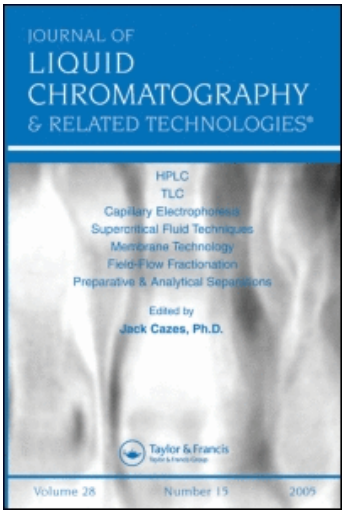

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Pseudo-Isotachophoresis: II. Visualization of the Stacking

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To cite this Article Shihabi, Zak(2006) 'Pseudo-Isotachophoresis: II. Visualization of the Stacking', Journal of Liquid Chromatography & Related Technologies, 29: 2, 175 — 183
To link to this Article: DOI: 10.1080/10826070500416411
URL: http://dx.doi.org/10.1080/10826070500416411

Journal of Liquid Chromatography & Related Technologies®, 29: 175–183, 2006

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070500416411

Pseudo-Isotachophoresis: II. Visualization of the Stacking

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Abstract: In order to investigate the stacking step by pseudo-isotachophoresis (p-ITP) completely apart from the separation step, wide capillaries 10–20 cm × 0.5–0.8 cm (I.D.) were completely filled with coloured analytes, such as Br-cresol green and ponceau S dissolved in both acetonitrile and salts and imaged at different times. The compounds concentrated visibly with efficiency of ~10–12. Chrmogenic salts, such as chromate (ions), migrated to the anode ahead of the Br-cresol green. It was apparent in this work that acetonitrile and salts are necessary for the stacking. Unexpectedly, the speed of the stacking and its association with some boundary separation were evident in this set-up. As the salt concentration in the sample is decreased, the stacking speed increased greatly. Also, as the concentration of the analytes in the sample decreased the speed of the stacking increased. Indirectly, EOF affected the concentration by moving the anodic band slowly to the cathode and prevented reaching equilibrium. In most cases, when two anionic compounds are present in the sample, there was concentration but without separation. However, as the length of the tubes (sample) is increased some boundary separation is present. As the sample concentration decreased the separation between the two compounds improved. Thus, the stacking step under proper conditions of analysis such as sample plug dimensions, buffers, and analyte concentration, can be accompanied by some separation. This simple approach should be helpful to investigate other types of stacking.

Keywords: Stacking, lsotachophoresis, Pseudo-isotachophoresis, Sample concentration

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INTRODUCTION

One major obstacle for the widespread use of CE for practical analysis of small molecules is the poor detection limits, because of the narrow light path of the capillaries and limited amount of the sample which can be injected. Sample stacking is a very simple and convenient means to overcome this problem, bringing the sensitivity of the CE closer to that of the HPLC.[1,2] Different methods of stacking have been described,[2] some are limited to few compounds while others are applicable for more compounds.[2] We have concentrated our efforts on stacking for small molecules present in biological fluids and complex samples by inclusion in the sample of both organic solvents and salts. The latter two compounds bring along special stacking termed as pseudo-isotachophoresis (p-ITP).[3]

It is difficult to follow the details of stacking in a commercial CE instrument where the stacking occurs rapidly, transiently, and simultaneously with the separation step. The CE instrument records only one particular event, at one particular moment in time, only at the detection window rather than imaging the entire capillary. These factors make the study of the early steps of stacking by p-ITP very difficult to follow by the traditional CE instrument. In the previous approach,[4] we eliminated to a great extent, the contribution of the separation step by moving the sample directly to the detector window. Some of the stacking events were investigated by this means. These studies revealed that the transient p-ITP stacking step can be associated with some separation.

Here, a different approach is undertaken to follow the stacking step in p-ITP, without contribution of the separation step. Coloured compounds filling the entire capillary and subjected to voltage in a home-made unit were imaged at different times. The aim of this work, is to show that when acetonitrile and salts together are present in the sample, concentration similar to that by ITP can be observed[5] and to investigate the early events of this step. This simple system showed several unexpected results. Furthermore, it is suitable for studying other types of stacking methods also.

EXPERIMENTAL

Chemicals

Br-cresol green was obtained from Fisher Scientific (Fair Lawn, NJ, USA), Br-cresol purple from Matheson Coleman and Bell (Norwood, OH, USA), ponceau S and p-nitro-phenylphsophate were obtained from Sigma Chemicals (Saint Louis, MO, USA), and 1-nitroso-2-naphthol from Eastman Kodak (Rochester, NY), glass tubing was obtained from SMI (Melville, CA, USA).

Tubing

Different materials of tubes (10 cm × 0.5–0.8 mm), such as glass, polyethylene, and Teflon were tried.

Instruments

A power supply Model EI20P00 (Glassman, White house Station, NJ) was used to deliver voltage to the tubing. The tubing was completely filled with sample, To prevent siphoning, the ends of the tube were connected to 2 cm × 50 μm (I.D.) silica tubing through a silicone sleeve. The current was kept around 20 μA to prevent excess heating and the formation of air bubbles, The voltage was 2–6 kV. Several tubings were analyzed at the same time and left for 2–8 hrs.

Buffers

Mainly stock borate-carbonate, 7 g/1 of each, pH 8.8, was used except where is indicated. The electrode ends were dipped into this buffer.

Methods

Several coloured compounds, such as bromophenol green, bromophenol purple, aminophenol, ponceal S, and nitrosonaphthol, were mixed separately, or in combinations with acetonitrile and small ions such as sodium chloride, borate ions, and potassium chromate. Teflon or glass capillary tubing was filled with the above solutions and subjected to 20 μA. The whole tubing was imaged with a 6.1 Mp model DX 7630 (Kodak, Rochester, NY, USA).

RESULTS

The stacking of coloured substances by entirely filling a glass tube should be a very simple and direct approach to investigate the progress of the stacking step in p-ITP. However, in practice it has several practical problems and limitations, especially in the present home-made unit. These limitations need to be addressed first. In order to observe visually the stacking process without special lenses, wide capillaries have to be used. Different materials such as glass, polyethylene, and teflon were tried. All these tubes produced stacking. However, because of the width of these tubes siphoning becomes a great problem. Thus, silica capillaries ~2 cm in length × 50 μm (I.D.) were connected to the ends through flexible silicon sleeves, as in Fig. 1.

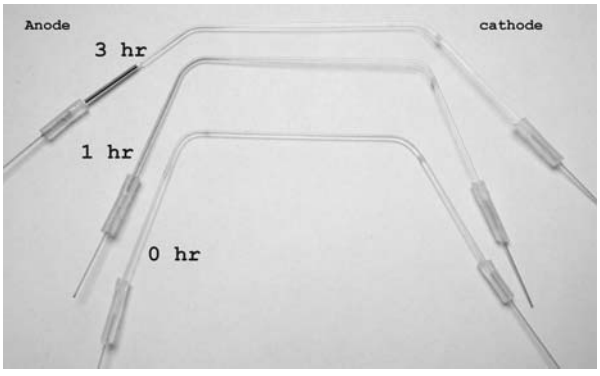

Figure 1. Stacking by p-ITP: tubes (glass 100 mm × 0.5 mm) were filled with sample containing (final concentration): Br-Cresol Green 3 μg/mL, 0.15% NaCl, 66% acetonitrile, borate-carbonate buffer diluted ten times. Separation time 0, 1, and 3 hrs.

Thus, the tubes used here have different degrees of EOF and also do not dissipate the heat fast enough necessitating operation under lower voltage, which leads to prolonged analysis times, ~1–10 hrs. They were also easily subjected to air bubble formation. The major aim here was to demonstrate that sample concentration by p-ITP occurs in these wide tubes; it can be visually examined; and furthermore, it can help to investigate the early steps of stacking. Regardless of these limitations, these tubes offered very useful insights and unexpected results about the p-ITP stacking.

When a single anionic compound, such as Br-cresol green, fills the entire capillary sample concentration occurs (Fig. 1). The bands start to migrate from the cathodic side towards the anodic side with the anodic edge slightly more stained. Finally the two edges merge at the anodic side with a heavy single stained band. Ultimately, the concentrated band either keeps moving into the buffer reservoir or it moves slowly under the EOF towards the cathode with diffusion. Thus, this system does not reach equilibrium.

The stacking efficiency was calculated by two different methods: The First method is the length of the analyte plug compared to the original length of the capillary. The second method is cutting the concentrated area and measuring its optical absorbable in a measured volume of diluent compared to a similar segment of a tube, which has not been subjected to the voltage. The calculations by the first and second method were 10.2 and 12.7, respectively. Other compounds such Br-cresol purple, ponceal S, p-nitro-phenylacetate, and nitroso-2-naphthol all concentrated too.

Both salts and analytes concentration in the sample affected the stacking efficiency, but more importantly affected the speed of the concentration. As the salts concentration decreased from 0.4 to 0.05% the speed of the stacking improved greatly from ~8 to ~2 hr. Since this system does not

reach equilibrium due to the presence of EOF and, also, have not been optimized, samples with low salt concentration, visually (apparently), seem to stack at a particular time, faster than the ones with higher salt concentration. However, in the previous work,[4] we have shown that increasing the salt concentration (to a certain limit) improves the stacking and the separation, similar to what is expected in ITP. The decrease in speed of the stacking with the high salt concentration reflects a decrease of the velocity of the molecules due to a decrease in the field strength. Another factor which affected the speed of the stacking is the concentrations of the analyte. In order to visualize the stacking high concentration of the analyte are used. As the concentration decreased the speed is increased and so the ability to visualize the colour of the band is diminished. It is not clear if the EOF contributes significantly to the stacking, since it tends to move the anodic edge of the band towards the cathodic edge. Further studies based on reversing the EOF will be necessary to study the importance of this factor. Stacking in a weak buffer and in absence of acetonitrile. (i.e., high field strength stacking)[6,7] is very slow here. Within a given time the stacking efficiency was almost 3–5 times more efficient with acetonitrile and the salts.

When two anionic dyes, such as Br-cresol green and ponceau S, are mixed and dissolved in sodium chloride and acetonitrile they concentrate as very sharp peaks and separate by CE (Fig. 2). In the home made instruments they start to concentrate at the anodic side without separation and slowly the band gets smaller and darker in colour. Removing the band and scanning it showed the characteristic spectra of the two compounds. In other words, there was sample concentration (stacking) here without separation. However, when increasing the length by joining two tubes together a boundary separation between the two compounds was observed. Depending on the buffer in the sample, different degrees of partial separation at the edges can be observed (Fig. 3), which is similar to that in ITP before reaching the equilibrium.[5] Initially (0 time) the tubes exhibit a single lavender colour across the length of the tube, Later on (after 6 hrs), three areas of different colours, each about 1 cm in length can be observed, A light blue area (representing Br-cresol), very dark blue colour representing the mixture, and a purple colour (representing the ponceau S) are observed, Fig 3. A decrease in the concentration of the analytes (by half) improved the resolution (visually) between the two bands.

Stacking works well with most salts, e.g., sodium chloride and potassium dichromate,[4] The yellow colour of the chromate migrated ahead of that of the Br-cresol green, close to the anode as a boundary (Fig. 4). However, most of the compounds used here have several ionizable groups and buffering is important, especially for visualizing the colour. Also, a low concentration of buffers gives better reproducibility and continuity of the current, In practice, the sodium chloride or the potassium chromate can be completely replaced by buffers, such as sodium borate or sodium phosphate, with good stacking as described earlier.[8]

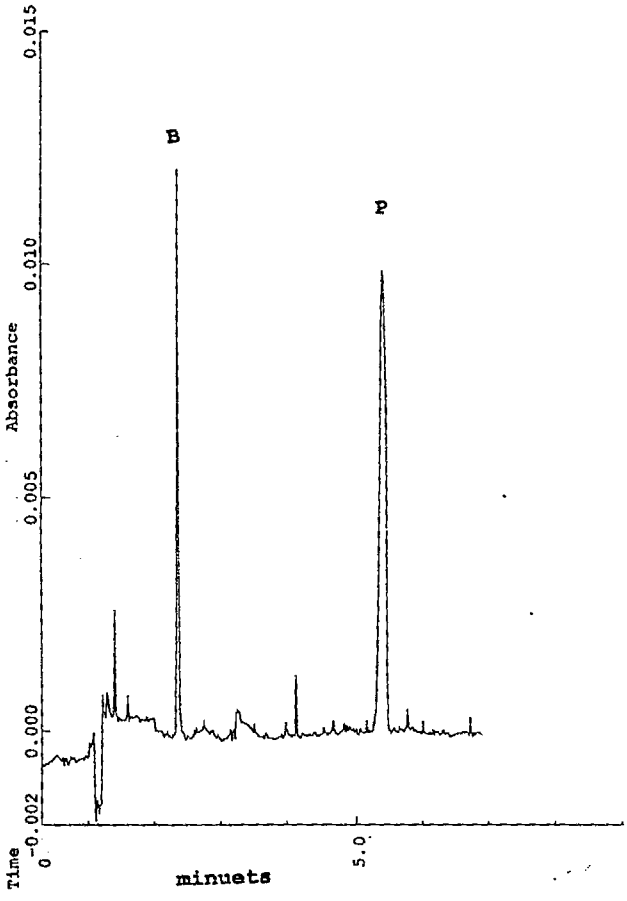

Figure 2. Separation in the capillary electrophoresis instrument (Beckman Model 2000) of a mixture of Br-creol green (50 mg/L) and ponceau (250 mg/L) in CE containing 66% acetonitrile and 0.3% NaCl (separation buffer borate: pH 9.2, 200 mmol/L; 14 kV; sample size 10% of the capillary, 200 nm; capillary 28 cm × 50 μm).

CONCLUSIONS

Despite the limitations of this approach it did confirm several critical points. Most importantly, it showed that sample concentration can be induced simply by dissolving the analytes in acetonitrile and salts and this can be visually confirmed. Regardless of optimization, ~10–12-fold concentration was obtained by this simple method. It also showed the importance of both the acetonitrile and the salts for the stacking. It showed that the salts (such as chromate) move faster and stack ahead of the other analytes. Salts

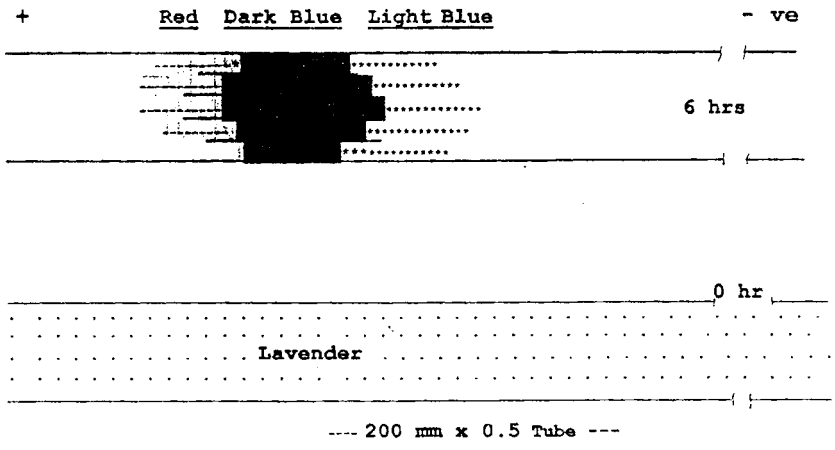

Figure 3. Separation of the mixture Br-cresol green and ponceau S of Fig 2 (containing 0.15% NaCl, 66% acetonitrile, and borate-carbonate buffer diluted ten times) in the home made instrument (at 0 and at 6 hrs). The tube (200 mm × 0.5 mm) was filled with the sample.

concentration in the sample affected the stacking speed of the concentration, which was not evident before. It showed the similarity of the p-ITP to the ITP: the overall concentration, the movement of the analyte as a boundary (not separated as a zone). On the other hand, there are few differences between

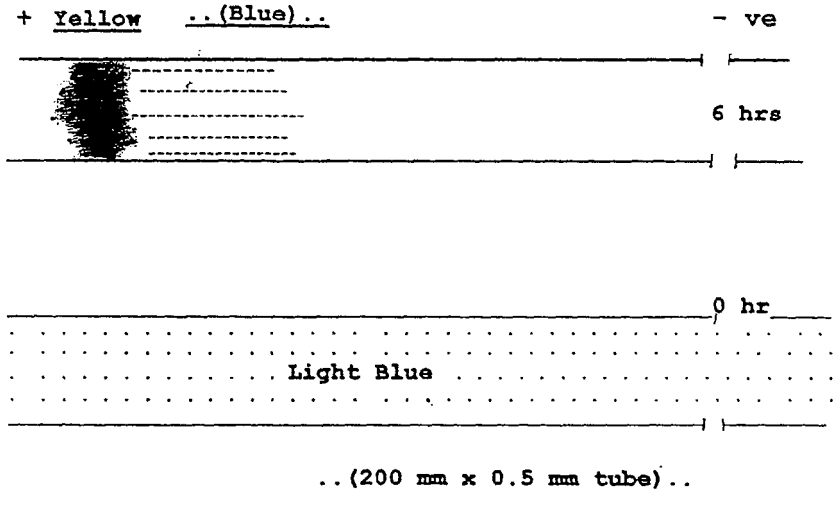

Figure 4. Migration of chromate (yellow colour) ahead of the Br-cresol green (blue colour). The tube (200 mm × 0.5 mm) was filled with sample containing 15 μg/mL Br-cresol green, 0.13% potassium bichromate, and 66% acetonitrile.

these two techniques. The major one is the absence of a terminating ion in p-ITP and also the ability to concentrate both anionic and cationic compounds at the same time in p-ITP.[9] The effect of EOF on stacking deserves further study.

Stacking usually is thought of in terms of concentration without separation; but as evident here and in the previous paper, this can be accompanied by some degree of separation.[4] This is true for the p-ITP as well as the ITP. In fact, the name of stacking is borrowed from a stack of coins,[10] which indicates some separation with bands touching each other. Optimizing the stacking so the separation can start early at this step will improve the overall separation in CE.

Since the resolution between two compounds in CE is based on the combination of both the stacking and the separation steps, the transient step of stacking has to occur within a certain frame of time for peak height. This depends on many factors such as buffer pH, sample size, and capillary diameter. Thus, based on this work, the optimum concentration of stacking as far as salts, voltage, and other factors will depend on so many variables that it is almost an empirical one.

The importance of the pseudo-ITP is the simplicity and the wide applicability of this technique for complex samples. It occurs as a transient step in capillary electrophoresis. ITP has many stringent requirements for success.[11,12] It is easy to select a leading ion in ITP, but finding a suitable terminating ion is far more difficult. On the other hand, p-ITP is much simpler to perform in practice.

With the exception of zwitterionic compounds, methods solely to concentrate small molecules by the electric current are limited. This method has the potential to concentrate these molecules. A better optimized design, which accommodates smaller diameter tubes, better temperature control, and a CCD camera in the UV range, may lead to constructing an instrument which can be used solely for concentrating and desalting of small molecules based on this principle.

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Received August 15, 2005
Accepted September 20, 2005
Manuscript 6703