A SPECIAL SAMPLING TECHNIQUE FOR ACCURATELY DETERMINING MOBILITIES OF BIOLOGICALLY ACTIVE SUBSTANCES IN THE TISELIUS ELECTROPHORESIS CELL

by

A. RANDRUP

State Veterinary Institute for Virus Research, Lindholm, near Kalvehave (Denmark)

When electrophoretic investigation of a biologically active material is attempted, it is essential to be able to determine the mobility of the active principle exclusively on the basis of biological measurements. Such determinations make it possible to control whether an optically demonstrable boundary actually corresponds to the biological principle, and if no boundary can be demonstrated optically, it will still be possible to measure the mobility of the biologically active principle.

In order to be able to perform biological measurements it is necessary to remove samples from the electrophoresis cell, but as far as is known to the author, there exists no method of sampling permitting accurate determination of the mobility. THEORELL[1] has constructed a special electrophoresis apparatus permitting quite good sampling, but this apparatus does not possess the many advantages of that of the TISELIUS type. However, information is also available about sampling from the TISELIUS electrophoresis cell. Most of the methods described are, however, preparative, their object being to sample whole fractions, as for example the methods devised by TISELIUS[2], SVENSSON[3], and SHEDLOVSKY *et al.*[4], whereas BOURDILLON AND LENETTE[5] have performed sampling for analytical purposes. If calculations of mobilities on basis of samples removed according to the methods mentioned are made, rather inaccurate results will be obtained. This is mainly due to the facts that the volumes removed are too large and the sources of error on the sampling are not investigated. When we wanted to perform electrophoresis of foot-and-mouth disease virus at Lindholm we therefore first tried to devise a method of sampling not possessing these drawbacks.

DESCRIPTION OF THE METHOD

For the sampling a special needle was constructed, shown in Fig. 1. The figure also shows how the needle can be lowered into the electrophoresis cell through one of the small centre plugs.

The holder of the needle is made of a folded copper sheet. The needle is pressed against the bent edge of the copper sheet by a spring placed between the two layers. The holder efficiently checks the tendency of the needle to vibrate, and makes a slow and quiet movement of the latter possible*.

* The needle and holder were devised by Mr. E. ANDERSEN, Lindholm.

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As the needle is lowered into the electrophoresis cell, complete prevention of the vibration can be obtained by sliding the needle along one of the broad sides of the rectangular channel. Since it is held firmly by the holder, the needle can conveniently be lowered to any desired point in the cell. In an apparatus equipped with the PHILPOT-SVENSSON optical system, the needle can be observed as a shadow on the screen, the entire electrophoretic diagram, however, remains visible.

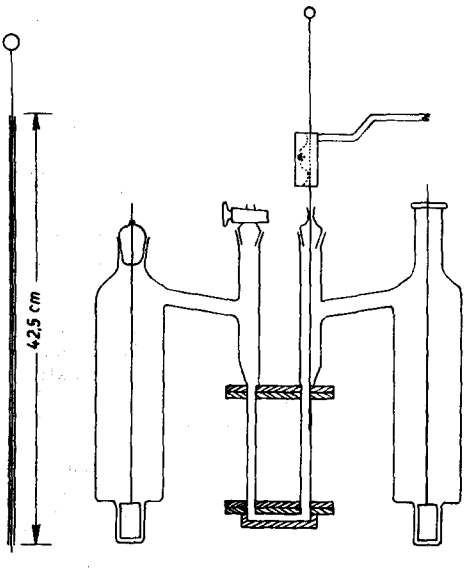

Fig. 1. The internal diameter of the needle is 1.0 mm. The diameter of the wire is 0.80 mm.

Before inserting the sampling needle, its lower end is sealed with a small amount of grease. "Lubriseal"*, which has a melting point of about 40° C, has been found to be satisfactory. The wire inside the needle protrudes a little out of the grease as seen in Fig. 1. In 7 tests we have kept the needle sealed in this way for some time in a coloured solution, and ascertained that no liquid had penetrated.

The sample is taken into the needle by slowly moving the wire upwards, whereby a hole is formed in the grease. Since the wire fits snugly it can be fixed at any desired level, and it is therefore possible to take samples of different volumes. When the wire has been moved upwards to the level chosen, the needle is sealed at the top by melted paraffin, so that the sample does not leak out when the needle finally is removed from the electrophoresis cell (paraffin cannot be used for sealing at the lower end, as it gets too hard by the low temperature in the electrophoresis cell). The entire sample taken can now easily be transferred to a small glass by drawing the wire out of the needle and blowing into the latter. The greatest volume which can be removed is 0.2 ml, the smallest about 0.05 ml.

INVESTIGATION OF SOURCES OF ERROR

It is impossible to know whether the sample originates exactly from the level of the cell at which the hole of the needle was placed, as the sampling may produce currents in the liquid around the point of the needle. This is the greatest source of error. By experiments we have attempted to determine from which layers the sample originates.

In order to examine the layers below the point of the needle a boundary was established between a concentrated solution of phenol-red in the centre section of the cell and water in the upper section, and samples were removed from the water at various levels above this boundary. The solution of phenol-red was so concentrated that even after a ten thousand fold dilution it was faintly reddish. Therefore it was possible to observe even a very small content of the red liquid in the samples. The amount of

* Obtained from ARTHUR H. THOMAS, Philadelphia, U.S.A.

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phenol-red in the samples could be determined by comparison with suitable dilutions of the original phenol-red solution.

In order to investigate the layers above the point of the needle, a boundary was established between a K_2SO_4 solution in the centre section of the cell, and a phenol-red solution in the upper section, and samples were removed from the K_2SO_4 solution at various levels below the boundary.

The phenol-red solution was made by dissolving 1 g phenol-red in about 100 ml water, and adding the amount of NaOH solution necessary for neutralising the acid dye.

For practical reasons the experiments were performed at room temperature without a thermostat. It was here necessary to operate with a rather great difference between the specific gravities of the coloured and the colourless liquids, about 0.011 g/ml, as otherwise the boundary was unstable. The specific gravity was measured by a gravity gradient tube[6]. With this apparatus the specific gravity is measured in proportion to water at the temperature of the apparatus during the measurement.

In the experiments in which samples were removed below the boundary, the coloured solution with a specific gravity of 1.0031 was placed in the upper section and a K_2SO_4 solution with a specific gravity of 1.0141 in the centre section. The difference between the specific gravities of the boundary then became 0.0110. In the experiments in which samples were taken above the boundary, a mixture of 2 parts phenol-red plus one part filtered cattle serum was placed in the centre section and water in the upper section. The specific gravity of the phenol-red mixture was 1.0115, and the difference between the specific gravities at the boundary therefore became 0.0115.

The boundary was established as usual by sliding the sections of the electrophoresis cell, and the sampling was made immediately after the establishment of the boundary, the latter being therefore as sharp during the sampling as in any way possible. It was less fortunate to operate with the boundary behind the ground glass edge of the sliding end plates of the cell sections. However, it was possible to look through the edge when paraffin oil was applied to it, and so it was possible to move the needle to the right position. In the experiments in which sampling was made above the boundary, the needle was moved to position before the boundary was established. The hole of the needle could always be placed at the desired distance from the boundary with an error of less than 0.2 mm.

All the samples were removed from the open side of the apparatus, whereas the other side was kept closed (see Fig. 1). However, it is also possible to remove samples from the closed side of the apparatus, just by sliding aside the bottom section of the cell and lowering the needle through the three-way stopcock. In all the experiments the boundary was established between the upper and the centre sections of the cell.

The volume of the sample was determined by weighing. For each sampling the needle was washed and dried.

The comparison between the densities of the colours of suitable dilutions of the sample and the initial solution could in most cases be made by the eye. Only when the difference between the densities of the colours was small, a spectrophotometer was used (wave length 550 mμ). Before the comparison a NaOH solution was always added to the liquids to ensure that exclusively the phenol-red base was present. By calculation of the dilutions the NaOH added was taken into consideration.

An *"Aminco"** electrophoresis apparatus was employed. The cross-section area of the cell employed was 2 × 15 mm^2 (by the company called *"Semimicro analytical cell"*).

The results of sampling above the boundary appear from Table I. It will be seen that in all the experiments less than 1% of the sample originates from layers at a level more than 2.2 mm lower than the hole of the needle (*i.e.* the hole formed in the grease when the wire is drawn out). The results of sampling below the boundary appear from Table II. It will be seen that in all the experiments less than 2% of the sample originates from layers originally at a level more than 2.2 mm above the hole of the needle.

As mentioned above, the sampling was made from the open side of the electrophoresis apparatus, whereas the other side remained closed, so that in the cases in which sampling was made below the boundary, the latter was descending. As previously mentioned the cross-section of the cell employed was 2 × 15 mm^2, and as a volume of about 0.05 ml was sampled, the boundary consequently descended about 1.7 mm during the sampling, and therefore almost reached the point of the needle. In certain experiments, for example in experiment 7 Table II, the boundary would seem

* Manufactured by the American Instrument Company, Silver Spring Maryland, U.S.A.

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to have passed the point of the needle, without any great amount of coloured solution having moved into the needle. In explanation of this it may be stated *that* the point of the needle may have been placed lower than recorded, up to 2.2 mm below the boundary (see above), *that* part of the sample originates from layers at a lower level than the point of the needle, and, finally, *that* the boundary may have been deformed by the currents of the liquid so that it bulges around the point of the needle. Thus the experiments suggest that if the boundary did not move, sampling might be made immediately below the latter without getting more than a small percentage of coloured solution in the sample.

TABLE I

SAMPLING ABOVE THE BOUNDARY

Above the boundary: distilled water spec. grav. 1,0000.
Below the boundary: phenol-red solution with serum.

Sample No.	*Sampling above boundary mm*	*Wire elevated mm*	*Vol. ml*	*Content of phenol-red solution*	*Spec. grav. of phenol-red solution*
1	1.5	40	0.070	1 %	
2	2.0	42	0.067	0.7 %	
3	2.0	42	0.056	0.05 %	
4	2.0	42	0.059	0.03 %	about 1.011
5	2.0	42	0.064	0.2 %	
6	2.0	42	0.058	0.05 %	
7	2.0	42	0.049	0.05 %	
8	2.0	42	0.066	0.1 %	
9	2.0	42	0.052	0.5 %	
10	2.0	42	0.045	0.2 %	
11	2.0	42	0.049	0.2 %	
12	2.0	42	0.064	0.2 %	1.0115
13	2.0	42	0.064	0.2 %	
14	2.0	42	0.062	0.2 %	
15	2.0	42	0.065	0.4 %	

TABLE II

SAMPLING BELOW THE BOUNDARY

Above the boundary: phenol-red solution spec. grav. = 1.0031.
Below the boundary: K_2SO_4 solution spec. grav. = 1.0141.

Sample No.	*Sampling below boundary mm*	*Wire elevated mm*	*Vol. ml*	*Content of phenol-red solution*
1	3	90	0.060	< 0.1 %
2	1	90	0.072	> 1 %
3	1.5	90	0.082	< 0.5 %
4	1.5	90	0.068	> 1 %
5	1.5	90	0.061	> 5 % < 10 %
6	2	90	0.058	ca 0.1 %
7	2	90	0.069	< 1 %
8	2	85	0.061	< 2 % > 1 %
9	2	85	0.060	< 2 % > 1 %
10	2	85	0.052	< 1 %
11	2	85	0.065	< 2 % > 1 %
12	2	85	0.064	< 2 % > 1 %
13	2	85	0.054	< 1 %

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Another two sources of error on the sampling were taken into consideration and their importance investigated by experiments.

As the volume removed is so small, it would seem possible that a rather large amount of the sample remained on the walls of the needle and on the wire after the needle had been emptied. The amount involved was determined by removing samples of phenol-red solution and afterwards washing the needle and wire thoroughly with 1 ml water. On basis of the density of the colour of the rinse water, its content of phenol-red could be determined. In 5 experiments a content of 1/1000 — 1/300 was found. As the volume of the rinse water was 1 ml, it appears that an amount of 0.0010–0.0035 ml of the sample was deposited on the walls of the needle and on the wire (after the needle had been emptied). As the volume of the entire sample was 0.05 ml, this corresponds to a deposit of 2 to 7%.

When the sample is in the needle there is a small space between the surface of the fluid in the needle and the paraffin sealing. As this space may "give" there is a possibility that the surface of the fluid in the needle may descend and a small amount of the sample leak out while the needle is removed from the apparatus. In order to determine the amount involved, several samples were taken with a glass pipette of almost the same internal diameter as the needle. The samples were taken from the densely coloured phenol-red solution, so that the fluid inside the pipette could be clearly seen. In 5 experiments it was observed that the surface of the fluid in the pipette descended 0.0–0.7 mm during the removal of the pipette from the container. Considering the dimensions of the needle, the pipette, and the wire, it is now possible to calculate that an amount of less than 0.0005 ml leaks out of the needle when this is removed from the electrophoresis apparatus. This corresponds to $< 1\%$ of a sample of 0.05 ml.

The two latter sources of error can therefore cause only small variations in the samples. In the experiments presented in Tables I and II, these sources of error have also influenced, and therefcre they do not give rise to any special adjustments.

INVESTIGATION OF SOURCES OF ERROR WHEN SEVERAL SAMPLES ARE REMOVED DURING THE SAME ELECTROPHORESIS EXPERIMENT

In many instances it will be of interest to remove several samples during the same electrophoresis experiment. When using our method the samplings should then be made in the order from the top to the bottom, so that the needle does not touch layers from which later on sampling is to be made. However, a sampling may also cause disturbance in layers at a lower level than the point of the needle, and in order to investigate this source of error, some experiments with a phenol-red solution were made.

The technique of these experiments was mainly the same as that described above. Therefore only a few special things will be mentioned here.

The boundary between the phenol-red in the upper section and the K_2SO_4 in the centre section, with a difference between the specific gravities of 0.011, is not quite stable as the dye slowly descends into the colourless solution. This was of no importance in the experiments in Table II, which were made very quickly, but the experiments in Table IV take more time, and in these experiments the boundary was therefore made more stable by increasing the difference between the specific gravities to 0.0177 (the specific gravity of the K_2SO_4 solution employed was 1.0227).

In the experiments presented in Table III, a boundary was established between a mixture of 2 parts phenol-red and 1 part cattle serum in the centre section, and distilled water in the upper section. In each experiment sampling was made first at a level

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2.8 mm above the boundary (all these samples were cclourless), and then sampling was made at a level 2 mm above the boundary (as in the experiments in Table I). It will be seen from Table III that the first sampling had caused so little disturbance that all of the second samples contained less than 1% of the coloured solution.

TABLE III

SAMPLES REMOVED SUCCESSIVELY IN SERIES OF TWO

First sample removed 2.8 mm above the boundary. Second sample 2 mm below the boundary.
Below the boundary: phenol-red solution with serum spec. grav. 1.0109.
Above the boundary: distilled water spec. grav. 1.0000.

Experiment No.	*1*	*2*	*3*	*4*	*5*	*6*
Content of phenol-red solution in 2nd sample	$< 1\%$	$< 1\%$	$< 1\%$	$< 1\%$	$< 1\%$	$< 1\%$

In the experiments presented in Table IV, a boundary was established between a K_2SO_4 solution in the centre section, and a phenol-red solution in the upper section. In each experiment sampling is made first at a level 2.8 mm above the boundary, and then from the K_2SO_4 solution at a level 2.8 mm below the boundary. It will be seen from Table IV that all of the second samples contained less than 3% phenol-red solution.

TABLE IV

SAMPLES REMOVED SUCCESSIVELY IN SERIES OF TWO

First sample removed 2.8 mm above the boundary. Second sample 2.8 mm below the boundary.
Above the boundary: phenol-red solution spec. grav. 1.0050.
Below the boundary: K_2SO_4 solution spec. grav. 1.0227.

Experiment No.	*1*	*2*	*3*	*4*	*5*
Content of phenol-red solution in 2nd sample	$< 1\%$	$< 1\%$	$< 2\%$	$< 2\%$	$< 3\%$

In the first series of experiments (Table III) the distance between the two samplings was 0.8 mm, and in the last (Table IV) 5.6 mm. Consequently, it is possible to remove several samples during the same electrophoresis experiment with a mutual distance of 6 mm only. Of each sample $< 1\%$ will then originate from layers which were more than 2.2 mm lower than the hole of the needle during the sampling in question, and $< 3\%$ will originate from layers which were more than 3 mm above the hole of the needle.

APPLICATION OF THE METHOD

CALCULATION OF THE ERROR ON DETERMINATIONS OF MOBILITY

The results from the experiments with the coloured solutions cannot be applied directly in the different cases met with in practice, for example, electrophoresis of protein solutions, especially because it is not certain that all the boundaries are equally stable. The best procedure will be, in each individual case, to control the method by

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experiments with coloured solutions under circumstances as similar as possible to those under which the method is to be applied. In the electrophoresis of protein solutions the differences between the specific gravities at the boundaries will often be considerably less than those employed in the above-described experiments. These experiments were performed at room temperature without a thermostat. However, a few experiments were also made with the cell in the thermostat of the electrophoresis apparatus at 2° C. These experiments showed that in the thermostat stable boundaries can be obtained with far smaller differences between the specific gravities of the coloured and the colourless solutions.

As mentioned above the experiments in this communication were performed with a cell of a cross-section area of 2×15 mm^2. It is obvious that the method of sampling may also be applied in connection with the more ordinary cell type with a cross section area of 3×25 mm^2, and that the greater cross-section area here gives a possibility of sampling from even thinner layers than 4.4 mm, or of taking a greater volume from a layer of this thickness.

The application of the method at Lindholm will be described in a later report on electrophoresis of foot-and-mouth disease virus (FM-virus). At this point just a calculation on basis of the results in Tables I–IV will be made in order to show the accuracy to be obtained by the sampling method on determination of the mobility of FM virus.

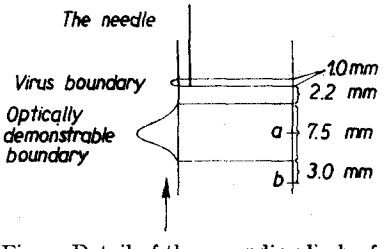

Fig. 2. Detail of the ascending limb of the electrophoresis cell (front view).

The strength of an FM virus solution is measured by inoculating a series of ten-fold dilutions in guinea pigs (titration). Because an error of up to one ten-fold dilution must be taken into consideration, the determination is very inaccurate. In spite of this, rather accurate determinations of the electrophoretic mobility may be obtained by simultaneously titrating samples removed during the electrophoresis and the initial solution subjected to electrophoresis.

In Fig. 2 the optically demonstrable boundary is supposed to be due to an inactive protein substance, whereas the virus is supposed to migrate a little quicker than the protein substance, and to be present in such a low concentration that it cannot be demonstrated optically.

If a sample is taken as shown in the figure, it will contain more than 10% undiluted virus solution, and its strength will therefore be below one ten-fold dilution less than the strength of the undiluted original virus solution (the initial solution). If, on the other hand, the virus had migrated together with the optically demonstrable boundary, the sample would according to Table I have contained less than 1% undiluted virus solution, and the strength of the sample would therefore have been more than two ten-fold dilutions less than the strength of the initial solution. This difference between the two cases is greater than the error in the virus titrations, and by the method of sampling it is therefore possible to demonstrate even such a small distance between the two components as that in question. It can easily be calculated how small a difference in mobility it will be possible to demonstrate as a consequence of this. The distance between the two boundaries is 6.5 mm, as will be seen from Fig. 2. In the "Aminco" electrophoresis apparatus 55 mm of the rectangular channel of the cell can be observed

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optically. If the optically demonstrable boundary has moved this distance when the sample is taken, the difference between the two mobilities is $6.5 \times 100\% = 11.8\%$.

If the sampling had been made as described above, and the sample had been more than two ten-fold dilutions weaker than the initial solution, it could conversely have been inferred that the mobility of the virus was less than 112% of the mobility of the optically demonstrable boundary.

If compensation is applied, the distance covered to be observed optically will be more than 55 mm, and thereby a correspondingly greater accuracy can be obtained. However, in some instances, especially when small differences in specific gravity exist at the boundary, material may stick to the walls of the cell during compensation and so obscure the results.

It will be seen by a similar reasoning that if the sample is removed at *a* in Fig. 2, there is a possibility of showing that the mobility of the virus is greater than $100 - 4.3 \times 100 = 92\%$ of the mobility of the optically demonstrable boundary. If the sample is removed at *b* in Fig. 2 there will in the same way be a possibility of showing that the mobility of the virus is greater than $100 - 9.5 \times 100\% = 82.5\%$ of the optically demonstrable boundary.

It will be seen from Tables III and IV that it is possible to remove successively the three samples mentioned, in the course of the very same electrophoresis experiment. Consequently, it is possible to obtain six good mobility determinations for each electrophoresis experiment, it being, as mentioned, possible to remove samples from both sides of the cell. Of the six samples three determine an upper limit of the mobility of the biological principle, and the three others a lower limit, the same deviations as described above being obtained, but in the opposite direction, by sampling in the descending limb of the cell (here, however, material may stick to the walls of the cell and cause trouble in the same way as in connection with compensation). It appears that the most exact results will be obtained from the samples removed from the middle of the optically demonstrable boundary. Removal of all six samples gives, however, simultaneously a pattern of how the concentration of the biological principle changes around the boundary.

If in the above-mentioned example there had been no optically demonstrable boundary for comparison, it might be possible with good samples and by reasoning as above to determine the electrophoretic mobility of the FM virus with an error of less than 10% (besides the usual errors on the determination of electrophoretic mobility). By application of compensation it might also here be possible to obtain greater accuracy.

SUMMARY

1. A needle has been constructed by means of which samples of 0.05–0.2 ml can be removed from a TISELIUS electrophoresis cell in such a way that it can be accurately determined from which level in the cell a sample originates.

2. The sources of error connected with the method of sampling have been investigated by experiments with coloured solutions.

3. It has also been proved by experiments with coloured solutions that several samples may be removed in the course of one electrophoresis experiment, and that the distance between the levels of sampling may be as small as 6 mm.

4. By calculations in which a very inaccurate biological method of assay is assumed it has been shown that the sampling method makes it possible to demonstrate differences as small as 10% between the mobilities of a biological principle and an optically demonstrable boundary. Further

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it has been shown that the method provides possibilities of determining electrophoretic mobilities exclusively on basis of biological measurements with an error of less than 10% (besides the usual errors on determinations of electrophoretic mobility).

RÉSUMÉ

1. Il a été exécuté une canule avec laquelle, d'après une méthode décrite en détailles, l'on peut prélever, d'une cellule d'électrophorèse-Tiselius, des échantillons de 0.05 à 0.2 ml, de telle sorte que l'on puisse établir, avec exactitude, le niveau de la cellule d'où provient un échantillon donné.

2. Les sources d'erreur de la méthode de prélèvement ont été examinées au cours d'expériences sur solutions colorées.

3. Par des expériences sur solutions colorées, il a été constaté également que l'on peut prélever plusieurs échantillons au cours de la même expérience d'électrophorèse et que l'intervalle entre les prélèvements peut être réduit à 6 mm.

4. Sur la base de ces expériences, on a exécuté des calculs prouvant que la méthode de prélèvement permet d'établir des écarts de moins de 10% entre les mobilités d'un principe biologique et d'une limite démonstrable par voie optique. Dans les calculs une méthode de mesure biologique très inexacte a été prévue. En outre, il a été établi que la méthode permet de déterminer des mobilités d'électrophorèse uniquement sur la base de mesures biologiques avec une erreur de moins de 10% (en dehors des erreurs habituelles dans les déterminations de mobilités d'électrophorèse).

ZUSAMMENFASSUNG

1. Es wurde eine Kanüle hergestellt, mit welcher man nach einer näher beschriebenen Methode Proben von 0.05–0.2 ml einer Tiselius-Elektrophoresezelle so entnehmen kann, dass sich genau feststellen lässt, von welchem Niveau in der Zelle eine Probe herrührt.

2. Die Fehlerquellen der Entnahme-Methode wurden durch Versuche mit gefärbter Lösung untersucht.

3. Durch Versuche mit gefärbter Lösung wurde ebenfalls gezeigt, dass man mehrere Proben bei demselben Elektrophorese-Versuch entnehmen kann und dass der Zwischenraum zwischen den Entnahmen auf 6 mm beschränkt werden kann.

4. Auf Grund dieser Versuche wurden Berechnungen vorgenommen, welche erweisen, dass die Entnahme-Methode ermöglicht, Abweichungen unter den Mobilitäten eines biologischen Prinzips und einer optisch nachweisbaren Grenze von 10% nachzuweisen. Bei den Berechnungen wurde eine sehr ungenaue biologische Messmethode vorausgesetzt. Ebenfalls wurde gezeigt, dass die Methode ermöglicht, elektrophoretische Mobilitäten lediglich auf Grund biologischer Messungen mit einem Fehler von weniger als 10% (ausser den gewöhnlichen Fehlern bei elektrophoretischen Mobilitätsbestimmungen) zu ermitteln.

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