SN

中华人民共和国出入境检验检疫行业标准

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肉及肉制品中己烯雌酚残留量检测方法 酶联免疫法

Determination of diethylstilbestrol residues in meat and meat product—Enzyme linked immunosorbent assay

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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：中华人民共和国天津出入境检验检疫局。

本标准主要起草人：孙俐、郑文杰、唐丹舟、魏亚东、张宏伟。

本标准系首次发布的出入境检验检疫行业标准。

肉及肉制品中己烯雌酚残留量检测方法 酶联免疫法

1 范围

本标准规定了肉及肉制品中己烯雌酚残留量的酶联免疫测定方法。

本标准适用于鸡肉、猪肉、牛肉、羊肉、猪肉饼中己烯雌酚残留量的检测。

2 试样的制备和保存

2.1 试样的制备

从所取全部样品中取出有代表性样品约 1 kg，充分搅碎，混匀，采用四分法，将样品分成两等份，装入洁净容器，加封并做标识。

2.2 试样的保存

试样放置－20℃～－18℃条件下保存。

3 测定方法

3.1 方法提要

本方法的测定基础是竞争性酶联免疫反应。己烯雌酚与己烯雌酚酶标记物共同竞争己烯雌酚抗体的结合位点，用酶标仪测量微孔溶液的吸光度值，己烯雌酚浓度与吸光度值成反比，按绘制的校正曲线定量计算。

3.2 试剂和材料

除另有规定外，所用化学试剂均为分析纯，或为更高级别。水为重蒸馏水。

3.2.1 己烯雌酚酶联免疫定量测定试剂盒（参见附录 A）。

3.2.2 叔丁基甲基醚：色谱纯。

3.2.3 石油醚。

3.2.4 二氯甲烷。

3.2.5 磷酸。

3.2.6 盐酸。

3.2.7 甲醇：色谱纯。

3.2.8 磷酸氢二钠（$Na_2HPO_4 \cdot 2H_2O$）。

3.2.9 磷酸二氢钠（$NaH_2PO_4 \cdot H_2O$）。

3.2.10 氯化钠。

3.2.11 三羟甲基氨基甲烷。

3.2.12 磷酸盐缓冲液（67 mmol/L，pH＝7.2）：称取 9.61 g 磷酸氢二钠，1.79 g 磷酸二氢钠和 8.7 g 氯化钠溶解于并定容至 1 000 mL 水中。

3.2.13 Tris 缓冲液（20 mmol/L，pH＝8.5）：称取 2.42 g 三羟甲基氨基甲烷溶解于 700 mL 水中，加入 200 mL 甲醇，用 5 mol/L 盐酸调节 pH 值至 8.5，加水定容至 1 000 mL。

3.2.14 洗涤用磷酸盐缓冲液（20 mmol/L，pH＝7.2）：称取 2.85 g 磷酸氢二钠，0.55 g 磷酸二氢钠，9 g氯化钠和 1 mL 吐温 20，溶解并定容至 1 000 mL 水中。

3.2.15 氢氧化钠溶液（1 mol/L）：称取 40 g 氢氧化钠溶于 1 000 mL 水中。

3.2.16 已烯雌酚标准品：标准品纯度≥98%。

3.2.17 已烯雌酚标准品溶液的配制：准确称取适量的已烯雌酚标准品，用甲醇配制成 1 mg/mL 标准贮备溶液，于 4℃～8℃条件下保存。

3.3 仪器和设备

3.3.1 酶标仪：波长 450 nm。

3.3.2 高剪切分散器。

3.3.3 涡旋振荡器。

3.3.4 天平。

3.3.5 离心机：3 000 r/min。

3.3.6 氮气吹干仪。

3.3.7 洗板机。

3.3.8 固相萃取仪。

3.3.9 水浴锅：70℃±2℃。

3.3.10 微量加样器：20 μL，50 μL，100 μL，200 μL。

3.3.11 微量多通道加样器：50 μL，100 μL。

3.3.12 RIDA C_{18}柱：100 mg，1 mL。

3.4 样品处理

3.4.1 试样提取

称取 5.0 g 试样至离心管中，加入 10 mL 67 mmol/L pH 7.2 磷酸盐缓冲液，均质，用 8 mL 叔丁基甲基醚提取 3.0 g 均质物，强烈振荡 20 min，3 000 r/min 离心 10 min。转移出上清液，再用 8 mL 叔丁基甲基醚重复提取沉淀物，将两次提取的醚相合并，70℃水浴蒸发至干。用 1 mL 70%甲醇溶解干燥的残留物，用 3 mL 石油醚洗涤甲醇溶液，均质 15 s，3 000 r/min 短时离心，吸除石油醚。70℃水浴蒸干甲醇溶液，用 1 mL 二氯甲烷溶解，用 3 mL 氢氧化钠(NaOH)溶液提取。用 300 μL 6 mol/L 磷酸中和提取液。

3.4.2 样品纯化

用 RIDA C_{18}柱纯化，流速均为 1 d/s。用 3 mL 无水甲醇洗涤柱子，用 2 mL 20 mmol/L pH 8.5 的 Tris 缓冲液平衡柱。将上述中和提取液加入柱中，用 2 mL 20 mmol/L pH 8.5 的 Tris 缓冲液洗涤柱，用 3 mL 40%甲醇洗涤柱子，用正压去除残留的液体并且用空气或氮气吹 2 min 以干燥柱子。用 1 mL 80%甲醇洗脱样品，用 1 mL 80%甲醇稀释洗脱液，加入 2 mL 蒸馏水进一步稀释，取 20 μL 进行分析。

3.5 酶联免疫测定

3.5.1 测定步骤[1)]

已烯雌酚试剂盒中所有试剂的温度均应回升至室温(20℃～25℃)后方可使用。测定中吸取不同的试剂和样品溶液时应更换吸头。

将测定需用的微孔条插入框架(标准液和样液、空白分别作平行试验测定)，记录标准液和样液的位置。分别吸取 20 μL 已烯雌酚标准溶液、样品溶液至各自的微孔，再加入 50 μL 已稀释的已烯雌酚抗体至每个微孔，持微孔板在台面上以圆周运动方式混匀后，然后用封口膜密封孔条以防溶液挥发。于 2℃～8℃避光孵育至少 20 h。微孔板恢复至室温，倒掉微孔中的液体，用洗涤缓冲液洗板操作 3 次。加入 50 μL 已稀释的已烯雌酚酶标记物，充分混匀，室温孵育 1 h。倒掉微孔中的液体，洗板操作 3 次。加入 50 μL 酶基质和 50 μL 发色剂至各微孔中，充分混匀，20℃～25℃暗处避光孵育 30 min。加入100 μL 反应终止液至每个微孔中，充分混匀，在 30 min 内测量并记录每个微孔溶液 450 nm 波长的吸光度值。

1) 给出该信息是为了方便本标准的使用者，并不表示对某一产品操作步骤的认可。如果其他产品的操作步骤有不同，需经实验评估后采用。

3.5.2 空白试验

除不称取试样外，均按上述步骤进行。

3.6 结果计算

按式(1)计算百分比吸光度值：

$$吸光度值 = \frac{B_{样品/标准} - B_{空白}}{B_0} \times 100\% \quad \cdots\cdots(1)$$

式中：

$B_{样品/标准}$——标准品或样品的平均吸光度值，%；

$B_{空白}$——空白孔的平均吸光度值；

B_0——零标准的平均吸光度值。

以百分比吸光度值为纵坐标，以己烯雌酚标准溶液的浓度(ng/mL)的对数为横坐标，绘制出校正曲线(参见附录B)。每次试验均应重新绘制校正曲线。从标准曲线上得到试样中相应的己烯雌酚浓度后，结果按式(2)进行计算：

$$X = \frac{c \times V \times 1\,000}{m \times 1\,000} \quad \cdots\cdots(2)$$

式中：

X——样品中己烯雌酚的残留量，单位为微克每千克(μg/kg)；

c——从标准曲线上得到的样品中己烯雌酚浓度，单位为纳克每毫升(ng/mL)；

V——样品溶液的最终定容体积，单位为毫升(mL)；

m——样品溶液所代表的最终试样质量，单位为克(g)。

也可以用各种酶标仪的数据处理软件进行计算，所得结果表示至一位小数。

4 检测限

本方法的检出限为0.5 μg/kg。

5 确证试验

如被测样品中己烯雌酚残留量的值大于限量要求时，应用其他方法进行确证。

6 回收率

回收率试验数据参见附录B。

附 录 A
（资料性附录）
己烯雌酚酶联免疫定量测定试剂盒

A.1 己烯雌酚酶联免疫定量测定试剂盒

本标准酶联免疫测定步骤中使用的试剂盒为德国 r-biopharm 公司产品[2)]，试剂盒包括：

a) 框架，96 孔板；
b) 标准溶液，40%己烯雌酚甲醇溶液：0，0.025，0.05，0.10，0.2，0.4 ng/mL；
c) 己烯雌酚酶标记物溶液：用稀释缓冲液以 1∶11 比例稀释浓缩液，充分混匀后使用；
d) 己烯雌酚抗体浓缩液：用稀释缓冲液以 1∶11 比例稀释浓缩液，充分混匀后使用；
e) 酶基质；
f) 发色剂；
g) 反应终止液；
h) 稀释缓冲溶液。

A.2 己烯雌酚校正曲线

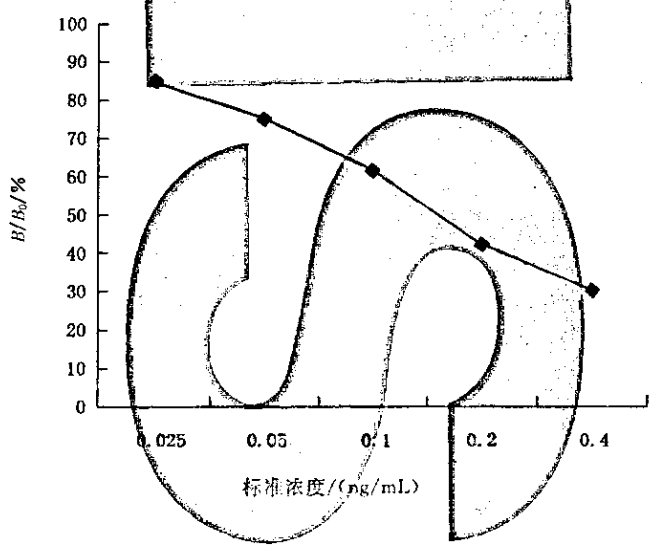


图 A.1 己烯雌酚校正曲线

2) 给出该信息是为了方便本标准的使用者，并不表示对某一产品的认可。如果其他产品具有相同的效果，需经实验评估后使用这些等效产品。

附 录 B
（资料性附录）
回 收 率

本方法中己烯雌酚添加浓度及回收率数据：

——添加量为 0.5 μg/kg 时，平均回收率为 85.2%～97.5%；

——添加量为 1.0 μg/kg 时，平均回收率为 82%～104.2%；

——添加量为 5.0 μg/kg 时，平均回收率为 84.5%～94.8%。

Foreword

Annex A and B of this standard are informative annex.

This standard was proposed by Certification and Accredditation Adiministration of the People's Republic of china.

This standard was mainly drafted by Tianjin Entry-Exit Inspection and Quarantine Bureau of the People's Republic of china.

This standard was mainly drafted by Sun Li, Wenjie Zheng , Danzhou Tang , Yadong Wei, Hongwei Zhang.

This standard is professional standard of Entry-exit inspection and quarantine promulgated for the first time.

Determination of diethylstilbestrol residues in meat and meat product —Enzyme linked immunosorbent assay

1 Scope

This standard specifies the determination by ELISA method of diethlstilbestrol residuse in meat and meat product.

This standard is applicable to the screen determination of diethlstilbestrol residuse in chicken meat, pork, beef,mutton and breaded pork.

2 Sample preparation and storage

2.1 sampling procedure

The representative sample should be taken out, and total weight is not less than 1 kg. Sample defated and homogenized thoroughly. At least each sample should be divided into equal portions. All samples should be placede in a clean and dry container to maintain the sample completeness and traceability, then change into a bigger clean container to seal up and transport. The label should be labeled out side of each sample container.

2.2 Storage of sample

After sampling,the test sample should be stored at −20℃～−18℃.

3 Method of determination

3.1 Principle of determination

The basis of the test is the competitive enzyme-linked immunoreactions. Diethylstilbestrol and diethylstilbestrol enzyme conjugate compete for the limited anti- diethylstilbestrol antibody. The absorbance value of each well solution is measured by microwell system. The diethylstilbestrol concentration is inversely proportional to the absorption value, and compare with the calibration curve for quantitative measurement.

3.2 Reagents and materials

In this standard, all the chemical reagents should be A. R. grade unless it identified specially, "water" is doubled distilled water.

3.2.1 Enzyme immunoassay kit for the quantitative analysis of diethylstilbestrol(see Annex A).

3.2.2 Tert-butylmethylether: chromatographic grade.

3.2.3 Petroleum ether.

3.2.4 Dichloromethane.

3.2.5 Phosphoric acid.

3.2.6 HCl.

3.2.7 Methanol: Chromatogram grade.

3.2.8 $Na_2HPO_4 \cdot 2H_2O$.

3.2.9 $NaH_2PO_4 \cdot H_2O$.

3.2.10 Sodium chloride.

3.2.11 Tris-(hydroxymethyl)-aminomethane

3.2.12 Phosphate buffer(67 mmol/L, pH = 7.2): Dissolve 9.61 g $Na_2HPO_4 \cdot 2H_2O$, 1.79 g $NaH_2PO_4 \cdot H_2O$ and 8.7 g Sodium chloride in 1 000 mL distilled water.

3.2.13 Tris-HCl-buffer(20 mmol/L, pH = 8.5): Dissolve 2.42 g tris-(hydroxymethyl)-aminomethanein approx. 700 mL distilled water, mix with 200 mL methanol, adjust pH 8.5 with 5 mol/L HCl, and fill up to 1 000 mL with distilled water.

3.2.14 Washing buffer(20 mmol/L, pH = 7.2): Dissolve 2.85 g $Na_2HPO_4 \cdot 2H_2O$, 0.55 g $NaH_2PO_4 \cdot H_2O$, 9 g NaCl and 1 mL Tween 20 in 1 000 mL distilled water.

3.2.15 1 mol/L NaOH: Dissolve 40 g sodium hydroxide in 1 000 mL distilled water.

3.2.16 The purity of diethylstilbestrol is over 98%.

3.2.17 preparation of solution: Dissolve some DES in methanol, and the concentration of the storage solutions is 1 mg/mL. Then store at 4℃～8℃.

3.3 Apparatus and equipment

3.3.1 Microwell system:450 nm.

3.3.2 Homogenizer.

3.3.3 Shaker.

3.3.4 Balance.

3.3.5 Centrifuge:3 000 r/min.

3.3.6 Nitrogen Evaporator.

3.3.7 Washing machine.

3.3.8 SPE cartridge.

3.3.9 Water bath boiler:70℃ ±2℃.

3.3.10 Pipettes:20 μL,50 μL,100 μL,200 μL.

3.3.11 Multichannel pipette:50 μL,100 μL.

3.3.12 RIDA C_{18} columns:100 mg, 1 mL.

3.4 Sample extraction and cleanup

3.4.1 Sample extraction

Weigh 5.0 g of the test sample into a centrifuge tube, add 10 mL 67 mmol/L pH 7.2 phosphate buffer, vibrate for 1 min, take 3.0 g of the above sample , with 8 mL Tert-butylmethylether, vibrate 20 min strongly. Centrifuge at room temperature 3 000 r/min for 10 min. Remove the ether supernatant into a glass centrifuge tube. Repeat the extraction with 8 mL Tert-butylmethylether. Combine the ether phase, the organic phase evaporated with nitrogen gas in water bath at 70℃. Dissolve the residue in 1 mL70% methanol, wash the methanolic solution with 3 mL petroleum ether, homogenize for 15 s, centrifuge 3 000 r/min shortly, aspirate and reject petroleum ether supernatant. The methanolic solution is then evaporated to dryness under a gentle stream of nitrogen. The residue is dissolved in 1 mL dichlormethane and extracted with 3 mL NaOH. Neutralize the extract with 300 μL 6 mol/L phosphoric acid.

3.4.2 Sample cleanup

The extract is further purified as following by means of RIDA C_{18} columns. The column is rinsed with

3 mL of methanol and equilibrated with 2 mL 20 mmol/L tris-HCl. The sample solution pass though the column at a rate of 1 drop/s, followed by rinsing with 2 mL of 2 mL 20 mmol/L tris-HCl and 3 mL 40% methanol in water. The fluid residue is removed by positive pressure, the column is dried for 2 min by floating it with air or nitrogen. Then elute sample with 1 mL 80% methanol in water and dilute the eluent with 1 mL 80% methanol in water, further dilute this solution with 2 mL doubled distilled water and employ 20 μL in the assay.

3.5 ELISA determination

3.5.1 Test procedure[1)]

Bring all reagents and the microtiter plate to room temperature before use(20℃～25℃). Pipette tips should be changed by using for different reagents and sample solutions.

Insert a sufficient amount of microtitre wells into the microwell holder for all standards and samples to be run in duplicate. Record standard and sample positions. Add 20 μL of six kinds of diethylstilbestrol standard solution and sample solution to separate duplicate wells. Add 50 μL of diluted anti-diethylstilbestrol antibody solution to each well, flap lightly and mix thoroughly, sealing all wells with adhesive paper to avoid solution evaporating, incubate for 20 h at 2℃～8℃ in darkness. The microtiter must be brought to room temperature. Pour the liquid out of the wells and wash the wells three times. Add 50 μL of diluted DES enzyme conjugate to the bottom of each well, mix gently and incubate for 1 h at 20℃～25℃. Pour the liquid out of the wells and wash the wells three times. Add 50 μL of substrate and 50 μL of chromogen to each well, flap lightly and mix thoroughly and incubate for 30 min at 20℃～25℃ in darkness. Add 100 μL of stop solution to each well, flap lightly and mix thoroughly. Measure and record the absorbance value of each well solution at 450 nm within 30 min.

3.5.2 Blank test

Follow the sample extraction and test procedure without sampling.

3.6 results expression

Calculate absorbance value in percentage according to the formula (1):

$$\text{absorbance value in percentage} = \frac{B_{\text{standard/sample}} - B_{\text{blank}}}{B_0} \times 100\% \quad \cdots\cdots\cdots\cdots (1)$$

where

$B_{\text{standard/sample}}$—mean absorbance value of standards and samples, %;

B_{blank}—mean absorbance value of blank;

B_0—Zero standard mean absorbance value.

---

1) Gives this information is in order to the user who facilitate this standard, does not express to some product sequence of operation approval. The testing process of another equal kits maybe a little differ, so it should be used after testing, evaluation and verification.

Make the absorbance value in percentage as ordinate, and diethylstilbestrol concentration (ng/mL) as abscissa, plot the calibration curve of the absorbance value in percentage and diethylstilbestrol standard concentration (see Annex B). A new calibration curve should be prepared for each determination. Read corresponding to the concentration of each sample from calibration curve and calculate according to the formula (2):

$$X = \frac{c \times V \times 1\,000}{m \times 1\,000} \qquad \cdots\cdots(2)$$

where

X—The diethylstilbestrol residues content of the test sample, μg/kg;

c—The diethylstilbestrol concentration value of sample read from calibration curve, ng/mL;

V—The total volume of sample solution, mL;

m—The total sample quality of sample solution, g.

Also may use each kind of the data processing software to carry on the computation, obtained result expression to a decimal.

4 Limit of determination

The limit of determination of this method is 0.5 μg/kg.

5 confirmation

If the diethylstilbestrol residue is over the limit of determination, it should be confirmed by another method.

6 Recovery

See Annex B.

Annex A
(Informative Annex)
Enzyme immunoassay kit for the quantitative analysis of diethylstilbestrol

A.1 Enzyme immunoassay kit for the quantitative analysis of diethylstilbestrol

The r-biopharm kit[2)] of Germany is used in this standard 3.5 ELISA determination, Each test kit contains:

a) Microtiter plate with 96 wells;

b) DES standard solution: 0, 0.025, 0.05, 0.10, 0.2, 0.4 ng/mL DES in 40% methanol, ready to use;

c) Conjugate: The DES enzyme conjugate is diluted 1 : 11 in buffer;

d) Anti-DES-antibody: The anti-DES antibody is diluted 1 : 11 in buffer;

e) Substrate;

f) Chromogen;

g) Stop solution;

h) Buffer.

A.2 diethylstilbestrol calibration curve

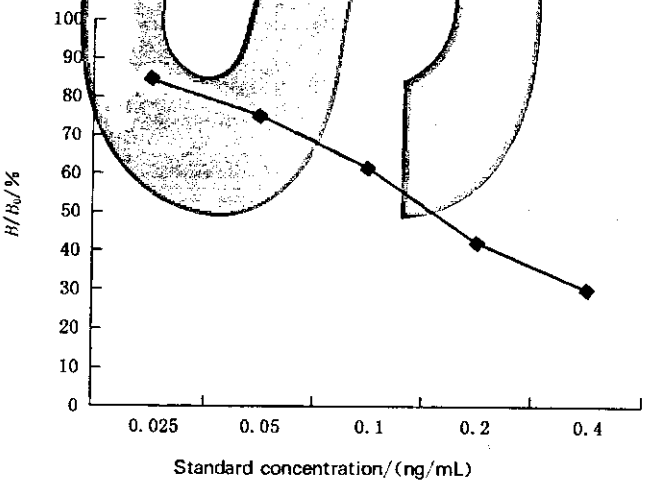


Figure A.1—Diethylstilbestrol calibration curve

2) Gives this information is in order to the user who facilitate this standard, does not express to some product sequence of operation approval. The testing process of another equal kits maybe a little differ, so it should be used after testing, evaluation and verification.

Annex B
(Informative Annex)
Recovery

The experimental data of these fortifying concentrations of diethylstilbestrol and recoveries are as follows:

—At 0.5 μg/kg, the recovery is 85.2%～97.5%;

—At 1.0 μg/kg, the recovery is 82%～104.2%;

—At 5.0 μg/kg, the recovery is 84.5%～94.8%.

中华人民共和国出入境检验检疫
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