THE DETERMINATION OF NITROIMIDAZOLE RESIDUES IN FISH AND FISH PRODUCTS

by

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Abstract

A method was developed and validated for the determination of nitroimidazoles (NIs) including 2-hydroxymethyl-1-methyl-5-nitroimidazole (HMMNI), ipronidazole (IPZ), 1-methyl-2-(2'-hydroxyisopropyl)-5-nitroimidazole (IPZ-OH), metronidazole (MNZ), 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole (MNZ-OH), ronidazole (RNZ) and dimetridazole (DMZ) in fish and crustaceans by ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS). The compounds were extracted with acidic acetonitrile, followed by solid phase extraction (SPE) C18 clean up and hexane washing. The validated method has a linear range from 0-50 ng/g in target representative species (tilapia, salmon and shrimp), with LOD from 0.07 – 1.0 ng/g and LOQ from 0.21 – 3.0 ng/g, depending on the analyte. Recoveries ranged from 87 to 121% for the analytes of interest. Method precision ranged from 6 to 26% RSD with HorRat values within typical limits of acceptability. The method successfully analyzed rainbow trout samples treated with MNZ in a depletion study under controlled conditions and is suitable for use in a regulatory monitoring program for residues of nitroimidazoles in seafood and aquacultured products.

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LIST OF ABBREVIATIONS

Chemicals

CIPRO	Ciprofloxacin
CV	Crystal Violet
DANO	Danofloxacin
DMZ-D3	Deuterated Dimetridazole
DMZ	Dimetridazole
ENRO	Enrofloxacin
HMMNI	2-Hydroxy-1-Methyl-5-Nitroimidazole
FLUM	Flumequine
IPZ	Ipronidazole
IPZ-OH	Hydroxy Ipronidazole
LCV	Leucocrystal Violet
LMG	Leucomalachite Green
MG	Malachite Green
MeCN	Acetonitrile
MeOH	Methanol
MNZ	Metronidazole
MNZ-OH	Hydroxy Metronidazole
NaCl	Sodium Chloride
NAL	Naladixic acid
NI	Nitroimidazole
OXO	Oxolinic acid

RNZ	Ronidazole
SARA	Sarafloxacin
TNZ	Tinidazole

Other

ADI	Acceptable Daily Intake
ANOVA	Analysis of Variation
APCI	Atmospheric Pressure Chemical Ionization
BEH	Ethylene Bridged Hybrid
BIO	Bedford Institute of Oceanography
CAC	Codex Alimentarius Commission
CCα	Decision Limit
CCβ	Detection Capability
CED	Clinical Evaluation Division
cELISA	competitive Enzyme – Linked Immunosorbent Assay
CFIA	Canadian Food Inspection Agency
CUSUM	Cumulative Sum
CVMP	Committee for Veterinary Medicinal Products
DAD	Diode Array Detector
Da	Dalton
DFO	Department of Fisheries and Oceans
EC	European Commission
ECD	Electron Capture Detector
ESC	Experimental Studies Certificate

ESI	Electrospray Ionization
FAO/WHO	Food and Agriculture Organization/ World Health Organization
FLD	Fluorescence Detector
GC	Gas Chromatography
h	Hours
HPLC	High Pressure Liquid Chromatography
HSD	Human Safety Division
HSS	High Strength Silica
IS	Internal Standard
ISO/IEC	International Organization of Standards / the International Electrotechnical Commission
JCGM	Joint Committee of Guides in Metrology
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantification
LOU	Letter of Understanding
MIP	Molecular Imprinted Polymer
MRL	Maximum Residue Limits
MS	Mass Spectrometry
MU	Measurement of Uncertainty
m/z	Mass to Charge Ratio
NPD	Nitrogen Phosphorus Detection

pp	polypropylene
RSD	Relative Standard Deviation
R^2	Correlation Coefficient
SD	Standard Deviation
SPE	Solid Phase Extraction
TOF	Time of Flight
UPLC	Ultra Performance Liquid Chromatography
UV	Ultra Violet

Chapter 1 – Introduction

The aquaculture (fish farming) industry has experienced rapid growth during the last decade (Diaz-Cruz et al., 2003). Infectious diseases are a major concern in this industry because of potential negative impacts on production and the potential for disease impacts on wild populations (Johnston and Santillo, 2002). In natural systems there is a low prevalence of infection, but in the aquaculture industry where stresses lower resistance and stocking density facilitates transmission of disease, impacts of disease outbreaks can be severe. Disease management therefore depends on chemotherapeutic agents. Even though chemical usage is widespread in the aquaculture industry, chemical residues have received little attention.

Nitroimidazoles are a group of veterinary drugs which have been used to treat infections in food producing animals (Huet et al., 2005). They are also used in the treatment of intestinal infections in fish caused by flagellates of the genus *Hexamita,* which has been associated with high mortality in young salmonids as well as aquarium fish and other marine species (Tojo and Santamarina, 1998). The most frequently used nitroimidazoles, dimetridazole (DMZ), ipronidazole (IPZ), metronidazole (MNZ) and ronidazole (RNZ), are suspected of being genotoxic, carcinogenic and mutagenic, as are their hydroxy metabolites which retain the original nitroimidazole ring (Huet et al, 2005). Their molecular structures can be seen in Figure 1.1.

Health Canada scientists have reviewed the toxicity data submitted by manufacturers and assessed the risks and benefits of the use of nitroimidazoles in food producing animals (Health Canada, 2003). Due to concerns raised about the safety of residues found in food products from animals treated with the 5-nitroimidazole class of

drug, it was considered prudent to ban the sale of this class of drug for administration to food producing animals, the sale of treated animals for food use and the sale of food products derived from treated animals, although some of these drugs remain available for therapeutic use in humans.

The Fish Program of the Canadian Food Inspection Agency (CFIA) has identified residue testing for the nitroimidazoles as a priority in their action plan with regard to EU requirements for fish exports (CFIA internal communication). The EU has required the CFIA to have a residue monitoring plan in place for nitroimidazoles in aquaculture products under Council Directive 96/23/EC (EC, 1996). To avoid trade disruptions, CFIA has committed to validating a method of analysis for nitroimidazoles in fish and fish products. The purpose of the validation studies is to establish method performance and demonstrate 'fitness for purpose' (CFIA Dartmouth Laboratory, SOP-DAR-CHE-001-00, 2009), including meeting requirements for inclusion of the method within the "scope of accreditation" of the Dartmouth Laboratory under ISO-17025 (ISO, 2005) as administered by the Standards Council of Canada.

To follow through with CFIA commitments and complete a thesis project, a two year study was initiated to satisfy the following objectives:

- Critical assessment of existing information on analytical methods for nitroimidazole residues which may be applicable to fish tissues and on the distribution and depletion of nitroimidazole residues in fish.
- Development and validation of a confirmatory method for the determination of nitroimidazole residues in tilapia, salmon and shrimp muscle tissues.

- Determination of stability of nitroimidazoles in solution, during sample processing/analysis and in fish tissue under typical conditions of storage.
- Depletion study of nitroimidazoles in muscle tissue on fish species under controlled conditions.

Based on these objectives a simple, rapid and robust method will be validated and used to monitor aquacultured products for the presence of nitroimidazole residues in a regulatory environment. Information from the depletion study will enable interpretation of residue findings.

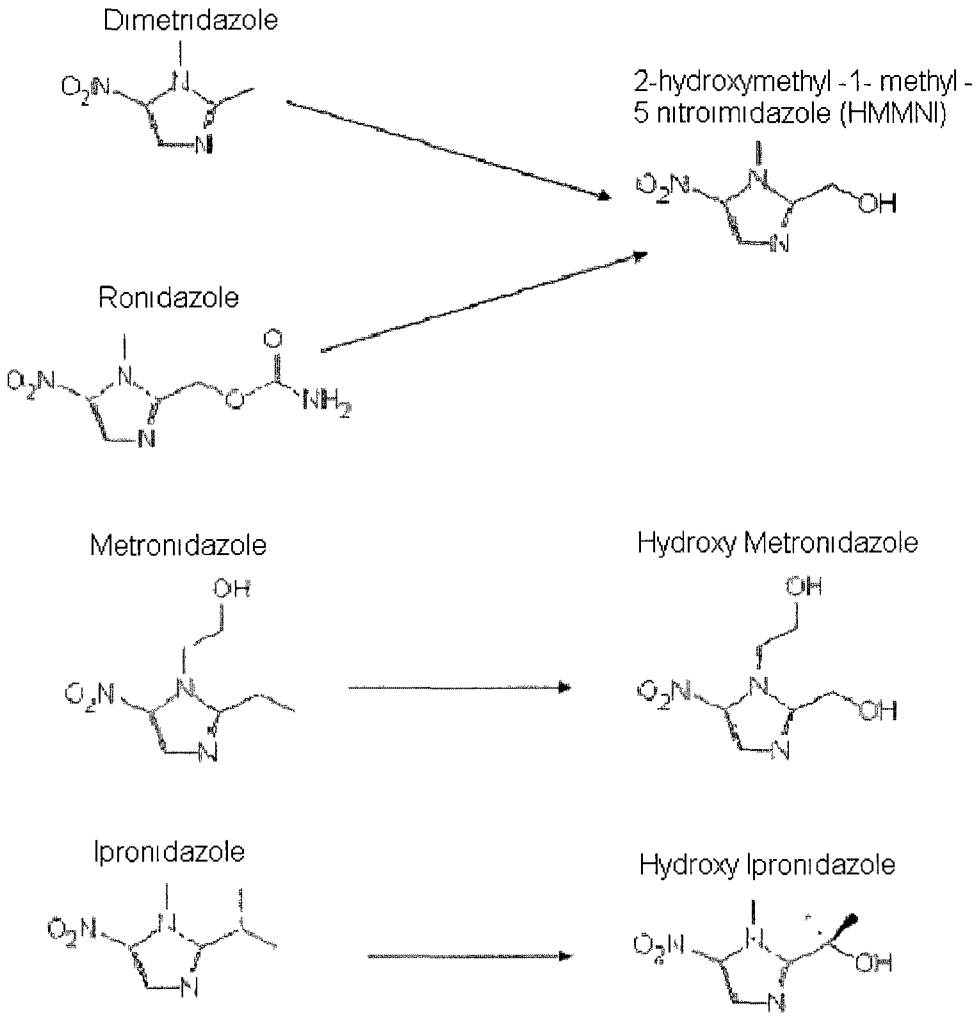


Figure 1.1 Chemical structures of nitroimidazole compounds and metabolites.

Chapter 2 – Literature Review

2.1 Background

An antibiotic complex isolated from a strain of streptomyces in Japan in 1953 was identified as 2-nitroimidazole in 1955 (Anon., 1978). This discovery lead to the preparation and testing of the more readily accessible isomeric 5-nitroimidazoles. In 1957 these isomers were found to be more active antiprotozoal agents than the natural product. The main properties of these compounds that account for their therapeutic success are their selective uptake by and cytotoxic action on anaerobic organisms (Lindmark and Muller, 1976). Their toxicity for aerobic microorganisms has been found to be low.

Metronidazole (MNZ) was determined to give the best compromise between activity and toxicity and was introduced as Flagyl (Anon., 1978). It became well established as the first agent to be systematically effective against many protozoa. During the 1960s, many therapeutic claims were made for treatment of many conditions in humans. The most significant claim was for the treatment of acute ulcerative gingivitis. MNZ has also been useful in preventing post operative infection caused by susceptible anaerobes. It has been a difficult drug to improve upon (Voogd, 1981). Resistance among trichomonids, for example, has very rarely, if ever, been responsible for therapeutic failure.

Research of nitroimidazoles has been concerned with important veterinary uses as well as applications in human medicine (Research Article, 1978). Dimetridazole (DMZ), which is more toxic than MNZ, was selected for treatment in animals (Voogd, 1981). Many other nitroimidazole compounds have been synthesized and investigated for therapeutic or prophylactic applications. Tinidazole, nimorazole and ornidazole are

applied as drugs in human medicine, while ronidazole (RNZ) and ipronidazole (IPZ) have veterinary applications.

Several nitroimidazoles, including DMZ, IPZ, MNZ and RNZ, were reviewed in a report by the Joint Food and Agriculture Organization / World Health Organization (FAO/WHO) Expert Committee on Food Additives (WHO, 1989), which recommends international standards for permitted concentrations of food additives, contaminants and residues of approved veterinary drugs in foods to the Codex Alimentarius Commission (CAC). The committee noted that there was increasing concern by regulatory authorities regarding the presence of residues of nitroimidazoles in food. They encouraged industry groups and national authorities to generate the necessary toxicological and residue data for safety assessment. They stressed the importance of examining the possible hazard to human health arising from the ingestion of residues of antimicrobial agents administered to food producing animals. Due to the gaps in available data, the committee was unable to establish an Acceptable Daily Intake (ADI) for these compounds, so no Maximum Residue Limits (MRLs) have been established for nitroimidazoles in foods by the Codex Alimentarius Commission.

DMZ, RNZ and MNZ are banned from use in food producing animals in the EU, US and China (Mottier et al., 2006). IPZ has never been authorized for use as a veterinary drug and is also considered a banned substance. The use of nitroimidazoles in food-producing animals (including fish) has been banned in Canada under the Food and Drug Act and Regulations (Department of Justice Canada, 2008).

2.2 Toxicity of Nitroimidazoles

The mutagenic action of nitroimidazoles against bacteria has been well documented for many years. The antimicrobial action of 5-nitroimidazoles is believed to be due to the metabolic reduction of the nitro group by microbial metabolism (Schmid and Schmid, 1999). A review by Voogd in 1981 showed that MNZ was the most investigated compound of the nitroimidazoles for mutagenicity and noted that MNZ was found to be mutagenic to bacteria. In a study by Voogd et al. (1974), DMZ was found to have similar activity to MNZ, but less than RNZ which even at low concentrations exerted a pronounced mutagenic effect against three types of bacteria. The activity of the hydroxy metabolite of MNZ has been shown to have approximately 65% of the activity of the parent drug on bacteria and the activity of MNZ was enhanced when combined with its hydroxy metabolite (Pendland et al., 1994). The reduction products of MNZ also cause DNA damage if the nitro group is reduced in the presence of DNA (Voogd, 1981). Studies have demonstrated that these drugs induce mutations even in bacteria resistant to their killing activity.

The mutagenic effect of nitroimidazoles on mammals has been less clear. However, in one study serum levels attained in man after therapeutic treatment with MNZ were sufficient to cause mutagenic activity in bacteria (Voogd, 1981). Mutagenic activity to bacteria can also be demonstrated in the urine of human subjects after treatment (Conner et al., 1977). The activity detected in the urine was significantly higher than could be accounted for by the presence of the administered drug. Chromatographic analysis indicated the presence of a metabolite which was found to be ten times more mutagenic than MNZ. A much lower dose of MNZ was required in humans than mice for significant amounts of metabolite to be detected in the urine. The metabolite was reported

to represent 35-40% of the nitroimidazole excreted in urine of treated patients. These findings not only established concern for use of the parent compounds, but also the metabolites that are formed.

In contrast, little or no mutagenic effect was shown on mammalian cells *in vitro* with MNZ (Voogd, 1981). When the potential toxicity of RNZ residues present in the tissues of food-producing animals was assessed, the data demonstrated that although RNZ is a potent mutagen, residues from it which may be present in the tissues of animals lack any mutagenic activity and are probably not hazardous to humans who consume them (Wilslocki et al, 1984). Reduced derivatives of DMZ were also shown to lack mutagenic activity. In other investigations, no teratological effects due to MNZ or most other nitroimidazoles have been observed (Elizondo et al., 1994). In a genotoxic study on human lymphocyte cells, MNZ did not induce changes in sister-chromatid exchanges (Voogd, 1981).

Even though no mutagenic activity was observed in mammalian cells *in vitro*, reduction products are formed by microbes in the gut or by mammalian cells under anaerobic conditions. Acetamide is a metabolite generated from MNZ by microbes in the gut of mammals and is carcinogenic to rats if applied in high dosages. MNZ was found to be carcinogenic in mice and rats, and dimetridazole in rats. MNZ was found to induce lung tumors in mice and its metabolism is similar in mice to that in humans (Voogd et al., 1974). It was concluded that long term effects on humans are not known and genetic damage cannot be excluded (Voogd, 1981). With the very mutagenic nitroimidazoles, serious genetic and carcinogenic effects are probable. Therefore, the recommendation was the prevention of unnecessary exposure of humans to these drugs. It was clear by 1981

that the data reviewed were beginning to cause serious concern about the safety of nitroimidazoles. The review paper by Voogd (1981), in particular, emphasized the importance of studying nitroimidazoles for their safety.

Another study suggested that there are reduced nitroimidazoles which are responsible for toxicity to mammalian cells (Noss et al., 1988). The metabolites retaining the nitroimidazole ring were found to be carcinogenic and mutagenic in some species (Mottier et al., 2006). Nitroimidazoles have been shown to be differentially cytotoxic towards hypoxic mammalian cells (Silver et al., 1986). A dose-dependant reduction of the nitro group in animals and aerobic microorganisms can be caused by very high doses of 5-nitroimidazoles. This explains the tumors found in laboratory animals exposed to high doses of nitroimidazole drugs (Schmid and Schmid, 1999). Research indicates that MNZ is considered to be mutagenic and cytotoxic in fish (Maher et al., 2008). However, it should not be assumed that therapeutic doses of all 5-nitrimidazoles pose a genotoxic hazard (Schmid and Schmid, 1999).

Genotoxic activity of MNZ was evaluated *in vitro* (human lymphocyte cells) and *in vivo* (mouse bone marrow cells) by Mudrey et al (1994). A genotoxic effect indicated that MNZ is a direct mutagenic and chromosome damaging agent. It was recommended that the consumption of MNZ should be controlled in order to protect the public health from its mutagenic/carcinogenic risk. Genotoxic effects of MNZ were evaluated in the hamster embryo and it was concluded that MNZ is capable of transmission of potential genotoxic effects to the fetus (Garry and Nelson, 1987). It was apparent that MNZ needs further evaluation for potential adverse reproductive outcomes. In reproductive studies, many 5-nitroimidazole compounds are reported to inhibit spermatogenesis and cause

infertility (McClain and Downing, 1988). Data from studies with nitroimidazoles suggest that these agents may be genotoxic as they induced chromosomal aberrations in human lymphocytes *in vitro* and *in vivo* at high doses (Committee for Veterinary Medicinal Products, 2008)

The most recent review of the toxicology of MNZ found in the literature search, published in 1994, discussed the major findings relating to biotransformation, genotoxicity and carcinogenicity of MNZ (Dobias et al.). The paper concluded that the present data available are insufficient to permit an accurate evaluation of the complete risk posed by MNZ to human health. There are many papers concerning MNZ genotoxicity, but further definitive studies are required before the real risks of MNZ on human health can be ascertained. MNZ is a chemical agent of great concern based on its toxicity and has been identified as a priority for future research, including analytical determination (Johnston and Santillo, 2002).

In the proceedings of a meeting of an expert group organized by the aquaculture authority, Government of India, it was stressed that the unscientific use of antibiotics, including nitroimidazoles, can have adverse impacts on human health and also the environment (The Aquaculture Authority, 2002). Fish farming is an important source of drugs in the environment. Even though it has been estimated that around 70% of the drugs administered in aquaculture are released into the environment, very little information is available about the fate and the potential effects of these drugs in the environment (Diaz-Cruz et al., 2003). In recent years it has become clear that the use and disposal of medicinal substances may have adverse effects on the environment (Lansky and Halling-Sorenson, 1987). The acute toxicity of MNZ was tested on freshwater and marine

organisms. The study demonstrated the potential ecotoxic effect of MNZ, suggesting the need for further investigations of the environmental effects resulting from exposure to medicinal substances.

The literature reviewed that is related to the toxicity and carcinogenicity of nitroimidazoles in bacteria, animals and humans and the potential impact on the environment emphasizes the need to monitor the use of these drugs in food producing animals to prevent unnecessary exposure for consumers. The importance of monitoring these drugs is reinforced by the contradictions and unknowns of the risks of nitroimidazoles in the reported studies. A great deal of unknown effects appears to be related to the oxidative and reductive products involved in the metabolism of nitroimidazoles. For this reason, metabolites as well as parent compounds need to be analyzed.

2.3 Stability of Nitroimidazoles

To provide an effective program for the control of nitroimidazole use in food-producing animals and their products (including fish and fish products) the conditions under which sampling takes place are of utmost importance (Polzer and Gowik, 2005). Factors such as matrix, temperature conditions and homogeneity of the sample have to be taken into account. These conditions can contribute significantly to the ability to detect the illicit use of substances. The investigation of information on stability of nitroimidazoles is important for the accuracy of results. It defines the requirements for storage of standards and also for the shipment and storage of laboratory samples.

The literature was assessed for existing information on the stability of nitroimidazoles in both biological matrices and solution. A working paper prepared by

scientists in the European Union notes that nitroimidazole residues are unstable in animal muscle, although no supporting data were provided (EU, 2006). In studies with pig muscle and plasma, good stability of both the DMZ and its metabolite HMMNI were shown for several days at 4°C and several months at -30°C (Carignan et al., 1988a). Conversely, thermal stress was shown to have an extreme impact on the concentration of nitroimidazoles in turkey muscle (Polzer and Gowik, 2005). Immediate freezing of muscle tissue samples after sample collection is essential for effective residue control. Stability studies of with DMZ, MNZ, RNZ and IPZ in treated turkeys demonstrated that nitroimidazoles are not stable in muscle or liver at room temperature and decelerated degradation is seen at 4°C (Polzer et al., 2004). Plasma and retina samples are stable under the same storage conditions and storage at -24°C considerably slows down degradation of analytes.

In a slightly different stability study, standards were mixed with plasma, rather than using incurred samples (Gibson et al., 1994). These were stored at -20°C and showed no loss of imidazole compounds (MNZ and its hydroxyl and acid metabolite and TNZ) after storage for as long as 2 weeks.

Another study was found to be particularly interesting since nitroimidazole residues are normally tested on raw tissue, but we normally eat our food after it is cooked (Rose et al., 1999). This study found that there is little evidence of any instability of DMZ, RNZ and their metabolite in chicken during normal cooking. In model aqueous and lipid solutions, DMZ and its 2-hydroxy metabolite, were relatively stable for times and temperatures normally encountered during cooking. RNZ in hot aqueous solutions was converted to the 2-hydroxy metabolite. This reinforces the idea that food for human

consumption should be tested for nitroimidazoles residues as they are not destroyed during the cooking process.

Stability of standards varied depending on the solvent used and storage temperature. Stock standards of DMZ and deuterated DMZ in methanol were stable for at least 3 months when stored at 4°C in amber colored vials, while dilute standard solutions prepared from stock solution in methanol were stable for at least a month when stored at 4°C (Cannavan and Kennedy, 1997). RNZ, MNZ and DMZ stock solutions were prepared in 50/50 v/v water/acetonitrile and stored at 4°C for a maximum of 1 month (Daeseleire et al., 2000). Another study showed that DMZ stock standard in acetonitrile-water (50/50 v/v) was stable for at least 8 months at -18°C (Mortier, 2005a). Standards of HMMNI (2-hydroxymethyl-1-methyl-5-nitroimidazole) and IPZ hydroxyl metabolite, IPZ-OH, prepared in ethyl acetate are stable for at least one year if refrigerated (United States Department of Agricultural Food Safety and Inspection Service, 2005). Stability for 1 year at 4°C was also seen in stock standards of RNZ, MNZ, IPZ, HMMNI, IPZ-OH and MNZ-OH (MNZ hydroxyl metabolite) in methanol (Fraselle et al., 2007). Intermediate and working standards from stock made up in 0.1% acetic acid/acetonitrile (93:7) were stable at 4°C for 6 months. Stock standard solutions of MNZ, DMZ, RNZ and TNZ in methanol stored in the dark at -18°C can be used for 2 months. The working standard should be prepared daily (Zhou et al., 2007). Lastly, two studies stored various nitroimidazole stock standards made up in methanol in dark glass bottles at -20°C for 6 months. The intermediate standard made up from stock with MeOH was stable for 2 months at 4°C in the dark and the working standard in methanol was good for 2 weeks in one study (Xia et al., 2008) and for 2 months in the other (Xia et al., 2006b).

The information on the stability of different nitroimidazoles in different solvents at various temperatures is useful. Although because of the variability reported in different studies, stability studies were repeated using conditions specific to the thesis project. Unfortunately, no information on the stability of these drugs in fish tissue was in any of the literature. Therefore, it was considered important to study the stability of nitroimidazoles in fish tissue under typical conditions of storage. Results of studies using muscle tissues of different species may be used as a guideline. Also, several of the methods recommend storage of standards in amber glassware or in the dark. However, there was no information specifically stating that the nitroimidazoles are susceptible to degradation by light with the exception of one paper. Hurtaud-Pessel et al. (2000) stated that nitroimidazoles are very light sensitive and therefore it is essential to protect the solutions and the extracts from light. This variable required further investigation.

In a recent paper by Xia et al (2009), stability of nitroimidazoles was determined in solvent, in matrix (swine kidney) and in final sample extract. For stability in solvent, stock solutions were tested monthly by injection of freshly prepared working solutions. Six kidney samples were fortified with nitroimidazoles (5 $\mu g\ kg^{-1}$), among which three were analyzed immediately and the others were stored at -20°C for four weeks before analysis to assess stability in matrix. To study the stability in extract, fortified samples were analyzed, then the final extracts were stored at -20°C for 7 days, thawed at room temperature, then analyzed again. There was no significant difference ($p > 0.05$) observed under the storage treatments of nitroimidazoles in solvent, matrix or final sample extract. A similar approach to this was followed to determine stability of nitroimidazoles in the

thesis project to better define requirements for storage of nitroimidazole standards and for shipment and storage of laboratory samples of fish species.

2.4 Depletion and Distribution of Nitroimidazoles

Depletion studies can be useful in determining the probable illegal treatment regimens with nitroimidazoles which may lead to detectable residues. Such studies were previously used before the ban of nitroimidazoles to determine withdrawal times required for residues in foods to be considered safe. During medication with DMZ, the mean concentration of the drug and its major metabolite depend on the animal species and the tissue (Carignan et al., 1988a). In pork muscle, the total concentration of drug and metabolites bearing the nitro group declines rapidly within hours after the medicated feed is withdrawn; within 72 hours it is below the limit of detection. A study to monitor the elimination of DMZ and its major metabolite in swine plasma and tissue showed very little HMMNI and no DMZ in the liver after 2 hours (h) of withdrawal (Carignan et al., 1988b). No drug or metabolite could be detected at 49 h in any of the tissues, including muscle and kidney. Most values for both substances found in muscle were close to those in plasma. The muscle of swine given DMZ in feed for 14 days contained no detectable levels of HMMNI at 12 h withdrawal time (Newkirk et al., 1990). DMZ, RNZ and HMMNI are all rapidly eliminated from poultry muscle tissue, with no detectable residues present after 5 days following the withdrawal period (Rose et al., 1999). In turkeys treated with DMZ, the analyte and hydroxy metabolites could be detected for a longer period of time in plasma and retina than in muscle and liver (Polzer et al., 2004).

After a withdrawal period of 5 days, DMZ and its metabolites can still be found in the muscle and liver.

DMZ is absorbed from the gastrointestinal tract in treated turkeys. About 88% of the administered dose is eliminated from turkeys within 3 days, whereas about 76% is eliminated from pigs within 7 days (Committee for Veterinary Medicinal Products, 2008). In depletion studies on turkeys and pigs, tissue-bound residues were evaluated using radiolabelled DMZ. About 50% of the total radioactivity was not extracted. In another depletion study summarized in the CVMP report, DMZ and its metabolite could be detected in skin/fat of pigs until 9 days and in turkeys until 12 days after treatment.

Depletion rates could potentially be much longer if bound residues are not extracted (Health Canada, 2003). Bound residues are a concern for the determination of nitroimidazole residues. There are insufficient data on the depletion rate of bound residues of the 5-ntiroimidazole drugs to enable appropriate withdrawal periods.

Nitroimidazole residues in eggs are the least related to the thesis project; however it was decided to include the information in case it may be useful. Nitroimidazoles are rapidly incorporated into eggs of laying hens, with residues detected 1 day after commencement of treatment (Rose et al., 1999). Depletion studies have shown that after one dose, residues are present for up to 8 days. After a single oral dose of IPZ, RNZ and DMZ to laying hens, residues of parent compound and/or the hydroxylated metabolites could be detected in eggs 5-8 days after dosing (Aerts et al., 1989). These findings are consistent with those reported for another veterinary drug, lasalocid, where it was demonstrated that exposure to the drug results in deposition of residues in eggs during

their formation, so that such residues are seen in eggs produced for up to 10 days after removal of the exposure to the drug (Kennedy, Hughes & Blanchflower, 1998).

Information on distribution of residues identifies appropriate tissues for analysis. Some distribution information for nitroimidazoles has been included with the depletion studies, such as the mention of bound residues. More information on distribution is included in the following. In turkeys treated with DMZ, MNZ, RNZ and IPZ, analytes were found to be present in considerably higher amounts in plasma and retina compared to muscle and liver (Polzer et al., 2004). Nitroimidazoles in incurred muscle samples showed a considerable inhomogeneity in their distribution (Polzer and Gowak, 2005). An inhomogeneous distribution of IPZ and RNZ and their metabolites in turkey muscle samples is seen irregardless of sampling procedures. For effective residue control, lyophilization is recommended to achieve homogeneous muscle tissue samples. After administration of feed containing DMZ to laying hens, residues will appear in both egg white and yolk (Kan and Petz, 2000). Deposition of the drug in egg white and yolk requires intestinal absorption and transport via blood (plasma). For both DMZ and its metabolite, much higher concentrations are found in the albumin than the yolk (Mortier et al., 2005b).

Information on the depletion and distribution of MNZ and MNZ-OH in trout was reported by Sorensen and Hansen (2000). Trout were given feed containing MNZ in an aquaculture pilot plant. Residues of MNZ and MNZ-OH were detected in muscle and skin tissues shortly after the administration period, but not 3 weeks later. The concentration levels in muscle and skin tissue were not significantly different. A MNZ depletion study was also done on tilapia. Farm raised adult tilapia were treated with MNZ for 5 days

followed by a 5 day withdrawal period (Maher et al, 2008). Relatively high levels of MNZ were present in the muscles during dosing; these levels decreased during withdrawal, however were still present at the end of the 5 day withdrawal period. This was the only information found on this topic for fish. Both of these fish depletion studies were taken into consideration when planning the study as part of the thesis project.

2.5 Metabolism of Nitroimidazoles

The 5-nitroimidazoles are rapidly metabolized with the main metabolite of DMZ, IPZ, and MNZ resulting from the oxidation of the side chain in the C-2 position of the imidazole ring (Rose et al., 1999). RNZ has a different degradation pathway and shows only a minor metabolite containing the imidazole ring, identical to that of DMZ. The hydroxylated derivative of DMZ has been recognized by the 34th Joint FAO/WHO Expert Committee on Food Additives (JECFA) as the major metabolite in tissues (WHO, 1989). This assessment was also used by the Committee for Veterinary Medicinal Products (CVMP, 2008). From measurements of the parent drug and the corresponding main hydroxyl-metabolite in various incurred materials from turkeys, it can be concluded that HMMNI should be chosen as the target analyte to prove a treatment with DMZ and the metabolite IPZ-OH used as proof for IPZ, while the parent drug is to be preferred for RNZ and MNZ (Polzer et al., 2004). In trout treated with MNZ, the fraction of the metabolite to the parent was found to be low, leaving the parent drug the analyte of interest (Mottier et al., 2006). Sorensen and Hansen (2000) had previously reported similar findings in trout treated with MNZ. They found the fraction of MNZ-OH to MNZ was less than 2% on the first day after the administration period. This ratio of MNZ-OH

to MNZ was also observed by Maher et al (2008) in tilapia with incurred residues resulting from treatment with MNZ.

During the plateau period for drug residues in eggs for DMZ and its metabolite, the metabolite/parent compound ratio equals 2.6 +- 0.2. This clearly indicates that the hydroxyl metabolite must be included when performing residue analysis for DMZ (Mortier et al, 2005b). In depletion studies of laying hens DMZ and IPZ were extensively metabolized to hydroxylated nitroimidazole metabolites; RNZ was excreted mainly as the parent compound (Aerts et al., 1991). From these studies of varying matrices it appears the parent drug is the target analyte for RNZ and MNZ residue monitoring. However, the metabolites HMMNI and IPZ-OH for DMZ and IPZ, respectively, are the target analytes. This was important information to consider for the thesis project and emphasizes the need to not only analyse for parent compounds, but to consider metabolites as well.

2.6 Methodology for the Determination of Nitroimidazoles

Methodologies for the determination of nitroimidazoles have been described for some time. In reviewing the literature on nitroimidazoles, the topic of methodologies was the most important for the project. JECFA reaffirmed the importance of reliable analytical methods in the regulatory control of veterinary drug residues in food-producing animals (WHO, 1989). The committee strongly recommended that increased efforts should be made to validate such methods and to obtain validation data. Few such data were available for consideration at the meeting.

Validation data provide information to determine if a method is fit for purpose (CFIA Dartmouth Lab, SOP-DAR-CHE-001-00, 2009). In order for a method to be fit for

purpose certain performance characteristics must be evaluated and met. The requirements for a quantitative method include the following parameters: analytical range; linearity; selectivity; matrix effects; limit of detection (LOD) and limit of quantification (LOQ); accuracy; stability of analyte in standard solution, matrix and extract; repeatability of detection system and method; intermediate precision; decision limit (CCα) and detection capability (CCβ); measurement uncertainty (MU) and ruggedness. The method to be validated is tested, modified and optimized as required to obtain the desired values for the parameters listed.

When new methods are developed, method selection, development, modification and adaptation (including characteristics of the analytical range, linearity, selectivity, initial demonstration of recovery and precision and preliminary LOD/LOQ) must be completed prior to validation activities. Method ruggedness must be determined and critical control points identified before method validation commences. Analytical methods selected for validation and implemented for regulatory use may be chosen from previously published methods in the scientific literature, methods supplied by technical organizations, drug or equipment manufacturers or from "in-house" methods development, but in all cases optimized parameter data must be demonstrated prior to routine use of the method.

To ensure human food safety, the development and improvement of residue analysis is an important task (Xia et al., 2007). Due to banning of the use of nitroimidazoles in food-producing animals, residue monitoring requires the sophisticated analytical techniques of gas chromatography (GC) or liquid chromatography (LC) coupled to mass spectrometry (MS) to provide confirmation of residue identity (Huet et

al., 2005). Owing to its high specificity and sensitivity, MS is well suited for confirming nitroimidazoles. LC coupled with MS or tandem MS (MS-MS) has become the most powerful approach for determining antibiotic residues in food (Xia et al., 2008).

The less selective methods such as GC coupled with nitrogen-phosphorus detection (NPD) or LC coupled with fluorescence detection (FLD) can be useful for preliminary screening of samples for nitroimidazole residues, followed by the use of a confirmatory method for non-compliant samples (Thompson et al., 2008). The European Commission considers that a confirmatory method "means methods that provide full or complementary information enabling the substance to be unequivocally identified and if necessary quantified at the level of interest" (European Commission, 2002).

Another important aspect of method development is investigating solvent extraction of the homogenized tissue followed by liquid partitioning and /or a solid phase extraction (SPE) clean up step. Tissue samples contain many diverse compounds in addition to possible traces of target analytes (Maher et al., 2008). It is very important to extract as much as possible of the target analytes and also to exclude interfering substances present in biological matrices. The majority of current methods employ a SPE step in order to purify the extract (Cronley et al., 2009). Extraction and clean up procedures selected from the literature were therefore identified to be evaluated and modified, as necessary, to determine the most appropriate procedures.

2.6.1 Methodology Using Gas Chromatography

In reviewing analytical methods for the determination of nitroimidazoles, GC methods predated and accounted for much fewer methods compared to LC. GC is now

less popular due to the required derivatization step to create volatile analytes. The derivatization step also results in the same derivatization product from RNZ and HMMNI (Wang, 2001). Most GC methods have been replaced by LC-MS due to lack of requirement for derivatization, applicability to parent compounds and metabolites, excellent detection capabilities and the potential to combine multiple classes of compounds in a single method. However, even though LC is the preferred technique for analysis of drug residues, it is not available in all labs. Two of the GC methods found are used in surveillance programs in other countries (Wang, 2001; Ho et al., 2005). It is important to know how other countries are monitoring these residues. In the first paper a method is developed and described for the determination of DMZ, RNZ and MNZ in chicken meat by GC-NPD. The author concludes the method is suitable for statutory residue testing and is used as a quick screening method in the National Residue Surveillance Plan in China. The other paper describes a method for the determination of DMZ and MNZ in poultry muscle and liver, and porcine liver and kidney by GC-MS. The method provides the means for fast turn around time and high sample throughput. The method was adopted for use in food surveillance programs in the Hong Kong Administrative Region.

Another GC-MS method reviewed presented a method that is well suited as a screening method for the surveillance of DMZ, RNZ, MNZ, IPZ and their corresponding metabolites in turkey and swine muscle (Polzer and Gowik, 2001). Furthermore, DMZ, RNZ and MNZ in muscle samples of turkey and swine can be confirmed by this method. An earlier publication described the determination of HMMNI in swine muscle by GC electron capture detector (ECD) (Newkirk et al., 1990). This method evaluated the SPE

clean up step and conducted recovery studies on the overall procedure as well as at various stages of the procedure. Single compound methods were widely used in the 1980s and 90s, before many were replaced by LC and LC/MS multi-nitroimidazole methods. LC-MS is the preferred method for the thesis work as it is readily available in the laboratory. Other aspects of these papers may however be useful, such as the extraction and clean up procedures.

2.6.2 Other Methodology

Other methodologies include those that do not require sophisticated and expensive instrumentation. These methods can be useful as screening methods followed by a confirmatory method for positive samples. Non-instrumental screening methods are not the focus of the thesis project, but they can play a role in a regulatory environment. As with other screening methods, the extraction procedures may be useful for incorporation into more advanced procedures.

The least impressive method was a high performance thin-layer chromatographic method for the fluorescence detection of DMZ, RNZ and HMMNI in pork and poultry tissue (Gaugain and Abjean, 1996). The paper concludes that the validation results prove the reliability of the method. However, the detection limits are subjective because the detection is visual. It is not apparent how a method that relies only on subjective visual detection can be reliable, as other researchers have noted variability of results recorded by different observers (Stead et al, 2005; Ashwin et al, 2009). In another method an antibody was generated that could bind MNZ (Huet et al., 2004). A direct competitive enzyme-linked immunosorbent assay (cELISA) was used to characterize binding of this antibody

to a number of nitroimidazole drugs. The development and validation of an immunoassay for determining five nitroimidazoles in chicken muscle and egg, based on an antibody capable of broad spectrum compound recognition, was reported. Although unfamiliar with these methods, it is interesting work.

The final two methods involve the use of an optical biosensor. The aim of the first paper was to develop a biosensor assay to detect a broad range of nitroimidazoles in chicken muscle (Connelly et al., 2007). The assay was developed and produced in a prototype kit format. Data generated from a multi-laboratory trial provided evidence the kit was easy to use and suitably sensitive to fulfill the standards required by international legislation. In the second study an optical biosensor screening assay was developed for the detection of nitroimidazoles in porcine, bovine and ovine kidney tissue (Thompson et al., 2008). It was further adapted to include avian liver, serum and eggs and bovine milk. The authors conclude that this method is capable of detecting low concentrations of a range of nitroimidazoles and their metabolites. It is used in labs as an initial screening program followed by confirmatory analysis for potentially non-compliant samples. These methods appear to be quick, inexpensive and apply to a wide range of species and matrices. Although they are not relevant to the current research, they may warrant investigation at a later time.

2.6.3 Methodology Using Liquid Chromatography

Of all the methods described in the literature, liquid chromatography is the current method of choice. Nitroimidazole residues have been determined in many different matrices using this technique. Honey, water and milk are probably the least similar matrices to fish, however, the methods are still worth reviewing in relation to the project.

The extraction procedures for these matrices are most likely not applicable, but the instrumentation parameters may be.

Two methods were found for the determination of nitroimidazoles in water (Capitan-Vallvay et al., 2002 and Tamtam et al, 2009). The first method successfully determined MNZ, DMZ, IPZ, RNZ and the metabolite HMMNI in water samples using LC-MS with electrospray ionization (ESI) following clean up with SPE. SPE is a widely used preparation technique in the determination of pharmaceutical compounds. The other method was an ultra performance LC-ESI tandem mass spectrometry method (UPLC-MS-MS) method for the determination of 17 antibiotics in natural waters, one of which was from the nitroimidazole group (ornidazole). Significant progress has been made in recent years in chromatographic analysis by the introduction of UPLC which provides higher peak capacity, greater resolution, lower detection limits and higher speed of analysis. Although the analysis of water used in farming fish would be interesting, it is not part of the proposed project. However, the conditions for LC-ESI-MS-MS were considered in the initial method development process.

The method developed and validated for the determination of veterinary drugs including nitroimidazoles in milk was based on ultra performance LC-time of flight-MS or UPLC-ToF-MS (Stolker, 2008). As previously mentioned, the technique of UPLC provides improved resolution while shortening run times. The selectivity of ToF should be greater than that of MS-MS due to higher resolution, but detection limits are usually higher. ToF instrumentation was not available for research purposes on the thesis project, but the information on UPLC separation was useful, particularly for demonstrating the selectivity and speed of analysis using this chromatographic separation technique.

A simple and sensitive method was developed for the determination of MNZ, DMZ, RNZ, tinidazole (TNZ) and HMMNI residues in honey by LC-UV (Zhou et al., 2007). While the accuracy and precision of this method meet the requirements for monitoring drug residues in honey, it was considered that the extraction conditions used were considered unlikely to be applicable to fish. In addition, LC-UV is not a confirmatory method.

Plasma, urine and eggs are also quite dissimilar to fish tissue; nevertheless several LC methods developed for these matrices were evaluated. Plasma has been determined to be the preferred target matrix for nitroimidazole residue control in some countries since the residues are homogeneously distributed and more stable compared with muscle tissue (Fraselle et al., 2007). A method using confirmatory analysis by LC coupled to atmospheric pressure chemical ionisation MS (LC-APCI-MS-MS) was developed and validated. LC-MS-MS was shown to be a good choice for the identification and quantification of nitroimidazoles in plasma with the exception of IPZ and IPZ-OH for which identification only was possible due lack of specifity. A reliable and sensitive method was also developed and validated for the determination of RNZ, MNZ, DMZ and HMMNI in swine urine by LC-MS-MS in positive-ion ESI mode (Xia et al., 2006b). The LC-MS-MS conditions in positive-ion ESI and APCI mode were helpful in planning analytical approaches to be evaluated in the project work.

Some LC methods have been described which are specifically for eggs, while others are for application to eggs and other matrices, such as poultry and swine tissues. In a fast, sensitive and very selective HPLC-MS-MS method for the detection of RNZ, MNZ and DMZ in eggs, the extract was filtered and directly injected into the LC-MS-MS

system (Daeseleire et al., 2000). Tuning of the LC-MS-MS instrument was performed with compound solutions in positive ESI mode. Fish tissue was considered likely to contain too many potential interferences for such a simple extraction procedure with no clean up step. Tuning of the LC-MS-MS in ESI (+) mode is useful. In another LC-ESI-MS-MS based method in eggs for the determination of nitroimidazoles, a molecular imprinted polymer (MIP) was synthesized and tested to extract four nitroimidazoles and three of their metabolites from egg powder samples (Mohamed et al., 2008). The use of MIP sorbents resulted in selective binding of the targeted analytes while removing interferences. MIP-SPE was also evaluated in another report for the extraction of nitroimidazole compounds from milk and egg samples and analyzed by LC-MS (Shimelis et al, 2009). Highly reproducible recoveries were observed using the MIP technology. MIP-SPE may warrant further investigation for use with fish tissue when these products are readily commercially available. Two more methods for eggs using LC-MS-MS for the determination of DMZ were reviewed (Mortier et al., 2003; Mortier et al., 2005). The method in the article from 2005 was also used for determination of DMZ in feed. This method was useful to determine levels in medicated feed for depletion studies.

The latest LC method for eggs, which was also for the determination of the highest number of nitroimidazole compounds, was by Cronley et al (2009). A rapid, confirmatory method was developed for the determination of 11 nitroimidazoles in egg using LC MS-MS. Egg samples were extracted with acetonitrile and NaCl added to remove contaminants. This was followed by a wash step with hexane.

A number of papers described LC methods which were for both eggs and animal tissue including turkey, poultry and swine. The first paper described a simple and rapid

method for the determination of DMZ in poultry meat and liver and in eggs by LC-MS after solvent extraction and SPE clean up (Cannavan and Kennedy, 1997). This method can be used to monitor residues caused by contaminated feed or illegal use. It provides mass-related data which are desirable for confirmatory methods. Since this method involves confirmatory MS it was taken into consideration for the project work. The extraction for poultry tissue is also more similar to fish tissue. Another paper describes the development of an HPLC-UV screening method for the presence of nitromidazoles as well as a confirmatory HPLC-APCI-MS method (Sams et al., 1998). The HPLC-UV method was validated on egg and chicken muscle samples and the HPLC-APCI-MS was validated on egg samples. Both of these procedures have been used for the analysis of samples as part of UK surveillance programs. The fact that this method involves a confirmatory method that is being used in the UK is helpful.

In another method which reported the determination of RNZ, MNZ, DMZ and HMMNI in poultry and swine muscle and eggs by LC-ESI-MS-MS, samples were extracted, filtered and the extract was directly injected into the LC-ESI-MS-MS system (Xia et al., 2006a). While it would be beneficial if this method could be applied to fish tissue, it is unlikely that fish tissue could be extracted and run without a cleanup step. Interference, in fact, was reported for the analysis of the muscle samples in this method. The problem was overcome by addition of NaCl, which could be attempted in fish tissue. The addition of NaCl with extraction solvent also allowed for greater removal of impurities and produced cleaner samples for analysis with egg samples (Cronley et al, 2009). Shao et al (2009) reported an additional method which directly injected sample extracts into a UPLC-ESI-MS-MS system. This method was developed for determining

14 coccidiostats (including RNZ, MNZ and DMZ) in eggs and chicken. It was reported that a purification step was not carried out due to poor recoveries of some analytes. This is contrary to what is usually reported by most papers.

One paper focused on clean up procedures to isolate residues including nitroimidazoles from the potential interferants in foods (Stubbings et al., 2005). A cation exchange clean up procedure was developed for use with acetonitrile extracts from chicken and turkey muscle and egg. The clean up procedure was employed within screening and confirmatory procedures by the reporting laboratory. The point of this paper was more related to sample extraction and clean up rather than instrumentation, but it is an important part of developing a method and aids in the project work. One of the problems especially with MS detection is the presence of interferences that can cause ion suppression and poor limits of detection (Diaz-Cruz et al., 2003). For this reason, advanced purification techniques, such as immunoaffinity SPE or molecular imprinted polymers (MIPs), are required to meet the challenge of analysing drugs in difficult matrices. Purification procedures can also include liquid-liquid extraction. Hexane is commonly used for lipid removal in the analysis of animal samples (Shao et al, 2009).

The final and most important method developed for chicken meat and egg was adapted for the analysis of fish (Mottier et al., 2006). The work in the paper described a method for the determination of four 5-nitroimidazoles and three metabolites using LC-MS-MS. The primary target matrices for validation were eggs and chicken meat, but the method was also adapted for analysis of fish. A precise and sensitive quantification was obtained for 5 of the 7 compounds, but MNZ and its metabolite were unable to be quantified. It was recommended they be quantified by means of a matrix matched

calibration quantification procedure. It was exciting to find a confirmatory method that was able to be adapted to fish.

Several LC methods were developed for meats only, such as poultry and swine. These matrices are probably the most closely related to fish tissue. Two of these methods are not confirmatory methods, but the extraction procedures were considered potentially useful. The first method was for the determination of MNZ, RNZ and DMZ and its metabolite in swine tissue by LC with diode array detector, LC-DAD (Shen et al., 2003). The SPE column was used to clean up tissues successfully with a minimum number of steps and small amount of solvents. In the second method, which is capable of the simultaneous determination of six nitroimidazoles and one metabolite in chicken and pork by SPE HPLC-UV, the extraction and cleanup conditions were investigated and optimized (Sun et al., 2007).

The other methods for the determination of nitroimidazoles in meat all used LC-ESI-MS. One used single MS (Hurtaud-Pessel et al., 2000) while the other four used tandem MS (Xia et al., 2007, 2008, 2009 and Sun et al, 2009). All five were confirmatory methods as even the single MS method monitored several ions for each nitroimidazole providing the specificity required for confirmatory analysis. The LC-MS method was used to determine DMZ, RNZ, MNZ and HMMNI in poultry meat (Hurtaud-Pessel et al., 2000). The first LC-ESI-MS-MS method confirmed DMZ, RNZ, MNZ and a metabolite in porcine liver at concentrations appropriate for monitoring illegal use of selected banned compounds in livestock production (Xia et al., 2007). In the second study LC-ESI-MS-MS was used for the simultaneous determination of nitrofurans and nitroimidazoles in pork (Xia et al., 2008). The nitroimidazole aspect of this method will be useful and

possibly the simultaneous determination may also be useful for future work beyond this project as nitrofurans are also veterinary drugs that are currently monitored in the lab.

One of the more recent methods found was for the simultaneous determination of four 5-nitroimidazoles (MNZ, RNZ, DMZ and IPZ) and their corresponding metabolites (MNZ-OH, IPZ-OH and HMMNI) in swine kidney (Xia et al, 2009) by LC-MS-MS after SPE. Another recent method which was considered to be for meat tissue from animal origin was for the determination of nitroimidazoles in natural casings by LC-MS-MS with SPE (Sun et al, 2009). Natural casing is obtained by extensive processing of fresh intestine from healthy livestock and is stuffed with different types of sausage for human consumption. This was the only method for the determination of nitroimidazoles in this type of tissue. Considering its selectivity, sensitivity and availability to my work LC-ESI-MS-MS was identified as the method of choice for the thesis project.

After a thorough search for methods specifically for fish tissue, only two were found. In the first paper a HPLC method based on SPE with UV detection was developed and validated for the determination of MNZ and MNZ-OH in muscle and skin tissue of rainbow trout (Sorenson and Hanson, 2000). It was noted that no validated method had previously been reported for the determination of MNZ and MNZ-OH in fish tissues. Unfortunately this is not a confirmatory method, but it is still useful, particularly for the extraction. Trout is a similar matrix to salmon, one of the representative matrices used to validate the project method. The second method was also an SPE-HPLC-UV method for the determination of MNZ and MNZ-OH (Maher et al., 2008). It was for tilapia which is the representative whitefish matrix used for method validation purposes. These two methods combined with the method by Mottier et al. (2006), which was adapted for fish

tissue and mentioned earlier, were considered to play the largest role in method development and validation.

2.7 Summary

This literature review on nitroimidazoles allowed a critical assessment of existing information on the following topics: toxicity; stability, depletion and distribution studies; the metabolism and methods of analysis for nitromidazoles. The literature has shown some of the toxic effects of nitroimidazoles and their metabolites as well as the potential risk to human health. The extent of the negative impact on humans, in large part, seems unknown. For these reasons, they are banned substances for use in food producing animals. The importance of the conditions surrounding sampling and analysis for stability purposes to provide reliable results have also been shown. Depletion and distribution studies in tissue indicate that target matrices and analytes (parent compound or metabolites) require consideration. Information on the metabolism of nitroimidazoles has indicated the need to monitor both parent compounds and their metabolites. The need for the determination of these residues in our food supply is evident as well. There were many methods presented for many different matrices for the analysis of nitroimidazole residues.

It was apparent that very little work had been done on the determination of nitroimidazoles in aquacultured fish even though they are used to treat infections in fish. Up to this point, only three methods have been found for nitroimidazoles in fish. One of these methods was a confirmatory LC-MS-MS method, however the authors failed to quantitate one of the most abundantly used nitroimidazoles and its metabolite (MNZ and

MNZ-OH). The next was a screening HPLC-UV method for MNZ and MNZ-OH only and the third was another screening HPLC-UV method for MNZ and a veterinary drug from another class.

Chapter 3 - Experimental Materials and Methods

3.1 Chemicals and Materials

The analytical standards MNZ, RNZ, DMZ, IPZ-OH, HMMNI and MNZ-OH were obtained from Sigma Aldrich (St. Louis, MO, USA). Deuterated dimetridazole (DMZ-D_3) and IPZ were purchased from Witega (Berlin, Germany). All pharmaceuticals were of analytical grade (>99%).

HPLC grade dichloromethane, methanol, acetonitrile and 2-propanol, hexane (distilled in glass) and laboratory grade formic acid (90%) were purchased from Fisher Scientific (Fairlawn, N.J, USA). Perchloric acid (60%) was from SEASTAR Chemicals Inc. (Sidney, B.C., Canada). Glacial acetic acid came from J.T. Baker (Phillipsburg, NJ, USA). Water was purified using a Milli Q water system from Millipore (Bedford, MA, USA).

Individual stock standards at 100µg/mL were prepared by dissolving 10 mg of standard in 100 mL of acetonitrile and were stored at 4°C for 1 year, at which time a fresh stock standard solution was prepared. A mixed intermediate standard solution (1µg/mL) was prepared by diluting 1 mL of each stock solution into 100 mL of 0.1% acetic acid. A working standard (100ng/mL) was prepared by diluting 5 mL of intermediate standard into 100 mL of 0.1% acetic acid. These were stored at 4°C for 12 months, at which time fresh mixed intermediate stock and working standard solutions were prepared. Internal standard stock, intermediate and working solutions were prepared at the same concentrations using the same dilutions and stored under the same conditions.

Waters Sep-Pak C-18 SPE cartridges were purchased from Waters (Milford, MA, USA). The 0.2 um nylon membrane syringe filters were purchased from Pall Corporation (Ann Arbour MI, USA).

Sample material including tilapia, salmon and shrimp muscle tissues were provided by the CFIA for method development and validation. Live rainbow trout (Oncorhynchus mykiss) for the depletion study were provided by the Nova Scotia Provincial Fish Hatchery (Caledonia, NS).

3.2 Sample Preparation

Prior to sample preparation the material was stored at -80°C. Precautions were taken to prevent contamination between samples. Before processing each sample, the cutting board, knives, spatulas and the immediate work area were carefully scrubbed to reduce the risk of cross-contamination. Fish were partially thawed to facilitate preparation, then placed on a cutting board for removal of sample material. For larger finfish, three cross sectional slices were removed from each side of the fish, one from just back of the pectoral fin, one just back of the vent and one midway between the other two. For smaller fish, one fillet from each side of the fish was removed for homogenate preparation. Skin, viscera and bones were removed and the tissue was homogenized using a domestic food processor. For crustaceans, the meat was removed from the shell and homogenized. The homogenate was stored in a sealed container below -20°C until analysis.

3.3 Extraction and Clean Up

A 4g test portion of homogenized tissue was weighed into a 50 mL polypropylene (pp) centrifuge tube. Test portions of blank tissue were fortified with a 200µL aliquot of mixed working standard for recovery determinations. An aliquot (100µL) of working internal standard was added to each test portion prior to addition of 16mL of 0.08% perchloric acid in acetonitrile solution. The tissue and acidic acetonitrile were then homogenized using a Polytron tissue homogenizer (PT10-35, Kinematica AG, Switzerland) until homogenous and the volume was adjusted to 25 mL with dichloromethane. Tubes were placed on a rotator (Glass Col, Terre Haute, USA) for 10 minutes, and then centrifuged (Allegra XR-15 with SX4750 rotor, Beckman Coulter) at 538g for 10 minutes. A 10 mL aliquot of supernatant was removed to a 14 mL pp Falcon tube. For matrix matched standards, three additional samples of blank tissue were extracted and five 10 mL aliquots were transported to individual 14 mL pp falcon tubes. Tube contents were evaporated to 2 mL under a stream of nitrogen at $\leq 45°C$.

A Waters Sep-Pak SPE cartridge was conditioned with 2 mL of acetonitrile. A 2 ml portion of concentrated extract was passed through the cartridge and the effluent was collected in a 16 x 100 mm disposable glass tube. The extract tube was rinsed with three 2 mL portions of acetonitrile, passing each rinse through the column and collecting the effluent. The SPE column was rinsed with 2 mL of acetonitrile and the effluent collected. The effluent was evaporated to dryness under a stream of nitrogen at $\leq 45°C$, and 1 ml of acetonitrile added to each tube. To remove fat, a 1 ml portion of hexane was added to the extract in acetonitrile and vortexed. This mixture was then centrifuged at 269 g for 5 minutes, the hexane layer removed and the acetonitrile again evaporated to dryness under a stream of nitrogen at $\leq 45°C$. For samples and spikes, the residue was reconstituted with

1000 µL of 0.1 % acetic acid solution and vortexed to ensure the residue was fully dissolved. Required volumes used for the preparation of the matrix matched standards are given in Table 3.1. The specified volume of working standard and internal standard was added and diluted with the specified volume of 0.1% acetic acid and vortexed to prepare each of the calibration standards. All samples, spikes and standards were filtered through a 0.2 µm nylon filter into an autosampler vial.

Table 3.1 Volumes of working standard (100 ng/mL), internal working standard (100ng/ml) and 0.1% acetic acid required to provide specified concentrations of the matrix matched standards.

Matrix Matched Standard Solution (ng/mL)	Tissue Equivalents (ng/g)	Neat Working Standard Added (uL)	Internal Working Standard Added (uL)	0.1% Acetic Acid Added (uL)
0	0	0	0	1000
2	1.25	20	40	940
4	2.5	40	40	920
10	6.25	100	40	860
50	31.25	500	40	460

3.4 UPLC-MS-MS Instrumentation

Chromatographic separation of nitroimidazoles was performed on a Waters Aquity Ultra Performance Liquid Chromatography system using a Waters Aquity HSS T3 C18 1.8µm particle size, 2.1mm id x 50mm (Waters, Milford, MA, USA) maintained at 35°C. The mobile phase consisted of 0.1% acetic acid in water (mobile phase A) and 0.1% acetic acid in acetonitrile (mobile phase B) pumped at a flow rate of 0.4 ml/min.

The initial conditions (0-0.5 min) were 95% A. Then the conditions changed to 70% A (0.5-5 min) and then to 5% A (5-10 min). The volume of sample injected was 20 μL.

The UPLC system was coupled to a Micromass Quattro Premier XE triple quadropole mass spectrometer (Waters Scientific, Milford, MA, USA) equipped with a source capable of operation in electrospray ionization (ESI) and atmospheric pressure ionisation (APCI) modes and controlled by MassLynx software (version 4.1). MS tune parameters for maximum intensity of precursor ions were as follows: capillary voltage, 2 kV; extractor voltage, 5V; source temperature, 130°C; desolvation temperature, 450°C; cone gas flow, 550L/hr. After initial comparison of performance in both ESI and APCI modes, the MS instrument was operated in the ESI positive mode for all compounds in subsequent experiments and the data were acquired in multiple reaction monitoring (MRM) mode. MRM transitions were optimized during tuning and are summarized in table 3.2, with optimized cone voltages and collision energies used in the experiments.

Table 3.2 Mass Transitions, cone voltages and collisions energies for various nitroimidazole compounds

Compound	Mass Transitions (Da)	Cone (V)	Collision (eV)
HMMNI	157.8>139.9	20	15
	157.8>55.2	20	20
IPZ	170.0>124.0	25	17
	170.0>109.0	25	25
IPZ-OH	186.0>168.0	20	15
	186.0>122.0	20	20
MNZ	171.8>127.9	25	15
	171.8>82.0	25	20
MNZ-OH	188.2>125.9	20	15
	188.2>122.9	20	15
RNZ	201.0>140.0	15	12
	201.0>55.1	15	15
DMZ	142.2>96.1	25	15
	142.2>81.2	25	25
DMZ-D_3	145.0>99.0	25	17
	145.0>83.1	25	23

3.5 Identification and Quantification

For each compound being analyzed, the parent ion and the two most intense daughter ions were monitored. The first mass transition was used for quantification and the second, in combination with the first, for confirmation. This information, as well as retention time, was used to ensure correct peak identification. Confirmation of a compound was based on the following: the signal to noise ratio for each product ion was

at least 3:1; the retention time of the product ions in the sample matched the product ions in the standards within 5 % and the ion ratios of the analytes in the samples were ± 20% the average ion ratios for the standards (EU, 2002).

A calibration curve was prepared by plotting concentration of matrix-matched standard (ng/mL) versus instrument response. Sample concentrations were calculated from the matrix-matched calibration curve correcting for dilutions, mass of extracted sample tissue and for recovery from fortified samples. To calculate internal standard recovery for each sample, the mean response (area) was calculated for the known concentration of internal standard in each calibration standard. From this information a calibration factor (area/concentration) was determined and used to multiply by the concentration of each standard to give predicted responses (area) for the standards. This was used to prepare an internal standard calibration curve by plotting the standard concentration (ng/ml) versus response (area). The internal standard concentration in each sample was calculated from this curve. Percent recovery was calculated by dividing the calculated concentration by the true concentration and multiplying by 100. In this instance, the internal standard was used as quality control check for each sample. The internal standard was also used to control variability during extraction, sample injection and ionization by preparing a standard curve plotting standard concentration (ng/ml) against the ratio of sample response to internal standard response. The following calculation was used to calculate sample concentration:

$$ng/g = \left(\frac{[C]X2.5}{Wt}\right) / \%\,\mathrm{Rec}$$

Where C = Concentration in ng/mL as determined from the standard curve
Wt = Weight of sample used in grams
% Rec = Percent Recovery
2.5 = Dilution Factor (25 mL/10 mL)

3.6 Method Development

Method development began with a search for suppliers to obtain pure standards for the analytes of interest which were determined to be MNZ, RNZ, DMZ, HMMNI, IPZ, IPZ-OH, MNZ-OH and DMZ- D_3 as the internal standard. After UPLC-MS-MS conditions and parameters were determined, the approach for method development described in Murphy (2009) was used for determining linearity, matrix effects, ruggedness and stability.

3.6.1 Mass Spectrometry (MS)

Each analyte was individually infused directly into the MS as a standard solution at a concentration of 50 ng/ml for tuning of MRM transitions. Tuning was based on available literature information (Mottier et al, 2006) for initial selection of target ions and transitions to determine mass transitions for quantification and analyte confirmation. The MS probe was capable of both ESI and APCI modes, so all compounds were tested using both ESI and APCI in positive and negative modes. APCI was achieved by installing the corona pin. Parameters such as cone voltage and collision energy were optimized during infusion for each analyte to obtain the maximum sensitivity with the highest amount of product ions available, and the two MRM transitions providing best response were determined for each molecule. Fragmentation pathways for each molecule and structures for each fragment were also proposed.

ESI positive mode was chosen for analysis because it gave the best results and it was also convenient since other methods for veterinary drugs were routinely performed in ESI mode on the same instrument. The appropriate dwell time was set to provide enough data points across the peak to give reproducible peak areas for quantification purposes and to maintain signal intensity (Hernando et al., 2007). To obtain a sufficient number of scans for each compound, dwell time was set for 0.05 seconds for each transition and interscan delay was 0.02s. Two separate time windows, one containing 5 compounds and the other containing 2 were created to also increase scan counts and improve analysis efficiency. All of these parameters were contained in a Tune file in MassLynx.

3.6.2 Liquid Chromatography (LC)

Liquid chromatography conditions were established and optimized based on information from literature and experimental evaluation. In the literature, chromatographic separation of nitroimidazoles was performed in gradient mode using water acidified with 0.1 % acetic acid (mobile phase A) and acetonitrile acidified with 0.1 % acetic acid (mobile phase B) at a flow rate of 0.25 µL/min (Cronly et al., 2009). These mobile phases were used for other veterinary drug procedures performed in the lab (CFIA Dartmouth Laboratory, SOM-DAR-CHE-037-03, 2007 and SOM-DAR-CHE-038-05, 2009). A flow rate of 0.4 ml/min was used and two main gradiant elution programs were considered from Xia et al (2009) and van de Reit at al (2005). Different variations of these gradient profiles were attempted until the best separation was obtained. Initially, separation was performed using an Aquity BEH C18 column (50mm x 2.1mm id 1.7µm particle size) as described by Tamtam et al. (2009). A Waters Aquity HSS T3 C18 1.8µm particle size, 2.1mm id x 50mm was also evaluated and equivalent performance was

obtained on both columns. A column temperature of 35°C was used as it was the standard temperature for several other veterinary drug methods. Use of a temperature slightly above laboratory room temperature eliminates minor variations in chromatography which may be caused by fluctuations in the laboratory room temperature during the course of an analytical run. Some controllers will not control at room temperature and need to be set 5-10 °C above or below room temperature.

3.6.3 Linear Range and Sensitivity of Calibration Curve

Instrumental linear range for the analytes was determined by the injection of standard solutions in order to determine at what concentration the instrument response no longer conforms to a linear equation (y= mx +b). The expected concentration range from routine samples was not known, however a predicted range was used. Six calibration solutions ranging from 10-100 ng/mL made up in 0.1% acetic acid were injected. The concentrations of the solutions were evenly spaced to determine the precise level at which the calibration curve is no longer linear. The standard concentration (ng/ml) was plotted against instrument response to determine the linear portion and sensitivity of the curve. The instrument linear range was used to determine the analyte concentration range for which the method was fit for purpose. Sensitivity was determined from the slopes of the calibration curves. Sensitivity describes the change in instrument response for a given concentration change (CAC, 2009b; Anon, 1998).

3.6.4 Preliminary Extraction and SPE

In the literature, tilapia fish tissue samples were extracted with a mixture of 0.2% orthophosphoric acid-methanol (6:4) followed by a clean-up procedure using a reversed

phase C18 Waters Sep Pak extraction cartridge (Maher et al, 2008). Ethyl acetate was used to extract fish tissue after the addition of 0.5 M K_2HPO_4 by Mottier et al (2006). A mixture of acetonitrile with hexane (2:1) was used for extraction of trout followed by clean up using a silica SPE cartridge (Sorenson and Hanson, 2000). Acetonitrile was also used for extraction of nitroimidazoles from other types of samples such as bovine plasma (Cronly et al., 2009). Two other veterinary drug residue methods for fish tissue currently used by CFIA (SOM-DAR-CHE-050-01 and SOM-DAR-CHE-039-07, 2009) used acetonitrile and 0.08% perchloric acid in acetonitrile with Oasis HLB and Waters C18 Sep Pak cartridges, respectively.

Using this information, fortified salmon was extracted using either acidic acetonitrile or 100% acetonitrile only followed by Waters Sep-Pak SPE. To determine if the Waters Sep Pak cartridges caused loss of analytes, extracts of blank salmon tissue were fortified with analytical standards both before and after SPE. Hexane was used in previous work with nitroimidazoles (Mottier et al, 2006 and Xia et al, 2007) to remove impurities, as well as in other veterinary drug residue methods for fish (Pearce et al, 2009). Therefore, fortified salmon samples were extracted with and without hexane wash for comparison. Several veterinary drug methods for fish tissue included a filtration step using 0.2μm nylon filters prior to LC analysis and these were used for this method to remove any remaining impurities remaining after extraction, SPE clean-up and hexane partitioning.

3.6.5 Matrix Effects and Method Selectivity

Three commodities were chosen to determine the effect of matrix on instrument response. Fish muscle from tilapia was representative of a low fat fish matrix, salmon a

high fat fish matrix and shrimp was the representative crustacean. The matrix can change the chromatographic profile or create an enhanced or suppressed response from the detector (Gosetti et al., 2010). To determine the matrix effect, calibration curves were prepared using both neat and matrix matched standards and compared. Three matrix fortified calibration curves were prepared using extracted blank tilapia, salmon and shrimp muscle. The extracts were fortified with appropriate aliquots of standards prior to reconstitution to give concentrations of 0, 2, 4, 10 and 50 ng/ml. A set of neat standards were prepared at the same concentrations.

Calibration curves for the neat and fortified standards were prepared by plotting the average response of the standard solution against the standard concentration. Significant differences (>10%) in the slope of the matrix fortified curves compared to that of the neat curve or changes in the elution profile would indicate that the matrix did affect the instrument response. If there were significant differences between the curves, matrix matched standards and/or an internal standard are used.

In addition to examination of chromatograms to ensure that there were no co-eluting matrix components which could interfere with the target analytes, other drugs that potentially could be present in samples were tested to ensure that their presence did not interfere with the analysis. Compounds tested included malachite green (MG), leucomalachite green (LMG), crystal violet (CV) and leucocrystal violet (LCV) from the triphenyl methane dyes class of drugs; naladixic acid (NAL), oxolinic acid (OXO) and flumiquine (FLUM) from the quinolones and the fluoroquinolones including ciprofloxacin (CIPRO), danofloxacin (DANO), enrofloxacin (ENRO) and sarafloxacin (SARA).

3.6.6 Preliminary Determination of Method LOD, Recovery and Precision

A preliminary estimate of LOD was calculated for each analyte in all three matrices based on signal-to-noise and the concentration of the standards analyzed. The LOD was estimated by dividing the known concentration of a standard by the signal-to-noise determined for the analyte peak in the standard and multiplying this by 3, which is the minimum signal-to- noise ratio required (Guidance for Industry, 2010). LOD was also determined by evaluating the noise level of a blank sample and calculating the concentration equivalent to 3 times the noise at the expected retention time for the analyte (Eurachem, 1998).

Preliminary recoveries were also calculated by spiking each commodity in duplicate at three concentrations, 1, 2 and 3 ng/g. Preliminary method repeatability was done at one concentration, 5 ng/g in tilapia and instrument repeatability was assessed by running five injections of each spike level and the fortified material.

3.6.7 Ruggedness (Robustness)

Ruggedness (also termed robustness) is a resistance to changes in the results produced by an analytical method when minor deviations are made from the experimental conditions described in the method (Eurachem, 1998). The ruggedness of the nitroimidazole method was tested by introducing small changes to the procedure and examining the effect on the results. It was not necessary to perform ruggedness testing on all three matrices as matrix effects were investigated previously in method development. Tilapia was chosen for ruggedness testing since it was considered representative of the most common matrices for the anticipated workload.

Seven variables were tested and are listed in Table 3.3. Youden's Factorial approach, where seven variables can be combined in a specific manner to determine the effects of all seven variables using eight combinations in a single experiment, was used (Youden and Steiner, 1975). The experiment was carried out in duplicate over two separate days to eliminate the chance of a single sample affecting the outcome. Blank tilapia tissue was fortified with a mixed standard to give a concentration of 5 ng/g of each analyte.

Table 3.3 Variables Tested for Ruggedness

	Original Condition		Alternate Condition
A	Add 16 ml of acidic acetonitrile	a	Add 10 ml of acidic acetonitrile
B	Dry extract to 2 ml	b	Dry extract completely
C	Evaporate eluent to dryness at 45°C	c	Evaporate eluent to dryness at 65°C
D	Use 0.1% acetic acid as the make solvent	d	Use water as the make up solvent
E	Wash with 1 ml of hexane	e	Wash with 0.5 ml hexane
F	Use 0.2 μm nylon filters	f	Use 0.2 μm Teflon filters
G	Condition SPE with 2ml acetonitrile	g	Do not condition SPE

The variables to be tested set up into various factor combinations were listed in table 3.4, each one giving a measurement of s to z.

Table 3.4: Various Factorial Combinations for Ruggedness

Sample	Factor Combinations	Measurement
1	A B C D E F G	s
2	A B c D e f g	t
3	A b C d E f g	u
4	A b c d e F G	v
5	A B C d e F g	w
6	A B c d E f G	x
7	A b C D e f G	y
8	A b c D E F g	z

To determine the effect of each individual factor, the following was calculated.

Effect of A and a: $[(s + t + u + v)/4] - [(w + x + y + z)/4] = J$
This simplifies to: $(4A/4) - (4a/4) = J$

Effect of B and b: $[(s + t + w + x)/4] - [(u + v + y + z)/4] = K$
Effect of C and c: $[(s + u + w + y)/4] - [(t + v + x + z)/4] = L$
Effect of D and d: $[(s + t + y + z)/4] - [(u + v + w + x)/4] = M$
Effect of E and e: $[(s + u + x + z)/4] - [(t + v + w + y)/4] = N$
Effect of F and f: $[(s + v + w + z)/4] - [(t + u + x + y)/4] = O$
Effect of G and g: $[(s + v + x + y)/4] - [(t + u + w + z)/4] = P$

The values calculated for the differences between factors J-P were examined. Factors which created statistically significant changes were determined by performing a two-sample t-test. Equal variance for each factor was assumed. If the p-value was <0.05 the factor was considered significant, if the p-value was >0.15, the factor was not significant and if $0.05<p<0.15$, the factor might have been significant. If factors were significant, the procedure was changed to reflect this.

3.6.8 Internal Standard

Initially an internal standard (IS) was added to the procedure during method development and was used as a quality control check for the method. The IS was added to each standard and sample and the percent recovery was calculated for each sample. The internal standard chosen was DMZ-D_3. It was not feasible to have IS for all seven compounds as that was too expensive. It was later decided, based on observations made during method validation, that internal standard responses would also be used in the calibration curve to determine sample concentrations.

3.6.9 Stability

The stability of nitroimidazoles in standard solution, in matrix and in sample extract was investigated. The investigation was initiated during method development and continued into method validation.

3.6.9.1 Light Sensitivity

Nitroimidazoles were reported to be very light sensitive in one paper (Hurtaud et al, 2000) and were stored in the dark and /or in amber glassware in several other methods (Ho et al, 2005 and Xia et al, 2008). However, light sensitivity was not reported for storage conditions on the certificate of analysis for the analytical standards. To determine if nitroimidazole standards were light sensitive in solution, a mixed working standard was prepared and divided into two portions. One portion was stored under ultraviolet light for 24 hours and the other portion was stored in the dark. After the 24 hour period, neat standards were prepared at 0, 2, 4, 10 and 50 ng/ml concentrations and calibration curves for each prepared and compared. If a slope difference of <10% was determined for an analyte there was considered to be no significant difference between the two sets of standards. Ten 20 ng/ml standards from both the light protected and the UV exposed working standard solutions were also prepared. These were analyzed and the average area counts calculated for each. The % RSD was calculated between the two averages.

3.6.9.2 Standard Solution Stability

Stability of analyte in standard solution was determined by a comparison of a working standard that was prepared fresh with one that had been stored at 4°C. Comparisons were made weekly for the first month, monthly for 6 months and then

bimonthly until a year. The % RSD was calculated between old and freshly prepared standard to determine if the stability of the old standard had been compromised.

3.6.9.3 Stability During Processing/Extract Stability

During method development, recoveries were monitored to determine if any significant loss of analyte occurred at any step(s) in the method and if any such losses observed could be attributed to stability issues. To determine extract stability, representative vials from recovery studies described in section 3.7.2 at each concentration level studied were analyzed daily for 5 days. The mean concentration, SD and % RSD was calculated for each compound at each concentration. The mean concentration at each time point was plotted with error bars representing the SD. The extracts were considered stable if the error bars remained within the range of one another and/or %RSDs were $\leq$ the %RSD seen during repeatability studies.

CUSUM charts were used to see any changes in the extracts over the 5 days. CUSUM values were calculated by using the first actual raw data value as the first CUSUM point. The second point for CUSUM takes the previous point (raw data value), adds the new data value, and subtracts the fixed average of raw data results. Control limits were set using two times the standard deviation of the results on day 1. If several points were found below the lower control limit, this indicated degradation of the analyte.

Paired t-tests and ANOVA (analysis of variance) were also applied to the extract results. The t-test was used to determine if there was a significant difference between day 1 results and each of the following days. If the 2 tailed p-value was <0.05, the results were considered significantly different. A p-value of 0.05 or greater indicated no difference between two groups of results. To compare the results from each day with one

another, the ANOVA test was used to determine if the variance between days was statistically significant. If the F statistic was determined to be smaller than the critical F value, the differences between values over time were not significant.

3.6.9.4 Tissue Stability

Analyte matrix stability was determined by analyzing samples with known concentrations biweekly over a 2 month period. Fortified samples of tilapia muscle were prepared at two concentrations, 1ng/g and 10 ng/g. Samples of both concentrations were pre weighed into 50 ml polypropylene tubes. Five replicates were weighed out for each time point over the course of the stability study for two temperature conditions, -20°C and -80°C. To represent the typical storage conditions and the effect of freeze/thaw cycles on prepared samples, sealed containers of sample at both concentrations were stored at -20°C and removed from the freezer, thawed and refrozen at each time point.

Results from this study were graphed to determine if concentrations changed over time. The average of the results from 5 replicates over a 2 month period at each time point was plotted on a graph. The standard deviation of the replicates was used as error bars on each point on the graph. This was done for each analyte at 2 different concentrations for all conditions. If the error bars were within the range of another set of error bars on the same graph, then the analyte in tissue was considered to be stable.

CUSUM charts were also used to display running totals of the differences in results from the average at time 0. Control limits were set using two times the standard deviation of the results at time 0.

The %RSD of replicate results from time 0 to 8 weeks was calculated and reviewed to determine if they were typical of those seen in repeatability studies. T-tests were used to identify any significant differences between time 0 results and the other time points. To see any significant differences within all time points ANOVA was used.

3.7 Method Validation

For the validation study, experimental parameters determined included LOD/LOQ, recovery, repeatability, intermediate precision and measurement uncertainty (MU). These parameters were determined based on a written standard operating procedure which outlined the validation of analytical methods by the Chemistry section of the CFIA Dartmouth Laboratory to meet required performance criteria of a method (SOP-DAR-CHE-001-00, CFIA), based on recommendations from the International Union of Pure and Applied Chemistry (Thompson et al, 2002). In addition, the parameters decision limit (CCα) and detection capability (CCβ) required in European Commission regulations for methods used in veterinary drug residue analysis were also determined (EC, 2002).

3.7.1 LOD and LOQ

LOD is the lowest concentration of analyte in a sample that can be detected, but not reliably quantified under the stated conditions of the test. LOD was determined for each analyte in all commodities including tilapia, salmon and shrimp muscle tissue. This was done by evaluating the noise level of 5 blank samples per run on four separate days (n=20). The LOD for each blank was determined by calculating the concentration equivalent to 3 times instrument noise at the expected retention time for the analyte. The LOQ was a mathematical determination based on the LOD and represents the smallest

amount of analyte in a test sample that can be quantitatively determined with suitable precision and accuracy under previously established method conditions. The LOQ was calculated by multiplying the LOD by 3 (Codex, 2009b).

3.7.2 Accuracy (Trueness and Bias)

Recovery studies were done to determine the accuracy of the method for the analytes being validated in the specified matrices. The fraction recovered of the total analyte added is usually expressed as a percentage; thus the per cent recovery represents the trueness of the method, while the difference between the recovery and the actual quantity of analyte added to the matrix represent the method bias, which may be positive or negative (Eurachem, 1998). A recovery greater than 100% indicates a positive bias, while a recovery less than 100% indicates a negative bias.

The recovery of each analyte in tilapia, salmon and shrimp muscle was determined by analyzing each matrix fortified with a specified amount of the analyte. This was carried out on three fortification levels. Five fortified samples were analyzed at each level on one day and this was repeated on two other days for a total of three runs. The mean, SD and % RSD for each of the three levels was calculated. Nitroimidazoles were banned in all food animals and therefore there were no regulated levels or established maximum residue limits (MRLs) for these compounds. The fortification levels chosen for each analyte were 3 X LOD (approximately the LOQ), 10 X LOD and the upper limit of the linear range of the calibration curve (CAC, 2009b).

3.7.3 Precision

Both instrument and method repeatability were determined. Instrument repeatability was determined by repeat injections of prepared standard used for the calibration curve as well as a fortified sample at each of the 3 fortification levels from the recovery study. Each sample was injected 5 times in random order to prevent any bias. The mean, SD and % RSD were calculated.

Method repeatability was determined by analyzing five replicate extractions of fortified material at the same levels that were used in the recovery study. This process was done three times on separate days. The mean, SD and % RSD were calculated.

Intermediate precision expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous samples under prescribed conditions, which typically include experiments conducted by different analysts, over extended timescales, within a single laboratory (Eurachem, 1998). Intermediate precision was performed by a second analyst with reagents prepared by the second analyst. This parameter was used to determine if there were analyst-related biases in the method. The same material prepared for method repeatability was used for intermediate precision. The second analyst prepared fresh reagent and samples were extracted and analyzed in replicate (5) over 3 separate days. The average, SD and %RSD were calculated both separately and with method repeatability results. HorRat values were calculated from these results (Horwitz and Alpert, 2006). The HorRat is the ratio of the reproducibility relative standard deviation to that calculated from the Horwitz equation (CAC, 2009b).

3.7.4 CCα and CCβ

The decision limit (CCα) is the limit at and above which it can be concluded with an error of probability of α that a sample contains the analyte (EC, 2002). CCα was determined by analyzing 5 blank samples on four analysis days. The concentration equivalent to 3 X the instrument noise was calculated at the expected retention time for each analyte using the regression equation obtained from the calibration curve. The average of the 20 determinations for each matrix was the CCα for that matrix.

Detection capability (CCβ) is defined as the smallest content of the substance that may be detected, identified and/or quantified in a sample with an error of probability of β (EC, 2002). A total of ten blank samples, fortified at the determined CCα for each matrix were prepared on two separate days. Samples were analysed and the analytes identified. The concentration of the samples was determined from the standard curve and the average, SD and %RSD were calculated. The detection capability was determined as the decision limit plus 1.64 times the standard deviation of the values obtained.

3.7.5 Measurement Uncertainty (MU)

MU is defined as a parameter associated with a result of a measurement that characterises the dispersion of the values that could reasonably be attributed to the measureand (JCGM, 2008). The uncertainty of this method was grouped into two categories; accuracy and precision. The MU was estimated by taking into account the recovery results which represented accuracy and the data sets that were assessed against precision were from intermediate precision results (Ellison et al, 2000). The calculation used for determining the relative uncertainty of the method was the square root of the sum of the squares of the respective relative uncertainties for accuracy and precision.

$$UofM = \sqrt{(RU(accuracy)^2 + RU(precision)^2}$$

3.8 Depletion Study

A depletion study under controlled conditions was conducted to determine depletion time in muscle tissue from initial treatment of the drug until residues were no longer detectable. The most commonly used nitroimidazole compound, metronidazole, was used to conduct the depletion study in rainbow trout. Only two previous papers have been published on the metabolism of MNZ in fish, one in trout (Sorenson and Hanson, 2000) and the other in tilapia (Maher et al., 2008).

The Bedford Institute of Oceanography (BIO) fish lab was the location chosen to complete the depletion study. Dr. Jocelyne Hellou from the Department of Fisheries and Oceans (DFO) provided help with conducting the exposure, plus advice and access to the fish lab facilities to prepare fish samples representative of real case ones. Rainbow trout and feed were provided by the Nova Scotia Provincial Government fish hatchery in Caledonia, NS. Feed originated from Corey Feed Mills Ltd in Fredericton, NB.

To use a banned substance such as metronidazole, it was required that an application for an Experimental Studies Certificate (ESC) for a veterinary drug be completed and sent to Health Canada for approval. The application was reviewed by the Clinical Evaluation Division (CED) and the Human Safety Division (HSD). An Animal Use Protocol form was also completed and submitted to the Maritimes/Gulf/CFIA Regional Animal Care Committee. Prior to submission of the form it was required that I complete an online Experimental Fish Course that was offered by the Canadian Aquaculture Institute at the University of Prince Edward Island.

Since more than one organization was involved in the depletion study it was necessary that an agreement in the form of a Letter of Understanding (LOU) was written and signed to outline the responsibilities of the two parties, CFIA and DFO. Security clearance for CFIA employees for entrance into BIO was also required.

3.8.1 Preparation of Medicated Diet

Medicated feed was prepared at the BIO facility, using the same feed pellets the fish were fed prior to the medication period and MNZ that was purchased from Sigma, at a concentration of 3 g/kg of feed. The MNZ was dissolved in acetone and the food pellets coated with the solution. The acetone was evaporated by air drying for 24 hours at room temperature leaving the MNZ residue on the pellets. The medicated pellets were stored at -20C until use. Prior to feeding, small quantities were thawed and refrigerated until fed.

A sample of the pellets was extracted and analyzed by LC-MS-MS to determine the concentration achieved. The pellets were crushed by mortar and pestle, then extracted as in section 3.3, except only 1 g of material was weighed out and 3 ml of water added. Dilutions of 10^5 and 10^6 times were done to avoid contamination and to keep concentrations within the standard curve. Non medicated feed was spiked at the expected concentration (3g/kg) of the medicated feed and treated the same to account for any losses during sample preparation and analysis. Samples were analyzed as in section 3.4 and results calculated as in section 3.5 taking into consideration the change in sample weight and the dilutions.

3.8.2 Preparation of Incurred Fish

Seventy three rainbow trout (approximately 200g in size) were transported from the Caledonia, NS fish hatchery to BIO fish labs by truck using aerated tanks. Once relocated to BIO, the fish were housed in two separate tanks capable of holding forty fish each and maintained under the same conditions (water temperature, dissolved oxygen, etc.). Water quality parameters were measured periodically. The Fish Laboratory Manager recorded the supply water temperature for the tanks daily at approximately 9:00 am. Faeces and any residue at the bottom of the tank was vacuumed out and placed in buckets to settle, then transferred in jars, autoclaved and disposed of by CFIA along with other lab waste chemicals.

Fish were fed commercial food pellets from Corey Feed, which was used at the hatchery, prior to exposure to the prepared medicated feed and during the withdrawal periods. Feeding was done once daily as required by the Animal Care Committee. Fish were acclimated for 2 weeks at BIO prior to the study to ascertain the amount of feed that would be consumed by the fish under new holding conditions. Fish were fed the medicated diet during two five day medicated periods and the drug free diet during acclimation and withdrawal periods at a level of 1% body weight. Feeding was the same as in a typical aquaculture farm. Fish were fed as a group in the tanks and access of individual fish to feed was not controlled.

3.8.3 Sampling of Fish

Immediately prior to the first feeding of the medicated diet, seven fish were sampled as controls. The fish were sacrificed with a blow on the head so as to not add potential interferences while undergoing chemical analysis. The first sampling was followed by 2 samplings during the 5 day medication period (after 3 and 5 days of

medication) and three samplings during the 5 day withdrawal period (after days 6, 8 and 10 from the medication, i.e. days 1, 3 and 5 of withdrawal). The sample size at each time point was 7. A second 5 day medication period was also done with a 16 day withdrawal period. Samples were taken on day 6, 12, 15, 18 and 22 after medication (i.e. days 1, 6, 9, 12 and 16 of withdrawal). The sample size at each time point was 5. All fish sampled were weighed and measured prior to being filleted and frozen at -80°C. Fish were sacrificed at BIO, placed on ice and transported immediately to the adjacent CFIA facility (1-2 minutes between facilities).

3.8.4 Chemical Analysis

Samples were prepared as per section 3.2. Homogenized samples were extracted using acidic acetonitrile followed by C-18 SPE column clean up explained in section 3.3. Analysis was performed in duplicate by UPLC-MS-MS following the parameters outlined in section 3.4. Quality assurance guidelines were maintained using spike recoveries and an internal standard.

Results were calculated from a calibration curve prepared as in section 3.5. The mean, SD and % RSD were calculated for the results at each time point. Results were plotted to show change in concentration with time.

3.8.5 Disposal of Waste from Tanks Following Depletion Study

Since nitroimidazoles are banned substances, it was necessary to avoid external contamination to the water system. Faeces and any residue at the bottom of the tank was vacuumed out and placed in buckets to settle, then transferred in jars, autoclaved and disposed of by CFIA along with other lab waste chemicals.

3.8.6 Tissue Disposal

Incurred tissue not required for analysis in the depletion study were maintained in frozen storage and for use as analyst training and quality control materials once routine analysis of CFIA survey samples commences. Any stored materials which become unfit will be disposed as laboratory waste.

Chapter 4 - Results and Discussion

4.1 Method Development

The development of an analytical method for very low concentrations of a residue or contaminant in a food usually involves three major steps. First, the detection parameters are determined for standards of the analyte. Second, a chromatographic separation is developed using standards of the analyte. Finally, procedures are investigated to extract the analyte from the matrix and to remove most co-extractives and potential interferences, using procedures such as solid phase extraction. When mass spectrometric detection is used in combination with liquid chromatographic separation, additional studies must be conducted to determine if matrix effects can enhance or suppress the response from the analyte, thus providing information on whether calibration curves may use pure standards or whether alternative approaches, such as fortification of blank matrix with standards or use of internal standards, is required (Matuszewski et al, 2003). The investigations conducted during methods development in this research followed this general approach and the results of these investigations are presented in the following sections.

During the development of a residue method it is important to perform extraction and clean up experiments. Typically methods to determine nitroimidazoles involve extraction with an organic solvent, followed by SPE, in order to clean up the extracts prior to determination (Mahugo-Santana et al., 2010). Sample extraction is necessary in order to isolate and concentrate the target compounds from complex matrices because most analytical instruments cannot handle the sample directly. Many nitromidazole residue methods use ethyl acetate or acetonitrile to extract the residues from biological

samples followed by SPE in order to clean up the extract. Also, to aid in eliminating impurities, agents such as hexane can be added.

To determine an extraction and clean up procedure, experiments were based on observations from literature as well as procedures used for other veterinary drug residue classes monitored in the CFIA Dartmouth laboratory. The organic solvents and SPE used which gave the best recoveries were chosen for the method.

LC-MS has become the instrument of choice for drug residue analysis including nitroimidazole residues (Mahugo-Santana et al., 2010). Single MS is being replaced by tandem MS to achieve confirmation of analyte identity. LC-MS-MS was chosen for development of the method for its confirmation capabilities.

UPLC takes advantage of technological strides made in particle-chemistry performance (Mahugo-Santana et al, 2010). The use of UPLC in the development of this method allowed the use of columns and instrumentation that are operated under higher pressures which resulted in increased resolution, sensitivity and speed of analysis. All seven nitroimidazole compounds investigated were separated in under 5 minutes. See table 4.2 in section 4.1.2 for retention time of the analytes.

Gradient elution is commonly used in LC using a binary system including an aqueous component and a less polar organic solvent with a weak acid buffer to maintain appropriate pH (Mahugo-Santana, 2010). A water-acetonitrile gradient with 0.1% acetic acid was chosen and shown to give good separation and resolution of the analytes included in the method.

Data results reported in this chapter were rounded according to Miller and Miller, (2005) and the raw data contained in the appendices were not rounded. As per Miller and

Miller, the standard deviation dictated the number of significant figures that were reported, with uncertainty being associated with the number indicated by precision. Therefore, a standard deviation of 0.1 indicates uncertainty at the first digit following the decimal and no additional figures are reported.

4.1.1 MS

The parent compounds MNZ, IPZ, RNZ and DMZ along with their metabolites MNZ-OH, IPZ-OH and HMMNI were monitored using MS-MS detection. Both APCI and ESI interfaces have been applied for LC-MS analysis of nitroimidazoles usually under positive ionization conditions (Sams et al, 1998 and Hurtaud-Pessel et al., 2000). ESI in positive mode gave the most favourable analyte responses during development. According to Commission Decision 2002/657/EC MSMS (EC, 2002) and Codex Alimentarius Commission Guideline CAC/GL 71-2009 (CAC, 2009a) methods are required to have a precursor (parent) ion plus two characteristic transition ions for confirmation of each target analyte. Mass spectra were recorded for each analyte at various cone voltages to give the best response followed by the application of different collision energies to select two daughter ions for each parent. Table 4.1 summarizes parent ions and their daughter fragments necessary for quantification and confirmation of the analytes.

Table 4.1 Parent and daughter fragmentations for nitroimidazoles and their metabolites

Compound	Parent ion m/z	Daughter m/z
MNZ	171.0	127.9 82.0
IPZ	170.0	124.0 109.0
RNZ	201.0	140.0 55.1
DMZ	142.2	96.1 81.2
MNZ-OH	188.2	125.9 122.9
IPZ-OH	186.0	168.0 122.0
HMMNI	157.8	139.9 55.2

Fragmentation pathways for nitroimidazole molecules were discussed in two previously published papers (Mottier et al, 2006 and Sun et al, 2009). The proposed fragmentation pathway of the mass fragments used for each molecule studied is explained in the following and shown in figure 4.1.

IPZ and IPZ-OH

IPZ (m/z 170) fragmented with a loss of NO_2 (46 Daltons (Da)) to produce m/z 124 and CH_3NO_2 (61 Da) to give m/z 109. According to Mottier et al., 2006, the CH_3 is released when the imidazole ring opens, while Sun et al, 2009 propose the CH_3 is released from outside the ring. The compound IPZ-OH (m/z 186) gave a fragment with m/z 168 following a loss of H_2O, then NO_2 was also eliminated giving a radical cation (m/z 122).

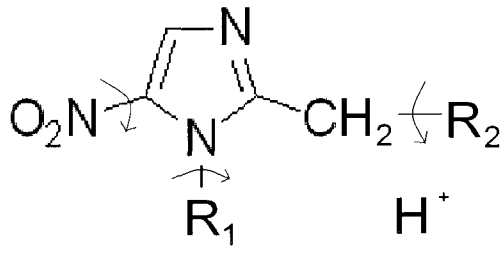
MNZ and MNZ-OH

The mass spectra for MNZ (m/z 172) contained fragment ions at m/z 128 and 82. The loss of C_2H_3OH (44 Da) led to m/z of 128, followed by a further loss of NO_2 from this fragment producing a radical cation with m/z 82. For the metabolite MNZ-OH (m/z 188), the main fragments were at m/z 126 and 123. The elimination of H_2O and C_2H_3OH (62 Da) gave m/z 126. The losses of NO_2 and H_2O followed by a cyclization of the residual fragment gave m/z 123.

DMZ, RNZ and HMMNI

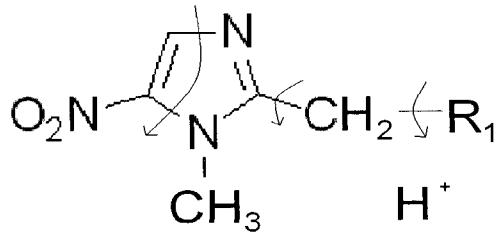
The fragmentation pathway for DMZ (m/z 142) was similar to IPZ. The fragment at m/z 96 represented the loss of NO_2 while a further loss of CH_3 produced a fragment at m/z 81. For RNZ (m/z 201), the ion observed at m/z 140 resulted from the loss of CH_3NO_2 (61 Da) and the ion at m/z 55 was due to a loss of $C_4H_6O_4N_2$ (146 Da) which results from the opening of the imidazole ring. The precursor molecular ion for HMMNI, which had a mass to charge ratio (m/z) of 158, had a fragment ion at m/z 140 from the loss of H_2O and an ion at m/z 55 from the loss of C_2HNO_2 and CH_3OH when the imidazole ring opens, for a total of 103 Da.

a)



	Fragment Loss			
Analyte (Da)	R_1 (Da)	R_2 (Da)	Other (Da)	Pathway
IPZ (170)	CH_3 (15)		NO_2 (46)	1) 170-46→124 2) 170-46-15→109
IPZ-OH(186)		H_2O (18)	NO_2 (46)	1) 186-18→168 2) 186-18-46→122
MNZ (172)		CH_2-CHOH (44)	NO_2 (46)	1) 172-44→128 2) 172-44-46→82
MNZ-OH (188)	CH_2=CHOH (44)	H_2O	NO_2 (46) + H_2O (18) + cyclization (1)	1) 188-44-18→126 2) 188-46-18-1→123
DMZ (142)	CH_3 (15)		NO_2 (46)	1) 142-46→96 2) 142-46-15→81

b)



	Fragment Loss		
Analyte (Da)	R1 (Da)	Other (Da)	Pathway
RNZ (201)	HO-CO-NH_2 (61)	Ring opens, loss of $C_4H_6O_4N_2$ (146)	1) 201-61→140 2) 201-146→55
HMMNI (158)	H_2O (18)	Ring opens, loss of NO_2-C=CH + CH_3OH (103)	1) 158-18→140 2) 158-103→55

(c)

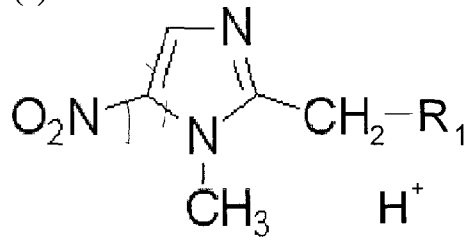


Figure 4.1 Proposed MS ion formation for IPZ, IPZ-OH, MNZ, MNZ-OH and DMZ (a), RNZ and HMMNI (b) and an alternative pathway for DMZ and IPZ (Mottier et al, 2006)

4.1.2 LC

Typical chromatograms of blank tilapia muscle and tilapia muscle spiked at 1 ng/g with HMMNI, IPZ, IPZ-OH, MNZ, MNZ-OH, RNZ and DMZ are shown in figure 4.2. Similar chromatograms of salmon and shrimp muscle are shown in figures 4.3 and 4.4. Chromatographic separation was achieved for all 7 analytes using the chosen gradient. Analytes were well separated within 5 minutes with a total run time of 10 minutes including re-equilibration. Retention times for compounds are shown in table 4.2.

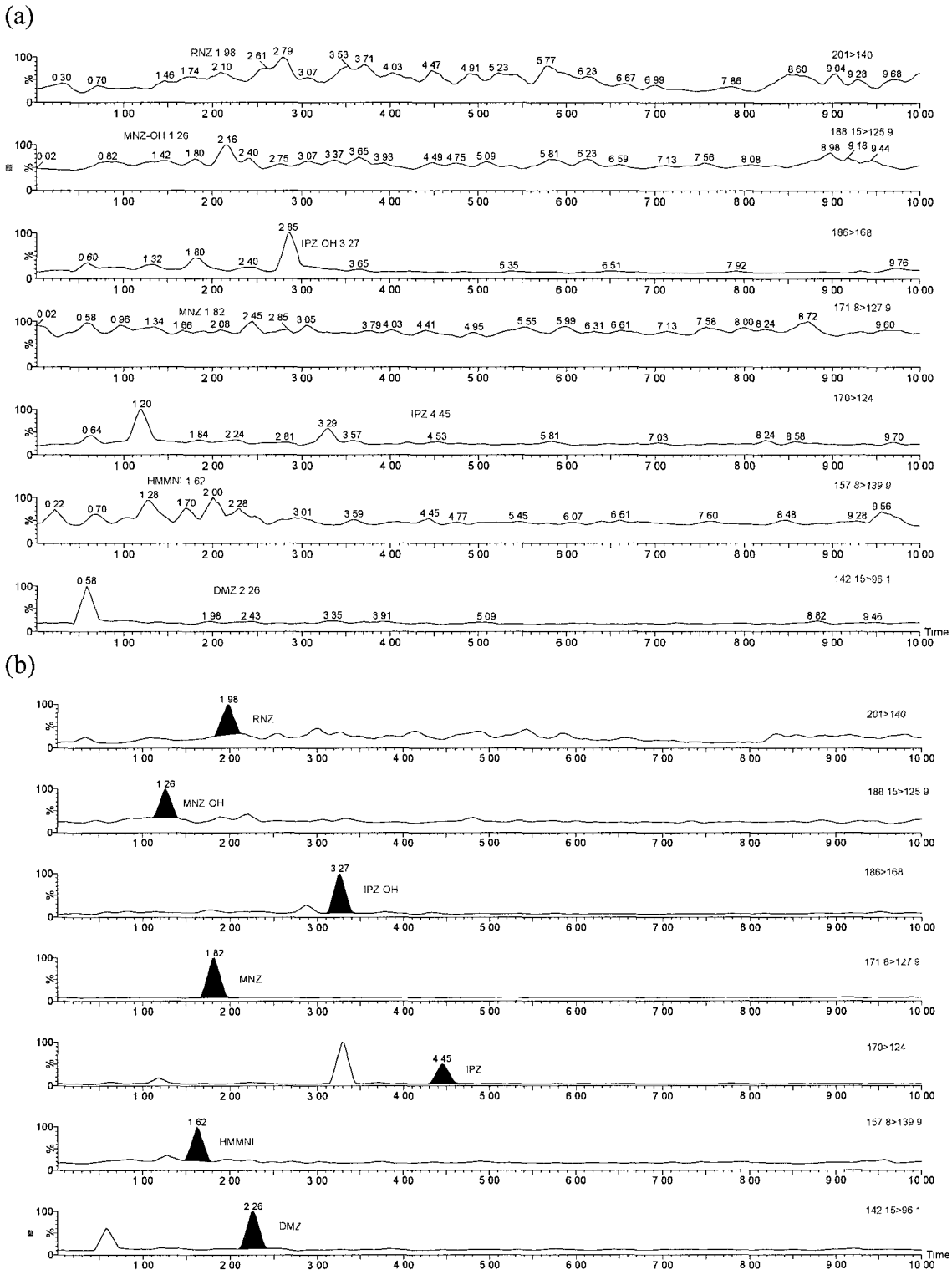


Figure 4.2 Chromatograms of tilapia fish tissue extract: blank tissue (a), tissue fortified with 1 ng/g nitroimidazoles (HMMNI, IPZ, IPZ-OH, MNZ, MNZ-OH, RNZ and DMZ (b). The blank chromatogram is labelled with expected retention times of analytes.

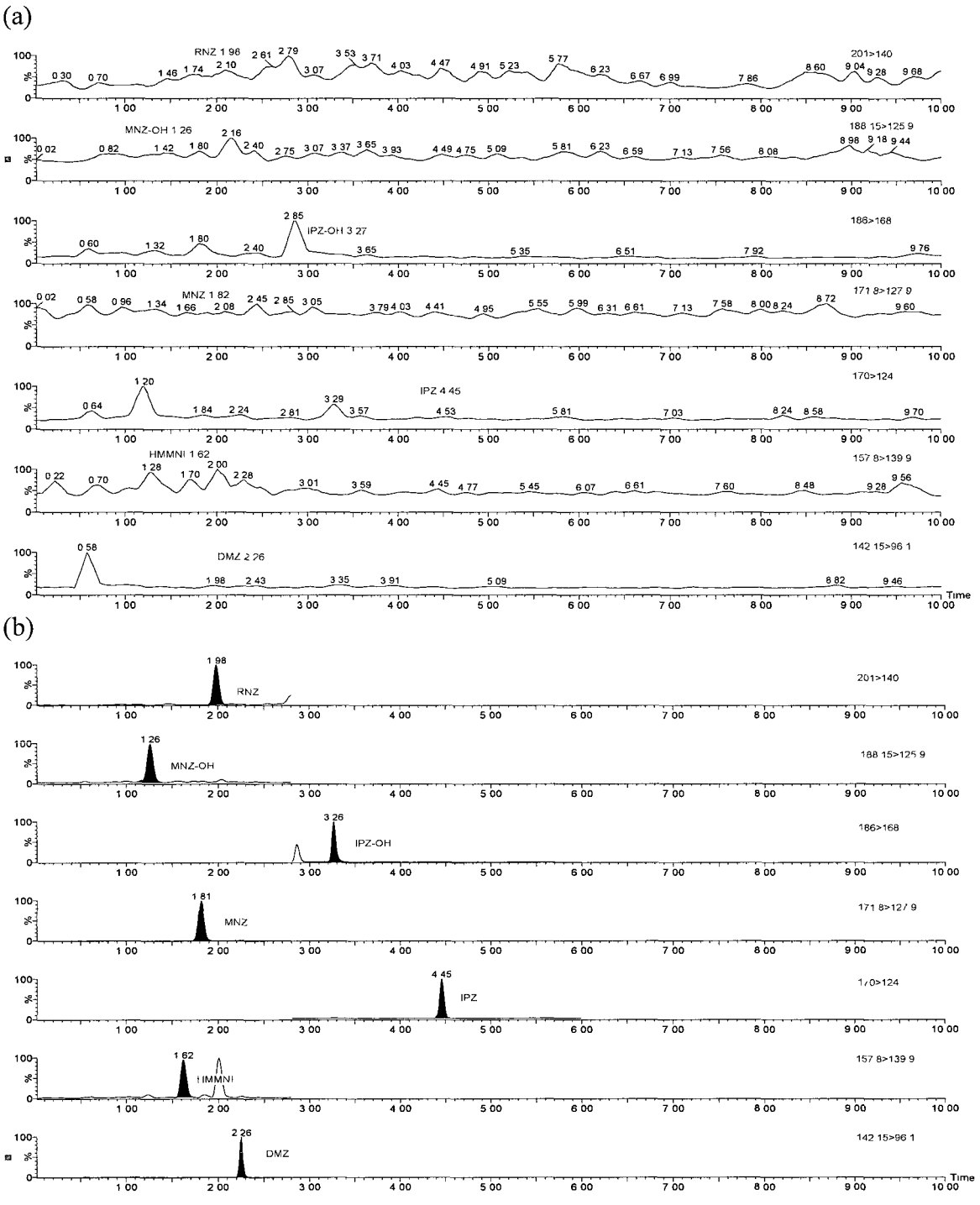


Figure 4.3 Chromatograms of salmon fish tissue extract: blank tissue (a), tissue fortified with 1 ng/g nitroimidazoles (HMMNI, IPZ, IPZ-OH, MNZ, MNZ-OH, RNZ and DMZ (b). The blank chromatogram is labelled with expected retention times of analytes.

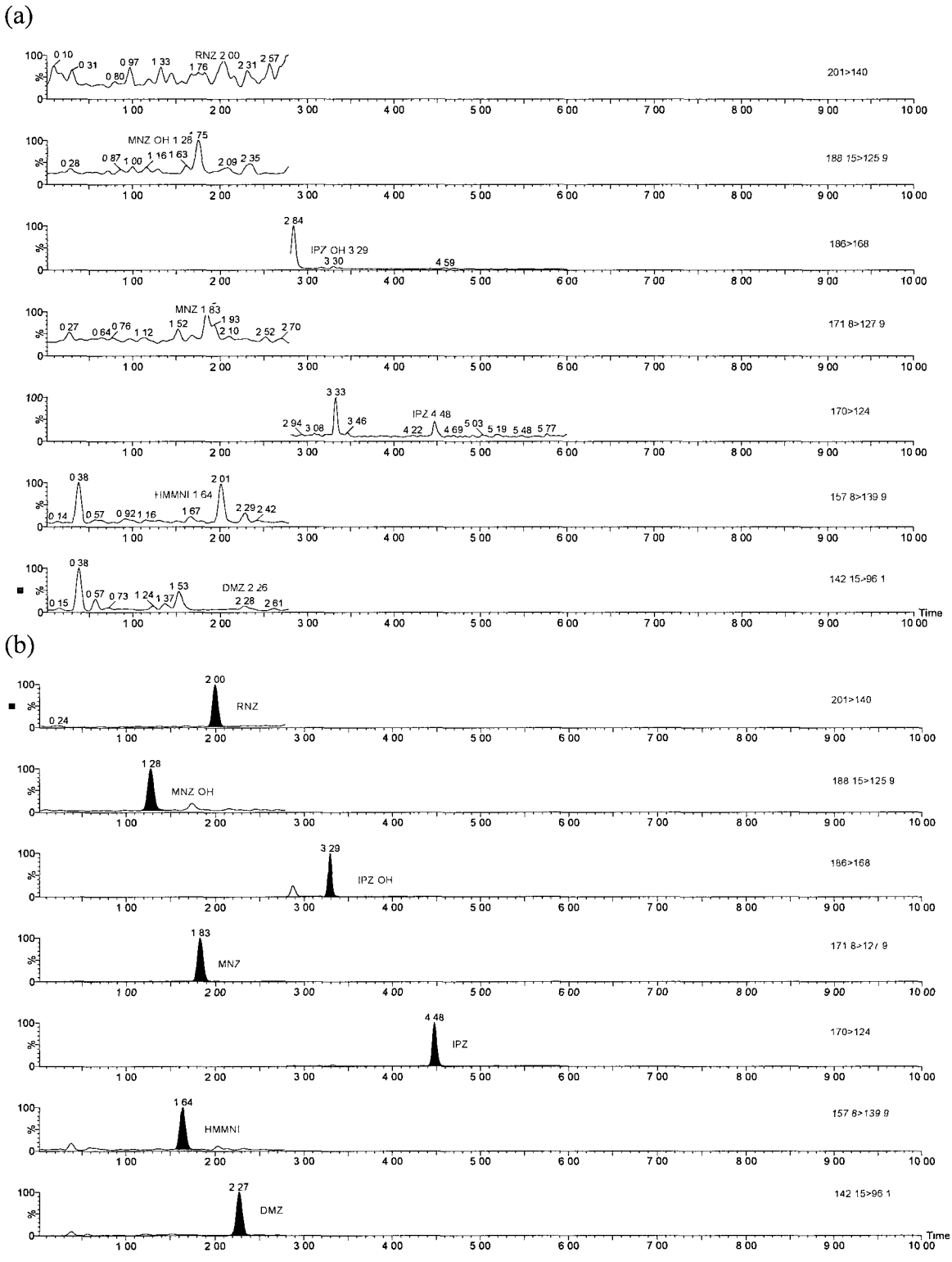


Figure 4.4 Chromatograms of shrimp fish tissue extract: blank tissue (a), tissue fortified with 1 ng/g nitroimidazoles (HMMNI, IPZ, IPZ-OH, MNZ, MNZ-OH, RNZ and DMZ (b). The blank chromatogram is labelled with expected retention times of analytes.

Table 4.2 Retention times, Correlation Coefficients (R^2) and equations from calibration curves for NI compounds

Analyte	Retention Time (minute)	Correlation Coefficient (R^2)	Calibration Equation
HMMNI	1.6	0.9975	y = 909.52x - 701.03
IPZ	4.5	0.9941	y = 2352.1x + 227.93
IPZ-OH	3.3	0.9985	y = 2585.6x + 4894.3
MNZ	1.8	0.9941	y = 1656.9x + 5502
MNZ-OH	1.3	0.9966	y = 458.98x + 217.58
RNZ	2.0	0.9969	y = 1094x + 1509.7
DMZ	2.3	0.9987	y = 1999.5x - 1370.1

4.1.3 Linear Range and Sensitivity

A standard curve was tested at 0, 10, 20, 40, 60, 80 and 100 ng/ml nitroimidazoles (NIs). The standard calibration curves were linear for all analytes with correlation coefficients (R^2) $\geq$ 0.9941. Correlation coefficients for each analyte are in table 4.2. From this information, it was decided calibration curves with standards 0, 2, 4, 10 and 50 ng/ml would be used when determining matrix effects and for routine analysis of samples. Linear range was determined to be 0-62.5 ng/g, which was used to determine the analytical range of 0.3 – 62.5 ng/g. This was further tested during validation studies.

Sensitivity was determined from the slopes of the calibration curves. Sensitivity describes the change in instrument response for a given concentration change (CAC, 2009b; Anon, 1998). It is represented by the slope of the calibration curve and can be determined by using samples containing various concentrations of the analyte. The slopes were neither too steep nor too shallow and were considered suitable to produce reliable analytical results. As shown in figure 4.5 the slope of the curve has an angle of inclination that lies between 30 and 60 degrees. This was true for all nitroimidazole analytes and the

calibration equations are in table 4.2. Consistent change was observed in detector response with concentration indicating good sensitivity.

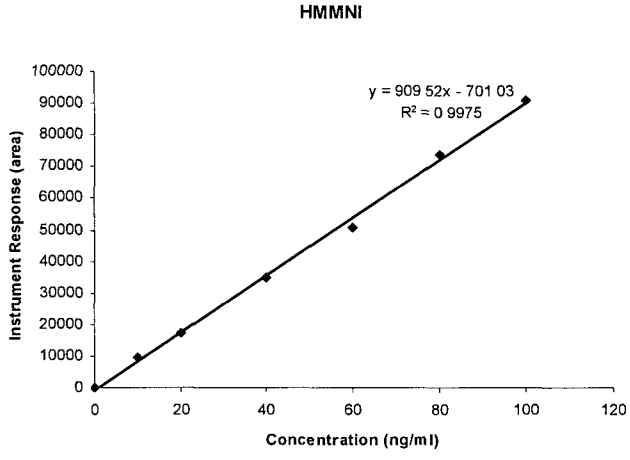


Figure 4.5 Calibration curve for HMMNI using neat standards

4.1.4 Preliminary Extraction and SPE

In the first experiment, spiked salmon muscle samples were extracted with either acidic MeCN (0.08% perchloric acid) or 100 % MeCN followed by SPE cleanup using C18 Waters Sep Pak cartridges. Very low recoveries resulted with the 100% MeCN extraction and better recoveries ranging from 41-62% were obtained with acidic MeCN.

To determine if the chosen SPE C18 Sep Paks caused the loss of analytes, spikes were added both before and after the SPE step. Acidic MeCN was used for extraction since it had improved recoveries compared to 100% MeCN. Recoveries were similar for both spiking before and after SPE, which indicated the SPE was not causing the loss of analyte.

In an attempt to improve recoveries, a hexane wash was added to remove fat and other impurities. This provided an improvement with mean recoveries for all analytes

with hexane wash ranging from 85 to 106%. Method development continued using acidic MeCN, C18 SPE and a hexane wash.

The intermediate precision "includes the same measurement procedure, same location, and replicate measurements on the same or similar objects over an extended period of time, but may include other conditions involving changes", such as "new calibrations, calibrators, operators, and measuring systems" (JCGM, 2008). During method validation a problem with the hexane wash step was discovered when a second analyst was analysing salmon to assess intermediate precision of the method. The hexane wash step was originally conducted after the final extract was made up in 0.1% acetic acid solution. However, vigorous vortexing of the hexane and 0.1% acetic acid with fatty extract such as salmon, caused emulsions to form. While the analytes are not fat soluble, the emulsion formation did interfere with recovery and analysis. This had not been a problem seen by the first analyst. To avoid future issues with emulsions that may not separate, the hexane wash was done while the extract was in acetonitrile, before 0.1% acetic acid was added to the final extract. The acetonitrile and hexane layers easily separated even after vigorous vortexing. Analyses with fortified salmon, shrimp and tilapia were repeated at concentrations of 1, 10 and 50ng/g with the change in the hexane wash step. The % RSDs calculated between results with the original and the new hexane steps were within the acceptable range for method repeatability (table 4.3). T-tests were also performed on the data to determine any significant differences. An example of a t-test and raw data for the hexane wash results are in Appendix 1. No significant difference between results was seen for shrimp muscle. As shown in table 4.3 all two tailed p-values were >0.05 for sample size of 15 (n=15). Some p-values for tilapia and salmon were

<0.05, however this was due to a smaller sample size (n=5). This method verification was accepted based on the shrimp p-values and the acceptable % RSDs for all commodities and was written into the procedure.

Table 4.3 RSDs and p-values calculated between nitroimidazole results obtained using the original and new hexane step for muscle from tilapia, salmon and shrimp. (See Appendix 1 for the analyte concentration results determined).

		Tilapia		Salmon		Shrimp	
Analyte	Fortified Concentration (ng/g)	RSD (%)	p-value	RSD (%)	p-value	RSD (%)	p-value
HMMNI	1	13	0.24	7.5	0.01	12	0.27
	10	7.2	0.59	15	0.03	8.6	0.61
	50	5.9	0.16	8.6	0.85	6.3	0.69
IPZ	1	9.8	0.02	20	0.01	11	0.50
	10	6.7	0.43	10	0.04	12	0.58
	50	9.3	0.05	15	0.00	11	0.30
IPZ-OH	1	6.7	0.09	12	0.03	5.4	0.54
	10	4.6	0.25	6.9	0.26	6.8	0.73
	50	4.2	0.05	7.6	0.10	6.2	0.75
MNZ	1	8.0	0.19	18	0.18	9.8	0.06
	10	8.0	0.003	8.8	0.10	9.2	0.17
	50	8.9	0.03	14	0.16	8.7	0.96
MNZ-OH	1	9.4	0.14	13	0.34	13	0.22
	10	4.3	0.53	9.3	0.51	11	0.28
	50	5.4	0.30	12	0.02	8.2	0.40
RNZ	1	8.8	0.38	8.1	0.73	19	0.39
	10	6.0	0.26	9.8	0.01	17	0.21
	50	7.9	0.15	7.0	0.15	16	0.08
DMZ	1	7.2	0.24	6.6	0.29	8.3	0.96
	10	6.8	0.03	10	0.54	10	0.85
	50	8.2	0.16	9.5	0.04	8.5	0.70

4.1.5 Matrix Effects and Selectivity

The common perception has been that MS-MS detection is highly selective and thus ion suppression or enhancement caused by sample matrix and interferences from metabolites is eliminated (Matuszewski, B.K. et al., 2003). However, co-eluting, undetected matrix components may affect the intensity of the analytes and effect reproducibility and accuracy of the method. Regulatory requirements include the need for the assessment of matrix effect during development and validation of LC-MS-MS methods. The recommended approach (Matuszewski, B.K. et al., 2003) is to compare the MS response for analytes spiked into extracts of blank matrix (matrix matched standards) with the response for pure standards to assess matrix effects, then to compare the response obtained for standards spiked into blank matrix (matrix fortified standards) prior to extraction with the response for standards spiked into extracts of blank matrix to assess recovery.

Matrix effects were revealed by comparison of the slopes of neat and matrix matched standard curves. The absolute values of the % differences between the slope of the neat standard curve versus matrix matched were > 10% for all analytes, which indicated it was necessary to perform analysis using matrix matched standards and/or an internal standard (IS). Correlation coefficients for both neat and matrix matched calibration curves for each analyte in all three matrices tested; along with calibration equations and slope differences are shown in table 4.4 for tilapia muscle, table 4.5 for salmon muscle and 4.6 for shrimp muscle. Responses of the standards for the neat calibration curves were higher than those of the matrix matched which indicated ion suppression from the detector. This can be seen in figure 4.6 for HMMNI in tilapia

muscle, figure 4.7 for HMMNI in salmon muscle and figure 4.8 for HMMNI in shrimp muscle, in which both curves are compared.

Table 4.4 Correlation Coefficients (R^2), calibration equations and slope differences for both neat and matrix calibration curves for tilapia muscle

	Neat		Matrix		
Analyte	Correlation Coefficent (R^2)	Calibration equation	Correlation Coefficent (R^2)	Calibration equation	Slope Difference (%)
HMMNI	1.000	y = 1419.3x - 24.861	1.000	y = 965.11x - 66.015	32
IPZ	0.9998	y = 2663.2x - 867.17	0.9997	y = 2368.2x - 431.65	11
IPZ-OH	1.000	y = 3427.2x + 337.27	0.9998	y = 2656x + 880.98	22
MNZ	0.9998	y = 2864.3x + 1124.9	0.9999	y = 2256.4x + 670.87	21
MNZ-OH	0.9999	y = 755.14x + 164.24	0.9999	y = 530.1x - 60.546	30
RNZ	0.9998	y = 1656.5x + 427.38	1.000	y = 1197.4x + 55.943	28
DMZ	0.9999	y = 2613.2x - 319.47	1.000	y = 1970.8x - 62.738	25

Table 4.5 Correlation Coefficients (R^2), calibration equations and slope differences for both neat and matrix calibration curves for salmon muscle

	Neat		Matrix		
Analyte	Correlation Coefficient (R^2)	Calibration equation	Correlation Coefficient (R^2)	Calibration equation	Slope Difference (%)
HMMNI	1.000	y = 1419.3x - 24.861	1.000	y = 963.10x + 205.69	32
IPZ	0.9997	y = 2663.2x - 867.17	1.000	y = 2104.6x - 193.08	21
IPZ-OH	1.000	y = 3427.2x + 337.27	1.000	y = 2935.5x + 221.43	14
MNZ	0.9998	y = 2864.3x + 1124.9	0.9997	y = 2249.1x + 863.46	22
MNZ-OH	0.9999	y = 755.14x + 164.24	0.9997	y = 562.63x + 69.507	26
RNZ	0.9998	y = 1656.5x + 427.38	0.9995	y = 1235.1x + 327.66	25
DMZ	0.9999	y = 2613.2x - 319.47	0.9999	y = 2118.8x - 120.88	19

Table 4.6 Correlation Coefficients (R^2) and slope differences for both neat and matrix calibration curves for shrimp muscle

	Neat		Matrix		
Analyte	Correlation Coefficient (R^2)	Calibration equation	Correlation Coefficient (R^2)	Calibration equation	Slope Difference (%)
HMMNI	0.9992	y = 1179.3x - 760.54	0.9996	y = 753.93x + 176.99	36
IPZ	0.9996	y = 3041.5x - 1420.4	0.9998	y = 1729x - 200.07	43
IPZ-OH	0.9996	y = 2912.7x + 130.53	0.9988	y = 2338.5x - 330.9	20
MNZ	0.9999	y = 2481.1x - 558.31	0.9999	y = 1857.8x + 301.25	25
MNZ-OH	0.9997	y = 604.87x - 50.277	0.9999	y = 408.59x - 96.095	32
RNZ	0.9994	y = 1358.7x - 475.95	0.9958	y = 867.77x + 580.45	36
DMZ	0.9993	y = 2145.2x - 1353.1	0.9984	y = 1853.1x - 1213.5	14

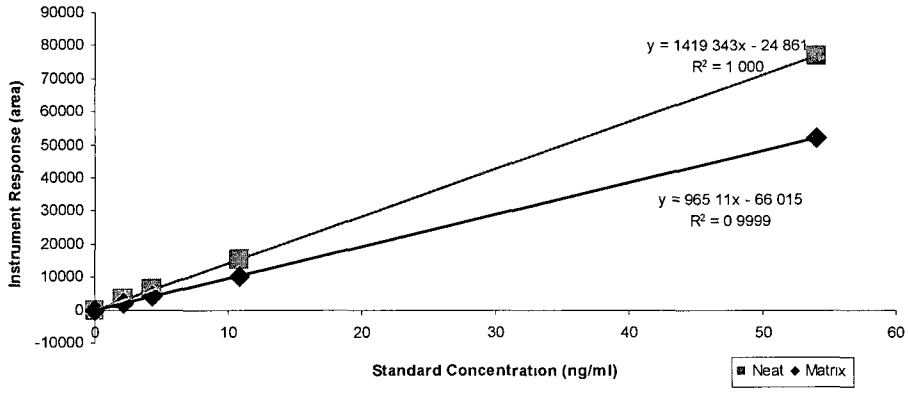


Figure 4.6 Comparison of neat and tilapia matrix curves for HMMNI

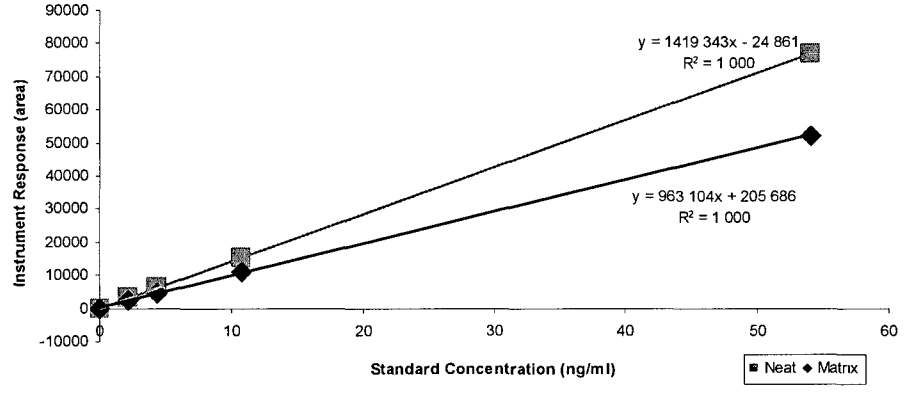


Figure 4.7 Comparison of neat and salmon matrix curves for HMMNI

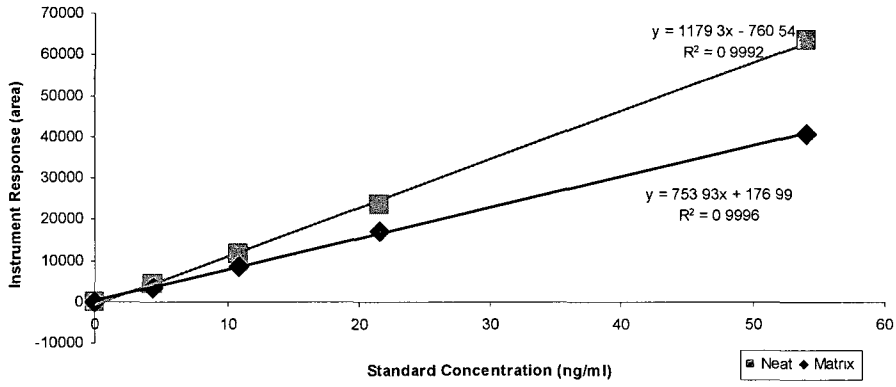


Figure 4.8 Comparison of neat and shrimp matrix curves for HMMNI

It was necessary to determine if there were any significant interferences in the identification and /or quantification of results (EC, 2002). It can be observed from figures 4.2, 4.3 and 4.4 that there were no co-eluting peaks in the matrices. The chromatogram in figure 4.9 of tilapia muscle spiked with fluoroquinolones, quinolones and triphenyl methane dyes showed there were no interferences from these veterinary drugs, which could be present in test materials. Trout material incurred with MNZ and MNZ-OH was also spiked with these other vet drug classes and compared with the incurred material without spike added. Similar results were obtained with or without the other classes of drugs present (table 4.7). The % RSD calculated from the mean and SD of both results was within acceptable repeatability results. In addition, recoveries did not suggest the presence of significant co-eluting peaks (see Section 4.1.6).

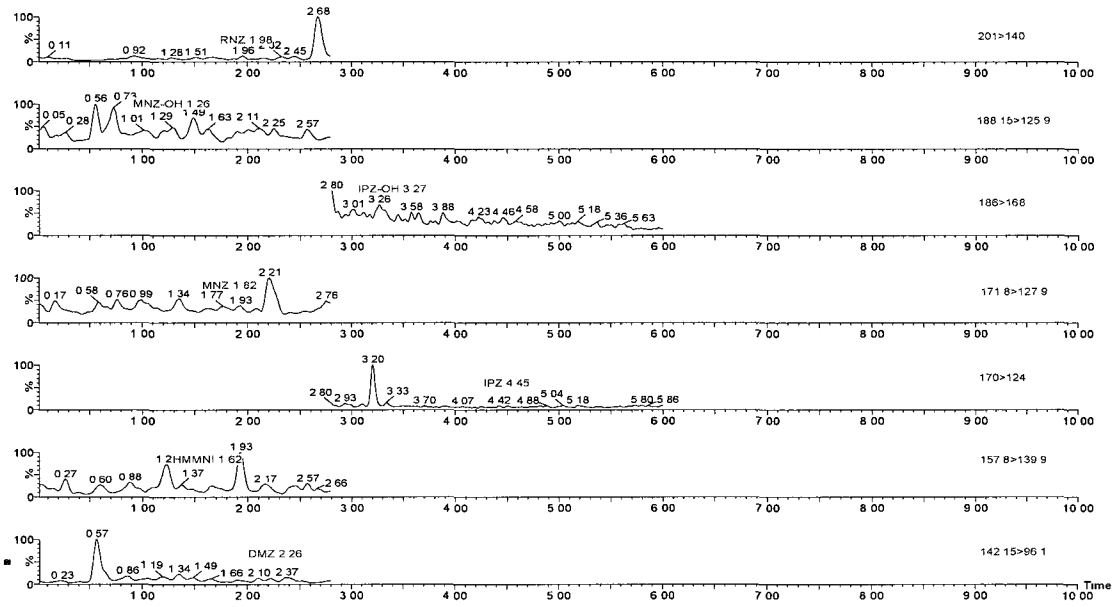


Figure 4.9 Chromatogram of blank tilapia tissue spiked with fluoroquinolones, quinolones and triphenyl methane dyes. The chromatogram is labelled with expected retention times of NI analytes.

Table 4.7 Comparison of MNZ and MNZ-OH results for incurred trout samples spiked and not spiked with fluoroquinolones, quinolones and triphenyl methane dyes

	Not spiked		Spiked		Combined results	
Analyte	MNZ	MNZ-OH	MNZ	MNZ-OH	MNZ	MNZ-OH
Mean (ng/g)	26	3.1	29	3.1	28	3.1
SD	4	0.4	1	0.1	3	0.3
%RSD	15	13	5.1	4.2	12	10

Each NI investigated for the method was identified with a precursor ion and two product ions. All analytes had different precursor and product ions providing selectivity. Also, other analytes from different classes of veterinary drugs which may be present in material being tested for NIs, have different precursor and product ions.

4.1.6 Preliminary LOD, Recovery and Precision

LOD was first estimated during method development for each analyte in all three matrices to be between 0.1 – 0.3 ng/g based on signal-to-noise and the concentration of standards analyzed. It was then determined to be approximately 0.1 - 0.3 ng/g by evaluating the noise level of a blank salmon, shrimp and tilapia muscle sample that had been analyzed and calculating the concentration equivalent to three times the noise expected at the retention time.

Preliminary recoveries for all analytes were determined during method development to be 85 to 117% for tilapia, 85 to 106% for salmon and 95 to 110% for shrimp muscle. Preliminary method repeatability on tilapia muscle fortified at 5ng/g gave %RSDs of 3 to 8% depending on the analyte. All of these parameters were further investigated in validation.

4.1.7 Ruggedness

Ruggedness is defined as a resistance to changes in the results produced by an analytical method when minor deviations are made from the experimental conditions described in the method (Eurachem, 1998; CAC, 2009b). Ruggedness was investigated using tilapia muscle fortified to contain a concentration of 5 ng/g NI. Several variables tested were determined to have created statistically significant changes determined by a two sample t-test with p-values < 0.05 (Appendix 2).The factors which were found to be significant included the volume of acidic MeCN used; continued drying after evaporation was complete; evaporation temperature; and the filter used for collection of the final extract in the autosampler vial. The procedure was changed to include additional

specifications for the volume of acidic MeCN used and the evaporation temperature, as divergence from the required volume or temperature specified could significantly affect final results. Excessive drying during evaporation and the type of filter used were identified as critical control points, as divergence from the written method could severely affect final results.

4.1.8 Internal Standard

DMZ-D_3 was introduced during method development as a quality control check for each sample analyzed in a run. The recovery of DMZ -D_3 for each sample was first calculated during a method repeatability and recovery experiment. The recoveries calculated ranged from 86 to 97%. These recoveries were useful for determining any analyte loss in a particular sample during sample preparation or analysis.

A deuterated internal standard is also commonly used in mass spectrometry to improve quantitative analysis, so the use of the internal standard for quantification was also investigated. In some cases, a single internal standard can be used for quantification of multiple analytes, provided the relative response factors of the analytes to the internal standard are well-established and recoveries of both the internal standard and the analytes are consistent. In many cases, however, it is preferable to use a deuterated version of each analyte as the internal standard for quantification of that analyte, since this eliminates the issues of recovery and consistency of response (it is generally considered that both analyte and deuterated internal standard will be subject to the same influences in any analysis, so recovery and response will be similarly affected for both). The use of the internal standard to improve quantification in this method is further discussed in Section 4.2.2 Accuracy (Trueness and Bias).

4.1.9 Stability

Stability testing of both analyte and analyte/matrix is a mandatory element for methods to meet the criteria of 2002/657/EC (EC, 2002). No degradation of analyte should occur during extraction, analysis or storage. It must be ensured that working standards are stable and a time period at which new ones must be prepared is established. It must also be determined if protecting the solution from light is required. In addition, stability in the presence of matrix has to be determined and can be combined with recovery experiments. To ensure sample stability, experiments are required to determine stability of the tissue during normal conditions of storage for a typical time period from sample receipt to analysis. It is necessary to use ≥ 5 replicates for each concentration and time point in a sample stability study.

4.1.9.1 Light Sensitivity

Since nitroimidazoles had been reported as very light sensitive by Hurtaud et al. (2000) and were stored in the dark and/or in amber glassware in several other methods including Ho et al. (2005) and Xia et al. (2008), a mixed working standard was prepared and divided into two portions and one portion was stored under UV light to mimic potential exposure during routine use and the other in the dark. When neat standards were prepared with the UV-exposed and UV-protected working standard and the calibration curves compared, slope differences of $< 10\%$ were determined for HMMNI, IPZ, IPZ-OH, MNZ and DMZ indicating no significant difference between the two sets of standards. The slope differences for MNZ-OH and RNZ were 11 and 15% indicating a

significant difference. Slope differences and calibration equations for all analytes are in table 4.8. A comparison of UV and non- UV calibration curves is shown in figure 4.10 for HMMNI.

Table 4.8 Calibration equations and slope differences for calibration curves prepared with UV exposed and non UV exposed working standards for NI analytes

Analyte	UV exposed calibration equation	Non UV exposed calibration equation	Slope difference (%)
HMMNI	y = 1126.2x + 362.02	y = 1068.8x - 136.59	5.4
IPZ	Y = 3286.2x - 137.60	y = 3209.1x - 453.91	2.4
IPZ-OH	Y= 3659.2x + 679.08	y = 3761.0x - 59.558	2.7
MNZ	Y= 2268.8x + 1329.8	y = 2224.1x + 406.22	2.0
MNZ-OH	y = 607.06x + 199.82	y = 682.26x - 176.82	11
RNZ	Y= 1674.6x + 60.790	y = 1455.7x + 456.78	15
DMZ	Y = 280.03x - 358.10	y = 2653.8x + 63.924	5.6

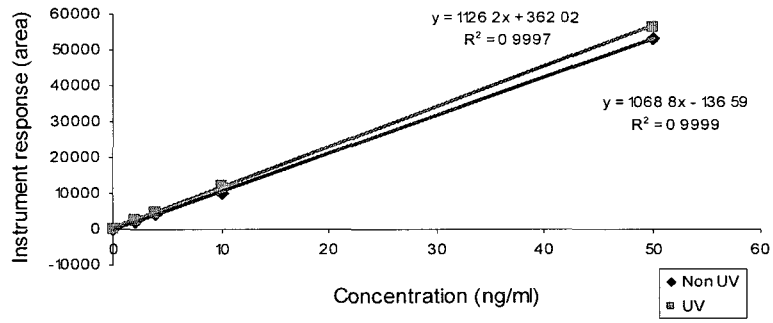


Figure 4.10 Comparison of calibration curves prepared with UV-exposed and non UV-exposed working standards for HMMNI

The two analytes, MNZ-OH and RNZ were further investigated by preparing ten 20 ng/ml standards from both the light-protected and the UV-exposed working standard solutions. These were analyzed and the average area counts calculated for each. The %RSD was calculated between the two averages to be 5% for MNZ-OH and 1% for RNZ. Both of these were within previous instrument repeatability results. Therefore, it appeared that the analytes were not light sensitive in solution and did not require light protection.

4.1.9.2 Standard Solution Stability

Stability of analyte in solution was determined by comparing a working standard prepared on the day of analysis to one that had been stored in an amber vial in the dark at 4°C. The %RSDs calculated between area counts of freshly prepared standards and 12-month old standards were $\leq$ 10% (table 4.9). These % RSDs remained within the range calculated during instrument repeatability. Therefore the working standard was considered stable under normal conditions of use for 12 months.

Table 4.9 Standard solution stability over 12 months: Comparison of old and freshly prepared standards by RSD for NI analytes

	%RSD						
Analyte	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
Day 7	0.37	3.9	2.1	1.4	2.2	1.1	1.2
Day 14	8.1	1.8	1.2	9.1	0.09	0.84	1.4
Day 28	3.2	2.4	1.3	4.7	1.1	10	2.3
Month 2	0.62	1.5	4.9	11	2.6	0.95	9.6
Month 3	3.3	4.0	8.6	0.94	3.1	2.1	3.2
Month 4	2.2	1.4	0.43	2.9	0.57	0.04	2.1
Month 5	2.7	3.2	3.7	1.5	3.9	2.2	4.5
Month 6	0.63	3.0	2.3	4.4	0.62	1.7	1.6
Month 8	1.7	4.3	3.7	1.2	8.6	4.1	0.04
Month 10	6.8	0.88	4.6	10	2.0	5.6	6.9
Month 12	1.4	5.9	0.92	2.8	5.8	1.8	9.6

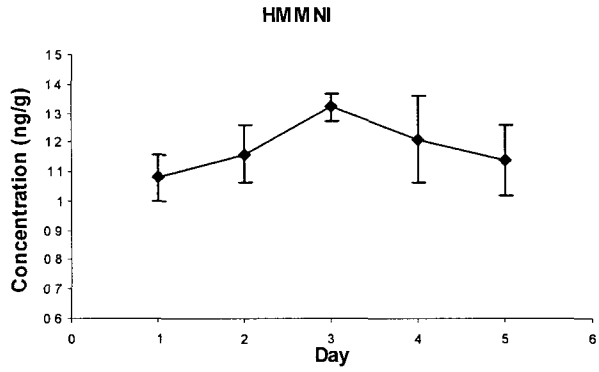
4.1.9.3 Stability During Processing/Extract Stability

To determine extract stability, the extracts from recovery studies were analyzed daily for 5 days. The mean concentration, SD and %RSD were calculated for each compound at the three concentration levels that had been used for the recovery studies (1, 10 and 50ng/g). The mean concentration and SD from each day were plotted on graphs to

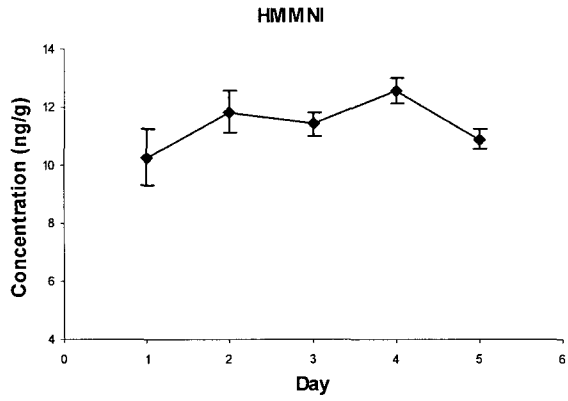
determine if the error bars overlapped from each time point. CUSUM charts, t-test and ANOVA were also used to detect any changes in the extracts.

When the average results from the 5 day stability study for the 1ng/g concentration were plotted for all analytes, the ranges encompassed by the error bars overlapped one another. For DMZ at 10ng/g, the day 4 error bar was not within the range of any other error bar; however at day 5 it was back within the range at day 3. All others were within the range of one another at 10ng/g. At 50ng/g, a few error bars were out of range, but they were back in the range of the initial time point at the following time point. From these graphs, all analytes in the extracts appeared stable for 5 days. See figure 4.11 for HMMNI graphs and Appendix 3 for all other analytes. The differences observed were attributed to sample homogeneity and/or expected analytical variation (measurement uncertainty).

(a)



(b)



(c)

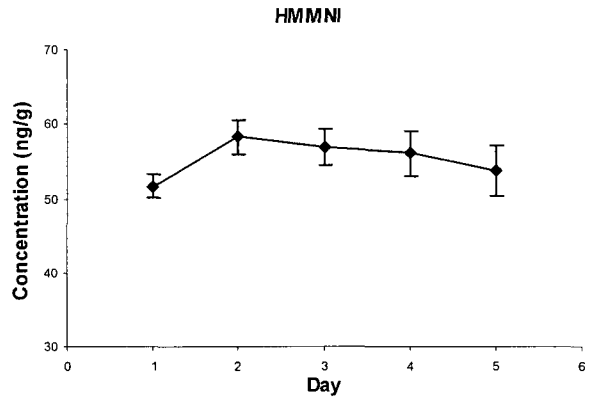
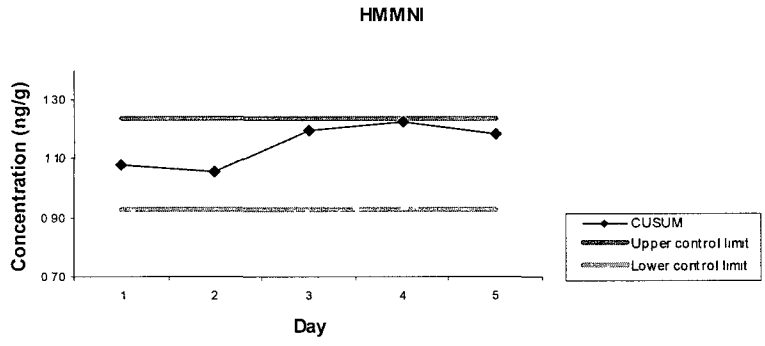


Figure 4.11 Charts representing tilapia muscle extract stability of HMMNI over 5 days at 1ng/g (a), 10 ng/g (b) and 50 ng/g (c)

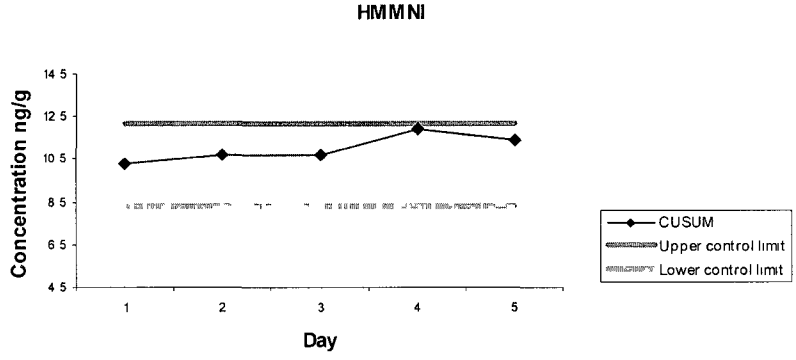
A CUSUM chart is a statistical tool used to monitor change (Page, 1954; Barnard, 1959). A CUSUM chart is constructed to show trends in change, so that random positive and negative changes in the quantity being monitored are expected. However, when the changes are consistently positive or negative and proceed outside the control limits established for the process, then the process is considered to be "out of control". In this case, where changes in concentration over time were being monitored, a continued decrease (or increase) in concentration of analyte from the concentration measured at the initial time point would suggest instability of the analyte or sample material.

From plotting CUSUM charts (figure 4.12 for HMMNI and Appendix 4 for all other analytes), which were used to reveal any changes in concentration of analyte in the extracts over the 5 days, a few points were found outside of the control limits, but all with the exception of one were back in on day 5. MNZ at 10ng/g was out on day 5, however this point was slightly above the upper control limit, so it didn't appear any degradation had occurred.

(a)



(b)



(c)

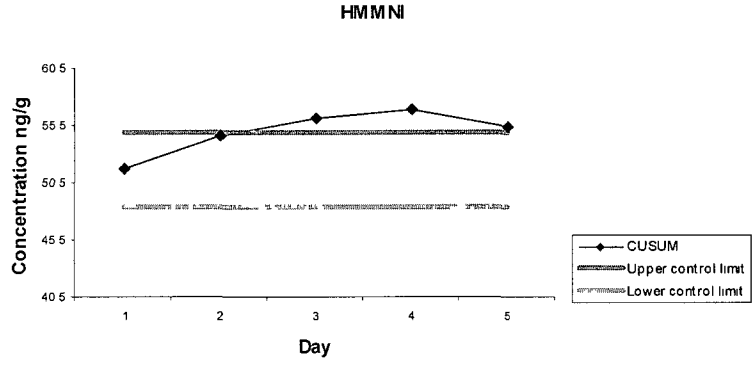


Figure 4.12 CUSUM charts representing stability of HMMNI in tilapia muscle extract over 5 days at 1ng/g (a), 10ng/g (b) and 50ng/g (c)

The t-tests applied to the extract results showed there were significant differences between time 0 results and some of the other time points with p-values < 0.05. When results at each time point were compared with one another for an analyte at a particular condition/concentration using ANOVA, significant differences were also seen. Some F statistics were determined to be larger than the critical F values. The %RSDs calculated on the results from the 5 days for each analyte at all concentrations were within the acceptability limits for repeatability of the method indicating the extracts were stable. The mean, SD and RSD are shown in table 4.10. The raw data and examples of t-tests and ANOVA are in Appendix 5. Since the t-test and ANOVA findings contradicted what was shown by %RSD and CUSUM, it was considered that the data sets used may not have been large enough to apply t-tests or ANOVA and the results do not prove instability of the analytes. Some authorities require 20 fish replicates per time point. In addition, no recovery losses attributable to analyte instability were observed during method development and validation experiments.

Table 4.10 Mean, SD and %RSD for stability of NI analytes in tilapia muscle extract over 5 days at 1, 10 and 50 ng/g

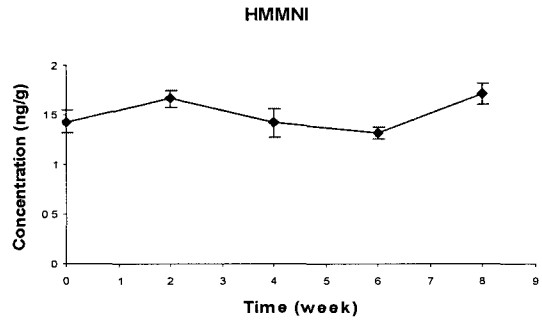
Analyte	Concentration(ng/g)	Mean Result (ng/g)	SD	%RSD
HMMNI	1	1.2	0.1	11
	10	11	1	8.4
	50	55	3	6.0
IPZ	1	1.1	0.1	7.9
	10	9.1	0.6	6.9
	50	58	4	6.6
IPZ-OH	1	1.3	0.1	6.0
	10	12	1	6.5
	50	64	5	8.4
MNZ	1	1.1	0.1	12
	10	11	1	10
	50	53	5	8.7
MNZ-OH	1	1.0	0.1	12
	10	9.3	0.8	8.2
	50	46	4	8.7
RNZ	1	1.2	0.1	9.8
	10	11	1	10
	50	54	6	12
DMZ	1	1.1	0.1	10
	10	10	1	9.6
	50	56	5	8.8

4.1.9.4 Tissue Stability

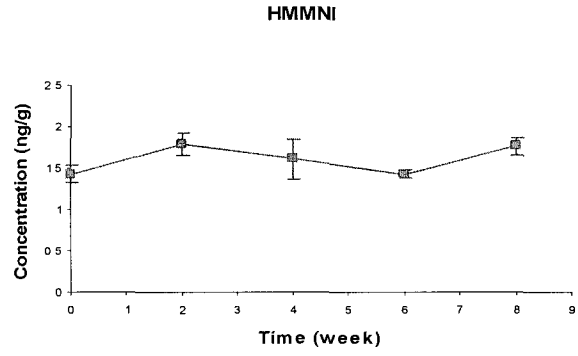
The stability of the analytes in tissue was determined by analysing tilapia muscle samples fortified at 1.5 and 10ng/g NIs and stored for 2 months at -20°C and -80°C. The material stored at -20°C included both pre-weighed samples and containers of sample which had to be thawed to take a portion for testing and refrozen to mimic how samples are typically treated in the lab. The samples stored at -80°C were pre-weighed. The mean, SD and %RSD was calculated for 5 replicates at each biweekly time point for 2 months at each concentration and condition studied. In addition, error bar and CUSUM charts were prepared and t-tests and ANOVA were used to determine changes in the tissue concentration.

On all graphs where the average of the results from 5 replicates over a 2 - month period at each time point was plotted with error bars for each analyte and condition, the error bars were within the range of one another for 1.5 and 10ng/g tissue. See figure 4.13 and 4.14 for HMMNI at 1.5 and 10ng/g, respectively and Appendix 6 and 7 for all other analytes. From this, all analytes were considered stable in tissue for 2 months under the tested conditions. When CUSUM charts were used to display running totals of the differences in results from the average at time 0, a few points were slightly outside the control limits, but none were below the lower control limit indicating there had been no degradation. Figure 4.15 shows results for 1.5 ng/g HMMNI and figure 4.16 shows 10ng/g HMMNI, while Appendix 8 and 9 contain charts for all other analytes.

(a)



(b)



(c)

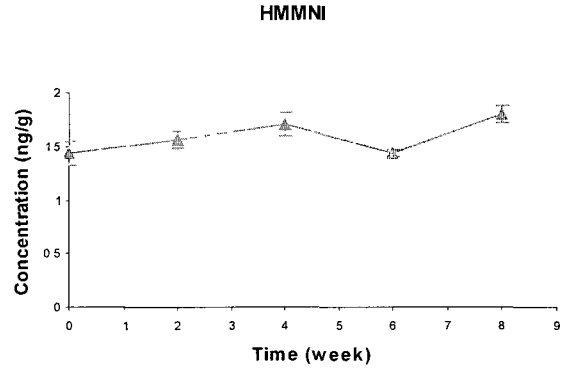
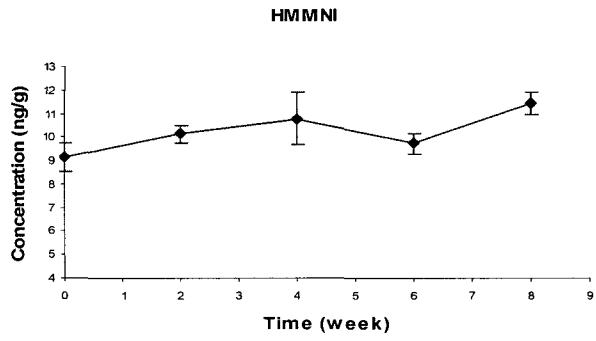
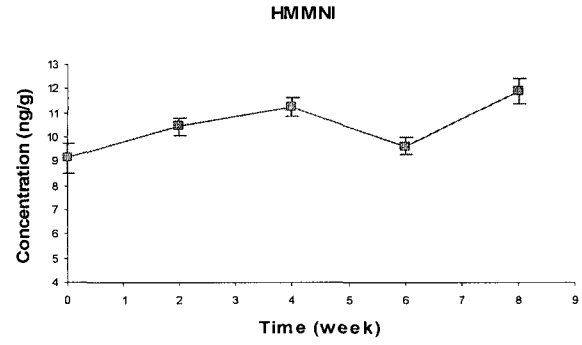


Figure 4.13 Error bar charts representing the stability of HMMNI in tilapia tissue over a 2 month period at a concentration of 1.5 ng/g with storage conditions of -20°C (a), -80°C (b) and -20°C freeze/thaw (c)

(a)



(b)



(c)

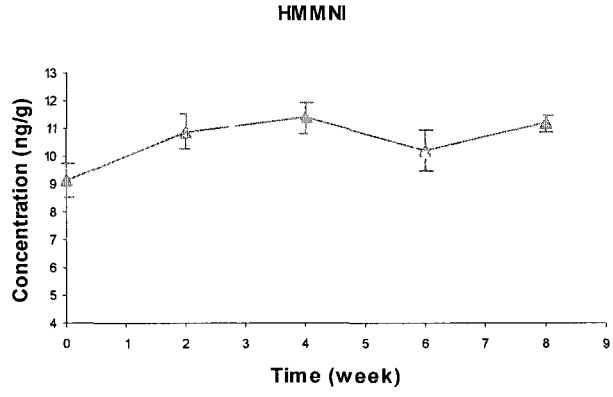
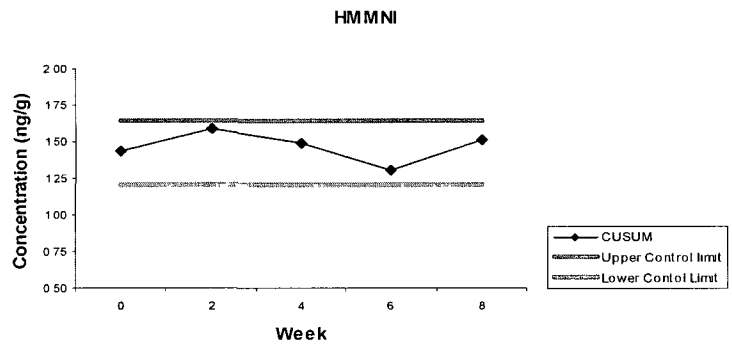
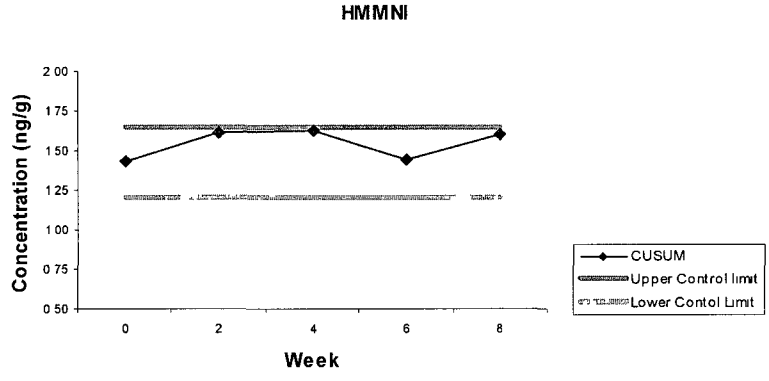


Figure 4.14 Error bar charts representing the stability of HMMNI in tilapia tissue over a 2 month period at a concentration of 10 ng/g with storage conditions of -20°C (a), -80°C (b) and -20°C freeze/thaw (c)

(a)



(b)



(c)

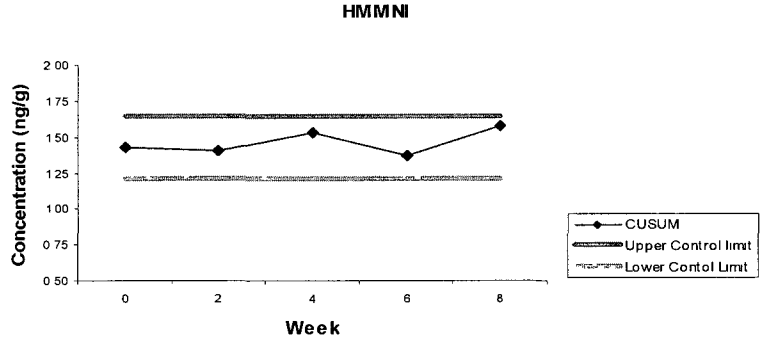
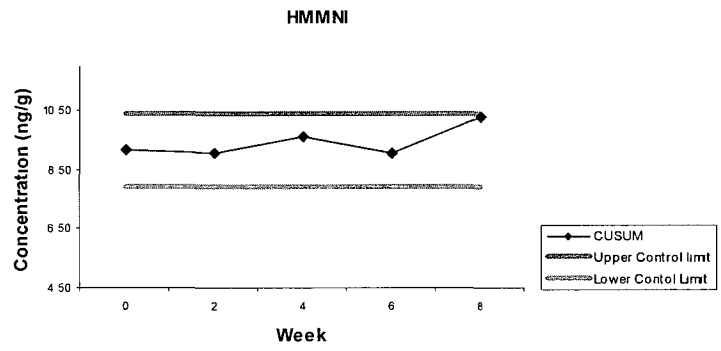
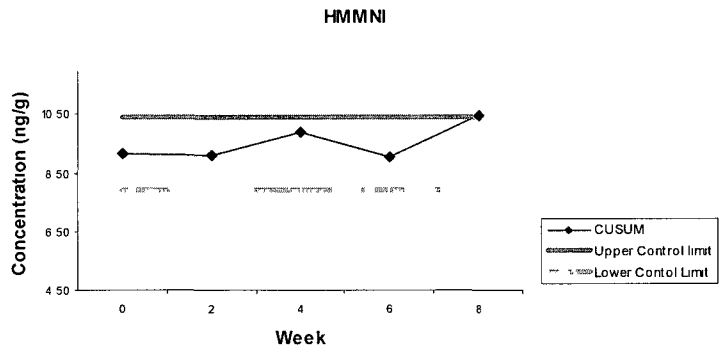


Figure 4.15 CUSUM charts representing the stability of HMMNI in tilapia tissue over 2 months for 1.5ng/g at storage conditions of -20°C (a), -80°C (b) and -20°C freeze/thaw (c)

(a)



(b)



(c)

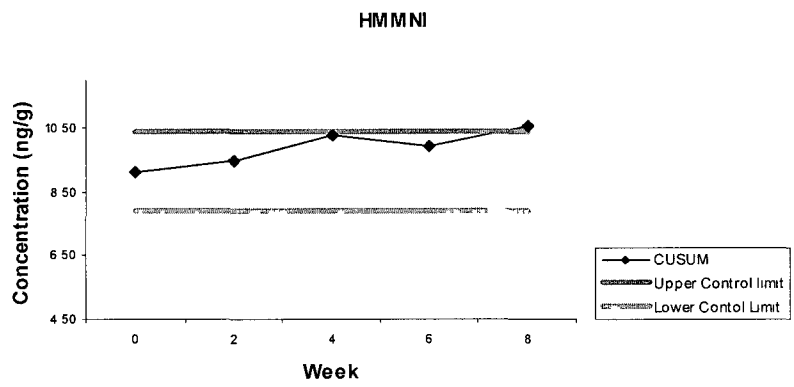


Figure 4.16 CUSUM charts representing the stability of HMMNI in tilapia tissue over 2 months for 10 ng/g at storage conditions of -20°C (a), -80°C (b) and -20°C freeze/thaw (c)

As with extract stability, t-tests and ANOVA applied to the results indicated some significant differences between time points, but data sets were not large enough. The % RSDs calculated for each analyte at the different conditions and concentrations were typical of those seen in validation repeatability studies. Therefore, analytes were stable at the conditions and concentrations studied. The mean, SD and % RSD are summarized in table 4.11, 4.12 and 4.13 for tissue stability at -20°C, -80°C and -20°C freeze/thaw, respectively. The raw data for tissue stability is shown in Appendix 10.

Table 4.11 Mean, SD and % RSD for tilapia tissue stability of NI analytes for 1.5 and 10 ng/g samples stored at -20°C

Analyte	Concentration (ng/g)	Mean (ng/g)	SD	%RSD
HMMNI	1.5	1.5	0.2	12
	10	10	1	9.9
IPZ	1.5	1.6	0.2	10
	10	11	1.2	11
IPZ-OH	1.5	1.5	0.1	9.5
	10	10	1	8.6
MNZ	1.5	1.5	0.1	8.5
	10	10	1	5.9
MNZ-OH	1.5	1.4	0.2	14
	10	9.5	1.3	13
RNZ	1.5	1.6	0.1	7.6
	10	10	1	5.8
DMZ	1.5	1.4	0.1	8.1
	10	10	1	5.6

Table 4.12 Mean, SD and % RSD for tilapia tissue stability of NI analytes for 1.5 and 10 ng/g samples stored at -80°C

Analyte	Concentration (ng/g)	Mean (ng/g)	SD	%RSD
HMMNI	1.5	1.6	0.2	13
	10	10	1	11
IPZ	1.5	1.6	0.2	12
	10	11	1	13
IPZ-OH	1.5	1.5	0.1	7.3
	10	10	1	8.4
MNZ	1.5	1.6	0.1	5.8
	10	10	1	4.7
MNZ-OH	1.5	1.5	0.2	14
	10	9.4	1.1	12
RNZ	1.5	1.6	0.2	9.2
	10	10	1	7.0
DMZ	1.5	1.4	0.1	8.4
	10	10	1	7.3

Table 4.13 Mean, SD and % RSD for tilapia tissue stability of NI analytes for 1.5 and 10 ng/g samples stored at -20°C freeze/thaw

Analyte	Concentration (ng/g)	Mean (ng/g)	SD	%RSD
HMMNI	1.5	1.6	0.2	11
	10	10	1	9.3
IPZ	1.5	1.6	0.2	12
	10	11	1	13
IPZ-OH	1.5	1.5	0.2	9.6
	10	10	1	9.3
MNZ	1.5	1.6	0.1	5.3
	10	10	1	6.4
MNZ-OH	1.5	1.4	0.2	14
	10	9.6	1.4	14
RNZ	1.5	1.6	0.1	7.1
	10	10	1	6.1
DMZ	1.5	1.4	0.1	9.6
	10	10	0.9	8.5

4.2 Method Validation

4.2.1 LOD and LOQ

LOD was determined for each analyte in tilapia, salmon and shrimp muscle by first measuring the height of the noise level in 20 blank samples (5 extracts/day over 4 days) in the quantifying chromatogram at the expected retention times for each analyte and calculating the concentration equivalent to the noise. The tissue concentration at instrument noise was averaged for each analyte and multiplied by 3 to give LODs ranging from 0.07 to 0.32 ng/g for tilapia, 0.08 to 0.41 ng/g for salmon and 0.08 to 0.24ng/g for shrimp, depending on the analyte. The LOQ was calculated by multiplying the LOD by 3 and ranged from 0.20 to 0.97 ng/g for tilapia, 0.24 to 1.2 ng/g for salmon and 0.23 to 0.74 ng/g for shrimp.

It was later realized that some LOD results were underestimated and the confirmation ion could not be seen at these levels. The LOD values had to be adjusted in order for confirmation to be achieved at the claimed LODs. Adjusted values included 0.40 ng/g for HMMNI, 0.30 ng/g for RNZ and 0.20 ng/g for DMZ in tilapia muscle; 1.0 ng/g HMMNI in salmon muscle; and 0.90 ng/g for HMMNI, 0.20 ng/g for RNZ and 0.10 ng/g for DMZ in shrimp muscle. LOQ values were also adjusted as necessary. It was decided the noise level of the confirmation chromatogram would be used for determining LOD to avoid this problem in the future. LOD and LOQ for tilapia, salmon and shrimp muscle including adjusted values are shown in tables 4.14. Raw data can be found in Appendix 11.

Table 4.14 LOD and LOQ values for NI analytes in tilapia, salmon and shrimp muscle

	Tilapia		Salmon		Shrimp	
Analyte	LOD	LOQ	LOD	LOQ	LOD	LOQ
HMMNI	0.40	1.2	1.0	3.0	0.90	2.7
IPZ	0.07	0.21	0.13	0.39	0.08	0.23
IPZ-OH	0.10	0.31	0.24	0.71	0.09	0.28
MNZ	0.07	0.20	0.08	0.24	0.10	0.30
MNZ-OH	0.32	0.97	0.40	1.2	0.25	0.74
RNZ	0.30	0.90	0.41	1.2	0.20	0.60
DMZ	0.20	0.60	0.10	0.30	0.10	0.30

4.2.2 Accuracy (Trueness and Bias)

The fortification levels chosen for recovery studies were 1, 10 and 50 ng/g in tilapia, salmon and shrimp muscle for all analytes. This was based on 3 X LOD (LOQ), 10 X LOD and the upper limit of the linear range (CAC, 2009b). LOQs varied depending on the analyte, therefore to simplify the method recovery experiment, concentrations were chosen based on the average of the LOQs. For example, 1ng/g was chosen for LOQs ranging from 0.2 to 3.0 ng/g for all analytes and matrices studied. Also, 50ng/g was chosen as the upper limit of the linear range even though the calibration curve was linear at higher concentrations because this was the range to be worked in during routine testing.

In tilapia muscle at a concentration of 1ng/g, average recoveries for analytes ranged from 97 to 124%. At 10ng/g, recoveries were seen from 95-122% and for 50ng/g results were 89-118%. For salmon muscle, recoveries were 94-123% at 1 ng/g; 87-119% at 10ng/g; and 81-119% at 50ng/g. Recoveries achieved from shrimp muscle were 98-124% at 1ng/g, 93-118% at 10ng/g and 93-110% for 50ng/g. Most recoveries were greater than 100%, indicating positive biases, while only a few were less than 100% (indicating negative bias).

The performance criteria that should have been met in recovery studies included the following: a range of mean % recovery from 60-120% for concentrations from 1 to10 ng/g and 70-110% for 50 ng/g (CAC, 2009b). Recovery results for shrimp muscle met the criteria for all analytes at all concentrations. For salmon muscle, three analytes, HMMNI (117%), MNZ (119%) and RNZ (111%) were out of range at 50 ng/g. Average IPZ-OH recovery of 118% was also out of range at 50ng/g for tilapia. These criteria were established for single analyte methods and therefore in a multi-analyte method these deviations were considered acceptable because conditions cannot be optimized for all individual analytes in a multi-analyte method. Use of appropriate ISs could bring performance of these analytes within the accepted criteria.

In an attempt to improve recoveries, they were corrected using the DMZ-D_3 IS by calculating a peak area ratio of analyte or standard response divided by IS area response to calculate recoveries. This allowed recoveries for tilapia and shrimp muscle to meet the criteria, but salmon muscle still had analytes out of the % recovery range, including HMMNI (120%), IPZ (111%) and IPZ-OH (112%) at 50ng/g. It appears correcting all

analytes using DMZ-D_3 as an IS may not be an improvement. This is further discussed in section 4.2.3.

The percent recoveries were also calculated for DMZ-D_3 spiked at 2.5ng/g in all matrices. DMZ-D_3 in tilapia muscle had a mean recovery of 103% while the average recovery was 105% and 118% for salmon and shrimp muscle, respectively. These recoveries met the performance criteria. Mean recoveries for each concentration tested including IS corrected mean recoveries for all analytes are summarized in tables 4.15 for tilapia muscle, 4.16 for salmon muscle and 4.17 for shrimp muscle. See Appendix 12 for more detailed raw recovery data.

Table 4.15 Accuracy for NI analytes including mean recovery, IS corrected recovery, SD and RSD in tilapia muscle

Analyte	Concentration (ng/g)	Mean Recovery (%)	SD	RSD (%)	Mean IS corrected recovery (%)	SD	RSD (%)
HMMNI	1	112	12	10	107	17	16
	10	109	10	9.4	107	12	11
	50	107	10	9.3	99	14	14
IPZ	1	104	7	7.0	99	11	11
	10	99	7	6.8	98	10	10
	50	108	10	9.0	103	12	11
IPZ-OH	1	124	8	6.9	116	14	12
	10	122	12	9.4	119	12	10
	50	118	10	8.1	110	15	14
MNZ	1	114	11	9.7	107	13	12
	10	112	12	11	109	12	11
	50	106	9	8.2	98	13	13
MNZ-OH	1	97	10	10	94	15	16
	10	95	7	7.5	96	9	9.3
	50	89	4	4.0	85	11	13
RNZ	1	115	11	9.2	113	15	13
	10	115	8	7.2	117	14	12
	50	104	5	4.7	101	14	14
DMZ	1	111	7	6.6	102	9	8.4
	10	105	5	4.6	99	7	7.4
	50	107	10	9.6	96	10	10
DMZ-D3	2.5	103	8	8			

Table 4.16 Accuracy for NI analytes including Mean recovery, IS corrected recovery, SD and RSD in salmon muscle

Analyte	Concentration (ng/g)	Mean Recovery (%)	SD	RSD (%)	Mean IS corrected recovery (%)	SD	RSD (%)
HMMNI	1	119	12	9.7	109	20	18
	10	119	18	15	114	17	15
	50	117	6.9	5.9	120	24	20
IPZ	1	113	7.7	6.8	102	14	14
	10	109	9.5	8.7	109	16	15
	50	107	10	9.8	111	19	17
IPZ-OH	1	123	13	11	105	14	13
	10	108	11	10	102	13	13
	50	107	14	13	112	14	13
MNZ	1	114	6.2	5.4	109	18	16
	10	97	11	11.3	108	17	16
	50	119	16	14	105	11	11
MNZ-OH	1	94	11	11	109	18	16
	10	87	15	17	108	17	16
	50	81	5.9	7.3	105	11	11
RNZ	1	114	4.1	3.6	89	16	18
	10	109	13	12	79	6	7.3
	50	111	17	16	86	14	17
DMZ	1	118	8.1	6.9	97	11	11
	10	110	9.1	8.2	109	12	11
	50	98	13	13	110	21	19
DMZ-D3	2.5	105	16	15			

Table 4.17 Accuracy for NI analytes including Mean recovery, IS corrected recovery, SD and RSD in shrimp muscle

Analyte	Concentration (ng/g)	Mean Recovery (%)	SD	RSD (%)	Mean IS corrected recovery (%)	SD	RSD (%)
HMMNI	1	101	15	15	90	11	12
	10	102	12	12	90	6	6.4
	50	97	13	14	87	3	3.9
IPZ	1	104	11	11	93	6	6.9
	10	107	6	6	94	5	5.1
	50	107	11	10	96	3	2.8
IPZ-OH	1	116	16	14	104	9	8.8
	10	118	9	8	103	3	2.9
	50	110	14	12	98	5	5.1
MNZ	1	112	17	15	100	9	9.4
	10	110	11	10	96	5	4.3
	50	101	14	13	90	4	4.7
MNZ-OH	1	98	15	15	88	10	12
	10	93	8	8.1	81	4	5.5
	50	93	11	12	83	4	5.3
RNZ	1	124	16	13	111	10	9.5
	10	119	9	7.7	105	4	4.1
	50	109	9	8.4	97	6	6.2
DMZ	1	105	11	11	94	6	6.7
	10	105	9	8.6	92	5	5.1
	50	103	15	14	92	4	4.8
DMZ-D3	2.5	118	7	6			

4.2.3 Precision

Instrument repeatability was determined by repeat injections of standards from the calibration curve and fortified samples at 1, 10 and 50ng/g concentrations. A summary of %RSD for the standards is provided in tables 4.18, 4.19 and 4.20 for tilapia, salmon and shrimp muscle tissues, respectively. The % RSD for repeat injections of fortified samples of tilapia, salmon and shrimp muscles are in tables 4.21, 4.22 and 4.23, respectively. For all analytes in tilapia muscle, % RSDs were $\leq 10\%$ which was considered to be within acceptable limits. For salmon muscle, all analytes had % RSDs $< 10\%$, except for MNZ-OH and RNZ, which had $\leq 15\%$. Shrimp muscle was similar to salmon muscle, having a %RSD for MNZ-OH as high as 18% and 11% for RNZ. Since the method was a multi analyte method, making less scans available per analyte, these repeatabilities were accepted. More work on the MS was planned to improve the repeatability for these analytes. It was also discovered that running certain other veterinary drug methods prior to nitroimidazole analysis on the same column affected repeatability. Methods such as CFIA SOM-DAR-CHE-050-01, 2009 (fluoroquinolones) and SOM-DAR-CHE-039-07, 2009 (triphenylmethane dyes), which use different mobile phases, caused the instrument response of standards to increase during an analytical run. In response to this issue, a column was designated for nitroimidazole analysis only.

Table 4.18 Instrument repeatability determinations (%RSD) of NI analytes in tilapia muscle by analysis of replicate injections of matrix matched standards

	Standard Concentrations (ng/g)			
Analyte	2	4	10	50
HMMNI	3.5	4.2	4.3	3.5
IPZ	7.2	5.7	6.5	6.0
IPZ-OH	7.1	3.3	3.6	4.9
MNZ	6.0	5.3	6.4	6.4
MNZ-OH	5.6	3.6	6.2	3.0
RNZ	9.5	2.3	2.8	3.9
DMZ	5.5	8.1	8.8	6.0

Table 4.19 Instrument repeatability determinations (%RSD) of NI analytes in salmon muscle by analysis of replicate injections of matrix matched standards

	Standard Concentrations (ng/g)			
Analyte	2	4	10	50
HMMNI	2.8	1.4	4.6	3.1
IPZ	2.6	5.3	4.1	4.6
IPZ-OH	2.2	3.0	2.8	3.4
MNZ	3.9	2.4	1.9	3.6
MNZ-OH	7.8	5.0	6.9	7.9
RNZ	5.3	3.7	6.1	3.7
DMZ	5.4	3.9	4.9	4.5

Table 4.20 Instrument repeatability determinations (%RSD) of NI analytes in shrimp muscle by analysis of replicate injections of matrix matched standards

	Standard Concentrations (ng/g)			
Analyte	2	4	10	50
HMMNI	7.2	4.9	5.5	8.3
IPZ	6.7	5.1	6.9	6.6
IPZ-OH	7.2	6.3	5.3	5.5
MNZ	6.3	7.0	7.9	5.2
MNZ-OH	13	8.7	5.5	10
RNZ	6.2	7.4	3.3	4.8
DMZ	7.6	8.4	8.6	10

Table 4.21 Instrument repeatability determinations (%RSD) of NI analytes in tilapia muscle by analysis of replicate injections of fortified samples

	Concentration (ng/g)		
Analyte	1	10	50
HMMNI	5.3	2.2	4.2
IPZ	7.0	4.8	6.2
IPZ-OH	2.7	0.8	4.9
MNZ	5.4	4.2	2.7
MNZ-OH	9.2	4.9	4.6
RNZ	5.6	2.4	4.0
DMZ	7.0	4.8	6.2

Table 4.22 Instrument repeatability determinations (%RSD) of NI analytes in salmon muscle by analysis of replicate injections of fortified samples

	Concentration (ng/g)		
Analyte	1	10	50
HMMNI	4.8	4.7	2.4
IPZ	8.4	3.8	4.7
IPZ-OH	3.3	3.5	2.6
MNZ	4.1	2.5	2.1
MNZ-OH	18	9.5	5.4
RNZ	11	4.4	2.4
DMZ	9.2	5	5.9

Table 4.23 Instrument repeatability determinations (%RSD) of NI analytes in shrimp muscle by analysis of replicate injections of fortified samples

	Concentration (ng/g)		
Analyte	1	10	50
HMMNI	9.0	6.0	6.2
IPZ	9.4	9.4	9.0
IPZ-OH	4.4	5.2	4.8
MNZ	5.4	4.2	2.7
MNZ-OH	11	11	10
RNZ	9.4	6.7	6.5
DMZ	15	15	15

Tilapia, salmon and shrimp muscle tissues fortified at 1, 10 and 50 ng/g were analyzed to determine method repeatability expressed as %RSD. Uncorrected data were first used to calculate %RSD followed by data corrected by spike recovery. Results for tilapia muscle are summarized in table 4.24, results for salmon muscle are in table 4.25 and results for shrimp muscle are in table 4.26. See Appendix 13 for raw data. The criteria used to determine the performance of an analytical method were derived from a statistical evaluation of data from several thousand collaborative studies (CAC, 2009b). The performance targets for precision recognized by Codex Alimentarius were $\leq 35\%$ for concentration of 1ng/g, 30% for 10ng/g and 20% for 50ng/g. The %RSD results for all analytes at each concentration in salmon and shrimp muscle tissues were within the target limits for both uncorrected and corrected data. In tilapia muscle tissues, the % RSD from uncorrected data for RNZ was the only result higher than the criteria at 21% for 50ng/g. However, the % RSDs calculated with the corrected data were within the criteria limits. Since the CAC recommends recovery-corrected data should be reported (CAC, 2009a), the results were considered acceptable for tilapia.

Table 4.24 Precision for NI analytes using uncorrected and spike corrected data in tilapia muscle

		Uncorrected data			Corrected data		
Analyte	Fortification level (ng/g)	Mean concentration (ng/g)	SD	RSD (%)	Mean concentration (ng/g)	SD	RSD (%)
HMMNI	1.0	1.2	0.1	8.5	1.1	0.1	7.6
	10	12	2	13	11	1	7.9
	50	54	5	9.7	50	2	4.7
IPZ	1	1.1	0.1	10	1.1	0.2	22
	10	10	1	7.1	11	2	22
	50	50	6	12	52	6	12
IPZ-OH	1.0	1.3	0.2	12	1.1	0.2	14
	10	13	2	12	11	1	8.1
	50	61	7	12	49	4	7.8
MNZ	1.0	1.2	0.1	12	1.1	0.2	15
	10	12	1	11	10	1	7.5
	50	53	3	6.6	47	4	8.9
MNZ-OH	1.0	1.0	0.1	9.5	1.1	0.1	10
	10	10	1.0	13	11	1	11
	50	46	6	13	52	4	8.2
RNZ	1.0	1.1	0.2	18	1.1	0.1	8.1
	10	11	2	21	10	1	4.7
	50	49	10	21	47	2	4.4
DMZ	1.0	1.2	0.1	8.8	1.0	0.1	12
	10	11	1	5.8	9.9	0.7	7.2
	50	55	5	9.1	48	2	5.2

Table 4.25 Precision for NI analytes using uncorrected and spike corrected data in salmon muscle

		Uncorrected data			Corrected data		
Analyte	Fortification level (ng/g)	Mean concentration (ng/g)	SD	RSD (%)	Mean concentration (ng/g)	SD	RSD (%)
HMMNI	1.0	1.0	0.1	13	1.0	0.2	16
	10	9.5	0.7	7.2	10	1	12
	50	42	4	10	44	7	16
IPZ	1	1.1	0.1	11	0.98	0.17	17
	10	10	1	9.1	9.0	1.7	19
	50	48	3	6.9	43	7	17
IPZ-OH	1.0	1.4	0.1	7.9	1.0	0.1	10
	10	13	1	6.5	9.5	0.9	9.9
	50	57	5	8.9	43	6	13
MNZ	1.0	1.1	0.1	7.5	1.0	0.1	12
	10	9	1	12	9	1	11
	50	46	3	7.4	44	6	13
MNZ-OH	1.0	0.9	0.1	14	1.0	0.2	20
	10	8	1	17	9	1	13
	50	37	4	11	41	8	20
RNZ	1.0	1.1	0.1	10	1.0	0.2	19
	10	10	1	5.6	9.4	1.3	14
	50	45	4	9.6	41	8	18
DMZ	1.0	1.1	0.1	9.1	1.0	0.1	12
	10	11	1	7.1	9.6	0.9	9.2
	50	53	4	8.0	47	5	11

Table 4.26 Precision for NI analytes using uncorrected and spike corrected data in shrimp muscle

		Uncorrected data			Corrected data		
Analyte	Fortification level (ng/g)	Mean concentration (ng/g)	SD	RSD (%)	Mean concentration (ng/g)	SD	RSD (%)
HMMNI	1.0	1.1	0.1	11	0.89	0.14	15
	10	10	1	5.4	8.8	0.9	10
	50	52	2	4.7	43	3	6.4
IPZ	1	1.1	0.1	6.4	0.92	0.09	9.3
	10	10	1	5.2	8.8	0.8	8.5
	50	52	6	11	46	3	5.6
IPZ-OH	1.0	1.4	0.1	5.4	0.92	0.05	5.3
	10	13	1	4.4	8.8	0.6	6.7
	50	63	3	4.8	43	2	5.6
MNZ	1.0	1.1	0.1	8.9	0.89	0.08	8.6
	10	11	1	8.7	8.6	0.7	9.2
	50	52	3	5.9	41	2	5.0
MNZ-OH	1.0	0.87	0.08	8.6	0.83	0.09	11
	10	9.2	0.6	6.6	8.7	1.1	13
	50	43	4	6.8	43	4	8.5
RNZ	1.0	1.1	0.1	12	0.99	0.24	25
	10	11	1	7.9	10	2	21
	50	50	4	7.2	43	2	4.7
DMZ	1.0	1.1	0.1	7.7	0.90	0.07	7.5
	10	11	1	10	8.8	1.0	11
	50	56	4	7.5	44	3	6.9

Intermediate precision was calculated from results generated by 2 analysts. The %RSDs for intermediate precision were expected to be 1-2% greater than those for a single analyst, as different analysts will typically have minor differences in the manner in which they perform the method. These results were also calculated based on both spike corrected and uncorrected data. All of the %RSDs for tilapia and shrimp were within the acceptability criteria. Salmon had a %RSD of 24% for IPZ at 50ng/g with uncorrected data, which was above the target limit. The % RSDs from the corrected data were within the target limits and therefore intermediate precision criteria were considered to be met.

HorRat values were also calculated for intermediate precision. The HorRat is the ratio of the reproducibility relative standard deviation to that calculated from the Horwitz equation (Horwitz & Albert, 2006; CAC, 2009b). The HorRat is indicative of method performance for a large majority of analytical methods in chemistry. Typical limits of HorRat acceptability are between 0.5 to 2.0 for between laboratory performance. The within laboratory RSD (repeatability) is typically one-half to two thirds of the between laboratory RSD, therefore HorRat acceptability limits of 0.3-1.3. The HorRat values calculated for salmon muscle were all within these acceptability limits for both uncorrected and spike corrected data. Values for shrimp and tilapia muscle were < 1.3 and some were biased low for acceptability limits (<0.3). This was believed to be due to the fact that the analysis done by different analysts was done on the same instrument. It is recognized that within-laboratory results for the HorRat may fall below the accepted range and this is usually attributed to good quality control of the analytical process, including factors such as analyst training and experience, use of dedicated analytical instruments and common reagents, etc., within the laboratory (Horwitz & Albert, 2006).

Salmon muscle had the higher HorRat values because the fortified salmon used by the second analyst was past the determined stability time and lower concentrations resulted. The degraded results were confirmed by a re-analysis of the tissues by analyst 1 at that time. A comparison of these results with % RSDs between the two sets of data are shown in table 4.27. Intermediate precision results are summarized in tables 4.28, 4.29 and 4.30 for tilapia, salmon and shrimp muscle respectively. The raw data results are in Appendix 13.

Table 4.27 Mean results of data from analyst 1 and 2 for degraded salmon muscle tissue

Analyte	Concentration (ng/g)	Mean result (ng/g)	SD	RSD (%)
HMMNI	1	0.56	0.04	7.5
	10	5.7	0.9	15
	50	33	3	8.6
IPZ	1	0.9	0.2	20
	10	8.3	0.9	10
	50	45	7	15
IPZ-OH	1	0.77	0.09	12
	10	7.4	0.5	6.9
	50	39	3	7.6
MNZ	1	0.7	0.1	18
	10	6.4	0.6	8.8
	50	39	5	14
MNZ-OH	1	0.55	0.07	13
	10	5.4	0.5	9.3
	50	34	4	12
RNZ	1	0.59	0.05	8.1
	10	6.0	0.6	9.8
	50	31	2	7.0
DMZ	1	0.88	0.06	6.6
	10	7.6	0.8	10
	50	40	4	9.5

Table 4.28 Intermediate precision results including combined mean, RSD and HorRat values from uncorrected and spike corrected tilapia muscle results

		Uncorrected Data				Corrected Data			
Analyte	Fortification level (ng/g)	Mean concentration (ng/g)	SD	RSD (%)	HorRat	Mean concentration (ng/g)	SD	RSD (%)	HorRat
HMMNI	1.0	1.2	0.1	8.7	0.19	1.1	0.1	9.3	0.21
	10	12	1	11	0.24	10	1	9.0	0.20
	50	54	4	8.2	0.18	48	3	7.2	0.16
IPZ	1	1.1	0.1	9.3	0.21	1.1	0.2	18	0.41
	10	10	1	8.7	0.19	11	2	17	0.38
	50	49	6	12	0.26	50	6	11	0.25
IPZ-OH	1.0	1.3	0.1	9.7	0.22	1.1	0.1	11	0.25
	10	13	1	9.1	0.20	11	1	7.1	0.16
	50	61	5	8.9	0.20	49	3	6.5	0.15
MNZ	1.0	1.1	0.1	10	0.23	1.0	0.1	12	0.27
	10	11	1	9.9	0.22	10	1	7.0	0.16
	50	52	3	5.9	0.13	47	3	7.4	0.17
MNZ-OH	1.0	0.98	0.09	9.4	0.21	1.1	0.1	11	0.24
	10	10	1	10	0.22	11	1	11	0.26
	50	45	4	9.7	0.22	49	5	9.9	0.22
RNZ	1.0	1.2	0.2	15	0.34	1.1	0.1	6.8	0.15
	10	11	2	18	0.39	10	1	5.0	0.11
	50	50	8	17	0.38	46	3	5.7	0.13
DMZ	1.0	1.1	0.1	8.8	0.20	1.0	0.1	13	0.0
	10	11	1	9.0	0.20	10	1	9.1	0.20
	50	52	6	11	0.24	48	3	6.4	0.14

Table 4.29 Intermediate precision results including combined mean, RSD and Horat values from uncorrected and spike corrected salmon muscle results

		Uncorrected Data				Corrected Data			
Analyte	Fortification level (ng/g)	Mean concentration (ng/g)	SD	RSD (%)	HorRat	Mean concentration (ng/g)	SD	RSD (%)	HorRat
HMMNI	1.0	1.0	0.2	24	0.53	1.0	0.2	23	0.52
	10	10	1	15	0.33	9	2	18	0.39
	50	46	8	18	0.40	45	8	17	0.39
IPZ	1	1.0	0.2	23	0.51	0.9	0.2	19	0.43
	10	9	2	22	0.50	9	2	20	0.45
	50	46	11	24	0.54	43	9	21	0.46
IPZ-OH	1.0	1.4	0.2	18	0.40	1.1	0.3	25	0.56
	10	12	2	12	0.28	10	2	18	0.39
	50	60	10	16	0.36	48	11	22	0.50
MNZ	1.0	1.1	0.2	17	0.39	1.0	0.2	20	0.44
	10	10	1	12	0.27	9	1	15	0.34
	50	49	6	13	0.28	45	6	14	0.32
MNZ-OH	1.0	0.9	0.2	26	0.58	0.9	0.2	25	0.55
	10	8	1	17	0.37	8	2	19	0.41
	50	40	7	18	0.41	40	7	19	0.41
RNZ	1.0	1.1	0.3	25	0.56	1.0	0.3	26	0.57
	10	10	2	17	0.38	9	2	18	0.39
	50	47	10	22	0.48	43	10	22	0.49
DMZ	1.0	1.1	0.2	20	0.45	1.0	0.2	14	0.32
	10	10	2	20	0.44	9	2	18	0.39
	50	51	11	22	0.49	47	7	15	0.34

Table 4.30 Intermediate precision results including combined mean, RSD and HorRat values from uncorrected and spike corrected shrimp muscle results

		Uncorrected Data				Corrected Data			
Analyte	Fortification level (ng/g)	Mean concentration (ng/g)	SD	RSD (%)	HorRat	Mean concentration (ng/g)	SD	RSD (%)	HorRat
HMMNI	1.0	1.0	0.1	8.5	0.19	0.90	0.11	12	0.26
	10	10	1	5.7	0.13	8.8	0.8	8.6	0.19
	50	51	3	6.3	0.14	43	3	6.3	0.14
IPZ	1	1.0	0.2	16	0.37	0.9	0.1	11	0.25
	10	9	2	19	0.42	8	1	12	0.27
	50	47	9	18	0.41	45	5	11	0.26
IPZ-OH	1.0	1.3	0.1	8.2	0.18	0.9	0.1	5.4	0.12
	10	12	1	8.3	0.18	8.8	0.6	6.8	0.15
	50	60	5	9.2	0.21	42	3	6.2	0.14
MNZ	1.0	1.1	0.1	8.5	0.19	0.9	0.1	9.8	0.22
	10	11	0.8	7.4	0.16	8.8	0.8	9.2	0.20
	50	51	4	7.5	0.17	42	3	7.2	0.16
MNZ-OH	1.0	0.8	0.1	12	0.26	0.9	0.1	13	0.29
	10	6.9	0.6	7.0	0.16	9	1	11	0.24
	50	43	4	8.8	0.20	44	4	8.2	0.18
RNZ	1.0	1.1	0.1	10	0.23	1.0	0.2	19	0.43
	10	11	0.8	7.1	0.16	9.6	1.6	17	0.37
	50	50	4	8.0	0.18	44	7	16	0.37
DMZ	1.0	1.1	0.1	7.8	0.17	0.9	0.1	8.3	0.19
	10	11	1	9.1	0.20	8.7	0.9	10	0.22
	50	55	5	8.9	0.20	44	4	8.5	0.19

Method repeatability %RSD was also calculated for concentrations corrected with the IS DMZ-D_3 area counts and are shown in table 4.31 for tilapia muscle, table 4.32 for salmon muscle and table 4.33 for shrimp muscle. The raw data can be seen in Appendix 13. These % RSDs for each commodity and concentration for all analytes were within the acceptable criteria. This was an improvement over the previous repeatability results from both corrected and uncorrected data. However, it was also noted that DMZ-D_3 did not appear appropriate to correct results for all analytes. In particular, IPZ-OH concentration results appeared to be overestimated. These results were similar to uncorrected results and higher than spiked corrected values. It was determined that DMZ-D_3 could not be applied to all analytes studied as an IS and further work is required with more internal standards.

Table 4.31 Precision for NI analytes using IS corrected data for residues in tilapia muscle

Analyte	Fortification level (ng/g)	Mean concentration (ng/g)	SD	RSD (%)
HMMNI	1.0	1.2	0.1	11
	10	12	1	10
	50	53	4	7.6
IPZ	1	1.0	0.1	9.2
	10	9.9	0.8	8.4
	50	49	3	5.9
IPZ-OH	1.0	1.3	0.1	6.5
	10	13	1	7.6
	50	59	2	3.3
MNZ	1.0	1.2	0.1	10
	10	11	1.0	8.9
	50	51	4	7.6
MNZ-OH	1.0	1.0	0.1	8.5
	10	9.7	1	9.9
	50	44	4	10
RNZ	1.0	1.1	0.2	18
	10	11	2	20
	50	47	9	20
DMZ	1.0	1.1	0.1	11
	10	11	1	7.8
	50	53	3	6.4

Table 4.32 Precision for NI analytes using IS corrected data for residues in salmon muscle

Analyte	Fortification level (ng/g)	Mean concentration (ng/g)	SD	RSD (%)
HMMNI	1.0	1.0	0.1	11
	10	9.6	0.7	7.4
	50	43	3	6.0
IPZ	1	1.1	0.1	13
	10	10	1	9.1
	50	49	3	7.1
IPZ-OH	1.0	1.4	0.1	8.9
	10	13	1	9.2
	50	58	3	5.0
MNZ	1.0	1.1	0.1	6.6
	10	9.7	0.9	9.2
	50	48	3	7.4
MNZ-OH	1.0	0.9	0.1	14.4
	10	8.3	0.9	10
	50	38	3	7.2
RNZ	1.0	1.1	0.1	10
	10	10	0.8	8.0
	50	46	2	5.4
DMZ	1.0	1.2	0.1	9.9
	10	11	1.0	9.4
	50	54	4	7.7

Table 4.33 Precision for NI analytes using IS corrected data for residues in shrimp muscle.

Analyte	Fortification level (ng/g)	Mean concentration (ng/g)	SD	RSD (%)
HMMNI	1.0	1.0	0.1	8.9
	10	9.4	0.6	6.7
	50	45	2	5.6
IPZ	1	1.0	0.1	5.7
	10	9.3	0.4	4.6
	50	48	3	5.9
IPZ-OH	1.0	1.2	0.1	8.0
	10	11	0.5	4.4
	50	55	3	6.0
MNZ	1.0	1.0	0.1	5.9
	10	9.6	0.5	5.4
	50	44	3	6.3
MNZ-OH	1.0	0.8	0.1	13
	10	8.1	0.4	5.1
	50	40	3	8.2
RNZ	1.0	1.0	0.1	12
	10	9.6	0.6	6.7
	50	44	4	9.0
DMZ	1.0	1.0	0.1	6.8
	10	9.8	0.6	6.5
	50	49	4	8.8

4.2.4 CCα and CCβ

The decision limit (CCα) is determined by using validation guidelines provided by the EU. Commission Decision 2002/6576EC, which sets guidelines for the validation of both screening and confirmatory analytical methods of analysis. The Commision Decision implements the Council Directive 96/23/EC concerning the method performance and interpretation of results, for the fulfillment of key requirements set by the EU. The directive applies not only to methods used by official regulatory laboratories for control of veterinary drug residues in foods within the EU, but also to laboratories conducting tests in other countries for foods to be exported to the EU. Performance characteristics such as detection capability must be assessed in order to classify a screening method as "fit for purpose"; i.e., capable of detecting and quantifying residues of a particular analyte. For the method to be classified as confirmatory, the decision limit must also be included in validation studies.

The decision limit was determined simultaneously with LOD as these are determined in the same manner for analytes such as the NIs which have no maximum residue limits set. The decision limits ranged from 0.07 to 1.0 ng/g depending on the analyte and matrix. Appendix 11 contains the raw data for CCα. These levels were used to spike the appropriate blank material to be analyzed. The SD of the concentration of samples was determined and used to calculate the CCβ. Both uncorrected and spike corrected results were used to calculate CCβ. In tilapia muscle, the CCβ ranged from 0.09 to 0.49 ng/g using uncorrected data and 0.09 to 0.50 ng/g using spike corrected data. For salmon muscle, uncorrected data gave CCβ ranging from 0.11 to 1.15 ng/g and spike corrected from 0.10 to 1.14ng/g. Shrimp muscle had CCβ values for uncorrected data

from 0.11 to1.02 ng/g and 0.11 to 1.03 for spike corrected. CCβ results were not affected by using uncorrected versus spike corrected results. Results were summarized in tables 4.34, 4.35 and 4.36 for tilapia, salmon and shrimp muscle, respectively. See Appendix 14 for CCβ raw data results.

Table 4.34 CCα and CCβ using uncorrected and corrected spike results obtained for NI analytes in tilapia muscle.

Analyte	CCα (ng/g)	CCβ (ng/g) Uncorrected	CCβ (ng/g) Corrected
HMMNI	0.40	0.49	0.49
IPZ	0.07	0.09	0.09
IPZ-OH	0.10	0.13	0.13
MNZ	0.07	0.09	0.09
MNZ-OH	0.32	0.41	0.43
RNZ	0.30	0.36	0.35
DMZ	0.20	0.25	0.24

Table 4.35 CCα and CCβ using uncorrected and corrected spike results obtained for NI analytes in salmon muscle.

Analyte	CCα (ng/g)	CCβ (ng/g) Uncorrected	CCβ (ng/g) Corrected
HMMNI	1.0	1.15	1.14
IPZ	0.13	0.18	0.18
IPZ-OH	0.24	0.29	0.28
MNZ	0.08	0.11	0.10
MNZ-OH	0.40	0.48	0.49
RNZ	0.41	0.52	0.50
DMZ	0.12	0.14	0.14

Table 4.36 CCα and CCβ using uncorrected and corrected spike results obtained for NI analytes in shrimp muscle.

Analyte	CCα (ng/g)	CCβ (ng/g) Uncorrected	CCβ (ng/g) Corrected
HMMNI	0.90	1.0	1.0
IPZ	0.08	0.09	0.09
IPZ-OH	0.10	0.11	0.11
MNZ	0.10	0.12	0.12
MNZ-OH	0.24	0.31	0.33
RNZ	0.20	0.26	0.25
DMZ	0.10	0.11	0.11

4.2.5 Measurement Uncertainty (MU)

ISO/IEC-17025 requires that an accredited lab assess the MU associated with each test method listed within the scope of the accreditation. This was included as part of the validation for the NI method. It included both accuracy (the closeness of the result to the true value) and precision (the variability associated with measurement). The "top down" approach was taken which used a direct determination of the combined contributions of uncertainty from method performance obtained during recovery studies and method precision (Ellison et al, 2000).

It would be expected that the MU for an analytical method for food analysis would be approximately 20-50% at the 95% confidence interval (k=2). MUs were calculated using both uncorrected and spike corrected data for intermediate precision. Results are summarized in table 4.37. For tilapia muscle, the MUs using uncorrected

results were within 20-50% accept for RNZ which was 60%. The MU for RNZ was 28% using corrected results, however IPZ was 57%. All other MUs with corrected results were within 20-50% for tilapia. MUs (uncorrected results) for shrimp muscle ranged from 36-40%, except for IPZ which was at 60%, while MUs (corrected results) were 34-45% except for RNZ which was 66%. All MUs for salmon muscle were higher than the expected range. The uncorrected result MUs ranged from 58-82% and the corrected from 55-81%. This was because of the increased %RSDs for intermediate precision due to the fortified salmon material degrading. Future experiments with IS should improve % RSDs for recovery and precision as well and therefore improve MU.

Table 4.37 MU for NI analytes using both uncorrected and spike corrected results for muscle tissues of tilapia, salmon and shrimp

	Tilapia		Salmon		Shrimp	
Analyte	MU (uncorrected)	MU (spike corrected)	MU (uncorrected)	MU (spike corrected)	MU (uncorrected)	MU (spike corrected)
HMMNI	38	36	71	72	36	42
IPZ	38	57	82	72	65	45
IPZ-OH	38	35	61	81	40	34
MNZ	38	38	58	64	40	42
MNZ-OH	37	40	75	76	40	44
RNZ	60	27	78	80	40	66
DMZ	37	38	76	55	38	39

4.3 Depletion Study

The application for an Experimental Studies Certificate (ESC) for the use of MNZ was reviewed by the Clinical Evaluation Division (CED) and the Human Safety Division (HSD) of Health Canada and it was deemed to be unnecessary to obtain an ESC for MNZ to be used in the depletion study. The online Experimental Fish Course by the Canadian Aquaculture Institute was successfully completed and the Animal Use Protocol was approved by the Animal Care Committee. The Letter of Understanding (LOU) was signed by both parties and security clearance was given for CFIA employees involved in the project to enter BIO to perform duties related to the depletion study.

4.3.1 Preparation of Medicated Diet

To account for residue loss during preparation of the medicated feed, a 10% excess of MNZ was added. Therefore, 3.3g of MNZ was added to 1kg of feed to get a concentration of approximately 3g/kg. It was determined that 4mls of acetone should be added per 10g of pellets for optimal absorption by the pellets. Therefore, 3.3g of MNZ was absorbed in 400ml of acetone for 1kg of feed.

The analysis of medicated feed was done in triplicate with three medicated pellet sample extracts being diluted 10^6 times and another 3 samples being diluted 10^5 times. Non-medicated pellet samples spiked at 3g/kg were also done in triplicate with 10^5 and 10^6 dilutions. Results are summarized in Table 4.38. The average concentration was determined to be 2.8g MNZ per kg of feed (RSD 5.6%) with an average spike recovery of 104%.

Table 4.38 Results of medicated feed analysis by LC-MSMS

Analyte	Dilution	Mean Result (g/kg)	SD	RSD %	Recovery %
MNZ	10^5	2.7	0.4	13	100
	10^6	2.9	0.3	10	109

4.3.2 Preparation of Incurred Fish

The fish were delivered by the Nova Scotia Provincial Government fish hatchery in Caledonia, NS on Sept 7, 2010 and placed in the holding tanks to be used for the depletion study at BIO. Fish were not fed on the first day of arrival as they were expected not to eat when stressed from travel. After that, they ate well each day during the 20-day acclimatization period at 1 % of their body weight/day. A mortality was found on day 8 after arrival. Water temperature was recorded each day at approximately 9:00 am by the Fish Laboratory Manager at BIO and the temperatures are shown in table 4.39 and figure 4.17 for the entire study including the acclimatization period. The mean temperature with SD from the day the fish arrived to the end of the depletion study was 18.2 ± 1.3 °C. The mean temperature from the first day of medication to the last day of withdrawal was 17.4± 0.94 °C.

Table 4.39 Daily Temperatures recorded during acclimatization and the depletion study

Date	Temperature (°C)
Sept 7	20.1
8	20.2
9	19.7
10	20.1
13	19.9
14	19.8
15	19.7
16	19.4
17	19.4
20	18.9
21	18.9
22	18.6
23	18.5
24	18.5
27	18.1
28	18.0
29	17.9
Oct 1	17.8
4	17.8
5	17.9
6	17.9
7	17.7
8	17.7
12	17.1
13	17.0
15	16.9
18	16.2
19	16.2
20	16.1
21	15.8
22	15.9

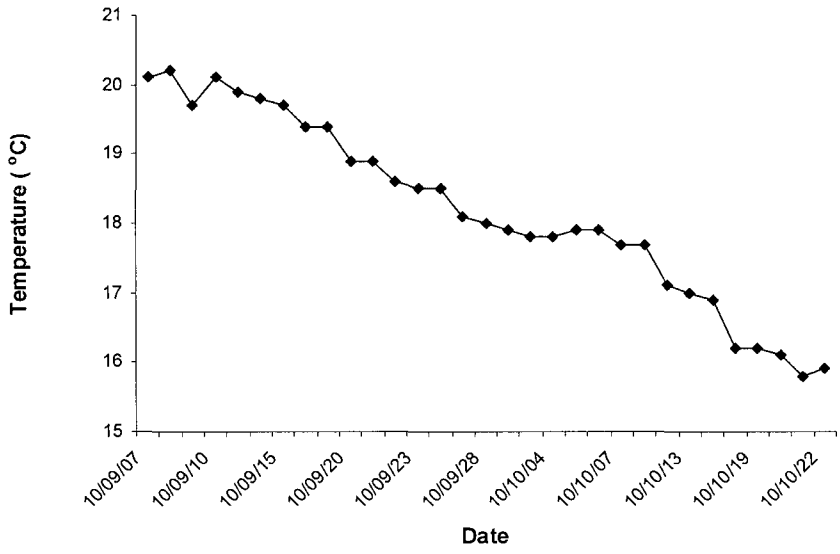


Figure 4.17 Daily Temperatures recorded during acclimatization and the depletion study

4.3.3 Sampling of Fish

On day 14 after the fish arrived, 7 control samples were taken. Since there was a larger range in the size of fish than anticipated, it was decided to take seven of the larger fish rather than random sampling so the depletion study would be done on fish closer in size. After sampling fish were measured and weighed. Mean weight and length with SD are summarized for each time point in table 4.40. All weights and lengths recorded are in Appendix 15. The remaining fish were given medicated feed at 1 % their body weight/day for the first 5 day medication period taking samples on the scheduled days. Withdrawal was originally to be 10 days, however fish were mistakenly fed medicated feed on the sixth day of withdrawal. It was then decided to do the second 5 day medication period with a 16 day withdrawal period. There were enough fish remaining to take a sample size of 5 at 5 time points. One fish died during the second medication

period. Four fish remained after all samplings which were sacrificed and used as quality control material.

Table 4.40 Mean weight and length of trout for each sampling time point

Date (yymmdd)	Mean weight (g)	SD	Mean length (cm)	SD
100920	334	100	28	2
100922	159	46	22	2
100924	188	57	24	3
100925	213	32	25	2
100927	169	38	22	2
100929	218	77	25	3
101007	202	48	24	3
101012	166	45	24	1
101015	199	48	25	2
101018	213	80	25	2
101022	234	84	26	4

4.3.4 Chemical Analysis

It is beneficial to test a method for detection of veterinary drug residues using actual dosed animal (incurred) samples as these samples are closer to what would be found in an actual monitoring situation than samples which have only been fortified (Maher et al, 2008). This was accomplished as a result of the depletion study as well as providing knowledge to enable interpretation of residue findings during routine analysis.

Each sample taken for each time point was analyzed for MNZ along with its metabolite MNZ-OH in duplicate and the mean, SD and % RSD were calculated. The results obtained for MNZ are in table 4.41 and table 4.42 for MNZ-OH for the first medication and withdrawal period. For the second withdrawal period, MNZ results are in table 4.43 and MNZ-OH results are in table 4.44. Raw data are shown in Appendix 16. If the %RSD for duplicates fell outside of typical method repeatability (>25%), the analysis was repeated. The results for all samples at each time point were also averaged and the SD and %RSD calculated. The mean results for the first medication and withdrawal period are in table 4.45 and the results for the second withdrawal period are in table 4.46. There were large ranges with high % RSDs between samples from the same time point. A Grubb's test was done to determine any outliers (Appendix 17). Only one result was found to be an outlier and the analysis was repeated. The range of results was expected due to the nature of working with live animals. The fish were the same age, but ranged in size and fed and metabolized the drug at different rates. Even though results from the same time points varied, an overall increase in concentration of MNZ and MNZ-OH was seen during the first medication period and a decrease during the withdrawal periods. The concentration of MNZ can be seen in figure 4.18 for the first medication and withdrawal period while the concentration of MNZ-OH is shown in figure 4.19. The depletion of MNZ and MNZ-OH during the second withdrawal period can be seen in figures 4.20 and 4.21, respectively.

Table 4.41 MNZ residue concentrations in trout muscle from the first medication and withdrawal period

Sample		Day 0	Day 3 dosing	Day 5 Dosing	Day 1 Withdrawal	Day 3 Withdrawal	Day 5 Withdrawal
1	Mean(ng/g) SD RSD (%)	<0.08	15847 917 5.8	30814 276 12	34503 671 1.9	1159 132 11	1387 16 1.2
2	Mean(ng/g) SD RSD (%)	<0.08	23972 889 3.7	1050 56 5.4	12072 1 0.0	7124 489 6.9	6314 724 11
3	Mean(ng/g) SD RSD (%)	<0.08	20062 1440 7.2	24964 2096 8.4	38862 3682 9.5	9637 946 9.8	7476 305 4.1
4	Mean(ng/g) SD RSD (%)	<0.08	21806 1874 8.6	26898 2421 9.0	29084 800 2.8	16471 1319 8.0	1191 142 12
5	Mean(ng/g) SD RSD (%)	<0.08	19255 267 1.4	14043 703 5.0	25324 4906 19	10666 769 7.2	5.4 0.5 9.9
6	Mean(ng/g) SD RSD (%)	<0.08	23359 526 2.2	22428 2039.3 9.1	15956 1216.1 7.6	11487 345 3.0	2874 141 4.9
7	Mean(ng/g) SD RSD (%)	<0.08	21125 433 2.0	28234 4116 15	36176 4624 13	12835 1711 13	6078 29 0.47

Table 4.42 MNZ-OH residue concentrations in trout muscle from the first medication and withdrawal period

Sample		Day 0	Day 3 dosing	Day 5 Dosing	Day 1 Withdrawal	Day 3 Withdrawal	Day 5 Withdrawal
1	Mean(ng/g) SD RSD (%)	<0.40	301 36 12	1378 53 3.8	1200 13 1.1	37 0 0.6	204 5 2.3
2	Mean(ng/g) SD RSD (%)	<0.40	512 28 5.6	6.3 0.6 8.8	267 18 6.6	366 15 4.0	352 64 18
3	Mean(ng/g) SD RSD (%)	<0.40	306 51 17	712 62 8.7	1779 149 8.4	618 45 7.3	281 53 19
4	Mean(ng/g) SD RSD (%)	<0.40	351 51 14	1020 150 15	610 50 8.2	979 51 5.2	162 2 1.0
5	Mean(ng/g) SD RSD (%)	<0.40	342 47 14	347 49 14	848 206 24	688 55 7.5	0.55 0.04 7.7
6	Mean(ng/g) SD RSD (%)	<0.40	392 14 3.7	814 150 18	100 5 5.0	843 41 4.9	221 24 11
7	Mean(ng/g) SD RSD (%)	<0.40	476 2 0.4	1070 10 1.0	1025 171 17	721 133 18	498 36 7.2

Table 4.43 MNZ residue concentrations in trout muscle from the second withdrawal period

Sample		Day 1 withdrawal	Day 6 withdrawal	Day 6 withdrawal	Day 12 withdrawal	Day 16 withdrawal
1	Mean(ng/g)	17132	1852	32	1.9	0.83
	SD	1158	236	4	0.0	0.11
	RSD (%)	6.8	13	13	0.4	13
2	Mean(ng/g)	14733	714	386	44	0.51
	SD	272	65	27	3	0.03
	RSD (%)	1.8	9.2	7.0	7.6	5.6
3	Mean(ng/g)	28903	983	28	194	0.66
	SD	2797	79	2	17	0.00
	RSD (%)	9.7	8.0	5.8	8.9	0.0
4	Mean(ng/g)	23078	289	406	6.6	1.1
	SD	672	65	44	0.6	0.1
	RSD (%)	2.9	23	11	9.8	7.4
5	Mean(ng/g)	17882	1455	333	9.5	5.9
	SD	156	242	45	0.2	0.3
	RSD (%)	0.9	17	13	2.2	5.2

Table 4.44 MNZ-OH residue concentrations in trout muscle from the second withdrawal period

Sample		Day 1 withdrawal	Day 6 withdrawal	Day 6 withdrawal	Day 12 withdrawal	Day 16 withdrawal
1	Mean(ng/g) SD RSD (%)	420 30 7.2	193 5 2.4	7.0 0.8 11	<0.40	<0.40
2	Mean(ng/g) SD RSD (%)	301 62 21	30 6 22	32 3 9.9	7.5 0.5 6.4	<0.40
3	Mean(ng/g) SD RSD (%)	930 41 4.4	89 6 7.1	3.2 0.5 17	24 0 1.4	<0.40
4	Mean(ng/g) SD RSD (%)	629 9 1.4	56 1 2.4	39 2 6.4	0.63 0.04 5.7	<0.40
5	Mean(ng/g) SD RSD (%)	480 32 6.8	47 3 7.0	33 5 15	1.4 0.3 19	0.99 0.03 2.9

Table 4.45 Mean concentrations of residues of MNZ and MNZ-OH in muscle of trout treated with MNZ during the first medication and withdrawal period

	MNZ Concentration (ng/g)			MNZ-OH Concentration (ng/g)			
Day	Min	Max	Mean ±SD	Min	Max	Mean ±SD	Fraction of MNZ-OH to MNZ (%)
0			<0.08			<0.40	
3	15847	23972	20775 ± 2744	301	512	329 ± 82	1.6
5	1050	30811	21205 ± 10387	6.3	1378	764 ± 464	3.6
6	12072	38862	27425 ± 10263	100	1779	833 ± 574	3.0
8	1159	16471	9911 ± 4814	37	979	607 ± 315	6.1
10	5.4	7476	3618 ± 2963	0.55	498	246 ± 156	6.8

Table 4.46 Mean concentrations of MNZ and MNZ-OH in muscle of trout treated with MNZ during the second withdrawal period.

	MNZ Concentration (ng/g)			MNZ-OH Concentration (ng/g)			
Day	Min	Max	Mean ±SD	Min	Max	Mean ±SD	Fraction of MNZ-OH to MNZ (%)
1	14733	28903	20346 ± 5670	301	930	552 ± 242	2.7
6	289	1852	1058 ± 613	30	193	83 ± 65	7.8
9	27	406	165 ± 188	3.2	39	23 ± 16	14
12	1.9	194	51 ± 81	0.51	6.0	6.7 ± 10	13
16	0.51	6.0	2 ± 2	0	0.99	<0.40	

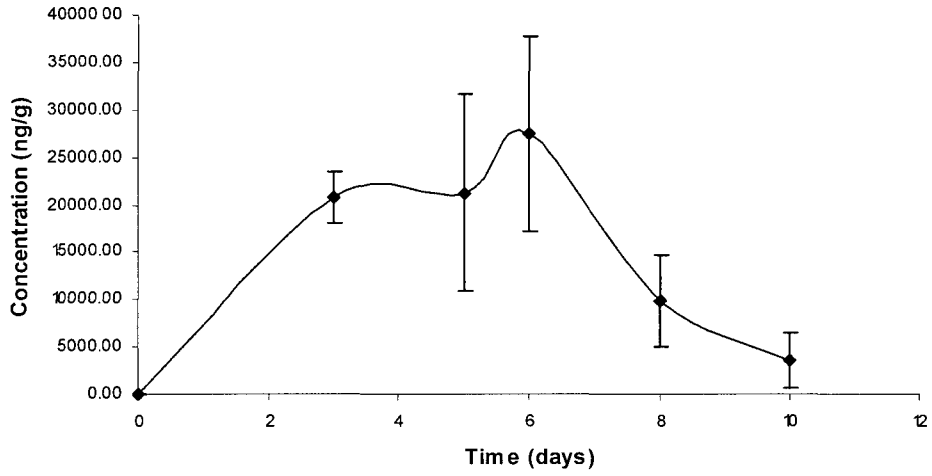


Figure 4.18 Depletion curve representing MNZ concentrations in trout tissue during the first MNZ medication and withdrawal period.

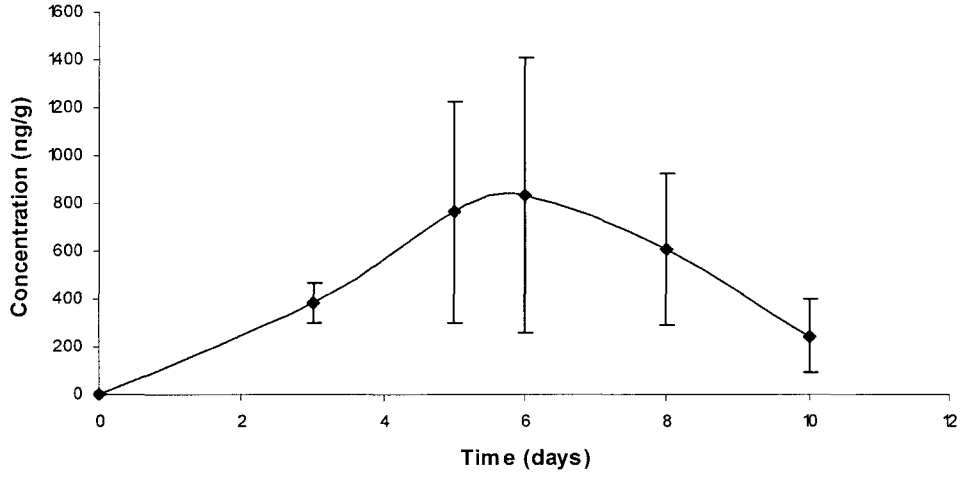


Figure 4.19 Depletion curve representing MNZ-OH concentrations in trout tissue during the first MNZ medication and withdrawal period.

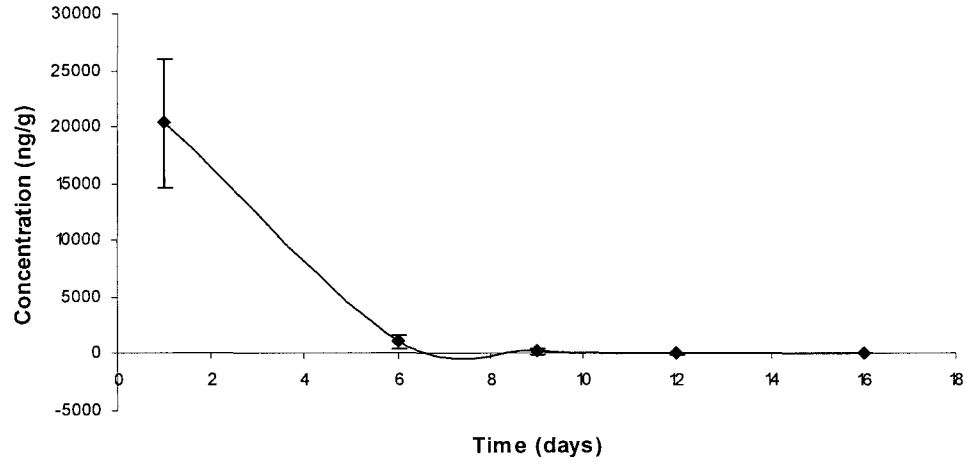


Figure 4.20 Depletion curve representing MNZ concentrations in trout tissue during the second withdrawal period.

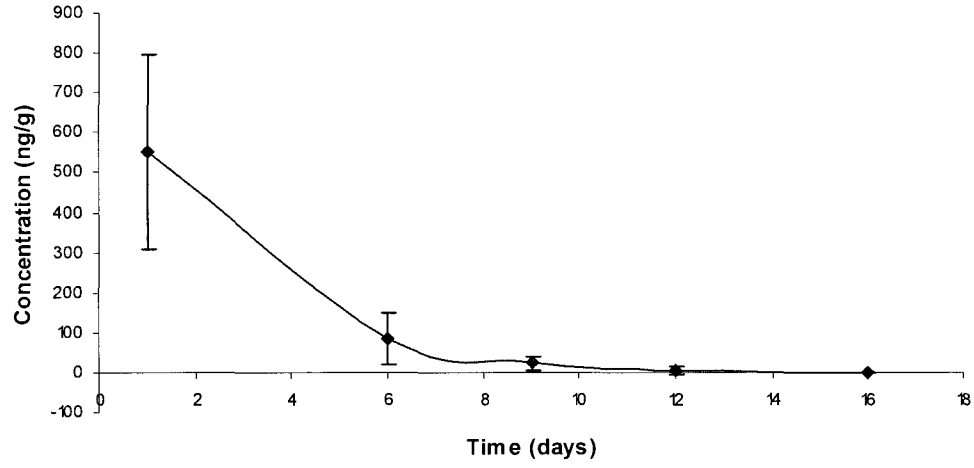


Figure 4.21 Depletion curve representing MNZ-OH concentrations in trout tissue during the second withdrawal period.

No residues were detected before the medication period and low concentrations of drug residues were still detectable after 16 days of withdrawal. The mean MNZ residue concentration peaked on day 1 of withdrawal at 27425 ± 10262 ng/g during the first study. MNZ-OH residue concentration also peeked on day 1 of withdrawal with a

concentration of 832 ± 573 ng/g. On day 5 of withdrawal the MNZ residue level was 3617 ± 2963 ng/g and MNZ-OH was 246 ± 156 ng/g. During withdrawal after the second medication period, the residue level was 20345 ± 5670 ng/g for MNZ on day 1 and MNZ-OH was 552 ± 242 ng/g. By day 16 of withdrawal, the MNZ concentration was 1.8 ± 2.3 ng/g and MNZ-OH was below the LOD (<0.40).

MNZ and MNZ-OH were detected in relatively high concentrations during the medication periods. As a result extracts from the samples were diluted appropriately in order for the drug levels to fall within the range of the calibration curve for the method. The values reported were corrected for these dilutions. The highest results obtained were 5 to 10 times higher than previous depletion studies seen (Maher et al, 2008 and Sorenson and Hansen, 2000). However, the trout in the study conducted by Sorenson and Hansen were fed a much lower dose of MNZ (approximately 6 times lower). In the Maher study, the dose was comparable, but it was a different species of fish (tilapia) so different results would be expected, especially considering the differences in fat content.

The fraction of MNZ-OH to MNZ was reported as less than 2 % on the first day after the administration period in the study by Sorensen and Hansen, 2000. The results observed in the Maher et al (2008) study were similar. In this study, on day 3 of the first medication period the fraction of MNZ-OH to MNZ was less than 2% as well, but it increased as the study continued and was 6.8% by day 10 of withdrawal. During the second withdrawal period, the level started at 2.7% on day 1 and increased as high as 13.8% on day 9 of withdrawal. Fractions of MNZ-OH to MNZ at each time point can be seen in table 4.45 for the first medication and withdrawal period and in table 4.46 for the second withdrawal period.

4.3.5 Tissue Disposal

The incurred tissue that remained after chemical analysis for the depletion study was stored at -80°C. All incurred tissue material was combined and diluted with the blank control material to obtain appropriate levels to be used for quality control materials during routine residue testing.

SUMMARY AND CONCLUSIONS

Chemical usage is widespread in the aquaculture industry. Nitroimidazoles are a class of veterinary drug that have been banned for use in food producing animals, including aquacultured fish, due to safety concerns. Residue testing for nitroimidazoles was identified as a priority by the CFIA to meet EU requirements for exports. To meet this requirement, a thesis project to develop and validate a residue method was undertaken and completed.

For project completion several objectives had to be satisfied. A review of available information on analytical methods for nitroimidazole residues and depletion studies in fish was completed. Limited information was found on nitroimidazole residues in fish. However, using the information and resources available, a simple, rapid and sensitive quantitative and confirmatory method was developed and validated that is suitable for monitoring of nitroimidazole residues and their hydroxy metabolites in fish tissue using UPLC-MS-MS. Good recoveries (87-121%) and acceptable RSDs (<26%) for method repeatability were obtained with low limits of quantitation, (0.21-3.0 ng), depending on the analyte. Working standards were determined to be stable for 12 months, while extracts were stable for 5 days and tissue for 2 months under appropriate storage conditions. The validated method meets performance requirements for use in a regulatory monitoring program for nitroimidazole residues in fish and crustaceans.

Since few reports had been published on the metabolism of nitroimidazoles in fish, a depletion study was undertaken using MNZ in feed provided to trout under controlled conditions. The effectiveness of the method was further shown by its determination of MNZ and MNZ-OH levels in the incurred trout fish muscle tissue.

Analyte concentrations were determined over a 5 day medication and 16 day withdrawal period. This information from the depletion study will enable interpretation of residue findings during regulatory monitoring.

FUTURE WORK

Although a method for the determination of NIs in fish and crustaceans was developed and validated for salmon, tilapia and shrimp muscle and MNZ and its metabolite were successfully analyzed in incurred trout material, challenges still remain. Future work including the investigation of the use of ISs and/or improved SPE clean up to eliminate matrix effects are recommended to deal with these challenges.

Ion suppression was identified for all compounds in the matrices studied. To correct for these matrix effects, a matrix matched calibration curve was used to quantitate analytes. However, it has been found that the degree of ion suppression/enhancement varies from matrix to matrix and also between different lots of the same sample (Matusweski et al., 2003). In a regulatory environment several different matrices may need to be analyzed in the same run. Salmon, shrimp and tilapia muscle are representative of many samples the CFIA analyzes, but many other species and processed samples require analysis as well. In addition, if different sources of the same matrices demonstrate different degrees of ion suppression or enhancement, as found by Matusweski, this would create a problem for matrix matched calibration.

The addition of an internal standard is largely used to compensate for matrix effects (Gosseti et al., 2010). In particular, the use of stable isotope-labelled analogues as ISs is highly recommended for diminishing suppression effects. These were not used for the method validation due to their expense. However, with the knowledge that the NI compounds studied are stable in solution for at least a year, it makes their purchase more feasible. Also, considering an increase over the last 2 years in the variety of matrices that

require regulatory testing, future work with ISs is recommended to diminish matrix effects.

Matrix effects may also be eliminated or minimized by modifications to the clean up process (Matusweski et al., 2003 and Gossetti et al, 2010). To lower matrix effects in the final extract, it is suggested to systematically determine the matrix effect after different sample pre-treatments. Strong cation exchange (SCX), OASIS HLB and more recently molecularly imprinted polymers (MIP) have been used to clean up extracts for NI analysis. Experiments utilizing various SPE clean up alternatives are recommended.

Since the NI method follows several of the steps of other established methods for other classes of veterinary drugs such as triphenyl methane dyes and fluoroquinolones, the possibility exists for the development of a multi-class residue method. Maximizing the number of analytes that may be determined by a single procedure or from a single portion of test material would be more cost-effective (Stolker et al, 2008). There is increasing interest in methods for the simultaneous analysis of several classes of veterinary drugs. Very few multi-class LC-MS-MS methods exist due to a number of analytical challenges to overcome. However, due to cost-effectiveness and time saving possibilities, investigation of the multi-class residue approach is therefore recommended as a potential area for future work.

REFERENCES

Aerts, R. Egberink, I., Kan, C., Cornelis, K., Keukens, H. and Beek, W. (1991) Liquid chromatographic multicomponent method for the determination of residues of ipronidazole, ronidazole and dimetronidazole and some relevant metabolite in eggs, plasma and feces and its use in depletion studies in laying hens. J.AOAC. 74(1):46-55.

Anonymous. (1978) The nitroimidazole family of drugs. Br J Vener Dis 54:69-71.

Anonymous. (1998) Peer reviewed methods program – manual on policies and prodecures. AOAC International.

The Aquaculture Authority. (2002) Proceedings of the expert group organized by aquaculture authority, Government of India on the use of antibiotics, drugs and chemicals in shrimp aquaculture and steps to be taken for their regulation. May 18, 2002. http://aquaculture.nic.in/pdf/antibiotics.pdf. November 13, 2008.

Ashwin, H., Stead, S., Caldow, M., Sharman, M., Stark, J., de Rijk, A., Keely, B.J. (2009) A rapid microbial inhibition-based screening strategy for fluoroquinolone and quinolone residues in foods of animal origin. Anal. Chim. Acta. 637:241-246.

Barnard, G.A. (1959). "Control charts and stochastic processes". *Journal of the Royal Statistical Society* **B (Methodological)** (21): 239–71.

Canadian Food Inspection Agency (2007) Standard operating method for analysis of florfenicol, florfenicol amine, thiamphenical and chloramphenicol in fish and crustaceans by LC, CFIA Dartmouth Laboratory (internal document) SOM-DAR-CHE-037-03.

Canadian Food Inspection Agency (2009) Standard operating procedure for method validation of routine methodologies, CFIA Dartmouth Laboratory (internal document) SOP-DAR-CHE-001-00.

Canadian Food Inspection Agency (2009) Standard operating method for determination of triphenylmethane dyes in salmon, shrimp and aquacultured products, CFIA Dartmouth Laboratory (internal document) SOM-DAR-CHE-039-07.

Canadian Food Inspection Agency (2009) Standard operating method for determination of nitrofuran metabolites in aquacultured fish and crustaceans by LC, CFIA Dartmouth Laboratory (internal document) SOM-DAR-CHE-038-05.

Canadian Food Inspection Agency (2009) Standard operating method for determination of fluoroquinolones in a variety of aquacultured products, CFIA Dartmouth Laboratory (internal document) SOM-DAR-CHE-050-01.

Cannavan, A. and Kennedy, D.G. (1997) Determination of dimetridazole in poultry tissues and eggs using liquid chromatography-thermospray mass spectrometry. Analyst. 122:963-966.

Capitan-Vallvay, L.F., Ariza, A., Checa, R. and Navas, N. (2002) Determination of five nitroimidazoles in water by liquid chromatography-mass spectrometry. J. Chrom. 978(1-2):243-248.

Carignan, G., Skakum, W. and Sven, S. (1988a) Dimetridazole residues in pork tissue. I. Assay by liquid chromatography with electrochemical detector. J.AOAC. 71(6):1141-1145.

Carignan, G., MacIntosh, A., Skakum, W. and Sved, S. (1988b) Dimetridazole residues in pork tissue. II. Application of liquid chromatographic method to monitor elimination of drug and its major methobolite. J.AOAC. 71(6):1146-1149.

CAC (2009a) CAC/GL 71-2009: Guidelines for the design and implementation of national regulatory food safety assurance programme associated with the use of veterinary drugs in food producing animals, Joint FAO/WHO Food Standards Program, Rome; http://www.codexalimentarius.net/download/standards/11252/CXG_071e.pdf; accessed April 19, 2010.

CAC (2009b) Guidelines on analytical terminology. CAC/GL 72-2009. Codex Alimentarius Commission, Joint FAO/WHO Food Standards Program, Rome; http://www.codexalimentarius.net/download/standards/11357/cxg_072e.pdf; accessed April 20, 2010.

Committee for Veterinary Medicinal Products (2008) Dimetridazole (3) Summary Report. The European Agency for the Evaluation of Medicinal Products. www.emea.europa.eu/pdfs/vet/mrls/dimetridazole3.pdf. November 28, 2008.

Connelly, L., Thompson, C.S., Haughey, S.A., Traynor, I.M., Tittlemeier, S. and Elliot, C.T. (2007) The development of a multi-nitroimidazole residue analysis assay by optical biosensor via a proof of concept project to develop and assess a prototype test kit. Anal. Chim. Acta. 598(1):155-161.

Conner, T., Stockel, M., Evrard, J. and Legator, M. (1997) The contribution of metronidazole and two metabolites to the mutagenic activity detected in urine of treated humans and mice. Cancer Research. 37:629-633.

Cronley, M., Behan, P., Foley, B. Malone, E. and Regan, L. (2009) Rapid confirmatory method for the determination of 11 nitroimidazoles in egg using liquid chromatography tandem mass spectrometry. J. Chrom. A. 1216: 8101-8109.

Daeseleire, E., De Ruyek, H., and Van Renterghem, R. (2000) Rapid confirmatory assay for the simultaneous detection of ronidazole, metronidazole and dimetridazole in eggs using liquid chromatography-tandem mass spectrometry. Analyst. 125:1533-1535.

Department of Justice Canada (2008) Food and Drugs Act and Regulations. Part B. Section B.01.048.2.http://laws.justice.gc.ca/en/showdoc/cr/C.R.C.-c.870/bo-ga:l_B. October 20, 2008.

Diaz-Cruz, M.S., Lopez de Alda, M.J. and Barcelo, D. (2003) Environmental behavior and analysis of veterinary and human drugs in soils, sediments and sludge. Trends in Analytical Chemistry. 22(6):340-350.

Dobias, L., Cerna, M., Rossner, P. and Sram, R. (1994) Genotoxicity and carcinogenicity of metronidazole. Mutation Research. 317:177-194.

Elizondo, G., Montero, R., Herrera, J.E., Hong, E. and Ostrosky-Wegman, P. (1994) Lymphocyte proliferation kinetics and sister-chromatid exchanges in individuals treated with metronidazole. Mutation Research. 305:133-137.

Ellison, S.L.R., Rosslein, M., Willians, A., ed. (2000) Quantifying Uncertainty in Analytical Measurement- EURACHEM/CITAC Guide CG 4, 2nd ed., http://www.eurachem.org/guides/QUAM2000-1.pdf; accessed January 20, 2010.

EC (1996) Council Directive 96/23/EC of 29 April 1996 on measures to monitor certain substances and residues thereof in live animals and animal products and repealing Directives 85/358/EEC and 86/469/EEC and Decisions 89/187/EEC and 91/664/EEC, Official Journal of the European Community L 125, 23.5., p. 10–32.

EC (2002) Commision Decision of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results (2002/657/EC). Off. J. European Communities L 221/8 (17.8.2002).

EC (2006) Commission staff working document on the implementation of National Residue Monitoring Plans in the member states in 2004 (Council Directive 96/23/EC). http://ec.europa.eu/food/food/chemicalsafety/residues/workdoc_2004_en.pdf; accessed February 26, 2009.

Eurachem (1998) The Fitness for Purpose of Analytical Methods. A Laboratory Guide to Method Validation and Related Topics. http://www.eurachem.org/guides/valid.pdf; accessed January 20, 2010.

Fraselle, S., Derop, V., Degroodt, J.M. and Van Loco, J. (2007) Validation of a method for the detection and confirmation of nitroimidazoles and the corresponding hydroxyl metabolites in pig plasma by high performance liquid chromatography-tandem mass spectrometry. Anal. Chim. Acta. 586:383-393.

Garry, V.F. and Nelson, R.L. (1987) Host mediated transformation:metronidazole. Mutation Research. 190:289-295.

Gaugain, M. and Abjean, J.P. (1996) High performance thin-layer chromatographic method for the fluorescence detection of three nitroimidazole residues in pork and poultry tissue. J. Chrom. A. 737:343-346.

Gibson, R.A., Lattanzio, L. and McGee, H. (1984) Optimized liquid-chromatographic determination of metronidazole and its metabolite in plasma. Clin.Chem. 30(5):784-787.

Gosetti, F., Mazzucco, E., Zampieri, D. and Gennaro, MC. (2010) Signal suppression/enhancement in high-performance liquid chromatography tandem mass spectrometry. J. Chrom A. 1217:3929-3937.

Guidance for Industry: Mass spectrometry for confirmation of the identity of animal drug residues. US Food and Drug Administration. http://fda.gov/downloads/animalveterinary/guidancecomplianceenforcement/guidanceforindustry/ucm052658.pdf; accessed March 24, 2011.

Health Canada (2003) Part II Canada Gazette – Food and Drug Regulations – Amendments will be published in Part II Canada Gazette of August 27, 2003 – Project No. 1227 (Prohibition of Certain Veterinary Drugs). Drug and Health Products. www.hc-sc.gc.ca/dhp-mps/vet/legislation/acts-lois/cg-gc_part2-eng.php. November 16, 2008

Hernando, MD, Suarez-Barcena, JM, Bueno, MJM, Garcia-Reyes, JF and Fernandez-Alba, AR. (2007) Fast separation liquid chromatography-tandem mass spectrometry for the confirmation and quantitative analysis of avermectin residues in food. J. Chrom A. 1155(1):62-73.

Horwitz, W., & Alpert, R. (2006) The Horwitz Ratio (HorRat): AUseful Index of Method Performance with Respect to Precision. J. AOAC Int. 89 (4): 1095-1109.

Ho, C., Sin, D., Wong, K.M. and Tang, H. (2005) Determination of dimetridazole and metronidazole in poultry and porcine tissue by gas chromatography electron capture negative ionization mass spectrophotometry. Anal.Chim. Acta. 530:23-31.

Huet, A.C., Mortier, L., Daesleire, E., Fodey, T., Elliot, C. and Delahaut, P. (2004) Development of an ELISA screening test for nitroimidazoles in egg and chicken muscle. Anal. Chim. Acta. 534:157-162.

Huet, A., Mortier, L., Daeslaire, E., Fodey, T., Elliot, C. and Delahaut, P. (2005) Development of an Elisa screening test for nitroimidazoles in egg and chicken muscle. Anal. Chim. Acta. 534:157-162.

Hurtaud-Pessel, D., Delepine, B. and Laurentie, M. (2000) Determination of four nitroimidazole residues in poultry meat by liquid chromatography-mass spectrometry. J. Chrom. A. 882:89-98.

JCGM (2008) JCGM 200:2008(E/F) - International vocabulary of metrology — Basic and general concepts and associated terms (VIM). Vocabulaire international de métrologie — Concepts fondamentaux et généraux et termes associés (VIM). Bureau International des Poids et Mesures. Available at: http://www.bipm.org/utils/common/documents/jcgm/JCGM_200_2008.pdf; accessed March 18, 2011.

Johnston, P. and Santillo, D. (2002) Chemical usage in aquaculture: Implications for residues in market products. Greenpeace Research Laboratories, Department of Biological Sciences. Technical Note 06/2002:1-16.

Kan, C. and Petz, M. (2000) Residues of veterinary drugs in eggs and their distribution between yolk and white. J. Agric. Food Chem. 48(12):6397-6403.

Kennedy, DG., Hughes, PJ., and Bleanchflower, WJ. (1998) Ionophore residues in eggs in Northern Ireland. Food Addit Contam. 15(5): 535-541.

Lansky, P.F. and Halling-Sorenson, B. (1997) The toxic effect of the antibiotic metronidazole on aquatic organisms. Chemosphere. 35(11):2553-2561.

Lindmark, D.G. and Muller, M. (1976) Antitrichromatid action, mutagency and reduction of metronidazole and other nitroimidazoles. Antimicrobial Agents and Chemotherapy. 10(3):476-482.

Maher, H.M., Youssef, R.M., Khalil, R.H. and El-Bahr, S.M. (2008) Simultaneous multiresidue determination of metromidazole and spiramycin in fish muscle using high performance liquid chromatography with UV detection. J. Chrom. B. 876:175-181.

Mahugo-Santana, C., Sosa-Ferrera, Z., Torres-Padron, M.E.and Santana-Rodriguez, J.J. (2010) Analytical methodologies for the determination of nitroimidazole residues in biological and environmental liquid samples: A review. Analytica Chimica Acta. 665:113-122.

Matuszewski, B.K., Contanzer, M.L. and Chavez-Eng, C.M. (2003) Strategies for the assessment of matrix effects in quantitative bioanalytical methods based on HPLC-MS/MS. Anal. Chem. 75:3019-3030.

McClain, R.M. and Downing, J.C. (1988) Reproduction studies in rats treated with ornidazole. Toxicology and Applied Pharmacology. 92:480-487.

Miller, J.N. and Miller, J.C. (2005) Statistics and Chemometrics for Analytical Chemistry, Fifth edition, Pearson Education Limited, Harlow.

Mohamed, R., Mottier, P., Treguier, L., Richoz-Payot, J. Yilmaz, E., Tabet, J.C. and Guy, P. (2008) Use of molecularly imprinted solid-phase extraction sorbent for the determination of four 5-nitroimidazoles and three of their metabolites from egg based samples before tandem LC-ESI-MS/MS analysis. J. Agric. Food Chem. 56:3500-3508.

Mortier, M., Daeseleire, E. and Delahaut, P. (2003) Simultaneous detection of five coccidiostats in eggs by liquid chromatography-tandem mass spectrometry. Anal. Chim. Acta. 483:27-37.

Mortier, L., Daeseleire, E. and Van Peteghem, C. (2005a) Liquid chromatographic tandem mass spectrometric determination of five coccidiostats in poultry eggs and feed. J. Chrom. B. 820:261-270.

Mortier, L., Huet, A., Daeslaire, E., Huyghebaert, G., Fodey, T., Elliot, C., Delahaut, P. and Van Peteghem, C. (2005b) Deposition and depletion of five anticcocidials in eggs. J. Agric. Food Chem 53(18):7142-7149.

Mottier, P., Hure, I., Gremand, E. and Guy, P.A. (2006) Analysis of four 5-nitroimidazoles and their corresponding hydroxymetabolated metabolites in egg, processed egg, and chicken meat by isotope dilution liquid chromatography tandem mass spectrometry. J. Agric. Food Chem. 54:2018-2026.

Mudry, M.D., Carballo, M., Labal de Vinuesa, M., Gonzalez Cid, M. and Larripa, I. (1994) Mutagenic bioassay of certain pharmacological drugs: III. Metronidazole (MNZ). Mutation Research. 305:127-132.

Newkirk, D.R., Righter, H.F., Schenck, F.J., Okrasinski Jr, J.L. and Barnes, C.J. (1990) Gas chromatography determination of incurred dimetridazole residues in swine tissues. J. AOAC. 73(5):702-704.

Noss, M., Panicucci, R., McClelland, R. and Rauth, A. (1988) Preparation, toxicity, and mutagenicity of 1-methyl-2-nitroimidazole. A toxic 2-nitroimidazole reduction product. Biochemical Pharmacology. 37(13):2585-2593.

Page, E. S. (June, 1954). "Continuous Inspection Scheme". *Biometrika* **41** (1/2): 100–115.

Pearce, J.N., Burns, B.G., van de Reit, J.M., Casy, M.D. and Potter, R.A. (2009) Determination of fluoroquinolones in aquaculture products by unltra-performance liquid chromatography-tandem mass spectrometry. Food Additives and Contaminants. 26(1):39-46.

Pendland, S., Piscotti, S., Schreckenberger, P. and Danziger, L. (1994) *In vitro* activities of metronidazole and its hydroxy metabolite against bacteroides spp. Antimicrobial Agents and Chemotherapy. 38(9):2106-2110.

Polzer, G. and Gowik, P. (2001) Validation of a method for the detection and confirmation of nitroimidazoles and corresponding hydroxyl metabolites in turkey and swine muscle by means of gas chromatography-negative ion chemical ionization mass spectrometry. J. Chrom. B. 761:47-60.

Polzer, J., Stachel, C. and Gowek, P. (2004) Treatment of turkeys with nitroimidazoles Impact of selection of target analytes and matrices on an effective residue control. Anal. Chim. Acta. 521:189-200.

Polzer, J. and Gowak, P. (2005) Nitroimidazoles in turkey muscle - the influence of sampling conditions on measurement results in residue control. Anal. Chim. Acta. 529(1-2):299-303.

Rose, M.D., Bygrave, J. and Sharman, M. (1999) Effect of cooking on veterinary drug residues in food Part 9. Nitroimidazoles. Analyst. 124:289-294.

Sams, M. J., Strutt, P.R., Barnes, K.A., Damant, A.P. and Rose, M.D. (1998) Determination of dimetridazole, ronidazole and their common metabolite in poultry muscle and eggs by high performance liquid chromatography with UV detection and confirmatory analysis by atmospheric pressure chemical ionisation mass spectrometry. Analyst. 123:2545-2549.

SCC, 2010. Accreditation Program for Laboratories, Standards Council of Canada; http://www.scc.ca/en/programs-services/laboratories; accessed August 16, 2010.

Schmid, A. and Schmid, H. (1999) Pharmaco-toxicological mode of action of antimicrobial 5-nitroimidazole derivatives. J. Vet. Med. A. 46:517-522.

Shao, B., Wu, X., Zhang, J., Duan, H., Chu, X. and Wu, Y. (2009) Development of a rapid LC MS-MS method for multiclass determination of 14 coccidiostat residues in effs and chicken. Chromatographia. 69:1083-1088.

Shen, J., Yhang, Y., Zhang, S., Ding, S. and Xiang, X. (2003) Determination for nitroimidazoles and their metabolites in swine tissue by liquid chromatography. J. AOAC Inter. 86(3):505-509.

Shimelis, O., Wihlborg, A., Rudolfsson, M., Boyd, B. and Trinh, A. (2009) Extraction of nitroimidazoles from milk and eggs using molecularly imprinted polymers. Supelco Analytical. 27.4.

Silver, A., McNeil, S., O'Neill, P., Jenkins, T. and Ahmed, I. (1986) Induction of DNA strand breaks by reduced nitroimidazoles. Biochemical Pharmacology. 35(22):3923-3928.

Sorenson, L.K. and Hanson, H. (2000) Determination of metronidazole and hydroxyl metronidazole in trout by a high-performance liquid chromatographic method. Food Additives and Contaminants. 17(3):197-203.

Standards Council of Canada. (2005) CAN-P-4E (ISO/IEC 17025:2005) General Requirements for the Competence of Testing and Calibration Laboratories.

Stead, S., Richmond, S., Sharman, M., Stark, J., Geijp, E. (2005) A new approach for detection of antimicrobial drugs in food. Premi®Test coupled to scanner technology. Anal. Chim. Acta. 529: 83-88.

Stolker, A.A.M., Rutgers, P., Oosterink, E., Lasaroms, J.J.P., Peters, R.J.B., van Rhijn, J.A. and Nielen, M.W.F. (2008) Comprehensive screening and quantification of veterinary drugs in milk using UPLC-ToF-MS. Anal. Bioanal. Chem. 391:2309-2322.

Stubbings, G., Tarbin, J., Cooper, A., Sharman, M., Bigwood, T. and Robb, P. (2005) A multi-residue cation-exchange clean up procedure for basic drugs in produce of animal origin. Anal. Chim. Acta. 547:262-268.

Sun, H.W., Wang, F.C. and Ai, L.F. (2007) Simultaneous determination of seven nitroimidazole residues in meat by using HPLC-UV detection with solid-phase extraction. J. Chrom. B. 857(2):296-300.

Tamtam, F., Mercier, F., Eurin, J., Chevreuil, M. and le Bot, B. (2009) Ultra performance liquid chromatography tandem mass spectrometry performance evaluation for the analysis of antibiotics in natural waters. Anal. Bioanal. Chem. 393: 1709-1718.

Thompson, C.S., Traynor, I.M., Fodey, T.L. and Crooks, S.R.H. (2008) Improved screening method for the detection of a range of nitroimidazoles in various matrices by optical biosensor. Anal. Chim. Acta. Doi10.1016/jaca 200809.040.

Thompson, M., Ellison, S.L.R. and Wood, R. (2002) Harmonized Guidelines for Singe-Laboratory Validation of Analytical Methods. Pure and Appl. Chem. 74:835-855.

Tojo, J. L. and Santamarina, M.T. (1998) Oral pharmacological treatments for parasitic diseases of rainbow trout *Occorhynchus mykiss*. I. *Heximita salmonis*. Dis Aquat Org. 33:51-56.

United States Department of Agricultural Food Safety and Inspection Service (2005) Screen for nitroimidazoles by HPLC. Office of Public Health Science:1-4.

Voogd, C.E., Van der Stel, J.J. and Jacobs, J.J.J.A.A. (1974) The mutagenic action of nitroimidazoles I. Metronidazole, nimorazole, dimetridazole and ronidazole. Mutation Research. 26:483-490.

Voogd, C.E. (1981) On the mutagenicity of nitroimidazoles. Mutation Research. 86:243-277.

Wang, J.H. (2001) Determination of three nitroimidazole residues in poultry meat by gas chromatography with nitrogen phosphorus detection. J. Chrom. A. 918:435-438.

Wislocki, P.G., Bagan, E.S., Cook, M.M., Bradley, M.O., Wolf, F.J. and Lu, A.Y.H. (1984) Drug residue formation from ronidazole, a 5-nitroimidazole. VI. Lack of mutagenic activity of reduced metabolites and derivatives of ronidazole. Chem. Biol. Interactions. 49:27-38.

World Health Organization (1989) Evaluation of certain veterinary drug residues in food. Technical Report Series 788. 34th Report of the Joint FAO/WHO Expert Committee on Food Additives. pp.8-63.

Xia, X., Li, X., Shen, J., Zhang, S., Ding, S. and Jiang, H. (2006a) Determination of four nitroimidazoles in poultry and swine muscle and eggs by liquid chromatography/tandem mass spectrometry. J. AOAC Inter. 89:94-99.

Xia, X., Li, X., Shen, J., Zhang, S., Ding, S. and Jiang, H. (2006b) Determination of nitroimidazole residues in porcine urine by liquid chromatography/tandem mass spectrometry. J. AOAC Inter. 89(4):1116-1119.

Xia, X., Li, X., Zhang, S., Ding, S., Jiang, H. and Shen, J. (2007) Confirmation of four nitroimidazoles in porcine liver by liquid chromatography-tandem mass spectrometry. Anal. Chim. Acta. 586:394-398.

Xia, X. Li, X., Shang, S., Ding, S., Jiang, H., Li, J., and Shen, J. (2008) Simultaneous determination of 5-nitroimidazoles and nitrofurans in pork by high performance liquid chromatography-tandem mass spectrometry. J. Chrom. A. 1208:101-108.

Xia, X., Li. X., Ding, S., Shang, S., Jiang, H., Li, J. and Shen, J. (2009) Determination of 5-nitroimidazoles and their corresponding hydroxy metabolites in swine kidney by ultra performance liquid chromatography couples to electrospray tandem mass spectrometry. Anal. Chim. Acta. 637: 79-86.

Youden, W.J., & Steiner, E.H (1975) Statistical Manual of the AOAC, AOAC International.

Zhou, J., Shen, J., Xue, X., Zhao, J., Li, Y., Zhan, J. and Zhang, J. (2007) Simultaneous determination of nitroimidazole residues in honey samples by high-performance liquid chromatography with ultraviolet detection. J. AOAC Inter. 90(3):872-878.

Appendices

Appendix 1 Comparison of hexane wash results with acetonitrile extract (analysis 1) and 0.1% acetic acid make up solvent (analysis 2) salmon and shrimp muscle at low, medium and high concentrations for all analytes. Also included is an example of a paired t-test for HMMNI in each commodity.

Tilapia muscle results at low concentration

		Concentration determined (ng/g)						
Analysis	Sample	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	0.98	1.17	1.3	0.98	1 03	1	1.1
	2	1.13	1 17	1.41	1.08	0.98	1.3	1 14
	3	1.08	0.99	1.24	0.9	0 87	1.1	1.18
	4	1.18	1.09	1 43	1.06	1.12	0.98	1.19
	5	1.04	0.94	1.31	1 06	0.85	1.13	1.02
2	1	1 07	1 02	1 31	1 17	0 95	1 24	0 94
	2	0 90	1 03	1 16	1 19	0 89	1 18	1 10
	3	1 14	0 93	1 22	1 07	0 89	1 17	1 05
	4	0 77	0 90	1 26	1 01	1 00	1 17	1 03
	5	0 89	0 92	1 21	1 03	0 86	1 08	1 14
	Mean	1.02	1.02	1.29	1.06	0 94	1.14	1.09
	SD	0.130792	0.0998	0 085797	0.084755	0.088217	0.099582	0.078804
	RSD	12.85%	9.82%	6 68%	8 03%	9.35%	8.77%	7.24%

Tilapia muscle results at medium concentration

		Concentration determined (ng/g)						
Analysis	Sample	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	9.73	9.57	12.09	8.99	8 73	10.08	10.14
	2	11.56	9.44	12.82	9.87	8.89	11.31	9.57
	3	9.49	8.31	11.5	9.4	9.03	10.16	10 28
	4	9.50	9.97	12.32	9.38	9.15	10.77	9.81
	5	11.04	9.33	13.28	9.73	8.49	10.56	9.9
2	1	10 45	8 74	11 79	10 60	9 00	10 59	10 70
	2	9 81	10 26	12 14	10 78	8 95	10 35	10 78
	3	10 69	9 05	11 77	11 52	8 30	11 62	10 34
	4	11 03	10 25	12 31	10 79	9 48	11 24	11 48
	5	11 04	9 62	12 91	10 71	9 52	12 03	11 78
	Mean	10.43	9.45	12.29	10.18	8.95	10 87	10 48
	SD	0.750692	0.631386	0.563088	0.812951	0.385953	0.651638	0.716237
	RSD	7.19%	6.68%	4.58%	7.99%	4.31%	5 99%	6 84%

Tilapia muscle results at high concentration

		Concentration determined (ng/g)						
Analysis	Sample	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	51.96	58.93	8.99	42 32	40 87	49 48	49.41
	2	53.70	61.58	9 87	48.22	43.06	52.35	54.64
	3	51 96	57.56	9 4	52 02	43.31	48.94	52.08
	4	51.53	59.94	9 38	49 2	46.39	53.82	57.65
	5	49 17	48.25	9.73	50.02	43.89	47.47	50.47
2	1	57 66	53 88	10 60	57 40	46 13	54 17	58 45
	2	51 41	53 75	10 78	55 02	48 23	59 37	60 32
	3	59 41	49 36	11 52	54 94	46 99	59 21	59 62
	4	51 39	48 42	10 79	52 62	41 80	49 14	51 03
	5	54 56	50 21	10 71	56 87	45 35	52 32	61 58
	Mean	53 28	54.19	10 18	51 86	44.60	52 63	55.53
	SD	3.146148	5.050133	0.812951	4.601802	2.387215	4 150374	4.537472
	RSD	5 91%	9.32%	7.99%	8.87%	5.35%	7.89%	8 17%

Example of t-test for tilapia muscle HMMNI results:

HMMNI

	Analysis 1	*Analysis 2*	*Analysis 1*	*Analysis 2*	*Analysis 1*	*Analysis 2*
Mean	1.082	0.954	10.264	10.604	51.664	54.886
Variance	0.00602	0.02223	0.93743	0.25828	2.63963	13.14313
Observations	5	5	5	5	5	5
Pearson Correlation	-0.65135		-0.50832		-0.1543	
Hypothesized Mean Difference	0		0		0	
df	4		4		4	
t Stat	1.375152		-0.58379		-1.71731	
P(T<=t) one-tail	0.120539		0.295348		0.080528	
t Critical one-tail	2.131846		2.131846		2.131846	
P(T<=t) two-tail	0.241079		0.590696		0.161057	
t Critical two-tail	2.776451		2.776451		2.776451	

Salmon muscle results at low concentration

		Concentration determined (ng/g)						
Analysis	Sample	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	0.56	1.00	0.77	1.03	0 65	0.59	0.92
	2	0.57	1.14	0.82	0.77	0 55	0.55	0.96
	3	0 52	1.06	0.85	0 72	0 66	0.63	0.91
	4	0.53	1.09	0.78	0.69	0 53	0.52	0.85
	5	0 49	0.98	0.88	0.74	0.52	0.66	0.87
2	1	0 58	0 58	0 57	0 53	0 40	0 56	0 76
	2	0 64	0 75	0 70	0 60	0 57	0 58	0 88
	3	0 57	0 80	0 71	0 67	0 54	0 60	0 86
	4	0 60	0 78	0 77	0 70	0 53	0 57	0 84
	5	0 58	0 87	0 82	0 71	0 57	0 67	0 94
	Mean	0 56	0.91	0.77	0.72	0.55	0 59	0.88
	SD	0.042479	0.178154	0.089449	0 130486	0.072388	0.048086	0 057629
	RSD	7 53%	19 69%	11 66%	18.22%	13 11%	8.11%	6.56%

Salmon muscle results at medium concentration

		Concentration determined (ng/g)						
Analysis	Sample	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	4.32	7.52	6.23	5.32	4.1	5.04	6.21
	2	5 42	9.31	7.46	6.32	5.71	5.76	7.61
	3	5.03	8.78	7.69	6.24	5.54	6.14	7.79
	4	5.03	9.21	7.49	6 05	5 65	5.57	7.64
	5	5.38	9 57	7 29	6.47	5.56	5.54	7.85
2	1	6 64	7 74	7 70	6 61	5 55	6 17	8 70
	2	6 83	8 02	8 08	7 46	5 70	7 04	8 79
	3	6 39	7 44	7 68	6 73	5 62	6 60	7 10
	4	6 65	7 68	7 75	6 72	5 77	6 49	7 80
	5	5 23	7 28	6 98	6 06	5 08	5 82	6 83
	Mean	5.69	8.26	7.44	6.40	5.43	6.02	7.63
	SD	0.865535	0.871528	0.515779	0.559798	0.504002	0.590236	0.783947
	RSD	15.21%	10.56%	6.94%	8.75%	9.29%	9.81%	10.27%

Salmon muscle results at high concentration

		Concentration determined (ng/g)						
Analysis	Sample	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	31.86	48.67	38 37	36 72	34.10	29.84	40.56
	2	32.32	51.18	40.6	39 92	37.38	33.49	42 9
	3	33.05	51.41	41.05	38 02	34.6	30.48	41.42
	4	30.89	49.72	40.24	40.05	36.13	29.82	43.61
	5	38.61	54.78	44 59	52 51	41 3	36.08	45.99
2	1	37 92	40 49	40 50	40 42	34 07	29 92	41 76
	2	29 61	37 35	36 32	35 45	29 16	29 51	37 25
	3	32 29	34 84	33 47	32 40	28 37	28 97	35 36
	4	34 87	41 44	37 92	36 06	30 91	30 44	33 86
	5	34 06	41 76	38 71	37 65	30 95	31 14	38 55
	Mean	33.55	45.16	39.18	38 92	33.70	30.97	40 13
	SD	2.896526	6.795968	2.998066	5 366654	3.981641	2.181892	3.822234
	RSD	8.63%	15.05%	7.65%	13.79%	11 82%	7.05%	9 53%

Example of t-test for salmon muscle HMMNI results:

HMMNI
t-Test: Paired Two Sample for Means

	Analysis 1	*Analysis 2*	*Analysis 1*	*Analysis 2*	*Analysis 1*	*Analysis 2*
Mean	0.534	0.594	5.036	6.348	33.346	33.75
Variance	0.00103	0.00078	0.19463	0.41512	9.27253	9.50265
Observations	5	5	5	5	5	5
Pearson Correlation	0.61919447		-0.36116823		-0.0702	
Hypothesized Mean Difference	0		0		0	
df	4		4		4	
t Stat	-5.0709255		-3.24953623		-0.20153	
P(T<=t) one-tail	0.0035629		0.015694746		0.425058	
t Critical one-tail	2.13184649		2.131846486		2.131846	
P(T<=t) two-tail	0.0071258		0.031389492		0.850117	
t Critical two-tail	2.77645086		2.776450856		2.776451	

Shrimp muscle results at low concentration

		Concentration determined (ng/g)						
Analysis	Sample	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	0 90	0 96	0 92	0 84	0 81	1 33	0 86
	2	0 83	0 92	0 93	0 91	0 71	1 29	0 88
	3	0 85	1 01	0 92	0 92	0 77	1 28	0 91
	4	0 87	0 96	0 91	0 85	0 76	1 23	0 89
	5	0 99	0 97	0 96	0 91	0 68	1 36	0 93
	6	0 62	0 80	0 87	0 81	0 97	0 77	0 74
	7	0 83	0 82	0 86	0 74	0 88	0 76	0 83
	8	0 86	0 83	0 91	0 90	0 77	0 87	0 87
	9	0 80	0 82	0 86	0 77	0 78	0 69	0 88
	10	0 64	0 82	0 86	0 92	0 77	0 68	0 89
	11	0 96	0 86	0 85	0 84	0 99	0 81	0 84
	12	1 04	1 02	1 00	0 98	0 89	0 97	0 98
	13	1 04	0 96	0 96	0 97	0 82	0 91	0 97
	14	1 05	1 03	0 99	0 96	0 83	0 94	1 00
	15	1 04	1 01	0 93	0 99	0 96	0 92	0 96
2	1	0 93	1 01	0 91	0 89	0 93	0 90	0 98
	2	0 97	0 94	0 86	0 90	0 95	0 90	0 96
	3	0 90	0 97	0 93	1 06	0 92	0 99	1 03
	4	0 92	0 95	0 90	1 08	1 09	0 87	0 92
	5	0 97	0 91	0 97	1 01	0 92	0 81	1 01
	6	0 94	0 58	0 83	0 95	0 67	0 87	0 81
	7	1 03	0 74	0 96	1 04	0 68	1 05	0 92
	8	0 95	0 98	0 96	0 95	1 09	0 89	0 93
	9	0 98	0 83	0 99	1 10	0 92	1 09	0 95
	10	0 86	0 83	0 91	1 06	0 97	1 01	0 86
	11	0 91	0 94	0 83	0 95	0 78	0 97	0 80
	12	0 81	0 97	0 89	0 87	0 82	0 83	0 81
	13	0 86	0 96	0 84	0 82	0 84	0 89	0 77
	14	0 85	1 00	0 89	0 86	0 92	0 89	0 82
	15	0 79	0 96	0 88	0 93	0 87	0 84	0 84
	Mean	0 90	0 91	0 91	0 93	0 86	0 95	0 89
	SD	0 11	0 10	0 05	0 09	0 11	0 18	0 07
	RSD	11 83%	11 00%	5 43%	9 76%	13 03%	19 11%	8 32%

Shrimp muscle results at medium concentration

		Concentration determined (ng/g)						
Analysis	Sample	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	7 88	8 12	8 12	7.98	7.53	11.49	8.07
	2	8.19	8 66	8.66	8.46	7 96	12.45	8.52
	3	9 21	9.13	9.13	9.03	8.20	13.62	9 51
	4	8.61	9.11	9.11	8.81	8.05	12.89	9 13
	5	8.51	8.75	8.75	9.04	7.96	12.41	8.61
	6	7.69	8.18	8.18	7 80	8 07	7.97	7.51
	7	7.89	8.16	8.16	7 92	8.05	7.91	8 19
	8	8.38	8.17	8.17	8.04	7.73	7.40	7 71
	9	7 44	8.41	8 41	7.08	8.51	7 89	8.11
	10	8.80	8.20	8.20	8.34	8.27	7.53	7.22
	11	10 10	9 37	9.37	9.06	9.06	9 30	9.68
	12	10.22	9 56	9.56	9.19	9.77	9.80	9.90
	13	9.52	9.57	9.57	9.48	10 84	9.52	9.17
	14	9 93	9.63	9 63	9.42	10.47	9.90	10.33
	15	9 60	9.37	9.37	8.95	10 49	10.16	9 64
2	1	9 02	9 3	8 51	8 94	9 43	8 09	8 59
	2	8 9	9 25	8 94	8 73	8 88	9 04	9 48
	3	9 38	9 88	9 35	9 21	10 13	8 80	9 54
	4	9 31	8 46	9 11	9 67	9 78	9 10	9 36
	5	8 81	9 64	9 45	9 22	9 90	8 85	9 86
	6	8 83	7 09	8 90	9 28	9 39	9 48	8 93
	7	9 52	7 37	9 18	10 61	9 69	10 02	9 10
	8	9 58	7 11	9 64	9 97	9 53	10 41	9 42
	9	9 96	6 42	8 94	10 28	10 50	10 10	8 66
	10	8 25	5 89	8 41	9 79	9 27	9 52	8 25
	11	8 04	7 99	7 45	7 95	7 99	7 98	7 14
	12	7 89	8 62	7 97	7 70	8 51	8 69	7 33
	13	8 97	9 63	8 70	8 25	8 42	9 00	8 35
	14	8 58	8 94	8 53	8 67	8 76	9 26	8 05
	15	8 57	8 48	7 88	8 39	8 24	8 93	8 12
	Mean	8.85	8.55	8.78	8.84	8.98	9 58	8.72
	SD	0.76	1.00	0 60	0.81	0 96	1.60	0.87
	RSD	8.55%	11.68%	6.78%	9.17%	10 69%	16.69%	9 96%

Shrimp muscle results at high concentration

		Concentration determined (ng/g)						
Analysis	Sample	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	41 69	47 95	42 77	41 58	39 64	58 46	43 48
	2	38 73	45 20	40 25	39 00	38 65	57 16	41 43
	3	43 38	48 66	43 03	42 35	41 86	58 84	45 95
	4	42 14	46 22	42 58	42 73	41 88	59 57	46 19
	5	42 75	46 96	42 14	42 39	42 14	59 29	45 01
	6	39 32	41 12	38 17	44 58	37 44	34 13	37 46
	7	40 41	46 42	40 41	48 28	39 05	38 10	41 02
	8	41 58	44 93	42 47	51 08	42 82	38 74	45 1
	9	43 46	42 31	40 6	50 22	42 26	40 67	44 86
	10	42 89	46 33	44 5	52 96	45 07	38 64	44 56
	11	42 97	42 90	40 56	38 12	43 23	40 40	41 67
	12	47 41	50 61	45 6	42 45	47 76	43 73	47 12
	13	47 57	48 88	46 52	41 04	48 28	45 60	49 07
	14	46 63	45 59	45 03	41 26	47 93	43 41	48 24
	15	46 46	47 74	45 37	40 75	48 37	41 52	46 82
2	1	42 28	45 56	43 10	40 75	41 94	37 88	43 97
	2	42 63	40 30	41 43	42 38	46 47	36 83	43 84
	3	45 30	48 64	45 33	44 17	47 89	40 87	50 74
	4	49 25	50 74	47 49	46 37	49 66	41 12	48 51
	5	47 57	53 15	45 63	48 19	48 30	41 43	51 01
	6	40 82	37 21	42 85	45 47	45 86	46 18	44 44
	7	39 25	26 67	35 45	41 38	40 12	38 44	35 57
	8	43 07	39 45	41 6	45 36	44 6	41 86	44 76
	9	45 54	38 65	44 1	48 76	48 23	46 3	46 63
	10	43 28	42 51	44	46 18	46 48	43 82	46 13
	11	42 64	47 88	39 93	40 93	38 12	39 95	39 65
	12	40 18	43 61	39 28	37 31	39 86	39 37	37 49
	13	42 97	48 07	42 56	42 81	42 23	42 38	43 38
	14	46 05	49 33	42 15	44 22	42 89	42 68	44 29
	15	42 39	47 96	40 52	43 71	43 04	40 48	40 22
	Mean	43 35	45 05	42 51	43 89	43 74	43 93	44 29
	SD	2 72	5 15	2 63	3 80	3 59	7 23	3 76
	RSD	6 27%	11 43%	6 19%	8 66%	8 22%	16 45%	8 48%

Example of t-test for shrimp muscle HMMNI results:

HMMNI
t-Test: Paired Two Sample for Means

	Analysis 1	*Analysis 2*	*Analysis 1*	*Analysis 2*	*Analysis 1*	*Analysis 2*
Mean	0.983333	1.011333	9.78333	9.908	43.15933	43.548
Variance	0.016124	0.005727	1.18203	0.708331	7.749121	7.46396
Observations	15	15	15	15	15	15
Pearson Correlation	0.67074		0.55216		0.100884	
Hypothesized Mean Difference	0		0		0	
df	14		14		14	
t Stat	-1.14564		-0.51473		-0.40701	
P(T<=t) one-tail	0.135575		0.30738		0.345079	
t Critical one-tail	1.761309		1.761309		1.761309	
P(T<=t) two-tail	0.271149		0.61476		0.690157	
t Critical two-tail	2.144789		2.144789		2.144789	

Appendix 2 Experimental results and calculations for ruggedness testing using tilapia muscle. An example of the full calculations including a t-test is given for HMMNI. For all other analytes (IPZ, IPZ-OH, MNZ, MNZ-OH, RNZ and DMZ) only experimental data results are shown.

HMMNI (ng/g)

EXPERIMENTAL RESULTS

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8
	s	t	u	v	w	x	y	z
1st rep	5.96	5.7	4.96	5.68	6.71	6.86	6.16	6.56
2nd rep	5.76	5.8	6.56	6	6.8	6.06	6.55	6.28
Mean	5.86	5 75	5.76	5 84	6.755	6.46	6.355	6.42
SD	0.141421356	0.070711	1.13137085	0.22627417	0.06363961	0.56568542	0.275771645	0.19799
%RSD	2.41%	1.23%	19.64%	3.87%	0.94%	8.76%	4.34%	3.08%

DIFFERENCES

Effect of A and a = [(s+t+u+v)/4] - [(w+x+y+z)/4] = (4A/4) - (4a/4) = J

FACTORS	ORIGINAL		ALTERNATE		DIFFERENCE	
A and a	(s+t+u+v)/4	5.80	(w+x+y+z)/4	6 50	J	0.70
B and b	(s+t+w+x)/4	6.21	(u+v+y+z)/4	6 09	K	-0.11
C and c	(s+u+w+y)/4	6.18	(t+v+x+z)/4	6 12	L	-0.07
D and d	(s+t+y+z)/4	6.10	(u+v+w+x)/4	6.20	M	0.11
E and e	(s+u+x+z)/4	6.13	(t+v+w+y)/4	6.18	N	0.05
F and f	(s+v+w+z)/4	6.22	(t+u+x+y)/4	6.08	O	-0.14
G and g	(s+v+x+y)/4	6.13	(t+u+w+z)/4	6.17	P	0.04
					Mean	**0.08**
					SD	**0.28**

USING tilapia tissue WITH 5 ng/g OF HMMNI

T-Tests

DATA (ng/g)

A	B	C	D	E	F	G
5.96	5.96	5.96	5.96	5.96	5.96	5.96
5.76	5.76	5.76	5.76	5.76	5.76	5.76
5.7	5.7	4.96	5.7	4.96	5.68	5.68
5.8	5.8	6.56	5.8	6.56	6	6
4.96	6.71	6.71	6.16	6.86	6.71	6.86
6.56	6.8	6.8	6.55	6.06	6.8	6.06
5.68	6.86	6.16	6.56	6.56	6.56	6.16
6	6.06	6.55	6.28	6.28	6.28	6.55

a	b	c	d	e	f	g
6.71	4.96	5.7	4.96	5.7	5.7	5.7
6.8	6.56	5.8	6.56	5.8	5.8	5.8
6.86	5.68	5.68	5.68	5.68	4.96	4.96
6.06	6	6	6	6	6.56	6.56
6.16	6.16	6.86	6.71	6.71	6.86	6.71
6.55	6.55	6.06	6.8	6.8	6.06	6.8
6.56	6.56	6.56	6.86	6.16	6.16	6.56
6.28	6.28	6.28	6.06	6.55	6.55	6.28

Two sample t-tests for ruggedness:

HMMNI

t-Test: Two-Sample Assuming Equal Variances

	A	*a*	*B*	*b*	*C*	*c*	*D*	*d*
Mean	5.8025	6.4975	6.20625	6.09375	6.1825	6.1175	6.09625	6.20375
Variance	0.196392857	0.089621	0.248026786	0.30676964	0.380021429	0.17959286	0.1193125	0.436112
Observations	8	8	8	8	8	8	8	8
Pooled Variance	0.143007143		0.277398214		0.279807143		0.2777125	
Hypothesized Mean Difference	0		0		0		0	
df	14		14		14		14	
t Stat	-3.675665104		0.427199453		0.245761559		-0.407981759	
P(T<=t) one-tail	0.00124735		0.33786563		0.404716571		0.344728983	
t Critical one-tail	1.76130925		1.76130925		1.76130925		1.76130925	
P(T<=t) two-tail	0.0024947		0.675731261		0.809433141		0.689457967	
t Critical two-tail	2.144788596		2.144788596		2.144788596		2.144788596	

HMMNI
t-Test: Two-Sample Assuming Equal Variances

	E	*e*	*F*	*f*	*G*	*g*
Mean	6.125	6 175	6.21875	6.13571429	6.12875	6.17125
Variance	0.352085714	0.208514	0 187841071	0.3962619	0.157183929	0.4038125
Observations	8	8	8	7	8	8
Pooled Variance	0.2803		0.284035302		0.280498214	
Hypothesized Mean Difference	0		0		0	
df	14		13		14	
t Stat	-0.188881077		0 301042041		-0.16049218	
P(T<=t) one-tail	0.42644839		0 38407169		0 437393067	
t Critical one-tail	1 76130925		1.770931704		1.76130925	
P(T<=t) two-tail	0.852896781		0.76814338		0.874786135	
t Critical two-tail	2.144788596		2.16036824		2.144788596	

IPZ (ng/g)

EXPERIMENTAL RESULTS

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8
	s	t	u	v	w	x	y	z
1st rep	6.04	5.8	5.14	4.49	5.31	4.54	5.73	5.04
2nd rep	5.47	6.39	5.17	3.99	4.89	3.77	4.59	4.47
Mean	5.755	6.095	5.155	4 24	5.1	4.155	5.16	4.755
SD	0.403050865	0 417193	0.021213203	0 35355339	0.296984848	0.54447222	0.806101731	0.403051
%RSD	7.00%	6.84%	0.41%	8.34%	5.82%	13.10%	15.62%	8.48%

IPZ-OH (ng/g)

EXPERIMENTAL RESULTS

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8
	s	t	u	v	w	x	y	z
1st rep	6.17	6.08	5.69	6.4	6.18	6.26	5.92	5.41
2nd rep	5.74	5.73	5.82	5.23	5.14	5.08	5.52	5.27
Mean	5.955	5.905	5.755	5.815	5.66	5 67	5.72	5.34
SD	0.304055916	0.247487	0.091923882	0.82731493	0.735391052	0.834386	0.2828427	0.098995
%RSD	5.11%	4.19%	1.60%	14.23%	12.99%	14.72%	4.94%	1.85%

MNZ (ng/g)

EXPERIMENTAL RESULTS

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8
	s	t	u	v	w	x	y	z
1st rep	6.11	5.16	5.23	5.86	6.03	5.65	5.6	5.6
2nd rep	5.54	5.41	4.85	5.06	5.81	6.13	5.94	5.05
Mean	5.825	5.285	5.04	5.46	5.92	5.89	5.77	5.325
SD	0.403050865	0.176777	0.268700577	0.56568542	0.155563492	0.33941125	0.2404163	0.388909
%RSD	6.92%	3.34%	5.33%	10.36%	2.63%	5.76%	4.17%	7.30%

MNZ-OH (ng/g)

EXPERIMENTAL RESULTS

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8
	s	t	u	v	w	x	y	z
1st rep	5.62	5.29	5.92	6.36	5.5	6.55	5.77	6.25
2nd rep	5 94	5.7	5.95	5.35	5.38	5.95	5.08	5.07
Mean	5.78	5 495	5.935	5.855	5.44	6.25	5.425	5.66
SD	0.226274	0.289914	0.021213203	0.714178	0.084852814	0.424264	0.487903679	0.834386
%RSD	3.91%	5.28%	0.36%	12.20%	1.56%	6.79%	8.99%	14.74%

RNZ (ng/g)

EXPERIMENTAL RESULTS

	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8
	s	t	u	v	w	x	y	z
1st rep	5.21	5.51	5.05	5.69	4.95	6.07	6.93	5.8
2nd rep	5.15	5.24	5.52	4.83	4.84	5.31	7.32	5.31
Mean	5.18	5.375	5.285	5 26	4 895	5.69	7.125	5.555
SD	0.042426407	0.190919	0.332340187	0.608112	0.077781746	0.537401	0.275771645	0.346482
%RSD	0.82%	3.55%	6.29%	11.56%	1.59%	9.44%	3.87%	6.24%

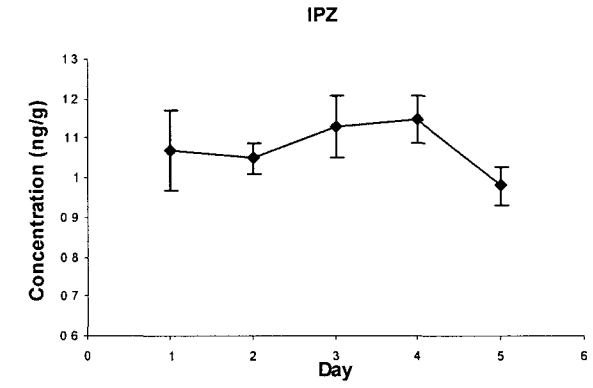
DMZ (ng/g)

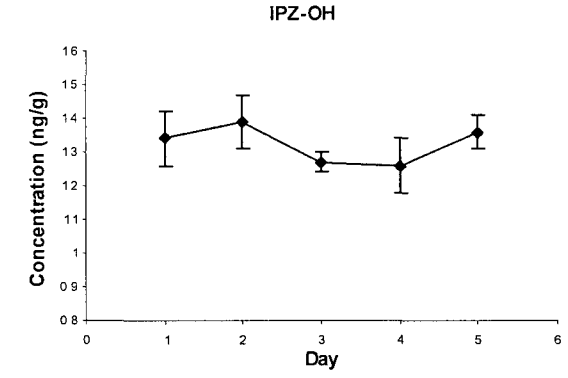
EXPERIMENTAL RESULTS

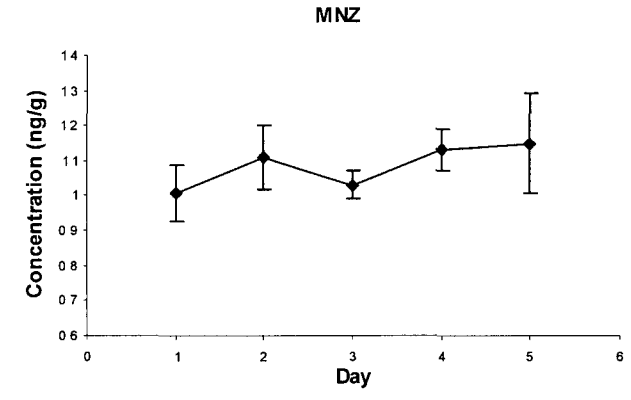
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8
	s	t	u	v	w	x	y	z
1st rep	6.66	5.74	5.59	5.02	6.2	4.81	7.49	4.95
2nd rep	5.9	6.44	5.88	5.05	5.82	5.06	5.23	4.87
Mean	6 28	6.09	5.735	5.035	6.01	4.935	6.36	4.91
SD	0.537401154	0.494975	0.205060967	0.021213	0.268700577	0.176777	1.598061325	0.056569
%RSD	8.56%	8.13%	3.58%	0.42%	4.47%	3 58%	25.13%	1.15%

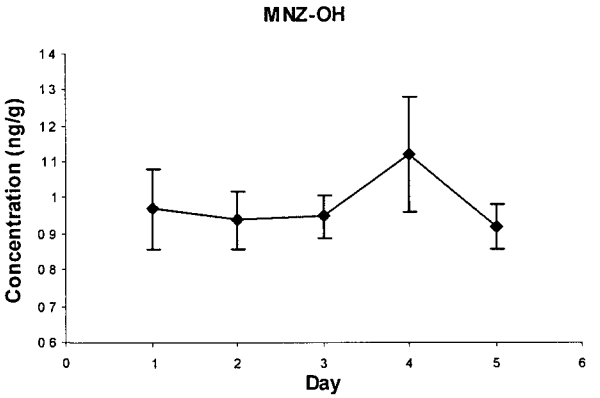
Appendix 3 Error bar charts representing tilapia muscle extract stability of NI analytes over 5 days at 1ng/g (a), 10 ng/g (b) and 50 ng/g (c).

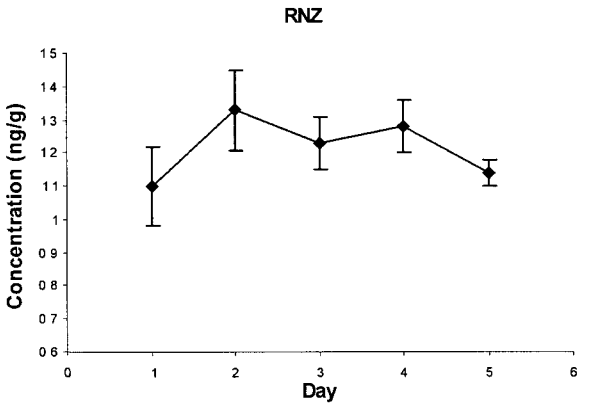
(a)

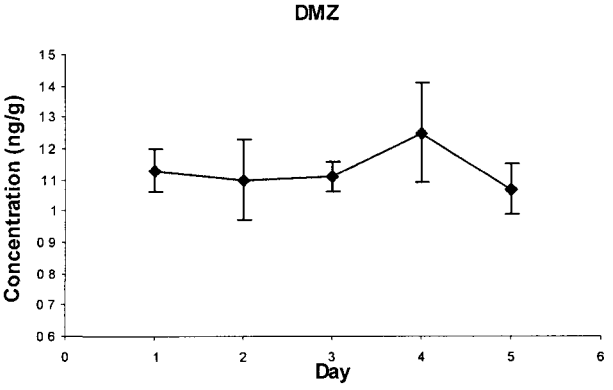




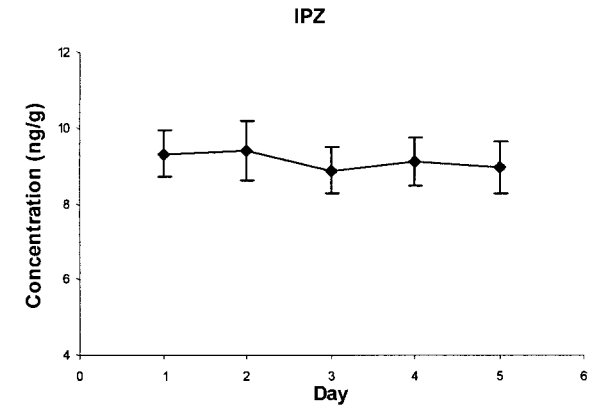


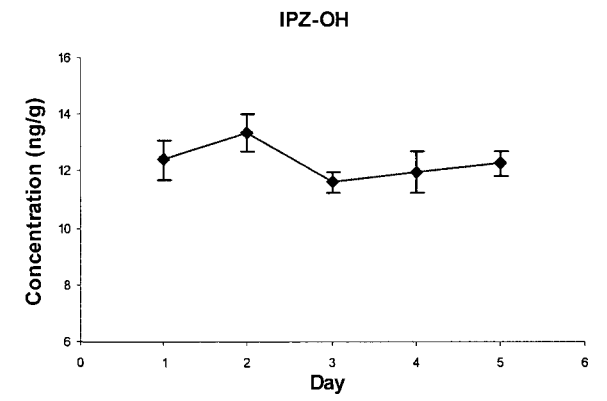

MNZ-OH
Concentration (ng/g)
Day

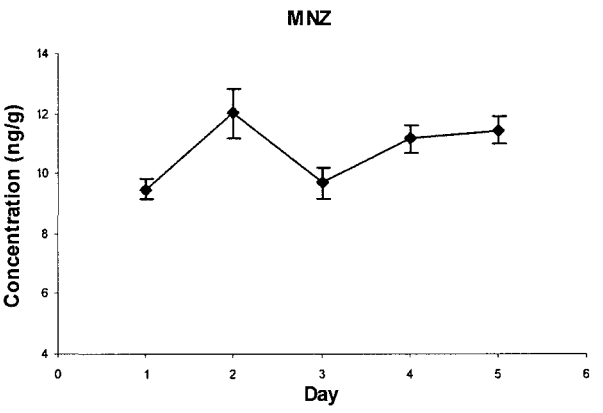

RNZ
Concentration (ng/g)
Day

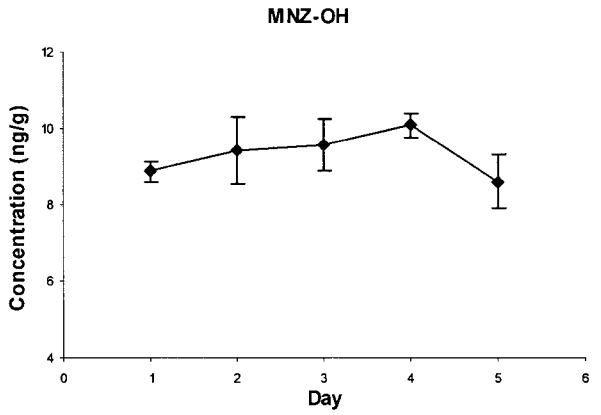

DMZ
Concentration (ng/g)
Day

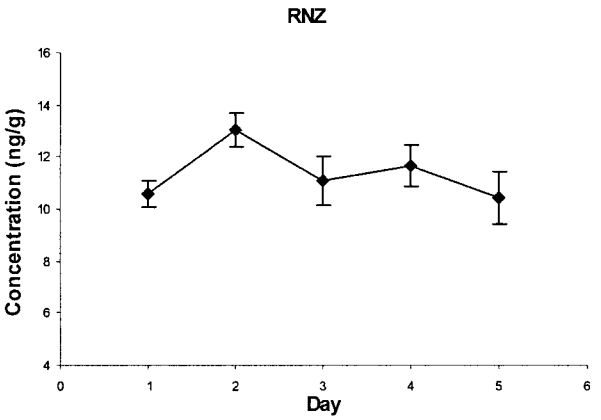
(b)

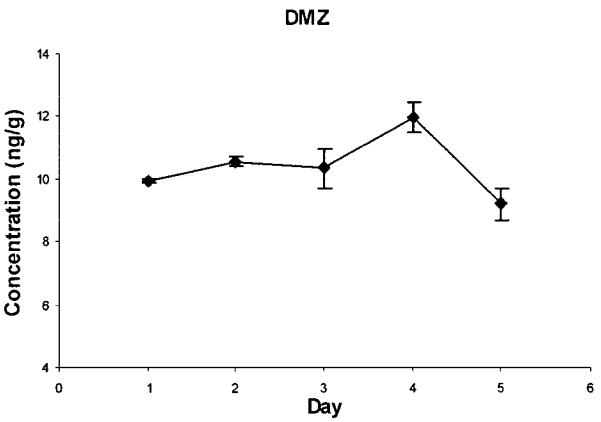

IPZ
Concentration (ng/g)
12
10
8
6
4
0
1
2
3
4
5
6
Day


IPZ-OH
Concentration (ng/g)
16
14
12
10
8
6
0
1
2
3
4
5
6
Day

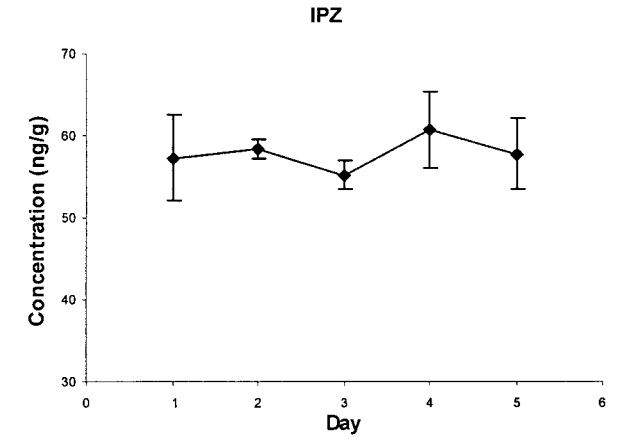

MNZ
Concentration (ng/g)
14
12
10
8
6
4
0
1
2
3
4
5
6
Day

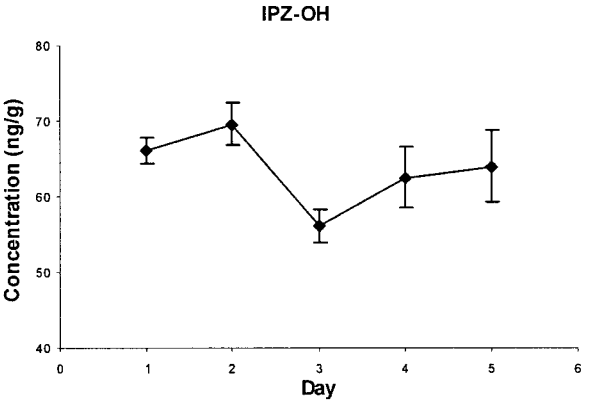

MNZ-OH
Concentration (ng/g)
Day

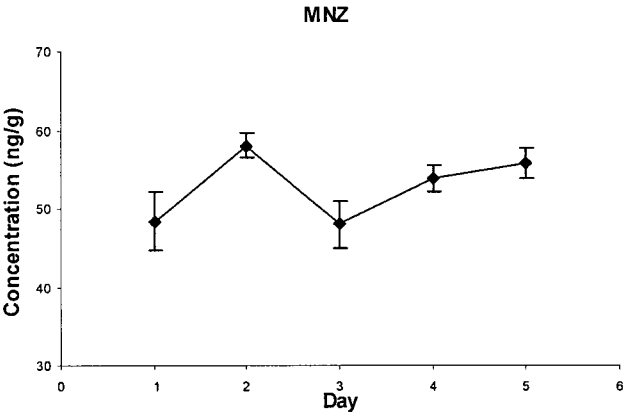

RNZ
Concentration (ng/g)
Day

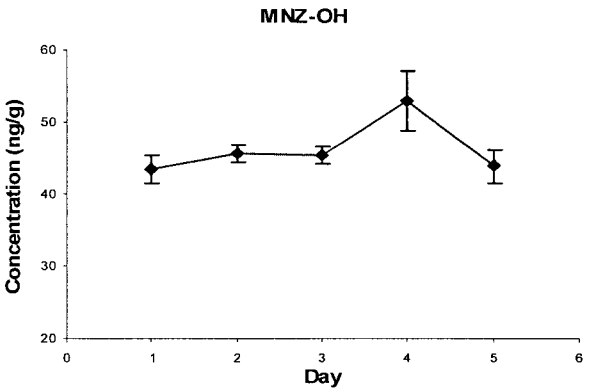

DMZ
Concentration (ng/g)
Day

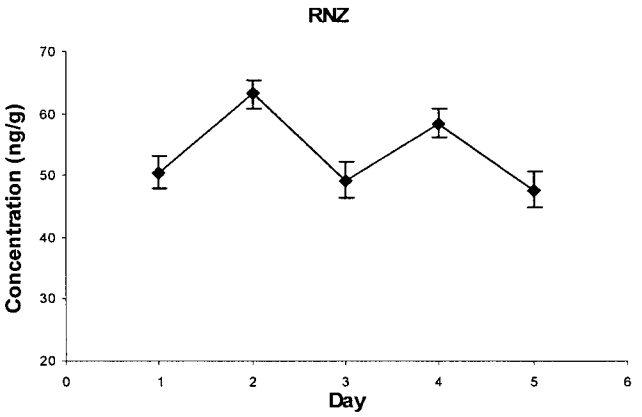
(c)

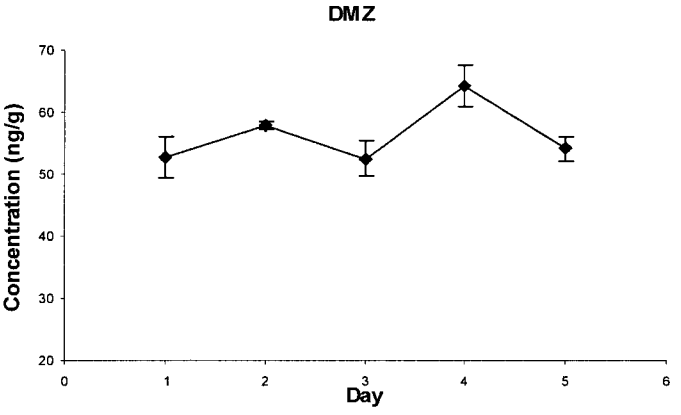

IPZ
Concentration (ng/g)
70
60
50
40
30
0
1
2
3
4
5
6
Day


IPZ-OH
Concentration (ng/g)
80
70
60
50
40
0
1
2
3
4
5
6
Day


MNZ
Concentration (ng/g)
70
60
50
40
30
0
1
2
3
4
5
6
Day

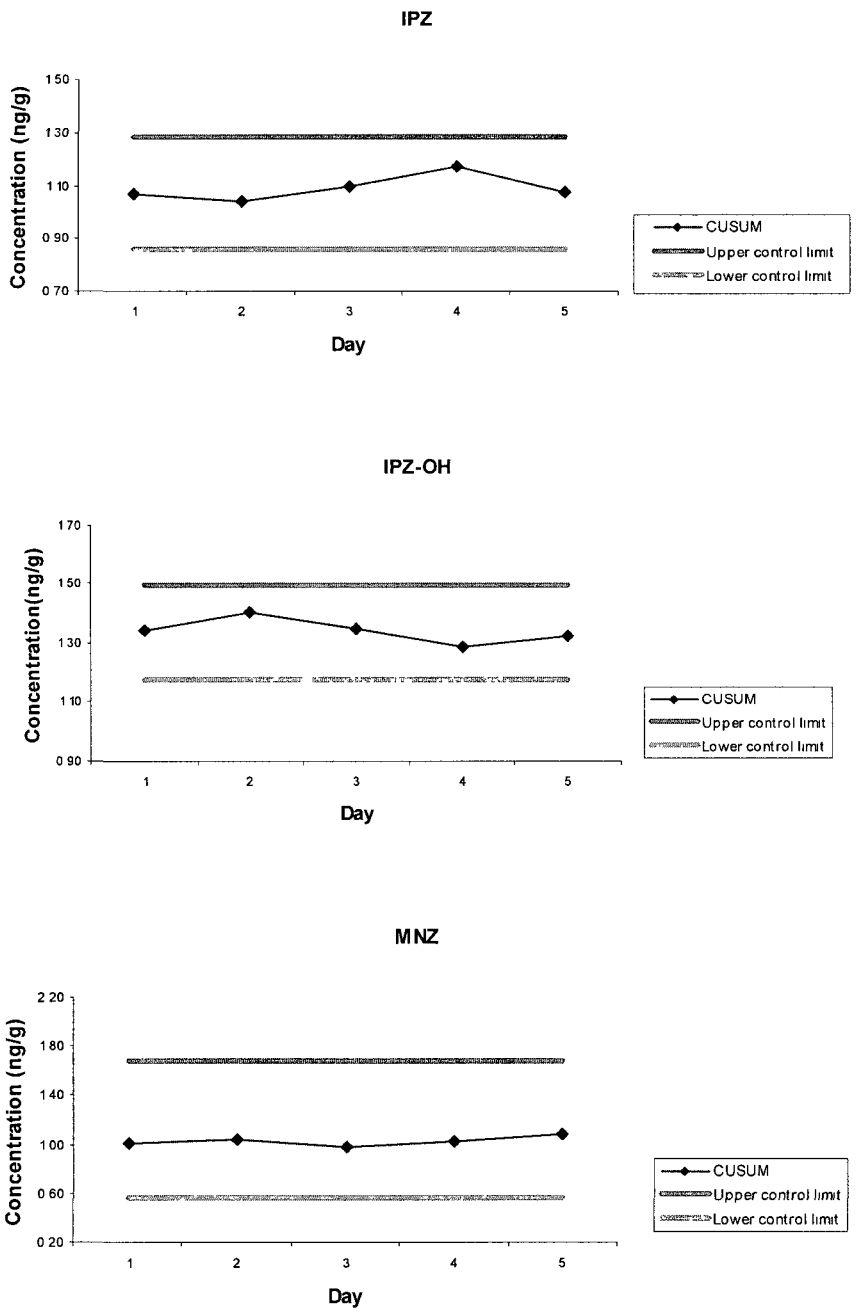

MNZ-OH
Concentration (ng/g)
60
50
40
30
20
0
1
2
3
4
5
6
Day

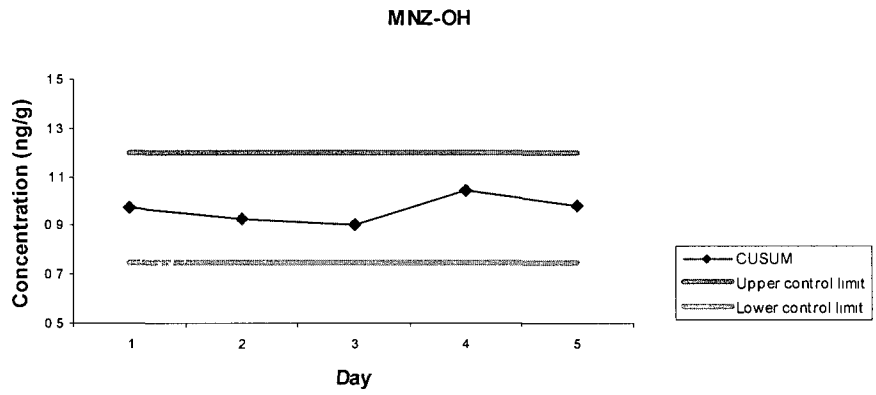

RNZ
Concentration (ng/g)
70
60
50
40
30
20
0
1
2
3
4
5
6
Day

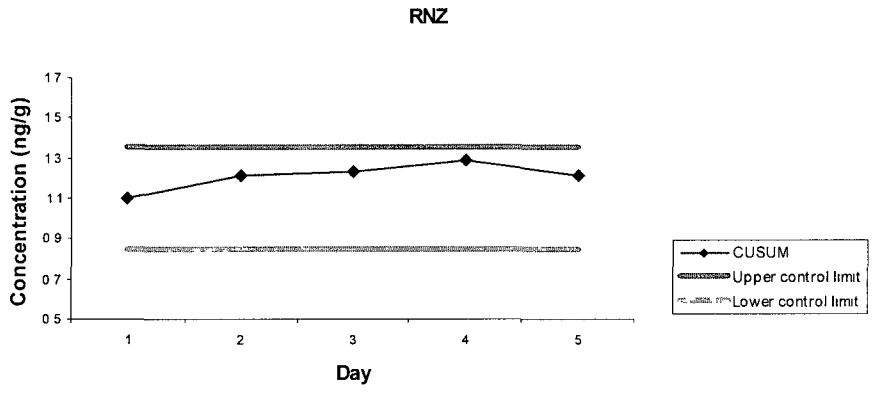

DMZ
Concentration (ng/g)
70
60
50
40
30
20
0
1
2
3
4
5
6
Day

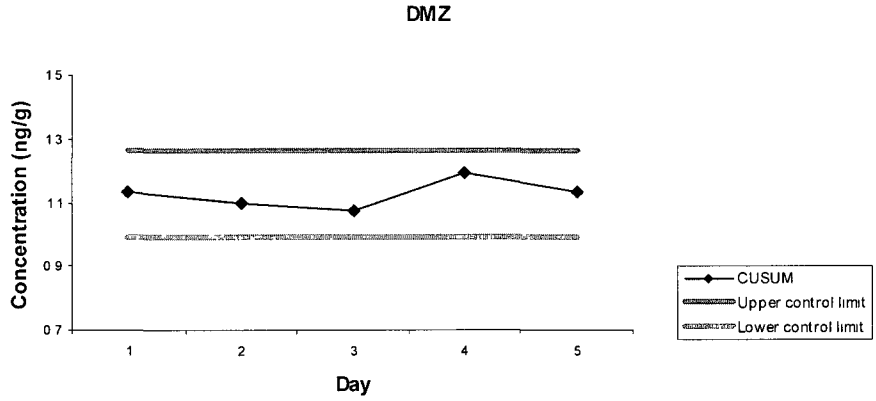
Appendix 4 CUSUM charts representing stability of NI analytes in tilapia muscle extract over 5 days at 1ng/g (a), 10 ng/g (b) and 50 ng/g (c).

(a)

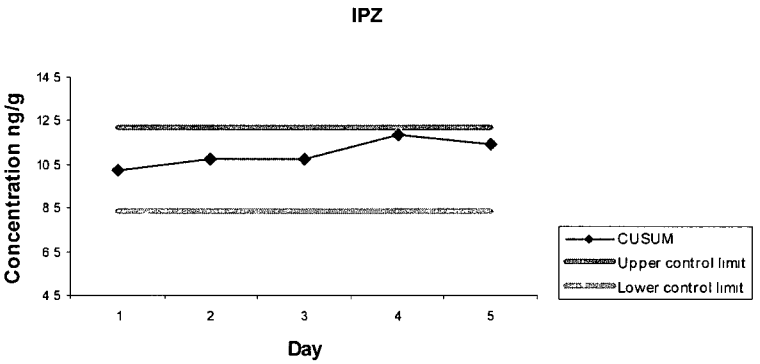


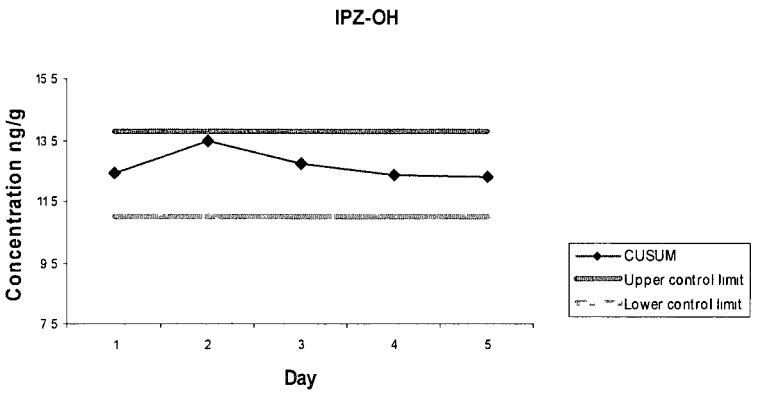

MNZ-OH
Concentration (ng/g)
15
13
11
09
07
05
1
2
3
4
5
Day
CUSUM
Upper control limit
Lower control limit

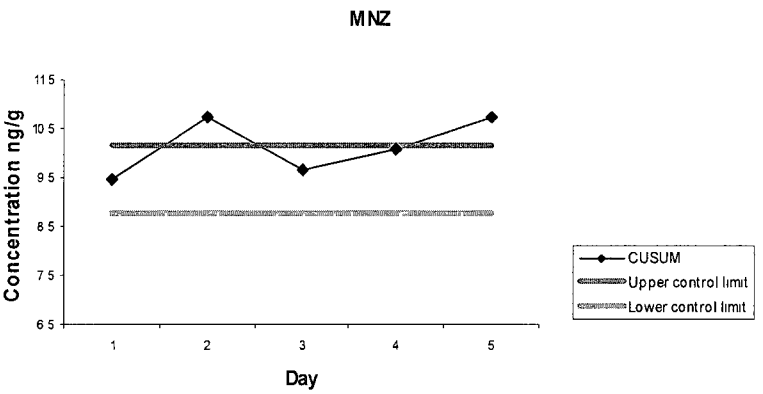

RNZ
Concentration (ng/g)
17
15
13
11
09
07
05
1
2
3
4
5
Day
CUSUM
Upper control limit
Lower control limit

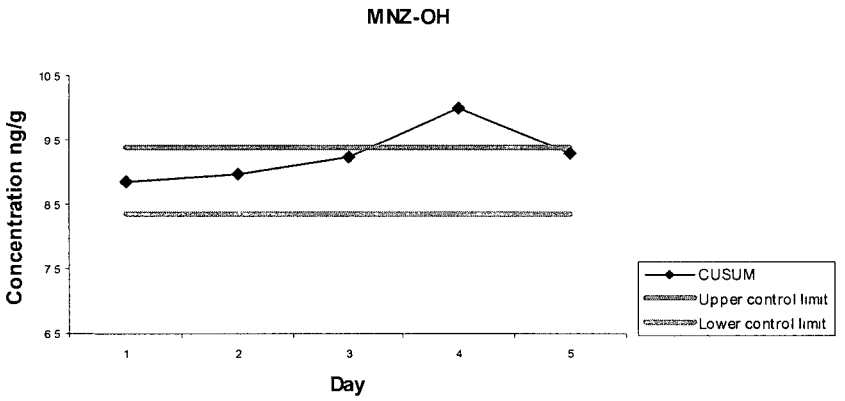

DMZ
Concentration (ng/g)
15
13
11
09
07
1
2
3
4
5
Day
CUSUM
Upper control limit
Lower control limit

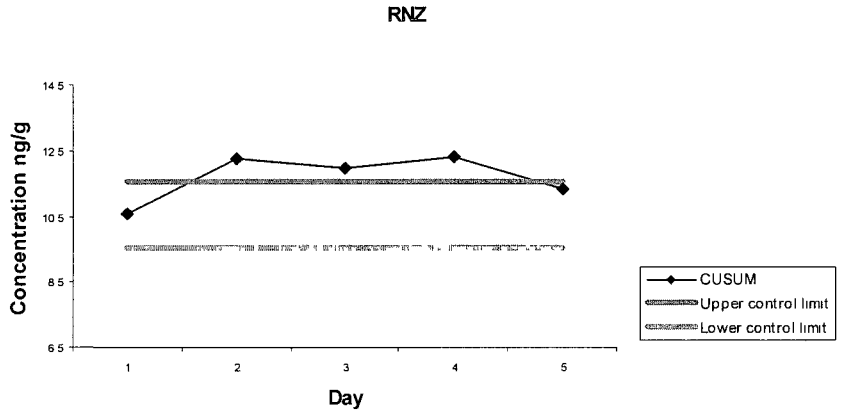
(b)

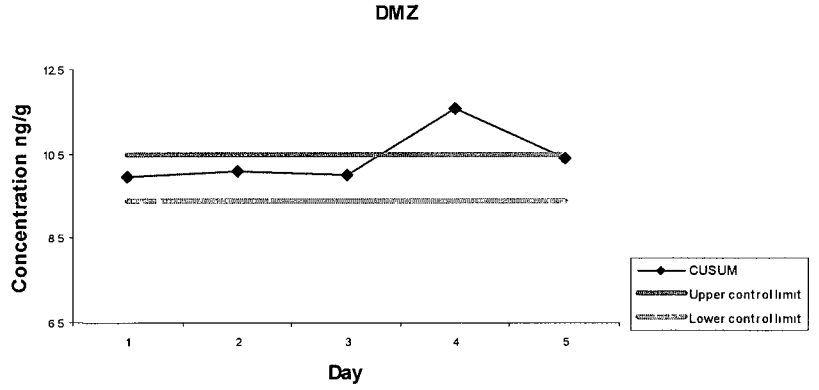

IPZ
Concentration ng/g
14 5
12 5
10 5
8 5
6 5
4 5
1
2
3
4
5
Day
CUSUM
Upper control limit
Lower control limit


IPZ-OH
Concentration ng/g
15 5
13 5
11 5
9 5
7 5
1
2
3
4
5
Day
CUSUM
Upper control limit
Lower control limit

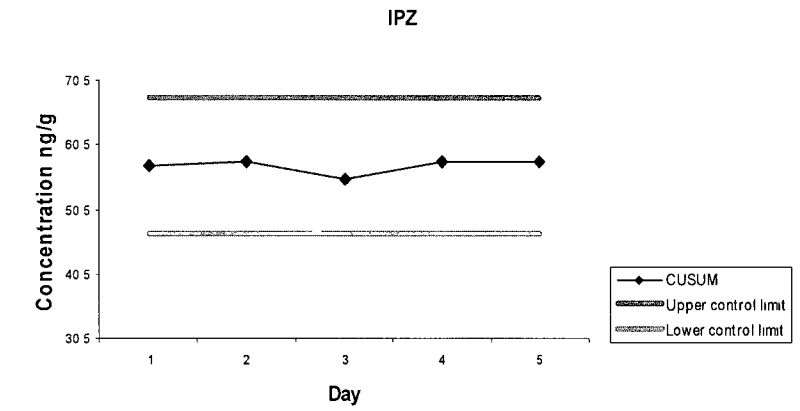

MNZ
Concentration ng/g
11 5
10 5
9 5
8 5
7 5
6 5
1
2
3
4
5
Day
CUSUM
Upper control limit
Lower control limit

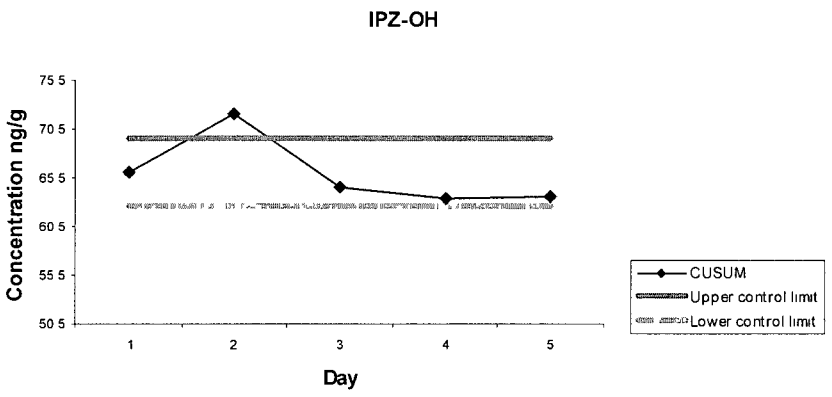

MNZ-OH
Concentration ng/g
10.5
9.5
8.5
7.5
6.5
1
2
3
4
5
Day
CUSUM
Upper control limit
Lower control limit

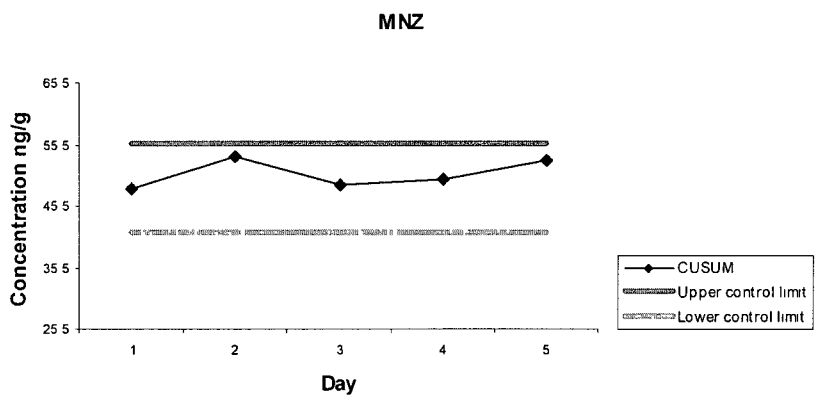

RNZ
Concentration ng/g
14.5
12.5
10.5
8.5
6.5
1
2
3
4
5
Day
CUSUM
Upper control limit
Lower control limit

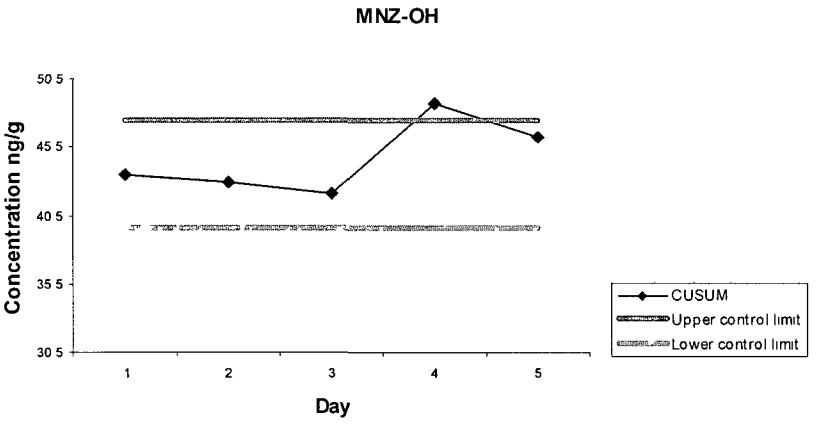

DMZ
Concentration ng/g
12.5
10.5
8.5
6.5
1
2
3
4
5
Day
CUSUM
Upper control limit
Lower control limit

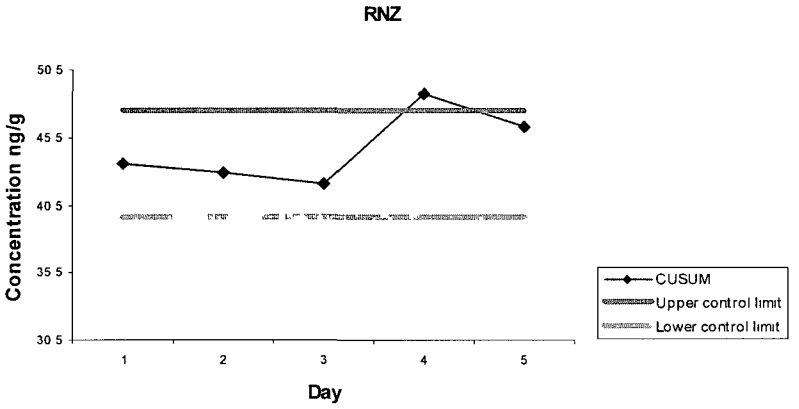
(c)

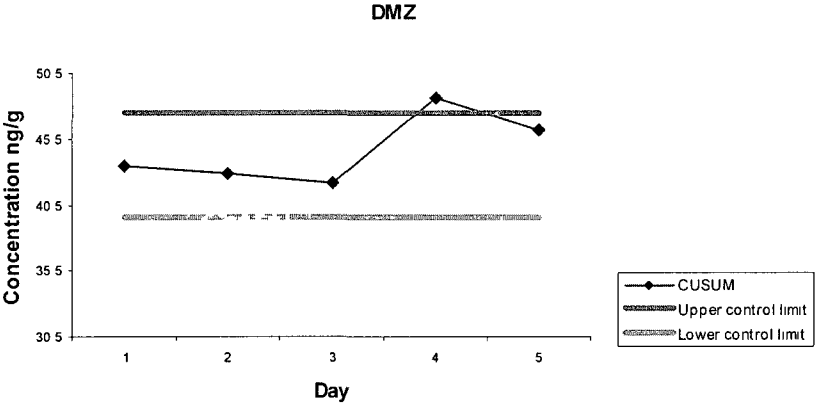

IPZ
70 5
60 5
50 5
40 5
30 5
Concentration ng/g
1
2
3
4
5
Day
CUSUM
Upper control limit
Lower control limit


IPZ-OH
75 5
70 5
65 5
60 5
55 5
50 5
Concentration ng/g
1
2
3
4
5
Day
CUSUM
Upper control limit
Lower control limit


MNZ
65 5
55 5
45 5
35 5
25 5
Concentration ng/g
1
2
3
4
5
Day
CUSUM
Upper control limit
Lower control limit


MNZ-OH
Concentration ng/g
50 5
45 5
40 5
35 5
30 5
1
2
3
4
5
Day
CUSUM
Upper control limit
Lower control limit


RNZ
Concentration ng/g
50 5
45 5
40 5
35 5
30 5
1
2
3
4
5
Day
CUSUM
Upper control limit
Lower control limit


DMZ
Concentration ng/g
50 5
45 5
40 5
35 5
30 5
1
2
3
4
5
Day
CUSUM
Upper control limit
Lower control limit

Appendix 5 Data from tilapia muscle extract stability (low, medium and high concentrations) with examples of a t-test and ANOVA for HMMNI at low concentration.

Low concentration results

		Concentration determined (ng/g)						
Day	Sample	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	0 98	1 17	1 30	0 98	1 03	1 00	1 10
	2	1 13	1 17	1 41	1 08	0 98	1 30	1 14
	3	1 08	0 99	1 24	0 9	0 87	1 10	1 18
	4	1 18	1 09	1 43	1 6	1 12	0 98	1 19
	5	1 04	0 94	1 31	1 06	0 85	1 13	1 02
2	1	1 12	1 04	1 29	1 05	0 82	1 19	0 99
	2	1 23	1 11	1 45	1 18	0 93	1 28	1 32
	3	1 13	1 06	1 33	1 15	1 00	1 36	1 07
	4	1 02	1 05	1 47	1 19	1 02	1 51	1 11
	5	1 29	1 00	1 43	0 98	0 91	1 33	1 02
3	1	1 30	1 14	1 29	1 04	0 98	1 13	1 13
	2	1 26	1 18	1 29	1 07	0 94	1 26	1 14
	3	1 30	1 09	1 25	0 99	0 84	1 21	1 10
	4	1 34	1 21	1 28	1 07	0 99	1 34	1 15
	5	1 41	1 02	1 23	1 00	0 98	1 21	1 02
4	1	1 30	1 14	1 26	1 19	1 12	1 22	1 28
	2	1 23	1 23	1 35	1 16	0 88	1 21	1 39
	3	1 02	1 07	1 13	1 05	1 13	1 30	1 06
	4	1 39	1 18	1 31	1 16	1 33	1 40	1 43
	5	1 10	1 11	1 24	1 07	1 15	1 26	1 11
5	1	1 02	0 96	1 34	1 04	0 89	1 15	1 16
	2	1 05	0 98	1 44	1 3	0 89	1 07	1 09
	3	1 15	1 03	1 33	1 01	0 97	1 15	0 97
	4	1 33	1 04	1 37	1 29	0 98	1 18	1 13
	5	1 13	0 91	1 32	1 09	0 85	1 16	1 00
	Mean	1 18	1 08	1 32	1 11	0 98	1 22	1 13
	SD	0 13	0 09	0 08	0 14	0 12	0 12	0 12
	RSD	10 62%	7 94%	6 05%	12 41%	11 99%	9 82%	10 22%

HMMNI

t-Test: Paired Two Sample for Means

	day 1	*day 2*	*day 1*	*day 3*	*day 1*	*day 4*	*day 1*	*day 5*
Mean	1.082	1.158	1.082	1.322	1.082	1.208	1.082	1.136
Variance	0.00602	0.01097	0.00602	0.00322	0.00602	0.02227	0.00602	0.01468
Observations	5	5	5	5	5	5	5	5
	-		-					
Pearson Correlation	0.359319918		0.148769916		0.311348598		0.716434229	
Hypothesized Mean Difference	0		0		0		0	
df	4		4		4		4	
	-				-			
t Stat	1.124723162		-5.22480413		1.940529476		-1.42006954	
P(T<=t) one-tail	0.161815609		0.003203194		0.06215086		0.114305666	
t Critical one-tail	2.131846486		2.131846486		2.131846486		2.131846486	
P(T<=t) two-tail	0.323631219		0.006406389		0.12430172		0.228611333	
t Critical two-tail	2.776450856		2.776450856		2.776450856		2.776450856	

HMMNI

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
day 1	5	5.41	1.082	0.00602
day 2	5	5.79	1.158	0.01097
day 3	5	6.61	1.322	0.00322
day 4	5	6.04	1.208	0.02227
day 5	5	5.68	1.136	0.01468

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.164824	4	0.041206	3.604444	0.022780322	2.866081
Within Groups	0.22864	20	0.011432			
Total	0.393464	24				

Medium concentration results

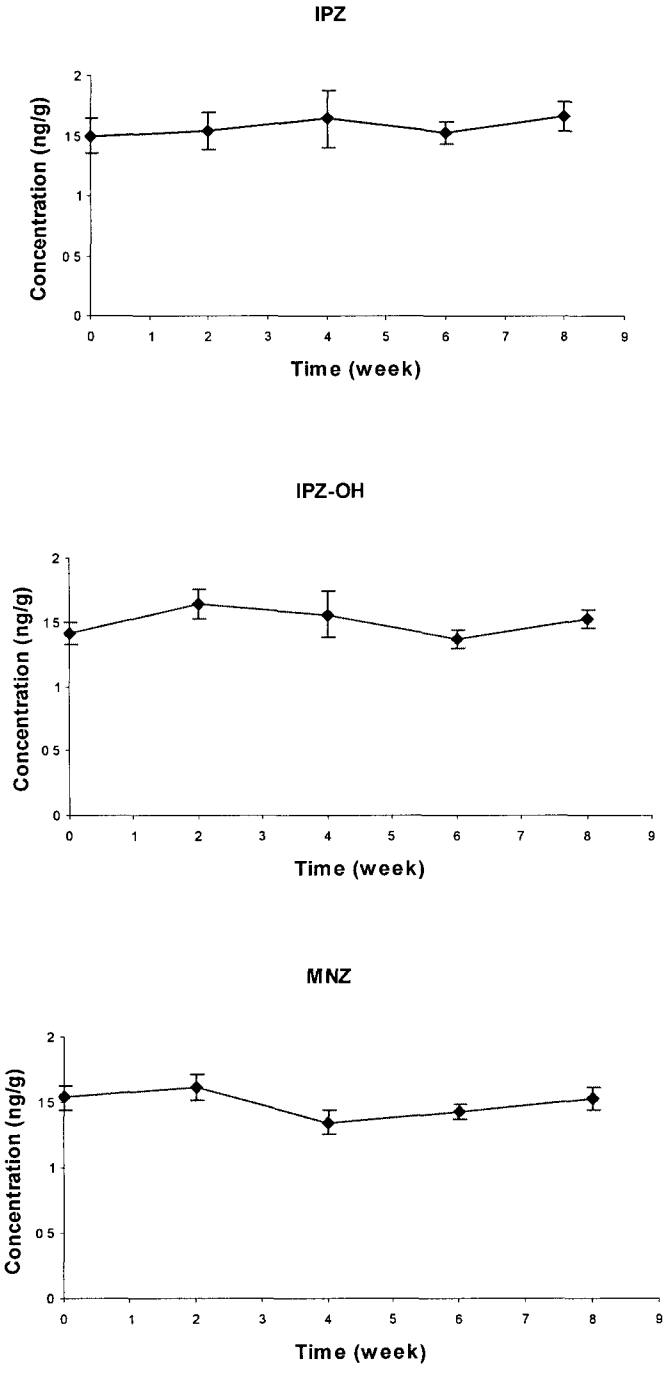
		Concentration determined (ng/g)						
Day	Sample	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	9 73	9 57	12 09	8 99	8 73	10 08	10 14
	2	11 56	9 44	12 82	9 87	8 89	11 31	9 57
	3	9 49	8 31	11 50	9 40	9 03	10 16	10 28
	4	9 50	9 97	12 32	9 38	9 15	10 77	9 81
	5	11 04	9 33	13 28	9 73	8 49	10 56	9 90
2	1	11 38	10 06	12 27	10 65	8 23	13 53	10 63
	2	12 59	9 66	13 73	12 59	9 63	13 35	10 69
	3	11 40	8 09	13 24	12 13	10 36	11 99	10 27
	4	11 18	9 94	13 82	12 00	8 79	12 99	10 49
	5	12 62	9 19	13 8	12 69	10 10	13 38	10 68
3	1	11 76	9 50	11 76	9 44	9 25	9 87	10 6
	2	11 40	9 40	11 88	10 23	10 08	11 82	10 94
	3	11 63	8 03	11 00	9 11	8 65	10 97	9 55
	4	10 70	8 88	11 74	9 39	9 56	10 61	10 72
	5	11 58	8 59	11 61	10 22	10 33	12 14	9 78
4	1	12 78	9 12	12 84	11 39	10 30	12 55	12 18
	2	11 87	10 08	10 82	11 57	9 87	10 70	12 00
	3	13 04	8 39	12 22	11 48	9 66	11 81	12 6
	4	12 43	8 79	11 95	10 56	10 12	11 01	11 75
	5	12 6	9 14	11 92	10 73	10 44	12 24	11 30
5	1	10 30	9 73	12 41	11 75	7 68	10 07	9 35
	2	11 13	8 92	12 44	11 52	9 06	10 18	9 26
	3	11 16	7 89	12 13	11 70	7 98	9 10	8 34
	4	10 91	8 96	11 62	10 64	8 94	10 92	9 77
	5	11 02	9 31	12 74	11 56	9 32	11 81	9 21
	Mean	11 392	9 1316	12 318	10 7488	9 3056	11 3568	10 3924
	SD	0 957071	0 630477	0 795402	1 111026	0 765872	1 185545	0 996162
	RSD	8 40%	6 90%	6 46%	10 34%	8 23%	10 44%	9 59%

High concentration results

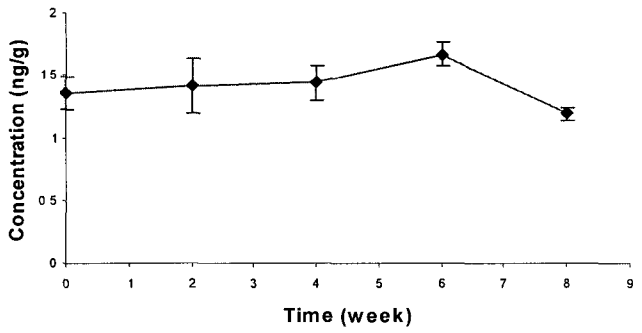
		Concentration determined (ng/g)						
Day	Sample	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	51 96	58 93	63 90	42 32	40 87	49 48	49 41
	2	53 70	61 58	67 49	48 22	43 06	52 35	54 64
	3	51 96	57 56	65 81	52 02	43 31	48 94	52 08
	4	51 53	59 94	65 12	49 40	46 39	53 82	57 65
	5	49 17	48 25	68 05	50 02	43 89	47 47	50 47
2	1	54 53	60 12	70 10	60 40	44 74	62 74	58 64
	2	59 82	57 98	71 96	56 76	45 08	64 14	57 35
	3	59 08	57 18	69 55	57 19	45 30	62 68	57 50
	4	57 65	58 89	65 13	58 96	45 17	65 94	58 07
	5	60 24	57 56	71 45	57 07	47 84	60 01	57 52
3	1	59 41	55 01	53 14	47 54	46 59	51 28	52 71
	2	59 31	57 53	57 14	46 00	46 78	49 01	50 62
	3	55 95	54 39	57 62	49 92	44 24	52 27	56 55
	4	56 02	53 01	58 33	51 93	45 46	44 89	54 08
	5	53 78	55 97	54 38	44 49	44 17	48 88	48 85
4	1	53 69	59 69	60 42	52 84	50 01	58 06	61 96
	2	52 87	63 84	64 09	56 41	47 46	57 07	62 25
	3	56 28	57 45	61 87	52 31	53 92	55 57	64 09
	4	60 56	66 97	68 35	52 89	58 13	61 83	69 85
	5	57 02	55 76	57 69	54 92	54 70	59 91	62 84
5	1	52 77	59 76	68 53	55 98	45 79	50 02	55 42
	2	51 21	60 62	62 39	53 06	43 34	49 74	53 75
	3	59 24	56 76	56 57	58 61	46 12	44 60	56 93
	4	54 84	60 90	66 51	55 71	43 45	49 59	51 82
	5	51 16	50 79	66 03	55 89	40 43	44 51	52 58
	Mean	55 35	57 8576	63 6648	52 8344	46 2496	53 792	56 3052
	SD	3 311477	3 816792	5 343046	4 610032	4 041598	6 401706	4 972762
	RSD	5 98%	6 60%	8 39%	8 73%	8 74%	11 90%	8 83%

Appendix 6 Error bar charts representing the stability of NI analytes in tilapia tissue over a 2 month period at a concentration of 1.5 ng/g with storage conditions of -20°C (a), -80°C (b) and -20°C freeze/thaw (c).

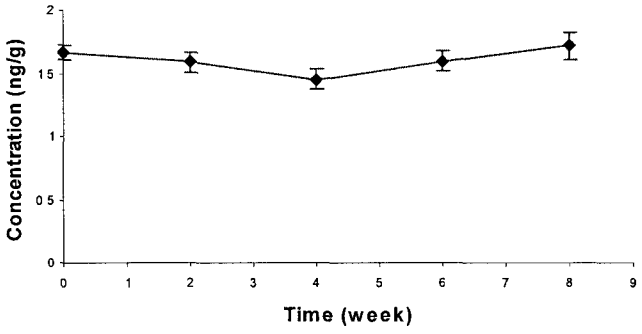
(a)



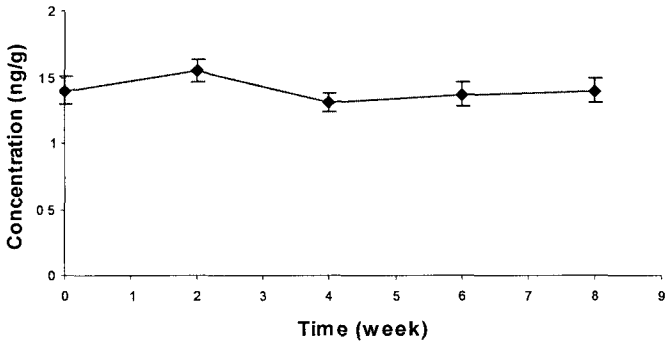
MNZ-OH



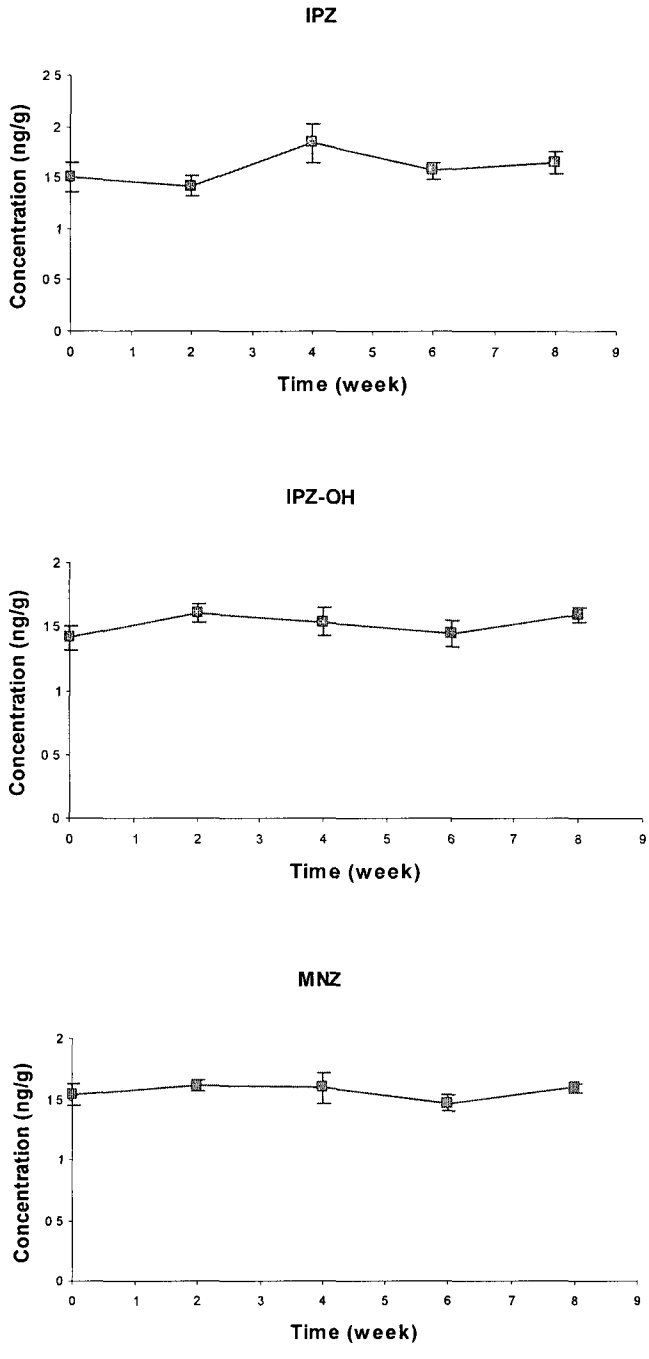
RNZ



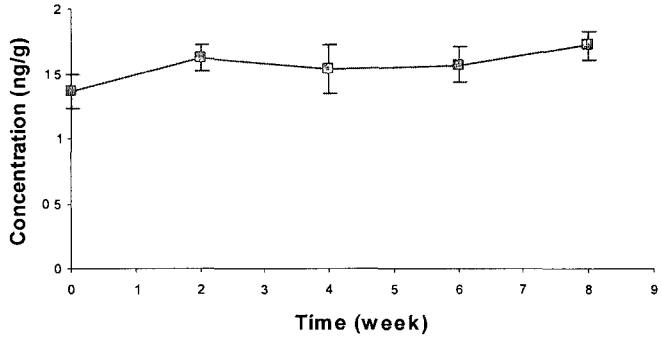
DMZ



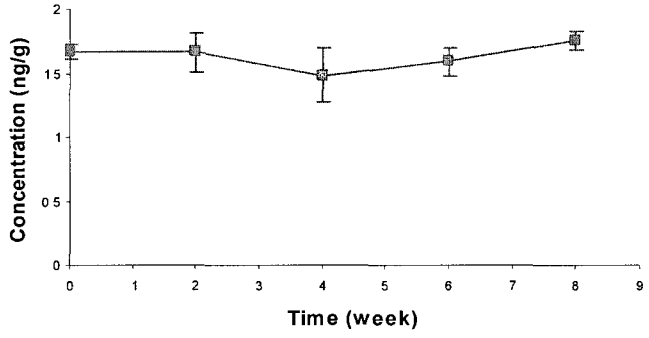
(b)


IPZ
Concentration (ng/g)
Time (week)
IPZ-OH
Concentration (ng/g)
Time (week)
MNZ
Concentration (ng/g)
Time (week)

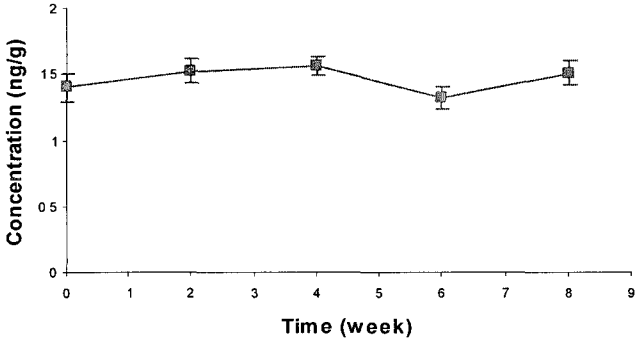
MNZ-OH



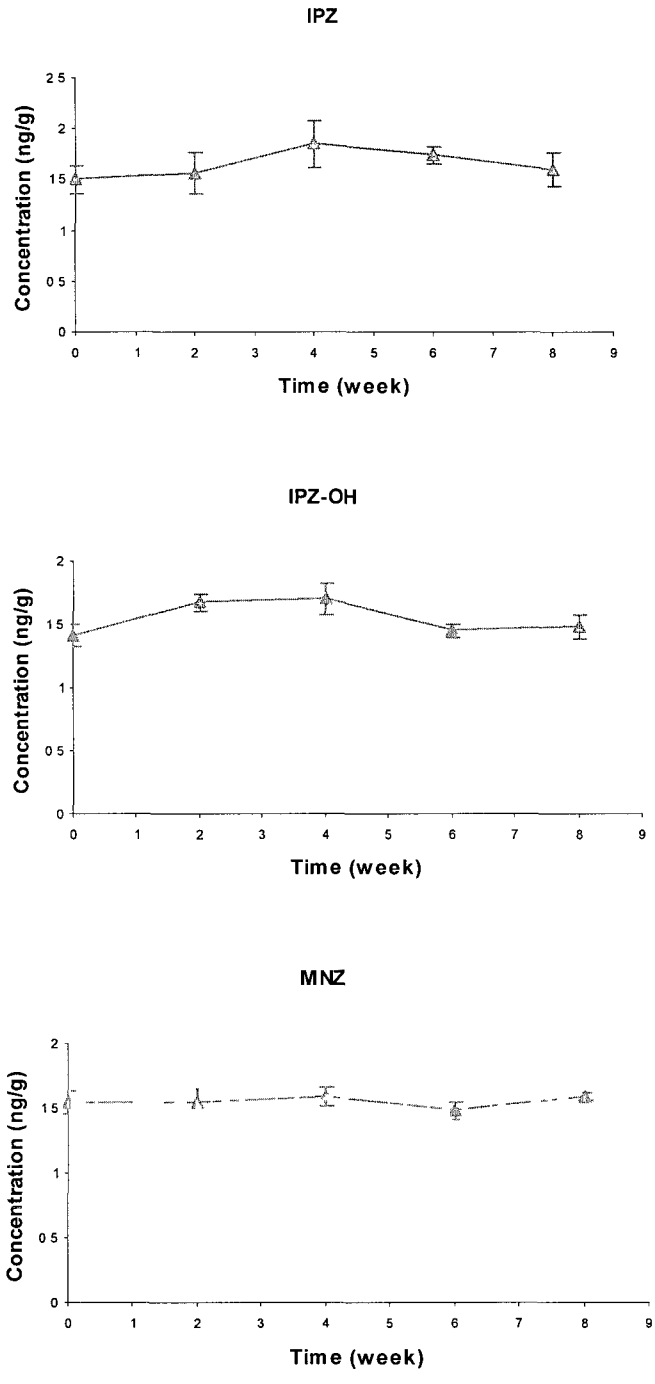
RNZ



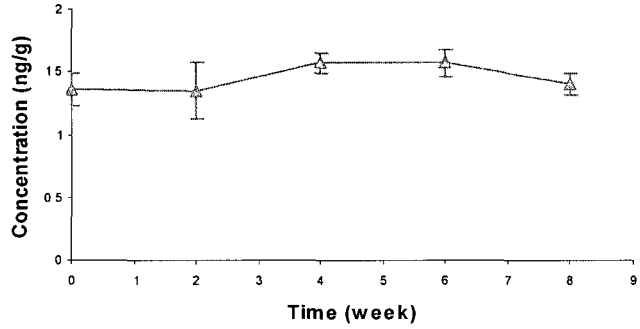
DMZ



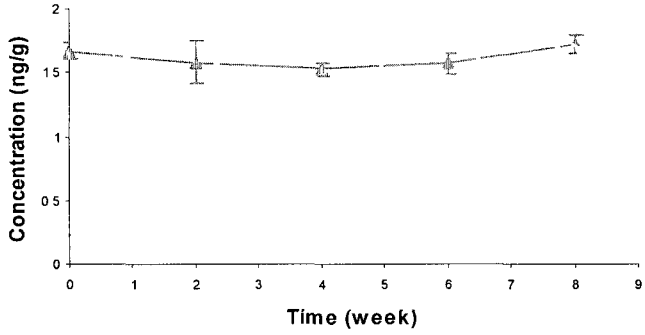
(c)


IPZ
Concentration (ng/g)
Time (week)
IPZ-OH
Concentration (ng/g)
Time (week)
MNZ
Concentration (ng/g)
Time (week)

MNZ-OH



RNZ



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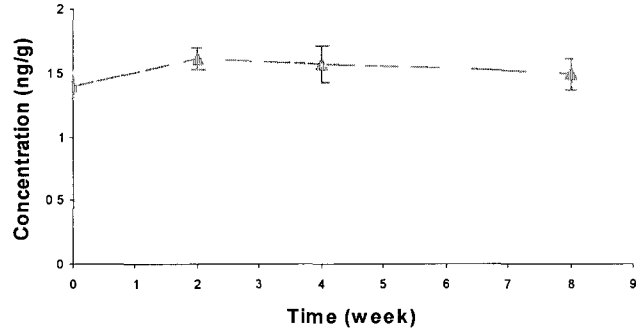
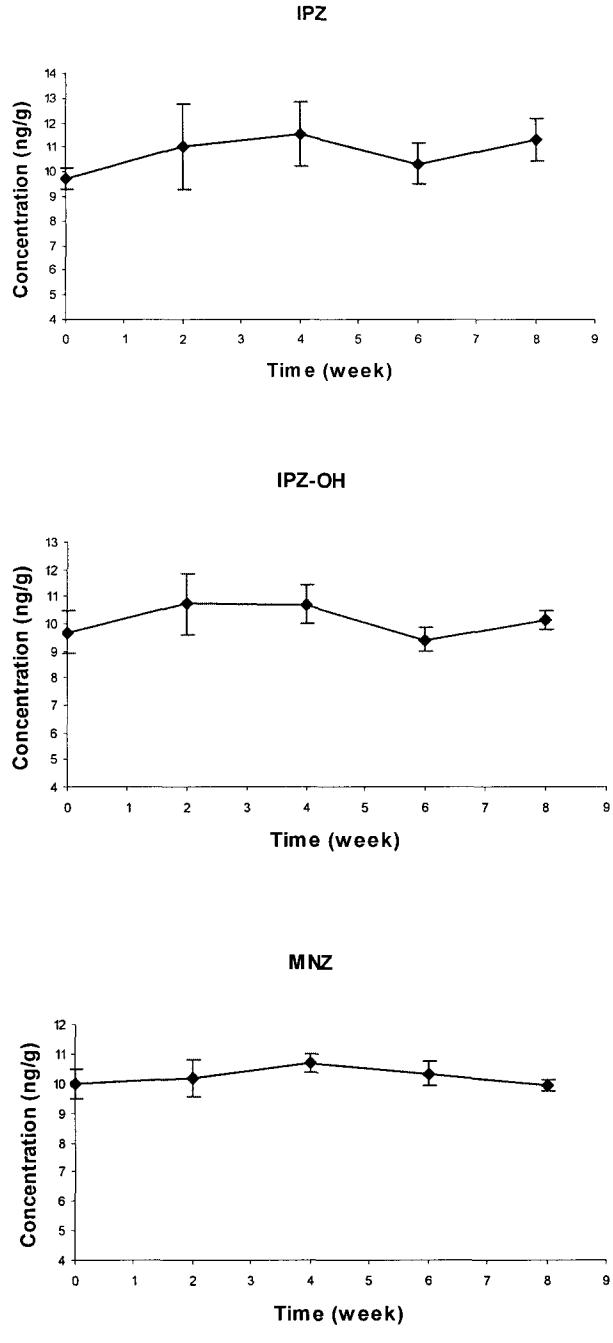
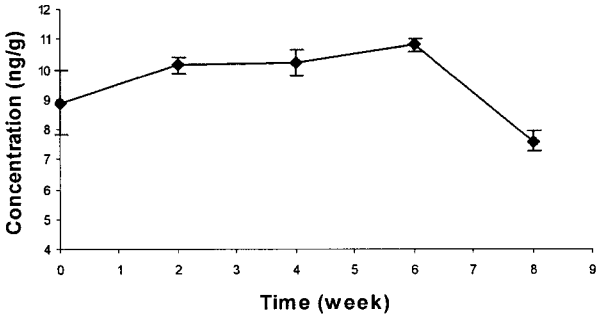


Appendix 7 Error bar charts representing the stability of NI analytes in tilapia tissue over a 2 month period at a concentration of 10 ng/g with storage conditions of -20°C (a), -80°C (b) and -20°C freeze/thaw (c).

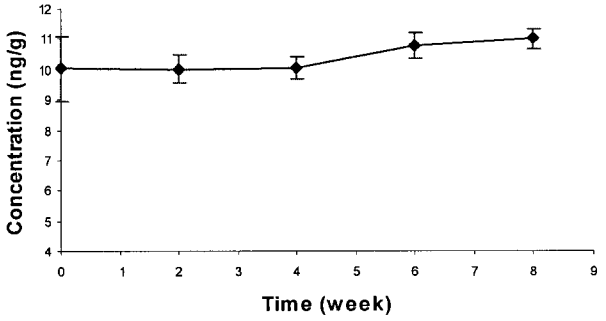
(a)



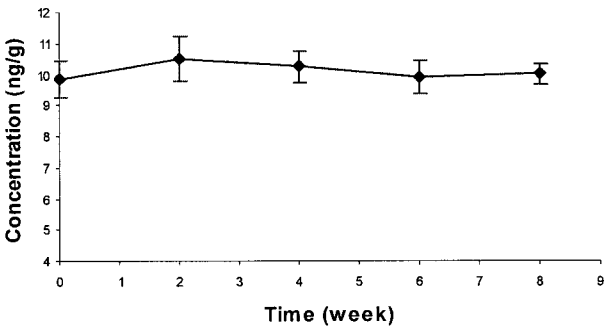
MNZ-OH



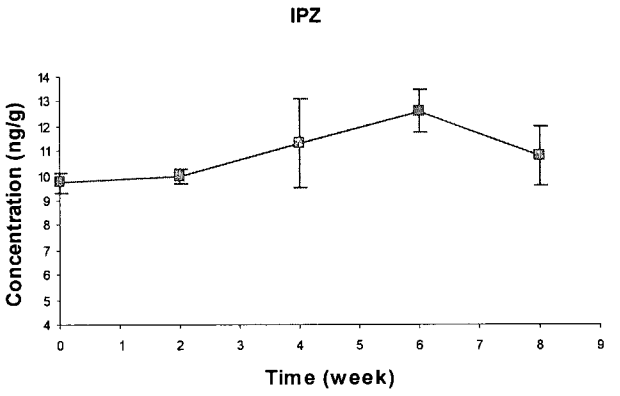
RNZ

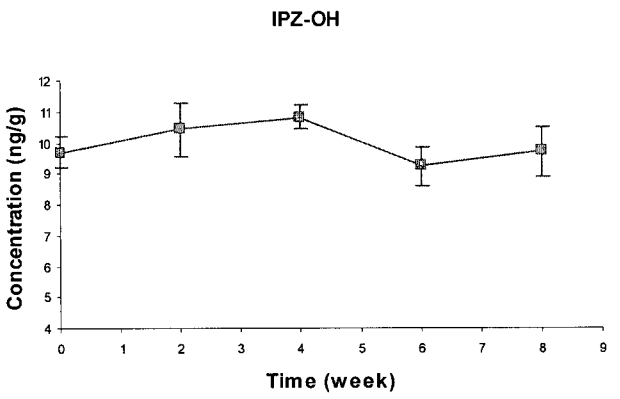


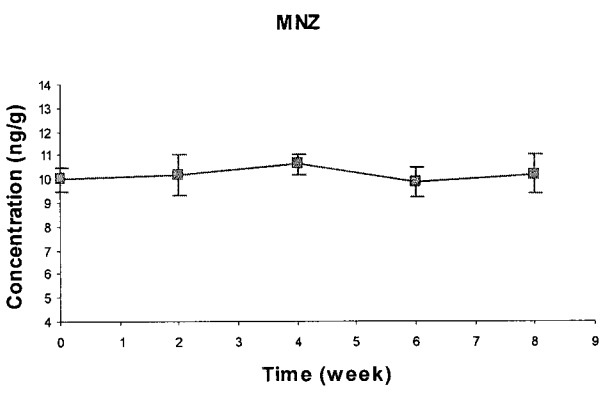
DMZ



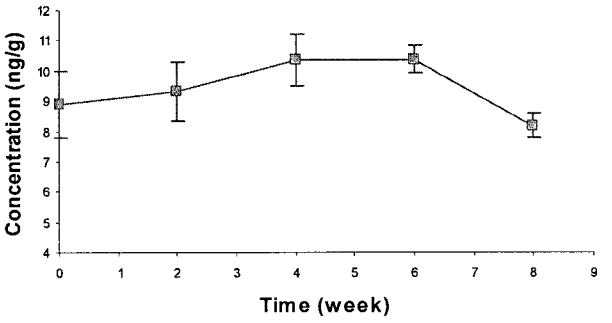
(b)


IPZ
Concentration (ng/g)
Time (week)

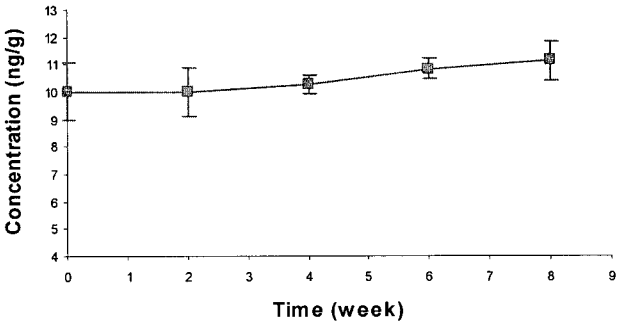

IPZ-OH
Concentration (ng/g)
Time (week)


MNZ
Concentration (ng/g)
Time (week)

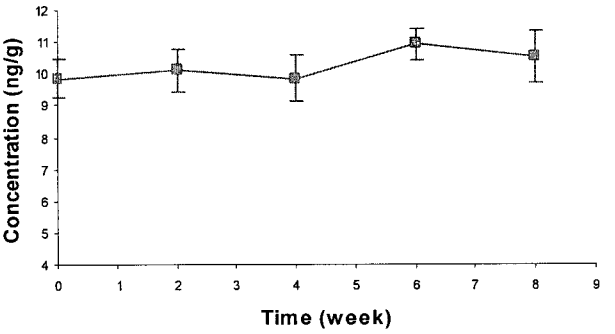
MNZ-OH



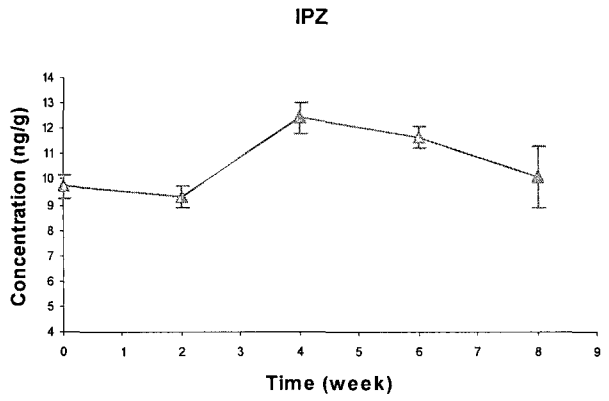
RNZ

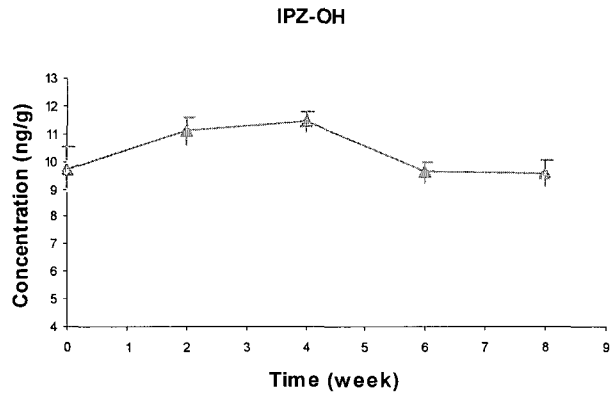


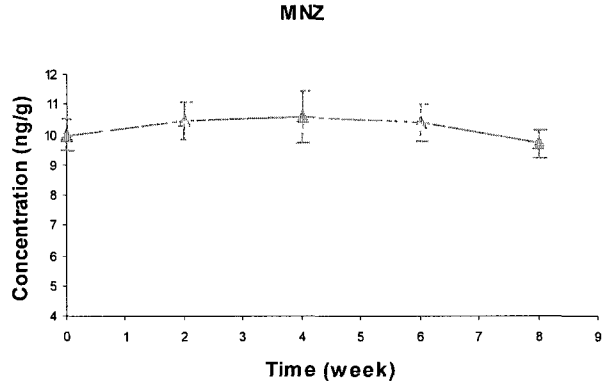
DMZ



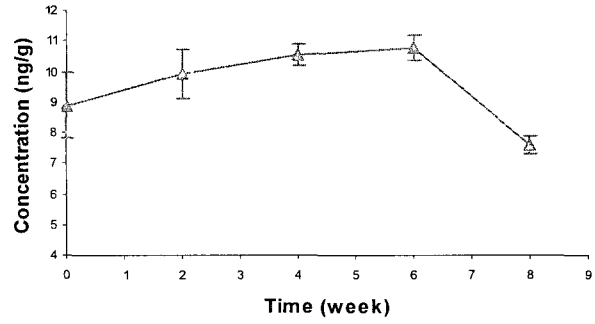
(c)


IPZ
Concentration (ng/g)
Time (week)

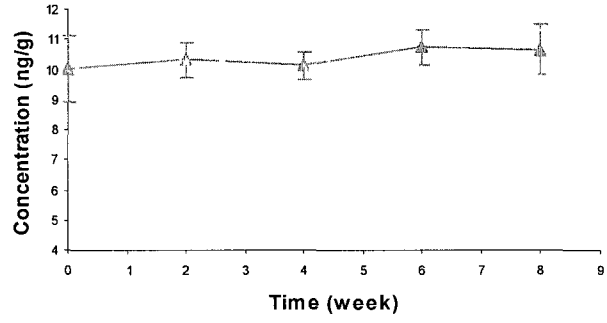

IPZ-OH
Concentration (ng/g)
Time (week)


MNZ
Concentration (ng/g)
Time (week)

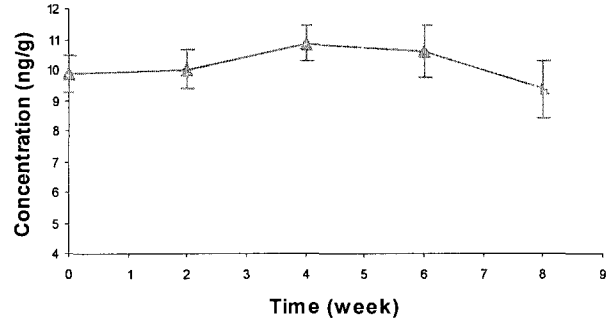
MNZ-OH



RNZ

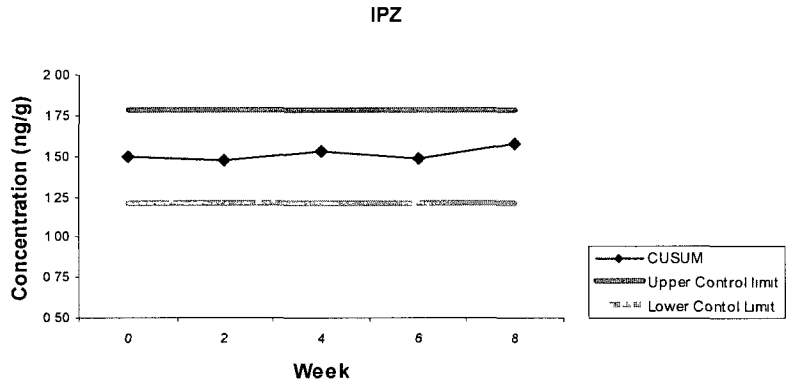


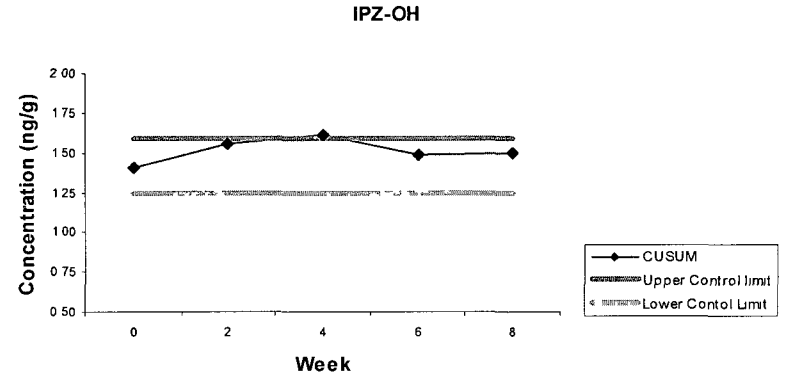
DMZ

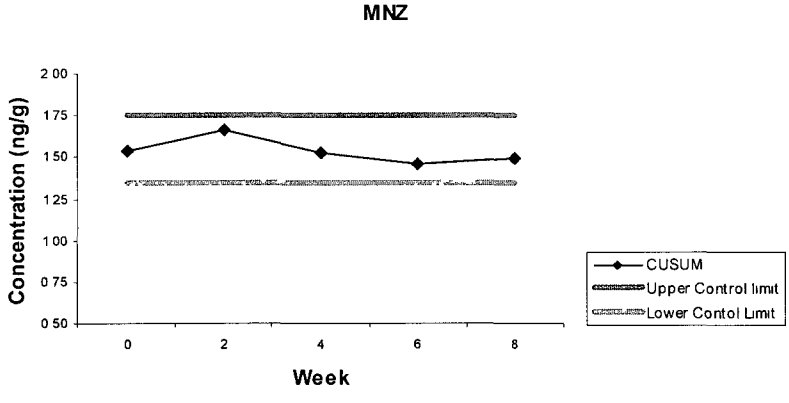


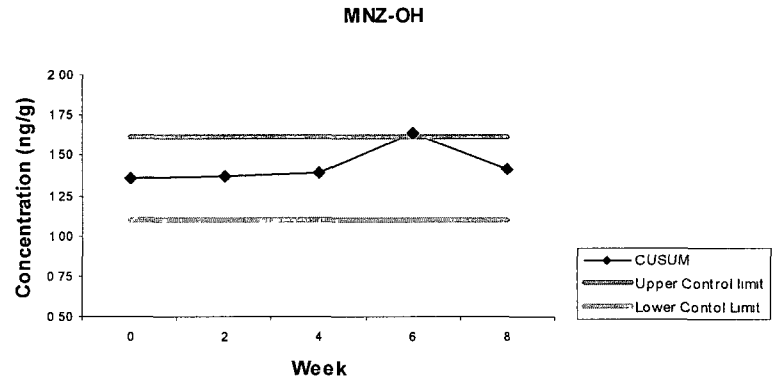
Appendix 8 CUSUM charts representing the stability of NI analytes in tilapia tissue over 2 months for 1.5 ng/g at storage conditions of -20°C (a), -80°C (b) and -20°C freeze/thaw (c).

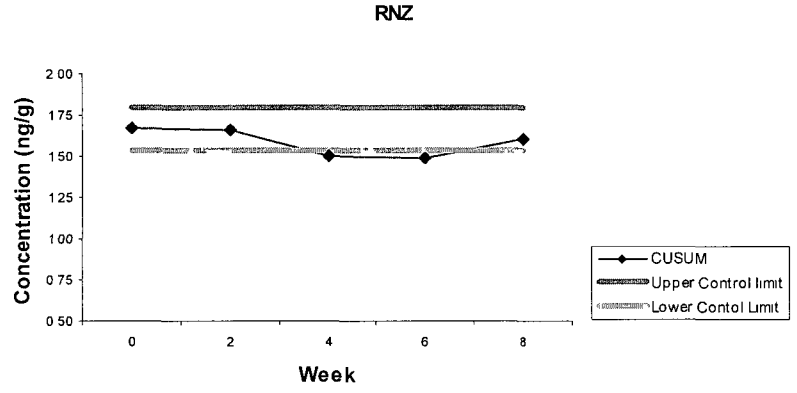
(a)

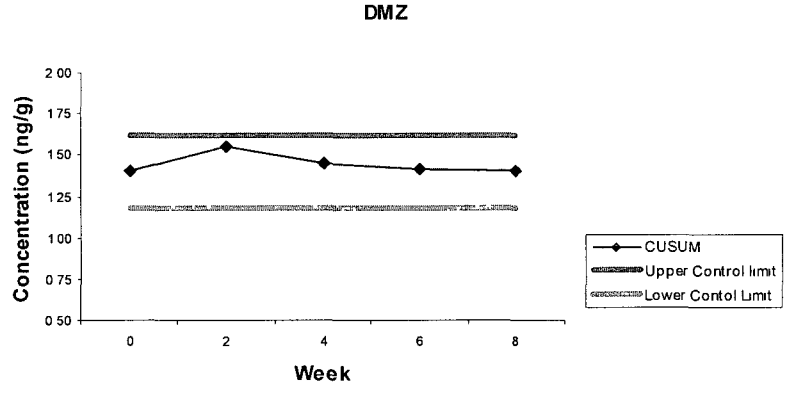




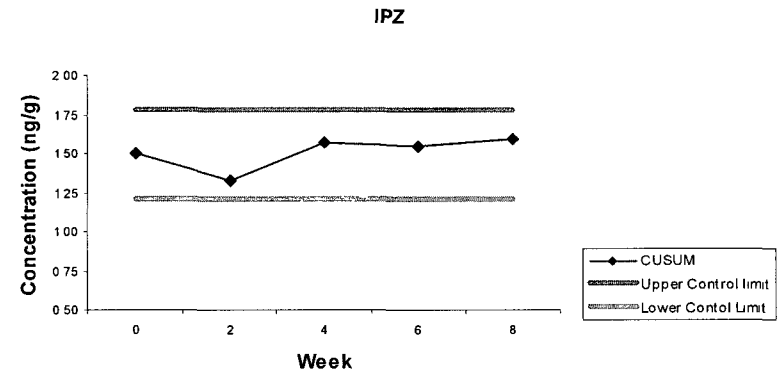


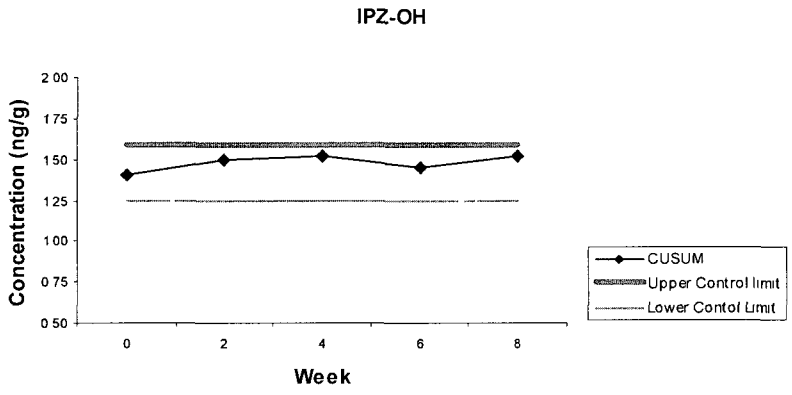

MNZ-OH
Concentration (ng/g)
2 00
1 75
1 50
1 25
1 00
0 75
0 50
0
2
4
6
8
Week
CUSUM
Upper Control limit
Lower Contol Limit

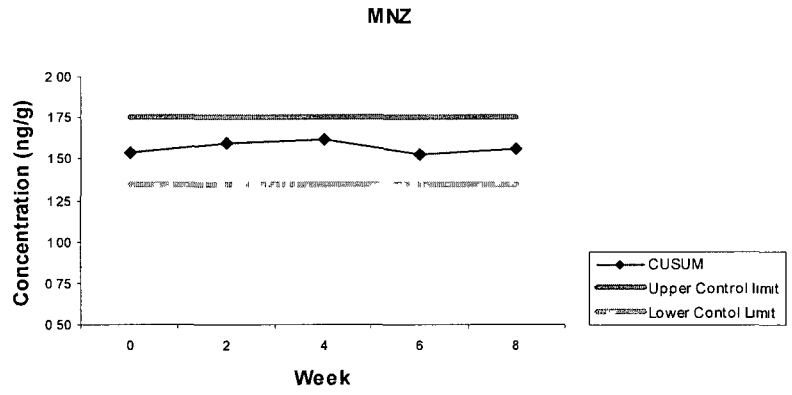

RNZ
Concentration (ng/g)
2 00
1 75
1 50
1 25
1 00
0 75
0 50
0
2
4
6
8
Week
CUSUM
Upper Control limit
Lower Contol Limit

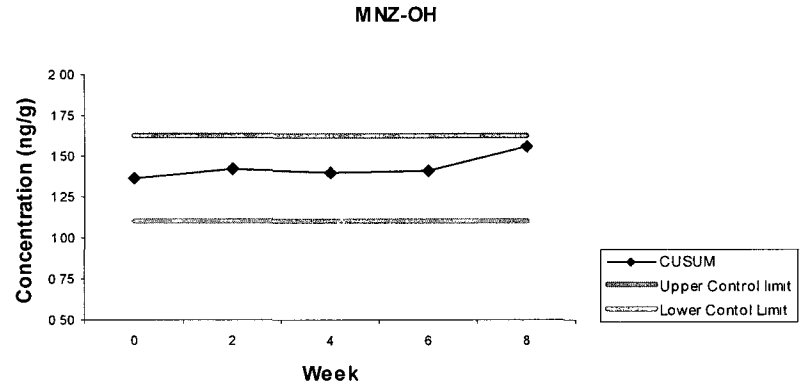

DMZ
Concentration (ng/g)
2 00
1 75
1 50
1 25
1 00
0 75
0 50
0
2
4
6
8
Week
CUSUM
Upper Control limit
Lower Contol Limit

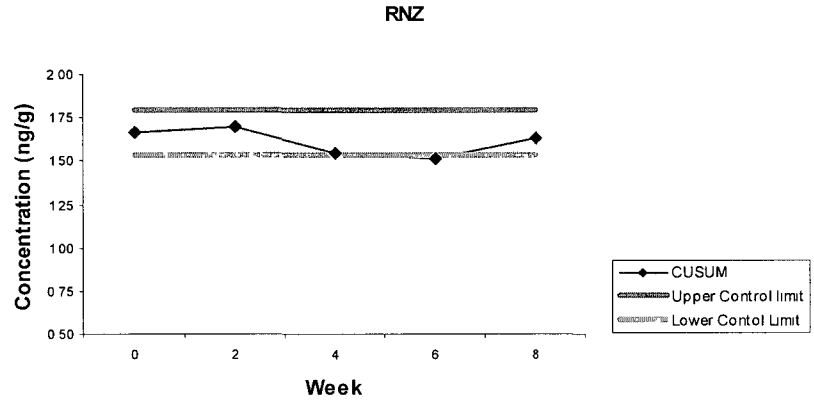
(b)

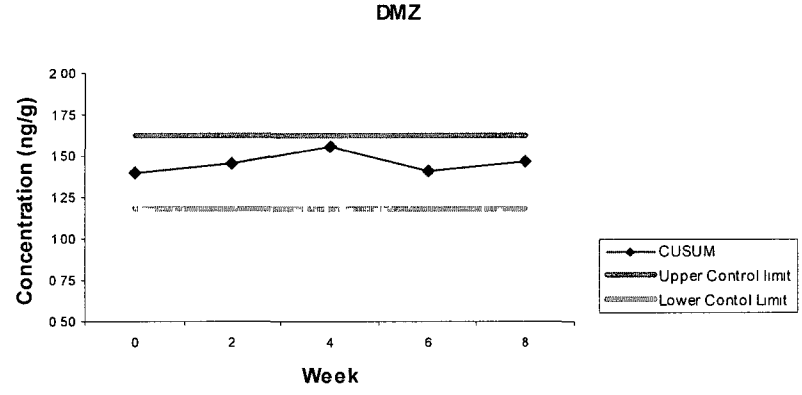

IPZ
Concentration (ng/g)
2 00
1 75
1 50
1 25
1 00
0 75
0 50
0
2
4
6
8
Week
CUSUM
Upper Control limit
Lower Contol Limit


IPZ-OH
Concentration (ng/g)
2 00
1 75
1 50
1 25
1 00
0 75
0 50
0
2
4
6
8
Week
CUSUM
Upper Control limit
Lower Contol Limit

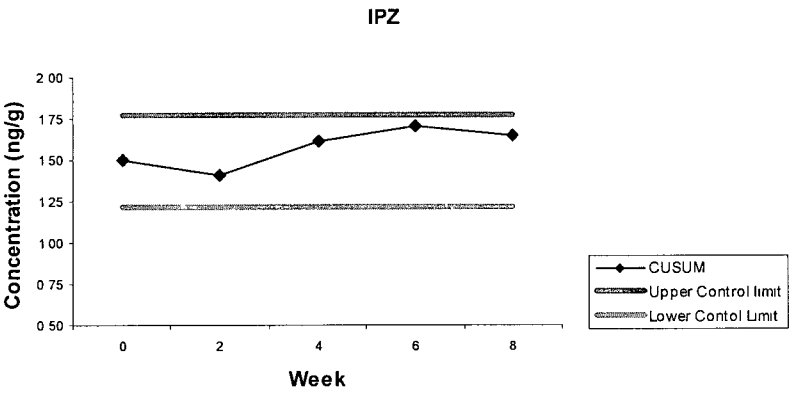

MNZ
Concentration (ng/g)
2 00
1 75
1 50
1 25
1 00
0 75
0 50
0
2
4
6
8
Week
CUSUM
Upper Control limit
Lower Contol Limit

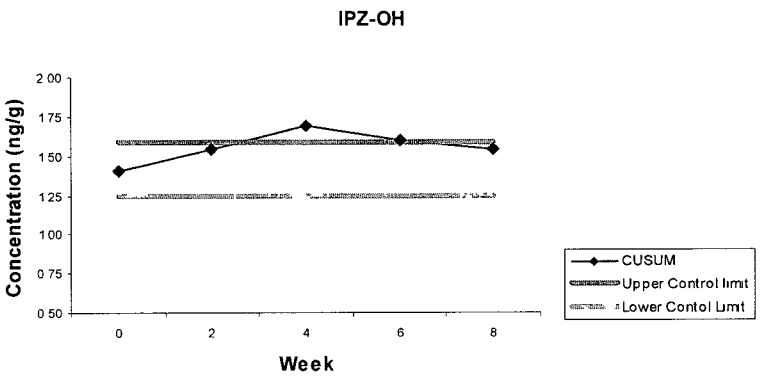

MNZ-OH
Concentration (ng/g)
2 00
175
150
125
100
0 75
0 50
0
2
4
6
8
Week
CUSUM
Upper Control limit
Lower Contol Limit

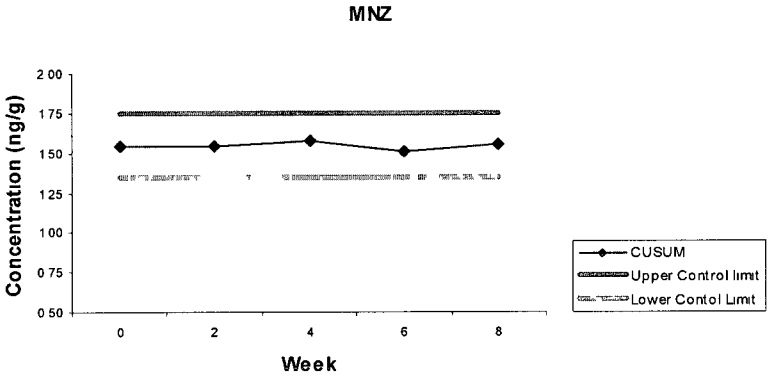

RNZ
Concentration (ng/g)
2 00
175
150
125
100
0 75
0 50
0
2
4
6
8
Week
CUSUM
Upper Control limit
Lower Contol Limit

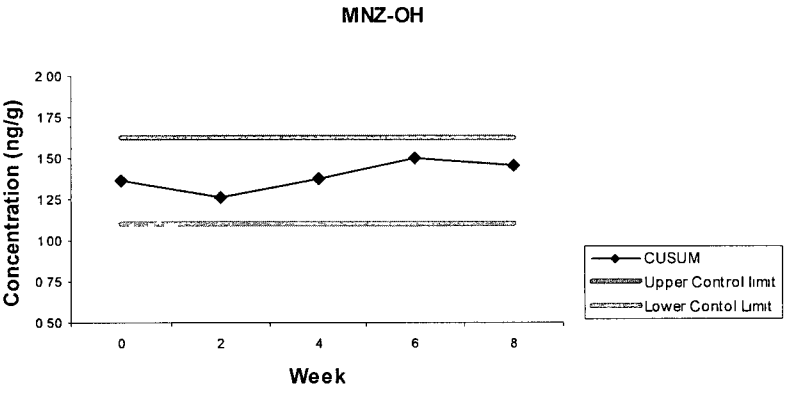

DMZ
Concentration (ng/g)
2 00
175
150
125
100
0 75
0 50
0
2
4
6
8
Week
CUSUM
Upper Control limit
Lower Contol Limit

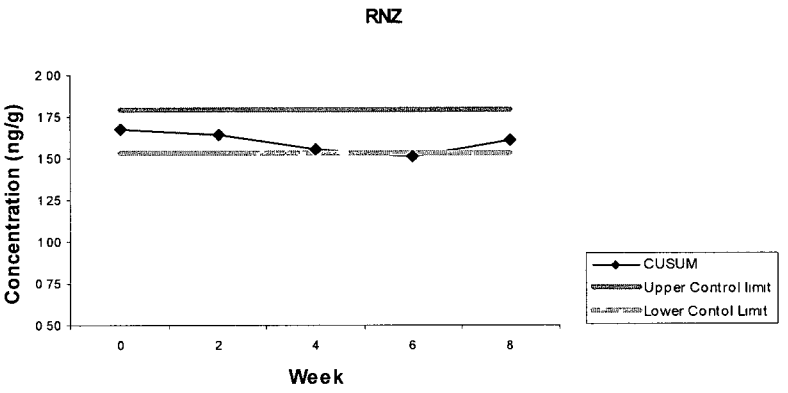
(c)

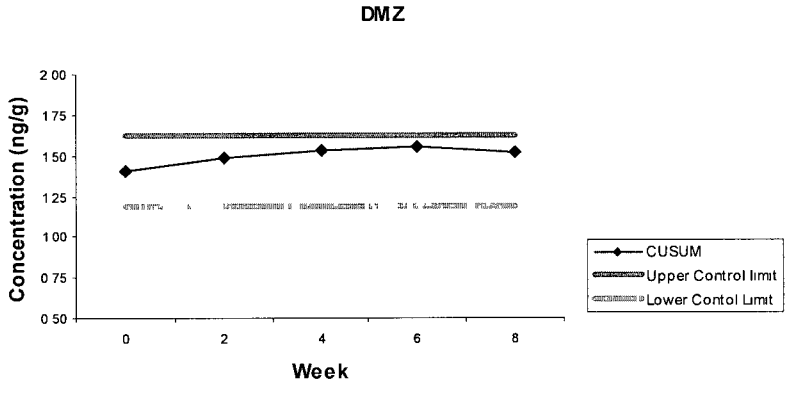

IPZ
Concentration (ng/g)
2 00
1 75
1 50
1 25
1 00
0 75
0 50
0
2
4
6
8
Week
CUSUM
Upper Control limit
Lower Contol Limit


IPZ-OH
Concentration (ng/g)
2 00
1 75
1 50
1 25
1 00
0 75
0 50
0
2
4
6
8
Week
CUSUM
Upper Control limit
Lower Contol Limit


MNZ
Concentration (ng/g)
2 00
1 75
1 50
1 25
1 00
0 75
0 50
0
2
4
6
8
Week
CUSUM
Upper Control limit
Lower Contol Limit

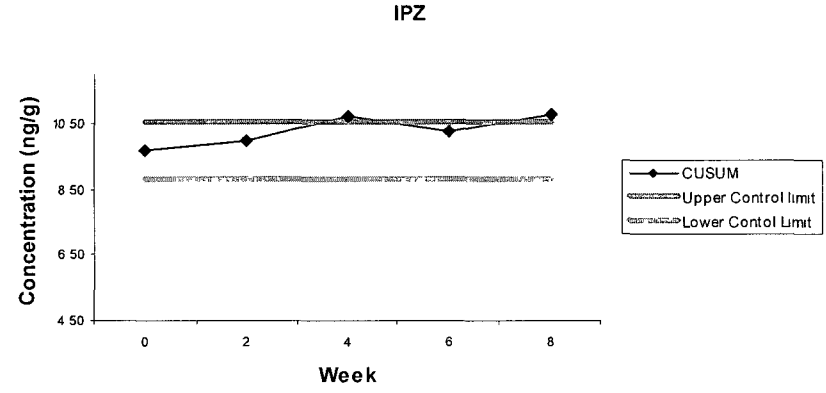

MNZ-OH
Concentration (ng/g)
2 00
1 75
1 50
1 25
1 00
0 75
0 50
0
2
4
6
8
Week
CUSUM
Upper Control limit
Lower Contol Limit

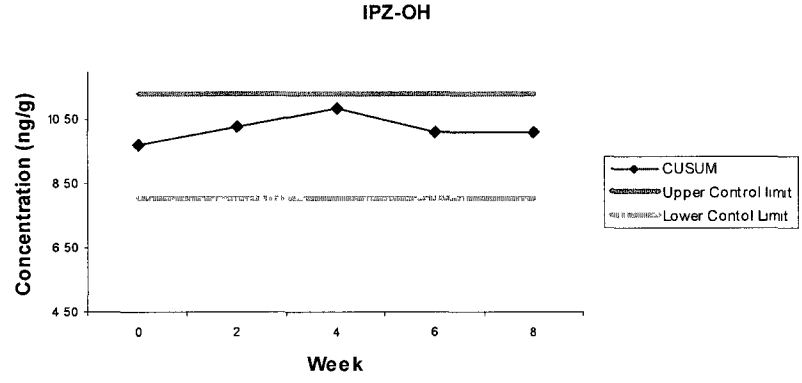

RNZ
Concentration (ng/g)
2 00
1 75
1 50
1 25
1 00
0 75
0 50
0
2
4
6
8
Week
CUSUM
Upper Control limit
Lower Contol Limit

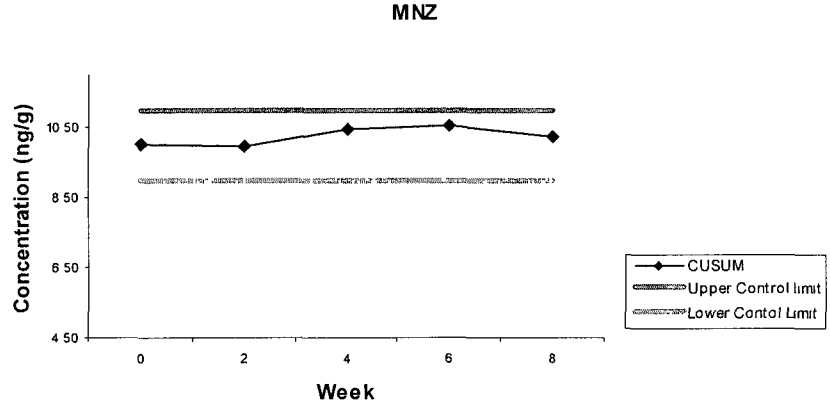

DMZ
Concentration (ng/g)
2 00
1 75
1 50
1 25
1 00
0 75
0 50
0
2
4
6
8
Week
CUSUM
Upper Control limit
Lower Contol Limit

Appendix 9 CUSUM charts representing the stability of NI analytes in tilapia tissue over 2 months for 10 ng/g at storage conditions of -20°C (a), -80°C (b) and -20°C freeze/thaw (c).

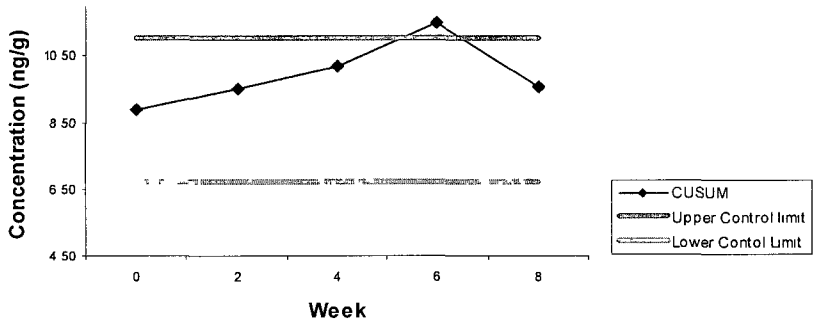
(a)



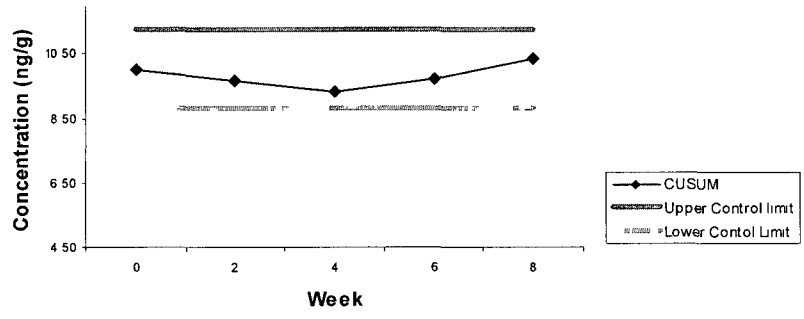




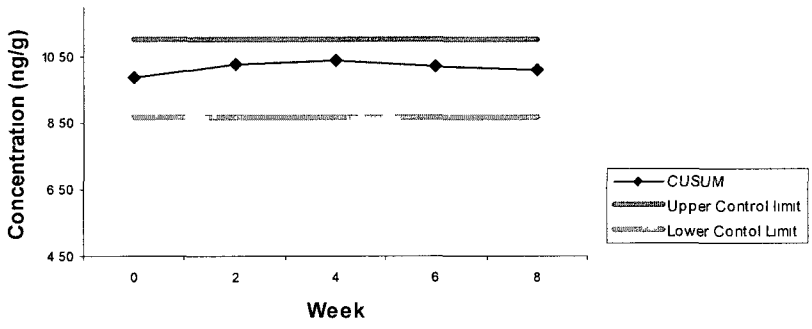
MNZ-OH



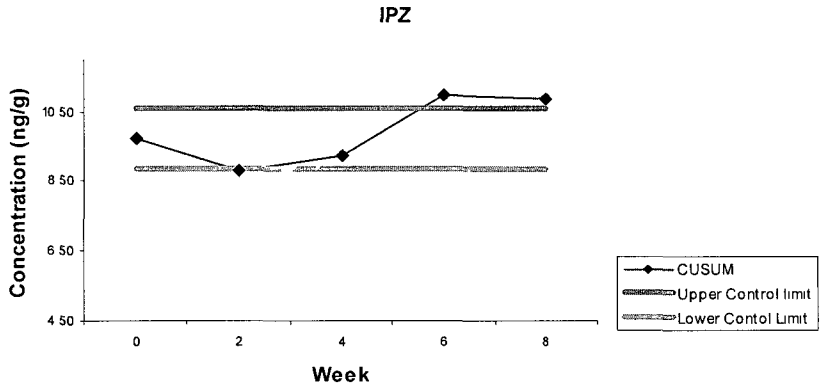
RNZ

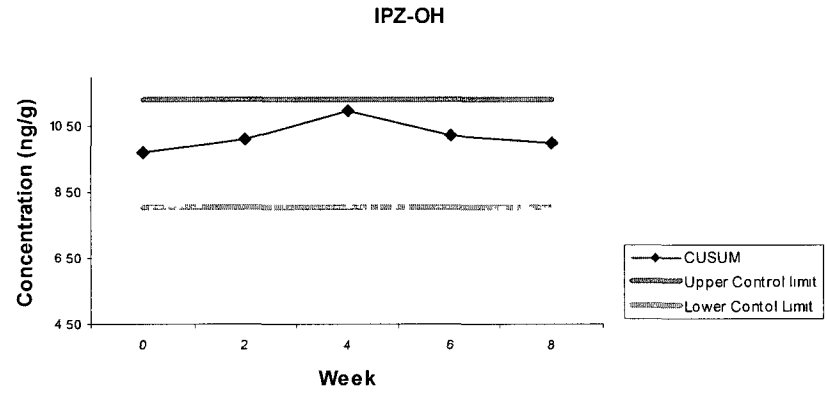


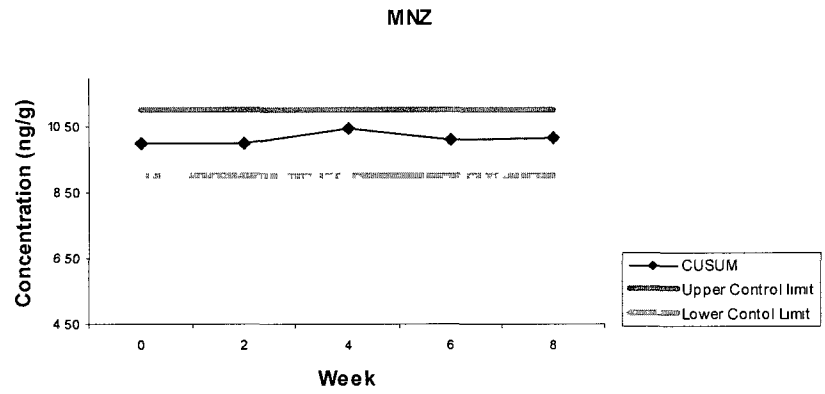
DMZ



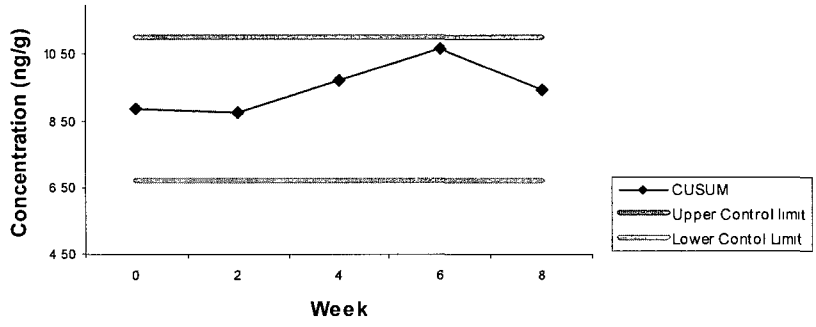
(b)


IPZ
Concentration (ng/g)
10 50
8 50
6 50
4 50
0
2
4
6
8
Week
CUSUM
Upper Control limit
Lower Contol Limit

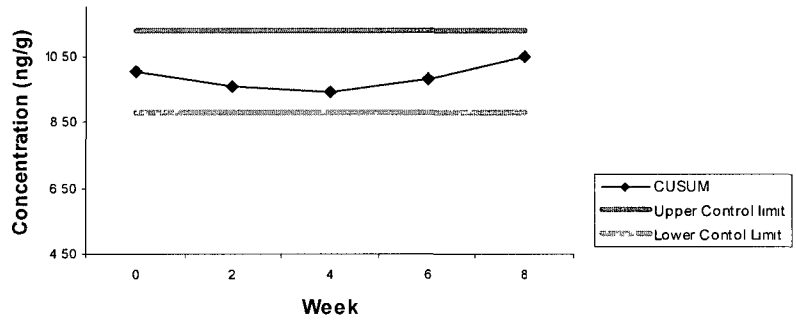

IPZ-OH
Concentration (ng/g)
10 50
8 50
6 50
4 50
0
2
4
6
8
Week
CUSUM
Upper Control limit
Lower Contol Limit


MNZ
Concentration (ng/g)
10 50
8 50
6 50
4 50
0
2
4
6
8
Week
CUSUM
Upper Control limit
Lower Contol Limit

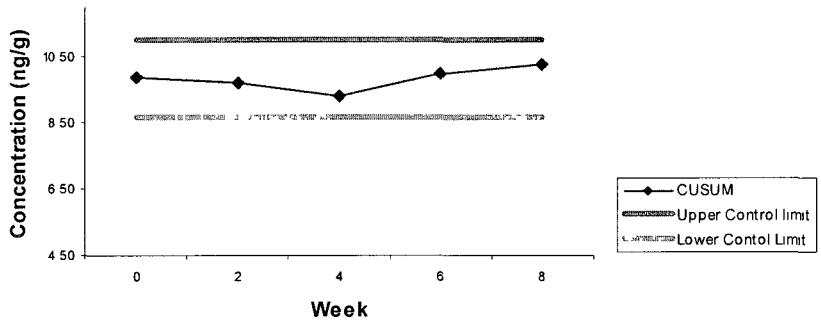
MNZ-OH



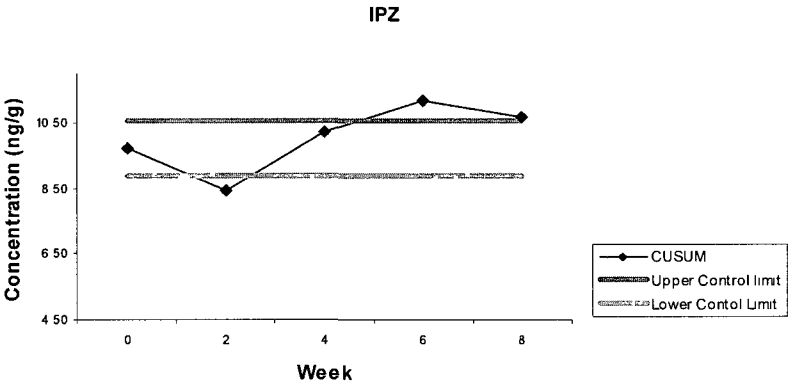
RNZ

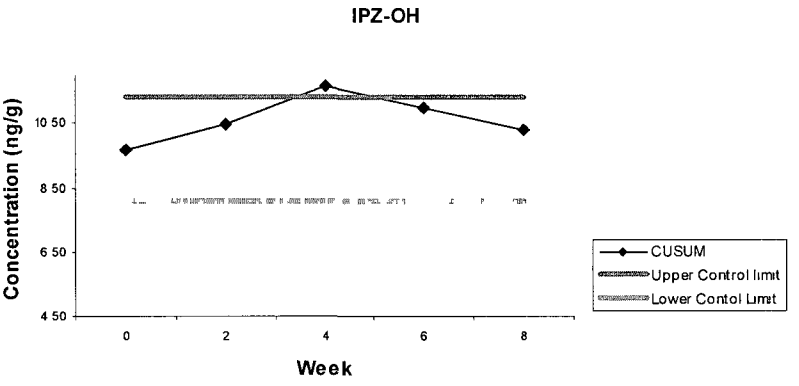


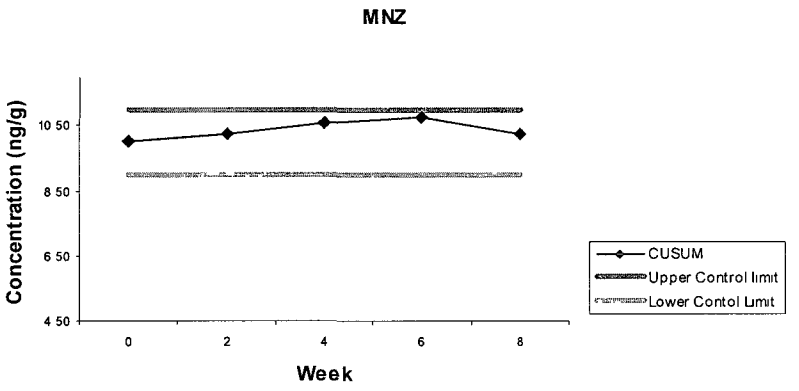
DMZ

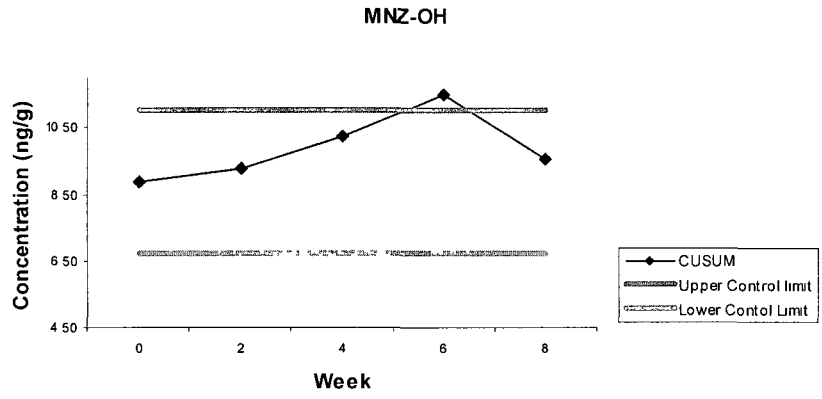


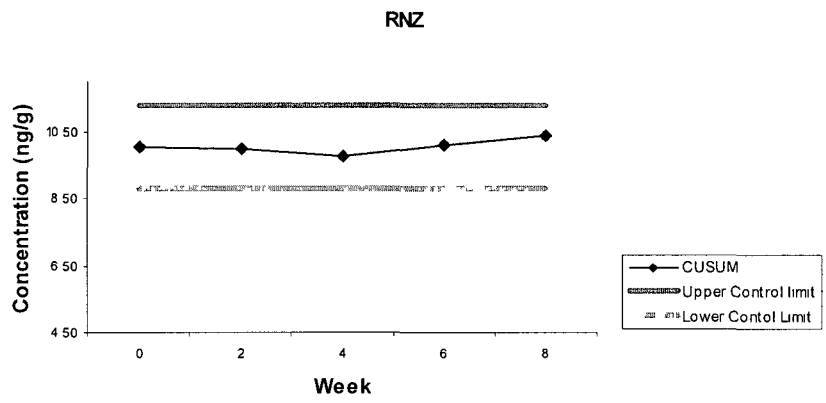
(c)

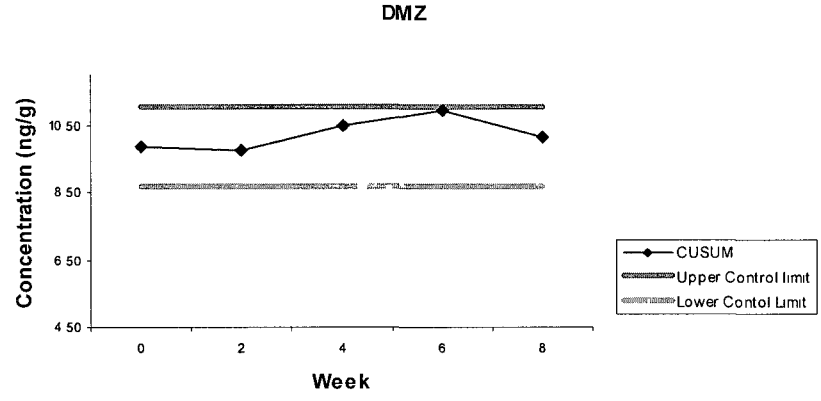

IPZ
Concentration (ng/g)
10 50
8 50
6 50
4 50
0
2
4
6
8
Week
CUSUM
Upper Control limit
Lower Contol Limit


IPZ-OH
Concentration (ng/g)
10 50
8 50
6 50
4 50
0
2
4
6
8
Week
CUSUM
Upper Control limit
Lower Contol Limit


MNZ
Concentration (ng/g)
10 50
8 50
6 50
4 50
0
2
4
6
8
Week
CUSUM
Upper Control limit
Lower Contol Limit


MNZ-OH
Concentration (ng/g)
10 50
8 50
6 50
4 50
0
2
4
6
8
Week
CUSUM
Upper Control limit
Lower Contol Limit


RNZ
Concentration (ng/g)
10 50
8 50
6 50
4 50
0
2
4
6
8
Week
CUSUM
Upper Control limit
Lower Contol Limit


DMZ
Concentration (ng/g)
10 50
8 50
6 50
4 50
0
2
4
6
8
Week
CUSUM
Upper Control limit
Lower Contol Limit

Appendix 10 Data from tilapia tissue stability for -20°C, -80°C and -20°C freeze/thaw conditions at concentrations of 1.5ng/g and 10ng/g. An example of a t-test and ANOVA is included on results for 1.5ng/g HMMNI at -20°C.

-20C 1 5 ng/g

		Concentration determined (ng/g)						
Week	Sample	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
0	1	1 39	1 26	1 41	1 52	1 20	1 72	1 25
	2	1 27	1 54	1 35	1 54	1 25	1 72	1 43
	3	1 45	1 59	1 56	1 39	1 43	1 56	1 50
	4	1 45	1 48	1 37	1 63	1 41	1 67	1 33
	5	1 57	1 61	1 37	1 64	1 51	1 67	1 50
2	1	1 67	1 79	1 70	1 64	1 42	1 61	1 69
	2	1 56	1 49	1 66	1 60	1 40	1 64	1 50
	3	1 63	1 54	1 78	1 60	1 23	1 67	1 54
	4	1 81	1 53	1 54	1 50	1 27	1 47	1 48
	5	1 65	1 33	1 52	1 77	1 78	1 54	1 52
4	1	1 64	1 75	1 88	1 37	1 53	1 57	1 42
	2	1 28	1 98	1 52	1 30	1 27	1 47	1 27
	3	1 40	1 48	1 51	1 50	1 38	1 35	1 27
	4	1 33	1 36	1 43	1 29	1 41	1 43	1 27
	5	1 45	1 64	1 45	1 27	1 62	1 42	1 34
6	1	1 33	1 65	1 46	1 51	1 66	1 67	1 47
	2	1 25	1 53	1 27	1 36	1 71	1 47	1 44
	3	1 30	1 49	1 40	1 40	1 60	1 58	1 27
	4	1 42	1 41	1 39	1 48	1 81	1 61	1 31
	5	1 30	1 51	1 36	1 42	1 55	1 66	1 34
8	1	1 60	1 63	1 55	1 37	1 12	1 54	1 39
	2	1 59	1 81	1 50	1 55	1 16	1 70	1 37
	3	1 81	1 50	1 41	1 61	1 21	1 77	1 27
	4	1 84	1 74	1 55	1 52	1 26	1 83	1 43
	5	1 73	1 64	1 60	1 58	1 21	1 77	1 53
	Mean	1 51	1 57	1 50	1 49	1 42	1 60	1 41
	SD	0 18	0 16	0 14	0 13	0 20	0 12	0 11
	RSD	12 18%	10 25%	9 47%	8 54%	14 16%	7 62%	8 06%

HMMNI

t-Test: Paired Two Sample for Means

	week 0	*week 2*	*week 0*	*week 4*	*week 0*	*week 6*	*week 0*	*week 8*
Mean	1.426	1.664	1.426	1.42	1.426	1.32	1.426	1.714
Variance	0.01188	0.00838	0.01188	0.01935	0.01188	0.00395	0.01188	0.01343
Observations	5	5	5	5	5	5	5	5
Pearson Correlation	0.4179322		0.257226615		0.350351897		0.615141	
Hypothesized Mean Difference	0		0		0		0	
df	4		4		4		4	
t Stat	4.8744299		0.087649634		2.256852163		-6.51524	
P(T<=t) one-tail	0.0040967		0.467183888		0.043491424		0.001433	
t Critical one-tail	2.1318465		2.131846486		2.131846486		2.131846	
P(T<=t) two-tail	0.0081935		0.934367776		0.086982848		0.002865	
t Critical two-tail	2.7764509		2.776450856		2.776450856		2.776451	

HMMNI

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
week 0	5	7.13	1.426	0.01188
week 2	5	8.32	1.664	0.00838
week 4	5	7.1	1.42	0.01935
week 6	5	6.6	1.32	0.00395
week 8	5	8.57	1.714	0.01343

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.582904	4	0.145726	12.78522548	2.52534E-05	2.866080706
Within Groups	0.22796	20	0.011398			
Total	0.810864	24				

-80°C, 1.5 ng/g

		Concentration determined (ng/g)						
Week	Sample	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
0	1	1 39	1 26	1 41	1 52	1 20	1 72	1 25
	2	1 27	1 54	1 35	1 54	1 25	1 72	1 43
	3	1 45	1 59	1 56	1 39	1 43	1 56	1 50
	4	1 45	1 48	1 37	1 63	1 41	1 67	1 33
	5	1 57	1 61	1 37	1 64	1 51	1 67	1 50
2	1	1 70	1 42	1 71	1 66	1 76	1 47	1 56
	2	1 83	1 51	1 59	1 55	1 52	1 79	1 60
	3	1 90	1 31	1 53	1 55	1 70	1 62	1 46
	4	1 61	1 33	1 62	1 63	1 57	1 83	1 47
	5	1 92	1 54	1 57	1 63	1 55	1 59	1 49
4	1	1 42	1 75	1 50	1 54	1 33	1 33	1 33
	2	1 59	1 77	1 52	1 50	1 44	1 48	1 48
	3	2 01	1 62	1 72	1 79	1 83	1 83	1 69
	4	1 40	2 11	1 43	1 52	1 50	1 47	1 62
	5	1 62	1 92	1 54	1 61	1 59	1 32	1 66
6	1	1 35	1 44	1 40	1 54	1 39	1 46	1 47
	2	1 43	1 65	1 38	1 41	1 46	1 51	1 44
	3	1 45	1 60	1 54	1 49	1 70	1 68	1 27
	4	1 46	1 56	1 59	1 52	1 61	1 60	1 31
	5	1 46	1 59	1 35	1 38	1 70	1 72	1 34
8	1	1 78	1 76	1 53	1 55	1 23	1 70	1 39
	2	1 57	1 52	1 53	1 56	1 16	1 85	1 37
	3	1 78	1 65	1 57	1 60	1 24	1 67	1 27
	4	1 84	1 75	1 68	1 65	1 12	1 79	1 43
	5	1 83	1 55	1 62	1 60	1 25	1 75	1 53
	Mean	1 60	1 59	1 52	1 56	1 46	1 63	1 45
	SD	0 21	0 19	0 11	0 09	0 20	0 15	0 12
	RSD	12 89%	11 72%	7 32%	5 80%	13 68%	9 20%	8 41%

-20°C freeze/thaw, 1.5 ng/g

		Concentration determined (ng/g)						
Week	Sample	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
0	1	1 39	1 41	1 41	1 52	1 20	1 72	1 25
	2	1 27	1 35	1 35	1 54	1 25	1 72	1 43
	3	1 45	1 56	1 56	1 39	1 43	1 56	1 50
	4	1 45	1 37	1 37	1 63	1 41	1 67	1 33
	5	1 57	1 37	1 37	1 64	1 51	1 67	1 50
2	1	1 45	1 69	1 69	1 65	1 28	1 54	1 49
	2	1 52	1 69	1 69	1 53	1 49	1 36	1 58
	3	1 54	1 75	1 75	1 64	1 37	1 82	1 64
	4	1 67	1 60	1 60	1 43	1 61	1 68	1 61
	5	1 60	1 64	1 64	1 50	1 02	1 51	1 73
4	1	1 85	2 04	1 84	1 63	1 62	1 57	1 42
	2	1 61	1 50	1 72	1 58	1 50	1 47	1 27
	3	1 75	1 74	1 54	1 51	1 68	1 59	1 27
	4	1 68	2 02	1 61	1 55	1 56	1 54	1 27
	5	1 59	1 96	1 82	1 70	1 49	1 49	1 34
6	1	1 42	1 67	1 47	1 44	1 39	1 61	1 42
	2	1 44	1 87	1 41	1 53	1 60	1 50	1 27
	3	1 38	1 70	1 45	1 57	1 70	1 51	1 27
	4	1 47	1 76	1 53	1 46	1 60	1 55	1 27
	5	1 46	1 69	1 40	1 40	1 56	1 68	1 34
8	1	1 85	1 66	1 47	1 54	1 17	1 76	1 39
	2	1 82	1 41	1 35	1 58	1 09	1 73	1 37
	3	1 79	1 73	1 56	1 63	1 17	1 60	1 27
	4	1 65	1 42	1 42	1 59	1 11	1 80	1 43
	5	1 84	1 77	1 59	1 59	1 19	1 72	1 53
	Mean	1 58	1 65	1 54	1 55	1 40	1 61	1 41
	SD	0 17	0 20	0 15	0 08	0 20	0 11	0 14
	RSD	10 56%	11 96%	9 60%	5 30%	14 46%	7 11%	9 65%

-20°C, 10 ng/g

		Concentration determined (ng/g)						
Week	Sample	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
0	1	9 25	9 96	9 72	9 77	9 74	10 63	9 34
	2	8 51	10 07	10 28	10 14	9 14	10 50	10 44
	3	9 88	8 97	9 48	10 79	9 98	9 20	9 95
	4	9 56	9 72	10 51	9 82	8 10	10 20	10 42
	5	8 50	9 81	8 43	9 48	7 46	9 61	9 16
2	1	10 58	11 79	9 64	9 39	9 76	9 65	11 23
	2	9 52	12 13	10 43	9 64	10 07	10 00	9 49
	3	10 09	8 77	9 99	9 43	10 36	9 55	10 32
	4	10 09	12 90	10 98	10 99	10 42	10 02	11 25
	5	10 38	9 57	12 55	11 43	10 08	10 73	10 27
4	1	10 18	10 00	9 74	10 30	9 80	9 87	9 36
	2	10 54	12 84	11 11	10 80	10 52	10 13	10 38
	3	10 04	11 96	11 61	10 72	10 17	9 61	10 61
	4	12 73	12 63	10 69	11 16	10 78	10 06	10 62
	5	10 49	10 34	10 40	10 48	9 80	10 61	10 27
6	1	9 65	10 08	9 38	9 55	10 53	10 99	9 49
	2	9 92	11 48	9 93	10 34	10 73	10 09	10 42
	3	9 83	9 21	8 69	10 56	11 06	11 17	9 18
	4	10 20	10 65	9 57	10 34	10 79	10 70	10 32
	5	9 02	10 27	9 52	10 84	10 99	11 01	10 19
8	1	11 01	11 46	10 08	9 46	7 65	10 43	9 75
	2	11 55	12 63	9 75	9 71	7 06	11 17	10 49
	3	10 98	10 34	10 48	9 74	7 75	11 27	9 72
	4	11 56	10 80	9 89	10 13	7 66	11 03	10 21
	5	12 07	11 28	10 42	10 47	7 89	11 17	9 96
	Mean	10 25	10 79	10 13	10 22	9 53	10 38	10 11
	SD	1 02	1 25	0 87	0 60	1 28	0 61	0 57
	RSD	9 91%	11 55%	8 56%	5 86%	13 45%	5 85%	5 62%

-80°C, 10 ng/g

		Concentration determined (ng/g)						
Week	Sample	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
0	1	9 25	9 96	9 72	9 77	9 74	10 63	9 34
	2	8 51	10 07	10 28	10 14	9 14	10 50	10 44
	3	9 88	8 97	9 48	10 79	9 98	9 20	9 95
	4	9 56	9 72	10 51	9 82	8 10	10 20	10 42
	5	8 50	9 81	8 43	9 48	7 46	9 61	9 16
2	1	10 83	10 04	11 04	11 11	8 81	9 50	10 81
	2	10 66	9 97	10 73	9 88	10 07	11 38	10 60
	3	10 43	10 17	9 68	10 47	10 56	10 39	9 27
	4	9 86	10 27	10 38	9 93	8 31	9 13	10 36
	5	10 40	9 42	10 26	9 48	8 76	9 72	9 53
4	1	10 95	12 04	10 06	10 74	10 27	9 91	10 60
	2	11 87	10 13	10 58	10 53	11 55	10 48	9 41
	3	11 00	9 52	10 07	10 78	10 88	10 15	9 64
	4	10 93	14 12	12 08	10 10	9 59	10 10	10 64
	5	11 44	10 79	11 31	10 87	9 58	10 79	9 03
6	1	9 26	13 87	9 49	10 02	10 75	10 17	11 12
	2	9 30	11 77	9 82	9 37	10 52	10 98	11 48
	3	9 55	12 63	8 92	10 25	10 31	11 09	11 18
	4	9 98	12 99	9 00	10 16	10 74	10 92	10 51
	5	10 05	11 81	8 94	9 45	9 59	11 08	10 30
8	1	12 72	10 90	9 43	10 24	8 46	11 26	11 90
	2	11 61	9 71	9 62	10 50	8 59	11 07	10 53
	3	11 37	10 42	10 49	10 07	8 06	10 54	9 68
	4	11 95	12 75	10 10	10 14	7 59	10 56	10 52
	5	11 85	10 04	8 90	10 06	8 15	12 27	10 05
	Mean	10 47	10 88	9 97	10 17	9 42	10 47	10 26
	SD	1 12	1 45	0 84	0 47	1 14	0 73	0 75
	RSD	10 67%	13 32%	8 43%	4 66%	12 11%	6 97%	7 28%

-20°C freeze/thaw, 10 ng/g

		Concentration determined (ng/g)						
Week	Sample	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
0	1	9 25	9 96	9 72	9 77	9 74	10 63	9 34
	2	8 51	10 07	10 28	10 14	9 14	10 50	10 44
	3	9 88	8 97	9 48	10 79	9 98	9 20	9 95
	4	9 56	9 72	10 51	9 82	8 10	10 20	10 42
	5	8 50	9 81	8 43	9 48	7 46	9 61	9 16
2	1	10 72	9 09	11 60	9 92	9 25	10 07	9 41
	2	11 68	9 57	10 52	9 99	9 28	9 45	9 65
	3	10 01	9 64	11 18	10 18	10 98	10 92	11 03
	4	11 21	9 62	11 55	10 93	9 46	10 53	9 79
	5	10 82	8 73	10 60	11 30	10 58	10 62	10 27
4	1	11 87	12 86	11 83	10 58	10 62	9 67	10 93
	2	11 16	12 86	11 69	10 20	9 97	10 30	10 48
	3	10 97	11 43	11 16	9 53	10 81	9 80	10 09
	4	10 84	12 53	10 96	11 81	10 69	10 15	11 33
	5	12 01	12 40	11 62	10 84	10 78	10 82	11 50
6	1	9 68	11 69	9 42	10 61	11 02	10 21	11 45
	2	10 68	11 83	10 13	11 12	10 94	10 93	10 77
	3	10 26	11 37	9 28	10 52	10 75	11 54	9 88
	4	11 10	12 18	9 92	10 38	10 12	10 88	11 33
	5	9 29	11 14	9 44	9 44	11 13	10 12	9 61
8	1	11 14	8 83	8 96	9 61	7 81	10 16	7 96
	2	10 84	9 50	9 24	9 09	7 41	9 90	8 99
	3	11 52	10 08	10 03	10 11	7 98	11 84	9 70
	4	11 02	12 05	10 03	9 52	7 54	10 18	10 41
	5	11 36	9 99	9 70	10 18	7 26	11 23	9 77
	Mean	10 56	10 64	10 29	10 23	9 55	10 38	10 15
	SD	0 98	1 36	0 96	0 66	1 35	0 64	0 86
	RSD	9 29%	12 80%	9 32%	6 43%	14 11%	6 12%	8 50%

Appendix 11 Raw data for LOD, LOQ and CCα for tilapia, salmon and shrimp muscle.

Tilapia muscle

Run 1			Blank1	Blank2	Blank3	Blank4	Blank5
HMMNI	Response	height	2490	1680	1660	2040	1590
	Tissue conc	ng/g	0 065	0 041	0 045	0 055	0 043
IPZ	Response	height	1570	1130	1370	1350	1960
	Tissue conc	ng/g	0 024	0 016	0 022	0 021	0 031
IPZ-OH	Response	height	4700	3850	4320	3720	3240
	Tissue conc	ng/g	0 039	0 031	0 037	0 032	0 028
MNZ	Response	height	1490	1910	1460	1580	1960
	Tissue conc	ng/g	0 016	0 020	0 017	0 018	0 022
MNZ-OH	Response	height	1460	1170	1480	2080	2210
	Tissue conc	ng/g	0 07	0 05	0 07	0 10	0 11
RNZ	Response	height	5160	4160	5170	4210	5370
	Tissue conc	ng/g	0 095	0 073	0 098	0 080	0 102
DMZ	Response	height	3270	2290	2150	2520	2880
	Tissue conc	ng/g	0 047	0 037	0 027	0 029	0 034
Run 2							
HMMNI	Response	height	2580	2030	1710	1630	1920
	Tissue conc	ng/g	0 075	0 055	0 047	0 047	0 055
IPZ	Response	height	1380	1700	1440	1380	2190
	Tissue conc	ng/g	0 018	0 021	0 018	0 018	0 028
IPZ-OH	Response	height	5690	4410	5340	5780	5400
	Tissue conc	ng/g	0 047	0 034	0 042	0 048	0 045
MNZ	Response	height	1900	1880	2210	1280	1440
	Tissue conc	ng/g	0 022	0 020	0 024	0 015	0 016
MNZ-OH	Response	height	1990	1990	4310	1930	1400
	Tissue conc	ng/g	0 096	0 090	0 194	0 093	0 067
RNZ	Response	height	5150	4260	4000	5290	4130
	Tissue conc	ng/g	0 090	0 070	0 066	0 093	0 072
DMZ	Response	height	2720	2320	1660	1670	2010
	Tissue conc	ng/g	0 047	0 037	0 027	0 029	0 034
Run 3							
HMMNI	Response	height	3350	2560	3080	3100	3440
	Tissue conc	ng/g	0 077	0 062	0 072	0 077	0 081
IPZ	Response	height	5470	1400	1670	2420	3600
	Tissue conc	ng/g	0 046	0 013	0 014	0 022	0 031
IPZ-OH	Response	height	5530	4060	3610	2750	4210
	Tissue conc	ng/g	0 036	0 028	0 024	0 019	0 028
MNZ	Response	height	4130	2110	2090	1500	1420
	Tissue conc	ng/g	0 035	0 019	0 018	0 014	0 012
MNZ-OH	Response	height	2000	1600	1560	2180	2670
	Tissue conc	ng/g	0 065	0 054	0 051	0 076	0 088
RNZ	Response	height	3230	2710	2590	4520	4570
	Tissue conc	ng/g	0 048	0 043	0 039	0 073	0 070
DMZ	Response	height	5430	2270	3100	2006	2350
	Tissue conc	ng/g	0 035	0 015	0 020	0 014	0 015
Run 4							
HMMNI	Response	height	3000	2250	1010	1680	1560
	Tissue conc	ng/g	0 217	0 151	0 070	0 118	0 110
IPZ	Response	height	1320	725	1210	739	840
	Tissue conc	ng/g	0 037	0 019	0 032	0 020	0 023
IPZ-OH	Response	height	3120	2490	2440	2630	2400
	Tissue conc	ng/g	0 043	0 032	0 032	0 035	0 032
MNZ	Response	height	1590	962	1670	998	2010
	Tissue conc	ng/g	0 037	0 021	0 038	0 023	0 046
MNZ-OH	Response	height	2040	1620	1530	1480	2230
	Tissue conc	ng/g	0 209	0 154	0 150	0 147	0 222
RNZ	Response	height	2540	2570	3380	3190	2640
	Tissue conc	ng/g	0 112	0 105	0 143	0 136	0 113
DMZ	Response	height	1620	3130	2000	982	1150
	Tissue conc	ng/g	0 024	0 043	0 029	0 014	0 017

Salmon Muscle

Run 1			Blank1	Blank2	Blank3	Blank4	Blank5
HMMNI	Response	height	897	1160	2180	1270	1130
	Tissue conc	ng/g	0 097	0 123	0 228	0 136	0 126
IPZ	Response	height	1320	670	1250	1250	1070
	Tissue conc	ng/g	0 059	0 031	0 057	0 059	0 053
IPZ-OH	Response	height	4640	2610	2420	1840	4140
	Tissue conc	ng/g	0 123	0 072	0 066	0 051	0 121
MNZ	Response	height	1710	410	1070	926	1020
	Tissue conc	ng/g	0 069	0 017	0 044	0 039	0 045
MNZ-OH	Response	height	862	1840	775	1570	1740
	Tissue conc	ng/g	0 124	0 276	0 114	0 237	0 274
RNZ	Response	height	3490	1920	2320	3920	1940
	Tissue conc	ng/g	0 177	0 102	0 121	0 209	0 111
DMZ	Response	height	2480	3120	1490	1940	2330
	Tissue conc	ng/g	0 076	0 100	0 047	0 063	0 079
Run 2							
HMMNI	Response	height	1700	2250	1430	2800	3210
	Tissue conc	ng/g	0 111	0 140	0 089	0 183	0 208
IPZ	Response	height	984	909	965	836	918
	Tissue conc	ng/g	0 026	0 023	0 024	0 022	0 023
IPZ-OH	Response	height	3004	3030	3380	2780	2830
	Tissue conc	ng/g	0 078	0 075	0 084	0 073	0 071
MNZ	Response	height	813	778	522	538	569
	Tissue conc	ng/g	0 028	0 026	0 017	0 019	0 019
MNZ-OH	Response	height	900	1530	1080	1010	736
	Tissue conc	ng/g	0 098	0 158	0 112	0 110	0 077
RNZ	Response	height	2210	1590	2350	2470	2730
	Tissue conc	ng/g	0 151	0 104	0 154	0 169	0 179
DMZ	Response	height	1360	1480	1200	1090	1010
	Tissue conc	ng/g	0 029	0 030	0 024	0 023	0 020
Run 3							
HMMNI	Response	height	2020	1810	1770	1930	1630
	Tissue conc	ng/g	0 174	0 158	0 149	0 164	0 145
IPZ	Response	height	1230	857	1220	767	816
	Tissue conc	ng/g	0 049	0 034	0 047	0 030	0 033
IPZ-OH	Response	height	5340	4950	1980	3200	5140
	Tissue conc	ng/g	0 134	0 126	0 049	0 079	0 133
MNZ	Response	height	916	558	828	872	728
	Tissue conc	ng/g	0 025	0 016	0 022	0 024	0 021
MNZ-OH	Response	height	1370	1570	1150	1220	1260
	Tissue conc	ng/g	0 149	0 173	0 122	0 131	0 141
RNZ	Response	height	3170	2650	4470	3500	2810
	Tissue conc	ng/g	0 149	0 126	0 206	0 163	0 136
DMZ	Response	height	1640	1580	1270	1370	1200
	Tissue conc	ng/g	0 043	0 042	0 033	0 036	0 033
Run 4							
HMMNI	Response	height	1740	1610	1340	1470	2710
	Tissue conc	ng/g	0 087	0 079	0 068	0 069	0 136
IPZ	Response	height	758	579	506	453	475
	Tissue conc	ng/g	0 081	0 061	0 055	0 046	0 051
IPZ-OH	Response	height	5222	4240	3460	4030	4290
	Tissue conc	ng/g	0 062	0 049	0 042	0 045	0 051
MNZ	Response	height	1050	1120	1140	718	1630
	Tissue conc	ng/g	0 019	0 020	0 021	0 012	0 030
MNZ-OH	Response	height	1190	1140	599	867	1560
	Tissue conc	ng/g	0 084	0 079	0 043	0 061	0 110
RNZ	Response	height	3360	3670	2350	3990	4180
	Tissue conc	ng/g	0 091	0 097	0 065	0 102	0 114
DMZ	Response	height	2050	2260	1380	1100	1060
	Tissue conc	ng/g	0 024	0 026	0 016	0 012	0 012

Shrimp Muscle

Run 1			Blank1	Blank2	Blank3	Blank4	Blank5
HMMNI	Response	height	1420	1460	1150	1800	1450
	Tissue conc	ng/g	0 070	0 074	0 056	0 087	0 074
IPZ	Response	height	1740	1310	1670	1910	1050
	Tissue conc	ng/g	0 027	0 021	0 026	0 029	0 017
IPZ-OH	Response	height	3370	3120	3540	4640	2980
	Tissue conc	ng/g	0 029	0 027	0 030	0 039	0 026
MNZ	Response	height	1810	1630	1930	3130	957
	Tissue conc	ng/g	0 027	0 025	0 028	0 046	0 015
MNZ-OH	Response	height	1180	1470	1720	1380	2080
	Tissue conc	ng/g	0 093	0 118	0 132	0 106	0 168
RNZ	Response	height	3330	1850	3220	5040	3120
	Tissue conc	ng/g	0 074	0 042	0 070	0 109	0 071
DMZ	Response	height	1520	3030	1660	2330	2740
	Tissue conc	ng/g	0 016	0 032	0 017	0 024	0 029
Run 2							
HMMNI	Response	height	2740	3170	1770	1370	2110
	Tissue conc	ng/g	0 121	0 138	0 078	0 060	0 092
IPZ	Response	height	4050	1460	2420	2110	2380
	Tissue conc	ng/g	0 061	0 021	0 036	0 031	0 035
IPZ-OH	Response	height	8820	5680	6800	5190	8010
	Tissue conc	ng/g	0 069	0 044	0 053	0 040	0 062
MNZ	Response	height	7600	2310	3590	3030	3850
	Tissue conc	ng/g	0 099	0 030	0 046	0 039	0 049
MNZ-OH	Response	height	1700	1030	1390	2220	1270
	Tissue conc	ng/g	0 121	0 072	0 098	0 157	0 089
RNZ	Response	height	4250	4090	3630	3200	2900
	Tissue conc	ng/g	0 094	0 089	0 080	0 071	0 063
DMZ	Response	height	5800	3620	5390	5320	5750
	Tissue conc	ng/g	0 053	0 033	0 049	0 049	0 052
Run 3							
HMMNI	Response	height	1300	1590	989	1100	2810
	Tissue conc	ng/g	0 038	0 049	0 030	0 033	0 083
IPZ	Response	height	1850	2650	3260	1950	2920
	Tissue conc	ng/g	0 026	0 023	0 028	0 017	0 024
IPZ-OH	Response	height	4100	3400	4060	3940	5570
	Tissue conc	ng/g	0 021	0 018	0 021	0 021	0 028
MNZ	Response	height	2590	2800	5470	2720	3940
	Tissue conc	ng/g	0 021	0 023	0 044	0 022	0 031
MNZ-OH	Response	height	911	1450	1390	1460	1080
	Tissue conc	ng/g	0 035	0 059	0 055	0 058	0 042
RNZ	Response	height	1690	2580	3960	2330	2520
	Tissue conc	ng/g	0 021	0 034	0 050	0 030	0 031
DMZ	Response	height	2460	3510	4050	4350	5430
	Tissue conc	ng/g	0 013	0 020	0 022	0 024	0 029
Run 4							
HMMNI	Response	height	1590	1720	1460	1010	1290
	Tissue conc	ng/g	0 045	0 049	0 040	0 028	0 035
IPZ	Response	height	2490	1320	1780	1630	3320
	Tissue conc	ng/g	0 022	0 012	0 016	0 014	0 029
IPZ-OH	Response	height	3780	3610	4290	3270	5070
	Tissue conc	ng/g	0 020	0 019	0 022	0 017	0 025
MNZ	Response	height	2080	2780	2410	2500	5810
	Tissue conc	ng/g	0 017	0 023	0 020	0 021	0 046
MNZ-OH	Response	height	684	828	1380	1200	900
	Tissue conc	ng/g	0 032	0 039	0 063	0 055	0 040
RNZ	Response	height	1660	1750	1770	2980	1910
	Tissue conc	ng/g	0 022	0 023	0 023	0 039	0 024
DMZ	Response	height	2090	2110	1310	2530	1820
	Tissue conc	ng/g	0 014	0 014	0 009	0 017	0 012

Appendix 12 Method recovery results for tilapia, salmon and shrimp muscle including matrix matched recovery, internal standard corrected recovery and DMZ-D_3 recovery.

Matrix matched recovery for tilapia muscle

Recovery Results									
Level	Day	Rep	Percent Recovery						
			HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1ng/g in 4g	1	1	92%	118%	114%	99%	103%	99%	109%
		2	102%	112%	118%	105%	94%	122%	108%
		3	106%	103%	113%	94%	90%	112%	122%
		4	104%	103%	117%	101%	105%	91%	111%
		5	102%	98%	119%	112%	88%	116%	105%
	2	1	117%	93%	129%	112%	114%	123%	102%
		2	100%	106%	117%	116%	92%	119%	113%
		3	107%	94%	114%	126%	103%	123%	99%
		4	119%	108%	126%	121%	97%	121%	125%
		5	130%	106%	126%	132%	100%	131%	111%
	3	1	109%	116%	140%	129%	89%	120%	123%
		2	120%	97%	126%	113%	102%	111%	112%
		3	121%	104%	131%	121%	96%	121%	110%
		4	130%	101%	126%	121%	73%	117%	110%
		5	124%	104%	138%	115%	105%	102%	107%
		$\bar{x}$	**112%**	**104%**	**124%**	**114%**	**97%**	**115%**	**111%**
		SD	**11.6%**	**7.3%**	**8.5%**	**11.1%**	**9.7%**	**10.6%**	**7.4%**
		%RSD	**10.4%**	**7.0%**	**6.9%**	**9.7%**	**10.1%**	**9.2%**	**6.6%**
10ng/g in 4g	1	1	92%	97%	106%	92%	88%	100%	102%
		2	106%	93%	109%	98%	87%	109%	93%
		3	92%	87%	104%	99%	94%	104%	106%
		4	93%	105%	113%	99%	96%	111%	102%
		5	103%	93%	115%	98%	84%	103%	98%
	2	1	108%	93%	123%	119%	104%	118%	104%
		2	117%	98%	130%	124%	102%	125%	103%
		3	118%	96%	124%	120%	109%	126%	106%
		4	112%	97%	120%	118%	97%	120%	106%
		5	112%	108%	119%	114%	101%	118%	108%
	3	1	110%	99%	142%	120%	89%	110%	110%
		2	126%	107%	133%	113%	92%	116%	106%
		3	117%	111%	128%	118%	95%	123%	108%
		4	117%	99%	127%	117%	88%	122%	113%
		5	112%	106%	140%	133%	97%	114%	107%
		$\bar{x}$	**109%**	**99%**	**122%**	**112%**	**95%**	**115%**	**105%**
		SD	**10.2%**	**6.8%**	**11.5%**	**11.9%**	**7.1%**	**8.2%**	**4.9%**
		%RSD	**9.4%**	**6.8%**	**9.4%**	**10.6%**	**7.5%**	**7.2%**	**4.6%**
50ng/g in 4g	1	1	100%	121%	114%	88%	84%	100%	101%
		2	103%	126%	120%	100%	88%	105%	111%
		3	98%	116%	115%	105%	87%	96%	104%
		4	96%	119%	112%	99%	91%	105%	113%
		5	92%	97%	119%	102%	88%	94%	100%
	2	1	101%	103%	112%	104%	88%	107%	98%
		2	107%	100%	115%	104%	83%	109%	97%
		3	101%	107%	113%	105%	90%	105%	96%
		4	107%	98%	103%	102%	87%	106%	95%
		5	102%	104%	106%	104%	89%	102%	97%
	3	1	115%	97%	125%	107%	87%	105%	114%
		2	119%	111%	139%	125%	94%	112%	117%
		3	127%	108%	127%	117%	93%	103%	118%
		4	111%	120%	130%	113%	93%	102%	126%
		5	120%	100%	126%	115%	95%	110%	120%
		$\bar{x}$	**107%**	**108%**	**118%**	**106%**	**89%**	**104%**	**107%**
		SD	**9.9%**	**9.8%**	**9.6%**	**8.7%**	**3.5%**	**4.9%**	**10.3%**
		%RSD	**9.3%**	**9.0%**	**8.1%**	**8.2%**	**4.0%**	**4.7%**	**9.6%**
		$\bar{x}$	**109%**	**104%**	**121%**	**111%**	**94%**	**111%**	**108%**
		SD	**11%**	**9%**	**10%**	**11%**	**8%**	**10%**	**8%**
		%RSD	**10%**	**8%**	**8%**	**10%**	**8%**	**9%**	**8%**

Matrix matched recovery for salmon muscle

Level	Day	Rep.	Recovery Results Percent Recovery HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1ng/g in 4g	1	1	101%	116%	107%	111%	81%	105%	105%
		2	108%	95%	117%	108%	82%	111%	111%
		3	119%	110%	117%	111%	95%	120%	114%
		4	107%	121%	114%	107%	128%	118%	105%
		5	142%	119%	117%	108%	92%	111%	114%
	2	1	115%	110%	111%	115%	97%	112%	112%
		2	119%	112%	117%	120%	95%	115%	114%
		3	100%	107%	121%	107%	88%	112%	120%
		4	113%	107%	117%	116%	90%	113%	122%
		5	124%	124%	114%	125%	95%	116%	122%
	3	1	128%	117%	143%	122%	96%	119%	128%
		2	128%	106%	136%	110%	91%	108%	126%
		3	125%	115%	154%	124%	94%	116%	115%
		4	126%	124%	135%	115%	96%	116%	133%
		5	126%	115%	131%	117%	89%	111%	122%
		$\bar{x}$	**119%**	**113%**	**123%**	**114%**	**94%**	**114%**	**118%**
		SD	**11.5%**	**7.7%**	**13.3%**	**6.2%**	**10.6%**	**4.1%**	**8.1%**
		%RSD	**9.7%**	**6.8%**	**10.8%**	**5.4%**	**11.3%**	**3.6%**	**6.9%**
10ng/g in 4g	1	1	99%	99%	96%	99%	82%	111%	99%
		2	110%	87%	99%	109%	99%	117%	113%
		3	113%	109%	102%	108%	111%	127%	113%
		4	95%	99%	109%	99%	105%	117%	96%
		5	146%	118%	112%	97%	104%	111%	98%
	2	1	99%	102%	94%	95%	66%	99%	101%
		2	114%	119%	106%	106%	79%	119%	110%
		3	114%	119%	92%	108%	75%	91%	118%
		4	111%	110%	105%	80%	72%	112%	114%
		5	140%	101%	103%	80%	73%	118%	106%
	3	1	111%	115%	123%	105%	78%	84%	118%
		2	114%	114%	118%	100%	91%	107%	114%
		3	149%	108%	119%	80%	73%	85%	105%
		4	127%	116%	123%	110%	99%	117%	124%
		5	142%	118%	122%	86%	98%	113%	124%
		$\bar{x}$	**119%**	**109%**	**108%**	**97%**	**87%**	**109%**	**110%**
		SD	**17.6%**	**9.5%**	**10.8%**	**11.0%**	**14.6%**	**13.0%**	**9.1%**
		%RSD	**14.8%**	**8.7%**	**10.0%**	**11.3%**	**16.8%**	**12.0%**	**8.2%**
50ng/g in 4g	1	1	109%	93%	110%	121%	85%	90%	75%
		2	117%	102%	90%	131%	72%	109%	111%
		3	119%	94%	114%	142%	84%	97%	99%
		4	102%	108%	111%	108%	85%	89%	83%
		5	113%	114%	122%	139%	85%	129%	88%
	2	1	114%	130%	106%	111%	91%	140%	120%
		2	120%	116%	115%	143%	80%	105%	108%
		3	121%	119%	74%	114%	71%	104%	107%
		4	115%	104%	114%	108%	82%	102%	103%
		5	129%	104%	94%	143%	74%	135%	101%
	3	1	112%	103%	115%	101%	73%	95%	81%
		2	119%	93%	89%	103%	78%	103%	98%
		3	127%	115%	106%	103%	82%	105%	84%
		4	124%	113%	122%	105%	86%	136%	107%
		5	115%	103%	123%	109%	81%	120%	99%
		$\bar{x}$	**117%**	**107%**	**107%**	**119%**	**81%**	**111%**	**98%**
		SD	**6.9%**	**10.5%**	**14.2%**	**16.2%**	**5.9%**	**17.1%**	**12.8%**
		%RSD	**5.9%**	**9.8%**	**13.3%**	**13.7%**	**7.3%**	**15.5%**	**13.1%**
		$\bar{x}$	**118%**	**110%**	**113%**	**110%**	**87%**	**111%**	**108%**
		SD	**13%**	**9%**	**15%**	**15%**	**12%**	**13%**	**13%**
		%RSD	**11%**	**9%**	**13%**	**13%**	**14%**	**11%**	**12%**

Matrix matched recovery for shrimp muscle

Recovery Results									
Level	Day	Rep.	Percent Recovery HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1ng/g in 4g	1	1	101%	99%	107%	98%	84%	106%	94%
		2	66%	75%	82%	76%	75%	90%	88%
		3	83%	96%	95%	87%	75%	109%	89%
		4	85%	99%	108%	105%	97%	134%	102%
		5	91%	90%	90%	92%	90%	109%	81%
	2	1	106%	108%	122%	119%	107%	123%	109%
		2	96%	104%	122%	118%	93%	119%	110%
		3	104%	115%	125%	121%	91%	124%	112%
		4	103%	110%	123%	119%	103%	123%	111%
		5	96%	109%	123%	117%	98%	129%	111%
	3	1	109%	106%	119%	111%	106%	144%	108%
		2	115%	106%	127%	127%	112%	124%	118%
		3	109%	112%	134%	129%	98%	146%	110%
		4	124%	117%	131%	130%	117%	137%	112%
		5	123%	119%	137%	127%	130%	144%	118%
		$\bar{x}$	**101%**	**104%**	**116%**	**112%**	**98%**	**124%**	**105%**
		SD	**15.4%**	**11.4%**	**16.4%**	**16.6%**	**15.0%**	**15.9%**	**11.4%**
		%RSD	**15.3%**	**10.9%**	**14.1%**	**14.9%**	**15.2%**	**12.8%**	**10.9%**
10ng/g in 4g	1	1	84%	107%	107%	96%	78%	113%	93%
		2	99%	108%	108%	101%	94%	121%	102%
		3	92%	108%	114%	100%	91%	116%	104%
		4	91%	104%	112%	103%	89%	110%	103%
		5	96%	97%	115%	113%	92%	107%	101%
	2	1	94%	101%	109%	100%	87%	110%	98%
		2	100%	106%	115%	106%	95%	117%	101%
		3	100%	102%	114%	105%	92%	117%	99%
		4	96%	100%	111%	101%	85%	112%	93%
		5	99%	104%	115%	106%	85%	116%	98%
	3	1	105%	110%	124%	116%	94%	123%	112%
		2	125%	115%	133%	128%	104%	136%	120%
		3	115%	111%	131%	124%	101%	133%	116%
		4	117%	115%	129%	120%	101%	130%	113%
		5	124%	121%	131%	129%	104%	131%	120%
		$\bar{x}$	**102%**	**107%**	**118%**	**110%**	**93%**	**119%**	**105%**
		SD	**12.3%**	**6.4%**	**9.1%**	**11.0%**	**7.5%**	**9.2%**	**9.1%**
		%RSD	**12.0%**	**6.0%**	**7.7%**	**10.0%**	**8.1%**	**7.7%**	**8.6%**
50ng/g in 4g	1	1	90%	97%	100%	93%	85%	105%	92%
		2	86%	102%	105%	97%	93%	113%	95%
		3	90%	106%	110%	99%	95%	114%	103%
		4	98%	100%	119%	101%	95%	115%	101%
		5	93%	102%	109%	97%	94%	107%	100%
	2	1	88%	101%	98%	86%	81%	100%	91%
		2	90%	106%	100%	90%	82%	103%	94%
		3	90%	98%	97%	89%	84%	101%	92%
		4	83%	94%	93%	85%	78%	93%	87%
		5	81%	93%	92%	85%	78%	93%	84%
	3	1	106%	120%	123%	113%	100%	114%	114%
		2	112%	124%	126%	117%	102%	115%	123%
		3	117%	122%	127%	119%	110%	119%	123%
		4	121%	121%	128%	121%	108%	121%	126%
		5	116%	122%	129%	119%	107%	118%	125%
		$\bar{x}$	**97%**	**107%**	**110%**	**101%**	**93%**	**109%**	**103%**
		SD	**13.4%**	**11.3%**	**13.7%**	**13.5%**	**11.0%**	**9.2%**	**14.9%**
		%RSD	**13.7%**	**10.5%**	**12.4%**	**13.4%**	**11.9%**	**8.4%**	**14.4%**
		$\bar{x}$	**100%**	**106%**	**115%**	**107%**	**95%**	**117%**	**104%**
		SD	**14%**	**10%**	**14%**	**14%**	**12%**	**13%**	**12%**
		%RSD	**14%**	**9%**	**12%**	**13%**	**12%**	**11%**	**11%**

Internal standard corrected recovery in tilapia muscle

Recovery Results									
Level	Day		Percent Recovery						
		Rep.	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1ng/g in 4g	1	1	88%	112%	108%	94%	98%	94%	104%
		2	92%	101%	107%	94%	85%	110%	98%
		3	94%	91%	100%	84%	80%	99%	108%
		4	108%	107%	121%	104%	108%	94%	115%
		5	104%	100%	121%	114%	90%	118%	108%
	2	1	94%	75%	104%	90%	92%	99%	82%
		2	85%	90%	100%	99%	78%	102%	97%
		3	99%	87%	105%	116%	94%	113%	91%
		4	104%	94%	111%	106%	85%	106%	110%
		5	112%	92%	109%	114%	86%	113%	96%
	3	1	99%	107%	125%	115%	88%	122%	101%
		2	128%	104%	132%	118%	119%	132%	108%
		3	131%	114%	138%	128%	112%	146%	107%
		4	124%	98%	117%	113%	76%	124%	95%
		5	141%	116%	148%	123%	124%	124%	105%
		$\bar{x}$	**107%**	**99%**	**116%**	**107%**	**94%**	**113%**	**102%**
		SD	**17.0%**	**11.3%**	**14.4%**	**12.9%**	**14.9%**	**14.9%**	**8.6%**
		%RSD	**15.9%**	**11.4%**	**12.3%**	**12.0%**	**15.8%**	**13.1%**	**8.4%**
10ng/g in 4g	1	1	82%	86%	95%	82%	79%	89%	91%
		2	111%	97%	115%	103%	91%	114%	98%
		3	94%	88%	106%	101%	95%	106%	108%
		4	89%	100%	108%	95%	92%	106%	98%
		5	106%	96%	118%	101%	87%	106%	100%
	2	1	105%	91%	120%	116%	101%	116%	102%
		2	108%	91%	119%	114%	94%	115%	94%
		3	110%	89%	116%	112%	102%	118%	99%
		4	116%	100%	124%	122%	100%	124%	110%
		5	111%	107%	118%	113%	101%	117%	108%
	3	1	109%	100%	138%	117%	96%	122%	98%
		2	119%	102%	122%	104%	95%	122%	90%
		3	133%	127%	142%	131%	118%	156%	110%
		4	106%	90%	112%	103%	86%	123%	91%
		5	104%	99%	127%	120%	97%	118%	89%
		$\bar{x}$	**107%**	**98%**	**119%**	**109%**	**96%**	**117%**	**99%**
		SD	**12.2%**	**10.1%**	**11.7%**	**12.2%**	**8.9%**	**14.2%**	**7.3%**
		%RSD	**11.4%**	**10.4%**	**9.9%**	**11.2%**	**9.3%**	**12.1%**	**7.4%**
50ng/g in 4g	1	1	93%	113%	107%	82%	78%	93%	94%
		2	95%	117%	111%	92%	81%	97%	103%
		3	81%	96%	96%	88%	72%	80%	86%
		4	84%	104%	98%	87%	80%	92%	99%
		5	93%	98%	120%	102%	88%	94%	101%
	2	1	114%	117%	126%	118%	99%	121%	111%
		2	94%	87%	100%	91%	72%	95%	85%
		3	87%	93%	98%	92%	78%	91%	84%
		4	91%	83%	87%	87%	74%	90%	81%
		5	94%	96%	97%	96%	82%	94%	89%
	3	1	112%	96%	119%	102%	92%	114%	100%
		2	124%	118%	142%	127%	106%	130%	109%
		3	123%	106%	120%	111%	98%	112%	103%
		4	102%	112%	117%	101%	92%	104%	99%
		5	0%	0%	0%	0%	0%	0%	0%
		$\bar{x}$	**99%**	**103%**	**110%**	**98%**	**85%**	**101%**	**96%**
		SD	**13.9%**	**11.5%**	**14.9%**	**12.9%**	**10.8%**	**13.8%**	**9.6%**
		%RSD	**14.0%**	**11.3%**	**13.6%**	**13.2%**	**12.7%**	**13.8%**	**10.0%**
		$\bar{x}$	**104%**	**100%**	**115%**	**105%**	**92%**	**110%**	**99%**
		SD	**15%**	**11%**	**14%**	**13%**	**12%**	**16%**	**9%**
		%RSD	**14%**	**11%**	**12%**	**13%**	**14%**	**14%**	**9%**

Internal standard corrected recovery in salmon muscle

Recovery Results									
Level	Day	Rep.	Percent Recovery HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1ng/g in 4g	1	1	88%	102%	94%	97%	71%	92%	92%
		2	101%	88%	108%	100%	76%	103%	103%
		3	102%	94%	100%	95%	82%	103%	98%
		4	92%	104%	98%	92%	110%	101%	90%
		5	113%	95%	94%	86%	73%	88%	92%
	2	1	111%	110%	107%	110%	91%	123%	110%
		2	94%	75%	85%	93%	85%	100%	97%
		3	99%	95%	89%	95%	97%	111%	99%
		4	105%	110%	121%	110%	117%	130%	107%
		5	172%	139%	132%	115%	123%	131%	116%
	3	1	109%	93%	110%	121%	85%	90%	75%
		2	117%	102%	90%	131%	72%	109%	111%
		3	119%	94%	114%	142%	84%	97%	99%
		4	102%	108%	111%	108%	85%	89%	83%
		5	113%	114%	122%	139%	85%	129%	88%
		$\bar{x}$	**109%**	**102%**	**105%**	**109%**	**89%**	**106%**	**97%**
		SD	**19.6%**	**14.4%**	**13.7%**	**17.6%**	**16.1%**	**15.3%**	**11.1%**
		%RSD	**18.0%**	**14.2%**	**13.0%**	**16.2%**	**18.1%**	**14.4%**	**11.4%**
10ng/g in 4g	1	1	99%	94%	95%	98%	83%	96%	96%
		2	95%	90%	94%	96%	76%	92%	91%
		3	91%	96%	109%	96%	79%	101%	108%
		4	97%	91%	99%	99%	77%	96%	104%
		5	90%	83%	83%	91%	69%	84%	89%
	2	1	112%	115%	106%	107%	75%	112%	114%
		2	125%	131%	116%	116%	87%	131%	121%
		3	125%	131%	101%	119%	83%	100%	130%
		4	124%	122%	117%	89%	80%	125%	127%
		5	154%	111%	114%	89%	81%	130%	117%
	3	1	114%	130%	106%	111%	91%	140%	120%
		2	120%	116%	115%	143%	80%	105%	108%
		3	121%	119%	74%	114%	71%	104%	107%
		4	115%	104%	114%	108%	82%	102%	103%
		5	129%	104%	94%	143%	74%	135%	101%
		$\bar{x}$	**114%**	**109%**	**102%**	**108%**	**79%**	**110%**	**109%**
		SD	**17.4%**	**16.0%**	**12.8%**	**17.2%**	**5.8%**	**17.5%**	**12.4%**
		%RSD	**15.3%**	**14.6%**	**12.5%**	**16.0%**	**7.3%**	**15.9%**	**11.3%**
50ng/g in 4g	1	1	94%	92%	100%	97%	75%	92%	100%
		2	109%	95%	109%	100%	82%	96%	113%
		3	90%	88%	104%	95%	72%	87%	87%
		4	88%	92%	89%	86%	71%	84%	98%
		5	100%	97%	98%	100%	75%	92%	102%
	2	1	122%	134%	127%	123%	91%	97%	136%
		2	122%	130%	119%	115%	103%	120%	128%
		3	178%	137%	134%	102%	92%	106%	132%
		4	141%	137%	129%	131%	116%	136%	145%
		5	153%	135%	123%	100%	112%	127%	141%
	3	1	112%	103%	115%	101%	73%	95%	81%
		2	119%	93%	89%	103%	78%	103%	98%
		3	127%	115%	106%	103%	82%	105%	84%
		4	124%	113%	122%	105%	86%	136%	107%
		5	115%	103%	123%	109%	81%	120%	99%
		$\bar{x}$	**120%**	**111%**	**112%**	**105%**	**86%**	**106%**	**110%**
		SD	**24.2%**	**18.9%**	**14.4%**	**11.2%**	**14.3%**	**17.3%**	**21.2%**
		%RSD	**20.2%**	**17.0%**	**12.8%**	**10.7%**	**16.7%**	**16.2%**	**19.3%**
		$\bar{x}$	**114%**	**107%**	**107%**	**107%**	**85%**	**108%**	**105%**
		SD	**21%**	**17%**	**14%**	**15%**	**13%**	**16%**	**16%**
		%RSD	**18%**	**16%**	**13%**	**14%**	**16%**	**15%**	**15%**

Internal standard corrected recovery in shrimp muscle

Recovery Results									
Level	Day	Rep.	Percent Recovery HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1ng/g in 4g	1	1	105%	103%	112%	102%	87%	111%	98%
		2	65%	73%	80%	75%	73%	88%	86%
		3	81%	93%	92%	85%	73%	106%	86%
		4	84%	98%	107%	104%	95%	132%	101%
		5	93%	93%	93%	94%	93%	112%	83%
	2	1	93%	95%	106%	104%	94%	108%	96%
		2	85%	92%	108%	105%	82%	105%	98%
		3	86%	95%	103%	100%	75%	102%	93%
		4	89%	95%	106%	103%	89%	106%	96%
		5	80%	91%	102%	98%	81%	107%	92%
	3	1	97%	94%	106%	98%	94%	128%	96%
		2	104%	96%	115%	114%	101%	111%	106%
		3	87%	90%	107%	103%	79%	116%	88%
		4	101%	95%	107%	106%	96%	112%	91%
		5	101%	98%	113%	105%	107%	119%	98%
		$\bar{x}$	**90%**	**93%**	**104%**	**100%**	**88%**	**111%**	**94%**
		SD	**10.8%**	**6.5%**	**9.1%**	**9.4%**	**10.4%**	**10.5%**	**6.3%**
		%RSD	**12.0%**	**6.9%**	**8.8%**	**9.4%**	**11.9%**	**9.5%**	**6.7%**
10ng/g in 4g	1	1	79%	100%	101%	91%	73%	106%	88%
		2	95%	104%	104%	97%	91%	116%	99%
		3	86%	101%	107%	93%	85%	108%	97%
		4	86%	98%	106%	97%	85%	104%	97%
		5	88%	90%	106%	104%	85%	99%	93%
	2	1	85%	90%	97%	89%	78%	99%	87%
		2	88%	93%	101%	93%	83%	103%	88%
		3	87%	89%	100%	91%	80%	102%	87%
		4	86%	90%	100%	91%	77%	101%	84%
		5	89%	94%	103%	95%	77%	104%	88%
	3	1	88%	92%	103%	96%	78%	102%	93%
		2	100%	92%	106%	102%	83%	108%	96%
		3	92%	89%	105%	100%	81%	107%	93%
		4	94%	92%	104%	97%	81%	105%	91%
		5	100%	98%	107%	104%	84%	106%	97%
		$\bar{x}$	**90%**	**94%**	**103%**	**96%**	**81%**	**105%**	**92%**
		SD	**5.7%**	**4.8%**	**3.0%**	**4.8%**	**4.5%**	**4.3%**	**4.7%**
		%RSD	**6.4%**	**5.1%**	**2.9%**	**5.0%**	**5.5%**	**4.1%**	**5.1%**
50ng/g in 4g	1	1	86%	94%	97%	89%	82%	101%	88%
		2	83%	98%	101%	93%	90%	109%	91%
		3	84%	98%	101%	92%	88%	105%	95%
		4	92%	94%	112%	95%	90%	109%	95%
		5	86%	94%	100%	89%	87%	98%	92%
	2	1	82%	95%	92%	81%	76%	94%	85%
		2	86%	101%	95%	86%	78%	98%	90%
		3	85%	93%	92%	84%	79%	96%	87%
		4	83%	96%	93%	85%	78%	93%	87%
		5	85%	97%	96%	89%	82%	97%	88%
	3	1	86%	96%	99%	91%	80%	92%	92%
		2	91%	101%	103%	96%	83%	94%	100%
		3	90%	94%	98%	91%	84%	91%	95%
		4	93%	93%	98%	94%	83%	93%	97%
		5	88%	93%	99%	91%	82%	91%	96%
		$\bar{x}$	**87%**	**96%**	**98%**	**90%**	**83%**	**97%**	**92%**
		SD	**3.4%**	**2.7%**	**5.0%**	**4.3%**	**4.4%**	**6.1%**	**4.4%**
		%RSD	**3.9%**	**2.8%**	**5.1%**	**4.7%**	**5.3%**	**6.2%**	**4.8%**
		$\bar{x}$	**89%**	**94%**	**102%**	**95%**	**84%**	**104%**	**93%**
		SD	**7%**	**5%**	**7%**	**8%**	**7%**	**9%**	**5%**
		%RSD	**8%**	**5%**	**6%**	**8%**	**9%**	**9%**	**6%**

DMZ-D_3 Recovery in tilapia, salmon and shrimp muscle

DMZ- D_3 Recovery Results					
Level	Day	Rep.	Percent Recovery		
			Tilapia	Salmon	Shrimp
2.5ng/g in 4g	1	1	101%	113%	105%
		2	106%	107%	112%
		3	108%	116%	113%
		4	92%	116%	111%
		5	94%	124%	107%
	2	1	120%	88%	105%
		2	113%	114%	112%
		3	105%	112%	113%
		4	110%	89%	111%
		5	112%	83%	107%
	3	1	110%	93%	114%
		2	93%	115%	112%
		3	92%	110%	126%
		4	105%	125%	124%
		5	91%	105%	123%
		$\bar{x}$	**104%**	**107%**	**113%**
		SD	**9.1%**	**13.1%**	**6.6%**
		%RSD	**8.8%**	**12.2%**	**5.8%**
2.5ng/g in 4g	1	1	107%	116%	117%
		2	91%	124%	114%
		3	94%	110%	118%
		4	100%	117%	116%
		5	93%	136%	119%
	2	1	99%	87%	117%
		2	105%	89%	114%
		3	103%	89%	118%
		4	94%	88%	116%
		5	97%	89%	119%
	3	1	100%	114%	121%
		2	105%	96%	127%
		3	88%	114%	126%
		4	111%	98%	126%
		5	108%	103%	125%
		$\bar{x}$	**100%**	**105%**	**119%**
		SD	**6.9%**	**15.2%**	**4.5%**
		%RSD	**6.9%**	**14.5%**	**3.7%**
2.5ng/g in 4g	1	1	103%	125%	114%
		2	103%	109%	113%
		3	115%	129%	119%
		4	109%	132%	116%
		5	95%	116%	119%
	2	1	85%	83%	114%
		2	111%	86%	113%
		3	111%	77%	119%
		4	114%	83%	116%
		5	105%	85%	119%
	3	1	103%	110%	126%
		2	96%	89%	124%
		3	103%	108%	132%
		4	109%	89%	131%
		5	no spike	118%	132%
		$\bar{x}$	**104%**	**103%**	**120%**
		SD	**8.2%**	**18.9%**	**6.7%**
		%RSD	**7.9%**	**18.4%**	**5.6%**
		$\bar{x}$	**103%**	**105%**	**118%**
		SD	**8%**	**16%**	**7%**
		%RSD	**8%**	**15%**	**6%**

Appendix 13 Method repeatability using uncorrected, spike corrected and internal standard corrected data for tilapia, salmon and shrimp muscle. Intermediate precision (analyst 1 and 2 combined results) is included for the uncorrected and spike corrected data.

Tilapia muscle uncorrected data

Level 1 1.0 ng/g

Day	Replicate	Analyst 1							Analyst 2						
		HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	1 17	0 95	1 00	1 19	0 89	1 12	1 11	1 30	1 20	1 42	1 07	1 17	1 44	1 15
	2	1 05	1 15	1 27	1 13	1 04	1 10	1 43	1 08	1 11	1 36	1 14	1 00	1 31	1 21
	3	1 13	1 02	1 07	1 12	0 98	0 99	1 16	1 17	1 16	1 40	1 33	1 04	1 40	1 12
	4	1 13	1 09	1 30	1 07	0 99	1 09	1 07	1 25	1 00	1 46	1 14	1 04	1 35	1 22
	5	1 21	0 90	1 06	1 21	0 82	1 12	1 00	1 21	1 16	1 34	1 10	1 05	1 36	1 20
	Average	1.14	1.02	1.14	1.14	0.94	1.08	1.15	1.20	1.13	1.40	1.16	1.06	1.37	1.18
	STD Dev	0.06	0.10	0.14	0 06	0.09	0 05	0.17	0 08	0.08	0 05	0 10	0.06	0.05	0 04
	% RSD	5.2%	9.9%	11 9%	4 9%	9.3%	5.0%	14 3%	6 9%	6 9%	3 4%	8.8%	6.1%	3.6%	3 6%
2	1	1 16	1 08	1 36	1 14	0 98	0 81	1 16	1.11	0 91	1 11	1 04	0 88	1 29	0 95
	2	1 30	1 19	1 48	1 33	1 06	0 92	1 16	1.36	1.04	1 25	1 20	1 00	1 14	1 06
	3	1 14	1 12	1 42	1 27	0 90	0 95	1 18	1.15	1 14	1 32	1 21	0 95	1 32	1 05
	4	1 31	1 20	1 55	1 19	0 96	0 92	1 10	1.26	1.09	1 33	1 10	1 09	1 24	1 02
	5	1 20	1 03	1 41	1 28	0 96	0 98	1 17	1.35	1.07	1 26	1 05	1 01	1 28	1 05
	Average	1.22	1.12	1.44	1.24	0 97	0.92	1.15	1.25	1 05	1.25	1.12	0 99	1.25	1.03
	STD Dev	0 08	0.07	0.07	0.08	0.06	0 06	0 03	0.11	0.09	0.09	0 08	0.08	0.07	0.05
	% RSD	6 5%	6 4%	5.1%	6.1%	5.9%	7 0%	2 7%	9.1%	8 2%	7 0%	7 2%	7.9%	5.6%	4 4%
3	1	1 28	1 27	1 31	0 84	0 91	1 24	1 14	1 10	1 10	1 33	0 99	0 82	1 06	1 23
	2	1 47	0 88	1 31	0 99	0 94	1 28	1 02	1 06	0 90	1 28	1 02	0 90	1 05	0 94
	3	1 29	1 04	1 35	1 08	1 10	1 34	1 19	1 27	1 05	1 39	1 05	0 99	1 13	1 17
	4	1 29	1 04	1 37	1 35	1 04	1 36	1 27	1 07	1 05	1 28	1 04	0 94	1 02	1 10
	5	1 19	1 19	1 51	1 05	1 20	1 52	1 18	1 05	1 15	1 29	1 02	0 84	1 07	1 11
	Average	1 30	1.08	1.37	1.06	1 04	1 35	1.16	1.11	1.05	1 31	1.02	0 90	1.07	1.11
	STD Dev	0.10	0.15	0.08	0.19	0.12	0 11	0.09	0.09	0.09	0.05	0.02	0.07	0.04	0.11
	% RSD	7.8%	13 9%	6.0%	17.5%	11 4%	8.0%	7 9%	8 2%	8 9%	3 6%	2 2%	7 8%	3 8%	9.8%
Individual	Average	1.22	1.08	1.32	1.15	0.98	1.12	1.16	1.19	1.08	1 32	1 10	0.98	1 23	1 11
	SD	0 10	0 11	0 16	0.13	0.09	0.20	0 10	0.11	0 09	0.08	0.09	0 09	0.14	0.09
	%RSD	8.5%	10 5%	12 4%	11.7%	9.5%	17.7%	8 8%	9.0%	8 2%	6.4%	8.3%	9.7%	11.4%	8.4%
Combined	Average	1.20	1.08	1.32	1.12	0 98	1.17	1.13							
	SD	0.11	0.10	0 13	0.12	0 09	0.18	0 10							
	% RSD	8 7%	9 3%	9.7%	10.3%	9.4%	15.2%	8.8%							
Horwiz ratio	PRSD(R)	44.774	44.774	44.774	44.774	44.774	44.774	44.774							
	HorRat	0 19	0.21	0 22	0.23	0.21	0 34	0.20							

Tilapia muscle uncorrected data

Level 2 10 0 ng/g

Day	Replicate	Analyst 1							Analyst 2						
		HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	9 78	10 02	11 18	9 84	8 13	9 55	9 64	11 25	11 47	14 44	10 52	9 70	13 03	12 09
	2	10 51	10 11	11 03	10 64	8 68	10 84	11 08	11 56	11 64	14 83	12 42	10 61	13 51	12 46
	3	11 02	8 53	11 40	11 58	8 70	11 16	10 32	12 04	12 09	13 82	12 00	10 14	12 62	11 91
	4	11 36	10 68	11 69	9 67	8 23	10 10	11 45	11 98	11 01	13 28	11 60	9 90	13 76	11 96
	5	9 62	9 80	11 42	10 06	9 87	10 37	11 57	11 62	11 09	13 97	11 04	9 88	13 69	11 77
	Average	10 46	9 83	11 34	10 36	8 72	10 40	10 81	11 69	11 46	14 07	11 52	10 05	13 32	12 04
	STD Dev	0 76	0 79	0 25	0 78	0 69	0 63	0 82	0 32	0 44	0 59	0 75	0 35	0 48	0 26
	% RSD	7 2%	8 1%	2 2%	7 5%	7 9%	6 0%	7 6%	2 8%	3 8%	4 2%	6 6%	3 5%	3 6%	2 2%
2	1	11 81	10 18	13 27	11 48	9 47	8 91	10 98	12 15	10 54	12 43	10 58	9 93	11 42	9 98
	2	11 40	10 61	13 81	11 29	10 17	8 39	11 22	12 08	11 13	13 34	11 99	10 57	12 34	10 35
	3	10 91	11 33	14 53	11 70	9 65	8 34	11 76	11 27	9 50	13 44	11 81	9 68	11 59	9 01
	4	12 09	10 76	13 74	11 48	9 57	8 55	11 03	11 43	9 88	12 91	11 05	9 68	12 81	9 10
	5	12 15	9 55	13 98	11 26	9 92	9 05	10 73	12 63	10 04	12 83	10 88	9 65	11 47	8 95
	Average	11 67	10 49	13 87	11 44	9 76	8 65	11 14	11 91	10 22	12 99	11 26	9 90	11 93	9 48
	STD Dev	0 52	0 67	0 46	0 18	0 29	0 32	0 39	0 56	0 63	0 41	0 61	0 39	0 62	0 64
	% RSD	4 4%	6 3%	3 3%	1 5%	2 9%	3 7%	3 5%	4 7%	6 2%	3 2%	5 4%	3 9%	5 2%	6 8%
3	1	13 29	10 27	13 80	13 27	11 46	13 50	11 23	10 19	9 48	12 48	9 82	8 94	9 58	10 88
	2	14 98	10 31	15 16	12 38	11 89	13 72	11 87	11 07	9 23	13 07	10 26	9 19	10 41	9 82
	3	13 79	11 14	16 32	13 65	11 91	13 24	12 08	9 79	8 86	12 81	9 97	8 88	9 51	10 50
	4	12 67	9 24	14 46	13 23	10 79	14 68	11 40	10 83	9 83	13 09	9 95	8 48	9 36	10 20
	5	14 19	9 94	13 93	13 31	11 16	14 66	11 95	11 01	8 73	13 39	10 75	9 12	10 26	10 20
	Average	13 78	10 18	14 73	13 17	11 44	13 96	11 71	10 58	9 23	12 97	10 15	8 92	9 82	10 32
	STD Dev	0 88	0 69	1 04	0 47	0 48	0 67	0 37	0 56	0 45	0 34	0 37	0 28	0 48	0 40
	% RSD	6 4%	6 7%	7 0%	3 6%	4 2%	4 8%	3 2%	5 3%	4 9%	2 6%	3 7%	3 1%	4 8%	3 8%
Individual	Average	11 97	10 16	13 31	11 66	9 97	11 00	11 22	11 39	10 30	13 34	10 98	9 62	11 69	10 61
	SD	1 58	0 72	1 61	1 30	1 25	2 35	0 65	0 76	1 06	0 68	0 83	0 61	1 57	1 18
	%RSD	13 2%	7 1%	12 1%	11 1%	12 6%	21 3%	5 8%	6 7%	10 3%	5 1%	7 5%	6 3%	13 4%	11 1%
Combined	Average	11 68	10 23	13 33	11 32	9 80	11 35	10 92							
	SD	1 25	0 89	1 22	1 12	0 98	1 99	0 99							
	% RSD	10 7%	8 7%	9 1%	9 9%	10 0%	17 5%	9 0%							
Horwiz ratio	PRSD(R)	44 774	44 774	44 774	44 774	44 774	44 774	44 774							
	HorRat	0 24	0 19	0 20	0 22	0 22	0 39	0 20							

Tilapia muscle uncorrected data

Level 3 50 ng/g

Day	Replicate	Analyst 1							Analyst 2						
		HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	47 67	41 53	49 06	47 34	36 25	44 72	48 77	53 51	49 85	61 83	52 13	43 54	56 32	53 89
	2	50 38	44 71	53 62	48 06	39 71	48 49	49 71	52 31	53 70	62 79	51 81	45 29	54 84	59 29
	3	50 54	40 71	52 29	49 55	44 27	48 22	49 05	50 90	51 58	64 65	53 47	43 37	54 95	58 54
	4	54 93	42 79	52 41	51 39	41 74	48 05	46 70	50 20	53 63	60 99	49 69	44 57	55 06	55 63
	5	49 12	44 00	52 22	51 52	45 1	48 59	51 31	55 44	52 00	64 2	57 22	48 36	59 13	56 65
	Average	50 53	42 75	51 92	49 57	41 41	47 61	49 11	52 47	52 15	62 89	52 86	45 03	56 06	56 80
	STD Dev	2 72	1 66	1 70	1 90	3 59	1 63	1 67	2 09	1 60	1 55	2 79	2 02	1 82	2 18
	% RSD	5 4%	3 9%	3 3%	3 8%	8 7%	3 4%	3 4%	4 0%	3 1%	2 5%	5 3%	4 5%	3 2%	3 8%
2	1	50 88	48 66	60 95	50 24	39 06	36 18	50 66	53 98	45 22	57 16	52 51	43 15	51 44	43 77
	2	50 71	53 28	63 96	54 24	42 63	38 08	59 03	57 13	47 56	60 2	48 75	47 77	52 54	45 95
	3	53 88	58 41	63 1	52 66	43 17	36 63	56 5	57 89	45 72	58 09	51 38	43 86	54 92	46 65
	4	53 14	56 17	67 61	54 62	44 48	39 32	54 92	56 41	47 25	62 36	49 67	43 76	55 27	46 4
	5	50 50	53 20	58 43	52 47	41 85	37 77	54 48	59 38	50 81	59 62	51 68	45 89	53 55	47 16
	Average	51 82	53 94	62 81	52 85	42 24	37 60	55 12	56 96	47 31	59 49	50 80	44 89	53 54	45 99
	STD Dev	1 57	3 67	3 43	1 74	2 02	1 24	3 06	2 00	2 19	2 01	1 54	1 91	1 61	1 31
	% RSD	3 0%	6 8%	5 5%	3 3%	4 8%	3 3%	5 6%	3 5%	4 6%	3 4%	3 0%	4 3%	3 0%	2 9%
3	1	57 63	55 19	65 59	52 42	53 65	56 84	60 19	46 00	41 68	56 59	49 36	44 24	43 99	47 78
	2	60 81	50 39	62 3	51 73	51 320	62 660	58 94	51 68	42 32	59 08	50 35	46 47	37 99	48 17
	3	62 06	57 04	68 51	57 76	49 060	63 040	62 6	54 90	40 42	58 35	51 19	43 21	45 16	47 41
	4	65 45	57 08	70 87	58 51	55 130	66 100	57 12	54 34	45 07	58 06	48 85	43 78	50 25	45 55
	5	57 22	53 85	70 52	58 49	55 400	59 020	59 61	48 95	39 55	61 5	47 11	44 09	43 14	45 43
	Average	60 63	54 71	67 56	55 78	52 91	61 53	59 69	51 17	41 81	58 72	49 37	44 36	44 11	46 87
	STD Dev	3 39	2 77	3 61	3 41	2 69	3 63	1 99	3 74	2 12	1 80	1 55	1 24	4 39	1 29
	% RSD	5 6%	5 1%	5 3%	6 1%	5 1%	5 9%	3 3%	7 3%	5 1%	3 1%	3 1%	2 8%	10 0%	2 7%
Individual	Average	54 33	50 47	60 76	52 73	45 52	48 91	54 64	53 53	47 09	60 36	51 01	44 76	51 24	49 88
	SD	5 26	6 23	7 34	3 48	6 02	10 40	4 97	3 60	4 75	2 51	2 41	1 66	5 96	5 30
	%RSD	9 7%	12 4%	12 1%	6 6%	13 2%	21 3%	9 1%	6 7%	10 1%	4 2%	4 7%	3 7%	11 6%	10 6%
Combined	Average	53 93	48 78	60 56	51 87	45 14	50 08	52 26							
	SD	4 45	5 71	5 39	3 07	4 36	8 41	5 60							
	% RSD	8 2%	11 7%	8 9%	5 9%	9 7%	16 8%	10 7%							
Horwiz ratio	PRSD(R)	44 774	44 774	44 774	44 774	44 774	44 774	44 774							
	HorRat	0 18	0 26	0 20	0 13	0 22	0 38	0 24							

Tilapia muscle spike corrected data

Level 1 1 0 ng/g

		Analyst 1							Analyst 2						
Day	Replicate	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	1 14	1 33	0 92	1 12	1 02	1 13	1 06	1 07	1 18	1 15	0 93	1 11	1 15	0 92
	2	1 03	1 61	1 18	1 07	1 18	1 11	1 37	0 89	1 09	1 10	0 99	0 95	1 05	0 97
	3	1 10	1 42	0 99	1 07	1 11	1 00	1 11	0 97	1 14	1 14	1 15	0 99	1 12	0 90
	4	1 11	1 52	1 20	1 01	1 13	1 10	1 03	1 03	0 99	1 19	0 99	0 98	1 08	0 98
	5	1 18	1 25	0 98	1 14	0 94	1 13	0 96	1 00	1 14	1 09	0 95	0 99	1 09	0 96
	Average	1 11	1 43	1 05	1 08	1 08	1 09	1 11	0 99	1 11	1 13	1 00	1 00	1 10	0 95
	STD Dev	0 06	0 14	0 13	0 05	0 10	0 05	0 16	0 07	0 07	0 04	0 09	0 06	0 04	0 03
	% RSD	5 0%	10 1%	12 1%	4 7%	8 9%	4 9%	14 2%	6 9%	6 6%	3 6%	8 7%	6 1%	3 5%	3 6%
2	1	1 14	0 98	1 16	1 12	1 23	1 00	1 06	0 90	0 83	0 86	0 90	0 91	1 07	0 95
	2	1 28	1 08	1 26	1 31	1 33	1 14	1 06	1 11	0 95	0 97	1 04	1 04	0 95	1 07
	3	1 13	1 02	1 21	1 25	1 13	1 18	1 08	0 94	1 04	1 03	1 05	0 99	1 10	1 06
	4	1 29	1 09	1 32	1 18	1 20	1 14	1 00	1 02	1 00	1 03	0 96	1 13	1 03	1 03
	5	1 18	0 93	1 20	1 26	1 21	1 22	1 07	1 10	0 97	0 98	0 91	1 05	1 06	1 06
	Average	1 20	1 02	1 23	1 22	1 22	1 14	1 05	1 01	0 96	0 97	0 97	1 02	1 04	1 03
	STD Dev	0 08	0 07	0 06	0 07	0 07	0 08	0 03	0 09	0 08	0 07	0 07	0 08	0 06	0 05
	% RSD	6 3%	6 6%	5 0%	6 1%	5 9%	7 3%	3 0%	9 2%	8 3%	7 1%	7 3%	7 9%	5 5%	4 8%
3	1	1 07	1 11	0 90	0 84	0 96	0 93	0 89	1 04	1 35	1 12	0 96	0 88	1 14	1 39
	2	1 22	0 77	0 90	0 77	0 99	0 96	0 80	1 00	1 10	1 08	1 00	0 98	1 13	1 06
	3	1 07	0 91	0 93	0 84	1 16	1 00	0 93	1 20	1 29	1 17	1 02	1 06	1 21	1 32
	4	1 07	0 91	0 95	1 05	1 09	1 02	1 00	1 02	1 29	1 08	1 01	1 01	1 09	1 24
	5	0 99	1 03	1 04	1 03	1 26	1 14	0 93	0 99	1 41	1 09	1 00	0 91	1 14	1 25
	Average	1 08	0 95	0 94	0 91	1 09	1 01	0 91	1 05	1 29	1 11	1 00	0 97	1 14	1 25
	STD Dev	0 08	0 13	0 06	0 13	0 12	0 08	0 07	0 09	0 12	0 04	0 02	0 07	0 04	0 12
	% RSD	7 7%	13 7%	6 1%	13 9%	11 3%	8 0%	8 0%	8 2%	9 0%	3 5%	2 3%	7 6%	3 8%	9 8%
Individual	Average	1 13	1 13	1 08	1 07	1 13	1 08	1 02	1 02	1 12	1 07	0 99	1 00	1 09	1 08
	SD	0 09	0 24	0 15	0 16	0 11	0 09	0 13	0 08	0 16	0 09	0 06	0 07	0 06	0 15
	%RSD	7 6%	21 6%	13 6%	14 8%	10 0%	8 1%	12 5%	7 9%	14 6%	8 1%	6 3%	7 1%	5 5%	14 1%
Combined	Average	1 08	1 12	1 07	1 03	1 06	1 09	1 05							
	SD	0 10	0 20	0 12	0 12	0 11	0 07	0 14							
	% RSD	9 3%	18 2%	11 0%	12 1%	10 7%	6 8%	13 4%							
Horwiz ratio	PRSD(R)	44 774	44 774	44 774	44 774	44 774	44 774	44 774							
	HorRat	0 21	0 41	0 25	0 27	0 24	0 15	0 30							

Tilapia muscle spike corrected data

Level 2 10 0 ng/g

Day	Replicate	Analyst 1							Analyst 2						
		HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	9 57	14 03	10 33	9 33	9 26	9 65	9 24	9 31	11 27	11 71	9 08	9 20	10 39	9 67
	2	10 28	14 16	10 20	10 09	9 88	10 96	10 62	9 56	11 43	12 02	10 73	10 06	10 78	9 97
	3	10 78	11 95	10 54	10 98	9 91	11 27	9 89	9 96	11 88	11 20	10 37	9 62	10 07	9 53
	4	11 11	14 95	10 81	9 17	9 36	10 21	10 97	9 91	10 82	10 76	10 02	9 39	10 97	9 57
	5	9 42	13 73	10 55	9 54	11 24	10 48	11 09	9 61	10 89	11 33	9 54	9 37	10 92	9 42
	Average	10 23	13 76	10 49	9 82	9 93	10 51	10 36	9 67	11 26	11 40	9 95	9 53	10 63	9 63
	STD Dev	0 74	1 11	0 23	0 73	0 79	0 63	0 78	0 27	0 43	0 48	0 65	0 33	0 39	0 21
	% RSD	7 2%	8 1%	2 2%	7 5%	7 9%	6 0%	7 5%	2 8%	3 8%	4 2%	6 6%	3 5%	3 6%	2 2%
2	1	11 66	9 24	11 32	11 33	11 89	11 05	10 00	9 86	9 60	9 64	9 19	10 31	9 50	10 08
	2	11 25	9 62	11 78	11 15	12 77	10 41	10 23	9 80	10 14	10 35	10 41	10 97	10 27	10 45
	3	10 77	10 28	12 39	11 55	12 12	10 34	10 72	9 15	8 66	10 42	10 25	10 05	9 65	9 10
	4	11 93	9 76	11 71	11 33	12 01	10 61	10 05	9 27	9 00	10 02	9 60	10 05	10 66	9 19
	5	11 99	8 65	11 92	11 11	12 45	11 22	9 78	10 25	9 15	9 95	9 45	10 02	9 55	9 04
	Average	11 52	9 51	11 82	11 29	12 25	10 73	10 16	9 67	9 31	10 08	9 78	10 28	9 93	9 57
	STD Dev	0 51	0 61	0 39	0 18	0 36	0 39	0 35	0 45	0 57	0 32	0 53	0 40	0 51	0 65
	% RSD	4 4%	6 4%	3 3%	1 6%	2 9%	3 6%	3 5%	4 7%	6 2%	3 1%	5 4%	3 9%	5 2%	6 8%
3	1	11 04	8 96	9 49	10 30	12 06	10 11	8 83	9 65	11 66	10 53	9 56	9 65	10 25	12 26
	2	12 44	8 99	10 44	9 61	12 52	10 28	9 34	10 49	11 35	11 02	9 99	9 92	11 13	11 06
	3	11 45	9 71	11 23	10 60	12 54	9 91	9 50	9 27	10 89	10 81	9 70	9 59	10 17	11 82
	4	10 52	8 06	9 95	10 27	11 36	10 98	8 96	10 25	12 08	11 04	9 68	9 15	10 02	11 50
	5	11 79	8 67	9 59	10 34	11 75	10 98	9 40	10 42	10 74	11 29	10 46	9 84	10 97	11 49
	Average	11 45	8 88	10 14	10 22	12 05	10 45	9 21	10 02	11 34	10 94	9 88	9 63	10 51	11 63
	STD Dev	0 73	0 60	0 71	0 37	0 51	0 50	0 29	0 53	0 55	0 28	0 36	0 30	0 50	0 45
	% RSD	6 4%	6 7%	7 0%	3 6%	4 2%	4 8%	3 2%	5 3%	4 9%	2 6%	3 7%	3 1%	4 8%	3 8%
Individual	Average	11 07	10 72	10 82	10 45	11 41	10 56	9 91	9 78	10 64	10 81	9 87	9 81	10 35	10 28
	SD	0 87	2 37	0 88	0 78	1 21	0 49	0 71	0 43	1 09	0 67	0 49	0 47	0 54	1 08
	%RSD	7 9%	22 1%	8 1%	7 5%	10 6%	4 7%	7 2%	4 4%	10 2%	6 2%	5 0%	4 8%	5 2%	10 5%
Combined	Average	10 43	10 68	10 81	10 16	10 61	10 46	10 09							
	SD	0 94	1 81	0 76	0 71	1 21	0 52	0 92							
	% RSD	9 0%	17 0%	7 1%	7 0%	11 4%	5 0%	9 1%							
Horwiz ratio	PRSD(R)	44 774	44 774	44 774	44 774	44 774	44 774	44 774							
	HorRat	0 20	0 38	0 16	0 16	0 26	0 11	0 20							

Tilapia muscle spike corrected data

Level 3 50 ng/g

Day	Replicate	Analyst 1 HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ	Analyst 2 HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	46 63	58 18	45 36	44 89	44 67	45 18	46 74	44 26	48 99	50 12	45 04	41 31	44 91	43 12
	2	49 29	62 63	49 57	45 58	45 19	48 99	47 64	43 27	52 77	50 9	44 76	42 97	43 73	47 44
	3	49 44	57 03	48 35	46 99	50 39	48 72	47 01	42 10	50 69	52 41	46 19	41 15	43 82	46 84
	4	53 73	59 95	48 46	48 73	47 51	48 55	44 76	41 53	52 70	49 44	42 93	42 29	43 91	44 51
	5	48 05	61 63	48 28	48 85	51 33	49 10	49 17	45 86	51 09	52 04	49 43	45 88	47 15	45 33
	Average	49 43	59 88	48 00	47 01	47 82	48 11	47 06	43 40	51 25	50 98	45 67	42 72	44 70	45 45
	STD Dev	2 66	2 33	1 57	1 79	2 99	1 65	1 60	1 73	1 57	1 25	2 41	1 92	1 45	1 75
	% RSD	5 4%	3 9%	3 3%	3 8%	6 3%	3 4%	3 4%	4 0%	3 1%	2 5%	5 3%	4 5%	3 2%	3 8%
2	1	50 22	44 14	51 98	49 58	49 04	44 87	46 16	43 80	41 21	44 35	45 6	44 79	42 82	44 21
	2	50 05	48 34	54 54	53 54	53 53	47 23	53 79	46 36	43 34	46 71	42 34	49 59	43 73	46 41
	3	53 18	52 98	53 82	51 98	54 2	45 42	51 48	46 97	41 67	45 07	44 62	45 52	45 72	47 08
	4	52 45	50 95	57 66	53 91	55 84	48 77	50 04	45 77	43 06	48 38	43 13	45 42	46 00	46 86
	5	49 84	48 26	49 83	51 78	52 55	46 84	49 64	48 18	46 31	46 25	44 88	47 63	44 58	47 63
	Average	51 15	48 93	53 57	52 16	53 03	46 63	50 22	46 22	43 12	46 15	44 11	46 59	44 57	46 44
	STD Dev	1 55	3 33	2 93	1 72	2 53	1 54	2 79	1 62	2 00	1 56	1 34	1 99	1 34	1 32
	% RSD	3 0%	6 8%	5 5%	3 3%	4 8%	3 3%	5 6%	3 5%	4 6%	3 4%	3 0%	4 3%	3 0%	2 8%
3	1	47 86	48 11	45 14	40 71	56 48	42 57	47 32	43 56	51 26	47 72	48 05	47 74	47 07	53 83
	2	50 49	43 93	42 87	40 17	54 03	46 93	46 34	48 94	52 04	49 82	49 02	50 15	40 64	54 27
	3	51 53	49 73	47 15	44 85	51 65	47 21	49 21	51 99	49 70	49 21	49 83	46 63	48 31	53 41
	4	54 35	49 76	48 78	45 43	58 04	49 50	44 90	51 45	55 43	48 96	47 55	47 24	53 76	51 31
	5	47 52	46 95	48 53	45 42	58 32	44 20	46 86	46 35	48 64	51 86	45 86	47 58	46 16	51 17
	Average	50 35	47 70	46 49	43 32	55 70	46 08	46 93	48 46	51 41	49 51	48 06	47 87	47 19	52 80
	STD Dev	2 81	2 41	2 49	2 64	2 83	2 72	1 57	3 54	2 61	1 52	1 51	1 34	4 70	1 46
	% RSD	5 6%	5 1%	5 4%	6 1%	5 1%	5 9%	3 3%	7 3%	5 1%	3 1%	3 1%	2 8%	10 0%	2 8%
Individual	Average	50 31	52 17	49 35	47 49	52 18	46 94	48 07	46 03	48 59	48 88	45 95	45 73	45 49	48 23
	SD	2 34	6 21	3 85	4 22	4 26	2 09	2 48	3 12	4 46	2 49	2 38	2 80	3 00	3 65
	%RSD	4 7%	11 9%	7 8%	8 9%	8 2%	4 4%	5 2%	6 8%	9 2%	5 1%	5 2%	6 1%	6 6%	7 6%
Combined	Average	48 17	50 38	49 12	46 72	48 96	46 21	48 15							
	SD	3 48	5 61	3 19	3 46	4 83	2 64	3 07							
	% RSD	7 2%	11 1%	6 5%	7 4%	9 9%	5 7%	6 4%							
Horwiz ratio	PRSD(R)	44 774	44 774	44 774	44 774	44 774	44 774	44 774							
	HorRat	0 16	0 25	0 15	0 17	0 22	0 13	0 14							

Tilapia muscle IS corrected data

Level 1 1.0 ng/g

Day	Replicate	Analyst 1						
		HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	1 40	1 14	1 20	1 42	1 07	1 34	1 33
	2	1 05	1 16	1 28	1 14	1 04	1 10	1 44
	3	1 16	1 04	1 10	1 16	1 00	1 02	1 19
	4	1 20	1 15	1 37	1 13	1 04	1 15	1 13
	5	1 35	1 00	1 18	1 35	0 92	1 25	1 11
	Average	1.23	1.10	1.23	1.24	1.01	1.17	1.24
	STD Dev	0.14	0.07	0.10	0.14	0.06	0.13	0.14
	% RSD	11.6%	6.6%	8.4%	10.9%	5.7%	10.7%	11.4%
2	1	1 10	1 03	1 29	1 08	0 93	0 77	1 10
	2	1 21	1 10	1 38	1 23	0 98	0 85	1 08
	3	1 07	1 05	1 33	1 19	0 84	0 89	1 10
	4	1 19	1 09	1 41	1 08	0 87	0 84	1 00
	5	1 04	0 89	1 22	1 11	0 83	0 85	1 01
	Average	1.12	1.03	1.33	1.14	0 89	0.84	1.06
	STD Dev	0.07	0.08	0.08	0 07	0.06	0.04	0.05
	% RSD	6 7%	8.2%	5 7%	6 0%	7.2%	5.2%	4.6%
3	1	1 21	1 20	1 24	1 02	0 86	1 17	1 07
	2	1 48	0 88	1 31	0 99	0 94	1 28	1 02
	3	1 21	0 97	1 26	1 01	1 03	1 25	1 11
	4	1 17	0 95	1 25	1 23	0 95	1 24	1 15
	5	1 05	1 04	1 33	1 16	1 05	1 34	1 04
	Average	1.22	1 01	1.28	1.08	0.97	1.26	1.08
	STD Dev	0.16	0.12	0.04	0.11	0.08	0.06	0.05
	% RSD	12.9%	12.1%	3.1%	9.9%	7.9%	4.9%	4.9%
Individual	Average	1.19	1.05	1.28	1.15	0.96	1 09	1.13
	SD	0.13	0.10	0.08	0.12	0.08	0.20	0.12
	%RSD	11.0%	9 2%	6 5%	10.4%	8.5%	18 5%	10 6%

Tilapia muscle IS corrected data

Level 2 10.0 ng/g

Day	Replicate	Analyst 1						
		HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	10 49	10 74	11 98	10 55	8 72	10 24	10 34
	2	11 16	10 73	11 72	11 31	9 22	11 52	11 77
	3	12 50	9 67	12 94	13 13	9 87	12 66	11 71
	4	12 18	11 45	12 54	10 37	8 82	10 83	12 28
	5	10 61	10 80	12 59	11 09	10 89	11 44	12 76
	Average	11.39	10.68	12.35	11.29	9 50	11.34	11.77
	STD Dev	0.91	0.64	0.49	1.10	0 90	0.90	0 91
	% RSD	8.0%	6.0%	4.0%	9.7%	9.4%	8.0%	7 7%
2	1	11 25	9 69	12 64	10 93	9 01	8 50	10 45
	2	10 02	9 33	12 14	9 93	8 94	7 39	9 87
	3	10 10	10 48	13 44	10 83	8 93	7 73	10 88
	4	10 84	9 65	12 31	10 29	8 58	7 68	9 89
	5	11 84	9 30	13 63	10 97	9 67	8 83	10 46
	Average	10 81	9.69	12.83	10.59	9.03	8.03	10.31
	STD Dev	0.77	0.48	0.67	0.46	0.40	0.61	0 43
	% RSD	7.1%	4.9%	5.2%	4.3%	4.4%	7.6%	4.2%
3	1	13 00	10 05	13 50	12 97	11 21	13 20	10 99
	2	14 02	9 66	14 20	11 58	11 13	12 84	11 12
	3	12 92	10 44	15 30	12 78	11 16	12 40	11 32
	4	11 84	8 63	13 52	12 35	10 08	13 69	10 65
	5	12 06	8 45	11 85	11 30	9 48	12 45	10 16
	Average	12 77	9.45	13.67	12.20	10.61	12.92	10 85
	STD Dev	0.87	0.87	1.26	0.73	0.79	0.54	0 46
	% RSD	6 8%	9.3%	9.2%	6.0%	7 4%	4.2%	4.2%
Individual	Average	11.66	9.94	12 95	11.36	9.71	10.76	10 98
	SD	1.16	0.84	0.98	1.01	0.96	2.21	0 86
	%RSD	10.0%	8 4%	7 6%	8.9%	9.9%	20.5%	7 8%

Tilapia muscle IS corrected data

Level 3 50 ng/g

Day	Replicate	Analyst 1						
		HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	55 72	48 55	57 36	55 34	45 88	52 27	57 01
	2	54 13	48 04	57 61	51 64	42 67	52 10	53 42
	3	54 78	44 13	56 68	53 7	47 99	52 26	53 17
	4	59 54	46 38	56 81	55 7	45 24	52 08	50 62
	5	57 77	51 74	61 41	60 59	53 04	57 15	60 35
	Average	56 39	47.77	57.97	55 39	46 96	53.17	54 91
	STD Dev	2.23	2 81	1.96	3.32	3.89	2.23	3.80
	% RSD	4 0%	5 9%	3 4%	6 0%	8.3%	4.2%	6.9%
2	1	46 81	44 76	56 07	46 22	35 93	33 34	46 61
	2	46 73	49 10	58 94	49 99	39 28	35 15	54 4
	3	49 13	53 26	57 54	48 02	39 36	33 45	51 52
	4	48 45	51 21	61 64	49 8	40 55	35 91	50 07
	5	49 92	52 59	57 76	51 87	41 37	37 39	53 85
	Average	48.21	50.18	58.39	49.18	39.30	35.05	51.29
	STD Dev	1.41	3 42	2.08	2 14	2 07	1.71	3 15
	% RSD	2 9%	6 8%	3.6%	4 4%	5 3%	4.9%	6 1%
3	1	52 10	49 91	59 34	47 36	48 51	51 38	54 43
	2	54 49	45 17	55 86	46 33	46 00	56 15	52 84
	3	54 79	50 37	60 52	50 95	43 32	55 64	55 27
	4	55 64	48 54	60 29	49 7	46 88	56 18	48 57
	5	49 16	46 28	60 62	50 22	47 60	50 69	51 23
	Average	53 24	48.05	59.33	48.91	46 46	54 01	52.47
	STD Dev	2 63	2 26	2 00	1 97	1 98	2.73	2 67
	% RSD	4.9%	4.7%	3.4%	4.0%	4.3%	5.1%	5.1%
Individual	Average	52.61	48.67	58.56	51.16	44 24	47.41	52.89
	SD	4.02	2 88	1.96	3 90	4 45	9 29	3.38
	%RSD	7.6%	5 9%	3.3%	7 6%	10 1%	19 6%	6 4%

Salmon muscle uncorrected data

Level 1 1 0 ng/g

		Analyst 1							Analyst 2						
Day	Replicate	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	1 01	1 03	1 40	1 08	0 95	1 24	1 26	1 28	1 23	1 68	1 45	1 22	1 49	1 46
	2	0 94	1 00	1 34	1 02	0 88	1 06	1 12	1 36	1 4	1 76	1 37	1 19	1 57	1 51
	3	1 04	1 05	1 36	1 13	0 84	1 18	1 13	1 2	1 36	1 72	1 41	0 98	1 51	1 53
	4	0 93	1 03	1 30	1 08	0 87	1 21	1 12	1 43	1 39	1 65	1 37	1 30	1 48	1 42
	5	1 09	0 96	1 44	1 12	0 93	1 21	1 16	1 4	1 31	1 79	1 26	1 33	1 45	1 46
	Average	1 00	1 01	1 37	1 09	0 89	1 18	1 16	1 33	1 34	1 72	1 37	1 20	1 50	1 48
	STD Dev	0 07	0 04	0 05	0 04	0 05	0 07	0 06	0 09	0 07	0 06	0 07	0 14	0 04	0 04
	% RSD	6 7%	3 5%	4 0%	4 0%	5 0%	6 0%	5 1%	7 0%	5 2%	3 3%	5 2%	11 4%	3 0%	3 0%
2	1	0 88	1 30	1 54	1 14	0 91	1 13	1 25	1 22	0 78	1 42	1 19	0 87	1 29	1 05
	2	0 97	1 17	1 54	1 23	0 91	1 14	1 27	1 16	0 83	1 47	1 16	0 94	1 31	1 15
	3	1 12	1 31	1 52	1 07	0 99	1 12	1 22	1 1	0 81	1 40	1 14	1 08	1 22	1 12
	4	1 22	1 23	1 52	1 13	1 08	1 20	1 27	1 27	0 87	1 51	1 17	1 03	1 42	1 12
	5	0 97	1 13	1 50	1 10	0 90	1 09	1 20	1 38	0 84	1 63	1 23	0 91	1 21	1 13
	Average	1 03	1 23	1 52	1 13	0 96	1 14	1 24	1 23	0 83	1 49	1 18	0 97	1 29	1 11
	STD Dev	0 14	0 08	0 02	0 06	0 08	0 04	0 03	0 11	0 03	0 09	0 03	0 09	0 08	0 04
	% RSD	13 2%	6 4%	1 1%	5 3%	8 1%	3 6%	2 5%	8 7%	4 1%	6 1%	2 9%	9 0%	6 6%	3 4%
3	1	0 97	0 99	1 34	0 95	0 62	0 94	1 06	0 64	0 51	0 76	0 65	0 40	0 60	0 67
	2	0 91	1 03	1 36	1 07	0 76	1 09	1 08	0 71	0 67	0 94	0 74	0 58	0 62	0 77
	3	0 89	0 99	1 27	1 00	0 79	0 95	1 07	0 64	0 71	0 95	0 82	0 55	0 64	0 75
	4	0 73	0 90	1 19	0 92	0 66	0 89	0 94	0 67	0 70	1 02	0 85	0 53	0 61	0 73
	5	0 80	1 09	1 31	1 01	0 76	0 93	0 98	0 64	0 87	1 09	0 87	0 58	0 72	0 82
	Average	0 86	1 00	1 29	0 99	0 72	0 96	1 03	0 66	0 69	0 95	0 79	0 53	0 64	0 75
	STD Dev	0 09	0 07	0 07	0 06	0 07	0 08	0 06	0 03	0 13	0 12	0 09	0 07	0 05	0 05
	% RSD	11 0%	6 9%	5 2%	5 8%	10 3%	7 9%	6 1%	4 7%	18 5%	12 9%	11 5%	14 1%	7 5%	7 3%
	Average	0 96	1 08	1 40	1 07	0 86	1 09	1 14	1 07	0 95	1 39	1 11	0 90	1 14	1 11
	SD	0 12	0 12	0 11	0 08	0 12	0 11	0 10	0 32	0 30	0 34	0 26	0 31	0 38	0 31
Individual	%RSD	12 8%	11 4%	7 9%	7 5%	14 2%	10 5%	9 1%	29 4%	31 4%	24 8%	23 4%	33 9%	33 6%	27 9%
	Average	1 02	1 02	1 39	1 09	0 88	1 12	1 13							
	SD	0 24	0 23	0 25	0 19	0 23	0 28	0 23							
Combined	% RSD	23 7%	23 0%	18 0%	17 5%	26 1%	25 0%	20 2%							
Horwiz ratio	PRSD(R)	44 774	44 774	44 774	44 774	44 774	44 774	44 774							
	HorRat	0 53	0 51	0 40	0 39	0 58	0 56	0 45							

Salmon muscle uncorrected data

Level 2 10.0 ng/g

Day	Replicate	Analyst 1							Analyst 2						
		HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	9 71	9 84	12 00	9 40	7 25	10 02	10 42	11 60	11 79	15 51	11 60	9 88	12 38	12 84
	2	9 93	9 49	12 41	9 50	7 92	10 22	10 53	11 02	11 86	14 25	11 82	9 63	12 12	12 46
	3	9 32	10 02	12 71	10 06	8 00	10 59	10 69	10 74	12 02	13 85	12 09	9 77	12 55	12 69
	4	10 18	11 24	13 55	10 30	8 39	11 15	11 06	10 82	12 12	14 66	10 82	9 24	11 43	12 75
	5	10 04	10 14	13 29	10 45	8 77	10 84	10 84	11 74	12 45	15 40	12 40	10 18	12 64	13 73
	Average	9.84	10.15	12.79	9.94	8.07	10 56	10.71	11.18	12.05	14 73	11.75	9.74	12 22	12.89
	STD Dev	0.34	0.66	0.63	0.47	0.57	0 46	0 25	0.46	0.26	0.72	0.60	0.35	0.49	0.49
	% RSD	3.4%	6 5%	4 9%	4.7%	7.0%	4.3%	2.4%	4.1%	2.2%	4 9%	5.1%	3 5%	4.0%	3.8%
2	1	9 76	10 91	13 18	10 36	8 96	10 34	11 38	10 57	7 75	12 67	10 33	8 16	10 92	9 71
	2	10 50	10 47	13 70	10 81	9 54	11 33	12 06	9 22	5 74	10 67	9 18	8 22	10 49	8 32
	3	9 79	10 82	13 39	10 45	8 92	10 79	11 27	9 24	6 01	10 97	9 48	7 67	10 06	8 83
	4	10 61	11 39	13 81	10 75	8 81	11 31	12 11	10 82	7 14	12 69	10 40	8 69	10 67	9 64
	5	9 58	10 20	13 44	9 90	8 17	10 41	11 17	10 40	6 67	12 38	10 40	8 37	10 92	9 72
	Average	10.05	10 76	13 50	10.45	8.88	10.84	11.60	10.05	6.66	11.88	9.96	8.22	10.61	9.24
	STD Dev	0.47	0 45	0.25	0 36	0 49	0.47	0.45	0.76	0.82	0.98	0.58	0.37	0.36	0.64
	% RSD	4.7%	4 2%	1.9%	3.5%	5.5%	4.4%	3 9%	7.6%	12.3%	8.2%	5.9%	4 5%	3.4%	6 9%
3	1	8 82	9 04	11 88	9 14	7 81	9 87	9 90	7 37	6 92	10 26	8 08	5 59	6 59	7 59
	2	8 82	9 48	12 25	9 41	7 70	10 02	10 39	7 58	7 16	10 77	9 12	5 74	7 52	7 67
	3	9 04	9 32	11 66	9 08	7 97	9 84	10 20	7 10	6 65	10 23	8 22	5 66	7 05	6 19
	4	8 59	9 45	12 12	9 21	7 59	9 95	10 13	7 39	6 86	10 33	8 22	5 81	6 93	6 81
	5	8 35	7 97	11 25	8 89	7 70	9 44	9 34	5 81	6 51	9 30	7 40	5 12	6 21	5 96
	Average	8.72	9.05	11 83	9.15	7.75	9.82	9 99	7.05	6.82	10.18	8.21	5.58	6.86	6.84
	STD Dev	0.26	0.63	0.40	0.19	0.14	0.23	0 40	0.71	0.25	0.54	0.61	0.27	0 49	0.78
	% RSD	3.0%	7.0%	3.3%	2.1%	1.9%	2.3%	4.0%	10 1%	3.7%	5 3%	7.5%	4 9%	7.2%	11.4%
Individual	Average	9.54	9 99	12.71	9.85	8 23	10.41	10.77	9.43	8.51	12.26	9.97	7.85	9.90	9 66
	SD	0.69	0 91	0.82	0.65	0.64	0.58	0.77	1.91	2.63	2.07	1.59	1.80	2.36	2.64
	%RSD	7.2%	9.1%	6.5%	6.6%	7.8%	5.6%	7.1%	20.2%	31.0%	16 9%	16.0%	23.0%	23.9%	27.4%
Combined	Average	9.48	9.25	12.49	9.91	8.04	10.15	10.21							
	SD	1 41	2.08	1 57	1.20	1.34	1 71	1 99							
	% RSD	14.9%	22 5%	12.5%	12 1%	16.7%	16.8%	19.5%							
Horwiz ratio	PRSD(R)	44 774	44 774	44 774	44.774	44.774	44.774	44.774							
	HorRat	0.33	0 50	0 28	0.27	0.37	0.38	0 44							

Salmon muscle uncorrected data

Level 3 50 ng/g

Day	Replicate	Analyst 1							Analyst 2						
		HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	44 40	44 39	56 54	44 16	37 25	45 64	50 60	58 58	62 78	76 17	59 6	50 21	61 41	67 13
	2	45 22	50 39	62 14	49 85	40 67	48 46	55 72	57 90	63 98	77 21	58 46	49 33	62 51	67 68
	3	47 25	50 55	63 81	49 42	42 61	50 45	57 90	59 15	68 57	81 07	61 08	51 98	64 39	69 48
	4	45 09	47 24	59 57	47 24	37 89	47 47	51 40	60 92	64 85	79 64	59 1	51 87	65 28	68 03
	5	50 29	53 48	67 37	53 21	44 79	52 95	58 01	58 03	64 10	71 76	60 13	51 41	62 00	64 46
	Average	46 45	49 21	61 89	48 78	40 64	48 99	54 73	58 92	64 86	77 17	59 67	50 96	63 12	67 36
	STD Dev	2 40	3 48	4 12	3 35	3 17	2 81	3 53	1 22	2 20	3 59	1 00	1 15	1 65	1 84
	% RSD	5 2%	7 1%	6 7%	6 9%	7 8%	5 7%	6 5%	2 1%	3 4%	4 7%	1 7%	2 3%	2 6%	2 7%
2	1	41 01	46 52	56 2	45 06	36 89	43 87	52 43	48 87	27 89	52 29	47 15	40 75	47 56	41
	2	44 06	48 29	58 06	47 73	40 69	46 29	54 06	52 26	34 26	59 48	53 44	42 39	52 64	47 06
	3	41 71	47 33	57 45	44 61	35 33	40 47	53 96	57 13	36 23	63 69	56 01	48 32	54 34	49 41
	4	44 83	50 54	60 62	47 22	39 76	47 87	58 42	52 28	31 19	57 69	49 61	43 15	51 68	43 28
	5	44 71	52 39	59 92	47 79	41 29	47 52	56 90	54 77	35 12	60 88	52 36	47 43	56 72	46 98
	Average	43 26	49 01	58 45	46 48	38 79	45 20	55 15	53 06	32 94	58 81	51 71	44 41	52 59	45 55
	STD Dev	1 78	2 41	1 81	1 53	2 57	3 08	2 44	3 09	3 39	4 25	3 43	3 30	3 40	3 36
	% RSD	4 1%	4 9%	3 1%	3 3%	6 6%	6 8%	4 4%	5 8%	10 3%	7 2%	6 6%	7 4%	6 5%	7 4%
3	1	34 13	42 27	47 03	39 65	30 14	37 57	45 94	42 11	36 16	53 98	49 41	34 33	31 94	36 43
	2	38 70	48 81	53 16	43 41	34 52	42 05	50 3	32 88	33 37	48 41	43 33	29 37	31 50	32 49
	3	37 48	45 97	54 82	43 75	33 31	40 86	49 66	35 86	31 13	44 6	39 61	28 58	30 90	30 84
	4	38 64	45 05	52 79	43 16	33 00	41 47	47 24	38 72	37 03	50 54	44 07	31 14	32 50	29 53
	5	37 68	43 35	52 46	43 17	33 21	40 70	46 96	37 82	37 31	51 59	46 02	31 18	33 25	33 63
	Average	37 33	45 09	52 05	42 63	32 84	40 53	48 02	37 48	35 00	49 82	44 49	30 92	32 02	32 58
	STD Dev	1 87	2 53	2 95	1 68	1 62	1 74	1 87	3 42	2 67	3 54	3 60	2 21	0 90	2 66
	% RSD	5 0%	5 6%	5 7%	3 9%	4 9%	4 3%	3 9%	9 1%	7 6%	7 1%	8 1%	7 2%	2 8%	8 2%
Individual	Average	42 35	47 77	57 46	45 96	37 42	44 91	52 63	49 82	44 26	61 93	51 96	42 10	49 24	48 50
	SD	4 34	3 29	5 10	3 40	4 17	4 32	4 21	9 71	15 32	12 30	6 97	8 91	13 53	15 06
	%RSD	10 3%	6 9%	8 9%	7 4%	11 1%	9 6%	8 0%	19 5%	34 6%	19 9%	13 4%	21 2%	27 5%	31 1%
Combined	Average	46 08	46 02	59 70	48 96	39 76	47 08	50 56							
	SD	8 31	11 03	9 53	6 19	7 24	10 11	11 07							
	% RSD	18 0%	24 0%	16 0%	12 6%	18 2%	21 5%	21 9%							
Horwiz ratio	PRSD(R)	44 774	44 774	44 774	44 774	44 774	44 774	44 774							
	HorRat	0 40	0 54	0 36	0 28	0 41	0 48	0 49							

Salmon muscle spike corrected data

Level 1 1 0 ng/g

Day	Replicate	Analyst 1 HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ	Analyst 2 HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	1 12	1 07	1 10	1 10	1 20	1 25	1 19	1 08	1 04	1 42	1 23	1 03	1 25	1 23
	2	1 05	1 03	1 05	1 04	1 12	1 06	1 06	1 15	1 19	1 48	1 16	1 01	1 32	1 27
	3	1 15	1 09	1 07	1 16	1 07	1 18	1 07	1 01	1 15	1 45	1 19	0 83	1 28	1 29
	4	1 03	1 07	1 03	1 10	1 10	1 22	1 06	1 21	1 17	1 39	1 16	1 10	1 25	1 20
	5	1 21	0 99	1 14	1 15	1 17	1 21	1 10	1 18	1 11	1 51	1 07	1 13	1 22	1 23
	Average	1 11	1 05	1 08	1 11	1 13	1 18	1 10	1 13	1 13	1 45	1 16	1 02	1 26	1 24
	STD Dev	0 07	0 04	0 04	0 05	0 05	0 07	0 06	0 08	0 06	0 05	0 06	0 12	0 04	0 04
	% RSD	6 6%	3 8%	4 0%	4 3%	4 6%	6 2%	5 0%	7 2%	5 2%	3 3%	5 1%	11 5%	3 0%	2 9%
2	1	0 93	1 18	1 16	1 10	0 94	1 04	1 10	1 18	0 76	1 37	1 15	0 85	1 25	1 02
	2	1 02	1 06	1 16	1 18	0 95	1 04	1 12	1 13	0 81	1 43	1 13	0 92	1 27	1 11
	3	1 18	1 19	1 14	1 04	1 03	1 03	1 08	1 07	0 79	1 36	1 11	1 05	1 18	1 08
	4	1 29	1 12	1 14	1 09	1 12	1 10	1 11	1 23	0 85	1 47	1 14	1 00	1 38	1 09
	5	1 02	1 03	1 13	1 07	0 93	1 00	1 06	1 34	0 81	1 58	1 19	0 88	1 18	1 10
	Average	1 09	1 12	1 15	1 10	0 99	1 04	1 09	1 19	0 80	1 44	1 14	0 94	1 25	1 08
	STD Dev	0 14	0 07	0 01	0 05	0 08	0 04	0 02	0 10	0 03	0 09	0 03	0 08	0 08	0 04
	% RSD	13 3%	6 4%	1 2%	4 8%	8 1%	3 5%	2 2%	8 6%	4 1%	6 2%	2 6%	8 9%	6 6%	3 3%
3	1	0 93	0 75	0 94	0 84	0 62	0 75	0 90	0 58	0 58	0 57	0 53	0 40	0 56	0 76
	2	0 87	0 79	0 96	0 94	0 76	0 86	0 91	0 64	0 75	0 70	0 60	0 57	0 58	0 88
	3	0 86	0 76	0 89	0 88	0 79	0 75	0 91	0 57	0 80	0 71	0 67	0 54	0 60	0 86
	4	0 71	0 68	0 84	0 81	0 66	0 71	0 79	0 60	0 78	0 77	0 70	0 53	0 57	0 84
	5	0 77	0 83	0 92	0 89	0 76	0 74	0 83	0 58	0 87	0 82	0 71	0 57	0 67	0 94
	Average	0 83	0 76	0 91	0 87	0 72	0 76	0 87	0 59	0 76	0 71	0 64	0 52	0 60	0 86
	STD Dev	0 09	0 06	0 05	0 05	0 07	0 06	0 05	0 03	0 11	0 09	0 08	0 07	0 04	0 07
	% RSD	10 5%	7 3%	5 2%	5 7%	10 3%	7 5%	6 3%	4 7%	14 3%	13 2%	11 8%	13 5%	7 4%	7 6%
Individual	Average	1 01	0 98	1 04	1 03	0 95	1 00	1 02	0 97	0 90	1 20	0 98	0 83	1 04	1 06
	SD	0 17	0 17	0 11	0 12	0 19	0 19	0 12	0 29	0 19	0 36	0 26	0 24	0 33	0 17
	%RSD	16 4%	17 2%	10 4%	11 9%	20 0%	19 0%	11 7%	29 4%	20 7%	30 3%	26 0%	29 2%	31 6%	16 1%
Combined	Average	0 99	0 94	1 12	1 00	0 89	1 02	1 04							
	SD	0 23	0 18	0 28	0 20	0 22	0 26	0 15							
	% RSD	23 2%	19 2%	24 9%	19 9%	24 8%	25 6%	14 4%							
Horwiz ratio	PRSD(R)	44 774	44 774	44 774	44 774	44 774	44 774	44 774							
	HorRat	0 52	0 43	0 56	0 44	0 55	0 57	0 32							

Salmon muscle spike corrected data

Level 2 10 0 ng/g

Day	Replicate	Analyst 1							Analyst 2						
		HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	10 79	10 20	9 48	9 60	9 20	10 05	9 85	9 79	9 96	13 10	9 79	8 34	10 45	10 84
	2	11 03	9 82	9 79	9 71	10 04	10 25	9 95	9 30	10 01	12 03	9 98	8 13	10 23	10 52
	3	10 36	10 38	10 03	10 29	10 14	10 62	10 11	9 07	10 15	11 69	10 20	8 25	10 59	10 71
	4	11 32	11 64	10 69	10 52	10 63	11 18	10 45	9 13	10 23	12 38	9 13	7 80	9 65	10 77
	5	11 16	10 50	10 49	10 68	11 12	10 87	10 25	9 91	10 51	13 00	10 46	8 59	10 67	11 59
	Average	10 93	10 51	10 10	10 16	10 23	10 59	10 12	9 44	10 17	12 44	9 91	8 22	10 32	10 89
	STD Dev	0 37	0 68	0 50	0 48	0 72	0 46	0 24	0 39	0 22	0 61	0 50	0 29	0 41	0 41
	% RSD	3 4%	6 5%	4 9%	4 8%	7 0%	4 3%	2 4%	4 1%	2 1%	4 9%	5 1%	3 5%	4 0%	3 8%
2	1	10 29	9 89	9 92	10 00	9 30	9 50	9 99	10 27	7 53	12 31	10 03	7 93	10 61	9 43
	2	11 07	9 49	10 31	10 44	9 90	10 41	10 59	8 95	5 57	10 37	8 92	7 99	10 19	8 08
	3	10 32	9 81	10 08	10 10	9 25	9 91	9 90	8 98	5 84	10 66	9 21	7 45	9 77	8 58
	4	11 18	10 33	10 40	10 38	9 14	10 39	10 64	10 51	6 94	12 33	10 10	8 45	10 37	9 36
	5	10 10	9 25	10 11	9 56	8 48	9 56	9 81	10 10	6 48	12 02	10 10	8 14	10 61	9 44
	Average	10 59	9 75	10 16	10 10	9 21	9 95	10 19	9 76	6 47	11 54	9 67	7 99	10 31	8 98
	STD Dev	0 50	0 41	0 19	0 35	0 51	0 44	0 40	0 74	0 80	0 95	0 56	0 36	0 35	0 62
	% RSD	4 7%	4 2%	1 9%	3 5%	5 5%	4 4%	3 9%	7 6%	12 3%	8 2%	5 8%	4 6%	3 4%	6 9%
3	1	8 49	6 89	8 35	8 05	7 80	7 82	8 38	6 64	7 74	7 70	6 61	5 55	6 17	8 70
	2	8 49	7 22	8 61	8 30	7 70	7 95	8 79	6 83	8 02	8 08	7 46	5 70	7 04	8 79
	3	8 70	7 10	8 19	8 00	7 96	7 80	8 64	6 39	7 44	7 68	6 73	5 62	6 60	7 10
	4	8 26	7 21	8 52	8 12	7 59	7 89	8 57	6 65	7 68	7 75	6 72	5 77	6 49	7 80
	5	8 04	6 08	7 91	7 84	7 69	7 48	7 91	5 23	7 28	6 98	6 06	5 08	5 82	6 83
	Average	8 40	6 90	8 32	8 06	7 75	7 79	8 46	6 35	7 63	7 64	6 72	5 54	6 42	7 84
	STD Dev	0 25	0 48	0 28	0 17	0 14	0 18	0 34	0 64	0 29	0 40	0 50	0 27	0 46	0 90
	% RSD	3 0%	6 9%	3 3%	2 1%	1 8%	2 3%	4 0%	10 1%	3 7%	5 3%	7 4%	4 9%	7 2%	11 4%
Individual	Average	9 97	9 05	9 53	9 44	9 06	9 45	9 59	8 52	8 09	10 54	8 77	7 25	9 02	9 24
	SD	1 22	1 68	0 94	1 06	1 16	1 29	0 88	1 69	1 67	2 25	1 58	1 29	1 94	1 44
	%RSD	12 2%	18 6%	9 9%	11 2%	12 7%	13 7%	9 2%	19 8%	20 6%	21 3%	18 0%	17 7%	21 5%	15 6%
Combined	Average	9 25	8 57	10 03	9 10	8 16	9 23	9 41							
	SD	1 63	1 72	1 77	1 37	1 51	1 63	1 19							
	% RSD	17 6%	20 0%	17 7%	15 0%	18 6%	17 7%	12 6%							
Horwiz ratio	PRSD(R)	44 774	44 77	44 774	44 774	44 774	44 774	44 774							
	HorRat	0 39	0 45	0 39	0 34	0 41	0 39	0 28							

Salmon muscle spike corrected data

Level 3 50 ng/g

		Analyst 1							Analyst 2						
Day	Replicate	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	49 35	45 97	44 64	45 14	47 22	45 77	47 83	49 45	52 99	64 30	50 31	42 39	51 84	56 67
	2	50 27	52 19	49 05	50 95	51 56	48 60	52 66	48 88	54 01	65 18	49 35	41 64	52 77	57 14
	3	52 53	52 35	50 37	50 51	54 03	50 60	54 73	49 93	57 88	68 44	51 56	43 88	54 35	58 65
	4	50 12	48 93	47 03	48 28	48 04	47 60	48 58	51 42	54 74	67 23	49 89	43 79	55 11	57 43
	5	55 89	55 39	53 18	54 39	56 79	53 10	54 83	48 99	54 11	60 58	50 76	43 40	52 33	54 42
	Average	51 63	50 97	48 85	49 85	51 53	49 13	51 73	49 73	54 75	65 15	50 37	43 02	53 28	56 86
	STD Dev	2 66	3 61	3 25	3 42	4 02	2 82	3 34	1 03	1 86	3 03	0 84	0 97	1 39	1 55
	% RSD	5 2%	7 1%	6 6%	6 9%	7 8%	5 7%	6 5%	2 1%	3 4%	4 7%	1 7%	2 3%	2 6%	2 7%
2	1	43 24	42 18	42 30	43 52	38 27	40 29	46 05	47 47	27 09	50 79	45 80	39 59	46 20	39 83
	2	46 46	43 78	43 69	46 09	42 20	42 51	47 48	50 77	33 28	57 78	51 91	41 17	51 14	45 72
	3	43 98	42 91	43 23	43 08	36 65	37 17	47 39	55 50	35 19	61 87	54 41	46 94	52 79	48 00
	4	47 26	45 83	45 62	45 60	41 24	43 97	51 31	50 79	30 30	56 04	48 19	41 92	50 21	42 05
	5	47 14	47 50	45 09	46 16	42 83	43 64	49 97	53 20	34 12	59 14	50 86	46 07	55 10	45 64
	Average	45 62	44 44	43 99	44 89	40 24	41 52	48 44	51 55	32 00	57 12	50 23	43 14	51 09	44 25
	STD Dev	1 87	2 19	1 36	1 48	2 66	2 82	2 14	3 01	3 29	4 13	3 33	3 20	3 30	3 26
	% RSD	4 1%	4 9%	3 1%	3 3%	6 6%	6 8%	4 4%	5 8%	10 3%	7 2%	6 6%	7 4%	6 5%	7 4%
3	1	32 84	32 23	33 05	34 95	30 12	29 78	38 88	37 92	40 49	40 50	40 42	34 07	29 92	41 76
	2	37 23	37 21	37 35	38 26	34 49	33 33	42 58	29 61	37 35	36 32	35 45	29 16	29 51	37 25
	3	36 06	35 04	38 52	38 56	33 28	32 39	42 03	32 29	34 84	33 47	32 40	28 37	28 97	35 36
	4	37 17	34 35	37 09	38 04	32 97	32 87	39 99	34 87	41 44	37 92	36 06	30 91	30 44	33 86
	5	36 26	33 05	36 86	38 05	33 19	32 26	39 75	34 06	41 76	38 71	37 65	30 95	31 14	38 55
	Average	35 91	34 38	36 57	37 57	32 81	32 13	40 65	33 75	39 18	37 38	36 40	30 69	30 00	37 36
	STD Dev	1 80	1 93	2 07	1 48	1 62	1 38	1 58	3 08	2 99	2 66	2 95	2 19	0 84	3 04
	% RSD	5 0%	5 6%	5 7%	3 9%	4 9%	4 3%	3 9%	9 1%	7 6%	7 1%	8 1%	7 2%	2 8%	8 1%
Individual	Average	44 39	43 26	43 14	44 11	41 53	40 93	46 94	45 01	41 97	53 22	45 67	38 95	44 79	46 16
	SD	6 99	7 49	5 66	5 65	8 42	7 55	5 32	8 61	10 16	12 46	7 21	6 41	11 04	8 73
	%RSD	15 8%	17 3%	13 1%	12 8%	20 3%	18 4%	11 3%	19 1%	24 2%	23 4%	15 8%	16 5%	24 7%	18 9%
Combined	Average	44 70	42 62	48 18	44 89	40 24	42 86	46 55							
	SD	7 71	8 79	10 80	6 41	7 47	9 50	7 12							
	% RSD	17 3%	20 6%	22 4%	14 3%	18 6%	22 2%	15 3%							
Horwiz ratio	PRSD(R)	44 774	44 774	44 774	44 774	44 774	44 774	44 774							
	HorRat	0 39	0 46	0 50	0 32	0 41	0 49	0 34							

Salmon muscle IS corrected data

Level 1 1.0 ng/g

Day	Replicate	Analyst 1						
		HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	1 01	1 04	1 40	1 08	0 95	1 24	1 26
	2	0 90	0 95	1 27	0 97	0 84	1 01	1 07
	3	1 01	1 02	1 32	1 09	0 81	1 14	1 10
	4	0 96	1 06	1 34	1 11	0 89	1 25	1 15
	5	1 03	0 91	1 36	1 06	0 87	1 14	1 10
	Average	0.98	1.00	1.34	1.06	0.87	1.16	1.14
	STD Dev	0.05	0 06	0.05	0 05	0.05	0 10	0 08
	% RSD	5.4%	6 4%	3 6%	5.1%	6.1%	8 4%	6 6%
2	1	0 92	1 36	1 61	1 14	0 95	1 18	1 31
	2	1 02	1 23	1 62	1 07	0 96	1 20	1 34
	3	1 13	1 33	1 54	1 11	1 01	1 13	1 24
	4	1 19	1 20	1 48	1 09	1 06	1 17	1 24
	5	0 97	1 13	1 50	1 10	0 90	1 10	1 21
	Average	1 05	1 25	1 55	1.10	0.98	1.16	1.27
	STD Dev	0.11	0 09	0 06	0.03	0.06	0.04	0.05
	% RSD	10.7%	7.6%	4.1%	2.3%	6 3%	3.5%	4 3%
3	1	0 89	0 91	1 24	0 87	0 57	0 87	0 98
	2	0 88	1 00	1 32	1 04	0 74	1 06	1 05
	3	0 88	0 98	1 26	0 99	0 78	0 93	1 06
	4	0 87	1 06	1 41	1 10	0 78	1 06	1 11
	5	0 81	1 11	1 32	1 02	0 77	0 94	0 99
	Average	0.87	1 01	1.31	1.00	0.73	0.97	1.04
	STD Dev	0 03	0 08	0.07	0.09	0.09	0 08	0 05
	% RSD	3.7%	7.6%	5 1%	8 5%	12 3%	8 7%	5 2%
Individual	Average	0 96	1.09	1.40	1.06	0.86	1 09	1 15
	SD	0.10	0 14	0.12	0.07	0.12	0.12	0 11
	%RSD	10.7%	13 0%	8.9%	6.6%	14.4%	10.5%	9 9%

Salmon muscle IS corrected data

Level 2 10.0 ng/g

Day	Replicate	Analyst 1						
		HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	8 80	8 93	10 89	8 52	6 58	9 09	9 45
	2	8 74	8 35	10 92	8 36	6 97	9 00	9 27
	3	8 49	9 12	11 57	9 16	7 28	9 64	9 73
	4	9 24	10 20	12 30	9 35	7 61	10 12	10 04
	5	9 19	9 28	12 16	9 57	8 03	9 92	9 93
	Average	8.89	9.18	11.57	8.99	7.29	9.55	9.68
	STD Dev	0.32	0.67	0.66	0.53	0.56	0 50	0.32
	% RSD	3.6%	7.3%	5.7%	5.9%	7.7%	5.2%	3 3%
2	1	9 55	10 67	12 89	8 80	8 76	10 11	11 12
	2	10 31	10 28	13 46	9 34	9 37	11 13	11 84
	3	9 56	10 58	13 08	9 84	8 71	10 55	11 01
	4	10 47	11 24	13 63	10 43	8 69	11 16	11 95
	5	11 15	11 88	15 65	11 93	9 52	12 13	13 01
	Average	10.21	10 93	13.74	10.07	9.01	11.02	11.79
	STD Dev	0.67	0.63	1 11	1.20	0 40	0.76	0 80
	% RSD	6.6%	5.8%	8.1%	11.9%	4.5%	6.9%	6.8%
3	1	9 56	9 79	12 87	9 90	8 46	10 69	10 73
	2	9 67	10 39	13 43	10 32	8 45	10 99	11 39
	3	9 69	9 99	12 50	9 73	8 54	10 55	10 93
	4	9 06	9 96	12 78	9 71	8 01	10 49	10 68
	5	9 83	9 38	13 24	10 46	9 06	11 10	10 99
	Average	9 56	9 90	12 96	10.02	8.50	10.76	10.94
	STD Dev	0 30	0 37	0 37	0.35	0 37	0 27	0.28
	% RSD	3 1%	3.7%	2 9%	3.4%	4.4%	2.5%	2.6%
Individual	Average	9.55	10.00	12 76	9.69	8 27	10.44	10.80
	SD	0.70	0 91	1 18	0.89	0.86	0.83	1.02
	%RSD	7.4%	9.1%	9.2%	9 2%	10.3%	8.0%	9 4%

Salmon muscle IS corrected data

Level 3 50 ng/g

Day	Replicate	Analyst 1						
		HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	43 71	43 70	55 67	43 48	36 67	44 94	49 81
	2	39 18	43 66	53 84	43 19	35 23	41 99	48 27
	3	44 65	47 77	60 30	46 70	40 27	47 68	54 71
	4	41 88	43 89	55 35	43 89	35 20	44 11	47 75
	5	45 51	48 40	60 98	48 16	40 54	47 93	52 50
	Average	42.99	45.48	57.23	45.08	37.58	45.33	50.61
	STD Dev	2.52	2.39	3.20	2 22	2.65	2.50	2.94
	% RSD	5.9%	5.2%	5.6%	4.9%	7 0%	5 5%	5.8%
2	1	43 94	49 84	60 22	46 67	39 53	47 01	56 17
	2	45 17	49 51	59 52	53 26	41 71	47 46	55 42
	3	45 07	51 15	62 08	52 45	38 18	43 73	58 31
	4	46 93	52 92	63 47	49 16	41 63	50 13	61 17
	5	45 76	53 62	61 32	54 52	42 26	48 63	58 23
	Average	45.37	51.41	61.32	51.21	40 66	47.39	57.86
	STD Dev	1.09	1.82	1.55	3.22	1.73	2.38	2 24
	% RSD	2.4%	3.5%	2.5%	6.3%	4 3%	5.0%	3.9%
3	1	42 57	52 71	58 66	49 45	37 59	46 85	57 29
	2	41 57	52 42	57 11	46 63	37 080	45 170	54 03
	3	38 94	47 75	56 96	45 46	34 610	42 450	51 59
	4	41 29	48 14	56 42	46 12	35 270	44 310	50 48
	5	39 52	45 46	55 02	45 28	34 840	42 680	49 24
	Average	40.78	49 30	56 83	46.59	35.88	44 29	52.53
	STD Dev	1.51	3.16	1.31	1.69	1 36	1.82	3 19
	% RSD	3.7%	6.4%	2.3%	3.6%	3 8%	4 1%	6.1%
Individual	Average	43.05	48.73	58.46	47.63	38.04	45.67	53 66
	SD	2.56	3.44	2 92	3.53	2 76	2.48	4.11
	%RSD	6.0%	7.1%	5.0%	7.4%	7 2%	5.4%	7.7%

Shrimp muscle uncorrected data

Level 1 1 0 ng/g

Day	Replicate	Analyst 1							Analyst 2						
		HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	1 13	1 15	1 41	1 09	0 96	1 24	1 15	1 04	0 93	1 26	1 04	0 87	1 15	1 18
	2	1 04	1 09	1 42	1 18	0 84	1 20	1 18	1 09	0 87	1 19	1 05	0 89	1 15	1 15
	3	1 07	1 20	1 41	1 20	0 91	1 19	1 22	1 01	0 89	1 28	1 24	0 86	1 26	1 23
	4	1 10	1 15	1 39	1 11	0 90	1 14	1 19	1 03	0 88	1 24	1 26	1 02	1 10	1 11
	5	1 25	1 16	1 47	1 18	0 80	1 27	1 25	1 09	0 84	1 33	1 17	0 86	1 04	1 22
	Average	1 12	1 15	1 42	1 15	0 88	1 21	1 20	1 05	0 88	1 26	1 15	0 90	1 14	1 18
	STD Dev	0 08	0 04	0 03	0 05	0 06	0 05	0 04	0 04	0 03	0 05	0 10	0 07	0 08	0 05
	% RSD	7 3%	3 4%	2 1%	4 2%	7 1%	4 1%	3 2%	3 5%	3 7%	4 1%	9 0%	7 6%	7 1%	4 2%
2	1	0 79	1 00	1 30	1 01	1 03	1 00	0 93	1 01	0 52	1 01	0 96	0 57	0 88	0 91
	2	1 06	1 03	1 29	0 93	0 93	0 98	1 03	1 11	0 67	1 17	1 06	0 57	1 06	1 04
	3	1 10	1 05	1 38	1 12	0 81	1 12	1 09	1 02	0 88	1 17	0 96	0 92	0 90	1 05
	4	1 02	1 03	1 30	0 97	0 82	0 90	1 09	1 06	0 74	1 21	1 11	0 78	1 11	1 07
	5	0 82	1 03	1 30	1 15	0 81	0 88	1 11	0 93	0 74	1 10	1 07	0 82	1 02	0 98
	Average	0 96	1 03	1 31	1 04	0 88	0 98	1 05	1 03	0 71	1 13	1 03	0 73	0 99	1 01
	STD Dev	0 14	0 02	0 04	0 10	0 10	0 10	0 07	0 07	0 13	0 08	0 07	0 16	0 10	0 07
	% RSD	14 9%	1 7%	2 8%	9 2%	11 1%	9 8%	7 0%	6 5%	18 4%	7 0%	6 6%	21 4%	10 1%	6 5%
3	1	1 03	0 98	1 18	1 06	0 93	0 91	1 00	1 15	0 95	1 16	1 21	0 79	1 21	1 09
	2	1 12	1 16	1 39	1 24	0 84	1 08	1 18	1 03	0 98	1 25	1 11	0 83	1 03	1 10
	3	1 12	1 10	1 33	1 22	0 77	1 02	1 16	1 09	0 98	1 18	1 05	0 85	1 11	1 04
	4	1 13	1 16	1 37	1 22	0 78	1 05	1 19	1 08	1 01	1 25	1 10	0 93	1 10	1 11
	5	1 12	1 14	1 29	1 25	0 90	1 03	1 15	1 00	0 97	1 23	1 19	0 88	1 05	1 14
	Average	1 10	1 11	1 31	1 20	0 84	1 02	1 14	1 07	0 98	1 21	1 13	0 86	1 10	1 10
	STD Dev	0 04	0 08	0 08	0 08	0 07	0 06	0 08	0 06	0 02	0 04	0 07	0 05	0 07	0 04
	% RSD	3 8%	6 8%	6 3%	6 5%	8 4%	6 3%	6 8%	5 4%	2 2%	3 4%	5 9%	6 2%	6 4%	3 3%
Individual	Average	1 06	1 10	1 35	1 13	0 87	1 07	1 13	1 05	0 86	1 20	1 11	0 83	1 08	1 09
	SD	0 12	0 07	0 07	0 10	0 08	0 12	0 09	0 05	0 14	0 08	0 09	0 12	0 10	0 09
	%RSD	11 1%	6 4%	5 4%	8 9%	8 6%	11 6%	7 7%	5 2%	15 9%	6 5%	8 4%	14 5%	9 4%	7 8%
Combined	Average	1 05	0 98	1 28	1 12	0 85	1 07	1 11							
	SD	0 09	0 16	0 11	0 10	0 10	0 11	0 09							
	% RSD	8 5%	16 5%	8 2%	8 5%	11 9%	10 4%	7 8%							
Horwiz ratio	PRSD(R)	44 774	44 774	44 774	44 774	44 774	44 774	44 774							
	HorRat	0 19	0 37	0 18	0 19	0 26	0 23	0 17							

Shrimp muscle uncorrected data

Level 2 10 0 *ng/g*

Day	Replicate	Analyst 1							Analyst 2						
		HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	9 93	10 35	12 42	10 38	8 89	10 68	10 81	10 09	8 57	11 73	10 42	8 82	10 33	10 32
	2	10 31	10 81	13 24	11 01	9 40	11 58	11 41	9 95	8 52	12 32	10 18	8 30	11 53	11 39
	3	11 60	11 58	13 97	11 76	9 68	12 67	12 74	10 50	9 11	12 89	10 74	9 47	11 23	11 47
	4	10 85	11 22	13 94	11 47	9 50	11 98	12 23	10 42	7 80	12 55	11 28	9 14	11 62	11 26
	5	10 71	10 48	13 38	11 77	9 39	11 53	11 53	9 86	8 88	13 02	10 75	9 26	11 29	11 85
	Average	10 68	10 89	13 39	11 28	9 37	11 69	11 74	10 16	8 58	12 50	10 67	9 00	11 20	11 26
	STD Dev	0 63	0 51	0 63	0 59	0 29	0 72	0 75	0 28	0 50	0 51	0 41	0 46	0 51	0 57
	% RSD	5 9%	4 7%	4 7%	5 2%	3 1%	6 2%	6 4%	2 8%	5 8%	4 1%	3 9%	5 1%	4 6%	5 0%
2	1	9 78	10 05	12 32	9 78	8 52	10 29	9 35	9 55	6 34	10 81	9 39	7 95	9 62	10 08
	2	10 04	9 89	12 29	9 93	8 50	10 22	10 19	10 30	6 59	11 14	10 74	8 20	10 17	10 28
	3	10 66	9 81	12 31	10 07	8 16	9 56	9 59	10 36	6 35	11 70	10 09	8 07	10 55	10 63
	4	9 46	9 83	12 66	8 87	8 98	10 19	10 09	10 78	5 74	10 86	10 40	8 89	10 24	9 77
	5	11 19	9 99	12 36	10 45	8 73	9 74	8 98	8 92	5 26	10 21	9 91	7 85	9 66	9 31
	Average	10 23	9 91	12 39	9 82	8 58	10 00	9 64	9 98	6 06	10 94	10 11	8 19	10 05	10 01
	STD Dev	0 70	0 10	0 15	0 59	0 30	0 33	0 51	0 74	0 54	0 54	0 51	0 41	0 40	0 50
	% RSD	6 8%	1 0%	1 2%	6 0%	3 5%	3 3%	5 3%	7 4%	9 0%	4 9%	5 0%	5 0%	4 0%	5 0%
3	1	10 89	10 26	13 00	11 48	8 53	10 38	11 58	10 19	8 08	10 43	10 20	8 09	9 92	9 69
	2	11 02	10 71	13 27	11 65	9 19	10 94	11 84	10 00	8 72	11 16	9 89	8 62	10 80	9 95
	3	10 26	11 02	13 29	12 00	10 20	10 64	10 97	11 36	9 75	12 19	10 60	8 52	11 18	11 34
	4	10 70	10 87	13 37	11 94	9 85	11 06	12 36	10 88	9 05	11 94	11 13	8 87	11 51	10 93
	5	10 35	10 72	13 01	11 33	9 87	11 34	11 53	10 86	8 58	11 04	10 77	8 34	11 09	11 03
	Average	10 64	10 72	13 19	11 68	9 53	10 87	11 66	10 66	8 84	11 35	10 52	8 49	10 90	10 59
	STD Dev	0 33	0 28	0 17	0 29	0 67	0 37	0 51	0 56	0 62	0 71	0 48	0 29	0 60	0 72
	% RSD	3 1%	2 7%	1 3%	2 5%	7 0%	3 4%	4 3%	5 2%	7 0%	6 3%	4 6%	3 5%	5 5%	6 8%
Individual	Average	10 52	10 51	12 99	10 93	9 16	10 85	11 01	10 27	7 82	11 60	10 43	8 56	10 72	10 62
	SD	0 57	0 54	0 57	0 95	0 60	0 85	1 15	0 60	1 40	0 88	0 50	0 50	0 69	0 77
	%RSD	5 4%	5 2%	4 4%	8 7%	6 6%	7 9%	10 4%	5 8%	17 8%	7 6%	4 8%	5 9%	6 5%	7 2%
Combined	Average	10 39	9 16	12 29	10 68	8 86	10 78	10 82							
	SD	0 59	1 72	1 02	0 79	0 62	0 77	0 98							
	% RSD	5 7%	18 7%	8 3%	7 4%	7 0%	7 1%	9 1%							
Horwiz ratio	PRSD(R)	44 774	44 774	44 774	44 774	44 774	44 774	44 774							
	HorRat	0 13	0 42	0 18	0 16	0 16	0 16	0 20							

Shrimp muscle uncorrected data

Level 3 50 ng/g

Day	Replicate	Analyst 1							Analyst 2						
		HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	52 50	57 32	65 41	54 12	46 8	54 35	58 24	47 30	41 98	59 40	47 52	39 20	48 35	52 86
	2	48 77	54 03	61 56	50 77	45 64	53 14	55 49	47 70	37 14	57 10	49 51	43 44	47 00	52 70
	3	54 64	40 71	65 81	55 13	49 43	54 70	61 54	50 69	44 82	62 47	51 49	44 77	52 16	61 00
	4	53 07	42 79	65 12	55 62	49 45	55 38	61 87	55 10	46 76	65 44	54 07	46 41	52 47	58 32
	5	53 84	44 00	64 46	55 18	49 75	55 12	60 29	53 22	48 98	62 88	56 18	45 15	52 88	61 33
	Average	52 56	47 77	64 47	54 16	48 21	54 54	59 49	50 80	43 94	61 46	51 75	43 79	50 57	57 24
	STD Dev	2 27	7 40	1 70	1 98	1 87	0 88	2 65	3 40	4 59	3 25	3 46	2 78	2 70	4 24
	% RSD	4 3%	15 5%	2 6%	3 6%	3 9%	1 6%	4 5%	6 7%	10 4%	5 3%	6 7%	6 3%	5 3%	7 4%
2	1	50 02	51 84	57 5	44 58	39 53	44 11	46 62	44 15	33 25	52 02	46 02	38 83	46 84	50 17
	2	51 41	58 51	60 88	48 28	41 23	49 23	51 05	52 45	23 84	43 04	41 87	33 97	38 99	40 15
	3	52 89	56 64	63 98	51 08	45 21	50 06	56 13	46 58	35 25	50 5	45 91	37 76	42 46	50 52
	4	55 28	53 34	61 17	50 22	44 62	52 55	55 84	49 25	34 54	53 54	49 35	40 84	46 96	52 63
	5	54 57	58 40	67 04	52 96	47 59	49 94	55 46	46 81	37 98	53 42	46 74	39 35	44 44	52 07
	Average	52 83	55 75	62 11	49 42	43 64	49 18	53 02	47 85	32 97	50 50	45 98	38 15	43 94	49 11
	STD Dev	2 18	3 02	3 59	3 19	3 23	3 10	4 14	3 14	5 39	4 35	2 68	2 59	3 33	5 11
	% RSD	4 1%	5 4%	5 8%	6 5%	7 4%	6 3%	7 8%	6 6%	16 3%	8 6%	5 8%	6 8%	7 6%	10 4%
3	1	46 33	48 68	56 31	48 29	40 66	45 12	49 84	54 04	48 46	55 92	52 55	38 6	49 65	53 85
	2	51 12	57 44	63 3	53 79	44 92	48 84	56 36	50 92	44 14	55 02	47 9	40 36	48 93	50 92
	3	51 29	55 48	64 58	52	45 41	50 93	58 7	54 45	48 65	59 6	54 96	42 76	52 67	58 91
	4	50 28	51 74	62 51	52 27	45 08	48 48	57 7	58 35	49 93	59 02	56 77	43 43	53 04	60 15
	5	50 10	54 18	62 98	51 63	45 50	46 37	55 99	53 71	48 54	56 75	56 12	43 59	50 31	54 62
	Average	49 82	53 50	61 94	51 60	44 31	47 95	55 72	54 29	47 94	57 26	53 66	41 75	50 92	55 69
	STD Dev	2 02	3 40	3 24	2 02	2 06	2 26	3 46	2 66	2 21	1 98	3 60	2 18	1 84	3 79
	% RSD	4 1%	6 4%	5 2%	3 9%	4 6%	4 7%	6 2%	4 9%	4 6%	3 5%	6 7%	5 2%	3 6%	6 8%
Individual	Average	51 74	52 34	62 84	51 73	45 39	50 55	56 07	50 98	41 62	56 41	50 46	41 23	48 48	54 01
	SD	2 44	5 80	2 99	3 03	3 09	3 63	4 22	3 95	7 66	5 60	4 54	3 36	4 16	5 48
	%RSD	4 7%	11 1%	4 8%	5 9%	6 8%	7 2%	7 5%	7 7%	18 4%	9 9%	9 0%	8 2%	8 6%	10 1%
Combined	Average	51 36	46 98	59 62	51 10	43 31	49 52	55 04							
	SD	3 25	8 62	5 49	3 85	3 81	3 98	4 92							
	% RSD	6 3%	18 3%	9 2%	7 5%	8 8%	8 0%	8 9%							
Horwiz ratio	PRSD(R)	44 774	44 774	44 774	44 774	44 774	44 774	44 774							
	HorRat	0 14	0 41	0 21	0 17	0 20	0 18	0 20							

Shrimp muscle spike corrected data

Level 1 1 0 ng/g

		Analyst 1							Analyst 2						
Day	Replicate	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	0 90	0 96	0 92	0 84	0 81	1 33	0 86	0 93	1 01	0 91	0 89	0 93	0 90	0 98
	2	0 83	0 92	0 93	0 91	0 71	1 29	0 88	0 97	0 94	0 86	0 90	0 95	0 90	0 96
	3	0 85	1 01	0 92	0 92	0 77	1 28	0 91	0 90	0 97	0 93	1 06	0 92	0 99	1 03
	4	0 87	0 96	0 91	0 85	0 76	1 23	0 89	0 92	0 95	0 90	1 08	1 09	0 87	0 92
	5	0 99	0 97	0 96	0 91	0 68	1 36	0 93	0 97	0 91	0 97	1 01	0 92	0 81	1 01
	Average	0 89	0 96	0 93	0 89	0 75	1 30	0 89	0 94	0 96	0 91	0 99	0 96	0 89	0 98
	STD Dev	0 06	0 03	0 02	0 04	0 05	0 05	0 03	0 03	0 04	0 04	0 09	0 07	0 07	0 04
	% RSD	7 1%	3 3%	2 1%	4 3%	6 9%	3 8%	3 0%	3 3%	3 9%	4 4%	9 0%	7 5%	7 3%	4 4%
2	1	0 62	0 80	0 87	0 81	0 97	0 77	0 74	0 94	0 58	0 83	0 95	0 67	0 87	0 81
	2	0 83	0 82	0 86	0 74	0 88	0 76	0 83	1 03	0 74	0 96	1 04	0 68	1 05	0 92
	3	0 86	0 83	0 91	0 90	0 77	0 87	0 87	0 95	0 98	0 96	0 95	1 09	0 89	0 93
	4	0 80	0 82	0 86	0 77	0 78	0 69	0 88	0 98	0 83	0 99	1 10	0 92	1 09	0 95
	5	0 64	0 82	0 86	0 92	0 77	0 68	0 89	0 86	0 83	0 91	1 06	0 97	1 01	0 86
	Average	0 75	0 82	0 87	0 83	0 83	0 75	0 84	0 95	0 79	0 93	1 02	0 87	0 98	0 89
	STD Dev	0 11	0 01	0 02	0 08	0 09	0 08	0 06	0 06	0 15	0 06	0 07	0 19	0 10	0 06
	% RSD	14 9%	1 3%	2 5%	9 6%	10 7%	10 1%	7 3%	6 5%	18 5%	6 8%	6 6%	21 4%	9 9%	6 5%
3	1	0 96	0 86	0 85	0 84	0 99	0 81	0 84	0 91	0 94	0 83	0 95	0 78	0 97	0 80
	2	1 04	1 02	1 00	0 98	0 89	0 97	0 98	0 81	0 97	0 89	0 87	0 82	0 83	0 81
	3	1 04	0 96	0 96	0 97	0 82	0 91	0 97	0 86	0 96	0 84	0 82	0 84	0 89	0 77
	4	1 05	1 03	0 99	0 96	0 83	0 94	1 00	0 85	1 00	0 89	0 86	0 92	0 89	0 82
	5	1 04	1 01	0 93	0 99	0 96	0 92	0 96	0 79	0 96	0 88	0 93	0 87	0 84	0 84
	Average	1 03	0 98	0 95	0 95	0 90	0 91	0 95	0 84	0 97	0 87	0 89	0 85	0 88	0 81
	STD Dev	0 04	0 07	0 06	0 06	0 08	0 06	0 06	0 05	0 02	0 03	0 05	0 05	0 06	0 03
	% RSD	3 6%	7 2%	6 4%	6 5%	8 5%	6 6%	6 7%	5 5%	2 3%	3 3%	6 0%	6 2%	6 3%	3 2%
Individual	Average	0 89	0 92	0 92	0 89	0 83	0 99	0 90	0 91	0 90	0 90	0 96	0 89	0 92	0 89
	SD	0 14	0 09	0 05	0 08	0 09	0 24	0 07	0 07	0 12	0 05	0 09	0 12	0 08	0 08
	%RSD	15 4%	9 3%	5 3%	8 6%	11 4%	24 7%	7 5%	7 3%	12 8%	5 7%	9 2%	13 7%	9 0%	9 3%
Combined	Average	0 90	0 91	0 91	0 93	0 86	0 95	0 89							
	SD	0 11	0 10	0 05	0 09	0 11	0 18	0 07							
	% RSD	11 8%	11 0%	5 4%	9 8%	13 0%	19 1%	8 3%							
Horwiz ratio	PRSD(R)	44 774	44 774	44 774	44 774	44 774	44 774	44 774							
	HorRat	0 26	0 25	0 12	0 22	0 29	0 43	0 19							

Shrimp muscle spike corrected data

Level 2 10 0 ng/g

Day	Replicate	Analyst 1							Analyst 2						
		HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	7 88	8 66	8 12	7 98	7 53	11 49	8 07	9 02	9 3	8 51	8 94	9 43	8 09	8 59
	2	8 19	9 04	8 66	8 46	7 96	12 45	8 52	8 9	9 25	8 94	8 73	8 88	9 04	9 48
	3	9 21	9 69	9 13	9 03	8 20	13 62	9 51	9 38	9 88	9 35	9 21	10 13	8 80	9 54
	4	8 61	9 39	9 11	8 81	8 05	12 89	9 13	9 31	8 46	9 11	9 67	9 78	9 10	9 36
	5	8 51	8 76	8 75	9 04	7 96	12 41	8 61	8 81	9 64	9 45	9 22	9 90	8 85	9 86
	Average	8 48	9 11	8 75	8 66	7 94	12 57	8 77	9 08	9 31	9 07	9 15	9 62	8 78	9 37
	STD Dev	0 50	0 43	0 41	0 45	0 25	0 78	0 56	0 25	0 54	0 37	0 35	0 49	0 40	0 47
	% RSD	5 9%	4 7%	4 7%	5 2%	3 1%	6 2%	6 4%	2 8%	5 8%	4 1%	3 9%	5 1%	4 6%	5 0%
2	1	7 69	7 98	8 18	7 80	8 07	7 97	7 51	8 83	7 09	8 90	9 28	9 39	9 48	8 93
	2	7 89	7 84	8 16	7 92	8 05	7 91	8 19	9 52	7 37	9 18	10 61	9 69	10 02	9 10
	3	8 38	7 78	8 17	8 04	7 73	7 40	7 71	9 58	7 11	9 64	9 97	9 53	10 41	9 42
	4	7 44	7 80	8 41	7 08	8 51	7 89	8 11	9 96	6 42	8 94	10 28	10 50	10 10	8 66
	5	8 80	7 93	8 20	8 34	8 27	7 53	7 22	8 25	5 89	8 41	9 79	9 27	9 52	8 25
	Average	8 04	7 87	8 22	7 84	8 13	7 74	7 75	9 23	6 78	9 01	9 99	9 68	9 91	8 87
	STD Dev	0 55	0 09	0 11	0 47	0 29	0 26	0 41	0 68	0 61	0 45	0 50	0 49	0 40	0 44
	% RSD	6 8%	1 1%	1 3%	6 0%	3 6%	3 3%	5 3%	7 4%	9 0%	5 0%	5 0%	5 0%	4 0%	5 0%
3	1	10 10	9 04	9 37	9 06	9 06	9 30	9 68	8 04	7 99	7 45	7 95	7 99	7 98	7 14
	2	10 22	9 44	9 56	9 19	9 77	9 80	9 90	7 89	8 62	7 97	7 70	8 51	8 69	7 33
	3	9 52	9 71	9 57	9 48	10 84	9 52	9 17	8 97	9 63	8 70	8 25	8 42	9 00	8 35
	4	9 93	9 57	9 63	9 42	10 47	9 90	10 33	8 58	8 94	8 53	8 67	8 76	9 26	8 05
	5	9 60	9 44	9 37	8 95	10 49	10 16	9 64	8 57	8 48	7 88	8 39	8 24	8 93	8 12
	Average	9 87	9 44	9 50	9 22	10 13	9 74	9 74	8 41	8 73	8 11	8 19	8 38	8 77	7 80
	STD Dev	0 31	0 25	0 12	0 23	0 71	0 33	0 42	0 44	0 61	0 51	0 38	0 29	0 49	0 53
	% RSD	3 1%	2 6%	1 3%	2 5%	7 0%	3 4%	4 3%	5 2%	7 0%	6 3%	4 6%	3 4%	5 6%	6 8%
Individual	Average	8 80	8 80	8 83	8 57	8 73	10 02	8 75	8 91	8 27	8 73	9 11	9 23	9 15	8 68
	SD	0 92	0 75	0 59	0 69	1 11	2 11	0 95	0 59	1 25	0 62	0 85	0 74	0 68	0 81
	%RSD	10 4%	8 5%	6 7%	8 1%	12 7%	21 0%	10 8%	6 6%	15 1%	7 1%	9 3%	8 0%	7 4%	9 4%
Combined	Average	8 85	8 54	8 78	8 84	8 98	9 58	8 72							
	SD	0 76	1 05	0 60	0 81	0 96	1 60	0 87							
	% RSD	8 6%	12 3%	6 8%	9 2%	10 7%	16 7%	10 0%							
Horwiz ratio	PRSD(R)	44 774	44 774	44 774	44 774	44 774	44 774	44 774							
	HorRat	0 19	0 27	0 15	0 20	0 24	0 37	0 22							

Shrimp muscle spike corrected data

Level 3 50 ng/g

Day	Replicate	Analyst 1							Analyst 2						
		HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	41 69	47 95	42 77	41 58	39 64	58 46	43 48	42 28	45 56	43 10	40 75	41 94	37 88	43 97
	2	38 73	45 20	40 25	39 00	38 65	57 16	41 43	42 63	40 30	41 43	42 38	46 47	36 83	43 84
	3	43 38	48 66	43 03	42 35	41 86	58 84	45 95	45 30	48 64	45 33	44 17	47 89	40 87	50 74
	4	42 14	46 22	42 58	42 73	41 88	59 57	46 19	49 25	50 74	47 49	46 37	49 66	41 12	48 51
	5	42 75	46 96	42 14	42 39	42 14	59 29	45 01	47 57	53 15	45 63	48 19	48 30	41 43	51 01
	Average	41 74	47 00	42 15	41 61	40 83	58 66	44 41	45 41	47 68	44 60	44 37	46 85	39 63	47 61
	STD Dev	1 80	1 37	1 11	1 52	1 58	0 94	1 98	3 04	4 98	2 36	2 99	2 97	2 12	3 52
	% RSD	4 3%	2 9%	2 6%	3 6%	3 9%	1 6%	4 5%	6 7%	10 4%	5 3%	6 7%	6 3%	5 3%	7 4%
2	1	39 32	41 12	38 17	35 59	37 44	34 13	37 46	40 82	37 21	42 85	45 47	45 86	46 18	44 44
	2	40 41	46 42	40 41	38 54	39 05	38 10	41 02	39 25	26 67	35 45	41 38	40 12	38 44	35 57
	3	41 58	44 93	42 47	40 77	42 82	38 74	45 1	43 07	39 45	41 6	45 36	44 6	41 86	44 76
	4	43 46	42 31	40 6	40 09	42 26	40 67	44 86	45 54	38 65	44 1	48 76	48 23	46 3	46 63
	5	42 89	46 33	44 5	42 28	45 07	38 64	44 56	43 28	42 51	44	46 18	46 48	43 82	46 13
	Average	41 53	44 22	41 23	39 45	41 33	38 06	42 60	42 39	36 90	41 60	45 43	45 06	43 32	43 51
	STD Dev	1 71	2 40	2 38	2 54	3 06	2 40	3 32	2 42	6 04	3 58	2 65	3 05	3 29	4 53
	% RSD	4 1%	5 4%	5 8%	6 4%	7 4%	6 3%	7 8%	5 7%	16 4%	8 6%	5 8%	6 8%	7 6%	10 4%
3	1	42 97	42 90	40 56	38 12	43 23	40 40	41 67	42 64	47 88	39 93	40 93	38 12	39 95	39 65
	2	47 41	50 61	45 6	42 45	47 76	43 73	47 12	40 18	43 61	39 28	37 31	39 86	39 37	37 49
	3	47 57	48 88	46 52	41 04	48 28	45 60	49 07	42 97	48 07	42 56	42 81	42 23	42 38	43 38
	4	46 63	45 59	45 03	41 26	47 93	43 41	48 24	46 05	49 33	42 15	44 22	42 89	42 68	44 29
	5	46 46	47 74	45 37	40 75	48 37	41 52	46 82	42 39	47 96	40 52	43 71	43 04	40 48	40 22
	Average	46 21	47 14	44 62	40 72	47 11	42 93	46 58	42 85	47 37	40 89	41 80	41 23	40 97	41 01
	STD Dev	1 87	2 99	2 33	1 59	2 19	2 02	2 89	2 10	2 18	1 42	2 80	2 16	1 48	2 79
	% RSD	4 1%	6 3%	5 2%	3 9%	4 6%	4 7%	6 2%	4 9%	4 6%	3 5%	6 7%	5 2%	3 6%	6 8%
Individual	Average	43 16	46 12	42 67	40 60	43 09	46 55	44 53	43 55	43 98	42 36	43 87	44 38	41 31	44 04
	SD	2 78	2 58	2 39	2 02	3 67	9 27	3 08	2 73	6 76	2 93	3 05	3 52	2 74	4 43
	%RSD	6 4%	5 6%	5 6%	5 0%	8 5%	19 9%	6 9%	6 3%	15 4%	6 9%	7 0%	7 9%	6 6%	10 0%
Combined	Average	43 35	45 05	42 51	42 23	43 74	43 93	44 29							
	SD	2 72	5 15	2 63	3 04	3 59	7 23	3 76							
	% RSD	6 3%	11 4%	6 2%	7 2%	8 2%	16 4%	8 5%							
Horwiz ratio	PRSD(R)	44 774	44 774	44 774	44 774	44 774	44 774	44 774							
	HorRat	0 14	0 26	0 14	0 16	0 18	0 37	0 19							

Shrimp muscle IS corrected data

Level 1 1 0 ng/g

Day	Replicate	Analyst 1						
		HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	1 04	1 06	1 30	0 99	0 88	1 14	1 06
	2	0 93	0 97	1 27	1 03	0 75	1 07	1 05
	3	0 94	1 05	1 23	1 03	0 80	1 03	1 06
	4	0 98	1 03	1 25	0 98	0 81	1 02	1 06
	5	1 11	1 04	1 32	1 04	0 72	1 13	1 12
	Average	1.00	1 03	1.27	1.01	0.79	1.08	1.07
	STD Dev	0.08	0.04	0.04	0 03	0.06	0.06	0.03
	% RSD	7.5%	3.4%	2.9%	2.7%	7.8%	5.1%	2.6%
2	1	0 79	1 01	1 31	1 02	1 03	1 00	0 93
	2	1 00	0 97	1 22	0 88	0 88	0 92	0 98
	3	1 04	0 99	1 30	1 06	0 77	1 06	1 03
	4	1 00	1 01	1 27	0 95	0 80	0 88	1 07
	5	0 80	1 02	1 28	1 13	0 80	0 87	1 09
	Average	0.93	1.00	1.28	1.01	0.86	0.95	1.02
	STD Dev	0.12	0.02	0.04	0.10	0.11	0.08	0.07
	% RSD	13.0%	2 0%	2.7%	9 6%	12.3%	8.6%	6 4%
3	1	0 90	0 85	1 02	0 92	0 81	0 79	0 87
	2	0 93	0 96	1 15	1 03	0 70	0 90	0 98
	3	0 94	0 91	1 11	1 02	0 64	0 85	0 96
	4	0 93	0 98	1 12	1 00	0 64	0 86	0 98
	5	0 92	0 94	1 06	1 03	0 74	0 85	0 95
	Average	0.92	0.93	1.09	1 00	0.71	0.85	0.95
	STD Dev	0 02	0.05	0.05	0 05	0.07	0.04	0.05
	% RSD	1.6%	5.5%	4.7%	4 6%	10.2%	4.6%	4 8%
Individual	Average	0 95	0.99	1 21	1.01	0.78	0.96	1.01
	SD	0 08	0.06	0 10	0.06	0.10	0.11	0.07
	%RSD	8.9%	5.7%	8.0%	5.9%	12.6%	11.7%	6.8%

Shrimp muscle IS corrected data

Level 2 10.0 ng/g

Day	Replicate	Analyst 1						
		HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	8 65	9 02	10 81	8 88	7 74	9 30	9 42
	2	8 98	9 42	11 53	9 42	8 19	10 08	9 94
	3	10 22	10 21	12 31	10 17	8 53	11 16	11 22
	4	9 23	9 55	11 86	9 59	8 09	10 20	10 41
	5	9 36	9 15	11 69	10 10	8 21	10 07	10 08
	Average	9.29	9.47	11.64	9.63	8.15	10.16	10 21
	STD Dev	0.59	0 46	0.55	0.53	0.28	0 66	0.67
	% RSD	6.3%	4.9%	4 7%	5.5%	3.5%	6.5%	6.5%
2	1	9 78	10 05	12 32	9 69	8 51	10 29	9 35
	2	9 57	9 43	11 73	9 47	8 11	9 75	9 72
	3	9 97	9 18	11 51	9 41	7 63	8 94	8 97
	4	8 91	9 25	11 92	8 35	8 46	9 59	9 50
	5	10 74	9 58	11 85	10 02	8 38	9 34	8 62
	Average	9 79	9 50	11.87	9 39	8.22	9 58	9 23
	STD Dev	0 66	0 35	0.30	0.63	0.36	0.50	0 44
	% RSD	6 8%	3.6%	2.5%	6.7%	4.4%	5 2%	4 7%
3	1	9 04	8 51	10 79	9 53	7 07	8 62	9 61
	2	9 69	9 41	11 66	10 25	8 08	9 62	10 41
	3	8 77	9 41	11 35	10 26	8 71	9 09	9 37
	4	8 74	8 87	10 90	9 74	8 03	9 03	10 09
	5	8 69	8 99	10 91	9 52	8 28	9 52	9 68
	Average	8.99	9.04	11.12	9.86	8 03	9.18	9 83
	STD Dev	0.42	0.38	0.37	0.37	0 60	0.40	0 41
	% RSD	4 6%	4.2%	3.3%	3 8%	7.5%	4.4%	4 2%
Individual	Average	9.36	9.34	11.54	9.63	8.13	9.64	9.76
	SD	0.63	0.43	0 50	0.52	0 41	0.65	0.64
	%RSD	6.7%	4 6%	4.4%	5.4%	5.1%	6.7%	6.5%

Shrimp muscle IS corrected data

Level 3 50 ng/g

Day	Replicate	Analyst 1						
		HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ
1	1	47 64	52 01	59 35	48 22	42 46	49 31	52 85
	2	44 39	49 18	56 02	45 38	41 53	48 37	50 51
	3	49 19	52 38	59 25	48 74	44 50	49 25	55 41
	4	46 66	48 58	58 57	48 02	43 47	48 69	54 40
	5	48 32	50 39	57 85	48 63	44 65	49 47	54 12
	Average	47.24	50.51	58.21	47.80	43.32	49.02	53.46
	STD Dev	1.84	1 68	1 36	1.38	1.33	0.47	1.88
	% RSD	3.9%	3.3%	2.3%	2.9%	3.1%	1 0%	3.5%
2	1	45 45	47 11	52 26	40 52	35 93	40 09	42 37
	2	44 06	50 16	52 18	41 39	35 34	42 20	43 76
	3	43 69	46 79	52 85	42 19	37 35	41 36	46 37
	4	46 82	45 18	51 81	42 54	37 79	44 51	47 29
	5	45 78	49 00	56 25	44 43	39 93	41 90	46 53
	Average	45 16	47.65	53.07	42.21	37.27	42.01	45 26
	STD Dev	1.28	1.95	1 82	1.46	1.79	1.61	2 10
	% RSD	2.8%	4.1%	3.4%	3.5%	4.8%	3 8%	4.6%
3	1	39 58	41 56	48 07	41 25	34 72	38 53	42 56
	2	41 81	46 96	51 73	43 99	36 72	39 93	46 09
	3	45 43	49 11	57 16	46 05	40 20	45 09	51 97
	4	43 92	45 17	54 56	45 66	39 36	42 33	50 38
	5	43 49	47 01	54 63	44 81	39 47	40 23	48 59
	Average	42.85	45.96	53 23	44.35	38.09	41.22	47.92
	STD Dev	2.24	2.83	3 47	1.91	2 30	2.55	3 71
	% RSD	5.2%	6.2%	6 5%	4.3%	6.0%	6.2%	7 7%
Individual	Average	45.08	48 04	54.84	44.79	39 56	44 08	48 88
	SD	2.51	2.82	3 32	2.81	3.26	3.98	4.32
	%RSD	5 6%	5.9%	6.0%	6.3%	8.2%	9.0%	8.8%

Appendix 14 Raw data for CCβ using uncorrected and spike corrected results for tilapia, salmon and shrimp muscle.

Tilapia muscle results using uncorrected data

	Day 1							Day 2							
	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ	
Result 1	0 450	0 060	0 120	0 060	0 290	0 340	0 200	0 390	0 110	0 190	0 060	0 450	0 440	0 280	
Result 2	0 420	0 050	0 120	0 070	0 300	0 340	0 190	0 550	0 070	0 140	0 080	0 440	0 440	0 210	
Result 3	0 460	0 060	0 160	0 060	0 250	0 340	0 170	0 520	0 080	0 150	0 090	0 400	0 370	0 250	
Result 4	0 490	0 080	0 150	0 070	0 360	0 300	0 220	0 380	0 070	0 190	0 080	0 500	0 420	0 300	
Result 5	0 450	0 080	0 110	0 070	0 290	0 330	0 200	0 370	0 090	0 150	0 080	0 320	0 390	0 180	
Result 6	0 450	0 090	0 150	0 080	0 370	0 350	0 180	0 530	0 070	0 160	0 080	0 380	0 370	0 220	
Result 7	0 580	0 080	0 160	0 070	0 370	0 350	0 210	0 430	0 080	0 170	0 090	0 410	0 360	0 260	
Result 8	0 530	0 080	0 120	0 080	0 430	0 330	0 160	0 470	0 090	0 170	0 100	0 430	0 490	0 220	
Result 9	0 490	0 090	0 140	0 100	0 290	0 450	0 210	0 380	0 080	0 140	0 080	0 400	0 440	0 240	
Result 10	0 510	0 090	0 140	0 070	0 270	0 350	0 150	0 370	0 080	0 150	0 110	0 320	0 410	0 220	
Average	0 483	0 076	0 137	0 073	0 322	0 348	0 189	0 439	0 082	0 161	0 085	0 405	0 413	0 238	
St Deviation	0 045	0 014	0 017	0 011	0 054	0 037	0 022	0 069	0 012	0 018	0 013	0 053	0 039	0 034	
% rel st Dev	9 3%	17 8%	12 7%	15 1%	16 8%	10 6%	11 7%	15 6%	14 2%	10 9%	15 1%	13 1%	9 4%	14 2%	
Indivdual CCß	0 474	0 091	0 133	0 086	0 411	0 360	0 236	0 513	0 088	0 133	0 089	0 410	0 364	0 255	ng/g
Combined CCβ	0 493	0 089	0 133	0 087	0 410	0 362	0 246	ng/g							

Tilapia muscle results using spike corrected data

	Day 1							Day 2							
	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ	
Result 1	0 420	0 060	0 090	0 070	0 350	0 310	0 210	0 400	0 110	0 140	0 060	0 540	0 360	0 240	
Result 2	0 400	0 050	0 100	0 080	0 370	0 320	0 210	0 570	0 070	0 110	0 080	0 540	0 370	0 190	
Result 3	0 430	0 060	0 130	0 070	0 300	0 310	0 180	0 530	0 080	0 110	0 090	0 480	0 310	0 220	
Result 4	0 470	0 080	0 120	0 080	0 430	0 280	0 240	0 400	0 070	0 140	0 090	0 600	0 350	0 260	
Result 5	0 420	0 080	0 090	0 080	0 350	0 310	0 220	0 380	0 090	0 120	0 090	0 390	0 330	0 150	
Result 6	0 430	0 090	0 120	0 090	0 450	0 320	0 200	0 550	0 070	0 120	0 080	0 460	0 310	0 190	
Result 7	0 560	0 080	0 130	0 080	0 350	0 320	0 230	0 450	0 080	0 130	0 090	0 420	0 300	0 220	
Result 8	0 510	0 070	0 100	0 100	0 530	0 310	0 180	0 480	0 090	0 130	0 100	0 520	0 410	0 190	
Result 9	0 460	0 090	0 110	0 120	0 360	0 410	0 230	0 390	0 080	0 100	0 080	0 490	0 360	0 200	
Result 10	0 480	0 090	0 110	0 080	0 330	0 330	0 170	0 390	0 080	0 110	0 110	0 390	0 340	0 190	
Average	0 458	0 075	0 110	0 085	0 382	0 322	0 207	0 454	0 082	0 121	0 087	0 483	0 344	0 205	
St Deviation	0 046	0 014	0 014	0 014	0 065	0 032	0 023	0 070	0 012	0 013	0 013	0 066	0 032	0 029	
% rel st Dev	10 1%	18 1%	12 9%	16 8%	17 0%	9 9%	11 0%	15 4%	14 2%	10 7%	14 6%	13 6%	9 2%	14 3%	
Indivdual CCß	0 476	0 091	0 127	0 091	0 429	0 352	0 237	0 514	0 088	0 126	0 089	0 431	0 352	0 248	ng/g
Combined CCβ	0 495	0 089	0 126	0 090	0 430	0 352	0 243	ng/g							

Salmon muscle results using uncorrected data

	Day 1							Day 2							
	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ	
Result 1	0 94	0 08	0 29	0 09	0 34	0 4	0 1	0 95	0 18	0 34	0 08	0 35	0 46	0 15	
Result 2	1 13	0 12	0 34	0 1	0 33	0 47	0 13	1 33	0 12	0 34	0 1	0 4	0 36	0 1	
Result 3	1 12	0 1	0 35	0 11	0 29	0 41	0 14	1 25	0 12	0 4	0 11	0 42	0 5	0 13	
Result 4	1 17	0 15	0 34	0 12	0 33	0 44	0 13	1 3	0 11	0 39	0 1	0 26	0 5	0 12	
Result 5	1 11	0 15	0 36	0 1	0 3	0 54	0 15	1 34	0 14	0 38	0 07	0 33	0 53	0 13	
Result 6	1 16	0 16	0 36	0 09	0 21	0 39	0 12	1 15	0 12	0 32	0 1	0 31	0 45	0 13	
Result 7	1 03	0 14	0 36	0 09	0 37	0 4	0 13	1 23	0 12	0 37	0 09	0 31	0 33	0 14	
Result 8	1 19	0 09	0 32	0 08	0 31	0 41	0 12	1 33	0 14	0 37	0 11	0 34	0 48	0 12	
Result 9	1 03	0 13	0 39	0 14	0 34	0 49	0 15	1 27	0 2	0 34	0 09	0 3	0 45	0 17	
Result 10	1 17	0 17	0 44	0 11	0 35	0 52	0 15	1 23	0 13	0 39	0 07	0 27	0 63	0 14	
Average	1.105	0.129	0 355	0 103	0 317	0.447	0.132	1.238	0.138	0.364	0.092	0 329	0.469	0 133	
St Deviation	0.076	0.029	0 038	0 017	0 042	0.052	0.015	0 111	0 028	0.026	0.014	0 049	0.080	0.018	
% rel st. Dev	6 9%	22 6%	10 7%	16 3%	13.3%	11 6%	11 6%	9.0%	20.2%	7.1%	15 2%	14 8%	17.0%	13 5%	
Indivdual CCß	1.125	0 177	0 300	0 108	0 471	0 494	0 140	1 182	0 175	0 280	0.103	0 481	0 540	0 144	ng/g
Combined CCβ	1 153	0.176	0.290	0.106	0.476	0 517	0 142	ng/g							

Salmon muscle results using spike corrected data

	Day 1							Day 2							
	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ	
Result 1	0 96	0 08	0 23	0 08	0 41	0 35	0 09	0 81	0 16	0 22	0 07	0 37	0 36	0 12	
Result 2	1 15	0 12	0 26	0 09	0 4	0 41	0 12	1 13	0 11	0 22	0 08	0 43	0 28	0 08	
Result 3	1 14	0 1	0 27	0 1	0 35	0 36	0 13	1 07	0 1	0 26	0 09	0 44	0 4	0 1	
Result 4	1 20	0 16	0 26	0 12	0 4	0 39	0 12	1 11	0 09	0 26	0 08	0 28	0 4	0 1	
Result 5	1 13	0 16	0 28	0 1	0 36	0 47	0 14	1 14	0 12	0 25	0 06	0 35	0 42	0 11	
Result 6	1 18	0 17	0 27	0 09	0 25	0 35	0 12	0 98	0 1	0 21	0 08	0 33	0 36	0 11	
Result 7	1 05	0 14	0 28	0 09	0 38	0 35	0 12	1 05	0 11	0 25	0 08	0 27	0 26	0 11	
Result 8	1 22	0 1	0 25	0 07	0 38	0 36	0 11	1 14	0 13	0 24	0 09	0 36	0 38	0 1	
Result 9	1 06	0 14	0 3	0 13	0 4	0 43	0 14	1 08	0 18	0 22	0 07	0 31	0 36	0 14	
Result 10	1 19	0 18	0 34	0 11	0 42	0 46	0 14	1 04	0 11	0 25	0 06	0 29	0 5	0 11	
Average	1 128	0 135	0 274	0 098	0 375	0 393	0 123	1 055	0 121	0 238	0 076	0 343	0 372	0 108	
St Deviation	0 077	0 032	0 028	0 017	0 047	0 045	0 015	0 095	0 027	0 018	0 010	0 056	0 065	0 015	
% rel st Dev	6 9%	23 7%	10 3%	17 6%	12 4%	11 3%	12 1%	9 0%	22 3%	7 5%	13 4%	16 3%	17 4%	13 6%	
Indivdual CCß	1 127	0 182	0 284	0 109	0 478	0 482	0 139	1 155	0 174	0 267	0 097	0 493	0 515	0 139	ng/g
Combined CCβ	1 141	0 178	0 276	0 103	0 486	0 498	0 139	ng/g							

Shrimp muscle results using uncorrected data

	Day 1							Day 2							
	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ	
Result 1	0 84	0 07	0 08	0 09	0 28	0 15	0 08	0 89	0 06	0 09	0 1	0 17	0 13	0 07	
Result 2	1 08	0 07	0 11	0 1	0 26	0 25	0 1	0 96	0 07	0 1	0 11	0 18	0 19	0 08	
Result 3	1 04	0 07	0 1	0 09	0 21	0 25	0 1	1 04	0 06	0 11	0 11	0 23	0 26	0 09	
Result 4	1 06	0 06	0 1	0 1	0 17	0 22	0 1	1 01	0 07	0 11	0 12	0 27	0 21	0 09	
Result 5	1 01	0 06	0 09	0 1	0 24	0 18	0 09	0 96	0 07	0 1	0 11	0 26	0 19	0 08	
Result 6	0 94	0 08	0 08	0 1	0 18	0 17	0 08	0 8	0 07	0 1	0 11	0 24	0 19	0 08	
Result 7	1 00	0 06	0 09	0 1	0 17	0 21	0 08	0 93	0 08	0 11	0 1	0 22	0 17	0 08	
Result 8	0 92	0 08	0 1	0 11	0 23	0 19	0 1	0 87	0 08	0 11	0 15	0 28	0 16	0 09	
Result 9	0 93	0 07	0 09	0 09	0 16	0 24	0 1	1 02	0 07	0 1	0 09	0 29	0 23	0 09	
Result 10	0 94	0 06	0 1	0 1	0 15	0 22	0 07	0 97	0 07	0 1	0 11	0 2	0 19	0 09	
Average	0 976	0 068	0 094	0 098	0 205	0 208	0 090	0 945	0 070	0 103	0 111	0 234	0 192	0 084	
St Deviation	0 071	0 007	0 009	0 006	0 043	0 033	0 011	0 071	0 006	0 006	0 015	0 040	0 034	0 007	
% rel st Dev	7 2%	11 0%	9 8%	6 1%	21 1%	15 8%	12 2%	7 5%	9 0%	6 2%	13 6%	16 9%	17 9%	7 9%	
Indivdual CCß	1 016	0 090	0 110	0 110	0 316	0 254	0 118	1 016	0 088	0 105	0 125	0 310	0 256	0 111	ng/g
Combined CCβ	1 016	0 089	0 107	0 118	0 313	0 255	0 114	ng/g							

Shrimp muscle results using spike corrected data

	Day 1							Day 2							
	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ	HMMNI	IPZ	IPZ-OH	MNZ	MNZ-OH	RNZ	DMZ	
Result 1	0 99	0 06	0 07	0 10	0 35	0 14	0 08	1 01	0 06	0 08	0 11	0 20	0 13	0 08	
Result 2	1 27	0 06	0 10	0 12	0 32	0 24	0 10	1 09	0 07	0 10	0 12	0 20	0 19	0 08	
Result 3	1 22	0 07	0 09	0 10	0 26	0 23	0 10	1 18	0 06	0 11	0 12	0 26	0 25	0 09	
Result 4	1 25	0 06	0 09	0 12	0 21	0 20	0 10	1 14	0 07	0 11	0 13	0 30	0 21	0 09	
Result 5	1 19	0 06	0 08	0 11	0 30	0 17	0 09	1 09	0 07	0 09	0 12	0 30	0 19	0 08	
Result 6	1 11	0 08	0 08	0 12	0 22	0 16	0 08	0 91	0 07	0 09	0 12	0 28	0 19	0 09	
Result 7	1 18	0 06	0 08	0 11	0 21	0 19	0 08	1 05	0 07	0 10	0 10	0 25	0 17	0 09	
Result 8	1 08	0 08	0 09	0 13	0 29	0 23	0 10	0 98	0 07	0 11	0 16	0 31	0 16	0 09	
Result 9	1 10	0 07	0 08	0 10	0 20	0 23	0 10	1 16	0 06	0 10	0 10	0 33	0 23	0 09	
Result 10	1 11	0 06	0 09	0 12	0 18	0 21	0 07	1 10	0 07	0 09	0 11	0 22	0 19	0 09	
Average	1 150	0 066	0 085	0 113	0 254	0 200	0 090	1 071	0 067	0 098	0 119	0 265	0 191	0 087	
St Deviation	0 082	0 008	0 008	0 010	0 055	0 033	0 011	0 080	0 005	0 010	0 016	0 044	0 032	0 005	
% rel st Dev	7 2%	12 1%	9 5%	8 9%	21 7%	16 3%	12 2%	7 5%	6 8%	10 0%	13 8%	16 7%	17 0%	5 3%	
Indivdual CCß	1 035	0 090	0 108	0 117	0 336	0 253	0 118	1 032	0 085	0 111	0 128	0 318	0 253	0 108	ng/g
Combined CCβ	1 033	0 088	0 109	0 122	0 327	0 253	0 113	ng/g							

Appendix 15 The weight and length of Trout samples at each time point during the depletion study.

Date (yymmdd)	Sample	Weight (g)	Length (cm)
100920	1	168 0	24
	2	361 1	29
	3	343 7	28
	4	463 9	30
	5	270 6	28
	6	432 5	30
	7	300 6	27
100922	1	63 4	18
	2	149 8	22
	3	181 3	24
	4	156 3	23
	5	197 7	23
	6	196 2	23
	7	167	23
100924	1	153 9	22
	2	119 7	22
	3	163 3	23
	4	219 3	26
	5	270 8	29
	6	245 7	27
	7	141 3	21
100925	1	183 9	24
	2	215 0	27
	3	241 6	28
	4	189 8	23
	5	252 4	27
	6	235 0	26
	7	170 7	22
100927	1	93 0	19
	2	196 3	25
	3	209 5	25
	4	185 8	22
	5	159 0	22
	6	172 9	21
	7	167 5	22
100929	1	165 0	23
	2	219 2	25
	3	324 2	30
	4	260 8	26
	5	101 6	22
	6	172 1	22
	7	283 6	29
101007	1	222 1	25
	2	250 3	28
	3	135 3	21
	4	169 1	23
	5	234 6	24
101012	1	182 3	25
	2	181 3	24
	3	218 3	26
	4	97 6	23
	5	147 9	24
101015	1	198 1	25
	2	261 7	27
	3	226 5	26
	4	167 4	22
	5	141 3	24
101018	1	142 7	24
	2	174 0	23
	3	265 3	28
	4	327 4	27
	5	153 7	22
101022	1	158 9	23
	2	358 4	32
	3	154 9	24
	4	266 8	27
	5	231 3	25

Appendix 16 Individual trout sample results from duplicate analyses during depletion study dosing and withdrawal periods.

Results (ng/g) from first dosing and withdrawal period												
	Day 0		Day 3 Dosing		Day 5 Dosing		Day 1 Withdrawal		Day 3 Withdrawal		Day 5 Withdrawal	
	MNZ	MNZ-OH	MNZ	MNZ-OH	MNZ	MNZ-OH	MNZ	MNZ-OH	MNZ	MNZ-OH	MNZ	MNZ-OH
Sample												
1	0 00	0 00	16494 96	275 55	31009 57	1340 73	34977 34	1190 30	1066 01	37 63	1397 95	207 59
1rep	0 00	0 00	15198 21	327 02	30619 24	1415 11	34029 03	1209 15	1252 77	37 32	1375 47	200 94
Mean	**0.00**	**0.00**	**15846.59**	**301.29**	**30814.41**	**1377.92**	**34503.19**	**1199.73**	**1159.39**	**37.48**	**1386.71**	**204.27**
SD	**0.00**	**0.00**	**916.94**	**36.39**	**276.00**	**52.59**	**670.56**	**13.33**	**132.06**	**0.22**	**15.90**	**4.70**
%RSD	**0.00%**	**0.00%**	**5.79%**	**12.08%**	**0.90%**	**3.82%**	**1.94%**	**1.11%**	**11.39%**	**0.58%**	**1.15%**	**2.30%**
2	0 00	0 00	24601 57	531 95	1010 71	5 92	12071 22	254 93	7469 22	355 86	5801 63	306 25
2rep	0 00	0 00	23343 10	491 72	1090 15	6 71	12071 87	279 83	6777 89	376 56	6825 66	397 46
Mean	**0.00**	**0.00**	**23972.34**	**511.84**	**1050.43**	**6.32**	**12071.55**	**267.38**	**7123.56**	**366.21**	**6313.65**	**351.86**
SD	**0.00**	**0.00**	**889.87**	**28.45**	**56.17**	**0.56**	**0.46**	**17.61**	**488.84**	**14.64**	**724.10**	**64.50**
%RSD	**0.00%**	**0.00%**	**3.71%**	**5.56%**	**5.35%**	**8.85%**	**0.00%**	**6.58%**	**6.86%**	**4.00%**	**11.47%**	**18.33%**
3	0 00	0 00	21080 20	341 52	23482 48	668 60	36258 44	1673 44	8968 26	585 91	7260 46	318 89
3rep	0 00	0 00	19043 35	269 67	26446 58	756 09	41466 15	1884 52	10305 62	649 73	7691 58	243 50
Mean	**0.00**	**0.00**	**20061.78**	**305.60**	**24964.53**	**712.35**	**38862.30**	**1778.98**	**9636.94**	**617.82**	**7476.02**	**281.20**
SD	**0.00**	**0.00**	**1440.27**	**50.81**	**2095.94**	**61.86**	**3682.41**	**149.26**	**945.66**	**45.13**	**304.85**	**53.31**
%RSD	**0.00%**	**0.00%**	**7.18%**	**16.63%**	**8.40%**	**8.68%**	**9.48%**	**8.39%**	**9.81%**	**7.30%**	**4.08%**	**18.96%**
4	0 00	0 00	23131 24	386 73	28609 58	1126 53	29649 55	574 53	15538 39	943 14	1291 10	160 42
4rep	0 00	0 00	20481 16	314 62	25186 32	913 96	28518 85	645 26	17404 04	1014 70	1090 08	162 72
Mean	**0.00**	**0.00**	**21806.20**	**350.68**	**26897.95**	**1020.25**	**29084.20**	**609.90**	**16471.22**	**978.92**	**1190.59**	**161.57**
SD	**0.00**	**0.00**	**1873.89**	**50.99**	**2420.61**	**150.31**	**799.53**	**50.01**	**1319.21**	**50.60**	**142.14**	**1.63**
%RSD	**0.00%**	**0.00%**	**8.59%**	**14.54%**	**9.00%**	**14.73%**	**2.75%**	**8.20%**	**8.01%**	**5.17%**	**11.94%**	**1.01%**
5	0 00	0 00	19443 43	308 46	14539 93	381 83	21854 79	702 29	10122 01	648 31	5 75	0 52
5rep	0 00	0 00	19066 32	375 26	13546 19	313 06	28793 57	993 30	11209 97	721 28	5 00	0 58
Mean	**0.00**	**0.00**	**19254.88**	**341.86**	**14043.06**	**347.45**	**25324.18**	**847.80**	**10665.99**	**684.80**	**5.38**	**0.55**
SD	**0.00**	**0.00**	**266.66**	**47.23**	**702.68**	**48.63**	**4906.46**	**205.78**	**769.30**	**51.60**	**0.53**	**0.04**
%RSD	**0.00%**	**0.00%**	**1.38%**	**13.82%**	**5.00%**	**14.00%**	**19.37%**	**24.27%**	**7.21%**	**7.53%**	**9.87%**	**7.71%**
6	0 00	0 00	23730 97	402 05	20986 17	708 62	15096 25	103 40	11242 78	814 04	2774 35	204 61
6rep	0 00	0 00	22987 18	381 52	23870 21	920 08	16816 11	96 36	11731 20	872 01	2973 59	238 46
Mean	**0.00**	**0.00**	**23359.08**	**391.79**	**22428.19**	**814.35**	**15956.18**	**99.88**	**11486.99**	**843.03**	**2873.97**	**221.54**
SD	**0.00**	**0.00**	**525.94**	**14.52**	**2039.32**	**149.52**	**1216.12**	**4.98**	**345.37**	**40.99**	**140.88**	**23.94**
%RSD	**0.00%**	**0.00%**	**2.25%**	**3.71%**	**9.09%**	**18.36%**	**7.62%**	**4.98%**	**3.01%**	**4.86%**	**4.90%**	**10.80%**
7	0 00	0 00	20818 40	477 69	25323 11	1062 58	32905 97	903 83	11625 05	626 55	6098 28	523 40
7rep	0 00	0 00	21430 76	475 16	31144 69	1077 47	39445 90	1146 25	14044 42	815 40	6057 69	472 51
Mean	**0.00**	**0.00**	**21124.58**	**476.43**	**28233.90**	**1070.03**	**36175.94**	**1025.04**	**12834.74**	**720.98**	**6077.99**	**497.96**
SD	**0.00**	**0.00**	**433.00**	**1.79**	**4116.48**	**10.53**	**4624.43**	**171.42**	**1710.75**	**133.54**	**28.70**	**35.98**
%RSD	**0.00%**	**0.00%**	**2.05%**	**0.38%**	**14.58%**	**0.98%**	**12.78%**	**16.72%**	**13.33%**	**18.52%**	**0.47%**	**7.23%**

Results (ng/g) from the second withdrawal period										
	Day 1 Withdrawal		Day 6 Withdrawal		Day 9 Withdrawal		Day 12 Withdrawal		Day 16 Withdrawal	
	MNZ	MNZ-OH	MNZ	MNZ-OH	MNZ	MNZ-OH	MNZ	MNZ-OH	MNZ	MNZ-OH
Sample										
1	16315 00	398 72	1684 71	196 42	34 96	6 51	1 93	0 00	0 75	0
1rep	17949 44	441 17	2018 62	189 93	29 02	7 57	1 92	0 00	0 9	0
Mean	**17132.22**	**419.95**	**1851.67**	**193.18**	**31.99**	**7.04**	**1.93**	**0.00**	**0.83**	**0.00**
SD	**1155.72**	**30.02**	**236.11**	**4.59**	**4.20**	**0.75**	**0.01**	**0.00**	**0.11**	**0.00**
%RSD	**6.75%**	**7.15%**	**12.75%**	**2.38%**	**13.13%**	**10.65%**	**0.37%**	**0.00%**	**12.86%**	**0.00%**
2	14540 59	345 01	667 98	34 24	366 68	29 34	41 41	7 19	0 49	0
2rep	14924 80	256 99	760 57	25 00	404 64	33 75	46 09	7 87	0 53	0
Mean	**14732.70**	**301.00**	**714.28**	**29.62**	**385.66**	**31.55**	**43.75**	**7.53**	**0.51**	**0.00**
SD	**271.68**	**62.24**	**65.47**	**6.53**	**26.84**	**3.12**	**3.31**	**0.48**	**0.03**	**0.00**
%RSD	**1.84%**	**20.68%**	**9.17%**	**22.06%**	**6.96%**	**9.89%**	**7.56%**	**6.39%**	**5.55%**	**0.00%**
3	30881 27	958 82	927 05	93 29	27 11	3 55	181 61	24 08	0 66	0
3rep	26925 03	900 90	1038 84	84 38	29 41	2 78	205 98	23 60	0 66	0
Mean	**28903.15**	**929.86**	**982.95**	**88.84**	**28.26**	**3.17**	**193.80**	**23.84**	**0.66**	**0.00**
SD	**2797.48**	**40.96**	**79.05**	**6.30**	**1.63**	**0.54**	**17.23**	**0.34**	**0.00**	**0.00**
%RSD	**9.68%**	**4.40%**	**8.04%**	**7.09%**	**5.75%**	**17.20%**	**8.89%**	**1.42%**	**0.00%**	**0.00%**
4	23553 66	623 23	335 03	57 12	375 07	37 48	7 07	0 65	1 08	0
4rep	22602 83	635 54	242 69	55 20	436 76	41 06	6 15	0 60	1 2	0
Mean	**23078.25**	**629.39**	**288.86**	**56.16**	**405.92**	**39.27**	**6.61**	**0.63**	**1.14**	**0.00**
SD	**672.34**	**8.70**	**65.29**	**1.36**	**43.62**	**2.53**	**0.65**	**0.04**	**0.08**	**0.00**
%RSD	**2.91%**	**1.38%**	**22.60%**	**2.42%**	**10.75%**	**6.45%**	**9.84%**	**5.66%**	**7.44%**	**0.00%**
5	17992 38	456 55	1283 45	44 66	301 56	29 64	9 32	1 18	5 73	0 97
5rep	17772 15	502 53	1625 95	49 29	365 12	36 77	9 62	1 55	6 17	1 01
Mean	**17882.27**	**479.54**	**1454.70**	**46.98**	**333.34**	**33.21**	**9.47**	**1.37**	**5.95**	**0.99**
SD	**155.73**	**32.51**	**242.18**	**3.27**	**44.94**	**5.04**	**0.21**	**0.26**	**0.31**	**0.03**
%RSD	**0.87%**	**6.78%**	**16.65%**	**6.97%**	**13.48%**	**15.18%**	**2.24%**	**19.17%**	**5.23%**	**2.86%**

Appendix 17 An example of a Grubbs test results for MNZ sample residues to determine any outliers.

Grubbs Test

Significance level:	0.05 (two-sided)
Critical value of Z:	2.02

	MNZ		
Row	**Value**	**Z**	***Significant Outlier?***
1	15846.59	1.7957	Furthest from the rest, but not a significant outlier (P>0.05)
2	23972.34	1.165	
3	20061.78	0.2599	
4	21806.2	0.3757	
5	19254.88	0.5539	
6	23359.08	0.9415	
7	21124.58	0.1273	