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中华人民共和国出入境检验检疫行业标准

SN/T 3032—2011

出口食品中三聚氰胺和三聚氰酸检测方法　液相色谱-质谱/质谱法

Determination of melamine and cyanuric acid in foodstuffs for export—HPLC-MS/MS method

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前　言

本标准按照 GB/T 1.1—2009 给出的规则起草。

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：中华人民共和国上海出入境检验检疫局、中国检验检疫科学研究院、中华人民共和国深圳出入境检验检疫局、中华人民共和国湖北出入境检验检疫局。

本标准主要起草人：邓晓军、赵善贞、郭德华、李淑娟、彭涛、靳宝辉、韩丽、盛永刚、赵晓亚、武巧彬、张旖。

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1　范围

本标准规定了食品中三聚氰胺和三聚氰酸残留量的制样和液相色谱-质谱/质谱测定。

本标准适用于鸡蛋、猪肉、猪肝、猪肾、肠衣、虾、蜂蜜、豆奶、豆粉、蛋白粉、液态奶、奶粉、炼乳、奶酪、奶油、冰淇淋、奶糖、饼干中三聚氰胺和三聚氰酸残留量的定性测定和确证。

2　方法提要

试样中三聚氰胺和三聚氰酸残留采用乙腈和水提取，盐酸调节 pH 值至 2.0～3.0 后，乙腈饱和正己烷脱脂，提取液经离子交换和硅胶复合固相萃取柱净化，液相色谱-质谱/质谱检测和确证，内标法定量。

3　试剂与材料

除非另有规定，均使用分析纯试剂，水为去离子水。

3.1　乙腈：液相色谱级。

3.2　甲醇：液相色谱级。

3.3　正己烷：液相色谱级。

3.4　甲酸：液相色谱级。

3.5　甲酸铵。

3.6　盐酸。

3.7　氨水。

3.8　二乙胺。

3.9　乙腈＋水(7＋3，V_1+V_2)：量取 700 mL 乙腈和 300 mL 纯水，混匀。

3.10　乙腈＋水(5＋5，V_1+V_2)：量取 500 mL 乙腈和 500 mL 纯水，混匀。

3.11　1 mol/L 盐酸：准确量取 83 mL 浓盐酸(3.6)用水稀释定容到 1 L。

3.12　5%氨水甲醇：量取 95 mL 甲醇和 5 mL 氨水，混匀。使用前配制。

3.13　100 mmol/L 甲酸铵＋乙腈(1＋9，V_1+V_2)pH 3.2 溶液：精确称取 6.3 g 甲酸铵(3.5)溶解于 100 mL 纯水中，加入 900 mL 乙腈混匀，用甲酸调节 pH 至 3.2±0.1。

3.14　含 0.1%甲酸的乙腈：量取 1 L 乙腈，加入 1 mL 甲酸，混匀。

3.15　标准物质：三聚氰胺(melamine，CAS 号：108-78-1)、三聚氰胺同位素内标($^{15}N_3$-melamine，CAS 号：287476-11-3)、三聚氰酸(cyanuric acid，CAS 号：108-80-5)、三聚氰酸同位素内标($^{13}C_3$-cyanuric acid，CAS 号：201996-37-4)，纯度均大于 98%。

3.16　标准储备液：分别精确称取三聚氰胺、三聚氰胺同位素内标的标准品(3.15)0.1 g(精确到 0.000 1 g)，用乙腈＋水(3.10)溶解，稀释并定容于 100 mL 容量瓶中，分别配制成浓度为 1 mg/mL 的标准储备溶液。该溶液应配制于棕色容量瓶中，－18 ℃以下避光保存。分别精确称取三聚氰酸、三聚氰酸同位素内标的标准品(3.15)0.1 g(精确到 0.000 1 g)，用少量二乙胺(3.8)溶解，用甲醇稀释并定容

于 100 mL 容量瓶中，分别配制成浓度为 1 mg/mL 的标准储备溶液。该溶液应配制于棕色容量瓶中，−18 ℃以下避光保存。

3.17 标准中间储备溶液：准确移取 100 μL 上述各标准储备液于 100 mL 容量瓶中，用乙腈稀释定容，分别配成 1 μg/mL 的三聚氰胺、三聚氰酸、三聚氰胺同位素内标以及三聚氰酸同位素内标标准溶液，该溶液应配制于棕色容量瓶中，−18 ℃以下避光保存。

3.18 标准工作曲线：准确移取适量上述标准中间储备溶液(3.17)，用乙腈甲酸铵溶液(3.13)配制成适当浓度的混合标准工作曲线溶液，参考线性浓度范围为 10 ng/mL～500 ng/mL，三聚氰胺同位素内标浓度为 100 ng/mL，三聚氰酸同位素内标浓度为 200 ng/mL，临用时现配。

3.19 微孔滤膜：0.22 μm，有机系。

3.20 Anpelclean MCT 柱(亲水性键合硅胶和阳离子交换树脂复合填料)[1]：3 mL，150 mg 或相当者。

4 仪器和设备

4.1 液相色谱-质谱/质谱仪：配有电喷雾离子源(ESI)。

4.2 分析天平：感量 0.000 1 g 和 0.01 g。

4.3 均质器。

4.4 涡旋混匀器。

4.5 超声波水浴。

4.6 冷冻离心机：10 000 r/min。

4.7 固相萃取装置。

4.8 氮吹仪。

5 试样制备与保存

5.1 猪肉、猪肝、猪肾、鸡蛋、虾、肠衣、饼干、奶糖

从所取全部试样中取出有代表性试样可食部分约 500 g，充分捣碎均匀，装入洁净容器中，密封，并标明标记，于−18 ℃以下冷冻存放。肠衣试样在提取前先用去离子水洗，去盐。

5.2 蜂蜜、液态奶、奶粉、豆奶、豆粉、蛋白粉、炼乳、奶酪、奶油、冰淇淋

从所取全部试样中取出有代表性试样约 500 g，装入洁净容器中，密封，并标明标记，于 4 ℃冷藏存放。

注：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

6.1.1 猪肉、虾、肠衣、猪肝、猪肾、鸡蛋

称取均质试样 2 g(精确至 0.01 g)，置于 50 mL 塑料离心管中，分别加入 200 μL 的三聚氰胺同位素内标溶液(3.17)和 400 μL 的三聚氰酸同位素内标溶液(3.17)，鸡蛋试样中添加 1 mL 去离子水和 7 mL

1) Anpelclean MCT 柱是上海安谱科学仪器有限公司所生产产品的商品名称。给出这一信息是为了给本标准的使用者提供方便，而不是标准主管部门对这一产品的认可。

乙腈，其余试样中加入 10 mL 乙腈＋水(3.9)涡旋混合 30 s，再加入一定量 1 mol/L 盐酸溶液(3.11)调节 pH 至 2.0～3.0，涡旋混合 2 min，超声 15 min。4 ℃下 8 000 r/min 离心 5 min，取上清液于 50 mL 塑料离心管中，加入 5 mL 乙腈饱和正己烷，涡旋混合 2 min，4 ℃下 8 000 r/min 离心 5 min，去正己烷层，样液过滤纸[滤纸预先用乙腈＋水(3.10)润湿]后取 2 mL 于 15 mL 玻璃试管中，加入 0.8 mL 去离子水，用乙腈＋水(3.10)稀释至 5 mL，待净化。

6.1.2 蜂蜜

称取均质试样 1 g(精确至 0.01 g)，置于 50 mL 塑料离心管中，分别加入 100 μL 的三聚氰胺同位素内标溶液(3.17)和 200 μL 的三聚氰酸同位素内标溶液(3.17)，加入 8 mL 乙腈＋水(5＋5，V_1+V_2)(3.10)涡旋混合 30 s，再加入一定量 1 mol/L 盐酸溶液(3.11)调节 pH 至 2.0～3.0，涡旋混合 2 min，超声 15 min，8 000 r/min 离心 5 min。上清液过滤纸[滤纸预先用乙腈＋水(3.10)润湿]于 15 mL 玻璃试管，用乙腈＋水(3.10)稀释至 8 mL 左右，待净化。

6.1.3 液态奶、炼乳、冰淇淋、豆奶

称取均质试样 2 g(精确至 0.01 g)置于 50 mL 塑料离心管中，加入 2 mL 去离子水，200 μL 的三聚氰胺同位素内标溶液(3.17)和 400 μL 的三聚氰酸同位素内标溶液(3.17)，加入 4 mL 乙腈，涡旋混合 30 s，再加入一定量 1 mol/L 盐酸溶液(3.11)调节 pH 至 2.0～3.0，涡旋混合 2 min，超声 15 min，4 ℃下 8 000 r/min 离心 5 min，上清液过滤纸[滤纸预先用乙腈＋水(3.10)润湿]于 15 mL 玻璃试管中，用乙腈＋水(3.10)稀释至 8 mL 左右，待净化。

6.1.4 奶油、奶酪、奶糖、饼干、奶粉、豆粉、蛋白粉

称取均质试样 1 g(精确至 0.01 g)，置于 50 mL 塑料离心管中，分别加入 100 μL 的三聚氰胺同位素内标溶液(3.17)和 200 μL 的三聚氰酸同位素内标溶液(3.17)，用 3 mL 去离子水溶解，加入 7 mL 乙腈，涡旋混合 30 s，再加入一定量 1 mol/L 盐酸溶液(3.11)调节 pH 至 2.0～3.0，涡旋混合 2 min，超声 15 min，4 ℃下 8 000 r/min 离心 5 min。奶油、奶酪、饼干试样用 5 mL 乙腈饱和正己烷脱脂，上清液过滤纸[滤纸预先用乙腈＋水(3.10)润湿]后取 2 mL 于 15 mL 玻璃试管中，加入 0.8 mL 去离子水，用乙腈＋水(3.10)稀释至 5 mL 左右，待净化。

6.2 净化

MCT 固相萃取柱(3.20)依次用 3 mL 甲醇、3 mL 乙腈＋水(3.10)过柱活化，保持柱体湿润。将(6.1)中所得样液转入 MCT 柱中，流速不能超过 1 滴/s，用 2 mL 乙腈＋水(3.10)润洗 15 mL 玻璃试管并过柱，在低真空条件下抽干小柱，依次用 2 mL 甲醇、4 mL 5%氨水甲醇(3.12)溶液洗脱柱上待分析组分，收集洗脱液，在不低于 40 ℃条件下氮吹至干，加入 1.0 mL 流动相(3.13)振荡溶解残渣，过 0.22 μm有机型滤膜(3.19)后，液相色谱-质谱仪进行测定。

6.3 测定

6.3.1 高效液相色谱条件

6.3.1.1 色谱柱：HILIC 柱(150 mm×2.1 mm，5 μm)，或相当者。

6.3.1.2 流动相：A：100 mmol/L 甲酸铵＋乙腈(1＋9，V_1+V_2)pH 3.2；B：含 0.1%甲酸的乙腈，梯度洗脱程序见表 1。

表 1　流动相及梯度洗脱程序

时间 min	100 mmol/L 甲酸铵＋乙腈(1＋9，V_1+V_2)pH3.2(流动相 A)	含 0.1%甲酸的乙腈 (流动相 B)
0	0	100
2.5	0	100
4	100	0
8	100	0
8.5	0	100
12	0	100

6.3.1.3　流速：0.4 mL/min。

6.3.1.4　进样量：10 μL。

6.3.1.5　柱温：室温。

6.3.2　质谱条件

6.3.2.1　离子源：电喷雾 ESI，正[illegible]式。

6.3.2.2　扫描方式：多反应监测 MRM。

6.3.2.3　雾化气(GS1)、气帘气(CUR)、辅助气(GS2)、碰撞气(CAD)均为高纯氮气或其他合适气体；使用前应调解各气体流量以及离子源温度(TEM)使质谱灵敏度达到检测要求，详细条件参见附录 A。

6.3.2.4　电喷雾电压(IS)、碰撞电压(CE)、去簇电压(DP)、碰撞室入口电压(EP)、碰撞室出口电压(CXP)应优化至最佳灵敏度，监测离子和定量离子等详细条件参见表 A.1。

6.3.3　定量测定

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，标准曲线工作液应有 5 个浓度水平，应包括零点。标准工作液和待测液中两种药物的响应值均应在仪器线性响应范围内。如果样品中待测物含量超过线性范围，应用流动相(3.13)稀释到合适浓度后分析。在上述色谱条件下的三聚氰酸、三聚氰胺的参考保留时间分别为 1.50 min、6.61 min，三聚氰酸、三聚氰胺标准溶液的多反应监测(MRM)色谱图参见图 B.1。

6.3.4　定性测定

按照液相色谱-质谱/质谱条件测定试样和标准工作溶液，如果试样中待测物质的保留时间与标准品一致，保留时间偏差在 5%之内；定性离子对的相对丰度，是用相对于最强离子丰度的强度百分比表示，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，则可判断试样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偏差

相对离子丰度	>50%	>20%～50%	>10%～20%	≤10%
允许的最大偏差	±20%	±25%	±30%	±50%

6.4 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式(1)计算试样中药物的残留含量，计算结果需扣除空白值：

$$X_i = \frac{R_i \times c_i \times V}{R_s \times m} \times \frac{1\,000}{1\,000} \quad \cdots\cdots(1)$$

式中：

X_i ——试样中三聚氰胺和三聚氰酸残留量，单位为微克每千克(μg/kg)；

R_i ——样液中的分析物与内标物峰面积的比值；

c_i ——从标准曲线上得到的药物残留量的溶液浓度，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)；

R_s ——标准曲线中分析物与内标物峰面积的比值；

m ——最终样液代表的试样质量，单位为克(g)。

8 方法的测定低限和回收率

8.1 测定低限(LOQ)

本方法的测定低限：液态奶以及鸡蛋、猪肉、猪肝、猪肾、肠衣、蜂蜜、虾等动物源性食品中三聚氰胺为 25 μg/kg，三聚氰酸为 50 μg/kg。

豆奶、豆粉、蛋白粉等植物源性食品，以及奶粉、炼乳、奶酪、奶油、冰淇淋、奶糖、饼干等乳制品与含乳制品中三聚氰胺为 50 μg/kg，三聚氰酸为 100 μg/kg。

8.2 回收率

采用本方法对鸡蛋、猪肉、猪肝、猪肾、肠衣、虾、蜂蜜、豆奶、豆粉、蛋白粉、液态奶、奶粉、炼乳、奶酪、奶油、冰淇淋、奶糖、饼干 18 种食品基质进行添加回收试验，两种待测物在 18 种食品基质中的回收率资料参见表 C.1。

附 录 A
（资料性附录）
API 4000 QTRAP 四级杆质谱仪参数[2)]

质谱仪参数：

a) 电喷雾电压(IS)：正模式 5 500 V；负模式−4 500 V；

b) 碰撞气压力(CAD)：Medium；

c) 雾化气压力(GS1)：517 kPa(75 Psi)；

d) 气帘气压力(CUR)：172 kPa(25 Psi)；

e) 辅助气压力(GS2)：414 kPa(60 Psi)；

f) 离子源温度(TEM)：550 ℃；

g) 监测离子对、碰撞电压(CE)、去簇电压(DP)、碰撞室入口电压(EP)、碰撞室出口电压(CXP)如表 A.1 所示。

表 A.1 定性、定量离子对以及 CE、DP、EP、CXP 参考值

化 合 物	母离子 (Q1)	检测模式	子离子 (Q3)	CE V	DP V	EP V	CXP V
三聚氰胺	127.1	正模式	85.5*	27	71	8	14
			68.4	43	71	11	10
$^{15}N_3$-三聚氰胺内标	130.1		87.4	27	61	8	14
三聚氰酸	127.9	负模式	42.0*	−14	−50	−10	−1
			85.0	−18	−50	−10	−1
$^{13}C_3$-三聚氰酸内标	130.9		43.0	−30	−45	−10	−1
注：* 为定量离子对。							

2) 非商业性声明：附录 A 所列参考质谱条件是在 AB API 4000 QTRAP 质谱仪上完成的，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）
三聚氰胺和三聚氰酸标准品色谱图

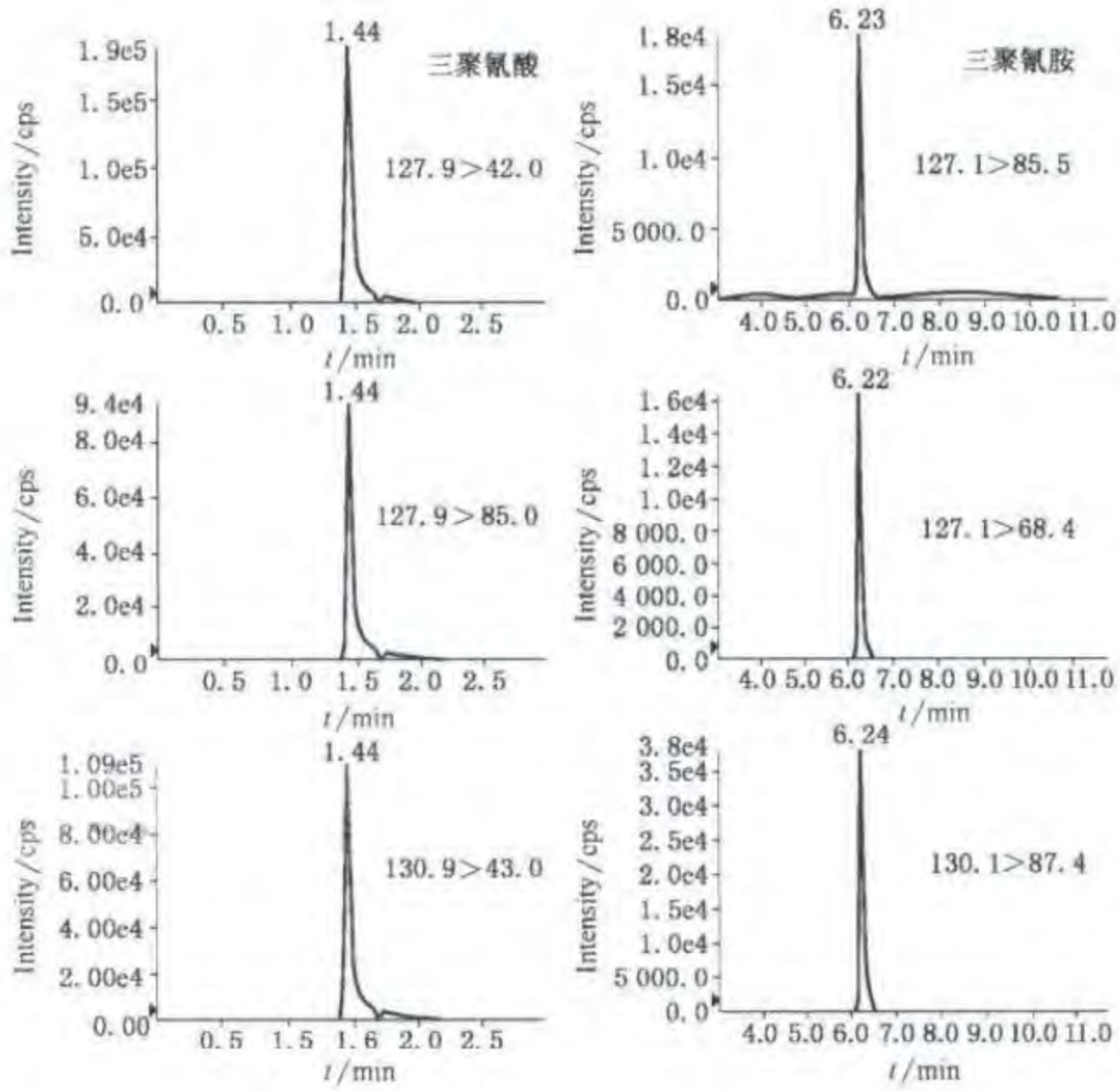

图 B.1 三聚氰酸(200 ng/mL)和三聚氰胺(100 ng/mL)标准溶液的多反应监测(MRM)色谱图

附 录 C
（资料性附录）
食品中三聚氰胺和三聚氰酸回收率范围

表 C.1 食品中三聚氰胺、三聚氰酸添加回收率范围

基质	化合物	添加水平 μg/kg	回收率 %	基质	化合物	添加水平 μg/kg	回收率 %
鸡蛋	三聚氰胺	25	92.8～107.6	虾肉	三聚氰胺	25	104.8～129.2
		50	95.6～109.4			50	93.0～122.0
		100	94.0～123.0			100	111.1～127.9
	三聚氰酸	50	73.8～101.8		三聚氰酸	50	81.8～115.6
		100	80.1～110.0			100	74.3～116.0
		200	104.0～117.5			200	106.0～127.0
猪肉	三聚氰胺	25	88.8～120.0	蜂蜜	三聚氰胺	25	78.8～100.8
		50	93.2～100.8			50	97.2～124.6
		100	94.1～111.1			100	99.6～105.0
	三聚氰酸	50	80.5～106.9		三聚氰酸	50	115.2～128.6
		100	73.6～85.5			100	112.0～128.0
		200	92.0～99.0			200	103.0～120.0
猪肝	三聚氰胺	25	104.6～123.2	猪肾	三聚氰胺	25	84.0～124.4
		50	108.6～128.6			50	82.4～111.4
		100	90.2～108.0			100	88.7～105.7
	三聚氰酸	50	76.6～91.8		三聚氰酸	50	97.9～117.5
		100	90.9～122.9			100	108.9～128.9
		200	82.0～90.5			200	90.9～112.9
肠衣	三聚氰胺	25	89.2～129.6	液态奶	三聚氰胺	25	99.6～124.8
		50	96.4～128.4			1 000	75.5～124.4
		100	79.8～92.8			2 500	72.6～93.4
	三聚氰酸	50	71.4～104.2		三聚氰酸	50	79.4～126.8
		100	78.6～107.1			250	82.4～123.2
		200	92.1～119.6			2 500	96.7～108.3
奶粉	三聚氰胺	50	70.0～94.0	饼干	三聚氰胺	50	72.8～88.4
		1 000	70.1～74.4			1 000	98.5～123.1
		2 500	70.3～83.2			2 500	91.4～98.6
	三聚氰酸	100	77.4～91.0		三聚氰酸	100	82.3～98.4
		250	73.2～86.0			250	88.9～103.2
		2 500	70.8～84.8			2 500	80.8～90.0

表 C.1（续）

基质	化合物	添加水平 μg/kg	回收率 %	基质	化合物	添加水平 μg/kg	回收率 %
冰淇淋	三聚氰胺	50	106.1～126.9	炼乳	三聚氰胺	50	82.2～120.4
		1 000	106.7～128.6			1 000	88.0～125.0
		2 500	89.7～113.7			2 500	101.2～115.2
	三聚氰酸	100	77.7～101.5		三聚氰酸	100	75.8～127.0
		250	92.2～108.0			250	76.0～111.2
		2 500	95.6～107.6			2 500	81.2～92.4
奶油	三聚氰胺	50	84.8～93.4	奶粉	三聚氰胺	50	89.2～127.6
		1 000	97.9～102.2			1 000	75.3～107.4
		2 500	96.7～101.9			2 500	80.5～95.7
	三聚氰酸	100	73.8～78.3		三聚氰酸	100	77.7～107.3
		250	93.5～103.9			250	73.7～88.5
		2 500	87.8～99.8			2 500	72.6～81.0
奶糖	三聚氰胺	50				50	70.6～123.4
		1 000	107.6～114.5		三聚氰胺	1 000	91.5～121.0
		2 500	98.1～104.6			2 500	93.5～103.5
	三聚氰酸	100	86.7～110.5	蛋白粉		100	73.6～123.7
		250	75.6～79.3		三聚氰酸	250	71.9～104.1
		2 500	96.1～108.5			2 500	107.8～129.8
豆粉	三聚氰胺	50	70.4～128.4	豆奶		50	73.6～128.4
		1 000	83.1～87.0		三聚氰胺	1 000	79.8～83.2
		2 500	76.0～84.8			2 500	78.4～86.0
	三聚氰酸	100	73.1～85.5			100	70.0～92.5
		250	74.8～90.4		三聚氰酸	250	99.4～117.0
		2 500	70.8～75.6			2 500	86.7～94.6

Foreword

This standard was drafted under the rules derived from GB/T 1.1—2009.

It is noted that some contents in this file may involve in patent authority. Agency regarding distribution and publication of this file does not be responsible for the identification of these patents.

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

The standard was drafted by Shanghai Entry-Exit Inspection and Quarantine Bureau of the People's Republic of China, Chinese Academy of Inspection and Quarantine, Shenzhen Entry-Exit Inspection and Quarantine Bureau of the People's Republic of China and Hubei Entry-Exit Inspection and Quarantine Bureau of the People's Republic of China.

The standard was mainly drafted by Deng Xiaojun, Zhao Shanzhen, Guo Dehua, Li Shujuan, Peng Tao, Jin Baohui, Han Li, Sheng Yonggang, Zhao Xiaoya, Wu Qiaobin and Zhang Yi.

Determination of melamine and cyanuric acid in foodstuffs for export—HPLC-MS/MS method

1 Scope

The standard specifies the method of sample preparation and determination of melamine and cyanuric acid in foodstuffs by HPLC-MS/MS.

The standard is applicable to the determination of melamine and cyanuric acid in egg, pork, liver, kidney, casing of pig, shrimp, honey, soybean milk, soybean powder, protein powder, liquid milk, milk powder, condensed milk, cheese, butter, icecream, toffee, biscuit by HPLC-MS/MS.

2 Principle

The residues of melamine and cyanuric acid were extracted using combination solution of acetonitrile with water followed by an adjustment of pH value to 2～3. The extraction was defatted using hexane and cleaned up with MCT SPE column. Melamine and cyanuric acid residues are determined by HPLC-MS/MS, and quantified by internal standard method.

3 Reagents and materials

Unless otherwise specified, the entire reagent used should be analytical grade, water is deionizer water.

3.1 Acetonitrile: HPLC grade.

3.2 Methanol: HPLC grade.

3.3 Hexane: HPLC grade.

3.4 Formic acid: HPLC grade.

3.5 Ammonium formate.

3.6 Hydrochloric acid (HCl).

3.7 Ammonium hydroxide

3.8 Diethyl amine.

3.9 Acetonitrile + water (7 + 3, $V_1 + V_2$): mix 700 mL of acetonitrile with 300 mL of water.

3.10 Acetonitrile + water (5 + 5, $V_1 + V_2$): mix 500 mL of acetonitrile with 500 mL of water.

3.11 1 mol/L HCl: mix 83 mL of HCl (3.6) with water to final volume of 1 L.

3.12 5% ammoniation-methanol: mix 5 mL of ammonia with 95 mL methanol to final volume of 100 mL.

3.13 100 mmol/L ammonium formate + acetonitrile (1 + 9, $V_1 + V_2$) pH 3.2: weigh accurately 6.3 g of ammonium formate and dissolve in 100 mL of water, add 900 mL of acetonitrile, adjust pH value to 3.2 ± 0.1 with formic acid.

3.14 0.1% formic acid-acetonitrile: mix 1 mL of formic acid with 1 L of acetonitrile.

3.15 Standards: Melamine (CAS No.: 108-78-1), $^{15}N_3$-melamine (CAS No.: 287476-11-3), Cyanuric acid (CAS No.: 108-80-5), $^{13}C_3$-cyanuric acid (CAS No.: 201996-37-4), purity of all chemicals was more than 98%.

3.16 Stock standard solution of melamine and cyanuric acid standards: weigh accurately appropriate amount standard of melamine and $^{15}N_3$-melamine and dissovle in acetonitrile + water (3.10) to final concentration of 1 mg/mL respectively. Dissolve appropriate amount standard of cyanuric acid and $^{13}C_3$-cyanuric acid (3.15) in diethyl amine and dilute to final concentration of 1 mg/mL with methanol respectively. All standards were refrigerated at −18 ℃.

3.17 Stock standard solution of intermediate standards: dilute the stock standard solution of melamine, $^{15}N_3$-melamine, cyanuric acid and $^{13}C_3$-cyanuric acid standards to final concentration of 1 μg/mL with acetonitrile respectively, store refrigerated at −18 ℃.

3.18 Calibration curve working solutions: to require as useful, prepare the calibration curve solution of melamine, $^{15}N_3$-melamine, cyanuric acid and $^{13}C_3$-cyanuric standards to final concentration from 10 ng/mL～500 ng/mL with isotope-labeled internal standard concentration of 100 ng/mL of $^{15}N_3$-melamine and 200 ng/mL of $^{13}C_3$-cyanuric acid by dilution of ammonium formate + acetonitrile (3.13) respectively. These working standard solutions are store refrigerated at −18 ℃, diluted directly before use.

3.19 Micropore film: 0.22 μm. organic.

3.20 Anpelclean MCT SPE columns (hydrophilic silica gel and cation exchange resin mixed sorbents)[1] :3 mL,150 mg or equalvent.

4 Apparatus and equipment

4.1 Liquid chromatography combined with electrospray ionization mass spectrometry (ESI).

4.2 Balances (0.000 1 g and 0.01 g).

4.3 Pulverizer.

4.4 Vortex mixer.

4.5 Ultrasonic apparatus.

4.6 Centrifuge.

4.7 Apparatus of SPE.

4.8 Nitrogen evaporator.

5 Preparation and storage of test sample

5.1 Meat,liver,kidney,egg,shrimp,casing,biscuit,sugar

The combined primary sample is reduced to 500 g which is totally minced and placed into a clean vessel as a test sample,which is sealed and labeled. The test sample should be stored at temperature of below −18 ℃. Casing sample was desalted before using.

5.2 Honey, milk, milk powder, soybean milk, soybean powder, protein powder, condensed milk, cheese,butter,ice cream

The combined primary sample is reduced to 500 g placed into a clean vessel as a test sample,which is sealed and labeled. The test sample should be stored at temperature of 4 ℃.

Annotation:in course of sampling and sample preparation,precaution should be taken to avoid con-

1) Anpelclean MCT SPE column for extraction of melamine and cyanuric acid is developed and produced by shanghai Anpel Scientific Instrument Co. The information given here is not that CNCA accredits the product,but for user's convenience.

tamination or any factor that may causes the change of residue content.

6 Procedure

6.1 Extraction

6.1.1 Meat shrimp, casing, liver, kidney, egg

Weigh 2 g (accurate to 0.01 g) sample in 50 mL plastic centrifuge tubes, add 200 μL of $^{15}N_3$-melamine and 400 μL of $^{13}C_3$-cyanuric acid solution (3.17) respectively, add 1 mL water and 7 mL acetonitrile in case of egg sample, add 10 mL acetonitrile + water (3.9) to other types of sample, vibrate for 30 s, add 1 mol/L HCl (3.11) to adjust pH value within the range of 2.0～3.0. mix and vibrate for 2 min followed by an ultrasonic extraction for 15 min, and then centrifuge at 8 000 r/min for 5 min. The supernatant is degreased by 5 mL of hexane, vibrate for 2 min and centrifuge at 8 000 r/min for 5 min, transfer the supernatant to filter into a 15 mL glass tube. Take 2 mL of sample solution and add 0.8 mL of water, and dilute to final volume of 5 mL with acetonitrile + water (3.10).

6.1.2 Sample of honey

Weigh 1 g (accurate to 0.01 g) sample in 50 mL plastic centrifuge tubes, add 100 μL of $^{15}N_3$-melamine and 200 μL of $^{13}C_3$-cyanuric acid solution (3.17) respectively, add 8 mL acetonitrile + water (3.10) and vibrate for 30 s, add 1 mol/L HCl (3.11) to adjust pH value within the range of 2.0～3.0, mix and vibrate for 2 min followed by an ultrasonic extraction for 15 min and then centrifuge at 8 000 r/min for 5 min, transfer the supernatant to filter into a 15 mL glass tube and adjust to 8 mL by acetonitrile + water (3.10).

6.1.3 Sample of milk, condensed milk, ice cream, soybean milk

Weigh 2 g (accurate to 0.01 g) sample in 50 mL plastic centrifuge tubes, add 200 μL of $^{15}N_3$-melamine and 400 μL of $^{13}C_3$-cyanuric acid solution (3.17) respectively, add 2 mL water and 4 mL acetonitrile, vibrate for 30 s, add 1 mol/L HCl (3.11) to adjust pH value within the range of 2.0～3.0, mix and vibrate for 2 min followed by an ultrasonic extraction for 15 min and then centrifuge at 8 000 r/min for 5 min. Transfer the supernatant to filter into a 15 mL glass tube and adjust to 8 mL by acetonitrile + water (3.10).

6.1.4 Sample of butter, cheese, sugar, biscuit, milk powder, soybean powder, protein powder

Weigh 1 g (accurate to 0.01 g) sample in 50 mL plastic centrifuge tubes, add 100 μL of $^{15}N_3$-melamine and 200 μL of $^{13}C_3$-cyanuric acid solution (3.17) respectively, dissolved by 3 mL water, add 7 mL acetonitrile, vibrate for 30 s, add 1 mol/L HCl (3.11) to adjust pH value within the range of 2.0～3.0,

mix and vibrate for 2 min followed by an ultrasonic extraction for 15 min, and then centrifuge at 8 000 r/min for 5 min. Butter, cheese and biscuit samples are defatted using 5 mL of hexane. The supematant is defatted by 5 mL of hexane, vibrate for 2 min and centrifuge at 8 000 r/min for 5 min, transfer the supernatant to filter into a 15 mL glass tube. Take 2 mL of sample solution and add 0.8 mL of water, and dilute to final volume of 5 mL with acetonitrile + water (3.10).

6.2 Cleaning up

The MCT (3.20) SPE cartridge is conditioned with 3 mL of methanol and 3 mL of acetonitrile + water (3.10). Load all the sample solution to column at gravity with flow no more than 1 d/s. Then, the column is washed with 2 mL of acetonitrile + water (3.10). After purge the cartridges at vacuum, the column is eluted with 2 mL of methanol and 4 mL 5% ammonia-methanol (3.12) subsequently. The elutes is concentrated to nearly dryness by gentle nitrogen under 40 ℃ and add 1 mL of mobile phase (3.13) to resolve the residence and filter by 0.22 μm film (3.19) before HPLC-MS analysis.

6.3 Determination

6.3.1 HPLC operating conditions

6.3.1.1 Column: Waters Atlantis HILIC (150 mm × 2.1 mm, 5 μm), or the equvalent.

6.3.1.2 Mobile phase: A 100 mmol/L ammonium formate + acetonitrile ($1+9$, V_1+V_2) pH 3.2, B 0.1% formic acid-acetonitrile.

Table 1—Gradient program of mobile phase

Time min	Mobile phase A	Mobile phase B
0	0	100
2.5	0	100
4	100	0
8	100	0
8.5	0	100
12	0	100

6.3.1.3 Rate: 0.4 mL/min.

6.3.1.4 Injection volume: 10 μL.

6.3.1.5 Column temperature: room temperature.

6.3.2 Mass spectral acquisition

6.3.2.1 Suorce:ESI (postive-negative swithed mode).

6.3.2.2 Monitor mode:multiple raction monitoring,MRM.

6.3.2.3 Nebulizer gas (GS1),curtain gas (CUR),auxiliary heater gas (GS2) and collision gas (CAD) are high purity nitrogen or equivalent,optimize the flow rate of each gas and ion source temperature to reach the equirement of the sensitivity of mass spectrometry. Detailed parameters are shown as annex A.

6.3.2.4 Collision energy (CE),decustering potential (DP),collision cell exit potential (CXP),collision cell entrance potential (EP)and electros pray capillary voltage (IS) should be optimized to the best sensitivity. Related parameters and qualifier and quantifier MRM are listed as table A.1.

6.3.3 Quantitation of HPLC-MS/MS

According to established HPLC-MS/MS operating condition,determine the sample solution and the standard working curve simultaneously. The standard working curve should contained 5 level of concentration including the zero point. If the determined sample is over the scope of standard working curve,the concentration of determined sample should diluted to a porper concentration by mobile phase (3.13). Under the above HPLC-MS/MS operating condition,the retention time of cyanuric acid and melamine are 1.50 min,6.61 min respectively,the MRM chromatograms of the standard are listed as figure B.1.

6.3.4 Confirmation of HPLC-MS/MS

Determinate under the established HPLC-MS/MS conditions,and calculated the intensity ration of two selected ion pairs of the sample solution and the standard working solution. If the retention times of sample chromatogram peaks are consistent with that of working solution and their windage is less than 5 min,and the relative abundance ratio tolerance is the same as listed (table 2),it is safe to conclude that this compound do exit in the sample.

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

Relative ion intensities	>50%	>20%~50%	>10%~20%	≤10%
Permitted relative tolerances	±20%	±25%	±30%	±50%

6.4 Blank test

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

7 Calculation and expression of result

Calculation the content of benzodiazepine residues in the test sample by HPLC-MS/MS data processor or according to the formula (1). The blank value should be subtracted from the above result of calculation.

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

Where:

X_i —the residue content of melamine and cyanuric acid, μg/kg;

R_i —the ration of peak areas between analytes and internal standard in sample solution;

c_i —the concentration of melamine and cyanuric acid residue from standard working curve, ng/mL;

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

R_s —the ration of peak areas between analytes and internal standards in calbiration curves;

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

8 Limit of quantification (LOQ) and recovery

8.1 Limit of quantification (LOQ)

The limit of quantification of the method in milk and foodstuffs of animal orgin as egg, meat, kidney, liver and casing of pig, shrimp and honey is 25 μg/kg for melamine and 50 μg/kg for cyanuric acid.

The limit of quantification of the method in foodstuffs of plant orgin as soybean milk, soybean powder, protein powder, and milk products contained products as milk powder, condensed milk, cheese, butter, ice cream biscuit and sugar is 50 μg/kg for melamine and 100 μg/kg for cyanuric acid.

8.2 Recovery

According to the experimental data, the fortified concentration and recovery ranges of melamine and cyanuric acid in milk, foodstuffs of animal orgin as egg, meat, kidney, liver and casing of pig, shrimp and honey, foodstuffs of plant orgin as soybean milk, soybean powder, protein powder, and milk products contained products as milk powder, condensed milk, cheese, butter, ice cream, biscuit and sugar are listed as table C. 1.

Annex A
(Informative)
Main mass parameters of API 4000[2)]

Main mass parameters:

a) Electros pray capillary voltage:positive mode 5 500 V; negative mode −4 500 V.

b) CAD:Medium.

c) GSI:517 kPa (75 psi).

d) CUR:172 kPa (25 psi).

e) GS2:414 kPa (60 psi).

f) TEM:550 ℃.

g) Qualifier and quantifier MRM,collision engry (CE),declustering potential (DP).

Table A. 1—Transitions for confirmation and quantification,CE,DP,EP,CXP

Compound	Precursor ion (Q1)	Mode	Production (Q3)	CE V	DP V	EP V	CXP V
Melamine	127.1	positive	85.5*	27	71	8	14
			68.4	43	71	11	10
$^{15}N_3$-melamine	130.1		87.4	27	61	8	14
Cyanuric acid	127.9	negative	42.0*	−14	−50	−10	−1
			85.0	−18	−50	−10	−1
$^{13}C_3$-cyanuric acid	130.9		43.0	−30	−45	−10	−1
Annotation: the symbol"*"represents the quantitative transition.							

2) Non-commercial statement:the equipment and their types involved in the standard method are not related to commercial aims,and it is encouraged to use equipment of different corporation or different type.

Annex B
(Informative)
MRM chromatogram of standard

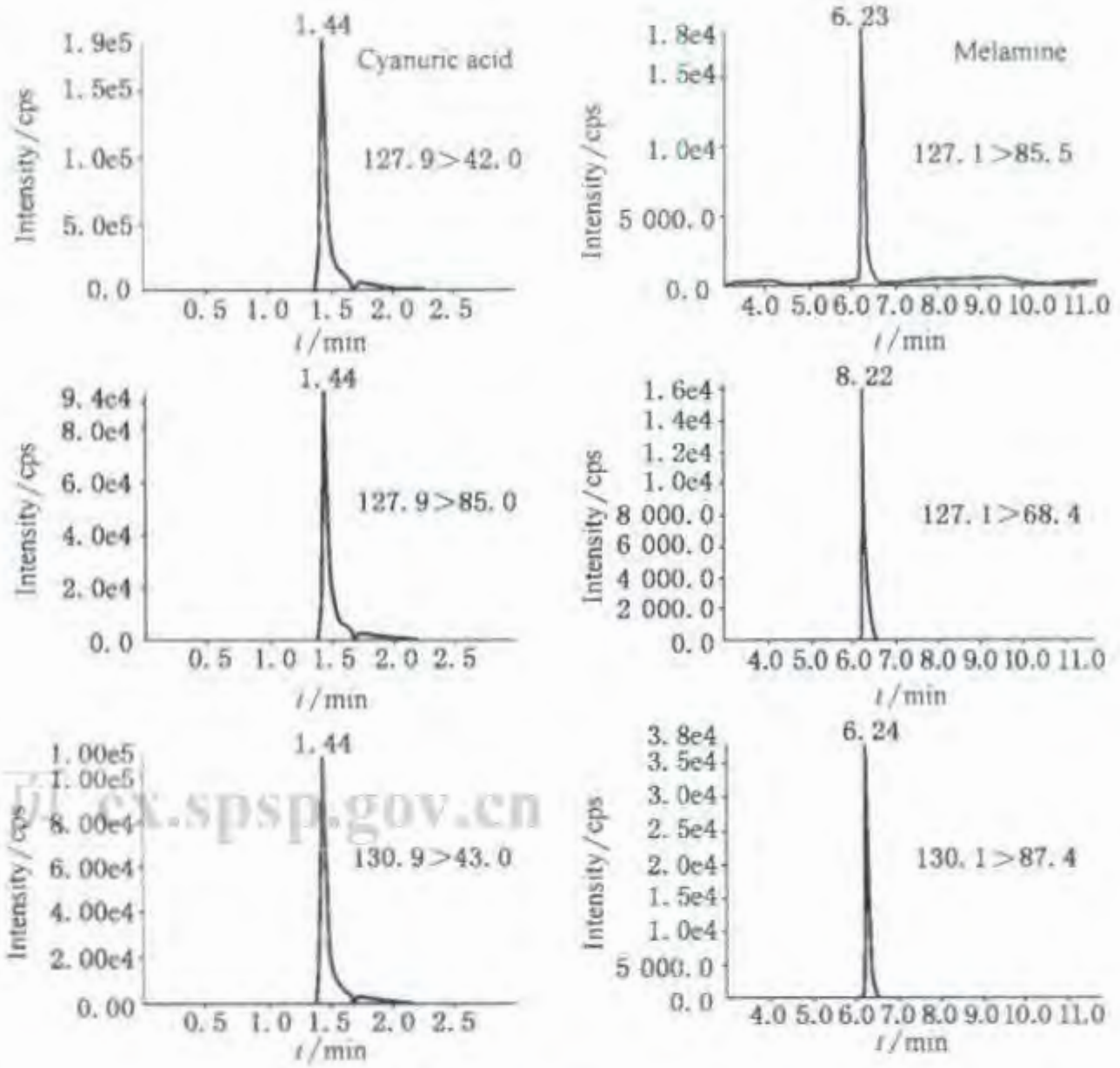

Figure B.1—MRM chromatogram of cyanuric acid (200 ng/mL) and melamine (100 ng/mL) standards

Annex C
(Informative)
Recovery ranges

Table C. 1—Recovery ranges

Matrix	Residuum	Fortified level μg/kg	Recovery %	Matrix	Residuum	Fortified level μg/kg	Recovery %
Egg	Melamine	25	92.0～107.6	Shrimp	Melamine	25	104.8～129.2
		50	95.6～109.6			50	93.0～122.0
		100	94.0～123.0			100	111.1～127.9
	Cyanuric acid	50	73.8～101.8		Cyanuric acid	50	81.8～115.6
		100	80.1～110.0			100	74.3～116.0
		200	104.0～117.5			200	106.0～127.0
Pork	Melamine	25	83.2～120.0	Honey	Melamine	25	78.8～100.8
		50	75.2～100.8			50	97.2～124.6
		100	93.5～119.5			100	99.6～105.0
	Cyanuric acid	50	80.5～101.9		Cyanuric acid	50	115.2～128.6
		100	73.5～85.5			100	112.0～128.0
		200	92.0～99.0			200	103.0～120.0
Liver	Melamine	25	101.6～123.2	Kindy	Melamine	25	84.0～124.4
		50	108.6～123.6			50	82.4～111.4
		100	90.2～103.0			100	88.7～105.7
	Cyanuric acid	50	76.6～91.8		Cyanuric acid	50	97.9～117.5
		100	89.9～122.9			100	108.9～128.9
		200	82.0～90.5			200	90.9～112.9
Casing	Melamine	25	89.2～129.6	Milk	Melamine	25	99.6～124.8
		50	96.4～128.4			1 000	75.5～124.4
		100	79.8～92.8			2 500	72.6～93.4
	Cyanuric acid	50	71.4～104.2		Cyanuric acid	50	79.4～126.8
		100	78.6～107.1			250	82.4～123.2
		200	92.1～119.6			2 500	96.7～108.3
Milk powder	Melamine	50	70.0～94.0	Biscut	Melamine	50	72.8～88.4
		1 000	70.1～74.4			1 000	98.5～123.1
		2 500	70.3～83.2			2 500	91.4～98.6
	Cyanuric acid	100	77.4～91.0		Cyanuric acid	100	82.3～98.4
		250	73.2～86.0			250	88.9～103.2
		2 500	70.8～84.8			2 500	80.8～90.0

Table C. 1 (continued)

Matrix	Residuum	Fortified level μg/kg	Recovery %	Matrix	Residuum	Fortified level μg/kg	Recovery %
Ice cream	Melamine	50	106. 1～126. 9	Condensed milk	Melamine	50	82. 2～120. 4
		1 000	106. 7～128. 6			1 000	88. 0～125. 0
		2 500	89. 7～113. 7			2 500	101. 2～115. 2
	Cyanuric acid	100	77. 7～101. 5		Cyanuric acid	100	75. 8～127. 0
		250	92. 2～108. 0			250	76. 0～111. 2
		2 500	95. 6～107. 6			2 500	81. 2～92. 4
Butter	Melamine	50	84. 8～93. 4	Cheese	Melamine	50	89. 2～127. 6
		1 000	97. 8～102. 2			1 000	75. 3～107. 4
		2 500	96. 7～101. 9			2 500	80. 5～95. 7
	Cyanuric acid	100	73. 8～78. 3		Cyanuric acid	100	77. 7～107. 3
		250	93. 5～103. 9			250	73. 7～88. 5
		2 500	87. 8～99. 8			2 500	72. 6～81. 0
Surger	Melamine	50	76. 4～86. 4	Protein powder	Melamine	50	70. 6～123. 4
		1 000	107. 9～111. 1			1 000	91. 5～121. 0
		2 500	98. 1～104. 6			2 500	93. 5～103. 5
	Cyanuric acid	100	86. 7～110. 5		Cyanuric acid	100	73. 6～123. 7
		250	73. 6～79. 3			250	71. 9～104. 1
		2 500	96. 1～108. 5			2 500	107. 8～129. 8
Soybean powder	Melamine	50	70. 4～128. 4	Soybean milk	Melamine	50	73. 6～128. 4
		1 000	83. 1～87. 0			1 000	79. 8～83. 2
		2 500	76. 0～84. 8			2 500	78. 4～86. 0
	Cyanuric acid	100	72. 1～85. 5		Cyanuric acid	100	70. 0～92. 5
		250	74. 8～90. 4			250	99. 4～117. 0
		2 500	70. 8～75. 6			2 500	86. 7～94. 6