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中华人民共和国出入境检验检疫行业标准

SN/T 2223—2008

进出口动物源性食品中硫粘菌素残留量检测方法 液相色谱-质谱/质谱法

Determination of tiamulin 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。

本标准起草单位：中华人民共和国辽宁出入境检验检疫局、中华人民共和国江西出入境检验检疫局、大连工业大学。

本标准主要起草人：孙兴权、林维宣、温志海、张玉苍。

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硫粘菌素残留量检测方法 液相色谱-质谱/质谱法

1 范围

本标准规定了进出口动物组织中硫粘菌素残留量的液相色谱-质谱/质谱测定方法。

本标准适用于猪肉、猪肝、猪肾中硫粘菌素残留量的检测和确证。

2 规范性引用文件

下列文件中的条款通过本标准的引用而成为本标准的条款。凡是注日期的引用文件，其随后所有的修改单(不包括勘误的内容)或修订版均不适用于本标准，然而，鼓励根据本标准达成协议的各方研究是否可使用这些文件的最新版本。凡是不注日期的引用文件，其最新版本适用于本标准。

GB/T 6682 分析实验室用水规格和试验方法。(GB/T 6682—2008，ISO 3696:1987，MOD)

3 方法提要

试样经乙酸乙酯提取，正己烷脱脂，固相萃取小柱净化后，液相色谱-质谱/质谱法测定和确证，外标法定量。

4 试剂和材料

除另有说明外，所用试剂均为分析纯，水为 GB/T 6682 规定的一级水。

4.1 乙酸乙酯：色谱纯。

4.2 正己烷：色谱纯。

4.3 甲醇：色谱纯。

4.4 乙腈：色谱纯。

4.5 乙酸铵。

4.6 0.1%乙酸铵：称取 1.0 g 乙酸铵，加水溶解并定容至 1 000 mL。

4.7 硫粘菌素标准物质(Tiamulin)：$C_{28}H_{47}NO_4S$，CAS No. 55297-95-5，纯度大于等于 98.8%。

4.8 硫粘菌素标准储备溶液(1.0 mg/mL)：称取适量的硫粘菌素标准物质(4.7)，用甲醇配成 1.0 mg/mL 的标准储备液。储备液置于－18 ℃避光贮存。

4.9 硫粘菌素标准工作溶液(1.0 μg/mL)：吸取适量的硫粘菌素标准储备溶液(4.8)，用甲醇配成 1.0 μg/mL的标准工作溶液。标准工作溶液置于 4 ℃避光贮存。

4.10 固相萃取小柱：Waters oasis HLB 柱，500 mg，6 mL，或相当者。

4.11 微孔滤膜：0.45 μm，有机相。

5 仪器和设备

5.1 液相色谱-串联四极杆质谱仪：配有电喷雾离子源。

5.2 分析天平：感量分别为 0.1 mg 和 0.01 g。

5.3 绞肉机。

5.4 涡旋混合器：2 000 r/min。

5.5 离心机:3 000 r/min。

5.6 旋转蒸发器。

5.7 固相萃取装置。

5.8 具塞离心管:聚四氟乙烯,15 mL 和 50 mL。

6 试样制备与保存

从所取动物肌肉、肝脏、肾脏全部样品组织中取出有代表性样品约 0.5 kg,用绞肉机充分绞碎混匀均分成两份。制备好的试样置于样品瓶中,密封,并标明标记,于−18 ℃以下冷冻存放。

试样制备与保存过程中应防止样品受到污染或发生残留物含量的变化。

7 测定步骤

7.1 提取

称取 5 g 试样(精确到 0.01 g)于 50 mL 离心管(5.8)中,加入 25 mL 乙酸乙酯(4.1),2 000 r/min 涡旋混匀 2 min,3 000 r/min 离心 5 min,收集提取液至浓缩瓶中。残渣再加入 20 mL 乙酸乙酯,重复上述抽提操作。合并提取液,50 ℃以下旋转浓缩至近干。用 4 mL 正己烷(4.2)和 4 mL 水依次将残渣转移至 15 mL 离心管(5.8)中,2 000 r/min 涡旋混匀 1 min,3 000 r/min 离心 3 min,弃去上层正己烷溶液,再加入 4 mL 正己烷,重复上述脱脂操作,所得水层提取液待净化。

7.2 净化

分别用甲醇(4.3)和水各 3 mL 对 Oasis HLB 固相萃取小柱(4.10)进行活化预处理,然后将测定步骤 7.1 所得水层提取液转移至小柱上,控制流出速度小于 2 mL/min。用 10 mL 水分次洗涤离心管,洗涤液淋洗 Oasis HLB 小柱后弃去。用 6 mL 甲醇洗脱,收集全部洗脱液,加水定容至 10 mL,混匀后过 0.45 μm 滤膜(4.11),供液相色谱-串联质谱测定。

7.3 硫粘菌素基质校准标准溶液的配制

称取七个基质空白样品(称样量为 5 g)于 50 mL 离心管(5.8)中,其中六个空白样品中分别加入适量硫粘菌素标准工作溶液(4.9),按照样品操作步骤 7.1、7.2 同步操作,使最终样品溶液中硫粘菌素的浓度分别为 2.5 ng/mL、5.0 ng/mL、10 ng/mL、20 ng/mL、50 ng/mL、100 ng/mL。所得系列硫粘菌素基质标准校准溶液即配即用,用以制作标准曲线。

7.4 测定

7.4.1 液相色谱条件

a) 色谱柱:ZORBAX Eclipse XDB-C_{18},150 mm×2.1 mm(内径),5 μm,或相当者;

b) 进样量:5.0 μL;

c) 流速:0.3 mL/min;

d) 流动相组成及梯度洗脱程序见表 1。

表 1 液相色谱梯度洗脱程序

时间/min	甲醇/%	0.1%乙酸铵/%	乙腈/%
0.0	40	20	40
2.0	30	40	30
4.0	30	40	30
4.01	40	20	40
7.0	40	20	40

7.4.2 质谱条件

a) 离子源:电喷雾离子源;

b) 扫描方式:正离子扫描;

c) 检测方式:多反应监测;

d) 雾化气、气帘气、辅助气、碰撞气均为高纯氮气;使用前应调节各参数使质谱灵敏度达到检测要求,质谱条件参见附录 A。

7.4.3 液相色谱-质谱/质谱测定

7.4.3.1 定性测定

在相同试验条件下,样品中待测物质与同时检测的浓度相近的基质校准标准溶液具有相同的保留时间,并且所选择的离子相对丰度比相一致或在表 2 规定的允许偏差范围内,则可判断样品中存在对应的被测物。在上述实验条件下,硫粘菌素的保留时间约为 2.74 min,定性离子对(m/z)为 494.2/191.9;494.2/163.4;494.2/118.8,定量离子对为 494.2/191.9,参考离子相对丰度比(%)分别为 100.00;32.92;28.31,相应的谱图参见附录 B。

表 2 定性确证时离子相对丰度比的最大允许偏差

相对离子丰度比/%	>50	>20～50	>10～20	≤10
允许的相对偏差/%	±20	±25	±30	±50

7.4.3.2 定量测定

在仪器最佳工作条件下,对硫粘菌素的基质校准标准溶液分别进样,以峰面积为纵坐标,系列基质校准标准溶液浓度为横坐标绘制标准工作曲线,用标准工作曲线对样品进行定量,样品溶液中待测物的响应值均应在仪器测定的线性范围内。

7.5 空白试验

除不加试样外,均按上述步骤操作。

8 结果计算

按式(1)计算试样中硫粘菌素残留量:

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

式中:

X——试样中硫粘菌素残留量,单位为微克每千克(μg/kg);

c——从标准曲线中得到的被测组分溶液浓度,单位为纳克每毫升(ng/mL);

c_0——从标准曲线中得到的空白试验中被测组分的溶液浓度,单位为纳克每毫升(ng/mL);

V——样品溶液最终定容体积,单位为毫升(mL);

m——最终样液所代表试样质量,单位为克(g)。

9 测定低限和回收率

9.1 测定低限

本方法的测定低限为 10 μg/kg。

9.2 回收率

不同样品基质,不同添加浓度下的硫粘菌素回收率范围数据见表 3。

表 3 不同基质中不同硫粘菌素添加水平的回收率范围

基质	添加水平/(μg/kg)	回收率/%
猪肉	10	76.3～87.8
	20	82.5～96.5
	40	92～99.5

表 3（续）

基质	添加水平/(μg/kg)	回收率/%
猪肝	10	78.3～85.7
	20	81～92.5
	40	86.3～94.8
猪肾	10	82～93.2
	20	83.5～92.5
	40	92～98.3

附 录 A
（资料性附录）
质 谱 条 件[1)]

质谱条件：

a） 电喷雾电压(IS)：4 500 V；

b） 雾化气压力(GS1)：137.9 kPa(20 psi)；

c） 气帘气压力(CUR)：130.425 kPa(15 psi)；

d） 碰撞气压力(CAD)：75.845 kPa(11 psi)；

e） 辅助气流速(GS2)：35 L/min；

f） 离子源温度(TEM)：600 ℃；

g） Q1、Q3 均为单位分辨率(Unit)；

h） 硫粘菌素的定性离子对、定量离子对、采集时间、去簇电压(DP)、入口电压(EP)、碰撞能量(CE)及碰撞室出口电压(CXP)等参数见表 A.1。

表 A.1 硫粘菌素的主要质谱参考条件参数

化合物	定性离子对(m/z)	定量离子对(m/z)	采集时间/ms	去簇电压(DP)/V	入口电压(EP)/V	碰撞能量(CE)/V	碰撞室出口电压(CXP)/V
硫粘菌素	494.2/191.9	494.2/191.9	200	106	10	32	14
	494.2/163.4		200	50	10	44	9
	494.2/118.8		200	50	10	32	10

1） 附录 A 所列参数是在 API4000 质谱仪上完成的，此处列出实验用仪器型号仅供参考，不涉及商业目的，鼓励标准使用者尝试不同厂家和型号的仪器。

附 录 B
（资料性附录）
XIC 和离子相对丰度比图谱

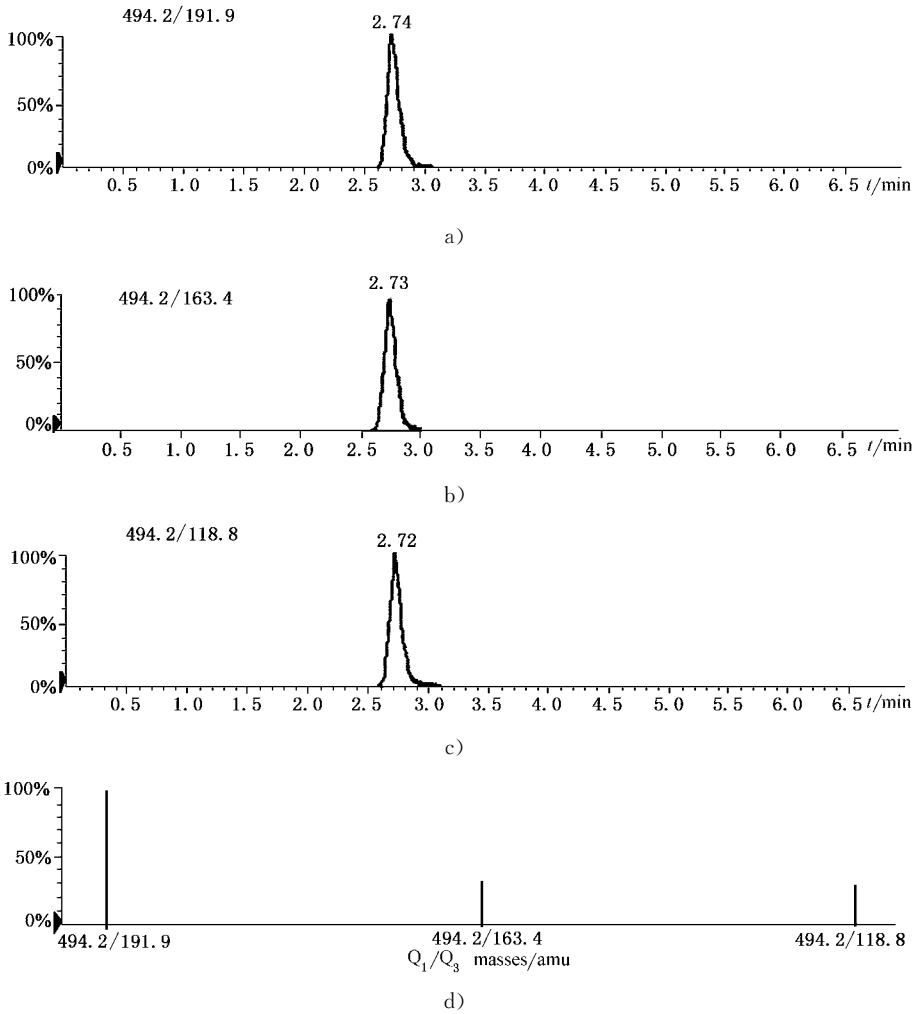

图 B.1 硫粘菌素的提取离子流图(XIC)以及离子相对丰度比图谱

Foreword

Annex A and Annex B of this standard are informative annexes.

This standard was proposed by and is under the charged of certification and accreditation administation of the People's Republic of China.

This standard was drafted by Liaoning Entry-Exit Inspection and Quarantine Bureau of the People's Republic of China，Jiangxi Entry-Exit Inspection and Quarantine Bureau of the People's Republic of China and Dalian Polytechnic University.

The standard was mainly drafted by Sun Xing-quan，Lin Wei-xuan，Wen Zhi-hai，Zhang Yu-cang.

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

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

1 Scope

The standard specifies the methods for determination of tiamulin residues in animal food by high performance liquid chromatography and tandem mass spectrometry.

The standard is applicable to the determination of tiamulin residues in muscle, liver and kidney of pig samples.

2 Reference

For the files with an indication of date, the sequencely modified versions (disincluding the contents of errata) or revised versions of them would not be applicable to this standard. However, it is encouraged for those have reached an agreement according to this standard to research whether to use the latest version of these documents. For the files without an indication of date, their lastest versions would be applicable to this standard.

GB/T 6682 Specification and test method for water used in analytical lab (GB/T 6682—2008, ISO 3696:1987, MOD)

3 Principle

Tiamulin residue is extracted from the sample with ethyl acetate. And the extract is defatted with *n*-hexane and cleaned up with hydrophilic—lipophilic balance (HLB) solid phase extraction column sequently. Finally the analyte is determinated by high performance liquid chromatography and tandem mass spectrometry, using external standard method.

4 Reagents and materials

Unless otherwise specified, all the reagent is analystic grade and "water" is deionized water.

4.1 Ethyl acetated: HPLC grade.

4.2 *n*-Hexane: HPLC grade.

4.3 Methanol: HPLC grade.

4.4 Acetonitril: HPLC grade.

4.5 Ammonium acetate (NH_4Ac).

4.6 0.1% NH_4Ac: Weigh 1.0 g NH_4Ac and desolve in 1 000 mL water.

4.7 Tiamulin($C_{28}H_{47}NO_4S$): CAS No: 55297-95-5, purity≥98.8%.

4.8 Stock solution of tiamulin: 1.0 mg/mL. Weigh certain amount of the standard accurately, and dissolve it with methanol to prepare a stock solution which concentration is 1.0 mg/mL. The solution could be available if it is stored at −18 ℃ in refrigerator avoiding light.

4.9 Working solution of tiamulin: 1.0 μg/mL. Certain amount of tiamulin stock solution of tiamulin was diluted with methanol into a kind of tiamulin working solution which concentration is 1.0 μg/mL. The solution could be available if it is stored at 4 ℃ in refrigerator avoiding light.

4.10 HLB SPE column: 500 mg, 6 mL or equivalent.

4.11 Film: 0.45 μm, Organic phase.

5 Apparatus and equipment

5.1 Liquid chromatography with electrospray ionization mass spectrometry.

5.2 Balance whose sensitivity should be 0.1 mg or 0.01 g.

5.3 Meat Mixer.

5.4 Vortex mixer.

5.5 Centrifuge: 3 000 r/min.

5.6 Rotary vacuum evaporator.

5.7 Apparatus for solid phase extraction.

5.8 Centrifuge tube with cap: 15 mL and 50 mL.

6 Preparation and storage of test sample

Take a representative portion whose weight is about 0.5 kg from the whole primary sample such as musculature, liver and kidney and ground it in a blender. Depart and keep the prepared sample into

two sample bottles equally before seal and label, then stored them in −18 ℃ refrigerator. It is important for the detecting analyte to avoiding pollution and keep its concentration without varied during the whole storing period.

7 Procedure

7.1 Extraction

Weigh 5 g of the test sample (accurate to 0.01 g) into a 50 mL centrifuge tube. Add 25 mL ethyl acetate into the tube and homogenize for 2 min at 2 000 r/min. Centrifuge the mixture at 3 000 r/min for 5 min, and the supernatant layer was displaced into a bottle for rotary evaporation. Repeat the extracting procedure with 20 mL ethyl acetate and combined the extracted solution. Then reduced pressure evaporate the solution to nearly dryness in a water bath blow 50 ℃. Add 4 mL *n*-hexane and 4 mL water to dissolve residues and transfer them into a 15 mL centrifuge tube sequencely. Blend the mixture for 1 min under 2 000 r/min, centrifuge for 3 min under 2 000 r/min, and discard the supernatant layer. Add 4 mL *n*-hexane to repeat the defatting procedure.

7.2 Clean up

Transfer the analyte solution in water layer into the HLB SPE column which has been processed with 3 mL methanol followed by 3 mL water sequencely, and making the flow rate below 2 mL/min in the control. Rinse the SPE column with 10 mL water, then discard the eluate. Collect all the eluted solution after elute the column with 6 mL methanol and adjust the elutate volume to 10 mL with water. Mix the solution and filter it through 0.45 μm film. The filtrate is ready for LC-MS/MS determination.

7.3 Preparation of Tiamulin standard solution calibrated by Matrix

Weigh seven negative matrix samples whose weight is 5 g respectively into 50 mL centrifuge tube, and spike a certain amount of tiamulin working solution into six of them. Then process these samples according to the analytic steps 7.1 and 7.2 as the procedure mensioned above to prepare the standard solutions which concentration are 2.5 ng/mL, 5.0 ng/mL, 10 ng/mL, 20 ng/mL, 50 ng/mL, 100 ng/mL respectively. The Tiamulin standard solutions calibrated by Matrix are made for obtaining a standard curve and should be used after prepared timely.

7.4 Determination

7.4.1 HPLC operating conditions

a) Column: ZORBAX Eclipse XDB-C_{18}, 150 mm × 2.1 mm (i.d.), 5 μm, or the equivalent;

b) Injection volume: 5.0 μL;

c) Flow rate: 0.3 mL/min;

d） Mobile phase gradient see table 1.

Table 1—Mobile phase gradient of tiamulin

Time/min	Methanol/%	0.1% Ammonium acetate/%	Acetonitrile/%
0.0	40	20	40
2.0	30	40	30
4.0	30	40	30
4.01	40	20	40
7.0	40	20	40

7.4.2 Mass spectral acquisition

a） Source：ESI；

b） Polarity：positive；

c） Mode：multiple reaction monitoring；

d） Gas1、Gas2 and Curtain gas are nitrogen. Adjust the parameters to satisfy the sensitivity need of mass spectrometry before used and the other detail parameters see annex A.1.

7.4.3 LC-MS/MS determination

7.4.3.1 Confirmation of LC-MS/MS

Under the same LC-MS/MS conditions，the standard working solutions and the test sample solutions are injected. If the retention time and the relative abundance ratios for the precursor and the product ion pairs of the test sample solution match that of the standard working solution which has calibrated by matrix within a proper range respectively（see table 2），the corresponding analyte must be presented in the test samples. At the test conditions montioned above，the retention time of tamulin is about 2.7 min；the precursor and the product ion pairs（m/z） for qualification are 494.2/191.9，494.2/163.4 and 494.2/118.8 respectively，the precursor ion pairs（m/z） for quantitation is 494.2/191.9，and the relative abundance ratio（%） of them are about 100.00，32.92 and 28.31 respectively. Those relative graphically information was presented in Annex B.

Table 2—Maxium allowable deviation of relative abundance ratio while confirmation

Relative abundance ratio/%	＞50	＞20～50	＞10～20	≤10
Maximum allowable deviation/%	±20	±25	±30	±50

7.4.3.2 Quantitation of LC-MS/MS

Under the optimal condition of LC-MS/MS，a serial of standard solutions of tiamulin which have been

calibrated by matrix is injected to obtain a standard working curve whose y-axis represents peak area and x-axis the concentration. The analyte concentration in the test samples solution should be quantified depending on the curve and its response value should be within the linear range of the instrument.

7.5 Blank test

The operation of the blank test is the same as the described in the method of determination, but with the omission of sample addition.

8 Calculation and expression of result

Calculation the content of tiamulin residue in the test sample according to the formula (1).

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

Where:

X—the residue content of tiamulin in the test sample, μg/kg;

c—the tiamulin concentration of test sample obtained from the standard working curve, ng/mL;

c_0—the tiamulin concentration of test sample in the blank test obtained from the standard working curve, ng/mL;

V—the final volume of the sample solution, mL;

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

9 Limit of quantification (LOQ) and recovery

9.1 Limit of quantification

The limit of quantification of tiamulin is 10 μg/kg.

9.2 Recovery

The recovery range of tiamulin with different spiking levels in different matrix see table 3.

Table 3—The recovery range of tiamulin with different spiking levels in different matrix

Matrix	Spiking level/(μg/kg)	Recovery/%
Muscle of pig	10	76.3～87.8
	20	82.5～96.5
	40	92～99.5
Liver of pig	10	78.3～85.7
	20	81～92.5
	40	86.3～94.8
Kidney of pig	10	82～93.2
	20	83.5～92.5
	40	92～98.3

Annex A
(informative)
Mass spectral acquisition[1)]

Mass spectral acquisition:

a) IS:4 500 V;

b) GS1:137.9 kPa(20 psi);

c) CUR:130.425 kPa(15 psi);

d) CAD:75.845 kPa(11 psi);

e) GS2:35 L/min;

f) TEM:600 ℃;

g) Q1 and Q3 scaned with unit resolution mode;

h) The other reference mass spectrometry parameters see the table A.1.

Table A.1 **Mass spectrometry parameters of tiamulin**

Compound	Ion pairs for qualitative analysis	Ion pairs for quantitative analysis	Dwell time/ms	DP/V	EP/V	CE/V	CXP/V
tiamulin	494.2/191.9	494.2/191.9	200	106	10	32	14
	494.2/163.4		200	50	10	44	9
	494.2/118.8		200	50	10	32	10

1) The parameter listed in annex A is obtained from the instrument of API 4 000 mass spectrometry. The instrument type amentioned above is aimed to proving some reference for user and do not involve in the commercial activity.

Annex B
（informative annex）

The mass chromatography and the reference relative abundance ratios for the precursor and the product ion ratio of tiamulin：

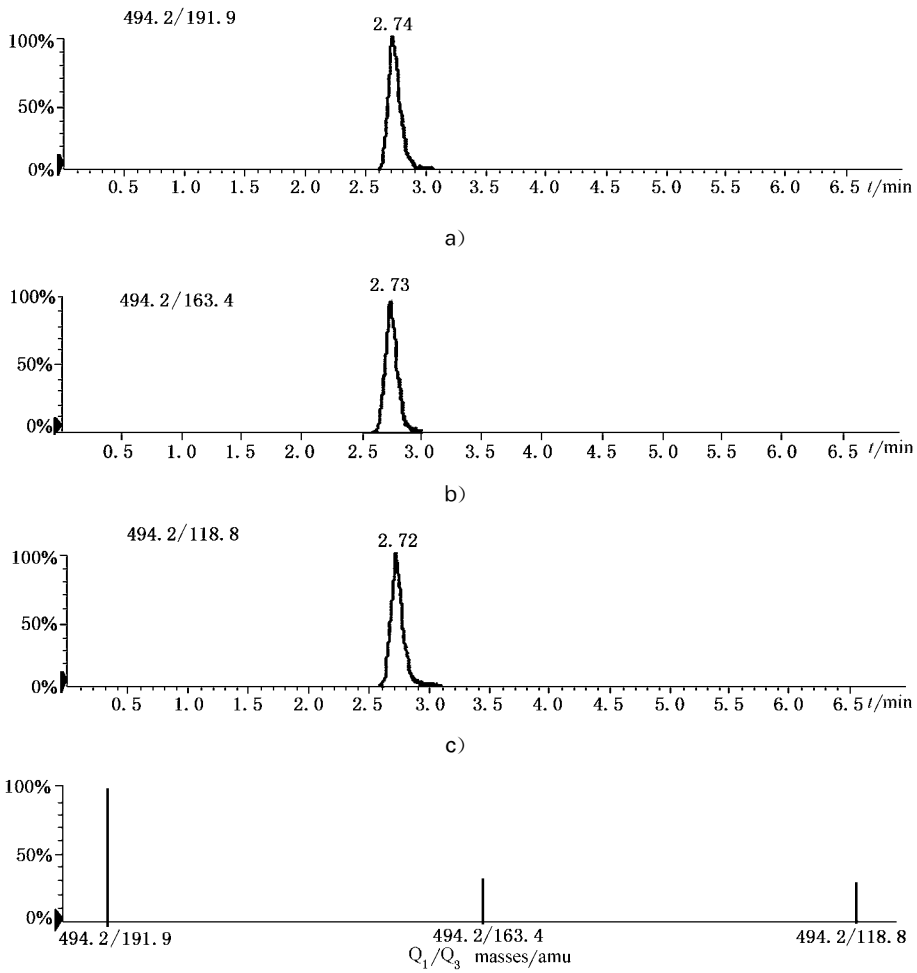

Figure B.1—Extract ion chromatography（XIC）
and the reference relative abundance ratios of tiamulin

中华人民共和国出入境检验检疫
行 业 标 准
进出口动物源性食品中硫粘菌素
残留量检测方法
液相色谱-质谱/质谱法
SN/T 2223—2008
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