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MULTIPARAMETRIC CURVE FITTING—XII

RESOLUTION CAPABILITY OF TWO PROGRAMS FOR ANALYSING MULTICOMPONENT SPECTRA, SQUAD(84) AND PSEQUAD(83)

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Summary—The resolving power of multicomponent spectra analysis and the computation reliability of the stability constants and molar absorptivities determined for nine variously protonated anions of three sulphonephthaleins and an impurity, by analysis of data for a mixture by programs SQUAD(84) and PSEQUAD(83), has been examined by use of synthetic and experimentally measured spectra containing severely overlapping spectral bands. The model mixture of Bromocresol Green, Phenol Red, Thymol Blue and azoxine as impurity, with five yellow, three blue and one red species in the pH range from 2 to 10, was used to examine the influence of precision of spectral data and of use of the spectra of the individual components, on the precision and accuracy of the estimated parameters when the chemical model is known. An efficient computation strategy has been found and both programs were shown to lead to the same values and reliability of the parametric estimates. Of the various diagnostics considered, the goodness-of-fit achieved is used as the criterion of whether the parameters found adequately represent the data.

Programs for analysis of multicomponent spectra[1–10] can facilitate the identification and resolution of individual components of a mixture and also determine the stability constants and molar absorptivities of various species in solution equilibria. Multiwavelength spectrophotometric data in general offer considerably more information than potentiometric data[8,9] about chemical equilibria. As shown earlier,[5] SQUAD(84)[5] and PSEQUAD(83)[7] are particularly reliable and efficient diagnostic tools. SQUAD(84) provides a systematic method of formulating and testing models of equilibrium systems. The resolving power of these two programs has now been tested by estimation of the very similar protonation constants of four acid/base indicators from spectral measurements on a mixture of all four; it should be noted that the spectral bands of the individual species severely overlap. The reliability of determination of stability constants and molar absorptivity was examined by the use of simulated and experimental data, as a function of the instrumental error of absorbance reading, with and without use of the spectra of the isolated individual components in the absorbance matrix, and with mixtures in which some species involved in the protonation equilibria were of the same colour. The efficiency of both programs has been verified and a strategy of efficient computation is suggested.

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THEORY

Multicomponent spectral analysis

The multicomponent spectral analysis program was described in Part X of this work.[5] It can adjust $\vec{\beta}_{pqr}$ and $\vec{\epsilon}_{pqr}$ for a given set of spectra by fitting the predicted absorbance-response surface to given spectral data, with one dimension representing the dependent variable (absorbance), and the other two dimensions representing the independent variables, *viz.* the total component concentrations (or pH) of n_s solutions, at n_w wavelengths. The parameters to be determined are (*i*) the stoichiometric indices, (*ii*) the stability constants (β_{pqr}) and molar absorptivities (ϵ_{pqr}) and (*iii*) the free concentrations, of all the species in the chemical model found. The general equations for the complexes are

$$p\mathrm{M} + q\mathrm{L} + r\mathrm{H} = \mathrm{M}_p\mathrm{L}_q\mathrm{H}_r$$

with

$$\beta_{pqr} = [\mathrm{M}_p\mathrm{L}_q\mathrm{H}_r]/([\mathrm{M}]^p[\mathrm{L}]^q[\mathrm{H}]^r)$$

where the charges are omitted for simplicity. A chemical model must always be hypothesized for calculation of the stability constants and molar ab-

sorptivities. When the estimated $\vec{\beta}_{pqr}$ and $\vec{\epsilon}_{pqr}$ values for the assumed chemical model have been refined, the agreement between the experimental and predicted data can be examined. 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.

Errors in spectral data

To test the ability of the programs to find true parametric estimates, examination of simulated data is useful, 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, $s_{inst}(A)$, are imposed on the calculated 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 indicators may have acid–base character, in which case the background colour will vary with pH. At low pH some indicators may separate from solution and/or be absorbed on the cuvette walls, and at higher concentrations oligomers and micelles may be formed and changes in ionic strength or reagent concentrations cause a systematic rather than a random error. All statistical tests in the program are based on the assumption that systematic errors are absent from the data.

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. To characterize the program performance, simulated data can be used.[11] 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 constants 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 on the precision and accuracy of parameter estimation, (*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 analysed[12] to test whether the refined parameters adequately represent the data, and should be randomly distributed about the predicted regression curve. To analyse the residuals, the following statistics are compared with those of the generated random errors to find whether both distributions are Gaussian in nature: the error mean $m_{e,1}$ with the residual mean $m_{r,1}$, the mean error $|\bar{e}|$ with the mean residual $|\bar{r}|$, the selected standard deviation $s(e)$ with that of the residuals $s(r)$, the skewness of the error set $m_{e,3}$ with that of the residual set $m_{r,3}$, the curtosis of the error set $m_{e,4}$ with that of the residual set $m_{r,4}$, and finally the Hamilton R-factor for relative fit, $R(e)$ with $R(r)$.

EXPERIMENTAL

Reagents

Analytical grade Bromocresol Green (BCG), Phenol Red (PR) and Thymol Blue (TB) were used without purification; chromatography on Whatman No. 2 paper with 1-butanol–acetone–26% ammonia solution (4:3:2 v/v) showed no spots of coloured impurities. Each indicator was dissolved in dilute sodium hydroxide solution and the solution was titrated with 1*M* perchloric acid. The concentration of each indicator was determined by potentiometric pH-titration with perchloric acid and re-titration with sodium hydroxide. The resulting pH-titration curve was analysed by the regression program ACBA[13] and the concentrations of the indicator and acid/base impurities, and the ionization constants of the individual indicators, alone or in mixtures, were estimated (Table 1). A goodness of fit test was applied as a criterion of the reliability of refinement. The BCG was found to be 97.8% pure, with a small amount of a coloured impurity; the purities of the other two sulphonephthaleins were 84.7% for PR and 91.3% for TB, and the impurities were inorganic. The 1*M* sodium hydroxide was found to contain about 6% of carbonate, even though prepared from a 50% solution by dilution in the customary manner. It was standardized with 1*M* perchloric acid which itself was standardized with mercuric oxide and potassium bicarbonate, with agreement to within 0.1%.

Instruments

A Zeiss Spekol 21 single-beam spectrophotometer was equipped for spectrophotometric titration. The accuracy and precision of the spectrophotometric measurements were checked with standard solutions of potassium chromate and copper(II) sulphate. Reproducibility of absorbance was ± 0.0005, photometric linearity was better than 0.5% ($0.1 \leq A \leq 1.0$), the wavelength accuracy was better than 1 nm and the spectral band-width was kept at 3 nm. A 10-mm path-length cuvette was used.

The pH was measured with a Radiometer PHM 4d pH-meter with a G202B glass electrode and K410 saturated calomel electrode; calibration was done with Radiometer standard buffers S1500, S1510 and S1316 (pa_H 6.865, 7.410 and 4.010 at 25°).

The titration cell was a jacketed constant-temperature glass vessel of 150 ml volume, closed with a bung carrying the glass and reference electrodes, argon inlet, thermometer, stirrer shaft and polyethylene capillary tip of the microburette.

The burette was a home-made micrometer syringe burette of 2500 μl capacity. Its polyethylene capillary tip was immersed in the solution during addition of reagent, but then withdrawn from it to avoid leakage of reagent from the

Table 1. Determination of protonation constant and sulphonephthalein concentration, $c_{L,calc}$, by the regression analysis of the pH-titration curve by the ACBA program

		Bromocresol Green	Phenol Red	Thymol Blue	Mixture of 3 sulphonephthaleins
Initial volume, V_0, *ml*		26.00	26.80	25.80	26.15
Ionic strength, *M*		0.042	0.068	0.039	0.060
BCG:	$\log \beta_{11}$	4.84 ± 0.02			4.88 ± 0.01
	$c_{L,w}$, *M*	0.00957			0.00962
	$c_{L,calc}$, *M*	0.00936			
	Purity, %	97.8			
PR:	$\log \beta_{11}$		7.81 ± 0.00		7.87 ± 0.01
	$c_{L,w}$, *M*		0.00928		0.00958
	$c_{L,calc}$, *M*		0.00786		
	Purity, %		84.7		
TB:	$\log \beta_{11}$			9.09 ± 0.02	9.12 ± 0.01
	$c_{L,w}$, *M*			0.00973	0.00975
	$c_{L,calc}$, *M*			0.00888	
	Purity, %			91.3	
$s(V)_{NaOH}$		0.0019	0.0014	0.0027	0.0042
Residual mean		1.93E-4	−2.04E-4	−1.12E-4	−3.69E-5
Mean residual		0.0015	0.0097	0.0018	0.0031
Standard deviation		0.0017	0.0013	0.0025	0.0040
Hamilton *R*-factor, %		0.76	0.66	0.97	0.75

tip during the pH and spectral measurements. The burette was calibrated and found precise to ± 0.5 µl.

Spectrophotometric multiple-wavelength pH-titrations

The absorption spectra of the individual sulphonephthaleins and their mixtures, as a function of pH, were measured by means of spectrophotometric multi-wavelength pH-titration as described earlier.[14]

Spectra tested

A number of models comprising synthetic data for mixtures of three sulphonephthaleins and an azoxine impurity, that would provide overlapping equilibra and spectral bands, were constructed to test the program performance.

Five protonation constants, one for each of the three sulphonephthaleins and two for the azoxine, were refined. The protonation equilibria involved nine variously protonated and light-absorbing species, over the pH range from 2 to 10, five of the species being yellow, three blue and one red. Figure 1 shows the strong overlap of the various spectra. The relevant pK_a values are 3.05 (azoxine), 4.7 (BCG), 6.9 (PR) and 9.2 (TB). A simplifying factor, however, is that none of the species interact with each other, and there are no-side reactions to consider.

Five sets of spectra were used, as follows:

(A) Synthetic spectra of a BCG + PR + TB + azoxine mixture and of the individual components.

For 37 ($=n_s$) systems containing one or all four ($n_z = 4$) of the basic components (BCG, PR, TB and azoxine) at selected pH values the total absorbances at 32 ($=n_w$) wavelengths ranging from 380 nm ($=\lambda_{start}$) to 690 nm ($=\lambda_{end}$), were calculated and then loaded with random errors. Fourteen of the spectra were for the BCG + PR + TB + azoxine mixture, 8 for the azoxine alone, and 5 for each of the other three indicators.

(B) Synthetic spectra of a BCG + PR + TB + azoxine.

Fourteen of the spectra were the same as those in set (A), and 18 for the BCG + PR + TB mixtures in various concentration ratios and over different pH ranges.

(C) Experimentally measured spectra of a BCG + PR + TB mixture and of the individual components.

The experimental spectra had a higher uncertainty than that with which the synthetic spectra were loaded, so testing was restricted to a mixture of all three sulphonephthaleins ($n_z = 3$) (18 spectra from $\lambda_{start} = 380$ nm to $\lambda_{end} = 690$ nm, Figs. 2 and 3), and 5 protonation equilibrium spectra for each of the individual components (total $n_s = 33$), with readings at 32 ($=n_w$) wavelengths).

(D) Experimentally measured spectra of a BCG + PR + TB mixture. The 18 spectra for the mixture in set (C) were used.

(E) Experimentally measured spectra of the three individual sulphonephthaleins.

The remaining 15 spectra sets from set (C) were used.

Computation

The protonation constants and molar absorptivities of the sulphonephthaleins were refined with two least-squares programs: SQUAD(84)[5] and PSEQUAD(83).[7] The purity of the indicators, the acid–base impurities and the ionization constants were determined with the regression program ACBA.[13] Computations were performed with the EC 1033 (500 K) computer in the Computing Centre, College of Chemical Technology, Pardubice, Czechoslovakia.

DISCUSSION

Analysis of synthetic data (Tables 2 and 3)

The performance of SQUAD(84)[5] and PSEQUAD(83)[7] was first tested with data sets (A) and (B), which allowed systematic variation of the spectral, equilibrium and noise characteristics.

The primary study was to determine the effect of precision of the absorbance data on the precision and accuracy of the estimated parameters. Higher imprecision of the absorbance data would be expected to result in a poorer fit. Estimation of the parameters would be inaccurate because of 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 supercurve, $U = U_{min} + s^2(A)$. The standard deviation of each parameter b_i defined by $s(b_i) = \max[(b_D - b_{min})_i]$ can be calculated as the maximum difference between the value for b_i at any

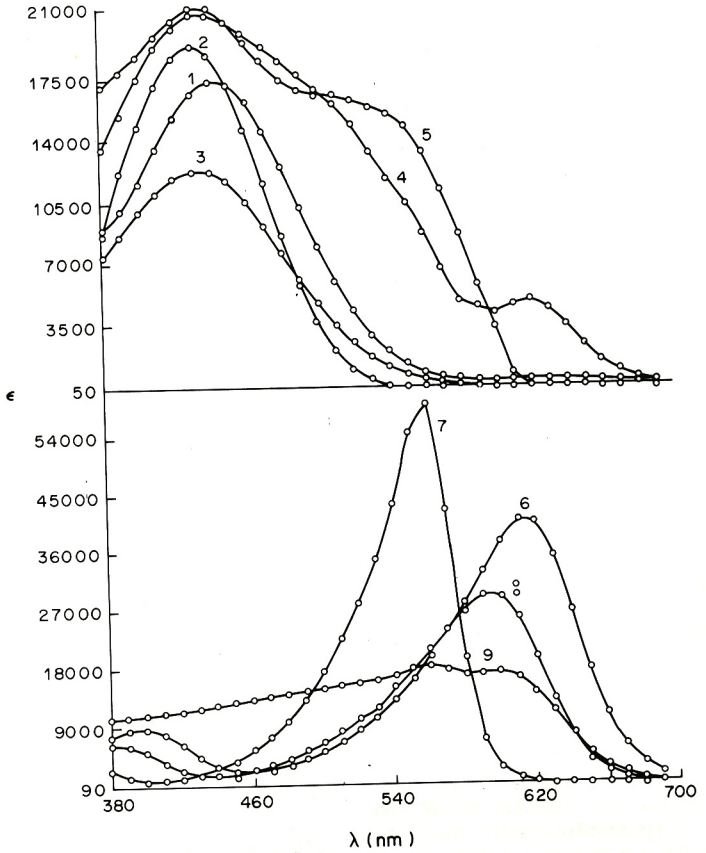

Fig. 1. Absorption spectra (molar absorptivities) of BCG, PR, TB and azoxine impurity in the forms LH^{2-}, (1) BCG, (2) PR, (3) TB, (4) azoxine; LH_2^-, (5) azoxine; L^{3-}, (6) BCG, (7) PR, (8) TB, (9) azoxine, used for simulation of spectral data.

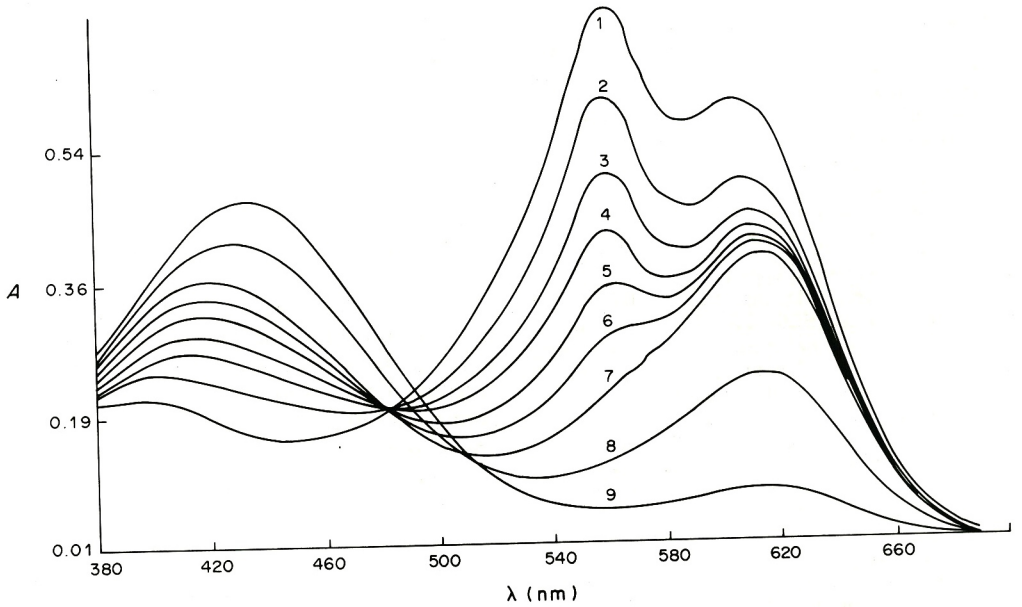

Fig. 2. Absorption spectra of a mixture of BCG + PR + TB for various pH values and a concentration ratio BCG:PR:TB = 0.983:0.578:1.331 where $c_{TB} = 9.89 \times 10^{-6} M$; $d = 1.000$ cm, $I = 0.015$, temperature = 25°. Values of pH: (1) 9.126, (2) 8.608, (3) 8.222, (4) 7.921, (5) 7.640, (6) 7.300, (7) 6.482, (8) 4.873, (9) 4.054.

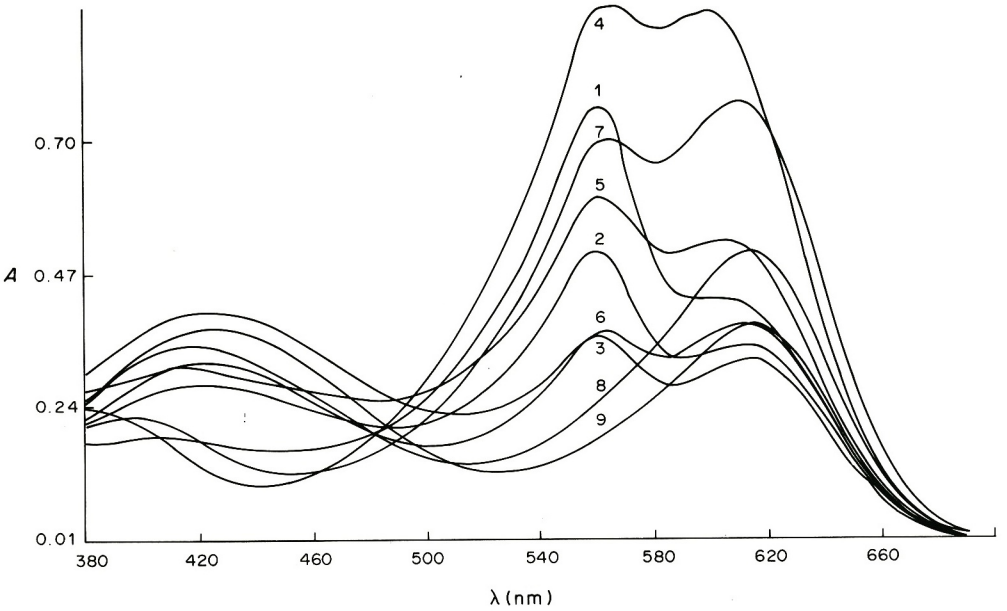

Fig. 3. Absorption spectra of a mixture of BCG + PR + TB for various pH values and a concentration ratio BCG:PR:TB = 0.739:0.827:1.000 for pH: (1) 8.949, (2) 8.023, (3) 7.622 and 0.739:0.414:1.952 with pH (4) 10.019, (5) 8.893, (6) 7.855, and 1.477:0.414:1.000 for pH: (7) 9.190, (8) 5.273, (9) 4.931. $c_{TB} = 9.89 \times 10^{-6} M$, $d = 1.000$ cm, $I = 0.015$, temperature = 25°.

point on the D-boundary, and the value for b_i at the minimum. As the last U contour of the D-boundary is then rather a large ellipse, the parametric standard deviations are larger, and the precision poorer.

SQUAD(84)[5] starts with data-smoothing of the spectra set, followed by factor analysis FA608.[2] The position of a break on the $s_k(A) = f(k)$ curve is calculated, and gives $k^* = 9$, with the corresponding co-ordinate $s_9^*(A)$ value (for terminology see Part X[5] of this series). Five protonation constants and nine molar absorptivities for 32 wavelengths constitute 293 parameters which are refined by the MR algorithm in the first run of the SQUAD program. In the second run, the NNLS algorithm makes the final refinement of all previously found parametric estimates, with all molar absorptivities kept non-negative. The reliability of the parametric estimates may be tested by use of the SQUAD(84) diagnostics.

The first diagnostic indicates whether all parametric estimates have physical meaning and realistic values. The accuracy of these estimates is shown as a systematic error calculated as the deviation from the true values of the parameters. The deviations for the pK_a values and molar absorptivities of set (A) were negligible: even the largest value of $s_{inst}(A)$, 0.015, caused <0.01 deviation in log β_{11} and 1.5% deviation in the molar absorptivities. For set (B), the accuracy for the pK_a estimates was the same as for set (A) for the sulphophthaleins but not for the azoxine. There was a larger effect of $s_{inst}(A)$ for set (B) than for set (A).

The second diagnostic tests whether all calculated free species concentrations have physical meaning, which proved to be the case. The low values of the standard deviations of all the estimated parameters (the third diagnostic) proved the estimates to be sufficiently precise.

The fourth diagnostic (the correlation coefficients) proved the absence of interdependence for any pair of protonation constants.

The degree-of-fit (the fifth diagnostic) proved that the $s(A)$ value reached at convergence agreed with the absorbance precision $s_{inst}(A)$, chosen for loading the random error, and the Hamilton R-factor confirmed a good degree of fit.

PSEQUAD(83) gave the same results for the parametric estimates and though PSEQUAD 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).

The synthetic data were also used for determination of the statistical quantities, $m_{r,1}$, $|\bar{r}|$, $m_{r,3}$, $m_{r,4}$ and the R-factor, which may be used as reference limits in further analysis of laboratory data. For the selected $s_{inst}(A)$ value of 0.001 the R-factor of the residuals was 0.2%, and for $s_{inst}(A) = 0.003$ it was 0.5–0.6%. This means that when the R-factor for laboratory data with a drift in pH and wavelength is less than 1%, the degree of fit may be considered realistic.

The effect (on the ability to find true parametric estimates) of including the spectra of the pure components in the absorbance matrix for the indicator mixture was estimated by comparing the results of sets (A) and (B). The estimation of well-conditioned protonation constants for the three sulphonephthaleins in set (B) seems to be of the same precision and accuracy as for set (A). A slight

Table 2. Determination of protonation constants and molar absorptivities of variously protonated forms of a mixture of three sulphonephthaleins and azoxine impurity by regression analysis of simulated spectra set (A) containing also spectra of basic components, parametric deviations estimated by SQUAD(84) and PSEQUAD(83), $d(\beta) = 10^3[(\log \beta_{11})_{calc} - (\log \beta_{11})_{true}]$ and $d(\epsilon) = \epsilon_{calc} - \epsilon_{true}$

Program used	SQUAD(84)	PSEQUAD(83)	SQUAD(84)	PSEQUAD(83)	SQUAD(84)	PSEQUAD(83)	SQUAD(84)	PSEQUAD(83)
$s_{inst}(A)$	0.001		0.003		0.008		0.015	
BCG: $\log \beta_{11} = 4.7$	$-(0.4 \pm 0.6)$	$-(0.4 \pm 0.6)$	$-(1.4 \pm 1.8)$	$-(1.1 \pm 1.8)$	$-(4.0 \pm 4.8)$	$-(3.7 \pm 4.7)$	$-(7.7 \pm 8.9)$	$-(7.5 \pm 8.7)$
440 nm, $\epsilon_{11} = 17399$	25	26	77	78	211	210	397	394
610 nm, $\epsilon_{10} = 40858$	-72	-77	-227	-231	-620	-468	1166	-596
PR: $\log \beta_{11} = 7.9$	0.8 ± 0.6	0.7 ± 0.6	2.2 ± 1.9	2.2 ± 1.9	6.0 ± 5.2	6.0 ± 5.1	11.6 ± 9.7	11.7 ± 9.5
430 nm, $\epsilon_{11} = 19401$	-23	-23	-68	-70	-184	-187	-343	-347
560 nm, $\epsilon_{10} = 58702$	9	3	-1	6	13	12	45	32
TB: $\log \beta_{11} = 9.2$	$-(0.4 \pm 0.6)$	$-(0.4 \pm 0.6)$	$-(1.3 \pm 1.8)$	$-(1.3 \pm 1.8)$	$-(3 \pm 5)$	$-(3 \pm 5)$	$-(5 \pm 9)$	$-(5 \pm 9)$
430 nm, $\epsilon_{11} = 12294$	4	5	13	13	35	36	65	66
600 nm, $\epsilon_{10} = 29199$	-6	-6	-28	2	-79	20	-141	-146
Azox.: $\log \beta_{12} = 9.95$	$-(0.8 \pm 3.2)$	0.1 ± 3.4	$-(2 \pm 90)$	$-(0.4 \pm 90)$	$-(1 \pm 26)$	$-(2 \pm 24)$	2 ± 46	8 ± 45
430 nm, $\epsilon_{12} = 21485$	19	18	55	54	-302	-301	273	272
$\log \beta_{11} = 6.9$	0.7 ± 1.0	0.6 ± 1.0	2 ± 3	2 ± 3	5 ± 8	5 ± 8	8 ± 14	10 ± 14
430 nm, $\epsilon_{11} = 21141$	5	6	16	16	491	482	82	78
600 nm, $\epsilon_{10} = 17371$	-15	-19	-58	-59	-157	-159	-305	-292
Factor analysis: k, $s_k(A)$	9, 0.0008		9, 0.0024		9, 0.0060		9, 0.0109	
Fitness test: $s(A)$	0.000915	0.000969	0.00276	0.00274	0.00735	0.00721	0.0137	0.0134
$m_{r,1}$ and $m_{e,1}$	2.01E-6	2.59E-6	3.23E-7	-8.28E-6	-2.69E-5	-8.30E-5	-7.89E-5	-2.52E-4
$\lvert\bar{r}\rvert$ and $\lvert\bar{e}\rvert$	0.00064	0.00074	0.00191	0.00221	0.00508	0.00383	0.00946	0.01084
$s(r)$ and $s(e)$	0.00092	0.00106	0.00276	0.00316	0.00735	0.00837	0.01370	0.01562
$m_{r,3}$ and $m_{e,3}$	0.017	-0.020	-0.022	-0.030	-0.049	-0.045	-0.057	-0.062
$m_{r,4}$ and $m_{e,4}$	2.150	2.214	2.232	2.240	2.288	2.273	2.308	2.294
$R(r)$ and $R(e)$, %	0.167	0.193	0.502	0.575	1.337	1.524	2.493	2.842

Table 3. Determination of protonation constants and molar absorptivities of variously protonated forms of a mixture of three sulphonephthaleins and azoxine impurity by regression analysis of simulated spectra set (B) without spectra of basic components; parametric deviations estimated by SQUAD(84) and PSEQUAD(83), $d(\beta) = 10^3[(\log \beta_{11})_{calc} - (\log \beta_{11})_{true}]$ and $d(\epsilon) = \epsilon_{calc} - \epsilon_{true}$

Program used	SQUAD(84)	PSEQUAD(83)	SQUAD(84)	PSEQUAD(83)	SQUAD(84)	PSEQUAD(83)	SQUAD(84)	PSEQUAD(83)
$s_{inst}(A)$	0.001		0.003		0.008		0.015	
BCG: $\log \beta_{11} = 4.7$	$-(0.2 \pm 1.4)$	$-(0.2 \pm 1.5)$	$-(0.5 \pm 4.3)$	$-(0.7 \pm 4.3)$	$-(0.5 \pm 11.4)$	$-(1.3 \pm 11.2)$	4.3 ± 20.5	2 ± 20
440 nm, $\epsilon_{11} = 17399$	−96	−85	−281	−274	−728	−715	−1373	−1500
610 nm, $\epsilon_{10} = 40858$	24	22	111	67	384	176	965	383
PR: $\log \beta_{11} = 7.9$	$-(0.6 \pm 1.6)$	$-(0.6 \pm 1.6)$	$-(2 \pm 5)$	$-(2 \pm 5)$	$-(6 \pm 13)$	$-(7 \pm 13)$	$-(15 \pm 24)$	$-(8 \pm 24)$
430 nm, $\epsilon_{11} = 19401$	173	165	508	508	1366	1359	2713	2740
560 nm, $\epsilon_{10} = 58702$	−39	−26	−103	−103	−318	−351	−986	−41
TB: $\log \beta_{11} = 9.2$	$-(0.2 \pm 1.8)$	(0.1 ± 1.8)	$-(1 \pm 5)$	$-(0.4 \pm 5)$	$-(3 \pm 15)$	$-(4 \pm 14)$	$-(6 \pm 27)$	4 ± 27
430 nm, $\epsilon_{11} = 12294$	17	19	50	53	133	135	251	220
600 nm, $\epsilon_{10} = 29199$	58	56	172	150	473	391	920	853
Azox.: $\log \beta_{12} = 9.95$	14 ± 35	12 ± 36	33 ± 98	14 ± 110	81 ± 209	56 ± 250	240 ± 263	162 ± 280
430 nm, $\epsilon_{12} = 21485$	−220	−223	−1671	−659	−1784	−1750	−3500	−3328
$\log \beta_{11} = 6.9$	6.4 ± 9.7	6.8 ± 9.8	19 ± 29	21 ± 29	73 ± 75	82 ± 76	281 ± 113	269 ± 130
430 nm, $\epsilon_{11} = 21141$	−449	−453	−1450	−1354	−3606	−3635	−7275	−7178
600 nm, $\epsilon_{10} = 17371$	−161	−148	−497	−420	−1228	−1023	−1309	−660
Factor analysis: k, $s_k(A)$	9, 0.00074		9, 0.00213		9, 0.00652		9, 0.01199	
Fitness test: $s(A)$	0.00093	0.00094	0.00278	0.00276	0.00745	0.00733	0.0139	0.0137
$m_{r,1}$ and $m_{e,1}$	1.56E-7	−8.83E-6	4.31E-7	−2.65E-5	1.25E-6	−8.21E-5	2.01E-6	−1.91E-4
$\lvert\bar{r}\rvert$ and $\lvert\bar{e}\rvert$	0.00063	0.00075	0.00188	0.00225	0.00502	0.00600	0.00936	0.01213
$s(r)$ and $s(e)$	0.00093	0.00109	0.00278	0.00328	0.00745	0.00876	0.01393	0.01639
$m_{r,3}$ and $m_{e,3}$	−0.047	−0.065	−0.045	−0.066	−0.082	−0.069	−0.079	−0.075
$m_{r,4}$ and $m_{e,4}$	2.129	2.026	2.059	2.039	2.132	2.033	2.133	2.043
$R(r)$ and $R(e)$, %	0.201	0.236	0.600	0.709	1.606	1.888	3.000	3.531

Table 4. Determination of protonation constants and molar absorptivities of variously protonated forms of a mixture of three sulphonephthaleins by regression analysis of experimental spectra sets (C), (D) and (E) by SQUAD(84) (in upper line) and PSEQUAD(83) (in lower line)

	Individual sulphonephthalein			Mixture of sulphonephthaleins		
	BCG	PR	TB	Set (C)	Set (D)	Set (E)
Basic components, n_z	2	2	2	3	3	3
Colour species, n_c	2	2	2	6	6	6
Solutions, n_s	5	5	5	33	18	15
Wavelengths, n_w	32	32	32	32	32	32
Fitted points, n	165	165	165	1089	594	495
Refined parameters, m	65	65	65	195	195	195
Degrees of freedom, $n-m$	100	100	100	894	399	300
				Values of refined parameters		
BCG: log β_{11}	4.655 ± 0.004			4.670 ± 0.010	4.730 ± 0.016	4.655 ± 0.007
	4.655 ± 0.004			4.671 ± 0.010	4.713 ± 0.022	4.655 ± 0.008
440 nm, ϵ_{11}	18248 ± 125			18147 ± 544	16518 ± 1998	18245 ± 279
	18245 ± 176			18144 ± 508	16343 ± 1540	18244 ± 345
610 nm, ϵ_{10}	41673 ± 334			41826 ± 569	40716 ± 1185	41675 ± 650
	41678 ± 179			41842 ± 416	41206 ± 868	41678 ± 350
PR: log β_{11}		7.862 ± 0.003		7.878 ± 0.012	7.863 ± 0.021	7.862 ± 0.008
		7.862 ± 0.003		7.879 ± 0.011	7.840 ± 0.022	7.862 ± 0.009
430 nm, ϵ_{11}		27002 ± 111		26516 ± 782	21880 ± 2741	27001 ± 432
		27006 ± 181		26517 ± 732	23004 ± 2000	27005 ± 546
560 nm, ϵ_{10}		73705 ± 562		73949 ± 1286	67482 ± 1848	73702 ± 977
		73725 ± 226		73980 ± 879	66796 ± 1720	73722 ± 682
TB: log β_{11}			9.180 ± 0.013	9.186 ± 0.011	9.264 ± 0.019	9.180 ± 0.007
			9.181 ± 0.013	9.187 ± 0.010	9.351 ± 0.025	9.180 ± 0.008
430 nm, ϵ_{11}			13284 ± 312	13677 ± 322	17500 ± 984	13284 ± 188
			13283 ± 384	13676 ± 301	17300 ± 714	13285 ± 237
590 nm, ϵ_{10}			31219 ± 947	32137 ± 556	38577 ± 1076	31217 ± 564
			31227 ± 442	32131 ± 393	39351 ± 895	31217 ± 274
				Factor analysis		
$k, s_k(A)$	2, 0.0018	2, 0.0008	2, 0.0009	6, 0.0010	6, 0.0011	6, 0.0004
				Fitness test		
$s(A)$	0.0054	0.0035	0.0173	0.0166	0.0122	0.0106
	0.0053	0.0035	0.0169	0.0164	0.0113	0.0104
Residual mean	4.17E-7	1.45E-7	2.31E-6	4.14E-5	1.01E-4	5.31E-6
Mean residual	0.0031	0.0017	0.0089	0.0091	0.0079	0.0046
Standard deviation	0.0054	0.0035	0.0173	0.0166	0.0122	0.0106
Skewness (=0)	0.261	−0.264	0.724	−1.617	−0.513	1.048
Curtosis (=3)	2.576	4.355	3.545	9.141	2.146	8.317
R-factor (%)	1.24	1.07	4.05	4.80	3.03	2.74

difference may be found for the ill-conditioned protonation constants of the azoxine and a significant error appears in the estimated molar absorptivities for set (B). It may be concluded that inclusion of the spectra of the pure basic components increases the accuracy and precision of the estimated parameters.

The programs employ different minimization algorithms, SQUAD(84) using conventional multiple regression for solving a set of overdetermined linear equations, which may be interchanged (during the program execution) with the Gauss–Newton non-negative least-squares algorithm which ensures that computed molar absorptivities are always positive. PSEQUAD(83) is based on the Gauss–Newton method. Both programs gave the same reliability and results, but SQUAD(84) offers more diagnostic tools for assessing the results and for chemical model building.

Analysis of laboratory data (Table 4)

Though both programs were proved satisfactory for analysis of data loaded with a random absorbance error, the experimental data may also suffer from drift in pH and observational wavelength.

The spectra of the isolated, individual sulphonephthaleins were measured first, and analysed, and the protonation constants and molar absorptivities ϵ_{11} and ϵ_{10} were used as reference values for comparison with those found by analysis of the mixture. The precision varied from one sulphonephthalein to another, being highest for Phenol Red and lowest for Thymol Blue. Solutions of Thymol Blue are alkaline and contain carbonate, so some drift of pH may be expected, unless precautions are taken to eliminate the carbonate.

Both programs were tested with data-sets (C), (D) and (E). Sets (C) and (E) gave the same parametric estimates, which were also in good agreement with those for the individual sulphonephthaleins. There were slight differences for set (D), however, which did not contain the spectra of the individual components.

Processing the laboratory data with SQUAD(84) indicated, through FA608, that $k^* = 6$ and gave the corresponding $s_6^*(A)$ values. This arose because of the very severe overlap of the individual spectra, the factor analysis then not being able to distinguish three of the coloured species from the other six.

The degree-of-fit test showed that the experimental data were less precise than the synthetic data. Nevertheless, both programs were able to find sufficiently true and accurate parameter estimates, with equal reliability.

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