



**NATIONAL STANDARD OF THE
PEOPLE'S REPUBLIC OF CHINA**

GB 5009.290-2023

**National food safety standard - Determination of Vitamin K2 in
foods**

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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 K2 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.

3.2 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 $\pm$ 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 μ m 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.

3.3.1 Menaquinone-4 (MK-4) standard substance (C₃₁H₄₀O₂, CAS number: 863-61-6):

the purity of $\geq 98\%$, or reference material with a national authentication and a Reference Material Certificate.

3.3.2 Menaquinone-7 (MK-7) standard substance (C₄₆H₆₄O₂, CAS number: 2124-57-

4): the purity of $\geq 98\%$, or reference material with a national authentication and a Reference Material Certificate.

3.3.3 Menaquinone-9 (MK-9) standard substance (C₅₆H₈₀O₂, CAS number: 523-39-7):

the purity of $\geq 98\%$, or reference material with a national authentication and a Reference Material Certificate.

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 degrees C +/- 2 degrees C 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 degrees C +/- 2 degrees C 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 degrees C +/- 2 degrees C away from light. The shelf life is 6 months.

3.4.2.1 MK-4 and MK-7 mixed standard intermediate solution (10 ug/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 ug/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) (m1) of the mixed powder sample into a 250 mL beaker, add 100 mL of water, record the mass (m2) of the solution after adding water, mix thoroughly, and dissolve; accurately weigh 6 g (accurate to 0.001 g) (m3) 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 degrees C $\pm$ 2 degrees C 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 $\pm$ 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) (ms) 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 $\pm$ 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 $\pm$ 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) (m1) of the homogenized sample and place it into a 250 mL beaker, add 100 mL of water, and record the mass (m2) of the solution after adding water. After homogenization with the homogenizer, immediately and accurately weigh

3 g (accurate to 0.001 g) (m3) 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 degrees C $\pm$ 2 degrees C 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 $\pm$ 2 °C for enzymatic hydrolysis for more than 4 hours (vortex for 1 minute every 30 minutes), allowing