the ligand for the presence of a dianion or for the interaction with the metal cation as well. This AMX system is therefore due to the different magnetic properties of the

![FIGURE 3](image-url) ¹H NMR spectra on solutions containing Ca²⁺ (M) and Cys(L) at CM = 7.5 mmol L⁻¹, CL = 10 mmol L⁻¹, t = 25 °C, J = 0.15 mol L⁻¹ in NaCl, 1.94 ≤ pH ≤ 10.46.

**TABLE 3** Comparison between the experimental formation constants of Ca²⁺-ligand species and protonation constants of GSSG obtained via ¹H NMR and potentiometry at t = 25 °C and J = 0.15 mol L⁻¹.

| Ligand | Species | ¹H NMR | Potentiometry |
|--------|---------|--------|---------------|
| Cys    | MLH₂    | 20.4 (2)b | 20.76 |
|        | MLH     | 12.4 (1)  | 12.50 |
| PSH    | MLH₂    | 21.0 (2)b | 20.60 |
|        | MLH     | 12.15    | 12.15 |
| GSH    | MLH₂    | 20.49 (8)b | 19.97 |
|        | MLH     | 10.9 (8)  | 11.02 |
| GSSG   | LH      | 9.3 (2)b  | 9.618 |
|        | LH₆     | 18.35 (9) | 18.442 |
|        | LH₉     | 22.22 (4) | 22.212 |
|        | LH₁₀    | 25.43 (9) | 25.319 |
|        | LH₁₅    | 27.467    | 27.467 |
|        | LH₁₀     | 28.987    | 28.987 |
|        | LH₂₀    | 30.18     | 30.18 |
|        | LH₂₅    | 28.16     | 28.16 |
|        | LH₃₀    | 25.12     | 25.12 |
|        | LH₃₅    | 21.21 (6)b | 21.19 |
|        | LH₄₀    | 12.04 (7) | 12.15 |

aOverall formation constants.
b≥95% of confidence interval.
two protons in 3 and consequent more complex coupling between the three protons H-2, H3-3, and Hb-3. The interaction with Ca2+ is evident from the comparison with the corresponding chemical shift values of Cys alone, under the same experimental conditions.

1H NMR spectra of Ca2+-PSH, Ca2+-GSH solutions were reported in Supplementary Figures S2-S3. Both ligands evidenced a similar behavior to Cys, as their spectra NMR showed a significant shift in signals at the change of pH. The interaction of each ligand with Ca2+ is highlighted by the comparison with the corresponding chemical shift values of the ligand in the absence of the metal cation under the same experimental conditions. The comparison of the formation constant values obtained via potentiometric and 1H NMR measurements (see Table 3) shows satisfactory correspondence.

Here the NMR analysis of the free GSSG ligand at different pH values is reported. 1H NMR spectra of GSSG in 10% D2O/H2O solution show only six signals due to the symmetry to the S-S bond. Table 3 shows the comparison between the protonation constant values obtained by potentiometric and 1H NMR measurements. It was possible to obtain the values relating to the first four protonation constants (LH, LH2, LH3, and LH4), while those relating to the LH5 and LH6 species were kept constant using the values obtained by potentiometry. The agreement among the results obtained by the two different techniques was excellent. 1H NMR spectra registered on Ca2+-GSSG solutions at t = 25 °C and I = 0.15 mol L−1, represented in Figure 4, show substantially the same signal pattern observed in the spectra relating to the solutions containing GSSG ligand (Supplementary Figure S4). In detail, at pH = 2.2 are present amide protons 4 and 13 at δ = 8.5 ppm (2 singlets), proton in 3 at 3.95 ppm (quartet), proton in 11 at δ = 3.85 ppm (multiplet), proton in 14 at δ = 3.22 ppm (multiplet), proton in 2 at δ = 2.93 ppm (dd), proton in 9 at δ = 2.50 ppm (multiplet), and proton in 10 at δ = 2.13 ppm (multiplet).

The chemical shift values of the individual species were calculated on the basis of formation percentages of each species in solution. These chemical shifts, reported in Supplementary Table S3, were used to determine the values of the formation constants of the complex species. In Table 3 these formation constant values obtained by 1H NMR titrations were reported, together with potentiometric ones. It is possible to notice a good agreement among the values determined by the two different techniques. For Ca2+-GSSG species only the values referring to MLH2 and MLH species were refined, keeping constant ones obtained by potentiometry related to MLH5, MLH4, and MLH3 species. The speciation model considered for all the systems is also confirmed by the complete overlap of the experimental and calculated chemical shift values shown in Figure 5. It should be noted that, at pH > 8, 1H NMR spectra on the solutions containing ligands in the presence of Ca2+ show significant differences with respect to the corresponding free ligands. At pH > 8, differences of Δδ between 0.05 and 0.10 ppm were calculated on average for all ligands, except for GSSG. From this experimental evidence, it can be assumed that Cys, PSH, GSH, and GSSG could behave as divalent ligands, binding Ca2+ and giving rise to cyclic complexes.

MALDI MS and MS/MS
Mass spectrometry combined with soft ionization methods as electrospray ionization (ESI) and matrix assisted laser desorption
ionization (MALDI) is currently becoming a strategic approach to clarify structures and coordination sites in compounds where metals are chelated by biological ligands (Cardiano et al., 2009; Furia et al., 2014; Aiello et al., 2017; Aiello et al., 2018a; Chilè et al., 2020). MALDI-TOF/TOF-MS platforms can be used for the highly sensitive analysis of low molecular weight compounds (Aiello et al., 2020a) in complex matrices (Aiello et al., 2018b; Aiello et al., 2020b; Imbrogno et al., 2019; Salvatore et al., 2020). In order to investigate whether calcium binding by Cys, PSH, GSH, and GSSG induces formation of complexes, a water solution of 2 equivalents of each ligand was added dropwise to 1 equivalent of CaCl2 and complex association was analyzed by MALDI MS using sinapinic acid as matrix. Signals corresponding to complex ML with 1:1 stoichiometry are the most intense signals in the spectrum for all investigated systems. The molecular masses derived from these measurements are in good agreement with the calculated mass (within 5 ppm, Table 4). The simplest systems, represented by Ca2+-Cys and Ca2+-PSH, will briefly be discussed. Both ligands hold multiple donor sites that are capable of intramolecular stabilization of the metal-ligand species. The carboxylic acids, bearing donor groups in their α or β positions, generally act as bidentate ligands giving rise to cyclic structures (Aiello et al., 2018a; Falcone et al., 2013). Accordingly, the formation of [MLH]+ species suggests that Cys and PSH act as bidentate ligands giving rise to six-membered cycles (Figure 6). The simplicity of the MS/MS spectra suggests that only few fragmentation pathways are allowed for the decomposition of complexes. MALDI MS/MS spectrum of the system Ca2+-Cys (Figure 6A) reveals that the main fragmentation pathways of the precursor [MLH]+ (m/z 159.97, [CaC3H6NSO2]+) consist in the loss of low molecular species such as NH2 (m/z 144.96 [CaC3H5SO2]+) and CH2NH (m/z 130.95 ([CaC2H3SO2]+)). However, some characteristic fragment ions can be found and correlate with the proposed structure. In particular, the formation of the ions of m/z 130.95 ([CaC2H3SO2]+), m/z 118.95 ([CaC2H7SO]+), and m/z 90.95 ([CaH3SO]+) arises from across ring fragmentation of a six-membered structure. Analogously, PSH leads to a cyclic structure ([MLH]+ of m/z 188.01 ([CaC6H10NO2S]+). Several distinguishing ion products were detected in the MS/MS spectra; all the peak assignments are described in Table 4 and Figure 6B. In agreement with the NMR data, it can be reasonably stated that Cys and PSH act as divalent ligands and that they bind the Ca2+ ion through O and S giving rise to six-membered cyclic complexes, as already observed for other ligands containing carboxylic and thiol groups (Cardiano et al., 2009).

GSH is a tripeptide bearing two free -COOH groups, a -NH2 group, and a -SH group; it provides a hydrophilic interface and a handle for further reactivity with other functional molecules as well as metal ions. The metal coordination ability of GSH is well documented, highlighting its multichelating nature. The speciation of both reduced and oxidized forms of GSH in MS/MS condition was considered. Information about molecular mass of the Ca2+-GSH as well as Ca2+-GSSG complex is easily obtained using 1:1 Ca2+-GSH molar ratios. The peak at m/z 346.04 corresponds to the ion [MLH]+ in which GSH is deprotonated.
TABLE 4 | Mass spectrometry data of Ca²⁺-L species, reported as m/z values, formula assignments, and MS/MS values for fragment ions.

| Formula | m/z   | Δppm |
|---------|-------|------|
| [M-Cys]⁺ | ![image](https://example.com/fig4a.png) | 159.97 | 5.0 |
| [CaC₄H₇NO₂S]⁺ | 144.96 | 4.5 |
| [CaC₄H₇SO₃]⁺ | 130.95 | 6.0 |
| [CaOH]⁺ | 118.95 | 5.3 |
| [CaH₂SO₄]⁺ | 90.95 | 4.8 |
| [CaOH]⁺ | 56.97 | 4.0 |
| [M-PSH]⁺ | ![image](https://example.com/fig4b.png) | 188.01 | 6.0 |
| [CaH₄O₃]⁺ | 156.96 | 4.5 |
| [CaH₄O₂S]⁺ | 161.00 | 5.5 |
| [CaH₂O]⁺ | 83.05 | 5.3 |
| [CaOH]⁺ | 56.97 | 4.8 |
| [M-GSH]⁺ | ![image](https://example.com/fig4c.png) | 346.04 | 5.0 |
| [CaC₈H₁₃N₂O₄]⁺ | 330.04 | 4.5 |
| [CaC₈H₁₃N₂O₃]⁺ | 312.05 | 5.5 |
| [CaC₈H₁₃N₂O₄]⁺ | 302.05 | 5.1 |
| [CaC₈H₁₃N₂O₃]⁺ | 271.01 | 4.8 |
| [CaC₈H₁₃N₂O₄]⁺ | 219.05 | 4.7 |
| [CaH₂N₂O₃]⁺ | 191.04 | 4.1 |
| [CaH₂NO₃]⁺ | 125.98 | 3.9 |
| [CaOH]⁺ | 56.97 | 5.3 |
| [M-GSSG]⁺ | ![image](https://example.com/fig4d.png) | 651.10 | 4.0 |
| [CaC₁₀H₁₄N₃O₆]⁺ | 635.60 | 4.2 |
| [CaC₁₀H₁₄N₃O₆]⁺ | 619.76 | 4.8 |
| [CaC₁₀H₁₄N₃O₆]⁺ | 576.07 | 5.2 |
| [CaC₁₀H₁₄N₃O₆]⁺ | 522.06 | 4.1 |
| [CaC₁₀H₁₄N₃O₆]⁺ | 379.07 | 4.7 |
| [CaC₁₀H₁₄N₃O₆]⁺ | 346.04 | 5.3 |
| [CaC₁₀H₁₄N₃O₆]⁺ | 312.05 | 5.5 |
| [CaC₁₀H₁₄N₃O₆]⁺ | 271.01 | 4.8 |
| [CaH₃NO₃]⁺ | 125.98 | 3.9 |

(i.e., GSH²⁻) and therefore presumably bound to Ca²⁺ via -COOH and -NH amino groups. The calcium complex of GSH (m/z 346.04 \([\text{Ca}_{34}H_{41}N_{5}S_{2}O_{4}²⁻]\)) decomposes to give, besides major H₂O and CO₂ and H₂S losses, small abundances of w₃b*, a₃*, b₂*, c₂*, b₁*, and d₂* calcium containing and z₁ non-calcium product ions (Figure 7A). Product ions, which contain the C terminus, are formed by losses of residues comprised of only one amino acid, suggesting that the primary binding site for the Ca²⁺ is the N terminus of the peptide. The formation of the ion of m/z 125.98 ([CaH₆CaNaNO₃]⁺)) and its counterpart m/z 219.05 ([CaH₁₄H₂N₂O₂S]⁺) indicates that Ghu is calcium-binding amino acid. The Ca²⁺-GSSG (m/z 651.10 \([\text{Ca}_{26}H_{31}CaNa_{6}O_{12}S_{2}²⁻]\)) complex decomposes giving a remarkably simple spectrum; it breaks down releasing Ghu (m/z 522.06 \([\text{Ca}_{15}H_{25}S_{2}N_{2}O_{3}²⁻]\)), Gly (m/z 576.07 \([\text{Ca}_{18}H_{26}N_{2}S_{2}O_{10}²⁻]\)), and OH (m/z 635.60 \([\text{Ca}_{20}H_{31}Na_{6}S_{4}O_{11}²⁻]\)) as neutrals. The further formation of the most informative calcium containing products of m/z 619.76, m/z 346.04, and m/z 379.07 (Figure 7B) is also observed. The breakage of CH₂-S and S-S bonds leads to the formation of the ions of m/z 379 and 346, respectively. Thereafter, both calcium containing species decompose giving rise to low intensity ion series. Appearance of small mass calcium containing ions, in MS/MS spectrum of Ca-GSSG peptide complex, is additional evidence that calcium binding is via N terminus of the peptide. Therefore, GSSG involves calcium in an "open" type complex, in which the metal ion is not coordinated from both glutamic acids, assuming a behavior like a simple amino acid. Finally, the simplicity of MS/MS spectra indicates that the binding of Ca²⁺ ions to GSH and GSSG is to the deprotonated glutamyl carboxylic residue and to the NH amino function. Ca²⁺-peptide complexes undergo fragmentation that are determined by the location of the Ca binding site.

**Speciation in Biological Fluids**

In order to evaluate the relevance of the systems under study under real conditions, two biological fluids were considered. The first application consists in the evaluation of formation percentages of Ca²⁺ complex species, by considering plasma concentration, temperature, and ionic strength conditions (t = 37 °C, I = 0.15 mol L⁻¹, C_Ca = 2.5 mmol L⁻¹, C_Cys = 0.01 mmol L⁻¹; C_GSH = 5.5 μmol L⁻¹, C_GSSG = 0.5 μmol L⁻¹, C_Cl = 0.1037 mol L⁻¹, C_SO₄ = 0.49 mmol L⁻¹, C_CO₃ = 24.9 mmol L⁻¹, C_POM = 1.6 mmol L⁻¹) (Lentner, 1983). In these conditions, at pH = 7.4 the main species is CaPO₄, with a percentage of 60.9%. The most important species among ones under study are CaCysH₂ and CaCysH, although their sum just reaches 10.3%, as shown in Figure 8A.

The second application is based on lens aqueous solution. In the human eye the aqueous humor is located between the lens and the cornea. It is a gelatinous fluid where antioxidants, such as GSH and Cys, are investigated widely, since they serve as markers for eye diseases and infections. In the lens, the antioxidant GSH and ascorbic acid have unusually high concentration (Pescosolido et al., 2016). The functions performed by GSH with ascorbic acid in the lens are manifold. Among them, very important is the protection of protein thiol groups against oxidation agents and the detoxification of hydrophobic species in reactions catalyzed by glutathione S-transferase enzymes. In cataractous lens as well as in the aging lens, calcium concentration increases, and destruction of ascorbic acid and reduction of GSH content also occur (Chandorkar et al., 1980; Pescosolido et al., 2016). Accordingly, two different simulations were performed considering the composition of electrolyte and biological ligands in normal and in cataractous lens water. The obtained results are very different. Figure 8B represents the pie plot of Ca²⁺ complex species at pH = 7.2, by considering normal lens water concentrations (C_Ca = 0.01 mmol L⁻¹, C_Cys = 0.0143 mmol L⁻¹; C_GSH = 3.28 mmol L⁻¹, C_GSSG = 0.095 mmol L⁻¹, C_Cl = 0.79 mmol L⁻¹, C_Ascorbic Acid = 1 mmol L⁻¹) (Chandorkar et al., 1980; Königsberger et al., 2015). In this case, among species formed by Ca²⁺-ligands under study, those containing GSH are the main species. Among them, very important is that the binding of Ca²⁺ ions to GSH and GSSG is to the deprotonated glutamyl carboxylic residue and to the NH amino function.
(Chandorkar et al., 1980; Kisic et al., 2012; Königsberger et al., 2015; Pescosolido et al., 2016). In this case, percentage of Ca\textsubscript{GSH} species drastically decreases while remaining significant (from 41.9 to 18%); Ca\textsubscript{GSSG} increases slightly while resulting in an irrelevant species. These simulations confirm the need of knowledge of reliable formation constants at different conditions to predict the relevance of the species in real systems.

**Dependence of Formation Constants on the Temperature**

Formation constant values of the complex species reported in Table 2, obtained by potentiometric measurements at \( t = 15, 25, 37 \) °C, were analyzed for the determination of the formation enthalpy changes of the species, via the van’t Hoff equation, already employed for several other systems (Cardiano et al., 2019; Cordaro et al., 2019; Foti and Giuffré, 2020; Giuffrè et al., 2019; Giuffrè et al., 2020):

\[
\log \beta_T = \log \beta_\theta + \Delta H^\circ \left( \frac{1}{\theta} - \frac{1}{T} \right) \text{Rln}10,
\]

where \( \log \beta_T \) is the formation constant at a specific ionic strength and temperature (expressed in Kelvin), \( \log \beta_\theta \) is the formation constant at \( T = 298.15 \) K, and \( \Delta H^\circ \) is the formation enthalpy change at \( T = 298.15 \) K in kJ mol\(^{-1}\), \( R = 8.314,472 \) J K\(^{-1}\) mol\(^{-1}\).

The values of formation enthalpy changes of all the species of Ca\textsuperscript{2+}-Cys, -PSH, -GSH, and -GSSG systems are collected in Table 5, together with entropy and free energy values. They are also shown as bar plot in Figure 9, to better highlight the contribution to the formation free energy of the enthalpy and entropy thermodynamic parameters. Since the interactions between Ca\textsuperscript{2+} and the ligands understudy are mainly of electrostatic nature, it is expected that the entropic term gives the highest contribution to the free energy change, due to the orientation disorder given by the solvation water molecules. This was found for most species (except for MLH one formed by the interaction with Cys ligand, MLH\(_2\), and MLH ones containing GSSG ligand).

**Sequestering Ability**

The sequestering capacity represents the tendency, in solution, of a ligand to complex metal cation forming metal-ligand species, which allow for reducing the concentration of the free metal cation in solution. The stability of the complex species formed in solution influences the concentration of the free metal ion. The higher the stability of the formed species, the lower the concentration of the free cation. Considering the whole pH range, different metal-ligand species are formed in solution; each of them contributes to the sequestration of the metal cation. In order to describe the sequestering capacity of a given ligand with respect to a metal cation, it is not enough to know the formation constant values and the formation percentages of the different metal-ligand species. It is necessary to consider that different metal-ligand systems, having formation constants different from each other, can...
show the same formation percentages at a given pH and vice versa. Furthermore, all the equilibria in which the ligand and the metal ion under study take part must be considered, namely, ligand protonation, metal ion hydrolysis reactions, and weak interactions with the background salt. For these reasons, an empirical parameter, \( pL_{0.5} \), was proposed, which represents the cologarithm of the ligand concentration necessary to sequester 50% of the metal cation present in traces. The traces are precisely the concentration conditions with which many metal cations are present in natural fluids. To evaluate for quantitative purposes the sequestering capacity of a ligand with respect to a metal cation, the following Boltzmann-type equation with asymptotes 0 for \( pL \rightarrow 0 \) and 1 for \( pL \rightarrow \infty \) was used (Gianguzza et al., 2012; Falcone et al., 2013; De Stefano et al., 2016):

\[
\chi = \frac{1}{1 + 10^{(pL - pL_{0.5})}},
\]

where \( \chi \) is the sum of the molar fractions of the metal-ligand species and \( pL \) is the cologarithm of the total ligand concentration. This parameter depends on system conditions, such as temperature, pH, and ionic strength.

In order to evaluate the sequestering capacity of Cys, PSH, GSH, and GSSG ligands toward Ca\(^{2+} \) under physiological conditions (pH = 7.4, \( t = 37 ^\circ C \), \( I = 0.15 \) mol L\(^{-1} \)). As can be seen, the sequestering capacities of the ligands toward Ca\(^{2+} \) under physiological conditions follow the order:

\[
pL_{0.5} (\text{GSSG}) > pL_{0.5} (\text{GSH}) > pL_{0.5} (\text{Cys}) > pL_{0.5} (\text{PSH})
\]

By comparing these data with those relating to the stepwise formation constants of Ca\(^{2+}\)-ligand species, obtained by potentiometric measurements under physiological conditions, it is possible to find a different order of stability for the MLH\(_2\) species:

\[
\log K (\text{PSH}) > \log K (\text{GSSG}) > \log K (\text{GSH}) > \log K (\text{Cys})
\]

and a further different order for the MLH species:

\[
\log K (\text{PSH}) > \log K (\text{GSH}) > \log K (\text{GSSG}) \approx \log K (\text{Cys})
\]

This underlines the importance of calculating the sequestering ability that, taking into account all the interactions, can be different with respect to the order of stability assessed for a single species and reveal the “real” trend of the ligands.

**Literature Comparisons**

In literature databases there are few thermodynamic data on interactions of ligands under study with Ca\(^{2+} \) (Martell et al., 2004;
As regards Ca\textsuperscript{2+}-Cys system, a paper reports at $t = 25^\circ$C and $I = 0.1$ mol L\textsuperscript{−1} log $\beta = 1.92$ for ML species and several ternary species with other ligands (Ramamoorthy and Manning, 1975). This only value cannot be compared with the results with this paper, since the speciation model is totally different. In the case of Ca\textsuperscript{2+}-GSH system, a speciation model at $t = 37^\circ$C and $I = 0.15$ mol L\textsuperscript{−1} with four species, namely, MLH\textsubscript{2}, MLH, ML, and MLOH, with log $\beta = 20.68$, 12.89, 3.84, -6.46, respectively, is reported (Touche and Williams, 1976). These values can be compared with ours, as regards the common species, i.e., MLH\textsubscript{2} and MLH, in the same experimental conditions (log $\beta = 20.14$, 11.66, respectively). The significant differences probably can be attributed to the different speciation model considered. In a paper of Singh, where formation constant values of GSH with several metal cations, namely, Ca\textsuperscript{2+}, Mg\textsuperscript{2+}, Cu\textsuperscript{2+}, Pb\textsuperscript{2+}, Ni\textsuperscript{2+}, Zn\textsuperscript{2+}, Co\textsuperscript{2+}, Cd\textsuperscript{2+}, and Mn\textsuperscript{2+}, are reported, only one formation constant value referred to ML species was obtained for each system, including one containing Ca\textsuperscript{2+} (Singh et al., 2001). For this reason, this formation constant value cannot be compared with results here reported.

In a more recent paper, a fairly similar speciation model with three species was found, namely, MLH\textsubscript{2}, MLH, and ML, where log $\beta = 19.27$, 11.08, 1.60, respectively ($t = 25^\circ$C, $I = 0.15$ mol L\textsuperscript{−1}) (Cigala et al., 2012). In this paper, the values obtained under the same conditions for MLH\textsubscript{2} and ML species are log $\beta = 20.39$, 11.53, respectively. The agreement in this case, mainly for MLH species, is quite satisfactory.

**CONCLUSION**

The main purpose of this study was obtaining consistent speciation models and reliable thermodynamic data referring to Ca\textsuperscript{2+}-bioligands systems, based on the results gained via different analytical techniques. Speciation models and stability formation constants obtained by potentiometry were confirmed by $^1$H NMR spectroscopy. Indeed, the comparative analysis of the chemical shift values of the studied bioligands allows for reasonably affirming that all of them act as chelating agents of Ca\textsuperscript{2+}. MALDI MS confirmed the formation of complexes and
MS/MS experiments and, moreover, indicated different complexing behaviors of the ligands toward Ca\(^{2+}\). The results suggest that Cys and PSH act as bidentate ligands giving rise to six-membered cycles via O and S; GSH and GSSG bind to Ca\(^{2+}\) ion via O and N. By potentiometry, formation constant values under different temperatures were evaluated. In this way were also obtained \(T\Delta S\) and \(\Delta H\) values, necessary to calculate formation constants at different temperatures. The sequestering ability of Cys, PSH, GSH, and GSSG toward Ca\(^{2+}\) was evaluated under different pH and temperature conditions, with particular attention to those simulating biological fluids, evidencing an interesting trend.

Finally, obtained stability data were crucial to gain simulations under biological fluid conditions, as blood and lens water, and pointed out the importance of reliable thermodynamic data for simulations useful for applications to real systems, characterized by variable composition and pH.

### DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author.

### AUTHOR CONTRIBUTIONS

OG planned the experiments, supervised and organized the analysis, performed speciation calculations and simulations, and wrote the manuscript. CF contributed to conception, design of the study, analysis of the results, and manuscript revision. FC performed the potentiometric measurements, prepared the solutions for \(^{1}H\)
NMR experiments, and contributed to spectra acquisition. MC performed the ¹H NMR experiments and the qualitative analysis of the spectra and contributed to ¹H NMR section. DA contributed to experimental design of the study. DA and AN performed MALDI MS and MS/MS experiments and wrote mass spectrometry discussion. All authors contributed to manuscript revision, read, and approved the submitted version.

**FUNDING**

Publication fees will be covered by the University of Messina FFABR 2020 funds, University of Calabria funds and by Frontiers discount (Discount Code: DSC-11002218503PRD).

**ACKNOWLEDGMENTS**

The authors OG and CF thank University of Messina for Research & Mobility 2017 funds (ARCADIA project) and for FFABR 2020 funds. The authors DA and AN thank University of Calabria for funds. All the authors thank Frontiers Fee Support Team for the discount granted.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fchem.2021.640219/full#supplementary-material.

Cardiano, P., Giacobello, F., Giuffrè, O., and Sammartano, S. (2016). Thermodynamics of Al³⁺-thiocarboxylate interaction in aqueous solution. J. Mol. Liq. 222, 614–621. doi:10.1016/j.molliq.2016.07.077

Cardiano, P., Giuffrè, O., Napoli, A., and Sammartano, S. (2009). Potentiometric, ¹H-NMR, ESI-MS investigation on dimethyl(IV) cation-mercaptocarboxylate interaction in aqueous solution. New J. Chem. 33, 2286–2295. doi:10.1039/b908114c

Cardiano, P., Giuffrè, O., Pellerito, L., Pettignano, A., Sammartano, S., and Scopelliti, M. (2006). Thermodynamic and spectroscopic study of the binding of dimethyl(IV) by citrate at 25°C. Appl. Organomet. Chem. 20, 425–435. doi:10.1002/aoc.1076

Cassone, G., Chilli, D., Giacobello, F., Giuffrè, O., Mollica Nardo, V., Ponterio, R. C., et al. (2019). Interaction between As(III) and simple thiocidox in water: an experimental and ab initio molecular dynamics investigation. J. Phys. Chem. B 123, 6090–6098. doi:10.1021/acs.jpcb.9b04901

Chandorkar, A. G., Bulakh, P. M., and Albal, M. V. (1980). Electrolyte composition in normal and cataractous lenses. Indian J. Ophthalmol. 28, 135–138.

Chilli, D., Aiello, D., Grasso, G. I., Giuffrè, O., Napoli, A., Sgarlata, C., et al. (2020). Complexation of As(III) by phosphonate ligands in aqueous fluids: thermodynamic behavior, chemical binding forms and sequestering abilities. J. Environ. Sci. (China) 94, 100–110. doi:10.1016/j.jes.2020.03.056

Cigala, R. M., Crea, F., De Stefano, C., Lando, G., Miele, D., and Sammartano, S. (2012). Modeling the acid-base properties of glutathione in different ionic media, with particular reference to natural waters and biological fluids. Amino Acids 43, 629–648. doi:10.1007/s00726-011-1110-0

Cordaro, M., Foti, C., Giacobello, F., Giuffrè, O., and Sammartano, S. (2019). Phosphonic derivatives of nitrolitriacetic acid as sequestering agents for Ca²⁺ in aqueous solution: a speciation study for application in natural waters. ACS Earth Space Chem. 3, 1942–1954. doi:10.1021/acsearthspacechem.9b00183

Crea, P., De Stefano, C., Kambarami, M., Miller, F. J., and Sharma, V. K. (2008). Effect of ionic strength and temperature on the protonation of oxidized glutathione. J. Solut. Chem. 37, 1245–1259. doi:10.1007/s10953-008-9310-2

Davis, C. D., and Hanumegowda, U. M. (2008). Drug metabolism handbook. Hoboken, NJ: John Wiley & Sons.

De Stefano, C., Foti, C., Giuffrè, O., and Miele, D. (2016). Complexation of Hg²⁺, CH₃Hg⁺, Sn⁴⁺, and (CH₃)₂Sn⁺ with phosphonic NTA derivatives. New J. Chem. 40, 1443–1453. doi:10.1039/C5NJ02531A

De Stefano, C., Foti, C., Giuffrè, O., and Sammartano, S. (2014). Acid-base and UV behaviour of 3-(3,4-dihydroxyphenyl)-propenoic acid (caffeic acid) and complexing ability towards different divalent metal cations in aqueous solution. J. Mol. Liquids 195, 9–16. doi:10.1016/j.molliq.2014.01.027

De Stefano, C., Sammartano, S., Mineo, P., and Rigano, C. (1997). “Computer tools for the speciation of natural fluids,” in Marine chemistry - an environmental analytical chemistry approach. Editors A. Gianguzza, E. Pelizzetti, and S. Sammartano (Amsterdam: Kluwer Academic Publishers), 71–83.

Dringen, R. (2000). Metabolism and functions of glutathione in brain. Prog. Neurobiol. 62, 649–671. doi:10.1016/s0031-0082(99)00060-x

REFERENCES

Aiello, D., Giambona, A., Leto, F., Passarella, C., Damiani, G., Maggio, A., et al. (2018b). Human coelomic fluid investigation: a MS-based analytical approach to prenatal screening. Sci. Rep. 8, 10973. doi:10.1038/s41598-018-29384-9

Aiello, D., Siciliano, C., Mazzotti, F., Di Donna, L., Athanassopoulos, C. M., and Napoli, A. (2020a). A rapid MALDI MS/MS based method for assessing saffron (Crocus sativus L.) adulteration. Food Chem 307, 125527. doi:10.1016/j.foodchem.2019.125527

Aiello, D., Cardiano, P., Cigala, R. M., Gans, P., Giacobello, F., Giuffrè, O., et al. (2017). Sequestering ability of oligophosphate ligands toward Al³⁺ in aqueous solution. J. Chem. Eng. Data 62, 3981–3990. doi:10.1021/acs.jced.7b00865

Aiello, D., Furia, E., Siciliano, C., Bongiorno, D., and Napoli, A. (2018a). Study of the coordination of ortho-tyrosine and trans-4-hydroxyproline with aluminum(III) and iron(III). J. Mol. Liq. 269, 387–397. doi:10.1016/j.molliq.2018.08.074

Aiello, D., Siciliano, C., Mazzotti, F., Di Donna, L., Risoluti, R., and Napoli, A. (2020b). Protein extraction, enrichment and MALDI MS and MS/MS analysis from bitter orange leaves (citrus aurantium). Molecules 25, 1485. doi:10.3390/molecules25071485

Alderighi, L., Gans, P., Ienco, A., Peters, D., Sabatini, A., and Vacca, A. (1999). Hyperquad simulation and speciation (HYSS): a utility program for the investigation of equilibria involving soluble and partially soluble species. Coord. Chem. Rev. 184, 311–318.

Brigham, M. P., Stein, W. H., and Moore, S. (1960). The concentrations of cysteine and cystine in human blood plasma. J. Clin. Invest. 39, 1633–1638. doi:10.1172/JCI104186

Brinthurst, F. R., and Potts, J. T. (1979). “Calcium and phosphate,” in Endocrinology. Editor L. J. e. a. D. Groot (New York: Grune & Stratton).

Braudus, A. E. (1993). “Physiological functions of calcium, magnesium and phosphorus and mineral ion balance,” in Primer on the metabolic bone diseases and disorders of mineral metabolism. Editor M. J. Favus (New York: Raven Press).

Cardiano, P., De Stefano, C., Giuffrè, O., and Sammartano, S. (2008). Thermodynamic and spectroscopic study for the interaction of dimethyl(IV) with L-cysteine in aqueous solution. Biophys. Chem. 133, 19–27. doi:10.1016/j.bpc.2007.11.005

Cardiano, P., Falcone, G., Foti, C., Giuffrè, O., and Napoli, A. (2013). Binding ability of glutathione towards alkyltin(IV) compounds in aqueous solution. J. Inorg. Biochem. 129, 84–93. doi:10.1016/j.jinorgbio.2013.09.004

Cardiano, P., Cordaro, M., Chilli, D., Foti, C., and Giuffrè, O. (2019). Interactions of inosine 5′-monophosphate with Ca²⁺ and Mg²⁺: a thermodynamic and spectroscopic study in aqueous solution. J. Chem. Eng. Data 64, 2859–2866. doi:10.1021/acs.jced.9b00231

Cardiano, P., Cucinotta, D., Foti, C., Giuffrè, O., and Sammartano, S. (2011). Potentiometric, calorimetric and ¹H-NMR investigation on Hg²⁺-mercaptocarboxylate interaction in aqueous solution. J. Chem. Eng. Data 56, 1995–2004. doi:10.1021/je101007n

Aiello et al. Ca²⁺ Complexation With Relevant Bioligands
Falcone, G., Foti, C., Gianguzza, A., Giuffrè, O., Napoli, A., Pettignano, A., et al. (2013). "Sequestration ability of some chelating agents towards methylmercury(II)." Anal. Bioanal. Chem. 405, 881–893. doi:10.1007/s00216-012-6336-5

Falcone, G., Giuffrè, O., and Sammartano, S. (2011). "Acid-base and UV properties of some aminophenol ligands and their complexing ability towards Zn2+ in aqueous solution." J. Mol. Liq. 159, 146–151. doi:10.1016/j.molliq.2011.01.003

Filella, M., and May, P.M. (2005). "Reflections on the calculation and publication of potentiometrically-determined formation constants. Talanta 65, 1221–1225.

Foti, C., and Giuffrè, O. (2020). "Interaction of ampicillin and amoxicillin with Mn2+; a speciation study in aqueous solution. Molecules 25, 3110. doi:10.3390/molecules25143110

Frasinetti, C., Ghelli, S., Gans, P., Sabatini, A., Moruzzi, M. S., and Vacca, A. (1995). "Calcium: controls and triggers," in The biological chemistry of the elements: the inorganic chemistry of life. Oxford University Press, 278–314.

Frasinetti, C., Ghelli, S., Gans, P., Sabatini, A., Moruzzi, M. S., and Vacca, A. (1995). "Calcium: controls and triggers," in The biological chemistry of the elements: the inorganic chemistry of life. Oxford University Press.

Labib, M., Sargent, E. H., and Kelley, S. O. (2016). "Electrochemical methods for the analysis of clinically relevant biomolecules." Chem. Rev. 116, 9001–9090. doi:10.1021/acs.chemrev.6b00220

Laurie, S. H., Prime, D. H., and Sarkar, B. (1979). "Analytical potentiometric and spectroscopic study of the equilibria in the aqueous nickel(II)-triethylenetetramine and nickel(II)-D-penicillamine systems. Can. J. Chem. 57, 1411–1417. doi:10.1139/v79-230

Lentner, C. (1983). Geigy scientific Tables. Basle, Switzerland: CIBA-Geigy.