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Ampholytic dyes for spectroscopic determination of pH in electrofocusing

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Abstract

Ampholytic dyes were studied for the purpose of spectroscopic monitoring of the local pH of solutions within capillary systems used for the focusing and separation of ampholytes. The dyes are compounds selected from previously described aminomethylnitrophenols and also a group of compounds based on aminomethylated sulfonphthaleins. The suggested ampholytic dyes are good ampholytes and are also pH indicators with colour transition around the p*I* value. The possibility of the spectroscopic monitoring of actual pH was verified in a system involving pH gradient ion chromatography with diode-array detection and on-line flow-through pH detection.

1. Introduction

In electrophoretic ampholyte focusing, the pH of the analyte environment for a series of compounds to be separated should be known for the experimental determination of the pH gradient and local pH. In ampholyte focusing, this can be achieved, e.g., with help of suitable internal standards or pH (p*I*) markers. Apart from their detectability, the p*I* markers should behave as "good" ampholytes. The term "good" ampholytes means that their effective charge vs. pH dependence should be sufficiently steep close to the marker p*I*. For flat-bed focusing formats, markers based on natural compounds are widely used. These include purified proteins [1–3], carbamylated proteins [4] and proteins stained by dyes [5]. In capillary focusing, it has been suggested that the p*I* be determined via gradient mobilization [6]. In addition to electrophoretic methods, ampholytes can be focused in a pH gradient in an ion-exchange column and by chromatofocusing. Also, focusing of ampholytes in isoelectric focusing field-flow fraction (IEF-FFF) has been described [7]. When performing such experiments in miniaturized format, on-line pH monitoring entails technical difficulties.

In the above free-flow focusing methods and, generally, in capillary analytical methods, on-line spectrophotometric multi-channel optical detection is advantageously used for the solute monitoring. Such detection offers the ability to determine the extent of compound protonation when the spectra of particular forms are sufficiently different from one another. For example, this information was used to calculate the equilibrium constants, pK_a, of pH indicators in proton–ligand systems by flow-injection analysis (FIA) with diode-array multi-dimensional spectral detection [8].

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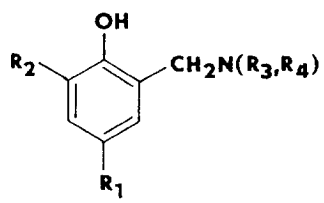
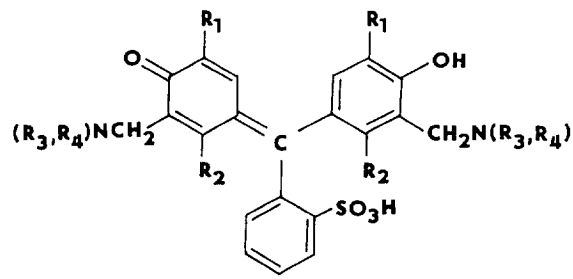
In principle the calculation can be reversed, i.e., the local pH and pH gradient steepness should be available from the monitored spectra provided that the spectral and acid–base characteristics of the compound are known. When applied to ampholyte focusing, the suitable compound should simultaneously be a good ampholyte [9] and a pH indicator with its colour transition close to its p*I*. For spectroscopic pH monitoring near the component p*I*, it is advantageous when the spectra of the isoelectric form and those of the negatively and positively charged forms are very different. In this way, ampholytic dyes that behave also as pH indicators with a colour transition close to the p*I* value may yield a deeper insight into the focusing of ampholytes in free-flow systems.



I

Compounds with selected spectroscopic properties were sought among low-molecular-mass p*I* markers, based on aminomethylated nitrophenols (**I**) [9], which are characterized by sufficient purity, stability and low hydrophobicity. These compounds have been successfully focused both in LC with a pH gradient [9] and isoelectric capillary and preparative free-flow separations [10–13]. Also, the p*I* values of these compounds have been determined independently of IEF [9]. The nitrophenol-based p*I* markers [9] investigated in this work were 4-methyl-6-nitro-2-(4-morpholinomethyl)phenol, 4-nitro-2-(4-morpholinomethyl)phenol and 2-chloro-6-nitro-4-(4-morpholinomethyl)phenol (see Table 1).

In this paper, a group of low-molecular-mass dyes was developed and investigated, namely aminomethylated sulfonphthaleins of general formula **II**, where groups R_1–R_4 are explained in Table 1.



II

The structures of these compounds can be regarded as modified known acid–base and complexometric indicators. Thus, the design of the aminomethylated sulfonphthaleins was based on well known indicators such as phthalein complexone [14], fluorexone [15], xylenol orange and other sulfonphthaleins [16,17], the acid–base properties of which have been reviewed [18,19].

Table 1
Structures of the studied ampholytic dyes of general formulae **I** and **II**

Formula	No.	R_1	R_2	$N(R_3R_4)$[a]	M_r
I	1	CH_3	NO_2	MOR	289
	2	NO_2	H	MOR	275
	3	4-$CH_2N(R_3R_4)$	2-Cl-6-NO_2	MOR	309
II	4	CH_3	H	MOR	561
	5	$CH(CH_3)_2$	CH_3	MOR	665
	6	$CH_2N(R_3R_4)$	H	MOR	679
	7	$CH(CH_3)_2$	CH_3	PIP	661
	8	$CH_2N(R_3R_4)$	H	PIP	671

[a] MOR = 4-Morpholinyl; PIP = 1-piperidyl.

Spectroscopic and acid–base studies [20–22] indicated several colour transitions with marked changes in the spectra over a broad pH range. Such spectral properties were also expected for the compounds developed here.

Additionally, the investigated ampholytic dyes included methyl red, which is a common pH indicator and p*I* marker, and 4-(4′-hydroxyphenylazo)-1-naphthylethylenediamine, used previously in IEF-FFF experiments [7]. The possibility of the spectroscopic monitoring of actual pH profiles was verified using a system involving pH gradient ion chromatography with diode-array detection (DAD) and on-line flow-through pH detection.

2. Experimental

2.1. Materials

The preparation and characterization of 4-methyl - 6 - nitro - 2 - (4 - morpholinomethyl)-phenol (**1**), 4-nitro-2-(4-morpholinomethyl)-phenol (**2**) and 2-chloro-6-nitro-4-(4-morpholinomethyl)phenol (**3**) were described previously [9]. The new compounds (Table 1) were 3′,3″ - bis(4-morpholinomethyl) - *o* - cresolsulfonphthalein (**4**), 3′,3″ - bis(4 - morpholinomethyl)-thymolsulfonphthalein (**5**), 3′,3″,5′,5″ - tetrakis(4 - morpholinomethyl)phenolsulfonphthalein (**6**), 3′,3″ - bis(1 - piperidinomethyl)thymolsulfonphthalein (**7**) and 3′,3″,5′,5″ - tetrakis(1 - piperidinomethyl)phenolsulfonphthalein (**8**). They were prepared from the commercially available sulfonphthaleins phenol red, *o*-cresol red and thymol blue (Fluka, Buchs, Switzerland) by means of the Mannich reaction [17,23]. The appropriate amine (50 mmol) was added portionwise, with cooling, to 37% aqueous formaldehyde (60 mmol) in 25 ml of ethanol. After addition of sulfonphthaleins (50 mmol), the reaction mixture was heated under reflux for 10 h. The aqueous ethanol was removed under reduced pressure, the residue was dissolved in 25 ml of methanol and 5 ml of concentrated hydrochloric acid were added portionwise to the resulting solution. After cooling, the products were separated by filtration or removal of the solvent under reduced pressure. The isolated hydrochlorides were recrystallized from methanol or aqueous ethanol. The purity of all compounds was checked by TLC and ion-exchange liquid chromatography with a pH gradient and diode-array UV–Vis detection [24].

The azo dyes methyl red (**9**) and 4-(4′-hydroxyphenylazo) - 1 - naphthylethylenediamine (**10**), both from Lachema (Brno, Czech Republic) were used as received.

2.2. Liquid chromatography

The conditions for ion-exchange chromatography with a wide-range pH gradient were described previously [9,24]. A PU 4100M liquid chromatograph (Philips, Cambridge, UK) equipped with a Model 7125 injection valve (Rheodyne, Cotati, CA, USA) and a PU 4021 multi-channel detector (Philips) were used. Data collection and post-run evaluation were controlled by PU 6003 v.3.0 diode-array detector software (Philips). The actual pH profile of the column effluent was monitored with an OP-0745P pH capillary flow-through electrode connected to an OP-208/1 pH meter (Radelkis, Budapest, Hungary) and a line recorder. A 150 × 2 mm I.D. Separon HEMA-BIO 1000 Q ion-exchange column (Tessek, Prague, Czech Republic) was used as received. The alkaline buffer (A) was an aqueous solution of 10 m*M* each of piperazine, L-histidine, ethylenediamine, 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) and 20 m*M* ammonia solution. The pH of buffer A was adjusted to 10.1 with 2 *M* potassium hydroxide solution. The acidic buffer (B) was 1.0 *M* formic acid. Chemicals used for the buffer preparation were obtained from Fluka and Merck (Darmstadt, Germany). The dyes were sampled in the alkaline buffer in a sample volume of 10 μl.

2.3. Determination of pK_a, *pI* and $[-dz/d(pH)]$

The acid–base properties of the prepared compounds **4**–**8** were evaluated by potentiomet-

ric titration using an MS 22 pH meter (Laboratory Instruments, Prague, Czech Republic), equipped with a Model 01-29 combined glass pH electrode (Crytur, Turnov, Czech Republic). The instrument was calibrated by means of commercial standard buffer solutions (Institute of Sera and Vaccines, SEVAC, Prague, Czech Republic). The temperature during titration was kept at 23°C. The titration curves obtained were evaluated both graphically and numerically to obtain pK_a, p*I* and $-dz/dpH$ values for each compound. The $-dz/dpH$ values reported in Table 2 were the tangents of the found pH dependence of the effective charge of the compound at the isoelectric point. Curve fitting was carried out with the program Eureka V. 1.0 (Borland, Scotts Valley, CA, USA). The whole procedure for pK_a determination was verified by determination of the pK_a of L-histidine monohydrochloride (Reanal, Budapest, Hungary) as a standard. The differences between the determined and tabulated [25] pK_a values of L-histidine were less than 0.1 pH unit.

2.4. Spectroscopy

The absorptivity in aqueous buffer solutions with pH corresponding to the compound p*I* value was determined with a Series 634 UV–Vis spectrophotometer (Varian Techtron, North Springvale, Australia). Spectra of some compounds were treated using LETAGROP PC software [26,27] to obtain pK_a values and the spectra of the individual protonated forms. Examples of the spectra obtained are shown in Figs. 1 and 2.

2.5. Determination of log P_{ow}

The partition coefficient between 1-octanol and water, P_{ow}, was determined spectroscopically by the shake-flask method as described [28]. The log P_{ow} values presented in Table 2 correspond to the pH of the water-rich phase equal to the p*I* value of the respective dye. The pH of the water-rich phase was adjusted with 0.1 *M* phosphate buffer. The absorptivity of the water-rich phase was determined at its λ_{max} in the visible spectrum; the solution was equilibrated for 3 h at 25°C with a known amount of water-saturated 1-octanol and the absorptivity of the aqueous phase was measured again.

3. Results and discussion

3.1. Characteristics of dyes

Nitrophenols

From the set of nitrophenol-based p*I* markers [9], compounds **1–3** (see Tables 1 and 2), were chosen owing to the variation of their spectra over a broad pH range; see Fig. 1a, where spectra of compound **1** are shown for several pH values in the range 4–8.6. The spectra of particular protonated forms of compounds **1–3** (see Fig. 2a–c) were extracted from composite spectra vs. pH dependence. The cross-over points of the spectra of positively charged and neutral forms (curves 1 and 2 in Fig. 2) and the spectra of neutral and negatively charged forms (curves 2 and 3 in Fig. 2) correspond to the isosbectic points. Although the difference between the spectra of the anionic and neutral forms of compound **3** is small, the spectra of compounds **1–3** support their applicability for the spectroscopic determination of pH close to the compound p*I*. In Fig. 2d, the spectra obtained for methyl red forms are depicted for comparison.

The acid–base characteristics summarized in Table 2 are, except where indicated otherwise in the footnotes, those determined by evaluation of the titration curves. Additionally, some dissociation steps can also be reliably evaluated spectroscopically. A comparison of the values obtained by the different methods can be made from the data summarized in Table 3, which includes the acid–base characteristics of nitrophenol compounds obtained both by titration and spectroscopically. Comparison of the pK_a values determined by different methods shows the need for further work if the pK_a value is to be known reliably with an accuracy of better than 0.1 pH unit. Despite the differences between values obtained by the different methods, the p*I* values in Table 2 are mostly those derived from the pH titration curves, which make the data more consistent. Moreover, the order of compound

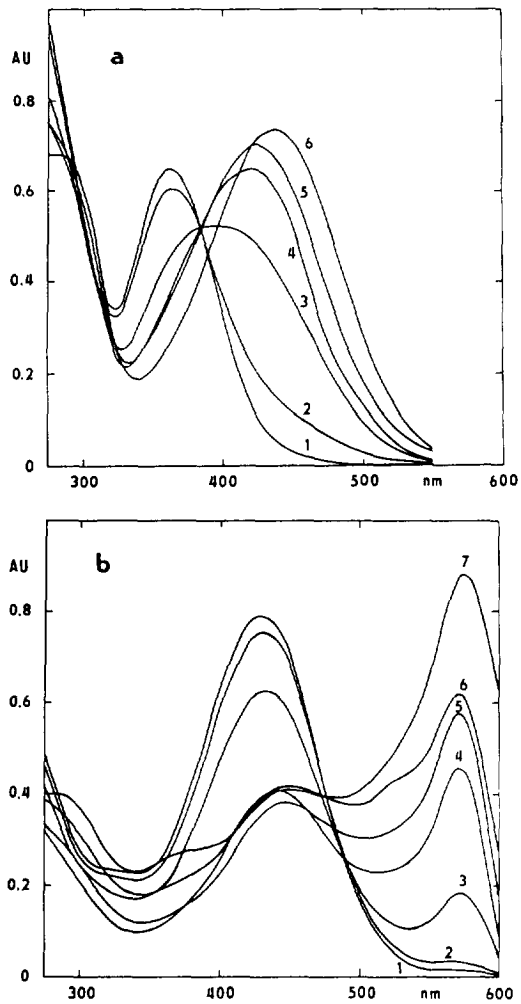


Fig. 1. Absorption curves of ampholytic dyes at various pH values. (a) Compound **1**: pH = (1) 4.03, (2) 5.14, (3) 6.08, (4) 7.08, (5) 7.88, (6) 8.60. Concentration of dye, $1.73 \cdot 10^{-4}$ *M*. (b) Compound **4**: pH = (1) 3.05, (2) 4.04, (3) 5.13, (4) 6.13, (5) 7.08, (6) 7.86, (7) 9.08. Concentration of dye, $6.3 \cdot 10^{-5}$ *M*.

zones focused in capillary IEF corresponds to the order of titration-based values even for p*I* differences of the separated compounds down to 0.1 pH unit [13].

Triphenylmethane dyes

The isoelectric point of compound **4** was determined both by titration (p*I* = 6.15, see Table 2) and by zone electrophoresis [30], which gave a p*I* value of 6.17 together with a $-d\mu/dpH$ value of $9.0 \cdot 10^{-9}$ m^2 V^{-1} s^{-1} pH^{-1} around the compound p*I* value. The dependence of spectra on pH is shown in Fig. 1b. As expected, the complexity of the molecule leads to the several colour transitions. The cationic form is yellow, the anionic form is violet and the isoelectrically focused neutral form appears in the red zone. However, the calculation of the properties of particular protonated forms would probably need a much larger number of measurements than summarized in Fig. 1b. Nevertheless, the lack of acid–base and spectroscopic data for a particular dye form does not hinder its use as a p*I* marker with sought spectroscopic properties. The dye focusing ability, good water solubility, visual detectability and colour vs. pH dependence was appreciated, e.g., in the development of a new capillary electrofocusing method [31].

The phenol red structure enables one to introduce up to four aminomethyl groups, leading to compounds **6** and **8** (see Table 1). The pK_a of the colour contrast yellow to violet transition was determined spectroscopically to be 3.0 in both instances. Throughout the entire pH range from 4 to 12, the spectrum showed no marked changes. Apparently, the dissociation of amino groups is accompanied only by marginal changes in the spectra; the respective pK_a values are in the region where the phenolic group is fully dissociated. On the other hand, the presence of four equivalent amino groups in the compound moiety generates a favourably large $-dz/dpH$ value close to the compound p*I*.

The more basic thymol blue-based indicators (**5** and **7**, Table 1) have their p*I* values in the region where the phenolic group is almost undissociated. This may be the origin of the low absorption coefficients at pH around the dye p*I*. The colour contrast transition is in a more alkaline region than the compound p*I*. The presence of aliphatic substituents causes an increase in log P_{ow} of these derivatives (see Table 2).

Although the number of ampholytic sulfonphthaleins characterized in Table 2 is limited, the values presented show that the suggested ampholytic sulfonphthaleins can have their p*I* in both acidic and alkaline regions. The effective charge vs. pH dependence at the compound p*I*,

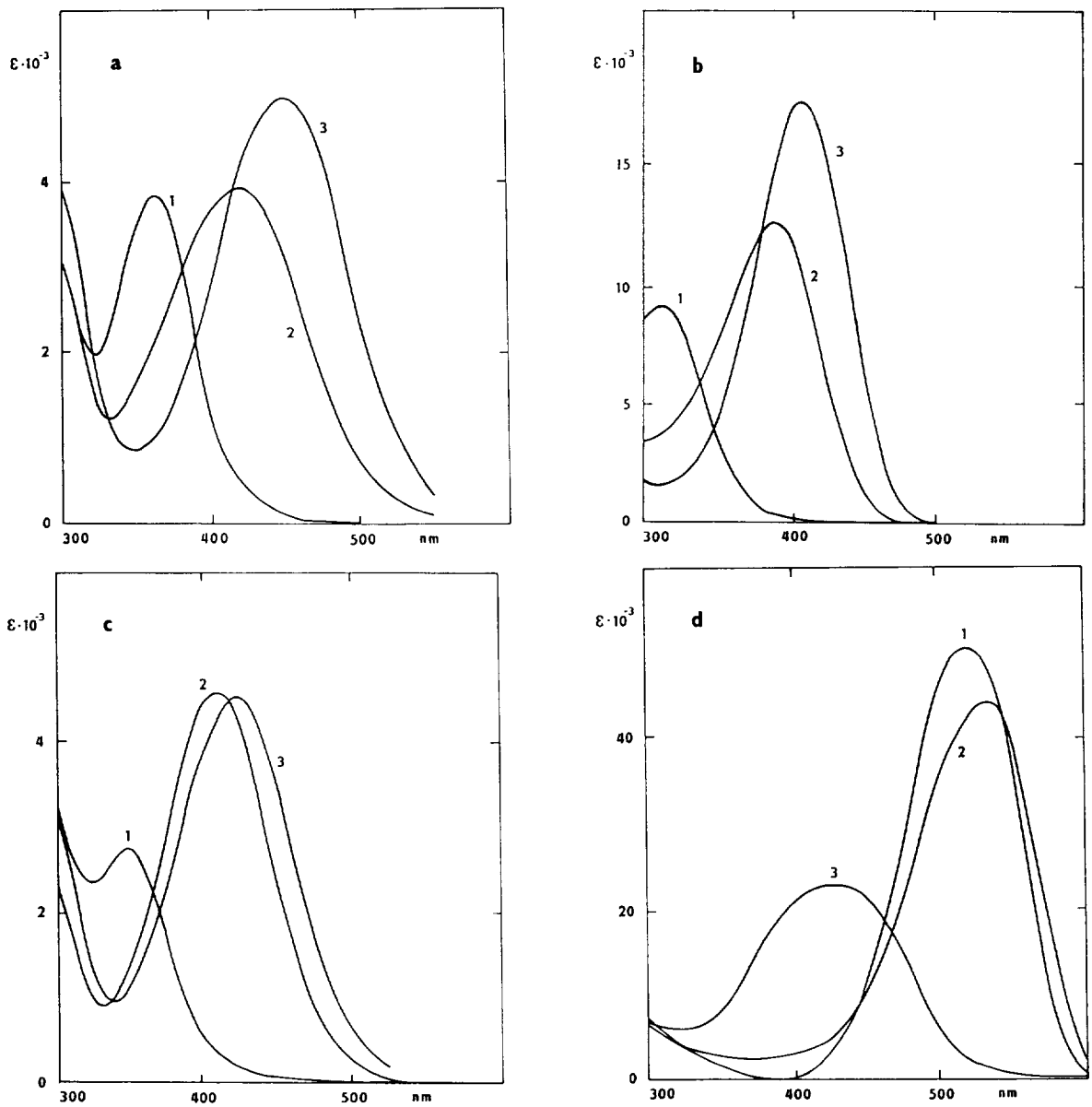


Fig. 2. Absorption curves of particular protonated forms. 1 = Cationic form; 2 = neutral form; 3 = anionic form. Compounds: (a) **1**; (b) **2**; (c) **3**; (d) **9** (methyl red).

$-dz/dpH$, is high enough for all compounds, which gives a good possibility of using them as p*I* markers in electrofocusing experiments. The log P_{ow} values indicate that a low component hydrophobicity can be achieved by a suitable choice of both the starting substrate and introduced amino groups. The low colour intensity of the thymolsulfonphthalein derivatives close to the compound p*I* is surprising.

In comparison with nitrophenol dyes, the spectral transitions of sulfonphthalein dyes are more complicated (see, e.g., Fig. 1b) and close together so that the reliable evaluation of the spectra of different protonated forms would need

Table 2
IEF, spectral and lipophilic characteristics of the studied ampholytic dyes

No.	p*I*	$(-dz/dpH)_{pI}$	λ_{max}(nm)[a]	$A^{1\%}$ [b]	log P_{ow} [c]
1[d]	7.2	0.15	416	162	1.05
2[d]	6.6	0.15	400	562	0.49
3[d]	5.3	0.12	409	142	−0.16
4	6.2	0.48	576	190	0.48
5	7.1	0.16	450	43	1.52
6	7.4	1.01	569	285	−0.01
7	8.1	0.24	582	33	1.09
8	10.3	1.05	570	330	0.88
9[e]	3.9[g]	0.20	523	1440	3.5
10[f]	9.5[g]	0.90	482	640	2.72

[a] Wavelength of absorption maximum in the visible spectrum of aqueous buffer solution at pH equal to the p*I* value.
[b] Absorptivity of a 1% aqueous buffer solution at pH equal to the p*I* value.
[c] Partition coefficient between 1-octanol and water at 25°C.
[d] Values from Ref. [9].
[e] Methyl red.
[f] Azo dye **10** (Ref. [7]).
[g] Determined spectroscopically.

more detailed study. Therefore, the development of a method of characterization similar to that previously described [8] is in progress.

An attempt was made to synthesize coloured ampholytes based on other aminomethylated triphenylmethane indicators. However, they were found to be unsuitable as soluble ampholytic testing dyes or p*I* markers. They included derivatives of phenolphthalein, which form white precipitates close to the pH of the expected p*I* value. The precipitate is soluble to give a colourless solution in acidic medium and a violet solution in alkaline medium. The products of aminomethylation of fluorescein and aurin were composed of a number (up to eight) of components distinguishable by gradient LC. Although they behaved well in electrophoretic focusing experiments, their purification to defined products was unsuccessful.

Table 3
Comparison of potentiometrically determined pK_a and spectroscopically determined pK_a (s) values of the selected nitrophenol dyes

No.	pK_{a_1}	pK_{a_1}(s)	pK_{a_2}	pK_{a_2}(s)
1	5.70	5.66	8.66	8.52
2	5.16	5.42[a]	8.07	8.13[b]
3	3.76	3.94	6.91	–[c]

[a] Ref. [29]: 5.53.
[b] Ref. [29]: 8.02.
[c] Not determined.

Azo dyes

The spectroscopically determined pK_a values of methyl red (compound **9**, see Table 2) are 2.61 and 5.17 ± 0.13 (3σ), which can be compared with the literature values of pK_{a_1} = 2.6 [32] and pK_{a_2} = 5.0 [18,32]. This indicator is known to be suitable for spectroscopic pH determination [18]. However, the p*I* value of methyl red lies relatively far into the acidic region so that its use as an internal standard in electrofocusing experiments is limited.

Azo dye **10** (see Table 2) has a satisfactory $-dz/dpH$ and its p*I* value of 9.5 fills the gap (8.6–10.1) in the pH range of nitrophenol dyes [9]. The spectroscopically determined pK_a of the colour contrast transition (yellow to violet) is 2.49 ± 0.21, which is far from the compound p*I*. The pK_a of dissociation of the phenolic group, 8.92 ± 0.10, is accompanied by a transition from yellow to orange.

The high hydrophobicity of compounds **9** and

10 can cause some complications in their use, e.g., they may adsorb on the parts of equipment made of plastics.

3.2. On-line spectroscopic pH monitoring

The properties specified above indicate that some of the dyes studied have potential for spectroscopic monitoring of pH by DAD close to the compound p*I*. The relationship between the observed actual spectrum of the compound in the capillary and the local pH can be reliably calculated from sufficiently different spectra and pK_a values of the ionized forms of the indicator. However, the calculations based on spectral and acid–base characteristics of different protonated forms of the indicator are complicated. Tautomeric forms can be expected close to the compound p*I* [29,33]. Further, the specification of individual forms of the sulfonphthalein ampholytes is not available at present. The use of the pH dependence of composite spectra is more straightforward and applicable for all the compounds studied. As the diode-array detector enables one to monitor the absorbance ratio, the pH dependence of the absorbance ratio at wavelengths suitably chosen from the composite spectrum should give sufficient information for continuous pH monitoring. Examples of the pH dependences of the absorbance ratio for selected ampholytic dyes are shown in Fig. 3. Based on the highest steepness obtained for the compounds **1** and **4**, suitable wavelength ratios were chosen, i.e. for **1** as 425/390 nm and for **4** as 475/400 nm (see Fig. 3). The wavelengths for the other two compounds, **2** and **3**, were selected so that the absorbance ratio was almost constant within the pH range of the compound elution.

The applicability of the above compounds in on-line spectroscopic monitoring of the local pH was verified in ion-exchange LC with DAD and a flow-through pH electrode. Indeed, the monitored absorbance ratios for compounds **2** and **3** are almost constant (see Fig. 4). On the other hand, the absorbance ratio within the peaks of compounds **1** and **4** are functions of time and, consequently, of the elution volume. It allows the spectroscopic determination the pH of compound elution and the local slope of the pH gradient. The profile of the local pH obtained graphically with the help of the curves in Fig. 3 is included in Fig. 4 (broken bold lines) together with the pH gradient monitored by the flow-through capillary glass pH electrode (solid bold lines). Corrections were made for the volume difference between the diode-array and on-line pH detectors and for the correlation between the output of the on-line pH detector and that of the pH meter used for off-line pH measurement of solutions used for recording spectra. In both figures, the differences between the spectroscopically and potentiometrically determined local pHs are within a few tenths of a pH unit. Such differences may probably be explained by the complicated comparison of both approaches. On the other hand, the results support the statement that the suggested dyes have potential for on-line spectroscopic pH monitoring close to the dye p*I*. This may be particularly important in miniaturized formats of focusing where changes in pH profiles with time are of interest. The potentiometric measurements of changes in pH profiles may suffer from a high time constant of the electrode response. It should be noted that in

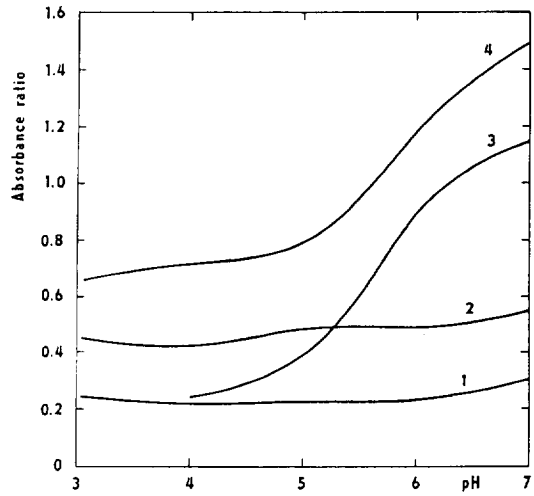


Fig. 3. pH dependences of some absorption ratios for selected dyes. $1 = A_{475}/A_{425}$ for compound **3**; $2 = A_{425}/A_{390}$ for compound **2**; $3 = A_{425}/A_{390}$ for compound **1**; $4 = A_{475}/A_{400}$ for compound **4**.

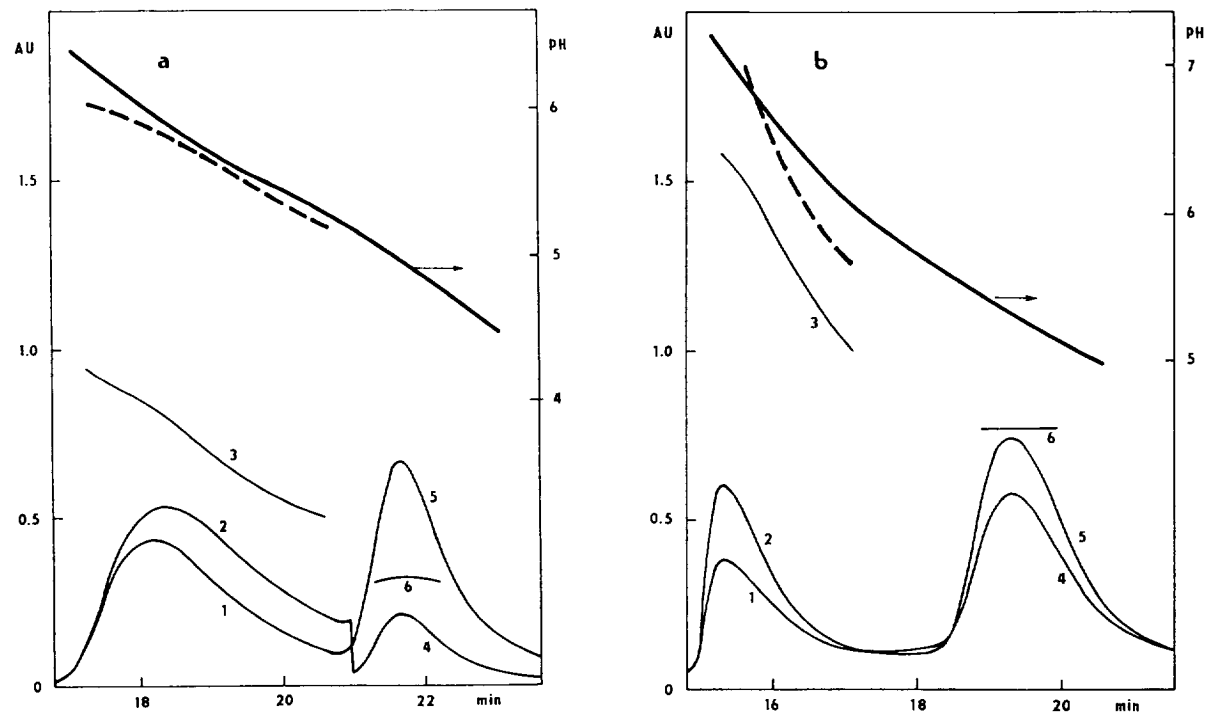


Fig. 4. Chromatograms of selected ampholytic dyes obtained by anion-exchange chromatography with pH gradient, multi-channel spectrophotometric detection and on-line pH detection. Column, 150 × 2 mm I.D. HEMA-BIO 1000 Q, 10 μm; flow-rate, 0.2 ml min^{-1}. Solutions (concentrations in water, mM): (A) piperazine 10, L-histidine 10, ethylenediamine 10, Tris 10, ammonia 20 (pH 10.1); (B) formic acid 1000. Gradient steepness: from 0 to 13% (v/v) B in A in 30 min. Spectrophotometric detector: PU 4021 multi-channel diode-array detector. pH detector: OP-0745P flow-through glass capillary electrode (15 μl) with OP-208/1 pH meter. Details of chromatogram of (a) compounds **1** and **3** and (b) compounds **4** and **2**. Curve numbers and corresponding wavelengths and wavelength ratios used for eluent monitoring (nm): (a) 1 = 400; 2 = 475; 3 = 475/400; 4 = 425; 5 = 390; 6 = 425/390. (b) 1 = 425; 2 = 390; 3 = 425/390; 4 = 475; 5 = 425; 6 = 475/425. Full bold lines, pH course monitored by on-line pH detector; Broken bold lines, pH course calculated from absorption ratios and curves 3 and 4 in Fig. 3, respectively.

LC, the pH of the compound elution is generally different from the compound pI.

4. Conclusions

The described sulfonphthalein ampholytic dyes have properties suitable for their use as low-molecular-mass pI markers. The hydrophilic ampholytic dyes selected from aminomethylnitrophenols and aminomethylsulfophthaleins have been shown to be able to monitor the local pH profile close to a compound pI by means of evaluation of collected multi-wavelength spectra. Although a more detailed characterization of the compounds is necessary to enhance the accuracy of the values obtained, the use of the pH dependence of dye composite spectra and the monitoring of selected two-wavelength ratios has the potential for on-line elucidation of the time dependence of actual pH profiles. Application of the selected compounds in capillary electrofocusing is in progress.

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