SN

中华人民共和国进出口商品检验行业标准

SN 0296—93

出口禽肉中莫能菌素残留量检验方法 生物自显影法

Method for the determination of monensin residues in poultry meat for export —Bioautography method

1993-12-28 发布　　1994-05-01 实施

中华人民共和国国家进出口商品检验局　发布

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1 主题内容与适用范围

本标准规定了出口禽肉中莫能菌素残留量的抽样、制样和生物自显影测定法。

本标准适用于出口冻鸡中莫能菌素残留量的检验。

2 抽样和制样

2.1 检验批

以不超过 2 500 件商品为一检验批。

同一检验批的商品应具有相同特征，如包装、标记、产地、规格和等级等。

2.2 抽样数量

批量，件	最低抽样数，件
1～25	1
26～100	5
101～250	10
251～500	15
501～1 000	17
1 001～2 500	20

2.3 抽样方法

按 2.2 规定的抽样件数随机抽取，逐件开启。每件至少取一袋作为原始样品，原始样品总量不少于 2 kg，放入清洁容器内，加封后标明标记，及时送交实验室。

2.4 样品制备

从每袋原始样品中取出部分有代表性样品，将可食部分放入高速组织捣碎机中捣碎均匀，充分混匀，用四分法缩分出不少于 1 kg 试样。装入清洁容器内，加封后标明标记。

2.5 样品保存

将试样于－18℃冷冻保存。

注：在抽样及制样过程中，必须防止样品受到污染或发生残留物含量的变化。

3 测定方法

3.1 方法提要

用甲醇提取肉样中莫能菌素，经四氯化碳萃取后浓缩至干，以甲醇溶解残留物，用薄层分离，再用生

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物自显影法测定。

3.2 设备和材料

3.2.1 微量注射器:50 μL。

3.2.2 薄层板:硅胶,Q/YT 257-85SG,5 cm×20 cm,使用前 110℃活化 2 h。

3.2.3 展开缸:240 mm×57 mm×32 mm。

3.2.4 游标卡尺:测量范围 0～200 mm,精度 0.02 mm 或使用抑菌圈测量仪测量。

3.2.5 离心机:转速 3 000 r/min。

3.2.6 旋转浓缩器。

3.2.7 恒温培养箱:37±1℃。

3.2.8 高压灭菌器。

3.2.9 长方形培养皿:20 cm×10 cm×5 cm。

3.2.10 均质器:带均质杯 250 mL。

3.3 试剂和培养基

3.3.1 试剂

3.3.1.1 甲醇:分析纯。

3.3.1.2 四氯化碳:分析纯。

3.3.1.3 乙酸乙酯:分析纯。

3.3.1.4 莫能菌素标准品:960 μg(效价)/mg(中国兽药监察所提供)。

3.3.1.5 试验菌种:枯草杆菌(*Bacillus subtilis*),菌种号 ATCC 6633(卫生部药品生物制品检定所提供)。

3.3.2 培养基

3.3.2.1 菌种用培养基(见附录 A 第 A1 章)。

3.3.2.2 生物自显影用培养基(见附录 A 第 A2 章)。

3.3.2.3 肉汤培养基(见附录 A 第 A3 章)。

3.4 测定步骤

3.4.1 工作液制备

3.4.1.1 莫能菌素标准贮备液

准确称取适量的莫能菌素标准品,用甲醇溶解配制成浓度为 500 μg(效价)/mL 的莫能菌素标准溶液。配制后于冰箱中保存,一周内使用。

3.4.1.2 莫能菌素标准工作液

吸取标准贮备液,用甲醇分别稀释成 2,3,4,5,10,和 15 μg(效价)/mL 的标准工作标准液。以上稀释液均须当日配制。

3.4.1.3 菌种培养及芽胞菌悬浮液制备

将菌种安瓿瓶的上部消毒后敲碎,加入少量肉汤培养基,使其溶解并移至肉汤管中混匀。置 37±1℃温箱中培养 24 h。将培养物接种于菌种培养基中,置于 37±1℃培养一周,镜检含芽胞菌数达 85% 以上,便可制备芽胞悬浮液。

用适量灭菌生理盐水冲洗菌苔,然后将该菌液转移至离心管中,充分摇匀后,于 3 000 r/min 离心 30 min,弃去上清液,再加入同样量的灭菌生理盐水重复离心一次。弃去上清液,再加入适量灭菌生理盐水,摇匀后于 65℃水浴中加热 30 min,然后,于 1 000 r/min 离心 5 min,取上清液并转入灭菌试管中,即为芽胞菌悬浮液,置于冰箱中保存,可使用一个月。

3.4.2 试液制备:准确称取绞碎试样 10 g(精确至 0.1 g)于均质杯中,加入 20 mL 甲醇,均质 2 min 后,移入离心管中,于 3 000 r/min 离心 20 min。取其上清液 15 mL,转入盛有 5 mL 蒸馏水的 250 mL 分液漏斗中,混合后加入 10 mL 四氯化碳,充分振摇,静置分层,放出四氯化碳层于 150 mL 茄形瓶中。用四

氯化碳再重复提取两次，合并四氯化碳至上述茄形瓶中，于 50～60℃水浴中用旋转蒸发器浓缩至干，加入 0.5 mL 甲醇溶解残留物供 TLC 实验用。此样液中相当于禽肉试样浓度为 15 g/mL。

3.4.3 测定

3.4.3.1 生物自显影

将每个样品及标准溶液分别点在四块薄层板上，先于每块薄层板下端 2 cm 处划一条基线，然后在这条基线上分别点 20 μL 试液和 3 μg(效价)/mL 莫能菌素标准溶液，试液和标准溶液点相距 2.5 cm。在展开缸中用乙酸乙酯进行展开，直至溶剂前沿线距板顶端 1.5 cm 处为止，取出薄层板，风干后备培养用。

将薄层板水平置于高压灭菌的长方形培养皿中，无菌操作将已熔化并冷却至 50～55℃的生物自显影用培养基均匀地喷在其表面上，然后用 10 mL 上述接种芽胞菌悬液的培养基铺满整个薄层板，保持水平，待其凝固，于 37±1℃培养 18 h。

3.4.3.2 对照试验

除不加试样外，按上述测定步骤进行。

3.5 结果计算和表述

经过培养后，在薄层板上与莫能菌素标准液产生抑菌圈的 R_f 值(约 0.38)相同位置上，显现抑菌圈者即为阳性。

当样品判定为阳性时，测量四块薄层板上的试液及标准溶液产生的抑菌圈直径，分别计算出平均值。当每组四个直径值的相对标准偏差(RSD)均不超过 5%时，并且试液的抑菌圈直径与标准溶液(20 μL)的抑菌圈直径近似(直径相差不超过±1 mm)，则其样品中莫能菌素含量按下式计算：

$$X = \frac{c}{m}$$

式中：X——样品中莫能菌素的含量，mg/kg；

c——试验中所用的标准溶液的浓度，μg/mL；

m——最终试液所代表的禽肉试样的浓度，g/mL。

4 测定低限、回收率

4.1 测定低限

本方法测定低限为 0.20 mg(效价)/kg。

4.2 回收率

回收率的实验数据：莫能菌素浓度在 0.20～0.67 mg(效价)/kg 范围内，回收率为 81%～100%。

附 录 A
培养基成分及制备方法
（补充件）

A1 菌种用培养基

蛋白胨	10.0 g
牛肉浸膏	5.0 g
氯化钠	2.5 g
琼脂	15.0 g
蒸馏水	1 000 mL

将上述各成分于蒸馏水中加热溶解，用 1 mol/L 氢氧化钠溶液或 10%盐酸调节 pH，使其灭菌后 pH 为 6.5±0.1，分装于试管或克氏瓶内，于 121℃15 min 高压灭菌，灭菌后制备成所需斜面备用。

A2 生物自显影用培养基

蛋白胨	10.0 g
酵母浸膏	2.5 g
葡萄糖	10.0 g
琼脂	15.0 g
蒸馏水	1 000 mL

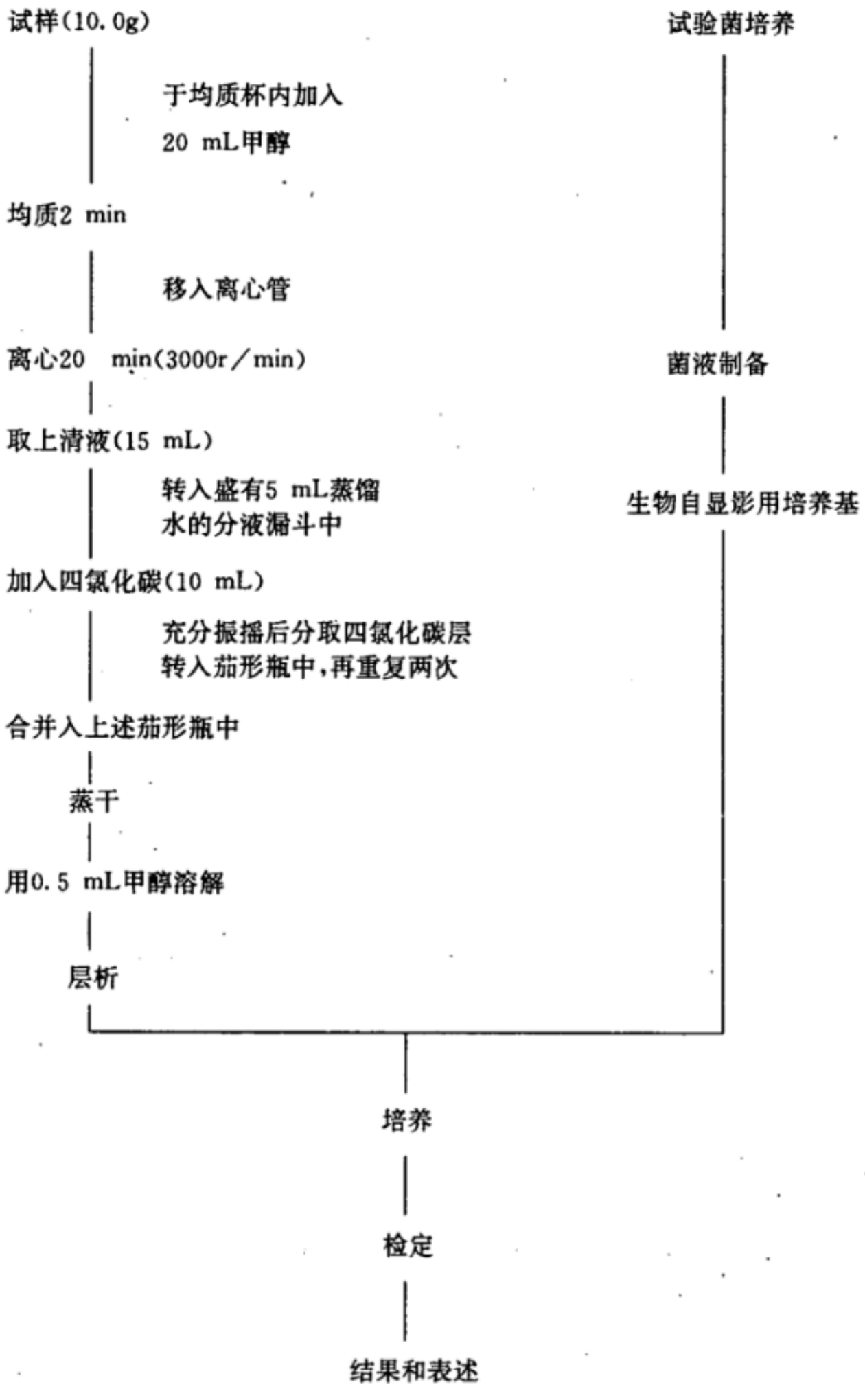
将上述各成分于蒸馏水中加热溶解，用 1 mol/L 氢氧化钠溶液或 10%盐酸调节 pH，使其灭菌后 pH 为 6.0±0.1，分装于锥形瓶内，于 121℃15 min 高压灭菌，备用。

A3 肉汤培养基

蛋白胨	10.0 g
牛肉浸膏	5.0 g
氯化钠	2.5 g
蒸馏水	1 000 mL

将上述各成分于蒸馏水中加热溶解，用 1 mol/L 氢氧化钠溶液或 10%盐酸调节 pH，使其灭菌后 pH 为 7.0±0.1，分装于试管中，于 121℃15 min 高压灭菌。

附 录 B
检验程序图
（补充件）



附加说明：

本标准由中华人民共和国国家进出口商品检验局提出。

本标准由中华人民共和国天津进出口商品检验局负责起草。

本标准主要起草人袁而森、王素琴、李剑影。

本标准等同采用日本厚生省检验方法：畜水产食品中の残留物质检查法(1990)。

Professional Standard of the People's Republic of China
for Import and Export Commodity Inspection

SN 0296—93

Method for the determination of monensin residues in poultry meat for export —Bioautography method

1 Scope and field of application

This standard specifies the method of sampling, sample preparation and determination by bioautography method of monensin residues in poultry meat for export.

This standard is applicable to the determination of monensin residues in chicken meat for export.

2 Sample and sample preparation

2.1 Inspection lot

The quantity of an inspection lot should not be more than 2 500 packages.

The characteristics of the cargo within the same inspection lot, such as packing, mark, origin, specification and grade, should be the same.

2.2 Quantity of sample taken

Number of packages in each inspection lot	Minimum number of packages to be taken
1—25	1
26—100	5
101—250	10
251—500	15
501—1 000	17
1 001—2 500	20

2.3 Sampling procedure

A number of packages specified in 2.2 are taken at random and opened one by one. From each at least one bag shall be taken as primary sample. The total weight of all primary samples should not be less than 2 kg, which shall be placed in a clean container, sealed, labeled and sent to laboratory in time.

2.4 Preparation of test sample

Part of representative sample is taken from each bag of the primary sample, the edible portions are homogenized by grinding in a meat grinder. The homogenized sample is thoroughtly mixed and reduced to at least 1 kg by quartering as test sample. The test sample is placed in a clean container which shall

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be sealed and labeled.

2.5 Storage of test sample

The test sample should be frozen and stored at −18℃.

Note: In the course of sampling and sample preparation, precaution must be taken to avoid contamination or any factor which may cause the change of residue content.

3 Method of determination

3.1 Principle of method

The monensin is extracted from the meat tissues with methanol, partitioned into carbon tetrachloride. After evaporation to dryness, the residues are dissolved in methanol. The prepared extract is chromatographed by thin layer chromotography and determined by bioautography method.

3.2 Apparatus and equipments

3.2.1 Micro-syringe: 50 μL.

3.2.2 Thin-layer plate: Silica gel, Q/YT 257-85SG, 5 cm × 20 cm, activate at 110℃ for 2 h before use.

3.2.3 Developing tank: 240 mm × 57 mm × 32 mm.

3.2.4 Vernier calliper: measuring range 0—200 mm, precision 0.02 mm or use measurer.

3.2.5 Centrifuge: 3 000 r/min.

3.2.6 Rotary evaporator.

3.2.7 Incubator: 37±1℃.

3.2.8 Autoclave sterilizer.

3.2.9 Rectanguler culture dish: 20 cm × 10 cm × 5 cm.

3.2.10 Homogenizer: with homogeneous cup (250 mL).

3.3 Reagents and media

3.3.1 Reagents

3.3.1.1 Methanol: Analytical grade.

3.3.1.2 Carbon tetrachloride: Analytical grade.

3.3.1.3 Ethyl acetate: Analytical grade.

3.3.1.4 Monensin standard: 960 μg(potency)/mg (Provided by Veterinary Drug Supervision Institute of China)

3.3.1.5 Bacterial strain

Bacillus subtilis ATCC 6633(Provided by Drug and Biological Product Inspection Institute of the State Ministry of Public Health).

3.3.2 Media

3.3.2.1 Medium for strain culture (See Appendix A1).

3.3.2.2 Midium for bioautography(See Appendix A2).

3.3.2.3 Broth medium(See Appendix A3).

3.4 Procedure of determination

3.4.1 Preparation of working solution

3.4.1.1 Monensin standard stock solution: Accurately weigh a proper quantity of monensin standard and dissolve in methanol to prepare 500 μg(potency)/mL standard stock solution. Store in a refrigerator, which can be used within a week.

3.4.1.2 Monensin standard working solution

Pipet a certain amount of monensin standard stock solution, and dilute with methanol to prepare 2, 3, 4, 5, 10, and 15 μg(potency)/mL standard working solutions respectively, all of above solution should be preparaed at the same day.

3.4.1.3 Culture of strain and preparation of spores suspension

After sterilizing the ampoule of the strain, cut off the top and add a small amount of broth medium in it to dissolve the content. Transfer into the above mentioned broth test-tube, mix well and incubate at 37±1℃ for 24 h. Transfer the incubated culture to medium for strain and incubate at 37±1℃ for a week. When the number of spores detected by microscope exceed 85%, it can be used for the preparation of spores suspension.

Wash down the spores with sterilized saline and transfer to a centrifugal tube, after thoroughly mixing, centrifuge at 3 000 r/min for 30 min. Discard the supernate and repeat above step once more. Discard the supernate, add a certain amount of sterilized saline and mix well, heat the suspension in a 65℃ water bath for 30 min. Centrifuge again at 1 000 r/min for 5 min. Transfer the supernate (the spores suspension) into sterile tubes. The spores suspension can be used within a month by storing in a refrigerator.

3.4.2 Preparation of sample solution

Accuately weigh 10.0 g of the chopped sample (accurate to 0.1 g) into a homogeneous cup. Add 20 mL of methanol and homogenize for 2 min. Transfer it into a centrifugal tube and centrifuge at 3 000 r/min for 20 min. Pipet 15 mL of supernate into a 250 mL separatory funnel containing 5 mL of distilled water, after mixing, add 10 mL of carbon tetrachloride and shake vigorously. Let stand to separate and drain carbon tetrachloride into a 150 mL Mojonner-type flask. Repeat this extraction step twice using carbon tetrachloride and combine these carbon tetrachloride into the above Mojonner-type flask. Evaporate the carbon tetrachloride to dryness in a water bath at 50—60℃ with a rotary evaporator, add 0.5 mL of methanol to dissolve the residue for TLC. In this solution, the concentration of poultry meat sample is equivalent to 15 g/mL.

3.4.3 Determination

3.4.3.1 Bioautography

Spot each sample and standard solution on four plates respectively. On each plate, at first scribe a baseline across the plate 2 cm from the bottom, then spot 20 μL sample extract and monensin standard solution〔3 μg(potency)/mL〕on the baseline, the sample spot shall be 2.5 cm away from the standard spot. Develop the TL plate in the tank using ethyl acetate as developing solution, until the front margin of the solvent is 1.5 cm from the top of plate. Remove the plate, after the plate is air-dried, it is ready for later incubation.

Place the thin-layer plate into a sterilized rectanguler culture dish horizontally. Aseptically spray the melted and cooled to 50—55℃ medium for bioautography onto the surface of the plate, then pour 10 mL of the above medium inoculated with *Bacillus subtilis* spores suspension over the surface of the plate. Allow the plate to keep on level surface until the agar solidifies and incubate at 37℃ for 18 h.

3.4.3.2 Contrast test

The operation of the contrast test is the same as that describe in the method of determination, but with omission of sample addition.

3.5 Calculation and expression of result

After incubation, the test is considered positive if the sample shows inhibit zone on the plate with the same R_f value(ca 0.38), by comparing with the inhibit zone of the monesin standard solution.

When the sample is showed positive result, measure the diameters of the positive inhibit zone on four plates, cause by the sample extraction and standard solution, calculate the averages respectively. When the relative standard deviation(RSD) values of each group are not more than 5%, and the diameters of inhibit zone of sample extraction is about the same(within ±1 mm)as that of monensin standard solution(20 μL), then the content of monensin can be calculated according to the equation below:

$$X = \frac{c}{m}$$

where

X—content of monensin in sample, mg/kg;

c—concentration of standard solution which is used in the test, μg/mL;

m—corresponding mass of the poultry meat in each milliliter of the final test solution, g/mL.

4 Limit of determination and recovery

4.1 Limit of determination

The limit of determination is 0.20 mg(potency)/kg.

4.2 Recovery

According to the experimental data, when the fortifying concentration of monensin is in the range of 0.20—0.67 mg(potency)/kg, the recovery is 81%—100%。

Appendix A
Ingredients of medium and preparation method
(Supplement)

A1 Medium for strain culture

Peptone	10.0 g
Beef extract	5.0 g
NaCl	2.5 g
Agar	15.0 g
Distilled water	1 000 mL

Dissolve each ingredients in distilled water. After heating and stirring, adjust the pH with sodium hydroxide solution (1 mol/L) or hydrochloric acid (10%) so that the value after autoclaving is 6.5 ±0.1. Dispense into test tubes or Kolle flask. Autoclave at 121℃ for 15 min, and prepare stant as required.

A2 Medium for bioautography

Peptone	10.0 g
Yeast extract	2.5 g
Glucose	10.0 g
Agar	15.0 g
Distilled water	1 000 mL

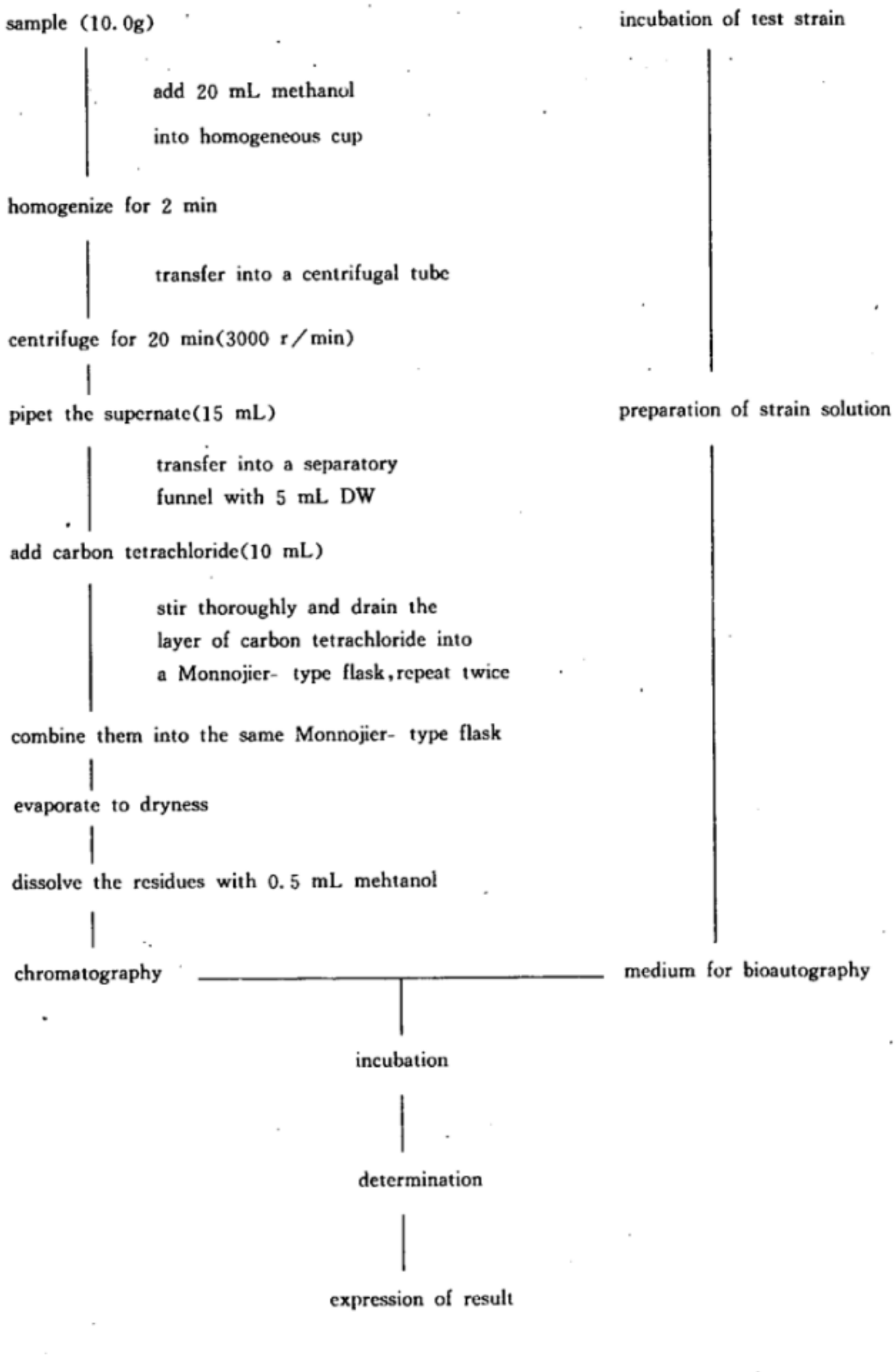
Dissolve each ingredients in distilled water. After heating and stirring, adjust the pH with sodium hydroxide solution (1 mol/L) or hydrochloric acid (10%) so that the value after autoclaving is 6.0 ±0.1. Dispense into conical flasks, autoclave at 121℃ for 15 min. Store for later use.

A3 Broth medium

Peptone	10.0 g
Beef extract	5.0 g
NaCl	2.5 g
Distilled water	1 000 mL

Dissolve each ingredients in distilled water. After heating and stirring, adjust the pH with sodium hydroxide solution (1 mol/L) or hydrochloric acid (10%) so that the value after autoclaving is 6.5 ±0.1. Dispense into tubes. Autoclave at 121℃ for 15 min.

Appendix B
Procedure of test
(Supplement)



Additional explanations:

This standard was proposed by the State Administration of Import and Export Commodity Inspection Bureau of the People's Republic of China.

This standard was drafted by the Tianjin Import and Export Commodity Inspection Bureau of the People's Republic of China.

This standard was mainly drafted by Yuan Ersen, Wang Suqing, Li Jianying.

This standard is identical with the determination method which is passed by The Public Health Department of Japan: 畜水产食品中の残留物质检查法(1990).

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