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pK_a determinations by using a HPLC equipped with DAD as a flow injection apparatus

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Abstract

A semi-automated method to determine pK_a values spectrophotometrically is described. The method uses the capabilities of a HPLC equipped with a diode array detector (DAD) as a flow injection apparatus. The advantages are low sample consumption, rapid sample throughput, high sensitivity, and precision. Experimental pK_a values obtained for two model compounds, benzoic acid (~4.0) and 2-aminopyridine (~6.8), are consistent with literature values. Constant ionic strength was maintained for a wide pH range. Solubilized samples in non-aqueous solvents were also investigated. The weakening in pK_a values, often seen when using non-aqueous solvents, was small (0.04–0.40 pH units) compared to conventional methods. © 1997 Elsevier Science B.V.

Keywords: Apparent pK_a; Automation; Constant ionic strength; DAD; Diode array detector; Experimental pK_a; FIA; Flow Injection Analysis; HPLC; Ionization constants; Non-aqueous solvents; Spectroscopic pK_a determination; Universal buffer; UV–Vis

1. Introduction

Although spectrophotometry is a very sensitive method for determining pK_a values [1], the method is very labor intensive and time consuming because it is based on analyzing wavelength shifts as a function of pH. Many buffer solutions of varying pH values have to be scanned to ensure an accurate determination. Benzoic acid and 2-aminopyridine were chosen as model compounds since they possess chromophores suitable for spectroscopic pK_a determinations and literature values for their experimental pK_a values are readily available [1–5]. Flow injection analysis (FIA) [6] methods to determine pK_a values have been previously reported in the literature. For example, FIA has been used to determine ionization constants of unstable compounds using single wavelength UV–Vis spectroscopy by taking advantage of the short residence times with FIA [7]. Other researchers used FIA with DAD in the visible region to determine ionizations constants of indicators [8,9]. FIA has also been used to determine pK_a values of weak acids with flow injection titration using potentiometric detection [10], which lacks the sensitivity of spectrophotometric methods [1]. These FIA methods do not easily

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lend themselves to automated, high throughput pK_a determinations, and their buffer preparation is not automated. In this study, a FIA spectrophotometric method is described that takes advantage of a HPLC equipped with diode array detection (DAD) [11]. We used a commonly available HPLC equipped with a DAD and take advantage of the instrument's built-in automation. Our method uses the HPLC autosampler's ability to automatically inject small volumes of sample solutions accurately into a flowing mobile phase buffer accurately formulated by the solvent delivery system of the HPLC and uses the DAD to obtain spectra rapidly. It will be shown that wavelength scans over the entire spectral range can easily be carried out under constant ionic strength conditions over a wide pH range.

2. Materials and methods

Benzoic acid and 2-aminopyridine were obtained from Aldrich (St. Louis, MO) and were used without further purification. All solvents were HPLC grade and reagent grade acetic acid, phosphoric acid, boric acid as well as KCl were obtained from EM Science (Gibbstown, NJ). Stock solutions of 2-aminopyridine and benzoic acid, (5 mg/100 ml), in water, methanol, ethanol, 1-propanol, acetonitrile and tetrahydofuran as well as 20% methanol: 80% water (v/v) and 20% 1-propanol: 80% water (v/v) were prepared. A 1 ml aliquot of each solution was sufficient for the FIA pK_a determinations. KOH (0.2 M) was prepared from KOH (1 M) certified by Fisher Scientific (Fair Lawn, NJ).

A Hewlett Packard HP 1090 HPLC equipped with a diode array detector (DAD) and a 4 nm slit was slightly modified as shown schematically in Fig. 1. As mentioned, our method has features common with FIA [6], and unlike conventional HPLC, does not employ chromatographic separation. Instead of a chromatographic column we used a 0.01 × 300 cm coil to allow for equilibration of the injected solutions and to increase the time from injection to peak elution. The HPLC autoinjector was used to accurately inject small volumes of sample solution into a continuous flow of mobile phase. Typical injection volumes and flow rates were 10 μl and 1.25 ml min^{-1}, respectively, unless otherwise noted. The HPLC's DAD was used for automated spectral detection of the injected sample solution. The pathlength of the DAD flowcell was 0.6 cm. The temperature of the HPLC's column chamber was maintained with a water bath at 25°C and was continuously monitored with a temperature probe. These studies were carried out using the HP ChemStation software version A.02.05 with a 3D absorbance-wavelength-time software.

The HPLC's ternary reservoir system was used to automatically prepare 50 mobile phase buffer solutions with pH values between 1.8 and 12.5 at a constant ionic strength of 0.146 M. Three solutions were used to prepare the mobile phase buffers. Reservoir *A* contained a universal buffer [12] at pH 1.8 which contained acetate (0.04 M), borate (0.04 M), phosphate (0.04 M), and KCl (0.15 M) which was added to increase the ionic strength. Reservoir *B* contained KOH (0.2 M) for pH adjustment. KOH was used instead of NaOH, to avoid a significantly larger sodium alkaline error at high pH [13]. Finally, reservoir *C* contained HPLC grade water for ionic strength adjustments. Varying proportions of solutions in *A* and *B* were used to change pH; and the water in *C* was used to dilute solutions *A* and *B* to a constant predetermined ionic strength of 0.146 M.

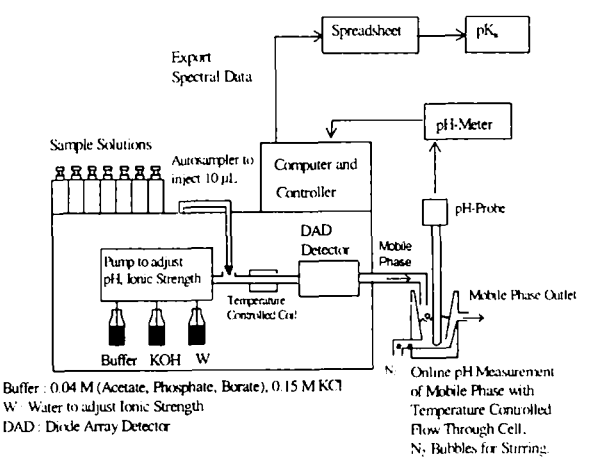

Fig. 1. Semi-automated pK_a determinations by using a HPLC equipped with DAD as a flow injection apparatus.

The ionic strength μ was calculated by $\mu = 0.5 * \Sigma_i'' m_i Z_i^2$, where m_i is the concentration of the ith charged species, and Z_i is the charge of the ith species. The hydrogen ion activity, obtained from pH measurements, was assumed to equal the hydrogen ion concentration and was used for the calculation of μ; m_i was calculated from 0.04 M using the ionization constants of acetic, phosphoric, and boric acid and the appropriate volume ratios of A:B:C, respectively.

A sequence of method programs from the HP Chemstation controlled the HP 1090's pump for the preparations of buffers with known pH and ionic strength. Since no chromatographic column was used, elution times were short (ca. 0.15 min) and run times per injection were nominally 1 min. The pH of the mobile phase buffer was measured in a temperature controlled flow-through-cell shown in Fig. 1, using a Fisher Accumet 25 pH meter and a Fisher Accu-pHast electrode. The flow through cell had an approximate fill volume of 2 ml and was covered to minimize evaporation. Prior to each use, the pH meter was standardized with pH 1, 7, and 11 calibrating buffers (Baxter, Deerfield, IL). The SoftwareWedge program (TAL Enterprises, Philadelphia, PA) was used to acquire pH readings from the pH meter every 5 min and transfer them into a Microsoft Excel worksheet. For adequate flushing 10 min at 1.25 ml min^{-1}, equivalent to ca. 500 loop volumes, were allowed for pH re-equilibration between buffer changes.

UV–Vis spectra of the sample solutions at different mobile phase buffer pH values were collected from 190 to 550 nm using the DAD. The elution time was typically 0.15 min and the chromatographic peak width at peak base was 0.11 min. The time interval of spectral data collection was 0.007 min so that typically 16 or more spectra were collected as the sample passed through the detector. The apex spectrum was chosen for pK_a calculation, since it was the spectrum with the highest intensity and consistency. pK_a values calculated from other locations are comparable. Spectral data were then exported from HP Chemstation data files to Microsoft Excel for pK_a calculations. All pK_a determinations were averages of multiple determinations at different pH values [1].

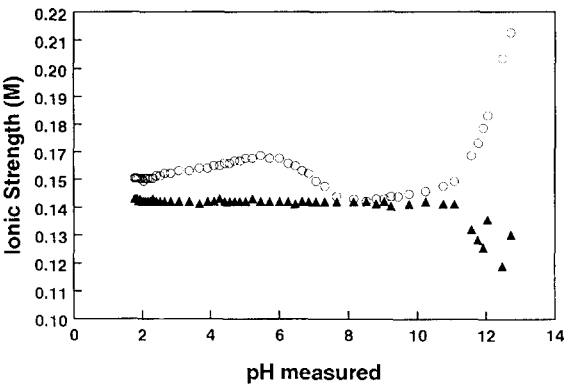

Fig. 2. Ionic strength profile of universal buffers prepared by the HP1090 HPLC and used for spectral pK_a determinations. (○) Ionic strength without adjustment by dilution with water, obtained by varying the ratio of solutions in A and B, from 100:0 to 50:50 (v/v) in increments of 1% (v/v), respectively, giving an ionic strength range of 0.146–0.165 M between pH 1.8 and 11.5. (▲) Ionic strength obtained by varying the ratio of solutions in A and B and adjusted to 0.146 M where possible, by dilution with water (reservoir C) between pH 1.8 and 11.5. Assumption for ionic strength calculation: $[H^+] = -\log pH$.

The pK_a values were calculated as described by Albert and Serjeant [1]. For a weak base, such as 2-aminopyridine, the pK_a was calculated by:

$$pK_a = pH + \log \frac{A_{obs} - A_M}{A_I - A_{obs}}$$

The pH of the mobile phase was measured online in the flow-through-cell, A_M and A_I are the absorbances of the unionized and ionized species at the analytical wavelength, respectively. A_{obs} is the observed absorbance of the sample at the measured mobile phase pH and analytical wavelength. Choice of analytical wavelengths is discussed elsewhere [1].

3. Results and discussion

Fig. 2 shows the ionic strength of the universal buffer as a function of pH. A 'third order type' of polynomial curve occurred when pH was varied by mixing only solutions from reservoir A and B. A more constant ionic strength was obtained by dilution with water from reservoir C. These dilu-

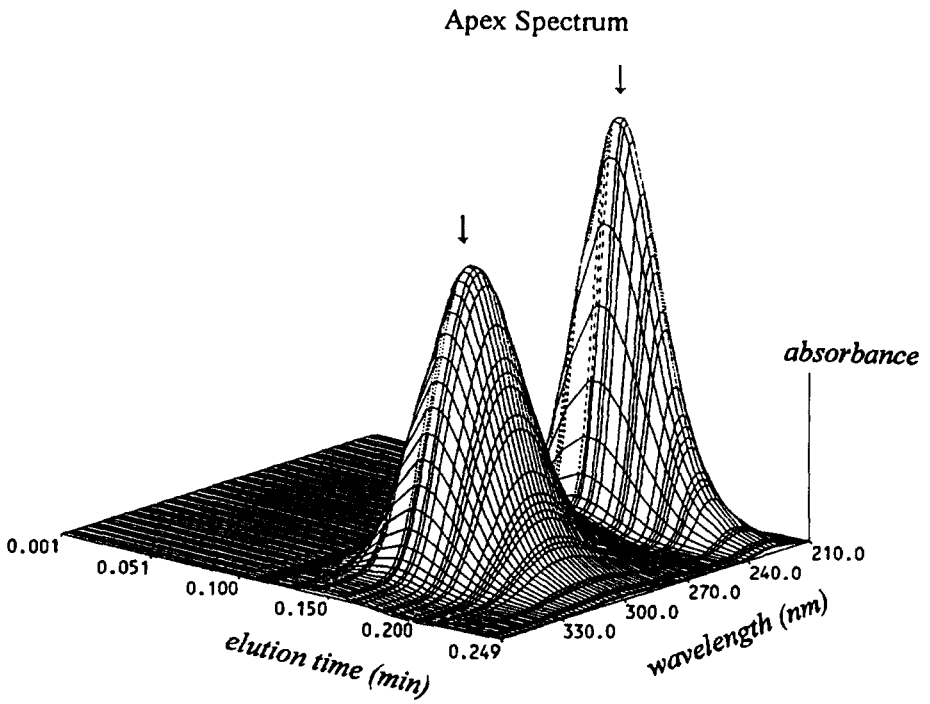

Fig. 3. 3D elution profile of 2-aminopyridine in water, 25 μl injected into a flow of 1.25 ml min^{-1} mobile phase buffer with pH 3.7. The UV spectrum at elution maximum (peak apex spectrum) was used for pK_a calculations. Analytical wavelength chosen was 300 nm.

tions were adjusted to maintain the lowest ionic strength of the system (0.146 M), which occurred at approximately pH 9. Using this technique, Fig. 2 shows that nearly constant ionic strength could be achieved up to pH 11.5 when dilutions were used. At higher pH's, our calculations overestimated the dilution required. Since pK_a values for 2-aminopyridine and benzoic acid can be accurately determined by spectra collected between 1.8 and 10.0, no further refinements were made in this study to correct for ionic strength above pH 11.5.

Fig. 3 shows a typical 3D elution profile of 2-aminopyridine (dissolved in water) obtained from the HP's 3D software. Although 45 UV-spectra were collected over approximately 0.25 min to ensure accurate detection of the apex spectrum, only the apex spectrum, at approximately 0.16 min was used for pK_a calculations. Figs. 4 and 5 show selected apex spectra at various pH for benzoic acid and 2-aminopyridine, respectively. For benzoic acid, the absorbance changes most rapidly with pH at the analytical wavelength of 230 nm. Above pH 6, no significant change in absorbance was observed. Similarly, for 2-aminopyridine, the analytical wavelength of 300 nm was chosen. One measure of the precision of the methodology can be seen from the sharp isosbestic points. A more quantitative measure of the precision is the relative standard deviation (RSD) of the absorbance at the analytical wavelength from six replicate injections at constant pH. For our method the RSD is very low. For example, the RSD of the measured absorbances for 2-aminopyridine at pH 6.2 was 0.67%; for benzoic acid, 0.22%.

Above pH 10.4, we noticed that spectra collected did not go through the isosbestic points (e.g. 225 nm for benzoic acid and 275 nm for 2-aminopyridine, respectively). This phenomena occurred even when the ionic strength was lowered by water dilution. When water blanks were examined, high background spectra were observed

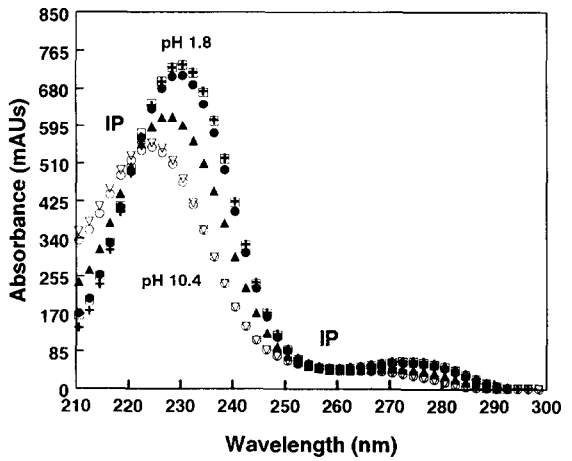

Fig. 4. Benzoic Acid dissolved in 100% Water. Representative spectra of benzoic acid from pH 1.8 to 10.4: (□) pH 1.80, (+) pH 2.04, (•) pH 3.03, (▲) pH 4.09, (○) pH 6.04, (▽) pH 10.42, $pK_a = 4.02$. To obtain spectra, 10 μl of benzoic acid solution (0.05 mg ml^{-1}) were injected into 1.25 ml min^{-1} mobile phase buffer. IP, Isosbestic Point and mAUs, milli absorbance units.

for pH > 10. Background substraction of a water blank above pH 10 led to sharp isosbestic points. No water background correction was needed for pH 1.8 through pH 10.

The experimental pK_a values obtained for benzoic acid and 2-aminopyridine are given in Table 1. The determinations of the pK_a values without a water ionic strength adjustment gives values that are slightly stronger than literature values. Thus for the weak base, 2-aminopyridine, we determined a pK_a of 6.84 whereas an average of literature values gives 6.76. When we maintained a constant ionic strength of 0.146 M, we obtained a value of 6.77 (average of two determinations). Similarly, our determination of benzoic acid's pK_a as 3.97 is slightly stronger than 4.01 (literature average). For constant ionic strength (~0.15 M), we obtained a pK_a of 4.02; which is in good agreement with a pK_a of 4.04 reported in the literature for the same ionic strength [5].

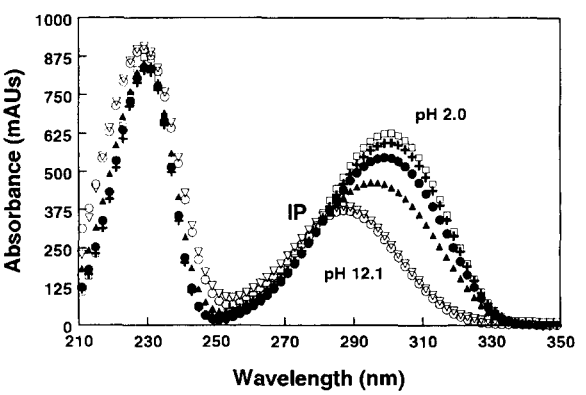

Fig. 5. 2-aminopyridine dissolved in 100% Water. Representative spectra from pH 2.0 to 12.1: (□) pH 2.04, (+) pH 4.09, (•) pH 6.04, (▲) pH 6.69, (○) pH 9.04, (▽) 12.14, $pK_a = 6.77$. To obtain spectra, 10 μl of 2-aminopyridine solution (0.05 mg ml^{-1}) were injected into 1.25 ml min^{-1} mobile phase buffer. IP, Isosbestic Point and mAUs, milli absorbance units.

Organic solubilizing agents with their lower dielectric properties would be expected to depress ionization of weak acids and bases. This 'weakening' effect should result in higher p_sK_a, the apparent pK_a values in mixed solvent systems for weak acids, and lower p_sK_a values for weak bases [1]. Table 2 shows that, in general, these 'weakening effects' occurred. These data were obtained by dissolving the samples in a variety of organic solvents and injecting 10 μl of these solutions into 1.25 ml min^{-1} aqueous buffer flow. The pH values were measured in aqueous solutions as discussed earlier using the flow-through cell and were not affected by the small amount organic solvent injected. Small expected increases in p_sK_a values for benzoic acid (0.05–0.15 pH units) and small decreases for 2-aminopyridine (0.04–0.40 pH units), respectively, were observed. Acetonitrile seems to be the exception to a dielectric constant [14–16] correlation with weakening of the ionization. Apparently, the interaction between acetonitrile and the substrate is greater than that of a typical solute and the bulk dielectric effect.

The weakening in the ionization constants due to organic solvent we observed are small compared to those observed with conventional potentiometric methods. Albert and Goldacre [17] found that ΔpK_a values for acridine bases in 50% ethanol (v/v) solutions were typically 0.43–1.49 pH units, i.e. the p_sK_a appeared lower than they were in pure water. Similarly, for 100% methanol, the p_sK_a value of benzoic acid is 9.44 [18], and decreases to 5.11 and 4.02 in 48.5 and 6.3%

Table 1
Comparison of experimental pK_a values for 2-aminopyridine and benzoic acid

	2-aminopyridine pK_a (300 nm) (mean ± SD)	Benzoic Acid pK_a (230 nm) (mean ± SD)
Samples in water, buffer not diluted with water, variable ionic strength, range: 0.146–0.165 M, $T = 25°C$	6.84 ± 0.03 ($n = 8$)	3.97 ± 0.03 ($n = 11$)
Samples in water, controlled ionic strength buffer 0.146 M, adjusted with water. $T = 25°C$	6.77 ± 0.02 ($n = 16$)	4.02 ± 0.04 ($n = 18$)
pK_a literature values [1–5]	6.82, 6.76[a], 6.71	3.99[b], 4.01[c], 4.04[d]

[a] Ionic strength 0.01 M,
[b] Ionic strength 0.11 M,
[c] Ionic strength 0.1 M,
[d] Ionic strength 0.15 M.

methanol/water (w/w), respectively [3]. With our method, the largest decrease we observed was 0.40 pH units with acetonitrile as a solvent for 2-aminopyridine. Aqueous methanol mixtures, on the other hand, caused the smallest weakening effect. These observations are in agreement with the literature [1]. When using 20% methanol (v/v), we found the depression to be nearly negligible (0.04 pH units). The relatively small weakening effect observed suggests that the compounds are in a largely aqueous environment which is the result of mixing a small volume (10 μl) of non-aqueous solution with a mobile phase buffer flow of 1.25 ml min^{-1}. This mixture evidently produces a solution in the DAD sample cell which is very close to an aqueous solution, with a pH fixed by the universal buffer system.

Our method covers a pH range from approximately 1.8 to 13 and is mainly a limitation of our universal buffer system. Based on the criteria outlined by Albert and Serjeant [1] pK_a values from 3.8 to 11 can be determined with our method. With nearly constant ionic strength pH values in the range of 1.8–11.5 can be used to calculate pK_a values. It is conceivable that other buffer systems [1] can be used to extend the useful pH range to determine more extreme pK_a values. The method should be applicable to slightly soluble compounds, possessing spectral characteristics that give wavelength shifts when the pH is varied, including zwitterions, provided that the sample possess analytically useful chromophores i.e. if the pH is varied, a change in the concentration of species results that is detectable with UV–Vis spectroscopy. The calculations may be a bit more complex, of course, as discussed in [1].

4. Conclusions

Spectrophotometric measurements of pK_a values are useful due to their inherent high sensitivity. However, accurate determinations requires that buffers of known pH at carefully controlled ionic strength be prepared so that the ionized/unionized forms of functional groups are controlled by the buffer pH. In this study, the automation and precision of a modern HPLC for several solutions of narrow pH range and the power of a DAD to take spectra rapidly, coupled with a flow-through-cell for measuring pH were used for accurate spectral pK_a measurements.

Manual buffer preparation of 30–50 buffers is replaced with pre-programmed mixtures of reservoirs *A* and *B*; these can be incremented to less than 0.05 pH units over an approximate pH range of 1.8–13. Maintaining constant ionic strengths can be carried out over a pH range from pH 1.8 to 11.5 by dilution using a third reservoir. This improves the reproducibility of pK_a determinations. Reliable pK_a values in the range of slightly less then 3.8 to slightly more then 11 can be determined at nearly constant ionic strength. Because modern HPLCs can inject 10 μl samples with a high degree of precision, very little sample is needed for pK_a determinations (approximately

Table 2
Comparison of experimental pK_a values and p_sK_a values for model compounds a variety of solubilizing agents[a]

Solubilizing agent	Dielectric constant[b]	2-aminopyridine $pK_a/p_sK_a^c$ ($7 \leq n \leq 11$) (mean ± SD)	Benzoic acid $pK_a/p_sK_a^c$ ($7 \leq n \leq 11$) (mean ± SD)
Water	78.3	6.84 ± 0.03	3.97 ± 0.03
20% methanol/ 80% H_2O (v/v)	71.4	6.80 ± 0.04	4.02 ± 0.02
20% 1-propanol/ 80% H_2O (v/v)	64.9	6.80 ± 0.05	4.05 ± 0.02
Methanol	32.7	6.63 ± 0.06	4.10 ± 0.02
Ethanol	24.6	6.62 ± 0.04	4.11 ± 0.04
1-propanol	20.5	6.61 ± 0.08	4.11 ± 0.03
Tetrahydrofuran	7.6	6.57 ± 0.14	4.10 ± 0.03
Acetonitrile	35.9	6.44 ± 0.04	4.12 ± 0.04

[a] Injection volume was 10 μl of sample solutions and mobile phase buffer flow was 1.25 ml min^{-1}, analytical wavelengths were 310 and 240 nm for 2-aminopyridine and benzoic acid, respectively. Ionic strength range 0.146–0.165 M. $T = 25°C$.
[b] Dielectric constants from references [14–16].
[c] Apparent pK_a values: p_sK_a values determined when using non-aqueous solvents.

0.5 mg). Duplicate or triplicate determinations are easily carried out using multiple injections at a given pH. Due to the capabilities of the HPLC's autosampler, automated pK_a determinations of a large number of compounds is feasible. The HPLC can easily be used for both our method and conventional chromatography since our modifications to the instrument are very minor. Automated sample handling, data acquisition, and the small impact of solubilizing solvents highlight the advantages of the FIA spectrophotometric method using a HPLC equipped with a DAD for pK_a determinations.

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