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RELIABILITY OF PROTONATION CONSTANTS OF SNAZOXS OLIGOMERS

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Summary—Concentration protonation constants of variously protonated oligomers of sulphoazoxine SNAZOXS were determined by regression analysis of potentiometric titration curves. The group and common parameters were estimated using different computational strategies of three regression programs, MINIQUAD, MIQUV and PSEQUAD. ANOVA proved that six various computational strategies of three regression programs have no significant influence on reliability of protonation constants estimated in comparison with the reproducibility of the titration. Chemical model of protonation equilibria L_2H^{5-}, $L_2H_2^{4-}$, $L_2H_3^{3-}$, and $L_2H_4^{2-}$ and reaction scheme of oligomers protonation for SNAZOXS was found.

Protonation and complex-forming equilibria of some sulphoazoxine have been studied systematically in our laboratory. Protonation constant of sulphoazoxine oligomers were evaluated by regression analysis of potentiometric electromotive force–*emf* titration curves.[1,2] While the program MINIQUAD,[3] MIQUV,[4] or PSEQUAD[5] refines the common parameters β_{qr} for species L_qH_r only, the program ESAB,[6] MAGEC[7] or SUPERQUAD[8] enables refinement of the group parameters standard potential E^0, Nernstian slope, concentrations of titrand and titrant, *etc.*

Protonation constants estimated by regression analysis of potentiometric titration curves are affected[2] by (1) the used instrumental technique; (2) temperature T; (3) ionic strength; and (4) the used strategy of experimental technique encompassing the titration procedure, the kind of electrodes, the standardization of glass electrode cell and the reliability on concentrations of basic components L and H; and (5) the computational strategy of the regression algorithm used.

It was proved that the precision of group parameters (E^0, Nernstian slope, concentration of titrand and titrant, *etc.*) has strong effect on reliability of protonation constant β_{qr} and therefore the group parameters should be refined simultaneously with common parameters.[9] As several regression programs under different computational strategies enable simultaneous refinement of common and group parameters it would be interesting to check if the computational strategy of regression analysis has a significant influence on the value of common parameters β_{qr}.

Dissociation of protonated sulphoazoxine SNAZOXS, 7-(4-sulpho-1-naphtylazo)-8-hydroxyquinoline-5-sulphonic acid (LH_5^{2+}) at concentration lower than 10^{-6} mol/dm^3 when a monomer prevails in solution[10] may be expressed by the following:

$$LH_5^{2+} = LH_4^{+} + H^{+} = LH_3 + H^{+}$$
$$= LH_2^{-} + H^{+} = LH^{2-} + H^{+} = L^{3-} + H^{+},$$

where protonated ions of SNAZOXS are LH_5^{2+} with protonation constant β_{15}, LH_4^{+} with β_{14}, neutral molecule LH_3 with β_{13}, and anions LH_2^{-} with β_{12}, LH^{2-} with β_{11} and L^{3-}. Above this concentration oligomers are formed and even in the range $2 < pH < 4$ some sulphoazoxine agglomerates and sometimes precipitation occurs.

The aim of this study was to determine the oligomers formed by the SNAZOXS and their protonation equilibria. The reliability of estimated protonation constants and the reproducibility of the titration are discussed.

THEORY

Determination of protonation equilibria of oligomers

Assume that protons (H) and SNAZOXS ligand (L) form various species according to the reaction

$$rH + qL = L_qH_r(\beta_{qr}) \qquad (1)$$

charges are omitted for the sake of simplicity. The protonation constant is given by

$$\beta_{qr} = [L_q H_r]/(l^q h^r) \quad (2)$$

where h and l are the free concentrations of SNAZOXS [L] and hydrogen [H^+], respectively. The mass balance equations are

$$L = l + q\Sigma\beta_{qr} l^q h^r \quad (3)$$

$$H = h + r\Sigma\beta_{qr} l^q h^r \quad (4)$$

The activity coefficients are assumed to be kept constant by the ionic medium. For potentiometric *emf* titrations, the following relationship holds for the total hydrogen ion concentration:

$$L_{exp} = (L_0 V_0 + L_T V_T)/(V_0 + V_T) \quad (5)$$

$$H_{exp} = (H_0 V_0 + H_T V_T)/(V_0 + V_T) \quad (6)$$

where H_0 (or L_0) is the total initial concentration of hydrogen ions (or SNAZOXS) in the titrand, H_T (or L_T) is the total initial concentration of hydrogen ions (or SNAZOXS) in the titrant (for hydroxide $-H_T$ is given), V_0 is the initial volume of the titrand and V_T is the volume of titrant added from burette.

When a glass-saturated calomel electrode (SCE) electrode is used, the potential readout (or *emf*) may be written as follows

$$\begin{aligned} E &= E_H + E_j - E_{SCE} \\ &= E^0 + (RT/F)\ln h + (RT/F)\ln \gamma_H \\ &\quad + j_a h - j_b K_w/h - E_{SCE} \\ &= E^{0\prime} + S \log h \end{aligned} \quad (7)$$

where $E^{0\prime}$ is the formal standard potential of the glass electrode, γ_H is the activity coefficient for hydronium, $h = [H^+]$, E_j is the liquid-junction potential ($j_a h - j_b K_w/h$), and S is the slope of the electrode response, $(RT/F)\ln 10$, for Nernstian response.

An explicit equation for the titration volume, expressing the relation between the volume of titrant added $V_{T,i}$, monitored *emf* E_i, and the common (β_{qr}) and group parameters (p) are given by

$$V_{T,i} = f(E_i; \beta_{qr}, p) \quad (8)$$

in which the vector of common parameters β_{qr} contains protonation constants of all SNAZOXS oligomers formed, the vector of group parameters $p = (E^{0\prime}, S, K_w, E_j, L_0, H_0, X_0, L_T, H_T, X_T)$ containing besides the constants of the Nernst equation, E^0, S, E_j, the initial concentration of SNAZOXS, L_0, and the initial concentration of hydrogen-ion, H_0 in the titrand, the concentration of acid–base impurities X_0 (*i.e.* carbonates) as well as the corresponding quantities for the titrant, L_T, H_T and X_T; K_w is the operational ion product of water. In most cases group parameters cannot be determined independently with sufficient accuracy.

In most regression programs for treating *emf* data the task is to find a model and a set of protonation constants that give the "best" fit to the experimetal data. In ESAB the parameters β_{lr} and p are refined by minimizing the residual-square sum

$$U = \sum_{i=1}^{n} w_i (V_{T,exp,i} - V_{T,calc,i})^2 = \text{minimum} \quad (9)$$

where w_i is the statistical weight equal to

$$w_i = \frac{1}{\sigma_{V,i}^2} + \left(\frac{\delta V_T}{\delta E}\right)_i \sigma_{E,i}^2. \quad (10)$$

In MINIQUAD only common parameters β_{qr} are refined, by minimizing the residual-square sum U

$$U = \sum_{i=1}^{n} w_i (C_{exp,i} - C_{calc,i})^2 = \text{minimum} \quad (11a)$$

where C_i is the total concentration of SNAZOXS (L) or proton (H) at the ith point of the titration curve.

The program MIQUV[4] estimates those values β_{qr} which minimize the residual-squares sum U, again taken over all experimental points:

$$U = \sum_{i=1}^{n} w_i (E_{exp,i} - E_{calc,i})^2 = \text{minimum}, \quad (11b)$$

the statistical weight w_i is defined by equation (10). In MIQUV only instrumental uncertainties in the *emf* readings, σ_E, and in the added titrant volume, σ_V, are taken into account.

The program PSEQUAD[5] solves the mass-balance equations (3) and (4) by using a derivative method of minimizing the residual-squares sum U:

$$\begin{aligned} U = &\sum_{i=1}^{n} w_i (V_{T,exp,i} - V_{T,calc,i})^2 \\ &+ \sum_{j=1}^{n} \sum_{i=1}^{n} w_i (E_{exp,i} - E_{calc,i})_j^2 = \text{minimum} \end{aligned} \quad (11c)$$

where $V_{T,i}$ leads to residuals in titrant volume per the ith point or residual in L or H, and E_i leads to residuals in *emf* for the ith point as measured by the jth electrode. PSEQUAD is able to evaluate simultaneously different types

of measurements carried out in solutions of varying composition but also in a group of solutions, where the member of component differs from one group to another. As mentioned, there are several options, minimization of volume or total concentrations as well as various *emf*s either alone or together, such as $V_{T,i}$ and E_i together (orthogonal regression).

A number of models can be tested and the one that gives the lowest U value and also fulfils some other statistical criteria is selected as the most plausible (degree-of-fit test).

The degree-of-fit is performed by statistical analysis of the residuals in L and H as given in equations (11). Residuals should be randomly distributed about the predicted regression curve. Systematic departures indicate that the model is not adequate and/or some other parametric estimates are not satisfactory. The arithmetic mean of the residuals, $\bar{e}$, should be close to zero and the residual standard deviation, $s(\hat{e})$, should be close to the instrumental error of the variable from which residuals are calculated. The Hamilton R-factor[15] of relative fitness is conveniently expressed in percent and permits a comparison of the fit obtained by different titrations.

Accuracy of protonation constants for oligomers

To classify an accuracy of protonation constants estimated the value of each protonation constants $\log \beta_{qr}$ is considered to consist of the "true" value μ and several sources of systematic error according to the relation

$$\log \beta_{qr} = \mu + \epsilon_{cell} + \epsilon_{conc} + \epsilon_{alg} + \epsilon \qquad (12)$$

where ϵ_{cell} is the systematic error due to uncertain values of electrochemical (group) parameters E^0, and S; ϵ_{conc} is the systematic error due to uncertain values of concentrations (group parameters) H_0, H_T and L_0, L_T; ϵ_{alg} is the systematic error due to failing or false minimization process of regression algorithm; ϵ is the random error.

To evaluate ϵ_{alg} various computational strategies of regression algorithms may be used. The systematic error ϵ_{alg} in protonation constants may be estimated as the factor α_i in the one-way analysis of variance. The objective of an analysis of variance (ANOVA) is to investigate the effect of various factors on the variability of protonation constants and to determine which part of variation in a population of protonation constants estimated is due to systematic causes called factors and which is due to *random effect*. The choice of a computational technique in regression estimation of protonation constants is called here the controlled factor. Moreover, the results of estimated protonation constants are subject to random errors. The analysis of variance compares both causes of error deciding whether or not the controlled factor has a significant effect.

The model of the response in one-factor ANOVA can be written

$$y_{ij} = \mu_i + \epsilon_{ij} \qquad (13)$$

where y_{ij} represents the jth protonation constant being repeated n_i times, $j = 1, 2, \ldots, n_i$, at the ith computational strategy of regression estimation of protonation constants, or shortly at the ith treatment. There are k different computational strategies examined, $i = 1, 2, \ldots, k$, and μ_i is the true response (mean) at a given computational strategy while ϵ_{ij} is the random error present in the jth protonation constant at the ith treatment. The mean μ_i may be divided into two parts

$$\mu_i = \hat{\mu} + \alpha_i, \qquad (14)$$

where $\hat{\mu}$ represents the estimation of an overall mean and α_i represents the effect of the ith treatment. The total number of estimated protonation constants is $n = n_1 + n_2 + \cdots + n_k$.

Let $\hat{\mu}_i$ denote the mean of protonation constants on the ith treatment

$$\hat{\mu}_i = \frac{1}{n_i} \sum_{j=1}^{n_i} y_{ij} \qquad (15)$$

The overall mean $\hat{\mu}$ of all protonation constants can be defined

$$\hat{\mu} = \frac{1}{k} \sum_{i=1}^{k} \hat{\mu}_i = \frac{1}{n} \sum_{i=1}^{k} \sum_{j=1}^{n_i} y_{ij} \qquad (16)$$

Equations (15) and (16) determine the estimates of means μ_i by equation (13) or μ in (14). The estimate of effect α_i was calculated by

$$\hat{\alpha}_i = \hat{\mu}_i - \hat{\mu}. \qquad (17)$$

In cases where we have the same sample size for each treatment, the following condition holds true

$$\sum_{i=1}^{k} \hat{\alpha}_i = 0. \qquad (18)$$

The null hypothesis that there is no treatment effect on protonation constant, *i.e.* the hypothesis of equal population means H_0: $\mu_1 = \mu_2 = \cdots = \mu_k = \mu$ is tested first. The analysis

begins by partitioning the sum of squared deviation from the overall $\hat{\mu}$ into two components, the component attributed to a computational strategy S_T and the component representing the variation due to random errors S_R. Here

$$S_T = \sum_{i=1}^{k} n_i(\hat{\mu}_i - \hat{\mu})^2, \tag{19}$$

represents the variability between individual treatments and

$$S_R = \sum_{i=1}^{k} \sum_{j=1}^{n_i} (y_{ij} - \hat{\mu}_i)^2 \tag{20}$$

represents the variability within all treatments. Unbiased estimate of variance of errors σ^2 is the mean square of residuals defined by

$$\hat{\sigma}^2 = S_R/(n-k). \tag{21}$$

The null hypothesis means an equality of all treatments, *i.e.* insignificance of effect $H_0: \alpha_i = 0$, $i = 1, \ldots, k$ while the alternative hypothesis being expressed $H_A: \alpha_i \neq 0$, $i = 1 \ldots, k$. The test is based on the fact that S_T/σ^2 has a χ^2-distribution with $(k-1)$ degrees of freedom whilst S_R/σ^2 has an independent χ^2-distribution with $(n-k)$ degrees of freedom. Their ratio follows the Fisher–Snedecor F-distribution with $(k-1)$ and $(n-k)$ degrees of freedom

$$F = \frac{S_T(n-k)}{S_R(k-1)}. \tag{22}$$

When F is greater than the quantile $F_{1-\alpha}(k-1, n-k)$, the null hypothesis is rejected, and the effect of computational strategy is taken as significant. Then the total variance σ^2 is only related to an uncontrolled (random) factor and may serve as the estimate for a replication variance.

EXPERIMENTAL

Chemicals and solutions

SNAZOXS of analytical-reagent grade was obtained from Spolana (Neratovice, Czech Republic) and purified as described previously.[2] The actual concentration of SNAZOXS in each *emf* titration was determined by *emf* titration with NaOH and evaluated by regression analysis of ESAB[6] and MAGEC[7] programs and the $\log h$ scale. Impurities in SNAZOXS were mostly inorganic salts.

Perchloric acid. A 1*M* solution was prepared by dilution of 70% $HClO_4$ of analytical-reagent grade with doubly distilled, de-ionized water and standardized against HgO and KI using the Gran method in MAGEC program.

Sodium hydroxide. A 1*M* solution and carbonate-free, was prepared by reaction of sodium in doubly distilled water, deionized and deaerated water in atmosphere of argon and under efficient cooling at temperature around 275 K.

Apparatus

All *emf* measurements were made at 298.0 ± 0.1 K, by means of an OP-208/1 digital voltmeter (Radelkis, Budapest) with a G202B glass electrode and an OP-0830P SCE reference electrode (Radelkis, Budapest). A water-jacketed 100 ml glass vessel, closed with a Teflon bunk carrying the electrodes argon inlet, thermometer, stirrer and the microburette capillary tip, was used for the titrations.

During the titrations a stream of argon was bubbled through the solution both for stirring and for maintaining an inert atmosphere. The argon was passed through the pure ionic medium before entering the equilibrium solution.

The burettes used were home-made syringe microburettes with micrometer screw, type PK1250 of capacity 1250 μl.

Procedure of "equilibrium titration"

To determine chemical model of protonation equilibria of sulphoazoxine SNAZOXS the procedure of following steps was applied.

Step (1). Standardization of perchloric acid: c_{HClO_4}. Perchloric acid was standardized on HgO and KI and titration curve was evaluated by Gran method (MAGEC).

Step (2). Calibration of glass electrode cell: $E^{0\prime}$, S, (pK_w), H_T (and X_T). The hydrogen concentration $[H^+] = h$ is known from an initial concentration H_0 and measured *emf*, E. From $E = E^{0\prime} + S \log h$ for each point $\{E, h\}$ of titration curve of known concentration of perchloric acid with standard sodium hydroxide, the group parameters E^0 and S were estimated.

Step (3). Determination of concentration of SNAZOXS and hydrogen ions: L_0, H_0. To analyze an *emf*-titration curve concerning a mixture of sulphoazoxine SNAZOXS and $HClO_4$ with NaOH by ESAB or MAGEC, the content of SNAZOXS L_0 and the content of protons H_0 were determined.

A mixture containing 10.00 ml 0.01*M* SNAZOXS and 0.265 ml 1*M* $HClO_4$ ($L_0^{(0)} = 0.01$ mol

/dm³, $H_0^{(0)} = 0.05$ mol/dm³) was titrated with 1.00*M* sodium hydroxide ($-H_T = 1.00$ mol/dm³) and the *emf*, *E*, was read. The temperature was kept constant at 298 ± 0.2 K.

Step (4). Protonation equilibria of SNAZOXS: $\{q, r\}$ and β_{qr}. To analyze a set of *emf*–titration curves concerning a mixture of sulphoazoxine SNAOXS and $HClO_4$ with NaOH by MINIQUAD when previously estimated values of group parameters E^0, S, H_0, H_T are used, the chemical model of a number of oligomers, their stoichiometry $\{q, r\}$ and protonation constants β_{qr} was determined.

Step (5). Accuracy of $\log \beta_{qr}$ by the analysis of variance: ϵ_{alg}. The null hypothesis H_0: $\epsilon_{alg} = 0$ *vs* H_A: $\epsilon_{alg} \neq 0$ in order to find the effects of programs on protonation constants are significant. When F is greater than the quantile $F_{1-\alpha}(k-1, n-k)$, the null hypothesis is rejected, and the effect of computational strategy is taken as significant.

All computations using regression programs MINIQUAD,[3] MAGEC,[7] ESAB[6] and CHEMSTAT[11] (Analysis of Variance) were done on IBM PC-AT.

RESULTS AND DISCUSSION

Search for the best model of oligomers

A mixture of SNAZOXS and perchloric acid was titrated with sodium hydroxide using the four steps of "equilibrium titration" cited above and the whole procedure was reproduced six times. Concentration of SNAZOXS was 0.1 mol/dm³. All six potentiometric $E = f(V_T)$ titration curves were analyzed by MINIQUAD to search for the best chemical model of protonation equilibria of oligomers and 20 different models were tried to fit the data. For each data set, distribution functions can be obtained in the printout such that in a certain concentration range only the species with major contributions are used in the model search.

In this search certain criteria have to be fulfilled: (i) the degree-of-fit is achieved by statistical analysis of residuals; (ii) all the species

Table 1. Search for the best model for the formation of oligomers in the system H^+-SNAZOXS by regression analysis of one potentiometric curve using the program MINIQUAD. Besides the estimated standard deviations in units of the last digits in $\log \beta_{qr}$ written in parentheses the highest percentage of each species concentration $[L_qH_r]_{max.}$ in equilibrium mixture is presented at actual value of pH

	Group parameters						
L [mol/dm³]	0.01100						
H [mol/dm³]	0.05406						
Range pH	1.804–9.829						
Points	58						
E^0 [mV]	368.7						
S [mV/pH]	58.54						
	Common parameters						
Species are described by $\log \beta_{qr}(s(\log \beta_{qr}))$, $[L_qH_r]_{max.}$ at pH value							
Hypothesis	A	B	C	D	E	F	G
LH	8.02(1) 98.3, 6.1	—	—	Negat.	—	—	—
LH_2	11.86(3) 99.1, 1.8	—	—	Negat.	—	—	—
L_2H	—	—	10.22(3) 36.9, 8.3	Posit.	Posit.	Posit.	Posit.
L_2H_2	—	18.17(5) 97.7, 6.4	18.17(2) 96.3, 6.1	Posit.	Posit.	Posit.	Posit.
L_2H_3	—	22.51(13) 63.0, 3.8	22.52(3) 63.2, 3.8	Posit.	Posit.	Posit.	Posit.
L_2H_4	—	25.80(14) 96.8, 1.8	25.80(3) 96.7, 1.8	Posit.	Posit.	Posit.	Posit.
L_2H_5	—	—	—	—	Negat.	—	—
L_3H_2	—	—	—	—	—	Negat.	—
L_3H_4	—	—	—	—	—	Negat.	—
L_4H_2	—	—	—	—	—	—	Negat.
L_4H_4	—	—	—	—	—	—	Negat.
Statistical analysis of residuals as the criterion of model search							
$U_{min} \times 10^5$	0.29	1.3	0.084				
$\lvert\bar{e}\rvert \times 10^5$	2.8	6.8	0.43				
$s(\hat{e}) \times 10^4$	1.3	3.4	0.85				
R-factor [%]	1.21	2.56	0.65				
Test of H_0	Rejec.	Rejec.	Accept.	as (C)	as (C)	as (C)	as (C)

found must have meaningful concentrations; and (iii) the standard deviations in $\log \beta_{qr}$ are examined and tested. In this way the following species were found: dimers L_2H, L_2H_2, L_2H_3, and L_2H_4 (charges have been omitted for simplicity). Table 1 shows an example of search for the best model when only seven models are present. Species that contribute less than 5% in the concentration range studied have been rejected. The first model A assumes that no aggregates are formed and only the protonation constants of LH and LH_2 are to be calculated. The constants $\log \beta_{11} = 7.5$ and $\log \beta_{12} = 10.2$ obtained by spectrophotometry in very dilute solution (1 μmol/dm^3) are used as input values. On minimization this model terminates with a poor fit to the experimental titration curve, indicating that the model is inadequate.

In the second model β_{11} and β_{12} are kept constant while β_{22}, β_{23}, and β_{24} are estimated and so on; in Table 1 another five models are tested. A low value of the Hamilton R-factor proving a good degree-of-fit achieved can be regarded as that giving species a physical meaning. As the total monomer content was always less than 1% they have been excluded from the final model. In addition to dimers, trimers and tetramers (models *E*, *F* and *G* were also tested but in most instances were rejected from the final model using the criteria mentioned above. The final model for SNAZOXS is described by model C.

Effect of computational strategy on protonation constants

To increase the reliability of protonation equilibria of SNAZOXS means to avoid or to minimize systematic errors ϵ_{cell}, ϵ_{conc} and ϵ_{alg} in β_{qr}, cf. equation (12). The ϵ_{cell} and ϵ_{conc} may be found by experimental strategy[9] while ϵ_{alg} by computational strategy of regression algorithms.

Besides regression program MINIQUAD also other different regression algorithms were used. Table 2 gives the results of treating the data for protonation of SNAZOXS with pro-

Table 2. Protonation constants $\log \beta_{qr}$ of oligomers estimated from six repeated titrations by seven regression techniques: A: MINIQUAD(L, H) B: PSEQUAD(pH); C: PSEQUAD(orto pH–V); D PSEQUAD(V); E: PSEQUAD (back) reverse calculation; F: PSEQUAD(orto V-pH); G$^+$: MIQUV(E), for $T = 298$ K and parameters $E^{0\prime}$ [mV], S [mV/pH] determined with MAGEC (for G$^+$ $S = 59.16$ mV/pH). The standard deviations $s(\log \beta_{qr})$ in units of last valid digits of $\log \beta_{qr}$ are in parentheses. The outliers denoted by a star * have not been used in calculation of means μ_t

Program		Titration 1	2	3	4	5	6
Points		60	69	66	61	58	63
$E^{0\prime}$ [mV]		367.9	369.0	368.2	368.4	368.7	366.7
S [mV/pH]		58.30	58.40	58.46	58.37	58.54	58.58
Range pH		1.8–10.0	1.7–9.8	1.9–10.0	1.8–9.7	1.8–9.8	1.8–10.1
Log β_{21}	A	10.27(3)	10.25(3)	10.31(5)	10.15(2)	10.22(3)	10.28(3)
	B	10.25(7)	10.08(6)	10.29(4)	10.13(3)	10.19(6)	10.28(6)
	C	10.23(4)	10.13(3)	10.23(5)	10.13(3)	10.19(3)	10.26(4)
	D	10.23(4)	10.12(3)	10.28(5)	10.13(3)	10.19(3)	10.25(4)
	E	10.23(4)	10.13(3)	10.20(5)	10.12(3)	10.20(3)	10.25(4)
	F	10.24(2)	—	10.23(24)	10.12(2)	10.20(2)	—
	G$^+$	10.1(5)	10.1(1)	10.1(2)	10.0(2)	10.1(1)	10.2(1)
Log β_{22}	A	18.19(2)	18.23(2)	18.28(3)	18.09(1)	18.17(2)	18.15(2)
	B	18.29(5)*	18.18(4)	18.24(3)	18.13(20)	18.29(5)*	18.28(6)
	C	18.17(2)	18.10(2)	18.14(3)	18.08(2)	18.17(2)	18.15(2)
	D	18.17(2)	18.11(2)	18.18(3)	18.08(2)	18.17(2)	18.13(2)
	E	18.17(3)	18.11(2)	18.12(3)	18.08(2)	18.18(2)	18.14(2)
	F	18.18(2)	—	18.15(3)	18.08(1)	18.18(2)	—
	G$^+$	18.0(2)	18.0(1)	18.1(1)	17.9(1)	18.0(1)	18.0(1)
Log β_{23}	A	22.55(3)	22.58(3)	22.73(5)	22.39(2)	22.52(3)	22.48(3)
	B	22.83(7)*	22.59(5)	22.62(4)	22.50(3)	22.83(7)*	22.81(7)*
	C	22.52(3)	22.39(2)	22.47(4)	22.39(2)	22.51(3)	22.47(3)
	D	22.53(3)	22.39(2)	22.53(4)	22.39(2)	22.51(3)	22.44(3)
	E	22.53(4)	22.39(2)	22.44(5)	22.38(2)	22.52(3)	22.45(3)
	F	22.55(2)	—	22.47(3)	22.39(2)	22.54(2)	—
	G$^+$	22.3(3)	22.3(1)	22.5(1)	22.1(1)	22.3(1)	22.28(1)
Log β_{24}	A	25.93(3)	25.92(3)	26.15(5)	25.74(2)	25.80(3)	25.84(3)
	B	26.12(8)*	25.80(6)	25.82(5)	25.82(4)	26.01(1)	26.07(8)
	C	25.89(3)	25.65(2)	25.63(4)	25.73(1)	25.79(2)	25.84(3)
	D	25.90(3)	25.66(2)	25.73(3)	25.73(2)	25.79(2)	25.81(3)
	E	25.90(3)	25.66(2)	25.59(5)	25.72(2)	25.80(3)	25.82(3)
	F	25.92(3)	—	25.67(3)	25.74(2)	25.81(3)	—
	G$^+$	25.6(3)	25.6(1)	25.8(1)	25.4(1)	25.5(1)	25.6(1)

Table 3. The treatment means μ_i and the effect of treatment α_i, $i = 1, \ldots, 6$, of repeated titrations evaluated by seven computational strategies: A: MINIQUAD(L, H); B: PSEQUAD (pH); C: PSEQUAD(orto pH–V); D: PSEQUAD(V); E: PSEQUAD(back) reverse calculation; F: PSEQUAD(orto V-pH); and G$^+$: MIQUV(E). Into the overall mean $\hat{\mu}$ found with ANOVA the protonation constant estimated with MIQUV was not included. ANOVA tests H_0: $\epsilon_{alg} \simeq 0$ *vs* H_A: $\epsilon_{alg} > 0$; $F_{crit}(0.95, 5, 25) = 2.558$

Computational strategy	$\log \beta_{21}$ μ_i ($\alpha_i \times 1000$)	$\log \beta_{22}$ μ_i ($\alpha_i \times 1000$)	$\log \beta_{23}$ μ_i ($\alpha_i \times 1000$)	$\log \beta_{24}$ μ_i ($\alpha_i \times 1000$)
A	10.247(42)	18.185(27)	22.544(46)	25.897(87)
B	10.203(−2)	18.207(49)	22.695(74)	25.904(94)
C	10.193(−12)	18.135(−23)	22.458(−37)	25.755(−55)
D	10.202(−3)	18.140(−18)	22.465(−31)	25.770(−40)
E	10.188(−17)	18.133(−25)	22.452(−44)	25.748(−62)
F	10.197(−8)	18.148(−11)	22.487(−8)	25.785(−25)
G$^+$	10.100	17.985	22.303	25.573
μ	10.205(63)	18.158(49)	22.496(77)	25.810(114)
F_{exp}	0.666	1.919	1.878	2.174
Conclusion	Accepted	Accepted	Accepted	Accepted

grams MINIQUAD (residuals in L and H), PSEQUAD (residuals in pH), PSEQUAD (residuals in pH and V, orthogonal regression), PSEQUAD (residuals in V), PSEQUAD (reverse calculation), PSEQUAD (residuals in V and pH) and MIQUV (residuals in E). The results obtained on treating each titration separately and for all six titration together are given. The mean of protonation constants on the ith treatment and an overall of the protonation constants were calculated and are given in Table 3. The analysis of variance was applied in order to investigate possible differences between six computational strategies used.

It is interesting that a much better fit with the experimental data is obtained with $\{E, V_T\}$ data compared with the normalized variables $\{pH, V_T\}$. It is recommended to use primary variable *emf* than the transformed pH as logarithmic transformation change an original distribution of random errors in response E. Outliers in $\log \beta_{qr}$ were found only in protonation constants estimated by program B:PSEQUAD (pH) when residuals are in pH. The effect of treatment (computational strategy) α_i in Table 3 is of magnitude of several hundreds in $\log \beta_{qr}$ what means small enough and comparable with experimental random errors. Statistical testing by the Fischer–Snedecor test leads to the conclusion that the differences between programs are not significant, *i.e.* ϵ_{alg} in β_{qr} may be taken as equal to zero.

Whenever an ANOVA F-test for simultaneously compared treatment means is performed it is also customarily of interest to determine which specific differences there are among the treatment means. Such specific comparisons may have been of interest to the investigator before the data were collected or may arise in completely exploratory studies only after the data have been examined. In either event, a seemingly reasonable first approach to making inferences about differences among the treatment means, would be to make several t-tests and to focus on all those tests found significant. Testing differences between treatment means in ANOVA setting the multiple comparisons technique are created. In Scheffe's multiple comparison procedure[12] the null hypothesis H_0: $\mu_i = \mu_j$ is rejected for all pairs of (i, j) treatments for which is valid

$$|\hat{\mu}_i - \hat{\mu}_j| \geq \sqrt{(k-1) \cdot \sigma^2 \cdot F_{1-\alpha}(k-1, n-k) \cdot [1/n_i + 1/n_j]} \quad (23)$$

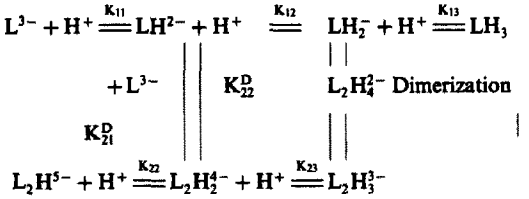
Table 4. Test specific difference among means μ_i, $i = 1, \ldots, 6$, estimated by different computational techniques in the following order of protonation constants $\log \beta_{21}$, $\log \beta_{22}$, $\log \beta_{23}$, $\log \beta_{24}$ and indicated in the table as (++++) where (+) means that the null hypothesis H_0: $\mu_i = \mu_j$ was accepted while (−) means rejected. For each combination of regression technique the agreement of four protonation constants $\log \beta_{qr}$ is tested by ANOVA in package CHEMSTAT

	B	C	D	E	F
A	++++	++++	++++	++++	++++
B		++−+	++−+	++−+	+−++
C			++++	++++	++++
D				++++	++++
E					++++

where n is the total number of protonation constants, k is number of treatment means, n_i and n_j are the sizes of the ith and jth treatments, respectively, $\hat{\sigma}^2$ is the estimate of variance calculated by equation (23). This equation is used for all pairs of indices (i,j) and results are in Table 4. Acceptance of the null hypothesis H_0 denoted by (+) means that two compared values of $\log \beta_{qr}$ estimated by different computational strategies are statistically identical. The only B: PSEQUAD (pH) differ in results from other computational strategies used.

CONCLUSIONS

It may be concluded that much better fit with the experimental data is obtained when residuals are in L, H or V_T and the group parameters are also refined. The influence of group parameters is much more significant than computational strategy of regression algorithm. Search of the best model of protonation equilibria leads to the following tentative reaction shown in Scheme 1. The stepwise protonation constants were obtained from $K_{12} = \beta_{12}/\beta_{11}$, $K_{13} = \beta_{13}/\beta_{12}$, $K_{22} = \beta_{22}/\beta_{21}$ and $K_{24} = \beta_{24}/\beta_{23}$. The dimerization constants were obtained from



Scheme 1.

$K_{21}^{D} = \beta_{21}/\beta_{11}$, $K_{22}^{D} = \beta_{22}/\beta_{11}^2$, $K_{23}^{D} = \beta_{23}/(\beta_{12}\beta_{11})$ and $K_{24}^{D} = \beta_{24}/\beta_{12}^2$.

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