

SN

中华人民共和国出入境检验检疫行业标准

SN/T 0535—2016
代替 SN 0535—1996

进出口饲料中棉酚的测定

Determination of gossypol in feed for import and export

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中 华 人 民 共 和 国
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前 言

本标准按照 GB/T 1.1—2009 给出的规划起草。

本标准代替 SN 0535—1996《出口饲料中棉酚检验方法液相色谱法》。与 SN 0535—1996 相比,除编辑性修改外主要技术变化如下:

——增加了确证方法;

——技术指标满足多种基质和限量要求。

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检验检疫技术中心,中华人民共和国上海出入境检验检疫局。

本标准主要起草人:张文华、黄超群、邹学权、陈玲玲、于卓然、童贇恺、陈丽、汤杭燕。

本标准所代替标准的历次版本发布情况为:

——SN 0535—1996。

进出口饲料中棉酚的测定

1 范围

本标准规定了饲料中棉酚的两种测定方法,高效液相色谱法(HPLC法)和液相色谱-质谱/质谱法(LC-MS/MS法)。

本标准适用于棉籽饼(粕)、猪饲料和鸡饲料中棉酚的定量测定;液相色谱-质谱/质谱法的定量确证。

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(包括所有的修改单)适用于本文件。

GB/T 6682 分析实验室用水规格和试验方法

3 第一法 高效液相色谱法(HPLC法)

3.1 原理

试样用丙酮水溶液提取,过滤膜,用高效液相色谱测定,外标法定量。

3.2 试剂和材料

除特殊注明外,所有试剂均为分析纯,水为 GB/T 6682 规定的一级水。

3.2.1 丙酮:色谱纯。

3.2.2 甲醇:色谱纯。

3.2.3 磷酸。

3.2.4 丙酮-水溶液(70+30,体积比):准确量取 70 mL 丙酮(3.2.1)和 30 mL 水,混匀后备用。

3.2.5 0.1%磷酸溶液:1 mL 磷酸溶解水中,并定容至 1 L,混匀过滤(0.45 μm)后备用。

3.2.6 棉酚标准品:CAS:303-45-7,纯度为 90.5%。

3.2.7 棉酚标准储备液:准确称取 10 mg(精确至 0.1 mg)棉酚标准品于 10 mL 容量瓶中,用丙酮(3.2.1)溶解并定容至刻度,配制成浓度为 1 mg/mL 的标准储备溶液,于 4 $^{\circ}\text{C}$ 避光保存。

3.2.8 微孔滤膜:0.22 μm ,有机系。

3.3 仪器和设备

3.3.1 高效液相色谱(HPLC)仪:配有紫外检测器。

3.3.2 电子天平:感量为 0.01 g 和 0.000 1 g。

3.3.3 离心机:转速不低于 4 000 r/min。

3.3.4 振荡仪:转速不低于 300 r/min。

3.3.5 涡旋混匀器。

3.3.6 塑料离心管:50 mL。

3.3.7 研钵。

3.4 试样处理

称取 5 g(精确至 0.01 g)试样于 50 mL 离心管中,加入 10 mL 水浸泡 1 h,加入丙酮(3.2.1)至 30 mL,振摇 20 min。离心 5 min 后,将上清液转移至 50 mL 棕色容量瓶中,用 10 mL 丙酮-水溶液(3.2.4)重复提取两次,用丙酮-水溶液(3.2.4)定容至 50 mL。静置 5 min,取上清液过微孔滤膜(3.2.8),供 HPLC 测定。

3.5 高效液相色谱测定

3.5.1 HPLC 参考条件

HPLC 参考条件如下:

- a) 色谱柱: C₁₈ 柱, 250 mm×4.6 mm(内径), 粒径 5.0 μm, 或功能相当者。
- b) 流动相: 甲醇(3.2.2)-磷酸溶液(3.2.5)(88+12, 体积比), 混匀。
- c) 流速: 1.5 mL/min。
- d) 波长: 238 nm。
- e) 柱温: 25 ℃。
- f) 进样量: 10 μL。

3.5.2 标准曲线绘制

用丙酮(3.2.1)将棉酚标准储备液逐级稀释得到的浓度为 1 μg/mL、2 μg/mL、4 μg/mL、6 μg/mL、10 μg/mL 的标准工作液, 浓度由低到高进样检测, 以峰面积-浓度作图, 得到标准曲线回归方程。棉酚的标准品色谱图参见附录 A 中图 A.1。

3.5.3 定量测定

待测样液中棉酚的响应值应在标准曲线线性范围内, 超过线性范围则应稀释后再进样分析。

3.5.4 空白试验

除不称取试样外, 均按上述测定条件和步骤进行。

3.5.5 结果计算

试样中棉酚的含量由色谱数据处理软件或按式(1)计算获得:

$$X = \frac{C \times V \times 1\,000}{m \times 1\,000} \dots\dots\dots (1)$$

式中:

X —— 试样中棉酚的含量, 单位为毫克每千克(mg/kg);

C —— 从标准工作曲线得到的棉酚溶液浓度, 单位为微克每毫升(μg/mL);

V —— 样液最终定容体积, 单位为毫升(mL);

m —— 最终样液代表的试样量, 单位为克(g)。

注: 计算结果需扣除空白。

3.6 方法测定低限

本方法的测定低限为 10 mg/kg。

3.7 回收率和精密度

饲料基质中不同添加浓度水平下的回收率范围见表 1。

表 1 饲料中棉酚添加回收率与精密度试验(n=6)

化合物	添加水平 (mg/kg)	回收率范围 /%	相对标准偏差 RSD/%
棉酚	10	89.9~99.2	3.4
	20	82.8~90.3	3.6
	40	82.3~87.1	2.3

3.8 阳性试样的重现性实验

三种阳性棉籽粕试样的重现性实验,结果见表 2。

表 2 测定不同阳性试样的重现性与精密度试验(n=6)

被测试样	测定值/mg/kg						平均值 mg/kg	相对标准偏差 RSD/%
	1	2	3	4	5	6		
棉籽粕 1	92.04	90.99	76.47	87.36	82.61	97.78	87.88	8.6
棉籽粕 2	29.31	29.23	32.00	30.55	28.66	33.22	30.50	5.9
棉籽粕 3	24.87	25.09	23.59	23.06	26.10	24.28	24.50	4.5

4 第二法 液相色谱-质谱/质谱法(LC-MS/MS 法)

4.1 原理

试样用丙酮水溶液提取,过滤膜,用液相色谱-质谱/质谱法测定和确证,外标法定量。

4.2 试剂和材料

除特殊注明外,所有试剂均为分析纯,水为符合 GB/T 6682 规定的一级水。

4.2.1 甲酸:色谱纯。

4.2.2 乙腈:色谱纯。

4.2.3 0.1%甲酸溶液:准确量取 1 mL 甲酸于 1 L 容量瓶中,加水并定容至刻度,混匀后备用。

4.2.4 其他同 3.2。

4.3 仪器和设备

4.3.1 液相色谱-质谱/质谱(LC-MS/MS)仪:配有电喷雾离子源(ESI)。

4.3.2 其他同 3.3。

4.4 试样处理

称取 1 g(精确至 0.01 g)试样于 50 mL 离心管中,加入 10 mL 水浸泡 1 h,加入丙酮(3.2.1)至 30 mL,振摇 20 min。离心 5 min 后,将上清液转移至 100 mL 棕色容量瓶中,用 30 mL 丙酮-水溶液(3.2.4)重复提取两次,用丙酮-水溶液(3.2.4)定容至 100 mL。静置 5 min,取上清液过微孔滤膜(3.2.8),供 LC-MS/MS 测定。

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4.5 液相色谱-质谱/质谱测定

4.5.1 LC 参考条件

- LC 参考条件如下：
- a) 色谱柱： C_{18} ，100 mm×2.1 mm(内径)；粒径 3.5 μm ，或功能相当者。
 - b) 流动相：乙腈(4.2.2)-甲酸溶液(4.2.3)，梯度洗脱程序参见表 3。
 - c) 流速：0.30 mL/min 或根据仪器条件优化。
 - d) 进样量：5 μL 。

表 3 梯度洗脱程序

时间/min	0.1%甲醇溶液/%	乙腈/%
0.0	50	50
2.0	50	50
4.0	10	90
6.0	10	90
7.0	50	50
10.0	50	50

4.5.2 MS/MS 参考条件

- MS/MS 参考条件如下：
- a) 电离方式：电喷雾离子源，负离子。
 - b) 电喷雾电压(IS)：3 500 V。
 - c) 鞘气：高纯氮气，6.5 L/min。
 - d) 辅助气：高纯氮气，5.0 L/min。
 - e) 碰撞气：高纯氩气，0.2 Pa。
 - f) 扫描模式：多反应监测(MRM)，母离子 m/z 517.1，定量离子 m/z 230.9，定性离子 m/z 471.1。
 - g) 离子源温度：300 $^{\circ}\text{C}$ 。
 - h) 毛细管温度：270 $^{\circ}\text{C}$ 。
 - i) 碰撞能量： m/z 517.1>230.9 为 43V， m/z 517.1>471.1 为 35 V。

4.5.3 标准曲线绘制

取空白试样按照 4.4 处理。用所得的试样溶液将棉酚储备液(3.2.7)逐级稀释得到的浓度为 0.01 $\mu\text{g/mL}$ 、0.02 $\mu\text{g/mL}$ 、0.04 $\mu\text{g/mL}$ 、0.06 $\mu\text{g/mL}$ 、0.1 $\mu\text{g/mL}$ 的标准工作液，浓度由低到高进样检测，以定量离子峰面积-浓度作图，得到标准曲线回归方程。基质匹配加标棉酚的标准溶液 LC-MS/MS 多反应监测质量色谱图参见附录 A 中的图 A.2。

4.5.4 定量测定

待测样液中棉酚的响应值应在标准曲线线性范围内，超过线性范围则应稀释后再进样分析。

4.5.5 定性测定

按照上述条件测定样液和标准工作溶液，如果样液中待测物的色谱峰保留时间与标准溶液一致(变

化范围在±2.5%之内),试样中目标化合物的两个子离子的相对丰度与浓度相当标准溶液的相对丰度一致,相对丰度偏差不超过表 4 的规定,则可判断试样中存在棉酚。

表 4 定性离子相对丰度的最大允许偏差

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

4.5.6 结果计算

同 3.5.4。

4.6 空白实验

除不称取试样外,均按上述测定条件和步骤进行。

4.7 方法测定低限

本方法的测定低限为 1 mg/kg。

4.8 回收率和精密度

饲料基质中不同添加浓度水平下的回收率范围见表 5。

表 5 饲料中棉酚添加回收率与精密度试验(n=6)

化合物	添加水平 (mg/kg)	回收率范围 /%	相对标准偏差 RSD/%
棉酚	1	88.0~102.0	5.3
	20	83.3~90.2	2.9
	40	82.4~86.3	1.7

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附录 A
(资料性附录)
棉酚标准品色谱图

图 A.1 和图 A.2 给出了棉酚标准溶液的 HPLC 色谱图和多反应监测图。

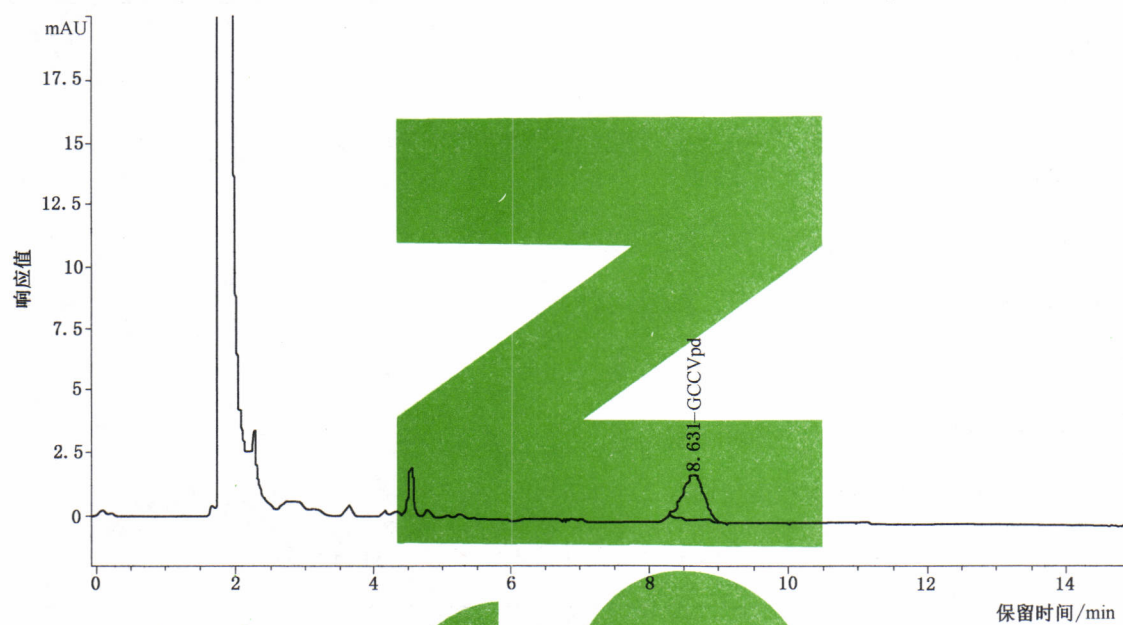


图 A.1 棉酚标准溶液的 HPLC 色谱图(1 $\mu\text{g/mL}$)

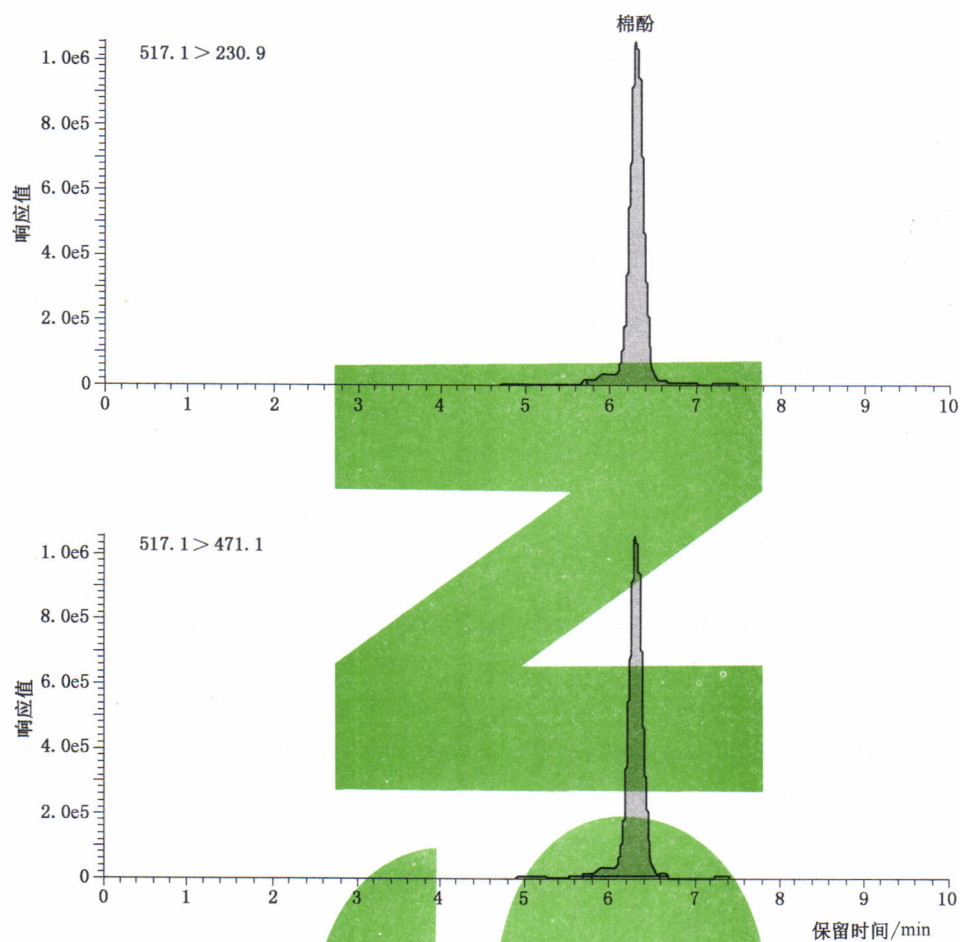


图 A.2 棉酚标准溶液的多反应监测图(10 ng/mL)

Foreword

This standard is drafted according to GB/T 1.1—2009 “Directives for standardization Part 1: Structure and drafting of standards” principle.

This standard is used to replace of SN 0535—1996 “Method for the determination of gossypol in fodders for Export-Liquid chromatographic method”. The main improvement from SN 0535—1996:

—LC-MS/MS is performed as a confirmatory method;

—The method is suitable for various matrixes and limits.

Some parts of the standard may have relationship with some patents. The release department has no responsibility to recognize these patents.

This standard was proposed by and is under the charged of the Certification and Accreditation and Administration 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.

This standard is mainly drafted by Zhang Wenhua, Huang Chaoqun, Zou Xuequan, Chen Lingling, Yu Zhuoran, Tong Yunkai, Chen Li, Tang Hangyan.

This part replaced the previous version of the release of the standard as follows.

—SN 0535—1996.

Determination of gossypol in feed for import and export

1 Scope

This standard specifies two kinds of determination methods, high performance liquid chromatography (HPLC) and liquid chromatography-tandem mass spectrometry (LC-MS/MS).

This standard is applicable to the determination and confirmation of gossypol in cottonseed cake (meal), pig feed and chicken feed.

2 Normative references

The following documents are necessary for this standard. For dated references, only dated editions shall apply to this standard. For undated references, the latest edition of the normative document (including subsequent amendments) referred to applies.

GB/T 6682 specification and test method for water used in analytical lab.

3 HPLC method

3.1 Principle

Gossypol is extracted from the sample with acetone-water (70 + 30, v/v), after dilution and filtration, the extract is determined by HPLC, and quantified by external standard method.

3.2 Reagent and materials

Unless otherwise specified, all the reagents used should be analytical grade. "water" is first-grade water prescribed by GB/T 6682.

3.2.1 Acetone: HPLC grade.

3.2.2 Methanol: HPLC grade.

3.2.3 Phosphoric acid.

3.2.4 Acetone-water (70 + 30, v/v): Dissolve 70 mL of acetone to final volume of 100 mL with water.

3.2.5 0.1% phosphoric acid solution: Dissolve 1 mL phosphoric acid to final volume of 1 000 mL with water.

3.2.6 Standards: gossypol, $C_{30}H_{30}O_8$, CAS No.: 303-45-7, purity $\geq 90.5\%$.

3.2.7 Stock standard solution: accurately weigh 0.01 ± 0.0001 g of gossypol standard and dissolve with acetone to make the standard stock solution of 1.0 mg/mL. This solution is stored in a refrigerator at 4 °C.

3.2.8 Membrane filter: 0.22 μ m, organic phase.

3.3 Apparatus and equipment

3.3.1 HPLC with ultraviolet detector.

3.3.2 Electronic balance: Accurate 0.01 g and 0.0001 g.

3.3.3 Centrifuge: 4 000 r/min equipped.

3.3.4 Vibrator: 300 r/min equipped.

3.3.5 Vortex mixer.

3.3.6 Plastic centrifuge tube: 50 mL.

3.3.7 Mortar.

3.4 Procedure

Weigh 5 ± 0.01 g of the test sample into a 50 mL polypropylene bottle, add 10 mL of water, stand for 1 h. Dissolve in acetone (3.2.1) to make the final volume to be 30 mL, blend for 20 min with vibrator (3.3.4), transfer the above solution to 50 mL brown volumetric flask, extract the residue with 10 mL acetone-water (3.2.4) twice more, dilute exactly the extract solution to final volume of 50 mL with acetone-water (3.2.4), stand for 5 min. After filtration, collect 1 mL for determination.

3.5 HPLC determination

3.5.1 HPLC condition

HPLC condition is as follows:

- a) LC column: C₁₈ column, 250 mm × 4.6 mm (i.d.) × 5.0 μm (film thickness), or equivalent column.
- b) Mobile phase: methanol (3.2.2) + phosphoric acid solution (3.2.5) (88 + 12, v/v).
- c) Flow rate: 1.5 mL/min.
- d) Excitation wavelength, 238 nm.
- e) Column temperature: 25 °C.
- f) Injection volume: 10 μL.

3.5.2 Preparation of the working curve

The gossypol stock standard solution is diluted by acetone (3.2.1). The concentration of these gossypol standard working solution are 1 μg/mL, 2 μg/mL, 4 μg/mL, 6 μg/mL, 10 μg/mL. Plot a working curve of peak area versus concentrations of gossypol. Chromatogram of the standard is listed as figure A. 1 in annex A.

3.5.3 Determination and confirmation by HPLC

The response of the analyte should be within the linear range of the instrument detection and quantified by external standard method.

3.5.4 Blank test

Perform the blank test with the same procedures as that described in the method of determination but without sample addition.

3.5.5 Calculation and expression of result

Calculation the content of gossypol in the test sample by HPLC data processor or according to the formula (1). The result of calculation should be deducted with blank value.

$$X = \frac{C \times V \times 1\,000}{m \times 1\,000} \dots\dots\dots (1)$$

Where:

X —the residue content of gossypol in the test sample, mg/kg;

C —the concentration of gossypol in the standard working solution, μg/mL;

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

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

3.6 Limit of quantitation

The limit of quantitation (LOQ) of the method for gossypol is 10 mg/kg.

3.7 Recovery and precision

The recovery range and precision of this method is shown in table 1.

Table 1 Recovery range and precision of gossypol in feed ($n=6$)

Compound	Spike /(mg/kg)	Recovery /%	RSD/%
Gossypol	10	89.9~99.2	3.4
	20	82.8~90.3	3.6
	40	82.3~87.1	2.3

3.8 Reproducibility of positive sample

Reproducibility test of three positive samples is listed in table 2.

Table 2 Reproducibility and precision of gossypol in Cottonseed meal ($n=6$)

Sample	Measured value/(mg/kg)						Average mg/kg	RSD/%
	1	2	3	4	5	6		
Cottonseed meal 1	92.04	90.99	76.47	87.36	82.61	97.78	87.88	8.6
Cottonseed meal 2	29.31	29.23	32.00	30.55	28.66	33.22	30.50	5.9
Cottonseed meal 3	24.87	25.09	23.59	23.06	26.10	24.28	24.50	4.5

4 LC-MS/MS method

4.1 Principle

Gossypol is extracted from the sample with acetone-water (70 + 30, v/v), after dilution and filtration, the extract is determined by LC-MS/MS, and quantified by external standard method.

4.2 Reagent and materials

Unless otherwise specified, all the reagents used should be analytical grade. “water” is first-grade water prescribed by GB/T 6682.

4.2.1 Formic acid: HPLC grade.

4.2.2 Acetonitrile: HPLC grade.

4.2.3 0.1% formic acid solution: Dissolve 1 mL formic acid to final volume of 1 000 mL with water.

4.2.4 Others are the same as 3.2.

4.3 Apparatus and equipment

4.3.1 Liquid chromatography-mass/mass spectrometry, equipped with electrospray ion source.

4.3.2 Others are the same as 3.3.

4.4 Procedure

Weigh 1 ± 0.01 g of the test sample into a 50 mL polypropylene bottle, add 10 mL of water, stand for 1 h. Dissolve in acetone (3.2.1) to make the final volume to be 30 mL, blend for 20 min with vibrator (3.3.4), transfer the above solution to 100 mL brown volumetric flask, extract the residue with 30 mL acetone-water (3.2.4) twice more, dilute exactly the extract solution to final volume of 50 mL with acetone-water (3.2.4), stand for 5 min. After filtration, collect 1 mL for determination.

4.5 LC-MS/MS determination

4.5.1 HPLC condition

HPLC condition is as follows;

- a) Column: C_{18} , 100 mm \times 2.1 mm (i.d.) \times 3.5 μ m (film thickness); equivalent.
- b) Mobile phase: acetonitrile (4.2.2) – 0.1% formic acid solution (4.2.3) (A), gradient elution condition is listed in table 3.
- c) Flow rate: 0.30 mL/min.
- d) Injection volume: 5 μ L.

Table 3 Gradient elution program

Time min	0.1% formic acid solution %	Acetonitrile %
0.0	50	50
2.0	50	50
4.0	10	90
6.0	10	90
7.0	50	50
10.0	50	50

4.5.2 MS/MS condition

MS/MS condition is as follows:

- a) Ionization mode: electrospray ionization source, negative ion mode.
- b) Ion spray voltage: 3 500 V.
- c) Sheath gas: high purity nitrogen, 6.5 L/min.
- d) Auxiliary gas: high purity nitrogen, 5.0 L/min.
- e) Collision gas: high purity argon, 0.2 Pa.
- f) Monitoring mode: multiple reaction monitoring (MRM), parent ion m/z 517.1, quantitative ion m/z 230.9, qualitative ion m/z 471.1.
- g) Ion source temperature: 300 °C.
- h) Capillary temperature: 270 °C.
- i) Collision energy: m/z 517.1 > 230.9 is 43 V, m/z 517.1 > 471.1 is 35 V.

4.5.3 Preparation of the working curve

Transfer adequate intermediate solution of standards to sample blank matrix for preparation of calibration curve. The concentration of these gossypol standard working solution are 0.01 µg/mL, 0.02 µg/mL, 0.04 µg/mL, 0.06 µg/mL, 0.1 µg/mL. Plot a working curve of peak area versus concentrations of gossypol. Multiple reaction monitoring chromatogram of the standard is listed as figure A.2 in annex A.

4.5.4 Determination and confirmation by LC-MS/MS

The responses of the analyte should be within the linear range of the instrument detection and quan-

tified by external standard.

4.5.5 Qualitative determination

Use the established LC-MS/MS parameters above for determination, and calculate the abundance ratio of two selected ion pairs of the same solution and the standard working solution. If the retention times of sample chromatogram peaks are consistent with that of working solution, and relative abundance ratio tolerance is listed in table 4, it is positive to conclude this pesticide do exist in the sample.

Table 4 Maximum permitted tolerances for relative ion intensities while confirmation

Relative intensity	>50%	>20% to 50%	>10% to 20%	≤10%
Permitted tolerances	± 20%	± 25%	± 30%	± 50%

4.5.6 Calculation and expression of result

This is the same as 3.5.4.

4.6 Blank test

Perform the blank test with the same procedures as that described in the method of determination but without sample addition.

4.7 Limit of quantitation

The limit of quantitation (LOQ) of the method for gossypol is 1 mg/kg.

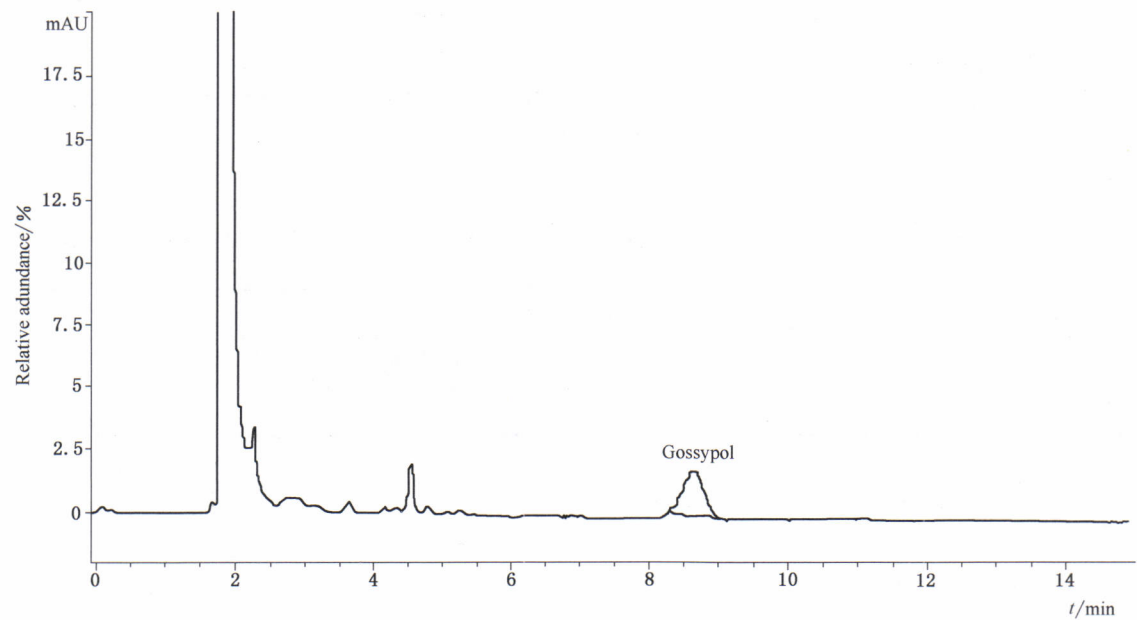
4.8 Recovery and precision

According to the experimental data, the spiked concentration of gossypol and its corresponding recoveries are listed in table 5.

Table 5 Recovery range and precision of gossypol in feed ($n=6$)

Compound	Spike /(mg/kg)	Recovery /%	RSD /%
Gossypol	1	88.0~102.0	5.3
	20	83.3~90.2	2.9
	40	82.4~86.3	1.7

Annex A
(Informative annex)



FigA.1 HPLC chromatogram of gossypol standard(1 µg/mL)

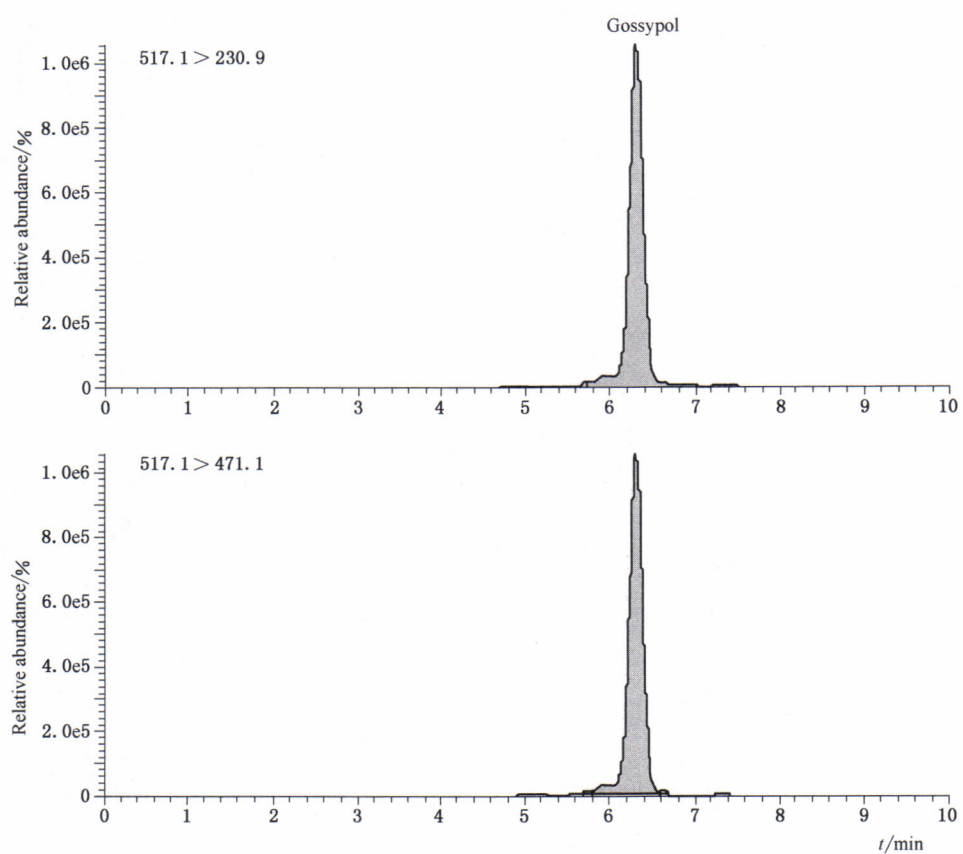


Fig A.2 Multiple reaction monitoring chromatogram of gossypol standard(10 ng/mL)

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