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METHOD FOR SEPARATING IONIZED SUBSTANCES

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ABSTRACT OF THE DISCLOSURE

A spacer for facilitating the detection or separation of the consecutive zones of a sample formed in an electrophoretic separation process is provided. The spacer consists of a mixture of a great number of substances, the ions of which have different mobilities, the mobility difference between consecutive ions being small and substantially equal.

The present invention refers to a method for separating ionized substances generally referred to as A^+X^- , where A^+ denotes the equivalent of a positive ion and X^- denotes the equivalent of a negative ion, at least one of the ion equivalents comprising ions the mobilities of which differ from each other the method consisting of a separation of the last mentioned ions based on the fact that these ions migrate with different velocity if disposed within an electrical field so as to form different consecutive zones in said field, the separation being facilitated by introducing so called spacers, the purpose of which is to obtain a distance between the different ions migrating in the electrical field.

The above described method is known per se. It could for instance be performed by moistening a filter paper having a width of a couple of centimeters and a length of 20 cm. in such a way that sodium chloride is supplied to one end of the paper and sodium ascorbate having the same molarity is the sodium chloride is supplied to the other end of the paper. The end of the paper which is moistened with sodium chloride is disposed in an electrode vessel, the anode vessel, which vessel contains sodium chloride and the end of the paper that is moistened with sodium ascorbate is disposed in a second electrode vessel, the cathode vessel, which contains sodium ascorbate. The boundary between the two solutions will then be located approximately at centre of the paper i.e. 10 cm. from each end. The anode vessel contains the $+$ pole and the cathode the $-$ pole. If a voltage is supplied to the poles, the sodium ions will migrate towards the cathode vessel. The chloride ions will migrate towards the anode vessel. The chloride ions will be followed by the ascorbate ions which also migrate towards the anode vessel. The mobility of the chloride ions is higher than that of the ascorbate ions which means that since the ions having the higher mobility will migrate in front of the ions with the lower mobility the boundary between the different ions will be sharp. If namely a faster ion for some reason would move into the zone occupied by the slower ions, the fast ions will very rapidly migrate back into the zone containing the faster ions due to the higher electrical field existing in the zone comprising the slower ions. In a corresponding way a slow ion that moves into the zone containing faster ions will migrate more slowly there due to the lower electrical field in the zone containing the faster ions. Thus the concentration of the fast ions will not vary during the experiment.

The concentration of the slower ions will be lower than that of the fast ions. This concentration is de-

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pendent upon the concentration of the fast ions and the different mobilities of the ions comprised in the system.

If a small amount of a solution containing a number of negative ions and a positive ion, the sodium ion, is disposed between the chloride ions and the ascorbate ions, and the negative ions in this solution have different mobilities, all mobilities however, being lower than that of the chloride ions and higher than that of the ascorbate ions, the chloride ions will lead the migration towards the anode. The chloride ions are followed by the mixture of ions and the mixture will in turn be followed by the ascorbate ions, the terminating ions. During the process the fastest ions in the mixture will form a zone immediately after and in contact with the chloride ions. The concentration of these ions will be somewhat lower than the concentration of the leading ions, the chloride ions. The length of this zone will be determined by the number of ions available. If only an infinitesimal amount of the ion is available, the zone will theoretically be infinitesimally thin. The other substances in the mixture will form zones having lower concentrations. The slowest ion in the mixture will migrate immediately in front of and in contact with the ascorbate ions.

In the above described separation method it is known per se to separate consecutive zones by means of added ions, so called spacers. Thus experiments have been carried out in which single ions have been added to either rather complicated natural mixtures of ions (e.g. beet root juice) or uncomplicated mixtures of metal ions. A spacer has for instance been disposed between zones of potassium and sodium ions, three different spacers have further been disposed between sodium and lithium ions.

In accordance with the present invention it has, however, been found out that a considerably improved result could be obtained by using a spacer mixture, consisting of a great number of substances, the mobility of which differ, the mobility difference of the consecutive ions being small and substantially equal.

In the electrophoresis system provided with the above described spacer mixture a number of different types of sample ions (n different ions) are disposed between the chloride ions and the ascorbate ions. The n different types of sample ions all have different mobilities. The chloride ions as in the above described experiment have the highest mobility in the system and the ascorbate ions have the lowest mobility. As in the experiment described above the electrical voltage supplied to the system is such that the chloride ions migrate towards the anode in front of the other negative ions. After some time when an equilibrium has been achieved the n different sample ions are arranged in n consecutive zones which are in contact with each other. In order to separate the n consecutive sample zones from each other $n-1$ spacers are required. The number of spacers might, however, be increased, e.g. in such a way that ten spacers are disposed between each zone, i.e. ten times $n-1$ spacers are required. In order to keep the distance between the n zones equal in both experiments the total amount of spacer ions should be about the same in both experiments. By using a mixture of a very great number of spacers a great number of consecutive zones could be separated. By using a mixture comprising a great number of spacers within a certain mobility range two zones formed by sample ions the mobilities of which are very close to each other, can be separated. The above discussed spacer could also be used when one is interested in obtaining narrow zones of the spacer between consecutive zones of the sample in order to facilitate the detection of the boundaries between the sample zones. The spacer is then added in a small chemical quantity. When the spacer is used for this purpose it could be defined as a marker.

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By using a mixture of a great number of ions having different mobilities something that could be called a "spacer gradient" is obtained. As the conductivity in the spacer gradient is highest in the front, i.e. close to the chloride ions, and then continuously decreases towards the ascorbate ions, the spacer gradient could also be defined as a conductivity gradient.

The velocity of a charged particle in an electrical field having unity strength (1 volt/cm.) is called the mobility of the particle. The value of the mobility is a function of the charge of the particle and the resistance obtained when the particle is transferred within the medium. This resistance is substantially proportional to the weight of the particle. Ion mixtures comprising a complete spectrum of mobilities could be obtained in different ways.

If all ions have the same charge a great number of ion weights must be present in the mixture. The ions must be dilutive in water (water-soluble) and should not give rise to viscous solutions. A type of ions that satisfy these requirements can be obtained from mixtures of polyethylene glycol by providing the polyethylene glycol molecules with one or more charges. One or both of the OH-groups at the end of the glycol molecules are made to react with a charged substance. The charged part of this substance can be bound to the glycol by means of an ether bond or an ester bond. Ester bonds are easily formed, e.g. by transformation of the glycol with succinic acid anhydride. A stable negative charge can also be obtained by oxidation of a glycolic OH-group to a carboxyl group. In such reactions, however, reactive side products might be formed (e.g. aldehyde or peroxide groups). These should be removed, e.g. by reduction.

Ether bonds can be formed by reacting a halogen compound with sodium alcoholates from glycols. The halogen compound can either be a halogen substituted acid or a halogen substituted amine. Another type of reaction which also leads to the formation of an ether bond is the reaction between an alcohol or an alcoholate with cyclic compounds, e.g. sultone, lactide or lactone.

Ester bonds can be formed by reacting an acid (e.g. sulphuric acid, phosphoric acid) or a halogen substituted acid (e.g. chlorosulphonic acid, phosphorous oxychloride) with glycol. As acylating agent an anhydride of a dibasic acid can also be used.

Instead of polyethylene glycol polypropylene glycol could be used. Because of their low solubilities and high melting points only polypropylene glycols having a low degree of polymerization can be used. Copolymers of ethyleneoxide and small amounts of propyleneoxide give rise to polyglycols having good solubility in water. Mixtures of polyethylene glycol and copolymers with different concentrations of polypropylene groups give a great variety of molecular weights. Such mixtures form an excellent initial material for making spacer mixtures.

By adding different ionized groups in a synthesis a great number of ions can be formed. Sodium glycolate could for instance be reacted with sodium salts of chloroacetic acid, 2-chloro-propionic acid, 3-chloro-propionic acid, bromo-ethanesulfonic acid and propane sultone.

Another method of increasing the number of different ions in a mixture consists therein that different parts of the synthesis mixture are deuterated to different degrees. The differently deuterated parts of the mixture will together comprise a great number of different ions.

Instead of polyethylene glycol other molecules might be used as the uncharged parts in mixtures of ions having the same charge. For instance polyvinyl alcohol with different degrees of polymerization, polyvinyl acetate and other polyvinyl alcohol esters having different degrees of polymerization and saponification, polyacrylamide and other vinylpolymers, soluble in water, can be used. Further oligo- and polysaccharides of different kinds can be used. All the above mentioned polymers, however, are not so suitable for this purpose, since they give rise

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to solutions which have a too high viscosity from the electrophoretic point of view.

Ions having different mobilities can be made from one and the same uncharged molecule by providing this with a different number of charges. An oligo-saccharide can be provided with carboxymethyl groups by a reaction with chloroacetic acid or with sulphopropyl groups by a reaction with propane sultone. Polyvinyl alcohol could be substituted in a similar way. The same number of substituents could be added at different parts of the uncharged polymer and thus a number of ions having different mobilities will be obtained.

A simple method for producing ions having different charge is to let a compound containing one or more hydroxyl groups react with polyphosphoric acid. In such reactions polyphosphoric esters having polyphosphoric chains of different length are formed. This type of esters could be rapidly hydrolysed at high pH-values. They are, however, sufficiently stable at pH-values below 9 to be used in experiments lasting for a few hours.

Furthermore, polyfunctional molecules could be provided with both positive and negative groups.

The invention will now be explained in conjunction with a few examples, the Examples 1-4, 7-8, and 10 defining a production of ion mixtures and the Examples 5, 6, 9, and 11 defining the use of ion mixtures as spacers.

EXAMPLE 1

300 g. polyethylene glycol, molecular weight 300
23 g. sodium
100 ml. toluene
102 g. propane sultone.

Polyethylene glycol and toluene are mixed in a 1-liter three-necked flask, provided with cooler, stirrer and heating jacket. ¼ of the sodium is added and dissolved while stirring and heating. The rest of the sodium is dissolved in the same way in three portions. Propane sultone is added in portions of 10-20 g. Between each addition the propane sultone is allowed to react completely. After the last addition the flask is heated during ½ hour in order to obtain an abundant reflux. The reaction product is then cooled. Water is added in small portions. The reaction product is transferred to an Erlenmeyer flask and an equal volume of water is added. The toluene phase is sucked away. Toluene could further be removed by evaporation in vacuum.

EXAMPLE 2

100 g. polyethylene glycol, molecular weight 400
75 g. polyethylene glycol, molecular weight 300
50 g. polyethylene glycol, molecular weight 200
35 g. sodium
100 ml. toluene
210 g. propane sultone, dissolved in the smallest possible amount of toluene.

The synthesis is analogous to that of Example 1. After adding the last portion of propane sultone the reaction product is heated during 3 hours with an abundant reflux. The reaction product is then cooled and kept at room temperature for 15 hours. Water is added as in Example 1.

EXAMPLE 3

100 g. polyethylene glycol, molecular weight 400
75 g. polyethylene glycol, molecular weight 300
50 g. polyethylene glycol, molecular weight 200
35 g. sodium
100 ml. toluene
35 g. butyrolactone
140 g. propane sultone dissolved in the smallest possible amount of toluene.

The synthesis is performed as in Example 2. The butyrolactone is added dropwise to the reaction mixture. The propane sultone solution is added in the same way.

5 EXAMPLE 4

6 g. triethylene glycol, technical quality
5 g. polyphosphoric acid.

The glycol and the phosphoric acid are mixed in a 25 ml. flask with a magnetic stirrer. The mixture is kept at a temperature of 80–90° C. for 2 hours and then diluted with ice water to a volume of 100 ml. The pH-value is adjusted to pH 7.2 with solid tris(hydroxymethyl)amino-

methane. The composition of the obtained spacer ion mixtures were investigated by a two-dimensional electrophoresis technique especially worked out for this purpose. A cellulose acetate strip having a width of 2–3 mm. was moistened with a solution containing chloride ions as fast ions and tris(hydroxymethyl)aminomethane forming the counter-ions. The strip was placed on a glass plate which via an anode wick was brought into contact with the anode solution (trischloride). The free end of the strip was placed at a distance of 3 cm. from the cathode wick. In the space between the strip and the cathode wick was placed a piece of acetate cellulose material having a fibrous structure. The diluted ion mixture to be investigated was applied on this piece. The solution further contained a small amount of a strongly negatively charged blue dye stuff. The test volumes did not exceed 50 μ l. As a cathode solution trisglycinate at pH 8.5 was used. At this pH-value glycine has its carboxyle group completely ionized (one negative charge). About 90% of the glycine molecules have their amino groups protonated (one positive charge). 10% of the amino groups are uncharged. The average charge is thus 0.1 negative charge. The molecular weight of glycine is 75. The glycine will then migrate as if it had an ion weight of 750. The electrophoresis was run until the blue dye stuff had reached a point a few cm. from the anode wick. 10 cm. of the electrophoretic strip was transferred to a 10 cm. wide cellulose layer moistened with 0.2 molar sodium ethylenediaminetetraacetate buffer having a pH-value of 6.1. The voltage was applied perpendicular to the cellulose acetate strip. The ions in the strip migrated into the thin layer. After the electrophoresis these layers were dried. The positions of the ions were determined with iodine vapor.

When positive ions were separated ϵ -ammoniumcapronic acid was used as the terminating ion and potassium as the leading ion. The counter-ion was formate and the pH-value was 4.5.

The electrophoretic separations to be described below were all carried out by using a technique similar to that used in the first electrophoretic two-dimensional procedure. The cellulose acetate strip, however, had a width of 12–14 mm.

EXAMPLE 5

Anodic buffer: 0.2 M tris chloride, pH 7.2
Cathodic buffer: 0.5 M tris glycerine pH 8.5
Sample: a mixture consisting of 20 μ l. beet root juice; 20 μ l. of a solution produced in accordance with Example 3; 5 μ l. of a solution of tetra-sulphonated indigo (Tetra) 0.1 mg./ml., a blue dye-stuff having high mobility.

The electrophoresis was run at 1000 v. (4–2 ma.) for 45 minutes and then at 1500 v. for 60–75 minutes. The experiment was run until the Tetra boundary had reached a point a few centimeters from the anode wick. A control experiment was run simultaneously with only 20 μ l. beet root juice.

The strip from the control experiment showed a number of red-violet and yellow zones close to each other. About 0.5–1 cm. in front of these zones a yellow zone was located somewhat closer to the anode.

On the strip where a spacer mixture was present the Tetrazone was located 3 cm. in front of a strongly coloured red-violet zone. Between this zone and the Tetra zone five sharp zones having different yellow colours were

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found. Behind the strongly coloured red-violet zone four zones having different red-violet or brown colours were found.

In a similar experiment 500 v. was applied during 1 hour after the initial 45 minute period at 1000 v. Clearly visible but somewhat diffuse zones were formed on the strip. After another 15 minutes at 1500 v. most of the zones had sharp boundaries and after another 10 minutes all zones were sharp.

EXAMPLE 6

Anodic buffer: 0.2 M tris chloride, pH 7.2

Cathodic buffer: 0.3 M tris ascorbate, pH 7.2.

Samples were applied to cellulose acetate strips (1.2 x 30 cm.) according to the scheme below.

	Strip number							
	1	2	3	4	5	6	7	8
20 μ l glucose-6-phosphate ^{-14}C	+	+	+	+	+	+	+	+
5 μ l β -hydroxi butyric acid ^{-14}C	+	+	+	+	+	+	+	+
5 μ l lactate ^{-14}C	+	+	+	+	+	+	+	+
5 μ l citrate.....	+	+	+	+	+	+	+	+
5 μ l succinate ^{-14}C	+	+	+	+	+	+	+	+
5 μ l product accord. to Example 4.....	+	+	+	+	+	+	+	+

This experiment was run at 1000 v. and 30 ma. for 110 min. After the electrophoresis the wet strips were mounted in a rack, which was placed in a freezer, and freeze-dried. The anodic part of the dried strips were evaluated by autoradiography. The substances migrated in the following order: Succinate, lactate, β -hydroxy butyric acid and glucose-6-phosphate, the last mentioned substance having the lowest mobility. In strip 1, i.e. the strip without spacer, the zones were situated adjacent to each other, whereas on the strips 2 and 8 the zones were clearly separated and equally distributed within 20 mm.

EXAMPLE 7

15 g. polyethylene glycol, mol. weight 300
10 g. succinic acid anhydride.

The glycol and the succinic acid anhydride are mixed in a 50 ml. flatbottomed flask. The mixture is stirred at 90–100° C. for 2 hours. The chilled reaction product is then diluted to 200 ml. with ice water. The pH-value is adjusted to 7.2 with solid tris(hydroxymethyl)aminomethane.

EXAMPLE 8

10 g. polyethylene glycol, mol. weight 200
5 g. succinic acid anhydride.

The glycol and the succinic acid anhydride are mixed in a 50 ml. flatbottomed flask. The mixture is then stirred at a temperature of 90–95° C. for 2 hours. The chilled reaction product is then diluted with ice water to 200 ml. The pH-value is adjusted to pH 7.2 with solid tris(hydroxymethyl)aminomethane.

EXAMPLE 9

Anodic buffer: 0.2 molar tris chloride, pH 7.2

Cathodic buffer: 10% solution of calcium lactobionate. The electrophoresis was carried out on a glass plate covered with parallel strips of cellulose layers. Each strip measured 1.2 x 40 cm. and the distance between the strips was 1 cm. The major part of each strip is moistened with the anodic buffer from the anodic side. The sample is added to a part of the strip which is not moistened and is situated close to the cathodic end of the strip.

Sample and spacer: 20 μ l. of a mixture consisting of equal parts of beet root juice and

- the reaction product in accordance with Example 7,
- the reaction product in accordance with Example 8,
- water

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was applied. After 3 hours of electrophoresis at a voltage of 1000–2000 v. the fastest coloured zones had migrated 25 cm.

On the control strip there was a 4 mm. zone composed of yellow and red bands. On the strips which contained spacers two main zones could be found, a faster yellow zone and a slower red zone, the distance between the zones being about 2 cm. In front of the yellow main zone three faint yellow zones could be seen. The red main zone was followed by two other red zones. Between the two main zones a number of red zones could be seen. Furthermore, in ultraviolet light a number of fluorescing zones could be discovered.

EXAMPLE 10

8 g. polyphosphoric acid (degree of polymerization 2.4)
4 g. phosphoric acid anhydride.

The substances are mixed for 40 min. at a temperature of 40° C. 32 g. of polyethylene glycol, having a mol. weight of 400 is added. The mixture is kept at 70° C. for 160 min. while stirring. The mixture is then diluted to 100 ml. with ice water and neutralized to pH 7.2 with solid tris(hydroxymethyl)aminomethane.

EXAMPLE 11

20 μ l. of the spacer mixture according to Example 10,
diluted 10 times and
20 μ l. of beet root juice

were placed on a 1.2 cm. wide and 40 cm. long cellulose thin layer strip.

After 3 hours of electrophoresis at 1000–2000 v. the fastest moving zones had migrated 25 cm. The distance between the two coloured main components, a faster yellow one and a slower red one, was 1 cm. Half a cm. in front of the yellow main component two faint yellow zones could be seen, the distance between these zones being $\frac{1}{2}$ cm. A control strip on which only 20 μ l. beet

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root juice was applied, showed yellow and red bands in contact with each other covering 4 mm. of the strip.

What is claimed is:

1. A method for separating ionized substances generally referred to as A^+X^- , where A^+ denotes the equivalent of a positive ion and X^- denotes the equivalent of a negative ion, at least one of the ion equivalents comprising ions the mobilities of which differ from each other, the method consisting in a separation of the last mentioned ions based on the fact that these ions migrate with different velocity if disposed within an electrical field so as to form different consecutive zones in said field, the separation being facilitated by the introduction of so called spacers, the purpose of which is to obtain a distance between the different ions migrating in the electrical field, characterized in, that as spacers there is used a mixture of a great number of substances, the mobilities of which differ, the mobility difference between consecutive ions being small and substantially equal.

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