Spectrochimica Acta Part A 86 (2012) 305–314

Contents lists available at SciVerse ScienceDirect

Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy

journal homepage: www.elsevier.com/locate/saa

Reliability of dissociation constants and resolution capability of SQUAD(84) and SPECFIT/32 in the regression of multiwavelength spectrophotometric pH-titration data

Milan Meloun[a,*], Zuzana Ferenčíková[a], Milan Javůrek[b]

[a] Department of Analytical Chemistry, University of Pardubice, CZ 532 10 Pardubice, Czech Republic
[b] Department of Process Control, University of Pardubice CZ 532 10 Pardubice, Czech Republic

ARTICLE INFO

Article history:
Received 1 June 2011
Received in revised form 23 August 2011
Accepted 17 October 2011

Keywords:
Spectrophotometric titration
Dissociation constant
SPECFIT/32
SQUAD(84)
Physostigmine salicylate

ABSTRACT

The resolving power of multicomponent spectral analysis and the computation reliability of the stability constants and molar absorptivities determined for five variously protonated anions of physostigmine salicylate by the SQUAD(84) and SPECFIT/32 programs has been examined with the use of simulated and experimental spectra containing overlapping spectral bands. The reliability of the dissociation constants of drug was proven with goodness-of-fit tests and by examining the influence of pre-selected noise level $s_{inst}(A)$ in synthetic spectra regarding the precision $s(pK)$ and also accuracy of the estimated dissociation constants. Precision was examined as the linear regression model $s(pK) = \beta_0 + \beta_1 s_{inst}(A)$. In all cases the intercept β_0 was statistically insignificant. When an instrumental error $s_{inst}(A)$ is small and less than 0.5 mAU, the parameters' estimates are nearly the same as the bias $\Delta pK = pK_{a,calc} - pK_{a,true}$ is quite negligible. In all four dissociation constants the bias seems to be quite small even though for pK_{a4} it is a little bit higher, i.e., +0.05 for $s_{inst}(A)$ about 1.0 mAU. In the interval of $s_{inst}(A)$ from 0.1 to 1.0 mAU all four dissociation constants pK_i are accurate enough. Of the various regression diagnostics considered, the goodness-of-fit is the most efficient criterion of whether the parameters found adequately represent the data. The magnitude of instrumental error $s_{inst}(A)$ only slightly affects the shape of a Cattel's scree graph $s_k(A) = f(k)$ to determine the true number of light-absorbing species in the equilibrium mixture.

1. Introduction

The programs for multicomponent spectral analysis [1–8] can facilitate the identification and resolution of individual components of a mixture and also determine the protonation constants and molar absorptivities of variously protonated species in solution equilibria. Multi-wavelength spectrophotometric pH-titration data in general offer considerably more information than potentiometric titration data about chemical equilibria. As shown earlier, SQUAD(84) [4–6] and SPECFIT/32 [8–12] are particularly reliable and efficient diagnostic tools.

In their previous work [13–23] the authors have shown that the spectrophotometric method in combination with suitable chemometric tools can be used to determine protonation constants β_r or acid dissociation constants K_a even for barely soluble drugs. Spectrophotometry is a convenient method for K_a determination in very diluted aqueous solutions (about 10^{-5}–10^{-6} M), providing that the compound possesses pH-dependent light absorption due to the presence of a chromophore in proximity to the ionization centre *cf.* Refs. [24–32]. There are many cases in which the spectral responses of two and sometimes even more components overlap considerably, and analysis is not straightforward [15]. Problems arise because of strong overlapping chemical components involved in the equilibrium, and uncertainties arising from the mathematical algorithms used to solve such problems [16]. In such cases, much more information can be extracted if multivariate and multiwavelength spectrophotometric data are analyzed by means of appropriate multivariate data-analysis software.

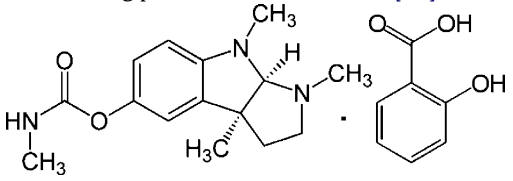
The most relevant algorithms are the hard-modelling programme SQUAD(84) [5–7] with the Newton–Raphson method in nonlinear least-squares minimization and the soft-modelling programme SPECFIT/32 [8–10,12] with the Levenberg–Marquardt minimization method. The resolving power of these two programs has now been tested by estimating four dissociation constants of physostigmine salicylate from spectral measurements; it should be noted that the spectral bands of the individual variously protonated anions overlap. The reliability of determining dissociation constants and molar absorptivity was examined using simulated and experimental data; this was done as a function of the instrumental error of absorbance reading for mixtures in which some

* Corresponding author. Tel.: +420 466037026; fax: +420 466037068.
E-mail address: milan.meloun@upce.cz (M. Meloun).

1386-1425/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.saa.2011.10.041

species involved in the protonation equilibria were of a similar colour. The efficiency of both programs has been verified and a strategy of efficient computation was used as described previously [14].

Recently, the alkaloid *physostigmine salicylate*, also known as eserine ($C_{15}H_{21}N_3O_2$) is the carbaminic acid ester of 2-hydroxy-(3aS-cis)-1,2,3,3a,8,8a-hexahydro-1,3a,8-trimethylpyrrolo[2,3-b]-indol was studied in our laboratory. The actual drug in this case is the salicylate of physostigmine (physostigminium salicylicum) having a molecular weight of 413.46 and a melting point of 185–187 °C, Ref. [33].



The published dissociation constant is $pK_a = 8.27$ at $I = 0.1$ ($NaClO_4$) and 25 °C, Ref. [17]. It belongs to the therapeutic category – cholinergic (anticholinesterase), miotic. The protonation scheme of physostigmine salicylate predicted with SPARC programme brings Fig. 1.

2. Theoretical

2.1. Procedure for the determination of the protonation/dissociation constants

An acid–base equilibrium of the drug studied is described in terms of the thermodynamic protonation of the Brönsted base L^{z-1} according to the equation $L^{z-1} + H^+ \rightleftarrows HL^z$ characterized by the protonation constant

$$K_H = \frac{a_{HL^z}}{a_L^{z-1} a_{H^+}} = \frac{[HL^z]}{[L^{z-1}][H^+]} \frac{y_{HL^z}}{y_{L^{z-1}} y_{H^+}} \quad (1)$$

where $y_{HL^z}, y_{L^{z-1}}, y_{H^+}$ are the activity coefficients of particular species. The protonation equilibria between the anion L (the charges are omitted for the sake of simplicity) of a drug and a proton H are considered to form a set of variously protonated species L, HL, H_2L, H_3L, etc., which have the general formula H_rL in a particular chemical model and which are represented by n_c the number of species, r_i, $i = 1, \ldots, n_c$ where index i labels their particular stoichiometry; the overall protonation (stability) constant of the protonated species, β_r, may then be expressed as

$$\beta_{qr} = \frac{[L_qH_r]}{[L^q][H]^r} = \frac{c}{l^q h^r} \quad (2)$$

where the free concentration $[L] = l$, $[H] = h$ and $[L_qH_r] = c$. For dissociation reactions realized at constant ionic strength the so-called "mixed dissociation constants" are defined as

$$K_{a,j} = \frac{[H_{j-1}L]a_{H^+}}{[H_jL]} \quad (3)$$

As each aqueous species is characterized by its own spectrum, for UV/vis experiments and the ith solution measured at the jth wavelength, the Lambert–Beer law relates the absorbance, A_{ij}, being defined as

$$A_{ij} = \sum_{n=1}^{n_c} \varepsilon_{j,n} c_n = \sum_{n=1}^{n_c} (\varepsilon_{r,j} \beta_r l h^r)_n \quad (4)$$

where $\varepsilon_{r,j}$ is the molar absorptivity of the H_rL species with the stoichiometric coefficient r measured at the jth wavelength and $q = 1$. The absorbance A_{ij} is an element of the absorbance matrix $\boldsymbol{A}$ of size $(n_s \times n_w)$ being measured for n_s solutions with known total concentrations of $n_z = 2$ basic components, c_L and c_H, at n_w wavelengths.

2.2. Multicomponent spectral analysis

The multicomponent spectral analysis programs with experimental and computational strategy were described previously [13–16]. The parameters to be determined are (i) the stoichiometric indices, (ii) the protonation stability constants β_r and molar absorptivities ε_r and (iii) the free concentrations of all the species in the estimated chemical model. A multicomponent spectral analysis program can adjust $\boldsymbol{\beta}_{qr}$ and $\boldsymbol{\varepsilon}_{qr}$ for absorption spectra by minimizing the residual-square sum function *RSS* denoted here as $U(b)$,

$$tU_b = \sum_{i=1}^{n_s}\sum_{j=1}^{n_w} (A_{\mathrm{exp},i,j} - A_{\mathrm{calc},i,j})^2 = \sum_{i=1}^{n_s}\sum_{j=1}^{n_w} \left(A_{\mathrm{exp},i,j} - \sum_{k=1}^{n_c} \varepsilon_{j,k} c_k \right)^2 = \text{minimum} \quad (5)$$

where $A_{i,j}$ represents an element of the experimental absorbance response-surface of size $n_s \times n_w$ and the independent variables c_k are the total concentrations of the basic components c_L and c_H being adjusted in n_s solutions. Unknown parameters are the best estimates of the protonation constants, $\boldsymbol{\beta}_{qr,i}$, $i = 1, \ldots, n_c$, which are adjusted by the regression algorithm. At the same time, a matrix of molar absorptivities $(\varepsilon_{qr,j}, j = 1, \ldots, n_w)_k$, $k = 1, \ldots, n_c$, as non-negative real numbers is estimated, based on the current values of protonation constants. For a set of current values of $\boldsymbol{\beta}_{qr,i}$, the free concentrations of ligand l for each solution are calculated, as h is known from the pH measurement. Then, the concentrations of all the species in the equilibrium mixture $[L_qH_r]_j$, $j = 1, \ldots, n_c$ are obtained; they represent n_s solutions of the matrix $\boldsymbol{C}$. If the agreement is not considered satisfactory, new chemical models are tried until a better fit with the experimental data is obtained. The present communication deals with the situation in which the chemical model is known and the reliability of parameter-determination by spectral analysis is examined.

2.3. Errors in spectral data

To test the ability of the programs to find true parametric estimates, the examination of simulated data is a useful tool, allowing systematic evaluation of the effect of noise levels in the data. Spectral data may be subject to three kinds of error: (i) normally distributed random errors, which cannot be eliminated from the data, (ii) systematic errors, which are sometimes difficult to identify and eliminate, and (iii) gross errors. When simulated data are used, wavelengths and concentrations are regarded as error-free, and random errors generated in accordance with the selected standard deviation of absorbance, $s_{\mathrm{inst}}(A)$ are imposed on the precisely calculated error-free values of absorbances. In experimental work, of course, random and systematic error can arise in both the wavelength settings and the reagent concentrations, and cannot usually be distinguished. The sources of systematic error in pH measurement are well known and documented. Coloured impurities in a drug may have an acid–base character, in which case the background colour will vary with pH. At low pH some species may separate from the solution and/or be adsorbed in the cuvette walls, and at higher concentrations oligomers and micelles of the drug molecule may be formed and changes in ionic strength or reagent concentrations cause a systematic rather than a random error. However, all statistical tests in the program are based on the assumption that systematic errors are absent from the data.

2.4. Spectra modelling with simulated data

Multicomponent spectral analysis programs can also be applied when an adequate chemical model is known and only resolution of the spectra by use of different algorithms is to be investigated.

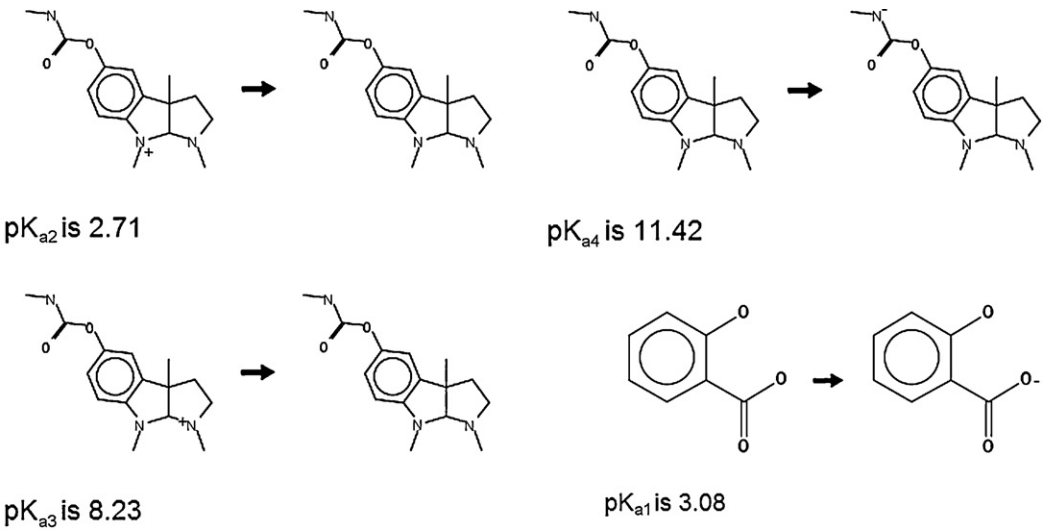


Fig. 1. Deprotonation pathway of physostigmine salicylate is taken from a prediction programme SPARC (http://archemcalc.com/sparc/pKa/).

To characterize the program performance, simulated data can be used. Model spectra of a mixture of acid/base pairs are simulated as the sum of Gaussian peaks, each generated from three arbitrary constants: the wavelength (λ_{max}), the molar absorptivity (ε_{max}) at this wavelength and the effective band-width (δ) at half-intensity. These characteristics also describe the degree of overlap of the spectra of the individual species. This approach allows examination of (i) the effect of the overall spectrophotometric error $s_{inst}(A)$ on the precision and accuracy of the parameter estimate, (ii) various regression algorithms, (iii) the sensitivity of each parameter in the model, and also allows establishment of an optimum computational strategy for efficient data treatment. The residuals are analyzed to test whether the refined parameters adequately represent the data, and should be randomly distributed about the predicted regression curve. To analyze the residuals, the following statistics are compared with those of the generated random errors in noise to find whether both distributions are Gaussian in nature: the random errors mean $m_{\epsilon,1}$ in comparison with the residual mean $m_{e,1}$, the mean random error $|\epsilon|$ in comparison with the mean residual $|e|$, the standard deviation of random errors $s(\epsilon)$ in comparison with that of the residuals $s(e)$, the skewness of the random error set $m_{\epsilon,3}$ in comparison with that of the residual set $m_{e,3}$, the kurtosis of the random error set $m_{\epsilon,4}$ in comparison with that of the residual set $m_{e,4}$, and finally the Hamilton R-factor for relative fit, $R(\epsilon)$ in comparison with $R(e)$.

2.5. Signal-to-noise ratio SER

Direct results from experimental and instrumental operations in a laboratory are always approximate, mainly because of the limited accuracy and precision of measuring instruments. The level of "experimental noise" should be used in the experiment as a critical factor. Therefore, it is necessary to have a consistent definition of the *signal-to-noise ratio SNR* so that the impact of this characteristic can be critically assessed. Traditional approaches to *SNR* are typically based on the ratio of the maximum signal to maximum noise value. As an alternative, the concept of instrumental error was again employed and the *signal-to-error ratio SER* is defined where the instrumental standard deviation of absorbance $s_{inst}(A)$ is used for an error. The plot of small absorbance changes in the spectrum when dissociation of the drug studied means that the value of the absorbance difference for the jth-wavelength of the ith-spectrum $\Delta_{ij} = A_{ij} - A_{i,acid}$ is divided by the instrumental standard deviation $s_{inst}(A)$, and the resulting ratios $SER = \Delta/s_{inst}(A)$ are plotted in dependence of wavelength λ for all absorbance matrix elements, where $A_{i,acid}$ is the initial spectrum of the acid form of the drug being measured for the starting pH value of the pH range studied. This *SER* ratio is then compared with the limiting *SER* value to test if the absorbance changes are significantly larger than the instrumental noise. The plot of the ratio $e/s_{inst}(A)$, i.e., the ratio of the residuals divided by the instrumental standard deviation $s_{inst}(A)$ depending on wavelength λ for all the residual matrix elements is for testing to ascertain if the residuals are of the same or similar magnitude as the instrumental noise to prove the best curve fitting achieved.

2.6. Reliability of estimated dissociation constants

The reliability of determined parameter estimates, b_j, $j = 1, \ldots, m$, for m unknown parameters (dissociation constants and molar absorption coefficients) may be examined by the goodness-of-fit test also called the fitness test, *cf.* page 101 in Ref. [24]. A source of problems may be found in components of a *regression triplet* [the data quality for a proposed model, the model for a given data set, and the method of estimation based on fulfilment of all least-squares assumptions] described previously [14].

3. Experimental

3.1. Chemicals and solutions

Hydrochloric acid, 1 M, was prepared from conc. HCl (p.a., Lachema Brno) using redistilled water and standardized against HgO and KI with reproducibility of less than 0.2%. *Potassium hydroxide*, 1 M, was prepared from pellets (p.a., Aldrich Chemical Company) with carbon dioxide-free redistilled water and standardized against standardized HCl with a reproducibility of 0.1%. The preparation of other solutions from analytical reagent-grade chemicals has been described previously [30–32]. *Physostigmine salicylate* 7×10^{-5} M, was prepared from solid samples [17] using redistilled water. The high purity of the substances (over 98%) was guaranteed by the supplier.

3.2. Apparatus and pH-spectrophotometric titration procedure

The apparatus Cintra 40 (GBC, Australia) spectrophotometer used and the pH-spectrophotometric titration procedure have been described previously [15,17]. The experimental and computation

scheme for the determination of the protonation constants of the multicomponent system is taken from Meloun et al., *cf.* page 226 in Ref. [24] and the five-step procedure is described in details elsewhere [15,16].

3.3. Software used

Computation relating to the determination of dissociation constants was performed by regression analysis of the UV/vis spectra using the SQUAD(84) [5] and SPECFIT/32 [8–10,12] programs. Most of graphs were plotted using ORIGIN 8 [34] and S-Plus [35]. The thermodynamic dissociation constant pK_a^T was estimated with the MINOPT nonlinear regression programme in the ADSTAT statistical system [36] (TriloByte Statistical Software, Ltd., Czech Republic). A qualitative interpretation of the spectra with the use of the INDICES program [19] aims to evaluate the quality of the dataset and remove spurious data, and to estimate the minimum number of *factors*, i.e., contributing aqueous species, which are necessary to describe the experimental data and determine the number of dominant species present in the equilibrium mixture.

3.4. Supporting information available

Complete experimental and computational procedures, input data specimens and corresponding output in numerical and graphical form for the programs INDICES, SQUAD(84) and SPECFIT/32 are available free of charge on line at http://meloun.upce.cz and in the DOWNLOAD and DATA blocks.

4. Discussion

4.1. Analysis of laboratory data

Recently, physostigmine salicylate studied in our laboratory represents a drug acid which exhibits quite small changes in spectra when pH changing. Other instrumental methods could not be used due to limited solubility in water. It is wise before starting a regression to analyze actual experimental spectra, to search for scientific library sources, to obtain a good default for the number of ionizing groups and numerical values for the initial guess as to the relevant protonation constants and the probable spectral traces of all the expected components in protonation equilibria mixture.

pH-spectrophotometric titration enables absorbance-response data (Fig. 2a and b) to be obtained for analysis by the least-squares nonlinear regression, and the reliability of parameter estimates (p*K*'s and ε's) can be evaluated on the basis of the goodness-of-fit test of residuals. Some changes in spectra (Fig. 2c) are small within deprotonation; in fact, both of the variously protonated species LH_2 and LH_3 exhibit quite similar absorption bands. The adjustment of pH value from 2 to 11 causes the absorbance to change only by 0.150 of the *A*-pH curve, so that the monitoring of five components L, LH, LH_2, LH_3 and LH_4 of the protonation equilibrium is rather unsure (Fig. 2c and d). As the changes in spectra are small, a very precise measurement of absorbance is necessary for reliable detection of the protonation equilibrium studied. Despite the fact that potentiometric determination of dissociation constants of physostigmine salicylate leads to only one $pK_a^T = 8.07(3)$, spectrophotometric determination enables an estimate of four dissociation constants and molar absorption coefficients of five variously protonated species L, LH, LH_2, LH_3, and LH_4 of physostigmine salicylate. The best curve fitting of experimental spectra set with the lowest residuals and the lowest value of the Hamilton *R*-factor of relative fitness was achieved in the case of 5 species and 4 dissociation constants (Table 1).

The chemical model of four dissociation constants and five molar absorptivities of physostigmine salicylate was calculated for 26 wavelengths to constitute and estimate $5 + (5 \times 26) = 135$ unknown parameters, which were estimated and refined by SQUAD(84) or SPECFIT/32 programs in the first run. The reliability of the parameter estimates found may be tested using the following five diagnostics of the regression triplet procedure:

The *1st diagnostic* indicates whether all of the parametric estimates $pK_{a,r}$ and ε_r have physical meaning and reach realistic values for 46 values of pH-spectra of physostigmine salicylate measured at 26 wavelengths. The SQUAD(84) programme terminates with the parameter estimates $pK_{a1} = 2.760(51)$, $pK_{a2} = 3.969(111)$, $pK_{a3} = 8.281(53)$ and $pK_{a4} = 10.960(69)$ at 25 °C and ionic strength $I = 0.023$ M KCl with $s(A) = 0.36$ mAU while the SPECFIT/32 program leads to estimates $pK_{a1} = 2.759(55)$, $pK_{a2} = 3.969(41)$, $pK_{a3} = 8.280(16)$, and $pK_{a4} = 10.970(18)$ at 25 °C and ionic strength $I = 0.023$ M KCl with $s(A) = 0.40$ mAU. As the standard deviations $s(pK_a)$ of parameters pK_a in brackets of each pK_a and $s(\varepsilon_r)$ of parameters ε_r are significantly smaller than their corresponding parameter estimates (Table 1), all the variously protonated species are statistically significant at a significance level $\alpha = 0.05$. The physical meaning of the dissociation constant $pK_{a,r}$, molar absorptivities ε_r, and stoichiometric indices *r* are examined in searching the protonation equilibria model in Table 1. The 1st hypothesis of the protonation model L, HL, is rejected, since poor fitness was achieved. The absolute values of $s(pK_{a,j}), s(\varepsilon_j)$ give information about the last *U*-contour of the hyperparaboloid in the neighbourhood of the pit, U_{min}. For well-conditioned parameters, the last *U*-contour is a regular ellipsoid, and the standard deviations are reasonably low. High *s* values are found with ill-conditioned parameters and a "saucer"-shaped pit, *cf.* Ref. [24]. The relation $s(\beta_j) \times F_\sigma < \beta_j$ should be met where F_σ is equal to 3. The graph Fig. 2c shows that the estimated molar absorptivities of all of the variously protonated species $\varepsilon_L, \varepsilon_{LH}, \varepsilon_{LH_2}, \varepsilon_{LH_3}$ and ε_{LH_4} of physostigmine salicylate depending on wavelength are realistic. Some spectra overlap and may cause some resolution difficulties in regression analysis. As some protonation models in the model search of Table 1 (1st model: L, LH, 2nd model: L, LH, LH_2, 3rd model: L, LH, LH_2, LH_3, 4th model: L, LH, LH_2, LH_3, LH_4) were tested, it may be concluded that regression spectra analysis can distinguish among these models, and on the basis of a very good spectra fitting the model L, LH, LH_2, LH_3, LH_4 was proven.

The *2nd diagnostic* tests whether all of the calculated free concentrations of the five variously protonated species on the distribution diagram of the relative concentration expressed as a percentage have physical meaning, which proved to be the case (Fig. 2d). The calculated free concentration of the basic components and variously protonated species of the protonation equilibria model should show molarities down to about 10^{-8} M. Expressed in percentage terms, a species present at about 1% relative concentration or less in an equilibrium behaves as numerical noise in a regression analysis. A distribution diagram of relative concentration shows the protonation equilibria of L, LH, LH_2, LH_3, LH_4 depending on pH in Fig. 2d and makes it easier to judge quickly the contributions of individual species to the total concentration. Since the molar absorptivities will generally be in the range 10^3–10^5 l mol^{-1} cm^{-1}, species present at less than *ca.* 0.1% relative concentration will affect the absorbance significantly only if their ε is extremely high.

The *3rd diagnostic* of the number of light-absorbing species was estimated using the INDICES algorithm [19] (Table 1, row k^* and $s_k(A)$). The number of light-absorbing species k^* can be predicted from the index function values by finding the point $k^* = k$ where the slope of index function $PC(k) = f(k)$ changes, or by comparing $PC(k)$ values to the instrumental error $s_{inst}(A)$. Very low value of $s_{inst}(A)$ in Table 1 proves that a sufficiently precise spectrophotometer and efficient experimental technique were used. The position of the break point on the $s_k(A) = f(k)$ curve in the factor analysis

Table 1
The search for a protonation model LH_r of physostigmine salicylate with dissociation constants $pK_{a,r}$ using the SQUAD nonlinear regression analysis at the ionic strength $I = 0.046$ and 25 °C. The standard deviations of the parameters estimated in the last digits are in brackets. The charges are for case of simplicity omitted. Because of working tool three decimal places of pK were used here.

q, r	pK_{qr}	pK_{qr}	pK_{qr}	pK_{qr}
1, 0	–	–	–	–
1, 1	7.973(28)	7.516(214)	3.065(61)	2.529(53)
1, 2	–	10.645(176)	7.768(56)	4.307(37)
1, 3	–	–	10.976(49)	7.864(25)
1, 4	–	–	–	10.887(20)
$s(A)$ [mAU]	7.54	7.09	1.16	0.54
s_k(A) [mAU] and k^*	5.99 and 2	0.84 and 3	0.26 and 4	0.17 and 5
$\|e\|$ [mAU]	4.76	3.73	0.68	0.31
$g_1(e)$	0.23	0.26	−0.95	0.32
$g_2(e)$	4.86	6.18	6.05	5.84
R-factor [%]	4.63	4.22	0.67	0.30
Hypothesis of model is	Rejected	Rejected	Rejected	Accepted

scree plot is calculated and gives $k = 5$ with the corresponding coordinate $s_5(A) = 0.17$ mAU, which may also be taken as the actual instrumental error $s_{inst}(A)$ of the spectrophotometer used, Fig. 6d.

The *4th diagnostic* concerns the goodness-of-fit (Table 1). The goodness-of-fit achieved is easily seen by examination of the differences between the experimental and calculated values of absorbance, $e_i = A_{exp,i,j} - A_{calc,i,j}$. Examination of the spectra and of the graph of the predicted absorbance response-surface through all the experimental points should reveal whether the results calculated are consistent and whether any gross experimental errors have been made in the measurement of the spectra. One of the most important statistics calculated is the standard deviation of absorbance, $s(A)$, calculated from a set of refined parameters at the termination of the minimization process. Although this statistical analysis of residuals gives the most rigorous test of the degree-of-fit, realistic empirical limits must be used. The statistical measures of all residuals e prove that the minimum of the elliptic hyperparaboloid U is reached with SQUAD(84): the residual standard deviation $s(e) = 0.32$ mAU always has sufficiently low values. Presentation of the residuals in Table 1 assists the detection of an outlier spectrum point, a trend in the spectrum residuals, or an abrupt shift of level in the spectra. The statistical measures of all the residuals prove that the minimum of the elliptic hyperparaboloid is reached: the mean residual $|\bar{e}| = 0.31$ mAU and the residual standard deviation $s(e) = 0.54$ mAU have sufficiently low values. The skewness $g_1(e) = 0.32$ is close to zero and proves a symmetric distribution of the residuals set, while the kurtosis $g_2(e) = 5.84$ is proving a symmetric Laplace distribution. The Hamilton R-factor of relative fitness is 0.30% calculated with SQUAD(84) only, thus proving an excellent achieved fitness, and the parameter estimates may therefore be considered quite reliable. If the Hamilton R-factor of relative fit, expressed as a percentage is <0.5%, the fit is excellent. The criteria of resolution used for the hypotheses were: (1) a failure of the minimization process in a divergence or a cyclization; (2) an examination of the physical meaning of the estimated parameters to ensure that they were both realistic and positive; and (3) the residuals should be randomly distributed about the predicted regression spectrum, and systematic departures from randomness

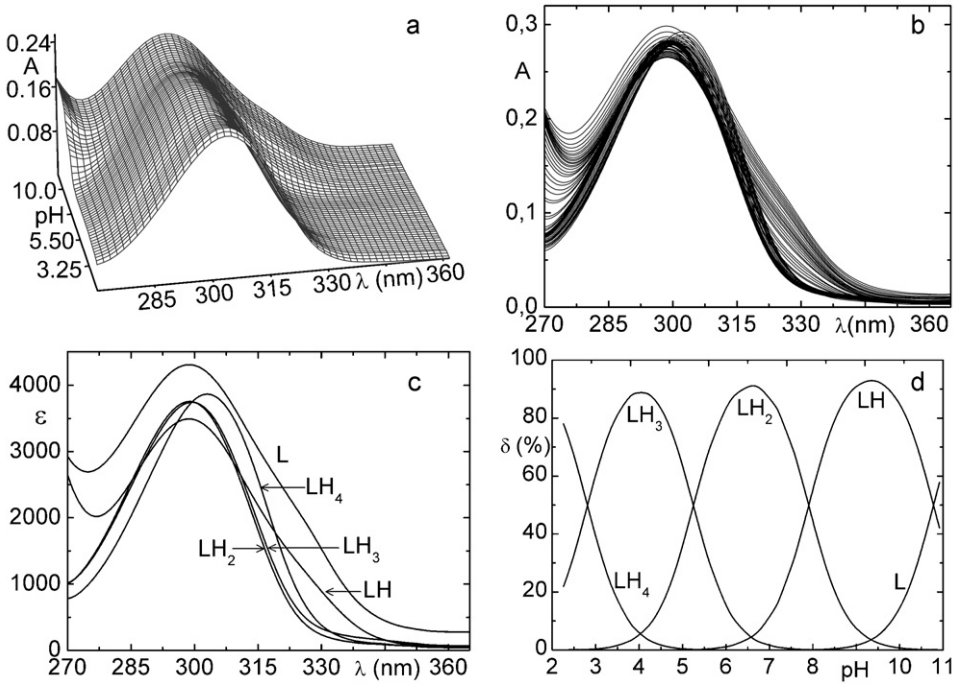


Fig. 2. (a) The 3D-absorbance-response-surface representing the measured multiwavelength absorption spectra of protonation equilibria for physostigmine salicylate depending on pH at 25 °C, (b) Absorption spectra of 7×10^{-5} M physostigmine depending on pH at 25 °C; (c) pure spectra profiles of molar absorptivities ε [mol^{-1} dm^3 cm^{-1}] *vs.* wavelengths λ [nm] for the variously protonated species L, HL, H_2L, H_3L and H_4L; (d) distribution diagram of the relative concentrations of all variously protonated species L, HL, H_2L H_3L and H_4L of physostigmine depending on pH at 25 °C, (SPECFIT/32, ORIGIN). The charges of species are omitted for the sake of simplicity.

Table 2
Thermodynamic dissociation constants for physostigmine salicylate at 25 °C and 37 °C. The standard deviations of the parameters estimated in the last digits are in brackets.

	25 °C	37 °C
pK_{a1}	2.93(8)	2.61(13)
pK_{a2}	3.95(8)	3.79(2)
pK_{a3}	8.43(2)	8.05(2)
pK_{a4}	10.04(6)	10.54(7)

were taken to indicate that either the chemical model or the parameter estimates were unsatisfactory.

To express and analyze small changes of absorbance in the spectral set, the absorbance differences for the j-th wavelength of the i-th spectrum $\Delta_i = A_{ij} - A_{i,\mathrm{acid}}$ were calculated so that the absorbance value of the acidic form was subtracted from the absorbance value of the spectrum measured at the actual pH. The absorbance difference Δ_i was then divided by the actual instrumental standard deviation $s_{\mathrm{inst}}(A)$ of the spectrophotometer used, and the resulting value represents the *signal-to-error* value *SER*. Fig. 3a shows a graph of the *SER* depending on wavelength in the measured range for the drug used. When the *SER* is larger than 10, a factor analysis is sufficiently able to predict the correct number of light-absorbing components in the equilibrium mixture. To prove that non-linear regression also can analyze such data of small absorbance changes, the residuals set was compared with the instrumental noise $s_{\mathrm{inst}}(A)$. If the ratio $e/s_{\mathrm{inst}}(A)$ is of similar magnitude, i.e., nearly equal to one, it means that sufficient curve fitting was achieved by the non-linear regression of the spectra set and that the minimization process found the minimum of the residual-square-sum function $U_{\min}$. Fig. 3b shows a comparison of the ratio $e/s_{\mathrm{inst}}(A)$ depending on wavelength for physostigmine salicylate measured. From the figure it is obvious that most of the residuals are of the same magnitude as the instrumental noise and thus indicates that changes of absorbance are 100 times larger and therefore sufficient reliability of the regression process is proven.

The thermodynamic dissociation constants of 4 unknown parameters pK_a^T at 25 °C and 37 °C were estimated by applying a Debye–Hückel equation to the data of Fig. 4; Table 2 shows point estimates of four thermodynamic dissociation constants with standard deviation in brackets of the physostigmine salicylate. Because of the narrow range of ionic strengths, the ion-size parameter $\mathring{a}$ and the salting-out coefficient C could not be estimated.

4.2. Analysis of simulated data

The performance of SQUAD(84) [5–7] and SPECFIT/32 [8–10] was first tested with a simulated data set of physostigmine salicylate, which allowed systematic variation of the spectral, equilibrium and noise characteristics. The primary study was to determine the effect of the precision of the absorbance data $s_{\mathrm{inst}}(A)$ on the precision $s(pK)$ and accuracy $\Delta pK = pK_{a,\mathrm{calc}} - pK_{a,\mathrm{true}}$ of the estimated parameters $pK_{a,i}$. Higher imprecision of the absorbance data would be expected to result in a poorer fit. Estimation of the parameters would be inaccurate due to uncertainty in the pit co-ordinates, as the hyperparaboloid response-surface would have a broad and indefinite minimum. The parametric precision is related to the D-boundary, by the super-curve, $U = U_{\min} + s^2(A)$. The standard deviation of each parameter b, defined by $s(b_i) = \max[(b_D - b_{\min}),]$ can be calculated as the maximum difference between the value for b_i, at any point on the D-boundary, and the value for b_i at the minimum. There is then a rather large ellipse as the last U contour of the D-boundary, the parametric standard deviations are larger, and the precision poorer.

For pre-selected ("true") values of parameters (i.e., 4 dissociation constants $pK_{a1} = 3.00$, $pK_{a2} = 6.10$, $pK_{a3} = 7.90$, $pK_{a4} = 10.50$, and the matrix of molar absorption coefficients of all five variously protonated species L, LH, LH_2, LH_3 and LH_4 of physostigmine salicylate depending on wavelength, the "theoretical spectra points" along the exact curves set were calculated. Each theoretical point was then transformed into an "experimental" one, also called the "*simulated point*", by the addition of a random error (having obviously a normal distribution) obtained with the aid of a random-number generator according to the optioned instrumental error of absorbance expressing the noise of spectrophotometer used, $s_{\mathrm{inst}}(A)$, and being chosen in the range from 10^{-8} and 0.1 mAU up to 1.0 mAU. All such resulting "experimental points" were thus corrupted with a random error ϵ. The error set ϵ can be then tested statistically for Gaussian distribution, independence and homogeneity. The statistical measures of random errors mentioned in residual analysis, $E(\epsilon)$, $|\epsilon|$, $s(\epsilon)$, g_1, g_2 *RSC*, Hamilton R-factor are tested in the 1st column of Table 3.

For the set of 46 pH values, absorbance spectra for 26 wavelengths from 274 to 362 nm were calculated, then corrupted with random errors. In Table 3 statistical measures of generated instrumental noise for each pre-selected $s_{\mathrm{inst}}(A)$ value are in the first column to prove that a set of random errors exhibit normal distribution. Corrupting the spectra points according to $s_{\mathrm{inst}}(A)$ with generated the high random error ϵ may, however, decrease the accuracy and precision of the parameter estimated (Fig. 5). When 4 dissociation constants are to be refined or ill-conditioned parameters in the model (i.e., molar absorption coefficients of species exhibiting quite similar $\varepsilon = f(\lambda)$ curve) are to be adjusted, spectra with a low precision $s_{\mathrm{inst}}(A)$ may result in erroneous values (so-called biased values in mathematical terminology) of the parameter estimates even if a reliable regression method is applied. In cases when the corruption $s_{\mathrm{inst}}(A)$ is small, the parameters minimizing the least-squares criterion are nearly the same as the pre-selected values and the bias $\Delta pK = pK_{a,\mathrm{calc}} - pK_{a,\mathrm{true}}$ is therefore small, though for very ill-conditioned models the bias ΔpK can be high (Table 3).

In the regression analysis of A-pH spectra, the reliability of regression process and estimates found can be classified mostly according to the precision of parameters estimated and also based on the goodness-of-fit achieved (Fig. 5). To test when the regression algorithm has found the best estimates of parameters without significant bias, the residuals should be randomly distributed about the predicted regression curve as the systematic departures from randomness indicate that the parametric estimates are not satisfactory. To analyze residuals, their statistics are compared with the statistics of imposed corrupting random errors; it is checked "whether both distributions are Gaussian in nature and/or sign". Even the degree-of-fit achieved by all regression methods is good enough and the minimization process was assumed to have terminated successfully as shown in Fig. 5.

The purpose of this paper is to demonstrate the procedure of investigating the reliability of the parameter estimation and how much two various minimization methods in SQUAD(84) and SPECFIT/32 affect the precision and accuracy of the parameter estimates when other conditions are strictly equal. The systematic deviation from its pre-selected and true value $pK_{a,\mathrm{true}}$ or bias $\Delta pK = pK_{a,\mathrm{calc}} - pK_{a,\mathrm{true}}$ is used to classify an accuracy of the parameter estimates caused by inaccuracy of data. In all four dissociation constants the bias seems to be quite small even though for pK_{a4} it is higher, i.e., +0.05 for $s_{\mathrm{inst}}(A)$ about 1.0 mAU. However, it is true that rarely such low precision spectra are analyzed. The accuracy of the pK_i estimates was therefore investigated on the basis of bias ΔpK_i, $i = 1, \ldots, 4$, being expressed in the linear regression model $\Delta pK_i = \beta_0 + \beta_1 s_{\mathrm{inst}}(A)$ as a function of the instrumental standard deviation $s_{\mathrm{inst}}(A)$. In the investigated interval of $s_{\mathrm{inst}}(A)$ from 0.1 to

Table 3
Determination of mixed dissociation constants of variously protonated forms of physostigmine salicylate by the nonlinear regression of simulated spectra set when the parametric deviation or the bias estimated by SQUAD(84) and SPECFIT are $\Delta pK_a = pK_{a,calc} - pK_{a,true}$ in pH units, $k^* = 5$ components for all values of $s_{inst}(A)$, $n_w = 26$, $n_s = 46$, $n_c = 5$. Because of working tool for a comparison of two programs four decimal places of ΔpK were used here.

$s_{inst}(A)$ [mAU]	SQUAD(84) SPECFIT 1.00E−08			SQUAD(84) SPECFIT 0.1			SQUAD(84) SPECFIT 0.2			SQUAD(84) SPECFIT 0.3			SQUAD(84) SPECFIT 0.4		
	Errors analysis		Residuals analysis	Errors analysis		Residuals analysis	Errors analysis		Residuals analysis	Errors analysis		Residuals analysis	Errors analysis		Residuals Analysis
ΔpK_1		0.0000	0.000		−4.00E−04	0.000		−0.0008	−0.0010		−0.0012	−0.0010		−0.0016	−0.0020
ΔpK_2		0.0000	0.000		−1.70E−03	−0.002		−0.0035	−0.0040		−0.0055	−0.0060		−0.0076	−0.0080
ΔpK_3		0.0000	0.000		8.00E−04	0.001		0.0015	0.0020		0.0023	0.0020		0.0030	0.0030
ΔpK_4		0.0000	0.000		2.80E−03	0.003		0.0055	0.0050		0.0081	0.0080		0.0107	0.0100
$s(pK_1)$		0.0000	1.362E−04		0.0088	3.040E−03		0.0176	0.0061		0.0264	0.0091		0.0351	0.0121
$s(pK_2)$		0.0000	2.361E−04		0.0082	5.262E−03		0.0164	0.0105		0.0245	0.0157		0.0326	0.0208
$s(pK_3)$		0.0000	5.732E−05		0.0065	1.278E−03		0.0130	0.0025		0.0195	0.0038		0.0260	0.0051
$s(pK_4)$		0.0000	3.275E−04		0.0061	7.317E−03		0.0123	0.0146		0.0184	0.0219		0.0245	0.0292
$s_k(A)$ [mAU]		0.00			0.088			0.176			0.264			0.352	
$s(A)$[mAU]	0.00	0.00	0.00	0.10	0.09	0.05	0.20	0.19	0.10	0.30	0.28	0.15	0.40	0.38	0.19
$E(\hat{e})$	−3.80E−10	2.73E−19	1.37E−10	−3.80E−06	3.36E−17	3.20E−11	−7.59E−06	−1.95E−18	3.11E−10	−1.14E−05	1.05E−17	8.38E−11	−1.52E−05	1.03E−17	3.83E−10
$\lvert\bar{e}\rvert$ [mAU]	0.00	0.00	0.00	0.08	0.07	0.04	0.15	0.14	0.08	0.23	0.21	0.11	0.30	0.28	0.15
$s(e)$ [mAU]	0.00	0.00	0.00	0.10	0.09	0.05	0.20	0.19	0.10	0.30	0.28	0.15	0.40	0.38	0.19
g_1	−0.19	−0.02	0.06	−0.19	−0.07	−0.14	−0.19	−0.07	−0.14	−0.19	−0.07	−0.13	−0.19	−0.07	−0.10
g_2	2.46	2.02	12.83	2.68	2.63	0.43	2.68	2.63	0.39	2.68	2.63	0.34	2.68	2.63	0.76
RSC	1.08E−13	8.76E−11	5.73E−09	1.08E−05	9.34E−06	2.86E−06	4.30E−05	3.74E−05	1.14E−05	9.68E−05	8.41E−05	2.55E−05	1.72E−04	1.50E−04	4.53E−05
R-factor [%]	0.00	0.00	–	0.07	0.07	–	0.14	0.13	–	0.21	0.20	–	0.28	0.26	–

$s_{inst}(A)$ [mAU]	SQUAD(84) SPECFIT 0.5			SQUAD(84) SPECFIT 0.6			SQUAD(84) SPECFIT 0.8			SQUAD(84) SPECFIT 0.1		
	Errors analysis		Residuals analysis	Errors analysis		Residuals analysis	Errors analysis		Residuals analysis	Errors analysis		Residuals analysis
ΔpK_1		−0.0020	−0.0020		−0.0025	−0.0030		−0.0065	−0.0030		−0.0046	−0.0040
ΔpK_2		−0.0099	−0.0100		−0.0122	−0.0130		−0.0174	−0.0180		−0.0226	0.0200
ΔpK_3		0.0037	0.0040		0.0043	0.0050		0.0056	0.0060		0.0069	0.0080
ΔpK_4		0.0132	0.0130		0.0157	0.0150		0.0207	0.0210		0.0260	0.0440
$s(pK_1)$		0.0438	0.0153		0.0524	0.0184		0.0692	0.0248		0.0857	0.0268
$s(pK_2)$		0.0407	0.0261		0.0487	0.0314		0.0644	0.0419		0.0797	0.0450
$s(pK_3)$		0.0324	0.0064		0.0389	0.0077		0.0515	0.0103		0.0639	0.0110
$s(pK_4)$		0.0306	0.0366		0.0367	0.0441		0.0486	0.0592		0.0604	0.0650
$s_k(A)$ [mAU]		0.440			0.528			0.701			0.871	
$s(A)$ [mAU]	0.50	0.47	0.24	0.60	0.56	0.29	0.65	0.75	0.40	1.00	0.93	0.43
$E(\hat{e})$	−1.90E−05	3.63E−17	8.00E−11	−2.28E−05	−2.00E−17	5.38E−12	−3.01E−05	−1.99E−17	3.12E−10	−4.10E−05	3.97E−18	2.28E−10
$\lvert\bar{e}\rvert$ [mAU]	0.38	0.35	0.18	0.45	0.42	0.22	0.60	0.56	0.30	0.75	0.70	0.32
$s(e)$ [mAU]	0.50	0.47	0.24	0.60	0.56	0.29	0.80	0.75	0.40	1.00	0.93	0.43
g_1	−0.19	−0.07	0.00	−0.19	−0.07	−0.01	−0.20	−0.08	0.02	−0.21	−0.09	−0.14
g_2	2.68	2.63	1.41	2.68	2.63	1.26	2.68	2.63	1.34	2.67	2.64	1.19
RSC	2.70E−04	2.34E−04	7.15E−05	3.87E−04	3.36E−04	1.04E−04	6.86E−04	5.96E−04	1.88E−04	1.07E−03	9.26E−04	2.19E−04
R-factor [%]	0.36	0.33		0.43	0.40		0.57	0.53		0.71	0.66	

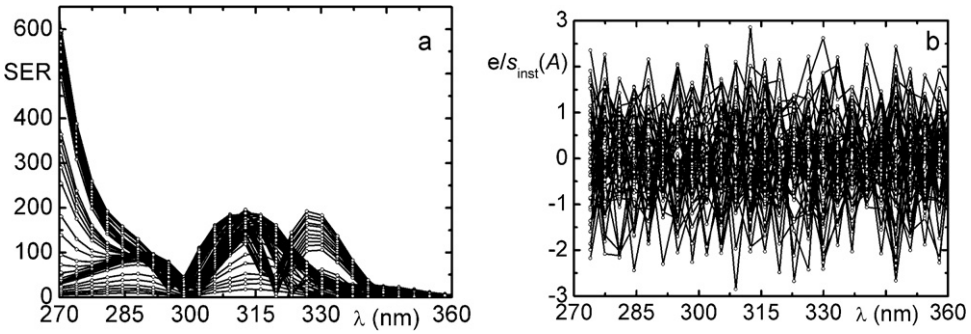


Fig. 3. (a) The plot of small absorbance changes in the spectrum of physostigmine salicylate means that the value of the absorbance difference for the *j*th-wavelength of the *i*th-spectrum $\Delta_{ij} = A_{ij} - A_{i,\text{acid}}$ is divided by the instrumental standard deviation $s_{\text{inst}}(A)$, and the resulting ratios $SER = \Delta/s_{\text{inst}}(A)$ are plotted in dependence of wavelength λ for all absorbance matrix elements, where $A_{i,\text{acid}}$ is the limiting spectrum of the acid form of the drug measured. This ratio is compared with the limiting *SER* value for the physostigmine salicylate to test if the absorbance changes are significantly larger than the instrumental noise. (b) The plot of the ratio $e/s_{\text{inst}}(A)$, i.e., the ratio of the residuals divided by the instrumental standard deviation $s_{\text{inst}}(A)$ depending on wavelength λ for all the residual matrix elements for physostigmine salicylate tests if the residuals are of the same magnitude as the instrumental noise, (SPECFIT/32, ORIGIN).

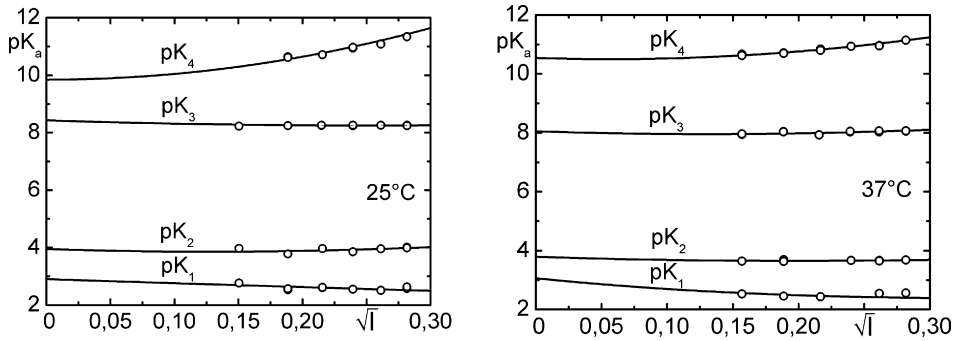


Fig. 4. Dependence of four mixed dissociation constants $pK_{a,i}$, $i = 1, \ldots, 4$ of physostigmine salicylate on the square root of ionic strength at 25 °C and 37 °C which leads to thermodynamic dissociation constants in Table 2.

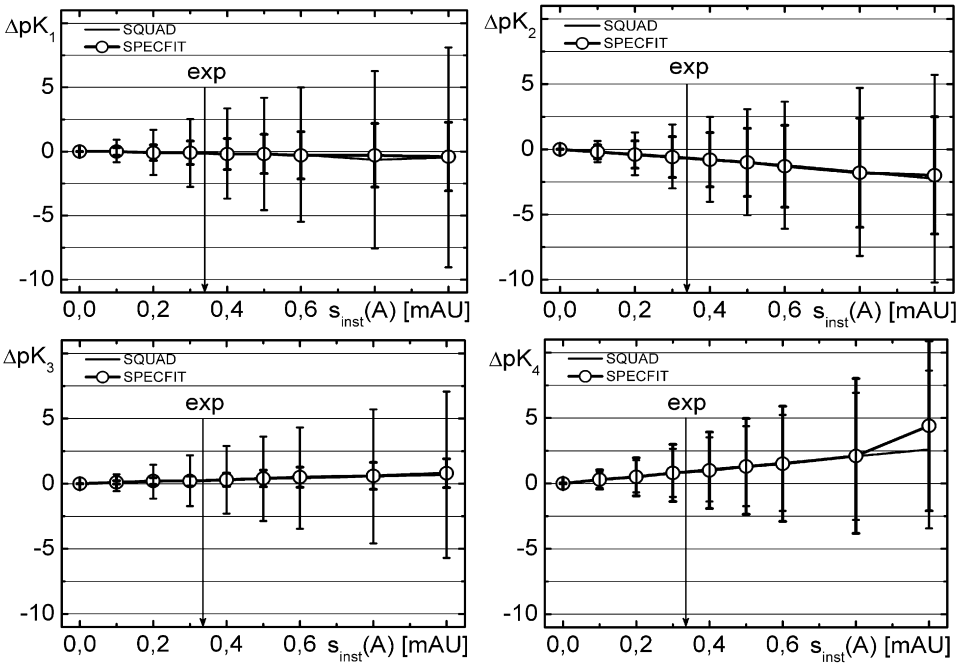


Fig. 5. The parametric bias in accuracy $\Delta pK_i = 100(pK_{i,\text{calc}} - pK_{i,\text{true}})$ denoted with circles of the point estimates and precision denoted with line segments of the interval estimates of four dissociation constants of physostigmine salicylate by regression analysis of simulated spectra sets. The parametric deviations are estimated by SQUAD(84) and SPECFIT/32. The arrow denotes an experimental standard deviation (**exp**) estimated from the absorbance data matrix with a Kankare's approach.

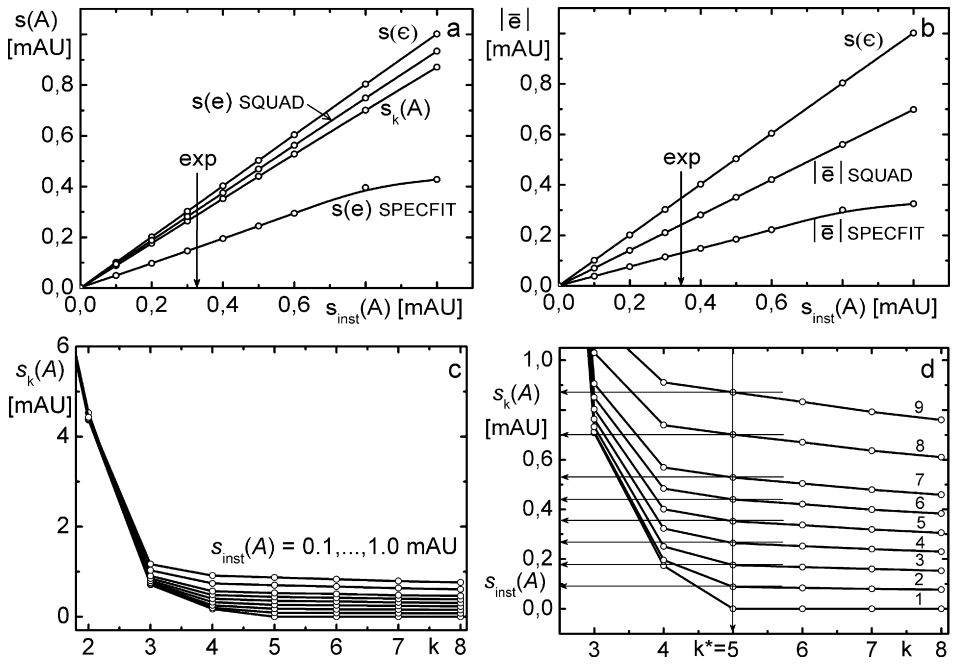


Fig. 6. The influence of the instrumental error $s_{inst}(A)$ on a goodness-of-fit represented with the statistical analysis of random errors ϵ and residuals e with the use of (a) standard deviation of random errors $s(\epsilon)$ and of residuals $s(e)$, (b) the mean deviation $|\bar{\varepsilon}|$ and residuals $|\bar{e}|$, (c) the Cattel's scree plot of the residual standard deviation of absorbance $s_k(A)$ depending on the number of the light-absorbing species in physostigmine salicylate equilibria mixture for nine various levels of instrumental noise (d). The detail view on the Cattel's scree plot enabling an evaluation from simulated spectra of the actual instrumental standard deviation $s_{inst}(A)$ for five components $k^* = 5$, (ORIGIN).

1.0 mAU it is valid that both parameter estimates b_0 and b_1 are not statistically significant, and so the dissociation constants pK_i are accurate for all values of $s_{inst}(A)$.

Precision was examined according to the estimated standard deviation of the dissociation constant s(pK) depending on the noise level $s_{inst}(A)$, being also expressed as the linear regression model $s(\mathrm{p}K) = \beta_0 + \beta_1 s_{inst}(A)$. In all cases the intercept was statistically insignificant. While parameter pK_1 is well-conditioned in the regression model as pK_1 is sensitive enough to the absorbance noise, the other three parameters pK_2, pK_3 and pK_4 are less sensitive to the magnitude of random errors, and are therefore worse-conditioned in the regression model (Table 3).

The noise level $s_{inst}(A)$ has an influence on the precision of the estimated parameters pK_i when close overlapping equilibria exist. This is the case when LH_2 and LH_3 species exhibit overlapping spectra, and therefore the estimation of pK_2 is more difficult and the estimate's precision depends on the noise level of the spectral data. In Table 3 the bias in accuracy and uncertainty of the dissociation constants is given for the noise level $s_{inst}(A) = 0.3$ mAU, which corresponds to common experimental data.

In Fig. 5 an arrow (**exp**) denotes a realistic experimental noise of the spectrophotometer used in our laboratory. In such case the bias in all four dissociation constants is about ±0.01 pH units. Parameters precision is considered from the standard deviation of estimates. Estimated standard deviation $s(\mathrm{p}K_{ai})$ is about ±0.02 for SQUAD(84) while for the SPECFIT/32 it is usually a little bit smaller.

Goodness-of-fit test in Table 3 and Fig. 6c and d analyses random errors ϵ and residuals e and indicates that a sufficiently close fit was achieved. As the statistical measures of residuals are close to those of random errors, the minimization procedure always terminated in the global minimum of the residual sum of squares. Moreover, the residual standard deviation $s(e) = 0.30$ mAU are of the same magnitude as the instrumental error $s_{inst}(A) = 0.3$ mAU leading to $s(\mathrm{p}K_{a,i}) = 0.02$. Certain underlying assumptions of regression analysis as an independence of random errors and residuals, normal distribution for errors and residuals as the skewness should be zero and the kurtosis should be 3. The residuals should possess all these statistics that agree or at least do not refute characteristics of errors. The Hamilton R-factor of relative fitness also enabling the monitoring of the regression process quality is in agreement with the errors and residuals magnitude.

Programme SQUAD(84) starts with data-smoothing of the spectra set, followed by factor analysis FA608, Ref. [2]. The position of a break on the $s_k(A) = f(k)$ curve is calculated, and gives $k^* = 5$, with the corresponding co-ordinate $s_5^*(A)$ value (Fig. 6c and d). To determined the instrumental error of the spectrophotometer used, $s_{inst}(A)$, the Wernimont–Kankare method [24] was applied. If there are five components in the solution, this means that the true rank of the absorbance matrix is equal to *five*, $k^* = 5$, and the corresponding residual standard deviation of absorbance $s_k(A)$ being estimated from the graph $s_k(A) = f(k)$ for $k = 5$ is equal to $s_{inst}(A)$ as shown in Fig. 6d. The magnitude of instrumental error of spectrophotometer used slightly affects the shape of the graph $s_k(A) = f(k)$ and also moderately the location of the break on this curve.

SPECFIT/32 gave the same results for the parametric estimates and, though it does not offer all the diagnostics that SQUAD(84) does, the degree-of-fit achieved proved the sufficient reliability of the estimates and the agreement with SQUAD(84). As a realistic experimental noise of the spectrophotometer used in our laboratory is about 0.3 mAU, the bias in all four dissociation constants is then about ±0.01 pH units. The estimated standard deviation $s(\mathrm{p}K_{a,i})$ is about ±0.02 for SQUAD(84) while for the SPECFIT/32 it is usually a little bit smaller.

5. Conclusions

The reliability of the dissociation constants of the drug physostigmine salicylate studied may be proven with regression diagnostics of goodness-of-fit tests of the absorption spectra at various pH. Regression diagnostics represent procedures for examining the *regression triplet* (data, model, method) for the identification of (a) the data quality for a proposed model; (b) the model quality for a given set of data; and (c) the fulfilment of all least-squares assumptions. When an instrumental error $s_{inst}(A)$ is small, the parameters

minimizing the least-squares criterion are nearly the same as preselected values and the bias $\Delta pK = pK_{a,calc} - pK_{a,true}$ is therefore quite negligible. In all four dissociation constants the bias seems to be quite small even though for pK_{a4} it is higher, i.e., +0.05 for $s_{inst}(A)$ about 1.0 mAU and in the interval of $s_{inst}(A)$ from 0.1 to 1.0 mAU all four dissociation constants pK_i are accurate enough. Precision was examined according to the estimated standard deviation of the dissociation constant $s(pK)$ depending on the noise level $s_{inst}(A)$, being also expressed as the linear regression model $s(pK) = \beta_0 + \beta_1 s_{inst}(A)$. In all cases the intercept was statistically insignificant. The magnitude of instrumental error $s_{inst}(A)$ slightly affects the shape of a Cattel's scree graph $s_k(A) = f(k)$ to determine the number of light-absorbing species in equilibrium mixture.

Acknowledgments

The financial support of the Grant Agency IGA MZ ČR (No. NS9831-4/2008) and of the Czech Ministry of Education (Grant No. MSM0021627502) is gratefully acknowledged.

References

[1] L.G. Sillen, B. Warnqvis, Equilibrium constants and model testing from spectrophotometric data using letagrop, Acta Chem. Scand. 22 (1968) 3032–3034.
[2] J.J. Kankare, Computation of equilibrium constants for multicomponent systems from spectrophotometric data, Anal. Chem. 42 (1970) 1322.
[3] D.J. Leggett, W.A.E. McBryde, General computer-program for computation of stability-constants from absorbance data, Anal. Chem. 47 (1975) 1065–1070.
[4] D.J. Leggett, Numerical analysis of multicomponent spectra, Anal. Chem. 49 (1977) 276–281.
[5] M. Meloun, M. Javůrek, J. Havel, Multiparametric curve fitting.10. A structural classification of programs for analyzing multicomponent spectra and their use in equilibrium-model determination, Talanta 33 (1986) 513–524.
[6] D.J. Leggett, S.L. Kelly, L.R. Shiue, Y.T. Wu, D. Chang, K.M. Kadish, A computational approach to the spectrophotometric determination of stability-constants. 2. Application to metalloporphyrin axial ligand interactions in non-aqueous solvents, Talanta 30 (1983) 579–586.
[7] D.J. Leggett, Computational Methods for the Determination of Formation Constants, Plenum Press, New York, 1985.
[8] H. Gampp, M. Maeder, C.J. Meyer, A.D. Züberbuhler, Calculation of equilibrium-constants from multiwavelength spectroscopic data. 1. Mathematical considerations, Talanta 32 (1985) 95–101.
[9] H. Gampp, M. Maeder, C.J. Meyer, A.D. Züberbuhler, Calculation of equilibrium-constants from multiwavelength spectroscopic data. 2. Specfit – 2 user-friendly programs in basic and standard Fortran-77, Talanta 32 (1985) 257–264.
[10] H. Gampp, M. Maeder, C.J. Meyer, A.D. Züberbuhler, Calculation of equilibrium-constants from multiwavelength spectroscopic data. 3. Model-free analysis of spectrophotometric and electron–spin-resonance titrations, Talanta 32 (1985) 1133–1139.
[11] H. Gampp, M. Maeder, C.J. Meyer, A.D. Züberbuhler, Calculation of equilibrium constants from multiwavelength spectroscopic data-IV model-free least-squares refinement by use of evolving factor analysis, Talanta 33 (1986) 943–951.
[12] SPECFIT/32, Spectrum Software Associates, 197M Boston Post Road West, Marlborough, MA, 01752 USA, 2004.
[13] M. Meloun, Z. Ferenčíková, L. Netolická, T. Pekárek, Thermodynamic dissociation constants of alendronic, ibandronic and risedronic acids by regression analysis of potentiometric data, J. Chem. Eng. Data 56 (2011) 3848–3854.
[14] M. Meloun, T. Syrový, S. Bordovská, A. Vrána, Reliability and uncertainty in the estimation of pK_a by least squares nonlinear regression analysis of multiwavelength spectrophotometric pH titration data, Anal. Bioanal. Chem. 387 (2007) 941–955.
[15] M. Meloun, S. Bordovská, T. Syrový, A. Vrána, Tutorial on a chemical model building by least-squares non-linear regression of multiwavelength spectrophotometric pH-titration data, Anal. Chim. Acta 580 (2006) 107–121.
[16] M. Meloun, S. Bordovská, T. Syrový, A novel computational strategy for the pK(a) estimation of drugs by non-linear regression of multiwavelength spectrophotometric pH-titration data exhibiting small spectral changes, J. Phys. Org. Chem. 20 (2007) 690–701.
[17] M. Meloun, P. Černohorský, Thermodynamic dissociation constants of isocaine, physostigmine and pilocarpine by regression analysis of potentiometric data, Talanta 52 (2000) 931–945.
[18] M. Meloun, D. Burkoňová, T. Syrový, A. Vrána, Thermodynamic dissociation constants of silychristin, silybin, silydianin and mycophenolate by the regression analysis of spectrophotometric data, Anal. Chim. Acta 486 (2003) 125–141.
[19] M. Meloun, T. Syrový, A. Vrána, Determination of the number of light-absorbing species in the protonation equilibra of selected drugs, Anal. Chim. Acta 489 (2003) 137–151.
[20] M. Meloun, T. Syrový, A. Vrána, The thermodynamic dissociation constants of ambroxol, antazoline, naphazoline, oxymetazoline and ranitidine by the regression analysis of spectrophotometric data, Talanta 62 (2004) 511–522.
[21] M. Meloun, T. Syrový, A. Vrána, The thermodynamic dissociation constants of losartan, paracetamol, phenylephrine and quinine by the regression analysis of spectrophotometric data, Anal. Chim. Acta 533 (2005) 97–110.
[22] M. Meloun, T. Syrový, A. Vrána, The thermodynamic dissociation constants of haemanthamine, lisuride, metergoline and nicergoline by the regression analysis of spectrophotometric data, Anal. Chim. Acta 543 (2005) 254–266.
[23] M. Meloun, M. Javůrek, J. Militký, Computer estimation of dissociation-constants. 5. Regression-analysis of extended Debye–Huckel law, Mikrochim. Acta 109 (1992) 221–231.
[24] M. Meloun, J. Havel, E. Högfeldt, Computation of Solution Equilibria: A Guide to Methods in Potentiometry, Extraction and Spectrophotometry, Ellis Horwood Chichester, England, 1988.
[25] M. Meloun, J. Havel, Computation of Solution Equilibria 1. Spectrophotometry, Folia Fac. Sci. Nat. Univ. Purkyn. Brunensis (Chemia), Brno, 1984.
[26] M. Meloun, J. Havel, Computation of Solution Equilibria 2. Potentiometry, Folia Fac. Sci. Nat. Univ. Purkyn. Brunensis (Chemia), Brno, 1985.
[27] J. Havel, M. Meloun, in: D.J. Leggett (Ed.), Computational Methods for the Determination of Formation, Constants, Plenum Press, New York, 1985.
[28] P. Gans, A. Sabatini, A. Vacca, Investigation of equilibria in solution. Determination of equilibrium constants with the HYPERQUAD suite of programs, Talanta 43 (1996) 1739–1753.
[29] K.Y. Tam, K. Takács-Novák, Multi-wavelength spectrophotometric determination of acid dissociation constants: a validation study, Anal. Chim. Acta 434 (2001) 157–167.
[30] M. Meloun, S. Bordovská, L. Galla, The thermodynamic dissociation constants of four non-steroidal anti-inflammatory drugs by the least-squares nonlinear regression of multiwavelength spectrophotometric pH-titration data, J. Pharm. Biomed. Anal. 45 (2007) 552–564.
[31] M. Meloun, S. Bordovská, Benchmarking and validating algorithms that estimate pK(a) values of drugs based on their molecular structures, Anal. Bioanal. Chem. 389 (2007) 1267–1281.
[32] M. Meloun, S. Bordovská, A. Vrána, The thermodynamic dissociation constants of the anticancer drugs camptothecine 7-ethyl-10-hydroxycamptothecine, 10-hydroxycamptothecine and 7-ethylcamptothecine by the least-squares nonlinear regression of multiwavelength spectrophotometric pH-titration data, Anal. Chim. Acta 584 (2007) 419–432.
[33] K. Florey, Analytical Profiles of Drug Substances, Academic Press, 1990.
[34] ORIGIN 8.5, OriginLab Corporation, One Roundhouse Plaza, Suite 303, Northampton, MA 01060, USA.
[35] S-PLUS. http://www.insightful.com/products/splus.
[36] ADSTAT 1.25, 3.0 (Windows 95), ADSTAT 1.25, 2.0, 3.0 (Windows 95), TriloByte Statistical Software Ltd., Pardubice, Czech Republic, 1990.