ISSN: 1995-4875

CRFM Special Publication. No.14

Manual on Laboratory Testing of Fishery Products

The SPS Project is funded by the European Union under the 10th Economic Development Fund and is being implemented by the Inter-American Institute for Cooperation on Agriculture (IICA) with the following regional Partners: the CARICOM Secretariat, the Caribbean Regional Fisheries Mechanism (CRFM), El Comité Nacional para la Aplicación de Medidas Sanitarias y Fitosanitarias de la República Dominicana (CNMSF) and CARIFORUM.

Manual on Laboratory Testing of Fisheries Products

Prepared by: Christine Froese, Megapesca Lda., December 2016, under contract to the Inter-American Institute for Cooperation on Agriculture (IICA), through the 10th EDF funded Sanitary and Phytosanitary Project

Correct Citation:

Froese, C, 2016. Manual on Laboratory Testing of Fishery Products. *CRFM Special Publication*. No.14. 89pp.

ISSN: 1995-4875

ISBN: 978-976-8257-38-3

Cover Photo: Fishery Product Testing Laboratory, Saint Vincent

Contents

List of Tables

List of Figures

ABBREVIATIONS

µg	Microgram
3-MCPD	3-monochloropropane-1,2-diol or 3-chloro-1,2-propanediol
AAS	Atomic Absorption Spectroscopy
AFNOR	Association Française de Normalisation
AOAC	Association of Analytical Chemists
ASP	Amnesic Shellfish Poisoning
ASPW	Alkaline saline peptone water
BAM	Bacteriological Analytical Manual
BSI	British Standards Institution
BMS	Bivalve Molluscan Shellfish
CAs	Competent Authorities
CCA	Chromogenic Coliform Agar
Cd	Cadmium
CEFAS	Centre for Environment, Fisheries & Aquaculture Science
CEN	European Committee for Standardisation
Cf	Correction Factor
CFU	Colony Forming Units
CFP	Ciguatera Fish Poisoning
SPE COOH	Solid Phase Extraction Purification of Carboxylic Acid Products
CREM	Ciguatoxin Rapid Extraction Method
CRFM	Caribbean Regional Fisheries Mechanism
CTX	Ciguatoxins
DNA	Deoxyribonucleic acid
DSP	Diarrheic shellfish poisoning
E. Coli	*Escherichia coli*
EC	European Commission
EDF	European Development Fund
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
EN	English Language
EPA	Environmental Protection Agency (US)
EU	European Union
FDA	Food and Drug Administration
GC-ECD	Gas chromatography with electron capture detection

GC-MS	Gas Chromatography with Mass Spectrometer
GLC	Gas Liquid Chromatograph
h	Hours
HABs	Harmful algal blooms
HACCP	Hazard Analysis and Critical Control Point
HCH	Hexachlorcyclohexan
Hg	Mercury
HPLC	High performance liquid chromatography
ICMSF	International Commission on Microbiological Specifications for Foods
IEC	International Electrotechnical Commission
ISO	International Organization for Standardization
IUPAC	International Union of Pure and Applied Chemistry
kg	Kilogram
LC-MS	Liquid Chromatography-Mass Spectrometry
LC-MS-MS	Liquid Chromatography–Tandem Mass Spectroscopy
MBA	Mouse Bio Assay
m-CP	Membrane *Clostridium perfringens*
MF	Membrane Filtration
MKTTn	Muller-Kauffmann Tetrathionate-Novobiocin
MPN	Most Probable Number
MRL	Maximum Residue Level
MRPL	Minimum Required Performance Limit
MU	Mouse Units
MUG	4-methylumbelliferyl-beta-D-glucuronide
NaCl	Sodium Chloride
NACMCF	National Advisory Committee for Microbiological Criteria for Foods
NF	Validation mark, European certification system by AFNOR based on EN ISO 16140 standard
NSP	Neurotoxic Shellfish Poisoning
Pb	Lead
PBS	Phosphate Buffered Saline
PCB	Polychlorinated Biphenyl
PCR	Polymerase Chain Reaction
ppm	Part per million
PSP	Paralytic Shellfish Poisoning
PTFE	Polytetrafluoroethylene

RVS	Rappaport-Vassiliadis Medium with Soya
SO_2	Sulphur Dioxide
SPE	Solid Phase Extraction
SPS	Sanitary and Phytosanitary
STX	Saxitoxin
TCBS	Thiosulphate Citrate Bile and Sucrose
TSA	Tryptone Soy Agar
TSC	Tryptose Sulfite Cycloserine
TSI	Triple Sugar Iron
TSYEA	Tryptone Soya Yeast Extract Agar
TSYEB	Tryptone Soya Yeast Extract Broth
UKAS	UK Accreditation Service
VRBL	Crystal Violet Neutral Red Bile Lactose
WHO	World Health Organization
XLD	Xylose Lysine Deoxycholate

FOREWORD

The fishery sector is of great importance for CARIFORUM States, as it provides employment for an estimated 121,000 persons, and contributes significantly to food security and export earnings. The marine capture sector is mostly characterized by a small-scale multi-gear fishery, but several countries have also developed distant water fleets of industrial vessels. Aquaculture is also becoming more important, with some large-scale investments in shrimp and tilapia production as well as numerous experimental and small-scale operations. The fishery sector of CARICOM countries also engages in significant international trade with combined exports worth US$390 million in 2015, with imports over US$180 million (which supply not only domestic markets, but also help to sustain our tourism sector). All this business, and the resulting benefits to the people of our region, depend wholly on the fishery products we produce and market being safe for human consumption. However, ensuring such safety against the background of a diversified and globally integrated fishery sector presents significant challenges, requiring not only considerable resources, but also a high level of expertise and knowledge.

The Caribbean Regional Fisheries Mechanism was formed in 2002 with the objective to promote and facilitate the responsible utilization of the Region's fisheries and other aquatic resources for the economic and social benefits of the current and future population of the region. In line with this aim, we are therefore pleased to present this Manual, which is one of a series, which provides valuable, up-to-date, regionally relevant and practical advice on ensuring the food safety of Caribbean fishery products. The Manuals are intended for use by both fishery sector operators, as well as those involved in protecting our consumers, through the implementation and enforcement of sanitary regulations. We are sure that these documents will help to provide a solid technical basis for the ensuring the continued and sustainable growth of our seafood sector.

1 INTRODUCTION

1.1 Background

This manual was developed within the framework of the EU funded 10th EDF Sanitary and Phytosanitary (SPS) Project, under the terms of a contract "Capacity Building of regulatory and industry stakeholders in Aquaculture and Fisheries Health and Food Safety to meet the SPS requirements of international trade", implemented by Megapesca Lda., Portugal.

The primary objective of the project is to:

> *Build capacities of CARIFORUM States in health and food safety requirements of fisheries and aquaculture (inland, marine) products and as such ensure safe food standards for fisheries products in the region, while meeting the requirements of the region's trading partners worldwide.*

The expected result is that capacities will be built at the national and regional levels for health and food safety requirements of fisheries and aquaculture (inland, marine) products that will also ensure safe food standards for fisheries products in the region, while meeting the requirements of the region's trading partners worldwide.

This operational manual is one of eight manuals aimed at providing structured guidelines to ensuring the safety of fish and fishery products for human consumption, in terms of best practices and official controls. The strengthening of sanitary conditions throughout the region is expected to lead to improved health and well-being of national populations, and increased international trade in fishery products.

1.2 About this manual

This manual provides practical guidelines for laboratory managers and analysts, regarding the requirements for development of a range of tests for assessing the food safety of fishery products.

The manual sets out the testing methods for fishery products, to be conducted by testing laboratories engaged in assessing chemical and microbiological food safety parameters applicable to fishery products. It will be especially useful in instructing Competent Authorities (CAs) and their designated laboratories on how to develop a comprehensive testing capacity for certification of fishery products for international trade.

The manual covers groups of test parameters, based on the typical hazards encountered in the Caribbean. For science-based information about the specific hazards encountered in the Caribbean, and their legal and control requirements, please refer to the CRFM Guide to Food Safety Hazards in Caribbean Fishery products. This sets out regulatory requirements (for example in relation to European Union (EU) limits to the levels of a particular contaminant) together with other requirements for the design of monitoring and control systems.

The specification for most of the testing methods for fish and fishery products recommended in this manual relates to the requirements of relevant EU regulations. These regulations define the limits for various substances, and the test methods and sampling plans to be used for their determination when exporting fishery products to the EU, but they are equally applicable to national public health measures. Where specific methods or procedures are not defined in the legislation, the manual seeks to provide recommended best practices and, if possible, performance criteria of the tests concerned. This document draws on previous work undertaken in 2005 and

2010 by the European Development Fund (EDF) funded project Strengthening Fishery Product Health Conditions in ACP and OCT Countries[1], with updates where appropriate.

1.3 How to use this manual

The guide is aimed at technical and managerial staff working at the Competent Authorities (CAs) laboratories. It will support laboratories in developing test methods for hygiene and food safety testing in fish and fishery products.

The manual details methods for laboratory testing of fishery and aquaculture products, and of the water used by processors and for production of ice. It includes test methods for analysis of potential food safety risks in the Caribbean related to:

- Microbiology of water
- Microbiology of food (fish and fishery products)
- Aquaculture residues (veterinary medicines, pesticides, environmental contaminants).
- Histamine
- Heavy metals (Lead, Cadmium, Mercury)
- Sulphur dioxide and sulphite residues
- Shellfish poisons
- Ciguatera toxins.

Test methods for the analysis of feedstuffs used in aquaculture are not included, nor are the simpler chemical techniques for determination of physical-chemical parameters of processing water (e.g. pH, turbidity, etc.), considered to be outside the scope of the manual.

The manual will be of particular value for those seeking to build national and regional testing capacities for these parameters. For each test parameter, the operation manual sets out the methodologies (official tests), sampling plans and references (where specified by EU) together with requirements for sample preparation, equipment, chemicals and reagents, requirements of standards, staff requirements, and reporting of results. Performance criteria are provided, when specified by legislation or international standards, to support the validation of the individual test methods. In addition, where they are available and applicable, the manual provides an overview of rapid screening methods to be used.

It should be noted that in some cases it may not be financially viable or justifiable to create capacity to undertake certain tests, particularly chemical tests, where sample numbers are limited, or where the nature of the test requires additional, expensive equipment. In these cases, the user will need to identify and employ suitable external laboratories.

Summaries of the methods with references are provided. Although the document is up to date at the time of writing, users should be aware that standards and legislative requirements are frequently updated, and they should use the most recent edition of each reference.

[1] Manual/Handbook Strengthening Fishery Products Manual for the Execution of Sanitary Inspection of Fishery Products for Human Consumption and the Guide to the Establishment of Environmental (EMP) and Residue (RMP) Monitoring Plan for the Execution of Sanitary Inspection of Fish as Raw Material and Fish-Products as Food for Human Consumption, Mission Ref: CA073GEN, May 2010, published by Strengthening Fishery Products Health Conditions in ACP/OCT Countries (Project No. 8ACPTPS137)

Procedures with an EN ISO number can generally be accessed through a National Bureau of Standards. Most are also available online from member organizations of the European Committee for Standardization (CEN, www.cen.eu) such as the British Standards Institution (BSI, www.bsi.com). CEN itself does not supply technical procedures. Procedures from published technical journals can generally be obtained from technical libraries, or online from the publishing journal referenced.

2 OVERVIEW OF REQUIREMENTS

2.1 Official control laboratories

Official laboratories are appointed by the CAs to undertake chemical analysis or microbiological examination of samples that have been taken for official control purposes.

The CA must designate an official laboratory or laboratories for official controls related to detection and enumeration of microorganisms and their toxins and metabolites. All official control laboratories should be accredited for the individual tests or groups of tests they are using for official control purposes. They are further obliged to satisfy standards of performance in external proficiency assessment schemes, to use validated analytical methods, and to employ suitably qualified persons to carry out analyses. The same is required of third country laboratories nominated for testing for official control.

Official laboratories, together with CAs, form an important structure to ensure the safety of foods. Close liaison between sampling inspectorates and food control laboratories is essential. This should recognise and acknowledge the mutual dependence required to achieve effective enforcement (control) and to optimise use of resources.

The objectives of the sampling programmes to be mutually agreed should recognise the requirements to establish the degree of certainty necessary to achieve these objectives. For example, that the appropriate sample sizes required to ensure representative samples are taken, and that contamination is avoided by selection of appropriate containers for storing samples. The food inspectors should be able to apply their initiative in sampling foods.

Sampling and analysis methods used in official controls should comply with relevant Community rules and/or with internationally recognised rules or protocols, e.g. European Committee for Standardisation (CEN) or those agreed in national legislation. In the field of food microbiology CAs, in accordance with Regulation (EC) No 882/2004[2], must e.g. verify food business operators' compliance with Commission Regulation (EC) No. 2073/2005[3] by specified test methods and sampling plans.

The use of alternative analytical methods is acceptable only when the methods are validated against the reference method and, if a proprietary method, certified by a third party in accordance with the protocol set out in EN/ISO standard 16140 or other internationally accepted similar protocols, is used. Other methods shall be validated according to internationally accepted protocols, and their use authorised by the CA.

The core competences of the official laboratory and the food inspectors are complementary in auditing and supervising Hazard Analysis Critical Control Points (HACCP) systems. The scientists

[2] Commission Regulation (EC) No. 882/2004 of 29 April 2004 on official controls performed to ensure the verification of compliance with feed and food law, animal health and animal welfare rules

[3] Commission Regulation (EC) No. 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs

of the official laboratories can give expert advice to inspectors on criteria for samples taken at critical control points of food processing and for end-products. The findings of the inspectors, along with the interpretation of the analyses of the process samples or samples of the end-products, are essential to the final professional judgement of food safety.

The data produced by an officially recognised laboratory must be seen to be independent of outside influence, and must be recognised by both consumers and the food industry as of the highest quality. These characteristics can only be achieved by a reliable blend of qualifications, expertise and experience applied in a well-equipped, accredited, and quality assured environment. A further characteristic is the requirement to be able to apply these skills over a very wide range of potential problems, (in contrast to the more limited range of many specialist laboratories).

Note that in the following method descriptions, only specific additional items of equipment relevant to the test method are detailed, assuming that the laboratory is fully equipped with all support items (e.g. balances etc.). Official food control laboratories should have adequate infrastructure, facilities, equipment, supplies and reference materials, and access to calibration and maintenance.

2.2 Staff requirements

For a laboratory accredited to carry out specific analytical tests, and to examine microbiological tests, an adequate number of suitably qualified food analysts with training, experience and integrity is required, as well as management and support staff.

At the technical level, there should be a competent technical manager or laboratory manager responsible for overseeing all the analyses performed, who can provide the necessary training and certify the competence of the staff conducting the tests.

All members of staff must be trained in every aspect of their duties, whether in the use of specific items of equipment or for full analytical procedures.

Tests for official controls should be performed or supervised by an experienced person, with a degree in chemistry and microbiology or the equivalent. Alternative qualifications may be sufficient where a member off staff has extensive relevant experience relating to the laboratories' standard of accreditation. Staff should have relevant practical work experience before working unsupervised. The technicians could be graduates, but this is not so critical provided they have some basic chemistry/microbiology qualifications (A level, diploma or equivalent) and receive appropriate on the-job training.

For a microbiology laboratory routinely testing fish, fishery products and water samples, experienced food microbiologists trained in working with pathogens are especially relevant. Microbiologists typically work with little supervision but are supported by technicians. They should be able to interpret results, work with dilutions, and understand microbiological principles, methods and new techniques.

Water sampling should be part of the accreditation. The sample taker must be adequately trained and competent in sampling. Good cooperation between the sampling inspectorate and the laboratory is essential for the accuracy of test results. If the background in food microbiology is weak, it can be augmented by training, either through appropriate courses or on the-job mentoring by a suitably experienced colleague.

Typically, the analysis for contaminates and residues in fish require sophisticated instrumentation to analyse at low detection limits, e.g. for heavy metals by use of atomic absorption spectroscopy (AAS). These analyses are usually undertaken in specialised laboratories, and such analyses require qualified chemists to prepare samples and to operate the equipment. Staff training is essential to ensure that system failures cannot be attributed to incompetence of the analyst. The analyst performing high performance liquid chromatography (HPLC) analysis must be competent

in the preparation of the mobile phase and test solutions and in assessing the analytical results. This will help to ensure that important critical method parameters are controlled (such as retention times of the analytes). For toxin analysis by animal testing or use of cell lines, the management of animals and cell lines would require specialist facilities and expertise.

For certain tests, it is recommended that specialised (reference) laboratories for contaminants and food-borne disease organisms are used.

2.3 Quality control and validation of test methods

Testing laboratories should have a clearly defined quality control system in place to ensure that the apparatus, reagents and techniques are suitable for the tests. In terms of quality control, the use of positive controls, negative controls and blanks should be an integral part of the tests.

For verification of quantitative methods, repeatability, measurement uncertainty, and limit of quantification should be determined, and for qualitative methods, the limit of detection.

Uncertainty is inherent in any test method, and can be assessed by the repeatability and reproducibility of test results. These should be monitored by control tests analysed beside sample tests, by in-house comparability testing between analysts, and by external inter-comparison exercises to discover uncertainties within the test methods.

A programme of periodic checks is necessary to demonstrate that variability is under control. The programme may involve the use of spiked samples, the use of reference materials (including proficiency testing scheme test materials), replicate testing, and replicate evaluation of test results, (e.g. counting of colonies in petri dishes by two analysts). All tests included in the laboratory's accreditation status need to be covered.

The internal quality control programme must be adapted to the actual frequency of tests performed by the laboratory. It is recommended that, where possible, tests should incorporate controls to monitor performance. In microbiological testing, it is advisable to conduct a performance test of the media used. More information on performance testing for the quality assurance of the culture medium on selectivity and productivity reference is provided in ISO/TS 11133-1[4].

Laboratories undertaking chemical tests and microbiological examination of official control samples of fish and fishery products are expected to take part in a Proficiency Testing (PT) scheme. For detailed information on available schemes and dates, see EPTIS database[5].

More information on validation of test methods is provided in the CRFM Manual Laboratory Quality Assurance. The Fitness for Purpose of Analytical Methods Guide from Eurachem: A Laboratory Guide to Method Validation and Related Topics, second edition (2014), is also a useful reference. For further reading reference is provided in Annex 1.

Regarding interpretation of test results, food business operators should regard all test results above the limits as unacceptable, regardless of the uncertainty involved. In official controls, this uncertainty should be taken into account to be sure beyond reasonable doubt that the batch in question does not comply with the criterion. For quantitative analyses, calculation of measurement

[4] Microbiology of food, animal feed and water -- Preparation, production, storage and performance testing of culture media

[5] https://www.eptis.bam.de/en/index.htm.

uncertainty in relation to each quantitative microbiological determination should be in line with ISO/TS 19036[6]:

3 MICROBIOLOGICAL PARAMETERS FOR WATER

Water-borne pathogens are a leading cause of disease and death worldwide. Routine microbiological testing of drinking water supplies and environmental waters is essential for the protection of public health. Maintaining an uncontaminated water supply requires constant attention and regular monitoring by testing. The concept of microbiological testing of drinking water supplies is built on the detection of indicator organisms in water supplies. *Escherichia coli* (*E.coli*) most closely matches the criteria for an ideal indicator species.

Tests for total coliforms and faecal coliforms are used routinely to screen samples for faecal indicator species. Other species present in faeces in lower numbers are also used as indicator organisms, notably enterococci and, to a lesser extent, *Clostridium perfringens*. There are some situations where it is necessary to test directly for water borne pathogens, such as Pseudomonas aeruginosa, especially in treated waters.

In addition to tests for indicator organisms and certain specific pathogens, non-selective colony counts are also routinely carried out to determine the population of heterotrophic bacteria present. Counts at two temperatures are typically performed to provide information on the general microbiological population of the water, and detect sudden changes in water quality.

Most probable number (MPN) tests for routine water microbiology have now been largely replaced by membrane filtration (MF) methods, that are more sensitive for the detection of indicator organisms and pathogens, although MPN may still be useful for occasional tests conducted in small laboratories or in the field, and commercial test kits based on MPN methods are available for coliforms and enterococci

Council Directive 98/83/EC[7] specifies methods to be used for testing the quality of water for human consumption.

The recommended procedures of Council Directive 98/83/EC are ISO Standards:

(a) *Escherichia coli* (E. coli) and coliform bacteria (EN ISO 9308-1 or EN ISO 9308-2

(b) Enterococci (EN ISO 7899-2)

(c) *Pseudomonas aeruginosa* (EN ISO 16266)

(d) Enumeration of culturable microorganisms — colony count 22 °C (EN ISO 6222)

(e) Enumeration of culturable microorganisms — colony count 36 °C (EN ISO 6222)

(f) *Clostridium perfringens* including spores (EN ISO 14189).

[6] Microbiology of food and animal feeding stuffs – Guide on estimation of measurement uncertainty for quantitative determination

[7] Council Directive 98/93/EC of 3 November 1998 on the quality of water intended for human consumption

3.1 Sampling for water analysis, sample handling and sample preparation

3.1.1 Sampling frequency

The Council Directive 98/93/EC (drinking water directive) dictates minimum frequency of sampling and analyses for water intended for human consumption supplied from a distribution network or from a tanker, or used in food-production.

Water quality monitoring is based on sampling of water from defined sampling points as determined by the CAs, and should meet the relevant requirements set out in the Directive 98/93/EC Annexes, which specifies parametric values of microbiological parameters. The samples must be taken at the points of compliance as defined in the Directive, to ensure that water intended for human consumption meets the requirements of the Directive.

3.1.2 Sampling methods

Sampling methods vary with the type of sample being taken and the location. Comprehensive official advice on sampling from distribution systems and other water sources is available in published guides, such as the UK Environment Agency booklet The Microbiology of Drinking Water (2010), Part 2 - Practices and procedures for sampling and the US EPA's (Environmental Protection Agency) Interactive Sampling Guide for Drinking Water System Operators. This includes procedures for microbiological sampling. Staff required to take water samples for microbiological analysis should be trained according to the principles outlined in such publications.

The taking of the sample should be in accordance with the instructions for sampling, handling and preservation as described by EN ISO 19458[8] and EN ISO 5667-1[9].

Obtaining representative water samples is a critical part of microbiological water analysis. Samples should be collected in sterile containers which, for chlorinated water, should contain an appropriate quantity of sodium thiosulphate to neutralise residual chlorine. It is also important to ensure that the sampler does not contaminate the inside of the sample container, and rubber gloves should be worn where necessary.

Ideally water from piped distribution systems or tanks should be taken from hygienically designed sample taps. Bacterial growth may occur in taps, and it is good practice to disinfect the tap by flaming, or with alcohol (70% isopropanol) or other suitable disinfectant before sampling. Water should be allowed to run through the tap for several minutes to flush out any contamination within the tap and ensure that the sample is representative.

The time between sampling and analysis at the lab should be as short as possible. The water quality analysis should start on the same day as sampling. Water supplied in closed containers should be examined within 12 h of bottling, the temperature of storage being maintained at 5 ± 3°C during this period. If transportation exceeds 8 h, the use of a temperature logger is recommended.

The equipment for sampling should include sterile single-use gloves, a gas burner, beaker, disinfection spray, a water resistant pen, forceps, a transport box with cooling aggregates, a calibrated thermometer, and means of transport.

[8] Water quality, sampling for microbiological analysis
[9] Water quality; sampling; guidance on the design of sampling programmes and sampling techniques

3.1.3 Sample preparation

For sample preparation, information on isolation and on inoculation of isolation media is provided by ISO 8199[10], ISO 11133[11] and ISO 7218[12].

Media and reagents should be prepared using water grade 3, as specified in ISO 3696[13], or water of equivalent purity, free from substances, which might inhibit growth under the conditions of the test. Composition and preparation of culture media and reagents are described in the relevant ISO standards (below).

3.2 Microbiological methods

3.2.1 Colony count at 22°C and 36°C (EN ISO 6222:1999)

Water quality is determined by enumeration of culturable micro-organisms (colony count) by inoculation in a nutrient agar culture medium.

Waters of all kinds invariably contain a variety of micro-organisms derived from various sources, such as soil and vegetation, and estimation of the overall numbers provide useful information for the assessment and surveillance of water quality. Separate counts are usually made of the micro-organisms which can grow and form colonies on nutrient agar media at 36°C and 22°C.

Colony counts are useful for assessing the integrity of ground-water sources, the efficacy of water treatment processes such as coagulation, filtration, and disinfection, and they provide an indication of the cleanliness and integrity of the distribution system. They can also be used to assess the suitability of a supply for the preparation of food and drink, where the water supply should contain few micro-organisms that might contaminate the product. The main value of colony counts lies in the detection of changes from those expected, based on frequent and long term monitoring. Any sudden increase in the count can be an early warning of serious pollution and calls for immediate investigation.

The method is intended to measure the operational efficiency of the treatment process of public drinking water supplies and for general application to all types of water. It is particularly applicable to the examination of water intended for human consumption, including water in closed containers and natural mineral waters.

Scope: The International Standard describes a method for the enumeration of culturable micro-organisms in water by counting the colonies formed in a nutrient agar culture medium after aerobic incubation at 36°C and 22°C .

Principle: Measured volumes or dilutions of water samples are mixed with yeast extract agar and one set of Petri dishes incubated at 22°C for 68 h, while the second is incubated at 36°C for 44 h. Colonies growing in the medium are counted.

Equipment: Use microbiological laboratory equipment and, in particular:

[10] Water quality -- General guidance on the enumeration of micro-organisms by culture
[11] Microbiology of food, animal feed and water – Preparation, production, storage and performance testing of culture media
[12] Microbiology of food and animal feeding stuffs - General requirements and guidance for microbiological examinations
[13] Water for analytical laboratory use -- Specification and test methods

a) Autoclave
b) Incubator at 36°C (±2°C)
c) Incubator at 22°C (±2°C)
d) Petri dishes
e) Water bath 45°C (±1°C)
f) Colony counter

Media/Reagents: For the media preparation use ingredients of uniform quality and chemicals of analytical grade. Use glass-distilled or deionised water for making media.

a) Peptone diluent (ISO 8199)
b) Yeast extract agar

Sampling handling: Examine water supplied in closed containers within 12 h of bottling, keeping the temperature of storage at 5°C (± 3°C) during this period.

Sample preparation: Water samples are treated with peptone diluent to prepare a set of dilutions. These are used to prepare pour plates in the yeast extract agar.

Procedure: No more than 2 ml of the diluted samples should be used per Petri dish. One set of plates is incubated at 22°C (±2°C) for 68 h and one at 36°C (±2°C) for 44 h. After incubation, the number of colonies present on each plate is counted, and used to calculate the number of Colony Forming Units (CFU) per ml water.

Expression of results and test report: Results are expressed as number of CFU per millilitre (CFU/ml) of the sample for each temperature of incubation. The test report should include the sample identification, technique (pour plate), medium used, and the time and temperature of incubation at minimum.

3.2.2 *Escherichia coli* and coliforms (EN ISO 9308-1:2014)

Water quality - Enumeration of *Escherichia coli* and coliform bacteria - Part 1: Membrane filtration method for waters with low bacterial background flora

The presence and extent of faecal contaminant is an important factor in assessing the quality of water, and the risk to human health from infection. The presence of *E. coli* (which normally inhabits the bowel of man and other warm-blooded animals), indicates such pollution. Examination for coliform bacteria can be more difficult to interpret, because some coliform bacteria live in soil and surface fresh water, and are not always intestinal. The presence of coliform bacteria, although not a proof of faecal contamination, may indicate failure in treatment, storage or distribution.

Scope: The International Standard describes a method for the enumeration of E. coli and coliform bacteria. It is based on membrane filtration and subsequent culture on a Chromogenic Coliform Agar (CCA) medium, and calculation of the target organism in the sample. The method is especially suitable for waters with low bacterial numbers producing less than 100 total colonies on CCA,

such as drinking water, disinfected water, or water after appropriate treatment. It is not suitable for surface waters or shallow well-waters, due to possible background growth on the agar medium interfering with the reliable enumeration *of* E. coli *and* coliform bacteria.

Principle: Filtration of a test portion of the sample through a membrane filter, which retains the organisms, and placement of the membrane filter on a chromogenic coliform agar plate. After incubation of the membrane filter at 36°C (± 2°C) for 21°C (± 3°C) h β-D-galactosidase positive colonies (pink to red) are counted as presumptive coliform bacteria that are not E. coli. To avoid false-positive results, the presumptive colonies shall be confirmed by a negative oxidase reaction. β-D-galactosidase and β-D-glucuronidase positive colonies (dark-blue to violet) are counted as E. coli. Total coliform bacteria are the sum of oxidase negative colonies with pink to red colour and all dark-blue to violet colonies.

Equipment and glassware:

a) Autoclave
b) Incubator at 36°C (±2°C)
c) pH meter
d) Membrane filtration equipment
e) Membrane filters (pore size 0.45 µm; sterile)
f) Sterile forceps with rounded ends
g) (Sterile loop)

Media/reagents:

Use ingredients of uniform quality and chemicals of analytical grade

For preparation of culture media use distilled water or deionized water

a) Chromogenic Coliform Agar
b) Oxidase reagent
c) Tryptone Soy Agar (TSA)

Sampling handling: Samples have to be transported and stored at 5°C (±3°C) in accordance with ISO 19458[14]. Under special circumstances, samples may be kept at 5°C (±3°C) for up to 24 h. In this case the storage time must be mentioned in the test report.

Sample preparation: Reference is provided by ISO 8199[15] for sample preparation, isolation, and inoculation on isolation media.

Procedure: Water samples (100 ml or more) are filtered through membrane filters, which are then placed on the required media. Following the standard method, filters are placed on CCA and incubated at 36°C (±2°C) for 21h (±3 h).

[14] Water quality – Sampling for microbiological analysis.

[15] ISO 8199:2005, Water quality -- General guidance on the enumeration of micro-organisms by culture

Colonies giving rise to a pink to red coloration of the medium are likely coliform bacteria that are not *E.coli* (positive reaction). Colonies giving a dark-blue to violet reaction are counted as *E.coli*.

An oxidase test is performed with all, or at least 10 of the pink or red colonies selected (ISO 8199 (see footnote 15), by use of a commercial oxidase test or fresh oxidase reagent to confirm that the coliform bacteria are not *E.coli*.

By use of fresh oxidase reagent, a representative number of colonies (at least 10) is transferred to a pre-treated filter paper by use of a loop. A positive oxidase reaction is shown by appearance of dark blue colour within 30 s and is identified as *E.coli*. This is not observed for coliform bacteria since they are oxidase negative.

Subcultures are prepared where too many colonies are grown, because closeness of colonies or too small colonies will not ensure that the oxidase test is carried out with pure cultures. Subcultures would be prepared onto a non-selective agar, e.g. TSA at 36°C (±2°C) for 21h (± 3 h).

Expression of result: Confirmed colonies are counted on the membrane filter, and calculated as numbers of coliforms and E. coli present in 100 ml water (or other filtered volume). The sum of all oxidase negative pink to red colonies, plus all dark-blue to violet colonies, is the count of coliform bacteria. E. coli are all dark-blue to violet colonies.

Performance criteria, quality assurance: The use of positive controls, negative controls, and blanks is part of the test.

Performance testing of the CCA should be carried out by use of control strains. The International Standard provides performance characteristics for the CCA. Data for the calculation of performance characteristics are usually collected from tests with potable water, thus it may be necessary for laboratories to carry out their own validation, depending on the type of water.

The international Standard also provides information on suitable control strains for performance testing of the oxidase test.

3.2.3 *Clostridium perfringens* – including spores (ISO 14189:2013)

Water quality — Enumeration of *Clostridium perfringens* – Method using membrane filtration

Clostridium perfringens (*C. perfringens*) is a valuable indicator for faecal pollution, and exists in the intestine both as spores and vegetative cells. Spores are also found in environmental samples. The spores of *C. perfringens* survive in water for months, much longer than vegetative faecal indicator bacteria and consequently their presence may indicate remote or intermittent faecal pollution. Monitoring of *C. perfringens* has proven useful for the assessment of the quality of water resources, and to check the stages of water treatment to evaluate the efficacy of the treatment. The spores are not always inactivated by routine disinfection procedures (e.g. chlorination).

Scope: The International Standard describes a method for the enumeration of *Clostridium perfringens* (including spores) in water for human consumption, by the membrane filtration method.

Principle: Samples of water are filtered through membranes that retain spores of *clostridia*. The membrane is incubated on a selective/differential agar (tryptose-sulfite-cycloserine agar) anaerobically at 44°C (± 1°C) for 21 h (± 3 h). *C. perfringens* usually produce black or grey to yellow brown colonies. Characteristic colonies are counted and confirmatory tests carried out.

The result is calculated as the colony count per sample volume. If a count of spores alone is required, the sample is first pre-treated at 60°C (±2°C) to inactivate vegetative bacteria.

Equipment:

a) Membrane filtration equipment
b) Membrane filters (pore size 0.45 µm)
c) Sterile filter funnels
d) Autoclave
e) Water bath and/or incubator at 44°C (±1°C)
f) Forceps, sterile
g) Anaerobic jar or similar equipment
h) Anaerobic generating equipment

Media/reagents:

a) Tryptose sulfite cycloserine agar (TSC)
b) Blood agar or Columbia agar base or another suitable nutrient-rich agar
c) Acid Phosphase reagent

For uniformity of results, it is important to use constituents of uniform quality and chemicals of recognized analytical grade in the preparation of media, and also glass-distilled water or deionized water.

Alternatively, use commercially available dehydrated complete medium and reagents, prepared and used according to the manufacturer's instructions. Other grades of chemicals may be used, provided they can be shown to lead to the same results.

Sample preparation: Samples should be cooled during transport, ideally at 5°C (± 3°C). Start examination as soon as possible after the collection of the sample, preferably within the same working day. The recommended maximum sample storage time (including transport), is for vegetative bacteria 12 h and for spores 24 h. The sample storage time including transport shall not exceed 18 h for vegetative bacteria and 72 h for spores.

When counting only spores, the sample (greater than the volume to be analysed) is mixed and heated to 60°C (± 2°C) in a water bath for 15 h (± 1min). The temperature should be exactly monitored.

A test volume of sample or dilution of it, after heat treatment if required, should be chosen to yield, if possible, between 10 and 80 colonies on a membrane 47 mm to 50 mm in diameter. Test volumes or dilutions should be prepared as described in ISO 8199.

Procedure: Water samples (100 ml) are filtered through membrane filters, which are then placed on a TSC agar plate by use of flame sterilised funnels as the spores of *C. perfringens* are more heat resistant. Record the volume filtered.

The time between placing the membrane on the TSC agar and starting incubation should not exceed 1 h and should be as short as possible. After incubation, anaerobically at 44°C (± 1°C) for

21h (± 3 h), calculate the quantity of *C.perfringens* by counting all colonies which show black or grey to yellow brown staining within 30 min after incubation. The black colour of the colonies rapidly fades and finally disappear. The plates should be checked jar by jar, or in portions, if the incubation was performed in an anaerobic incubator.

For confirmation of *Clostridium perfringens,* subculture all colonies for counts of 1 to 10, and at least 10 colonies for counts above 10, taken randomly onto blood agar plates. When this is impracticable, all typical colonies from a sub-area of the membrane should be examined. Columbia agar base or another nutrient-rich agar (e.g. TSA) could be used for subculture, incubated anaerobically in an incubator at 36°C (± 2°C) for 21 h (± 3 h).

Acid phosphatase test: Colonies grown anaerobically on blood or nutrient agar plates are spread on filter paper, and 2 to 3 drops of acid phosphatase reagent are placed onto the colonies. A purplish colour developed within 3 min to 4 min is considered a positive reaction. *C. perfringens* produces black or grey to yellow brown colonies on TSC agar, even if the colour is faint, and acid phosphatase is present.

The plates are incubated at 44°C (±1°C) for 21 h (±3 h). Opaque yellow colonies that turn pink/red after exposure to ammonium hydroxide are identified as *C. perfringens*.

Expression of results: From the numbers of total and confirmed colonies, the numbers of presumptive *C. perfringens* and the number of spores, if applicable, present in 100 ml of the filtered volume are calculated, in accordance with ISO 8199 (see footnote page 8). Counts are expressed as CFU. *C. perfringens* per 100 ml water. The test report should contain the number of colonies of presumptive *C. perfringens* (optional), the number of colonies confirmed as *C. perfringens*, and whether the result represents the total number of *C. perfringens* (vegetative cells and spores) or spores only. Where required, the variability of the test results should be evaluated.

Performance criteria, quality assurance: Include a blank control in each batch of analyses by use of 100 ml of sterile water, and treat it like a sample but without pasteurization. No colonies should be visible after incubation.

For the confirmation step performed by acid phosphatase test, include positive and negative control strains. The International standard provides information on *C. perfringens* strains to be used as positive control for media control and the confirmation test. For performance testing on TSC agar, calculate productivity and selectivity by comparing with a non-selective reference medium.

For quantitative process quality control, use a suspension of *C. perfringens* to compare recovery with that on a non-selective agar such as blood agar. Alternatively, use reference materials. Select the volume filtered to contain between 10 to 80 CFU.

Include an appropriate control for correct anaerobic conditions (e.g. anaerobic indicator strip), whenever anaerobic incubation is performed.

3.2.4 Intestinal enterococci (EN ISO 7899 -2:2000)

Water quality - Detection and enumeration of intestinal enterococci - Part 2: Membrane filtration method

In the EU, enterococci are used as indicators of drinking water contamination. Enterococci are not permitted in a 100 ml sample of tested drinking water that flows from a tap, and they are not permitted in a 250 ml sample of bottled water.

For purposes of water examination, enterococci can be regarded as indicators of faecal pollution. However, it should be noted that some enterococci found in water can occasionally originate from other habitats.

Scope: The International Standard describes a method for the calculation of culturable intestinal enterococci microorganisms in water by membrane filtration, but this is not suitable if the water contains large amounts of suspended matter, or if levels of interfering microorganisms are too high. It is suitable for the examination of large volumes of water with low levels of intestinal enterococci.

Principle: Water samples are filtered through membranes that retain microorganisms. The membrane is placed on a selective medium containing sodium azide to inhibit growth of Gram-negative organisms, and 2,3,5-triphenyltetrazolium chloride, a colourless dye reduced to red formazan by intestinal enterococci. Confirmation is carried out by transferring membranes with typical colonies on to bile aesculin azide agar, pre-heated at 44°C. The aesculin in the medium is hydrolyzed by intestinal enterococci in 2h. The end product, 6,7-dihydroxycoumarin, combines with iron (III) ions to give a tan to black coloured compound that diffuses into the medium.

Equipment:

a) Autoclave
b) Incubator at 36°C (±2°C)
c) Incubator at 44°C (±0.5°C)
d) Filtration equipment
e) Membrane filters (pore size 0.45 µm)
f) Sterile forceps
g) Hotplate or water bath maintained at 100°C

Media/reagents:

a) Slanetz and Bartley medium
b) Bile aesculin azide agar

Sample preparation: Filter volumes of the water sample or portions of the dilution, through a sterile membrane filter with a rated pore diameter equivalent to 0,4 µm. As specified in ISO 8199 (see page 8), place each membrane on a Petri dish containing Slanetz and Bartley medium.

Procedure: Water samples are filtered through membrane filters that are then placed on Slanetz and Bartley medium. The plates are incubated at 36°C (±2°C) for 44 h (±4 h). Typical colonies are red, maroon or pink, in the middle or throughout. Membranes with typical colonies are transferred without inverting, and using sterile forceps, on to bile aesculin azide agar that has been preheated to 44°C. Plates are incubated at 44°C (±0.5°C) for 2 h. Colonies showing a black or tan colour in the surrounding medium are counted as intestinal enterococci.

Results: Counts are expressed as CFU intestinal enterococci per 100 ml water.

Performance criteria, quality assurance: The use of positive controls, negative controls and blanks is part of the test.

3.2.5 *Pseudomonas aeruginosa* (ISO 16266:2006)

Water quality - Detection and enumeration of *Pseudomonas aeruginosa* - Method by membrane filtration

Pseudomonas aeruginosa (*P. aeruginosa*) is an opportunistic pathogen of man that is capable of growth in water at very low nutrient concentrations. Water for human consumption may sometimes be tested for *Pseudomonas aeruginosa* for reasons of public health. In these cases, it is typical to examine 100 ml volumes.

Scope: The international Standard describes a method for the isolation and enumeration of *Pseudomonas aeruginosa* in samples of bottled water by a membrane filtration technique. This method can also be applied to other types of water with a low background flora, for example, pool waters and waters intended for human consumption.

Principle: Samples of water are filtered through membranes that retain microorganisms. The membrane is placed on a selective medium and incubated. Pyocyanin-producing colonies are considered as confirmed *P. aeruginosa*, but other fluorescing or reddish-brown colonies require confirmation. Colonies requiring confirmation are sub-cultured on to nutrient agar. After incubation, colonies that did not fluoresce initially are tested for the oxidase reaction. Oxidase-positive cultures are tested for fluorescein production and the ability to produce ammonia from acetamide. Cultures that were fluorescent to start with are tested for the ability to produce ammonia from acetamide.

Equipment:

a) Autoclave
b) Incubator at 36°C (±2°C)
c) Incubator at 44°C (±0.5°C)
d) Filtration equipment
e) Membrane filters (pore size 0.45 µm)
f) UV lamp

Media/Reagents: Use reagents of analytical reagent quality in the preparation of culture media and diluents, unless otherwise specified. Prepare the medium as described by the International Standard or use commercially available media and reagents prepared according to the manufacturer's instructions.

a) Pseudomonas-CN agar
b) Kings B medium
c) Acetamide broth
d) Nutrient agar
e) Oxidase reagent

f) Nessler reagent

Sample preparation: Filter volumes of the water sample or portions of the dilution through a sterile cellulose ester membrane filter with a rated pore diameter equivalent to 0, 45 µm. As specified in ISO 8199 (see footnote page 8), place each membrane on a Petri dish containing CN agar, ensuring no air is trapped beneath the membrane.

Procedure: Water samples are filtered through membrane filters, which are then placed on Pseudomonas-CN agar. The plates are incubated at 36°C (±2°C) for 44 h (±4 h). They are examined for growth after 22 h (±2 h) and 44 h (±4 h). Confirmed colonies of *P. aeruginosa* are all those that produce a blue/green colour (pyocyanin).

Any non-pyocyanin colonies on the membrane that fluoresce under UV light are counted as presumptive *P. aeruginosa*, and are confirmed using acetamide broth.

All other reddish-brown colonies are counted as presumptive *P. aeruginosa* and are confirmed using the oxidase reaction, acetamide broth and King's B medium. Colonies requiring confirmation are picked off the membrane and grown on nutrient agar at 36°C (±2°C) for 22 h (±2 h). All cultures are checked for purity before carrying out the confirmatory tests.

Colonies that are reddish brown are initially tested for the oxidase reaction. The remaining two confirmatory tests are carried out on all cultures that are oxidase positive.

Reddish-brown colonies are also subcultured on to King's B medium and incubated at 36°C (±2°C) for up to 5 days. The plates are examined under UV light daily and any fluorescence is noted. Cultures appearing to fluoresce up to 5 days after inoculation are recorded as positive.

Finally, all fluorescent, non-pyocyanin-producing colonies, and reddish-brown colonies, are tested in acetamide broth. Tubes of the broth are inoculated with the subculture and incubated at 36°C (±2°C) for 22 h (±2 h). One to two drops of Nessler reagent are added to the tubes and if ammonia is produced they change colour from brick red to yellow depending on the concentration. *P. aeruginosa* gives a positive reaction in acetamide broth.

Expression of results: All the characteristic colonies are added together to give a count for *P. aeruginosa* in a given volume of water. From the number of characteristic colonies counted on the membranes, and taking account of the proportion of confirmatory tests performed, calculate the number of confirmed *Pseudomonas aeruginosa* present in a specific volume of the water. For mineral water, spring water and other bottled waters, the volume should be 250 ml; for other waters, the volume should usually be 100 ml.

Performance criteria, quality assurance: For calculation of recovery, the International Standard provides mean recoveries (%) relative to the count on nutrient agar after dilution in distilled water and filtration for Pseudomonas aeruginosa. Data result from a trial in six laboratories from five countries.

The International standard recommends strains to be used as positive control and as negative control for all stages.

Rapid methods: Although most official methods for microbiological water analysis still rely on traditional culture methods and membrane filtration methods, the long-time taken to obtain results has focused attention on alternative rapid methods.

These are based on growth based methods, direct measurement (e.g. flow cytometry), cell component analysis as indirect measure of microbial presence (e.g. genotypic methods), optical

spectroscopy e.g. 'real time' airborne particle counters, nucleic acid amplification Polymerase Chain Reaction (PCR), flow cytometry and immuno-magnetic separation. Combining MF with quantitative PCR detection and enumeration has been shown to be a particularly rapid and effective means of analysing water samples. However, the main disadvantage of this method is that it may detect non-viable cells and overestimate the population. A number of chromogenic media-based detection methods/enzyme based methods, allowing quantification within 24 hours and less, are available for most water quality tests. In Table 1 some of them are listed. Colilert technique has been shown to correlate very well with the traditional membrane filter and MPN methods when used to test freshwater. Some of the tests are validated. All providers advertise their products via the internet. For more information, see the supplier websites shown in Table 1.

TABLE 1: COMMERCIAL PRODUCT TESTS IN WATER QUALITY

Coliforms		
Test	**Principle, test time**	**Supplier**
Lamotte coliform	Tablet nutrient based; presence/absence; 40-48 h	LaMotte Company, USA www.lamotte.com
***E.coli* and coliform**		
Test	**Principle, test time**	**Supplier**
Rapid Hicoliform Test Kit	Combination of chromogenic and fluorogenic substrates; 18-24 h	HiMedia Laboratories www.himedialabs.com
Enterolert, Colisure, and Colilert®-18[16] With Quanti-Tray and Quanti-Tray/2000	Patented substrate technology (DST), nutrient-indicator; < 18 h	IDEXX, USA www.idexx.com
m-ColiBlue by HACH	Broth, 24 h	HACH www.hach.com
CHROMagar™ ECC	Chromogenic media; 24 h	CHROMagar www.CHROMagar.com/contact
Modified Colitag™	Presence-absence test; 16 - 22 h; EPA approved	Hach www.hach.com
Charm E*Colite	Fluorescence; US EPA-approved "bag" test; 28 – 48 h	Charm Sciences Inc. www.charm.com
Readycult® Coliforme 100	Fluorogenic media; 18-24 h	Merck Millipore www.merckmillipore.com
ColiComplete®	Enzymatic detection; 28-48 h AOAC Official Method 992.30	BioControl Systems www.biocontrolsys.com

[16] Colilert -18/Quanti-Tray is set to become the new ISO worldwide standard for detecting total coliforms and *E. coli* in water under ISO 9308-2:2012 Colilert -18

3M™ Petrifilm™ Coliform Count Plates[17]	Official Methods of Analysis (OMA); AFNOR validated	3M www.3m.com
EC Blue 100 by ROTH	Specific Enzyme Substrate Culture Medium Method; 24 h	Car Roth GmbH & Co KG, www.carlroth.com
REBECCA™ CF Water	Chromogenic medium; 21- 44 h; AFNOR validated	bioMerieux www.biomerieux-industry.com
Pseudomonas aeruginosa		
Test	**Principle, test time**	**Supplier**
Rapid *P. aeruginosa* Agar	Selective chromogenic medium; 22 - 24 h; NF validated	Bio Rad http://www.bio-rad.com
VIT® *Pseudomonas aeruginosa* (kit)	Fluorescence microscope; 3 h	Vermicon www.vermicom.com
Quanti-Tray® & Quanti-Tray®/2000 Pseudalert*	Fluorescence, 24 h	IDEXX www.idexx.com
Enterococci		
Test	**Principle, test time**	**Supplier**
Enterolert® Rapid Enterococci Test	Fluorescence, 24 h AFNOR validated	IDEXX www.ca.idexx.com
HiEnterococci Test Kit	Chromogenic substrate; 24 -48 h	HiMedia Laboratories www.himedialabs.com
Readycult® Enterokokken 100	Fluorogenic media, 18 - 24 h	Merck Millipore www.merchmillipore.com
Compact Dry ETC	Selective agents (X-glucoside, antibiotics); food and water; 20 - 24 h	R Biopharm www.r-biopharm.com
Colony count 22 °C, 36°C		
Test	**Principle, test time**	**Supplier**
MicroSnap Total delivers TVC test, quantified with the EnSURE luminometer. MicroSnap	Bioluminogenic test; 7 h AOAC-RI Performance Tested Method # 031501	Hygiena www.hygiena.com
TTC/*E.coli* & Coliform dipslide	Chromogenic agar; 24-48 h	Lovibond www.lovibond.com
Clostridium perfringens		

[17] Official Methods of Analysis, published by AOAC International

Test	Principle, test time	Supplier
m-CP Medium chromogenic agar substrates	Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption recommends m-CP Medium for testing; 24 h	Thermo Scientific Limited www.thermofisher.com

Source: Internet search, 2016

4 MICROBIOLOGICAL PARAMETERS FOR FISHERY PRODUCTS

4.1 Sources of data

Microbiological criteria for fish, fishery products, sea shellfish and molluscs have been produced both in international (Codex Committee on Food Hygiene,) and European legislation. The mandatory requirements on microbiological criteria, including samples, plans and methods of analysis, are laid down in Commission Regulation (EC) No. 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs (2073/2005). This sets quantitative limits on the counts of *Escherichia coli*, thermotolerant coliform, mesophilic aerobic bacteria and *Vibrio parahaemolyticus* during the production and, at the finished product stage, for pathogens such as Staphylococcus aureus, *Salmonella* and Listeria. New requirements for live bivalve molluscs, echinoderms, tunicates and marine gastropods apply from 1 January 2017 onwards[18].

The following sections describe the required methods for microbiological testing for fish, fishery products, sea shellfish and molluscs, for parameters most likely to be required in the Caribbean region.

4.2 Sampling

4.2.1 Sampling methods

Since sampling for microbiological testing is crucial for the accuracy of the test results, instructions for sampling, sample handling, and preparation of the test sample are provided in the descriptions below. Detailed sampling requirements for each test are set out in Regulation 2073/2005[19].

In case there are no specific international or legal standards for sampling, it is recommended that the laboratory and parties concerned come to an agreement in advance. In such cases, relevant standards of ISO and the guidelines of the Codex Alimentarius should be used as reference methods, such as Codex Alimentarius: General guidelines on sampling, CAC/GL 50-2004; ISO 7218[20], also NMKL (Nordic Committee on Food Analysis) Procedure No. 12: Guide on Sampling

[18] Commission Regulation (EU) 2015/2285 of 8 December 2015 amending Annex I to Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs)

[19] Commission Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs

[20] Microbiology of food and animal feeding stuffs – General rules for microbiological examinations

for Analysis of Foods (www.nmkl.org). ISO 18593[21] is the EU reference sampling method for sampling of processing areas and equipment.

Samples must be handled and labelled in such a way as to guarantee both their legal and analytical validity. For official control, it is important that the laboratory receives a sample truly representative of the product that has not been damaged or changed during transport or storage. Incorrect sampling can lead to false negative or false positive results.

Whenever possible, samples are submitted to the laboratory in the original unopened containers, or representative portions are transferred to sterile containers under aseptic conditions. Sample taking is always by use of sterile sampling equipment and use of aseptic technique. Containers used in sampling must be clean, dry, leak-proof, wide-mouthed, sterile, and of a size suitable for samples of the product. Sterile plastic bags (for dry, unfrozen materials only) or plastic bottles, are useful containers for line samples. Each sample unit should be identified with a properly marked strip of masking tape.

Whenever possible, at least 100 g for each sample unit should be obtained. Open and closed controls of sterile containers should be submitted with the sample.

The samples should be delivered promptly to the laboratory, with the original storage conditions maintained as nearly as possible. For transport of samples, they should be kept under conditions which prevent alteration in the number of microorganism present. The fastest means of transport should be preferred.

Frozen or refrigerated products are transported in approved insulated containers of rigid construction, so that they will arrive at the laboratory unchanged. Frozen samples are collected in pre-chilled containers. Refrigerated samples should be cooled in ice at 0-4°C, and transported in a sample chest with suitable refrigerant, capable of maintaining the sample at 0-4°C until arrival at the laboratory. When collecting liquid samples, an additional sample as a temperature control should be taken.

The temperature of the control sample should be checked at the time of collection, and on receipt at the laboratory. The times and dates of collection and of arrival of all samples at the laboratory should be recorded. Dry or canned foods that are not perishable and are collected at ambient temperatures need not be refrigerated.

The following storage temperature should be observed: fresh and refrigerated products between 0 and 4°C, frozen or deep frozen products below -18°C fresh fish and sensitive products between 0 and 2°C, spoiled stable units between 0 and 4°C and transport in closed packaging.

4.2.2 Sample reception and handling

The conditions of the sample on receipt in the laboratory should be checked. If the samples are insufficient or their conditions is unsatisfactory, the laboratory should refuse the samples. Accepted samples are documented. Samples awaiting storage should be stored in a way to prevent any alteration in the number of microorganism present. It is important to have appropriate storage temperatures, and to keep examination deadlines, e.g. for fresh and refrigerated products within 24 h after receipt; for longer storage periods immediately freeze the sample at -18°C.

Upon receipt at the laboratory, the analyst should note the general physical condition of the sample. If the sample cannot be analysed immediately, it should be stored. Frozen samples should be stored at -20°C until examination. Refrigerate unfrozen perishable samples at 0-4°C not longer than 36 h. Non-perishable, canned, or low-moisture foods are stored at room temperature until

[21] Microbiology of food and animal feeding stuffs - Horizontal methods for sampling techniques from surfaces using contact plates and swabs

analysis. Place containers in a freezer long enough to chill them thoroughly. Keep frozen samples solidly frozen at all times. Do not freeze refrigerated products. Unless otherwise specified, refrigerated samples should be analysed within 36 h after collection.

4.2.3 Sample preparation

Sample preparation is to be performed according to appropriate parts (applicable to fish and seafood) of:

- **EN ISO 6887-1:** Microbiology of food and animal feeding stuffs - Preparation of the test samples, of initial suspension and of decimal dilutions for microbiological examination - Part 1: General rules for the preparation of the initial suspension and decimal dilutions
- **ISO 6887-3:** Microbiology of food and animal feeding stuffs - Preparation of test samples, initial suspension and decimal dilutions for microbiological examination - Part 3: Specific rules for the preparation of fish and fishery products
- **ISO 7218:** Microbiology of food and animal feeding stuffs - General requirements and guidance for microbiological examinations.

When handling product, aseptic technique is always used. Before handling or analysis of the sample, the immediate and surrounding work areas must be cleaned. In addition, the immediate work area should be swabbed with commercial germicidal agent.

Preferably, frozen samples should not be thawed before analysis. If necessary to temper a frozen sample to obtain an analytical portion, thaw it in the original container, or in the container in which it was received in the laboratory. Whenever possible, transferring the sample to a second container for thawing should be avoided. Normally, a sample can be thawed at 2-5°C within 18 hours.

Various degrees of non-uniform distribution of microorganisms are to be expected in any food sample. To ensure more even distribution, liquid samples must be shaken thoroughly and, if practical, dried samples should be mixed using sterile spoons or other utensils before withdrawing the analytical unit from a sample of 100 g or greater.

An analytical unit of 50 g of liquid or dry food is used to determine aerobic plate count value and MPN of coliforms. Other analytical unit sizes (e.g., 25 g for Salmonella) may be recommended, depending on the specific analysis to be performed. Use an analytical unit size and diluent volume as recommended by the method in use. If contents of package are obviously not homogeneous, macerate the entire contents of the package and withdraw the analytical unit, or, preferably, analyse each different food portion separately, depending on the purpose of test.

Samples can be prepared by the use of a high speed blender or a stomacher. In case of using the blender, tare the high-speed blender jar; then aseptically and accurately (± 0.1 g) weigh unthawed food (if frozen) into the jar. If the entire sample weighs less than the required amount, weigh a portion equivalent too one-half of the sample and adjust the amount of diluent or broth accordingly. Total volume in the blender must completely cover blades. For the sample preparation by mechanical blending equipment, requirements are a sterile glass or metal high-speed blender jar with cover, balance, sterile beakers, sterile graduated pipets, knives, forks, spatulas, forceps, scissors, tablespoons, and tongue depressors (for sample handling).

4.3 Microbiological methods

4.3.1 Colony count at 30°C (EN ISO 4833-1:2013)

Microbiology of the food chain - Horizontal method for the enumeration of microorganisms - Part 1: Colony-count at 30°C by the pour plate technique

Scope: The International Standard describes a method for the enumeration of microorganisms by counting the colonies growing in a solid medium after incubation at 30°C under aerobic conditions. The method is applicable to products intended for human consumption, animal feed, and environmental samples in production and handling.

The ISO is applicable to products that require a reliable count when a low limit of detection is specified, or for products expected to contain spreading colonies that obscure colonies of other organisms.

Principle: Decimal dilutions of the sample are used to prepare pour plates in plate count agar (PCA). The Petri dishes are incubated at 30°C for 72 h after which the colonies growing in the medium are counted.

Equipment:

a) Autoclave
b) Incubator at 30°C (±1°C)
c) Petri dishes
d) Pipettes (1 ml)
e) Water bath (capable of operating at 44–47°C)
f) Colony counter
g) pH meter
h) Test tubes, flasks or bottles

Media/Diluents: For preparation, production and performance testing of culture media follow ISO11133.

a) Use the diluent as specified in ISO 6887 (see section 3.2.3)

General diluents

Peptone salt solution

Buffered peptone water

The addition of 3.5–4.0% sodium chloride (isotonic with sea water) is recommended when examining raw, unprocessed marine fish for their natural marine microbial flora.

Diluents for special purposes

Peptone-salt solution with bromocresol purple solution may be used with certain acidic products so that adjustment of pH can be carried out without the use of a sterile pH probe. Peptone solution may be used for bivalve molluscs, gastropods and other shellfish.

(b) Plate count agar.

Sample preparation: Use a 50g analytical unit of liquid or dry food to determine aerobic plate count value and MPN of coliforms.

Add dilution water to blender jar containing 50 g analytical unit and blend 2 min. Make dilutions of original homogenate promptly, using pipettes that deliver required volume accurately. Not more than 15 min should elapse from the time.

Prepare decimal dilutions, as appropriate, of food homogenate by transferring 10 ml of previous dilution to 90 ml of diluent. Shake all dilutions.

Procedure: Having prepared decimal dilutions in the appropriate diluent, 1 ml aliquots of each appropriate dilution are transferred to two Petri dishes.

Dilutions are selected that will give colony counts of between 15 and 300 colonies per plate. Some 12–15 ml PCA that has been cooled to 44 – 47°C is poured into each Petri dish. The time between preparing the sample and pouring the medium into the Petri dishes should be no longer than 45 min. The contents of the Petri dishes are carefully mixed and then allowed to solidify on a level surface. If it is suspected that the colonies will overgrow the surface of the medium, about 4 ml of an overlay medium at 44 – 47°C can be poured on to the surface of the solidified, inoculated medium. The overlay is allowed to solidify. The Petri dishes are incubated inverted at 30°C (±1°C) for 72 h (±3 h). Dishes should not be stacked more than six high, and must be separated from one another, and from the walls and top of the incubator. After incubation, the number of colonies present on each plate is counted and used to calculate the number of CFU per g or ml sample.

Expression of results: The number of colonies present on each plate is counted and used to calculate the number of CFU per g or ml sample. The calculation is executed by following the procedure in ISO 7218 (see above).

Performance criteria, quality assurance: For performance testing of the culture media PCA productivity should be tested according to ISO 11133 (see 2.1.3). Information on control strains, criterion for the quantitative control method and reference medium are provided by the International Standard.

Performance criteria for precision, repeatability and reproducibility are provided by the International Standards.

Rapid tests: A number of chromogenic media based detection methods/enzyme based methods, fluorescent methods allowing quantification within 24 -48 hours are available. Some of them are listed in Table 2.

TABLE 2: COMMERCIAL PRODUCT TESTS COLONY COUNT

Coliforms		
Test	**Principle, test time**	**Supplier**
Compact Dry TC (Total Count)	Chromogenic media; 48 h; can also be used for surface monitoring	R-Biopharm www.r-biopharm.com
3M™ Petrifilm™ Rapid Aerobic Count Plates	Contain nutrients, gelling agent, dual-sensing indicator; 24 h	3M www.3m.com
BioLumix system plus Aerobic Count Vial*	Fluorescence; same day, overnight and up to 48 hours	BioLumix www.mybiolumix.com

Source: Internet search, 2016

4.3.2 *Salmonella* spp. (EN ISO 6579:2002/Amd 1:2007) [22]

Microbiology of food and animal feeding stuffs - Horizontal method for the detection of *Salmonella* spp.

Scope: The International Standard describes a method for the detection of *Salmonella* spp., including *S. Typhi* and *S. Paratyphi* in products intended for human consumption, animal feeds, and environmental samples from food production and food handling areas.

Principle: Four successive stages are involved in the detection of Salmonella. In the first, a nonselective, pre-enrichment stage, buffered peptone water is inoculated with the sample and incubated at 37°C (±1°C) for 18 h (±2 h). The pH must not fall below 4.5 if acid products are being examined. The second stage is a selective enrichment in liquid media. Rappaport-Vassiliadis medium with soya (RVS) broth and Muller-Kauffmann tetrathionate-novobiocin (MKTTn) broth are inoculated with the pre-enrichment culture. The samples in RVS broth are incubated at 41.5°C (±1°C) for 24h (±3 h) and the samples in MKTTn broth are incubated at 37°C (±1°C) for 24h (±3 h). Each of the selective enrichments is plated out on to two selective solid media. One of these media must be xylose lysine deoxycholate (XLD) agar, which is incubated at 37°C (±1°C) and examined after 24 h (±3 h). The second selective agar is chosen by the laboratory, but should be suitable for isolating lactose-positive *Salmonella* and *S. Typhi* and *S. Paratyphi* strains. Media that can be used include brilliant green agar and bismuth sulphite agar. They should be incubated according to the manufacturer's instructions. Presumptive *Salmonella* colonies are subcultured, plated out, and their identity confirmed using biochemical and serological tests.

Equipment:

a) Autoclave

b) Drying cabinet or oven (capable of operating at 37–55°C)

[22] Since 2008, EN-ISO 6579, including annex D, is under revision. Detection of Salmonella will become part 1 of the new CEN-ISO 6579 series. CEN ISO 6579-1 Microbiology of the food chain -- Horizontal method for the detection, enumeration and serotyping *of* Salmonella -- Part 1: Horizontal method for the detection of Salmonella spp. is expected to be published before Summer 2016.

c) Incubator at 37°C (±1°C)

d) Water bath or incubator at 41.5°C (±1°C)

e) Water bath (capable of operating at 44–47°C)

f) Water bath at 37°C (±1°C)

g) Sterile loops (diameter 3 mm or 10 µl), or sterile pipettes

h) pH meter

i) Test tubes or flasks

j) Graduated pipettes or automatic pipettes

k) Petri dishes

Media/Reagents:

a) Buffered peptone water

b) RVS broth

c) Muller-Kauffmann tetrathionate-novobiocin (MKTTn) broth

d) Xylose lysine deoxycholate (XLD) agar

e) Second medium (selective plating out medium)

Nutrient agar

Triple sugar iron (TSI) agar

Urea agar

L-Lysine decarboxylation medium

f) Reagents for detection of β-galactosidase

g) Reagents for Voges-Proskauer reaction

VP medium

Creatine solution

1-naphthol, ethanolic solution

Potassium hydroxide solution

h) Reagents for indole reaction

Tryptone/tryptophan medium

Kovac's reagent

i) Semi-solid nutrient agar

j) Physiological saline solution

Sera: Commercial available types of agglutinating sera containing antibodies for one or several O-antigens.

Sampling[23]: A 2-class sampling plan for official controls of products of live bivalve molluscs, live echinoderms, tunicates and marine gastropods, cooked crustaceans, and mollusc shellfish intended

[23] Changing in 2017 as to sampling plan

for human consumption (placed on the market during their shelf-life) is applicable. The sampling plan consists of 5 samples, of which none should contain pathogens in 25 g of shellfish for human consumption.

Sample preparation: Each sample is analysed for the presence of Salmonella. A 25 g analytical unit is taken at random from each 100 g sample unit. The remainder of the sample unit is kept in a sterile container for compliance requirements.

The samples are taken using sterile tools, e.g. spoons, scalpels, knives, spatulas and pipettes. Frozen products should be first thawed at below 5°C (for not longer than 12 hours). In the case of deeply frozen samples, sterile drills are used for sampling.

Crustaceans and fish: Preferably, frozen samples are not thawed before analysis. If frozen samples must be tempered to obtain analytical portions, they are thawed within 12-18 h at 2-5°C. 25 g samples are aseptically weighed into a sterile blending container and blended for 2 min. Sterile broth is added and blended for 2 min. The homogenized mixture is aseptically transferred to a sterile screw-cap jar, or other appropriate container, and let stand for 60 ± 5 min at room temperature with jar securely capped. It is mixed well by swirling and the pH is determined with test paper.

Procedure: A sample of 25 g (when possible) is mixed with the pre-enrichment medium to yield a 1:10 dilution and incubated at 37°C (±1°C) for 18h (±2 h). It is important for acid foods that the pH does not fall below 4.5, so a double strength buffered peptone water may be used.

Following the pre-enrichment, 0.1 ml of the culture is transferred to a tube containing 10 ml RVS broth and 1 ml to a tube containing MKTTn broth. The inoculated RVS broth is incubated at 41.5°C (±1°C) for 24 h (±3 h) and the inoculated MKTTn broth at 37°C (±1°C). The incubation temperature must not exceed 42.5°C.

Following incubation, both broths are plated out on to XLD agar, and a second agar of choice. Well separated colonies should be obtained on the plates. The inoculated XLD agar plates are incubated inverted at 37°C for 24 h (±3 h), and the plates containing the agar of choice are incubated according to the manufacturer's instructions. After the incubation period, the plates are examined for colonies typical of Salmonella, as well as those that are not typical. On XLD agar, typical *Salmonella* colonies have a black centre and a lightly transparent zone of a reddish colour due to the colour change of the indicator. Salmonella hydrogen sulphide (H_2S)-negative variants (e.g. *S. Paratyphi* A) on XLD agar are pink with a darker pink centre. Lactose-positive *Salmonella* on XLD agar are yellow with or without blackening.

For confirmation, at least one colony should be selected, if it is considered to be typical or suspect, and a further four colonies if the first one is negative. The colonies are streaked on to nutrient agar plates in a manner allowing well separated colonies to develop. The plates are incubated at 37°C (±1°C) for 24 h (±3 h). Pure cultures are used for biochemical and serological confirmation. If shown to be reliable, commercially available biochemical identification kits can be used following the manufacturer's instructions. If not available, the following tests should be carried out.

Slanted tubes of TSI agar are inoculated by streaking the slant surfaces and stabbing the butts. The tubes are incubated at 37°C (±1°C) for 24 h (±3 h). Typical *Salmonella* cultures show alkaline (red) slants and acid (yellow) butts with gas formation (bubbles) and, in 90% of cases, the formation of hydrogen sulphide (blackening of the agar). A lactose-positive *Salmonella* will have an acid (yellow) slant.

The surfaces of the urea agar slants are streaked, incubated at 37°C (±1°C) for 24 h (±3 h) and examined at intervals. A positive reaction is shown by the colour of phenol red changing to rose-

pink and later to deep cerise, due to the splitting of urea thereby releasing ammonia. The reaction can be seen after 2–4 h.

Tubes of L-lysine decarboxylation medium are inoculated just below the surface of the liquid medium and incubated at 37°C (±1°C) for 24 h (±3 h). A positive reaction is indicated by turbidity and a purple colour, whereas a yellow colour indicates a negative reaction.

To check for β -galactosidase activity, a loopful of the suspect culture is suspended in a tube containing 0.25 ml saline solution. One drop of toluene is added and the tube shaken. The tube is placed in a water bath set at 37°C and left for approximately 5 min. A volume of 0.25 ml of the β galactosidase reagent is added to the tube and mixed. The tube is placed back in the water bath and left for 24 h (±3 h). The tube is examined at intervals. A yellow colour indicates a positive reaction and is often visible after 20 minutes.

To check the Voges-Proskauer reaction, a loopful of the suspect culture is suspended in a sterile tube containing 3 ml VP medium. The tube is incubated at 37°C (±1°C) for 24 h (±3 h). After incubation, 2 drops of the creatine solution are added, 3 drops of the alcoholic solution of 1-naphthol, followed by 2 drops of potassium hydroxide solution, shaking well after each addition. A pink to bright red colour forming within 15 min indicates a positive reaction.

To test for the indole reaction, a tube containing 5 ml tryptone/tryptophan medium is inoculated with a loopful of the suspect culture. The tube is incubated at 37°C (±1°C) for 24 h (±3 h) and 1 ml of Kovac's reagent is then added. The formation of a red ring indicates a positive reaction, whereas a yellow brown ring indicates a negative reaction.

Table 3 shows the usual reactions given by Salmonella, but some strains may give uncharacteristic reactions.

Further confirmation of *Salmonella* isolates can be obtained by carrying out serological tests. *Salmonella* O-, Vi- and H-antigens can be detected using slide agglutination from pure colonies using appropriate antisera, after auto-agglutinable strains have been eliminated.

Isolates considered to be *Salmonella* or that may be *Salmonella* should be sent to a recognized *Salmonella* reference centre for definitive typing.

TABLE 3: TYPICAL *SALMONELLA* BIOCHEMICAL REACTIONS

	***Salmonella* strain**				
Test	Most *Salmonella* strains	*S. Typhi*	*S. Paratyphi* A	*S. Paratyphi* B*	*S. Paratyphi C**
TSI acid from glucose	++	++	++	+	+
TSI gas from glucose	+	–	++	+	+
TSI acid from lactose	–	–	‡	–	–
TSI acid from sucrose	–	‡	‡	–	–
TSI H_2S produced	+	+	–	+	+
Urea hydrolysis	–	‡	‡	–	–
Lysine decarboxylation	+	+	–	+	+
β -galactosidase reaction	–	‡	‡	–	–
Voges-Proskauer reaction	‡	‡	‡	–	–
Indole produced	–	‡	‡	–	–

++ 100% of isolates tested in this group gave a positive reaction.

+ 92–98% of isolates tested in this group gave a positive reaction.

‡ 100% of isolates tested in this group gave a positive reaction.

– 98–99% of isolates tested in this group gave a negative reaction.

* Not known what percentage of isolates in this group gave this reaction.

Expression of results: Determination of *Salmonella* spp. in food products always consists in detecting the presence of those bacteria in a specified amount of the product (25g/ml), but the number of those microorganisms in food is not determined. The results of the tests are expressed as a presence or absence of *Salmonella* in the mass or volume of the sample tested. The test report should contain any deviation in enrichment media or the incubating conditions used.

Performance criteria, quality assurance: Introduce reference samples into control flasks of the pre-enrichment medium, to check the ability to detect *Salmonella* with the methods and media as described. Proceed with the control flasks as for the test cultures.

Precision data obtained from an inter-laboratory trial are provided in an Annex of the International Standard. Use reference material to calculate accuracy.

An analytical control is required for each sample tested.

Rapid tests: Since it takes at least three to five days to obtain a result using traditional methods of detection for *Salmonella* spp, a substantial number of alternative rapid screening methods have been developed to produce results more quickly for food and environmental samples. Many of these are available commercially, and have been successfully validated by the Association of

Analytical Chemists (AOAC) and/or Association Française de Normalisation (AFNOR). The AOAC database of performance tested methods contains more than 40 products for the rapid detection of *Salmonella (*www.eoma.aoac.org/methods/result.asp).

Salmonella rapid test and screening kits utilise several different technologies, including novel culture techniques, immunomagnetic separation, enzyme immunoassay (EIA) - and ELISA (enzyme-linked immunosorbent assay)-based assays incorporating fluorescent or colorimetric detection, simple lateral flow assays incorporating immunochromatographic technology, and molecular techniques such as Deoxyribonucleic acid (DNA) hybridisation and PCR (polymerase chain reaction)-based assays, many of which now include real-time detection. Some methods can be automated to screen large numbers of samples. Almost all rapid test protocols include a selective enrichment stage, and then apply concentration and/or rapid detection techniques to replace culture on selective agars and further confirmatory tests. Most can claim to produce a result in approximately 48 hours or less, depending on the enrichment protocol.

Table 4 provides an overview on different rapid test for *Salmonella* in food/fish.

TABLE 4: COMMERCIAL PRODUCT TESTS FOR SALMONELLA

Cell separation/concentration from matrix		
Test	**Supplier**	**Website**
3M Tecra Unique *Salmonella*	3 M Food Safety	http://www.3m.com
VIDAS *Salmonella*	bioMerieux	http://www.biomerieux.com
Captivate	Lab M	http://www.labm.com
Pathatrix Auto System	Life Technologies	http://www.thermofisher.com
Dynabeads	Life Technologies	http://www.thermofisher.com
Concentrating Pipette	InnovaPrep	http://innovaprep.com
Chromogenic Agar		
Test	**Supplier**	**Website**
Rapid Salmonella	Bio-Rad Laboratories S.A.	http://www.bio-rad.com
Chromic ID Salmonella ISO, AOAC validated	bioMerieux	http://www.biomerieux.com
Harlequin TM Salmonella ABC	Lab M	http://www.labm.com
Chromatic Salmonella	Liofilchem srl	http://www.liofilchem.net
Compact Dry SL	R-Biopharm	http://www.r-biopharm.com
Hi Chrome Salmonella Agar	Sigma Aldrich	www.sigmaaldrich.com
Oxid Brillance Salmonella Agar	Thermo Scientific	www.thermofisher.com
BBL Chromagar	BD Diagnostic Systems	http://www.bd.com/
Salmonella Chromogenic Agar	Conda S.a.	http://www.conda.es/
Molecular methods		
Test	**Supplier**	**Website**
3 M Molecular detection system	3 M Food safety	http://www.3m.com
Foodproof Salmonella Detection Lyokit	BioTecon Diagnostics	www.bc-diagnostics.com
Gene Up	bioMerieux	http://www.biomerieux.com/
IQ Check Salmonella	Bio-Rad Laboratories	http://www.bio-rad.com/
Sure Fast Salmonella	R-Biopharm	http://www.r-biopharm.com/
Bax System PCR Assay AOAC 2003.09	Du Pont Qualicon	http://www.dupont.com/
SureFast Salmonella	R-Biopharm	http://www.r-biopharm.com/
ANSR Salmonella spp detection	Neogen Corp	http://www.neogen.com/
Vit-Salmonella gen probe	Vermicon AG	http://www.vermicon.com/
Sure Tec Salmonella Species Assay	Thermo Scientific	www.thermofisher.com/

Source, Internet search 2016

4.3.3 *Clostridium perfringens* (EN ISO 7937:2004)

Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of *Clostridium perfringens* — Colony-count technique

Clostridium perfringens, an important food-borne pathogen to consider in fish trade, is found in the gut and thus indicates faecal contamination, although spores also occur in the environment. It is uncommon to detect this organism in properly handled ready-to-eat foods. Illness is caused by the ingestion of large numbers of viable vegetative bacteria, which sporulate in the lower small intestine and produces enterotoxin induced diarrhoea. This enterotoxin is not produced in foods. Spores are common in the environment and may survive the cooking process, so that low level contamination of the final product may occasionally occur. Control is achieved by preventing spore germination and growth in food and by rapid cooling. Adequate cold storage and adequate reheating of food are of paramount importance. *C. perfringens* will grow between 15°C and 52°C with virtually no growth below 12°C. Not all *C. perfringens* produce enterotoxin and these non-toxigenic isolates (irrespective of the numbers of bacteria present) will not produce food-borne disease. However, the presence of high numbers of non-toxigenic *C. perfringens* in a ready-to-food is unsatisfactory and indicates poor processing, particularly during cooling.

Scope: The International Standard describes a method for the enumeration of viable *Clostridium perfringens* in products intended for human consumption and animal feeds, or environmental samples from food production and food handling areas.

Principle: Petri dishes are inoculated with a specified quantity of the initial suspension and appropriate decimal dilutions. A selective medium is added to the plates, followed by an overlay of the same medium. The plates are incubated anaerobically at 37°C for 20 (±2) h. Characteristic colonies are counted.

Equipment:

a) Autoclave
b) Incubator at 37°C (±1°C)
c) Anaerobic jars plus gas packs
d) pH meter
e) Loops and inoculating needles
f) Filtration units for sterilization of solutions (syringes and filters)
g) Test tubes, flasks or bottles
h) Durham tubes
i) Pipettes or micropipettes (capacity 1 ml and 10 ml)
j) Petri dishes
k) Water bath (capable of being maintained at 44–47°C)
l) Water bath at 46°C (±0.5°C)
m) Rubber bulbs

Media/Reagents:

a) Diluent (EN ISO 6887-3:2003)

b) Sulphite-cycloserine agar

c) Fluid thioglycollate medium

d) Lactose sulphite medium (optional)

e) Nitrate motility medium (optional)

f) Nitrate detection reagent (optional)

g) Zinc dust (optional)

h) Lactose-gelatin medium (optional)

Sampling: Sample the entire portion of food (whole fish) or take representative samples of 25 g each from different parts of the suspect food, because contamination may be unevenly distributed. Transport and examine samples promptly without freezing, if possible, and store at about 10°C until examined.

If analysis cannot be started within 8 h, or if the sample must be shipped to the laboratory for analysis, treat it with sterile buffered glycerine-salt solution, store immediately at -70 to - 80°C, and transport it to the laboratory with dry ice to maintain temperature as low as possible during shipment.

Sample preparation: Thaw samples at room temperature and transfer sample and solution to sterile blender jar. Transfer 25 g portion of sample to a sterile blender jar. Add dilution fluid to blender jar and homogenize 1-2 min at low speed. Obtain uniform homogenate with as little aeration as possible and proceed with examination.

Procedure: Having prepared decimal dilutions in the appropriate diluent, 1 ml aliquots of each appropriate dilution are transferred to two Petri dishes. Dilutions are selected that will give fewer than 150 colonies. Some 10–15 ml sulphite-cycloserine agar cooled to 44 – 47°C is poured into each Petri dish. The contents of the Petri dishes are carefully mixed and then allowed to solidify on a level surface. When the medium has solidified, about 4 ml of an overlay of the same medium is added. The overlay is allowed to solidify. The Petri dishes are placed in anaerobic jars and incubated at 37°C for 20 h (±2 h) under anaerobic conditions. A longer incubation may result in excessive blackening of the plates.

All plates containing fewer than 150 colonies are selected after the incubation period. Characteristic colonies of *C. perfringens* are black, caused by the reduction of sulphite to sulphide. All characteristic colonies on a plate should be counted and five selected from each plate for further confirmation tests.

Confirmation can be carried out in lactose sulphite medium. The reaction is very specific for *C. perfringens* and *C. absonum* at 46°C and it is, therefore, not imperative to ensure that the black colonies picked from the agar are pure before inoculation into the thioglycollate medium and subsequently into the lactose sulphite medium. Each selected colony is inoculated into the fluid thioglycollate medium and incubated at 37°C for 18–24 h under anaerobic conditions. After incubation, 5 drops of the thioglycollate culture are transferred without delay to the lactose sulphite medium using a sterile pipette. The tubes are incubated at 46°C for 18–24 h in a water bath under aerobic conditions.

The tubes of lactose sulphite medium are examined for the production of gas, and the presence of a black precipitate (iron sulphite). Durham tubes more than a quarter full of gas, and tubes with a black precipitate, are considered positive.

If there is a doubt, for example, when the Durham tube in the blackened medium is less than a quarter full of gas, transfer without delay 5 drops of the culture to a new tube of lactose sulphite medium. Incubate the tube in a water bath at 46°C for 18–24 h and examine the tube as previously described. Bacteria that form characteristic colonies in the sulphite-cycloserine medium, and give a positive confirmation with the lactose sulphite medium are considered to be *C. perfringens*. All other tubes are considered to be negative.

An alternative confirmation technique uses nitrate motility medium and lactose-gelatin medium. This technique requires well-isolated characteristic colonies. If it is not possible to pick off well separated colonies, inoculate five characteristic colonies into pre-de-aerated fluid thioglycollate medium. Incubate the medium at 37°C for 18–24 h. Streak the colonies on to sulphite-cycloserine base agar plates and add an overlay of 10 ml of sulphite-cycloserine base agar. After the agar has solidified, incubate the plates anaerobically at 37°C for 18–24 h. Select from each plate at least one typical and well separated colony. If necessary, repeat the streaking and inoculation on sulphite-cycloserine base agar plates until well isolated and characteristic colonies are obtained.

The selected colonies are stab inoculated into freshly de-aerated nitrate motility medium. The tubes are incubated at 37°C for 24 h and then examined for growth along the stab line. Motility is demonstrated if there has been diffuse growth in the medium away from the stab line. The presence of nitrite is tested for by adding 0.2–0.5 ml nitrite detection reagent to each tube of nitrate motility medium. This should be carried out under a fume hood. The formation of a red colour confirms the reduction of nitrate to nitrite. If no red colour is formed within 15 min, a small amount of zinc dust is added and allowed to stand for 10 min. If a red colour forms after the addition of zinc dust, no reduction of nitrate has occurred.

Selected colonies are inoculated into freshly de-aerated lactose-gelatine medium, which is then incubated at 37°C for 24 h under anaerobic conditions. The tubes are examined for gas and a yellow colour (due to acid formation), indicating the fermentation of lactose. The tubes are chilled for 1 h at 5°C and checked for gelatine liquefaction. If the medium has solidified, re-incubate for another 24 h and check again for gelatine liquefaction.

Bacteria that produce black colonies in sulphite-cycloserine medium, are non-motile, usually reduce nitrate to nitrite, produce acid and gas from lactose, and liquefy gelatine in 48 h are considered to be *C. perfringens*. Cultures that show a faint reaction for nitrite (a pink colour) should be eliminated as *C. perfringens* gives consistent intense and immediate reactions.

Expression of results: Determination of the number of culturable and confirmed *Clostridium perfringens* bacteria per millilitre or per gram of sample.

Performance criteria, quality assurance: Two dilution series are set to get replicated results.

Rapid tests: Some commercial test products for *C. perfringens* are summarised in Table 5.

TABLE 5: COMMERCIAL TEST PRODUCTS FOR *CLOSTRIDIUM PERFRINGENS*

Test kit	Analytical Technique	Approximate Test time	Supplier
Clostridium perfringens test presumptive	Uses prepared traditional media	48 hours	Biomedix http://www.biomedix.com
ISO-GRID*	MF with selective culture medium	24 - 48 (72) hours	Neogen Cooperation www.neogen.com
PET-RPA TD30	Reversed passive latex agglutination	24 - 48 hours	Oxoid Inc Thermo Fisher http://www.oxoid.com
CP ChromoSelect Agar	Chromogenic agar	26 hours	Sigma Aldrich www.sigmaaldrich.com

* ISO-GRID disposable units and ISO-GRID membranes are filtration systems used on food, beverage and water samples for the detection, confirmation and enumeration of microorganisms including total plate counts, Coliforms, generic *E. coli, E. coli* O157:H7, Salmonella, *Listeria monocytogenes*, Staphylococcus and more.

ISO-GRID approvals: Total Bacterial Count AOAC No. 986.32; Total Coliform/E. coli Count AOAC No. 990.11; *Salmonella* Detection AOAC No. 991.12; E. coli O157:H7 Count AOAC No. 997.11

Source: Internet search, 2016

4.3.4 Coliforms (ISO 4832:2006)

Microbiology of food and animal feeding stuffs — Horizontal method for the enumeration of coliforms — Colony-count technique

Scope: The International Standard gives general guidelines for the enumeration of coliforms in products intended for human consumption and animal feeds, or environmental samples from food production and food handling areas, by counting colonies on a solid medium that has been incubated at either 30°C or 37°C. This technique is recommended if samples are expected to contain more than 100 CFU per g or ml of sample.

Principle: A specified quantity of the initial suspension and appropriate decimal dilutions are used to prepare pour plates in an appropriate selective medium. The plates are incubated aerobically at 30°C or 37°C (as agreed) for 24 h. Characteristic colonies are counted and, if required, a number of colonies can be confirmed by testing for fermentation of lactose.

Two poured plates are prepared using a solid selective culture medium, with a specified quantity of the test sample if the initial product is liquid, or with a specified quantity of an initial suspension in the case of other products. Other pairs of poured plates are prepared under the same conditions, using decimal dilutions of the test sample or of the initial suspension. 4.2 The plates are incubated at or (as agreed) for 4.3 The characteristic colonies are counted and, if required, a number of colonies are confirmed by fermentation of lactose.

Equipment:

a) Autoclave

b) Incubator at 30°C (±1°C) or 37ªC (±1°C)

c) Petri dishes

d) Pipettes (total delivery 1 ml)

e) Water bath (capable of being maintained at 44–47°C)

f) Water bath at 100°C

g) Colony counter

h) Test tubes

i) Durham tubes

j) Bottles or flasks

k) pH meter

l) Loop (3 mm diameter or disposable)

Media/Reagents:

a) Diluent (EN ISO 6887-3:2003)

b) Crystal violet neutral red bile lactose (VRBL) agar

c) Brilliant green lactose bile broth

Sampling: Sampling should be carried out in accordance with the specific International Standard appropriate to the product concerned. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject. For sampling techniques from surfaces using contact plates and swabs see ISO 18593 (footnote page 20)

Sample preparation: Weigh 50 g of fish/food into a sterile high-speed blender jar. Frozen samples can be softened by storing for <18 h at 2-5°C, but do not thaw. Add buffered water and blend for 2 min. If <50 g of sample is available, weigh portion that is equivalent to half of the sample and add sufficient volume of sterile diluent to make a 1:10 dilution. The total volume in the blender jar should completely cover the blades.

Prepare decimal dilutions with sterile diluent or equivalent. Number of dilutions to be prepared depends on anticipated coliform density.

Procedure: Having prepared decimal dilutions in the appropriate diluent, 1 ml aliquots of each appropriate dilution are transferred to two Petri dishes. About 15 ml VRBL agar that has been cooled to 44–47°C is poured into each Petri dish. The contents of the Petri dishes are carefully mixed and then allowed to solidify on a level surface. The time between preparing the initial dilution and pouring the plates should not exceed 15 min. When the medium has solidified about 4 ml of an overlay of the same medium is added. The overlay is allowed to solidify. The Petri dishes are incubated inverted at 30°C or 37°C for 24 h (±2 h).

All plates containing 10 or more colonies and fewer than 150 colonies are selected after the incubation period. Colonies are counted if they are purplish red with a diameter of at least 0.5

mm, and are sometimes surrounded by a reddish zone of precipitated bile. These are considered as characteristic colonies and require no further confirmation.

Atypical colonies (i.e. colonies smaller than 0.5 mm in diameter) should also be counted and confirmed immediately after the incubation period. Five atypical colonies of each type are inoculated into tubes of brilliant green lactose bile broth and incubated at 30°C or 37°C (as agreed) for 24 h (±2 h). Cultures that produce gas (collected in a Durham tube) are counted as coliforms. These should be added to the confirmed colonies when calculating the count.

Take the results into account in the calculation.

Results: The number of coliforms per millilitre or per gram of sample is calculated from the number of characteristic colonies obtained in the plates chosen.

For calculation of results see ISO 7218 (see 2.1.3).

Performance criteria: Given a Poisson distribution of microorganisms in the substrate, the confidence limits of this method vary according to the count of colonies examined. More information on calculating confidence limits, repeatability and reproducibility is provided by the International Standard. For performance testing relating to crystal violet neutral red bile lactose (VRBL) agar see ISO/TS 11133-2:2003[24], Table B.1. If repeatability has been checked, the final quoted results obtained.

Rapid tests: Various rapid tests for coliforms (food and environment) are available. In Table 6 some of them are listed.

TABLE 6: COMMERCIAL TEST PRODUCTS FOR COLIFORM BACTERIA (FOOD AND ENVIRONMENT)

Test	Principle	Approx. Total Test Time	Supplier
3M™Petrifilm™	Nutrients and indicator; AFNOR certification	6 - 14; 24 h	3M http://www.3m.com
BioLumix system CC: Coliform Vial	Colour, florescent change	Same day, overnight	BioLumix Neogen Corporation www.mybiolumix.com
ISO-GRID	MF with selective culture medium	24 - 48 (72) h	Neogen Cooperation www.neogen.com
MicroSnap Coliform AOAC validated (#071302)	Bioluminogenic reaction Bioluminescent swab test	<10 CFU in 8 h 6 h quant. results 8 h presence/absence	Hygiena http://www.hygiena.com

Source: Internet search, 2016

[24] http://www.iso.org/iso/iso_catalogue/catalogue_ics/catalogue_detail_ics.htm?csnumber=34501

4.3.5 *Escherichia coli* (ISO 16649-3:2015)

Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of β - glucuronidasepositive *Escherichia coli* Part 3: MPN technique using 5-bromo-4-chloro-3-indolyl-β - D-glucuronide

The consumption of sewage-polluted bivalve molluscan shellfish (BMS) may cause illness in the consumer through ingestion of faecal-borne pathogens. Enteric bacteria, such as E. coli, are used as indicator organisms to assess the sanitary quality of shellfish and their growing waters, and to predict the risk of exposure to enteric pathogenic microorganisms. In the EU, the criteria for laying down the microbiological standards for BMS are set out in Regulation (EC) 854/2004[25] and Regulation (EC) 2073/2005 stipulating conditions for the production and placing on the market of live BMS. Across the EU, E. coli in shellfish flesh is used as an indicator of faecal contamination of BMS. The EU reference method for the determination of E. coli in BMS is ISO 16649-3.

Scope: The International Standards describes a method for the detection and enumeration of β - glucuronidase positive E. coli, by means of a liquid-medium culture technique, and calculation of the MPN after incubation at 37°C (± 1°C), followed by 44°C (± 1°C). It is applicable to products intended for human consumption and animal feeds or environmental samples from food production and food handling areas. The method is suitable for the calculation of cells of E. coli that have been subjected to stress arising from dehydration, freezing, exposure to a saline environment or damage by disinfectants such as chlorine-containing products. The acceptability of the method is limited by a large degree of variability. The method is intended to be applied and the results interpreted in the light of the information given by the International Standard.

Principle: The method used to enumerate E. coli in BMS is a two-stage, five-tube three-dilution MPN method. The first stage of the method is a resuscitation step, requiring inoculation of minerals modified glutamate broth (MMGB) with a series of diluted shellfish homogenates and incubation at 37°C (±1°C) for 24 h (±2 h). The presence of *E. coli* is confirmed by sub-culturing acid producing tubes onto agar containing 5-bromo-4-chloro-3-indolyl-β -D glucuronide and detecting β -glucuronidase activity.

Note: E. coli O157 and some other strains of *pathogenic E. coli, will not* be detected by this method.

Equipment:

a) Autoclave
b) Incubator at 37°C (±1°C)
c) Incubator at 44°C (±1°C)
d) Drying cabinet or ventilated oven (capable of being maintained at 25°C (±1°C) to 50°C (±1°C), or laminar flow cabinet
e) Refrigerator 5°C (±3°C)
f) pH meter
g) Test tubes
h) Pipettes (total delivery 1 ml and 10 ml)

[25] Regulation (EC) 854/2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption

i) Loops - sterile, 10µl

j) Petri dishes

k) Sterile glassware

l) Waring blender and jars or stomacher and stomacher bags

m) Shucking knife, Oyster cracker or other suitable equipment for opening shellfish

n) Electric top pan balance

Media:

a) 0.1% peptone (0.1% P)

b) Peptone salt solution (PSS)

c) Minerals modified glutamate broth (MMGBx1, MMGBx2)

d) Tryptone bile glucuronide agar (TBGA/TBX)

Sampling: A pooled sample comprising a minimum of 10 individual animals.

Sample reception: Samples must be received in an intact food-grade plastic bag, and properly packed in a cool box with ice packs with recorded temperature at the time of sampling. Samples are regarded as unsatisfactory if on receipt at the laboratory the sample is frozen, the container is leaking, the shellfish are covered in mud, or immersed in water or mud/sand. Use of alternate sample transport criteria may be acceptable, where acceptable verification studies have been undertaken.

Sample preparation: Upon receipt in the laboratory, the temperature of the samples is recorded. Samples should preferably be examined immediately. If storage in the laboratory is necessary, then samples should be stored at 5°C (±3°C). Choose shellfish that are alive and discard all dead shellfish and those with obvious signs of damage. More shellfish can be used, if necessary, to produce the required volumes for each analysis.

Mud and sediment adhering to the shell should be removed prior to opening by rinsing/scrubbing under cold, running tap water of potable quality. Shellfish should not be re-immersed in water as this may cause them to open. Open all selected shellfish with a flame-sterilised shucking knife or equivalent and empty meat and liquor into a sterile container. If sterilised by heating allow the knife to cool before using. When opening shellfish, ensure that the hand holding the shellfish is protected with a heavy-duty safety glove to prevent cuts.

Dilution and homogenisation: Add 2ml of sterile 0.1% P or PSS per 1g of shellfish. Homogenise at high speed for approximately 1 minute and decant the contents back into the sterilised container.

Procedure: Decimal dilution series is prepared such that the final dilution yields a negative result. Generally, three tubes of the selective enrichment medium are used for each dilution, although with some products, for example live shellfish, special products or where greater accuracy is required, five tubes should be inoculated for each dilution.

10 ml of the test sample if liquid, or 10 ml of the initial suspension in the case of other products, is added to 10 ml of double strength mineral-modified glutamate medium. 1 ml of the test sample if liquid, or 1 ml of the initial suspension in the case of other products, is added to 10 ml of single strength mineral-modified glutamate medium. 1 ml of further dilutions is added to 10 ml of single

strength mineral-modified glutamate medium. The contents of all tubes are mixed and incubated at 37°C for 24 h (±2 h).

Each tube that shows yellow coloration indicating acid formation, is streaked on to a plate of tryptone bile glucuronide agar in order to obtain separate colonies. The plates are incubated at 44°C for 20–24 h. Petri dishes should not be stacked more than three high.

After incubation, the plates are examined for any colonies that are dark, light blue or blue green, indicating β -glucuronidase-positive E. coli. Tubes should be considered positive if they give rise to blue or blue-green colonies on tryptone bile glucuronide agar. The number of positive tubes at each dilution is counted and an MPN table used to determine the MPN of E. coli in the sample.

Results: Results should be reported as the MPN per 100g of flesh and intra-valvular liquid. Estimated MPN values given for tube combinations of 0, 0, 0 and 5, 5, 5 should be assigned values of <18 and >18,000 per 100g of flesh and intra-valvular liquid respectively. Both the probability category and MPN result can be determined (using the Excel spreadsheet MPN calculator as shown on the website of the European Reference Laboratory CEPAS, website address below) and the results from all dilutions tested should be used. Only values determined from combinations that give a category 1 or 2 profile should be recorded. Category 3 values should be recorded as 'Void'.

Performance criteria: Measurement uncertainty can be assessed by the repeatability and reproducibility of test results. These should be monitored through control tests analysed alongside sample tests, through in-house comparability testing between analysts, and through external inter-comparison exercises, that would highlight any uncertainties within the test methods.

The inclusion of the measurement of uncertainty or confidence limits for the result is recommended. An example for assessing quantitative methods is the use of Lenticule ™ discs (www.sigmaaldrich.com).

The International Standard provides information on reference strains to be used in performance testing. It is recommended to include an un-inoculated bottle of single strength MMGB as a negative control.

Reference: National Reference Laboratory for monitoring bacteriological and viral contamination of bivalve molluscs, The Centre for Environment, Fisheries & Aquaculture Science (CEFAS). Generic protocol Enumeration of *Escherichia coli* in bivalve molluscan shellfish by the MPN technique (based on ISO 16649-3) Issue 12: 25.02.16 (https://www.cefas.co.uk/nrl/information-centre/nrl-laboratory-protocols/enumeration-of-escherichia-coli-in-molluscan-bivalve-shellfish/.

Rapid tests: There are many other methods for enumerating coliforms *and E. coli*, including several that use fluorogenic reagents like MUG (4-methylumbelliferyl-beta-D-glucuronide) or other chromogenic substrates, for presumptive detection and identification of coliform and *E. coli* in foods. Many of these tests, such as the Petrifilm dry rehydratable film, the hydrophobic grid membrane filter/MUG (HGMF/MUG) method, ColiComplete, and Colilert, have been evaluated by collaborative studies and adopted as official first or final action by the AOAC. There are also many modifications of the membrane filtration assays that have been developed for testing for coliform, faecal coliform and E. coli and used mostly for water, environmental waters, and shellfish harvest waters analysis.

Some of the commercial product tests to be used in fish are listed in Table 7.

TABLE 7: COMMERCIAL PRODUCT TESTS FOR *ESCHERICHIA COLI*

Test	Principle	Approx. Total Test Time	Supplier
3M™ Petrifilm™ (w/ Coliforms)	Nutrients, indicators	24-48 hours.	3M www.3m.com
ColiComplete AOAC Official Method 992.30	Enzymatic	28-48 hours	Biocontrol www.biocontrolsys.com
Compact Dry EC (E. coli and coliforms)*	Chromogenic enzyme substrates	24 hours	R-Biopharm www.r-biopharm.com
Colilert *E. coli* (also coliforms)	Substrate Technology	24 hours	Idexx www.idexx.com
Colitag-S" medium	Fluorescence signal	30 (16-48hours)	Neogen www.foodsafety.neogen.com

*MicroVal, NordVal, AOAC validated

AOAC inclusion of Colilert® as an approved method (991.15) for the detection and enumeration of total coliforms and E coli in water

Source: Internet search, 2016

4.3.6 Coagulase-positive staphylococci – Staphylococcus aureus and other species (ISO 6888-1:1999/Amd. 1:2003)

Microbiology of food and animal feeding stuffs - Horizontal method for the enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) - Part 1: Technique using Baird-Parker agar medium

Staphylococcus aureus (S. aureus) is highly vulnerable to destruction by heat treatment and nearly all sanitizing agents. Thus, the presence of this bacterium or its enterotoxins in processed foods or on food processing equipment is generally an indication of poor sanitation. *S. aureus* can cause severe food poisoning. Foods are examined for the presence of S. aureus and/or its enterotoxins to confirm that S. aureus is the causative agent of foodborne illness, to determine whether a food is a potential source of "staph" food poisoning, and to demonstrate post-processing contamination, which is generally due to human contact or contaminated food-contact surfaces. Conclusions regarding the significance of S. aureus in foods should be made with circumspection. The presence of a large number of *S. aureus* organisms in a food may indicate poor handling or sanitation; however, it is not sufficient evidence to incriminate a food as the cause of food poisoning. The isolated S. aureus must be shown to produce enterotoxins.

Scope: The International Standard describes a method for the enumeration of coagulase-positive staphylococci in products intended for human consumption or animal feeds by counting colonies growing on Baird-Parker medium after aerobic incubation at 35°C or 37°C.

Principle: Decimal dilutions of the sample are inoculated on to the surface of Baird-Parker agar plates, incubated at 35°C or 37°C and examined after both 24 h and 48 h. Typical and atypical colonies are selected so as to give a significant result and are confirmed by a positive coagulase test result.

Equipment:

e) Autoclave

f) Incubator at 35°C (±1°C) or 37°C (±1°C)

g) Drying cabinet or ventilated oven (capable of being maintained at 25°C (±1°C) to 50°C (±1°C), or laminar flow cabinet

h) Water bath at 47°C (±2°C)

i) Test tubes, flasks or bottles with screw caps

j) Petri dishes

k) Straight wire and Pasteur pipette

l) Pipettes (total delivery 1 ml, 2 ml and 10 ml)

m) Spreaders

n) pH meter

Media/Reagents:

a) Diluent (EN ISO 6887-3:2003)26

b) Baird-Parker agar

c) Brain heart infusion broth

d) Rabbit plasma

Sampling: Sampling should be carried out in accordance with the specific International Standard appropriate to the product concerned. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject. For swabbing of surfaces etc. the ISO standard should be used, see above

Sample preparation: Transfer 50 g of the analytical unit in a blender jar, add buffered dilution water and blend 2 min. Homogenise for between 30 seconds and three minutes in a stomacher. The homogenisation time required will depend on the manufacturer's instructions and the type of sample being examined.

Prepare all decimal dilutions.

If possible, dilutions should be selected that will give colony counts of between 10 and 300 colonies per plate.

Procedure: From decimal dilutions in the appropriate diluent, 0.1 ml aliquots of each appropriate dilution are spread on the surface of each of two Baird-Parker agar plates. If low numbers of Coagulase-Positive Staphylococci are required to be counted, 1 ml of the test sample, if liquid, or the initial suspension, can be spread on to the surface of a large (140 mm) agar plate or three 90 mm agar plates, in duplicate. Plates are allowed to dry for some 15 min with their lids on at laboratory temperature.

[26] Microbiology of the food chain - Preparation of test samples, initial suspension and decimal dilutions for microbiological examination - Part 3: Specific rules for the preparation of fish and fishery products

The plates are incubated inverted at 35°C or 37°C for 24 h (±2 h). After this time the bottoms of plates are marked to show the positions of any typical colonies. The plates are re-incubated at 35°C or 37°C for a further 24 h (±2 h) and the positions of any new colonies are marked. Atypical colonies should also be marked at this point. Typical colonies are black or grey, shiny and convex, and are surrounded by a clear zone that may be partially opaque. After incubation for at least 24 h, an opalescent ring immediately in contact with the colony may appear in this clear zone. The colonies are 1–1.5 mm in diameter after 24 h, and 1.5–2.5 mm in diameter after 48 h incubation. Atypical colonies are similar in size to typical colonies, but may be shiny black colonies with or without a narrow white edge, the clear zone is absent or barely visible and the opalescent ring is absent or hardly visible, or grey colonies without a clear zone.

Colonies should only be counted on plates containing a maximum of 300 colonies with 150 typical and/or atypical colonies at two successive dilutions. One plate should contain at least 15 colonies. Select five typical colonies for confirmation if there are only typical colonies, five atypical colonies if there are only atypical colonies, or five of each if both types are present, from each plate.

Using a wire, remove some of each selected colony from the surface and inoculate into a separate tube of brain heart infusion. Incubate this at 35°C or 37°C for 24 h (±2 h). After incubation, add 0.1 ml of each culture to 0.3 ml rabbit plasma in sterile haemolysis tubes and incubate at 35°C or 37°C. Examine the plasma for clotting after 4–6 h incubation by tilting the tube. If there is no positive reaction, re-examine the tube after 24 h incubation. Suppliers of rabbit plasma may provide their own instructions that should be followed. The test is positive if the volume of the clot occupies more than half the original volume of liquid. A negative control should be set up by adding sterile brain heart infusion to the recommended quantity of rabbit plasma.

Expression of results: Count and record the number of typical colonies. If several types of colonies are observed that appear to be *S. aureus* on selected plates, count number of colonies of each type and record counts separately. When plates of the lowest dilution contain <20 colonies, these may be used. Report as Coagulase Positive-Staphylococci.

If plates containing >200 colonies have colonies with the typical appearance of S. aureus and typical colonies do not appear at higher dilutions, use these plates for the enumeration of S. aureus, but do not count no typical colonies. Select > 1 colony of each type counted and test for coagulase production. Add number of colonies on triplicate plates represented by colonies giving positive coagulase test and multiply by the sample dilution factor. Report this number as number of *S. aureus*/g of food tested.

Calculate the number of coagulase positive Staphylococci per g as follows:

$$\text{Count per g} = \frac{\text{No. of colonies confirmed}}{\text{No. of colonies tested}} \times \frac{\text{Presumptive count}}{\text{Volume tested x dilution}}$$

For spiral plates, calculate the colony count per ml of dilution plated by adding together the counts from the two segments and dividing the total by the volume constant for the segment counted. Alternatively, use the tables supplied by the manufacturer. To obtain the colony count per g or ml multiply the count by the dilution factor.

Note: For samples showing less than 35 colonies on a plate, the confidence limits will be wide, and it is recommended that the result is reported as less than 7×10^3 CFU per gram, per ml or another sample portion.

Performance criteria, quality assurance: Precision data are included in the International Standard.

The use of duplicate plates at each dilution to achieve a weighted mean is not considered essential where the focus is on identifying bacterial levels that pose a risk to public health. The impact of plating variation is addressed by determining method uncertainty. Official control samples that have been submitted strictly in accordance with sampling plans and formal samples are tested in duplicate and weighted mean counts determined.

Quality control of media and internal quality assurance checks should be performed according to control organisms. The international standards give information on positive control S. aureus and negative control.

4.3.7 Vibrio cholerae and *Vibrio parahaemolyticus (ISO/TS 21872-1:2007)*

Microbiology of food and animal feeding stuffs -- Horizontal method for the detection of potentially enteropathogenic Vibrio spp. - Part 1: Detection of *Vibrio parahaemolyticus* and *Vibrio cholera.*

Scope: The Technical Specification describes a method for detection of *Vibrio parahaemolyticus* and *Vibrio cholerae* in products intended for human consumption or animal feeds and environmental samples from food production and food handling areas.

Principle: The detection of *V. parahaemolyticus* and *V. cholerae* requires four successive stages. An enrichment medium, alkaline saline peptone water (ASPW), is inoculated with the test sample and incubated at 37°C for 6 h (±1 h) for deep frozen products, or 41.5°C for 18 h (±1 h). This is then used to inoculate the ASPW for a second enrichment stage. The medium is incubated at 41.5°C for 18 h (±1 h). The cultures obtained from both of the enrichment stages are used to inoculate thiosulphate citrate bile and sucrose (TCBS) agar and a second solid selective medium of choice. The TCBS plates are incubated at 37°C and examined after 24 h (±3 h). The medium of choice is incubated according to the manufacturer's instructions. Presumptive colonies are subcultured and confirmed using appropriate biochemical tests.

Equipment:

a) Autoclave
b) Incubator at 37ªC (±1°C)
c) Incubator or water bath at 41.5°C (±1°C)
d) Water bath adjustable from 44°C to 47°C
e) Water bath at 37°C (±1°C)

Media/Reagents:

a) ASPW
b) Thiosulphate citrate bile and sucrose agar (TCBS)
c) Selective agar of choice
d) Saline nutrient agar

e) Oxidase reagents

f) Saline triple sugar iron (TSI) agar

g) Saline medium for detection of ornithine decarboxylase

h) Saline medium for detection of lysine decarboxylase

i) Saline medium for detection of arginine dihydrolase

j) Reagent for detection of β-galactosidase

k) Saline medium for detection of indole

l) Saline peptone waters

m) Sodium chloride solution

Sampling: Sampling should be carried out in accordance with the specific International Standard appropriate to the product concerned. If there is no specific International Standard, it is recommended that the parties concerned come to an agreement on this subject. For sampling techniques from surfaces using contact plates and swabs see ISO 18593 (footnote page 20).

Samples must be received in an intact food grade plastic bag and properly packed in a cool box with ice packs to reach a temperature of less than 8˚C within 4 hours and then maintain this for at least 24 hours. Such samples should not be received frozen.

Vibrio spp. can grow very rapidly in seafood at ambient temperature, and samples must be chilled to below 10°C immediately and then analysed as quickly as possible. However, the cells are easily damaged by rapid cooling and samples should not be cooled by direct contact with ice.

Samples from harvesting areas should have been rinsed, but not immersed, and drained at time of sampling, and should be regarded as unsatisfactory if they are received in the laboratory with the sample container leaking, the shellfish covered in mud, or immersed in water or mud/sand. Choose shellfish that are alive and discard all dead shellfish and those with obvious signs of damage. Select the appropriate number of shellfish depending on the species. More shellfish can be used, if necessary, to produce the required volumes for each analysis. Samples should be examined immediately or stored at 3°C (±2˚C) for no more than 24 hours until examination.

Sample preparation: Sample preparation procedures for shellfish typically require pooling 10-12 individual animals. The pooled sample is then homogenised using a sterile high-speed blender. If sample dilutions are required they should be prepared with a diluent containing salt, such as phosphate buffered saline (PBS).

Mud and sediment adhering to the shellfish should be removed prior to opening the shellfish by rinsing/scrubbing under cold, running tap water of potable quality. Shellfish should not be re-immersed in water as this may cause them to open. Open all selected shellfish as described below with a flame-sterilised shucking knife, and empty meat and liquor into a beaker. To flame sterilise the shucking knife, place the knife in the beaker of ethanol and sterilise using an electric Bunsen system. Allow the knife to cool before using. When opening shellfish ensure that the hand holding the shellfish is protected with a heavy-duty safety glove to prevent cuts.

Oysters and Clams - Insert the knife between the two shells towards the hinge end of the animal. Push the knife further into the animal and prise open the upper shell, allowing any liquor to drain into the beaker. Push the blade through the animal and sever the muscle attachments by sliding across the animal. Remove the upper shell and scrape the contents of the lower shell into a beaker.

Mussels and Cockles - Insert the knife between the shells of the animal and separate the shells with a twisting motion of the knife. Collect the liquor from the animal in the beaker, then cut the muscle between the shells and scrape the contents into a beaker.

Stomacher procedure: For homogenisation weigh 25g (±1g) of shellfish flesh and intra-valvular fluid into at least three stomacher bags, to avoid small pieces of shell from puncturing the bags. Remove excess air from the bag and operate the stomacher for 3 minutes at normal speed. Add sterile ASPW. Stomach at 'normal' speed for a further 3 minutes.

Homogenisation: According to the sensitivity required, a portion of the sample is homogenized in ×9 the volume of ASPW. If there is a large quantity, the ASPW should be warmed to 37°C prior to inoculation with the test portion.

If the dilution and incubation cannot be carried out on the same day, the initial suspension should be stored at 5°C (±3°C) until the following day. Storage at refrigeration temperatures should be avoided or kept to a minimum as this will reduce levels of *V. parahaemolyticus* and *V. cholerae* in the sample. The initial suspension is incubated at 37°C for 6 h (±1 h) for deep-frozen products or 41.5°C for 6 h (±1 h) for fresh, dried, or salted products. Care should be taken to apply the whole method to products with a high salt content, as the final salt concentration in the medium might alter the characteristics.

Procedure: For the second selective enrichment, 1 ml from the surface of the first selective enrichment culture is transferred to a tube containing 10 ml ASPW. The tube is incubated at 41.5°C for 18 h (±1 h).

The cultures obtained from both the first and second selective enrichments are streaked on to TCBS and the second isolation medium plates so that well separated colonies develop. The TCBS plates are incubated inverted at 37°C for 24 h (±3 h) and examined for typical colonies. *V. parahaemolyticus* colonies are smooth, green (sucrose negative) and 2–3 mm in diameter. *V. cholerae* colonies are smooth, yellow (sucrose positive) and 2–3 mm in diameter. The plates of the second medium are incubated for the time/temperature recommended by the manufacturer, and also examined for typical colonies of *V. parahaemolyticus* or *V. cholerae*.

At least five typical colonies of each Vibrio species being tested for should be picked off selective agar plates and streaked on to plates of saline nutrient agar or used to inoculate slants of saline nutrient agar. The plates or slants are incubated at 37°C for 24 h (±3 h).

For confirmation, appropriate commercially available biochemical test kits may be used. The bacterial inoculums must always be prepared in a sufficiently saline medium or dilution fluid. Alternatively, colonies can be confirmed using the following tests. For a first screening for presumptive *V. parahaemolyticus* or *V. cholerae* cultures, the following tests are carried out.

The oxidase test should be carried out on the isolates. Gram stains and motility tests should also be carried out on the cultures. Any Gram-negative, motile cultures giving positive oxidase tests should be retained for further tests.

A slope of saline TSI agar is stabbed to the bottom of the agar butt and streaked longitudinally along the slope. It is incubated at 37°C for 24 h (±3 h). *V. parahaemolyticus* typically produces a red (alkaline) slant and a yellow (acid) butt, without the formation of or gas. V. cholerae typically produces a yellow (acid) slant and a yellow (acid) butt, without the formation of hydrogen sulphide or gas. The incubation time should not exceed 24 h as the yellow slant of V. cholerae may turn red after that time.

Saline ornithine decarboxylase medium is inoculated just below the surface, and 1 ml sterile mineral oil is layered on top of the medium. It is incubated at 37°C for 24 h (±3 h). Turbidity and violet colour after incubation indicate a positive reaction (bacterial growth and decarboxylation of ornithine). A negative reaction is indicated by a yellow colour.

Saline lysine decarboxylase medium is inoculated just below the surface, and 1 ml sterile mineral oil is layered on top of the medium. It is incubated at 37°C for 24 h (±3 h). Turbidity and violet colour after incubation indicate a positive reaction (bacterial growth and decarboxylation of lysine). A negative reaction is indicated by a yellow colour.

Saline arginine dihydrolase medium is inoculated just below the surface and 1 ml sterile mineral oil is layered on top of the medium. It is incubated at 37°C for 24 h (±3 h). Turbidity and violet colour after incubation indicate a positive reaction (bacterial growth and dihydrolation of arginine). A negative reaction is indicated by a yellow colour.

To test for β -galactosidase activity, a colony is suspended in 0.25 ml saline solution. A drop of toluene is added and the tube is shaken. The tube is placed in a water bath at 37°C and allowed to stand for approximately 5 min. Then 0.25 ml β -galactosidase reagent is added and the contents of the tube mixed. The tube is placed back in the water bath at 37°C and left for 24 h (±3 h), examining it from time to time. A yellow colour indicates the presence of β -galactosidase. Often, this reaction is visible after 20 min. An absence of colour after 24 h indicates a negative reaction. Commercially available paper disks may also be used.

To carry out the indole test, 5 ml tryptone-trytophan saline medium is inoculated with the suspect colony. It is incubated at 37°C for 24 h (±3 h). After incubation, 1 ml Kovac's reagent is added. The formation of a red ring indicates a positive reaction (formation of indole), whereas a yellow-brown ring signifies a negative reaction.

To test halotolerance a series of peptone waters with increasing salt concentration is prepared (0%, 2%, 4%, 6%, 8% and 10%). A suspension of a colony is prepared and each of the tubes is inoculated.

The tubes are incubated at 37°C for 24 h (±3 h). Growth is indicated by turbidity in the tube.

Typically, V. parahaemolyticus strains decarboxylate ornithine and lysine, give a negative reaction for arginine dihydrolase and β -galactosidase activity, and a positive indole reaction. V. parahaemolyticus requires salt for growth and will grow in up to 8% salt concentrations but not at 10%.

Typically, V. cholerae strains decarboxylate ornithine and lysine, give a negative reaction for arginine dihydrolase and positive reactions for β -galactosidase activity and production of indole. V. cholerae will grow in peptone water containing 0% and 2% salt, but will not grow at salt levels of 6% and higher.

Confirmation tests should be carried out on cultures that do not show growth in 10% saline peptone water and which give a negative arginine dihydrolase reaction.

Final confirmation and determination of pathogenicity factors are complicated, and best performed by sending cultures to a specialist/reference laboratory. Cultures should be sent on saline nutrient agar slopes.

Results: If at least one colony from the sample tested conforms to the expected criteria of *V. parahaemolyticus*, *V. cholerae* then report the sample as "*V. parahaemolyticus V. cholerae* detected in 25 g". If no colonies from the sample tested conforms to the expected criteria of *V. parahaemolyticus V. cholerae* report the sample as "*V. parahaemolyticus V. cholerae* not detected in 25 g".

Performance criteria, quality assurance: Apply positive (expected results as turbid growth) and negative controls. For information on strains to be used see the CEFAS procedure below.

References:

- 8914:1990 Microbiology – General guidance for the detection of *Vibrio parahaemolyticus*
- Generic standard operating procedure detection of *Vibrio parahaemolyticus* in bivalve molluscan shellfish issued by technical manager, microbiological food safety of the European Community Reference laboratory for monitoring bacteriological and viral contamination of bivalve molluscs. The Centre for Environment, Fisheries & Aquaculture Science (CEFAS) Weymouth Laboratory, Barrack Road, The Nothe, Weymouth, European Community Reference laboratory for monitoring bacteriological and viral contamination of bivalve molluscs Dorset,

  (https://eurlcefas.org/; https://eurlcefas.org/media/6232/crl_sop_vibrio_17_11_07.pdf).

Rapid methods: Additional detection and confirmation methods are well established and typically take 4-7 days for completion. Biochemical confirmation can be accomplished using commercial identification systems such as the API 20E test strip from bioMérieux and Remel's RapID™ NF PLUS System. However, it is important to ensure that cultures are suspended in a saline medium to ensure the growth of halophilic species. Immunological identification and confirmation tests based on immunoassay (EIA) and ELISA for pathogenic Vibrio's are not widely available in the form of commercial tests kits. A latex agglutination test, the VET-RPLA Kit (Oxoid), is available for cholera toxin detection in culture filtrates. A number of molecular methods for confirmation have been developed, notably PCR assays for the identification of Vibrio species. Protocols for several of these methods are provided in the US Food and Drug Administration Bacteriological Analytical Manual (FDA BAM).

Some commercial rapid test kits for *Vibrio* spp. are available. Selective chromogenic media are mostly used for the isolation of most Vibrio species for the analysis of products of human consummation and feed and for monitoring of environmental samples in food production and handling. It particular enables the presumptive identification of *V. parahaemolyticus* and *V. cholera.* Table 8 provides an overview on some products for Vibrio detection in food/fishery products.

TABLE 8: COMMERCIAL PRODUCTS FOR VIBRIO RAPID DETECTION

Molecular methods			
Test	**Principle**	**Approximate Total Test Time**	**Supplier**
foodproof® Vibrio Detection LyoKit	Real time PCR	< 24 h	BIOTECON Diagnostics http://www.bc-diagnostics.com
CeeramTools	Molecular detection system	< 24 h	bioMérieux www.ceeramtools.com
BAX® Vibrio	Real-Time PCR Assay	24 h	Dupont Qualicon www.dupont.com
QuickBlue PCR kits	Real-Time PCR Kits	< 24h	Bioanalytic GmbH www.q-bioanalytic.net
Culture media			
Test	**Principle**	**Approximate Total Test Time**	**Supplier**
HardyCHROM™ Vibrio	Chromogenic medium; fluorescence	/	Hardy Diagnostics www.hardydiagnostics.com
ChromID™ Vibrio	Chromogenic substrat, enzymatic	24h	bioMérieux www.biomerieux.com
Compact Dry VP	Chromogenic Compact dry plates	20-24 h	R-Biopharm www.r-biopharm.com
RIDA®STAMP Vibrio	Agar, surface, product	24 - 48 h	R-Biopharm www.r-biopharm.com

Source: Internet search, 2016

4.3.8 *Listeria monocytogenes* (ISO 11290-1:1996/Amd.1:2004)

Microbiology of food and animal feeding stuffs - Horizontal method for the detection and enumeration of *Listeria monocytogenes* - Part 1: Detection method

Amd 1:2004 Modification of the isolation media and the haemolysis test, and inclusion of precision data.

Scope: The International Standard describes a method for the detection of *Listeria monocytogenes* applicable to products intended for human consumption and animal feeds.

Principle: Detection of *L. monocytogenes* is carried out following four successive stages. A primary enrichment is carried out in a selective medium containing one volume of lithium chloride and half a volume of both acriflavine and nalidixic acid (half Fraser broth), which is also used as a diluent for the test portion. The primary enrichment is incubated at 30°C for 24 h. Secondary enrichment is carried out in full strength Fraser broth incubated at 35°C or 37°C for 48 h. Both the primary and secondary enrichment cultures are plated out on to two selective solid media. The first is

Oxford agar and the second PALCAM agar incubated at 30°C, 35°C or 37°C and examination after 24 h, and if necessary after 48 h to check for characteristic colonies.

Presumptive colonies are subcultured, and confirmed by carrying out appropriate morphological, physiological and biochemical tests.

Equipment:

a) Autoclave or oven for dry sterilisation
b) Drying cabinet or incubator (capable of being maintained at 25°C (±1°C) to 50°C (±1°C),
c) Incubator at 25°C (±1°C)
d) Incubator at 30°C (±1°C)
e) Incubator at 35°C (±1°C) or 37°C (±1°C)
f) Water bath at 47°C (±2°C)
g) Loops and wires
h) pH meter
i) Test tubes or flasks
j) Measuring cylinders
k) Pipettes (total delivery 10 ml and 1 ml)
l) Petri dishes
m) Anaerobic jars and gas packs (5–12% carbon dioxide, 5–15% oxygen and 75% nitrogen) - optional
n) Equipment for the Henry Illumination test -optional
o) o) Microscope

Media/reagents:

a) Half Fraser broth
b) Fraser broth
c) Oxford agar
d) PALCAM agar
e) Tryptone soya yeast extract agar (TSYEA)
f) Tryptone soya yeast extract broth (TSYEB)
g) Sheep blood agar
h) Carbohydrate utilization broth (rhamnose and xylose)
i) Motility agar (optional)
j) CAMP medium and test strains
k) Hydrogen peroxide solution
l) Phosphate-buffered saline (PBS)

Sampling: It is Important that the laboratory receives a sample which is truly representative and has not been damaged or changed during transport

The sampling procedure has to take into account the heterogeneity of the production. For this purpose, ISO Standards and Codex Alimentarius General Guidelines on Sampling (CAC/GL 50-2004) should be used as reference methods, as indicated by Regulation (EC) No. 2073/2005. When no information on the structure of the batch is available, the most objective way to draw test units is to give all the test units of the production the same chance to be drawn. The simple random sampling is recommended to estimate the proportion of test units above the limit of 100 CFU/g.

Sample preparation: Prepare the test sample in accordance with the specific International standard appropriate to the product concerned. If there is no specific international standard, it is recommended that the parties concerned come to an agreement on the subject.

The analytical sample size is generally 25 g, and this can be from individual units or as part of a sample composite.

Sample analysis should be initiated as soon as possible upon sample receipt. If sample analysis must be delayed, store frozen samples, frozen, at -20°C (± 5°C); store non-perishable, canned or low-moisture foods at room temperature, and store refrigerated, unfrozen perishable foods at 4 °C (± 2°C) until sample analysis is initiated.

Qualitative detection from foods: 25 g representative portion is blended or stomached and then enrichment is continued as described. Certain foods may require different sample set-up procedures such as soaking and rinsing. A 50g portion of the sample should be reserved for possible pathogen enumeration. Store it at 5° C if it is not frozen or, if frozen, in a non-defrosting freezer. Refer to applicable sampling compliance guidance documents for additional instructions.

Composite sample analysis: Composites may be used to analyse multiple sub units from a single sample. Generally, two composites are prepared from a sample consisting of 10 sub-samples. 50g or ml representative portions from each of the 5 sub-samples are pooled and selective agents is added and then blended or stomached. A 50g portion of this composite blend (equivalent to 25g food plus 25 ml buffered Listeria enrichment broth) is combined with 200 ml of basal buffered Listeria enrichment broth. The composite is then inoculated. An aliquot (100 ml) of the composite blend should be retained, preferably at 5°C and not below 0° C, for possible pathogen enumeration.

Procedure: The initial suspension is prepared by adding an appropriate amount of the sample to the selective primary enrichment medium (half Fraser broth), to obtain a 1:10 ratio sample to medium. The suspension is incubated at 30°C for 24 h (±2 h).

A 0.1 ml aliquot is taken from the primary enrichment and added to 10 ml of the secondary selective enrichment medium, Fraser broth. This is incubated at 35°C or 37°C for 48 h (±2 h).

After the incubation of the primary medium and secondary broths, both cultures are streaked on to separate plates of Oxford and PALCAM -selective plating–out medium, to obtain well separated colonies. This procedure should also be repeated for the second selective solid medium with the two selective plating-out media.

The dishes obtained from the above (Agar plates and the second selective medium plates) are incubated inverted at 30°C, 35°C or 37°C. PALCAM agar plates are incubated either micro-aerobically in a jar containing the gas mixture (see equipment), or aerobically.

After incubation for 24 h and an additional 18-24 h, if growth is weak or no colonies are observed, the dishes/plates are examined for typical Listeria colonies.

On Oxford agar, Listeria spp. are small (1 mm diameter), greyish colonies with black halos after 24 h. After 48 h the colonies become darker with a possible greenish sheen, about 2 mm in diameter, with black halos and sunken centres.

PALCAM plates should be exposed to the air for 1 h if they have been incubated under micro-aerobic conditions, to allow the medium to regain its pink to purple colour. After 24 h, typical Listeria spp. appear as small or very small greyish or olive green colonies, 1.5–2 mm in diameter, sometimes with black centres, but always with black halos. After 48 h, colonies are green, 1.5–2 mm in diameter, with a central depression and surrounded by a black halo.

Five typical colonies should be picked off each plate of each selective media for confirmation tests. Selected colonies are streaked on to TSYEA plates so that well separated colonies develop. The plates are incubated at 35°C or 37°C for 18–24 h.

Cultures are tested for their catalase reaction and a Gram stain is carried out. They are also tested for motility by suspending a colony in a tube containing TSYEB. The tube is incubated at 25°C for 8–24 h until a cloudy medium is observed. The culture is used to prepare a hanging drop and observed under the microscope. *Listeria* spp. appear as slim, short rods with tumbling motility. Motility can also be checked using motility agar. Cultures that are Gram-positive short rods, catalase positive with tumbling motility can be tested further to confirm whether they are *L. monocytogenes*.

A haemolysis test is carried out. Cultures are stabbed into sheep blood agar plates using a wire. After incubation at 35°C or 37°C for 24 h (±2 h), *L. monocytogenes* shows narrow, clear, light zones (β haemolysis). A positive and negative (*L. innocua*) control should be set up to ease interpretation. *L. innocua* shows no clear zone around the stab, *L. seeligeri* shows a weak zone of haemolysis and *L. ivanovii* shows a wide, clearly delineated zone of haemolysis. The haemolytic reaction may also be carried out by dispersing a colony in 150 µl of TSYEB, which is then incubated at 37°C for 2 h. A 150 µl suspension of sheep red blood corpuscles is then added and incubated at 37°C for 15–60 min. The suspension is then kept at 3°C (±2°C) for about 2 h. The tube is examined for haemolysis. If there is no reaction, the tube can be left at 3°C (±2°C) for up to 24 h (±3 h).

Utilization of rhamnose and xylose is tested by inoculating each carbohydrate broth with a culture from TSYEB. The broths are incubated at 35°C or 37°C for up to 5 days. A yellow colour (acid production) indicates a positive reaction and usually occurs mostly within 24–48 h.

The CAMP test is carried out on sheep blood agar using *Staphylococcus aureus* and *Rhodococcus equi*. The test strain is streaked at right angles to streaks of *S. aureus and R. equi*, ensuring that the streaks do not touch. The plates are incubated at 35°C or 37°C for 12–18 h. An enhanced zone of haemolysis at the intersection of the test strain with each of the cultures is a positive reaction. *L. monocytogenes* gives a positive reaction against S. aureus and a negative reaction against *R. equi*.

L. monocytogenes gives a positive haemolysis reaction, produces acid from rhamnose but not from xylose and gives a positive CAMP reaction against *S. aureus* but not against *R equi.*

Expression of results: Presence or absence of *Listeria monocytogenes* in the test portion are reported in grams or the millilitres of the sample tested.

Confirmation of one or more *L. monocytogenes* isolates from an enrichment indicates that *L. monocytogenes* is present at ≥ 1 CFU per sample size analysed or present on environmental swab or sponge sample.

Performance criteria, quality assurance: To check the quality of the enrichment and identification media on selective growth a dilution of the reference culture of recently isolates strains of Listeria and negative control strains in a control flask of the selective primary enrichment

medium is introduced. Add 10-100 *Listeria monocytogenes* cells or negative control strains per flask and proceed with the control flasks as for the test cultures to demonstrate that the positive control is recovered. The International Standards provides information on the control strains that can be used.

Rapid tests: Alternatively, rapid test kits with their respective enrichment media approved as AOAC Official Methods of Analysis (OMA) may be conditionally used to screen for the presence of Listeria contaminants. Putative Listeria isolates on selective agars from standard or screen positive enrichments are purified on non-selective agars and confirmed by conventional identification tests or by a battery of such tests in kit form. Isolates may be rapidly confirmed as *L. monocytogenes* (or not) by using specific test kits or PCR procedures. Subtyping of *L. monocytogenes* isolates is generally expected, which includes serological typing and pulsed-field gel electrophoresis (PFGE).

Various AOAC Official Method rapid screening methods for Listeria spp.in seafood are described; for an overview see e.g. the analytical manual (BAM) of the Food and Drug Administration.

http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm071400.htm).

These tests are based on different methods.

Alternatively, purified isolates may be rapidly identified by using commercial kits or real-time PCR and AOAC rapid methods for the detection of *L. monocytogenes* may be used. Some of these commercial available tests are listed in Table 9.

TABLE 9: COMMERCIAL PRODUCT TESTS FOR *LISTERIA MONOCYTOGENES*

Test	Principle	Approx. Total Test Time	Supplier
API Listeria Requires an additional β-haemolysis test.	Miniature biochemical tests	18-72 hours	bioMerieux www.biomerieux.com
Remel™ Micro-ID™ Listeria Identification System	Reagent impregnated paper disks (15 biochemical tests for differentiation)	24 hours	Thermo Fisher www.thermofisher.com
BAX® Automated System *	DNA-based detection, PCR assay	Next day results	Du Pont www.dupont.com
Real-time PCR, requires an additional β-haemolysis test. CAMP test is optional.	PCR analysis	24 hours	Dupont Qualicon www.dupont.com
The foodproof® Listeria Genus Detection Kits	Real-time PCR		Biotecon Diagnostics www.bc-diagnostics.com
Compact Dry LS	Chromogenic Compact Dry plates	Incubation time 24 ± 2 h at 35 – 37 °C	R-Biopharm www.r-biopharm.com
VIDAS UP Listeria (LPT)	Phage recombinant protein technology a	Next day results – 48 hours ISO, AOAC validated	bioMerieux www.biomerieux-industry.com/food/vidas-detection-listeria-spp

*Also for Salmonella, E. coli, Campylobacter

Source: Internet search, 2016

5 CHEMICAL PARAMETERS

5.1 Introduction

The major chemical hazards with a concern for public health to be controlled and tested chemically in fish and fishery products from the Caribbean region include:

a. Heavy metals, manmade and environmental accumulated in fish
b. Histamines in certain fish species. Histamine fish poisoning results from the consumption of inadequately preserved and improperly refrigerated fish.
c. Marine biotoxins in a range of fishery products, originating from phytoplankton
d. Sulphite salts that are used as a means of controlling melanosis in raw crustaceans such as shrimp and lobster.

For these risks to public health legal maximum limits are set by European Regulations. Batches of fishery products in which the levels of contaminants or residues exceed the maximum limits as indicated, shall be regarded as unfit for human consumption. More information on the nature of these hazards and the applicable limits are set out in the CRFM to Food Safety Hazards in Caribbean Fishery Products.

This section describes the testing methods which must be followed if the results are to be considered valid, and accepted as evidence of compliance. The methods set out also include reference to sampling plans and instructions for sample preparation are specified. Where no specific methods for the determination of contaminants in foodstuffs are prescribed, laboratories may select any validated method of analysis. Where possible, the validation should include a certified reference material.

5.2 Heavy metals

5.2.1 Sampling

Sampling of products should be risk based, with a higher proportion of samples taken from species most susceptible to contamination. Heavy metal content should be recorded with information regarding species, size/age of fish, catch location, and season, to allow the operator to build up a picture of the distribution.

Commission Regulation (EC) No 333/2007 of 28 March 2007 laying down the methods of sampling and analysis for the official control of the levels of lead, cadmium, mercury, inorganic tin, 3-MCPD and benzo(a)pyrene in foodstuffs, covers the methods of sampling and performance criteria for methods of analysis for the official control of the maximum levels of these metals. Sampling plans are also provided as well as the minimum number of incremental samples to be taken from the lot or sub-lot. The weight of an incremental sample should be at least 100 grams or 100 millilitres, resulting in an aggregate sample of at least about 1 kg or 1 litre.

Sampling should be performed by an authorised person, and 10 incremental samples per lot should be taken. Each lot or sublot that is to be examined should be sampled separately. Any changes that would affect the levels of contaminants, adversely affect the analytical determination, or make the aggregate samples unrepresentative, should be avoided and precautions taken. As far as possible, incremental samples should be taken at various places distributed throughout the lot or sublot.

Each sample should be placed in a clean, inert container offering adequate protection from contamination, from loss of analytes by adsorption to the internal wall of the container, and from

damage in transit. All necessary precautions should be taken to avoid any change in composition of the sample which might arise during transportation or storage. Each sample taken for official use should be sealed at the place of sampling. A record should be kept of each sampling, identification, date and place of sampling, together with any additional information likely to be of assistance to the analyst.

5.2.2 Sample preparation procedures

A representative and homogeneous laboratory sample without introducing secondary contamination must be obtained. All of the sample material received by the laboratory should be used for the preparation of the laboratory sample, since compliance with Commission Regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs, should be established on the basis of the levels determined in the laboratory samples.

The fish sample taken for analysis should reflect the portion to be consumed, normally the muscle, separated from any skin and bone. Clean water should be used to wash the sample prior to analysis and blending of the sample such that a representative subsample can be taken. Where the whole fish is consumed (e.g. anchovies, whitebait, etc.), whole fish should be blended together and the analytical subsample taken.

The analyst cuts off the contaminated front of the fish samples and takes 100 g of each incremental sample. 10 x 100 g of sample are homogenized.

For sample preparation of fish, rinse the fish with water. A final rinse with de-ionised water is recommended, rinsing water being removed from the samples (soft tissue etc.). The fish is cut with a ceramic knife, and care is taken during cutting not to damage the abdominal wall, to avoid contamination of the muscles with the guts. Skin and bones are discarded and only the muscles without skin and bones are homogenised. Fish fillets are used in their entirety, while fish species normally intended for eating with bones and skin, e.g. sardines, anchovies, sprats and whitebait, should be blended without de-boning. Frozen fish may be homogenised completely before defrosting. Care should be taken to avoid liquid loss during defrosting. If frozen fish is thawed to room temperature, proceed as for fresh fish. For canned fish, separate fish and other parts of the product if possible, and homogenise the content of the can. For smoked fish, proceed as for fresh fish.

The aggregated sample is made up by uniting all incremental samples and should be at least 1 kg, finely ground (where relevant), and thoroughly mixed, using a process that has been demonstrated to achieve complete homogenisation e.g. by use of a high speed blender.

The analyst should ensure that samples do not become contaminated during sample preparation. Wherever possible, apparatus and equipment coming into contact with the sample should not contain the metals to be determined, and be made of inert materials e.g. plastics such as polypropylene, polytetrafluoroethylene (PTFE) etc. These should be acid cleaned to minimise the risk of contamination. High quality stainless steel may be used for cutting edges. For operations, such as cutting and weighing, special equipment such as ceramic knives, porcelain or quartz spatula, agate grinders, should be used. Pressure digestion vessels should be made of quartz or similar inert material. For cleaning, use a mixture of nitric acid and water. All disposable plastic articles should be rinsed with acid before use.

5.2.3 Cadmium and lead (BS EN 14084:2003)

Foodstuffs. Determination of trace elements. Determination of lead, cadmium, zinc, copper and iron by atomic absorption spectrometry (AAS) after microwave digestion

Scope: The European Standard method is applicable for residues of lead and cadmium (also zinc, copper and iron) in foodstuffs.

Principle: The sample is oxidized by wet ashing in a microwave digester, using hydrogen peroxide and nitric acid. Lead and cadmium are determined by graphite absorption spectrometry, following dilution of the sample extract.

Equipment: All glassware should be carefully cleaned and rinsed, following the procedure in EN 13804[27].

a) Microwave digester complete with digestion vessels
b) Atomic absorption spectrometer (AAS) with graphite furnace, burners for flame analysis, an autosampler, and full data measurement and recording system.
c) Lead lamp
d) Cadmium lamp
e) Graphite tubes
f) Plastic bottles
g) Ceramic knives for cutting the fish
h) Warring Blender (mill/grinder for homogenising samples)
i) Tubes and vials made from PP, PTFE
j) Analytical Balance
k) Knife
l) Chopping Board
m) Sampling Bottle
n) Tin Opener

Reagents:

a) Nitric acid 0.1 mol/litre (7 ml concentrated nitric acid to 1000 ml), and 3 mol/litre (200 ml concentrated acid to 100 ml)
b) Hydrogen peroxide, 30%
c) Lead certified reference standard, 1000 mg/litre
d) Cadmium certified reference standard, 1000 mg/litre

Standard and calibration solutions: Commercial certified reference standards are recommended, rather than the use of standard solutions prepared in-house.

Method: A portion of sample equivalent to between 0.2g and 0.5g of dry fish (1,0–1.5 g wet weight) is digested with 5 ml nitric acid (0.1 mol/litre) and 2 ml hydrogen peroxide (30%) in a sealed vessel in a microwave digestion oven, using the conditions (power/time) recommended by

[27] BS EN 13804:2013: Foodstuffs. Determination of elements and their chemical species. General considerations and specific requirements

the manufacturer for the particular system in use (generally between 20 min and 30 min). After cooling, the sample is measured and made up to volume (minimum 25 ml) with distilled water.

The metal content in the sample is determined by AAS using flame or graphite furnace mode, depending upon the metal concentrations present. The graphite furnace technique is generally used for cadmium and lead. Metal content is calculated from a calibration curve, using a minimum of three calibration standards, of which at least two should be addition standards (a standard in the presence of sample matrix at the same concentration as in the test sample). The sample extract should be diluted as necessary.

A sample blank must be run on each occasion. The method should be read in conjunction with BS EN 13804 (see footnote page 59).

Performance criteria: General guidance with regard to performance criteria (i.e. applicability, precision, specificity, trueness and limit of quantification) and sample preparation are reviewed in BS EN 13804.

The criteria for performing the method follow standard provisions, using a validated procedure for which performance data are available from published reports of inter-laboratory testing.

The veracity of the procedure is confirmed by testing a certified reference material. The specificity is confirmed by the absence of interfering materials, when testing samples and reagents without residues are present. Precision is generally accepted as Horwitz ratio (HorRat) *R* index ≤2, and the limit of quantification, established practically by the laboratory, should be appropriate for testing at and below the specified acceptable residue limits.

Performance criteria for methods of analysis for lead and cadmium are provided for applicability, limit of detection, limit of quantification, precision, recovery and specificity. The laboratory should participate in appropriate proficiency testing schemes.

Expression of results: Compliance with maximum levels laid down in Regulation (EC) No 1881/2006[28] should be established based on the levels determined in the laboratory samples. If no extraction step is applied in the analytical method (e.g. in case of metals), the result may be reported uncorrected for recovery, as long as evidence is provided (by using suitable certified reference material) that the certified concentration allowing for the measurement uncertainty is achieved

The analytical result should be reported with measurement uncertainty (MU), as: x +/– U,

where x is the analytical result, and U is the expanded measurement uncertainty, using a coverage factor of 2, giving a level of confidence of approximately 95 % (U = 2u).

5.2.4 Mercury (BS EN 13806:2002)

Foodstuffs. Determination of trace elements. Determination of mercury by cold-vapour atomic absorption spectrometry (CVAAS) after pressure digestion.

The method is based on cold-vapour atomic absorption spectrometry (CVAAS) after pressure digestion.

Scope: The method is specifically for the determination of residues of mercury in foodstuffs.

[28] Commission Regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs

Principle: Test solutions are prepared by pressure digestion of the sample using the procedure of BS EN 13805:2014[29], and residues determined using cold-vapour AAS after reduction of the mercury with sodium borohydride or divalent tin, and measurement of the mercury content of the vapour as it is flushed into the measuring cuvette of the spectrometer.

Equipment:

a) Pressure digester with quartz glass sample holders although PTFE can be used

b) Atomic absorption spectrometer (AAS) with hydride generator, accessories for the cold vapour technique and appropriate data measurement and recording systems

c) Mercury lamp

Reagents:

a) Hydrochloric acid, concentrated

b) Nitric acid, concentrated

c) Stannous chloride (SnCl2.2H20), (50g in 100 ml of concentrated hydrochloric acid diluted to 500 ml with water)

d) Sodium borohydride (3g plus 1 g of sodium hydroxide to 100 ml in water)

e) Potassium permanganate (0.4g in water, diluted to 10 ml; note that this reagent should be prepared fresh each working day)

f) Potassium dichromate solution (5g in 500 ml of concentrated nitric acid and diluted to 1 litre with water).

g) Sodium hydroxide

h) Mercury reference standard solution, 1000mg/litre (a certified commercial reference standard is recommended)

i) Zero member compensation solution

Method: Sample capacity and operation (temperature/pressure) depend on the design of the digester, but generally a sample of 1–1.5g is digested with 3 ml of nitric acid. Digestion time may be up to 3 h, but this is significantly reduced in a microwave system (maximum normally 30 min). With pressure digestion, common conditions are for the sample to be heated to 150°C over a period of 60 min, then the temperature raised to 300°C in 40 min, and kept at 300°C for a further 60 min. In a microwave system, digestion is started at low power, and the power then raised in stages, with 5-min holding periods, to 1000 W and held for a further 10 min.

Mercury residues in the digested sample are reduced, using a stannic chloride/hydrochloric acid solution or sodium borohydride (3% in 1% sodium hydroxide), and then the mercury vapour is flushed into the measuring cuvette of the AAS, where mercury residues are measured at 253.7 nm. For operating procedures, consult the manual for the specific hydride generator in use.

The international standard gives examples for CVAAS determination and for background correction.

[29] Foodstuffs. Determination of trace elements. Pressure digestion

As an analytical quality control reference samples having reliable known mercury contents should be analysed in parallel with testing samples at all steps in the method. Blank solutions should also be determined.

Calculate the mass fraction of mercury, w, in milligrams per kilogram of sample by using the equation: $w = \frac{a \times V}{V1 \times m \times 1000}$

Where a is the absolute mass of mercury, in nanograms found in the test solution used; V I the volume of the digestion solution after being made up in millilitres; V1 is the volume of test solution used in millilitres, and m is the initial sample mass in grams.

If necessary, subtract the result of the blank solution from the content of mercury.

The limit of quantification for a test portion of 0.5g in a final digestion volume of 20 ml is between 0.002 and 0.2mg/kg.

Performance criteria: General guidance with regard to performance criteria (i.e. applicability, precision, specificity, trueness and limit of quantification) and sample preparation are reviewed in BS EN 13806:2002.

The criteria for performing the method follow standard provisions using a validated procedure where performance data are available from published reports of inter-laboratory testing.

Precision, repeatability and reproducibility of the method should be established and confirmed by an interlaboratory comparison, with results provided by the standard. Precision is generally accepted as Horwitz ratio (HorRat) *R* index ≤2, and the limit of quantification, established practically by the laboratory, should be appropriate for testing at and below the specified acceptable residue limits.

Performance criteria for methods of analysis for lead, cadmium, mercury are provided for calculation of applicability, limit of detection, limit of quantification, precision, recovery and specificity. Laboratories should participate in appropriate proficiency testing schemes, and apply quality control, such as quality control charts.

Expression of result, performance criteria and quality assurance as see above.

Reference: BS EN 13804:2013: Foodstuffs. Determination of elements and their chemical species. General considerations and specific requirements. British Standards Institution.

5.3 Histamine

According to Commission Regulation (EC) No. 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs, histamine assays (in fish and fishery products) must be conducted by reliable methods, such as the HPLC method, enabling quantitative determination of biogenic amines following descriptions by AOAC publications[30]: The EU reference method for sampling and determination of histamine in fish and fishery products is described here, followed by a description of the rapid test methods available for screening or HACCP plan implementation.

[30] Malle, P.,Valle, M. and Bouquelet, S. (1996) Assay of biogenic amines involved in fish decomposition. Journal of the Association of Official Analytical Chemists International 79(1): 43–49 and Duflos G., Dervin C., Malle P., Bouquelet S. Relevance of matrix effect in determination of biogenic amines in plaice (*Pleuronectes platessa*) and whiting (*Merlangus merlangus*). J. AOAC Internat. 1999, 82, 1097-1101

5.3.1 Sampling

In Regulation 2073/2005 for histamine analysis, a 3 –class-sampling plan applies. The normal sampling plan for histamine from fishery products consists of nine samples to be taken, in which the average histamine content must be 100mg/kg or less. No more than 2 samples may have levels between 100mg and 200mg/kg, and no sample may have a level above 200mg/kg.

These limits apply to fish from the following families only:

- *Scombridae*: tuna, mackerel, bonito, etc.
- *Clupeidae:* herring sardines, etc.
- *Coryphaenidae*: anchovies
- *Pomatomidae*
- *Scomberedocidae.*

A consignment of fishery products comprising a fish species susceptible to the production of histamine, should not be placed on the market if the level of histamine in nine samples selected at random from the consignment, exceeds the minimum levels specified below.
Fish from these families, which have undergone enzyme-ripening treatment in brine, are permitted higher histamine levels, but not more than twice the above values.

Maximum level of histamine in fish sauce produced by fermentation of fishery products is established at 400 mg/kg, and a new single sample sampling plan should be applied for histamine in fish sauce placed on the market during its shelf-life. If the single samples are found to contain more than 400 mg/kg the whole batch should be deemed unsafe.

5.3.2 Sample preparation

In trace element analysis, only the part intended for eating should be tested, and results are related to this part. For preparation of the test sample, 200g should be available from the eatable part of the laboratory sample.

The fish is rinsed with water. A final rinse with de-ionised water is recommended, and rinsing water removed from the samples (soft tissue etc.). Care should be taken during cutting so as not to damage the abdominal wall to avoid contamination of the muscles with the guts. Cut the fish with a ceramic knife.

Homogenise only the muscles without skin and bones. Fish fillets should be used in their entirety. Fish species normally intended for eating with bones and skin, e.g. sardines, anchovies, sprats and whitebait, should be blended without de-boning.

For canned fish, separate fish and other parts of the product if possible, and homogenise the contents of the can. For smoked fish, proceed as for fresh fish.

Frozen fish may be homogenised completely before defrosting. Care should be taken to avoid liquid loss during defrosting.

Fully homogenise the test material by use of a high-speed blender.

Weigh exactly 5.0g of test material into a centrifugation tube, and cool in crushed ice until the test material reaches approx. 4°C. Add 10 ml perchloric acid previously cooled to 2°C, then add 100 µl of 1,3-diaminopropane solution and blend for 1 minute. Centrifuge with 12000g at 4°C for 5 min.

Pipette 100 µl of the supernatant into a glass vial, add 300 µl of Na2CO3 solution and 400 µl of dansylchloride solution and close the tube tight. Swirl and incubate for 5 min at 60°C in the dark. Cool tube to room temperature and add 100 µl proline solution. Swirl and place the tube in the

dark for 15 min. Add 500 µl toluene, swirl again and keep in the freezer at -18°C for at least 30 min.

Collect all the non-frozen, organic substance in a new tube, and evaporate the solvent under nitrogen flow at room temperature. Re-dissolve the evaporated residue in 200µl acetonitrile and swirl well. Filter through inline syringe filter and inject 10 µL into the HPLC.

Standard derivatization: Add 1 ml of each of the dilute working standards to separate, labelled 5 ml volumetric flasks, and then proceed as above, without the addition of the 1 ml of sample extract.

5.3.3 Determination of histamine by HPLC-UV

Scope: The protocol describes the determination of histamine in fish (fresh tuna, herring, and canned tuna) in a working range up to 400 mg/kg with UV-HPLC. The procedure is suitable for the determination of histamine in fresh, frozen, canned, and smoked fish.

For further information, the following references should be consulted:

- Histamine in seafood: AOAC Official Method 977.13 – Fluorometric method
- Equivalence testing of histamine methods. Final Report, Stroka et al. (2014) Publications Office of the European Union

  https://ec.europa.eu/jrc/en/publication/eur-scientific-and-technical-research-reports/equivalence-testing-histamine-methods-final-report.

Principle: Histamine is extracted from fish tissue after acid precipitation of the insoluble protein, derivatized with dansyl chloride and then determined using HPLC with a UV/visible wavelength detector.

The test samples are extracted with perchloric acid (0.2M) and derivatised with dansyl chloride. Surplus derivatisation agent is neutralized with proline. Histamine is separated on a reversed phase C18 column using a water/acetonitrile gradient. Histamine is identified by retention time as a baseline resolved peak. Quantification is carried out by UV absorption at 254 nm against an internal standard (1,3 dihydrochloride diaminopropane).

Equipment:

a) High speed blender (e.g. Ultra Turrax)
b) Analytical and laboratory balances (0.01g and 0.0001g resolution)
c) Centrifuge tubes with screw caps
d) Pipettes of various volumes: f.i. 20-200 µl and 100-1000 µl
e) Refrigerated centrifuge
f) Glass tubes of 10 ml
g) Vortex mixer
h) Water bath suitable for maintaining a temperature of 60°C (±1°C)
i) Laboratory freezer capable of maintaining -18°C (±5°C)
j) Evaporation block
k) Syringes (2 ml)
l) Syringe needles (20G, 0.9 mm)
m) Luer lock filters 0.2 µm

n) Flask, vial, insert, cap

o) HPLC system (injector, pump, UV-detector)

p) C18 chromatography column, 5µm, inside diameter 4.6 mm, length 250mm (or Agilent ZORBAX SB-C18, 3.5 µm particle, 4.6 x 150mm)

Reagents and Standards:
All reagents are of analytical grade unless stated differently

a) Water (ISO grade)

b) Perchloric acid 65%

c) Perchloric acid solution 0.2 M: either dilute 19.5 ml (65%

d) HClO4) or 17.2 ml (70% HClO4) to 1 litre with water.

e) Acetone

f) Acetonitrile (HPLC grade)

g) 1,3-diaminopropane dihydrochloride

h) 1,3-diaminopropane solution (0.8 mg/ml): dissolve 40.0 mg in 50 ml water. The solution is stable for 3 weeks at 5±3°C.

i) Sodium carbonate solution (Na2CO3): dissolve 110g (accuracy 0.1g) until complete saturation in approximately 150ml Millipore® water.

j) Dansylchloride

k) Dansyl chloride solution (7.5 mg/ml): dissolve 375.0 mg dansylchloride in 50 ml of acetone. The solution can be stored in the dark at -18°C for 3 weeks.

l) L-Proline

m) L-proline solution (100 mg/ml): dissolve 1.000g proline in 10 ml of water. The solution can be stored at 5±3°C for 3 weeks.

n) Toluene

o) Histamindichloride

Chromatographic conditions (HPLC separation):
Flow rate: 1.4 mL/min
Column temperature: 45°C
Chromatographic conditions: A 250 × 4.6 mm, 5 µm Luna C18 column is installed in the HPLC with a 4 × 3 mm Securiguard cartridge
Wavelength UV/visible detector: 254 nm
Pump is programmed for an acetonitrile/water gradient at 1 ml/min as below:

Gradient elution (Malle et. al)

Time (min)	Water (%)	Acetonitrile (%)
0	40	60
6	25	75
8	25	75
13	25	75
20	5	95
20.01	40	60
30	40	60

The above conditions are a guide, and adjustments may be necessary to optimize separation of the derivatized histamine from other amines.

Calibration curve: A five-point calibration curve is constructed, plotting histamine content (x axis) against response (y axis).

Calculation: histamine content, mg/kg = $\frac{C \times 25}{W}$

where C is the histamine content read from the calibration curve, and W is the weight of the sample (g).

Validation samples: Treat replicate samples of homogenized fish tissue with (i) 0.1 ml of the histamine stock solution and (ii), 0.5 ml of the histamine stock solution, giving treatment levels equivalent to 20 and 100 µg/g, respectively. Use the above method to extract, derivatize and analyse the spiked samples. Calculate the percentage of recovered histamine.

Note: Malle et al. (1996) report that the use of a cryostat to cool the sample to -20°C during extraction improves sample stability.

5.3.4 Determination of histamine by Rapid tests:

Histamine can be measured in a number of ways, including the use of test kits, ELISA, a chemical fluorescent procedure and HPLC.

Histamine test kits may be qualitative, semi-quantitative, or fully quantitative. They come with an easy-to-follow supplier's instructions. It is recommended to use validated tests and to verify them for the use at the laboratory in official controls. In the Guide to Food Safety Hazards in Caribbean Fishery Products, a selection of some of the commercial testing systems available for rapid testing of histamine in fishery products is provided.

5.4 Sulphur dioxide and sulphite residues

5.4.1 Sampling

Sampling should be carried out, based on an agreement on this subject by the parties concerned.

Prepare the sample and weigh a portion into the distillation flask. The quantity of sample will vary with the expected level of sulphur dioxide as follows in Table 10:

TABLE 10: EXPECTED SULPHUR DIOXIDE (PPM) LEVEL

Expected sulphur dioxide (ppm)	Sample weight (g)	Added water (ml)
10	50	20
10–100	25	30
100	10	40

When the sample has been added, add the appropriate volume of distilled water as given above. Add 50 ml of methanol and thoroughly mix by swirling.

5.4.2 Official method for determination of sulphite residues (BS EN 1988-1:1998)

The EU recommended procedure is a European Standard procedure.

Reference: Foodstuffs – Determination of Sulfite – Part 1: Optimized Monier-Williams Method. BS EN 1988-1: 1998. British Standards Institution. A summary of the method is provided here.

Scope: The procedure allows for the determination of sulphite as sulphur dioxide in a wide range of foodstuffs at concentrations greater than 10 mg/kg.

Principle: Sulphite residues are converted to sulphur dioxide by refluxing with hydrochloric acid. The sulphur dioxide is distilled, in a flow of nitrogen, and trapped in hydrogen peroxide solution. The sulphuric acid thus produced is determined by titration with sodium hydroxide solution.

Equipment:

a) Distillation apparatus for optimized Monier-Williams method (commercially available)
b) Burette, 10 ml capacity, designed to maintain carbon dioxide free atmosphere over the contents (sodium hydroxide)
c) Chilled water circulator
d) Heating mantle, 1 litre capacity
e) Gas regulator for nitrogen supply, with oxygen absorbing trap

Reagents:

a) Hydrochloric acid, 4 mol/litre
b) Hydrogen peroxide solution
c) Sodium hydroxide solution, 0.010 mol/litre, standardized
d) Methyl red indicator, 0.25% in ethanol
e) Ethanol
f) Ethanol/water mixture, 5+95
g) Water, distilled
h) Nitrogen gas, high purity

Sampling: Sampling should be carried out based on an agreement by the parties concerned.

Sample Preparation: Homogenize 50g of sample with 100 ml of the ethanol/water mixture, until the sample is reduced sufficiently to pass through the neck of a round-bottomed flask.

Method: Assemble the apparatus (see Figure 1), add 400 ml of water to the round-bottomed flask through the dropping funnel, and close the funnel tap.

Pour 90 ml of hydrochloric acid solution into the dropping funnel. Add 30 ml of hydrogen peroxide solution to the receiver tube/flask. Apply nitrogen to the system at a rate of 200 ml/s and allow the system to purge for 15 min.

Add the macerated, aqueous sample to the distillation flask, reassemble the apparatus, and ensure that all joints are sealed. Use grease if necessary around the outside of the joints.

Open the tap of the dropping funnel to allow the acid to enter the distillation flask, using pressure (if necessary), applied through the adapter attached to the funnel. Do not drain the acid entirely as this acts as a liquid barrier to prevent the escape of any liberated sulphur dioxide. Ensure that the coolant is flowing through the condenser, then apply heat to the distillation flask and boil the contents at a steady rate for 105–110 min.

Disconnect the receiver flask/tube, and immediately titrate with the standardized sodium hydroxide solution, using the methyl red indicator until an endpoint is reached where the yellow colour lasts for longer than 20 s.

Expression of results: Calculation: The concentration of sulphur dioxide, in mg/kg, is calculated using:

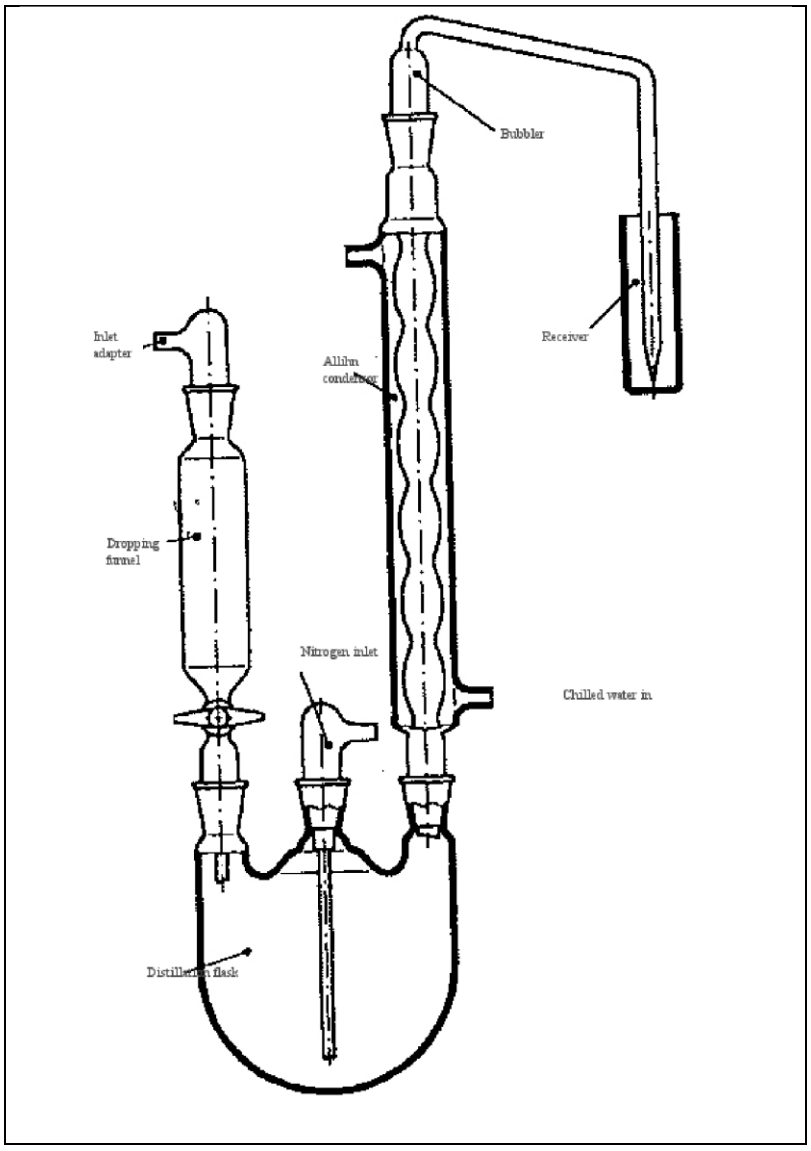
$$\text{mg/kg} = \frac{32.03 \times V \times N \times 1000}{\text{Sample weight}}$$

where 32.03 is the milliequivalent weight of sulphur dioxide in g/mol, N is the molarity of the sodium hydroxide solution (mol/litre), V is the volume of sodium hydroxide used in the titration, and 1000 is a factor to convert milliequivalents to microequivalents.

Quality assurance: Prepare a reagent blank and run the test again, titrating the distillate and correcting the sample test result as appropriate.

Based on an inter-laboratory test conducted by the US Food and Drug Administration in 1986, for a seafood sample with a determined mean value of 10.41mg/kg sulphur dioxide, the repeatability limit was calculated to be 4.12mg/kg and the reproducibility limit to be 7.8mg/kg.

FIGURE 1: MONIER-WILLIAMS APPARATUS FOR SULPHITE RESIDUES



5.5 Shellfish poisons (PSP, ASP, DSP)

Food business operators must ensure that live bivalve molluscs, and other shellfish (including marine gastropod molluscs), placed on the market for human consumption, do not contain marine biotoxins in total quantities (measured in the whole body or any part edible separately) that exceed the regulatory limits. These are set out in the CRFM Guide to Food Safety Hazards in Caribbean Fishery Products.

European Commission Regulation (EC) No. 2074/2005 of 5 December 2005 specifying implementing measures for certain products, states that CAs should use the biological (mouse) testing method to detect for paralytic shellfish poisoning (PSP). The reference method for the domoic acid group (the causative agent of Amnesic shellfish poisoning), is based on high-performance liquid chromatography (HPLC). The mouse bioassay (MBA) is the reference method for lipophilic toxins. Alternative procedures based on HPLC could be used as alternative or supplementary test procedures, but these can only be applied if they are validated following an internationally recognised protocol to provide an equivalent level of public health protection. There are no validated rapid methods suitable for dockside, nor commercial testing of lots of fish for any of these toxins.

5.5.1 Sampling and sample preparation

Shellfish samples should be collected from identified harvest sites at the stated frequency. The sites and collection frequency may change depending upon the prevalence of algal toxins but any changes must be notified beforehand. Ideally, shellfish samples should be collected twice a week

Shellfish sample size should be such that at least 200g of meat can be provided for the Amnesic shellfish poisoning (ASP), Diarrheic shellfish poisoning (DSP) and PSP assays.

Shellfish must be placed in polythene bags and the bags tied, with an air space above the shellfish to allow the animals to breathe. The sample submission form should be completed noting date of collection, site identification number and production area, name of collector, state of tide, water temperature, weather conditions etc. The bagged sample and form should then be placed in the container provided, along with frozen cool packs and foam. The box must be sealed with adhesive tape.

5.5.2 Determination of Paralytic shellfish poison

The mouse bioassay has historically been the most universally applied technique for examining shellfish (especially for PSP). Unfortunately, the dose-survival times for the DSP toxins in the mouse assay fluctuate considerably, and fatty acids interfere with the assay, giving false-positive results. In recent years, considerable effort has been applied to development of chemical assays to replace these bioassays.

A HPLC procedure has been developed to identify individual PSP toxins (detection limit for saxitoxin = 20 fg/100 g of meats, 0.2 ppm (known as the Lawrence method,[31] as published in AOAC Official Method 2005.06). This is defined as a permitted reference method by Commission Regulation (EC) No 1664/2006 of 6 November 2006 amending Regulation (EC) No 2074/2005, regarding implementing measures for certain products of animal origin intended for human consumption, and repealing other implementing measures.

5.5.2.1 Determination by mouse bioassay

Scope: This test method details the procedure for the determination of PSP toxicity in molluscan shellfish using the MBA. The specificity of this method is high. The use of hydrochloric acid and subsequent boiling ensures the complete extraction of analysis of PSP toxins.

Note that: (i) the high salt content of a sample suppresses the toxic effect of PSP toxins on mice, as it also reacts with the nerve impulses of mice. Zinc has also been shown to lead to death in mice for PSP levels that have no health threats to humans. (ii) When the analysis of the sample is not possible on the day of receipt, the sample may be stored either whole or homogenized by refrigeration at 4°C (±3°C), or frozen below -20°C overnight or over the weekend.

Principle: The acute toxic effect of a hydrochloric acid extract of a shellfish sample injected intraperitoneally into an albino mouse is assessed. The calculation of toxicity is based upon the death time of the mice injected. This is followed by converting the time to toxicity in mouse units (MU) using Sommer's table (Table 11).

[31] Journal of AOAC INTERNATIONAL *(Vol. 88, No. 6),* "Quantitative Determination of Paralytic Shellfish Poisoning Toxins in Shellfish Using Pre-chromatographic Oxidation and Liquid Chromatography with Fluorescence Detection"

Equipment:

a) Shucking knife

b) No. 10 sieve

c) pH paper in increments of 0.5 pH units

d) Disposable hypodermic 26 gauge sterile syringe, 1 ml, with needles

e) Reagents/materials

f) Hydrochloric acid concentrated GPR grade

g) Hydrochloric acid (0.1 N)

h) Hydrochloric acid (5 N)

i) Deionized water

j) 0.1 N sodium hydroxide

k) Certified calibration solution of saxitoxin (STX)

Male albino mice from a standard stock colony (of the same strain), weighing 20 (±1) g. Mice should be weighed to two decimal places immediately prior to injection.

Sample Preparation: Once the shellfish have been removed from the shells, the bulk sample is thoroughly homogenized using a high speed blender.

Extraction: Weigh 100 + 1 g of the tissue homogenate into a 600 ml beaker. Add 100 ml of 0.1 N hydrochloric acid from a measuring cylinder, stir and check pH with a pH test paper; the pH should be <4.0.

Heat the mixture to boiling with constant stirring, boil gently for 5 min then remove from the heat and cool to room temperature.

Adjust pH to 2–4 with 5 N hydrochloric acid or 0.1 N sodium hydroxide. Add distilled water to make up weight to 200 g with constant stirring to prevent local alkalinization and consequent destruction or conversion of PSP toxins.

Allow the homogenate to settle and centrifuge 5 ml of supernatant at about 4°C for 5 min at 3500 rpm.

Procedure:

Mouse bioassay: Albino male mice weighing 20 (± 1) g should be used for the assay. Mice should be acclimatised in the test area for a minimum of 8 h prior to injection, under controlled environmental conditions.

All mice should be examined prior to inoculation to establish the health of the animals, and recorded on the relevant worksheet. This should include observation of:

- Activity in cage (i.e. not listless or fatigued)
- The coat should be smooth and the eyes should be bright
- There should be no signs of discharge from the nose, mouth, genitals or anus
- There should be no signs of injury or bite marks on the back, flanks, legs, head or ventral surfaces.

Prepare the injection by drawing 1 ml of the centrifuged extract into a 1 ml sterile hypodermic syringe. Ensure that the needle does not come into contact with the beaker during filling as this may dull the point of the needle and cause trauma during injection.

Inject 1 ml intraperitoneally into each of three mice. Only operators who are trained to carry out such procedures should carry out these injections.

Note the time of inoculation and the time of death (last gasping breath) by a stop clock. If median death time is less than 5 min, prepare dilutions of the sample extract to obtain a death time of 5–7 min. Three mice should be used per sample if the death time is more than 7 min. If large dilutions are needed, the pH should be adjusted drop-wise using dilute hydrochloric acid to a pH 2– 4 (never above 4.5).

The correction factor (CF) should be calculated once a week (when the samples are being analysed) using five mice. Mice are injected with a known concentration of STX to cause them to die within 5–7 min. The deaths and observations are recorded. The CF (from Table 11) is applied and the CF is then calculated accordingly.

The mice are observed for 60 min and the mean death time is noted, and observations are recorded.

The death time of each mouse is converted into MU using Sommer's table (see Table 12). The following formula is used to calculate the PSP toxicity of the sample:

μg STX_{eq}/100 g flesh = median corrected MU × CF × dilution factor × 200

The death time of mice surviving beyond 60 min is considered to be <0.875 MU.

TABLE 11: CORRECTION TABLE FOR WEIGHT OF MICE

Weight of mice (g)	Mouse unit factor (MU)	Weight of mice (g)	Mouse unit factor (MU)
10.00	0.500	19.10	0.973
11.00	0.560	19.20	0.976
12.00	0.620	19.30	0.979
13.00	0.675	19.40	0.982
14.00	0.730	19.50	0.985
14.50	0.760	19.60	0.988
15.00	0.785	19.70	0.991
15.50	0.810	19.80	0.994
16.00	0.840	19.90	0.997
16.50	0.860	20.00	1.000
17.00	0.880	20.10	1.003
17.10	0.885	20.20	1.006
17.20	0.890	20.30	1.009
17.30	0.895	20.40	1.012
17.40	0.900	20.50	1.015
17.50	0.905	20.60	1.018
17.60	0.910	20.70	1.021

17.70	0.915	20.80	1.024
17.80	0.920	20.90	1.027
17.90	0.925	**21.00**	1.030
18.00	0.930	21.10	1.032
18.10	0.934	21.20	1.034
18.20	0.938	21.30	1.036
18.30	0.942	21.40	1.038
18.40	0.946	21.50	1.040
18.50	0.950	21.60	1.042
18.60	0.954	21.70	1.044
18.70	0.958	21.80	1.046
18.80	0.962	21.90	1.048
18.90	0.966	22.00	1.050
19.00	0.970	23.00	1.070

Source: Table 95908B of the AOAC MBS method of 2000

TABLE 12: SOMMER'S TABLE RELATING MOUSE DEATH TIMES TO TOXICITY UNITS

Death time (minutes)	Mouse units (MU)	Death time (minutes)	Mouse units (MU)	Death time (minutes)	Mouse units (MU)
1:00	100.0	**4:00**	2.500	5:25	1.770
1:10	66.20	4:05	2.440	5:26	1.764
1:15	38.30	4:10	2.380	5:27	1.758
1:20	26.40	4:15	2.320	5:28	1.752
1:25	20.70	4:20	2.260	5:29	1.746
1:30	16.50	4:25	2.210	5:30	1.740
1:35	13.90	4:30	2.160	5:31	1.735
1:40	11.90	4:35	2.120	5:32	1.730
1:45	10.40	4:40	2.080	5:33	1.725
1:50	9.330	4:45	2.040	5:34	1.720
1:55	8.420	4:50	2.000	5:35	1.715
		4:55	1.960	5:36	1.710
2:00	7.670			5:37	1.705
2:05	7.040	**5:00**	1.920	5:38	1.700
2:10	6.520	5:01	1.914	5:39	1.695
2:15	6.060	5:02	1.908	5:40	1.690

2:20	5.660	5:03	1.902	5:41	1.686
2:25	5.320	5:04	1.896	5:42	1.682
2:30	5.000	5:05	1.890	5:43	1.678
2:35	4.730	5:06	1.884	5:44	1.674
2:40	4.480	5:07	1.878	5:45	1.670
2:45	4.260	5:08	1.872	5:46	1.664
2:50	4.060	5:09	1.866	5:47	1.658
2:55	3.880	5:10	1.860	5:48	1.652
		5:11	1.854	5:49	1.646
3:00	3.700	5:12	1.848	5:50	1.640
3:05	3.570	5:13	1.842	5:51	1.636
3:10	3.430	5:14	1.836	5:52	1.632
3:15	3.310	5:15	1.830	5:53	1.628
3:20	3.190	5:16	1.824	5: 54	1.624
3:25	3.080	5:17	1.818	5:55	1.620
3:30	2.980	5:18	1.812	5:56	1.616
3:35	2.880	5:19	1.806	5:57	1.612
3:40	2.790	5:20	1.800	5:58	1.608
3:45	2.710	5:21	1.794	5:59	1.604
3:50	2.630	5:22	1.788		
3:55	2.560	5:23	1.782	**6:00**	1.600
		5:24	1.776		
6:01	1.596	6:39	1.450	**11:00**	1.075
6:02	1.592	6:40	1.447	11:30	1.060
6:03	1.588	6:41	1.443		
6:04	1.584	6:42	1.440	**12:00**	1.050
6:05	1.580	6:43	1.437		
6:06	1.576	6:44	1.433	**13:00**	1.030
6:07	1.572	6:45	1.430		
6:08	1.568	6:46	1.427	**14:00**	1.015
6:09	1.564	6:47	1.425		
6:10	1.560	6:48	1.422	**15:00**	1.000
6:11	1.556	6:49	1.419		
6:12	1.552	6:50	1.417	**16:00**	0.990
6:13	1.548	6:51	1.414		

6:14	1.544	6:52	1.411	**17:00**	0.980
6:15	1.540	6:53	1.409		
6:16	1.536	6:54	1.406	**18:00**	0.972
6:17	1.532	6:55	1.403		
6:18	1.528	6:56	1.401	**19:00**	0.965
6:19	1.524	6:57	1.398		
6:20	1.520	6:58	1.395	**20:00**	0.960
6:21	1.516	6:59	1.393		
6:22	1.512			**21:00**	0.954
6:23	1.508	**7:00**	1.390		
6:24	1.504	7:15	1.350	**22:00**	0.948
6:25	1.500	7:30	1.310		
6:26	1.496	7:45	1.280	**23:00**	0.942
6:27	1.492				
6:28	1.488	**8:00**	1.250	**24:00**	0.937
6:29	1.484	8:15	1.220		
6:30	1.480	8:30	1.200	**25:00**	0.934
6:31	1.477	8:45	1.180		
6:32	1.473			**30:00**	0.917
6:33	1.470	**9:00**	1.160		
6:34	1.467	9:30	1.130	**40:00**	0.898
6:35	1.463				
6:36	1.460	**10:00**	1.110	**60:00**	0.875
6:37	1.457	10:30	1.090		
6:38	1.453				

For the evaluation of toxicity, after the conversion using Sommer's table, a value of 80 µg STXeq /100 g tissue is normally accepted as the permissible level of PSP toxin concentration. Values in shellfish greater than this are considered hazardous and such shellfish are not suitable for human consumption.

Quality control: The mice used in the CF calculation should be treated the same as mice used for test samples. After 5–7 min the mice are observed. Any deaths and observations are recorded. The CF is then calculated accordingly. The CF should be within ± 20% of the value of the first CF.

Reference: (1995) Cunniff, P.A. (ed.) Paralytic shellfish poison: Biological method. Sec. 35.1.37, Method 959.08. In Official Methods of Analysis of AOAC International, 16th ed., pp. 22–23. AOAC International, Gaithersburg, MD.

5.5.2.2 Determination by HPLC (Lawrence Method)

Scope: This test method details the procedure for the determination of PSP toxicity caused by SXT and analogues in molluscan shellfish by HPLC with fluorescence detection (HPLC-FD). The procedure has a high specificity.

Principle: The determination of STX and its analogues/derivatives is based on the HPLC method of Lawrence *et al.* (2005). The toxins are extracted by heating the sample with dilute acetic acid solution, then cleaned using solid phase extraction (SPE) and after periodate and peroxide oxidation, analysed by HPLC-FD. Most toxins (STX, dcSTX, C1, 2, B1 and GTX2, 3) can be quantified after simple SPE C18 clean-up. Extracts containing the toxins NEO, GTX1, 4 and B2 must be further purified by using Solid Phase Extraction Purification of Carboxylic Acid Products (SPE COOH). Toxin content is calculated by the comparison of peak areas in the test sample with the responses given by solutions of certified reference standards.

Note that when analysis of the sample is not possible on the day of receipt, the sample may be stored either whole or homogenized by refrigeration at 4 (□3)□C; or frozen below -20°C overnight or over the weekend.

Equipment:

a) No. 10 sieve
b) Graduated centrifuge tube (50 ml)
c) Parafilm
d) Ultra-Turrax® disperser with 8 mm diameter shaft head
e) Solid phase extraction (SPE) extraction manifold
f) Sonicator
g) HPLC with C18 column (15 cm long × 4.6 mm i.d. packed with 5–10 µm bonded silica gel with guard column)
h) HPLC vials, amber with screw cap

Reagents:

a) Glacial acetic acid AR grade (99% purity)

b) Sodium chloride, 0.3 M and 0.05 M

c) Ammonium formate, 0.3 M

d) Periodic acid AR grade, 0.03 M

e) Ammonium acetate, 0.01 M

f) Acetonitrile

g) Methanol

h) Hydrogen peroxide, 30%

i) Acetic acid, 1%

j) Disodium hydrogen phosphate, 0.3 M

k) Periodate oxidizing reagent: 5 ml 0.03 M periodic acid + 5 ml 0.3 M ammonium formate + 5 ml disodium hydrogen phosphate. Mix thoroughly and adjust the pH to 8.2 by the addition of 1 M sodium hydroxide (µl quantities) with mixing. Keep refrigerated (4°C ± 1°C); the reagent must be used within 8 h of preparation

l) Peroxide oxidizing reagent: 8.33 ml of hydrogen peroxide to 25 ml with deionized water; mix thoroughly. Keep refrigerated (4°C ± 1°C); the reagent must be used within 8 h of preparation

m) Carboxyl weak cation exchange SPE cartridges 0.5g/3 ml

n) C18 SPE cartridges, 0.5 g, 6 ml

o) Certified PSP stock and working calibration standards

p) Deionized water

Sample preparation: Once the shellfish have been removed from the shells, the bulk sample is thoroughly homogenized using a high speed blender. Add 2.5 (±0.05) g of homogenized shellfish to a 50ml centrifuge tube. Add 1.5 ml of 1% acetic acid to the tube, screw on the cap lightly and swirl mix for 3 min. Place in a water bath with boiling water and boil for 5 min exactly. Remove the tube from the bath, swirl mix for a further 2 min, and then cool the tubes in a refrigerator (~ 4°C) for 5 min. Centrifuge at 3500 rpm (20°C) for 15 min. Transfer the supernatant to a 5ml volumetric flask and label with sample details. Add another 1.5 ml of 1% acetic acid to the shellfish material and swirl mix for 2 min. Centrifuge at 3500 rpm (20°C) for 15 min. Transfer the supernatant to the volumetric flask and carefully add deionized water to the 5ml mark. Invert the flask five times to mix the extract. Immediately transfer the 5ml extract to a centrifuge tube, screw on the cap and label. If clean-up is not to take place immediately, store at -20°C in a freezer. The concentration of shellfish extract is 0.5 g/ml shellfish material.

Clean-up: Condition a C18 SPE cartridge with 6 ml of methanol; do not allow the cartridge to dry (leave a thin layer of methanol above the upper surface of the C18 sorbent mass). Drain off the residual methanol and immediately add 6 ml of deionized water; again, do not allow the cartridge to dry. Place a 15ml centrifuge tube under the cartridge. Add 1ml of acetic acid shellfish extract and allow the extract to flow through the SPE cartridge slowly and to collect in the tube.

As soon as the extract approaches the upper surface of the C18 sorbent, add 2ml of deionized water to the SPE cartridge and allow the water to flow through the SPE cartridge slowly and collect in the tube. Once all the water has passed through the C18 cartridge, dispose of the cartridge, and then remove the tube and test the pH of the C18 cleaned-up extract. The pH will

be approximately 3.3 and should be adjusted to 6.5 ± 0.05 by adding 1 M sodium hydroxide, and mix by gently swirling. Retest the pH.

Once the pH is 6.5 ± 0.05, make up the extract to exactly 4 ml with deionized water.

Screw on the cap, swirl mix for 5 s and place in a rack ready for COOH SPE clean-up. The concentration of shellfish extract is 0.125 g shellfish material/ml.

Carboxyl phase (weak cation exchange) SPE clean-up of the C18 SPE extract: Add 10 ml (1 ml + 3 ml + 3 ml + 3ml) of 0.01 M ammonium acetate to the cartridge and allow the solution to pass through the cartridge slowly; do not allow the cartridge to dry.

Place a 15ml centrifuge tube under the cartridge. Using a pipette, add 2 ml of the C18 acetic acid extract to the cartridge and allow the extract to pass slowly through the cartridge and collect in the centrifuge tube. Add 4 ml of deionized water to the cartridge and allow it to pass through slowly and collect in the same tube. When all the water has passed through the cartridge, remove the tube and with a pipette, add deionized water and make up exactly to 6.0 ml. Label the tube as tube "1". Place a second centrifuge tube under the cartridge. Add 4 ml of 0.05 M sodium chloride to the cartridge and allow the solution to slowly pass through the cartridge and collect in the centrifuge tube. When all the 0.05 M sodium chloride has passed through the cartridge, remove the tube and add 0.05 M sodium chloride to the tube with a pipette and make up exactly to 4 ml. Label as tube "2". Place a third centrifuge tube under the cartridge. Add 5 ml of 0.3 M sodium chloride to the cartridge and allow the solution to slowly pass through the cartridge and collect in the tube. When all the 0.3 M sodium chloride has passed through the cartridge, remove the tube and add 0.3 M sodium chloride to the tube with a pipette and make up exactly to 5 ml. Label tube as tube "3".

- Tube 1 contains the C1, C2, C3 and C4 PSP toxins.
- Tube 2 contains GTX1, 4, GTX2, 3, GTX5 (B1), dcGTX2, 3 and B2 PSP toxins.
- Tube 3 contains STX, dcSTX and NEO PSP toxins.

Oxidation of COOH extracts by periodate and peroxide reagents: Periodate oxidation is for the derivatization of GTX1, 4 in tube 2 and NEO in tube 3.

Transfer 200 µl of the extract (or reference standard solution) to an HPLC autosampler vial, add 200 µl of deionized water and 1 ml of the periodate reagent. Seal the vial and thoroughly mix the contents. Allow to stand at room temperature for 1 min and then add 10 µl of glacial acetic acid. Reseal the vial and swirl the mix. Allow to stand for approx. 10–15 min prior to analysis by HPLC.

Procedure: Peroxide oxidation is for the derivatization of GTX2, 3 and GTX5 (B1) in tube 2 and STX and dcSTX in tube 3. Add 50 µl of peroxide reagent to an autosampler vial and 500 µl of 1M sodium hydroxide solution. Seal the vial and thoroughly mix the contents.

Add 200 µl of extract (or reference standard solution) and re-seal the vial and thoroughly mix the contents.

Allow to stand for 2 min at room temperature. Add 40 µl of glacial acetic acid to the contents of the vial, re-seal and again thoroughly mix the contents. The sample is now ready for analysis by HPLC.

It is important that the HPLC analysis should be performed no later than 8–10 h after sample oxidation.

HPLC determination

Column: 150 × 4.6 mm (5 µm) C18 LC

Flow rate: 2 ml/min

Column temperature: 30°C

Fluorescence excitation wavelength: 340 nm

Fluorescence emission wavelength: 395 nm

Temperature controlled autosampler: 20°C

Injection volume: periodate treated – 100 µl, peroxide treated – 50 µl

Mobile phase composition, phase A: dissolve 6.5 (±0.1) g of ammonium formate in 800 ml of HPLC grade water and sonicate for 2 min; add 4 ml of 0.1 M acetic acid; check the pH and adjust to 6 with

0.1 M acetic acid as necessary; make up the volume to 1000 ml and thoroughly mix.

Mobile phase composition, phase B: dissolve 6.5 (±0.1) g of ammonium formate in 800 ml of HPLC grade water, add 50 ml of acetonitrile and sonicate for 2 min; add 4 ml of 0.1 M acetic acid; check the pH and adjust to 6 with 0.1 M acetic acid as necessary; make up the volume to 1000 ml and thoroughly mix.

Elution gradient:

Time (min)	A (%)	B (%)
0.0	100	0
5.0	95	5
9.0	30	70
11.0	100	0
15.0	100	0

Toxin elution: the toxins elute in the following order:

PSP toxin	Number of peaks	Peak number (#)	Retention time (min)	
			Periodate extract	Peroxide extract
STX	1	-	9.1	9.1
NEO	3	#1	4.7	-
		#2	9.1	-
		#3	3.1	-
GTX1, 4	3	#1	1.6	-
		#2	2.4	-
		#3	5.9	-
GTX2, 3	1	-	5.9	5.9
GTX5	1	-	-	7.8
dcSTX	2	#1	4.1	4.1
		#2	4.7	4.7

Retention times are approximate and will vary between systems but are given as an indicator of elution.

Quantification of PSP toxins: Calibration curves should be prepared using solutions prepared from certified reference materials. Suggested toxin levels in calibration standards after periodate oxidation (100 µl injection volume):

PSP toxin	Periodate working calibration standards (ng on column)				
	Level 1	Level 2	Level 3	Level 4	Level 5
STX-e					
NEO-b	0.060	0.12	0.24	0.47	0.94
GTX1, 4-b	0.10	0.20	0.40	0.80	1.6
GTX2, 3-b					
GTX5-b (B1)					
dcSTX	0.14	0.28	0.56	1.1	2.2

Suggested toxin levels in calibration standards after peroxide oxidation (50 µl injection volume):

PSP toxin		Peroxide working calibration standards (ng on column)			
	Level 1	Level 2	Level 3	Level 4	Level 5
STX-e	0.063	0.13	0.26	0.51	1.02
NEO-b					
GTX1, 4-b					
GTX2, 3-b	0.13	0.26	0.51	1.0	2.1
GTX5-b (B1)	0.10	0.20	0.40	0.80	1.6
dcSTX	0.12	0.25	0.50	0.99	2.0

Reporting of results: The toxin concentrations are determined using the following equation:

$$\text{toxin concentration} = \frac{\text{ng [toxin] on column (ng/g)}}{\text{g [shellfish tissue on column]}}$$

concentration = µg [toxin]/kg [shellfish tissue] where the mass of the shellfish tissue on the column can be found in Table 13.

As the regulatory limit is defined in STX equivalents per kg shellfish flesh, the concentrations of the different toxins found in a sample must be converted to STX equivalents. Each toxin will be converted using the relative toxicity in Table 14 and the final values summed to give the final concentration as:

concentration = µg [STXeq]/kg [shellfish tissue]

TABLE 11: MASS OF SHELLFISH TISSUE ON COLUMN (G) AFTER PERIODATE AND PEROXIDE OXIDATIONS FOR QUANTIFICATION OF STX TOXIN

COOH extract	After periodate (100 µl injection) (g)	After peroxide (50 µl injection) (g)
Tube 1 (6.0 ml)	0.0042	0.0021
Tube 2 (4.0 ml)	0.0063	0.0032
Tube 3 (5.0 ml)	0.0050	0.0025

TABLE 12: PSP CONCENTRATIONS AT THE ACTION LIMIT AND RELATIVE TOXICITIES TO STX

PSP toxin	Relative toxicity to STX	Concentration at action limit* (µg/g)
NEO	0.9243	0.87
GTX1	0.994	0.80
GTX4	0.7261	1.10
GTX1, 4	0.8601	0.95
STX	1	0.80
dcSTX	0.5131	1.56
GTX5	0.0644	12.42
GTX2	0.3592	2.23
GTX3	0.6379	1.25
GTX2, 3	0.4986	1.74

*Based on limit of 80 µg [STX equivalence]/100 g

Note that all samples, COOH extracts (tubes 2 and 3 only) must be oxidized with both periodate and peroxide for the detection and quantitation of STX, NEO, GTX1,4, GTX2, 3, GTX5 (B1) and dcSTX.

Evaluation of toxicity: The regulatory limit for PSP toxins in shellfish is 80 µg STXequivalent/100 g tissue; any values exceeding this are considered dangerous and the sample not fit for human consumption.

Report the results in milligrams of PSP toxin per kilogram.

Quality assurance: A reagent blank (control) should be taken through the whole extraction, clean-up and oxidation process and analysed. If this blank gives a response(s) higher than the limit of detection, sample analysis should be repeated due to possible contamination.

A spiked sample should be prepared and processed as a sample. Normal recoveries should be within 85– 115% recovery of the true value. The r^2 of the working calibration standards should be >0.95 in order to accept sample results.

Reference:

Lawrence, J. (2005) Paralytic Shellfish Poisoning Toxins in Shellfish Prechromatographic Oxidation and Liquid Chromatography with Fluorescence Detection. AOAC International, Gaithersburg, MD.

5.5.3 Amnesic shellfish poisoning by HPLC-UV

Scope: This test method is suitable for the determination of amnesic shellfish poisoning (ASP) toxicity caused by domoic acid in molluscan shellfish by HPLC with UV detection (HPLC-UV). The specificity of the method is high.

Principle: Domoic acid analysis is based on the HPLC-UV method by Quilliam *et al.* (1995). A 50% methanol solution is used to extract the domoic acid in a homogenized sample followed by a saxitoxin (SAX)-solid phase extraction (SPE) sample clean-up. This extract is then analysed using isocratic liquid chromatography with photo-diode array and the domoic acid content determined using UV detection at 242 nm. The results are quantified using certified reference materials of domoic acid.

Note that when analysis of the sample is not possible on the day of receipt, the sample may be stored either whole or homogenized by refrigeration at 4°C (±3°C), or frozen below -20°C overnight or over the weekend.

Equipment:

a) No. 10 sieve
b) Parafilm
c) Tissues
d) HPLC with C18 column (25 cm long × 4.6 mm i.d. packed with 5–10 µm bonded silica gel with guard column)

Reagents/Material:

a) Acetonitrile HPLC grade (99% purity)
b) Methanol HPLC grade (99% purity)
c) Formic acid analytical grade
d) Trifluoroacetic acid (99% purity)
e) Citric buffer solution: dissolve 8.08 g of citric acid in approximately 60 ml of deionized water. Add 2.8 g of tri-ammonium citrate and a further 20 ml of deionized water. Mix, ensure that both salts have dissolved and add 10 ml of acetonitrile. Make up the volume to 100 ml and mix thoroughly.
f) SAX cartridges containing 500 mg of silica derivatized with quaternary ammonium silane
g) Deionized water (HPLC grade)
h) Domoic acid working solutions made up from certified calibration standards
i) Certified ASP muscle tissue reference material (quality control sample)

Sample Preparation: Once the shellfish have been removed from the shells, the bulk sample is thoroughly homogenized using a high speed blender.

Extraction: Accurately weigh out 4.0 $\pm$ 0.1 g of the tissue homogenate into a 50ml graduated centrifuge tube labelled with the sample code.

Add 16 ml of a methanol: water mix (1:1) and homogenize the mixture using an Ultra-Turrax® blender at medium speed for 1 min.

Centrifuge the sample at 3000 rpm for 10 min at about 4°C.

Filter the supernatant through a dry methanol-compatible 0.45 µm filter using a syringe into a labelled centrifuge tube. Remove 6 ml of supernatant and freeze at lower than -12°C for re-testing if necessary.

Clean-up: Condition an SAX cartridge by passing through 6 ml methanol, then 3 ml water followed by 3 ml of the 1:1 methanol: water mixture. The flow rate should be approximately 1 drop/s. Do not allow the cartridge to go dry during this process.

Load 5 ml of the filtered supernatant on to the cartridge and allow to drain at approximately 1 drop/s.

Stop the flow when the sample meniscus reaches the top of the cartridge bed. Discard the eluate.

Add 5 ml of a mixture of acetonitrile: water (1:9) and allow to drain at approximately 1 drop/s. Stop the flow when the sample meniscus just reaches the top of the cartridge bed. Discard the eluate.

Add 0.5 ml citrate buffer eluent; allow to drain at approximately 1 drop/s. Stop the flow when the sample meniscus reaches the top of the cartridge bed. Discard the eluate.

Elute the domoic acid by using the citrate buffer eluent. Collect 2 ml of eluate in a tube. Mix the solution and withdraw an aliquot for HPLC analysis.

Samples should not be stored in the refrigerator for longer than 1 week prior to analysis. Do not freeze the SAX eluent extraction as the domoic acid will decompose under these conditions.

Procedure

HPLC determination: Establish the HPLC with a 250 × 4.6 mm C18 LC column maintained at 40°C and with a mobile phase of acetonitrile at 10.0 % and trifluoroacetic acid at 0.1 % at a flow rate of 1 ml/min.

Determine the domoic acid content of the sample extracts by comparison with reference solutions of domoic acid prepared from a certified reference, using an injection volume of 20 µl.

Reporting of result: In milligrams of domoic acid per kilogram.

Quality assurance: Normal quality assurance measures should be implemented. The acceptable r^2 value should be not less than 0.9990.

References:

Hardstaff, W.R., Jamieson, W.D., Milley, J.E., Quilliam, M.A. and Sim, P.G. (1990) Reference materials for domoic acid, a marine neurotoxin. Fresenius' Journal of Analytical Chemistry **338**: 520–525.

Lawrence, J.F., Charbonneau, C.F. and Menard, C. (1991) Liquid chromatographic determination of domoic acid in mussels, using AOAC paralytic shellfish poison extraction procedure: Collaborative study. Journal of the Association of Official Analytical Chemists International **74**: 68–72.

Lawrence, J.F., Charbonneau, C.F., Menard, C., Quilliam, M.A. and Sim, P.G. (1989) Liquid chromatographic determination of domoic acid in shellfish products using the AOAC paralytic shellfish poison extraction procedure. Journal of Chromatography **462**: 349–356.

Quilliam, M.A. (2003) Chemical methods for domoic acid, the amnesic shellfish poisoning (ASP) toxin. In Manual on Harmful Marine Microalgae, Monographs on Oceanographic Methodology, Vol. 11, Hallegraeff, G.M., Anderson, D.M. and Cembella, A.D. (eds), pp. 247–266. Intergovernmental Oceanographic Commission (UNESCO), Paris.

Quilliam, M.A., Xie, M. and Hardstaff, W.R. (1995) A rapid extraction and cleanup procedure for the liquid chromatographic determination of domoic acid in unsalted seafood. Journal of Association of Official Analytical Chemists International **78**: 543–554.

5.5.4 Determination of diarrheic shellfish toxins

Scope: The procedure allows for the determination of diarrheic shellfish poisoning (DSP) toxicity in molluscan shellfish by mouse bioassay (MBA). The method is semi-quantitative and specificity is low. The method detects all the lipophilic components historically included in the DSP complex: okadaic acid and analogues, dinophysis toxins and pectenotoxins. Other acetone soluble toxins such as domoic acid, azaspiracids, brevetoxins, gymnodimine, spirolides, pinnatoxin, ciguatoxin, palytoxin, aplysiatoxins and lyngbyatoxin can also be detected.

Note that although a higher specificity is desirable, there are neither analytical methods nor standards/reference materials available to regulatory laboratories that allow the identification or confirmation of the presence of most of the co-extracted active substances.

Principle: The MBA procedure of Yasumoto *et al.* (1978) is based on the acute toxic effect of an acetone extract of the analytical sample (i.e. molluscan shellfish hepatopancreas), partitioned between diethyl ether and water to remove interference from polar toxins, evaporated and suspended in an aqueous solution of 1% Tween 60. A portion of this extract is injected intraperitoneally into male albino mice with the evaluation of toxicity based upon the death time of the mice.

Note that when analysis of the sample is not possible on the day of receipt, the sample may be stored either whole or homogenized by refrigeration at 4°C (±3°C), or frozen below -20°C overnight or over the weekend.

Equipment:

a) Strainer
b) Filter paper (24 cm diameter)
c) Parafilm or similar material
d) Disposable hypodermic 26 gauge sterile syringes, 1 ml, with needles
e) Tissues

Reagents/material:

a) Acetone GPR grade
b) Diethyl ether GPR grade
c) Aqueous Tween 60 solution, 1%
d) Deionized water
e) Certified mussel reference material for DSP toxins

Male albino mice from a standard stock colony, weighing 20 (±1) g. These mice should be weighed to two decimal places immediately prior to injection.

Sample Preparation: Once the shellfish have been removed from the shells, the hepatopancreas is separated from each and the bulked samples thoroughly homogenized, using a high speed blender.

Extraction: Weigh 25 (±0.2) g of separated hepatopancreas into a maceration vessel and add 50 ml of acetone.

Homogenize the sample using an Ultra-Turrax® blender on low speed for 2–3 min. Chilling may be necessary to reduce the loss of acetone if the system becomes warm. Filter the homogenate through a filter paper in a glass funnel into a pre-weighed labelled 1 litre round-bottom flask (weighed to 0.01 decimal places).

After filtration, remove any sample material shellfish extract from the filter paper using a spatula and return it to the maceration vessel. Add a further 50 ml of acetone to the residue and homogenize for a further 2–3 min at low speed, then filter again through the same filter paper. Repeat these steps until the extraction has used a total volume of 150 ml of acetone.

The acetone extract is evaporated to dryness using a rotary evaporator under gentle vacuum at 40 ± 1°C.

Using a pipette, add 10 ml of distilled water to the evaporation flask, followed by 50 ml of diethyl ether. Mix the suspension by swirling the flask and transfer the mixture to a separation funnel.

Mix the solution by gently rocking the funnel back and forward 8–10 times (releasing the gas pressure build up when necessary), allow the water phase to separate from the diethyl ether phase, and drain off the water (bottom) layer back into the evaporation flask.

Add a further 50 ml of diethyl ether to the evaporation flask, mix to dissolve remaining residue, and add to the separation funnel. Repeat this step to give a total of 150 ml of diethyl ether in the separation funnel plus the aqueous phase.

Allow the water phase to separate from the diethyl ether phase and drain off the water (bottom) layer to a waste beaker.

Add 20 ml of distilled water to the diethyl ether in the separation funnel and mix. Allow to separate, and drain off the water as waste. Repeat this step.

Collect the ether phase in the 1 litre flask and rotary evaporate to dryness under gentle vacuum at 40°C (±1°C).

Calculations: Re-weigh the evaporation flask and calculate the weight of residue. Using a lipid density of 0.93, calculate the volume of the lipid residue. (Divide the weight of residue by 0.93).

The volume of 1% Tween 60 needed to make up a final volume of 5 ml is calculated by subtraction of the value above from 5 ml.

The residue is suspended in this volume of 1% Tween 60 solution, using an automatic pipette to make up 5 ml of inoculum solution, so that a final concentration of 5 g hepatopancreas is obtained.

The residue in the flask should be removed from the flask walls using a spatula and suspended in the 1% Tween 60 solution.

All traces of the residue should be suspended in the 1% Tween 60, so that when the flask is inverted the suspension can be decanted to a labelled suitable container. Sufficient time (5–10 min) should be allowed to let all the contents drain into the container.

The flask should then be checked visually to ensure that there are no significant amounts of residue remaining.

If immediate injection is not possible the suspension is covered (with parafilm or a similar material) and stored overnight in the fridge at 4°C (±2°C). If injection is not possible for a longer period, the suspension can be stored in the fridge at 4°C (±2°C) overnight or frozen below -20°C for 2 nights.

The suspension can be homogenized for 5–10 s to ensure complete suspension of any particles of residue and to ensure that no blockages occur in the hypodermic needle.

The homogenizer head should be rinsed between operations with distilled water, and excess water wiped off from the head with a clean tissue.

Mouse bioassay

Albino male mice weighing 20g (±1g) should be used for the assay. These should be from a standard stock colony and acclimatized in the test area for a minimum of 12 h prior to injection, under appropriate environmental conditions.

All mice should be examined prior to inoculation to establish the health of the animals. This should include observation of:

- Activity in cage (i.e. not listless or fatigued)
- The coat should be smooth and the eyes should be bright
- There should be no signs of discharges from nose, mouth, genitals or anus
- They should have no signs of injury or bite marks on the back, flanks, legs, head or ventral surfaces.

Prepare the injections by drawing 1 ml of the suspension into a 1 ml sterile hypodermic syringe. Ensure that the needle does not come into contact with the container during filling, as this may dull the point of the needle and cause trauma during injection. It is recommended that 26 gauge needles are used where possible to minimize trauma.

1 ml of the sample extract is injected intraperitoneally into each of three mice. This should only be carried out by a trained operator.

The mice are observed for the first 30 min, and then at least every hour for the first 3 h. Any DSP symptoms are documented during this time. After 24 h the mice are observed and any deaths are recorded.

Evaluation of toxicity: The death of two out of three or more mice within 24 h is interpreted as a positive result for the presence of DSP toxins and the shellfish considered not suitable for human consumption.

Quality assurance: One batch of mice should be injected using the above bioassay procedure every time a new 1% Tween 60 solution is prepared. This is defined as the negative control.

A positive control sample prepared using a certified reference material should be taken through the whole analytical procedure at least once every 4 months. The mice should be treated the same as those used for test samples. After 24 h the mice are observed and any deaths and observations are recorded.

References:

McMahon, B.M. and Sawyer, L.D. (eds) (1968) Section 232.4. In *Pesticide Analytical Manual*, Vol. I. US Food and Drug Administration, Washington, DC.

Yasumoto, T., Murata, M., Oshima, Y., Matsumoto, G.K. and Clardy, J. (1984) Diarrhetic shellfish poisoning. In *Seafood Toxins*, Ragelis, E.P. (ed.), pp. 207–214. American Chemical Society, Washington, DC.

Yasumoto, T., Oshima, Y. and Yamaguchi, M. (1978) Occurrence of a new type of shellfish poisoning in the Tohoku District. *Bulletin of the Japanese Society Of Fisheries Science* 44(11): 1249–1255.

5.6 Ciguatera toxin

5.6.1 Introduction

Ciguatera Fish Poisoning (CFP) is a human intoxication caused by the consumption of fish that contain ciguatoxins (CTXs). CFP is found endemically within the western Atlantic Ocean including the Caribbean Sea. CTXs are secondary metabolites produced by the marine benthic dinoflagellate of the genus *Gambierdiscus*. Ciguatoxin-group toxins (CTXs) found mainly in the Caribbean are classified as, (C) or (I) – CTX-group toxins. The toxins are lipid soluble polyether compounds with over 10 analogues identified for the Caribbean. The main Caribbean ciguatoxin (C-CTX-1) is less polar and tenfold less toxic in humans than the main Pacific cigatera P-CTX-1. Outbreaks of ciguatoxin poisoning are mostly localized.

Whilst no regulatory limits or methods of analysis are set within Europe, EC regulations do require that checks are made to ensure that fishery products do not contain biotoxins such as CTX.

Ciguatera poisoning is a clinical diagnostic since there is as yet no confirmatory test. Therefore, it is important to have adequate CTX quantification methods to diagnose CFP cases, and to prevent intoxications through the analysis of consumable fish. For more information, see Guide to food safety hazards in Caribbean Fishery products, 2016 CRFM Special Publication. No.11.

CTXs, along with their presence at trace levels in fish tissue, make their reliable detection and quantification in fish flesh difficult. Numerous methodologies based upon different approaches (e.g., toxicological symptoms, antibody recognition, mass spectrometry, etc.) have been developed for CTX determination. The in vivo mouse bioassay (MBA), has historically been the most universally applied technique for examining used for the surveillance and determination of CTX-contaminated fish. However, ethical reservations concerning its use and its poor specificity makes it inappropriate as a method of analysis. In recent years, considerable effort has been applied to develop chemical assays to replace these bioassays.

5.6.2 HPLC method: Rapid extraction combined with LC tandem mass spectroscopy

Most current developments have focused on the application of LC-MS-MS for the detection of the toxin, such as a HPLC-MS-MS analysis following a ciguatoxin rapid extraction method (CREM) to simplify the analysis with a limit of quantification of 0,1 ng/g. This CREM-LC-MS-MS method simplifies the detection and quantitation of clinical relevant levels of ciguatoxin in crude extract of fish. It provides significant improvements in sensitivity and is designed to allow multiple analysis a day. Given the similar physical and chemical properties of the different Pacific Ocean, Caribbean Sea and Indian Ocean ciguatoxins CREM–LC-MS-MS may have applicability to the extract of multiple CTXs prior to analysis. Its applicability to detect and quantify clinically relevant levels of C-CTX-1 in suspect fish from the Caribbean is yet to be determined. Further development and validation is required.

Scope: This test method is suitable for the detection and quantification of ciguatoxin in fish flesh by gradient reversed-phase liquid chromatography–tandem mass spectroscopy (LC-MS-MS) after rapid ciguatoxin extraction (CREM). The limit of quantification is 0,1 ng/g.

***Principle*:** P-CTX-1 analysis is based on a ciguatoxin rapid extraction method (CREM). The initial extraction by methanol/hexane is followed by orthogonal solid phase extraction (SPE) clean-up steps, filtration and evaporation under N2 stream prior to gradient reversed-phase LC-MS-MS. Conditions are established using pure P-CTX1 for quantification of results.

Equipment:

a) High speed blender, e.g. Ultraturrax
b) Centrifuge
c) Pipettes, Pasteur
d) Millipore membrane filter 0,45 µm
e) Glass vial
f) C18 SPE cartridge
g) Falcon tube
h) Silica SPE cartridge
i) Syringe
j) LC system with C18 column (5 µm Phenomenex Luna 2.1 x 250 mm) fitted with a pre-column (Phenomenex C18, 4 x 2.1 mm, 5 µm)
k) Two triple-quadrupole mass spectrophotometer with Turbo-Ion-Spray Ionisation (AB Sciex Instruments)
l) AP 4000 (OTRAP)

Reagents/Material:

a) Acetonitrile HPLC grade (99% purity)
b) 1M NaCL
c) N2
d) Chloroform 99,8% pure (Ajex Finechem), containing ethanol, methanol, or alkyl stabilizers at < 0,2%
e) Methanol HPLC grade (99% purity)
f) Ammonium formate
g) Formic acid
h) Formic acid analytical grade
i) Deionized water (HPLC grade)
j) Solvent A: 2mM ammonium formate and 0,1% formic acid
k) Solvent B: 95% Acetonitrile with 2 mM ammonium formate and 0,1% formic acid.
l) P-CTX-1 spiked raw fish, flesh, crude extract; suspect ciguatera fish flesh sample

Sample Preparation:

Extraction: Cook 2 g minced fish at 70°C for 20 min in capped 50 ml Falcon tubes. Cool before homogenisation with methanol/hexane 3:1 until no lumps of fish remain. Centrifuge the homogenate and transfer the lower aqueous methanol with a syringe, filtered into a glass vial.

Adjust the crude extract to 50-55% aqueous methanol by addition of 2 ml H20. Precondition a 900 mg C18 SPE cartridge with 4 ml of water and load the sample and rinse with 65% methanol, eluate with 80% methanol. An additional SPE clean-up step is added by collecting the 80% methanol fraction in a Falcon tube adding 1 M NaCl before extraction with chloroform. Centrifuge at 2000 rpm and transfer the lower chloroform phase to a vial and N2 dried at low heat.

Precondition a silica cartridge with chloroform. The sample is loaded in chloroform and eluted with chloroform methanol 9:1. For both SPE clean-up steps syringe assisted positive displacement is used. Samples, including unspiked and spiked samples for validation are evaporated under N2 and low heat before re dissolved in 200 µl of 50% aqueous methanol 50% prior to LC/MS/MS analysis.

Procedure

HPLC determination: Gradient reversed-phase liquid chromatography –tandem mass spectroscopy (LC-MS-MS).

Establish the LC system with a C18 Colum fitted with a pre column.

Eluate the column at 400 µl/min with a linear gradient from 35% B to 100% B over 5 min. Then equilibrate the column for 5 min with 35% B prior to the next run.

Detection by two triple-quadrupole mass spectrophotometer with Turbo-Ion-Spray Ionisation (AB Sciex Instruments).

MS-MS conditions were established using pure P-CTX-1 dissolved in 50 % B and injected directly in the mass spectrometer at 10 µl/min. For the MS/MS signal optimisation and analysis conditions by API 4000 (OTRAP) reference is provided to the original publication (see below).

The content of the sample extracts is determined by comparison with spiked samples/reference solutions of P-CTX-1 prepared from a reference, using an injection volume of 20 µl.

Performance criteria: Validation of method by un-spiked (negative control) and spiked samples and confirmation with suspected fish. For validation results see the original publication (reference below).

Reporting of results: In µg P-CTX-1 equivalents/kg

Reference: Richard J. Lewis et al. (2009): Rapid extraction combined with LC tandem mass spectroscopy for the determination of ciguatoxins in ciguateric fish flesh, Toxin 54, 62-66.

6 ADDITIONAL TESTS REQUIRED FOR PRODUCTS OF AQUACULTURE

The implementation of residue monitoring programmes for aquaculture products will require some of the tests indicated above, but in addition may require additional tests for a wide range of banned and permitted substances, in line with risk-based requirements (defining banned substances, permitted substances and environmental contaminants,) according to national regulations, or those applicable in export countries. A wide range of possible test parameters may be required to fulfil these requirements, to reflect the design of the monitoring system and the aquatic animal health status of the country. These are described in the Guide to Food Safety Hazards in Caribbean Fishery Products. There is frequently scope for proposing a more limited testing regime.

It should be noted that for most of the substances indicated, the EU does not specify particular procedures, but requires that minimum performance levels apply. The specific tests for each parameter are too numerous to define in detail here. However, the analytical method typically used to obtain these levels of analytical performance is liquid chromatography-mass spectrometry (LC-MS), a technique that combines the physical separation capabilities of liquid chromatography (HPLC) with the mass analysis capabilities of mass spectrometry. LC-MS is a powerful technique used for many applications that has very high sensitivity and specificity. Generally, its application is oriented towards the specific detection and potential identification of chemicals in the presence of other chemicals (in a complex mixture).

Some of the specific substances which can be tested using this approach are set out below.

Tetracycline analysis is usually by reversed phase HPLC with UV detection or LC-MS. The analysis can be difficult and variable recoveries can be an issue. Sulphonamides can be analysed by GC-MS, reversed phase HPLC with UV detection or LC-MS. The extraction and separation can be long and tedious. Analysis of other drugs such as anthelminthics is by HPLC with UV detection or LC-MS, although analysis of Ronidazole is by reversed-phase HPLC with UV detection or by LC-MS after extraction and clean-up using strong cation exchange.

Specific minimum required performance limit (MRPL) for the testing method have been established in Commission Decision 2002/657/EC implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results.

MRPLs are defined as "*minimum content of an analyte in a sample, which at least has to be detected and confirmed*" and are the reference point for action in relation to the evaluation of consignments of food. To date MRPLs have been established for the following substances (Table 15).

TABLE 13: MINIMUM REQUIRED PERFORMANCE LIMIT FOR TESTING CERTAIN SUBSTANCES IN FOOD

Substance and/or metabolite	Matrices	MRPL	Reference
Chloramphenicol	Meat, Eggs, Milk, Urine, Honey Aquaculture products	0,3 µg/kg	Commission Decision 2003/181/EC
Nitrofuran metabolites: - furazolidone - furaltadone - nitrofurantoin - nitrofurazone	Poultry meat for all Aquaculture products	1 µg/kg	
Sum of malachite green and leucomalachite green	Meat of aquaculture products	2 µg/kg	Commission Decision 2004/25/EC

Source: Commission Decision 2002/657/EC

ANNEX 1: FURTHER READING

This guide is based on a number of different sources of information. These are listed below, and may be consulted for additional information regarding the nature and characterisation of the different hazards identified.

EURL: Methods –Equivalent testing of histamine methods -Final Report Joerg Stroka et al, 2014, Report EUR 26605 EN, European Commission, Joint Research Centre Institute for Reference Materials and Measurements. Science and Policy Report by the Joint Research Centre of the European Commission.

https://ec.europa.eu/jrc.

Strengthening Fishery Products Health Conditions in ACP/OCT Countries Module 2: Strengthening Fishery Laboratories and Technical Institutes Guide to the Development and Maintenance of Fishery Product Testing Laboratories (LTI040GEN), October 2010, Health Conditions in ACP/OCT countries, Secretariat of the ACP Group of States SFP-ACP/OCT Management Unit, REG/70021/000

http://www.megapesca.com/files/manual.rar

Residue (RMP) Monitoring Plan for the Execution of Sanitary Inspection of Fish as Raw Material and Fish-Products as Food for Human Consumption, Mission Ref: CA073GEN, May 2010, published by Strengthening Fishery Products Health Conditions in ACP/OCT Countries (Project No. 8ACPTPS137), Health Conditions in ACP/OCT countries, Secretariat of the ACP Group of States SFP-ACP/OCT Management Unit, REG/70021/000

http://www.megapesca.com/files/manual.rar

Enumeration of coagulase-positive staphylococci (*Staphylococcus aureus* and other species) National Infection Service Food Water and Environmental Microbiology Standard Method FNES8. Issued by PHE National Infection Service Food, Water & Environmental Microbiology Methods Working Group Document number FNES.8 Version number 4 Effective Date 19.08.2016 DOCUMENT

https://www.gov.uk/government/publications/enumeration-of-coagulase-positive-staphylococci-standard-method

B. Magnusson and U. Örnemark (eds.) Eurachem Guide: The Fitness for Purpose of Analytical Methods – A Laboratory Guide to Method Validation and Related Topics, (2nd ed. 2014). ISBN 978-91-87461-59-0. Available from

www.eurachem.org.

European Union Reference Laboratory (EURL) for monitoring bacteriological and viral contamination of bivalve molluscs. For reports, procedures and other relevant documents relating to bivalve shellfish sanitation.

https://eurlcefas.org

EPTIS database for proficiency testing (PT) scheme for laboratories.

https://www.eptis.bam

International Organization for Standardization. ISO

http://www.iso.org

EU Legislation:

COMMISSION REGULATION (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs

http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2005:338:0001:0026:EN:PDF

Commission Regulation (EC) No 1441/2007 of 5 December 2007 amending Regulation (EC) No 2073/2005 on microbiological criteria for foodstuffs.

http://eur-lex.europa.eu/legal-content/EL/TXT/PDF/?uri=CELEX:32007R1441&from=EN

COMMISSION REGULATION (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs

http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32006R1881&from=en

Council Regulation 2377/90 laying down a Community procedure for the establishment of maximum residue limits of veterinary medicinal products in foodstuffs of animal origin

http://ec.europa.eu/health/files/eudralex/vol-5/reg_1990_2377/reg_1990_2377_en.pdf