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NATIONAL STANDARD OF THE
PEOPLE'S REPUBLIC OF CHINA

GB 5009.282-2020

**National food safety standard - Determination of 1-methylimidazole, 2-methylimidazole and 4-methylimidazole
in food**

食品安全国家标准

食品中 1-甲基咪唑、2-甲基咪唑及 4-甲基咪唑的测定

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State Administration for Market Regulation.**

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National food safety standard - Determination of 1-methylimidazole, 2-methylimidazole and 4-methylimidazole in food

1 Scope

This standard specifies the method for the determination of 1-methylimidazole, 2-methylimidazole, and 4-methylimidazole in food by liquid chromatography-tandem mass spectrometry.

This standard applies to the determination of the content of 1-methylimidazole, 2-methylimidazole, and 4-methylimidazole in condensed milk, frozen drinks, jams, soy products, cocoa and chocolate products, candy, wheat flour products, biscuits, fillings and surface slurries, meat products, flavored syrups, condiments (soy sauce, vinegar, sauce and sauce products, cooking wine, compound seasoning), beverages, wine, jelly, and puffed food.

2 Principles

After adding the isotope internal standard to the sample, carry out the extraction with water or acid water, perform the purification and concentration by a solid-phase extraction column, determine by a liquid chromatography-tandem mass spectrometer, and use the isotope internal standard method for quantification.

3 Reagents and materials

Unless otherwise specified, the reagents used in this method are all analytical reagents, and the water is the grade I water specified in GB/T 6682.

3.1 Reagents

3.1.1 Acetonitrile (CH_3CN): chromatographically pure.

3.1.2 Methanol (CH_3OH): chromatographically pure.

3.1.3 Formic acid (HCOOH): chromatographically pure.

3.1.4 Ammonia water (NH_4OH): The concentration shall be 25%~28%.

3.1.5 N-hexane ($\text{CH}_3(\text{CH}_2)_4\text{CH}_3$): chromatographically pure.

3.1.6 Ammonium acetate ($\text{CH}_3\text{COONH}_4$): chromatographically pure.

3.1.7 Sodium chloride (NaCl).

3.2 Reagent preparation

3.2.1 2% formic acid aqueous solution: Measure out 20 mL of formic acid, add it to 980 mL of water, and mix well.

3.2.2 Ammonia methanol solution: Measure out 50 mL of ammonia water, add it to 950 mL of methanol, and mix well.

3.2.3 Ammonium acetate solution (5 mmol/L): Weigh 0.39 g of ammonium acetate, dissolve it in 1000 mL of water, and mix well.

3.2.4 Acetonitrile-ammonium acetate solution (9+1): Mix acetonitrile and ammonium acetate solution (5 mmol/L) uniformly in a volume ratio of 9:1.

3.3 Standards

3.3.1 1-methylimidazole standard ($\text{C}_4\text{H}_6\text{N}_2$, CAS number: 616-47-7): purity $\geq 98\%$, or a standard substance certified by the country and granted a standard substance certificate.

3.3.2 2-methylimidazole standard ($\text{C}_4\text{H}_6\text{N}_2$, CAS number: 693-98-1): purity $\geq 98\%$, or a standard substance certified by the country and granted a standard substance certificate.

3.3.3 4-methylimidazole standard ($\text{C}_4\text{H}_6\text{N}_2$, CAS number: 822-36-6): purity $\geq 98\%$, or a standard substance certified by the country and granted a standard substance certificate.

3.3.4 Isotope internal standard 1-methylimidazole- D_6 ($\text{C}_4\text{H}_6\text{N}_2$, CAS number: 285978-27-0): purity $\geq 98\%$.

3.3.5 Isotopic internal standard 2-methylimidazole- D_6 ($\text{C}_4\text{H}_6\text{N}_2$, CAS number: 1173022-19-9): purity $\geq 98\%$.

3.3.6 Isotope internal standard 4-methylimidazole- D_6 ($\text{C}_4\text{H}_6\text{N}_2$, CAS number: 1219804-79-1): purity $\geq 98\%$.

3.4 Standard solution preparation

3.4.1 Standard stock solution (1 mg/mL): Accurately weigh 25 mg of 1-methylimidazole standard (the weight shall be accurate to 0.01 mg), add 1 mL of water

to dissolve it, and transfer it with acetonitrile to a 25 mL brown volumetric flask; make up to the mark and shake well. Accurately weigh 25 mg of 2-methylimidazole standard and 4-methylimidazole standard respectively (the weight shall be accurate to 0.01 mg), dissolve them with acetonitrile, and transfer to 25 mL brown volumetric flasks; respectively dilute to the mark and shake well. Keep them sealed at -20 °C and away from light, then they are valid for 6 months.

3.4.2 Mixed standard working solution (2.0 µg/mL): Pipette 100 µL of each of the above standard stock solutions into the same 50 mL brown volumetric flask, add acetonitrile to dilute to the mark, and shake well; then, the mixed standard solution of 2.0 µg/mL is obtained. Keep this solution sealed at 4 °C and away from light, then it is valid for 3 months.

3.4.3 Isotope internal standard stock solution (1 mg/mL): Accurately weigh 10 mg of 1-methylimidazole-D₆, 2-methylimidazole-D₆, and 4-methylimidazole-D₆ respectively (the weight shall be accurate to 1 mg), dissolve them with acetonitrile, and transfer to 10 mL brown volumetric flasks; dilute to the mark and shake well. Keep them sealed at -20 °C and away from light, then they are valid for 6 months.

3.4.4 Isotope internal standard mixed working solution (1.0 µg/mL): Pipette 100 µL of each of the above isotope internal standard stock solutions into the same 100 mL brown volumetric flask, dilute to the mark with acetonitrile, and shake well; then, the isotopic internal standard mixed working solution of 1.0 µg/mL is obtained. Keep this solution sealed at 4 °C and away from light, then it is valid for 3 months.

3.4.5 Standard series solutions: Pipette 25 µL, 75 µL, 150 µL, 300 µL, 600 µL, and 1000 µL of mixed standard working solutions into 5 mL brown volumetric flasks, respectively, add 250 µL of isotope internal standard mixed working solution, and use acetonitrile- ammonium acetate solution (9+1) to dilute the mixed solution to the scale; then, the mixed standard series solution of 1-methylimidazole, 2-methylimidazole and 4-methylimidazole are prepared, and the mass concentration is 10 ng/mL, 30 ng/mL, 60 ng/mL, 120 ng/mL, 240 ng/mL, 400 ng/mL respectively. In the actual sample determination, the added amount and dilution multiple of the isotope internal standard mixed working solution can be adjusted according to the sample concentration.

3.5 Materials

3.5.1 Mixed cation exchange solid phase extraction column: N-vinylpyrrolidone-divinylbenzene copolymer matrix-sulfonic acid group, 150 mg, 6 mL, or equivalent. Before use, activate it with 5 mL methanol first, and then rinse it with 5 mL water to balance.

3.5.2 Homogeneous particle: It shall be suitable for 50 mL centrifuge tubes.

3.5.3 Organic filter membrane: The pore size shall be 0.22 µm.

volume to 20 mL, and shake well; put it in a centrifuge tube, and centrifuge at 10,000 r/min for 5 min; the supernatant is to be purified later.

5.1.2.2 Semi-fluid samples (such as jam, oyster sauce, flavored syrup, frozen drinks) and gum-based candy, tapioca balls

Weigh 2 g of uniformly mixed sample (the weight shall be accurate to 0.001 g), and put it in a 50 mL centrifuge tube; add 100 μ L of 1.0 μ g/mL isotope internal standard mixed working solution, and mix well; add 20 mL of water accurately, vortex for 1 min, and heat it in a water bath at 70 °C for 10 min. After it cools to room temperature, perform the ultrasonic extraction for 20 min, and centrifuge at the speed of 10,000 r/min and the temperature of 4 °C for 5 min; the supernatant is to be purified later.

5.1.2.3 Jelly

Weigh 2 g of uniformly mixed sample (the weight shall be accurate to 0.001g), and put it in a 50 mL centrifuge tube; add 100 μ L of 1.0 μ g/mL isotope internal standard mixed working solution, and mix well; add 2 g of sodium chloride, accurately add 20 mL of water, vortex for 1 min, and heat it in a 70 °C water bath for 10 min. After it cools to room temperature, perform the ultrasonic extraction for 20 min, and centrifuge at the speed of 10,000 r/min and the temperature of 4 °C for 5 min; the supernatant is to be purified later.

5.1.2.4 Condensed milk, cocoa and chocolate products

Weigh 2 g of uniformly mixed sample (the weight shall be accurate to 0.001 g), and put it in a 50 mL centrifuge tube; add 100 μ L of 1.0 μ g/mL isotope internal standard mixed working solution, and mix well; accurately add 20 mL of 2% formic acid aqueous solution, and heat it in a 60 °C water bath for 5 min; add 2 homogeneous particles, vortex for 5 min, and centrifuge at 10,000 r/min for 5 min; the supernatant is to be purified later.

5.1.2.5 Other samples such as bean products, sauces and sauce products

Weigh 2 g of uniformly mixed sample (the weight shall be accurate to 0.001 g), and put it in a 50 mL centrifuge tube; add 100 μ L of 1.0 μ g/mL isotope internal standard mixed working solution, and mix well; add 20 mL of water accurately, homogenize for 1 min, and centrifuge at the speed of 10,000 r/min and the temperature of 4 °C for 5 min; the supernatant is to be purified later.

5.1.3 Sample purification

5.1.3.1 Soy sauce, vinegar, beverages, wine, condensed milk, cocoa and chocolate products

Pipette 10 mL of the supernatant, pass it through a solid-phase extraction column, rinse

with 5 mL of 2% formic acid aqueous solution and 5 mL of methanol in turn, and elute it with 10 mL of ammonia methanol solution; collect all the eluent, and blow with nitrogen below 45 °C to near dryness. Accurately add 1 mL of acetonitrile-ammonium acetate solution (9+1), vortex for 30 s, filter it through a 0.22 µm organic filter membrane, and use the filtrate for the determination with a liquid chromatograph mass spectrometer.

5.1.3.2 Oyster sauce

Pipette 10 mL of the supernatant, add 200 µL of formic acid to mix and acidify, and filter it if necessary; pass it through a solid phase extraction column, rinse with 5 mL of 2% formic acid aqueous solution and 5 mL of acetonitrile in turn, and drain; rinse twice with 1.5 mL of n-hexane and drain again; then, elute it with 10 mL of ammonia methanol solution, collect all the eluent, and blow with nitrogen below 45 °C to near dryness; add 1 mL of acetonitrile-ammonium acetate solution (9+1) accurately, vortex for 30 s, filter it through a 0.22 µm organic system membrane, and use the filtrate for the determination with a liquid chromatograph mass spectrometer.

5.1.3.3 Other substrates except those mentioned in 5.1.3.1 and 5.1.3.2

Pipette 10 mL of the supernatant, add 200 µL of formic acid to mix and acidify, and filter it if necessary; pass it through a solid phase extraction column, rinse with 5 mL of 2% formic acid aqueous solution and 5 mL of methanol in turn, and elute with 10 mL of ammonia methanol solution; collect all the eluent, and blow with nitrogen below 45 °C to near dryness; add 1 mL of acetonitrile- ammonium acetate solution (9+1) accurately, vortex for 30 s, filter it through a 0.22 µm organic filter membrane, and use the filtrate for the determination with a liquid chromatograph mass spectrometer.

5.2 Instrument reference conditions

5.2.1 Liquid chromatography reference conditions

The liquid chromatography reference conditions are listed below:

- a) Liquid chromatography column: HILIC column (2.1 mm×100 mm, 3.5 µm), or a column with equivalent performance.
- b) Chromatography column temperature: 35 °C.
- c) Mobile phase: (A) acetonitrile; (B) 5 mmol/L ammonium acetate solution; see Table 1 for elution gradient reference conditions.
- d) Flow rate: 0.6 mL/min.
- e) Injection volume: 2 µL.

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