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中华人民共和国出入境检验检疫行业标准

SN/T 1768—2006

水产品中孔雀石绿和结晶紫及其代谢产物的快速测定方法

Determination of malachite green, crystal violet and the corresponding leuco compounds in the aquatic products

2006-04-25 发布　　2006-11-15 实施

中华人民共和国
国家质量监督检验检疫总局　发布

中华人民共和国出入境检验检疫
行 业 标 准

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中国标准出版社出版
北京复兴门外三里河北街16号
邮政编码:100045
网址 www.bzcbs.com
电话:68523946 68517548
中国标准出版社秦皇岛印刷厂印刷

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开本 880×1230 1/16 印张 1.25 字数 30 千字
2006年8月第一版 2006年8月第一次印刷
印数 1—2 000

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前　言

本标准附录A、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：中国检验检疫科学研究院、中华人民共和国北京出入境检验检疫局、中华人民共和国辽宁出入境检验检疫局、中华人民共和国福建出入境检验检疫局、中华人民共和国江西出入境检验检疫局、中华人民共和国湛江出入境检验检疫局、中华人民共和国舟山出入境检验检疫局。

本标准主要起草人：贾东芬、张顺合、张维、徐超一、林维宣、田苗、李耀平、占春瑞、陈伟、周向阳。

本标准系首次发布的出入境检验检疫行业标准。

水产品中孔雀石绿和结晶紫及其代谢产物的快速测定方法

1 范围

本标准规定了水产品中孔雀石绿及其代谢物隐色孔雀石绿、结晶紫及其代谢物隐色结晶紫残留量的高效液相色谱、液相色谱-串联质谱的快速测定方法。

本标准适用于鲜活水产品及其制品中孔雀石绿及其代谢物隐色孔雀石绿、结晶紫及其代谢物隐色结晶紫残留量的测定。

2 液相色谱法

2.1 原理

试样中的残留的孔雀石绿、结晶紫及其代谢物用孔雀石绿、结晶紫快速检测前处理试剂盒提供的试剂提取、浓缩后用液相色谱进行检测，外标法定量。

2.2 试剂和材料

除另有规定外，所有试剂均为分析纯，水为重蒸馏水。

2.2.1 乙腈：色谱纯。

2.2.2 无水乙酸钠。

2.2.3 冰乙酸：色谱纯。

2.2.4 异丙醇。

2.2.5 对-甲苯磺酸。

2.2.6 乙酸盐缓冲液：称取 4.950 g 无水乙酸钠及 0.950 g 对-甲苯磺酸，溶解于 950 mL 水中，用冰乙酸调节溶液 pH 到 4.5，最后用水定容到 1 000 mL。

2.2.7 标准品：孔雀石绿、隐色孔雀石绿、结晶紫、隐色结晶紫纯度大于 98%。

2.2.8 标准贮备溶液：准确称量孔雀石绿、隐色孔雀石绿、结晶紫、隐色结晶紫各 10 mg，用乙腈分别定容于 100 mL 容量瓶中，配制成 100 μg/mL 的标准贮备液。

2.2.9 混合标准溶液：用流动相稀释标准贮备溶液，配制成孔雀石绿、隐色孔雀石绿、结晶紫、隐色结晶紫均为 10 μg/mL 的混合标准溶液。0℃～4℃避光保存。

2.2.10 孔雀石绿、结晶紫快速检测前处理试剂盒[1)]。

2.3 仪器和设备

2.3.1 高效液相色谱仪：配紫外-可见光可变波长检测器；检测波长时间程序可控。

2.3.2 匀浆机。

2.3.3 旋转蒸发仪。

2.3.4 离心机：4 000 r/min、8 000 r/min。

2.3.5 振荡器。

2.3.6 超声波水浴。

2.3.7 聚四氟乙烯离心管：2.5 mL，50 mL，具塞。

2.3.8 微孔滤膜：0.45 μm。

1) 孔雀石绿、结晶紫快速检测前处理试剂盒是由中国检验检疫科学研究院研发北京陆桥商检新技术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。

2.4 测定步骤

2.4.1 提取、净化

准确称取已捣碎的样品 5.00 g 于 50 mL 离心管中，先加入孔雀石绿、结晶紫快速检测前处理试剂盒中的提取剂[2)]（液体 20.0 mL）、用匀浆机以 8 000r/min 的速度均质 30 s，再加入提取剂 2[3)]（固体粉末），振荡 1 min，4 000 r/min 离心 5 min，取上清液 10.0 mL 于鸡心瓶中再加入异丙醇 10 mL 50℃～55℃水浴旋转蒸干。残留物用 1.0 mL 流动相溶解，溶解液放入 2.5 mL 离心管中，8 000 r/min 离心 5 min后，用微孔滤膜过滤，滤液供仪器测定。

2.4.2 绘制标准工作曲线

移取孔雀石绿、隐色孔雀绿、结晶紫、隐色结晶紫混合标准溶液，用流动相稀释成 2 ng/mL、50 ng/mL、250 ng/mL、500 ng/mL 标准工作溶液，用高效液相色谱仪测定，得出标准工作曲线。

2.4.3 测定

2.4.3.1 液相色谱条件

a) 色谱柱：C_{18}柱，250 mm×4.6 mm（内径），粒度 5 μm；

b) 流动相：乙腈＋乙酸盐缓冲液（80＋20，体积分数）；

c) 流速：1.0 mL/min；

d) 柱温：室温；

e) 检测波长时间程序：0 min～5.0 min，618 nm；5.0min～12 min，588 nm；12 min～18 min，267nm；孔雀石绿的检测波长为 618 nm，结晶紫的检测波长为 588 nm，隐色孔雀石绿和隐色结晶紫的检测波长为 267 nm；

f) 进样量：50 μL。

2.4.3.2 色谱测定

根据样液中孔雀石绿、隐色孔雀绿、结晶紫、隐色结晶紫的含量情况，选定峰面积相近的标准工作溶液。标准工作溶液和样液中孔雀石绿、隐色孔雀绿、结晶紫、隐色结晶紫的响应值均应在仪器的检测线性范围内。在上述色谱条件下，孔雀石绿、隐色孔雀绿、结晶紫、隐色结晶紫的保留时间约为孔雀石绿 4.0 min、隐色孔雀石绿 13.6 min、结晶紫 5.4 min 和隐色结晶紫 14.7 min。标准品色谱图参见附录 A 中图 A.1。

2.4.4 空白实验

除不加试样外，按上述测定步骤进行。

2.5 结果计算

用色谱数据处理机或按式(1)分别计算供试样品中的孔雀石绿、隐色孔雀石绿、结晶紫和隐色结晶紫残留量。

$$\omega = \frac{2 \times c_i \times V}{m} \qquad \cdots\cdots(1)$$

式中：

ω——水产品中孔雀石绿、隐色孔雀石绿、结晶紫和隐色结晶紫残留量，单位为微克每千克（μg/kg）；

c_i——标准曲线上查出试样溶液中孔雀石绿、隐色孔雀石绿、结晶紫和隐色结晶紫标准工作溶液的浓度，单位为微克每升（μg/L）；

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2) 提取剂 1 是孔雀石绿、结晶紫快速检测前处理试剂盒中试剂商品名称，由有机化学试剂组成的混合液体试剂，其作用是将样品中的待测物有效地提取出来。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。

3) 提取剂 2 是孔雀石绿、结晶紫快速检测前处理试剂盒中试剂商品名称。由无机化学组成的混合粉状试剂，其作用是助提及去除杂质和脂肪。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。

V——最终定容体积数，单位为毫升(mL)；

2——换算常数；

m——供试试料样品质量，单位为克(g)。

本方法分别计算孔雀石绿及代谢产物隐色孔雀石绿，并分别报告孔雀石绿及代谢产物隐色孔雀石绿结果。

本方法分别计算结晶紫及代谢产物隐色结晶紫，并分别报告结晶紫及代谢产物隐色结晶紫结果。

3 测定低限、回收率

3.1 检测限

本方法孔雀石绿、隐色孔雀石绿、结晶紫、隐色结晶紫的检测限均为 2.0 μg/kg。

3.2 回收率

本方法孔雀石绿、隐色孔雀石绿、结晶紫、隐色结晶紫回收率为：85%～105%。

4 液相色谱-串联质谱法

4.1 原理

试样中残留的孔雀石绿、结晶紫及其代谢物用孔雀石绿、结晶紫快速检测前处理试剂盒提供的试剂提取、浓缩后用液相色谱-串联质谱进行检测，外标法定量。

4.2 试剂和材料

除另有规定外，所有试剂均为分析纯，水为重蒸馏水。

4.2.1 乙腈：色谱纯。

4.2.2 无水乙酸铵。

4.2.3 冰乙酸：色谱纯。

4.2.4 异丙醇。

4.2.5 5 mmol/L 乙酸铵缓冲溶液：称取 0.385 g 无水乙酸铵，溶解于大约 980 mL 水中，用冰乙酸调节 pH 到 4.5，最后用水定容到 1 000 mL，过 0.2 μm 滤膜。

4.2.6 标准品：孔雀石绿、隐色孔雀石绿、结晶紫、隐色结晶紫纯度大于 98%。

4.2.7 标准贮备溶液：准确称取孔雀石绿、隐色孔雀石绿、结晶紫、隐色结晶紫各 10 mg，用乙腈分别溶解定容于 100 mL 容量瓶中，配制成 100 μg/mL 的标准贮备液。

4.2.8 混合标准溶液：用流动相稀释标准贮备溶液，配制成孔雀石绿、隐色孔雀石绿、结晶紫、隐色结晶紫均为 10 μg/mL 的混合标准溶液。0℃～4℃避光保存。

4.2.9 孔雀石绿、结晶紫快速检测前处理试剂盒。

4.3 仪器和设备

4.3.1 高效液相色谱-串联质谱联用仪：配有电喷雾(ESI)离子源。

4.3.2 匀浆机。

4.3.3 旋转蒸发仪。

4.3.4 离心机：4 000 r/min、8 000 r/min。

4.3.5 振荡器。

4.3.6 超声波水浴。

4.3.7 聚四氟乙烯离心管：2.5 mL、50 mL，带塞。

4.3.8 微孔滤膜：0.45 μm。

4.4 测定步骤

4.4.1 提取和净化

准确称取已捣碎的样品 5.00 g 于 50 mL 离心管中，先加入孔雀石绿、结晶紫快速检测前处理试剂

盒中的提取剂1(液体20.0 mL),用匀浆机以8 000 r/min的速度均质30 s,再加入提取剂2(固体粉末),振荡1 min,4 000 r/min离心5 min,取上清液10.0 mL于鸡心瓶中再加入异丙醇10 mL,50℃～55℃水浴旋转蒸干。残留物用2.5 mL流动相溶解,溶解液放入2.5 mL离心管中,8 000 r/min离心5 min后,用微孔滤膜过滤,滤液供仪器测定。

4.4.2 绘制标准工作曲线

移取孔雀石绿、隐色孔雀绿、结晶紫、隐色结晶紫混合标准溶液,用流动相稀释成0.1 ng/mL、0.2 ng/mL、0.5 ng/mL、1.0 ng/mL、5.0 ng/mL、10 ng/mL标准工作溶液,用高效液相色谱-串联质谱仪测定,得出标准工作曲线。

4.4.3 测定

4.4.3.1 高效液相色谱-串联质谱条件

a) 色谱柱:C_{18}柱,150 mm×2.1 mm(内径),粒度5 μm;

b) 流动相:乙腈+5 mmol/L乙酸铵(75+25,V/V);

c) 流速:0.2 mL/min;

d) 柱温:35℃;

e) 进样量:10 μL;

f) 离子源:电喷雾ESI,正离子;

g) 扫描方式:多反应监测MRM;

h) 雾化气、窗帘气、辅助加热气、碰撞气均为高纯氮气;使用前应调节各气体流量以使质谱灵敏度达到要求;

i) 喷雾电压、去集簇电压、碰撞能等电压值应优化至最优灵敏度;

j) 监测离子对:孔雀石绿m/z 329/313(定量离子)、329/208;隐色孔雀石绿m/z 331/316(定量离子)、331/239;结晶紫m/z 372/356(定量离子)、372/251;隐色结晶紫m/z 374/358(定量离子)、374/238。

4.4.3.2 液相色谱-串联质谱定量测定

按照4.4.3.1液相色谱-串联质谱条件测定样品和标准工作溶液,标准曲线法测定样液中的孔雀石绿、隐色孔雀石绿、结晶紫、隐色结晶紫含量。样品中被测物残留量应在标准曲线范围之内,如果含量超出标准曲线范围,应用流动相适当稀释。在上述色谱条件下,孔雀石绿、结晶紫 、隐色孔雀石绿、隐色结晶紫的保留时间约为1 .89 min、5.35 min、2.47 min和5.51 min。标准溶液的液相色谱-电喷雾质量色谱图参见附录B中的图B.1。

4.4.3.3 液相色谱-串联质谱定性测定

按照4.4.3.1液相色谱-串联质谱条件测定样品和标准工作溶液,分别计算样品和标准工作溶液中两对离子对色谱峰面积的比值,仅当两者数值的相对偏差小于25%时方可确定两者为同一物质。

4.5 空白试验

除不加试样外,均按上述测定步骤进行。

4.6 结果计算表述

按式(2)分别计算供试样品中的孔雀石绿、隐色孔雀石绿、结晶紫和隐色结晶紫残留量,计算结果需扣除空白。

$$\omega = \frac{2 \times c_i \times V}{m} \qquad \cdots\cdots(2)$$

式中:

ω——水产品中孔雀石绿、隐色孔雀石绿、结晶紫或隐色结晶紫残留量,单位为微克每千克(μg/kg);

c_i——标准曲线上查出试样溶液中孔雀石绿、隐色孔雀石绿、结晶紫和隐色结晶紫的标准工作溶液浓度,单位为微克每升(μg/L);

V——最终定容体积数，单位为毫升(mL)；

2——换算常数；

m—— 供试试料样品质量，单位为克(g)。

本方法分别计算孔雀石绿及代谢物隐色孔雀石绿，并分别报告孔雀石绿及代谢物隐色孔雀石绿结果。

本方法分别计算结晶紫及代谢物隐色结晶紫，并分别报告结晶紫及代谢物隐色结晶紫结果。

5 测定低限、回收率

5.1 测定低限

本方法孔雀石绿、隐色孔雀石绿、结晶紫、隐色结晶紫的检测低限均为 0.5 μg/kg。

5.2 回收率

本方法孔雀石绿、隐色孔雀石绿、结晶紫、隐色结晶紫回收率均为：85%～105%。

附 录 A
（资料性附录）
标准品色谱图

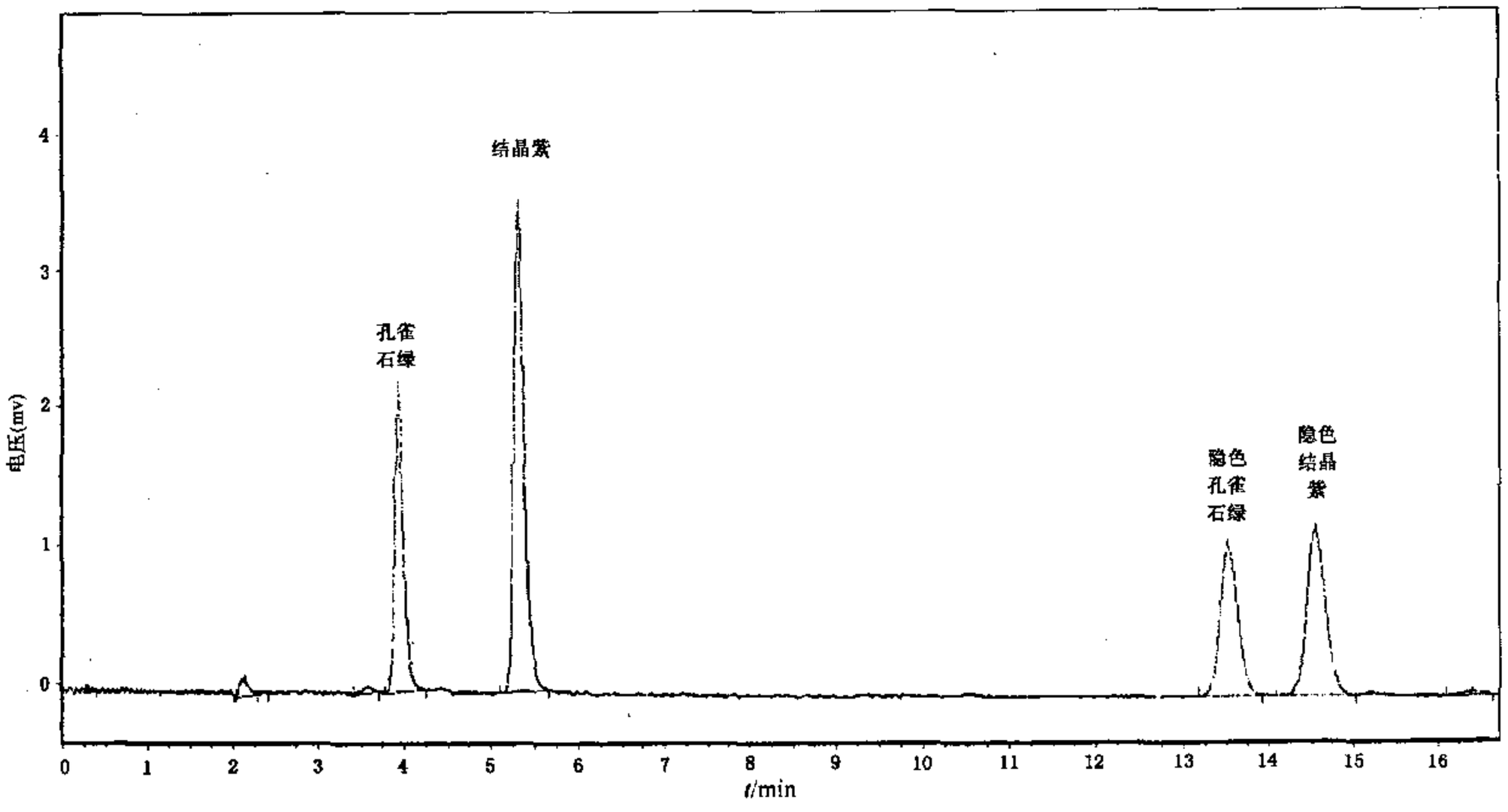

图 A.1 孔雀石绿、隐色孔雀石绿、结晶紫、隐色结晶紫混合标准品的液相色谱图

附 录 B
（资料性附录）
标准品液相色谱-电喷雾质量色谱图

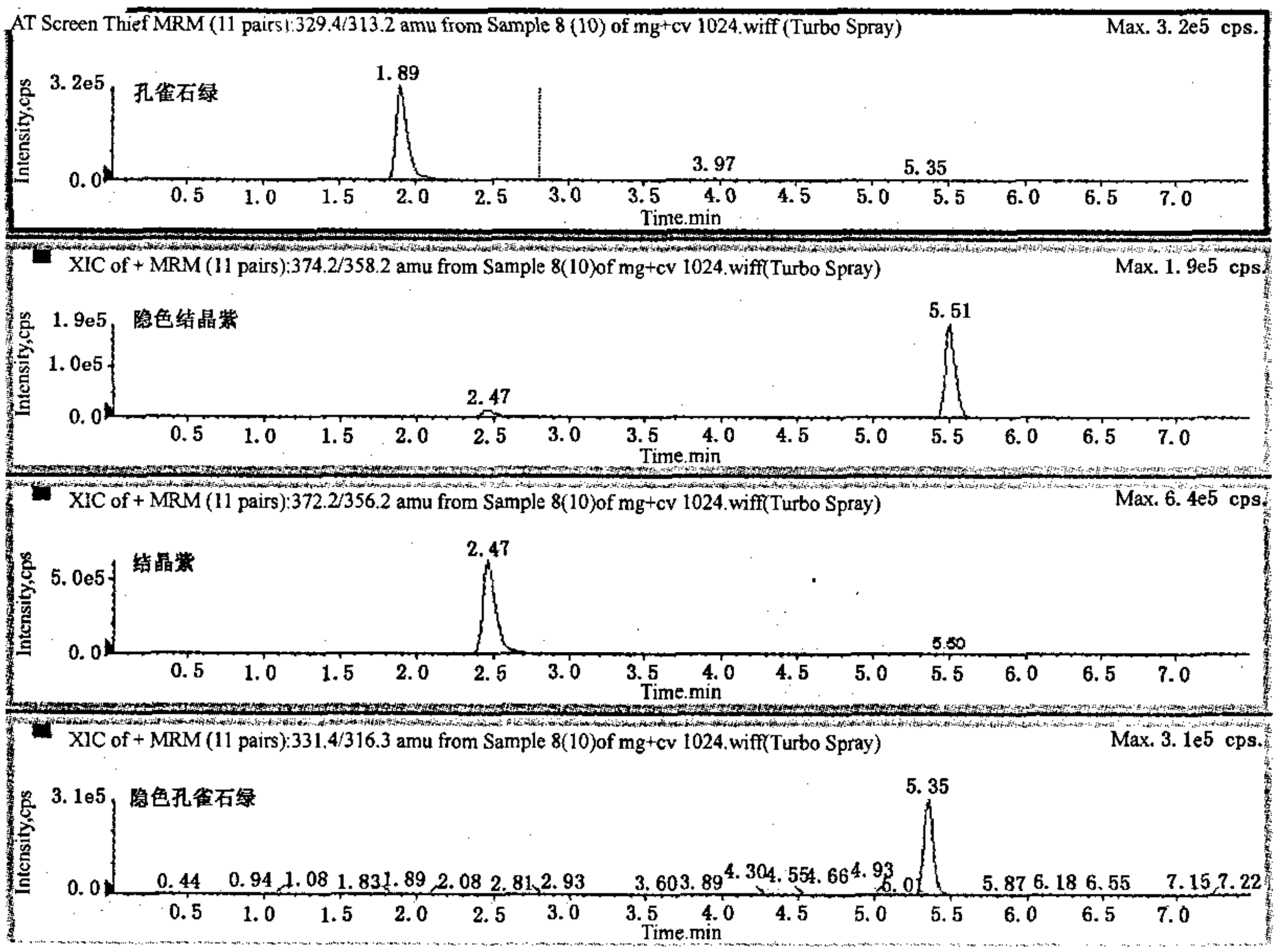

图 B. 1 孔雀石绿、隐色孔雀石绿、结晶紫、隐色结晶紫液相色谱-电喷雾质量色谱图

Preface

The appendix A and the appendix B are reference materials.

The standard is put forward and managed by the Certification and Accreditation Administration of the People's Republic of China(CNCA).

This standard was drafted by Chinese Academy of Inspection and Quarantine, Beijing Entry-Exit Inspection and Quarantine Bureau, Liaoning Entry-Exit Inspection and Quarantine Bureau, Fujian Entry-Exit Inspection and Quarantine Bureau, Jiangxi Entry-Exit Inspection and Quarantine Bureau, Zhanjiang Entry-Exit Inspection and Quarantine Bureau, and Zhoushan Entry-Exit Inspection and Quarantine Bureau.

This main drafters of this standard are Jia Dongfen, Zhang Shunhe, Zhang Wei, Xu Chaoyi, Lin Weixuan, Tian Miao, Li Yaoping, Zhan Chunrui, Chen Wei, Zhou Xiangyang.

The Entry-Exit professional standard is issued for the first time.

Determination of malachite green, crystal violet and the corresponding leuco compounds in the aquatic products

1 Scope

The standard provides a rapid determination method for malachite green (MG), leucomalachite green (LMG), crystal violet (CV) and leucocrystal violet(LCV) residues exiting in the raw and processed aquatic products by HPLC and LC-MS/MS.

The standard is used to rapidly determine the residues of MG and LMG, CV and LCV in the raw and processed aquatic products.

2 HPLC method

2.1 principle

The residuces, after the sample extracted by a pre-treatment kit from samples and concentrated, are determined by HPLC, and quantified by the external standard method.

2.2 Reagents and materials

All the reagents are analytial purity and water is redistilled unless otherwise mentioned.

2.2.1 Acetonitrle: chromatographically pure.

2.2.2 Anhydrous sodium acetate.

2.2.3 Glacial acetic acid: chromatographically pure.

2.2.4 Isopropyl alcohol.

2.2.5 *p*-methylbenzosulfonic acid.

2.2.6 Sodium acetate buffer solution: 4.950 g of anhydrous sodium acetate and 0.950 g p-methylbenzosulfonic acid are weighed out and disolved into 950 mL of water. Adjust pH of the solution to 4.5 with glacial acetic acid and make volume of the solution to 1 000 mL with water.

2.2.7 Standard reagents: MG, LMG, CV and LCV, whose purity is more than 98%.

2.2.8 A standard stock solution at a concentration of 100 μg/mL in acetonitril is prepared by weigh out 10 mg of MG standard which will be dissolved into acetonitril. The volume of 100 mL is made. The same process is followed for LMG, CV and LCV standard stock solution preparation.

2.2.9 Mixed standard solution is prepared by diluting the stock solutions with mobile phase, in which the concentration of each standard reagent is 10 μg/mL. Keep the mixed standard solution at 0℃～4℃ and away from light.

2.2.10 Pre-treatment kit[1] for the extraction of MG, LMG, CV and LCV.

2.3 Instruments and equipment

2.3.1 HPLC with UV-VIS scanning detector, and detection wave and time are controlled by programme.

2.3.2 blender.

2.3.3 rotary evaporator.

2.3.4 centrifuge, with 4 000 r/min, 8 000 r/min.

2.3.5 vortex.

2.3.6 ultr-sonic water bath.

2.3.7 Beflon centrifuge tubes, 2.5 mL, 50 mL, with plugs.

2.3.8 microbore screen filter, ϕ 0.45 μm

2.4 Procedure

2.4.1 Extraction and cleaning up

Five g of a sample are weighed out into a 50 mL centrifuge tube, extract 1[2] (liquid, about 20.0 mL) from the pre-treatment kits is added, and the tissue is homogenized in the tube at 8 000 r/min for 30 s and then extract 2[3] (powder) is added in the same tube. After shaking the tube for 1 min, the sample is centrifuged (4 000 rpm, 5 min), and 10.0 mL of the supernatant is added to a pear-shaped flask containing 10 mL of isopropyl alcohol and the solvent is removed by roto-evaporation with water bathing at 50℃～55℃. The residue is dissolved in 1.0 mL of mobile phase, which is then transferred to a 2.5 mL centrifuge tube and centrifuged (8 000 r/min, 5 min), the supernatant filters through 0.45 μm, and the filtrate is used for the determination by HPLC.

2.4.2 Drawing up calibration curve

Standard working solution is peprepared by making dilutions of mixed standard solution with mobile phase into serial concentrations of 2 ng/mL, 50 ng/mL, 250 ng/mL, 500 ng/mL. The calibration curve is drawn up based on the data determined by HPLC.

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1) Pre-treatment kit for the extraction of MG, LMG, CV and LCV is developed by Chinese Academy of Inspection and Quarantine (CAIQ) and produced by Beijing Land Bridge Commodity Inspection New Technology Co. The information given here is not that CNCA accredits the product, but for user's convenience.

2) Extract 1, composed of liquid organic chemicals, which plays a role of the extraction of residues of MG, LMG, CV and LCV in aquatic samples, is commodity name. The information given here is not that CNCA accredits the product, but for user's convenience.

3) Extract 2, composed of powder inorganic chemicals, which plays a role of assisting extraction and the removal of fat and other components, is commodity name. The information given here is not that CNCA accredits the product, but for user's convenience.

2.4.3 Determination

2.4.3.1 HPLC Instrumental conditions.

a) Column: C_{18}, 250 mm × 4.6 mm (i. d.), grain size 5 μm;
b) Mobile phase: acetonitrile + sodium acetate buffer solution (80 + 20, *V*/*V*);
c) Flow rate: 1.0 mL/min;
d) Column temperature: room temperature;
e) Programme for detection wave and time: 0 min~5.0 min, 618 nm; 5 min~12 min, 588 nm; 12 min~18 min, 267 nm. 618 nm for the determination of MG, 588 nm for CV, 267 nm for both LMG and LCV;
f) Injection volume: 50 μL.

2.4.3.2 Determination by HPLC

According to MG, LMG, CV and LCV content in the filtrate, a standard working solution which generates the almost same peak area as the filtrate is chosen. Area response of MG; LMG, CV and LCV between the standard working solution and the filtrate should be within linear range. Under above the conditions retention time of MG, LMG, CV and LCV is respectively about 4.0 min, 13.6 min, 5.4 min, and 14.7 min. Chromatograms of the standard solution by HPLC are shown in figure A1 of appendix A.

2.4.4 Blank test

Follow all the above steps without samples added.

2.5 Calculation

The residue amount of MG, LMG, CV and LCV is respectively calculated with HPLC data processor or formula(1). The amount of each residue is equal to the calculated result minus value of the blank test result.

$$\omega = \frac{2 \times c_i \times V}{m} \qquad \text{.....(1)}$$

Where

ω——residue amount of MG, LMG, CV and LCV in aquatic samples, μg/kg;

c_i——concentrations of MG, LMG, CV and LCV read from calibration curve, μg/L;

V——final volume, mL;

2——constant;

m——weight of samples.

MG and LMG residues are respectively calculated and reported by the standard method.

CV and LCV residues are respectively calculated and reported by the standard method.

3 Determination limit and recovery

3.1 Determination limit

The standard method provides 2.0 μg/kg determination limit for residues of MG, LMG, CV and LCV in aquatic samples.

3.2 Recovery

The standard method provides a recovery of 85%～105%.

4 LC-MS/MS method

4.1 Principle

The residuces, after the sample extracted by a pre-treatment kit from samples and concentrated, are determined by LC-MS/MS, and quantified by the external standard method.

4.2 Reagents and materials

All the reagents are analytial purity and water is redistilled unless otherwise mentioned.

4.2.1 Acetonitrle: chromatographically pure.

4.2.2 Anhydrous ammonium acetate.

4.2.3 Glacial acetic acid: chromatographically pure.

4.2.4 Isopropyl alcohol.

4.2.5 5 mmol/L ammonium acetate buffer solution: weigh out 0.385 g of anhydrous ammonium acetate, disolve it into about 980 mL of water, then adjust pH of the solution with glacial aceticacid to 4.5 and *make volume of the solution* to 1 000 mL, filter it through 0.2 μm membrance.

4.2.6 Standard reagents: There are MG, LMG, CV and LCV. Each is more than 98% in purity.

4.2.7 A standard stock solution at a concentration of 100 μg/mL in acetonitril is prepared by weighing out 10 mg of MG standard which will be dissolved into acetonitril. The volume of 100 mL is made. The same process is followed for LMG, CV and LCV standard stock solution preparation.

4.2.8 Mixed standard solution is prepared by diluting the stock solutions with mobile phase, in which the concentration of each standard reagent is 10 μg/mL. Keep the mixed standard solution at 0℃～4℃ and away from light.

4.2.9 Pre-treatment kit for the extraction of MG, LMG, CV and LCV.

4.3 Instruments and equipment

4.3.1 LC-MS/MS, with ESI ionic source.

4.3.2 Blender.

4.3.3 Rotary evaporator.

4.3.4 Centrifuge, with 4 000 r/min, 8 000 r/min.

4.3.5 Vortex.

4.3.6 Ultra-sonic water bath.

4.3.7 Beflon centrifuge tubes, 2.5 mL, 50 mL, with plugs.

4.3.8 Microbore screen filter, ϕ 0.45 μm.

4.4 Procedure

4.4.1 Extraction and cleaning up

Five g of a sample are weighed out into a 50 mL centrifuge tube, extract 1 (liquid, about 20.0 mL) from the pre-treatment kits is added, and the tissue is homogenized in the tube at 8 000 r/min for 30 s and then extract 2 (powder) is added in the same tube. After shaking the tube for 1 min, the sample is centrifuged (4 000 rpm,5 min), and 10.0 mL of the supernatant is added to a pear-shaped flask containing 10 mL of isopropyl alcohol and the solvent is removed by roto-evaporation with water bathing at 50℃～55℃. The residue is dissolved in 2.5 mL of mobile phase, which is then transferred to a 2.5 mL centrifuge tube and centrifuged (8 000 rpm, 5 min), the supematant filters through 0.45 μm, and the filtrate is used for the determination by LC-MS/MS.

4.4.2 Drawing up calibration curve

Standard working solution is peprepared by making dilutions of mixed standard solution with mobile phase into serial concentrations of 0.1 ng/mL, 0.2 ng/mL, 0.5 ng/mL, 1.0 ng/mL, 5.0 ng/mL, 10 ng/mL. The calibration curve is drawn up based on the data determined by LC-MS/MS.

4.4.3 Determination

4.4.3.1 LC-MS/MS Instrumental conditions.

a) Column: C_{18}, 150 mm×2.1 mm (i.d.), grain size 5 μm;
b) Mobile phase: acetonitrile+ 5 mmol/L of ammonium acetate buffer solution (75+25, *V*/*V*);
c) Flow rate: 0.2 mL/min;
d) Column temperature: 35℃;
e) Injection volume: 10 μL;
f) Ionization source: ESI, positive ion;
g) Scanning model: MRM;
h) Atomization gas, curtain gas, auxiliary heating gas and collision gas are high grade nitrogen gas; Adjust flow rate of gases for making MS meet requirements for sensitivity;
i) Spray voltage, de-cluster voltage, collision energy voltage, etc. are optimized in sensitivity;
j) Detection ion pair: MG: m/z 329/313 (quantitative ion), 329/208; LMG: m/z 331/316(quantitative ion), 331/239; CV: m/z 372/356 (quantitative ion), 372/251; LCV: m/z 374/358(quantitative ion), 374/238.

4.4.3.2 Quantitative Determination by LC-MS

MG, LMG, CV and LCV both in the filtrate and in the standard working solution are determined with LC-MS/MS parameters (4.4.3.1), and quantified by calibration curve. The quantity of the residues in the filtrate should be within calibration curve range. If the quantity is beyond the curve range, the filtrate should be diluted with mobile phase in a proper ratio. Under the above parameters retention time of MG, LMG, CV and LCV is respectively about 1.89 min, 5.35 min, 2.47 min, and 5.51 min.

Chromatograms of the standard solution by LC-MS/MS (ES) are shown in figure B1 of appendix B.

4.4.3.3 Qualitative Determination by LC-MS

MG, LMG, CV and LCV both in the filtrate and in the standard working solution are determined with *LC-MS/MS parameters* (4.4.3.1). The ratio of two peak areas (quantitative ion and un-quantitative ion) from the filtrate and the standard working solution is calculated. When deviation between the two values is less than 25%, the same compound can be confirmed.

4.5 Blank test

Follow all the above steps without samples added.

4.6 Calculation and result expression

The residue amount of MG, LMG, CV and LCV is respectively calculated with formula (2). The amount of each residue is equal to the calculated result minus value of the blank test result.

$$\omega = \frac{2 \times c_i \times V}{m} \qquad \cdots\cdots(2)$$

Where

ω——residue amount of MG, LMG, CV and LCV in aquatic samples, μg/kg;

c_i——concentrations of MG, LMG, CV and LCV reading calibration curve, μg/L;

V——*final volume*, mL;

2——constant;

m——weight of samples.

MG and LMG residues are respectively calculated and reported by the standard method.

CV and LCV residues are respectively calculated and reported by the *standard method*.

5 *Determination limit and recovery*

5.1 Determination limit

The standard method provides 0.5 μg/kg determination limit for residues of MG, LMG, CV and LCV *in aquatic samples*.

5.2 Recovery

The standard method provids a recovery of 85%～105%.

Annex A
(informative)
Chromatogram of the standard

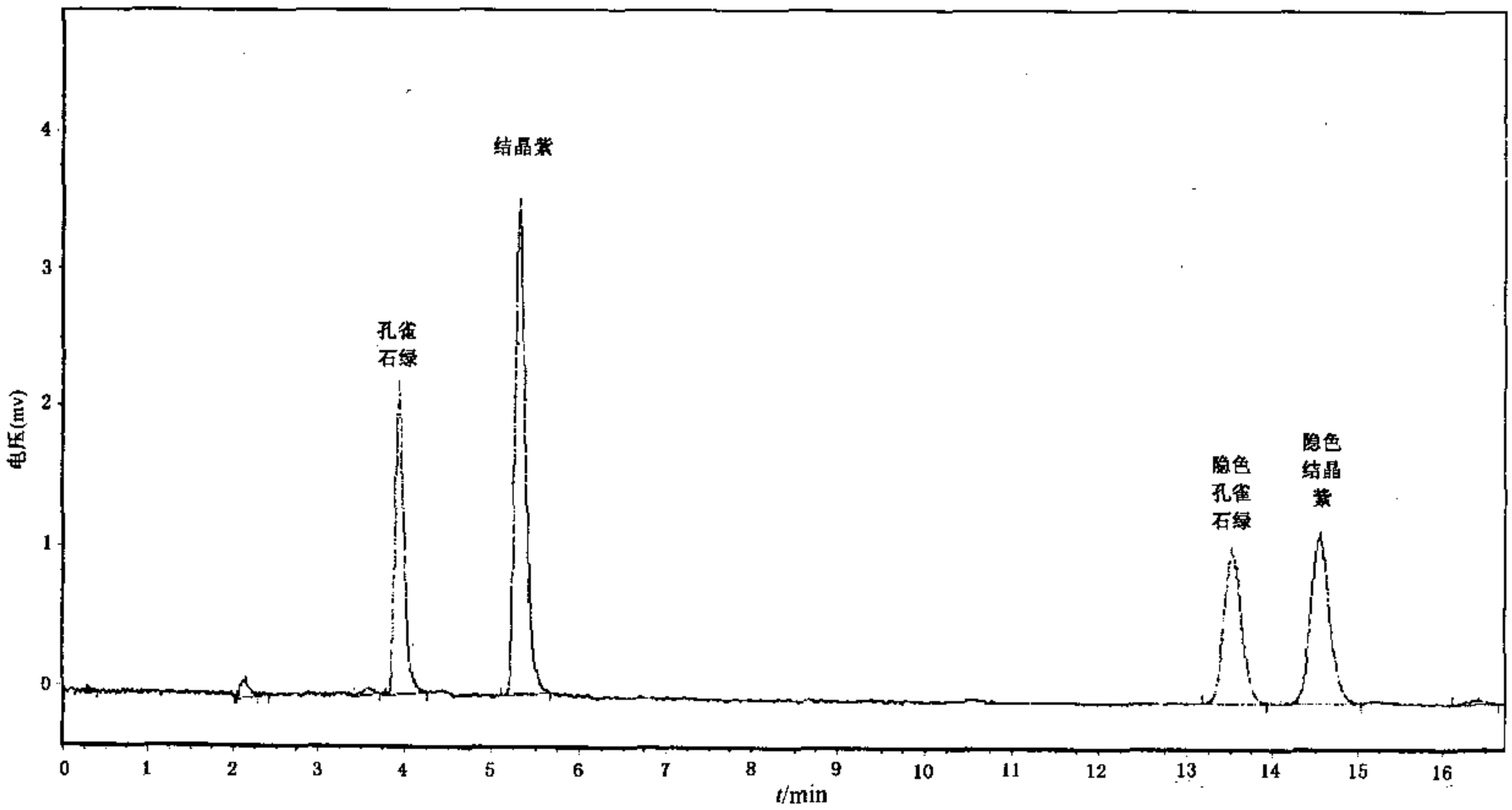

Figure A. 1 Total chromatograms of MG, LMG, CV and LCV in the standard working solution from HPLC

Annex B
(informative)
LC-MS(ES) of the standard

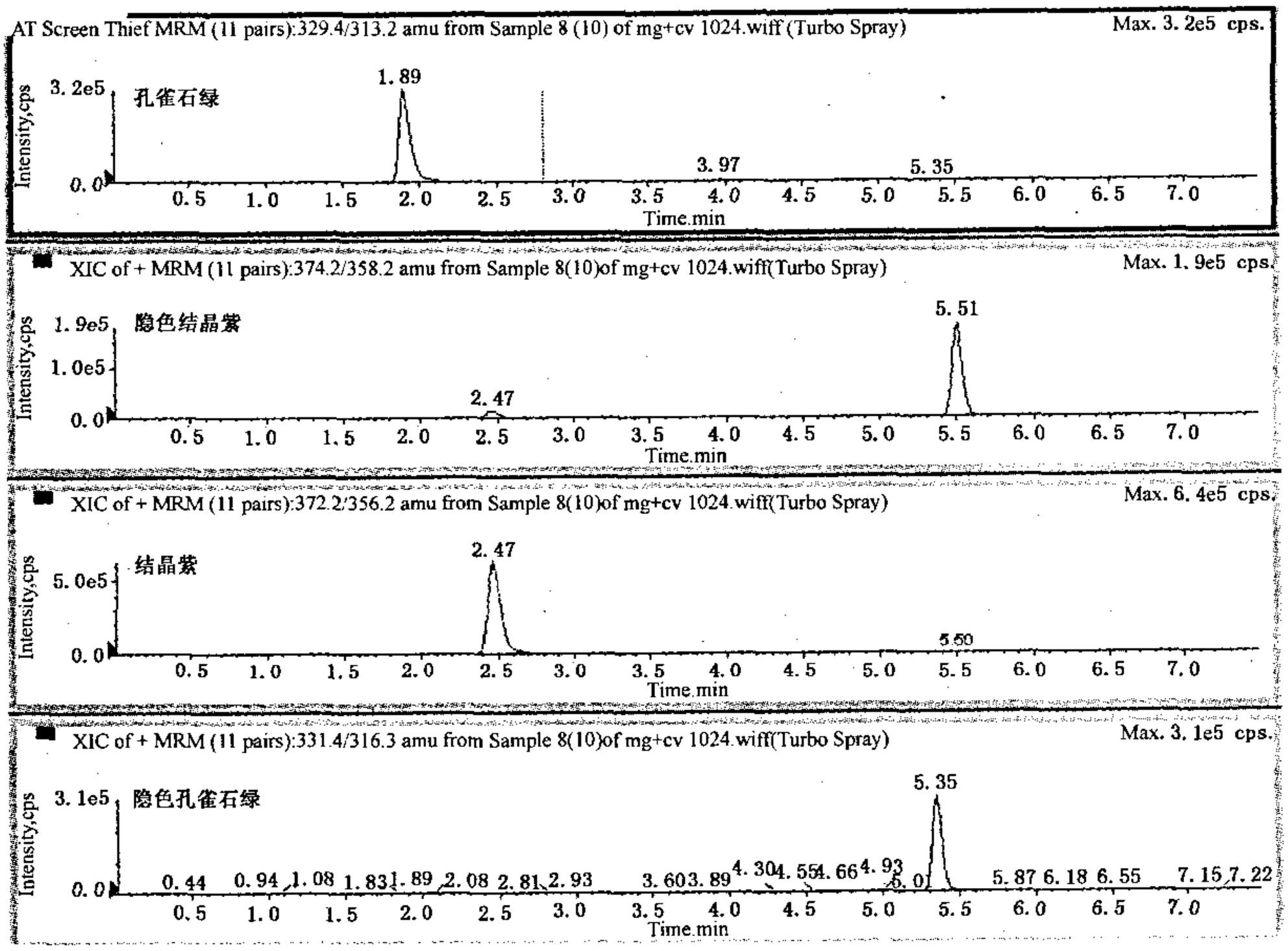

Figure B. 1 Total ion chromatograms of MG, LMG, CV and LCV in the standard working solution from LC-MS(ES)

SN/T 1768-2006

书号:155066 • 2-17032