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# Soft- and Hard-Modeling Approaches for the Determination of Stability Constants of Metal–Peptide Systems by Voltammetry

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**The $Zn^{2+}$–glutathione system is studied as a model for metal–peptide systems where some critical factors must be considered when using voltammetric techniques for the determination of stability constants. These factors are the presence of side reactions (in this case, both the protonation of glutathione and the hydrolysis of $Zn^{2+}$), the association–dissociation rates of the complexes compared with the time scales of the measurements (which makes the complexes electrochemically labile or inert), and the electron transfer kinetics on the electrode surface (which makes the metal ion reduction reversible or irreversible). For the study of these factors, three data treatment approaches have been applied: (i) the electrochemical hard-modeling approach (modelization of both chemical equilibrium and electrochemical processes), (ii) a chemical hard-modeling approach (modelization of chemical equilibria only, based on the least-squares curve-fitting program SQUAD), and (iii) a previously developed model-free soft-modeling approach based on multivariate curve resolution with a constrained alternating least-squares optimization. By analyzing differential pulse polarographic data obtained under different experimental conditions, the influence of the mentioned factors on every approach is discussed and, if possible, the corresponding stability constants are computed. The results of this study showed the potential usefulness of voltammetry in combination with hard- and soft-modeling data analysis for the study of peptide complexation equilibria of metal ions such as Zn which have neither relevant spectroscopic properties nor proper isotopes for NMR measurements.** 

The binding of metal ions by peptides and proteins is of high interest in biological systems. Formation constants of metal complexes are determined by a variety of methods, both spectroscopic and potentiometric playing key roles (1). Although these methods are very well established, they are limited by the relatively high concentrations required which cannot always be reached. In these cases, alternative methods are of highest interest.

Among the electrochemical techniques (2), voltammetric have been widely used to study the interactions between metal ions and a diversity of ligands (3). Historically, polarography, i.e., voltammetry at dropping mercury electrodes, has been the most widely used technique because of its very high reproducibility and reliability at low concentrations (4). This is especially true for the more sophisticated techniques such as differential pulse polarography (DPP)[3] (5).

Glutathione (GSH) is considered an essential constituent of all living cells. Indeed, GSH is usually the most abundant intracellular nonprotein thiol (6). GSH presents eight binding sites: two carboxylic acid groups, an amino group, a sulfhydryl group, and two peptide linkages. Because all sites cannot be simultaneously coordinated to a single metal ion, the coordination chemistry of GSH is characterized by the formation of protonated and polynuclear complexes. Most metal–GSH complexation studies in solution have

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[3] Abbreviations used: DPP, differential pulse polarography; MCR-ALS, multivariate curve-resolution method with alternating least-squares optimization.

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been done through potentiometry. However, those measurements alone provide little or no information on the nature of the complexes at the molecular level, since a macroscopic property is measured. These problems have been resolved to some extent using information at the molecular level provided by spectroscopy. The acid–base chemistry of the four acidic groups of GSH, whose knowledge is necessary for quantitative studies of metal binding, has been characterized at the molecular level (7, 8).

The binding of $Zn^{2+}$ by GSH has been the subject of a number of studies, mostly by pH titration techniques. However, there is a lack of agreement on which $Zn^{2+}$–GSH species are formed as well as on the magnitude of their stability constants (6, 9–11). The use of electroanalytical techniques is especially important for the study of Zn complexes because of the lack of (i) relevant spectroscopic properties of Zn and (ii) a proper Zn isotope for NMR measurements.

The complexation between $Zn^{2+}$ and GSH was recently studied by DPP (12). During the DPP titrations of $Zn^{2+}$ with GSH most voltammograms did not have the regular shape observed for $Zn^{2+}$ in the absence of GSH. They seemed to be formed by overlapping of simpler signals. This means that $Zn^{2+}$ ions bound to the different sites of GSH did not dissociate appreciably during the voltammetric measurement and, as a consequence, they were independently reduced at different potentials yielding different DPP signals, which due to their proximity produce overlapping peaks. The complexity of the highly overlapping polarograms prevented the use of classical voltammetric hard-modeling approaches (4) and the direct interpretation of the signals. Moreover, secondary phenomena like hydrolysis of $Zn^{2+}$ or losses of electrochemical reversibility of the $Zn^{2+}$ reduction were detected in some cases, thus making even more difficult treatment by electrochemical hard modeling.

Then, data analysis was done through a soft-modeling approach based on factor analysis that had been previously developed by Tauler *et al.* for the study of spectrophotometric data (13) and that was later applied to voltammetric signals (14, 15). It basically consists of a multivariate curve-resolution method with alternating least-squares optimization (MCR-ALS) which does not assume any a priori chemical model. The MCR-ALS approach was recently proposed as a very useful tool for the study of the metal-binding properties of the peptides by voltammetric means (15).

The study of the Zn–GSH system by MCR-ALS (12) clearly indicated the presence of three main species (the free metal ion and two complexes). However, the above-mentioned secondary phenomena prevented the use of a closure constraint forcing the total metal concentration to be equal to a well-known value (if hydrolysis is present, the total metal concentration in solution is different from that initially added, since one part of the metal is precipitated as insoluble hydroxide). The MCR-ALS method provided a set of pure theoretical voltammograms for each electrochemical process (forced to have the same height) and a set of concentration profiles which indicated the relative changes in the concentration of every species but, as a consequence of the lack of closure constraint, not the absolute values of them. Obviously, this may hinder the calculation of stability constants. Regardless, the comparison of the shapes of the concentration profiles obtained in our previous study (12) suggested the 1:1 and 1:2 Zn:GSH stoichiometries for the two main complexes. In fact, some structural considerations, jointly with the shape of pure signals, suggested that the 1:1 complex could actually be a 2:2 molecule, but this hypothesis is difficult to confirm unless structural information can be obtained by complementary techniques.

In the present work, the Zn–GSH system has been used as a model to cope with such methodological problems which can be present in a high variety of metal–ligand systems, including metal–peptide. Moreover, three different approaches have been used to determine the corresponding stability constants. Under the different experimental conditions studied, this system has demonstrated to be a valuable model to compare the advantages and drawbacks of these three different approaches used for computing stability constants: (i) the "classical" electrochemical hard modeling (modelization of both chemical equilibrium and electrochemical processes); (ii) the already commented MCR-ALS soft modeling, including a closure constraint in the ALS optimization; and (iii) a kind of chemical hard modeling (i.e., the modelization of the chemical equilibria only) by using a least-squares curve-fitting program (SQUAD) (16) originally designed for spectrophotometric data that, to our knowledge, has not been applied before for the analysis of voltammetric signals.

## THEORY

### *Electrochemical Hard Modeling*

The voltammetric signal measured for a metal ion in the presence of complexing agents is determined by the phenomena taking place in three different regions of the measuring device (2).

In the bulk solution (the majority of the sample, not disturbed by the electrodic processes) there is usually chemical equilibrium, so that the concentration of every species is constant and verifies the full set of stability constants of the system.

In the diffusion layer (a thin region around the working electrode) the concentrations of the species are affected by the electrodic processes and change progressively as a function of time, applied potential, and distance to the electrode. This concentration change is

mainly determined by the rate of consumption of the electroactive species in the electrode, the rate of transport by diffusion of the species toward the electrode (the bigger the diffusion coefficient the higher the rate), and the extension of the reactions that can transform one species into another as they are transported along the diffusion layer. The extension of this last phenomenon is determined by the relation between the kinetics of association–dissociation of the complexes and the time taken by the voltammetric measurement. If the kinetics is much slower than the measurement, there is no conversion between species in the diffusion layer and every species can be independently transported and reduced (as it happens, for instance, in a mixture of $Zn^{2+}$ and $Cd^{2+}$ ions). In this situation, the complexes are termed electrochemically inert. On the contrary, if kinetics is much faster than the measurement, every variation in the concentration of a species is immediately counteracted by association or dissociation of other species. This means that there is a continuous interconversion of species that ensures the fulfillment of all stability constants along the diffusion layer and produces a single signal which is the average of the reduction of all the metal species. Then, the complexes are named electrochemically labile.

Finally, on the electrode surface, for each applied potential a current is measured which is proportional to the flux of species being reduced there. This flux is determined by both the electrochemical equilibrium (according to Nernst's law) and the rate of the electron transfer on the electrode surface and also by the abovementioned rate of transport of the electroactive species in the vicinity of the electrode. The fulfillment of Nernst's law combined with the transport by diffusion is responsible for the sigmoidal or peak shape of most voltammetric signals. Concerning the electron transfer, if the process is fast and can be easily produced in the opposite direction, such process is called reversible and, on the contrary cases, irreversible.

Then, it is clear that the resulting voltammetric signal is related to the concentrations in the bulk solution, but also that in many cases the existing relationship is not as straightforward as, for instance, in spectrophotometric measurements. To find this relationship, electrochemical hard modeling postulates theoretical models considering both the complexation in the bulk solution and the set of processes taking place in the diffusion layer and on the electrode surface. The mathematical resolution of every model produces equations containing a set of parameters, stability constants among them that can be fitted to experimental data.

The simplest case that can be studied by electrochemical hard modeling is that of a metal, which can be reversibly reduced, forming electroinactive inert complexes. Then, the signal of the free metal always appears at a fixed half-wave or peak potential with a peak or limiting current directly proportional to the concentration of the free metal ion, and this makes calculations very easy. If the complexes are electroactive, new signals appear at fixed half-wave or peak potentials (usually more negative than that of the free metal signal) with peak or limiting currents also being directly proportional to the concentrations of the respective complexes. In such a case, calculations are still easy as far as the signals can be measured individually, i.e., they do not overlap with each other or with the free metal signal. The presence of overlapping signals makes necessary the use of deconvolution techniques or, much better, soft-modeling approaches for multivariate curve resolution.

When the system includes a reversibly reduced metal ion and a set of successive labile complexes, a single reduction signal is observed which is shifted to more negative potentials as the ligand concentration increases and whose limiting or peak current can also be decreased if the diffusion coefficients of the complexes are considerably lower than that of the free metal ion. In the presence of a sufficient excess of the ligand, the method by De Ford and Hume (4) allows the calculation of the corresponding stability constants from the ratio between the limiting or peak current obtained for the metal in the presence ($I$) and in the absence of the ligand ($I_0$) and the shift in the half-wave or peak potential ($\Delta E$) caused by the addition of the ligand:

$$F_0 = \exp\left(-\frac{nF}{RT}\Delta E - \ln\frac{I}{I_0}\right) = 1 + \sum_i \beta_i (c_L)^i, \quad [1]$$

where $nF/RT$ equals 77,88 $V^{-1}$ for $n = 2$ and at 25°C, $F_0$ is the Leden function of order zero, $c_L$ is the bulk concentration of the ligand, and $\beta_i$ is the overall stability constant of the $ML_i$ complex. Although this method was initially developed for small-size ligands, the model by de Jong *et al.* (17–19) showed the validity of Eq. [1] for the systems including labile macromolecular complexes, excess ligand, and absence of adsorption phenomena. A further extension of the model (20–22) also proved the validity of the equation for mixtures of labile and inert complexes, including all the possible overall stability constants in the sum.

An interesting consequence of these facts is the possibility of taking into account the side reactions of both metal and ligand in the medium considered (as metal hydrolysis, ligand protonation, or metal complexation by the components of the buffer). In this case, Eq. [1] is still valid (21), but now $\beta_i$ are conditional stability constants that must be corrected by the corresponding side-reaction coefficients of both metal and ligand ($\alpha_M$ and $\alpha_L$, respectively). Moreover, it is possible to estimate voltammetrically the value of $\alpha_M$ by comparison

between the currents and potentials obtained for a metal solution in the absence of side reactions ($I_0$, $E_0$) and those measured for the same concentration of metal in the medium under study ($I_0'$, $E_0'$) (21):

$$\alpha_M \approx \exp\left( -\frac{nF}{RT}(E_0' - E_0) - \ln\frac{I_0'}{I_0}\right). \qquad [2]$$

The side-reaction coefficient for the protonation of the ligand cannot be determined voltammetrically, but it can be estimated from the protonation constants in the literature. For the particular case of GSH, this is given by the equation

$$\alpha_L = 1 + K_{p1}[H^+] + K_{p1}K_{p2}[H^+]^2 + K_{p1}K_{p2}K_{p3}[H^+]^3 + K_{p1}K_{p2}K_{p3}K_{p4}[H^+]^4 \qquad [3]$$

with the literature values of $\log K_{p1} = 9.53$, $\log K_{p2} = 8.64$, $\log K_{p3} = 3.48$, and $\log K_{p4} = 2.08$, measured at 25°C and at ionic strength of 0.15 mol liter$^{-1}$ (11).

A more problematic situation is found when the association–dissociation kinetics of the complexes is of the same order of the measurement rate. Then, from an electrochemical point of view, the complexes are neither inert nor labile. This still produces separate reduction signals for every species, but with half-wave or peak potentials which are not constant and limiting or peak currents not proportional to the bulk concentrations. Thus, the signal of the free metal is higher and moves to more negative potentials, compared to the inert situation, since it is produced not only by the reduction of the originally free metal ions but also by the metal ions coming from complex dissociation. In this particular case, there are no rigorous simple expressions to compute stability constants, but it has been shown that in some cases Eq. [1] can still produce reasonable results (23).

An additional important complication that can be encountered is the irreversible reduction of the metal and/or the complexes. This is caused by a slow electron transfer on the electrode surface, and it produces both a broadening and a shift (to more negative potentials) of the sigmoidal signals that preserve the same limiting currents that would have been observed in the reversible case. For peak-shaped signals, the irreversibility produces a shift of the peak toward more negative potentials, a decrease of the peak current, and an increase of the peak width (5). From the point of view of electrochemical hard modeling, the (usual) irreversible type of the inert complex signals is not a serious problem, but the irreversibility of the metal ion signal has dramatic consequences, since there are no simple methods to separate the irreversibility contribution from that of complexation on the observed potential shift and current decrease, so Eq. [1] is no longer valid.

*Soft Modeling (MCR-ALS)*

In contrast with electrochemical hard modeling, the soft-modeling MCR-ALS method does not impose any model for the metal complexation nor for the electrochemical processes involved in voltammetric measurements. Instead, it determines concentrations and pure signals for every electroactive species just by analysis of signal variation at different metal-to-ligand ratios. This method has been described in previous woks (13–15). The only hypothesis assumed is the linearity of the signals with respect to the concentration of the electroactive species. This means that the overall signal should be the sum of the signals that would be obtained for every species alone (pure signals) multiplied by the corresponding species concentration. As discussed before, this linearity is only warranted in electrochemically inert systems, but for some labile systems the signal is also close to linear (14).

For carrying out MCR-ALS, the individual voltammograms recorded at different metal-to-ligand ratios are arranged in a data matrix of currents ***I,*** with as many rows as recorded voltammograms at each metal-to-ligand ratio and as many columns as potentials scanned during the current measurements. First, a singular value decomposition (SVD) of matrix **I** is applied to determine the number of linearly independent contributions, i.e., the number of independent electroactive species linearly contributing to the current. Then, the data matrix **I** is decomposed as a product of a matrix **C** (containing the concentrations of every electroactive species) and a matrix **V** (containing the corresponding pure voltammograms) plus an error matrix **X** (13):

$$\mathbf{I} = \mathbf{CV} + \mathbf{X}. \qquad [4]$$

This matrix decomposition is carried out using an ALS optimization which imposes several restrictions (constraints) during the iterative process. The more important constraints are selectivity (only one component is present in a potential region or at a given metal-to-ligand ratio), nonnegativity (of concentrations and/or signals), unimodality (unimodal shape of concentration profiles and/or pure voltammograms), signal shape (fitting of pure voltammograms to a parametric equation which imposes a predetermined shape), and closure (the sum of the metal or ligand concentrations in some or all species is equal to a well-known value or a series of values). The relative error of the matrix decomposition is expressed as a percentage of lack of fit (lof), according to the equation

$$\text{lof} = \sqrt{\frac{\sum_{i,j} (a_{ij} - \hat{a}_{ij})^2}{\sum_{i,j} a_{ij}^2}} \cdot 100, \qquad [5]$$

where $a_{ij}$ are the experimental values of matrix **I** and $\hat{a}_{ij}$ are the corresponding calculated values.

Additional details of the MCR-ALS method (13) and its application to voltammetric data are described elsewhere (14, 15). MCR-ALS has been implemented using a MATLAB computer graphics environment program (24).

Concerning the MCR-ALS method, a critical point should be clearly understood for further interpretation of the results. During the MCR-ALS analysis of experimental data, a reduced number of components is resolved, and one of the basic assumptions is that these components are associated with the different chemical sources of data variation. In the interpretation of previous results (12, 15) it was proved that every resolved component by MCR-ALS was related to an electroactive site yielding an electrochemical signal and not necessarily to a pure chemical species. Thus, in some cases an electroactive site and a chemical species are coincident (for instance, free metal ions). However, in some other cases, an electroactive site can be inside a chemical species, or even two or more different electroactive sites can be included in the same chemical species (for instance, in metal clusters) (12, 15).

As a drawback, it must be mentioned that the need for linearity makes the MCR-ALS approach very sensitive to the inert/labile character of the systems (25). In contrast, MCR-ALS has the advantage of not requiring electrochemical models, which are not available or difficult to manage for the treatment of overlapping and/or irreversible signals and in intermediate situations between labile and inert complexation. An additional advantage is the possibility of working with poorly defined species, such as those of ill-defined macromolecular ligands, that cannot be treated by hard-modeling approaches.

### *Chemical Hard Modeling (SQUAD)*

The chemical hard-modeling approach is intermediate between the hard-electrochemical and the soft-modeling approaches. As the first, it assumes a chemical model (i.e., a certain number of species with their stoichiometries) but, concerning electrochemistry, it only imposes the hypothesis of linearity between concentrations and currents (the same as in soft modeling). In SQUAD, a set of postulated stability constants are optimized using a nonlinear least-squares curve-fitting approach. From these stability constants, mass-action law, and mass-balance equations, matrix **C** is first estimated. Then matrix **V** is calculated by linear least squares from **I** and **C** matrices and the process is repeated (see Ref. 16). Thus, the data matrix decomposition is similar to that of MCR-ALS but imposes the fulfillment of the mass-action law associated to every species and the mass-balance equations for the metal and the ligand. In this way, the values of the (postulated) stability constants are optimized by means of an iterative procedure. It is important to notice that, in the presence of side reactions, the stability constants determined are, in fact, conditional constants that can be corrected by using the corresponding side-reaction coefficients.

In the present work, a FORTRAN version of the least-squares curve-fitting program SQUAD (stability quotients from absorbance data) has been applied (16). Since SQUAD is almost exclusively used for spectrophotometric data, the introduction of voltammetric data implies the treatment of potentials as wavelengths and the treatment of currents as absorbances. In this analogy, the pure spectra (molar absorptivities) would be equivalent to the pure voltammograms. Lack of fit can be calculated in the same way as for MCR-ALS.

As in soft modeling, the requirement of linearity can make chemical hard modeling very sensitive to the inert/labile character of the complexes but the absence of electrochemical restrictions favors, in principle, the treatment of overlapping and/or irreversible signals.

## MATERIALS AND METHODS

### *Materials*

Reduced GSH (>99% iodometric purity, confirmed by both elemental analysis and HPLC with spectrophotometric detection), $KNO_3$, $Na_2B_4O_7 \cdot 10\ H_2O$, $Zn(NO_3)_2 \cdot 4H_2O$, $HNO_3$, and NaOH were of analytical grade supplied by Merck. The reagents were dissolved in ultrapure water obtained from a Milli-Q Plus 185 attached to a RiOS Elix3 system (Millipore). DPP measurements were performed in an Autolab system (Eco-Chemie, The Netherlands) attached to a Metrohm 663 VA stand (Metrohm, Switzerland) and a personal computer with GPES3 data-acquisition software (EcoChemie). For the automatic additions of the GSH solution, the system was also connected to a Metrohm 665 dosimat. All experiments were carried out in a glass cell at room temperature (25°C) under a purified nitrogen atmosphere. The working, reference, and auxiliary electrodes were the static mercury drop electrode (SMDE), with a drop area of 0.40 $mm^2$, Ag/AgCl, KCl (3 M), and glassy carbon, respectively. A pulse time of 40 ms, pulse amplitude of 50 mV, drop time of 0.6 s, and step potential of 4 mV were used.

*Methods*

Twenty-five milliliters of freshly prepared 0.15 mol liter$^{-1}$ borate buffer at pH 8.5 was placed in the voltammetric cell. The solution was deaerated with pure nitrogen for 20 min and the DPP polarogram registered. An aliquot of the standard $Zn^{2+}$ solution was added to the cell to obtain a $1 \times 10^{-5}$ mol liter$^{-1}$ solution. After deaeration for 1 min, with mechanical stirring, a new DPP curve was recorded. Then successive aliquots of the standard GSH solution (oxygen-free, fresh, and containing the same borate buffer solution) were added to the cell in order to obtain different GSH-to-$Zn^{2+}$ concentration ratios until a large excess of GSH was reached. All these solutions were deaerated with pure nitrogen and stirred for 1 min after each addition and before recording the respective DPP polarogram. For determining the $Zn^{2+}$ side-reaction coefficients, an acidic solution (pH 4.5) of $1 \times 10^{-5}$ mol liter$^{-1}$ $Zn^{2+}$ in 0.15 mol liter$^{-1}$ $KNO_3$ was also measured.

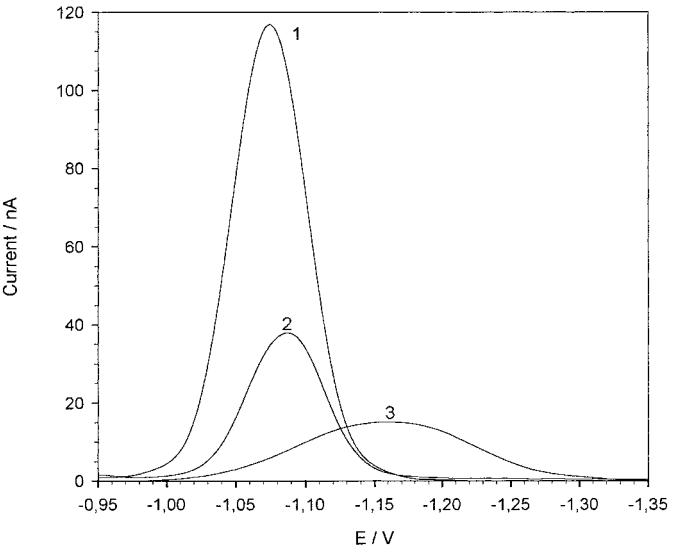


**FIG. 1.** DPP voltammograms of $1 \times 10^{-5}$ mol liter$^{-1}$ $Zn^{2+}$ solution in (1) 0.15 mol liter$^{-1}$ $KNO_3$ at pH 4.5, (2) 0.015 mol liter$^{-1}$ borate buffer at pH 8.5, and (3) 0.15 mol liter$^{-1}$ borate buffer at pH 8.5.

## RESULTS AND DISCUSSION

### *$Zn^{2+}$ Hydrolysis and Irreversibility of the Reduction Process*

As pointed out in the introductory paragraphs, two main experimental problems arise in the study of the $Zn^{2+}$–GSH system, as they can appear in many other metal–ligand systems: the hydrolysis of $Zn^{2+}$ (or the corresponding metal ion) and the losses of electrochemical reversibility of the measured signals. As a consequence, the proper choice of the buffer solution (composition, concentration, and pH) and the total metal concentration are critical in order to obtain a reliable picture of the metal–ligand system from voltammetric data.

Figure 1 illustrates these phenomena. DPP polarogram 1 was obtained for a $Zn^{2+}$ solution in a $KNO_3$ salt medium at pH 4.5. This acidic pH prevents the formation of hydroxo complexes (26) and the $KNO_3$ medium allows the system to be electrochemically reversible, as proved by the half-peak width of 62 mV, in good agreement with the usual reversibility criteria for DPP (5). Signal 2 in Fig. 1 was obtained for a $Zn^{2+}$ solution at pH 8.5 in 0.015 mol liter$^{-1}$ borate buffer. Under these conditions the reduction of $Zn^{2+}$ is still reversible (half-peak width of 62 mV), but there is a decrease of the peak current and a shift of the peak potential to the negative direction compared to signal 1. This fact is due to the formation of $Zn^{2+}$ hydroxo complexes at pH 8.5 (26): those that are soluble and labile are responsible for the potential shift, whereas those that are inert or insoluble cause the current decrease. Taking into account that neither nitrate nor borate complexes $Zn^{2+}$, the comparison of polarograms 1 and 2 by means of Eq. [2] provides an estimate of the side-reaction coefficient for the hydrolysis of $Zn^{2+}$. Finally, when $Zn^{2+}$ is at pH 8.5 in 0.15 mol liter$^{-1}$ borate buffer, the DP polarogram (signal 3) suffers an important current decrease, a peak broadening (the half-peak width is increased up to 147 mV), and a large shift to more negative potentials compared to signal 1. A small part of the current decrease and the potential shift is due to the same side reactions of hydrolysis taking place in the case of signal 2 (the value of $\alpha_M$ should be practically identical). Nevertheless, the majority of the current decrease and the potential shift, jointly with the increase of the peak width, are caused by the fact that the $Zn^{2+}$ reduction becomes irreversible (2, 4, 5). It must be pointed out that the $Zn^{2+}$ ion has an intermediate electron transfer rate at mercury electrodes, so that small changes in the electric structure of the double layer around the electrode can modify the $Zn^{2+}$ reduction from reversible to irreversible. Among the factors that can modify such behavior, the nature and concentration of the supporting electrolyte (and/or the buffer) seem to play a key role (27), which explains the change of reversibility when increasing the borate concentration from 0.015 to 0.15 mol liter$^{-1}$.

Borate medium has been chosen because no evidence on the complexation of Zn(II) by borate is reported on the stability constants tables and compendia. Different buffer solutions were tested in order to choose the best medium for working at very negative potentials (ca. $-1.3$ V), as is the case when Zn complexes are involved. From those tested, borate buffer was idoneous, allowing a linear relationship peak current vs $Zn^{2+}$ concentration inside a wide range (0–60 $\mu$M) at pH 8.5 and

absence of electrochemical interferent signals in the region of potentials considered.

*Analysis of the Zn–GSH System*

From these considerations, it is interesting to find experimental conditions that, being close to those required (for instance, the physiological ones), would allow the system to be reversible and free from metal hydrolysis. Unfortunately, for the Zn–GSH system it is not possible to prevent hydrolysis completely, since at low pH values GSH hardly complexes $Zn^{2+}$ (6).

Although interactions between glutathione and borate cannot be fully refused, previous studies on glutathione (12) and ($\gamma$-Glu-Cys)$_2$-Gly and ($\gamma$-Glu-Cys)$_3$-Gly (28) under voltammetric conditions indicate that relevant borate interactions with glutathione are not observed.

With respect to the electrochemical reversibility, several studies were done, which are summarized in Fig. 2. At low concentrations of borate buffer, reversible $Zn^{2+}$ signals are obtained (Fig. 2a) but the evolution of the polarograms suggests that the association–dissociation kinetics of the complexes is intermediate between inert and labile behavior. In fact, only two signals are obtained, which do not seem to be the sum of simpler peaks (the half-peak widths remain practically constant), and they can be related in principle to the reduction of free $Zn^{2+}$ and of an inert $Zn^{2+}$–GSH complex. Nevertheless, the progressive potential shift (toward more negative values) of the $Zn^{2+}$ peak evidences that such a signal contains a nonnegligible contribution of $Zn^{2+}$ coming from complex dissociation. This phenomenon can be explained in different ways. For instance, formation of a labile 1:1 complex (responsible for the potential shift of the $Zn^{2+}$ peak) and an inert 1:2 Zn–GSH complex (reducing the more negative peak and responsible for the decrease of the first peak because of the formation of the 1:2 complex when GSH is added) would be possible. In this case, the electrochemical hard modeling of Eq. [1] would be fully applicable to the first peak, whereas both soft and chemical hard modeling could be affected to some degree by the lack of linearity caused by labile complexation. Nevertheless, alternative explanations are also possible. Then, any of the formed complexes could have an intermediate behavior between inert and labile, thus being partially reduced in the first peak (the fraction of metal dissociated during measurement) and partially reduced in the second peak (the undissociated complex fraction). If this happens, all three modeling approaches can be affected by a significant error.

Under other experimental conditions (especially for higher $Zn^{2+}$ concentrations), $Zn^{2+}$ reduction is initially not reversible but its reversibility is progressively increased as GSH concentration increases. This is evidenced by the decrease of the signal width and of the shift of the peak toward more positive potentials (Fig. 2b). Taking into account that both proposed soft-modeling and chemical hard-modeling approaches are only possible if the pure signals remain at a constant potential along the experiments, this situation does not seem a priori very appropriate for any of them.

In contrast, Fig. 2c shows the DPP polarograms obtained under conditions similar to those used in the previous study (12), with a high concentration of borate buffer and a low $Zn^{2+}$ concentration that ensure a fully inert behavior of the complexes. Then, the $Zn^{2+}$ signal is not reversible but maintains its shape along the whole experiment. As pointed out under Theory, the nonreversibility of $Zn^{2+}$ prevents the use of electrochemical hard modeling but this is not a problem for MCR-ALS nor SQUAD, which only require constant and additive pure signals (which is fulfilled by inert complexes) and do not impose any restriction about the shape or position of voltammetric signals.

SVD analysis of these data matrices indicates in all cases the presence of three major components (Fig. 3), i.e., three different types of $Zn^{2+}$, in good agreement with previous results (12). This implies that, in principle, all systems behave close to linearly (an important deviation from the linear behavior would result in a larger number of components). Note, however, that the data set of Fig. 2b yields a second singular value that is lower and more similar to the third value than in the other two cases. This suggests that in such an experiment it would be more difficult to distinguish the contributions of the second and third factors.

The application of electrochemical hard modeling is only possible for the data set shown in Fig. 2a (the only one having a $Zn^{2+}$ signal reversible and not overlapped). Figure 4 shows the experimental $F_0$ values obtained as a function of the total ligand concentration and the curve fitted according to Eq. [1] (taking into account only the values with enough excess ligand). The best fitting was obtained with a polynomial of degree 2, thus producing (conditional) stability constants for the 1:1 and 1:2 complexes, which are shown in Table 1. It must be mentioned that the DeFord and Hume method does not consider polynuclear complexes, so it is not possible to compute the stability constant of the hypothetical 2:2 complex.

With respect to soft modeling, Fig. 5 compares the concentration profiles (matrix **C**) and the pure voltammograms (matrix **V**) obtained in the MCR-ALS decomposition of all three data sets given in Figs. 2a, 2b, and 2c when the constraints of selectivity (no complexation of the metal in the absence of ligand), nonnegativity (for concentrations and signals), and closure (total metal concentration is practically constant and equal to the value in the absence of ligand) are applied. For all three data sets, the lack of fit achieved was very

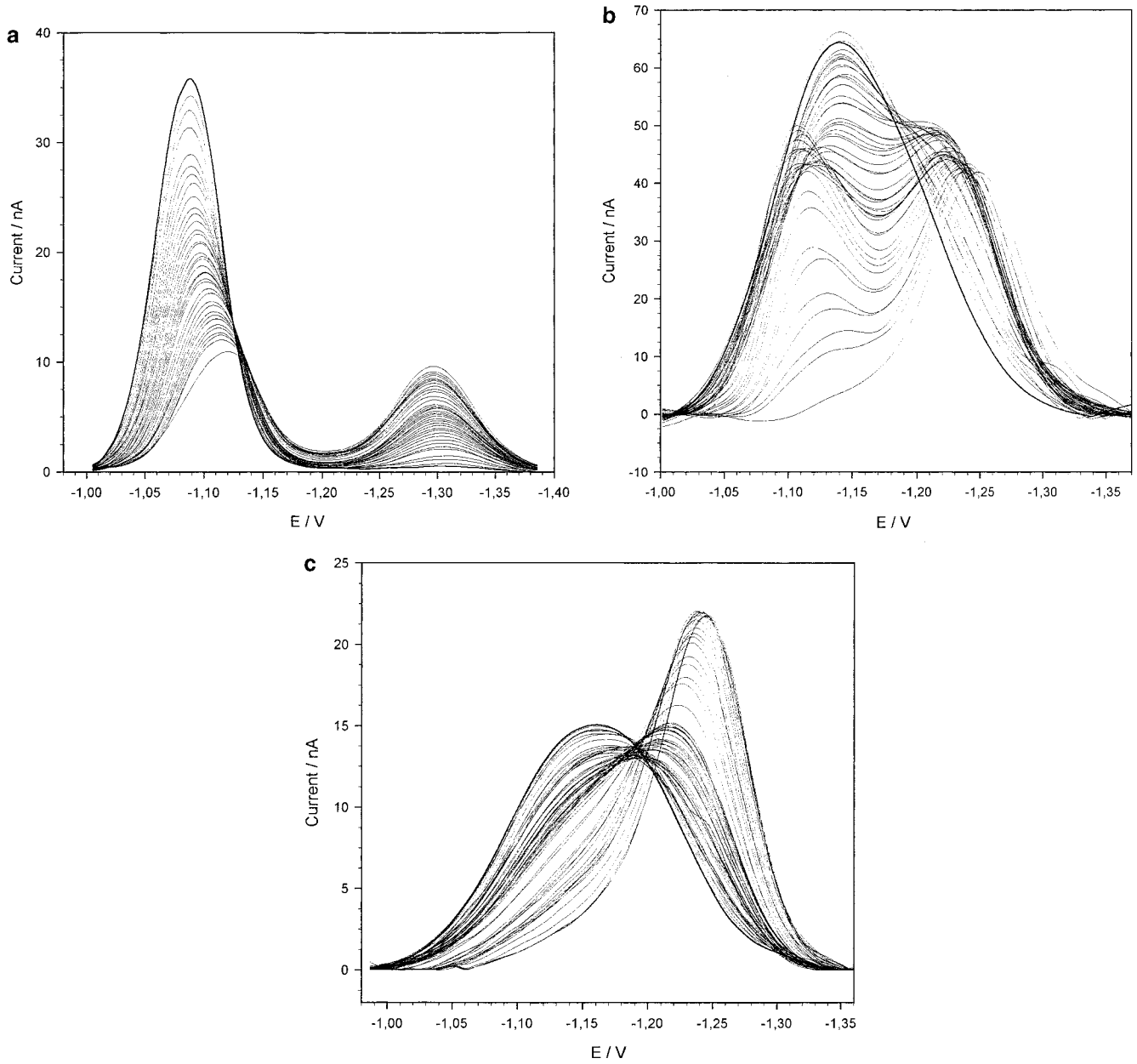


**FIG. 2.** DPP data sets for the titration of a $Zn^{2+}$ solution with GSH at pH 8.5 under the conditions: (a) $1 \times 10^{-5}$ mol liter$^{-1}$ $Zn^{2+}$ and 0.015 mol liter$^{-1}$ borate buffer, (b) $5 \times 10^{-5}$ mol liter$^{-1}$ $Zn^{2+}$ and 0.15 mol liter$^{-1}$ borate buffer, and (c) $1 \times 10^{-5}$ mol liter$^{-1}$ $Zn^{2+}$ and 0.15 mol liter$^{-1}$ borate buffer.

good (1.48, 2.15, and 3.92% for matrices in Figs. 2a, 2b, and 2c, respectively), which confirms a reasonable linearity of the data in the three cases. It is important to notice that MCR-ALS decomposition does not use any chemical model or stoichiometry. Use of a closure constraint equal to the total metal concentration gives **C** and **V** matrices that contain concentrations and currents per mole of $Zn^{2+}$. Further transformation of such concentrations and voltammograms into actual species concentrations requires the assumption of a particular stoichiometry.

When analyzing the shape of the pure voltammograms, many double peaks are found, especially in Figs. 5B and 5D. As pointed out before, a description of the system where every factor corresponds to a single electrochemical process implies that every unit signal should be a single peak. For this purpose, MCR-ALS has been applied with an additional unimodality con-

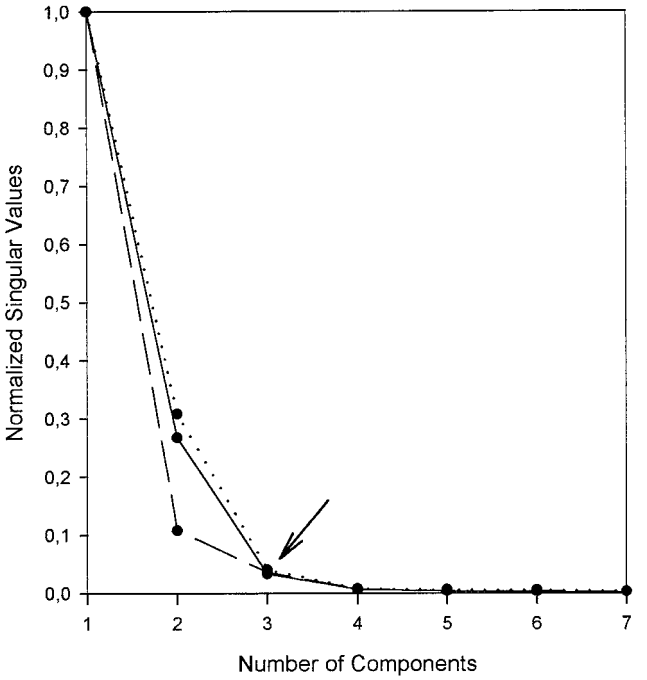


**FIG. 3.** Singular value decomposition (SVD) plot for the data sets in Figs. 2a (—), 2b(- - -), and 2c (· · ·). Singular values are normalized to maximum value (first singular value) equal to 1.

straint for the voltammograms (and, eventually, for the concentration profiles). In the analysis of the data set of Fig. 2c, this produces slight changes in the concentration profiles (not shown) with a minor increase of the error (from 3.92 to 4.06%). Thus, the system appears to behave linearly even in terms of separate electrochemical processes. In contrast, the use of unimodality hinders a reasonable MCR-ALS fitting of the data shown in Figs. 2a and 2b (divergence, large errors, or unrealistic concentration profiles are encountered), which evidences the impossibility of a linear description of such systems in terms of single unimodal signals relating each of them to an electrochemical process.

For using chemical hard modeling with SQUAD, two alternative chemical models are proposed: model I considers the presence of M and complexes ML and $ML_2$, with overall stability constants $\beta_{11}$ and $\beta_{12}$, respectively, whereas model II considers species M, $M_2L_2$, and $ML_2$, with constants $\beta_{22}$ and $\beta_{12}$. These stability constants are defined as

$$\beta_{11} = c_{ML}/(c_M\, c_L) \quad [6]$$

$$\beta_{22} = c_{M2L2}/(c_M^2\, c_L^2) \quad [7]$$

$$\beta_{12} = c_{ML2}/(c_M\, c_L^2). \quad [8]$$

The application of SQUAD to the three data matrices produces quite different results: the iterative process does not converge for the matrices in Figs. 2a and 2b, whereas a good fit is obtained for the matrix in Fig. 2c (lof = 3.70% for both models I and II). The stability constants obtained for both complexation models are shown in Table 1. As expected, analysis of these data does not allow one to decide if the intermediate complex is 1:1 or 2:2.

It is interesting to note that despite the good result of MCR-ALS decomposition without unimodality, analysis of the data sets in Figs. 2a and 2b by SQUAD (which does not use unimodality either) was not successful. This means that the concentrations obtained by MCR-ALS cannot be accurately related through equilibrium constants. This could be because of unresolved rotational ambiguities (13) (the concentrations obtained for some of the species are linear combinations of their actual values giving similar data fits). It could be also possible that one of the species involved in the equilibrium becomes electroinactive or precipitates and, hence, is not properly considered in the postulated solution equilibrium. Finally, and more probable, SQUAD could be more sensitive to small departures from linearity than MCR-ALS. Regardless, it is clear that the **C** and **V** matrices obtained by soft modeling for systems of Figs. 2a and 2b are a good mathematical solution but they are an unrealistic (electro)chemical solution since they do not follow the mass-action law nor a scheme of single electrochemical processes.

Figure 6 compares, for each chemical model, the concentration profiles and the pure voltammograms

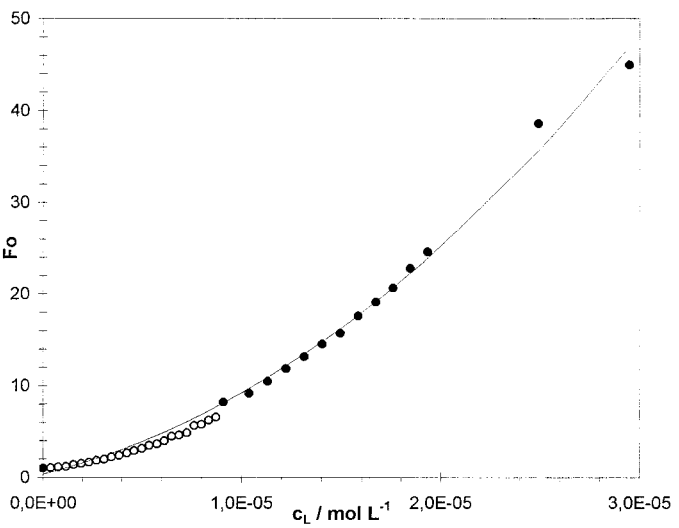


**FIG. 4.** Values of the $F_0$ Leden function computed from the data in Fig. 2a as a function of the total ligand concentration added ($c_L$). Symbols indicate experimental points, whereas the solid line corresponds to the curve fitted to a second-degree polynomial according to Eq. [1] (considering only the points marked in black).

**TABLE 1**

Logarithms of the Conditional Stability Constants (Log $\beta'$) Obtained for the Zn–GSH Complexes Assuming Model I (M, ML, and $ML_2$) and Model II (M, $M_2L_2$, and $ML_2$)

| Experimental conditions[a] | Chemical model | Calculation method | lof (%) | Conditional stability constants | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Log $\beta'_{11}$ | SD | Log $\beta'_{22}$ | SD | Log $\beta'_{12}$ | SD |
| 1 | I | Eq. [1][b] | — | 5.72 | (0.11) | — | — | 10.55 | (0.05) |
| 2 | I | MCR-ALS | 3.69 | 4.87 | (0.04) | — | — | 8.40 | (0.20) |
| | | SQUAD | 3.70 | 4.69 | (0.01) | — | — | 8.56 | (0.02) |
| 2 | II | MCR-ALS | 3.69 | — | — | 14.82 | (0.16) | 8.40 | (0.20) |
| | | SQUAD | 3.70 | — | — | 14.69 | (0.02) | 8.83 | (0.02) |

*Note.* The corresponding standard deviations are also given (SD), as well as the lack of fit (lof) achieved by MCR-ALS and SQUAD.
[a] 1: $Zn^{2+}$, $1 \times 10^{-5}$ mol liter$^{-1}$, 0.015 mol liter$^{-1}$ borate buffer, pH 8.5; 2: $Zn^{2+}$, $1 \times 10^{-5}$ mol liter$^{-1}$ 0.15 mol liter$^{-1}$ borate buffer, pH 8.5.
[b] Electrochemical hard modeling (DeFord–Hume method).

obtained in the analysis of experimental data of Fig. 2c by SQUAD and by MCR-ALS without unimodality (for a better comparison with SQUAD, where unimodality cannot be imposed). All these curves are expressed in terms of chemical species concentrations and not per atom of Zn as before (Fig. 5). As can be seen, there is good agreement between the results of both approaches for the two postulated chemical models, and the lack of fit is very similar for both approaches (MCR-ALS and SQUAD). It must be noted that SQUAD is able to optimize the log $\beta'$ values along the whole range of ligand concentrations, whereas analysis of MCR-ALS concentration profiles allows only a rough calculation point by point at restricted concentration ranges. As shown in Table 1, the stability constants determined by MCR-ALS (in the above-mentioned manner) are of the same order as those obtained by SQUAD, but quite less precise.

Table 2 shows the values of the overall stability constants obtained by correcting the conditional $\beta'$ constants for the side reactions of $Zn^{2+}$ with $OH^-$ ions and those of GSH with $H^+$ ions according to

$$\log \beta_{11} = \log \beta'_{11} + \log \alpha_M + \log \alpha_L \quad [9]$$

$$\log \beta_{22} = \log \beta'_{22} + 2 \log \alpha_M + 2 \log \alpha_L \quad [10]$$

$$\log \beta_{12} = \log \beta'_{12} + \log \alpha_M + 2 \log \alpha_L, \quad [11]$$

where $\alpha_M$ and $\alpha_L$ are the side-reaction coefficients of $Zn^{2+}$ and GSH, computed according to Eq. [2] (by comparison of an acidic $KNO_3$ blank and a borate blank at pH 8.5) and Eq. [3], respectively.

As Table 2 indicates, the application of electrochemical hard modeling to DPP data obtained at 0.015 mol liter$^{-1}$ borate (Fig. 2a) produces reasonable stability constants which are of the same order as those in the literature, although somewhat lower. These results are quite good taking into account, on one hand, that Eq. [1] is not strictly applicable (since there is no warranty for fully inert and fully labile behavior of the complexes) and, on the other hand, that the experimental conditions are not strictly the same (by comparing the literature values, it seems that there is an important variability of such constants with respect to the temperature and the ionic strength). Moreover, it must be pointed out that it is not clear whether the DPP signals of the complexes are sensitive to the "single" ML (or $M_2L_2$) and $ML_2$ complexes or to their "overall" concentration, including contributions from protonated or hydroxylated forms. In this last situation, the (overall) stability constants determined would be an average of different stability constants and would not be comparable to the $\beta_1$ and $\beta_2$ values in the literature.

As expected, SQUAD or MCR-ALS cannot distinguish from the two models (due to the same lack of fit) and the constants for the two possible situations are given in Tables 1 and 2. It must be mentioned that structural considerations about the different possible intermediate complexes suggested that model II including the $M_2L_2$-type complex is more reliable than model I (12, 29, 30). Regardless, additional experiments by complementary structural techniques are required to fully confirm this point.

## CONCLUSIONS

The results obtained so far indicate that the soft-modeling MCR-ALS method and the chemical hard-modeling SQUAD method can be complementary and very useful for the voltammetric study of metal–peptide complexes. This is especially true when some experimental problems (quite usual in this kind of systems) hinder an appropriate application of the classical methodologies of electrochemical hard modeling. Among these problems, the overlapping of signals, the intermediate association–dissociation kinetics of the complexes, metal ion hydrolysis, and the losses of elec-

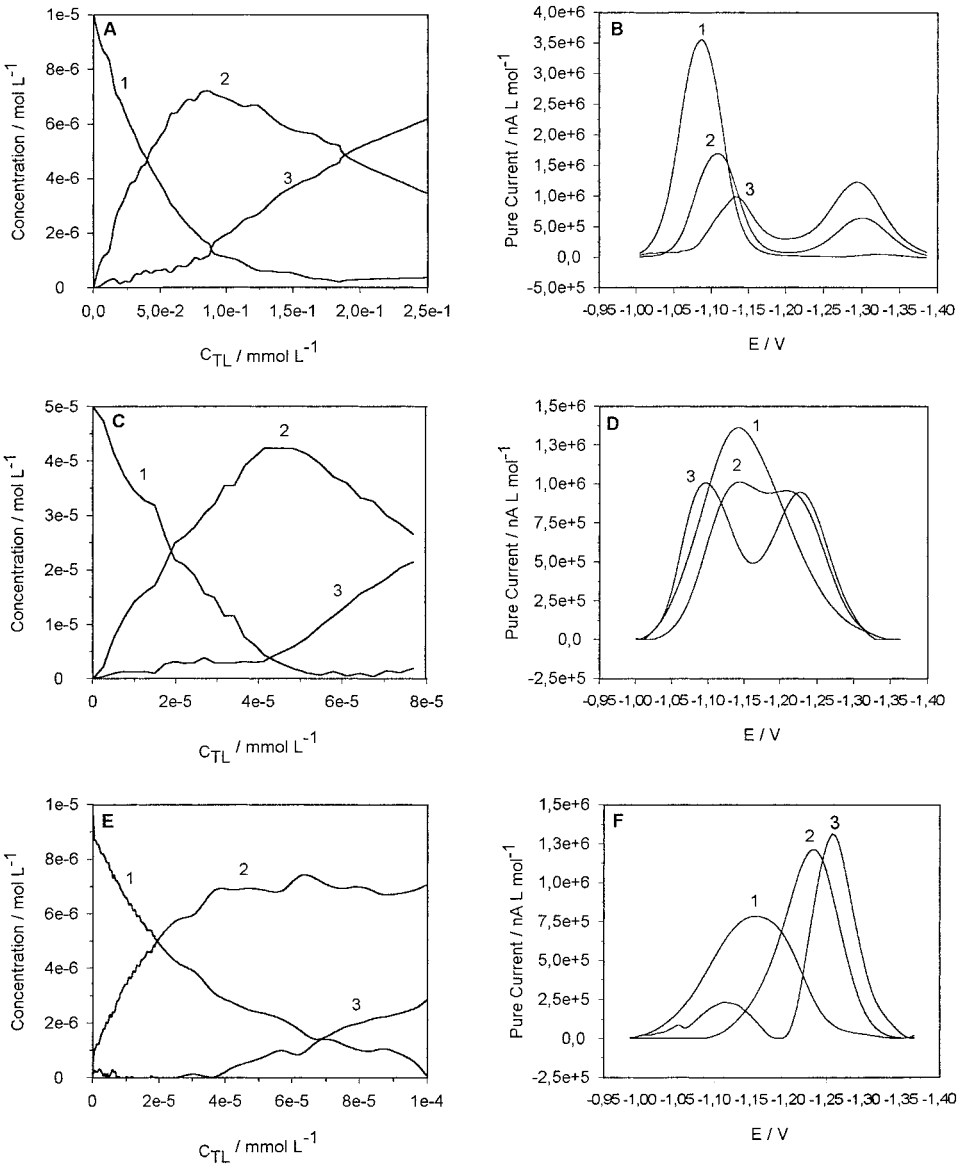


**FIG. 5.** Concentration profiles (left) and pure voltammograms (right) obtained in the MCR-ALS decomposition of the data matrices shown in Fig. 2 by imposing selectivity, nonnegativity, and closure constraints in ALS optimization. In the figure, couples A and B, C and D, and E and F correspond to data from Figs. 2a, 2b, and 2c, respectively.

trochemical reversibility of the metal reduction can be mentioned.

As voltammetric signals are not always linear with respect to the concentration, SVD and MCR-ALS are good tools to detect if such linearity exists. Linear systems provide a good lack of fit in both the SVD and ALS decompositions with a number of components equal to the number of chemical species. In addition, soft-modeling MCR-ALS yields concentration profiles and pure voltammograms that are a good description of the evolution of the species along the experiment.

In some cases it is not possible to improve such a semiquantitative description of the system by MCR-ALS because the concentration of some species is not well defined. If all species can be related through equilibrium constants, the determination of such constants from MCR-ALS concentration profiles can be somewhat inaccurate. Then, MCR-ALS results can be refined by assuming a chemical model and imposing the fulfillment of the mass-action law. This is what can be obtained by using computer programs like SQUAD, which have been initially designed for obtaining stability constants from spectroscopic data. In this approach, voltammetric data behave in a linear manner, as it happens for absorbances in Beer's law, and the total current can be expressed as the sum of the con-

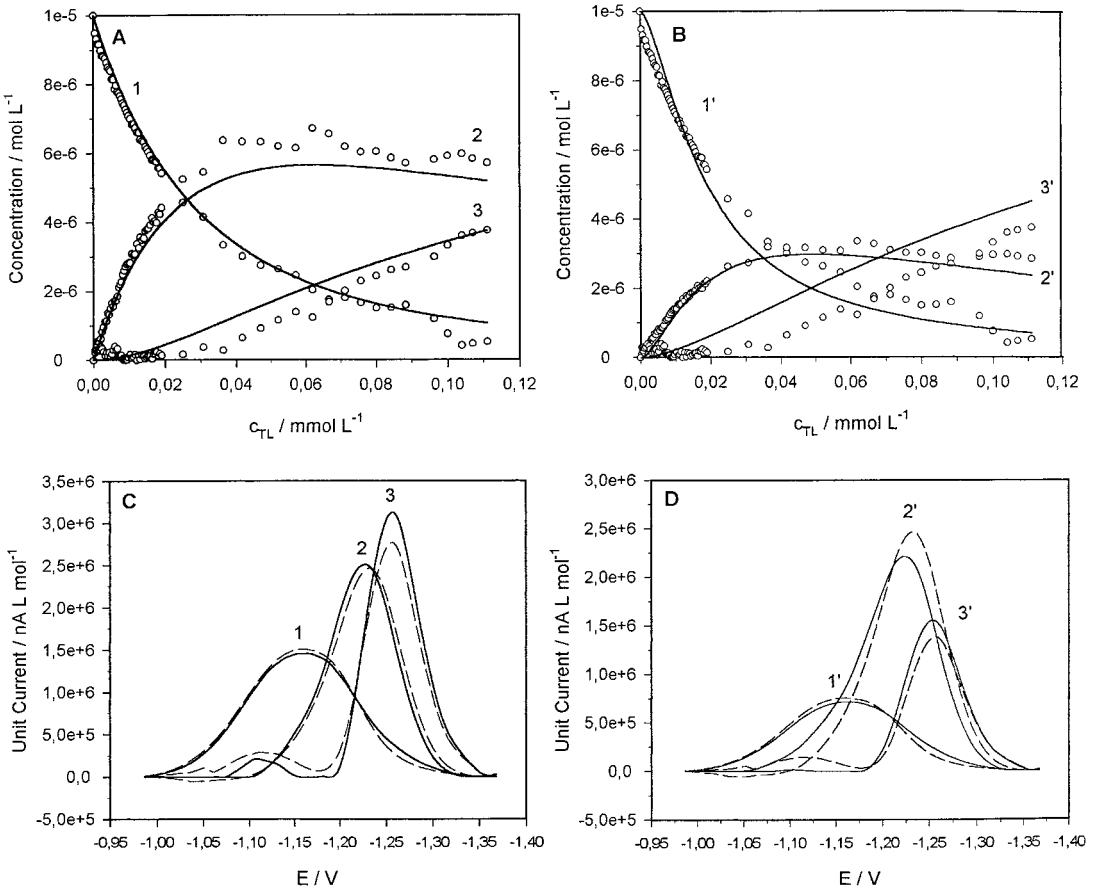


**FIG. 6.** Concentration profiles obtained by the application of MCR-ALS (○) and SQUAD (—) and pure DPP voltammograms by MCR-ALS (- - -) and SQUAD (—) assuming model I (A and C) and model II (B and D). Curve 1 corresponds to free $Zn^{2+}$ and curves 2 and 3 to complexes ML and $ML_2$, respectively, while curve 1′ corresponds to free $Zn^{2+}$ and curves 2′ and 3′ to complexes $M_2L_2$ and $ML_2$, respectively. Data analyzed are those from Fig. 2c ($1 \times 10^{-5}$ mol liter$^{-1}$ $Zn^{2+}$ and 0.15 mol liter$^{-1}$ borate buffer).

centration of every species multiplied by its characteristic current per unit of concentration (the equivalent of the molar absorptivity).

The application of the proposed methodologies to the $Zn^{2+}$–GSH system made possible an accurate determination of the $\beta_{12}$ stability constant for the 1:2 complex

**TABLE 2**

Logarithms of the Overall Constants (Log $\beta$) of the Zn–GSH Complexes Obtained after Correction of the Values of Table 1 for Side Reactions

| Experimental conditions[a] | Chemical model | Calculation method | Stability constants | | |
|---|---|---|---|---|---|
| | | | Log $\beta_{11}$ | Log $\beta_{22}$ | Log $\beta_{12}$ |
| 1 | I | Eq. [1] | 7.7 | — | 13.9 |
| 2 | I | MCR-ALS | 6.9 | — | 11.8 |
| | | SQUAD | 6.7 | — | 12.0 |
| 2 | II | MCR-ALS | — | 18.8 | 11.8 |
| | | SQUAD | — | 18.7 | 12.2 |
| 3 | I | Ref. (9) | 8.57 | — | 13.59 |
| 4 | I | Ref. (10) | 7.98 | — | 12.5 |

*Note.* Data taken from Refs. (9 and 10) are also given for comparative purposes.

[a] 1: 25°C, 0.015 mol liter$^{-1}$ borate buffer, DPP; 2: 25°C, 0.15 mol liter$^{-1}$ borate buffer, DPP; 3: 25°C, 3 mol liter$^{-1}$ $NaClO_4$, potentiometry with glass electrode; 4: 37°C, 0.15 mol liter$^{-1}$ $NaClO_4$, potentiometry with glass electrode.

(quite independent of the chemical model) and reasonable estimates of $\beta_{11}$ and $\beta_{22}$ for the 1:1 and 2:2 complexes, depending on the chemical model considered.

## ACKNOWLEDGMENTS

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