



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

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National food safety standard - Determination of bixinin foods

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1 Scope

This Standard specifies the method of liquid chromatography for the determination of bixin (bixin and norbixin) in foods.

This Standard is applicable to the determination of bixin and norbixin in cheese, processed cheese and similar products, margarine and similar products, non-dairy creamers, frozen drinks, jams, chocolate and chocolate products, candies, grains and grain products, baked goods, western-style ham, meat sausages, compound seasonings, beverages, jelly and puffed food.

2 Principle

Use ammonia ethanol solution to extract the specimen. After degreasing with petroleum ether and acidification with acetic acid, use dichloromethane to extract it. Then, adopt liquid chromatography to separate it; use diode array detector or ultraviolet detector to detect it. Use the external standard method to quantify it.

3 Reagents and Materials

Unless it is otherwise specified, the reagents used in this Method are analytically pure; the water is Grade-1 water specified in GB/T 6682.

3.1.5 Dichloromethane (CH₂Cl₂). chromatographically pure.

3.1.6 2,6-di-tert-butyl-4-methylphenol (C₁₅H₂₄O, BHT for short).

3.2.1 BHT-methanol solution. weigh-take 0.5 g of BHT (3.1.6); use 500 mL of methanol to

dissolve it.

3.2.2 BHT-ethanol solution. weigh-take 0.5 g of BHT (3.1.6); use 500 mL of anhydrous ethanol

to dissolve it.

3.2.3 70% aqueous acetonitrile. measure-take 350 mL of acetonitrile and 150 mL of water, mix them up; add 0.5 g of BHT (3.1.6); shake to dissolve it.

3.2.4 5% ammonia ethanol solution. measure-take 50 mL of ammonia water and 950 mL of anhydrous ethanol, mix them up; add 1 g of BHT (3.1.6); shake to dissolve it.

3.2.5 BHT-petroleum ether solution. weigh-take 0.5 g of BHT (3.1.6); use 500 mL of petroleum

ether to dissolve it.

3.2.6 BHT-dichloromethane solution. weigh-take 0.5 g of BHT (3.1.6); use 500 mL of

dichloromethane to dissolve it.

3.2.7 2% acetic acid aqueous solution. measure-take 20 mL of glacial acetic acid; add it to 980 mL of water; mix it up.

3.3.1 Norbixin (C₂₄H₂₈O₄, CAS. 542-40-5). purity ? 98.0%, or reference substances certified

by the state and awarded with a reference substance certificate.

3.3.2 Bixin (C₂₅H₃₀O₄, CAS. 6983-79-5). purity ? 98.0%, or reference substances certified by

the state and awarded with a reference substance certificate.

3.4.1 Standard stock solution (100 mg/L). accurately weigh-take 10.0 mg of norbixin reference

substance (3.3.1) with the converted content into a 100 mL brown volumetric flask; add BHT-methanol solution (3.2.1) to dissolve and reach a constant volume, then, mix it up. Accurately weigh-take 10.0 mg of bixin reference substance (3.3.2) with the converted content into a 100 mL brown volumetric flask; add BHT-ethanol solution (3.2.2) to dissolve

and reach a constant volume, then, mix it up. Store the standard stock solution at -18°C in the dark. The shelf life is 6 months.

3.4.2 Mixed standard working solution. respectively absorb-take 0.05 mL, 0.1 mL, 0.2 mL, 0.5

mL, 1.0 mL, 2.0 mL and 5.0 mL of the norbixin and bixin standard stock solution (3.4.1) into a

10 mL brown volumetric flask. Add 70% aqueous acetonitrile (3.2.3) and reach a constant volume; mix it up. The concentration of the norbixin and bixin mixed standard series working solutions is respectively. 0.5 mg/L, 1.0 mg/L, 2.0 mg/L, 5.0 mg/L, 10 mg/L, 20 mg/L and 50 mg/L. Prepare them right before use.

Weigh-take 0.5 g ~ 5 g (accurate to 0.01 g) of specimen into a 50 mL plastic centrifuge tube with a stopper. Add 2 g ~ 5 g of diatomaceous earth; use a glass rod to thoroughly mix the sample and diatomaceous earth; stir, until it becomes loose and granular. Add 20 mL of 5% ammonia ethanol solution (3.2.4). The following steps are the same as 5.1.2.1.

5.1.2.3 Liquid samples Weigh-take 0.5 g ~ 5 g (accurate to 0.01 g) of specimen into a 50 mL plastic centrifuge tube with a stopper. Add 5 mL of 5% ammonia ethanol solution (3.2.4). Reserve it for purification.

5.1.3 Sample purification Add 2 mL of ammonia water to the specimen extract; use water to dilute to about 20 mL. Add

20 mL of BHT-petroleum ether solution (3.2.5); perform vortex oscillation for 1 min; centrifuge it and discard the upper layer of solution. Repeatedly add 20 mL of BHT-petroleum ether solution (3.2.5); perform vortex oscillation for 1 min; centrifuge it and discard the upper layer of solution. Add 2 mL of glacial acetic acid and 25 mL of BHT-dichloromethane solution (3.2.6)

to the lower layer of solution; perform vortex oscillation for 1 min; centrifuge it and discard the upper layer of solution. Add 1 mL of BHT-ethanol solution (3.2.2) to the lower layer of dichloromethane solution. At 35°C , adopt the nitrogen concentration device to concentrate to the volume of about 1 mL. Use BHT-ethanol solution (3.2.2) to assist the transferring of the residual solution to a 5 mL brown volumetric flask; reach a constant volume and shake it well.

Use 0.45 μm filter membrane (3.5.2) to filter it into an injection vial. Reserve it for liquid chromatographic determination.

NOTE. avoid light during the pre-processing.

5.2 Instrument Reference Conditions

5.2.1 Chromatographic column. C18 chromatographic column, column length. 150 mm,

inner diameter. 4.6 mm, particle size. 5 μ m, or equivalent chromatographic column.

5.2.2 Mobile phase. acetonitrile + 2% aqueous acetic acid = 70 + 30.

5.2.3 Flow rate. 1.0 mL/min.

5.2.4 Column temperature. 30 $^{\circ}$ C.

5.2.5 Injection volume. 10 μ L.

5.2.6 Detection wavelength. 458 nm.

5.3 Drawing of Standard Curve Respectively inject the mixed standard working solution prepared in 3.4.2 into the liquid chromatograph (see Figure A.1 in Appendix A for the liquid chromatogram of the cis-norbixin and cis-bixin standard solutions). Use the retention time for qualitative determination to determine the corresponding peak area; take the mass concentration of the mixed standard