The United Nations University
FISHERIES TRAINING PROGRAMME

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PREPARATION OF A STANDARD OPERATION PROCEDURE FOR VALIDATION OF LABORATORY METHODS FOR TRACE METAL ANALYSIS IN SEAFOOD FOR NATIONAL AQUATIC RESOURCES RESEARCH AND DEVELOPMENT AGENCY (NARA), SRI LANKA

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ABSTRACT

Fisheries are important in Sri Lanka as a food sector as well as an export sector. The main export fish species is yellowfin tuna and main markets are Japan, EU countries and the USA. Yellowfin tuna and swordfish are high in the aquatic food web, therefore accumulation of trace metals can occur. The export goods quality and safety should comply with the WTO, national, regional and international food standards. The food testing laboratories have a very critical role for this purpose. So that the analytical and calibration laboratory follows the accreditation procedure, international regulations like ISO/IEC 17025 and method validation is important required. The analytical chemical laboratory NARA is in the process of accrediting their chemical analysis range and method validation is one of the lacking factors. The objective of this project is to develop a standard operation procedure (SOP) for the method validation, then using mercury analysis in fish as a case study to test the SOP. The SOP is prepared base on the IUPAC, EURACHEM and ICH guideline. In the SOP parameters for method validation are acquired i.e. specificity, selectivity, precision, accuracy, linearity and range, limit of detection, limit of quantification, robustness/ruggedness, and uncertainty. Mercury analysis in fish using a cold vapor atomic absorption spectroscopy (CV-AAS) was used as a case study for the applicability of the SOP developed. The results showed that the method validation characters were within acceptance range and suitable for analysis up to 5 ppm level with uncertainty of ± 21%.

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Abbreviations

AAS	Atomic Absorption Spectrometry
AOAC	Association of Analytical Communities
AR	Analytical region
ASTM	American Society for Testing and Materials
CITAC	Cooperation on International Tractability in Analytical Chemistry
CRM	Certified Reference Material
GDP	Gross Domestic Production
EEZ	Exclusive Economic Zone
EU	European Union
EURACHEM	European analytical chemical organization
FAO	Food and Agriculture Organization
FDA	Food and Drug Administrative of the United States
GMP	Good Manufacture Practices
GTA	Graphite Tube Atomizer
HACCP	Hazard Analysis Critical Control Point
HFP	Histamine Food Poisoning
HPLC	High Performance Liquid Chromatography
ICH	International Conference on Harmonization
IDL	Instrumental Detection Limit
IEC	International Electro technical Commission
ISO	International Standard Organization
IUPAC	International Union of Pure and Applied Chemistry
LoD	Limit of Detection
LoQ	Limit of Quantification
MDL	Method Detection Limit
NARA	National Aquatic Resources and Research Development Agency
ND	Not Detected
PT	Proficiency Test
RASSF	Rapid Alert System for Food and Feed
RSD	Relative standard Deviation
SD	Standard Deviation
SEAFDEC	South East Asian Fisheries Development Centre
SLAB	Sri Lanka Accreditation Board
SLR	Sri Lankan Rupee (1 USD ≈ 110 SLR)
SOP	Standard Operation Procedure
UNU	United Nations University
USP	United States Pharmacopeia
VGA	Vapor Generation Accessory
WTO	World Trade Organization

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1 INTRODUCTION

1.1 Fisheries in Sri Lanka and export of seafood

Sri Lanka is a small tropical island in the Indian Ocean off the southern tip of India and has an exclusive economic zonal (EEZ) area of 517,000 km^2 (Figure 1). Capture fisheries produced 293,170 MT and total fisheries production including aquaculture was 339,170 MT in 2009 (NARA Sri Lanka 2009). This was an increase in seafood production by 6.8% compared to the previous year.

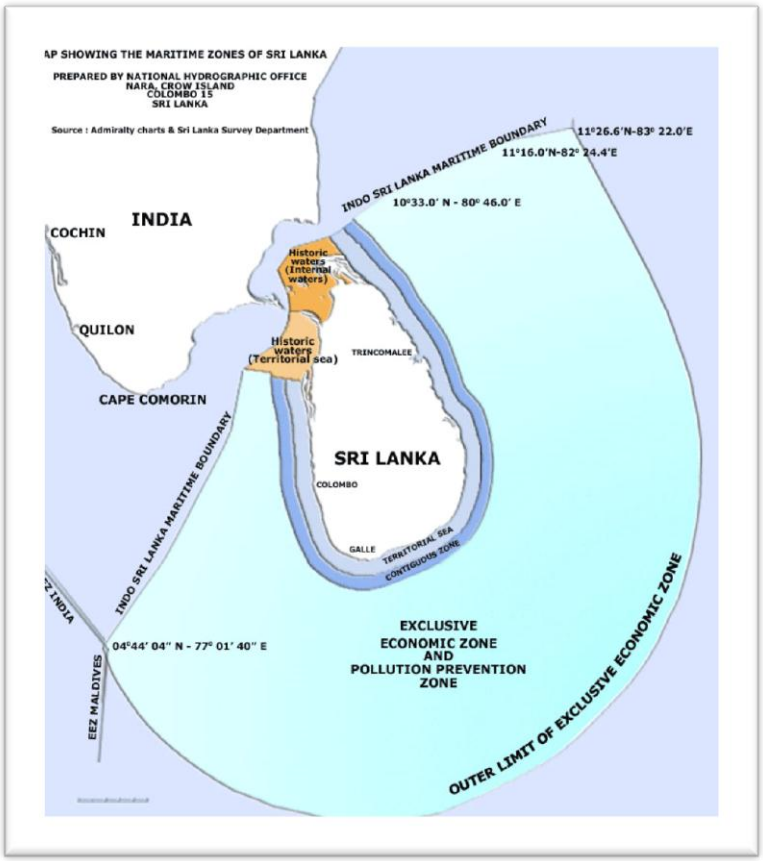

Figure 1: Map showing Exclusive Economic Zone of Sri Lanka (source: MOFAR, 2010).

The fisheries sector contributed a significant income to the national economy. The total contribution of the fisheries sub-sector to the gross domestic production (GDP) was 1.7% in 2009 (NARA 2009). The fisheries sector is an important source of local employment generation and provides about 475,000 employment opportunities directly and indirectly. This is about 8.5% of total employment in the country (NARA 2009). Yellowfin tuna (*Thunnus albacares*), swordfish (*Xiphias gladius*), skipjack tuna (*Katsu wonuspelamis*) and marlin (*Makira* sp.) are the most important export fishes in Sri Lanka (FAO 2009), and make a significant contribution to foreign earnings. Annual production of yellowfin tuna in Sri Lanka in 2008 was 33,027 MT while for swordfish it was 779 MT, skipjack tuna 78,860 MT and marlin was 2,408 MT (FAO 2009). The annual fish production of Sri Lanka increased steadily up to 2004, then it suddenly decreased year 2005, following the tsunami disaster, however now it is again increasing (Figure 2).

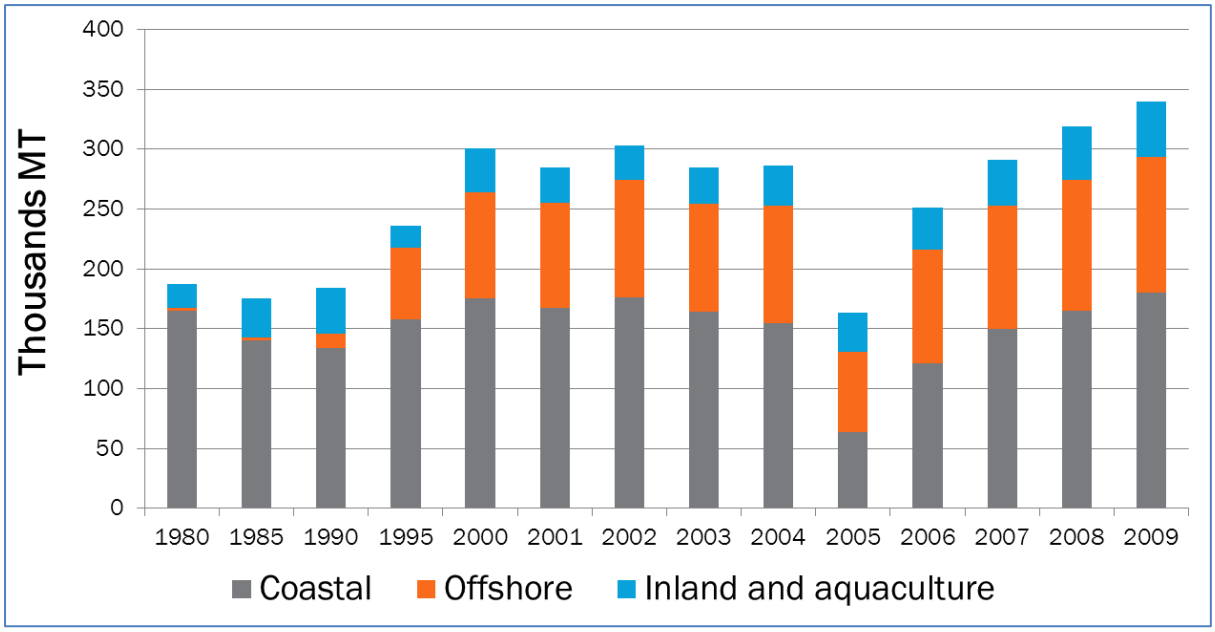

Figure 2: Annual seafood production of Sri Lanka (source: MOFAR, 2010).

The Sri Lankan fisheries sector is an important contributor to export value; it is also the main source of animal protein for the Sri Lankan population. In 2009, Sri Lanka exported 5.5% of its total landings (about 18,715 MT) and earned 21 million USD. Of this, 15,014 MT were fresh fish, mainly yellowfin tuna with an export value of 15 million USD. Other seafood such as prawns, lobster, crab, beach de mar, chunk shell, mollusks, shark fins and ornamental fish are also exported and make up about 20% of total fisheries export in quantity, as well as 25% of total export earnings (NARA 2009). In 2008 yellowfin tuna contributed 45.8% to total fish exports and was mainly exported to Japan, a market that requires first grade products for the Sashimi fish market. Sri Lanka's yellowfin tuna and other species are also imported by countries within the European Union, USA, and other non-EU countries (NARA 2009).

1.1.1 Problems related to chemical contaminants such as trace metals in seafood from Sri Lanka

The main chemical contaminant problems of seafood export in Sri Lanka are histamine and trace metals. Histamine food poisoning (HFP) mainly occurs after eating spoiled fish of tuna, mackerel, mahi-mahi and other fish in the Scombroidae family. Histamine is a biogenic amine which is normally produced by decarboxylation process of an amino acid called hisidine. This process is accelerated by increase in temperature, bacteria and histidine decarboxylase enzyme. Normally yellowfin tuna has high level of histidine and therefore needs much attention during the post-harvest handling and processing (Kerr *et al.*, 2002). Histamine poisoning can be prevented by proper handling of the fish at the time of capture and during subsequent storage, processing and distribution where fish should be chilled as rapidly as possible after capture (Bell 2003). Histamine can caused allergy, respiratory, gastrointestinal and neurological disturbance effects to humans (Lehane and Olley 1999).

Most aquatic eco-systems contain trace metals to some extent released from domestic, industrial and other anthropogenic activities as well as natural phenomena like volcanic activity (Vinodini and Narayan 2008). Some metals like copper (Cu) and zinc (Zn) are essential for fish metabolism while other metals like mercury (Hg), lead (Pb) and cadmium

(Cd) have no known biological role. Fish takes up essential and non-essential metals during the normal metabolic mechanism, taken up to the body through foods, gills and skin and accumulating in their body tissues (Kamaruzzaman *et al.,* 2010). Predators like swordfish, yellowfin tuna and sharks are at the top of the food web, therefore large amounts of metals may accumulate in their bodies (Yilmaz 2009). Further carnivorous fish species especially those in high in the food web can bio-accumulate trace metals, and contain high level of trace metals that can be harmful to consumers' health. The European Union's (EU's) rapid alert system for food and feed (RASFF) notified 28 cases for Sri Lanka exports in 2009, in some of which contaminants exceeded the maximum allowed concentration. In the last few years the number of alerts regarding heavy metal concentration in fish imported into EU from Sri Lanka has increased. The rapid alert notifications mainly concerned mercury that was found to be higher than the maximum permissible level. Swordfish from Sri Lanka was most often reported to exceed the maximum permissible level for mercury (Jinadasa *et al.,* 2010).

1.1.2 How to ensure seafood safety related to chemical risk factor

The World Trade Organization (WTO) agreement, to which Sri Lanka is a signatory, requires the export or import country to certify that the product is of good quality and safe before it is consumed (WTO 2010). In addition there are many international, regional and national regulations regarding seafood safety. Sri Lanka has yet to implement appropriate procedures to ascertain the quality of seafood before it is exported or put on the local market. The export regulation of seafood products from Sri Lanka is based on EU regulations. The maximum acceptable concentration of trace metals differs from one seafood to another based on risk assessment. The EU has established maximum permitted levels for three trace metals i.e. cadmium, lead and mercury in seafood and Sri Lanka follows this regulation. According to the EU regulation 2073/2005, 1881/206, 629/2008and Sri Lanka export regulation (No 1528/7) the maximum level of contaminants in target fish species in this study are shown in in Table 1.

Table 1: The maximum level of contaminants of selected fish species according to EU and Sri Lanka regulations

Contaminants	**Fish species**	**Maximum level of contaminants**
Hg	yellowfin tuna, swordfish, marlin and skipjack tuna	1 mg/kg of wet weight
Cd	yellowfin tuna, skipjack tuna	0.1 mg/kg of wet weight
	swordfish	0.3 mg/kg of wet weight
	marlin	0.05 mg/kg of wet weight
Pb	yellowfin tuna, swordfish, marlin and skipjack tuna	0.03 mg/kg of wet weight
Histamine	yellowfin tuna, swordfish, marlin and skipjack tuna	mean histamine value of nine fish in each batch should not exceed the 100 mg/kg of wet weight

Two measures have been enforced in Sri Lanka to ensure the export seafood quality;

1. In 1999, a fisheries quality control unit was established under the Ministry of Fisheries and Aquatic Resources. This unit has the responsibility to ensure the quality of seafood that is exported from Sri Lanka according to international requirements, especially the EU food safety legislation. This unit has the responsibility to inspect and audit seafood

factories, ice plants, fish landing centers and boats, water used for seafood production, aquaculture farms and official analytical laboratories. The unit prepares an annual sampling, auditing and inspection plan to implement as mentioned above. Since it does not have testing laboratory facility to analyze the samples, the samples are delivered to the seafood testing laboratory for analysis.

2. All seafood exporting companies should have minimum HACCP certification and they also need to fulfill their buyers' requirements. To fulfill this they also have sampling procedures and send samples on a regular basis to the official analytical laboratories for various analyses. In addition they have their own daily routine procedure including rapid testing.

1.1.3 Role and weakness of NARA chemical laboratory related to chemical food safety standards

The quality control unit of the Ministry of Fisheries and Aquatic Resources in Sri Lanka approved three laboratories for official seafood testing for the seafood exporters. One of them is the quality control laboratory, National Aquatic Resources Research and Development Agency (NARA) which is the main official laboratory under the Ministry of Fisheries and Aquatic Resources. The NARA quality control laboratory has both microbiology and chemistry units. The NARA microbiology laboratory is accredited according to ISO/IEC 17025 while the chemical laboratory is not. The analytical chemical laboratory needs to take measures to receive accreditation according to the ISO/IEC 17025 guideline in order to receive international recognition. The ISO/IEC 17025 standard gives guidelines for the operation of analytical or calibrating laboratories. It covers all types of testing, sampling and calibration laboratories. Laboratories seek accreditation after fully implementing the requirements mentioned in the ISO/IEC standard. The chemical laboratory at NARA is aiming for ISO/IEC 17025 accreditation, for trace metal and histamine analysis of seafood by 2011. To achieve this, it requires validation of the analytical as well as internal quality audit checks on analytical results, as this is a prerequisite in seeking accreditation.

For this purpose several managerial and technical requirements need to be fulfilled. The analytical method validation is one of requirements that the laboratory should fulfill for accreditation, but to date the unit has not been able to validate the chemical methods used to measure trace elements such as Hg, Pb, Cd and histamine.

1.2 Objectives and goal of the project

The overall goal of this study is the validation of chemical methods used at the chemical laboratory at NARA, Sri Lanka in order to comply with accreditation under ISO/IEC 17025.
The objectives of this project are;

- Prepare a standard operation procedure (SOP) for method validation of trace metal analysis that can be applied by the chemical laboratory, NARA.
- Apply the SOP to data obtained using the Hg detection by cold vapor atomic absorption spectroscopy at chemical laboratory, NARA as a case study and prepare a sample validation report for Hg based on the results obtained for suitable report to be presented to the Sri Lanka Accreditation Board (SLAB).

2 CHEMICAL METHOD VALIDATION

All methods used in analytical chemistry are subject to error. Therefore the methods should be evaluated as well as tested to confirm that it is suitable for the intended purpose. The main task of method validation is measurement of specific method validation characteristics. Other essential tasks of method validation include using the internal quality control procedures, participation in suitable proficiency schemes and accreditation of the laboratory according to international standards like ISO/IEC 17025 (Boqué *et al*., 2002). Method validation is carried out for several reasons such as good manufacturing practices (GMP) legislation, good economics and good science practices.

Method validation is defined as follows: "validation is the confirmation by examination and the provision of objective evidence that the particular requirements for a specific intended use are fulfilled, a process of evaluating method performance and demonstrating that it meets a particular requirement" (ISO/IEC 17025:2005, close 5.4.5). Laboratories accredited or intending to seek accreditation under ISO/IEC 17025 are expected to have validated methods employed in the accreditation process. The method validation is the process of acquiring the necessary information to assess the ability of method to perform its intended task. It is given the information to obtain results reliably, determine the condition under such results can be obtained and determine the limitation of the method.

Validation of analytical methods used in analytical laboratories is a requirement to meet the ISO/IEC 17025 accreditation, standard and to ensure that the test method gives correct and reliable results. Method validation is an essential part of analytical method development procedures. Therefore the margin of where method development finishes and method validation begins is not always very clear. Many method validation parameters are evaluated as a part of method development (Taverniers I. *et al*., 2004).

2.1 Who should do the method validation and how?

Normally analytical laboratories use several types of analytical methods; some of them are validated by international organizations like ASTM, AOAC, or they develop new methods or make a few adjustments to internationally approved methods. Nevertheless the laboratory needs to verify that that method is suitable for its intended purpose. The method validation procedure can be carried out according to several schemes, e.g. an alternative comparative analytical method, using proficiency scheme or using certified reference materials (IUPAC, 2002). "The ongoing reliability and comparability of data can be guaranteed only through the implementation of quality assurance system including the application of method validation according to international accepted procedures and performance criteria. But some analysts see method validation as something that can only be done by collaborating with other laboratories and therefore do not do it" (Eurachem 1998). The different categories of validation and to what extent validation needs to be done on an analytical method are described in Table 2.

Table 2: Different methods categories and the degree of validation and recommendation

Degree of external validation by independent bodies	**Recommended internal validation by the laboratory concerned**
The method is externally validated in a method performance study	Verification of accuracy and precision
The method is externally validated but is used on a new matrix or using new instruments	Verification of accuracy and precision, possibly also detection limits
Well established, but not tested method	Verification, possibly a more extensive validation
The method is published in the scientific literature and has stated important performance characteristics	Verification, possibly a more extensive validation
The method is published in the scientific literature without presentation of performance characteristics	The method needs to be fully validated
The method was internally developed by another organization	The method needs to be fully validated

The analytical methods used in the chemical laboratory. NARA were all either developed by another organization (South East Asian Fisheries Development Centre, SEAFDEC) and/or are published in scientific literature without presentation of performance characteristics. Therefore we need to carry out the full validation procedure.

2.2 Characteristics to be considered in method validation

The method validation character or parameters vary depending on the guidelines. The parameters, as define by ICH and other organizations are summarized in Table 3.

Table 3: Parameters for method validation with reference to ICH, USP and ISO 17025

Parameter	**Comments**
Specificity	USP, ICH
Selectivity	ISO 17025
Precision	USP, ICH
------Repeatability	ICH, ISO 17025
------Intermediate precision	ICH
------Reproducibility	ICH, defined as ruggedness in USP, ISO 17025
Accuracy	USP, ICH, ISO 17025
Linearity	USP, ICH, ISO 17025
Range	USP, ICH
Limit of detection	USP, ICH, ISO 17025
Limit of quantitation	USP, ICH, ISO 17025
Robustness	USP, Included in ICH as method development activity, ISO
Ruggedness	USP, defined as reproducibility in ICH

(Source: Hurber 2010)

The typical process that is followed in an analytical method validation is listed chronologically below (McPolin 2009);

1. Design a protocol and allocate a person, time frame, chemical, equipment, budget etc.
2. Evaluate the method validation character as follows, specificity, precision, accuracy, linearity and range, detection limit, quantification limit, robustness and uncertainty
3. Evaluation of validation results
4. Documentation and reporting – this document should include the information of equipment, condition, reagent preparation, procedure of standard curve and quality control sample preparation, system suitability, summary of the validation data, summary of the back calculation data of the standard curve, standard curve plot and special methods note etc.
5. Continuous monitoring

The parameters that have to be checked for each method type are different. Table 4 presents the method validation parameters that need to be verified for the chemical test methods used at NARA. Nevertheless the parameters can change from one occasion to another and should be considered on a case by case basis; this depends on the nature and extent of the validation required as well as the purpose.

Table 4: Method validation parameters that need to be verified for the chemical test method used at NARA

Type of analytical procedure Characteristics	**Specific test (determination of trace metal level, histamine level etc.)**
Specificity	Yes
Precision	Yes
Intermediate precision	No
Accuracy	Yes
Detection limit	Yes
Quantitation limit	Yes
Linearity	Yes
Range	Yes
Robustness	Yes

2.2.1 Specificity

ICH defines specificity as "the ability to assess unequivocally the analyte in the presence of components which may be expected to be present. Typically this might include impurities, degradants, matrix, etc." (ICH 2000). IUPAC and AOAC also used the same definition for the term of selectivity with some comments. Specificity is assuring the accuracy of the determination and the quality of analysis. For all types of analytical methods a check of specificity is required. The separation power of the analyte and closely related substances (isomers, degradation products, endogenous substances, matrix constituents) is very important in analytical chemistry. Therefore suitable techniques, potential interferences substances and blank sample should be analyzed to identify possible interferences. Jorgen *et al.,* (2001) mentioned that the term of selectivity and specificity is often used interchangeably in the analytical chemistry literature. But IUPAC has given the clarification that "specificity is the ultimate of selectivity", while the guidelines of the FDA refer only to selectivity

(IUPAC 2002). Generally specificity is considered 100% selectivity, but this is not always true.

In atomic absorption spectrometry (AAS), the specificity to the reaction takes place in the flame, graphite tubes or reaction cell (when analyzing the Hg, Se, As etc.). Every element absorbs at a specific wave length. Interferences can results from anions or matrix. The main interference anion is a chloride. The matrix effects can be of two types; mask effects or background effects. At a certain ratio between the concentrations of the analyte and the interfering ions, the influence of anion is not significant, but in small concentrations those can be significant and avoiding that influence uses the modifiers which are recommended by producer methods "cookbook" (Smith and Shanahan 2004). Some analytical methods such as Kjeldahl methods are self-defining. Such methods do not need to determine specificity (McPolin 2009). For spectrophotometry methods like high performance liquid chromatography (HPLC), specificity is quite a difficult task, because finding pure and consistent peak is necessary and this depends on several parameters such as mobile phase and column parameters (Hurber 1997).

2.2.2 Precision

ICH defines "the precision of an analytical procedure as the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions" (ICH 2000). Normally precision is expressed as a variance, standard deviation or percent relative standard deviation (RSD). The sample preparation techniques (homogeneity), weighing, pipetting, dilution and extraction method also contribute to the precision value. Precision is divided into three categories: repeatability, intermediate precision (or intermediate reproducibility) and reproducibility (ICH 2000). But some consider system precision as the fourth type of precision. As an example in Hg analysis by AAS, this measurement can be peak height or peak area value.

Repeatability is the result of the method over a short time interval under the same conditions like same sample, same instruments, same reagents and same analysts. Repeatability is also termed intra-assay precision. Repeatability can be used as reproducibility when the sample is analyzed by a number of laboratories (this is the largest measure of precision).

Intermediate precision is the result from within laboratory variations due to variation such as different days, analysts, and equipment. ICH mention only these three conditions, but chemicals (reagents), column condition etc. also affects the intermediate precision. Formally intermediate precision is known as ruggedness.

The reproducibility refers to the results of collaborative studies between laboratories. The variation factors are similar with intermediate precision except for the different locations. To be statistically meaningful, at least six laboratories must be involved in a proficiency testing (PT) program, analyzing at least three identical samples.

The performance criteria depend on the type of analysis. Precision value is less than 1.5 in validation collaborative trial in HorraT_r or T_R value (EU 2001/22/EC). That value is calculated according to the Horvitz equation. Precision for biological samples performance criteria are more like 15% at the concentration limits and it can vary between 2% and more than 20% in environmental samples. The AOAC manual for the Peer-Verified Methods program and IUPAC present a table with acceptable relative standard deviation for repeatability precision data as a function of different analyte concentration (Table 5).

Table 5: Analyte concentration versus precision (IUPAC 2002).

Analyte %	**Analyte ratio**	**Unit**	**RSD%**
100	1	100%	1.3
10	10^{-1}	10%	2.8
1	10^{-2}	1 %	2.7
0.1	10^{-3}	0.1%	3.7
0.01	10^{-4}	100 ppm	5.3
0.001	10^{-5}	10 ppm	7.3
0.0001	10^{-6}	1 ppm	11
0.00001	10^{-7}	100 ppb	15
0.000001	10^{-8}	10 ppb	21
0.0000001	10^{-9}	1 ppb	30
0.00000001	10^{-10}	0.1 ppb	43

Normally food trace metal level is below 1 ppm concentration; this means its RSD% value for precision is normally more than 11.

2.2.3 Accuracy

ICH defines the accuracy of an analytical procedure as "the closeness of agreement between the conventional true value or an accepted reference value and the value found" (ICH 2000). Sometime this is termed as trueness. The method accuracy is also dependent on the systematic errors which are inherent either within the method itself, in the way the method is used and the environment in which the method is used. These systematic errors cause biased results. The bias of a method is an expression of how close the mean of aset of results (produced by the method) is to the true value. Bias can cause either elevation or lowering of test results. Bias is usually determined by analyzing certified reference materials (CRM) or by spiking samples or alternative validation study. If a CRM is not available, a laboratory can use the proficiency samples or in-house prepared reference materials. The recovery of spiking test is a more common method. The expected recovery (Table 6) depends on the sample matrix, the sample processing procedure and the analyte concentration. The AOAC manual for the Peer-Verified Methods program includes a table with estimated recovery data as a function of analyte concentration (Table 6).

Table 6: Analyte recovery at different concentrations (AOAC 2002)

Active ingredient (%)	Analyte Ratio	Unit	Mean Recovery (%)
100	1	100%	98-101
10	10^{-1}	10%	95-102
1	10^{-2}	1 %	92-105
0.1	10^{-3}	0.1%	90-108
0.01	10^{-4}	100 ppm	85-110
0.001	10^{-5}	10 ppm	80-115
0.0001	10^{-6}	1 ppm	75-120
0.00001	10^{-7}	100 ppb	70-120
0.000001	10^{-8}	10 ppb	70-125
0.0000001	10^{-9}	1 ppb	40-120

The trace metal levels of seafood are normally around 1 ppm (range of few ppb levels up to few ppm). Therefore their recovery value ranges between 75-120% of trace metal analysis. Nevertheless, the EU commission regulation No 2001/22/EC mentions that recovery between 80 – 120% are acceptable for analysis of the levels of lead, cadmium, mercury and 3-MCPD in foodstuffs (EU 2001/22/EC).

2.2.4 Limit of Detection

ICH defines the "Limit of Detection (LoD) of an individual analytical procedure as the lowest amount of analyte in a sample which can be detected but not quantitated as an exact value. The LoD is a characteristic for the limit test only" (ICH 2000). When analyzing low concentrations, measurements like trace metal or trace pesticides, it is important to know the lowest concentration of the analyte that can be confidently detected by the method. Concentrations below this limit may not be detected. However it may be unnecessary to estimate the LoD when evaluating analytical methods for the determination of the components which are present in high levels. Sometimes two types of detection limits have been considered i.e. instrumental detection limit (IDL) and method detection limit (MDL). IDL is similar to LoD and it is the lowest that the instrument can detect. MDL is also similar to IDL, but the difference is that MDL is based on samples which have gone through the entire sample preparation scheme prior to analysis.

There are several methods available for determine the LoD (ICH 2000),e.g.:

1. Based on visual evaluation - this is normally used in non-instrumental methods.
2. Based on signal noise ratio - it is expressed as a concentration at a certain specified signal-to-noise ratio, usually two-or three to one (3 or 2:1).
3. Based on the standard deviation of the response and slope - here LoD is expressed based on the standard deviation of the response (σ) and the slope of the calibration curve (S).

In the EU regulation the performance criterion of LoD is no more than one tenth of the value of specification (EU 2001/22/EC).

2.2.5 Limit of Quantification

ICH defines the "limit of quantitation (LoQ) of an individual analytical procedure as the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy" (ICH 2000). The term of practical quantification limit (PQL) is another term for LoQ. The LoQ is expressed as concentration. In general, the LoQ of a method is associated with its LoD.

LoQ can be determined using several methods, e.g.:

1. Based on visual inspection.
2. Based on signal noise ration - in chromatographical analysis the LoQ is commonly expressed as ten times higher than the base signal noise.
3. Based on the standard deviation of the response and the slope (same as the LoD).

In practice LoQ is estimated to be 5-10 times LoD. If the analyte concentration is below the LoQ value, results are reported as non-detectable (ND). Therefore this is a very important parameter in risk assessment (Corley 2002). In the EU regulation the performance criterion of LoQ is no more than one fifth of the value of specification (EU 2001/22/EC).

2.2.6 Linearity and range

ICH defines "The linearity of an analytical procedure as its ability (within a given range) to obtain test results that are directly proportional to the concentration (amount) of analyte in the sample" (ICH 2000). When analyte concentration and test results are directly proportionate they are linear. This may be true within a given range. It is generally reported as the variance of the slope of a regression line.

ICH defines "the range of an analytical procedure as the interval between the upper to the lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity" (ICH 2000). For any quantitative method it is necessary to determine the range of analyte concentration to which a method may be applied. Both ends of the analytical range have some limitations. At the lower end there is the value of the limit of detection or limit of quantification and at the upper there may be various effects depending on the instrument response system (EUARCHEM 2000).

The ICH recommends the linearity curve's correlation coefficient, y-intercept, slope of the regression line and residual sum of squares and plot of the data are evaluated.

2.2.7 Robustness

ICH defines "the robustness of an analytical procedure as a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage" (ICH 2000). The robustness test examines the effect that operational parameters have on analysis results. Therefore it is sometimes considered in the method development stage. Many of the robustness parameters are related to the equipment and methods.

Ruggedness is defined by the USP that it "measure the degree of reproducibility of the results obtained under a variety of conditions, expressed as %RSD". The conditions which are

considered in ruggedness evaluation are different laboratories, analysts, instruments, reagents, days, operators and materials. In the ICH documents ruggedness is not addressed, because it is replaced it by reproducibility.

In here are mentioned some possible causes for the robustness.

- Variables that need to be considered when using methods based on AAS – sample matrix, different acid, digestion procedure etc.
- Variables that need to be considered when using a HPLC method – pH of mobile phase, column condition, temperature, different solvent etc.

2.2.8 Uncertainty

Uncertainty is defined as "a parameter associated with the result of a measurement that characterizes the dispersion of the values that could reasonably be attributed to the measurand" (EURACHEM / CITAC Guide CG 4, 2000). This is not the same as error, which is defined as the "difference between an individual result and the true value" (EURACHEM/CTAC Guide CG 4, 2000). Laboratories that are seeking accreditation according to ISO/IEC 17025 should estimate the uncertainty value of their method. Many procedures have been proposed for estimating uncertainty in analytical measurements (EURACHEM / CITAC Guide CG 4, 2000). These involve the identification of all the possible sources of uncertainty for the method, the estimation of their magnitude and the combination of these individual uncertainties to give standard and expanded estimates. Four steps to calculate uncertainty are described in the EUARCHEM/CITAC CG 4, 2000 guideline.

- Specify what is being measured.
- Identify what causes the result to change.
- Quantify the uncertainty components.
- Calculate the combined and expanded uncertainty.

The first step should mention the type of analyte. It can be organic, in organic or of any other type. For example, it can be total mercury in fish and fishery products. In the second step the laboratory should identify what are the suitable sources that affect the results obtained. These sources can be sampling strategy, sample collection and sample homogeneity, instrumental and environmental factors etc.

Reasonable levels of uncertainty for chemical analysis according to EEC regulation No 315/93 is listed in Table 7.

Table 7: Value of the uncertainty in difference concentration range (EC. 315/93).

Concentration	**Expanded Uncertainty**
100 g/100 g	4%
10 g/100 g	5%
1 g/100 g	8%
1 g/ kg	11%
100 mg/kg	16 %
10 mg/kg	22%
1 mg/kg	32%
<100 µg/kg	44%

Trace metal value in seafood is normally around 1 mg/kg to <100 µg/kg. Therefore the uncertainty value can be up to ± 44% of the read value.

2.3 Trace metal analysis

There are several instrumental methods and detectors for the trace metal analysis; these are: Inductively Coupled Plasma Spectrometry (ICP), Optical Emission Spectrometry (OES), Mass Spectrometry (MS), Gas Chromatography (GC), Gas Chromatography Inductively Coupled Plasma Mass Spectrometry (GC-MS), X-Ray Fluorescence Spectrometry (XRF), Atomic Absorption Spectrometry (AAS), Graphite Furnace Atomic Absorption Spectroscopy (GF-AAS), Automated combustion techniques, Pyrolysis Infra-red detectors, Fluorescence detectors, Cold vapor atomic absorption (VG AAS).

2.3.1 Atomic absorption Spectrometry, used at NARA

Atomic absorption spectrometry (AAS) is a fairly universal analytical method for determination of metallic elements when present as a trace or in higher concentrations. AAS is a spectro-analytical procedure for the qualitative and quantitative determination of chemical elements employing the absorption of optical radiation (light) by free atoms in the gaseous state. In analytical chemistry the technique is used for determining the concentration of a particular element (the analyte) in a sample to be analyzed. AAS can be used to determine over 70 different elements. In Atomic Absorption Spectrometry, the sample solution is first vaporized and atomized in a flame. Then it transforms it to unexcited ground state atoms, which absorb light at specific wavelengths. A light beam from a lamp whose cathode is made of the element in question is passed through the flame. Radiation is absorbed, transforming the ground state atoms to an excited state. The amount of radiation absorbed depends on the amount of the sample element present. Absorption at a selected wave length is measured by the change in light intensity striking the detector and is directly related to the amount of the element in the sample.

Flame atomic absorption methods are referred to as direct aspiration determinations. They are normally completed as single element analyses and are relatively free of inter element

spectral interferences. For some elements, the temperature or type of flame used is critical. Graphite furnace atomic absorption spectrometry replaces the flame with an electrically heated graphite furnace. The major advantage of this technique is that the detection limit can be extremely low.

Cold vapor technique has been especially useful for the determination of mercury level in fish. The hydride generation method is especially suitable for arsenic, antimony and selenium determinations (Ramasamy 1995). In this method water (H_2O) is used as an acid. Stannous chloride ($SnCl_2$) is used as a reductant and it helps to release the Hg into the sample cell.

3 MATERIALS AND METHODS

3.1 Preparation of a standard operation procedure (SOP) for method validation

The main purpose of the preparation of an SOP for method validation is to have a document that the laboratory staff of the quality control laboratory-chemistry unit NARA can apply for validation of the analytical procedure used for trace metal analysis at NARA as a step towards acquiring ISO/IEC-17025 accreditation. The SOP presents a summarization of the characteristics that should be considered during the validation of the analytical procedures and it is based on the following documents.

- The fitness of purpose of analytical method, a laboratory guide to method validation and related tropics, EURACHEM guide 1998.
- ICH Q2B, Validation of Analytical Procedures: Methodology, Geneva, 1996.
- IUPAC Technical Report, Harmonized Guidelines for Single-Laboratory Validation of Methods of Analysis, Journal of Pure Appl. Chem., Vol. 74, No. 5, pp. 835–855, 2002.
- EURACHEM / CITAC Guide CG 4, Quantifying Uncertainty in Analytical Measurement, 2000.

3.1.1 General principle

The discussion of the validation of analytical procedures is directed to the one of the most common types of analytical procedures: quantitative tests for contaminants content. At chemical laboratory NARA, three types of methods are used for trace metal analysis: one of them is a standardized method (i.e. AOAC method); another one is based on modification of an established method; and the third one is an analytical procedure that is used by several laboratories in Sri Lanka and Asia. The methods are used for trace metal and histamine analysis and are categorized under the third type of analytical procedure. The extent of the method validation and character depends on which category the analytical procedure in question falls under.

The factor affecting the test results and their uncertainty can be grouped into three main categories.

- Instrument and technical factors (sampling, homogeneity, test method, equipment)
- Human factors
- Environmental factors

Instrument and technical factors are related to various causes. In order to minimize their effects the following measures should be taken;

- Maintain equipment under SOP
- Maintain daily and annual calibration procedures

Human factor is related to the competence and training of laboratory staff. This issue can be dealt with in a numbers of ways;

- Provide internal and external training opportunities.
- Assess staff competence internally every year (e.g. using internal control samples).
- Participate in external proficiency testing schemes.

Environmental factors are controlled through regular checks according to the instruction of the catalogs, e.g. for AAS, the laboratory temperature should be maintained between 20-25 ± 2°C and 8-80% humidity.

3.2 Validation procedure

The validation procedure is very important for the laboratory as well as the accreditation body. Method validation is often based on a combination of the validation procedures as described in detail in chapter 2 and its subsections. The chemical laboratory NARA intends to apply this SOP to evaluate the performance characteristic to validate chemical methods used at NARA.

3.3 Validation characteristics

The objective of the analytical procedure should be clearly understood since this will govern the validation characteristics which need to be evaluated. Typical validation characteristics which should be considered are listed below:

- Specificity
- Accuracy
- Precision
- Repeatability
- Reproducibility
- Limit of Detection (LoD)
- Limit of Quantification (LoQ)
- Linearity and range
- Robustness
- Uncertainty

3.3.1 Specificity

Specificity of trace metal analysis was determined as below (Table 8).

Table 8: Procedure for specificity measurement

What do you do?	**How many times?**	**What to do with data?**
Analyze spiked samples, reference materials by test method and/or other independent methods.	At least 7 at each of 3 concentrations	Use the results from the confirmatory techniques to assess the ability of the method to confirm the analyte identity and its ability to measure the analyte in isolation from other interference.
Analyze samples containing various suspected interference in the presence of the analyte of interest.		Examine effect of interference - does the presence of the interference enhance or inhibit detection or quantification of the measurands?

3.3.2 Precision

Two types of precision should be measured,i.e. repeatability precision and reproducibility precision were determined as in Table 9.

Table 9: Procedure for precision measurement

What do you do?	**How many times?**	**What to do with data?**
Run standard, reference material, spiked sample		
Same analyst, equipment, laboratory, short time scale, 3 concentrations in range	10 independent trials	Estimate repeatability standard deviation in each concentration
Difference analyst, equipment*, same laboratory, difference time scale, 3 concentrations in range		Estimate intra laboratory reproducibility standard deviation in each concentration
Difference analyst, laboratories*, extended time scales, 3 concentrations in range		Estimate inter laboratory reproducibility standard deviation

* depending on availability

Standard deviation (S) and relative standard deviation (RSD) are calculated using the equation below.

$$S = \sqrt{\sum (xi - \bar{x})2 / (n - 1)}$$

S = standard deviation
$\bar{x}$ = mean value
x_i= actual value
n = number of determination

RSD % = (s/$\bar{x}$) x 100
RSD = Relative standard deviation

3.3.3 Accuracy

Accuracy should be assessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range (e.g. 3 concentrations /3 replicates each of the total analytical procedure). Accuracy was calculated as in Table 10.

Table 10: Procedure for accuracy measurement

What do you do?	**How many times?**	**What to do with data?**
Analyze blank and CRM using the candidate method.	At least 10 independent measurements	Estimate the difference between the mean certified value of the CRM to the value obtained in the test.
Or can follow, reagent blank and references/ test material using alternate standard method		Estimate the difference between the results with the candidate method and the alternate standard method.

3.3.4 Limit of detection

Limit of detection (LoD) is based on the standard deviation of the response and the slope. The detection limit (LoD) may be expressed as:

DL = 3.3 σ/S

Where σ = the standard deviation of the response (peak height, peak area etc.)
S = the slope of the calibration curve

- The slope S may be estimated from the calibration curve of the analyte.
- For this purpose the "blank + 3S" approach will usually suffice.
- S = standard deviation of sample blank or fortified sample blank value.

Table 11: Procedure for limit of detection measurement

What do you do?	**How many times?**	**What to do with data?**
Measure the result corresponding to the sampling blank.	Minimum 10 independent measurements	Express LoD as a concentration corresponding to mean + 3 s, where s is the sample standard deviation.
Measure the result corresponding to sample blank fortified at lowest acceptable concentration.		

3.3.5 Limit of quantification

Limit of quantification (LoQ) based on the standard deviation of the response and the slope. The quantitation limit (QL) may be expressed as:

QL = 10 σ/S

Where σ = the standard deviation of the response (peak height, peak area etc.)
S = the slope of the calibration curve

- The slope S may be estimated from the calibration curve of the analyte.
- For this purpose the "blank + 5S, 6S or 10S" approach will usually suffice.
- S = standard deviation of sample blank or fortified sample blank value.

Table 12: Procedure for limit of quantification measurement.

What do you do?	**How many times?**	**What to do with data?**
Measure the results of sample blanks.	10 independent measurements	Estimate LoQ as a 5x, 6x or 10x standard deviation of the mean.
Fortify aliquots of sample blanks at various concentration close to LoD.	At least 3 concentration and 10 replicate measurements of each concentration	Calculate the value of s of each concentration and plot against concentration, then assign LoQ.

3.3.6 Linearity and range

A linear relationship should be evaluated across the range of the analytical procedure. For any quantitative method, it is necessary to determine the range of analyte concentrations or property values over which the method may be applied.

Table 13: Procedure for linearity and range of measurement

What do you do?	**How many times?**	**What to do with data?**
Analyze blank + CRM or fortified sample blanks at various concentrations.	At least 6 concentrations + blanks (independently prepared) Proceed step (2)	Plot the conc. vs results and identify approximate working and linear range.
(2) Analyze CRM or fortified sample blanks within the linear range.	At least 6 concentration to be tried	Calculate regression coefficients in the linear range. Calculate the residual plot and establish the linearity.

Slope and linearity of the calibration curve (forced zero)

$$Y = mx + c$$

$$m = \frac{\sum[(xi - \bar{x})\,(yi - \bar{y})}{\sum(xi - \bar{x})2}$$

$$C = \bar{y} - m\bar{x}$$

$$r = \frac{\sum[(xi - \bar{x})\,(yi - \bar{y})}{\sum(xi - \bar{x})2 \sum(yi - \bar{y})}$$

m = slope of the line
c = intercept
r = correlation coefficient

Any other method is also justified

3.3.7 Robustness

Robustness was measured as in Table 14.

Table 14: Procedure for robustness measurement

What do you do?	**How many times?**	**What to do with data?**
Identified variables which could have significant effect on the method. Conduct experiments to monitor the effect of each variable on accuracy and precision.	Analyze each set of experimental conditions once.	Estimate the effect of each change in condition on the mean. Design quality control in order to control the critical variables.

Sample matrix is the main source effect for robustness and it is measured by recovery of trace metals analysis. It was measuredas in Table 15.

Table 15: Procedure for recovery measurement.

What to do?	How many times?	What to do with data?
Analyze blanks and samples unfortified and fortified with the analyte of interest at a range of seafood desired to validate or CRM samples	6 independent measurements	Determine recovery of analyte at the various concentration.

Recovery is calculated by the following equation.

$$\textbf{Percentage recovery} = \frac{(\mathbf{C1 - C2})}{\mathbf{C3}} \mathbf{X\ 100}$$

Where,
C1= Corrected concentration of spiked sample in µg/g
C2 = Corrected concentration of non-spiked sample in µg/g
C3 = Concentration of spike added into sample in µg/g

The concentration of spiked and non-spiked sample value is corrected subtracted by blank value.

3.3.8 Uncertainty

Estimation of uncertainty in chemical analysis is a very important indicator of the quality of analytical measures. It gives the confidence interval for a test result given in the form of '±'. All analytical methods involve a number of steps and each step is characterized by certain uncertainty. According to the ISO TAG4 'Guide to the Expression of Uncertainty in Measurement' and the EURACHEM/CITAC Guide 'Quantifying Uncertainty in Analytical Measurement', the overall measurement uncertainty is a function of all the uncertainties in each step of the analytical process. According to EURACHEM/CITAC guideline all analytical uncertainty calculation is based the following four steps.

- Specify what is being measured.
- Identify what causes the result to change (sampling, instrument, reagent etc.).
- Quantify the uncertainty components.
- Calculate the combined and expanded uncertainty.
  U = k x Uc
  U = expanded uncertainty
  k = coverage factor
  Uc = combined standard uncertainty

There are some other methods also available for the calculation of uncertainty measurement, e.g. use of proficiency data and precision data. According to Sanco/10232/2006 uncertainty is

a value of combined RSD analytical and RSD sample matrix weight. RSD analytical value should be calculated as above.

$$\textbf{RSD } \boldsymbol{total} = \sqrt{[(\textbf{RSD } \boldsymbol{analytical})^2 + (\textbf{RSD } \boldsymbol{sample\ mix\ weight})^2)]}$$

In the literature, RSD sample mix weight is considered as10%.

$$\textbf{Overall Uncertainty} = \textbf{2 X RSD } \boldsymbol{total}$$

Normally the uncertainty value is more than 20% of total reading.

3.4 Sample and sample preparation

The certified reference samples (CRM) used were from Fapas United Kingdom (canned fish) for the method validation procedure. Yellowfin Tuna, Swordfish, Marlin and Skipjack Tuna fish were collected from the main fish market in Sri Lanka.

The fish samples were chopped with a plastic knife and mixed well using a stainless steel homogenizer. Reference sample and homogenized fish samples were digested using Mars CEM XP-1500 (model No 927065) microwave digester. The number of reference samples and fish samples were decided according to the SOP (Appendix 1) and duplicate samples were taken from each fish. Around 1 g measured to four decimal places of homogenized sample was weighed in a Teflon vessel. Then the duplicates of a fish sample were spiked with respectively 0.25ml of 1 ppm mercury standard solution, 0.25ml of 1 ppm cadmium standard solution and 0.25ml of 1 ppm lead standard solution and 5ml of 65% conc. HNO_3 (AR, made from Sigma chemicals, USA) was added and allowed to stand for 15 minutes in fume hood for pre digestion. Then the Teflon vessel was connected to a microwave digester and turned on. The digested fish samples were transferred to 50 ml volumetric flask and made up to the mark with deionized water. A blank sample was treated the same way. The detailed digestion procedure is described in Appendix2.

3.5 Analysis of trace metals in fish samples by Atomic Absorption Spectrometer

The chemical method used to measure trace metals and histamine in NARA is based on the "compilation of key regional laboratories validated methods in Southeast Asia, 2008 (SEAFDEC)"with a small modification based on the facilities and requirements in NARA. Cold vapor generation method, Varian Atomic Absorption Spectrophotometer with Vapor generation accessory (Varian VGA 77) was used for analysis of mercury in different types of fish. The detailed method description of mercury analysis of fish is given in Appendix 2. Lead and cadmium in fish on the other hand were analysed using a Varian Atomic Absorption Spectrophotometer with graphite tube atomizer (Varian GTA 120).

3.6 Data analysis

Data analysis was done by using Microsoft Excel-2010 and Effivalidation-3 software.

3.7 Resources and costs for the method validation

The resources and costs for the method validation of trace metals (Hg, Cd and Pb) are estimated as follows;

Table 16: Estimated cost for method validation procedure of trace metals

Item	Cost per unit (SLR)	No of unit	Total cost (SLR)
Reference samples	10,500	3	31,500
Fish samples	500	10	5000
Analysis cost	3000	40	120,000
Proficiency testing program			25,000
Other			10,000
Total			191,500 (1740 USD) 1 USD = 110 SLR

This cost was allocated by the annual project (2011) call to upgrade the NARA laboratory.

4 RESULTS AND DISCUSSION OF THE CASE STUDY: VALIDATION OF METHOD FOR ANALYZING MERCURY IN FISH

This section contains the results and discussion of a case study where the SOP developed for method validation of trace metal analysis was applied to data obtained for the analysis of mercury (Hg) by cold vapor atomic absorption spectroscopy method at the chemistry laboratory in NARA. It is considered to be an example of a validation report for trace metal analysis that could be presented to the Sri Lanka Accreditation Board (SLAB).

Throughout the case study, a quality control procedure was maintained. When operating the AAS, a calibration curve is constructed and the absorbance value of the mid standard (15 ppb) recorded in a control chart. This value was maintained between upper and lower warning limits (± 2SD). The intensity of a hollow cathode lamp, temperature and humidity of the instrument room was recorded as well registered in a record table and maintained within the limits prescribed in the instrument manual instructions (temperature 20-25 ± 2°C and relative humidity 8-80%).

4.1 Example of calculation of method validation parameter

The CRM used in this case study was canned fish (T-0774) obtained from Fapas, United Kingdom and the official value of Hg concentration was 19.9µg/kg and accepted range was 11.2 – 28.7 µg/kg. This CRM was used throughout the method validation procedure.

4.1.1 Specificity

The specificity of the analytical method was evaluated by addition of different concentrations of a mercury standard to the CRM. The following tests were carried out. Run CRM, (sample 1), CRM + 0.25 ml of 1 ppm Hg std. (sample 2) and CRM + 0.5 ml of 1 ppm Hg std. (sample 3) and the recovery value calculated and recorded in Table 17.

Table 17: Results and calculation of specificity for the analysis of mercury (Hg) by cold vapor atomic absorption spectroscopy

Trial No	**Recovery (%)**		
	Sample 1	**Sample 2**	**Sample 3**
1	104.10	101.18	100.03
2	99.28	104.16	98.32
3	100.46	101.78	101.47
4	101.85	97.04	102.75
5	96.22	98.17	100.42
6	95.28	97.78	99.44
7	98.20	103.14	98.98
Mean value ($\bar{x}$)	**99.34**	**100.46**	**100.20**
S of x value (s1)	3.10	2.81	1.52
RSD = ($s/\bar{x}$)*100	3.12	2.79	1.51

The mean concentrations of samples 1, 2 and 3 were 19.77, 245.39 and 511.56 µg/kg. The data for the selectivity calculation were extracted from NARA, AAS database, for calculation purposes only. This parameter needs to be repeated as described in the SOP and the results incorporated into the method validation document.

The measurements of trace elements by AAS may be disturbed by the presence of other components, e.g. some metals interference, matrix effects and ionization effects. But those can be overcome by using a different technique. Interference can be overcome by using chemical modifiers e.g. phosphoric acid or ammonium di-hydrogen phosphate modifier for Pb and Cd. The mutual interferences can be overcome by adding excess of an easily ionisable element. The design of instruments also helps to overcome the problem by using monochromators and filters.

The evaluation of selectivity is a difficult task in spectro-photometric instruments to ascertain whether the peaks within a sample chromatogram are pure or consist of more than one compound. Case study results showed that the mean recovery value of CRM was 100.02 and mean RSD 2.47%.

4.1.2 Precision and accuracy

The precision and accuracy of the analytical method was evaluated by addition of different concentrations of a mercury standard to the CRM. The following tests were carried out. Precision calculation**; run 10 CRM and spiked sample as before and calculated recovery value and accuracy; run 10 CRM on different days and by different analysts. Results are presented in Table 18 and 19.

Table 18: Results and calculation of precision for the analysis of mercury (Hg) by cold vapor atomic absorption spectroscopy

Trial No	Recovery (%)		
	Sample 1	**Sample 2**	**Sample 3**
1	105.23	101.18	108.06
2	104.10	114.70	100.03
3	99.28	104.16	105.20
4	100.46	101.78	98.32
5	94.82	97.04	101.47
6	101.85	95.89	102.75
7	96.22	98.17	100.42
8	95.28	108.47	99.44
9	98.20	97.78	98.98
10	93.71	103.14	103.21
Mean value ($\bar{x}$)	**98.91**	**102.23**	**101.79**
STDV of x value (s1)	3.98	5.81	3.06
RSD = ($s/\bar{x}$)*100	4.03	5.68	3.01
Mean RSD	**4.24**		

The calculation concentrations of samples 1, 2 and 3 (standard deviation of reading) were 19.68 (±0.79), 250.39 (±25.46) and 507.71 (±29.78) µg/kg respectively. As described in the SOP it is necessary to carry this test out in three concentrations, but at the moment data was only available for one concentration and therefore this was used for the calculation purpose in this case study.

Table 19: Results and calculation of repeatability for the analysis of mercury (Hg) by cold vapor atomic absorption spectroscopy

Trial No	Day 1		Day 2	
	Analyte value (ppb)	**Recovery (%)**	**Analyte value (ppb)**	**Recovery (%)**
1	20.94	105.23	21.24	106.72
2	20.72	104.10	20.92	105.12
3	19.76	99.28	18.92	95.09
4	19.99	100.46	19.07	95.81
5	18.87	94.82	19.82	99.61
6	20.27	101.85	20.30	102.03
7	19.15	96.22	20.04	100.69
8	18.96	95.28	19.91	100.03
9	19.54	98.20	19.76	99.29
10	18.65	93.71	19.32	97.06
Mean value ($\bar{x}$)	19.68	98.91	19.93	100.14
S of x value (s1)	0.79	3.98	0.75	3.75
RSD = ($s/\bar{x}$)*100	4.03	4.03	3.75	3.75

****** As described in the SOP this test should be run at three concentration levels, but in practice this was only done once in the case study.

The trace elements intended for accreditation are commonly below 1 ppm in the samples that will be analyzed with this method. According to IUPAC the accepted criteria value of RSD% is more than 11 in 1 ppm concentration range (IUPAC 2002). The acceptable range of precision of biological sample is up to 15% accepted by EU/2001/22 EC, but in this case study it was 4.24%.

When calculating accuracy, repeatability calculations are supposed to be obtained from three different concentrations, whereas in the present report only one concentration was available from NARA and therefore used for calculation purpose. These parameters should be calculated again for the method validation purpose. According to AOAC the accuracy value should be 75-120% (AOAC 2002) when the concentration is in the 1 ppm range and according to EU food contaminants regulation No 2001/22/EC, the recovery value should be 80-120%. In this case study the results are showed that all recovery values were 99.53% and therefore within the accepted range.

The method employed in NARA for analysis of Pb and Cd in seafood is based on a graphite tube atomizer (GTA) AAS method. When using pyrolytic graphite tube, ash can form and affect the precision and accuracy (Garnys 1975). Therefore when analyzing Pb and Cd with GTA-AAS, the time temperature program must be carefully concerned. Other important factors are purity of the reagents and standards used to calibrate the AAS instrument. It is crucial to the analytical accuracy and precision of the results. In NARA's chemical laboratory, analytical reagent (AR) chemicals are used, but supra pure reagent chemicals are more suitable. Due to the high price of supra pure reagent chemicals, the manager for the laboratory decided to use AR chemicals, as this was considered adequate for the intended purpose of the analytical method at NARA's chemical laboratory. Another important factor is the water used to for prepare acids and standards as well as diluting the samples. If the water is contaminated with e.g. trace elements, it will cause poor accuracy and precision when measuring these elements in the test results. Therefore, high quality reagent water is needed.

4.1.3 Limit of detection and limit of quantification

The limit of detection (LoD) and limit of quantification (LoQ) of the analytical method were evaluated as follows. Ten blank samples were run and absorbance value recorded. Results are presented in Table 20.

Table 20: Results and calculation of LoD and LoQ for the analysis of mercury (Hg) by cold vapor atomic absorption spectros copy.

Trial No	Blank absorbance value
1	0.0019
2	0.0023
3	0.0028
4	0.0031
5	0.0038
6	0.0007
7	0.0015
8	0.0025
9	0.0052
10	0.0036
Mean value ($\bar{x}$)	0.0027
S of x value (s1)	0.0013
LoD ($\bar{x}$ + 3S), mg/kg	**0.0066**
LoQ ($\bar{x}$ + 10S), mg/kg	**0.0155**

In food analysis, especially in the analysis of trace elements and contaminants, there is a need to accurately measure low levels. Modern equipment offers excellent possibilities for this purpose. The equipment should be selected according to the level of detection. AAS technology can measure few ppb and up to ppm levels, but it unable to go beyond that to small levels like nano range. To evaluate the quality of an analytical method the limit of detection (LoD) and limit of quantification (LoQ) are frequently used. LoD and LoQ have to be determined separately for each sample type (matrix). Further the LoD and LoQ vary between different laboratories and instrument manufacturers.

The EU regulation No. 1528/7 indicates that the LoD and LoQ should be no more than one tenth and one fifth of the value of specification. The maximum allowable mercury limit of the selected fish species are 1 mg/kg and the one tenth value is 0.1 mg/kg and one fifth value is 0.2 mg/kg in wet weight basis. In this case study, LoD of Hg was calculated to be 0.0066 mg/kg and LoQ was 0.0155 mg/kg. If the sample concentrations are below LoQ, the laboratory does not necessary have to mention the LoQ in the test report. In this case the results should be expressed as below LoQ or not detected (ND).

4.1.4 Linearity and range

The linearity of the analytical method was evaluated as follows. Solutions were prepared with 5, 10, 15, 20 and 25 ppb concentration and made to a calibration curve. Results are presented in Table 21.

Table 21: Results and calculation of linearity for the analysis of mercury (Hg) by cold vapor atomic absorption spectroscopy

Standard (ppb)	Reading	Calculated value Y =0.0145*X
5	0.0675	0.0725
10	0.1433	0.1450
15	0.2162	0.2175
20	0.2888	0.2900
25	0.3676	0.3625

The graph was plotted and the coefficient of determination (R^2) value calculated (Figure 3).

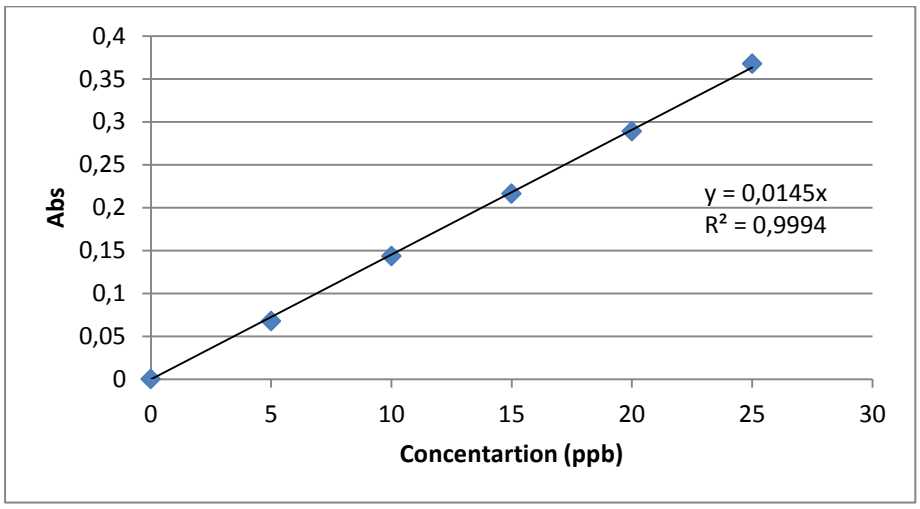

Figure 3: Calibration graph of mercury analyzed with cold vapor atomic absorption spectroscopy.

For the calculation of linearity range, standards below the first standard used to evaluate the linearity of the analytical method i.e. 1, 2, 3, 4 ppb and above the highest standard i.e. 25 to 100 ppb were run and the absorbance value recorded. Results are presented in Table 22 and Figure 4.

Table 22: Absorbance value for standards above and below the working range for the analysis of mercury (Hg) by cold vapor atomic absorption spectroscopy.

Standard (ppb)	Reading (abs)
1	0.0124
2	0.0260
3	0.0457
4	0.0598
30	0.4191
40	0.5820
50	0.6782
60	0.9230
70	1.1013
80	1.1700
90	1.2204
100	1.2928

The above data were plotted and R^2 calculated. It showed that up to 50 ppb concentration level the R^2 value maintains above 0.99 (Figure 5). Calculations based on the absorbance value of this range showed that the range of analysis for the analytical method of mercury (Hg) by cold vapor atomic absorption spectroscopy is between LoD up to 5 ppm.

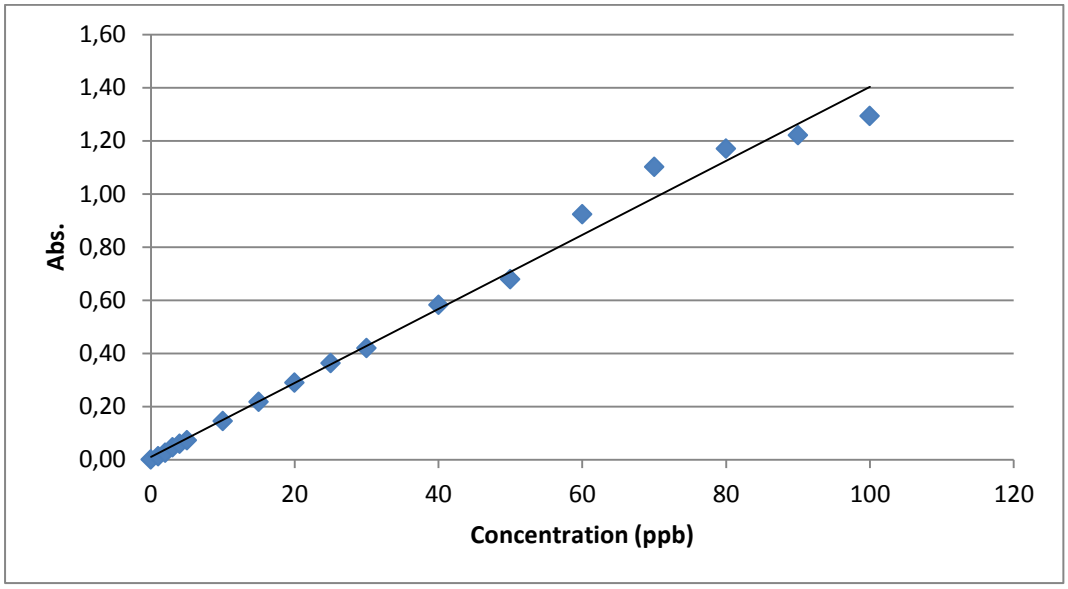

Figure 4: The graph showing the absorbance value of standards in the lower and upper working range for the analysis of mercury (Hg) by cold vapor atomic absorption spectroscopy.

For most analytical methods the working range is known from previous experience. When introducing a new method or a modification of a laboratory method it is necessary to define the analytical range. However the ideal calibration curve when using absorption technique is defined by Bear's law; according to that the absorbance of an analyte is proportional to its concentration.

The instrumental working range was obtained based on running 5 concentrations (5, 10, 15, 20 and 25 ppb). Normally the R^2 value of the calibration graph should be above 0.99 and in this case study results showed that it was 0.9994. According to the results this instrument was suitable to analysis of sample LoD up to 5 mg/kg of Hg concentration. In practice, the sample value is maintained between 20-80% of the calibration range.

4.1.5 Robustness

The main variable of interest in trace metal analysis is probably the sample matrix that may have substantial effect on ruggedness and recovery. To evaluate the ruggedness and recovery four types of fish and spiked samples were analyzed and the recovery calculated, while all other conditions were controlled. Results are presented in Table 23.

Table 23: Results and calculation of robustness and recovery for the analysis of mercury (Hg) by cold vapor atomic absorption spectroscopy.

Fish species	**Hg concentration (ppb)**	**Recovery (%)**
Yellowfin tuna	374.64	90.04
Marlin	455.56	95.21
Swordfish	886.48	95.42
Skipjack tuna	47.87	101.30
Overall recovery of the method		**95.49**
Overall standard deviation (±)		**9.81**

Variables that affect the final results are e.g. sample matrix, different acid production and digestion procedure. Sample digestion was performed by the assistance of microwave digestion method for every digestion cycle the temperature, time and pressure level were recorded and maintained according to the method application. Nitric acid (HNO_3) from Sigma chemical (produced by United States, grade-AR) was used for the trace metal analysis. This was done in order to avoid the effect of variation of acid type on the final results.

To determine the effectiveness of a method, a recovery experiment can be carried out. This case study considers only 4 species: Yellowfin tuna, Swordfish, Marlin and Skipjack tuna. The recovery value of this case study was 95.49%, which is within the acceptable range according to the criteria of EU food contaminants regulation No 2001/22/EC, where it is stated that the recovery should be 80-120%. Further validation should be carried out with other seafood and storage conditions of samples like frozen versus fresh fish.

4.1.6 Uncertainty

The uncertainty in the determination of the mercury (Hg) concentration by the cold vapor atomic absorption spectroscopy method was evaluated by addition of different concentrations of a mercury standard to the CRM. The following tests were carried out; nine CRM samples run as follow;

9 CRM sample, without spiking (set -1)
9 CRM spiked with 0.25 ml, 1 ppm Hg standard (set-2)
9 CRM spiked with 0.50 ml, 1 ppm Hg standard (set -3)

The results were calculated and presented in Table 24.

Table 24: Results and calculation of uncertainty in the determination of the mercury (Hg) concentration by the cold vapor atomic absorption spectroscopy.

	Sample-set 1	Sample-set 2	Sample-set 3
	20.9409	228.9463	555.5368
	20.7151	251.6957	485.8440
	19.7564	244.1825	508.1794
	19.9910	260.1505	524.2569
	18.8698	217.6303	535.7953
	20.2688	265.1859	525.6926
	19.1470	260.8672	516.7116
	18.9603	235.7231	484.4237
	19.5418	231.8578	484.7491
Mean value ($\bar{x}$)	**19.7990**	**244.0266**	**513.4655**
S of x value (s)	0.7467	16.5505	25.0101
RSD = ($s/\bar{x}$)*100	3.7716	6.7823	4.8709

The uncertainty calculates as follow.

RSD *analytical* calculate = $\sqrt{\boldsymbol{\varepsilon}(\boldsymbol{RSD1 + RSD2 + RSD3})}\boldsymbol{/3}$

= 2.2675

RSD sample mix weight is considered to be 10%

Thus, **RSD *total*** = $\sqrt{\boldsymbol{\varepsilon}(\boldsymbol{RSD\ analytical})^{\boldsymbol{2}} + (\boldsymbol{RSD\ sample\ mix\ weight})^{\boldsymbol{2}}}$

= 10.2539

Overall uncertainty **= 2 x RSD total**

= **20.51%**

The concept of "uncertainty" is introduced to evaluate the reliability of analysis results. There are several methods available to calculate the uncertainty. In the case study, uncertainty value calculation is based on SANCO/10232/2006 method. This is quite a simple method compared to other published methods like EURACHEM/CITAC CG4. In this method, the RSD of sample mix and weighing was considered to be 10%, including the variation of volume, weight, balance, drift of signals, noice etc. Therefore the uncertainty value using this method is always greater than 20%, and in this case study it was ±21%. The acceptance criterion is up to 32%.

Other activities to increase personal competence regarding trace element analysis

In order to increase personal competence part of the training period included participation in sample analysis at MATIS laboratories to increase knowledge and experience. Additionally, the training helped to fully appreciate the use of different analytical procedures in the method at NARA chemical laboratory. The most important lessons learned that are applicable in Sri Lanka are the following;

- Fish sample homogenization and handling for analysis of contaminants, freeze drying procedure prior to trace metal analysis using inductively coupled plasma- mass spectrometry (ICP-MS)/AAS.
- Traceability procedure for sample and standard preparation procedures.

- Cleaning procedure for the microwave components (vials) and instrument.
- The flow plan of laboratory to avoid cross contamination of standards and samples.

5 CONCLUSION AND RECOMMENDATION

The SOP procedure developed in this study is suitable for the planned method validation of activities NARA for trace metals analysis and histamine analysis in the chemical laboratory and it can be used as a basic document for the preparation of other method validations related to analytical chemistry. Base on this SOP validation of other analytical methods used at NARA chemical laboratory i.e. for trace metals such as Pb and Cd as well as for the chemical contaminant histamine are recommended.

The analytical method used for the case study i.e. Hg analysis of fish by using CV-AAS, needs additional laboratory work as dummy data were used to evaluate some method validation characteristic, nevertheless preliminary evaluated results indicate that the method is suitable for its intended purpose. In the case study only four important fish species are considered. Therefore, further work is need to evaluate whether the analytical methods suitable for other seafood as well.

As mention above, dummy data were used to evaluate some method validation characteristic in the case study and these validation characteristic have to be prepared before applying the accreditation. Further the NARA chemical laboratory should participate in a suitable international proficiency testing scheme to comply with ISO/IEC 17025.

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APPENDIX 1

Standard Operation Procedure (SOP) for the chemical laboratory, NARA

In this SOP the trace metal analysis of fish and fishery products are mainly considered, but it can be applicable for any chemical analysis with small difference. Before method validation procedure read the sample preparation and method; refer to the method manual of chemical laboratory and instrument operation procedure; refer to the SOP for the instruments.

1. Quality control procedure for method validation
   Record the value of mid of standard and maintain that within the upper and lower acceptable range.

2. Selectivity/ Specificity
   Prepare and analyse sample of following
   1. 07 CRM sample
   2. 07 CRM , spiking with 0.25 ml of 1 ppm Hg standard
   3. 07 CRM, spiking with 0.50 ml of 1 ppm Hg standard
   - Condition for other method: Run 3 levels of samples through the calibration range as low, middle and high value, spiked volume change with the range.

   Calculate the mean value ($\bar{x}$), standard deviation (s) and relative standard deviation (RSD) [$(s/\bar{x})*100$]

3. Accuracy
   Run 10 CRM sample within short time period and calculate the results
   Estimate the difference between received value and CRM value (between the acceptable ranges)

4. Precision (Repeatability and reproducibility)
   Run 10 samples of CRM and two other spiked samples (as No 2) as follows
   - Within one day, same analyst
   - Within different day, different analyst

   Calculate the $\bar{x}$, s and RSD as previous

5. Linearity and range
   Run blanks and standards as described in methods, ex. for mercury analysis of fish, 5 standards run as 5, 10, 15, 20 and 25 ppb
   Plot a graph concentration against absorbance and calculate R^2 value of the graph
   Run 6 standards below and lower the above standards and calculate to which extent that graph is linear ($R^2>0.99$), and calculate at what concentration can read between the linearity range

6. Limit of detection (LoD) and Limit of quantification (LoQ)
   Calculate the standard deviation of above standard (σ) and slope of the calibration curve (S), or run 10 blank samples or fortified samples and calculate as follows;
   - **LoD = (3.3 σ) / S**
   - **LoQ = (10 σ) / S**

7. Robustness/Ruggedness and Recovery
   Estimate what are the sources of effects onthe results

E.g. trace metal analysis by AAS: sample matrix
HPLC: pH of mobile phase, flow rate, column etc.

Run the five samples of each selected fish species in different types (e.g. lean fish, fat fish etc.) and spike the sample and calculate the recovery of each species

$$\textbf{Percentage recovery} = \frac{(\mathbf{C1 - C2})}{\mathbf{C3}} \mathbf{X\ 100}$$

C1 = corrected concentration of spiked sample
C2 = corrected concentration of non-spiked sample
C3 = concentration of spike added to sample

8. Uncertainty
   Prepare the sample as follows

	CRM	Yellowfin tuna	Swordfish	Skipjack tuna
Non spiked sample	n = 3	n = 3	n = 3	n = 3
Add 0.25 ml of 1 ppm standard	n = 3	n = 3	n = 3	n = 3
Add 0.50 ml of 1 ppm standard	n = 3	n = 3	n = 3	n = 3

Calculate RSD value as previous in each group.

$$\boldsymbol{RSD\ analytical} = \sqrt{\boldsymbol{\varepsilon}}(\boldsymbol{RSD1} + \cdots + \boldsymbol{RSD12})/\mathbf{12}$$

$$\boldsymbol{RSD\ total} = \sqrt{[(\boldsymbol{RSD\ analytical})^{\mathbf{2}}) + (\boldsymbol{RSD\ sample\ mix\ weight}^{\mathbf{2}})]}$$

Generally, RSD sample mix weight is consideredto be 10%

$$Overall\ Uncertainty = 2\ X\ RSD\ total$$

APPENDIX2

Method for sample preparation for analysis of trace metals in fish sample by microwave digester

Scope:

- ✓ Preparation of the fish sample for analysis of trace metal by microwave assisted acid digestion and analysis by AAS.

Safety:

- ✓ Avoid contact of HNO_3 with skin
- ✓ Carry out the digestion in fume hood.

References:

- ✓ Textbook of qualitative chemical analysis Vogel (5^{th} edition)
- ✓ Laboratory procedure book-International Atomic Energy Agency, Marine Environmental laboratory-MC 98000, Monaco
- ✓ CEM application note for acid digestion- sample type: Fish tissue

Principle:

- ✓ Biological sample are treated with concentrated HNO_3 in order to decompose the samples and solubilise all metals.

Reagent:

- ✓ Nitric acid (HNO_3), 65%, 'AR'
- ✓ Deionised water (>18 MΩ cm)

Instrument:

- ✓ Microwave digester (CEM)
- ✓ Electronic balance

Procedure:

- ✓ Sample should be chopped with a plastic or stainless steel knife and well mixed prior to taking the test portion.
- ✓ Weigh accurately to 4 decimal places 1g of homogenized sample into microwave digester tube (if using wet sample), unless;
- ✓ Weigh the dry clean petri dish to 4 decimal places and record the weight on the record sheet.
- ✓ Place the wet sample (thoroughly mixed minced fish sample) in duplicates, into the dried petri dish. Spread the sample evenly with spatula. Weigh the sample and dish to 4 decimal places and record the weight on record sheet. Determine the actual weight of the wet sample.
- ✓ Dry the sample in the oven at 102°C overnight (≈18 hrs); partially cover the dish with foil.
- ✓ After drying, transfer the dish to the desiccator to cool for at least 30 mins. Weigh the dried sample and dish to 4 decimal places and record the weight on the record sheet. Determine the weight of the dried sample and hence its moisture content.

- ✓ Transfer the dried sample, from the petridish into a mill or a mortar. Grind the dried sample into powder form. Mix the sample thoroughly and transfer approximately 0.5 g (weigh sample to 4 decimal places) of the sample into alabelled Teflon reactor.
- ✓ If sample needs to be spiked, the spiking solution should be added into the Teflon reactor before placing it in the microwave digester. Spiked volume is as below.

Element	Conc. of standard (ppm)	Vol. of standard added to sample (ml)
Cadmium (Cd)	1	0.25
Lead (Pb)	1	0.25
Mercury (Hg)	1	0.25

- ✓ Add 5 ml of concentrated HNO_3, and allow sample to predigest open for 15min before sealing the vessels.
- ✓ Run the microwave digestion program as given below in no 10.0.
- ✓ Allow sample to cool to room temperature then release pressure carefully by opening the valve, and then open the reactor.
- ✓ Transfer the sample into labelled 50 ml one mark volumetric flask through a No. 542 Whatman filter paper.
- ✓ Rinse the Teflon tubes and filter paper 3 times with deionised water and make up to the mark with deionised water. This solution is appropriate to determine the respective trace element by AAS.

Reagent blanks:

- ✓ At least one blank should be prepared for each batch of analysis. They are prepared in a similar manner as samples, except that no sample is added to the digestion vessels.

Reference materials:

- ✓ At least one certified reference material (or spiked sample) should be used and prepared in duplicate for each batch. These digestions are prepared in a similar manner as samples. A reference material of similar composition and concentration range should be used.

Microwave heating program:

Stage	Power		Ramp time mm : ss	Pressure (psi-limit)	Temp °C	Stir	Hold time mm : ss
	Level	%					
1	400 W	100	15.00	800	200	Off	10.00

APPENDIX 3

Method for determination of mercury (Hg) in fish by using Cold Vapour AAS technology

Scope

- ✓ Preparation of the fish sample for analysis of Mercury (Hg) by cold vapour atomic absorption method and analysis by AAS

Safety

- ✓ Avoid contact of conc. nitric acid with skin.
- ✓ Carry out the digestion in a fume hood and follow the microwave digestion safety precautions.
- ✓ Avoid spilling the standard to open laboratory sink

Reference documents

- ✓ Mercury in fish. Alternative Flameless Atomic Absorption Spectrophotometric Method. 9.2.23. First action 1977. Final action 1978. In: Official Methods of Analysis of AOAC International 2000. 17th Ed. Volume I. Chapter 9, p.36.
- ✓ Compilation of Key Regional Laboratories Validated Methods in Southeast Asia – SEAFDEC – Singapore
- ✓ Laboratory procedure book- International Atomic Energy Agency, Marine Environmental Laboratory-MC-98000, Monaco

Reagent

- ✓ Deionised water, (>18 MΩ cm)
- ✓ Nitric acid (65%), 'AR'
- ✓ 5% (v/v) HNO_3 solution {diluent}
  Dilute 50ml of conc. HNO_3 in deionised water and make up to1000 ml.
- ✓ Mercury (Hg), standard solution, 1000 mg/l,
- ✓ Primary stock solution: 1,000 mg/l (ppm)
- ✓ Secondary (i) stock solution: 10 mg/l (ppm)
  Pipette 1 ml of 1000 ppm primary stock solution into a 100 ml volumetric flask and make up with 5% (v/v) solution. This standard solution can be stored for a month in a polypropylene bottle.
- ✓ Secondary (ii) stock solution: 1 mg/l (ppm)
  Pipette 10 ml of 10 ppm secondary (i) stock solution into a 100 ml volumetric flask and make up with 5% (v/v) HNO_3 solution. This standard solution can be stored for a month in a polypropylene bottle.
- ✓ Secondary (iii) stock solution: 100µg/l (ppb)
  Pipette 10ml of 1 ppm secondary (ii) stock solution into a 100 ml volumetric flask and make up with 5% (v/v) HNO_3 solution. This standard solution can be stored for a week in a polypropylene bottle.
- ✓ Working standard solution: 5, 10, 15, 20, 25 µg/l (ppb)
  Pipette 5, 10, 15, 20, and 25 ml of 100 ppb secondary (iii) stock solution into 100 ml volumetric flask respectively make up with 5% (v/v) HNO_3 solution. These are to be freshly prepared.

Apparatus/Equipment

- ✓ Atomic absorption spectrometer (AAS-Varian)
- ✓ VGA unit (Varian) with flow through cell

- ✓ Hollow Cathode lamp (Hg-Varian)

Sample preparation

- ✓ Refer to the sample preparation method in appendix A.

AAS procedure

- ✓ Refer to VGA-AAS procedure explained below.

Calculation

- ✓ Concentration of total mercury in fish µg/g (ppm)

$$= \frac{(\text{Sample conc.} - \text{Blank conc.}) \times \text{Dilution factor} \times \text{Volume}}{\text{Sample wt}}$$

- ✓ Where,

Sample conc. = Concentration of sample in µg/l (ppb)
Blank conc. = Concentration of blank in µg/l (ppb)
Volume = Final volume of sample solution prepared (l)

$$\text{Dilution factor} = \frac{\text{Volume of diluted sample solution (ml)}}{\text{Volume of aliquot taken for dilution (ml)}}$$

Sample wt = Weight of sample (g)

Operation procedure for VG-AAS for Hg analysis of seafood

Reagent solutions:

- ✓ Deionised water (>18MΩ cm)
- ✓ Hydrochloric acid (HCl), fuming 37%, 'AR'
- ✓ Nitric acid (cleaning solution)

20% w/v $SnCl_2$ in 20% v/v HCl (200 ml):

- ✓ Weigh accurately 40 g of $SnCl_2$ into a clean glass beaker using a plastic spatula (beaker and spatula are used only for $SnCl_2$)
- ✓ Add 40 ml of concentrated HCl directly to the $SnCl_2$ and transfer to a 200 ml volumetric flask. Mix and wait for complete dissolution of $SnCl_2$.
- ✓ Add deionised water to the mark.
- ✓ With older stock of $SnCl_2$ it may be necessary to warm up the solution on a hot plate to obtain complete dissolution of $SnCl_2$ (do not allow to boil).
- ✓ In case of low concentrations samples if $SnCl_2$ is found to be contaminated, it should be purged with nitrogen for 30 mins before use.

Nitric acid 10% v/v (500 ml):

- ✓ Put about 400 mlof deionised water into a 500 ml volumetric flask.
- ✓ Add carefully 50 ml of concentrated nitric acid.
- ✓ Make up to the mark with deionised water.

- ✓ Shake well; this solution can be stored if kept in a tightly closed flask.

General operation:

- ✓ Switch on the instrument.
- ✓ Make sure the lamp is on.
- ✓ Before beginning optimization, wait approximately 15 min so that the lamp is stable.
- ✓ Optimize the lamp position without the cell in order to get maximum energy. Record the gain in the logbook.
- ✓ Optimize the burner position with the cell, the maximum energy should be read.
- ✓ Make instrument zero.
- ✓ Switch on the argon.
- ✓ Put each of the 3 Teflon capillary tubes into the appropriate solutions;
  - ✓ $SnCl_2$ solution
  - ✓ Deionised water (Acid container)
  - ✓ Rinse solution (10% HNO_3)
- ✓ Switch on the VGA and slowly tighten the pressure adjusting screw on the peristaltic pump until the liquids are pumped.
- ✓ Check that there are no leaks.
- ✓ Check the flow rate; it should be 1 ml/min for rinse solution and $SnCl_2$ and 7 ml/min for sample.
- ✓ Let the system run for about 10 min in order to clean the system. Disconnect the black tube from the quartz absorption cell if the system has not been running for a while to prevent contamination of the cell.

Calibration curve:

- ✓ Prepare standard solution with five standards plus one zero. The zero calibration is prepared as standard solution without adding the standards.
- ✓ If samples are not within the calibration curve, dilute them in the same matrix, or prepare a new calibration curve.

Running a sequence:

- ✓ Make the instrument zero without connecting the VGA to the cell.
- ✓ Connect the VGA to the cell.
- ✓ Set up the delay time (about 45 s for VGA Varian), this can be optimized under the optimized signal, aspirate a standard solution and measure the time needed to reach the maximum (stable) signal.
- ✓ Measure as sample the signal, obtained when only $SnCl_2$ and deionised water is aspirating. It should be zero.
- ✓ Measure as sample the signal, obtain all three solution are measured, it should be zero, so the next instrument zero can be done on that.
- ✓ Measure the zero calibration as a sample and record the absorbance in calibration, because while the zero calibration is set up, the instrument automatically subtracts it from all measurements. If the absorbance of the zero solution is high, it is necessary to check for the source of contamination before beginning analysis.
- ✓ Run a calibration curve.
- ✓ At least one blank and one reference material or one checks standard are measured before the sample.
- ✓ Run the samples, a zero calibration and re-slope should be measured every 5 samples.

Shutdown procedure:

- ✓ Rinse all tubing with deionised water for about 20 min. (make sure to keep separate the tube for the $SnCl_2$ solution from the other tubes).
- ✓ Turn off the VGA system and computer.
- ✓ Release the tension from the tubing.
- ✓ Turn off gas and instrument.
- ✓ Empty the waste bottle.

VGA-AAS condition:

Element	**Mercury (Hg)**
Lamp/Current	4 mA
Wave length	253.7
Slit	0.5
Measurement mode	Peak height
Integration time	20 sec
Baseline correction time	2 sec
Argon gas supply pressure	3.6 bar or 43-57 psi or 300-400 kPa
Argon gas flow rate	70-85 ml/min
Reductant solution/volume	20% w/v $SnCl_2$ in 20% v/v HCl / 1 ml/min
Calibration point	5,10,15,20,25 µg/L (ppb)
Flame condition	No flame, room temperature
Cell	Flow through cell