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Effect of pH and ions in the sample on stacking in capillary electrophoresis

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Abstract

Concentrating the sample on the capillary called "stacking" is a simple technique that overcomes the poor detection limits of capillary electrophoresis (CE). Understanding the factors that affect the stacking mechanism is vital. Two general stacking methods are used in CE: (1) low ionic strength buffer in the sample (LISS) and (2) stacking by inclusion of acetonitrile (AS) in the sample. As the sample volume, especially in the latter method, is greatly increased we show that the pH, buffer type and ionic strength of the sample affect greatly the plate number, resolution and migration time. Surprisingly, we find inclusion of a pH, molarity or ion type in the sample, different from that of the separation buffer, can greatly improve the resolution and the stacking for some components of the sample, especially for the acetonitrile induced stacking. © 1997 Elsevier Science B.V.

Keywords: Stacking; Sample handling; Ionic strength; pH effects; Buffer composition; Acetaminophen; Iopamidol; Iothalamic acid

1. Introduction

Sample concentration on the capillary known as "stacking" is a simple technique to enhance the sensitivity in CE. It can be achieved by two general methods for capillary zone electrophoresis: preparing the sample in the same separation buffer but at a lower (~10 times less) ionic strength (LISS) [1,2], or by including acetonitrile in the sample, called "acetonitrile stacking" (AS) [3–5]. These two methods are different. LISS deteriorates with increasing ionic strength in the sample, while on the contrary, AS stacking is improved by an increase of the ionic strength in the sample [1,2], but it requires acetonitrile presence in the sample itself [4,5]. Acetonitrile stacking is used for samples with high concentrations of salts or proteins such as in the case of serum or food, since it eliminates the proteins which can interfere in the analysis. The stacking is limited to small molecules.

In both types of stacking large volumes of sample can be injected onto the capillary, especially in the AS where the sample contributes to about one third of the capillary volume. It is customary to dilute the sample in the same separation buffer but at 10 times less concentration for the LISS [1,2]. Because of the large volume injected in stacking, the sample pH, buffer type and ionic strength theoretically can all modulate the overall separation.

In this work we investigate the effect of the pH, ionic strength and type of buffer in the sample itself on the two types of stacking. We demonstrate that these factors affect greatly the plate number, resolution and migration. Contrary to the traditional

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practice of preparing the sample in the same separation buffer, we demonstrate that better separation can be obtained, in some instances, if the buffer, pH or molarity of the sample is different from that of the electrophoresis buffer.

2. Experimental

2.1. Chemicals

Iopamidol was obtained from Squibb Diagnostics (Princeton, NJ, USA); iothalamic acid from Malinckrodt (St. Louis, MO, USA); acetaminophen and all other chemicals from Sigma (St. Louis, MO, USA).

2.2. Instrument

A Model 4000 Quanta (Waters Associates, Milford, MA, USA) was set at 214 nm. The capillary was 32 cm×50 μm. The separation buffer was 300 m*M* borate buffer, pH 9.5, except where indicated. Samples were electrophoresed at 8 kV with detection at 214 nm. Samples were injected hydrodynamically, for 120 s, in most of the experiments, filling 8% of the capillary volume to the detector. In some experiments the injection time was increased to 240 s (16%), or 500 s (33%) of the capillary volume as indicated.

2.3. Stock solutions

A mixture of acetaminophen 2 mg, iopamidol 2 mg and iothalamic acid 1 mg/ml was used. This solution was diluted ten fold in the different buffers.

2.4. Acetonitrile stacking

The sample (10 μl) was mixed with 100 μl buffer at 250 m*M* (or as specified) and 200 μl acetonitrile.

2.5. Low ionic strength stacking

The sample (10 μl) was mixed with 300 μl of the separation buffer at 25 m*M*.

2.6. Theoretical plate number (N)

The calculation was based on the formula, $N=5.5$ (migration time/peak width at half height)^2 [6,7].

2.7. Percentage of the capillary volume injected

This was determined as described previously [8].

3. Results and discussions

3.1. Non-stacking

Under non-stacking conditions small volumes (less than 1% of the capillary) can be injected with good separation but with poor detection signal (Fig. 1A). Increasing the sample volume to 8%, under non- stacking conditions, caused poor peak shape and loss of the separation due to sample overloading (Fig. 1B). The inclusion of acetonitrile in the sample (66%) gives improved peak shape and separation of the three compounds with about 7 fold increase in detector signal [5], especially when salts are present in the sample (Fig. 1C). In the absence of salts, acetonitrile produces less stacking as previously described [4].

3.2. Acetonitrile stacking

The two most common buffers in CE are borate and phosphate. These two buffers have low absorbency at 200 nm. Previous studies with AS were performed with borate as the separation buffer. Unfortunately, phosphate as an electrophoresis buffer generates much Joule heating, limiting its use to low molarity. A high molarity of phosphate dictates a low voltage which slows the analysis. Stacking is favored by a high ionic strength for the separation buffer [5,7]. For this reason, borate has an advantage as a separation buffer for sample stacking, because it generates less Joule heating.

3.3. Borate, 300 mM, pH 9.5 as the separation buffer

In this set of experiments a borate buffer, 300 m*M*, pH 9.5, is used as the separation buffer while the

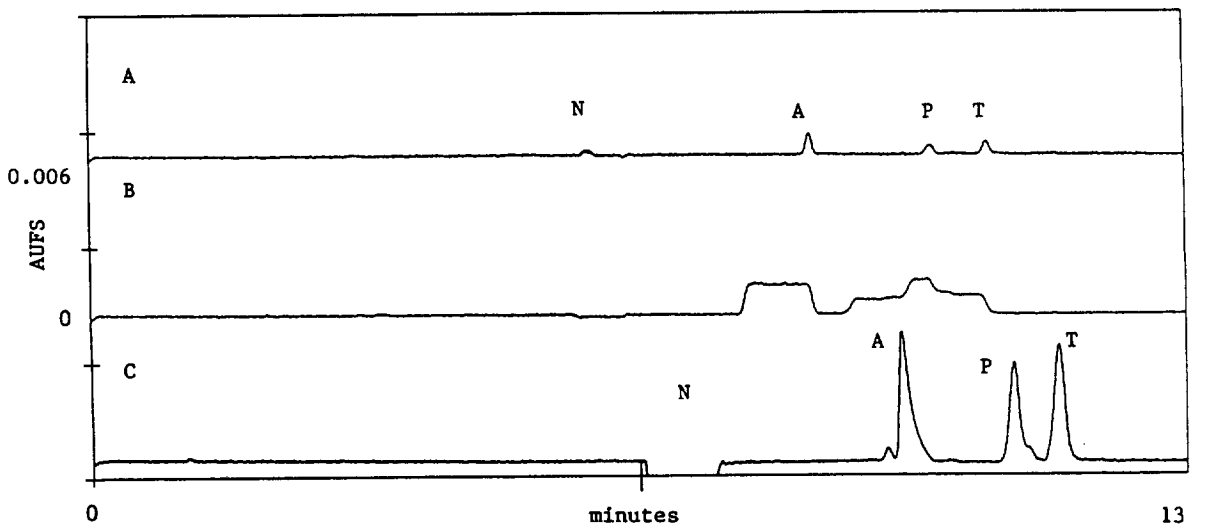

Fig. 1. Comparison of non-stacking to stacking: Sample, a mixture of acetaminophen (A), iopamidol (P) and iothalamic acid (T) dissolved in the electrophoresis buffer (300 m*M*) and injected at: (A) 0.5% and (B) 8% loading, and (C) the sample dissolved in 1% saline containing 66% acetonitrile at 8% loading. N=neutral compounds.

sample buffer, molarity and pH were varied. Dissolving the sample in borate buffer at a concentration close to that of the separation buffer, 250 m*M*, but with 66% acetonitrile produced baseline separation of the three compounds. A low pH (about 8) in the sample, despite being different from that of the electrophoresis buffer, surprisingly produced a higher plate number *N* (Fig. 2). The separation deteriorated at pH 10 in the sample. The migration time was also affected by the pH of the sample. Since these compounds are weakly acidic, lowering the pH in the sample slows their anodic migration in favor of stacking.

An increase in the molarity of the borate buffer in the sample at a constant sample pH of 8, caused an increase of *N* (Fig. 3). Interestingly, a molarity of 400 m*M* in the sample, above that of the separation buffer (300 m*M*), gave the highest *N* (Fig. 3). In the absence of acetonitrile in the sample, the high salts deteriorate the separation. A high sample buffer concentration, in the presence of acetonitrile, decreases band diffusion and, similar to a low pH in the sample, slows the migration, leading to better stacking, Fig. 3.

Changing the type of buffer in the sample affects the separation. For example, at pH 8.0, 250 m*M*, borate gave better stacking than phosphate buffer, for the first peak, acetaminophen (Fig. 4). Tris also gave good separation for the iothalamate but because it has an absorption in the ultraviolet range, it can cause interference.

At 120 s injection time only 8% of the capillary is filled with sample. Increasing the sample volume further to 33% of the capillary also gave baseline separation of the three compounds with a further increase of 2.5 times in detection signal (Fig. 5B) resulting in about a 20 times increase over the non-stacking injection (Fig. 1A). The width of the neutral molecules (*N*, Fig. 5B) is also greatly increased to a few minutes instead of seconds. However, the width of the three sample peaks is much smaller than that of the neutral molecules, reflecting the degree of stacking achieved. Because of the decrease in the effective capillary length due to the large sample volume, there is a loss of resolution as judged by the increase in peak width (Fig. 5B compared to Fig. 5A).

3.4. Borate, 300 mM, pH 8.5 as the separation buffer

Lowering the pH of the electrophoresis buffer from 9.5 to 8.5 speeded up the analysis slightly and moved the first peak (acetaminophen) close to the

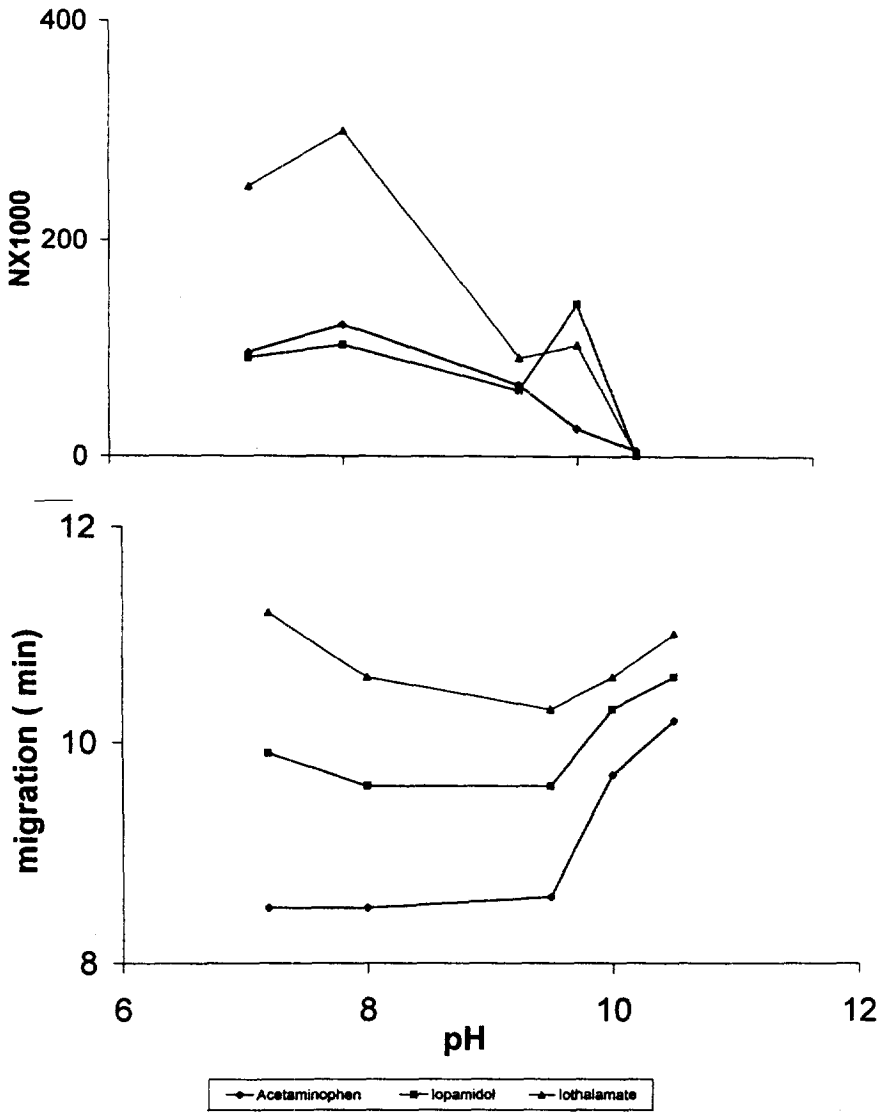

Fig. 2. Effect of sample pH on theoretical plate number (N) for acetonitrile stacking. Acetaminophen, iopamidol and iothalamic acid were prepared in borate buffer 250 mM at different pH. The sample contained 100 μl of the buffer mixed with 200 μl acetonitrile. The separation buffer was borate 300 mM, pH 9.5.

neutral peak. At pH 7.2 in the sample, the stacking remains, in general, good. The pH in the sample affected the N and migration time similarly to that observed previously for the electrophoresis buffer of pH 9.0. As the pH of the sample is raised from pH 7.2 to 8.5, 9.0 and 10, the N for the iopamidol peak decreased from 160 to 85, 45 and $19 \cdot 10^3$; while the migration time increased from 8.8 to 9.0, 9.4 and 9.6 min.

Increasing the sample volume (from 8 to 16% of the capillary volume) using the electrophoresis buffer of pH 8.5 was different from that observed earlier with pH 9.5. Unexpectedly, the N for the iopamidol peak increased greatly from 160 000 to about 800 000; while that for iothalamate decreased from 85 000 to 40 000, indicating that factors other than simple stacking contribute to these effects. As the sample volume increases, the amount of salts also

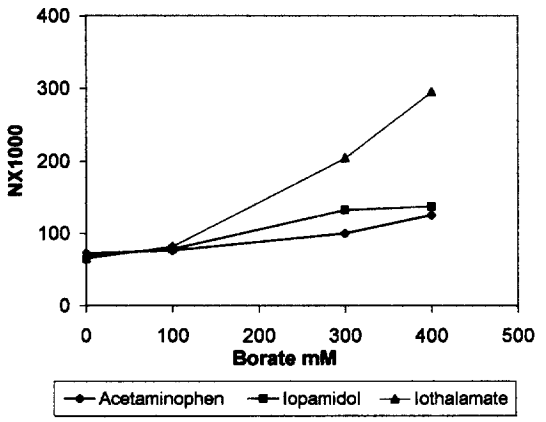

Fig. 3. Effect of the sample molarity (0–400 m*M*) on plate number (*N*) for acetonitrile stacking at pH 8.0, while other conditions as in Fig. 2.

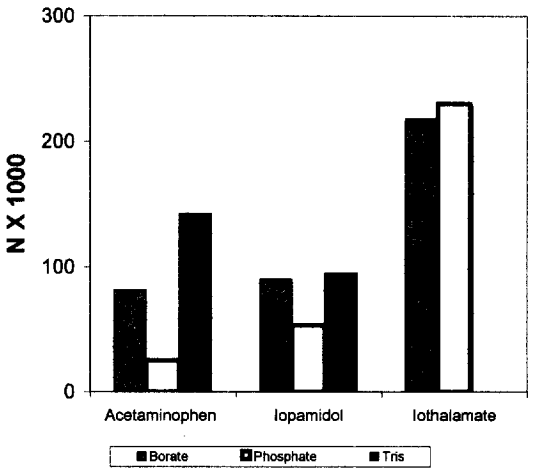

Fig. 4. Effect of the type of buffer (at 250 m*M*, pH 8.0) in the sample on plate number (*N*) for acetonitrile stacking: borate, phosphate, and Tris; while other conditions as in Fig. 2.

increases, leading in some instances to conditions suitable for a transient isotachophoresis favorable for the iopamidol.

3.5. Stacking in phosphate as the separation buffer

Phosphate buffers generate too much Joule heating; however, it is important to test if other buffers, such as phosphate, can yield acetonitrile stacking similar to that obtained in borate buffer. In order to obtain separation of the three compounds in phosphate buffer, different conditions had to be used. A molarity of 400 m*M*, pH 8.2 at 5.5 kV was required for the analysis. The low voltage is used in order to avoid an excess of heat in the capillary. A lower pH, more favorable for the buffering capacity of phosphate, caused the first two peaks to co-elute. The separation of these compounds is good at a higher pH, in spite of the long analysis time of 40 min (about three times that of borate) (Fig. 6). Diluting

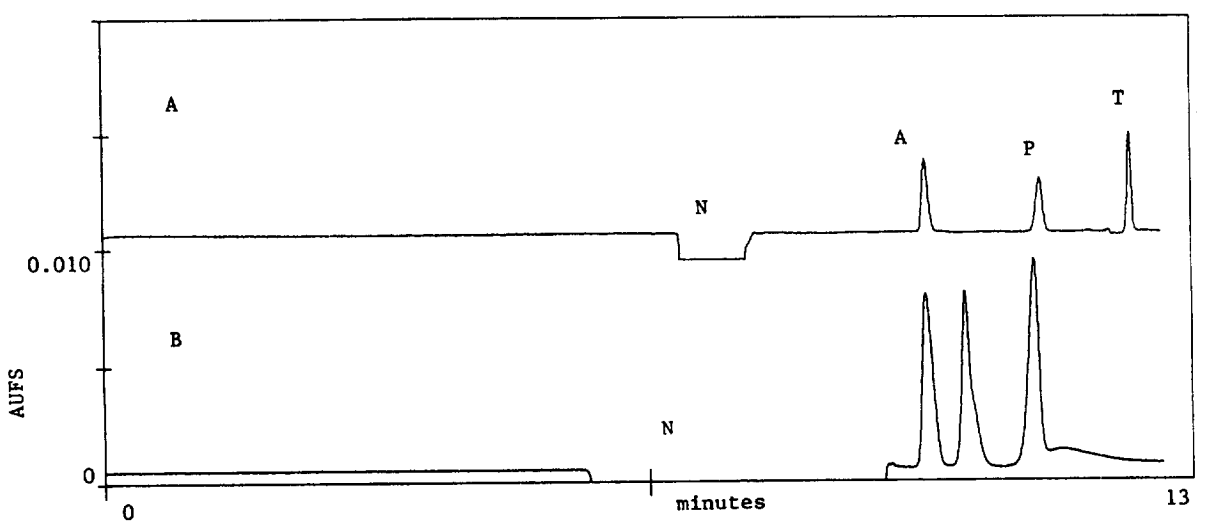

Fig. 5. Effect of sample size on acetonitrile stacking. Sample volume (A) 8% of the capillary and (B) sample volume 32% of the capillary. Sample buffer is borate 250 m*M*, pH 8; while other conditions as in Fig. 2.

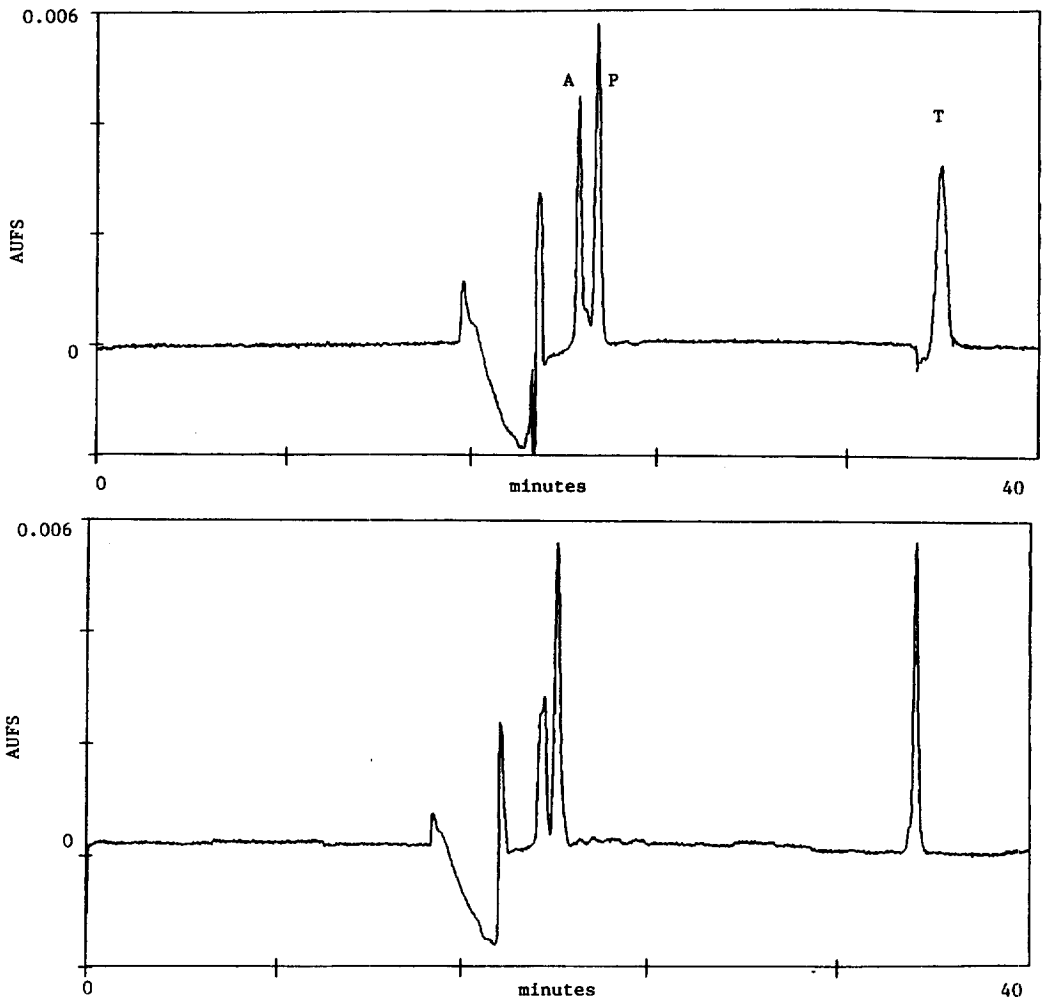

Fig. 6. Effect of the type of buffer in the sample on acetonitrile stacking: (top) phosphate 250 m*M*, pH 8.0; and (bottom) borate 250 m*M*, pH 8.0, using phosphate 400 m*M* at pH 8.0 as the separation buffer [acetaminophen (A), iopamidol (P) and iothalamic acid (T)].

the sample in phosphate, 250 m*M* at pH 8.0 (Fig. 6, top) compared to that of borate (Fig. 6, bottom) shows that the stacking is slightly different. The phosphate favored the stacking of the two early peaks while the borate favored the stacking of the late peak (iothalamic acid). The *N* for iothalamic acid in borate and phosphate buffer in the sample are 172 000 and 61 000, respectively. Changing the pH or the type of buffer also affected the separation as the borate separation buffer did earlier.

3.6. Low-ionic buffer stacking

Using a low ionic strength buffer (10 times lower molarity) in the sample in absence of acetonitrile, produced very poor separation with 8% loading, especially for acetaminophen where the peak eluted with the neutral compounds. However, the pH of the buffer affected the separation, in this type of stacking, similar to that of AS. As the pH is increased from 7.2 towards 10, the stacking decreased with an increase in the migration time (Fig. 7). Furthermore, the buffer type in the sample affected the separation. Borate buffer in the sample favored the stacking of the late peak of iothalamate compared to phosphate buffer.

LISS was worse when the separation buffer was borate compared to phosphate. In order to get better

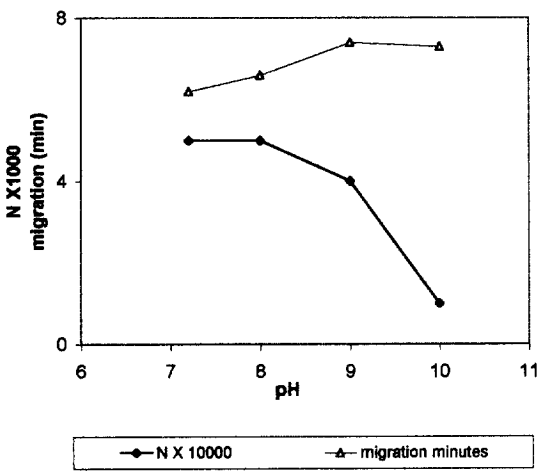

Fig. 7. Effect of sample pH (borate 250 m*M*) on plate number (*N*) for low-ionic strength stacking of the iothalamate peak. The electrophoresis buffer is phosphate 400 m*M*, pH 8.0.

peak shape when borate is used as a separation buffer, the sample volume had to be decreased from 8 to 3% of the capillary volume. Again, the type of buffer affected the stacking greatly. The use of carbonate in the sample, a buffer different from that of the separation buffer, compared to borate, improved the shape of the first peak (acetaminophen).

3.7. Precision of the injection

The R.S.D. for the migration time did not change by increasing the sample volume. However, the R.S.D. for the peak height and area improved by increasing the injection volume (Table 1). The accuracy of the integration in general is improved by an increase of the area as described earlier [9].

4. Concluding remarks

From the above data, with large injections, many factors in the sample can affect the separation, especially in the acetonitrile stacking. When several compounds are analyzed by CE, the conditions in the separation buffer might not be ideal for all the compounds. Because many factors such as ionic strength, type of the salts, pH, amount of acetonitrile and voltage, can affect the solubility, ionization and migration of the different compounds in the sample, the stacking can be different for each compound. Furthermore, the stacking here can also be modulated by other factors such as transient isotachophoresis or focusing effects. Thus, different conditions in the sample relative to that of the separation buffer can influence, and in some instances, can improve the separation. Based on this work, the sample does not have to be dissolved in the same separation buffer. A buffer or a pH different from that used in the separation can be desirable in order to change the resolution or the plate number for a particular peak/component. This is true for both types of stacking, low LISS and AS.

In many instances one compound is of primary interest. As demonstrated in Figs. 2,4,6 and 7, the stacking of one compound relative to others can be enhanced based on the choice of the conditions in the sample. Adjusting the electrophoresis and the sample buffers allows large volumes to be injected with better sensitivity. Depending on the required resolution between peaks and their migration from the neutral compounds, one third of the capillary can be filled with sample. In practical analysis, the numerous compounds present in complex samples, such as found in serum or food, limit the sample loading to about 10% of the capillary volume. This is enough to allow many drugs in serum to be detected at levels close to 1 mg/l [10].

LISS is suited for analysis of samples with a clean matrix while the AS is better suited for analysis of samples with complex matrices such as those of food, industrial or biological origin [10]. AS counteracts the deleterious effect of high salts, eliminates the proteins in the sample [10] and furthermore improves the precision of the analysis. On the other

Table 1
Precision (R.S.D. %) of the injection (% of the capillary volume) for iothalamic acid

Injection (%)	*n*	Migration time	Peak height	Peak area
1	18	0.7	5.8	6.2
8	18	0.8	3.9	3.1

hand, acetonitrile is limited to small molecules and some peptides and is not suitable for proteins.

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