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Traditional Chinese medicine — Microscopic examination of medicinal herbs

Médecine traditionnelle chinoise — Examen microscopique des herbes médicinales

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Foreword

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This document was prepared by Technical Committee ISO/TC 249, *Traditional Chinese medicine*.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Traditional Chinese medicine — Microscopic examination of medicinal herbs

1 Scope

This document specifies the methods for microscopic examination of medicinal herbs. It covers the equipment, sampling, preparation and observation methods. This document is applicable to medicinal herbs used in traditional Chinese medicine, including Chinese materia medica (whole medicinal materials) and decoction pieces derived from plants. It is not applicable to medicinal materials derived from animals or minerals.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
microscopic examination
examination of a test specimen by microscope with a magnification of generally × 50 to × 500

[SOURCE: ISO 17639:2003, 3.2, modified.]

3.2
medicinal herbs
raw materials derived from various parts of plants for drugs used in herbal medicine

Note 1 to entry: Herbal medicine includes traditional Chinese medicine, Korean medicine and Kampo.

3.3
slide
flat rectangular plate of glass on which an object is mounted for microscopic examination

[SOURCE: ISO 10934-1:2002, 2.133]

3.4
cover glass
rectangular or circular piece of thin glass used to cover a microscopical preparation

[SOURCE: ISO 10934-1:2002, 2.34, modified — Note 1 to entry removed.]

3.5
micrometer
device for measuring small lengths

[SOURCE: ISO 10934-1:2002, 2.96]

4 Sampling

Small-sized, cut or powdered material (50 g to 250 g) samples shall be taken after mixing thoroughly. Large-sized or whole material (250 g to 500 g) samples shall be taken after mixing thoroughly. After that, select a representative sample of the material. If necessary, the samples should be preserved in airtight containers.

5 Apparatus

Use the usual laboratory apparatus and, in particular, the following:

5.1 Optical microscope or slide scanner.

5.2 Optical or in-software micrometer.

5.3 Imaging devices such as drawing attachments, embedded camera or digital imaging sensor for the microscope.

5.4 Slides and cover glasses.

5.5 Botanical dissecting instruments such as tweezers, surgical knife, razor blade, microtome.

6 Preparation for microscopic examination

6.1 Cross-section or longitudinal-section slides

a) According to the sample condition, moisturizing, fixation or maceration process can be added. See A.1 to A.3 for additional information.

b) Select representative pieces of the material being examined and cut into suitable lengths.

c) After softening, cut the material with a razor blade or a microtome to a thickness of 10 µm to 20 µm.

d) Place a section on a slide glass, add two or three drops of a mounting agent or chloral hydrate solution and place a cover glass over it, taking precautions against the inclusion of bubbles.

e) Embed the material in hard paraffin for cutting, if necessary. See A.4.

6.2 Powder slides

a) Place about 0,1 g of powdered sample in a watch glass containing two or three drops of a swelling agent or chloral hydrate solution, stir well with a small rod to prevent the inclusion of bubbles and allow to stand for more than 10 min to swell the sample.

b) Using a small glass rod, smear the slide glass with a small amount of the swollen sample, add one drop of the mounting agent and place a cover glass on it so that the tissue sections spread evenly without overlap, taking precautions against the inclusion of bubbles.

6.3 Mounting and swelling agents

Mounting and swelling agents may be made of a mixture of glycerine and water (1:1) or a mixture of glycerine, 95 % ethanol and water (1:1:1) as mounting and swelling agents. Other agents which have characteristics of mounting and swelling agents can be used.

7 Observation of components

Observation can be conducted in the order of the outer portion, inner portion and cell contents. In case of a powdered sample, observation can be made in the order of characteristic component, matter present in large amounts, rarely existing matter and cell contents. For histochemical detection of the sample, see A.6.

8 Test report

The test report shall include the following information:

a) all information necessary for the complete identification of the sample;

b) the sampling method used;

c) the test method used, with reference to this document, i.e. ISO/TS 21310:2020;

d) the test result(s) obtained;

e) all operating details not specified in this document, or regarded as optional, together with details of any incidents which may have influenced the test result(s);

f) any unusual features (anomalies) observed during the test;

g) the date of the test.

Annex A
(informative)

Preparation methods for microscopy

A.1 Moisturizing

Dried parts of a plant may require softening before preparation for microscopy, preferably by soaking in water. 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 need to be soaked in water or in 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 can sometimes be necessary.

Any water-soluble contents can be removed from the cells by soaking in water. Starch grains can be gelatinized by heating in water. In certain cases, material can be moistened with water for a few minutes to soften the surfaces and allow sections to be cut.

A.2 Fixation

Fixation is the process of preserving the tissue by placing the tissue in fixatives. The permeation of fixatives into the tissue can be dependent upon the size of the sample. Before fixation, it is recommended that samples be cut smaller than 6 mm × 6 mm × 6 mm.

The following water solutions are used as fixatives:

— ethanol: 50 % to 70 %

— formalin: under 5 %

— acetic acid: approximately 100 %

— chromic acid: approximately 1 %

— F.A.A. solution: formalin 5 ml, acetic acid 5 ml, 50 % to 70 % ethanol 90 ml

— Craf III solution: 1 % chromic acid 30 ml, 10 % acetic acid 20 ml, formalin 10 ml, water 40 ml.

A.3 Maceration

A.3.1 General

For maceration, cut or slice the sample into small pieces about 2 mm in thickness. Depending on the feature of the material, one of the following three methods can be used. For medicinal herb samples with only a few or scattered woody tissues or with parenchyma tissues, use the potassium hydroxide method. For hard materials mainly composed of woody tissues or woody tissues grouped into bundles, use the chromic-nitric acid or potassium chlorate method.

A.3.2 Potassium hydroxide method

Place the sample in a test tube, add an adequate quantity of aqueous potassium hydroxide solution (a volume fraction of 5 %), then heat until the residue can be easily separated when pressed with a glass rod. Decant the alkaline solution and wash the residue with water. Transfer a small amount of

macerated material onto a slide and tease it out with a needle. Mount in dilute glycerine and examine under a microscope.

A.3.3 Chromic-nitric acid method

Place the sample in a test tube and add an adequate quantity of chromic-nitric acid test solution, then leave to stand until the material can be easily separated when pressing with a glass rod. Decant the acidic solution, wash the residue with water and prepare the slide as directed in A.3.2.

A.3.4 Potassium chlorate method

Place the sample in a test tube, add dilute nitric acid (volume fraction of 50 %) and a few crystals of potassium chlorate. Warm gently until the effervescence subsides, then add a few crystals of potassium chlorate to maintain a slight effervescence. When the tissue shows a tendency to disintegrate, break the material with a glass rod. Decant the acidic solution, wash the macerated material with water and prepare the slide as directed in A.3.2.

A.4 Sectioning

A.4.1 Thin section (free hand section)

Grasp the sample to be sectioned between the top of the thumb and forefinger of one hand. Holding a single-edge razor blade or a microtome blade with the thumb and forefinger of the opposite hand, rapidly draw the blade through the plant material in a smooth continuous sliding motion (not sawing). The sections will accumulate on the flat part of the razor blade. After cutting a few sections, dip the razor blade into water standing in a watch glass and the sections will float off the blade. Select several of the best sections to observe.

A.4.2 Paraffin embedding section

A.4.2.1 Dehydration

The series of tert-butanol (TBA) is the most common method for dehydration, but a lot of other methods can be applied. If the case of using TBA series, keep pure TBA in a warm place before use (e.g. on the top of an incubation oven) as it freezes below 25 °C. An example of TBA series is shown in Table A.1.

Table A.1 — Example of TBA series

Stage	t-butanol %	95 % ethanol %	Water %
1	10	40	50
2	20	50	30
3	35	50	15
4	55	45	0
5	75	25	0
6	100	0	0
Dehydrate tissue at each stage for 1 h to 1 d, depending on tissue size (e.g. root tips for 1 h, anthers or leaves for 2 h at each stage). Stages 1 to 5 are processed at room temperature and stage 6 in the incubation oven at 56 °C to 60 °C.			

A.4.2.2 Embedding section

a) Keep molten paraffin wax and plastic pipettes in incubation oven.

b) Add paraffin wax in vials half-filled with TBA. Alternatively, add an equal volume of molten wax to the volume of TBA in the vials.

c) Place in the oven at 56 °C to 60 °C with the vial cap attached and mix a few times until the wax is completely melted.

d) Leave in the oven overnight. The next day, pour or pipette away the TBA/wax mixture into a waste container.

e) Add fresh molten wax and leave in the oven overnight until the TBA evaporates. The following day, exchange the wax with new wax two or three times (use a warm transfer pipette or decant).

Trim the wax block around the sample with a single-edged razor blade so that the upper and lower edges are parallel, the left and right edges are at an angle, and the lower edge of the block is longer than the upper. Cut the block with the razor blade away from the sample in clean cuts from the top to the bottom of the block.

Fix the embedding ring with the sample in the sample holder on the microtome. Set the section thickness (usually 10 µm to 16 µm) and cutting angle (around 7 degrees). Start sectioning. The first few sections will not be useful, so remove them with a brush, always away from the blade not towards it. When the surface of the sample evens, a ribbon will start to form. Place the cut sections on a dark-coloured tray. Cut several ribbons until reaching the region of interest, then examine the sections with a stereomicroscope to locate sections containing the region of interest.

Label the objective slides with a pencil, put them on the slide-warmer and apply some water (to almost cover them). Put the sections on a warm slide with an area smaller than the cover glass and make duplications (one for the experiment, the other for the control).

Leave the sections on the hot plate overnight to dry and attach to the slides. Store them in slide boxes at room temperature or in the refrigerator at 4 °C.

A.4.3 Frozen section

If the sample slide does not need to be kept permanent, frozen section method (also known as cryosection) may be chosen. In order to use this method, a cryostat (also known as cryotome) set that is a microtome inside a freezer is needed.

The sample specimen is placed on a metal tissue disc which is then secured in a chuck and frozen rapidly to about –20 °C to –30 °C. The specimen is embedded in a gel-like medium called optimal cutting temperature (OCT) compound. At this temperature, most tissue becomes rock solid. Usually a lower temperature is required for fat or lipid-rich tissues. Each tissue has a preferred temperature range for processing.

The preparation of this sample type is much faster than the paraffin-embedding method. However, the technical quality of these sections is much lower.

A.5 Staining

Generally, the overall structure is observed through dyeing using safrain O, fast green FCF and hematoxylin, and a histochemical method is used to identify specific cell contents. If a sample is made using the paraffin-embedding method, a deparaffinization process is performed using xylene or other solvents before proceeding with the staining process.

A.6 Histochemical detection

A.6.1 Cell walls

A.6.1.1 Lignified cell wall

Add one or two drops of phloroglucinol test solution to the specimen on the slide, allow to stand for about 30 s then add one drop of hydrochloric acid. Lignified cell walls are stained red or purplish-red according to the extent of lignification.

A.6.1.2 Suberized or cuticular cell wall

Add one or two drops of Sudan III test solution to the specimen on the slide, then allow to stand for a few minutes or warm gently. Suberized or cuticular cell walls are stained orange-red or red.

A.6.1.3 Cellulose cell wall

Add one or two drops of zinc chloride-iodine test solution and allow to stand for few minutes. Alternatively, add one or two drops of iodine test solution, allow to stand for a while and add dilute sulphuric acid (a volume fraction of 66 %). Cellulose cell walls are stained blue or purple.

A.6.1.4 Siliceous cell wall

Add one or two drops of sulphuric acid and no change is observed.

A.6.2 Cell contents

A.6.2.1 Starch

Add iodine test solution. A blue or purple colour is observed.

A.6.2.2 Aleurone

a) Add iodine test solution. A brown or yellowish-brown colour is observed.

b) Add mercuric nitrate test solution. A brick red colour is observed. If the material is oily, render it fat-free by immersing in and washing with ether or petroleum ether before carrying out the test.

A.6.2.3 Fatty oil, volatile oil, or resin

a) Add iodine test solution. A brown or yellowish-brown colour is observed.

b) Add mercuric nitrate test solution. A brick red colour is observed. If the material is oily, render it fat-free by immersing in and washing with ether or petroleum ether before carrying out the test.

A.6.2.4 Inulin

Add α-naphthol in ethanol (10 % mass/volume) and then add sulphuric acid. The crystals of inulin turn purplish-red and dissolve rapidly.

A.6.2.5 Mucilage

Add ruthenium red test solution. A red colour is observed.

A.6.2.6 Calcium oxalate crystals

a) Insoluble in dilute acetic acid (a volume fraction of 6 %), soluble in dilute hydrochloric acid (a volume fraction of 9,5 % to 10,5 %) without effervescence.

b) Dissolve gradually in dilute sulphuric acid (a volume fraction of 50 %).

A.6.2.7 Calcium carbonate (stalactite)

Soluble in dilute hydrochloric acid (a volume fraction of 9,5 % to 10,5 %) with effervescence.

A.6.2.8 Silica

Insoluble in sulphuric acid.

A.6.3 Test solutions for histochemical detection

A.6.3.1 Chloral hydrate test solution

Dissolve 50,0 g chloral hydrate in a mixture of 15 ml of water and 10 ml of glycerine.

A.6.3.2 Cuoxam test solution

Add a sufficient amount of water to 0,5 g of copper carbonate and grind in a mortar, then add 10 ml of strong ammonia solution to dissolve.

A.6.3.3 Ferric chloride test solution

Dissolve 1,0 g of ferric chloride in water and make up to 100 ml.

A.6.3.4 Fuchsin glycerine gelatin

Dissolve 1,0 g of animal gelatin in 6 ml of water, then add 7 ml of glycerine and heat with gentle stirring until well mixed. Then filter through a piece of gauze into a Petri dish, add a sufficient amount of basic fuchsin solution (dissolve 0,1 g of basic fuchsin in 600 ml of absolute ethanol and 80 ml of camphor oil), mix well and allow to solidify.

A.6.3.5 Glycerol-acetic acid test solution

Mix well equal volumes of glycerine, glacial acetic acid and water.

A.6.3.6 Iodine test solution

Use 0,1 M iodine solution directly.

A.6.3.7 Mercuric nitrate test solution

Add 3 ml of fuming nitric acid to 4,5 g of mercury. When the reaction is completed, dilute with an equal volume of water. Preserve in an amber-coloured glass bottle with a glass stopper and protect from light.

A.6.3.8 α-Naphthol test solution

To 10,5 ml of a solution of α-naphthol in ethanol (a mass fraction of 15 %) gently add 6,5 ml of sulphuric acid and mix well. Then add 40,5 ml of ethanol, 4 ml of water and mix well.

A.6.3.9 Phloroglucinol test solution

Dissolve 1,0 g of phloroglucinol in 100 ml of ethanol (90 %) and then filter. Preserve in an amber-coloured glass bottle and protect from light.