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 sulfate R.*

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

(5) TEMED: *tetramethylethylenediamine R.*

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 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.

01/2009:20233

2.2.33. NUCLEAR MAGNETIC RESONANCE SPECTROMETRY

INTRODUCTION

Nuclear magnetic resonance (NMR) spectrometry is an analytical method in particular suitable for the elucidation of the chemical structure of organic molecules by means of interpretation of their NMR spectra, arising from, for example, ^{1}H or the X-nuclei ^{13}C, ^{19}F, ^{15}N, ^{31}P. The spectra can be used for qualitative and quantitative purposes.

Under suitable experimental conditions, the integrated NMR intensities of the signals are directly proportional to the number of nuclear spins of the molecular group responsible for the signal. These integrals can be used for quantitative analysis.

GENERAL PRINCIPLES

Placing an ensemble of nuclei with angular momentum and a magnetic moment in a static magnetic field (B_0) causes the nuclei to arrange themselves in different, quantum-mechanically controlled orientations in relation to the axis of the magnetic field. These orientations are different in energy. An oscillating high-frequency magnetic field (B_1), perpendicular to B_0, will cause transitions between these orientations with net energy absorption. According to the resonance condition $\omega_0 = \gamma B_0$ (γ = gyromagnetic ratio, ω_0 = Larmor frequency), either the B_0 magnetic field or the frequency (ω_1) of the B_1 field may be varied to achieve a spectrum (continuous wave (CW) method). Nowadays the B_1 irradiation is achieved by the use of a radiofrequency (RF) pulse (Fourier transform (FT) method). The coherent radiation emitted during the return to the initial state is observed in the form of a decay curve, called the free induction decay (FID). Subsequent Fourier transformation gives the spectrum in the frequency domain, providing information about the molecular structure. Additional radiofrequency fields may be applied during acquisition of the FID signal to suppress scalar (through-bond) interactions between nuclei (called 'decoupling'). One- and multi-dimensional techniques can be applied for qualitative and quantitative purposes, on samples in either the liquid or the solid state.

Important structural information is derived from the following spectroscopic features:

resonance frequency	kind of nuclei observed
number of resonance signals (singlets, multiplets)	number of chemically distinct groups of nuclei
chemical shift δ (ppm)	chemical nature and environment of the structural group observed
intensity of resonance signals	relative number of resonant nuclei per chemically distinct group
multiplicity of coupling pattern	number of nuclei that are scalar coupled to the observed nucleus
coupling constant ^{n}J (Hz)	number of bonds in the coupling pathway, and its geometry

Correlations of different spectral parameters (e.g. chemical shift and coupling constant, or chemical shifts of different nuclei within one molecular system) can be performed by homo- and hetero-nuclear two- and higher-dimensional methods. Information about the relaxation times T_1 and T_2, nuclear Overhauser effects (NOEs) and the kinetics of time-dependent processes are also accessible from appropriate experiments.

APPARATUS

A high-resolution NMR spectrometer consists of at least the following parts:

- a magnet to deliver the constant magnetic field B_0;
- a temperature-controlled probe to contain the sample, to deliver the radiofrequency pulse and to detect radiation emitted by the sample;
- an electronic console to generate high-power radiofrequency pulses and to collect and digitise the FID signal; this unit also maintains the stability of the instrument electronics;
- a data acquisition and processing unit (computer);

and may also include:

- a continuous flow cell for coupled liquid chromatographic-NMR or flow injection analysis;
- a system for pulsed field gradient NMR.

The high magnetic field is generated by a superconducting coil in a Dewar flask filled with liquid helium. The probe typically contains the sample in a 5 mm-outer-diameter sample tube or in a flow cell, and is connected to the electronics cabinet by RF cables carrying lock, ^{1}H-, and X-nucleus frequencies. Additional devices for tuning and matching the electronic circuits are essential, and sample temperature control is often used.

The NMR spectrometer should be demonstrated to be operating correctly. Appropriate tests to demonstrate this are, typically, measurement of linewidths at half height for defined peaks under defined acquisition conditions, signal-to-noise ratios (*S/N*) for standard mixtures, pulse power (measured as a 90° pulse width), and pulse reproducibility. All instrument manufacturers publish specifications and measurement protocols for these parameters for specific instrument/probe combinations, and compliance with these specifications should be demonstrated.

FOURIER TRANSFORM NMR (FT-NMR)

Contemporary spectrometers generally operate according to the Fourier transform (FT) principle: after exciting the sample with a radiofrequency pulse of appropriate frequency (ν), amplitude (B_1) and duration (τ_p) and a succeeding short dead time (t_d) (to enable the electronics to recover), the amplified analogue FID signal is sampled during the acquisition time (t_{ac}) and digitised with an analogue-to-digital converter (ADC), and the results are stored in the spectrometer memory. The receiver output is amplified prior to digitisation to maximise sensitivity without saturating the ADC. In case of observation of X-nuclei, the standard experiment includes, if necessary, broadband ^{1}H decoupling, i.e. irradiation of all the protons during the experiment. To increase the *S/N*, multiple FID signals may be accumulated coherently and summed. Fourier transformation of this time-domain data gives the frequency-domain spectrum.

PARAMETERS

The following acquisition parameters influence the result of an FT experiment, and should be adjusted and controlled.

Pulse width (τ_p). The excitation pulse is directed along the x-axis of the so-called rotating frame, its duration (or 'width', τ_p) determines the flip angle (θ) and thus the intensity (*I*) of the resonance signal:

$$\theta = \gamma' \times B_1 \times \tau_p \quad (1)$$

$$M_y = M_o \times \sin\theta \quad (2)$$

The observed magnetisation M_y is maximum at θ = 90°. The pulse duration should be short to guarantee that all signals in the spectral width (*SW*) are excited to a similar degree. The magnetisation decays due to relaxation processes.

Dead time (t_d). The dead time is the time between the end of the pulse and start of the acquisition, it is necessary for technical reasons and care should be taken as it may influence signal intensities and peak phase. Rapidly decaying signals (giving rise to broad spectral lines) are reduced in intensity by more than slowly decaying signals (which give rise to narrow spectral lines).

Acquisition time (t_{ac}). The acquisition time (t_{ac}) is related to the spectral width (i.e. the whole observed region) and the number of digital data points (*DP*) collected during signal acquisition.