1.15 Electrophoresis

Electrophoresis is a physical method of analysis permitting the separation of compounds that are capable of acquiring en electrical charge in a conducting electrolyte. In this medium the ionized particles move more or less rapidly under the influence of an electrical field.

The electrophoretic mobility is the rate of migration of the substance measured in cm/s under the influence of a potential gradient of 1 V/cm, and is expressed in $cm^2 \cdot V^{-1} \cdot s^{-1}$.

The measurement of electrophoretic mobility is significant only where experimental conditions have been precisely defined. This mobility depends on the characteristics of the substance, its nature, size, form, and electrical charge. It also depends on the composition of the conducting liquid, its nature, concentration, pH, the presence of additional solvents and viscosity. The direction of migration depends on the sign of the electrical charge of the particle as it moves towards the electrode of opposite sign.

According to the methods used, the electrophoretic mobility is either measured directly or compared with that of a reference substance.

Moving Boundary (Free-flow) Electrophoresis

This technique, used exclusively for the determination of the mobility, is particularly suitable for substances of high molecular weight with poor diffusion properties.

The boundaries are usually measured both before and after the application of an electrical field by a physical method, such as refractometry or conductometry. The concentration of the substance in the conducting liquid, the characteristics of the latter and the details of the procedure, including quantitative evaluation of the fractions, are specified in the monographs.

Zone Electrophoresis (Electrophoresis using a Supporting Medium)

This method uses only small sample sizes. The nature of the supporting medium (for example, paper, cellulose acetate, starch-gel, agar-gel, polymethacrylamide, mixed gel) introduces additional factors influencing the mobility. The rate of migration depends on the mobility of the particles and also on the electro-endosmotic current (in the case of carriers with polar properties), on the currents due to evaporation (caused by heat generated through the Joule effect), and on the gradient of the electrical field.

In practice, the mobility of the electrophoretic zones and their signs are ignored; the zones are located by experience or by comparison with those given by a reference substance treated in the same way.

After separation of the constituents the position of colourless substances may be determined by treating the electropherogram with a reagent that will convert them to coloured or fluorescent derivatives. For quantitative purposes, the spot (zone) may be carefully separated, the substance eluted with a suitable solvent and then determined by a sufficiently sensitive method, such as spectrophotometric measurement, either directly or after a chemical reaction. In another quantitative procedure after conversion to a coloured derivative, the zone intensity can be measured with the aid of a scanning densitometer.

An apparatus for electrophoresis on a supporting medium is composed of:

- A source of direct current, preferably of stabilized voltage.

- A chamber for electrophoresis, generally in the form of a parallelepiped, made from glass or rigid plastic material with an airtight lid ensuring the maintenance of an atmosphere of saturated humidity. Two insulated electrical leads are sealed through the walls of the chamber, one at either end, each lead having an internal connector to which are attached electrodes of platinum wire. The chamber should be fitted with suitable safety devices to ensure that the electrical supply is disconnected when the lid is removed. Two double troughs provided with a central lengthwise partition are inserted in the chamber, one at each end. Alternatively, the troughs may be integral parts of the chamber. One platinum electrode is laid along the bottom of each outer trough compartment. The electrodes are connected through external insulated cables to an electrical power source having an output of not less than 450 V D.C. at 150 mA. The power source should be provided with a means of indicating and controlling the voltage and of indicating the current consumption. Additional circuitry may be incorporated to stabilize the voltage.

- A holder device. When paper or cellulose acetate is used, the carrier strips impregnated with the conducting liquid are stretched by an appropriate arrangement and the ends immersed into the electrode troughs. In gel electrophoresis, an adherent on an even layer of gel is placed on glass and the electrical connections are attached at each end.

- A device to locate and measure the spots.

Recommended procedure

Paper electrophoresis

A chamber about 50 cm long, 38 cm wide and 4.5 cm deep, with troughs about 37 cm long externally, 5 cm wide and 2 cm in depth internally, is suitable.

The electrophoresis paper consists of suitable filter-paper (Whatman 3 MM or similar grade is suitable) that has been washed chromatographically with a suitable solvent if so specified in the monograph. The paper is cut into strips of appropriate size and a baseline is drawn across the paper about 13 cm from one end.

Fill the troughs of the apparatus with the conducting liquid specified in the monograph. Place strips of electrophoresis paper (about 30 cm by 5 cm) in the troughs so as to form bridges between the outer and inner compartments and ensure that the electrodes are fully immersed in the conducting liquid in the outer compartments.

Apply separately to points along the baseline of the electrophoresis paper, at least 1 cm from the edge of the paper and not less than 2.5 cm apart, the volumes of solutions prepared as specified in the monograph.

Allow the spots to dry and then place the end of the paper nearest the baseline in the inner compartment of the trough connected to the anode and the other end in the inner compartment of the trough connected to the cathode. Wet the paper with the conducting liquid, using a brush, starting from the ends of the paper and working towards the baseline. Do not wet the strip that includes the applied substance. Close the lid, allow the liquid to diffuse across the baseline, if necessary cover the apparatus so as to exclude light, connect the cables to the power supply and switch on the current. Adjust the voltage to about 20 V per cm of paper between the troughs and allow electrophoresis to proceed for the time indicated or until the marker substances have moved the specified distances. Switch off the current, remove the paper, dry in a current of air protected from light if necessary, and examine the resulting electropherogram under the conditions prescribed in the monograph. When the use of marker substances is specified, the test is valid only if the marker substances move to the specified distances from the baseline. If the intensity of any subsidiary spots derived from the tested substance is less than that of the spot obtained from the reference solution, the substance conforms to the requirements. When specified in the monograph, spray the paper uniformly on both sides with the reagent, carry out any further prescribed treatment to complete the reaction, and apply the same criteria to the resulting spots.

Electrophoresis on cellulose acetate strips

It is preferable to use a smaller chamber than for paper electrophoresis; one measuring about 25 cm by 24 cm with troughs of 10 cm by 23 cm is suitable.

Use cellulose polyacetate strips of suitable quality, measuring 2.5 cm by 17 cm, which are immersed in the conducting liquid for approximately 1 hour before use.

Apply the solutions, prepared as specified in the monograph and in the volume indicated, 8 cm from one end of the strip, then carry out electrophoresis as described under paper electrophoresis. Colour the bands, wash them and render them transparent by the methods specified in the monographs, which also give the method of evaluation to be used.

Gel electrophoresis

The inert carrier consists of a 1-2 mm thick layer of an agar or starch gel of suitable consistency and shaped as an elongated rectangle.

The conducting liquid is either incorporated into the gel layer or sprinkled on it, until it is well moistened, after it has already been formed. The solution of the substance is placed on the surface of the gel layer or inside the holes bored for that purpose in the layer. The gel layer is connected at both its narrower ends with two troughs containing the conducting liquid, the connection being made by wicks composed of a double layer of absorbent lint moistened with the conducting liquid. The gel layer on its support and the connections are then placed in a suitable chamber.

The electrophoretic process is effected by application of direct electric current. To remove heat generated by the Joule effect of the current, water or other suitable cooling liquid should be circulated in the course of the process through the supporting plate.

When the process has been completed, the resulting spots or areas of migration are located by a suitable method (for example, as an inhibition zone after suitable incubation, if the tested substance is an antibiotic and an appropriate test organism has been incorporated into the gel layer, or by a chemical method), as specified in the individual monographs.