LC-MS/MS method for Quantification of Animal Blood Products

1. Scope

The standard specifies the sample preparation and quantification of animal blood products of pig, sheep, bovine, chicken and duck 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 20 g of representative samples should be taken from all samples, then homogenized by the homogenizer, put in suitable clean container. The fresh blood of pig, sheep, bovine, chicken and duck were from the local slaughterhouse. After natural sedimentation, the blood was sterilized under high temperature at 121 °C for 30 min. After being sealed and labeled, the samples should be stored at below -18 °C 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 1.0 g test sample (accurate to 0.01 g) was weighed in a 50 mL plastic centrifuge tube. Then, 10 mL protein extraction solution (3.2.3) pre-cooled in advance was added and carefully homogenized by the homogenizer. The extract was centrifuged at 12,000 rpm for 20 min at 4 °C.

5.2.2 Digestion

100 μL of the extract (5.2.1) was transferred to a 4-mL centrifuge tube, followed by addition of 200 μL extraction buffer solution, reduced with 60 μL of DTT solution (3.2.4) at 56 °C for 40 min in a thermomixer and then alkylated by adding 60 μL of IAA solution (3.2.5) for 30 min in the dark. Afterwards, samples were diluted with 2.7 mL Tris-HCl (3.2.6) and 50 μL trypsin solution (3.2.9) and then incubated at 37 °C for 2 h. The digestion was stopped by adding TFA (3.1.12) to regulate pH<2.0.

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 0.4 g pure pig, bovine, chicken, sheep and duck blood (accurate to 0.01 g) and placed in the 50 mL plunger centrifuge tube. The exact step (5.2.1) was the same. 20, 50, 100, 200, and 300 μL the extract was pipetted into centrifuge tubes, corresponding to obtain the final concentrations of 0.4, 1.0, 2.0, 4.0 and 6.0 mg/mL for blood 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	97	3
0.2	90	10
15.8	60	40
16.8	20	80

17.3	20	80
18.3	97	3
23	97	3

e）Flow rate：0.2 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.

Each species has three specific peptides, one of which is quantitative peptide and the other two are auxiliary qualitative peptide.

Table 2 MRM parameters of porcine-specific peptides

Species	**Markers**	**Peptide**	**Parent ion (*m/z*)**	**product ion (*m/z*)**	**Collision energy (V)**
sheep	sheep-1	AAVTGFWGK	468.75	695.3*/594.3/390.2	21
	sheep-2#	VKVDEVGAEALGR	448.25	416.3*/545.3/345.2	20
	sheep-3	VGGNAGAYGAEALER	717.85	908.4*/1036.5/745.4	26
bovine	bovine-1	AAVTAFWGK	475.76	390.2*/608.3/143.1	21
	bovine-2	VGGHAAEYGAEALER	510.58	488.3*/617.3/417.2	22
	bovine-3#	VKVDEVGGEALGR	443.58	416.3*/345.2/232.1	20
pig	pig-1#	VGGQAGAHGAEALER	474.91	745.4*/441.7/882.4	21
	pig-2	SVEFTGFDPR	577.78	272.2*/316.2/187.1	23
	pig-3	QGLLPVLENLK	612.37	812.5*/282.1/299.2	24
duck	duck-1	LAPVAQELK	484.79	392.7*/588.3/784.5	21
	duck-2#	VVEQLSNLR	529.30	859.5*/489.3/602.4	22
	duck-3	VAGHQEEFGSEALQR	553.27	487.3*/416.3/616.3	23
chicken	chicken-1	MTPLVQEFR	560.80	444.7*/678.4/888.5	23
	chicken-2	GIPQASEYQAK	596.30	511.3*/796.4/329.2	23
	chicken-3#	LISFLDELQK	603.34	979.5*/227.2/745.4	24

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 blood 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 blood in the sample is according to Formula (1).

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

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 calculated by quantitative peptide, 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 blood: 20 g/100g-100 g/100g, Recovery: 70%-130%.

Annex A

(Informative)

LC-MS/MS chromatogram of peptides.