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Draft Indian Standard
METHODS OF SAMPLING AND TESTS FOR DYE INTERMEDIATES

(*Second Revision of* IS 5299)
(ICS No. 71.080.80)

Dyes Intermediates Sectional Committee PCD 26	Last date for comment is **16 October 2023**

FOREWORD
(*Formal clauses to be added later*)

This Indian standard was originally published in 1969 when it was prepared taking into consideration the views of the manufacturers, consumers, testing laboratories as well as the technologists in the field. The methods of test incorporated were mainly those which were routinely used in industrial laboratory for assessing quality of dye intermediates.

The Committee responsible for the preparation of this standard decided to revise it in the light of the experience gained during the long span of period. In the first revision, instrumental methods of testing were incorporated to keep pace with the ongoing trends in the industry.

This revision (*second*) has been undertaken to update the standard by updating the cross referred standards.

Determination of crystallizing or freezing point has been aligned with ISO 1392:1977 'Determination of crystallizing point—General method, on the subject'.

In reporting the result of a test or analysis made in accordance with this standard, if the final value, observed or calculated, is to be rounded off, it shall be done in accordance with IS 2:2022 'Rules for rounding off numerical values *(second revision)'*.

1 SCOPE

This standard prescribes the methods of sampling and tests for dye intermediates.

2 REFERENCES

The following Indian Standards contain provisions which, through reference in this text constitute provisions of this standard. At the time of publication, the editions indicated were valid. All standards are subject to revision and parties to agreement based on this standard are encouraged to investigate the possibility of applying the most recent editions of the standards given below:

Sl. No.	IS No.	Title
1	264 : 2005	Nitric acid — Specification (*third revision*)
2	265 : 2021	Hydrochloric acid — Specification (*fifth revision*)
3	266 : 1993	Sulphuric acid — Specification (*third revision*)
4	695 : 2020	Acetic acid — Specification (*fourth revision*)
5	1574 : 1980	Specification for Glass weighing bottles (*first revision*)
6	2362: 1993	Determination of water by Karl Fischer method — Test method (*Second revision*)
7	4730: 1994	Method for determination of density of liquids (*first revision*)
8	4905: 2015/ISO 24153: 2009	Random sampling and randomization procedures (*first revision*)
9	5298: 2013	Method for determination of distillation range and distillation yield (*second revision*)
10	5762: 1970	Methods for determination of melting point and melting range
11	8883 (Part 1) : 2005	Methods of sampling of chemical and chemical products: Part 1 General requirements and precautions (*first revision*)
12	8883 (Part 2/ Sec 1) :1978	Methods of sampling chemicals and chemical products: Part 2 Sampling equipment, Section 1 For solids
13	8883 (Part 2/ Sec 2) : 1978	Methods of sampling of chemicals and chemical products: Part 2 Sampling equipment, Section 2 For liquid
14	8883 (Part 2/ Sec 3):1978	Methods of sampling chemical and chemical products: Part 2 Sampling equipment, Section 3 For gases

3 TERMINOLOGY

For the purpose of this standard, the following definitions shall apply.

3.1 Approved Sample

The sample accepted by the indentor or inspection authority as the basis for supply. When a sample is tested and approved by the purchaser or inspection authority, the result of such tests as permits the supplier to meet the limits imposed by specification for deliveries shall be made available to the supplier.

3.2 Composite Sample

The quantity of material obtained by mixing portions of material taken from each of the sample units shall be termed as a composite sample representing the lot as a whole and meant for testing to ascertain the conformity of the lot to the specified requirements.

3.3 Increment

The quantity of material taken by a single operation of the sampling implement.

3.4 Individual Sample

The material collected from a single sample unit and set apart as representing that unit for the purpose of testing to ascertain the conformity of unit to specified requirements.

3.5 Lot

All the units of the same size and type in a single consignment of the material and belonging to the same batch of manufacture shall constitute a lot. If consignment is known to consist of different batches of manufacture or of different sizes or types of unit, the units belonging to the same batch and of the same size and type shall be grouped together and each such group shall constitute a separate lot.

3.6 Sample Unit

Any unit withdrawn from a lot for the purpose of testing the quality of the lot.

3.7 Tender Sample

The sample submitted by the supplier with his tender.

3.8 Unit

Any form of packing of the material, such as a can, a carboy, a cask, a barrel, a drum, a bag, a crate, a tank or a tanker.

3.9 Initial Boiling Point

The temperature at the instant the first drop falls from the end of the condenser.

3.10 Dry Point

The temperature at the instant the last drop of liquid evaporates from the lowest point in the distillation flask, any liquid on the side of the flask being disregarded.

4 SAMPLING

4.1 General Requirements of Sampling

In drawing, preparing, storing and handling test samples the precautions and directions as given in IS 8883 (Part 1) shall be observed.

4.2 Scale of Sampling

4.2.1 For ascertaining the conformity of the material to the requirements of relevant material specifications, each lot as defined in 3.5 shall be examined separately.

4.2.2 For this purpose, from all the units comprising the lot, the number of sample unit shall be chosen in accordance with Table 1 and 4.2.3

Table 1 Sampling of Dye Intermediates

Sr. No.	Total Number of Units in the Lot N	Number of Sample Units to be Selected n
01	2 to 15	2
02	16 to 25	3
03	26 to 50	4
04	51 to 100	5
05	101 to 150	6
06	151 to 300	7
07	301 and above	8

4.2.3 The sample units shall be selected at random from the lot and to ensure the randomness of selection, random number tables (*see* IS 4905) shall be used. In case such tables are not available, the following procedure may be adopted.

Starting from any unit, count them in one order as 1, 2, 3,... up to *r* and so on, where r is the integral part of N/n (N being the total number of units in the lot and n being the number of sample units to be selected). Every *r* th unit thus counted shall be withdrawn to constitute a sample.

4.3 Preparation of Test Samples and Referee Sample

The preparation of test samples and referee sample may be done in line with IS 8883 (Part 2/ Sec 1); IS 8883 (Part 2/Sec 2); and IS 8883 (Part 2/Sec 3) depending upon the form of the material.

4.4 Sample Containers

4.4.1 The sample containers used for storing an individual sample or a composite sample shall have a suitable cap, stopper or lid.

4.4.2 The sample container shall be made of a material (glass metal or any other) which is suitable for preserving the properties of the material without itself affecting them in any manner. The size of the sample container shall be such that it shall be almost but not completely filled by the individual sample or composite sample as the case may be.

4.4.3 Labelling of Sample Containers

Each sample container shall carry a label with particulars necessary for clear identification of the source of the sample, such as consignment number, the lot number, the name of the product, the batch number, and the like. In addition it will carry the date of sampling and the signatures of the supplier and the purchaser or their representatives in whose presence the samples had been taken.

5 DETERMINATION OF DENSITY

5.1 General

Density shall be determined with a density bottle or pyknometer as per procedure given in IS 4730. The temperature at which the relative density is determined shall be 27/27°C or at specified temperature as mentioned in the relevant specification.

6 DETERMINATION OF BULK DENSITY

6.1 Apparatus

6.1.1 The apparatus is shown in Fig. 1. The base of the measuring cylinder A shall be ground flat and the empty measuring cylinder A together with the rubber bung shall weigh 250 ± 5 g. It shall be accurately calibrated to 250 ml with an error of less than 1 ml. The distance between zero and 250 ml graduation on the measuring cylinder shall be

not less than 220 mm and not more than 240 mm. The distance between the flat ground part of the base of the measuring cylinder A and the rubber base pad B, when the measuring cylinder A is raised to the full height, shall be 25± 2 mm.

6.1.2 Balance or Scales

Of a type providing easy access to the pans being at least 10 cm in diameter. It is necessary that the pointer should show a significant deflection for a change in load of 0.25 g.

6.1.3 The rubber base pad B shall have a Shore hardness of 35 to 50.

6.1.4 A timing device to indicate seconds.
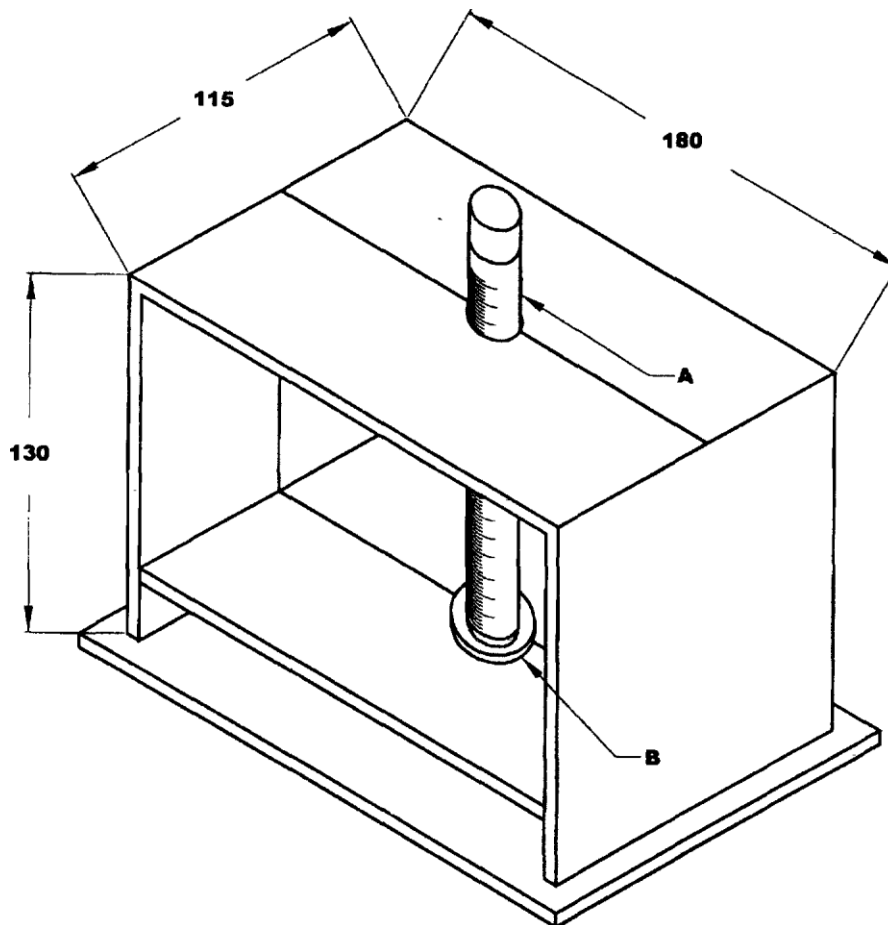
All dimensions in millimeters
FIG.1 APPARATUS FOR THE DETERMINATION OF BULK DENSITY

6.2 Procedure

Weigh M g (*see* Note) of the material, which has been freshly pulverized, sieved and collected in the containers (or drums) of the pulverizing or the sieving machine. The weighing should be done on a piece of black glazed paper (25cm × 25 cm), with two parallel creases to form a channel 12.5 mm down the middle of the paper. Counterpoise the paper on the balance before weighing the material. Pick up the paper and form it. Pick up the paper and form it into a chute. Allow it to lie between the thumb and fingers on the palm of the hand and introduce it into the cylinder held at 45° to the vertical. Slip the powder gently and smoothly into the measuring cylinder without knocking or squeezing. Assemble the measuring cylinder as shown in Fig. 1. With the thumb and forefinger of one hand, gently grasp the upper part of the cylinder and within one second, lift it as far as 25 mm, do not jerk the cylinder by knocking it against the upper stop. At the start of the next second, release the cylinder smoothly. Continue lifting and

dropping until 50 complete drops have been given. Once in every two seconds, a gentle twist of about 10 degrees should be given to the cylinder. When 50 drops are completed, raise the cylinder to the eye level and read the volume of the material (V).

> NOTE — The mass M may vary from substance to substance. It will be less for a very light (fluffy) material and more for a very heavy material. An arbitrary figure may be chosen by taking a preliminary rough reading of volume falling from 250 to 150 ml.

6.3 Calculation

Bulk density, g/ml $= \frac{M}{V}$

Where
M = massing of the material taken for test, and
V = volume in millilitres of the material after tapping.

7 DETERMINATION OF DISTILLATION (BOILING) RANGE/ YIELD

Determine the distillation range/yield by the method as prescribed in IS 5298.

8 DETERMINATION OF CRYSTALLIZING OR FREEZING POINT

8.1 Scope

Method for the determination of crystallizing points in the range from about -50 to about + 250°C.

The crystallizing point can be determined directly on the sample as received, or on the dried sample, or on both. In which of these conditions the sample is to be tested and, if the determination is to be made with the dried sample, what method of drying is to be used, will be stated in the specific test method for each material.

8.2 Principle

Cooling the liquid or liquefied sample, and determination of the crystallizing point by observation of the temperature during crystallization under defined conditions.

8.3 Reagents
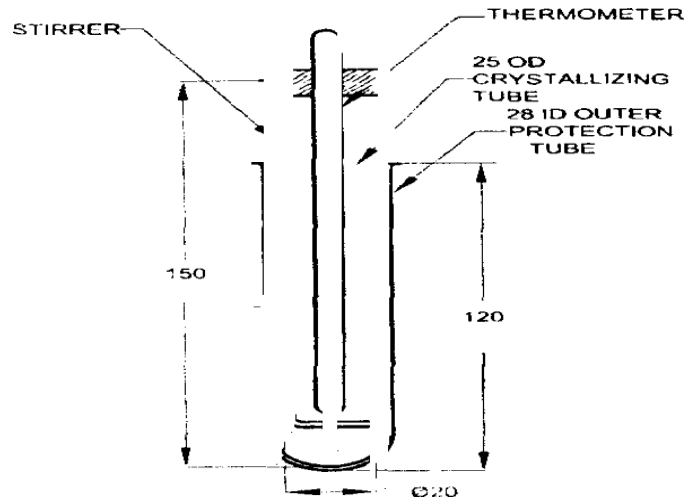
8.3.1 Acetone

8.3.2 Solid Carbon Dioxide

8.3.3 Ice

8.3.4 Calcium Sulphate

Dried at about 170°C. Dry calcium sulphate dihydrate ($CaSO_4.2H_2O$) for 24 h at about 170°C, allow to cool in a desiccator and then store it in an air-tight container.

8.4 Apparatus

Ordinary laboratory apparatus and the apparatus shown in Fig. 2, 3, 4 and 5 comprising the following items.

All dimensions in millimeters.
FIG. 2 APPARATUS FOR DETERMINATION OF CRYSTALLIZING POINT

8.4.1 Crystallizing Tube

External diameter approximately 25 mm and length approximately 150 mm.

8.4.2 Outer Protection Tube

Internal diameter approximately 28 mm, length approximately 120 mm and wall thickness approximately 2 mm.

8.4.3 Stirrer

Glass or stainless steel, with a loop approximately 20 mm in diameter; it may be operated by hand or mechanically, to provide approximately one 30 mm stroke per second.

8.4.4 Precision Thermometer

Graduated at intervals of 0.1°C with a known maximum scale error of 0.1°C, and with the range stated in the specific test method for the particular material.

8.4.5 Dewar Vessel

Approximately 500 ml capacity, containing the appropriate cooling mixture (carbon dioxide/acetone or ice water or water) and provided with a suitable laboratory thermometer. An example of such a Dewar vessel is given in Fig. 3, but other vessels of the same capacity may also be used.
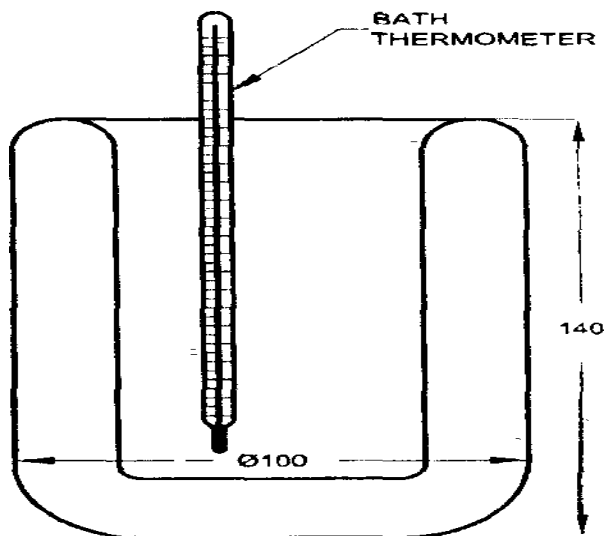
All dimensions in millimetres.
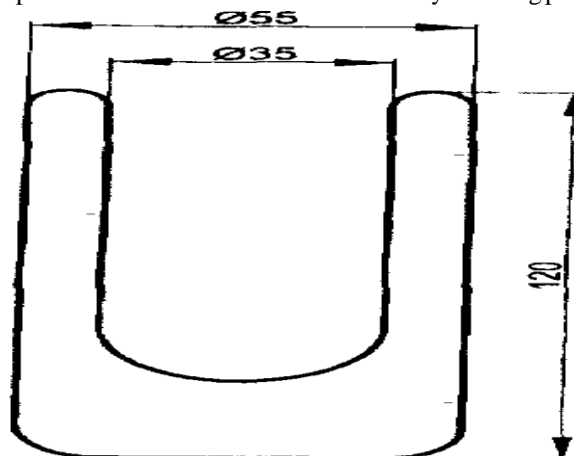

FIG. 3 DEWAR VESSEL

8.4.6 Dewar Vessel

As shown in Fig. 4 (It is not necessary for the inner surfaces of the vessel to be silvered).

8.4.7 *Heating Bath*

As shown in Fig. 5, containing silicon oil or other suitable liquid heating medium, and provided with a suitable laboratory thermometer.
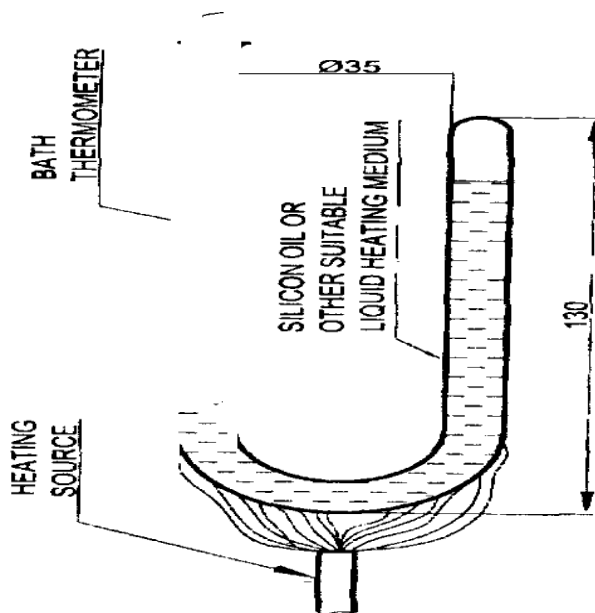
8.5 Procedure

8.5.1 Preparation of the sample for the direct determination of the crystallizing point on the sample as received.

All dimensions in millimeters
FIG. 4 DEWAR VESSEL

All dimensions in millimeters
FIG. 5 HEATING BATH

8.5.1.1 Liquid products

Fill the crystallizing tube **(8.4.1)** to a depth of approximately 60 mm with the untreated sample and proceed as specified in **8.5.3.**

8.5.1.2 Solid products

Before the determination of the crystallizing point of these products, they shall be melted in a water bath, drying oven or oil bath [this can be carried out in the crystallizing tube (8.4.1), using the heating bath (8.4.7)], care being taken to ensure that the temperature of the molten product does not exceed its melting point by more than a few degrees. Fill the crystallizing tube to a depth of approximately 60 mm with the molten sample and proceed as specified in **8.5.3.**

8.5.2 Preparation of the sample for the determination of the crystallizing point on the dried sample.

8.5.2.1 Liquid products

Liquid products of normal water content [that is, less than or equal to 2 percent (m/m)] shall be dried in the crystallizing tube **(8.4.1)** by addition of calcium sulphate. Fill the crystallized tube to a depth of approximately 60 mm with the liquid sample, add the calcium sulphate **(8.3.4)** (2 to 5 g are usually required) and proceed as specified in **8.5.3.**
In some cases, other methods of drying may be required; these will be specified in the specific test method for the particular material.

8.5.2.2 Solid products

The drying method for solid products depends on the water content of the sample and on the value of the crystallizing point.

a) Products with a lower water content [that is, less than or equal to 2 percent (m/m)]. Solid products with melting points below approximately 150°C shall be dried with calcium sulphate. Fill the crystallizing tube **(8.4.1)** to a depth of approximately 60 mm with the molten sample, add the calcium sulphate **(8.3.4)** (2 to 5 g are usually required) and proceed as specified in **8.5.3.** Solid products with melting points above approximately 150° C shall be dried in an oven at 60° C, or under vacuum, or by air drying, and the determination of the crystallizing point then carried out on the molten sample. Fill the crystallizing tube

(**8.4.1**) to a depth of approximately 60 mm with the molten sample and proceed as specified in **8.5.3**. For these higher melting point samples, the method and time of drying will be indicated in the specific test method for the particular material; in some cases, alternative drying methods may be given for a material, whatever its melting point.

b) Products with higher water content

Samples with a higher water content (for example, pastes) shall in every case dried before determination of the crystallizing point, for example, in an oven at 60° C or under vacuum, etc. The determination is then carried out on the molten sample. Fill the crystallizing tube (**8.4.1**) to a depth of approximately 60 mm with the molten sample and proceed as specified in **8.5.3**.

In addition, with products melting below approximately 150°C add some calcium sulphate (**8.3.4**) (normally 2 to 5g), in the crystallizing tube before commencing the determination.
The method of drying, the time of drying or alternative drying methods will be indicated in the specific test method for the particular material.

NOTE — Before the determination of the crystallizing point, solid samples shall be melted in a water-bath, drying oven or oil bath [this can be carried out in the crystallizing tube using the heating bath (**8.4.1**)] care being taken   to ensure that the temperature of the molten sample does not exceed its melting point by more than a few degrees.

8.5.3 Preparation of the Apparatus

Insert the stirrer (**8.4.3**) into the crystallizing tube (**8.4.1**) prepared as specified in **8.5.1** and **8.5.2**. Secure the specified thermometer (**8.4.4**) vertically in the liquid or molten product with its bulb approximately 15 mm above the bottom of the crystallizing tube. Fit the outer tube (**8.4.2**) to this assembly (if necessary by means of a cork shive or a rubber sleeve), and place the whole in position as follows:

a) For crystallizing points in the range frog room temperature down to approximately - 50^0C: in the Dewar vessel (**8.4.5**) filled with the appropriate cooling mixture (carbon dioxide acetone or ice water or water) at a temperature 3 to 5° C below the crystallizing point to be determined;

b) For crystallizing points in the range from room temperature up to approximately 100 ^{0}C: in the Dewar vessel (**8.4.6**) and

c) For crystallizing points in the range from approximately 100 to 250°C in the heating bath (**8.4.7**) at a temperature 5 to 7°C below the crystallizing point to be determined.

8.5.4 Determination

Check that the sample is still liquid at this stage; stir the sample and take temperature readings. These should decrease uniformly at first, then rise suddenly as the substance ccrystallizes; sometimes the temperature remains constant for a short time. If the temperature rise exceeds 1 to 2° C, this indicates that excessive super cooling has occurred. In this case, the determination should be repeated, seeding the liquid or the melt to prevent excessive super cooling. Read the highest temperature attained after crystallization and adjust the reading for scale error and emergent stem correction. Record this temperature to the nearest 0.1°C as the crystallizing point of the product under test.

NOTES

1) The stirrer may be omitted and the stirring carried out by hand using the thermometer but care should be taken that the thermometer does not touch the walls of the crystallizing tube.
2) For the correct determination of the crystallizing point of a solid sample, it is necessary that the product should melt during the test without any decomposition. That this condition is fulfilled may be checked by repeating the test and comparing the two results. If the two crystallizing temperatures are the same, this indicates that the above condition has been met.

8.6 Results

Record the crystallizing point thus determined to the nearest 0.1°C, indicating the condition of the sample, that is, whether tested in the dried or the undried condition, or in both.

9 DETERMINATION OF MELTING POINT

Determine the melting point by method as prescribed in IS 5762.

10 DETERMINATION OF MOISTURE CONTENT

10.0 General

Three methods are given, the choice of the method depends upon the material of which the moisture content is to be estimated.

10.1 Dean and Stark Method

10.1.1 Apparatus

The apparatus consists of a glass flask heated by suitable means and provided with a reflux condenser discharging into a trap and connected to the flask. The connections between the trap, the condenser and the flask should be interchangeable ground-glass joints. The trap serves to collect and measure the condensed water, and to return the solvent to the flask. The assembly of the apparatus is shown in Fig. 6 and the various components are described below.

a) Flask — A 500 to 1000-ml capacity flask of the shape shown in Fig. 6, made of hard resistance glass well-annealed and as free as possible from striae and similar defects.

b) Condenser - A glass water-cooled reflux type condenser, of the design and dimensions shown in Fig. 7. The only mandatory dimensions for the condenser are the external diameters of the inner tube and of the jacket, which shall be 16 to 17 mm and 23 to 25 mm respectively. The joints A and B should be neatly finished as shown in Fig. 7 particularly the bore at B shall have the minimum disturbance. The shoulder above the cone of joint D shall be elongated as shown in the figure to avoid a sharp re-entrant shape which may restrict the free flow of liquid down the inner wall. The cone shall be extended beyond the length appropriate to the joint D and the lower end ground at an angle of approximately 60° to the axis. The drainage tip shall be at the front of the condenser when the lower water connection is to the left, and the finish shall be either smooth or fire-polished. When inserted into the trap the tip of the condenser shall be 6 to 7 mm above the surface of the liquid in the trap after distillation conditions have been established. The nominal dimensions of the joint D are given below.

Nominal dial of Large end of ground zone	Nominal dial of Small end of ground zone	Nominal dial of Length ground zone measured axially
mm	mm	mm
18.8	16.2	26
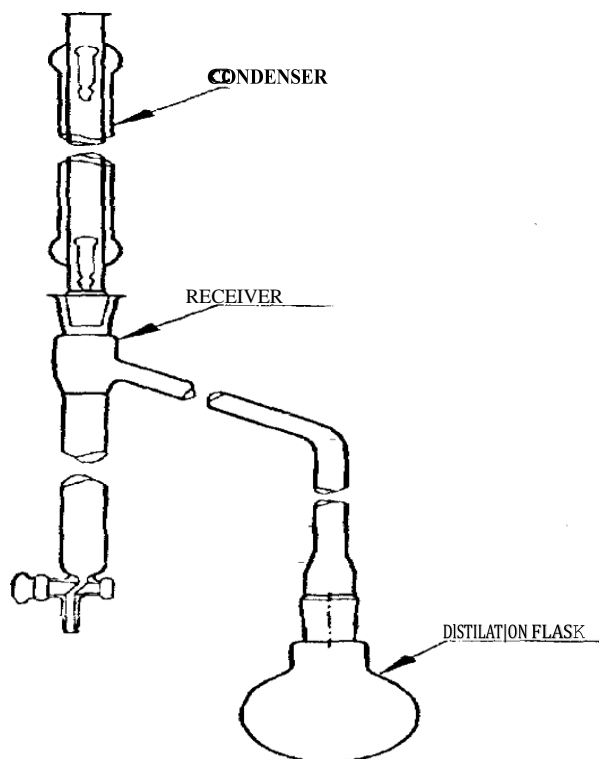
FIG. 6 TYPICAL ASSEMBLY OF DEAN AND STARK APPARATUS

c) Receivers — Also called the trap, made of hard resistance glass, well-annealed and as free as possible from striae and similar defects, provided with ground-glass joints, with the shape, dimension's and tolerances given in Fig. 8 and consisting essentially of the upper chamber, together with the tube and ground joint leading to the flask, and the graduated tube.

The receiver shall be of two sizes, namely, 2-ml capacity and 10-ml capacity (see Fig. 8 and 9). The mandatory dimensions and tolerances for the receiver shall be as given in Table 2
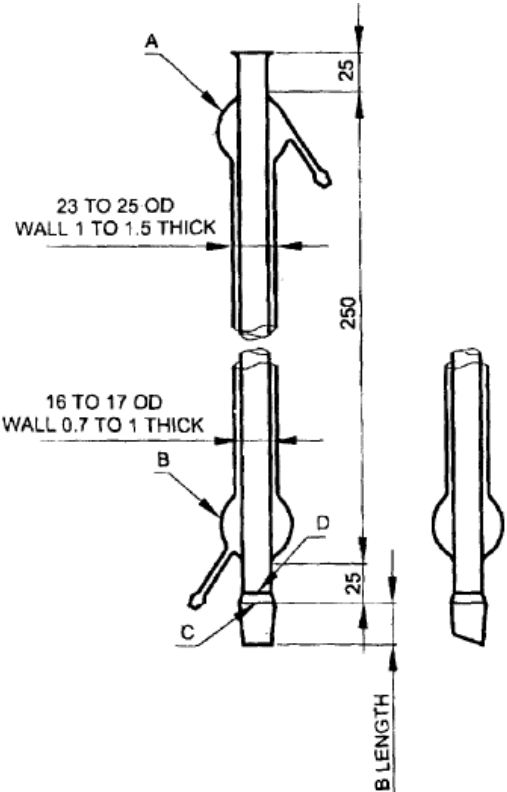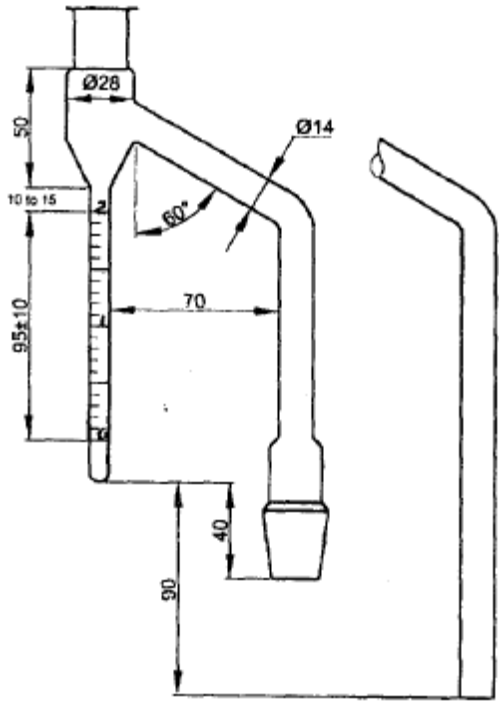
FIG. 7 CONDENSER

FIG. 8 2-ml RECEIVER

The shoulder of the upper chamber of the receiver immediately below the conical joint shall be finished square, as shown in Fig. 8 and 9. The graduated portion of the receiver shall be cylindrical throughout its length. The bottom of the graduated tube of the 2-ml receiver shall be sealed, the end of the tube being approximately Hhemispherical in shape. The graduated scales on the receivers shall be numbered and subdivided as shown in Fig. 8 and 9. The graduation marks shall be fine cleanly etched permanent lines of uniform thickness lying in the planes at right angles to the axis of the tube. The graduation marks shall be confined to the cylindrical portion of the tube and there shall be no evident irregularity in their spacing. In these receivers the numbered graduation marks shall be carried completely round the tube, the shortest graduation marks shall be carried halfway round the tube, and the graduation marks of intermediates length shall be carried approximately two-thirds of the way round the tube and shall project equally at each end beyond the shortest graduation marks.
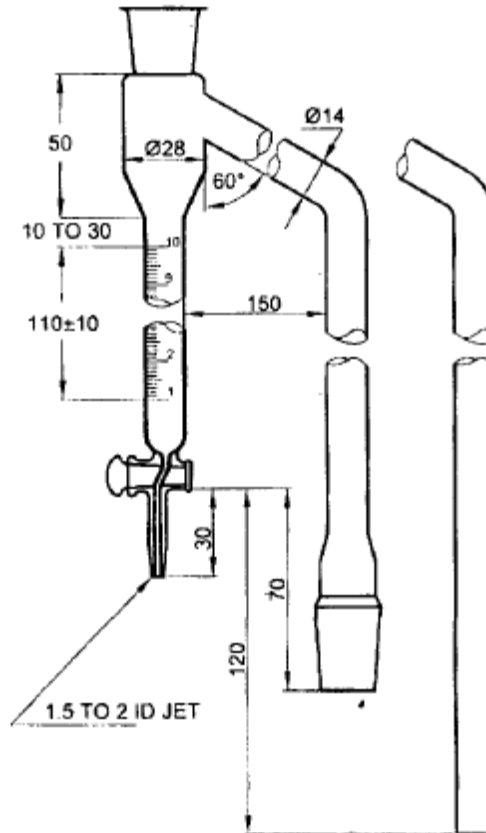
All dimensions in millimeters
FIG. 9 10-ml RECEIVER

The capacity corresponding to any graduation mark is defined as the volume of water at 27°C, expressed in milliliters, required to fill the graduated portion to that mark at 27° C, the axis of the graduated portion being vertical and the lowest point of the water meniscus being set on the graduation mark. In the case of 10 ml receiver, the volume of the bore of the stop-cock key and the volume of the jet below the stop cock shall not be included as part of the measured volume. The error at any point on the receiver scale, and also the difference between the errors at any two points on the scale, shall not exceed the figures given for the receiver in Table 2.

For the 10 ml receiver, the stop-cock shall be of the 2 mm oblique bore having the general design shown in Fig. 8 and 9. The rate of leakage, tested with the stop-cock free from grease, the barrel and key wetted with water, the receiver filled initially with water to the top of the scale, and the key in either of the fully shut-off position, shall not exceed the figures given in Table 2.

Each receiver shall have permanently and legibly marked on it.

1) The abbreviation, ml;
2) The inscription '27°C' to indicate that the receiver is graduated for content at 27°C; and

3) An identification number shall also appear on the key.

d) *Heat source* — The source of heat may be either an oil-bath or an electric heater provided with a sliding rheostat or other means of heat control. The temperature of the oil in the bath should not be very much higher than the boiling point of xylene or toluene, whichever solvent is used.

e) *Copper wire* — Long enough to extend through the condenser, with one end twisted into a spiral. The diameter of the spiral should be such that it fits snugly within the graduated portion of the receiver and yet may be moved up and down.

Table 2 Dimensions and Tolerances for Receiver
[*Clause* 10.1.1(c)]

Sl. No.	Characteristic	Receiver	
		2 ml	10 ml
(1)	(2)	(3)	(4)
i)	Volume, equivalent to smallest subdivision, ml	0.05	0.1
ii)	Scale length, mm	95 ±10	110 ± 10
iii)	Length of cylindrical tube above upper graduation mark, mm	10 to 15	10 to 30
iv)	Tolerance on capacity, ml	± 0.002	± 0.006
v)	Maximum permissible leakage rate of stop-cock, ml/min	---	0.004

10.1.2 Reagent

10.1.2.1 Xylene or toluene

Saturate the xylene or toluene by shaking with a small quantity of water, and distillate Use the distillate for the determination of moisture.

10.1.3 Procedure

Clean the entire apparatus with chromic acid cleaning solution to minimize the adherence of water droplets to the sides of the condenser and the receiver. Rinse thoroughly with water and dry completely before using. The quantity of material taken for the test is determined by the amount of moisture present (m/m), as indicated below:

Moisture Range	Quantity of Material (Approximately)
Less than 1 percent	200 gm
1 to 5 percent	100 gm
Moisture in excess of 5 percent	Proportionally smaller quantity

Place the specified quantity of material, accurately weighed, in the distillation flask, add an equal volume of xylene or toluene, as desired or at least 100 ml if less than 100 g of the material is used, and swirl well to mix. Assemble the apparatus and fill the receiver with the solvent by pouring it through the condenser until it begins to overflow into the distillation flask. Insert a loose cotton plug at the top of the condenser to prevent condensation of atmospheric moisture within the tube. In order that the refluxing may be under control, wrap the flask and the tube leading to the receiver with asbestos cloth. Heat the flask so that the distillation rate is about 100 drops per minute. When the greater part of the water has distilled over, increase the distillation rate to about 200 drops per minute and continue until no more water is collected. Purge the reflux condenser occasionally during the distillation with 5 ml portions of xylene or toluene to wash down any moisture adhering to the walls tithe condenser.

The water in the receiver may be made to separate from the xylene or toluene by moving the spiral copper wire up and down in the condenser and receiver occasionally, thus causing the water to settle at the bottom of the receiver. Reflux until the water-level in the receiver remains unchanged for 30 minutes and then shut-off the source of heat. Flush the condenser with either xylene or toluene, as required, making use of the spiral copper wire to discharge any moisture droplets. Immerse the receiver in water at about 27^0C for at least 15 minutes or until the xylene or toluene layer is clear, and then read the volume of water.

10.1.4 Calculation

$$\text{Moisture content, percent by mass} = \frac{100 \times V \times D}{M}$$

Where
   V = volume in millilitres of water,
   D = density of water at the temperature at which the volume of water is read, and
   M = massing of the material taken for test.

10.2 Karl Fischer Method

This method shall be used for determination of water content up to 0.5 percent according to procedure prescribed in IS 2362.

10.3 Air-Oven Method

10.3.1 Apparatus

10.3.1.1 Weighing bottle

Squat type, conforming to IS 1574.

10.3.1.2 Desiccator

Containing an efficient desiccant, such as phosphorous pentoxide.

10.3.1.3 Air-oven

Preferably electrically heated, with temperature control device.

10.3.2 Procedure

Weigh accurately about 10 g of the material into a weighing bottle which has been dried, cooled in the desiccator and then weighed. Place the weighing bottle in the air-oven for approximately one hour at 105 ±1°C. Remove the weighing bottle from the oven, cool in the desiccator to room temperature and weigh. Repeat this procedure but keep the weighing bottle in the oven only for half an hour each time until the difference between the two successive weighing does not exceed one milligram.

10.3.3 Calculation

$$\text{Moisture content, percent by mass} = \frac{M_2 \times 100}{M_1}$$

Where
   M_1 = massing of the material taken for the test, and
   M_2 = loss in mass in gm of the material upon drying.

11 DETERMINATION OF SOLUBILITY / MATER IN SOLUBLES

11.1 Solubility in Acid

Prepare 2 percent solution of the sample in 10 percent hydrochloric acid (v/v) or according to the procedure described in the individual material specification. Heat to specified temperature or to about 75 to 80^0 C to dissolve. Observe the opalescence. If the insoluble matter is more than a trace, filter through previously weighed sintered crucible of porosity G4, wash free of acid, dry and weigh.

11.1.1 Calculation

Acid insoluble matter, percent by mass $= \frac{M_2}{M_1} \times 100$

Where
    M_1 =mass in gm of the material taken for the test and
    M_2 =mass in gm of the residue.

11.2 Solubility in Alkali

Prepare 2 percent solution of the sample in 10 percent sodium carbonate or sodium hydroxide solution or according to the procedure given in the individual material specification. Heat to specified temperature of about 75 to 80^0C to dissolve. Observe the opalescence. If the insoluble matter is more than a trace, filter through a sintered crucible of porosity G4, wash thoroughly, dry, weigh and calculate the percentage of the alkali-insoluble matter according to the formula given in **11.1.1**.

11.3 Solubility in Solvent

Dissolve the sample as prescribed in individual material specification in 2 percent concentration in specified solvent. Warm if necessary, and observe the clarity of solution. If the insoluble matter is more than a trace, filter through a sintered crucible of porosity G4, wash thoroughly with the solvent, dry, weigh and calculate the percentage of solvent insoluble according to formula given in **11.1.1.**

12 DETERMINATION OF ASH

12.0 General

Two methods are given. The first method gives the ash left on ignition whereas the second method gives the Sulphated ash left on ignition.

12.1 Method A

Heat a silica or platinum crucible, cool it in a desiccator and weigh. Weigh accurately about 2 g of sample into the crucible and heat it gently on a burner until most of the sample has burnt into ash. Then ignite it in a muffle furnace maintained at about 800° C, until no more carbon particles are visible. Cool in a desiccator and weigh. Heat again in a muffle furnace, then cool in a desiccator and weigh. Repeat the process until constant mass is obtained.

12.1.1 Calculation

Ash, by mass $= \frac{M_1 \times 100}{M_2}$

Where
    M_1 =mass in gm of ash, and
    M_2 =mass in gm of the sample taken for test.

12.2.1 Method B

In this method heating of the material is carried out in the presence of concentrated sulphuric acid and the resulting ash is determined as Sulphated ash.

12.2.1 Reagent

12.2.1.1 Concentrated sulphuric acid (See IS 266).

12.2.1.2 Concentrated nitric acid (See 1S 264).

12.2.2 Procedure

Heat a silica or platinum crucible. Cool in a desiccator and weigh. Then weigh accurately about 2 gm of the sample into the clean, dry crucible. Add to this 1 ml of concentrated nitric acid and a few drops of concentrated sulphuric acid just enough to wet the sample. Cover the crucible partly with a lid, if necessary to avoid spurting. Heat the crucible gently on a small flame till evolution of fumes ceases. Cool the residue, add a few more drops of concentrate sulphuric acid and heat slowly, gradually raising the temperature till evolution of fumes has stopped. Repeat this procedure, namely, the addition of sulphuric acid and heating until the residue in the crucible is free from carbon particles. Put the crucible in a muffle furnace at 800°C for one hour. Cool in a desiccator and weigh. Repeat heating cooling and weighing till constant mass is obtained.

12.2.3 Calculation

Sulphated Ash, by mass $= \frac{M_1 \times 100}{M_2}$

Where
   M_1 =mass in gm of the Sulphated ash, and
   M_2 =mass in gm of the sample taken for test.

13 DETERMINATION OF AROMATIC PRIMARY AMINES

13.0 General

Aromatic primary amines react quantitatively with nitrous acid in acid medium to form diazonium salts. The amount of nitrous acid thus used up may be expressed in terms of amount of the amine present.

13.0.1 For strongly basic amines, such as aminophenol's, halogenated amines, benzidines, toluidine, xylidines naphthylamines, etc, the direct diazotization method is frequently used. Here a standard solution of sodium nitrite is added from a burette to the solution or slurry of the amine in mineral acid maintained cold between 0 to 5°C till end point is reached.

13.0.1.1 The strength of sodium nitrite solution, to be used depends on several factors, such as speed of reaction, sharpness of end point, loss of nitrous acid, etc (*See* Note).

   NOTE — A 0.1 N solution gives poor end point, whereas with 1N solution of sodium nitrite, there is too much loss of nitrous acid

13.0.2 For weakly basic amines, such as naphthionic acid and amino benzoic acid, the indirect diazotization method is frequently used. Here a known excess of sodium nitrite is mixed with the amine solution and then quickly acidified. After allowing time for completion of reaction, excess sodium nitrite is back titrated with a standard solution of an amine.

13.0.2.1 For weakly basic mononitroanilines and halonitroanilines, it is necessary to first dissolve the amine in sulphuric acid, the amine is then reprecipitated by adding ice. The finely precipitated amine is redissolved in hydrochloric acid and then it is titrated against the standard solution of sodium nitrite.

13.1 Direct Diazotization Method

13.1.1 Apparatus

13.1.1.1 Mechanical stirrer

13.1.1.2 Burette

13.1.1.3 Beakers

13.1.1.4 Thermometer

13.1.1.5 Thistle funnel

13.1.2 Reagents

13.1.2.1 Sodium nitrite solution of suitable strength *(see* **13.0.1.1).** This solution shall be freshly standardized against pure sulphanilic acid *foll*owing the procedure given in **13.1.3.**

13.1.2.2 *Concentrated hydrochloric acid — See I*S 265.

13.1.2.3 *Acetic acid — See* IS 695.

13.1.2.4 *Starch iodide test papers*

13.1.2.5 *Potassium bromide solution — 25 percent (m/v)*

13.1.3 *Procedure*

13.1.3.1 Take an accurately weighed sample of an amine, large enough to give about 30 ml titration reading with standard sodium nitrite solution of chosen strength, into a one-litre beaker. Dissolve in 500 ml of water and 50 ml of concentrated hydrochloric acid with heating, if necessary. If an amine contains an acid group, such as sulphonic, dissolve by adding requisite quantity of sodium carbonate and water and then add concentrated hydrochloric acid equivalent to sodium carbonate added and 50 ml more. If the amine is not soluble in dilute hydrochloric acid or dilute sodium carbonate solution, dissolve first in glacial acetic acid and then add hydrochloric acid and water.

13.1.3.2 Immerse the beaker containing sample within about 2.5 cm of the rim in crushed ice and water. Keep the beaker immersed until the temperature is about 0 to 5°C. While stirring mechanically, add through thistle funnel, as shown in Fig. 10, the solution of standard sodium nitrite from the burette as rapidly as the spot test permits keeping the temperature of the solution below 5°C. Test the solution by spotting on starch- iodide paper. If the rate of diazotization is slow, add the solution of nitrite slowly, avoiding a large excess of nitrite at any stage. In many cases where diazotization is slow, it may be considerably accelerated by addition of few' millilitres of potassium bromide solution and sharper end-point may be obtained. The end-point is reached when an immediate faint blue coloured ring appears when spotted on starch-iodide paper and persists for a period of 10 minutes or so without further addition of nitrite solution.

13.1.4 *Calculation*

Aromatic primary amine, percent by mass $= \dfrac{V \times N \times M}{10\ W \times A}$

Where

    $V =$ volume in millilitres of sodium nitrite solution used,
    $N =$ normality of the sodium nitrite solution,
    $M =$ molecular mass of the amine,
    $W =$ mass in g of the sample taken for the test, and
    $A =$ number of amino groups per molecule of the amino

13.2 Indirect Diazotization Method

13.2.1 Apparatus — same as in **13.1.1.**

13.2.2 Reagents — same as in **13.1.2.**

13.2.2.1 Sodium sulphanilate solution

0.5N, Standardize against a standard sodium nitrite solution following the procedure given in **13.1.3.**

13.2.3 *Procedure*

Take an accurately weighed sample which is large enough to give about 30 ml titration reading with standard sodium nitrite solution of chosen strength and transfer to a well-stoppered flask. Add water and sodium carbonate necessary to dissolve and to make the solution slightly alkaline. Add standard sodium nitrite solution in excess but not more than 3 to 5 ml. cool the flask in a ice bath. Add about 50g of ice and as quickly as possible, 50 ml of concentrated hydrochloric acid. Stopper the flask immediately. Mix well, allow the flask to stand for 10 to 15 minutes in the ice-bath and shake the contents of the flasks at intervals. Titrate the excess sodium nitrite solution with standard sodium sulphanilate and add 2 to 3 ml in excess. Finally back titrate the excess sodium sulphanilate with standard sodium nitrite solution to a distinct blue coloured ring test on starch-iodide paper that can be repeatedly obtained during a period of 10 to 15 minutes without further addition of nitrite solution.

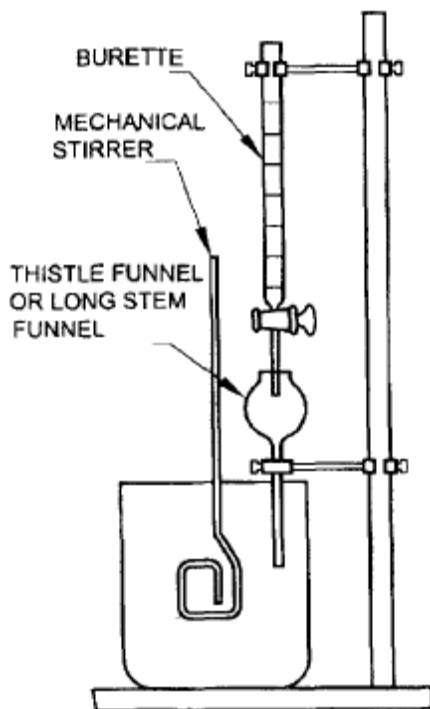**Fig. 10 TITRATION ASSEMBLY**

13.2.4 Calculation

Aromatic primary amine percent by mass $= \dfrac{(V_1 N_1 - V_2 N_2)M}{W \times 10 \times A}$

Where

    V_1 = total volume (initial and final) in millilitres of sodium nitrite solution used,

N_1= normality of sodium nitrite solution,
V_2= volume in millilitres of sodium sulphanilate solution used,
N_2 = normality of sodium sulphanilate solution,
M = molecular mass of the compound,
W = mass in g of the sample taken for the test, and
A = number of amino groups per molecule.

14 DETERMINATION OF COUPLING VALUE

14.0 General

Most of phenols, naphthols, aromatic amines and compounds with active methylene groups couple in either alkaline or acidic medium quantitatively with a diazonium compound and from the consumption of diazonium compound, purity of above compound may be estimated and this estimated value is known as coupling value.

14.0.1 The coupling takes place in the para position on the phenol or amine unless this position is occupied in which case the coupling may take place on ortho position, though the reaction at the ortho position will usually be considerably slower. Some phenols and amines will couple first in para position and then will commence to couple in ortho position. The compounds containing active methylene groups will couple at these positions. The coupling procedures vary somewhat depending on the reactivity of the compounds involved. Some compounds couple in a solution at pH 5, others will couple only in very alkaline solution, the faster the coupling, better the analysis; since the diazonium compound is unstable. The more alkaline the solution, the faster the coupling. However, the more alkaline the solution, the faster the diazonium salt will decompose. In general, salts like sodium acetate and sodium bicarbonate will be sufficiently alkaline for most couplings. Sodium carbonate is sometimes used while sodium hydroxide is seldom resorted to, since it decomposes the diazonium salt too readily.

14.1 Apparatus

14.1.1 *Mechanical Stirrer*

14.1.2 *Beakers*

14.1.3 *Burette* — Amber coloured, jacketed.

14.1.4 *Pipettes*

14.1.5 *Graduated Flasks*

14.2 Reagents

14.2.1 *Tetrazodianisidine Solution*

Dissolve 2 g of dianisidine hydrochloride in 7 ml of hydrochloric acid. Heat, if necessary. Cool to 0°C and titrate with 1 N sodium nitrite solution to just complete the reaction. Make up the solution to 100 ml in a volumetric flask. Store this solution in an ambercoloured bottle at a cool place.

14.2.2 *H-Acid (1-Amino-8-Hydroxy-Naphthalene-3, 6-Disulphonic Acid) Indicator Solution*

Dissolve 0.5 g H-Acid in 100 ml of 1 percent sodium carbonate solution.

14.2.3 *Standard Diazonium Solution*

Either of the following diazonium solutions described in **14.2.3.1** and **14.2.3.2** may be used depending on the pH of the coupling medium.

14.2.3.1 *p-Toluene diazonium chloride standard solution* (0.1 N)

Dissolve 53 g of p-toluidine in 131 ml of concentrated hydrochloric acid and dilute to 1 litre. Add 100 ml of 0.5 N p-toluidine solution into a 500-ml volumetric flask, and cool the flask to 15 to 20°C. Add 50 ml of 1 N sodium nitrite solution maintained at a temperature of 15 to 20°C and dilute the resultant solution to 500 ml. Shake solution to assure thorough mixing. A slight excess of nitrous acid should be indicated on starch-iodide paper. Store the solution in the dark in an ice- bath. After 30 minutes, it is ready for use. Standardize the solution by using resublimed m-toluenediamine or recrystallized 1-phenyl-3-methyl-pyrazolone-5. The solution of diazonium salt should not be used after 5 h because of its instability.

14.2.3.2 *p-Nitrobenzene diazonium chloride standard solution* (0.1 N)

Take 6.9 gm of p-nitro aniline in 500-ml beaker, add 20 ml of concentrated hydrochloric acid and about 50 ml of water. Heat to 85 to 90°C to dissolve completely. Dilute the contents to approximately 200 ml with water. Add about 100 gm ice to cool it to 5°C. Keep the contents under mechanical stirring and to it add previously cooled 50 ml of sodium nitrite solution (1 N) in one lot. Stir for 15 to 20 minutes. It should give positive test on starch-iodide paper and congo red paper. Remove the excess sodium nitrite by adding few ml of sulphamic acid solution. Filter the diazo solution. Make up the volume to 500 ml with ice water in volumetric flask. Store the solution in ice-bath in the dark. Standardize the solution freshly before use. P-Nitrobenzene diazonium chloride solution is preferred for coupling in weakly acidic medium.

14.2.3.3 *p-Chloroaniline diazonium chloride standard solution* (0.1 N)

Take 6.5 g of p-chloroaniline in 500-ml beaker. Add 20 ml of concentrated hydrochloric acid and about 50 ml water and heat to 80 to 85°C to dissolve it completely. Dilute the content to approximate 200 ml with water and add 100 g ice to cool it to 5°C. Keep the content under mechanical stirring and add previously cooled 50 ml of sodium nitrite (1 N) rapidly in one lot. Stir for 30 minutes, cool it externally, if needed to maintain the temperature. It should give positive test on starch- iodide paper and Congo red paper. Remove the excess sodium nitrite by adding few ml of 10 percent (*m/v*) sulphamic acid solution. Filter the diazo solution and make up the volume with ice cold water to 500 ml in a volumetric flask. Store the diazonium chloride solution in ice-bath in the dark. Standardize the solution freshly before use *p*-chloroaniline diazonium chloride solution is preferred in weakly alkaline to a moderately alkaline coupling reaction up to 8 to 9 *p*H.

14.2.3.4 *Standardization of diazonium solution*

Weigh accurately about 0.6 g of resublimed *m*-toluilenediamine or about 0.7 g of recrystallized 1- phenyl-3-methyl-5-pyrazolone into 1-litre beaker. Add 20 ml distilled water. Dissolve with the help of few ml of 10 percent hydrochloric acid or 10 percent sodium hydroxide solution respectively. Clear solution. Add to this 100 ml water. Neutralize with soda ash solution or acetic acid respectively. Add 10 ml of saturated sodium acetate solution and 10 ml of 1 N acetic acid. Cool the solution to about 10°C.

Agitate the solution with a mechanical stirrer and add the diazonium solution from a burette equipped with a water jacket through which water is circulating at about 10°C. The burette or the glass jacket should be of amber glass to minimize any decomposition of the diazonium salt by light. Add the diazonium solution as rapidly as coupling takes place, but never in excess since there is danger of decomposition, which leads to high results. To test for excess diazonium salt in the solution, place a few drops of the solution on a piece of filter paper. About a centimeter away from the edge of the liquid mark, place a few drops of H-Acid indicator .solution. Where the two liquid portions meet on the filter paper, a colour will develop if excess diazonium salt is present.

To test for excess coupling agent (*m*-toluilenediamine or 1-phenyl-3-methyl-.5-pyrazolone) in the reaction mixture place a few drops of tetrazodianisidine hydrochloride solution on a piece of filter paper about a centimeter away from the edge of a drop of reaction mixture, also placed on the filter paper. If a colour is developed, presence of unreacted coupling agent is indicated in the reaction mixture.

Add the diazonium standard solution in portions, depending on the rate of coupling with the particular compound contained in the sample. Test the reaction mixture for excess coupling agent and diazonium salt after each addition. The additions of diazonium solution should be in the increments of 0.5 ml when near the end point. The end point is

taken as the point at which negative test for coupling agent and a negative test for diazonium salt are obtained. When this point is reached, read the burette, add a few drops more of diazonium solution and test for diazonium salt. If a positive test is obtained, take the previous reading. If the reaction product is water soluble and the colour interferes with the spot tests, add sodium chloride to salt it out to show clear end point.

14.3 Procedure

14.3.1 Analyze the samples in the same manner in which the diazonium solution are standardized except that the sample is substituted for the m-toluilenediamine or the pyrazolone. The sample should contain 0.003 to 0.005 moles of coupling material. Also, if the acetate - acetic buffer is not sufficiently alkaline for a satisfactory coupling rate, use 100 ml of 2 N sodium carbonate. Use sodium hydroxide for materials which couple very slowly or for samples soluble only in sodium hydroxide. Use the same base for standardization and analysis.

14.4 Calculation

$$\text{Coupling value, percent by mass} = \frac{V \times N \times M}{W \times 10}$$

Where

   $U =$ volume in milliliters of diazonium solution consumed,
   $N =$ normality of diazonium solution used,
   $M =$ molecular mass of coupling component (sample), and
   $W =$ mass in g of the sample taken for test

   NOTE — for the compounds known to couple at both ortho and para positions, the calculation shall be modified by a factor of 0.5.

15 DETERMINATION OF PURITY OF NITRO COMPOUNDS

15.0 Principle of the Method

Aromatic nitro compounds are quantitatively reduced to amino compounds with zinc dust and from the amount of sodium nitrite solution consumed, purity of nitro aromatic compounds may be calculated.

15.1 Apparatus

15.1.1 *Round-Bottomed Flask*

15.1.2 *Graudated Flask*

15.1.3 *Pipettes*

15.2 Reagents

15.2.1 *Zinc Dust*

15.2.2 *Hydrochloric Acid — See* I5 265

15.2.3 *Sodium Nitrite Solution* – 0.1 N

15.2.4 *Potassium Bromide*

15.2.5 *Starch-Iodide Paper*

15.2.6 *Methanol*

15.3 Procedure

Weigh accurately about 1.0 g of the sample into the round-bottomed flask. Add 25 ml of methanol and shake to dissolve. Place the flask in the heating mantle, fix it with the condenser through which cold water is circulated. Through the other opening add 5 g of zinc dust with the help of a funnel. Then add 20 ml of hydrochloric acid. Close the opening with a ground glass stopper and reflux. When the zinc dust is consumed stop heating and allow the flask to cool. Add another installment of zinc dust and hydrochloric acid and reflux again. Continue the same operation for a third time. Observe the colour of the mixture. It should be colorless or light reddish but not yellow. Cool and filter.

15.3.2 Wash the residue three to four times with distilled water. Collect the filtrate and washings quantitatively. Add 2 g of potassium bromide and titrate against 0.1 N sodium nitrite using starch-iodide paper as an external indicator. The end point is reached when a drop of the reaction mixture placed on the starch- iodide paper strip produces an instantaneous light blue colour which persists for five minutes.

15.4 Calculation

Assay, percent by mass = $\dfrac{V \times N \times M}{W \times 10}$

Where

    V = volume in milliliters of sodium nitrite solution used,
    N = normality of sodium nitrite solution,
    M = molecular mass of the nitro compound, and
    W = mass in g of the sample taken for test.

16 DETERMINATION OF AZO COMPOUNDS

16.0 Principle of the Method

The material is boiled with an excess of titanous chloride solution in presence of hydrochloric acid and the excess titanous chloride solution is back titrated with standard ferric ammonium sulphate solution using ammonium thiocyanate solution as indicator. The reaction is indicated by the following equation:

$$RN = NR_1 + 4\,H \longrightarrow RNH_2 + R_1NH_2$$
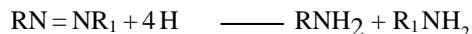
16.1 Apparatus

16.1.1 *Reduction Flask* — as shown in Fig. 11.

FIG. 11 REDUCTION FLASK

N$_2$ OR CO$_2$
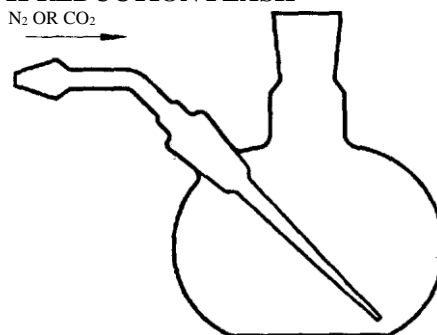
16.1.2 *Graduated Flask*

16.1.3 *Pipettes*

16.2 Reagents

16.2.1 *Concentrated Hydrochloric Acid*

16.2.2 *Concentrated Sulphuric Acid*

16.2.3 *Ammonium Thiocyanate Solution* — 20 percent (*m/v*)

16.2.4 *Titanium Trichloride Solution*

Take 100 ml of 15 percent titanium trichloride solution and filter through a thick pad of glass wool. Add this ttitanium trichloride solution to a previously boiled (to remove dissolved oxygen) mixture of 100 ml of concentrated AR grade hydrochloric acid, and 700ml of distilled water. Mix by passing the current of an inert gas, such as carbon dioxide or nitrogen for some time. Adjust the final volume to 1 litre with distilled water. Store the reagent in a bottle coated with black paint (outside) to protect the solution from sunlight.

16.2.5 *Standard Ferric Ammonium Sulphate Solution*

Dissolve 50 g of ferric ammonium sulphate (AR Grade) in a mixture of 300 ml of water and 6 ml of concentrated (AR grade) sulphuric acid. Dilute to 1000 ml with distilled water.

16.2.5.1 *Standardization of the solution*

Measure accurately about 40 ml of the solution into a glass stoppered flask. Add 5 ml of 5 N hydrochloric acid, mix and add a solution of 4 g of potassium iodide dissolved in 10 ml of distilled water. Stopper, allow to stand for 10 minutes. Then titrate the liberated iodine with sodium thiosulphate solution (0.1 N), adding starch as indicator, as the end point is approached. Correct for a blank run on the same quantities of the same reagents, and calculate the normality. Store the standard ferric ammonium sulphate solution in tight containers protected from light.

16.2.6 *Potassium Iodide* — AR Grade.

16.2.7 *Sodium Thiosulphate* — AR Grade.

16.3 Procedure

16.3.1 Accurately weigh 1.0 g sample and dissolve in cold or hot water or ethyl alcohol or acetic acid as required and dilute to 500 ml with the same solvent at room temperature.

16.3.2 Pipette a 50 ml aliquot in case of water soluble compounds and 10-ml aliquot in case of water insoluble compounds into a 500-ml reduction flask containing 50 ml of dilute hydrochloric acid and 50 ml water which have been previously brought to boil. Start passing carbon dioxide gas over the solution and heat to boiling again. Pass carbon dioxide gas in the storage bottle containing titanium trichloride solution and immediately draw 50 ml titanium trichloride solution by pipette through which carbon dioxide gas has been passed previously, and add it to the dye solution. Note the time when titanium trichloride solution is completely added. Boil the contents of the flask for 10 to 15 minutes for complete reduction. Then cool it in an ice- bath maintaining a continuous flow of carbon dioxide throughout the experiment. Add 10 ml of ammonium thiocyanate indicator solution and back titrate the mixture against standard ferric ammonium sulphate solution to a permanent colour change. Record volume of ferric ammonium sulphate solution as *A*.

> NOTE — This reading should preferably be not less than 10 ml.

16.3.3 Determine blank reading exactly following the procedure as stated in **16.3.2** with 50 ml of water or 10 ml of solvent in place of sample solution and taking the same amount of all the reagents and the same reduction time. Record the volume of titre as *B*.

16.4 Calculation

Azo compound, percent by mass $= \frac{(B-A) \times N \times M}{4 \times E \times W \times 10}$

Where

   A = volume in millilitres of ferric Ammonium sulphate solution required for the sample,
   B = volume in millilitres of ferric Ammonium sulphate solution required for the blank,
   N = normality of ferric Ammonium sulphate solution,
   M = molecular mass of the material,
   E = number of azo groups present in the compound, and
   W = mass in g of the sample present in the aliquot taken for the test.

17 DETERMINATION OF ASSAY/PURITY OF DYE INTERMEDIATES AND ESTIMATION OF THEIR IMPURITIES

17.0 Four methods, namely, Gas Liquid Chromatography (GLC), High Performance Liquid Chromatography (HPLC), Thin Layer Chromatography (TLC) and Paper Chromatography have been stipulated for the determination of purity/assay of various dye intermediates as well as estimation of the impurities present.

17.1 Gas Liquid Chromatography Method

17.1.1 *Principle of the Method*

In GLC the mobile phase is an inert gas and the stationary phase is either a solid or a fixed liquid coated on a solid support with the help of solvents. The carrier gas drives the mixture through the column wherein the solutes partition between the gas and the stationary liquid. In addition to partition, adsorption of the solutes on the solid support and at the gas liquid surface is also a factor in many cases. Differential migration results if the distribution coefficients of the solute are different enough to allow the sorbent to selectively retard them. However, the mobile phase selection and variable temperature conditions aid in achieving resolution in gas Chromatography.

17.1.2 *Apparatus*

Any gas Chromatography with:

   a) High pressure gas cylinder for carrier gas;
   b) Pressure regulator and flow control valves to obtain a uniform rate of gas flow;
   c) Injection port with self-sealing septa for injecting the sample;
   d) Oven which can accommodate glass and stainless steel columns;
   e) A high sensitive detector having a linear response over a large concentration range and relatively insensitive to flow and temperature variations, like Flame ionization and Thermal conductivity; and
   f) A recorder or an integrator to record the signal generated by the detector and computing the area under each peak.

17.1.2.1 *Injection* system

The injection port is kept hot enough to vaporize the sample rapidly but not so hot as to decompose it. The temperature of 10 to 50°C above that of the column is often recommended.

17.1.2.2 *Column selection*

The choice of the column packing is the most critical in GC. The column can be one of two types, either packed or capillary. Packed columns contain an inert solid support with a thin coating of liquid phase. Small and uniform particle sizes (40-60 to 100-120 mesh) give the highest efficiency. The support is usually diatomaceous earth according to their polarity. The, polar ones (for example, FFAP, Carbowaxes, etc.), and the non-polar ones (for example SE—30, OV-1, Squalene, etc.). Separations are best achieved by matching the solute and liquid types. Some liquid phases react chemically with certain solutes and are very selective for these compounds. Selective liquid phases give larger differences in retention time and require shorter columns to achieve a desired separation. Capillary columns can be used depending upon the compounds to be separated.

17.1.2.3 *Column temperature*

Lowering the oven temperature usually improves resolution while increasing the temperature will decrease the analysis time. The temperature chosen for an isothermal separation must therefore be a compromise. If the liquid coating is reduced a lower temperature, can be used, a condition which could be beneficial for the separation of heat-labile solutes. The column temperature should never be high enough to either change the sample or to decompose or cause excessive vaporization of the stationary liquid. A column temperature around the average boiling point of the major component of the mixture can be tried. For wide boiling range mixtures, better resolution at the low end and faster elution at the higher end is obtained if temperature programming is employed. This method which usually involves a controlled linear increase in temperature during a run, sharpens the later peaks and makes the sensitivity of the analysis the same for high and low boiling components.

17.1.2.4 *Detector*

The most widely used detectors are:

   a) Flame Ionization Detector (FID), and
   b) Thermal Conductivity Detector (TCD).

17.1.2.5 *Flame Ionization Detector*

Components in the column effluents are ionized by burning in hydrogen-air flame. This allows the gas in the detector to conduct an electrical current. Above the flame, a collector, electrode, to which a DC potential is applied, measures this conductivity. The combustion of organic compounds increases the conductivity and the resultant current is amplified and fed to a recorder or a computing integrator. The detector is highly sensitive and its range of linear response to increasing concentration is very wide. It responds to almost all compounds, except inorganic gas, and Carbon- disulphide. The response is based on carbon weight percentage in the molecule.

17.1.2.6 *Thermal Conductivity Detector*

This detector consists of two identical metal cells, each containing a tungsten, rhenium-tungsten or gold sheathed wire filament or a thermistor. The column effluent flows through one-cell and pure carrier gas with a high thermal conductivity (hydrogen or helium) through the other. The temperature of the wires, and their resistance, depends upon the composition of the gas flowing over them. When pure gas flows through both cells, the temperature are same and the detector is balanced. This is initially arranged as a part of a balanced Wheatstone bridge circuit; so that the resistance increase will cause an imbalance in the bridge and give rise to an electrical signal which is amplified and fed to a recorder or computing integrator.

17.1.3 Quantification

Calculate the peak areas of individual components and quantification can be done by the following methods:

   a) Area percentage,
   b) Area normalization,
   c) Internal standard, and
   d) External standard.

17.1.4 Typical analysis by gas chromatography and calculations by area percentage is as follows:

Column material	stainless steel
Length	2 m.
Outer Diameter	1/8 "
Column	10 percent x E60 on chromosorb WAW/DMCS (MESH 80/100)
Carrier gas	Nitrogen
Flow Rate:	30 ml/min

Injector temperature	200°C
Detector temperature	200°C
Oven temperature	Initial temperature: 50°C isothermal for 3 minutes and
	Final temperature: 100°C with a programming rate of 3°C per minute
Detector:	Flame ionization
Chart speed	5 mm/min
Sample size	0.5 micro litter of 10 percent solution in solvent.

17.1.4.1 *Procedure*

Conduct the flow of the carrier gas and inject 0.5 micro litter of the sample at the injection port where it is vaporized and well mixed with the carrier gas. It then passes into the cinematographic column wherein the vaporized constituents of the sample are separated by the virtue of their differing interaction with the stationary phase. For an efficient separation, the column should be maintained at the temperature suggested through the time required for the resolution of the constituents. As each component enters the detector, it gives a signal corresponding to the amount of the particular component leaving the column. The detector signal after amplification is transmitted to the integrator or a recorder for plotting the curves. From the specific area under various peaks corresponding to specific components the quantities of different components are determined.

17.1.4.2 *Calculations*

Calculate the peak area of individual component and calculate the amount of each component as given below:

A_1 = area under peak 1,
A_2 = area under peak 2, and
A_3 = area under peak 3, etc.

$$\text{Assay, percent} = \frac{A_1 \times 100}{A_1 + A_2 + A_3 + --- An} \times 100$$

n = number of peaks.

17.2 High Performance Liquid Chromatography

17.2.1 *Principle of the Method*

HPLC is a liquid chromatographic technique involving a liquid mobile phase and a solid stationary phase. The high performance here refers to rapid separation and quantification of many components at trace level and involving only few microliters of sample volume. This process is achieved, in principle, by having very small particles with a diameter of few micrometers, typically in the range 5 to 10 μm, packed uniformly into small columns of stainless steel (typically 10 to 25 cm in length and 4-5 mm i.d.) which can withstand high pressures. A high pressure solvent delivery system is required to have a reasonable flow through the column and is achieved with the help of a peristaltic pump. The sample is injected on the top of the column (sample volumes typically 5 to 25 μl) and eluted out by pumping the desired solvent under pressure. The eluent passes through a flow through detector (typical capacity 20 μl) which continuously monitors the concentration of separated components. The detector then feeds the data to a recorder and a plot of detector response against time is recorded and is called a chromatogram.

In the conventional liquid chromatography, a polar stationary phase is used and the mobile phase is non- polar. This is known as normal phase in HPLC when a polar stationary phase is used. In the reverse phase chromatographic process (R_{pc}), the stationary phase used is non-polar and a polar mobile phase is used. The order of elution of the components in the reverse phase is opposite of normal phase Chromatography, that is, the polar component is eluted first.

17.2.2 *Equipment for HPLC*

The essential features of a liquid and cinematographic system consists of: (a) A solvent delivery system consisting of a pump, pressure and flow controls and a filter in the inlet to remove suspended particles; (b) A sample delivery system; (c) The column; (d) The detector; and (e) Data handling device.

The entire system is available commercially and the main characteristics of the individual components or the system are briefly given below. However, the manufacturer's instructions must be consulted for exact details of the particular system.

a) *Solvent delivery System*

The solvent is to be delivered to the system at high pressure with a constant flow and is achieved by using a pulse-free pump. Variation of flow rate generally affects the separation speed and the retention time. In general, the commercially available pump systems easily meet the requirements of flow rate and pressure. A combination of more than one pump can be used for using two or more solvents for separation. Both constant volume and constant pressure pumps are available commercially and the most commonly used being single or multihead reciprocating pumps of constant volume type.

b) *Sample delivery system*

Introduction of sample on the column is generally done either by using a syringe or by using a sample delivery valve. Septum injectors allow sample introductions by a high pressure syringe through self sealing elastor septum. However, the most common system used is micro volume sampling valves which give better reproducibility into pressurized columns. The sample is introduced into an external loop in the valve at atmospheric pressure and by appropriate rotation the same is introduced into the mobile phase. The volume of the sample introduced normally is small and ranges in the 5 to 25 μl range.

c) *The column*

The columns employed in HPLC are normally made from precision bore polished stainless steel tubing with typical dimensions of 10 -30 cm long and 4 to 5 mm i.d. The stationary phase is retained at each end by thin stainless steel frits with a mesh size of 2 μm or less. The packings consist of small rigid particles (diameter less than 10 μm and with a narrow particle size range) are normally used as column packing. To overcome some of the problems associated with conventional silica gel or coatings of other materials on porus packing, the stationary phase can be chemically bonded to supporting material silica gel are reacted with substituted chlorosilanes and di and tri chlorosilanes producing either monomeric or polymeric bonded phases. In Analytical HPLC the most important bonded phase are the non-polar **C-5** and **C-18** type in which the modifying groups are either octyl or octadecyl hydrocarbon chain.

d) *Detector*

The function of detector in HPLC is to monitor mobile phase as it comes out of the column. As in GLC, there is no universal detector in HPLC. The available HPLC detectors are based on either bulk property of the mobile phase or on the property of components to be separated. The bulk property detectors are based on the principle of measuring the differences of the property of added components in the mobile phase compared to the mobile phase alone. These type of detectors, in principle, can be used for all types of applications but the sensitivity is very poor. The detectors based on measurement of refractive index and conductivity fall in this category. The second type of detectors based on the property of components to be separated, are ultraviolet / visible spectrophotometric and fluorescence detectors which is based on the property of ultraviolet/visible light absorption and light emission in the case of fluorescence detectors. Both UV and fluorescence detectors have high sensitivity and their response is independent of nature of the mobile phase. The most commonly employed detector is UV detector, both single and double beam instrument are commercially available and cover the wavelength range of 210 - 800 nm.

e) *Data handling device*

The quantitative analysis by HPLC requires that a relationship is established between the magnitude of the detector signal and the concentration of a particular product in the sample. The plot of response of detector signal against time, leads to different peaks for different components present in the sample. The peak height and peak area are both

related to the concentration of the component present through the relative response of the detector for that particular component. Quantitative analysis can be done by first establishing the response of the component relative to that of a similar compound added in a known amount as an internal standard. Alternatively the system can be calibrated for each component by injecting known amounts of pure components separately (external standard quantification).

17.2.3 *General Procedure for Operation*

a) *Phase selection*

The LC mode of separation to be followed is normally chosen according to the solubility of the substance, its molecular weight and ionic behavior. Organic samples that are soluble in hexane or chloroform but not in water can be separated in the reverse phase mode whereas water sensitive substances and substances soluble in organic solvents can be separated by the normal phase mode.

b) *Column selection*

The type of the column required for a particular separation depends on the polarity of the substance to be separated. Silica gel columns are most commonly employed in the normal phase and bonded silica gel phases are commonly employed in the reverse phase.

c) *Solvent selection*

The solvent or the mobile phase used must be specially purified and free from all impurities. The presence of dissolved gases in the solvent also affect the separation process and hence must be removed by degassing. Separations sometime can be improved by using mixture of more than one mutually compatible solvents. By using gradient mixers the composition of mobile phase can be continuously changed during analysis. The flow rate of the solvent also affects separation and should be adjusted to obtain a good separation in a reasonable time.

d) *Sample preparation*

The sample to be analyzed normally should have some solubility in the mobile phase chosen and normally the sample is dissolved in the mobile phase. If this is not possible, a small amount of polar solvent such as dimethyl sulphoxide and dimethyl formamide (DMSO or DMF) can be used to dissolve the substance followed by dilution with mobile phase to required concentration before using. Insoluble and suspended particles must not be present and is to be removed by filtration.

e) *Separation*

The extent of separation that can be achieve depends on a number of operational parameters of the system including the nature of stationary phase, nature of mobile phase, temperature and flow rate. Good separation between components is measured not only by their distance between each other but also by the breadth of the peaks. Peaks must be sharp and symmetrical and the operation parameters must be adjusted so as to set a good separation and sharp peaks.

Typical analysis procedure (*reverse phase*)

Reagents	HLPC grade solvent double distilled water
Apparatus	HPLC chronographs equipped with a reverse phase column. Typical parameter for water's make HPLC system are given below:

Column	Stainless steel 125 mm length, 4.9 mm i.d. Nucleosil C 18.5 μm particle size
Temperature	25°C
Flow rate	1 ml/min
Injection Vol	5 μl

Chart speed (Detector)	5 mm/min
Attenuation	1.0
Wavelength (Detector)	254 nm

17.2.3.1 *Procedure*

a) *Preparation of sample solution*

Weigh accurately about 0.03 gm of the sample and transfer to a 100 ml volumetric flask and dissolve the sample in mobile phase and make up the mark with the mobile phase, stopper and shake well.

b) *Preparation of standard solution*

Weigh accurately 0.03 gm of the pure sample (standard) and transfer to a 100 ml volumetric flask and prepare the solution by making up to mark with the mobile phase stopper and shake well.

c) *Determination of purity of substance*

Start the pump and allow the mobile phase to flow through the column and adjust the flow rate and detector parameters. When a steady base line is obtained inject 5.0 µl of each of standard solution and also inject 5.0 µl of sample solution. Compute the area of each peak and calculate using the following equation:
Percent, purity =

$$\frac{Concentration\ of\ standard \times Area\ of\ peak\ of\ sample\ \times Purity\ of\ standard}{Concentration\ of\ sample\ \times Area\ of\ peak\ of\ standard}$$

17.3 Thin Layer Chromatography

17.3.1 *Principle of the Method*

In Chromatography techniques, physical phenomena of adsorption and partition is employed; only practical techniques are different for its use in different forms as paper, column, high pressure Liquid and gas Chromatography. In Thin Layer Chromatography (TLC) stationary phase consists of a thin layer of sorbent (silica gel, alumina, cellulose powder, etc) coated on an inert, rigid, backing material such as glass plate, aluminium or plastic so that separation process occurs on a flat essentially two dimensional surface. TLC is widely used for qualitative analysis; it does not provide quantitative information of high precision and accuracy. With the development of High Performance Thin Layer Chronograph (HPTLC), separations and quantitative measurements has become possible.

17.3.2 *State of the Art of TLC*

TLC technique consists of preparation of plates, sample application, development of plates and location of spots.

17.3.2.1 *Preparation of plates*

In TLC, variety of coating materials are available, but silica gel is most frequently used. A slurry of adsorbent (silica gel, alumina, cellulose powder, etc) is spread uniformly by means of commercial spreader or manually. The recommended thickness of adsorbent layer is 150- 250 mm. After air drying overnight or even drying at 80-90°C for about 30 min, the plate is ready for use. The plates can be also prepared by dipping into slurry of adsorbent in volatile solvents (like chloroform) and air drying.

Precoated ready plates are also commercially available which can be cut to any size required.

17.3.2.2 Sample application

The accuracy and precision with which the sample spots are applied is very important when quantitative analysis is required. The origin line, to which the sample solution is applied is usually located 2-2.5 cm from the bottom of the plate. Small volumes of 1, 2 or up to 5 μl are applied using calibrated syringe. Care must be taken to avoid disturbing the surface of the adsorbent as this causes distorted shapes of the spots on subsequently developed chromatogram. Use of low boiling solvent clearly aids drying and helps to ensure compact spots.

17.3.2.3 *Development of plates*

TLC chromatograms are developed by ascending techniques in which plate is immersed in developing solvents to a depth of 0.5 cm. The developing chamber is preferably lined with sheet of filter paper which dip into the solvent in the base of the chamber, for ensuring the chambers saturation with solvent vapours. Development is allowed to proceed until the solvent front has travelled the required distance (80 percent of the plate length). The plate is then removed from the chamber and solvent front is marked with pointed object.

Plate is allowed to dry in an oven or otherwise taking into consideration heat and light sensitivity of separated compound.

17.3.2.4 *Location of spots*

Positions of separated solutes can be located by various methods. Coloured substances can be directly viewed, while colourless solutes may usually be detected by spraying the plate with appropriate reagent which produces coloured areas in the regions which they occupy. Some compound fluorescent in U.V. (Plate required will be HF-254) light may be located in this way. Visualization of colourless separated solutes is also achieved by placing the plate in an iodine vapour chamber for few seconds. Iodine forms reversible complexes with most of the organic substances, thus dark spots are developed in those areas containing sample material.

17.3.2.5 *Rf values*

Elution characteristics are reported by Rf values. The Rf value is a measure of the travel of substance on the plate during the chromatogram relative to the solvent front. The values are defined by length of migration of substance divided by the solvent front distance.

17.3.2.6 *Quantitative evaluation*

This is done by removing separated components by scraping relevant adsorbent portion, extracted in suitable solvent and after removal of solvent analyzed by appropriate technique (UV, GLC) more precisely by measuring photo densities of spots directly on TLC using densitometer.

17.3.3 *Conclusion*

TLC is used in number of applications. The speed of technique makes it quite useful for monitoring large scale chromatograms. Analysis of fractions can guide decisions on solvent elution sequence. By comparing the colours of unknown (developed by using specific spray reagents) against various concentration of calibrated samples, impurities and purities can be qualitatively predicted (for example, ONPT impurity in MNPT).

17.3.4 *High Performance Thin Layer Chromatography*

17.3.4.1 *Principle of the method*

In recent years Quantitative Thin Layer Chromatography (QTLC) has gained importance. In modern analytical laboratories. TLC had become an instrumental technique for precise estimation of purities and impurities. After availability of TLC systems "high resolution adsorbent materials, the innovative approach of TLC appeared in the form of HPTLC (High Performance Thin Layer Chromatography). HPTLC differs from conventional TLC in a number of ways but mainly in the size of the particles used in the manufacture of plates and precise instrumental technique to get the quantitative results. Unlike in GLC, 100 percent sample is accountable in TLC.

On HPTLC layers, chromatography takes place in the fast capillary flow range of developing solvents. The advantages of HPTLC over conventional TLC are: (a) better separation efficiency, (b) better economy with very low running costs, (c) very simple sample preparation, (d) unknown samples chromatographed and quantitatively analyzed in very short time, and (e) applications in diverse fields with impurity profile and finger printing.

For HPTLC the particle size is usually between 5 μm and 10 μm with much tighter control over the size distribution. Layers are 0.1 to 0.2 mm thick with rapid separation using development distance of 5-10 cm. The small particle size and more uniform nature of silica gel results in greater chromatographic efficiency than found in ordinary TLC. In TLC detection is based on spiny reagent and use of plates impregnated with fluorescent indicators but in HPTLC for quantitative estimations. TLC scanner is used, which operates in U.V. visible modes. This scanner can measure absorbance, fluorescence and capable of obtaining spectra of individual spots with precise control in all stages of sample application to Chromatography through innovative instrumentation the importance of HPTLC, has potentially increased which also complements other chromatography methods.

17.3.4.2 *State of the art of HPTLC technique*

Sample application, development of chromatogram, densitometric chromatogram evaluation and TLC scanner are primary stages involved in getting quantitative results.

17.3.4.2.1 *Sample application*

Sample can be applied in band or spot made. Uniform sample applications on plate provides optimum conditions for densitometric measurements. Sample can be applied manually or by using automatic TLC samplers. Automatic samplers make the analysis more reliable. 0.1 to 1.0 μg sample for spot applications and 0.5 to 5.0 μg for bond applications is recommended on HPTLC plates. Different concentrations of calibration standards against unknown are applied for QTLC.

17.3.4.2.2 *Chromatogram development*

Very small amount of mobile phase (20 ml for 20 cm × 20 cm plate and 4 ml for 10 cm × 10 cm plate) is sufficient for development of plates. Plate is developed in the solvent chamber with pre-equilibrium mobile phase vapours. Low cost reagent grade solvents can be used for chromatogram development only if solvent mixtures behave like a one component solvent. Gradient elutions can also be done by repeatedly developing the chromatogram with ascending order of solvent polarities. Automated multiple development system (AMD) can be used for reproducible gradient elutions. Gradient elution provides separation of widely differing polar substances on chromatogram. New dimension in chromatographic resolution can be obtained by reversed phase gradient elution.

17.3.4.2.3 *Densitometric chromatogram evaluation (DCE)*

Densitometric chromatogram evaluation consists of four steps: Scanning, integration, calibration and result computation. Methods and raw data created and stored can be retrieved for later use at any time.

DCE is done in the reflectance mode. Diffusely reflected light is measured by the photosensor and the difference between the optical signal from the sample free background and that from the sample zone is correlated with the amount of respective fraction of calibration standards chromatographed on the same place. Densitometer scans in a linear way by absorbance and fluorescence with a spectral range from 200 to 800 nm. Computing integrator consists of simple personal computer with software tailored for TLC evaluation.

Absorption spectra as well as fluorescence excitation spectra are measured and recorded. Multiwavelength scanning also permits optical resolution of fractions insufficiently separated by chromatography which is applicable everywhere.

Scanning is done in direction of chromatography. Scanner searches first in the *Y* direction (direction of chromatography) until a signal maximum is detected; then, at this *Y* position in the *X* direction until that maximum is found. Only then is the quantification run of that fraction performed.

17.3.4.3 *Conclusion*

Thus by doing various combination of instrumental techniques, valuable information is obtained through High Performance Thin Layer Chromatography. Almost all dyes and intermediates are analyzed using quick HPTLC method with affordable running cost to small laboratories. HPTLC is a powerful and valid method of quantitative analysis and simultaneously a most valuable partner in multidimensional separation technique. HPTLC systems comply with all modem GLP requirements.

Following general procedure is involved in development and validation of a new method:

Step l - Optimization of the chromatographic procedure,
Step 2 - Create statistical record on reliability and
Step 3 - Implement routine evaluation

All these three steps are depicted in following block diagram: (*see* Fig. 12)

17.4 Paper Chromatography

17.4.1 *Principle of the Method*

Paper chromatography is a form of partition chromatography in which stationary phase is a thin liquid film adsorbed on the surface of an essentially inert support. Mobile phase may be either a liquid (liquid - liquid partition chromatography) or a gas (gas- liquid partition chromatography or gas chromatography - GLC). Separation depends largely upon partition between the two phases. Paper chromatography is an important example of partition chromatography in which fine quality filter papers serve as a support by the cellulose molecules for the immobile liquid phase.

17.4.2 *State of the Art of Paper Chromatography*

Paper chromatography consists of spotting samples on ready papers, developing in mobile phases by ascending or descending techniques and location of spots and calculating *Rf* values and evaluation against standards either qualitatively or quantitatively.
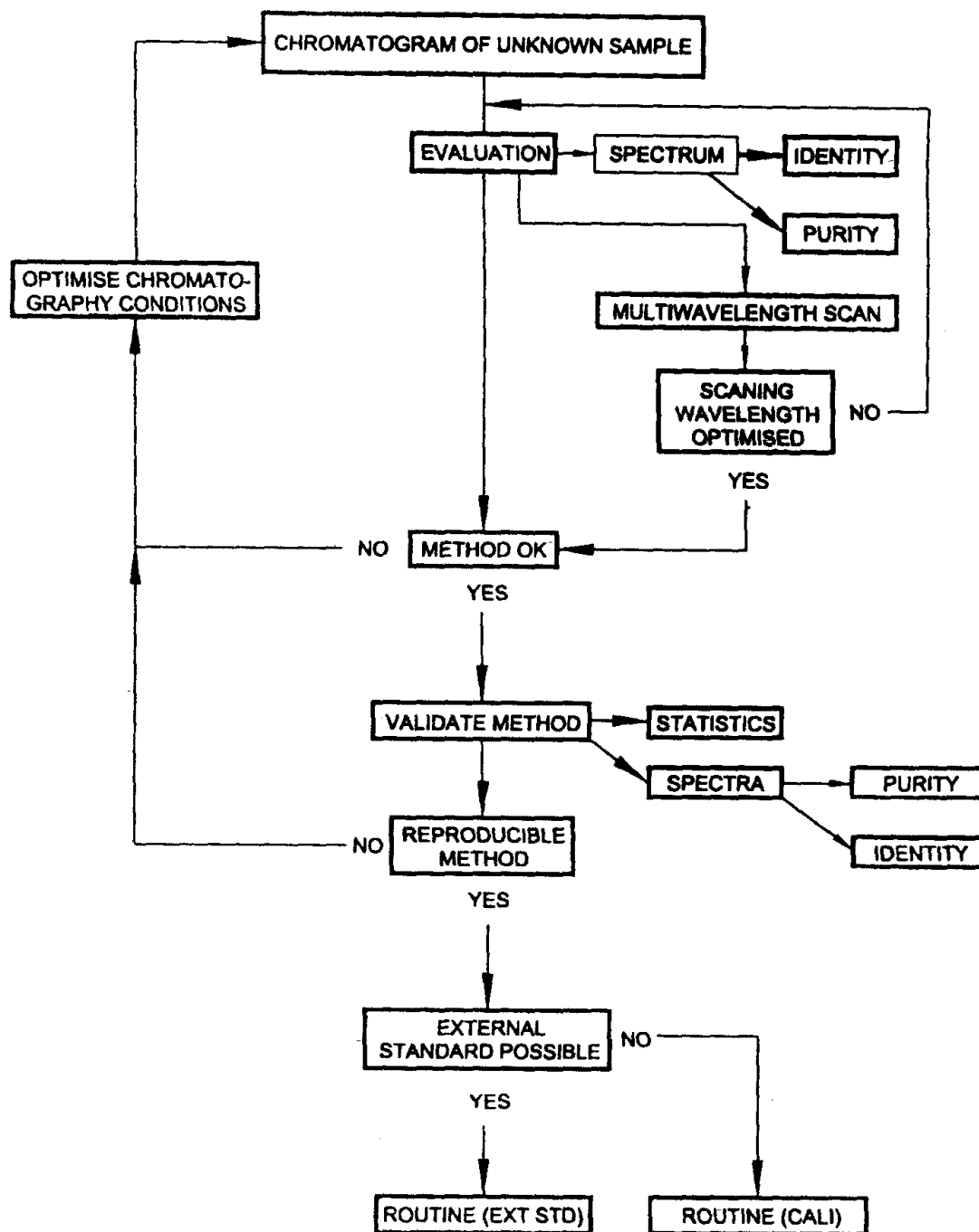
FIG. 12 BLOCK DIAGRAM FOR HPTLC

17.4.2.1 *Selection of stationary phase*

Different grades of filter papers are used (for example, zero and 1 to 4 Number Whatman Papers). When speed of development is important in that case, Paper No. 3 is suitable. No. 1 is most suitable for general use. When heavy loading and good resolution is required, thick quality No. 20 is used. Modified cellulose papers such as silicone treated papers are useful for non-aqueous phases for reversed phase chromatography. Ion exchange cellulose papers are used for separation of ionic-organic and inorganic substances.

17.4.2.2 *Selection of mobile phase*

Generally, mixture of one or more organic solvents and water is used. Water immiscible organic solvents like phenol, acetic acid are used as one of the organic components to increase the proportion of water in mobile phase. From two-phase system, settled organic phase, saturated with water is used as mobile phase. The aqueous layer being used to saturate the atmosphere of the vessel in which chromatogram is subsequently developed.

For getting good resolution in chromatography of amphoteric substances like amino acids, the spotted paper is dipped into aqueous buffer solution, air dried and this paper pre-equilibrated with buffer solution is then developed.

17.4.2.3 *Sample application*

Drops of solution of substances to be chromatographed are individually applied to the paper by means of a capillary tube. Spots are applied with accuracy and precision with calibrated syringes in quantitative analysis. Spotted paper is developed with mobile phase (downward by gravity — descending technique or upward by capillarity — ascending technique).

14.4.2.4 *Development of chromatogram*

Chromatogram is developed in mobile phase either by descending or ascending technique. When solvent has travelled required distance, the paper is removed from container, solvent front position marked and paper is dried. If spots are not coloured, their location is determined by spraying with suitable chromogenic reagent.

Rf value is calculated as:

$$Rf = \frac{Distance\ moved\ by\ substance}{Distance\ moved\ by\ solvent\ front}$$

In ascending technique, paper is supported vertically in a closed jar. In descending technique, specially designed jar is used. The paper is supported at the top of the tank and which contains the mobile phase. Paper end is held by glass rod. Mobile phase flows regularly and even down the paper. Descending technique may be used with advantage when a two phase solvent system is to be employed. In such cases aqueous phase is placed in bottom of jar and allowed to saturate the atmosphere. Paper loaded with sample is equilibrated and then mobile phase is poured at top lid for development of paper.

17.4.2.5 *Quantitative evaluation*

This is done by removing separated components by cutting relevant adsorbent portion of paper extracted in suitable sol vent and after removal of solvent, analyzed by appropriate technique (UV, GLC) more precisely by measuring photodensities of spots directly on paper using densitometer.

17.4.3 *Applications*

17.4.3.1 *Separation of amino acid by descending technique*

Hydrochloride of amino acid solutions are made in water. Spotted papers are developed by solvent (Butanol: Glacial acetic acid: Water 4:1:5). Paper is sprayed with ninhydrin (2 percent solution in butan-2-ol) and develop colour by heating paper in oven for 5-10 minutes at 100°C. Amino acids ate identified based on *Rf* values in comparison with standards.

17.4.3.2 *Separation of monosaccharides by descending technique*

Monosaccharide solutions are made in water. Spotted papers are developed by solvent (butan-1-ol, acetone, water 4:5:1). Spray the paper with an ethanolic solutions of naphtho-resorcinol. Paper dried in oven at 100°C for 5-10 minutes till spots become visible. Unknowns are identified knowing the *Rf* values.

17.4.4 *Special Applications*

Complex mixtures having close *Rf* values may not be separated by above ascending or descending techniques. In such case two dimensional paper chromatography is used with advantage. Single spot of the mixture is applied near to one corner of paper and the chromatogram is developed in one direction as usual. The paper is dried and chromatogram is redeveloped in second solvent system so that the direction of flow is at right angles with respect to the first. In both solvent systems, components have wide range of *Rf* values achieving good separation. Such component is characterized by two *Rf* values.

17.4.5 Conclusion

Paper chromatography is used for the separation of number of organic compounds-ionic, organic and inorganic compounds. As compared to TLC, this technique is time consuming; but the components having very close Rf values can be separated by two dimensional technique which is not possible in TLC. Quantitative estimations can be done using standard substances.