Method. Prepare and examine the sample in the same manner as for the establishment of the database. A suitable mathematical transformation of the Raman spectrum may be calculated to facilitate spectrum comparison or quantitative prediction.

Comparison of the spectra or transforms of the spectra or quantitative prediction of properties or amounts in the material in question may involve the use of a suitable chemometric or statistical classification or calibration technique.

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2.2.49. FALLING BALL VISCOMETER METHOD

The determination of dynamic viscosity of Newtonian liquids using a suitable falling ball viscometer is performed at 20 ± 0.1 °C, unless otherwise prescribed in the monograph. The time required for a test ball to fall in the liquid to be examined from one ring mark to the other is determined. If no stricter limit is defined for the equipment used the result is valid only if 2 consecutive measures do not differ by more than 1.5 per cent.

Apparatus. The falling ball viscometer consists of: a glass tube enclosed in a mantle, which allow precise control of temperature; six balls made of glass, nickel-iron or steel with different densities and diameters. The tube is fixed in such a way that the axis is inclined by 10 ± 1° with regard to the vertical. The tube has 2 ring marks which define the distance the ball has to roll. Commercially available apparatus is supplied with tables giving the constants, the density of the balls and the suitability of the different balls for the expected range of viscosity.

Method. Fill the clean, dry tube of the viscometer, previously brought to 20 ± 0.1 °C, with the liquid to be examined, avoiding bubbles. Add the ball suitable for the range of viscosity of the liquid so as to obtain a falling time not less than 30 s. Close the tube and maintain the solution at 20 ± 0.1 °C for at least 15 min. Let the ball run through the liquid between the 2 ring marks once without measurement. Let it run again and measure with a stop-watch, to the nearest one-fifth of a second, the time required for the ball to roll from the upper to the lower ring mark. Repeat the test run at least 3 times.

Calculate the dynamic viscosity η in millipascal seconds using the formula:

$$\eta = k\,(\rho_1 - \rho_2) \times t$$

- k = constant, expressed in millimeter squared per second squared,
- ρ_1 = density of the ball used, expressed in grams per cubic centimetre,
- ρ_2 = density of the liquid to be examined, expressed in grams per cubic centimetre, obtained by multiplying its relative density d_{20}^{20} by 0.9982,
- t = falling time of the ball, in seconds.

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2.2.54. ISOELECTRIC FOCUSING

GENERAL PRINCIPLES

Isoelectric focusing (IEF) is a method of electrophoresis that separates proteins according to their isoelectric point. Separation is carried out in a slab of polyacrylamide or agarose gel that contains a mixture of amphoteric electrolytes (ampholytes). When subjected to an electric field, the ampholytes migrate in the gel to create a pH gradient. In some cases gels containing an immobilised pH gradient, prepared by incorporating weak acids and bases to specific regions of the gel network during the preparation of the gel, are used. When the applied proteins reach the gel fraction that has a pH that is the same as their isoelectric point (pI), their charge is neutralised and migration ceases. Gradients can be made over various ranges of pH, according to the mixture of ampholytes chosen.

THEORETICAL ASPECTS

When a protein is at the position of its isoelectric point, it has no net charge and cannot be moved in a gel matrix by the electric field. It may, however, move from that position by diffusion. The pH gradient forces a protein to remain in its isoelectric point position, thus concentrating it; this concentrating effect is called "focusing". Increasing the applied voltage or reducing the sample load result in improved separation of bands. The applied voltage is limited by the heat generated, which must be dissipated. The use of thin gels and an efficient cooling plate controlled by a thermostatic circulator prevents the burning of the gel whilst allowing sharp focusing. The separation is estimated by determining the minimum pI difference (ΔpI), which is necessary to separate 2 neighbouring bands:

$$\Delta\mathrm{pI} = 3 \times \sqrt{\frac{D\,(\mathrm{dpH}/\mathrm{d}x)}{E\,(-\mathrm{d}\mu/\mathrm{dpH})}}$$

- D = diffusion coefficient of the protein,
- $\frac{\mathrm{dpH}}{\mathrm{d}x}$ = pH gradient,
- E = intensity of the electric field, in volts per centimetre,
- $-\frac{\mathrm{d}\mu}{\mathrm{dpH}}$ = variation of the solute mobility with the pH in the region close to the pI.

Since D and $-\frac{\mathrm{d}\mu}{\mathrm{dpH}}$ for a given protein cannot be altered, the separation can be improved by using a narrower pH range and by increasing the intensity of the electric field.

Resolution between protein bands on an IEF gel prepared with carrier ampholytes can be quite good. Improvements in resolution may be achieved by using immobilised pH gradients where the buffering species, which are analogous to carrier ampholytes, are copolymerised within the gel matrix. Proteins exhibiting pIs differing by as little as 0.02 pH units may be resolved using a gel prepared with carrier ampholytes while immobilised pH gradients can resolve proteins differing by approximately 0.001 pH units.

PRACTICAL ASPECTS

Special attention must be paid to sample characteristics and/or preparation. Having salt in the sample can be problematic and it is best to prepare the sample, if possible, in deionised water or 2 per cent ampholytes, using dialysis or gel filtration if necessary.

The time required for completion of focusing in thin-layer polyacrylamide gels is determined by placing a coloured protein (e.g. haemoglobin) at different positions on the gel surface and by applying the electric field: the steady state is reached when all applications give an identical band pattern. In some protocols the completion of the focusing is indicated by the time elapsed after the sample application.

The IEF gel can be used as an identity test when the migration pattern on the gel is compared to a suitable standard preparation and IEF calibration proteins, the IEF gel can be used as a limit test when the density of a band on IEF is compared subjectively with the density of bands appearing in a standard preparation, or it can be used as a quantitative test when the density is measured using a densitometer or similar instrumentation to determine the relative concentration of protein in the bands subject to validation.

APPARATUS

An apparatus for IEF consists of:

- a controllable generator for constant potential, current and power; potentials of 2500 V have been used and are considered optimal under a given set of operating conditions; a supply of up to 30 W of constant power is recommended;
- a rigid plastic IEF chamber that contains a cooled plate, of suitable material, to support the gel;
- a plastic cover with platinum electrodes that are connected to the gel by means of paper wicks of suitable width, length and thickness, impregnated with solutions of anodic and cathodic electrolytes.

ISOELECTRIC FOCUSING IN POLYACRYLAMIDE GELS: DETAILED PROCEDURE

The following method is a detailed description of an IEF procedure in thick polyacrylamide slab gels, which is used unless otherwise stated in the monograph.

PREPARATION OF THE GELS

Mould. The mould (see Figure 2.2.54.-1) is composed of a glass plate (A) on which a polyester film (B) is placed to facilitate handling of the gel, one or more spacers (C), a second glass plate (D) and clamps to hold the structure together.

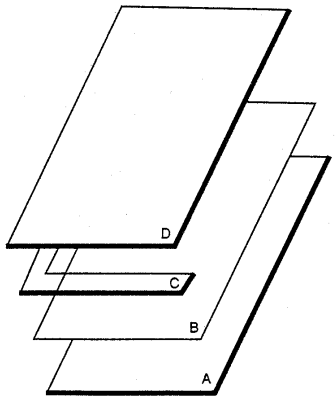

Figure 2.2.54.-1 – *Mould*

7.5 per cent polyacrylamide gel. Dissolve 29.1 g of *acrylamide R* and 0.9 g of *methylenebisacrylamide R* in 100 ml of *water R*. To 2.5 volumes of this solution, add the mixture of ampholytes specified in the monograph and dilute to 10 volumes with *water R*. Mix carefully and degas the solution.

Preparation of the mould. Place the polyester film on the lower glass plate, apply the spacer, place the second glass plate and fit the clamps. Before use, place the solution on a magnetic stirrer and add 0.25 volumes of a 100 g/l solution of *ammonium persulphate R* and 0.25 volumes of *tetramethylethylenediamine R*. Immediately fill the space between the glass plates of the mould with the solution.

METHOD

Dismantle the mould and, making use of the polyester film, transfer the gel onto the cooled support, wetted with a few millilitres of a suitable liquid, taking care to avoid forming air bubbles. Prepare the test solutions and reference solutions as specified in the monograph. Place strips of paper for sample application, about 10 mm × 5 mm in size, on the gel and impregnate each with the prescribed amount of the test and reference solutions. Also apply the prescribed quantity of a solution of proteins with known isoelectric points as pH markers to calibrate the gel. In some protocols the gel has pre-cast slots where a solution of the sample is applied instead of using impregnated paper strips. Cut 2 strips of paper to the length of the gel and impregnate them with the electrolyte solutions: acid for the anode and alkaline for the cathode. The compositions of the anode and cathode solutions are given in the monograph. Apply these paper wicks to each side of the gel several millimetres from the edge. Fit the cover so that the electrodes are in contact with the wicks (respecting the anodic and cathodic poles). Proceed with the isoelectric focusing by applying the electrical parameters described in the monograph. Switch off the current when the migration of the mixture of standard proteins has stabilised. Using forceps, remove the sample application strips and the 2 electrode wicks. Immerse the gel in *fixing solution for isoelectric focusing in polyacrylamide gel R*. Incubate with gentle shaking at room temperature for 30 min. Drain off the solution and add 200 ml of *destaining solution R*. Incubate with shaking for 1 h. Drain the gel, add *coomassie staining solution R*. Incubate for 30 min. Destain the gel by passive diffusion with *destaining solution R* until the bands are well visualised against a clear background. Locate the position and intensity of the bands in the electropherogram as prescribed in the monograph.

VARIATIONS TO THE DETAILED PROCEDURE (SUBJECT TO VALIDATION)

Where reference to the general method on isoelectric focusing is made, variations in methodology or procedure may be made subject to validation. These include:

- the use of commercially available pre-cast gels and of commercial staining and destaining kits,
- the use of immobilised pH gradients,
- the use of rod gels,
- the use of gel cassettes of different dimensions, including ultra-thin (0.2 mm) gels,
- variations in the sample application procedure, including different sample volumes or the use of sample application masks or wicks other than paper,
- the use of alternate running conditions, including variations in the electric field depending on gel dimensions and equipment, and the use of fixed migration times rather than subjective interpretation of band stability,
- the inclusion of a pre-focusing step,
- the use of automated instrumentation,
- the use of agarose gels.

VALIDATION OF ISO-ELECTRIC FOCUSING PROCEDURES

Where alternative methods to the detailed procedure are employed they must be validated. The following criteria may be used to validate the separation:

- formation of a stable pH gradient of desired characteristics, assessed for example using coloured pH markers of known isoelectric points,

– comparison with the electropherogram provided with the chemical reference substance for the preparation to be examined,
– any other validation criteria as prescribed in the monograph.

SPECIFIED VARIATIONS TO THE GENERAL METHOD

Variations to the general method required for the analysis of specific substances may be specified in detail in monographs. These include:

– the addition of urea in the gel (3 M concentration is often satisfactory to keep protein in solution but up to 8 M can be used): some proteins precipitate at their isoelectric point; in this case, urea is included in the gel formulation to keep the protein in solution; if urea is used, only fresh solutions should be used to prevent carbamylation of the protein;
– the use of alternative staining methods;
– the use of gel additives such as non-ionic detergents (e.g. octylglucoside) or zwitterionic detergents (e.g., CHAPS or CHAPSO), and the addition of ampholyte to the sample, to prevent proteins from aggregating or precipitating.

POINTS TO CONSIDER

Samples can be applied to any area on the gel, but to protect the proteins from extreme pH environments samples should not be applied close to either electrode. During method development the analyst can try applying the protein in 3 positions on the gel (i.e. middle and both ends); the pattern of a protein applied at opposite ends of the gel may not be identical.

A phenomenon known as cathodic drift, where the pH gradient decays over time, may occur if a gel is focused too long. Although not well understood, electroendoosmosis and absorption of carbon dioxide may be factors that lead to cathodic drift. Cathodic drift is observed as focused protein migrating off the cathode end of the gel. Immobilised pH gradients may be used to address this problem.

Efficient cooling (approximately 4 °C) of the bed that the gel lies on during focusing is important. High field strengths used during isoelectric focusing can lead to overheating and affect the quality of the focused gel.

01/2008:20255

2.2.55. PEPTIDE MAPPING

Peptide mapping is an identity test for proteins, especially those obtained by rDNA technology. It involves the chemical or enzymatic treatment of a protein resulting in the formation of peptide fragments followed by separation and identification of these fragments in a reproducible manner. It is a powerful test that is capable of identifying almost any single amino acid changes resulting from events such as errors in the reading of complementary DNA (cDNA) sequences or point mutations. Peptide mapping is a comparative procedure because the information obtained, compared to a reference substance similarly treated, confirms the primary structure of the protein, is capable of detecting whether alterations in structure have occurred, and demonstrates process consistency and genetic stability. Each protein presents unique characteristics which must be well understood so that the scientific and analytical approaches permit validated development of a peptide map that provides sufficient specificity.

This chapter provides detailed assistance in the application of peptide mapping and its validation to characterise the desired protein, to evaluate the stability of the expression construct of cells used for recombinant DNA products and to evaluate the consistency of the overall process, to assess product stability as well as to ensure the identity of the protein, or to detect the presence of protein variant.

Peptide mapping is not a general method, but involves developing specific maps for each unique protein. Although the technology is evolving rapidly, there are certain methods that are generally accepted. Variations of these methods will be indicated, when appropriate, in specific monographs.

A peptide map may be viewed as a fingerprint of a protein and is the end product of several chemical processes that provide a comprehensive understanding of the protein being analysed. 4 principal steps are necessary for the development of the procedure: isolation and purification of the protein, if the protein is part of a formulation; selective cleavage of the peptide bonds; chromatographic separation of the peptides; and analysis and identification of the peptides. A test sample is digested and assayed in parallel with a reference substance. Complete cleavage of peptide bonds is more likely to occur when enzymes such as endoproteases (e.g., trypsin) are used, instead of chemical cleavage reagents. A map must contain enough peptides to be meaningful. On the other hand, if there are too many fragments, the map might lose its specificity because many proteins will then have the same profiles.

ISOLATION AND PURIFICATION

Isolation and purification are necessary for analysis of bulk drugs or dosage forms containing interfering excipients and carrier proteins and, when required, will be specified in the monograph. Quantitative recovery of protein from the dosage form must be validated.

SELECTIVE CLEAVAGE OF PEPTIDE BONDS

The selection of the approach used for the cleavage of peptide bonds will depend on the protein under test. This selection process involves determination of the type of cleavage to be employed, enzymatic or chemical, and the type of cleavage agent within the chosen category. Several cleavage agents and their specificity are shown in Table 2.2.55.-1. This list is not all-inclusive and will be expanded as other cleavage agents are identified.

Pretreatment of sample. Depending on the size or the configuration of the protein, different approaches in the pretreatment of samples can be used. If trypsin is used as a cleavage agent for proteins with a molecular mass greater than 100 000 Da, lysine residues must be protected by citraconylation or maleylation; otherwise, too many peptides will be generated.

Pretreatment of the cleavage agent. Pretreatment of cleavage agents, especially enzymatic agents, might be necessary for purification purposes to ensure reproducibility of the map. For example, trypsin used as a cleavage agent will have to be treated with tosyl-L-phenylalanine chloromethyl ketone to inactivate chymotrypsin. Other methods, such as purification of trypsin by high performance liquid chromatography (HPLC) or immobilisation of enzyme on a gel support, have been successfully used when only a small amount of protein is available.

Pretreatment of the protein. Under certain conditions, it might be necessary to concentrate the sample or to separate the protein from added substances and stabilisers used in formulation of the product, if these interfere with the mapping procedure. Physical procedures used