ProMCC: An all-in-one tool for trace metal complexation studies

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A B S T R A C T
A new windows-based, user friendly program (ProMCC) for (i) the determination of metal complexation parameters (ligand concentration (L) and conditional stability constants (K′)) and for (ii) theoretical simulation of metal complexometric titration, assuming discrete ligand model, is developed. Although primarily intended for treatment of experimental data obtained either by anodic stripping voltammetry (ASV) or competitive ligand exchange adsorptive cathodic stripping voltammetry (CLE-AdCSV), it could manage titration-type data of other techniques (e.g. ISE, sorption isotherm). Currently, the program is capable to process (fit and/or simulate) titration data up to three discrete ligand classes. Procedure for adjustment of “true” analytical sensitivity incorporated in ProMCC was found to provide reasonably good estimates of sensitivity either for one-ligand or two-ligand system. The particular feature of ProMCC is that it incorporates two complementary fitting methodologies: (1) a non-linear fitting of conventional linearized transformations (e.g. Rutić/Van Den Berg, Langmuir/Gerringa) and (2) a “complete complexation model” — a matrix based optimization of mass balance equations. Comparison test of different non-linear fitting modes and titration types revealed that a slight underestimation of ligand concentration and overestimation of conditional stability constant may occur if titration is performed in logarithmic mode, mainly due to unfavorable noise distribution. An advantage of implemented “complete complexation model” fitting mode is that it allows simultaneous analysis of titrations obtained at multiple detection windows as unified dataset (multi-detection window approach), providing complexation parameters for up to three ligand classes. A new alternative “RAL-approach” in analyzing complexometric titrations obtained at multiple detection windows for copper–salicylaldoxime (Cu–SA) system is suggested. It assumes that the analytical sensitivity is changing along the titration curve respecting the true speciation of Cu–SA in sample. An adapted empirical equation for calculation of relative intensity (RAL) is proposed. Flexibility in adjusting parameters, immediate graphical feedback and visualizations make ProMCC handy for treatment of large set of experimental data, and a tool for research in refinement of the methods of metal complexing capacity determination which is continuously improving.

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1. Introduction

It is well recognized that the speciation of trace elements in natural waters is a key factor in understanding their reactivity, toxicity, bioavailability and/or toxicity for micro-organisms (Bruland et al., 1991; Gledhill and Buck, 2012; Hirose, 2007). In oceanographic community, a research focus is directed toward elements (mainly metals) having known biological function (e.g. Fe, Cu, Zn, Co) (Baars and Croot, 2011; Bruland et al., 2000; Buck et al., 2012; Ellwood and Van Den Berg, 2001; Gledhill and Buck, 2012; Ibisammi et al., 2011; Thuroczy et al., 2010; Town and Filella, 2000; Van Den Berg and Dharmvani, 1984). While the inorganic speciation of elements is known and predictable, still the challenging task is understanding of metal vs. natural organic matter (NOM) interactions (either of mainly terrestrial origin (coastal regions) or autochthon one produced in water column). The great efforts are particularly directed toward understanding of Fe speciation, due to its role in primary production in oceans (Gledhill and Buck, 2012). In spite of low solubility in marine waters (~0.01 nM) (Liu and Millero, 2002), dissolved Fe concentration could exceed this limit mainly because of Fe complexation with organic ligands, producing complexes with high stability constants (Boyd and Ellwood, 2010; Boye et al., 2010; Cullen et al., 2006; Hunter and Boyd, 2007). While in open ocean conditions, biologically derived ligands (e.g. siderophores, exopolysaccharides) are found to largely control Fe speciation (Barbeau et al., 2003; Hassler et al., 2011; Hirose, 2007; Hunter and Boyd, 2007; Velasquez et al., 2011), complexation with humic material may dominate in coastal regions (Batchelli et al., 2010; Laglera et al., 2007; Laglera and Van Den Berg, 2009). From the other side, Cu is a micronutrient at its low concentration levels, while it can be toxic for some micro-organisms at higher concentrations (Duran and Beiras, 2013; Sunda et al., 1987). The range of the “optimal” Cu concentrations is relatively narrow. For both elements (Fe, Cu) it is reported that in oceans they exist predominantly in a form of strong organic complexes.

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Understanding the speciation of trace elements at their ambient concentration in the ocean and predicting their speciation in relation to increased concentrations (e.g. due to anthropogenic pollution) or change of physico-chemical conditions (e.g. ocean acidification/climate change) are the main interests of complexation studies (Hirose, 2006; Millero et al., 2009). The main purpose of metal speciation analysis, i.e. the determination of complexation parameters, is to estimate (I) the metal speciation at actual concentration of the natural sample and (II) the ability of the “sample” to complex metals at their increased concentration (to predict the metal speciation).

Although different methods and protocols of direct characterization of metal–organic complex are reported in literature (Wiramadjen et al., 2008), due to very low concentration of metals in seawater and experimental limitations of separation, extraction and measurement of different metal complexes, an alternate indirect approach in characterization of metal–organic ligand interactions is usually practiced by marine chemists (Bruoland et al., 2000; Buck et al., 2012; Campos and Van Den Berg, 1994; Capodaglio et al., 1995; Gerringa et al., 1995; Louis et al., 2009a; Monticelli et al., 2010; Omanović et al., 1996; Plavšić et al., 2009; Ružić, 1982; Van Den Berg, 1982). It is based on the titration of the sample by the target metal at natural pH. Upon addition, metal is redistributed between different species, among them one (or group) of them is used as an “active” component for measurement, whereas the rest is considered to represent undetectable organic complexes. The most utilized technique for quantification of “active” components is electrochemical techniques due to their good sensitivity and selectivity. Basically two methods/protocols exist: anodic stripping voltammetry (ASV) and competitive ligand exchange adsorptive cathodic stripping voltammetry (CLE-AdCSV).

There are two distinct concepts in ASV methodology. The first assumes that after the addition, metal is redistributed between the strong inert organic complexes (which will not be reduced at selected accumulation potential) and other labile electroactive species having comparable diffusion coefficients (inorganic and some weak organic complexes) (Bruoland et al., 2000; Croot et al., 1999; Garnier et al., 2004b; Lorenzo et al., 2007; Plavšić et al., 2009). The second concept assumes that all chemical species are electroactive at selected accumulation potential, but having different diffusion coefficients and that the curvature shape of titration curve is a product of average diffusion coefficients which is changing along the titration curve (Chakraborty et al., 2007; Town and Filella, 2000). The first approach is widely accepted, however it is certain that neither of the models by itself is “correct”, and an effort should be made in order to resolve how the mutual behavior is translated to the titration curve and calculation of complexation parameters. A crucial step for both concepts is selection of adequate deposition accumulation/reduction potential. A pseudopolarographic (PP) “fingerprint” of the sample, displaying one or more distinct PP waves, provides not only the basis for selection of adequate accumulation potential, but additional quantitative and qualitative information of the metal complexation (Croot et al., 1999; Cullen et al., 2006; Gibbon-Walsh et al., 2012; Louis et al., 2008, 2009a; Omanovic and Branca, 2003, 2004; Omanovic et al., 1996; Town and Filella, 2000; Town and van Leeuwen, 2006). The potential 100–200 mV more negative than the redox potential of labile (inorganic) metal is usually sufficient. As an alternative to ASV, a potentiometric stripping analysis (PSA) (known also as stripping chronopotentiometry, SCP) could be applied (Waeles et al., 2008).

In CLE-AdCSV method a known concentration of competing ligand (CL), forming metal complex(es) with known stability constants, is added to titrated sample (Campos and Van Den Berg, 1994; Donat and Van Den Berg, 1992; Ellwood and Van Den Berg, 2001; Laglera et al., 2007; Monticelli et al., 2010; Rue and Bruland, 1995; Van Den Berg and Huang, 1984). An equilibrium redistribution between added ligand (AL) and the natural ligand (L) is established at each titration point of added metal. The extent of formed metal complex(es) with AL is measured by their reduction. The high sensitivity of AdCSV method is based on the ability of the formed metal–AL complex to adsorb and accumulate on the surface of electrochemical sensor (usually mercury drop). In most cases, an accumulation potential more positive than the reduction of metal–AL complexes is selected. However to increase the sensitivity, a potential more negative than the reduction of metal–AL complexes is used as well (Campos and Van Den Berg, 1994). The concentrations of added competing ligand determine the so-called “detection window” (DW) of the CLE-AdCSV method (Bruoland et al., 2000; Donat and Van Den Berg, 1992; Gledhill and Buck, 2012; Hirose, 2006; Sander et al., 2011; Van Den Berg and Huang, 1984). Although the shape of the titration curve depends on the chosen DW, calculated complexation parameters should not depend on it. However, due to experimental uncertainties no consistent data could be obtained for the same sample (Campos and Van Den Berg, 1994; Hudson et al., 2003; Sander et al., 2011). To overcome problems associated with the single DW and to provide more reliable complexation parameters, titrations at multiple DW or multiple analytical window (MAW) (Bundy et al., 2014; Hudson et al., 2003; Sander et al., 2011; Wells et al., 2013) were suggested.

Although the range of metal binding sites of different strength exists (heterogeneity of NOM) (Dzombak et al., 1986), a discrete model, representing sites of “similar characteristics”, is usually exploited in complexation studies in seawater. In practice one, two or even three ligand classes, forming 1:1 metal–ligand complexes, are considered to reliably represent the titration curve, i.e. to describe the scenario of the metal complexation with organic ligands. With increasing metal additions, a free organic ligand is progressively saturated, producing linear range at the high-end of titration curve, representing non-complexing behavior. Although in theory the “linear range” is only asymptotically approaching a non-complexing relationship, it is often used to represent the sensitivity of the method (called “internal calibration”), and it is used to transform signal intensities to measured concentrations (needed for further calculations). The problem of “internal calibration” approach comes to the fore when an additional (e.g. third) weak organic ligand is present in solution and is progressively saturated in the linear range of the titration curve (Hudson et al., 2003; Kogut and Voelker, 2001; Laglera et al., 2013; Turoczy and Sherwood, 1997; Voelker and Kogut, 2001; Wu and Jin, 2009).

Once the titration curve is acquired, the next step is calculation of complexation parameters (total ligand concentrations and conditional stability constants) by fitting experimental data on appropriate model/relationship. Either direct fitting of titration curve or fitting of Langmuir/Gerringa (Gerringa et al., 1995), linearized Ružić/Van Den Berg (Ružić, 1982; Van Den Berg, 1982) or Scatchard (Scatchard, 1949) transformations is performed. In case of one ligand model (1L-model), Ružić/Van Den Berg and Scatchard transformations linearize the data and fitting is easily performed. However, for direct fitting of “1L” titration curve or fitting of “1L” Langmuir/Gerringa transformation, as well as for two (or more) ligand models (2L, 3L) a non-linear fitting is favored. Calculation of complexation parameters in such cases is performed by fitting data on chosen “analytical solution” and/or by numerical “complete complexation model” fitting (Duran and Nieto, 2011; Garnier et al., 2004c; Gerringa et al., 1995; Hudson et al., 2003; Laglera et al., 2013; Lorenzo et al., 2007; Sander et al., 2011; Wells et al., 2013). The satisfactory accuracy and precision of measured data are essential in obtaining reliable final parameters. Deviations from the predicted fitting model may lead to non-convergent or unreliable results.

Apart from using equilibrium principles in estimation of the complexation parameters by above described approaches, an alternative kinetic approach has been shown to provide comparable results, which also provide results of association/dissociation kinetics of the studies chemical system (Louis et al., 2009a). An additional point which could be raised in the overall protocol, which is largely overlooked in the literature, is the treatment of the primary voltammetric curves, i.e. determination of peak heights (Cobelo-Garcia et al., 2014; Pižeta et al., 1999).
has been shown that improper treatment could, lead to appearance of additional strong ligand, which is not originally present in the solution (Omanovic et al., 2010).

In this paper we present an all-in-one, user friendly software for metal complexation studies that addresses and tries to cover different approaches mentioned above. A demonstration of its options and capabilities of (I) determination (calculation/optimization) of complexation parameters by using, both “analytical solutions” and “complete complexation model” approaches and of (II) simulations of complexometric titrations will be provided through different artificial titration experiments. An influence of the different fitting modes on optimized complexation parameters, a simple solution of overcoming the problem of the analytical sensitivity determination, for both 1L and 2L-models and a new insight into problem of variable sensitivity in AdCSV method of Cu–SA spectrometry methodology will be discussed. Simulation part in an easy way provides to user the control over their intended experiments, and in more, the software offers “unlimited space of play” for users in order to explore different aspects of metal–organic ligand interactions (“what if…”). Although the methodology of metal complexation parameters estimation is primarily discussed in relation to metal speciation in natural waters where the “discrete” binding sites simplification is applied, there is still a large interest to perform such experiments in simple model solutions of known chemical composition. Thus, we envisage that some aspects discussed in this paper will find attention of modelers, as well.

2. Calculation of complexation parameters — theoretical background

2.1. Exact analytical solutions

There are several assumptions and model simplifications that should be outlined when considering determination of metal speciation by common approach using electrochemical methods. As mentioned above, all calculations are based assuming discrete model of binding strengths (one or more ligand classes, L_i) with 1:1 metal–ligand stoichiometry. The next assumption is that full equilibrium is attained before measurement and that measured intensities are related only to specie(s) predicted by the model (e.g. no kinetical contribution in ASV). The effect of other competitive reactions as consequence of increased metal concentration, is neglected.

In general, the starting point for metal speciation calculations is the mass balance equations of metal (M) and ligands (L_i):

\[ |M|_T = |M|_f + \sum |MX_i| + \sum |ML_i| \]  
\[ |L_i|_T = |L_i|_f + \sum |ML_i| + \left( \sum_{ij} |M_i L_j| \right) \]

where T denotes total dissolved metal/ligand, f — free metal/ligand, \( \sum |MX_i| \) — sum of all inorganic complexes, \( \sum |ML_i| \) — sum of all organic complexes with studied metal, and \( \sum_{ij} |M_i L_j| \) — sum of all other complexes of L with other cations (usually omitted in mass balance equations).

Stability constant (K') of metal complex with L, valid for a given solution composition, is given by:

\[ K'_{ML} = \frac{|ML|}{|M|_T |L_i|} \]

where \( |L_i'| \) is the concentration of non-bound ligand by the metal.

In practice the sum of free metal concentration and its inorganic species is denoted as M', which is related to free metal concentrations via inorganic side reaction coefficient, \( \alpha_m = |M'|/|M| \). Conditional stability constants of formed metal complexes are expressed either against |M'| or |M| so the conversion between the two is \( \alpha_{PC} \). There is not an accepted convention of the notation of conditional stability constants, but it should be clearly indicated what is the basis of expression. In case of Fe organic complexes (FeL), the difference from these 2 approaches is \( 10^{10} \) (and so easy to distinguish), while for Cu and some other metals the difference is up to two orders of magnitude. K'_{FeL,Fe} and K'_{FeL,Tn} are examples of clearly expressed stability constants.

For one ligand model (1L) by mathematical transformations, the above equations could be transformed into well-known Ružič/Van Den Berg (R/VDB) (Eq. (4)), Scatchard (SC) (Eq. (5)) and Langmuir/Gerringa (L/G) (Eq. (6)) transformations (Gerringa et al., 1995; Ružič, 1982; Van Den Berg, 1982):

\[ \frac{|M'|}{|ML|} = \frac{|M'|}{|L_f|} + \frac{1}{K'_{ML}} \]  
\[ \frac{|ML|}{|M'|} = -K'_{ML} + K'_{ML} |L_f| \]  
\[ |ML| = \frac{K'_{ML} |L_f| |M'|}{1 + K'_{ML} |M'|} \]

While R/VDB and SC transformations produce linear relationship, from which total ligand concentration and conditional stability constant could be calculated from the slope and the intercept, the L/G relationship produces curvature shape, and non-linear fitting is needed.

For two or more ligand models, R/VDB (Eq. (7)) and L/G (Eq. (8)) equations could be extended for additional members on the right side:

\[ \frac{|M'|}{\sum |ML_i|} = \frac{1}{\left( \sum |M_i|_f + 1/K'_i \right)} + \frac{|L_j|}{\left( \sum |M_i|_f + 1/K'_i \right)} + \ldots \]  
\[ \sum |ML_i| = K'_{i |L_f|^j |M'|} + K'_{2 |L_f|^j |M'|} + \ldots \]

Both transformations produce curvature shape, providing information on number of ligand classes present. Estimation of complexing parameters could be performed either by separately treating different segments of a titration curve (quasi linear parts) (Bruland et al., 2000; Wu and Jin, 2009) or by non-linear fitting (Duran and Nieto, 2011; Garnier et al., 2004b; Gerringa et al., 1995; Monticelli et al., 2010; Pižeta et al., 1999; Voelker and Kogut, 2001; Wu and Jin, 2009). An explicit analytical solution for more than one ligand model does not exist for Scatchard transformation.

Above relationships attained wide popularity because of their simplicity and relatively straightforward graphical visualization of number of ligand classes, however fitting the data in those transformations is not mathematically acceptable as the X and Y axes through the transformations became dependent. This problem could be overcome if explicit analytical relationships of |M'| vs. |M| are used for data fitting. For both, 1L and 2L-models, such relationships exist and are used for calculation of complexation parameters in literature (Duran and Nieto, 2011; Gerringa et al., 2014; Hudson et al., 2003; Lorenzo et al., 2007).

For 1L-model the following analytical solution is valid:

\[ |M'| = a + \sqrt{a^2 + 4|M'|_f/K'} \]

where \( a = (-|M|_f + |L_f| + 1/K') \). Eq. (9) is a well known solution for roots of quadratic equation. Similarly, the explicit solution for 2L-model is the equation for roots of cubic equation, however due to its complexity.
it is not reported here, and authors are guided to the literature (Hudson et al., 2003; Pižeta and Branica, 1997).

The above equations apply directly to the titrations performed by ASV, where measured current corresponds directly to [M].

In CLE-AdCSV method, a competing ligand is added in solution and Eq. (1) is extended for additional member:

\[ [M]^i_f = [M]^i_f + \sum_i [MX]^i + \sum_i [ML]^i + \sum_i [MAL]^i, \]

(10)

where \( \sum_i [MAL]^i \) is the sum of concentrations of all metal species formed by added ligand, each defined by conditional stability constant which has to be known:

\[ K'_{MAL} = \frac{[MAL]^i}{[M]^i_f [AL]^i_f}. \]

(11)

It is assumed that the concentration of AL is sufficiently high that the inorganic species ([M]') could be neglected. In CLE-AdCSV, measured signal is related to the reduction of accumulated [MAL], complex(es). From Eq. (11) it follows that

\[ [MAL]^i = K'_{MAL} [M]^i_f [AL]^i_f. \]

(12)

Conditional stability constants of MAL complexes are expressed either against [M] or [M]' for a given solution composition. Additionally, it is assumed that the unbound concentration of added ligand ([AL]'i) is equal to its total concentration ([AL]T), because its concentration is much higher than that of metal along the titration curve.

Like for inorganic side reaction coefficient, the conversion factor between labile ([M]'i) or free ([M]'i) metal and [MAL] is the side reaction coefficient of AL defined as:

\[ \alpha_{MAL} = K'_{MAL} [AL]^i_f, \]

or \[ \alpha'_{MAL} = K'_{MAL} [AL]^i_f. \]

(13)

In case that more than one complex with M is formed with AL both should be considered in calculation of \( \alpha_{MAL} \). Using \( \alpha_{MAL} \), measured currents corresponding to [MAL] are transformed into [M]' or [M]T, so that all above equations for calculation of complexation parameters could be used for CLE-AdCSV method as well.

It should be clearly pointed out once more that these equations are valid only for sufficiently large concentration of AL compared to total metal and under assumption that the distribution of metal species with AL (MAL and M(AL)'2) is constant along the titration. These conditions are mainly fulfilled in case of Fe speciation, where concentration of added ligand is usually at least two orders of magnitude higher than total Fe, however for Cu speciation for instance in estuarine samples these conditions are not fulfilled. This problem will be discussed in more details later in the text.

2.2. Complete complexation model

The above described approach of complexation parameters determination is based on exact analytical solutions. However, an alternate methodology respecting the true mass–balance equilibrium reactions of all components included in the system is described (Garnier et al., 2004c; Hudson et al., 2003; Kogut and Voelker, 2001; Sander et al., 2011; Wells et al., 2013). In this approach an optimization of complexation parameters is performed by solving mass balance equations taking measured concentrations (dependent) and [M]T and [AL]T (independent) as input variables. While Garnier et al. (2004c), Sander et al. (2011), and Wells et al. (2013) used in-house developed codes, Kogut and Voelker (2001) used FITEQL based code. It was shown that the benefit of using this approach is treatment of experimental data obtained at different “detection windows” as unified set of data, leading to reliable estimation of complexation parameters (Hudson et al., 2003; Sander et al., 2011; Wells et al., 2013).

3. Description of the ProMCC

ProMCC is developed to serve as a tool for metal complexation studies based on titration experiment assuming the above elaborated discrete model of complexing sites. Intended to be used from both, experienced and non-experienced users, it is designed to allow users to follow logical sequence and easy managing of the complete procedure (“select and push the button”). It is assumed that users are familiar with the basic principles of the applied methodologies. ProMCC operates basically in two distinct modes, which are interconnected:

- simulation of complexometric titration, and
- calculation of complexing parameters ([k] and [L]) from experimental titration data sets.

The two abovementioned approaches (the exact analytical solutions and the complete complexation model) are implemented in both modes.

For calculation of complexing parameters using exact analytical solutions, a well-known Levenberg–Marquardt algorithm for nonlinear fitting is applied. Uncertainties of fitted parameters are provided by the used algorithm in form of standard error (SE) and are expressed as 95% confidence limits by multiplying SE by the t-value (Student's t-distribution) calculated according to number of data. The accuracy and precision of used algorithm (available as free DLL file at: http://fitting.datamaster2003.com/) were checked by nonlinear regression NIST Statistical Reference Data Sets (http://www.itl.nist.gov/div898/strd/nls/nls_main.shtml). Fitted parameters as well as corresponding uncertainties (standard error) showed matching for at least 8 significant digits with certified values. It should be clearly noted that uncertainties provided by the ProMCC assume dependent/independent variables in data pair to be fitted (specific problems in uncertainty will be elaborated more later in the text).

For fitting data using “complete complexation model” a PROSECE (Programme d’Optimisation et de Spéciation Chimique dans l’Environnement) code (Garnier et al., 2004a, 2004c) is implemented in ProMCC. Original PROSECE program is written on the basis of the numerical calculation program Octave. Although originally developed and used as flexible fitting tool for calculation of complexation parameters for multiple variables (Louis et al., 2009b), in the actual version of ProMCC the code is adapted to be used only for calculation of metal complexation parameters and modeling of complexometric titrations and to bring this approach in a form of user friendly environment. Shortly, PROSECE combines speciation calculation subroutine and iterative optimization loop as a fitting methodology. The matrix-based speciation calculation is using Newton–Raphson optimization method in solving mass balance equations, according to predefined Morel’s table definition representing all chemical reactions of the system. The optimization subroutine (based on modified simplex) adjusts selected parameters, giving a new set of parameters for the new speciation calculation. This process is iteratively repeated until minimal fitting error is reached.

ProMCC is developed within Embarcadero Delphi programming environment. It is Windows-based software and is currently not supported by Mac OS or Linux. The latest software information and updates are available at: https://sites.google.com/site/mccprosece/.

3.1. Graphical user interface

The fore-front of ProMCC is its graphical user interface (GUI) with four separate graphs (Fig. 1):

1. Original titration curve, [M]found vs. [M]T, (Fig. 1, A).
2. Langmuir transformation, (MAL and M(AL)'2) from experimental titration data sets.
3. Ružič/Van Den Berg transformation, \([M]_{\text{bound}}/\sum[M\ell]\) vs. \([M]_{\text{bound}}\) (Fig. 1, C).

4. Scatchard transformation, \(\sum[M\ell]/[M]_{\text{bound}}\) vs. \(\sum[M\ell]\) (Fig. 1, D).

\([M]_{\text{bound}}\) is referred as “observed” or measured concentration (obtained from the measured intensities by dividing with sensitivity factor (S)).

ProMCC is fully interactive, providing immediate graphical representation of the performed actions. All four plots could be presented either in “Lin–Lin” or “Log–Log” mode allowing easy way of identifying:

- number of ligands present, i.e. selection of model on which data will be fitted,
- occurrence of outliers (which could be discarded), and
- visual evaluation of applied treatments and goodness of fit.

The benefit of examining all four relationships is clearly presented in Fig. 1, where examples of simulated titration curves (no noise introduced) for 1L and 2L ligand models are plotted, as appear in ProMCC. It is obvious that A and B plots in “Linear” scales could not be used to resolve the number of ligands. Although for such ideal data Ružič/Van Den Berg transformation provides indication of two ligand classes, only Scatchard plot undoubtedly shows existence of one or two ligand classes. To distinguish more than two ligand classes, “Logarithmic” mode is preferable, however it should be noted that the “resolution” between two ligand classes depends on their stability constants and concentrations.

### 3.2. Simulations of complexometric titrations

The main purposes of complexometric titration simulations are to acquire a meaningful bunch of titration data sets as a base for (1) design of real titration experiment, (2) development/improvement of data analysis methodology and (3) prediction of metal speciation under relevant natural conditions. Although well-established speciation modeling programs are available free of charge (e.g. Visual Minteq, PHREEQC, CHEAQS, etc.), they do not allow easy adaptation of internal parameters/setup and are not suitable for building large set of data in relatively short time. Purpose-developed speciation modeling tools, such as ProMCC, are thus of great support for a devoted group of users. Ideally, simulated virtual experiments should mimic real experiments, avoiding as much as possible simplifications of adopted model.

ProMCC offers theoretical modeling (simulations) of complexometric titrations, as seen experimentally by either ASV or CLE-AdCSV, for up to three ligand classes (1L, 2L and 3L models). Fig. SI-1A shows input simulation window with almost all available options. As is mentioned previously, measured signal in ASV is proportional to the concentration of “labile” species ([M‘]), which are assumed to correspond to free metal and its inorganic species, whereas the signal in AdCSV is proportional to the new formed complex(es) with added ligand (AL). The latter denotes that competing ligand should be selected for modeling. For this purpose, a database containing stability constants of metal complexes with AL could be created. For both techniques, a side reaction coefficient(s) (SRC) could/should be specified, respecting the basis of conditional stability constants (K’) expression. In case of ASV, SRCN is 1 if K’ is expressed against labile metal (M‘), while it is 1 if expressed against free metal ([Mfree]) for a given sample composition. Selection of AL is mandatory for AdCSV titration simulation, and the same rule for K’ expression should be respected as above specified for ASV (see Table 1 for examples, in a way how database is organized within ProMCC).

Simulation of ASV titration is performed by an iterative bisection routine (Pizeta and Branica, 1997), solving polynomial equations of second, third and fourth orders. Precision is set to 10⁻²⁵, which was found sufficient for all simulations. There is not any difference in generated data regardless if bisection method is used or exact analytical solutions (quadratic/cubic) mentioned previously. It is assumed that inorganic side reaction coefficient (if applied in simulation) is not changing along the titration curve. This assumption corresponds to real ASV experiment, as the inorganic speciation of metal is not changing in relation to total or labile (measured) metal concentration. This is due

<table>
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<th>ID</th>
<th>No MAL spec.</th>
<th>logKMAL</th>
<th>logKMALAL</th>
<th>Inorg. alfa</th>
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<td>0</td>
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</table>
to sufficiently large concentration of inorganic ligands (OH\(^-\), Cl\(^-\), CO\(_3\)\(^{2-}\), SO\(_4\)\(^{2-}\), etc.) present either in seawater or freshwater.

Simulation of CLE-AdCSV titration could be performed either using the same protocol as for ASV, assuming “constant” side reaction coefficient of AL, or by solving mass balance equations (complete complexation model). Because of the nature of AdCSV, the latter method is preferred, and is applied if option “Include AL speciation” is selected within ProMCC.

Once the basic input parameters (initial metal concentration, sensitivity, number of titration points, number of repetitions for each metal concentration, end-titration metal concentration) are specified, the type of titration should be selected. Available options are: (1) linear (equimolar) additions, (2) logarithmic addition or (3) user defined input. It is already demonstrated that the type of titration could impact the complexity parameters determination (Garnier et al., 2004c; Laglera et al., 2013). In common practice a (quasi)logarithmic mode is preferred as it could provide reasonable number of points for calculation of more than one ligand classes if properly distributed along the titration.

In order to mimic real experimental conditions, a random noise could be added to data. In generating titration curves, the authors used either constant or variable noise distributed along the titration curve (Garnier et al., 2004c; Laglera et al., 2013; Sander et al., 2011; Turoczy and Sherwood, 1997; Voelker and Kogut, 2001). Although the real experimental noise could not be fully reproduced, based on experimental evidences, a prediction of noise structure could be done. At low end of the titration curve, where the concentration of electroactive metal specie(s) is low, the variability of signal intensity is much higher than at the end of the titration curve. The main reasons of higher signal variability at low labile metal are low signal to noise ratio (S/N ratio) and/or instability of “signal to background current relationship”. Other sources of signal variability (e.g. Hg drop size and/or stirring reproducibility) are not depended on the measured concentration. ProMCC offers selection of either constant or variable noise distribution. Variable noise is based on an equation given by Garnier et al. (2004c). Its distribution along the titration curve could be adapted and is dependent on predefined limit of the quantification (LOQ) (see Fig. SI-1A).

Once data set for virtual titration experiment is generated, it could be further treated by switching to “Optimization” part in order to calculate complexation parameters.

3.3. Optimization — calculation of complexation parameters

Once experimental data set is acquired or virtual titration is generated by ProMCC, the determination of complexing parameters (\(K'_i, [L_i]\)) could be performed. Detailed description and the presentation of graphical window are omitted from the main text, but for easier tracing of explained procedure/methodology readers are advised to see Fig. SI-1B or ProMCC user manual. The first most critical step in overall calculation procedure is assignment of the method sensitivity. In order to generate four plots, an initial “internal calibration” (IC) slope (\(S^0\)) is by default calculated based on the last 5 titration points. Number of points can be adapted, while also the “predefined” sensitivity can be manually specified or adjusted by assigned up/down control. The option of automatic adjustment the sensitivity (“Auto Adjust”) is also implemented. The “auto-adjust” procedure and its efficiency will be elaborated and discussed in ProMCC application — results and discussion section. Regardless of ASV or AdCSV titration data, side reaction coefficient(s) should be specified in the same way as described in modeling subsection.

After the sensitivity is defined, initial complexation parameters (“Initial guess”) can be specified manually or automatically. For automatic initial guess quasi-linear sections of Scatchard (stronger ligand) and Ružič/Van Den Berg (weaker ligand) transformations are used, applying simple linear fitting to determine complexation parameters. A number of points used for “Initial Guess” are automatically assigned or manually adjusted. As previously mentioned, the two fitting approaches are available: (1) classical nonlinear fitting of selected relationship and (II) “complete complexation model” fitting.

3.3.1. Non-linear fitting

Depending on the model on which data will be fitted (1L, 2L, 3L), data can be fitted either on:

- Langmuir transformation;
- Ružič/Van Den Berg transformation;
- fitting of \([M]_\text{bound} \text{ vs. } [M]_i\) relationship (only 1L-model, direct analytical solution, Eq. (9));
- fitting of \([M]_i' \text{ vs. } [M]_\text{bound}\) relationship (“inverse” approach — see below).

In the current stage, direct fitting of \([M]_\text{bound} \text{ vs. } [M]_i\) (cubic analytical solution) for 2L-model is not implemented due to the restrictions in applied DLL (Dynamic Link Library) (length of defined function should be less than 256 characters). Thus an “inverse” fitting is implemented, so that \([M]_\text{bound}\) is specified to be independent variable and \([M]_i\) as dependent variable, represented by the following equation:

\[
[M]_i' = [M]_i + \sum_{i=1}^{n} \frac{[L_i]K_i[M]_i'}{1 + K_i[M]_i'}. \tag{14}
\]

Such an alternative is also suggested by Gerringa et al. (2014) and is also used by Laglera et al. in estimation of sensitivity by non-linear fitting method (Laglera et al., 2013).

For all four options, three separate fitting modes are offered: (I) linear, (II) logarithmic and (III) weighted. In linear mode (Lin–Lin), a relationship with XY data in linear scale is fitted, while XY pairs are converted to logarithmic values for logarithmic fitting (Log–Log). When weighted fitting is applied (regardless of Lin–Lin or Log–Log data), a “1/N\(^2\)” weights are assigned to each Y value.

From our experience, based on real and computer generated titrations, in cases where fitting by Lin–Lin mode could not converge, Log–Log and/or weighted fitting provide reasonable estimates of complexing parameters. An example of the efficiency of different fitting combination is provided in ProMCC application — results and discussion section.

3.3.2. Complete complexation model fitting

The detailed comparison of non-linear and “complete complexation model” fitting is provided by Garnier et al. (2004c). It was found that “complete complexation model” fitting, made by PROSECE program, gave more accurate results than linear and non-linear methods in most of the examined cases. The PROSECE code is implemented in ProMCC and is offered as fitting option for both ASV and AdCSV titration experiments. Although AdCSV titration data could be fitted by the above described nonlinear methods, due to the nature of AdCSV experiment, where added ligand is included in mass balance equation and because its speciation could be changed during titration experiment, a “complete complexation model” fitting is recommended for AdCSV data. In “complete complexation model” fitting mode, uncertainty of each optimized parameter is calculated as the maximal absolute variation (in percentage) of the parameter value corresponding to a 10% increase of the optimal fitting error.

Recently, a concept of multi-window detection as an approach in estimation of complexation parameters was highlighted (Bundy et al., 2014; Hudson et al., 2003; Sander et al., 2011; Wells et al., 2013). Namely, the “detection window” in AdCSV could be adjusted in order to improve accuracy and precision of complexation parameters determination of more than one distinct ligand classes. The effect of changing detection window is reflected on a titration curve shape, which may lead to inconsistency in determined parameters (which from the theoretical point of view should be the same) (Campos and Van Den Berg, 1994). Discrepancies are mainly caused by the non-ideal data points
(considering only repeatability in this case). However, this is unavoidable in real experiment. To partly overcome this problem it was suggested that fitting is performed on unified set of data for all detection windows at once, taking into account empirically adjusted sensitivities based on RAL approach (Hudson et al., 2003; Sander et al., 2011; Wells et al., 2013). This new methodology is suggested and tested up to now by only few authors, mainly due to the restrictions related to accessible algorithms/programs.

ProMCC is providing full support of this methodology through the “complete complexation model” fitting mode.

4. ProMCC application—results and discussion

In order to demonstrate possibilities of ProMCC, several simulated experiments and corresponding fittings will be presented, being at the same time examples of how this program could be used. Note that complexation parameters used in these examples represent very limited range of possibilities which could occur when working with real samples, and thus, the related conclusions are valid primarily for the reported datasets (not necessary applicable for other datasets having different complexation parameters).

4.1. An effect of non-linear fitting modes on estimation of complexation parameters

Although new sophisticated methodologies in estimation of metal complexing parameters are recently developed and applied (Garnier et al., 2004b, 2004c; Hudson et al., 2003; Sander et al., 2011; Wells et al., 2013), still the classical linear and non-linear fitting of titration curves are the two most applied approaches (Bruland et al., 2000; Campos and Van Den Berg, 1994; Duran and Nieto, 2011; Gerrina et al., 2014; Laglera et al., 2013; Rue and Bruland, 1995; Wu and Jin, 2009). While classical linear fitting of Ružič/Van Den Berg or Scatchard transformations is providing reasonable parameters for one ligand system (1L), its use is limited in multi-ligand system, where estimation of complexation parameters should be done in an iterative way considering separate portions of the titration curve for a stronger and a weaker ligand (Bruland et al., 2000; Laglera-Baquer et al., 2001; Laglera et al., 2013; Wu and Jin, 2009). For fitting of Langmuir transformation, a nonlinear fitting is needed in either case. Originally all equations, whether in transformed form or as direct analytical solutions, are expressed in a form of a linear scale. In general, there is not so much of details in the literature on how these equations are further handled/adapted, other than application of multiplication factor (usually 10^x) in order to avoid calculations with small numbers (Garnier et al., 2004c; Laglera et al., 2013). Here we wanted to inspect whether mathematical transformation of converting data to logarithmic values or application of weighted fitting could indicate which “combination” is providing better estimates of complexation parameters.

A series of virtual titration experiments are generated, for both 1L- and 2L-models. For 1L-model the following parameters were selected: [L] = 15 nM, logK = 8.5; and for 2L-model: [L1] = 10 nM, logK1 = 10; [L2] = 40 nM, logK2 = 8. For both models, titration was simulated respecting logarithmic and linear addition modes. Initial concentration of metal was 1 nM and titration was composed of 15 points (with two replicates at each metal concentration) on which realistic variable noise from ±30% to ±5% was added (see part 3.2). Sensitivity was predefined as 1 nA/nM and it was assumed as known parameter in fitting procedure. In total 20 titrations were generated for each titration mode, and each combination of fitting protocol (Log–Log, Lin–Lin and Weighted) was applied on every separate titration.

Fig. 2 shows box plots of obtained results for 1L-model. As a measure of parameters estimate variability (accuracy and precision), for each fitting mode the number of cases (of total 20) having parameter estimate higher than 5% for [L] and 15% for K comparing to preset values is indicated above the X-axis. In overall it is clear that all fitting modes gave relatively good estimates of complexation parameters when titration is performed in linear mode (i.e. with equidistant 3 nM metal additions). Among three modes of fitting, Lin–Lin produced the lowest average variability for each of four examined fitting models, while variability of Log–Log and Weighted fitting was similar. Although less than 2%, it is evident that fitting data on Ružič/Van Den Berg transformation in linear mode consistently underestimated [L] and overestimated logK. The highest variability in parameters is found for “inverse” transformation ([M]T vs. [M]bound) when fitted in Log–Log and Weighted mode, despite the fitting is performed using “virtually” dependent–independent variables. This experiment revealed that, although based on different equations, the two pairs (indicated by dotted connected lines in Fig. 2) produced exactly the same results: (Langmuir_Lin = ([M]T vs. [M]bound)_Lin) and (Langmuir_Log = Ružič/Van Den Berg Log). We cannot provide exact mathematical explanation for this occurrence, but it is clear that internal handling of the fitting algorithm “considered” equations in a same manner. Taking into account that Langmuir, Scatchard and Ružič/Van Den Berg transformations provide X and Y data which are mutually dependent (which is mathematically incorrect) and prone to behave thus more scattered, it would be expected that fitting by these transformations provides more biased estimation of complexation parameters. Our virtual experiment did not confirm this probability, i.e. fitting on exact analytical solution (AS) (true dependent/independent variables) did not produce better estimates of complexation parameters, nor their lower uncertainty. However, it is very important here to mention that uncertainty of parameter estimates obtained by using transformation methods does not account for X/Y variables dependency (strictly speaking, mathematically they are incorrect), i.e. in this stage, ProMCC is not having incorporated methodology to account for problem of propagated uncertainty of this kind. Thus, potential practitioners should be aware that real uncertainty is higher than reported by the program. Principally, the consequence of “real” uncertainty could be potentially reflected in incorrect conclusions as to the statistical significance of a parameter, i.e., is a ligand truly “detected”. Due to the inconsistency in methodology in calculation of correct parameter uncertainties, the further detailed discussion is omitted from the manuscript, however as an information, the distribution (box-plot) of uncertainties for different fitting modes is presented in Fig. SI-2A.

Fitting in Log–Log mode showed consistent underestimation of ligand concentration for all tested combinations, and overestimation of conditional stability constant. Probably the applied variable noise structure which is differently distributed along the titration curve for two titration modes caused such anomaly. Namely, while in logarithmic titration mode noise higher than ±5% (and ±3%) was applied to 6 points located at low-end titration segment, in linear titration mode only one point was affected by such high noise. To check if the noise structure is a major reason of ligand concentration underestimation, logarithmic titration experiment is performed additionally with constant noise up to ±5%. As expected, no systematic deviation from the preset values was observed in [L] and logK estimation (Fig. SI-2B). It should be clearly pointed out, that although apparent, deviations of the estimated complexation parameters from the true values are truly minimal, however they show the tendency of the applied methodology.

Similar virtual experiments were performed for 2L-model system. Corresponding plots are given in Supporting Information document (Fig. SI 3A and 3B). For linear titration, no obvious general trend is detected in any of the parameters, however fitting by Ružič/Van Den Berg transformation in Lin–Lin mode, leads to an underestimation of [L1] and overestimation of [L2], logK1, logK2 (with highest variability among “combinations”). This is consistent with previous observations where it was found that Ružič/Van Den Berg method is more affected than other fitting modes. The same performance for Ružič/Van Den Berg Lin–Lin method is found also when titration is performed in
logarithmic mode. The same as for 1L-model, a slight underestimation of \( L_1 \) was obtained, caused probably also by the noise structure.

4.2. Tuning of analytical sensitivity

Estimation of correct analytical sensitivity which is then applied to convert observed/measured intensities to metal concentrations is found to be the trickiest part in the overall procedure of complexation parameters determination. To overcome this problem some authors used separate calibrations performed in UV digested samples (Campos and Van Den Berg, 1994; Voelker and Kogut, 2001), however due to other influences on the measured signal (e.g. surface active substances), the protocol was found as inadequate. The further attempt was to use last few titration points in (quasi)linear, high-end segment to estimate the sensitivity (Bruland et al., 2000; Kogut and Voelker, 2001; Plavšić et al., 2009; Voelker and Kogut, 2001; Wu and Jin, 2009). This approach a priori assumes that natural ligand is fully saturated. Although strictly theoretically speaking this assumption is never fulfilled, it could be a relatively good approximation of sensitivity in cases when relatively strong ligand is dominant in considered concentration range (for ASV experiment). Although not perfect, this method is still applied, but under well controlled and tested measurement conditions (Bundy et al., 2014). For a titration data set of a good quality, this method usually underestimates the sensitivity (Hudson et al., 2003; Kogut and Voelker, 2001; Laglera et al., 2013; Voelker and Kogut, 2001). In case when determination of complexation parameters is performed by AdCVS, this range depends on ratio of \( \alpha_{ML}/\alpha_{MAL} \) (Wu and Jin, 2009). The effective solution for correct sensitivity estimation for 1L-model was proposed by Turoczy and Sherwood (1997). It is based on iterative adaptation of sensitivity until \( L_1 \) and \( \log K' \) converge to a stable value. The next step in advancing the sensitivity estimation was a combined use of iterative Turoczy and Sherwood method by iterative linear (Laglera et al., 2013) and/or non-linear fitting (Laglera et al., 2013; Wu and Jin, 2009). An additional alternative approach is to incorporate sensitivity (S) as an unknown parameter in fitting procedure. An advanced method of simultaneous fitting of complexing parameters and sensitivity, as well as alternate “inverse” fitting approach is detailed in paper of Gerringa et al. (2014). An “overload” titration is proposed for improvement of sensitivity in Cu–SA system (Kogut and Voelker, 2001). In this approach sensitivity is determined at very high concentration of added ligand, and because for Cu–SA system sensitivity is dependent on the concentration of added AL, a backward correction of applied sensitivity is needed (Hudson et al., 2003; Kogut and Voelker, 2001). Correction factors are expressed as RAL values and are obtained by normalizing sensitivities at different total SA concentrations with those at highest AL concentrations all obtained in UV digested sample. This methodology was first suggested by Kogut and Voelker (2001) as the basis for the overload titration and was mathematically formalized by
Hudson et al. (2003) in order to handle multiple windows, and thereafter refined by Sander et al. (2011) and Wells et al. (2013).

Any of the mentioned approaches of sensitivity adjustment is based on error minimization protocol. The protocols differ in a feature used to track error minimization. If it is reported, the explanation of error minimization is expressed as convergence of \([L]\), \(\log K'\) or \(S^\text{TE}\) to stable values (Laglera et al., 2013; Turoczy and Sherwood, 1997; Wu and Jin, 2009). In ProMCC an iterative method of sensitivity adjustment, based on minimization of average value of relative errors (RAE) (in %) 2009). In ProMCC an iterative method of sensitivity adjustment, based on minimization of average value of relative errors (RAE) (in %) is implemented. The method is based on a fact that the average error should tend to minimum as the slope is approaching to “true” value. The following simple protocol is employed. First an initial sensitivity (\(S^\text{INI}\)) is manually adjusted at the value so that \(S\) is evidently underestimated. Underestimation of sensitivity could be easily spotted if three transformation plots are visually examined: a decreasing trend of high-end Langmuir plot, concave-up shape of the Ružić/Van Den Berg plot and an opposite direction of values for high-end titration points for Scatchard plot. An example is provided in Fig. SI-4. After the \(S^\text{INI}\) is adjusted, a non-linear fitting (e.g. Langmuir_\text{Lin}) is performed providing set of first estimates of complexation parameters from which \([M]_{\text{FIT}}\) and RAE are calculated. In the next step sensitivity is increased by defined factor (selectable) and a new non-linear fitting is performed providing new RAE. This protocol is repeated until defined number of iterations (adjustable) is reached. In this way a relatively large range of sensitivities is “scanned”, with one RAE value identified as minimum. Corresponding sensitivity will be then assigned as “true” value. Fig. 3 demonstrates how the protocol works. For computer generated titrations without added noise, the method is absolutely accurate for both 1L and 2L-models (indicated by full lines in Fig. 3). The precision of estimates depends on the step of “increase factor”, and thus a two stage process is implemented, first with bigger and then smaller step of “increase factor”. As expected, for slightly more realistic titration data points (±3% random noise), a preset sensitivity (\(S = 1\)) is not reached, but the estimated sensitivity agrees within 1% with the expected one.

To inspect efficiency of the method in estimation of sensitivity and optimized complexation parameters for “real” experiments, a series of titration experiments, assigning more realistic noise structure (±5 to ±30%) to titration data, were generated. Titrations are performed for both 1L- and 2L-model systems with additions of metal respecting logarithmic distribution. For each ligand model 30 separate titration experiments were generated, each with its own noise structure. The objective of this experiment was also to inspect whether the upper range of added metal has an influence on the estimation of “true” sensitivity, as well as on resulting complexation parameters and [Fe]_{\text{inorg}}. Thus, 10 sets (each composed of 30 simulated titrations) at increasing [Fe]_{\text{max}} were generated, each titration having its own random variable noise distribution. Determination of complexation parameters of Fe with TAC as a competing (added) ligand was chosen as a virtual model. For 1L-model [L] = 1.5 nM, \(\log K' = 12.5\) were preselected, and for 2L-model [L] = 2 nM, \(\log K' = 12.5\); [L] = 8 nM, \(\log K' = 11\). For both models \([Fe]_\text{ini} = 0.1 \text{ nM}\), \([TAC] = 10 \, \mu\text{M}\) (\(\alpha_{\text{Fe:TAC}}\), Fe = 263), Limit of Quantification, LOQ = 0.01 nM. Titrations were performed at all total metal concentrations, with duplicate “measurement” (30 data XY pairs). Langmuir_\text{Lin} and Langmuir_\text{Log} modes were used for data fitting (indicated in figures). Fig. 4 presents results obtained for 1L-model. For each [Fe]_{\text{max}}, although with slight general overestimation, the method provided quite correct average estimates of the sensitivity, with maximal relative error below 7% (except the lowest [Fe]_{\text{max}} which was 10.5%). The variability in sensitivity estimates is diminishing with increasing [Fe]_{\text{max}}. Such trend was expected and is related to the structure and distribution of the added noise. Namely, as the titration was composed always of 15 titration points, and as logarithmic distribution of added metal was applied, there were more points with larger noise at lower [Fe]_{\text{max}}, causing higher inaccuracy and variability in sensitivity adjustment (see Fig. SI-5). Surprisingly, the lowest variability (highest precision) in conditional stability constant estimates was obtained for lowest [Fe]_{\text{max}}. Although with the unfavorable noise, the number of points covering the saturation of natural ligands was sufficiently large to provide fairly good estimates in logK. In overall, slightly higher average values of sensitivity caused noticeable underestimation of logK and overestimation of [L] (Fig. 4C and 4D). However, a combination of under- and over-estimated parameters, which is expressed by \(\alpha_{\text{Fe:TAC}}\), Fe caused absence of tendency in [Fe]_{\text{max}} estimation (Fig. 4B). Except from getting lower variability of sensitivity estimation, the increase of maximal concentration of total metal did not significantly improve neither accuracy nor precision of the complexation parameters estimates for 1L-model.

Good estimates of sensitivity for 1L-model were fairly expected as this was already demonstrated by other authors who used several adjustment methods (Laglera et al., 2013; Turoczy and Sherwood, 1997; Wu and Jin, 2009). The challenge was to check method efficiency for model with two distinct ligands. For total ligand concentrations of 10 nM, titration was performed by increasing [FeT]_{\text{max}} by factors 2 to 10. Wu and Jin (2009) found that the conventional nonlinear fitting cannot converge if incorrect sensitivity is used. Although our virtual experiments were performed with realistic noise structure of titration data, convergence problem occurred only in cases when initial sensitivity was set so low that visual observation of the “linearization” transformations clearly indicated the incorrect. Fig. 5 presents the testing results. A pretty good overall performance of sensitivity adjustments was obtained with relative errors less than 8%. While underestimation of average sensitivity was registered for the two lowest titration ranges (6% and 3%, respectively), other estimates were within 1%, demonstrating promising capability of applied methodology, at least on simulated data sets with defined complexation parameters. Sensitivity underestimation for two lowest titration ranges was most probably affected by the decreased number of points in the range where second ligand is dominant, i.e. the titration finished at the point where ligand was not saturated (Wu and Jin, 2009). Generally, nonlinear fitting performed on data with underestimated sensitivity causes subsequently underestimation of calculated [L]_1 and [L]_2, and overestimation of \(\log K'\) and \(\log K''\). This trend is apparent for two lowest titration ranges, however the drift is an obvious general characteristic for all scanned [Fe]_{\text{max}}. To exclude possible influence of the sensitivity adjustment and its variability on the mentioned characteristic, an equivalent set of titrations is generated, but with the difference that data were fitted by fixed (correct) sensitivity (1). The same trends were observed, implying that sensitivity variability was not the reason for under/over estimation.

**Fig. 3.** Dependence of the average error of fitting (average difference between fitted and experimental value of [M]_{\text{meas}}) on sensitivity. Lines represent error-free titration while lines with symbols represent results when 3% of fixed error is introduced in titration data. Following data were used for simulation of titrations: \([L] = 10 \, \text{nM}\), \(\log K' = 8.5\) for 1L-model and \([L] = 10 \, \text{nM}\), \(\log K' = 10\); \([L] = 50 \, \text{nM}, \log K' = 8\) for 2L-model.
of complexation parameters (see Fig. SI-6A). It should be noted that results presented in Fig. 5 are obtained by fitting titration data using Langmuir_Log mode. As we already noted previously (Fig. 2) for 1L-model system, an underestimation of \([L_1]\) and overestimation of \(\log K'_1\) was observed for titrations performed in logarithmic mode. Closer inspection of that figure revealed that there is a difference between data obtained by two modes of Langmuir fitting, Log–Log and Lin–Lin. Namely, Lin–Lin fitting produced more accurate estimate of complexation parameters than Log–Log mode. Thus, we performed a third titration set, which was treated by Langmuir Lin–Lin mode. The obtained results (presented in Fig SI-6B) confirmed previous observation that Lin–Lin mode of Langmuir model provides more accurate complexation parameters. Under/over estimation of parameters in Log–Log fitting mode leads to slight underestimation of average \([Fe]_{\text{inorg}}\) for all \([Fe]_{\text{max}}\) (maximal relative error up to ~15%). Lin–Lin mode in general provided correct averages of \([Fe]_{\text{inorg}}\).

Based on results obtained on simulated data sets, it could be concluded that proposed method provides reasonable estimates of analytical sensitivity adjustment/correction for 1L and 2L complexation models. Laglera et al. (2013) showed that if an additional ligand (L3) is present in the solution and not considered by the applied model, an iterative correction of sensitivity (treating data as 2L-model) provides an underestimated sensitivity even for error-free titration data. Reproducing their experiment, our adjusted S was within the range of their best estimates (data not shown). In case that first ligand is almost fully saturated at early beginning of the titration curve (as is the case for Laglera data (Laglera et al., 2013)), the removal of few first titration points in the sensitivity adjustment procedure, strongly improve the accuracy of the sensitivity estimate, and could be recommended as an option in the overall procedure of sensitivity adjustment. The efficiency of iterative sensitivity adjustments depends on the “distributions” of the stability constants, ligand concentrations and titration points, as well as on the quality of experimental data. The repetitive measurement (i.e. at least duplicate, better triplicate) of each titration point is thus strongly recommended in order to improve overall accuracy and precision of complexation parameters estimates.

An important point which should be mentioned again is the uncertainties of the parameter estimates. Namely, aside of the already mentioned underestimation of uncertainties when fitting of transformed data is undertaken, the problem of correct uncertainties is further associated also with the application of analytical sensitivity (when transforming signal intensities to measured concentrations). Common two step procedure in estimation of complexation parameters (first adjustment of sensitivity, followed by fitting of transformed data) does not take into account that the sensitivity is a "floating" variable. This means that the uncertainty of analytical sensitivity is not incorporated in uncertainties of parameter estimates, i.e. the obtained uncertainties are (further) underestimated (and statistically incorrect). This leads us to conclusion that uncertainties propagated in two steps (transformation and sensitivity) may have much higher influence on the statistical significance of the complexation parameter estimates, than if propagation of uncertainty is not taken into account (which is a common praxis in the literature). This problem is related exclusively to the two-step fitting approach and not the fitting algorithms by themselves or fitting program (ProMCC or e.g. Sigma Plot). The problem of propagated uncertainties is rarely discussed in the literature, despite the fact of great importance.
The simplest solution to overcome the problems of incorrect uncertainties is to incorporate the sensitivity as a variable and to fit truly dependent/independent relationship, current intensity versus total metal. As noted previously, analytical solutions exist for both, 1L and 2L models. The current version of ProMCC does not have implemented this methodology in nonlinear fitting mode, but we are in the process of incorporating it fully in the next version. The fitting of sensitivity will be also incorporated within “complete complexation model” fitting, similar to those implemented in original PROSECE program for fluorescence quenching (Michon et al., 2010).

4.3. ProMCC multi-detection window method and RAL titration concept

Due to the lack of larger sample volumes and/or relatively long time needed to accomplish measurements, the determination of complexation parameters by CLE-AdCSV method was commonly accomplished by performing single detection window titrations, i.e. at one concentration of competing ligands. Although pioneering studies did not always provide consistent complexation parameters (Bruland et al., 2000; Buck et al., 2012; Campos and Van Den Berg, 1994), multiple detection window approach has been recently recognized as a great potential in analyzing complexometric titration data (Bundy et al., 2014; Sander et al., 2011). Multi-window principle is acknowledged by Hudson et al. (2003) who suggested new approach based on simultaneous treatment of all data as unified set. They demonstrated that accuracy in parameters determination is greatly increased, while uncertainty was decreased. Further advances in multi-detection window approach, by treating unified set of data were made by Sander et al. (2011) and Wells et al. (2013). While, Hudson’s approach was based on analytical solutions, capable of solving 1L and 2L-models, Sander and Wells developed numerical approach (initially presented by Garnier et al. (2004a, 2004c)), with possibility of solving up to five ligand classes. The new approach was initially named as “automated multi-window optimization” (AMO) and thereafter as Sander–Wells (S–W) method (Sander et al., 2011; Wells et al., 2013).

Respecting advantages of treating multi-detection window unified data set, an effort has been made to implement that approach into ProMCC. As is mentioned before, a PROSECE code is used as an optimization methodology. Basically there are no functional differences in treatment of single or multi-detection window data in ProMCC. For

![Fig. 5. Iterative sensitivity, correction and corresponding complexation parameters and [Fe]=max for 2L-model (Fe-TAC) obtained by varying maximal concentration of total Fe in a titration ([Fe]=max). Preset values indicated by the horizontal dashed line are: [L1] = 2 nM, logK1’ = 12.5, [L2] = 8 nM, logK2’ = 11. [Fe]ini = 0.1 nM, [TAC] = 10 μM (αFe(TAC), Fe’ = 263), LOQ = 0.01 nM.](image-url)
demonstration purposes of ProMCC efficiency in analyzing unified data set obtained by multi-detection window titrations, a set of single detection window titrations simulating Cu–SA complexation titration were generated under following conditions: [L1] = 30 nM, logK′1 = 12.5, [L2] = 100 nM, logK′2 = 10.5, [SA]1 = 0.5, 1, 2, 5 and 15 μM, [Cu]in = 1 nM. Logarithmic titrations were generated with maximal concentration of Cu, [Cu]max = 400 nM. Each titration was composed of 15 titration points on which real experimental noise structure (±30%; ±5%) is applied. The arbitrary sensitivity for each titration (detection window) was set as one, and as such, obtained currents correspond directly to measured concentrations. Determination of complexation parameters were performed on each single titration separately as well as on unified data set (multi-detection window). Table 2 lists the obtained results. Among the single detection window titrations the fitting of first two (0.5 and 1 μM SA) provided correct estimates of all complexation parameters, 3rd and 4th (2 and 5 μM SA) gave good estimates for only first ligand, while the 5th (15 μM SA), although not dramatic, failed in estimation of all parameters. Optimization performed on unified data set, as expected, provided the most correct complexation parameters. Fig. SI-7 shows an example of unified data fitting as observed in ProMCC.

A detailed examination (simulation and fitting) of the above multiple detection titrations of Cu–SA system, revealed a specific behavior of the Cu–SA speciation along the titration. Namely, it is already shown (Bruland et al., 2000; Campos and Van Den Berg, 1994; Kogut and Voelker, 2001) that the sensitivity of the titration is dependent on the concentration of added SA. The change in sensitivity authors ascribed to the change in Cu(SA)2 concentration. Thus, for sensitivity estimation by “overload” titration a correction factor was needed, normalizing sensitivity in dependence of the added SA concentrations (Voelker and Kogut, 2001). By fitting experimental data of several authors, Hudson et al. have shown that the relative (normalized) sensitivity (RAL) in relation to [SA]1 could be expressed by empirical equation (Hudson et al., 2003). When unified data set is used for estimation of complexation parameters, the Hudson’s RAL approach is applied (Hudson et al., 2003; Sander et al., 2011; Wells et al., 2013).

The fact that Cu(SA)2 is identified as “driving” force of sensitivity change, leads us to an assumption that the Hudson’s relationship might be related to the change in CuSA and Cu(SA)2 concentration ratio and also, that this ratio is changing not only with different total concentration of SA, but also along the titration range. Usually it is assumed that the side reaction coefficient M-AL is constant, because much larger concentration of AL is added than the maximal concentration of added metal. This is largely fulfilled in case of Fe speciation or Cu speciation in ocean samples (e.g. 33.1 nM and 1 μM of total Cu and SA, respectively (Campos and Van Den Berg, 1994)), however for Cu speciation in e.g. estuarine samples, this condition is not certain for the titrations performed at the low concentration of added SA (e.g. ~2 μM). Consequently, this raised the question to modify RAL value not only in function of total SA but in relation to true Cu partitioning between CuSA and Cu(SA)2 species. Having this in mind, a modified equation is proposed to adjust the relative sensitivities:

$$ RAL = \frac{S_{\text{max}}}{S} \propto K \times \frac{R}{1 + K \times \frac{R}{T}} $$

where $K = 1.1, R = \frac{[CuSA]}{[Cu(SA)2]}$, and $T = [L1] = 30$ nM, $logK′1 = 12.5, [L2] = 100 nM, logK′2 = 10.5, [SA]′1 = 0.5, 1, 2, 5 and 15 μM, [Cu]in = 1 nM; logarithmic titrations with [Cu]max = 400 nM using the empirical equation proposed by Hudson et al. (2003) and the Eq. (15).

Parameters of this function ($\propto, K$) were firstly optimized by fitting the RAL values obtained by Hudson’s equation (Excel solver®), and the corresponding Cu chemical speciation, considering the lowest total Cu concentrations for each titration (i.e. being closer to the conditions of previously published studies). In second stage the two parameters were re-adjusted to final values of $\propto = 1.1$ and $K = 10$ to fulfill condition that RAL tend to 0 and 1 for no added SA and the extremely high SA concentration, respectively. As shown in Fig. 6A, Eq. (15) satisfactorily simulated the RAL variations vs. [SA]′, as the one proposed by Hudson et al. (2003). However, contrarily to the Hudson’s empirical equation which assumes a constant RAL for a defined [SA]′ (i.e. whatever the variation of Cu(SA)2 proportion), Eq. (15) suggests that the RAL should vary significantly over the titration, especially for the titrations performed at the lowest SA concentrations at relatively high total Cu concentrations (Fig. 6B).

Fig. 7 shows obtained calibration plots (no organic ligand is present), which clearly demonstrate the consequences of RAL variability along the titration. While the calibration at highest concentration of added SA is fully linear, a clear deviation from linearity is observed for the lowest SA concentrations. At high added Cu concentration even a decreasing trend is predicted. An extra example showing possible performance of

<table>
<thead>
<tr>
<th>[SA]′1 (μM)</th>
<th>[L1] (nM)</th>
<th>logK′1</th>
<th>[L2] (nM)</th>
<th>logK′2</th>
</tr>
</thead>
<tbody>
<tr>
<td>True</td>
<td>30</td>
<td>12.5</td>
<td>100</td>
<td>10.5</td>
</tr>
<tr>
<td>SW1 0.5</td>
<td>29.9 ± 0.4</td>
<td>12.47 ± 0.02</td>
<td>104.8 ± 3.1</td>
<td>10.50 ± 0.02</td>
</tr>
<tr>
<td>SW2 1</td>
<td>30.3 ± 0.7</td>
<td>12.49 ± 0.03</td>
<td>97.8 ± 8.8</td>
<td>10.52 ± 0.05</td>
</tr>
<tr>
<td>SW3 2</td>
<td>28.4 ± 0.2</td>
<td>12.51 ± 0.01</td>
<td>142.3 ± 6.4</td>
<td>10.42 ± 0.03</td>
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<tr>
<td>SW4 5</td>
<td>30.4 ± 0.9</td>
<td>12.48 ± 0.03</td>
<td>403.0 ± 103.0</td>
<td>9.95 ± 0.13</td>
</tr>
<tr>
<td>SW5 15</td>
<td>22.6 ± 0.6</td>
<td>12.62 ± 0.02</td>
<td>131.4 ± 21.8</td>
<td>10.90 ± 0.10</td>
</tr>
<tr>
<td>MW –</td>
<td>29.8 ± 0.8</td>
<td>12.49 ± 0.03</td>
<td>103.5 ± 8.3</td>
<td>10.51 ± 0.05</td>
</tr>
</tbody>
</table>

Fig. 6. (A) Simulation of RAL values by Hudson equation and by the Eq. (15), (B) variations of RAL along the different titrations from a multi-detection window experiment ([L1] = 30 nM, logK′1 = 12.5, [L2] = 100 nM, logK′2 = 10.5, [SA]′1 = 0.5, 1, 2, 5 and 15 μM, [Cu]in = 1 nM; logarithmic titrations with [Cu]max = 400 nM using the empirical equation proposed by Hudson et al. (2003) and the Eq. (15).
titration curves assuming both variable and constant RAL along the titration is presented in Fig. SI-8. We did not find in the literature confirmation of such behavior; however, some statements could be indicative. Kogut and Voelker (2001) mentioned that addition of high concentration of Cu, in order to estimate “overload” sensitivity, is not possible in practice because of loss of the linearity. In absence of published experimental evidences, such tendency obviously remains hypothetical and has to be confirmed by further experimental studies. However, it suggests that a correction of RAL only based on [SA]T could not be sufficient and would need more advanced approach in treatment of the titration data in order to estimate complexation parameters. Although still not implemented, ProMCC is suitable to easily accept a proposed variable RAL approach.

5. Final notes and recommendations

The ProMCC is a tool developed over the years in which traditional, but also new methodologies in handling complexometric titration data for calculation of metal complexation parameters in natural waters (i.e. interaction of metals and organic ligands) are incorporated in a user friendly form. However due to related specificities and complexity of the subject, it should NOT be used in a simple manner: “press the button and get the value”, meaning that users should critically evaluate the obtained results based on their own experience. The particular feature of ProMCC is its graphical interface, facilitating visualization of data using graphs of multiple transformations, and as such, helps in decision of accepting the model and goodness of fit by using “expert eye” approach.

Design of an experiment with adequate distribution of titration points and acquisition of data of good quality (low scatter/noise) are the first important steps in obtaining reliable complexation parameters. Most commonly, additions of titering metal are selected in a way to represent “logarithmic” distribution. As the number of ligands and the range of ligand concentrations is a priori not known, this titration mode provides the highest probability of covering more than one ligand class. The recurrent question is about the sufficient number of titration points. As for any other analytical method, the repetitive measurement is strongly recommended. We recommend measurement of each titration point least in triplicate. If regular drift in signal intensity (increase or decrease) with repetitive measurement at each titration point is observed (e.g. non-equilibrium conditions), the titration should be declared as non-valid and the sources of drift should be discovered and eliminated, and titration repeated. There is no exact number of titration points that is optimal. For example, it is certain that in case of one-ligand model, a lower number of titration points is required than in case of a two-ligand model. However, both cases imply that the adequate titration range is covered. Based on literature data and on our experience, the optimal number of titration points ranges between 10 and 15. In case of applying multiple detection window approach and analyzing all data as a unique dataset, the optimal number of titration points for each separate detection window could be lower (and different among the detection windows), however we advocate of performing separate titrations with at least 10 titration points. In order to benefit in this subject, users are advised to perform simulated titration experiments offered within ProMCC.

Once titration data (true experimental or simulated) are acquired, the second important step is the selection of the correct complexation model on which data will be fitted, i.e. one-, two- or three-ligand model. Visualization of the transformed data would strongly help in the decision, and thus, users are advised to carefully examine all transformation plots in both, linear and logarithmic axis scales.

The next crucial and very complex issue in obtaining reliable complexation parameters is the analytical sensitivity which is needed to transform measured signal intensities to concentrations. As it is a priori not known, users should follow one of the existing approaches (e.g. internal calibration, recursive iterative adjustment, fitted parameter). While it was found that “internal calibration” method tends to underestimate the sensitivity, iterative approach in sensitivity adjustment (implemented in ProMCC) provides reasonable estimates. The alternative/complementary approach is to incorporate sensitivity as unknown parameter in the fitting/optimizing process. Although from the statistical point of view this approach provides most correct fitting parameters uncertainties, it does not necessary lead to the most correct values. As the TRUE sensitivity will remain basically unknown for the real complexometric titration experiment, we suggest that users examine different approaches in sensitivity adjustment and to choose the one for which they think it is the most representative.

The ProMCC is developed in order to be used by both, experts and non-experts in data treatment, however as for any other software/instrument, user is referred to the “User manual” in which the program features and instructions of use are described. ProMCC is permanently under development, respecting feedbacks of users, as well as incorporating newly developed theories/methodologies. Further versions of ProMCC will offer also a “master-version” for the “complete complexation model” fitting tool, allowing to fit data of various experimental approaches (adsorption isotherm, metal titration, acid–base titration, fluorescence quenching, ...), and of more complex chemical systems (e.g. more than 3 ligands, bi- or multi-dentate ligands, competition effects, ...), while also simultaneously optimizing others parameters (e.g. sensitivity, RAL, ...), as allowed in original PROSECE version.

ProMCC is available free of charge. At current stage, licensing is not needed to use ProMCC, however, users will be invited to provide some basic information to authors. The latest version of ProMCC can be found at: https://sites.google.com/site/mccprosece/.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.marchem.2014.10.011.


