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EUROPEAN UNION REFERENCE LABORATORY FOR MARINE BIOTOXINS

EURLMB Standard Operating Procedure for PSP toxins by Mouse Bioassay

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Coordination:
European Union Reference Laboratory for Marine Biotoxins (EURLMB)
Agencia Española de Consumo, Seguridad Alimentaria y Nutrición (AECOSAN)
c/ Fonte das Abelleiras, 4 - Edificio CITEXVI - 36310 Vigo, Spain
E-mail: eurlmb@msssi.es
http://www.aesan.msssi.gob.es/en/CRLMB/web/home.shtml

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INTRODUCTION

The regulatory structure in the European Union (EU) includes a group of regulations for the control of PSP toxins. Thus, Regulation (EC) Nº 853/2004 of the European Parliament and of the Council lays down specific hygiene rules for food of animal origin. With regard to the "Health standards for live bivalve mollusks", it indicates that the bivalve mollusks placed on the market for human consumption must not contain marine biotoxins (Paralytic Shellfish Poison: PSP) in total quantities (measured in the whole body or any part edible separately) that exceed 800 micrograms per kilogram.

Regarding methodologies, Commission Regulation (EC) Nº 2074/2005 amended by Commission Regulations (EC) Nº 1664/2006, (EC) Nº 1244/2007 and (EU) Nº 15/2011 indicates the recognized testing methods for marine biotoxins for the purpose of Regulations (EC) Nº 853/2004 and Nº 854/2004. With regard to the PSP detection method it is specified that: "*The paralytic shellfish poison (PSP) content of edible parts of mollusks (the whole body or any part edible separately) must be detected in accordance with the biological testing method or any other internationally recognised method. The so-called Lawrence method may also be used as an alternative method for the detection of those toxins as published in AOAC Official Method 2005.06 (Paralytic Shellfish Poisoning Toxins in Shellfish)*"

1. Purpose

The purpose of this Standard Operating Procedure (SOP) is to detail the conduct of the analysis in the determination of PSP toxins in bivalve mollusks, echinoderms, marine gastropods and tunicates by Mouse Bioassay.

2. Scope

The method described is the biological method applicable to determine PSP toxins in AOAC (Paralytic Shellfish Poison, Biological method, Final Action, Method 959.08, in Official Methods of Analysis (1995) of the Association of Official Analytical Chemists (AOAC), Natural Toxins, Chapter 49, page 46)

3. Apparatus and Material

- High-speed blender or homogenizer (minimum 13500 rpm)
- Analytical balance, accuracy to the nearest 0.1 mg
- Balance, accuracy to the nearest 0.01 mg
- Heat block
- Centrifuge (minimum speed 3000 rpm)

- Extraction cabinet
- Filter paper (Whatman Nº1 or similar)
- Instruments for sample preparation, knives, spatulas, scissors, stainless steel sieve, plastic jars
- Round bottom flasks (400 – 1000 mL)
- Volumetric flask 100 mL, 250 mL
- Test tubes 100 mL
- Glass tubes min 10 mL
- Adjustable automatic pipettes
- Magnetic stirrer
- Disposable 1 mL sterile syringes with 22-26 gauge needle
- Stop watch, range of measurement minimum 60 min. and accuracy to the nearest 1/10 second
- pH-meter

4. Mice

Use albino mice Swiss strain (CD1 is recommended) preferably weighing between 19 g and 21 g, from stock colony used for routine assays. Do not use mice >23 g nor <17 g and do not re-use mice.

5. Reagents and Reference Materials

- Paralytic shellfish poison standard solution
- Paralytic shellfish poison working standard solution.- 1 µg/mL. Dilute 1 mL standard solution to 100 mL with distilled H_2O. Solution is stable several weeks at 3-4ºC.
- Distilled H_2O
- H_2O pH 3: Add HCl 37% drop wise to distilled H_2O until adjust final pH 3
- Hydrochloric acid (HCl) 37%
- Hydrochloric acid 0.1 N: Take 8.3 mL HCl 37% and make up to 1 L distilled H_2O
- Hydrochloric acid 5 N: Take 41.5 mL HCl 37% and make up to 100 mL distilled H_2O
- Sodium Hydroxide (NaOH) 99%
- Sodium Hydroxide 0.1 N: Dissolve 0.4 g NaOH in distilled H_2O and make up to 100 mL

Note: Solutions HCl 0.1N, HCl 5N and NaOH 0.1N will be renewed at least once a year.

6. Analytical procedure

6.1 Standardization of Bioassay: Conversion factor (CF) calculation

Dilute 10 mL aliquots of 1 µg/mL standard solution with 10, 15, 20, 25 and 30 mL H_2O, respectively, until intraperitoneal injection of 1 mL doses into few (minimum 2) mice causes median death time of 5-7 min. pH of dilutions should be 2-4 and must not be >4.5, it is recommended using H_2O pH 3.

Test additional dilutions in 1 mL increments of H_2O, e.g., if 10 mL diluted with 25 mL H_2O kills mice in 5-7 min, test solutions diluted 10 + 24 and 10 + 26.

Inject group of 10 mice with each of 2 or preferably 3 dilutions that fall within median death time of 5-7 min. Give 1 mL dose to each mouse by intraperitoneal injection and determine death time as time elapsed from completion of injection to last gasping breath of mouse.

Repeat assay 1 or 2 days later, using dilutions prepared above which differed by 1 mL increments of H_2O. Then repeat entire test, starting with testing of dilutions prepared from newly prepared working standard solution.

Calculate median death time for each group of 10 mice used on each dilution. If all groups of 10 mice injected with any dilution gave median death time <5 or >7 min, disregard results from this dilution in subsequent calculations. On other hand, if any groups of 10 mice injected with dilution gave median death time falling between 5 and 7 min, include all groups of 10 mice used on that dilution, even though some of median death times may be <5 or >7min. From median death time or each group of 10 mice in each of selected dilutions, determine number of mouse units/mL from Sommer's Table. Divide calculated µg poison/1 mL by mouse units/1mL to obtain conversion factor (CF value) expressing µg poison equivalent to 1 mouse unit (MU). Calculate average of individual CF values, and use this average value as reference point to check routine assays.

6.2 Periodical check CF

Inject 5 mice with appropriate dilution of working standard such that median death time falls within 5-7min. CF value thus determined should check with average CF value within ±20%.

If it does not check within this range, complete group of 10 mice by adding 5 mice to the 5 mice already injected, and inject a second group of 10 mice with same dilution of standard. Average CF value determined for second group with that of first group. Take resulting value as new CF value.
Variation of >20% represents significant change in response of mice to poison, or in technique of assay. Changes of this type require change in CF value.

6.3 Sample preparation

Clams, oysters, mussels, cockles, razor clams and similar: Thoroughly clean outside of shellfish with fresh H_2O. Open by cutting adductor muscles. Rinse inside with fresh H_2O to remove sand or other foreign material. Remove meat from shell by separating adductor muscles and tissue connecting at hinge.
Transfer meats to stainless sieve or to filter paper and let drain. Collect 100-150 g meats and homogenise.

Other types of shellfish: Separate edible portion and apply test to this portion alone. Drain and homogenise as in previous paragraph.

Canned shellfish: Place entire contents of can (meat and liquid) in blender and blend until homogeneous.
For large cans, drain meat a few minutes on a sieve and collect all liquid. Determine weight of meat and volume of liquid. Recombine test portion of each in proportionate amounts. Blend test recombined portions in blender until homogeneous.

6.4 Extraction

Weigh 100 g well-mixed material into tared baker. Add 100 mL 0.1N HCl, stir thoroughly, and write down weight of sample and HCl 0.1N. Check pH (pH should be <4.0 preferably ca 3.0). If necessary, adjust pH as indicated below. Heat mixture, boil gently 5 min, and let cool to room temperature. Adjust cooled mixture to pH 2.0-4.0 (never >4.5).
To lower pH, add 5N HCl drop wise with stirring; to raise pH, add 0.1N NaOH drop wise with constant stirring to prevent local alkalinization and consequent destruction of poison. Add distilled H_2O pH 3 up to obtain previous weight of sample and HCl 0.1N.
Stir and check pH. Let settle until portion of supernatant is translucent and can be decanted free of solid particles large enough to block hypodermic needle. If necessary centrifuge mixture to supernatant 5 min at 3000 rpm. Only enough liquid to perform bioassay is necessary.

6.5 Mouse test

Intraperitoneally inoculate each test mouse with 1 mL acid extract. Note time of inoculation and observe mice carefully for time of death as indicated by last gasping breath. Record death time from stopwatch. One mouse may be used for initial determination, but 2 or 3 are preferred. If death time or median death time of several mice is <5 min, make dilution to obtain death times of 5-7min adding H_2O pH 3. Inject at least 3 mice with dilution that fall median death time of 5-7 min.
If death time of 1 or 2 mice injected with undiluted sample is >7 min, total of ≥3 mice must be inoculated to establish toxicity of sample.

7. Calculation and Interpretation of Toxicity

Mice should be observed 60 min. Determine median death times of mice, including survivors, and from Sommer's Table determine corresponding number of mouse units.

Note: Consider death time of survivor mice >60 min or equivalent to <0.875 mouse units.

Correct mouse units obtained for each mouse corresponding to death time by multiplying mouse units by weight correction factor from Sommer's Table.

Then determine median mouse unit for group.

PSP toxicity, expressed as µg equivalents STX diHCl / kg meat is calculated by the function:

µg equivalents STX diHCl / kg = MU corrected x CF x dilution factor x 2000

The result will be expressed without decimals.

Result >800 µg equivalents STX diHCl/kg is considered as positive result, if the result falls within ≥400 and <800 µg equivalents STX diHCl/kg will be a negative result and if result <400 µg equivalents STX diHCl / kg should be considered no detected.