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Capillary electrophoresis screening of poisonous anions extracted from biological samples

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Abstract

A method was developed for screening human biological samples for poisonous anions using capillary electrophoresis (CE) employing indirect UV detection. The run buffer consisted of 2.25 mM pyromellitic acid, 1.6 mM triethanolamine, 0.75 mM hexamethonium hydroxide and 6.5 mM NaOH at pH 7.7. Biological samples were pretreated using solid phase extraction. The method was applied to the analysis of human blood, plasma, urine, and intestinal contents. Twenty-nine different anions were detectable at aqueous concentrations of 1 part per million (ppm) with a typical analysis time less than 20 min. Intraday migration time R.S.D. and peak area R.S.D. for blood samples were less than 1.1% and 6.3%, respectively. Interday migration time R.S.D. for plasma samples ranged from 7.5% to 10.4%. The new method produced efficient separations of various target anions extracted from complex biological matrices.

Keywords: Capillary electrophoresis; Anions; Screening; Poison; UV detection; Urine; Blood; Plasma; Intestinal contents

1. Introduction

More than 2.3 million poisonings were reported in the U.S. in the year 2002. Over one half percent (14,137) of these were classified as malicious or contaminant/tampering [1]. The United States 49th Code of Federal Regulations (CFR) 173.132 defines a poisonous solid as a solid with an LD_{50} for acute oral toxicity of not more than 200 mg/kg [2]. Familiar poisons, such as sodium fluoroacetate, sodium cyanide, and sodium azide qualify as poisonous solids under this standard (Table 1). However, with LD_{50} values greater than 200 mg/kg both sodium thiocyanate (764 mg/kg) and sodium bromide (3500 mg/kg) do not qualify as poisons under the definition of the code (Table 1). The dose of the poison is an important consideration, which may dictate the feasibility of its use. For an average 70 kg adult human, the estimated lethal doses of sodium fluoroacetate, sodium cyanide, and sodium azide are all below 2 g (Table 1). For sodium thiocyanate and sodium bromide the lethal doses are so large, 53 g and 245 g, respectively, that the use of these two compounds for human poisoning becomes less practical (Table 1). However, children can be the victims of both intentional and accidental poisonings. For a child, the lethal dose would be much smaller than an adult, so compounds with lower toxicity are still of forensic interest.

The common occurrence of poisonings underscores the importance of developing a general screening method for anions extracted from human biological samples. A reliable screening method would facilitate the detection of small inorganic and organic anions extracted from complex matrices by minimizing the number of individual tests needed. Several methods for the analysis of anions in biological fluids are described in the literature [3–11] with reviews covering the sample preparation of biological samples for analysis by CE and analysis of various analytes in biological samples [12–14]. A recent review by Galli, et al., described the analysis of anions in multiple sample types, including biological samples, by CE [15]. However, these methods usually detect no more than 15 analytes in a single assay. A more useful screening method would discriminate a larger number of analytes in a single assay. One approach to anion analysis has been the use of ion chromatography (IC) [16–20]. IC has been used to achieve the simultaneous analysis of inorganic anions and organic acids [16]. In recent years, capillary

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Table 1
Material safety data sheet LD_{50} values used; estimated lethal doses calculated using LD_{50} values with a 70 kg bodyweight

Chemical	Oral toxicity		
	LD_{50} (mg/kg)	Lethal dose (g)	
Sodium fluoroacetate	0.1	0.007	CFR poison
Sodium cyanide	6.4	0.45	CFR poison
Sodium azide	27	1.9	CFR poison
Sodium nitrite	85	6	CFR poison
Sodium thiocyanate	764	53	
Sodium bromide	3500	245	

electrophoresis (CE) has become as reliable as IC for anion separation. The high resolution, straightforward sample preparation, short run times and small sample volumes have made CE a desirable alternative for anion analysis [4,6–11,21–30].

For this study, an established CE buffer system was applied to the analysis of extracts from human blood, plasma, urine and intestinal contents. The buffer composition was developed originally by Harrold et al. and consists of a pyromellitic acid/hexamethonium electrolyte [31]. Detection of the analytes is accomplished by indirect UV. The pyromellitic acid buffer system was previously used as a test method for the analysis of poisonous anions in beverages and foodstuffs [18]. The focus of the present study was the development of a reliable sample treatment and screening test by CE for anions of forensic interest extracted from biological fluids utilizing a pyromellitic acid/hexamethonium electrolyte. Anions of high and low toxicity were tested in order to evaluate the potential of the method for screening various anions.

2. Experimental

2.1. Reagents and standards

The CE run buffer used for all analyses in this study was 2.25 mM pyromellitic acid (Fluka Chemical Corp., Milwaukee, WI, USA), 1.6 mM triethanolamine (Fisher Scientific Co., Fair Lawn, NJ, USA), 0.75 mM hexamethonium hydroxide (Fluka Chemical Corp.), and 6.5 mM NaOH (Fisher Scientific Co.) at pH 7.7, as described by Harrold et al. [31]. Blood and plasma samples were obtained from Valley Biomedical (Winchester, VA, USA). The sample of intestinal contents was obtained from an adjudicated casework sample provided by the Chemistry Unit, FBI Laboratory Division, Washington, DC. Urine was obtained from an anonymous donor.

The CE run buffer was prepared from individual stock solutions of 1.0 M NaOH, 1.07 M triethanolamine, and 0.1 M hexamethonium hydroxide (as purchased from Fluka). All solutions were prepared using 18 MΩ deionized water. The pH was adjusted to pH 7.7 with 0.1 M NaOH. The run buffer was vacuum-filtered using a 0.45 μm CA (cellulose acetate) filter system (Corning Inc., Corning, NY, USA). Primary stock anion standards were prepared at 1000 ppm anion concentrations each using the potassium salts of chloride, nitrite, nitrate, chlorate, perchlorate, pyrosulfate, sulfate, tetrathionate, thiocyanate (Sigma, St. Louis, MO, USA), azide, bromide, chromate (Fisher Scientific Co.), bromate, iodide (Baker & Adamson, New York, NY, USA), oxalate (Mallinckrodt, St. Louis, MO, USA), and phosphate (Aldrich, Milwaukee, WI, USA). The sodium salts of acetate, carbonate, fluoride, formate, sulfide, sulfite, tartrate, thiosulfate (Fisher Scientific Co.), bicarbonate (Sigma), chlorite (Alfa Products Thiokol/Ventron Division, Danvers, MA, USA), and fluoroacetate (Aldrich), and the acid forms of benzoic acid (Sigma), citric acid (Fisher Scientific Co.), and phthalic acid (Eastman Kodak Co., Rochester, NY, USA) were used to prepare the remaining primary standards. Composite working standards containing 10 ppm each of the desired anions were prepared from the primary anion standards.

2.2. Equipment

The CE instruments used in this study were the Spectra Phoresis models 500 and 1000 (Thermo Separation Products, San Jose, CA, USA). Separations were performed using a CElect FS50 (Supelco, Bellefonte, PA, USA) bare fused silica capillary with a 50 μm inner diameter and an effective length of 57 cm to the detector. A new capillary was conditioned by flushing it with 0.05 M NaOH and with run buffer for 30 min each. Initially, voltage was applied without an injection at −30 kV and 25 °C for 30 min to remove residual manufacturing impurities from the capillary. The capillary was flushed for five minutes with run buffer between each run. Standard separation conditions were −30 kV at 25 °C. Samples were introduced onto the capillary by hydrodynamic injection for 10 s at 1.5 psi. Indirect UV detection was performed simultaneously at 200 and 250 nm, respectively.

2.3. Sample preparation

All biological samples were pretreated prior to analysis to reduce matrix interference. One milliliter of blood or plasma was mixed with 2 mL of acetone (AlliedSignal Burdick & Jackson, Muskegon, MI, USA). The extract was vortexed for 1 min, and centrifuged for 5 min at 10,000 rpm. Twenty milligrams of intestinal contents were mixed with 2 mL of deionized water. The blood or plasma acetone extract, dilution of intestinal contents, or five mL of neat urine were filtered through 0.5 mL Maxi-Clean IC-RP solid phase extraction cartridges (Alltech, Deerfield, IL, USA). The cartridges were conditioned by washing them with 5–10 mL of HPLC grade methanol (Fisher Scientific Co.) followed by 10 mL of deionized water. Each sample was introduced onto a cartridge and eluted at a rate of approximately one drop per second. The first 0.5 mL fraction of the eluate, which was residual from the water wash, was discarded. Approximately 0.5 mL of deionized water was added to the cartridge when the last of the sample had reached the top of the packing material to elute the remaining sample. The filtrates were diluted 10–100-fold with deionized water.

3. Results and discussion

A total of twenty-nine different anions have been detected using the pyromellitic acid CE system. Several anion standards

were analyzed to determine the migration order, or any comigration, for all of the anions. These standards contained anions with similar migration times which allowed the determination of the over all migration order of the anions. The relative migration order of the twenty nine anions is as follows: thiosulfate ($S_2O_3^{2-}$), bromide (Br^-), chromate (CrO_4^{2-}), iodide (I^-), chloride (Cl^-), sulfate (SO_4^{2-}), sulfite (SO_3^{2-}), pyrosulfate ($S_2O_7^{2-}$), sulfide (HS^-), nitrite (NO_2^-), nitrate (NO_3^-), oxalate ($C_2O_4^{2-}$), perchlorate (ClO_4^-), azide (N_3^-), thiocyanate (SCN^-), tetrathionate ($S_4O_6^{2-}$), chlorate (ClO_3^-), citrate ($C_6H_5O_7^{3-}$), tartrate ($C_4H_4O_6^{2-}$), fluoride (F^-), bromate (BrO_3^-), formate (HCO_2^-), monohydrogen phosphate (HPO_4^{2-}), chlorite (ClO_2^-), phthalate ($C_8H_4O_4^{2-}$), bicarbonate (HCO_3^-), fluoroacetate ($C_2H_2FO_2^-$), acetate ($C_2H_3O_2^-$), and benzoate ($C_7H_5O_2^-$). Other than benzoate, the migration times of the anions were less than 20 min. The 29 anions, representing both target analytes and possible interferants, demonstrated a limit of detection of 1 ppm in standard aqueous solutions. A representative separation, from this study, of an aqueous solution containing 16 anions is shown in Fig. 1. In approximately twelve minutes, the 16 anions are resolved with low noise, good peak shape, and a stable baseline. Under standard separation conditions (−30 kV at 25 °C) the following sets of anions demonstrated comigration: (1) chromate, iodide and chloride; (2) sulfate, sulfite, pyrosulfate, and sulfide; (3) perchlorate and azide; (4) tetrathionate and thiocyanate; (5) bromate and fluoride.

The results of individual standards or of standards containing a mixture of bicarbonate and carbonate revealed the presence of only one peak at approximately 13 min. The two pK_as of carbonic acid (H_2CO_3) are 6.35 and 10.33, respectively [32]. At pH 7.7 the relative concentrations of bicarbonate and carbonate are approximately 96% and 4%, respectively. At any time 96% of the ions will be in the form of bicarbonate and 4% will be carbonate; however, the same ions will not always be bicarbonate and carbonate all of the time. This is because the time scale of the protonation–deprotonation equilibrium is much faster than the time scale of the separation. Therefore, the carbonate and bicarbonate ions are not distinguished from one another and only one peak is seen.

Relative migration time, peak shape, and peak position on the baseline were the criteria used for tentatively identifying the anions in biological samples. The identities of the unknowns were tested by standard addition of a specific anion with the sample specimen. The presence of a single larger intensity peak indicated a positive screening test of the unknown anion.

For biological fluids, sample preparation is necessary to remove proteins from the sample matrix for two reasons. First, if the sample is left untreated, the proteins can change the electroosmotic flow (EOF) characteristics in the capillary by adsorbing onto the capillary wall. Changes in the EOF along the capillary can lead to band broadening and decreased peak symmetry, column efficiency and reproducibility [33]. Second, it is important to remove the proteins from the matrix to ensure that any potentially bound anions are separated from them.

The biological samples tested consisted of human intestinal contents, urine, blood, and plasma. All biological samples were mixed with anion standards and pretreated as previously described prior to analysis. The concentrations of oxalate, fluoroacetate, nitrate, azide, thiocyanate, and bromide added to the biological samples were chosen to correspond to toxic or lethal values reported in the literature [7,8,35–37].

Analysis of intestinal contents showed the presence of chloride and sulfate in addition to several unknown components. A sample of the intestinal contents was mixed with oxalate to a concentration of 50 ppm and diluted 10-fold. The electropherogram for this run shows a completely resolved oxalate peak at 6.63 min (Fig. 2).

Analysis of urine showed the presence of chloride, sulfate, oxalate, citrate, monohydrogen phosphate, and bicarbonate. The presence of these ions supports the results obtained using other CE methods in the literature [7,8]. Fluoroacetate was added to a urine sample to a concentration of 65 ppm and then diluted 10-fold for analysis. The fluoroacetate peak appears at 17.45 min without interference (Fig. 3).

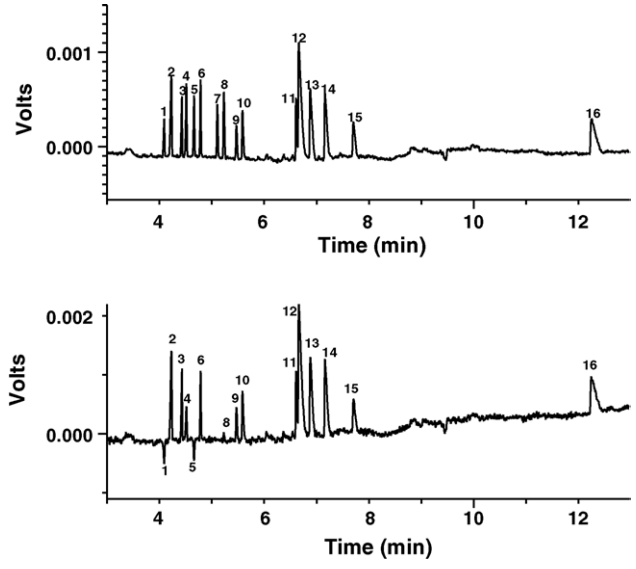

Fig. 1. Sixteen anion standard. Conditions: −30 kV, 25 °C, 10 s injection at 1.5 psi, upper trace 250 nm, lower trace 200 nm. Peaks: (1) bromide; (2) chloride; (3) sulfate; (4) nitrite; (5) nitrate; (6) oxalate; (7) azide; (8) thiocyanate; (9) chlorate; (10) citrate; (11) tartrate; (12) fluoride; (13) formate; (14) monohydrogen phosphate; (15) chlorite; (16) acetate.

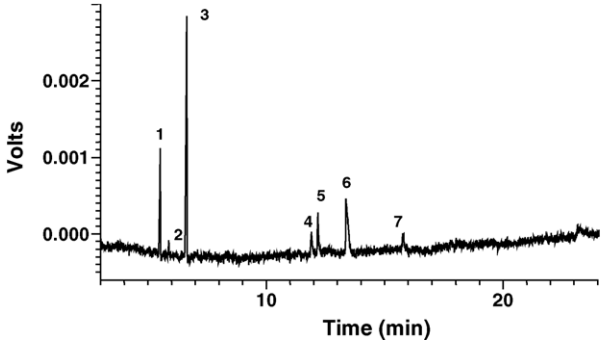

Fig. 2. Intestinal contents with 5 ppm added oxalate. Conditions: −30 kV, 25 °C, 10 s injection at 1.5 psi, 200 nm. Peaks: (1) chloride; (2) sulfate; (3) oxalate; (4–7) unknown.

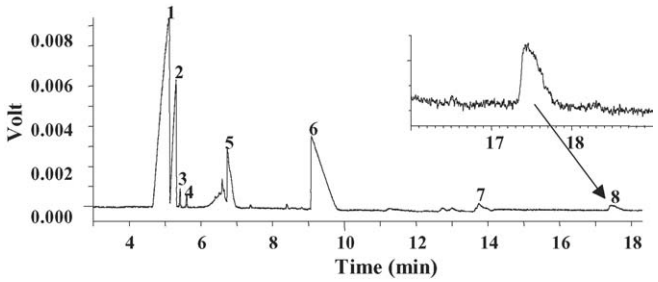

Fig. 3. Urine with 7 ppm added fluoroacetate. Conditions: −30 kV, 25 °C, 10 s injection at 1.5 psi, 250 nm. Peaks: (1) chloride; (2) sulfate; (3) nitrate; (4) oxalate; (5) citrate; (6) monohydrogen phosphate; (7) bicarbonate; (8) fluoroacetate.

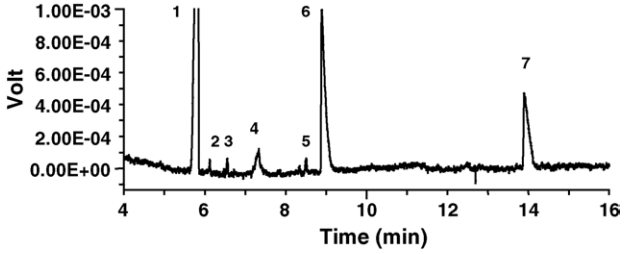

Fig. 4. Blood with 2.0 ppm added nitrate. Conditions: −30 kV, 25 °C, 10 s injection at 1.5 psi, 250 nm. Peaks: (1) chloride; (2) sulfate; (3) nitrate; (4, 5) unknown; (6) citrate; (7) monohydrogen phosphate.

Analysis of blood and plasma samples showed the presence of chloride, sulfate, citrate, monohydrogen phosphate and bicarbonate. The blood and plasma samples were preserved with citrate, a conventional additive for blood collection vials. Nitrate, at 50 ppm, was added to a blood sample, which was then diluted 30-fold, including sample pretreatment. The nitrate peak was resolved at 6.49 min (Fig. 4). Plasma with the addition of azide at 300 ppm and thiocyanate at 150 ppm was diluted 30-fold, similar to the blood sample. The azide and thiocyanate peaks were resolved at 5.69 and 5.85 min, respectively (Fig. 5). In addition, blood containing 1500 ppm bromide and urine containing 50 ppm azide and thiocyanate were analyzed with successful separation and detection of the anions.

Positive peaks in indirect UV detection represent a decrease in absorbance relative to the run buffer and negative peaks represent an increase in absorbance relative to the run buffer. The peaks of bromide, iodide, nitrate, phthalate, and benzoate exhibit negative deflections at 200 nm. The negative peaks of bromide and nitrate at 200 nm are shown in Fig. 1. At 200 nm the absorbance of azide, peak 7 in Fig. 1, is nearly equal to the run buffer. Therefore, at 200 nm no peak for azide is seen in Fig. 1. The analysis of a sample using two wavelengths, at 200 and 250 nm, allows for the spectral resolution of some closely migrating anions, such as chromate, iodide, and chloride, because iodide exhibits a negative peak at 200 nm while chromate and chloride exhibit positive peaks.

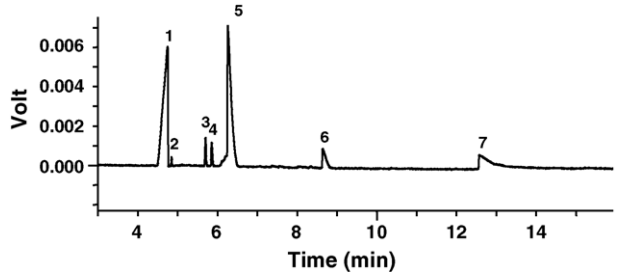

Fig. 5. Plasma with the addition of 10 ppm azide and 5 ppm thiocyanate. Conditions: −30 kV, 25 °C, 10 s injection at 1.5 psi, 250 nm. Peaks: (1) chloride; (2) sulfate; (3) azide; (4) thiocyanate; (5) citrate; (6) monohydrogen phosphate; (7) bicarbonate.

Two milliliters of acetone were added to prepare blood and plasma samples for analysis in order to precipitate the proteins for removal. A 10-fold dilution of a treated blood or plasma sample contains approximately 6.6% acetone by volume. Normally, organic modifiers are added to the run buffer to decrease the EOF, and reduce wall interactions thereby affecting the selectivity of the system [34]. The presence of acetone on the column had no noticeable adverse effects on the separation efficiency, selectivity, or the reproducibility of the system.

Intraday reproducibility values (Table 2) were obtained from five hydrodynamic injections of a pretreated blood sample for 10 s each at 1.5 psi. For chloride, citrate, and phosphate the intraday R.S.D. for migration times and peak areas were below 1.1% and 6.3%, respectively (Table 2). The sample injections were run at −30 kV and 25 °C.

Interday reproducibility values were obtained from 84 injections of a pretreated plasma sample. The injections were made on seven non-consecutive days. Table 3 shows the average, standard deviation, and R.S.D. values for peak migration time, asymmetry, baseline plate count and baseline resolution for the 84 runs. Migration time, peak width and asymmetry factor values were obtained using the instrument's PC1000 software (Thermo Separation Products). The R.S.D. values for migration times were between 7.5% and 10.4%. Peak asymmetry R.S.D. values were between 0.83% and 10.6%. Plate count R.S.D. values were between 11% and 19%. For resolution the R.S.D. values were between 8.2% and 12%. The R.S.D. values for migration time, peak asymmetry, and resolution correspond well to those reported by Ehmann et al. who used the same mobile phase for the analysis of aqueous anion standards using hydrodynamic injection [29]. Our reproducibility values were obtained from pretreated plasma samples so higher R.S.D.s than those obtained from aqueous standards is expected. As shown in Fig. 5, all of the peaks in the plasma sample are baseline resolved. The low value for the resolution between chloride and sulfate is due to

Table 2
Pretreated blood sample

	Chloride	Citrate	Phosphate
t_m (min)			
Average ($n = 5$)	4.75	6.28	8.42
S_x	0.04	0.05	0.09
R.S.D. (%)	0.84	0.80	1.1
Area (mAU s)			
Average ($n = 5$)	69000	32000	7100
S_x	4000	2000	400
R.S.D. (%)	5.8	6.25	5.63

Run conditions: −30 kV, 25 °C, 10 s injection at 1.5 psi, 250 nm.

Table 3
Interday reproducibility values obtained from 84 injections of a pretreated plasma sample

	Cl^-	SO_4^{2-}	N_3^-	SCN^-	Citrate
Average					
t_m	5.1	5.3	6.3	6.5	7.0
Peak asymmetry	0.54	0.81	1.34	1.37	7.60
Baseline plate count	4500	320000	180000	170000	15000
Baseline resolution		0.67	21	3.2	3.3
S_x					
t_m	0.39	0.40	0.59	0.62	0.73
Peak asymmetry	0.0045	0.057	0.064	0.090	0.80
Baseline plate count	690	62000	24000	22000	1600
Baseline resolution		0.078	2.4	0.30	0.27
%R.S.D.					
t_m	7.5	7.7	9.3	9.6	10.4
Peak asymmetry	0.83	7.0	4.8	6.6	10.6
Baseline plate count	15	19	14	13	11
Baseline resolution		12	11	9.4	8.2

For the baseline resolution values the peak pairs are as follows: chloride and sulfate, sulfate and azide, azide and thiocyanate, and thiocyanate and citrate.

the high peak asymmetry of chloride. The calculated resolution value therefore does not reflect the complete baseline resolution that is achieved. The low plate count for the chloride and citrate peaks are due to their high peak asymmetry, their average values being 0.54 and 7.6 for chloride and citrate, respectively. The peak asymmetry of chloride and citrate is due to electromigration dispersion, which will be explained later. The interday reproducibility results show the separation was reproducible.

The presence of high concentrations of anions, such as chloride, phosphate, and citrate in biological samples could lead to problems in identifying other closely migrating target anions present at lower concentrations. However, as demonstrated in Figs. 2–5, target analytes at lower concentrations can be separated and tentatively identified. If the presence of target analytes that migrate close to other anions is suspected, then the run temperature can be decreased to 15 °C to enhance resolution of the peaks. By changing the run temperature the mobilities of the ions in the run buffer will be changed, but to differing degrees for each ion [38]. Resolution between some closely migrating ions may be increased but may also be decreased between other ions. At 15 °C iodide is resolved from chloride and bromate is resolved from fluoride. However, lowering the temperature to 15 °C resulted in the comigration of nitrite and sulfite. Lowering the temperature is the preferred alternative, as it does not change the analyte concentrations in the sample. If the sample is diluted or the injection volume decreased, care must be exercised to ensure that the target analytes are not missed due to the final concentrations being below the detection limit of the system.

The differences seen in peak shape and migration times of anions in various biological samples can be explained by sample ionic strength and capillary condition. The velocity of a sample zone, and therefore an ion's migration time, is a function of its concentration [39]. The high salt concentrations of biological samples can lead to zone broadening known as electromigration dispersion. As the analyte's concentration increases, zone broadening and peak deformation occur due to the solution conductivity increasing, which will lead to peak tailing, or the solution conductivity decreasing, which will lead to peak fronting [39]. Electromigration dispersion is more pronounced with increasing sample amounts. This can be seen in Figs. 3 and 4 for chloride, citrate, and monohydrogen phosphate and in Fig. 5 for chloride, citrate, monohydrogen phosphate, and bicarbonate. The variations in peak profiles and migration times from sample to sample can be caused by electromigration dispersion due to the high salt concentrations in the biological samples analyzed.

The sample preparation performed was done to remove particulate matter in addition to proteins and any other large biological molecules that might interfere with the CE analysis. The endogenous salts should be unaffected by the sample preparation and present in high concentrations. However, the chemical condition of the capillary wall can affect EOF. The surface charge of the silica capillary can be changed by adsorption of matrix components from previous samples [39]. This will affect the repeatability and reproducibility of migration times. The longer a column has been used and the more samples analyzed, the greater the chance of sample matrix components being adsorbed onto the capillary's wall. For instance, the runs in Figs. 2 and 4, and Figs. 3 and 5 were all approximately a month apart. In addition, the runs in Figs. 3 and 5 were on a different capillary from the runs in Figs. 2 and 4. The main source of migration time irreproducibility is due to inconsistencies in the EOF. These inconsistencies can occur between different capillaries or different runs on the same capillary [39].

4. Conclusion

The pyromellitic acid CE run buffer system has been demonstrated to be an effective method of screening human biological samples for potentially poisonous anions. Separation and reproducibility were obtained from anions extracted from human blood, plasma, urine, and intestinal contents. Dual wavelength detection enabled closely migrating species to be distinguished. Some comigration problems were resolved merely by changing

the run temperature. Sample preparation, which was fast and simple, enhanced reproducibility of the results for the detection of anions in biological samples. Electromigration dispersion was seen in the biological samples analyzed, which affected the migration time reproducibility and peak profiles. The described method can reliably screen biological samples for the presence of 29 different anions.

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