molecules between the solvent of the mobile phase and the same solvent in the stagnant liquid phase (stationary phase) within the pores of the packing material. The pore-size range of the packing material determines the molecular-size range within which separation can occur.

Molecules small enough to penetrate all the pore spaces elute at the *total permeation volume* (V_t). On the other hand, molecules apparently larger than the maximum pore size of the packing material migrate along the column only through the spaces between the particles of the packing material without being retained and elute at the *exclusion volume* (V_0 void volume). Separation according to molecular size occurs between the exclusion volume and the total permeation volume, with useful separation usually occurring in the first two thirds of this range.

Apparatus. The apparatus consists essentially of a chromatographic column of varying length and internal diameter (Ø), if necessary temperature-controlled, packed with a separation material that is capable of fractionation in the appropriate range of molecular sizes and through which the eluent is passed at a constant rate. One end of the column is usually fitted with a suitable device for applying the sample such as a flow adapter, a syringe through a septum or an injection valve and may also be connected to a suitable pump for controlling the flow of the eluent. Alternatively the sample may be applied directly to the drained bed surface or, where the sample is denser than the eluent, it may be layered beneath the eluent. The outlet of the column is usually connected to a suitable detector fitted with an automatic recorder which enables the monitoring of the relative concentrations of separated components of the sample. Detectors are usually based on photometric, refractometric or luminescent properties. An automatic fraction collector may be attached, if necessary.

The packing material may be a soft support such as a swollen gel or a rigid support composed of a material such as glass, silica or a solvent-compatible, cross-linked organic polymer. Rigid supports usually require pressurised systems giving faster separations. The mobile phase is chosen according to sample type, separation medium and method of detection. Before carrying out the separation, the packing material is treated, and the column is packed, as described in the monograph, or according to the manufacturer's instructions.

Criteria for assessing the suitability of the system are described in the chapter on *Chromatographic separation techniques* (*2.2.46*). The extent to which adjustments of parameters of the chromatographic system can be made to satisfy the criteria of system suitability are also given in this chapter.

DETERMINATION OF RELATIVE COMPONENT COMPOSITION OF MIXTURES

Carry out the separation as stated in the monograph. If possible, monitor the elution of the components continuously and measure the corresponding peak areas. If the sample is monitored by a physico-chemical property to which all the components of interest exhibit equivalent responses (for example if they have the same specific absorbance), calculate the relative amount of each component by dividing the respective peak area by the sum of the peak areas of all the components of interest. If the responses to the property used for detection of the components of interest are not equivalent, calculate the content by means of calibration curves obtained with the calibration standards prescribed in the monograph.

DETERMINATION OF MOLECULAR MASSES

Size-exclusion chromatography may be used to determine molecular masses by comparison with appropriate calibration standards specified in the monograph. The retention volumes of the calibration standards may be plotted against the logarithm of their molecular masses. The plot usually approximates a straight line within the exclusion and total permeation limits for the separation medium used. From the calibration curve, molecular masses may be estimated. The molecular-mass calibration is valid only for the particular macromolecular solute/solvent system used under the specified experimental conditions.

DETERMINATION OF MOLECULAR SIZE DISTRIBUTION OF POLYMERS

Size-exclusion chromatography may be used to determine the distribution of the molecular size of polymers. However, sample comparison may be valid only for results obtained under the same experimental conditions. The reference substances used for the calibration and the methods for determination of the distribution of molecular sizes of polymers are specified in the monograph.

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2.2.31. ELECTROPHORESIS

GENERAL PRINCIPLE

Under the influence of an electrical field, charged particles dissolved or dispersed in an electrolyte solution migrate in the direction of the electrode bearing the opposite polarity. In gel electrophoresis, the movements of the particles are retarded by interactions with the surrounding gel matrix, which acts as a molecular sieve. The opposing interactions of the electrical force and molecular sieving result in differential migration rates according to sizes, shapes and charges of particles. Because of their different physico-chemical properties, different macromolecules of a mixture will migrate at different speeds during electrophoresis and will thus be separated into discrete fractions. Electrophoretic separations can be conducted in systems without support phases (e.g. free solution separation in capillary electrophoresis) and in stabilising media such as thin-layer plates, films or gels.

FREE OR MOVING BOUNDARY ELECTROPHORESIS

This method is mainly used for the determination of mobility, the experimental characteristics being directly measurable and reproducible. It is chiefly employed with substances of high relative molecular mass and low diffusibility. The boundaries are initially located by a physical process such as refractometry or conductimetry. After applying a given electric field for an accurately measured time, the new boundaries and their respective positions are observed. The operating conditions must be such as to make it possible to determine as many boundaries as there are components.

ZONE ELECTROPHORESIS USING A SUPPORTING MEDIUM

This method requires the use of small samples only.

The nature of the support, such as paper, agar gel, cellulose acetate, starch, agarose, methacrylamide, mixed gel, introduces a number of additional factors modifying the mobility:

a) owing to channelling in the supporting medium, the apparent distance covered is less than the real distance,

b) some supporting media are not electrically neutral. As the medium is a stationary phase it may sometimes give rise to a considerable electro-endosmotic flow,

c) any heating due to the joule effect may cause some evaporation of the liquid from the supporting medium which, by capillarity, causes the solution to move from the ends towards the centre. The ionic strength therefore tends to increase gradually.

The rate of migration then depends on four main factors: the mobility of the charged particle, the electro-endosmotic flow, the evaporation flow, and the field strength. Hence it is necessary to operate under clearly defined experimental conditions and to use, wherever possible, reference substances.

An *apparatus* for electrophoresis consists of:

– a *generator supplying direct current* whose voltage can be controlled and, preferably, stabilised,

– an *electrophoresis chamber*. This is usually rectangular and made of glass or rigid plastic, with two separate compartments, the anodic and the cathodic, containing the electrolyte solution. In each compartment is immersed an electrode, for example of platinum or graphite. These are connected by means of an appropriately isolated circuit to the corresponding terminal of the power supply to form the anode and the cathode. The level of the liquid in the two compartments is kept equal to prevent siphoning.

  The electrophoresis chamber is fitted with an airtight lid which maintains a moisture-saturated atmosphere during operation and reduces evaporation of the solvent. A safety device may be used to cut off the power when the lid is removed. If the electrical power measured across the strip exceeds 10 W, it is preferable to cool the support.

– a *support-carrying device*:

  Strip electrophoresis. The supporting strip, previously wetted with the same conducting solution and dipped at each end into an electrode compartment is appropriately tightened and fixed on to a suitable carrier designed to prevent diffusion of the conducting electrolyte, such as a horizontal frame, inverted-V stand or a uniform surface with contact points at suitable intervals.

  Gel electrophoresis. The device consists essentially of a glass plate (for example, a microscope slide) over the whole surface of which is deposited a firmly adhering layer of gel of uniform thickness. The connection between the gel and the conducting solution is effected in various ways according to the type of apparatus used. Precautions must be taken to avoid condensation of moisture or drying of the solid layer.

– measuring *device or means of detection*.

Method. Introduce the electrolyte solution into the electrode compartments. Place the support suitably impregnated with electrolyte solution in the chamber under the conditions prescribed for the type of apparatus used. Locate the starting line and apply the sample. Apply the electric current for the prescribed time. After the current has been switched off, remove the support from the chamber, dry and visualise.

POLYACRYLAMIDE ROD GEL ELECTROPHORESIS

In polyacrylamide rod gel electrophoresis, the stationary phase is a gel which is prepared from a mixture of acrylamide and *N,N'*-methylenebisacrylamide. Rod gels are prepared in tubes 7.5 cm long and 0.5 cm in internal diameter, one solution being applied to each rod.

Apparatus. This consists of two buffer solution reservoirs made of suitable material such as poly(methyl methacrylate) and mounted vertically one above the other. Each reservoir is fitted with a platinum electrode. The electrodes are connected to a power supply allowing operation either at constant current or at constant voltage. The apparatus has in the base of the upper reservoir a number of holders equidistant from the electrode.

Method. The solutions should usually be degassed before polymerisation and the gels used immediately after preparation. Prepare the gel mixture as prescribed and pour into suitable glass tubes, stoppered at the bottom, to an equal height in each tube and to about 1 cm from the top, taking care to ensure that no air bubbles are trapped in the tubes. Cover the gel mixture with a layer of *water R* to exclude air and allow to set. Gel formation usually takes about 30 min and is complete when a sharp interface appears between the gel and the water layer. Remove the water layer. Fill the lower reservoir with the prescribed buffer solution and remove the stoppers from the tubes. Fit the tubes into the holders of the upper reservoir and adjust so that the bottom of the tubes are immersed in the buffer solution in the lower reservoir. Carefully fill the tubes with the prescribed buffer solution. Prepare the test and reference solutions containing the prescribed marker dye and make them dense by dissolving in them *sucrose R*, for example. Apply the solutions to the surface of a gel using a different tube for each solution. Add the same buffer to the upper reservoir. Connect the electrodes to the power supply and allow electrophoresis to proceed at the prescribed temperature and using the prescribed constant voltage or current. Switch off the power supply when the marker dye has migrated almost into the lower reservoir. Immediately remove each tube from the apparatus and extrude the gel. Locate the position of the bands in the electropherogram as prescribed.

SODIUM DODECYL SULPHATE POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Scope. Polyacrylamide gel electrophoresis is used for the qualitative characterisation of proteins in biological preparations, for control of purity and quantitative determinations.

Purpose. Analytical gel electrophoresis is an appropriate method with which to identify and to assess the homogeneity of proteins in pharmaceutical preparations. The method is routinely used for the estimation of protein subunit molecular masses and for determining the subunit compositions of purified proteins.

Ready-to-use gels and reagents are widely available on the market and can be used instead of those described in this text, provided that they give equivalent results and that they meet the validity requirements given below under Validation of the test.

CHARACTERISTICS OF POLYACRYLAMIDE GELS

The sieving properties of polyacrylamide gels are established by the three-dimensional network of fibres and pores which is formed as the bifunctional bisacrylamide cross-links adjacent polyacrylamide chains. Polymerisation is catalysed by a free radical-generating system composed of ammonium persulphate and tetramethylethylenediamine.

As the acrylamide concentration of a gel increases, its effective pore size decreases. The effective pore size of a gel is operationally defined by its sieving properties; that is, by the resistance it imparts to the migration of macromolecules. There are limits on the acrylamide concentrations that can be used. At high acrylamide concentrations, gels break much more easily and are difficult to handle. As the pore size of a gel decreases, the migration rate of a protein through the gel decreases. By adjusting the pore size of a gel, through manipulating the acrylamide concentration, the resolution

of the method can be optimised for a given protein product. Thus, a given gel is physically characterised by its respective composition in acrylamide and bisacrylamide.

In addition to the composition of the gel, the state of the protein is an important component to the electrophoretic mobility. In the case of proteins, the electrophoretic mobility is dependent on the pK value of the charged groups and the size of the molecule. It is influenced by the type, concentration and pH of the buffer, by the temperature and the field strength as well as by the nature of the support material.

DENATURING POLYACRYLAMIDE GEL ELECTROPHORESIS

The method cited as an example is limited to the analysis of monomeric polypeptides with a mass range of 14 000 to 100 000 daltons. It is possible to extend this mass range by various techniques (e.g. gradient gels, particular buffer system) but those techniques are not discussed in this chapter.

Denaturing polyacrylamide gel electrophoresis using sodium dodecyl sulphate (SDS-PAGE) is the most common mode of electrophoresis used in assessing the pharmaceutical quality of protein products and will be the focus of the example method. Typically, analytical electrophoresis of proteins is carried out in polyacrylamide gels under conditions that ensure dissociation of the proteins into their individual polypeptide subunits and that minimise aggregation. Most commonly, the strongly anionic detergent sodium dodecyl sulphate (SDS) is used in combination with heat to dissociate the proteins before they are loaded on the gel. The denatured polypeptides bind to SDS, become negatively charged and exhibit a consistent charge-to-mass ratio regardless of protein type. Because the amount of SDS bound is almost always proportional to the molecular mass of the polypeptide and is independent of its sequence, SDS-polypeptide complexes migrate through polyacrylamide gels with mobilities dependent on the size of the polypeptide.

The electrophoretic mobilities of the resultant detergent-polypeptide complexes all assume the same functional relationship to their molecular masses. Migration of SDS complexes is toward the anode in a predictable manner, with low-molecular-mass complexes migrating faster than larger ones. The molecular mass of a protein can therefore be estimated from its relative mobility in calibrated SDS-PAGE and the occurrence of a single band in such a gel is a criterion of purity.

Modifications to the polypeptide backbone, such as *N*- or *O*-linked glycosylation, however, have a significant impact on the apparent molecular mass of a protein since SDS does not bind to a carbohydrate moiety in a manner similar to a polypeptide. Thus, a consistent charge-to-mass ratio is not maintained. The apparent molecular mass of proteins having undergone post-translational modifications is not a true reflection of the mass of the polypeptide chain.

Reducing conditions. Polypeptide subunits and three-dimensional structure is often maintained in proteins by the presence of disulphide bonds. A goal of SDS-PAGE analysis under reducing conditions is to disrupt this structure by reducing disulphide bonds. Complete denaturation and dissociation of proteins by treatment with 2-mercaptoethanol or dithiothreitol (DTT) will result in unfolding of the polypeptide backbone and subsequent complexation with SDS. In these conditions, the molecular mass of the polypeptide subunits can be calculated by linear regression in the presence of suitable molecular-mass standards.

Non-reducing conditions. For some analyses, complete dissociation of the protein into subunit peptides is not desirable. In the absence of treatment with reducing agents such as 2-mercaptoethanol or DTT, disulphide covalent bonds remain intact, preserving the oligomeric form of the protein. Oligomeric SDS-protein complexes migrate more slowly than their SDS-polypeptide subunits. In addition, non-reduced proteins may not be completely saturated with SDS and, hence, may not bind the detergent in a constant mass ratio. This makes molecular-mass determinations of these molecules by SDS-PAGE less straightforward than analyses of fully denatured polypeptides, since it is necessary that both standards and unknown proteins be in similar configurations for valid comparisons. However, the staining of a single band in such a gel is a criterion of purity.

CHARACTERISTICS OF DISCONTINUOUS BUFFER SYSTEM GEL ELECTROPHORESIS

The most popular electrophoretic method for the characterisation of complex mixtures of proteins involves the use of a discontinuous buffer system consisting of two contiguous, but distinct gels: a resolving or separating (lower) gel and a stacking (upper) gel. The two gels are cast with different porosities, pH, and ionic strengths. In addition, different mobile ions are used in the gel and electrode buffers. The buffer discontinuity acts to concentrate large volume samples in the stacking gel, resulting in improved resolution. When power is applied, a voltage drop develops across the sample solution which drives the proteins into the stacking gel. Glycinate ions from the electrode buffer follow the proteins into the stacking gel. A moving boundary region is rapidly formed with the highly mobile chloride ions in the front and the relatively slow glycinate ions in the rear. A localised high-voltage gradient forms between the leading and trailing ion fronts, causing the SDS-protein complexes to form into a thin zone (stack) and migrate between the chloride and glycinate phases. Within broad limits, regardless of the height of the applied sample, all SDS-proteins condense into a very narrow region and enter the resolving gel as a well-defined, thin zone of high protein density. The large-pore stacking gel does not retard the migration of most proteins and serves mainly as an anticonvective medium. At the interface of the stacking and resolving gels, the proteins experience a sharp increase in retardation due to the restrictive pore size of the resolving gel. Once in the resolving gel, proteins continue to be slowed by the sieving of the matrix. The glycinate ions overtake the proteins, which then move in a space of uniform pH formed by the tris(hydroxymethyl)aminomethane and glycine. Molecular sieving causes the SDS-polypeptide complexes to separate on the basis of their molecular masses.

PREPARING VERTICAL DISCONTINUOUS BUFFER SDS POLYACRYLAMIDE GELS

Assembling of the gel moulding cassette. Clean the two glass plates (size: e.g. 10 cm × 8 cm), the polytetrafluoroethylene comb, the two spacers and the silicone rubber tubing (diameter e.g. 0.6 mm × 35 cm) with mild detergent and rinse extensively with water. Dry all the items with a paper towel or tissue. Lubricate the spacers and the tubing with non-silicone grease. Apply the spacers along each of the two short sides of the glass plate 2 mm away from the edges and 2 mm away from the long side corresponding to the bottom of the gel. Begin to lay the tubing on the glass plate by using one spacer as a guide. Carefully twist the tubing at the bottom of the spacer and follow the long side of the glass plate. While holding the tubing with one finger along the long side twist again the tubing and lay it on the second short side of the glass plate, using the spacer as a guide. Place the second glass plate in perfect alignment and hold

the mould together by hand pressure. Apply two clamps on each of the two short sides of the mould. Carefully apply four clamps on the longer side of the gel mould thus forming the bottom of the gel mould. Verify that the tubing is running along the edge of the glass plates and has not been extruded while placing the clamps. The gel mould is now ready for pouring the gel.

Preparation of the gel. In a discontinuous buffer SDS polyacrylamide gel, it is recommended to pour the resolving gel, let the gel set, and then pour the stacking gel since the composition of the two gels in acrylamide-bisacrylamide, buffer and pH are different.

Preparation of the resolving gel. In a conical flask, prepare the appropriate volume of solution containing the desired concentration of acrylamide for the resolving gel, using the values given in Table 2.2.31.-1. Mix the components in the order shown. Where appropriate, before adding the ammonium persulphate solution and the tetramethylethylenediamine (TEMED), filter the solution if necessary under vacuum through a cellulose acetate membrane (pore diameter 0.45 µm); keep the solution under vacuum by swirling the filtration unit until no more bubbles are formed in the solution. Add appropriate amounts of ammonium persulphate solution and TEMED as indicated in Table 2.2.31.-1, swirl and pour immediately into the gap between the two glass plates of the mould. Leave sufficient space for the stacking gel (the length of the teeth of the comb plus 1 cm). Using a tapered glass pipette, carefully overlay the solution with water-saturated isobutanol. Leave the gel in a vertical position at room temperature to allow polymerisation.

Preparation of the stacking gel. After polymerisation is complete (about 30 min), pour off the isobutanol and wash the top of the gel several times with water to remove the isobutanol overlay and any unpolymerised acrylamide. Drain as much fluid as possible from the top of the gel, and then remove any remaining water with the edge of a paper towel.

In a conical flask, prepare the appropriate volume of solution containing the desired concentration of acrylamide, using the values given in Table 2.2.31.-2. Mix the components in the order shown. Where appropriate, before adding the ammonium persulphate solution and the TEMED, filter the solution if necessary under vacuum through a cellulose acetate membrane (pore diameter: 0.45 µm); keep the solution under vacuum by swirling the filtration unit until no more bubbles are formed in the solution. Add appropriate amounts of ammonium persulphate solution and TEMED as indicated in Table 2.2.31.-2, swirl and pour immediately into the gap between the two glass plates of the mould directly onto the surface of the polymerised resolving gel. Immediately insert a clean polytetrafluoroethylene comb into the stacking gel solution, being careful to avoid trapping air bubbles. Add more stacking gel solution to fill the spaces of the comb completely. Leave the gel in a vertical position and allow to polymerise at room temperature.

Table 2.2.31.-1. – *Preparation of resolving gel*

Solution components	Component volumes (ml) per gel mould volume of							
	5 ml	l0 ml	15 ml	20 ml	25 ml	30 ml	40 ml	50 ml
6 per cent acrylamide								
Water R	2.6	5.3	7.9	10.6	13.2	15.9	21.2	26.5
Acrylamide solution(1)	1.0	2.0	3.0	4.0	5.0	6.0	8.0	10.0
1.5 M Tris (pH 8.8)(2)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
100 g/l SDS(3)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/l APS(4)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED(5)	0.004	0.008	0.012	0.016	0.02	0.024	0.032	0.04
8 per cent acrylamide								
Water R	2.3	4.6	6.9	9.3	11.5	13.9	18.5	23.2
Acrylamide solution(1)	1.3	2.7	4.0	5.3	6.7	8.0	10.7	13.3
1.5 M Tris (pH 8.8)(2)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
100 g/l SDS(3)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/l APS(4)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED(5)	0.003	0.006	0.009	0.012	0.015	0.018	0.024	0.03
10 per cent acrylamide								
Water R	1.9	4.0	5.9	7.9	9.9	11.9	15.9	19.8
Acrylamide solution(1)	1.7	3.3	5.0	6.7	8.3	10.0	13.3	16.7
1.5 M Tris (pH 8.8)(2)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
100 g/l SDS(3)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/l APS(4)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED(5)	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02

Solution components	Component volumes (ml) per gel mould volume of							
	5 ml	l0 ml	15 ml	20 ml	25 ml	30 ml	40 ml	50 ml
12 per cent acrylamide								
Water R	1.6	3.3	4.9	6.6	8.2	9.9	13.2	16.5
Acrylamide solution(1)	2.0	4.0	6.0	8.0	10.0	12.0	16.0	20.0
1.5 M Tris (pH 8.8)(2)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
100 g/l SDS(3)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/l APS(4)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED(5)	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
14 per cent acrylamide								
Water R	1.4	2.7	3.9	5.3	6.6	8.0	10.6	13.8
Acrylamide solution(1)	2.3	4.6	7.0	9.3	11.6	13.9	18.6	23.2
1.5 M Tris (pH 8.8)(2)	1.2	2.5	3.6	5.0	6.3	7.5	10.0	12.5
100 g/l SDS(3)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/l APS(4)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED(5)	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02
15 per cent acrylamide								
Water R	1.1	2.3	3.4	4.6	5.7	6.9	9.2	11.5
Acrylamide solution(1)	2.5	5.0	7.5	10.0	12.5	15.0	20.0	25.0
1.5 M Tris (pH 8.8)(2)	1.3	2.5	3.8	5.0	6.3	7.5	10.0	12.5
100 g/l SDS(3)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
100 g/l APS(4)	0.05	0.1	0.15	0.2	0.25	0.3	0.4	0.5
TEMED(5)	0.002	0.004	0.006	0.008	0.01	0.012	0.016	0.02

(1) *Acrylamide solution: 30 per cent acrylamide/bisacrylamide(29:1) solution R.*

(2) 1.5 M Tris (pH 8.8): *1.5 M tris-hydrochloride buffer solution pH 8.8 R.*

(3) 100 g/l SDS: a 100 g/l solution of *sodium dodecyl sulphate R.*

(4) 100 g/l APS: a 100 g/l solution of *ammonium persulphate R.* Ammonium persulphate provides the free radicals that drive polymerisation of acrylamide and bisacrylamide. Since ammonium persulphate solution decomposes slowly, fresh solutions must be prepared weekly.

(5) TEMED: *tetramethylethylenediamine R.*

Mounting the gel in the electrophoresis apparatus and electrophoretic separation. After polymerisation is complete (about 30 min), remove the polytetrafluoroethylene comb carefully. Rinse the wells immediately with water or with the *SDS-PAGE running buffer R* to remove any unpolymerised acrylamide. If necessary, straighten the teeth of the stacking gel with a blunt hypodermic needle attached to a syringe. Remove the clamps on one short side, carefully pull out the tubing and replace the clamps. Proceed similarly on the other short side. Remove the tubing from the bottom part of the gel. Mount the gel in the electrophoresis apparatus. Add the electrophoresis buffers to the top and bottom reservoirs. Remove any bubbles that become trapped at the bottom of the gel between the glass plates. This is best done with a bent hypodermic needle attached to a syringe. Never pre-run the gel before loading the samples, since this will destroy the discontinuity of the buffer systems. Before loading the sample carefully rinse the slot with *SDS-PAGE running buffer R*. Prepare the test and reference solutions in the recommended sample buffer and treat as specified in the individual monograph. Apply the appropriate volume of each solution to the stacking gel wells. Start the electrophoresis

Table 2.2.31.-2. – *Preparation of stacking gel*

Solution components	Component volumes (ml) per gel mould volume of							
	1 ml	2 ml	3 ml	4 ml	5 ml	6 ml	8 ml	10 ml
Water R	0.68	1.4	2.1	2.7	3.4	4.1	5.5	6.8
Acrylamide solution(1)	0.17	0.33	0.5	0.67	0.83	1.0	1.3	1.7
1.0 M Tris (pH 6.8)(2)	0.13	0.25	0.38	0.5	0.63	0.75	1.0	1.25
100 g/l SDS(3)	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
100 g/l APS(4)	0.01	0.02	0.03	0.04	0.05	0.06	0.08	0.1
TEMED(5)	0.001	0.002	0.003	0.004	0.005	0.006	0.008	0.01

(1) Acrylamide solution: *30 per cent acrylamide/bisacrylamide (29:1) solution R.*

(2) 1.0 M Tris (pH 6.8): *1 M tris-hydrochloride buffer solution pH 6.8 R.*

(3) 100 g/l SDS: a 100 g/l solution of *sodium dodecyl sulphate R.*

(4) 100 g/l APS: a 100 g/l solution of *ammonium persulphate R.* Ammonium persulphate provides the free radicals that drive polymerisation of acrylamide and bisacrylamide. Since ammonium persulphate solution decomposes slowly, fresh solutions must be prepared weekly.

(5) TEMED: *tetramethylethylenediamine R.*

using the conditions recommended by the manufacturer of the equipment. Manufacturers of SDS-PAGE equipment may provide gels of different surface area and thickness. Electrophoresis running time and current/voltage may need to vary as described by the manufacturer of the apparatus in order to achieve optimum separation. Check that the dye front is moving into the resolving gel. When the dye is reaching the bottom of the gel, stop the electrophoresis. Remove the gel assembly from the apparatus and separate the glass plates. Remove the spacers, cut off and discard the stacking gel and immediately proceed with staining.

DETECTION OF PROTEINS IN GELS

Coomassie staining is the most common protein staining method with a detection level of the order of 1 µg to 10 µg of protein per band. Silver staining is the most sensitive method for staining proteins in gels and a band containing 10 ng to 100 ng can be detected.

All of the steps in gel staining are done at room temperature with gentle shaking (e.g. on an orbital shaker platform) in any convenient container. Gloves must be worn when staining gels, since fingerprints will stain.

Coomassie staining. Immerse the gel in a large excess of *Coomassie staining solution R* and allow to stand for at least 1 h. Remove the staining solution.

Destain the gel with a large excess of *destaining solution R*. Change the destaining solution several times, until the stained protein bands are clearly distinguishable on a clear background. The more thoroughly the gel is destained, the smaller is the amount of protein that can be detected by the method. Destaining can be speeded up by including a few grams of anion-exchange resin or a small sponge in the *destaining solution R*.

NOTE: the acid-alcohol solutions used in this procedure do not completely fix proteins in the gel. This can lead to losses of some low-molecular-mass proteins during the staining and destaining of thin gels. Permanent fixation is obtainable by allowing the gel to stand in a mixture of 1 volume of trichloroacetic acid R, 4 volumes of methanol R and 5 volumes of water R for 1 h before it is immersed in the Coomassie staining solution R.

Silver staining. Immerse the gel in a large excess of *fixing solution R* and allow to stand for 1 h. Remove the fixing solution, add fresh fixing solution and incubate either for at least 1 h or overnight, if convenient. Discard the fixing solution and wash the gel in a large excess of *water R* for 1 h. Soak the gel for 15 min in a 1 per cent *V/V* solution of *glutaraldehyde R*. Wash the gel twice for 15 min in a large excess of *water R*. Soak the gel in fresh *silver nitrate reagent R* for 15 min, in darkness. Wash the gel three times for 5 min in a large excess of *water R*. Immerse the gel for about 1 min in *developer solution R* until satisfactory staining has been obtained. Stop the development by incubation in the *blocking solution R* for 15 min. Rinse the gel with *water R*.

DRYING OF STAINED SDS POLYACRYLAMIDE GELS

Depending on the staining method used, gels are treated in a slightly different way. For Coomassie staining, after the destaining step, allow the gel to stand in a 100 g/l solution of *glycerol R* for at least 2 h (overnight incubation is possible). For silver staining, add to the final rinsing a step of 5 min in a 20 g/l solution of *glycerol R*.

Immerse two sheets of porous cellulose film in *water R* and incubate for 5 min to 10 min. Place one of the sheets on a drying frame. Carefully lift the gel and place it on the cellulose film. Remove any trapped air bubbles and pour a few millilitres of *water R* around the edges of the gel. Place the second sheet on top and remove any trapped air bubbles. Complete the assembly of the drying frame. Place in an oven or leave at room temperature until dry.

MOLECULAR-MASS DETERMINATION

Molecular masses of proteins are determined by comparison of their mobilities with those of several marker proteins of known molecular weight. Mixtures of proteins with precisely known molecular masses blended for uniform staining are available for calibrating gels. They are obtainable in various molecular mass ranges. Concentrated stock solutions of proteins of known molecular mass are diluted in the appropriate sample buffer and loaded on the same gel as the protein sample to be studied.

Immediately after the gel has been run, the position of the bromophenol blue tracking dye is marked to identify the leading edge of the electrophoretic ion front. This can be done by cutting notches in the edges of the gel or by inserting a needle soaked in India ink into the gel at the dye front. After staining, measure the migration distances of each protein band (markers and unknowns) from the top of the resolving gel. Divide the migration distance of each protein by the distance travelled by the tracking dye. The normalised migration distances so obtained are called the relative mobilities of the proteins (relative to the dye front) and conventionally denoted as R_F. Construct a plot of the logarithm of the relative molecular masses (M_r) of the protein standards as a function of the R_F values. Note that the graphs are slightly sigmoid. Unknown molecular masses can be estimated by linear regression analysis or interpolation from the curves of log M_r against R_F as long as the values obtained for the unknown samples are positioned along the linear part of the graph.

VALIDATION OF THE TEST

The test is not valid unless the proteins of the molecular mass marker are distributed along 80 per cent of the length of the gel and over the required separation range (e.g. the range covering the product and its dimer or the product and its related impurities) the separation obtained for the relevant protein bands shows a linear relationship between the logarithm of the molecular mass and the R_F. Additional validation requirements with respect to the solution under test may be specified in individual monographs.

QUANTIFICATION OF IMPURITIES

Where the impurity limit is specified in the individual monograph, a reference solution corresponding to that level of impurity should be prepared by diluting the test solution. For example, where the limit is 5 per cent, a reference solution would be a 1:20 dilution of the test solution. No impurity (any band other than the main band) in the electropherogram obtained with the test solution may be more intense than the main band obtained with the reference solution.

Under validated conditions impurities may be quantified by normalisation to the main band using an integrating densitometer. In this case, the responses must be validated for linearity.

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2.2.32. LOSS ON DRYING

Loss on drying is the loss of mass expressed as per cent *m/m*.

Method. Place the prescribed quantity of the substance to be examined in a weighing bottle previously dried under the conditions prescribed for the substance to be examined. Dry the substance to constant mass or for the prescribed time by one of the following procedures. Where the drying