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.

**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.

**DRIYING 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 RF. Construct a plot of the logarithm of the relative molecular masses (M) of the protein standards as a function of the RF 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 against RF, 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 RF values. 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.

**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
temperature is indicated by a single value rather than a range, drying is carried out at the prescribed temperature ± 2 °C.

a) “in a desiccator”: the drying is carried out over diphosphorus pentoxide R at atmospheric pressure and at room temperature;

b) “in vacuo”: the drying is carried out over diphosphorus pentoxide R, at a pressure of 1.5 kPa to 2.5 kPa at room temperature;

c) “in vacuo within a specified temperature range”: the drying is carried out over diphosphorus pentoxide R, at a pressure of 1.5 kPa to 2.5 kPa within the temperature range prescribed in the monograph;

d) “in an oven within a specified temperature range”: the drying is carried out in an oven within the temperature range prescribed in the monograph;

e) “under high vacuum”: the drying is carried out over diphosphorus pentoxide R at a pressure not exceeding 0.1 kPa, at the temperature prescribed in the monograph.

If other conditions are prescribed, the procedure to be used is described in full in the monograph.

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2.2.33. NUCLEAR MAGNETIC RESONANCE SPECTROMETRY

Nuclear magnetic resonance (NMR) spectrometry is based on the fact that nuclei such as 1H, 13C, 19F, 31P possess a permanent nuclear magnetic moment. When placed in an external magnetic field (main field), they take certain well-defined orientations with respect to the direction of this field which correspond to distinct energy levels. For a given field value, transitions between neighbouring energy levels take place due to absorption of electromagnetic radiation of characteristic wavelengths at radio frequencies.

The determination of these frequencies may be made either by sequential search of the resonance conditions (continuous-wave spectrometry) or by simultaneous excitation of all transitions with a multifrequency pulse followed by computer analysis of the free-induction decay of the irradiation emitted as the system returns to the initial state (pulsed spectrometry).

A proton magnetic resonance spectrum appears as a set of signals which correspond to protons and are characteristic of their nuclear and electronic environment within the molecule. The separation between a given signal and that of a reference compound is called a chemical shift (δ) and is expressed in parts per million (ppm); it characterises the kind of proton in terms of electronic environment. Signals are frequently split into groups of related peaks, called doublets, triplets, multiplets; this splitting is due to the presence of permanent magnetic fields emanating from adjacent nuclei, particularly from other protons within two to five valence bonds. The intensity of each signal, determined from the area under the signal, is proportional to the number of equivalent protons.

Apparatus. A nuclear magnetic resonance spectrometer for continuous-wave spectrometry consists of a magnet, a low-frequency sweep generator, a sample holder, a radio-frequency transmitter and receiver, a recorder and an electronic integrator. A pulsed spectrometer is additionally equipped with a pulse transmitter and a computer for the acquisition, storage and mathematical transformation of the data into a conventional spectrum.

Use a nuclear magnetic resonance spectrometer operating at not less than 60 MHz for 1H. Unless otherwise prescribed, follow the instructions of the manufacturer.

Before recording the spectrum, verify that:

1) The resolution is equal to 0.5 Hz or less by measuring the peak width at half-height using an adequate scale expansion of:

   a) either the band at δ 7.33 ppm or at δ 7.51 ppm of the symmetrical multiplet of a 20 per cent V/V solution of dichlorobenzene R in deuterated acetone R,

   b) or the band at δ 0.00 ppm of a 5 per cent V/V solution of tetramethylsilane R in deuterated chloroform R.

2) The signal-to-noise ratio (S/N), measured over the range from δ 2 ppm to δ 5 ppm on the spectrum obtained with a 1 per cent V/V solution of ethylbenzene R in deuterated chloroform R, is at least 25.1. This ratio is calculated as the mean of five successive determinations from the expression:

\[
S = \frac{2.5A}{H}
\]

\[A\] = amplitude, measured in millimetres, of the largest peak of the methylene quartet of ethylbenzene centred at δ 2.65 ppm. The amplitude is measured from a base line constructed from the centre of the noise on either side of this quartet and at a distance of at least 1 ppm from its centre.

\[H\] = peak to peak amplitude of the base line noise measured in millimetres obtained between δ 4 ppm and δ 5 ppm.

3) The amplitude of spinning side bands is not greater than 2 per cent of the sample peak height in a tube rotating at a speed appropriate for the spectrometer used.

4) For quantitative measurements verify the repeatability of the integrator responses, using a 5 per cent V/V solution of ethylbenzene R in deuterated chloroform R. Carry out five successive scans of the protons of ethyl groups and determine the mean of the values obtained. None of the individual values differs by more than 2.5 per cent from the mean.

Method. Dissolve the substance to be examined as prescribed and filter; the solution must be clear. Use a chemical shift internal reference compound, which, unless otherwise prescribed, is a solution containing 0.5 per cent V/V to 1.0 per cent V/V of tetramethylsilane R (TMS) in deuterated organic solvents or 5 g/l to 10 g/l of sodium tetradeuteriodimethylsilapentanoate acid R (TSP) in deuterium oxide R. Take the necessary quantity and record the spectrum.

CONTINUOUS-WAVE SPECTROMETRY

Adjust the spectrometer so that it is operating as closely as possible in the pure absorption mode and use a radio-frequency setting which avoids saturation of the signals. Adjust the controls of the spectrometer so that the strongest peak in the spectrum of the substance to be examined occupies almost the whole of the scale on the recorder chart and that the signal of the internal reference compound corresponds to a chemical shift of δ 0.00 ppm.

Record the spectrum over the prescribed spectral width and, unless otherwise specified, at a sweep rate of not more than 2 Hz per second. Record the integral spectrum over the same spectral width and at a suitable sweep rate according to the instrument used. When quantitative measurements are required, these should be obtained as prescribed.