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Laboratory manual of standardized methods for the analysis of pesticide and antibiotic residue in aquaculture products

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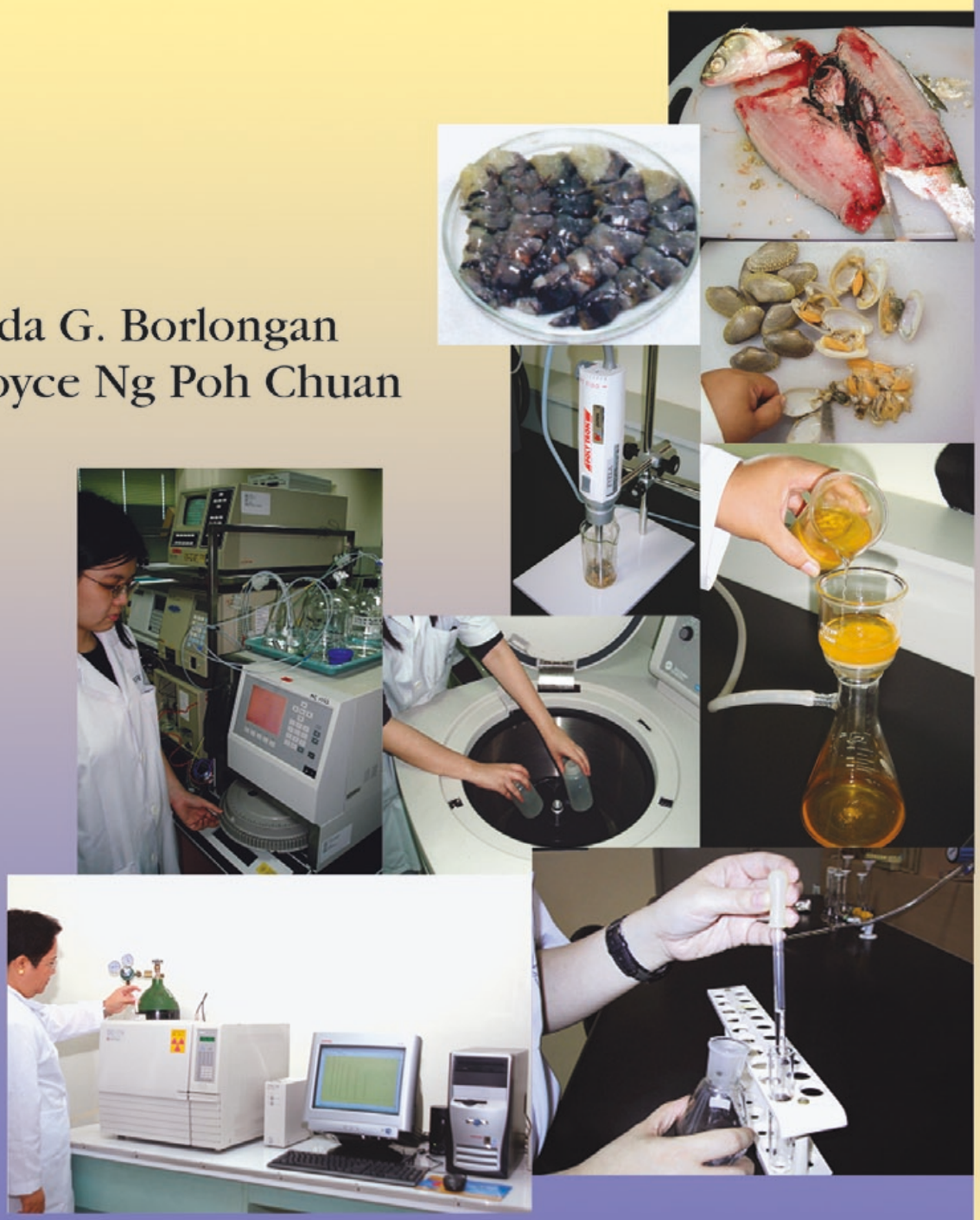
Joyce Ng Poh Chuan



SOUTHEAST ASIAN FISHERIES DEVELOPMENT CENTER
Aquaculture Department and Marine Research Department

GOVERNMENT OF JAPAN TRUST FUND

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On the Cover

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PREFACE

Aquaculture production in Southeast Asia has grown rapidly and is now a major contributor to food supply worldwide. The use of chemicals in aquaculture systems for various purposes is widely recognized. While aquaculturists acknowledge that some operations are reliant on chemical usage, they also realize the potential danger associated with chemical misuse. The increasing use of chemicals in aquaculture has led to widespread public concern because pesticide and antibiotic residues could eventually end up in aquaculture products. Because of their potential adverse effect on human health, governments worldwide set limits on allowable levels of chemical residues in food and animal feeds and monitor these levels. The monitoring and enforcement actions are, however, dependent on the technical capability to detect residues. Realizing this need, the Government of Japan through the Trust Fund's Fish Disease Project funded a project to standardize methodologies for detection of pesticide and antibiotic residues in aquaculture products. Two separate studies were implemented by the Aquaculture Department of the Southeast Asian Fisheries Development Center (SEAFDEC/AQD, Philippines) and the Marine Fisheries Research Department (SEAFDEC/MFRD, Singapore) on pesticide and antibiotic residues, respectively. The results of these studies are the basis for this manual, which will benefit all those involved in the monitoring and enforcement aspects of chemical residue limits in aquaculture products in the region.

Kazuya Nagasawa

Dr. Kazuya Nagasawa
Fish Disease Project Leader
Government of Japan Trust Fund

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CONTENTS

CHAPTER 1

Detection of Antibiotic Residues in Aquaculture Products

Prepared by

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INTRODUCTION

Aquacultured animals are under constant threat from bio-aggressors such as viruses, bacteria, parasites and fungi. These organisms harm either spontaneously or through aquatic animal husbandry practices, and often both. Indeed, it is generally recognized that disease problems follow the development of techniques for animal production.

Consequently, fish culture uses a variety of chemicals that represent potential threats to the health of the cultured animal, indigenous biota, and even humans. Chemicals employed in aquaculture include the following:

- Drugs used to treat disease (chemical therapeutants)
- Chemicals introduced through construction materials
- Hormones used to alter reproductive viability, sex, and growth rates

Of these, chemotherapeutic drugs are the most harmful. Chemotherapeutic treatments are initiated after clinical signs of a disease appear in a population of fish. Chemicals used in construction and hormones are not considered because they are relatively non-toxic.

The use of chemical therapeutants obviously leads to the transit of drugs and to their persistence in products intended for human consumption. It also leads to the release of drugs or their metabolites to the aquatic environment. Hence the criticisms raised in the press against the use of chemotherapy in aquaculture, and the restrictive legislation set up in many countries under pressure of public opinion. It sometimes appears that people would believe that drug resistance of bacteria responsible for human infections originates exclusively, or almost exclusively, from consumption of animal products such as those provided by aquaculture.

It should be noted that in addition to the chemicals that are deliberately used, fish raised in aquaculture are also susceptible to contamination via pesticides present in feed, agriculture run-off water, and sediments. The magnitude of human exposure to these sources has not yet been fully assessed and should be examined periodically in light of the growth and change in this sector of the seafood industry.

Determination of Oxolinic Acid by HPLC-Fluorescence Method

Principle

Oxolinic acid (OXA) is a powerful synthetic antibacterial agent used in aquaculture in curing or preventing diseases caused by certain species of Yersinia, Aeromonas and Vibrio.

The methods for the detection of the bactericide, oxolinic acid, can be generally divided into two categories, namely biological and physiochemical. The biological method, such as bioassay, lacks sensitivity and specificity. The physiochemical method, based mainly on HPLC with UV or fluorescence detection, is much faster, more specific and more sensitive than the biological method.

Apparatus

a. High Performance Liquid Chromatograph (HPLC): WATERS Isocratic pump system, WATERS in-line degasser, 600E Multisolvent Delivery System, 600E System Controller, 717 Plus Autosampler equipped with 470 Scanning Fluorescence Detector capable of monitoring emission at 365 nm and excitation at 337 nm.

b. Chromatographic column: Reverse phase, TSK-GEL ODS-8OTM (150 x 4.6mm)

Operating condition: Flow rate set at 0.5 mL/min.

Injection volume: 20 ml.

As a part of the system shut-down at the end of the experiment, HPLC grade water is pumped through the column for a minimum of twenty min followed by a twenty min rinse with methanol:water (7:3) at 0.5mL/ min

c. Filter Unit: GL, Chromatodisc 13P, 0.4 mm

d. Glass centrifuge bottle (150 ml) and tubes (15 mL)

e. Separatory flasks (125 mL)

f. Florentine flask (100 mL)

g. Tissue homogenizer

h. Centrifuge

i. Rotary evaporator

j. Ultrasonic water bath

Reagents

a. Acetonitrile (HPLC grade)

b. Water (HPLC grade, Diamond-Q)

c. 1-propanol (GR grade)

d. n-hexane (GR grade)

e. Sodium sulfate, anhydrous (GR grade)

f. Methanol (HPLC grade)

g. Sodium chloride (GR grade)

h. 0.1 M citric acid (HPLC grade): Dissolve 21.01 g of monohydrate salt of citric acid in 1L of HPLC grade water.

i. Oxolinic acid (Sigma Chemical Company): All standard solutions are stored below 10° C. Stock solution is stable for at least 3 months, but diluted solutions should be kept no longer than 2 weeks.

Stock solution (100 ppm): Weigh accurately 10 mg of oxolinic acid and make up to 100 mL in a volumetric flask using acetonitrile:water (1:1) solution.

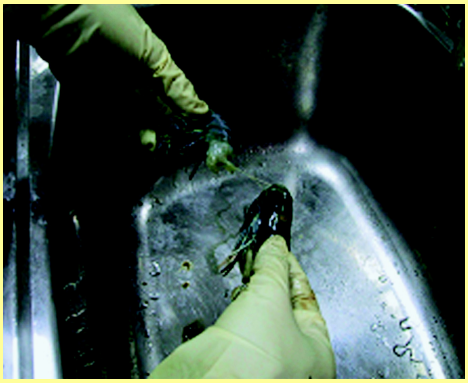
Intermediate solution (10 ppm): Pipette accurately 5 mL of the 100 ppm stock solution into a 50 mL volumetric flask and make up volume with acetonitrile:water (1:1) solution.

Working solution (1 ppm): Pipette accurately 5 mL of the 10 ppm intermediate solution into a 50 mL volumetric flask and make up volume with acetonitrile-water (1:1) solution.
The working solution should not be used after 1 month of refrigerated storage and fresh working solution should be prepared.

j. Mobile Phase: (Acetonitrile:methanol) - 0.1M citric acid solution (2:2:3), filtered through polyvinylidene fluoride membrane (0.45 mm). The mobile phase should be prepared daily.

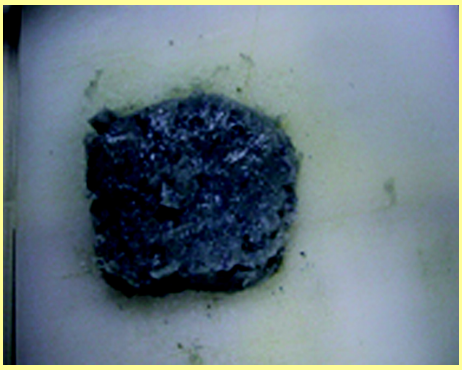
Sampling Procedures

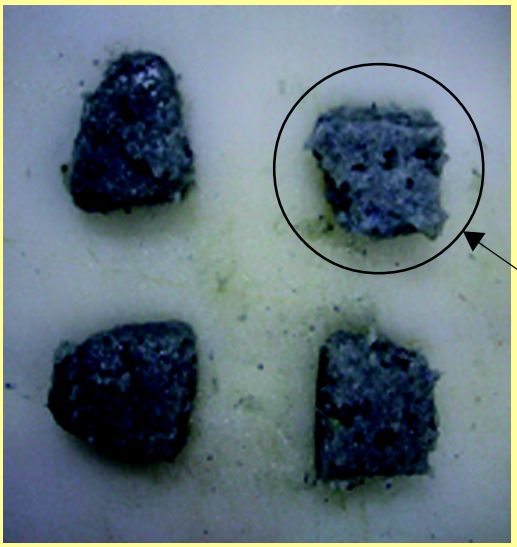
1. Place frozen shrimps sample at 5° C overnight. Remove the head and shell.



2. Mince the sample rapidly and thoroughly with chopper. Remove unground material from the blade of chopper and mix thoroughly with ground material and mince thoroughly again.

3. Turn the mince into the shape of a burger and divide into four equl portions.

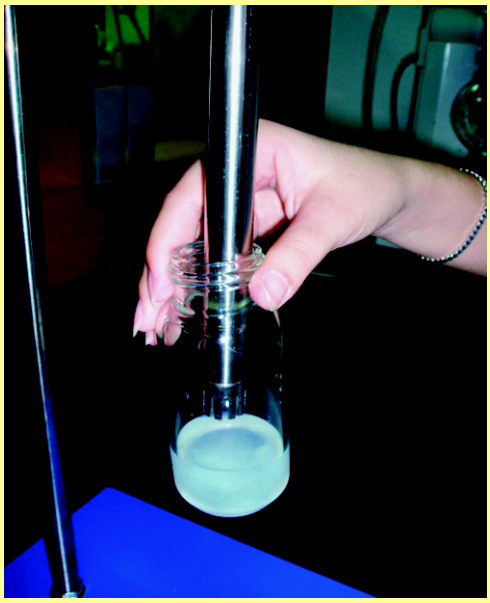




4. Take the first quadrant of minced meat for testing.

Procedure

1. Accurately weigh 5 g of the minced sample (edible portion of the tiger prawn, not de-veined) into a 150 ml glass ceentrifuge bottle. Spike test sample at this stage with 2 ml of 1 ppm working solution.



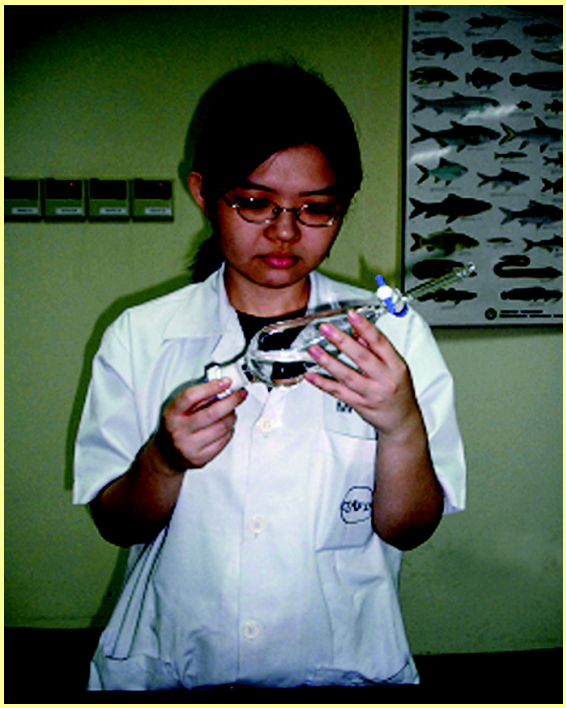
2. Add 25 ml of acetonitrile and 10 g of anhydrous soduim sulfate into the sample and homogenize for 1 min.

3. Centrifuge at room temperature for 5 min at 2,500 rpm.

4. Filter the supernatant through Whatman No.1 filter paper into a 125 ml separatory funnel containing 25 ml acetonitrile - saturated n-hexane.

5. Add another 25 ml of acetonitrile to the homogenate and sonicate for 30 sec. gently mixing the sample with a glass rod during this process.

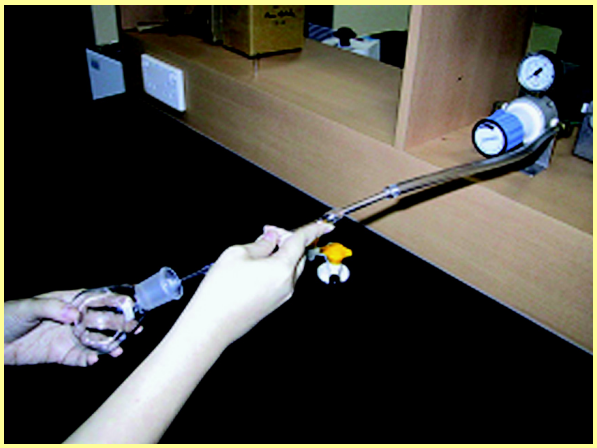
6. Centrifuge at room temperature for 5 min at 2,500 rpm and filter the supernatant into the earlier 125 ml separatory funnel (Step 4). Rinse the filter paper with acenotrile and allow washing to drain into separatory funnel



7. Shake flask vigorously for 10 min. Allow to stand for separation ito layers.

8. Slowly drain off the acetonitrile (lower) layer into a 100 ml florentine flask.

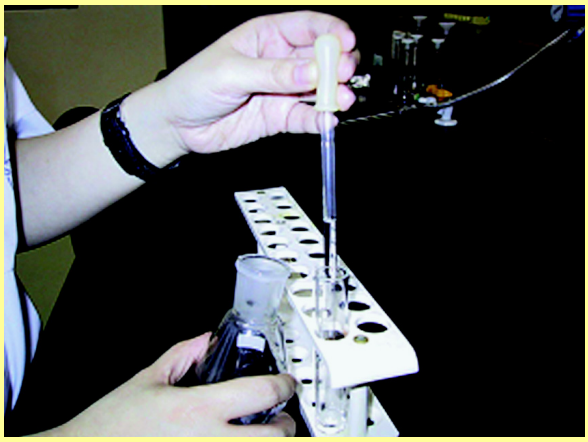
9. Add approximately 5 ml of 1-propanol (add more if mixture boils too vigorously) and evaporate the acetonitrile layer to dryness using the rotary evaporator at 40° C.



10. Flush flask with nitrogen gas to remove any trace of propanol.

11. Add accurately 2 ml of acetonitrile:water (3:7) and sonicate until the residue is dissolved.

12. Transfer the the sonicated solution into a 15ml glass centrifuge tube. Add 100 ml of soduim chloride and 1 ml of acetonitrile- saturated n-hexane and sonicate the mixture. At this stage, an orange-layer develops at the top. This is a sign of lipids moving on to the top n-hexane layer.



13. Centrifuge at room temperature for 5 min at 2,500 rpm.

14. Very carefully, using a pasteur pipette, pipette out the acetonitrile (lower, aqueous) layer into a plastic syringe (fixed with filter Chromatodisc 13P) and filter the extract into a glass vial.

15. The filtered sample is then ready for injection into HPLC. Inject 20 µl sample using a flow rate of 0.5 ml/min with detector excitation set at 337 nm and emission at 365 nm.

Calculation

Concentration of oxolinic acid in sample (ppm) =

$$\text{Std conc.(mg)} \times \frac{\text{Sample peak area}}{\text{Std. peak area}} \times \frac{\text{Final vol. of extract}}{\text{Sample weight}} \times F \times \frac{\text{Inj. vol.(sample)}}{\text{Inj.vol.(std)}}$$

where F = dilution factor = 1

Method Validation

Method validation was performed in compliance with regulations to ensure that the analytical methodology is accurate, specific, reproducible, and adaptable over the specified range of an analyte.

System suitability tests are used to verify that the resolution and reproducibility of the system are adequate for the analysis to be performed. The system suitability was checked by injecting 2 ppm of oxolinic acid standard for 10 times into the HPLC. Percentage relative standard deviation calculated from the 10 injections is 1.8 % (criteria: %RSD < = 2%). This indicates that the autosampler of HPLC is suitable for use.

All the criteria like Number of theoretical plates (N), Tailing factor (T), Precision (%RSD) and Resolution (R) were met. The results are shown in Table 1. The retention time of oxolinic acid is about 6.5 min. Analysis time per injection is only 9 min, which is practical. Thus, this shows that flow-rate, column length, and solvent composition are suitable for this application. Relative standard deviation of retention time between replicates is < 1%. T, was determined by calculating the asymmetrical factor derived from generated chromatogram. Calculated T was found to be about 1.6 (criteria: 0.5 < = T = < 2) and N, was 3306 (general criteria: N > = 2000). The N and T values indicate that the column used, TSK-GEL ODS-80 TM, is suitable for this application.

Table 1. System suitability of the HPLC-fluorescence detector system for oxolinic acid determination.

Parameters	Oxolinic acid*
Retention Time (min)	6.481± 0.018 %RSD = 0.28
Area	4697763 ± 84561 %RSD = 1.80
Height	229946 ± 4287 %RSD = 1.86
$W_{1/2}$ (min)	0.266 ± 0.008 %RSD = 2.90
A (min)	0.317 ± 0.000 %RSD = 0.00
B (min)	0.205 ± 0.013 %RSD = 6.15
Tailing Factor (min)	1.60 ± 0.10 %RSD = 6.00
Resolution (R)	7.41 ± 0.02 %RSD = 0.28
Theoretical Plates (N)	3306 ± 203 %RSD = 6.13

* Mean ± Standard Deviation

Criteria:

1. Number of theoretical plates, N >= 2000 [N=16(tR/W)^2]
2. Tailing Factor, 0.5<= T =<2
3. Precision RSD<= 2 % in 10 injections of the standard
4. Resolution, R>=2 between adjacent peaks [R = (tR1-tR2)/ 0.5(w1+w2)]

Calibration curves were generated using 0.2, 0.5, 1.0 and 2.0 ppm of the oxolinic acid standards. A good correlation (r = 0.999) was obtained between concentration and peak area.

Recovery tests were carried out for 7 blanks, 7 spiked blanks, and 7 spiked tiger prawn samples (spiked with 2mL of 1 ppm standard solution). (Table 2). No oxolinic residues were detected for the 7 blank samples. The recoveries for the 7 spiked blanks and 7 spiked tiger prawn samples ranged from 86.50 – 88.60%. The limit of quantification (LOQ) was 0.06 mg/g and the limit of detection (LOD) was 0.02 mg/g. The precision, expressed as percentage relative standard deviation, was below 4.53 %.

Table 2. Percentage recovery of oxolinic acid in spiked blanks and spiked tiger prawn samples.

Oxolinic Acid (2 µg per 5 g sample)	% Recovery*
Spiked blanks	86.50 ± 7.04
Spiked samples	88.60 ± 4.01

* Mean ± Standard Deviation

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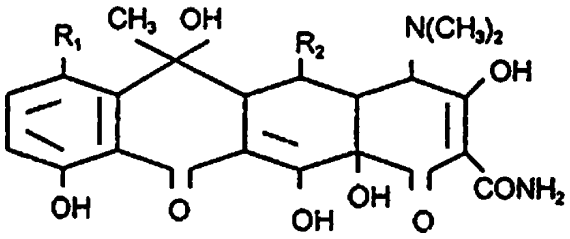
Guide to Food Hygiene Examination. 1993. Japan Food Hygiene Association.

Method Validation – A Laboratory Guide 1996, EURACHEM Working Group Secretariat, Laboratory of the Government Chemist, Teddington, Ltd. UK.

Determination of Oxytetracycline, Tetracycline and Chlortetracycline by HPLC - Flourescence Method

Principle

Tetracycline is widely used in the culture of fish as an antibacterial agent against various fish diseases such as vibriosis, furunculosis, etc., and is generally used along with other drugs or antibiotics. However, high dosage and improper use of antibiotics have resulted in levels of antibiotic residues that are higher than permitted, raising concern over the effects of antibiotics on the environment and on consumer health. The 3 main antibiotics under the tetracycline family that are commonly used as feed additives are oxytetracycline (OTC), tetracycline (TC) and chlortetracycline (CTC). The quantitative method used for the determination of these residues during this study was High Performance Liquid Chromatography (fluorescence detector method).



where

Oxytetracycline	:	R1 = H,	R2 = OH
Tetracycline	:	R1 = H,	R2 = H
Chlortetracycline	:	R1 = Cl,	R2 = H

Apparatus

a. Liquid Chromatography: WATERS Isocratic pump system, WATERS in-line degasser, 600E Multisolvent Delivery System, 600E System Controller, 717 Plus Autosampler equipped with 470 Scanning Fluorescence Detector capable of monitoring emission at 520 nm and excitation at 380 nm.

b. Chromatographic column: Reverse phase, TSK-GEL ODS-8OTM (150 x 4.6mm)

   Operating condition: Flow rate set at 1 mL/min.

   Injection volume: 20 µl

As a part of the system shut-down at the end of the experiment, HPLC grade water is pumped through the column for a minimum of 20 min followed by a 20-min rinse with methanol:water (7:3) at 0.5mL/min.

c. Sep-Pak C18 cartridge (WATERS) with 10 ml plastic syringe
d. Glass centrifuge bottle (150 mL) and tubes (15 mL)
e. Propylene centrifuge bottle (250 mL)
f. Florentine flask (500 mL and 100 mL)
g. Tissue homogenizer
h. Centrifuge
i. Rotary evaporator
j. Ultrasonic water bath

Reagents

a. Oxytetracycline.HCl Standard (Sigma)
b. Tetracycline.HCl Standard (Sigma)
c. Chlortetracycline.HCl Standard (Sigma)
d. Methanol, HPLC grade
e. Magnesium acetate, GR grade
f. Tetrahydrate salt of magnesium acetate, GR grade
g. Disodium salt of Ethylene Diamine Tetraacetic Acid (EDTA), GR grade
h. Monohydate salt of citric acid
i. Dihydrate salt of disodium hydrogen phosphate, GR grade
j. Petroleum ether, GR grade
k. 5% Disodium salt of Ethylene Diamine Tetraacetic Acid (EDTA): Dissolve 5 g EDTA in 100 mL pure Diamond Q water.
l. Mcllvaine buffer (pH 6.0): Prepare a 0.2 M solution of dihydrate salt of disodium hydrogen phosphate by dissolving 22.71 g of the solute in 800 mL of distilled water. Prepare a 0.1 M solution of monohydrate salt of citric acid by dissolving 10.50 g of the solute in 500 mL of distilled water. To the 500 mL of 0.1 M citric acid solution, slowly add 0.2 M solute of dihydrate salt of disodium hydrogen phosphate and adjust the pH of the buffer to 6.0.

m. 0.1 M EDTA in McIlvaine buffer (pH 5.5): Dissolve 37.224 g of disodium salt of EDTA in 1 L of McIlvaine buffer and check that pH is 5.5.

n. Extraction solution: Mix 0.1 M EDTA in McIlvaine buffer (pH 5.5) and methanol in ratio of 3:7 by volume. It is important to prepare the extraction solution fresh each time before use as white precipitates could form almost immediately, after mixing.

o. 1 M Imidazole buffer (pH 7.2): Dissolve 68.08 g imidazole, 10.72 g magnesium acetate and 0.37 g disodium salt of EDTA in 800 ml of HPLC grade water. Adjust to pH 7.2 using acetic acid and make up to 1 L with HPLC grade water.

p. Mobile Phase solution: Mix 1 M imidazole and HPLC grade methanol in the ratio of 80:20 by volume. It is very important to filter the Mobile Phase solution through a 0.45 µm Millipore filter.

q. Oxytetracycline, Tetracycline and Chlortetracycline (Sigma Chemical Company): All standard solutions are stored below 10° C. Stock solution is stable for at least 3 months, but diluted solutions should be kept no longer than 2 weeks.

Stock solution (1000 ppm): Weigh accurately 100 mg of oxytetracycline, tetracycline, and chlortetracycline standards into three separate 100 mL volumetric flask. Make up to volume with HPLC grade methanol:water (1:1) solution.

Intermediate solution (100 ppm): Pipette accurately 10 mL of the 1000 ppm stock solution into a 100 mL volumetric flask and make up to volume with HPLC grade methanol:water (1:1) solution.

Working solution (5 ppm): To get a mixed standard solution of oxytetracycline, tetracycline and chlortetracycline, pipette 5 mL of each of the three standards (intermediate solutions) to a 100 mL volumetric flask and make up to volume with HPLC grade methanol:water (1:1) solution.
It is best to prepare fresh working solution after one month.

Sampling Procedures

1. Place frozen shrimp sample in 5° C overnight.
2. Remove head and shell.
3. Mince the sample rapidly and thoroughly with a chopper.
4. Remove unground material from blade of chopper and mix thoroughly with ground material and mince thoroughly again.
5. Turn mince into the shape of a burger and divide into equal quarters.
6. Take first quadrant of minced meat for testing.

Procedure

1. Weigh 10 g of the minced sample (edible portion of tiger prawn, not de-veined) into a 250 ml plastic centrifuge bottle. (For spiked sample, spike with 2 ml of 5 ppm standard working solution).

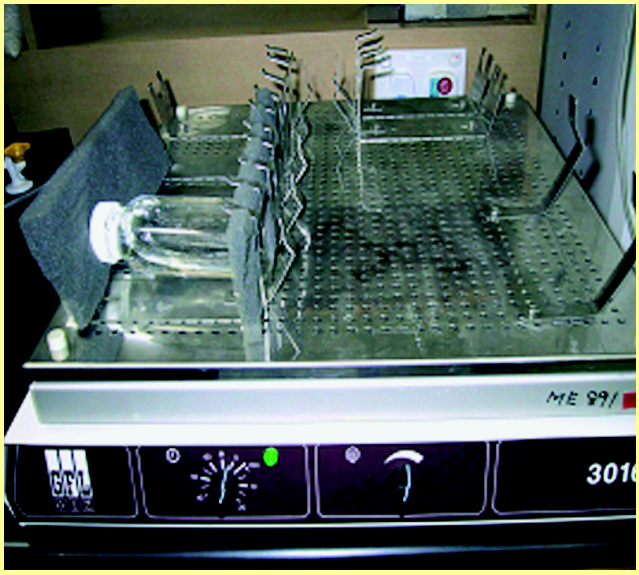
2. Add 60 ml of the freshly prepared extraction solution into the centrifuge bottle and homogenize for 1 min.

3. Centrifuge for 5 min at 3,000 rpm.

4. Filter supernatant through Whatman filter paper, No. 41, into a 500 ml florentine flask.

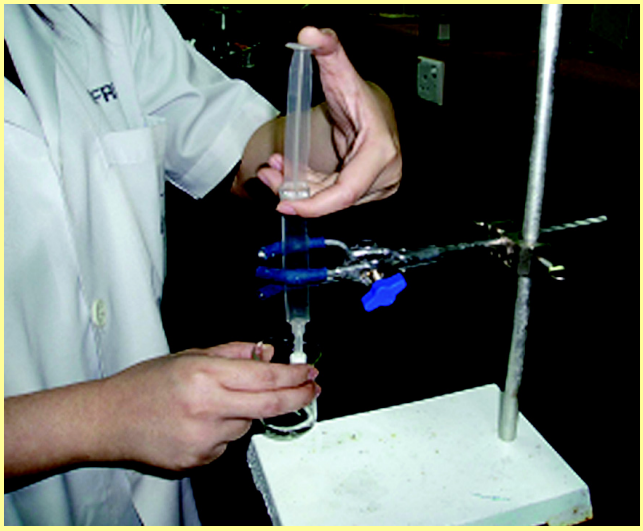
5. Wash residue with another 30 ml of extraction solution and sonicate for 30 sec. Gently mix the sample during this process using a glass rod. Centrifuge for 5 min at 3,000 rpm and filter the supernatant into the same florentine flask.

6. Rinse filter paper twice, with 10 ml of extrraction solution each time. Evaporate pooled filtrate to approximately 5 ml, in a water bath set at 40° C.



7. Transfer the concentrated filtrate to a 150 ml centrifuge bottle. Rinse florentine flask with 20 ml of Milli-Q water and pool rinse into the same centrifuge bottle containing the filtrate. Add approximately 20 ml of petroleum ether and shake the mixture for 10 min.

8. Centrifuge for 5 min at 3,000 rpm. Use Komagome pipette to remove most of the petroleum (upper) layer, leaving behind only aqeous layer.



9. To condition the Sep-pak C18 cartridge, attach it to the glass syringe at one end, followed by the following washing steps:
   - Inject the cartridge with 10 ml of methanol and discard the eluate.
   - Elute with 10 ml of Milli-Q water and discard the eluate.

10. Transfer the aqeous layer (from Step 8), into the plastic syringe attached with the conditioned Sep-Pak C18 cartridge (from Step 9). This is the absorption stage of any tetracycline residue to the cartridge.

11. Elute the tetracycline residues with 30 ml of methanol at a flow rate of 1.5 ml/min. Collect the eluate in a 100 ml florentine flask.

12. Evaporate the eluate to complete dryness using the rotary evaporator at 40° C and by flushing with nitrogen gas.

13. Add 2 ml of mobile phase to the dried residue. Sonicate until clear before injecting into the HPLC.

Calculation

Concentration of tetracycline in sample (ppm):

$$\text{Std conc.(mg)} \times \frac{\text{Sample peak area}}{\text{Std. peak area}} \times \frac{\text{Final vol. of extract}}{\text{Sample weight}} \times F \times \frac{\text{Inj. vol.(sample)}}{\text{Inj.vol.(std)}}$$

where F = dilution factor = 1

Method Validation

The system suitability was checked by injecting a 5ppm mixed standard (oxytetracycline, tetracycline, and chlortetracycline) for 10 times into the HPLC.

System suitability is the checking of a system to ensure performance before or during the analysis of unknowns. Parameters such as tailing factors, resolution and reproducibility (%RSD retention time and area for replicates) were determined and compared against the specifications set for the method.

All the criteria like Number of theoretical plates (N), Tailing factor (T), Precision (%RSD) and Resolution (R) were met. The results are shown in Table 3. The standard solution at 5 ppm produced 3 distinct peaks within 25 min after injection. This shows that flow-rate, column length and solvent composition is suitable for this application. Resolution between oxytetracycline and tetracycline peaks is 2.0 and at least the same for chlortetracycline with reference to either oxytetracycline or tetracycline (Table 3). The % RSD for retention time ranged from 0.27 to 0.78. The number of theoretical plates and tailing factor indicate that the column used, TSK-GEL ODS-8OTM, is suitable for this application.

Table 3. System suitability of the HPLC-fluorescence detector system for OTC, TC and CTC determination

Parameters	OTC*	TC*	CTC*
Retention Time (min)	5.063 ± 0.013 %RSD = 0.27	7.777 ± 0.025 %RSD = 0.32	23.950 ± 0.187 %RSD = 0.78
Area	3014317 ± 56586 %RSD = 1.88	3422706 ± 50176 %RSD = 1.47	1271583 ± 31481 %RSD = 2.48
Height	143573 ± 15163 %RSD = 1.06	137345 ± 1590 %RSD = 1.16	21901 ± 359 %RSD = 1.64
$W_{1/2}$ (min)	0.26 ± 0.01 %RSD = 3.70	0.342 ± 0.026 %RSD = 7.55	0.87 ± 0.02 %RSD = 2.34
A (min)	0.32 ± 0.04 %RSD = 11.58	0.37 ± 0.02 %RSD = 6.15	1.06 ± 0.11 %RSD = 10.00
B (min)	0.51 ± 0.03 %RSD = 6.04	0.39 ± 0.03 %RSD = 7.53	0.88 ± 0.14 %RSD = 15.58
Tailing Factor (min)	1.63 ± 0.26 %RSD = 15.87	1.07 ± 0.14 %RSD = 13.39	0.84 ± 0.18 %RSD = 21.15
Resolution (R)	4.78 ± 0.16 %RSD = 3.45	5.65 ± 0.15 %RSD = 2.68	72.19 ± 3.57 %RSD = 4.95
Theoretical Plates (N)	2139 ± 168 %RSD = 8.67	2700 ± 234 %RSD = 7.86	3523 ± 340 %RSD = 9.66

* Mean ±Standard deviation

Criteria

1. Number of theoretical plates, N >= 2000 [N=16(tR/W)^2]
2. Tailing Factor, 0.5<= T =<2
3. Precision RSD<= 2 % in 10 injections of the standard
4. Resolution, R > =2 between adjacent peaks [R = (tR1-tR2)/ 0.5(w1+w2)]

The calibration curves were generated for individual components using 0.5, 2.0, 5.0 and 10.0 ppm of the individual standards. A good correlation (r = 0.999) was obtained between concentration and peak area.

Table 4. Correlation between concentration and peak area

Antibiotics	Coefficient of correlation, r
Oxytetracycline	0.999
Tetracycline	0.999
Chlotetracycline	0.999

Recovery tests were carried out for 7 blanks, 7 spiked blanks, and 7 spiked tiger prawn (spiked with 1 ug/g standard solution). No tetracycline residues were detected for the 7 blank samples. The recoveries for the 7 spiked blanks and spiked tiger prawn were satisfactory. The percentage recoveries for OTC, TC, and CTC were 78.62%, 79.89%, and 68.26% respectively (Table 5).

Table 5. The mean percentage recovery of 7 spiked tiger prawn samples

Antibiotics (10 ug per 10 g sample)	% Recovery*
OTC	78.62 ± 1.44
TC	79.89 ± 0.54
CTC	68.26 ± 2.14

* Mean ± Standard Deviation

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CHAPTER 2

Detection of Pesticide Residues in Aquaculture Products

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INTRODUCTION

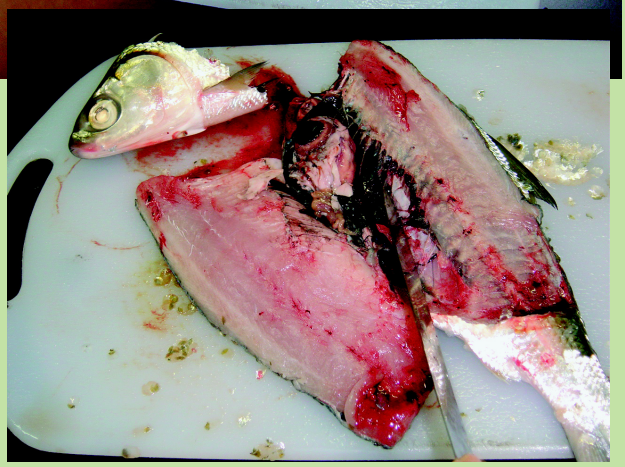
Agricultural chemicals such as fertilizers and pesticides have made an important contribution to agriculture. Pesticides protect crops from pests and diseases. They have brought about large yield increases, and have helped ensure that the rise in food production has kept well ahead of the rise in population. However, there is a growing concern about the safe use of these chemicals, and the potential dangers to farmers who use them, the environment, and consumers. There is particular concern about pesticides, since almost all chemicals that can kill pests are also potentially damaging to human health.

Legislation requires that pesticide use is appropriately controlled and maximum residue levels (MRLs) not be exceeded. The level of pesticide residues in food raw materials is a measurable standard. But while residue analysis is essential for companies wishing to assure themselves that their products have been produced in accordance with best practice and within the law, it can be used to greatest effect when targeted at samples most likely to contain residues.

Reliable residue analytical methods are necessary to measure the magnitude of residue in a seafood, and to enforce legal residue limits (tolerances). Sample preparation and extraction, clean up of extracts and pesticide detection are the main procedures in pesticide residue analysis. There is an interplay among these factors which should be considered in the choice of a particular method.

Preparation of Samples

The fish sample is scaled (if scaly) and the head removed. Using a stainless knife, the flesh and other edible portions are removed from the bone and entrails. These edible portions are cut into small cubes or pieces and frozen until analyses.



Head and shells of tiger shrimp samples are removed and edible portions separated. These edible portions are cut into small cubes or pieces and frozen until analysed.

Mollusks (oysters, clams and mussels) are shucked or de-shelled (snails) and meat are separated and cut into small pieces and frozen until analysed.

Oysters

Snails

Clams

Seaweed samples are chopped using a knife or cut into small pieces using a pair of scissors and frozen until analysed.

The frozen samples are allowed to thaw under room temperature and then ground in a meat chopper. The ground samples are thoroughly mixed and representative samples are taken for analysis.

Multi-residue Method

Principle

The sample is blended with anhydrous sodium sulfate to disintegrate the solid and for it to combine with water in the sample. Fat is extracted from sample using petroleum ether. Extracts are purified by petroleum ether-acetonitrile partitioning, followed by chromatography on Florisil column. For some fractions, further clean up in Magnesia column may sometimes be needed. Pesticide residues in concentrated eluates are measured by gas chromatography.

Reagents

a.) Acetonitrile (CH_3CN) - Some lots of reagent grade CH_3CN are impure and require distillation. Generally vapors from such lots will turn moistened red litmus paper blue when held over the mouth of a storage container. Purify CH_3CN as follows: To 4 L CH_3CN, add 1 mL phosphoric acid (H_3PO_4), 30 g phosphorus pentoxide (P_20_5) and boiling chips, and distill in all glass apparatus at 81-82^0 C. Do not exceed 82^0 C.

b.) Acetonitrile saturated with petroleum ether – Saturate CH_3CN with redistilled petroleum ether

c.) Methanol – UCP, reagent grade or ACS

d.) Alcoholic alkali solution (2%) - Dissolve 2 g potassium hydroxide (KOH) in methanol, and dilute to 100 mL.

e.) Eluting solvent (6%)- Dilute 60 mL ethyl ether to 1 L with redistilled petroleum ether.

f.) Eluting solvent (15%)- Dilute 150 mL ethyl ether to 1 L with redistilled petroleum ether.

g.) Eluting solvent (50%)- Dilute 500 mL ethyl ether to 1 L with redistilled petroleum ether.

h.) Ethyl ether- Redistilled at 34-35° C, and stored under nitrogen (N_2). Must be peroxide-free by test.

i.) Florisil, 60/100 PR grade, activated at 675° C, available from Floridin Co., 3 Pennsylvania Center, Pittsburgh, PA 15235. When 675 ° C activated Florisil is obtained in bulk, transfer immediately after opening to 500 mL glass jars or bottles, with glass-stoppered or foil-lined, screw-top lids, and store in the dark. Heat for 5 h or more at 130° C before use. Store at 130° C in glass stoppered bottles or in air-tight dessicator at room temperature and reheat at 130°C after 2 days.

j.) Hexane-Reagent grade, redistilled in all glass distillation apparatus

k.) Magnesium oxide (MgO) - Adsorptive magnesia (Fisher Scientific Co. No. S-120). Treat as follows: Mix 500 g MgO with water to form a slurry. Heat on steam bath for about 30 min, and filter with suction. Dry overnight at 105-130° C and pulverize to pass No. 60 sieve. Store in jar with cover.

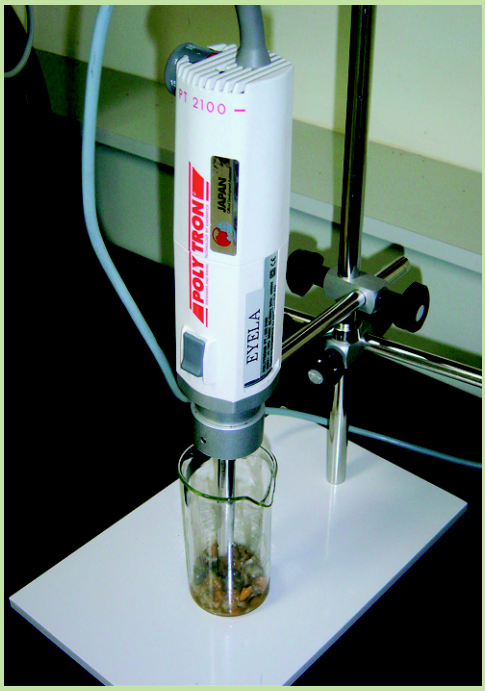
l.) Magnesia-Celite mixture- Mix treated MgO with Celite 545 (1:1, w:w).

m.) Petroleum ether- Reagent grade, redistilled in all glass distillation apparatus at 30-60° C.

n.) Sodium sulfate- Anhydrous, granular

Extraction

1. Weigh 25-50 g of thoroughly ground and mixed sample in a homogenizing beaker.



2. Homogenize the sample in high speed blender or homogenizer until thoroughly mixed.

3. Add 100 g anhydrous sodium sulfate ($Na_2 SO_4$) to combine with water and to disintegrate sample

4. Mix with a spatula and blender until well mixed. Scrape down the sides of blender jar and break up the caked material with a spatula.

5. Add 150 ml petroleum ether and blend at high speed for 2 min.

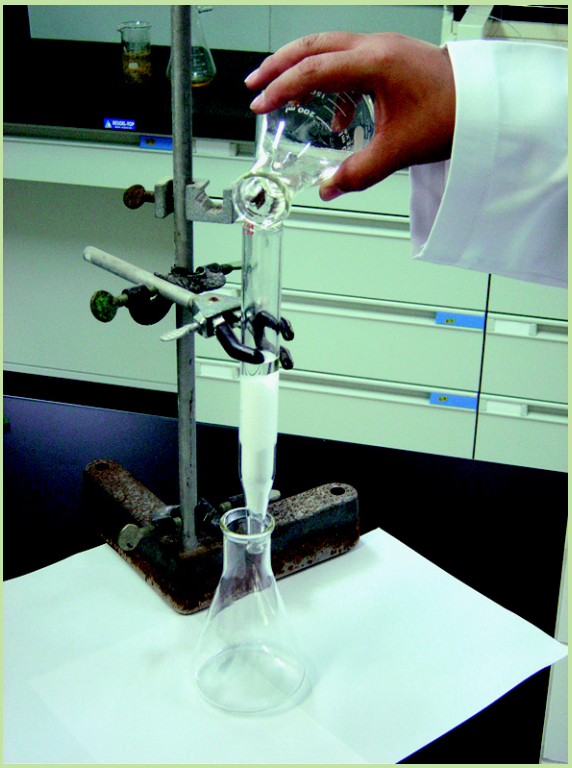
6. Decant petroleum ether supernatant into a glassi fritted funnel with filter paper, into a 500 ml flask fitted with a suction apparatus.

7. Extract residues in blender with two additional 100 mL portions of petroleum ether, blending 2 min each time.

8. Decant petroleum ether supernatants through fritted funnel and combine with the first extract.

9. After last blending, transfer residue from blender jar to funnel, rinsing blender jar and materials in funnel with several small portions of petroleum ether.

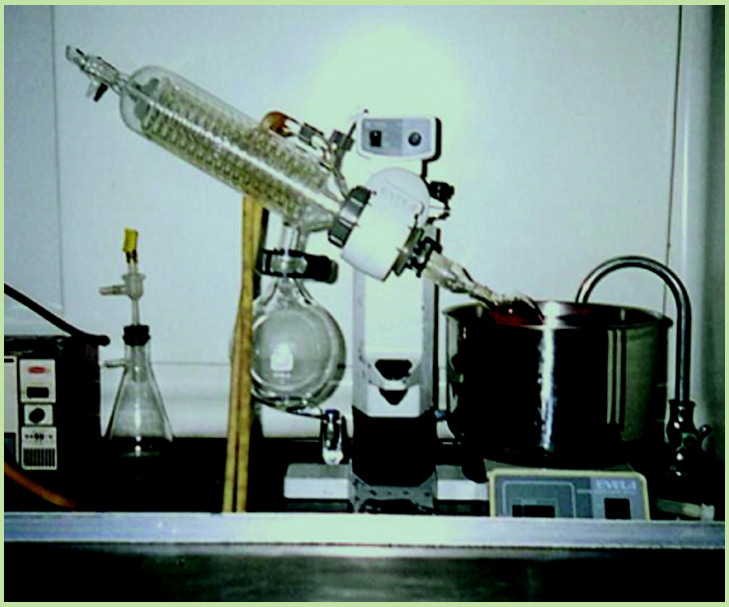
10. Pour combined extracts and rinses into a column (25 mm x 50 mm long) of anhydrous Na_2SO_4 and collect the petroleum ether extract.



11. Evaporate most of petroleum ether in a Kuderna-Danish concentrator.

12. Transfer the fat solution to tared pear-shaped flask using small amount of petroleum ether.

13. Evaporate petroleum ether at steam bath temperature using a rotary evaporator to obtain fat.



14. When petroleum ether is completely removed, weigh and record weight of fat extracted.

15. Take about 3 g fat for ether-acetonitrile partitioning.

16. Record weight of fat taken for clean-up using the formula:

$$\text{Wt. sample analyzed} = \frac{\text{(wt. fat for clean-up)}}{\text{(wt. fat extracted)}} \times \text{(wt. original sample)}$$

Clean-up

a.) Petroleum ether-Acetonitrile Partitioning

1. Weigh about 3 g fat into a 125 mL separatory funnel.
2. Add 15 mL petroleum ether and 30 mL acetonitrile saturated with petroleum ether.

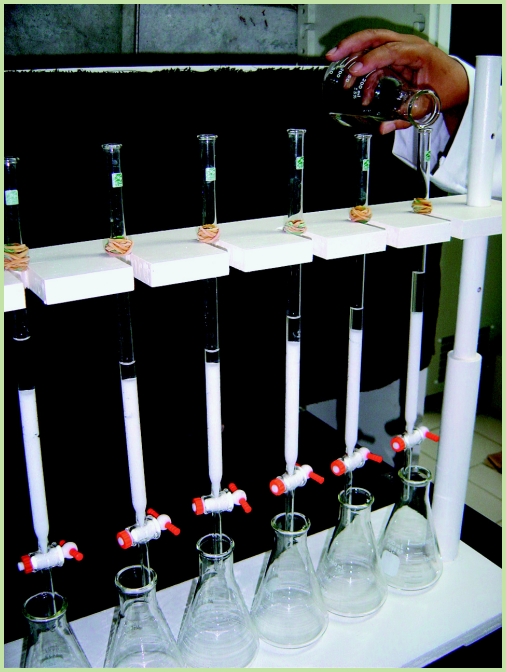
3. Shake the funnel vigorously for 1 min and allow the layers to separate.
4. Drain the acetonitrile into a 1 L separatory funnel containing 650 mL distilled H_20, 40 mL saturated NaCl solution, and 100 mL petroleum ether. Set aside.
5. Extract the petroleum ether layer in 125 mL separatory funnel with 3 additional 30 mL portions of acetonitrile saturated with petroleum ether, shaking vigorously 1 min each time and combine all extracts in the 1L separatory funnel.
6. Hold the 1 L separatory funnel in horizontal position and mix thoroughly for 30-45 sec. Allow the layers to separate and drain the aqueous layer into another 1 L separatory funnel.
7. Add 100 mL petroleum ether to the second 1 L separatory funnel, shake vigorously for 15 sec and allow the layers to separate. Discard the aqueous layer, combine petroleum ether extract with petroleum ether in the first separatory funnel and wash with two 100 mL portions of water. Discard the washing.

8. Drain the petroleum ether layer through a column (25 mm x 50 mm long) of anhydrous Na_2SO_4 into a 500 mL Kuderna-Danish concentrator.

9. Evaporate the extract to 5-10 mL in Kuderna-Danish concentrator and transfer the extract to a Florisil column.

b.) Florisil Column Clean-up

1. Prepare a column (10 mm i. d. x 120 mm long) and fill it with 4 g activated Florisil topped with anhydrous Na_2SO_4. to about 2 cm. Pre-wet the column with 40-50 mL petroleum ether.



2. Transfer the petroleum ether solution of sample extract to the column. Rinse the container with two, 5 mL portions of petroleum ether and transfer rinsings to column.

3. Elute the column at about 5 mL/min with 200 mL of 6% ethyl ether/petroleum ether eluant.
4. Change the receiver and elute at about 5 mL/min with 200 mL of 15% ethyl ether/petroleum ether eluant.
5. Do step #4 again, this time using 50% ethyl ether/petroleum ether eluant.
6. Concentrate each eluate to a suitable definite volume (1-2 mL) in a Kuderna-Danish concentrator.
7. The concentrated eluate is now ready for injection into a gas chromatograph.

Notes: The first eluate (6%) contains organochlorine pesticides (aldrin, BHC, DDE, DDD, o,p'- and p,p'-DDT, heptachlor, heptachlor epoxide, lindane, methoxychlor, mirex, and ethylan), industrial chemicals (polychlorinated biphenyls (PCB) and organophosphorus pesticides (ethion and ronnel) and is usually suitable for gas chromatography directly.

The second eluate (15%) contains organochlorine pesticides (dieldrin and endrin) and organophosphorus pesticides (diazinon, methyl parathion and parathion).

The third eluate (50%) contains organophosphorus pesticide (malathion).

c.) Magnesia Column Clean-up

(Applicable only to organochlorine pesticides in 15% eluate when additional clean-up is necessary).

1. Transfer about 10 g Mg0 celite mixture to a chromatographic tube without stopcock and using the vacuum to pack.

2. Pre-wash the column with about 40 mL petroleum ether, discard pre-wash, and place a Kuderna-Danish concentrator under the column.

3. Transfer the15% Florisil eluate which has been concentrated to about 5 mL, to column, rinsing with small portions of petroleum ether.

4. Elute with 100 mL petroleum ether.

5. Concentrate the eluate to a suitable volume (1-2 ml). This is now ready for injection into a gas chromatograph.

Detection and Quantitation by Gas Chromatography

Detection method is the process of identifying the pesticides and determining their concentrations. With the aid of an analytical instrument, such as a gas chromatograph (GC), we can obtain information about the original sample by running a standard solution containing the pesticide(s) at a known concentration and comparing it with the sample. In order for a sample to run through the GC, the pesticide must be extracted from the sample into a high purity solvent. This extracted portion is referred to as the sample extract. Upon entering the GC, the extract is vaporized in a thermally controlled injection port. The vapor containing the pesticide then passes into a temperature-and pressure-controlled oven within the GC where it is separated in the column. The vapor enters the column and is eventually transported, with the help of a carrier gas, to a thermally controlled detector which detects the pesticide as it flows through it. The time it takes for the pesticide to move through a column and reach the detector is its retention time and the same pesticide will always have the same retention time on the same column. All data transmitted from the detectors is collected and stored on a computer which compiles the data into a chromatogram as the GC is running.

Gas Chromatographic Conditions

Gas chromatograph Model: Shimadzu GC-17A, equipped with ^{63}Ni-electron capture detector, attached to a CBM-102 Chromatopak recorder system

Detector: Electron capture detector (ECD)

Column: SPB-608 (Supelco), Capillary (Fused silica), 30m x 0.25mm I. D., 0.25 µm film

Column Oven Temperature: 150° C (4 min) to 290° C at 8° C/min, hold 10 min.

Detector temperature: 300° C

Injector temperature: 220 ° C

Carrier gas: Nitrogen (N_2) at flow rate of 40 cm/sec

Procedure

1. Study the operating manual of the gas chromatograph available in your laboratory. Turn on and set the specified chromatographic conditions.
2. Inject a suitable aliquot (3-8 µL) of concentrated eluate from Florisil or MgO-celite column containing an amount of compound within the linear range into the gas chromatograph.
3. Tentatively identify the residue peaks on basis of retention times.
4. Measure the area or height of residue peak(s) and determine the residue amount by comparison to peak area or height obtained from a known amount of appropriate reference material(s).
5. To ensure valid measurement of residue amount, the size of peaks from residue and reference standard should be within ± 25%.
6. Chromatograph reference material(s) immediately after samples.

Determination of pesticide residues in non-fatty samples

Principle

Non-fatty samples are blended with acetone and filtered. Extracts are transferred from aqueous filtrate to organic phase by shaking with petroleum ether and methylene chloride.

Reagents

a.) Solvents: acetone, methylene chloride (CH_2Cl_2), petroleum ether- redistilled in glass

b.) Sodium sulfate- Anhydrous, granular

c.) Glass wool- Rinse with acetone and alcohol several times and dry. Washed glass wool will be somewhat brittle.

d.) Reference standards- Prepare all standards in glass-distilled acetone

Extraction

1. Weigh 100 g chopped sample into high-speed blender jar and add 200 mL acetone. Blend for 2 min at high speed.

2. Filter with suction through a Buchner funnel fitted with filter paper and collect the extract in 500 mL suction flask.

3. Place 80 mL sample extract in 1 L separatory funnel, and add 100 mL petroleum ether and 100 mL CH_2Cl_2. Shake vigorously for 1 min and let stand to allow layers to separate.

4. Transfer lower aqueous layer to a second 1 L separatory funnel. Set aside.

5. Dry the upper organic layer in first separatory funnel by passing through 1 ½ inch column of Na_2SO_4 supported on washed glass wool in 4 inch funnel, and collect the eluate in 500 mL Kuderna-Danish concentrator fitted with volumetric flask or calibrated receiving tube.

6. To the aqueous phase in Step 4, add 7 g NaCl and shake vigorously until most NaCl is dissolved. Add 100 mL CH_2Cl_2, shake for 1 min, and dry lower organic phase through same Na_2SO_4 column.
7. Extract the aqueous phase again with additional 100 mL CH_2Cl_2 and dry as above. Rinse Na_2SO_4 with 50 mL CH_2Cl_2.
8. Attach a Snyder column on Kuderna-Danish concentrator (boiling chips may be added) and start evaporation slowly by placing only receiver tube into steam.
9. After 100-150 mL has evaporated, concentrator may be exposed to more steam and concentrated further to about 2 ml.
10. Add 50 mL petroleum ether and repeat concentration step.
11. Add 20 mL acetone and reconcentrate to about 2 ml.

Note: Do not let solution go to dryness during any concentration step.

12. Adjust the volume of extract to suitable definite volume with acetone.
13. Inject a measured amount into the GC.

Calculation of Equivalent Sample Weight

$$\frac{\text{mg sample equivalent}}{\text{uL final extract}} = 100 \times \frac{80}{200 + W - 10} \times \frac{1}{\text{mL final volume}}$$

where: 200 = mL acetone blended with 100 g sample
W = amount (mL) water present in original sample
10 = adjustment for water-acetone volume contraction

Note: Thus, when sample contains 85% water (85ml/100g) and final extract volume is 7 mL, each uL contains:

$$100 \times \frac{80}{200 + (85-10)} \times \frac{1}{7} = 4.15 \text{ mg sample equivalent/uL final extract}$$

Determination of Polychlorinated Biphenyl Residues

Extraction

1. Weigh 20 g of thoroughly ground and mixed sample.
2. Moisten 40 g granular Na_2SO_4 with petroleum ether and add to the sample. Mix and let stand for 20 min and mix again.
3. Add 100 mL petroleum ether to the sample and blend for 1-2 min. Let stand for 10 min.
4. Plug funnel with glass wool, overlay with 20 g granular Na_2SO_4, and place funnel in 250 mL volumetric flask. Decant petroleum ether extract through Na_2SO_4 into the volumetric flask.
5. Repeat the extraction of the sample using 100 mL petroleum ether.
6. Repeat extraction using 70 mL petroleum ether.
7. Dilute the filtrate by the addition of petroleum ether so that the total final volume is 250mL.
8. Transfer 25 mL aliquot to a tared pear-shaped flask and evaporate the solvent in a rotary evaporator.
9. Weigh the flask and determine % fat.
10. For fish containing <10% fat, transfer a 25 mL aliquot to 125 mL K-D concentrator.
    For fish containing > 10% fat, take an aliquot to contain not > 200 mg fat.
11. Concentrate the sample to about 3 ml on a steam bath and transfer to a Florisil column.

Clean-up (Florisil Column)

1. Place 4 g Florisil into (120 x 10 mm id) chromatographic tube. Add anhydrous Na_2SO_4 to a height of 2 cm above the Florisil.
2. Open the stopcock fully, tap the tube to allow the adsorbent to settle, and mark the tube at 1 cm above the Na_2SO_4 layer.

3. Wash the Florisil column with 20-25 mL petroleum ether.

   (Note: Solvent level must not go down below the 1-cm mark.)

4. Place 125 mL K-D flask under the column.
5. Using a disposable Pasteur pipet, transfer 3 mL sample to column. Temporarily close stopcock if necessary.
6. Add 35 mLpetroleum ether-ether mixture (94 + 6) and elute.
7. Concentrate the eluate to about 2 mL.
8. Inject measured amount into GC.

Determination of Carbamate Residues

Principle

The carbamate group of pesticide residues includes carbonolate, carbaryl (Sevin), carbofuran, and propoxur (Baygon). The residue is extracted from the sample with acetonitrile. The extract is purified by partitioning with petroleum ether and coagulating in a H_3PO_4-NH_4Cl solution. Phenolic impurities are largely eliminated by partitioning CH_2Cl_2 extract with KOH solution. Carbamate residues are treated with 1-fluoro-2,4-dinitrobenzene to form their corresponding derivatives.

Reagents

a.) Borax - 5% aqueous solution. Dissolve 5 g in 95 mL water.

b.) Diatomaceous earth - Wash thoroughly with acetone and dry for 2 h at 110° C.

c.) Coagulating solution:

Stock solution- Dissolve 20 g NH_4Cl and 40 mL H_3PO_4 in 360 mL distilled water.

Working solution- Dilute 100 mL stock solution to 1 L for coagulation.

d.) 1-fluoro-2,4 - dinitrobenzene solution - Redistill at 128° C and 1 mm pressure. Dissolve 1.5 mL in 25 mL acetone.

e.) Pesticide standards - Best quality obtained from manufacturer, analytical grade when available

f.) Potassium hydroxide solution - 0.5N aqueous solution

g.) Sodium chloride solution - 30% aqueous solution

h.) Solvents - acetone, methylene chloride, isooctane, acetonitrile and petroleum ether distilled in glass, acetophenone, and methanol (analytical grade)

Extraction and Clean-up

1. Weigh 100 g sample in a beaker and add 200 mL CH_3CN.
2. Homogenize in blender operated at moderate speed for 2 min.
3. Filter with suction into 500 mL round-bottom flask through filter paper in 12 cm Buchner funnel.
4. Transfer 100 mL aliquot into 250 mL separatory funnel.
5. Add 25 mL NaCl solution and shake.
6. Drain and discard the aqueous phase and repeat the treatment with fresh NaCl solution.
7. Add 100 mL petroleum ether, and shake for 30 sec.
8. Drain CH_3CN into 1 L separatory funnel.
9. Strip petroleum ether by shaking for 20 sec with 50 and 10 mL portions of CH_3CN, draining each into the 1 L separatory funnel.
10. Add 300 mL distilled water, 25 mL NaCl solution, and 50 mL methanol.
11. Extract the mixture with 100 mL and two 25 mL portions of CH_2Cl_2, shaking each for 20 sec.
12. Drain the lower layer into 500 mL round-bottom flask.
13. Add 2 drops acetophenone, and evaporate in rotary evaporator under reduced pressure.

Note: During evaporation keep water bath within 40-50^o C range and remove flask from water bath when the volume of the extract has been reduced to a few ml, so that final evaporation to dryness takes place at low temp.

14. Add 5 mL acetone, and swirl the flask to dissolve residue.
15. Add 50 mL coagulating solution, and swirl to mix.
16. Add 1-2 g diatomaceous earth, and swirl again to mix.
17. Pour the solution into a 150 mL suction filter of medium porosity and collect the filtrate in a 500 mL round-bottom flask.
18. Rinse the sides of flask with 5 mL acetone, swirl and repeat the coagulation.
19. When the filtration is completed, transfer the filtrate to 250 mL separatory funnel.
20. Extract the carbamate residue by shaking for 20 sec with three 25 mL portions of CH_2Cl_2.
21. Drain the CH_2Cl_2 (lower layer) extract into another 250 mL separatory funnel. The solution may be kept overnight at this point.
22. Add 40 mL distilled water and 10 ml 0.5 N KOH, mix by gently and briefly swirling the flask, and shake for 20 sec.
23. Drain the CH_2Cl_2 layer through granular anhydrous Na_2SO_4 supported by glass wool in a filter funnel, and collect the filtrate in a 250 mL Erlenmeyer flask.
24. Add 100 mL distilled water, 2 mL 0.5 N KOH and 1 mL 1-fluoro-2,4-dinitrobenzene solution. Stopper, and mix for 20 min at high speed on a mechanical agitator.
25. Add 10 mL 5% borax, swirl to mix, and heat on a steam bath for 20 min.
26. Cool to room temperature by placing the flask in shallow water bath for 10 min.
27. Add 5 mL isooctane, stopper, shake for 3 min at high speed, and pour into 250 mL separatory funnel.
28. Drain the aqueous phase and rinse twice with distilled water.
29. Drain the isooctane solution into a funnel containing a 6 mm glass wool plug and into a test tube. Cover the test tube with a glass stopper. The solution may be kept overnight at this point.
30. Inject 10 µL sample into a gas chromatograph. If it is necessary to dilute the sample, transfer 1 ml of the isooctane extract to another test tube, dilute to exact volume with isooctane, and shake to mix.

Method Validation

The recovery of each pesticide residue was determined by spiking tiger shrimp or fish muscle tissue with 0, 5, 10, 20, 30, 40, 50, 100, 150 and 200 ng/g of each pesticide standard, and extracting the samples as described previously. Each determination was replicated 5 times. The recoveries ranged from 75 to 99%. Precision, expressed as percentage relative standard deviation, was below 2.5%.

Limits of Detection

A method detection limit (MDL) was determined by running and analyzing a series of quality control samples (EPA STANDARDS, Spikes) to determine the lowest concentration of the pesticide that can be consistently and reliably measured using a given instrument. A spike is a standard containing the pesticide at a known concentration and goes through the entire extraction process like a real sample. Since spikes mimic real samples, we can measure the efficiency of the extraction method as well as the instrument performance and establish the (MDL) of the pesticide in various sample media. Laboratories should not report a pesticide at a concentration lower than the method detection limit.

An instrument detection limit (IDL) is determined by running a series of low level standards. The IDL gives us an idea of the lowest concentration of the pesticide residue that can be detected by the instrument. The IDL is consistently lower than the MDL because there is no extraction involved. We only use IDLs to monitor the instrument, but the IDLs have little relevance to the concentration of the actual sample extract.

The MDLs of 29 pesticide residues established using the methods described previously are summarized in the table next page:

Method Detection Limit (MDL) of 29 Pesticide Residues

Pesticides	MDL µg/g	Pesticides	MDL µg/g
Aldrin	0.075	Endrin ketone	0.024
a- BHC	0.025	Heptachlor	0.010
b- BHC	0.010	Heptaclor epoxide	0.015
g- BHC	0.010	Methoxychlor	0.050
d- BHC	0.015	Chloroneb	0.50
a-Chlordane	0.0015	Chlorothanonil	0.025
g- Chlordane	0.0015	Etridiazole	0.025
p.p' DDD	0.0025	Propachlor	0.050
p,p' DDE	0.010	Trifluralin	0.025
p.p' DDT	0.060	Simazine	0.50
Dieldrin	0.020	Aldicarb	1.0
Endosulfan I	0.015	Carbaryl (Sevin)	2.0
Endosulfan II	0.015	Carbofuran	1.5
Endosulfan sulfate	0.015	Propour (Baygon)	1.0
Endrin	0.025		

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