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Traditional Chinese medicine — Fermented *Cordyceps* powder

Médecine traditionnelle chinoise — Poudre de Cordyceps *fermenté*

Reference number
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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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

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

Introduction

Cordyceps sinensis, known as “冬虫夏草”, Chinese Pinyin *Dong Chong Xia Cao* (winter worm, summer plant), is a rare raw material used in traditional Chinese medicine. *Cordyceps sinensis* enjoys equal popularity with ginseng and velvet and ranks first among these three tonic medicines. The use of *Cordyceps sinensis* can be traced to AD 863, during the Tang Dynasty, in the Youyang Essays by Duan Chengshi. In recent years, the efficacy of *Cordyceps sinensis* has been further confirmed and its extensive and significant efficacy is acknowledged worldwide.

The bioactivities of *Cordyceps sinensis* include immunoregulation, anti-bacteria, anti-cancer, anti-oxidation, anti-aging, blood sugar control and fat reduction. Due to the specific environment required for the growth of *Cordyceps sinensis*, the resources of wild *Cordyceps sinensis* are limited. Increasing market demand has therefore resulted in high prices. Driven by increased interest, wild *Cordyceps sinensis* is facing extinction due to plunder digging, which impacts its natural ecological environment. The culture of *Cordyceps sinensis* has become a hot topic of research, with a focus on liquid fermentation technology. Fermented *Cordyceps* powder is manufactured with strain extracted from wild *Cordyceps sinensis* by low-temperature liquid fermentation, simulating the growth environment of wild *Cordyceps sinensis.* Quality control for fermented *Cordyceps* powder is complicated. There are many functional active substances in fermented *Cordyceps* powder, including polysaccharide compounds, alkaloids (Cordycepin), peptide compounds, sterols, terpenoids and other secondary metabolites. The content of these substances in fermented *Cordyceps sinensis* determines to a large extent the quality and efficacy of fermented *Cordyceps powder*. Traditional analytical methods vary and depend highly on experience. It is difficult to determine the quality of fermented *Cordyceps* powder with existing methods.

A guarantee of the quality of fermented *Cordyceps* powder is crucial to ensure the quality and safety of medicines and health products made from fermented *Cordyceps* powder. Exploring quality control methods of fermented *Cordyceps* powder and creating a standard combining traditional Chinese medicine characteristics and current technology will have profound influence on the industry of fermented *Cordyceps* powder.

As national implementation can differ, national standards bodies are invited to modify the values given in 4.5, 4.8 and 4.9. Reference values of national and regional limits of heavy metals, microbiological examination and aflatoxins are given in Annex E.

Traditional Chinese medicine — Fermented *Cordyceps* powder

1 Scope

This document specifies a set of requirements and a test method to control the quality of fermented *Cordyceps* powder, including test items such as identification, assay, water content, residue on ignition, microbial limit and heavy metals.

It is applicable to fermented *Cordyceps* powder which is produced by liquid fermentation with extracted strain of *Ophiocordyceps sinensis* (Berk.).

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements 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.

ISO 1762, *Paper, board, pulps and cellulose nanomaterials — Determination of residue (ash content) on ignition at 525 °C*

ISO 6673, *Green coffee — Determination of loss in mass at 105 degrees C*

ISO 13903:2005, *Animal feeding stuffs — Determination of amino acids content*

ISO 18664, *Traditional Chinese Medicine — Determination of heavy metals in herbal medicines used in Traditional Chinese Medicine*

ISO 22283, *Traditional Chinese medicine — Determination of aflatoxins in natural products by LC-FLD*

ISO 22467, *Traditional Chinese medicine — Determination of microorganisms in natural products*

3 Terms and definitions

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

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

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

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

3.1
***Ophiocordyceps sinensis* (Berk.)**
sac fungi parasite in bat moth larvae

3.2
fermented *Cordyceps* powder
dried powder of mycelium obtained by liquid fermentation of *Ophiocordyceps sinensis* (Berk.)

4 Recommendations and requirements

4.1 Characteristics

The powder should be grey to greyish yellow, slightly fishy and salty.

4.2 Thin-layer chromatography (TLC) identification

The test solution shall present the spots with the same position and colour as that of the reference powder solution and reference solution under sunlight and ultraviolet light.

4.3 High-performance liquid chromatography (HPLC) identification

The retention times of six peaks obtained with the test solution and the reference powder solution should be concordant with each other. The retention time of adenosine and uridine in the test solution should correspond to that of the reference solutions of adenosine and uridine, respectively.

4.4 Amino acid analyser identification

The retention time of the peaks in the test solution should be concordant with that of tyrosine, lysine, histidine and arginine in the reference solution.

4.5 Heavy metals

The contents of heavy metals such as arsenic, lead, cadmium and mercury shall be determined.

4.6 Loss on drying

Loss on drying should not be more than 6,0 %.

4.7 Residue on ignition

Residue on ignition should not be more than 7,0 %.

4.8 Microbial limit

Total aerobic microbial count (TAMC), total combined yeast and mould count (TYMC) and *Escherichia coli (E. coli)* shall be determined.

4.9 Aflatoxins

The contents of aflatoxins such as aflatoxin B_1 (AFB_1) and the sum of Aflatoxin B_1 (AFB_1), Aflatoxin B_2 (AFB_2), Aflatoxin G_1 (AFG_1) and Aflatoxin G_2 (AFG_2) shall be determined.

4.10 Content of total amino acids

Total amino acids should not be less than 30,0 %.

4.11 Content of mannitol

Mannitol ($C_6H_{14}O_6$) should not be less than 7,0 %.

4.12 Content of adenosine

Adenosine ($C_{10}H_{13}N_5O_4$) should not be less than 0,08 %.

5 Test method

5.1 Characteristics

Take an appropriate amount of the sample on clean white paper, observe at a distance of 25 cm to 30 cm under adequate lighting, smell and taste.

5.2 TLC identification

See Annex A for additional information.

5.3 HPLC identification

See Annex B for additional information.

If TLC and HPLC identification are not sufficient to distinguish similar products, the reference test method specified in Annex C should be applied.

5.4 Amino acid analyser identification

The testing method specified in ISO 13903 shall be applied.

5.5 Determination of heavy metals

The testing method specified in ISO 18664 shall be applied.

5.6 Determination of loss on drying

The testing method specified in ISO 6673 shall be applied.

5.7 Determination of residue on ignition

The testing method specified in ISO 1762 shall be applied.

5.8 Microorganism examination

The testing method specified in ISO 22467 shall be applied.

5.9 Determination of aflatoxin

The testing method specified in ISO 22283 shall be applied.

5.10 Determination of total amino acids

The testing method of total amino acids specified in ISO 13903:2005, 2.2 shall be applied.

5.11 Determination of mannitol

Accurately weigh 1 g of the sample into a 150-ml round-bottomed flask, add precisely 100 ml of ethanol, weigh, heat under reflux for 2h, cool, replenish the weight loss with ethanol, mix well and filter. Measure 5 ml of successive filtrate into an iodine flask, add 50 ml of sodium (potassium) periodate solution, heat in a water bath for 15 min, cool, add 10 ml of potassium iodide TS, stopper tightly and allow to stand for 5 min. Titrate with 0,05 mol/l sodium thiosulfate VS, towards the end of titration add starch IS and continue to titrate until the blue colour disappears. 1 ml of 0,05 mol/l sodium thiosulfate is equivalent to 0,910 9 mg of mannitol ($C_6H_{14}O_6$).

5.12 Determination of adenosine

See Annex D for additional information.

6 Test report

The test report shall include the following:

a) the sample;

b) a reference to this document, i.e. ISO 4754:2022;

c) the method used;

d) the result(s), including a reference to the clause which explains how the results were calculated;

e) any deviations from the procedure or unusual features observed during the test;

f) the date of the test.

Annex A
(informative)

Test method for identification by thin layer chromatography (TLC)

A.1 Preparation of test solution

Take 0,5 g of the sample, add 10 ml of methanol, ultrasonicate for 1 h, filter and use the filtrate as the test solution.

A.2 Preparation of reference powder solution

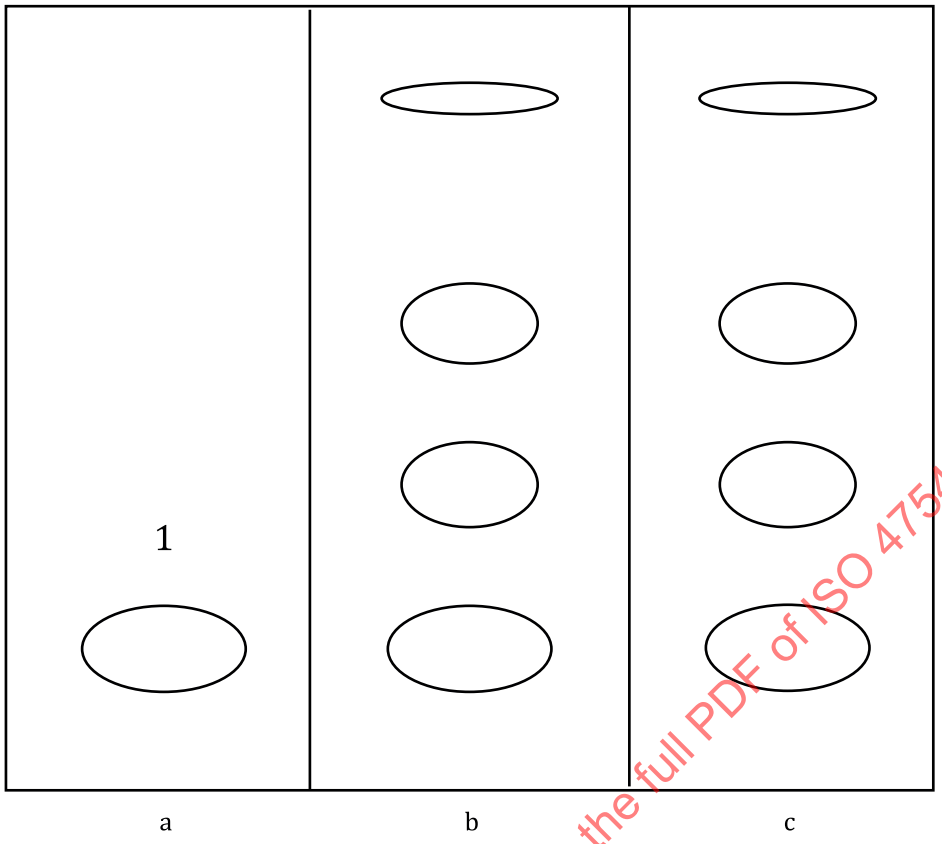
Take 0,5 g of fermented *Cordyceps* reference powder, add 10 ml of methanol, ultrasonicate treat for 1 h, filter and use the filtrate as the reference powder solution.

A.3 Preparation of reference solution

Dissolve ergosterol chemical reference substance (CRS) in methanol to produce a solution containing 0,4 mg per ml as the reference solution.

A.4 Identification by TLC

Use silica gel G as the coating substance and a mixture of petroleum ether (60 °C to 90 °C), ethyl acetate and formic acid in the ratio of 5:1:0,1 (volume fraction) as the mobile phase. Apply separately 10 µl of each of the three solutions to the plate. After developing and removal of the plate, dry in air. Spray with 10 % solution of sulphuric acid in ethanol, heat at 105 °C until the spots clear and observe under sunlight and ultraviolet light (365 nm). A typical TLC chromatogram is shown in Figure A.1.



Key

1 ergosterol
a ergosterol reference solution
b reference solution of fermented *Cordyceps* powder
c sample solution of fermented *Cordyceps* powder

Figure A.1 — Schematic diagram of typical TLC identification chromatograms of fermented *Cordyceps* powder

Annex B
(informative)

Test method for identification by high-performance liquid chromatography (HPLC)

B.1 Preparation of test solution

Accurately weigh 0,5 g of the sample into a conical flask with a stopper, add 20 ml of ether, stopper tightly, macerate for 30 min, filter and discard the ether. Expel the solvent to dryness, put into a stoppered conical flask along with the filter paper, accurately add 50 ml of 0,5 % phosphoric acid solution, stopper tightly and weigh accurately. Ultrasonicate for 30 min, cool and weigh again, replenish the weight loss with 0,5 % phosphoric acid solution, mix well and allow to stand. Take the supernatant, filter and use the subsequent filtrate as the test solution.

B.2 Preparation of reference powder solution

Prepare a reference powder solution using the same procedure described for the preparation of test solution in B.1, except replacing the sample with fermented *Cordyceps* reference powder.

B.3 Preparation of uridine reference solution

Add uridine CRS in 10 % methanol to obtain a solution containing 5 µg per ml as the uridine reference solution.

B.4 Preparation of adenosine reference solution

Accurately weigh a quantity of adenosine CRS and dissolve in 0,5 % phosphoric acid solution to prepare a solution containing 12 µg per ml as the adenosine reference solution.

B.5 Apparatus and chromatographic conditions

Using octadecylsilane bonded silica gel as the stationary phase, acetonitrile as mobile phase A and 0,04 mol/l potassium dihydrogen phosphate solution as mobile phase B, elute according to Table B.1.

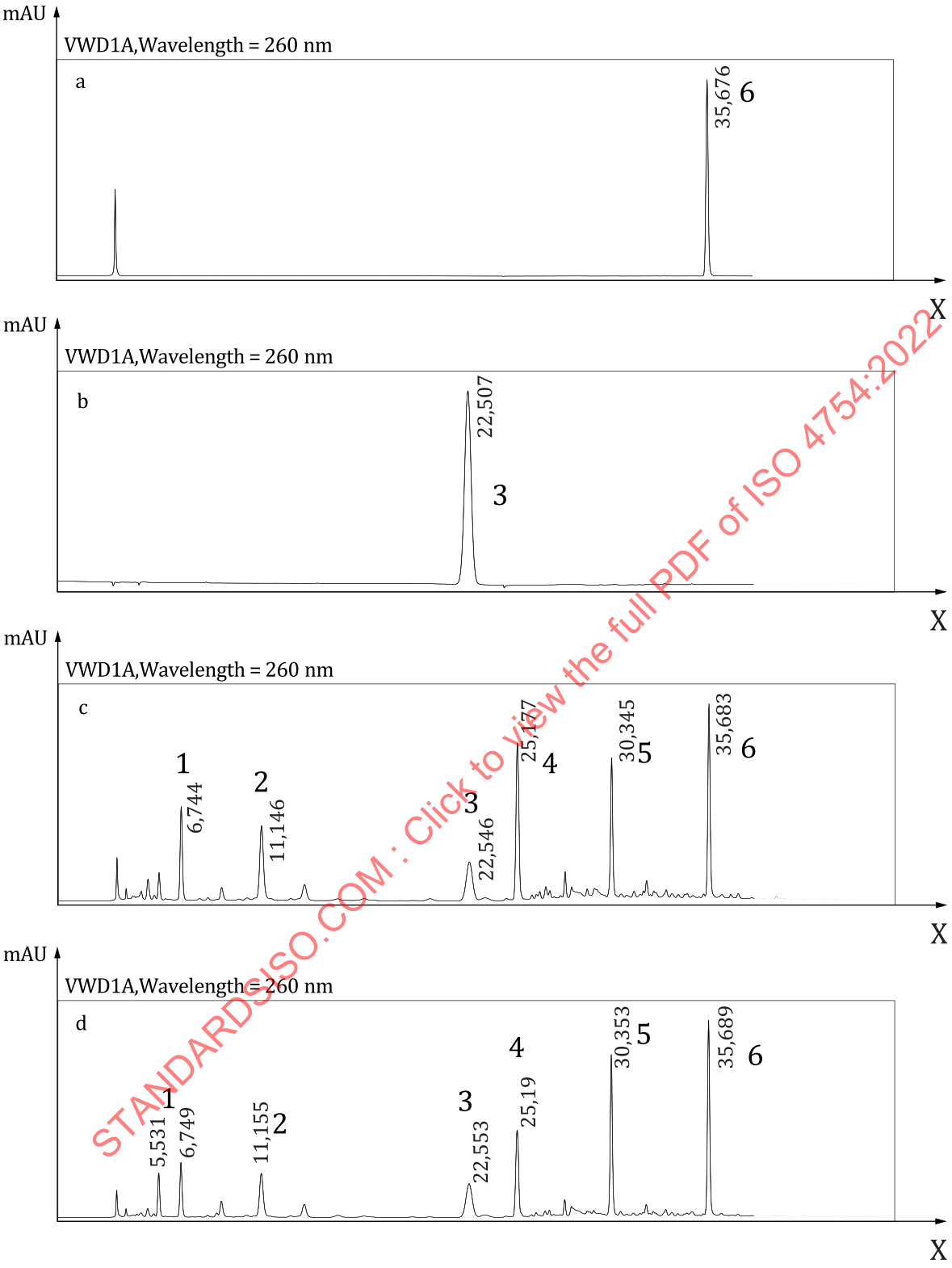
Table B.1 — Apparatus and chromatographic conditions

Time min	Mobile phase A %	Mobile phase B %
0 to 15	0	100
15 to 45	0 to 15	100 to 85

Detector wavelength is 260 nm, theoretical plate number should be not less than 3 000, calculated with the peak of adenosine reference.

B.6 Identification by HPLC

Inject 20 µl of each of the four solutions into the chromatograph. Typical reference HPLC identification chromatograms are shown in Figure B.1.



Key

X retention time (min)
1 uridylic acid
2 guanylic acid
3 uridine
4 adenylic acid
5 guanosine

6 adenosine
a adenosine reference solution
b uridine reference solution
c reference solution of fermented *Cordyceps* powder
d sample solution of fermented *Cordyceps* powder

Figure B.1 — Typical reference HPLC identification chromatograms of fermented *Cordyceps* powder

Annex C
(normative)

Test method for deoxyribonucleic acid (DNA) sequencing

C.1 Extraction of genomic DNA

Weigh 0,5 g of the sample into a mortar, add 20 ml of liquid nitrogen and grind. Repeat the addition of liquid nitrogen and grinding three times. Transfer 0,1 g of the sample into a 1,5-ml centrifuge tube and use a fungal genome extraction kit to extract and obtain the DNA template.

C.2 Polymerase chain reaction (PCR) amplification

Amplify the DNA template with universal 18S rDNA primers NSIF (AGTCATATGCTTGTCTC) and FungR (TTCCCCGTTACCCGTTG).

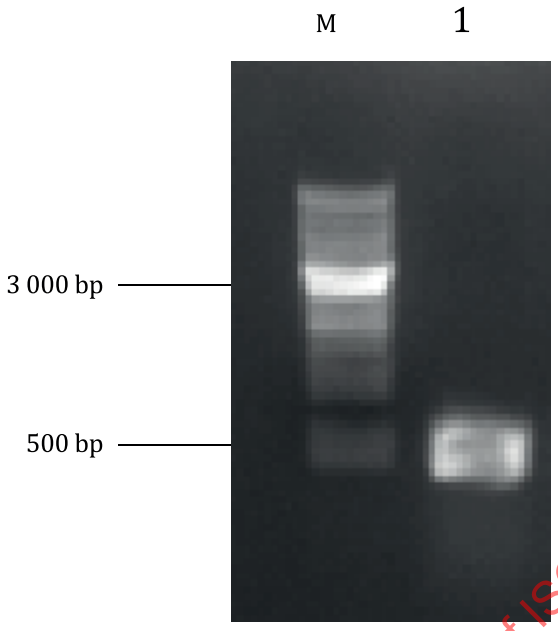
A reaction system with a total volume of 50 µl contains 0,5 µl of Phusion enzyme (2 000 units/ml), 1,5 µg of DMSO (100 %), 1 µl of dNTP (10 mmol/l), 10 µl pf 5X HF buffer, 2 µl of NSIF (10 µmol/l), 2 µl of FungR (10 µmol/l), 0,5 µl of the DNA template and 32,5 µl of ddH_2O.

The cycling protocol is as follows:

Denaturation	1 cycle	95 °C for 5 min
PCR amplification	34 cycles	98 °C for 10 s 55 °C for 30 s 72 °C for 30 s
Final extension	1 cycle	72 °C for 10 min

C.3 Electrophoresis

Separate the PCR products by agarose gel electrophoresis and visualize with DNA green dye. A single DNA strip should be present at 500 bp. A typical agarose gel electrophoresis pattern is shown in Figure C.1.



Key

M 1kb DNA marker

1 PCR amplification fragment of fermented *Cordyceps* powder

Figure C.1 — Agarose gel electrophoresis pattern

C.4 18S rDNA sequencing

The sequence has the highest similarity with the *Ophiocordyceps sinensis* strain in GenBank; the reference sequence is as follows:

TTCGGGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAGTTGTTGTGGTTAAAAAGCTCG-
TAGTTGAACCTTGGGCCTGGCTGCCGGTCCGCCTCACCGCGTGTACTGGTCCGGCCGGGCCTTTCCCTCTGTG-
GAACCCCATGCCCTTCACTGGCCGTGGCGGGGAAACAGGACTTTTACTTTGAAAAAATTAGAGTGCTCCAGG-
CAGGCCTATGCTCGAATACATTAGCATGGAATAATGAAATAGGACGCGCGGTTC6TATTTTGTTGGTTTCTAG-
GACCGCCGTAATGATTAATAGGGACAGTCGGGGGCATCAGTATTCAATGGTCAGAGGTGAAATTCTTGGATC-
CATTGAAGACTAACTACTGCGAAAGCATTTGTCAAGGATGTTTTCATTAATCAGGAACGAAAGTTAGGG-
GATCGAAGACTGCTAAA

The similarity should not be less than 99 %.

Annex D
(informative)

Test method for adenosine by HPLC

D.1 Apparatus and chromatographic conditions

Stationary phase: octadecylsilane bonded silica gel.

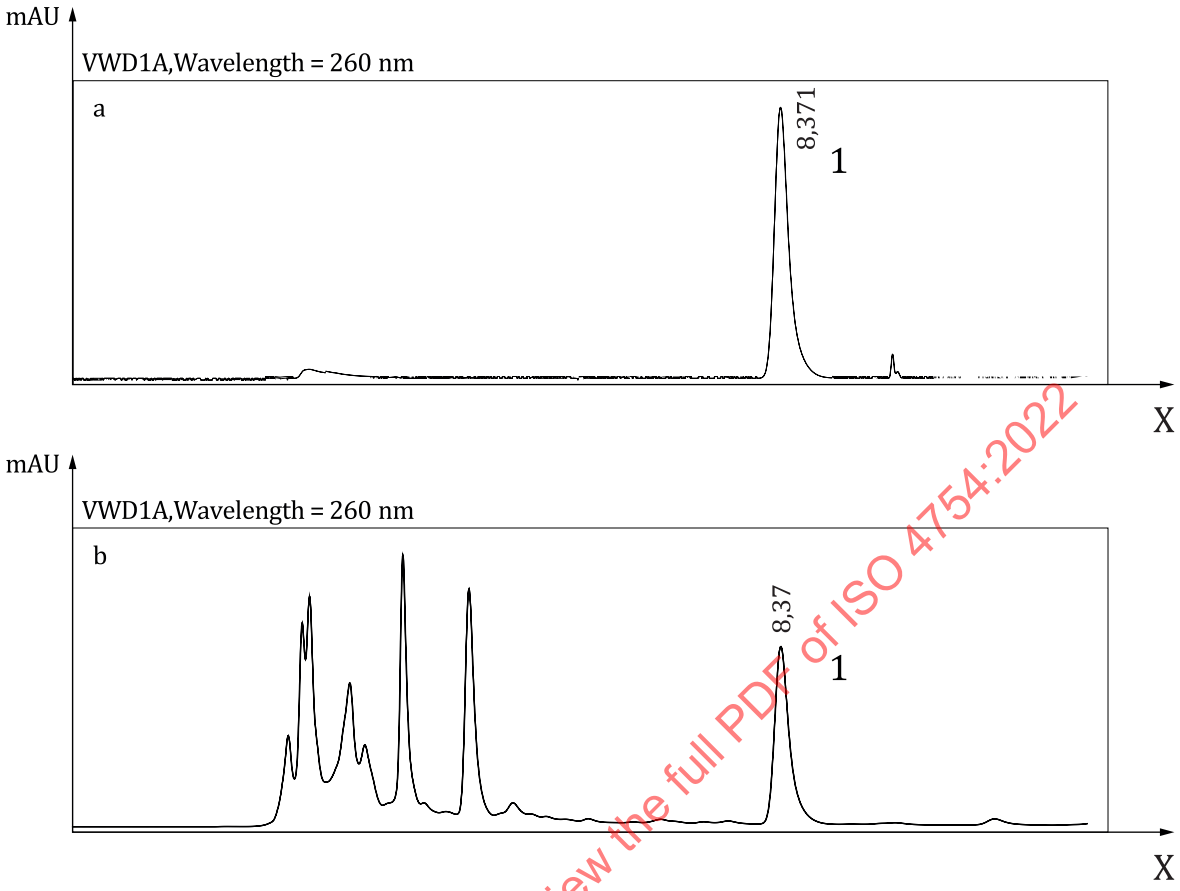
Mobile phase: a mixture of acetonitrile and 0,04 mol/l of potassium dihydrogen phosphate solution (5:95).

Detector wavelength: 260 nm.

System suitability: the number of the theoretical plate of the column is not less than 3 000, calculated with the peak of adenosine reference.

D.2 Determination of adenosine

Inject accurately 10 µl of both adenosine reference solution and test solution into the chromatograph in accordance with Annex B, record the chromatograms and calculate the content. Typical reference HPLC chromatograms are shown in Figure D.1.



Key

X retention time (min)
1 adenosine
a adenosine reference solution
b sample solution of fermented *Cordyceps* powder

Figure D.1 — Typical HPLC chromatograms of adenosine