# ⟨563⟩ IDENTIFICATION OF ARTICLES OF BOTANICAL ORIGIN

## INTRODUCTION

Identification of raw plant material intended for use in the manufacture of pharmaceuticals, excipients, or dietary supplements is carried out by examining the morphological and histological features of the article under test and by performing diagnostic chemical tests on the article. The botanical and chemical characteristics of the test article are then compared to the known botanical and chemical characteristics of the plant species. Reference articles may be specified to assist in the proper botanical and chemical identification of the plant and plant part. A reference article may be either a USP Authenticated Reference Material, which may be used for both botanical and chemical identification, or a USP Reference Standard, which is used for chemical identification only.

## USP AUTHENTICATED REFERENCE MATERIALS

USP Authenticated Reference Materials are plant organs or tissues certified to have come from a plant that has been properly identified as belonging to the species listed on the label. The authentication is performed by botanical taxonomists, plant anatomists, phytochemists, or other plant scientists contracted by USP. A USP Authenticated Reference Material is typically a dried, pulverized plant organ or tissue, and it may be obtained from USP. Herbarium samples that may include roots, stems, leaves, flowers, fruits, and seeds of the authenticated plants are archived and made available for examination upon request. Standard herbarium samples usually consist of the entire mature plant. USP Authenticated Reference Materials undergo the same botanical and chemical diagnostic tests as those applied to test raw materials. A test article must have all botanical and chemical characteristics specified and found in the USP Authenticated Reference Material. To serve its intended purpose, each USP Authenticated Reference Material is properly stored, handled, and used. Generally, USP Authenticated Reference Materials are stored in their original containers under cool and dry conditions and protected from light and insect infestation. Where special storage conditions are necessary, directions are given on the label. Active principles and marker compounds typically degrade with time; therefore, expiration dates are assigned to USP Authenticated Reference Materials for their use in chemical identification. USP Authenticated Reference Materials are not intended for use in the manufacture of pharmaceuticals, excipients, or dietary supplements.

## BOTANICAL IDENTIFICATION

The botanical identification of raw plant materials used in the manufacture of pharmaceuticals, excipients, or dietary supplements consists of ascertaining the macroscopic characteristics of the plant part, such as root, stem, leaf, flower, fruit, or seed, used in the manufacture of the article, as well as ascertaining its histological (microscopic) features. It may also include the inspection of organoleptic features of the botanical tissue such as the presence or absence of a characteristic odor. Individual compendial monographs may include botanical information on possible adulterant species to help ensure their absence in the raw material. For a proper identification of the plant, plant organ, or plant tissue, it is necessary to have a basic knowledge of plant anatomy.

### Diagnostic Plant Morphology and Anatomy

This section exclusively addresses the diagnostic morphological and anatomical features of vascular plants and the various plant parts, such as roots, stems, leaves, flowers, fruits, and seeds, from which pharmaceuticals, excipients, or dietary supplements are derived. Vascular plants include pteridophytes (ferns and fern allies; for example, genera *Aspidium*, *Equisetum*, and *Lycopodium*), gymnosperms (seed plants, in which the seed is not enclosed within a fruit; for example, genera *Ephedra*,*Gingko*, and *Pinus*), and angiosperms (seed plants, in which the seed is enclosed within a fruit; for example, genera *Allium*, *Digitalis*, *Panax*, *Matricaria*, and *Rauwolfia*). Anatomical diagnostic features that are specified in an individual monograph (see *Botanic characteristics* in individual monographs) may include, but are not limited to, the presence of a particular tissue within an organ; the arrangement and type of cells within a tissue; the presence and type of secretory canal, oil, or resin duct or laticifers within an organ; the number of epithelial cells surrounding a secretory canal; and the presence and type of ergastic substances such as starch, inulin, fat globules, essential oils, calcium oxalate crystals, cystoliths, polyphenols, fluids, or other materials occurring in the cytoplasm, organelles, vacuoles, cavities, or cell wall.

#### ROOTS

The tissues present in young roots, starting with the most external tissue, include an epidermis with root hairs, cortex, endodermis, pericycle, phloem, xylem, and, in some species, pith. In some species, the outermost layer or layers of cortex are distinct from the inner layers, in which case they are referred to as a hypodermis. In species that undergo secondary growth in the roots, it is typical for all tissues external to the pericycle to be sloughed off. Roots that exhibit secondary growth have a periderm or bark, composed of a phellum (cork), phellogen (cork cambium), and phelloderm as the outermost tissue. Underneath the periderm, remnants of primary phloem, secondary phloem, vascular cambium, primary xylem, and secondary xylem can be found. Secondary vascular tissues have medullary rays separating clusters of the principal conducting cells of phloem (sieve elements or sieve cells) and the principal conducting cells of xylem (vessels and tracheids). Most species of plants that undergo secondary growth lack pith in the root. The type and arrangement of the principal conducting cells of the vascular tissues may be diagnostic of the species. Roots of many species develop into food storage organs. Abundant parenchyma and

large amounts of starch or other polysaccharides characterize these types of roots. The presence, type, and arrangement of fibers, sclereids, and other tissues, and the presence and location of ergastic material, may also be diagnostic features. Morphologically, roots may be distinguished from rhizomes (the underground stems) primarily by the absence of nodes and internodes, which are present in rhizomes.

## STEMS

Several external macroscopic features of stems that may be diagnostic of the species include the attributes of the nodes, internodes, leaf scars, vascular bundle scars, lenticles, and buds; the growth pattern of the buds; position and arrangement of the leaves along the stem; and the presence of tendrils, spines, thorns, or prickles. Starting with the outermost tissue, the internal arrangement of tissues in the young stems of most species is epidermis, cortex, a concentric ring of vascular bundles separated from each other by parenchymatous medullary rays, and pith. Depending on the species, stomata or trichomes or both structures may be present in the epidermis. The cortex of some species may include a hypodermis or an endodermis or both. In most monocotyledons, the vascular bundles are not arranged concentrically; instead, they are scattered throughout a mass of parenchyma tissue internal to the epidermis. Because of this arrangement, neither cortex, medullary rays, nor pith can be discerned. In woody plant stems that undergo secondary growth, it is typical for the epidermis to be sloughed off and replaced by a periderm composed of a phellum, phellogen, and phelloderm. Some species are characterized by having multiple periderms (rhytidome). Lenticles may be present in the periderm and their attributes may serve as diagnostic features. Underneath the periderm are the remnants of the cortex, primary phloem, secondary phloem, vascular cambium, secondary xylem, primary xylem, and pith. Medullary rays are also present. As in the root, the type and arrangement of the principal conducting cells of the vascular tissues; the presence, type, and arrangement of fibers, sclereids, and other tissues; and the presence and location of ergastic material may also be diagnostic features. Rhizomes may have some morphological characteristics similar to those of roots and therefore they may be mistaken for roots. However, rhizomes can be correctly identified as stems because they have distinct nodes and internodes.

## LEAVES

Several macroscopic features of leaves that may be diagnostic of the species include the attributes of the leaf blade, petiole, and stipules and the phyllotaxy. The outermost tissue of a leaf blade is the epidermis, followed by mesophyll and vascular tissues. Microscopic diagnostic features of epidermal cells include the cuticle thickness and markings, the shape and arrangement of stomata and guard cells, the arrangement and size of subsidiary cells, stomatal number (number of stomata per unit area), and stomatal index (number of stomata per unit number of epidermal cells). Additional features useful in the identification of leaf material include types and arrangement of trichomes (plant hairs) present; type and arrangement of mesophyll and vascular tissues; palisade mesophyll ratio; presence and appearance of accessory tissues such as parenchymatous or sclerenchymatous bundle sheaths, paraveinal mesophyll, endodermis, and transfusion tissue; type and arrangement of the principal conducting cells of the vascular tissues; presence, type, and arrangement of fibers, sclereids, and other tissues; and presence, location, and physical appearance of ergastic material.

## FLOWERS

Flowers are the best diagnostic morphological features of any flowering plant, and the floral structure is the principal criterion used in plant taxonomy. The diagnostic features of flowers include type of inflorescence; presence, number, and appearance of the primary floral parts (sepals, petals, stamens, and carpels); type of symmetry displayed by the floral parts; relative position of the ovaries in regard to the other parts of the flower; the number of ovules per ovary; type of placentation of the ovary; physical appearance of the pollen grains; presence of nectaries; presence of covering or glandular trichomes; and physical features of accessory structures such as the receptacle and bracts. The histological features and the presence of ergastic materials in the tissues of floral parts are also diagnostic of the species.

## FRUITS

The identification of the species of plant from which a fruit was derived may be determined by observing several macroscopic criteria. These criteria include the number of pistils found in the fruit; the number of carpels within each pistil; the number of seeds within each carpel; the placentation of the fruit; and the determination of whether the fruit is dehiscent, indehiscent, or fleshy. Additional diagnostic features include the number of sutures in a dehiscent fruit, the determination of whether the seeds are fused to or free from the pericarp wall, physical features of the three layers of the pericarp of fleshy fruits (epicarp, mesocarp, and endocarp), and presence and physical appearance of accessory tissues such as the receptacle and bracts. Histological features of fruit tissues may aid in identification. The characteristics of the seeds within the fruit are also diagnostic features of the species.

## SEEDS

The macroscopic features of seeds used in identification include the shape and size of the seed; appearance of the seed-coat surface; placement of the hilum and micropyle; and presence of accessory structures of the seed coat such as the arils, caruncle, or oil bodies. Physical features of the embryo, such as its size, shape, position, and the number and appearance of the cotyledons, as well as the presence and appearance of accessory nutritive tissues such as the remnants of a megagametophyte (in gymnosperms), perisperm (nucellus), or endosperm, are also diagnostic of the species. Histological features of the seed coat and other structures and tissues of the seed may also be used for species identification.

## Microtechnique

Histological analysis of botanical specimens can be performed on whole plant material or plant powder. The use of cytological stain or other reagents may be necessary to visualize certain histological features. Crossed polarizers can be used to detect structures that rotate plane-polarized light. These structures include starch grains, calcium oxalate crystals, some fibers, and grains of sand (present as a contaminant) that can be observed as bright objects against a dark background. One polarizer is commonly placed in the condenser or the light source, and the second polarizer is placed in the ocular. Light entering the slide from below is plane polarized, permitting only some light waves in a specific plane to pass through. When the two polarizers are aligned, the field becomes bright; when the two polarizers are crossed, the field becomes dark.

### PROCEDURE FOR TEMPORARY MOUNTS AND POWDERED MATERIAL

**General procedure:** Plant samples are observed under the microscope by employing different mounting media, stains, or other solutions to assist in the correct identification of the test article. If a USP Authenticated Reference Material is available, prepare it with the same mounting media or reagent solutions used for the test article. Place 1 or 2 drops of water, *Glycerin–alcohol solution*, *Chloral hydrate solution*, or another reagent solution (see *Preparation and use of reagent solutions, optical devices, and mountants*) in the center of a clean slide. Transfer a small plant tissue section or a portion of plant powder into the mountant or reagent solution, and cover with a clean coverslip. (For specific preparation techniques, see *Preparation of temporary mounts and hand sections*, *Maceration*, or *Preparation of powdered materials*, as appropriate.) To prevent the formation of air bubbles, the coverslip may be carefully placed at an appropriate angle with its edge making the first contact with the slide and then pressed until it covers the specimen. Using a piece of filter paper, remove excess fluid from the margin of the coverslip. Air bubbles can be removed by placing the slide in a vacuum desiccator. When using chloral hydrate, air bubbles can be removed by gently boiling the sample over a small flame such as that from an alcohol lamp. To replace the mountant or reagent solution, place drops of the new mountant or reagent solution on one edge of the coverslip. Place a strip of filter paper at the opposite edge of the coverslip to remove the old mountant or reagent solution and to cause the new mountant or reagent solution to be drawn over the powdered material or tissue. Plant oils can be also washed away from the tissue in this manner when solvent hexane or acetone are washed through the slide followed by water and, if necessary, *Chloral hydrate solution*. Do not use *Chloral hydrate solution* immediately after treating the plant tissue with flammable solvents without thoroughly washing the tissue with water. This is to avoid setting fire to residual solvent when the microscope slide is later placed over a small flame to boil the tissue. Care must be taken when using reagent solutions that are volatile or corrosive to the microscope. To prevent drying of aqueous or chloral hydrate solutions during observation, add a small drop of glycerin to the slide. Observe the mounted sample under an optical microscope (see *Optical Microscopy* ⟨*776*⟩), and examine for histological features.

**Preparation and use of reagent solutions, optical devices, and mountants:** The following reagents, optical devices, and mounting media are used to assist in the identification of cells, tissues, structural features, and ergastic substances in the tissue or powdered material (see *Table 1* and *Table 2*).

**Table 1. The Use of Reagent Solutions and Optical Devices**

| Detection | Reagent Solutions and Optical Devices |
|---|---|
| Calcium carbonate concretion | *Diluted acetic acid* |
| Calcium oxalate crystals | *Crossed polarizers* |
| Cellulose | *Carmine alum–methyl green solution*<br>*Hydriodic acid*<br>*Zinc chloride–iodine solution* |
| Cytoplasm | *Alcoholic picric acid solution* |
| 1,8-Dihydroxyanthra-quinones | *1 M Potassium hydroxide solution* |
| Essential oils | *Osmium tetroxide solution*<br>*Sudan III solution* |
| Inulin | *Naphthol–sulfuric acid solution* |
| Lignin | *Carmine alum–methyl green solution*<br>*Phloroglucinol–hydrochloric acid solution*<br>*Universal reagent* |
| Lipids (cutin, waxes, and suberin included) | *Carmine alum–methyl green solution*<br>*Osmium tetroxide solution*<br>*Sudan III solution*<br>*Universal reagent* |
| Pectin and mucilage | *Ruthenium red solution*<br>*Thionine solution*<br>*Toluidine blue solution* |
| Phytoglycogen | *Ruthenium red solution* |
| Protein bodies | *Alcoholic picric acid solution*<br>*Osmium tetroxide solution* |

**Table 1. The Use of Reagent Solutions and Optical Devices** *(continued)*

| Detection | Reagent Solutions and Optical Devices |
|---|---|
| Saponin | *Blood–gelatin mixture*<br>*Iodine–glycerin solution* (confirm by testing with *Blood–gelatin mixture*) |
| Starch | *Crossed polarizers*<br>*Iodine solution*<br>*Universal reagent* |
| Tannins and other polyphenols | *Ferric chloride solution*<br>*Osmium tetroxide solution* |

**Table 2. Bleaching and Clarifying Agents and Mountants**

| Use | Mountants and Agents |
|---|---|
| Bleaching agents | *Sodium hypochlorite solution* |
| Clarifying agents | *Chloral hydrate solution*<br>*Lactochloral solution*<br>*Lactophenol solution* |
| Mountants | *Glycerin*<br>*Glycerin–alcohol solution*<br>*Glycerin–gelatin mixture*<br>*Water* |

*Alcoholic picric acid solution*—Prepare a 1% solution of picric acid in alcohol. Picric acid is useful to stain cells having dense cytoplasm such as aleurone cells in seeds. Place a small amount of powdered plant material in a test tube, and shake with about 1 mL of solvent hexane to remove plant oils, which would interfere with the reaction. Centrifuge, and discard solvent hexane. Soak the plant powder in *Alcoholic picric acid solution* for about 30 min. Transfer a portion of the powder to a microscope slide, and observe under a microscope. Cytoplasm and protein bodies turn bright yellow. [**CAUTION**—Picric acid is explosive when dry. Handle appropriately.]

*Blood–gelatin mixture*—Add 4.5 g of gelatin powder to 100 mL of a 0.9% sodium chloride solution, and allow to swell for 30 min. Heat the gel, while stirring, to about 80° in a water bath. Cool to 40°, and add 6 mL of defibrinated bovine blood. Heat to 45°–50°, and pour onto a microscope slide in a thin layer of about 1 mm while the slide is in a horizontal position. To prevent loss of blood-gelatin mixture from the sides, seal the microscope slide edge with a 1-cm wide adhesive tape to form a tray. After cooling and solidification, it is ready for use. [NOTE—Store in a humid chamber for NMT 1–2 days at 3°–4°.] To test for saponins, place small clusters of the powdered plant material on the blood–gelatin layer, spacing them a few mm apart from each other, transfer to a humidifier for a few h, and observe. Saponin-containing particles will cause light-transparent zones to appear in the blood–gelatin.

*Carmine alum–methyl green solution*—Boil 1.5 g of carmine for 30 min in a 15% solution of aluminum potassium sulfate. Cool, filter, and add 10 mL of a 0.75% methyl green solution while stirring. Add 1–2 drops to the plant material. Lignin and suberin turn green and cellulose turns red-violet.

*Chloral hydrate solution*—Use chloral hydrate TS. When using the solution as a clarifying agent, add a few drops to the plant material, and boil briefly over a small flame. Chloral hydrate dissolves cellular contents and intercellular substances and allows cell walls and shapes to be easily observed. It can be used to assist in the identification of cork, fibers, vessels, calcium oxalate crystals (with the aid of crossed polarizers), trichomes, stomata, and pollen.

*Crossed polarizers*—This optical device is used to detect calcium oxalate crystals and starch grains (amyloplasts). In polarized light, calcium oxalate crystals and starch grains appear as bright, birefringent objects on a dark background. Starch grains observed under polarized light will also have a Maltese-cross effect with the arms of the cross intersecting at the hilum. Calcium oxalate crystals are usually best viewed after the sample has been clarified with *Chloral hydrate solution* or another clarifying agent.

*Diluted acetic acid*—Add 1–2 drops to the plant material, and immediately observe under a microscope. Calcium carbonate deposits dissolve with effervescence.

*Ferric chloride solution*—Dilute 1 mL of ferric chloride TS with 9 mL of water. For the detection of phenol hydroxyl groups, such as tannins and flavonoids, from the side of the coverslip add the solution to the aqueous sample. Tannins and other polyphenols become blue-black to green.

*Glycerin*—Use as a mountant to prevent the drying of aqueous and chloral hydrate solutions.

*Glycerin–alcohol solution*—Mix equal volumes of glycerin and alcohol. Use as a mounting medium.

*Glycerin–gelatin mixture*—Add 10.0 g of powdered gelatin to 60 mL of water. Allow to stand for 2 h, and add 70 mL of glycerin containing 1.5 g of dissolved phenol. Heat in a water bath, and filter through a preheated funnel containing glass wool. The filtered mixture is liquefied before use, and it serves as a mounting medium. Add a few drops to the cut or powdered plant material, and cover with a heated coverslip. This preparation is used for long-term storage of specimen mounts. The margins of the coverslip may be sealed with Canada balsam after a few months of drying.

*Hydriodic acid*—Add 1–2 drops to plant material. Cellulosic cell walls become blue to blue-violet.

*Iodine solution*—Add 1–2 drops of 0.1 N iodine VS to the plant material. Starch particles become dark-blue to blue-violet; this reaction is reversible on heating. [NOTE—Proteins, lipids, and cellulose turn yellow to brown; guaiac powder particles become green to blue, but this reagent is not used for diagnostic identification of these features.]

*Iodine–glycerin solution*—Dissolve 0.3 g of iodine and 1.0 g of potassium iodide in a small quantity of water, and add 10 mL of a mixture of glycerin and water (1:1). Add 1–2 drops to the powdered plant material. Samples containing saponins form

yellow lumps or aggregates. If a sample tests positive for saponin, the result has to be confirmed by testing the sample with *Blood–gelatin mixture* as well.

*Lactochloral solution*—Dissolve 50.0 g of chloral hydrate in 50 mL of lactic acid with gentle heating. Add a few drops to the plant material. Place the microscope slide in a small vacuum desiccator if it is necessary to eliminate air bubbles. *Chloral hydrate solution* and *Lactochloral solution* are used for the same type of identification, except that *Lactochloral solution* is a stronger clarifying agent and it is used for plant material that is more difficult to clarify.

*Lactophenol solution*—Mix 20 g of lactic acid, 40 g of glycerin, and 20 mL of water. Add 20 g of phenol, and mix. This is a strong clarifying agent suitable for the examination of pollen grains.

*Naphthol–sulfuric acid solution*—Prepare a 20% solution of 1-naphthol in alcohol. To the plant material add 1 drop of 1-napthol solution and 1 drop of sulfuric acid. Inulin crystals turn brownish red and then dissolve.

*Osmium tetroxide solution*—Dissolve 0.1 g of osmium tetroxide in 5 mL of distilled water. Add 1–2 drops of the solution so obtained to the plant material. Essential oils, fatty oils and other lipids, tannins, and protein bodies become brown to black.

*Phloroglucinol–hydrochloric acid solution*—This solution is used for the identification of lignin and other hydroxyphenylpropane derivatives; lignified tissues, such as sclereids, vessels, fibers, and stone cells; and lignified parenchyma. Moisten the powder or the cut sample with phloroglucinol TS, and allow to dry for 2–3 min before placing the coverslip. Add a few drops of a 25% hydrochloric acid solution, and cover with the coverslip. Lignified cell walls turn carmine red. [NOTE—This stain is not stable.] Cells with hydroxyphenylpropane derivatives, such as vanillin and ferulic acid, also turn red. Alternatively, hydroxyphenylpropane derivatives can be extracted from the plant material and the plant material then examined. To extract hydroxyphenylpropane derivatives repeatedly immerse the untreated material in alcohol, mix on a vortex mixer, centrifuge, and discard the alcohol between washings. Then treat the plant material as specified previously, beginning with the addition of phloroglucinol TS.

*1 M Potassium hydroxide solution*—Add 1 drop to plant material. Cells containing 1,8-dihydroxyanthraquinones will stain red.

*Ruthenium red solution*—Add a few drops of ammonium hydroxide to ruthenium red TS. [NOTE—Store the solution protected from light.] Add 1–2 drops to the plant material. Pectin-containing cell membranes, acidic mucilage, and phytoglycogen turn red.

*Sodium hypochlorite solution*—This solution is used to bleach deeply colored sections. Immerse the plant material in the solution for a few min until sufficiently bleached. Wash the tissue with water, and mount with a suitable mounting agent. [NOTE—Sodium hypochlorite will extract lignin; plant tissue so treated will test negative for lignin.]

*Sudan III solution*—Dissolve 0.5 g of sudan III in 50 mL of alcohol or isopropyl alcohol with reflux boiling. Cool, filter, and add 50 mL of glycerin. Add 1–2 drops of this solution to plant powder. Essential oils, waxes, cutin, suberin, and fatty oils and other lipids combine with this lipophilic colorant and become orange-red to red after a short time.

*Thionine solution*—Prepare a 0.2% thionine acetate solution in 25% alcohol. Immerse the dry sample in this solution. After about 15 min, wash out the excess of stain with 25% alcohol. Mucilage will have swollen into spherical globules and turned red-violet, while cellulose, pectin, and lignified septa will turn blue or blue-violet.

*Toluidine blue solution*—Using toluidine blue, proceed as directed for *Thionine solution*.

*Universal reagent*

SOLUTION A: Dilute 20 mL of a lactic acid-saturated solution of sudan III with 30 mL of lactic acid.

SOLUTION B: Dissolve 0.55 g of aniline sulfate in 35 mL of water.

SOLUTION C: Dissolve 0.55 g of potassium iodide and 0.05 g of iodine in 5 mL of water, and add 5 mL of alcohol.

PROCEDURE: Combine *Solution A*, *Solution B*, and *Solution C*, and add 2.5 mL of hydrochloric acid while stirring. [NOTE—The solution is used without filtering.] For identification, add 2–3 drops to the sample, and gently boil over a small flame. If necessary, small amounts of *Universal reagent* may be added during boiling. Cover with the coverslip. Lignified elements turn yellow, suberin turns red-brown, lipids turn red, and starch turns blue-violet.

*Water*—Use as a mounting medium. [NOTE—All grades of water are acceptable for this purpose.]

*Zinc chloride–iodine solution*—Dissolve 20.0 g of zinc chloride and 6.5 g of potassium iodide in 10.5 mL of water. Add 0.5 g of 0.1 N iodine VS, and shake for 15 min. Filter if necessary. Store in low-actinic glassware. Add 1–2 drops to the plant material, and allow to stand for a few min. Cellulosic cell walls are stained blue to blue-violet.

**Preparation of temporary mounts and hand sections:** When using the dry plant tissue, soak or gently boil in water until soft. Do not soften too much. Material can then be treated like fresh plant material. When appropriate, use the mountants or reagent solutions listed for use with plant powder to help visualize features of the tissue (see *Preparation and use of reagent solutions, optical devices, and mountants*).

To make an epidermal peel of the leaf, petal, sepal, bract, and other leaf-like appendages, roll the tissue into a cylinder, and nick with a sharp, polytef-coated razor blade that has been wetted with water. Grasp the nicked piece of tissue with forceps, and strip back removing a clear section of the epidermis. Mount in water on a microscope slide, place a coverslip over the tissue, and examine under a microscope.

If it is difficult to obtain an epidermal peel using the previous procedure, proceed as follows. Soak the tissue in a 40%–60% nitric acid solution at 60° for 3–4 min or until the epidermis can be easily peeled. The peel is then washed 3–5 times in water to remove the excess of nitric acid. Neutralize the tissue in a 1% potassium hydroxide solution or a 1% sodium hydroxide solution. Wash the tissue again with water, mount in water on a microscope slide, place a coverslip over the tissue, and examine under a microscope.

An alternative method of preparing leaf tissue for the examination of the epidermis is to heat a leaf fragment (about 5 mm × 5 mm) for 15 min in *Chloral hydrate solution* on a water bath. Transfer the tissue to a microscope slide, add a drop of water, and cover with a coverslip. These procedures can be used to determine the stomatal type, distribution, number, and index.

Stomatal number is determined by counting the number of stomata per unit area of a microscopic field. Determine the stomatal number on at least 10 different sites of the specimen, and calculate a mean value. Keep track of which leaf surface is being observed, abaxial or adaxial, as the stomatal number for different surfaces is frequently significantly different.

To calculate the stomatal index, the specimen is observed under a microscope at a low magnification. The size of the surface is determined with a calibrated micrometer ocular, and the number of stomata and the number of epidermal cells for that area are determined. The stomatal index is calculated:

$$\text{Result} = (100 \times S)/(E + S)$$

$S$ = number of stomata for a given area
$E$ = number of epidermal cells of the same area

Determine the stomatal index on at least 10 different sites of the specimen, and calculate a mean value. Again, keep track of which leaf surface is being observed, abaxial or adaxial, as the stomatal indices for different surfaces is frequently significantly different.

To make a cross section of a leaf or thin roots, stems, or other thin appendages, lay the appendage to be sectioned on a microscope slide. Place another microscope slide over the appendage with a portion of the tissue exposed. Using a sharp, polytef-coated razor blade that has been wetted, cut straight down along the edge of upper slide. Without moving the upper slide, cut down again with the razor blade at an angle. Some practice may be necessary to be able to get sections thin enough so that when they are mounted and covered with a coverslip, these sections can be used to determine tissue arrangements (for instance, the number of palisade layers in leaf, thickness of cuticle, types of trichomes, types of vascular bundles, and the like). Because razor blades dull quickly, they have to be replaced frequently.

Use the cross section of leaf tissue so obtained to determine the palisade mesophyll ratio. Alternatively, boil leaf fragments of about 2 $mm^2$ in *Chloral hydrate solution*, mount, cover with a coverslip, and observe under a microscope. Identify groups of four adaxial epidermal cells, and count the palisade mesophyll cells that are lying below and are at least 50% covered by the epidermal cells. This value divided by 4 is the palisade mesophyll ratio. Determine the palisade mesophyll ratio of at least 10 groups of epidermal cells, and calculate a mean value. Palisade mesophyll ratio can also be determined on powdered leaf material.

To make a cross section of thick stems, roots, or other plant parts, including woody tissues, hold the tissue in one hand and using a sharp, polytef-coated razor blade that has been wetted with water, shave a cross section from the appendage. Mount in water, another medium, or reagent solution, place a coverslip over the material, and examine under a microscope. Sections thin enough to determine vascular tissue arrangement, ray type, parenchyma distribution, presence of crystals, and the like can usually be made with a little practice.

**Maceration:** For the proper identification of a plant material, it is sometimes necessary to macerate the tissue into its individual cells before microscopic examination. This can be an especially useful technique for woody or other hard tissues. The material is cut into small pieces of about 2-mm thickness and 5-mm length or sliced into pieces of about 1-mm thickness. Depending on the nature of the cell wall, one of the following methods is used. For hard or highly lignified tissues, use *Method I*. For tissues that are not extensively lignified, use *Method II*.

*Method I*

SOLUTION A: Use 4 N nitric acid solution.

SOLUTION B: Prepare a mixture of 1.2 M chromium trioxide solution and sulfuric acid (7:4).

PROCEDURE: Place the plant material in a test tube containing about 5 mL of a mixture of *Solution A* and *Solution B* (1:1). Heat in a water bath for 20 min. Wash the tissue repeatedly with water, and transfer to a microscope slide. Tease the tissue apart with a dissecting needle, add 1–2 drops of mountant, cover with a coverslip, and examine under a microscope. If necessary, cells can be further separated from each other by pressing down on the coverslip with a gentle, sliding motion. The macerated tissue will test negative for lignin.

*Method II*

PROCEDURE: Place the plant material in a test tube containing about 5 mL of 2 M potassium hydroxide solution. Heat in a water bath for 30 min. Wash the tissue repeatedly with water, and transfer to a microscope slide. Add 1–2 drops of mountant. Place a coverslip over the tissue, press down, squashing the tissue, and examine under a microscope. The macerated tissue will test negative for lignin.

**Preparation of powdered materials:** Place 1 or 2 drops of water, another mountant, or a reagent solution in the center of a clean slide. Moisten the tip of a dissecting needle with water, and dip into the powder under test. Transfer a small amount of material that adheres to the needle into the fluid on the slide, and stir thoroughly and carefully. Cover with a clean coverslip. Because the arrangement of the tissue structures within the plant tissue has been destroyed, the important features for observation of the powdered plant material are the chemical and physical features of tissues and cell types, as well as the presence and chemical and physical features of ergastic substances. The specific tissues, cells, and ergastic substances to be examined are specified in the individual monograph.

## PROCEDURE FOR THIN, PERMANENT MOUNTS

When it is necessary to reveal detailed histological features of a plant specimen, thin tissue sections have to be obtained. The sections need to be thin enough to transmit light, and they have to be cut in such a plane that the desired features are exposed. The plant material is properly killed, fixed, dehydrated, and embedded in paraffin or other embedding media. The embedding medium is used as a solid-support matrix during tissue sectioning. After sectioning and mounting, staining of the specimen is frequently performed to aid in the differentiation of certain structures. [NOTE—The process of fixing, dehydration, embedding, and staining can be significantly expedited by utilizing a microwave oven specifically designed for histological work.]

**Killing and fixation:** As a first step in preparing plant material for sectioning, living cells are killed, and the tissue is preserved. This is most frequently done by employing a chemical fixative. A good general purpose fixative for plant material is a mixture of formaldehyde, acetic acid, and alcohol (FAA).

*FAA solution*—Mix 50 mL of alcohol, 5 mL of glacial acetic acid, 10 mL of formaldehyde solution, and 35 mL of water. [NOTE—Periodically prepare fresh solution, as it loses effectiveness with storage.]

*Procedure*—Completely immerse the plant material in the *FAA solution*. Allow the material to remain immersed for 18–24 h at room temperature. Plant material can be kept indefinitely in *FAA solution*, as long as it remains completely immersed and is not allowed to dry out. Certain plant tissues may require vacuum infiltration to facilitate the penetration of the fixative. Vacuum infiltration is required if the tissue has abundant air spaces or epidermal hairs or if it floats on top of the fixative solution. Place the tissue in a small vial containing the fixative. Place the uncapped vial into a bell jar or desiccator that is connected to a vacuum

source, preferably an oil-sealed vacuum pump. The vacuum is vented into a fume hood to prevent fixative vapors from filling the room. Slowly turn on the vacuum. Do not use a strong vacuum because the fixative may start to boil and damage the tissue. As residual air is pulled from the tissue, it will rise to the surface. Turn the vacuum on and off through several cycles until the tissue stays at the bottom of the container during an "on" cycle.

**Tissue dehydration:** Paraffin and other embedding media are hydrophobic; thus, water must be removed from the plant tissue after fixation. This is accomplished by immersing the fixed tissue in dehydration solutions, which are a series of mixtures of alcohol and water with increasing alcohol concentration. The final solution in the series is dehydrated alcohol. Begin by washing the fixed tissue once or twice with fresh 50% alcohol to remove traces of FAA. Remove this solution, and subsequently remove any other dehydration solution, by decanting the solution or removing it with the aid of a glass pipet. Add the first dehydration solution (70% alcohol) to the vial, completely immersing the tissue. The graded alcohol–water series and the suggested times for tissue immersion are listed in *Table 3*.

**Table 3**

| Dehydration Solution | Time (h) |
|---|---|
| 50% alcohol | 1–2 |
| 70% alcohol | 1–2 |
| 90% alcohol | 1–2 |
| 95% alcohol | 1–2 |
| Dehydrated alcohol containing 0.1% of safranin O | 2–4 |
| Dehydrated alcohol | 1 |

Safranin O is added to the penultimate dehydration solution in the series to visualize the tissue when it has become embedded in paraffin. If the tissue to be sectioned is hard or woody, the time for each step in the series may need to be increased to up to 24 h. If necessary, the tissue can be stored for several days in 70% alcohol or in solutions of even higher alcohol concentrations.

**Embedding**

*Preparation for embedding*

ALCOHOL REMOVAL: Paraffin is the most common embedding medium, although other embedding media are available. After dehydration, alcohol is removed from the tissue by using a graded series of dehydrated alcohol–xylene solutions, because paraffin is not soluble in alcohol. The graded dehydrated alcohol–xylene series and the suggested times for tissue immersion are listed in *Table 4*.

**Table 4**

| Alcohol Removal Solution | Time (h) |
|---|---|
| A mixture of dehydrated alcohol and xylene (3:1) | 1 |
| A mixture of dehydrated alcohol and xylene (1:1) | 1 |
| A mixture of dehydrated alcohol and xylene (1:3) | 1 |
| Xylene | 1 |
| Xylene | 1 |

XYLENE REMOVAL: Once xylene has completely replaced alcohol, paraffin is added slowly to infiltrate the tissue and remove xylene. Proceed as follows:

1. For each mL of xylene add about 1 paraffin chip to the tissue vial, cap, and allow to stand at room temperature for 4 h. Add additional paraffin chips until no more chips dissolve.
2. Place the tissue in an oven maintained at 42°–45°. Add 2–3 paraffin chips every h until no more chips dissolve at that temperature.
3. Pour off one-third of the volume, and replace with an equal volume of melted paraffin. Do not cap, and transfer the vial to an oven maintained at 58°–60°.
4. After the paraffin remelts (about 4 h later) pour off one-half of the volume, and replace with an equal volume of melted paraffin. Transfer the vial to the oven maintained at 58°–60° if the paraffin begins to solidify.
5. Repeat the fourth step twice more, then pour off the entire volume of paraffin–xylene. Replace with pure melted paraffin. About 4 h later, pour off the paraffin, and replace with fresh pure melted paraffin. Repeat the pouring off and replacement 4 h later, and allow to stand overnight. [NOTE—Transfer the vial to the oven maintained at 58°–60° if the paraffin begins to solidify at any point.]

*Embedding procedure*—Pour the tissue with the paraffin to an embedding boat. Paraffin has to completely cover the tissue by about 3–5 mm. Place the embedding boat on top of a preheated warming platform that is designed for histology work. Adjust the tissue in the boat to its proper orientation for sectioning. Slowly cool the paraffin by sliding the boat down to the cool side of the platform until the paraffin has solidified. Immerse the paraffin block in ice water to rapidly cool the block and to prevent paraffin crystals from forming. Store the paraffin block at 4°.

**Sectioning and mounting:** Cut the paraffin block into pieces, each containing one tissue sample. Trim the paraffin block, as close to the tissue mass as possible, to form a rectangle or a slight trapezoid. Such trimming will prevent sectioning problems due to excess paraffin around the tissue. To make transverse sections, orient the tissue at a right angle to a wooden tissue block

of which the face has been soaked in melted paraffin. Affix the paraffin block to the face of the tissue block. Add a small amount of melted paraffin to the base of the paraffin block to help form a tighter seal. Cool the block to 4°.

Properly mount and adjust the tissue and paraffin block in a microtome. Use a sharp stainless-steel microtome knife that has been properly honed. Set the microtome to cut sections 8–15 μm thick (10-μm thickness is optimal for most tissues). Cut individual or serial sections. Prepare a microscope slide as follows. An adhesive may be prepared as a solution containing 1% of gelatin and 0.5% of sodium benzoate that is heated to 30°–35° to dissolve the gelatin. Smear a thin film of the adhesive so obtained onto the slide, allow to dry, rinse with a 4% solution of formaldehyde TS, and add a small amount of water. Plate the cut sections upside down on the slide, so that they float on water, and flood with a 4% solution of formaldehyde TS. The sections will immediately spread out and wrinkles will disappear.

Place the slide on a warming platform, maintained at 42°, to relax the sections. Pipet, and blot the excess water and formaldehyde solution. Dry overnight in an oven at 42° to ensure adherence of the tissue section to the slide.

**Staining**

*Preparation for staining*—Immerse the microscope slide with the affixed tissue twice into xylene, each time for 10–15 min, to remove paraffin. Then immerse the slide into the following sequence of solutions, leaving it in each solution for 5 min and taking care not to dislodge the tissue: a mixture of dehydrated alcohol and xylene (1:1), dehydrated alcohol, alcohol, and a 70% alcohol solution. The tissue is bleached prior to staining if it is opaque because of the presence of tannins or other ergastic materials. To bleach, dip the slide into a 1% potassium permanganate solution for 1 min, rinse with water, dip into a 5% oxalic acid solution for 1 min, and rinse thoroughly with water. The material is ready for staining. One of the following two staining procedures is recommended for most botanical identification work. The first staining procedure uses safranin O counterstained with fast green. An alternative staining procedure uses safranin O counterstained with orange G.

*Safranin O–fast green staining*

SAFRANIN O STAINING SOLUTION: Prepare a mixture of methoxyethanol, dehydrated alcohol, water, and formaldehyde solution (50:25:25:2). Add a sufficient quantity of sodium acetate to obtain a solution containing 1% of sodium acetate, and mix. Add a sufficient quantity of safranin O to obtain a solution containing 1% of safranin O, and mix.

FAST GREEN STAINING SOLUTION: Prepare a mixture of methoxyethanol, dehydrated alcohol, and methyl salicylate (1:1:1) containing 0.05% of fast green FCF.

PROCEDURE: Once the tissue has been rehydrated to 70% alcohol as described in *Preparation for staining*, immerse for 2–24 h, depending on the tissue, in *Safranin O staining solution*. Remove excess stain by immersing the slide in water several times. Transfer the slide to an alcohol solution containing 0.5% of picric acid for 2–10 s to further remove excess stain from the section and to assist in differentiation of the tissue structures. To stop the action of the picric acid, transfer the slide for 10 s to 1 min to an alcohol solution containing 4 drops of ammonium hydroxide in each 100 mL of alcohol. Transfer the slide to dehydrated alcohol for 10 s. Visually inspect the stained tissue under a microscope to see if further destaining with picric acid is necessary. Counterstain for 10–15 s in *Fast green staining solution*. Transfer slide through two changes of a mixture of methyl salicylate, dehydrated alcohol, and xylene (2:1:1), each change lasting for 5–10 s. Then transfer the slide to a mixture of xylene and dehydrated alcohol (95:5) for 1 min. Transfer through two changes of xylene. Store in xylene until ready to mount the coverslip. Chromosomes, nuclei, and lignified, cutinized, or suberized cell walls will be stained red. Cytoplasm and cellulosic cell walls will be stained green to blue, depending on the pH of the tissue.

*Safranin O–orange G staining*

SAFRANIN O STAINING SOLUTION: Prepare a 0.004% solution of safranin O.

ORANGE G STAINING SOLUTION: Dissolve 2 g of orange G, 5 g of tannic acid, and 4 drops of hydrochloric acid in water, and dilute with water to 100 mL.

PROCEDURE: Once the tissue has been rehydrated to 70% alcohol as described in *Preparation for staining*, sequentially transfer the slide through the series of solutions in *Table 5*.

**Table 5**

| Solution | Time |
|---|---|
| 35% alcohol | 5 min |
| A filtered 2% zinc chloride solution | 1 min |
| Water | 5 s |
| *Safranin O staining solution* | 5 min |
| Water | 5 s |
| *Orange G staining solution* | 1 min |
| Water | 5 s |
| A filtered 5% tannic acid solution | 5 min |
| Water | 3 s |
| A 1% ferric ammonium sulfate solution | 2 min |
| Water | 15 s |
| 45% alcohol | 10 s |
| 90% alcohol | 10 s |
| Dehydrated alcohol | 10 s |
| A mixture of dehydrated alcohol and xylene (1:1) | 1–2 min |

Finally, store in xylene until ready to mount the coverslip. Cellulosic cell walls will stain blue-black, nuclei will stain yellow, starch grains will stain black, and lignified cell walls will stain red.

**Mounting the coverslip:** The mounting of a coverslip over the tissue completes the preparation of the slide. Canada balsam, diluted with a small portion of xylene, can be used as an adhesive. Other mountants are also commercially available. Upon drying of the mountant, the slide can then be examined under a microscope. The entire process of making permanent microscope slides can be expected to take 5 or more days.

**Scanning electron microscopy:** Botanicals in commerce are often encountered in the form of powder or in pieces, thus making authentication by the routine method of cross-sectioning of the article difficult and often impossible. Structures such as xylem vessels and trachids may be broken into smaller bits, making detection of pitting and lignifications on the walls difficult if not impossible using an optical microscope. Structures that are resistant to these processes are most useful in identification. Scanning electron microscopy (SEM) is useful for characterizing the size and morphology of microscopic specimens. The more detailed differential characteristics in the structure of trichomes and peculiar elements in the epidermis, along with superficial granular material containing specific compounds, can be observed and identified with SEM, which assists in the identification of particular species. SEM has been used extensively to investigate surface topology of a wide variety of plant materials. It can play a vital role in authentication of an entire botanical, those in powder form, distinguishing between closely related species, and can be used to examine a mixture of powders.

Introduction and general information about SEM as applied to pharmacopeial articles can be found in general information chapter *Scanning Electron Microscopy* ⟨1181⟩.

SEM produces a higher resolution compared to that possible using an optical microscope, and the images obtained are three-dimensional. SEM has the advantage of providing images with a large depth of field, which allows a substantial thickness of the sample to be in focus at one time. It allows the analysis of specimens as large as 50 mm, making it possible to produce detailed topographical electron micrographs of an object clearly visible to the naked eye. The maximum resolution for SEM (minimum distance by which the two objects can be separated and observed as distinct objects) is 10–20 nm compared to 200–300 nm for optical microscopy. Typical SEM magnification ranges from ×10 to ×300,000. Commercial SEM instruments also are available with magnifications as low as ×5 and as high as ×2,000,000. In comparison, typical modern optical microscopes have a magnification range of ×10 to ×2000. At low magnification, images obtained with SEM provide more information than those of optical microscopy. SEM can produce images for which contrast is based on compositional variations of specimens.

***Change to read:***

## CHEMICAL IDENTIFICATION

To help ensure the authenticity of the article, chemical identification is performed in conjunction with the botanical identification outlined previously. Chemical identification typically employs chromatographic procedures to detect the presence of marker compounds specified in the individual monograph. Spectroscopic or chromatographic profiles can be used to achieve chemical identification by fingerprint comparison against that of a reference sample or standard. Examples of spectroscopic methods include ultraviolet (UV), infrared (IR), and Fourier transformed IR (see ▲*Spectroscopic Identification Tests* ⟨197⟩▲ (CN 1-May-2020)). Examples of chromatographic methods include high-pressure liquid chromatography (HPLC), thin-layer chromatography (TLC), two-dimensional-TLC, and gas chromatography (GC) (see *Chromatography* ⟨621⟩). Analytical methods used for fingerprinting should be capable of detecting as many chemical constituents as possible. Multiple fingerprints, using a combination of analytical methods with different separation principles and test conditions, may be useful. In addition to the spectroscopic chromatographic methods, qualitative wet-chemistry methods may also be specified in the individual monograph.

### Chemotaxonomy

Chemotaxonomy is the classification of the plants based on their chemical constituents and it may be useful in botanical articles identification. Metabolic compounds found within plant tissues can be divided into two broad categories based on their functions. The first category comprises primary metabolites—metabolites involved in the physiological plant processes that are absolutely necessary for life and ubiquitous throughout the plant kingdom. These processes include photosynthesis; respiration; and nucleic acid, protein, carbohydrate, and lipid metabolism. The second category comprises secondary metabolites—compounds that are thought not to be absolutely necessary for plant processes, although they may have important functions in the plant's interactions with other organisms, such as allelopathic interactions; in chemical defense against herbivores and plant pathogens; and in signaling to attract pollinating and seed-dispersing animals. Many secondary metabolites are known to have pharmacological activity. They are also the basis for the chemotaxonomy of plants. Secondary metabolites fall into several different chemical classes such as nonprotein amino acids, flavonoids, xanthones, coumarins, polyacetylenes, cyclic polyketides, monoterpenes, sesquiterpenes, iridoids, triterpenes, sterols, nitrogen-containing terpenes, and alkaloids. These chemical classes are not ubiquitous throughout the plant kingdom, but tend to be specific to certain botanical classes, orders, and families. Moreover, many chemical subclasses and individual secondary compounds are specific to certain subfamilies, genera, or species. It is these chemical subclasses and individual compounds that can be used as marker compounds to aid in the proper identification of plant material.

### Active Principles and Marker Compounds

For chemical identification of botanical articles, extracts are prepared. Such extracts are usually complex mixtures of several chemical constituents. For a large majority of botanical extracts it is not known with certainty which of the various components is responsible for the reported pharmacological effect. It is generally believed that several constituents act synergistically to provide the reported effect. For articles for which compendial monographs are provided, certain chemical constituents of the

article are chosen and quantitative test procedures for determining their content are provided. The choice of such constituents, known generally as marker compounds, is based on certain considerations. Currently, the following types of marker compounds are specified in compendial monographs and may be identified in raw materials:

ACTIVE PRINCIPLES: These are constituents that have proven clinical activity. A minimum content or range for the active principles is usually specified in the individual monograph. A quantitative determination of active principles during stability studies of botanical dosage forms provides necessary information for arriving at suitable expiration dates.

ACTIVE MARKERS: These are constituents that have known pharmacological activity contributing in some extent to efficacy. However, the clinical efficacy for these constituents may not be proven. A minimum content or range for active markers is usually specified in individual monographs. A quantitative determination of active markers during stability studies of botanical dosage forms provides necessary information for arriving at suitable expiration dates.

ANALYTICAL MARKERS: Where neither defined active principles nor active markers are known, other constituents of the botanical extract amenable to quantitative determination are chosen. These markers aid in the positive identification of the article under test. In addition, maintaining a minimum content or a specified range of the analytical markers helps to achieve standardization of the plant extract and to arrive at a suitable expiration date during stability studies.

NEGATIVE MARKERS: These are constituents that may have allergenic or toxic properties, rendering their presence in the botanical extract undesirable. For example, ginkgolic acids from ginkgo belong to this category. A stringent limit for these negative markers may be specified in individual monographs.

### Use of USP Reference Articles

Reference articles are used to assist in the identification of marker compounds within the test article. Reference articles are either USP Authenticated Reference Materials or USP Reference Standards (see *USP Reference Standards* ⟨11⟩), whichever is specified in the individual monograph. USP Reference Standards used to identify marker compounds in the test articles may be a single purified chemical entity, a mixture of purified chemical entities, or a standardized extract prepared from the authenticated plant article. USP Reference Standards may also be used to quantitate marker compounds, as specified in the individual monograph.

A pulverized test article undergoes a specified extraction procedure (see *Methods of Extraction* in *Botanical Extracts* ⟨565⟩) and is prepared for chromatographic or wet-chemistry analysis. If a USP Authenticated Reference Material is available, then it undergoes the same extraction procedure as the test article. The test preparation and reference articles then undergo the same chromatographic or wet-chemistry procedure specified in the individual monograph. The response of the test preparation is compared to the response of reference articles to determine the presence of the marker compounds in the test article.

## DNA-BASED METHODS FOR AUTHENTICATION OF ARTICLES OF BOTANICAL ORIGIN

Because morphological identification often is not possible when the original plant material consists of dried, cut and shifted, or processed plant parts or when the material consists only of a whole, single plant part containing no taxonomic characters, additional identification methods, such as DNA-based identification, often are required for these sample types. DNA-based methods have been shown to be efficient in distinguishing genuine plant materials from adulterants in complex botanical matrixes and can complement traditional botanical identification methods that rely on morphological features or chemistry. In addition, DNA-based methods often are more reliable than traditional methods, especially when applied to single-organ specimens that lack diagnostic taxonomic characters, to powdered materials in which the distinguishing characteristics are no longer visible, or when it is difficult to distinguish among closely related or morphologically similar species.

### DNA Barcoding

DNA barcoding is a particular type of DNA sequence-based identification method that uses short sequences of specific nuclear or plastid DNA loci for identification of plant species. The assays rely on comparison of nucleotide sequences from a specific stretch of DNA (DNA sequences or DNA barcode) to perform DNA sequence-based identification. Further, DNA-based methods, such as next-generation sequencing (NGS) technologies, are able to identify multiple species in a mixture, including expected and unexpected species.

### Botanical Identification Using DNA (Sanger) Sequencing

The process for botanical identification using DNA (Sanger) sequencing includes marker selection, DNA extraction, polymerase chain reaction (PCR) primers and amplification, DNA sequencing, and comparison with reference materials, as described in the following sections. See *Nucleic Acid-Based Techniques—Extraction, Detection, and Sequencing* ⟨1126⟩, *Nucleic Acid-Based Techniques—Amplification* ⟨1127⟩, and *Nucleic Acid-Based Techniques—Genotyping* ⟨1129⟩ for additional information.

#### MARKER SELECTION

The chosen sequence must be sufficiently specific to capture any potential primary and adulterant species in the sample but also sufficiently universal to avoid false-negative reactions for closely related species. For example, a species-specific primer is not appropriate for most identification procedures because adulterants cannot be detected and amplification failure can result because of either the absence of the species or degraded DNA. In many cases, a single marker may be sufficient for identification, but multiple markers from different parts of the genome (e.g., plastid or nuclear material) ensure that hybrids can be detected.

Typically, regions used for DNA sequence identification range from 100–1500 base pairs in length. Smaller DNA fragments may be less susceptible to DNA degradation.

## DNA EXTRACTION

Before amplification of the desired marker can be performed, the total genomic DNA must be extracted. The suitability of a procedure for genomic extraction depends on the starting material and the purity of the DNA required for downstream applications. The principal procedures are described as follows, and several commercial kits are available to accommodate different sample types and applications.

Total genomic DNA must be extracted from ground plant material. Plant materials can be homogenized manually using a mortar and pestle, mechanical grinder, or other apparatus depending on the nature of the material. Total genomic DNA extraction and purification can be challenging because of the abundance of secondary metabolites (polysaccharides, tannins, essential oils, phenolics, alkaloids, and waxes) in many medicinal plant species. Some of the secondary metabolites may coprecipitate with DNA during extraction and may inhibit further enzymatic reactions, including restriction digestion and PCR. In particular, large amounts of complex polysaccharides can make extraction of usable DNA impossible, rendering the aqueous portion of the extract too viscous to allow efficient separation of DNA from the contaminating polysaccharides. This type of contamination can lead to poor DNA yield and can prevent access by modifying enzymes.

Numerous commonly used DNA extraction methods are appropriate for a wide range of fresh and dried plant materials, including cetyl trimethylammonium bromide, silica-based methods, and a variety of commercially available kits that use silica columns or glass-coated magnetic beads. Although many of these methods work well on both fresh and dried materials and on any plant part, those that are degraded or contain significant levels of secondary compounds or other PCR inhibitors may require minor adjustments to standard extraction protocols.

## PCR PRIMERS AND AMPLIFICATION

Typically, PCR primers are between 18 and 30 bases in length and amplify a region between 100 and 1500 base pairs in length. As noted, PCR primers may be universal, meaning they are capable of amplifying all potential organisms that are present in a test sample (including fungi, plants, and animals or an important and predicable subset), or they are taxon specific, meaning they have been designed to amplify only organisms in a targeted set (i.e., family, genus, species, or subspecies). A number of nuclear, mitochondrial, and plastid gene regions are used for universal amplification, including nrITS, nrITS1, nrITS2, *matK*, *rbcL*, *psbA–trnH* intergenic spacer, *cox3*, *COI* (also known as *cox1*), external transcribed spacer, 18S, 5S, *trnL–trnF* intergenic spacer, and *trnL* intron. Taxon-specific primers may be designed on the basis of proteins found in specific plant groups (e.g., the soy lectin gene found in soy) or by obtaining sequences of any variable DNA region for the target taxon and designing primers that specifically bind only to sequences from the taxon of interest.

## DNA SEQUENCING

Most commonly, DNA sequencing is conducted using the Sanger protocol that has been modified to use fluorescent dye terminators on a capillary electrophoresis apparatus, although a number of emerging sequencing technologies [e.g., next-generation sequencing (NGS)] are now taking root. Once fluorescent dye has been incorporated into the amplified DNA, the bases are identified by their emission of light at different wavelengths. The resulting data are a chromatogram that can be visualized and analyzed by several sequence-analysis computer programs.

## COMPARISON WITH REFERENCE MATERIALS

DNA sequences of test articles are compared to sequences obtained from multiple reference materials in an aligned matrix (commonly referred to as an alignment), which allows the sequences to be visually inspected to identify diagnostic nucleotide positions. Although numerous computer programs are able to automate comparisons between sequences from test articles and reference sequences, performance varies widely. Investigators should always manually check the results suggested by computer programs to confirm identity. Positive identifications are not possible when the sequences from test articles fall outside the range of known variation represented in the reference sequences. If a sufficiently large number of reference materials have been used to develop the assay, most test materials should be identified without ambiguity based on DNA sequence data.