Quality Management for
Aquacultured Shrimp
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ASEAN-CANADA FISHERIES POST-HARVEST TECHNOLOGY PROJECT - PHASE II

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The ASEAN-Canada Fisheries Post-Harvest Technology Project - Phase II started in April 1992. The Project's objectives are to strengthen and upgrade fisheries product quality and fish inspection services within ASEAN countries; to assist in the development and implementation of improved methods and technologies in fish processing, preservation and packaging, on the basis of regional collaborative efforts, to enhance the transfer/adoption of appropriate technologies to the fish processing industries through training and extension services.

The Project activities are coordinated and administered by the ASEAN Executing Agency (AEA) which is incorporated in the Marine Fisheries Research Department (MFRD) of the Southeast Asian Fisheries Development Centre (SEAFDEC). In cooperation with the ASEAN governments, the Project established regional centres for fish processing technology (RC-FPT, Singapore), fish inspection and quality control (RC-FIQC, Indonesia), and information preparation and dissemination (RC-IPD, Malaysia) and developed work programs of national importance and regional interest for all ASEAN countries.

Each ASEAN country except Malaysia conducts two activities on either seafood processing or quality control in order to develop technical training manuals/materials and assist the RC-IPD in the production of extension materials based on these Project activities. The technologies developed are then transferred to the fish processing industries in the region through end-of-activity seminars/demonstrations and dissemination of information/training materials by government and private sector extension personnel.

The contribution of the Canadian International Development Agency (CIDA) for providing funds to assist the development of this work and its publication is gratefully acknowledged.

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Preface

Shrimp aquaculture in Thailand has developed since mid 1980s. The production of shrimp from aquaculture has increased dramatically from 45,000 tons in 1983 to 300,000 tons in 1993. Estimated figure for production of aquacultured black tiger shrimp in 1994 and 1995 were 263,000 tons and 280,000 tons respectively, while marine shrimp production were 100,000 and 105,000 tons. The latest statistic showed that aquaculture production of shrimp accounted for 73% of production.

The value of total shrimp production from 1983 to 1993 increased from US$760,000 to US$1,464 million. The volume increased from 1.2 million tons in 1986 to 2.3 million tons in 1995. The industry involves 80% small scale farmers and 20% large scale farmers. Shrimp has become a major export commodity as well as major source of economic income to those people involved in the industry. The success of shrimp farming is based on suitability of site (climate), availability of wild broodstock, experience in aquaculture, well established infrastructure and supporting industries, and small scale shrimp farmer co-operation

The Thai shrimp aquaculture industry has an excellent record for the production of safe products of consistent quality. The product has gained acceptance and a remarkable market share in various markets. However, as aquaculture production expanded, the industry and the Department of Fisheries was alerted to factors which can impact on the public health safety of aquaculture products.

The Department of Fisheries together with the aquaculture industry and processing industry jointly developed preventative approaches to assure control over raw materials, the manufacturing process, the production environment, and personnel. It is based on total raw material quality control. The activities of the ASEAN Canada Fisheries Post harvest Technology Project focused on the identification of potential hazards , the application of control measures at CCPs, and the monitoring and verifying of CCPs; thereby enabling the assurance of food safety during aquaculture and processing.

Thailand has successfully conducted a pilot project on Improved Quality of Aquaculture shrimp with the support of Canadian International Development Agency through the ASEAN Canada Fisheries Post Harvest Technology Project. Specialists were provided to the Department of Fisheries Inspection Agencies, to strengthen their inspection and development program. The experience gained is a basis for this manual, which we hope it will benefit all those involved in the shrimp industry in ASEAN region.

Dr. Plodprasop Suraswadi
Director General
Department of Fisheries, Thailand

Acknowledgment and Introduction

Pilot Project to Improve Quality of Aquacultured Shrimp
Department of Fisheries
Thailand

The Department of Fisheries of Thailand has conducted two pilot projects, under ASEAN-Canada Fisheries Post-Harvest Technology Project, emphasizing improvement and management of seafood quality. The pilot project "Improve Quality of Aquacultured Shrimp" is of particular interest and importance as shrimp is a major commodity which generates foreign income in many ASEAN countries. Several problems and setbacks have been encountered with aquacultured shrimp ; mostly relating to farming practices, quality and safety issues.

Thus, the pilot project was initiated with the aims :

- to obtain information on black tiger shrimp farming practices as well as the shrimp quality levels
- to overcome problems faced by the industry especially on quality and safety point of view
- to develop sensory and microbiological techniques
- to study and develop drug residue determination techniques
- to establish a Hazard Analysis and Critical Control Point or HACCP plan for good farming practice and shrimp processing and
- to disseminate the knowledge gained through training offered to Thai fish inspectors and industry personnel.

ACTIVITIES AND ACHIEVEMENTS

Establishment of HACCP Plan

In the HACCP generic model, three critical control points of the shrimp industry, based on safety aspect, were identified as follows:

- raw materials, where chemical hazards can be found due to misuse of drugs
- water quality where microbiological hazards can be caused by *Salmonella*, Coliform, *E. coli*, etc., and
- temperature control, which could lead to microbiological hazard.

HACCP Plan for Aquaculture Shrimp

The Department of Fisheries HACCP team has established a HACCP plan for aquaculture shrimp. The critical control points identified were farm condition, water quality, pond conditions and the use of drugs and other chemicals.

Shrimp Farm Sanitation Check List

The pilot project has designed a shrimp farm sanitation check list. The rating sheet was developed based on Codex Alimentarius guidelines. The check list is now in use by fishery

officers. Shrimp farms are regularly inspected with an aim to improve farm sanitation in order to reduce microbiological hazard.

Drug Residue Determination

Methods for determining drug residue analysis were studied; for example: High Performance Liquid Chromatography or HPLC is widely used and well recognized by its accuracy and sensitivity. The ASEAN-Canada Fisheries Project contracted Dr. Thakor Patel of Memorial University, Newfoundland, Canada, to extend his expertise on HPLC and Gas Chromatography (GC) to the project staff. The Department of Fisheries is now in the process of developing screening tests for Oxytetracycline and Oxolinic Acid.

Problems on Decomposition

Another problem regularly faced by the industry is decomposition. The sensory profile of black tiger shrimp and effects of handling practices on quality were studied.

According to the HACCP plan, raw material quality was identified as one of the critical control points which could lead to decomposition problems. Freshness of shrimp can be maintained by lowering temperature to 0° to 5°C immediately after harvest and at all times during processing. As a result of the study on shrimp handling, acceptable quality shrimp products can be obtained if storage time of raw materials in ice does not exceed 6 days.

- **Sensory assessment**

  Sensory assessment proved effective and reliable for raw material inspection. Mr. Klaus Schallie, sensory specialist of the Canadian Department of Fisheries and Oceans or DFO, was attached to the pilot project for a period of one month. During his consultancy, the black tiger shrimp decomposition pattern was identified. Frozen shrimp of various quality levels were prepared and subsequently used in workshops organized for Thai inspectors and the industry. Since sensory technique is widely used on imported seafood inspection in both USA and Canada, contact between Thailand and Canadian DFO and US Food and Drug Administration or USFDA regarding the matter was established.

- **Microbiological analysis**

  Another output of this pilot project was in the area of microbiology. Microbiological techniques were demonstrated by Mr. Conrad Powell, microbiological consultant from the Canadian DFO. During his consultancy, he extended to the Thai officers the knowledge on determination of pathogenic bacteria such as *Salmonella, Listeria monocytogenes*, etc. Regarding laboratory management, current methodology employed in the laboratory was reviewed. Appropriate sample handling techniques were recommended. Water quality determination was demonstrated.

Training

Training was considered a very important part of the project especially for the industry. During the conduct of this project, several training sessions and workshops were organized. There were

workshops on sensory assessment of frozen shrimp, drug residues determination, water quality determination and HACCP application. Approximately 200 production and Quality Control Personnel attended the events.

CONCLUSIONS

The outputs of this project can be concluded as follows:

- Inspection and quality control procedures were established.
- Department of Fisheries Inspectors were trained both nationally an internationally.
- The manual on "Aquaculture Shrimp Quality Management Manual" was prepared.
- Several Training Courses were offered to the industry.
- Problems on drug residues were addressed and resolved to a certain extent.
- The trade volume between Thailand, Canada, USA and other countries has increased.
- Linkage among inspection agencies of Thailand, Canada, USA and ASEAN countries was strengthen.
- Technology has been transferred to the industry in Thailand and in member countries.

The Department of Fisheries sincerely appreciated the assistance of Canadian International Development Agency (CIDA) in providing valuable support in this activity.

The staff, Program Manager Mr. Leonard G. Limpus and Project Director Mr. Tan Sen Min at the AEA are highly appreciated for their support and assistance, to make this program a success.

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ASEAN-Canada Fisheries Post-Harvest Technology Project - Phase II

Chapter I

Hazard Control for Aquaculture Shrimp Products

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Hazard Control for Aquaculture Shrimp Products

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Shrimp aquaculture industry has an excellent record for the production of safe products of consistent quality. However, as aquaculture production expands, the industry and regulatory agencies is alert to factors which can impact on the public health safety of aquaculture products. Specific problems which may be encountered in shrimp aquaculture products include:

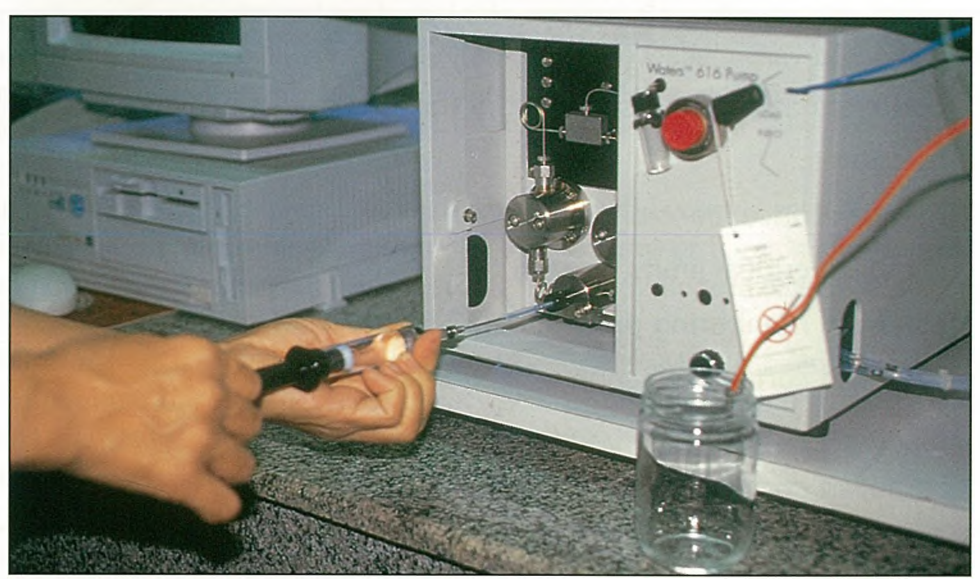
- contamination by bacterial and viral pathogens *e.g.* Salmonella, *Vibrio cholerae*.
- presence of veterinary drugs (and other substances) which may have potentially hazardous effects on consumers, handlers, and the environment.
- residues of aquaculture chemicals or other environmental contaminants.

Under the surveillance program for aquacultured shrimp conducted by the Department of Fisheries, the HACCP concept is being applied into all operations, including the production and handling of raw materials, processing operations, the processing environment, handling and storage practices, and distribution activities. This approach minimizes the reliance on analytical tests and reduces the need for comprehensive inspection of finished aquaculture products and can identify and dealt with hazards before they create an impact on processors and consumers. Hazards in aquacultured shrimp include microbiological hazards as well as chemical hazards associated with the inappropriate use of drugs and chemicals in aquaculture.

The Department of Fisheries together with the aquaculture industry and processing industry jointly developed preventative approaches to assure control over raw materials, the manufacturing process, the production environment, and personnel. It is based on the identification of potential hazards, applying of control measures at CCPs (critical control points), and the monitoring and verifying of CCPs; thereby enabling the assurance of food safety during aquaculture and processing.

Shrimp Aquaculture in Thailand

Raw material quality control units located in 22 major shrimp culture areas; the unit concentrated on aquaculture shrimp quality monitoring at production sites



INTRODUCTION

Techniques such as surveillance and inspection of final products do little to assure the safety of the food supply. The hazard analysis critical control point system (HACCP) enables aquaculturists and processors to exercise control over food safety. HACCP is essentially a technique based upon anticipation and prevention of food safety hazards and it may be applied throughout the food chain from producer through to final consumer, leading to enhanced food safety and better use of resources.

Under surveillance program for aquaculture shrimp conducting by the Department of Fisheries, the HACCP concept is being introduced into all operations, including the production and handling of raw materials, processing operations, the processing environment, handling and storage practices, and distribution activities. This approach minimizes the reliance on analytical tests and reduces the need for comprehensive inspection of finished aquaculture products and to identify and deal with hazards before they create an impact on processors and consumers. Hazards in shrimp aquaculture include microbiological hazards as well as chemical hazards associated with the inappropriate use of drugs and chemicals in aquaculture.

HACCP PRINCIPLES

1. Conduct hazard analysis, identify hazards and specify control measures.
2. Identify critical control points (CCP).
3. Establish critical limits at each CCP.
4. Establish monitoring procedures.
5. Establish corrective action procedures.
6. Establish verification procedures.
7. Establish documentation procedures.

This chapter will review the use of HACCP concept to prevent impact on product safety. Some examples will be elaborated.

HACCP PRINCIPLES

The HACCP system consists of seven principles which outline how to establish, implement, and maintain a HACCP Plan. The HACCP principles have been published by the Codex Alimentarius Commission and have gained international acceptance.

The implementation of the HACCP system requires technical expertise and entails a systematic study of all operations, processing, packaging, storage, distribution, and subsequent handling by the consumer. When HACCP is applied at an early stage it will result in significantly reduced food safety risks.

1. Conduct Hazard Analysis

Potential hazards in aquaculture must be identified, hence all activities associated with production, harvesting, processing, storage, distribution, and marketing of aquaculture products must be evaluated. This includes a review of:

- the use of antibiotics, drugs and other veterinary chemicals.
- potential sources and specific points of bacterial and parasitic contamination during production and processing.
- the potential for microorganisms to survive or multiply in aquaculture products.
- the risks and the severity of all hazards identified.

Furthermore, it is necessary to establish whether:

- pathogenic microorganisms/toxins may be present in raw materials.
- pathogens may contaminate aquaculture products after harvest.
- aquaculture products will be held warm, chilled, frozen, or at ambient temperatures.

An important aid to hazard analysis is the process flow chart which documents all the major steps in an aquaculture operation. Process flow charts may take many forms, with some incorporating symbols to indicate sources of contamination, CCPs, inspection activities, etc.

The next step is to identify all the hazards which could occur at each stage and to describe preventative measures for their control. The severity of hazards and the probability of their occurrence is also evaluated according to epidemiological data about the foodstuff.

Hazard analysis is a vital component of the HACCP system. It requires a good knowledge of aquaculture operations, access to technical literature and epidemiological data, and a sound knowledge of the production environment (achieved by observation and discussion with farmers or workers).

2. Identify Critical Control Points (CCP)

The CCP is unique to the HACCP system, as all preventative and control measures are aimed at hazards which have been identified during the hazard analysis step. A CCP must be identified for each hazard.

To be a CCP, an operation must be such that appropriate action will prevent, control, or minimize the hazard. Cooking shrimps will control risks associated with contamination by food poisoning bacteria. Potential contamination of the shrimp pond environment may be minimized by using dry pelleted feeds (rather than using fresh manure) or controlling farm hygiene.

To aid in deciding what operations are CCPs, a decision tree has been developed as shown in *Figure 1.* The decision tree contains a logical series of questions which are asked for each hazard at each processing step. The answer to each question leads the HACCP team to a decision whether or not a procesing step is a CCP.

3. Establish Critical Limits for Each CCP

The most important phase in the establishment of a HACCP system is translating CCP information into surveillance procedures which can be used in the production and processing environment.

Critical limits define the boundaries between safe and unsafe products (and practices), hence they must be associated with a factor which can be measured and monitored on a routine basis.

This involves defining product and process variables and their tolerances at each CCP. For example, application of drug or chemical during the growing period could be a CCP, with critical limit include the use of approved drug and chemical and drug MRL. Other examples of critical limits include pesticide MRL, allowable levels of heavy metals, chlorine levels, minimum particle size for filth, storage temperatures for chilled products, concentration and dipping time for metabisulphite dips, etc.

Information for determining critical limits may be drawn from published information, expert advice, experimental data, and mathematical modelling.

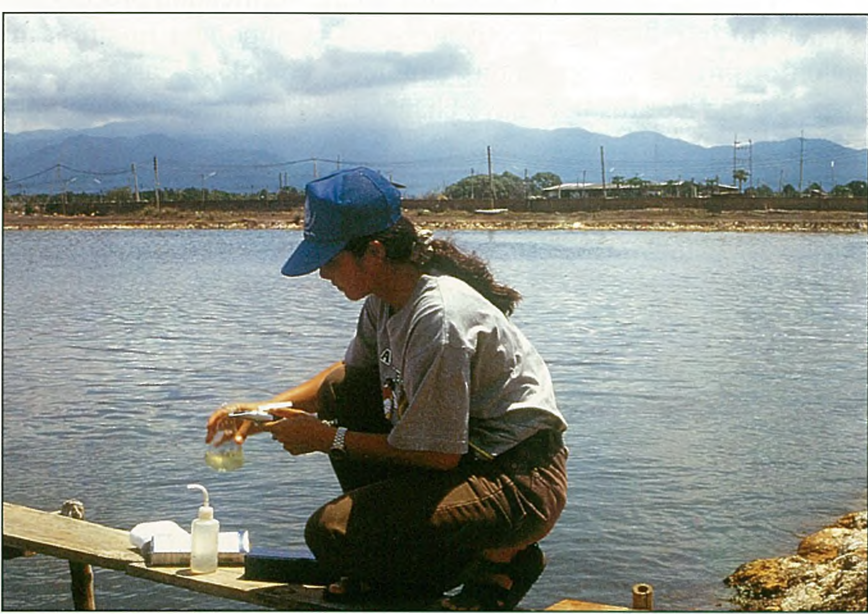
Microbiological specifications should be avoided as the test results are not available for several days. HACCP is based on the ability to take instant action when the process deviates, so microbiological analysis is typically used only for verification purposes. Where rapid microbiological methods are available they may be used for monitoring CCP, however they need to be truly rapid (minutes rather than hours).

4. Establish Monitoring Procedures

If HACCP is to function effectively, a regular schedule for monitoring each CCP must be

established. Monitoring should be undertaken by persons involved in the operation, and involve making observations, taking measurements, and testing of samples. When establishing the monitoring program it is necessary to:

- carefully establish the frequency of testing *i.e.* daily, once per shift, continuous, etc.
- ensure sampling is based on statistical sampling plans.
- document suitable monitoring methods.
- assign staff to take responsibility for monitoring and recording of results.



Monitoring activities must provide rapid results, so the appropriate corrective action may be promptly implemented. Therefore, measurement of pH, salinity, time, temperature, physical and chemical parameters are favoured over lengthy and complex determinations such as microbiological analysis.

5. Establish Corrective Action Procedures

When monitoring indicates deviation from the specified range (critical limits) immediate action must be taken to rectify the situation and get the process back under control.

Under HACCP, corrective action is clearly defined beforehand so the person knows how to respond to any process deviations. This includes advice on how to correct the problem and bring the process back under control, and guidance on isolating all affected product. All suspect product should be placed on hold until it can be tested to assess its safety. There is also the need to implement action to review the process and the corrective action in order to prevent a recurrence of the deviation and the hazard. Examples of corrective actions in aquaculture practices includes extending of withdrawal period or thorough washing of harvested shrimp.

6. Establish Verification Procedures

The HACCP system should be audited to assess whether it complies with the documented HACCP plan. This verification process assists in improving the HACCP system and determines whether the HACCP system achieves its goals. The types of questions which may be asked during the verification process include:

- Have the correct CCP(s) been selected?

- Have effective criteria for control been specified?
- Are control measures in place?
- Are the monitoring activities effective?

Verification may also involve a thorough review of documentation as well as examination of all microbiological, chemical, and physical test data to ensure production operations are fully controlled.

HACCP systems are in a constant state of evolution, so the verification process assists in fine tuning the system to improve its effectiveness. It is important to stress that any change in production or processing operations requires a complete reassessment of the HACCP system because the hazards may have changed and this necessitates a review of the critical control points.

7. Establish Documentation Procedures

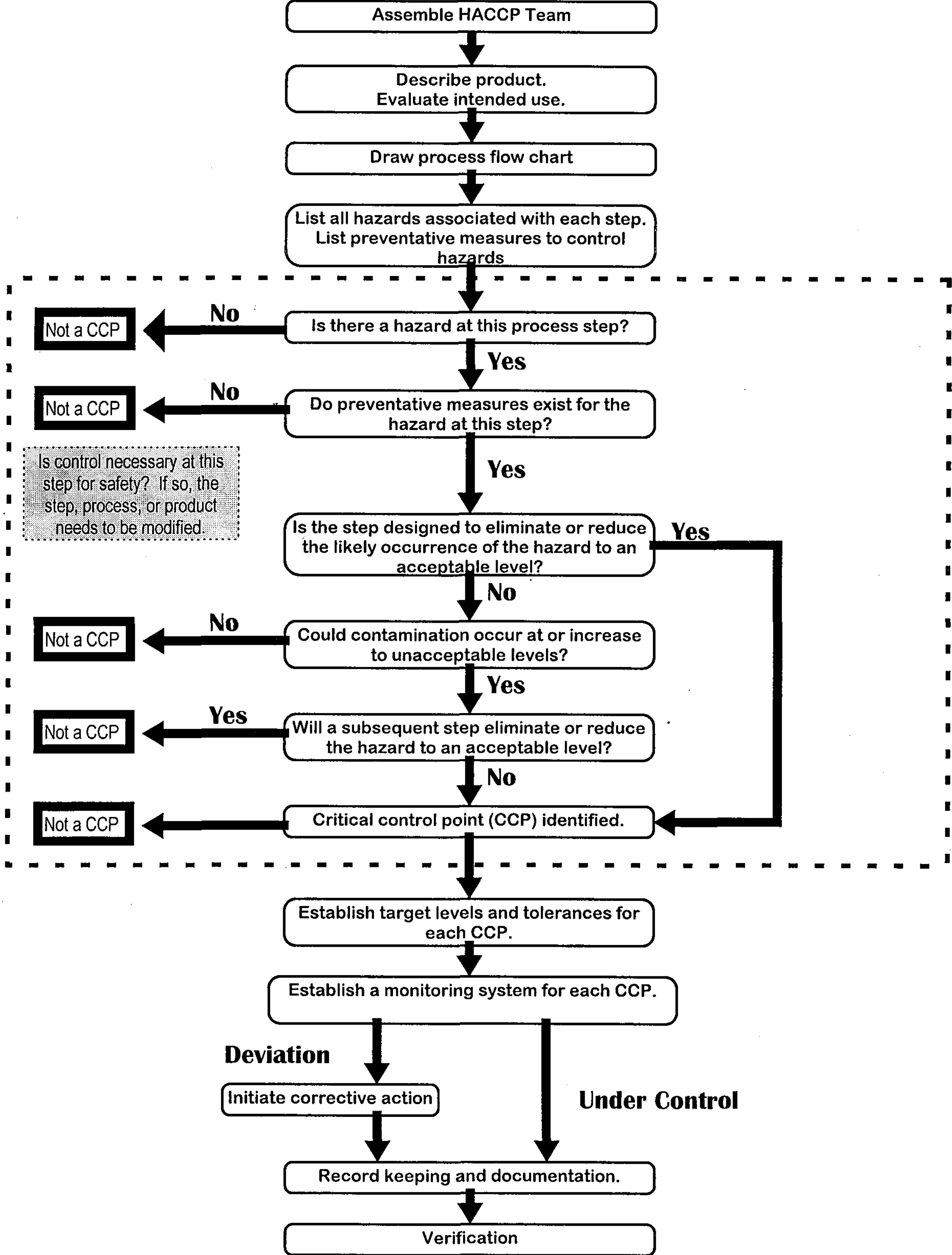
Under HACCP, aquaculturists must maintain records of all CCP monitoring activities, including records of raw materials (fry, feed, water sources, chemical and drug), production data, and monitoring activities. The collection and collation of this data assists in carrying out verification activities, as well as in trouble shooting to determine the cause of production problems, data analysis for production improvements, and to review production history where products have been the subject of customer complaints or action by regulatory agencies.

The application of the HACCP technique commences with the formation of a HACCP team whose task is to oversee the development, implementation, and maintenance of the HACCP system. *Figure 1* documents the various stages in the process, which integrates steps **1** to **7** above, in a logical sequence.

APPLICATION OF HACCP TO AQUACULTURE

CODEX Committee on food hygiene recommended the steps for HACCP application as appear in *Figure 1*.

Figure 1: The CCP Decision Tree



A HACCP team

THE HACCP TEAM

HACCP is normally implemented by a multidisciplinary team of people (the HACCP team).

Members of the team should be drawn from personnel having knowledge and experience in the following areas: HACCP, quality assurance, aquaculture production or processing, and engineering. Personnel in the team must have a real working knowledge of what happens in aquaculture production and be able to analyse and interpret data.

Each team must have a HACCP expert who understands the principles of HACCP and can effectively communicate the concepts to all staff. While many staff will find HACCP a difficult and complicated system, the role of the expert will be to train staff about HACCP and its common sense approach to managing food safety. Training is an important element of HACCP, and the team should receive training to develop their level of technical expertize.

AQUACULTURE PRODUCTS DESCRIPTION

The first task of developing a HACCP Plan is to describe the species.

Black tiger shrimp (*Peneaus monodon*), cultured for three months in an earth pond, wet and pellet feed are given. Water was changed continually, throughout culture period.

Antibiotics or chemicals may be used to treat shrimp at larvae stage or when shrimp was found infected. Drugs may be added in the feed or applied directly.

Shrimp is harvested manually; harvested shrimp was put in ice with in 15 minutes after caught; it is then sorted and packed in ice and transport to processing factory.

PROCESS FLOW CHART

Process flow chart for shrimp production is shown in *Figure 2*.

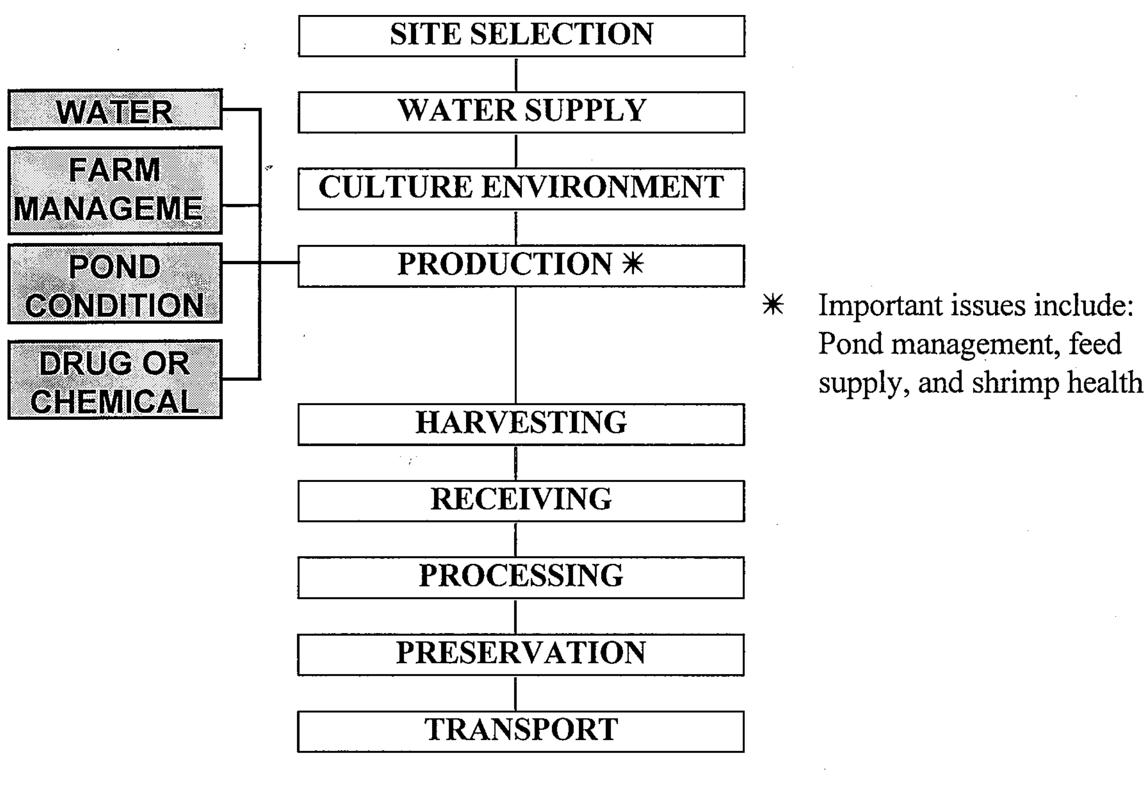


Figure 2: Generic Aquaculture Process Flow Chart

HAZARD ANALYSIS

A hazard is defined as a biological, chemical, or physical agent with the potential to cause an adverse health effect. The first stage in the HACCP process is to conduct a comprehensive hazard analysis of the food (aquaculture product) relative to its intended end-use, including a review of raw materials, ingredients, production and processing operations, consumer usage, etc. The HACCP team must determine the type and range of hazards that may be encountered during the production and processing of an aquaculture product. This process may be assisted by describing the product, evaluating its intended use, and preparing a process flow chart which describes the steps in producing and processing the product. The flow chart may alert the team to the opportunity for contamination or product abuse at different stages in the production chain.

Potential hazards in aquaculture may be identified as biological hazards, chemical, and physical hazards. Hazards can enter an aquaculture product at any time during production and processing *e.g.* occur as a result of using pesticide contaminated feed; inappropriate use of veterinary chemicals; pollution of the aquaculture environment by pathogenic bacteria or viruses; contamination during the processing of aquaculture products, etc. *Table 1* explores some of the types of hazards that may be encountered.

Hazard analysis were conducted. Specific hazards which may be encountered in shrimp aquaculture products are shown in *Table 1*:

Table 1: Hazards Associated With Aquaculture Products

CATEGORY	EXAMPLES OF HAZARDS	
Biological hazards	Pathogenic bacteria	Salmonella, Shigella, *E.coli*, *Vibrio cholerae*, *V.parahaemolyticus*, *V.vulnificus*, *Aeromonas hydrophila*, *Listeria monocytogenes*, etc
	Parasites and Protozoa	Larva of parasites such as trematodes, cestodes, or nematodes. *e.g. Clonorchis sinensis*, *Anisakis* sp., *Capillaria philippinensis*, etc
	Viruses	Hepatitis A, Norwalk virus, etc
	Mycotoxins	Aflatoxins
Chemical hazards	Veterinary residues	Hormones, growth regulators, antibiotics
	Pesticide residues	Herbicides, fungicides, insecticides, etc
	Heavy metals	Mercury, lead, cadmium, copper, etc
Physical hazards	Glass, wood, metal, etc	

The microbiological safety of aquaculture products has been the subject of much research in recent years. It is largely accepted that the microbiological quality of the production environment, impacts on the microbiological quality of the fish and ultimately the processed

product. This is no more apparent than with molluscan shellfish, as they have been implicated in numerous outbreaks of foodborne disease. They represent a threat to human health when they are consumed raw, hence there is need for control over production, harvesting, processing, and distribution.

On the other hand, aquaculture production of finfish and crawfish has not presented a major health hazard for consumers in the United States (NOAA, 1991). Literature on the potential hazards from Salmonella and Vibrio species in the framing of shrimp is conflicting. Salmonella and *Vibrio cholerae* were found to be present as part of the natural flora of brackish cultured shrimp, and pose a major concern for processors and exporters (Reilly and Twiddy, 1992). In contrast, Salmonella was not recovered from shrimps or shrimp ponds in a recently completed study in Thailand (Dalsgaard *et al.*, 1995).

Clearly, the aquaculture production of finfish, crustaceans, and molluscs may present a threat to public health if they are not grown and harvested under strictly hygienic conditions. Once harvested, aquaculture species are at risk from contamination in the processing plant with a wide range of pathogenic bacteria derived from the processing environment, water used in processing, equipment, and food handlers. The HACCP team needs to review the scientific literature to identify potential hazards and to quantify the risks.

At this stage the HACCP team will need to prepare a simple process flow chart to assist in identifying the steps where hazards are introduced or become a potential problem. The process flow chart is therefor an important aid to hazard analysis, documenting all the major steps in an aquaculture operation. *Figure 2* is a generic model for commercial aquaculture production. It would need to be tailored to meet the needs of specific aquaculture operations *i.e.* shrimp production, catfish farming, etc.

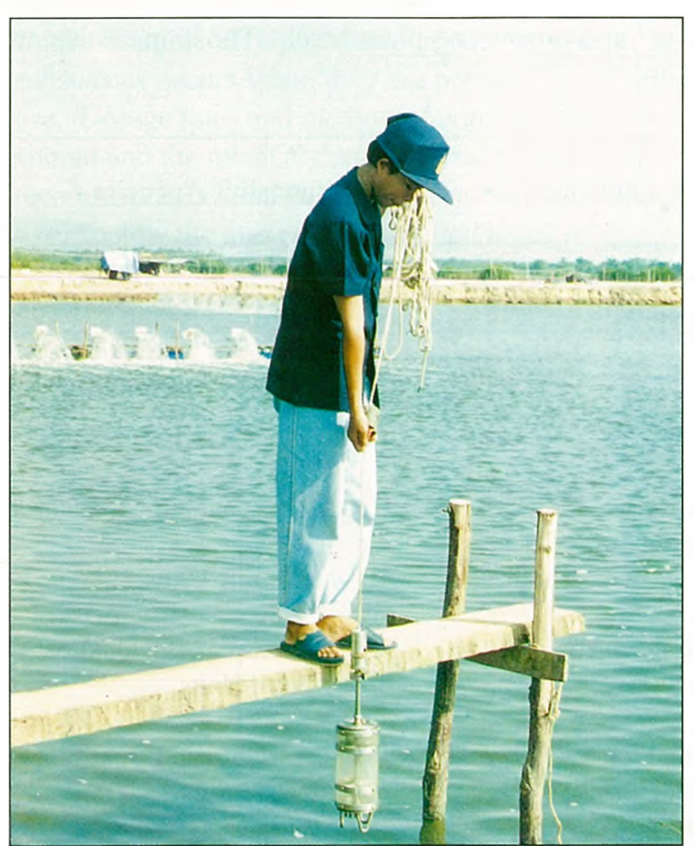
The HACCP team must identify all known hazards, nominate the step(s) in the process flow chart where each hazard may occur, and describe preventative measures for their control. This stage requires much thought and often expert assistance in determining what are the real hazards. Increasingly risk analysis techniques are being applied to this step, to ensure that the hazards are sufficiently real. For example the team needs to review:

- use of antibiotics, drugs and veterinary chemicals.
- potential sources of bacterial and parasitic contamination.
- potential for microorganisms to survive or grow in aquaculture products.

Table 2: Hazard Analysis For Shrimp Aquaculture Production

PRODUCTION STEP	HAZARD	SEVERITY	RISK	SIGNIFI-CANT Yes/No	CONTROL AT
SITE SELECTION	chemical contamination microbiological contamination	medium/ high	low/ medium	yes	prerequisite control
GROWING • pond condition	chemical contamination microbiological contamination			yes	prerequisite control by good farming practices
• water supply	Salmonella	high	medium	yes	CCP
• feed/fertilizer	Salmonella	high	medium	yes	CCP
• uses of chemical and drugs					CCP
HARVESTING	Salmonella re-contamination Glass, wood	high high	medium medium	yes yes	CCP CCP

Site Selection



Growing Period

Harvesting

PREVENTIVE MEASURES DEVELOPED

Recognizing that the control at aquaculture level are not an easy task, the Department of Fisheries, therefore, since 1991, has taken several measures to control the above identified hazards which could be harmful to consumers and caused rejection from importing countries.

The preventive measures, based on HACCP preventive control concepts, were developed specifically to prevent drug and chemical residue in aquaculture products and to prevent

microbiological contamination at farm level and processing plant level. Those measures are outlined as follows:

Potential Critical Control Points	Preventive Controls	Responsible Agencies
Farm management	• farm registration • farm sanitation and practices	• Coastal Aquaculture Division
Feed production	• feed control	• Feed Control Division
Farming practices	• control uses of vet. drug • monitor quality of water, antibiotic residue, shrimp disease • monitoring residue in raw material	• Coastal Aquaculture Division • 18 Mobile Units • 22 Raw material Quality Control Units
Processing plants	• control sanitation hygiene, processing practices at processing establishment • inspection of finished products	• Fish Inspection and Quality control Division • 6 Fish Inspection centers

IDENTIFICATION OF CRITICAL CONTROL POINTS (CCPs)

CCPs relate to specific processing steps where a hazard can be controlled. CCPs can be found by using knowledge of the process and all the possible hazards to decide on the best preventative measures for their control. The decision tree in *Figure 1* can be used to assist in deciding what operations are a CCP, however many HACCP teams find it rather complicated.

A major concern in aquaculture relates to the use of veterinary substances such as antibiotics, drugs, anesthetics, and growth promotants. If drug residues above the MRL are considered a hazard, the decision tree would be used to determine the critical control point as in *Table 2.*

Therefore, the production (growing) step is a Critical Control Point for misuse of registered chemicals such as drugs. Accordingly, the HACCP team must now examine the preventative measures that need to be put in place to avoid such a hazard occurring, and to develop monitoring methods to ensure correct procedures are adhered to.

The use of drugs is especially important, as relatively few drugs have been approved for use in aquaculture. Hence a number of drugs are being used without approval. Clearly this is illegal and raises many difficulties especially when aquaculture products enter foreign markets.

Contamination of aquaculture products with excess levels (exceeding MRLs) of approved substances occurs when they are not used in accordance with instructions for safe and effective use. Dosage rates and method of administration with vary depending on the type and age of the animal and the medical condition being treated. The aquaculture producer must then follow the recommended withdrawal period, to enable the animal to metabolically reduce the drug level in tissue below the maximum permitted level.

Table 2: Decision Tree

STEP HAZARD	DECISION TREE QUESTION	PROCESS STEP (from flow chart): PRODUCTION
GROWING DRUG RESIDUE	1. Is there a hazard at this production step? (GROWING - 4 MONTHS)	YES (drug residue exceeds MRL)
	2. Do preventative measures exist for the identified hazard?	YES (use approved drug at correct dosage)
	3. Is the step specifically designed to eliminate or reduce the likely occurrence of the hazard to an acceptable level?	NO (a growing period is design to grow shrimp to marketable size)
	4. Could contamination occur at or increase to unacceptable levels?	YES (during growing period, the drug may be added in feed or directly applied)
	5. Will a subsequent step or action eliminate or reduce the hazard to an acceptable level?	NO (drug or chemical can not be removed by any processing method)

HACCP PLAN

The following HACCP plan were developed by a HACCP team consisted of:

- Aquaculture technologists
- Processing plants quality control personnel
- Food technologists
- HACCP specialist

The plan is developed based on the work conducted under the ASEAN-Canada Fisheries Post-Harvest Technology Project - Phase II, to be used as generic model for further development.

REFERENCE:

Mahony, 1995. HACCP in Aquaculture: Papers prepared for PAEC/DOF Seminar on Quality Assurance for Aquaculture Products. Queen Sirikit National Convention Centre, Bangkok

Major Activities of Raw Material Quality Control Unit

- Mobile units
- Water quality testing
- Drug residue determination (HPLC and Microassay)

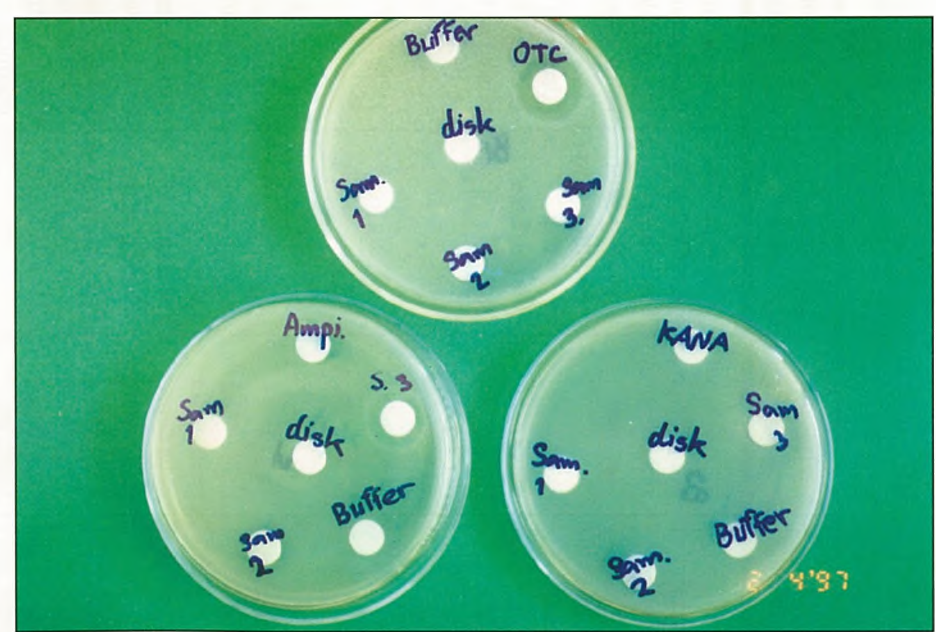
Raw Material Unit

Mobile Unit

Water Quality Testing

Drug Residue Determination (HPLC)

Drug residue Determination (Microassay)



HACCP PLAN FOR AQUACULTURED SHRIMP

AQUACULTURE FARM______________ DATE:__________ PAGE NO.:__________

Critical Operation	Hazard	Control measures	Monitoring Procedure	Recorded	Monitoring Frequency	Specifications	Person Responsible	Corrective Action
A production or processing operation as described in the process flow chart.	Identify what can go wrong at this critical operation.	What variables associated with this critical operation must be controlled to prevent or minimize the hazard?	List methods used to monitor the variables listed under CCP. (Usually a documented procedure).	Indicate where the results are recorded.	List the frequency of monitoring activities.	List maximum and minimum values for each variable monitored.	Nominate who is responsible for the monitoring activity.	What action is to be taken if the CCP deviates from the permitted range? Who is responsible for correcting the situation? Who must be notified?
e.g. preparing ponds, loading water, stocking fries, feeding shrimp, treating shrimp with antibiotics, harvesting and packaging, etc.	*e.g.* Salmonella *or* other micro organism contamination, drug residue exceeding MRL, etc.	*e.g.* Drug dosage, hygiene, temperature during shrimp storage, etc.	*e.g.* Core temperature using thermometer, pH meter, data logger, visual assessment, rapid test for drug and chemical residue etc.	*e.g.* Log for raw material uses, production record log, etc.	*e.g.* Every week, day or hour, etc.	*e.g.* Nil tolerance of foreign matter, MRL for drugs, -18°C ± 1°C, etc.	*e.g.* Farm worker, technician, etc.	**Corrective action must:** • correct the problem • isolate product • determine cause.

HACCP PLAN FOR AQUACULTURED SHRIMP

AQUACULTURE FARM__________ DATE:________ PAGE NO.:__________

Critical Operation	Hazard	Control measures	Monitoring Procedure	Recorded	Monitoring Frequency	Specifications	Person Responsible	Corrective Action
Water used in aquaculture	Contaminated water : Pathogenic bacteria Pesticides	Farm hygiene control In-coming water quality In-coming water treatment pond	Farm hygiene inspection Monitor water quality Monitor pesticide level in nearby water sources (obtain information from govt. monitoring program)	Farm log book ■ farm hygiene record ■ water quality record	Every week Fortnightly Fortnightly	Acceptable according to farm hygiene rating scale (Department of Fisheries, Thailand) Pathogenic bacteria - not detected MRL pesticide	Farm technician or trained worker	Correct deficiencies identified If water contains high bacterial count or pathogenic bacteria, increase frequency of monitoring during grow out period Re-evaluate the source of water Retain water at longer period in treatment pond before use Farm workers should report to farm manager

HACCP PLAN FOR AQUACULTURED SHRIMP

AQUACULTURE FARM________________ DATE:__________ PAGE NO.:______

Critical Operation	Hazard	Control measures	Monitoring Procedure	Recorded	Monitoring Frequency	Specifications	Person Responsible	Corrective Action
Farm management	Contaminated feed, fry, shrimp diseases	Farming practices Stock density	Monitor water physical quality Monitor disease	Farm log book water quality record	Every week Fortnightly	CODEX Guidelines water quality for aquaculture Acceptable - DOF Farm Rating Scale	Farm manger or trained worker	Treat disease appropriately in consultation with fish pathologist Farm workers should report to farm manager
Drug chemical residue	Use of un-approved drug or chemical Residue exceeding acceptable level	Identify appropriate application level of drug or chemical used in farm Appropriate withdrawal period applied	Review record of drug application Screening check using appropriate test kits	Drug application record	Every week throughout growth period	Oxytetracycline 0.5 ppm Others: no tolerance Withdrawal period 21 days	Trained worker	Apply appropriate withdrawal period Mark lot of raw material for further verification Send raw material for residue check-up 14 days before harvesting

DEPARTMENT OF FISHERIES
FARM INSPECTION RATING REPORT

Farm Name ______________________ Location ______________________

Date ______________________ Inspector ______________________

ITEMS	RATING				COMMENTS
	VG	G	F	NI	
1. GENERAL CONDITION 1.1 Land based establishment 1.1.1 Located on a hygienic water source 1.1.2 Sited at safe distance from housing and/or factories 1.1.3 Site protected from flooding 1.1.4 Soil for construction meets heavy metal and/or other chemical limits 1.2 Water based establishment 1.2.1 Located on a hygienic water source 1.2.2 Sited at safe distance from housing and/or factories 1.2.3 Site is protected from waterborne traffic 1.2.4 Good circulation of water					
2. LAYOUT AND CONSTRUCTION 2.1 Site has separate area and a proper hygienic operation for: a) Hatchery b) Feed storage c) Sizing and packing 2.2 Site access can be controlled 2.3 Floors - in good repair, clean, properly sloped, water proof and non-absorbent (where appropriate), without crevices and easy to clean 2.4 Walls - smooth, in good repair, light coloured, clean, water proof, non-absorbent (where appropriate) and without crevices 2.5 Ceiling - smooth, in good repair and easy to clean 2.6 Drains - smooth and impervious, and of sufficient capacity					

VG - VERY GOOD, G - GOOD, F - FAIR, NI - NEED IMPROVEMENT

ITEMS	RATING				COMMENTS
	V	G	F	NI	
3. WATER USED IN AQUCULTURE 3.1 Appropriate quality for rearing, treated properly before use 3.2 Microbiological quality meets prescribed limit 3.3 Properly treated before draining					
4. HYGIENE REQUIREMENTS 4.1 Water/ice used with product 4.1.1 Microbiological quality meets prescribed limit 4.1.2 Contains suitable residual chlorine 4.2 Toilets 4.2.1 Located at safe distance from areas where rearing, feed storage and packing are done 4.3 Cleanliness 4.3.1 Handwashing facilities are provided 4.3.2 There is hygienic removal of waste matters 4.4 Waste and offal 4.4.1 Containers with lids are provided 4.4.2 Containers are frequently removed from areas 4.5 Pest control 4.5.1 A program to control birds, rodents and other animals is in place					
5. EQUIPMENT AND UTENSILS 5.1 Made of approved material 5.2 Properly designed to facilitate cleaning and disinfection					

ITEMS	RATING				COMMENTS
	V	G	F	NI	
6. FARM PRACTICES 6.1 GENERAL CLEANLINESS 6.1.1 Area is kept clean 6.1.2 Equipment and utensils are washed before using 6.1.3 Utensils are proper stored 6.2 Rearing and Handling 6.2.1 Records are kept and maintained for: a) Water and uses of water b) Feed and feeding c) Diseases and their control d) Drugs and chemicals, indicating: i) A list of drugs and chemicals used ii) A record of time(s) / amount(s) used iii) A record of withdrawal period(s) 6.2.2 Proper harvesting techniques are used 6.2.3 The product is washed with hygienic quality water 6.2.4 The product is at a temperature close to melting ice 6.2.5 Efforts are made to prevent contamination during harvesting, sorting and transporting 6.2.6 Delays during handling is kept to a minimum.					

Overall rating :

Comments :

Inspector :	Verified by :

Chapter II

Sensory Assessment of Black Tiger Shrimp (*Penaeus Monodon*)

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Department of Fisheries
Thailand

Sensory Assessment of Black Tiger Shrimp (Penaeus Monodon)

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Chief, Sensory and Physical Quality Subdivision
Fish Inspection and Quality Control Division
Department of Fisheries, Thailand

INTRODUCTION

Export of Thai frozen shrimp accounted for US$6.9 billion and totaled 450,278 metric tonnes in 1995. From 1985, the marked expansion of black tiger shrimp aquaculture was the major contributor to Thailand's becoming one of the world's largest frozen shrimp exporters. Approximately 80% of total export is derived from the aquaculture sector while wild catch has decreased substantially due to over exploitation of natural resources. In addition, the quality of the wild catch is rather low due to poor handling practices and lengthy storage time on board fishing vessels without proper icing. Major markets of Thai frozen shrimps are USA and Japan. Export to Canada accounted for approximately 4% of the total figure. However, Thailand has been a major supplier with a market share of around 42% in 1995.

Sensory assessment is officially enforced on imported food products by USA and Canada. Objective judgments can be achieved through extensive training especially by sensory experts of the import authorities. Sensory assessment is recognized as an effective monitoring procedure to evaluate quality of raw materials and for in-process quality control in Hazard Analysis and Critical Control Point (HACCP) based programs. Decomposition is a problem commonly found in low quality seafood products, which results in rejection of the shipment. These characteristics can be directly perceived by way of sensory tests. In order to confirm the initial sensory results, indole, a product of tryptophan degradation, is currently used by the US Food and Drug Administration (USFDA) to validate the sensory evaluation of shrimp decomposition. They have recommended a defect action level of ≥ 25 µg/100g.

In order to obtain a wide range of samples of diferent qualities for sensory training and establishing sensory profiles, preparation of authentic samples should be conducted through controlled spoilage runs. Inspectors should be trained using prepared samples with a known spoilage background as well as commercial samples. The following is the sample preparation step for the establishment of a sensory profile for black tiger shrimp.

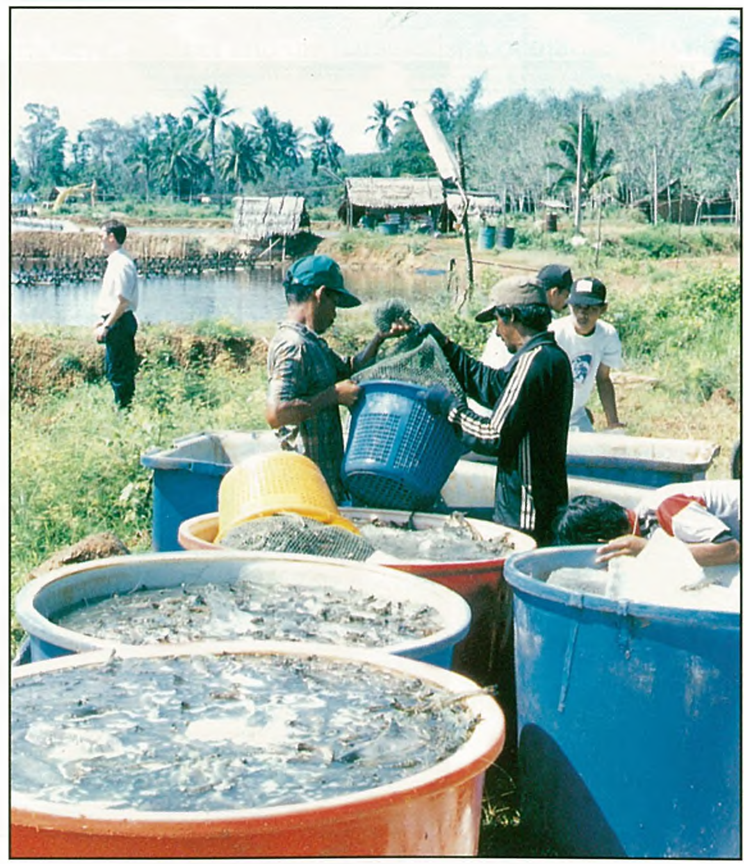
PREPARATION OF FROZEN BLACK TIGER SHRIMP SAMPLES

Live black tiger shrimp from a culture pond were shocked to death in iced water. The shrimp samples were then transferred in ice to a freezing establishment. One set of shrimp samples was stored at ambient temperature whilst another was stored between layers of ice. Shrimp were drawn at various intervals for processing into frozen raw and cooked peeled headless tail-on product. For ambient storage, shrimp samples to be processed into frozen raw products were drawn for analyses at 0, 6, 9, 12, 16, 20, 27, 32 and 43 hours and at 0, 3, 6, 9, 20, 27, 32 and 43 hours for frozen cooked products. For ice storage, black tiger shrimp were

drawn at 0, 1, 3, 4, 6, 8 and 10 days of storage for processing into frozen raw products. The frozen shrimp samples were stored at -18°C for further sensory and indole analyses.

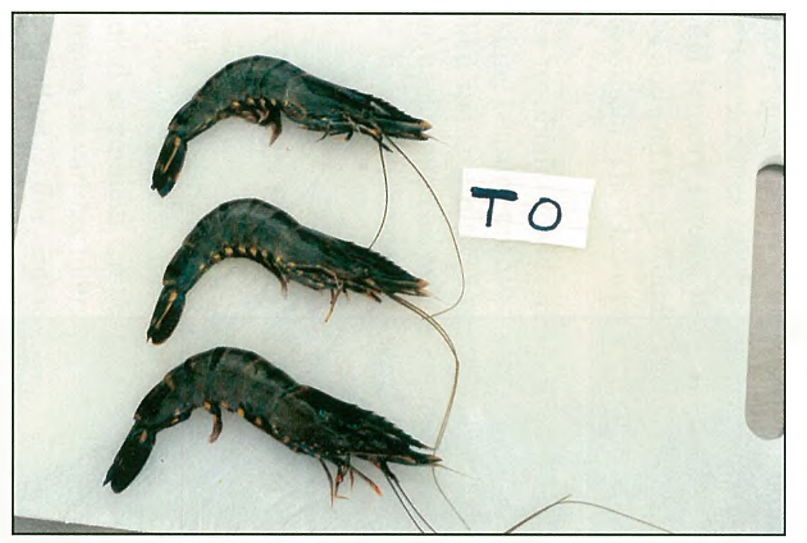
Shrimp of the best quality is obtained from the farm and iced immediately

Farm Site



Icing of Shrimp at Farm

Samples were withdrawn at different hours in order to establish sensory quality profile



SENSORY EVALUATION TECHNIQUES

Sensory experts should demonstrate to trainee inspectors sensory evaluation techniques and explain the characteristics of acceptable and reject quality. This method is called "Descriptive Analysis", in which a group of highly trained or expert analysts examine the attributes of a product and provides a detailed descriptive profile of it (which can be put in a standard). With training and knowledge, sensory analysts can use descriptive analysis to communicate with each other regarding the qualities of a particular product. In the case of shrimp, or any seafood for that matter, odours and flavours can be easily grouped into four categories:

1. Odours that are typical of the species in question, these are the naturally occurring odours that are present at the moment of capture or harvest before there is any noticeable loss of quality;

2. Persistent, distinct and uncharacteristic odours and flavours described in the standard as taint which can be further divided into rancidity due to the oxidation of oils present in the product and abnormal odours which may result from the type of feed, environmental conditions (e.g. poor water quality or algae blooms) or processing conditions (e.g. scorched, acrid or metallic);

3. Persistent, distinct and uncharacteristic odours and flavours described in the standard as decomposed including but not limited to the following: ammonia, musty, yeasty, vegetable, sour, faecal, hydrogen sulphide, putrid;

4. Persistent, distinct and uncharacteristic odour and flavour of any material which has not been derived from the product and which posed a threat to human health (e.g. solvents, fuel oil, etc.) is categorised as critical foreign material.

It is important to remember that the "Descriptors" employed by a standard must be meaningful to the analysts who are expected to use the standard to evaluate a given product. This may require the development of other terms and descriptors when sensory attributes are translated into different languages or are used by analysts coming from a different cultural background. It should be kept in mind that when samples are examined, descriptors developed should be useful to everyone.

Chemical analysis for the concentration of indicators of decomposition is often used to confirm the results of sensory analysis. In the case of shrimp, indole is widely recognised as a good indicator of certain types of spoilage. It is generally accepted that samples showing levels of indole above 20 to 30 micrograms per 100 grams are decomposed and that trained sensory analysts will usually reject such samples for the presence of odours and/or flavours indicating decomposition. The absence of these indicators does not necessarily meant that the product is not decomposed since some indicators are only formed in the presence of certain types of bacteria, at certain temperatures.

To determine sensory profile of frozen black tiger shrimp, the frozen shrimp samples previously prepared were thawed and evaluated by placing the samples in order form best to lowest quality. Sensory changes of both raw and cooked products were observed and

decision on acceptability was made by the assessors. The samples were also analysed for indole contents as well, using the method specified in AOAC (1990). *Tables 1* to *3* exhibit indole contents and sensory characteristics of frozen raw and cooked black tiger shrimp processed from raw material stored at ambient temperature and in ice respectively.

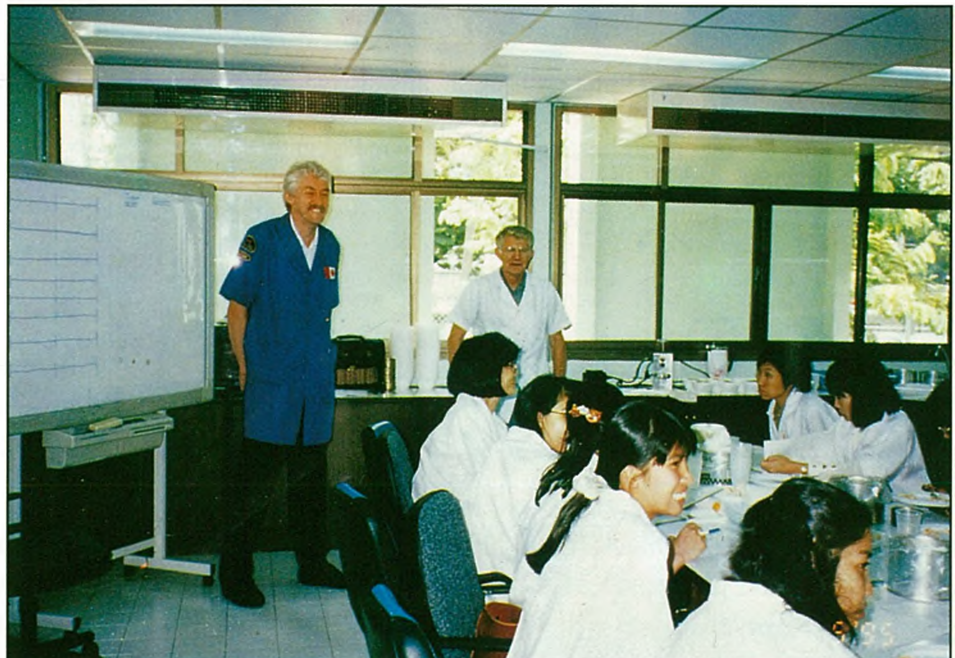
Table 1. Sensory Characteristics And Indole Contents of Frozen Raw Black Tiger Shrimp Processed From Raw Material Stored At Ambient Temperature

Storage hour(s)	Indole (μg/100g)	Sensory quality level	Characteristics
0	0.3	Acceptable	Firm, resilient, bright, glossy, grassy or seaweedy odour
6	0.2	Acceptable	Firm, resilient, uniform colour, fresh odour
9	0.3	Acceptable	Firm, slightly faded, slightly discoloured, fresh odour
12	0.3	Acceptable	Slightly soft, slightly slimy, slightly discoloured, slightly stale
16	0.1	Acceptable	Slightly opaque, slightly discoloured
20	0.6	Acceptable	Slightly discoloured, neutral odour,
27	0.5	Acceptable	Firm, slightly discoloured, slightly stale
32	0.4	Borderline acceptable	Not resilient, some red discoloured, slightly opaque, slightly slimy, slightly putrid
43	5.6	Borderline fail	Not resilient, discoloured, opaque, musty, slightly putrid, slightly ammonical

Table 2. Sensory Characteristics And Indole Contents Of Frozen Cooked Peeled Black Tiger Shrimp Processed From Raw Material Stored At Ambient Temperature

Storage hour(s)	Indole (μg/100g)	Sensory quality level	Characteristics
0	0.7	Acceptable	Bright, uniform, fresh and sweet odour
3	0.5	Acceptable	Bright, uniform, fresh and sweet odour, slightly cold storage odour
6	0.4	Acceptable	Neutral odour
9	0.2	Acceptable	Slightly opaque, slightly stale, slightly musty
20	0.2	Acceptable	Opaque, slightly stale
27	0.2	Acceptable	Some translucent, some opaque, bleached, slightly stale
32	0.3	Acceptable	Slightly stale, slightly musty
43	7.3	Borderline acceptable	Discoloured, stale, old odour, slightly musty

Sensory experts and panels exzmining black tiger shrimp



Sensory experts and panels working on descriptors

Sensory testing in progress

Table 3. Sensory Characteristics And Indole Contents Of Frozen Raw Black Tiger Shrimp Processed From Raw Material Stored In Ice

Storage hour(s)	Indole (µg/100g)	Sensory quality level	Characteristics
0	0.4	Acceptable	Firm, resilient, bright and uniform colour, fresh and sweet odour
1	0.4	Acceptable	Firm, resilient, slightly discoloured, fresh and sweet odour
3	0.2	Acceptable	Firm, slightly discoloured, slightly stale
4	0	Acceptable	Opaque, bleached, slightly discoloured, neutral odour
6	0.2	Acceptable	Slightly discoloured, milky appearance
8	1.7	Borderline reject	Opaque, slightly discoloured, musty, slightly putrid
10	174.4	Reject	Soft, opaque, bleached, red discoloured, strong putrid

From the above tables, it was found that black tiger shrimp stored at ambient temperature for more than 32 hours should not be processed as a raw product, since the samples could be rejected for decomposition. However, it was still found that frozen cooked peeled black tiger shrimp made from raw material stored at ambient temperature for 43 hours were borderline acceptable. For ice storage, black tiger shrimp should not be stored for more than 6 days.

When storage time increases, deterioration begins and the following characteristics could be found; discolouration, soft texture, slimy surface, milky appearance, stale, sour, musty and putrid odour. The discolouration, red or orange off-colour development in shrimp, is due to denaturation of astaxanthin-protein complexes and oxidation of red astaxanthin to orange astaxin and/or to the presence of a cryptaxanthin-like yellow pigment. Off-odour development in the shrimp appears to be divided into two categories: (a) musty and cooked shrimp odours due to chemical and/or enzymatic activity, and (b) putrid and sour odours due to bacterial activities. Cooked shrimp and musty odours occur in some samples which have little increase in bacterial levels, while putrid shrimp odours occur only in shrimp with high bacterial levels.

The indole contents in *Tables 1* to *3* show an increasing trend. The significant changes should be caused by substantial growth of indole forming bacteria. *Proteus* is believed to be responsible for the formation of indole. It has the ability to convert tryptophan in shrimp to indole. From the above tables, the values in some reject samples are much lower than the actionable level of 25 µg/100g established by USFDA. Thus, indole is of value in assessing the history of shrimp if high temperature abuse is suspected. The index is of less value if the spoilage has occurred in ice or at low temperature. It can be concluded that while indole levels indicate decomposition, decomposed shrimp may not contain indole. The level of indole in frozen shrimp is an indicator of pre-freezing quality and not the result of a substantial increase during frozen storage.

SENSORY PROFILE OF BLACK TIGER SHRIMP

Judgment on shrimp sensory quality is basically made based upon odour of decomposition. However, other characteristics such as appearance and texture could be used in association with odour for making more accurate decision. *Table 4* shows the sensory profile of black tiger shrimp of various quality levels which can be used as a guideline for shrimp product inspection.

Headless Shrimp

Cooked Shrimp

Table 4. Sensory Profile Of Frozen Black Tiger Shrimp

Quality level	Characteristics			
	Odour	**Appearance**	**Texture**	**Colour**
Passable	Fresh, sweet, neutral, grassy-seaweedy	Translucent	Firm	Uniform, colour typical of species
Borderline passable	Slightly stale, slightly yeasty, slightly fishy	Translucent	Slightly soft	Slightly discoloured
Borderline fail	Musty, slightly sour, fishy, old sock odour	Slightly opaque, black spots	Tough, soft, dehydrated	Bleached or faded
Fail	Putrid, ammonical, faecal, chemical-fuel contaminants	Opaque, cooked appearance	Soft, mushy	Discoloured

LOT ACCEPTANCES

Decision is made to inspect a shipment of frozen shrimp products in accordance with the standard for sensory attributes. Samples will be drawn in a random and representative manner based on the number of units in each lot using an AQL 6.5 sampling plan established by Codex Alimentarius Commission. Samples to be evaluated for quality will be thawed under controlled conditions to minimise any additional quality loss. All of the shrimp in each sample unit will be evaluated and categorised for compliance with the standard. When the total number of shrimp in a sample unit which are determined to be tainted and /or decomposed exceeds 10% then that unit will be considered to be defective. A lot will be considered unacceptable when the number of defective units exceeds the acceptance number for the sample size designated in the sampling plan (note that the acceptance number for decomposition is lower than for taint). A lot will be considered unacceptable when any single instance of critical foreign material occurs.

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Chapter III

A Simple Microbiological Method for Determining Presence and for Classifying of Antibiotic Residues in Marine Food Products

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A Simple Microbiclogical Method for Determining Presence and for Classifying of Antibiotic Residues in Fish Products

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SIMPLE EXAMINATION METHOD FOR ANTIBIOTIC RESIDUES IN MARINE FOOD PRODUCTS

Drug residue in aquacultured shrimp present concerns to health authorities and consumers. The best measure to prevent a health threat to consumers is the proper management of farm, and if drugs are needed, they are to be used with care, correct amount and type. In circumstances where control is in the hand of processors, determination of drug residue in raw material fifteen to twenty one days before harvesting for residue level in raw material is recommended. Detection of residue when the raw material is received at the factory is only good for establishing a suppler history.

Drug residues can be detected by either a simple microbiological assay or sophisticated chemical methods.

The following method is a simple microbiological assay developed by the Ministry of Health and Welfare, Japan for testing a few groups of antibiotics, and could be utilised by processors and regulators for screening purposes.

1. Materials

I) Assay Strain

- a) *Micrococcus luteus* ATCC 9341 (hereinafter called *M. luteus*)
- b) *Bacillus subtilis* ATCC 6633 (hereinafter called *B. subtilis*)
- c) *Bacillus mycoides* ATCC 11778 (hereinafter called *B.mycoides)*

II) Culture Medium

- a) Agar medium for storage and maintenance Ordinary agar medium (Nutrient Agar)*[1)]
- b) Liquid medium for propagation Bouillon for measurement of sensitivity (Sensitive Test Broth)*[1)]
- c) Medium for mixing with assay strain
  (1) Antibiotic Medium 5 (Difco) (hereinafter called AM5)
  (2) Antibiotic Medium 8 (Difco) (hereinafter called AM8)

*[1)] Nissui Seiyaku's products or similar mediums.

III) Petri Dish

Sterilised Petri dish with an inner diameter of 86±1mm.

IV) Paper Disk

Paper disks with a diameter of 10mm and thickness of 1.1-1.2 mm are used*2).

The paper disks are sterilized for 15 min at 121°C and are completely dried before use.

*2) Toyo Roshi's (filter) products for Antibiotics assay paper disks of meat or a paper disk of required thickness cut to size.

V) Buffer Solution

a) *Citric Acid Acetone Buffer Solution*

Prepare the buffer solution by mixing of 30vol.% distilled water, 35vol.% acetone, and 35vol.% mixture of 0.2M citric acid solution and 0.5M potassium hydroxide solution in the same volume.

Prepare the 0.2M citric acid solution with 4.2g of monohydrate citric acid ($C_6H_8O_7$:MW210.14) which is dissolved in distilled water with a total volume of 100ml.

Prepare the 0.5M potassium hydroxide solution with 2.8g potassium hydroxide (KOH:MW56.11) which is dissolved in distilled water with a total volume of 100ml.

b) *pH 4.5 Phosphate Buffer Solution*

Prepare the pH4.5 buffer solution with 13.6g of potassium dihydrogen phosphate (KH_2PO_4:MW136.09) which is dissolved in distilled water with a total volume of 1000ml.

c) *pH 6.0 Phosphate Buffer Solution*

Prepare the pH6.0 buffer solution with 8.0g potassium dihydrogen phosphate (KH_2PO_4: MW136.09) and 2.0g dipotassium hydrogenphosphate (K_2HPO_4:MW174.18) which are dissolved in distilled water with a total volume of 1000ml.

d) *pH 8.0 Phosphate Buffer Solution*

Prepare the pH 8.0 buffer solution with 0.523g potassium dihydrogen phosphate (KH_2PO_4:MW136.09) and 16.73g dipotassium hydrogenphosphate (K_2HPO_4:MW174.18) which are dissolved in distilled water with a total volume of 1000ml.

Note: The phosphate buffer solutions of pH 4.5, 6.0 and 8.0 are preferred to be prepared just before they are used. The solutions should be stored under sealed condition after being sterilised for 15 min at 121°C under high pressure. Solutions with precipitates and turbidity can not be used.

2. Storage and Maintenance of Assay Strain

I) *M. luteus*

a) Incubate the assay strain on a slant nutrient agar at 30°C for 18 hours. After being confirmed that *M. luteus* spreads on the medium, then seal it with a sterilized rubber stopper and store in a refrigerator. The transplantation interval of maintenance is one month or one and a half months.

b) Seed purely cultured thick fresh bacteria (multiplied on nutrient agar medium plate) into 1 ml. nutrient broth with 10-20% defated (skim) milk or with glycerin and seal it with a sterilized rubber stopper and store it in a freezer*3). It can be kept (stored) for longer duration than a slant nutrient agar medium.

c) Keep the assay strain in a refrigerator or freezer using freeze-drying method.

*3) The concentration of glycerin is 10-16% when it is preserved at -70°C, 40% when at -20°C.

II) *B. subtilis*

a) Incubate the assay strain on a slant nutrient agar medium at 30°C for 18 hours. After being confirmed that *B. subtilis* spreads on the medium, seal it with a sterilized rubber stopper and store in a refrigerator. The transplantation interval of maintenance is one month or one and a half months.

b) Subdivide the assay strain which is produced by (3. Preparation of Assay Strain Solution); those for short storage within one month are put in a refrigerator, those for longer storage are put in a freezer.

c) Keep the assay strain in a refrigerator or a freezer using freeze-drying method.

III) *B. mycoides*

a) The assay strain is incubated on a slant nutrient agar medium at 30°C for 18 hours. After being confirmed that *B. mycoides* spreads on the medium, it is then sealed with a sterilized rubber stopper and stored in a refrigerator. The transplantation interval of maintenance is one month or one and a half months.

b) Subdivide the assay strain which is produced by (3. Preparation of Assay Strain Solution); those for short storage within on month are put in a refrigerator, those for longer storage are put in a freezer.

c) Keep the assay strain in a refrigerator or a freezer using freeze-drying method.

Note: It's convenient when these assay strains are maintained and stored in several different methods. Each method mentioned above can be used for the maintenance. The best way is using all the 3 methods to maintain the assay strains.

3. Preparation of Assay Strain Solution

I) *M. luteus*

Inoculate the stored assay strain into the Sensitivity test broth, incubate it for 18 hours at 30°C , maintain three generations, use the medium solution of the third generation as assay strains.

II) *B. subtilis*

Apply the stored assay strain on a nutrient agar medium plate, culture it for one week at 30°C to produce spores*[4)]. Scrape the grown bacteria on the plate, put it into sterilized physiological saline solution and heat it up for 30 min at 65°C. Then centrifuge it for 20 min at 3000rpm, throw away the supernatant. Put the sediment into sterilized physiological saline solution again, and use it as the spore solution.

Produce serial dilutions of the spore solution, mix a 1% dilution into AM5 which is kept at 50°C flow 8ml of the mixture into a petri dish to form a plate. Put a paper disk wetted with 0.5μg/ml kanamycin solution on each plate and culture them for 18 hours at 30°C . Find out the concentration whose inhibition zone is 14±1 mm in diameter. Produce the dilution with the same concentration as the assay strain solution. The number of spores in the assay strain solution is about 10^7-10^8/ml.

III) *B. mycoides*

Apply the stored assay strain on a nutrient agar medium plate , culture it for one week at 30°C to produce spores*[4)]. Scrape the grown bacteria on the plate, put it into sterilized physiological saline solution and heat it up for 30 min at 65°C. Then centrifuge it for 20 min at 3000rpm, throw away the supernatant. Put the sediment into sterilized physiological saline solution again, and use it as the spore solution.

*[4)] Dye the spores, see them under a microscope and confirm that there are 80% spores in a visual field of microscope. If the productivity of spores is not enough, incubate the plate for few more days to multiple spores. If even after an incubation period of more than 10 days, the number of spores is still not enough, it is considered that there are something wrong with the assay strain and do not use such assay strain.

Produce dilutions with gradual decreasing method from the spore solution, mix a 1% dilution into AM8 which is kept at 50°C, flow 8ml of the mixture into a petri dish and to form a plate. Put a paper disk wetted with 0.25μg/ml oxytetracycline

solution on each plate and culture them for 18 hours at 30°C. Find out the concentration whose inhibition zone is 14±1mm in diameter. Produce the dilution with the same concentration as the assay strain solution. The number of spores in the assay strain solution is about 10^7-10^8/ml.

4. Preparation of Assay Plates

I) *M. luteus* Plate

Add *M. luteus* assay strain solution into five times AM5 which was sterilized for 15 min at 121°C under high pressure and was then kept at 50°C. After well mixing pour 8ml of the mixture into each petri dish and coagulate them evenly. However, we have to put a paper disk wetted with 0.025μg/ml ampicillin solution on the plate, incubate it for 18 hours at 30°C, the diameter of inhibition zone must be 14±1mm. The sensitivity test must be carried out on more than one plate for one lot, there is no necessity to test all plates.

Do not use plates from the lot with inhibition zone greater or smaller than 14±1mm in diameter*[5].

II) *B. subtilis* Plate

Add *B. subtilis* assay strain solution into one hundred times AM5 which was sterilized for 15 min at 121°C under high pressure and was then kept at 50°C. After well mixing pour 8ml of the mixture into each petri dish and coagulate them evenly. However, we have to put a paper disk wetted with 0.5μg/ml kanamycin solution on the plate, and incubate it for 18±1 hours at 30°C, that the diameter of inhibition zone must be 14±1mm. The sensitivity test must be carried out on more than one plate for one lot, there is no necessity to test all plates.

Do not use plates from the lot with inhibition zone greater or smaller than 14±1mm in diameter*[5].

III) *B. mycoides* plate

Add *B. mycoides* assay strain solution into one hundred times AM8 which was sterilized for 15 min at 121°C under high pressure and was then kept at 50°C. After well mixing pour 8ml of the mixture into each Petri dish and coagulate them evenly. However, we have to put a paper disk wetted with 0.25μg/ml oxytetracycline solution on the plate, culture it for 18±1 hours at 30°C, the diameter of inhibition zone must be 14±1 mm. The sensitivity test must be carried out on more than one plate for one lot, there is no necessity to test all plates.

Do not use plates from the lot with inhibition zone greater or smaller than 14±1mm in diameter*[5].

*[5] When the following reasons: (1) a variation in assay strain, (2) a mistake in amount of bacteria to be inoculated, (3) a mistake in preparation of assay medium plates, and (4) wrong

concentrations of antibiotic in paper disks, etc., are considered, the preparation should be carried out again.

Note: The inhibition zones which form in the plates of *M. luteus*, *B. subtilis* and *B. mycoides* should have a clear boundary and has no colony inside the zone and the assay strain on the plate must be smooth without spots. The plates which do not meet the above conditions cannot be used since they may have a variation and may be contaminated.

5. Standard Solution of Antibiotics

Prepare a standard solution having a potential titre equivalent to 1000μg/ml of the standard antibiotics. Use antibiotics with a known factor (in the unit of 0.1mg) for this preparation. Using a sterilized messpipette, dilute the standard solution with the phosphate buffer solutions following minimum inhibition concentration method to prepare the working standard solution. The messpipette should be changed every time to dilute different solutions. Basically, the 1000μg/ml standard solution is prepared for every measurement.

The working solution can also be prepared using 10 times dilution method.

Example: Prepare standard solution of ampicillin.
If the potential on the label of bottle containing sodium ampicillin is 840g, weight exactly 10mg of the powder and dissolve it in 8.4ml sterilized distilled water, then a solution with titre of 1000μg/ml is formed.

Prepare the following working solution with the same method:

I) Ampicillin Standard Solution
Prepare the 1000μg/ml ampicillin standard solution by dissolving sodium ampicillin in sterile distilled water. Use pH6.0 phosphate buffer solution for dilution.

II) Kanamycin Standard Solution
Prepare the 1000μg/ml kanamycin standard solution by dissolving kanamycin sulfate in sterile distilled water. Use pH8.0 phosphate buffer solution for dilution.

III) Oxytetracycline Standard Solution
At first, dissolve oxytetracyclin hydrochloride in a small amount of 0.1N HCl and add necessary amount of sterile distilled water to prepare a standard solution of oxytetracycline of 1000μg/ml. Use pH4.5 phosphate buffer solution for dilution.

P.S. Dissolve macrolide antibiotics in a small amount of methanol first, then add sterilized distilled water to prepare standard of each macrolide antibiotic solution of 1000μg/ml . For dilution, use pH8.0 phosphate buffer solution.

6. Preparation of Sample Solution

Weigh 5g of muscles, meat or internal organs, add 20ml citric acid-acetone buffer solution, homogenize it, filter it with a filter paper, and the filtrate is the sample solution. If the filtering is difficult, centrifuge it for 15 min at 3000rpm, filter the supernatant.

7. Testing Method

Dip a paper disk into the sample solution, put the paper disk on a assay plate*[6], and press down gently with a pair of forceps. Keep it in a refrigerator for more than 30 min, then incubate it for 18 hours at 30°C.

Use the paper disk wetted with citric acid-acetone buffer solution as a negative control.

*[6] For one sample solution, use more than two paper disks for respective assay plates.

8. Judgement

When the diameter of inhibition zone is greater than 12mm, the result is positive*[7]. Confirm that the negative control of citric acid-acetone result is negative.

*[7] Clear inhibition zone shows that the result is positive and indicates the presence of inhibitory substances to the test organisms in the sample, otherwise it is negative.

CLASSIFYING AND ESTIMATING METHOD FOR ANTIBIOTIC RESIDUES IN MARINE FOOD PRODUCTS

1. Materials

I) Assay Strain

- a) *Micrococcus luteus* ATCC 9341 (hereinafter called *M. luteus*)
- b) *Bacillus subtilis* ATCC 6633 (hereinafter called *B. subtilis*)
- c) *Bacillus mycoides* ATCC 11778 (hereinafter called *B. mycoides*)

II) Culture Medium

- a) Agar medium for storage and maintenance
  Ordinary agar medium (Nutrient agar)*[8]
- b) Liquid medium for propagation
  Bouillon for measurement of sensitivity (Sensitivity test broth)*[8]
- c) Medium for mixing with assay strain
  - (1) Antibiotic Medium 5 (Difco) (hereinafter called AM5)
  - (2) Antibiotic Medium 8 (Difco) (hereinafter called AM8)

*8) Nissui Seiyaku's products or similar mediums.

III) Petri Dish

Sterilised petri dish with an inner diameter of 86±1mm.

IV) Paper Disk

Paper disks with a diameter of 10mm and thickness of 1.1-1.2mm are used*9).
The paper disks are sterilized for 15 min at 121°C and are completely dried before use.

*9) Toyo Roshi's (filter) products for Antibiotics assay paper disks of meat or a paper disk of required thickness cut to size.

V) Buffer Solution

a) *pH4.5 Phosphate Buffer Solution*
Same as mentioned in session {b) pH4.5 Phosphoric Acid Buffer Solution of V) Buffer Solution of (1. Materials)} in "Simple Examination Method for Antibiotic Residues in Livestock and Marine Food Products (Revised)".

b) *pH8.0 Phosphate Buffer Solution*
Same as mentioned in the session {d) pH8.0 Phosphoric Acid Buffer Solution of V) Buffer Solution of (1. Materials)} in "Simple Examination Method for Antibiotic Residues in Livestock and Marine Food Products (Revised)".

c) *pH4.0 Macllvaine Buffer Solution*
To prepare this buffer solution, mix 12.29ml of 0.1M citric acid solution and 7.71ml of 0.2M disodium hydrogenphosphate solution.

d) *pH4.0 Macllvaine Buffer Solution with Containing 0.01M EDTA-2Na*
Add ethylene diamine tetraacetate disodium salt (EDTA-2Na) in pH4.0 Macllvaine buffer solution to prepare its 0.01M solution.

e) *pH3.0 Macllvaine Buffer solution*
Prepare this buffer solution by mixing 15.86ml of 0.1M citric acid solution and 4.11ml of 0.2M disodium hydrogenphosphate solution.

VI) Column

a) SEP-PAK C18 Cartridge*10) (hereinafter called C18 Column)
C18 Column is treated with 5ml methanol, 5ml distilled water and 5ml saturated EDTA-2Na solution. The flow rate for the treatment is 1.5 ml/min.

b) Baker 10 CARTRIDGE carboxylic acid extraction column*11)
First pump 5ml of hexane into the COOH column and vacuate it for about 1 min. Then the column is treated with 5ml of methanol, 5ml of distilled water and 5ml of pH 4.0 Macllvaine buffer solution. The flow rate for the

treatment is about 1.5ml/min. The column is wetted by pH4.0 Macllvaine buffer solution until charge with the sample.

*[10] The product of Waters (Millipore Co.) or the similar product.

*[11] The product of J.T. Baker Inc. or the similar product.

2. Storage and Maintenance of Assay Strain

Same as mentioned in session (2.Storage and Maintenance of Assay Strain) in "Simple Examination Method For Antibiotic Residues in Livestock and Marine Food Products (Revised)".

3. Preparation of Assay Strain Solution

Same as mentioned in session (3. Preparation of Assay Strain Solution) in "Simple Examination Method for Antibiotic Residues in Livestock and Marine Food Products (Revised)".

4. Preparation of Assay Plates

Same as mentioned in session (4. Preparation of Assay Plates) in "Simple Examination Method for Antibiotic Residues in Livestock and Marine Food Products (Revised)".

5. Preparation of Sample Solution

Add a 10g sample in 30ml of pH4.0 Macllvaine buffer solution which contains 0.01M EDTA. Homogenize it and centrifuge it for 15 min at 3000rpm. Gather the supernatant, add 10ml hexane into it, mix them well by stirring, then centrifuge the mixture for 15 min at 3000rpm. Gather the aqueous layer, add 30ml chloroform into it, mix them well by stirring, then centrifuge the mixture for 15 min at 3000rpm. Gather chloroform layer, evaporate it to dryness under reduced pressure at less than 40°C. Dissolve the residue in 1ml of pH8.0 phosphate buffer solution. The resulted solution is the sample solution A.

On the other hand, flow the aqueous layer into the C18 column, then into the COOH Column. Wash the C18 column with 10ml distilled water, then flow with methanol. Throw the first 0.5ml water away and gather 5ml effluent (methanol). Evaporate it to dryness under reduced pressure at less than 40°C. Dissolve the residues into 1ml pH4.5 phosphoric acid buffer solution. The resulted solution is the sample solution B.

Pass pH3.0 Macllvaine buffer solution into the COOH Column, gather the 5ml effluent. Use 5N NaOH and 1N NaOH solutions to adjust the pH of the effluent to pH7.5. The effluent is the sample solution C.

6. Testing Method

Put the paper disks wetted with the samples solution on the assay plates of *B. subtilis, M. luteus* and *B. mycoides* and press down gently with a pair of forceps. Keep these

plates with the paper disks in a refrigerator for 30 min, then incubate them for 18 hours at 30°C.

7. Judgement

When the diameter of inhibition zone is greater than 12mm, the result is positive. Estimate the type of antibiotics remained in the livestock and marine food products from the sensitivity pattern of the three assay strains shown in *Table 1*.

Furthermore, penicillin is inactivated with penicillinase.

Note: When there are inhibition zones formed in all the three plates with, dilute the sample solution to a suitable concentration and test again.

Table 1: Classification and Estimation of Antibiotics by Sensitivity Pattern of Assay Strains

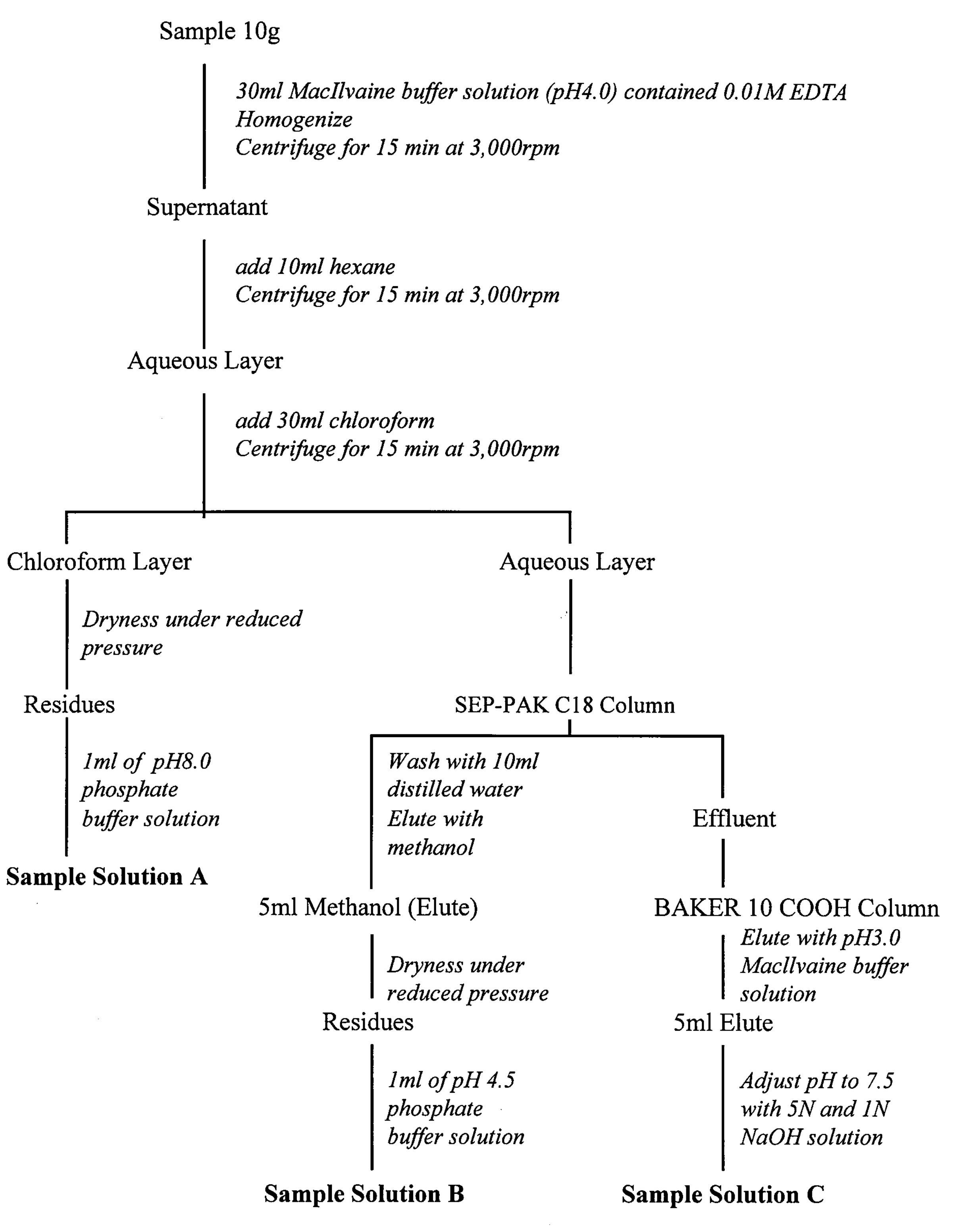
Sample Solution	Assay Strain			Antibiotics
	B. subtilis	*M. luteus*	*B. mycoides*	
A	+ -	++ +	- -	Macrolides
B	+ -	- -	++ +	Tetracyclines
	+ -	++ +	- -	Penicillins
C	++ +	- -	+ -	Aminoglycosides

Note:

++ represents bigger inhibition zone (diameter) than that shown by + .

\- means no inhibition zone formed.

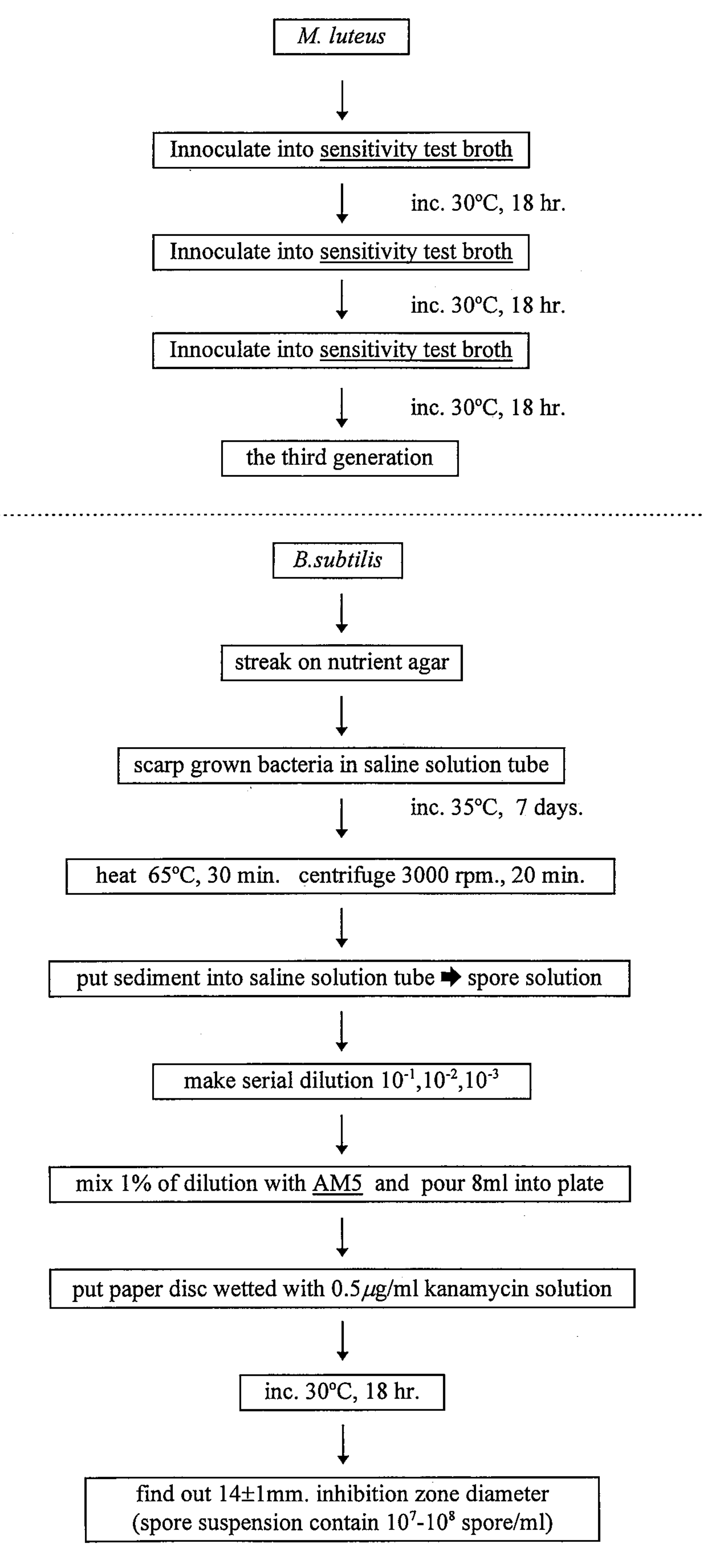
Figure1: Preparation Of Sample Solutions For Estimating Antibiotics in Livestock And Marine Food Products

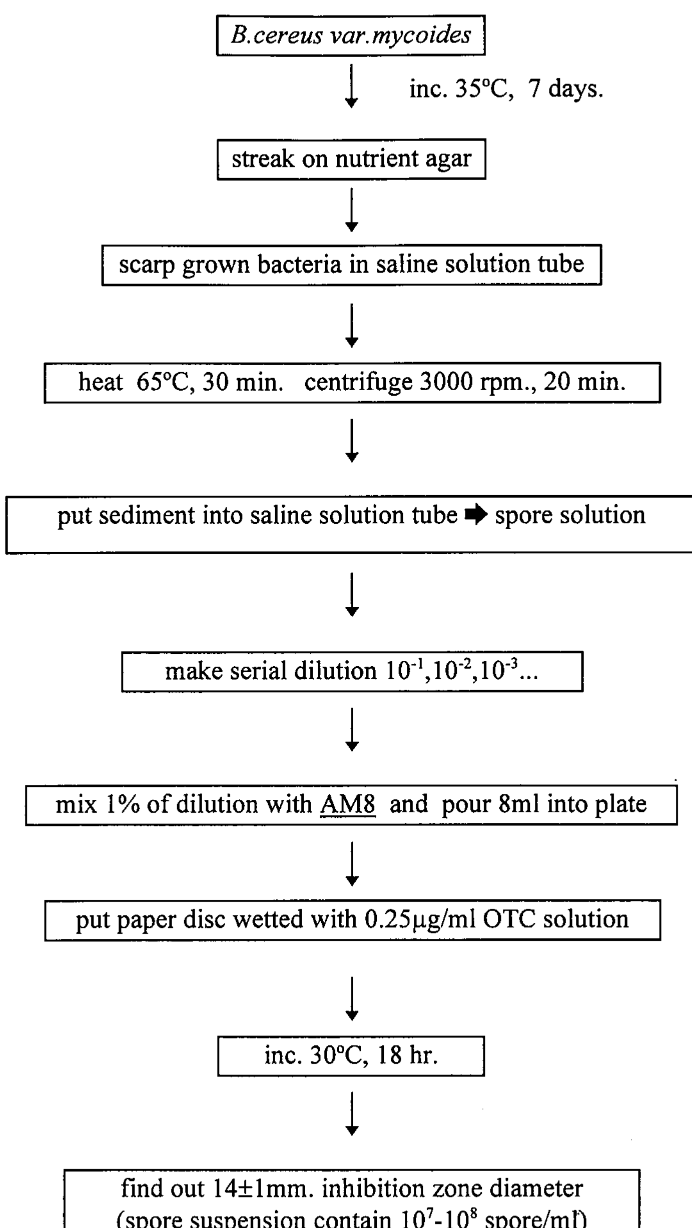


Reference:

Method developed by the Ministry of Health and Welfare, Japan.

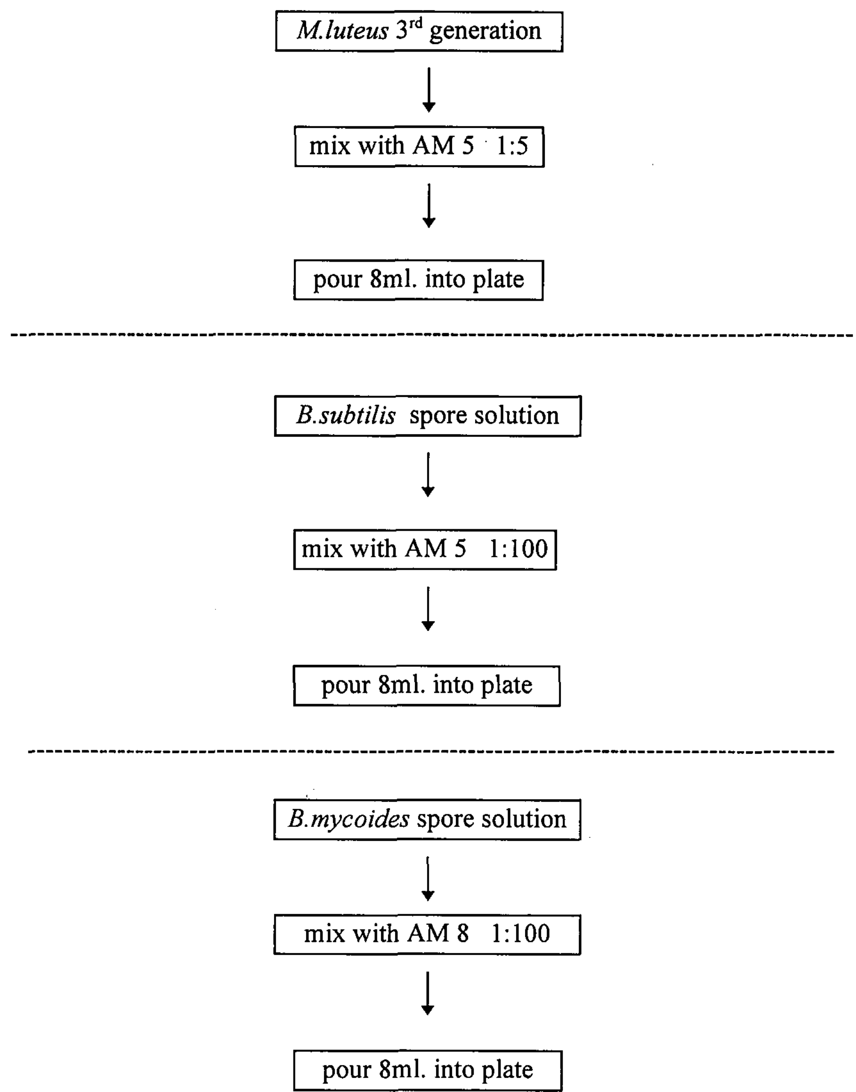
Preparation of Assay Strain Solution





B.cereus var.mycoides
inc. 35°C, 7 days.
streak on nutrient agar
scarp grown bacteria in saline solution tube
heat 65°C, 30 min. centrifuge 3000 rpm., 20 min.
put sediment into saline solution tube ➡ spore solution
make serial dilution 10-1,10-2,10-3...
mix 1% of dilution with AM8 and pour 8ml into plate
put paper disc wetted with 0.25µg/ml OTC solution
inc. 30°C, 18 hr.
find out 14±1mm. inhibition zone diameter
(spore suspension contain 10⁷-10⁸ spore/ml)

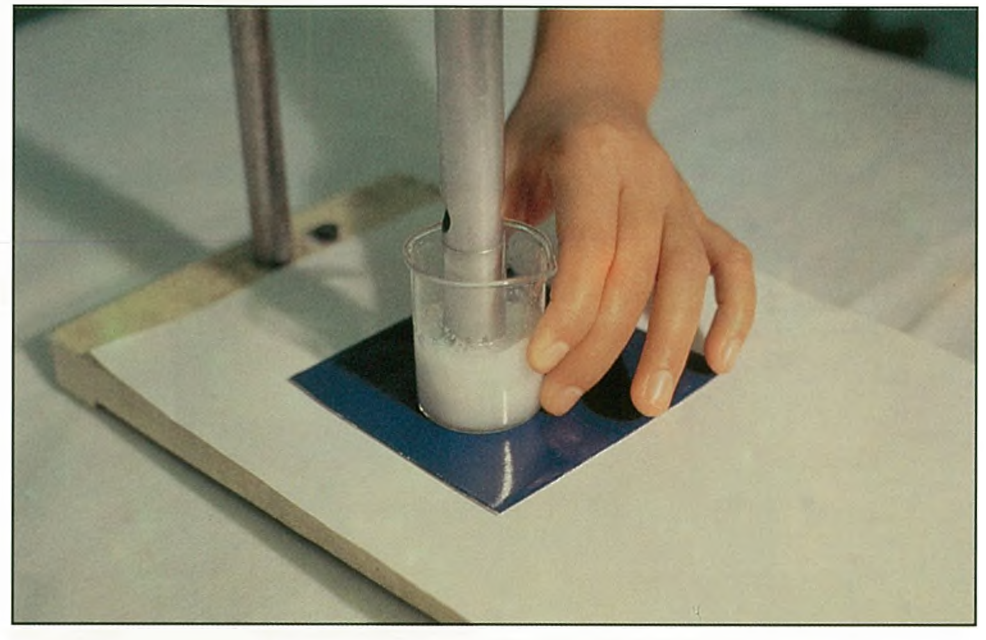
Preparation of Assay Plates



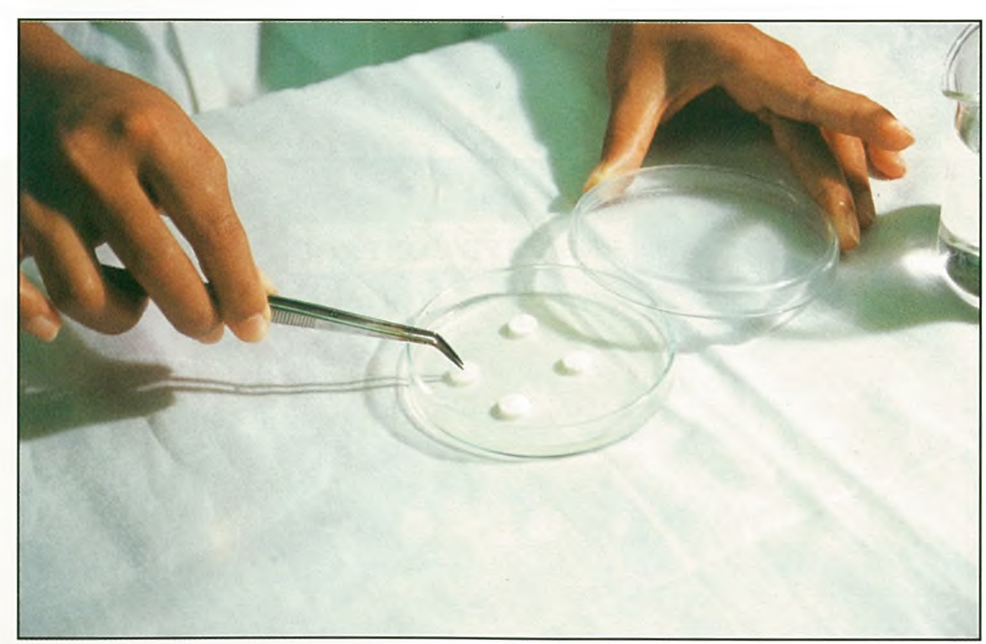
Determination of Oxytetracycline Residues by Microbiological Assay

Shrimp Sample

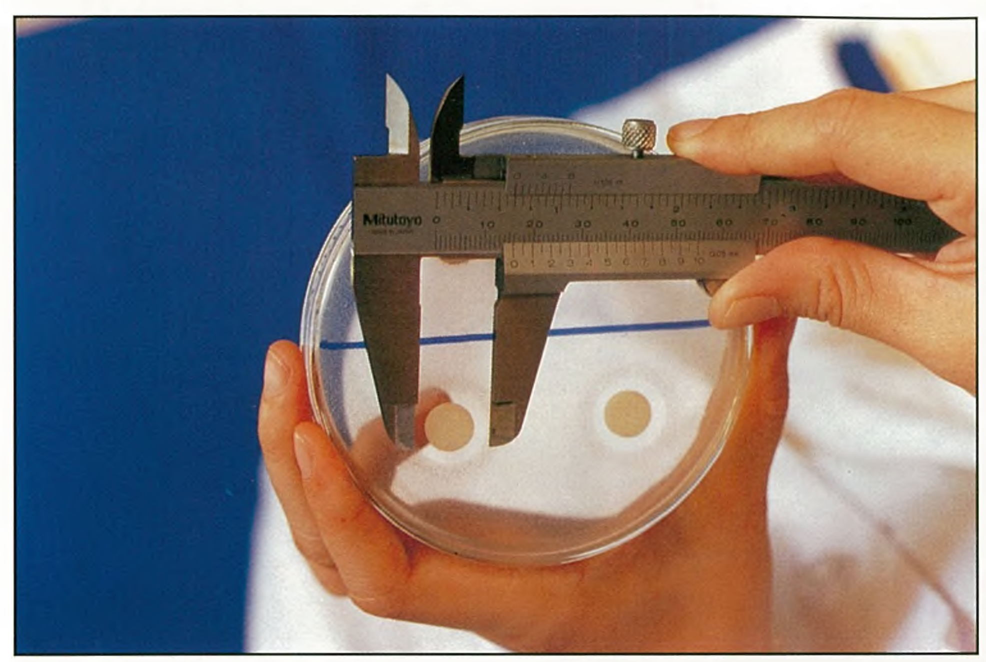
(1) Peeled and blend shrimp sample.	→	(2) Weigh 5.0 g. of samples + 20ml citric acid-acetone buffer solution.



(3) Homogenize at 5,000 rpm for 1 min. → (4) Filter through filter paper Whatman No.1



(5) Dip a paper disk into the sample solution. Put disk on an agar plate and → (6) use the paper disk wetted with citric acid-acetone buffer solution as a negative control. → (7) Incubate 30°C 18 hours.



(8) If diameter of inhibition zone is greater than 12 mm. the result is positive.

→

(9) Confirm that negative control of citric acid-acetone result is negative.

Positive sample, negative control

Chapter IV

Determination of Drug and Chemical Residue by HPLC

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Determination of Drug and Chemical Residue by HPLC

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OXOLINIC ACID

1. Principle

The Quinolone oxolinic acid (5-ethyl-5,8-dihydro-8-oxo-1,3-dioxolo[4,5-g] quino-line-7-carboxylic acid) is a commonly used antibiotic for treatment of infectious, diseases in fish farming. Tissue is extracted by homogenizing with dry ethyl acetate the organic phase is evaporated, the residue partitioned between aqueous oxalic acid and hexane. The aqueous phase is chromatographed using fluorometric detection. The LC is based on the method described by Ikai et. al.(1989).

2. Apparatus

2.1 Liquid Chromatography: Isocratic pump system, injector, fluorometric detector capable of monitoring emission at 369 nm and excitation at 327 nm.

2.2 Chromatographic column: Reverse phase, Nova-Pak C18 (150 mm x 3.9 mm). Operating condition: set flow rate 1.0 ml/min. As a part of the system shut-down at the end of the day, the mobile phase is pumped through the column for a minimum of fifteen minutes followed by a fifteen-minute rinse with methanol at 1 ml/min.

2.3 Homogenizer

2.4 Centrifuge

2.5 Rotary Evaporator

2.6 Filter Unit: Hyperclean Syringe Filters, 0.45μm Nylon, Non-Sterile, 13mm id. (Shandon Cat. No. 66982331)

2.7 Centrifuge tube: Oak Ridge Centrifuge Tube, PA (Nalgene Cat. No. 3119-0010)

3. Reagents

3.1 Anhydrous Na_2SO_4

3.2 Ethyl acetate AR-grade

3.3 n-Hexane AR-grade

3.4 Methanol HPLC-grade

3.5 Acetonitril HPLC-grade

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

Primary Standard Solution (100 μg/ml): Weigh Oxolinic acid 0.01g into a 100ml volumetric flask. Add ca. 10ml of dimethyl sulfoxide and swirl until completely dissolved. Dilute to 100ml with acetonitril and mix.

Secondary Standard Solution (10 μg/ml) : Pipette 10ml of Primary Standard Solution into 100ml volumetric flask and dilute to volume with acetonitril.

Working Standard Solution (1 μg/ml): Pipette 10ml of Secondary Standard Solution into 100ml volumetric flask and dilute to volume with acetonitril.

3.7 Oxalic acid 0.01M: Weigh 1.26g Oxalic acid dihydrate to 1 litre volumetric flask and dilute to volume with water, using 3N NaOH to adjust pH of 0.01M Oxalic acid to pH3.3.

3.8 Mobile Phase : Methanol:Acetonitril:0.01M Oxalic acid (pH3.3) = 1:3:6, filtered through Nylon-66 membrane. Mobile phase should be prepared daily and stored in a glass container.

4. Procedure

4.1 Weigh homogeneous sample 5.0g into centrifuge tube and add 10.0 of anh. Na_2SO_4 and 30ml of Ethyl acetate.

4.2 Homogenize with Polytron Homogenizer at 5,000rpm for 1 minute. Rinse probe with 5ml Ethyl acetate.

4.3 Centrifuge at 5,000rpm for 5 minutes. Filter the supernatant through filter paper Whatman No. 541 to 250ml Round Bottom Flask (RBF).

4.4 Rehomogenize the sediments with another 30ml Ethyl acetate and rinse probe well with 5ml of Ethyl acetate. Centrifuge at 5,000rpm for 5 minutes and combine the supernatant with the supernatant from the first extraction.

4.5 Evaporate the solvent in 250ml RBF to dryness by using Rotary Evaporator at 40°C until an oily liquid residue remains.

4.6 Redissolve residue with 5ml of mobile phase and 1ml of n-Hexane. Tube is capped and liquid is swirled, making sure that walls of the tube is thoroughly rinsed.

4.7 Centrifuge at 5000rpm for 5 minutes. Discard upper layer (n-Hexane) and transfer only clear aqueous liquid into a HPLC Autosampler vial. Sample preparations are stable in a refrigerator for 2-3 days.

4.8 Inject 200μl aliquots using a flow rate of 1 ml/min. with detector set at 327 nm excitation and 369 nm emission.

4.9 At the end of each day, rinse the system for 15 - 30 minutes with Methanol at 1 ml/min.

5. Calculation

$$\text{Oxolinic acid } (\mu g/g) = \frac{\text{Peak Area sample}}{\text{eak Area Std.}} \times \text{Conc. Std}$$

6. Method Validation

The recovery of Oxolinic acid from shrimp tissue was determined by spiking shrimp muscle tissue with 50, 80, 120, 160, 200 and 240 ng/g of Oxolinic acid, and submitting the samples to the described procedure. Each amount was added to five replicate portions. The recoveries ranged from 79 - 98%. The limit of quantification was 20 ng/g. Precision, expressed as percentage relative standard deviation, was below 4.82%.

The linearity of the method was checked by spiking the tissue with Oxolinic acid in the range 50 - 240 ng/g. Least-squares linear regression analysis of the data gave the equation: $y = 13.93x + 0.0087$, where y was the peak area and x was the amount of Oxolinic acid added. The correlation coefficient (r) was 0.9997.

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2. Ikai, Y., II. Oka, N. Kawamura, M. Yamada, K. Harada, M. Suzuki and H. Nakazawa (1989) J.Chromatography, 477, 397-406.

OXYTETRACYCLINE

1. Scope and Application

1.1 The method is applicable to fish and fish products.

2. Principle

This method permits the detection and identification of Oxytetracycline in animal tissues. The tetracyclines are extracted from the tissues with McIlvaine Buffer (pH4) and the filtered extract is then passed through a conditioned C-18 minicolumn (Sep-Pak C18). The tetracyclines are removed from the Sep-Pak C18 with methanol, the solvent evaporated and the residues dissolved in the HPLC mobile phase. The solution is chromatographed using fluorometric detection based on Japan Frozen Food Inspection Corporation.

3. Interferences

3.1 There are no known significant interferences.

4. Sampling Procedure And Storage

4.1 Take a representative sample from the product lot and store so as to maintain sample integrity.

5. Sample Preparation

5.1 Sample preparation should take into account the type of product and how it is used and prepared by the consumer.

5.2 For raw fish, fresh or thawed: pass the sample through a grinder a sufficient number of times to obtain a homogeneous blend.

5.3 For processed products containing no separable liquid: thaw in the package (if frozen) and pass the sample through a grinder a sufficient number of times to obtain a uniform mix.

5.4 Collect the homogenized sample into a thoroughly cleaned, sealable plastic cup or glass bottle. Store the sample in a refrigerator or freezer until required. Ensure that the prepared sample is still homogeneous prior to weighing. If liquid separates from the sample, thoroughly reblend before use.

6. Apparatus

6.1 50ml disposable polypropylene centrifuge tubes.

6.2 Polytron Homogenizer: Ika - Labortechnik Ultra - Turrax T25.

6.3 Centrifuge (capable of 5,000 rpm): Tomy Seiko Co., Ltd. with Rotor No. 9N.

6.4 Pasteur pipette, disposable.

6.5 Bond Elute C-18 disposable columns (Sep-Pak Plus C18), Part No. 20515.

6.6 Sample filter, Hyperclean Syringe Filter, 0.45 μm, Nylon membrane 13 mm.

6.7 Liquid Chromatography: Waters system consisting of 510 pump, fluorometric detector 470 capable of monitoring emission at 380 nm and excitation at 520 nm.

6.8 Chromatographic column: Reverse phase, Nova-Pak C18 (150 mm x 3.9 mm). Operating condition: set flow rate 0.8 ml /min. As a part of the system shut-down at the end of the day, the mobile phase is pumped through the column for a minimum of fifteen minutes, followed by a fifteen-minute rinse with methanol at 1 ml/min.

7. Reagents

7.1 Methanol HPLC-grade.

7.2 Imidazole AR-grade.

7.3 Disodium Ethylene Diamine Tetra-acetic acid AR-grade (Na_2EDTA).

7.4 Disodium hydrogen phosphate AR-grade (Na_2HPO_4).

7.5 McIlvaine Buffer/EDTA solution (pH = 4): Dissolve 28.41g of Na_2HPO_4 in distilled water in a 1 litre flask. Dilute to volume and mix. Dissolve 21.01g of citric acid monohydrate in distilled water in a 1 litre flask. Dilute to volume and mix. Combine 1 litre of the citric acid solution with 625ml of the Na_2HPO_4 solution in a 2 litres flask. Check the pH of this mixture; it should be 4.0 + 0.05.

Make the McIlvaine Buffer to 0.1 M EDTA by adding the appropriate weight and dissolving. For example, 37.224g EDTA/L x 1.625 L = 60.49 g.

7.6 Mobile Phase: 1 M Imidazole-Methanol. Dissolve Imidazole 68.08g, Magnesium acetate 10.72g and Na_2EDTA 0.37g with 800ml of deionized water, then adjust pH to 7.2 with Glacial Acetic acid. Adjust volume to 1 litre with deionized water. Mix the solution with Methanol at ratio 77:23.

7.7 Oxytetracycline (Sigma Chemical Company): Store the standard solutions below 10°C. Stock solution is stable for at least 3 months, but diluted solution should be kept no longer than 2 weeks.

Primary Standard Solution (100μg/ml): Weigh 0.01g of Oxytetracycline and into a 100ml volumetric flask. Add 0.01 N HCl a little and swirl until completely dissolved, dilute to volume with 0.1 N HCl.

Secondary Standard Solution (10μg/ml): Pipette 10ml of Primary Standard Solution into 100ml volumetric flask and dilute to volume with 0.1 N HCl.

Working Standard Solution (1μg/ml): Pipette 10ml of Secondary Standard Solution into 100ml volumetric flask and dilute to volume with 0.1 N HCl.

8. Procedure

8.1 Weigh 5.0g of whole or freshly homogenized tissues (see Note 1) into a 50ml polypropylene centrifuge tube.

8.2 Add 25ml of McIlvaine Buffer/EDTA solution (pH4) and homogenize with Polytron Homogenizer at 5,000rpm for 1 minute.

8.3 Centrifuge at 3,500rpm for 10 minutes. Filter the supernatant through filter paper Whatman No. 1 to 100ml beaker.

8.4 Rehomogenize the sediments with another 25ml McIlvaine Buffer / EDTA solution (pH = 4) and rinse probe well with 5ml of McIlvaine Buffer / EDTA solution. Centrifuge at 3.500rpm for 10 minutes and combine the supernatant with the one from the first extraction.

8.5 Load the extract on to the Sep-Pak C18 which had previously been deactivated by passing 10ml of Methanol, followed by 10ml of water dropwise through column. Regulate flow rate at ca 1 to 2 drops/sec. using valve to intriduce air leak into system.

8.6 Rinse the beaker with 10ml water and add to the reservoir when the extract is loaded on the column. Allow the column to run dry when the water rinse is conpleted and continue to draw air through the column for 5 minutes with vacuum at maximum.

8.8 Elute oxytetrscycline with 10ml of methanol to 125ml RBF. Evaporate the methanol by using Rotary Evaporator. Redissolve the residues with 5ml of mobile phase.

8.9 Filter the samples and standards through the Hyperclean Syringe Filter 0.45 um Nylon Membrane into HPLC vials. Inject 50μl to LC system at flow rate of 0.8ml/min. with detector set at 380 nm excitation and 520 nm emission.

8.10 At the end of each day, rinse the system for 15-30 min. with methanol at 1 ml/min.

9. Calculation

$$\text{Tetracyclines } (\mu g/g) = \frac{\text{Peak Area sample}}{\text{Peak Area Std.}} \times \text{Conc. Std.} \times \text{DF}$$

where: DF = Dilution Factor

10. Appendix

10.1 Note 1

It is preferable to use whole or freshly homogenized tissue as pre-homogenized tissue tends to cause the minicolumns to become plugged. This may be due to altered proteins in the homogenate. If pre-homogenized tissue is used, split the extract into two equal volumes and filter. changing filters between volumes.

11. Method Validation

The recovery of Oxytetracycline from shrimp tissue was determined by spiking shrimp muscle tissue with 50, 100, 200, 400, 800 and 1,600 ng/g of OTC, and submitting the samples to the described procedure. Each amount was added to five replicate portions. The recoveries ranged from 78 to 120%. The limit of quantification was 10 ng/g. Precision, expressed as percentage relative standard deviation, was below 15.1%.

The linearity of the method was checked by spiking the tissue with OTC in the range 50 - 1,600 ng/g. Least-squares linear regression analysis of the data gave the equation:
$y = 8.4957x + 0.1220$, where y was the peak area and x was the amount of OTC added. The correlation coefficient (r) was 0.9948.

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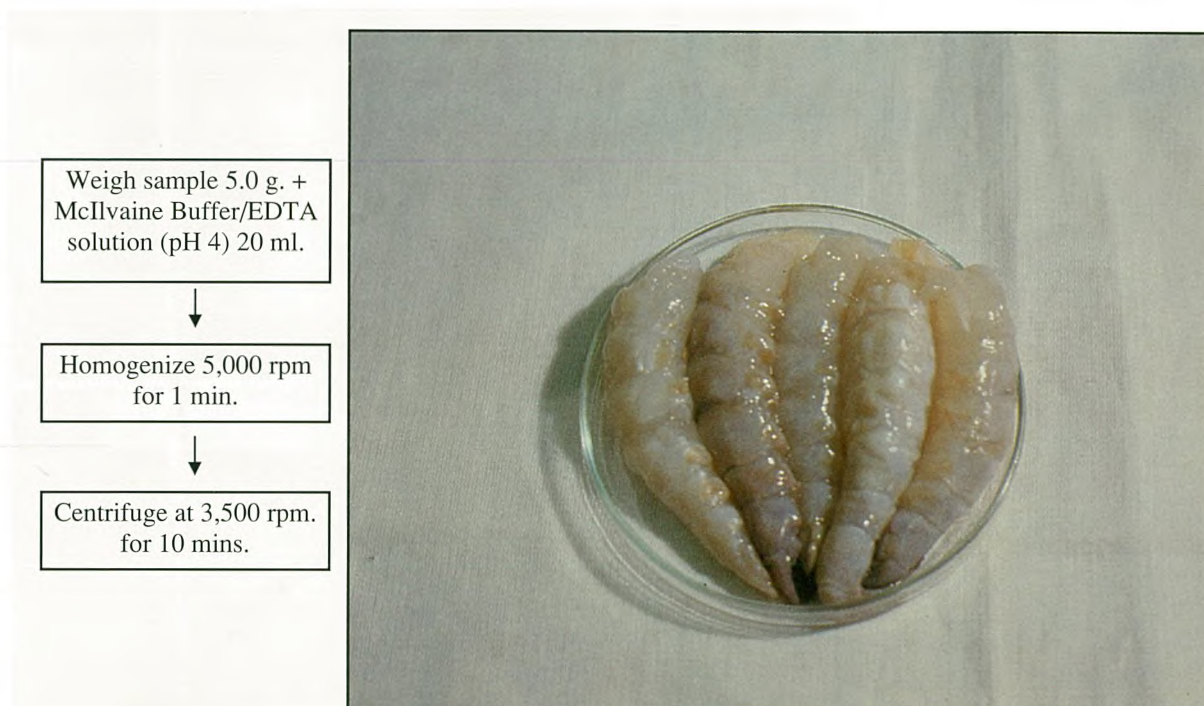
Determination of Oxytetracycline by HPLC



Re-homogenize the sediments with another 20 ml. McIlvaine Buffer/EDTA solution (pH 4)

↓

Centrifuge at 3,500 rpm. for 10 mins.

↓

Combine the supernatant with the supernatant from the first extraction.

Load the extract on to the Sep-Pak C18 which had previously been deactivated by padding 10 ml. of Methanol,

↓

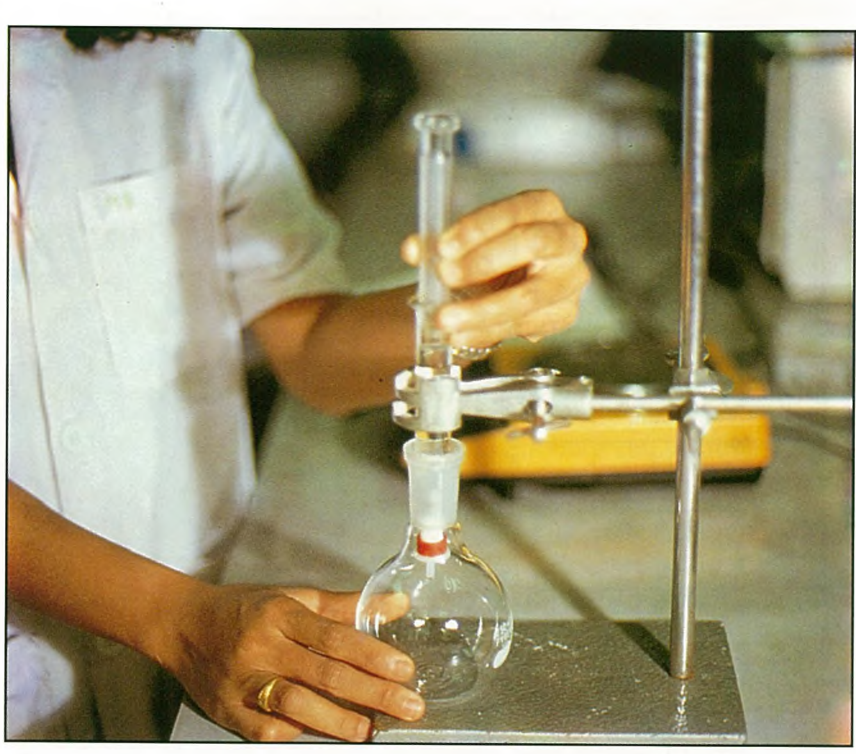
followed by 10 ml. of water dropwise through column. Regulate Flow Rate at ca 1-2 drops/sec.

↓

Elute Oxytetracycline with 10 ml. of methanol to 250 ml. RBF.

↓

Evaporate the methanol by using Rotary Evaporator at 40°C.



Re dissolve residue with 5.0 ml. of mobile phase.

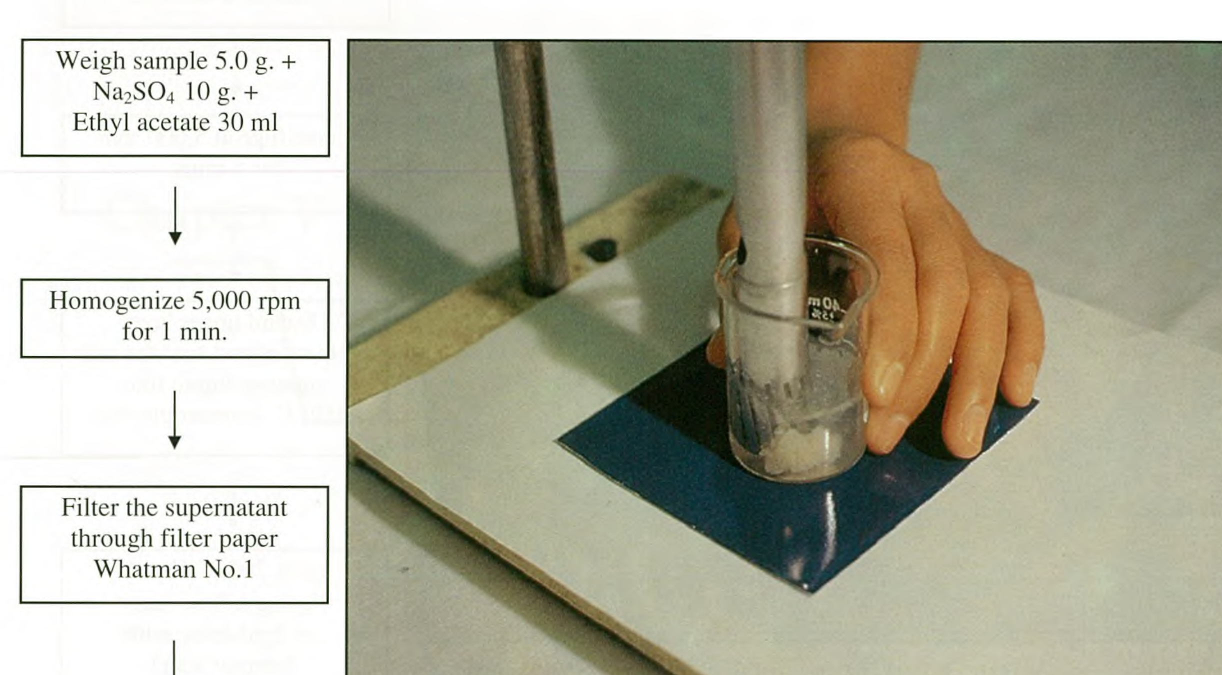
↓

Filter the samples through the Hyperclean Syringe Filter 0.45 µm Nylon menbrane into HPLC vials.

↓

Inject 50 µl to LC system at flow rate of 0.8 ml./min. with detector set at 380 nm. excitation and 520 nm. emission.

Determination of Oxolinic Acid by HPLC



Weigh sample 5.0 g. + Na_2SO_4 10 g. + Ethyl acetate 30 ml

↓

Homogenize 5,000 rpm for 1 min.

↓

Filter the supernatant through filter paper Whatman No.1

↓

Rehomogenize the sediments with another 30 ml. Ethyl acetate.

→

Combine the solvent in 250 ml. RBF. to dryness by using Rotary Evaporator at 40°C until an liquid remains.

Add solvent

Redissolve residue with 5 ml. of mobile phase and 1 ml. of n-Hexane.

↓

Centrifuge at 5,000 rpm for 5 mins.

↓

Discard upper layer (n-Hexane) and transfer only aqueous liquid into a HPLC Autosample vial.

↓

Inject 200μl aliquots using a flow rate of 1 ml./min. with detector set at 327 nm. excitation and 369 nm. emission.

Prepare for reading

Chapter V

Analysis of Antibiotic and Drug Residues in Agrifoods and Seafoods

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A. INTRODUCTION

Regulatory Overview

In United States the US Food and Drug Administration's Centre for Veterinary Medicine, Department of Health and Human Services, has the authority to regulate veterinary drug products in foods. The US Federal Food, Drug, and Cosmetic Act (FFDCA) covers food safety, medical devices, and other public health matters. Section 512 of the FFDCA deals with general safety provisions for new animal drugs while Section 409 and 706, respectively, cover safety aspects of food additives and colour additives. The code of Federal Regulation (CFR) subpart E relates directly to the safety of foods consumed by humans.

In Canada the Bureau of Veterinary Drugs, Health Drugs, Health Protection Branch, Federal Department of Health, is responsible for regulating use of veterinary drugs in food-producing animals. This process closely resembles the registration process in US as provided under a harmonisation initiatives of US-Canada Free Trade Agreement.

Regulatory authorities in US and Canada have established maximum residue limits (MRLs) for edible tissues. Unlike Canada, US has procedures for establishing an MRL for non-edible tissues when this tissues is the best target tissue for marker residue. The most common target tissue is liver or kidney.

Although the regulatory authority approving substances in US lies with FDA's Centre for Veterinary Medicine (CVM), the regulatory authority for determining compliance of animal-derived foods is the US Department of Agriculture, Food Safety and Inspection Services (FSIS). The residue control programs established by FSIS include the National Residue Program (NRP) major component of which includes antibiotic residue testing.

In Canada residue monitoring of drug residues in edible tissues is the function of Canadian Meat Inspection system of the Food Production and Inspection system of the Food Production and Inspection Branch, Federal Department of Agriculture and Agrifoods. Monitoring for antibiotic residues in cultured finfish is the responsibility of the Inspection Service Branch, Federal Department of Fisheries and Oceans.

There are less than 30 antimicrobial drugs approved by FDA for use in food-producing animals in the US. The tolerance of antimicrobial residues in foods permitted by the FDA ranges from approximately 0 - 10 ppm.

Although the average intake of meat has increased after World War II, the consumption of fish in Japan remains high when compared to other countries[1]. The different categories of feed additives in use in Japan include antioxidants, and anti-fungal agents (to prevent spoilage of feed) as well as 7 amino acids, 28 vitamins, and 32 minerals (used as dietary supplements). A separate group of 7 synthetics antibacterial with 21 antibiotics is used as feed additives to improve the efficiency of feeds[1].

In Japan use of antibiotics as feed additives and veterinary medicinal drugs are regulated by the Pharmaceutical Affairs Law and the Law Concerning Safety Assurance and Quality Improvement of Food. Another essential law is the Japanese Food Sanitation Law which states that no food should contain any antibiotic or synthetic antibacterial substances in meat, eggs, fish, shellfish, milk and dairy products.

In the European Union (EU) veterinary medicinal products are regulated as two distinct groups. The conventional therapeutic drugs, including those for prophylactic treatments, vaccines, and immunological products, are regulated under the Veterinary Medicine Directives 85/851/EEC and 85/852/EEC. The growth promoting antibiotics, the peptides, carbodox, coccidiostats, and olaquindox are regulated under Directive 70/524/EEC. Recent development in EU is the establishment of European Medicine Evaluation Agency (EMEA) based in United Kingdom. This agency authorises the use of drugs and many decisions are binding on Member States. Special EU legislation deals with the establishment of MRLs and are established by the Committee for Veterinary Medicinal Products (CVMP) through its Working Group on Safety of Residues.

Screening Methods

In both Canada and the US, testing programs based on evaluating animals at slaughter houses for the presence of antibiotic residues utilise one of the two commercially available microbial growth inhibition tests. The program uses either the Swab Test on Premises (STOP)[2] or the Calf Antibiotic and Sulfa Test (CAST)[3]. Both tests were originally developed by scientists at the USDA-FSIS. These tests although similar in principle use different growth media and different test organisms, providing a different range of test sensitivities to various antibiotic residues that may be present in samples[4].

In Canada, STOP and CAST test kits are used in random surveys of slaughter house animals to test for the prevalence of violative antibiotic residues. The suspect carcasses are detained pending on laboratory testing of kidney and muscle tissues.

The FSIS Microbiology Division has recently developed a new antimicrobial screening test designed to detect the presence of antibiotic and sulfonamide residues in animal tissues. The new test, Fast Antimicrobial Screen Test (FAST) was designed to read an in-plant test result in a shorter time than either STOP or CAST. FAST is currently implemented in calf slaughtering plants in the US.

Other screening tests used in US and Canada include screening of eggs, egg-products, and dairy products using Brilliant Black Reduction Test (BBR Test), the Charm II tests, Delvo Tests, and LacTek Tests.

In Japan a method based on microbiological assay[5] is used for detecting and classifying antibacterial residues. This method applies a filter paper disc method using Bacillus subtilis (ATCC 6637), Bacillus cereus var mycoides (ATCC 11778), and Micrococcus luteus (ATCC 9341) in identifying antibacterial residues in fractions of samples obtained by chemical separation. Fractions A, B, and C contained different antibiotics that give different growth inhibition patterns which allow their rapid identification and classification. The purpose of a screening is to quickly determine whether or not an analyte is present at or near the level of concern in the target sample.

Amongst member states of European Community the Four Plate Test[6] is very popular. For this method, also known as Frontier Post Test, uses four plates with three different organisms (Bacillus subtilis, two different species, and Micrococcus luteus). The organisms are grown at different pH conditions such that they respond differently to different antibiotics. Two discs of meat or offal removed from the sample with sterile cork borer are placed on the plates in duplicates. Discs of standard penicillin, streptomycin, sulfadimidine, and erythromycin are used as controls to indicate that the test has been successfully performed.

A test is positive when an annular zone greater than 2 mm appears around the discs taken from the food samples. The purpose of running four plates is to attempt to obtain some indication of the class of antibiotic that may be present. From the size and the pattern of the annular zones of inhibition it is suggested that some guidance can be obtained regarding the class of antibiotic in question.

Chemical Method

The use of chemical methods for antibiotic residues is limited because microbial inhibition methods are commonly used for regulatory purposes. Application of high-performance liquid chromatography methods for antimicrobials have been described[7-9]. The current range of chemical analysis techniques such HPLC, GC-MS, and HPLC-MS are powerful and useful for drug residue analysis; however complete facilities for analysis are expensive to set up. Currently HPLC methods are used for official analysis of synthetic antimicrobials.

There are two chemical methods for penicillin used by the EU Member States. One is the GC method of Miltschen and Petz[10] and the other is HPLC method of Boison et al[11].

The main chemical methods used for tetracyclines (tetracycline, chlortetracycline, and oxytetracyclin) are those of Ikai et al[12], Farrington et al[13], and Blanchflower[14].

Aminoglycosides pose problems in chemical analysis. Their solubility properties and their lack of distinctive chromaphores offer obstacle to an analyst. However, a very

recent publication of a method for streptomycin and dihydrostreptomycin[15] has appeared. A method for gentamycin[16] has been published in the EU Manual[17] that may be useful for neomycin.

Chemical methods for chloramphenicol available in EU utilise either GC (with or without coupled MS) or HPLC-GC methods[18,19]. There are several published methods for chloramphenicol[20,21].

Very little attention is being paid to develop chemical methods for aminoglycosides, macrolides, peptides antibiotics and ionophores. For monensin TLC and HPLC methods are available[22, 23].

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B. DETECTION OF DRUG AND ANTIBIOTIC RESIDUES USING RAPID KITS

Scope:

Antibiotics are used frequently in modern agriculture and aquaculture practices, both as additives to feed and water to enhance weight gain, improve feed efficiency and to prevent diseases; in higher dosages they are useful in treating specific diseases in terrestrial as well as aquatic animals. Consequences of such usage is contamination not only of food products but also the environment. Thus, routine inspection of food products has become an important function of public health agencies and regulatory authorities all around the world.

Not only has there been a great leap in the production of food globally but also international food trade has increased considerably. This has generated growing need for more and rapid testing methods. Public health and regulatory agencies as well as producers need help to cope up with increased volume of testing; they are looking for rapid and more cost-effective mechanisms to face the problem of food testing and meet the consumer demand for products free of antibiotic or chemical residues.

Biological assays introduced in early 1970s had appropriate properties required to meet high-volume testing. These microbiological assays, however, failed to provide the desired specificity in the detection of specific antibiotics. Today both microbiological and immunological assays are used with improved specificity and sensitivity.

Principle:

a. Microbiological Inhibition Tests

Microbiological assays involve growth (either on solid agar or liquid broth medium) and are based on definable parameters which characterise response of test organisms in the presence of antibiotics. The microbial response to a series of standard antibiotic concentrations with specific strain of a test organism is used to determine the levels of an antibiotic in an unknown sample.

Agar diffusion tests are conducted by inoculating a nutrient agar with specific test organism and applying a solution containing the drug residue to the agar surface in more than one way. The growth medium is then incubated for a specific period of time at a specified temperature.

In biological assays the response of the test organism is determined by measuring the zone of inhibition (ZI) formed in plates in the presence of antimicrobials. Tests that include measurement of ZI include Live Animal Swab Test (LAST), Calf Antibiotic and Sulfa Test (CAST), Swab Test on Premises (STOP), and Bacillus stearothermophilus disc assay (BSDA).

Another method to test inhibition of microbes is by incorporating redox indicators in the test medium. Examples of such test include Brilliant Black Reduction Test (BBR), Arla Micro Test, and Rapid Antibiotic Test (LUMAC). A redox indicator

like Brilliant Black is purple in its oxidised form and yellow when reduced. Thus organisms growing in the absence of inhibitors will cause a colour change from purple to yellow. In the presence of antimicrobials the lack of metabolism will result in no colour change (negative test).

Inhibition tests which involve colour changes are dependent on acid-base indicators. For example bromocresol purple yields yellow colour at pH5.2 and a purple colour at pH 6.8. Microbial metabolism in the presence of fermentable sugars added to the medium results in acid production causing the pH to drop. This change results in alteration of colour of the medium. Tests that make use of colour change of the medium include Charm Farm Test, Fast Antibiotic Screen Test (FAST), Charm Inhibition Assay (CIA), Delvotest and Delvotest SP, BSDA, and Valio T101.

b. Immunological Assays

The principles of immunoassays are similar regardless of whether isotopic or non-isotopic techniques are used. Radioimmunoassays are based on the competition between a labelled (with radioactive or non-radioactive) analyte (antibiotic or drug) for a binding site on an antibody. The presence of a large quantities of unlabeled analyte in a sample results in less radioactivity bound to the antiserum. A comparison of the ratio of the bound to the free labelled analyte with that obtained from a series of standards permits to quantitate the analyte in an unknown sample.

Enzyme immunoassays (EIAs) are non-isotopic immunoassays that use enzymes as labels. Enzyme-linked immunosorbent assays (ELISA) include separation of an enzyme-labelled antigen or antibody complex from a free enzyme-labelled antigen or antibody. ELISA tests have been developed in which wells or microtiter polystyrene wells or plates or membranes such as polystyrene , nylon, and nitrocellulose are used as solid phase to immobilise either antibodies or antigens. These membrane are coated onto single tubes, cups, dipsticks, or discs upon which tests are conducted. Commercially available ELISA test kits for detecting antibiotic residues in food and biological fluids include Cite Probe (Idexx), LacTek, Beta-Lactum, Lactek Ceftiofur, LacTek Tetracycline (Idetek), Penyme/Penzyme III, Signal (SmithKline Beecham), Ridascreen Tetracyline (R-Biopham Gmbh), SingleStep Block, and SingleStep ELISA (Environ. Diagnostic).

In these tests antibodies against specific drugs are immobilised on to a solid phase. A specific volume of a sample containing the free drug of interest is added to the immobilised antibodies followed by the addition of an enzyme conjugate. The mixture is allowed to incubate for a specified time during which the free drug competes with the enzyme-labelled drug (hapten) for the antibody binding sites. After incubation period, any unbound free drug or enzyme-bound drug are washed away; an enzyme substrate solution is added and the test system is usually chosen so that it is colourless initially but yields a green or blue coloured products when acted upon by the enzyme. The blue or green colour development indicates that there was no significant amounts of antigen (drug residue) in the test sample

(negative test). On the other hand no colour formation indicates the presence of the residue (positive test).

C. MICROBIAL INHIBITION RAPID TESTS (STOP, CAST, AND LAST)

Scope:

These tests kits are manufactured by Iditek Inc., Burlington, North Carolina, USA., and are used for screening animals for the presence of antibiotics and sulfa residues. The sample containing drug residues may represent animal tissues (kidney, muscle, liver), animal fluids (urine, serum, or tissue fluids) or antimicrobials extracted from animal/fish tissues, grains, and animal/fish feed.

Principle:

The scientific basis of these tests includes inhibition of test organisms in the presence of antimicrobial substances. An agar plate is uniformly seeded with spore suspension of Bacillus subtilis (STOP, LAST) and Bacillus megaterium (CAST). As the spores germinate, a uniform lawn of bacterial colonies grow on the surface of the agar.

A cotton swab saturated with a sample is positioned on the agar surface. If antimicrobials are present in the sample, these will diffuse out into the agar layer and prevent or inhibit the growth of bacterial colonies in an area around the swab. A clear zone of inhibition (ZI) around the swab indicates the presence of antimicrobial substance (positive test). A uniform lawn of bacterial colonies around the swab head reveals the opposite (negative test).

Sample Preparation:

These tests are primarily intended for testing poultry and cattle kidney tissues (carcasses) for the presence of antibiotic residues. However, the tests work equally well using liquid samples containing residues extracted from animal, or fish tissues and animal feed samples.

Materials:

Most of the materials needed to perform the tests are included in the kits. Agar plate: Ten plates per kit.

Spore suspensions: Spore suspensions of test organisms for LAST, CAST, and STOP, 4 ml vial per kit (Bacillus subtilis for LAST and STOP; Bacillus megaterium for CAST).

Swabs: Sterile cotton swabs, 30 per kit.

Felt-tip marking pen: One per kit.

Forceps: One per kit.

Neomycin discs: Ten per kit

Plastic ruler: One 6 in ruler per kit.

Sample containers: Twenty containers per kit.

Note: Always keep agar plates, vials of spore suspensions, and neomycin discs in a refrigerator at all times except when in use. Materials not supplied but may be needed include, a knife or a pair of scissors, clean paper towels, and incubators, 44-45°C (for B. megaterium) and 27-29°C (for B. subtilis).

Procedure:

(a) Remove one agar plate and a vial of spore suspension from the refrigerator. Allow them to warm up to the room temperature.

(b) Select one of the test samples (urine, kidney, muscle tissue or liver, or liquid sample of a residue extracted from a test sample).

(c) For a kidney tissue sample make a smooth incision, using a clean knife, about 1-2 cm deep and two-thirds the length of the kidney. Follow the same procedure for a muscle or liver tissues.

(d) Select one sterile cotton-tipped swab and gently swab the incised surface with it. For liquid samples soak the swab. Ensure that the swab is completely saturated with the sample. Carefully and gently re-swab the incised surface or the muscle tissue.

(e) Using a marking pen, mark the sidewall of the agar plate with an "X". Rotate the plate so that the "X" mark is on the far side of the plate.

(f) Select an appropriate vial of the spore suspension and shake it vigorously to thoroughly mix the contents.

(g) Select one sterile cotton-tipped swab and dip it into the spore suspension to thoroughly saturate the tip.

(h) Withdraw the swab from the vial, recap the vial, and set it aside. Carefully hold the swab so it does not touch anything.

(i) Lightly swab back and forth across the width of the agar plate. Entire agar surface must be covered with the spore suspension.

(j) Using a pair of clean thumb forceps remove a neomycin disc and position it on the agar surface of the swabbed plate beside the "X" mark on the side wall of the

plate. Lightly touch the neomycin disc with the tip of the forceps to firmly seat the disc on the surface of the agar.

(k) Carefully break the swab stick about 1.5 cm below the saturated tip. Discard the swab stick. Position the saturate swab on the agar surface directly across from the neomycin disc. Gently press the swab with forceps to firmly seat it on the surface of the agar.

(l) Repeat Step 11 if additional samples have been prepared. Two swabs can be placed in one plate provided they are properly positioned and identified as shown in the illustration.

(m) Incubate the agar plates at the appropriate temperatures for at least hours but not more than 24 hours.

(n) Carefully examine the agar surface around the neomycin disc. The area around the disc that is clear of bacterial growth (zone of inhibition, ZI) indicates that the positive control is effective. Lack of ZI suggests that there is a possible problem with the material or the procedures.

(o) A ZI around the head of the swab indicates that antibiotics present in the saturated swab head have diffused through the agar and inhibited bacterial growth in the ZI.

(p) The ZI around the swab head indicates that the test is positive. The absence of ZI around the head of the swab indicates that the test is negative.

(q) For interpretation of results see the illustration.

Interpretation of Results:

A. ANTIBIOTIC NEGATIVE B. ANTIBIOTIC POSITIVE C. TEST INCONCLUSIVE

STOP

	A. Antibiotic Negative	B. Antibiotic Positive	C. Test Inconclusive
IF YOU HAVE...	Opaque bacterial growth right up to the swab tips	Clear zones around swab tips	Opaque bacterial growth right up to the swab tips
AND YOU HAVE...	A clear zone around the Neomycin disc 20 - 25mm	Clear zone around Neomycin disc between 20 - 25mm	Clear zone around Neomycin disc that is less than 20mm
THEN TAKE THIS ACTION...	Animal is ready for market	Animal has antibiotic residues	Rerun test

LAST

	A. Antibiotic Negative	B. Antibiotic Positive	C. Test Inconclusive
IF YOU HAVE...	Opaque bacterial growth right up to the swab tips	Clear zones around swab tips	Opaque bacterial growth right up to swab tips
AND YOU HAVE...	A clear zone around the Neomycin disc 16 - 24mm	Clear zone around Neomycin disc between 16 - 24mm	Clear zone around Neomycin disc that is less than 16mm
THEN TAKE THIS ACTION...	Animal is ready for market	Animal has antibiotic residues. Retest in 2 to 3 days	Rerun test

CAST

	A. Antibiotic Negative	B. Antibiotic Positive	C. Test Inconclusive
IF YOU HAVE...	Opaque bacterial growth right up to the swab tips	Clear zones around swab tips	Opaque bacterial growth right up to swab tips
AND YOU HAVE...	A clear zone around the Neomycin disc 26 - 29mm	Clear zone around Neomycin disc (N5) between 26 - 29mm	Clear zone around Neomycin disc (N5) that is less than 24mm
THEN TAKE THIS ACTION...	Animal is ready for market	Animal has antibiotic residues. Retest in 2 to 3 days	Rerun test

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3. Johnson, R.W. and et al (1981) J. Food Protect. 44: 828.

4. Association of Official Analytical Chemists, Official Methods of Analysis, 15th ed.(Arlington, VA: AOAC,1990) Sections 982.16A- 982.16D.

5. Richardson, G.H.(1985). Standard Methods of Analysis, 15th. ed., (Washington D. C.: American Public Health Association, 1985).

6. Thaker, N.H. and et al (1993). Fast Antimicrobial Screen Test (FAST). In-Plant Study. (Washington D. C.: United States Department of Agriculture, food Safety and Inspection Services, 1993).

D. DETECTION OF ANTIMICROBIALS USING IMMUNOLOGICAL METHODS

1. Singlestep Block Sulfamethazine (SMZ) and Sulfadimethoxine (SDM)

Scope:

These rapid tests are for determining the presence of SMZ and SDM extracted from feed, tissues, serum, milk, or urine with methanol/water or dilution buffer, clarified by filtration and diluted in dilution buffer provided with the kits.

Principle:

These tests are based on solid phase competitive immunoassays. In these tests SMZ and SDZ are coupled to an enzyme. this enzyme-bound drug competes with the free drug in the test sample for the antibody binding site. The presence of significant levels of the drug residue in the sample prevents the enzyme-bound drug from binding to antibody coated in the reaction sites and is washed out. Upon the addition of the substrate the enzyme (if bound to the antibody) acts on the substrate to give a blue coloured product. The presence of SMZ and SDZ in the test sample is indicated by the absence of the blue colour.

Materials:

Each kit contains:

(1) Foil wrapped testing devices. Each device contains 4 reaction sites.
(2) One red capped dropper bottle of Sulfamethazine enzyme.
(3) One green capped dropper bottle of Negative control.
(4) One yellow capped dropper bottle of Substrate A
(5) One blue capped dropper bottle of Substrate B.
(6) One white capped dropper bottle of wash solution.
(7) One bottle of dilution buffer.

Materials required but not included in the kits:

(1) Blender for grinding and mixing grain, feed, and nut, samples.
(2) Methanol/water solution (80:20), 100 ml per grain sample.
(3) Various size pipettes capable of transferring from 100 to 1000 μl volumes.
(4) Timer.
(5) Test tube or a small container to prepare solutions.

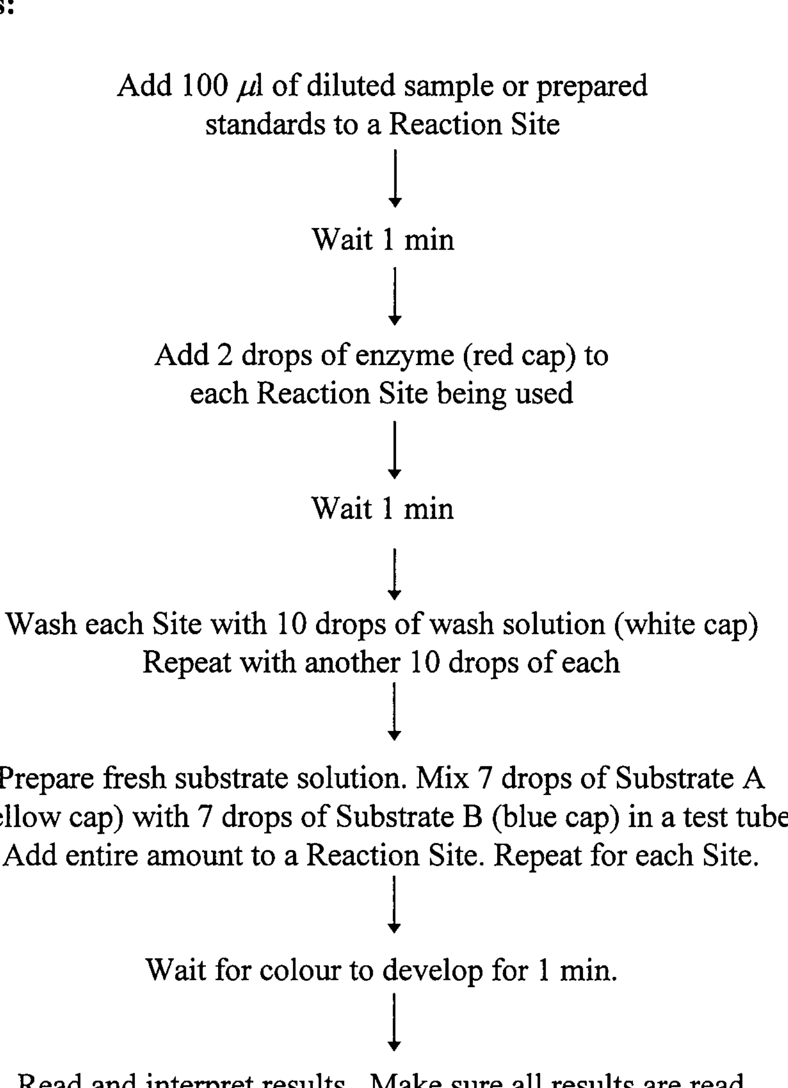
Preparation Of Standard Solutions Of Analytes:

(a) Dissolve separately 1 mg equivalent of SDM or SDZ (sodium salts) in 1 ml of methanol to prepare a 1 mg/ml solution. SDM and SDZ are available from Sigma Chemical Co., catalogue No. S-7385 and S-5637.
(b) Perform serial dilution in dilution buffer to attain the required level standard concentration.

Sample Preparation:

Follow the instructions provided with the kits.

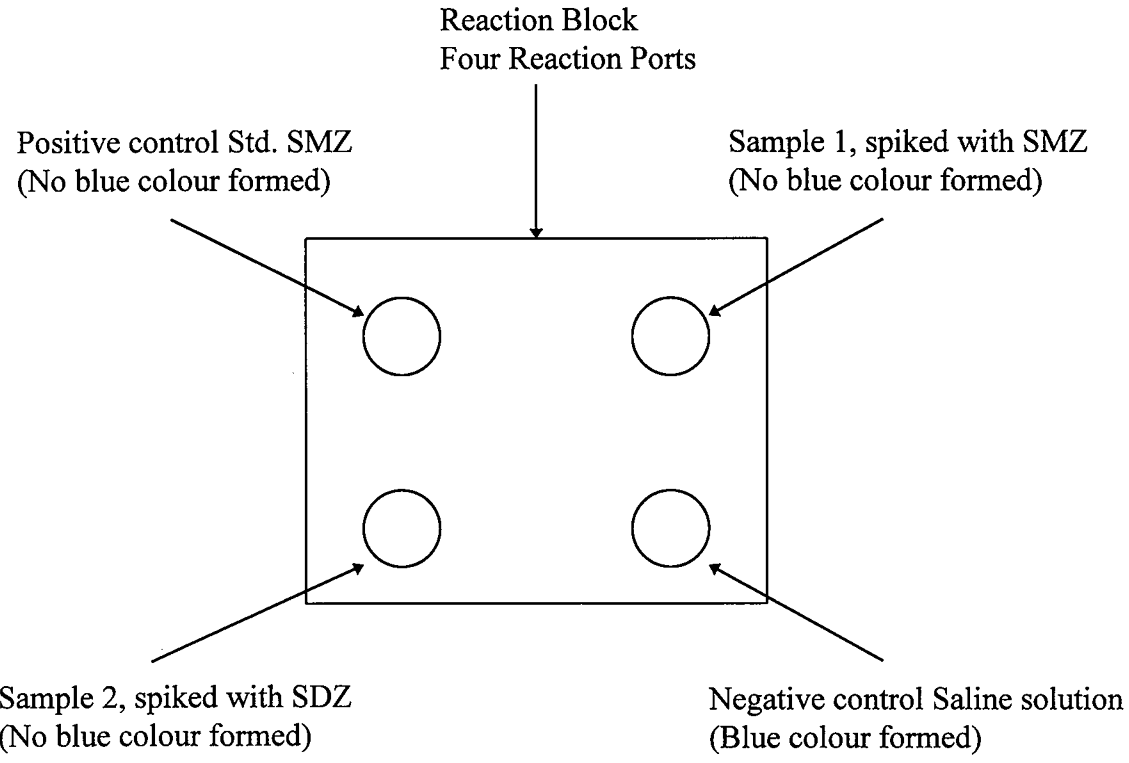
Procedures:



Interpretation of Results:

Blue colour indicates negative test (no residue in a sample) while no colour indicates positive test (presence of residue in a sample).

FOUR REACTION SITES ON A REACTION BLOCK



Sketch of A Testing Device (Reaction Block)
Illustrating the Positioning of the Reaction Sites

2. EZ-SCREEN Sulfamethazine and Sulfadimethoxine (QUICK CARD TEST)

Scope :

The tests are qualitative enzyme immunoassays for the detection of sulfamethazine or sulfadimethoxine in urine, serum, extracted samples of meat, fish, or feed. This screening procedure is intended to serve as an indicator of the presence of these residue levels that may be violative as defined by the USDA-FSIS's National Residue Program (NRP).

To assure the absence of potentially hazardous levels of the above drugs in edible foods, the Food and Drug Administration requires that all animals be drawn from the drugs for at least 15 days prior to slaughter. During this withdrawal period the residues are gradually cleared from the animal tissues and eliminated in the urine. Tolerance levels for uncooked edible tissues have been set at 0.1 ppm for cattle, swine, and poultry.

Principle:

The drug residues are extracted from samples with methanol/water, clarified by filtration and diluted in buffer provided with the kit or presented as a urine or serum sample and diluted in buffer provided with the kit. The diluted sample and negative control are added to the indicated QUICK-CARD test ports. Following absorption, enzyme conjugate is added to the test ports followed by a wash reagent and the substrate reagent. The analyte present in the sample competes with the enzyme-residue conjugate for the antibody bound to the QUICK-CARD. The presence of a significant level of a residue is indicated by the absence of colour at the test port.

Materials:

Each kit contains reagents or testing two samples and includes the following:

(1) QUICK-CARD: Two reaction sites per card are coated with rabbit antibody to a residue (sulfamethazine or sulfadimethoxine).
(2) ENZYME-RESIDUE CONJUGATE: The enzyme conjugate dropper tube contains a sealed ampule and reconstitution diluent. The glass ampule contains horseradish peroxidase conjugated to sulfamethazine or sulfadimethoxine. The lyophilized conjugate is reconstituted by breaking the glass ampule and gently shaking to mix.
(3) NEGATIVE CONTROL: The negative control dropper tube contains phosphate buffered saline solution.
(4) SUBSTRATE: The substrate dropper tube contains a sealed glass ampule and reconstitution diluent. The glass ampule contains substrate 4-chloro-1-naphthol and urea peroxide substrate. Reconstitute the tableted reagent by breaking the glass ampule and shaking to mix. The reconstituted reagent is stable for 8 hours at room temperature.
(5) DILUENT BUFFER: Two plastic screw-capped tubes containing 4.5 ml of phosphate buffered saline solution, pH 7.2.

(6) PIPPETTES: Four disposable pipettes. The pipettes deliver a drop having 50μl (0.05ml) when filled to a level of 1/2 inch.
(7) COTTON SWABS: Two cotton swabs.

Sample Preparation:

As per instructions provided with the Kits.

Test Procedure:

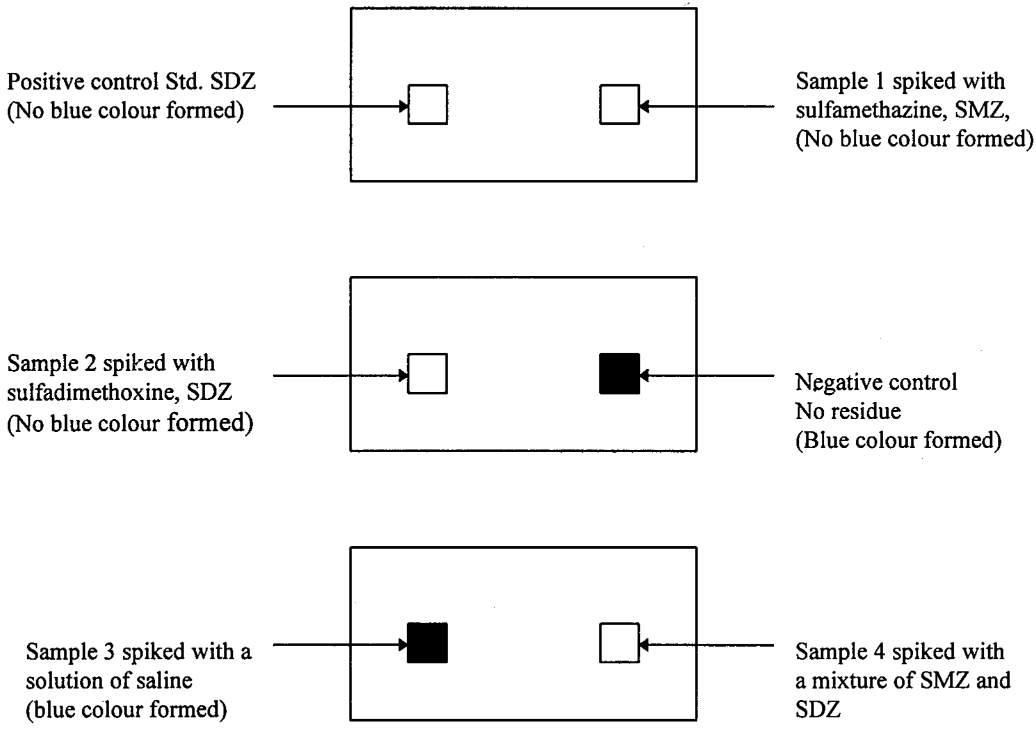
(a) Remove foil pouch from refrigerator and allow all reagents in the pouch to reach ambient temperature before opening.
(b) Prepare the Negative control by removing the plastic shrink seal from around the dropper cap.
(c) Prepare the Enzyme by squeezing the plastic dropper to break the inner glass ampule. Tilt the tube back and forth for approximately 20 seconds to re-hydrate and mix the contents. Remove the plastic shrink seal from around the dropper cap.
(d) Prepare the Substrate by squeezing the plastic dropper tube to break the inner glass ampule. Shake the tube vigorously. Remove the plastic shrink seal from around the dropper cap.
(e) Place the test card on a clean, flat surface and label it with the sample number and the date.
(f) When applying sample to the card, do not touch the sample pipette tip to the port. Hold the pipette so that the tip is about ½ inch above the port and allow the drop to fall freely.
(g) Apply one drop of Negative control to the "Control" port of the card.
(h) Apply one drop of a sample to the second port on the card. Allow the sample and control drops to absorb into the test ports before proceeding to the next step.
(i) Discard the first drop of Enzyme and then apply one drop to both of the ports on the card.
(j) Allow the Enzyme to absorb into the test ports before proceeding to the next step.
(k) Apply two drops of the Substrate to both the ports. Set a timer for 5 min.
(l) When the timer goes off, read the test results. If colour is visible in both the "Control" and "Sample" ports the sample is negative for sulfamethazine or sulfadimethoxine.

Interpretation of Results:

The sample is considered to be NEGATIVE for sulfamethazine or sulfadimethoxine when the "Sample" port develops readily detectable colour (light grey, grey-blue or blue) over the surface of the port.

The sample is considered to be POSITIVE for the residues when the "Sample" port fails to develop readily detectable colour (remains colourless).

Three QUICK CARDS used to test the presence of sulfamethazine and sulfadimethoxine in sample spiked with kinon concentration residues.



3. RAPID SINGLESTEP ELISA for General Sulfa, Sulfadimethoxine and Sulfa methazine

Scope:

These ELISA tests are solid phase immunoassays designed to rapidly detect and quantify a specific compound in a sample by means of a high affinity capture antibody. Liquid sample may represent extracts from animal tissues, seafoods, grains, or animal/fish feeds.

Principle:

Enzyme-conjugate (horseradish peroxidase is conjugated to a drug residue e.g. sulfadimethoxine or sulfamethazine) and antibody to this conjugate is prepared. The ELISA plate wells are coated with this antibody. A sample is added to the wells followed by and enzyme conjugate. During the incubation period, the conjugate competes with the drug residue in the sample for the binding sites on the antibody coated well. After a wash step to remove any unbound material, substrate is added for the final colour development. The colour intensity is inversely proportional to the amount of residue present in the sample. Those samples which contain the residue will inhibit binding of the enzyme conjugate to the antibody resulting in less colour than the negative control.

Materials:

The following materials are included in the kit:

(1) Antibody coated wells of a microtiter plate: The microtiter plate contains wells (twelve rows of 8 wells in each).
(2) Enzyme (lyophilized): Contains compound conjugated to the horseradish peroxidase enzyme.
(3) Enzyme diluent: Used to reconstitute lyophilized enzyme. Contains stabilised PBS.
(4) Sample and Standard Diluent: Contains 0.1% BSA in PBS.
(5) Standards (lyophilized): Reconstitute to 5 ml with sample and standard diluent.
(6) Wash solution: contains Tween-20 in PBS.
(7) Substrate: Contains 3,3,5,5- Tetramethylbenzidine.
(8) Stop solution: Contains 3N H_2SO_4.

Other materials required but not included in the kit:

(1) Micropipettes and tips.
(2) Disposable reagent troughs for pipetting reagent
(3) Glass culture tubes for dilutions.
(4) Microplate reader. Requires a 450 nm or 650 nm filter.

Assay Procedure:

Prior to performing test, allow one hour for all reagents to reach room temperature. Mix all reagents by gentle inversion.

(a) Reconstitute enzyme by adding 12 ml of enzyme diluent to the bottle of lyophilized enzyme. Let stand for 5 min, then mix by gentle inversion.
(b) To reconstitute standard, add 5 ml of sample and standard diluent to each bottle of lyophilized standard. Let stand for 5 min, then gently invert 10 times.
(c) Pipette 20 μl of sample and standard diluent (as negative control) into designated wells.
(d) Pipette 20 μl of the prepared standard(s) into adjacent wells.
(e) Pipette 20 μl of each prepared sample in the next available wells.
(f) Add 100 μl of the reconstituted enzyme to all wells. Mix by tapping the plate.
(g) Incubate the plate at room temperature for 10 min.
(h) Discard the solution from all the wells by inverting over absorbent paper. Pipette 400µl of wash solution in all the wells and discard by inverting the plate over an absorbent paper. Repeat this three times.
(i) Immediately add 150 μl of substrate to each well. Do not allow the wells to dry. Mix the reagents by tapping.
(j) Incubate at room temperature for 10 - 15 min or until a blue colour appears in the negative control wells.
(k) Add 150 μl of stop solution to all wells. Mix by tapping .
(l) Read results using a microplate reader at 450 nm wavelength.

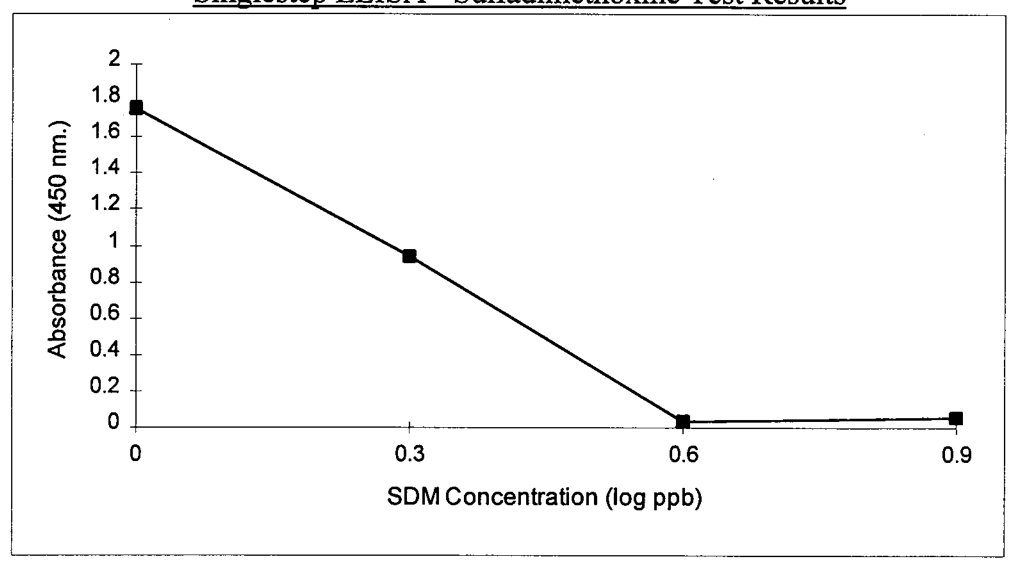
Interpretation of Results:

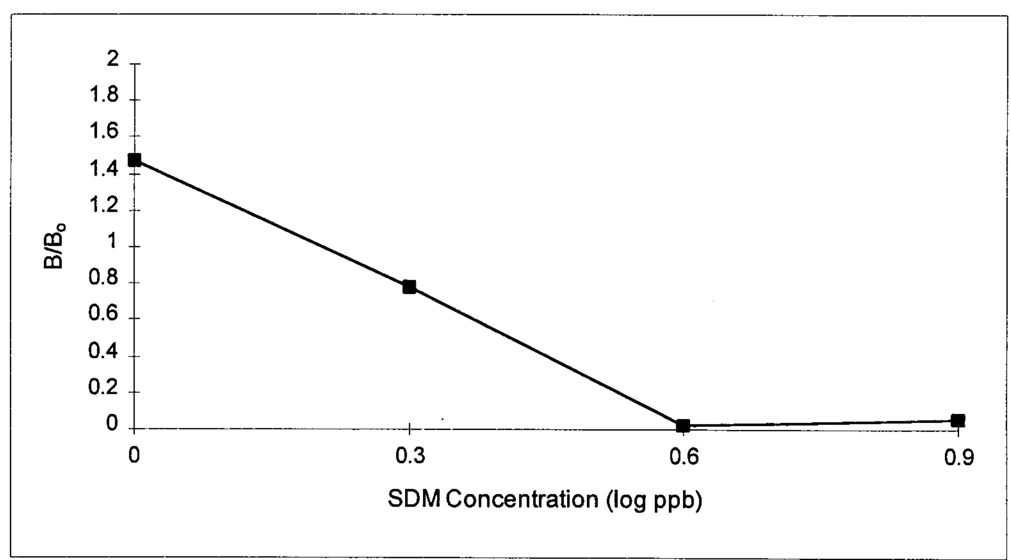
For the test to be valid, wells containing negative control samples must have a medium blue colour (unstopped) or a bright yellow colour (stopped).

Results:

Absorbency of the reactions in the microtiter plate wells was measured at 650nm using a plate reader. The results obtained are summarised.

Singlestep ELISA - Sulfadimethoxine Test Results





Std. SDM (ppb)	Absorbance (450 nm)	B/B_o
0	1.76	1.47
2	0.94	0.78
4	0.03	0.02
8	0.05	0.05

Computing Programs MicroELISA System Version 1.7 was used to read the microtiter plate.

E. RAPID ELISA TEST (RIDASCREEN) FOR SULFAMETHAZINE

Scope:

The test is competitive enzyme immunoassay for the quantitative analysis of sulfamethazine in milk and meat and for the qualitative analysis of sulfamethazine in meat. Sulfaemthazine extracted from various foods including seafoods can also be analysed. Sulfonamides are widely used as feed additives for fattening of animals. Combined with inhibitors of dihydrofolate reductase such as trimethoprim, tetromoprim, or pyrimethamine sulfonamides are also used in veterinary medicine for the treatment of intestinal infections, mastitis, pulmonitis and other diseases. Sulfonamide residues may therefore occur in food of animal origin such as milk and meat. The carcinogenic sulfamethazine represents a threat to human health. The recently published EC Regulation No. 675/92 established a maximum residue limit (MRL) of 100 ppb for sulfonamides in meat and a preliminary MRL of 100 ppb in milk.

For the detection of sulfamethazine physico-chemical methods such as HPLC are used because the microbial inhibition assays lack sensitivity and desired specificity. But due to high cost and instrumentation associated with HPLC procedures its use for routine screening has been limited.

Using RIDASCREEN Sulfamethazine test it is possible to detect as little as 10 ppb of this residue in samples. RIDASCREEN and RIDA are trade marks of R-Biopharm GmbH, Germany.

Principle:

The basis of the test is the antigen-antibody reaction carried out in an ELISA mirotiter plate. The microtiter wells are coated with sheep antibodies directed against anti-sulfamethazine rabbit IgG (i.e. antibodies to antibodies against the enzyme-sulfamethazine conjugate). Anti-sulfamethazine antibodies, sulfamethazine-enzyme conjugate, and the sulfamethazine standard or sample solution are added. Free sulfamethazine and sulfamethazine-enzyme conjugate compete for the anti-sulfamethazine antibody binding sites. At the same time , the anti-sulfamethazine antibodies are also bound by the immobilised sheep antibodies. Any unbound enzyme conjugate is then removed in a washing step. Enzyme substrate (urea peroxide) and chromogen (tetramethylbenzidine) are added to the wells and incubated. Bound enzyme conjugate converts the colourless chromogen into a blue product. The addition of the stop reagent changes the colour from blue to yellow. The colour intensity is measured at 450nm. The absorption is inversely proportional to the sulfamethazine concentration in the sample.

Materials:

Each kit contains sufficient materials for 96 measurements. Each kit contains:

(1) Microtiter plate, one.

(2) Sulfamethazine standard concentrates, 300μl each; 0, 10, 30, 90, 270, and 810 ppb.
(3) Conjugate. Peroxidase -sulfamethazine conjugate.
(4) Anti-sulfamethazine antibody concentrate.
(5) Substrate. 7ml solution.
(6) Chromogen. 7ml solution.
(7) Stop reagent. 14ml solution. Contains 1M sulphuric acid.
(8) Buffer 1 (20-fold concentrate).
(9) Buffer 2, concentrate, standard dilution buffer for sample.

Other material required but not provided with the kit:

Equipment: microtiter plate reader, centrifuge, rotary evaporator, stomacher, shaker, graduated pipettes, and micropipettes.

Preparation of Samples:

As per instructions provided with the kit.

Preparation of Working Solutions:

As per instructions provided with the kit.

Test Procedures:

(a) Add 50μl of diluted enzyme conjugate to the bottom of each well.
(b) Add 50μl of standard or prepare sample to the separate wells.
(c) Add 50μl of diluted antibody solution to each well. Mix well and incubate for 2 hours at room temperature.
(d) Pour out the contents of the wells by inverting over an absorbent paper.
(e) Fill all the wells with 250μl distilled water and pour out the liquid as in step 5. Repeat 2 more times.
(f) Add 50μl of substrate and 50μl of chromogen to each well. Mix and incubate min at room temperature in the dark.
(g) Add 100μl of stop solution to each well. Mix and measure the absorbance at nm against an air blank.

Results:

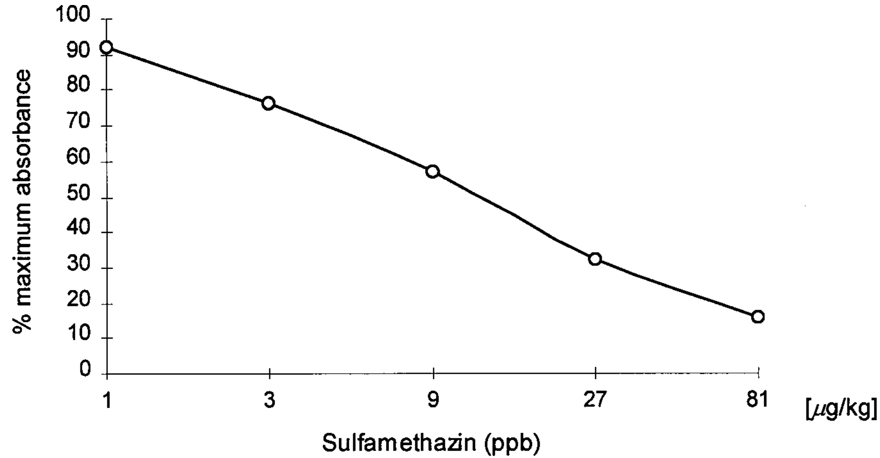
The mean values of the absorbance values obtained for the standard and the samples are divided by the absorbance value of the first standard (zero standard) and multiplied by 100. The zero standard is thus is made equal to 100% and the absorbance values are quoted in percentages.

The values calculated for the standard are plotted as shown in the following figure. The calibration curve should be virtually linear in the 1 - 27 μg/kg(ppb) range.

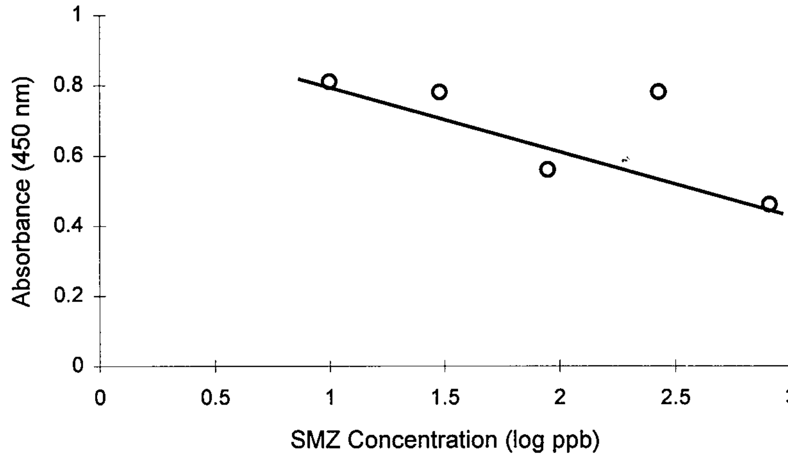
$$\frac{\text{Absorbance standard (or sample)}}{\text{Absorbance zero standard}} \times 100 = \%\ \text{Absorbance}$$

The sulfa methazine concentration in µg/kg corresponding to the absorbance each sample can be read from the calibration curve.

Calibration curve of RIDASCREEN Sulfamethazine kit



Test Results of RIDASCREEN Sulfamethaxine Kit



Std. SDZ (ppb)	SMZ (log ppb)	Absorbance (450 nm)
10	1.0	0.81
30	1.48	0.78
90	1.95	0.56
279	2.43	0.78
810	2.91	0.46

Chapter VI

Chemical Determination of Drug Residues in Seafoods Using High Performance Liquid Chromatography (HPLC)

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A. INTRODUCTION

The classical methods for determining antibiotics are inhibition bioassays. In most instances, these bioassays are primary procedures for residue screening and quantitative analysis.

One of the advantages of microbial inhibition assays is that they detect almost all members of several antibiotic classes simultaneously at a relatively low cost. However, drawbacks may occur because some analytes have a reduced detection sensitivity to test organisms or an inhibition by possible coextractant such as lipids from animal tissues or lysozyme.

The use of chemical methods for antibiotic determination is limited, particularly for older antibiotics, because microbial inhibition tests are commonly used for regulatory purposes. In many instances chemical methods are only used for chemical identifications.

In past two decades numerous chemical methods (gas chromatography, GC; thin-layer chromatography, TLC; high-performance liquid chromatography, PHLC) have been developed (1-3). These physicochemical methods tend to be more specific, more precise, and have lower limits of detection (i.e. they are more sensitive compared to microbiological methods).

Looking to the future, we can expect to see a continued evolution of test methods for antibiotics in food animals in both field tests and confirmatory tests (chemical) applied in laboratories. With the signing of the new General Agreement on Tarrifs and Trade (GATT) agreement, increased recognition of validated laboratory methods can be expected. The GATT will focus on the Codex Alimentarius Commission and its subsidiaries for scientific support in the resolution of disputes. Other bodies such as AOAC INTERNATIONAL and the International Union of Pure and Applied Chemistry

(IUPAC) will play important role in reviewing validated methods involving both microbiological bioassays and chemical analysis.

The past decade has brought a shift from a total reliance on microbiological assays for the detection and identification of antibiotics to an increased use of chemical separation techniques. New techniques, such as supercritical fluid extraction, capillary zone electrophoresis, and the various combinations of separation techniques with mass spectrometry (MS) and tandem mass spectrometry, will bring further evolution to the regulatory program tests in all countries. Other developments in immunochemical and biochemical analysis will offer opportunities for fast, sensitive, and selective screening tests. There will be more collaboration amongst regulatory laboratories (agencies) around the world. The evolution of new methodologies will take into consideration the international implications and national and international requirements will have to be fully respected.

B. SIMULTANEOUS DETERMINATION OF SULFADIAZINE, SULFADIMETHOXINE TRIMETHOPRIM AND ORMETOPRIM IN SHRIMPS

Scope:

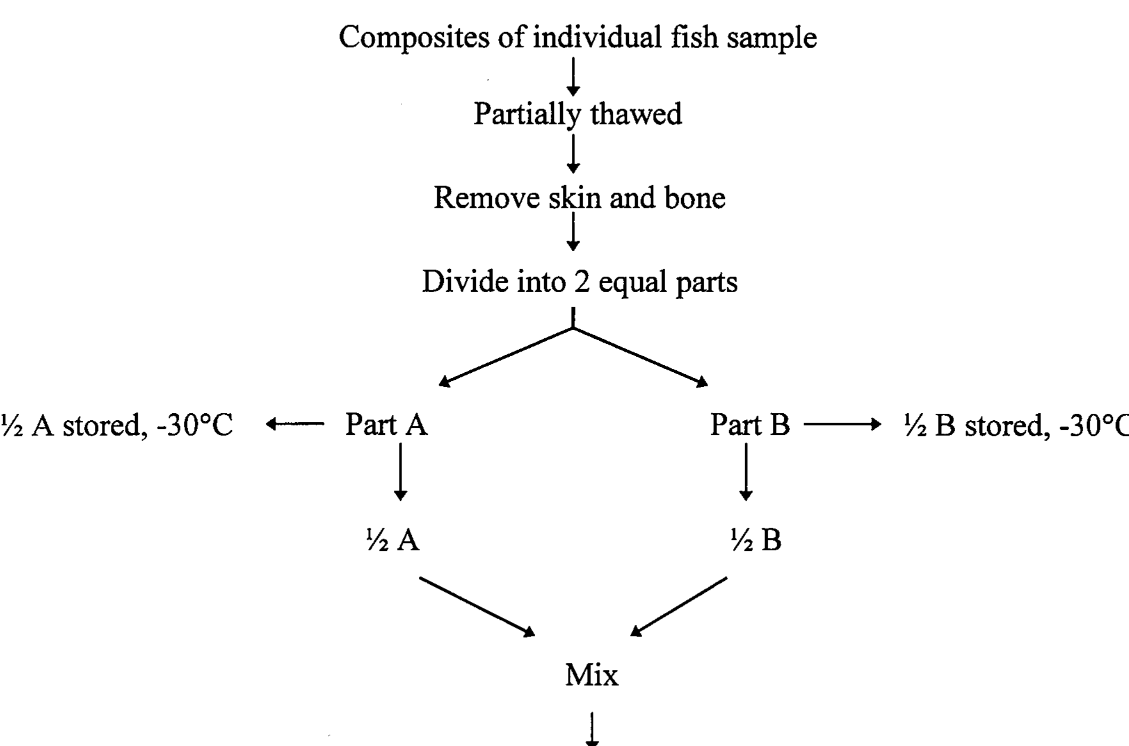
Romet 30 and Tribrissen are frequently used in the treatment of diseases in aquacultured fish. Romet 30 contains sulfadimethoxine (SDM), and ormetoprim (OMP), a potentiator while Tribrissen carries sulfadiazine (SDZ) and trimetoprim (TMP).

Chemical methods for the analysis of these residues have been reported and include TLC[1-4], GC[5], and HPLC[6-9]. The method described here involves simultaneous detection and determination of four sulfa residues.

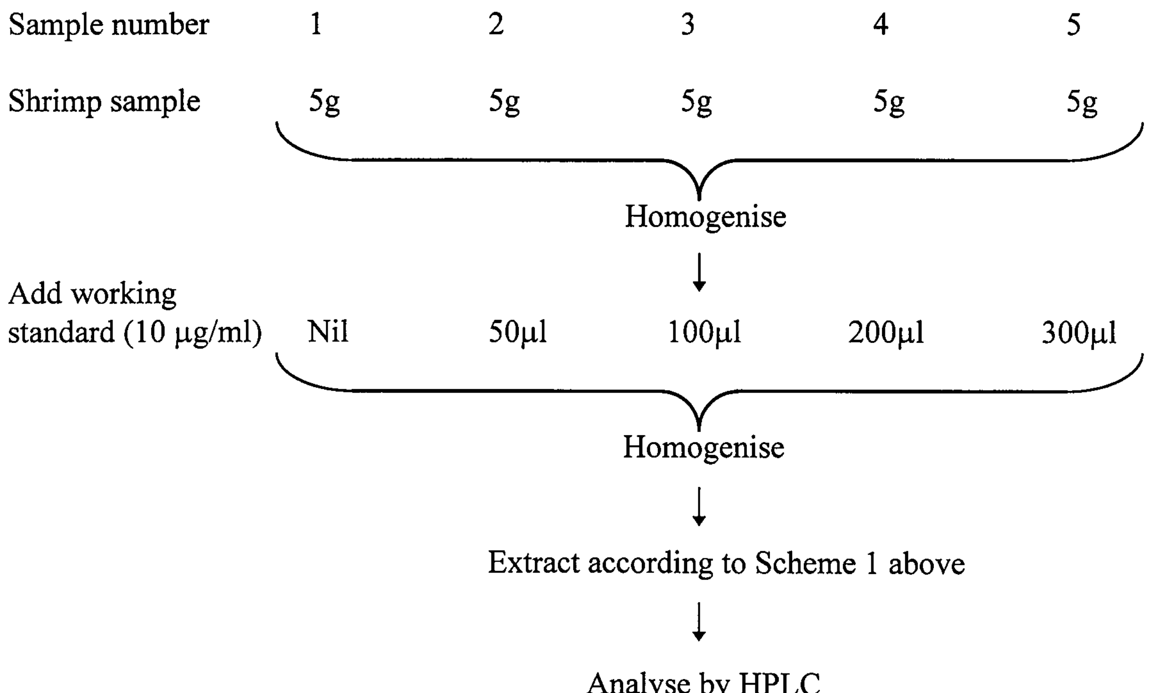
Sample Preparation:

Sample of large fish such as salmon and trout should be taken as steaks (minimum 5 samples) following the procedure outlined by AOAC. A representative sample from the product lot (shrimp, fish, crab, lobster etc.) should be collected and stored at -30 to -40°C to maintain sample integrity. The samples are prepared according to Scheme 1.

Scheme 1. Preparation of Samples for Extraction of Drug Residues



Scheme 2. Fortification and Extraction of Furazolidone from Spiked Shrimp Samples



Apparatus:

(1) HPLC System: The system used included a Waters 717 plus Autosampler,Waters 486 Tunable Absorbance Detector, Waters Temperature Control Module and Oven.

(2) Pump System: Waters 6510 HPLC pumps with capacity to generate rapid gradient and at least 4000 psi.

(3) Column: Vydac 201T54, 25 x 4.6 mm, Id., was used without a guard.

(4) Column Oven: Waters , Model Code CHM, Serial No. MX4MM7468M.

(5) Data Processing: Millennium 2010 Chromatography Manager equipped with Millennium computer software and coupled to COMPQ (Prolinear 4/66) Data Station with a NEC Printer (Model P1200) (NEC Technology, Hong Kong Ltd.).

(6) Effluent Monitor: Waters 486 Absorbance detector has sufficient sensitivity between 280-288nm and is an UV-Visible monitor.

(7) Syringes: Plastic disposable 5ml with 26g x 1.5 inch needle.

(8) Filters: Nylon 13mm syringe filters with 0.2 μ pore size.

(9) Centrifuge: Sorval model.

(10) Homogenizer: Brinkman Polytron.

(11) Glassware: Polyethylene centrifuge tubes, 50ml; glass centrifuge tubes, 50ml; round bottomed flasks, 50 or 100ml.

Reagents:

(1) Solvents: Acetonitrile (HPLC grade), glass distilled water, all other solvents ACS grade or better.

(2) Stock solutions of Standards (0.10 mg/ml): SDM and OMP standards were obtained from Hoffman-LaRoche while TMP and SDZ were products from Sigma Chemical Company. Weigh 10mg of each standard into a 100 ml volumetric flask. Dissolve the standard and bring each flask to volume with acetonitrile. TMP may require a drop or two of trifluoroacetic acid.

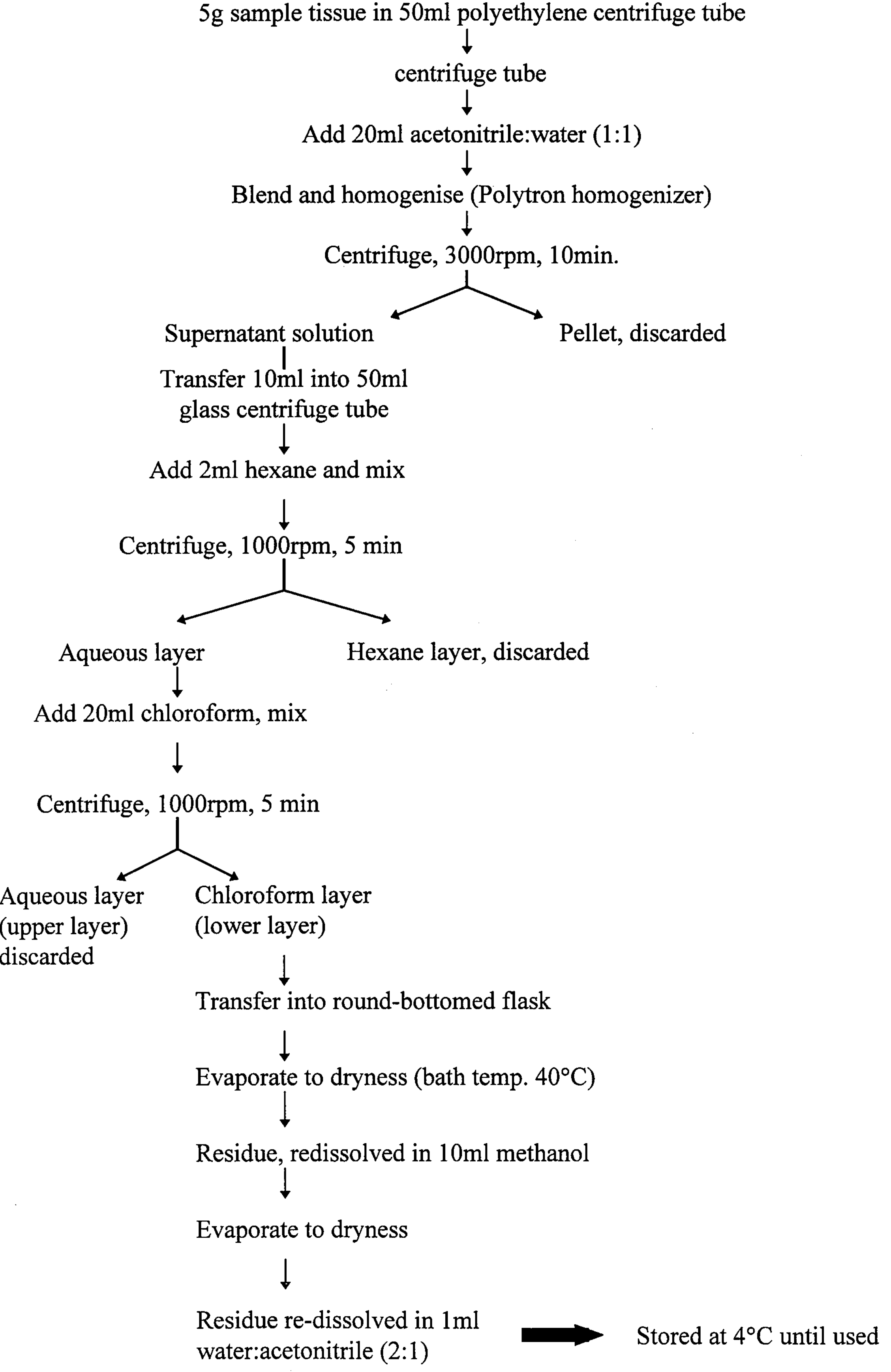
(3) Working standards (0.001 mg/ml): Pipette 1.0ml of each stock solution into a 100ml flask and dilute to volume with glass distilled water:acetonitrile (2:1).

(4) Mobile Phase: Solvent A - Glass distilled water, degassed.
Solvent B - Acetonitrile with 0.1% trifluoroacetic acid (TFA).

Procedure:

Extraction and clean up: See the flow diagram (*Scheme 3*).

Scheme 3



HPLC Analysis:

Preparation for HPLC: Use 0.2 μ syringe to directly filter sample into a 1.5ml autosampler vial.

Chromatographic conditions: The column system was operated at 35°C. The system was stabilised with 100% solvent A pumped at 1.0 ml/ml. The gradient used was as follows:

Time(min)	% Solvent A	% Solvent B
	100	0.0
0.1	92.0	8.0
7.0	85.0	15.0
24.0	30.0	70.0
30.0	100.0	0.0
35.0	100.0	0.0

After the gradient is over equilibrate for 11 min and then return the column to initial conditions over a period of 6 min and allow to equilibrate for 5 min before the next run is assumed.

Analysis: The system was standardised for peak height and retention time by at least two repeat injections of the mixed standard. Each sample (50μl) was injected and peaks identified by comparison of retention times with standards.

Calculations: To determine the μg of a drug in the sample formula of Burns et al[10] was used.

$$\frac{PH}{PHS} \times D = \text{Total drug in the original sample tissue}$$

where PH = Peak height/ 50μl injection

PHS = Peak height per μg standard

D = Dilution factor

The concentration of drug is normally expressed on a wet-basis such as μg/g wet weight (ww). To obtain this value simply divide the total analyte in μg by the weight of the original tissue extracted (W) in grams.

When 50 μl of sample is injected the dilution factor is 50.

$$\mu g \text{ drug/g ww} = \frac{PH \times 50}{PHS \times W}$$

Sample calculation for SDZ:

(1) $$\frac{18.71}{19.20} \times \frac{50}{50} \times 100 = 97.4\,\%$$

(2) $$\frac{15.61}{19.20} \times \frac{100}{100} \times 100 = 81.3\,\%$$

(3) $$\frac{8.91}{19.20} \times \frac{200}{200} \times 100 = 81.4\,\%$$

(4) $$\frac{12.65}{19.20} \times \frac{300}{300} \times 100 = 66.0\,\%$$

Results And Discussion:

Different concentrations of standard SDM, SDZ, TMP, and OMP were used to establish separately calibration curves. *Figures 1, 2, 3,* and *4* depict the calibrations curves. These curves indicate that the method used is sensitive enough to simultaneously detect residues of interest. The average retention times for SDZ, SDM, TMP and OMP were found to be, respectively, 9.61, 18.45, 21.43 and 21.91 minutes. The slopes of the calibration curves were linear with correlation coefficient values ranging between 0.9984 and 0.9988. Standard errors in these analysis were found to be 5.3%(TMP), 8.8%(SDZ), 10%(OMP), and 13.8%(SDM). *Figures 5, 6, 7,* and *8* depict elution profiles of these residues on a Vydac column used in this work. *Figure 9* is a chromatogram showing an elution pattern of the same analytes when injected as a mixture and chromatographed on the same HPLC column under similar conditions.

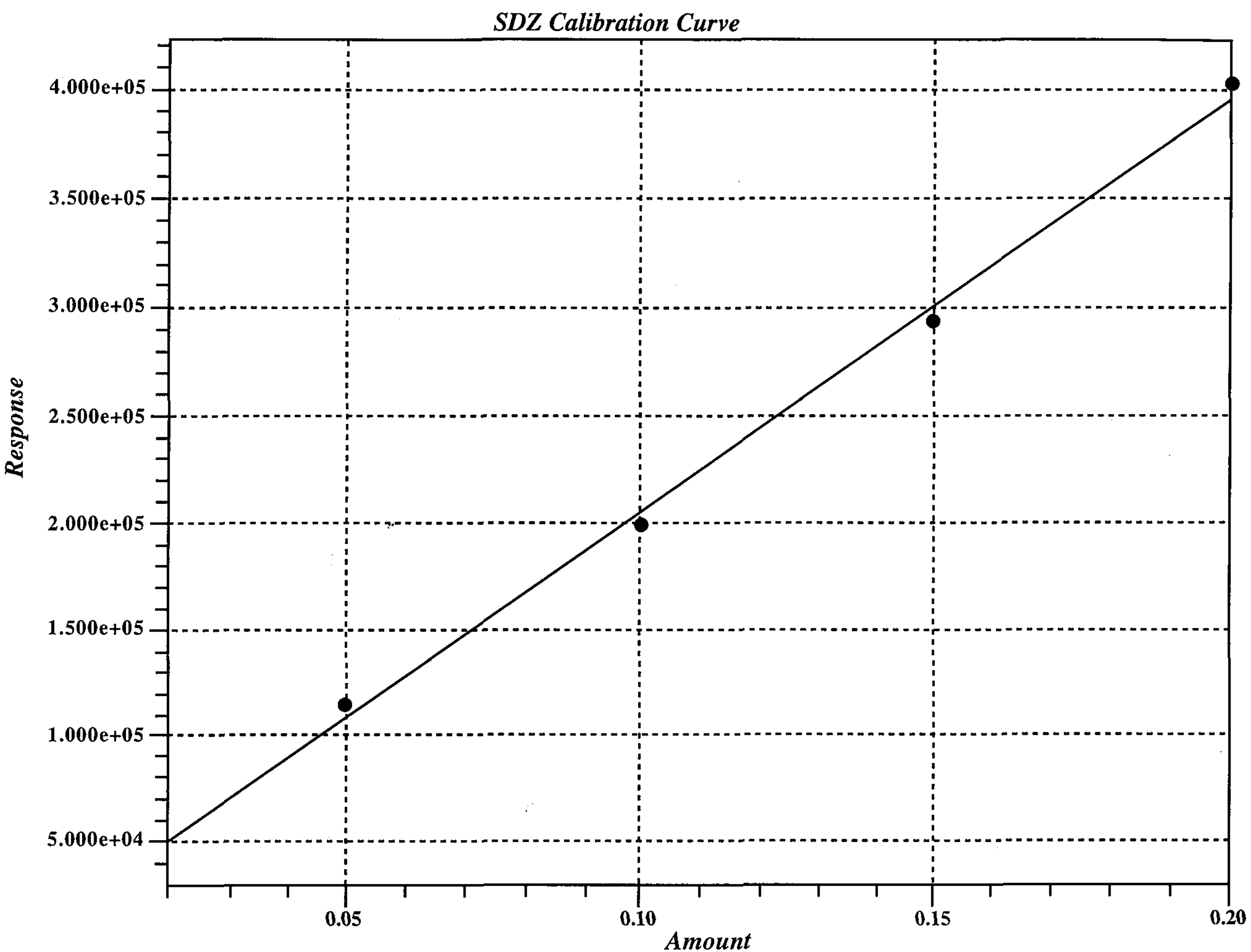
Table 1 summarises the results of an experiment in which sample of shrimp were fortified with known concentrations of the drug residues and then extracted using the approved protocol outlined above. Per cent recoveries of individual residues were calculated and were found to be 81.5%, 77.3%, 84.5%, and 109% for SDZ, SMZ, TMP, and OMP, respectively. Some component of shrimp origin which may have been co-extracted with the residues caused difficulty in estimating precisely the per cent recovery of TMP and OMP. Since these co-extractants perhaps absorbed light at

wavelengths similar to those for TMP and OMP the values calculated for their recoveries were higher than 100% as is evident from *Table 1*.

Table 1. Recovery of Analyte Residues from Fortified Shrimp Samples

Analyte	Amount added (ng)	Amount recovered (ng)	% Recovery
SDZ	50	48.7	97.4
	100	81.3	81.3
	200	162.7	81.4
	300	197.7	66.0
	Average: 81.5%		
SDM	50	30.0	60.0
	100	81.0	81.0
	200	152.0	76.0
	300	275.0	92.0
	Average: 77.3%		
TMP	50	32.3	64.5
	100	101.0	101.0
	200	316.0	158.0
	300	264.6	88.8
	Average: 84.5%		
OMP	50	34.4	68.6
	100	107.0	107.0
	200	337.0	168.0
	300	282.0	94.0
	Average: 109.4%		

Figure 1. Calibration Curve for Sulfadiazine (SDZ)



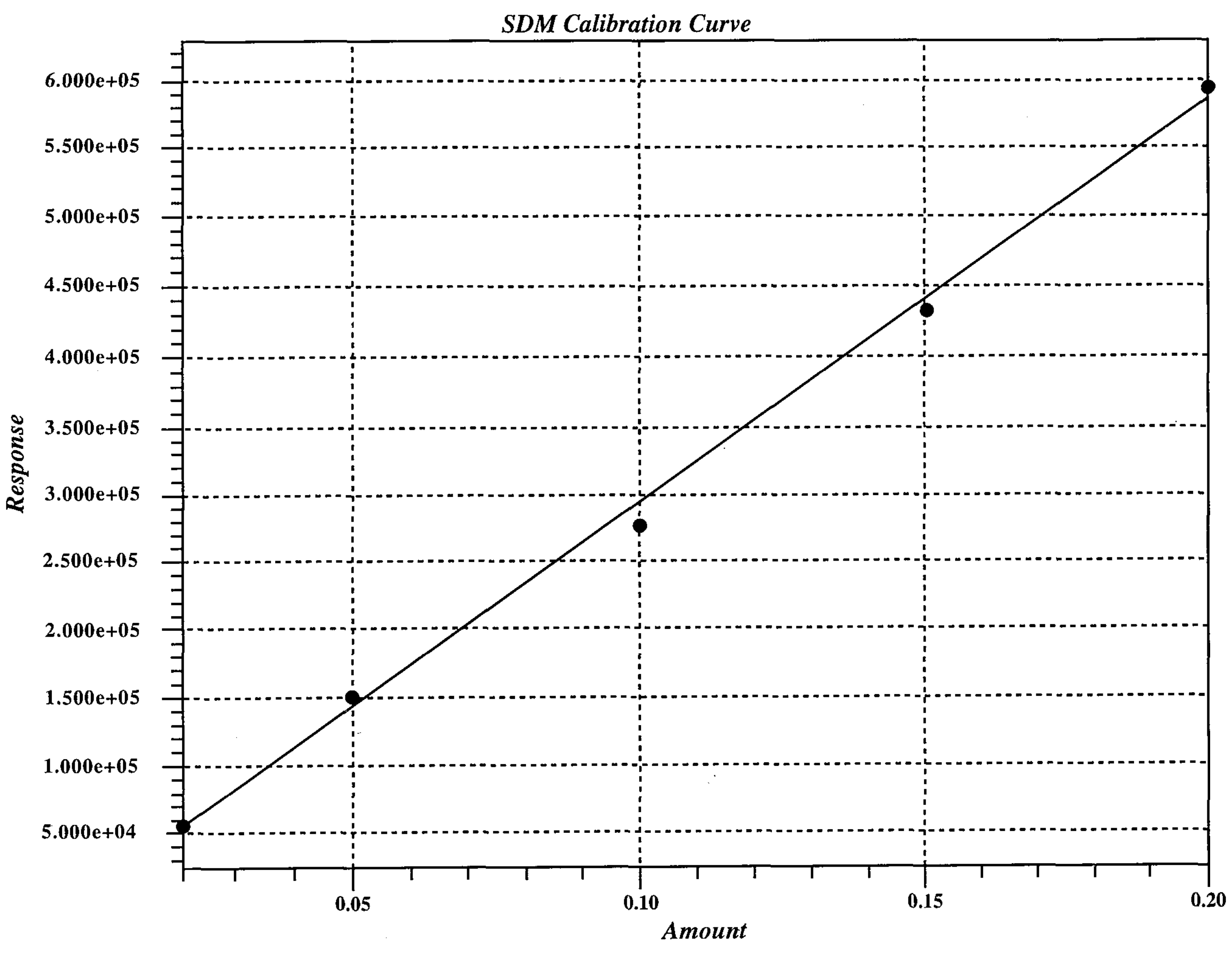
SDZ Calibration Information

Processing Method	Sulfa	System	Sulfa
Channel	486	Date	17-DEC-96
Type	LC	Name	SDZ
Retention Time	9.733 min	Order	1
A	10536.256098	B	1935559.210600
C	0.000000	D	0.000000
E	0.000000	F	0.000000
R	0.998537	R^2	0.997077
Standard Error	8835.200296		

SDZ Point Table

	Amount	Response	Calc. Amount	% Deviation	Manual
1	0.020000	49550.270000	0.020156	0.782	Yes
2	0.050000	114368.468000	0.053645	7.289	Yes
3	0.100000	197228.952000	0.096454	-3.546	Yes
4	0.150000	292343.647000	0.145595	-2.937	Yes
5	0.200000	405680.733000	0.204150	2.075	Yes

Figure 2. Calibration Curve for Sulfadizine (SDM)



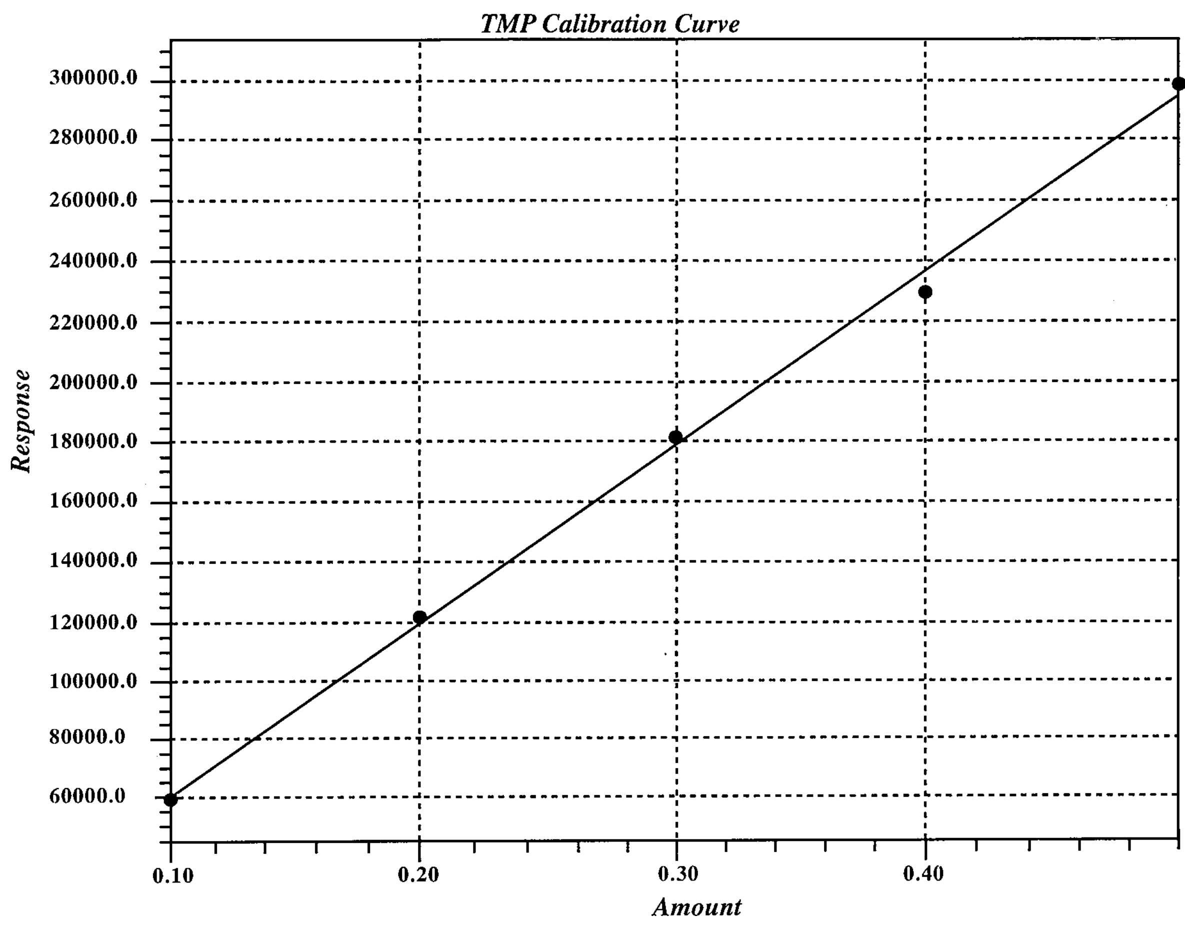
SDM ***Calibration Information***

Processing Method	Sulfa	System	Sulfa
Channel	486	Date	18-DEC-96
Type	LC	Name	SDM
Retention Time	18.433 min	Order	1
A	-5544.190171	B	2976050.376642
C	0.000000	D	0.000000
E	0.000000	F	0.000000
R	0.998484	R^2	0.996970
Standard Error	13831.721025		

SDM Point Table

	Amount	Response	Calc. Amount	% Deviation	Manual
1	0.020000	56160.883000	0.020734	3.669	Yes
2	0.050000	154063.998000	0.053631	7.262	Yes
3	0.100000	276077.752000	0.094629	-5.371	Yes
4	0.150000	432549.913000	0.147207	-1.862	Yes
5	0.200000	600972.699000	0.203799	1.900	Yes

Figure 3. Calibration Curve for Trimethoprim (TMP)



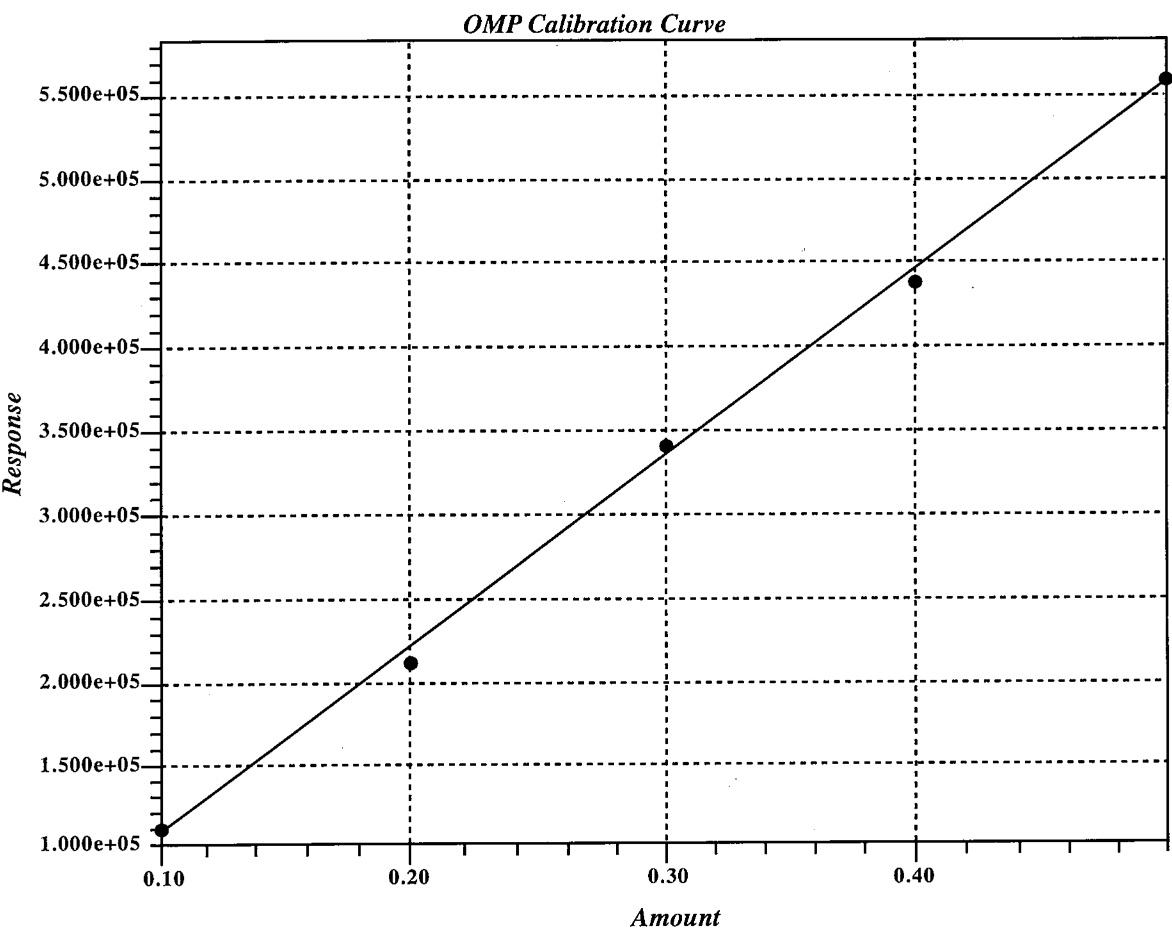
TMP Calibration Information

Processing Method	Sulfa	System	Sulfa
Channel	486	Date	18-DEC-96
Type	LC	Name	TMP
Retention Time	21.150 min	Order	1
A	-825.516703	B	598510.737408
C	0.000000	D	0.000000
E	0.000000	F	0.000000
R	0.998844	R^2	0.997689
Standard Error	5259.392120		

TMP Point Table

	Amount	Response	Calc. Amount	% Deviation	Manual
1	0.100000	57820.741791	0.097987	-2.013	Yes
2	0.200000	121226.679199	0.203926	1.963	No
3	0.300000	181338.263137	0.304362	1.454	No
4	0.400000	231126.776751	0.387549	-3.113	No
5	0.500000	302126.061719	0.506176	1.235	No

Figure 4. Calibration Curve for Ormetoprim (OMP)



OMP Calibration Information

Processing Method	Sulfa	System	Sulfa
Channel	486	Date	18-DEC-96
Type	LC	Name	OMP
Retention Time	21.567 min	Order	1
A	-6583.610951	B	1125397.937000
C	0.000000	D	0.000000
E	0.000000	F	0.000000
R	0.998610	R^2	0.997223
Standard Error	10843.623381		

OMP Point Table

	Amount	Response	Calc. Amount	% Deviation	Manual
1	0.100000	110052.021000	0.103639	3.639	Yes
2	0.200000	210282.364000	0.192702	-3.649	Yes
3	0.300000	340988.369744	0.308844	2.948	Yes
4	0.400000	431927.849000	0.389650	-2.587	Yes
5	0.500000	561928.247000	0.505165	1.033	Yes

Figure 5. Representative Chromatogram of 20ng of Sulfadiazine detected by UV Detector at 288nm under the conditions employed in this work.

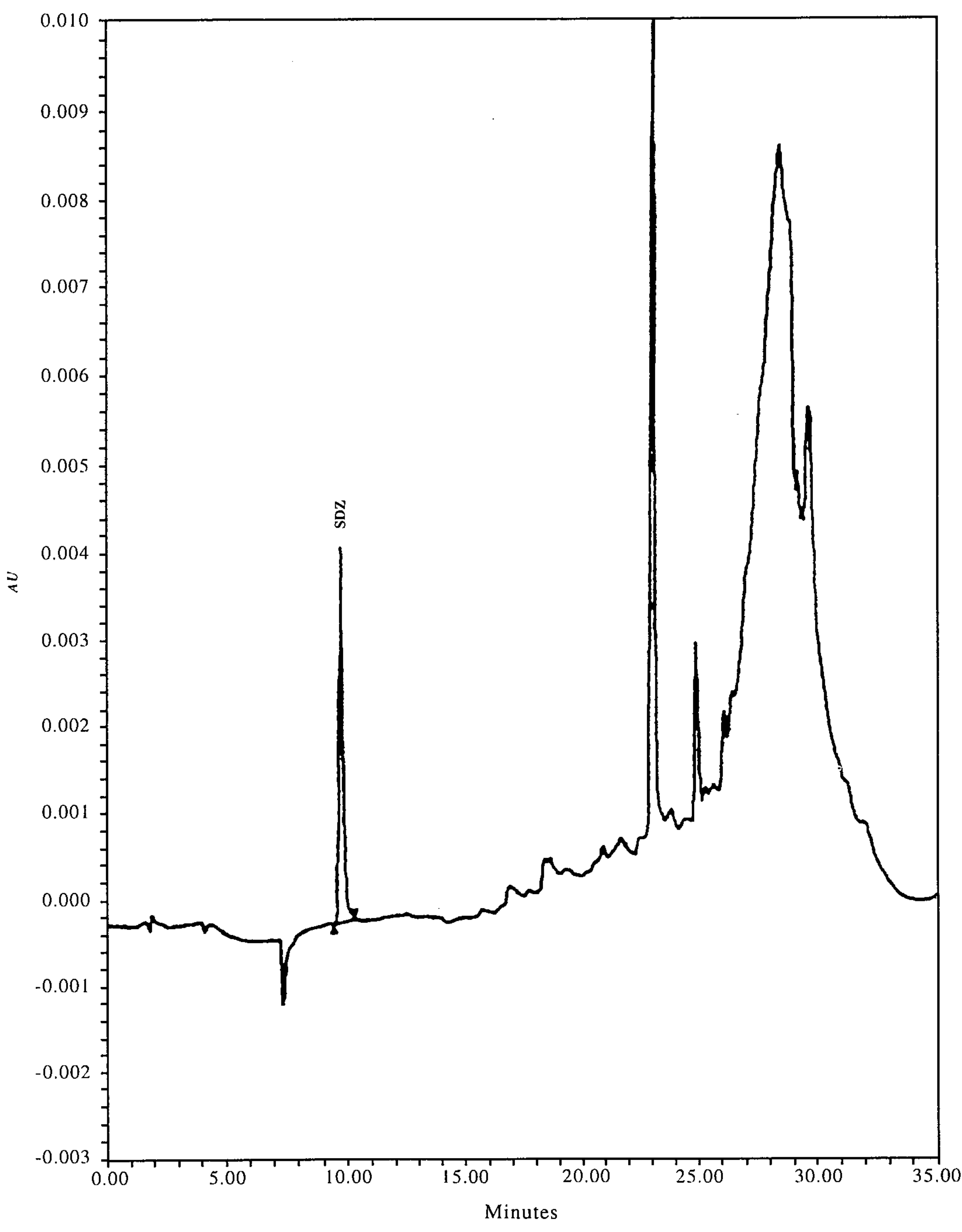


Figure 6. Representative Chromatogram of 20g of Sulfadimethoxin detected at 288nm under the conditions described in the text.

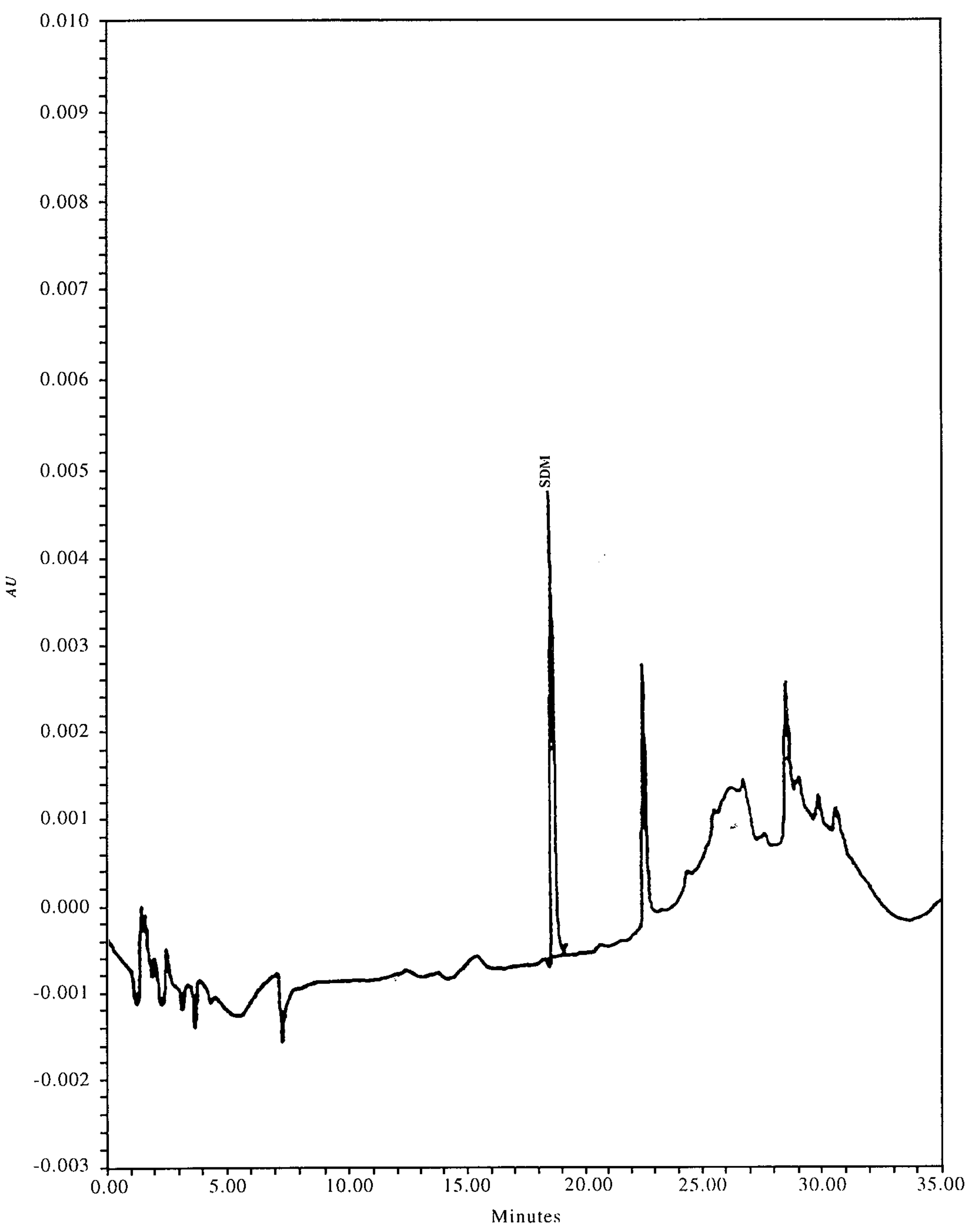


Figure 7. Representative Chromatogram of 200ng of Trimethoprim detected at 288nm under the conditions outlined in the text.

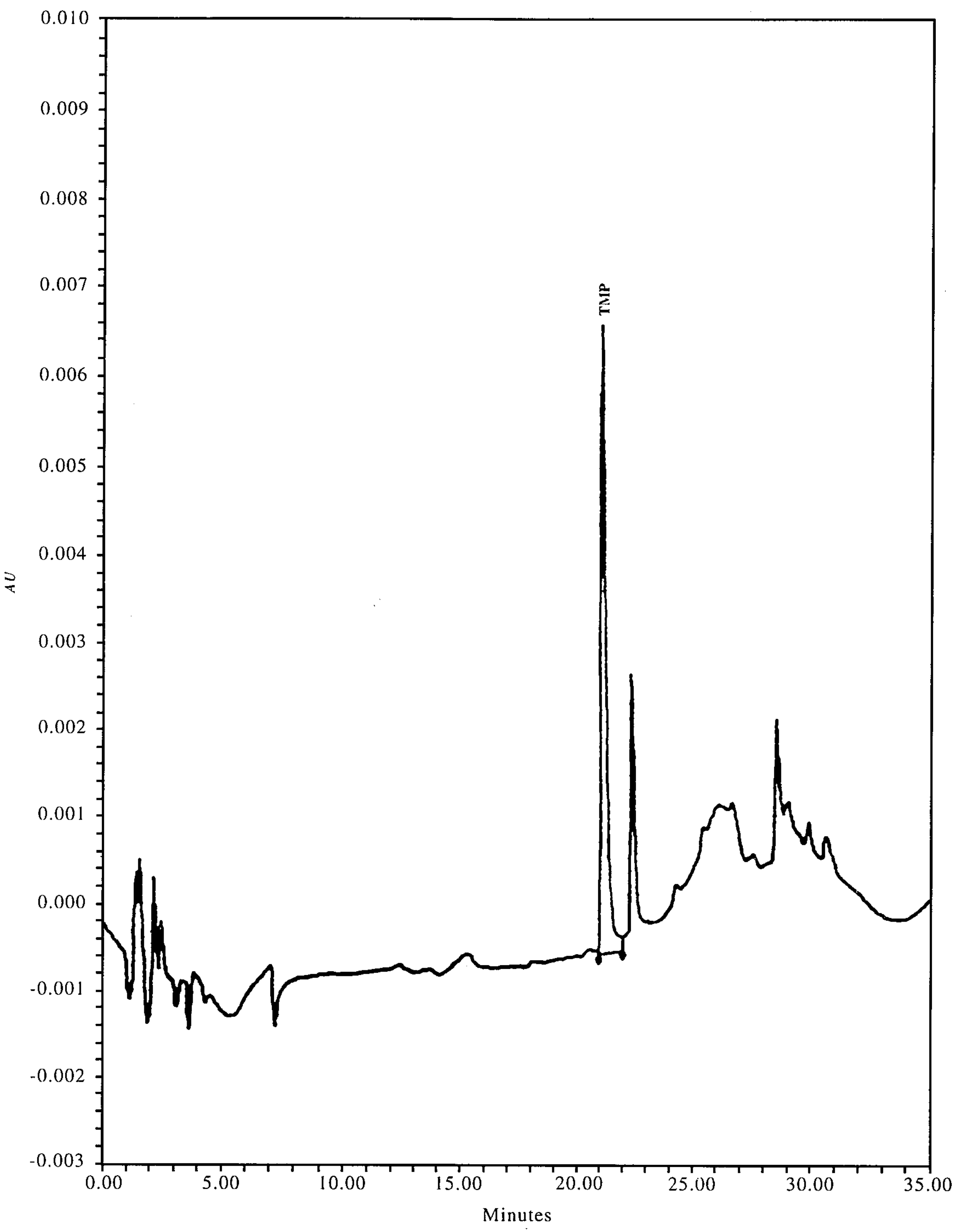


Figure 8. Representative Chromatogram of 200ng of Ormetoprim under the conditions described in the text.

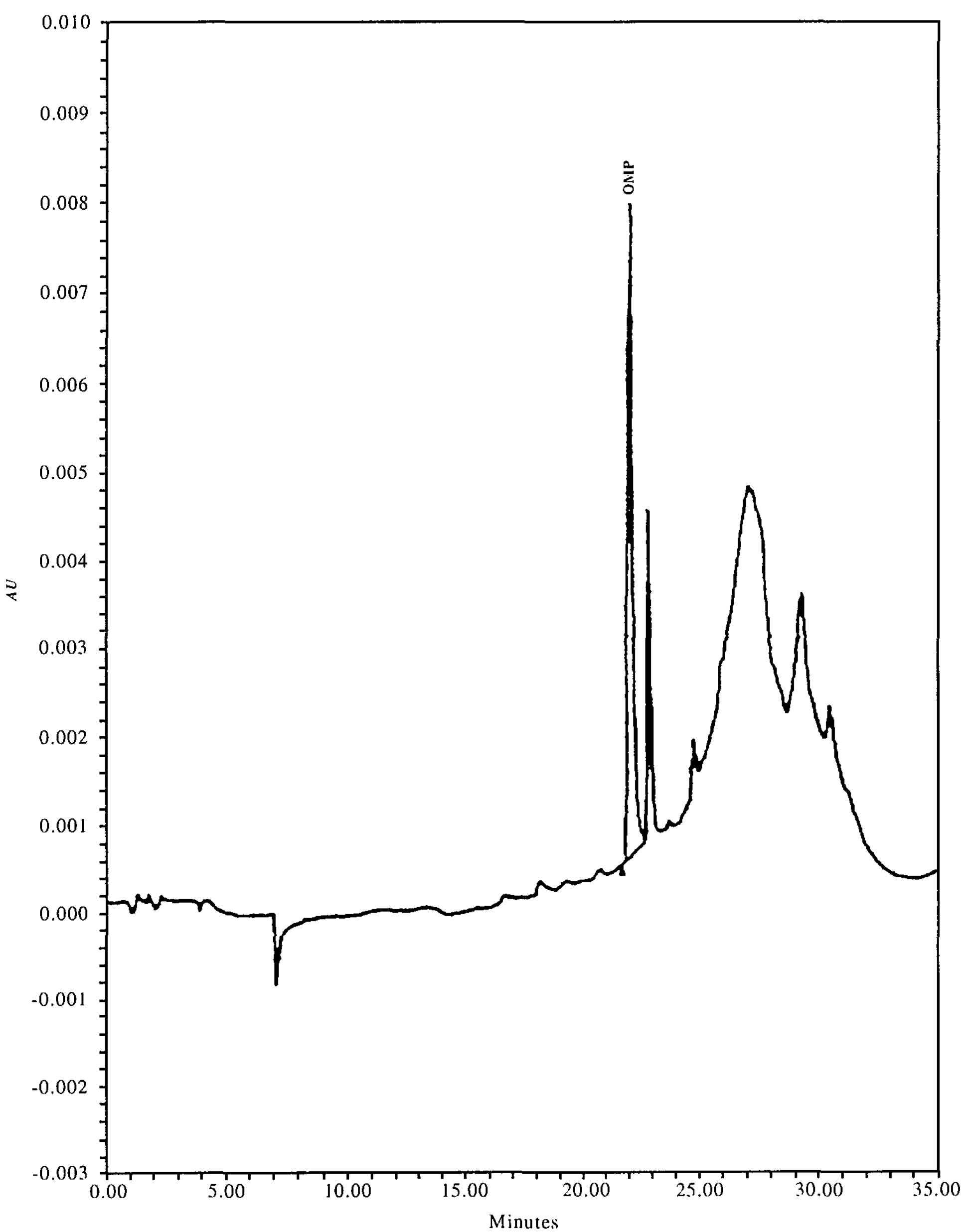
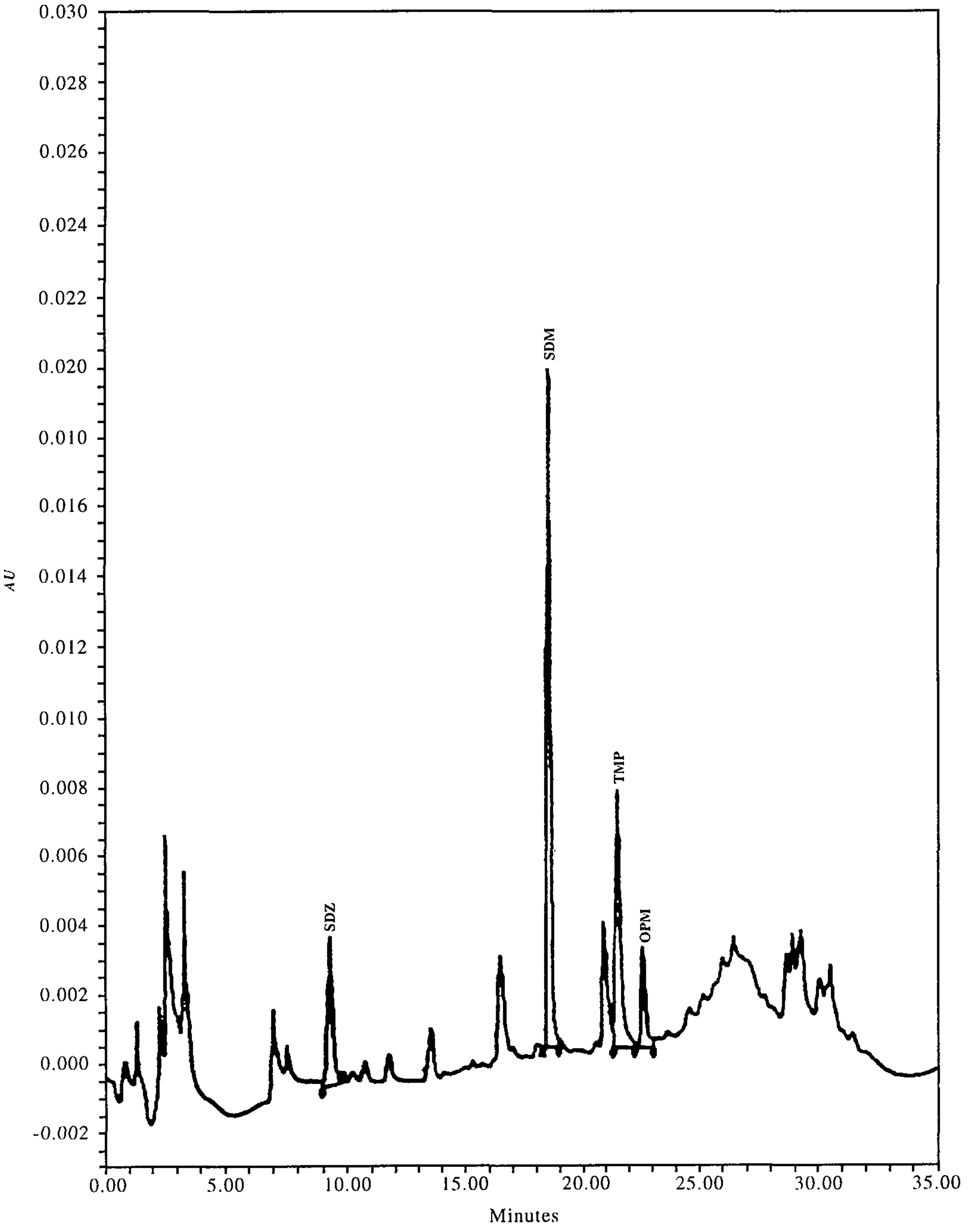


Figure 9. Liquid Chromatogram of 100ng of each of SDZ, SDM, TMP, and OPM obtained on Vyadac 201T54 Column under the conditions described in the text. UV Detection was monitored at 288nm.



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C. DETERMINATION OF FURAZOLIDONE IN SHRIMP USING HPLC METHOD

Scope:

Furazolidone is a synthetic nitrofuran antimicrobial that is effective in treating bacterial and fungal infections in fish and shrimp[1]. The use of this is prohibited in United States and Canada because of its carcinogenic properties. There are no laws to restrict its use in many other countries. Thus, residues of this drug may be present in aquaculture products and may represent a threat to human health.

In recent years fish for food have been artificially cultivated on a large scale in both fresh water and sea water in many countries. Oxolinic acid, nalidixic acid, and piromidic acid are antibacterial agents that are widely used in the cultivation of fish such as salmon, rainbow trout, sweetfish, carp, eel, and yellowtail.

In Japan the Food Safety Law established a zero residue level for all antimicrobial agents in foods in 1971. Hence, routine screening of above drug residues in cultivated fish is necessary, but the available methods of analysis are limited. Microbiological assays for oxolinic acid lack sensitivity and specificity.

Chemical methods for quantitation of furazolidone in tissues of terrestrial and aquatic animals have been developed[2-5]. The method described here is one reported by Stehly et al.[5].

Principle:

The HPLC method described involves extraction of furazolidone from shrimp using acetonitrile, and the extract is taken to dryness. The residue is dissolved in acetonitrile , and the solution is passed through alumina and C-18 cleanup columns. The eluate is taken to dryness and re-dissolved in a suitable solvent for reverse phase (C-18) liquid chromatography with UV detection at 365nm.

Materials:

(1) Blender: Warring 2-speed commercial blender, 50-250 ml container.

(2) Centrifuge tubes: Polepropylene, 15 and 50ml capacity.

(3) Homogenizer: Brinkmann PT 10-35, (Brinkmann Instruments, Inc. Westbury, NY.)

(4) Boiling flasks: Round-bottom flask, 100ml, and a pear shaped flask, 25ml.

(5) Filter: Millex-FG, 0.2µm, 13mm filter unit (Millipore Products Division, Bedford, MA).

(6) HPLC system: The system included A Waters 717 plus Autosampler, Waters 486 Tunable Absorbance Detector, Water Temperature Control Module and Oven.

(7) Pump system: Waters 510 HPLC pumps with capacity to generate rapid gradient and at least 4000 psi.

(8) HPLC column: Beckman 5 um Ultrasphere (254 x 4.6mm, id.) with 5μm Adsorbosphere C18 (10 x 4.6mm) guard column (Althech Associates Inc., Deerfield, Il., USA)

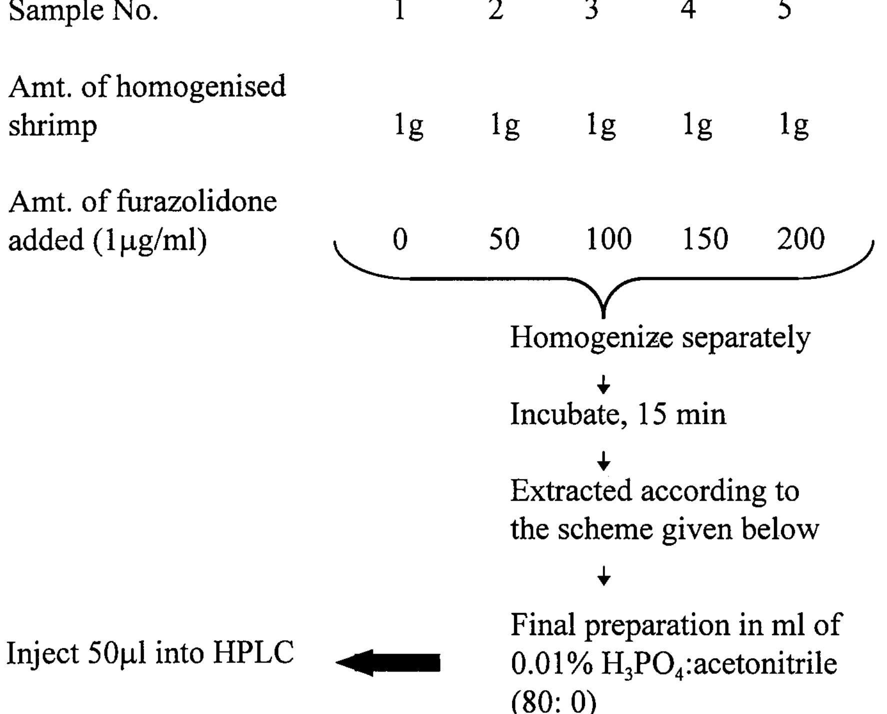
(9) Solvents: LC reagent-grade acetonitrile (J. T. Baker Inc., Philipsburg, PA., USA)

(10) Sodium sulfate: Anhydrous powder, reagent grade (J. T. Baker)

(11) Standard: Furazolidone (Sigma Chemical Co., St. Louis, MO)

Procedure:

(a) Fortification and Recovery Experiment:



Scheme 1

Sample Preparation

↓

Sample (cleaned shrimp)

↓

Remove tail and chitin shell

↓

Homogenize

↓

Weigh 1g in 50 ml polypropylene centrifuge tube

↓

Add 0.05g anhyd. Na_2SO_4 and 35 ml acetonitrile

↓

Incubate, 10 min

↓

Homogenize, 15 sec.

↓

Wash homogenizer probe with 3 ml acetonitrile

↓

Homogenize, 15 sec.

↓

Filter homogenate through pre-washed extraction column (15 ml with 20 um, polyethylene frits)

↓

Collect effluent into 100 ml boiling flask

↓

Evaporate to dryness (35°C)

↓

Residue, redissolved in 2 ml acetonitrile

↓

Pass through prewashed C18 column connected below an alumina column

Alumina column

C18 Column

↓

Collect filtrate in a 25 ml flask (total 6 ml)

↓

Evaporate to dryness

↓

Residue, redissolved in 500 µl solution of 0.01% H_3PO_4 : acetonitrile (80 : 20)

↓

Sonicate (30-60 sec) and pass through Millex filter → Inject 50 µl into HPLC

(b) Liquid chromatography: Prepare standard solutions containing 5, 10, 40, 80, 100, and 150ng furazolidone in 0.01% H_3PO_4:acetonitrile (80:20). The solutions are stable for one week if protected from direct light and refrigerated when not in use.

Set UV detector at 365nm and use mobile phase 0.01% H_3PO_4 - acetonitrile (70 + 30) at a flow rate of 1 ml/min to elute furazolidone standards at ca. 6.5 min. Plot standard curve from 50µl injections of the standards. Determine concentrations of furazolidone in shrimp by comparing integrated peak area for injected extract with standard curve.

Temperature of the HPLC column was maintained at room temperature and effluent was monitored at 365nm using Water Tunable Absorbance Detector.

(c) Data Processing: Millennium 2010 Chromatography Manager equipped with Millennium computer software and coupled to COMPQ (Prolinear 4/66) Data Station with a NEC Printer (Model P1200) (NEC Technology, Hong Kong Ltd).

Results:

Table 2 shows recovery of furazolidone from spiked shrimp homogenates while *Table 3* summarises the results obtained with different concentrations of standard furazolidone. Recovery values calculated for spiked samples were consistent between 50 and 200ng/g range and averaged 83%. Other workers have reported recovery values ranging between 77 and 90%.

Although in the present work lower levels (5 - 40ng/g) were not tested Stehly et al[5] have successfully demonstrated that this method is suitable for the quantitation of low levels of furazolidone extracted from shrimp.

Retention time for standard furazolidone under the conditions used ranged between 6.2 and 6.53 min while the average value was found to be 6.53 min. Under the conditions the values obtained for the spiked samples ranged between 6.15 and 6.30 min with an average value of 6.2 min.

Figure 10 depicts an elution profile of 80ng sample of standard furazolidone injected into HPLC and a similar peak was obtained from the residue extracted from spiked shrimp samples as seen in *Figure 11*. A calibration curve obtained by injecting different concentration of standards is shown in *Figure 12*. The plot reveals a linear relationship between the concentration of the drug and the peak height. The correlation coefficient value obtained agrees with the theoretical value expected.

Table 2. Recovery of Furazolidone from Spiked Shrimp Homogenates

Sample No.	Amount Added (ng)	Peak Area	Peak height	% Recovery	Retention time (min)
1.	Nil	--	--	--	--
2.	50	4252	207	114	6.22
3.	100	5252	361	71	6.18
4.	150	9649	521	86	6.17
5.	200	8992	603	60	6.15
STD	50	3724	332	100	6.30

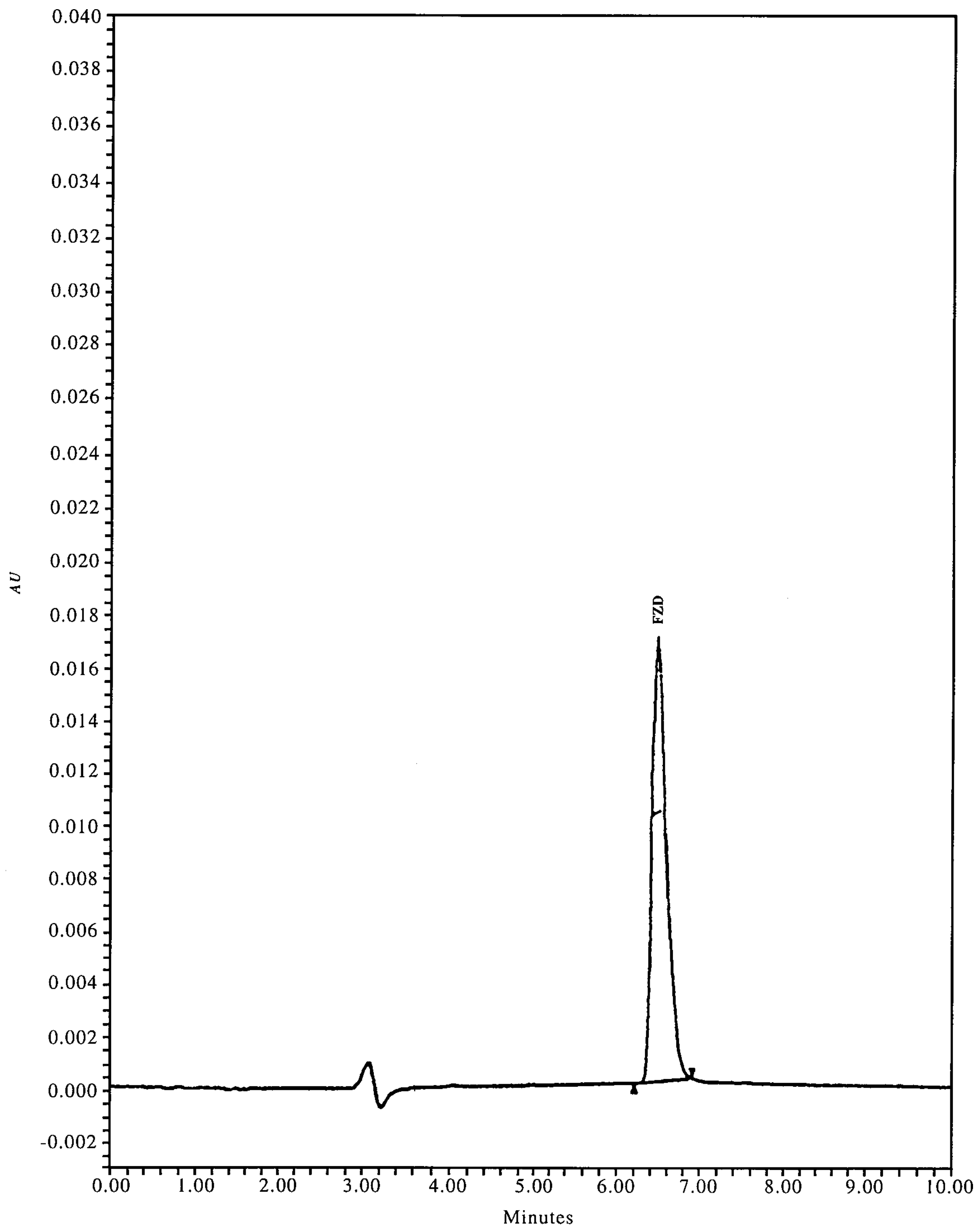
Average % Recovery = 83
Average retention time = 6.2 min

Table 3. Calibration Curve for the Standard Furazolidone

Amout injected (ng)	Peak height	Peak Area	Retention time (min)
5	971	11156	6.38
10	2134	24402	6.42
20	4211	48387	6.43
40	8536	102361	6.45
80	16838	206177	6.48
100	20204	256557	6.48
150	30104	390638	6.53

Correlation coefficient (R) = 0.9999
Average retention time = 6.53 min.
Coefficient of determination 9R (R) = 0.9999

Figure 10. Representative chromatogram of 80ng of Furazolidone from an Ultraphere column under the conditions described in the text. UV detection was monitored at 365nm.



Sample Name: Std. FZD 80ng Vial: 1 Inj: 1 Ch: 486 Type: Standard

Figure 11. Representative chromatogram of spiked shrimp homogenate (20ng/g). UV detection was monitored at 365nm.

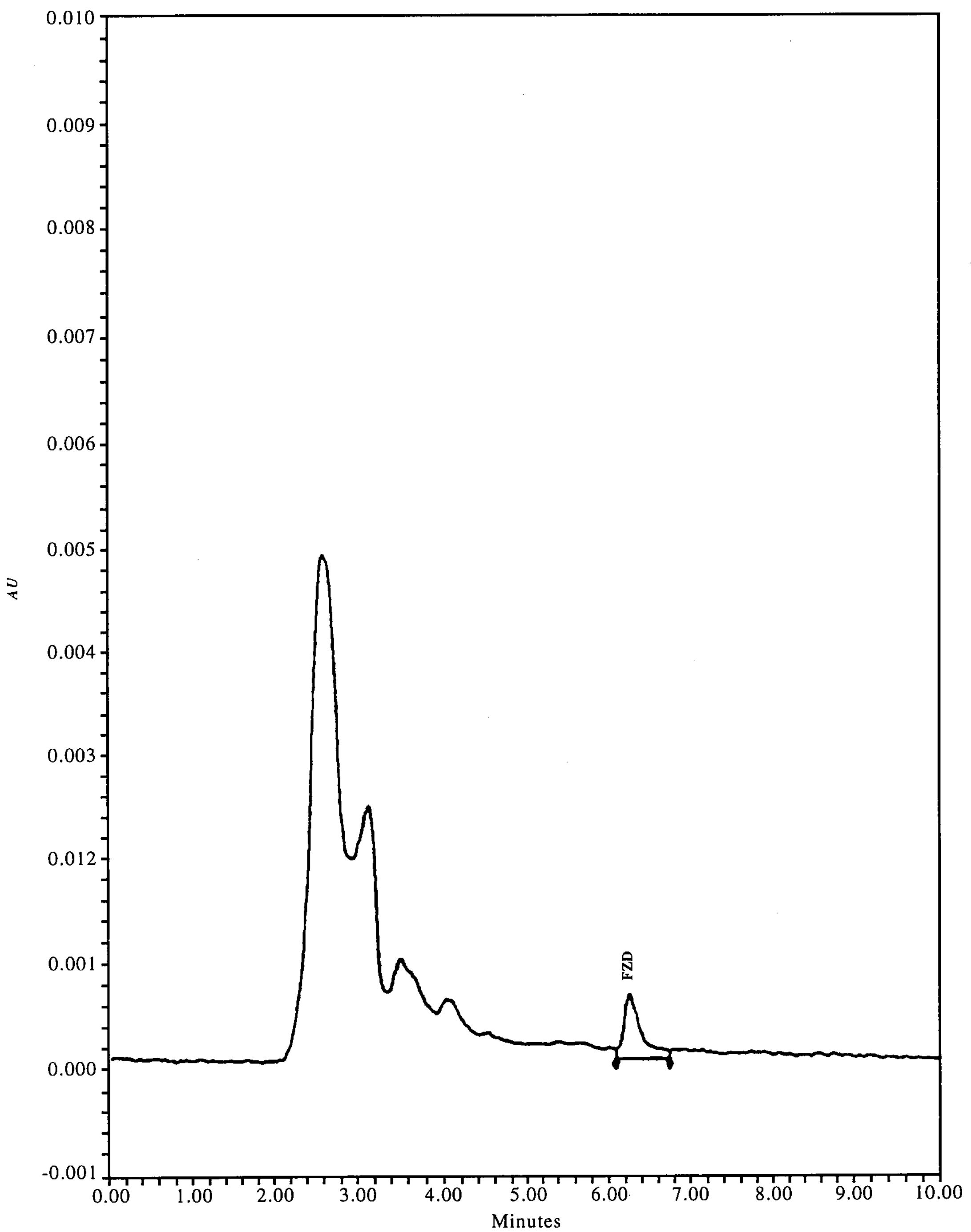
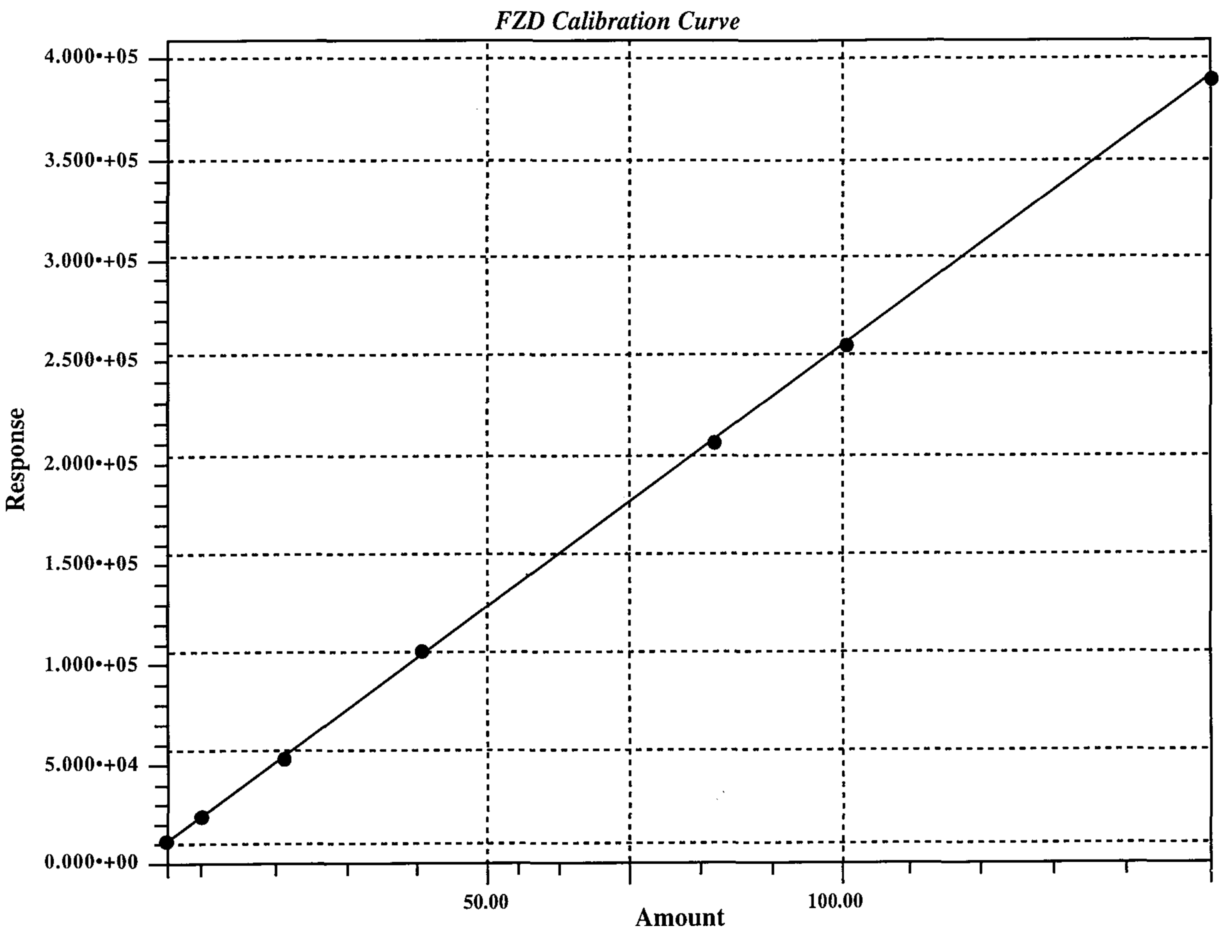


Figure 12. Calibration Curve Obtained with Different Concentrations of Standard Furazolidone



OMP Calibration Information

Processing Method	Default	System	EDTA
Channel	486	Date	24-DEC-96
Type	LC	Name	FZD
Retention Time	6.383 min	Order	1
A	-2604.654023	B	2612.124164
C	0.000000	D	0.000000
E	0.000000	F	0.000000
R	0.999961	R^2	0.999922
Standard Error	1366.719624		

FZD Point Table

	Amount	Response	Calc. Amount	% Deviation	Manual
1	5.000000	11156.377529	5.26138	5.363	No
2	10.000000	24401.500333	10.338771	3.388	No
3	20.000000	48387.096757	19.521182	-2.394	No
4	40.000000	102360.997409	40.184021	0.460	No
5	80.000000	206177.158689	79.927982	-0.090	No
6	100.000000	256556.582025	99.214746	-0.785	No
7	150.000000	390637.995539	150.515160	0.363	No

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D. DETECTION AND QUANTITATION OF MALACHITE GREEN IN SPIKED SHRIMP SAMPLES

Scope:

Malachite green (MG) has been used as an effective treatment for external fungal and protozoan infections in fish[1-2]. MG belongs to the triphenylmethane class of dyes, some of which are carcinogens[3]. Scientists have demonstrated that MG causes significant development of abnormalities when administered to eggs of rainbow trout and pregnant New Zealand white rabbits.

In animals, MG is reduced to its colourless form, leuco MG[4-5]. The leuco form of MG is a precursor of the chemical during production and could be a contaminant in commercially prepared dye.

Principle:

The chromatic and leuco forms of MG have been determined simultaneously by Bauer et al[6]. The authors split the fish tissue samples into half and oxidised one half with the lead oxide (PbO_2). The oxidised sample was analysed for chromatic MG. The amount of leuco MG in the sample was determined by the difference between unaltered and oxidised subsamples. Chromatic MG and leuco MG can be analysed by HPLC system using visible spectrophotometry after postcolumn oxidation of leuco form to chromatic form. The method described here is that of Allen et al[7] and involves conversion of leuco to chromatic form by the treatment with lead dioxide.

Apparatus:

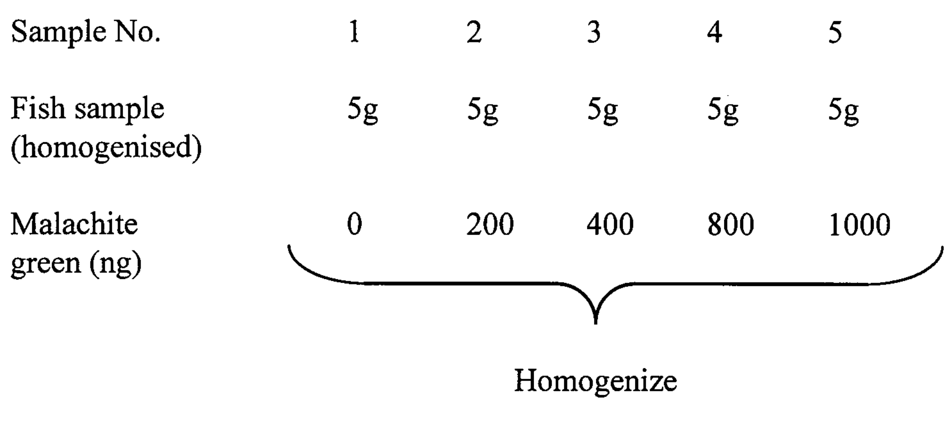
LC System:	Waters 717 system with Autosampler, Waters 486 tunable Absorbance Detector, Waters Temperature Control Module and Oven.
LC Pump system:	Waters 6510 pumps with capacity to generate rapid gradient and at least 4000 psi.
LC Column:	uBondapak C18, 300 x 3.9mm, id., particle size 10μm.
Spectrophotometer:	Waters 486 Tunable Absorbance Detector. Mg was monitored at 600nm instead of at 618nm because of the specification of the detector used.
Postcolumn reactor:	Postcolumn oxidation of leuco form. Stainless steel tube 32 x 4mm, id, packed with 10% lead dioxide (PbO_2) suspended in Celite 545 (PbO_2 is previously dry-mixed with Celite to give uniform mixture) and capped with 2μm frits. As the reactor is being packed with PbO_2 in Celite, gently tap it to prevent the formation of voids. Place postcolumn reactor in line between the HPLC column and the spectrophotometer detector.

Homogenizer:	Any high speed homogenizer.
Blender:	Warring or equivalent, equipped with a stainless steel cup.
Filtration column:	Bakerbond spe 6ml disposable filtration columns.

Chemicals And Reagents:

LC Mobile Phase:	Consisting of 85 + 15 mixture of methanol to aqueous acetate buffer (0.05 M sodium acetate and 0.1M glacial acetic acid in water)
Malachite green oxalate:	Cat. No. 1264 (Eastman Kodak Co., Rochester, NY)
Leuco malachite green:	Cat. No. 3620 (Eastman Kodak).
Anhydrous acetic acid:	cat.No.24,124-5 (Aldrich Chemical Co., Inc., Milwaukee, WI)
Anhydrous sodium bicarbonate:	Cat. No.S-8875 (Sigma Chemical Co., St. Louis, MO)
Sodium acetate (acetic acid, sodium salt, anhydrous):	Cat. No. 24,124-5 (Aldrich)
Anhydrous sodium sulfate:	Cat.No.7757-82-6 (Fisher Scientific, Pittsburgh, PA)
Solvents:	LC grade solvents, including glacial acetic acid, water, methanol, chloroform, and acetonitrile (J. T. Baker Inc.)
Lead oxide:	Cat. No.5727, ACS grade (Mallinckrodt Inc., Science Product Division, St. Louis, MO)
Celite 545:	Cat. No. C-212 (Fisher Scientific)
Malachite green oxalate and leuco malachite green stock solutions:	1mg/ml in methanol. Prepare standard solutions of malachite oxalate and leuco malachite green at concentrations of 200, 400, 800 and 1000ng/ml fresh daily in mobile phase for LC retention time markers.

Test Sample Preparation (fortification) and Extraction (recovery):



Sample No.	1	2	3	4	5
Fish sample (homogenised)	5g	5g	5g	5g	5g
Malachite green (ng)	0	200	400	800	1000

Homogenize

↓

Incubate, 30 min, room temperature

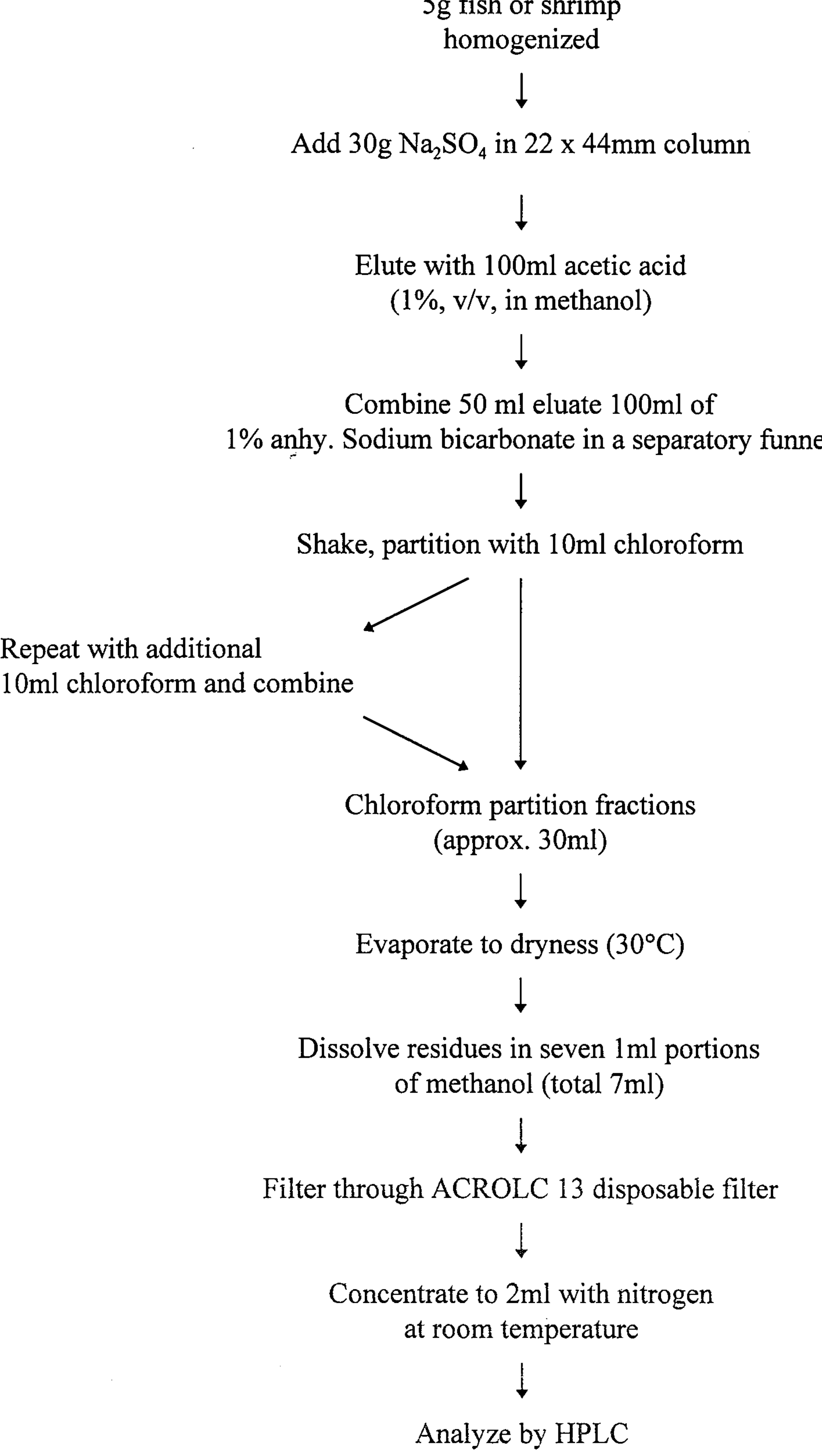
↓

Extract according to the scheme shown below

↓

Final preparation
HPLC analysis

Sample Preparation:



Results:

Figure 13. Chromatogram of 100ng of chromatic malachite green on a uBondapak C18 column with 1 ml/min of methanol-aqueous acetate buffer (85:15)

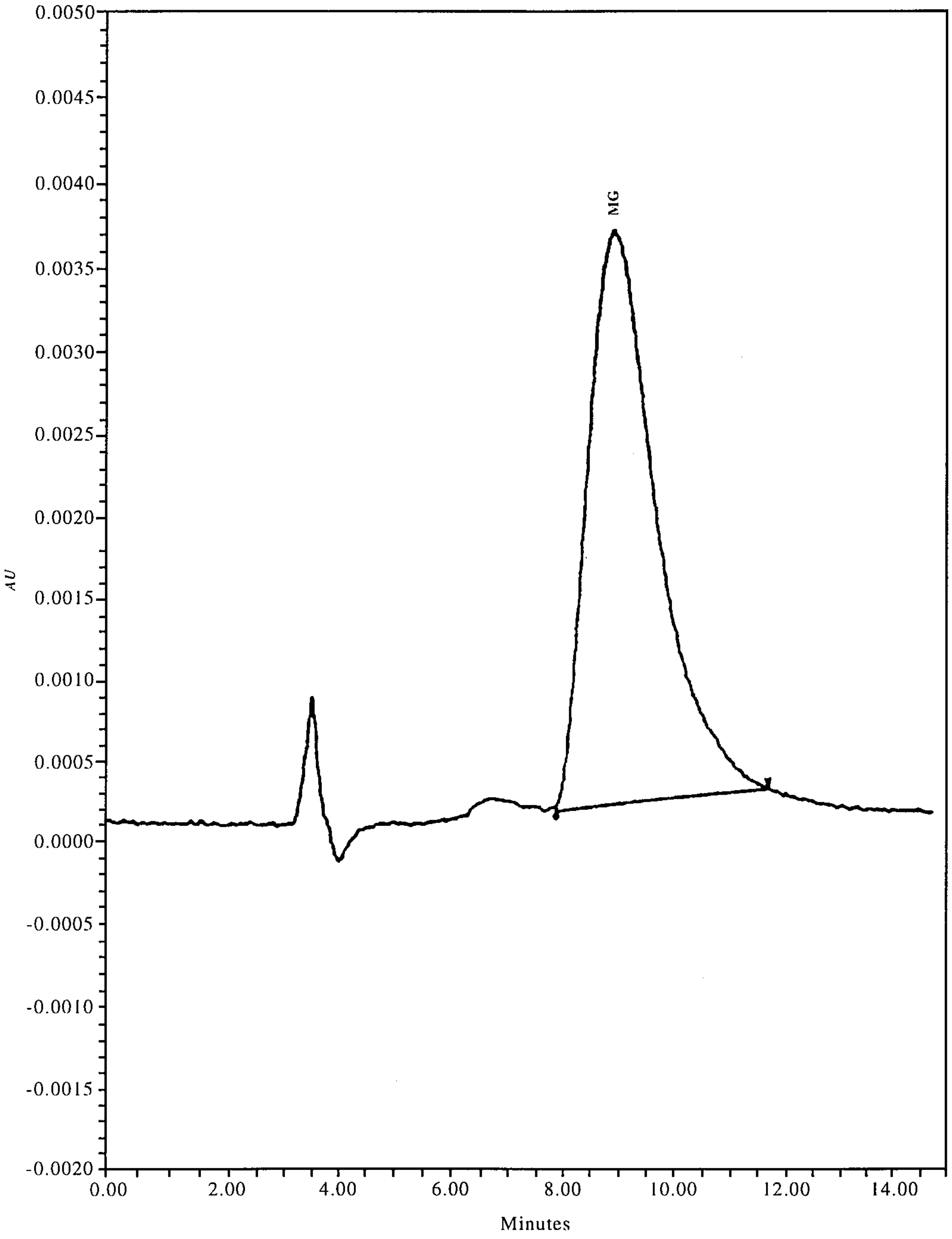
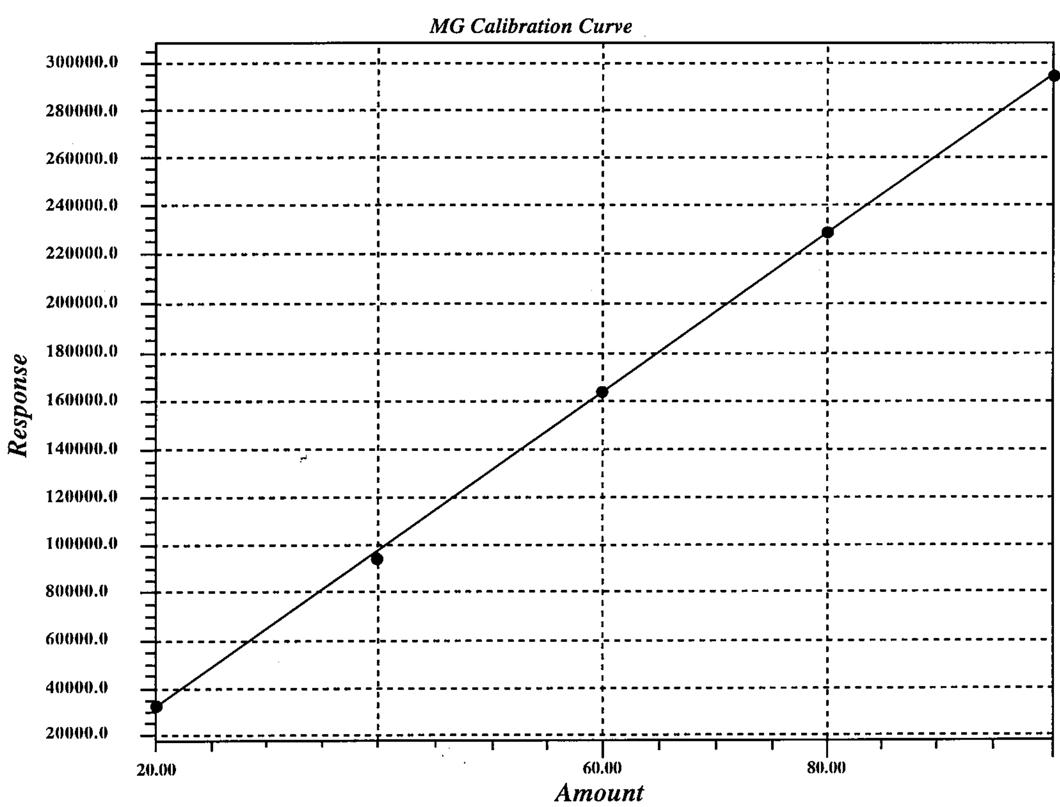


Figure 14: Calibration Curve for Malachite Green



MG Calibration Information

Processing Method	MG	System	EDTA
Channel	486	Date	02-JAN-97
Type	LC	Name	MG
Retention Time	9.333 min	Order	1
A	-34205.456400	B	3295.140210
C	0.000000	D	0.000000
E	0.000000	F	0.000000
R	0.999891	R^2	0.999782
Standard Error	1775.816900		

MG Point Table

	Amount	Response	Calc. Amount	% Deviation	Manual
1	20.000000	33189.682000	20.452889	2.264	Yes
2	40.000000	95094.384000	39.239556	-1.901	Yes
3	60.000000	163828.323000	60.098741	0.165	Yes
4	80.000000	230302.994000	80.272290	0.340	Yes
5	100.000000	295099.398000	99.936523	-0.063	Yes

Fortification and Recovery:

Total Residue Recoveries As Percent Of Total Fortification For Extracts Of Rainbow Muscle Tissue

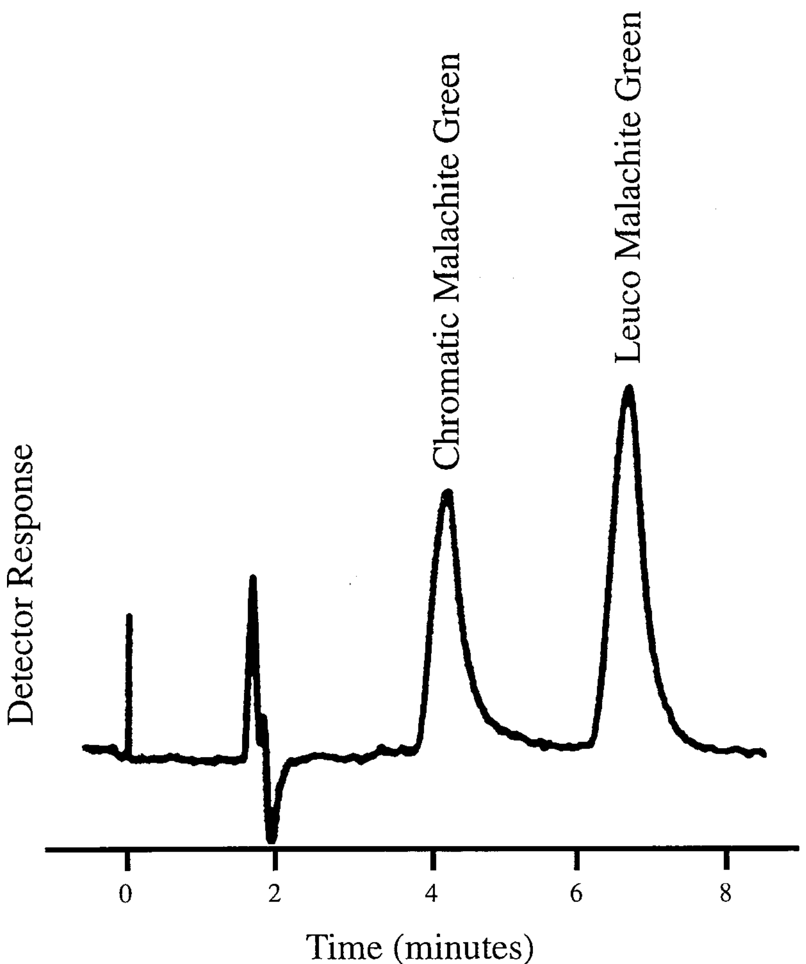
Fortification (ng/g)	Sample No.	Leuco MG	Chromatic MG
1000	1	85	15
	2	100	00
	3	100	00
	4	75	25
	5	70	30
	6	94	06

Source: Allen et al (1994). J.AOAC, 77: 553

It is interesting to note that the mean recovery of leuco MG and chromatic MG in Allen et al[7] were 89% and 11%, respectively. The standard deviation for the two residues were about 10.5%. The living cells in fish tissue tends to convert chromatic form into leuco form and hence the recovery of the former is usually low. However use of postcolumn oxidation of leuco form by lead dioxide chromatic form can facilitate the determination of chromatic as well as leuco form by the difference.

Allen et al[7] have successfully chromatographed simultaneously leuco and chromatic forms of MG using methanol-water (81:19), buffered with 0.05M sodium acetate and 0.05M glacial acetic acid.

Chromatogram of 10ng each of chromatic MG, and leuco MG on C18 column with 1.5ml/min of methanol:water (81:19), buffered with 0.05M sodium acetate and 0.05 M glacial acetic acid at 618nm.



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