Technical Completion Report of a USAID-funded Project

on

Developing analytical methods for histamine, mercury and lead in fish and related products

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Abbreviations

AIFST -	Australian Institute of Food Science and Technology
CRMs -	Certified reference materials
EPA -	Environmental Protection Agency
EU -	European Union
FAO -	Food and Agriculture Organization
FAPAS -	Food Analysis Performance Assessment Scheme
FDA -	Food and Drug Administration
HACCP -	Hazard Analysis and Critical Control Points
HPLC -	High Performance Liquid Chromatography
IANZ -	International Accreditation New Zealand
IEC -	International Electrotechnical Commission
ILCP -	Inter-laboratory Competency Programme
ISO -	International Organization for Standardization
LOD -	Limits of detection
LOQ -	Limits of quantification
LOR -	Limits of reporting
NATA -	National Association of Testing Authorities
NHS -	National Health Services
PDTTI -	Provisional Daily Total Tolerable Intake
PHAMA -	Pacific Horticultural & Agricultural Market Access
QC -	Quality Control
r -	Repeatability
R -	Reproducibility

RSD -	Relative standard deviation
s -	Standard deviation
SOP -	Standard Operating Procedures
SROS -	Scientific Research Organisation of Samoa
USA -	United States of America
USFDA -	United States Food and Drug Administration
WCPFC -	Western and Central Pacific Fisheries Commission

Summary

Fish and its related products provide a substantial portion of the essential macro (fat and protein) and micronutrients that are important for a balanced diet for Pacific people like Samoans. Ensuring a sustainable food supply requires food to be available in sufficient quantity and food that must also be safe for consumption. Potential sources of toxicity related to the consumption of fish on a global scale include contamination with heavy metals like mercury and lead as well scombroid or histamine poisoning resulting from fish spoilage.

The SROS in 2011 was successful in securing funds from the United States Embassy Office in Wellington, New Zealand to the value of USD$20,000, to develop the capacity of the laboratory to test for mercury, histamine and lead in fish and related products. The specific project objectives SROS set out to achieve are:

1. To establish relevant methods to analyse for the three chemical contaminants (mercury, histamine and lead) in fish and fishery products;
2. To validate and confirm the reliability and accuracy of the test methods; and
3. To collect data for methods in development as part of the accreditation process.

Although staff established the methods in their first attempt, there was great variation in the data gathered which led to the taking of a second approach in the project. A chemistry technical expert was seconded from Cawthron Institute in New Zealand who then provided advanced training for the staff in specific method development and validation. Mercury and histamine were prioritised due to their regulated status and due to shortage of time only these two methods were fully validated.

For histamine, SOP C20 was developed, entitled "Analysis of Histamine in Fish Products using Ion Pairing HPLC". The validation parameters used were:

- Method precision – for repeatability and reproducibility;
- Accuracy; and,
- Limits of reporting (LOR) and regulatory limit.

For mercury, SOP C21 was developed, entitled "Determination of Mercury in Fish and Shellfish by Cold Vapour Atomic Absorption Spectrometer". The validation plan involved analysis for evaluation of the following parameters:

- Calibration linearity and range;
- Accuracy;
- Precision – r and R were determined; and
- LOD and LOQ.

The histamine method satisfied all the acceptance criteria used for validation, while the mercury method requires a bit more work to confirm accuracy through participation in future inter-laboratory proficiency programmes. Both methods were assessed by the accrediting body International Accreditation New Zealand in August 2014, and both were deemed fit for purpose and were recommended to be added on SROS's scope of accredited tests.

SROS now has the competency to carry out analysis for these critical contaminants in fish and should inform key stakeholders of this capacity. Trained SROS technical staff can now also look to develop and validate more methods for contaminants of health significance to add to SROS's accredited test scope in the near future.

1. Introduction

Seafood such as crustaceans and fish constitutes an important source of protein and healthy fats in the diets of Samoans and other Islanders in the Pacific. The fish are not only consumed locally by households, food outlets and hotels, but exported to overseas markets.

There is much emphasis nowadays on food security and for a small island nation like Samoa, it is important to ensure that its food supply is not only sufficient in quantity but is also safe for consumption. A food safety issue is the potential for the local catch of fish to be the source of chemical toxins, as cases have been reported and treated at the National Health Services (NHS). Globally, toxicity due to heavy metals like mercury (Hg) and lead (Pb) has significant health impacts, while scombroid or more accurately, histamine and ciguatera poisoning, resulting from spoilage is a top concern for fish from tropical and sub-tropical regions (AIFST, 2003).

In late 2011 the Scientific Research Organisation of Samoa (SROS) received funding from the Embassy of the United States of America in Wellington, New Zealand, to extend the scope of the testing capacity of its chemistry laboratory to include three chemical contaminants in fish, namely histamine, Hg and ciguatoxin. However, it was identified during the early stages of the project that the only test kit for ciguatoxin testing was no longer available, so Pb testing was added in its place.

This report starts with a brief discussion of the global and local fishery situation as well as the export market for fish. It then discusses the quality aspects of fish with particular focus on the three chemical contaminants, Hg, Pb and histamine along with their international and local regulatory limits. It then presents the objectives of the study, and the approaches taken in establishing and developing the analytical methodologies with a focus on validation and parameters used as well as the application to the International Accreditation New Zealand (IANZ) for inclusion in SROS's accredited testing scope. The validation results are then discussed in detail along with the IANZ recommendation, and the report concludes with findings and recommendations for future work.

2. Background

2.1 Global supply of fish

The production and supply of fish worldwide has improved due to sustained increases in aquaculture production as well as improved distribution channels. In 2010 capture fisheries as well as aquaculture provided the population of the world with 148 million tonnes of fish, and even higher volumes were expected in 2011 with preliminary data showing 154 million tonnes (FAO, 2012).

Table 1 shows a gradual increase in fish volume from 2006 to 2011. Fish was reported to provide around 16% of the animal protein intake of the world's population (FAO, 2012). During the same period, aquaculture contribution to the provision of fish for human consumption shows a gentle trend, and has consistently supplied around 41 to 48% of the world total, with about two-thirds from inland production.

Marine fisheries, the main sources of fish for this study, have increased markedly in supply over the past decades from 16.8 million tonnes in 1950, to 86.4 in 1996 and stabilising at around 77.4 million tonnes in 2010. This notably coincides with the increasing proportion of marine fishery resources being overexploited (FAO, 2012).

Fish provides a valuable source of protein as well as essential micronutrients that are important for a balanced diet. The trade of fish and fish products has increased globally, and the importance of providing fresh and quality fish cannot be overemphasised.

Table 1: World fishery and aquaculture production and utilisation (in 10^6 tonnes)

	2006	2007	2008	2009	2010	2011
PRODUCTION						
Capture						
Inland	9.8	10	10.2	10.4	11.2	11.5
Marine	80.2	80.4	79.5	79.2	77.4	78.9
Total capture	**90**	**90.3**	**89.7**	**89.6**	**88.6**	**90.4**
Aquaculture						
Inland	31.3	33.4	36	38.1	41.7	44.3
Marine	16	16.6	16.9	17.6	18.1	19.3
Total aquaculture	**47**	**49.9**	**52.9**	**55.7**	**59.9**	**63.6**
Total World Fisheries	**137.3**	**140.2**	**142.6**	**145.3**	**148.5**	**154**
UTILISATION						
Human consumption	114.3	117.3	119.7	123.6	128.3	130.8
Non food uses	23	23	22.9	21.8	20.2	23.2
Population (billions)	6.6	6.7	6.7	6.8	6.9	7
Per capita food fish supply (kg)	17.4	17.6	17.8	18.1	18.6	18.8

Source: FAO, 2012

2.2 Local supply of fish

2.2.1 Fishery Operations in Samoa

Both offshore and inshore fishing are undertaken in Samoa. Although Samoa has an abundance of fish species from inshore fishing, this assessment focused on offshore fish because these constitute the bulk of exports and have the higher potential for heavy metal contamination from offshore sources and histamine contamination from spoilage during transport.

Offshore fish are caught within Samoa's 120,000 km^2 exclusive economic zone (EEZ) using longline or troll fishing. Fish caught using longline include albacore tuna, bigeye tuna, yellowfin and several marlin species, which are destined for the export market. Fishing for tuna occurs all year round, but intensifies during the albacore season which is from May to October. Imo and Sua (2004) reported that 79% of Samoa's tuna catch is albacore servicing both the local and

overseas markets. Table 2 shows the annual catch figures for each species from 2007 to 2011. The fish were frozen and sold to American Samoa, New Zealand, Australia, Hawaii and mainland USA. Most of the Pacific target the export of yellowfin to the sashimi markets in USA and Japan (WCPFC, 2012).

Troll fishing (Trolling) uses baited lines towed through the water to catch fish. The fish caught include skipjack tuna, yellowfin tuna and other pelagic fish. This type of fishing mostly services the local fish markets, associated with much smaller catches compared to longline and sales at the ports upon landing (Imo and Sua, 2004). Although fishery yields have declined in Samoa over the years because of the rising cost of imported fuel, fish continue to be the major commodity export for Samoa. Albacore is by far the most predominant catch for Samoa, followed by Yellowfin and Bigeye. Overfishing is not a reported issue for Samoa compared to the global situation.

Table 2: Longline annual catch estimates (metric tonnes) for Samoa for years 2007-2011.

SPECIES	2007	2008	2009	2010	2011	Total
Albacore	3,113	2,342	2,816	2,529	1,415	12,215
Yellowfin	305	317	412	386	395	1,815
Bigeye	101	106	117	107	71	502
Skipjack	40	31	77	66	51	265
Stripped Marlin	21	21	7	16	4	69
Black Marlin	13	15	13	15	5	61
Blue Marlin	21	16	9	6	7	59

Source: WCPFC, 2012

2.2.2 Export

Fish over the past years have been a major export earning commodity for the country, as shown in the 12 months from October 2011 to September 2012 in table 3. The Central Bank of Samoa in October (2012) reported fresh fish exports for the previous twelve months to be valued at $11.1M, highlighting the importance of this commodity as a foreign currency earner for Samoa.

Table 3: Fresh fish export for Samoa (total value in SAT$000, fob)

	Oct 11	**Nov 11**	**Dec 11**	**Jan 12**	**Feb 12**	**Mar 12**	**Apr 12**	**May 12**	**Jun 12**	**Jul 12**	**Aug 12**	**Sep 12**
Month	Oct 11	Nov 11	Dec 11	Jan 12	Feb 12	Mar 12	Apr 12	May 12	Jun 12	Jul 12	Aug 12	Sep 12
Volume (MT)	179	223	107	105	91	90	166	153	168	180	113	193
Total Value	1,069	1,283	662	657	571	560	1,011	995	1,399	1,079	701	1,115
Unit Value	5,966	5,754	6,214	6,239	6,246	6,226	6,099	6,504	8,345	5,994	6,211	5,783

Source: Central Bank of Samoa, 2012

2.3 Fish quality and safety

The global as well as local data indicate fish to be a highly sought after and traded commodity. The importance of fish for both trade and provision of nutrients for good health, either as fresh produce or processed food, cannot be overemphasised. For this reason the quality and safety issues surrounding fish and its derived products are quite stringent, especially when trying to enter into certain overseas markets like the European Union (EU) and Japan.

2.3.1 Fish Contamination

The fishing industry has adopted the use of Hazard Analysis and Critical Control Points (HACCP), a preventative approach to ensure food remains safe for human consumption after exposure to potential physical, chemical and biological hazards throughout the production process. Fish and seafood can be contaminated by various inorganic [e.g., Pb, Hg, cadmium

(Cd)], organic (e.g., dioxins, insecticides) and process–related (e.g., drug residues, sulphites) compounds (Suppin *et al.,* 2005). It can also be contaminated through the action of spoilage bacteria as in the case of histamine development in fish.

Results from past toxicological and environmental studies have prompted the need to test food for toxic elements. Mercury, Pb and histamine are some of the potential toxins commonly associated with consumption of fish and their derived products. Heavy metals like Pb and Hg are conservative pollutants as they cannot be broken down by bacteria, thus essentially making them permanent pollutants. Animals and plants can regulate their metal content to a certain extent, however metals that cannot be excreted will build up or bio-accumulate within these organisms (Clark, 2001).

2.3.1.1 Mercury

Mercury is a toxic environmental pollutant which can be found in three different forms, namely metallic element, inorganic salts and organic compounds (e.g. methyl mercury, ethyl mercury, phenyl mercury) (Rahimi and Behzadnia, 2011). The health problems associated with mercury and its compounds in fish are well known, with many studies and cases confirming the adverse health effects of contaminated seafood (Groth, 2005; Augelli *et al.,* 2007). Mercury is released into the environment in its inorganic form by natural sources (e.g., volcanoes) and human activities (e.g., waste incineration), and later converted to the organic form, methyl mercury ($Hg(CH)_2$), by bacteria in water and marine sediments (Rahimi and Behzadnia, 2011). This form is then readily up-taken by aquatic organisms and is bio-magnified in food webs through the actions of predatory and larger fish. Methyl mercury diffuses easier into animal fatty tissues and can also form soluble compounds making it the most health hazardous form of mercury (Augelli *et al*., 2007).

The United States Food and Drug Administration (USFDA) standards for methyl mercury recommend the limit for human consumption to be 1 ppm, making it a regulated food toxin. This concentration is frequently recorded in large fish predators such as swordfish, sharks, marlin, king mackerel and certain species of large tuna. Mercury poisoning affects the human

neurological system with children, pregnant mothers and females of child bearing age being the most vulnerable group (AIFST, 2003, Rahimi and Behzadnia, 2011). Reaction towards toxic doses of the poison includes paresthesia (numbness and tingling sensation around lips, fingers and toes) followed by stumbling and incoherent speech to physical impairment in worst cases (Suppin *et al*., 2005; Clark, 2001).

2.3.1.2 Lead

Lead is present in quite a few everyday products like paints, food wrappers and containers, insecticides, dyes as well as natural soil (AIFST, 2003). It was also a main additive to petrol as well as solder used to make cans, but these additives are now prohibited in both of these products thus reducing consumer exposure. Ground fish which feed mostly from the bottom of water bodies were also discussed by Srebocan *et al*. (2001) as a source of Pb contamination in predatory fish. Fish are also known to absorb Pb from contaminated waters. So in light of its extensive use, large quantities of Pb have been dispersed in the environment and eventually seeped into the surrounding oceans to contaminate the marine resource.

Lead is listed as one of the toxic elements of concern in food because when ingested by humans and animals, impairment of the nervous system could follow. USFDA does not regulate Pb levels in food products but have set a Provisional Daily Total Tolerable Intake (PDTTI) for high risk groups. Children are especially susceptible to Pb exposure due to high gastrointestinal uptake, and toxic levels cause learning disabilities and they shorten the life span of red blood cells leading to anaemia. So although the levels of Pb in food stuff are not at an alarming level the food regulators are still taking the precautionary approach in ensuring that allowable levels are maintained as low as possible.

2.3.1.3 Histamine

Scombroid poisoning or histamine fish poisoning is one of the most prominent form of food borne illnesses associated with consumption of fish (predominantly from Scombroidae family).

Histamine is produced post capture of fish, through the actions of contaminating bacteria under abusive conditions for temperature and time (AIFST, 2003,). For this reason histamine usually serves as an indicator of the freshness of the fish, and when present in very high levels become a health hazard. The most common fish implicated in scombroid poisoning are tuna, mackerel, herring and sardine, which all have naturally high levels of histidine. A range of bacterial species including *Morganella morganii*, *Morganella psychrotolerans, Klebsiella pneumoniae* and *Enterobacter aerogene* are capable of converting histidine to histamine with *M. morganii* being the most prolific histamine producer (Shin-Hee *et al*., undated; Fletcher, 2010).

HACCP principles if applied properly will eliminate the occurrence of histamine from handling and storage. To control histamine production, fish must be kept at 4.4 °C or lower to reduce growth and activity of spoilage bacteria that produce histamine (USFDA, 2001). USFDA set a maximum recommended level of 50 ppm for histamine while UK set a maximum mean limit of 100 ppm histamine in fish portions tested. Histamine is not uniformly distributed throughout the fish's body and if the tested portion contains 50 ppm, there is a possibility that other parts of the body have much higher concentrations (Fletcher, 2010). Levels exceeding 500 ppm are highly likely to be harmful to consumers. The symptoms from this poisoning will depend on the sensitivity of the individual to histamine but include nausea, and flushing as well as allergic type reactions.

2.3.2 Samoa General Food Standards

The proposed Food Regulations and Standards for Samoa (National Codex Committee, 2012) have combined the Food Hygiene Standard and Food Labelling Standard, and imposes standards applicable to all food available in the country (imported, processed and exported). Part 2 of these Food Regulations for General Food Standards contains schedules one and six which specify the following limits for the contaminants of interest:

- Schedule one on "maximum permitted levels of chemical contaminants" stipulates permitted levels of **Hg** in fish and fishery products to range from 0.5 to 1 mg/kg (depending on produce);

- Schedule one also indicates that the maximum level of allowable **Pb** for fish and various fishery products to range from is 0.2 – 1 mg/kg (depending on produce); and
- Schedule 6 indicates that for **histamine** no fish product shall exceed 20 mg per 100 g.

The above levels provide a guide on the sensitivity required of test methods as well as preferred detection limits in order to cater for our local food standards.

2.3.3 Overseas Regulations and Guideline levels

The three contaminants are regulated at slightly different levels by different international regulatory bodies (table 4).

Table 4: Overseas guidelines and regulations on permitted levels for histamine, Hg and Pb.

Guideline Source	Product	Histamine	Hg	Pb
FDA/EPA Safety Levels in Regulations and Guidelines - Sec 540 525	Tuna, mahi mahi and related fish	50 mg/kg defect action level 500 mg/kg based on toxicity		
	All fish		Methyl mercury – 1 mg/kg	
	Clams, oysters, mussels			1.7 mg/kg

2.4 Analytical method development and validation

The reliability of results provided by analysts is critical to not only the clients or decision makers but also the regulators involved. For this reason any test method developed must be fit for purpose, and method validation provides the objective evidence that the requirements for a particular intended use are satisfied (NATA, 2009).

SROS's microbiological and chemical laboratories are already accredited to ISO/IEC 17025 for testing laboratories, and it is a requirement of this Standard that laboratories demonstrate the validity of all methods whether they be in-house or modified standard methods. If standard methods are employed without modifications, then they need to be verified (NZS, 2005; IANZ, 2008).

The extent of the validation required however depends on the status of the method under consideration and the needs relating to its intended applications (NATA, 2009). The performance characteristics selected for the validation exercises undertaken for histamine Hg and Pb are briefly described below.

2.4.1 Validation parameters

The NATA Technical Note 17 (2009) and the IANZ Technical Guide 5 (2003) detail the guidelines for the validation and verification of chemical test methods from which the following parameters were selected.

a) Linearity: the range of instrumental response (y) as a function of concentration (x) is linear within a stated range and calculated using the equation $y = a + bx$. A simple plot of the data should indicate the nature of the relationship and the acceptable range is $r^2 > 0.99$.

b) Accuracy: this measures the quality of the result and has two components:
   i) Precision is a measure of random error in measurements, reflected by two aspects, repeatability (r) and reproducibility (R), and it is usually stated as the standard deviation or

relative standard deviation (RSD) of replicate measurements. Repeatability refers to tests performed under the same conditions (same analysts, lab, time, etc), while reproducibility refers to a series of measurements made under different conditions (different dates, labs, analysts, etc). Acceptable ranges for r and R are %RSD < 10 and < 15, respectively.

ii) Trueness describes how close a result is to the accepted reference value for the quantity measured. This requires an understanding of the various sources of bias affecting trueness of results which are attributable to the method used, the laboratory itself as well as the specific analytical run. Analytical recovery is a bias usually associated with sample preparation steps before determination. Analysing certified reference materials (CRMs) or spiking samples with known concentrations are the usual ways of determining bias in a method. A percentage recovery between 80 -120 % is an acceptable range as a measure of trueness. Participating in various inter-laboratory proficiency programmes (ILCP) for tests also gives an indication of how close results are to accepted reference values.

c) Limits of detection (LOD) – this is the lowest amount or concentration of an analyte which can be determined with acceptable level of uncertainty using a method and it is greater than the uncertainty associated with it.

d) Limits of quantification (LOQ) – this is the lowest concentration of analyte which can be determined with an acceptable level of uncertainty and it is commonly recommended to be equal to three times the LOD. The LOD and LOQ validation parameters are required for samples with low concentration of analytes, for example heavy metals like Hg and Pb.

There is a large list of possible sources of variation which affect results, with some being random and others systematic in nature. The combined effect of these variations for each method needs to be determined at the typical analyte levels of samples, before a method is guaranteed to be fit for purpose (IANZ, 2008).

3. Project Objectives

The objectives of the project are as listed below:

1. To establish relevant methods to analyse for the three chemical contaminants (Hg, histamine and Pb) in fish and fishery products;
2. To validate and confirm the reliability and accuracy of the test methods; and
3. To collect data for methods in development as part of the accreditation process.

4. Project Approach

Because the technical staff had experience in adopting standard methods and/or developing in-house analytical methods, the first approach was to internally develop the three relevant test methods and use them for analysis and data collection. The staff encountered difficulties in getting reliable and consistent results because of the sensitivity level required of the methods. A second approach was then taken, whereby an external expert for chemistry analytical methods was sought to assist in method development and validation.

4.1 Approach 1

Newly developed in-house methods face the difficult requirement of having to be vigorously validated. The Standard Operating Procedures (SOP) developed in-house included modifications to existing methods validated elsewhere. These methods were developed and trialled to analyse for Hg, histamine and Pb are not included in this report as they were substantially reviewed and amended for use in Approach 2.

4.2 Approach 2

A chemist from Cawthron Institute in New Zealand, a provider of research based advice and analytical services, conducted two separate training sessions of three weeks duration, in method development and validation for elements and histamine. The Australian programme for Pacific Horticultural & Agricultural Market Access (PHAMA) funded all the cost associated with the chemist's travel and contracted services, while this project paid for all the required consumables and equipment.

The training was provided to chemistry staff involved in the daily analysis of samples for technical services and involved the following:

1. establishment and validation of a method for histamine;

2. review of existing analytical methods for elements including heavy elements Hg and Pb, and implement and validate appropriate methods; and
3. development and implementation of quality control procedures relating to these tests.

The precision (repeatability, reproducibility) of results and spike recoveries achieved were the key parameters used to assess the validity and applicability of the methods developed. A validation plan was developed for each method, incorporating samples from the inter-laboratory proficiency programmes (ILCPs) along with spiked samples for method recoveries. All fish fillets used were bought from the local fish market and supermarkets.

4.2.1 Histamine analysis method

The SOP C20 for the histamine method, entitled "Analysis of Histamine in Fish Products using Ion Pairing HPLC", was developed. The validation plan involved extraction and analysis of histamine to determine the following methodology parameters:

a. Method precision – for repeatability and reproducibility;
b. Trueness; and
c. Limits of reporting (LOR) and regulatory limit.

4.2.2 Mercury analysis method

The SOP C21 for the Hg method, entitled "Determination of Mercury in Fish and Shellfish by Cold Vapour Atomic Absorption Spectrometer", was developed. The validation plan involved analysis for evaluation of the following parameters:

a. Calibration linearity and range;
b. Trueness;
c. Precision – r and R were determined; and
d. LOD and LOQ.

4.2.3 Lead analysis

Priority was given to histamine and Hg analysis due to the relatively higher incidence of these contaminants, so the chemist only had sufficient time to review the testing protocol developed in-house. He made recommendations concerning its further development with considerations given to the capabilities of the laboratory in terms of staffing and equipment.

4.2.4 Application for IANZ accreditation

At the end of the training by the chemist, sufficient time was provided for SOP amendments, data collection as well as further validation of both methods. An application was then sent with all the relevant documents to IANZ to request for the new chemistry methods (histamine and Hg) to be audited and added to SROS's accredited scope of tests.

5. Results & Discussions

5.1 Approach 2 Results

The results produced using the amended SOPs during the chemist's training are discussed in detail below.

5.1.1 Histamine analysis results

a) Method precision

For repeatability and reproducibility samples from three different species of fish (albacore, skipjack and yellowfin) were analysed for three consecutive days in triplicates (Table 5).

Results for albacore and skipjack samples showed good repeatability and reproducibility over the 3 days. An issue resulting from a carry over effect was identified in Day 1 from the use of the same blender for yellowfin after analysing albacore that had higher concentrations. The procedure for cleaning the blender between extractions was reviewed and amended after Day 1, and resulted in consistent results which were all lower than the reporting limits of the method.

An interesting observation is that yellowfin species of tuna appears to be less susceptible to histamine contamination compared to albacore and skipjack.

Table 5: Histamine method r and R results

Sample:	Albacore (mg/kg)			n	Mean	S.D.	RSDr
	4497	4519	4439	3	4485	41	0.91%
	4575	4489	4490	3	4518	49	1.08%
	4000	4293	4434	3	4242	221	5.21%
				Mean	4415		2.40%
				RSDr	3.0%		
				RSDR	4.6%		
Sample:	**Skipjack (mg/kg)**			**n**	**Mean**	**S.D.**	**RSDr**
	10545	10593	10687	3	10608	72	0.68%
	11351	11245	10959	3	11185	203	1.81%
	11902	11234	10791	3	11309	559	4.94%
				Mean	11034		2.48%
				RSDr	3.1%		
				RSDR	4.6%		
Sample:	**Yellowfin (mg/kg)**			**n**			
	58	10	5	3			
	<5	<5	<5	3			
	<5	<5	<5	3			

b) Trueness

Two samples from the FAPAS proficiency programme was analysed (table 6) and the same samples were analysed by the Cawthron Institute for comparison (table 7). For assessing method recovery, samples of yellowfin were spiked at a concentration equivalent to the regulatory limit of 50 mg/kg.

Results for both FAPAS samples were within the acceptance range on two of the three days of testing and all results were at the higher end of the acceptable range. It is possible the high bias may be attributable to poor sample handling (e.g., leaving the sample at room temperature for extended periods). The method reproducibility of the two FAPAS samples over the three days was very good, with a %RSD of 3.2 and 4.0%, respectively.

Table 6: Results for FAPAS proficiency samples

Date of analysis	SROS result FAPAS 27132 Histamine mg/kg	SROS result FAPAS 27137 Histamine mg/kg
5/6/14	137	225
6/6/14	146	243
7/6/14	141	232
Mean	**141**	**233**
SD	4.4	9.4
% RSD	3.2	4.0
Assigned value	126.7	212
Acceptable range	107.2 – 143.6	182 - 242

Table 7: Comparing SROS results with Cawthron Institute, NZ

Sample	***SROS***	***Cawthron***	***% difference***
FAPAS 27132	141.4	148	-4.7
FAPAS 27137	233.3	233	0.1
Albacore	4497	4623	-2.8
Skipjack	10545	10649	-1.0
Yellowfin	57.5	51	11.3

The method results compared well with those obtained by Cawthron Institute (table 7). Comparable amounts for histamine were determined for the various samples by the two laboratories. This further confirms the accuracy of the method as well as the suspected contamination of the yellowfin sample when first homogenised as detailed in table 5.

The results showed good recoveries with the first two days being much better than the last day's recovery (table 8). This three-day validation trial also provided valuable training exercise for the staff on all aspects of this test. Amendments were made to the SOP, including modifications to the extraction and cleaning procedure, post-run data processing and quality control requirements.

From a validation perspective the only short-coming was in the concentration levels measured. Because the skipjack and albacore samples were of such a high concentration and not of a typical level, a fourth single day analysis was instigated to focus on low concentration levels (5 and 50 mg/kg) at the range of LOR and regulatory limits.

Table 8: Histamine spike recoveries for yellowfin samples spiked with 10µg/ml

Date of analysis	Unspiked concentration µg/ml	Spiked concentration µg/ml	Spike recovery (%)
5/6/14	0.66	10.30	96.1
6/6/14	1.58	11.19	95.0
7/6/14	1.86	10.08	82.2

c) LOR and regulatory limits

A fresh fish sample was spiked at 5 mg/kg (1 µg/ml on the instrument) and 50 mg/kg (10 µg/ml on the instrument). Three replicates were analysed at each concentration. The low spike, 5 mg/kg is expected to be the LOR, while the 50 mg/kg spike is at the regulatory limit.

This trial was conducted after the training period and so was carried out by the staff themselves. It was a good test of the SROS staff's understanding and competence with the procedure while working on their own and as indicated in table 9, the recoveries and repeatability at the two concentrations were found to be very good.

The histamine method overall produced good consistent results within the acceptable ranges for R (<15% RSD) and r (<10% RSD). The method recovery was also good and covered both the regulatory limit as well as the expected LOR.

Table 9: Results for low concentration analysis of histamine

	Unspiked µg/ml	10 µg/ml	1 µg/ml
	0.023	10.04	0.98
		9.97	0.92
		10.13	0.94
mean		10.05	0.95
Std dev		0.081	0.029
%RSD		0.8%	3.1%
Spiked value		10	1.0
recovery		100%	95%

5.1.2 Mercury analysis results

a) Calibration: Linearity and Range

The linearity of the calibration curve for the method developed was determined for concentrations ranging from 0.001 – 0.20 mg/L, with triplicate readings taken for three consecutive days. The calibration curve was found to be linear over the calibration range, with a coefficient determination $R^2 > 0.996$ (figure 1). Thus the instrumental response as a function of concentration indicates the suitability of the method to be used within this range (0.001 – 0.20 mg/L). The NATA (2009) guidelines however recommend the use of six or more standards that are evenly spaced over the expected concentrations for samples. Having only four standards was a limitation to the applicable range and the number should be extended in the future.

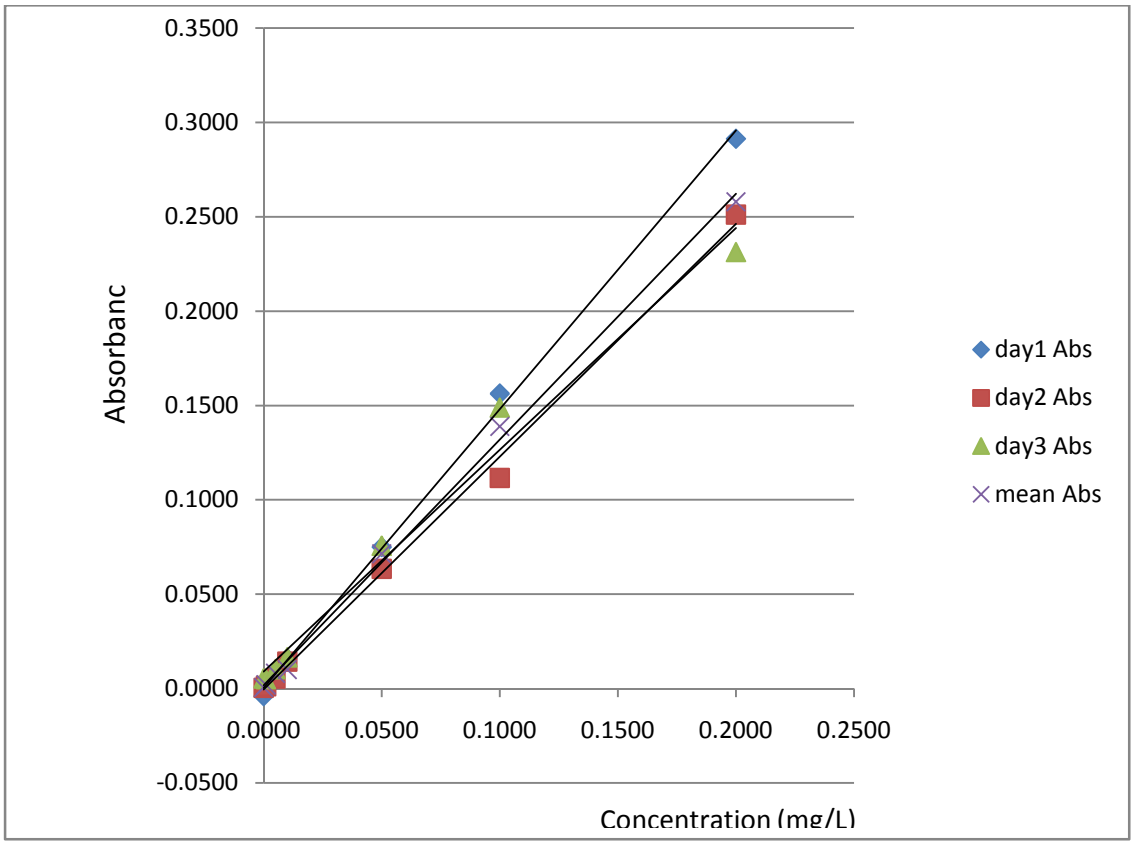

Figure 1: Method linearity results in the concentration range of 0.001 to 0.20 mg/L

b) Trueness

For method trueness, four reference samples with known values were analysed: DORM-4 (commercial QC), FAPAS 07189 (inter-laboratory proficiency sample), fishmeal QC (FMQC) and mussel powder (from Cawthron Institute). For evaluation of recovery two mussel powder samples were spiked with a 0.2 mg/kg of Hg. The recovery for the spiked samples showed a bit of variation but was still within the acceptable range (table 10). The reference samples also indicated variable results with the FMQC sample achieving the highest trueness when comparing daily results. The DORM 4 sample only had one result out of three that was close to the actual known value. Steps to achieve improvements were noted and incorporated for the dilution steps for the SOP.

From this trial it was determined that new ILCP samples and a certified reference material need to be procured for further investigations of the method trueness.

Table 10: Results for recoveries of spiked samples and accuracy using reference samples

Samples	Content Determined (mg/kg)				Actual Content (mg/kg)	Recovery (%)
	Day 1	Day 2 Spiked	Day 3	Unspiked		
T17368		0.390		0.23		85
T15644		0.36		0.15		105
Reference samples	Day 1	Day 2	Day 3	Mean		Std Dev
Dorm 4	0.30	0.30	0.47	0.36	0.41	0.09
FAPAS 07189	219	275	408	301	303	97.1
FMQC	0.49	0.50	0.65	0.55	0.48	0.10

c) Precision

The r of the method was determined by analysing three replicates for one species of fish (skipjack) and the FAPAS 07189 sample on the same day (table 11). For R another three replicate samples of the same were analysed on two consecutive days.

Table 11: Repeatability (r) results

Sample	Replicate			Mean	STD Dev	% RSD
	1	2	3			
Skipjack tuna	0.23	0.22	0.24	0.23	0.01	4
FAPAS 07189	0.38	0.43	0.42	0.41	0.03	7

The repeatability of the method averaged at 6% RSD and this is within the acceptance criteria of % RSD <10 (Table 11). The reproducibility averaged at 10% which is also within the acceptance criteria of %RSD < 15 (table 12).

Table 12: Reproducibility (R) results

Samples	Day 1					Day 2					RSD (%)
	R1	R2	R3	Mean	Std Dev	R1	R2	R3	Mean	Std Dev	
FAPAS 07189	0.293	0.308	0.223	0.275	0.045	0.358	0.171	0.145	0.225	0.116	14
Skip jack tuna	0.143	0.142	0.159	0.148	0.009	0.183	0.172	0.123	0.160	0.032	6

d) Limit of Detection/Limit of Quantification

For LOD, six replicates with low concentration of 0.001 mg/L were digested and analysed. The standard deviation of the six results was then multiplied by 3.33 to get LOD. The LOQ was obtained by LOD multiplied by the typical weight-volume ratio used in the method, which is 33.33. The LOD and LOQ established for the method are 0.003 and 0.100 mg/kg, respectively, which is below the minimum regulatory limit of 0.5 mg/kg.

For the Hg method, although most of the above results were acceptable, the method trueness still needs to be confirmed through the laboratory participation in the ILCP programme for Hg.

5.2 IANZ Accreditation for mercury and histamine methods

On the 6th and 7th of August 2014, an IANZ assessor and a technical expert conducted a follow up assessment after the full audit in November 2013, specifically to look at SROS's application for test scope extension. The two new methods analysing for histamine and Hg as below-listed:

- Analysis of Histamine in Fish and Fish Products by HPLC (In-house Method); and
- Determination of Hg in Fish and Shellfish by Atomic Absorption (USEPA 245.6 modified),

were submitted with supporting data for consideration by IANZ. The assessors recommended that these two methods be accredited upon the clearance of two simple corrective actions, which will be reviewed during an upcoming routine assessment in November 2014.

Thus the recommendation for accreditation for the above by the IANZ assessors confirms conformity with the requirements of NZS ISO/IEC 17025: 2005, the IANZ published Procedures and Conditions for Accreditation and relevant applicable technical criteria.

6. Conclusions & Recommendations

Monitoring for food contaminants as stipulated by food standards cannot be implemented without the capacity to accurately determine their levels in food sources. Histamine and Hg are two regulated contaminants which can affect the global trade of fish and related products.

This project has further strengthened the capability of SROS as an internationally accredited laboratory, to enable testing for Hg and histamine contaminants in food. It has also built staff capacity in the areas of method development and validation.

For the histamine method sufficient data were gathered to allow the method precision ($r < 10\%$ RSD; $R < 15\%$ RSD), trueness as well LOR and regulatory limits to be confidently determined within the accepted ranges and expected reporting limits. The Hg method showed a linear calibration curve for concentration ranges from 0.001 to 0.20 mg/L. This range needs to be further extended to cover the local allowable regulatory limit of 1 mg/L. Although precision in terms of repeatability and reproducibility were acceptable, the trueness of the method needs to be further investigated through participation in more ILCP programmes. The LOD and LOQ attained for the method were also acceptable.

The recommendation by the IANZ assessors to include the two methods in SROS laboratory accredited scope of tests is evidence that they were well developed and validated. All in all, the two analytical methods were considered fit for purpose because they satisfied the selected validation parameters. SROS is also now in a position to support the regulators in monitoring the proposed Food Regulations for Hg and histamine in fish and related products.

This newly acquired capacity should be made known to key stakeholders in the fishing and other relevant industries for projects of mutual interests to be undertaken. The SROS staff now needs to use the skills and knowledge acquired from this project to develop and validate more analytical methods which can test for other contaminants of health significance (e.g., Pb, Cd, etc). This will benefit the country as a whole, by providing local capacity to monitor more contaminants in food and the environment in general.

REFERENCES

AIFST (2003) *Foodborne microorganisms of public health significance,* 6th edition, AIFST Incorporated, Australia.

Augelli, M.A., R.A.A Munoz, E.M. Richter, M.I. Cantagallo, and L. Anges (2007) 'Analytical procedure for total mercury determination in fishes and shrimps by chronopotentiometric stripping analysis at gold film electrode after microwave digestion'. *Food Chemistry* 10(1), pp. 579 -584.

Central Bank of Samoa (2012) Update on the Samoan economy- First twelve months of 2011/2012, First two months of 2012/13, and outlook for 2012/13. http://www.cbs.gov.ws.

Chesher, D. (2008) 'Evaluating Assay Precision'. *Clinical Biochemical Review*, Vol 29, Supl (i).

Clark, R.B (2001) *Metals in: Marine Pollution*, 5th edition, pp. 98 -125, Oxford University Press, Oxford.

FAO (2012) State of World fisheries and aquaculture, FAO Fisheries and Aquaculture Department of Food and Agriculture, Rome. http://www.fao.org/docrep/016/i2727e/i2727e00.htm.

FAO (2012) FAO Fisheries & Aquaculture –Fish contaminants. http:www.fao.org/fishery/topic/14815/en.

FDA & EPA (2011) FDA & EPA safety levels in regulations and guidance, 3rd edition.

Fletcher, G.C. (2010) Research of relevance to histamine poisoning in New Zealand – A review. MAF Technical Paper: 2011/70, Ministry of Agriculture, New Zealand.

Groth, E. (2005) Risks and benefits of fish consumption: Yes, Mercury is a problem. Oceania and Mercury Policy Project.

IANZ (2003) Technical guide -Uncertainty of Measurement, Precision and Limits of Detection in Chemical and Microbiological Testing Laboratories, International Accreditation New Zealand.

IANZ, 2008. Technical Guide:Working Thermometers Calibration Procedures, 2nd edn.

Imo, R.T. and S.T. Sua (2004) Samoa tuna fisheries report. Samoa Fisheries Division, MAFFM, Apia, Samoa.

NATA (2009) Technical Note 17 - Guidelines for the validation and verification of chemical test methods. National Association of Testing Authorities, Australia.

National Codex Committee (2012) Food Regulations and Standards for Samoa (draft), Apia, Samoa.

NZS ISO/IEC 17025:2005, General Requirements for the Competence of Testing and Calibration Laboratories

Rahimi, E. and A. Behzadnia (2011) 'Determination of Mercury in Fish (*Otollithes ruber)* and canned tuna fish in Khuzestan and Shiraz, Iran'. *World Applied Sciences Journal* 15 (11), pp 1553 -1556.

Srebocan, E., J. Pompe-Gotal, A. Prevendar-Crnic and Z. Spacir (2001) 'Effect of sublethal lead concentrations in feed on α-aminolevulinic acid dehydratase activity in young carp'. *Veterinarski Arhiv* 71 (6), pp. 337 – 344

Suppin, D., R. Zahlbruckner, C.H. Krapfenbauer-Cermak and C.H. Hassan-Hauser (2005) 'Mercury, lead and cadmium content of fresh and canned fish collected from Austrian retail operations'. *Nutrition:* Vol 29/NR 11.

USFDA (2001) Guidance for industry, Bioanalytical method validation, US Department of Health and Human Services, Food and Drug Administration, Centre for Drug Evaluation and Research, USFDA, USA.

WCPFC (2012) Annual report to the commission. Part 1: Information on fisheries, research and statistics, Scientific Committee eighth regular session, WCPFC, Busan, Korea.