Application of Capillary Isotachophoresis in Food Analysis

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1) INTRODUCTION

Isotachophoresis (ITP) as one of the electrophoretic techniques is a modern analytical method with wide spectrum of use in the analytical practice. ITP as a working method is relatively young technique, although the laws which govern isotachophoretic separation were laid down in 1897. At that time the German chemist Kohlrausch published a theoretical treatment of the conditions at migrating boundary between two salt solutions and showed that the concentrations of ions at the boundary were related to their effective mobilities (Kohlrausch’s regulating function). Even though several research groups had worked over a long period with systems which were regulated by the Kohlrausch function, it was not until the 1960s that Kendall’s so-called ionic migration technique received full attention. In 1962 Konstatinov and Oshurkova developed a "moving boundary method" for the microanalysis of metal ions. Independently, in 1963, Everaerts and Martin started to work systematically with the technique of ITP from both theoretical and practical points of view. In 1968, Verheggen and Everaerts built a capillary tube apparatus for analytical ITP. This became the basis for the commercial application of ITP in food analysis.
development and production of isotachophoretic equipment by LKB-Produkter AB of Bromma, Sweden.

Up to 1970 several names had been used for what Kendall initially called the ionic migration technique - moving boundary method, displacement electrophoresis, steady state stacking, cons electrophoresis, and ionophoresis. In 1970 Haglund, together with a group of researcher in this field, introduced a name upon an important phenomenon of the electrophoretic technique, namely the identical velocities of the sample zones at equilibrium, isotachoelectrophoresis (from Greek: *isos*, equal; *tachos*, speed) or isotachophoresis for short.

2) PRINCIPLES OF ISOTACHOPHORESIS

It is very known that ionic species move under the influence of an applied electric field $E$ with a velocity $v$ of

$$v = m \cdot E \quad (1)$$

where $m$ is the effective mobility of the ionic species, which depends on several factors, e.g. pH, complex formation, type of solvent, etc. Differences in effective mobilities cause differences in velocities and, by utilising, this effect, the ionic species can be separated. Separation techniques based on this principle are called electrophoretic techniques, which can be divided into three main types, viz., zone electrophoresis, moving-boundary electrophoresis and isotachophoresis.

In case of the capillary isotachophoresis the separation of ionic species carries out in a capillary. Either anions or cations can be separated in one run. For the separation, e.g., of anionic species, the capillary and anode compartment are filled with the so-called leading electrolyte, the anion $L$ of which must have a mobility that is higher than that of any of the sample anionic species. The cation $P$ of the leading electrolyte must have a buffering capacity at the pH at which the analyses will be performed. The cathode compartment is filled with the terminating electrolyte, the anion $T$ of which must have a mobility that is lower than that of any of the sample anionic species. The sample is introduced between the leading and terminating electrolyte, e.g., by means of a sample tap or a micro-syringe.

When an electric current is passed through such a system (see Fig. 1a), a uniform electric field strength over the sample zone occurs and hence each sample anionic species will have a different migration velocity according to eqn. (1). The sample anionic species with highest effective mobility will run forwards and those with lower mobilities will remain behind. Hence, both in front of and behind the original sample zone, the moving boundary procedure results in a mixed zone $A + B$ (see Fig. 1b). In this way, the sample zones are sandwiched between the leading and terminating electrolyte. In the mixed zone of the sample, the separation continues and, after some time, when the separation is complete, a series of zones is obtained in which each zone contains only one anionic species of the sample if no anionic species with identical effective mobilities are present in the sample (see Fig. 1c). After this stage, no further changes to the system occur and a steady state has been reached. For this steady state, all zones must have identical migration velocities, determined by the migration velocity of the anionic species of the leading electrolyte. As the anionic species are arranged in order of decreasing effective mobilities, e.g., $m_L > m_A > m_B > m_T$, the electric field strengths $E$ increase on the rear side (see...
Working at a constant driving current, the electric resistance also increases (the conductivity decreases) on the rear side.

The increase in the voltage gradients in the consecutive zones induces two important characteristics of isotachophoretic systems.

![Figure 1](image)

**Figure 1** Separation of a mixture of anions A⁻ and B⁻ according to the isotachophoretic principle a), b), c) and graphical representation of potential \( V \), electric field strength \( E \) and concentration \( c \) for the different zones, moving in the steady state of an isotachophoretic analysis d).

The first characteristic is the "self-correction" of the zone boundaries. When a zone has attained the steady state, the boundary will not broaden further, which again is in contrast to zone electrophoresis, where the peaks are unsharp and broad owing to diffusion phenomena. If an anion remains behind in a zone with a higher electric field strength, then it will acquire a higher
migration velocity according to eqn. (1), until it reaches its own zone. If it diffuses into a preceding zone, where the electric field strength is lower than the value that corresponds to its velocity, its velocity will decrease and it will be overtaken by its proper zone. The second characteristic is the increase in resistance in the preceding zones, and by this feature the zones can be detected with a conductivity detector.

The concentration of all zones is determined by the concentration of the leading electrolyte according to Kohlrausch's regulating function

\[
\frac{C_L}{C_A} = \left(\frac{m_L}{m_L + m_P}\right)\left(\frac{m_A + m_P}{m_A}\right)
\]

where \(C_L\) and \(C_A\) represent the concentration of anionic species L and A in their own zones respectively; \(m_L\), \(m_A\) and \(m_P\) represent the effective mobilities of L, A and P respectively.

**Figure 2** The concentration adaptation

It can be stated that the concentrations in the zones are constant in a given system and that the ionic concentrations decrease to the rear side. The concentration do not depend on the composition of the sample. If the sample is very dilute, then during analyses by other techniques (e.g., zone electrophoresis, HPLC or GLC), the concentrations will be further decreased. In ITP, however, the concentration always attains a value fixed by the composition of the leading electrolyte. A dilute component in a sample is concentrated according to eqn. (2) and correspondingly the concentrated sample is diluted (see Fig. 2). The concentrating effect has extraordinary meaning especially in trace analyses. Up to thousandfold concentrating is usually reached during the ITP separation.
The importance of this phenomenon is clear when it is realised that, because the concentrations in the zones are constant, the length of a zone is a direct measure of the concentration of the sample ionic species.

3) CHOICE OF ELECTROLYTE SYSTEMS

When selecting a system for the isotachophoretic separation of ionic species, it is necessary to obtain differences in effective mobilities of the participating ions. The effective mobility is defined by

$$m_{\text{eff}} = \sum_i \alpha_i \beta_i \gamma_i m_i \quad (3)$$

where $\alpha_i$ is degree of dissociation, $\beta_i$ is degree of complexation with counter or co-counter ion, $\gamma_i$ is a correction factor for influence of relaxation and retardation effects and $m_i$ is the absolute ionic mobility.

The degree of dissociation is the parameter which mainly influences the effective mobility. It is also the factor which in practical work is most liable to be affected experimentally. It is, therefore, of vital importance to control the degree of dissociation when optimising the electrolyte solution for the separation. The degree of dissociation is directly related to the pH and for a weak electrolyte, HA, this can be expressed be the well-known equation

$$\text{pH} = \text{pK} + \log \frac{[A^-]}{[HA]} \quad (4)$$

where K is the dissociation constant for weak acid HA. When the pH is increased, the electrolyte HA is further dissociated and thereby its effective mobility is increased.

According to Kohlrausch equation, the electrolyte conditions in the leading zone determined all the parameters in the succeeding zones. The pH in the leading zone thus determines the degree of dissociation and, thereby, the effective mobility of the following zones.

An analogous situation is in the case of degree of complexation with counter or co-counter ions.

The correction factor for influence of relaxation and retardation effects describes the deceleration effect due to surrounding atmosphere of counter ions and solvation effects depending on used solvent.

The basic rules for the selection of electrolyte systems are described in the Table I. The $\text{H}^+$ or $\text{OH}^-$ ions must be considered as the potential terminator for each cationic and anionic system, respectively.

### TABLE I Basic rules for the selection of electrolyte systems

<table>
<thead>
<tr>
<th>suitable leading ion</th>
<th>Cationic ITP</th>
<th>Anionic ITP</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\text{K}^+$, $\text{NH}_4^+$, $\text{Na}^+$</td>
<td>$\text{Cl}^-$</td>
<td></td>
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### 4) INSTRUMENTATION

The isotachophoretic equipment consists of a narrow-bore tube made of an insulating material (PTFE, FEP, glass) with an inside diameter under 1 mm and with length ca 100 - 500 mm. The applied driving current varies between 1 to 500 mA. According to dimensions of capillary and the conductivity of leading electrolyte (ca 0.01 mol/l) the voltage at the end of analyses reaches up to 30 kV. The detectors that are available can be divided into three main classes:

- **Universal detectors** (conductimeter, potential gradient and thermometer)
- **Specific detectors** (UV absorption, fluorescence, electrochemical, etc.)
- Combinations of both.

When a universal detector is used, the information obtained is directly proportional to the effective mobilities of the ionic constituent, and the information derived therefore has a continuous stepwise character.

The information obtained from a specific detector (e.g., UV detector) is not directly proportional to the effective mobilities of the ionic constituents. The signals from specific detectors depend on the properties of the ionic species in the zones (not depending on the conditions of the leading electrolyte), one cannot always obtained quantitative and qualitative information from which the ionic species can be defined. It is clear that the combination of a universal and a specific detector will give the maximum amount of information in isotachophoretic separations.

### 5) QUALITATIVE AND QUANTITATIVE ASPECTS

The linear trace from the conductivity detector (generally from universal detectors) is a measure of the conductivities of the zones. Hence it is a measure of the effective mobilities of the ionic species in the zones and characterises the ionic species. The "step height" $h_A$ that can be found in the linear trace can be used for the identification of the anion $A$. In practice, the relative step height $\text{RSH}_A$ is used in place of $h_A$. The $\text{RSH}_A$ is ratio of $h_A / h_T$ (see Fig. 3).
Figure 3  The detection of the anions $A^-$ and $B^-$ by a conductimeter and a UV detector (the anion $A^-$ is UV absorbing)

From the height of a step, qualitative information can be deduced. Simultaneous, differentiated recording of conductivity is effective for accurate measurement of zone lengths ($l_A$, $l_B$), i.e., the distances between boundaries of zones. Measurement of peak-to-peak distances in differentiated recording gives accurate zone lengths. The length of a step provides quantitative information. UV rays are made incident from an external source, and each component ion gives specific absorbance. Non-UV absorbing components are not detected. Zones wider than slit width of the detector cell are recorded as trapezoidal peaks, while zones narrower than slit width as triangular peaks. The former are quantitated from the width $l$, and the latter from the height. The Response Factor (RF) is used for the quantitation. The RF equals the concentration (injected by a valve with fixed volume) giving such zone which passes the detector for one second.

6) APPLICATION OF CAPILLARY ISOTACHOPHORESIS IN FOOD ANALYSIS

ITP can be used only for analyses of ionic compounds; in one analytical run either cations or anions are separated and analysed. Non-ionic compounds (sugars, starch, fibre, etc.) which are frequently bulk components of the sample in question, do not move electrophoretically, and thus do not interfere with the analysis of ionic compounds. At present, ITP is predominantly carried out in water or in the mixture of water with some organic solvents and is applied mainly to water-soluble compounds.
The capillary ITP has advantages, such as high resolution, short analysis time (< 45 min), sensitivity (detection limit ~ 10^{-11} mol), easy quantification, excellent reproducibility (RSD < 2% rel.), the least necessity of sample pretreatment (typically only dilution and filtration), and low running cost.

Compounds which are usually determined in foodstuffs can be divided into three groups:

A) Naturally occurring food constituents

- macroelements (potassium, sodium, calcium, magnesium)
- inorganic acids (halides, sulphate, sulphite, nitrate, nitrite, phosphate)
- organic acids (oxalic, citric, isocitric, lactic, malic, tartaric, succinic, formic, acetic, propionic, butyric
- aminoacids (aspartic, glutamic, asparagine, glutamine, lysine, arginine)
- water soluble vitamins (ascorbic and dehydroascorbic acid, thiamine (B1), riboflavin (B2), pyridoxine (B6), niacin)
- biogenic amines (histamine, putrescine, cadaverine)
- proteins (lysozyme, ovalbumine)
- antinutrients, toxins (phytic acid, glucosinolates, tetrodotoxin)

B) Food additives

- preserves (benzoic, sorbic, salicylic, formic and propionic acids, esters of p-hydroxybenzoic acid, EDTA)
- flavour enhancers (glutamate, guanosine-5'-phosphate, inosine-5'-phosphate, saccharin, cyclamate, Acesulfam K, Aspartame, quinine)
- food water soluble artificial dyes (tartrazine, amaranth)

C) Food contaminants

- pesticides (alkanephenoxycarboxylic acids, triazines, pyrethroides)
- heavy metals (Cd, Zn, Cu, Pb)

A) Naturally occurring food constituents

By the help of ITP technique macroelements such as potassium, sodium, calcium and magnesium could be determined practically in all kinds of foodstuffs. An extraction of solid samples by water or mineral acid (HNO_3, HCl) or a dilution of liquid samples are the only treatment of samples prior to ITP analysis. As example of such analysis is determination of elements mentioned above in raw juice from sugar factory (Fig. 4) carried out in cationic ITP analysis using 7.5 mM - sulphuric acid as leading electrolyte and 10 mM - tri-lithium citrate as terminating one. Within 20 minutes one gets information about K, Na, Ca and Mg content in sample.
Figure 4 Isotachophoretic determination of alkali and alkaline earth metals.
Isotachophoregram of raw juice (5g/100 ml). Leading electrolyte consisted of 7.5 mM
- sulphuric acid and 10 mM - tri-lithium citrate as terminating electrolyte. Initial
driving current was 120 mA and detection one 60 mA.

Inorganic acids, such as halides, sulphate, sulphite, nitrate, phosphate are easily
determined by ITP in tap water, mineral water, vegetable, fruits and milk. As example the
determination of such compound in tap water is given in Fig. 5. The leading electrolyte consists
of 10mM - HCl + beta-Alanine + Bis-Tris Propane, pH 3.6 and the terminating one of 10 mM -
Citric acid. In one analysis chloride, nitrate, sulphate, fluoride and phosphate are determined
within 25 minutes.
Figure 5  Analysis of drinking tap water on inorganic anions content. Analysis were done by the help of a column coupling isotachophoregraph. A solution of 10 mM - HCl + 3 mM - Bis Tris Propane + □ - Alanine, pH 3.55 served as leading electrolyte and 10 mM - Citric acid as terminating one.

For the determination of organic acids in various foodstuffs ITP has been widely used. Lactic, citric, isocitric, malic, succinic, tartaric, oxalic, formic, acetic, propionic, butyric, etc. were determined in wines, beverages, vegetable, fruits, breads and other food samples. In Fig. 6 the ITPgram of Tokay wine is given. Leading electrolyte with chloride as leading ion and glycyglycine, pH 3 and benzoic acid as terminator were used for such analysis. The acid spectrum is important for control of wine maturing.

The water soluble vitamins, such as ascorbic and dehydroascorbic acid (C), thiamine (B1), pyridoxine (B6) and niacin (PP) could be analysed by ITP in juices, fruits and concentrates for beverages. As example the determination of ascorbic acid in lemonade GREPUS is showed on Fig.7. Besides ascorbic acid the determination of other acids is possible (citric) as well as the sorbic acids as preserving agent. One analysis takes less than 30 minutes. Analysis were done with 10 mM - HCl + beta-Alanine, pH 3.8 as leading and 10 mM - caproic acid as terminating electrolyte.
Figure 6 Isotachophoretic determination of organic acids in Tokay wine. Leading electrolyte consisted of 10 mM - HCl + Glycylglycine, pH 3 and terminating one of 10 mM - Benzoic acid. Phosphoric (1), tartaric (2), citric (3), malic (4), citramalic (5), lactic (6), gluconic (7) and succinic acid (8) were determined in one run.
Determination of vitamin C (ascorbate) in lemonade GREPUS. Besides ascorbate determination of sorbate (preserve) is possible in one analysis. For composition of electrolyte system see in text.

In fish and fish’s products the determination of histamine and other biogenic amines (cadaverine, putrescine) is easily done by cationic ITP analysis at high pH (9.5).

Using cationic electrolyte system at pH 5 the determination of lysozyme in eggs is possible and employing anionic electrolyte system (high pH 9) ovalbumine can be determined.

Capillary ITP was used for determination of natural antinutrients such as phytic acid and glucosinolates. In Fig. 8 the ITPgram of anionic analysis of glucosinolates sinalbin and sinigrin in mustard seeds is given. Glucosinolates were extracted from seeds by hot water and after filtration directly analyse by ITP. The leading electrolyte consists of mixture of hydrochloric acid and glycylglycine as counter ion giving pH 3 and caproic acid serves as terminating electrolyte.

Figure 7

In Fig. 8 the ITPgram of anionic analysis of glucosinolates sinalbin and sinigrin in mustard seeds is given. Analysis conditions see in text.

Toxin called tetrodotoxin (perhydroquinazoline) occurring in sea-fish that are very popular in Japan and China was determined by cationic ITP using potassium acetate buffer (pH 6) as leading electrolyte and acetic acid as terminating on. The mixture of water and dioxane (50 %) was used as solvent.

B) Food additives

Figure 8

Isotachophoregrams of anionic analysis of glucosinolates sinalbin and sinigrin in mustard seeds. Analysis conditions see in text.
Plenty of chemical substances serve as food additives such as preserves, artificial sweeteners, flavour enhancers, artificial dyes, stabilisers, etc. There are limits (qualitative and/or quantitative) of addition of such compounds varying from country to country. To some foodstuffs (e.g. addition of preserves into beer) is not allowed to apply any additives. So, that is why, to have a control over the addition of compounds in question. Capillary ITP offers the determination of such compounds.

Organic acids such as benzoic, sorbic, formic or propionic acids are commonly used as food preserves for beverages, jam, mayonnaise and other foodstuffs. These compounds could be easily determined by anionic ITP after extraction of solid sample (jam, bread) with water or after dilution of a liquid sample. EDTA is commonly used as a stabiliser for mayonnaise and salad dressings. This compound is determined as complex with Fe++ by anionic ITP. Water soluble artificial dyes (tartrazine, amaranth) are easily determined after extraction of a sample by anionic ITP. Glutamate, guanosine-5'-monophosphate and inosine-5'-monophosphate are added to soups, sauces, salad dressings as flavour enhancers. After extraction of solid samples with hot or cold water or after dilution of liquid samples these compounds could be very easily determined by anionic ITP in one run at 10 ppm levels. Quinine in Tonic water were determined after degassing (removal of carbon dioxide) by cationic ITP analysis. Artificial sweeteners Acesulfam K, saccharine or Aspartame (NutraSweet) were determined by anionic ITP in low-calories products or in foods for diabetics. As an example of such kind of analysis the determination of Aspartame in Coca-Cola depicted on Fig. 9 is given.
C) Food contaminants

Some of pesticides as food contaminants can be easily determined by capillary ITP. The pesticides in question could be divided into three groups according their behaviour in electric field, i.e., anionic, cationic and non-ionic. Anionic capillary ITP was used for the determination of alkanephenoxy-carboxylic acid, alkanephosphonic acids etc.. On Fig. 10 the determination of glyphosate (GLYPH) and its metabolite aminomethane phosphonic acid (AMPA) in apples is given. The solution of 10 mM - HCl + L-histidine, pH 6 served as leading electrolyte and morpholinoethanesulphonic acid served as terminator. Triazines as representative of cationic pesticides were determined by cationic ITP in milk and other foodstuffs. Pyrethroids are non-ionic pesticides. The determination of such compound could be carried out indirectly after alkaline hydrolysis. Phenoxybenzoic, dichlorochrysanthemic or chrysanthemic acids as products of such hydrolysis were determined by anionic ITP at leading electrolyte pH 4.5.
Heavy metals (Cd, Cu, Pb, Zn) and aluminium were determined by cationic ITP. The metals are releases from solid samples by nitric acid (mineralization) and after pH adjusting by ammonium acetate trapped and concentrated on selective sorbent (Spheron-OXIN). In the case of liquid samples the mineralization step is omitted and metals are concentrated on selective sorbent (Solid Phase Extraction). The trapped metals are eluted from sorbent by 1 M-HCl, evaporate to dryness and dissolved in water. The obtained solution is analysed by cation ITP. ITPogram of a model mixture is given on Fig. 11.

![ITPogram of a model mixture](image)

**Figure 11** Isotachophoregram of model mixture of 12 elements (0.1mM of each). Leading electrolyte was 20 mM - ammonium acetate + acetic acid + 20 mM - α-hydroxyisobutyric acid (HIBA), pH 4.3 and terminating electrolyte comprised of 10 mM - acetic acid.