QUALITY CONTROL METHODS FOR MEDICINAL PLANT MATERIALS

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PREFACE

Plant materials are used throughout developed and developing countries as home remedies, over-the-counter drug products and as raw material for the pharmaceutical industry. Since they represent a substantial proportion of the global drug market it is vital that internationally-recognized guidelines exist for assessment of their quality. A number of World Health Assembly resolutions, — WHA30.49 (1977), WHA31.33 (1978), WHA40.33 (1987), WHA42.43 (1989), — emphasized the need to ensure the quality of medicinal plant products by using modern control techniques and applying suitable standards. These guidelines are designed primarily for national drug quality control laboratories in developing countries to complement the The International Pharmacopoeia which provides quality specifications for only those few plant materials which are included in the WHO Model List of Essential Drugs (1). It does not constitute a herbal pharmacopoeia, but a collection of test procedures to support the development of national standards based on conditions of the local market with due regard to existing national legislation or/and national/regional norms. For specifications and standards, including those related to the food industry, see, in particular, the references and bibliography at the end of the text.

The present collection of test methods has been compiled as a preliminary guideline. They represent the state-of-the-art, and further improvements and additional tests will be incorporated at a later stage. The text will also be regularly updated to reflect developments on the work being carried out at national and regional levels. A list of projects will be kept under review to improve and expand this collection of tests and methods including replacement of toxic reagents, etc. It is expected however that their realization may take some more years.

In addition to the test methods, some suggestions regarding general limits for contaminants are also included. They should be considered as a basis for establishing national limits. WHO is not presently able to recommend limits of contaminants since these are too diverse and there is a lack of global consensus. For instance, the draft proposal for limits published in Pharmeuropa vol. 5, No. 1 March 1993 for some of the pesticides is far more restrictive than the one proposed here.

The test procedures are not framed to take account of all possible impurities. It is not to be presumed that any unusual substance which is not detectable by means of the prescribed tests is to be tolerated if common sense and good pharmaceutical practice require that it be absent.

The analysis of medicinal plant materials is not restricted to those methods discussed or recommended in this text and many techniques similar to those used for the analysis of synthetic drugs are also frequently implemented, (e.g. volumetric analysis, gravimetric determinations, gas chromatography, column chromatography, high performance liquid chromatography and spectrophotometric methods). Details of all these methods can be found in The International Pharmacopoeia, 3rd edition.
GENERAL NOTICES

General considerations

The metric system is used throughout the text. All temperatures are expressed in degrees Celsius (°C).

Tests are normally carried out at room temperatures (between 15 and 25 °C, or up to 30 °C in some climatic zones), unless otherwise indicated.

Any glassware used in the tests should be of suitable quality. Graduated and volumetric vessels should be calibrated at room temperature.

When a water-bath is referred to in the text, boiling water of about 100 °C is to be used, unless a specific water temperature is given.

Unless otherwise specified, all solutions indicated in the tests are prepared with distilled or demineralized water of adequate purity.

Nomenclature

Reagents and solutions used must conform to the requirements specified under "Reagents and test solutions" and are designated as follows:

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>Reagents</td>
</tr>
<tr>
<td>TS</td>
<td>Test solutions</td>
</tr>
<tr>
<td>VS</td>
<td>Volumetric solutions</td>
</tr>
</tbody>
</table>

Systematic names of plants or microorganisms are written in italics.

Precision

a) Quantities and volumes

The quantities and volumes of substances, materials and reagents to be used in the tests have to be measured with adequate precision which is indicated in the following way:

- a value of 20.0 is not less than 19.5 and not greater than 20.5,
- 2.0 is not less than 1.95 and not greater than 2.05,
- 0.20 is not less than 0.195 and not greater than 0.205.

b) Temperature measurement

Temperature measurement is indicated in a manner similar to that given for the quantities and volumes.

Storage conditions given in general terms refer to the following equivalent temperatures:

- in a refrigerator: 0 to 6 °C
- cold or cool: 6 to 15 °C
- room temperature: 15 to 25 °C, or up to 30 °C depending on climatic zones.

c) pH values

pH values are indicated in a manner similar to that given for the quantities and volumes.
Calculation of results

The results of tests and assays should be calculated to one decimal place more than indicated in the requirement and then rounded up or down as follows:

- if the last figure calculated is 5 to 9, the preceding figure is increased by 1;
- if it is 4 or less, the preceding figure is left unchanged.

Other calculations – for example, in the standardization of volumetric solutions – are carried out in a similar manner.

In the determination of ashes, extractable material, bitter and haemolytic activities, tanninoids, swelling index and assays, calculations must be made with reference to an undried sample.

If the material must be dried before it can be reduced to a powder for use in a determination, a correction is made to take the loss on drying into account, and the amount of active principle is calculated with reference to the undried sample.

Limits by statistical methods

Reasonable limits may be established using simple statistical methods, e.g. control chart techniques. Analytical results from about 20 successive batches are pooled together, the grand average and the "three sigma limits" (3 standard deviations from the grand average) are calculated. (Such calculations are applicable when more than one individual or independent sample per batch is analyzed (2, 3).

Solubility

Unless otherwise indicated, the approximate solubility of medicinal plant materials is evaluated at 20 °C. The expression "part" describes the number of millilitres (ml) of solvent represented by the stated number of parts in which 1 gram (g) of solid is soluble.

<table>
<thead>
<tr>
<th>Solubility</th>
<th>Parts</th>
<th>Less than</th>
<th>1 part</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very soluble</td>
<td></td>
<td>10 parts</td>
<td></td>
</tr>
<tr>
<td>Freely soluble</td>
<td></td>
<td>30 parts</td>
<td></td>
</tr>
<tr>
<td>Soluble</td>
<td></td>
<td>100 parts</td>
<td></td>
</tr>
<tr>
<td>Sparingly soluble</td>
<td></td>
<td>1000 parts</td>
<td></td>
</tr>
<tr>
<td>Slightly soluble</td>
<td></td>
<td>10000 parts</td>
<td></td>
</tr>
<tr>
<td>Very slightly soluble</td>
<td></td>
<td>100000 parts</td>
<td></td>
</tr>
<tr>
<td>Practically insoluble</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Storage

Medicinal plant material must be stored under specified conditions in order to avoid contamination and deterioration.

a) Containers

The container and its closure must not interact physically or chemically with the material within in any way which would alter its quality. The following terms include general requirements for the permeability of containers:

Well-closed container must protect the contents from extraneous matter or from loss of the material under normal conditions of handling, shipment, or storage.

Tightly closed container must protect the contents from extraneous matter, from loss of the material, and from efflorescence, deliquescence, or evaporation under normal conditions of handling, shipment, or storage. If the container is intended to be opened on several occasions, it needs to be designed to be airtight after reclosure.
b) Protection from light

Medicinal plant materials requiring protection from light should be maintained in a light-resistant container that — either by reason of the inherent properties of the material from which the container is composed, or because a special coating has been applied to the container — shields the contents from the effects of light. Alternatively, the container may be placed inside a suitable light-resistant (opaque) covering and/or stored in a dark place.

c) Temperature

Materials that require storage conditions at temperatures other than room temperature should be labelled accordingly.

d) Humidity

Low moisture may be maintained, if necessary, by the use of a desiccant in the container provided that direct contact with the product is avoided. Care must be taken when the container is opened in damp or humid conditions.

Size of Cut

Medicinal plant materials are used either whole, cut or powdered.

Cut medicinal plant materials are prepared by cutting or crushing the plant into small pieces, and the terms in the following table are used to indicate the degree of coarseness of the cut material:

to pass through a sieve
with a mesh size of

<table>
<thead>
<tr>
<th>Term</th>
<th>Size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>coarse cutting</td>
<td>4.00</td>
</tr>
<tr>
<td>medium cutting</td>
<td>2.80</td>
</tr>
<tr>
<td>fine cutting</td>
<td>2.00</td>
</tr>
</tbody>
</table>
UNITS OF MEASUREMENT AND THEIR ABBREVIATIONS

Names and symbols for units of measurement conform with those used in The International Pharmacopoeia and those of the "Système international d'Unités" (International System of units: SI), developed by the General Conference of Weights and Measures (CGPM) in collaboration with international organizations (4).

A. Basic SI-Units

<table>
<thead>
<tr>
<th>Units of length</th>
<th>Units of time</th>
</tr>
</thead>
<tbody>
<tr>
<td>metre</td>
<td>year</td>
</tr>
<tr>
<td>[m]</td>
<td>[a]</td>
</tr>
<tr>
<td>centimetre</td>
<td>day</td>
</tr>
<tr>
<td>[cm]</td>
<td>[d]</td>
</tr>
<tr>
<td>millimetre</td>
<td>hour</td>
</tr>
<tr>
<td>[mm]</td>
<td>[h]</td>
</tr>
<tr>
<td>micrometre</td>
<td>minute</td>
</tr>
<tr>
<td>[µm]</td>
<td>[min]</td>
</tr>
<tr>
<td>nanometre</td>
<td>second</td>
</tr>
<tr>
<td>[nm]</td>
<td>[s]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Units of volume (capacity)</th>
<th>Units of electric current</th>
</tr>
</thead>
<tbody>
<tr>
<td>litre</td>
<td>ampere</td>
</tr>
<tr>
<td>[l]</td>
<td>[A]</td>
</tr>
<tr>
<td>millilitre</td>
<td>milliampere</td>
</tr>
<tr>
<td>[ml]</td>
<td>[mA]</td>
</tr>
<tr>
<td>microlitre</td>
<td></td>
</tr>
<tr>
<td>[µl]</td>
<td></td>
</tr>
<tr>
<td>1 drop (non-SI unit)</td>
<td></td>
</tr>
<tr>
<td>~0.05 ml</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Units of mass</th>
<th>Units of amount of substance</th>
</tr>
</thead>
<tbody>
<tr>
<td>kilogram</td>
<td>mole</td>
</tr>
<tr>
<td>[kg]</td>
<td>[mol]</td>
</tr>
<tr>
<td>gram</td>
<td></td>
</tr>
<tr>
<td>[g]</td>
<td></td>
</tr>
<tr>
<td>milligram</td>
<td></td>
</tr>
<tr>
<td>[mg]</td>
<td></td>
</tr>
<tr>
<td>microgram</td>
<td></td>
</tr>
<tr>
<td>[µg]</td>
<td></td>
</tr>
</tbody>
</table>

B. Derived SI-Units

<table>
<thead>
<tr>
<th>Units of radioactivity</th>
<th>Units of electric potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>becquerel</td>
<td>volt</td>
</tr>
<tr>
<td>[bq]</td>
<td>[V]</td>
</tr>
<tr>
<td></td>
<td>millivolt</td>
</tr>
<tr>
<td></td>
<td>[mV]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Units of absorbed dose equivalent</th>
<th>Unit of resistance</th>
</tr>
</thead>
<tbody>
<tr>
<td>sievert</td>
<td>ohm</td>
</tr>
<tr>
<td>[Sv]</td>
<td>[Ω]</td>
</tr>
</tbody>
</table>
POWDER FINENESS AND SIEVE SIZE

Powders

The coarseness or fineness of a powder is classed according to the nominal aperture size expressed in μm of the mesh of the sieve through which the powder is able to pass.

Terms used to describe particle size

- **Coarse powder (2000/355).** A powder where all the particles pass through a No. 2000 sieve, and not more than 40% through a No. 355 sieve.

- **Moderately coarse powder (710/250).** A powder where all the particles pass through a No. 710 sieve, and not more than 40% through a No. 250 sieve.

- **Moderately fine powder (355/180).** A powder where all the particles pass through a No. 355 sieve, and not more than 40% through a No. 180 sieve.

- **Fine powder (180).** A powder where all the particles pass through a No. 180 sieve.

- **Very fine powder (125).** A powder where all the particles pass through a No. 125 sieve.

Sieves

The wire sieves used to sift powdered medicinal plant materials are classified by numbers as mentioned above which indicate the nominal aperture size expressed in μm.

Sieves are made of wire of uniform circular cross-section. They have the following specifications:

<table>
<thead>
<tr>
<th>Number of sieve</th>
<th>Nominal size of aperture [μm]</th>
<th>Nominal diameter of wire [mm]</th>
<th>Approximate screening area [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000</td>
<td>2.00</td>
<td>0.90</td>
<td>48</td>
</tr>
<tr>
<td>710</td>
<td>0.710</td>
<td>0.450</td>
<td>37</td>
</tr>
<tr>
<td>500</td>
<td>0.500</td>
<td>0.315</td>
<td>38</td>
</tr>
<tr>
<td>355</td>
<td>0.355</td>
<td>0.224</td>
<td>38</td>
</tr>
<tr>
<td>250</td>
<td>0.250</td>
<td>0.160</td>
<td>37</td>
</tr>
<tr>
<td>212</td>
<td>0.212</td>
<td>0.140</td>
<td>36</td>
</tr>
<tr>
<td>180</td>
<td>0.180</td>
<td>0.125</td>
<td>35</td>
</tr>
<tr>
<td>150</td>
<td>0.150</td>
<td>0.100</td>
<td>36</td>
</tr>
<tr>
<td>125</td>
<td>0.125</td>
<td>0.090</td>
<td>34</td>
</tr>
<tr>
<td>90</td>
<td>0.090</td>
<td>0.063</td>
<td>35</td>
</tr>
<tr>
<td>75</td>
<td>0.075</td>
<td>0.050</td>
<td>36</td>
</tr>
<tr>
<td>45</td>
<td>0.045</td>
<td>0.032</td>
<td>34</td>
</tr>
</tbody>
</table>

The nominal size of aperture of wire mesh sieves has been selected principally from among those recommended by ISO standard 565-1972.
1. GENERAL ADVICE ON SAMPLING

The reliability of any conclusions drawn from the analysis of a sample will depend upon how truly that sample represents the whole batch. General recommendations for sampling of pharmaceutical materials in connection with quality control are provided in the thirty-first report of the WHO Expert Committee on Specifications for Pharmaceutical Preparations (5).

Due to the specific characteristics of medicinal plant materials, in particular their inhomogeneity, special handling procedures are required in relation to sampling. The following procedures should be observed when selecting and preparing an average sample from a batch of material.

**Recommended procedures**

**Sampling of material in bulk**

Inspect each container/packaging unit (pack etc.) for conformity with pharmacopoeial monographs or other requirements regarding packaging and labelling. Check the integrity of the outer package and note any defects which may influence the quality or stability of the contents (physical damage, moisture etc.).

Damaged containers are sampled individually.

If initial inspection indicates that the batch is uniform, take the following samples:

When a batch consists of 5 containers/units, take a sample from all of them; and from a batch of 6-50 units, a sample from 5 packages. In the case of a batch of over 50 containers - sample 10% of the units - rounding up the number of units to the next highest figure of ten. For example, 51 units would be sampled as for 60.

After opening, inspect containers selected for sampling for:

- organoleptic characteristics (colour, texture and odour);
- presentation of the material (raw, cut, crushed, compressed);
- the presence of admixtures, foreign matter (sand, glass particles, dirt), mould, or signs of decay;
- the presence of insects;
- the presence of packaging material originating from poor or degraded containers.

From each container/package selected, take 3 original samples, taking care to avoid fragmentation. Samples should be taken from the upper, middle and lower parts. In the case of sacks and packages, 3 individual samples are taken by hand from a depth of not less than 10 cm from the top, and after cutting into the side of the package from the middle and lower parts. Samples of seeds are withdrawn with a grain probe. Material in boxes is first sampled from the upper layer; then approximately half of the contents is removed and samples are taken again. Finally after further removal of material, another sample is taken from the bottom. Samples should be as uniform as possible in mass. Individual samples are combined into a pooled sample which should be mixed carefully.

The average sample is obtained by quartering. The process of quartering consists of placing the sample, adequately mixed, formed as an even and square-shaped heap and dividing it diagonally into four equal parts. The two opposite parts are then taken diagonally, and carefully mixed. The process is repeated as necessary until the required quantity is obtained.

Pooled samples are quartered until the required amount remains which should be within ±10% (100-200 g for flowers and up to 10 kg for certain roots). Any remaining material should be returned to the batch.

The average sample is then quartered again and final samples are assembled and tested for the following characteristics:

- degree of fragmentation (sieve test);
- identity and level of impurities;
- determination of moisture and ash content;
- assay of active ingredients, where possible.
A portion of the final sample should be retained to serve as reference material, which may also be used for the purpose of checking quality control tests, if necessary.

**Sampling of material in retail packages**

From each wholesale container (boxes, cartons, etc.) selected for sampling, take at random two consumer packages. From small batches (1-5 boxes), take 10 consumer packages. Prepare the **pooled sample** by mixing the content of selected consumer packages and proceed as described above for the final sample.

**2. DETERMINATION OF FOREIGN MATTER**

Medicinal plant materials should be entirely free from visible signs of contamination by moulds, insects, and other animal contamination, including animal excreta. No abnormal odour, discoloration, slime or signs of deterioration should be detected.

It is seldom possible to obtain marketed plant materials that are entirely free of some form of innocuous foreign matter. However, no poisonous, dangerous or otherwise harmful foreign matter or residue should be allowed.

During storage, products should be kept in a clean and hygienic place, so that no contamination occurs. Special care should be taken to avoid formation of moulds, since they may produce aflatoxins.

A macroscopical method of examination can conveniently be employed for whole or cut plant materials, however microscopy is indispensable for powdered plant materials.

Before the medicinal plant materials are cut, ground or powdered, soil, stones, sand, dust and other foreign inorganic matter must be removed.

**Definition**

Foreign matter is material consisting of any or all of the following:

(a) Parts of the medicinal plant material or materials other than those named with specified limits;

(b) Any organism, part or product of an organism, other than that named in the definition and description;

(c) Mineral admixtures not adhering to the medicinal plant materials, such as soil, stones, sand, and dust.

**Sample size to be taken**

It is difficult to prepare a pooled sample since most foreign matter adheres to the medicinal plant materials which is intrinsically nonuniform. Special procedures requiring considerable practice are therefore necessary. The problem is especially difficult when samples of unbroken crude medicinal plant materials are selected which are too small. Thus, it is crucial that the size of the sample should be sufficiently large to be representative.

**Recommended procedures**

**Foreign matter in whole or cut medicinal plant materials**

Weigh the quantity of material as given below unless otherwise given in the instructions. Spread it in a thin layer and sort into groups of foreign matter either by visual inspection, using a magnifying lens (6x or 10x) or with the help of a suitable sieve, according to the requirements for the specific plant material.
Sift the remainder of the sample through a No. 250 sieve; “dust” is regarded as mineral admixture. Weigh the portions of this sorted foreign matter to within 0.05 g. Calculate the content of each group in g per 100 g of air-dried sample.

For some medicinal plant materials where the foreign matter may closely resemble the material itself, it may be necessary to take a pooled sample of each individual element and apply a critical test, either chemical, physical or by microscopy. The proportion of foreign matter is calculated from the sum of the portions which fail to respond to the test.

The following quantities constitute a sample, unless otherwise given in the test procedure:

- Roots, rhizomes and bark: 500 g
- Leaves, flowers, seeds and fruit: 250 g
- Cut medicinal plant materials (average weight of each fragment less than 0.5 g): 50 g

3. VISUAL EXAMINATION AND INSPECTION BY MICROSCOPY

Medicinal plant materials are categorized according to sensory, macroscopic and microscopic characteristics. Since the majority of information on the identity, purity and often quality of the material can be drawn from these observations, they are of primary importance before any further testing can be carried out.

A sensory, macroscopic and microscopic description should be elaborated for each individual medicinal plant material. Definition of the characteristics of the material is thus the first step towards the establishment of identity and the degree of purity. Wherever possible, authentic specimens of the material in question and samples of pharmacopoeial quality should be available to serve as a reference.

Visual inspection provides the simplest and quickest means by which to establish identity, purity and, possibly, the quality. If a sample is found to be devoid of, or significantly different from, the specified colour, consistency, odour and taste, it is considered as not fulfilling the requirements. However, judgement must be exercised when considering odour and taste due to the variability from person to person and at different times.

Macroscopic identity of medicinal plant materials is based on shape, size, colour, surface characteristics, texture, fracture and appearance of the cut surface. This identification is useful in judging the material in its entirety. Since these characteristics are mostly subjective and substitutes or adulterants exist which closely resemble the genuine material, it is often necessary to substantiate the findings of the macroscopic examination by microscopy and/or physicochemical analysis.

Inspection by microscopy of medicinal plant materials is indispensable for the identification of broken or powdered materials. The specimen may have to be treated with chemical reagents. However, an examination by microscopy alone cannot always provide complete identification, though when used in association with data from other analytical methods it can frequently provide invaluable supporting evidence.

Comparison with a reference material will often reveal characteristics not described in the requirements which would otherwise have been regarded as foreign matter, rather than normal constituents.

Any additional useful information for the preparation or the analysis of plant materials should also be included in the requirements, such as the determination of vein-islets and the palisade ratio.

VISUAL EXAMINATION AND ODOUR

No preliminary treatment is necessary for the sample. However, wrinkled and contracted leaves, herbs or flowers should be softened and stretched flat (see below "Preliminary treatment of medicinal plant materials"). Certain fruits and seeds may require softening before dissection and observation of internal characteristics.
**Recommended procedures**

**Size** — A graduated ruler in millimetres is adequate for the measurement of the length, width and thickness of crude materials. Small seeds and fruits may be measured by aligning 10 of them on a sheet of calibrated paper, 1 mm apart between the lines. The result obtained is divided by 10.

**Colour** — Examine the untreated sample under diffuse daylight. If necessary, an artificial light source with wavelengths similar to that of daylight may be used. The colour of the sample should be compared with that of a reference material.

**Surface characteristics, texture and fracture** — Examine the untreated sample. If necessary, a magnifying lens (6x to 10x) may be used. Wetting with water or reagents, as required, may be necessary to observe the characteristics of a cut surface. Touch the material to determine if soft or hard; bend and rupture it to provide information on the brittleness and appearance of the fracture plane — whether it is fibrous, smooth, rough, granular, etc.

**Odour** — If the material is expected to be innocuous, examine a small portion of the sample placed in the palm of the hand or a beaker of suitable size, by slow and repeated inhalation of the air over the material. If no distinct odour is perceptible, crush the sample between the thumb and index finger, between the palms of the hands, using gentle pressure or, if the material is known to be dangerous, by other suitable means such as pouring a small quantity of boiling water onto the crushed sample placed in a beaker. First, determine the strength of the odour (none, weak, distinct, strong) and then the odour sensation (aromatic, fruity, musty, mouldy, rancid, etc.). A direct comparison of the odour with a defined substance is most advisable (e.g. peppermint should have an odour similar to menthol, cloves an odour similar to eugenol).

**Taste** — This test should only be applied if specifically required for a given plant material.

**INSPECTION BY MICROSCOPY**

Once the material has been examined and grouped according to external characteristics, inspection by microscopy can be carried out as the next step on those groups where identity has not been established.

**Equipment**

(a) A microscope equipped with lenses providing a wide range of magnification and a sub-stage condenser, a graduated mechanical stage, objectives with a magnification of 4x, 10x. Colour filters of ground glass, blue-green. High eyepoint eyepieces are preferred for wearers of spectacles.

(b) A lamp, either separate or incorporated into the microscope.

(c) A set of polarizing filters.

(d) A stage micrometer and an ocular micrometer to be inserted into a 6x eyepiece and placed on the diaphragm or preferably, a micrometer eyepiece.

(e) A set of drawing attachments for microscopy.

(f) A microburner (Bunsen type).

(g) Slides and cover-glasses of standard size.

(h) A set of botanical dissecting instruments.

**Recommended procedures**

**Preliminary treatment of medicinal plant materials**

Select a representative sample of the material. Dried parts of a plant may require softening before preparation for microscopy by exposing the sample to a moist condition, which is to be preferred wherever possible, or soaking in water. For small quantities of material, put a wad of cotton-wool moistened with water into the bottom of a test-tube and cover with a piece of filter-paper. Place the material being examined on the paper, stopper the tube and allow to stand overnight or until the
material is softened and suitable for cutting. Use a desiccator for larger quantities of material, placing water into the lower part instead of the drying agent.

Bark, wood and other dense and hard materials usually need soaking in water or equal parts of water, ethanol and glycerol for a few hours or overnight until they are soft enough to be cut. Boiling in water for a few minutes may sometimes be necessary.

Any water-soluble contents may be removed from the cells by soaking in water. Starch grains are gelatinized by heating in water. In certain cases, moistening of a piece of material with water for a few minutes softens the surfaces to facilitate cutting into sections.

Preparation of specimens

For powdered materials – Place 1 or 2 drops of water, glycerol-ethanol TS or chloral hydrate TS on a glass slide; moisten the tip of a needle with water and take a little quantity of the powdered material that adheres to the needle tip. Thoroughly, but carefully, stir the powder in the drop of fluid, apply a cover-glass, slightly press the cover-glass with the handle of the needle, and remove excess fluid from the margin of the cover-glass with a strip of filter-paper. Other fluids may be used, if necessary, in the same manner.

If the specimen is to be freed from air bubbles, carefully boil the powder with the fluid on the slide until the particles are clear and the air is completely removed. Care should be taken to replace the fluid which evaporates so that the space beneath the cover-glass is completely filled with the fluid at the conclusion of the operation.

For surface of leaves and flowers – To render pieces of thin leaves transparent, boil them directly on a slide. Cut the piece of leaf into 2 halves, turn one upper side down and add chloral hydrate TS. Boil the specimen carefully over a small flame of a microburner and, as soon as bubbles escape, remove the slide from the flame. When the bubbles have ceased to appear, boil again until the fragments are transparent.

If necessary, cut square pieces of papery leaves, about 6 mm from the edge of the leaf, if not otherwise required. The pieces should be taken one-third to half of the way from the leaf-base and include a midrib or large vein as well as 1 or 2 pieces from the edge including 1 or 2 indentations, where appropriate. For broken or cut leaves take suitable fragments as described above. Put the fragments into a test-tube containing chloral hydrate TS and boil for a few minutes until they become transparent. Transfer a fragment to a slide and cut it into 2 equal halves. Turn 1 of the halves upside down and align both halves so that both upper and lower surfaces can be observed under the microscope. Add 1–2 drops of chloral hydrate TS and apply a cover-glass.

For thicker leaves, not transparent enough when prepared by the method described above, clarify fragments of a leaf by boiling in a tube and transfer a fragment onto a slide. Cut it into 2 equal halves and turn one upper side down. Scrape the surface of the halves by means of a scalpel until only 1 layer of epidermis is left. Wash the epidermis with drops of chloral hydrate TS or glycerol-ethanol TS to remove any residues and, if possible, turn both parts of the epidermis upper side down, and add one of the above fluids.

For even thicker or fleshy leaves, pull off the upper and lower parts of epidermis by winding the softened leaf around the index finger, pressing with the thumb and the middle finger against the index finger and carefully incising, catching the incised part with forceps, and bending the epidermis backwards carefully.

Petals and sepals of flowers may be treated similarly.

For sections – Select representative pieces of the material being examined and cut into suitable lengths, one end of which is smoothed after softening.

Cross or transverse sections are prepared by cutting with a razor blade at a right angle to the longitudinal axis of the material. Longitudinal sections are prepared by cutting in parallel with the
longitudinal axis, either in the radial direction (radial section) or in the tangential direction (tangential section).

Thick materials, such as wood, woody stems, rhizomes and roots can be cut by holding the softened material between the thumb and index finger, supported by the middle finger or by holding it in the central hole of a hand microtome. Thin materials such as leaves, petals and slender stems should be bound between two halves of a piece of elder-pith or other suitable support. If necessary, moisten the surface to be cut and the razor blade with ethanol (-375 g/l) TS. Cut the sections as thin and even as possible. Transfer the sections with a brush moistened with ethanol (-150 g/l) TS to a dish containing the above fluid. Select satisfactory sections for the preparation of the slides. For certain materials a sliding microtome may be used.

Seeds and fruits that are too flat, or are small and spherical and cannot be held in the manner described above may be inserted into a notch cut into a small rubber stopper or embedded in hard paraffin as follows: Prepare a hard paraffin block, rectangular in shape, about 7x7x15 mm, and melt a small hole in the centre of one end by means of a heated needle or thin glass rod. Press the material, which is preferably dry or softened by exposure to a moist condition, into this hole.

For the examination of mucilage, aleurone grains or sphere-crystals of inulin, cut the material without using water.

Clarification of microscopic particles

The presence of certain cell contents such as starch grains, aleurone grains, plastids, fats, oils, etc., often renders sections non-translucent and obscures certain characteristics. In such cases, reagents that dissolve certain of the above cell contents, are used in order to make the remaining parts stand out clearly or produce a penetrating effect. This renders the section more transparent and reveals details of the structures.

The action of clarifying agents depends on the refractive index; if it is close to that of the cell structure, the material being tested becomes almost invisible; if it differs appreciably, the material becomes markedly evident.

The most frequently used clarifying agents are the following (for the methods of preparation refer to "Reagents and test solutions"):

**Chloral hydrate TS** – On gentle heating it dissolves starch grains, aleurone grains, plastids, volatile oils, and expands collapsed and delicate tissue without any undue swelling action upon the cell walls and distortion of the tissue. It has a refractive index of $n_2^P = 1.44$–1.48, and is the best reagent for rendering calcium oxalate clearly evident and is particularly useful for small crystals. However, this reagent has a slow dissolving power upon calcium oxalate due to an increase in acidity when allowed to stand.

**Lactochloral TS** – It has a similar use to chloral hydrate TS, but is usually applied to sections that are difficult to clarify. It may be used cold. Before use, any air present in the specimen should be removed by placing in a desiccator and applying a vacuum.

**Lactophenol TS** – It may be used cold or upon heating. It has a refractive index of $n_2^P = 1.44$ and is useful for the preparation of fungi, pollen grains, most non-oily powders, and parasites such as mites, nematode worms, etc. Sizes of starch grains can be measured accurately, but the concentric rings are usually invisible when prepared in this reagent. Crystals of calcium oxalate are clearly visible in lactophenol and shine brightly when illuminated with polarized light. This reagent dissolves calcium carbonate deposits with a slow effervescence, due to the presence of lactic acid.

**Sodium hypochlorite TS** – it is used for bleaching deeply-coloured sections. Immense the sections in the solution for a few minutes until sufficiently bleached, wash with water and prepare the mount with glycerol-ethanol TS. The bleached sections give a negative reaction to lignin.

**Solvents used for fats and oils** – Xylene R and light petroleum R can be used to remove fats and oils from oily powders or sections. Immerse the material in the solvent for a short time, decant the liquid and wash the material with fresh solvent.
Histochemical detection of cell walls and contents

The application of reagents to a powdered sample or a section on a slide, as described below, can be carried out either by:

- adding drops of the reagent to the sample and applying a cover-glass, followed by irrigation, or
- placing drops of the reagent on one side of the cover-glass of a prepared specimen and removing the fluid under the cover-glass by suction, by placing a strip of filter-paper at the opposite side of it.

Using the second application, the progress of the reaction may be observed under a microscope. Care should be taken to avoid using reagents or vapours that could attack the lenses or stages of the microscope.

Cellulose cell walls – Add 1–2 drops of iodinated zinc chloride TS and allow to stand for a few minutes, or alternatively, add 1 drop of iodine (0.1 mol/l) VS, allow to stand for 1 minute, remove excess reagent with a strip of filter-paper and add 1 drop of sulfuric acid (~1160 g/l) TS; cellulose cell walls are stained blue to blue-violet.

On the addition of 1–2 drops of cuoxam TS, the cellulose cell walls will swell and gradually dissolve.

Lignified cell walls – Moist the powder or section on a slide with a small volume of phloroglucinol TS and allow to stand for about 2 minutes or until almost dry. Add 1 drop of hydrochloric acid (~420 g/l) TS and apply a cover-glass; lignified cell walls are stained pink to cherry red.

Suberized or cuticular cell walls – Add 1–2 drops of sudan red TS and allow to stand for a few minutes or warm gently; suberized or cuticular cell walls are stained orange-red or red.

Aleurone grains – Add a few drops of iodine/ethanol TS; the aleurone grains will turn yellowish brown to brown. Then add a few drops of ethanolic trinitrophenol TS; a yellow colour appears. Add about 1 ml of mercuric nitrate TS and allow to dissolve; the colour of the solution turns brick red.

If the specimen is oily, render it fat-free by immersing and washing it in an appropriate solvent before carrying out the above tests.

Calcium carbonate – Crystals or deposits of calcium carbonate dissolve slowly with effervescence when acetic acid (~60 g/l) TS or hydrochloric acid (~70 g/l) TS is added.

Calcium oxalate – Crystals of calcium oxalate are insoluble in acetic acid (~60 g/l) TS but dissolve in hydrochloric acid (~70 g/l) TS without effervescence (if applied by irrigation the acid should be more concentrated); they also dissolve in sulfuric acid (~350 g/l) TS, but needle-shaped crystals of calcium sulfate separate on standing after about 10 minutes. In polarized light, calcium oxalate crystals are birefringent. Calcium oxalate is best viewed after clearing the sample, e.g. with chloral hydrate TS.

Fats, fatty oils, volatile oils and resins – Add 1–2 drops of sudan red TS and allow to stand for a few minutes or heat gently, if necessary. The fatty substances are stained orange-red to red. Irrigate the preparation with ethanol (~750 g/l) TS and heat gently; the volatile oil and resins dissolve in the solvent, while fats and fatty oils (except castor oil and croton oil) remain intact.

Hydroxyanthraquinones – Add 1 drop of potassium hydroxide (~55 g/l) TS; cells containing 1,8-dihydroxyanthraquinones are stained red.

Inulin – Add 1 drop of each of 1-naphthol TS and sulfuric acid (~1760 g/l) TS; sphaero-crystals of inulin turn brownish red and dissolve.

Mucilage – Add 1 drop of Chinese ink TS to the dry sample; the mucilage shows up as transparent, spherically dilated fragments on a black background. Alternatively, add 1 drop of thionine TS to the dry sample, allow to stand for about 15 minutes, then wash with ethanol (~188 g/l) TS; the mucilage turns violet-red (cellulose and lignified cell walls are blue and bluish violet).

Starch –
- Add a small volume of iodine (0.02 mol/l) VS; a blue or reddish blue colour is produced.
• To a few starch grains add a small volume of glycerol-ethanol TS and examine between crossed polars; birefringence is observed giving a Maltese cross effect with the cross intersecting at the hilum.

_Tannin—Add 1 drop of ferric chloride (50 g/l) TS; it turns bluish black or greenish black._

**Disintegration of tissues**

Cut the material into small pieces, about 2 mm thick, 5 mm long or into slices, of about 1 mm thick. (Tangential longitudinal sections are preferred for woods or xylem from stems)

Use the following method according to the nature of the cell walls. For tissues with lignified cell walls use either method 1 or 2. For tissues where lignified cells are few or occur in small groups, use method 3.

**Method 1—Nitric acid and potassium chlorate method**

Place the material in a test-tube containing about 5 ml of nitric acid (500 g/l) TS and heat to boiling. Add a small quantity of powdered potassium chlorate R and allow to react, warming gently if necessary to maintain a slight effervescence; add fresh quantities of powdered potassium chlorate R as needed. When the tissue appears to be almost completely bleached and shows a tendency to disintegrate, apply pressure with a glass rod to the material to see if it breaks. If so, interrupt the reaction by pouring the contents of the test-tube into water. Allow the material to settle, decant it and wash it with fresh quantities of water until the acidity is removed. Transfer the material onto a slide and tease it out with a needle. Add 1 drop of glycerol-ethanol TS and apply a cover-glass. The disintegrated material gives a negative reaction for lignin.

**Method 2—Nitric and chromic acid method**

Place the material in a small dish and heat with nitro-chromic acid TS until the material breaks easily when pressure is applied with a glass rod. Wash the material repeatedly with water and transfer onto a slide; tease out and proceed as stated above. The disintegrated material gives a negative reaction for lignin.

This treatment can also be carried out on a slide. Place a moderately thick section of the material on a slide, add the reagent and, allow to stand for about 20 minutes. Separate the cells by applying gentle pressure, or with a sliding movement of the cover-glass. This process is especially useful when the disintegration of the tissues of a section under the microscope needs to be observed to ascertain from where the isolated cells are derived.

**Method 3—Caustic alkali method**

Place the material in a test-tube containing about 5 ml of potassium hydroxide (110 g/l) TS or sodium hydroxide (80 g/l) TS, heat on a water-bath for 15–30 minutes until a portion breaks easily when pressure is applied with a glass rod. Decant the liquid and wash the softened material several times with fresh quantities of water and heat to boiling. This method is particularly useful for the disintegration of bark, seeds, leaves and flowers, facilitating the elimination of fibres, scleroids, lactiferous tissues and epidermis. The disintegrated material gives a negative reaction for lignin.

**Measurement of specimens**

**Equipment**

Use a microscope with an ocular micrometer to measure the size of an object. The scales should be calibrated by means of a stage micrometer, consisting of a glass slide of usual size, upon which a scale is engraved or photographed, usually 1 or 2 mm in length, in 0.1 and 0.01 mm (= 10 μm) divisions. The ocular micrometer consists of a small disk of glass which is placed upon the diaphragm of a Huygenian eyepiece. Across the diameter of the disk a 100-line scale is engraved or photographed.
Calibration of the ocular micrometer (see figures below)

Focus the ocular micrometer scale by moving the lens until a sharp definition is obtained. Superimpose the stage micrometer on the scale of ocular micrometer and line-up on the scale divisions. Both scales should now be sharply defined in the field of view. By turning the eyepiece they are placed exactly in a parallel position, and if necessary, the stage micrometer is moved until the starting lines of both scales coincide. Count the number of ocular micrometer divisions corresponding to a certain length of the stage micrometer, in order to determine the length which is equivalent to 1 division of the ocular micrometer scale e.g., 100 divisions of the ocular micrometer scale are equal to 30 divisions of the stage micrometer; since the divisions of the stage micrometer are 0.01 mm apart, 100 ocular micrometer divisions are equivalent to 0.30 mm and each small division of the ocular micrometer represents 3.0 μm. Since the micrometer value applies only for a particular lens combination, it is advisable to determine and record the micrometer values for the most frequently used lens combinations.

Measurement of the size of an object

Place the specimen on the microscope stage and focus on the object. Superimpose the ocular micrometer scale on the object and read its dimensions with the ocular micrometer. Multiply the number of scale divisions by the micrometer value to give the actual dimension in μm. By this method, using a 40x objective and a 6x eyepiece, measurements are correct to the nearest 2 μm, e.g., a dimension of 20 μm is liable to an error of 2 μm, or 10%, or ±5%; a dimension of 100 μm is liable to an error of 2% or ±1%.

For curved and elongated objects, the measurement of lengths may be made by the use of a microscope equipped with a drawing apparatus or a camera-lucida. The instrument should be set up properly to clearly focus with the image of the object, the drawing paper and the pencil simultaneously. With the stage micrometer in place, trace the lines of the stage micrometer scale upon the paper fastened on a drawing board. Tilt the drawing board, if necessary, until the divisions drawn upon the paper are equally spaced. The magnification is determined by measuring the distance between selected lines on the drawing paper and dividing by the distance between the corresponding lines on the stage micrometer. Place the specimen on the microscope stage and trace the image of the object on the paper. Superimpose a coloured thread along the length of the object drawn on the paper and after stretching it, measure the length of the thread by means of a ruler graduated in mm; divide the length determined by the magnification to give the actual length of the object.

Types of stomata

Four significantly different types of stomata (see figure below) are distinguished by their form and arrangement of the surrounding cells, especially of subsidiary cells, as seen in a mature leaf. In describing an epidermis where certain stomata differ from the predominant type, the term applying to the majority of stomata is used.
(a) The anomocytic or ranunculaceous (irregular-celled) type, where the stoma is surrounded by a varying number of cells, generally not different from those of the epidermis.

(b) The anisocytic or cruciferous (unequal-celled) type; the stoma is usually surrounded by 3 or 4 subsidiary cells, one of which is markedly smaller than the others.

(c) The diacytic or canephylloaaceous (cross-celled) type; the stoma is accompanied by two subsidiary cells, the common wall of which is at right angles to the stoma.

(d) The paracytic or rubiaceous (parallel-celled) type; the stoma has two subsidiary cells, of which the long axes are parallel to the axis of the stoma.

Determination of the stomatal index

The stomatal index is the percentage of stomata existing in relation to epidermal cells.

Place fragments of leaves, about 5x5 mm in size, in a test-tube containing about 5 ml of chloral hydrate TS and heat on a water-bath for about 15 minutes or until the fragments are transparent. Transfer a fragment to a slide and prepare it as described earlier, the lower epidermis uppermost, in chloral hydrate TS and place a small drop of glycerol-ethanol TS on one side of the cover-glass to prevent the material from drying. Examine under a microscope with a 40x objective and a 6x eyepiece, equipped with a drawing apparatus. Mark on the drawing paper a cross (x) for each epidermal cell and a circle (o) for each stoma. Calculate the stomatal index as follows:

\[
\text{stomatal index} = \frac{S \times 100}{E + S}
\]

where
- \(S\) is the number of stomata in a given area of leaf and
- \(E\) is the number of epidermal cells (including trichomes) in the same area of leaf.

For each sample of a leaf carry out no fewer than 10 determinations and calculate the average index.
Microsublimation

A small, square metal plate, about 4x4 cm in size, is mounted on a square of asbestos board from which a central hole, about 1 cm in diameter, has been cut. Place a metal ring, about 1 cm in diameter and 8 mm in height, at the centre of the metal plate aligned with the hole of the asbestos board. Place a small quantity, about 0.1–0.2 g, of the powdered material on the bottom of the ring to form an even layer, about 2 mm thick. Cover the ring with a clean slide. Heat gently and gradually over a small flame of a Bunsen burner or a spirit lamp. Change the slide if a large amount of moisture or sublimate is observed. Usually 4–5 slides are prepared. Remove the slide from the ring, set it aside until the sublimate has dried and then examine under a microscope without adding any fluid or cover-glass.

A heating stage allows the temperature of the sublimation to be recorded.

4. THIN-LAYER CHROMATOGRAPHY

Thin-layer chromatography is particularly valuable for the qualitative determination of small amounts of impurities (6). The technique is easy to perform, effective and requires inexpensive equipment; therefore, it is frequently used for evaluating medicinal plant materials and their preparations.

The following parameters should be determined on the basis of published pharmacopoeial monographs and/or established experimentally for the analysis of each individual plant material:

(a) type of adsorbent and method of activation; if the latter is not mentioned, heat at 110 °C for 30 minutes;

(b) method of preparation and concentration of the test and reference solutions;

(c) volume of the solutions to be applied on the plate;

(d) mobile phase, and the distance of migration;

(e) condition of drying, temperature and method of detection;

(f) for the results obtained observe the spots:
- number and approximate position, or the Rf values if necessary,
- fluorescence and/or colour.

A. CLASSICAL METHOD

Recommended procedures

The method assumes the use of a laboratory-prepared chromatographic plates but precoated plates, activated if necessary, may be used provided that they have proven to be suitable for that particular application.

For identifying and determining the purity of a medicinal plant material, a powdered specimen of pharmacopoeial quality may be used as the reference material. The preparation of the solution to be tested and the reference solutions should be carried out simultaneously in exactly the same way. If a test for the presence of certain active principles of a medicinal plant material is to be carried out, a chemical reference substance identical to that principle should be used. The reference solutions prepared should be of known concentration. If the quantitative ratios of the chemical substance in the reference solution are selected in accordance with the composition of a typical material, comparison of the spot size offers valuable additional information. The same solvent should be used wherever possible for the preparation of test and reference solutions. The solvent system should be indicated in the test procedure for the individual material being examined. A tricolour mixture (e.g. 0.01% solutions in toluene of indophenol blue, sudan red G and dimethyl yellow) run together, permits a rapid control of the prevailing chromatographic conditions.
The chamber in which chromatography takes place should be protected from light if it is suspected that the materials being examined are unstable. In any case, the chromatographic chamber should always be in a position where the direct rays of the sun cannot fall on it. These rays may be refracted to different degrees owing to imperfections in the glass walls of the chamber. This may give rise to areas of elevated temperature on the chromatographic plate and result in erratic flow of the mobile phase.

Preparation of the samples

Prior to testing, carry out a rapid extraction process with the plant material being examined.

To 0.1–1.0 g of the powdered plant material add 1–10 ml of solvent, extract either by stirring, shaking the mixture for 3–30 minutes, or heating to boiling and allowing to cool. Remove the insoluble matter by centrifugation, or filter through a small funnel with filter-paper or a cotton plug. If necessary, evaporate the filtrate on a water-bath using just the time required to remove the solvent and dissolve the residue in a small volume of solvent (e.g. 0.1–0.2 ml). If necessary, purify the test solution repeating the extraction with solvent and using a different pH or by sublimation, distillation, etc.

Apparatus

The equipment consists of:

(a) glass plates of uniform thickness throughout their entire area, length, 15–20 cm, and wide enough to accommodate the required number of test and reference solutions;

(b) a device for spreading a uniform layer of coating material of desired thickness onto glass plates;

(c) a rack to hold the prepared plates during the drying period or for transportation and storage; normally to accommodate 10 plates with set spacings; the dimension should be such as to permit placing of the rack in a drying oven and desiccator;

(d) a chromatographic chamber of transparent material, usually glass with a tightly fitting lid, of suitable size to accommodate the test plates;

(e) a suitable spraying implement for the reagent with a fine spray nozzle and made of a resistant material;

(f) an ultraviolet light source emitting short (254 nm) and long (365 nm) wavelengths.

Before use, clean the plates scrupulously by immersion in a suitable cleaning liquid, rinsing thoroughly until the water runs off the plates without leaving any visible water marks or oily spots, and then dry. The plates must be completely free of lint or dust when the coating material is applied.

Method

Preparation of the adsorbent

Unless otherwise indicated in the test procedure, prepare a slurry of the coating material and water or an aqueous solution (see "Specifications for adsorbents"), using the spreading device, coat the cleaned plates with a layer about 0.25 mm thick. Dry the coated plates in air and heat them to activate at 110 °C for 30 minutes, then allow to cool. Inspect the uniformity of the distribution in transmitted light and the texture of the adsorbent layer in reflected light. If the plates are not to be used immediately, store them in a desiccator containing silica gel, desiccant, R. To form an edge remove a narrow strip (2–5 mm) of the coating material from the vertical sides of the plate.

If acid, alkaline or buffered layers are required, use diluted acid, base or salt mixtures instead of water for the preparation of the slurry, as indicated in the test procedure. An aqueous solution of 5–7 g of sodium carboxymethylcellulose R could replace the water, if the adsorbent does not already contain a binder.
**Saturation of the chromatographic chamber**

Unless otherwise given for the individual plant material, the chromatography is carried out in a saturated chamber. To achieve this, line at least half of the total area of the inside walls of the chamber with filter-paper and place in it a sufficient quantity of the mobile phase to form a 5 mm layer. Close the chamber and allow to stand for at least 1 hour at room temperature.

When the plate is exposed to air all operations should preferably be carried out at a relative humidity of 50–60%, and the plates should be handled with care.

**Applications of the test and reference solutions**

Using a micropipette or a syringe graduated into µl, place the solutions being examined onto the starting line, about 15 mm from the lower edge and not less than 15 mm from the vertical sides of the plate, and not less than 15 mm apart from each other. They should form a line parallel to the lower edge of the plate. The spots should be as small as possible and preferably not more than 4 mm in diameter; if necessary, apply the solution in portions, drying between applications. Mark, as indicated in the test procedures the desired distance the mobile phase is intended to ascend, usually 10–15 cm from the starting line.

Results of separation can often be improved by applying the solutions to form a horizontal band of 10–15 mm long and not more than 5 mm wide.

**Development of the chromatograms**

Allow the spots to dry, place the plate as nearly vertical as possible into the chamber, ensuring that the points of application are above the surface of the mobile phase. Close the chamber, develop the chromatogram allowing the solvent to ascend the specified distance at room temperature, unless otherwise indicated in the test procedures. Remove the plate, mark the position of the solvent front and allow the solvent to evaporate at room temperature or as given for the individual plant material.

**Observation and interpretation of the chromatograms**

In the first instance, observe the spots produced in daylight, then under short-wave and/or long-wave ultraviolet light. Mark the centre of each spot with a needle. Measure and record the distance from the centre of each spot to the point of application, and indicate for each spot the wavelength under which it was observed. If indicated in the test procedures, spray the spots with the indicated reagent, observe and compare the spots with those of a reference material.

If required calculate the \( R_f \) or \( R_r \) values as follows:

\[
R_f = \frac{a}{b} \quad \quad \quad R_r = \frac{a}{c}
\]

where

- \( a \) is the distance between the point of application and the centre of the spot of the material being examined;
- \( b \) is the distance between the point of application and the solvent front;
- \( c \) is the distance between the point of application and the centre of the spot of reference material.

\( R_f \) values are only given for information and they may differ slightly with each experiment due, for example to different saturation conditions of the chromatographic chamber, or the activity of the adsorbent layer, or the composition of the mobile phase.
B. MICROMETHOD

Recommended procedures

The chromatograms can be developed either vertically or horizontally. Unless otherwise given for an individual plant material, thin-layer chromatography is performed on small plates using the ascending technique.

1. Ascending technique

Apparatus

The equipment consists of:

(a) specially-made plates, length, not more than 100 mm, width, not more than 100 mm, that permit a distance of development of at least 60 mm;

(b) micropipettes, 1 or 2 μl, accuracy not exceeding ±10% of the stated volume;

(c) a chromatographic chamber with a tight-fitting lid and a flat base. The size of the chamber must be adapted to that of the plates and the volume of the mobile phase.

Method

Place a sufficient quantity of a previously-mixed and homogeneous mobile phase into the chromatographic chamber to form a 5 mm layer. (Mobile phase mixtures should be discarded after the development of a plate). Close the chamber and allow to stand at constant room temperature, protected from draughts and direct sunlight for 15 minutes.

Using a micropipette, apply the solutions being examined onto the starting line, about 10 mm from the vertical and lower edges and not less than 5–10 mm from each other. They should form a line parallel to the lower edge of the plate. The spots should be as small as possible and preferably no more than 2 mm in diameter. Mark, as indicated in the test procedures the desired distance the mobile phase is intended to ascend, usually 60 mm from the starting line.

Allow the spots to dry, place the plate as nearly vertical as possible into the chamber, ensuring that the points of application are above the surface of the mobile phase. The side edges of the plate must not come into contact with the wall of the chamber. Close the chamber, develop the chromatogram allowing the solvent to ascend the specified distance at room temperature, unless otherwise indicated in the test procedure. Remove the plate, mark the position of the solvent front and allow the solvent to evaporate at room temperature or as given for the individual plant material.

2. Horizontal technique

Apparatus

The equipment consists of:

(a) specially-made plates*, length 50 mm, width, 50 mm;

(b) micropipettes, 0.5 or 1 μl, accuracy not exceeding ±10% of the stated volume;

(c) a chromatographic chamber for horizontal development; commercially available chambers consist of a solvent-proof body with a tray for the mobile phase and a tight-fitting glass lid; the mobile phase is transferred from the tray to the layer via an exchangeable sintered-glass plate.

* See 4A. Classical method, page 18.
Method

Protect the chromatographic chamber from draughts and direct sunlight, and keep it at constant room temperature. Place a clean, dry sintered-glass plate into the chamber. (The sintered-glass plate should be cleaned with acetone R and dried, after each use).

If saturation is required, line the floor of the chamber with filter paper and place the required quantity of saturation liquid into it. Should more intensive saturation of the chamber be required use, in addition, a ready-made silica gel plate, cut to size, and saturated with the liquid. As an alternative a sandwich-type plate can be used with a dry silica gel plate.

Using a micropipette, apply the solutions being examined onto the starting line, about 7 mm from the edges of the plate and not less than 5–10 mm from each other. They should form a line parallel to the lower edge of the plate. The spots should be as small as possible and preferably no more than 1 mm in diameter. Mark, as indicated in the test procedures the desired distance the mobile phase is intended to travel, usually 40 mm from the starting line.

Allow the spots to dry, place the plate into the chamber with the coating downwards as to be in contact across the whole width with the sintered-glass plate. The points of application should reach about 3 mm from the edge of the sintered-glass plate. Close the chamber with the lid leaving the trough for the mobile phase open. Using a pipette place the required volume of previously-mixed homogeneous mobile phase, usually 1–2 ml into the trough and immediately close the chamber. Develop the chromatogram allowing the solvent to travel the specified distance at room temperature, unless otherwise indicated in the test procedure. Remove the plate, mark the position of the solvent front and allow the solvent to evaporate at room temperature or as given for the individual plant material.

Diagram of horizontal-separating chamber

5. Determination of Ash

The presence of ash in medicinal plant materials is determined by three different methods: total ash, acid-insoluble ash or water-soluble ash.

1. Total ash

This test is designed to measure the amount of material remaining after ignition. "Physiological ash" is derived from the plant tissue itself and "non-physiological" ash is the residue after ignition of the extraneous matter (e.g. sand and soil) adhering to the surface. The present procedure determines both kinds of ashes and is referred to as the "total ash" test.
2. Acid-insoluble ash

Acid-insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid, and igniting the washed insoluble matter left on the filter. This determination measures the presence of silica, especially sand and siliceous earth.

3. Water-soluble ash

Water-soluble ash is the calculated difference in weight between the total ash and the residue remaining after treatment of the total ash with water.

Recommended procedures

Total ash

Weigh accurately into a previously ignited and tared crucible, usually platinum or silica, about 2–4 g of the ground material. Spread the material in an even layer in the crucible, ignite the material by gradually increasing the heat to 500–600 °C until free from carbon, cool in a desiccator and weigh. If carbon-free ash cannot be obtained in this manner, cool the crucible and moisten the residue with about 2 ml of water or a saturated solution of ammonium nitrate R. Dry on a water-bath, then on a hot-plate and ignite to constant weight. Allow the residue to cool in a suitable desiccator for 30 minutes and weigh without delay. Calculate the content of total ash in mg per g of air-dried material.

Acid-insoluble ash

To the crucible containing the total ash, add 25 ml of hydrochloric acid (−70 g/l) TS, cover with a watchglass and boil gently for 5 minutes. Rinse the watchglass with 5 ml of hot water and add this liquid to the crucible. Collect the insoluble matter on an ashless filter-paper and wash with hot water until the filtrate is neutral. Transfer the filter-paper containing the insoluble matter to the original crucible, dry on a hot-plate and ignite to constant weight. Allow the residue to cool in a suitable desiccator for 30 minutes and weigh without delay. Calculate the content of acid-insoluble ash in mg per g of air-dried material.

Water-soluble ash

To the crucible containing the total ash, add 25 ml of water and boil for 5 minutes. Collect the insoluble matter in a sintered-glass crucible or on an ashless filter-paper. Wash with hot water and ignite for 15 minutes at a temperature not exceeding 450 °C. Subtract the weight of this residue in mg obtained from the weight of total ash. Calculate the content of water-soluble ash in mg per g of air-dried material.

6. DETERMINATION OF EXTRACTABLE MATTER

This method determines the amount of active constituents in a given amount of medicinal plant material extracted with solvents. It is employed for those materials for which as yet no suitable chemical or biological assay method exists.

Recommended procedures

Method 1 (Hot extraction method)

Weigh accurately, about 4.0 g of coarsely-powdered air-dried material to a glass-stoppered conical flask. Add 100 ml of water and weigh. Shake well and allow to stand for 1 hour. Attach a reflux condenser to the flask and boil gently for 1 hour, cool and weigh. Readjust to the original weight with the required solvent for the given plant material. Shake well and filter rapidly through a dry filter. Transfer 25 ml of the filtrate to a tared flat-bottomed dish and evaporate to dryness on a water-bath. Dry at 105 °C for 6 hours, cool in a desiccator for 30 minutes and weigh without delay. Calculate the content of extractable matter in mg per g of air-dried material.
Method 2 (Cold maceration method)

Weigh accurately, about 4.0 g of coarsely-powdered air-dried material to a glass-stoppered conical flask. Macerate with 100 ml of the required solvent for the given plant material for 6 hours, shaking frequently, then allowing to stand for 18 hours. Filter rapidly taking care not to lose any solvent, transfer 25 ml of the filtrate to a tared flat-bottomed dish and evaporate to dryness on a water-bath. Dry at 105 °C for 6 hours, cool in a desiccator for 30 minutes and weigh without delay. Calculate the content of extractable matter in mg per g of air-dried material.

For ethanol-soluble extractable matter, use the concentration of solvent as indicated in the test procedures; for water-soluble extractable matter, use water as the solvent. The use of other solvents is indicated in the test procedures.

7. DETERMINATION OF WATER AND VOLATILE MATTER

An excess of water in medicinal plant materials will lead to microbial growth, the presence of fungi or insects, and deterioration following hydrolysis. Therefore, limits for the amount of water should be set for every given plant material. This is especially important for material which absorbs moisture easily or deteriorates quickly in the presence of water.

The azeotropic method

This method gives a direct measurement of the water present in the material being examined. When the sample is distilled together with an immiscible solvent, such as toluene R or xylene R, the water present in the sample is absorbed by the solvent, they are distilled together and separated in the receiving tube on cooling. If the solvent is anhydrous, water may be absorbed in it and the results might be false. Therefore it is advisable to saturate the solvent with water before use.

Loss on drying

This test determines both water and volatile matter. Drying can be carried out either by heating to 100–105 °C or by drying in a desiccator over phosphorus pentoxide R under atmospheric or reduced pressure at room temperature for a specified period of time. The desiccation method is especially useful for materials that melt to a sticky mass at elevated temperatures.

Recommended procedures

Preparation of material being examined

Prepare a suitable quantity of the sample by cutting, granulating or shredding the unground or unpowdered material, so that the thickness of the parts does not exceed 3 mm. Seeds or fruits smaller than 3 mm should be cracked. Avoid the use of high-speed mills in preparing the sample, and exercise care so that no appreciable amount of moisture is lost during preparation. It is important that the portion taken is large enough to be a representative sample.

Azeotrophic method (toluene distillation)

The apparatus (see below) consists of a glass flask (A) connected by a tube (D) to a cylindrical tube (B) fitted with a graduated receiving tube (E) and a reflux condenser (C). The receiving tube (E) is graduated in 0.1 ml subdivisions so that the error of readings does not exceed 0.05 ml. The preferred source of heat is an electric heater with a rheostat control, or an oil-bath. The upper portion of the flask and the connecting tube may be insulated.

Thoroughly clean the receiving tube and the condenser of the apparatus, rinse with water and dry.
Introduce 200 ml of toluene R and about 2 ml of water into a dry flask. Heat the flask and distil over a period of 2 hours, allow to cool for about 30 minutes and read off the volume of water to an accuracy of 0.05 ml (1st distillation).

Weigh accurately a quantity of the material expected to give about 2–3 ml of water and transfer to the flask. (For weighing material having a pasty character, use a boat of metal foil). Add a few pieces of porous material and heat the flask gently for 15 minutes. When boiling begins, distil at a rate of 2 drops per second until most of the water has distilled over, then increase the rate of distillation to about 4 drops per second. As soon as the water has been completely distilled, rinse the inside of the condenser tube with toluene R. Continue the distillation for 5 more minutes, remove the heat, allow the receiving tube to cool to room temperature and dislodge any droplets of water which adhere to the walls of the receiving tube. Allow the water- and toluene-layers to separate and read off the volume of water (2nd distillation). Calculate the content of water in % using the formula:

\[
\frac{100 \cdot (n^1 - n)}{w}
\]

where
- \( w \) = the weight in g of the material being examined
- \( n \) = the number of ml of water obtained in the first distillation
- \( n^1 \) = the total number of ml of water obtained in both distillations

Apparatus used for the azeotropic method
(dimensions in mm)

The drawing will be included in the printed text.
Loss on drying (gravimetric determination)

Weigh accurately about 2–5 g of the prepared material, or the quantity given in the test procedures, in a previously dried and tared flat weighing bottle. Dry the sample by one of the following techniques:

(a) dry in an oven at 100–105 °C for 5 hours;
(b) dry in a desiccator over phosphorus pentoxide R under atmospheric pressure or reduced pressure and at room temperature;

Dry until two consecutive weighings do not differ by more than 5 mg, unless otherwise required in the test procedure. Calculate the loss of weight in mg per g of air-dried material.

8. DETERMINATION OF VOLATILE OILS

Volatile oils are characterized by their odour, oil-like appearance and ability to volatilize at room temperature. Chemically, they are usually composed of mixtures, such as monoterpenes, sesquiterpenes and their oxygenated derivatives. Aromatic compounds may also occur and predominate in certain volatile oils.

Since they were considered as the "essence" of the plant material, often having biological activities, they are also known as "essential oils". The term "volatile oil" is preferred because it is more specific and describes the physical properties.

In order to determine the volume of oil, the plant material is distilled with water and the distillate is collected in a graduated tube. The aqueous portion separates automatically and is returned to the distillation flask. If volatile oils possess a mass density higher than or near to that of water, or are difficult to separate from the aqueous phase due to the formation of emulsions, a solvent with a low mass density and a suitable boiling point may be added to the measuring tube. This will permit volatile oils to dissolve and thus bring them afloat in the aqueous phase.

Recommended procedure

Carry out the determination by steam distillation (see apparatus below). Collect the distillate in a graduated tube, using xylene R or the given solvent for the individual material, and allow the aqueous phase to recirculate into the distillation flask. For all determinations read the rate of distillation from the marks engraved on the apparatus.

Apparatus

The apparatus is constructed using resistant glass with a low expansion coefficient and the following dimensions:

(a) a round-bottomed, short-necked flask with a size of 500 or 1000 ml, the internal diameter of the ground-glass neck being 34.35–35.65 mm at the widest end;
(b) the apparatus consists of the following sections fused into one piece:

- a vertical tube (AC), 210–260 mm in length and an internal diameter of 13–15 mm;
- a bent tube (CDE), in which the distances between CD and DE are each 145–155 mm long and having an internal diameter of 7–8 mm;
- a bulb-condenser (FG), 145–155 mm in length, having a diameter of 8–10 mm at the restricted ends, and the bulb having a diameter of 15 mm;
- a vented stopper (K) and an orifice (K) with an internal diameter of 7.42–7.58 mm, the wide end being of ground-glass. The external diameter of the ground stopper should be 4.95–5.05 mm;
- a vented stopper (K') and an orifice (K) with an internal diameter of 7.42–7.58 mm, the wide end being of ground-glass. The external diameter of the ground stopper should be 4.95–5.05 mm;
- a tube (GH) having an internal diameter of 7–8 mm and 30–40 mm in length, bent to an angle of 30–40° (GHK) together with a similar tube (HK);
- a pear-shaped bulb (J) having a volume of 5 ml;
- a tube having a 1-ml volume (JL), graduated over 110–120 mm with subdivisions of 0.05 ml;
- a bulb-like swelling (L), having a volume of about 2 ml;
- a three-way tap (M);
- a connecting tube (BM), with an internal diameter of 7–8 mm, which is fitted in the middle with a security tube (N). The junction (B) should be placed 20–25 mm higher than the uppermost graduation;
- the condenser should fit into the flask by means of a ground-glass insert, not less than 34 mm in length and at its narrower end having an external diameter of 31.0–31.2 mm;

(c) either a burner allowing a fine control and fitted with a flue, or an electrical heating device;

(d) a vertical support with a horizontal ring covered with an insulating material.

Before use, clean the apparatus thoroughly by successive washings e.g. with acetone R, a suitable detergent, then rinse with water, drain the apparatus and assemble it in a suitable place.

**Apparatus**

(dimensions in mm)
Preparation of the sample

The preparation of the sample depends on the texture of the material and the location of the volatile oils. Hard and compact plant material (e.g. bark, roots or rhizomes) or material containing volatile oils in the cells or small cavities of the tissue, should be coarsely powdered; thick leaves should be finely cut or lightly bruised; materials such as bitter orange peel and lemon peel are preferably crushed under water, as the volatile oils contained in large schizolygogenous cavities is easily lost during the process of comminution. Material consisting of thin floral parts or thin lamina or containing volatile oils in the epidermal glands should only be distilled as a whole.

Method

Place the volume of distillation liquid given for the individual plant material in the flask, add a few pieces of porous porcelain and join the condenser to the apparatus. Introduce water by tube (N) until it reaches level (B). Remove stopper (K') and introduce the volume of xylene R or the solvent given for the material, using a graduated pipette with its tip at the bottom of tube (K). Replace stopper (K'), heat the liquid in the flask until it begins to boil and adjust the distillation rate to 2–3 ml per minute, unless otherwise given in the test procedure.

For the determination of the distillation rate, lower the level of water while distilling by means of a three-way tap until the meniscus is at the level of the lower mark (see Figure 2). Close the tap and simultaneously start a stop-watch. As soon as the level in the bulb reaches the level of the upper mark, stop the watch and note the time necessary to fill the bulb between the two marks. Open the tap and continue the distillation. Stop the heating after 30 minutes, turn off the heater and at least 10 minutes later, record the volume of solvent (xylene) collected in the graduated tube.

Introduce the required quantity of the individual plant material into the flask and continue the distillation as described above for the time and rate given in the instructions. After a further 10 minutes record the volume of oil collected in the graduated tube and subtract the volume of solvent (xylene) previously noted. The difference represents the volume of volatile oils in the weight of plant material taken. Calculate the content in ml of oil per 100 g of plant material.

9. DETERMINATION OF BITTERNESS VALUE

Medicinal plant materials which have a strong bitter taste ("bitters") are employed in therapeutics for their bitter effect and are utilized mostly as appetizing agents. The bitter effect stimulates secretions in the gastrointestinal tract, especially of gastric juice. Other, lesser effects of some "bitters" have also recently been identified.

Bitter substances can be determined chemically. However, since they are mostly composed of two or more constituents with various degrees of bitterness, it is first necessary to measure the total bitterness by using a biological method.

The bitter properties of plant material are determined by comparing the threshold bitter concentration of an extract of the materials with that of a diluted solution of quinine hydrochloride R. The bitterness value is expressed in terms of units, equivalent to a diluted solution, containing 1 in 2000, of quinine hydrochloride R.

Safe drinking water should be used as a vehicle for the extraction of plant materials and for the mouthwash after each tasting. Taste buds dull quickly if distilled water is used. The hardness of water rarely has any significant influence on the bitterness.

Sensitivity to bitterness is variable from person to person, and even for the same person it may vary at different times (due to fatigue, smoking, or after eating strongly flavoured food). Therefore, the determination of the threshold bitter concentration of the material to be tested against quinine hydrochloride must be carried out by the same person within a short space of time. The bitter sensation is not felt by the whole surface of the tongue, but is limited to the upper and lateral parts of the base of the tongue. The person carrying out the test requires a certain amount of training to determine the threshold concentration of the solution. A determination is first made of the threshold concentration of quinine hydrochloride R, and then of the material to be tested. A person who does
not appreciate a bitter sensation when tasting a solution containing 0.058 mg of quinine hydrochloride R is not suitable for this determination.

The preparation of the stock solution of each individual plant material (Sₜ) should be given in the test procedure. In single test series, unless otherwise indicated, always start the determination with the lowest concentration in order to retain sufficient sensitivity of the taste buds.

**Recommended procedure**

*Caution: This test should not be carried out until the identity of the plant material is confirmed.*

**Preparation of solutions**

- Stock and diluted quinine hydrochloride solutions

Dissolve 0.100 g of quinine hydrochloride R in sufficient safe drinking water to produce 100 ml. Further dilute 5 ml of this solution to 500 ml with safe drinking water. This stock solution of quinine hydrochloride (Sₚ) contains 0.01 mg/ml. Use 9 test-tubes for the serial dilution as indicated in the table below for the initial test.

**Initial test**

<table>
<thead>
<tr>
<th>tube no.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sₚ [ml]</td>
<td>4.2</td>
<td>4.4</td>
<td>4.6</td>
<td>4.8</td>
<td>5.0</td>
<td>5.2</td>
<td>5.4</td>
<td>5.6</td>
<td>5.8</td>
</tr>
<tr>
<td>safe drinking water [ml]</td>
<td>5.8</td>
<td>5.6</td>
<td>5.4</td>
<td>5.2</td>
<td>5.0</td>
<td>4.8</td>
<td>4.6</td>
<td>4.4</td>
<td>4.2</td>
</tr>
<tr>
<td>quinine hydrochloride in 10 ml of solution (= c)[ml]</td>
<td>0.042</td>
<td>0.044</td>
<td>0.046</td>
<td>0.048</td>
<td>0.050</td>
<td>0.052</td>
<td>0.054</td>
<td>0.056</td>
<td>0.058</td>
</tr>
</tbody>
</table>

- Stock and diluted solutions of the plant material

Prepare the solution as given in the test procedure for each individual material (Sₜ). Use 10 test-tubes for the serial dilutions as indicated in the table below for the second test.

**Second test**

<table>
<thead>
<tr>
<th>tube no.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sₜ (= b) [ml]</td>
<td>1.00</td>
<td>2.00</td>
<td>3.00</td>
<td>4.00</td>
<td>5.00</td>
<td>6.00</td>
<td>7.00</td>
<td>8.00</td>
<td>9.00</td>
<td>10.0</td>
</tr>
<tr>
<td>safe drinking water [ml]</td>
<td>9.00</td>
<td>8.00</td>
<td>7.00</td>
<td>6.00</td>
<td>5.00</td>
<td>4.00</td>
<td>3.00</td>
<td>2.00</td>
<td>1.00</td>
<td>-</td>
</tr>
</tbody>
</table>

**Method**

After rinsing the mouth with safe drinking water, taste 10 ml of the dilution swirling it in the mouth mainly near the base of the tongue for 30 seconds. Unless otherwise indicated in the test procedure, always begin with the lowest concentration of the serial dilution. If the bitter sensation is no longer felt, withdraw the solution and wait for 1 minute to ascertain that there is no delayed sensitivity. Then rinse with safe drinking water. The next highest concentration of dilution should not be tasted until at least
10 minutes have passed. The threshold bitter concentration is the lowest concentration of dilution at which a material still provokes a bitter sensation. After the first series of tests, rinse the mouth thoroughly with safe drinking water, until no bitter sensation remains and wait for at least 10 minutes before carrying out the second series of tests.

In this series of testing and in order to save time, it is advisable to first ascertain whether the solution in tube no. 5 (containing 5 ml of $S_T$ in 10 ml) gives a bitter sensation. If noted, find the threshold bitter concentration of the material by tasting the dilutions in tubes nos. 1–4. If the solution in tube no. 5 does not give a bitter sensation, find the threshold bitter concentration in the dilutions of tubes nos. 6–10.

All solutions and the safe drinking water for mouthwashing should be at 20–25 °C.

Calculation

\[
\frac{2000 \times c}{a \times b} = \text{bitterness value in units/g}
\]

where

- $a = \text{the quantity of material in mg/ml of } S_T$,
- $b = \text{the volume of } S_T \text{ in ml per 10 ml of the dilution of threshold bitter concentration, and}$
- $c = \text{the quantity of quinine hydrochloride } R \text{ in mg per 10 ml of the dilution of threshold bitter concentration.}$

10. DETERMINATION OF HAEMOLYTIC ACTIVITY

Many medicinal plant materials, especially those derived from Caryophyllaceae, Araliaceae, Sapindaceae, Primulaceae, and Dioscoreaceae contain saponins. The most characteristic property of saponins is their ability to cause haemolysis. Haemoglobin diffuses into the surrounding medium when a change occurs in the erythrocyte membranes.

The haemolytic activity of plant materials, or a preparation containing saponins, is determined by comparison with that of a reference material of saponin $R$ which has a haemolytic activity of 1000 units per g. A suspension of red blood cells is mixed with equal volumes of a serial dilution of the plant material extract. The lowest concentration to effect complete haemolysis is determined, after allowing to stand for a given period of time. A similar test is carried out simultaneously with saponin $R$.

Procedures proposed for the determination of the haemolytic activity of saponaceous medicinal plant material are all based on the same principle with few varying details, e.g. the source of red blood cells, methods for the preparation of the red blood cell suspension and the plant material extract, the defined haemolytic activity of the reference material of saponin, and the experimental method. In order to obtain reliable results, it is essential to standardize the experimental conditions, and especially to determine the haemolytic activity by comparison with that of saponin $R$.

**Recommended procedure**

For the preparation of the blood suspension fill a glass-stoppered flask to 1/10th of its volume with sodium citrate (36.5 g/l) TS, swirling to ensure that the inside of the flask is thoroughly moistened. Introduce the freshly shed blood of a healthy ox and shake immediately. The citrated blood prepared as such can be stored for about 8 days at 2–4 °C. Using a 50–ml volumetric flask carefully dilute 1 ml of the citrated blood with sufficient phosphate buffer pH 7.4 TS to volume. This diluted blood suspension (2% solution) can be used as long as the supernatant fluid remains clear and colourless. It must be stored at a cool temperature.

For the reference solution, transfer about 10 mg of saponin $R$, accurately weighed, to a volumetric flask and add sufficient phosphate buffer pH 7.4 TS to produce 100 ml. This solution should be freshly prepared.
The extract of plant materials and dilutions should be prepared as given in the test procedures for each individual material, using phosphate buffer pH 7.4 TS.

Preliminary test

Prepare a serial dilution of the plant material extract with phosphate buffer pH 7.4 TS and blood suspension (2%) using 4 test-tubes as given in the following table:

<table>
<thead>
<tr>
<th>tube no.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>plant material extract [ml]</td>
<td>0.10</td>
<td>0.20</td>
<td>0.50</td>
<td>1.00</td>
</tr>
<tr>
<td>phosphate buffer pH 7.4 TS [ml]</td>
<td>0.90</td>
<td>0.80</td>
<td>0.50</td>
<td>-</td>
</tr>
<tr>
<td>blood suspension (2%) [ml]</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>

As soon as the tubes have been prepared, gently invert them to mix and avoid the formation of foam. Shake again after a 30-minute interval and allow to stand for 6 hours at room temperature. Examine the tubes and record in which dilution total haemolysis has occurred, indicated by a clear, red solution without any deposit of red cells.

- If total haemolysis is only observed in tube no. 4, use the original plant material extract directly for the main test.
- If total haemolysis is observed in tubes 3 and 4, prepare a two-fold dilution of the original plant material extract with phosphate buffer pH 7.4 TS.
- If total haemolysis is observed in tubes 2, 3 and 4, prepare a five-fold dilution as described above.
- If, after 6 hours, all 4 tubes contain a clear, red solution, prepare a ten-fold dilution and carry out the preliminary test as described above.
- If total haemolysis is not observed in any of the tubes, repeat the preliminary test using a more concentrated plant material extract.

Main test

Prepare a serial dilution of the plant material extract, undiluted or diluted as determined by the preliminary test, with phosphate buffer pH 7.4 TS and blood suspension (2%) using 13 test-tubes as given in the following table:

<table>
<thead>
<tr>
<th>tube no.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>drug extract (diluted if necessary) [ml]</td>
<td>0.40</td>
<td>0.45</td>
<td>0.50</td>
<td>0.55</td>
<td>0.60</td>
<td>0.65</td>
<td>0.70</td>
<td>0.75</td>
<td>0.80</td>
<td>0.85</td>
<td>0.90</td>
<td>0.95</td>
<td>1.00</td>
</tr>
<tr>
<td>phosphate buffer pH 7.4 TS [ml]</td>
<td>0.60</td>
<td>0.55</td>
<td>0.50</td>
<td>0.45</td>
<td>0.40</td>
<td>0.35</td>
<td>0.30</td>
<td>0.25</td>
<td>0.20</td>
<td>0.15</td>
<td>0.10</td>
<td>0.05</td>
<td>-</td>
</tr>
<tr>
<td>blood suspension (2%) [ml]</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
</tbody>
</table>
Carry out the dilutions and evaluations as in the preliminary test but observe the results after 24 hours. Calculate the amount of medicinal plant material in g, or the preparation in g or ml, which produces total haemolysis (b).

**Test for saponin**

To eliminate the effect of individual variations in resistance of the blood suspension to saponin solutions, prepare a series of dilutions of saponin R in the same manner as described above for the plant material extract. Calculate the quantity of saponin R in g which produces total haemolysis (a).

**Calculation**

The haemolytic activity of the medicinal plant material is obtained using the following formula:

\[
\text{Haemolytic activity} = 1000 \times \frac{a}{b}
\]

where

- 1000 = the defined haemolytic activity of saponin R in relation to ox blood.
- a = quantity of saponin R in g
- b = quantity of plant material in g

**11. DETERMINATION OF TANNINS**

Tannins (or tanning substances) are substances capable of turning animal hides into leather by binding proteins to form water-insoluble substances which are resistant to proteolytic enzymes. This process, when applied to living tissue, is known as an "astringent" action and is the reason for the therapeutic application of tannins.

Chemically, tannins are complex substances; they usually occur as mixtures of polyphenols that are very difficult to separate and crystallize. They are easily oxidized and polymerized in solution and if this happens they lose much of their astringent effect and are therefore of little therapeutic value.

**Recommended procedure**

For the preparation of the plant material extract, introduce the given quantity in the test procedure of each individual material, previously powdered to a known fineness and weighed accurately, into a conical flask. Add 150 ml of water and heat over a boiling water-bath for 30 minutes. Cool, transfer the mixture to a 250–ml volumetric flask with water and dilute to volume with water. Allow the solid material to settle and filter the liquid through a filter-paper, diameter 12 cm, discarding the first 50 ml of the filtrate.

Determine the total amount of material extractable into water, by evaporating 50.0 ml of the plant material extract to dryness, and drying the residue in an oven at 105 °C for 4 hours and weigh (T1).

Determine the amount of plant material not bound to the hide powder extractable into water by taking 80.0 ml of the plant material extract, add 6.0 g of hide powder R and shake well for 60 minutes. Filter and evaporate 50.0 ml of the clear filtrate to dryness. Dry the residue in an oven at 105 °C and weigh (T2).

Determine the solubility of hide powder by taking 6.0 g of hide powder R, add 80.0 ml of water and shake well for 60 minutes. Filter and evaporate 50.0 ml of the clear filtrate to dryness. Dry the residue in an oven at 105 °C and weigh (T0).
Calculation:

\[
\text{Quantity of tannins in } \% = \frac{[T_1 - (T_2 + T_0)] \times 500}{w}
\]

where \( w \) = the weight of the plant material in g.

12. DETERMINATION OF SWELLING INDEX

Many medicinal plant materials are of specific therapeutic or pharmaceutical utility for their swelling properties, especially gums and those containing an appreciable amount of mucilage, pectin and hemicellulose.

The swelling index is the volume in ml taken up by the swelling of 1 g of plant material under specified conditions.

The determination is based on the addition of water or a swelling agent as given in the test procedure for each individual plant material (either whole, cut or pulverized). Using a glass-stoppered measuring cylinder, the material is shaken repeatedly for 1 hour and allowed to stand for a required period of time. The volume of the mixture (in ml) is then read.

The dimensions of the measuring cylinders should be given, especially the internal diameter of the graduated part, and the width of the scale.

The mixing of whole plant material with the swelling agent is easy to perform, but cut or pulverized material requires rigorous shaking at determined intervals to ensure even distribution of the material in the swelling agent.

Recommended procedure

Carry out simultaneously not less than 3 determinations of any material. Introduce the quantity of the individual plant material, previously reduced to the required fineness and accurately weighed, into a 25-ml glass-stoppered measuring cylinder. The length of the graduated portion of the cylinder should be about 125 mm, the internal diameter about 16 mm subdivided in 0.2 ml and marked from 0 to 25 ml in an upwards direction. Add 25 ml of water unless otherwise indicated in the test procedure, and shake the mixture thoroughly at intervals of every 10 minutes for 1 hour. Allow to stand for 3 hours at room temperature, or as otherwise given. Measure the volume in ml occupied by the plant material, including any sticky mucilage. Calculate the mean value of the individual determinations, related to 1 g of plant material.

13. DETERMINATION OF FOAMING INDEX

Many medicinal plant materials contain saponins that can cause a persistent foam when an aqueous decoction is shaken. In order to measure the foaming ability of an aqueous decoction of plant materials and their extracts a foaming index is established.

Recommended procedure

Reduce about 1 g of the plant material to a coarse powder (sieve size no. 1250), weigh accurately and transfer to a 500-ml conical flask containing 100 ml of boiling water. Maintain at moderate boiling for 30 minutes. Cool and filter into a 100-ml volumetric flask and add sufficient water through the filter to dilute the volume to 100 ml.

Place the above decoction into 10 stoppered test-tubes (height 16 cm, diameter 16 mm) in a series of successive portions of 1, 2, 3, up to 10 ml and adjust the volume of the liquid in each tube with water
to 10 ml. Stopper the tubes and shake them in a lengthwise motion for 15 seconds, 2 frequencies per second. Allow to stand for 15 minutes and measure the height of the foam.

- If the height of the foam in every tube is less than 1 cm, the foaming index is less than 100.
- If in any tube a height of foam of 1 cm is measured, the dilution of the plant material in this tube [a] is the index sought. If this tube is the first or second tube in a series, it is necessary to have an intermediate dilution prepared in a similar manner to obtain a more precise result.
- If the height of the foam is more than 1 cm in every tube, the foaming index is over 1000. In this case the determination needs to be made on a new series of dilutions of the decoction in order to obtain a result.

Calculation

$$\text{Foaming index} = \frac{1000}{a}$$

where a is the volume in ml of the decoction used for preparing the dilution in the tube where foaming is observed.

14. Determination of Pesticide Residues

Medicinal plant materials are liable to be affected by pesticide residues which accumulate from agricultural practices of spraying, treating soils during cultivation, and through the administration of fumigants during storage. In this context it is recommended that every country producing medicinal plant materials (naturally grown and/or cultivated) should have at least one control laboratory available capable of performing the determination of pesticides in accordance with the procedure below.

Since many medicinal preparations of plant origin are taken over long periods of time, limits for pesticide residues should be established following the recommendations of the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) (Codex Alimentarius) which have already been established for food and animal feed (7). These recommended guidelines include the analytical methodology for the assessment of specific pesticide residues.

Classification of Pesticides

Different classifications of pesticides exist (8, 9): A classification based on the chemical composition and/or structure of the pesticide is more useful for analytical chemists, e.g.:

- chlorinated hydrocarbons and related pesticides: aldrin, benzene hexachloride (BHC) or hexachlorocyclohexane (HCH), heptachlor, hexachlorobenzene (HCB), chlordane, DDT*, dieldrin, endrin, heptachlor, lindane, methoxychlor, toxaphene (camphorarlochlouren)
- chlorinated phenoxyalkanoic acid herbicides: 2,4-D; 2,4,5-T
- organophosphorus pesticides: carbophenothion*, chlorothion, coumaphos*, demeton, dichlorvos, dimethoate, ethion, fenchlorphos*, malathion, methyl parathion, parathion
- carbamate insecticides: carbaryl*
- dithiocarbamate fungicides: ferbam, maneb, nabam, thiram, zineb, ziram
- inorganic pesticides: aluminium phosphide, calcium arsenate, lead arsenate

* The International Nonproprietary Name (INN) of these substance are carbaril, carbofenthion, clofenotane, coumaryls, fencloros, respectively.
- miscellaneous: bromopropylate, chloropiparin, ethylene dibromide, ethylene oxide, methyl bromide.
- pesticides of plant origin: tobacco leaf and nicotine; pyrethrum flower, extract and pyrethroids; Derris root and rotenoids

Only the chlorinated hydrocarbons and related pesticides (e.g. aldrin, BHC, chlordane, dieldrin, DDT) and a few organophosphorus pesticides (e.g. carbofuran) retain a long residual action. Most other pesticides have very short residual actions. Therefore it is suggested, that where the length of exposure to pesticides is unknown, the medicinal plant material should be tested for the presence, or the content determined, of organically-bound chlorine and phosphorus.

Alternative classification of pesticides may be based on their intended use:
- insecticides
- fungicides and nematocides
- herbicides
- other pesticides (e.g. acaricides, molluscicides, rodenticides)
- fumigants (e.g. ethylene oxide, ethylene chlorohydrin, methyl bromide)

**Methods for the determination of pesticide residues**

Chromatography (mostly column and gas) is recommended as a principal method for the determination of pesticide residues. Samples are extracted by a standard procedure, impurities are removed by partition and/or adsorption, and the presence of a moderately-broad spectrum of pesticides is measured in a single determination. However, these techniques are not universally applicable. Some pesticides are carried through the extraction and clean-up procedures satisfactorily, others are recovered with a poor yield, and some are lost entirely. In chromatography the separations may not always be complete, pesticides may decompose or metabolize, and many of these products are still unknown. Consequently, as a result of limitations in the analytical technique and incomplete knowledge of pesticide interaction with the environment, it is not yet possible to apply an integrated set of methods which will satisfy all situations.

Therefore, it is desirable to be able to test plant materials of unknown history for broad groups of compounds rather than testing for individual pesticides. A variety of methods meet these requirements. Chlorinated hydrocarbons and other pesticides containing chlorine in the molecule, for example, can be detected by the measurement of total organic chlorine; insecticides containing phosphate can be measured by analysis for total organic phosphorus, while pesticides containing arsenic and lead can be detected by measurement of total arsenic or lead, respectively. Similarly, the measurement in a sample of total bound carbon disulfide will provide information on whether residues of the dithiocarbamate family of fungicides are present.

On the other hand, if the pesticide to which the plant material has been exposed is known or can be identified by suitable means, a well-established method for the determination of that particular pesticide residue should be employed.

**General aspects on the analytical methodology**

1. After collecting the samples they should be tested as quickly as possible before any physical and chemical changes occur. If prolonged storage is envisaged, the samples should preferable be stored in air-tight containers under refrigeration.

2. Light can cause degradation of many pesticides, therefore it is advisable to protect the samples and any extracts or solutions from undue exposure.

3. The type of container or wrapping material used should not interfere with the sample or cause erroneous analytical results.

4. Solvents and reagent used in the analytical method should be free of substances that may interfere with the reaction, alter the results or that may provoke degradation of the pesticide residue in the sample. Usually, it is necessary to employ specially purified solvents or to freshly distil them in an all-
glass apparatus. Blank determinations with the solvents should be carried out, concentrating and testing them as described in the specified method.

5. The simplest and quickest procedure should be used to separate unwanted material from the sample (clean-up procedure) in order to save time when many samples have to be tested.

6. The process of concentrating solutions should be made with great care, especially during the evaporation of the last traces of solvent in order to avoid losses of pesticide residues. For this reason, it is often not advisable to remove the last traces of solvent. Agents, such as mineral oil or other oils of low volatility that may help to preserve the solution could be added to retard the loss of the relatively volatile pesticides, especially when the last traces of solvent have evaporated. However, these agents, while satisfactory in colorimetric procedures, are usually not desirable in gas chromatographic methods. Heat-labile compounds may require a rotary-vacuum apparatus to evaporate them.

Maximum limit of pesticide residues for medicinal plant materials

A maximum residue limit (MRL) for medicinal plant materials, including their preparations such as tinctures, extracts, oils, etc. may be defined in line with the limits of pesticide residues set by the FAO/WHO Codex Alimentarius at the lowest level acceptable for vegetable food products. Since medicinal plant materials are usually taken in much smaller quantities than other food products MRL can be calculated based on the maximum acceptable daily intake of pesticides for humans (ADI) and the maximum daily dose of the medicinal plant material (MDD).

Where the nature of the pesticide to which the plant material has been exposed is unknown, it is necessary to determine only the content of total chlorine, and to base the calculation on the MRL of the most toxic chlorine-containing pesticide (e.g. aldrin or dieldrin).

Limits can be determined in two ways:

1. According to the formula:

\[
\frac{\text{ADI} \times 60 \text{ kg (bw)} \times \text{extraction factor}}{\text{MDI} \times \text{safety factor} 100}
\]

where:

- \(\text{ADI}\) = Acceptable Daily Intake, value of FAO/WHO
- \(\text{bw}\) = body weight
- \(\text{MDI}\) = Mean Daily Intake of drug
- \(\text{extraction factor}\) = transition rate of the pesticide from the drug into the preparation
- \(\text{safety factor}\) = arbitrary figure, defined as 100, taking into account of patient at risk

2. In accordance with certain national requirements, such as:

- The Order of Maximum Limits for Pesticides of the Federal Republic of Germany of 24 June 1982, as amended on 18 April 1984 and on 16 October 1989, with special reference to the maximum permissible quantities for tea and products similar to tea; and

- The 1986 guideline values, issued by the Central Determination and Appraisal Unit (ZEBS) of the Federal Health Office in Berlin, for lead, cadmium and mercury.

Despite the high amounts of residue present in certain plant materials, only smaller quantities remain after an extraction process due to low solubility in water or ethanol. The toxicological evaluation of residues in medicinal plant materials can be done according to the intake of material by a patient. The ratio between material and food consumption should not exceed 1% of the permissible total intake for man. For example, it would not be admissible if 30–50% of the acceptable daily intake of heavy metals or pesticide residues, which primarily derive from food and drinking water, were to be accounted for by the additional consumption of medicinal plant materials. These values are reached if the calculation is based on the extremely high residue levels of pesticides or the heavy metals in medicinal plant materials. Therefore, it is important to determine the quantity of residues actually consumed by the
subject in the final dosage form. The quantity of residues in medicinal plant materials is of toxicological significance only if it is ingested in large quantities, such as is the case with linseed.

Similarly, for heavy metal residues, the "theoretical" maximum permissible limits, the Provisional Tolerable Weekly Intake (PTWI) values of FAO/WHO, should be calculated using the following formula:

\[
\frac{\text{ADI (mg/kg) } \times 80 \text{ kg body weight } \times \text{extraction factor}}{\text{average daily drug consumption (kg) } \times 100 \text{ safety factor}} = \text{theoretical MRL}
\]

This formula is based on the acceptable daily intake (ADI) determined by FAO and WHO.

**DETERMINATION OF TOTAL CHLORINE AND PHOSPHORUS**

Most pesticides contain organically-bound chlorine or phosphorus.

**Recommended procedure**

**Preparation of the samples**

Reduce the material to a fine powder, and extract with a mixture of water and acetonitrile R. Pesticides are mostly soluble in this solvent, while most cellular constituents (e.g., cellulose, proteins, amino acids, starch, fats and related compounds) are sparingly soluble and are thus removed. A number of polar and moderately polar compounds may also be dissolved. Therefore, it is necessary to transfer the pesticides to light petroleum R. For pesticides containing chlorine, further purification is seldom required; but for those containing phosphorus, further purification may be necessary by column chromatography, eluting with mixtures of light petroleum R and ether R.

**Preparation of the column**

Use the following support:

Florisil R (grade 60/100 PR), activated at 650 °C. If such material is obtained in bulk, transfer it immediately after opening to a glass-stoppered 500 ml glass jar or bottle or has a foil-lined, screw-top lid. Store in the dark. Before use, heat at not less than 130 °C, cool in a desiccator to room temperature and heat once again to 130 °C after 2 days.

Prepare a Florisil column (22 mm, external diameter) which contains after settling, 10 cm of activated Florisil topped with about 1 cm of anhydrous sodium sulfate R. Pre-wet the column with 40–50 ml of light petroleum R. Place a graduated flask under the column to receive the eluate.

**Procedure**

Grind the material to pass sieve No. 710 or 840 and mix thoroughly. Place 20–50 g of the ground sample into a blender, add 350 ml of acetonitrile R with a water content of 35% (to 350 ml of water add sufficient acetonitrile R to produce 1000 ml). Blend for 5 minutes at a high speed. Filter under vacuum through an appropriate funnel, diameter 12 cm, and fitted with filter-paper, into a 500–ml suction flask.

Transfer the filtrate to a 250–ml measuring cylinder and record the volume. Transfer the measured filtrate to a 1-litre separating funnel and carefully add 100 ml of light petroleum R. Shake vigorously for 1–2 minutes, add 10 ml of sodium chloride (400 g/l) TS and 600 ml of water. Hold the separating funnel in a horizontal position and mix vigorously for 30–45 seconds. Allow to separate, discard the aqueous layer and gently wash the solvent layer with two portions, each of 100 ml of water. Discard the washings, transfer the solvent layer to a 100–ml glass-stoppered cylinder, and record the volume. Add about 15 g of anhydrous sodium sulfate R and shake vigorously. The extract must not remain in contact with this reagent longer than 1 hour. Transfer the extract directly to a Florisil column, or if necessary, reduce the volume first to 5–10 ml. Allow it to pass through the column at a rate of not
more than 5 ml per minute. Carefully rinse the cylinder with about two portions, each of 5 ml of light petroleum R, transfer them to the column, rinse with further small portions of light petroleum R, if necessary, and then elute keeping the same rate with 200 ml of ether/light petroleum TS1. Change the receiver and elute with 200 ml of ether/light petroleum TS2. Again change the receiver and elute with 200 ml of ether/light petroleum TS3.

Evaporate each eluate to a suitable volume, as required, for further testing.

- The first eluate contains chlorinated pesticides (aldrin, BHC, DDE, DDD (TDE), α,α′- and ρ,ρ′- DDT, heptachlor, heptachlor epoxide, lindane, methoxychlor), polychlorinated biphenyls (PCB), and phosphated pesticides (carbophenothion, ethion, and fenchlorphos).
- The second eluate contains chlorinated pesticides (dieldrin and endrin) and phosphated pesticides (methyl parathion, and parathion).
- The third eluate contains phosphated pesticide (malathion).

Combustion of the organic matter

Combustion of the organic matter in oxygen is the preparatory step for the determination of chlorine and phosphorus.

The pesticide is extracted from the sample and purified, if necessary. The extract is concentrated, evaporated to dryness, transferred to a sample holder, and burned in a suitable conical flask flushed with oxygen. The gases produced during combustion are then absorbed in a suitable solution, and the absorbed chlorine as chloride or the absorbed phosphorus as orthophosphate, both determined colorimetrically.

Apparatus

The combustion is carried out in a 1-litre conical flask made of borosilicate glass into the stopper of which is fused one end of a piece of platinum wire about 1 mm in diameter. To the free end of the wire is attached a piece of platinum gauze measuring about 1.5×2 cm to provide a means of holding the sample clear of the absorbing liquid during combustion.

a) Sample holder for chlorine-containing residues

For a small quantity of material, use a sample holder made from a piece of halide-free filter-paper about 5 cm long and 3 cm wide; for a small volume, preferably use a sample holder in the form of a cone made from cellulose acetate film. Prepare the cone as follows: wearing cloth gloves and using a cardboard template cut the film in a shape (a 4 cm radius). Manually pin the two edges together to form a cone. Seal the joined edges using heat and the seam formed is about 5 mm wide. Immerse the seam into acetone R to about one-half of its width for 10 seconds. Remove and dry it immediately in a stream of hot air. Using forceps, wash each cone by dipping them in a 1-litre beaker containing warm sodium hydroxide (240 g/l) TS for 10 seconds at a temperature of about 60 °C. Rinse the cone thoroughly with water and allow to drain dry on a piece of aluminium foil. Place each cone in a clean funnel (diameter 65 mm).

b) Sample holder for phosphorus-containing residues

Use a piece of halide-free filter-paper about 4 cm square as the sample holder.

Combustion of chlorine-containing residues

Transfer an aliquot of the extract as prepared above onto the sample holder which is placed in a funnel using a solvent that will not dissolve the sample holder. Allow the solvent to evaporate. Wearing rubber gloves, remove the sample holder and its dry contents from the funnel, and fold it over and up to form a small packet, about 1 cm² in area, place and secure it in the centre of the platinum gauze. Insert a narrow strip of filter-paper, about 1×3 cm, as a fuse into the top of the holder between the folds of the packet. Add 30 ml of water into the combustion flask. Moisten the neck of the flask with water. Fill the
flask thoroughly with oxygen by means of a tube with its end just above the liquid. Ignite the free end of the paper-strip and immediately insert the stopper. Hold the stopper firmly in place. When vigorous burning has begun, tilt the flask to prevent incompletely burned material from falling into the liquid. Immediately after combustion is completed, shake the flask vigorously for 10 minutes to completely dissolve the combustion products, place a small quantity of water around the rim of the flask, carefully withdraw the stopper. Rinse the stopper, platinum wire, platinum gauze and sides of the flask with water. Transfer the liquid and liquids used for rinsing to a 50-ml volumetric flask and dilute to volume with water.

Combustion of phosphorus-containing residues

Dip the sample holder made from filter-paper into methanoic sodium hydroxide TS, then suspend it in a current of heated air. Immediately transfer about 0.2 ml of an aliquot of the extract as prepared above to the sample holder with the aid of 0.2 ml portions of chloroform R using a micropipette. Allow the solvent to evaporate from the paper, fold it to about 1 cm² size and place it in the platinum gauze. Insert a strip of filter-paper, about 1x3 cm, as a fuse into the top of the holder between the folds of the packet. Add 10 ml of sulfuric acid (−37 g/l) TS to the combustion flask and continue with the combustion as described above. Transfer the solution and the liquid used for rinsing to a 25-ml volumetric flask and dilute to volume with water.

DETERMINATION OF CHLORIDES

Apparatus

A spectrophotometer capable of measuring the absorbance at 460 nm using absorption cells with path-lengths of 2-cm and 10-cm.

Procedure

Place 15 ml of the solution obtained after combustion in a 50-ml conical flask together with 1 ml of ferric ammonium sulfate (0.25 mol/l) VS and 3 ml of mercuric thiocyanate TS. Swirl the contents of the flask and allow to stand for 10 minutes. Transfer a portion of the solution to a 2-cm cell and measure the absorbance at 460 nm using water in the reference cell. The reading should be made promptly to minimize absorption of chloride from the air.

Prepare a standard solution of sodium chloride R containing 5 μg of chloride per ml. Transfer aliquots of this solution (0 ml, 2 ml, 4 ml, 6 ml, 8 ml, and 10 ml) into a series of 50-ml conical flasks and dilute the volumes to 15 ml with water. Develop the colour and measure the absorbances as described above. Prepare a curve, plotting the absorbances against the μg of chlorides per ml of the dilutions and interpolate the content of chlorides in the solutions of the material tested.

DETERMINATION OF PHOSPHATES

The phosphomolybdate method is based on the reaction of phosphate ions with ammonium molybdate to form a molybdophosphate complex, which is subsequently reduced to form a strongly blue-coloured molybdenum complex. The intensity of the blue colour is measured spectrophotometrically. This method is applicable for the determination of any phosphates that have undergone a prior separation procedure.

Naturally-occurring phosphates are present in most samples, and are often not removed during the clean-up procedure. Therefore, in order to obtain background values, it is necessary to proceed with the determination for all samples, even those containing no phosphate-pesticides. These values should be subtracted from the results obtained on testing pesticide residues. Extracts of most uncontaminated materials contain about 0.05–0.1 ppm of phosphorus. Therefore, no contamination of organophosphate pesticides can be assumed for in the range of 0.05–0.1 ppm.
Apparatus

A spectrophotometer capable of measuring the absorbance at 820 nm using an absorption cell with a path-length of 1-cm.

Procedure

Place an aliquot (7 ml) of the solution obtained after combustion in a calibrated 10 ml test-tube. Add 2.2 ml of sulfuric acid (300 g/l) TS and mix the solution well. Add 0.4 ml of ammonium molybdate (40 g/l) TS and swirl the contents of the flask. Then add 0.4 ml of aminophthalosulfonic acid TS and swirl the flask again. Heat the solution to 100 °C for 12 minutes ±2 minutes, cool, and transfer a portion of it to a 1-cm cell. Measure the absorbance at 820 nm using water in the reference cell.

Prepare standard dilutions with a known content of phosphate and measure the absorbance as described above. Prepare a curve, plotting the absorbances against the µg of phosphates per ml of the dilutions and interpolate the content of phosphates in the solutions of the material tested.

QUALITATIVE AND QUANTITATIVE DETERMINATION OF ORGANOCHLORINE PESTICIDES

Recommended procedure

Preparation of sample

Place 20 g of powdered plant material (mesh size 180), accurately weighed, in a 500-ml beaker (tall form), mix with 98 ml of water and allow to macerate for at least 30 minutes. Add 200 ml of acetone R and extract during 5 minutes, while cooling and using a high-speed mixer. Filter the homogenized mixture through a porcelain filter (Büchner funnel, 70 mm diameter) fitted with a filter-paper using a slight vacuum, into a 250-ml graduated cylinder, allowing the process to last no longer than 1 minute or to break it up after this time span, and then measure the volume [V] of the filtrate in ml.

Procedure

Transfer the above filtrate to a 500-ml separating funnel. Add a quantity of sodium chloride R in g equivalent to one-tenth of the volume of the filtrate and 100 ml of dichloromethane R. Shake vigorously for 5 minutes, allow the phases to separate and discard the lower (aqueous) layer. Dry the acetone-dichloromethane phase, transfer it to a 500-ml conical flask, add 25 g of anhydrous sodium sulfate R and swirl occasionally. Next, filter the solution into a 500-ml flask with a ground-glass stopper using a glass funnel (100 mm diameter) containing purified glass-wool and anhydrous sodium sulfate R. Rinse the separating funnel, the conical flask and glass funnel twice with 10 ml of ethyl acetate R. Add 5 ml of 2,2,4-trimethylpentane R, and concentrate the crude extract to about 2 ml in a rotary vacuum evaporator and a water-bath at 30–40 °C. Expel the remaining solvent in a gentle stream of air.

For the purification by gel chromatography, macerate 50 g of suitable beads (e.g. S–X3 bio-beads) in an elution mixture of cyclohexane R and ethyl acetate R (1:1) and pour them into a chromatographic column (length 600 mm, diameter, 25 mm). Rinse the gel bed with the elution mixture under air-free conditions, the column being adapted to a vacuum pump. Dissolve the extract in the flask with 5.0 ml of ethyl acetate R. Add 2 g of anhydrous sodium sulfate R, swirl gently and add 5.0 ml of cyclohexane R. Filter the completely dissolved crude extract through a rapid filter into a ground glass stoppered 10-ml test-tube and close the tube immediately. Then transfer 5.0 ml of the filtrate onto the gel column. Elute the pesticides with the elution mixture at an average rate of 5.0 ml/minute. The components of the plant materials to be separated leave the gel column first, followed by the active pesticidal ingredients. Fractionation must be determined again for each column, using appropriate reference substances.

Discard the first fraction (about 100 ml) containing the impurities. Collect the organochlorine pesticides appearing in the next eluate (about 70 ml) in a ground-glass stoppered flask. Add 10 ml of 2,2,4-trimethylpentane R and concentrate the solution to about 5 ml in a rotary vacuum evaporator and a
water-bath at 30–40 °C. Pipette another 5 ml of 2,2,4-trimethylpentane R into the flask and carefully evaporate the solution to about 1 ml (do not allow to become completely dry).

Calculation:

\[
\text{volume [V] of filtrate} \times \text{sample weight in (g)}
\]

\[
\frac{(\text{g}) \text{ of plant material in the purified extract}}{590}
\]

For the subsequent purification, transfer 1 g of previously deactivated silica gel for column chromatography (70–230 mesh) containing 1.5% of water, to a tube for chromatography (length, 25 cm; internal diameter, 7 mm). Top the column with 10 mm of anhydrous sodium sulfate R and cover with purified glass-wool. Before use rinse the column with 5 ml hexane R. Allow the solvent to reach the surface of the column filling, then transfer quantitatively, by means of a pipette, the purified extract obtained by gel chromatography from the flask to the prepared silica gel column and rinse with 1 ml hexane R. Set the flask aside for subsequent elutions.

Use a 10-ml volumetric flask as the receiver, elute any residues of polychlorinated biphenyls from the column with 10 ml hexane R (= eluate 0). Add 2 ml of an elution mixture composed of toluene R/hexane R (35:65) to the flask and swirl. Quantitatively transfer the solution to the column. Using another 10-ml volumetric flask as the receiver, elute the majority of the organochlorine pesticides from the silica gel column using 6 ml of the same elution mixture. Dilute the flasks to volume with the elution mixture (= eluate 1).

Rinse the flask with 2 ml of toluene R and quantitatively transfer it to the column. Collect the eluate in a third 10-ml volumetric flask. Add 8 ml of toluene R to the flask, swirl and transfer the solution to the silica gel column, elute the remaining organochlorine pesticides using the same receiver. Dilute the flask to volume with toluene R (= eluate 2).

Evaluate the test solutions by capillary gas chromatography using an Electron capture detector (ECD). Confirm the findings obtained for the main column (first separation system) with a second capillary column of different polarity (second separation system).

**Determination by gas chromatography**

A capillary gas chromatograph with an ECD is used for the measurement. Helium R is used as the carrier gas and a mixture of 85.5 of argon-methane R as an auxiliary gas for the detection.

*First separation system*

Silica column, vitreous, 30 m (internal diameter 0.25 mm) packed with a chemically-bound phase of 5% phenyl/95% methyl-polysiloxane* (e.g. DB-5 is a suitable grade, obtainable from J & W Scientific) and using the following temperature programme:

- 60 °C for 0.5 minutes;
- heating rate – 30 °C/minute → to 160 °C maintained at this temperature for 2 minutes;
- heating rate – 2 °C/minute → to 250 °C maintained at this temperature for 5 minutes.

Sample solution to be injected:

- use a "split/split-free" injector and maintain the injection port at a temperature of 240 °C;
- inject a volume of 1 μl at a rate of 30 seconds ("split-free").

Detector temperature: 300 °C.

*Second separation system*

Silica column, vitreous, 15 m (internal diameter 0.25 mm) packed with a chemically-bound phase of 7% cyanopropyl, 7% phenyl, 86% methyl-polysiloxane. Use the following temperature programme:

* DB 1701 is a suitable grade, obtainable from J & W Scientific.
— 60 °C for 0.2 minutes;
— heating rate = 30 °C/minute – to 180 °C maintained at this temperature for 1 minute;
— heating rate = 2 °C/minute – to 250 °C maintained at this temperature for 5 minutes.

Sample solution to be injected:
— use an on-column injector;
— inject a volume of 1 μl.

Detector temperature: 300 °C

Use the "external standard" method for the qualitative and quantitative evaluation of the organochlorine pesticides in the test solutions with reference solutions of the following pesticides:

α-, β-, γ- and δ-hexachlorocyclohexane (HCH); hexachlorobenzene; quintozene; aldrin; dieldrin; endrin; a- and b-endosulfan; endosulfan sulfate; heptachlor, heptachlorepoxide; toxaphene (camphorchlor); DDD, DDE and DDT (both α,α′ and α,α′,α′ isomers); methoxychlor.

The concentration of the residues is calculated by measuring the peak height of the pesticides obtained in the chromatograms:

\[
\text{Residue content (mg/kg)} = \frac{h_t \times 10}{w} \times \frac{w_r}{h_r}
\]

where:

- \(h_t\) = peak height obtained for the test solution in mm
- \(w\) = quantity of sample in the purified extract
- \(w_r\) = quantity in ng of pesticide in the reference solution injected
- \(h_r\) = peak height obtained for the reference solution in mm.

**ANALYSIS OF ESTERS OF ORGANOPHOSPHORUS COMPOUNDS**

The extraction and the clean-up procedures can be performed as described above, but the detection requires a Phosphorus flame ionization Detector (P-FID).

**DETERMINATION OF SPECIFIC PESTICIDE RESIDUES IN PLANT MATERIAL**

**General recommendations**

For the total determination mix thoroughly 1 kg of plant material.

In order to obtain reliable chromatographic results

— repeat the separation using another column;
— use a different separation system;
— use a different detector system;
— apply a coupling technique;
— prepare a derivation;
— perform chromatography with a mixture of the sample and a reference substance;
— change the sample preparation;
— use a fractionated elution during the column-chromatography clean-up procedure of the plant extract and test every fraction by chromatography; and
— compare the distribution coefficient of the material with that of a reference substance.

Prior to the quantitative determination of the material to be tested, check whether linearity exists between the values obtained in the range of 0.1–to 2–fold concentration. Otherwise, prepare another concentration range or evaluate the results by using a reference curve. Use any suitable mechanical or manual technique for the chromatographic determination.
Store the reference solutions protected from light to prevent decomposition. Use glass vessels closed with glass stoppers and keep them in a container saturated with the solvent employed to avoid any increase in concentration due to evaporation. Check the loss by evaporation by interim weighing of the vessels.

Use concentrated reference solutions no longer than 6 months and diluted reference solutions no longer than 4 weeks.

Rate of recovery

The rate of recovery (R) is the fraction in % of the reference material added to the plant material, determined under the conditions of the method described below.

**DETERMINATION OF DESMETRYN, PROMETRYN AND SIMAZINE RESIDUES**

Use the extracts as indicated below for the following plant materials:

<table>
<thead>
<tr>
<th>no.</th>
<th>material</th>
<th>no.</th>
<th>material</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Flores Calendulae</td>
<td>10</td>
<td>Fructus Foeniculi</td>
</tr>
<tr>
<td>2</td>
<td>Flores Chamomillae</td>
<td>11</td>
<td>Herba Millefolii</td>
</tr>
<tr>
<td>3</td>
<td>Folia Melissae</td>
<td>12</td>
<td>Herba Plantaginis ancoolutae</td>
</tr>
<tr>
<td>4</td>
<td>Folia Menthae piperitae</td>
<td>13</td>
<td>Radix Althaeeae</td>
</tr>
<tr>
<td>5</td>
<td>Folia Salviae</td>
<td>14</td>
<td>Radix Angelicae</td>
</tr>
<tr>
<td>6</td>
<td>Folia Thymi</td>
<td>15</td>
<td>Radix Levistici</td>
</tr>
<tr>
<td>7</td>
<td>Fructus Carvi</td>
<td>16</td>
<td>Radix Petroselini</td>
</tr>
<tr>
<td>8</td>
<td>Fructus Coriandri</td>
<td>17</td>
<td>Radix Valerianae</td>
</tr>
<tr>
<td>9</td>
<td>Fructus Cynobasti</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For materials no. 1 and 2, use an especially purified extract, whereas for materials no. 3–17 use a purified extract.

Determination of the rate of recovery

Prepare 5 individual samples as follows:

I. Place 10.0 g of powdered plant material into a 500-ml conical flask and add 1.0 ml of solution S2. For solution S2 dissolve separately 0.040 g of each of the following reference substances – desmetryn R, prometryn R and simazine R – in sufficient acetone R to produce 100.0 ml. Place 5.0 ml of each solution into a 100-ml volumetric flask and dilute the mixture to volume with acetone R. Shake this mixture mechanically for 60 minutes, if necessary, repeat the operation manually and then proceed with the "Preparation of the plant material extract". Either use the purified or especially purified extract for the determination by gas chromatography, as specified for a given plant material.

II. Treat 10.0 g of powdered plant material as described under "Preparation of the plant material extract" (see below). Either use the purified or especially purified extract for the determination by gas chromatography, as given in the test procedure for each individual plant material.
Calculate the rate of recovery (R) as follows:

\[
WR = \frac{2000 (a-b)}{c} \text{ %}
\]

where

- \(a\) = average quantity of the 5 residues in mg/kg obtained using procedure I,
- \(b\) = average quantity of the 5 residues in mg/kg obtained using procedure II,
- \(c\) = quantity of reference substances added to solution S2 in mg under procedure I.

The rate should be within the range of 70–120%.

Preparation of the plant material extract

Place 10.0 g of powdered plant material in a 500-ml conical flask and add 125.0 ml of chloroform R. Shake the mixture for 60 minutes and filter under reduced pressure through a filter-paper (medium grade) into a round-bottom flask. Wash the residue with 3 successive volumes each of 25.0 ml of chloroform R.

Procedure

Concentrate the combined filtrates to a volume of 3–5 ml, using a rotary vacuum evaporator and a water-bath at 40 °C. Transfer the extract to column for chromatography as prepared below, rinsing the round-bottom flask twice with 5.0 ml of chloroform R.

Preparation of the column for chromatography.

Use a glass tube (internal diameter, 20–22 mm) having a restricted orifice and protected with a sintered glass plate (e.g. P10 or P16, glass filter G4 or P40, glass filter G3). Fill the column with chloroform R, then pour purified aluminium oxide R into it to form a 100-mm thick layer. The support material should remain covered with chloroform R. After transferring the extract and the rinsing liquids to the column, elute with 150.0 ml of chloroform R, at a rate of 1–2 drops per second, collecting the eluate in a round-bottom flask. The first purifying process is completed when no eluate drips off the column any longer.

Evaporate the eluate to dryness using a rotary vacuum evaporator and a water-bath at 40 °C. To the residue add 10.0 ml light petroleum R and transfer the mixture to a column for chromatography containing a layer of purified aluminium oxide R, 50 mm thick, in light petroleum R. Elute the mixture with 90.0 ml of light petroleum R while rinsing the round-bottom flask, at a rate of 1–2 drops per second. Discard the eluate. Dissolve the remaining residue undissolved in light petroleum R with 10.0 ml of a mixture composed of 60 volumes of chloroform R and 40 volumes of light petroleum R and transfer the solution to the column. Rinse twice more the round-bottom flask with 10.0 ml of the solvent mixture. Transfer the liquid used for rinsing to the column. Elute with 120.0 ml of the above solvent mixture, at a rate of 1–2 drops per second and collect the eluate in a round-bottom flask. The second purifying process is completed when no eluate drips off the column any longer.

Evaporate the eluate to dryness using a rotary vacuum evaporator and a water-bath at 40 °C. Either dissolve the residue in sufficient acetone R to produce a volume of 10.0 ml (purified extract) to be used for the determination by gas chromatography or, treat it as described below.

To the residue add 10.0 ml of light petroleum R and 10.0 ml dimethyl sulfoxide R. Shake the mixture and transfer it to a separating funnel. Extract the dimethyl sulfoxide-layer twice with 10.0 ml of light petroleum R. Discard the petroleum ether extract. Then add 100 ml of water to the dimethyl sulfoxide-layer and extract 3 times, each with 20.0 ml of chloroform R. Extract the combined chloroform extracts twice with 20.0 ml of water and evaporate them to dryness using a rotary vacuum evaporator and a water-bath at 40 °C. Transfer the residue along with a mixture of 10.0 ml of light petroleum R and 10.0 ml of hydrochloric acid (1 mol/l) VS to a separating funnel and extract the mixture first with 10.0 ml and then with 5.0 ml of hydrochloric acid (1 mol/l) VS. Discard the petroleum ether layer and adjust the pH to between 7 and 8 of the combined aqueous solutions using sodium hydroxide (1 mol/l) VS. Extract the solution 3 times, each with 20.0 ml of chloroform R. Dry the combined chloroform extracts...
with anhydrous sodium sulfate R and filter into a round-bottom flask while rinsing the funnel 3 times each with 10.0 ml of chloroform R. Evaporate the filtrate to dryness using a rotary vacuum evaporator and a water-bath at 40 °C. Dissolve the residue in sufficient acetone R to produce 10.0 ml (especially purified extract) to be used for the determination by gas chromatography.

**Determination by gas chromatography**

Perform the determination as described under "Gas chromatography" (IP, vol.1, p.94).

**Apparatus**

- **Column packing** —
  - Material: glass
  - Internal diameter: 2 mm
  - Length: 1.2 m
  - A suitable stationary liquid phase (e.g. Carbowax 20 M 3.0% is suitable)
  - A suitable diatomaceous support (e.g. Chrom WAW D-MCS, 150–180 μm is suitable)

- **Mobile phase** —
  - Nitrogen R
  - Flow rate: 30.0 ml/min

- **Temperatures** —
  - Sample injection block: 230 °C
  - Column: 190 °C
  - Detector: 300 °C

- **Detector** —
  - Nitrogen selective

- **Sample solution to be injected** —
  - Volume: 2.0 μl

- **Separation characteristics** —
  - \( h \leq 1.2 \times 10^{-3} \) for desmetryn R
  - \( R_S \geq 1.2 \) for prometryn R/simazine R

- **Relative standard deviation (precision of chromatographic system)** —
  - \( s \leq 0.05 \) for desmetryn R, prometryn R and simazine R

**Procedure**

**Chromatogram T:**

To determine the separation characteristics, inject solution \( S_2 \) (For the preparation of solution \( S_2 \) see "Determination of the rate of recovery").

**Chromatograms A1–A5:**

To determine the relative standard deviation, inject solution \( S_2 \) and repeat the determination 5 times.

**Chromatogram \( S_2 \):**

- inject 1.0 ml of solution \( S_2 \) for the determination of the rate of recovery.
- dilute 1.0 ml of solution \( S_2 \) to 10.0 ml with acetone R and inject it for the chromatographic determination. On the chromatogram the peaks occur in the following sequence: prometryn, simazine, desmetryn.

**Chromatogram \( P_2 \):**

Inject the purified extract or the especially purified extract.

**Evaluation method**

**Determination by using an external standard:** \( a = 0.0005 \)

To convert the values obtained in percentage per weight, multiply the concentration in mg/kg by the factor of \( 10^4 \).

The total maximum permissible amount of residues due to desmetryn, prometryn and simazine should not exceed 2 mg/kg of plant material.
15. DETERMINATION OF ARSENIC AND HEAVY METALS

Contamination of medicinal plant materials with arsenic and heavy metals can be attributed to many causes such as environmental pollution and traces of pesticides.

LIMIT TEST FOR ARSENIC

Medicinal plant materials can contain traces of arsenic due to their treatment with certain pesticides. The limit is set in terms of μg of arsenic per g of plant material.

An estimate of the amount of arsenic in the material is made by matching the depth of colour of a series of standard stains. A stain produced with 1 ml of standard stain solution (dilute arsenic AsTS = 10 μg of As/ml) compared with a stain produced using 10 g of material indicates the presence of 1 μg of As per g of plant material.

Recommended procedure

Preparation of the sample by acid digestion

Place 35–70 g of coarsely ground material, accurately weighed, in a 800–1000 ml Kjeldahl flask. Add 10–25 ml of water and 25–50 ml of nitric acid (~1000 g/l) TS and then carefully add 20 ml of sulfuric acid (~1760 g/l) TS. Heat cautiously so that no excessive foaming takes place. Gradually add nitric acid (~1000 g/l) TS, drop by drop, until all the organic matter is destroyed. This is achieved when no further darkening of the solution is observed on continued heating, and a clear solution with copious vapours of sulfur trioxide is obtained. Cool, add 75 ml of water and 25 ml of ammonium oxalate (25 g/l) TS. Heat again until sulfur trioxide vapours develop. Cool, transfer with the help of water to a 250–ml volumetric flask, and dilute to volume with water.

Apparatus

A suitable type of apparatus is described below.

A wide-mouthed bottle of about 120 ml-capacity is fitted with a rubber bung through which passes a glass tube. The latter, made from ordinary glass tubing, has a total length of about 200 mm and an internal diameter of exactly 6.5 mm (external diameter about 8 mm). The lower end of the tube is drawn out to an internal diameter of about 1 mm, and has a hole not less than 2 mm in diameter blown in the side of the tube, near the constricted part. The tube is passed through the bung which fits into the bottle so that, when inserted in the bottle containing 70 ml of liquid, the constricted end of the tube is above the surface of the liquid and the hole in the side is below the bottom of the bung. The upper end of the tube has a flat, ground surface at right-angles to the axis of the tube and is slightly rounded off.

One of two rubber bungs (about 25 mm x 25 mm), each with a hole bored centrally of exactly 6.5 mm in diameter, is fitted at the upper end of the tube. The other bung is fitted with a piece of glass tube about 3 mm long and of exactly 6.5 mm internal diameter with a similar ground surface. One end of both tubes are flush with the larger end of the bungs, so that when the larger ends of the two bungs are held tightly together with a rubber band or a spring clip, the openings of the two tubes meet to form a true tube. Alternatively, the two bungs may be replaced by any suitable construction satisfying the conditions described in the test.

Method

Lightly pack the tube which fits into the wide-mouthed bottle with cotton-wool that has previously been moistened with lead acetate (80 g/l) TS and dried, at a depth not less than 25 mm from the top. Between the flat surfaces of the tubes, place a piece of mercuric bromide paper AAs that is large enough to cover their openings (15 mm x 15 mm).
As an alternative, the mercuric bromide paper AsR, can be fitted by any other means provided that
- the whole of the evolved gas passes through the paper,
- the portion of the paper in contact with the gas is a circle 6.5 mm in diameter and
- the paper is protected from sunlight during the test.

Place an aliquot (25–50 ml) of the solution being tested, prepared as described above, in the wide-mouthed bottle, add 1 g of potassium iodide AsR and 10 g of granulated zinc AsR, and place the prepared glass tube assembly quickly in position. Allow the reaction to proceed for 40 minutes. Compare any yellow stain which is produced on the mercuric bromide paper AsR, with a standard stain, produced in a similar manner with a known quantity of dilute arsenic AsTS. Examine the test and standard stains without delay in daylight; the stains fade with time.

The most suitable temperature for carrying out the test is generally about 40 °C but, as the rate of evolution of the gas varies somewhat with different batches of granulated zinc AsR, the temperature may have to be regulated, to obtain an even evolution of gas. The reaction may be accelerated by placing the apparatus on a warm surface, care being taken to ensure that the mercuric bromide paper AsR remains dry throughout.

Between successive tests, the tube must be washed with hydrochloric acid (~250 g/l) AsTS, rinsed with water and dried.

**Standard stain**

Prepare a solution by adding 10 ml of stannated hydrochloric acid (~250 g/l) AsTS and 1 ml of dilute arsenic AsTS, to 50 ml of water. The resulting solution, when treated as described in the general test, yields a stain on the mercuric bromide paper AsR, referred to as the standard stain (10 μg of As).

**LIMIT TEST FOR CADMIUM AND LEAD**

The choice of heating procedure and the determination is left to the analyst. Nevertheless, the determination must be consistent and sensitive enough to obtain the certified lead and cadmium content in order to compare with a reference material.

The following maximum amounts in dried plant materials are proposed which are based on the ADI values:

- Lead — 10 ppm
- Cadmium — 0.3 ppm

**Recommended procedure**

**Apparatus**

Clean scrupulously with nitric acid (~1000 g/l) TS the digestion vessel and all other equipment that may be used for the determination, rinse thoroughly several times with water and dry at 120 °C.

**Materials used**

- **Digestion vessels** — Vitreous silica crucible (DIN 12904), "tall form"; height, 62 mm; diameter, 50 mm; content 75 ml, with vitreous silica crucible cover*.
- **Digestion mixture** — 2 parts by weight of nitric acid (~1000 g/l) TS and 1 part by weight of perchloric acid (~1170 g/l) TS.

---

* A suitable vessel is obtainable from QGT, Bad Harzburg, Germany.
Reference materials
- Olive leaves (Olea europaea)∗
- Hay powder∗

Preparation of the sample

For the wet digestion method in an open system, weigh accurately 200–250 mg of air-dried plant material, finely cut and homogeneously mixed, into a cleaned silica crucible. Add 1.0 ml of the digestion mixture, cover the crucible without exerting pressure and place it in an oven with a controlled temperature and time regulator (computer-controlled, if available).

Heat slowly to 100 °C and maintain at this temperature up to 3 hours; then heat to 120 °C and maintain at this temperature for 2 hours; raise the temperature very slowly to 240 °C avoiding losses due to possible violent reactions especially in the temperature range of 160–200 °C and maintain at this temperature for 4 hours. The remaining dry inorganic residue can then be used for the heavy metals determination. Dissolve it in 2.5 ml of nitric acid (~1000 g/l) TS.

Every sample should be tested in parallel with a blank determination.

Method

The contents of lead and cadmium are determined either by:

- inverse voltametry (DPASV), or
- atomic absorption spectrophotometry (EAAS), or
- Zeemann atomic absorption spectrophotometry.

16. DETERMINATION OF MICROORGANISMS

Medicinal plant materials normally carry a great number of bacteria and moulds, often of soil origin. While a large range of bacteria and fungi form the naturally-occurring microflora of herbs, aerobic spore-forming bacteria frequently predominate. Current practices of harvesting, handling and production often cause additional contamination and microbial growth. The determination of E. coli and moulds may indicate good production and harvesting practices.

Methods for decontamination are restricted. For example, the use of ethylene oxide has been forbidden within the EC- countries. Treatment with ionizing irradiation requires a special registration procedure or is also forbidden in some countries.

In addition, the presence of aflatoxins in plant material can cause health hazards if absorbed even in very small amounts. Therefore, they should be determined after using a suitable clean-up procedure.

TEST FOR SPECIFIC MICROORGANISMS

The conditions of the test for microbial contamination are designed in such a manner as to minimize accidental contamination of the material being examined and the precautions taken must not adversely affect any microorganisms that could be revealed.

∗ BCR reference material CRM No. 62 Community Bureau of Reference - BCR, Directorate-General XII, Commission of the European Communities, 200 rue de la Loi, B-1049 Brussels, Belgium.
** IAEA-V-10, International Atomic Energy Agency, Analytical Quality Control Services, Laboratory Gelberdorf, P.O. box 100, A–Vienna, Austria.
Recommended procedures

1. Pretreatment of the material being examined

Depending on the nature of the crude medicinal plant material, grind, dissolve, dilute, suspend or emulsify the material being examined using a suitable method and eliminate any antimicrobial properties by dilution, neutralization or filtration.

- **Water-soluble materials** — dissolve or dilute 10 g or 10 ml of material, unless otherwise indicated in the test procedure, in lactose broth or another suitable medium proven to have no antimicrobial activity under the conditions of the test and adjust the volume to 100 ml with the same medium. (Some materials may require the use of a larger volume). If necessary, adjust the pH of the suspension to about 7.

- **Non-fatty materials insoluble in water** — suspend 10 g or 10 ml of material, unless otherwise indicated in the test procedure, in lactose broth or another suitable medium proven to have no antimicrobial activity under the conditions of the test and dilute to 100 ml with the same medium. (Some materials may require the use of a larger volume). If necessary, divide the material being examined and homogenize the suspension mechanically. A suitable surfactant, such as a 1 mg/ml solution of polysorbate 80 R may be added. If necessary, adjust the pH of the suspension to about 7.

- **Fatty materials** — homogenize 10 g or 10 ml of material, unless otherwise indicated in the test procedure, with 5 g of polysorbate 20 R or polysorbate 80 R. If necessary, heat to not more than 40 °C. (A temperature of not more than 45 °C, for the shortest possible time, might be required). Mix carefully while maintaining the temperature in a water-bath or in an oven. Add 85 ml of lactose broth or another suitable medium proven to have no antimicrobial activity in the conditions of the test, heated to not more than 40 °C, if necessary. Maintain this temperature for the shortest time necessary until formation of an emulsion and in any case, for not more than 30 minutes. If necessary, adjust the pH of the emulsion to about 7.

2. *Enterobacteriaceae* and certain other Gram-negative bacteria

- **Detection of bacteria**

Homogenize the pretreated material appropriately and incubate at 30–37 °C for a sufficient length of time for reactivation of the bacteria but not sufficient to encourage multiplication of the organisms (usually 2–5 hours). Shake the container, transfer 1 g or 1 ml of the homogenized material to 100 ml of *Enterobacteriaceae* enrichment broth—Mossel and incubate at 35–37 °C for 18–48 hours. Prepare a subculture on a plate with violet-red bile agar with glucose and lactose. Incubate at 35–37 °C for 18–48 hours. The material passes the test if no growth of colonies of Gram-negative bacteria are detected on the plate.

- **Quantitative evaluation**

Inoculate a suitable amount of *Enterobacteriaceae* enrichment broth—Mossel with quantities of homogenized material prepared as described under "Detection of bacteria", appropriately diluted as necessary, containing 1.0 g, 0.1 g and 10 μg or 1.0 ml, 0.1 ml and 10 μl of the material being examined. Incubate at 35–37 °C for 24–48 hours. Prepare a subculture of each of the cultures on a plate with violet-red bile agar with glucose and lactose in order to obtain selective isolation. Incubate at 35–37 °C for 18–24 hours. The growth of well-developed colonies, generally red or reddish in colour, of Gram-negative bacteria constitutes a positive result. Note the smallest quantity of material that gives a negative result. Determine from the table below the probable number of bacteria.
<table>
<thead>
<tr>
<th>Result for each quantity or volume</th>
<th>Probable number of bacteria per gram of material</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0 g or 1.0 ml</td>
<td></td>
</tr>
<tr>
<td>0.1 g or 0.1 ml</td>
<td></td>
</tr>
<tr>
<td>0.01 g or 0.01 ml</td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>more than $10^2$</td>
</tr>
<tr>
<td>+</td>
<td>less than $10^2$ but more than 10</td>
</tr>
<tr>
<td>+</td>
<td>less than 10 but more than 1</td>
</tr>
<tr>
<td>+</td>
<td>less than 1</td>
</tr>
</tbody>
</table>

3. *Escherichia coli*

Transfer a quantity of the homogenized material in lactose broth containing 1 g or 1 ml of the material being examined, prepared and incubated as for the test for "Enterobacteriaceae and certain other Gram-negative bacteria", to 100 ml of MacConkey broth and incubate at 43–45 ºC for 18–24 hours. Prepare a subculture on a plate with MacConkey agar and incubate at 43–45 ºC for 18–24 hours. Growth of red, generally non-mucoid colonies of Gram-negative rods, sometimes surrounded by a reddish zone of precipitation, indicates the possible presence of *Escherichia coli*. This may be confirmed by the formation of indole at 43.5–44.5 ºC or by other biochemical reactions. The material passes the test if no such colonies are detected or if the confirmatory biochemical reactions are negative.

4. *Salmonella*

Incubate the solution, suspension or emulsion of the pretreated material as described above at 35–37 ºC for 5–24 hours, as appropriate for enrichment.

Primary test: Transfer 10 ml of the enrichment culture to 100 ml of tetraionate bile brilliant green broth and incubate at 42–43 ºC for 18–24 hours. Prepare a subculture on at least two of the following three agar media: deoxycholate citrate agar, xylose, lysine, deoxycholate agar and brilliant green agar. Incubate at 35–37 ºC for 24–48 hours. Carry out the secondary test if any colonies are produced that conform to the description given in the table below.

Secondary test: Prepare a subculture of any colonies showing the characteristics as in the table below on the surface of triple sugar iron agar and using the deep inoculation technique. (This can be achieved by first inoculating the inclined surface of the culture medium followed by a stab culture with the same inoculating needle and incubating at 35–37 ºC for 18–24 hours.) The test is positive for the presence of *Salmonella* if in the deep culture, but not in the surface culture, a change of colour from red to yellow is observed and usually the formation of gas, with or without production of hydrogen sulfide in the agar. Confirmation is obtained by appropriate biochemical and serological tests.

The material being examined passes the test if, cultures of the type described do not appear in the primary test, or if, the confirmatory biochemical and serological tests are negative in the secondary test.

<table>
<thead>
<tr>
<th>Medium</th>
<th>Description of colony</th>
</tr>
</thead>
<tbody>
<tr>
<td>deoxycholate citrate agar</td>
<td>well-developed, colourless</td>
</tr>
<tr>
<td>xylose, lysine, deoxycholate agar</td>
<td>well-developed, red with or without black centres</td>
</tr>
<tr>
<td>brilliant green agar</td>
<td>small, transparent and colourless, or opaque, pink or white (frequently surrounded by a pink to red zone)</td>
</tr>
</tbody>
</table>
5. *Pseudomonas aeruginosa*

Pretreat the material being examined as described above but using buffered sodium chloride-peptone solution pH 7.0, or another suitable medium shown not to have antimicrobial activity under the conditions of the test, in place of lactose broth. Inoculate 100 ml of Soybean-casein digest medium with a quantity of the solution, suspension or emulsion thus obtained containing 1 g or 1 ml of the material being examined. Mix and incubate at 35–37 °C for 24–48 hours. Prepare a subculture on a plate of cetrimide agar and incubate at 35–37 °C for 24–48 hours. If no growth of microorganisms is detected, the material passes the test. If growth of colonies of Gram-negative rods, usually with a greenish fluorescence, occurs, apply an oxidase test and test the growth in soybean-casein digest medium at 42 °C, e.g. using the following method: Place 2 or 3 drops of a freshly prepared 0.01 g/ml solution of *N,N',N''-tetrarmethyl-p-phenylenediamine* dihydrochloride R on filter-paper (Whatman No. 1 is suitable) and apply a smear of the suspected colony; the test is positive if a purple colour is produced within 5–10 seconds. The material passes the test if cultures of the type described do not appear or if the confirmatory biochemical test is negative.

6. *Staphylococcus aureus*

Prepare an enrichment culture as described for *Pseudomonas aeruginosa*. Prepare a subculture on a suitable medium such as Baird-Parker agar. Incubate at 35–37 °C for 24–48 hours. The material passes the test, if no growth of microorganisms is detected. Black colonies of Gram-positive cocci often surrounded by clear zones may indicate the presence of *Staphylococcus aureus*. For catalase-positive cocci confirmation may be obtained, for example, by coagulate and deoxyribonuclease tests. The material passes the test, if cultures of the type described do not appear or if the confirmatory biochemical test is negative.

7. Nutritive and selective properties of the media and validity of the test for specific microorganisms

If necessary, grow separately the test strains listed below in the table on the culture media indicated at 30–35 °C for 18–24 hours. Dilute portions of each of the cultures using buffered sodium chloride-peptone solution pH 7.0 for the test suspensions to contain about 10^5 visible microorganisms per ml. Mix equal volumes of each suspension and use 0.4 ml (approximately 10^2 microorganisms of each strain) as an inoculum in tests for *Escherichia coli*, *Salmonella*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, in the presence and absence of the material being examined, if necessary. The validity of the test method should give a positive result for the respective strain of microorganism.

<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Strain number</th>
<th>Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>such as NCIMB 8545 (ATCC 8739, CIP 53.126)</td>
<td>lactose broth</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>such as NCIMB 8826 (ATCC 9027, CIP 82.118)</td>
<td>soybean-casein digest medium</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em></td>
<td>no strain number is recommended. Salmonella not pathogenic for man, such as <em>Salmonella abortus</em> (NCTC 6017, CIP 80.39), may be used</td>
<td>lactose broth</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>such as NCIMB 8625 (ATCC 6538 P, CIP 53.156) or NCIMB 9518 (ATCC 6538, CIP 4.83)</td>
<td>soybean-casein digest medium</td>
</tr>
</tbody>
</table>
TOTAL Viable AEROBIC COUNT

1. Pretreatment of the material being examined

Pretreat the material as described in the "Test for specific microorganisms", but in place of lactose broth, use buffered sodium chloride-peptone solution pH 7.0, or another suitable medium shown not to have antimicrobial activity under the conditions of the test.

2. Examination of the material being examined

The total viable aerobic count of the material being examined is determined, as given in the test procedures, either by membrane filtration, the plate-count or the serial-dilution method. Prepare a suitable dilution in order to obtain a number of colony-forming units to be within the limits suggested for the method used. Proceed with 10 g or 10 ml of the material, unless otherwise indicated in the test procedure.

- Membrane filtration

Use membrane filters having a nominal pore size not greater than 0.45 μm, the effectiveness of which in retaining bacteria has been established. For example, cellulose nitrate filters are used for aqueous, oily and weakly alcoholic solutions and cellulose acetate filters for strongly alcoholic solutions. The technique described uses filter disks of about 50 mm in diameter. For filters of a different diameter, adjust accordingly the volumes of the dilutions and washings. The filtration apparatus and the membrane are sterilized by appropriate means and are designed to permit the solution being examined to be introduced and filtered under aseptic conditions, as well as the membrane to be transferred to the culture medium.

Transfer 10 ml or a quantity of each dilution containing 1 g of the material to each of two membrane filters and filter immediately. If necessary, dilute the pretreated material to obtain an expected colony count of 10–100. Wash each membrane, filtering three or more successive quantities of approximately 100 ml of a suitable liquid such as buffered sodium chloride-peptone solution pH 7.0. For fatty materials, a suitable surfactant may be added, such as polysorbate 20 R or polysorbate 80 R. Transfer one of the membrane filters, intended primarily for the enumeration of bacteria, to the surface of a plate of casein soybean digest agar and the other, intended primarily for the enumeration of fungi, to the surface of a plate with Sabouraud glucose agar with antibiotics. Incubate the plates for 5 days, unless a more reliable count is obtained in a shorter time, for the detection of bacteria at 30–35 °C and for the detection of fungi at 20–25 °C. Count and calculate the number of colonies formed. Calculate the number of microorganisms per g or per ml of the material tested, if necessary counting bacteria and fungi separately.

- Plate count

For bacteria: Use Petri dishes 9–10 cm in diameter. To one dish add a mixture of 1 ml of the pretreated material and about 15 ml of liquefied casein soybean digest agar at a temperature not exceeding 45 °C. Alternatively, spread the pretreated material on the surface of the solidified medium in a Petri dish having the same diameter. If necessary, dilute the pretreated material as described above to obtain an expected colony count of not more than 300. Prepare at least two such plates using the same dilution and incubate them at 30–35 °C for 5 days, unless a more reliable count is obtained in a shorter period of time. Count the number of colonies formed and calculate the results using the plate with the largest number of colonies, but taking as the maximum 300 colonies per plate.

For fungi: Use Petri dishes 9–10 cm in diameter. To one dish add a mixture of 1 ml of the pretreated material and about 15 ml of liquefied Sabouraud glucose agar with antibiotics at a temperature not exceeding 45 °C. Alternatively, spread the pretreated material on the surface of the solidified medium in a Petri dish having the same diameter. If necessary, dilute the pretreated material as described above to obtain an expected colony count of not more than 100. Prepare at least two such plates using the same dilution and incubate them at 20–25 °C for 5 days, unless a more reliable count is obtained in a shorter period of time. Count the number of colonies formed and calculate the results using the plate with not more than 100 colonies.
### Serial dilution

Prepare a series of 12 tubes each containing 9–10 ml of soybean-casein digest medium. To each of the first three tubes add 1 ml of the dissolved, homogenized and diluted material in a proportion of 1–10, as described above. To the next three tubes add 1 ml of a 1–100 dilution of the material and to the next three tubes add 1 ml of a 1–1000 dilution of the material. To the last three tubes add 1 ml of the diluent. Incubate the tubes at 30–35 °C for at least 5 days. No microbial growth should appear in the last three tubes. If the reading of the results is difficult or uncertain owing to the nature of the material being examined, prepare a subculture of a liquid or a solid medium, the results being evaluated after a further period of incubation. Determine the most probable number of microorganisms per g or ml of the material according to the table below.

<table>
<thead>
<tr>
<th>Number of tubes with microbial growth for quantities or volumes of material examined</th>
<th>Most probable number of microorganisms per g or ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mg or 0.1 ml per tube</td>
<td>10 mg or 0.01 ml per tube</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
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<tr>
<td>3</td>
<td>3</td>
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<tr>
<td>3</td>
<td>2</td>
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<td>3</td>
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<tr>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
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<tr>
<td>3</td>
<td>1</td>
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<tr>
<td>3</td>
<td>1</td>
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<tr>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

If, for the first column, the number of tubes showing microbial growth is two or less, the most probable number of microorganisms per g or per ml is likely to be less than 100.

### 3. Effectiveness of the culture medium and validity of the counting method

The following strains are normally used:

- **Staphylococcus aureus** – NCIMB 8625 (ATCC 6538 P, CIP 53.156) or NCIMB 9518 (ATCC 6538, CIP 4.83)
- **Bacillus subtilis** – NCIMB 8054 (ATCC 6633, CIP 52.62)
- **Escherichia coli** – NCIMB 8545 (ATCC 8739, CIP 53.126)
- **Candida albicans** – ATCC 2091 (CIP 1180.79) or ATCC 10 231 (NCPF 3179, CIP 48.72)

Allow the test strains to grow separately in tubes containing soybean-casein digest medium at 30–35 °C for 18–24 hours, except for **Candida albicans** which needs a temperature of 20–25 °C for 48 hours.

Dilute portions of each of the cultures using buffered sodium chloride-peptone solution pH 7.0 to obtain test suspensions containing about 100 viable microorganisms per ml. Use the suspension of each microorganism separately as a control of the counting methods, in the presence and absence of the material being examined, if necessary.
For the validation of the method, a count for the test organism should be obtained differing by not more than a factor of 10 from the calculated value for the inoculum. To test the sterility of the medium and the diluent, as well as the aseptic performance, carry out the total viable aerobic count method using sterile buffered sodium chloride-peptone solution pH 7.0 as the test preparation. Growth of microorganisms should not be observed.

4. Interpretation of results

Limits should be interpreted as follows:

\[ 10^2 \text{ microorganisms} = \text{maximum limit of acceptance: } 5 \times 10^2; \]
\[ 10^3 \text{ microorganisms} = \text{maximum limit of acceptance: } 5 \times 10^3; \text{ etc.} \]

MICROBIAL CONTAMINATION LIMITS IN MEDICINAL PLANT MATERIALS

Different limits are set according to the use of the material and the material itself:

a) Contamination of "crude" plant material intended for further processing (including additional decontamination by any physical or chemical process):

The limits (10) are given for untreated plant material harvested under acceptable hygienic conditions per gram —

- maximum \( 10^4 \) *Escherichia coli*
- maximum \( 10^5 \) mould propagules

b) Plant materials that have been pretreated (e.g. boiling water as used for herbal teas and infusions) or if the material is used for topical dosage forms:

per gram —

- maximum \( 10^7 \) aerobic bacteria
- maximum \( 10^4 \) yeasts and moulds
- maximum \( 10^2 \) *Escherichia coli*
- maximum \( 10^4 \) other enterobacteria
- no *Salmonellae*

c) Other plant materials for internal use:

per gram —

- maximum \( 10^5 \) aerobic bacteria
- maximum \( 10^3 \) yeasts and moulds
- maximum \( 10^1 \) *Escherichia coli*
- maximum \( 10^3 \) other enterobacteria
- no *Salmonellae*

TEST FOR AFLATOXINS

This test is designed to detect the possible presence of aflatoxins B₁, B₂, G₁ and G₂, which are highly dangerous contaminants in any material of plant origin.

Reccomended procedure

Preparation of the samples

Grind or reduce not less than 100 g of crude medicinal plant material to a moderately fine powder (sieve no. 355/180). The larger the sample size, such as 500 g–1 kg or more, the greater the potential to detect pockets of contamination.
Weigh and transfer 50 g of the powdered material to a conical glass-stoppered flask, and add 170 ml of methanol R and 30 ml of water. Using a mechanical device, shake vigorously for not less than 30 minutes. Filter through a medium porosity filter-paper. If a special clean-up procedure is required (see below), collect from the start of flow 100 ml of filtrate (A), otherwise discard the first 50 ml and collect 40 ml of filtrate (B).

In order to eliminate interfering plant pigments use the special clean-up procedure, and transfer 100 ml of filtrate (A) to a 250-ml beaker. Add 20 ml of zinc acetate/aluminium chloride TS and 80 ml of water. Stir, allow to stand for 5 minutes, add 5 g of a filter aid, such as diatomaceous earth, mix and filter through a medium porosity filter-paper. Discard the first 50 ml and collect 80 ml of filtrate (C).

Transfer either filtrate B or C to a separatory funnel. Add 40 ml of sodium chloride (100 g/l) TS and 25 ml of light petroleum R, and shake for 1 minute. Allow the layers to separate and transfer the lower layer to a second separatory funnel. Extract twice with 25 ml of dichloromethane R and shake for 1 minute. Allow the layers to separate and combine each of the lower layers in a 125-ml conical flask. Add several boiling chips and evaporate almost to dryness on a water-bath. Cool the residue, cover the flask and keep it for the determination by thin-layer chromatography or for a further clean-up procedure by column chromatography.

If necessary, remove further interferences using a column, 300 mm x 10 mm, internal diameter, with stopper and either a medium pore sintered disc or a glass wool plug. Slurry 2 g of silica gel G with 10 ml of a mixture of 3 volumes of ether R and 1 volume of light petroleum R, pour into the column and wash with 5 ml of the same solvent mixture. Allow the adsorbent to settle and add to the top of the column a layer of 1.5 g of anhydrous sodium sulfate R. Dissolve the residue from above in 3 ml of dichloromethane R and transfer it to the column. Rinse the flask twice with 1-ml portions of dichloromethane R and add them to the column, eluting at a rate not faster than 1 ml/min. Then add successively to the column 3 ml of light petroleum R, 3 ml of ether R and 3 ml of dichloromethane R, and elute at a rate not faster than 3 ml/min. Discard the eluates. Add to the column 6 ml of a mixture of 9 volumes of dichloromethane R and 1 volume of acetone R and elute at a rate not faster than 1 ml/min, preferably without using vacuum. Collect this eluate in a small vial, add a few boiling chips and evaporate just to dryness on a water-bath.

**Method**

To either of the residues obtained above, add 0.2 ml of a mixture of 98 volumes of chloroform R and 2 volumes of acetonitrile R, close the vial and shake vigorously until the residues are dissolved, preferably using a Vortex type mixer.

Carry out the test as described under "Thin-layer chromatography", using silica gel G as the coating substance and a mixture of 85 volumes of chloroform R, 10 volumes of acetone R and 5 volumes of 2-propanol R as the mobile phase. Apply separately to the plate 2.5 μl, 5 μl, 7.5 μl and 10 μl of aflatoxin mixture TS, then apply three volumes, each of 10 μl of the sample residues. Further superimpose on one of these spots 5 μl of aflatoxin mixture TS. Place the plate in an unsaturated chamber. After removing the plate from the chromatographic chamber, allow it to dry in air and examine the chromatogram in a dark room under ultraviolet light (365 nm).

Four clearly-separated blue fluorescent spots are obtained from the aflatoxin mixture. Observe any spot obtained from the solutions of the residues that coincides in hue and position with that of the aflatoxin mixture. Any spot obtained from the solutions of the residues with the superimposed aflatoxin mixture should be more intense than the corresponding spot alone, and should show no sign of separation or tailing, which would be a sign of dissimilar compounds.

**Interpretation of results**

No spots of aflatoxins should be detected in any of the sample residues. If any spot is obtained, match the position of each fluorescent spot of the solutions of the residues with those of the aflatoxin mixture to identify the type of aflatoxin present. An approximate estimation in the sample may be obtained by comparing the intensity of the spots with those of the aflatoxin mixtures.
17. RADIOACTIVE CONTAMINATION

A certain amount of exposure to ionizing radiation cannot be avoided since there are many sources of radiation, including naturally-occurring radionuclides contained in the ground and the atmosphere. These sources are described extensively in the booklet "Facts about low-level radiation" (11).

Irradiation, may also be used as a procedure for microbial decontamination and sterilization of plant materials (after harvest), packaging materials, intermediate products, bulk material and finished products.

Dangerous contamination may equally be the consequence of a nuclear accident. The World Health Organization in close collaboration with several other international organizations has developed guidelines for use in the event of widespread contamination by radionuclides resulting from a major nuclear accident (12). This publication, emphasizes health risks from food accidentally contaminated by radionuclides that depend not only on the specific radionuclide and the level of contamination but also on the quantity of food consumed.

The range of radionuclides that may be released into the environment as the result of a nuclear accident might include long-lived and short-lived fission products, actinides, and activation products. The nature and the intensity of radionuclides released may differ markedly and depend on the source (reactors, reprocessing plants, fuel fabrication plants, isotope production units, etc.).

Dose per unit intake factor*

The amount of exposure to radiation depends on the intake of a radionuclide and other variables such as age, metabolic kinetics, and weight of the individual, e.g. the age-specific dose per unit intake factor (Sv/Bq) for different radionuclides is given in the following table:

<table>
<thead>
<tr>
<th>radionuclide</th>
<th>one-year-olds</th>
<th>dose per unit</th>
<th>adults</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10-year-olds</td>
<td>10-year-olds</td>
<td></td>
</tr>
<tr>
<td>Strontium-90**</td>
<td>1.1 × 10⁻⁷</td>
<td>4.0 × 10⁻⁸</td>
<td>3.6 × 10⁻⁷</td>
</tr>
<tr>
<td>Iodine-131***</td>
<td>3.6 × 10⁻⁶</td>
<td>1.0 × 10⁻⁶</td>
<td>4.4 × 10⁻⁷</td>
</tr>
<tr>
<td>Caesium-134**</td>
<td>1.2 × 10⁻⁸</td>
<td>1.2 × 10⁻⁸</td>
<td>2.0 × 10⁻⁸</td>
</tr>
<tr>
<td>Caesium-137**</td>
<td>1.0 × 10⁻⁶</td>
<td>1.0 × 10⁻⁶</td>
<td>1.3 × 10⁻⁶</td>
</tr>
<tr>
<td>Plutonium-239***</td>
<td>2.4 × 10⁻⁶</td>
<td>1.4 × 10⁻⁶</td>
<td>1.3 × 10⁻⁶</td>
</tr>
</tbody>
</table>

Any significant risk is associated with consumption of quantities over 20 kg per year and any health risk is most unlikely to be encountered given the amount of plant materials which would need to be ingested. Additionally, the level of contamination might be reduced during the manufacturing process and therefore, it is not envisaged here to suggest levels for contamination.

Method of measurement

Since radionuclides from accidental discharges vary with the type of facility involved, a generalized method of measurement is so far not available. However, should such contamination be of concern, suspected samples can be analyzed by a competent laboratory. Detailed laboratory techniques are available from the International Atomic Energy Agency (IAEA).***

* Also known as the "dose conversion factor".
** Committed effective dose equivalent.
*** Committed dose equivalent to the thyroid.
**** International Atomic Energy Agency (IAEA), Analytical Quality Control Services, Laboratory Seibersdorf, P.O. Box 100, A–Vienna, Austria
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6. The principles of thin-layer chromatography, as well as their application in pharmaceutical analysis are described in The International Pharmacopoeia (IP), third edition, Volume 1, WHO, Geneva, 1979.


10. Limits adapted according to the "Provisional guidelines on the microbial contamination of spices, herbs and vegetable seasonings", International consultative group on food irradiation, Consultation on microbiological criteria for foods to be further processed including irradiation, FAO, IAEA and WHO, Geneva, 1989, WHO/EHE/FOS/89.5, p. 21.


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ANNEX 1

CULTURE MEDIA AND STRAINS OF MICROORGANISMS

Culture media

The following media are satisfactory, but other media may be used if they have similar nutritive and selective properties for the microorganisms to be tested.

Baird-Parker agar

Procedure. Dissolve 10.0 g of pancreatic digest of casein R, 5.0 g of beef extract R, 1.0 g of water-soluble yeast R, 5.0 g of lithium chloride R, 20.0 g of agar R, 12.0 g of glycine R, 10.0 g sodium pyruvate R in sufficient water to produce 950 ml. Heat to boiling for 1 minute, shaking frequently, and adjust the pH with sodium hydroxide (0.5 mol/l) VS to 6.6–7.0. Sterilize in an autoclave at 121 °C for 15 minutes, cool to 45–50 °C and add 10 ml of a sterile 0.01 g/ml solution of potassium tellurite R and 50 ml of egg-yolk emulsion.

Brilliant green agar

Procedure. Dissolve 10.0 g of dried peptone R (meat and casein), 3.0 g of water-soluble yeast extract R, 5.0 g of sodium chloride R, 10.0 g of lactose R, 10.0 g of sucrose R, 20.0 g of agar R, 0.08 g of phenol red R, 12.5 mg of brilliant green R in sufficient water to produce 1000 ml. Heat to boiling for 1 minute. Adjust the pH with sodium hydroxide (0.05 mol/l) VS to 6.7–7.1. Immediately before use, sterilize in an autoclave at 121 °C for 15 minutes, cool to 50 °C and pour into Petri dishes.

Buffered sodium chloride-peptone solution pH 7.0

Procedure. Dissolve 3.56 g of potassium dihydrogen phosphate R, 7.23 g of disodium hydrogen phosphate R, 4.30 g of sodium chloride R, 1.0 g of dried peptone R (meat and casein) in sufficient water to produce 1000 ml. A quantity of 0.001–0.01 g/ml of polysorbate 20 R or polysorbate 80 R may be added. Sterilize in an autoclave at 121 °C for 15 minutes.

Casein soybean digest agar

Procedure. Dissolve 15.0 g of pancreatic digest of casein R, 3.0 g of papaic digest of soybean meal R, 5.0 g of sodium chloride R, 15.0 g of agar R in sufficient water to produce 1000 ml. Adjust the pH with sodium hydroxide (0.05 mol/l) VS to 7.1–7.5. Sterilize in an autoclave at 121 °C for 15 minutes.

Cetrimide agar

Procedure. Dissolve 20.0 g of pancreatic digest of gelatin R, 1.4 g of magnesium chloride R, 10.0 g of potassium sulfate R, 0.3 g of cetrimide R, 13.6 g of agar R, 10.0 ml of glycerol R in sufficient water to produce 1000 ml. Heat to boiling for 1 minute with shaking. Adjust the pH with sodium hydroxide (0.05 mol/l) VS to 7.0–7.4 and sterilize in an autoclave at 121 °C for 15 minutes.

Deoxycholate citrate agar

Procedure. Dissolve 10.0 g of beef extract R, 10.0 g of dried peptone R (meat), 10.0 g of lactose R, 20.0 g of sodium citrate R, 1.0 g of iron(III) citrate R, 5.0 g of sodium deoxycholate, 13.6 g of agar R, 20 mg of neutral red R in sufficient water to produce 1000 ml. Heat gently to boiling for 1 minute, cool to 50 °C and adjust the pH with sodium hydroxide (0.05 mol/l) VS to 7.1–7.5. Pour into Petri dishes. Do not heat in an autoclave.
Enterobacteriaceae enrichment broth-Mossel

Procedure. Dissolve 10.0 g of pancreatic digest of gelatin R, 5.0 g of glucose hydrate R, 20.0 g of dehydrated ox bile R, 2.0 g of potassium dihydrogen phosphate R, 8.0 g of disodium hydrogen phosphate R, 15 mg of brilliant green R in sufficient water to produce 1000 ml. Heat to boiling for 30 minutes and cool immediately. Adjust the pH with sodium hydroxide (0.05 mol/l) VS to 7.0–7.4.

Lactose broth

Procedure. Dissolve 3.0 g of beef extract R, 5.0 g of pancreatic digest of gelatin R, 5.0 g of lactose R in sufficient water to produce 1000 ml. Adjust the pH with sodium hydroxide (0.05 mol/l) VS to 6.7–7.1, and sterilize in an autoclave at 121 °C for 15 minutes.

MacConkey agar

Procedure. Dissolve 17.0 g of pancreatic digest of gelatin R, 3.0 g of dried peptone R (meat and casein), 10.0 g of lactose R, 5.0 g of sodium chloride R, 1.5 g of bile salts R, 13.5 g of agar R, 30 mg of neutral red R, 1.0 mg of crystal violet R in sufficient water to produce 1000 ml. Adjust the pH with sodium hydroxide (0.05 mol/l) VS to 6.9–7.3. Heat to boiling for 1 minute with constant shaking then sterilize in an autoclave at 121 °C for 15 minutes.

MacConkey broth

Procedure. Dissolve 20.0 g of pancreatic digest of gelatin R, 10.0 g of lactose R, 5.0 g of dehydrated ox bile R, 10 mg of brom cresol purple R in sufficient water to produce 1000 ml. Adjust the pH with sodium hydroxide (0.05 mol/l) VS to 7.1–7.5, and sterilize in an autoclave at 121 °C for 15 minutes.

Sabouraud glucose agar with antibiotics

Procedure. Dissolve 10.0 g of dried peptone R (meat and casein), 40.0 g of glucose hydrate R, 15.0 g of agar R in sufficient water to produce 1000 ml. Adjust the pH with acetic acid (-60 g/l) TS to 5.4–5.8 and sterilize in an autoclave at 121 °C for 15 minutes. Immediately before use, add sterile solutions of 0.10 g of benzylpenicillin sodium R and 0.10 g of tetracycline R per litre of medium, or alternatively before sterilization, add 0.050 g of chloramphenicol R per litre of medium.

Soybean-casein digest medium

Procedure. Dissolve 17.0 g of pancreatic digest of casein R, 3.0 g of papaic digest of soybean meal R, 5.0 g of sodium chloride R, 2.5 g of dipotassium hydrogen phosphate R, 2.5 g of glucose hydrate R in sufficient water to produce 1000 ml. Adjust the pH with sodium hydroxide (0.05 mol/l) VS to 7.1–7.5 and sterilize in an autoclave at 121 °C for 15 minutes.

Tetrathionate bile brilliant green broth

Procedure. Dissolve 8.6 g of dried peptone R, 8.0 g of dehydrated ox bile R, 6.4 g of sodium chloride R, 20.0 g of calcium carbonate R1, 20.0 g of potassium tetrathionate R, 0.070 g of brilliant green R in sufficient water to produce 1000 ml. Adjust the pH with sodium hydroxide (0.05 mol/l) VS to 6.8–7.2 and heat just to boiling; do not reheat.

Triple sugar iron agar

Procedure. Dissolve 3.0 g of beef extract R, 3.0 g of water-soluble yeast extract, 20.0 g of dried peptone R (casein and beef), 5.0 g of sodium chloride R, 10.0 g of lactose R, 10.0 g of sucrose R, 1.0 g of glucose hydrate R, 0.3 g of brown ammonium iron(III) citrate R, 0.3 g of sodium thiosulfate R, 25 mg of phenol red R, 12.0 g of agar R in sufficient water to produce 1000 ml. Heat to boiling for 1 minute with shaking. Adjust the pH with sodium hydroxide (0.05 mol/l) VS to 7.2–7.6. Distribute in
tubes and sterilize in an autoclave at 121 °C for 15 minutes. Allow to set in an inclined position covered with a butt.

**Violet-red bile agar with glucose and lactose**

Procedure. Dissolve 3.0 g of water-soluble yeast extract R, 7.0 g of pancreatic digest of gelatin R, 1.5 g of bile salts R, 10.0 g of lactose R, 5.0 g of sodium chloride R, 10.0 g of glucose hydrate R, 15.0 g of agar R, 30 mg of neutral red R, 2.0 mg of crystal violet R in sufficient water to produce 1000 ml. Heat to boiling and adjust the pH with sodium hydroxide (0.05 mol/l) VS to 7.2–7.6. Do not heat in an autoclave.

**Xylose, lysine, deoxycholate agar**

Procedure. Dissolve 3.5 g of xylose R, 5.0 g L-lysine R, 7.5 g of lactose R, 7.5 g of sucrose R, 5.0 g of sodium chloride R, 3.0 g of water-soluble yeast extract R, 0.08 g of phenol red R, 13.5 g agar R, 2.5 g sodium deoxycholate R, 6.8 g sodium thiosulfate R, 0.8 g brown ammonium iron(III) citrate R in sufficient water to produce 1000 ml. Adjust the pH with sodium hydroxide (0.05 mol/l) VS to 7.2–7.6. Heat just to boiling, cool to 50 °C and pour into Petri dishes. Do not heat in an autoclave.

**Strains of microorganisms**

The strains of microorganism referred to throughout the text are suitable, but others may be used if they have similar properties. Their abbreviations are the following and may be obtained from:

- **ATCC** American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, USA
- **CIP** Collection de l’Institut Pasteur, Service de la Collection Nationale de Cultures de Microorganismes, (CNCM), 25, rue du Docteur Roux, F 75015 Paris, France
- **NCIMB** National Collection of Industrial and Marine Bacteria, Tony Research Station, PO Box 31, 135 Abbey Road, Aberdeen AB9 8DG, Scotland
- **NCPF** National Collection of Pathogenic Fungi, London School of Hygiene and Tropical Medicine, Keppel Street, London WC1E 7HT, England
- **NCTC** National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London NW9 5HT, England
ANNEX 2

SPECIFICATIONS FOR ADSORBENTS
FOR USE IN THIN-LAYER CHROMATOGRAPHY

Cellulose

Description — A fine, white, homogeneous powder with an average particle size of less than 30 μm.

Preparation — Suspend 15 g in 100 ml of water and homogenize for 60 seconds in an electric mixer. Carefully coat cleaned plates with a layer 0.25 mm thick using a spreading device. Allow the coated plates to dry in air.

Separating power — Apply separately to the adsorbent layer 10 μl of 0.25 mg/ml solutions containing respectively brilliant black BN R, amaranth S R, fast yellow AB R and tropaeolin O R in a mixture of equal volumes of methanol R and water. Develop the plate using a mixture of 50 volumes of 1-propanol R, 10 volumes of ethyl acetate R and 40 volumes of water over a distance of 10 cm. The chromatogram shows 4 clearly-separated spots in an increasing order of Rf values.

Cellulose, microcrystalline

Description — A fine, white, homogeneous powder with an average particle size of less than 30 μm.

Preparation — Suspend 25 g in 90 ml of water and homogenize for 60 seconds in an electric mixer. Carefully coat cleaned plates with a layer 0.25 mm thick using a spreading device. Allow the coated plates to dry in air.

Separating power — Apply separately to the adsorbent layer 10 μl of 0.25 mg/ml solutions containing respectively brilliant black BN R, amaranth S R, fast yellow R and tropaeolin O R in a mixture of equal volumes of methanol R and water. Develop the plate using a mixture of 50 volumes of 1-propanol R, 10 volumes of ethyl acetate R and 40 volumes of water over a distance of 10 cm. The chromatogram shows 4 clearly-separated spots in an increasing order of Rf values.

Cellulose F_{254}

Description — A fine, white, homogeneous powder with an average particle size of less than 30 μm containing a fluorescent indicator having an optimal intensity of 254 nm.

Preparation — Suspend 25 g in 100 ml of water and homogenize for 60 seconds using an electric mixer. Carefully coat cleaned plates with a layer 0.25 mm thick using a spreading device. Allow the coated plates to dry in air.

Separating power — Apply separately to the adsorbent layer 10 μl of 0.25 mg/ml solutions containing respectively brilliant black BN R, amaranth S R, fast yellow R and tropaeolin O R in a mixture of equal volumes of methanol R and water. Develop the plate using a mixture of 50 volumes of 1-propanol R, 10 volumes of ethyl acetate R and 40 volumes of water over a distance of 10 cm. The chromatogram shows 4 clearly-separated spots in an increasing order of Rf values.

Fluorescence — Prepare a 1.0 mg/ml solution of benzoic acid R in a mixture of 9 volumes of 2-propanol R and 1 volume of formic acid R. Apply to the adsorbent layer at 10 points of applications, increasing quantities from 1–10 μl of the solution. Develop the chromatogram using a mixture of 9 volumes of 2-propanol R and 1 volume of formic acid R. Allow the solvents to evaporate and examine the chromatogram in ultraviolet light at 254 nm. The benzoic acid appears as dark spots on a fluorescent background in the upper third of the chromatogram for concentrations of 2 μg and larger.
Kieselguhr G

Description – A fine, greyish white powder with an average particle size of between 10 and 40 μm containing about 150 g of calcium sulfate, hemihydrate per kg. (The grey colour becomes more pronounced on triturating the powder with water).

Content of calcium sulfate – Place about 0.25 g, accurately weighed, in a flask with a ground-glass stopper, add 3 ml of hydrochloric acid (~70 g/l) TS and 100 ml of water and shake vigorously for 30 minutes. Filter through a sintered glass filter and wash the residue. Using the combined filtrate and washings, carry out the assay by complexometry for calcium (IP, vol. 1, page 128). Each ml of disodium edetate (0.05 mol/l) VS is equivalent to 7.26 mg of CaSO₄·1/2H₂O (MW 145.1).

pH – Shake 1 g with 10 ml of carbon-dioxide-free water R for 5 minutes. Measure the pH potentiometrically (IP, vol. 1, page 96); pH, between 7 and 8.

Preparation – Suspend 30 g in 60 ml of sodium acetate (1.6 g/l) TS shaking vigorously for 30 seconds. Carefully coat cleaned plates with a layer 0.25 mm thick using a spreading device. Allow the coated plates to dry in air.

Separating power – Apply separately to the adsorbent layer 5 μl of 0.10 mg/ml solutions containing respectively lactose R, sucrose R, glucose R, fructose R and galactose R in pyridine R. Develop the plate using a mixture of 65 volumes of ethyl acetate R, 23 volumes of 2-propanol R and 12 volumes of water. After removing the plate from the chromatographic chamber, dry it in an oven at 105–110 °C and allow to cool. Spray the plate with about 10 ml of anisaldehyde TS and heat to 100–105 °C for 5–10 minutes. The chromatogram shows 5 clearly-separated spots without tailing.

Kieselguhr GF 254

Description – A fine, greyish white powder with an average particle size of between 10 and 40 μm containing about 150 g of calcium sulfate, hemihydrate per kg and a fluorescent indicator having an optimal intensity of 254 nm. (The grey colour becomes more pronounced on triturating the powder with water).

Content of calcium sulfate – Place about 0.25 g, accurately weighed, in a flask with a ground-glass stopper, add 3 ml of hydrochloric acid (~70 g/l) TS and 100 ml of water and shake vigorously for 30 minutes. Filter through a sintered glass filter and wash the residue. Using the combined filtrate and washings, carry out the assay by complexometry for calcium (IP, vol. 1, page 128). Each ml of disodium edetate (0.05 mol/l) VS is equivalent to 7.26 mg of CaSO₄·1/2H₂O (MW 145.1).

pH – Shake 1 g with 10 ml of carbon-dioxide-free water R for 5 minutes. Measure the pH potentiometrically (IP, vol. 1, page 96); pH, between 7 and 8.

Preparation – Suspend 30 g in 60 ml of sodium acetate (1.6 g/l) TS shaking vigorously for 30 seconds. Carefully coat cleaned plates with a layer 0.25 mm thick using a spreading device. Allow the coated plates to dry in air.

Separating power – Apply separately to the adsorbent layer 5 μl of 0.10 mg/ml solutions containing respectively lactose R, sucrose R, glucose R, fructose R and galactose R in pyridine R. Develop the plate using a mixture of 65 volumes of ethyl acetate R, 23 volumes of 2-propanol R and 12 volumes of water. After removing the plate from the chromatographic chamber, dry it in an oven at 105–110 °C and allow to cool. Spray the plate with about 10 ml of anisaldehyde TS and heat to 100–105 °C for 5–10 minutes. The chromatogram shows 5 clearly-separated spots without tailing.

Fluorescence – Prepare a 1.0 mg/ml solution of benzoic acid R in a mixture of 9 volumes of 2-propanol R and 1 volume of formic acid R. Apply to the adsorbent layer at 10 points of applications, increasing quantities from 1–10 μl of the solution. Develop the chromatogram using a mixture of 9 volumes of 2-propanol R and 1 volume of formic acid R. Allow the solvents to evaporate and examine the chromatogram in ultraviolet light at 254 nm. The benzoic acid appears as dark spots on a fluorescent background in the upper third of the chromatogram for concentrations of 2 μg and larger.
Kieselguhr H

Description — A fine, greyish white powder with an average particle size of between 10 and 40 μm. (The grey colour becomes more pronounced on triturating the powder with water).

pH — Shake 1 g with 10 ml of carbon-dioxide-free water R for 5 minutes. Measure the pH potentiometrically (IP, vol. 1, page 96); pH, between 6.4 and 8.0.

Preparation — Suspend 30 g in 60 ml of sodium acetate (1.6 g/l) TS shaking vigorously for 30 seconds. Carefully coat cleaned plates with a layer 0.25 mm thick using a spreading device. Allow the coated plates to dry in air.

Separating power — Apply separately to the adsorbent layer 5 μl of 0.10 mg/ml solutions containing respectively lactose R, sucrose R, glucose R, fructose R and galactose R in pyridine R. Develop the plate using a mixture of 65 volumes of ethyl acetate R, 23 volumes of 2-propanol R and 12 volumes of water. After removing the plate from the chromatographic chamber, dry it in an oven at 105–110 °C and allow to cool. Spray the plate with about 10 ml of anisaldehyde TS and heat to 100–105 °C for 5–10 minutes. The chromatogram shows 5 clearly-separated spots without tailing.

Silica gel G

Description — A fine, white, homogeneous powder with an average particle size of between 10 and 40 μm containing about 130 g of calcium sulfate, hemihydrate per kg.

Content of calcium sulfate — Place about 0.25 g, accurately weighed, in a flask with a ground-glass stopper, add 3 ml of hydrochloric acid (~70 g/l) TS and 100 ml of water and shake vigorously for 30 minutes. Filter through a sintered glass filter and wash the residue. Using the combined filtrate and washings, carry out the assay by complexometry for calcium (IP, vol. 1, page 128). Each ml of disodium edetate (0.05 mol/l) VS is equivalent to 7.26 mg of CaSO₄·1/2H₂O (MW 145.1).

pH — Shake 1 g with 10 ml of carbon-dioxide-free water R for 5 minutes. Measure the pH potentiometrically (IP, vol. 1, page 96); pH, about 7.

Preparation — Suspend 30 g in 60 ml of water, shaking vigorously for 30 seconds. Carefully coat the cleaned plates with a layer 0.25 mm thick using a spreading device. Allow the coated plates to dry in air.

Separating power — Apply separately to the adsorbent layer 10 μl of 0.10 mg/ml solutions containing respectively indophenol blue R, sudan red G R and dimethyl yellow R in toluene R. Develop the plate with the same solvent over a distance of 10 cm. The chromatogram shows 3 clearly-separated spots, the spot of indophenol blue near the points of application, that of dimethyl yellow in the middle of the chromatogram, and that of sudan red G between the two.

Silica gel GF254

Description — A fine, white, homogeneous powder with an average particle size of between 10 and 40 μm containing about 130 g of calcium sulfate, hemihydrate per kg and about 150 g of a fluorescent indicator per kg and having an optimal intensity of 254 nm.

Content of calcium sulfate — Place about 0.25 g, accurately weighed, in a flask with a ground-glass stopper, add 3 ml of hydrochloric acid (~70 g/l) TS and 100 ml of water and shake vigorously for 30 minutes. Filter through a sintered glass filter and wash the residue. Using the combined filtrate and washings, carry out the assay by complexometry for calcium (IP, vol. 1, page 128). Each ml of disodium edetate (0.05 mol/l) VS is equivalent to 7.26 mg of CaSO₄·1/2H₂O (MW 145.1).

pH — Shake 1 g with 10 ml of carbon-dioxide-free water R for 5 minutes. Measure the pH potentiometrically (IP, vol. 1, page 96); pH, about 7.
Preparation — Suspend 30 g in 60 ml of water, shaking vigorously for 30 seconds. Carefully coat the cleaned plates with a layer 0.25 mm thick using a spreading device. Allow the coated plates to dry in air.

Separating power — Apply separately to the adsorbent layer 10 μl of 0.10 mg/ml solutions containing respectively indophenol blue R, sudan red G R and dimethyl yellow R in toluene R. Develop the plate with the same solvent over a distance of 10 cm. The chromatogram shows 3 clearly-separated spots, the spot of indophenol blue near the point of application, that of dimethyl yellow in the middle of the chromatogram, and that of sudan red G between the two.

Fluorescence — Prepare a 1.0 mg/ml solution of benzoic acid R in a mixture of 9 volumes of 2-propanol R and 1 volume of formic acid (−1080 g/l) TS. Apply to the adsorbent layer at 10 points of applications, increasing quantities from 1–10 μl of the solution. Develop the chromatogram using a mixture of 9 volumes of 2-propanol R and 1 volume of formic acid (−1080 g/l) TS. Allow the solvents to evaporate and examine the chromatogram in ultraviolet light at 254 nm. The benzoic acid appears as dark spots on a fluorescent background in the upper third of the chromatogram for concentrations of 2 μg and larger.

Silica gel H

Description — A fine, white, homogeneous powder with an average particle size of between 10 and 40 μm.

pH — Shake 1 g with 10 ml of carbon-dioxide-free water R for 5 minutes. Measure the pH potentiometrically (IP, vol. 1, page 96); pH, about 7.

Preparation — Suspend 30 g in 60 ml of water, shaking vigorously for 30 seconds. Carefully coat the cleaned plates with a layer 0.25 mm thick using a spreading device. Allow the coated plates to dry in air.

Separating power — Apply separately to the adsorbent layer 10 μl of 0.10 mg/ml solutions containing respectively indophenol blue R, sudan red G R and dimethyl yellow R in toluene R. Develop the plate with the same solvent over a distance of 10 cm. The chromatogram shows 3 clearly-separated spots, the spot of indophenol blue near the point of application, that of dimethyl yellow in the middle of the chromatogram, and that of sudan red G between the two.

Silica gel HF254

Description — A fine, white, homogeneous powder with an average particle size of between 10 and 40 μm containing about 150 g of a fluorescent indicator per kg and having an optimal intensity of 254 nm.

pH — Shake 1 g with 10 ml of carbon-dioxide-free water R for 5 minutes. Measure the pH potentiometrically (IP, vol. 1, page 96); pH, about 7.

Preparation — Suspend 30 g in 60 ml of water, shaking vigorously for 30 seconds. Carefully coat the cleaned plates with a layer 0.25 mm thick using a spreading device. Allow the coated plates to dry in air.

Separating power — Apply separately to the adsorbent layer 10 μl of 0.10 mg/ml solutions containing respectively indophenol blue R, sudan red G R and dimethyl yellow R in toluene R. Develop the plate with the same solvent over a distance of 10 cm. The chromatogram shows 3 clearly-separated spots, the spot of indophenol blue near the point of application, that of dimethyl yellow in the middle of the chromatogram, and that of sudan red G between the two.

Fluorescence — Prepare a 1.0 mg/ml solution of benzoic acid R in a mixture of 9 volumes of 2-propanol R and 1 volume of formic acid (−1080 g/l) TS. Apply to the adsorbent layer at 10 points of applications, increasing quantities from 1–10 μl of the solution. Develop the chromatogram using a mixture of 9 volumes of 2-propanol R and 1 volume of formic acid (−1080 g/l) TS. Allow the solvents to evaporate and examine the chromatogram in ultraviolet light at 254 nm. The benzoic acid appears as dark spots
on a fluorescent background in the upper third of the chromatogram for concentrations of 2 µg and larger.

_Silica gel, HF_{254}, silanized_

Description — A fine, white, homogeneous powder which, after shaking with water, floats on the surface because of its water-repellent properties.

Preparation — Suspend 30 g with 60 ml of a mixture of 2 volumes of water and 1 volume of methanol R shaking vigorously for 2 minutes. Carefully coat the cleaned plates with a layer 0.25 mm thick using a spreading device. Allow the coated plates to dry in air, then dry for 30 minutes in an oven at 100–105 °C.

Separating power — Prepare a mixture containing 0.1 g quantities of each of methyl laurate R, methyl myristate R, methyl palmitate R and methyl stearate R. Then add 40 ml of a 0.3 g/ml decanted solution of potassium hydroxide R in ethanol (~710 g/l) TS and heat under reflux on a water-bath for 1 hour. Cool, add 100 ml of water, acidify with hydrochloric acid (~70 g/l) TS and extract with three 1-ml volumes of chloroform R. Dry the combined chloroform extracts over anhydrous sodium sulfate R, filter and evaporate to dryness. Dissolve the residue in 50 ml of chloroform R.

Apply separately to the adsorbent layer, three 10 µl portions of this solution and develop the chromatogram in a mixture of 65 volumes of dioxan R, 25 volumes of water and 10 volumes of glacial acetic acid R.

After removing the plate from the chromatographic chamber, heat it in an oven at 120 °C for 30 minutes. Allow to cool, spray with a solution containing 35 mg of phosphomolybdic acid R per ml of 2-propanol R and heat at 80 °C until the spots become visible. Expose the plate to ammonia vapour until the adsorbent turns white. The chromatogram shows 4 clearly-separated spots.

Fluorescence — Prepare a 1.0 mg/ml solution of benzoic acid R in a mixture of 9 volumes of 2-propanol R and 1 volume of formic acid (~1080 g/l) TS. Apply to the adsorbent layer at 10 points of applications, increasing quantities from 1–10 µl of the solution. Develop the chromatogram using a mixture of 9 volumes of 2-propanol R and 1 volume of formic acid (~1080 g/l) TS. Allow the solvents to evaporate and examine the chromatogram in ultraviolet light at 254 nm. The benzoic acid appears as dark spots on a fluorescent background in the upper third of the chromatogram for concentrations of 2 µg and larger.
ANNEX 3

REAGENTS AND TEST SOLUTIONS

Acetic acid, glacial, R. C₂H₄O₂; d-1.048.
Suitable commercially-available reagent.

Acetic acid (~300 g/l) TS. A solution of glacial acetic acid R containing about 300 g/l of C₂H₄O₂
(approximately 5 mol/l); d-1.037.

Acetic acid (~60 g/l) TS. Acetic acid (~300 g/l) TS, diluted to contain about 60 g/l of C₂H₄O₂
(approximately 1 mol/l); d-1.008.

Acetone R. C₃H₆O.
Suitable commercially-available reagent.

Acetonitrile R. Methyl cyanide, C₂H₃N.
Description. A clear, colourless liquid.
Miscibility. Freely soluble with water.
Suitable commercially-available reagent.

Aflatoxin mixture TS.
Procedure. Prepare a mixed working standard in a mixture of 98 volumes of chloroform R and 2
volumes of acetonitrile R, containing per ml 0.5 μg of each of aflatoxins B₁ and G₁, and 0.1 μg of each
of aflatoxins B₂ and G₂.
Note: Aflatoxins are highly toxic and should be handled with care. National legal requirements should
be followed.
Aflatoxins are suitable commercially-available working standards.

Aluminium chloride R. AlCl₃.6H₂O.
Suitable commercially-available reagent.

Aluminium oxide, purified, R. Al₂O₃.
Suitable commercially-available reagent for column chromatography.

Amaranth S R. C.I. 16185; acid red 27; C₂₀H₁₁N₂N₂O₅₂S₃.
Description. A deep brown or deep reddish-brown, fine powder.
Suitable commercially-available reagent.

1,2,4-Aminonaphtoilsulfonic acid R. C₁₉H₁₀NO₄S.
Description. A white to slightly brownish pink powder.
Solubility. Sparingly soluble in water.
Suitable commercially-available reagent.

Aminonaphtholsulfonic acid TS.
Procedure. Add 0.25 g of 1,2,4-aminonaphtholsulfonic acid R to 100 ml of freshly-prepared sodium
metabisulfite (150 g/l) TS with mechanical stirring. After stirring for 15 minutes, add 0.5 g of
anhydrous sodium sulfite R. After stirring for an additional 5 minutes, filter the mixture.
Storage. Keep in a brown bottle.
Note. This reagent should be prepared freshly every week.

Ammonia (~260 g/l) TS. d ~0.894.
Suitable commercially-available reagent.

Ammonia (~100 g/l) TS. Ammonia (~260 g/l) TS, diluted to contain about 100 g/l of NH₃
(approximately 6 mol/l); d ~0.956.
Ammonium iron(III) citrate, brown, R. Ferric ammonium citrate, brown, soluble ferric citrate. Contains about 9% of NH₃, 16.5–18.5% of Fe, and about 65% of hydrated citric acid. 

Description. Reddish brown granules, garnet-red transparent scales, or brownish yellow powder; odourless or slight odour of NH₃. Very deliquescent.

Solubility. Very soluble in water; practically insoluble in ethanol (≈750 g/l) TS.

Storage. Store in a well-closed container, protected from light. Suitable commercially-available reagent.

Ammonium molybdate R. H₂₄Mo₇O₂₄·4H₂O.

Suitable commercially-available reagent.

Ammonium molybdate (40 g/l) TS. A solution of ammonium molybdate R containing about 40 g of H₂₄Mo₇O₂₄·4H₂O per litre.

Ammonium nitrate R. NH₄NO₃.

Suitable commercially-available reagent.

Ammonium oxalate H. C₂H₃N₂O₄·H₂O.

Suitable commercially-available reagent.

Ammonium oxalate (25 g/l) TS. A solution of ammonium oxalate R containing about 27 g of C₂H₃N₂O₄ per litre.

Ammonium thiocyanate R. CH₄N₂S.

Suitable commercially-available reagent.

Ammonium thiocyanate (75 g/l) TS. A solution of ammonium thiocyanate R containing about 75 g of CH₄N₂S per litre (approximately 1 mol/l).

Anisaldehyde R. 4-Methoxybenzaldehyde; C₈H₈O₂.

Description. A colourless to pale yellow, oily liquid with an aromatic odour.

Boiling point. About 248 °C.

Mass density. ρ₂₀ = about 1.125 kg/l.

Suitable commercially-available reagent.

Anisaldehyde TS.

Procedure. Mix in the following order 0.5 ml of anisaldehyde R, 10 ml of glacial acetic acid R, 85 ml of methanol R and 5 ml of sulfuric acid (≈1760 g/l) TS.

Argon R. Ar. Contains not less than 999.95 ml/l of Ar.

Suitable commercially-available reagent.

Argon-methane R.

Suitable commercially-available reagent.

Arsenic, dilute, AsTS. One ml contains 10 µg of arsenic.

Procedure. Dilute 1 ml of strong arsenic AsTS with sufficient water to produce 100 ml.

Note. Dilute arsenic AsTS must be freshly prepared.

Arsenic, strong, AsTS.

Procedure. Dissolve 0.132 g of arsenic trioxide R in 6 ml of sodium hydroxide (≈80 g/l) TS, by gentle heating. Dilute the cooled solution to 20 ml of water, and add 50 ml of hydrochloric acid (≈250 g/l) TS, and add sufficient water to produce 100 ml.

Arsenic trioxide R. As₂O₃.

Suitable commercially-available reagent.

Beads for gel chromatography.

Suitable commercially-available material for gel chromatography.
**Beef extract R.** A residue from beef broth obtained by extracting fresh, sound, lean beef by cooking with water and evaporating the resulting broth at a low temperature, usually under reduced pressure until a thick pasty residue is obtained. Suitable commercially-available reagent.

**Benzoic acid R.** C₇H₆O₂. Contains not less than 99.8% of C₇H₆O₂.
*Description.* Colourless, light, feathery crystals or a white, microcrystalline powder; odour, characteristic, faint.

*Solubility.* Slightly soluble in water; freely soluble in ethanol (~750 g/l) TS, ether R, and chloroform R.

*Method-insoluble substances.* Dissolve 20 g in 200 ml of methanol R and digest under complete reflux for 30 minutes. Filter through a tared filtering crucible, wash thoroughly with methanol R, and dry at 105 °C; it leaves a residue of not more than 1.0 mg.

*Assay.* Dissolve about 0.5 g, accurately weighed, in 15 ml of ethanol (~750 g/l) TS, previously neutralized to phenol red/ethanol TS, add 20 ml of water and titrate with sodium hydroxide (0.1 mol/l) VS, using phenol red/ethanol TS as indicator. Repeat the operation without the substance being examined and make any necessary correction. Each ml of sodium hydroxide (0.1 mol/l) VS is equivalent to 12.21 mg of C₇H₆O₂.

**Benzylpenicillin sodium R.** C₁₆H₁₇N₂Na₄O₄S. Quality of substance conforms to the monograph of The International Pharmacopoeia, vol. 2, p. 51.

**Bile salts R.**
*Description.* A concentrate of beef bile, the principal constituent of which is sodium desoxycholate, determined as cholic acid.

*Solubility.* Soluble in water and in ethanol (~750 g/l) TS.

*Acidity.* pH of 0.02 g/ml solution, 5.8-6.2.

Suitable commercially-available reagent.

**Brilliant black BN R.** C₂₈H₁₇N₅Na₄O₁₄S₄. C.I. 28440.
*Description.* A bluish-violet or greyish black powder or fine crystals.

*Solubility.* Freely soluble in water, practically insoluble in ethanol (~750 g/l) TS, acetone R, chloroform R and ether R.

Suitable commercially-available reagent.

**Brilliant green R.** Malachite green G; basic green 1; C.I. 42040; C₂₇H₃₄N₂O₄S.
*Description.* Small, glistening golden crystals.

*Solubility.* Soluble in water and ethanol (~750 g/l) TS.

Suitable commercially-available reagent.

**Bromine R.** Br₂.
Suitable commercially-available reagent.

**Bromine AsTS.**
*Procedure.* Dissolve 30 g of potassium bromide R in 40 ml of water, add 30 g of bromine R and dilute with sufficient water to produce 100 ml. The solution complies with the following test: Evaporate 10 ml nearly to dryness on a water-bath, add 50 ml of water, 10 ml of hydrochloric acid (~250 g/l) AsTS, and sufficient stannous chloride AsTS to reduce the remaining bromine, and apply the general test for arsenic. The colour of the stain produced is not more intense than that produced from a 1-ml standard stain, showing that the amount of arsenic does not exceed 1 µg/ml.

**Bromocresol purple R.** C₂₁H₁₈Br₂O₅S.
Suitable commercially-available reagent.

**Calcium carbonate R1.** CaCO₃.
Suitable commercially-available reagent.

**Calcium carbonate R2.** Calcium carbonate R1 of suitable quality to serve as a primary standard for the standardization of disodium edetate solutions.
Suitable commercially-available reagent.
Calcon R. Monosodium salt of 2-hydroxy-1-[(2-hydroxy-1-naphthylazo)naphthalene-4-sulfonic acid; C.I. mordant black 17, C.I. 15705, eriochrome blue black R, solochrome dark blue; C_{26}H_{13}N_{2}NaO_{5}S. Suitable commercially-available reagent.

Calcon carboxylic acid R. 2-Hydroxy-1-[(2-hydroxy-4-sulfo-1-naphthylazo)-3-naphthoic acid; C_{21}H_{14}N_{2}O_{7}S_3H_{2}O. Description. A dark brown powder with a violet tint. Solubility. Practically insoluble in water; slightly soluble in methanol R and in ethanol (−750 g/l) TS; freely soluble in solution of alkaline hydroxides. Suitable commercially-available reagent.

Calcon carboxylic acid Indicator mixture R. Procedure. Mix 0.1 g of calcon carboxylic acid R with 10 g of anhydrous sodium sulfate R.

Calcon Indicator mixture R. Procedure. Mix 0.1 g of calcon R with 10 g of anhydrous sodium sulfate R.

Cellulose for thin-layer chromatography. Quality specified under "Specifications for adsorbents for use in thin-layer chromatography".

Cetrimide R. Contains not less than 98.0% and not more than 101.0% of alkyltrimethylammonium bromide, calculated as C_{17}H_{33}BrN with reference to the dried substance. Description. A white or almost white, voluminous, free-flowing powder; odour, slight and characteristic. Solubility. Soluble in 2 parts of water; freely soluble in ethanol (−750 g/l) TS. Suitable commercially-available reagent.

Chinese ink TS. Indian ink. Suitable commercially-available reagent. Note. Before use, dilute 1 ml of black Chinese ink TS with 2 ml of water; if necessary, further dilute up to 1:10. It must be freshly prepared.

Chloral hydrate R. C_{2}H_{3}Cl_{3}O_{2}. Description. Colourless, hygroscopic crystals with a sharp odour. Melting temperature. About 55 °C. Suitable commercially-available reagent.

Chloral hydrate TS. Procedure. Dissolve 50 g of chloral hydrate R in 20 ml of water.

Chloramphenicol R. C_{11}H_{12}Cl_{2}N_{2}O_{5}. Quality of substance conforms to the monograph of The International Pharmacopoeia, vol 2, p. 64.

Chloroform R. CHCl_{3}. Suitable commercially-available reagent.

Chromic acid TS. Procedure. Dissolve 84 g of chromium trioxide R in 700 ml of water and add slowly while stirring in 400 ml of sulfuric acid (−1760 g/l) TS.

Chromium trioxide R. CrO_{3}. Suitable commercially-available reagent.

Copper(II) sulfate R. CuSO_{4}.5H_{2}O. Suitable commercially-available reagent.

Crystal violet R. C_{25}H_{30}ClN_{3}. Suitable commercially-available reagent.
Cuoxam TS (Tetramine copper dihydroxide, Schweizer's reagent).

**Procedure.** Dissolve 10 g of copper(II) sulfate R in 100 ml of water, and add sufficient sodium hydroxide (~240 g/l) TS until alkaline. Filter the precipitate and wash with cold water, previously made sulfate-free. To the moist copper hydroxide add, while stirring, ammonia (~100 g/l) TS until dissolved. Note: Cuoxam TS must be freshly prepared.

**Cyclohexane R.** C₆H₁₂.
Suitable commercially-available reagent.

**Desmetryn R.** C₉H₁₇N₅S₂. 2-Methylmercuri-4-methylamino-6-isopropylamino-S-triazine.
Suitable commercially-available reagent to be used as reference material.

**Dichloromethane R.** Methylene chloride, CH₂Cl₂.
**Description.** A clear colourless, mobile liquid.
**Miscibility.** Freely miscible with ethanol (~750 g/l) TS and ether R.
**Boiling range.** Not less than 95% distils between 39 and 41 ºC.
**Residue on evaporation.** Leaves, after evaporation on a water-bath and drying at 105 ºC, not more than 0.5 mg/ml.
Suitable commercially-available reagent.

**Dimethyl sulfoxide R.** C₅H₆OS.
**Description.** A colourless liquid; odourless or with a slight, but unpleasant odour.
**Mass density.** ρ₂₀ 1.10 kg/l.
Suitable commercially-available reagent.

**Dimethyl yellow R.** C₁₁H₁₈N₃. 4-dimethylaminoazobenzene; C₁₄H₁₅N₃.
**Description.** It produces in moderately acidic alcoholic solutions a red colour and in weakly acidic and alkaline solutions a yellow colour (pH range, 2.8-4.6).
**Caution.** Dimethyl yellow R is carcinogenic.
**Homogeneity.** Carry out the method for thin-layer chromatography, using silica gel G as the coating substance and dichloromethane R as the mobile phase. Apply 10 µl of a 0.1 mg/ml solution in dichloromethane R. After removing the plate from the chromatographic chamber allow it to dry in air. Only one spot appears on the chromatogram.
Suitable commercially-available reagent.

**Dioxan R.** 1,4-Dioxane, C₆H₈O₂.
**Caution.** It is dangerous to determine the boiling range or the residue on evaporation before complying with the test for peroxides.
**Description.** A clear, colourless liquid.
**Miscibility.** Miscible with water, ethanol (~750 g/l) TS and ether R.
**Boiling range.** Not less than 95% distils between 101 and 105 ºC.
**Melting temperature.** Solidifies when cooled in ice and does not completely remelt at temperatures below 10 ºC.
**Residue on evaporation.** Evaporate on a water-bath and dry to constant weight at 105 ºC; it leaves a residue of not more than 0.1 mg/ml.
**Mass density.** (ρ₂₀) = about 1.031 kg/l.
**Water.** Determined by the Karl Fischer method, not more than 5.0 mg/ml.
**Peroxides.** Add 5 ml to a mixture of 1 g of potassium iodide R dissolved in 10 ml of water, 5 ml of hydrochloric acid (~70 g/l) TS, and 2 ml of starch TS, and mix; not more than a faint blue or brown colour is produced.
Suitable commercially-available reagent.

**Dipotassium hydrogen phosphate R.** K₂HPO₄.
Suitable commercially-available reagent.

**Disodium edetate R.** C₁₀H₁₄N₂Na₂O₈·2H₂O.
Suitable commercially-available reagent.
**Disodium edetate (0.05 mol/l) VS.** Disodium edetate R, dissolved in water to contain 16.81 g of C₁₀H₁₄N₂Na₂O₈ in 1000 ml.

*Method of standardization.* Ascertain the exact concentration by an appropriate method. The following method is suitable: Transfer about 200 mg of calcium carbonate R₂, accurately weighed, to a 400-ml beaker, add 10 ml of water, and swirl to form a slurry. Cover the beaker with a watch glass and introduce 2 ml of hydrochloric acid (−70 g/l) TS from a pipette inserted between the lip of the beaker and the edge of the watch glass. Swirl the contents of the beaker to dissolve the calcium carbonate. Wash down the sides of the beaker, the outer surface of the pipette, and the watch glass with water, and dilute with water to about 100 ml. While stirring the solution, preferably with a magnetic stirrer, add about 30 ml of the disodium edetate solution from a 50-ml burette. Add 10 ml of sodium hydroxide (−80 g/l) TS and 0.3 g of calcio indicator mixture R or of calcio carboxylic acid indicator mixture R and continue the titration with the disodium edetate solution to a blue endpoint. Each 5.005 mg of calcium carbonate is equivalent to 1 ml of disodium edetate (0.05 mol/l) VS.

**Disodium hydrogen phosphate R. Na₂HPO₄.12H₂O.**
Suitable commercially-available reagent.

**Ethanol (−750 g/l) TS.**
Suitable commercially-available reagent.

**Ethanol (−710 g/l) TS.** A solution of about 950 ml of ethanol (−750 g/l) TS diluted with water to 1000 ml.

**Ethanol (−375 g/l) TS.** A solution of about 525 ml of ethanol (−750 g/l) TS diluted with water to 1000 ml.

**Ethanol (−188 g/l) TS.** A solution of about 260 ml of ethanol (−750 g/l) TS diluted with water to 1000 ml.

**Ethanol (−150 g/l) TS.** A solution of about 210 ml of ethanol (−750 g/l) TS diluted with water to 1000 ml.

**Ether R. C₄H₁₀O.**
Suitable commercially-available reagent.

**Ether/light petroleum TS1.**
*Procedure.* Dilute 60 ml of ether R with sufficient distilled light petroleum R to produce 1000 ml.

**Ether/light petroleum TS2.**
*Procedure.* Dilute 150 ml of ether R with sufficient distilled light petroleum R to produce 1000 ml.

**Ether/light petroleum TS3.**
*Procedure.* Dilute 500 ml of ether R with sufficient distilled light petroleum R to produce 1000 ml.

**Ethyl acetate R. C₄H₈O₂.**
Suitable commercially-available reagent.

**Fast yellow R. C.I. 13015; E105; C₁₂H₆N₃Na₂O₆S₂.**
*Description.* An orange-yellow to red powder.
*Solubility.* Freely soluble in water; slightly soluble in ethanol (−750 g/l) TS; practically insoluble in ether R and chloroform R.
Suitable commercially-available reagent.

**Ferric ammonium sulfate R. FeH₄NO₈S₂.12H₂O.** This reagent should be free of chlorides.
Suitable commercially-available reagent.

**Ferric ammonium sulfate (0.25 mol/l) VS.** Ferric ammonium sulfate R, dissolved in nitric acid (−750 g/l) TS to contain 120.5 g of FeH₄NO₈S₂.12H₂O in 1000 ml.
*Procedure.* Dissolve 120.5 g of ferric ammonium sulfate R in a sufficient quantity of nitric acid (−750 g/l) TS to produce 1000 ml. The reagent should be free of chlorides.
Ferric chloride R. FeCl₃·6H₂O. Suitable commercially-available reagent.

Ferric chloride (50 g/l) TS. Procedure. Dissolve 5 g of ferric chloride R in 100 ml of water.

Floril R. Suitable commercially-available material for column chromatography.

Formic acid (−1080 g/l) TS. CH₂O₂; d = 1.2. Suitable commercially-available reagent.

D-Fructose R. C₆H₁₂O₆. Description. A white, crystalline powder. Melting point. About 103 °C with decomposition. Specific optical rotation. Use a 0.10 g/ml solution in water containing 0.05 ml of ammonia (−100 g/l) TS; [α]D° = about −92 °. Suitable commercially-available reagent.

D-Galactose R. C₆H₁₂O₆. Description. A white, crystalline powder. Melting point. About 164 °C. Specific optical rotation. Use a 0.10 g/ml solution in water; [α]D° = about +80 °. Suitable commercially-available reagent.


Glucose hydrate R. Monohydrate of α-D-glucopyranose, C₆H₁₂O₆·H₂O. Contains not less than 99.0% and not more than 101.5% of C₆H₁₂O₆, calculated with reference to the dried substance. Description. Colourless crystals or a white crystalline or granular powder; odourless. Solubility. Soluble in about 1 part of water and in about 60 parts of ethanol (−750 g/l) TS; more soluble in boiling water and in boiling ethanol (−750 g/l) TS. Acidity. Dissolve 5 g in 50 ml of carbon-dioxide-free water R. It requires for neutralization not more than 0.5 ml of carbon-free sodium hydroxide (0.02 mol/l) TS, phenolphthalein/ethanol TS being used as indicator. Specific optical rotation. Dissolve 100 mg, previously dried to constant weight, in 1 ml of water, and add a few drops of ammonia (−100 g/l) TS; [α]D° = +52 to +53°. Soluble starch or sulfites. Dissolve 1 g in 10 ml of water and add 1 drop of iodine TS; the liquid is coloured yellow. Loss on drying. Dry to constant weight at 105 °C; it loses not less than 80 mg/g and not more than 100 mg/g. Sulfated ash. Not more than 1.0 mg/g. Assay. Dissolve about 0.1 g, accurately weighed, in 50 ml of water, add 30 ml of iodine (0.1 mol/l) VS, 10 ml of sodium carbonate (50 g/l) TS and allow to stand for 20 minutes. Add 15 ml of hydrochloric acid (−70 g/l) TS and titrate the excess of iodine with sodium thiosulfate (0.1 mol/l) VS, using starch TS as indicator. Perform a blank determination and make any necessary corrections. Each ml of iodine (0.1 mol/l) VS is equivalent to 9.008 mg of C₆H₁₂O₆.

Glycerol R. Propane-1,2,3-triol with small amounts of water, C₃H₈O₃. Contains not less than 970 g/kg of C₃H₈O₃. Description. A clear, almost colourless, syrupy and hygroscopic liquid; odourless. Miscibility. Miscible with water and ethanol (−750 g/l) TS; practically immiscible with ether R and chloroform R. Mass density. (ρ₂₀) not less than 1.256 kg/l. Refractive index (nD) not less than 1.469. Acrolein and other reducing substances. Mix 1 ml with 1 ml of ammonia (−100 g/l) TS and heat in a water-bath at 60 °C for 5 minutes; the liquid is not coloured yellow. Remove from the water-bath and add 3 drops of silver nitrate (40 g/l) TS; the liquid does not become coloured within 5 minutes. Sulfated ash. Not more than 0.5 mg/g. Suitable commercially-available reagent.
Glycerol-ethanol TS.

Procedure. Mix equal volumes of glycerol R, water and ethanol (-750 g/l) TS.

Glycine R. Aminoacetic acid, C₂H₅NO₂.
Suitable commercially-available reagent.

Helium R. He. Contains not less than 999.95 ml/l of He.
Suitable commercially-available reagent.

Hexane R. n-Hexane, C₆H₁₄.
Description. A colourless, mobile, highly inflammable liquid.
Boiling range. Distils completely over a range of 1 °C between 67.5 and 69.5 °C.
Mass density. ρ₂₀ 0.658-0.659 kg/l.
Refractive index. (n₀²₀) 1.374-1.375.
Suitable commercially-available reagent.

Hide powder R.
Suitable commercially-available reference material.

Hydrochloric acid (-420 g/l) TS. d -1.18.
Suitable commercially-available reagent.

Hydrochloric acid (-250 g/l) TS. A solution of hydrochloric acid (-420 g/l) TS in water, containing approximately 250 g/l of HCl; d -1.12.

Hydrochloric acid (-250 g/l) AsTS. Hydrochloric acid (-250 g/l)TS that complies with the following tests A and B:
(A) Dilute 10 ml with sufficient water to produce 50 ml, add 5 ml of ammonium thiocyanate (75 g/l) TS and stir immediately; no colour is produced.
(B) To 50 ml add 0.2 ml of bromine AsTS, evaporate on a water-bath until reduced to 16 ml, adding more bromine AsTS if necessary to ensure that an excess, as indicated by the colour, is present throughout the evaporation. Add 50 ml of water and 5 drops of stannous chloride AsTS and apply the general test for arsenic. The colour of the stain produced is not more intense than that produced from a 0.2-ml standard stain, showing that the amount of arsenic does not exceed 0.05 μg/ml.

Hydrochloric acid (-250 g/l), stannated, AsTS.

Procedure. Dilute 1 ml of stannous chloride AsTS with sufficient hydrochloric acid (-250 g/l) AsTS to produce 100 ml.

Hydrochloric acid (-70 g/l) TS.

Procedure. Dilute 260 ml of hydrochloric acid (-250 g/l) TS with sufficient water to produce 1000 ml (approximately 2 mol/l); d -1.035.

Hydrochloric acid (1 mol/l) VS. Hydrochloric acid (-250 g/l) TS, diluted with water to contain 36.47 g of HCl in 1000 ml.

Method of standardization. Ascertain the exact concentration of the 1 mol/l solution in the following manner: Dissolve about 1.5 g, accurately weighed, of anhydrous sodium carbonate R, previously dried at 270 °C for 1 hour, in 50 ml of water and titrate with the hydrochloric acid solution, using methyl orange/ethanol TS as indicator. Each 52.99 mg of anhydrous sodium carbonate R is equivalent to 1 ml of hydrochloric acid (1 mol/l) VS.

Indian ink, see Chinese ink TS.
Indophenol blue R. C.I. 49700; C₁₈H₂₆N₂O₆.

Description. A violet-black powder.

Solubility. Practically insoluble in water; soluble in chloroform R.

Homogeneity. Carry out the method for thin-layer chromatography, using silica gel G as the coating substance and dichloromethane R as the mobile phase. Apply 10 μl of a 0.1 mg/ml solution in dichloromethane R. After removing the plate from the chromatographic chamber allow it to dry in air. Only one spot appears on the chromatogram. Suitable commercially-available reagent.

Iodine R. I₂.
Suitable commercially-available reagent.

Iodine TS.

Procedure. Dissolve 2.6 g of iodine R and 3 g of potassium iodide R in sufficient water to produce 100 ml (approximately 0.1 mol/l).

Iodine (0.1 mol/l) VS. Iodine R and potassium iodide R, dissolved in water to contain 25.38 g of I₂ and 36.0 g of KI in 1000 ml.

Method of standardization. Ascertain the exact concentration of the 0.1 mol/l solution by titrating 25.0 ml with sodium thiosulfate (0.1 mol/l) VS, using starch TS as indicator.

Iodine (0.02 mol/l) VS. Iodine R and potassium iodide R, dissolved in water to contain 5.076 g of I₂ and 7.2 g of KI in 1000 ml.

Method of standardization. Ascertain the exact concentration of the solution following the method described under iodine (0.1 mol/l) VS.

Iodine/ethanol TS.

Procedure. Dissolve 1 g of iodine R in sufficient ethanol (–750 g/l) TS to produce 1000 ml.

Iron(III) citrate R. Ferric citrate, C₆H₅FeO₆.2H₂O.
Suitable commercially-available reagent.

Kieselguhr for thin-layer chromatography.
Quality specified under "Specifications for adsorbents for use in thin-layer chromatography".


Lactochloral TS.

Procedure. Dissolve 50 g of chloral hydrate R in 50 g of lactic acid R by gentle heating.

Lactophenol TS.

Procedure. To a mixture of 20 g of lactic acid R and 40 g of glycerol R dissolved in 20 ml of water, add 20 g of phenol R and mix.

Lactose R. C₁₂H₂₂O₁₁.
Suitable commercially-available reagent.

Lead acetate R. C₄H₆O₄Pb.3H₂O.
Suitable commercially-available reagent.

Lead acetate (80 g/l) TS. A solution of lead acetate R in freshly-boiled water containing about 80 g/l of C₄H₆O₄Pb (approximately 0.25 mol/l).

Lithium chloride R. LiCl.

Description. White, deliquescent crystals or granules.

Solubility. Freely soluble in water; soluble in acetone R and ethanol (–750 g/l) TS.

Storage. Store in a tightly closed container.

Suitable commercially-available reagent.
L-Lysine R. C₆H₁₄N₂O₂.
Description. Crystalline needles or hexagonal plates.
Solubility. Soluble in water; very slightly soluble in ethanol (~750 g/l) TS; insoluble in ether R.
Melting point. About 213 °C with decomposition.
Specific optical rotation. Dissolve 0.2 g in 10 ml of hydrochloric acid (~250 g/l) TS; [α]D²⁰ = about +21.5°.
Suitable commercially-available reagent.

Magnesium chloride R. MgCl₂·6H₂O.
Suitable commercially-available reagent.

Mercuric bromide R. HgBr₂.
Suitable commercially-available reagent.

Mercuric bromide AsTS.
Procedure. Dissolve 5 g of mercuric bromide R in sufficient ethanol (~750 g/l) TS to produce 100 ml.

Mercuric bromide paper AsR.
Procedure. Use smooth, white filter-paper weighing 65–120 g/m². The thickness of the paper in mm should be approximately equal numerically to the weight expressed as above, divided by 400. Soak pieces of filter-paper, not less than 25 mm in width, in mercuric bromide AsTS, decant the superfluous liquid, suspend the paper over a non-metallic thread and allow it to dry, protected from light.
Storage. Store the mercuric bromide paper AsR in stoppered bottles in the dark.
Note. Paper which has been exposed to sunlight or to vapours of ammonia must not be used as it produces only a pale stain or no stain at all.

Mercuric nitrate TS. (Millon's reagent, nitric acid solution of mercury).
Procedure. Dissolve 1 ml of mercuric R in 9 ml of fuming nitric acid R, keeping the mixture well cooled during the reaction. When the reaction is complete, dilute the solution with an equal volume of water. It should be protected from light and used within two months of preparation.

Mercuric thiocyanate R. C₂H₉N₂S₂.
Suitable commercially-available reagent.

Mercuric thiocyanate TS. A saturated solution of mercuric thiocyanate R in ethanol (~750 g/l) TS.

Mercury R. Hg.
Suitable commercially-available reagent.

Methane R. CH₄.
Suitable commercially-available reagent.

Methanol R. CH₄O.
Suitable commercially-available reagent.

Methyl laurate R. C₁₃H₂₆O₂.
Description. A colourless or pale yellow liquid.
Mass density. ρ₂₀ = about 0.87 kg/l.
Suitable commercially-available reagent.

Methyl myristate R. C₁₅H₃₀O₂.
Description. A colourless or slightly yellow liquid.
Mass density. ρ₂₀ = about 0.87 kg/l.
Suitable commercially-available reagent.

Methyl orange R. Sodium salt of 4'-dimethylaminoazobenzene-4-sulfonic acid, C₁₄H₁₄N₃NaO₃S.
Suitable commercially-available reagent.

Methyl orange/ethanol TS.
Procedure. Dissolve 0.04 g of methyl orange R in sufficient ethanol (~150 g/l) TS to produce 100 ml.
Methyl palmitate R. Methyl hexadecanoate; C\textsubscript{17}H\textsubscript{34}O\textsubscript{2}.

Description. A colourless, waxy solid.

Freezing point. About 27 °C.

Mass density. p\textsubscript{20} = about 0.86 kg/l.

Suitable commercially-available reagent.

Methyl stearate R. C\textsubscript{19}H\textsubscript{38}O\textsubscript{2}.

Description. A white or pale yellow, crystalline mass.

Melting point. About 38 °C.

Suitable commercially-available reagent.

1-Naphthol R. C\textsubscript{10}H\textsubscript{8}O.

Description. Colourless crystals or a white, crystalline powder; odour; characteristic.

Solubility. Soluble in 5 parts of ethanol (−750 g/l) TS (may form a slightly opalescent, colourless or almost colourless solution).

Melting range. 93–96 °C.

Sulfated ash. Not more than 0.5 mg/g.

Suitable commercially-available reagent.

1-Naphthol TS.

Procedure. Dissolve 20 g of 1-naphthol R in 100 ml of ethanol (−750 g/l) TS. Protect from light and use within a few days of preparation.

Neutral red R. C.I. 50040; C.I. Basic red; C\textsubscript{15}H\textsubscript{17}CIN\textsubscript{4}.

Suitable commercially-available reagent.

Nitric acid, fuming, R. HNO\textsubscript{3}.

Suitable commercially-available reagent.

Nitric acid (−1000 g/l) TS. d = 1.41.

Suitable commercially-available reagent.

Nitric acid (−750 g/l) TS.

Procedure. Dilute 750 ml of nitric acid (−1000 g/l) TS with sufficient water to produce 1000 ml (approximately 9 mol/l).

Nitric acid (−500 g/l) TS.

Procedure. Dilute 500 ml of nitric acid (−1000 g/l) TS with sufficient water to produce 1000 ml (approximately 8 mol/l).

Nitric acid (225 g/l) TS.

Procedure. Dilute 220 ml of nitric acid (−1000 g/l) TS with sufficient water to produce 1000 ml (approximately 3.5 mol/l).

Nitro-chromic acid TS.

Procedure. Mix equal volumes of nitric acid (225 g/l) TS and chromic acid TS.

Nitrogen R. N\textsubscript{2}.

Suitable commercially-available reagent.

Ox bile, dehydrated, R. Dehydrated, purified fresh bile.

Suitable commercially-available reagent.

Pancreatic digest of casein R.

Suitable commercially-available reagent.

Pancreatic digest of gelatin R.

Suitable commercially-available reagent.

Papic digest of soybean meal R.

Suitable commercially-available reagent.
Paraffin, liquid, R.  
Suitable commercially-available reagent.

Peptone, dried, R. A variety of peptones are available from casein, meat, beef or a mixture of these.  
Suitable commercially-available reagent.

Perchloric acid (−1.170 g/l) TS. d~1.67.  
Suitable commercially-available reagent.

Petroleum, light, R.  
Suitable commercially-available reagent.

Phenol R. C₆H₅O.  
Description. Colourless, or at most faintly pink, cohering or separate acicular crystals, or crystalline masses; odour, characteristic. Corrosive, and blanches the skin and mucous membranes.  
Solubility. Soluble in about 15 parts of water and in about 100 parts of liquid paraffin R; freely soluble in ethanol (−750 g/l) TS, ether R and chloroform R.  
Completeness of solution. 1.0 g dissolves completely in 15 ml of water at 15 °C.  
Congealing temperature. Not below 40.5 °C.  
Residue on evaporation. Evaporate on a water-bath and dry to constant weight at 105 °C; leaves not more than 0.5 mg/g of residue.  
Suitable commercially-available reagent.

Phenolphthalein R. C₂₀H₁₄O₄.  
Suitable commercially-available reagent.

Phenolphthalein/ethanol TS.  
Procedure. Dissolve 1.0 g of phenolphthalein R in sufficient ethanol (−750 g/l) TS to produce 100 ml.

Phenol red R. Phenolsulfonphthalein, C₁₉H₁₄O₅S.  
Suitable commercially-available reagent.

Phenol red/ethanol TS.  
Procedure. Dissolve 0.05 g of phenol red R in a mixture of 2.85 ml of sodium hydroxide (0.05 mol/l) VS and 5 ml of ethanol (−710 g/l) TS. Warm the solution slightly and after cooling dilute with sufficient ethanol (−150 g/l) TS to produce 250 ml.

Phloroglucinol R. Benzene-1,3,5-triol; C₉H₆O₃.2H₂O.  
Description. White or yellowish white crystals or a crystalline powder.  
Solubility. Slightly soluble in water; soluble in ethanol (−750 g/l) TS and ether R.  
Melting point. About 218 °C.  
Suitable commercially-available reagent.

Phloroglucinol TS.  
Procedure. Dissolve 1 g of phloroglucinol R in 100 ml of ethanol (−750 g/l) TS.

Phosphate buffer pH 7.4 TS.  
Procedure. Dissolve 6.8 g of potassium dihydrogen phosphate R in 250 ml of water and add 393.4 ml of sodium hydroxide (0.1 mol/l) VS.

Phosphomolybdic acid R. H₃PO₄.12MoO₃.24H₂O.  
Suitable commercially-available reagent.

Phosphorus pentoxide R. P₂O₅.  
Suitable commercially-available reagent.
Polysorbate 20 R. The mono-ester of lauric acid and tripolyethylene glycol 300-sorbitan ether.

**Description.** A yellow or brownish-yellow, oily liquid.

**Miscibility.** Miscible with water, ethanol (-750 g) TS, ethyl acetate R, methanol R and dioxan R; insoluble in mineral oil.

**Mass density.** (p20) = about 1.10 kg/l.

Suitable commercially-available reagent.

Polysorbate 80 R. The mono-ester of oleic acid and tripolyethylene glycol 300-sorbitan ether.

**Description.** Lemon to amber coloured, oily liquid.

**Miscibility.** Miscible with water, producing an odourless and nearly colourless solution. Miscible with ethanol (-750 g/l) TS, ethyl acetate R, and vegetable oils; immiscible with mineral oils.

**Mass density.** (p20) = about 1.10 kg/l.

Suitable commercially-available reagent.

**Potassium bromide R. KBr.**
Suitable commercially-available reagent.

**Potassium chlorate R. KClO3.**
Suitable commercially-available reagent.

**Potassium dichromate R. K2Cr2O7.**
Suitable commercially-available reagent.

**Potassium dichromate (0.0167 mol/l) VS.** Potassium dichromate R, dissolved in water to contain 4.904 g of K2Cr2O7 in 1000 ml.

**Method of standardization.** Ascertain the exact concentration of the (0.0167 mol/l) solution in the following manner: Place 25.0 ml of the potassium dichromate solution into a glass-stoppered flask, add 2 g of potassium iodide R, dilute with 200 ml of water, add 5 ml of hydrochloric acid (-420 g/l) TS, allow to stand for 10 minutes in a dark place, and titrate the liberated iodine with sodium thiosulfate (0.1 mol/l) VS, adding 3 ml of starch TS as the endpoint is approached. Correct for a blank determined on the same quantities of the same reagents.

**Potassium dihydrogen phosphate R. KH2PO4.**
Suitable commercially-available reagent.

**Potassium hydrogen phthalate R. C8H5KO4.**
Suitable commercially-available reagent.

**Potassium hydroxide R. KOH.**
Suitable commercially-available reagent.

**Potassium hydroxide (-110 g/l) TS.** A solution of potassium hydroxide R containing about 112 g of KOH per litre (approximately 2 mol/l).

**Potassium hydroxide (-55 g/l) TS.** A solution of potassium hydroxide R containing about 56 g of KOH per litre (approximately 1 mol/l).

**Potassium iodide R. KI.**
Suitable commercially-available reagent.

**Potassium iodide AsR.** Potassium iodide R that complies with the following test: Dissolve 10 g of potassium iodide R in 25 ml of hydrochloric acid (-250 g/l) AsTS and 35 ml of water; add 2 drops of stannous chloride AsTS and apply the general test for arsenic; no visible stain is produced.

**Potassium iodide (80 g/l) TS.** A solution of potassium iodide R containing about 83 g/l of KI (approximately 0.5 mol/l).

**Potassium sulfate R. K2SO4.**
Suitable commercially-available reagent.

**Potassium tetraethionate R. K2S4O6.**
Suitable commercially-available reagent.
Prometryn R. C_{10}H_{19}N_{5}S.
Suitable commercially-available reagent to be used as reference material.

1-Propanol R. n-Propanol; propan-1-ol, C_{3}H_{8}O.
Description. A clear, colourless liquid.
Miscibility. Miscible with water and ethanol (~750 g/l) TS.
Boiling range. Not less than 95% distils between 95 and 98 °C.
Mass density, \( \rho_{20} \) = about 0.803 kg/l.
Residue on evaporation. Evaporate on a water-bath and dry to constant weight at 105 °C; it leaves a residue of not more than 0.1 mg/g.
Suitable commercially-available reagent.

2-Propanol R. Isopropyl alcohol; C_{3}H_{8}O.
Suitable commercially-available reagent.

Propylene glycol R. Propane diol, C_{3}H_{8}O_{2}.
Suitable commercially-available reagent.

Pyridine R. C_{5}H_{5}N.
Suitable commercially-available reagent.

Quinine hydrochloride R. C_{20}H_{24}N_{2}O_{2}.HCl,2H_{2}O. Quality of substance conforms to the monograph of The International Pharmacopoeia, vol. 2, p. 248.

Saponin R.
Suitable commercially-available reference material.

Silica gel for thin-layer chromatography.
Quality specified under "Specifications for adsorbents for use in thin-layer chromatography".

Silica gel R.
Suitable commercially-available material for column chromatography.

Silica gel, desiccant, R.
Description. An amorphous, partly hydrated SiO_{2}, occurring in glassy granules of varying sizes. It is frequently coated with a substance that changes colour when the capacity to absorb water is exhausted. Such coloured products may be regenerated (i.e., may regain their capacity to absorb water) by heating at 110 °C until the gel assumes the original colour.
Loss on drying. Ignite 2 g, accurately weighed, at 950 ±50 °C to constant weight; the loss is not more than 60 mg/g.
Water absorption. Place about 10 g in a tared weighing-bottle, and weigh. Then place the bottle, with the cover removed, for 24 hours in a closed container in which 80% relative humidity is maintained by being in equilibrium with sulfuric acid having a relative density of 1.19. Weigh again; the increase in weight is not less than 310 mg/g.
Suitable commercially-available reagent.

Silver nitrate R. AgNO_{3}.
Suitable commercially-available reagent.

Silver nitrate (40 g/l) TS. A solution of silver nitrate R containing about 42.5 g/l of AgNO_{3} (approximately 0.25 mol/l).

Simazine R. C_{7}H_{12}ClN_{5}.
Suitable commercially-available reagent to be used as reference material.

Soda lime R.
Suitable commercially-available reagent.

Sodium acetate R. C_{2}H_{3}NaO_{2}.3H_{2}O.
Suitable commercially-available reagent.
Sodium acetate (1.6 g/l) TS. A solution of sodium acetate R containing about 1.64 g of C₂H₃NaO₂ per litre (0.02 mol/l).

Sodium carbonate R. Na₂CO₃.10H₂O.
Suitable commercially-available reagent.

Sodium carbonate, anhydrous, R. Na₂CO₃.
Suitable commercially-available reagent.

Sodium carbonate (50 g/l) TS. A solution of sodium carbonate R containing about 50 g of Na₂CO₃ per litre (approximately 0.5 mol/l).

Sodium carboxymethylcellulose R.
Suitable commercially-available material for chromatography.

Sodium chloride R. NaCl.
Suitable commercially-available reagent.

Sodium chloride (400 g/l) TS. A solution of sodium chloride R containing about 400 g of NaCl per litre.

Sodium chloride (100 g/l) TS. A solution of sodium chloride R containing about 100 g of NaCl per litre.

Sodium citrate R. C₆H₅Na₃O₇.2H₂O. Quality of substance conforms to the monograph of The International Pharmacopoeia, vol. 3, p. 192.

Sodium citrate (36.5 g/l) TS. A solution of sodium citrate R containing about 36.5 g of C₆H₅Na₃O₇ per litre.

Sodium deoxycholate R. C₂₃H₃₉NaO₄. Containing not less than 90% of C₂₃H₃₉NaO₄.
Suitable commercially-available reagent.

Sodium hydroxide R. NaOH.
Suitable commercially-available reagent.

Sodium hydroxide (−240 g/l) TS. A solution of sodium hydroxide R containing about 240 g of NaOH per litre of carbon-dioxide-free water R.

Sodium hydroxide (−80 g/l) TS. A solution of sodium hydroxide R containing about 80 g/l of NaOH (approximately 2 mol/l).

Sodium hydroxide (1 mol/l) VS. Sodium hydroxide R, dissolved in water to contain 40.01 g of NaOH in 1000 ml.
Method of standardization. Ascertain the exact concentration of the 1 mol/l solution in the following manner: Dry about 5 g of potassium hydrogen phthalate R at 105 °C for 3 hours and weigh accurately. If the potassium hydrogen phthalate is in the form of large crystals, they should be crushed before drying. Dissolve in 75 ml of carbon-dioxide-free water R and titrate with the sodium hydroxide solution, using phenolphthalein/ethanol TS as indicator. Each 0.0424 g of potassium hydrogen phthalate is equivalent to 1 ml of sodium hydroxide (1 mol/l) VS. Standard solutions of sodium hydroxide should be restandardized frequently.
Storage. Solutions of alkali hydroxides absorb carbon dioxide when exposed to air. They should therefore be stored in bottles with suitable non-glass, tightly-fitting stoppers, provided with a tube filled with soda lime R.

Sodium hydroxide (0.1 mol/l) VS. Sodium hydroxide R, dissolved in water to contain 4.001 g of NaOH in 1000 ml.
Method of standardization. Ascertain the exact concentration of the solution following the method described under sodium hydroxide (1 mol/l) VS.
Sodium hydroxide (0.05 mol/l) VS. Sodium hydroxide R, dissolved in water to contain 2.000 g of NaOH in 1000 ml.
Method of standardization. Ascertain the exact concentration of the solution following the method described under sodium hydroxide (1 mol/l) VS.

Sodium hydroxide (0.02 mol/l), carbonate-free, VS. Sodium hydroxide R, dissolved in water to contain 0.8001 g of NaOH in 1000 ml.
Method of standardization. Ascertain the exact concentration of the solution following the method described under sodium hydroxide (1 mol/l) VS.

Sodium hydroxide, methanolic TS.
Procedure. Dissolve 2.5 g of sodium hydroxide R in 10 ml carbon dioxide-free water R. Add 1 ml of propylene glycol R and dilute to 100 ml with methanol R.

Sodium hypochlorite TS. Containing 100–140 g/l of available chlorine.
Description. A yellowish liquid; odour of chlorine.
Suitable commercially-available reagent.

Sodium metabisulfite R. Na₂O₅S₂.
Suitable commercially-available reagent.

Sodium metabisulfite (150 g/l) TS. A solution of sodium metabisulfite R containing about 150 g of Na₂O₅S₂ per litre.

Sodium pyruvate R. C₃H₅NaO₂.
Description. An almost white to white powder or a crystalline powder.
Solubility. Soluble in water.
Suitable commercially-available reagent.

Sodium sulfate, anhydrous, R. Na₂SO₄.
Suitable commercially-available reagent.

Sodium thiosulfate R. Na₂S₂O₃·5H₂O.
Suitable commercially-available reagent.

Sodium thiosulfate (0.1 mol/l) VS. Sodium thiosulfate R, dissolved in water to contain 15.82 g of Na₂S₂O₃ in 1000 ml.
Method of standardization. Ascertain the exact concentration of the 0.1 mol/l solution in the following manner: Transfer 30.0 ml of potassium dichromate (0.0167 mol/l) VS to a glass-stoppered flask and dilute with 50 ml of water. Add 2 g of potassium iodide R and 5 ml of hydrochloric acid (−250 g/l) TS, stopper and allow to stand for 10 minutes. Dilute with 100 ml of water and titrate the liberated iodine with the sodium thiosulfate solution, using starch TS as indicator. Sodium thiosulfate solutions should be restandardized frequently.

Stannous chloride R. SnCl₂·2H₂O.
Suitable commercially-available reagent.

Stannous chloride TS.
Procedure. Dissolve 330 g of stannous chloride R in 100 ml of hydrochloric acid (−250 g/l) TS and sufficient water to produce 1000 ml.

Stannous chloride AsTS.
Procedure. Prepare from stannous chloride TS by adding an equal volume of hydrochloric acid (−250 g/l) TS, boiled down to the original volume, and filtered through a fine-grained filter-paper.
Test for arsenic. To 10 ml add 6 ml of water and 10 ml of hydrochloric acid (−250 g/l) AsTS, and distil 16 ml. To the distillate add 50 ml of water and 2 drops of stannous chloride AsTS and apply the general test for arsenic. The colour of the stain produced is not more intense than that produced from a 1–ml standard stain, showing that the amount of arsenic does not exceed 1 μg/ml.

Starch R.
Suitable commercially-available reagent.
Starch, soluble, R.
Suitable commercially-available reagent.

Starch TS.
Procedure. Mix 0.5 g of starch R or of soluble starch R with 5 ml of water, and add this solution, with constant stirring, to sufficient water to produce about 100 ml; boil for a few minutes, cool, and filter. Note. Starch TS should be freshly prepared.

Sucrose R. C₁₂H₂₂O₁₁.
Suitable commercially-available reagent.

Sudan red G R. 1-(4-Phenylazophenylazo)-2-naphthol; Sudan III; Solvent red 23; C.I. 26100; C₂₂H₁₆N₄O₂.
Description. A reddish brown powder.
Solubility. Practically insoluble in water; soluble in chloroform R.
Suitable commercially-available reagent.

Sudan Red TS.
Procedure. Dissolve 0.5 g of sudan red G R in 100 ml of glacial acetic acid R.

Sulfuric acid (~1760 g/l) TS. d ~1.84.
Suitable commercially-available reagent.

Sulfuric acid (~1160 g/l) TS.
Procedure. Add 660 ml of sulfuric acid (~1760 g/l) TS to sufficient water to produce 1000 ml.

Sulfuric acid (~350 g/l) TS.
Procedure. Add 200 ml of sulfuric acid (~1760 g/l) TS to sufficient water to produce 1000 ml.

Sulfuric acid (~300 g/l) TS.
Procedure. Add 171 ml of sulfuric acid (~1760 g/l) TS to sufficient water to produce 1000 ml (approximately 3 mol/l).

Sulfuric acid (~37 g/l) TS.
Procedure. Add 21.5 ml of sulfuric acid (~1760 g/l) TS to sufficient water to produce 1000 ml (approximately 0.375 mol/l).

Tetracycline R. C₂₂H₂₄N₂O₈.
Suitable commercially-available reagent.

N,N,N',N'-Tetramethyl-p-phenylenediamine dihydrochloride R. C₁₀H₁₆N₂·2HCl.
Description. Whitish-grey crystals.
Suitable commercially-available reagent.

Thionine R. C.I. 52000; C₁₂H₁₀ClN₃S.
Description. Blackish green glistening crystals.
Solubility. Freely soluble in hot water.
Suitable commercially-available reagent.

Thionine TS.
Procedure. Dissolve 0.2 g of thionine R in 100 ml of ethanol (~188 g/l) TS.

Toluene R. C₇H₈.
Suitable commercially-available reagent.

2,2,4-Trimethylpentane R. C₆H₁₈.
Suitable commercially-available reagent.

Trinitrophenol R. C₆H₃N₃O₇.
Suitable commercially-available reagent.
Trinitrophenol, *ethanolic*, TS.
*Procedure.* Dissolve 1 g of trinitrophenol R in 100 ml of ethanol (−750 g/l) TS.

Tropaeolin O R. C.I. 14270; E103: resorcin yellow; chrysoidin S; sulphone orange; acid orange 6; C₁₂H₁₀N₂NaO₅S.
*Description.* It produces in moderately alkaline solutions a yellow colour and in strongly alkaline solution an orange colour (pH range 11.0–12.7).
Suitable commercially-available reagent.

Water, carbon-dioxide-free, R. Water that has been boiled vigorously for a few minutes and protected from the atmosphere during cooling and storage.

Xylene R. C₈H₁₀.
Suitable commercially-available reagent.

D-Xylose R. C₅H₁₀O₅.
*Description.* A white, crystalline powder.
*Specific optical rotation.* Dissolve 1 g in 10 ml of water; [α]₂₀D = +20°.
Suitable commercially-available reagent.

Yeast extract, water-soluble, R.
Suitable commercially-available reagent.

Zinc R. Zn; granulate, powder, or dust.
Suitable commercially-available reagent.

Zinc, granulated, AsR. Granulated zinc R that complies with the following tests:
*Limit of arsenic.* Add 10 ml of stannated hydrochloric acid (~250 g/l) AsTS to 50 ml of water, and apply the general test for arsenic; use 10 g of granulated zinc R and allow the reaction to continue for 1 hour; no visible stain is produced.
*Test for sensitivity.* Repeat the test for arsenic with the addition of 0.1 ml dilute arsenic AsTS; a faint, but distinct yellow coloured stain is produced.

Zinc acetate R. C₄H₁₀O₄Zn·2H₂O.
Suitable commercially-available reagent.

Zinc acetate/aluminium chloride TS.
*Procedure.* Dissolve 200 g of zinc acetate R and 5 g of aluminium chloride R in sufficient water to produce 1000 ml.

Zinc chloride R. ZnCl₂.
Suitable commercially-available reagent.

Zinc chloride, iodinated, TS.
*Procedure.* Dissolve 40 g of zinc chloride R and 13 g of potassium iodide R in 21 ml of water. Add 1 g of iodine R and shake for 15 minutes. Filter if necessary.
*Storage.* It should be protected from light.

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