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2.2.27. THIN-LAYER CHROMATOGRAPHY

PRINCIPLE

Thin-layer chromatography is a separation technique in which a stationary phase consisting of an appropriate material is spread in a uniform thin layer on a support (plate) of glass, metal or plastic. Solutions of analytes are deposited on the plate prior to development. The separation is based on adsorption, partition, ion-exchange or combinations of these mechanisms and is carried out by migration (development) of solutes (solutions of analytes) in a solvent or a suitable mixture of solvents (mobile phase) through the thin layer (stationary phase).

EQUIPMENT

Plates. The chromatography is carried out using pre-coated plates as described under *Reagents* (*4.1.1*). In a monograph in which both normal and high-performance plates may be used, the particle size of the silica gel is indicated in parentheses ( ) after the name of the reagent.

Pre-treatment of the plates. It may be necessary to wash the plates prior to separation. This can be done by migration of an appropriate solvent. The plates may also be impregnated by procedures such as development, immersion or spraying. At the time of use, the plates may be activated, if necessary, by heating in an oven at 120 °C for 20 min.

Chromatographic tank with a flat bottom or twin trough, of inert, transparent material, of a size suitable for the plates used and provided with a tightly fitting lid. For horizontal development the tank is provided with a trough for the mobile phase and it additionally contains a device for directing the mobile phase to the stationary phase.

Micropipettes, microsyringes, calibrated disposable capillaries or other application devices suitable for the proper application of the solutions.

Fluorescence detection device to measure direct fluorescence or the inhibition of fluorescence.

Visualisation devices and reagents. Suitable devices are used for derivatisation to transfer to the plate reagents by spraying, immersion or exposure to vapour and, where applicable, to facilitate heating for visualisation of separated components.

Documentation. A device may be used to provide documentation of the visualised chromatogram, for example a photograph or a computer file.

PROCEDURE

Sample application. Apply the prescribed volume of the solutions at a suitable distance from the lower edge and from the sides of the plate and on a line parallel to the lower edge; allow an interval of at least 10 mm (5 mm on high-performance plates) between the centres of circular spots and 5 mm (2 mm on high-performance plates) between the edges of bands. Apply the solutions in sufficiently small portions to obtain circular spots 2-5 mm in diameter (1-2 mm on high-performance plates) or bands 10-20 mm (5-10 mm on high-performance plates) by 1-2 mm.

In a monograph in which both normal and high-performance plates may be used, the working conditions for high-performance plates are given in brackets [ ] after those for normal plates.

Vertical development. Line the walls of the chromatographic tank with filter paper. Pour into the chromatographic tank a sufficient quantity of the mobile phase for the size of the tank to give after impregnation of the filter paper a layer of appropriate depth related to the dimension of the plate to be used. For saturation of the chromatographic tank, replace the lid and allow to stand at 20-25 °C for 1 h. Unless otherwise indicated in the monograph, the chromatographic separation is performed in a saturated tank. Apply the prescribed volume of solutions as described above. When the solvent has evaporated from the applied solutions, place the plate in the chromatographic tank, ensuring that the plate is as vertical as possible and that the spots or bands are above the surface of the mobile phase. Close the chromatographic tank, maintain it at 20-25 °C and protect from sunlight. Remove the plate when the mobile phase has moved over the prescribed distance, measured between the points of application and the solvent front. Dry the plate and visualise the chromatograms as prescribed.

For two-dimensional chromatography, dry the plates after the first development and carry out a second development in a direction perpendicular to that of the first development.

Horizontal development. Apply the prescribed volume of the solutions as described above. When the solvent has evaporated from the applied solutions, introduce a sufficient quantity of the mobile phase into the trough of the chamber using a syringe or pipette, place the plate in the chamber after verifying that the latter is horizontal and connect the mobile phase direction device according to the manufacturer's instructions. If prescribed, develop the plate starting simultaneously at both ends. Close the chamber and maintain it at 20-25 °C. Remove the plate when the mobile phase has moved over the distance prescribed in the monograph. Dry the plate and visualise the chromatograms as prescribed.

For two-dimensional chromatography, dry the plates after the first development and carry out a second development in a direction perpendicular to that of the first development.

Parameters for assessing the suitability of the system are described in general chapter *2.2.46. Chromatographic separation techniques*. The extent to which adjustments of the chromatographic conditions can be made are also given in that general chapter.

VISUAL EVALUATION

Identification. The principal spot in the chromatogram obtained with the test solution is visually compared to the corresponding spot in the chromatogram obtained with the reference solution by comparing the colour, the size and the retardation factor (R_F) of both spots.

The retardation factor (R_F) is defined as the ratio of the distance from the point of application to the centre of the spot and the distance travelled by the solvent front from the point of application.

Verification of the separating power for identification. The performance of the plate, given by the test for chromatographic separation in the description of the corresponding reagent in general chapter *4.1.1. Reagents*, is usually sufficient. An additional performance criterion may be prescribed in the individual monograph to control the system suitability.

Related substances test. The secondary spot(s) in the chromatogram obtained with the test solution is (are) visually compared to either the corresponding spot(s) in the chromatogram obtained with the reference solution containing the impurity(ies) or the spot in the chromatogram obtained with the reference solution prepared from a dilution of the test solution.

Verification of the separating power. The requirements for the verification of the separating power are prescribed in the monographs concerned.

Verification of the detecting power. The detecting power is satisfactory if a spot or band is clearly visible in the chromatogram obtained with the most dilute reference solution.

QUANTITATIVE MEASUREMENT

The requirements for resolution and separation are prescribed in the monographs concerned.

Substances separated by thin-layer chromatography and responding to UV-Vis irradiation can be determined directly on the plate, using appropriate instrumentation. While moving the plate or the measuring device, examine the plate by measuring the reflectance of the incident light. Similarly, fluorescence may be measured using an appropriate optical system. Substances containing radionuclides can be quantified in 3 ways: either directly by moving the plate alongside a suitable counter or vice versa (see *Radiopharmaceutical preparations (0125)*), by cutting the plates into strips and measuring the radioactivity on each individual strip using a suitable counter or by scraping off the stationary phase, dissolving it in a suitable scintillation cocktail and measuring the radioactivity using a liquid scintillation counter.

Equipment. The equipment for direct measurement on the plate consists of:

- a device for exact positioning and reproducible dispensing of the amount of substances onto the plate;
- a mechanical device to move the plate or the measuring device along the *x*-axis or the *y*-axis;
- a recorder and a suitable integrator or a computer;
- *for substances responding to UV-Vis irradiation*: a photometer with a source of light, an optical device able to generate monochromatic light and a photo cell of adequate sensitivity, for the measurement of reflectance or transmittance; if fluorescence is measured, a suitable filter is required to prevent light used for excitation from reaching the detector while permitting emitted light or a specific portion thereof to pass;
- *for substances containing radionuclides*: a suitable counter for radioactivity; the linearity range of the counting device is to be verified.

Procedure. Prepare the solution of the substance to be examined (test solution) as prescribed in the monograph and, if necessary, prepare the reference solutions of the substance to be determined using the same solvent as in the test solution. Apply the same volume of each solution to the plate and develop.

Substances responding to UV-Vis irradiation. Prepare and apply not fewer than 3 reference solutions of the substance to be examined, the concentrations of which span the expected value in the test solution (about 80 per cent, 100 per cent and 120 per cent). Treat with the prescribed reagent, if necessary, and record the reflectance, transmittance or fluorescence in the chromatograms obtained with the test and reference solutions. Use the measured results for the calculation of the amount of substance in the test solution.

Substances containing radionuclides. Prepare and apply a test solution containing about 100 per cent of the expected value. Determine the radioactivity as a function of the path length and report the radioactivity in each resulting peak as a percentage of the total amount of radioactivity.

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2.2.28. GAS CHROMATOGRAPHY

PRINCIPLE

Gas chromatography (GC) is a chromatographic separation technique based on the difference in the distribution of species between two non-miscible phases in which the mobile phase is a carrier gas moving through or passing the stationary phase contained in a column. It is applicable to substances or their derivatives that are volatilised under the temperatures employed.

GC is mainly based on mechanisms of adsorption or mass distribution.

EQUIPMENT

The equipment typically consists of:

- an injector;
- a chromatographic column contained in an oven;
- one or more detector(s);
- a data acquisition system.

The carrier gas flows through the column and then through the detector at a controlled rate or pressure.

The chromatography is carried out either at a constant temperature or according to a given temperature programme.

INJECTORS

Injection may be carried out either into a vaporisation chamber, which may be equipped with a stream splitter, or directly at the head of the column using a syringe or an injection valve.

Injections of vapour phase may be effected by static or dynamic head-space injection systems.

Dynamic head-space (purge and trap) injection systems include a sparging device by which volatile substances in solution are swept into an adsorbent column maintained at a low temperature. Retained substances are then desorbed into the mobile phase by rapid heating of the adsorbent column.

Static head-space injection systems include a thermostatically controlled sample heating chamber in which closed vials containing solid or liquid samples are placed for a fixed period of time to allow equilibration of the volatile components of the sample between the non-gaseous phase and the vapour phase. After equilibration, a predetermined amount of the head-space of the vial is flushed into the gas chromatograph.

STATIONARY PHASES

Stationary phases are contained in columns that may be:

- a capillary column whose stationary phase may be a solid coating the inner surface of the column (e.g. macrogol 20 000) or a liquid deposited on the inner surface (e.g. dimethylpolysiloxane); in the latter case it may be chemically bonded to the inner surface;
- a column packed with the stationary phase that may be a solid phase (e.g. alumina, silica) or an inert solid support (usually a porous polymer) impregnated or coated with a liquid.

Capillary columns, made of fused silica, are 0.1 mm to 0.53 mm in internal diameter (Ø) and at least 5 m in length. The stationary phase is a film 0.1 µm to 5.0 µm thick.

Packed columns, made of glass or metal, are usually 1 m to 3 m in length with an internal diameter (Ø) of 2 mm to 4 mm.

MOBILE PHASES

Retention time and peak efficiency depend on the carrier gas flow rate; retention time is directly proportional to column length and resolution is proportional to the square root of the column length.

The carrier gas flow rate is usually expressed in millilitres per minute at atmospheric pressure and at the stated temperature. Flow rate is measured at the detector outlet, either with a calibrated mechanical device or with a bubble tube, while the column is at operating temperature.

The linear velocity of the carrier gas through a column is inversely proportional to the square of the internal diameter of the column for a given flow volume.

Helium, nitrogen and hydrogen are commonly used carrier gases.