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Arlinger

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[54] **METHOD AT ISOTACHOPHORETICAL SEPARATION TO DETECT SPECTROPHOTOMETRICALLY ZONE BOUNDARIES OBTAINED**

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[52] **U.S. Cl.** ..... **204/180 R;** 204/299

[51] **Int. Cl.²** **B01K 5/00**

[58] **Field of Search** ..... 204/180 R, 180 S, 299

[56] **References Cited**

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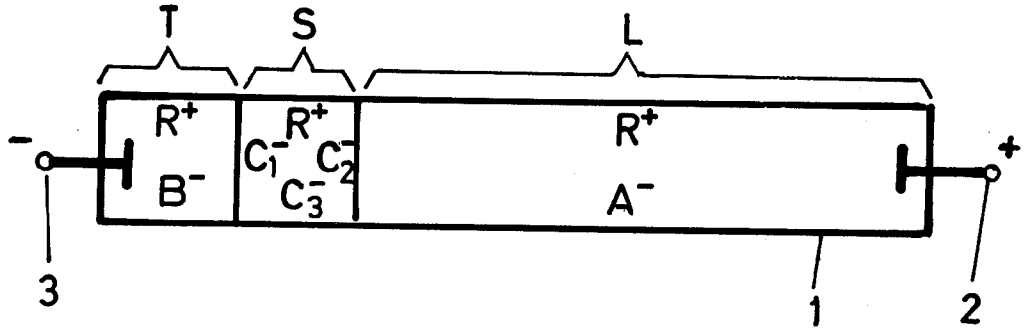
Assistant Examiner—A. C. Prescott

[57] **ABSTRACT**

A method for spectrophotometrically detecting constituents in an isotachophoretical column, consists in employing a counter-iron whose molar absorptivities differ within the range between acidic and basic conditions existing in the column.

2 Claims, 10 Drawing Figures



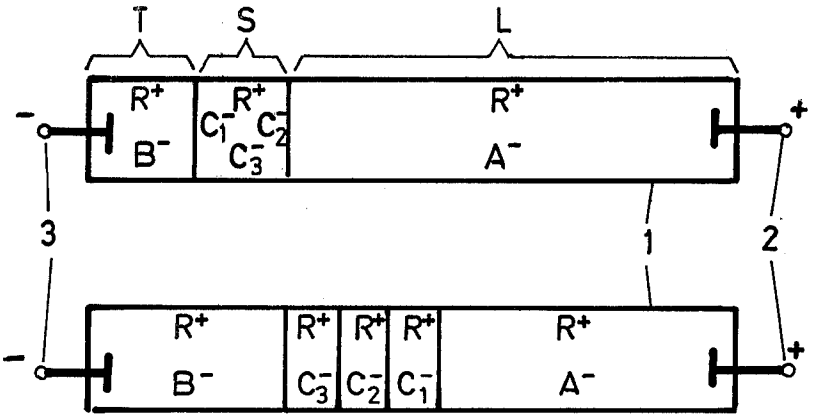


Fig.1

Fig.2

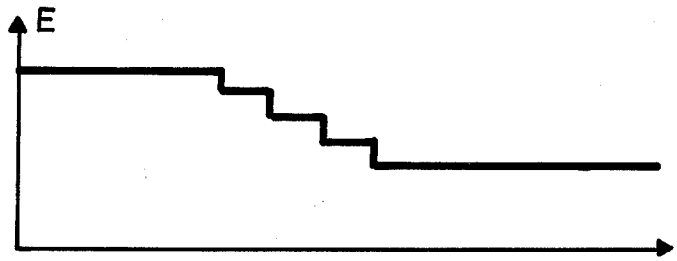


Fig.3

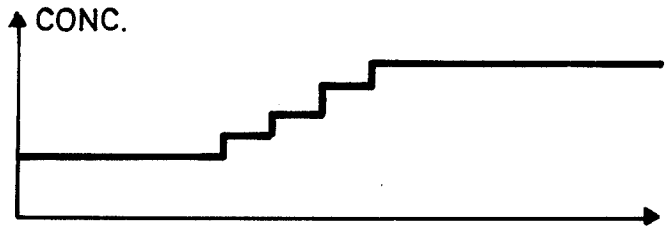


Fig.4

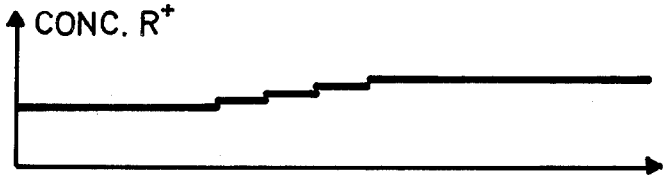


Fig.5

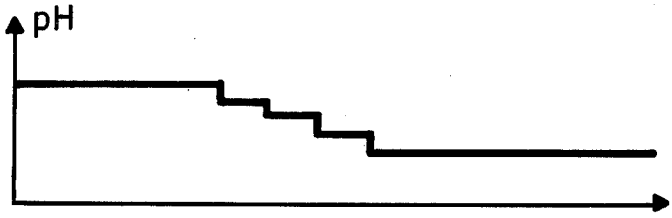
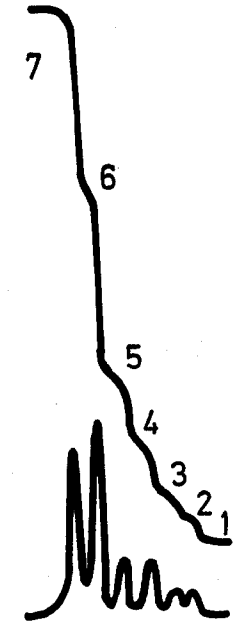
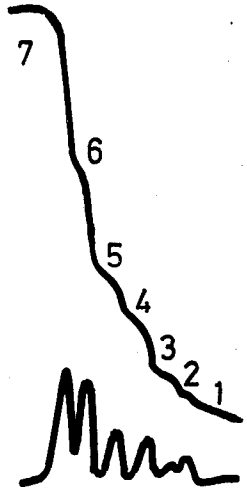


Fig.6





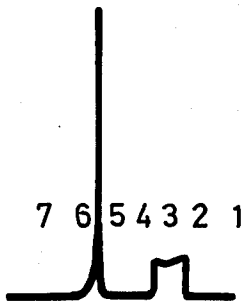


Fig. 7a

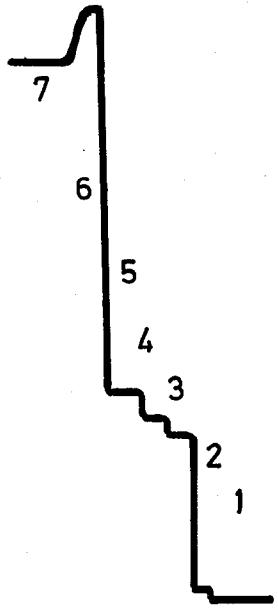


Fig. 7b

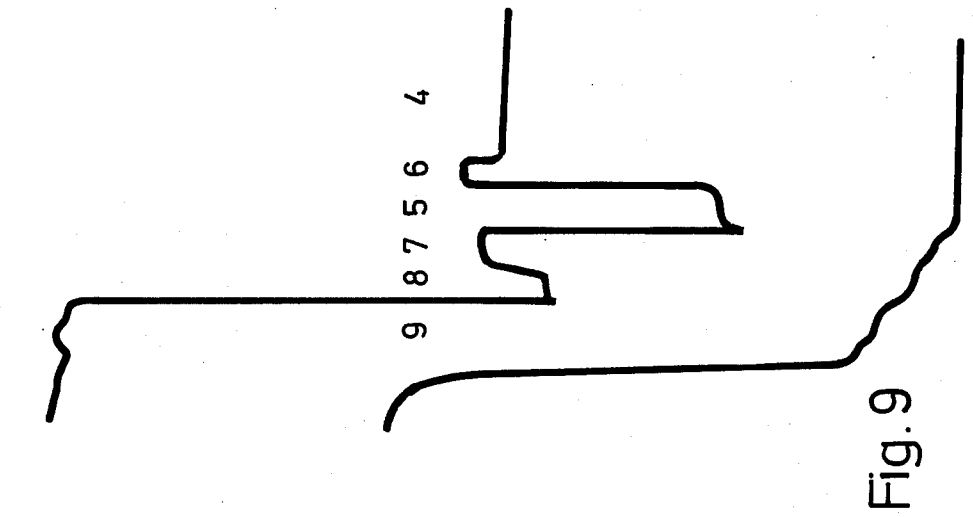


Fig. 9

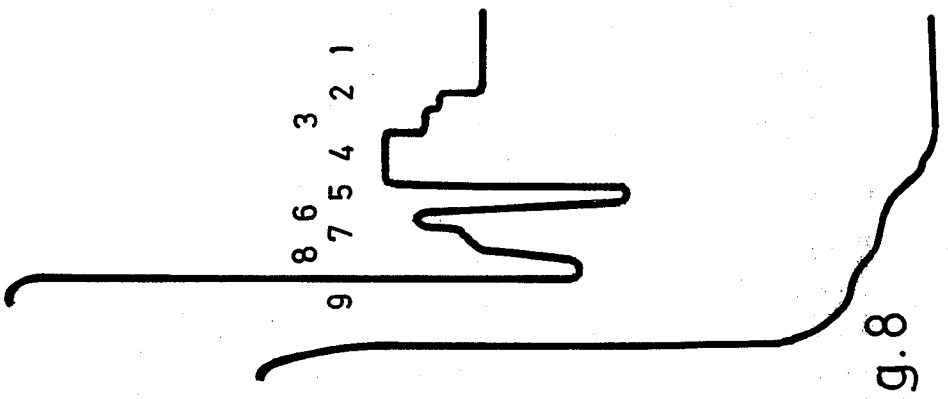


Fig. 8

METHOD AT ISOTACHOPHORETICAL SEPARATION TO DETECT SPECTROPHOTOMETRICALLY ZONE BOUNDARIES OBTAINED

The present invention relates to a method at isotachphoretical separation to detect spectrophotometrically within a wave-length interval zone boundaries obtained.

At isotachphoresis a separation of an ionized sample containing ions of a certain polarity is carried out in that way that the sample is introduced into a column, arranged between two electrodes, a leading electrolyte being introduced into that part of the column which is present between the sample and the electrode towards which said ions are migrating when a voltage is applied to the electrodes, said leading electrolyte containing ions of the same polarity but with higher mobility than the sample ions, and a terminating electrolyte being introduced into that part of the column which is present between the sample and the other electrode, said terminating electrolyte containing ions of said polarity with lower mobility than those of the sample ions. Throughout the whole column is also introduced an ion species having opposite polarity, a so-called counter-ion. The counter-ion suitably has buffering properties. Isotachophoresis is more closely described e.g. in Analytica Chemica Acta 38 (1967) pp 233–237, termed "Displacement Electrophoresis" and in the patent specification . . . . . (corresponding to Swedish Pat. No. 340.376).

At an isotachophoretical separation of ions, sharp boundaries between the zones formed by the ions are obtained. When an isotachophoretical separation is carried out some kind of detector is usually arranged at the column for detection of the zone boundaries obtained. An object of this detection is to indicate when sharp zone boundaries have been formed between all sample zones, indicating completed separation. Another purpose of such a detection is to govern a counter flow, utilized in several cases, in order that the zone boundary between leading electrolyte and sample mixture is kept stationary in the column as is described in the above mentioned patent specification.

The detection methods which have come to practice are principally thermal detection, based on the fact that the heat emission is different in the different zones and is increasing in the direction from leading towards terminating electrolyte, and spectrophotometrical detection of the different zones. The latter method provides faster measurement than the first one, which necessarily have to work with a time lag of about 5 seconds. Further the spectrophotometrical detection allows a far greater resolution than the thermal detection, more exactly some 50–100 times greater. A prerequisite of the spectrophotometrical detection is however that the various separated ion species show absorbance. The number of substances which show absorbance within the visible spectrum is very small. On the contrary a great number of substances show absorbance within the UV-range and therefore spectrophotometrical detection within the UV-range is more generally applicable. Within many chemical fields, e.g. biochemistry it is consequently advantageous to work with UV-detection. It is often however desired to separate by isotachophoresis substances which are not UV-absorbent. Usually such isotachophoretical separations

are carried out alternating with separations of UV-absorbing substances. It may also happen that a number of substances, only a few of them being UV-absorbing, should be separated by isotachophoresis. Previous methods do not allow spectrophotometrical detection in such cases.

The purpose of the present invention is to provide for spectrophotometrical detection of zones of substances, which do not show absorbance, and to allow the greater rapidity and greater resolution characteristic of spectrophotometrical detection as compared to thermal detection.

It is also a purpose of the present invention to provide a method for UV-detection of non-UV-absorbing substances, and to allow the greater rapidity and greater resolution which is possible at UV-detection as compared to thermal detection.

The characteristics of the invention are obvious from the claims following the specification.

The invention will now be further explained with reference to the attached drawings, showing embodiments of the invention by way of example. The invention should not be restricted thereto.

FIG. **1** schematically shows a column prior to an isotachophoretical separation,

FIG. **2** shows the same column after achieved equilibrium,

FIG. **3** shows schematically the electrical field strength E along the column,

FIG. **4** shows the concentration of the different anions at equilibrium,

FIG. **5** shows the concentration of the counter-ion R^+ along the column,

FIG. **6** shows schematically the pH-course along the column,

FIGS. *7a–9* show detector curves of isotachophoretical separations, shown as examples.

In FIGS. **1** and **2** is denoted by **1**, a column in which an anode **2** and a cathode **3** are introduced. In FIG. **1** the sample to be separated is introduced in that part of the column which is denoted by S, the sample consisting of salts containing three different anions C_1^-, C_2^- and C_3^-, of which C_1^- is assumed to have greater mobility than C_2^-, which in its turn is assumed to have greater mobility than C_3^-. That part of the column which is denoted by L is filled with the above mentioned leading electrolyte, which consists of anions A^-, having greater mobility than all anions in the sample. That part of the column which is nearest to the cathode, T, is filled with an electrolyte containing an anion B^- having a mobility which is smaller than those of all anions in the sample. Throughout all the column there is a cation species, common to all anions, a so-called counter-ion R^+, which suitably has buffering properties. When a direct voltage is applied to the electrodes **2** and **3** the anions will migrate towards the anode **2.** As a consequence of the different mobilities of the anions the electrical field strength over the zones L, S and T, respectively, will increase stepwise over the respective zones. This will however bring about that the anions present in the zone S will be separated according to their mobilities, so that the ions C_1^- having the greater mobility will form a zone nearest to the leading electrolyte, followed by a zone consisting of C_2^- and finally by a zone consisting of C_3^- next to the terminating electrolyte, which is shown in FIG. **2.** Along these zones then also the electrical field strength will increase stepwise.

This is shown in FIG. 3. The zones thus formed, will be very stable and sharply limited, as an anion which attempts to diffuse from one zone into an anterior zone where a lower electrical field strength is prevailing will obtain a lower migration velocity and will therefore be caught up by its original zone. In the same way an anion which attempts to diffuse into a posterior zone will be brought back to its original zone by the higher electrical field strength, prevailing in the posterior zone. Thus a very good self stabilizing of the zone boundaries will be achieved.

The conditions at a zone boundary between two salt solutions having an ion in common and being subject to an electrical field has been given by Kolrausch. (Ann. Phys. Leipzig 62, 209 (1897)):

$$\frac{C_A}{C_A} = \frac{U_A}{(U_A + U_R)} \cdot \frac{(U_B + U_R)}{U_B} : \frac{L_B}{L_A},$$

where

C = concentration

U = mobility (cm^2/volt, sec)

L = electrical charge

where indexes A, B and R are directed towards the ions A^-, B^- and R^+, respectively.

According to this relationship there is a concentration stage between the different anions at the different zone boundaries. This is shown in FIG. 4. The counter-ion should suitably have buffering properties. If so, the total concentration of the counter-ions will show considerably smaller stages at the different zone boundaries, as is hinted in FIG. 5. Also the pH is changing at the different zone boundaries, e.g. as is shown schematically in FIG. 6.

According to the present invention there is utilized a counter-ion R^+ having buffering properties, the counter-ion being chosen in that way that its molar absorptivities at acid and basic conditions, respectively, differ at a wave-length, suitable for measurement. The pH-course shown in FIG. 6 then will bring about an absorbance course along the column.

In FIGS. 7*a* and 7*b* are illustrated a separation of five anions by isotachophoresis. FIG. 7*a* illustrates a separation according to the prior art, while FIG. 7*b* shows the result of a separation according to the present invention. Each of FIGS. 7*a* and 7*b* shows from top to bottom detector readings from a thermal detector, a differential thermal detector and a spectrophotometrical detector, respectively. In the different curves a certain section corresponding to a certain ion is denoted by a number corresponding to that ion. In the example illustrated in FIGS. 7*a* and 7*b* the leading electrolyte, denoted by **1**, is 0.01M Cl^-, and the terminating ion, denoted by **7**, is capronate. Five ions are separated, **2** = ClO_3^-, **3** = oxalate, **4** = tartrate, **5** = citrate, **6** = acetate. In FIG. 7*a* the counter-ion is 0.0465M β-alanine, while in FIG. 7*b* the counter-ion is 0.012M creatinine. The leading electrolytes had a pH of 4.1 in both cases. The spectrophotometrical detection is made at 254 nm. It can be seen that the spectrophotometrical detection gives almost no output signal in the first case. When using creatinine as counter-ion in the second case the spectrophotometrical detection gives a very good picture. It can be seen that the resolution of the spectrophotometrical curve in FIG. 7*b* shows greater resolution than the thermal curves of the same figure. The peak in FIG. 7*a* between sample components **5** and **6** is due to a contamination in the sample.

Another separation is shown as an example in FIG. 8. The figure shows from top to bottom a curve from a spectrophotometrical detector at 254 nm and a curve from a thermal detector. The system to be separated in the example of FIG. 8 is as leading electrolyte 0.01M $(CH_3)_4NCl$ in methanol, saturated with sulfanilic acid and adjusted to pH 4.4 (as shown by an ordinary calomel-KCl electrode containing water) with $(CH_3)_4NOH$. Terminating electrolyte is 0.2M zinc acetate in methanol, and as counter-ion is used sulfanilic acid. Designations in the figure: **1** = $(CH_3)_4N^+$, **2** = NH_4^+, **3** = K^+, **4** = Na^+, **5** = Ba^{2+}, **6** = Li^+, **7** = Mg^{2+}, **8** = Ca^{2+}, **9** = Zn^{2+}. The concentration of Na^+ is 0.0015M and of the other sample ions 0.03M.

The example illustrated in FIG. 9 relates to a separation of the same ions as in the example of FIG. 8, in a methanolic system. In the example of FIG. 9 the leading electrolyte is 0.0089M NaCl + 0.0007M $NaOCOCH_3$ in methanol, saturated with sulfanilic acid and with a pH of 5.0 (as measured with an ordinary calomel-KCl electrode containing water). The terminating electrolyte and the counter-ion are the same as in the example of FIG. 8. The spectrophotometrical reading is made at 254 nm.

From the figures the considerably higher resolution at spectrophotometrical detection, made possible by the method according to the present invention, as compared to thermal detection, can be seen.

Thus FIGS. 7*a* and 7*b* shows an isotachophoretical separation in a water system, while FIGS. 8 and 9 show separations in methanolic systems.

The example of FIGS. 7*a* and 7*b* also shows separation and detection according to the present invention, of anions, while FIGS. 8 and 9 show separations and detection according to the present invention, of cations.

By choice of a counter-ion having such properties it is thus made possible to detect zones of sample components, which have no absorbance of their own.

Especially such a counter-ion could be chosen which is showing different molar absorptivities at acid and basic conditions at some wave-lengths within the UV-range and thus allow spectrophotometrical detection within the UV-range of substances which are not UV-absorbing. As mentioned above the method according to the present invention could be used as well for spectrophotometrical detection of sample zones, some of which show absorbance. Further the invention could often advantageously be used also for detection of sample zones after an isotachophoretical separation of the sample mixture, where each sample component is showing absorbance at some wave-length, but where the spectrophotometrical detection according to the present invention, e.g. with still another suitably chosen wave-length, will give a considerably more clear-cut result.

The difference in molar absorptivity at the counter-ion in acidic and basic conditions, respectively, of course have to be detectable by the spectrophotometer, used at the measurement. The smallest difference in absorbance which is detectable by hitherto known instruments is about $10^{-3} - 10^{-6}$ units. Measurements of smaller differences in absorbance by still more accurate instruments which may be developed in the future should not however fall outside the scope of the present invention.

I claim:

1. In a method of spectrophotometrical detection using an isotachophoretic column containing a leading and a terminating electrolyte with two electrodes spaced from each other along the length of the column electrically charged at opposite polarities, wherein the ions of higher mobility migrate toward one of said electrodes and the ions of lower mobility migrate toward the other of said electrodes, the improvement which comprises the step of adding to the column an electrolyte containing counter-ions whose molar absorptivities within the spectral range employed differ in accordance with the pH values.

2. The method of claim **1,** wherein the spectral range is within the range of UV light.

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