SN

中华人民共和国出入境检验检疫行业标准

SN/T 2239—2008

进出口动物源性食品中氮氨菲啶残留量检测方法 液相色谱-质谱/质谱法

Determination of isometamidium residue in foodstuffs of animal origin for import and export—LC-MS/MS method

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前　言

本标准的附录A和附录B均为资料性附录。

本标准由国家认证认可监督管理委员会提出并归口。

本标准由中华人民共和国重庆出入境检验检疫局、中国检验检疫科学研究院、中华人民共和国山东出入境检验检疫局和中华人民共和国黑龙江出入境检验检疫局负责起草。

本标准主要起草人：王国民、陈冬东、李贤良、周启明、李应国、张雷、唐柏彬、王建华、康庆贺。

本标准系首次发布的出入境检验检疫行业标准。

进出口动物源性食品中氮氨菲啶残留量检测方法 液相色谱-质谱/质谱法

1 范围

本标准规定了动物源性食品中氮氨菲啶残留量的制样和液相色谱-质谱/质谱检测方法。

本标准适用于牛肉、牛肝、牛肾、牛脂肪、羊肝、鸡肝、鱼肉及牛奶中氮氨菲啶残留量的测定和确证。

2 方法提要

试样中的氮氨菲啶用乙腈和甲酸铵甲醇混合溶液提取，提取液经浓缩、脱脂后，用液相色谱-质谱/质谱仪测定，外标峰面积法定量。

3 试剂和材料

所有试剂除特殊注明外，均为分析纯，水为二次蒸馏水。

3.1 甲醇：高效液相色谱级。

3.2 甲酸：高效液相色谱级。

3.3 乙腈：高效液相色谱级。

3.4 正己烷。

3.5 无水硫酸钠：650 ℃灼烧 4 h，在干燥器内冷却至室温，贮于密封瓶中备用。

3.6 甲酸铵：优级纯或相当者。

3.7 甲酸铵-甲醇溶液(0.25 mol/L)：称取 15.8g 甲酸铵溶于 1 000 mL 甲醇(3.1)中。

3.8 80%甲醇溶液：取甲醇(3.1)800 mL，加水稀释至 1 000 mL，混匀。

3.9 提取液：取乙腈(3.3)和甲酸铵-甲醇溶液(3.7)进行 1∶1 混合(体积比)。

3.10 氮氨菲啶盐酸盐(Isometamidium Chloride，CAS 号：34301-55-8，分子式：$C_{28}H_{26}ClN_7$)标准品：纯度大于或等于 97.0%。

3.11 氮氨菲啶标准储备液：准确称取适量氮氨菲啶盐酸盐标准品，用甲醇配制成氮氨菲啶浓度为 100 μg/mL 的标准储备液。此溶液在 0 ℃～4 ℃避光保存，可使用 3 个月。

3.12 标准工作溶液：根据需要用空白样品提取液将标准储备液稀释成 5.0 ng/mL、10 ng/mL、20.0 ng/mL、50 ng/mL、100 ng/mL、500 ng/mL 的标准工作溶液。置于 0 ℃～4 ℃避光保存，可使用 3 天。

3.13 0.22 μm 微孔滤膜，有机系。

4 仪器和设备

4.1 高效液相色谱-质谱/质谱仪，配电喷雾离子源(ESI)。

4.2 天平：感量为 0.01 g 和 0.000 1 g。

4.3 超声波清洗器。

4.4 旋涡混匀器。

4.5 氮吹仪。

4.6 离心机：5 000 r/min。

4.7 均质器：10 000 r/min。

4.8 容量瓶：100 mL、50 mL、10 mL。

4.9 具塞塑料离心管：50 mL。

5 试样的制备与保存

肌肉、内脏和鱼：取有代表性样约500 g，用组织捣碎机捣碎，装入洁净容器作为试样，密封并做好标识，于－18 ℃冰箱内保存。

奶：取有代表性样品约500 g，搅拌均匀后装入洁净容器内密封并做好标识，于4 ℃冰箱内保存。

制样操作过程中应防止样品受到污染或发生残留物含量的变化。

6 测定步骤

6.1 提取与净化

6.1.1 肌肉、内脏(肝脏、肾脏)、脂肪及鱼肉

准确称取5 g试样(精确到0.01 g)于50 mL具塞离心管中，加入2 g无水硫酸钠混匀，再加入20 mL提取液(3.9)，用均质器均质2 min，4 000 r/min离心3 min，将有机相转移至50 mL容量瓶中，残渣再用2×10 mL提取液(3.9)均质提取。离心合并有机相，用提取液(3.9)定容至50 mL，混匀后取2.00 mL用氮吹仪在35 ℃下浓缩至干。定量加入2.00 mL 80%甲醇溶液(3.8)溶解残渣，加入2 mL正己烷(3.4)旋涡混匀2 min后，2 500 r/min离心3 min，将下层过0.22 μm滤膜后，供液相色谱-质谱/质谱仪测定。

6.1.2 奶

准确称取5 g试样(精确到0.01 g)于50 mL具塞离心管中，加入20 mL提取液(3.9)，盖上盖混匀，超声提取15 min，冷却后将有机相过滤转移至50 mL容量瓶中，残渣再用2×10 mL提取液(3.9)超声提取，离心合并有机相。接下来按照6.1.1中“离心合并有机相”后的步骤进行。

6.2 测定

6.2.1 液相色谱-质谱/质谱条件

a) 液相色谱-串联质谱仪；

b) 色谱柱：Acquity UPLC™ BEH C_{18}柱，2.1 mm(内径)×100 mm，1.7 μm，或相当者；

c) 流动相：甲醇-0.2%甲酸，梯度洗脱程序见表1；

表1 流动相梯度洗脱程序

时间/min	甲醇/%	0.2%甲酸/%
0	10.0	90.0
3.00	90.0	10.0
4.00	90.0	10.0
4.50	10.0	90.0
5.00	10.0	90.0

d) 流速：0.20 mL/min；

e) 柱温：35 ℃；

f) 进样量：10 μL；

g) 离子源：电喷雾离子源；

h) 扫描方式：正离子；

i) 检测方式：多反应监测(MRM)；

j) 质谱/质谱参考条件参见附录A。

6.2.2 定性、定量测定

按照6.2.1液相色谱-质谱/质谱条件测定样液和标准工作溶液，外标标准曲线法测定样液中的氮氨菲啶残留量。样品中待测物残留量应在标准曲线范围之内，如果残留量超出标准曲线范围，应用空白样品提取液进行适当稀释。在上述色谱条件下，氮氨菲啶的质量色谱峰保留时间约为3.0 min。标准品的多反应监测色谱图参见附录B中的图B.1。

在相同实验条件下，样品与标准工作液中待测物质的质量色谱峰相对保留时间在2.5%以内，并且在扣除背景后的样品质量色谱图中，所选择的离子对均出现，同时与标准品的相对丰度允许偏差不超过表2规定的范围，则可判断样品中存在对应的被测物。

表2 使用液相色谱-质谱/质谱定性时相对离子丰度最大容许误差

相对丰度(基峰)/%	液相色谱-质谱/质谱定性时相对离子丰度最大允许误差/%
>50	±20
大于20至小于等于50	±25
大于10至小于等于20	±30
小于等于10	±50

6.2.3 空白试验

空白样品，按上述测定步骤进行。

7 结果计算和表述

用数据处理软件中的外标法，或绘制标准曲线，按照式(1)计算样品中氮氨菲啶残留量。

$$X = \frac{(c - c_0) \times V}{m \times 1\,000} \qquad \cdots\cdots(1)$$

式中：

X——试样中氮氨菲啶的残留量，单位为毫克每千克(mg/kg)；

c——由标准曲线得到的样液中氮氨菲啶的浓度，单位为纳克每毫升(ng/mL)；

c_0——由标准曲线而得的空白实验中的氮氨菲啶的浓度，单位为纳克每毫升(ng/mL)；

V——样液最终定容体积，单位为毫升(mL)；

m——最终样液所代表的试样质量，单位为克(g)。

8 测定低限、回收率

8.1 测定低限

本方法氮氨菲啶的测定低限为0.005 mg/kg。

8.2 回收率

8.2.1 牛肌肉中氮氨菲啶添加浓度及其回收率数据：

——添加浓度在0.005 mg/kg时，回收率在70.0%～84.0%之间；

——添加浓度在0.010 mg/kg时，回收率在71.0%～86.0%之间；

——添加浓度在0.020 mg/kg时，回收率在72.5%～85.0%之间。

8.2.2 牛肝脏中氮氨菲啶添加浓度及其回收率数据：

——添加浓度在0.005 mg/kg时，回收率在78.0%～104.0%之间；

——添加浓度在0.010 mg/kg时，回收率在74.0%～103.0%之间；

——添加浓度在0.020 mg/kg时，回收率在74.5%～103.0%之间。

8.2.3 牛肾脏中氮氨菲啶添加浓度及其回收率数据：

——添加浓度在0.005 mg/kg时，回收率在68.0%～80.0%之间；

——添加浓度在0.010 mg/kg时，回收率在67.0%～82.0%之间；

——添加浓度在0.020 mg/kg时，回收率在71.0%～92.0%之间。

8.2.4 牛脂肪中氮氨菲啶添加浓度及其回收率数据：

——添加浓度在0.005 mg/kg时，回收率在86.0%～104.0%之间；

——添加浓度在0.010 mg/kg时，回收率在81.0%～93.0%之间；

——添加浓度在0.020 mg/kg时，回收率在85.5%～101.5%之间。

8.2.5 牛奶中氮氨菲啶添加浓度及其回收率数据：

——添加浓度在0.005 mg/kg时，回收率在76.0%～94.0%之间；

——添加浓度在0.010 mg/kg时，回收率在75.0%～94.0%之间；

——添加浓度在0.020 mg/kg时，回收率在83.5%～101.5%之间。

8.2.6 羊肝中氮氨菲啶添加浓度及其回收率数据：

——添加浓度在0.005 mg/kg时，回收率在82.0%～102.0%之间；

——添加浓度在0.010 mg/kg时，回收率在84.0%～101.0%之间；

——添加浓度在0.020 mg/kg时，回收率在84.5%～100.5%之间。

8.2.7 鸡肝中氮氨菲啶添加浓度及其回收率数据：

——添加浓度在0.005 mg/kg时，回收率在84.0%～102.0%之间；

——添加浓度在0.010 mg/kg时，回收率在83.0%～101.0%之间；

——添加浓度在0.020 mg/kg时，回收率在83.5%～100.5%之间。

8.2.8 鱼肉中氮氨菲啶添加浓度及其回收率数据：

——添加浓度在0.005 mg/kg时，回收率在78.0%～102.0%之间；

——添加浓度在0.010 mg/kg时，回收率在68.0%～81.0%之间；

——添加浓度在0.020 mg/kg时，回收率在70.5%～79.0%之间。

附 录 A
（资料性附录）
参考质谱条件[1)]

参考质谱条件：

a） 毛细管电压：1.2 kV

b） 离子源温度：110 ℃

c） 脱溶剂气温度：350 ℃

d） 锥孔气流量（氮气）：45 L/h

e） 脱溶剂气流量（氮气）：600 L/h

f） 碰撞气压力（氩气）：0.22 mL/min

g） 碰撞室压力（氩气）：3.03 e^{-3}（mbar）

h） 定性离子对、定量离子对、锥孔电压、碰撞能量、驻留时间见表 A.1。

表 A.1 待测物定性离子对、定量离子对、锥孔电压、碰撞能量、驻留时间

化合物	母离子（m/z）	子离子（m/z）	锥孔电压/V	碰撞能量/ev	驻留时间/s
氮氨菲啶	460	298[a]	31.0	25	0.30
		313	31.0	25	0.30
[a] 离子为定量离子。					
注：对于不同质谱仪器，仪器参数可能存在差异，测定前应将质谱参数优化到最佳。					

1） 非商业性声明：附录 A 所列参考质谱条件是在 Waters UPLC/Priemer 型液质联用仪上完成的，此处列出试验用仪器型号仅为提供参考，并不涉及商业目的，鼓励标准使用者尝试不同厂家或型号的仪器。

附　录　B
（资料性附录）
氮氨菲啶的多反
应监测（MRM）离子色谱图

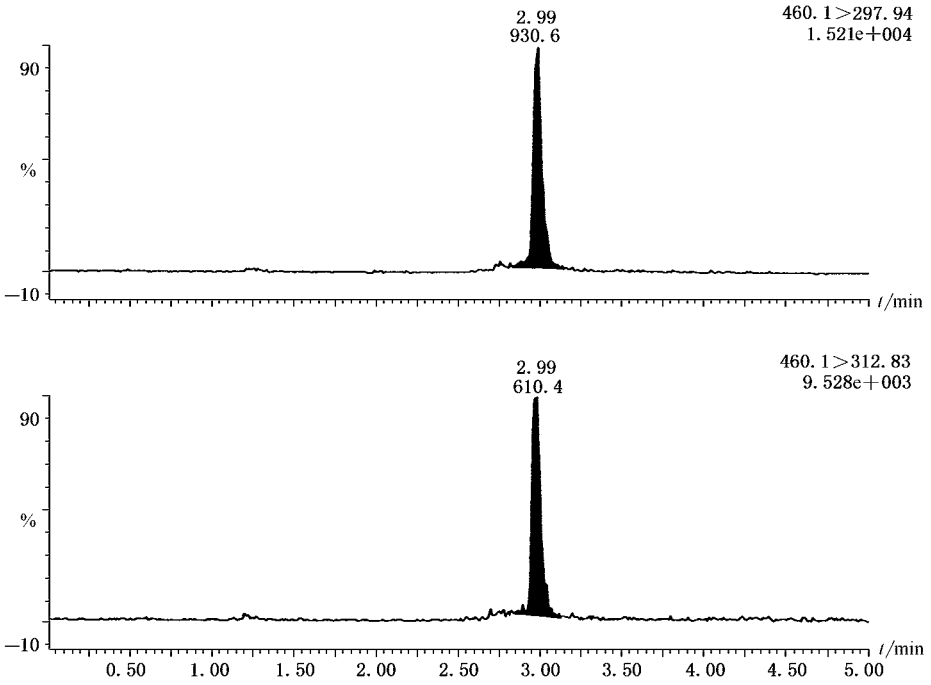

图 B.1　氮氨菲啶标准溶液（5 μg/L）多反应监测离子色谱图

Foreword

Annex A and B of this standard are informative annexes.

This standard was proposed by and was under the charge of National Regulatory Commission for Certification and Accreditation of the People's Republic of China.

The standard was drafted by Chongqing Entry-Exit Inspection and Quarantine Bureau of the People's Republic of China, Chinese Academy of Inspection and quarantine, Shandong Entry-Exit Inspection and Quarantine Bureau of the People's Republic of China, and Heilongjiang Entry-Exit Inspection and Quarantine Bureau of the People's Republic of China.

This standard was mainly drafted by Wang Guoming, Chen Dongdong, Li Xianliang, Zhou Qiming, Li Yingguo, Zhang Lei, Tang Baibin, Wang Jianhua, and Kang Qinghe.

This standard is a professional standard for entry-exit inspection and quarantine promulgated for the first time.

Note: This English version, a translation from the Chinese text, isonly for reference.

Determination of isometamidium residues in foodstuffs of animal origin—LC-MS/MS method

1 Scope

This standard specifies the methods of sample preparation and qualified and quantified determination by liquid chromatography-mass spectrometry of isometamidium residues in foodstuffs of animal origin.

This standard is applicable to the determination of isometamidium residues in beef, beef liver, beef kideny, beef fat, goat liver, poultry liver, fish and milk.

2 Principle

The residues of isometamidium residues in the test sample are extracted with the mixed solution of acetonitrile and ammonium formiate methanol. After being concentrated and reconstituted, the residues are determined by liquid chromatography-mass spectrometry, quantified by external standard method.

3 Reagents and materials

Unless specified, all reagents should be of analytical grade; "Water" is the distilled water.

3.1 Methanol: HPLC grade.

3.2 Formic acid: HPLC grade.

3.3 Acetonitrile: HPLC grade.

3.4 *n*-hexane.

3.5 Sodium sulfate: Dried at 650 ℃ for 4 h, cool to room temperature in a desiccator and store in sealed container.

3.6 Ammonium formiate: GR or the equivalent.

3.7 0.25 mol/L ammonium formiate methanol solution: Weigh 15.8 g ammonium formiate, dissolved in 1 000 mL methanol.

3.8 80% methanol solution: Dilute methanol (3.1) 800 mL with water to the volume of 1 000 mL.

3.9 Extractant: Dilute acetonitrile (3.3) with ammonium formiate methanol solution by 1∶1.

3.10 Isometamidium Chloride: CAS: 34301-55-8, Purity≥97.0%.

3.11 Stock standard solution: Accurately weight an adequate amount of Isometamidium Chloride standard in an volumetric flask and dissloved with methanol to form a Isometamidium stock standard solution of 100 μg/mL. The stock standard solution can be preserved at the temperature 0 ℃～4 ℃ for three months.

3.12 Standard working solution: according to the requirement, dilute standard solution with blank matrix extract solution . The concentration of the solution is 5 ng/mL、10 ng/mL、20 ng/mL、50 ng/mL、100 ng/mL and 500 ng/mL. It can be preserved at the temperature 0 ℃～4 ℃ for three days.

3.13 Membrane filter: 0.22 μm, organic.

4 Apparantus and equipment

4.1 High performance liquid chromatography triple-quadrupole tandem mass spectrometer equipped with electrospray ionization source (ESI).

4.2 Balance, sensitivity: 0.01 g, 0.000 1 g.

4.3 Ultrasonic extractor.

4.4 Vortex mixer.

4.5 Nitrogen evaporator.

4.6 Centrifuge: 5 000 rpm/min.

4.7 Homogenizer: 10 000 r/min.

4.8 Volume flask: 100 mL、50 mL、10 mL.

4.9 Plastic centrifuge tube with cap: 50 mL.

5 Sample preparation and storage

Muscle, liver, kidney and fish products: About 500 g representative samples should be taken from all

samples, then grinded and blended by a tissue blender to produce homogenous samples, put in suitable clean container. After being sealed and labeled, the samples should be stored at below −18 ℃.

Milk: About 500 g representative samples should be taken from all sample, then mixed to produce homogenous samples, and put in suitable clean containers. After being sealed and labeled, the samples should be stored at 0 ℃～4 ℃.

Certain measures should be taken to prevent contamination of the samples or decomposition of the residues during the sample preparation procedure.

6 procedure

6.1 Extract and Clean-up

6.1.1 Muscle, liver, kidney and fat

Accurately weigh 5 g of the test sample (accurate to 0.01 g) into a 50 mL polypropylene centrifuge tube with cap, add 2 g anhydrous sodium sulfate and 20 mL extractant (3.9), homogenize for 2 min, then centrifuge at 4 000 r/min for 3 min. Transfer the supernatant to a 50 mL volumetric flask, the residue was homogenized and extracted with 2 × 10 mL extractant (3.9). After centrifuging, combine the supernatant to the volumetric flask, mixed sufficiently and dilute to mark with extractant (3.9). Transfer the extract solution 2.00 mL to a 10 mL centrifuge tube, evaporate the extract to dryness on N-Evap below 35 ℃. The residue is dissolved with 2.00 mL of 80% methanol solution (3.8) and 2 mL n-hexane (3.4). Vortex 2 min and centrifuge at 2 500 r/min for 3 min. Filter the nether layer through a 0.22 μm membrane into HPLC vials. The solution is ready for LC-MS/MS determination.

6.1.2 Milk

Accurately weigh 5 g of the test sample (accurate to 0.01 g) into a 50 mL polypropylene centrifuge tube with cap, add 20 mL extractant (3.9), cover with cap and vortex, then extract in ultrasonic extractor for 15 min. After cooling, transfer the supernatant to a 50 mL volumetric flask, the residue was vortexed and extracted with 2 × 10 mL extractant (3.9). After centrifuging, combine the supernatant to the volumetric flask. Then, deal with the sample according to the step of 6.1.1 "After centrifuging, combine the supernatant to the volumetric flask".

6.2 Determination

6.2.1 LC-MS/MS operation condition

a) LC-MS/MS: Waters UPLC Quattro premier XE or equivalent;

b) LC column:Acquity UPLC™ BEH C_{18},2.1 (i.d.) × 100 mm,1.7 μm,(or other conformable column);

c) Mobile phase:methanol:0.2% Formic acid,the elution gradient is listed in Table 1;

Table 1—The elution gradient

Time/min	Methanol/%	0.2% Formic acid/%
0	10.0	90.0
3.00	90.0	10.0
4.00	90.0	10.0
4.50	10.0	90.0
5.00	10.0	90.0

d) Flow rate:0.20 mL/min;

e) Column temperature:35 ℃;

f) Injector volume:10 μL;

g) Ion source:ESI;

h) Scanning model:positive ion;

i) Monitoring model:multiple reaction monitoring (MRM);

j) MS/MS reference conditions listed in annex A.

6.2.2 Qualification and quantification test

According to the method of 6.2.1,detect the residues of Isometamidium in the test sample solution, the standard working solution. The response of Isometamidium should be in the linear range of the instrumental detection. If the response is out of the linear range,the sample should be diluted with the blank matrix extract solution to suitable concertration. Under the above chromatograph conditions,the reference retention time of Isometamidium is about 3.0 min. Reconstituted ion chromatogram of standard working solution is listed in annex B.

Under the same conditions of experiment,the retention time of the unknown sample is the same as the standard working solution; the qualification ions for every compound must be found. For the same analysis batch and the same compound,the variation range of the ion ratio between the two daughter ions for the unknown sample and the standard working solution at the similar concentration cannot be out of range of Table 2.

Table 2—Maximum permitted tolerances for relative ion intensities while confirmation

Relative intensity/%	>50	>20～50	>10～20	≤10
Permitted tolerances/%	±20	±25	±30	±50

6.2.3 Blank test

The operation of the blank test is the same as that is described while there is no sample invovled.

7 Calculation and expression of result

Calculating the content of Isometamidium residue concentration in the sample is carried out by LC/MS/MS data processor or according to the formula (1):

$$X = \frac{(c - c_0) \times V}{m \times 1\,000} \qquad \cdots\cdots(1)$$

Where:

X—the residue content of Isometamidium in the test sample, mg/kg;

c—the concentration of Isometamidium in the test sample calculated by calibration curve, ng/mL;

c_0—the concentration of Isometamidium in the blank test calculated by calibration curve, ng/mL;

V—the final volume of sample solution, mL;

m—the corresponding mass of test sample in the final sample solution, g.

8 Limit of quantification and recovery

8.1 Limit of quantification

The limit of quantification for Isometamidium is 0.005 mg/kg.

8.2 Recovery

8.2.1 According to the experimental date, the fortifying concentration of Isometamidium in musle and its corresponding recoveries are:

—0.005 mg/kg, the recovery is 70.0%～84.0%;

—0.010 mg/kg, the recovery is 71.0%～86.0%;

—0.020 mg/kg, the recovery is 72.5%～85.0%.

8.2.2 According to the experimental date, the fortifying concentration of Isometamidium in liver and its corresponding recoveries are:

—0.005 mg/kg, the recovery is 78.0%～104.0%;

—0.010 mg/kg, the recovery is 74.0%～103.0%;

—0.020 mg/kg, the recovery is 74.5%～103.0%.

8.2.3 According to the experimental date, the fortifying concentration of Isometamidium in kidney and its corresponding recoveries are:

—0.005 mg/kg, the recovery is 68.0%～80.0%;

—0.010 mg/kg, the recovery is 67.0%～82.0%;

—0.020 mg/kg, the recovery is 71.0%～92.0%.

8.2.4 According to the experimental date, the fortifying concentration of Isometamidium in fat and its corresponding recoveries are:

—0.005 mg/kg, the recovery is 86.0%～104.0%;

—0.010 mg/kg, the recovery is 81.0%～93.0%;

—0.020 mg/kg, the recovery is 85.5%～ 101.5%.

8.2.5 According to the experimental date, the fortifying concentration of Isometamidium in milk and its corresponding recoveries are:

—0.005 mg/kg, the recovery is 76.0%～94.0%;

—0.010 mg/kg, the recovery is 75.0%～94.0%;

—0.020 mg/kg, the recovery is 83.5%～101.5%.

8.2.6 According to the experimental date, the fortifying concentration of Isometamidium in sheep liver and its corresponding recoveries are:

—0.005 mg/kg, the recovery is 82.0%～102.0%;

—0.010 mg/kg, the recovery is 84.0%～101.0%;

—0.020 mg/kg, the recovery is 84.5%～100.5%.

8.2.7 According to the experimental date, the fortifying concentration of Isometamidium in chicken liver and its corresponding recoveries are:

—0.005 mg/kg, the recovery is 84.0%～102.0%;

—0.010 mg/kg, the recovery is 83.0%～101.0%;

—0.020 mg/kg, the recovery is 83.5%～100.5%.

8.2.8 According to the experimental date, the fortifying concentration of Isometamidium in fish and its corresponding recoveries are:

—0.005 mg/kg, the recovery is 78.0%～102.0%;

—0.010 mg/kg, the recovery is 68.0%～81.0%;

—0.020 mg/kg, the recovery is 70.5%～79.0%.

Annex A
(Informative)
Referenced conditions[1)]

Referenced conditions:

a) Capillary voltage:1.2 kV;

b) Source Temperature:110 ℃;

c) Desolvation Temperature:350 ℃;

d) Cone Gas Flow:45 L/h(N_2);

e) Desolvation Gas Flow:600 L/h(N_2);

f) Collosion Gas Flow:0.22 mL/min(He);

g) Collosion Cell Pressure:3.03e^{-3}(mbar)(He);

h) Transitions for confirmation and quantification, Cone Voltage, Collision energy and dwell time see table A.1.

Table A.1—Transitions for confirmation and quantification, Cone, Collision energy, dwell time

Target analyte	Q1 m/z	Q3 m/z	Cone/V	Collision energy/eV	dwell time/s
Isometamidium	460	298[a]	31.0	25	0.30
		313	31.0	25	0.30
[a] Quantiation ion pair.					
Note: The analysts are encouraged to use equipments of different corporation or different type.					

1) Non-commercial statement: the reference mass parameters in Annex A are accomplished by Waters UPLC Quattro premier XE LC/MS/MS, the equipment and its type involved in the standard method is only for reference and not related to any commercial aim.

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Annex B

(informative)

LC/MS/MS extracting ion current(XIC)of+MRM chromatograms for the standard

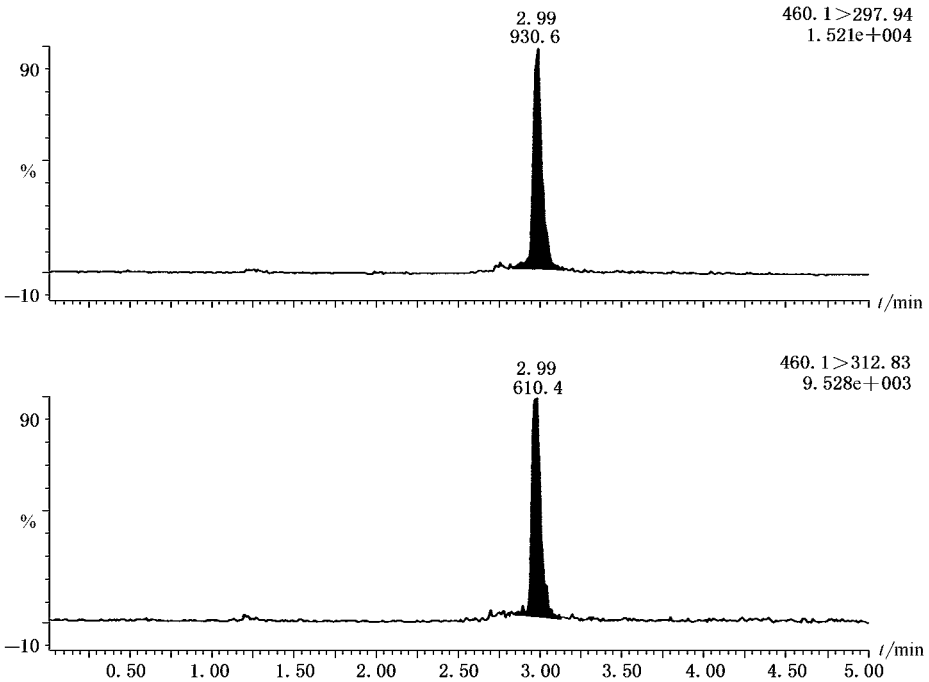

Figure B.1—LC/MS/MS extracting ion current(XIC)of+MRM chromatograms for Isometamidium standard

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