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National food safety standard - Determination of vitamin K_2 in food

食品安全国家标准　食品中维生素 K_2 的测定

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National food safety standard - Determination of vitamin K_2 in food

1 Scope

This standard specifies the liquid chromatography method for the determination of menaquinone-4, menaquinone-7, and menaquinone-9 content in food.

This standard is applicable to the determination of the three vitamin K_2 types of menaquinone-4, menaquinone-7, and menaquinone-9 in milk and dairy products, special dietary foods, fermented soy products, and meat and meat products.

2 Principle

The sample is enzymatically hydrolyzed by lipase, amylase, or protease, and n-hexane is used to extract menaquinone-4, menaquinone-7, and menaquinone-9. The extract is concentrated, separated by reversed-phase liquid chromatography, carried out Post-column derivatization with a zinc reduction column, detected with a fluorescence detector, and quantitatively analyzed with the external standard method.

3 Reagents and materials

Unless otherwise stated, the reagents used in this method are of analytical grade and the water is first-grade water specified in GB/T 6682.

3.1 Reagents

3.1.1 Methanol (CH_3OH): chromatographically pure.

3.1.2 Absolute ethanol (C_2H_6O).

3.1.3 n-hexane (C_6H_{14}).

3.1.4 Potassium carbonate (K_2CO_3).

3.1.5 Dichloromethane (CH_2Cl_2): chromatographically pure.

3.1.6 Tetrahydrofuran (C_4H_8O): chromatographically pure.

3.1.7 Glacial acetic acid ($C_2H_4O_2$).

3.1.8 Zinc chloride ($ZnCl_2$).

3.1.9 Anhydrous sodium acetate ($C_2H_3O_2Na$).

3.1.10 Potassium dihydrogen phosphate (KH_2PO_4).

3.1.11 Amylase: (CAS number: 9000-92-4) enzyme activity is ≥4000 U/g.

3.1.12 Lipase: (CAS number: 9001-62-1) enzyme activity is ≥40 U/g.

3.1.13 Protease: (CAS number: 9001-73-4) enzyme activity is ≥6000 U/mg.

3.1.14 Potassium hydroxide (KOH).

3.2 Reagent preparation

3.2.1 400 g/L potassium hydroxide solution: Weigh 20.0 g of potassium hydroxide and place it into a 100 mL beaker, dissolve it in 20 mL of water, and cool it; transfer it to a 50 mL volumetric flask, add water to make the volume up to the mark, mix well, and store in a polyethylene flask.

3.2.2 Phosphate buffer solution (pH 8.0): Weigh 54.0 g of potassium dihydrogen phosphate and place it into a 500 mL beaker, dissolve it in 300 mL of water, and adjust the pH to 8.0±0.2 with 400 g/L potassium hydroxide solution (3.2.1); transfer to a 500 mL volumetric flask, add water to make the volume up to the mark, and mix well.

3.2.3 Mobile phase: Weigh 1.5 g of zinc chloride and 0.5 g of anhydrous sodium acetate and place them into a 1000 mL beaker, add 500 mL of methanol, 100 mL of dichloromethane (or tetrahydrofuran), and 0.3 mL of glacial acetic acid, and transfer to a 1000 mL volumetric flask. After ultrasonic dissolution, add methanol to make the volume up to the mark, mix well, and filter with a 0.22 μm filter membrane.

NOTE: Adding methylene chloride can shorten the retention time, and the maximum should not exceed 150 mL/L.

3.2.4 Dichloromethane-methanol solution (volume ratio of 10+90): Accurately draw 20 mL of dichloromethane into a 200 mL volumetric flask, dilute with methanol and adjust the volume up to the mark, and mix well.

3.3 Standard products

3.3.1 Menaquinone-4 (MK-4) standard substance ($C_{31}H_{40}O_2$, CAS number: 863-61-6): the purity of ≥98%, or reference material with a national authentication and a Reference Material Certificate.

3.3.2 Menaquinone-7 (MK-7) standard substance ($C_{46}H_{64}O_2$, CAS number: 2124-57-4): the purity of ≥98%, or reference material with a national authentication and a

Reference Material Certificate.

3.3.3 Menaquinone-9 (MK-9) standard substance ($C_{56}H_{80}O_2$, CAS number: 523-39-7): the purity of ≥98%, or reference material with a national authentication and a Reference Material Certificate.

3.4 Preparation of standard solution

3.4.1 Standard stock solution

3.4.1.1 MK-4 standard stock solution (1.0 mg/mL): Accurately weigh 25 mg of MK-4 standard substance (accurate to 0.1 mg), dissolve it with dichloromethane methanol solution (3.2.4), make the volume up to 25 mL, and mix well. Store the solution in a brown glass container at -20 ℃±2 ℃ away from light. The shelf life is 6 months.

3.4.1.2 MK-7 standard stock solution (1.0 mg/mL): Accurately weigh 25 mg of MK-7 standard substance (accurate to 0.1 mg), dissolve it with dichloromethane methanol solution (3.2.4), make the volume up to 25 mL, and mix well. Store the solution in a brown glass container at -20 ℃±2 ℃ away from light. The shelf life is 6 months.

3.4.1.3 MK-9 standard stock solution (0.5 mg/mL): Accurately weigh 25 mg of MK-9 standard substance (accurate to 0.1 mg), dissolve it with dichloromethane methanol solution (3.2.4), make the volume up to 50 mL, and mix well. Store the solution in a brown glass container at -20 ℃±2 ℃ away from light. The shelf life is 6 months.

3.4.2 Standard intermediate solution

3.4.2.1 MK-4 and MK-7 mixed standard intermediate solution (10 μg/mL): Accurately pipette 0.50 mL of MK-4 and MK-7 standard stock solutions respectively into a 50 mL volumetric flask, use dichloromethane methanol solution (3.2.4) to dilute and adjust the volume up to the mark, and mix well. Store the solution in a brown glass container at -20 °C±2 °C away from light. The shelf life is 6 months.

3.4.2.2 MK-9 standard intermediate solution (50 μg/mL): Accurately pipette 5.00 mL of MK-9 standard stock solution into a 50 mL volumetric flask, dilute it with dichloromethane methanol solution (3.2.4) and adjust the volume up to the mark, and mix well. Store the solution in a brown glass container at -20 °C±2 °C away from light. The shelf life is 6 months.

3.4.3 Standard working solution

Accurately pipette 0.025 mL, 0.050 mL, 0.10 mL, 0.30 mL, 0.50 mL, and 1.0 mL of MK-4 and MK-7 mixed standard intermediate solution into 10 mL volumetric flasks respectively, and accurately pipette 0.010 mL, 0.020 mL 0.040 mL, 0.060 mL, 0.10 mL, and 0.20 mL of MK-9 standard intermediate solutions into the above-mentioned 10 mL volumetric flasks, use mobile phase (3.2.3) to make the volume up to the mark, and mix

edible parts of meat and meat products shall be crushed and homogenized; other solid and semi-solid samples shall be crushed and homogenized. Liquid samples shall be mixed evenly before measurement.

NOTE: The processing process shall be kept away from light as much as possible. After preparing a uniform sample, avoid light and detect it as soon as possible.

5.2 Sample processing

WARNING: Avoid direct exposure to UV light during processing, and operate in a place as dark as possible.

5.2.1 Enzymatic hydrolysis

5.2.1.1 Milk, dairy products, and special dietary foods

Weigh 50 g (accurate to 0.1 g) (m_1) of the mixed powder sample into a 250 mL beaker, add 100 mL of water, record the mass (m_2) of the solution after adding water, mix thoroughly, and dissolve; accurately weigh 6 g (accurate to 0.001 g) (m_3) of the prepared solution and place it into a 50 mL centrifuge tube, add 11 mL of water, then add 5 mL of phosphate buffer (3.2.2), and mix well; ultrasonicate in a water bath at 50 ℃±2 ℃ for 15 min, cool to room temperature, add 0.5 g of lipase, then add 0.2 g of amylase, cover with a cap, vortex for 2 to 3 minutes, mix well, and place in a constant temperature water bath at 37 °C±2 °C for enzymatic hydrolysis for more than 2 hours (vortex for 1 minute every 30 minutes), allowing it to be fully enzymatically hydrolyzed.

For liquid samples, weigh 1 g~10 g (accurate to 0.001 g) (m_s) of the mixed sample, place it into a 50 mL centrifuge tube, and add water to 15 mL; add 5 mL of phosphate buffer (3.2.2) to the extract, mix well, and ultrasonicate in a water bath at 50 °C±2 °C for 15 minutes. After cooling to room temperature, add 0.5 g of lipase and 0.2 g of amylase, cover with a cap, and vortex for 2 to 3 minutes. After mixing, place the enzyme in a constant-temperature water bath at 37 °C±2 °C, and enzymatically hydrolyze for more than 2 hours (vortex for 1 minute every 30 minutes) to allow full enzymatic hydrolysis.

5.2.1.2 Fermented soy products

Weigh 50 g (accurate to 0.1 g) (m_1) of the homogenized sample and place it into a 250 mL beaker, add 100 mL of water, and record the mass (m_2) of the solution after adding water. After homogenization with the homogenizer, immediately and accurately weigh 3 g (accurate to 0.001 g) (m_3) of the homogenate sample and place it into a 50 mL centrifuge tube, add 13 mL of water, add 5 mL of phosphate buffer (3.2.2), and mix well; ultrasonicate in a 50 ℃±2 ℃ water bath for 15 min, and cool to room temperature. Then, add 0.2 g of lipase, then add 0.1 g of protease, cover with a cap, vortex for 2 to 3 minutes, mix well, and place in a constant-temperature water bath at 37 °C±2 °C for

enzymatic hydrolysis for more than 4 hours (vortex for 1 minute every 30 minutes), allowing it to be fully enzymatically hydrolyzed.

5.2.1.3 Meat and meat products

Weigh 50 g (accurate to 0.1 g) (m_1) of the homogenized sample and place it into a 250 mL beaker, add 100 mL of water, and record the mass (m_2) of the solution after adding water. After homogenization with the homogenizer, immediately and accurately weigh 15 g (accurate to 0.001 g) (m_3) of the homogenate sample and place it into a 50 mL centrifuge tube, add 5 mL of water, then add 5 mL of phosphate buffer (3.2.2), and mix well; ultrasonicate in a 50 ℃±2 ℃ water bath for 15 min, and cool to room temperature. Then, add 0.2 g of lipase, then add 0.1 g of protease, cover with a cap, vortex for 2 to 3 minutes, mix well, and place in a constant-temperature water bath at 37 ℃±2 ℃ for enzymatic hydrolysis for more than 4 hours (vortex for 1 min every 30 min), allowing it to be fully enzymatically hydrolyzed.

5.2.2 Extraction

Take out the enzymatically hydrolyzed sample, add 10 mL of absolute ethanol and 1.0 g of potassium carbonate respectively, and mix well; then add 10 mL of n-hexane, vortex at 3000 r/min for 2 min~3 min or oscillate to extract at ≥250 r/min for 10 min, and centrifuge at 6000 r/min for 5 min, and transfer the supernatant to a 100 mL rotary evaporation bottle.

NOTE: To ensure complete extraction, if necessary, add 10 mL of n-hexane to the lower layer solution, repeat the operation once, and combine the supernatant into the above-mentioned rotary evaporation bottle.

5.2.3 Concentration

Rotate and evaporate the above n-hexane extract in a water bath at 40 ℃±2 ℃ until it is dry (if there is any residual liquid, lightly blow it with nitrogen until it is dry), use 5.0 mL of mobile phase (3.2.3) to fully dissolve the concentrate, and filter this solution with a 0.22 μm filter membrane; this filtrate is the prepared sample solution.

NOTE: After concentration and nitrogen blowing, if the sample concentrate is not easily dissolved at room temperature, the dissolution temperature can be increased to 40 ℃±2 ℃ until it is completely dissolved. For samples with high MK-7 content such as natto, the metered volume (5.2.3) can be appropriately increased to ensure that the concentration of the prepared sample solution is within the range of the standard curve.

5.3 Instrument reference conditions

5.3.1 Chromatographic column: C_{18} (5 μm, 4.6 mm×150 mm) or chromatographic column with equivalent performance.

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