SN

中华人民共和国出入境检验检疫行业标准

SN/T 2063—2008

进出口蜂王浆中氯霉素残留量的检测方法 液相色谱串联质谱法

Determination of chloramphenicol residue in royal jelly for import and export—LC-MS/MS

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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：中华人民共和国浙江出入境检验检疫局。

本标准主要起草人：谢文、丁慧瑛、钱艳、奚君阳、陈笑梅、黄雷芳。

本标准系首次发布的出入境检验检疫行业标准。

进出口蜂王浆中氯霉素残留量的检测方法 液相色谱串联质谱法

1 范围

本标准规定了蜂王浆中氯霉素残留量的制样和液相色谱串联质谱测定方法。

本标准适用于蜂王浆中氯霉素残留量的检测。

2 方法提要

样品用甲醇沉淀蛋白质，再用乙酸乙酯提取，经硅胶和 Oasis HLB 固相萃取小柱净化，液相色谱串联质谱测定和确证，同位素内标法定量。

3 试剂和材料

除另有规定外，所有试剂均为分析纯，水为二次蒸馏水。

3.1 乙酸乙酯：高效液相色谱级。

3.2 甲醇：高效液相色谱级。

3.3 甲苯：高效液相色谱级。

3.4 氯化钠。

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 甲醇水溶液：甲醇-水(2+8，体积比)。

3.7 甲苯-乙腈(9+1，体积比)。

3.8 甲苯-乙腈(4+6，体积比)。

3.9 氯霉素标准品(chloramphenicol，CAS NO. 56-75-7，$C_{11}H_{12}Cl_2N_2O_5$)：纯度大于等于 99%。

3.10 氯霉素-d5 标准品：纯度大于等于 99%。

3.11 氯霉素标准储备溶液：称取适量标准品(3.9)，用甲醇溶解，溶液浓度为 100 μg/mL。1℃～4℃冷藏保存。有效期 6 个月。

3.12 氯霉素-d5 标准储备溶液：称取适量标准品(3.10)，用甲醇溶解，溶液浓度为 100 μg/mL，1℃～4℃冷藏保存。有效期 6 个月。

3.13 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 0.04 ng/mL、0.12 ng/mL、0.24 ng/mL、0.32 ng/mL、0.64 ng/mL 的标准工作溶液，相当于样品中含有 0.1 μg/kg、0.3 μg/kg、0.6 μg/kg、0.8 μg/kg、1.6 μg/kg 氯霉素，同位素内标氯霉素-d5 浓度均为 0.25 ng/mL。

3.14 无水硫酸钠柱：80 mm×40 mm(内径)筒形漏斗，底部垫 5 mm 脱脂棉，再装 40 mm 无水硫酸钠。

3.15 硅胶固相萃取柱：500 mg 或相当者，使用前用 10 mL 甲苯-乙腈(9+1，体积比)预洗。

3.16 Oasis HLB 固相萃取柱：500 mg，或相当者，使用前用甲醇 7 mL 和水 10 mL 预洗。

4 仪器和设备

4.1 高效液相色谱-串联质谱仪：配有电喷雾离子源。

4.2 旋转蒸发器。

4.3 旋涡混合器。

4.4 固相萃取装置。

4.5 高速离心机：7 000 r/min。

4.6 氮吹仪。

5 试样制备与保存

取500 g代表性蜂王浆样品，在室温下解冻，等样品全部融化后搅匀，将试样均分成两份，分别装入样品瓶中，密封，并标明标记。一份作为试验样，另一份在－18℃保存。

在制样的操作过程中，应防止样品污染或发生残留物含量的变化。

6 测定步骤

6.1 提取

称取4 g试样(精确到0.01 g)置于50 mL具塞离心管中，准确加入0.5 mL氯霉素-d5(5 ng/mL)内标溶液和15 mL水，混匀，静置5 min，加甲醇至30.0 mL，于旋涡混合器上以2 000 r/min，混匀1 min，以6 000 r/min离心5 min，移取15.0 mL上层液置于另一个50 mL具塞离心管中，加入2 g氯化钠和20 mL乙酸乙酯，于旋涡混合器上以2 000 r/min，混匀1 min，以4 000 r/min离心5 min，将上层乙酸乙酯过无水硫酸钠柱(3.14)收集于浓缩瓶中，再加入20 mL乙酸乙酯，重复上述操作，合并乙酸乙酯提取液，再用5 mL乙酸乙酯洗涤无水硫酸钠柱，在40℃以下水浴减压浓缩至近干。

6.2 净化

用10 mL乙腈-甲苯(1＋9，体积比)溶液溶解残渣，将溶解液分次转移至硅胶净化柱(3.15)中，弃去流出液，用6 mL乙腈-甲苯(6＋4，体积比)洗脱，在50℃以下水浴下平缓氮气吹干，加入5 mL水溶解残渣，将溶液转移至Oasis HLB柱(3.16)中，再用10 mL水和5 mL甲醇水溶液(3.6)依次洗涤离心管，洗涤液过固相萃取小柱，弃去流出液。在负压下减压抽干，最后用5 mL甲醇洗脱，控制流速1 mL/min～2 mL/min，收集全部洗脱液于10 mL离心管中，洗脱液在50℃以下水浴下平缓氮气吹至约2.5 mL，用水定容至5.0 mL，混匀，将溶液通过0.45 μm滤膜，供液相色谱-串联质谱仪测定。

6.3 测定

6.3.1 液相色谱串联质谱条件

a) 色谱柱：C_8柱，5 μm，150 mm×4.6 mm(内径)或相当者；

b) 流动相见表1；

表1 流动相梯度洗脱

时间/min	水/%	甲醇/%
0	35	65
4	75	25
5.9	75	25
6	35	65
8	35	65

c) 流速：400 μL/min；

d) 进样量：20 μL；

e) 离子源：电喷雾离子源；

f) 扫描方式：负离子扫描；

g) 检测方式：多反应监测；

h) 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，参考条件参见附录A；

i) 监测离子对(m/z):氯霉素 321.0/256.9(定量离子)、321.0/152.0、321.0/194.0、321.0/175.8;氯霉素-d5 326.0/157.1。

6.3.2 液相色谱串联质谱测定

根据试样中被测样液的含量情况,选取待测物的响应值在仪器线性响应范围内的浓度进行测定,如超出仪器线性响应范围应进行稀释。在上述色谱条件下氯霉素、氯霉素-d5 的参考保留时间约为 6.1 min,标准溶液的选择性离子流图参见附录 B 中图 B.1。

6.3.3 液相色谱串联质谱确证

按照液相色谱-串联质谱条件测定样品和标准工作溶液,样品中待测物质的保留时间与标准溶液中待测物质的保留时间偏差在±2.5%之内。定量测定时采用同位素内标标准曲线法。定性时应当与浓度相当标准工作溶液的相对丰度一致,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 定性确证时相对离子丰度的最大允许偏差

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

6.3.4 空白试验

除不加试样外,均按上述操作步骤进行。

7 结果计算和表述

用色谱数据处理机或按下式计算试样中氯霉素残留含量,计算结果需扣除空白值:

$$x = (y - a)/b \qquad \cdots\cdots(1)$$

$$b = n\sum x_i y_i - \sum x_i \sum y_i / \sqrt{n\{\sum x_i^2 - (\sum x_i)^2\}} \qquad \cdots\cdots(2)$$

$$a = (\sum x_i - b\sum x_i)/n \qquad \cdots\cdots(3)$$

式中:

x——样品中氯霉素的残留量,单位为微克每千克(μg/kg);

x_i——标准溶液中氯霉素的含量,单位为微克每千克(μg/kg);

a——工作曲线的截距;

b——工作曲线的斜率;

y——样品溶液中氯霉素峰面积/氯霉素-d5 峰面积;

y_i——标准溶液中氯霉素峰面积/氯霉素-d5 峰面积;

n——工作曲线包括不同浓度的数目。

8 测定低限(LOQ)和回收率

8.1 测定低限(LOQ)

测定低限为 0.3 μg/kg。

8.2 回收率

回收率的实验数据(在不同添加浓度范围内)如下:

——添加浓度在 0.3 μg/kg 时,回收率范围为 83.1%～106.7%;

——添加浓度在 0.6 μg/kg 时,回收率范围为 82.3%～106.8%;

——添加浓度在 0.8 μg/kg 时,回收率范围为 82.6%～107.2%。

附 录 A
（资料性附录）
API 4000 LC-MS/MS 系统电喷雾离子源参考条件[1)]

监测离子对及电压参数：

a) 电喷雾电压(IS)：−4 500 V；

b) 雾化气压力(GS1)：262.01 kPa(38 Psi)；

c) 气帘气压力(CUR)：186.165 kPa(27 Psi)；

d) 辅助气流速(GS2)：310.275 kPa(45 Psi)；

e) 离子源温度(TEM)：525℃；

f) 碰撞气(CAD)：41.37 kPa(6 Psi)；

g) 离子对、去簇电压(DP)、碰撞气能量(CE)及碰撞室出口电压(CXP)见表 A.1。

表 A.1 离子对、去簇电压(DP)、碰撞气能量(CE)及碰撞室出口电压(CXP)

待 测 物	离子对 m/z	去簇电压(DP)/V	碰撞气能量(CE)/V	碰撞室出口电压(CXP)/V
氯霉素	321.0/256.9[a]	−65	−16	−10
	321.0/152.0	−65	−25	−10
	321.0/194.0	−65	−17	−10
	321.0/175.8	−65	−23	−10
氯霉素 d5	326.0/157.1[a]	−65	−25	−10
[a] 为定量离子对。				

1) 非商业性声明：附录 A 所列参数是在 API 4000 质谱仪完成的，此处列出试验用仪器型号仅是为了提供参考，并不涉及商业目的，鼓励标准使用者尝试不同厂家和型号的仪器。

附 录 B
（资料性附录）
氯霉素标准品选择性离子流图

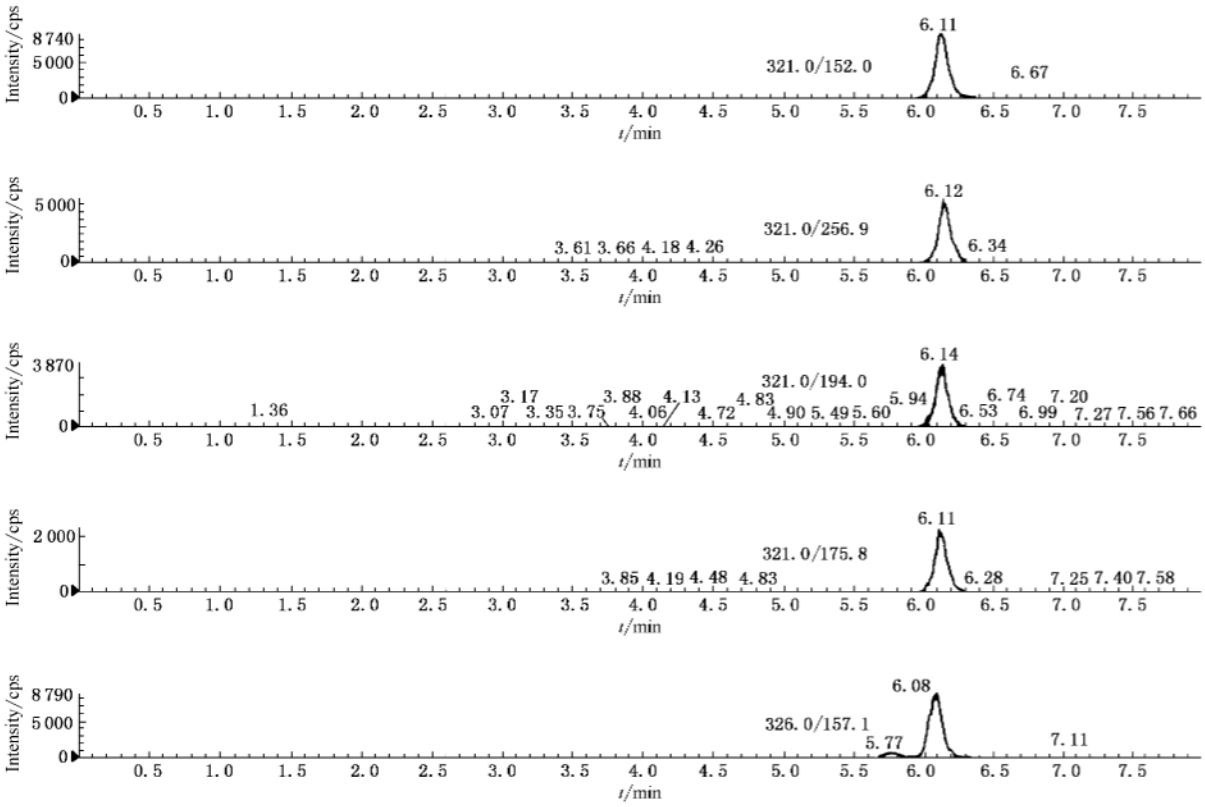

图 B.1 氯霉素(0.24 ng/mL)和氯霉素-d5(0.25 ng/mL)标准品的选择性离子流图

Foreword

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

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 Zhejiang Entry-Exit Inspection and Quarantine Bureau of the People's Republic of China.

The standard was mainly drafted by Wen Xie, Hui-yin Ding, Yan Qian, Jun-yang Xi, Xiao-mei Chen, Lei-fang Huang.

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 solely for guidance.

Determination of chloramphenicol residue in royal jelly for import and export—LC-MS/MS

1 Scope

The standard specifies the methods of sample preparation and determination by LC-MS/MS of chloramphenicol in royal jelly.

This standard is applicable to the determination of chloramphenicol residue in royal jelly sample.

2 Principle

Methanol is used to precipitate protein. The upper layer is extracted with ethyl acetate Then it is evaporated and cleaned up with silica and Oasis HLB columns. Finally it is determined and confirmed by LC-MS/MS. Internal standard method is used.

3 Reagents and materials

Unless otherwise specified, all reagents used should be analytical grade, "water" is double distilled water.

3.1 Ethyl acetate: HPLC grade.

3.2 Methanol: HPLC grade.

3.3 Toluene: HPLC grade.

3.4 Sodium chloride.

3.5 Anhydrous sodium sulphate: Ignite for 4 h at 650℃, cool to room temperature in desiccator and keep in a tightly closed container.

3.6 Methanol water solution: methanol-water (2+8, V/V)

3.7 Toluene-acetonitrile (9+1, V/V)

3.8 Toluene-acetonitrile (4+6, V/V)

3.9 Chloramphenicol (CAS NO. 56-75-7, $C_{11}H_{12}Cl_2N_2O_5$): Purity≥99%.

3.10 Chloramphenicol-d5: Purity≥99%.

3.11 Stock standard solution of chloramphenicol: Accurately weigh appropriate standard (3.9), dissolve with methanol, the concentration of solution is 100 μg/mL. should be stored at 1℃～4℃ in refrigerator. Stock standard solution is stable for 6 months.

3.12 Stock standard solution of chloramphenicol-d5: Accurately weigh appropriate standard (3.10), dissolve with methanol, the concentration of solution is 100 μg/mL. Should be stored at 1℃～4℃ in refrigerator. Stock standard solution is stable for 6 months.

3.13 Calibration curve standard working solutions: Working solutions were prepared in methanol at five concentration levels, 0.04 ng/mL, 0.12 ng/mL, 0.24 ng/mL, 0.32 ng/mL, 0.64 ng/mL. The concentration of CAP-d5 is 0.25 ng/mL. It is same as 0.1 μg/kg, 0.3 μg/kg, 0.6 μg/kg, 0.8 μg/kg, 1.6 μg/kg chloramphenicol in sample.

3.14 Column of anhydrous sodium sulfate: 80 mm × 40 mm (i. d.) cylinder funnel, pack with ca 5 mm absorbent cotton at the bottom of the column and fill in 40 mm anhydrous sodium sulfate.

3.15 Silica column: 500 mg, or equivalent. It is conditioned with 10 mL toluene-acetonitrile (9+1, *V*/*V*).

3.16 Oasis HLB column: 500 mg, or equivalent. It is conditioned with 7 mL methanol followed by 10 mL water.

4 Apparatus and equipment

4.1 Liquid chromatography combined with electrospray ionization mass spectrometry.

4.2 Rotary vacuum evaporator.

4.3 Vortex mixer.

4.4 SPE-12G Column Processor.

4.5 High speed centrifuge: 7 000 r/min.

4.6 Nitrogen evaporator.

5 Preparation of test sample

Royal jelly is about 500 g. The sample is melted under room temperature. Keep the prepared sample into two sample bottles, seal and label. The test sample is stored at room temperature. The rest sample is stored in −18℃ refrigerator.

In the course of sample preparation, precautions must be taken to avoid contamination or any factors, which may cause the change of residue content.

6 Analytical Procedure

6.1 Extraction

Weigh ca 4 g of the test sample (accurate to 0.01 g) into a 50 mL centrifuge tube, add 0.5 mL CAP-d5 (5 ng/mL) and 15 mL water, and mix the solution, standing for 5 min. Adjust volume to 30.0 mL with methanol. Vortex for 1 min under 2 000 r/min, centrifuge for 5 min under 6 000 r/min. Transfer 15.0 mL the supernatant layer into a 50 mL centrifuge tube, add 2 g sodium chloride and 20 mL ethyl acetate. Vortex for 1 min under 2 000 r/min, centrifuge for 5 min under 4 000 r/min. The supernatant layer was passed through anhydrous sodium sulfate column into flask (3.14). Repeat the extraction in the same way with 20 mL ethyl acetate and combined the solution. Ethyl acetate is evaporated to nearly dryness in a water bath below 40℃.

6.2 Clean up

Add 10 mL toluene-acetonitrile (9 + 1, V/V) to dissolve residues. Transfer the solution into silica column (3.15). Discard the eluate. Elute the column with 6 mL toluene-acetonitrile (4 + 6, V/V). The above solution is under a gentle stream of nitrogen gas to nearly dryness. Add 5 mL water to dissolve residues. Transfer the solution into Oasis HLB column (3.16). Rinse the tube and Oasis HLB column with 10 mL water and followed by 5 mL methanol water solution (3.6), discard the eluate. The cartridge is evacuated continuously to "dryness". Elute the column with 5 mL methanol, flow rate is 1 mL/min～2 mL/min, and collect eluted solution. The above solution is under a gentle stream of nitrogen gas to 2.5 mL at 50℃. Adjust volume of eluate to 5.0 mL with water. The solution is passed through 0.45 μm filter. The solution was used for LC-MS/MS determination.

6.3 Determination

6.3.1 LC-MS/MS operating conditions

a) Column: C_8, 5 μm, 150 mm × 4.6 mm(i.d), or the equivalent;

b) Mobile phase: table 1;

Table 1—Gradient of mobile phase

Time/min	Water/%	Methanol/%
0	35	65
4	75	25
5.9	75	25
6	35	65
8	35	65

c) Flow rate: 400 μL/min;

d) Injection Volume:20 μL;

e) Source:ESI;

f) Polarity:Negative;

g) Mode:Multiple reaction monitoring;

h) Carrier gas: Nitrogen (purity>99.999%). Instrumental settings may be optimized. See table A.1 in annex A;

i) Transitions (m/z): CAP 321.0/256.9 (quantification), 321.0/152.0, 321.0/194.0, 321.0/175.8; CAP-d5 326.0/157.1 (quantification).

6.3.2 LC-MS/MS determination

According to the concentrations of analyte in sample solution, contains should be within the linear range of the calibration curve. If it is over the range, the solution should be diluted. Under the above LC-MS/MS operating condition, the retention time of CAP and CAP-d5 are about 6.1 min, selected ion chromatograms of the standards see Figure B.1 in annex B. Internal standard method is used.

6.3.3 LC-MS/MS confirmation

Under LC-MS/MS conditions, the working solution and sample solution are injected. The retention time of the analyte in sample solution shall correspond to that of the analyte in standard solution. Tolerance is within ±2.5%. Calibration curve method with isotope internal standard is used for quantitative measurement. The relative intensities of sample transitions shall correspond to those of standard solution transitions for confirmation. The concentration of standard solution should be same with those of sample solution. The permitted tolerances listed in table 2, then the corresponding analyte must be present in sample.

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

6.3.4 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.

7 Calculation and expression of result

Calculation the content of chloramphenicol residue in the test sample by LC-MS/MS data processor or according to the formula. The blank value should be subtracted from the above result of calculation:

$$x = (y - a)/b \qquad \cdots\cdots(1)$$

$$b = n\sum x_i y_i - \sum x_i \sum y_i / \sqrt{n\{\sum x_i^2 - (\sum x_i)^2\}} \qquad \cdots\cdots(2)$$

$$a = (\sum x_i - b\sum x_i)/n \qquad \cdots\cdots(3)$$

where

x—chlormaphenicol concentration in the sample, μg/kg;

x_i—chlormaphenicol concentration of the standard, μg/kg;

a—intercept of the calibration curve;

b—slope of the calibration curve;

y—area ration of chloramphenicol peak area to chloramphenicol-d5 peak area of the sample;

y_i—area ration of chloramphenicol peak area to chloramphenicol-d5 peak area of the standard;

n—number of analyses per concentration range.

8 Limit of quantification (LOQ) and recovery

8.1 Limit of quantification

The limit of quantification for chloramphenicol is 0.3 μg/kg.

8.2 Recovery

According to the experimental data, the corresponding recoveries of fortifying concentrations are:

—Spike 0.3 μg/kg, the recovery is 83.1%～106.7%;

—Spike 0.6 μg/kg, the recovery is 82.3%～106.8%;

—Spike 0.8 μg/kg, the recovery is 82.6%～107.2%.

Annex A
(informative annex)
API 4 000 LC-MS/MS conditions[1)]

LC-MS/MS conditions:

a) IS: −4 500 V;

b) GS1:262.01 kPa(38 Psi);

c) CUR:186.165 kPa(27 Psi);

d) GS2:310.275 kPa(45 Psi);

e) TEM:525℃;

f) CAD:41.37 kPa(6 Psi);

g) Transitions,DP,CE,CXP see table A.1.

Table A.1—Transitions,DP,CE,CXP

Compound	Transitions m/z	DP/V	CE/V	CXP/V
chloramphenicol	321.0/256.9[a]	−65	−16	−10
	321.0/152.0	−65	−25	−10
	321.0/194.0	−65	−17	−10
	321.0/175.8	−65	−23	−10
chloramphenicol-d5	326.0/157.1[a]	−65	−25	−10
[a] quantification.				

1) Non-commercial statement: the equipments and their models involved in the standard method are not related to commercial motive. The analysts are encouraged to use different equipments and models.

Annex B
(informative annex)
Selected ion chromatograms of chloramphenicol and chloramphenicol-d5 standards.

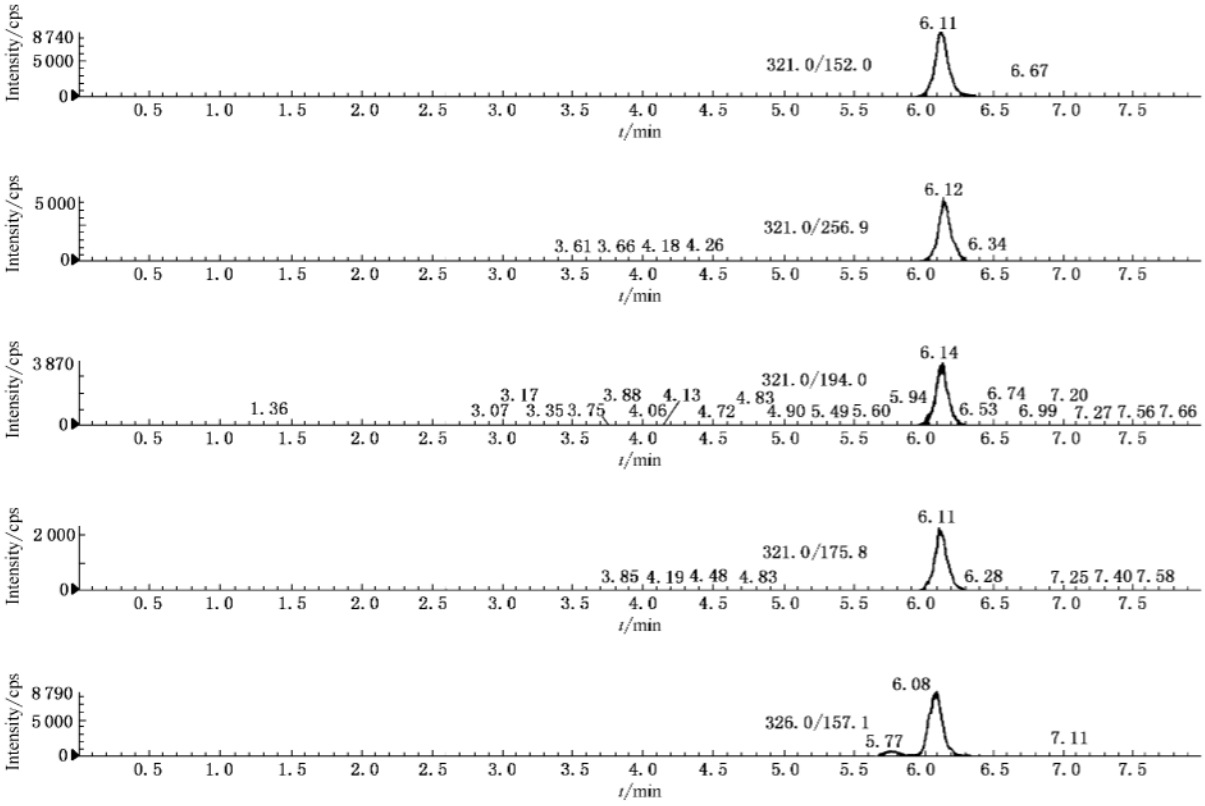

Figure B.1—Selected ion chromatograms of chloramphenicol(0.24 ng/mL)
and chloramphenicol-d5(0.25 ng/mL)