LC-MS/MS method for determination of pork content in processed meat products

1. Scope

The standard specifies the sample preparation and determination of pork content in processed meat products by LC-MS/MS.

2. Principle

The samples were detected by LC-MS / MS after protein extraction, enzymolysis and purification, and quantified by external standard method.

3. Reagents and materials

Unless otherwise specified, all the reagent used should be analytical grade, water is the first grade water prescribed by GB/T 6682.

3.1. Reagents

3.1.1 Methanol (CH_3OH): HPLC grade.

3.1.2 Acetonitrile (CH_3CN, ACN): HPLC grade.

3.1.3 Formic acid (HCOOH): HPLC grade.

3.1.4 Acetic acid (CH_3COOH): HPLC grade.

3.1.5 Dithiothreitol (DTT).

3.1.6 Iodoacetamide (IAA).

3.1.7 Trypsin: BioReagent.

3.1.8 Tris.

3.1.9 Hydrochloric acid (HCl).

3.1.10 Urea.

3.1.11 Thiourea.

3.1.12 Trifluoroacetic acid (TFA).

3.2. Preparation of reagents

3.2.1 Tris solution (0.2 mol/L): Weigh 6.05 g Tris (3.1.8) and dissolve it with appropriate amount of water. After cooling, dilute it with water to 250 mL and mix well.

3.2.2 HCl solution (0.2 mol/L): Transfer 4.1 mL HCl (3.1.9) to a 250 mL volumetric flask, dilute with water and mix well.

3.2.3 Protein extraction solution (7 mol/L urea, 2 mol/L thiourea, 0.05 M Tris-HCl，pH 8.0): Weigh 105.1 g urea (3.1.10) and 38.06 thiourea (3.1.11), and dissolve them with 62.5 mL Tris solution (3.2.1) and 33.5 mL HCl solution (3.2.2). Transfer the above solution to a 250 mL volumetric flask, dilute with water and mix well.

3.2.4 DTT solution (50 mmol/L): Weigh 0.0154 g DTT (3.1.5) and dissolve it with 2 mL water. DTT solution should be freshly prepared just before use.

3.2.5 IAA solution (100 mmol/L): Weigh 0.037 g IAA (3.1.6) and dissolve it with 2 mL water. IAA solution should be freshly prepared just before use.

3.2.6 Tris-HCl solution (25 mmol/L, pH 8.0): Transfer 12.5 mL Tris solution (3.2.1) and 6.7 mL HCl solution (3.2.2) to a 100 mL volumetric flask, dilute with water and mix well.

3.2.7 Acetic acid solution (1 mL/100 mL): Transfer 1 mL acetic acid (3.1.4) to a 100 mL volumetric flask, dilute with water and mix well.

3.2.8 Acetic acid solution (0.01 mL/100 mL): Transfer 1 mL acetic acid solution (3.2.7) to a 100 mL volumetric flask, dilute with water and mix well.

3.2.9 Trypsin solution (2 mg/mL): weigh 2 mg trypsin (3.1.7) and dissolve it with 2 mL acetic acid solution (3.2.8). Trypsin solution should be freshly prepared just before use.

3.2.10 Acetonitrile solution (50%): Transfer 50 mL acetonitrile (3.1.2) to a 100 mL volumetric flask, dilute with water and mix well.

3.2.11 Acetic acid solution (0.5%): Transfer 1.0 mL acetic acid (3.1.4) to a 200 mL volumetric flask, dilute with water and mix well.

3.2.12 Acetonitrile-Acetic acid solution (60+40): Transfer 60 mL acetonitrile (3.1.2) and 40 mL acetic acid solution (3.2.11) to a 100 mL volumetric flask and mix well.

3.2.13 TFA solution (0.1%): Transfer 1.0 mL TFA (3.1.12) to a 100 mL volumetric flask, dilute with water and mix well.

3.2.14 Formic acid solution (1 mL/1000 mL): Transfer 1 mL formic acid (3.1.3) to a 1000 mL volumetric flask, dilute with water and mix well.

3.2.15 Formic acid-Acetonitrile solution (1 mL/1000 mL): Transfer 1 mL formic acid (3.1.3) to a 1000 mL volumetric flask, dilute with acetonitrile (3.1.2) and mix well.

3.3. SPE Column

Oasis HLB cartridges, 60 mg/3 mL, or equivalent performance parameters.

4. Apparatus and equipment

4.1 High Performance Liquid Chromatography-Mass Spectrometer equipment (LC-MS/MS): equipped with electrospray ionization source (ESI).

4.2 Analytical balance: sensibility reciprocal is 0.01 g.

4.3 Centrifuge: ≥10000r/min.

4.4 Solid phase extraction device.

4.5 Vortex mixer.

4.6 High-speed grinding homogenizer.

5. Procedure

5.1. Sample preparation and storage

About 50 g of representative samples should be taken from all samples, then homogenized by the homogenizer, put in suitable clean container. After being sealed and labeled, the samples should be stored at below -18℃ in dark. Certain measures should be taken to prevent contamination of samples or decomposition of the residues during the sample preparation procedure.

5.2. Sample processing

5.2.1 Extract

Accurately 2 g test sample (accurate to 0.01 g) was weighed in a 50 mL plastic centrifuge tube. Then, 4 steel pillars and 20 mL protein extraction solution (3.2.3) pre-cooled in advance were separately added and carefully homogenized in a high-speed grinding homogenizer by setting 1000 rad/min 9 s; 1200 rad/min 9 s; 2600 rad/min 120 s; 2 cycles totally. The extract was centrifuged at 12,000 rpm for 20 min at 4 °C.

5.2.2 Digestion

200 μL of the extract (5.2.1) was reduced with 30 μL of DTT solution (3.2.4) at 56 °C for 40 min in a thermomixer and then alkylated by adding 30 μL of IAA

solution (3.2.5) for 30 min in the dark. Afterwards, samples were diluted with 900 μL Tris-HCl (3.2.6) and 60 μL trypsin solution (3.2.9) and then incubated at 37 °C for 4 h. The digestion was stopped by addition of 80 μL acetic acid (3.1.4).

5.2.3 Purification

Oasis HLB cartridge was activated with 3 mL ACN (3.1.2), 3 mL acetonitrile solution (3.2.10) and 3 mL TFA solution (3.2.13) and keep wet. All Enzymolysis solution (5.2.2) was transferred into the SPE column, and the column was washed with 3 mL TFA solution (3.2.13) and 3 mL acetic acid solution (3.2.11). The column was eluted with 2 mL Acetonitrile-Acetic acid solution (3.2.12). The elute solution was vortexed for 0.5min, filtered with 0.22μm filter membrane and injected into the LC-MS/MS system.

5.3. Preparation of standard working curve

Accurately weigh 2 g pure pork sample (accurate to 0.01 g) and placed in the 50 mL plunger centrifuge tube. The exact step (5.2.1) was the same. 10, 20, 50, 100, 200, and 300 μL the extract was pipetted into centrifuge tubes, corresponding to obtain the final concentrations of 0.5, 1.25, 2.5, 5.0, 10 and 15 mg/mL for pork content and appropriate amounts of extraction buffer solution were added to ensure that the mixed solution volume was 300 μL in each tube. The following steps were the same.

5.4. Apparatus operating condition

5.4.1 HPLC operating condition

a）Column: C_{18}, 2.1 mm × 100 mm, 1.9 μm or equivalent.

b）Column temperature：40℃；

c）Injection volume：10 μL；

d）Mobile phase: see Table 1.

Table 1 Mobile phase and gradient elution program

Time /min	Mobile phase A（Formic acid solution, 3.2.14), %	Mobile phase B（Formic acid-Acetonitrile solution, 3.2.15）, %
0.0	95	5
0.5	95	5

8.0	20	80
9.5	20	80
10	95	5
13	95	5

e）Flow rate：0.25 mL/min；

5.4.2 MS/MS operating condition

Ion source: electrospray ionization source (ESI); Scan mode: Positive-ion mode; Monitor mode：multiple reaction monitoring (MRM)；Spray voltage: 3500V; sheath gas flow: 35 Arb; auxiliary gas flow: 15 Arb; capillary temperature: 275 °C; vaporizer temperature: 380 °C; acquisition cycle: 0.5 s; collision gas pressure: 1.5 mTorr; Q1 and Q3 resolution: 0.7. Main MS parameters of target compound are listed in Table 2. Three pig specific peptides were selected, of which pep1 and pep2 were used for quantitative analysis and pep3 for qualitative analysis.

Table 2 MRM parameters of porcine-specific peptides

Markers	**Peptide**	**Parent ion (*m/z*)**	**product ion (*m/z*)**	**Collision energy (V)**
pep1[#]	EPITVSSDQMAK	653.321	766.340* /865.410/966.457	26
pep2#	GGPLTAAYR	453.246	212.103/480.257/581.304*/694.389	22
pep3	HDPSLLPWTASYDPGSAK	647.984	459.256*/550.263/663.346/824.377	25

Note: * The product ion is used for quantification. [#]The peptide is used for quantification.

5.5. Qualitative determination

Under the same determination conditions, the variation range of the retention time for the peak of analyte in unknown sample and in the standard working solution cannot be out of range of ±2.5%.

5.6. Quantitation determination

Under the optimized instrument working conditions, different working standard solutions were injected. Using peak area as y-axis and the concentration as x-axis, the concentration of pig in sample is quantified by standard calibration curve. The response of peptides in the sample solution should be in the linear range of the instrument detection.

6. Calculation and expression of the result

The calculation of pig in the sample is according to Formula (1).

$$X = \frac{C \times V \times 100}{m \times 1000} \times f \text{.....(1)}$$

where：

X—the content of pig in the test sample, g/100g;

C—the concentration of pig which is quantified by standard calibration curve, mg/mL;

V—the final volume of sample solution, mL;

m—the corresponding mass of test sample, g;

f—dilution ratio of sample solution.

The result was the average of the results of two peptides, expressed as the arithmetic mean of two independent determinations obtained under repeatability conditions and rounded to two decimal places.

7. Precision

The absolute difference of two independent determinations obtained under repeatability conditions shall not exceed 20% of the arithmetic mean.

8. Limit of quantitation

The limit of quantification is 0.5 g/100 g.

9. Recovery

The content of pork: 10 g/100g-90 g/100 g, Recovery: 60%-130%.

Annex A

(Informative)

LC-MS/MS chromatogram of peptides.