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Rilbe et al.

[11] **3,915,839**

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[54] **APPARATUS FOR ISOELECTRIC FOCUSING**

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

[51] **Int. Cl.²** **B01K 5/00**

[58] **Field of Search** ..... 204/180 R, 180 G, 299; 356/105

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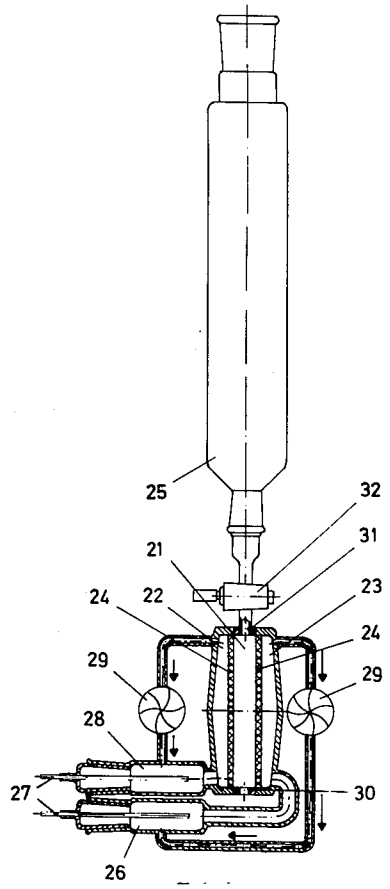
[57] **ABSTRACT**

A method for reducing the time required to obtain separation with a great resolution of components in a sample solution by isoelectric focusing consists of applying an electrical field between a pair of electrodes positioned at the top and bottom of density gradient columns in successive steps, a succeeding step being performed with a density gradient column having a smaller cross-section perpendicular to the electrical field than that of the column used in the preceding step.

16 Claims, 5 Drawing Figures



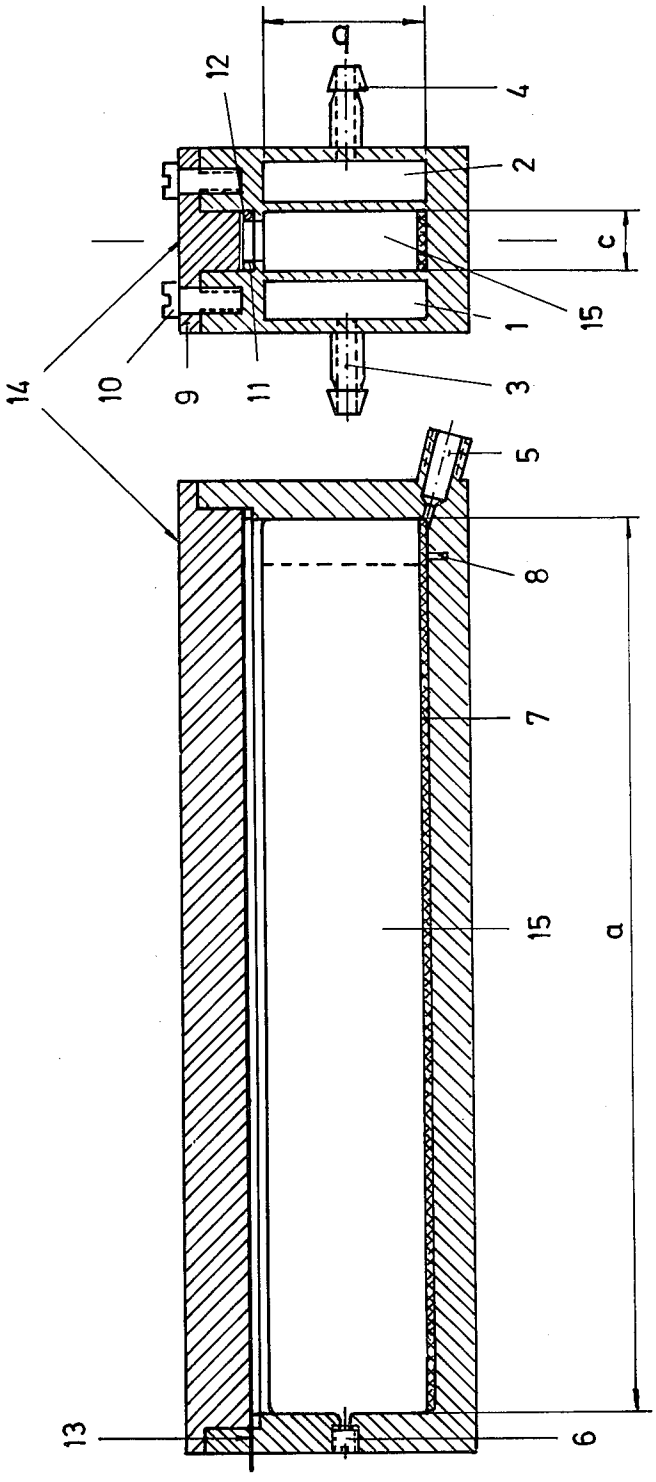


FIG. 1a

FIG. 1

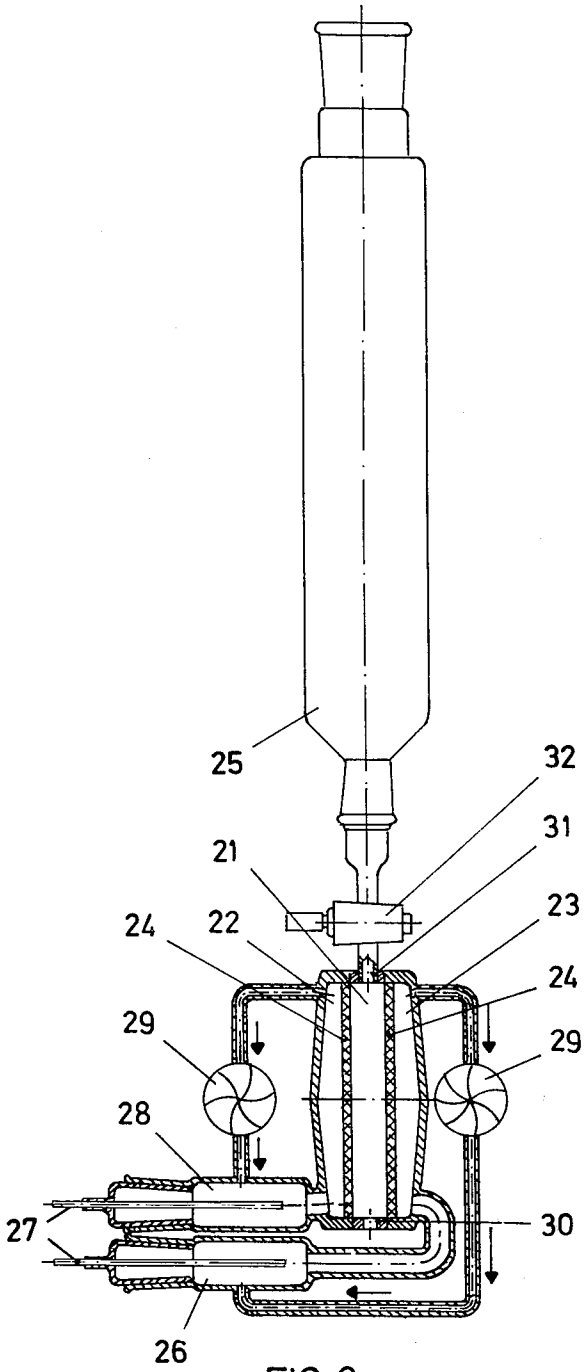


FIG. 2

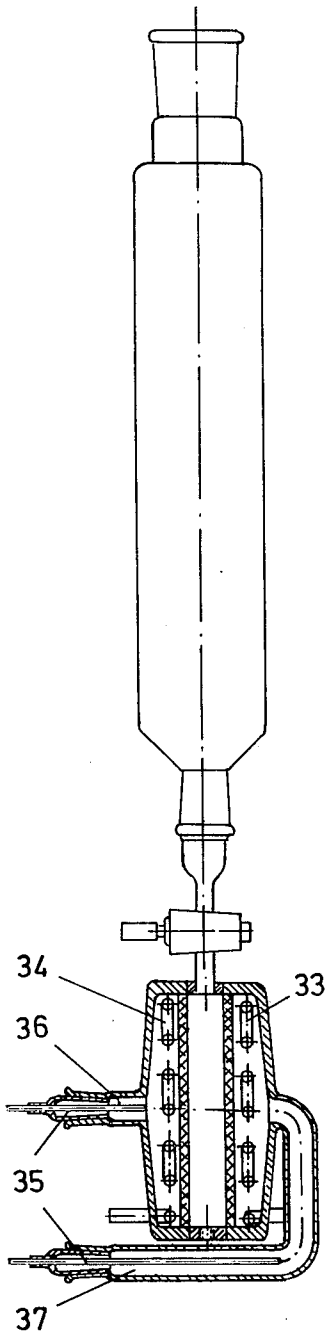


FIG. 3

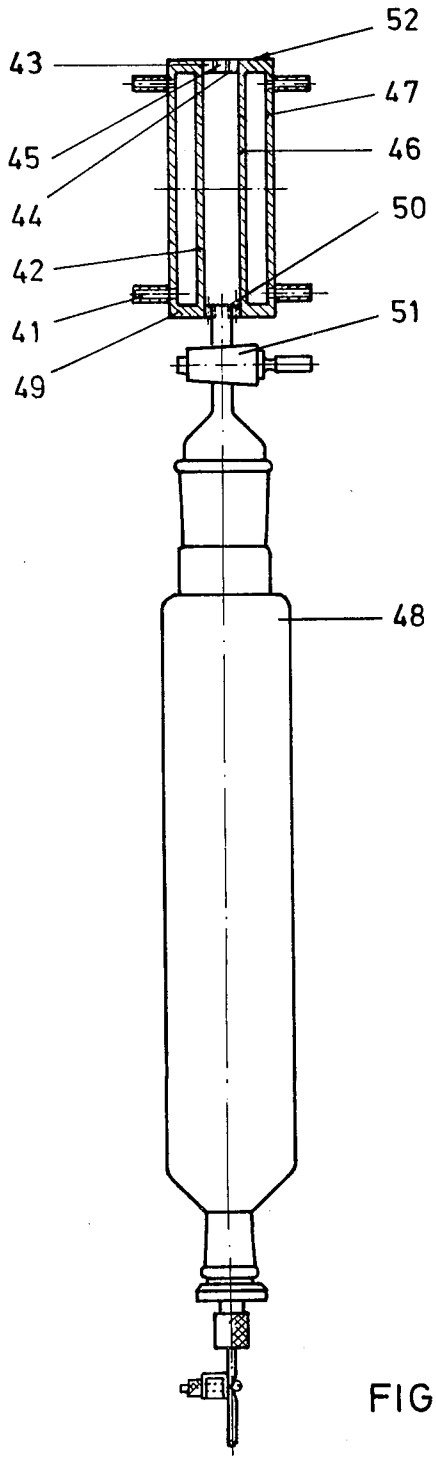


FIG. 4

APPARATUS FOR ISOELECTRIC FOCUSING

The present invention relates to a method in separation of components of a sample solution by isoelectric focusing in a focusing chamber which is subject to a direct current electrical field, and to an apparatus for accomplishing the method.

Isoelectric focusing is an electrophoretic method of separation, based on differences in isoelectric points of the sample components to be separated, and which is performed in an electrical current path having a varying pH. Isoelectric focusing is generally characterized in that after equilibrium has been achieved, the separated substances are located in that point in the pH-gradient which corresponds to the isoelectric point of each substance, respectively, and that each substance there has zero mobility. A survey of isoelectric focusing as separation method has been given by Haglund in Methods of Biochemical Analysis, 19, page 1–104, John Wiley & Sons (1971).

The pH-gradient, requisite in isoelectric focusing, can be obtained in two principally different ways. Thus, the pH-gradient could be artificial or natural. The artificial pH-gradient is achieved by a mechanically obtained mixture in gradually changed proportion of solutions of varying pH. The natural pH-gradient is obtained by the influence of the electrical field on a number of ampholytical substances having different isoelectric points. Thus, the natural pH-gradient has the advantage after having been formed by influence of the electrical field to be stabilized thereby. The theory of natural pH-gradients was discussed by Svensson in Acta Chem. Scand. 15, page 325–341 (1961), and in Acta Chem. Scand. 16, page 456–466 (1962).

In order to achieve practically a natural pH-gradient, suitable so called carrier ampholytes are required, the isoelectric points of which are narrowly and evenly distributed over the desired pH-interval and which possess in their respective isoelectric points buffering as well as electrically conducting properties. The lack of good carrier ampholyte was the limitation of this method until such substances were made available by an invention, made by Vesterberg, disclosed in the U.S. Pat. No. 3,485,736 (corresponding to Swedish Pat. No. 314,227). The disclosed mixture of carrier ampholytes are marketed by LKB-Produkter AB, Bromma, under the Trade Mark Ampholine.

The present invention can be performed by utilization of an artificial or a natural pH-gradient. It is preferred by the performance of the present invention to use a natural pH-gradient.

Separation of substances by isoelectric focusing in a natural pH-gradient is generally described by Svensson in Protides of the Biological Fluids, 15, page 515–522, Elsevier Publishing Company, Amsterdam (1968).

As appears from the mentioned literature some kind of convection stabilizing measures are required at isoelectric focusing. When performing the present invention the isoelectric focusing is carried out in a density gradient. This method implies that the relatively small density differences depending on temperature differences as well as those depending on the concentrations of the zone components are "drowned" in considerable greater, artificial density differences. Generally, the density gradient should be parallel or anti-parallel to the applied electrical field. Usually, the density of the solution is continuously increasing downwards. This is

achieved by using a heavier and a lighter solution, the proportion of the heavier solution increasing downwards in the column in which the isoelectric preparation is performed, while the proportion of the lighter solution decreases. Both solutions contain electrolytes, the heavier one in addition sugar to an amount of 500 grams/liter. The total density difference will amount to about 0.2 grams/cm^3.

Substances which are suitable for separation by isoelectric focusing are ampholytes. An important group of such substances are proteins.

A criteria of a good separation is the resolving power of the separation method. In isoelectric focusing the term resolving power is directed towards the smallest difference in isoelectric points of two components of the sample solution, which will result in two perceivable zones after focusing. In Acta Chem. Scand., 20, page 820–834 (1966), Vesterberg and Svensson have theoretically deduced and experimentally confirmed the following relationship concerning the resolving power of the isoelectric focusing method:

$$\Delta pI = 3 \sqrt{\frac{D\,(dpH/dx)}{E\,(-du/dpH)}}$$

where ΔpI is the smallest difference in isoelectric points, D is the diffusion coefficient of the sample substance, E the electrical field strength, prevailing in the zones, dpH/dx the pH-gradient present in the zones, and du/dpH the slope of the mobility curve (as a function of pH) at the isoelectric point. It should be observed that the pH change per length unit, not per volume unit, should be put into this relationship.

In order to achieve the best possible resolution, i.e. the smallest possible ΔpI, dpH/dx should consequently be given as low a value as possible. In other words the available natural pH-gradient should be distributed over as long a distance as practically possible. D and du/dpH are constant for a given substance, while an increase in E theoretically should result in an increased resolution. An increase in E, however, will entail an increased heat evolvation in the sample solution which would imply increased risk of heat convection. Thus, E is limited by the ability of the prevailing density gradient to "cover" the convection caused by heat evolvation.

If, at performing an isoelectric focusing, the pH-slope is made very flat, this will entail that also the density change is distributed over a longer distance, which will decrease its convection stabilizing capacity. Consequently, the electrical field strength has to be kept sufficiently low in order that the generation of heat convection is avoided. This will result in low ion migration velocities, which will bring about long separation time. The pH-gradient extended over a long distance moreover will entail long migration paths for the majority of the samples components, which will contribute to a prolonged focusing time. Consequently, the best possible resolution is achieved at the cost of slow focusing.

An apparatus for separation of substances by isoelectric focusing is shown in the above mentioned article of Svensson in Protides of the Biological Fluids, 15, page 515–522. This apparatus, which is marketed by LKB-Produkter AB, Bromma, is a development of an apparatus, which was described by Vesterberg, Wadstrom, Svensson and Malmgren in Biochim, Biophys. Acta, 133, page 435–445 (1967). The apparatus is designed

for isoelectric focusing in natural pH-gradient and in a density gradient. It is characterized by a relatively tall and narrow column with chilled vertical walls. Experiments in order to determine the resolving power of this apparatus were described by Vesterberg and Svensson in Acta Chem. Scand., 20, page 820–834(1966). According to this publication it is possible to obtain a resolving power of $\Delta pI = 0.02$, i.e. the apparatus described permits a very high resolution. On the other side isoelectric focusing in this apparatus suffers from the above mentioned disadvantage that only a relatively low field strength can be applied, resulting in long separation periods. In the experiments, mentioned above, the isoelectric focusing was continued for 48 hours, but a focusing period of 24–28 hours is stated as being normal. Moreover, the focusing period in some instances could be still longer, up to 3 days. Thus, the described apparatus requires very long focusing periods but will in return give a very good resolution.

By reducing the migration distances of the sample components the focusing period could be shortened. Furthermore, the density gradient is made stronger, whereby a higher field strength and considerably greater electrical current can be applied, which will result in greater migration velocities. Such a technique in isoelectric focusing has been described by Kolin in Methods of Biochemical Analysis, 6, page 259–288 (1958). It is stated that, by means of this technique, the separation period can be reduced to a few minutes. However, the obtainable resolution has not been specified, lesser been documented, and has to be assumed to be far inferior to that which can be achieved by means of the apparatus, described above. Kolin mentions (loc. cit. page 264, second paragraph) that a flatter pH-gradient should give an increased resolution. The considerably longer distance, over which the pH-gradient can be extended in the above mentioned apparatus, will however intail a superior resolution, while the focusing also embraces a wider pH-interval.

The isolation of fractions after completed focusing constitutes a special problem. Sample zones which are not too narrow, in a column with a relatively small cross-section, can easily be isolated for micropreparative purposes by sucking out through a capillary tube, as the horizontal inflow to this tube could be restricted to some centimeter or less. The fractions are also often run off through the bottom of the column, which one then is designed in that way that the cross-section of the column gradually is reduced to the diameter of the outlet tube. The last mentioned method is applied in the by LKB-Produkter AB marketed apparatus, described above. The density gradient then is exerting a stabilizing action against convection and in favour of laminar flow, bringing the fractions to flow out in correct order and with a minimum of remixing. In the apparatus, described by Kolin, the isolation of fractions is said to take place by sucking out through a capillary tube, but considerable practical difficulties would be at hand with the very thin sample zones which are obtained at focusing in said apparatus. Focusing according to Kolin's method would accordingly find an application only for analytical purposes as far as the separated zones in some way can be photographed and then quantitatively be evaluated.

If it is desired, starting from the apparatus described by Kolin, to increase for preparative purposes the capacity of the method by increasing the cross-section of the column with retained strong density gradient and steep pH-course, sucking out through a capillary tube will be practically impossible to perform, as the horizontal inflow then will be so long that the mixing with over and under lying liquid layers will be inevitable. Kolin has suggested, as described in loc.cit. page 277–278, that the separated zones are pressed apart by a plunger which diminishes the cross-section of the column, whereby the risks of remixing by sucking out through the capillary tube undoubtedly are reduced. The inserting of the plunger will not, however, increase the resolution as defined in the expression for ΔpI above, then the unsharpness of the zones will be magnified to the same extent as their mutual distances. A reduced diffusive remixing after disconnecting of the focusing current is however gained, since the diffusive mass transport is proportional to the cross-section and the concentration gradient, which are both reduced by introduction of the plunger. Remixing by diffusion is particularly rapid for narrow zones as the widening of zones is proportional to the square root of the time measured from an infinitely narrow zone.

If, for preparative purposes, the cross-section is increased in a very short column, in which a great field strength is applied, the generated heat must be diverted, in order that the density differences, generated by the heat, should not exceed the artificial density gradient. Philpot showed in Trans. Faraday Soc. 36, page 38–46 (1940), that radial cooling will give rise to radial density gradients, which will constitute a limiting factor at the electrophoretical separation. According to Philpot the heat, generated, should be axially diverted, whereby the artificial density gradient on the other hand can be strengthened.

Further, the apparatus, described by Philpot, hasa the same limitations as to the isolation of fractions as the above mentioned apparatus, described by Kolin.

Kolin's statement, that cooling of the focusing columns should not be necessary, would relate to the very small amounts of sample which were separated in the apparatus, described by Kolin, and the consequently small total heat generation in the column.

It is a purpose of the present invention to provide a method for the separation by isoelectric focusing of components in a sample solution, at which the focusing is accomplished in a short period of time and with a high resolution.

It is another purpose of the present invention to provide a method for separation by isoelectric focusing of components in a sample solution, at which the focusing is accomplished in a short period of time and with a great resolution over a wide pH-interval.

It is another purpose of the present invention to provide an apparatus, by means of which components in a sample solution can be separated by isoelectric focusing in a short period of time and with a great resolution.

It is another object of the present invention to provide a method for isoelectric separation of proteins for analytical purposes, at which the separation is accomplished rapidly and with a great resolution.

It is another object of the present invention to provide a method for isoelectric separation of proteins for preparative purposes, at which the separation is accomplished fast and with a great resolution.

It is another object of the present invention to provide a method for isoelectric focusing, in which the fo-

cusing is performed in several steps with a step-by-step increasing resolution in the separation.

It is another object of the present invention to provide an apparatus for isoelectric focusing, in which the focusing is performed in several steps with a step-by-step increasing resolution in the separation.

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

The purpose of the invention is accomplished by performing a focusing step with a mean cross-section of the focusing chamber, perpendicular to the electrical direct current field which is smaller than the corresponding mean cross-section in the nearest preceding focusing step.

More closely one focusing step is performed with a greater relation between column height, i.e. that distance in the column over which the electrical field is acting, and cube root of the column volume that in the nearest preceding focusing step.

The simplest form of a column device having varying mean cross-section perpendicular to the electrical field consists of one parallelepipedical column with the linear measures a, b and c, $a > b > c$, in which the isoelectric separation according to the invention can be accomplished in three steps, the first one with c, the second one with b and the third one with a as vertical dimension. The intermediate step can be omitted. Such a column requires different electrodes for different spatial orientations. Therefore, the electrodes have to be detachable.

The invention can also be performed by using a column with vertical walls made by an elastic material, like rubber, which permits a continuous varying of the mean cross-section perpendicular to the electrical field with retained column volume.

The invention can finally be performed by using at least two columns having different mean cross-section perpendicular to the electrical field, situated in sequence, one above another, and provided with connecting tubes between the top of a lower situated column and the bottom of the next higher situated one. The connecting tubes should be mainly vertical in order to allow, after completed prefocusing in one column, a transport free of convection of the column content to the next column for fine focusing.

In a column having a large cross-section, a plane bottom and a plane top, the outflow through an outlet tube, situated centrally in the bottom or in the top, will not be sufficient as it will imply a horizontal fluid transport along a path as long as the radius of the cylindrical column. A lag in the outflow from the peripheral parts of such a column can not be avoided, which will result in a certain remixing of separated components. This can be counteracted by means of a very slow outflow, but then the diffusion will be of increased importance, and the essential purpose of the invention to speed up the separation with retained great resolution cannot be completely realized.

The outflow can be improved by making top and bottom conical, with the outlet tube situated in the apex of the cone. This is, however, no good settlement, as it will entail a higher field strength and current density in the peripheral parts of the column than in its central parts. This will lead to a horizontal temperature gradient followed by a horizontal density gradient contribution which will easily result in thermical convection. Consequently, only a very slight conicity could be allowed in the top and in the bottom of the column.

A considerably better way is to completely dispense with an axial outflow of short and wide columns. In contrast, an outlet tube is applied at approximately half the height of a vertical column wall and on the opposite wall a similar tube for inflow of fluid or air to substitute the outflowing column content. After complete prefocusing the whole column is turned 90° in order that the two tubes will get positions at the very top and at the very bottom of the column tilted on its edge. The density gradient in the column will then, apart from some lag at the walls, retain its vertical direction whereby the vertical section of the column will get a geometry which is much more favourable for the fluid transport out of the column. If the column is cylindrical the two tubes are situated on diametrically opposite points on half the heigth of the cylindrical envelope surface. If the column is parallelepipedical the two tubes are situated in two opposite corners of a rectangle making a horizontal section of the column in focusing position.

The tilting of the column 90° before emptying should be carried out by means of suitable mechanical means, preferably by motor operation, and the tilting should not be carried out faster than during a few minutes. Manual turning could hardly be accomplished sufficiently slowly and carefully.

The fluid transport from a prefocusing column to a fine focusing column of the same volume as the prefocusing column could be carried out upwards as well as downwards. At transportation upwards a mainly vertical connection tube between the uppermost point of the prefocusing column and the bottom of the fine focusing column is utilized. The upward transport is accomplished by pumping into the bottom of the prefocusing column a fluid with at least as great a density as the greatest density in the sample solution. The column content is thereby lifted with a minimum of disturbance into the originally empty fine focusing column. Alternatively, it is possible without use of a fluid pump to allow the lower connection tube of the prefocusing column to dip into a supply of a fluid with at least as great a density as the greatest density in the sample solution, whereupon a suitable vacuum is applied to the fine focusing column. The column content will then be sucked up from the prefocusing column into the fine focusing column. The transportation velocity is adjusted to a low value, either by adjusting the vacuum in the upper column or by throttling of one of the connection tubes.

At transportation downwards the fine focusing column is first filled with a fluid with at least as great a density as the greatest density in the sample solution. Then a mainly vertical tube connection, free of air, is established between the upper end of the fine focusing column and the bottom of the prefocusing column. By opening of a bottom valve of the fine focusing column the fluid content of the prefocusing column is caused to flow down spontaneously into the fine focusing column with a minimum of disturbance.

The auxiliary fluid having an at least as great density as the greatest density in the sample solution does not need to be a water solution. Liquids, insoluble in water, such as trichloroethylene or carbontetrachloride are very suitable for the purpose.

It is not recommendable to allow the fluid content of the prefocusing column to flow down along the walls of

an empty fine focusing column, as this method gives rise to unnecessarily extensive remixing of separated components.

In certain focusing experiments it occurs that only a limited pH-interval is of interest for fine focusing. Then it could be advantageous to transfer only this fraction of interest into a fine focusing column having a volume, adapted to the volume of the fraction.

The electrode system could be designed in many different ways. To place the electrodes in separate electrode vessels of considerable volume compared to the column volume was introduced into the classical electrophoresis research by Michaelis in Biochem. Z., 16, page 81 (1909) and meant at that time an important progress as possible disturbing influence of electrode reaction products in the column thereby was prevented. Such disturbing influence could be brought about by ion migration, by diffusion or by convection from the electrodes to the column. The principle of electrodes in separate electrode vessels could advantageously be utilized also in the present invention. In that case the electrode vessels should be in electrolytically conducting connection to the column device, one to its top and the other to its bottom. In order to prevent undesired ion migration throughout the column the anolyte must consist of an acid or of a mixture of acids, while the catholyte must consist of a base or of a mixture of bases.

In a preferred embodyment of the present invention the column content is delimited from anolyte and catholyte by convection breaking membranes, permeable to electrical current. Such membranes could consist of glass filter discs, other porous discs, cellophane, relatively thick paper membranes, animal membranes, natural or synthetic gel discs, etc. These membranes should be strictly horizontal and should completely cover the cross-section of the column without leakage.

The use of such membranes implies the advantage that the chilling effect of the cooling system could be exerted on anolyte and catholyte either by bringing, by means of a circulation pump, the anolyte and the catholyte to circulate between the column device and a tube coil, immersed into a cooling reservoir, or by correspondingly bringing the cooling medium to circulate through tubes installed in the anolyte and catholyte chambers outside the membranes. Such a cooling system generates in the column a vertically directed temperature gradient which entail diversion of current heat in axial direction. This cooling principle, which was recommended by Philpot in Trans. Faraday Soc. 36, page 38–46 (1940) is of course of greatest importance in very short and wide columns, and particularly at their bottom, where the density gradient will receive a strengthening deriving from the temperature gradient which is directed upwards. At the top of the column a corresponding weakening of the density gradient is caused. These side effects can be compensated when preparing the artificial density gradient by strengthening the density gradient in the upper part of the column at the cost of a certain weakening in the lower part of the column.

If relatively high concentrations of strong acid and strong base are choosen, the anolyte and the catholyte will obtain conductivities which are some 10-powers greater than the conductivities generally prevailing in the column. The connection tubes between electrode vessels and column could therefore be rather slender and do not require particular cooling.

There are great freedom in the choice of electrode type when using an apparatus having separate electrode vessels. Gasing as well as non-gasing electrodes could be utilized, as can electrodes of noble metals, carbon, silver, mercury etc.

In the apparatus, showed by Svensson in Arch. Biochem. Biophys. Suppl. 1, page 132–138 (1962), noble metal electrodes were used in direct contact with the top and the bottom of the column. This is possible owing to the principle of the natural pH-gradient. As this electrode type generates the gases hydrogen and oxygen, Svensson suggests for the bottom electrode a gas outlet tube centrally situated in the cylindrical column. This is simply accomplished in columns with a great quotient between height and cube root of volume, i.e. columns with relatively moderate cross-sections. Attempts to apply the same principle to wide columns will result in a very uneven current density. An electrode situated in the bottom of a wide column therefore mainly has to fill the bottom surface of the column, and then the electrode must not be gasing, as rising gas bubbles should bring about convective disturbance in the column.

In order that an electrode should be non-gasing it is required that at the anode there is a reducing agent sufficiently strong to prevent evolvation of oxygen and at the cathode an oxidation agent sufficiently strong to prevent evolvation of hydrogen gas. One exception from this rule is constituted by palladium which in pure form or as a silver alloy is able to absorb large quantities of hydrogen. On the other hand a palladium electrode, saturated with hydrogen, could be utilized as a non-gasing anode as hydrogen in that case is present in the metal as a reducing agent. This property of palladium is well known since long, but it was not made use of in electrophoretical apparatus until 1960 by Neihof and Schuldiner (Nature 185, page 526 (1960)). In an important embodyment of the present invention the hydrogen absorbing ability of palladium is also utilized.

The reducing agent, which has to be present at a non-gasing anode, could either be the metal itself or the anolyte acid or some non-electrolyte which also is part of the anolyte. An example of the metal itself being reducing agent is a silver electrode in an anolyte of hydrogen halide acid, yet not hydrofluoric acid. The silver halide, formed at the anode oxidation, is almost completely unsoluble and will consequently not give rise to any migration of silver ions into the column. Another example is a lead anode in an anolyte environment of sulphuric acid. A third example is mercury in hydrogen halide acid environment, but mercury could only be used as a bottom electrode.

An example of the anolyte acid as reducing agent is hydrogen bromide acid or hydrogen iodide acid in contact with a noble metal or carbon anode. (Philpot, loc.cit., used ammonium bromide in contact with a carbon anode.) The oxidation at the anode generates free liquid bromide, which however may diffuse into the column and cause damage on proteins there. A more harmless, reducing acid is ascorbic acid.

The oxidating agent, requisite at a non-gasing cathode, can also be present on the cathode itself or in the catholyte. Silver, covered by an adhering layer of silver halide, is a known example, but such a cathode, when working, will evolve halide ions which will migrate into

the column. A bottom electrode of mercury, covered by a layer of calomel works in a corresponding way. Oxidating, soluble bases are not known. Consequently, if the oxidation agent should be present in the catholyte it has to be a non-electrolyte. Only palladium is able to function as non-gasing cathode without generating foreign anions which will migrate into the column.

By way of example the following drawings show a number of possible embodyments of the invention. The invention should not be restricted thereto.

In the drawings,

FIGS. **1** and **1***a* are, respectively, longitudinal and transverse cross-sectional elevations of a preferred form of one form of separation chamber;

FIG. **2** is a vertical cross-section of a modified form of prefocusing column combined with a final focusing column;

FIG. **3** is a cross-section of a column similar to FIG. **2** but having a modified cooling system; and

FIG. **4** is similar to FIGS. **2** and **3**, but modified to place the final column below the prefocusing column.

FIG. **1** shows a parallelepipedical combination column **14** comprising a focusing chamber **15** having internal measures *a*, *b*, and *c* length units, $a>b>c$. Outside the two largest surfaces, *ab* units or surface, there are two cooling jackets **1,2**, each provided with two pipe sockets **3,4**. Each of the two smallest walls, internally *bc* units of surface, is provided with a threaded opening **5,6** in which alternatively a threaded stopper, a threaded tube or a threaded electrode attachment could be inserted.

In this combination column the attachment of detachable electrodes constitutes the most difficult problem. Therefore, the construction shown in the figure is restricted to the realization of current transport along the dimensions *b* and *a*, which then should be vertical dimensions as the current direction has to be parallel or antiparallel to the density gradient. In the first case the column stands on a face having the internal measures *ac* units of surface, and in that position the prefocusing is accomplished. In the latter case the column is situated on a face having the internal measures *bc* units of surface, and in this position the final focusing is accomplished. The column could also be layed on a face having the internal measures *ab* units of surface, i.e. with *c* as vertical dimension. This position is suitably utilized at preparation of the density gradient as described by the Inventors in Separation Science 3, page 535 (1968). According to this method a small number of solutions having rising density are layered one under the other in a column, whereupon the column is turned down to a horizontal position in order to enlarge the diffusion area as well as the concentration gradient. The mass transport due to free diffusion thereby is increased to such an extent that only a short while is required for development of a continuous density variation in the column.

The problem of the bottom electrode could be solved very easily by using mercury, which always is spreading over the bottom independent of the spatial orientation of the column. Mercury **7** as an anode is non-gasing if it is present in an environment of hydrogen halide acid or sulphuric acid, as difficultly soluble mercury I salts are formed. Mercury as cathode is non-gasing in an environment of alkali hydroxides due to the formation of alkali metal amalgam. The external connection of a mercury electrode is also most simple. A platinum wire **8** will suffice, going through the column wall and arriving at an arbitrary corner of the column. In the figure the lead-through is intended to be bent at an angle and leading through a column face of internal measures *ab* units of surface to make possible that the column is placed upon a column face of internal measures *ac* units of surface.

Every non-gasing electrode must have a large area in order to keep the current density as low as possible. When the column is in prefocusing position (*b* vertical) a non-gasing top electrode consequently must have an area of the size of *ac* units of surface. The most suitable material is palladium-silver-alloy as cathode and the same alloy, saturated with hydrogen, as anode. Any fluid space between such an electrode and the column wall is non-desirable. Then it remains nothing but to coat this column wall with palladium-silver-alloy, and then the whole wall has to be detachable. A constructively simple way to make this is shown in the figure. The column wall **9** itself, made of plastic, having a T-shaped cross-section, is fastened by means of a number of screws **10** and is tightened by means of a O-ring **11**. A thin sheet of palladium-silver-alloy with the dimensions $c \times (a+d)$ units of surface, where *d* is slightly greater than the thickness of one of the end walls with internal measure *b* units of surface, constitutes the electrode **12**. The end wall has a cut out slit **13** having the electrode **12** protruding out of it to make possible to be connected to the voltage source. After completed prefocusing, the current is disconnected and all the screws **10** are loosened. The electrode **12** then can be pulled out whereupon all the screws **10** are once again tightened. The column then is carefully turned upright in order that *a* will become vertical dimension. Through the treaded opening **6** a platinum wire is introduced, fastened in a threaded electrode attachment provided with a gas outlet hole, whereupon voltage could be applied for final focusing. After completed final focusing the top electrode is replaced by an attachment having a tight threaded stopper, and the stopper which till then has been situated in the treaded hole **5** is replaced by a threaded outlet tube having a regulating valve. The stopper in the hole **6** now can be removed. The column is slightly tilted in order that the mouth of the hole **5** will be the lowest point of the column. After that the column can be emptied from its content, which is at that divided into fractions for pH-measurement and chemical and/or biological analysis.

The column can alternatively be made of quartz, whereby UV-absorption analysis can be accomplished directly in the column during the performing of focusing, which is of great advantage. The fastening of the column wall **9** by screws then must be substituted by a device for clamp fastening, but besides, the same construction can be retained. Two different sensitivity degrees in the optical analysis can be achieved by allowing the light to pass the path *b* or the path *c* in the column. The shift between these degrees of sensitivity is simply accomplished by turning the column 90°.

To utilize mercury as anode can be associated with certain risks if it is considered that mercury ions could go into solution and migrate through the column and transform the proteins in the column by complex formation. It is true that the mercury I halides are as sparingly soluble that they would hardly give rise to deleterious concentrations of mercury I ions, but at the anodical oxidation of mercury also small amounts of

mercury II salts, which are soluble are formed. However, these are so weakly dissociated that their cathodical migration will be very slow. Anyhow, there are doubtless cases in which mercury anode is completely inconceivable. The combination column then must be used with the cathode in the bottom and a sufficient reserve of alkali metal ions, or mercury as an electrode has to be completely abandoned. In the latter case the column construction has to be modified.

Principally this can be made by a symmetrical design in which the two column walls having an internal area of *ac* units of surfaces are made detachable as described in FIG. **1** for one of the walls. The palladium-silver-anode then should be saturated with hydrogen and the palladium-silver-cathode be free of hydrogen, and both the electrodes should be removed after prefocusing. A remaining problem then is how to realize a non-gasing bottom electrode with an area of *bc* units of surface during the final focusing. Presumably, the best way to achieve this is to prepare an extra column wall having the internal measure *ac* units of surface and in one end of this constructional element attach a palladium-silver-electrode, which is slightly smaller than *bc* units of surface. When the prefocusing is completed, the bottom electrode is pulled out with a temperary loosening of the screws **10,** and then the top wall with its electrode is completely removed and substituted by the extra wall which in its one end makes the attachment for the bottom electrode in final focusing position.

FIG. **2** shows a column device comprising a prefocusing column **21** and a final focusing column **25** in the position of the device during the transport of prefocused material from one column to the other and during the final focusing. During the prefocusing the device is turned 90° around an axis perpendicular to the plane of the paper so that the final focusing column **25** then will have a horizontal axis. The final focusing column **25** in this example is represented by the above mentioned apparatus, marketed by LKB-Produkter AB. As described above, this apparatus comprises in addition to the column noble metal electrodes, a central gas outflow tube as well as a cooling jacket.

As these constructional details are known as such and are not part of the invention, only the outer contours of this column have been drawn in the figure. The volumes of the two columns are equal.

The central part of prefocusing column is the focusing chamber **21** itself, limited at the top and at the bottom by two electrolytically conducting but convection breaking membranes **24,** e.g. glass filter discs. Laterally the focusing chamber is restricted by vertical walls, e.g. by a short elliptical or circular cylinder. In two diametrically opposite points there are threaded holes **30, 31** in which alternatively threaded stoppers or tubes can be inserted. During prefocusing there is in the hole **30** a stopper, in the hole **31** a tube which via a cock **32** forms the connection to the final focusing column.

Outside the membrane **24** there is an anolyte chamber **22** or **23** and a catholyte chamber **23** or **22** which via tubes and circulation pumps **29** is connected each to one electrode vessel **26,28** containing each an electrode **27.** Each tube-pipe comprises a coil, immersed into a cooling reservoir, which details, however, are not shown in the figure. A suitable clamping device, which is neither shown, is holding in a leakage free manner anolyte chamber, focusing chamber, and catholyte chamber together.

By operation of this column device first the membranes **24** are thoroughly moistened in order to be impermeable to air. Then into the hole **30** a tube is inserted which is connected to a density gradient mixing device, and the cock **32** is opened. With the whole device in final focusing position a vacuum is applied to the final focusing column in order that the prefocusing chamber **21** is filled with a solution of desired density variation. The cock **32** is closed, and the tube in the hole **30** is substituted by a stopper. Whole the device is turned 90° around an axis perpendicular to the plane of the paper, the electrode vessels are filled with anolyte and catholyte, respectively, and the circulation pumps **29** are started. Voltage is applied to the electrodes for prefocusing.

After completed prefocusing the current is disconnected, and the device is carefully turned 90° around an axis perpendicular to the plane of the paper. Anolyte and catholyte then are allowed to flow out through the gas outlet holes of the electrode attachments. It is also possible to completely remove the electrodes with their attachments. After the device has been raised the stopper in the hole **30** is substituted by a tube, filled with and dipping into a fluid having greater a density than the solution in the bottom of the column. This tube should also be provided with a cock or, if rubber or plastic tubes are used, a ligature clamp. With the latter one closed, the cock **32** is opened. By applying a suitable vacuum to the final focusing column **25** and by governing the cock or ligature clamp of the tube inserted in **30,** the column content in the chamber **21** is replaced by the said heavier fluid, and the prefocused material then will ascend into the final focusing column, the cooling jacket of which during this operation being throughflown by cooling medium. When the fluid transport is completed, voltage is applied to the final focusing column after the cock **32** has been closed. The prefocusing column is removed, and after the final focusing the column content can be fractioned by careful outflow through the cock **32.**

FIG. **3** shows a column device distinguished from that one shown in FIG. **2** only concerning the cooling system. In this device the anolyte and catholyte chambers are made larger in order that there is room for cooling coils **33,34** through which cooling medium from a cooling reservoir is made to circulate. This device has the advantage compared to that one shown in FIG. 2 that the circulation pumps are not under a voltage. The electrodes **35** can be attached in rather slender tubes **36,37** which are centrally leading out into the anolyte and catholyte chambers.

The utilization of column devices according to FIG. **2** and FIG. **3** have in common that the anolyte should consist of a solution of a strong acid and the catholyte of a solution of a strong base. Their concentrations should be between 0.1 and 1 equivalents per liter. Their conductivities then will be several 10-powers greater than that one prevailing in the central parts of the column at focusing. This is why it is possible in FIG. **2** and **3** to have as narrow tubes for supplying of current to the anolyte and catholyte chambers. There is no risk of uneven current density caused by the local falling out of the tubes into the anolyte and catholyte chambers.

FIG. **4** shows how a column device could be arranged in which the electrodes are in direct contact with the

prefocusing column and in which the fluid transport between the two columns is carried out from the top downwards. The figure is showing the device being in its position during fluid transport from one column to the other and during final focusing. During the prefocusing the device is turned 90° around an axis perpendicular to the plane of the paper, in order that the final focusing column **48** then will have a horizontal axis. Also in this example the final focusing column is represented by the above mentioned apparatus marketed by LKB-Produkter AB. As described above, this apparatus comprises, besides the column itself, noble metal electrodes, a centrally situated gas outflow tube, and a cooling jacket. As these constructional details are known as such and are not making part of the invention, only the outer countours of this column have been drawn in the figure. The volumes of the two columns are equal.

The prefocusing column **52** is at its top and bottom limited by two non-gasing electrodes **42** and **46**, each joint by soldering to one external wall of two cooling chambers **47** and **49**, each provided with two pipe sockets **41**. Laterally the prefocusing column is restricted by vertical walls **44**, e.g. being a short elliptical or circular cylinder. In two diametrically opposite points there are threaded holes **45,50** in which alternatively threaded stoppers or tubes could be inserted. During prefocusing there is in the hole **45** a stopper, in the hole **50** a tube which via a cock **51** forms the connection to the final focusing column **48**. The prefocusing column is tightened by means of O-rings **43** combined with a clamping device acting on the outer walls of the two cooling chambers.

When operating this column device it is first placed in final focusing position (as in the figure), the final focusing column is filled with a fluid with greater a density than the bottom solution to be used in the prefocusing column, whereupon the bottom cock of the final focusing column and the cock **51** between the columns are closed. The prefocusing column then is filled with an amfolyte solution containing the protein sample, out of a density gradient mixer, whereupon the hole **45** is closed by means of a stopper. The column device then is turned slowly 90° around an axis perpendicular to the plane of the paper, the cooling water flow is started and electrical voltage is applied for prefocusing. After completed prefocusing the current is disconnected and the column device is turned backwards 90°. The cocks in both ends of the final focusing column are opened, and the stopper in the hole **45** is removed. By means of one of the cocks the flow rate downwards is adjusted to a suitable, low value. After the fluid content of the prefocusing column has flowed down into the final focusing column, the bottom cock of the latter is closed, and the prefocusing column is removed. Voltage is applied to the final focusing column, and after completed final focusing the current is disconnected and the column content is divided into fractions flowing out through the bottom cock.

In a typical experiment with the device according to FIG. **1** the prefocusing required 1.5 hours and the final focusing 3 hours.

In an experiment with the device according to FIG. **4** the prefocusing was carried out during 1 hour, whereupon the column content was transferred to the final focusing column during 15 minutes. The final focusing was then carried out during 5 hours. By using any of these apparatuses it is possible to obtain the resolution power $\Delta pI = 0.02$, stated above.

With the device according to FIG. **4** it is most likely that this resolution can be achieved in a prefocusing period of only 10 minutes and a final focusing period of 3 hours. Thus, the focusing period is decreased from 24–48 hours to about 3.5–6.5 hours. Isoelectric focusing with this high resolution in this short period of time means a decided improvement compared to hitherto known technique.

We claim:

1. Apparatus for the separation of components in a sample solution by isoelectric focusing in which said focusing is performed in at least two successive focusing steps comprising, closed container means to be disposed in a first position during said first focusing step and in a second position during the second focusing step, the interior thereof defining a focusing chamber, at least two pairs of oppositely disposed walls, the mean area of one pair of said walls being less than the mean area of the other pair of said walls, the mean distance between said one pair of walls being greater than the mean distance between said other pair of walls, an electrode system for selectively generating a vertical direct current electrical field across said sample between the respective inner surfaces of each pair of said two pairs of oppositely disposed walls in either of said first or second positions of the container means.

2. Apparatus according to claim **1** which includes means for externally cooling said focusing chamber.

3. The invention defined in claim **1**, wherein said closed container means is rotated bodily about a single axis for movement between said first and second positions.

4. The invention defined in claim **1**, wherein said electrode system includes a separate vessel contianing an electrode and an electrolyte solution therein, said separate vessel and said closed container means being separated from each other by a convection breaking electrolytically conducting membrane, said membrane defining at least one of said two pairs of walls, said membrane separating the sample solution from the electrolytic solution.

5. The invention defined in claim **4**, wherein said separate vessel includes means for cooling said electrolytic solution.

6. The invention defined in claim **5**, wherein said means for cooling said electrolytic solution includes conduit means in said separate vessel for conducting a cooling medium in heat exchanging relationship through said electrolytic solution.

7. The invention defined in claim **1**, wherein said electrode system includes at least one electrode element in direct contact with said sample solution in one position of said container means.

8. The invention defined in claim **7**, wherein said electrode element comprises palladium.

9. The invention defined in claim **7**, wherein said electrode element comprises silver.

10. The invention defined in claim **7** wherein said electrode element comprises lead.

11. The invention defined in claim **7** wherein said electrode element comprises mercury.

12. The invention defined in claim **11** wherein said mercury electrode element is disposed at the bottom of said focusing chamber on a respective one of said two pairs of oppositely disposed walls in either of said first

and second positions of the container means, said means also including means for cooling said mercury.

13. The invention defined in claim **7**, wherein at least one wall of at least one of said two pairs of walls includes a flat electrode extending over the entire area of the inner surface of said one wall.

14. The invention defined in claim **13** wherein said closed container means includes a removable section, the inner surface of said section defining said one wall, and means for detachably connecting said section in fluid tight engagement with the remainder of the container means.

15. The invention defined in claim **14** wherein said closed container means also includes at least one cooling chamber in heat exchanging relationship with said sample solution.

16. The invention defined in claim **1** wherein said closed container means comprises two focusing chambers in communication with each other, and valve means for confining said sample solution in one of said focusing chambers while in said one position and for transferring the sample solution to the other of said focusing chambers when the container means is in said second position.

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UNITED STATES PATENT OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 3,915,839

DATED : October 28, 1975

INVENTOR(S) : Svante Harry Rilbe and Jarl Sune Pettersson

It is certified that error appears in the above-identified patent and that said Letters Patent are hereby corrected as shown below:

On the cover page, please add the following:

--Foreign Application Priority Data

May 16, 1972 SWEDEN.....6377/72--

Signed and Sealed this

second Day of March 1976

[SEAL]

Attest:

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Attesting Officer

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