ICS 67.050
C 53

SN

中华人民共和国出入境检验检疫行业标准

SN/T 1626—2019
代替 SN/T 1626—2005

出口肉及肉制品中甲硝唑、替硝唑、奥硝唑、洛硝哒唑、二甲硝咪唑、塞克硝唑残留量测定方法 液相色谱-质谱/质谱法

Determination of metronidazole, tinidazole, ornidazole, ronidazole, dimetridazole and secnidazole residues in meat and meat products for export—LC-MS/MS method

2019-10-25 发布　　2020-05-01 实施

中华人民共和国海关总署　发布

前　言

本标准按照 GB/T 1.1—2009 给出的规则起草。

本标准代替 SN/T 1626—2005《进出口肉及肉制品中甲硝唑、替硝唑、奥硝唑、洛硝哒唑、二甲硝咪唑、塞克硝唑残留量测定方法 高效液相色谱法》。

本标准与 SN/T 1626—2005 相比，主要技术变化如下：

——适用范围增加了牛肉，香肠，罐头，酱牛肉；

——测定物质增加了甲硝唑代谢物即羟基甲硝唑；

——简化了前处理步骤；

——将检测手段由高效液相色谱法改为高效液相色谱串联质谱法，修改了检测方法的测定低限；

——甲硝唑、羟基甲硝唑、洛硝哒唑、二甲硝咪唑及 HMMNI 改为采用同位素内标法定量。

请注意本文件的某些内容可能涉及专利。本文件的发布机构不承担识别这些专利的责任。

本标准由中华人民共和国海关总署提出并归口。

本标准负责起草单位：中国人民共和国石家庄海关。

本标准主要起草人：艾连峰、马育松、张海超、张婧雯、窦彩云、郭春海、陈瑞春、李玮。

本标准所代替标准的历次版本发布情况为：

——SN/T 1626—2005。

出口肉及肉制品中甲硝唑、替硝唑、奥硝唑、洛硝哒唑、二甲硝咪唑、塞克硝唑残留量测定方法 液相色谱-质谱/质谱法

1 范围

本标准规定了出口肉及肉制品中甲硝唑、羟基甲硝唑(甲硝唑代谢物)、洛硝哒唑、二甲硝咪唑、1-甲基-2-羟甲基-5-硝基咪唑(洛硝哒唑与二甲硝咪唑的共同代谢物,简写为 HMMNI)、替硝唑、奥硝唑、塞克硝唑 8 种硝基咪唑残留量的液相色谱-质谱/质谱测定方法。

本标准适用于鸡肉、猪肉、牛肉、香肠、罐头、酱牛肉中甲硝唑、羟基甲硝唑、洛硝哒唑、二甲硝咪唑、HMMNI、替硝唑、奥硝唑、塞克硝唑 8 种硝基咪唑残留量的测定和确证。

2 规范性引用文件

下列文件对于本文件的应用是必不可少的。凡是注日期的引用文件,仅所注日期的版本适用于本文件。凡是不注日期的引用文件,其最新版本(包括所有的修改版)适用于本文件。

GB/T 6682 分析实验室用水规格和试验方法

3 方法提要

试样中的硝基咪唑类药物采用乙酸乙酯提取,提取液经硅胶基质的强阳离子交换固相萃取柱净化,用液相色谱-质谱/质谱仪检测和确证,替硝唑、奥硝唑及塞克硝唑采用外标法定量,其余药物采用内标法定量。

4 试剂和材料

除另有规定外,所用试剂均为分析纯,水为符合 GB/T 6682 规定的一级水。

4.1 乙酸乙酯:液相色谱级。

4.2 甲醇:液相色谱级。

4.3 丙酮:液相色谱级。

4.4 乙腈:液相色谱级。

4.5 氨水:含量(质量分数浓度)为 25%～28%。

4.6 甲酸:液相色谱级。

4.7 乙酸:液相色谱级。

4.8 无水硫酸钠:分析纯。

4.9 乙酸-乙酸乙酯(5+95,体积比):量取 5 mL 乙酸,加入 95 mL 乙酸乙酯,混匀备用。

4.10 氨水-乙腈(5+95,体积比):量取 5 mL 氨水,加入 95 mL 乙腈,混匀备用。

4.11 0.1%甲酸水溶液:量取 1 mL 甲酸(4.6),用水稀释至 1 000 mL。

4.12 8种硝基咪唑及代谢物标准物质：标准品纯度均≥98.0%。各化合物基本信息参见附录A表A.1。

4.13 标准储备溶液：准确称取硝基咪唑标准品10 mg(准确至0.1 mg)，分别用甲醇溶解，并定容到10 mL棕色容量瓶中，配制溶液最终浓度为1 mg/mL，置于冰箱中1 ℃～4 ℃下避光保存。

4.14 混合标准中间液Ⅰ：取羟基甲硝唑和HMMNI标准储备液(4.13)各2 mL，其余标准储备液(4.13)各1 mL，移入100 mL棕色容量瓶中，用甲醇定容。配制混和标液中的羟基甲硝唑和HMMNI终浓度为20 μg/mL，其余药物终浓度为10 μg/mL，置于冰箱中1 ℃～4 ℃下避光保存。

4.15 混合标准中间液Ⅱ：取1 mL混合标准中间液Ⅰ(4.14)于100 mL棕色容量瓶中，用水稀释至刻度。该溶液羟基甲硝唑和HMMNI终浓度为200 ng/mL，其余标准物质浓度为100 ng/mL。置于冰箱中1 ℃～4 ℃下避光保存。

4.16 同位素内标标准物质：甲硝唑-$^{13}C_2{}^{15}N_2$(CAS：1173020-03-5)，羟基甲硝唑-D_2，洛硝哒唑-D_3(CAS：101855-87-4)，二甲硝咪唑-D_3(CAS：64678-69-9)，HMMNI-D_3(CAS：1015855-78-3)标准品纯度均≥98.0%。

4.17 同位素内标储备液：准确称取同位素内标标准品10 mg(准确至0.1 mg)，分别用甲醇溶解，并定容到50 mL棕色容量瓶中，配制终浓度为200 μg/mL储备液，置于冰箱中1 ℃～4 ℃下避光保存。

4.18 同位素内标中间液：移取适当体积各同位素内标储备液(4.17)用甲醇稀释至1 μg/mL，置于冰箱中1 ℃～4 ℃下避光保存。

4.19 同位素内标工作液：移取适当体积同位素内标中间液(4.18)用水稀释至100 ng/mL，置于冰箱中1 ℃～4 ℃下避光保存。

4.20 基质空白溶液：将不同基质的阴性空白样品除不添加内标工作液步骤外分别按照7.1和7.2净化处理后得到的溶液。

4.21 基质标准工作溶液：根据实验需要吸取一定量的混合标准中间液Ⅱ(4.15)和同位素内标工作液(4.19)，用基质空白溶液(4.20)稀释成所需浓度。

4.22 硅胶基质强阳离子交换固相萃取柱：分别用3 mL甲醇和3 mL乙酸-乙酸乙酯(4.9)预洗。整个过程应保持柱体湿润，最后柱上保留约1cm溶液，关闭活塞，柱上端连接贮液器，贮液器底部塞小块脱脂棉，备用。

4.23 微孔滤膜：0.22 μm，有机相型。

5 仪器和设备

5.1 高效液相色谱-质谱/质谱仪：配电喷雾离子源(ESI)。

5.2 组织捣碎机。

5.3 分析天平：感量分别为0.1 mg和0.01 g。

5.4 离心机：转速不低于5 000 r/min。

5.5 均质器，转速不小于10 000 r/min。

5.6 涡旋混匀器。

5.7 氮气吹干仪。

5.8 固相萃取装置。

6 样品制备和保存

6.1 制样要求

在制样的过程中，应防止样品受到污染或发生残留物含量的变化。

6.2 试样制备

取代表性样品500 g，将其可食部分切碎后，用组织捣碎机将样品充分捣碎均匀，装入洁净的盛样容器内，密封并标明标记，于－18 ℃以下冷冻存放。

7 测定步骤

7.1 提取

称取5.0 g(精确至0.01 g)试样，置于50 mL具螺旋盖的聚丙烯离心管中，添加100 μL同位素内标工作液(4.19)离心管中，加入25 mL乙酸乙酯(4.1)，5 g无水硫酸钠(4.8)，10 000 r/min均质1 min，以5 000 r/min离心5 min，所得上层清液转移至另一离心管中，加入0.5 mL乙酸(4.7)涡旋混合，待净化。

7.2 净化

将7.1所得上清液转移至已条件化的硅胶基质的阳离子交换柱(4.22)上的贮液器中，打开活塞，以小于2 mL/min流速滴下。接着依次用3 mL丙酮(4.3)，3 mL甲醇(4.2)淋洗，弃去淋洗液。最后用6 mL氨水乙腈(4.10)洗脱并收集(此过程流速小于2 mL/min)。洗脱液在45 ℃下氮气吹至近干，用水定容至1 mL，涡旋振荡或超声溶解，过0.22 μm滤膜供液相色谱-质谱/质谱仪测定。

7.3 测定

7.3.1 液相色谱参考条件

液相色谱参考条件如下：

a) 色谱柱：C_{18}柱，100 mm×2.1 mm(内径)，粒度1.7 μm，或相当者；
b) 流动相：梯度洗脱程序见表1；
c) 流速：350 μL/min；
d) 柱温：40 ℃；
e) 进样量：2 μL。

表1 流动相梯度洗脱程序

时间/min	0.1%甲酸/%	乙腈/%
0.00	90	10
0.50	90	10
4.00	88	12
6.00	10	90
7.01	10	90
8.01	90	10
9.00	90	10

7.3.2 质谱参数

质谱参数如下：

a) 离子化模式：电喷雾电离(ESI)正离子模式。

b) 质谱扫描方式：多反应监测(MRM)。

c) 其他质谱条件参见附录 B。

7.3.3 定性测定

按照上述色谱和质谱条件下进行测定，试液中待测物色谱峰保留时间与基质标准工作溶液保留时间偏差在±2.5%之内，各离子对的相对丰度应与标准品的相对丰度一致，且样品中各离子对的相对丰度与浓度接近的基质标准工作液中对应的离子对的相对丰度进行比较，偏差不超过表 2 规定的范围，则可判定为样品中存在对应的待测物。

表 2 定性确证时相对离子丰度的最大允许误差

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

7.3.4 定量测定

本方法中甲硝唑及其代谢物羟基甲硝唑、洛硝哒唑、二甲硝咪唑及其共同代谢物 HMMNI 采用同位素内标法定量测定，替硝唑、奥硝唑、塞克硝唑采用基质外标法定量。根据样液中被测物的含量情况，选定响应值相近的混合标准工作液。标准工作溶液和样液中分析物的响应值均应在仪器的检测线性范围内。对标准工作溶液和样液等体积参差进样测定。在上述仪器条件下，各分析物的参考保留时间为：羟基甲硝唑 1.14 min、羟基甲硝唑-D_3 1.14 min、甲硝唑内标 1.33 min、甲硝唑 1.34 min、HMMNI-D_3 1.40 min、HMMNI 1.41 min、二甲硝咪唑-D_3 1.64 min、二甲硝咪唑 1.66 min、洛硝哒唑-D_3 1.72 min、洛硝哒唑 1.74 min、塞克硝唑 2.18 min、替硝唑 2.75 min、奥硝唑 3.84 min；多反应离子监测色谱图参见附录 C。

7.4 空白试验

除不称取样品外，均按上述相同条件和步骤进行。

7.5 结果计算和表述

试样中的甲硝唑、羟基甲硝唑、洛硝哒唑、二甲硝咪唑及 HMMNI 残留含量采用同位素内标法定量，替硝唑、奥硝唑、塞克硝唑残留含量采用基质匹配外标法定量，定量结果利用数据处理系统计算或按式(1)计算：

$$X_i = \frac{C_i \times V}{m} \times \frac{1\,000}{1\,000} \qquad \cdots\cdots(1)$$

式中：

X_i ——试样中被测物含量，单位为微克每千克(μg/kg)；

C_i ——从标准曲线上得到的样液中待测物的含量，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 ——试样溶液所代表试样的质量，单位为克(g)。

注：计算结果应扣除空白值。

8 测定低限和回收率

8.1 测定低限

本方法中甲硝唑、洛硝哒唑、二甲硝咪唑、替硝唑、奥硝唑及塞克硝唑的测定低限为 0.5 μg/kg，羟基

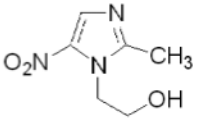
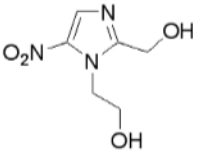
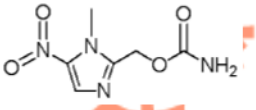
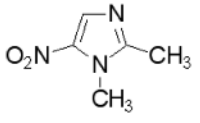
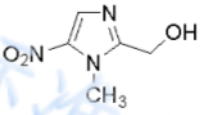
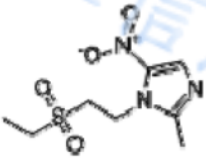
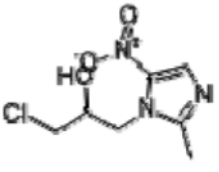
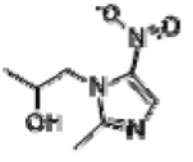
甲硝唑及 HMMNI 的测定低限为 1.0 μg/kg。

8.2 回收率

鸡肉、猪肉、牛肉、香肠、罐头及酱牛肉中各硝基咪唑药物的添加浓度及回收率数据参见附录 D。

附 录 A
（资料性附录）

表 A.1 硝基咪唑类药物的结构式、分子式、分子量和美国化学文摘登记号

名称	英文名	结构式	CAS No.	分子式	分子量
甲硝唑	Metronidazole		443-48-1	$C_6H_9N_3O_3$	171.15
羟基甲硝唑（MNZOH）	Metronidazole-OH		4812-40-2	$C_6H_9N_3O_4$	187.15
洛硝哒唑	Ronidazole		7681-76-7	$C_6H_8N_4O_4$	200.15
二甲硝咪唑	Dimetridazole		551-92-8	$C_5H_7N_3O_2$	141.13
1-甲基-2-羟甲基-5-硝基咪唑（HMMNI）	1-Methyl-5-Nitro-2-Hydroxymethylimidazole		936-05-0	$C_5H_7N_3O_3$	157.13
替硝唑	Tinidazole		19387-91-8	$C_8H_{13}N_3O_4S$	247.27
奥硝唑	Ornidazole		16773-42-5	$C_7H_{10}ClN_3O_3$	219.63
塞克硝唑	Secnidazole		3366-95-8	$C_7H_{11}N_3O_3$	185.18

附 录 B
（资料性附录）
参考质谱条件

B.1 参考条件

质谱参考条件如下：

a） 电喷雾电压：0.5 kV；
b） 锥孔电压：20 V；
c） 去溶剂气温度：500 ℃；
d） 去溶剂气流速：1 000 L/Hr；
e） 锥孔反吹气流速：150 L/Hr；
f） 雾化气压力：7 Bar；
g） 碰撞气：高纯氩气；
h） 碰撞气流速：1.7 mL/min。

表 B.1 被测物的参考保留时间、监测离子对和裂解能量

分析物	保留时间 (min)	离子对(m/z)	碰撞能量 (CE)/eV	内标化合物名称
甲硝唑	1.34	172.2/82.1	22	甲硝唑-$^{13}C_2{}^{15}N_2$
		172.2/128.0*	15	
羟基甲硝唑	1.14	188.2 /123.1*	12	羟基甲硝唑-D_2
		188.2 /126.0	22	
洛硝哒唑	1.74	201.2/55.2	24	洛硝哒唑-D_3
		201.2/140.0*	12	
二甲硝咪唑	1.66	142.1/81.0	22	二甲硝咪唑-D_3
		142.1/96.1*	14	
HMMNI	1.41	158.2/55.0	16	HMMNI-D_3
		158.2/140.1*	13	
替硝唑	2.75	248.2/121.1*	16	—
		248.2/128.0	20	
奥硝唑	3.84	220.1/82.06	28	—
		220.1/128.0*	14	
塞克硝唑	2.18	186.1/82.0	24	—
		186.1/128.0*	14	
甲硝唑-$^{13}C_2{}^{15}N_2$	1.33	176.2/86.3	22	—
羟基甲硝唑-D_2	1.14	190.2/125.0	13	—

表 B.1（续）

分析物	保留时间（min）	离子对（m/z）	碰撞能量（CE）/eV	内标化合物名称
洛硝哒唑-D_3	1.72	204.2/143.1	10	—
二甲硝咪唑-D_3	1.64	145.2/99.2	15	—
HMMNI-D_3	1.40	161.2/58.1	19	—
注：* 为定量离子对，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。				

非商业性声明：附录 B 所列参考质谱条件是在 Waters TQ-S 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 C
（资料性附录）
标准物质多反应监测质量色谱图

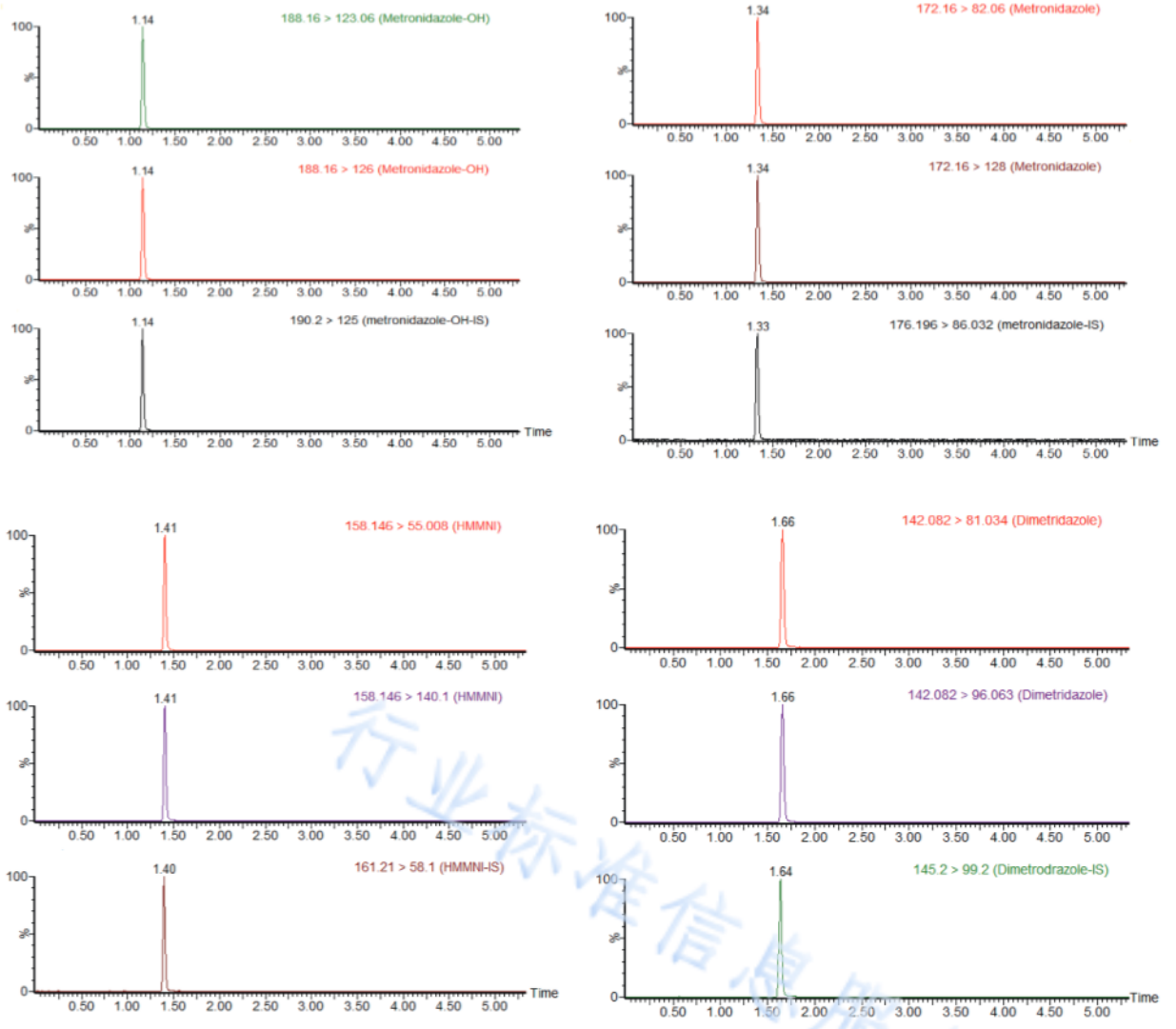


图 C.1 8 种硝基咪唑药物标准溶液多反应监测色谱图（代谢物 10 ng/mL，其余 5.0 ng/mL）

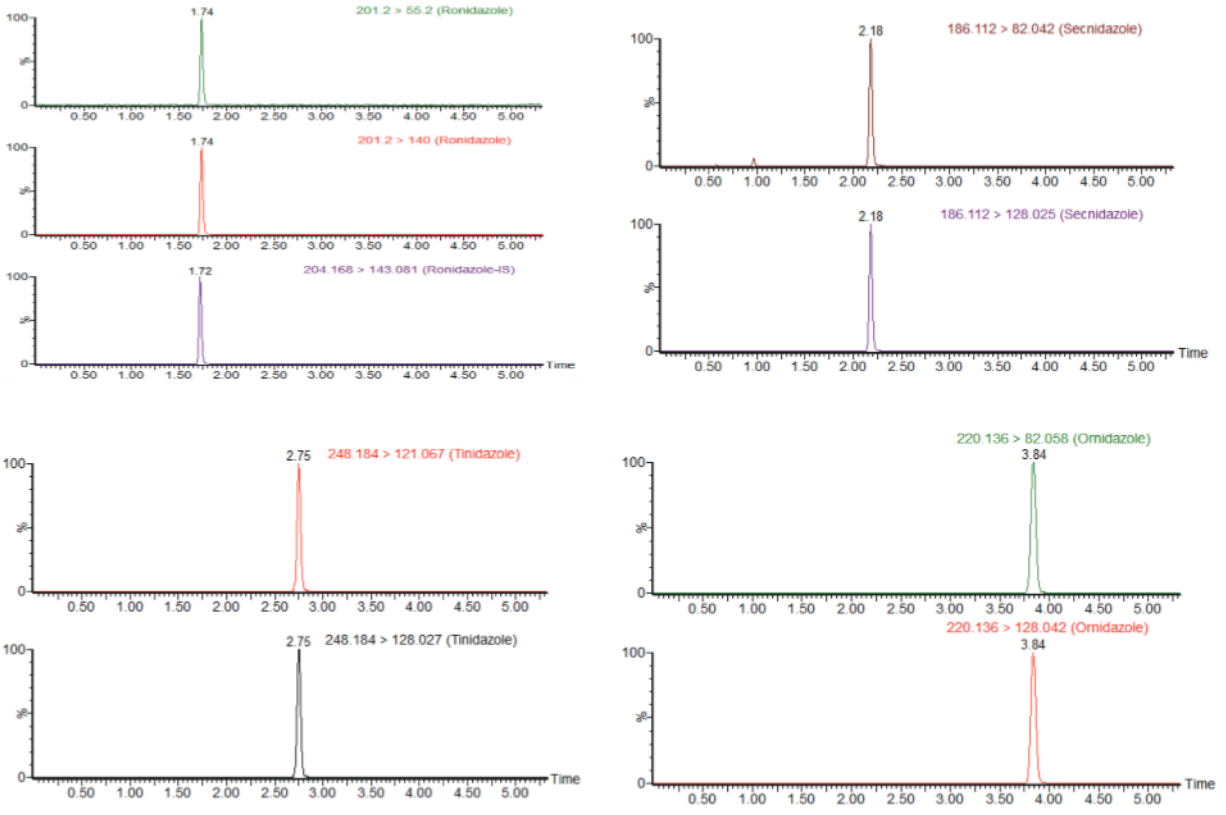


图 C.1（续）

附 录 D
（资料性附录）
回 收 率

表 D.1 硝基咪唑类药物在 6 种样品基质中添加浓度及回收率数据

目标化合物	添加水平 μg/kg	各基质中添加回收率范围/%					
		鸡肉	猪肉	牛肉	香肠	罐头	酱牛肉
甲硝唑	0.5	97.3-109.4	90.4-96.1	97.8-107.4	107.6-116.9	100.9-104.7	92.8-97.2
	1.0	94.9-101.4	92.5-96.5	102.9-108.3	109.7-115.8	99.3-107.2	97.8-101.4
	3.0	99.1-103.9	95.1-96.7	105.0-108.5	103.3-110.7	101.8-104.2	98.6-100.6
羟基甲硝唑	1.0	92.6-100.2	90.6-98.7	95.4-98.5	92.8-99.8	98.8-101.4	93.2-98.1
	2.0	95.6-99.3	93.4-98.4	98.5-103.9	92.9-103.7	99.7-101.8	97.5-101.3
	6.0	98.4-100.4	95.3-100.1	98.1-106.1	96.1-111.0	96.8-99.9	97.7-101.9
洛硝哒唑	0.5	87.2-92.4	87.9-91.7	96.9-105.5	83.8-97.8	89.2-98.7	94.3-100.8
	1.0	88.5-94.2	87.5-91.9	97.9-102.7	92.8-102.2	93.9-102.1	95.0-101.7
	3.0	88.6-93.1	88.6-91.1	96.0-102.0	92.6-103.0	97.0-101.6	95.2-101.0
二甲硝咪唑	0.5	90.5-100.0	92.2-101.3	95.2-101.4	85.1-98.8	91.4-97.5	102.4-118.4
	1.0	91.9-100.6	93.0-99.5	96.6-101.1	92.6-104.5	94.8-100.7	98.2-118.0
	3.0	96.0-100.7	97.5-102.3	99.5-102.4	97.7-108.7	97.7-100.5	96.3-99.2
HMMNI	1.0	93.8-102.5	92.6-98.1	98.1-112.3	94.7-105.7	94.7-105.7	113.8-119.8
	2.0	96.6-103.2	93.4-97.5	106.8-114.6	97.9-106.5	97.9-106.5	112.1-119.2
	6.0	98.2-103.0	95.2-98.9	106.8-115.7	95.2-101.8	95.2-101.8	112.6-117.1
替硝唑	0.5	81.8-90.9	86.8-96.4	102.7-106.9	81.6-99.0	83.4-91.1	80.2-86.3
	1.0	90.2-99.2	95.8-101.2	103.2-114.1	94.8-100.1	84.3-95.5	80.6-87.8
	3.0	86.6-94.7	96.7-105.7	97.2-104.8	93.3-100.5	84.8-96.2	79.3-88.3
奥硝唑	0.5	81.2-87.7	81.4-91.6	84.1-87.4	93.9-115.3	73.7-86.4	79.6-83.7
	1.0	85.9-93.0	92.1-101.1	83.9-87.2	105.1-112.5	83.5-86.0	77.5-83.5
	3.0	82.9-90.1	92.5-99.3	84.4-88.1	101.5-114.2	77.5-85.8	76.6-81.3
塞克硝唑	0.5	81.0-86.1	71.0-79.2	98.4-101.5	78.2-93.3	83.9-87.6	81.5-87.0
	1.0	88.0-93.8	80.2-83.2	97.8-106.1	92.8-100.8	83.9-90.4	81.4-85.6
	3.0	74.2-82.8	81.1-86.6	96.1-100.3	89.4-101.3	84.1-87.7	80.3-84.2

Foreword

This standard was drafted according to GB/T 1.1—2009《Directives for standardization—Part 1:Rules for the structure and drafting of stands》,GB/T 20001.4—2001《Rules for drafting standards—Part 4: Methods of chemical analysis》and SN/T 0001—1995《General rules for drafting the standards of biological method for the determination of pesticide veterinary drug residues and biotoxins in commodities for export》.

This standard reviews and combines the former standard,the method for determination of metronidazole,tinidazole,ornidazole,ronidazole,dimetridazole and secnidazole residues in meat and meat products for export—HPLC(SN/T 1626—2005).Compared with the above mentioned standard,this standard broadens the matrix of beef,sausage,canned food and sauced beef,increase the determination of metronidazole metabolites that hydroxyl metronidazole,and simplified the pre-treatment method,and uses liquid chromatography-tandem mass spectrometry instead of HPLC to increase sensitivity.Metronidazole,hydroxyl metronidazole,ronidazole,dimetridazol and HMMNI use isotope internal standard method to quantitative.

Attention is required to the certain contents of this text which might be related to some patents.This file is not responsible to identify these.

This standard was proposed by and is under the charge of General Administration of Customs P.R. China.

This standard was drafted by Shijiazhuang Customs District,P.R.China.

Main drafters of this standard are: AI Lianfeng, Ma Yusong, Zhang Haichao, Zhang Jingwen, Dou Caiyun,Guo Chunhai,Chen Ruichun,Li Wei.

All previous releases of this standard replaced standards as follows:

—SN/T 1626—2005.

Note: This English version,a translation from the Chinese text,is solely for guidance.

Determination of metronidazole, tinidazole, ornidazole, ronidazole, dimetridazole and secnidazole residues in meat and meat products for export—LC-MS/MS method

1 Scope

This standard specifies the determination method for residues of metronidazole, hydroxyl metronidazole, ronidazole, dimetridazole, 1-methyl-5-nitro-2-hydroxymethylimidazole (the common metabolites of ronidazole and dimetridazole, abbreviated as HMMNI), tinidazole, ornidazole, and secnidazole residues by liquid chromatography-tandem mass spectrometry.

This standard is applicable to the determination and confirmation of metronidazole, hydroxyl metronidazole, ronidazole, dimetridazole, HMMNI, tinidazole, ornidazole and secnidazole in chicken, pork, beef, sausage, canned food and sauce beef.

2 Normative references

The items of the following listed standard become the items of this standard due to the quotation by this standard. The cited references with date would not apply to this standard if their amendment (not including corrected printing errors) or revision appear. However, it is encouraged to study if the newest edition of these references can be used. The newest edition is applicable to this standard if the references are not quoted with date.

GB/T 6682 water for laboratory use—Specifications

3 Abstract of this method

Nitroimidazoles residues in samples are extracted with ethyl acetate. The extraction is purified by Silica gel matrix of the strong cation-exchange solid phase extraction column. The final solution are determined and confirmated by LC-MS/MS. Tinidazole, ornidazole, and secnidazole use external standard method to quantitative, the others use internal standard method to quantitative.

4 Reagents and materials

Unless specifically noted, all reagents used should be of analytically grade; "water" is redistilled water.

4.1 Ethyl acetate: HPLC grade.

4.2 Methanol: HPLC grade.

4.3 Acetone: HPLC grade.

4.4 Acetonitrile: HPLC grade.

4.5 Ammonium hydroxide: Content is 25%～28% (mass fraction concentration).

4.6 Formic acid: HPLC grade.

4.7 Acetic acid: HPLC grade.

4.8 Anhydrous sodium sulfate: AR grade.

4.9 Acetic acid-ethyl acetate (5+95): transfer 5 mL acetic acid (4.7) into 95 mL ethyl acetate (4.1), mix adequately.

4.10 Ammonium hydroxid-acetonitrile (5+95): transfer 5 mL ammonium hydroxide (4.5) into 95 mL acetonitrile (4.4), mix adequately.

4.11 0.1% formic acid solution: Accurately measure 1.0 mL formic acid (4.6) into a 1 000 mL volumetric flask, dilute with water to 1 000 mL.

4.12 Standard of 8 nitroimidazoles and metabolites: purity ≥98.0%. The compound basic informations can be found in the appendix A table A.1.

4.13 Stock standard solution: Accurately weigh 10 mg (accurate to 0.1 mg) standard (4.12), dissolve with methanol to scale in a 10 mL brown volumetric flask individually. The concentrations of the solution are 1 mg/mL. The solutions should be stored at 1 ℃～4 ℃ in dark.

4.14 Mixed medium standard solution Ⅰ: Accurately transfer 2.00 mL stock standard solution (4.13) of hydroxyl metronidazole and HMMNI and 1.00 mL stock standard solution of others (4.13) respectively into a 100 mL brown volumetric flask, dilute with methanol to the scale. The concentration of hydroxyl metronidazole and HMMNI is 20 μg/mL, the concentration of others is 10 μg/mL. The solutions should be stored at 1 ℃～4 ℃ in dark.

4.15 Mixed medium standard solution Ⅱ: Accurately transfer 1.00 mL Mixed medium standard solution Ⅰ (4.14) into a 100 mL brown volumetric flask, dilute with methanol to the scale. The concentration of hydroxyl metronidazole and HMMNI is 200 ng/mL, the concentration of others is 100 ng/mL. The solutions should be stored at 1 ℃～4 ℃ in dark.

4.16 Isotope internal standard: Metronidazole-$^{13}C_2{}^{15}N_2$ (CAS: 1173020-03-5), Metronidazole-OH-D_2, Ronidazole-D_3 (CAS: 101855-87-4), Dimetridazole-D_3 (CAS: 64678-69-9), HMMNI-D_3 (CAS: 1015855-78-3) purity ≥98.0%.

4.17 Stock isotope internal standard solution: Accurately weigh 10 mg (accurate to 0.1 mg) standard (4.16), dissolve with methanol to scale in a 50 mL brown volumetric flask individually. The concentrations of the solution are 200 μg/mL. The solutions should be stored at 1 ℃～4 ℃ in dark.

4.18 Medium isotope internal standard solution: Prepare a standard working solution of 1 μg/mL by diluting the above stock solution (4.17) with methanol. The solutions should be stored at 1 ℃～4 ℃ in dark.

4.19 Isotope internal working standard solution: Prepare a standard working solution of 100 ng/mL by diluting the above stock solution (4.17) with water. The solutions should be stored at 1 ℃～4 ℃ in dark.

4.20 Blank matrix solution: The solution obtainedby the negative blank samples of different matrix except adding internal working standard solution steps in accordance with 7.1 and 7.2 respectively after purification treatment.

4.21 Matrix standard working solution: According to the requirement, dilute middle standard solution (4.15) and isotope internal working standard solution (4.19) to appropriate concentration with blank matrix solution (4.20).

4.22 Slica gel matrix of the strong cation-exchange solid phase extraction column: 3 mL methanol and 3 mL acetic acid-ethyl acetate pass the cartridge respectively to condition. In this process, the column cartridge should keep wet and leave about 1 cm high solution in cartridge. Then connect a reservoir the bottom of which is stuffed with degrease cotton to the top of cartridge.

4.23 Membrane filter: 0.22 μm, organic type.

5 Apparatus and equipment

5.1 Liquid chromatography-mass spectrograph, equipped with electrospray ion source.

5.2 Organ blender.

5.3 Balance: accuracy to 0.01 g and 0.1 mg.

5.4 Centrifuge, speed of no less than 5 000 r/min.

5.5 Homogenizer, speed of no less than 10 000 r/min.

5.6 Vortexmixer.

5.7 N_2 evaporator.

5.8 Solid-phase extraction.

6 Preparation and storage of test sample

6.1 Requirement

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

6.2 Preparation of test sample

Take representative samples of 500 g, triturate the sample with a comminuter after chopping the edible part. And then place in clean containers, seal and indicate the tag, storage below −18 ℃.

7 Procedure

7.1 Extraction

Weigh 5.0 g (accurate to 0.01 g) samples in a 50 mL centrifuge tube, then add 100 μL isotope internal working standard solution (4.19), 25 mL ethyl acetate (4.1) and 5 g anhydrous sodium sulfate (4.8) to the sample and then homogenize it for 1 min (10 000 r/min), and then centrifuge the sample solution for 5 min at 5 000 r/min. Transfer the supernatants into an another 50 mL centrifuge tube and add 0.5 mL acetic acid (4.7). Vortex blending and wait for purification.

7.2 Cleaning-up

Transfer the extraction obtained from 7.1 into the reservoir above the conditioned column (4.22). Then let the solution pass the cartridge at the speed of 2 mL/min. After drops over, wash the cartridge with 3 mL acetone (4.3), 3 mL methanol (4.2) in turn. Discard the washings. Finally elute the cartridge with 6 mL of ammonium hydroxid-acetonitrile (4.10) at the speed of 2 mL/min. Then collect the residue and blow to dryness under a nitrogen flow in a water bath below 45 ℃. Residues are dissolved with 1.0 mL water. Then the solution is passed through 0.22 μm filter and ready for LC-MS/MS analysis.

7.3 Determination

7.3.1 HPLC operating conditions

HPLC operating conditions are as follows:

a) Column：C_{18} 100 mm × 2.1 mm(i.d.)，1.7 μm particle size or equivalent；

b) Mobile phases：gradient elution conditions are listed in table 1.

c) Flow rate：350 μL/min；

d) Column temperature：40 ℃；

e) Injection volume：2 μL.

Table 1—Mobile phase and gradient elution condition

Time/min	Water(0.1% formic acid)/%	Acetonitrile/%
0.00	90	10
0.50	90	10
4.00	88	12
6.00	10	90
7.01	10	90
8.01	90	10
9.00	90	10

7.3.2 MS conditions

MS conditions are as follows：

a) Ionization mode：ESI；

b) scan mode：multiple reaction monitoring MRM；

c) other reference mass operating conditions are listed in appendix B.

7.3.3 Qualitative analysis

If the deviation of retention time for analytes between test sample and standard solution is within ±2.5% under the same experiment conditions，and the difference of relative ion ration of analytes between test sample and standard solution is also within the error allowed（the max deviation allowed for relative ion ratio is listed in table 2，corresponding analytes would be considered to be in the sample.

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

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

7.3.4 Quantitative analysis

Metronidazole, hydroxyl metronidazole, ronidazole, dimetridazole and HMMNI use internal standard method to quantitative, tinidazole, ornidazole and secnidazole use matrix external standard method to quantitative. According to the amount of analytes in the sample liquid, selected response value of similar mixed standard working solution. The responses of the analytes in the standard working solution and the sample solution should be within the linear range of the instrument detection. Under the above operating condition, the standard working solution should be randomly injected in-between the injections of the sample solution of equal volume. Under the above operating condition, retention time of hydroxyl metronidazole, hydroxyl metronidazole-D_3, metronidazole-$^{13}C_2{}^{15}N_2$, metronidazole, HMMNI-D_3, HMMNI, dimetridazole-D_3, dimetridazole, ronidazole-D_3, ronidazole, secnidazole, tinidazole, and ornidazole is 1.14 min, 1.14 min, 1.33 min, 1.34 min, 1.40 min, 1.41 min, 1.64 min, 1.66 min, 1.72 min, 1.74 min, 2.18 min, 2.75 min and 3.84 min, respectively. The chromatogram of the standards can be found in the appendix C.

7.4 Blank test

The operation of the blank test is the same as the described in the method of determination, but without sample addition.

7.5 Calculation an expression of result

Metronidazole, hydroxyl metronidazole, ronidazole, dimetridazole and HMMNI residues in samples use internal standard method to quantitative, tinidazole, ornidazole and secnidazole residues use matrix matching external standard method to quantitative. Calculation the content of fumagillin residues in test sample by data processor or according to formula(1)

$$X_i = \frac{C_i \times V}{m} \times \frac{1\,000}{1\,000} \qquad \cdots\cdots(1)$$

where:

X_i —the residue content of analyte in test sample, μg/kg;

C_i —the concentration of analyte got from the matrix standard curve, ng/ml;

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

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

Note：the blank value should be subtracted from the result of calculation.

8 Limit of determination and recovery

8.1 Limit of determination

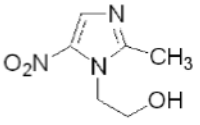
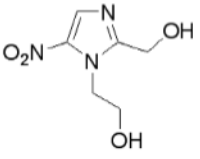
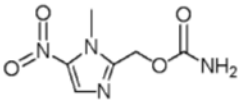
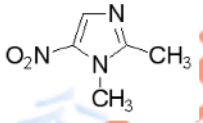
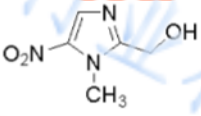
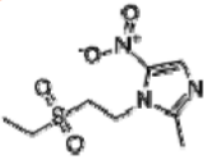
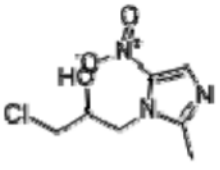
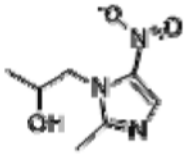
The limit of quantification of metronidazole，ronidazole，dimetridazole tinidazole，ornidazole and secnidazole is 0.5 μg/kg.The limit of quantification of hydroxyl metronidazole and HMMNI is 1.0 μg/kg.

8.2 Recovery

The ranges of recovery in chicken，pork，beef，sausage，canned food and sauce beef are showed in appendix D.

Appendix A
（Informative）

Table A.1 The molecular formula，molecular weight and the American chemical abstract registration number of nitroimidazoles

Analyte	Structural formula	CAS No.	Molecular formula	Molecular weight
Metronidazole		443-48-1	$C_6H_9N_3O_3$	171.15
Metronidazole-OH		4812-40-2	$C_6H_9N_3O_4$	187.15
Ronidazole		7681-76-7	$C_6H_8N_4O_4$	200.15
Dimetridazole		551-92-8	$C_5H_7N_3O_2$	141.13
1-Methyl-5-Nitro-2-Hydroxymethylimidazole（HMMNI）		936-05-0	$C_5H_7N_3O_3$	157.13
Tinidazole		19387-91-8	$C_8H_{13}N_3O_4S$	247.27
Ornidazole		16773-42-5	$C_7H_{10}ClN_3O_3$	219.63
Secnidazole		3366-95-8	$C_7H_{11}N_3O_3$	185.18

Appendix B
（Informative）
Reference mass conditions

B.1 Reference conditions

Reference conditions are as follows:

a) Capillary voltages:0.5 kV;

b) Cone voltages:20 V;

c) Desolvation temperatures:500 ℃;

d) Desolvationg gas flow rate:1 000 L/Hr;

e) Cone back purge gas flow rate:150 L/Hr;

f) Nebuliser gas pressure:7 Bar;

g) Collision gas:high-purify argon gas;

h) Collision gas flow:1.7 mL/min

Table B.1 The scan segment，ion pairs and collision energy of the analytes

Compound	Retention time (min)	Ion pairs (m/z)	Collision energy (eV)	Internal standard compound
Metronidazole	1.34	172.2/82.1	22	Metronidazole-$^{13}C_2{}^{15}N_2$
		172.2/128.0*	15	
Metronidazole-OH	1.14	188.2 /123.1*	12	Metronidazole-OH-D_2
		188.2 /126.0	22	
Ronidazole	1.74	201.2/55.2	24	Ronidazole-D_3
		201.2/140.0*	12	
Dimetridazole	1.66	142.1/81.0	22	Dimetridazole-D_3
		142.1/96.1*	14	
HMMNI	1.41	158.2/55.0	16	HMMNI-D_3
		158.2/140.1*	13	

Table B.1 (continued)

Compound	Retention time (min)	Ion pairs (m/z)	Collision energy (eV)	Internal standard compound
Tinidazole	2.75	248.2/121.1*	16	—
		248.2/128.0	20	
Ornidazole	3.84	220.1/82.06	28	—
		220.1/128.0*	14	
Secnidazole	2.18	186.1/82.0	24	—
		186.1/128.0*	14	
Metronidazole-$^{13}C_2{}^{15}N_2$	1.33	176.2/86.3	22	—
Metronidazole-OH-D_2	1.14	190.2/125.0	13	—
Ronidazole-D_3	1.72	204.2/143.1	10	—
Dimetridazole-D_3	1.64	145.2/99.2	15	—
HMMNI-D_3	1.40	161.2/58.1	19	—

Note: * mark is the quantification ion pair. for the different MS equipment, the parameters may be different, and the MS parameters should be optimiazed to the best before analysis.

Non-commercial statement: the reference mass parameters in Appendix B are accomplished by Waters TQ-S LC-MS/MS, the equipment and its type involved in the standard method is only for reference and not related to any commercial aim, and the analysts are encouraged to use equipment of different corporation or different type.

Appendix C
（Informative）
MRM chromatogram of standard

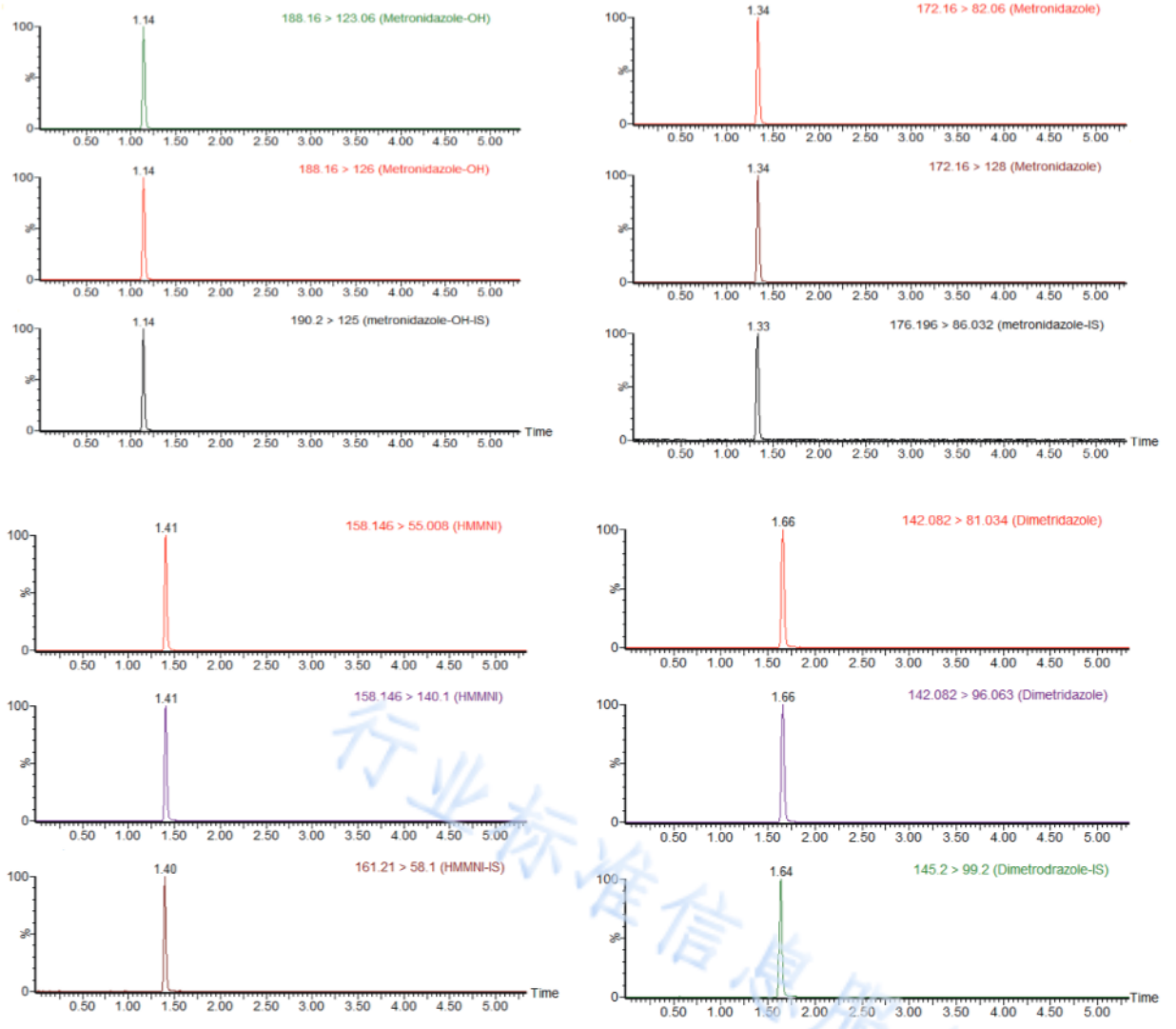


Fig.C.1　the MRM chromatogram of 8 nitroimidazoles standards
（metabolins at 10 ng/mL，the others at 5.0 ng/mL）

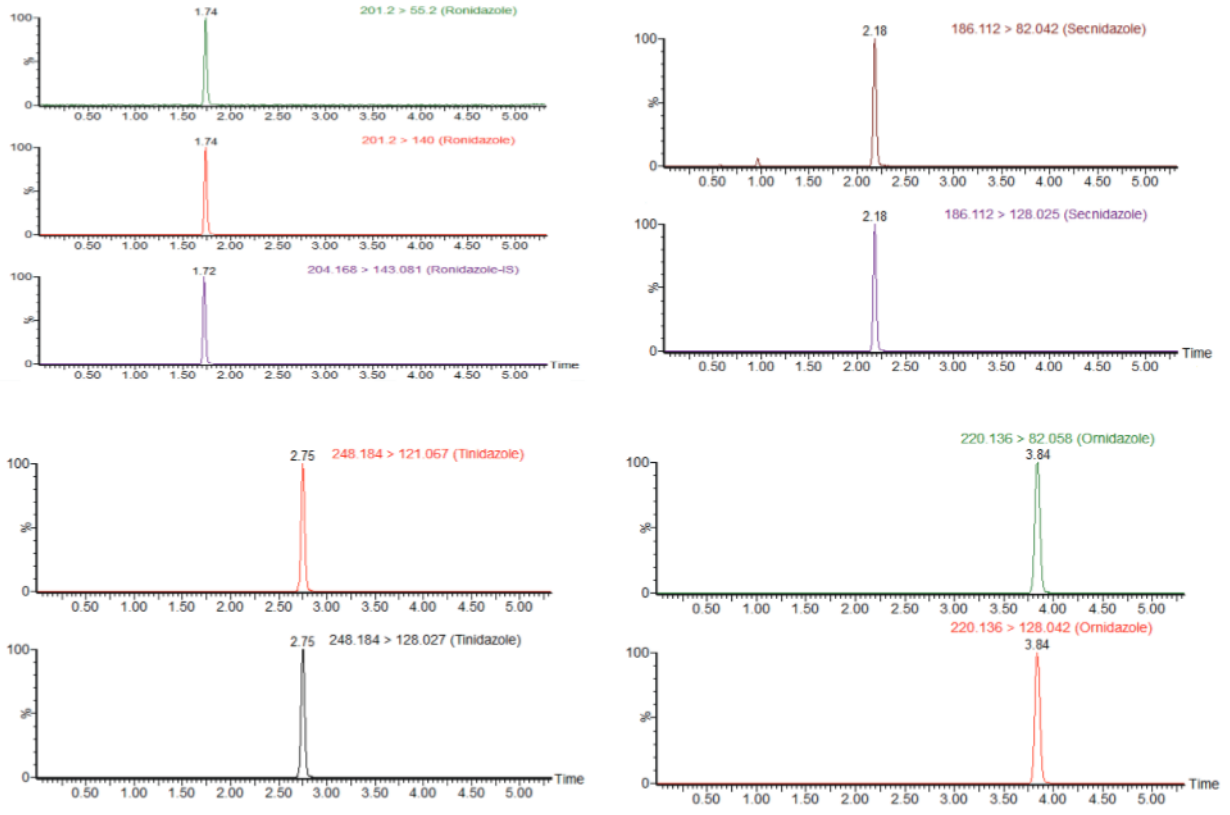


Fig.C.1（continued）

Appendix D
（Informative）
Recovery ranges

表 D.1　硝基咪唑类药物在 6 种样品基质中添加浓度及回收率数据

Compound	Fortified level μg/kg	Range of recovery/%					
		Chicken	Pork	Beef	Sausage	Canned food	Sauce beef
Metronidazole	0.5	97.3-109.4	90.4-96.1	97.8-107.4	107.6-116.9	100.9-104.7	92.8-97.2
	1.0	94.9-101.4	92.5-96.5	102.9-108.3	109.7-115.8	99.3-107.2	97.8-101.4
	3.0	99.1-103.9	95.1-96.7	105.0-108.5	103.3-110.7	101.8-104.2	98.6-100.6
Metronidazole-OH	1.0	92.6-100.2	90.6-98.7	95.4-98.5	92.8-99.8	98.8-101.4	93.2-98.1
	2.0	95.6-99.3	93.4-98.4	98.5-103.9	92.9-103.7	99.7-101.8	97.5-101.3
	6.0	98.4-100.4	95.3-100.1	98.1-106.1	96.1-111.0	96.8-99.9	97.7-101.9
Ronidazole	0.5	87.2-92.4	87.9-91.7	96.9-105.5	83.8-97.8	89.2-98.7	94.3-100.8
	1.0	88.5-94.2	87.5-91.9	97.9-102.7	92.8-102.2	93.9-102.1	95.0-101.7
	3.0	88.6-93.1	88.6-91.1	96.0-102.0	92.6-103.0	97.0-101.6	95.2-101.0
Dimetridazole	0.5	90.5-100.0	92.2-101.3	95.2-101.4	85.1-98.8	91.4-97.5	102.4-118.4
	1.0	91.9-100.6	93.0-99.5	96.6-101.1	92.6-104.5	94.8-100.7	98.2-118.0
	3.0	96.0-100.7	97.5-102.3	99.5-102.4	97.7-108.7	97.7-100.5	96.3-99.2
HMMNI	1.0	93.8-102.5	92.6-98.1	98.1-112.3	94.7-105.7	94.7-105.7	113.8-119.8
	2.0	96.6-103.2	93.4-97.5	106.8-114.6	97.9-106.5	97.9-106.5	112.1-119.2
	6.0	98.2-103.0	95.2-98.9	106.8-115.7	95.2-101.8	95.2-101.8	112.6-117.1
Tinidazole	0.5	81.8-90.9	86.8-96.4	102.7-106.9	81.6-99.0	83.4-91.1	80.2-86.3
	1.0	90.2-99.2	95.8-101.2	103.2-114.1	94.8-100.1	84.3-95.5	80.6-87.8
	3.0	86.6-94.7	96.7-105.7	97.2-104.8	93.8-100.5	84.8-96.2	79.3-88.3
Ornidazole	0.5	81.2-87.7	81.4-91.6	84.1-87.4	93.9-115.3	73.7-86.4	79.6-83.7
	1.0	85.9-93.0	92.1-101.1	83.9-87.2	105.1-112.5	83.5-86.0	77.5-83.5
	3.0	82.9-90.1	92.5-99.3	84.4-88.1	101.5-114.2	77.5-85.8	76.6-81.3
Secnidazole	0.5	81.0-86.1	71.0-79.2	98.4-101.5	78.2-93.3	83.9-87.6	81.5-87.0
	1.0	88.0-93.8	80.2-83.2	97.8-106.1	92.8-100.8	83.9-90.4	81.4-85.6
	3.0	74.2-82.8	81.1-86.6	96.1-100.3	89.4-101.3	84.1-87.7	80.3-84.2