

Status: Currently Official on 15-Feb-2025
 Official Date: Official as of 01-Aug-2015
 Document Type: Dietary Supplement Monographs
 DocId: GUID-CD91A454-BF61-4A0F-A10A-2961063AE728_2_en-US
 DOI: https://doi.org/10.31003/USPNF_M8409_02_01
 DOI Ref: rh76n

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***Bacillus subtilis* subsp. *subtilis* Menaquinone-7 Extract**

DEFINITION

Bacillus subtilis subsp. *subtilis* Menaquinone-7 Extract is the product obtained by supercritical carbon dioxide (CO₂) extraction of cultures of *Bacillus subtilis* subsp. *subtilis* strains that are capable of fermenting soybean protein isolate (soybean peptone) from soybeans to produce natto. The Extract is a brown oil, consisting mainly of fat (>97%), that contains NLT 1.5% and NMT 5.0% of menaquinone-7 (C₄₆H₆₄O₂), and NLT 0.014% and NMT 0.15% of menaquinone-6 (C₄₁H₅₆O₂). It contains no organic solvents.

IDENTIFICATION

• A. THIN-LAYER CHROMATOGRAPHY

Standard solution: Transfer 10 mg of [USP Menaquinone-7 RS](#) to a 10-mL volumetric flask, add 1 mL of tetrahydrofuran, and dissolve and dilute with the same solvent to volume.

Sample solution: Transfer 1.0 g of Extract to a 10-mL volumetric flask, and add 8 mL of tetrahydrofuran. Shake and allow the mixture to settle, centrifuge if necessary, and use the supernatant.

Chromatographic system

(See [Chromatography \(621\), Thin-Layer Chromatography.](#))

Mode: HPTLC

Adsorbent: Nano silica gel F₂₅₄ for HPTLC¹

Application volume: 5 µL

Developing solvent system: Hexane and ethyl acetate (95:5)

Spray reagent: Transfer 0.2 g of cerium sulfate and 4.2 g of ammonium molybdate to a 100-mL volumetric flask. Dissolve with 6.6 mL of 98% (w/w) sulfuric acid, and dilute with deionized water to volume. Stir to complete dissolution. Cool, and store the solution at 0°. Allow the spray reagent to achieve room temperature before use.

Analysis

Samples: *Standard solution* and *Sample solution*

Develop the chromatogram in the *Developing solvent system* until the solvent front has moved about three-fourths the length of the plate. Remove the plate from the chamber, and air-dry. Examine the plate under visible light and short-wavelength UV light, and record the spots. Spray the plate with the *Spray reagent*, and heat at 100°–110° for 5 min. Examine the plate under white light, and record the spots.

Acceptance criteria: Under visible light and short-wavelength UV light, the spots from the *Sample solution* correspond in color (light yellow), shape, and *R_F* value to those from the *Standard solution*. After applying the *Spray reagent*, under white light, the spots from the *Sample solution* correspond in color (dark blue), shape, and *R_F* value to those from the *Standard solution*. [NOTE—The *Sample solution* should also contain intense spots at lower *R_F* values due to the brown oil and its components.]

• B. The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the test for *Content of Menaquinone-7 and Menaquinone-6*.

COMPOSITION

• CONTENT OF MENAQUINONE-7 AND MENAQUINONE-6

[NOTE—Use low-actinic glassware.]

Mobile phase: Dehydrated alcohol and water (97:3)

[NOTE—Protect the following *Standard solution* and *Sample solution* from light, and inject immediately after preparation.]

Standard solution: Transfer 12.5 mg of [USP Menaquinone-7 RS](#) into a 25-mL volumetric flask, add 0.5 mL of tetrahydrofuran, and dilute with dehydrated alcohol to volume. Transfer 5.0 mL of this solution into a 25-mL volumetric flask, and dilute with dehydrated alcohol to volume.

Sample solution: Transfer 100 mg of Extract, accurately weighed, to a 25-mL volumetric flask, and add 2 mL of tetrahydrofuran. Dissolve and dilute with dehydrated alcohol to volume. Pass this solution through a filter of 0.45-µm pore size.

Chromatographic system

(See [Chromatography \(621\), System Suitability.](#))

Mode: LC

Detector: UV 268 nm

Column: 4.6-mm × 10-cm; 2.6-μm packing L1

Column temperature: 25°

Flow rate: 0.7 mL/min

Injection volume: 10 μL

Run time: At least 3 times the retention time of menaquinone-7

System suitability

Sample: *Standard solution*

[NOTE—The relative retention times for menaquinone-6 and menaquinone-7 are 0.81 and 1.00, respectively.]

Suitability requirements

Relative standard deviation: NMT 3.0% for six replicate injections

Analysis

Samples: *Standard solution* and *Sample solution*

Calculate the percentage of menaquinone-7 ($C_{46}H_{64}O_2$) in the portion of Extract taken:

$$\text{Result} = (r_{U7}/r_S) \times (C_S/C_U) \times 100$$

r_{U7} = peak response of menaquinone-7 from the *Sample solution*

r_S = peak response of menaquinone-7 from the *Standard solution*

C_S = concentration of [USP Menaquinone-7 RS](#) in the *Standard solution* (mg/mL)

C_U = concentration of Extract in the *Sample solution* (mg/mL)

Calculate the percentage of menaquinone-6 ($C_{41}H_{56}O_2$) in the portion of Extract taken:

$$\text{Result} = (r_{U6}/r_S) \times (C_S/C_U) \times 100$$

r_{U6} = peak response of menaquinone-6 from the *Sample solution*

r_S = peak response of menaquinone-7 from the *Standard solution*

C_S = concentration of [USP Menaquinone-7 RS](#) in the *Standard solution* (mg/mL)

C_U = concentration of Extract in the *Sample solution* (mg/mL)

Acceptance criteria

Menaquinone-7: 1.5%–5.0%

Menaquinone-6: 0.014%–0.15%

IMPURITIES

• [ELEMENTAL IMPURITIES—PROCEDURES \(233\).](#)

Acceptance criteria

Arsenic: NMT 2.0 μg/g

Cadmium: NMT 1.0 μg/g

Lead: NMT 3.0 μg/g

Mercury: NMT 0.1 μg/g

• ISOMERIC PURITY

Mobile phase: Methanol, tetrahydrofuran, dehydrated alcohol, and water (80:10:15:1)

Sample solution [NOTE—Protect the solution from light, and inject immediately after preparation.] Transfer 100 mg of Extract into a 25-mL volumetric flask, and add 2 mL of tetrahydrofuran. Dissolve and dilute with dehydrated alcohol to volume. Pass through a filter of 0.45-μm pore size.

Chromatographic system

(See [Chromatography \(621\), System Suitability.](#))

Mode: LC

Detector: UV 268 nm