4.1. Optimization of the Temperature and Voltage Conditions for the pK_a Determination

To investigate the effects of temperature variations in the determination of pK_a values of some weak acids and weak bases, the maximum voltage was first identified. This was done by running different buffers (Table 1) in the capillary electrophoresis set to an increasing voltage conditions at each studied temperature. For instance, for pH 2 buffer at 20°C the voltages of 2 to 30 kV at 2 kV interval were applied. Using the same buffer and the same rate of increasing voltage, the temperature was increased to 25°C, 30°C, 40°C, 45°C and 50°C. At each temperature the current (intensity) corresponding to the programmed voltages were obtained and used to construct the Ohm's law plot. Figures 2-4 are some examples of the graphs (the rest are in the appendices section). As long as there is a linear relationship between the intensity and voltage, the slope will be constant. In this study the linear relationship was defined to have an $r^2 \geq 0.999$. These procedures were adopted to all the prepared buffers.

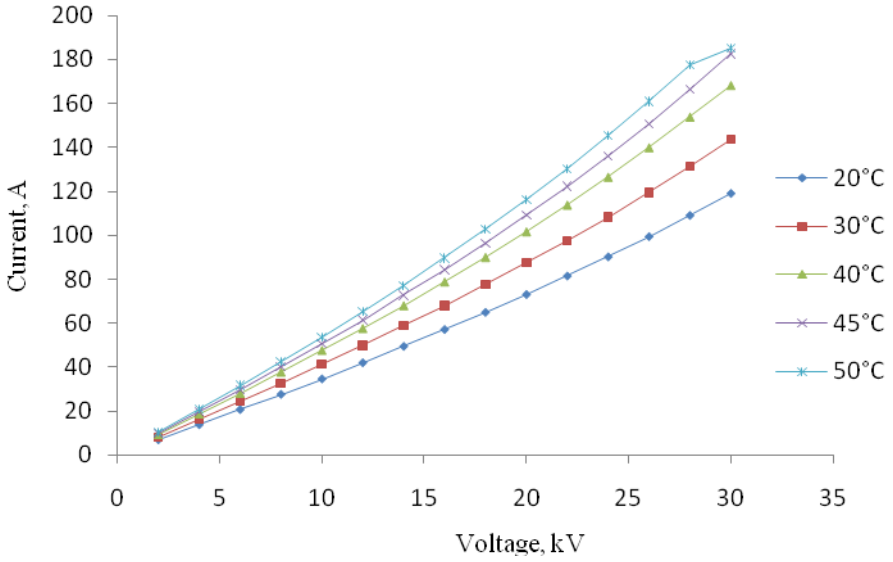

Figure 2. Ohm's law plot for pH 2 H_3PO_4/ $H_2PO_4^-$ buffer solution.

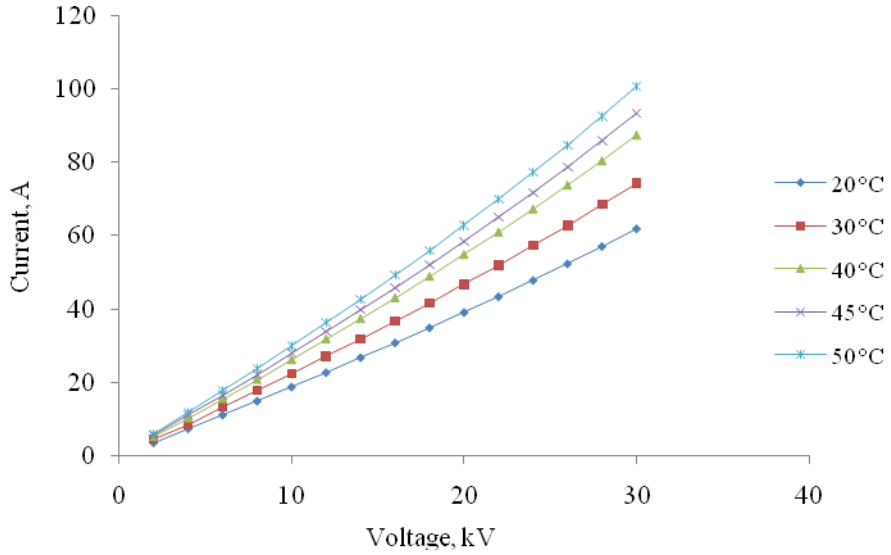

Figure 3. Ohm's law plot for pH 7 Bis-TrisH^+/ Bis-Tris buffer solution.

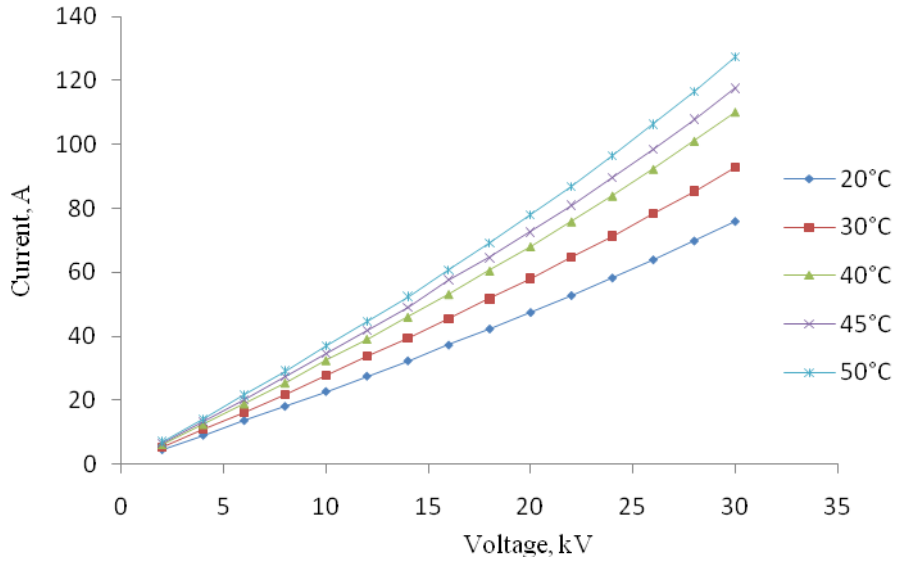

Figure 4. Ohm's law plot for pH 12 NaOH buffer solution.

It can be observed from the figures above that the curves are not perfectly straight, which implies that at some points deviation from the Ohm's law took place. This deviation occurs when the heat, referred as the Joule heat, in the capillary electrophoresis instrument is not well-dissipated. This observation means that 30 kV could not be the maximum working voltage in all the temperature conditions. To determine the maximum voltage a straight line that passes through the origin was drawn in each of the Ohm's law plot constructed in each buffer at all temperatures. The highest point in the graph that showed an $r^2 \geq 0.999$ was considered as the maximum voltage. The error related with this decision was expressed as percentage error. This was determined by comparing the experimental intensity, I_{expt} with the theoretical intensity, I_{calc} (equation 17). The I_{calc} was obtained by taking the product of the slope, m of the straight line previously constructed and the experimental voltage, V (I_{calc} = mV). The results are shown in Table 3.

$$\%\text{error} = \frac{I_{expt} - I_{calc}}{I_{calc}} \qquad (17)$$

For extremely high and low pH buffers (2 and 12) the maximum allowable voltages are comparatively lower than the rest of the buffer solutions. Even so, a maximum voltage of 18 kV was chosen since at lower voltages the time and efficiency of analysis would be sacrificed. Another reason is that the highest voltages of pH 2 and 12 are not significantly different from the other buffer solutions. There was an absence of a general trend to describe the relationship between voltage and temperature from all the buffers. This can be accounted for the differences in the nature and composition of these buffers. For pH 5 buffer solutions electrophoretic runs with and without applied pressure were performed. It can be noticed that there is no wide variations between the maximum voltages obtained from the two, hence the optimized condition was generalized to be applied with pressure during the electrophoretic run. After the optimization procedure a maximum voltage of 18 kV was decided to be used in all the electrophoretic runs at 20°C, 25°C, 30°C, 35°C, 40°C, 45°C and 50°C, and with a pressure of 1 psi.

Table 3. The maximum voltage (kV) obtained for each buffer at different temperatures and the % errors associated with them.

pH		Temperature, °C				
		20	30	40	45	50
2	V_{max}	18	16	16	14	14
	Error	13.89	14.38	11.77	16.48	12.39
3	V_{max}	20	22	24	30	30
	Error	13.66	12.16	8.41	10.22	8.89
4	V_{max}	18	22	24	24	24
	Error	11.94	11.89	11.22	12.21	12.67
5	V_{max}	24	18	20	12	14
	Error	11.21	9.91	13.10	8.55	9.42
5[a]	V_{max}	18	22	22	16	20
	Error	6.27	11.29	11.77	10.80	11.83
6	V_{max}	18	22	20	20	18
	Error	9.38	10.83	10.48	11.52	10.92
7	V_{max}	18	18	18	20	18
	Error	9.70	9.71	10.83	12.25	11.75
8	V_{max}	18	18	18	20	18
	Error	9.29	9.41	10.34	11.54	10.96
9	V_{max}	18	20	20	20	18
	Error	10.07	10.89	11.57	11.23	11.39
10	V_{max}	30	26	24	22	20
	Error	7.83	9.55	11.58	11.07	10.55
11	V_{max}	28	28	28	28	30
	Error	10.03	10.75	10.75	9.20	9.54
12	V_{max}	22	18	16	14	16
	Error	12.77	11.20	12.12	10.33	13.70

[a]with pressure of 1 psi

4.2. Determination of the pK_a of Analyte Using Internal Standard of Similar pK_a at Different Temperatures

The method used in the measurement of pK_a of some compounds that are either weak acids or weak bases was adopted from Fuguet et al [12]. The applicability of this method was tested by extending its use to the determination of pK_a at temperatures other than 25°C using internal standard with pK_a value close to the analyte. The selection was made by comparing their pK_a values at 25°C since databases are mostly available at this temperature.

The sample that contains the internal standard, analyte, and EOF marker were injected at different temperatures from 20°C to 50°C at 5°C interval with a voltage of 18 kV using the appropriate BGE. Their migration times in every temperature were recorded afterwards their mobilities were calculated from these using equation 10. For the calculation of the pK_a of the analyte (equations 12 or 13) the pK_a of the internal standard always correspond to the temperature at which it was employed. The calculated pK_a of the analyte was compared with its literature pK_a value available at each temperature. For compounds (either the internal standard or the analyte) which pK_a values at all temperatures are not available, the unavailable pK_a from the database were either extra/interpolated from the available data or were simply evaluated by comparing with the literature values at 25°C. Tables 4-7 show the calculated pK_a of the analytes at different temperatures, the respective literature pK_a values of the analytes, the literature pK_a values of the internal standard at different temperatures, and the difference between the literature pK_a values and the calculated ones.

The experiments were grouped into (IS-AN): base-base, acid-acid, base-acid and acid-base. Some compounds were used as internal standard or analyte more than once. It is expected that since the internal standard and the analytes have comparable pK_a values, there will not be large difference between the literature and calculated pK_a values of the analyte used in all the temperatures applied during the determination.

4.2.1. Basic Internal Standard and Basic Analyte

In this group the limiting mobility was measured at a pH of BGE 2 units below the pK_a of either the internal standard or the analyte while the effective mobility was determined at not higher than 0.5 pH units of the pK_a. The results are in Table 4. Aniline was used as the internal standard to determine the pK_a values of five analytes: 2-methoxyaniline, 2-methylaniline, 4-methylaniline, pyridine and acridine. It is highly noticeable that for the first four analytes the difference between the literature and the experimental pK_a is within 0-0.07 units. Aniline was analyzed previously in the same laboratory by the same method used in this experiment at 25°C and the calculated pK_a value is 4.62. The literature value of pK_a of aniline used as a reference for this experiment is 4.60 [28] which is not significantly different from the previous one.

Similar to aniline, pyridine was previously analyzed at 25°C giving a pK_a value of 5.29, while in this experiment 5.25 was obtained which are not so different from each other. A noticeable difference between the experimental and the literature pK_a values in all the temperatures were observed for acridine. At 25°C its literature pK_a is 5.6. It was compared with the pK_a of acridine obtained by the same method in the same laboratory also at 25°C which is 5.56. In this current study its calculated pK_a at 25°C is 5.54. Even more in this current research countercheck using classical method was done which gave a pK_a of 5.65. The graph for this is shown in Figure 5 in which the fittest curve was determined by Table Curve. In either case no systematic error was incurred, the literature pK_a values of acridine at all temperatures are all lower than the calculated pK_a of acridine. By taking into consideration all the pK_a values of acridine obtained from all the mentioned references at 25°C, the deviations from one another are small which implies that possibly the literature values in other temperatures are wrong. In the same way when pyridine was used as the internal standard for acridine, the same significant differences between the literature pK_a values and the experimental ones were observed. This supports the previous assumption that the literature pK_a values of acridine used in the comparison are wrong. In all the samples studied at temperatures other that 25°C all the differences between the literature and the calculated pK_a are acceptable except for acridine which means that the similarities in the pK_a is generally a suitable basis for choosing internal standard in the determination of acid-ionization constants at different temperatures.

Table 4. Results of the determination of acidity constants using basic analyte and basic internal standard at different temperatures.

aniline (IS) + 2-methoxyaniline (AN)

Temperature °C	pH 2 $\mu_{BH+, AN}$	pH 2 $\mu_{BH+, IS}$	pH 4.5 $\mu_{eff, AN}$	pH 4.5 $\mu_{eff, IS}$	Q_{AN}	Q_{IS}	$pK_{a,AN\ calc}$	$pK_{a,AN\ lit}$ [29]	$pK_{a,IS\ lit}$ [28]	$pK_{a,AN\ calc}$- $pK_{a,AN\ lit}$
20	139.01	155.21	103.62	124.05	0.34	0.25	4.57	4.61	4.70	-0.04
25	153.63	171.38	107.16	129.59	0.43	0.32	4.47	4.52	4.60	-0.05
30	168.96	187.73	109.65	133.76	0.54	0.40	4.38	4.45	4.51	-0.07
35	183.92	203.95	110.61	135.89	0.66	0.50	4.30	4.35	4.42	-0.05
40	198.83	220.38	110.23	137.55	0.80	0.60	4.21	4.28	4.34	-0.07
45	214.72	238.74	107.59	136.24	1.00	0.75	4.15	4.20	4.27	-0.05
50	230.58	254.48	105.67	133.20	1.18	0.91	4.07	4.13	4.19	-0.06

aniline (IS) + 2-methylaniline (AN)

Temperature °C	pH 2 $\mu_{BH+, AN}$	pH 2 $\mu_{BH+, IS}$	pH 4.5 $\mu_{eff, AN}$	pH 4.5 $\mu_{eff, IS}$	Q_{AN}	Q_{IS}	$pK_{a,AN\ calc}$	$pK_{a,AN\ lit}$ [30]	$pK_{a,IS\ lit}$ [28]	$pK_{a,AN\ calc}$- $pK_{a,AN\ lit}$
20	140.19	155.22	99.13	123.65	0.41	0.26	4.49	4.52	4.70	-0.03
25	155.00	171.78	102.67	130.21	0.51	0.32	4.40	4.44	4.60	-0.04
30	170.58	188.12	104.88	134.32	0.63	0.40	4.32	4.36	4.51	-0.04
35	186.03	205.39	104.70	136.54	0.78	0.50	4.23	4.27	4.42	-0.04
40	200.27	220.79	104.46	137.93	0.92	0.60	4.16	4.18	4.34	-0.02
45	215.69	237.59	101.53	136.22	1.12	0.74	4.09	4.11	4.27	-0.02
50	230.55	254.43	98.82	135.10	1.33	0.88	4.01	4.03	4.19	-0.03

(continuation)

aniline (IS) + 4-methylaniline (AN)

Temperature °C	pH 2 $\mu_{BH+, AN}$	pH 2 $\mu_{BH+, IS}$	pH 5 $\mu_{eff, AN}$	pH 5 $\mu_{eff, IS}$	Q_{AN}	Q_{IS}	$pK_{a,AN\ calc}$	$pK_{a,AN\ lit}$ [30]	$pK_{a,IS\ lit}$ [28]	$pK_{a,AN\ calc}$- $pK_{a,AN\ lit}$
20	140.80	155.39	107.01	77.44	0.32	1.01	5.20	5.16	4.70	0.04
25	155.35	171.57	112.46	77.68	0.38	1.21	5.10	5.07	4.60	0.03
30	171.71	188.70	115.66	76.49	0.48	1.47	4.99	4.99	4.51	0.00
35	186.03	204.52	117.36	74.63	0.59	1.74	4.89	4.90	4.42	-0.01
40	202.42	221.10	118.09	71.09	0.71	2.11	4.81	4.83	4.34	-0.02
45	217.51	237.89	116.15	67.81	0.87	2.51	4.73	4.75	4.27	-0.02
50	233.50	255.19	115.35	65.61	1.02	2.89	4.64	4.68	4.19	-0.04

aniline (IS) + pyridine (AN)

Temperature °C	pH 2 $\mu_{BH+, AN}$	pH 2 $\mu_{BH+, IS}$	pH 5 $\mu_{eff, AN}$	pH 5 $\mu_{eff, IS}$	Q_{AN}	Q_{IS}	$pK_{a,AN\ calc}$	$pK_{a,AN\ lit}$ [29]	$pK_{a,IS\ lit}$ [28]	$pK_{a,AN\ calc}$- $pK_{a,AN\ lit}$
20	207.27	155.18	167.74	77.87	0.24	0.99	5.32	5.28	4.70	0.04
25	226.99	171.59	178.05	77.46	0.27	1.22	5.25	5.23	4.60	0.02
30	247.98	188.53	188.04	76.11	0.32	1.48	5.18	5.19	4.51	-0.01
35	268.81	205.09	196.47	73.89	0.37	1.78	5.10	5.12	4.42	-0.02
40	289.19	220.96	203.27	71.50	0.42	2.09	5.03	5.07	4.34	-0.04
45	308.16	237.47	209.63	68.43	0.47	2.47	4.99	5.00	4.27	-0.01
50	331.45	253.41	216.89	64.97	0.53	2.90	4.93	4.94	4.19	-0.01

(continuation)

aniline (IS) + acridine (AN)

Temperature °C	pH 2 $\mu_{BH+, AN}$	pH 2 $\mu_{BH+, IS}$	pH 5 $\mu_{eff, AN}$	pH 5 $\mu_{eff, IS}$	Q_{AN}	Q_{IS}	$pK_{a,AN\ calc}$	$pK_{a,AN\ lit}$ [30]	$pK_{a,IS\ lit}$ [28]	$pK_{a,AN\ calc}$- $pK_{a,AN\ lit}$
20	123.86	155.16	112.02	78.34	0.11	0.98	5.67	5.35	4.70	0.32
25	137.92	170.71	121.35	77.90	0.14	1.19	5.54	5.24	4.60	0.30
30	152.64	187.97	129.89	76.38	0.18	1.46	5.43	5.14	4.51	0.29
35	167.56	204.52	138.46	74.47	0.21	1.75	5.34	5.04	4.42	0.30
40	181.56	220.43	145.98	71.93	0.24	2.06	5.27	4.95	4.34	0.32
45	195.99	237.16	151.52	68.49	0.29	2.46	5.19	4.86	4.27	0.34
50	211.47	253.76	160.25	66.47	0.32	2.82	5.13	4.77	4.19	0.37

2,4-Dimethylpyridine (IS) + 2-methylaniline (AN)

Temperature °C	pH 2 $\mu_{BH+, AN}$	pH 2 $\mu_{BH+, IS}$	pH 6 $\mu_{eff, AN}$	pH 6 $\mu_{eff, IS}$	Q_{AN}	Q_{IS}	$pK_{a,AN\ calc}$	$pK_{a,AN\ lit}$ [30]	$pK_{a,IS\ lit}$ [29]	$pK_{a,AN\ calc}$- $pK_{a,AN\ lit}$
20	183.18	154.62	94.89	130.55	0.93	0.18	6.15	6.03	6.85	0.12
25	200.98	170.79	105.49	143.68	0.91	0.19	6.06	5.96	6.74	0.10
30	220.03	188.04	116.57	157.91	0.89	0.19	5.97	5.88	6.64	0.09
35	238.73	205.03	127.58	171.41	0.87	0.20	5.90	5.81	6.55	0.09
40	258.23	221.42	137.87	184.94	0.87	0.20	5.80	5.74	6.45	0.06
45	276.47	239.68	149.33	200.87	0.85	0.19	5.72	5.68	6.37	0.05
50	273.56	256.40	161.84	215.03	0.69	0.19	5.74	5.61	6.29	0.12

(continuation)

pyridine (IS) + acridine (AN)

Temperature °C	pH 2 $\mu_{BH+, AN}$	pH 2 $\mu_{BH+, IS}$	pH 5 $\mu_{eff, AN}$	pH 5 $\mu_{eff, IS}$	Q_{AN}	Q_{IS}	$pK_{a,AN\ calc}$	$pK_{a,AN\ lit}$ [30]	$pK_{a,IS\ lit}$ [29]	$pK_{a,AN\ calc}$- $pK_{a,AN\ lit}$
20	124.43	206.69	107.78	161.61	0.15	0.28	5.53	5.35	5.28	0.19
25	138.36	226.93	116.61	173.04	0.19	0.31	5.45	5.24	5.23	0.21
30	152.21	246.61	125.49	181.19	0.21	0.36	5.41	5.14	5.19	0.27
35	166.31	267.16	132.11	189.18	0.26	0.41	5.32	5.04	5.12	0.27
40	181.37	287.43	139.29	197.48	0.30	0.46	5.24	4.95	5.07	0.30
45	196.44	308.26	143.05	200.79	0.37	0.54	5.15	4.86	5.00	0.30
50	211.21	329.66	147.34	202.14	0.43	0.63	5.10	4.77	4.94	0.33

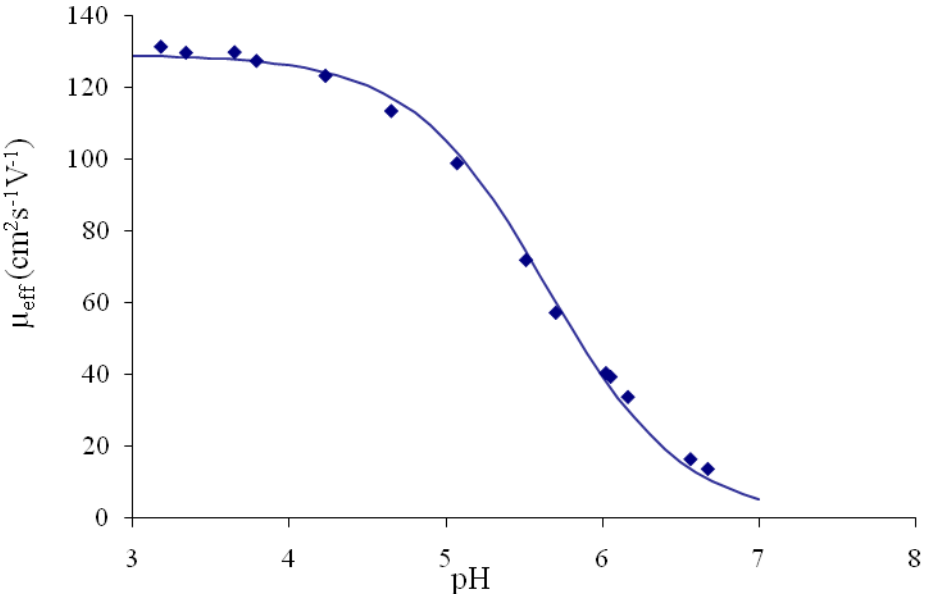

Figure 5. Effective mobilities of acridine at 25°C obtained through the classical method.

4.2.2. Acidic Internal Standard and Acidic Analyte

For this group of samples, the same method as the above was employed the only difference is that for the measurement of the limiting mobilities the BGE with 2 pH units above the pK_a of the compounds under study was used. The results are in Table 5. When the discrepancy between the literature and the experimental pK_a values was taken, in general, the results are up to 0.21. However there are some exceptions to this trend. When benzoic acid was used as the internal standard for some analytes, except with 3-bromobenzoic acid, all the errors obtained were always higher than the literature values. This indicates the probable presence of systematic error which may be due to the performance of the experiments to obtain the literature pK_a values. There are some analytes which literature pK_a values are available only at 25°C. In this case only the calculated pK_a values at 25°C were compared with the literature pK_a values which gave small differences. In addition, the calculated pK_a of these analytes at different temperatures did not vary much from their literature pK_a at 25°C.

Some phenolics were also used as internal standards and analytes. There are some errors with regards to the calculated values which, like the previous samples, can also be associated with the current study or with the literature experiments. The most observable deviations upon comparing the literature from the calculated pK_a values was observed from the sample containing phenol and 4-tert-butylyphenol, which is within 0.33-0.46 that are always lower than the literature. In this experiment the ionization constant obtained at 25°C is 9.98. This was counterchecked by the classical method (see Figure 6) which data was processed by Table Curve software, where a value of 10.31 which is the same as the one obtained by Rived, F. [31] was acquired. Phenols are generally unstable. It can be noted that in this experiment high pH of BGE were used (10 and 12) that might caused the degradation of both the internal standard and the analyte. These could be the reason for the errors incurred in this experimental study. Generally, in all the temperatures the differences between the literature and the calculated pK_a values were not that large which implies that this method with the internal standard having the same ionization constant as the analyte is reliable in spite of the high temperature conditions employed in the analysis.

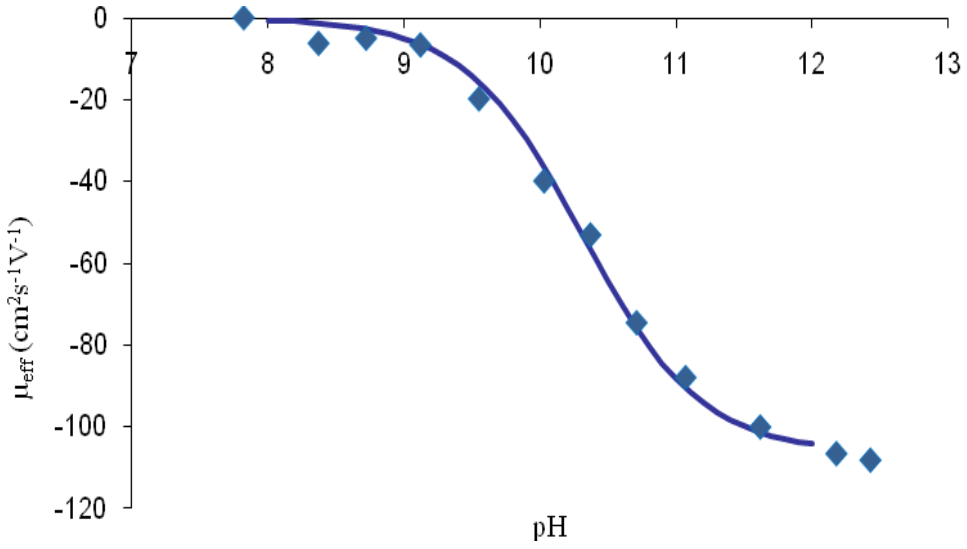

Figure 6. Effective mobilities of 4-tert-butylphenol at 25°C obtained through the classical method.

Table 5. Results of the determination of acidity constants using acidic analyte and acidic internal standard at different temperatures.

benzoic acid (IS) + 2,4-dimethylbenzoic (AN)

Temperature °C	pH 8		pH 4.5					$pK_{a,AN\ lit}$	$pK_{a,IS\ lit}$	
	$\mu_{A-,\ AN}$	$\mu_{A-,\ IS}$	$\mu_{eff,\ AN}$	$\mu_{eff,\ IS}$	Q_{AN}	Q_{IS}	$pK_{a,AN\ calc}$	[28]	[28]	$pK_{a,AN\ calc}$- $pK_{a,AN\ lit}$
20	-115.44	-139.04	-79.07	-91.92	0.46	0.51	4.16	3.95	4.21	0.21
25	-128.25	-154.17	-86.92	-101.91	0.48	0.51	4.17	3.99	4.20	0.18
30	-142.64	-170.74	-91.06	-108.18	0.57	0.58	4.19	4.02	4.20	0.17
35	-156.18	-186.65	-100.95	-120.54	0.55	0.55	4.22	4.05	4.22	0.17
40	-170.11	-202.82	-108.76	-130.73	0.56	0.55	4.23	4.07	4.22	0.16
45	-185.16	-219.98	-116.40	-140.67	0.59	0.56	4.24	4.11	4.22	0.13
50	-199.64	-237.01	-122.21	-148.90	0.63	0.59	4.25	4.14	4.22	0.11

4-nitrophenol (IS) + 3-nitrophenol (AN)

Temperature °C	pH 11		pH 7.5					$pK_{a,AN\ lit}$	$pK_{a,IS\ lit}$	
	$\mu_{A-,\ AN}$	$\mu_{A-,\ IS}$	$\mu_{eff,\ AN}$	$\mu_{eff,\ IS}$	Q_{AN}	Q_{IS}	$pK_{a,AN\ calc}$	[31]	[31]	$pK_{a,AN\ calc}$- $pK_{a,AN\ lit}$
20	-133.93	-139.17	-20.04	-107.28	5.68	0.30	8.50	8.42	7.22	0.08
25	-149.34	-155.71	-21.75	-118.06	5.87	0.32	8.44	8.36	7.18	0.08
30	-164.72	-172.02	-24.87	-131.40	5.62	0.31	8.36	8.28	7.10	0.08
35	-179.91	-188.76	-27.25	-143.51	5.60	0.32	8.30	8.23	7.05	0.07
40	-194.57	-204.41	-29.66	-155.92	5.56	0.31	8.25	8.17	7.00	0.08
45	-210.16	-219.83	-32.16	-168.34	5.54	0.31	8.22	8.11	6.96	0.11
50	-225.57	-238.17	-36.83	-182.45	5.13	0.31	8.13	8.08	6.91	0.05

(continuation)

phenol (IS) + 4-tertbutylphenol (AN)

Temperature °C	pH 12		pH 10					$pK_{a,AN\ lit}$ [31]	$pK_{a,IS\ lit}$ [31]	
	$\mu_{A-,\ AN}$	$\mu_{A-,\ IS}$	$\mu_{eff,\ AN}$	$\mu_{eff,\ IS}$	Q_{AN}	Q_{IS}	$pK_{a,AN\ calc}$			$pK_{a,AN\ calc}$- $pK_{a,AN\ lit}$
20	-42.38	-72.56	-22.87	-40.62	0.85	0.79	10.13	10.46	10.09	-0.33
25	-49.05	-83.50	-25.24	-42.61	0.94	0.96	9.98	10.31	9.99	-0.33
30	-57.09	-97.41	-25.52	-44.07	1.24	1.21	9.96	10.33	9.95	-0.37
35	-65.99	-109.80	-26.91	-46.07	1.45	1.38	9.87	10.27	9.85	-0.40
40	-72.50	-123.23	-27.50	-45.92	1.64	1.68	9.77	10.22	9.78	-0.45
45	-84.80	-140.40	-29.07	-47.70	1.92	1.94	9.71	10.17	9.72	-0.46
50	-93.94	-154.60	-27.14	-46.95	2.46	2.29	9.69	10.12	9.66	-0.43

4.2.3. Acidic/Basic Internal Standard and Basic/Acidic Analyte

For the study of the basic internal standard and acidic analyte (or acidic internal standard and basic analyte), three pH buffers were used in the electrophoretic run. One buffer was used to cause the partial ionization of both compounds thus facilitating the measurement of their effective mobilities and the remaining two buffers were used to cause their full ionization to facilitate the measurement of their limiting mobilities. The results for this experiment are illustrated in Tables 6 and 7, respectively.

Aniline was used as the internal standard to determine the ionization constants of benzoic acid and 2,4-dimethylbenzoic acid. Both of the samples did not differ so much from their literature values in all the temperatures used in the study. The errors are in the units of 0.01-0.13. The pK_a of benzoic acid was previously determined in the same laboratory by the same method and a value of 4.21 was obtained at 25°C. This differs by 0.10 from the pK_a value calculated in this experiment at the same temperature which is 4.11. This can be seen from Table 6. Phenol was used as internal standard for (+)-ephedrine while 4-nitrophenol was used as internal standard for 2,4-dimethylpyridine. The difference obtained here is relatively larger than the latter which is within the range of 0.23-0.43. In all the cases, the literature acidity constants are always greater than the experimental value and are almost of the same magnitude within the series of temperature studied. This implies that this could be attributed to the wrong literature pK_a values used in the calculations. All the literature values were corrected for ionic strength using the Debye-Hückel equation.

The small differences between the literature and the calculated pK_a of the analytes at different temperature of measurements mean that the choice of internal standard for this method would undeniably produce reliable results.

Table 6. Results of the determination acidity constants using acidic analyte and basic internal standard at different temperatures.

phenol (IS) + (+)-ephedrine (AN)

Temperature	pH 12	pH 9.5		pH 7						
°C	$\mu_{A-,\ IS}$	$\mu_{eff,\ AN}$	$\mu_{eff,\ IS}$	$\mu_{BH+,\ AN}$	Q_{AN}	Q_{IS}	$pK_{a,IS\ lit}$ [31]	$pK_{a,AN\ calc}$	$pK_{a,AN\ lit}$ [30]	$pK_{a,AN\ calc}$- $pK_{a,AN\ lit}$
20	-132.91	72.30	-22.84	93.87	0.30	4.82	10.10	9.84	10.09	0.26
25	-144.57	80.28	-22.97	106.39	0.33	5.29	9.92	9.70	9.99	0.23
30	-157.04	87.50	-24.90	119.84	0.37	5.31	9.83	9.57	9.95	0.26
35	-169.04	93.69	-25.81	132.47	0.41	5.55	9.66	9.43	9.85	0.23
40	-179.56	101.65	-25.52	144.68	0.42	6.04	9.54	9.31	9.78	0.23
45	-187.60	108.73	-27.25	155.75	0.43	5.88	9.48	9.19	9.72	0.30
50	-196.54	115.28	-26.70	169.00	0.47	6.36	9.36	9.07	9.66	0.29

4-nitrophenol (IS) + 2,4-dimethylpyridine (AN)

Temperature	pH 11	pH 7		pH 2						
°C	$\mu_{A-,\ IS}$	$\mu_{eff,\ AN}$	$\mu_{eff,\ IS}$	$\mu_{BH+,\ AN}$	Q_{AN}	Q_{IS}	$pK_{a,AN\ calc}$	$pK_{a,AN\ lit}$ [29]	$pK_{a,IS\ lit}$ [31]	$pK_{a,AN\ calc}$- $pK_{a,AN\ lit}$
20	-134.57	61.63	-67.88	155.21	1.52	0.98	7.22	6.87	7.22	0.35
25	-149.29	61.64	-78.46	171.66	1.78	0.90	7.14	6.76	7.18	0.39
30	-165.00	66.49	-86.32	188.70	1.84	0.91	7.05	6.66	7.10	0.39
35	-179.87	72.11	-89.75	206.56	1.86	1.00	6.95	6.57	7.05	0.38
40	-195.52	73.66	-101.33	223.86	2.04	0.93	6.89	6.47	7.00	0.43
45	-210.23	82.26	-99.80	241.29	1.93	1.11	6.80	6.38	6.96	0.42
50	-228.07	89.75	-98.78	259.42	1.89	1.31	6.69	6.31	6.91	0.38

Table 7. Results of the determination acidity constants using basic analyte and acidic internal standard at different temperatures.

aniline (IS) + benzoic acid (AN)

Temperature °C	pH 2 $\mu_{BH+, IS}$	pH 4.5 $\mu_{eff, AN}$	pH 4.5 $\mu_{eff, IS}$	pH 8 $\mu_{A-, AN}$	Q_{AN}	Q_{IS}	$pK_{a,AN\ calc}$	$pK_{a,AN\ lit}$ [28]	$pK_{a,IS\ lit}$ [28]	$pK_{a,AN\ calc}$- $pK_{a,AN\ lit}$
20	155.08	-91.83	117.50	-139.71	0.52	0.32	4.09	4.21	4.70	-0.12
25	173.56	-102.42	122.32	-155.28	0.52	0.42	4.11	4.20	4.60	-0.09
30	187.63	-111.59	125.13	-171.96	0.54	0.50	4.11	4.20	4.51	-0.09
35	207.72	-122.24	127.78	-187.06	0.53	0.63	4.11	4.22	4.42	-0.11
40	224.26	-134.04	127.75	-203.34	0.52	0.76	4.10	4.22	4.34	-0.12
45	241.78	-145.28	125.73	-220.60	0.52	0.92	4.12	4.22	4.27	-0.10
50	253.07	-156.41	123.80	-237.78	0.52	1.04	4.09	4.22	4.19	-0.13

aniline (IS) + 2,4-dimethylbenzoic acid (AN)

Temperature °C	pH 2 $\mu_{BH+, IS}$	pH 4.5 $\mu_{eff, AN}$	pH 4.5 $\mu_{eff, IS}$	μ_{A-} pH 8 $\mu_{A-, AN}$	Q_{AN}	Q_{IS}	$pK_{a,AN\ calc}$	$pK_{a,AN}$ lit [28]	$pK_{a,IS\ lit}$ [28]	$pK_{a,AN\ calc}$- $pK_{a,AN\ lit}$
20	155.28	-79.18	120.71	-119.44	0.51	0.29	4.03	3.95	4.70	0.08
25	171.69	-87.68	126.38	-132.54	0.51	0.36	4.03	3.99	4.60	0.04
30	188.50	-95.96	130.45	-146.61	0.53	0.45	4.05	4.02	4.51	0.03
35	208.39	-104.14	133.29	-160.78	0.54	0.56	4.08	4.05	4.42	0.03
40	222.23	-112.39	134.26	-175.42	0.56	0.66	4.08	4.07	4.34	0.01
45	238.15	-120.01	134.39	-188.95	0.57	0.77	4.09	4.11	4.27	-0.02
50	255.23	-127.06	131.65	-205.72	0.62	0.94	4.12	4.14	4.19	-0.01

4.3. Determination of Acidity Constant of the Analyte at 25°C Using Internal Standard of Similar and Different Standard Enthalpy Change

The similarities in the ΔH at 25°C, in addition to the similarities in the literature pK_a of some compounds, were used as the bases for selecting the appropriate internal standard for the determination of their acid ionization constants in varying temperatures in this research study. The analytes and the internal standard having close or similar ΔH values will show comparable van't Hoff plots which means that their equilibrium constants response to change in temperature in the same manner.

When performing pK_a measurements at a specific temperature in CZE sometimes there are fluctuations in temperature (decrease or increase) during the experiment. In this study, the determination of pK_a values of the analytes was done in different temperatures, and from these data the pK_a values at 25°C were calculated. Even though the mobilities were taken at different temperatures the literature pK_a of the internal standard used in the calculation was held constant at 25°C to verify if the analyte remains constant accordingly. Since they have the same ΔH no matter what the temperature at which data for the analyte are obtained, the pK_a will not change if the temperature of the internal standard is not also changed. The difference between the literature value at 25°C and the experimental values at all temperatures was computed. If these temperature ranges have no effect in the determination of the ionization constant of an analyte, it could be expected that the discrepancies from these two in all the temperatures studied are not significant. For this reason temperature control during pK_a determination is not critical within a temperature range. In addition the comparability of the ΔH of the compounds could be a basis for choosing internal standard for the investigation of the effect of variations in the temperature during the measurement of pK_a at a specific temperature of a compound.

The calculated pK_a values of analytes at different temperatures and their difference from the literature value at 25°C are shown in Table 8. Most of the literature values are obtained in the laboratory by the same method employed in this experiment at 25°C.

Table 8. Determination of the pK_a values of the analytes at 25°C using the data obtained from different temperatures.

Benzoic acid (IS) ($pK_{a25°C}$=4.21 ΔH=0.09-0.15[28]) **and 3-bromobenzoic acid (AN) ($pK_{a25°C}$=3.79 ΔH=0.168**[28])

Temperature, °C	$pK_{a,AN\ calc}$	difference at 25°C
20	3.80	0.01
25	3.78	-0.01
30	3.78	-0.01
35	3.78	-0.01
40	3.78	-0.01
45	3.78	-0.01
50	3.77	-0.02

Benzoic acid (IS) ($pK_{a25°C}$=4.21 ΔH=0.09-0.15 [28]) **and 4-aminobenzoic acid (AN) ($pK_{a25°C}$=4.87** [32] **ΔH=0.7**[32])

Temperature, °C	$pK_{a,AN\ calc}$	difference at 25°C
20	4.93	0.06
25	4.92	0.05
30	4.90	0.03
35	4.89	0.02
40	4.89	0.02
45	4.88	0.01
50	4.88	0.01

4-nitrophenol (IS) ($pK_{a25°C}$=7.10 ΔH=4.53-4.70[28]) **and 3,5-dichlorophenol ($pK_{a25°C}$=8.19 ΔH=4.88**[28])

Temperature, °C	$pK_{a,AN\ calc}$	difference at 25°C
20	8.12	-0.07
25	8.11	-0.08
30	8.09	-0.10
35	8.10	-0.09
40	8.11	-0.08
45	8.09	-0.10
50	8.08	-0.11

Phenol (IS)($pK_{a25°C}$=9.89 ΔH=4.8-5.8[28]) **and 4-bromophenol (AN) ($pK_{a25°C}$=9.28 ΔH=5.74**[28])

Temperature, °C	$pK_{a,AN\ calc}$	difference at 25°C
20	9.45	0.17
25	9.47	0.19
30	9.50	0.22
35	9.51	0.23
40	9.53	0.25
45	9.54	0.26
50	9.60	0.32

(continuation)

Phenol (IS) ($pK_{a25°C}$=9.89 ΔH=4.8-5.8[28]**) and**
4-chlorophenol (AN) ($pK_{a25°C}$=9.43[31] **ΔH=5.6**[32]**)**

Temperature, °C	$pK_{a,AN\ calc}$	difference at 25°C
20	9.53	0.10
25	9.54	0.11
30	9.56	0.13
35	9.59	0.16
40	9.60	0.17
45	9.61	0.18
50	9.66	0.23

4-nitrophenol (IS) ($pK_{a25°C}$=7.10 ΔH=4.53-4.70[28]**) and**
3-nitrophenol (AN) ($pK_{a25°C}$=8.36[31] **ΔH=4.705**[28]**)**

Temperature, °C	$pK_{a,AN\ calc}$	difference at 25°C
20	8.38	0.02
25	8.36	0.00
30	8.36	0.00
35	8.35	-0.01
40	8.35	-0.01
45	8.36	0.00
50	8.32	-0.04

Phenol (IS) ($pK_{a25°C}$=9.89 ΔH=4.8-5.8[28] **)and**
tryptophan (AN)(($pK_{a25°C}$=9.51 [32]**ΔH=10.7**[32]**)**

Temperature, °C	$pK_{a,AN\ calc}$	difference at 25°C
20	9.50	-0.01
25	9.44	-0.07
30	9.39	-0.12
35	9.34	-0.17
40	9.23	-0.28
45	9.27	-0.24
50	9.22	-0.29

2,4-dinitrophenol (IS) ($pK_{a25°C}$=4.12 ΔH=4.11[32]**) and**
3-bromobenzoic acid (AN) ($pK_{a25°C}$=3.79 ΔH=0.168[28]**)**

Temperature, °C	$pK_{a,AN\ calc}$	difference at 25°C
20	3.80	0.01
25	3.82	0.03
30	3.84	0.05
35	3.87	0.08
40	3.88	0.09
45	3.88	0.09
50	3.93	0.14

(continuation)

benzoic acid (IS) ($pK_{a25°C}$=4.21 ΔH=0.09-0.15[28]**) and 2,4-dinitrophenol (AN) ($pK_{a25°C}$=4.12 ΔH=4.11**[32]**)**

Temperature, °C	$pK_{a,AN\ calc}$	difference at 25°C
20	4.23	0.11
25	4.15	0.03
30	4.12	0.00
35	4.10	-0.02
40	4.07	-0.05
45	4.05	-0.07
50	4.02	-0.10

Phenol (IS) (($pK_{a25°C}$=9.89 ΔH=4.8-5.8[28]**) and (+)ephedrine (AN) ($pK_{a25°C}$=9.71 ΔH=11.037**[28]**)**

Temperature, °C	$pK_{a,AN\ calc}$	difference at 25°C
20	9.73	0.02
25	9.65	-0.06
30	9.60	-0.11
35	9.53	-0.18
40	9.48	-0.23
45	9.48	-0.23
50	9.42	-0.29

4-nitrophenol (IS) ($pK_{a25°C}$=7.10 ΔH=4.11[28] **and 2,4-dimethylpyridine (AN) ($pK_{a25°C}$=6.82 ΔH=8.14**[28]**)**

Temperature, °C	$pK_{a,AN\ calc}$	difference at 25°C
20	6.93	0.12
25	6.89	0.08
30	6.88	0.07
35	6.83	0.02
40	6.82	0.01
45	6.77	-0.04
50	6.71	-0.10

*Most pK_a values were obtained in the same laboratory using the same method

It can be observed that generally when the ΔH of both the internal standard and the analyte are similar, the difference in the literature pK_a at 25°C and experimental pK_a at different temperatures is lower than 0.10. Significant differences can be observed for samples containing phenolic groups. The large error for this can be accounted for the instability of phenolic compounds with time resulting to their degradation. On the other hand when the ΔH of the internal standard and the analytes have widely different values the calculated pK_a show relatively large difference from the reference value at 25°C. Examples are phenols and tryptophan, benzoic acid and 2,4-dinitrophenol, phenol and (+)-ephedrine, 4-nitrophenol and 2,4-dimethylpyridine and 2,4-dinitrophenol and 3-bromobenzoic acid. Specifically, when the ΔH of the internal standard is lower than

the ΔH of the analyte, the calculated difference between the literature and the experimental pK_a values is increasing with negative value as the temperature increases. On the contrary when the ΔH of the internal standard is higher than the ΔH of the analyte the calculated difference is increasing with positive value relative to the temperature increase.

To sum up, when the effect of temperature variations during the electrophoretic run has to be determined in the measurement of ionization constants, the use of internal standard possessing a ΔH similar with the ΔH of the analyte is recommended to avoid large errors brought about by temperature changes.