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Determination of anionic feed additives by two-dimensional capillary isotachophoresis

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Abstract

An isotachophoretic method was developed for the determination of organic and inorganic acids (benzoic, citric, formic, fumaric, lactic, malic, phosphoric and tartaric acid) in feed additive preparations. The resulting isotachophoretic measurements were carried out two-dimensionally in a column-coupling instrument equipped with contact conductivity (in both capillaries) and UV absorbance (in the analytical capillary) detectors, respectively. Preseparation was run in the first capillary at pH 6.1 of the leading electrolyte. The pH of the leading electrolyte of the second capillary was 2.5, with β-cyclodextrin added to improve the separation selectivity for tartrate and fumarate. The terminating electrolyte was 5 mmol l^{-1} caproic acid in all cases. The precision of the determinations including sample pretreatment, expressed as the relative standard deviation, varied between 3.3 and 7.9%, depending on the concentration levels of the analytes. The accuracy, determined by the recovery of analytes added, was typically in the order of 94 to 98%.

Keywords: Feed additives; Benzoic acid; Citric acid; Formic acid; Fumaric acid; Lactic acid; Malic acid; Phosphoric acid; Tartaric acid; Organic acids

1. Introduction

Additives are substances which are merged with animal feeds in order to influence the character of the nutrient or to effect animal growth. Many kinds of feed additives are applied, e.g. antioxidants, appetizing or aromatic substances, colourants, emulsifiers, preservatives, vitamins or medical compounds. According to the European Community Directive 70/524/EEC [1], qualitative and quantitative methods must be provided for the determination of the additives in premixtures and feeding stuffs.

Mixtures of various organic and inorganic acids (benzoic, citric, formic, fumaric, lactic, malic, phosphoric and tartaric) serve as feedstuff preservatives and/or regulators of acidity. These additives support digestion and contribute to the stabilization of the digestion microflora.

For the determination of these anions, classical analytical methods (volumetry and spectrophotometry) can be used [2–4]. For some acids (citric, lactic, malic, tartaric and lactic) enzymatic methods have been developed also [5,6]. These analytical methods, however, only allow the determination of

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individual species and often show interferences by other sample components. More advantageous is the use of methods where several compounds can be separated and determined simultaneously, e.g. gas chromatography of the analyte derivatives [7] or ion-exclusion chromatography [8].

Different modes of capillary electrophoresis can also be applied for the determination of anions in various mixtures, as described in fundamental reviews concerning these methods [9]. It must be pointed out, however, that all anions of the group considered has not been obtained with any method described so far and consequently, no validation for feed additive samples has been carried out.

In this paper an isotachophoretic method for the analysis of such commercial feed additive mixtures was established. As it was found that the analytes cannot be separated and therefore determined in a simple buffering system, because their effective mobilities are too similar, approaches to increase the separation selectivity were investigated by complexation with several inorganic and organic complexing cations, and formation of host–guest complexes. These approaches allowed us to establish separation conditions based on two-dimensional isotachophoresis which enabled us to determine all the analytes quantitatively.

2. Experimental

2.1. Chemicals

The following chemicals were used: hydrochloric acid, sodium hydroxide (both Titrisol, 0.1 mol l^{-1} solutions, analytical-reagent grade, Merck, Darmstadt, Germany), benzoic acid, citric acid, D,L-malic acid, sodium dihydrogenphosphate, L-tartaric acid, sodium formate, lithium lactate (all p.a., Merck); 1,3-bis[Tris(hydroxymethyl)methylamino]propane (bis–Tris–propane, BTP 99+%), caproic acid (99.5+%) (Aldrich, Steinheim, Germany); glycine (p.a.), methylhydroxyethylcellulose (MHEC, 30 000, all Serva, Heidelberg, Germany); β-cyclodextrin (Sigma, Deisenhofen, Germany), fumaric acid (p.a., Loba Chemie, Vienna, Austria). Double distilled water was used for the preparation of buffers.

Samples of feed additives (regulators of acidity and preservatives) for analysis were purchased from commercial suppliers.

2.2. Apparatus

The column-coupling instrument (ItaChrom EA 101, Merck) was equipped with two serially coupled capillaries made of a fluorinated ethylene–propylene copolymer (preseparation capillary 160 mm length, 0.8 mm I.D., analytical capillary 90 mm length, 0.3 mm I.D.). Detection was carried out with contact conductivity detectors (at the preseparation and at the analytical capillary) and with a UV absorbance detector (254 nm, at the analytical capillary), respectively. The samples were injected via a sample valve of 25 μl fixed volume.

2.3. Conditions of analysis

The electrolyte systems are described in Table 1. In all cases preseparation in the first capillary was carried out with system 1. The analytical capillary

Table 1
Buffering electrolyte systems applied

System	Leading electrolyte (LE)[a]	Additive in LE	pH of LE	Terminating electrolyte
1	10 mmol l^{-1} HCl +5.6 mmol l^{-1} BTP	none	6.1	5m*M* caproic acid
2	20 mmol l^{-1} HCl +30 mmol l^{-1} Gly	none	2.5	5m*M* caproic acid
3	20 mmol l^{-1} HCl +30 mmol l^{-1} Gly	5 mmol l^{-1} β-CD	2.5	5m*M* caproic acid
4	20 mmol l^{-1} HCl +30 mmol l^{-1} Gly	10 mmol l^{-1} β-CD	2.5	5m*M* caproic acid
5	20 mmol l^{-1} HCl +30 mmol l^{-1} Gly	15 mmol l^{-1} β-CD	2.5	5m*M* caproic acid
6	20 mmol l^{-1} HCl +30 mmol l^{-1} Gly	20 mmol l^{-1} β-CD	2.5	5m*M* caproic acid
7	20 mmol l^{-1} HCl +30 mmol l^{-1} Gly	25 mmol l^{-1} β-CD	2.5	5m*M* caproic acid

[a] MHEC (methylethylhydroxy-cellulose) was added to all leading electrolytes at a concentration of 0.1% in order to reduce the zeta potential.

was run with systems 1–7. The driving current was initially 250 μA and was 150 μA during detection in the preseparation capillary. It was initially 50 μA for system 1 and 70 μA for the other systems in the analytical capillary, respectively. During detection, the current was reduced to 40 μA (system 1) or to 30 μA (other systems).

2.4. Calibration

An external standard calibration method was used with free acids or the respective salts (see Section 2.1). Calibration analyses were carried out in both capillaries. In the preseparation capillary, five calibration points were measured within the calibration range 0.05 mmol l^{-1}–2.5 mmol l^{-1}. In the analytical capillary, six calibration points were measured within the range 0.02 mmol l^{-1}–0.12 mmol l^{-1}. The upper calibration limits were those concentrations where particular isotachophoretic zones remained mixed.

2.5. Sample preparation

100 mg of a sample was weighed into a 500-ml volumetric flask. A 10-ml volume of a 0.1 mol l^{-1} NaOH solution was added and the mixture was placed into an ultrasonic bath for 10 min. Then, the volumetric flask was filled to 500 ml and was placed again into the ultrasonic bath for another 20 min.

3. Results and discussion

3.1. Migration properties of the anionic analytes in different electrolyte systems

In order to get information about the migration behaviour of the analytes with regard to the pH of the buffer, a computer program was used to simulate their relative migration properties, the RE values, in the pH range between 2 and 10. The counter-ions used were glycine (pH 2.0–2.5), β-alanine (pH 3.0–3.5), ε-aminocaproic acid (pH 4.0–4.5), creatinine (pH 5.0), histidine (pH 5.5–6.0), imidazole (pH 6.5–7.0), Tris (pH 7.5–8.0), ammediol (8.5–9.0) and ethanolamine (pH 9.5–10.0). Aqueous solutions of the leading electrolyte have been taken into account for this simulation, and no interaction of the counter-ion with the analyte, e.g., by complexation, was considered. The computer program was based on the mathematical model published by Hirokawa and Kiso [10]. As a result of these calculations, the dependence of the RE values (which is the ratio of the conductivity of the leading electrolyte and the sample zone, respectively) on the pH of the leading electrolyte is plotted in Fig. 1. The RE value serves as the criterion for the separability of two isotachophoretic zones. If the difference between the

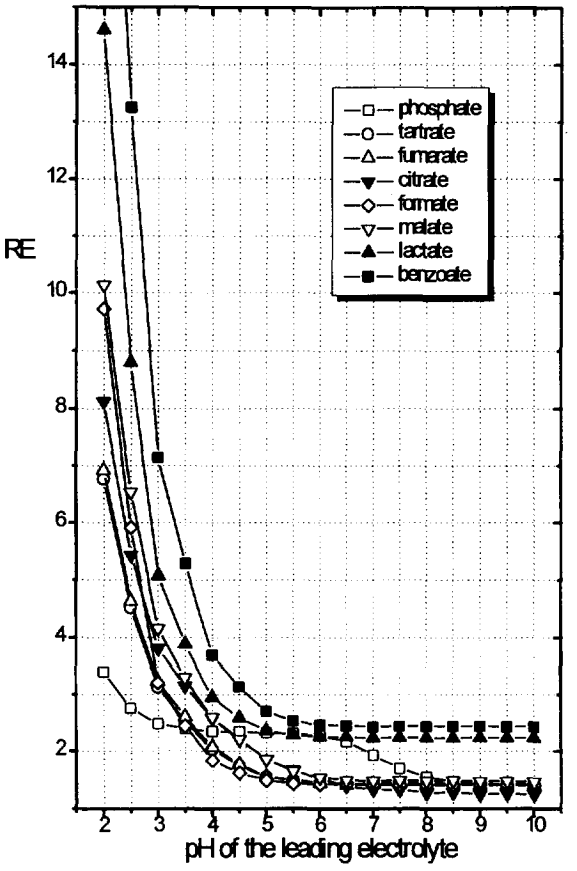

Fig. 1. Results of the computer simulation of the migration behavior of the considered anions as a function of the pH of the leading electrolyte. The RE value (i.e. the ratio of the conductivity of the leading electrolyte and sample zone, respectively) serves as the criterion for the separation capacity of the isotachophoretic zones. For better visualisation of the migration properties, the RE value of benzoate at pH 2.0 (RE=21.9) is not displayed. A difference in the RE values of less than 0.15 for a certain pair of solutes indicates that the ions are almost inseparable.

RE values is less than 0.15 there is a low probability that the zones will be separated, as pointed out in [11].

From this picture it is apparent, that benzoic and lactic acid are separated from the other anions over the full pH range. It can also be derived that the degree of separation of the anions under consideration decreases with increasing pH of the leading electrolyte, in accordance with findings based on chemometric methods [12]. In the pH range of the leading electrolyte (between 2 and 2.5), nearly all anions are likely to be separated; fumarate and tartrate, however, will comigrate in a mixed zone. In the pH range between 2.5 and 5, the migration order changes for several solutes and the probability of the occurence of mixed zones is rather high. At higher pH values of the leading electrolyte, the RE values of citrate, malate, tartrate, fumarate and formate are nearly identical and therefore the separation of these anions cannot be established. From pH 8 on, phosphate is found also in this group of unresolved solutes, because of its practically complete dissociation to the second acidic function.

The most important information obtained by this simulation is that the solute pair, tartaric and fumaric acid, will not be separated by varying the pH of the leading electrolyte. This is due to the very similar pK values of these solutes (3.019 and 4.384 for fumaric acid and 3.036 and 4.366 for tartaric acid) and by their very similar absolute mobilities ($31.0 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1} and $61.2 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1} for fumaric acid and $32.6 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1} and $60.7 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1} for tartaric acid [11]. It should be mentioned in this context that the separation of tartaric and fumaric acid has not been published yet by any capillary electrophoretic method.

3.1.1. One-dimensional isotachophoresis

Several approaches can be carried out to enhance the separation selectivity with regard to fumaric and tartaric acid, for example, complexation with bi- and oligovalent metal or organic cations. In the present investigation aluminium-, calcium-, copper-, mercury (II)-ions and bis–Tris–propane at pH 6.1 have been applied for this purpose. Resolution of fumaric and tartaric acid was, however, not reached in any case.

As an example, the isotachopherogram of a mixture of all anions obtained in a separation system with bis–Tris–propane as the counter-ion with complexing ability (number 1 in Table 1) is presented in Fig. 2, showing the results from the conductivity and the UV absorbance detectors. This system is normally used for wine analysis [13] and was also previously applied to the determination of phytic acid and phosphate in cereals and feeds [14]. The stationary mixed zone of tartaric and fumaric acid is apparent from both detector records (fumaric acid significantly absorbs UV radiation at 254 nm, in contrast to tartaric acid). Except for this pair, all other anions are clearly separated and form stable isotachophoretic zones. This fact can also be concluded from the calibration results given in Table 2, because the correlation coefficients indicate the high linearity of the graphs.

3.1.2. Two-dimensional isotachophoresis

For tartaric and fumaric acid, the structural differences of these solutes was taken as a tool for the adjustment of the separation selectivity. At low pH, the degree of dissociation of these anions is reduced, supporting preferred hydrophobic interaction of fumaric acid (based on the C–C double bond) with

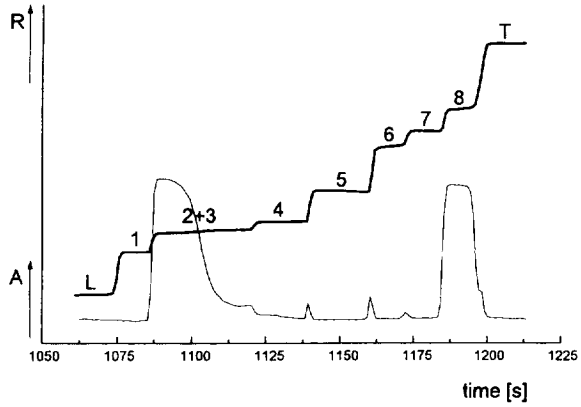

Fig. 2. Separation of a model anion mixture (0.1 mmol l^{-1} each) by the serially coupled preseparation and analytical capillary. Both capillaries were filled with electrolyte system 1 (Table 1). The thick line shows the record from the contact conductivity detector (R, resistance), the thin line gives the record from the UV absorbance detector (A). L = leading ion (chloride), 1 = formate, 2 and 3 = mixed zone of tartrate and fumarate, 4 = malate, 5 = citrate, 6 = lactate, 7 = phosphate, 8 = benzoate and T = terminating ion (caproate).

Table 2
Results of the calibration of the anions using the zone lengths measured with the contact conductivity detectors positioned in the first (preseparation) capillary and in the second (analytical) capillary, respectively

	First detector			Second detector		
Anion	n	Calibration equation	r	n	Calibration equation	r
Benzoate	5	$y = 42.10\,x + 0.32$	0.99997	6	$y = 241.3\,x + 0.5^a$	0.99844
Citrate	5	$y = 79.20\,x + 0.82^a$	0.99944	4	$y = 413.5\,x - 0.1^a$	0.99929
Formate	5	$y = 34.14\,x - 0.17^a$	0.99985	4	$y = 217.0\,x + 0.3^a$	0.99866
Fumarate	-	m	m	6	$y = 347.6\,x + 0.6^a$	0.99951
Lactate	5	$y = 42.19\,x + 0.28^a$	0.99960	6	$y = 256.0\,x + 1.9^a$	0.99793
Malate	4	$y = 66.03\,x + 0.26^a$	0.99998	6	$y = 338.6\,x + 2.5$	0.99793
Phosphate	5	$y = 47.4\,x + 0.81$	0.99997	6	$y = 364.1\,x + 0.4^a$	0.99988
Tartrate	-	m	m	6	$y = 370.1\,x + 1.7$	0.99990

The calibration relates the step length in s (y) to the concentration of the anion (x) in mmol l^{-1}.
m = anions comigrated in the mixed zone, n = number of calibration points, r = correlation coefficient.
[a] = Intercept not significantly different from zero.

an appropriate additive like cyclodextrine (CD) in the leading electrolyte, compared to the more hydrophilic character of tartaric acid.

Cyclodextrines are well known for the separation of positional [15,16] and optical [17] isomers. α-CD has been used also in isotachophoresis in order to improve the selectivity of separation for various inorganic anions [18]. In this work however, we tried to use β-CD to increase the degree of separation of tartrate and fumarate, based on the different hydrophobicities of these solutes at low pH. A higher affinity of fumaric acid to β-CD compared to tartaric acid was expected.

Thus, isotachophoretic experiments were carried out two-dimensionally by transferring the zones after the first, preseparation capillary (where the zones were recorded by the first conductivity detector) to the analytical one. Preseparation (first dimension) was run with electrolyte system 1 (at pH 6.1) and the analytical capillary (second dimension) with those at pH 2.5 (systems 2–7 in Table 1).

Because of the significantly smaller volume of the analytical capillary compared to the preseparation one (see Section 2.2.), the concentration of hydrochloric acid in systems 2–7 was increased to 20 mmol l^{-1} to enhance the separation capacity of the whole system. Another way of increasing the separation capacity would be the use of a longer capillary or a capillary with the same diameter as the preseparation one (which was, however, not part of the instrumental configuration). The use of a longer capillary would be rather problematic, because even the short analytical capillary caused a high voltage value when run with caproic acid as the terminating electrolyte in systems with a pH of 2.5. Also, caproic acid interacted very strongly with β-CD. Both effects led to a very low effective mobility of this terminating anion. The converted conductivity signal difference between the leading ion (chloride) and the terminating ion (caproate), measured on the lower detector, increased from 2.5 V (at 0 mmol l^{-1} β-CD) to 9.2 V (at 25 mmol l^{-1} β-CD). Thus the voltage at the end of the analysis was higher than 12 kV (at a concentration of β-CD of 25 mmol l^{-1}).

The result of the investigation is presented in the Fig. 3. From the UV detector record, it is apparent that even a concentration of 5 mmol l^{-1} of β-CD causes the formation of isotachophoretically stable zones of tartaric and fumaric acids. The conductivity difference is, however, not sufficiently large to get a differentiation on the conductivity detector. The conductivity differences increase with increasing concentration of β-CD and at 15 mmol l^{-1} a differentiated conductivity signal is obtained. The best resolution of tartaric and fumaric acid was achieved at a β-CD concentration of 25 mmol l^{-1} (system 7). This system is, however, rather problematic, because the high concentration of β-CD results in an oversaturated solution; after a certain time, crystals of β-CD are precipitated in the leading electrolyte. To avoid such problems, system 6 with 20 mmol l^{-1} β-CD was used instead [19].

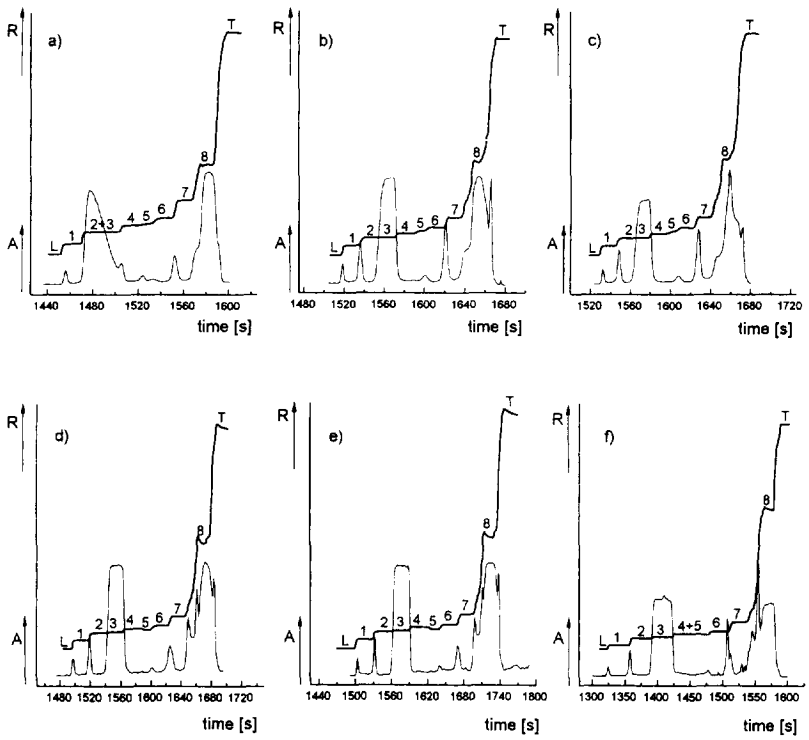

Fig. 3. Two-dimensional separation of a model anion mixture (0.05 mmol l^{-1} each) by coupling the preseparation capillary (filled with system 1, pH 6.1) and the analytical capillary (with systems 2–7 at pH 2.5) in series. Electrolyte in the analytical capillary contains (a) 0 mmol l^{-1}, (b) 5 mmol l^{-1}, (c) 10 mmol l^{-1}, (d) 15 mmol l^{-1}, (e) 20 mmol l^{-1} and (f) 25 mmol l^{-1} β-CD. Only records from the analytical capillary are shown. The thick line gives the record from the contact conductivity detector, the thin line shows the record from the UV absorbance detector (254 nm). L = leading ion (chloride), 1 = phosphate, 2 = tartrate, 3 = fumarate, 4 = citrate, 5 = formate, 6 = malate, 7 = lactate, 8 = benzoate and T = terminating ion (caproate).

From the picture it is apparent that benzoic acid exhibits the strongest interaction of all anions investigated.

It was further found that the separation capacity increases with increasing concentration of β-CD. For all these reasons a concentration of 20 mmol l^{-1} β-CD (according to system 6) was chosen as a compromise for the analysis of the real samples. With this system the calibration was carried out (see Section 2.4.) and from the correlation coefficients of the calibration equation (see Table 2) it can be stated that the isotachophoretic zones are stable.

3.2. Analysis of real samples in the one- or two-dimensional mode

The solid samples of feed additives were analysed after dissolution and occasional dilution. The preseparation capillary, run with system 1 (as the first dimension), enabled the separation and the determination of formic, malic, citric, lactic, phosphoric and benzoic acid to be carried out. The analytical capillary, run with system 6 (as the second dimension), enabled the separation of all selected acids to be carried out. The practical use of system 6 as a one-dimensional ITP is, however, very limited due to its low separation capacity for citrate and formate. A two-dimensional isotachophoretic approach is more advantageous. If samples do not contain fumaric and/or tartaric acid (which can be concluded already from the isotachopherogram obtained from the preseparation capillary), it is not necessary to use the second dimension for each sample. Such an example is given in Fig. 4, where the analysis of sample A is shown. As no zone corresponding to tartaric and/or fumaric acid was found, zone transfer to the ana-

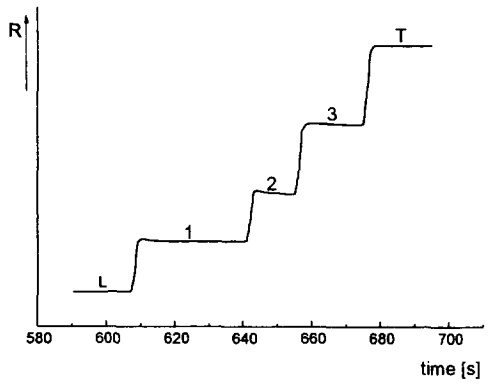

Fig. 4. Analysis of a real feed additive, sample A. The isotachopherogram from the preseparation capillary is shown. In this case no transfer to the analytical capillary was necessary (no fumarate or tartrate is present in the sample). L = leading anion (chloride), 1 = formate, 2 = citrate, 3 = phosphate and T = terminating anion (caproate).

lytical capillary was not necessary, and the whole analysis took less than 12 min.

In Fig. 5, the analysis of sample B is shown. In this case, zone "2" corresponds to tartaric and/or fumaric acid and therefore the sample was transferred to the analytical capillary, where this zone was identified as consisting only of fumaric acid. Such a two-dimensional analysis takes about 25 or 30 min, depending on the composition of the sample. Table 3 summarizes results of real sample analyses.

For the validation of the isotachophoretic method,

Table 3
Content of acids in real samples

Acid	Content % (w/w)	
	Sample A	Sample B
Benzoic acid	–	15.0 ± 1.2
Citric acid	20.0 ± 1.5	–
Formic acid	21.8 ± 0.5	16.5 ± 0.7
Fumaric acid	–	42.9 ± 1.5
Lactic acid	–	–
Malic acid	–	–
Phosphoric acid	19.2 ± 0.5	–
Tartaric acid	–	–

– = not present in the sample.
The values given are the average of three independent measurements.

the accuracy and precision of the analysis was determined. As a result, the typical recovery of the anions was from 94 to 98%, when the amounts of anions added did not exceed about half of the determined content. The only exception was citric acid, for which 82% recovery was found, for reasons that were not determined further. This lower recovery may be caused by a loss due to the complexing ability of this acid for bi- or oligovalent ions possibly present in the sample.

The precision of the method, obtained from ten repetitive determinations (including sample pretreatment) expressed as the relative standard deviation measured with sample B was 4.1% for formic acid, 3.3% for fumaric acid and 7.9% for benzoic acid.

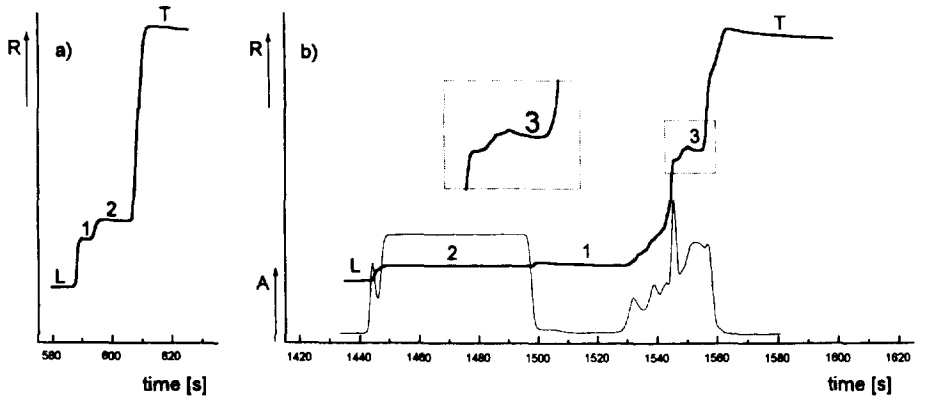

Fig. 5. Analysis of a real feed additive, sample B. The preseparation capillary was run with system 1 (pH 6.1), the analytical capillary with system 6 (pH 2.5, 20 mmol l^{-1} of β-CD added). All zones from the preseparation capillary (a) were transferred to the analytical one (b). Thick lines are records from the contact conductivity detectors and the thin line is the record from the UV absorbance detector (254 nm). L = leading anion (chloride), 1 = formate, 2 = fumarate, 3 = benzoate and T = terminating anion (caproate).

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