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BHUTAN STANDARD

Yartsa Guenbub (Cordyceps)

ICS 11.120.10

BHUTAN STANDARDS BUREAU

The National Standards Body of Bhutan

THIMPHU 11001

……, 2022

Price group B

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FOREWORD

This Bhutan Standard for Cordyceps was adopted by Bhutan Standards Bureau after the draft finalized by the Sub-committee on Cordyceps (TC 05/ SC 03) and Pharmaceuticals and Traditional Medicines Technical Committee (TC 05) and approved by the Bhutan Standards Bureau Board (BSB Board) on ….. 2022.

This standard is subject to systematic review after five years to keep pace with the market trends, industrial and technological developments. Any suggestions and further information may be directed to the concerned Technical Committee.

BTS XXX: 2022

INTRODUCTION

Ophiocordyceps sinensis, formerly known as *Cordyceps sinensis,* is also known as caterpillar fungus in English. In Bhutan, it is called “Yartsa Guenbub” translated as “Winter Worm, Summer Grass”, while it is called Dōng chóng xià cǎo in Chinese and Tōchūkasō in Japanese. “Winter Worm, Summer Grass” refers to the fact that it parasitizes and consumes a hibernating caterpillar over the winter and then produces a grass blade-like fruiting body in the late spring.

Cordyceps grows at altitudes ranging from 3500 to 4800 meters above sea level in cold, grassy, alpine meadows on the Himalayan ranges of Bhutan, India, Nepal, and Tibetan plateau.

Cordyceps has been described as a medicine in Sowa Rigpa based Bhutanese traditional medicine. It has been used as super food and medicine across the world since at least 5000 BC.

The Ministry of Agriculture and Forests initiated a study on Cordyceps in 1998 and submitted the recommendations of sustainable harvesting and marketing. Accordingly, Royal Kasho was issued in 2004 to legalize the harvesting and marketing of Cordyceps. Since then, it has become an important source of income for highlanders in the Cordyceps growing areas.

Over the past decade, Cordyceps trading has been increasing and there is a demand for certification from importers. Currently the certification of the Cordyceps is solely based on the physical appearances using the MoAF common standard of quality, 2019. However, the grading standard lacked other quality parameters to fully assess the quality of Cordyceps.

Therefore, this national standard is developed to guide national authority in certification of Cordyceps from Bhutan.

This standard has been prepared in consultation with stakeholders to suit the intended purposes. It is the responsibility and at the discretion of each individual or a company to adopt or comply with this standard. The standard organization or the technical committee will not be liable for any untoward events either health or material losses.

BTS XXX: 2022

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BHUTAN STANDARD

Yartsa Guenbub (Cordyceps)

1 Scope

This standard applies to Yartsa Guenbub (*Ophiocordyceps sinensis* [Berk] Sac) in dried form. Products from cultured cordyceps and in other forms are not covered under this standard.

2 Normative References

The following referenced documents are indispensable for the application of this document. For dated references only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

- BTS 139:2019 SARS 0014:2018, Food Hygiene -General Principles - Code of Practice

3 Terms and Definition

For the purpose of this standard the following definitions shall apply:

3.1 Adulteration - Adulteration is an act of intentionally debasing the originality of product either by the admixture or substitution with inferior substances or by the removal of some valuable ingredients.

3.2 Contaminants - Any biological/chemical/physical, or any other substances not intentionally added to the product, which may compromise the quality.

3.3 Cultured Cordyceps – Cordyceps grown artificially in culture medium in a controlled environment.

3.4 Other forms – Forms other than the dried Cordyceps such as extracts, powder, capsules, tea etc.

3.5 Solid impurities - Insoluble extraneous matter found in the product. It may consist of but not limited to dirt and miscellaneous debris, mineral matter, nitrogenous materials of animal or plant origin and carbohydrate substances such as vegetable fibres.

3.6 Sowa Rigpa – Formal traditional medicines system practiced in Bhutan and other Himalayan region.

3.7 "Yartsa Guenbub" - Local vernacular name for Ophiocordyceps sinensis.

4 Acronyms and Symbols

µl	: microlitre
BP	: British Pharmacopeia
CFU/g	: Colony Forming Unit per gram

cm	: centimetre
g	: grams
g/l	: gram/ litre
mg	: milligram
ml	: millilitre
mm	: millimetre
NLT	: Not Less Than
nm	: nanometre
NMT	: Not More Than
MoAF	: Ministry of Agriculture and Forests
ppm	: parts per million
TLC	: Thin Layer Chromatography
v/v	: volume by volume
v/w	: volume by weight
w/w	: weight by weight
≤	: Less than or equal to
>	: More than

5 Requirements

5.1 Description

Ophiocordyceps sinensis is commonly referred to as Cordyceps or caterpillar fungus and it is commonly used and traded in dried whole form. Cordyceps is one of the well-known tonics and an important component in Bhutanese traditional medicine. It is a composite consisting of the stromata of the fungus, parasitized on the caterpillar of some species of ghost moths (Family: Hepialidae).

5.1.1 Caterpillar is around 1.5 - 5 cm long and 3 - 8mm in diameter. Externally it is intense golden yellow to dark brown with 20-60 annulations and those near the head are relatively fine. There are 8 pairs of feet (3 pairs near the head, 4 pairs in the middle and 1 pair near the tail), only the 4 pairs of feet in the middle are conspicuous. Its texture is fragile, easily broken and the cross section is slightly even, yellowish-white with dark brown V-shaped clefts.

5.1.2 The head of the caterpillar can be differentiated into reddish-brown, reddish black or yellow colour variations.

5.1.3 The stroma is deep brown to brown in colour, thin and cylindrical, approximately 2 - 7cm long and 3 mm in diameter, with fine longitudinal wrinkles and the upper part is slightly swollen on maturation. The texture is soft and the cross section has whitish fibres.

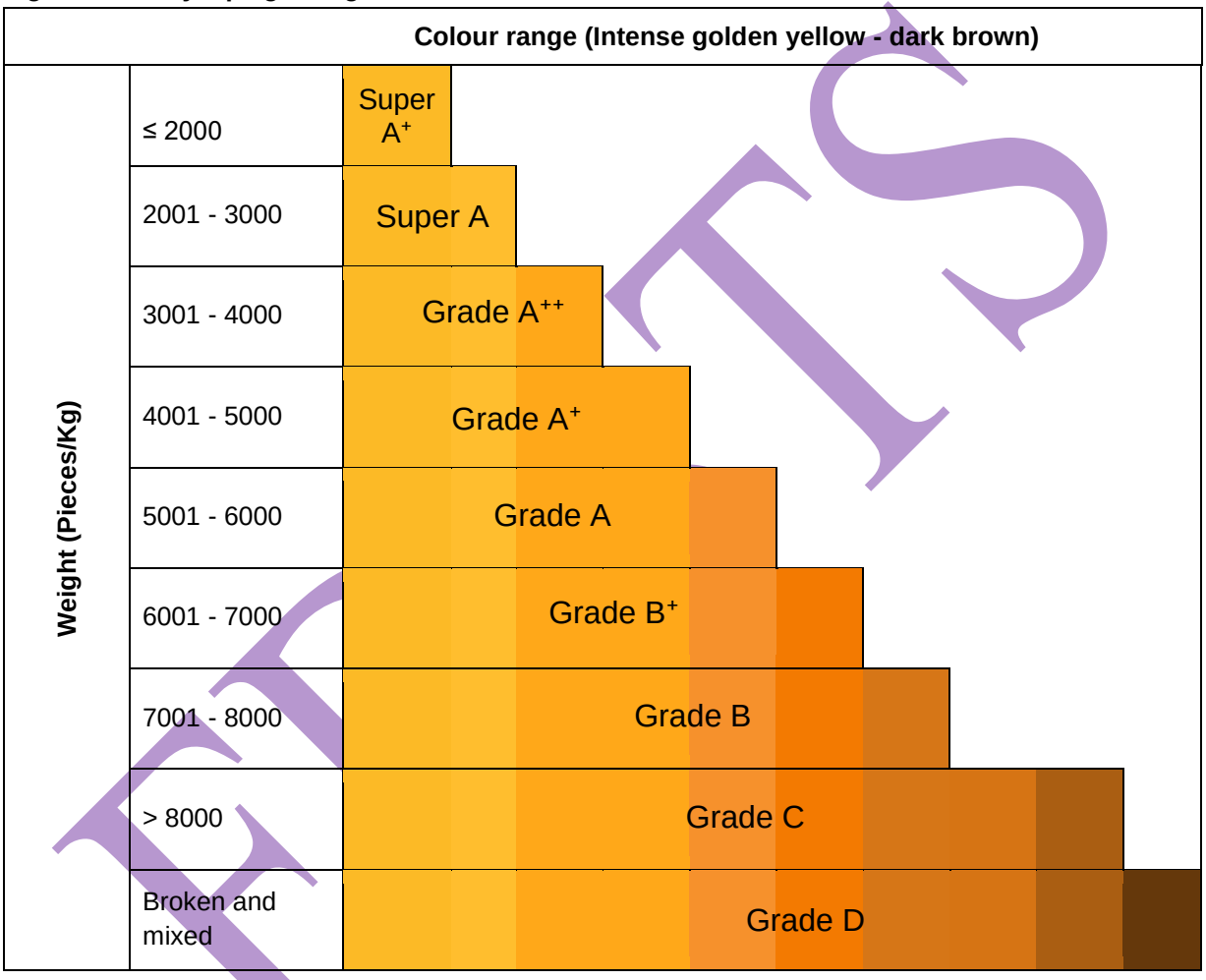
5.2 Scientific classification

Kingdom	: Fungi
Division	: Ascomycota
Class	: Sordariomycetes
Order	: Hypocreales
Family	: Ophiocordycipitaceae
Genus	: Ophiocordyceps
Species	: Sinensis

5.3 Classification

Cordyceps are classified into 9 different grades based on weight and colour. Super A^{+} is considered as the best grade and class D as the lowest grade. Class D consists of mixed and broken pieces of cordyceps irrespective of the colour. The colour of the dried Cordyceps is influenced by the way it is cleaned, dried and stored. The following grading chart (Figure 1) is based on the MoAF standard and recommendation from the sub-committee.

Figure 1: Cordyceps grading chart



5.4 Production

Dried Cordyceps are caterpillar fungus obtained from the wild. Suitable collection, harvesting, drying and storage conditions are essential to guarantee their quality. Dried cordyceps should, as far as possible, be free of any foul smell and taste, free from solid impurities such as soil, dust, dirt and other foreign material and animal contaminants. It should be practically free of any signs of deterioration and rehydration.

5.5 Tests

5.5.1 Identification Test

Thin Layer Chromatography (TLC) Method: The chromatogram of the test sample when examined under UV 254nm and 366nm, should correspond in position, colour and intensity to that of the reference sample zones as per method described in Annexure A.

5.5.2 Moisture content and volatile substances

Not more than 7% v/w when determined as per Annexure B, Method 1.2 or not more than 6% w/w determined as per Annexure B, Method 1.3.

5.5.3 Foreign matter

Not more than 2% w/w determined as per Annexure C.

5.5.4 Total ash

Not more than 5% w/w determined as per Annexure D, Method 1.1.

5.5.5 Acid insoluble ash

Not more than 2.5% w/w determined as per Annexure D, Method 1.2.

5.5.6 Water soluble ash

Not more than 2% w/w determined as per Annexure D, Method 1.3.

5.5.7 Extractable matter

Not less than 5% w/w determined as per Annexure E.

5.5.8 Heavy Metals Limits

The material shall be free from heavy metals in amounts which may represent a hazard to health and shall not exceed the limits specified in Table 1. The specified methods for determination of the listed heavy metals are not prescribed. The laboratories may use any validated methods for analysis, provided the selected methods meet the specific performance criteria.

Table 1: Heavy metals limits for Cordyceps
(Clause 5.5.8)

Heavy Metals	Limit (ppm)
Cadmium	NMT1.0
Lead	NMT 5.0
Mercury	NMT 0.1
Arsenic	NMT 2.0

5.5.9 Microbial Contaminations

Microbiological examination should be performed according to the methods given in BP 2019, Volume V, general chapters 2.6.12 and 2.6.31.
Acceptance criteria based upon the total aerobic microbial count (TAMC) and the total combined yeast/ moulds count (TYMC) are given below:

Table 2: Microbiological acceptance limit for Cordyceps
(Clause 5.5.9)

TAMC (2.6.12)	Acceptance criteria: 10^7 CFU/g Maximum acceptable count: 50000000 CFU/g
TYMC (2.6.12)	Acceptance criteria: 10^5 CFU/g Maximum acceptable count: 500000 CFU/g
E. Coli (2.6.31)	Acceptance criteria: 10^3 CFU/g
Salmonella (2.6.31)	Absence (25 g)

5.5.10 Aflatoxins

The product shall be free from mycotoxins in amounts which may represent a hazard to health and shall not exceed the limits specified in Table 3. Laboratories may use any validated methods for analysis provided the selected methods meet the specific performance criteria.

Table 3: Mycotoxins limits for Cordyceps
(Clause 5.5.10)

Mycotoxins	**Maximum level (ppm)**
Aflatoxin B1	0.01
Total aflatoxins	0.02

6 Hygiene

It is recommended that the products covered by the provisions of this Standard be prepared and handled in accordance with the section of BTS 139:2019 SARS 0014:2018.

7 Storage

For short duration

In an airtight container, protected from direct light and moisture.

For long duration

The properly dried Cordyceps are vacuum packed in Zip lock plastic bag. Then it is wrapped by a soft paper to protect from cold burns. This is further packed in airtight container/Zip lock plastic bags and is refrigerated at -10◦ to -15 degree Celsius.

8 Packaging

The content of each package must be uniform and of the same class. The visible part of the contents of the package must be representative of the entire contents.

Cordyceps shall be packed in a hermetically sealed container suitable for preventing the possible adulteration and contamination. The materials used inside the package must be clean to avoid causing any external or internal damage to the produce. The packaging materials should not contaminate the produce in any way.

9 Labelling

9.1 General

Shall fulfil following labelling requirements:

a) The name of the product
b) Grade
c) Net Weight
d) Origin of produce
e) Date of packaging
f) Expiry date
g) Full address of the exporter
h) Disclaimer
i) Storage conditions

9.2 Labelling prohibitions

9.2.1 Follow the latest Bhutan Medicines Rules and Regulations for any medicinal property claim.

9.2.2 The use of any statement or of any pictorial device which may be graphic or create confusion in the mind of the public or in any way mislead the public about the nature, origin, composition and properties of Cordyceps put on sale is prohibited.

10 Sampling

10.1 Scale of sampling

10.1.1 In a single consignment, all packages belonging to the same batch shall be grouped together and each group shall constitute a lot.

10.1.2 For ascertaining the conformity of the material to the requirements of the specification, samples shall be tested from each lot separately.

10.1.3 The number of containers to be sampled or taken from the lot depends on the size of the lot and shall be in accordance with column I and II of Table 4. From each selected package approximately equal number or pieces shall be taken from each packet so as to constitute the required sample size.

10.1.4 The required number of packages from each selected lot and the required quantity or pieces from each selected packet shall be chosen at random.

10.2 Sampling procedure

10.2.1 Draw a sample from the upper, middle and lower part of a randomly sampled container or packet.

10.2.2 Combine individual samples to make pooled samples and mix them thoroughly.

10.2.3 Quarter the pooled sample to obtain the average sample. While quartering, place the material in an even square shape and divide it diagonally into four equal parts and take two equal diagonal parts.

10.2.4 Quarter the average sample again to get the final sample.

10.3 Tests and Criteria for Conformity

Each sample selected according to 10.1 and 10.2 shall be examined for physico-chemical characteristic requirements. A sample failing to satisfy any of these requirements shall be considered as defective. The lot shall be considered as conforming to the standard if all requirements are satisfied.

Table 4: Scale of sampling

(Clause 10.3)

	Column I	**Column II**
Total No. of packages/ containers in a lot	Number of packages to be sampled	Quantity to be sampled in grams
Up to 5	All of them	10
6 and above	5	10

Annex A
(Clause 5.5.1)
Thin-layer chromatography Method

Thin-layer chromatography (TLC) is particularly valuable for the qualitative determination of small amounts of impurities. The principles of thin-layer chromatography and application of the technique in pharmaceutical analysis are described in the international pharmacopoeia.

Two thin-layer chromatography methods are described below:

1. Classical method

1.1. Recommended procedure

The method outlined below assumes that chromatographic plates prepared in the laboratory are used; however, precoated plates, activated, if necessary, may be used provided that they have proved suitable for the application concerned. A powdered specimen of pharmacopeial quality may be used as the reference material. If a test for the presence of a known active principle of an herbal material is to be carried out, a chemical reference substance identical to that principle should be used. The test and reference solutions should be prepared simultaneously in exactly the same way. The reference solutions should be of known concentration. If the relative concentrations of the chemical substances in the reference solution are selected in accordance with the composition of a typical material, comparison of the spot size offers valuable additional information. The solvent system should be specified in the test procedure for the individual material being examined. A three-colour mixture (e.g., 0.01% solutions in toluene of indophenol blue, Sudan red G and dimethyl yellow), run together, permits a rapid check on the prevailing chromatographic conditions. If it is suspected that the materials being examined are unstable, the chamber in which chromatography takes place should be protected from light. In any case, the chromatographic chamber should always be kept out of direct sunlight. Otherwise, the rays of the sun may be refracted to different degrees owing to imperfections in the glass walls of the chamber, giving rise to areas of elevated temperature on the chromatographic plate and erratic flow of the mobile phase.

1.2. Preparation of samples

Prior to testing, prepare an extract of the herbal material to be examined, using a rapid extraction process, as follows. To 0.1–1.0 g of the powdered herbal material, add 1–10 ml of solvent and extract either by stirring, shaking the mixture for 3–30 minutes, or heating to boiling and allowing to cool. Remove the insoluble matter by centrifugation, or filter through a small funnel with filter-paper or a cotton plug. If necessary, evaporate the filtrate on a water-bath for just as long as is required to remove the solvent, then re-dissolve the residue in a small volume of solvent (e.g., 0.1–0.2 ml). If necessary, purify the test solution by repeating the extraction with solvent at a different pH, or by sublimation, distillation, or other appropriate method.

1.3. Equipment

The equipment consists of:

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

- a device for spreading a uniform layer of coating material of desired thickness onto the glass plates;
- a rack to hold the prepared plates (normally 10 plates with set spacings) during the drying period or for transportation and storage; the rack should be small enough to fit in a drying oven and desiccator;
- a chromatographic chamber of transparent material, usually glass, with a tightly fitting lid, of suitable size to accommodate the test plates;
- a suitable spraying implement with a fine spray nozzle, made of a material resistant to the reagents to be used;
- an ultraviolet light source emitting short (254 nm) and long (365 nm) wavelengths.

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

1.4. Preparation of the adsorbent

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

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

1.5. Saturation of the chromatographic chamber

Unless otherwise specified in the test procedure, the chromatography is carried out in a saturated chamber. To achieve saturation, line at least half of the total area of the inside walls of the chamber with filter-paper, pour into the chamber a sufficient quantity of the mobile phase to saturate the filter-paper and form a layer about 5 mm deep. Close the chamber and allow it to stand for at least 1 hour at room temperature. All operations during which the plate is exposed to the air should preferably be carried out at a relative humidity of 50–60%, and the plates should be handled with care.

1.6. Application of the test and reference solutions

Using a micropipette or a syringe graduated in µl, place spots of the test and reference solutions onto the starting line, which should be parallel to and about 15 mm above the lower edge. The spots should be at least 15 mm from the sides of the plate, and at least 15 mm apart. They should be as small as possible, preferably not more than 4 mm in diameter; if necessary, apply the solution in portions, drying between applications. Mark the distance the mobile phase is intended to ascend as specified in the test procedure, usually 10–15 cm from the starting line. The results of separation can often be improved by applying the solutions to form a horizontal band 10-15 mm long and not more than 5 mm wide.

1.7. Development of chromatograms

Allow the spots to dry, place the plate — as nearly vertical as possible — into the chamber, ensuring that the points of application are above the surface of the mobile phase. Close the chamber. Develop the

chromatogram at room temperature, unless otherwise specified in the test procedure, allowing the solvent to ascend the specified distance. Remove the plate, mark the position of the solvent front and allow the solvent to evaporate at room temperature or as specified.

1.8. Observation and interpretation of the chromatograms

Observe the spots produced in daylight, then under short-wave and long-wave ultraviolet light. Mark the centre of each spot with a needle. Measure and record the distance from the centre of each spot to the point of application, and indicate for each spot the wavelength under which it was observed. If indicated in the test procedure, spray the spots with the specified reagent, and observe and compare the spots with those of a reference material. If required, calculate the ratio of the distance travelled on the adsorbent by a given compound to that travelled by the leading edge of the solvent (the Rf value) or the ratio of the distances moved by a compound and a stated reference substance (the Rr value) as follows:

$$R_f = \frac{a}{b} \qquad R_r = \frac{a}{c}$$

Where:

a = the distance between the point of application and the centre of the spot of the material being examined;
b = the distance between the point of application and the solvent front;
c = the distance between the point of application and the centre of the spot of reference material.

R_f values may vary with each experiment depending on the saturation conditions in the chromatographic chamber, the activity of the adsorbent layer, and the composition of the mobile phase.

2. Micro method

The chromatograms can be developed either vertically or horizontally. Unless otherwise specified in the test procedure for the herbal material concerned, thin layer chromatography is performed on small plates using the ascending technique.

2.1. Ascending technique

2.1.1. Equipment

The equipment consists of:

- precoated or specially prepared plates, not more than 100 mm long, and 100 mm wide, that permit development over at least 60 mm;
- 1-µl or 2-µl micropipettes accurate to ±10% of the stated volume;
- a chromatographic chamber with a tightly fitting lid and a flat base; the chamber must be of such a size to accommodate the plates and the appropriate volume of the mobile phase.

2.1.2. Method

Place a sufficient quantity of a previously mixed and homogeneous mobile phase into the chromatographic chamber to form a layer 5 mm deep (Mobile phase mixtures should be discarded after the development of

a plate). Close the chamber and allow it to stand at constant room temperature, protected from draughts and direct sunlight, for 15 minutes.

Using a micropipette, apply spots of the solutions being examined onto the starting line, which should be parallel to and about 10 mm above the lower edge of the plate. The spots should be at least 10 mm from the sides of the plate, and 5–10 mm apart. They should be as small as possible, preferably no more than 2 mm in diameter. Mark the distance the mobile phase is intended to ascend as specified in the test procedure for the herbal material concerned, usually 60 mm from the starting line.

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

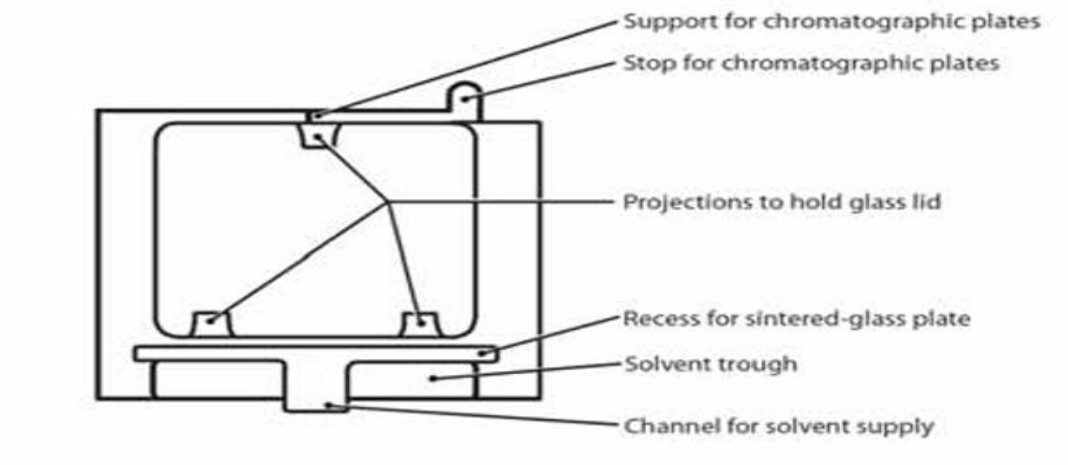
2.2. Horizontal technique

2.2.1. Equipment

The equipment consists of:

- specially made plates 50 mm long, 50 mm wide;
- 0.5-µl or 1-µl micropipettes, accurate to ±10% of the stated volume;
- a chromatographic chamber for horizontal development (Figure 1); commercially available chambers consist of a solvent-proof body with a trough for the mobile phase and a tightly fitting glass lid; the mobile phase is transferred from the trough to the adsorbent layer via an exchangeable sintered-glass plate.

Figure 1. Chromatographic chamber for horizontal development (horizontal cross-section)



2.2.2. Method

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

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

Using a micropipette, apply the volumes of the solutions to be examined onto the starting line of the chromatographic plate, which should be parallel to the lower edge of the plate. The spots produced should be at least 7 mm from the sides of the plate and not less than 5 mm apart. The spots should be as small as possible, preferably no more than 1 mm in diameter. Mark the distance the mobile phase is intended to travel as specified in the test procedure, for the herbal material concerned, usually 40 mm from the starting line.

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

Annex B
(Clause 5.5.2)
Determination of moisture and volatile substances

An excess of water in herbal materials will encourage microbial growth, the presence of fungi or insects, and deterioration following hydrolysis. Limits for water content should therefore be set for every given herbal material. This is especially important for materials that absorb moisture easily or deteriorate quickly in the presence of water.

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

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

Recommended procedure

1.1. Preparation of material

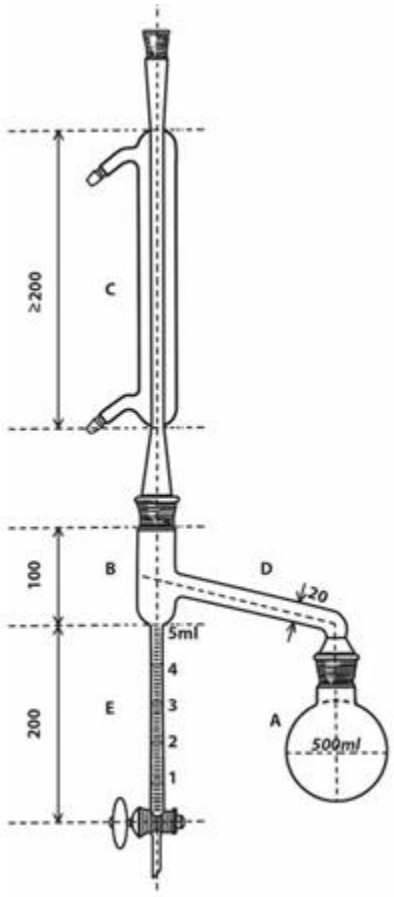
Prepare a suitable quantity of the sample by cutting, granulating or shredding the unground or unpowdered material, so that the thickness of the parts does not exceed 3 mm. Seeds or fruits smaller than 3 mm should be cracked. Avoid the use of high-speed mills in preparing the sample, and take care that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

1.2. Azeotropic method (toluene distillation)

The apparatus (Figure 2) consists of a glass flask (A) connected by a tube (D) to a cylindrical tube (B) fitted with a graduated receiving tube (E) and a reflux condenser (C). The receiving tube (E) is graduated in 0.1-ml divisions so that the error of readings does not exceed 0.05 ml. The preferred source of heat is an electric heater with a rheostat control, or an oil-bath. The upper portion of the flask and the connecting tube may be insulated.

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

Figure 2. Apparatus used to determine water content by the azeotropic method (dimensions in mm)



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

$$\frac{100(n_1 - n)}{w}$$

Where:

w = the weight in g of the material being examined;
n = the number of ml of water obtained in the first distillation;
n_1 = the total number of ml of water obtained in both distillations.

1.3. Loss on drying (gravimetric determination)

Place about 2–5 g of the prepared air-dried material, or the quantity specified in the test procedure for the herbal material concerned, accurately weighed, in a previously dried and tared flat weighing bottle. Dry the sample by one of the following techniques:

- in an oven at 100-105°C;
- in a desiccator over phosphorus pentoxide under atmospheric pressure or reduced pressure and at room temperature.

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

$$\frac{100(M_0 - M_1)}{M_0}$$

Where:

M_0 = the mass of sample before drying;
M_1 = mass of sample after drying;

Annex C
(Clause 5.5.3)

Determination of foreign matter

Materials should be entirely free from visible signs of contamination by moulds or insects, and other animal contamination, including animal excreta. No abnormal odour, discoloration, slime or signs of deterioration should be detected. It is seldom possible to obtain marketed plant materials that are entirely free from some form of innocuous foreign matter. However, no poisonous, dangerous or otherwise harmful foreign matter or residue should be allowed. During storage, products should be kept in a clean and hygienic place, so that no contamination occurs. Special care should be taken to avoid formation of moulds, since they may produce aflatoxins. Macroscopic examination can conveniently be employed for determining the presence of foreign matter in whole or cut plant materials. Any soil, stones, sand, dust and other foreign inorganic matter must be removed before herbal materials are cut or ground for testing.

Recommended procedures

1. Weigh a sample of herbal material, taking the quantity indicated above unless otherwise specified in the test procedures for the herbal material concerned.
2. Spread it in a thin layer and sort the foreign matter into groups either by visual inspection, using a magnifying lens (6× or 10×), or with the help of a suitable sieve, according to the requirements for the specific herbal material.
3. Sift the remainder of the sample through a No. 250 sieve; dust is regarded as mineral admixture.
4. Weigh the portions of this sorted foreign matter to within 0.05 g.
5. Calculate the content of each group in grams per 100 g of air-dried sample. For some herbal materials where the foreign matter may closely resemble the material itself, it may be necessary to take a pooled sample of the herbal material and apply a critical test either chemical or physical. The proportion of foreign matter is calculated from the sum of the portions that fail to respond to the test.

Annex D
(**Clause 5.5.4 – 5.5.6**)

Determination of ash

The ash remaining following ignition of herbal materials is determined by three different methods which measure total ash, acid-insoluble ash and water-soluble ash.

The total ash method is designed to measure the total amount of material remaining after ignition. This includes both “physiological ash”, which is derived from the plant tissue itself, and “non-physiological” ash, which is the residue of the extraneous matter (e.g., sand and soil) adhering to the plant surface.

Acid-insoluble ash is the residue obtained after boiling the total ash with dilute hydrochloric acid, and igniting the remaining insoluble matter. This measures the amount of silica present, especially as sand and siliceous earth.

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

1. Recommended procedure

1.1. Total ash

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

1.2. Acid-insoluble ash

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

1.3. Water-soluble ash

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

Annex E
(**Clause 5.5.7**)

Determination of extractable matter

This method determines the amount of active constituents extracted with solvents from a given amount of herbal material.

1. Hot extraction

Place about 4.0 g of coarsely powdered air-dried material, accurately weighed, in a glass-stoppered conical flask. Add 100 ml of water and weigh to obtain the total weight including the flask. Shake well and allow to stand for 1 hour. Attach a reflux condenser to the flask and boil gently for 1 hour; cool and weigh. Readjust to the original total weight with the solvent specified in the test procedure for the plant material concerned. Shake well and filter rapidly through a dry filter. Transfer 25 ml of the filtrate to a tared flat-bottomed dish and evaporate to dryness on a water-bath. Dry at 105°C for 6 hours, cool in a desiccator for 30 minutes, then weigh without delay. Calculate the content of extractable matter in mg per g of air-dried material.

2. Cold maceration

Place about 4.0 g of coarsely powdered air-dried material, accurately weighed, in a glass-stoppered conical flask. Macerate with 100 ml of the solvent specified for the plant material concerned for 6 hours, shaking frequently, then allow to stand for 18 hours. Filter rapidly, taking care not to lose any solvent, transfer 25 ml of the filtrate to a tared flat-bottomed dish and evaporate to dryness on a water-bath. Dry at 105°C for 6 hours, cool in a desiccator for 30 minutes and weigh without delay. Calculate the content of extractable matter in mg per g of air-dried material. For ethanol-soluble extractable matter, use the concentration of solvent specified in the test procedure for the herbal material concerned; for water-soluble extractable matter, use water as the solvent. Use other solvents as specified in the test procedure.

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