Supporting Information

Cannabis Inflorescence for Medical Purposes – USP Considerations for Quality Attributes

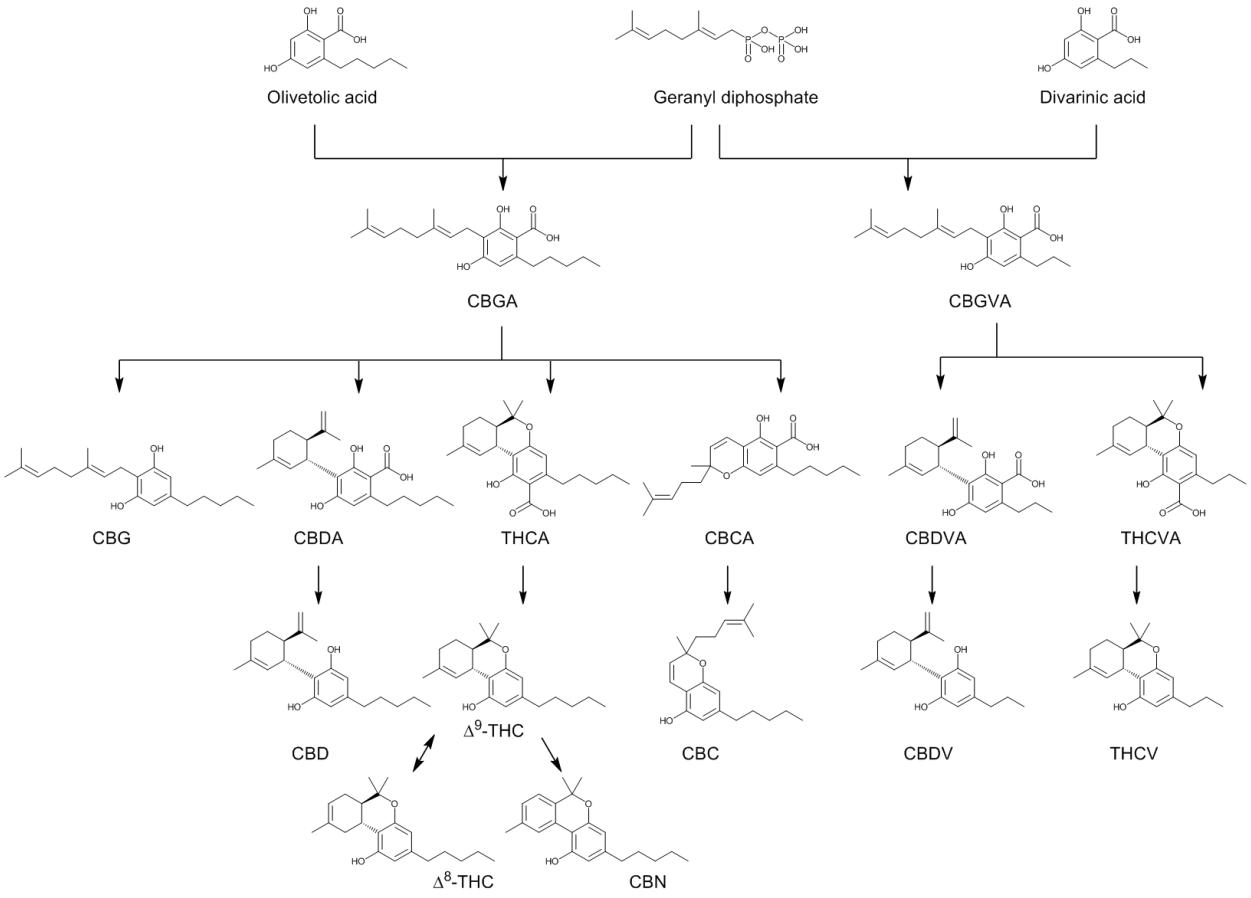

Figure S1. Cannabinoid biosynthetic pathway

APPENDIX 1. BOTANICAL CHARACTERISTICS

The primary literature source for the following description of macroscopic and microscopic botanical characteristics of cannabis is the American Herbal Pharmacopoeia's monograph on *Cannabis* inflorescence,[1] quoted extensively with the permission of the Editor-in-Chief. Additional details from other sources have been added, as indicated by their citations.

The vernacular word "cannabis" has evolved as a generic abstraction from the genus name *Cannabis*, conventionally italicized. Non-italicized, cannabis is employed as a noun and adjective, and frequently (often loosely) used both for cannabis plants and/or any or all of the intoxicant preparations made from them.[2] In the description that follows, the term "cannabis" refers strictly to the cannabis plant.

Macroscopic Features:

For macroscopic examination of material that is stuck together, soak the material in strong alcohol (70%) to dissolve the resin. Then pour off the alcohol and soak in water. The leaves, stems, bracts, flowers, and fruit can then be separated. However, material prepared in this manner should not be used for quantitative analysis due to constituent loss.[1]

Cannabis raw material has various characteristic morphological features. Those described here are generally visible with a hand lens of 10× or 20× power. In a few cases, such as some of the features of the very diagnostic trichomes, additional fine details are described, which might be difficult to see clearly without a 40× microscope. The visual detection of every fine detail described here is not required. The intent of providing this additional information is to facilitate the interpretation and understanding of what can be seen with a hand lens, such as how the readily visible resin chamber is formed and what makes the reddish tip of a multicellular trichome that can be seen when its glandular head has fallen off.

Cannabis is most often supplied as variously sized (1.5–15 cm or longer) branches and branchlets, which are sometimes broken up from the dried inflorescences of pistillate plants. Cannabis is generally dioecious, meaning that the pistillate (female) and staminate (male) flowers occur on separate plants, although monoecious (male and female flowers on the same plant) "races" have been created by hemp breeders, and monoecious individuals sometimes also occur in normally dioecious "races."[1,3,4]

The pistillate inflorescence segments, colloquially known as "buds," are often closely trimmed by hand or machine, sometimes leaving portions of the leaf bases and stiff petioles (Figure A1-1).

Figure A1-1. Cannabis dried pistillate inflorescences ("buds"): top and middle are trimmed, bottom are untrimmed and show subtending leaves (photo courtesy of Roy Upton, American Herbal Pharmacopoeia).

The segments are generally light to dark green, various shades of purple to dark purple, or from green-brown to brown. The segments may include whole or fragments of reduced upper leaves, stems, bracts, bracteoles, rudimentary calyx, immature ovules, styles, and glandular and non-glandular trichomes. In response to demand for very high levels of THC, there has been human selection for congested female inflorescences with the production of numerous, well-formed "buds" (Figure A1-2).

Figure A1-2. Cannabis "buds" showing brownish color variation, congested morphology and fragments of stems (photo courtesy of Ronan Yu, British Columbia Institute of Technology).

Thus, cannabis plants vary with regard to the length of the internodes within the inflorescence. Those of short length have a denser cluster of flowers so that the segment pieces appear more rounded; those of a longer length have a greater distance between individual flowers. Variation in the size and prominence of the various parts exists between cannabis groups and are influenced by human selection as well as environmental factors including light, water, nutrients, and methods of cultivation.

Color: Color is influenced by human selection and the methods of cultivation, handling, harvest, and curing. Pistillate inflorescence parts vary in color from bright, light green to deeper, dark green through dark purple (due to accumulation of anthocyanin pigments), to light yellow-gold to brown. Sometimes flowers have long reddish-orange to brown styles and stigmas. Indoor-grown material is often lighter green to bright purple, while material cultivated outdoors tends to be darker green to green-brown to dark purple (Figure A1-3). The color should be consistent throughout each sample and should not show signs of gray or black, which are indicators of fungal infection. Inflorescence parts with a high density of glandular and non-glandular trichomes can appear bright whitish and crystalline.[1]

Figure A1-3. Examples of color variation among chemotypes of cannabis dried pistillate inflorescences: (a) “SFV OG”, (b) “Magna”, (c) “Laaaav”, (d) “Glamour” (photos courtesy of Josh Wurzer, SC Labs).

Stems: The stems may be light brown, pale green, variously mottled, or entirely purple in color. Stems within inflorescences are often cut just below the node. Stems branch freely and repeatedly but the extent of branching is dependent on environmental and hereditary factors and the method of cultivation. Nodes and internodes are distinct with alternate branches and can be of varying length. The stem texture is fibrous and the surface is longitudinally furrowed with short, stiff hairs. The cortex and xylem are thin, and the pith is white and porous. Larger diameter (≥ 3 mm) branch pieces are often sourced from terminal shoots. Material with thinner stems is most often from lateral inflorescence branches or from side branches cut from terminal inflorescences.[1]

Upper Leaves: The upper leaves are rarely present in cultivated plants as these are often removed through mechanical or hand trimming. When present, the upper leaves are light to dark green, sometimes purple or mottled purple in color, or brown; they also appear dried and shriveled and sometimes clasping the inflorescence. After trimming, only the base of the petioles is typically left as stiff remnants at the nodes.[1]

Pistillate (Female) Inflorescence: Pistillate inflorescences are small, obscure, congested, axillary spicate cymes of approximately 1–5 cm in length and in width, with slightly protruding bracts. Development of the flowers proceeds from the base upward to the top of the inflorescence[5] (Figure A1-4). The tightly compacted inflorescences are the so-called “buds” of cannabis.

Figure A1-4. Pistillate inflorescence with senesced reddish-brown styles and stigmas, an indicator of inflorescence maturity (photo courtesy of Roy Upton, American Herbal Pharmacopoeia).

Bracts: Bracts are light to dark green or brownish-green and scabrous due to the presence of trichomes. Bracts are also numerous, alternating with overlapping edges and narrow stipules at the base. Some bracts are simple and others tripartite, but in both cases, the segments are lanceolate with an acuminate apex and a serrate or entire margin. Bracts subtending the spikes are often divided into five linear leaflets. Those subtending the individual flowers usually have three-minute leaflets. The bracts enclose the female flower except the exserted stigmas. Bracts and stipules both show a marked tendency to shrivel upon drying; in some cases, only the veins of the bracts remain intact. Bract proximal upper surfaces are densely covered by capitate stalked glandular trichomes that are readily seen with magnification (10×). Trichomes are absent from the distal region. Numerous non-glandular trichomes are also visible[1] (Figure A1-5).

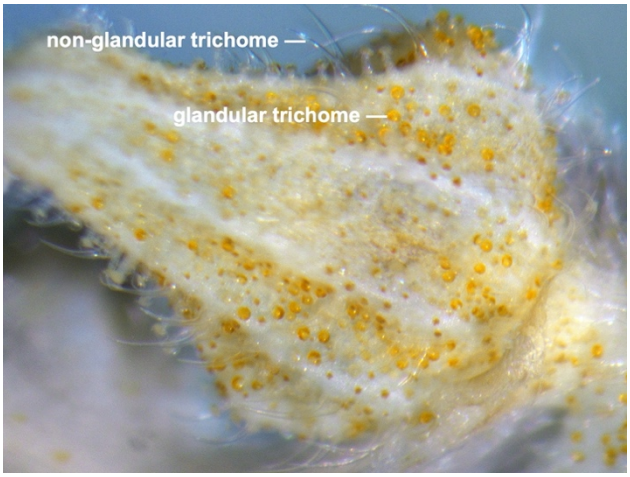

Figure A1-5. Outer surface of a bract showing numerous glandular trichomes with yellow contents and clear non-glandular trichomes (photo courtesy of ElSohly group, University of Mississippi).

Perigonal Bracts: Perigonal bracts are also called bracteoles or floral bracts. They are light to dark green or brownish-green, sometimes purple to red and/or mottled or streaked. Perigonal bracts are 4–8 mm long and form in pairs in the axil of a bract.

Perigonal bracts are ovate with an acute apex, entirely incurved margin, and are fused at the base to form a conical cup-like sheath that completely envelops the ovary and loosely encloses the mature fruit. Perigonal bracts are densely hispid or pilose. With 10× magnification, numerous resinous glandular trichomes and non-glandular trichomes are observed on the abaxial (outer or lower) surface.[1] In sinsemilla production, where seeds do not develop due to prevention of pollination, the perigonal bracts remain quite small and are very densely covered with glandular trichomes.

Pistillate Flowers: The very short pedicel bears a bract that subtends the perigonal bract. A single flower approximately 5–10 mm long is formed in the axil of each perigonal bract, thus appearing essentially sessile. Because the perigonal bracts are in pairs, the pistillate flowers also appear in pairs. The true perianth develops from the base of the ovary, initially divergent from the developing ovary but soon adhering closely to it and covering about two-thirds of the ovary at maturity. The hyaline (thin, translucent or transparent) membrane appears simple, smooth, or slightly fringed along the margin, and is often marbled by patches of pigmented cells.[6] The ovary is superior, composed of two carpels (bicarpellate) united to form a single chamber (uniloculate), may be 1–2 mm long, and whitish, and contains a single campylotropous (so curved that both ends of the embryo are close to each other) ovule. Each flower has a short apical style with two caducous (easily detached and shed), long filiform stigmatic branches (styles are often three-branched in sinsemilla material)[7] spreading at the apex and projecting well above the bracteole, and densely covered with long club-shaped papillae. The style plus stigma is up to 1 cm in length and of a dark reddish-brown to orange color[1] (Figure A1-6).

Figure A1-6. Close-up showing spreading stigmatic branches of pistillate flowers, starting to senesce by shriveling and turning from yellow to brown; surrounding bracts are covered in white glandular trichomes (photo courtesy of Roy Upton, American Herbal Pharmacopoeia).

Commercial cannabis samples made from pistillate inflorescences should be lacking staminate flowers. The staminate flower is on a pedicel, 2–4 mm, nodding; calyx of five tepals: yellowish-green to whitish, imbricate, ovate to lanceolate, 2.5–4 mm, membranous, with sessile trichomes petals absent, stamens five, opposite the tepals and prominent on filaments 0.5–1 mm and straight in bud but flaccid at maturity, anthers

oblong, with sparse, sessile glandular trichomes in the furrow, pore appearing near the apex and spreading into longitudinal dehiscence at maturity; rudimentary pistil small.[1,7] Adhering cannabis pollen may be present occasionally. Pollen grains (about 25–30 µm in diameter)[4] are triporate and have a smooth exine.[8]

Fruit: The fruit is an achene (Figure A1-7) and, together with the single enclosed seed, is commonly referred to as the "seed." When cannabis is grown under the sinsemilla cultivation technique, exposure to pollen is prevented; therefore, the pistillate flowers remain unfertilized throughout maturity and do not develop achenes. Unless specifically desired, achenes should be lacking from properly harvested material. Achenes separate easily from dry samples. The achene is 3–4 mm in diameter (larger in cultivated plants versus ruderal plants[6]) and about 4–6 mm long, solitary, ovoid to oblong in outline, and somewhat compressed (lenticular) in cross-section. The achene may appear glossy, off-white, gray, green, brown-green, or yellowish-green, often mottled in purple—the color is typically darker in chemotypes bred for medicinal use versus fiber or seed crops.[5] In ruderal plants, the base of the achene is elongated.[4] The achene is enclosed within the enlarged, persistent perigonal bract. The perianth at this stage constitutes merely an obscure papery sheath or fragments initially covering 60–90% of the mature achene, the perianth is often missing or present only at the base of the achene after drying. There is generally less adherence of the perianth to the achene in domesticated plants.[6] The thin wall of the ovary (pericarp) tightly covers the coat of the seed. The pericarp is dry, brittle, and finely reticulate. The endosperm is fleshy and oily, and the cotyledons are also fleshy. The embryo is strongly curved.

Figure A1-7. Achenes of cannabis (photo courtesy of ElSohly group, University of Mississippi).

Trichomes: Two primary categories of trichomes are present: glandular, cannabinoid-producing trichomes and non-glandular, non-cannabinoid-producing trichomes. Both can be observed with 10×–20× magnification.

Glandular trichomes are present mainly in three forms: capitate sessile with a multicellular head but no visible stalk, bulbous with a short stalk, and capitate stalked with a multicellular head and multicellular stalk. Staminate flowers have a fourth type of glandular trichome: sessile antherial glandular trichomes.[9,10] Secretory cells at the head

of the stalk form a secretory cavity (resin chamber) by separating an outer zone of their peripheral walls from the cuticle and the subsequent reinforcement of the subcuticular wall and thickening of the cuticle. This cavity fills with membrane-bound secretory vesicles containing cannabinoids and terpenes in an intervesicular matrix.[11] The glandular trichome head has shown increased size with selection for high-THC chemotypes.[12]

Capitate sessile glandular trichomes are the most abundant type, occurring on all aerial epidermal surfaces of mature plants. They are especially abundant on the underside surface of leaves, bracts, and abaxial surface of perigonal bracts. Despite their name, they are not actually sessile. The stalk is one cell high and 2–4 cells thick, but it is hidden beneath the radially-arranged eight cells of the glandular head.[9,10] The glandular head is typically 30–70 µm in diameter and approximately 15–20 µm high.[10,13,7] Two sizes can be distinguished: larger ones are found on the pistillate flowers while smaller ones are found on pistillate flowers, leaves, and stems. The glandular head is comprised of a disc of eight or more secretory cells at the base, above which the resin chamber forms. The glandular head's resin and terpenoid-rich essential oil contents are clear during early stages of development but become opaque-white with maturity and eventually age to become brown.[10] Detached capitate sessile glands can be seen in commercial samples.

Bulbous glandular trichomes, approximately 10–20 µm in diameter and 15–30 µm high,[10,13,7] are also widespread on all epidermal surfaces of the aerial parts, with the highest density on stems and the lowest on the bracts. Most have a two-celled head and a stalk that appears to be one or two cells long and one or two cells thick, but the structure is variable. The stalk may appear to be one-celled but actually has two cells, the upper much larger than the lower; larger ones have a stalk two cells thick. The glandular head may be simple and spherical or complex and multi-compartmented, varying in size from one to four secretory cells. The contents of the resin chamber may be clear or brown in some chemotypes.[10]

Capitate multicellular stalked glandular trichomes are generally abundant, forming a pubescence on both upper and lower epidermal surfaces of the petioles, bracts, and mainly on the abaxial surface of the perigonal bracts; they are rare on staminate plants. During development, stalked glandular trichomes arise first along the veins of the bracts and later spread over the entire surface; they are initiated later than bulbous or capitate sessile glandular trichomes. The stalks, produced by elongation of hypodermal cells, can attain a length of 100–200 µm.[7] The secretory cell disk at the base of the glandular head is about 30 µm in diameter and 15 µm in height and forms a dome-shaped to eventually spherical resin chamber, which is typically 50–100 µm[10,13,7] but may be as large as 129 µm in diameter in some THC-dominant chemotypes.[12] This is compared to industrial hemp cultivars that are 80 µm in diameter[12] with resin and essential oil contents that are clear during early stages of development but become opaque-white with maturity and eventually age to become orange-brown. As the trichomes age it is common for the resin head to become detached from the stalk at an abscission region, leaving the trichome with a tip of supportive neck cells that are reddish brown in color[14] due to the presence of flavonoids.[10] Fragments of multicellular glandular trichomes including portions of the stalk are also seen in commercial samples.

Non-glandular trichomes are all unicellular. The major types are distinguished by differences in size and location.

Stigmas have pollen-trapping, club-shaped trichomes (papillae) approximately 90–180 µm long with rounded ends,[1,5] which often become detached and are found scattered in the powder of commercial samples.[14]

The surfaces of stems, leaves and bracts have abundant covering trichomes of various types; they are all conical, unicellular, and highly silicified, but some contain cystoliths and others do not. A cystolith is a well-defined concretion (sometimes described as grape-like in shape) of calcium carbonate in cannabis (calcium oxalate in some other plants). As described further in the Microscopic Identification section below, calcium oxalate crystals are present in cannabis, but they are found scattered through the tissues, not in the trichomes. The presence of silica on the outer wall surface and cystoliths of calcium carbonate inside helps non-glandular trichomes to persist and to be useful for identifying cannabis material even when it has been burned to ash. The trichomes located on or near the major veins have a verrucose surface (i.e., with warts of cellulose and cutin), whereas those occurring between the veins have a slightly warty or smooth surface.[14]

The cystolithic trichomes are either elongated, not exceptionally enlarged at the base and having a distinctly warted wall, or they are short and much enlarged at the base.[14] The elongated cystolithic trichomes are approximately 150–220 µm long, sharply pointed, and with little enlargement of the base. They often have a distinctly thickened, verrucose wall and are found mainly on the adaxial surface of the leaf, always pointing to the distal part, giving the surface a rough texture.[10]

Very short (approximately 50–125 µm) cystolithic trichomes with a highly enlarged base and smooth or verrucose surfaces are found on the adaxial surface of the bract.[9,7] Some are also found on the abaxial surface of the perigonal bracts.[4] At the base of each cystolithic trichome is the cystolith. Detached fragments of cystolithic trichomes with a warty surface are seen in commercial samples.

Simple, slender unicellular non-cystolithic trichomes approximately 250–370 µm long (some as long as 500 µm) are abundant on the stems and the abaxial surface of leaves, bracts, and to a lesser extent, on the perigonal bracts forming a pubescence much less abundant on the upper surface. They are lying almost flat and oriented toward the distal end.[9,10] Those on the adaxial (inner or upper) surface of the perigonal bract have smooth surfaces and are completely flattened; some have a more cylindrical shape. On the abaxial surface, some covering trichomes, such as those on the lower epidermis of the perigonal bract, are fairly short, rigid, verrucose, slightly enlarged at the base, and abruptly tapered to the apex. Meanwhile, other covering trichomes, such as those on the veins and edges of the perigonal bracts, are larger and more elongated, sometimes having an enlargement at the base that gradually tapers to the apex. Some covering trichomes are bent and some have branches that join (anastomose) with neighboring trichomes.[15] Detached fragments of covering trichomes are seen in commercial samples.

Figure A1-8 shows the heavy coverage of trichomes of various types on the pistillate inflorescence's leaves and bracts. To aid in interpretation of what can be seen with a

hand lens, Figure A1-9 provides labeled photographs, taken with a light microscope and a scanning electron microscope (SEM), of the various trichome types.

Figure A1-8. Cannabis "bud" showing leaf upper surfaces bearing capitate sessile glandular trichomes, bulbous glandular trichomes and cystolithic trichomes; leaf lower surfaces bearing non-glandular non-cystolithic trichomes and capitate sessile glandular trichomes; and bracts bearing capitate stalked glandular trichomes, capitate sessile glandular trichomes and non-glandular non-cystolithic trichomes (photo courtesy of Josh Wurzer, SC Labs).

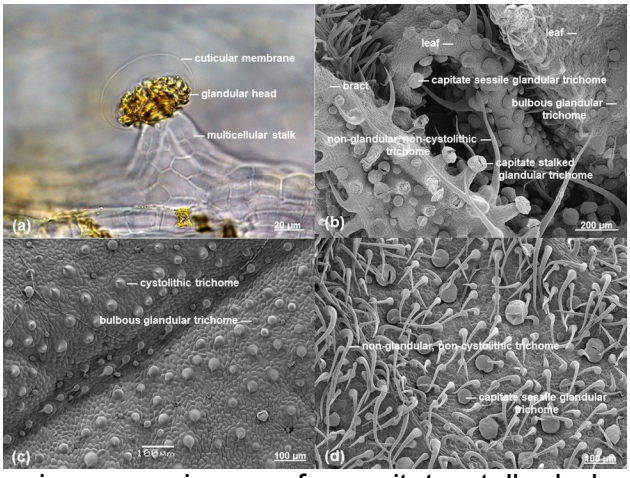

Figure A1-9. (a) Light microscope image of a capitate stalked glandular trichome; (b) SEM image of leaves and bracts of a pistillate inflorescence showing various types of trichomes; (c) SEM image of an adaxial (upper) leaf surface with its characteristic trichomes; (d) SEM image of an abaxial (lower) leaf surface with its characteristic trichomes (photos courtesy of ElSohly group, University of Mississippi).

Powder: Powder may be dull light to dark green, brownish or sometimes purplish. When viewing coarsely ground material under 20× magnification, small fragments of stems, upper leaves, bracts and bracteoles are present, with both attached and detached glandular and non-glandular trichomes, and orange to reddish-brown fragments of stigmas.[14] Fragments of the lower epidermis of leaves contain wavy vertical walls and oval stomata, while upper epidermis pieces have straight vertical walls

and no stomata. Most characteristics require higher magnification, for example, when viewing finely ground powder.[1]

Summary of Raw Material Diagnostic Macroscopic Characteristics: Characteristics of cannabis include the simultaneous presence of the following: rigid, curved cystolithic non-glandular trichomes on the upper leaf and bract surfaces; fine, slender non-cystolithic non-glandular trichomes on both lower and (to a lesser extent) upper surfaces; and capitate sessile glandular trichomes mainly on the lower surface of the leaflets, bracts and perigonal bracts.

Microscopic Features:
For microscopic examination, leaves, bracts, and twigs can be mounted in alcohol, water, or chloral hydrate solution. Some compounds may be diluted or lost when prepared in this manner so these samples should not be used for quantitative analysis.[1]

Bracts and Leaves: Microscopically, transverse sections of the leaflets and bracts show a dorsiventral structure. The palisade typically consists of a single layer (rarely two layers) of cylindrical cells and the spongy tissue of 2–4 layers of rounded parenchyma. Cluster crystals of calcium oxalate are present in all parts of the mesophyll. The upper epidermis cells bear unicellular, sharply pointed, curved and conical trichomes, approximately 150–220 µm long with enlarged bases containing cystoliths of calcium carbonate. The lower epidermis bears conical trichomes, which are longer (approximately 340–500 µm), more slender, and do not have cystoliths. Both upper and lower epidermises bear numerous glandular trichomes, and on the underside glandular trichomes are especially abundant over the midrib. The glandular trichomes are of three types: a long multicellular stalk and a multicellular head with approximately eight radiating club-shaped cells; a short unicellular stalk and a bicellular (rarely four-cell) head[14] but they may merely represent stages in the development of the normal stalked glandular trichomes.[16] There are also sessile (without stalk) glandular trichomes with a multicellular head.[16] Both upper and lower epidermises in the midrib region are followed by a few layers of collenchyma. The vascular bundle is composed of phloem, which is made up of small cells and xylem vessels arranged in radial rows. The lower epidermis displays numerous trichomes of three types: non-glandular, non-glandular cystolithic, and glandular. Clusters of calcium oxalate crystals, about 25–30 µm in diameter, are scattered in the cortical parenchyma tissues of the mesophyll and the palisade layer.[7] The simultaneous presence of cystolithic trichomes on the upper surface and non-cystolithic trichomes and sessile glandular trichomes on the lower surface of the leaflets is characteristic of cannabis.

Bracteoles: Bracteoles have an undifferentiated mesophyll of about four cell layers, the lower hypodermal layer having a cluster crystal of calcium oxalate in almost every cell. The abaxial surface bears numerous bulbous, sessile, and stalked glandular trichomes as well as unicellular conical trichomes. These trichomes are most numerous where the bracteole curves in to enclose the flower or fruit.[1]

Flowers: Fragments of the stigmas are fairly abundant in cannabis powder. They are orange to reddish-brown. In the stigmatic epidermis, nearly every cell has an extended papilla about 90–180 µm long, thin-walled, cylindrical, with a rounded apex.[1] Many papillae become detached and are found scattered in the powder. The sclerenchymatous layer of the pericarp, when viewed from above, shows cells that are very thick-walled and markedly sinuous, with striations and numerous pits. When viewed from below there is no apparent lumen and the surface is covered with minute, circular pits; a fairly large lumen becomes visible on focusing slightly downwards. These fragments are brown and not very numerous. Occasional fragments of the perianth can be seen. In surface view, they are composed of small, very thin-walled parenchymatous cells. The shape of the cells varies; in some fragments the cells are straight-walled and polygonal; in others they are irregularly elongated and the walls may be markedly sinuous.[14]

Stem: Fragments of the stem's epidermis have long, warty, cystolithic covering trichomes and glandular trichomes. Large, unbranched laticiferous tubes can be seen, coming from the stem phloem; they are elongated, unbranched, thin-walled tubes containing dark orange-brown granular secretions. Well-developed bundles of pericyclic fibers are present, coming from the interior of the phloem. The stem vessels are fairly large and occur in small groups; the walls are lignified and show annular or reticulate thickening. These tissues are found associated with pith and cortex parenchyma fragments containing calcium oxalate cluster crystals, about 25–30 µm in diameter.[1,14]

Images of the microscopic characteristics of cannabis inflorescence powder are provided in Figure A1-10.

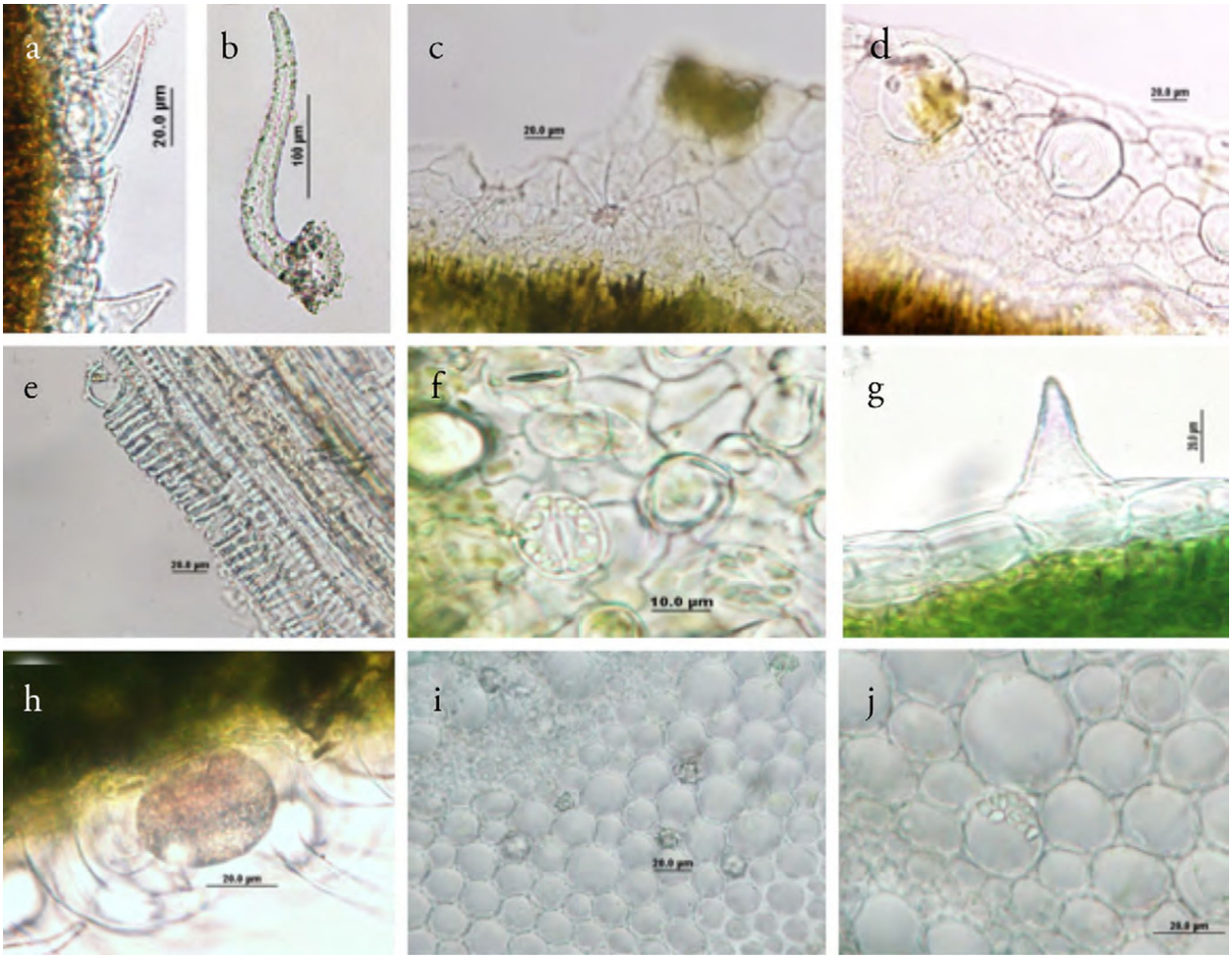

Figure A1-10. Microscopic characteristics of cannabis inflorescence powder: (a) non-glandular conical trichomes on the upper epidermis of leaflet; (b) cystolithic trichome with warty cuticle; (c) head of glandular trichome showing cells radiating from basal cells; (d) surface view of epidermis showing trichomes and cystoliths; (e) fragments of vessel elements showing spiral wall thickenings; (f) lower epidermis showing anomocytic stomata; (g) non-glandular conical trichome with cystolith; (h) head of a glandular trichome covered with cuticle; (i) cortical parenchyma showing crystals of calcium oxalate; (j) cortical parenchyma showing simple starch grains (photos courtesy of ElSohly group, University of Mississippi).

Figure A1-11 provides labeled photographs, taken with a light microscope and an SEM, of key characteristics of the microscopic anatomy of cannabis.

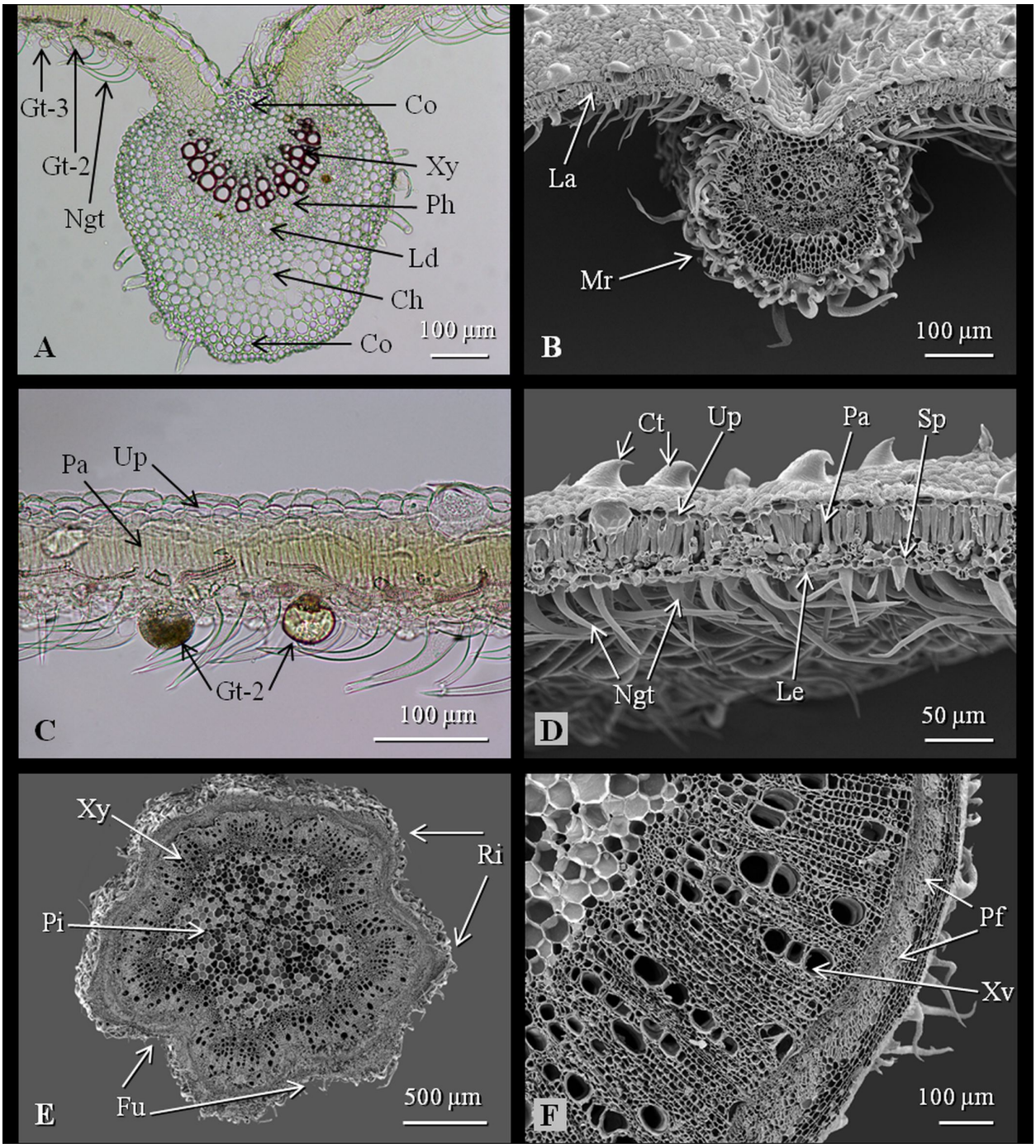

Figure A1-11. Anatomy of cannabis: (A) light microscope and (B) SEM images of a transection (TS) of a leaf through the midrib; (C) light microscope and (D) SEM images of a TS of a leaf through the lamina (blade); (E) SEM image of the TS of a stem, with a portion enlarged (F). Abbreviations: Ch – chlorenchyma, Co – collenchyma, Ct – cystolith trichome, Fu – furrows, Gt-2 – capitate sessile glandular trichome, Gt-3 – bulbous glandular trichome, La – lamina, Ld – laticifer duct, Le — lower epidermis, Mr – midrib, Ngt – non-glandular trichome, Pa – palisade, Pf – pericyclic fibers, Ph – phloem,

Pi – pith, Ri – ridges, Sp – spongy tissue, Up – upper epidermis, Xy – xylem (photos courtesy of ElSohly group, University of Mississippi).

Appendix 1 Photo Credits: The authors gratefully acknowledge the contribution of photographs by Roy Upton of the American Herbal Pharmacopoeia, Scotts Valley, CA; Ronan Yu of the British Columbia Institute of Technology, Burnaby, BC; Joshua H. Wurzer of SC Laboratories LLC., Santa Cruz, CA; and Mahmoud A. ElSohly and colleagues of the National Center for Natural Products Research, School of Pharmacy, University of Mississippi, University, MS.

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APPENDIX 2. HPTLC CHROMATOGRAPHIC PROFILE

Neutralized USP Cannabinoid Acids Mixture RS: Mix USP Cannabinoid Acids Mixture RS with formic acid (4:1).

Standard solution 1: Neutralized USP Cannabinoid Acids Mixture RS, and USP Cannabinoids Mixture RS.

Standard solution 2: USP Delta-9-Tetrahydrocannabinol RS, and USP Cannabidiol Solution RS (or USP Cannabidiol RS prepared as 1 mg/mL solution in methanol) diluted to 0.25 mg/mL in acetonitrile.

Sample solution: Transfer 500 mg of dried cannabis inflorescence, finely powdered and homogenized, into a centrifuge tube and add 5 mL of the mixture of methanol and hexane (9:1, v/v). Vortex the solution for 10 second and sonicate for 15 min in an ultrasonic bath at the room temperature (22±5°) interrupted by additional agitation for 10 seconds on a vortex every 5 minutes. Centrifuge and cool down to the room temperature. Transfer 1.0 mL of supernatant into dark vials.

Decarboxylated sample: The solution in one vial is evaporated to dryness under a stream of nitrogen at the room temperature. The vial is tightly closed and heated at 200° in an oven for 15 min. Cool down to the room temperature and reconstitute with a mixture of methanol and hexane (9:1, v/v) using vortex.

Chromatographic system

See *USP* General Chapter <203>, *High-Performance Thin-Layer Chromatography Procedure for Identification of Articles of Botanical Origin*[1]

Stationary Phase: Reverse phase C_{18} plates with an average particle size of 5 µm (Merck RP-18 HPTLC plates or similar). The plates were pre-washed by developing in methanol then dried at 120^0 for 30 minutes before use.

Application volume: 2 µL of *Standard solutions* and *Sample solution*; as 8-mm bands, 8 mm from the bottom of the plate.

Relative Humidity: Condition the plate to a relative humidity of about 33% using a suitable device.

Developing solvent system: Methanol, water, and glacial acetic acid (80:10:10).

Developing distance: 6 cm

Derivatization reagent: Vanillin-sulfuric acid prepared by dissolving 1 g of vanillin in 100 mL mixture of ethanol (95%) and sulfuric acid (96% ACS) (98:2)

Analysis

Samples: *Standard solutions* and *Sample solution*

Apply the samples as bands to a suitable (HPTLC) plate and dry in air. Develop the chromatograms in a saturated chamber. Treat the plates with the *Derivatization reagent*, heat at 100° for 3 min, and examine under white light.

System Suitability: The *Standard solutions* shows the cannabinoid bands with the order of increasing R_F: tetrahydrocannabinolic acid (THCA), cannabichromene (CBC), tetrahydrocannabivarinic acid (THCVA), delta-8-tetrahydrocannabinol (Δ^8-THC), delta-9-tetrahydrocannabinol (Δ^9-THC), cannabinol (CBN), cannabigerolic acid (CBGA), tetrahydrocannabidivarin (THCV), cannabidiolic acid (CBDA), cannabidiol (CBD), cannabigerol (CBG), cannabidivarinic acid (CBDVA), and cannabidivarin (CBDV).

Acceptance criteria: The *Sample solution* of the Cannabis Inflorescence labeled as THC-dominant chemotypes show the most intense band corresponding to THCA in the *Standard solution* and absence of bands corresponding to CBD and CBDA. The *Sample solution* of Cannabis Inflorescence labeled as CBD-dominant chemotype shows the most intense band corresponding to CBDA in the *Standard solution* and absence of bands corresponding to THC and THCA. The *Sample solution* of Cannabis Inflorescence labeled as a THC/CBD- intermediate chemotype shows intense bands corresponding to Δ^9-THC/THCA and CBD/CBDA of similar intensity. The *Decarboxylated sample* of THC-dominant and CBD-dominant chemotypes show complete disappearance of THCA and CBDA, respectively, and show the bands corresponding to the THC or CBD, respectively.
The *Decarboxylated sample* of a THC/CBD intermediate chemotype shows the bands corresponding to the THC and CBD and the complete disappearance of THCA and CBDA bands.

REFERENCE FOR APPENDIX 2

1. USP. General Chapter <203> High-Performance Thin-Layer Chromatography Procedure for Identification of Articles of Botanical Origin. 2019; https://hmc.usp.org/about/general-chapters/ Accessed March 12, 2020.

ACKNOWLEDGEMENT: The authors acknowledge HPTLC data from University of Mississippi and CAMAG.

APPENDIX 3. CONTENT OF CANNABINOIDS

[Note: Perform either Procedure 1 or Procedure 2. The requirements may be met by following any one of the specified procedures; the procedure used being stated in the labeling only if *Procedure 1* is not used.]

A. Procedure 1

Solution A: 0.1% Formic acid in water

Solution B: 0.1% Formic acid in acetonitrile

Mobile phase: See *Table 1*.

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	26	74
3.5	26	74
6.5	15	85
7.0	15	85
7.01	26	74
8.5	26	74

Standard solution A: USP Delta-9-Tetrahydrocannabinol RS, 1 mg/mL solution, diluted 1 in 10 with methanol to prepare 0.1 mg/mL solution.

Standard solution B: USP Cannabidiol Solution RS 1 mg/mL (or USP Cannabidiol RS prepared as 1 mg/mL solution in methanol) diluted 1:10 with methanol.

Neutralized USP Cannabinoid Acids Mixture RS: Mix USP Cannabinoid Acids Mixture RS with formic acid (4:1).

Standard solution C: Mix 1:1 USP Cannabinoids Mixture RS with Neutralized USP Cannabinoid Acids Mixture RS.

Sample solution: Transfer 0.5 g of Cannabis Inflorescence into a 50 mL conical vial containing an 11 mm stainless steel ball bearing. Add 20 mL of methanol and place the sealed conical vial in a high-throughput homogenizer for 1 minute at 1500 rpm. Allow the conical vial to cool to room temperature. Dilute 1 in 20 with methanol and mix well. Filter through submicron pore filter if necessary.

If desired, a 1 mg/mL solution of butyl-4- hydroxybenzoate in methanol can be used as internal standard instead of methanol to dissolve the reference standards and as extraction solvent for the inflorescence to compensate dilution and solution

transference errors. Peak response ratio of the internal standard (the reference peak) by the response factor of analyte would have to be included in the calculation in such case. [Response factor = peak area / concentration].

Chromatographic system

See *USP* General Chapter <621> *Chromatography*, *System Suitability*[1]

Mode: LC

Detector: UV with Diode Array Detection, 222 nm

Column: 4.6-mm × 15-cm, 2.7 µm C18, hard core with superficially porous shell, L1 (similar to Restek ARC-18)

Column temperature: 40º

Flow rate: 1.5 mL/min

Injection volume: 5 µL

System suitability

Sample: *Standard solution A* and *Standard solution C*

Suitability requirements

Verification of UV max: The maximum of absorbance of the UV spectrum at the apex of the peak for CBDA is at 222 ± 2 nm, *Standard solution C*

Resolution: NLT 1.0 between CBG and CBD; and, Δ^9-THC and Δ^8-THC, *Standard solution C*

Tailing factor: NMT 2.0 for Δ^9-THC peak, *Standard solution A*

Relative standard deviation: NMT 2.0% for Δ^9-THC peak in repeated injections, *Standard solution A*

Chromatogram similarity: The chromatogram of *Standard solution C* is similar to the typical chromatogram provided with the lot of USP Cannabinoid Acids Mixture RS and USP Cannabinoids Mixture RS.

Analysis

Samples: *Standard solution A*, *Standard solution B*, *Standard solution C* and *Sample solution*.

Using the chromatogram of *Standard solution A, Standards solution B* and *Standard solution C*, identify the retention times of the peaks corresponding to each cannabinoids in the *Sample solution* chromatogram. The relative retention times and Conversion Factors against cannabidiol are provided in Table 2.

Table 2

Analyte	Relative retention time	Conversion factor
CBDVA	0.62	0.68
CBDV	0.68	0.94
CBDA	0.85	0.70
CBGA	0.89	0.69
CBG	0.94	0.99
CBD	1.00	1.00
THCV	1.06	1.03
THCVA	1.35	0.68
CBN	1.43	0.52
Δ^{9}-THC	1.77	1.03
Δ^{8}-THC	1.82	1.21
CBC	2.04	0.67
THCA	2.17	0.73

[Notes: (1) As variability in relative response can occur between HPLC instruments, laboratories should verify the system suitability with regard to accuracy of wavelength for maximum of absorbance and establish response factors for each instrument. (2) The conversion factors listed above can be used as a guide. Conversion Factors in this appendix are derived by dividing the response factor of CBD (the reference peak) by the response factor of analyte.]

Calculate the amount (mg/g) of each cannabinoid in the portion of Cannabis Inflorescence taken:

$$\text{Result} = (r_U/r_S) \times C_S \times (V/W) \times F$$

r_U = peak response of cannabinoid from the *Sample solution*

r_S = peak response of CBD from the *Standard solution B*

C_S = concentration of CBD in the *Standard solution B* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Cannabis Inflorescence taken to prepare the *Sample solution* (g)

F = Conversion Factor for the analyte (Table 2)

Calculate total THC (mg/g)

$$\text{Result} = (\text{THCA} \times 0.877) + \Delta^9\text{-THC}$$

Calculate total CBD (mg/g)

$$\text{Result} = (\text{CBDA} \times 0.877) + \text{CBD}$$

Calculate the percentage of the labeled amount of cannabinoids

$$\text{Result} = (P/L) \times 100$$

P = amount of total THC or total CBD as determined above

L = labeled amount of cannabinoids

Acceptance criteria:

The following are the acceptance criteria for the cannabis chemotypes:

- THC-dominant chemotype:
  - Contains NLT 80% and NMT 120% of the labeled amount (in mg/g) of the total THC
  - The ratio of the total THC content to total CBD content is NLT 5:1, containing less than 10 mg/g of total CBD.
  - Contains NLT 80% and NMT 120% of the labeled amount of all other cannabinoids measured (in mg/g). Cannabis inflorescence must be labeled with the name and amount of any cannabinoid present in an amount of 10 mg/g or more.
  - The content of CBN is NMT 2% of the total content of THC. No unidentified peak in the *Sample solution* chromatogram exceeds the area of the CBN peak.

- CBD-dominant chemotype
    - Contains NLT 80% and NMT 120% of the labeled amount (in mg/g) of the total CBD
    - The ratio of the total THC content to total CBD content is NMT 1:5, containing less than 10 mg/g of total THC.
    - Contains NLT 80% and NMT 120% of the labeled amount of all cannabinoids measured (in mg/g). Cannabis inflorescence must be labeled with the name and amount of any cannabinoid present in an amount of 10 mg/g or more.
- THC/CBD intermediate chemotype:
    - Contains NLT 80% and NMT 120% of the labeled amount (in mg/g) of the total THC and total CBD.
    - The ratio of the total THC content to total CBD content is NLT 0.2:1 and NMT 5:1.
    - Contains NLT 80% and NMT 120% of the labeled amount of all cannabinoids measured (in mg/g). Cannabis inflorescence must be labeled with the name and amount of any cannabinoid present in an amount of 10 mg/g or more.
    - The content of CBN is NMT 2% of the total content of THC. No unidentified peak in the *Sample solution* chromatogram exceeds the area of the CBN peak.

B. Procedure 2

Extraction solution: acetonitrile and methanol (8:2)

Internal standard solution: 10 µg/mL of 4-androstene-3,17-dione in *Extraction solution*.

Standard solution A: Dilute USP Delta-9-Tetrahydrocannabinol RS to 20 µg/mL with methanol.

Standard solution B: Dilute USP Cannabidiol Solution RS (or USP Cannabidiol RS prepared as 1 mg/mL solution in methanol) to 20 µg/mL with methanol.

Standard solution C: Combine 400 µL of USP Cannabinoid Acids Mixture RS and 400 µL of USP Cannabinoids Mixture RS in a 4-dram vial. Transfer 8 µL to a derivatization vial, add 100 µL of 2% dimethylaminopyridine (DMAP) in *Extraction solution* and 500 µL of internal standard solution. Evaporate to dryness under stream of nitrogen at 50°C. Derivatize the residue with 100 µL of N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) for 30 min at 70°C. Cool the vial to room temperature before injection.

Sample solution: Transfer 300 mg of Cannabis Inflorescence to a centrifuge tube and add 2.5 mL of *Extraction solution*. Sonicate for 20 min and centrifuge for 5 min at 4000 rpm. The extraction is repeated four times and all supernatants are combined

into a 10 mL volumetric flask. Make up to the volume with *Extraction solution*. Transfer 10 µL of this solution to a derivatization vial, and add 50 µL of *Internal standard solution* and 10 µL of 2% DMAP in *Extraction solution*. Vortex and evaporate to dryness under a stream of nitrogen at 50º. Derivatize the residue with 100 µL of BSTFA for 30 min at 70º. Cool the vial to room temperature before injection.

Chromatographic system

See *USP* General Chapter <621> *Chromatography*, *System Suitability*[1]

Mode: GC

Detector: FID

Column: 0.25-mm × 15-m fused silica capillary; 0.25-µm film of G2 phase coating (Agilent DB-1ms or similar)

Temperature

Injector: 275º

Detector: 300º

Column: See *Table 3*.

Table 3

Initial Temperature (º)	Temperature Ramp (º/min)	Final Temperature (º)	Hold Time at Final Temperature (min)
190	-	190	1
190	30	230	2
230	5	250	1
250	20	300	2.75
190	-	190	10.75

Carrier gas: Helium

Flow rate: 0.8 mL/min

Injection size: 3 µL

Split mode: Split 20:1

System suitability

Sample: *Standard solution A* and *Standard solution C*

Suitability requirements

Resolution: NLT 1.0 between any two cannabinoid peaks, *Standard solution C*

Relative standard deviation: NMT 2.0% for Δ^9-THC and CBD peaks, *Standard solution A and Standard solution B*

Chromatographic similarity: The chromatogram of *Standard solution C* is similar to the reference chromatogram provided with the lot of USP Cannabinoid Acids Mixture RS and USP Cannabinoids Mixture RS being used.

Analysis

Samples: *Standard solution A*, *Standard solution B, Standard solution C* and *Sample solution*.

Using the chromatogram of *Standard solution A, Standard solution B* and *Standard solution C*, identify the retention times of the cannabinoid peaks corresponding to each cannabinoid in the *Sample solution* chromatogram. The relative retention times against internal standard and relative response factors against CBD are provided in Table 4.

Table 4

Analyte	Relative retention time
CBDV	0.43
THCV	0.48
CBD	0.63
CBC	0.69
Δ^8-THC	0.71
Δ^9-THC	0.74
CBDVA	0.77
CBG	0.81
CBN	0.83
THCVA	0.90
CBDA	0.94
Internal standard	1.00

THCA	1.12
CBGA	1.19

[Note: Conversion factors may be used. In such case the conversion factors should derived by dividing the response factor of CBD (the reference peak) by the response factor of analyte at the same concentration levels.]

Calculate the amount (mg/g) of each cannabinoid in the portion of Cannabis Inflorescence taken:

$$\text{Result} = (R_U/R_S) \times C_S \times (V/W) \times F$$

R_U = peak response ratio of cannabinoid relative to internal standard from the *Sample solution*

R_S = peak response ratio of CBD relative to internal standard from the *Standard solution B*

C_S = concentration of the CBD in *Standard solution B* (mg/mL)

V = volume of the *Sample solution* (mL)

W = weight of Cannabis Inflorescence taken to prepare the *Sample solution* (g)

F = Conversion Factor for the analyte

Calculate total THC (mg/g)

$$\text{Result} = (\text{THCA} \times 0.877) + \Delta^9\text{-THC}$$

Calculate total CBD (mg/g)

$$\text{Result} = (\text{CBDA} \times 0.877) + \text{CBD}$$

Calculate the percentage of the labeled amount of cannabinoid

$$\text{Result} = (P/L) \times 100$$

P = amount of total THC or total CBD as determined above

L = labeled amount of cannabinoid

Acceptance criteria:

The following are the acceptance criteria for the cannabis chemotypes:

- THC-dominant chemotype:
  - Contains NLT 80% and NMT 120% of the labeled amount (in mg/g) of the total THC
  - The ratio of the total THC content to total CBD content is NLT 5:1, containing less than 10 mg/g of total CBD.
  - Contains NLT 80% and NMT 120% of the labeled amount of all other cannabinoids measured (in mg/g). Cannabis inflorescence must be labeled with the name and amount of any cannabinoid present in an amount of 10 mg/g or more.
  - The content of CBN is NMT 2% of the total content of THC. No unidentified peak in the *Sample solution* chromatogram exceeds the area of the CBN peak.
- CBD-dominant chemotype
  - Contains NLT 80% and NMT 120% of the labeled amount (in mg/g) of the total CBD
  - The ratio of the total THC content to total CBD content is NMT 0.2, containing less than 10 mg/g of total THC.
  - Contains NLT 80% and NMT 120% of the labeled amount of all cannabinoids measured (in mg/g). Cannabis inflorescence must be labeled with the name and amount of any cannabinoid present in an amount of 10 mg/g or more.
- THC/CBD intermediate chemotype:
  - Contains NLT 80% and NMT 120% of the labeled amount (in mg/g) of the total THC and total CBD.
  - The ratio of the total THC content to total CBD content is NLT 0.2:1 and NMT 5:1.
  - Contains NLT 80% and NMT 120% of the labeled amount of all cannabinoids measured (in mg/g). Cannabis inflorescence must be labeled with the name and amount of any cannabinoid present in an amount of 10 mg/g or more.
  - The content of CBN is NMT 2% of the total content of THC. No unidentified peak in the *Sample solution* chromatogram exceeds the area of the CBN peak.

APPENDIX 4. CONTENT OF TERPENES

Internal Standard Stock Solution: 1000 µg/mL *n*-Tridecane in ethyl acetate

Internal standard solution: 100 µg/mL *n*-Tridecane in ethyl acetate

Standard stock solution: Mix β-caryophyllene, D-limonene, β-myrcene, α-pinene, and α-terpinolene to make 1.0 mg/mL each in Internal Standard Stock Solution

Standard solutions: Dilute the Standard Stock Solution to the concentration of 100 µg/mL using ethyl acetate.

Sample solution: Transfer 1 g of powdered Cannabis Inflorescence into a 15 mL centrifuge tube and extracted with 10 mL of *Internal standard solution*. Sonicate for 15 min. Centrifuge for 5 min and use the aliquot.

Chromatographic system

See *USP* General Chapter <621> *Chromatography*, *System Suitability*.[1]

Detector: FID

Column: 0.25-mm × 30-m fused silica capillary; 0.25-µm film of G27 phase coating (Agilent DB-5ms or similar)

Temperature

Injector: 250°

Detector: 300°

Column: See *Table 5*.

Table 5

Initial Temperature (°)	Temperature Ramp (°/min)	Final Temperature (°)	Hold Time at Final Temperature (min)
70	-	70	2
70	2	85	-
85	3	165	-

Carrier gas: Helium

Flow rate: 1.2 mL/min

Injection size: 2 µL

Split mode: Split 15:1

System suitability

Sample: *Standard solution*

Suitability requirements

Resolution: NLT 2.0 between any two peaks

Relative standard deviation: NMT 2.0%, *Standard solution*

Analysis

Samples: *Standard solution* and *Sample solution*

Using the chromatogram of *Standard solution*, identify the retention times of the terpene peaks corresponding to each terpene in the *Sample solution* chromatogram. The relative retention times are provided in Table 6.

Table 6

Analyte (groups)	Relative Retention Time
α-Pinene	0.72
β-Myrcene	0.86
D-Limonene	1.00
α-Terpinolene	1.18
β-Caryophyllene	2.26

Calculate the percentage of terpenes in the portion of Cannabis Inflorescence taken:

$$\text{Result} = (R_U/R_S) \times C_S \times (V/W) \times 100$$

R_U = peak response ratio of terpene relative to the internal standard from the *Sample solution*

R_S = peak response ratio of terpene relative to the internal standard from the *Standard solution*

C_S = concentration of the terpene in the *Standard solution* (mg/mL)

V = volume of the terpene in the *Sample solution* (mL)

W = weight of Cannabis Inflorescence taken to prepare the *Sample solution* (mg)

Calculate the relative content of each terpene with respect of total detected area between 0.1 and 2.5 RRT with respect to limonene:

$$\text{Result} = (Ai/TDA) \times 100$$

Ai = peak area of each terpene as determined above

TDA = total detected area between 0.1 and 2.5 RRT with respect to limonene

Acceptance criteria:

- For cannabis labeled as α-Pinene dominant: The relative content of α-Pinene is more than 2 times the content of any other peak
- For cannabis labeled Myrcene dominant: The relative content of Myrcene is more than 2 times the content of any other peak
- For cannabis labeled Limonene dominant: The relative content of Limonene is more than 2 times the content of any other peak
- For cannabis labeled Terpinolene dominant: The relative content of Terpinolene is more than 2 times the content of any other peak
- For cannabis labeled ß-Caryophyllene dominant: The relative content of ß-Caryophyllene is more than 2 times the content of any other peak
- For cannabis labeled Myrcene- β-Caryophyllene codominant: Myrcene and β-Caryophyllene are the mayor peaks in the chromatogram and the ratio of relative content of Myrcene/β-Caryophyllene is between 0.5 and 2
- For cannabis labeled Myrcene-Limonene codominant: Myrcene and Limonene are the mayor peaks in the chromatogram and the ratio of relative content of Myrcene/Limonene is between 0.5 and 2
- For cannabis labeled Limonene- β-Caryophyllene codominant: Limonene and β-Caryophyllene are the mayor peaks in the chromatogram and the ratio of relative content of Limonene/β-Caryophyllene is between 0.5 and 2
- For cannabis labeled Myrcene-Limonene- β-Caryophyllene codominant: Myrcene, Limonene and β-Caryophyllene are the mayor peaks in the chromatogram and the ratio of relative content of any pair of the three terpenes is between 0.5 and 2

REFERENCES FOR APPENDICES 3 and 4:

1. USP. General Chapter <621> Chromatography. 2019; https://hmc.usp.org/about/general-chapters/ Accessed March 12, 2020.