SN

中华人民共和国出入境检验检疫行业标准

SN/T 2225—2008

进出口动物源性食品中硫普罗宁及其代谢物残留量的测定　液相色谱-质谱/质谱法

Determination of tiopronin and its metabolites residues 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、附录B、附录C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：中华人民共和国江苏出入境检验检疫局、中华人民共和国湖北出入境检验检疫局、中华人民共和国湖南出入境检验检疫局、中华人民共和国辽宁出入境检验检疫局。

本标准主要起草人：徐锦忠、林宏、张晓燕、丁涛、黄娟、刘艳、张婧、戴华、胡小钟、林维宣、吴斌、蒋原、陶宏锦。

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普罗宁及其代谢物残留量的测定　液相色谱-质谱/质谱法

1　范围

本标准规定了进出口动物源性食品中硫普罗宁及其代谢物 2-巯基丙酸残留量测定的液相色谱-质谱/质谱检测方法。

本标准适用于进出口牛肉、牛奶，牛肝及牛肾中硫普罗宁及其代谢物 2-巯基丙酸残留的测定和确证。

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

试样中残留的硫普罗宁及其代谢物在三羟甲基氨基甲烷-乙二胺四乙酸二钠(Tris-EDTA)缓冲体系中用丙烯酸甲酯衍生化，调节 pH 值后，用乙酸乙酯提取，再经阴离子固相萃取小柱净化，用液相色谱-串联质谱进行定量和确证，外标法定量。

4　试剂和材料

除非另有说明，所用试剂均为分析纯，水为 GB/T 6682 规定的一级水。

4.1　甲酸：HPLC 级。

4.2　甲醇：HPLC 级。

4.3　乙腈。

4.4　乙酸乙酯。

4.5　盐酸。

4.6　三氯乙酸。

4.7　三羟甲基氨基甲烷(Tris)：生化试剂。

4.8　丙烯酸甲酯。

4.9　二水合乙二胺四乙酸二钠(EDTA)。

4.10　1 mol/L 盐酸溶液：量取 40 mL 浓盐酸，用水稀释至 500 mL。

4.11　7%丙烯酸甲酯乙腈溶液：量取 7 mL 丙烯酸甲酯加入 93 mL 乙腈中混匀。

4.12　5%甲酸甲醇溶液：量取 5 mL 甲酸加入 95 mL 甲醇中混匀。

4.13　40%甲醇水溶液：量取 40 mL 甲醇加入 60 mL 水中混匀。

4.14　Tris-EDTA 缓冲溶液：称取 6 g Tris 和 0.93 g EDTA 溶于 400 mL 水中，用 1 mol/L 盐酸(4.10)调 pH 到 9.1 后稀释至 500 mL。

4.15　20%三氯乙酸溶液：称取 20 g 三氯乙酸加入 80 mL 水中溶解混匀。

4.16　阴离子交换萃取小柱：混合型阴离子交换填料(MAX)，60 mg/3 mL 或相当者。

4.17 硫普罗宁(CAS号:1953-02-2,分子式:$C_5H_9NO_3S$)标准品,纯度大于等于99.0%。

4.18 2-巯基丙酸(CAS号:79-42-5,分子式:$C_3H_6O_2S$)标准品,纯度大于等于99.0%。

4.19 标准储备液:准确称取适量的硫普罗宁及其代谢物,分别用甲醇分别配制成1 mg/mL的标准储备液,−20 ℃避光保存。再用乙腈稀释配制1.0 μg/mL的混合标准溶液,4 ℃避光保存。有效期1个月。

4.20 空白样品提取液:用不含硫普罗宁及其代谢物的样品,按照7.1和7.2制备空白样品溶液。

4.21 标准工作溶液:根据需要用空白样品溶液将标准储备液稀释成10 ng/mL、20 ng/mL、50 ng/mL、100 ng/mL、200 ng/mL的混合标准工作溶液,现配。

4.22 滤膜:0.45 μm,有机相。

5 仪器和设备

5.1 液相色谱-质谱/质谱联用仪,配有电喷雾(ESI)源。

5.2 分析天平:感量0.1 mg,0.01 g。

5.3 涡旋混合器。

5.4 离心机:5 000 r/min。

5.5 无油真空泵。

5.6 旋转蒸发仪。

5.7 固相萃取装置。

5.8 高速均质器。

5.9 氮气吹干仪。

5.10 超声波发生器。

6 试样制备和保存

牛肉、牛肝和牛肾取样品中有代表性的约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。

牛奶: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。

7 测定步骤

7.1 提取

称取2 g试样(精确到0.01 g)于50 mL离心管中,加入10 mL Tris-EDTA缓冲液(4.14),再加入0.4 mL 7%丙烯酸甲酯乙腈(4.11)溶液,涡旋30 s,室温超声30 min。加入1 mL 20%三氯乙酸水溶液(4.15),混匀后加入10 mL乙酸乙酯(4.4),涡旋1 min,离心5 min(4 000 r/min),将上清液转移到50 mL离心管中,用10 mL乙酸乙酯(4.4)重复提取一次,合并上清液。在40 ℃减压旋转蒸发至干。

7.2 净化

依次用3 mL甲醇活化,3 mL水平衡阴离子固相萃取小柱(4.16)。用6 mL Tris-EDTA(4.14)分三次(每次2 mL)涡旋振荡溶解7.1提取液残留物,并依次过柱,控制固相萃取柱上样流速约为1.0 mL/min,依次用3 mL水、3 mL甲醇淋洗固相萃取柱,弃去上述滤液。用3 mL甲酸甲醇溶液(4.12)洗脱,洗脱流速约为1 mL/min,收集洗脱液,在40 ℃氮气吹干,用1.0 mL甲醇水溶液(4.13)定容,充分振荡溶解残渣,用0.45 μm滤膜过滤,待测。

7.3 测定

7.3.1 色谱条件

a) 色谱柱:Ultimate AQ C_{18}柱150 mm×2.1 mm(内径),5 μm,或相当者;

b） 柱温：25 ℃；

c） 进样量：25 μL；

d） 梯度洗脱，洗脱程序参见附录 A 表 A.1。

7.3.2 质谱条件

a） 离子源：电喷雾源（ESI），负离子；

b） 扫描方式：选择反应监测（SRM）；

c） 雾化气、气帘气、碰撞气均为高纯氮气或其他高纯气体；使用前应调节各气体流量以使质谱灵敏度达到检测要求；

d） 喷雾电压、去集簇电压、碰撞能等电压值应优化至最优灵敏度。

7.3.3 色谱测定

根据试样中被测物的含量情况，选取响应值适宜的标准工作液进行色谱分析，标准工作液应有六个浓度水平（包括零点）。标准工作液和待测样液中硫普罗宁及其代谢物 2-巯基丙酸的响应值均应在仪器线性响应范围内。上述色谱条件下，硫普罗宁衍生物的保留时间约为 7.8 min，代谢物 2-巯基丙酸衍生物的保留时间约为 9.4 min、母离子和子离子参见附录 B 中表 B.2。标准品选择离子流图参见附录 C 中图 C.1。

7.3.4 定性测定

进行样品测定时，如果检出的质量色谱峰保留时间与标准样品一致，并且在扣除背景后的样品谱图中，各定性离子的相对丰度与浓度接近的同样条件下得到的标准溶液谱图相比，最大允许相对偏差不超过表 1 中规定的范围，则可判断样品中存在对应的被测物。

表 1 定性确证时相对离子丰度的最大允许相对偏差

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

7.3.5 空白实验

除不称取样品外，均按上述测定条件和步骤进行。

8 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，按式（1）计算试样中硫普罗宁及其代谢物药物的残留量：

$$X_i = \frac{(c_i - c_{0i}) \cdot V}{m \times 1\,000} \qquad \cdots\cdots\cdots\cdots (1)$$

式中：

X_i——试样中硫普罗宁药物及其代谢物残留量，单位为毫克每千克（mg/kg）；

V——样品最终定容体积，单位为毫升（mL）；

c_i——由标准曲线而得的样液中硫普罗宁药物及其代谢物药物的含量，单位为微克每升（μg/L）；

c_{0i}——由标准曲线而得的空白实验中硫普罗宁药物及其代谢物药物的含量，单位为微克每升（μg/L）；

m——试样量，单位为克（g）。

9 测定低限与回收率

9.1 测定低限

本方法的测定牛奶中低限硫普罗宁及其代谢物均为：0.010 mg/kg。

本方法的测定牛肉、牛肝和牛肾中低限硫普罗宁及其代谢物均为：0.050 mg/kg。

9.2 回收率

回收率见表2。

表2 硫普罗宁及其代谢物添加回收结果

样品	添加水平/(mg/kg)	回收率/%	
		硫普罗宁	2-巯基丙酸
牛奶	0.010	75.2～91.5	71.8～94.4
	0.020	73.4～92.7	71.5～85.2
	0.040	76.1～100.3	75.4～93.3
牛肉	0.050	71.7～89.3	73.2～87.1
	0.100	72.3～82.2	71.4～92.3
	0.200	77.2～94.8	73.6～83.4
牛肝	0.050	71.1～89.5	70.4～85.3
	0.100	74.9～95.1	70.7～93.3
	0.200	71.8～84.8	71.6～88.9
牛肾	0.050	64.8～97.3	75.1～84.3
	0.100	71.4～102.5	72.2～90.6
	0.200	63.7～82.9	68.7～89.1

附　录　A

（资料性附录）

液相色谱-质谱/质谱法与高效液相色谱法梯度洗脱条件

表 A.1　液相色谱-质谱/质谱法梯度洗脱程序

时间/min	流速/(μL/min)	甲醇/%	0.1%甲酸/%
0	250	10	90
3.00	250	30	70
4.00	250	30	70
7.50	250	90	10
10.00	250	90	10
10.10	250	10	90
12.00	250	10	90

附　录　B
（资料性附录）
液相色谱-质谱/质谱法仪器参数与监测离子

表 B.1　质谱参数

离子模式	ESI，负离子
源温度/℃	300
气帘气	35
辅助气	5
碰撞气	1.5
喷雾电压/V	3 500 V
数据采集参数	Q1＝0.20，Q3＝0.70

表 B.2　选择离子及碰撞能量

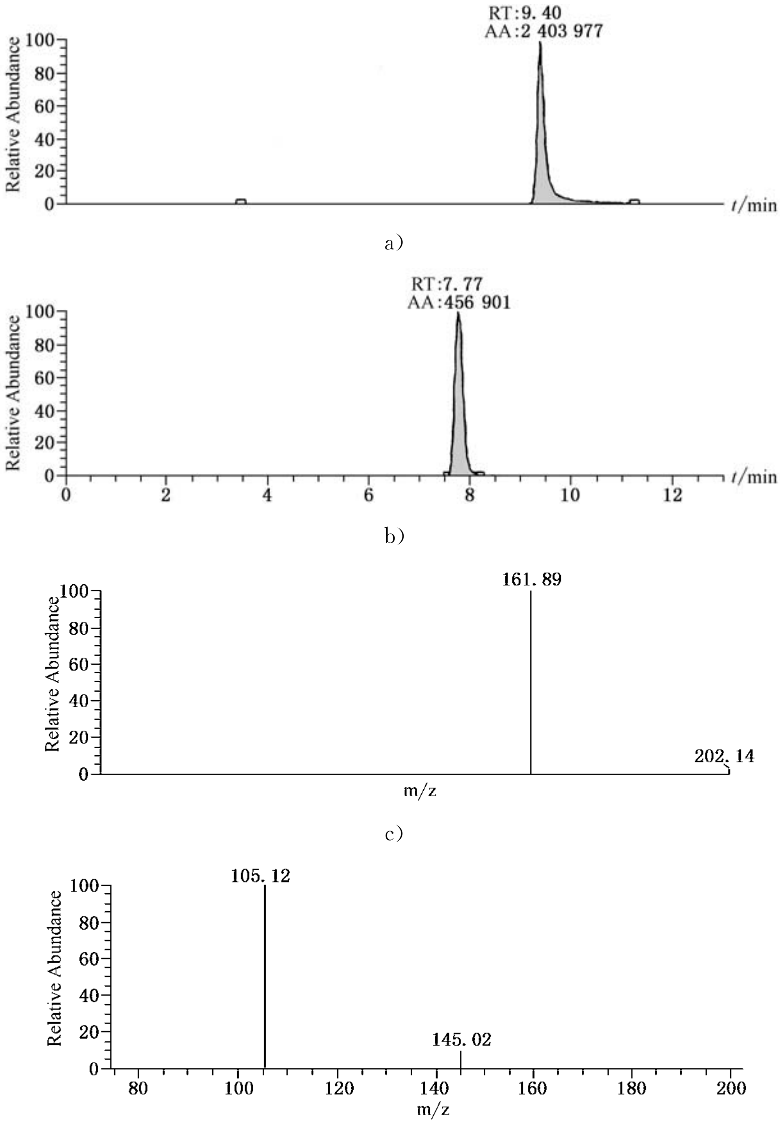
中文名称	简　　写	母离子(m/z)	子离子(m/z)	碰撞能量/eV
硫普罗宁衍生物	TP-MA	248	162[a]	12
			202	12
			74	20
2-巯基丙酸衍生物	2-MPA-MA	191	105[a]	12
			145	10

[a] 为定量离子。

附　录　C

（资料性附录）

液相色谱-质谱/质谱法与液相色谱法标准品谱图



a）

b）

c）

d）

图 C.1　硫普罗宁及其代谢物 2-巯基丙酸标准品衍生物液相色谱-质谱/质谱图

Foreword

Annex A,annex B and annex C of this standard are informative annex.

This standard was proposed by and was under the charge of the Certification and Accreditation Administration of the People's Republic of China.

This standard was drafted by Jiangsu Entry-Exit Inspection and Quarantine Bureau of the People's Republic of China,Hunan Entry-Exit Inspection and Quarantine Bureau of the People's Republic of China,Hubei Entry-Exit Inspection and Quarantine Bureau of the People's Republic of China,Liaoning Entry-Exit Inspection and Quarantine Bureau of the People's Republic of China.

The standard was mainly drafted by Xu Jinzhong,Lin Hong,Zhang Xiaoyan,Ding Tao,Huang Juan,Liu Yan,Zhang Jin,Dai Hua,Hu Xiaozhong,Lin Weixuan,Wu Bin,Jiang Yuan,Tao Hongjin.

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,is only for reference.

Determination of tiopronin and its metabolites residues in foodstuffs of animal origin for import and export—LC-MS/MS method

1 Scope

The standard specifies the method of determination of tiopronin and its metabolites 2-mercaptopropionic acid residues in foodstuffs of animal origin by LC-MS/MS.

The method is applicable to beef, milk, bovine liver and kidney products.

2 Normative references

The following normative documents contain provisions which, through reference in this text, constitute provisions of this standard. For dated references, subsequent amendments to, or revisions of, any of these publications do not apply. However parties to agreements based on this standard are encouraged to investigate the possibility of applying the most recent editions of the normative documents indicated below. For undated references, the latest edition of the normative document referred to applies.

GB/T 6682 Water for analytical laboratory use—Specification and test methods (GB/T 6682—2008, ISO 3696:1987, MOD)

3 Principle

Derivation of the tiopronin and its metabolites residues with methyl acrylate in aminotris(hydroxymethyl)methane-ethylenediaminetetraaceticacid disodium (Tris-EDTA) buffer solution first, and extraction with ethyl acetate, then concentration and dissolution, cleaned up by the anion-exchange cartridge. Determination and confirmation is made by means of liquid chromatography-tandem mass spectrometry, and quantitation is by the external standard method.

4 Reagents and Materials

All reagents shall be of recognized analytical quality unless specified, "water" is the first grade water prescribed by GB/T 6682.

4.1 Formic acid: HPLC grade.

4.2 Methanol: HPLC grade.

4.3 Acetonitrile.

4.4 Ethyl acetate.

4.5 Hydrochloric acid.

4.6 Trichloroacetic acid.

4.7 Aminotris(hydroxymethyl)methane (Tris): biochemical reagent.

4.8 Methyl acrylate.

4.9 Ethylenediamine tetraaceticacid disodium salt (EDTA).

4.10 1 mol/L hydrochloric acid solution: Add 8 mL hydrochloric acid to 92 mL water.

4.11 7% Methyl acrylate acetonitrile solution: Add 7 mL methyl acylate into 93 mL acetonitrile.

4.12 5% Formic acid methanol solution: Add 5 mL formic acid into 95 mL methanol.

4.13 40% methanol water solution: Add 40 mL methanol to 60 mL water.

4.14 Tris-EDTA buffer solution: Weigh 6 g Tris and 0.93 g EDTA and dissolve in 400 mL water, adjust the pH to 9.1±0.1 with 1 mol/L hydrochloric acid (4.10) and dilute to 500 mL with water.

4.15 20% Trichloroacetic acid solution: Dissolve 20 g trichloroacetic acid into 80 mL water.

4.16 Anion exchange cartridge: MAX, 60 mg/3 mL.

4.17 Tiopronin (CAS: 1953-02-2, Molecular formula: $C_5H_9NO_3S$) standard: Purity≥99.0%.

4.18 2-mercaptopropionic acid (CAS: 79-42-5, Molecular formula: $C_3H_6O_2S$) standard: Purity ≥99.0%.

4.19 Stock solutions: weigh appropriate tiopronin and its metabolite standard materials, dissolve with methanol solution to standard solution of 100 μg/mL. And then dilute with methanol solution to standard solution of 1 μg/mL. Store at 2 ℃～8 ℃ in black.

4.20 Blank matrix extract solution: According to 7.1, 7.2 extract the test sample without tiopronin and 2-mercaptopropionic.

4.21 Stock working solutions: dilute the stock solution to the followed five concentrations of 200.0 ng/mL, 100.0 ng/mL, 50.0 ng/mL, 20.0 ng/mL, 10.0 ng/mL. Diluting while use.

4.22 Teflon filter:0.45 μm orgnic filter.

5 Apparatus

5.1 Liquid Chromatography-Mass Spectrometer equipment:equipped with elelectrospray (ESI) ion source.

5.2 Analytical balances:sensitive 0.1 mg,0.01 g.

5.3 Vortex mixer.

5.4 Centrifuge:5 000 r/min.

5.5 Vacuum pump.

5.6 Rotary evaporator.

5.7 Solid phase extraction instrument.

5.8 Tissue triturator.

5.9 Nitrogen evaporator.

5.10 Ultrasonic cleaner.

6 Preparation and storage of test sample

For beef,liver and kidney samples,about 500 g representative edible should be taken from all samples and mixed well by homogenizer,then sealed in clean containers and marked The test sample should be stored at temperature of below −18 ℃.

About 200 g of milk sample should be taken from all samples. Mix throughly and then divide into two equal portions. Each portion of the test sample is put into a clean container,then sealed in clean containers and marked The test sample should be stored at temperature of below −4 ℃.

7 Procedure

7.1 Extraction

Accurately weigh 2 g of the prepared test sample (accurate to 0.01 g) into the 50mL centrifuge tubes,then add 10 mL Tris-EDTA solution(4.14) and 0.4 mL 7% methyl acrylate acetonitrile solution (4.11),vortex for 30 s and sonicate for 30 min at room temperature. Add 1 mL 20% trichloroacetic acid solution (4.15) and mix,then add 10 mL ethyl acetate (4.4) and vortex for 1 min. After that, the tubes are centrifuged at 2 000 r/min for 5 min. Transfer the supernatant to 50 mL centrifuge tube. Ethyl acetate (10 mL) is added to the tube and the sample is extracted, centrifuged and decanted again into the centrifuge tube. Then the extract is evaporated to dryness under reduced press while heating the flsak in a water bath set an 40 ℃.

7.2 Clean-up

MAX cartidge is pre-washed with 3 mL methanol and balanced with 3 mL water. A 3×2 mL Tris-EDTA(4.14) solution is added into the flask which is then swirled to dissolve the residue. The sample extracts is added to the cartidge at the flow rate of 1.0 mL/min. 3 mL of water, 3 mL of methanol are used to rinse the MAX cartridge. The fractions are also discarded. The residues are eluted from the MAX cartridge with 4 mL of 5% formic-methanol (4.12) at the flow rate of 1.0 mL/min and collected in 10 mL centrifuge tube. The collected eluate is dried under a stream of nitrogen gas at 40 ℃. Finally, residual material is dissolved in 1 mL of a mixture of 40% methanol (4.13). After being filtrated with a 0.45 μm filter, the solution is ready for analysis by LC-MS/MS.

7.3 Determination

7.3.1 LC operation conditions

a) Column: Ultimate AQ C_{18} column 150×2.1 mm (i. d.), 5 μm;

b) Column temperature: 25 ℃;

c) Injection volume: 25 μL;

d) Gradient elution procedure: Program pump for the following gradient (annex A table A.1).

7.3.2 MS operation conditions

a) Ion source: ESI, negative ionisation mode;

b) Scan mode: multiple reaction monitoring(SRM) mode;

c) Nebulizer Gas, curtain gas, collision gas and auxiliary gas were all gas of high-purity (nitrogen or other conformable gas). Mass spectrometer sensitivity was optimized by adjusting the gas flow parameters, Referenced conditions seen Annex B table B.1;

d) Calibrate the mass spectrometer and electrospray interface according to the manufacturer's specifications. Optimum parameters for MS/MS.

7.3.3 LC-MS/MS analysis

Prepare standard solutions containing tiopronin and 2-mercaptopropionic acid at appropriate concentrations according to the analyte in sample extracts. At least five levels(including zero) should be used in the construction of the calibration curve. The responses of the calibration solutions and sample solutions should be within the linear range. The retention time was 7.8 min of the tiopronin derivant and 9.4 min of the 2-mercaptopropionic acid derivant. The parent ions and daughter ions of the compounds list in Table B.2. Annex C Figure C.1 is the selected ion chromatogram of the standard solutions.

7.3.4 Qualification

The test samples and standard working solutions are determined at the same conditions above-mentioned. The variation range of the retention time for the peak of analyte in unknown sample and in the standard working solution can not be out of range of ±0.25 min. The qualification ions for every compound must be found, and at least include one precursor ion and two daughter ions. 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 can not be out of range of table 1.

Table 1—Maximum permitted tolerances of relative ion intensities for confirmation

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

7.3.5 Blank test

The reagent blank determination is taken the same complete analytical procedure applied without the test portion or using an equivalent amount of suitable solvent in place of the test portion.

8 Calculation and expression of the result

The calculation of tiopronin and its metabolite residue concentration in the sample is according to the following formula, the blank value should be subtracted from the result of calculation:

$$X_i = \frac{(c_i - c_{0i}) \cdot V}{m \cdot 1\,000} \quad \cdots\cdots (1)$$

Where:

X_i—tiopronin and its metabolite content in the test sample, mg/kg;

V—final volume of sample extract, mL;

c_i—the concentration of tiopronin and its metabolite in aliquot of sample solution injected into LC-MS/MS system, determined from the calibration curve, μg/L;

c_{0i}—the concentration of tiopronin and its metabolite in aliquot of blank solution injected into LC-MS/MS system, determined from the calibration curve, μg/L;

m—mass of sample, g.

9 Limit of quantification and recovery

9.1 Limit of quantification

The limit of quantification of tiopronin and its metabolites is 0.010 mg/kg for milk, and 0.050 mg/kg for bovine beef, liver and kidney.

9.2 Recovery

Recovery see table 2.

Table 2—Recovery

Sample	Spike level/(mg/kg)	Recovery/%	
		Tiopronin	2-Mercaptopropionic Acid
Milk	0.010	75.2～91.5	71.8～94.4
	0.020	73.4～92.7	71.5～85.2
	0.040	76.1～100.3	75.4～93.3
Beef	0.050	71.7～89.3	73.2～87.1
	0.100	72.3～82.2	71.4～92.30
	0.200	77.2～94.8	73.6～83.4
Bovine liver	0.050	71.1～89.5	70.4～85.3
	0.100	74.9～95.1	70.7～93.3
	0.200	71.8～84.8	71.6～88.9
Bovine kidney	0.050	64.8～97.3	75.1～84.3
	0.100	71.4～102.5	72.2～90.6
	0.200	63.7～82.9	68.7～89.1

Annex A

（informative）

LC gradient elution procedure of the LC-MS/MS method

Table A. 1—LC gradient elution procedure of the LC-MS/MS method

Time/min	Flow rate/（μL/min）	Methanol/%	0. 1% Formic acid/%
0	250	10	90
3. 00	250	30	70
4. 00	250	30	70
7. 50	250	90	10
10. 00	250	90	10
10. 10	250	10	90
12. 00	250	10	90

Annex B
(informative)
The condition parameters of the mass detector and the selected ion

Table B. 1—The condition parameters of the mass detector

Ionization mode、polarity	ESI，negative ion
Source Temperature/℃	300
Sheath Gas	35
Auxiliary Gas	5
Collision Gas	1. 5
Spray Voltage/V	3 500
Data acquisition parameters	Q1 ＝0. 20，Q3 ＝0. 70

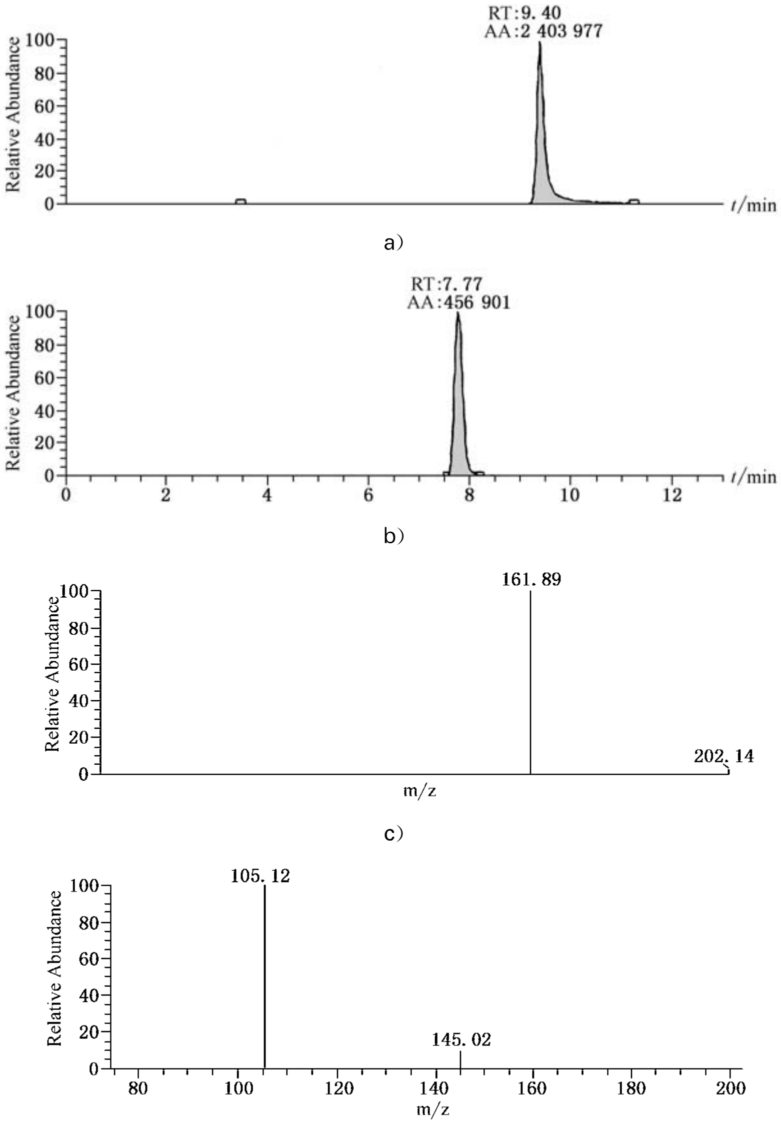
Table B. 2—The selected ion and the collision energy

Analyte	Abbreviation	Parent ion (m/z)	Daughter ions (m/z)	Collision Energy/eV
Tiopronin derivant	TP-MA	248	162[a]	12
			202	12
			74	20
2-Mercaptopropionic acid derivant	2-MPA-MA	191	105[a]	12
			145	10
[a] The daughter ion used for quantification is shown with asterisks.				

Annex C

（informative）

LC-MS/MS chromatogram



a）

b）

c）

d）

Figure C.1—LC-MS/MS chromatogram of the derivants of tiopronin and its metabolites 2-mercaptopropionic acid standard at 50 ng/mL

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