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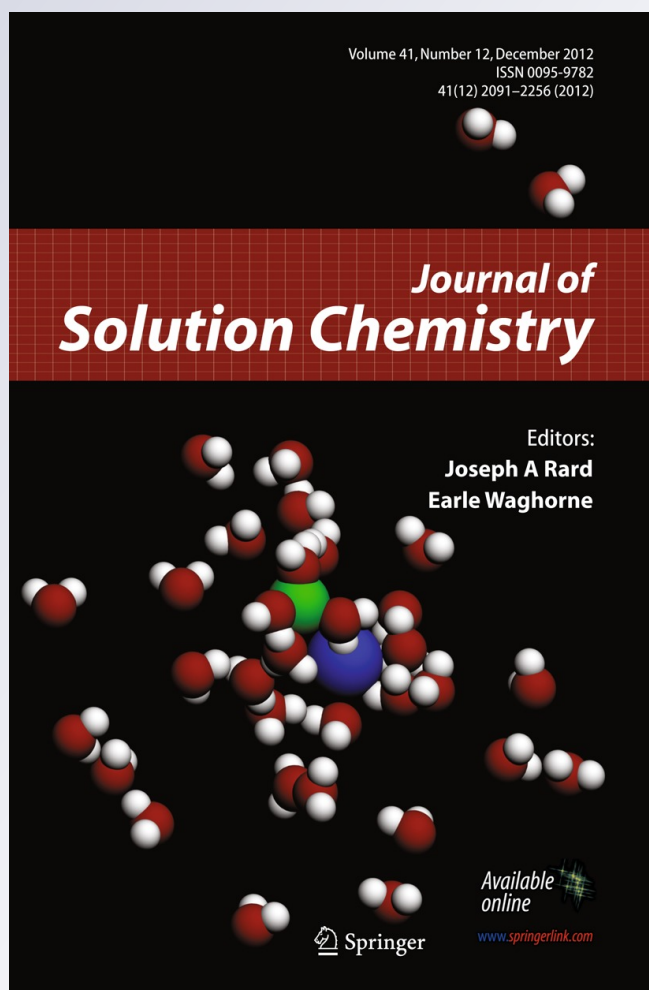
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# Dissociation Enthalpies and Thermodynamic Constants of Sildenafil Citrate by the Regression of Multiwavelength pH-spectrophotometric Titration Data

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**Abstract** pH-spectrophotometric titration data were used to determine the mixed dissociation constants of sildenafil citrate at different ionic strengths  $I$  at temperatures of 288.15, 298.15 and 310.15 K, with the use of two different multiwavelength and multivariate treatments of spectral data, SPECFIT32 and SQUAD(84) nonlinear regression analyses, and INDICES factor analysis. The reliability of the dissociation constants of this drug was proven with goodness-of-fit tests of the pH-spectra. The thermodynamic dissociation constants  $pK_{a,i}^T$  were estimated by a nonlinear regression of  $(pK_a, I)$  data using the Debye-Hückel equation:  $pK_{a,1}^T = 2.79$  (1), 3.03 (3) and 3.53 (1);  $pK_{a,2}^T = 4.97$  (2), 5.23 (2) and 5.34 (1);  $pK_{a,3}^T = 8.14$  (2), 7.93 (1) and 7.47 (1);  $pK_{a,4}^T = 9.47$  (2), 9.30 (1) and 9.13 (4); and  $pK_{a,5}^T = 10.73$  (5), 10.75 (3) and 10.79 (5) at  $T = 288.15, 298.15$  and  $310.15$  K, respectively, where the numbers in parentheses are the standard deviations in the last significant digits. Concurrently, the experimentally determined five thermodynamic dissociation constants are in a good agreement with their computational prediction of the SPARC program based on knowledge of the chemical structures. The factor analysis of spectra in the INDICES program predicts the correct number of light-absorbing components when the instrumental error is known and when the signal-to-error ratio  $SER$  is higher than 10. A rough estimation of the dissociation enthalpies  $\Delta H^0$  ( $\text{kJ}\cdot\text{mol}^{-1}$ ) and entropies  $\Delta S^0$  ( $\text{J}\cdot\text{K}^{-1}\cdot\text{mol}^{-1}$ ) has been obtained from the temperature variation of the thermodynamic dissociation constants by means of the van't Hoff equation.

**Keywords** Spectrophotometric titration · Dissociation constant · Sildenafil citrate · SPECFIT · SQUAD

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## 1 Introduction

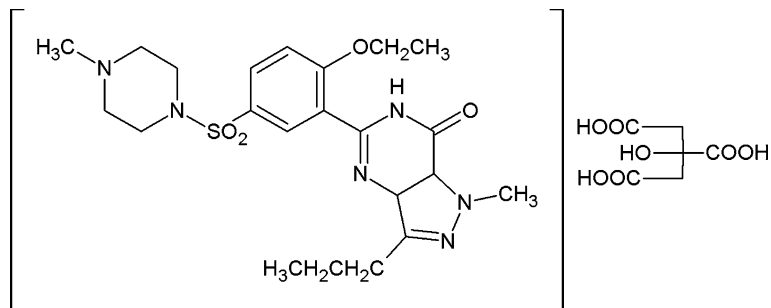
Sildenafil citrate is designated chemically as 1-[[3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo [4,3d] pyrimidin-5-yl)-4-ethoxyphenyl] sulfonyl]-4-methyl-piperazine citrate [CAS 139755-83-2] and has the structure shown in Fig. 1. Its formula is  $C_{28}H_{38}N_6O_{11}S$  and molecular mass: base  $474.6 \text{ g}\cdot\text{mol}^{-1}$ , citrate  $666.7 \text{ g}\cdot\text{mol}^{-1}$ .

Sildenafil citrate is used to treat male erectile dysfunction and pulmonary arterial hypertension. This commercially available aphrodisiac is a selective inhibitor of cyclic guanosine monophosphate (cGMP) specific phosphodiesterase type 5 (PDE5), which affects the local regulation of the erectile function and acts as a competitive binding agent of PDE5 in the corpus cavernosum, resulting in more cGMP [1–4].

Some methods have been reported for quantitative determination of sildenafil citrate, including screen-printed and conventional carbon paste electrodes for determining sildenafil citrate in tablets [5, 6], and voltammetric [7, 8], spectrophotometric [9] and chromatographic methods [10–16] for determining sildenafil citrate in pure form and in pharmaceutical formulations.

Dissociation constants are very important both in the analysis of drugs and in the interpretation of their mechanisms of action. Gobry et al. [17] determined  $pK_{a1} = 6.78$  and  $pK_{a2} = 9.12$  of sildenafil citrate at  $25 \text{ }^\circ\text{C}$  and  $I = 0.15 \text{ mol}\cdot\text{dm}^{-3}$  (KCl) potentiometrically, in water/MeOH solutions, after extrapolation to zero content of MeOH. Al Omari et al. [18] determined  $pK_{a1} = 7.10$  and  $pK_{a2} = 9.84$  spectrophotometrically at  $310 \text{ nm}$  at  $30 \text{ }^\circ\text{C}$  and in  $0.05 \text{ mol}\cdot\text{dm}^{-3}$  citrate buffer. It has been shown that the spectrophotometric method can be used in combination with suitable chemometric tools for the determination of dissociation constants  $pK_a$ , even for sparingly soluble drugs [24, 27, 28]. The reported enthalpy increments ( $\Delta H^0$ ) for dissociation processes in aqueous solutions often exhibit discrepancies among the values determined by different techniques and laboratories. The van't Hoff equation is often used to estimate  $\Delta H^0$  values of dissociation from the available dissociation constants at different temperatures. This procedure is based on the assumption that  $\Delta H^0$  is constant in the temperature range considered. The measurements and calculations are commonly carried out at room temperature but the instruments and associated software are able to determine  $pK_a$  values at higher temperatures such as  $310.15 \text{ K}$ .

In this study, pH-spectrophotometric titration was used due to the poor water solubility of sildenafil citrate which does not permit potentiometric titration with higher concentrations. Concurrently, the experimental determination of dissociation constants was combined with their computationally predicted values based on knowledge of the chemical structures.



**Fig. 1** Chemical structure of sildenafil citrate

## 2 Theoretical

### 2.1 Determination of the Protonation Model

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

$$\beta_r = [H_rL]/([L][H]^r) = c/(lh^r) \quad (1)$$

where the free concentration  $[L] = l$ ,  $[H] = h$  and  $[H_rL] = c$ . For dissociation reactions studied at constant ionic strength the “mixed dissociation constants” are defined as:

$$K_{a,j} = \frac{[H_{j-1}L] a_{H^+}}{[H_jL]} \quad (2)$$

As each aqueous species is characterized by its own spectrum, for UV/VIS experiments with the  $i$ th solution measured at the  $j$ th wavelength, the Lambert–Beer law relates the absorbance,  $A_{i,j}$ , to experimental quantities by:

$$A_{i,j} = \sum_{n=1}^{n_c} \varepsilon_{j,n} c_n = \sum_{n=1}^{n_c} (\varepsilon_{r,j} b_r l h^r)_n \quad (3)$$

where  $\varepsilon_{r,j}$  is the molar absorptivity of the  $H_rL$  species with the stoichiometric coefficient  $r$  measured at the  $j$ th wavelength. The absorbance  $A_{i,j}$  is an element of the absorbance matrix  $\mathbf{A}$  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 [19, 20]. To determine the dissociation constant of sildenafil citrate the programs SQUAD(84) [21, 22] and SPECFIT/32 [23–25] were used and the procedure has already been published [26].

### 2.2 Determination of the Number of Light-absorbing Species

For the study of protonation equilibria it is important to know the number of light-absorbing species. If we know this number, we can suggest the correct chemical model from which the non-linear regression provides estimates of the dissociation constants. The factor analysis feature in the INDICES algorithm [27] is an efficient instrument for determining the number of light-absorbing species. The source matrix is the absorbance matrix  $\mathbf{A}$ , whose columns represent the measured pH and the lines represent wavelengths. The source matrix  $\mathbf{A}$  with dimensions  $n \times m$  can be expressed as  $\mathbf{A} = \mathbf{L} \mathbf{F} + \mathbf{E}$  where  $\mathbf{L}$  with dimensions  $n \times k$  represents the factor score matrix,  $\mathbf{F}$  with dimensions  $k \times m$  is the matrix of factor weight, and  $\mathbf{E}$  with dimensions  $n \times m$  expresses the matrix of error factors. The various indicator function  $PC(k)$  techniques in the INDICES algorithm, developed to deduce the exact size of the true component space, can be classified into two general categories that were previously described in detail [27]: (a) precise methods based upon knowledge of experimental error of the absorbance data  $s_{insr}(A)$ , and (b) approximate methods requiring no knowledge of the experimental error. In general, most methods are based on the procedure of finding the point where the slope of the indicator function  $PC(k) = f(k)$  changes.

### 2.3 Determination of the Thermodynamic Protonation/Dissociation Constant

The dependence of the mixed dissociation constant  $K_a = a_{H^+} [L^{z-1}]/[HL^z]$  on ionic strength, when both ions  $HL^z$  and  $L^{z-1}$  have roughly the same ion-size parameter  $a$  in the dissociation equilibrium  $HL^z \rightleftharpoons L^{z-1} + H^+$  with the thermodynamic dissociation constant  $K_a^T = a_{H^+} a_{L^{z-1}}/a_{HL^z}$ , and supposing that the overall salting-out coefficients is given by  $C = C_{HL} - C_L$ , is expressed by the following extended Debye-Hückel equation (EDH):

$$pK_a = pK_a^T - (A(1-2z)\sqrt{I})/(1 + Ba^\circ\sqrt{I}) + CI \quad (4)$$

where  $A = 0.5112 \text{ mol}^{-1/2} \cdot \text{L}^{1/2} \cdot \text{K}^{3/2}$  and  $B = 0.3291 \times 10^{10} \text{ mol}^{-1/2} \cdot \text{m}^{-1} \cdot \text{L}^{1/2} \cdot \text{K}^{1/2}$  for aqueous solutions at  $T = 298.15 \text{ K}$ . The mixed dissociation constant  $pK_a$  represents a dependent variable while the ionic strength  $I$  is the independent variable. Three unknown parameters  $\mathbf{b} = \{pK_a, a^\circ, C\}$  are to be estimated by minimizing the sum of the squared residuals [28]:

$$U(\mathbf{b}) = \sum_{i=1}^n w_i [pK_{a,\text{exp},i} - pK_{a,\text{calc},i}]^2 = \sum_{i=1}^n w_i [pK_{a,\text{exp},i} - f(I; pK_a^T, a^\circ, C)]^2 = \text{minimum} \quad (5)$$

where the statistical weight  $w_i$  is usually equal to 1. The nonlinear estimation problem is simply a problem of optimization in the parameter space, in which the  $pK_a$  and  $I$  values are known and given while the parameters  $pK_a^T$ ,  $a^\circ$ , and  $C$  are unknown variables to be estimated. However, for small values of the ionic strength, only  $pK_a$  can be estimated.

### 2.4 Signal-to-noise Ratio *SER*

The level of “experimental noise” present in the experiments is 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 parameter can be critically assessed. Traditional approaches to *SNR* are typically based on the ratio of the maximum signal to maximum noise. As an alternative, the concept of instrumental error is again employed and the signal-to-error ratio *SER* is defined, where the error is assumed to be the instrumental standard deviation of absorbance  $s_{\text{inst}}(A)$ . The plot of small absorbance changes in the spectrum of the drug studied is the value of the absorbance difference for the  $j$ th-wavelength of the  $i$ th-spectrum,  $\Delta_{ij} = A_{ij} - A_{i,\text{acid}}$ , divided by the instrumental standard deviation  $s_{\text{inst}}(A)$ , and the resulting ratios  $SER = \Delta/s_{\text{inst}}(A)$  are plotted for each wavelength  $\lambda$  for all absorbance matrix elements, where  $A_{i,\text{acid}}$  is from 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.

Plots of the ratio  $e/s_{\text{inst}}(A)$ , i.e. the ratio of the residuals divided by the instrumental standard deviation  $s_{\text{inst}}(A)$  for each wavelength  $\lambda$  for all the residual matrix elements, are used for tests to determine whether the residuals are of the same or similar magnitude as the instrumental noise, to assist in achieving the best curve fitting.

### 2.5 Determination of the Enthalpy and Entropy Changes

A spontaneous process is the time-evolution of a system in which it releases energy, usually as heat, and moves to a lower, more thermodynamically stable energy state. For a reaction at constant temperature and pressure,  $\Delta G^0$ , the Gibbs energy is given by:

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \quad (6)$$

The sign of  $\Delta G^0$  depends on the signs of the changes in enthalpy  $\Delta H^0$  and entropy  $\Delta S^0$ , as well as on the absolute temperature ( $T$ , in K).  $\Delta G$  changes from positive to negative (or vice versa) when  $T = \Delta H^0/\Delta S^0$ . When  $\Delta G^0$  is negative, the process occurs spontaneously in the forward direction. When  $\Delta G^0$  is positive, the process proceeds spontaneously in reverse. When  $\Delta G^0$  is zero, the process is already in equilibrium, with no net change taking place over time. The enthalpy change  $\Delta H^0$  ( $\text{kJ}\cdot\text{mol}^{-1}$ ) for the dissociation process is calculated using the van't Hoff equation:

$$d \ln K/dT = \Delta H^0/RT^2 \quad (7)$$

From the Gibbs energy change  $\Delta G^0$  ( $\text{kJ}\cdot\text{mol}^{-1}$ ) and the enthalpy change  $\Delta H^0$  ( $\text{kJ}\cdot\text{mol}^{-1}$ ), the entropy change  $\Delta S^0$  ( $\text{J}\cdot\text{K}^{-1}\cdot\text{mol}^{-1}$ ) can be calculated by:

$$\Delta G^0 = -RT \ln K \quad (8)$$

$$\Delta S^0 = (\Delta H^0 - \Delta G^0) / (T/K), \quad (9)$$

where  $R$  (ideal gas constant) =  $8.314 \text{ J}\cdot\text{K}^{-1}\cdot\text{mol}^{-1}$ ,  $K$  is the thermodynamic dissociation constant, and  $T$  is the absolute temperature.

### 3 Experimental

#### 3.1 Chemicals and Solutions

Sildenafil citrate, donated by ZENTIVA k.s. with declared purity (0.98) by HPLC, was used without further purification. Hydrochloric acid,  $1 \text{ mol}\cdot\text{dm}^{-3}$ , was prepared by diluting concentrated HCl (p.a., Lachema Brno) with redistilled water and standardization against HgO and KI with a reproducibility better than 0.002 % according to the reactions  $\text{HgO} + 4 \text{KI} + \text{H}_2\text{O} \rightleftharpoons 2 \text{KOH} + \text{K}_2[\text{HgI}_4]$  and  $\text{KOH} + \text{HCl} \rightleftharpoons \text{KCl} + \text{H}_2\text{O}$ . Potassium hydroxide,  $1 \text{ mol}\cdot\text{dm}^{-3}$ , was prepared from the exact weight of pellets (p.a., Aldrich Chemical Company) with carbon-dioxide-free redistilled water (agitated for 50 min prior to that in an ultrasonic bath). The solution was stored for several days in a polyethylene bottle under an argon atmosphere. This solution was standardized against a solution of potassium hydrogen phthalate using the derivative method with reproducibility 0.001 %. Mercury oxide, potassium iodide and potassium chloride, p.a. Lachema Brno, were not further purified. Twice-redistilled water for the preparation of solutions was agitated for 50 min in a sonographic bath prior to use.

#### 3.2 pH-spectrophotometric Titration Procedure

The spectral measurements were carried out by using a spectrophotometer model GBC Cintra 40 with 1 cm optical path length. A Hanna 3120 pH meter with combined Theta HC 103-VFR glass electrode was used for measurements. The ionic strength from 0.019 to  $0.072 \text{ mol}\cdot\text{dm}^{-3}$  was adjusted with the use of  $3 \text{ mol}\cdot\text{dm}^{-3}$  KCl. The pH-spectrophotometric titration procedure has been described in detail previously [19, 20, 29]. The experimental and computation scheme used to determine the protonation constants of the multicomponent system was taken from Meloun et al., cf. page 226 in ref. [30], and the five considerations have been described in detail elsewhere [19, 20]: (1) instrumental error of



absorbance measurements,  $s_{\text{inst}}(A)$ , (2) experimental design, (3) number of light-absorbing species, (4) choice of computational strategy, and (5) diagnostics to indicate the correct chemical model. When a minimization process terminates, some curve-fitting diagnostics are examined to determine whether the results should be accepted: the physical meaning of the estimated parameters, the physical meaning of the species concentrations, the goodness-of-fit test and the deconvolution of spectra.

### 3.3 Computation and Software

Computations related to the determination of dissociation constants were performed by regression analysis of the UV/VIS spectra using the SQUAD(84) [21, 22] and SPECFIT/32 [31] programs. A qualitative interpretation of the spectra with the use of the INDICES program [27] in S-Plus [32] 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. Most graphs were plotted using ORIGIN 8 [33] and S-Plus [32]. The thermodynamic dissociation constant  $pK_a^T$  values were estimated with the MINOPT nonlinear regression program in the ADSTAT statistical system (TriloByte Statistical Software, Ltd., Czech Republic) [34]. SPARC (SPARC Performs Automated Reasoning in Chemistry) [35] predicts numerous physical properties and chemical reactivity parameters for a large number of organic compounds, strictly from molecular structure. After entering the compound's topological structure descriptors graphically,  $pK_a$  values of organic compound are predicted using approximately hundreds of Hammett and Taft equations and quantum chemistry calculations. The macroscopic properties of chemical compounds clearly depend on their microscopic structural descriptors, and the development of a Quantitative Structure/Property Relationship QSPR based on theoretical descriptors is a powerful tool for the prediction of the chemical, physical and biological properties of compounds. SPARC applies a mechanistic perturbation method to estimate the  $pK_a$  through a number of models that account for electronic effects, solvation effects, hydrogen bonding effects, and the influence of temperature. The user needs to know only the molecular structure of the compound to predict the property of interest. Unfortunately, to date no reliable method has been made available for predicting  $pK_a$  value over a wide range of molecular structures, either for simple compounds or for complex molecules such as drugs and dyes.

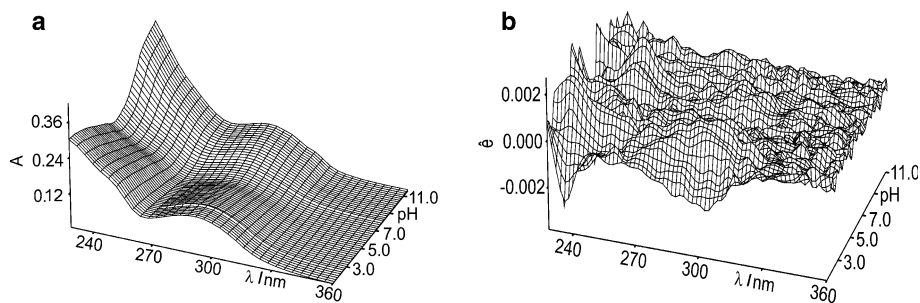
### 3.4 Supporting Information Available

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

## 4 Results and Discussion

The sildenafil citrate structure indicates five protonation equilibria. The pH-spectrophotometric titration provides absorbance–response 3D-data (Figs. 2a, 3a) serving for the non-linear regression with the SPECFIT and SQUAD(84) programs. The reliability of the





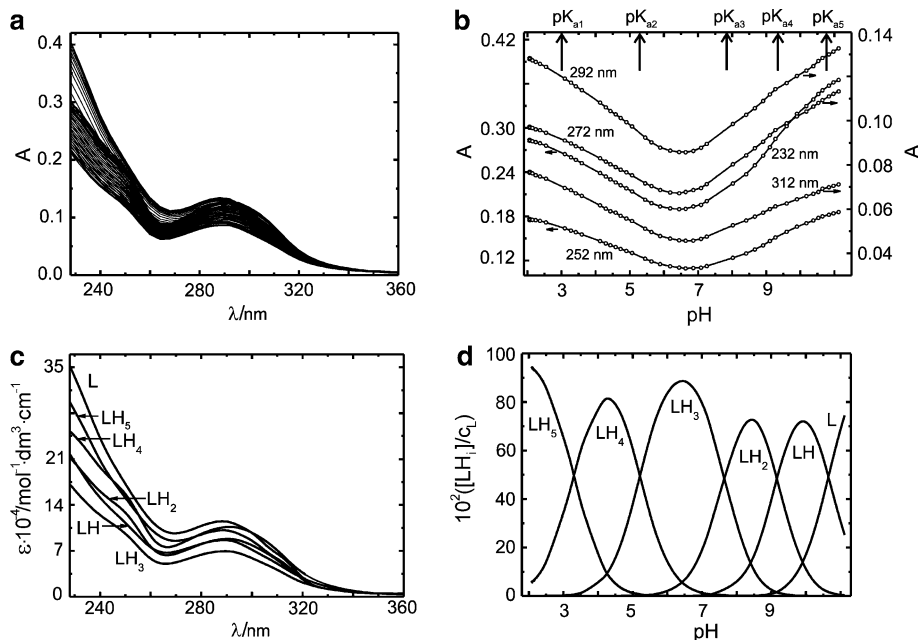
**Fig. 2** **a** The 3D-absorbance-response-surface representing the measured multiwavelength absorption spectra for sildenafil citrate depending on pH at  $T = 298.15$  K. **b** The 3D-residuals map after a non-linear regression performed with the SPECFIT/32 and SQUAD(84) programs (S-Plus)

estimated parameters ( $pK$  and  $\epsilon$ ) can be evaluated on the basis of the goodness-of-fit test of residuals (Fig. 2b). Other instrumental methods for determining dissociation constants do not seem to be suitable for sildenafil citrate because of its limited solubility in water.

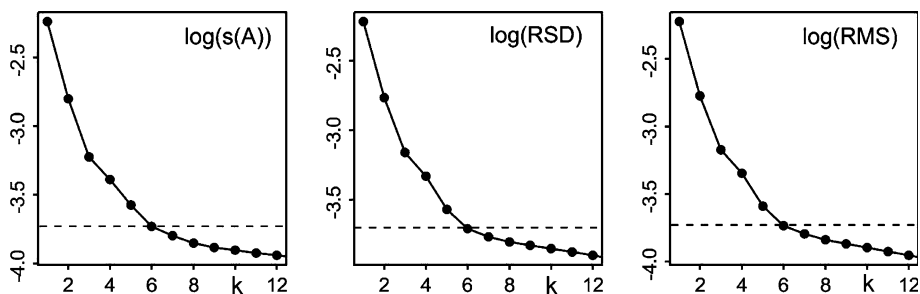
Changes in spectra are quite small from deprotonation (Fig. 3b); however, all of the variously protonated species exhibit similar absorption bands and therefore a very precise measurement of absorbance is required for the reliable estimation of parameters for the studied deprotonation equilibria. The spectrophotometric analysis of protonation equilibria of sildenafil citrate contains several steps. The first step involves the determination of the number of light-absorbing species. The hard-modeling method {e.g. SQUAD(84)} requires knowing the value of the number of light-absorbing species, their stoichiometry, and an initial approximation of the dissociation constants. The SQUAD(84) program contains Kankare's modification of Cattell's scree plot of eigenvalues and, depending on the number of components, factor analysis using INDICES algorithm was applied. The method of Kankare's residual standard deviation  $s(A)$  (Fig. 4,  $\log_{10} \{s(A)\}$ ), residual standard deviation  $RSD$  {Fig. 4,  $\log_{10} (RSD)$ }, and root mean square error  $RMS$  (Fig. 4,  $\log_{10} (RMS)$ ), identically lead to a significant break point on curve at  $k^* = 6$  with corresponding co-ordinates  $\log_{10} s_k^*(A) = -3.72$ , i.e.  $s_k^*(A) = 0.00019$ , which also represents the actual instrumental error  $s_{inst}(A)$  of the spectrophotometer used.

The next step is analyzing the reliability of the chemical model. The goodness-of-fit achieved is easily seen by examining 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 all of the experimental points should reveal whether the calculated results are consistent and whether any gross experimental errors have been made in the measurement of the spectra. One of the most important calculated statistics is the standard deviation of absorbance,  $s(A)$ , calculated from a set of refined parameters at the termination of the minimization process. The reliability of the proposed chemical model is considered on the basis of several criteria of the goodness-of-fit test:

- (a) Estimation of the standard deviation of absorbance  $s(A)$  after regression analysis should have a value close to the residual standard deviation of absorbance  $s_k(A)$  from the factor analysis of INDICES method. This quantity is usually compared to the standard deviation of absorbance calculated, and if  $s(A) \leq s_k(A)$ , or  $s(A) \leq s_{inst}(A)$ , then the instrumental error of the spectrophotometer  $s_{inst}(A)$  is used and the fit is considered to be statistically acceptable.



**Fig. 3** **a** Absorption spectra of  $3 \times 10^{-5}$  mol·dm<sup>-3</sup> of sildenafil citrate depending on the pH at  $T = 298.15$  K. **b** Plot of absorbance versus pH curves for  $\lambda = (232, 252, 272, 292, 312)$  nm and their dependence on pH at  $T = 298.15$  K. **c** Pure spectra profiles of molar absorptivity versus wavelength for the variously protonated species L, LH, LH<sub>2</sub>, LH<sub>3</sub>, LH<sub>4</sub> and LH<sub>5</sub>. **d** Distribution diagram of the relative concentrations of all of the protonated species L, LH, LH<sub>2</sub>, LH<sub>3</sub>, LH<sub>4</sub> and LH<sub>5</sub> of sildenafil citrate and their dependence on pH at  $T = 298.15$  K. The charges of species are omitted for the sake of simplicity (SPECFIT, SPLUS, ORIGIN)



**Fig. 4** The logarithmic dependence of the Cattell's index plot of eigenvalues in the form of three indicated modifying methods as a function of the number of principal components  $k$  for the pH-absorbance matrix: Kankare's residual standard deviation  $s_k(A)$ , residual standard deviation  $RSD$ , and root mean square error  $RMS$ . These three methods lead to six light-absorbing species in a pH-equilibrium mixture (S-PLUS)

- (b) The accepted chemical model should be the hypothesized model with a sufficiently low estimated arithmetic mean of residuals  $|\hat{e}|$  (Fig. 2b).
- (c) The chemical model found should have the lowest value of the Hamilton  $R$ -factor.
- (d) The residual skewness  $\hat{g}_1(\hat{e})$  should have a value equal to zero and the residual kurtosis  $\hat{g}_2(\hat{e})$  should have a value equal to three.

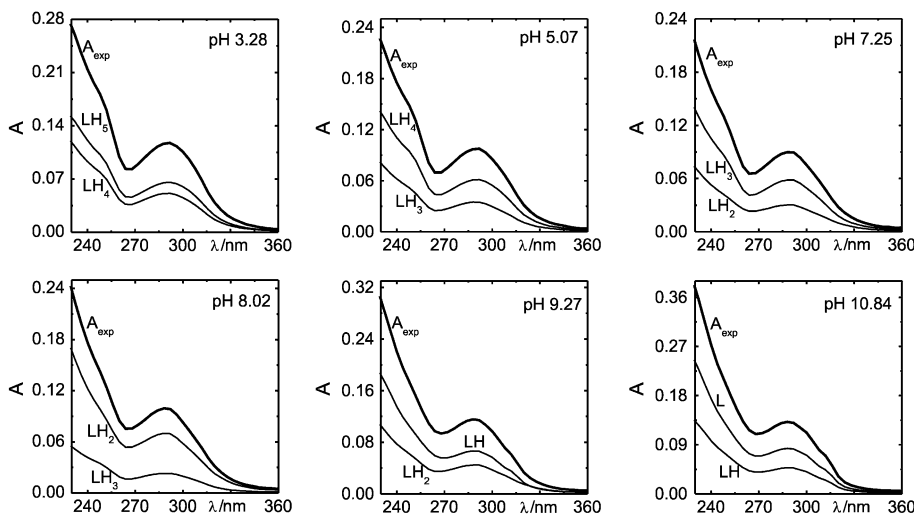
**Table 1** The search for a protonation model for sildenafil citrate using a SQUAD(84) analysis of the 3D absorbance–response surface at  $I = 0.018 \text{ mol}\cdot\text{dm}^{-3}$  and  $T = 298.15 \text{ K}$ . The charges of species are omitted for the sake of simplicity. Because of the computational method, three decimal places of  $\text{p}K_{qr}$  are reported here. Standard deviations of the estimated parameters in the last reported digits are given in parentheses

Hypothesized protonation models $L_qH_r$ with dissociation constants $\text{p}K_{qr}$					
$q, r$	$\text{p}K_{qr}$	$\text{p}K_{qr}$	$\text{p}K_{qr}$	$\text{p}K_{qr}$	$\text{p}K_{qr}$
1, 1	9.543 (52)	4.277 (17)	4.362 (58)	4.373 (240)	3.304 (42)
1, 2	–	9.083 (11)	8.404 (58)	8.099 (240)	5.207 (41)
1, 3	–	–	10.027 (34)	9.270 (195)	7.634 (42)
1, 4	–	–	–	10.623 (119)	9.162 (34)
1, 5	–	–	–	–	10.649 (23)
$s(A) \times 10^3$	13.35	3.59	2.56	2.50	0.53
$s_k(A) \times 10^3$	1.5, 2	0.46, 3	0.28, 4	0.17, 5	0.15, 6
$ \bar{l}  \times 10^3$	7.79	2.39	1.62	1.42	0.34
$s(e) \times 10^3$	13.35	3.59	2.56	2.50	0.53
$g_1(e)$	–0.52	–0.28	0.10	0.54	–0.46
$g_2(e)$	5.97	5.72	5.08	5.72	5.12
$R$ -factor (%)	10.6	2.82	1.98	1.91	0.40
Hypothesis is:	Rejected	Rejected	Rejected	Rejected	Accepted

A hypothesized chemical model that complies with these criteria is considered to be the best regression model showing the best goodness-of-fit of calculated absorbance–response surface of measured spectra. Table 1 shows the testing of various chemical hypotheses for sildenafil citrate at  $I = 0.018 \text{ mol}\cdot\text{dm}^{-3}$  and  $T = 298.15 \text{ K}$ . From testing of the five proposed protonation models, it was concluded that regression spectra analysis can distinguish among these models on the basis of very good spectral fitting, and that the protonation model of six species L, LH, LH<sub>2</sub>, LH<sub>3</sub>, LH<sub>4</sub> and LH<sub>5</sub> was proven. The statistical measures of all residuals from Fig. 2b prove that the minimum of the hyperparaboloid is reached: the mean residual  $|\bar{l}| = 0.00034$  and the residual standard deviation  $s(e) = 0.00053$  have sufficiently low values. The skewness  $g_1(e) = -0.46$  is close to zero and proves a nearly symmetric distribution of the residuals, while the kurtosis  $g_2(e) = 5.12$  is close to 6 proving a symmetric Laplace distribution. The Hamilton  $R$ -factor of relative fitness is 0.40 % calculated with SQUAD(84) only, proving an excellent achieved fitness, and the parameter estimates may therefore be considered as reliable enough.

Five dissociation constants and the six molar absorptivities  $\varepsilon$  ( $\text{dm}^3\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ ) of sildenafil citrate, i.e.  $\varepsilon_L$ ,  $\varepsilon_{LH}$ ,  $\varepsilon_{LH_2}$ ,  $\varepsilon_{LH_3}$ ,  $\varepsilon_{LH_4}$  and  $\varepsilon_{LH_5}$ , calculated for 39 wavelengths from 45 spectra (Fig. 2a, 3a) constitute  $(6 \times 39) + 5 = 239$  unknown regression parameters, which are estimated and refined with SQUAD(84) or SPECFIT32 programs in the first run. Figure 3 shows an overview of the steps of regression analysis of the spectra to determine the dissociation constants of sildenafil citrate. Factor analysis (Fig. 4) of the absorption spectra (Fig. 3a) leads to six light-absorbing species. The graph of molar absorptivity (Fig. 3c) for variously protonated species L, LH, LH<sub>2</sub>, LH<sub>3</sub>, LH<sub>4</sub> and LH<sub>5</sub> indicates that the selected chemical model is reliable. An important result of regression analysis is the distribution diagram (Fig. 3d) of the relative concentration of all different protonated species with dependence on pH.

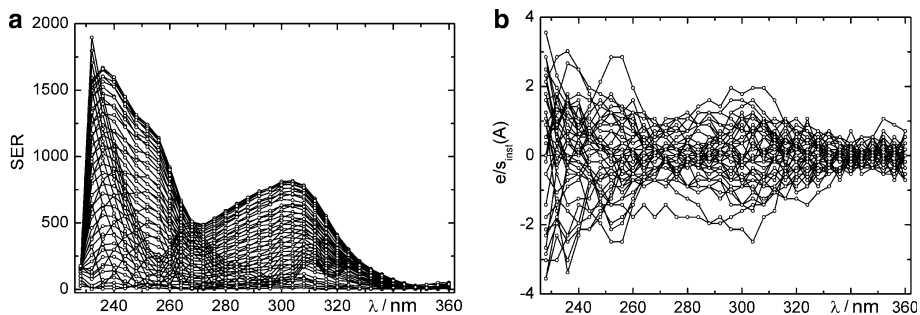
Figure 5 shows the deconvolution of the experimental spectrum into the individual spectra of variously protonated species to examine whether the experimental design is



**Fig. 5** Deconvolution of the experimental absorption spectrum of sildenafil citrate for 39 wavelengths into the spectra of the various individual protonated species L, LH, LH<sub>2</sub>, LH<sub>3</sub>, LH<sub>4</sub> and LH<sub>5</sub> in solution of each particular absorption spectrum at selected pH values of: 3.28, 5.07, 7.25, 8.02, 9.27 and 10.84. The charges of species are omitted for the sake of simplicity (SQUAD, ORIGIN)

efficient. If, for a particular pH range, the spectrum consists of just a single component, measuring further spectra for that range would be redundant, although they could improve the precision of the results. In pH ranges where more components contribute significantly to the spectrum, several spectra should be measured. Such spectra provide sufficient information for a regression analysis that monitors at least two species in equilibrium where none of them is a minor species. A minor species is defined as having a relative concentration in a distribution diagram of less than 5 % of the total concentration of the basic component. When, on the other hand, only one species prevails in the solution, the spectrum yields quite poor information for a regression analysis and the parameter estimate is rather uncertain and definitely not reliable enough. Spectral deconvolution seems to be quite a useful tool in a proposal for an efficient experimentation strategy.

To express small changes of absorbance in the spectral set, the absorbance differences for the  $j$ -th wavelength of the  $i$ -th spectrum  $\Delta_i = A_{ij} - A_{i,acid}$  are calculated, so that from the absorbance value of the spectrum measured at the actual pH the absorbance value of the acidic form is subtracted. The absorbance difference  $\Delta_i$  is then divided by the actual instrumental standard deviation  $s_{inst}(A)$  of the spectrophotometer used, and the resulting value represents the signal-to-error value  $SER$ . Figure 6a is a graph of the  $SER$  depending on wavelength in the measured range for sildenafil citrate. When the  $SER$  is larger than 10, a factor analysis is sufficient to predict the correct number of light-absorbing species in the equilibrium mixture. To prove that non-linear regression can analyze such data, the residual set is compared with the instrumental noise  $s_{inst}(A)$ . If the ratio  $e/s_{inst}(A)$  is of similar magnitude, i.e. nearly one, then it means that sufficiently good curve fitting was achieved by the non-linear regression of the spectra set and that the minimization process found the global minimum of the residual-square-sum function  $U_{min}$ . Figure 6b shows a comparison of the ratio  $e/s_{inst}(A)$  depending on wavelength used for studying for the sildenafil citrate. From Fig. 6 it is obvious that most of the residuals are of the same



**Fig. 6** The plot of absorbance changes in the spectrum (left part) 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 as a function of the wavelength  $\lambda$  for all absorbance matrix elements where  $A_{i,\text{acid}}$  denotes the limiting spectrum of the acid form of the drug. This ratio is compared to the limiting  $SER$  value for sildenafil citrate to test if the absorbance changes  $\Delta_{ij}$  are significantly larger than the instrumental noise  $s_{\text{inst}}(A)$ . The plot of the ratio  $e/s_{\text{inst}}(A)$ , i.e. the ratio of residuals divided by the instrumental standard deviation  $s_{\text{inst}}(A)$  as a function of wavelength  $\lambda$  for all residual matrix elements (right part) for sildenafil citrate, tests whether the residuals  $e$  are of the same magnitude as the instrumental noise  $s_{\text{inst}}(A)$

**Table 2** Mixed dissociation constants of sildenafil citrate at  $T = 288.15$  K at various ionic strengths  $I$  ( $\text{mol}\cdot\text{dm}^{-3}$ ), estimated by non-linear regression using the programs SPECFIT and SQUAD. Standard deviations of the estimated parameters in the last reported digits are given in parentheses

$I$ ( $\text{mol}\cdot\text{dm}^{-3}$ )	0.018	0.028	0.038	0.048	0.058	0.068
SPECFIT						
$\text{p}K_{\text{a}1}$	2.84 (2)	2.74 (3)	2.72 (2)	2.70 (2)	2.71 (2)	2.72 (4)
$\text{p}K_{\text{a}2}$	5.13 (4)	5.02 (2)	5.03 (2)	5.16 (3)	5.08 (3)	5.13 (6)
$\text{p}K_{\text{a}3}$	8.13 (3)	8.21 (2)	8.12 (5)	8.15 (3)	8.19 (1)	8.21 (1)
$\text{p}K_{\text{a}4}$	9.45 (4)	9.42 (5)	9.34 (3)	9.48 (7)	9.46 (5)	9.50 (5)
$\text{p}K_{\text{a}5}$	10.78 (3)	10.59 (3)	10.79 (4)	10.85 (2)	10.85 (3)	10.91 (4)
$s(A) \times 10^3$	0.28	0.32	0.31	0.29	0.22	0.21
SQUAD						
$\text{p}K_{\text{a}1}$	2.84 (1)	2.74 (2)	2.72 (2)	2.71 (1)	2.72 (1)	2.72 (2)
$\text{p}K_{\text{a}2}$	5.14 (1)	5.02 (1)	5.03 (1)	5.17 (1)	5.08 (1)	5.13 (2)
$\text{p}K_{\text{a}3}$	8.14 (3)	8.21 (4)	8.12 (5)	8.15 (8)	8.20 (3)	8.21 (3)
$\text{p}K_{\text{a}4}$	9.46 (2)	9.43 (3)	9.33 (4)	9.45 (5)	9.46 (3)	9.51 (4)
$\text{p}K_{\text{a}5}$	10.78 (1)	10.57 (1)	10.79 (2)	10.84 (6)	10.84 (2)	10.91 (2)
$s(A) \times 10^3$	0.31	0.36	0.35	0.33	0.25	0.26

magnitude as the instrumental noise and thus prove that the regression process was sufficiently reliable.

Applying the extended Debye-Hückel equation to the data in Tables 2, 3 and 4, according to the regression criterion, the unknown parameter  $\text{p}K_{\text{a}}^{\text{T}}$  was estimated at the three experimental temperatures. Table 5 reports estimated values of the thermodynamic dissociation constants of sildenafil citrate studied at three temperatures and calculated changes of enthalpy and entropy. Because of the small range of ionic strengths, the ion-size parameter  $\bar{a}$  and the salting-out coefficient  $C$  could not be estimated. Fig. 7.

**Table 3** Mixed dissociation constants of sildenafil citrate at  $T = 298.15$  K and various ionic strengths  $I$  ( $\text{mol}\cdot\text{dm}^{-3}$ ) estimated by non-linear regression using the programs SPECFIT and SQUAD. Standard deviations of the estimated parameters in the last reported digits are given in parentheses

$I$ ( $\text{mol}\cdot\text{dm}^{-3}$ )	0.018	0.029	0.040	0.051	0.062	0.072
SPECFIT						
$\text{p}K_{\text{a}1}$	3.19 (3)	3.27 (2)	3.38 (8)	3.47 (3)	3.70 (3)	3.96 (3)
$\text{p}K_{\text{a}2}$	5.10 (2)	5.25 (1)	5.33 (4)	5.42 (2)	5.44 (5)	5.49 (3)
$\text{p}K_{\text{a}3}$	7.82 (3)	7.87 (6)	7.87 (2)	7.95 (4)	7.82 (3)	7.86 (2)
$\text{p}K_{\text{a}4}$	9.28 (3)	9.17 (2)	9.31 (2)	9.36 (2)	9.38 (1)	9.38 (2)
$\text{p}K_{\text{a}5}$	10.74 (3)	10.81 (4)	10.80 (3)	10.96 (4)	11.04 (3)	11.03 (8)
$s(A) \times 10^3$	0.25	0.18	0.16	0.20	0.12	0.15
SQUAD						
$\text{p}K_{\text{a}1}$	3.19 (1)	3.27 (0)	3.33 (2)	3.46 (1)	3.70 (1)	3.96 (2)
$\text{p}K_{\text{a}2}$	5.10 (1)	5.25 (1)	5.32 (1)	5.42 (1)	5.44 (2)	5.49 (1)
$\text{p}K_{\text{a}3}$	7.82 (3)	7.86 (5)	7.86 (3)	7.95 (4)	7.82 (4)	7.86 (3)
$\text{p}K_{\text{a}4}$	9.24 (2)	9.17 (2)	9.31 (2)	9.36 (3)	9.38 (2)	9.38 (3)
$\text{p}K_{\text{a}5}$	10.75 (2)	10.81 (2)	10.80 (1)	10.96 (2)	11.04 (1)	11.03 (4)
$s(A) \times 10^3$	0.30	0.23	0.21	0.28	0.15	0.20

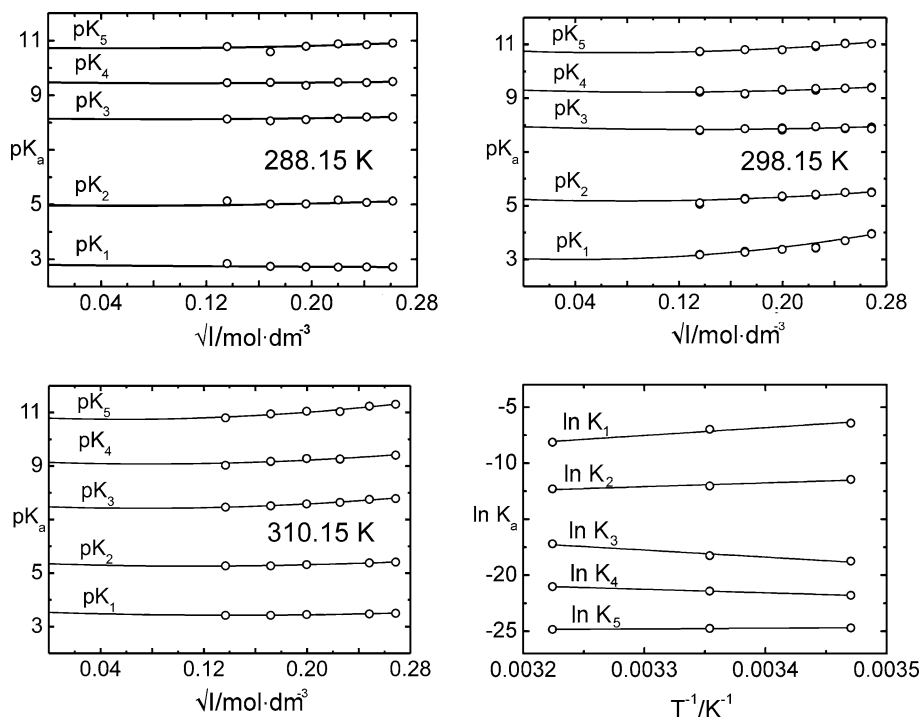
**Table 4** Mixed dissociation constants of sildenafil citrate at  $T = 310.15$  K and various ionic strengths  $I$  ( $\text{mol}\cdot\text{dm}^{-3}$ ) estimated by the non-linear regression using programs SPECFIT and SQUAD. Standard deviations of the estimated parameters in the last reported digits are given in parentheses

$I$ ( $\text{mol}\cdot\text{dm}^{-3}$ )	0.019	0.029	0.040	0.051	0.062	0.072
SPECFIT						
$\text{p}K_{\text{a}1}$	3.42 (3)	3.42 (3)	3.45 (2)	3.46 (2)	3.48 (4)	3.50 (2)
$\text{p}K_{\text{a}2}$	5.27 (2)	5.27 (2)	5.32 (2)	5.32 (2)	5.38 (4)	5.41 (2)
$\text{p}K_{\text{a}3}$	7.46 (3)	7.51 (2)	7.59 (3)	7.65 (4)	7.75 (5)	7.79 (4)
$\text{p}K_{\text{a}4}$	9.03 (2)	9.17 (1)	9.29 (1)	9.27 (1)	9.29 (2)	9.41 (1)
$\text{p}K_{\text{a}5}$	10.80 (2)	10.95 (3)	11.05 (6)	11.04 (3)	11.24 (5)	11.31 (4)
$s(A) \times 10^3$	0.19	0.21	0.13	0.24	0.34	0.21
SQUAD						
$\text{p}K_{\text{a}1}$	3.42 (2)	3.42 (2)	3.45 (1)	3.46 (1)	3.48 (2)	3.50 (1)
$\text{p}K_{\text{a}2}$	5.27 (1)	5.27 (1)	5.32 (1)	5.32 (1)	5.38 (1)	5.41 (1)
$\text{p}K_{\text{a}3}$	7.47 (3)	7.51 (2)	7.59 (3)	7.65 (3)	7.76 (4)	7.79 (1)
$\text{p}K_{\text{a}4}$	9.03 (2)	9.17 (2)	9.29 (4)	9.27 (2)	9.30 (3)	9.41 (2)
$\text{p}K_{\text{a}5}$	10.80 (1)	10.95 (1)	11.05 (3)	11.03 (1)	11.24 (3)	11.31 (2)
$s(A) \times 10^3$	0.25	0.26	0.19	0.29	0.44	0.28

As  $\Delta G^0$  values for all five dissociation constants in Table 5 are positive, the dissociation process occurs spontaneously in the reverse direction. The term exothermic describes a process that releases energy from the system, usually in the form of heat. The opposite of an exothermic process is an endothermic process, one that absorbs energy in the form of heat. In Table 5 four cases within the above rules may be distinguished just by examining the signs of the two terms  $\Delta H^0$  and  $\Delta S^0$  on the right side of the Gibbs energy equation:

**Table 5** The thermodynamic dissociation constants of sildenafil citrate at  $T = 288.15$  K,  $298.15$  K and  $310.15$  K and calculated changes of enthalpy  $\Delta H^0$  ( $\text{kJ}\cdot\text{mol}^{-1}$ ), entropy  $\Delta S^0$  ( $\text{J}\cdot\text{K}^{-1}\cdot\text{mol}^{-1}$ ) and Gibbs energy  $\Delta G^0$  ( $\text{kJ}\cdot\text{mol}^{-1}$ ). Standard deviations of the estimated parameters in the last reported digits are given in parentheses

	288.15 K	298.15 K	310.15 K	SPARC prediction	$\Delta H^0$ ( $\text{kJ}\cdot\text{mol}^{-1}$ )	$\Delta S^0_{298,15}$ ( $\text{J}\cdot\text{K}^{-1}\cdot\text{mol}^{-1}$ )	$\Delta G^0_{298,15}$ ( $\text{kJ}\cdot\text{mol}^{-1}$ )
$\text{p}K_{a1}$	2.79 (1)	3.03 (3)	3.53 (1)	3.71	-57.86	-252.06	17.29
$\text{p}K_{a2}$	4.97 (2)	5.23 (2)	5.34 (1)	4.90	-28.53	-195.83	29.85
$\text{p}K_{a3}$	8.14 (2)	7.93 (1)	7.47 (1)	7.28	52.40	23.95	45.26
$\text{p}K_{a4}$	9.47 (2)	9.30 (1)	9.13 (4)	9.03	26.41	-89.44	53.08
$\text{p}K_{a5}$	10.73 (5)	10.75 (3)	10.79 (5)	11.65	-4.69	-221.52	61.36



**Fig. 7** Dependence of the mixed dissociation constant  $\text{p}K_{a,r}$  of sildenafil citrate on the square root of the ionic strength, leading to the thermodynamic dissociation constant  $\text{p}K_a^T$  at  $T = 288.15$ ,  $298.15$  and  $310.15$  K, using the Van't Hoff equation approach (ADSTAT, ORIGIN)

- a)  $\Delta H$  is negative and the dissociation of citrate is exothermic:  $\text{p}K_{a1}$ ,  $\text{p}K_{a2}$ ,  $\text{p}K_{a5}$ .  
 b)  $\Delta H$  is positive and the dissociation of sildenafil is endothermic:  $\text{p}K_{a3}$ ,  $\text{p}K_{a4}$ .

## 5 Conclusions

When drugs are poorly soluble in water, pH-spectrophotometric titration may be used with nonlinear regression of the absorbance–response surface data. The reliability of the



dissociation constants of the drug sildenafil citrate is proven with goodness-of-fit tests of the absorption spectra measured at various pHs. The thermodynamic dissociation constants  $pK_{a,i}^T$  were estimated by a nonlinear regression of ( $pK_{a,i}$ ,  $I$ ) data using an extended Debye-Hückel equation:  $pK_{a,1}^T = 2.79$  (1), 3.03 (3) and 3.53 (1);  $pK_{a,2}^T = 4.97$  (2), 5.23 (2) and 5.34 (1);  $pK_{a,3}^T = 8.14$  (2), 7.93 (1) and 7.47 (1);  $pK_{a,4}^T = 9.47$  (2), 9.30 (1) and 9.13 (4); and  $pK_{a,5}^T = 10.73$  (5), 10.75 (3) and 10.79 (5) at  $T = 288.15$ , 298.15 and 310.15 K, respectively, where the numbers in parentheses are the standard deviations in the last significant digits.

Concurrently, the experimentally determined five thermodynamic dissociation constants were combined with their computational predictions of the SPARC program, based on knowledge of the chemical structures of the drug, and they are in good agreement with its experimental values. Most indices always predict the correct number of components even when the signal-to-error ratio *SER* is much higher than 10.

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