Sample stacking techniques in capillary electrophoresis

or

How to make peaks out of nothing

What is it?

Sample stacking is a process occurring inside the electrophoretic capillary during sample injection and/or initial stage of electrophoretic separation. During this process, analytes present in the injected sample are concentrated at the interface between sample zone and background electrolyte inside the electrophoretic capillary.

Why is it useful?

Thanks to the stacking process the analytes are focused in a very narrow zone at high concentration. The concentration of analytes in this stacked zone can be several orders of magnitude higher than their concentration in the original sample. This is advantageous for two reasons. Frist, the increase of analyte concentration can lower the detection limit of the method substantially. Second, focusing of the analyte into a narrow zone improves separation efficiency and results in narrow peaks that, apart from higher sensitivity, also mean higher precision of quantification.

Task 1 – peak shape

Briefly explain (using words or figures or combination of both) why quantification is more precise when peaks of analytes are narrow and high.

How the heck can it happen?

The sample stacking effects arise from the condition of continuity that have to be fulfilled in the capillary.

Continuity of electric current – equal electric current must flow through cross-section of the capillary at any point along the capillary length because charge carriers (cations and anions) cannot disappear at one place and appear at another one.

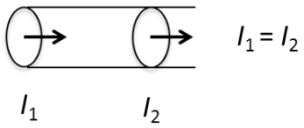


Figure 1 Continuity of electric current in the capillary

The river stream analogy

If we imagine a river that narrows in one place we can see the implications of the continuity condition. Let's suppose that the water in **Figure 2** would flow with the same linear velocity in all parts of the river. In that case lower amount of water per time would be flowing through the part where the river is narrow. As a result, water would be held up in the left wider part and water would be missing in right wider part because (as ions in the capillary) water cannot disappear at one spot

and appear at another one. For these reasons, the linear velocity of the flow is higher where the river is narrower.

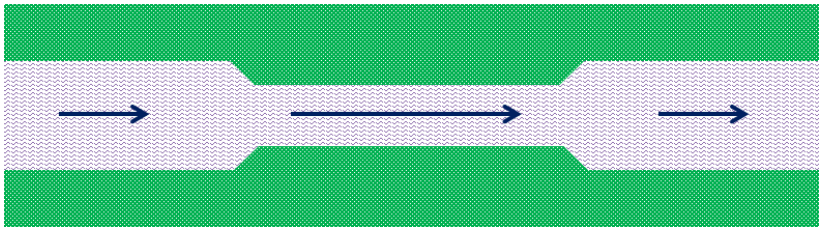


Figure 2 The river stream analogy

Soooo how it works inside a capillary

Inside an electrophoretic capillary, electric current is carried by ions. Analogously to the water flow in the river the electric current (charge transferred per time) must be constant in all parts along the capillary because charge carriers (ions) cannot appear and disappear. If there is a zone with lower concentration of ions (middle part of the capillary in **Figure 3**), this zone will have lower ability to carry electric charge (to lead electric current), i.e. this zone will have lower electric conductivity. Similarly to the river example, ions must migrate faster in the part of the capillary with lower conductivity in order to carry the same charge per time as their counterparts in more conductive parts for the capillary.

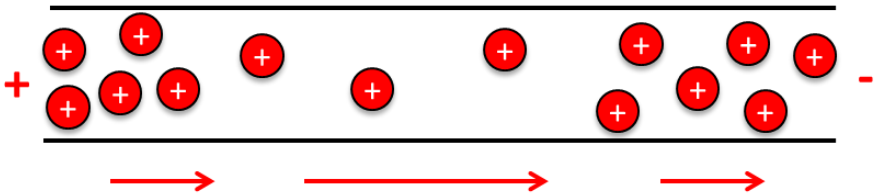


Figure 3 Continuity of electric current inside the capillary

But how do they do that? Electrophoretic mobility is dependent on charge, size and shape of ions, not on the conductivity of the solution.

In fact, velocity of migration *v* is a product of electrophoretic mobility μ and electric field intensity *E*, $v = \mu E$. Because the mobility does not change with conductivity of the solution, it must be the electric field intensity that changes locally and allows ions to migrate faster.

We can imagine it as three resistors connected in series (**Figure 4**). Value of electric current must be equal at all three resistors (analogously to what we said above). Therefore, the potential difference between both ends will be distributed unevenly. Resistor with higher resistivity (lower conductivity) will get higher potential gradient ("larger part of the voltage applied will be spend here"). This will cause the same electric current as smaller potential gradient at resistors with lower resistivity (which is in accordance with Ohm's law).

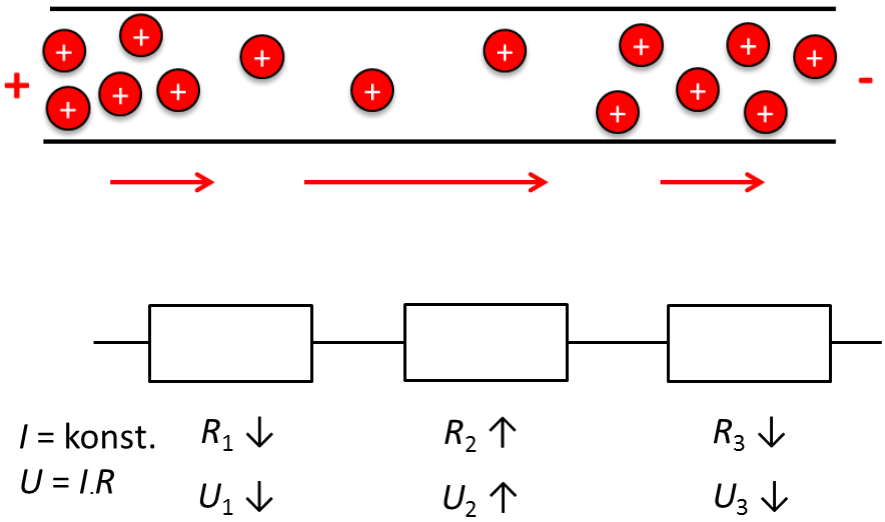


Figure 4 Representation of current continuity inside the capillary with resistors connected in series

Electromigration dispersion – unwanted fruit

One of the manifestations of the above mentioned effects in capillary electrophoresis is **electromigration dispersion**. Let's imagine a capillary (**Figure 5**) and let's focus on cations only. There are, of course, anions present to compensate the cations' charge but for the sake of simplicity we will not look at them now.

The capillary is filled with a background electrolyte whose cations are red-colored in the **Figure 5**. In the middle part of the capillary there is a sample zone containing blue-colored cations of similar concentration but larger hydrodynamic radius and thus lower electrophoretic mobility $\mu_{sample} < \mu_{BGE}$.

Thanks to that resistance of the "blue" sample zone is higher $R_{sample} > R_{BGE}$. If the potential gradient were distributed evenly along the capillary Ohm's law would imply that there would be lower electric current in the sample zone. As we have understood earlier, this cannot happen as the continuity of the electric current must be conserved. Therefore, the potential gradient is distributed along the capillary in such a way that in the sample zone there is higher intensity of the electric field $E_{sample} > E_{BGE}$. The higher field intensity forces the blue sample cations to migrate faster.

The sample cations migrate fast in the high-intensity field of the sample zone. When they reach the background electrolyte in front of them they enter low-intensity electric field, which slows them down. In this way, they are focused at the interface between the background electrolyte and the sample zone.

At the rear end of sample zone, the opposite process takes place and the sample cations are spreading and decreasing their concentration. This results in the typical triangular-shaped peaks that are resulting from electromigration dispersion, in this case tailing.

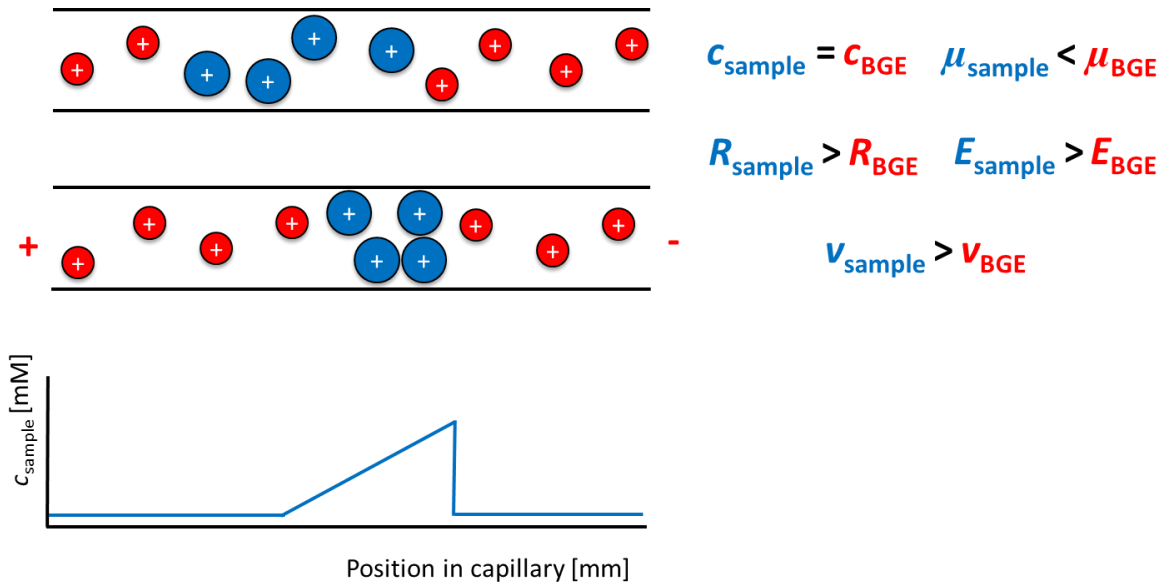


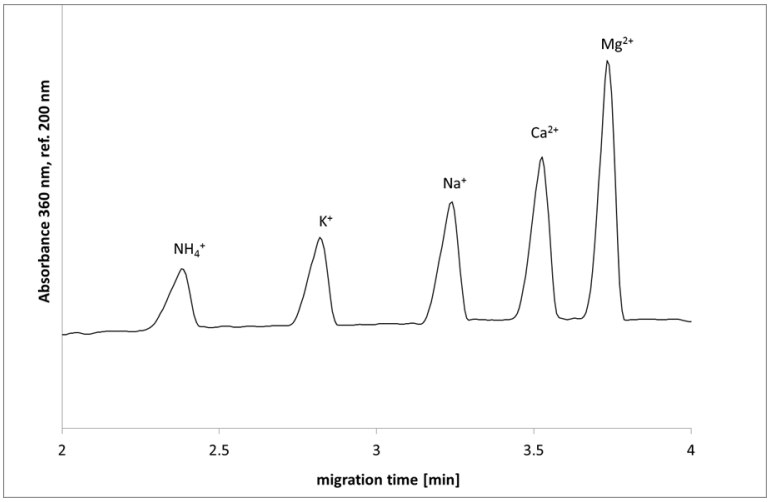
Figure 5 Electromigration dispersion

Of course if sample cations have higher mobility than background electrolyte cations, the opposite effects occur and the resulting triangular peaks are not tailing but fronting.

Task 2 – Electromigration dispersion

*Inspect the electropherogram, background electrolyte composition and ionic mobilities of the cations involved in **Figure 6** and explain:*

a) why the electromigration dispersion occurs in this case.
b) why the electromigration dispersion is most significant for ammonium cations and least significant for magnesium cations.
c) why the peaks are fronting in this case.
d) how we can eliminate electromigration dispersion of certain analyte.



Background electrolyte:

4 mM $CuSO_4$

4 mM HCOOH

4 mM 18-crown-6

Cation	μ [10^{-9} $m^2.V^{-1}.s^{-1}$]
NH_4^+	76.2
Ca^{2+}	61.7
Mg^{2+}	55.0
Cu^{2+}	55.6

Figure 6 Task 2 – electromigation dispersion

Sample stacking – appreciated effect

If the sample is highly diluted the low concentration causes low conductivity of the sample regardless of analyte's mobility. Therefore, one of the solutions to electromigration dispersion is making sure that conductivity of sample is much lower than conductivity of background electrolyte. In this case, electric field is strongly amplified in the sample zone. That causes analytes to migrate quickly and thus they concentrate at the interface of sample and background electrolyte. In this way, the analyte concentration can be increased by several orders of magnitude.

If sample is injected hydrodynamically by pressure (**left panel in Figure 7**), a given amount of sample is injected and then the analytes are stacked into a narrow zone during the initial phase of separation.

If sample is injected electrokinetically by voltage (**right panel in Figure 7**) analytes are being stacked at the interface between background electrolyte and sample already during the injection. The injection can be very long and large amounts of the analyte can be concentrated at the interface.

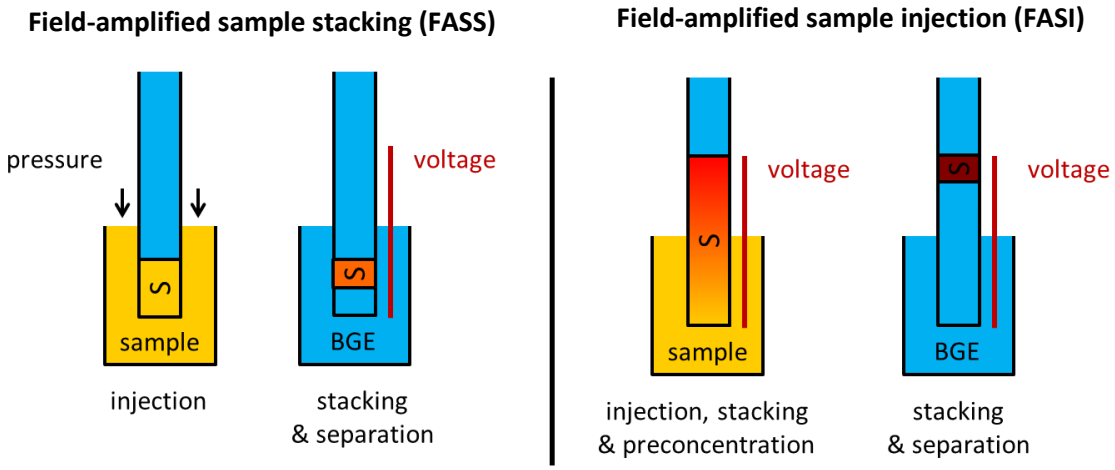


Figure 7 Field-amplified sample stacking and injection

Real-life example of difference between hydrodynamic injection by pressure (**left panel**) and long electrokinetic injection of low-conductivity sample employing the FASI (**right panel**) is shown in **Figure 8**.

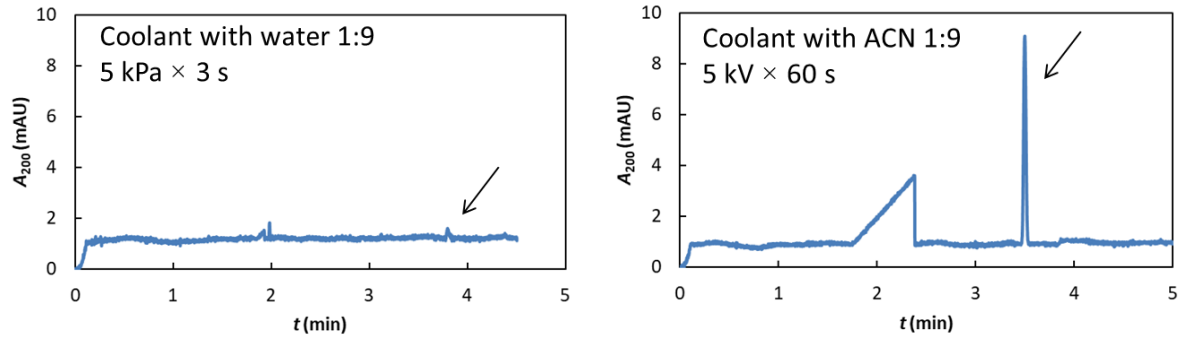
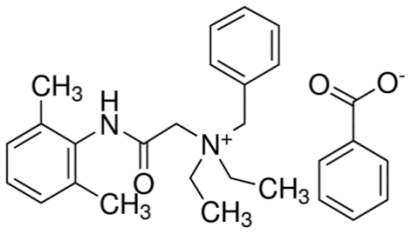


Figure 8 Electropherograms from determination of denathonium cation* (peak marked by an arrow) in engine coolant sample. **Left panel:** Sample of engine coolant diluted 1:9 with water injected hydrodynamically. **Right panel:** The same sample diluted 1:9 with acetonitrile (to decrease conductivity) and injected electrokinetically by 5 kV for 60 s.

* Denathonium benzoate is probably the most bitter compound in the world and is added to engine coolants in order to prevent accidental ingestion.



Drawbacks of FASI

An obvious drawback of the FASI is that the injected amount of the analyte is strongly dependent on conductivity of the sample. Therefore, when the samples differ in electric conductivity, an internal standard must be used to eliminate the errors.

Modifications and alternatives to FASI

- FASS/FASI – sample of low conductivity is required
- Large-volume sample stacking (LVSS) – sample of low conductivity is required
- pH-mediated sample stacking – can be used for high-conductivity samples
- Isotachophoresis (ITP) – stacking between leading and terminating electrolyte
- Chromatographic preconcentration – short segment of the capillary is packed with a stationary phase

How to make your sample a low-conductivity sample

Very elegant way is addition of an organic solvent (acetonitrile) to the sample. This decreases conductivity of the sample significantly. In case of biological samples, addition of acetonitrile also precipitates proteins that often cause problems in electrophoresis. Example of application of this approach can be seen in **Figure 8**.

Large-volume sample stacking

This is only a modification of classical FASI. In this case, we perform an extremely long electrokinetic injection so that up to ¾ of the capillary length are filled with the sample. Analytes are stacked at the front end of the sample zone. After the injection, we apply opposite pressure and push most of the injected sample matrix back out of the capillary through the inlet end. The analytes are concentrated in the front part of the sample zone which is not pushed out but stays at the beginning of the capillary. Then separation voltage is applied and pre-concentrated analytes are separated and detected.

pH-mediated sample stacking

In this approach, we decrease conductivity of the sample zone by suppression of dissociation of weak acid or suppression of protonation of weak base present in the sample.

In the example below (**Figure 9**), we inject sample containing analyte (cation A^+) with highly conductive chloride counterions. Thanks to electrokinetic injection analytes migrate into the capillary while chlorides migrate in the opposite direction and do not enter the capillary.

Thus we obtain a zone of analyte A^+ accompanied with acetate counterions (**Figure 9, bottom part**).

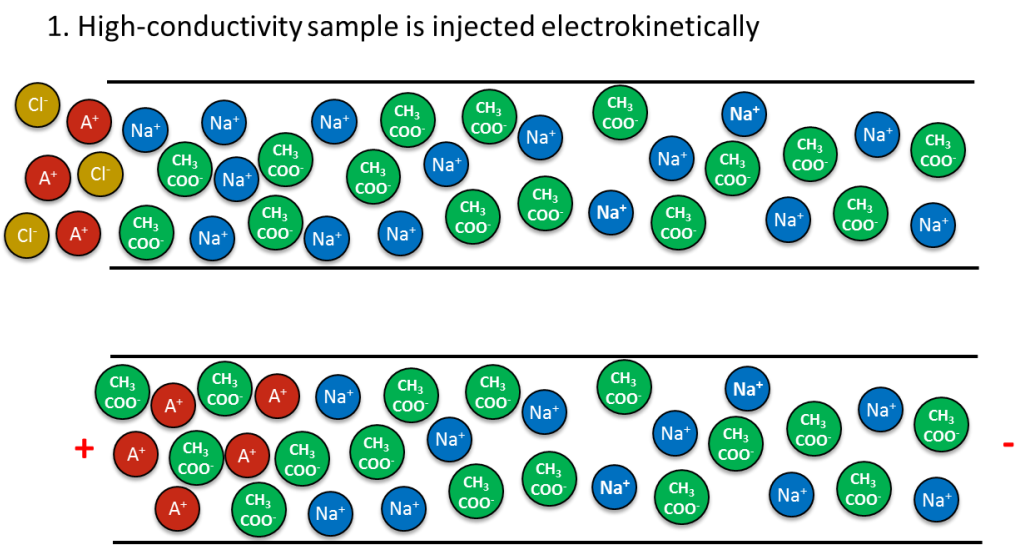


Figure 9 pH-mediated sample stacking – step 1

In the second step (**Figure 10**), we inject a strong acid (HCl) also electrokinetically. In this case, chlorides migrate again out of the capillary and H^+ ions migrate in the capillary. In the capillary they protonate the weak acetic acid, which loses its charge and the zone containing the analyte becomes less conductive. Sample stacking can thus occur in it.

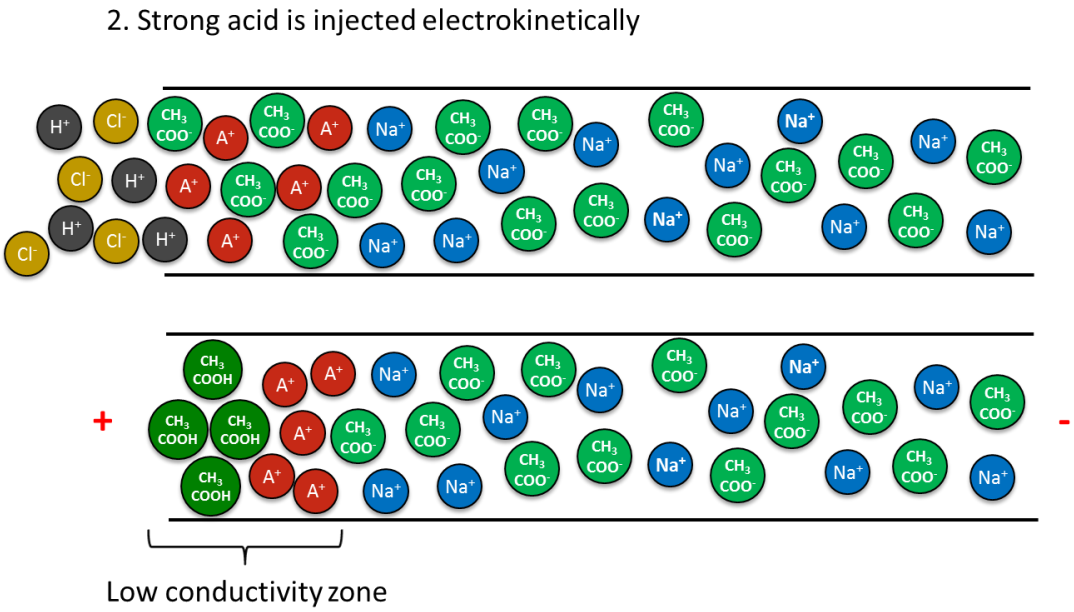


Figure 10 pH-mediated sample stacking – step 2

Task 3 – pH-mediated sample stacking

Study the paper by Y. Zhao and C. E. Lunte: Anal. Chem. 71 (1999) 3985 and explain briefly the principle of their pH-mediated sample stacking.

Transient ITP-CZE

Figure 11 explains the principle of isotachophoresis (ITP). The figure depicts situation when analytes are cationic. Then the sample is introduced between so called "leading electrolyte" that contains cations of very high mobility and "terminating electrolyte" that contains cations of low electrophoretic mobility. Thanks to the above mentioned condition of continuity, potential gradient is distributed unevenly over the length of capillary. The part of the capillary with high-mobility ions has lower electric field intensity and vice versa. Due to this, all cations are separated in zones that migrate in the same velocity (from which the name "isotachophoresis" originates).

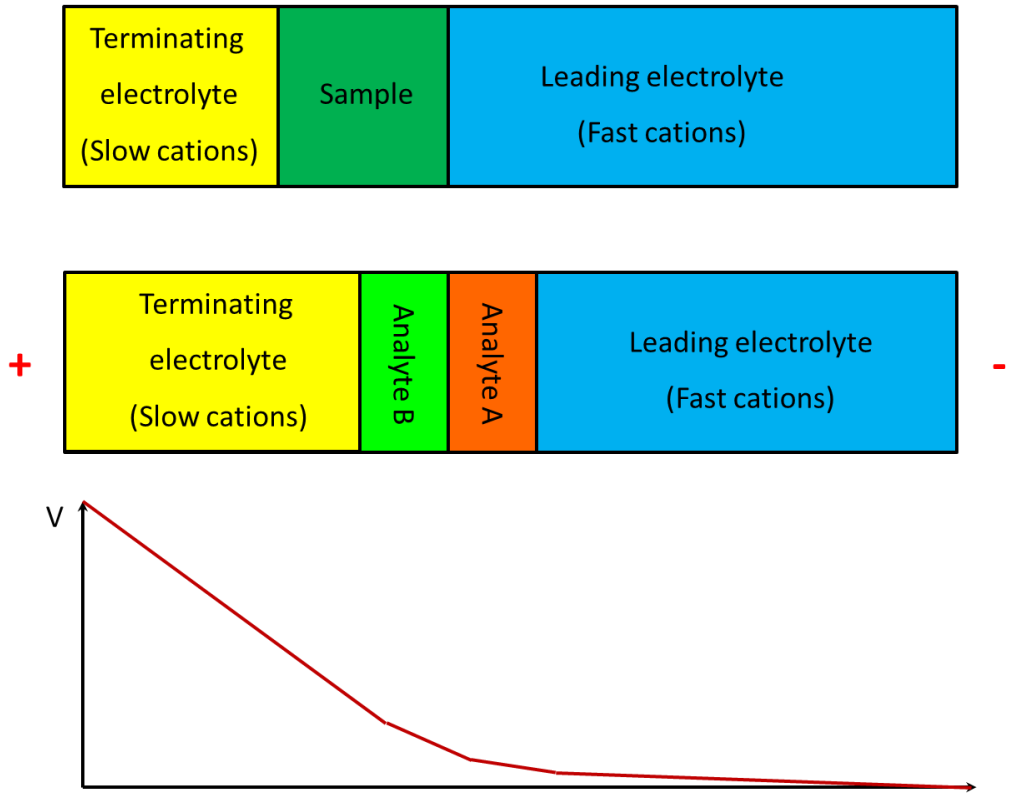


Figure 11 Principle of isotachophoresis

During the isotachophoretic part of the experiment, analytes are focused into narrow zones. Then terminating electrolyte is replaced with leading electrolyte and experiment continues with standard electrophoretic separation (**Figure 12**).

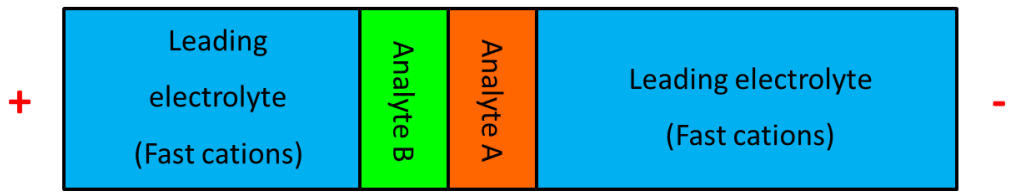


Figure 12 Electrophoretic separation following after isotachophoretic stacking

An example of application of the ITP-CZE method is in the article published by Schoots et al.: Clin. Chem. 36/3 (1990) 435. They developed a method for separation of hippuric, *p*-hydroxyhippuric and uric acids accumulated in blood serum of patients with chronical renal failure. As the sample of blood plasma naturally contains high concentration of fast migrating chloride anions, they used these anions as leading electrolyte that is already present in the sample. This is called self-induced ITP-stacking. As BGE, MES-His buffer, pH 6 was used, where the bulky MES anions work as the slow migrating anions of terminating electrolyte. **Figure 14** shows how increasing concentration of chlorides in the sample increases peak heights of the analytes.

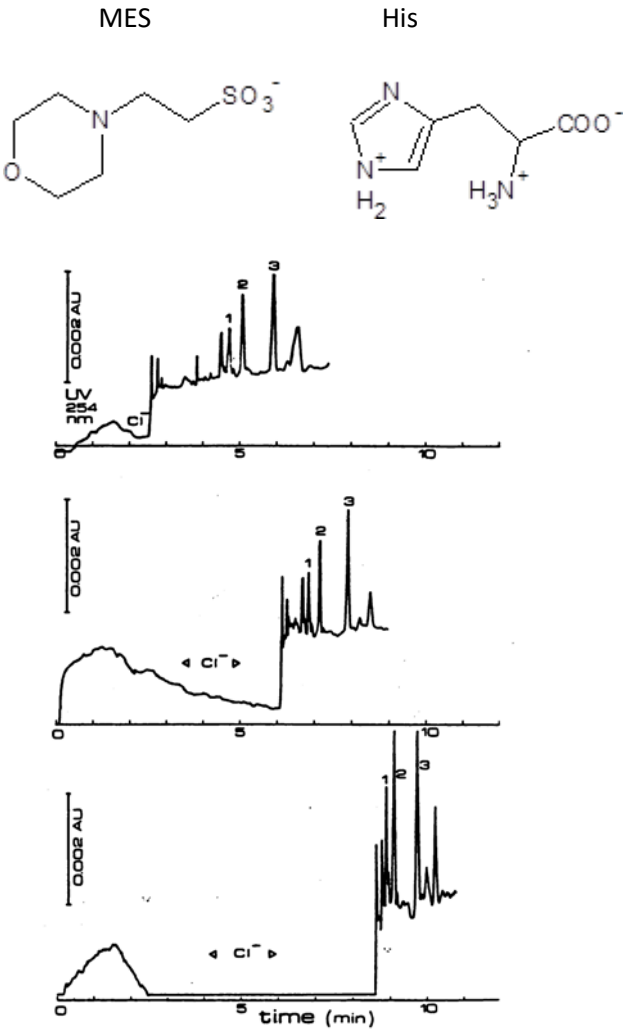


Figure 13 Increasing peak height of analytes with increasing concentration of chlorides in sample.
* Schoots et al.: Clin. Chem. 36/3 (1990) 435