3.1. Preparation of Buffer Solutions

A volume of 50 mL of each buffer solution covering the pH range ($pK_a \pm 2$) of the compound to be analyzed was prepared. For the classical method of pK_a determination, the prepared buffers possessed about 0.3-0.5 pH units interval within the coverage pH. For the new method of pK_a determination wherein internal standard is used, only the buffers that cause the partial and full ionizations of the analytes and the internal standards were prepared. The pK_a values, the coverage pH range and the stock solutions used in the preparation of each of the buffers are listed in Table 1. These were all filtered through a 0.45 μm nylon filter before use or they were stored in dark bottles at 4°C until the analysis was carried out. The pH was measured using the GLP 22 Crison pH meter with 5014 electrode at 25°C before use. The ionic strength of the background electrolyte (BGE) is an important factor to consider in the analysis. An ionic strength of 50mM was chosen because this provides a well buffering capacity and reduces the problem of Joule heating. The ionic strength of the buffers was maintained at 50mM through the use of appropriate amounts of the stock solutions and 0.5M NaOH, 0.5M HCl or 0.5M KCl solutions. Fresh buffers were employed for every analysis.

Table 1. Composition of the stock solutions and the pKa and pH ranges of the BGE [4].

Buffer	**pK_a**	**Coverage pH range**	**Stock solutions for buffers**
H_3PO_4/ $H_2PO_4^-$	1.27	1.51-3.11	0.1M NaH_2PO_4, 0.5M HCl
HCOOH/$HCOO^-$	3.75	2.60-4.80	0.1M HCOONa + 0.5M HCl
CH_3COOH/ CH_3COO^-	4.76	3.70-5.80	0.1M CH_3COONa + 0.5M HCl
$BisTrisH^+$/ BisTris	6.48	5.50-7.50	0.1M BisTrisHCl + 0.5M NaOH
$TrisH^+$/Tris	8.08	7.00-9.00	0.1M TrisHCl + 0.5M NaOH
CHES/$CHES^-$	9.50	8.40-10.10	0.1M CHESNa + 0.5M HCl
CAPS/$CAPS^-$	10.40	9.40-11.60	0.1M CAPSNa + 0.5M HCl
NaOH		11.80-13.00	0.5M NaOH + 0.5M KCl

3.2. Instrumentation

All the experiments were performed in a Beckman (Palo Alto, CA, USA) capillary electrophoresis. The P/ACE 5500 is equipped with a photo diode-array detector (PDA) and a 32 Karat version 5 software from Beckman Coulter for the

integrator. A fused silica capillary with a dimension of 51 cm total length and 47 cm length from the inlet to the detector, and a 50 μm internal diameter and 75 μm external diameter was used. The instrument has 4 trays: the left trays correspond to the buffer inlet tray (front), and the sample inlet tray (back) where the buffer solution and sample vials are placed, respectively. The right trays are the buffer outlet tray (front) and the sample outlet tray (back) that function to hold the vials as receivers of used liquids. The electrode and the capillary are always immersed in the inlet and the outlet vials. In the capillary the sample and the buffer flow. These flowing liquids passed to the detector window before finally reaching the outlet vial. To the detector the computer system is attached to convert the signal to useful information using the software mentioned previously. To the immersed electrode is where the applied high voltage power supply is directed to. The capillary electrophoresis is also equipped with a vacuum pump to hasten the flow of the liquids in the capillary. The equipment is thermally controlled as well. To mention, the temperatures used in the analysis were 20°C, 25°C 30°C, 35°C, 40°C, 45°C and 50°C and the voltage was 18 kV. All injections were done with a pressure of 1 psi.

3.3. Conditioning of Capillary

The conditioning of the capillary was always done at 25°C. This procedure of rinsing the capillary before the analysis is carried out to clean and remove all the residues left and the adsorbed materials from the previous experiments, and to rehydrate the surface silanols on the capillary. For new capillary, conditioning is primarily done to activate the surface silanols.

Conditioning of the new capillary was made by filling one Supelco bottle with milli-Q water and the other with 1M NaOH. These were placed in the inlet tray and the third bottle was put in the outlet tray to receive the used liquids. The instrument was programmed to do the rinsing of 10 minutes with milli-Q water, 10 minutes with 1 M NaOH, and 10 minutes with milli-Q water. Before the sample injection the conditioned capillary was always rinsed with the rinsing buffer solution for 30 minutes. For the used capillary or for daily conditioning, shorter time is employed. The same bottles were

used and the time program was as follows: 5 minutes for milli-Q water, 5 minutes for 0.1 M NaOH, 5 minutes for milli-Q water, and lastly 20 minutes for the rinse buffer. Forward pressure of 20 psi was applied throughout the conditioning and rinsing steps. Only fresh milli-Q water and NaOH solution were used in all the conditioning and analyses done. At the end of each analysis, which is referred to as the sequence, rinsing with water and NaOH was done as well.

3.4. Optimization Procedure

For each buffer solution, three Supelco bottles were utilized. The first bottle served as the rinsing buffer, the second one was for the observation and the third one was used as the outlet of the two bottles. For each buffer, the change in current with increasing voltage was noted at a given temperature. The instrument was programmed at 20°C and the voltage was allowed to increase from 2 kV to 30 kV. Afterwards the temperature was increased to 30°C and the same voltage was applied. This whole process was repeated several times until 50°C was attained at every 10°C interval. This was performed in all the prepared pH buffers. After all the observations were obtained, the capillary was rinsed with water to remove all the traces of the remaining buffers.

The current (intensity) obtained for every set voltage at each temperature was recorded. Using these data the Ohm's law plot was constructed. This was done for all the buffer solutions. The maximum voltage for each buffer was chosen using the criterion of $r^2 \geq 0.999$. Any voltage and current combinations which did not satisfy this criterion were not considered. This is in relation with the V=IR relationship of the Ohm's law. In general, the maximum voltage was found to be 18kV and was worked with 20°C, 25°C, 30°C, 35°C, 40°C, 45°C and 50°C in all the pH buffer solutions. These conditions were used for the determination of the dissociation constants of some compounds.

3.5. Preparation of Sample

Stock solutions for every analyte with a concentration of 1000µg/mL were prepared by either weighing using analytical balance or measuring the desired volume of the reagents. The reagents used, the manufacturer and their purity are shown in Table

Table 2. Analytes and internal standards used for pKa determination.

Chemical Name	Source	Purity
4-nitroaniline	Merck	>99%
2,4-lutidine (2,4-dimethylpyridine)	Aldrich	99%
o-tolidine	Baker	-
4-picoline (4-methylpyridine)	Aldrich	98%
Acridine	Sigma	97%
Benzoic acid	Baker	99.99%
2,4-dimethylbenzoic acid	Aldrich	98%
3-bromobenzoic acid	Merck	98%
4-aminobenzoic acid	Fluka	99%
4-nitrophenol	Fluka	>99%
3-nitrophenol	Aldrich	99%
2-nitrophenol	Fluka	>99%
3,5-dichlorophenol	Aldrich	97%
4-bromophenol	Aldrich	99%
4-chlorophenol	Synthesis	
(+)-ephedrine	Carlo Erba	-
L-tryptophan	Sigma	98%
2,4-dinitrophenol	Doesder	-
4-tertbutylphenol	Aldrich	99%
o-anisidine (2-methoxyaniline)	Aldrich	99+%
Aniline	Baker	99%
2,5-dichloroaniline	Merck	97%

2. Each was dissolved and diluted up to the 25 mL mark of the volumetric flask using either fresh milli-Q water or water-methanol solution as a solvent depending on the nature of the solute. The prepared stock solutions were transferred into dark bottles and stored in refrigerator to minimize degradation. In spite of this, some chemicals degrade quickly so that they were used right after their preparation; examples are the phenols.

For the preparation of sample to be subjected to the new method of pK_a determination, an appropriate volume of 1000µg/mL stock solution was drawn using a volumetric pipette. This was added with 1000µg/mL each of internal standard and

benzyl alcohol and diluted to make a volume of 25 mL which concentration is 100µg/mL. Benzyl alcohol served as the EOF marker or neutral marker. The internal standard was chosen based on the closeness of its pK_a value with the analyte and/or based on the similarities and differences of its standard enthalpy change (ΔH) with the analyte at 25°C. To assign the peaks on the electropherogram another solution containing either the analyte or the internal standard and benzyl alcohol with a concentration of 100µg/mL (prepared as the above) was injected after the sample run. For the classical method the same concentrations of the samples without the internals standard were prepared. All solutions were filtered in 0.45 µm nylon filter before use. Only fresh samples are used in every sequence performed.

3.6. Sample Analysis

3.6.1. Use of internal standard method

The prepared sample containing the analyte (AN), the internal standard (IS) and the EOF marker was injected. This was followed by the injection of the appropriate buffer solution to facilitate the flow and the measurement of the mobilities of each component. At least two buffers were used: one that caused the full ionization to calculate the limiting mobility, μ_{A^-} and another that caused the partial ionization to calculate the effective mobility, μ_{eff} of both the AN and IS. The vial containing only the EOF marker and the IS was injected in the same way as the sample injection. All the runs were done at 20°C, 25°C, 30°C, 35°C, 40°C, 45°C and 50°C. The mobility and the pK_a calculations were done by following equations 8-12.

3.6.2. Classical method

This method was used to countercheck for the pK_a of some analytes. In this method only the sample containing the analyte and the EOF marker was injected. All injections were done to get the retention times at all pH within the $pK_a \pm 2$ of the analyte at an interval of 0.3-0.5 pH units at 25°C . The µ was calculated employing equation 10. The calculated mobilities were plotted against the pH of the buffers. The inflection point

of the best fit curve by nonlinear regression is the pK_a of the analyte. Table Curve software was used.

In both methods fresh sample was injected from the inlet vial to the outlet vial by the application of a pressure of 1 psi (6894.76 Pa) at duration of 3 seconds. If a buffer was to be used for different samples or if the same sample and same buffer but in different temperature setting, the capillary was rinsed for 5 minutes with the current rinsing buffer before injection. When a buffer was to be changed, the capillary was rinsed for 5 minutes with water and for 10 minutes with the new buffer. The buffers could only be used for a maximum of 120 minutes. Each electrophoretic run took not more than 8 minutes. The response, that is, the migration time (in minutes) was obtained at 214 nm wavelength. The migration times were encoded and the mobilities were calculated in a Microsoft Excel spreadsheet.