**Trigonella foenum-graecum Seed**

**Final Authorized Version 1.0**

*Trigonella foenum-graecum* Seed

**DEFINITION**
The article consists of the dried ripe seeds of *Trigonella foenum-graecum* L. (Family Fabaceae). It contains NLT 0.2% of 4-hydroxyisoleucine, calculated on the dried basis.

**SYNONYMS**
*Foenum-graecum officinale* Moench var. *tibetanum* Alef.
*Trigonella foenum-graecum* subsp. *gladiata* (M. Bieb.) P. Fourn.
*Trigonella tibetana* (Alef.) Vassilcz.

**POTENTIAL CONFOUNGING MATERIALS**
None known

**SELECTED COMMON NAMES**
Arabic: حلبة
Chinese: 胡卢巴
Danish: Almindelig bukkehorn
English: Fenugreek, Greek hay, Greek clover, Greek hay seed
French: Fenugrec
German: Bockshornklee
Indian: Methi (Punjabi), menthe and mente (Kannada), uluva (Malayalam), mendium and ventaiyam (Tamil), mentulu (Telego)
Japanese: フェヌグリーク (fenugriiku), コロハ (koroha)
Korean: 호로파 (horopa)
Pinyin: Hu lu ba
Portugese: Feno-greco (Brazil)
Russian: пажитник греческий
Spanish: Alholva, heno griego, fenogreco
Swedish: Bockhornsklöver

**CONSTITUENTS OF INTEREST**
Amino acids: 4-Hydroxyisoleucine, 4-hydroxyisoleucine lactone, arginine, histidine, and lysine
Steroidal saponins: Trigoneoside IIA, IIB; graecunins H, I, J, K, L, M, N; trigofoenosides A, D, F, G; protogracillin; protodioscin; and diosgenin
Alkaloids: Trigonelline, gentianine, and carpaine
Flavonoids: Apigenin, luteolin, orientin, quercetin, vitexin, and isovitexin
Carbohydrates: Mainly mucilage forming polysaccharides

IDENTIFICATION

• A. Botanical Characteristics

Macroscopic: Seeds are oblong, flattened, or irregularly rhomboidal, with rounded edges; 3–5 mm long, 2–3 mm wide, about 2 mm thick; hard; yellowish-brown outside, yellow inside; nearly in the center of one of the long narrow sides is a small depression exhibiting a white point, the hilum, lying adjacent to the micropyle and raphae; the depression extends diagonally, in the form of a furrow on the widest surfaces and divides the seed into two parts of different sizes; the larger holds the cotyledons and the smaller the radicle.

Microscopic

Transverse cut: Shows yellowish embryo consisting of two large cotyledons encircled by dark, translucent endosperm filled with mucilage, and a crescent-shape radicle in a small pocket.

Transverse section: Testa—the outermost layer is covered with cuticle and composed of thick-wall, cylindrical, lignified cells with conical projections, through which a white line, linea lucida, extends across; middle layer of testa composed of cells with thick walls and wide intercellular spaces near their tops; inner section of testa composed of a few layers of narrow, tangentially elongated, compact, thin-wall parenchyma cells. Endosperm—outer layer consists of rectangular to polygonal thick-wall cells, full of aleurone grains; multiple layers of cells of various shapes, large, full of mucilage. Cotyledons—a layer of epidermal cells with thick walls; 3–4 layers of cells containing oil globules; a few layers of spongy tissue.

• B. Thin-Layer Chromatography—Amino Acids Profile

Standard solution A: 0.5 mg/mL of USP 4-Hydroxyisoleucine RS in methanol
Standard solution B: 50 mg/mL of USP Trigonella foenum-graecum Seed Dry Extract RS in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.

Sample solution: About 1.0 g of Trigonella foenum-graecum Seed, finely powdered, in 5.0 mL of methanol, heat in a water bath at 60° for 5 min, and filter.

[Note—Save the Sample solution for use in Identification C and the Specific Tests—Presence of Trigonelline.]

Chromatographic system
(See Chromatography <621> [1], Thin-Layer Chromatography.)

Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
Application volume: 2 µL each of Standard solution A and Standard solution B, and 4 µL of Sample solution, as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Temperature: 25°
Developing solvent system: n-Butanol, acetic acid, and water (7:2:1)
Developing distance: 6 cm
Derivatization reagent: Ninhydrin reagent created by combining 0.3 g of ninhydrin, 95 mL of isopropanol, and 5 mL of glacial acetic acid.

Analysis

Samples: Standard solution A, Standard solution B, and Sample solution
Apply the Samples as bands to a suitable HPTLC plate and dry in air. Develop the chromatograms in a saturated chamber, remove the plate from the chamber, and dry. Treat with Derivatization reagent, heat for 2 min at 100°–105°, and examine under visible light and UV light at 366 nm.

System suitability: Under visible light, the chromatogram of Standard solution B exhibits, in the lower
half, five bands: the most intense band at an \( R_f \) corresponding to the 4-hydroxyisoleucine band of Standard solution A, one minor band just below the 4-hydroxyisoleucine band, and three minor bands above the 4-hydroxyisoleucine band.

Under UV light at 366 nm, the chromatogram of Standard solution B exhibits, in the lower half, four bands: the most intense band as a very dark band at an \( R_f \) corresponding to the 4-hydroxyisoleucine band of Standard solution A, one minor dark band just below the 4-hydroxyisoleucine band, and two dark bands above the 4-hydroxyisoleucine band. The chromatogram of Standard solution B exhibits, in the upper half, three bright yellow bands, the one with the lowest \( R_f \) appears diffuse.

**Acceptance criteria:** Under visible light, the chromatogram of the Sample solution exhibits, in the lower half, the most intense band at an \( R_f \) corresponding to the 4-hydroxyisoleucine band of Standard solution A, and four additional brown bands corresponding to similar bands of Standard solution B: a minor band at an \( R_f \) below that of the 4-hydroxyisoleucine, and three less intense bands above the 4-hydroxyisoleucine band.

Under UV light at 366 nm, the chromatogram of the Sample solution exhibits, in the lower half, the most intense band at an \( R_f \) corresponding to the 4-hydroxyisoleucine band of Standard solution A, and three additional bands corresponding to similar bands of Standard solution B: a minor dark band at an \( R_f \) below that of the 4-hydroxyisoleucine, and two less intense bands above the 4-hydroxyisoleucine band. The chromatogram of the Sample solution exhibits, in the upper half, three bright yellow bands corresponding to similar bands of Standard solution B.

**C. Thin-Layer Chromatography—Steroidal Saponins Profile**

**Standard solution:** 50 mg/mL of USP Trigonella foenum-graecum Seed Dry Extract RS [2] in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.

**Sample solution:** Use the Sample solution prepared in Identification B.

**Chromatographic system**

(See Chromatography <621> [3], Thin-Layer Chromatography.)

**Adsorbent:** Chromatographic silica gel mixture with an average particle size of 5 \( \mu \)m (HPTLC plates)

**Application volume:** 2 \( \mu \)L, as 8-mm bands

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

**Temperature:** 25°

**Developing solvent system:** Dichloromethane, methanol, and water (18:8:1)

**Developing distance:** 6 cm

**Derivatization reagent:** Anisaldehyde reagent created by combining 85 mL of ice-cooled methanol mixed with 10 mL of glacial acetic acid, 5 mL of sulfuric acid, and 0.5 mL of \( \rho \)-anisaldehyde.

**Analysis**

**Samples:** Standard solution and Sample solution

Apply the Samples as bands to a suitable HPTLC plate and dry in air. Develop the chromatograms in a saturated chamber, remove the plate from the chamber, and dry. Treat with Derivatization reagent, heat for 3 min at 100°, and examine under visible light and UV light at 366 nm.

**System suitability:** Under visible light, the chromatogram of the Standard solution exhibits, in the lower half, five intense bands in the following order of increasing \( R_f \): a brown band close to the origin, a brown band due to fructose, a brown band due to protogracillin, a brown band due to protodioscin, and a violet band. The chromatogram of the Standard solution exhibits, in the upper half, two violet bands and a third violet band close to the solvent front.

Under UV light at 366 nm, the chromatogram of the Standard solution exhibits, in the lower third, three blue fluorescent bands interspersed with two brown bands due to saponins; a broad blue fluorescent band at about the middle of the run; and a pink band in between.
Acceptance criteria: Under visible light, the chromatogram of the Sample solution exhibits, in the lower half, three intense brown bands and a violet band corresponding in $R_f$ to similar bands of the Standard solution, and three violet bands in the upper half corresponding in $R_f$ to similar bands of the Standard solution.

Under UV light at 366 nm, the chromatogram of the Sample solution exhibits, in the lower third, three blue fluorescent bands interspersed with two brown bands corresponding in $R_f$ to similar bands of the Standard solution; a pink band about in the middle, and a broad blue fluorescent band below it corresponding in $R_f$ to similar bands of the Standard solution.

ASSAY

• CONTENT OF 4-HYDROXYISOLEUCINE

Solution A: 0.1% Phosphoric acid in water (v/v)

Solution B: Acetonitrile

Mobile phase: See Table 1.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Solution A (%)</th>
<th>Solution B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>80</td>
<td>20</td>
</tr>
<tr>
<td>20</td>
<td>40</td>
<td>60</td>
</tr>
</tbody>
</table>

Reagent: Acetonitrile, water, and triethylamine (40:12:8)

Diluent: Methanol and water (1:1)

Standard solution: Transfer about 2.0 mg of USP 4-Hydroxyisoleucine RS, accurately weighed, to a 100-mL volumetric flask, dissolve in 10 mL of Reagent, add 500 µL of phenyl isothiocyanate, and shake for 5 min. Add 60 mL of methanol, complete to volume with water, and mix.

Sample stock solution: Transfer about 2.0 g of Trigonella foenum-graecum Seed, finely powdered and accurately weighed, to a centrifuge tube, add 8 mL of Diluent, heat in a water bath at 65° for 5 min, sonicate for 5 min, and centrifuge. Transfer the supernatant into a 25-mL volumetric flask. Repeat the extraction two additional times and combine the extracts into the 25-mL volumetric flask. Complete to volume with Diluent and mix.

Sample solution: Transfer 5.0 mL of Sample stock solution to a 50-mL volumetric flask, add 10 mL of Reagent and 500 µL of phenyl isothiocyanate, and shake for 5 min. Add 30 mL of methanol, complete to volume with water, and mix. Before injection, pass through a membrane filter of 0.45-µm or finer pore size, discarding the first few mL of the filtrate.

Chromatographic system

(See Chromatography <621> II, System Suitability.)

Mode: LC

Detector: UV 254 nm

Column: 4.6-mm × 15-cm; 5-µm packing L1 (Similar to Zorbax Eclipse XD-C18, Luna C18(2) and Cosmosil C18-MS-II)

Flow rate: 1.5 mL/min

Injection volume: 20 µL

System suitability

Sample: Standard solution
Suitability requirements

- **Tailing factor:** NMT 2.0 for the 4-hydroxyisoleucine peak
- **Relative standard deviation:** NMT 2.0% determined from the 4-hydroxyisoleucine peak in repeated injections

Analysis

**Samples:** Standard solution and Sample solution

Using the chromatogram of the Standard solution, identify the retention time of the peak corresponding to 4-hydroxyisoleucine in the Sample solution chromatogram.

Calculate the percentage of 4-hydroxyisoleucine in the portion of Trigonella foenum-graecum Seed taken:

\[
\text{Result} = \left( \frac{r_U}{r_S} \right) \times C_S \times \left( \frac{V}{W} \right) \times d \times 100
\]

- \(r_U\) = peak area of 4-hydroxyisoleucine from the Sample solution
- \(r_S\) = peak area of 4-hydroxyisoleucine from the Standard solution
- \(C_S\) = concentration of 4-hydroxyisoleucine in the Standard solution (mg/mL)
- \(V\) = volume of the Sample stock solution (mL)
- \(W\) = weight of Trigonella foenum-graecum Seed taken to prepare the Sample stock solution (mg)
- \(d\) = dilution factor to prepare the Sample solution from the Sample stock solution

**Acceptance criteria:** NLT 0.2% on the dried basis

CONTAMINANTS

- **Elemental Impurities—Procedures <233>** [4]

**Acceptance criteria**

- **Arsenic:** NMT 2.0 µg/g
- **Cadmium:** NMT 0.5 µg/g
- **Lead:** NMT 5.0 µg/g
- **Mercury:** NMT 0.2 µg/g

- **Articles of Botanical Origin** [5], General Method for Pesticide Residues Analysis <561>: Meets the requirements

- **Microbial Enumeration Tests <61>** [6]: The total aerobic bacterial count does not exceed \(10^5\) cfu/g, the total combined molds and yeasts count does not exceed \(10^3\) cfu/g, and the bile-tolerant Gram-negative bacteria does not exceed \(10^3\) cfu/g.

- **Tests for Specified Microorganisms <62>** [7]: Meets the requirements of the tests for the absence of *Salmonella* species and *Escherichia coli*

SPECIFIC TESTS

- **Presence of Trigonelline**

  **Standard solution A:** 1.5 mg/mL of USP Trigonelline RS [8] in methanol

  **Standard solution B:** 50 mg/mL of USP Trigonella foenum-graecum Seed Dry Extract RS [2] in methanol. Sonicate for 10 min, centrifuge, and use the supernatant.

  **Sample solution:** Use the Sample solution prepared in Identification B.

**Chromatographic system**

(See Chromatography <621> [3], Thin-Layer Chromatography.)
Adsorbent: Chromatographic silica gel mixture with an average particle size of 5 µm (HPTLC plates)
Application volume: 5 µL, as 8-mm bands
Relative humidity: Condition the plate to a relative humidity of about 33% using a suitable device.
Temperature: 25°
Developing solvent system: Isopropyl alcohol, methanol, and water (4:1:4)
Developing distance: 6 cm

Analysis
Samples: Standard solution A, Standard solution B, and Sample solution
Apply the Samples as bands to a suitable HPTLC plate and dry in air. Develop the chromatograms in a saturated chamber, remove the plate from the chamber, dry, and examine under UV light at 254 nm.

System suitability: Under UV light, the chromatogram of Standard solution B exhibits, in the lower half, a quenching band at an \( R_f \) similar to the trigonelline band of Standard solution A. A strong quenching band is near the solvent front of the chromatogram.

Acceptance criteria: Under UV light, the chromatogram of Standard solution B exhibits, in the lower half, a quenching band corresponding in \( R_f \) to the trigonelline band of Standard solution A.

Swelling Index
Sample: 1.0 g of Trigonella foenum-graecum Seed, finely powdered
Analysis: Place the Sample in a 25-mL graduated cylinder, moisten with 1.0 mL alcohol, add 25 mL water, close the cylinder, and shake vigorously every 10 min for 1 h. Allow to stand for 3 h.
Acceptance criteria: Volume in milliliters occupied by 1 g of seed is NLT 6.

Articles of Botanical Origin [5], Foreign Organic Matter [561]: NMT 2.0%
Articles of Botanical Origin [5], Alcohol-Soluble Extractives, Method 1 [561]: NLT 14.0%
Articles of Botanical Origin [5], Water-Soluble Extractives, Method 1 [561]: NLT 28.0%

Loss on Drying [731]
Sample: 1.0 g of Trigonella foenum-graecum Seed, finely powdered
Analysis: Dry the Sample at 105° for 2 h.
Acceptance criteria: NMT 5.0%

Articles of Botanical Origin [5], Total Ash [561]
Analysis: 2.0 g of Trigonella foenum-graecum Seed, finely powdered
Acceptance criteria: NMT 5.0%

Articles of Botanical Origin [5], Acid-Insoluble Ash [561]
Analysis: 6.0 g of Trigonella foenum-graecum Seed, finely powdered
Acceptance criteria: NMT 1.0%

Additional Requirements
Packaging and Storage: Preserve in well-closed containers, protected from light and moisture, and store at room temperature.
Labeling: The label states the Latin binomial and the part(s) of the plant contained in the article.
USP 4-Hydroxyisoleucine RS [1]
USP Trigonella foenum-graecum Seed Dry Extract RS [2]
USP Trigonelline RS [8]

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