

# Complexation Equilibria of Some Sulphoazoxines

## Part VI.\* Protonation Constants of 7-(4-Carboxyphenylazo)-8-hydroxyquinoline-5-sulphonic Acid by Computer-assisted Multi-component Spectra Analysis

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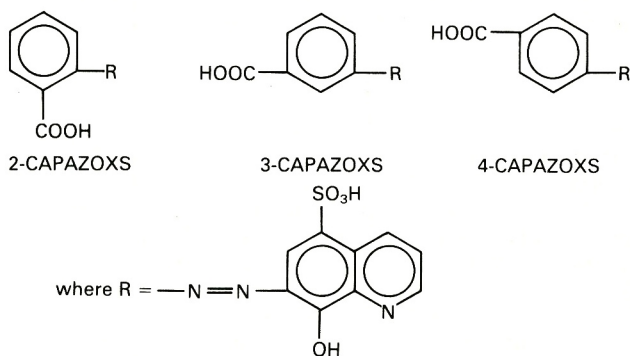
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The protonation constants and molar absorptivities for the formation of the monomers  $L^{3-}$ ,  $LH^{2-}$ ,  $LH_2^-$  and  $LH_3$ , and the dimers  $L_2H_2^{4-}$ ,  $L_2H_3^{3-}$  and  $L_2H_5^-$  of the metallochromic indicator 7-(4-carboxyphenylazo)-8-hydroxyquinoline-5-sulphonic acid (4-CAPAZOXS) have been determined in 0.1 M  $NaClO_4$  using the SPOPT non-linear regression program for absorbance - pH curves at a constant wavelength and by EY608, PSEQUAD(83) and SQUAD(84) analysis of absorbance - pH spectra. The reliability of absorbance - pH curve analysis for overlapping protonation equilibria and the existence of ill-conditioned parameters in the model were examined using the diagnostic SPOPT program. The efficiency of the search for a chemical model of oligomer equilibria by multi-component spectra analysis was proved by the three programs. A reaction scheme for the monomer - dimer equilibria during protonation is suggested.

**Keywords:** Computer assisted multi-component spectra analysis; multi-parametric curve fitting; 7-(4-carboxyphenylazo)-8-hydroxyquinoline-5-sulphonic acid; protonation constants; complexation equilibria

The protonation equilibria of the *ortho*-, *meta*- and *para*-isomers of the metallochromic indicator 7-(2-, 3- or 4-carboxyphenylazo)-8-hydroxyquinoline-5-sulphonic acid (CAPAZOXS) using the dissociation constants and molar absorptivities of the coloured and protonated  $L^{3-}$ ,  $LH^{2-}$  and  $LH_2^-$  forms have been determined in 0.1 M  $NaClO_4$  using absorbance - pH curves and absorbance - pH spectra analysis.<sup>1</sup>



Whereas the spectrophotometric measurements were carried out in a 1-cm cell with a concentration of  $4.5 \times 10^{-5}$  M for 2- and 3-CAPAZOXS, for 4-CAPAZOXS it was necessary to use a substantially lower concentration of  $6.68 \times 10^{-6}$  M and a 5-cm path length. Between pH 2 and 4 the 4-CAPAZOXS isomer exhibits an anomalous series of pH curves, probably owing to the formation of agglomerates, micelles or precipitates. These agglomerates are not formed at lower concentrations, e.g., at  $10^{-6}$  M, and the series of A - pH curves for 4-CAPAZOXS is then analogous to that of 2- and 3-CAPAZOXS.<sup>1</sup>

Recently, the efficiencies of the multi-component spectra analysis programs FA608 + EY608,<sup>2</sup> PSEQUAD(83)<sup>3</sup> and SQUAD(84)<sup>4-7</sup> for chemical model determinations were compared<sup>8</sup> and the guide of A - pH curve analysis, and the computational strategy of SPOPT program<sup>9</sup> was illustrated using the protonation equilibria of 3-CAPAZOXS. The computational conditions found may also be used to interpret the more complicated oligomer-forming protonation equilibria of 4-CAPAZOXS.

This paper discusses the determination of a chemical model for protonation equilibria in which oligomers appear, and illustrates the anomalous behaviour of 4-CAPAZOXS in acid solution. A reaction scheme for monomer - dimer equilibria during protonation is suggested.

### Experimental

#### Computer-assisted Interpretation of Absorbance - pH Spectra

If the protonation equilibria between a ligand, represented by the anion, L, of 4-CAPAZOXS (the charges are omitted for the sake of simplicity) and a proton, H, is considered to form a set of protonated species L, LH,  $LH_2$ ,  $LH_3$ ,  $L_2H_2$ ,  $L_2H_3$ , etc., which have a general formula  $L_qH_r$  in a particular chemical model and are represented by  $n_c$ , the number of species, and  $(q, r)_j$ ,  $j = 1, n_c$ , their stoichiometry, then the over-all protonation constant of the protonated species,  $\beta$ , may be expressed as

$$\beta_{qr} = [L_qH_r]/([L]^q [H]^r) = c/(l^q h^r) \dots \quad (1)$$

where free concentration  $[L] = l$  and  $[H^+] = h$ .

For the  $i$ th solution measured at the  $k$ th wavelength, the absorbance,  $A_{i,k}$ , is defined as

$$A_{i,k} = \sum_{j=1}^{n_c} \epsilon_{k,j} \times c_j = \sum_{j=1}^{n_c} (\epsilon_{qr,k} \beta_{qr} l^q h^r)_j \dots \quad (2)$$

\* For Parts I-V see references 1 and 16-19.



where  $\varepsilon_{qr,k}$  is the molar absorptivity of the  $L_qH_r$  species with stoichiometric coefficients  $q, r$ , measured at the  $k$ th wavelength. The absorbance  $A_{i,k}$  is the element of the absorbance matrix  $A$  of size  $(n_s \times n_w)$  being measured for  $n_s$  solutions with known total concentrations of the basic components,  $c_L$  and  $c_H$ , at  $n_w$  wavelengths.

The multi-component spectra analysing programs FA608 + EY608,<sup>2</sup> PSEQUAD(83)<sup>3</sup> and SQUAD(84)<sup>7</sup> may adjust  $\beta_{qr}$  and  $\varepsilon_{qr}$  for pH-absorption spectra by minimising the residual-square-sum function,  $U$ ,

$$U = \sum_{i=1}^{n_s} \sum_{k=1}^{n_w} (A_{\text{exp},i,k} - A_{\text{calc},i,k})^2$$

$$= \sum_{i=1}^{n_s} \sum_{k=1}^{n_w} (A_{\text{exp},i,k} - \sum_{j=1}^{n_c} \varepsilon_{k,j} c_j)^2 = \text{minimum} \quad (3)$$

where  $A_{i,k}$  represents the element of the experimental absorbance matrix of size  $(n_s \times n_w)$  and the independent variables are the total concentrations of the basic components  $c_L$  and  $c_H$  being adjusted in  $n_s$  solutions.

Unknown parameters may be divided into two equal groups<sup>10,11</sup>: (1) the chemical model input supplied by the user should contain (i) a number of light-absorbing species in solution,  $n_c$ , and (ii) the species stoichiometry indices,  $(q, r)_j$ ,  $j = 1, n_c$ . An estimate of the species stoichiometry may be substituted by the more rigorous ESI method<sup>12</sup> of straight stoichiometric indices determination. This substitution represents an efficient tool recently included in the SQUAD(84)<sup>7</sup> program. (2) The best estimates of the stability constants,  $\beta_{qr,j}$ ,  $j = 1, n_c$ , are adjusted by various regression algorithms, e.g., the Chandler-Stepit in EY608,<sup>2</sup> the Newton-Raphson method in PSEQUAD<sup>3</sup> and the multiple regression and non-negative linear least-squares method in SQUAD(84).<sup>7</sup> At the same time a matrix of molar absorptivities  $(\varepsilon_{qr,k}, k = 1, n_w)_j$ ,  $j = 1, n_c$ , is estimated, based on the current values of stability constants. Only SQUAD(84) estimates the molar absorptivities as non-negative reals.

The experimental and computation scheme for the determination of the protonation constants of multi-component systems was taken from Meloun *et al.*<sup>7</sup> The number of light-absorbing species,  $n_c$ , may be determined by factor analysis of the absorbance matrix.<sup>2</sup> In order to find the rank of the absorbance matrix,  $k^*$ , equal to a number of coloured species,  $n_c$ , the calculated absorbance error,  $s_k(A)$  is compared with the instrumental absorbance error of the spectrophotometer used,  $s_{\text{inst}}(A)$ , determined again by factor analysis of Beer's law dependence spectra for some spectrophotometric standard. If the condition  $s_k^*(A) \approx s_{\text{inst}}(A)$  is met, then the rank of the absorbance matrix equals the number of coloured species in the solution, i.e.,  $n_c = k$ .

Various hypotheses of chemical models with refined parameters have been tested and the statistical characteristics describing the test-of-fit of regression spectra through experimental points have been calculated. The elucidation of individual statistics may be found elsewhere.<sup>7,13,14</sup>

### Computer-assisted Interpretation of Absorbance - pH Curves

The regression programs used for analysing the  $A$  - pH curves at a constant wavelength in order to determine dissociation constants and molar absorptivities have been discussed comprehensively elsewhere.<sup>9-11,15</sup> The reliability of the estimated parameters has been commented on and the limits of precision and accuracy for the estimated parameters have been found for an example of overlapping equilibria and ill-conditioned parameters in a model.<sup>9,11</sup>

The SPORT<sup>9</sup> program, being based on the minimisation of the residual-square-sum function,  $U$ , expressed by equation (3), also calculates various diagnostic and statistical tests. The minimisation block contains a library of 30 optimisation algorithms, both derivative and non-derivative, which may be

called from the input. The error-analysis block calculates the confidence interval of parametric estimates and the fitness test rigorously examines the degree of fit obtained by the statistical analysis of residuals. The output block prints out parameter estimates, their standard deviations, the curve-fitting graph and the computer-drawn hyperparaboloid surface  $(1 - U)$  in optioned parametric co-ordinates in order to investigate the conditioning of both parameters in the model.

### Chemicals and Solutions

The preparations of 4-CAPAZOXS was described in the preceding papers of this series.<sup>1,16-19</sup> The purity was checked by paper chromatography using a Whatman 2 paper impregnated with EDTA in a descending arrangement in a water-pyridine-ethanol (2 + 1 + 4) system. Solutions of 4-CAPAZOXS in alkaline and neutral media are stable for up to 200 h, whereas acidic solutions begin to decolourise after about 20 h owing to the slow precipitation of salted-out indicator.

The preparation of buffers and other solutions from analytical-reagent grade chemicals has also been described earlier.<sup>1</sup>

Absorbance matrices of 4-CAPAZOXS at various pH values were analysed. The first set of spectra was obtained at a low 4-CAPAZOXS concentration ( $6.676 \times 10^{-6}$  M) in a 5-cm path length cell in which no oligomers were proved, whereas the second set was obtained at a concentration 7 times higher, measured in a 1-cm cell with oligomers in solution.

### Spectrophotometric Measurements

The pH-spectrophotometric titration was performed outside the cuvette with the use of a VSU2-G spectrophotometer (Zeiss, Jena, GDR) and was carried out as described in the first paper of this series.<sup>16</sup>

### Computation

Data treatment has been performed using the least-squares program SPORT<sup>9</sup> ( $A$  - pH curve analysis) and by the regression spectra analysing programs FA608 + EY608,<sup>2</sup> PSEQUAD(83)<sup>3</sup> and SQUAD(84).<sup>7</sup> The computation was performed on the EC 1033 (500K) computer in the Computing Centre, College of Chemical Technology, Pardubice, Czechoslovakia.

### Discussion

Solutions of 4-CAPAZOXS differing in pH contain four light-absorbing species,  $L^{3-}$ ,  $LH^{2-}$ ,  $LH_2^-$  and  $LH_3$ . The dissociation of the sulphonic group cannot be studied owing to its low solubility in strongly acidic media. The protonation equilibria were studied spectrophotometrically at two concentrations, at  $6.676 \times 10^{-6}$  M in a 5-cm cuvette and at  $4.506 \times 10^{-5}$  M in a 1-cm cuvette.

4-CAPAZOXS shows two isosbestic points in spectral curves, one at 473 nm and the other at 496 nm, but the spectra of solutions at pH 3.2 and 4 do not cross either of these isosbestic points. One overlap of two protonation equilibria ( $LH_2^- - LH_2^-$  and  $LH_2^- - LH_3$ ) exists in this pH region.

The current evaluation of  $A$  - pH curves by the regression SPORT program is based on a search for a best fit of the calculated regression curve through experimental  $A$  - pH points and this objective fitness test represents a resolution criterion between two tested chemical models (Table 1). The reliability of seven parameters,  $pK_{a1}$ ,  $pK_{a2}$ ,  $pK_{a3}$ ,  $\varepsilon_L$ ,  $\varepsilon_{LH}$ ,  $\varepsilon_{LH_2}$  and  $\varepsilon_{LH_3}$ , is considered by the values obtained for the residual mean,  $m_{r,1}$ , and the mean residual,  $|\bar{r}|$ , which should be close in value to the instrumental error of the spectrophotometer used,  $s_{\text{inst}}(A)$  [for the VSU2-G spectrophotometer used, the value of  $s_{\text{inst}}(A)$  is about 0.003 as obtained from a



**Table 1.** Non-linear regression of the  $A$  - pH curve of  $6.676 \times 10^{-6}$  M 4-CAPAZOXS at 480 nm by the algorithmic minimisation of the Rosenbrock method using the SPORT program. Free concentrations are relative to the total ligand concentration,  $c_L$ . Experimental conditions:  $6.676 \times 10^{-6}$  M 4-CAPAZOXS; path length, 5 cm;  $I = 0.1$  (NaClO<sub>4</sub> + Na<sub>3</sub>PO<sub>4</sub> + HClO<sub>4</sub> + EDTA); temperature = 25 °C:  $s_{\text{inst}}(A) = 0.003$  (VSU2-G, Zeiss, Jena, GDR). Minimisation terminated at  $U_{\text{min}} = 1.53 \times 10^{-4}$  with  $s(A) = 0.0030$ .

*Estimated values of parameters—*

EPS(L) $\times 10^{-4}$	$= 1.705 \pm 0.017$	
EPS(LH) $\times 10^{-4}$	$= 1.873 \pm 0.006$	
$pK_{a_1}$	$= 7.453 \pm 0.108$	$\log \beta_{11} = 7.453 \pm 0.108$
EPS(LH <sub>2</sub> ) $\times 10^{-4}$	$= 2.052 \pm 0.019$	
$pK_{a_2}$	$= 3.616 \pm 0.185$	$\log \beta_{12} = 11.069 \pm 0.185$
EPS(LH <sub>3</sub> ) $\times 10^{-4}$	$= 1.574 \pm 0.010$	
$pK_{a_3}$	$= 2.722 \pm 0.158$	$\log \beta_{13} = 13.791 \pm 0.158$

$i$	$pH_i$	$A_{\text{exp}}$	$A_{\text{calc}}$	Residual	[L], %	[LH], %	[LH <sub>2</sub> ], %	[LH <sub>3</sub> ], %
1	8.225	0.5780	0.5772	0.0008	85.53	14.47	0.00	0.00
2	8.030	0.5800	0.5809	-0.0009	79.05	20.95	0.00	0.00
3	7.857	0.5840	0.5850	-0.0010	71.70	28.30	0.00	0.00
4	7.662	0.5900	0.5906	-0.0006	61.78	38.21	0.00	0.00
5	7.516	0.5960	0.5952	0.0008	53.60	46.40	0.01	0.00
6	7.387	0.6020	0.5994	0.0026	46.19	53.81	0.01	0.00
7	7.194	0.6070	0.6055	0.0015	35.49	64.49	0.02	0.00
8	6.975	0.6080	0.6114	-0.0034	24.94	75.03	0.03	0.00
9	6.520	0.6180	0.6196	-0.0016	10.43	89.46	0.11	0.00
10	4.997	0.6300	0.6276	0.0024	0.33	95.67	3.97	0.02
11	3.930	0.6430	0.6425	0.0005	0.02	66.13	32.04	1.81
12	3.742	0.6450	0.6460	-0.0010	0.01	55.19	41.22	3.58
13	3.551	0.6440	0.6477	-0.0037	0.01	43.18	50.06	6.76
14	3.423	0.6480	0.6470	0.0010	0.00	35.22	54.84	9.94
15	3.305	0.6490	0.6446	0.0044	0.00	28.34	57.89	13.77
16	3.140	0.6430	0.6379	0.0051	0.00	19.90	59.44	30.66
17	3.068	0.6280	0.6338	-0.0058	0.00	16.74	59.03	24.22
18	2.892	0.6200	0.6211	-0.0011	0.00	10.48	55.42	34.10
19	2.616	0.5940	0.5968	-0.0028	0.00	4.43	44.21	51.36
20	2.442	0.5820	0.5813	0.0007	0.00	2.40	35.70	61.91
21	2.257	0.5700	0.5668	0.0032	0.00	1.18	27.03	71.78
22	2.006	0.5530	0.5514	0.0016	0.00	0.43	17.37	82.20
23	1.758	0.5400	0.5412	-0.0012	0.00	0.15	10.65	89.20
24	1.587	0.5350	0.5364	-0.0014	0.00	0.07	7.45	92.48

*Statistical analysis of residuals—*

Residual mean	.. ..	$4.0 \times 10^{-10}$
Mean residual	.. ..	0.0020
Standard deviation	.. ..	0.0025
Skewness	.. ..	-0.07
Curtosis	.. ..	2.86
Hamilton R-factor, %	.. ..	0.42

CPU time/s 1319.5

plot of the relationship  $s_{\text{inst}}(A) = f(A)$ . Other statistical quantities indicate the quality achieved for an actual distribution of residuals. Because the skewness,  $m_{r,3} = -0.07$ , is close to zero, the kurtosis,  $m_{r,4} = 2.86$ , is close to 3 and Pearson's chi-squared test value,  $\chi^2_{\text{exp}} = 8.67$ , is less than the critical value ( $\chi^2_{0.05,6} = 12.60$ ) for 6 degrees of freedom and a 0.05 significance level,<sup>14</sup> it may be concluded that the actual residual distribution is of a Gaussian normal nature. The Hamilton R-factor of relative fitness with respect to absorbance values reaches 0.42% and proves the excellent degree of fit achieved.

Table 2 shows the results of eight  $A$  - pH curve analyses at selected "analytical" wavelengths from the original absorbance matrix for  $6.675 \times 10^{-6}$  M 4-CAPAZOXS. The reliability of an estimation of ill-conditioned parameters depends on the choice of minimisation algorithm. As four parameters,  $pK_{a_1}$ ,  $\epsilon_L$ ,  $\epsilon_{LH}$  and  $\epsilon_{LH_3}$  are well conditioned in the mathematical model of equation (3), the paraboloid of a residual - square - sum function exhibits a well developed minimum. The remaining three parameters,  $pK_{a_2}$ ,  $pK_{a_3}$  and  $\epsilon_{LH_2}$ , are ill-conditioned in the model of equation (3), and the resulting paraboloid is of a "flat-bottomed saucer" shape and it is not

possible to indicate an obvious pit (as was possible in Figs. 3–6 of reference 9). A search for the true estimates of parameters is then unreliable and most minimisation algorithms fail. The careful choice of a successful minimisation subroutine, its computation strategy, parameter restrictions, minimisation steps, termination criteria avoiding a cyclisation or divergency, etc., may be carried out using the SPORT library of minimisation algorithms.<sup>9</sup>

Table 2 also shows selected algorithms for a particular  $A$  - pH curve analysis, which, even when started from various different initial parameter estimations, always led to the same minimum. As estimates of three ill-conditioned parameters differ slightly in the values obtained by various algorithms, the whole pH - absorbance matrix was also analysed by three regression spectra analysing programs and the values compared (Tables 2 and 3). This set of absorption spectra enables more information to be obtained about the analysed solution equilibria system. To check the estimates of protonation constants obtained above, 24 absorbance - pH spectra measured at 19 wavelengths from 390 to 570 nm were analysed by FA608 + EY608, PSEQUAD(83) and SQUAD(84) (Table 3).



**Table 2.** Non-linear regression of  $A$  - pH curves of  $6.676 \times 10^{-6}$  M 4-CAPAZOXS for eight selected wavelengths using the SPOPT program algorithms. The upper row of each pair of values shows the molar absorptivity estimated by the SPOPT program and the lower row that estimated by SQUAD(84). Experimental conditions as in Tables 1 and 3

	Algorithm used							
	Rosenbrock 430 nm	Rosenbrock 440 nm	Gradient 450 nm	Rosenbrock 480 nm	Grid 500 nm	Grid 510 nm	Rosenbrock 520 nm	Gradient 540 nm
EPS(L) $\times 10^{-4}$ ..	1.068 $\pm$ 0.013 1.068 $\pm$ 0.033	1.212 $\pm$ 0.014 1.208 $\pm$ 0.034	1.331 $\pm$ 0.024 1.303 $\pm$ 0.039	1.705 $\pm$ 0.017 1.701 $\pm$ 0.030	1.643 $\pm$ 0.012 1.655 $\pm$ 0.030	1.470 $\pm$ 0.014 1.475 $\pm$ 0.032	1.211 $\pm$ 0.012 1.220 $\pm$ 0.035	0.599 $\pm$ 0.021 0.598 $\pm$ 0.034
EPS(LH) $\times 10^{-4}$	0.780 $\pm$ 0.006 0.779 $\pm$ 0.028	0.912 $\pm$ 0.007 0.910 $\pm$ 0.029	1.120 $\pm$ 0.017 1.118 $\pm$ 0.033	1.873 $\pm$ 0.006 1.873 $\pm$ 0.025	2.056 $\pm$ 0.011 2.073 $\pm$ 0.024	1.909 $\pm$ 0.011 1.922 $\pm$ 0.026	1.851 $\pm$ 0.014 1.864 $\pm$ 0.029	0.966 $\pm$ 0.006 0.963 $\pm$ 0.028
pK <sub>a1</sub> .. .. .	7.504 $\pm$ 0.052	7.528 $\pm$ 0.058	7.512 $\pm$ 0.185	7.453 $\pm$ 0.108	7.569 $\pm$ 0.048	7.541 $\pm$ 0.049	7.534 $\pm$ 0.032	7.492 $\pm$ 0.062
EPS(LH <sub>2</sub> ) $\times 10^{-4}$	0.874 $\pm$ 0.015 0.855 $\pm$ 0.036	1.032 $\pm$ 0.017 1.007 $\pm$ 0.037	1.241 $\pm$ 0.017 1.232 $\pm$ 0.042	2.052 $\pm$ 0.019 2.038 $\pm$ 0.032	2.129 $\pm$ 0.047 2.193 $\pm$ 0.031	1.994 $\pm$ 0.059 2.063 $\pm$ 0.034	1.879 $\pm$ 0.029 1.930 $\pm$ 0.037	1.034 $\pm$ 0.026 1.037 $\pm$ 0.037
pK <sub>a2</sub> .. .. .	10.855 $\pm$ 0.203	10.935 $\pm$ 0.194	11.081 $\pm$ 0.201	11.069 $\pm$ 0.185	12.165 $\pm$ 1.453	11.923 $\pm$ 0.888	12.574 $\pm$ 1.403	11.173 $\pm$ 0.054
EPS(LH <sub>3</sub> ) $\times 10^{-4}$	0.516 $\pm$ 0.007 0.510 $\pm$ 0.029	0.592 $\pm$ 0.008 0.583 $\pm$ 0.030	0.743 $\pm$ 0.015 0.739 $\pm$ 0.034	1.574 $\pm$ 0.010 1.568 $\pm$ 0.026	2.380 $\pm$ 0.012 2.379 $\pm$ 0.025	2.368 $\pm$ 0.009 2.369 $\pm$ 0.027	2.262 $\pm$ 0.013 2.263 $\pm$ 0.030	2.533 $\pm$ 0.007 2.496 $\pm$ 0.030
pK <sub>a3</sub> .. .. .	13.610 $\pm$ 0.159	13.705 $\pm$ 0.156	13.784 $\pm$ 0.166	13.791 $\pm$ 0.158	14.998 $\pm$ 1.620	14.700 $\pm$ 1.025	15.308 $\pm$ 1.459	13.845 $\pm$ 0.056
U <sub>min</sub> $\times 10^4$ .. ..	1.83	1.93	2.61	1.53	1.17	2.04	2.08	2.14
s(A) $\times 10^3$ .. ..	3.28	3.37	3.92	3.00	2.63	3.46	3.50	3.55
Statistical analysis of residuals—								
Residual mean ..	-5.6 $\times 10^{-9}$	-7.9 $\times 10^{-9}$	-6.1 $\times 10^{-4}$	4.0 $\times 10^{-8}$	-7.1 $\times 10^{-6}$	-2.6 $\times 10^{-4}$	2.7 $\times 10^{-7}$	-2.0 $\times 10^{-3}$
Mean residual ..	0.0025	0.0024	0.0024	0.0020	0.0016	0.0022	0.0022	0.0023
Standard deviation	0.0028	0.0028	0.0033	0.0025	0.0022	0.0029	0.0029	0.0030
Skewness .. ..	-0.02	-0.16	0.12	-0.07	1.57	1.46	1.06	0.72
Curtosis .. ..	1.72	2.47	4.55	2.86	5.73	5.38	3.71	3.20
Hamilton R-factor(%) ..	1.03	0.91	0.89	0.42	0.31	0.43	0.47	0.65
Pearson $\chi^2$ test	Accepted	Accepted	Rejected	Accepted	Accepted	Accepted	Accepted	Accepted
CPU time/s .. ..	1024.3	1290.4	116.7	1319.8	1868.9	1881.9	1888.7	118.1

**Table 3.** Stoichiometry and stability constants of the stepwise protonation of 4-CAPAZOXS obtained by using three regression multi-component spectra analysing programs. Experimental conditions as in Table 1;  $n_z = 2$ ;  $n_c = 4$ ;  $n_s = 24$ ;  $n_w = 19$ ;  $\lambda_{\text{start}} = 390$  nm;  $\lambda_{\text{end}} = 570$  nm; estimated matrix rank  $k^* = 4$  for  $s_k^*(A) = 0.0010$

	Program				
	EY608	PSEUAD(83)	SQUAD(84)		
Log $\beta_{11}$ .. .. .	7.499 $\pm$ 0.055	7.486 $\pm$ 0.019	7.501 $\pm$ 0.014	7.804 $\pm$ 0.271	7.431 $\pm$ 0.253
490 nm, $\epsilon_{10} \times 10^{-4}$ .. ..	1.730	1.733 $\pm$ 0.011	1.730 $\pm$ 0.031	1.725 $\pm$ 0.032	1.727 $\pm$ 0.032
490 nm, $\epsilon_{11} \times 10^{-4}$ .. ..	2.109	2.111 $\pm$ 0.008	2.109 $\pm$ 0.026	2.232 $\pm$ 0.028	2.108 $\pm$ 0.027
[L] range .. .. .	$6 \times 10^{-6.5} \times 10^{-15}$	$6 \times 10^{-6.5} \times 10^{-15}$	$6 \times 10^{-6.5} \times 10^{-15}$	$6 \times 10^{-6.1} \times 10^{-14}$	$6 \times 10^{-6.8} \times 10^{-15}$
[LH] range .. .. .	$6 \times 10^{-6.4} \times 10^{-9}$	$6 \times 10^{-6.4} \times 10^{-9}$	$6 \times 10^{-9.4} \times 10^{-9}$	$6 \times 10^{-6.3} \times 10^{-9}$	$6 \times 10^{-6.6} \times 10^{-9}$
Log $\beta_{12}$ .. .. .	11.164 $\pm$ 0.051	11.143 $\pm$ 0.072	11.186 $\pm$ 0.041	11.537 $\pm$ 0.739	10.749 $\pm$ 0.475
490 nm, $\epsilon_{12} \times 10^{-4}$ .. ..	2.237	2.241 $\pm$ 0.011	2.193 $\pm$ 0.035	2.372 $\pm$ 0.035	2.240 $\pm$ 0.035
[LH <sub>2</sub> ] range .. .. .	$4 \times 10^{-6.3} \times 10^{-11}$	$4 \times 10^{-6.3} \times 10^{-11}$	$4 \times 10^{-6.3} \times 10^{-11}$	$4 \times 10^{-6.3} \times 10^{-11}$	$4 \times 10^{-6.9} \times 10^{-11}$
Log $\beta_{13}$ .. .. .	13.840 $\pm$ 0.056	13.823 $\pm$ 0.073	13.854 $\pm$ 0.042	14.053 $\pm$ 0.997	13.497 $\pm$ 0.534
540 nm, $\epsilon_{13} \times 10^{-4}$ .. ..	2.493	2.492 $\pm$ 0.011	2.496 $\pm$ 0.030	2.598 $\pm$ 0.031	2.482 $\pm$ 0.029
LH: $q_1, r_1$ .. .. .	1, 1	1, 1	1, 1	1.06 $\pm$ 0.05, 1	1, 0.99 $\pm$ 0.04
LH <sub>2</sub> : $q_2, r_2$ .. .. .	1, 2	1, 2	1, 2	1.06 $\pm$ 0.10, 2	1, 1.89 $\pm$ 0.11
LH <sub>3</sub> : $q_3, r_3$ .. .. .	1, 3	1, 3	1, 3	1.05 $\pm$ 0.11, 3	1, 2.92 $\pm$ 0.14
U <sub>min</sub> $\times 10^3$ .. .. .	3.91	8.10	3.59	3.54	3.54
s(A) .. .. .	0.0040	0.0045	0.0030	0.0030	0.0030
Fitness test by statistical analysis of residuals—					
Residual mean .. .. .	-7.57 $\times 10^{-7}$	—	-1.40 $\times 10^{-16}$	-2.02 $\times 10^{-14}$	-1.96 $\times 10^{-16}$
Mean residual .. .. .	0.0025	—	0.0021	0.0021	0.0021
Standard deviation .. ..	0.0040	—	0.0030	0.0030	0.0030
Skewness .. .. .	0.855	—	-0.274	-0.267	-0.262
Curtosis .. .. .	13.326	—	3.033	2.976	3.003
Hamilton R-factor, % ..	0.824	—	0.594	0.591	0.590
CPU time/s .. .. .	52.1	52.6	123.2	481.9	243.6

In the first step of FA608 + EY608 or SQUAD(84) spectra treatment, the factor analysis FA608 is applied and the matrix rank of the absorbance matrix,  $k^*$ , is determined (here equal to 4), and the corresponding absorbance error,  $s_k^*(A)$  (here equal to 0.0010). Following the minimisation, the program refines all the requested parameters and when a termination criterion is fulfilled, the process ends at the minimum of the residual - square - sum function,  $U_{\text{min}}$ . The standard deviation of absorbance after termination,  $s(A)$ , is calculated from the  $U$  value using the equation  $s(A) = (U_{\text{min}}/D.f.)^{1/2}$ , where

D.f. are degrees of freedom, equal to the difference of elements in the absorbance matrix and the number of parameters to be refined. As the instrumental absorbance error of the spectrophotometer used [ $s_{\text{inst}}(A) = 0.003$ ], and the absorbance error estimated by factor analysis [ $s_k^*(A) = 0.001$ ] are in agreement with the calculated standard deviation of absorbance after termination,  $s(A) = 0.003$ , the minimisation process can be considered to be successful.

In accordance with the Guide to Diagnostics Examination,<sup>7</sup> the refined stability constants and their standard deviations



have a physical meaning and the estimated curves of molar absorptivities *versus* wavelength for all the protonated species also have physical meaning as no values are negative and also no values reach unrealistically high values. The calculated free concentrations of all the protonated species are realistic because every concentration range starts or ends at a concentration greater than  $10^{-8}$  M. The correlation coefficients show that there is a strong correlation between the two protonation constants of overlapping equilibrium  $\log \beta_{12}$  and  $\log \beta_{13}$ , but that there is negligible correlation between the protonation constants  $\log \beta_{11}$  and  $\log \beta_{12}$  or  $\log \beta_{11}$  and  $\log \beta_{13}$ .

Rigorous fitness tests by objective statistics prove that the calculated regression spectra fit sufficiently through the experimental points.

All three programs, FA608 + EY608, PSEQUAD(83) and SQUAD(84), were in agreement over the refined parameters. Two runs of SQUAD(84) in Table 3 give an estimation of the stoichiometric indices  $q$  and  $r$  in  $L_qH_r$  obtained by the ESI method<sup>12</sup> simultaneously with the stability constants and molar absorptivities of all the protonated species. Estimated reals for  $q$  and  $r$  are very close to integer values and describe the L, LH, LH<sub>2</sub> and LH<sub>3</sub> species. No oligomers were found.

**Table 4.** A search of the most probable hypothesis of chemical model in oligomer-forming protonation equilibria of 4-CAPAZOXS by regression spectra analysis using the SQUAD(84) program. Experimental conditions:  $4.506 \times 10^{-5}$  M 4-CAPAZOXS;  $I = 0.1$  (NaClO<sub>4</sub> + Na<sub>3</sub>PO<sub>4</sub> + HClO<sub>4</sub> + EDTA); temperature, 25°C; path length, 1 cm; pH, various;  $s_{\text{inst}}(A) = 0.003$  (VSU2-G, Zeiss, Jena, GDR);  $n_z = 2$ ;  $n_c = 4$ ;  $n_s = 22$ ;  $n_w = 19$ ;  $\lambda_{\text{start}} = 390$  nm;  $\lambda_{\text{end}} = 570$  nm; estimated matrix rank  $k^* = 4$  for  $s_k^*(A) = 0.0015$

Run	Hypothesis of chemical model	$s(A)$	R-factor	Remark	Model testing
1	LH, LH <sub>2</sub> , LH <sub>3</sub>	0.0227	Not calculated	Failing*	Rejected
2	LH, LH <sub>2</sub> , LH <sub>3</sub> , L <sub>2</sub> H <sub>2</sub>	0.2213	32.90		Rejected
3	LH, LH <sub>2</sub> , LH <sub>3</sub> , L <sub>2</sub> H <sub>2</sub> , L <sub>2</sub> H <sub>3</sub>	0.0585	9.0	Divergence	Rejected
4	LH, LH <sub>2</sub> , LH <sub>3</sub> , L <sub>2</sub> H <sub>2</sub> , L <sub>2</sub> H <sub>3</sub> , L <sub>2</sub> H <sub>5</sub>	0.0558	8.35	Negative absorptivity	Rejected
5	LH, LH <sub>2</sub> , LH <sub>3</sub> , L <sub>2</sub> H <sub>2</sub> , L <sub>2</sub> H <sub>3</sub> , L <sub>2</sub> H <sub>5</sub> , L <sub>3</sub> H <sub>4</sub>	Not calculated	Not calculated	Failing*	Rejected
6	LH, LH <sub>2</sub> , L <sub>2</sub> H <sub>5</sub>	0.0218	3.18	Negative absorptivity	Rejected
7	LH, L <sub>2</sub> H <sub>2</sub> , L <sub>2</sub> H <sub>5</sub>	0.0200	2.82	Negative absorptivity	Rejected
8	LH, L <sub>2</sub> H <sub>2</sub> , L <sub>2</sub> H <sub>3</sub> , L <sub>2</sub> H <sub>5</sub>	0.0224	3.17	Negative absorptivity	Rejected
9	L <sub>2</sub> H <sub>2</sub> , L <sub>2</sub> H <sub>3</sub> , L <sub>3</sub> H <sub>5</sub>	0.1084	Not calculated	Failing*	Rejected
10	L <sub>2</sub> H <sub>5</sub> , L <sub>3</sub> H <sub>5</sub> , L <sub>3</sub> H <sub>3</sub>	0.0599	Not calculated	Failing*	Rejected
11	L <sub>2</sub> H <sub>2</sub> , L <sub>2</sub> H <sub>3</sub> , L <sub>2</sub> H <sub>5</sub> , L <sub>3</sub> H <sub>4</sub>	0.0703	Not calculated	Failing*	Rejected
12	LH <sub>2</sub> , L <sub>2</sub> H <sub>2</sub> , L <sub>2</sub> H <sub>3</sub>	0.0251	3.66	Negative absorptivity	Rejected
13	L <sub>2</sub> H <sub>2</sub> , L <sub>2</sub> H <sub>5</sub>	0.0296	4.43	Local minimum by MR† method	Accepted
14	LH <sub>2</sub> , L <sub>2</sub> H <sub>2</sub> , L <sub>2</sub> H <sub>5</sub>	0.0202	2.95	Minimum by NNLS† method	Accepted
15	L <sub>2</sub> H <sub>2</sub> , L <sub>2</sub> H <sub>3</sub> , L <sub>2</sub> H <sub>5</sub>	0.0274	3.99	Local minimum by MR† method	Accepted

\* Failing means that the minimisation process was interrupted in the intermediate part.

† The NNLS algorithm calculates non-negative molar absorptivities whereas the MR algorithm is a multiple regression which has no negative protection.

**Table 5.** Two accepted hypotheses of tested chemical models in the oligomer-forming protonation equilibria of 4-CAPAZOXS by regression spectra analysis of the SQUAD(84) program. Experimental conditions as in Table 4

Minimisation method	Tested hypothesis of chemical model		
	L <sub>2</sub> H <sub>2</sub> , L <sub>2</sub> H <sub>3</sub> , L <sub>2</sub> H <sub>5</sub>	LH <sub>2</sub> , L <sub>2</sub> H <sub>2</sub> , L <sub>2</sub> H <sub>5</sub>	
	Multiple regression	Multiple regression	Non-negative least-squares
Log $\beta_{12}$ .. .. .	—	10.941 ± 0.060	10.951 ± 0.057
490 nm, $\epsilon_{10} \times 10^{-4}$ .. .. .	—	2.742 ± 0.035	2.734 ± 0.035
490 nm, $\epsilon_{12} \times 10^{-4}$ .. .. .	—	3.166 ± 0.165	3.126 ± 0.161
[L] range .. .. .	—	$4 \times 10^{-5}$ – $5 \times 10^{-13}$	$4 \times 10^{-5}$ – $4 \times 10^{-13}$
[LH <sub>2</sub> ] range .. .. .	—	$1 \times 10^{-5}$ – $2 \times 10^{-11}$	$1 \times 10^{-5}$ – $2 \times 10^{-11}$
Log $\beta_{22}$ .. .. .	19.034 ± 0.095	18.939 ± 0.065	18.943 ± 0.066
490 nm, $\epsilon_{22} \times 10^{-4}$ .. .. .	5.316 ± 0.107	5.310 ± 0.070	5.296 ± 0.070
[L <sub>2</sub> H <sub>2</sub> ] range .. .. .	$2 \times 10^{-5}$ – $1 \times 10^{-10}$	$2 \times 10^{-5}$ – $1 \times 10^{-10}$	$2 \times 10^{-5}$ – $1 \times 10^{-10}$
Log $\beta_{23}$ .. .. .	23.201 ± 0.028	—	—
490 nm, $\epsilon_{23} \times 10^{-4}$ .. .. .	5.565 ± 0.242	—	—
[L <sub>2</sub> H <sub>3</sub> ] range .. .. .	$1 \times 10^{-5}$ – $4 \times 10^{-12}$	—	—
Log $\beta_{25}$ .. .. .	30.904 ± 0.198	30.595 ± 0.081	30.601 ± 0.082
500 nm, $\epsilon_{25} \times 10^{-4}$ .. .. .	2.750 ± 0.058	2.455 ± 0.080	2.444 ± 0.079
[L <sub>2</sub> H <sub>5</sub> ] range .. .. .	$2 \times 10^{-5}$ – $1 \times 10^{-21}$	$2 \times 10^{-5}$ – $5 \times 10^{-22}$	$2 \times 10^{-5}$ – $5 \times 10^{-22}$
$U_{\text{min}} \times 10$ .. .. .	3.02	1.65	1.66
$s(A)$ .. .. .	0.0274	0.0203	0.0204
<i>Fitness test by statistical analysis of residuals—</i>			
Residual mean .. .. .	$-5.13 \times 10^{-5}$	$-3.77 \times 10^{-6}$	$-1.52 \times 10^{-3}$
Mean residual .. .. .	0.0177	0.0138	0.0138
Standard deviation .. .. .	0.0277	0.0203	0.0204
Skewness .. .. .	-0.298	-0.381	-0.647
Curtosis .. .. .	3.373	2.884	3.023
Hamilton R-factor, % .. .. .	3.994	2.950	2.965
CPU time/s .. .. .	305.6	257.6	489.6



To examine the anomalous series of A - pH curves obtained for higher concentrations of 4-CAPAZOXS and to determine the oligomers, the absorbance matrix for 22 solutions and 19 wavelengths and for a concentration seven times higher than the previous example was analysed. Table 4 shows a search carried out by SQUAD(84) for the best chemical model. Fifteen hypotheses of chemical models were tested in order to find the one which best represented the data. The criteria of resolution used for the hypotheses were: (1) a failure of minimisation process in a divergency or a cyclisation; (2) an examination of the physical meaning of the estimated parameters if they were both realistic and positive; and (3) the residuals should be randomly distributed about the predicted regression spectrum and systematic departures from randomness were taken to indicate that either the model or parameter estimates were not satisfactory.

The first hypothesis proves that not only monomers are present. The second to fifth hypotheses proved that some oligomers were added to three monomers, but these and resulting models were rejected. Hypotheses of run numbers 13-15 were accepted because (i) the parameters found had a physical meaning, and (ii) a good fit was achieved.

Table 5 shows a survey of the best chemical models proved by the SQUAD(84) program. The estimated stability constants of three oligomers  $L_2H_2$ ,  $L_2H_3$  and  $L_2H_5$  ( $\log \beta_{22} = 18.943 \pm 0.066$ ,  $\log \beta_{23} = 23.201 \pm 0.242$  and  $\log \beta_{25} = 30.601 \pm 0.081$ , respectively) are in agreement with those found by poten-

tiometric titration by Hynková and co-workers<sup>20,21</sup> for concentrations 510 times higher ( $c_L = 2.3 \times 10^{-2} M$ ,  $\log \beta_{22} = 18.50 \pm 0.01$  and  $\log \beta_{23} = 23.44 \pm 0.02$ ).

Factor analysis was used to calculate the absorbance matrix rank,  $k^*$ , and the number of light-absorbing species in solution,  $n_c = k^* = 3$ , and the corresponding absorbance error,  $s_f^2(A) = 0.0037$ . The minimisation process terminated at a higher value of absorbance standard deviation,  $s(A) = 0.020$ , indicating some drift in data. This drift may be explained by some competitive equilibria, perhaps a formation of higher oligomers, micelles and the salting-out of the 4-CAPAZOXS. These species are present in low concentrations, and the situation is well known in equilibria studies in which there is enough of a species to interfere, but not enough to determine. Even when the spectra treated by computer analysis are from spectrophotometers of high precision, the selection of a certain range of data was necessary. Spectra of the solutions in which precipitation, even in a colloidal form, was observed were excluded from data treatment.

Graphs of the estimated molar absorptivities of protonated forms of 4-CAPAZOXS versus wavelength show no oligomers for dilute solutions (Fig. 1), whereas for concentrations higher than  $4.506 \times 10^{-5} M$ , three protonated dimers appear (Fig. 2).

The distribution diagrams of the relative population of protonated species of the acid - base equilibrium versus pH demonstrate the conditions under which only monomers exist (Fig. 3) and those under which dimers are formed (Fig. 4).

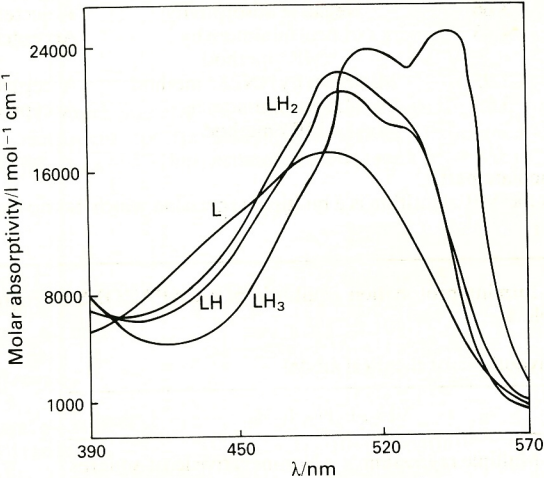


Fig. 1. Molar absorptivity of protonated monomers of 4-CAPAZOXS versus wavelength

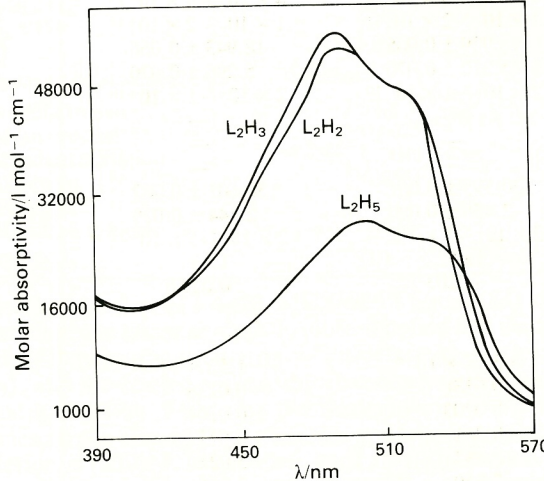


Fig. 2. Molar absorptivity of protonated dimers of 4-CAPAZOXS versus wavelength

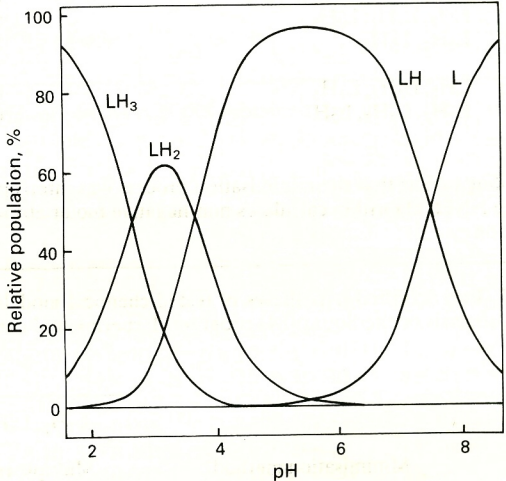


Fig. 3. Distribution diagram of relative population of protonated monomers of 4-CAPAZOXS versus pH

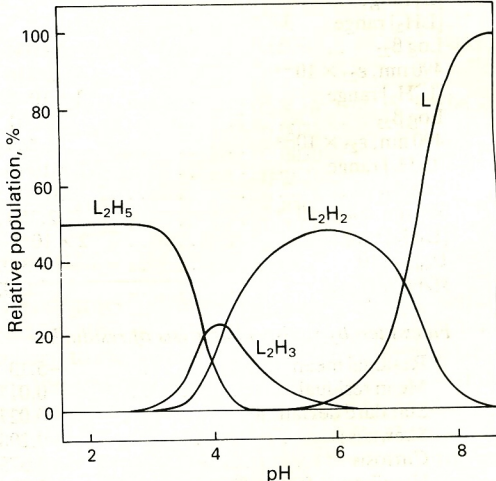


Fig. 4. Distribution diagram of relative population of protonated dimers of 4-CAPAZOXS versus pH

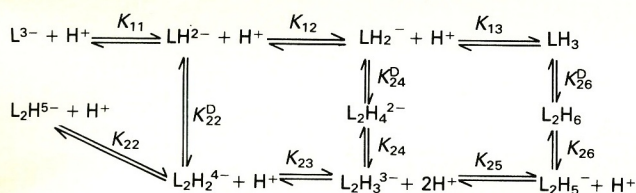


**Table 6.** Comparison of final and consecutive protonation and dimerisation constants of the oligomer-forming protonation equilibria of 4-CAPAZOXS, estimated by regression spectra analysis for  $c_L = 4.5 \times 10^{-5}$  M (oligomers determined) and for  $c_L = 6.676 \times 10^{-6}$  M (monomers) in this paper (upper row of each pair of values) and by regression analysis of potentiometric data for  $c_L = 2.3 \times 10^{-2}$  M in references 20 and 21 (lower row of each pair of values) oligomers only

Protonation constants		Dimerisation constant
Final	Consecutive	
Log $\beta_{11} = 7.453 \pm 0.108$ 7.45	Log $K_{11} = 7.453$ 7.45	
Log $\beta_{12} = 11.069 \pm 0.185$ 10.43	Log $K_{12} = 3.616$ 2.98	
Log $\beta_{13} = 13.791 \pm 0.158$ 13.17	Log $K_{13} = 2.722$ 2.74	
Log $\beta_{22} = 18.943 \pm 0.066$ 18.43 $\pm 0.07$	Log $K_{22} = 8.813$ 8.30	Log $K_{22}^D = 3.524$ 3.53
Log $\beta_{23} = 23.201 \pm 0.242$ 23.31 $\pm 0.17$	Log $K_{23} = 4.258$ 4.88	Log $K_{23}^D = 4.679$ 5.43
Log $\beta_{24} =$ Not indicated 28.00 $\pm 0.16$	Log $K_{24} =$ Not indicated 4.69	Log $K_{24}^D =$ Not indicated 7.14
Log $\beta_{25} = 30.601 \pm 0.081$ Not indicated	Log $K_{25} = 7.40$ Not indicated	Log $K_{25}^D = 5.741$ Not indicated
Log $\beta_{21} =$ Not indicated 10.13 $\pm 0.09$	Log $K_{21} =$ Not indicated —	Log $K_{21}^D =$ Not indicated 2.68

## Conclusions

Regression spectra analysis of the protonation equilibria of 4-CAPAZOXS proved that for diluted solutions of less than  $6.676 \times 10^{-6}$  M 4-CAPAZOXS the protonated monomers  $L^{3-}$ ,  $LH_2^-$ ,  $LH_2^-$  and  $LH_3$  prevail in solution. For concentrations higher than  $4.5 \times 10^{-5}$  M, three dimers,  $L_2H_2^{4-}$ ,  $L_2H_3^{3-}$  and  $L_2H_5^-$  were indicated and their stability constants and molar absorptivities were also estimated. A comparison of protonation constants found by spectrophotometric and potentiometric<sup>20,21</sup> data analysis is shown in Table 6. At the low concentrations used in spectrophotometric determination some oligomers were not seen. On the basis of this comparison, the following reaction scheme for the monomer-dimer equilibria during protonation is suggested:



in which the consecutive protonation constants are calculated by the equations  $K_{12} = \beta_{12}/\beta_{11}$ ,  $K_{13} = \beta_{13}/\beta_{12}$ ,  $K_{22} = \beta_{22}/\beta_{21}$ ,  $K_{23} = \beta_{23}/\beta_{22}$  and  $K_{24} = \beta_{24}/\beta_{23}$ .

The dimerisation constants were calculated by the equations  $LH_2^- + L^{3-} = L_2H_5^-$ ,  $K_{21}^D = \beta_{21}/K_{11}$ ,  $2LH_2^- = L_2H_3^{3-}$ ,  $K_{22}^D = \beta_{22}/K_{11}^2$ ,  $LH_2^- + LH_2^- = L_2H_3^{3-}$ ,  $K_{23}^D = \beta_{23}/(\beta_{12}\beta_{11})$ ,  $2LH_2^- = L_2H_2^{4-}$ ,  $K_{24}^D = \beta_{24}/\beta_{12}^2$ ,  $LH_3 + LH_2^- = L_2H_5^-$  and  $K_{25}^D = \beta_{25}/(\beta_{13}\beta_{12})$ .

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