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Method of Test for Ovalbumin from Chicken Eggs in Foods

1. Scope

This method is applicable to identification of ovalbumin in foods.

2. Method

After extraction and purification, ovalbumin is identified by liquid chromatography/tandem mass spectrometry (LC-MS/MS).

2.1. Equipment

2.1.1. Liquid chromatograph/tandem mass spectrometer

2.1.1.1. Ion source: electrospray ionization, ESI.

2.1.1.2. Column: ACQUITY UPLC CSH130 C18, 1.7 μm, 2.1 mm i.d.× 15 cm, or an equivalent product.

2.1.2. Centrifuge: centrifugal force > 12000 ×g, temperature control < 4°C.

2.1.3. Homogenizer.

2.1.4. Vortex mixer.

2.1.5. Horizontal shaking bath: automatic temperature control to within ± 2°C.

2.1.6. Shaker: Speed > 250 rpm.

2.1.7. Ultrasonicator.

2.1.8. Centrifugal vacuum concentrators.

2.1.9. Solid phase vacuum extraction manifolds.

2.2. Chemicals

Formic acid, HPLC grade;
Acetonitrile, HPLC grade;
Acetone, reagent grade;
Urea, reagent grade;
Ammonium bicarbonate, reagent grade;
Sodium hydroxide, reagent grade;
Dithiothreitol, reagent grade;
Iodoacetamide, reagent grade;
Trifluoroacetic acid, reagent grade;
Deionized water, resistivity ≥ 18 MΩ•cm (at 25°C);
Bradford reagent protein quantitative kit (note);
Trypsin (13000-20000 BAEE units/mg);
Ovalbumin, reference standard.

Note: The use of commercially protein quantitative kits can be self-assessed according to demand.

2.3. Apparatus and materials

2.3.1. Centrifuge tube: 15 mL and 50 mL, PP.

2.3.2. Microcentrifuge tube: 1.5 mL and 2 mL, PP.

2.3.3. Solid phase extraction cartridge: Oasis HLB cartridge, 6 mL, 500 mg, or an equivalent product.

2.3.4. Membrane filter: 0.22 μm, PTFE.

2.4. Reagents

2.4.1. 1 M sodium hydroxide

Dissolve and dilute 2 g of sodium hydroxide with deionized water to 50 mL.

2.4.2. 50 mM ammonium bicarbonate (pH 8.0)

Dissolve 3.95 g of ammonium bicarbonate with deionized water 900 mL, adjust pH with 1 M sodium hydroxide to 8.0, and dilute with deionized water to 1000 mL.

2.4.3. 8 M urea in 50 mM ammonium bicarbonate

Dissolve and dilute 240.24 g of urea with 50 mM ammonium bicarbonate (pH 8.0) to 500 mL.

2.4.4. 1 M dithiothreitol

Dissolve and dilute 154.3 mg of dithiothreitol with deionized water to 1 mL.

2.4.5. 500 mM iodoacetamide

Dissolve and dilute 92.5 mg of iodoacetamide with deionized water to 1 mL.

2.4.6. 1 mg/mL trypsin

Dissolve and dilute 10 mg of trypsin with deionized water to 10 mL.

2.4.7. 0.5% trifluoroacetic acid

Dilute 5 mL of trifluoroacetic acid with deionized water to 1000 mL.

2.4.8. 0.1% formic acid in acetonitrile

Dilute 1 mL of formic acid with acetonitrile to 1000 mL.

2.4.9. 75% acetonitrile

Dilute 750 mL of acetonitrile with deionized water to 1000 mL.

2.4.10. 0.1% formic acid in 5% acetonitrile

Mix 0.1 mL of formic acid, 5 mL of acetonitrile and 94.9 mL of deionized water.

2.5. Mobile phase

2.5.1. Solvent A

Dilute 1 mL of formic acid with deionized water to 1000 mL. Filter with membrane filter, and take the filtrate as the mobile phase A.

2.5.2. Solvent B

Dilute 1 mL of formic acid with acetonitrile to 1000 mL. Filter with membrane filter, and take the filtrate as the mobile phase B.

2.6. Standard solution preparation

Accurately weigh equivalent 5 mg of ovalbumin standard, dissolve and dilute with 50 mM ammonium bicarbonate (pH 8.0) to 5 mL, as standard solution, and then store under freezing until use.

2.7. Sample solution preparation

2.7.1. Protein precipitation

Transfer about 0.5 g of the homogenized sample accurately weighed into a 50 mL centrifuge tube, and add 3 mL of 50 mM ammonium bicarbonate (pH 8.0), mix by a vortex mixer, shake in the horizontal shaking bath at 100 rpm for 30 minutes at 37°C, and then shake for 30 minutes by the shaker. Centrifuge at 5500 ×g for 10 minutes, and collect the supernatant. Add 4 times the volume of pre-cooled acetone for 3 hours at 4°C, centrifuge at 5500 ×g for 10 minutes at 4°C, and discard the supernatant. Wash the wall of the centrifuge tube with 1 mL of acetone, centrifuge at 5500 ×g for 10 minutes at 4°C, discard the supernatant, and place the sediment in the fume hood to air dry for 15 minutes.

2.7.2. Protein quantification

Take the air-dried sediment from section 2.7.1, add 1 mL of 8 M urea in 50 mM ammonium bicarbonate, mix by a vortex mixer, and vibrate by ultrasonicator to fully dissolve, as the sample stock solution. Transfer 10 μL of the sample stock solution and dilute 10 times with 50 mM ammonium bicarbonate (pH 8.0), and quantified with Bradford reagent protein quantification kit.

2.7.3. Enzyme hydrolysis

Take the remaining sample stock solution from section 2.7.2 and mix with 10 μL of 1 M dithiothreitol, vortex-mix, and shake sample in a water bath for 60 minutes at 60°C. Add 60 μL of 500 mM iodoacetamide into sample and incubate for 30 minutes in the dark. Add 9 mL of 50 mM ammonium bicarbonate (pH 8.0), and then add an appropriate amount of 1 mg/mL

trypsin(note), mix by a vortex mixer, and shake at 100 rpm in a water bath for 16 to 18 hours at 37°C. Add 50 μL of formic acid, mix by a vortex mixer, react in a water bath for 30 minutes at 37°C, centrifuge at 5500 ×g for 10 minutes, and collect the supernatant for purification.

Note: The ratio of the amount of trypsin added to the protein content of the sample stock solution is 1:50 (w/w).

2.7.4. Purification

Transfer the supernatant for purification from section 2.7.3 into a solid phase extraction cartridge pre-rinsed with 5 mL of 0.1% formic acid in acetonitrile and 5 mL of 0.5% trifluoroacetic acid and discard the eluent. Wash the cartridge with 5 mL of 0.5% trifluoroacetic acid and 5 mL of deionized water and discard the eluent. Add 5 mL of 75% acetonitrile, and collect the eluent into a 15 mL centrifuge tube. Evaporate the eluent to dryness by centrifugal vacuum concentrators. Dissolve the residue with 500 μL of 0.1% formic acid in 5% acetonitrile, and centrifuge at 12000 ×g for 10 minutes. Take the supernatant and filter with a membrane filter. Take the filtrate as the sample solution.

2.8. Reference solution preparation

Transfer about 0.5 g of the homogenized blank sample accurately weighed into a 50 mL centrifuge tube, add 1 μL of the standard solution, and then follow the procedure described in section 2.7 to obtain the reference solution.

2.9. Identification

Accurately inject 10 μL of the sample solution and the reference solution into LC-MS/MS separately and operate according to the following conditions. Identify ovalbumin based on the retention time and the MRM of relative ion intensities (note 1).

LC-MS/MS operating conditions (note 2):

Column: ACQUITY UPLC CSH130 C18, 1.7 μm, 2.1 mm i.d. × 15 cm.

Column temperature: 40°C.

Mobile phase: a gradient program of solvent A and solvent B is as follows:

Time (min)	A (%)	B (%)
0.0 → 0.5	95 → 90	5 → 10
0.5 → 10.0	90 → 69	10 → 31
10.0 → 10.5	69 → 0	31 → 100

10.5 → 14.0	0 → 0	100 → 100
14.0 → 14.5	0 → 95	100 → 5
14.5 → 21.0	95 → 95	5 → 5

Flow rate: 0.3 mL/min.

Injection volume: 10 µL.

Ionization mode: ESI^+.

Nebulizer: 30 psi.

Capillary voltage: 4 kV.

Desolvation flow rate: 15 L/min.

Desolvation gas temperature: 250°C.

Sheath gas flow rate: 11 L/min.

Sheath gas temperature: 300°C.

Detection mode: multiple reaction monitoring (MRM). Detection ion pair, fragmentor voltage and collision energy are shown as follows.

Ovalbumin specific peptides (note 3)	Ion pair: Precursor ion (*m/z*) > product ion (*m/z*)	Fragmentor voltage (V)	Collision energy (eV)
GGLEPINFQTAADQAR	844.4 > 1331.7	166	31
	844.4 > 1121.5	166	31
ELINSWVESQTNGIIR	930.0 > 1116.6	166	29
	930.0 > 1017.5	166	37
LTEWTSSNVMEER	791.4 > 1052.5	166	25
	791.4 > 951.4	166	25

Note 1: Relative ion intensities are calculated by the ratio of peak areas of two different ion pairs (≤ 100%). Maximum permitted tolerances of relative ion intensities are as follows:

Relative ionic intensity (%)	Tolerance (%)
> 50	± 20
> 20-50	± 25
> 10-20	± 30
≤ 10	± 50

Note 2: All the parameters can be adjusted depending on different instruments if the above conditions are not applicable.

Note 3: According to EU technical document, the precise identification of a protein should detection at least two or more specific peptides.

Remark

1. Limit of detection (LOD) for ovalbumin is 2 ppm.
2. Sample solution should be diluted according to the situation to avoid carryover in the column.
3. When different samples are tested, methanol should be injected to clean the column, and if necessary, 0.1% formic acid in 5% acetonitrile should be injected to confirm the situation of cleaning of the column and eliminate interference between the samples.
4. After the column is used, it is recommended to flush column with acetonitrile for more than 60 minutes.
5. Further validation should be performed when interfering compounds appear in samples.

Reference

1. Gavage, M., Van Vlierberghe, K., Van Poucke, C., De Loose, M., Gevaert, K., Dieu, M., Renard, P., Arnould, T. and Gillard, N. 2019. Selection of egg peptide biomarkers in processed food products by high resolution mass spectrometry. J. Chromatogr. A 1584: 115-125.
2. Planque, M., Arnould, T., Delahaut, P., Renard, P., Dieu, M. and Gillard,N. 2019. Development of a strategy for the quantification of food allergens in several food products by mass spectrometry in a routine laboratory. Food Chem. 274: 35-45.
3. Planque, M., Arnould, T. and Gillard, N. 2017. Food allergen analysis: detection, quantification and validation by mass spectrometry. Allergens. pp. 7-41. Intech.
4. General European OMCL Network (GEON). 2016. Interpretation of screening results for unknown peptides and proteins by mass spectrometry based methods. PA/PH/OMCL (15) 04 2R.

Reference chromatogram

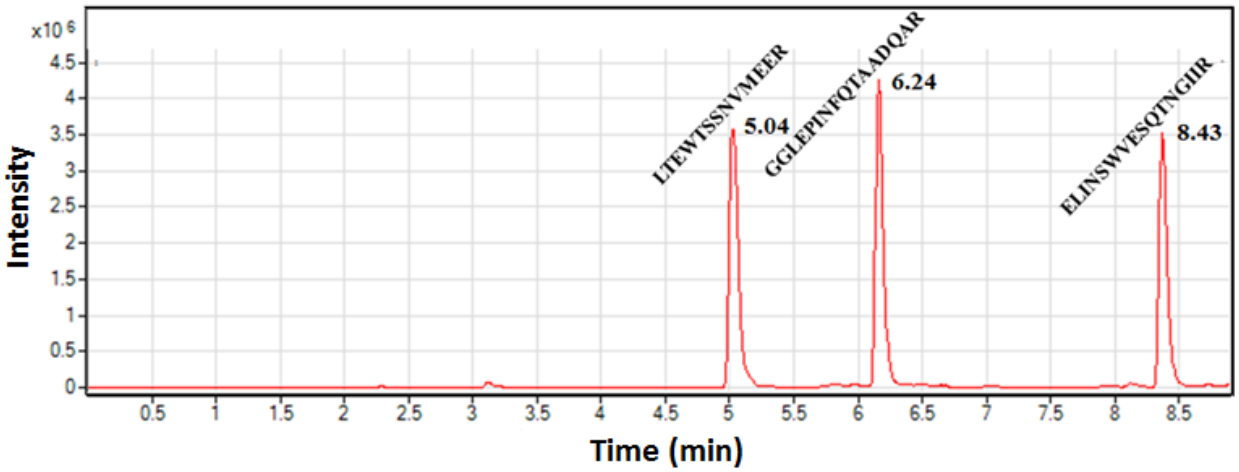

Figure. MRM chromatogram of ovalbumin specific peptides analyzed by LC-MS/MS.