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Enzyme-linked immunosorbent assay (ELISA) for the screening of dioxins in fish samples

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ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE SCREENING OF DIOXINS IN FISH SAMPLES

by

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ABSTRACT

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Dioxins are environmental contaminants that are toxic to humans. The conventional analytical method for dioxins, gas chromatography – high resolution mass spectrometry, is extremely time-consuming and expensive. Research is needed to find alternative methods that will increase sample throughput while decreasing time and costs associated with dioxin detection.

Dioxins readily accumulate in fish tissue and fish are a common food source for humans. Thus, the goal of this research was to develop a screening technique for dioxins in fish samples using enzyme-linked immunosorbent assay (ELISA). Three approaches, each with a different fish sample purification method but all using ELISA detection, were undertaken. This research concluded that the approach of Florisil® cleanup followed by ELISA detection (Florisil®-ELISA) was suitable as a screening technique. The other two approaches, one using gel permeation chromatography (GPC-ELISA) and the other using acid silica and carbon columns (acid silica/carbon-ELISA) for fish sample cleanup, were not suitable.

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This thesis is dedicated to my parents, Kuo-Hsing Chen and Louisa Chen.

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1 Introduction

Dioxins are environmental contaminants that are potentially toxic to living organisms. They exist everywhere in the environment and are characterized by high lipophilicity, persistence, and bioaccumulative properties. Their presence and exposure has been attributed to a broad spectrum of effects in humans such as chloracne, endocrine disruption and enzyme induction. The threat posed by dioxins has resulted in a strong demand for its monitoring by both the government and public. It is especially vital to monitor dioxins in common food sources susceptible to dioxin bioaccumulation such as fish since the majority of human exposure to dioxins is via ingestion. The Sport Fish Contaminant Monitoring Program exists in Ontario for the purpose of protecting public health.

Dioxin analysis is considered to be the most difficult in analytical chemistry. The current method of detection, gas chromatography – high resolution mass spectrometry (GC-HRMS), for dioxins in all environmental matrices, including fish, is very time-consuming and expensive. This can be attributed to the need for extensive extraction, meticulous cleanup and extremely sensitive detection methods for trace analysis of dioxins (Nording et al., 2006). The cost to analyze one sample, regardless of whether the result is dioxin-positive or dioxin-negative, is estimated to be $1,900 USD.

At the Ontario Ministry of the Environment (OMOE), dioxin analysis for fish involves a triple extraction procedure, thorough chromatographic cleanup using acid silica, alumina and carbon adsorbents, followed by instrumental GC-HRMS analysis. The ubiquitous presence of dioxins presents a problem since dioxins need to be detected at ultra-trace levels. To counter this ubiquitous presence, dioxin analysis must be performed in a specially-designed lab, dedicated solely to dioxin analysis. This specially-designed dioxin lab with negative air pressure is also

necessary for safety reasons. Thus, under optimum conditions, only 10 fish samples can be analyzed in 8 working days at OMOE (OMOE, 2006b).

Research is needed in the area of dioxin detection. Alternative methods to GC-HRMS should be sought in order to decrease the time and money spent on dioxin analysis, and increase sample throughput. Research is also needed in the area of sample preparation (extraction and cleanup) for the alternative methods so that compatibility with the alternative test is guaranteed from the outset (Harrison & Eduljee, 1999). Successful and complete extraction is necessary to ensure the separation of dioxins from the rest of the sample matrix and successful cleanup ensures that dioxins are separated from other organic coextractives (Reiner et al., 2006). These two combined – extraction and cleanup – are of utmost importance for the end results for any detection method (Nording et al., 2006). Although any new alternative method, even if comparable, may not replace the gold standard GC-HRMS method, the new method could complement or be used as a screen test and flag only the dioxin-positive samples for GC-HRMS determination.

A variety of alternative dioxin detection methods have already been explored by the scientific community. Among them are bioanalytical methods such as chemically activated luciferase gene expression (CALUX), polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA). The application of ELISA, which is a technique widely used in the medical industry, has a good track record for its adaptation for use in measuring a wide range of environmental contaminants in a variety of matrices.

Regarding ELISA for dioxin detection, studies have validated its use in an assortment of matrices such as soil, sediment, and breast milk. Evidently, a United States Environmental Protection Agency (US EPA) method exists for dioxin determination in soil using ELISA

(method 4025) (see US EPA, 2002a). There have been very few studies on dioxin analysis in fish using ELISA due in part to the effort required to remove lipids from the sample. Thus, the front-end aspect of fish sample cleanup is also important since an effective cleanup method is necessary to ensure the accurate quantification of dioxins. Sample cleanup methods for dioxin ELISA are generally less exhaustive and are variations of those used in conventional GC-HRMS analysis. For the few known studies of dioxin ELISA for fish, preliminary results show promise for the assay to be used as a screening test.

Dioxin contamination is ubiquitous in the environment. Hence, the Laboratory Services Branch (LSB) at the OMOE needs to analyze for dioxins in all the environmental sample matrices including soil, food and drinking water. The requirement for detection level is parts per billion (ppb) for soil, parts per trillion (ppt) for food (such as fish) and parts per quadrillion (ppq) for drinking water (Table 1.1). See Appendix A, Table A1, for mass units used in dioxin analysis.

Table 1.1 – Detection requirements for dioxins in various environmental matrices*.

Environmental Matrix	Detection Level	Reference
Soil	ppb	Guidance on Site Specific Risk Assessment for use at contaminated sites in Ontario. Appendix E (OMEE, 1996)
Food	ppt	Guide to Eating Ontario Sport Fish (OMOE, 2007b)
Drinking Water	ppq	Ontario Regulation 169/03

*Excerpted from Zhang, 2008, thesis submitted to Ryerson University.

Despite the promise of cost-effectiveness of ELISA, the method validation as US EPA method 4025 (see US EPA, 2002a) was based on soil samples at ppb level. In order to be useful for food analysis, the detection level needs to be a thousand-fold lower than the US EPA method. When the resolution is magnified a thousand-fold, then potential problems associated

with interfering substances in the sample matrix and background contamination of labware are also amplified. A portion of this study addresses the former problem of sample cleanup while the latter problem with labware contamination is addressed by another thesis (Zhang, 2008, thesis submitted).

The ultimate goal of this study is to develop an ELISA method to screen for dioxins in fish samples. This research topic was chosen in attempt to find a solution since currently, dioxins are potentially highly toxic to living beings but their detection is the most expensive and difficult to carry out in analytical chemistry. A successful method will increase sample throughput while reducing the time and costs associated with current dioxin analysis. In particular, the turnaround time and costs for dioxin-negative samples analyzed by GC-HRMS will be reduced. Since more research is needed to find effective fish sample cleanup methods for proper ELISA detection, there will be 3 objectives to achieve the goal; this research will be broken up into 3 separate studies or methods, with each study employing a different sample cleanup technique, followed by ELISA detection. The first study, termed GPC-ELISA, will employ gel permeation chromatography (GPC) for fish sample cleanup to remove lipids and other interferences, followed by ELISA detection. GPC was chosen for cleanup because it is automated and relatively inexpensive when compared to other automated methods.

The second study, termed acid silica/carbon-ELISA, will use an acid silica and carbon column combination for fish sample cleanup, followed by ELISA. Acid silica and carbon adsorbents were chosen since they are commonly used in the industry for fish sample cleanup. Additionally, these adsorbents are conveniently pre-packed by the manufacturer of the ELISA kits and readily available for purchase.

The third study, termed Florisil®-ELISA, will employ Florisil® to clean fish samples, followed by ELISA detection. Florisil® was chosen as a cleanup method because it is used to clean fish samples for polychlorinated biphenyl (PCB) and organochlorine (OC) analyses at the OMOE. If the Florisil®-ELISA technique were successful, leftover cleaned fish sample extracts from PCB and OC analyses would be used instead of discarded. Additionally, Florisil®-ELISA was chosen for study because preliminary results of a previous study done at the OMOE (see Lo et al., 2005) for two quality control (QC) samples analyzed in replicate were encouraging.

Success of the methods explored will be determined by the agreeability of the ELISA results with the corresponding GC-HRMS results for each fish sample. Although sample extraction is of equal importance to sample cleanup for successful dioxin analysis, it will not be of focus in this thesis research.

Since only 3 studies were found during the literature search on the topic of using ELISA as a means to detect for dioxins in fish, this research will contribute to the knowledge of a less explored area in the scientific community. Moreover, 2 of the 3 studies found were conducted at the OMOE and thus this research will further reinforce Canada, particularly Ontario, as the world leader in this field of study.

This research is a joint effort with the OMOE; thus, if a method proves to be successful, the OMOE can apply the new method to its benefit and realize immediate cost and time savings. Furthermore, the increased throughput with the new method would increase the number of dioxin-positive samples tested by GC-HRMS and thus allow for a better representation of dioxin concentrations in lake fish, as published in the *Guide to Eating Ontario Sport Fish*.

This research is presented in 5 chapters. The first and current chapter, the introduction, gives a brief overview of dioxins, identifies the research needs and outlines the objectives and

scope of this research. Chapter 2, the literature review, will give essential background information related to this research; it is divided into 3 sections: i) dioxins and furans, ii) ELISA, and iii) sample cleanup. Chapter 3 describes the materials and methods used for each of the 3 approaches explored to achieve the ultimate goal. Chapter 4 presents the results and discussion of each of the 3 approaches. Chapter 5 draws conclusions from the 3 approaches and discusses any recommendations.

2 Literature Review

2.1 Dioxins and Furans

2.1.1 Properties and Toxicity

Known collectively as "dioxins", the formal names for dioxins and furans are polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) (Harrison & Eduljee, 1999). Dioxins are toxic chemicals that are hydrophobic (Environment Canada, 2003) and extremely persistent (Schecter et al., 2006). Thus, they accumulate in the fatty tissues of living organisms (Government of Canada, 1990) and bioaccumulate up the food chain (WHO, 2007). They bind strongly to organic carbon in soils and sediments (Government of Canada, 1990; Environment Canada, 2003). Dioxins are very stable in the environment (US EPA, 2006); they exhibit very low vapour pressures, low solubilities in water, and high octanol/water partition coefficients (Environment Canada, 2003). Figure 2.1 shows the general structure of dioxins and furans.

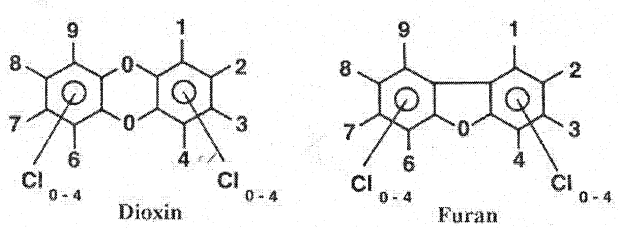


Figure 2.1 – Structure of dioxins and furans
(Government of Canada, 1990)

Of the 210 dioxin congeners – 75 dioxin congeners and 135 furan congeners – that exist, only 17 of them are considered to be toxic (Environment Canada, 2003), and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) is the most toxic (Government of Canada, 1990; Reiner et al., 2006; Schecter et al., 2006).

Most of the toxic effects of dioxins are mediated through the aryl hydrocarbon receptor (AhR) cellular protein. The AhR plays a role in the regulation of the expression of numerous genes (Schecter et al., 2006) such as those for normal physiology and development (Tijet et al., 2006). Dioxin and dioxin-like chemicals exert their toxic effects when they bind to the AhR (Okey et al., 2005; Schecter et al., 2006), dysregulating the expression of key genes under AhR control (Okey et al., 2005). 2,3,7,8-TCDD has the highest affinity for the AhR (Grassman et al., 1998) and thus, is the most toxic dioxin congener.

Since each congener differs in its toxicity, and dioxins in the real world occur as mixtures of congeners (Reiner et al., 2006), a concept called toxic equivalents (TEQ) is used to express the mixtures of different congeners as one concentration (Roberts & Roe, 2003). This TEQ approach is possible since all dioxins act on the AhR (Okey et al., 2005) and was first proposed by the OMOE in 1984 (Haws et al., 2006). In 1997, the World Health Organization (WHO) expert panel arrived at consensus in assigning to each toxic congener, a toxic equivalency factor (TEF) (van den Berg et al., 1998) relative to the most toxic congener, 2,3,7,8-TCDD, which is assigned a TEF of 1.0. TEQ is calculated by multiplying the concentration of each congener with its TEF and then adding them together (Safe, 1997/98). TEFs are assigned based on *in vivo* and *in vitro* toxicity data, with data from *in vivo* studies given more weight (van den Berg et al., 1998). TEF values are reevaluated on a regular basis as more data become available (WHO, 2008). The WHO human/mammalian TEFs for the 17 toxic congeners are found in Appendix B. As can be seen from Appendix B, some TEFs have been updated from 1998 to 2005. The TEFs for fish and birds can also be found in Appendix B.

2.1.2 Effects on Health

Dioxins have been shown to be toxic to animals in laboratory studies. In some studies, very minute amounts of dioxins have caused death in animals (Schecter et al., 2006), thus leading dioxins to be described as one of the most toxic man-made chemicals (OMOE, 2004; Schecter et al., 2006). Sensitivity to dioxins widely varies between species (Mukerjee, 1998). The lethal dose which kills half (LD_{50}) of the organisms tested is about 1 μg/kg of body weight for guinea pigs. However, for hamsters, the LD_{50} is about 1000 μg/kg of body weight (Schecter et al., 2006). Aside from lethality, other adverse effects associated with dioxin exposure in animals include: wasting, cardiotoxicity, adult neurotoxicity, hepatotoxicity, chloracne, lymphoid and gonadal atrophy, endocrine disruption, biochemical alterations, effects on developing nervous, immune and reproductive systems (Birnbaum & Tuomisto, 2000), and increased likelihood of tumours (OMOE, 2004).

Dioxin was deemed responsible for the extirpation of Ontario lake trout during the middle of the 20th century. This assertion was based on a vigorous model that took into account correlation of the extirpation period with heavy dioxin loading into Lake Ontario, historic record of dioxin content in sediments and TEQ levels causing early life stage toxicity and survival of lake trout sac fry. The model was successful in discounting other chemical or non-chemical stressors such as sea lamprey predation. Prediction of the model was consistent with restoration of lake trout by restocking programs, together with evidence of improved water quality through environmental control policies (Cook et al., 2003).

Dioxins are potentially toxic to humans. Of the adverse effects associated with dioxin exposure, chloracne, a skin disease, is the only proven effect (Dickson & Buzik, 1993; Bertazzi et al., 1998). Other adverse health effects associated with dioxin exposure include cancer,

diabetes, thyroid disease, reproductive and developmental toxicity, dermal toxicity, immune suppression, hepatotoxicity, and thymic atrophy (Hu & Bunce, 1999).

Controversy exists as to whether dioxins cause cancer because of human exposure to other unrelated chemicals (Hu & Bunce, 1999) which may confound the causes or contributions to the chronic illness. However, since 1997, dioxins have been classified as a "human carcinogen" (group 1) by the International Agency for Research on Cancer (IARC) (Mandal, 2005).

Dioxins cause enzyme induction (Hu & Bunce, 1999) such as the induction of cytochrome P450 1A1 (CYP1A1), which is a xenobiotic metabolizing enzyme (Mandal, 2005). Enzyme induction does not necessarily cause toxic effects (Mukerjee, 1998); however, it increases the risk of metabolizing precursor chemicals, thus causing the production of other chemicals which may be more biologically active (AEA Technology, 1999).

Exposure to dioxins has also been associated with endocrine disruption (Mukerjee, 1998) as seen by some of the above-mentioned effects associated with growth, development and maturation. For example, females exposed to furan-contaminated rice oil in Japan had irregular menstrual cycles (Aoki, 2001) and dioxin concentrations at or near background levels have been shown to affect thyroid hormone status (AEA Technology, 1999). As with this endocrine-disrupting effect and all other adverse effects associated with dioxin exposure except chloracne, the extent to which dioxin plays a role is unclear because of human exposure to complex mixtures of chemicals (AEA Technology, 1999).

As mentioned previously, the AhR has multiple functions in normal physiology by modulating approximately 400 genetic targets (392 ProbeSets), in the hepatic cells of male adult mice (Tijet et al., 2006). As described by Tijet et al. (2006), dioxin significantly altered the

expression of about 460 genes (456 ProbeSets) in an AhR-dependent manner, including some genes previously thought to be uninducible, such as flavin-containing monooxygenases. Furthermore, an estrogen receptor and two related genes exhibited AhR-dependent expression. Hence, the hormone effect of dioxin can be linked to the estrogen receptor pathways. The tumour-promoting effect of dioxin could be due to dioxin's ability to down-regulate the p53, which is a tumour suppressing protein (Tijet et al., 2006).

2.1.3 Sources, Fate and Levels

Dioxins are ubiquitous in the environment (Nording et al., 2006). They are byproducts from incomplete combustion and various industrial processes (Environment Canada, 2003; OMOE, 2007b). They are also produced through natural processes such as forest fires (Environment Canada, 2003). Dioxins have no known use (Interdepartmental, 1983) and are present in a variety of matrices such as soil, air, water, sediments (Environment Canada, 2003), fatty tissues of organisms, and food (fish and milk) (Sakurai et al., 1996). The sources of dioxins are mostly anthropogenic (Reiner et al., 2006) and most enter the environment through air (Health Canada, 2006). In 1999, 88% of dioxins released into the Canadian environment were released into the air from combustion sources, 10% were released into the soil and 2% were released into water (Environment Canada, 2003). The general pathways for dioxins in the environment are illustrated in Figure 2.2.

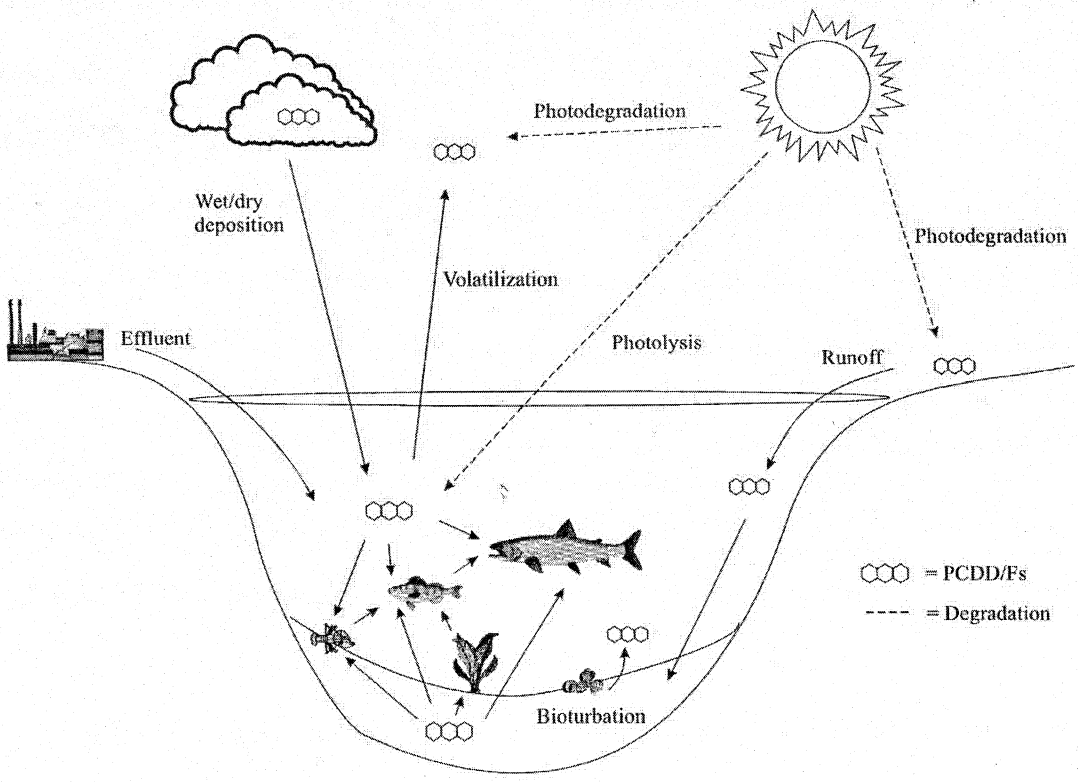


Figure 2.2 – General pathways for dioxins in the environment
(Environment Canada, 2003)

The ultimate sinks for dioxins are sediments and soils; when attached to these matrices, they degrade slowly, with a half-life of 10 years or longer for 2,3,7,8-TCDD (Government of Canada, 1990). In biological tissue, they also have long half lives (Government of Canada, 1990); it is estimated that the half-life of dioxin is 7-11 years in humans (Mukerjee, 1998).

In Ontario, the sources of dioxins include incinerators and other combustion activities, some petroleum refineries, and kraft pulp and paper mills that use chlorine (OMOE, 2004). In 2002, the dioxin emissions from incinerators (municipal, biomedical, sewage sludge and hazardous waste) in Ontario were approximately 10.5 g TEQ/year, down from approximately 23 g TEQ/year in 1999 (CCME, 2004). In some cases, dioxin-contaminated wastes can be found in

wood preservation sites and chemical industries. Although no longer manufactured in Ontario, the long-term effect of the manufacturing processes of herbicides such as 2,4,5-T and 2,4-D (Interdepartmental, 1983) still linger (OMOE, 2004).

Precipitation samples in Ontario show that levels of dioxins and furans are low in the province (Environment Canada, 2003). In Lake Ontario, the lake-wide average concentration of dioxin in sediments is 111 picograms (pg) TEQ/g. This level is five times higher than the probable effect level according to the Canadian Sediment Quality Guidelines. The high levels can be attributed to the hazardous waste facilities existing in the Niagara River watershed (Marvin et al., 2003).

2.1.4 Guidelines and Limits

The Canadian Government has set maximum allowable levels of dioxins in various matrices to protect public and environmental health. The Canadian Tissue Residue Guidelines (CTRG) to protect mammals that eat aquatic prey is 0.71 nanograms (ng) TEQ_{mam}/kg prey (wet weight; ww). The CTRG to protect birds that eat aquatic prey is 4.75 ng TEQ_{bird}/kg prey (ww) (Environment Canada, 2005c). The interim Canadian Sediment Quality Guidelines (CSeQG) for both freshwater and marine sediments is 0.85 ng TEQ/kg dry sediment; this level is considered safe for biota that live in or around the sediments (Environment Canada, 2005a). The Canadian Soil Quality Guidelines (CSoQG) states that 4 ng TEQ/kg soil (agricultural, residential and parkland, commercial, and industrial soils) is safe for biota living in or around Canadian soils (Environment Canada, 2005b). Currently, dioxins are scheduled for virtual elimination under the Canadian Environmental Protection Act (CCME, 2007).

In Ontario, the standard for ambient air (24-hour) is 5 pg TEQ/m^3; the maximum allowable levels in drinking water is 15 pg TEQ/L; the standard for residential surface soil is 1000 pg TEQ/g; and the standard for agricultural surface soil is 10 pg TEQ/g (OMOE, 2004).

The tolerable human daily intake of dioxins, according to the Joint Expert Committee on Food Additives, an expert group of the WHO and the Food and Agriculture Organization of the United Nations, is 2.3 pg/kg body weight per day. Studies done in 1998 and 1999 show that the average daily intake of dioxins by Canadians is 0.62 pg/kg body weight per day (Health Canada, 2006).

2.1.5 Sport Fish Contaminant Monitoring Program

The potential threat posed by dioxins has resulted in a strong demand for its monitoring by both the government and public (Sugawara et al., 2002). According to Health Canada (2006), 90% of human exposure to dioxins comes from food. Fish are a common food source for humans. However, due to bioaccumulation, dioxin levels in fish are 51,300 to 1,700,000 times greater than levels found in the surrounding environment (Environment Canada, 2003). In Ontario, fish are an important food source for both members of the aboriginal community and recreational fishers in the 250,000 lakes and water bodies, including the Great Lakes (OMOE, 2007b). There are more than one million anglers that fish for the purpose of recreation and consumption in the Ontario Great Lakes, inland lakes and rivers (Cole et al., 1997).

The Sport Fish Contaminant Monitoring Program tests toxins, including dioxins, in fish and issues consumption advisories based on the tests. This program, which started in 1976, is the largest of its kind in North America with approximately 5,000 fish tested each year from 1,700 water bodies in Ontario. Through the joint effort of the OMOE and the Ontario Ministry of Natural Resources (OMNR), consumption advisories for each fish species, based on

guidelines set out by Health Canada, are published every other year in the *Guide to Eating Ontario Sport Fish* (OMOE, 2007a). In the *Guide,* the contaminant levels in the fish are given based on the size and species of the fish and consumption advisories are separate for the general population and the sensitive population (children and women of child-bearing age). The fish selected for testing are from locations that are either popular for anglers, important food sources for local inhabitants, from known or suspected sources of pollution, or part of a monitoring program for contaminants in fish (OMOE, 2007b).

Since dioxins accumulate in fatty tissues, fish species with high lipid contents such as carp, trout, salmon and catfish are selected for testing. The portion of fish tested is the boneless, skinless dorsal fillet (OMOE, 2007b). Since dioxin analysis is especially costly, fish samples selected for dioxin analysis are all from the Great Lakes, where there is known contamination of dioxins (Emily Awad, March 2008, personal communication). According to the 2007-2008 edition of the *Guide*, sport fish consumption restrictions begin when dioxin concentrations are at 2.7 ppt TEQ, and total restrictions are advised when dioxin levels are above 21.6 ppt TEQ (OMOE, 2007b). It must be noted, however, that these levels are based on the analysis of only the fillet and excludes all other edible portions that may be eaten by individuals.

As part of the Sport Fish Contaminant Monitoring Program, lake trout or lake whitefish collected between 1989 and 2003 in the Canadian Great Lakes were measured for dioxins. It was found that the highest levels of dioxins were found in lake trout from Lake Ontario, with levels of 22-54 pg TEQ/g, which are above the dietary guidelines. Encouraging is the observation on temporal data from 1989 to 1999 that the TEQ in lake trout from Lake Ontario is decreasing at 1.5 pg/g per year (Bhavsar et al., 2008, in press). The TEQs measured in lake trout from the other Canadian Great Lakes showed that concentrations were 60-95% lower than those

from Lake Ontario. The five most dominant congeners measured from the fish were 2,3,7,8-TCDD, 2,3,7,8-tetrachlorodibenzofuran (2,3,7,8-TCDF), 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (1,2,3,7,8-PeCDD), 1,2,3,7,8-pentachlorodibenzofuran (1,2,3,7,8-PeCDF), and 2,3,4,7,8-pentachlorodibenzo-*p*-dioxin (2,3,4,7,8-PeCDD) (Bhavsar et al., 2008, in press).

2.2 Enzyme-Linked Immunosorbent Assay (ELISA)

2.2.1 Background

ELISA is an analytical tool commonly used in the medical field to detect infectious diseases. Since the 1970s, attempts have been made to develop ELISAs for dioxin detection (Harrison & Eduljee, 1999). However, the first ELISA method for dioxin detection did not come out until 2002 for the screening of dioxins in soil (see US EPA, 2002a). The extremely low solubility of dioxins – 1,000-fold lower than PCBs and polyaromatic hydrocarbons (PAHs) and 1,000,000-fold lower than some pesticides – presented a challenge for developing an ELISA for dioxins since ELISA is mainly performed in aqueous media (Harrison & Eduljee, 1999). Some other challenges encountered in the past included developing an ELISA with detection specific for the 17 toxic dioxin congeners and developing an ELISA sensitive enough to detect trace levels of dioxins (Harrison & Carlson, 1998).

2.2.2 Principles

The ELISA used for dioxin analysis is the competitive ELISA. In competitive ELISA, the target analyte, which, in this study, are dioxins in the sample, and the competitor-HRP enzyme conjugate, which, in this study, are enzyme-labelled dioxin fragments, compete for binding sites on antibodies immobilized on the walls of a tube. The antibodies are specific and only bind dioxins. The more binding sites that are occupied by the target analyte, the fewer sites

there are available for the competitor-HRP enzyme conjugate to bind. A series of wash steps removes excess competitor-HRP enzyme conjugate as well as other materials and compounds. A substrate is then added, which reacts with the bound competitor-HRP enzyme conjugate and produces a colour signal. The strength of the colour signal is inversely proportional to the concentration of dioxins in the sample (Neogen Corporation, n.d.). Thus, a darker colour would mean that less dioxins are present in the sample and a lighter colour would mean that more dioxins are present in the sample. Figure 2.3 illustrates the dioxin ELISA process. The anti-dioxin antibody in this figure is the antibody as referred to in this thesis.

The binding of the dioxins to the antibodies is based on structure. The antibody recognizes compounds which have both the 2,3,7,8- chlorination pattern and the dioxin/furan core structure. Generally speaking, 2,3,7,8-TCDD would have the highest affinity to the antibody and the other toxic dioxin congeners would have lower affinity for the antibody since they deviate from the pattern (US EPA, 2002a). Non-toxic dioxin congeners and dioxin-like polychlorinated biphenyls (dlPCBs) would have very low affinity to the antibody since they also deviate from the pattern. Non-dioxin compounds would not bind to the antibody since their structures are different. See Appendix C for the cross-reactivities of the toxic dioxin congeners to the antibody.

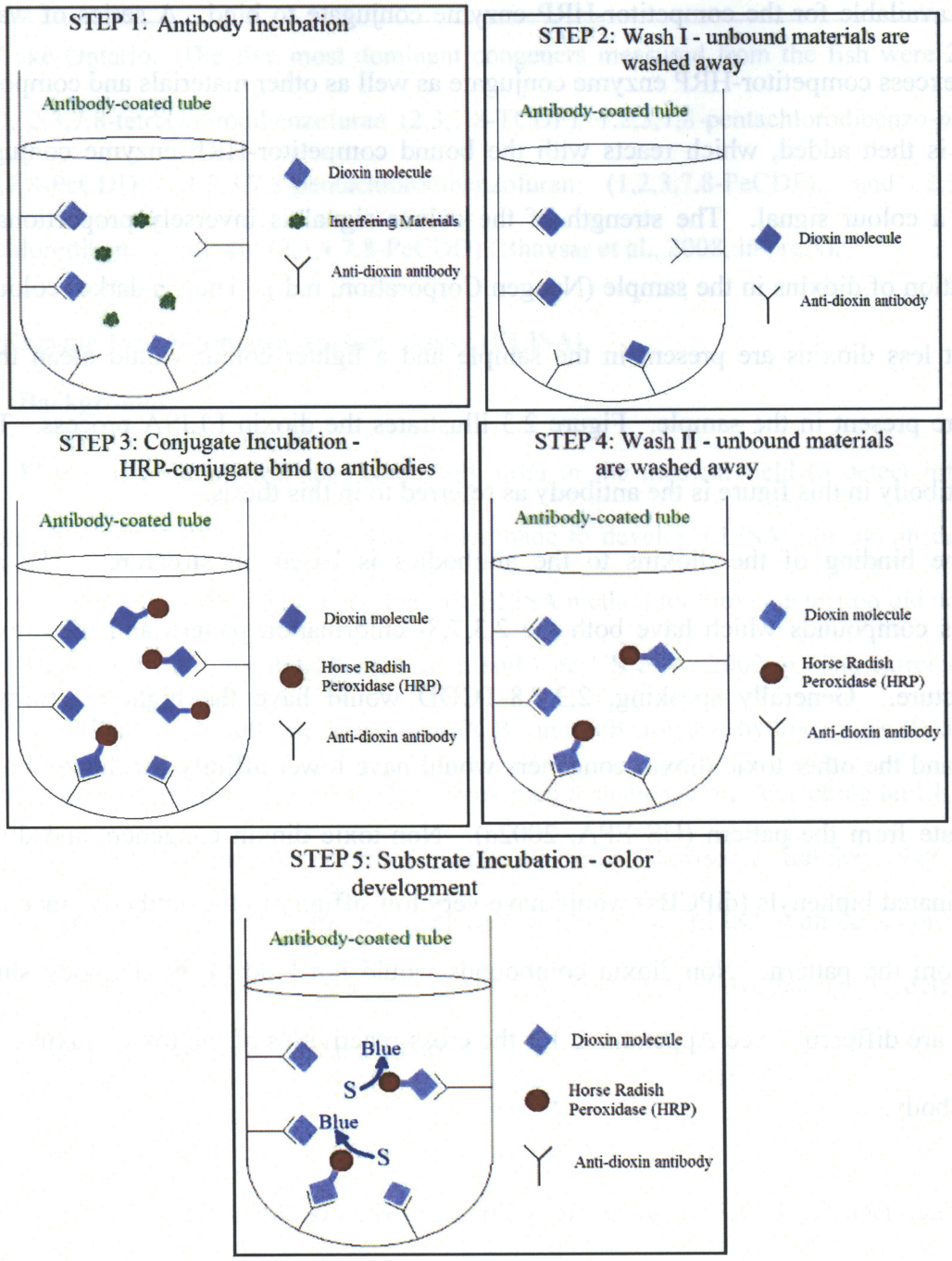


Figure 2.3 – ELISA for dioxin analysis
(Zhang, 2008, thesis submitted)

2.2.3 Advantages

ELISA, a bioanalytical test, is an alternative dioxin detection method to the conventional GC-HRMS method. GC-HRMS is considered the "gold standard" because it is accurate, precise, sensitive and selective (Reiner et al., 2006). This method, however, is time-consuming, lengthy (Schecter, 1998), and requires highly trained analysts (Sugawara et al., 2002) and expensive equipment (Shan et al., 2001). As a result, only a handful of laboratories worldwide are qualified for GC-HRMS analysis of dioxins (Schecter, 1998).

In contrast to GC-HRMS, ELISA offers distinct advantages. It is simple, inexpensive, fast, and allows for batch processing of samples (Harrison & Eduljee, 1999). Additionally, entry-level analysts can be easily trained to perform ELISA and ELISA is field portable (Billets, 2005).

The advantages of ELISA were seen during the US EPA Superfund Innovative Technology Evaluation (SITE) Program, where 209 soil, sediment and extract samples were tested for dioxins using different commercially-available technologies and were compared to results obtained from the reference method, GC-HRMS. It took 8 months and cost $398,029 USD to analyze all 209 samples (approximately $1,900 USD per sample) by GC-HRMS whereas it only took an average of 3 weeks and $59,234 USD (approximately $300 USD per sample) to analyze the same samples by ELISA (Billets, 2005; US EPA, 2005). Furthermore, unlike GC-HRMS where individual dioxin congener concentrations are obtained and then converted to TEQs using TEFs (see 2.1.1), results obtained from ELISA are directly in the TEQ form; although not quite identical, ELISA TEFs roughly mimic the TEFs used in GC-HRMS analysis (Reiner et al., 2006).

2.2.4 Other Alternative Bioanalytical Detection Methods

Alternative bioanalytical detection methods to GC-HRMS and different from ELISA exist, such as the human reporter gene system (HRGS), chemically activated luciferase gene expression (CALUX) and polymerase chain reaction (PCR).

When dioxin-like chemicals contact the AhR, the enzyme Cytochrome P450 is produced. The HRGS assay uses the human cell line 101L, integrated with a plasmid to contain the human CYP1A1 promoter (Cytochrome P450) with the firefly luciferase gene downstream. When dioxin-like compounds and/or high molecular weight PAHs contact the AhR, the enzymes *Cytochrome P450 and firefly luciferase are produced by the cells. When the cells are exposed to* luciferin, the reaction between luciferase and luciferin causes light to be emitted at a certain wavelength; the greater the intensity of light, the greater the concentration of dioxin-like compounds (US EPA, 2002b)

CALUX is analogous to HRGS, except that it uses a recombinant mouse cell line instead of a human cell line. The concentrations are measured in TEQ (US EPA, 2002b).

The PCR used for dioxin screening is called AhR-PCR. In this AhR-PCR assay, dioxins bind to AhR proteins that are extracted from mammalian cells. This binding causes the AhR to form an activated receptor complex with another AhR protein and DNA. This activated receptor complex is captured onto a well in a microplate and the DNA is amplified using real time PCR. The concentration of dioxins, in TEQ, can then be measured (US EPA, 2007).

ELISA was chosen over HRGS, CALUX and PCR for dioxin screening because ELISA does not require the care of cell culturing needed by HRGS and CALUX, and is not subject to the extreme care that is needed to prevent contamination in the PCR assay. ELISA was also the detection method of choice for this thesis research because the intent was to use an established

method for dioxin screening and apply it to the fish matrix. At the initial stages of this thesis research, US EPA method 4025 for the screening of dioxins in soil by ELISA (see US EPA, 2002a) was the only approved method. Towards the end of 2007, US EPA method 4430 for dioxin screening using PCR was approved (see US EPA, 2007); by this time, this thesis research had already been completed.

2.2.5 Validation of Dioxin ELISA for Environmental Matrices

ELISA has been validated for dioxin analysis using several environmental matrices. Analyzing dioxin in sediments, Nichkova et al. (2004) found a strong linear relationship between GC-HRMS and ELISA methods (correlation coefficient = 0.974, slope = 1.06) (see Figure 2.4). As a safety precaution, 2,3,7-trichloro-8-methyldibenzo-*p*-dioxin (TMDD) was used as a surrogate standard for the toxic 2,3,7,8-TCDD. The sample size used for the study was small (n = 13) and therefore Nichkova et al. (2004) concluded that ELISA could be used as a screening tool for dioxins but more validation studies needed to be done. Similarly, in a study of dioxin detection in soils, Nording et al. (2006), obtained a strong linear relationship (correlation coefficient = 0.90) between ELISA and GC-HRMS methods. The use of ELISA is approved by the US EPA's Method 4025 (US EPA, 2002a) for the screening of dioxins in soil at levels of 500 ppt.

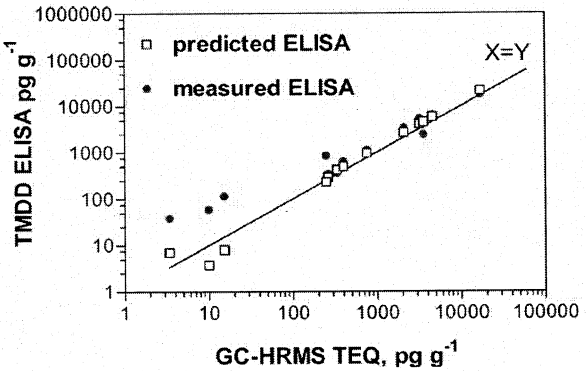


Figure 2.4 – Relationship between ELISA and GC-HRMS reported by Nichkova et al. (2004)

Most studies on the application of ELISA for dioxin analysis are based on matrices with higher reporting levels for dioxins. Studies where dioxin detection is essential in low concentrations to meet reporting requirements, such as for biological matrices, are lacking (Sugawara et al., 2002) because of the time required to remove lipids from the sample prior to analysis (Focant et al., 2001). Sugawara et al. (2002) obtained a strong linear relationship (correlation coefficient = 0.920) between ELISA and gas chromatography-mass spectrometry (GC-MS) for dioxin detection in human milk samples (n = 17) (see Figure 2.5), thus showing that ELISA is sensitive in that it can detect low levels of dioxins and also indicating that ELISA could be used as a screening tool.

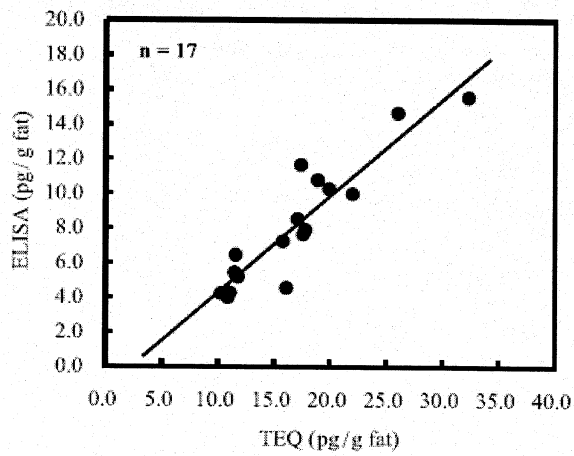


Figure 2.5 – Relationship between ELISA and GC-HRMS reported by Sugawara et al. (2002)

ELISA application for dioxin analysis in fish has been attempted in the past. Preliminary results by Kolic et al. (1998) to develop ELISA as a pre-screening tool for dioxin analysis in fish showed that ELISA results were comparable to GC-HRMS at concentrations above 9 ppt TEQ; ELISA results were biased high at levels below 9 ppt TEQ. Kolic et al. (1998) indicated that their method could be used as a pre-screening technique if the high bias below 9 ppt TEQ could be resolved.

Preliminary results by Lo et al. (2005) suggested that ELISA could be used as a screening tool. Analysis of a fish certified reference material (CRM) in quadruplicate by ELISA yielded favourable results (mean 24.10 ± 5.12 pg TEQ/g) compared to GC-HRMS (mean 26.36 ± 4.13 pg TEQ/g; n = 5) and the CRM (21.17 ± 7.83 pg TEQ/g). Five replicates performed on a fish sample that had gone through international QC showed that ELISA produced results (7.4 pg TEQ/g) that were 28% higher than the reference value (5.8 pg TEQ/g) (Lo et al., 2005).

A study by Shan et al. (2001) showed that dioxin results in fish and egg samples obtained by ELISA agreed with GC-MS results (R^2 = 0.89; n = 12) (see Figure 2.6). However, it did not

state the exact number of fish samples out of the 12 samples tested. No other studies on dioxin analysis in fish using ELISA have been found.

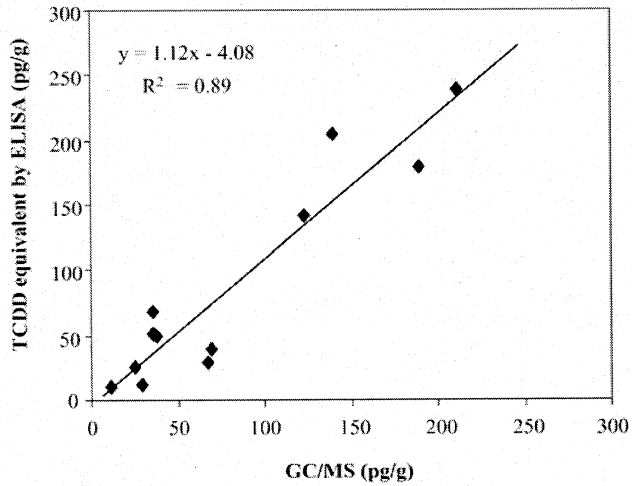


Figure 2.6 – Relationship between ELISA and GC-MS reported by Shan et al. (2001)

2.2.6 Validation of Other ELISAs for Environmental Matrices

ELISA has been shown to be a useful tool for the detection of other environmental contaminants such as pesticides, industrial chemicals, metals, endocrine disruptors, and algal toxins in various matrices.

Only a few prominent manufacturers of ELISA kits for environmental analyses exist because it is a small niche market. A brief internet search of 2 of these manufacturers returned well over 30 commercially-available ELISA kits for environmental analyses in a variety of matrices. A list of some of these ELISA kits for various contaminants is listed in Appendix D. Over 10 of these kits pertain to pesticides or industrial contaminant analyses in fish. However, the majority of these are concerned with detecting the contaminants in fish plasma; only 3 pertain to detection of contaminants [PCBs, dlPCBs and polybrominated diphenyl ethers (PBDEs)] in fish tissue.

Based on this promising track record of ELISA performance, as seen by the numerous available ELISA kits for different contaminants, there was encouragement to explore ELISA for the most difficult analyte (dioxin) for this thesis research.

2.3 Sample Cleanup

2.3.1 Role of Sample Cleanup

One of the most challenging tasks in analytical chemistry is the cleanup of sample extracts for dioxin analysis (Reiner et al., 2006). Cleanup techniques must be designed with care because of the propensity of dioxins to bind tightly to organic material and surfaces (Harrison & Eduljee, 1999); subsequent detection must then be at the ppt or ppq level, rather than in the ppb or parts per million (ppm) range of most other contaminants (Harrison & Carlson, 1998). Sample cleanup removes interfering and coextractable compounds from the sample (Reiner et al., 2006), such as PCBs and PBDEs, and concentrates the sample to improve sensitivity of the analysis (Harrison & Eduljee, 1999). Conventional GC-HRMS analysis often employs meticulous and complicated (Nichkova et al., 2004) sample cleanup methods using classical adsorbents such as silica, alumina, Florisil® and carbon (Reiner et al., 2006). Regarding ELISA detection, sample cleanup methods used are often variations of the conventional GC-HRMS cleanup methods (Harrison & Eduljee, 1999).

2.3.2 Cleanup Methods Used in Conventional Dioxin Analysis for Fish Samples

Sample cleanup for fish is usually executed by means of adsorption chromatography, which works on the basis of polarity. According to Reiner et al. (2006), the majority of current cleanup procedures are based on the "Dow" (see Lamparski et al., 1979) and "Smith-Stallings" (see Smith et al., 1984) procedures developed several decades ago. Table 2.1 describes some

cleanup procedures that have been applied for dioxin analysis in fish. Automated cleanup procedures also exist and in the last 20 years, there have been attempts to automate procedures to reduce time and analysis costs (Reiner et al., 2006).

Table 2.1 – Fish sample cleanup techniques used in conventional GC-HRMS analysis

Application	Adsorbent/Cleanup	Elution solvent	Notes	Reference
Fish	44% H_2SO_4 on silica (column 1)	Hexane	Removes bulk of lipids, oxidizable components.	Lamparski, et al., 1979
	Basic alumina (column 1)	Dichloromethane (DCM)	Separates non-polar species. Solvent exchange: 2,3,7,8-TCDD transferred from larger volume of hexane to smaller volume of DCM (reduce evaporation).	
	10% $AgNO_3$ on silica (column 2)	Hexane	Removes DDE, chlorinated aliphatic hydrocarbons, and sulfides.	
	Basic alumina (column 2)	50% (v/v) CCl_4:hexane; hexane; 50% DCM:hexane	Separates PCBs from dioxins.	
	Reversed-phase HPLC		Removes contaminants (ex. PCBs, DDE, phthalates) and compounds similar to dioxins (ex. chlorinated benzyl-phenylethers).	
Fish	*Part I* Potassium silicate (column 1) Silica gel (column 1)	50% cyclohexane:DCM	Removes acidic and highly polar coextractables. Silica gel & potassium silicate adsorb lipids. Silica retains compounds with pKa acidity constants ≤ 10; eg. Phenolic and carboxylic acid compounds, sulfonamides, hydroxyl PCBs.	Smith et al., 1984
	Cesium or potassium silicate (column 2)		Further adsorbs lipids.	

	Silica gel (column 2)		Further adsorbs lipids.	
	Carbon (column 3)	50% cyclohexane:DCM; DCM / methanol / benzene (75/20/5); toluene	Retains dioxins, while interferences such as DDE, PCBs, methoxy PCBs, PCDPEs pass through. Reverse elution.	
	Part II			
	Cesium silicate (column 4)	Hexane	Removes trace residues of acidic compounds.	
	40% H_2SO_4 on silica (column 4)		Retains PAHs.	
	Acid alumina (column 5)	Hexane; 2% DCM:hexane; 5% DCM:hexane; 8% DCM:hexane	Separates dioxins from polychlorinated napthalenes (PCNs), trace residuals of PCBs and other polychlorinated aromatic compounds.	
Animal tissue	Gel permeation chromatography	50% DCM:hexane	Removes lipids.	Norstrom et al., 1986
	Alumina	1% DCM:hexane	(Fraction 1) PCBs, DDT, mirex, non-polar OC compounds elute with solvent (discard).	
		50% DCM:hexane	(Fraction 2) Dioxins eluted out with this solvent.	
	Florisil® (apply to Fraction 2 from alumina cleanup above)	hexane	(Fraction 1) Remaining OC compounds contained in this fraction (discard).	
		5% DCM:hexane	(Fraction 2) Remaining OC compounds contained in this fraction (discard).	

		25% DCM:hexane	(Fraction 3) most dioxins contained in this fraction.	
		DCM	(Fraction 4) contains remainder of dioxins.	
	Alumina (optional; apply to Fractions 3 and 4 from Florisil® cleanup above)	1% DCM:hexane 50% DCM:hexane	Separates any remaining PCBs from dioxins.	
Fish	44% H_2SO_4 on silica	Hexane	Removes lipids and other oily compounds.	OMOE, 2006b
	Basic alumina	Hexane 10% CCl_4:hexane DCM	This fraction is discarded. This fraction is discarded. This fraction is collected.	
	Carbon	25% DCM:hexane Toluene	Reverse elution to collect dioxins.	
High-fat biological samples (including mackerel fish)	HCDS column (High Capacity Disposable Silica; consists of layer of acid, basic, and neutral silica)	Hexane	Removes fats.	Focant et al., 2001
	Silica (acidic, basic, neutral)	Hexane	Removes fats.	
	Alumina	2% DCM:hexane	Removes low polar interferences (some PCBs).	
		50% DCM:hexane	Elutes dioxins onto the next column.	
	Carbon	Toluene	Reverse elution to collect dioxins.	

Silica, an adsorbent with acidic properties (Anderson, 1987), is often the first adsorbent used for cleanup of fish samples, and may be impregnated with acid or base. Based on the literature reviewed in Table 2.1, silica removes the majority of fats, lipids, and other oily compounds in the sample. Only one out of the five procedures reviewed did not use silica as the first step of cleanup. In that one exception, an automated procedure that works on the principle of size exclusion, GPC, was used instead to remove lipids.

Alumina, an adsorbent with basic properties (Anderson, 1987), was employed in all of the procedures reviewed in Table 2.1. Alumina may also be impregnated with acid or base and separates dioxins from other polyaromatic compounds such as PCBs and other OC pesticides.

Florisil®, made of magnesium silicate with basic properties (US EPA, 2000), was also employed to separate OC compounds from dioxins. Carbon retains dioxins while allowing other interferences such as PCBs to pass through. High performance liquid chromatography (HPLC) was used to remove contaminants and compounds similar to dioxins. Fish sample cleanup is generally performed manually, although automated cleanup techniques (see Focant et al., 2001) have become more common.

Different adsorbents have different abilities to adsorb compounds. For the adsorbents silica, alumina and Florisil®, the increasing order of polarity, and thus adsorptivity, is alumina > Florisil® > silica (Vogel, 1989) (See Appendix E, Table A5 for a more comprehensive list). Adsorption can be physical, via van der Waals forces, or chemical, via chemical bonding such as hydrogen bonding (Anderson, 1987). Different solvents were also employed in the reviewed cleanup procedures in Table 2.1. Generally, adsorption occurs most readily when non-polar solvents such as hexane are used, and least readily when polar solvents are used. Thus, in order for separation of dioxins from other compounds to occur, the polarity of the eluting solvent could

be progressively increased. The eluatropic series lists solvents in order of increasing polarity used in adsorption chromatography, and can be found in Appendix E, Table A6. The boiling points of all the solvents in the eluatropic series are relatively low, allowing for quick evaporation of the eluted material (Vogel, 1989).

2.3.3 Cleanup Methods Compatible with ELISA for Dioxin Analysis in Fish

According to Harrison and Eduljee (1999), more research is needed in the area of sample extract cleanup so that compatibility with ELISA detection is guaranteed from the outset. A literature search on fish sample cleanup methods compatible with ELISA for dioxin analysis did not yield any results. A reason that no ELISA-specific sample cleanup methods have been developed is the lack of commercial potential shown by previous dioxin ELISAs (Harrison & Carlson, 1998). Where ELISA has been validated or shown promising results for dioxin analysis in fish, it can be assumed that the cleanup method described is compatible with ELISA. In the preliminary study by Kolic et al. (1998), acid silica was used for sample cleanup. In the study by Lo et al. (2005), Florisil® was used to clean up the fish. The study by Shan et al. (2001) validated ELISA for fish and egg samples; however, the cleanup method was only described as a modified multicolumn cleanup of the US EPA method. For the study by Sugawara et al. (2002) using breast milk – which contains lipids – as the matrix, a three-layer acid silica column was used.

Since PCBs, dlPCBs, and PBDEs have chemical structures and bioaccumulative properties similar to dioxin, fish tissue cleanup methods prior to ELISA detection for the former might be applicable to the latter. However, methods to clean fish for the three above-mentioned contaminants could not be found in the manufacturer's literature (see section 2.2.6 and Appendix

D). This finding is expected since the manufacturer's focus is on ELISA detection and not sample cleanup.

The cleanup methods employed by Kolic et al. (1998) and Lo et al. (2005) for use with ELISA were simpler compared to the exhaustive multicolumn cleanup needed for GC-HRMS detection. It appears that ELISA can tolerate a less thorough cleanup and still work (see Nording et al., 2006; Sugawara et al., 2002). This is likely because of the different nature of the ELISA and GC-HRMS detection methods. For ELISA, only compounds with the dioxin structure will bind to the antibodies while all other compounds are excluded and subsequently washed away. For GC-HRMS, components other than dioxins interfere with detection by producing unwanted and unknown peaks in the mass spectrum. The study by Sugawara et al. (2002) is especially encouraging because, like fish, the matrix analyzed, human breast milk, also contains high amounts of lipids.

2.4 Summary

Dioxins are environmental contaminants that are lipophilic, persistent and bioaccumulative. They are potentially highly toxic and exert a wide range of negative effects on living organisms. Thus, guidelines and limits exist to control the presence of dioxins and protect the health of living beings. The Sport Fish Contaminant Monitoring Program tests for dioxins in fish from lakes across Ontario and issues consumption advisories when dioxin levels are above the acceptable limit.

There is a need to find alternative dioxin detection methods because the current GC-HRMS method is time-consuming and extremely expensive. ELISA is one such alternative dioxin detection method. The application of ELISA for dioxin analysis is advantageous because it is less time-consuming, less expensive, and simpler than GC-HRMS. The application of ELISA as

a screening tool has been successful for a variety of environmental contaminants, including dioxins, in various matrices. In regards to ELISA for dioxin analysis in fish, preliminary studies have shown promise.

The cleanup of sample extracts for dioxin analysis is one of the most challenging tasks in analytical chemistry. Current sample cleanup techniques for dioxin ELISA are variations of those used for GC-HRMS analysis. These cleanup methods include adsorption chromatography using silica, alumina, carbon and Florisil®, and automated methods such as GPC. In order to ensure compatibility with ELISA detection from the outset, more research is required in the area of sample extract cleanup.

3 Materials and Methods

3.1 Materials

3.1.1 General

All solvents [dichloromethane (DCM), toluene, methanol, isooctane, concentrated hydrochloric acid and hexane] used in this study were purchased from Caledon Laboratories, Ltd., Ontario. Additional equipment included a digital dry bath (AccuBlock™, Labnet International, Inc., USA) and nitrogen generator (Parker Balston NitroVap-2LV, Parker Hannifin Corporation, Haverhill, MA, USA) and other equipment (spatula, forceps, disposable glass Pasteur pipettes, aluminum foil, Parafilm®, vortex, microdispensers, thermometer, scissors, 25-mL and 100-mL volumetric flasks, absorbent paper towels, beakers, Eppendorf repeat pipettor, test tube racks, and 16 x 100 mm disposable borosilicate glass tubes).

3.1.2 Extraction

Analytical balance (Mettler PM 200); rotator (Glas-Col® Rugged Rotator, USA); centrifuge (MSE Mistral® 2000); centrifuge tubes (Zymark Corporation, USA); sodium bicarbonate ($NaHCO_3$); and sodium sulphate (Na_2SO_4).

3.1.3 Cleanup

A GPC-ELISA

GPC instrument (AccuPrep MPS™, J2 Scientific, Columbia, Missouri, USA), SX-3 biobeads (Biorad); GPC calibration standard solution (AccuStandard, Inc., CLP-027, lot B5010071, USA, purchased from Chromatographic Specialties, Inc.); 200-mL TurboVap® vials and trays (Zymark Corporation, USA); and dioxin surrogate (1,3,6,8-TCDD) (AccuStandard, Inc., D-405N, lot 940121, USA, purchased from Chromatographic Specialties, Inc.).

B Acid Silica/Carbon-ELISA

Acid silica columns (CAPE Technologies, USA); carbon mini-columns (CAPE Technologies, USA); and tetradecane (certified, supplier #04595-500, Fisher Scientific).

C Florisil®-ELISA

Florisil® (Standard Activation Grade, 100-200 mesh, Caledon Laboratories Ltd., Ontario).

3.1.4 Detection

ELISA kits (High Performance Dioxin/Furan Immunoassay Kit, Product Number DF1-60) were purchased from CAPE Technologies, ME, USA; 2,3,7,8-TCDD standards (DF1-SK2) were purchased from Wellington Laboratories (Guelph, Ontario). Other equipment included a centrifuge (Sorvall Legend RT, Kendro Laboratory Products, USA) and spectrophotometer (Photometric Analyzer, Abraxis LLC, Warminster, PA, USA).

3.2 Methods

3.2.1 Sampling

In support of this program, the OMNR and the Environmental Monitoring and Reporting Branch (EMRB) of the OMOE are responsible for obtaining the fish samples from the various water bodies in Ontario. Preliminary sample preparation, which includes filleting, homogenizing and freezing the fish tissue, and sample identification/documentation are performed before the fish samples are referred to the LSB of the OMOE for dioxin analysis.

The fish samples are contained in glass jars that have been solvent rinsed and dried, and are capped with a Teflon® or aluminum foil-lined lid. The fish samples are kept frozen at a

maximum of -4°C until ready for use. The holding time for these frozen fish samples is indefinite (OMOE, 2006b).

For this thesis research, only fish samples with known GC-HRMS results as per OMOE method E3418 (OMOE, 2006b) were selected. The Laboratory Information Management System (LIMS) was accessed and a list of fish samples with a range of dioxin concentration levels was compiled. The list was given to appropriate personnel at the OMOE and only fish samples that were still available were retrieved. Since the three studies (GPC-ELISA, acid silica/carbon-ELISA, Florisil®-ELISA) were not done simultaneously, this process occurred on three separate occasions.

3.2.2 Extraction

Using an analytical balance, five grams of fish tissue (ww) were weighed out for each sample and put into a centrifuge tube. Concentrated hydrochloric acid (19.5 mL) and 20.5 mL of a 25% DCM:hexane mixture were added into the centrifuge tube containing the 5 g of fish. The contents in the centrifuge tubes were left to stand overnight to digest the fish. The centrifuge tubes were tumbled on a rotator at 45 rpm for 45 minutes and then centrifuged at 3,000 rpm for 6 minutes. The supernatant (extract) was passed through a 40% sodium bicarbonate:sodium sulphate mixture and into either a 25-mL or 100-mL volumetric flask. Either DCM was added (GPC cleanup) or 25% DCM:hexane was added (acid silica/carbon column cleanup and Florisil® cleanup) into the volumetric flask to make up the volume to either the 25-mL or 100-mL mark.

3.2.3 Cleanup

The amount of fish extract loaded onto each column (GPC, acid silica/carbon and Florisil®) was selected according to the lipid content of the fish as indicated in Table 3.1.

Table 3.1 – Choosing a volume of fish extract for cleanup.

Fish Lipid content	Fish weight chosen	mL Cleanup volume from 25-mL extract	mL Cleanup volume from 100-mL extract
0 – 4.99 %	1.5 g	7.5 mL	30 mL
5 – 9.99 %	1 g	5 mL	20 mL
10 – 19.99 %	0.5 g	2.5 mL	10 mL
20 – 29.99 %	0.25 g	1.25 mL	5 mL
>30%	0.1 g	0.5 mL	2 mL

The percent lipid contents of the fish were previously determined by OMOE method E3136 (see OMOE, 2006a) and obtained through LIMS.

A GPC-ELISA

Forty-nine lake fish samples were subjected to GPC cleanup (Figure 3.1). Seventy grams of SX-3 biobeads were packed into a glass column with internal diameter of 700 x 25 mm. The general procedure followed was as outlined in EPA Method 3640A, Gel Permeation Cleanup (US EPA, 1994). The fish sample extracts were evaporated down to 8 mL for injection into the GPC. DCM was used as the mobile phase. The flow rate of the mobile phase was set at 5 mL/min and the run time of each sample was 60 minutes, with the fraction collect time being at 28 to 47 minutes. The fraction collection time was based on the elution time, seen on the UV chromatogram, of the dioxin surrogate (Appendix F). The cleaned fractions (95 mL) were collected in the 200-mL TurboVap® vials.

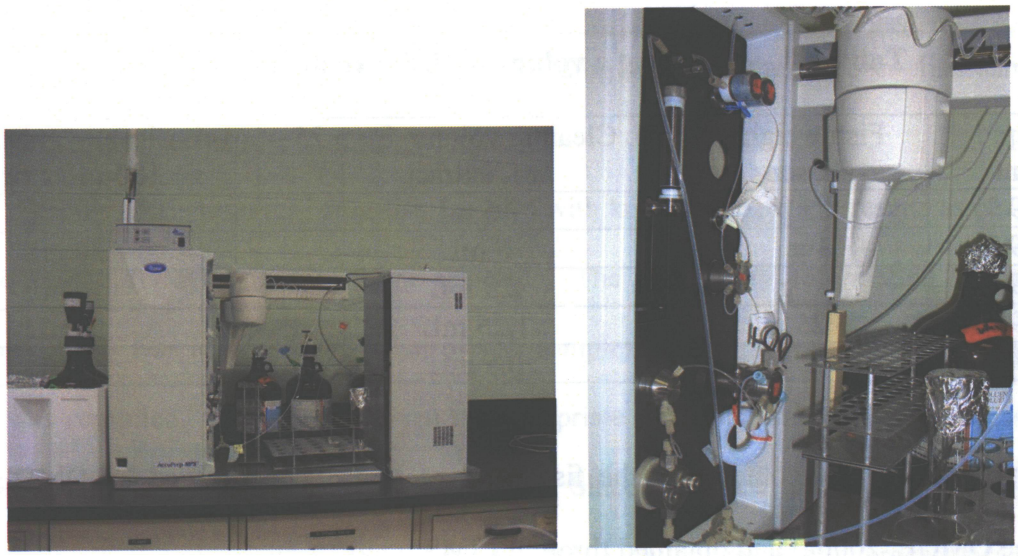


Figure 3.1 – GPC instrument (left) and close-up of automated sampler arm of GPC (right).

B Acid Silica/Carbon-ELISA

Sixty lake fish samples were subjected to cleanup by acid silica and carbon. Tetradecane (0.5 mL) was added as a keeper, along with fish extract, into a 16 x 100 mm borosilicate glass tube for each sample and evaporated to 0.5 mL for cleanup. The cleanup procedure was performed as per CAPE Technologies' Application Note AN-008, available at http://www.cape-tech.com/. Briefly, each acid silica/carbon dual column (CAPE Technologies, ME, USA) was wetted with 10 mL of hexane prior to sample loading. After sample loading, 25 mL of hexane was added (Figure 3.2). The carbon mini-column was then removed from the acid silica column and attached onto a glass reservoir. Hexane (10 mL), and then 6 mL of a 50% hexane:toluene mixture was run through the column to wash out PCBs. The carbon mini-column was then reversed and eluted with 10 mL of toluene into a 16 x 100 mm borosilicate glass tube containing 62.5 µL of detergent keeper, collecting the dioxins (Figure 3.3).

Figure 3.2 – Acid silica and carbon column cleanup of fish samples.

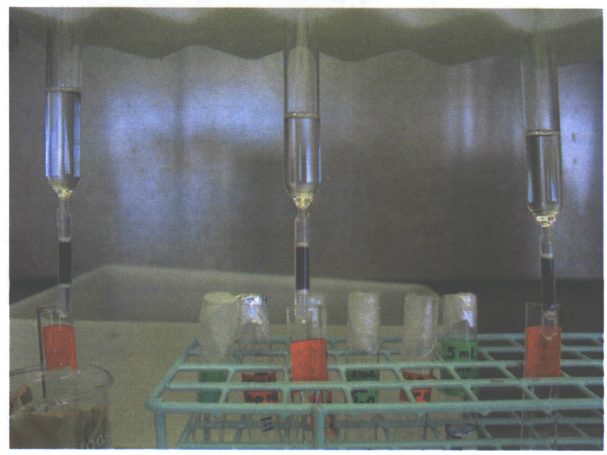


Figure 3.3 – Reverse elution with toluene to collect dioxins in the sample.

C Florisil®-ELISA

Eighteen lake fish samples were subjected to Florisil® cleanup (Figure 3.4). Each Florisil® column was packed to 24 cm thickness. The cleanup procedure was as per OMOE method E3136 (OMOE, 2006a) with a slight modification. Briefly, isooctane (2,2,4-trimethylpentane) keeper (2 mL) was added to each of the fish sample extracts and evaporated down to 1 – 1.5 mL. Each concentrated extract was loaded onto a Florisil® column, eluted with 25 mL of a 25% DCM:hexane mixture, and collected as a single fraction. Isooctane (2 mL) was

added to the 25 mL eluate and evaporated down to 3 – 5 mL. The volume was then made up to 10 mL using isooctane and 62.5 µL of detergent keeper was added.

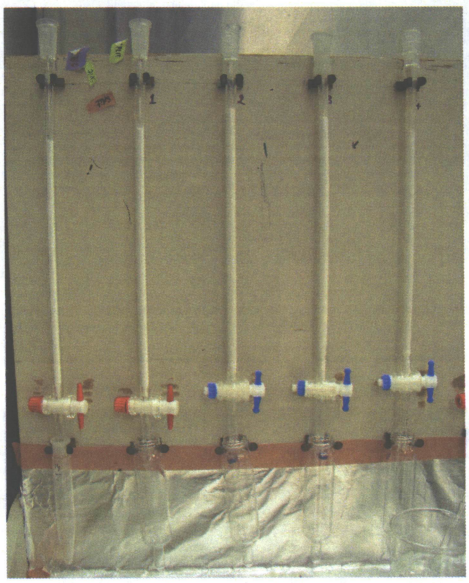


Figure 3.4 – Florisil® cleanup of fish samples.

3.2.4 Detection

Prior to ELISA detection, all cleaned sample extracts were evaporated at specific temperatures under a nitrogen stream of 1 psi, and then reconstituted in methanol.

Procedures for ELISA were as outlined in the ELISA kit insert IN-DF1 (CAPE Technologies), also available at http://www.cape-tech.com/. Briefly, antibodies were incubated overnight. After a wash step, conjugate was added and incubated for 15 minutes. The solution for this first wash step contained detergent [0.01% v/v Triton X-100 in autoclaved deionized distilled water (DDW)]. Following another wash step with DDW, substrate was added and incubated for 30 minutes. Absorbance readings were taken using a spectrophotometer.

3.2.5 Data analysis

For each fish sample, the ELISA result was compared to its corresponding GC-HRMS result. Correlation and linear regression analyses were performed. A square of correlation value of 0.66 or 66% was chosen as being acceptable for screening. This value was chosen in consideration of a fish CRM (Wellington Laboratories, Catalogue # WMF-01) whereby 70 GC-HRMS determinations (duplicates from 35 laboratories worldwide) were carried out. The mean of that CRM was 21.17 ± 7.83 pg TEQ/g.. In this case, one standard deviation from the mean, which is considered excellent, was roughly within 33% of the mean. Two standard deviations from the mean – in this case, 66% within the mean – is acceptable. Thus, this rationale was the basis for choosing a square of correlation of 66% as being acceptable for screening.

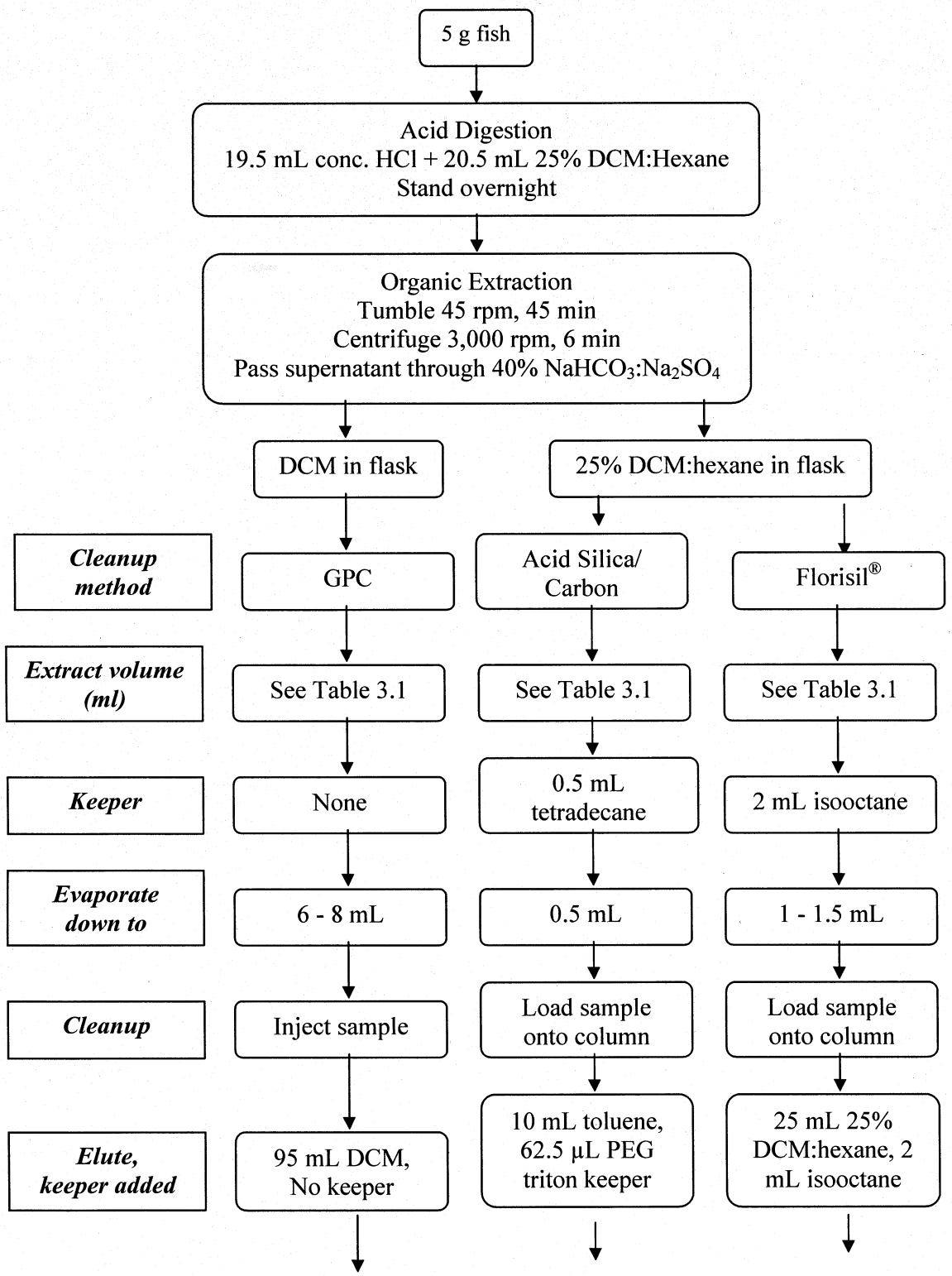
All statistics were performed using Minitab student release 12 for Windows (Minitab, Inc.).

3.2.6 Quality Control

ELISA determinations were done to ensure the cleanliness of all glassware and solvents used for this thesis research. Method blanks and spikes were also carried out as routine QC measures.

3.3 Summary

A summary of the procedures for the three approaches (GPC-ELISA, acid silica/carbon-ELISA and Florisil®-ELISA) is illustrated in Figure 3.5.


5 g fish
Acid Digestion
19.5 mL conc. HCl + 20.5 mL 25% DCM:Hexane
Stand overnight
Organic Extraction
Tumble 45 rpm, 45 min
Centrifuge 3,000 rpm, 6 min
Pass supernatant through 40% NaHCO3:Na2SO4
DCM in flask
25% DCM:hexane in flask
Cleanup method
GPC
Acid Silica/ Carbon
Florisil®
Extract volume (ml)
See Table 3.1
See Table 3.1
See Table 3.1
Keeper
None
0.5 mL tetradecane
2 mL isooctane
Evaporate down to
6 - 8 mL
0.5 mL
1 - 1.5 mL
Cleanup
Inject sample
Load sample onto column
Load sample onto column
Elute, keeper added
95 mL DCM, No keeper
10 mL toluene, 62.5 µL PEG triton keeper
25 mL 25% DCM:hexane, 2 mL isooctane

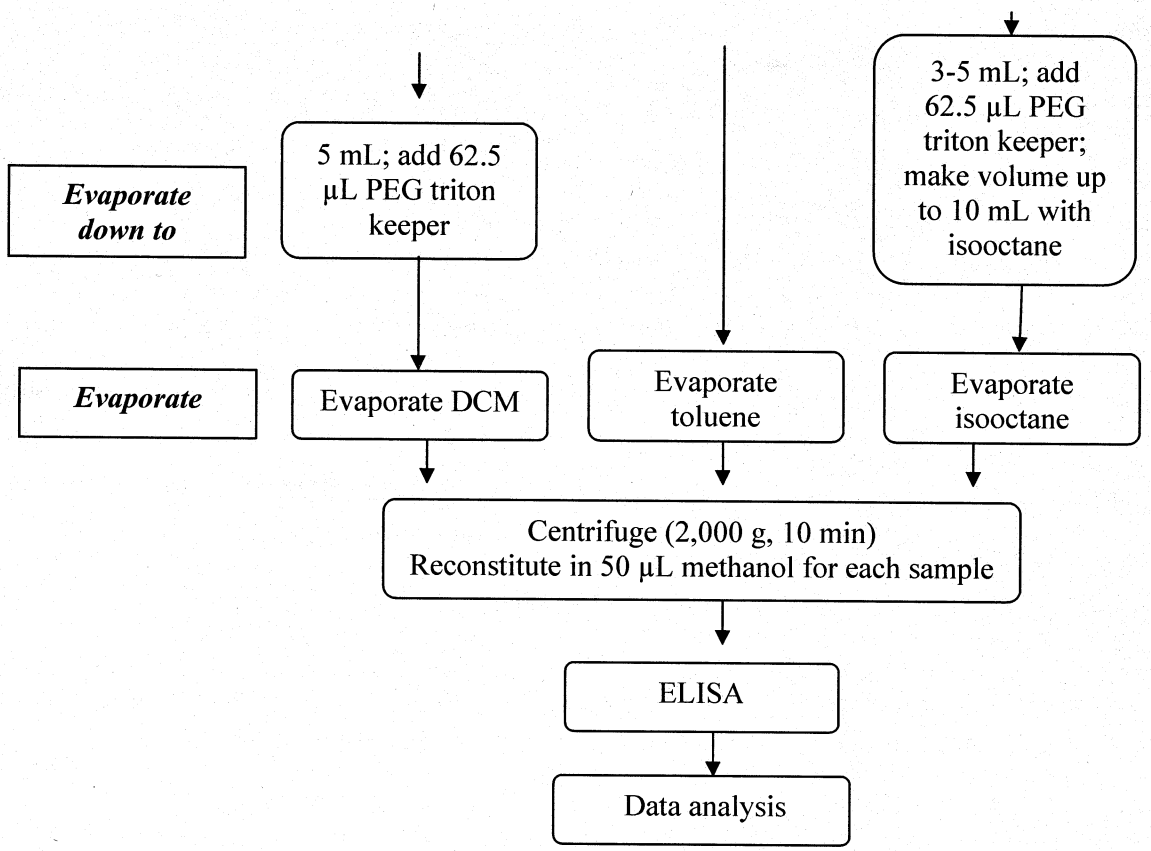


Figure 3.5 – Summary of methodology used in this research.

4 Results and Discussion

4.1 Individual Approach

A GPC-ELISA

Two of the 49 fish samples were lost due to laboratory accidents; thus, only 47 fish samples were analyzed by ELISA. ELISA reported 18 out of 47 fish samples (38%) with TEQ greater than 100 pg. These ELISA results were considered invalid since the valid range of the 2,3,7,8-TCDD calibrator is 0 – 100 pg.

It was noted that the validity of the results was connected to the amount of contaminants eluting around the same time as lipids (within the first 25 minutes), as seen in the UV chromatograms (see Figure 4.1). Generally, a low contaminant UV peak yielded valid ELISA results and a high contaminant UV peak yielded invalid ELISA results.

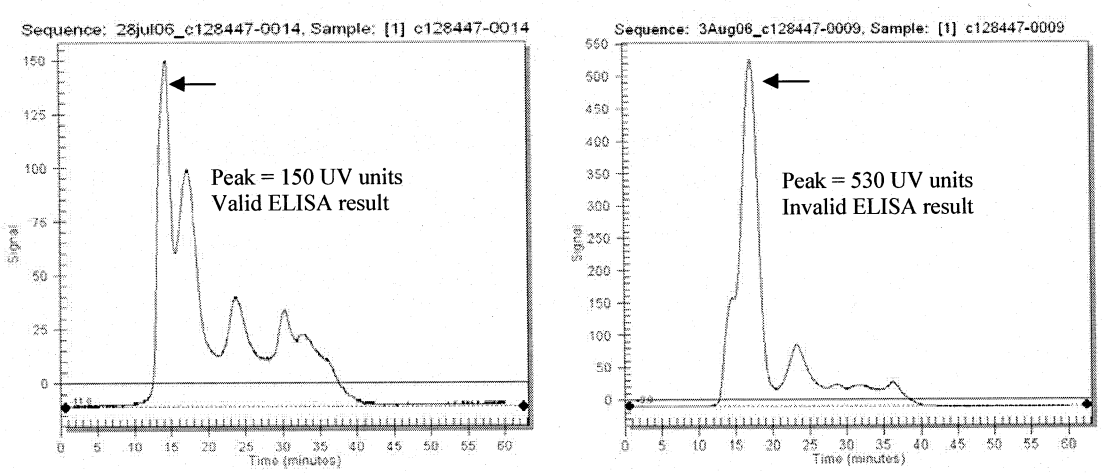


Figure 4.1 – Profiles of a fish sample with a low (left) and a high (right) contaminant UV peak.

A peak height of 250 UV units was arbitrarily set as a cut-off value between low and high levels of contaminants. Table 4.1 categorizes the number of valid ELISA results when the UV

signal was < 250 UV units (low amounts of contaminants) and the number of valid results when the UV signal was ≥ 250 UV units (high levels of contaminants).

Table 4.1 – Validity of ELISA results for fish samples.

UV signal (254 nm)	ELISA within valid range of 0 – 100 pg TEQ	
	Yes	No
≥ 250	7	17
< 250	21	1

A chi-square test determined the 2 groups to be statistically significant in terms of whether they fall within the measurable ELISA range or not ($X^2 = 21.2$, $p < 0.0005$). Note that the sample size according to Table 4.1 is n = 46 rather than n = 47, as a UV chromatogram was lost for one of the fish samples. Refer to Appendix G for the UV chromatograms of the 46 fish samples.

The first group in Table 4.1 consisted of 24 fish samples, among which 17 samples, or 71%, with a maximum peak height of ≥ 250 UV units, were not within the valid range of ELISA. These 17 fish samples were omitted from further analysis, rendering n = 7 for this group. There was no linear relationship between ELISA and GC-HRMS results (n = 7; r = -0.07) (Figure 4.2).

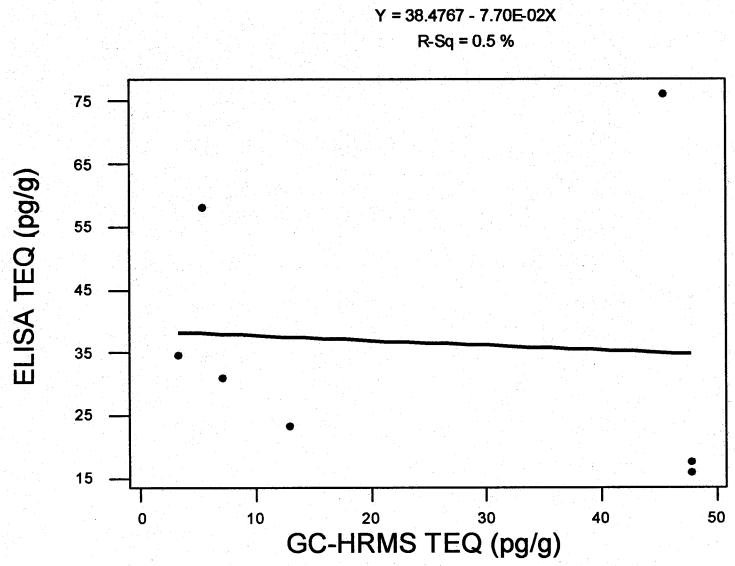


Figure 4.2 – A relationship between ELISA and GC-HRMS results when contaminant peaks are ≥ 250 UV units.

In the second group (Table 4.1), which contained 22 fish, all but one fish sample with contaminant peak heights < 250 UV units, or 95.5% of n = 22, were within the valid range of ELISA. The one invalid result was omitted from further analysis, rendering n = 21 for this group. For this group of fish samples, there was also no linear relationship between ELISA and GC-HRMS results (n = 21; r = 0.008) (Figure 4.3). It must be noted that the lack of linear relationship appears to be heavily influenced by the data points whose GC-HRMS TEQ values are greater than 10 pg/g. If a line were drawn where x = y (correlation coefficient = 1), it can be seen that when GC-HRMS TEQ values are less than 10 pg/g, ELISA is overestimating, and when GC-HRMS TEQ values are greater than 10 pg/g, ELISA is under-reporting.

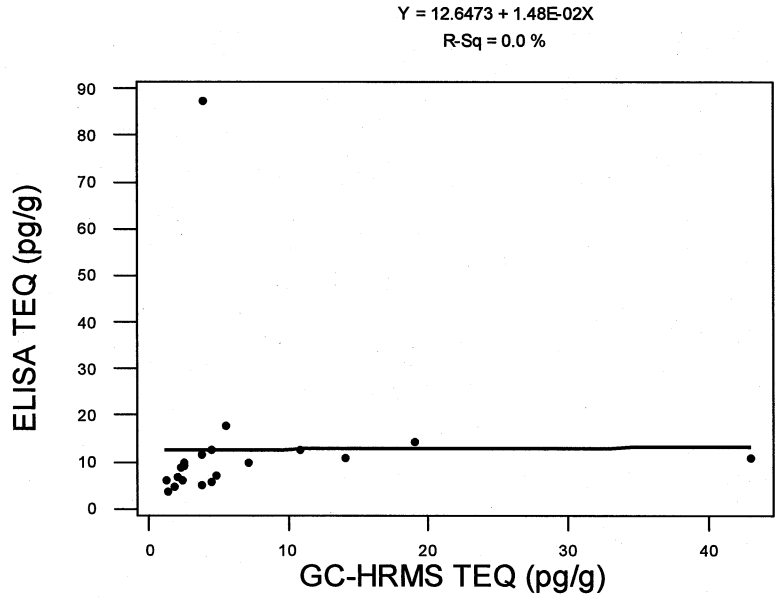


Figure 4.3 – A relationship between ELISA and GC-HRMS results when contaminant peaks are < 250 UV units.

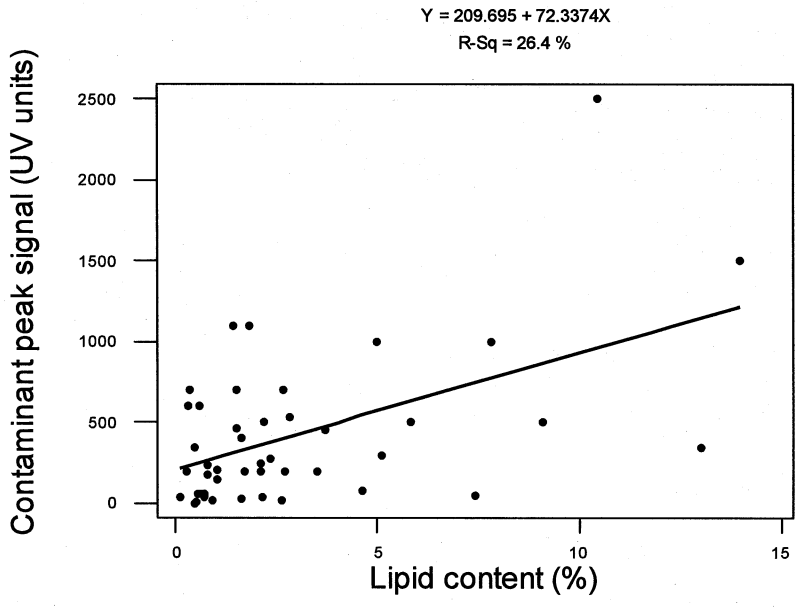


Figure 4.4 – A relationship between contaminant UV peak height and the percent lipid content of the fish sample.

An examination of percent fish lipid contents showed that there was a weak linear relationship between percent lipid and the contaminant UV peak height (n = 46; r = 0.514). A linear regression of contaminant UV peak height (dependent variable) and the % lipid content (independent variable) of the 46 fish samples indicated that only 26% of the variation of contaminant peak height could be explained by the model (Figure 4.4).

The large percentage of fish samples (38%) yielding invalid ELISA results was not surprising since all samples, when pipetted into the ELISA buffer, exhibited a cloudy appearance and/or lipid froth (Figure 4.5), indicating poor sample cleanup, and ultimately poor ELISA results.

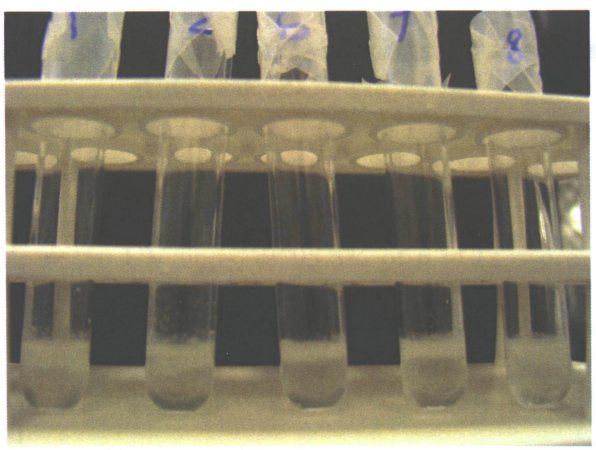


Figure 4.5 – GPC-cleaned fish samples after initial entry into ELISA tubes.

One possible cause of the poor cleanup could be that some smaller lipids were collected in the experimental run since the eluate was collected between 28 to 47 minutes of the run. The first 25 minutes of the run removes 80% of fat from biological tissue (Focant et al., 2001), so this possibility is likely. The adjustment of collect times of the sample extracts would not be an option in this case as doing so would risk losing dioxin analyte.

The above findings (Figures 4.2, 4.3 and 4.4) suggest that other compounds of similar sizes to lipids contributed to the observed contaminant UV peaks and were interfering components in the ELISA. In other words, there was a matrix effect. In the case of the first group with high contaminant peak heights, there were too many interferences rendering invalid ELISA results for most fish samples and producing poor results for the remaining fish (Figure 4.2). In the case of the second group with low contaminant peak heights, there were likely less contaminants, resulting in measurable ELISA results for all but one fish; however, there were still enough interferences to generate poor results (Figure 4.3). Hydrophobic binding of lipid to the plastic ELISA tube may be a possible cause of interference. The lipid could hinder the accessibility of the antibody to the competitor-HRP enzyme conjugate. Reduced conjugate binding will result in less colour development. Yet, competitive ELISA works on the principle of reduced conjugate binding in the presence of dioxin analyte. Therefore, lipid interference will result in an artifact signal of dioxin. In other words, the ELISA will report high dioxin amounts to the extent that it is out of the maximum ELISA range of 100 pg.

According to Harrison and Eduljee (1999), a key goal of sample cleanup is to remove bulk organic materials which, if not removed, will overwhelm the aqueous medium and interfere with antibody-analyte interactions. In the case of GPC, the cleanup was insufficient for the purpose of testing fish. This was clearly evident by the appearance of cleaned sample extracts in the ELISA tubes, even after ELISA wash steps (Figure 4.6); the two middle tubes containing fish samples still exhibited a cloudy appearance with lipid froths on top whereas the two outer tubes containing the method blanks were clear. The lipids and other organic materials that were not removed likely interfered with the accessibility of the dioxin antibodies to the conjugate, as well

as disrupted absorbance readings through the spectrophotometer, ultimately causing invalid ELISA results.

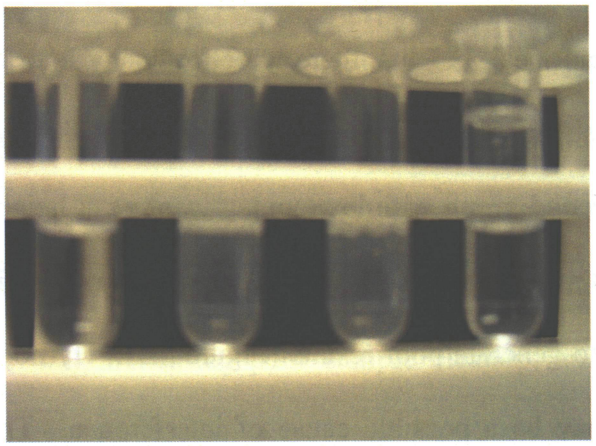


Figure 4.6 – Appearance of cleaned fish sample extracts after the first ELISA wash step.

GPC was chosen for this study because it is an automated cleanup method which can potentially help save on time and labour costs. Regarding conventional analysis, GPC is often employed as a first-step cleanup and followed by other column cleanup (see Focant et al., 2001 and Norstrom et al., 1986). This procedure was not an option in this study because the ultimate goal was to find a quick, simple, high throughput method for screening dioxins in fish. It was also assumed that ELISA would be able to tolerate a less exhaustive cleanup. However, this was not the case in this study.

The manufacturer of the GPC instrument used in this study has recently developed a dioxin module, which contains disposable carbon cartridges, for use with the GPC (Figure 4.7). This module has yet to be tested.

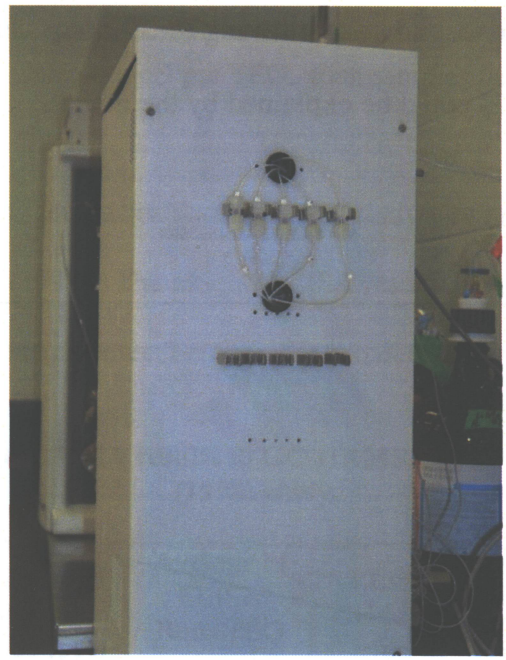


Figure 4.7 – New dioxin module for GPC.

B Acid Silica/Carbon-ELISA

Although 60 fish samples were cleaned with this method, only 22 of them were analyzed by ELISA. The remainder of the fish samples was lost due to problems associated with the home-made solvent evaporating apparatus. On several occasions, holes in some of the tubing caused nitrogen gas to escape. On another occasion, toluene vapours contacted the plastic portion on the top of the home-made instrument, causing plastic to melt and drip into the fish samples underneath. These trials and errors are typical during the developmental phase of any new method or instrumentation.

For the 22 fish samples analyzed, the correlation coefficient between ELISA TEQ and GC-HRMS TEQ was $r = 0.32$, indicating a weak linear relationship between the two detection methods. A simple linear regression of the data showed that ELISA was under-reporting (46% recovery) (Figure 4.8), and thus might lead to the reporting of false-negative results. This

under-reporting was likely due to analyte loss during the cleanup. Furthermore, only 10% of the variation of the data (R^2 = 10.2) could be explained by the model.

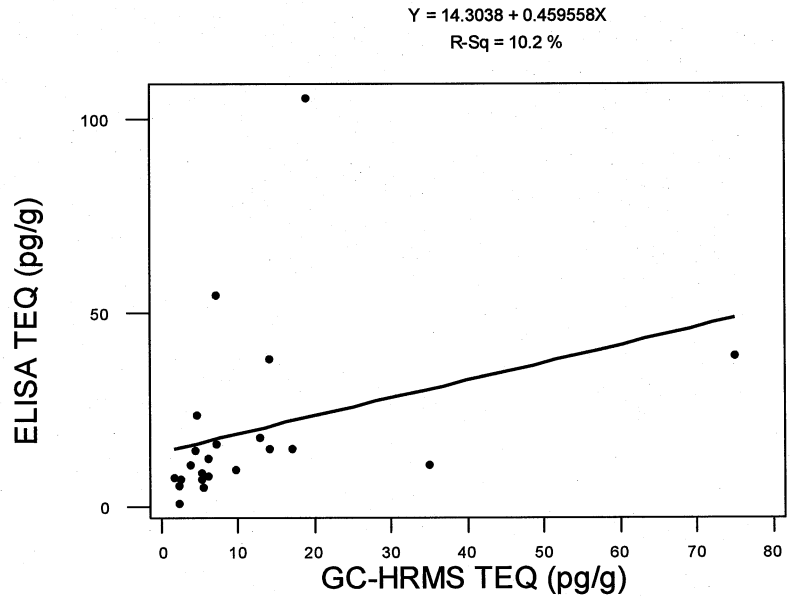


Figure 4.8 – Correlation of acid silica/carbon-ELISA and GC-HRMS results.

It must be noted that correlation is not resistant. Some outlying data points – those with TEQ values at or above 20 pg TEQ/g as measured by GC-HRMS – affected the correlation. The outliers could not be removed from analysis since each fish analyzed by ELISA had a corresponding result by GC-HRMS and thus there were no parameters to determine which data points were outliers. An option would be to test more fish with higher levels of dioxins in order to obtain a more reliable correlation.

Kolic et al. (1998) also employed acid silica to clean fish samples in their study; however, they did not state that carbon cleanup was employed. Their preliminary results revealed that ELISA results were generally comparable (within a factor of 2) to GC-HRMS

results for fish samples that were above 9 ppt TEQ. Fish samples below 9 ppt TEQ showed high bias.

The same pattern was seen when data from this study was presented in the same manner as presented by Kolic et al. (1998). The ratio of ELISA result to GC-HRMS result was used for each fish sample to determine agreement between the two detection methods.

Table 4.2 – Comparison of ELISA results to GC-HRMS sorted results from Kolic et al.'s (1998) study.

Fish Tissue Samples	Runs Per Sample	GC-HRMS Analysis TEQs (ppt)	Average of ELISA Runs TEQ (ppt)	ELISA / GC-HRMS ratio	Mean of ELISA / GC-HRMS ratio	Standard Deviation
1	3	2	13	6.5	5.7	2.90
2	2	2	9.6	4.8		
3	4	3	29	9.7		
4	2	4	7.5	1.9		
5	3	5	17	3.4		
6	2	7	55	7.9		
7	2	9	10.6	1.2	1.24	0.46
8	2	14	14	1		
9	4	20	39	2		
10	3	27	33	1.2		
11	4	66	52	0.8		

In Kolic et al.'s (1998) study, the mean of the ELISA to GC-HRMS ratio for fish samples below 9 ppt TEQ was 5.7 ± 2.9 (n = 6). For fish samples above 9 ppt TEQ, the mean of the ELISA to GC-HRMS ratio was 1.2 ± 0.5 (n = 5) (Table 4.2). It should be noted that the ratios presented by Kolic et al. (1998) represent means of fish samples that were run in replicates (duplicates, triplicates or quadruplicates). Thus, the mean ratios above were calculated by taking the mean of data that were previously averaged. Ideally, the raw data should be obtained to prevent calculations obtained by averaging previously averaged data. The raw data could not be obtained from this study but even if the raw data were available, it is not expected to drastically

change the calculations above. The actual sample sizes were n = 16 for fish samples below 9 ppt TEQ and n = 15 for fish samples above 9 ppt TEQ.

In this study, the mean of the ELISA to GC-HRMS ratio for fish samples below 9 ppt TEQ was 2.8 ± 1.9 (n = 14). For fish samples above 9 ppt TEQ, the mean of ELISA to GC-HRMS was 1.7 ± 1.7 (n = 8) (Table 4.3).

Table 4.3 – Comparison of acid silica/carbon-ELISA results to GC-HRMS sorted results.

Fish Tissue Samples	Runs Per Sample	GC-HRMS Analysis TEQs (ppt)	ELISA TEQ (ppt)	ELISA / GC-HRMS ratio	Mean of ELISA / GC-HRMS ratio	Standard Deviation
1	1	1.7	7.5	4.4	2.8	1.9
2	1	2.2	5.7	2.5		
3	1	2.2	1.0	0.5		
4	1	2.5	7.2	2.9		
5	1	3.8	11.0	2.9		
6	1	4.5	14.8	3.3		
7	1	4.6	23.8	5.2		
8	1	5.3	7.2	1.4		
9	1	5.3	8.9	1.7		
10	1	5.5	5.2	0.9		
11	1	6.0	7.9	1.3		
12	1	6.1	12.7	2.1		
13	1	7.1	54.7	7.7		
14	1	7.1	16.2	2.3		
15	1	9.7	9.6	1.0	1.7	1.7
16	1	12.9	18.0	1.4		
17	1	14.0	37.9	2.7		
18	1	14.0	14.9	1.1		
19	1	17.0	15.2	0.9		
20	1	19.0	105.4	5.5		
21	1	35.0	10.7	0.3		
22	1	75.0	38.9	0.5		

Kolic et al. (1998) stated that the high bias they obtained at lower dioxin concentration levels could be due to 3 reasons. The first reason of microbial contamination in their reagents did not occur in this experiment. The second reason of potentially having a high concentration

of a trichlorinated dioxin congener, 2,7,8-trichlorodibenzo-*p*-dioxin (2,7,8-T_3CDD) could apply to this study. The cross-reactivity of 2,7,8-T_3CDD is 24%, relative to 2,3,7,8-TCDD (see Appendix C). Therefore, a high concentration of this congener in a sample would result in a positive interference, increasing the ELISA TEQ measurement since this congener is not measured by GC-HRMS for its toxicity (Kolic et al., 1998; Nichkova et al., 2004). That is, 2,7,8-T_3CDD, is not one of the 17 toxic dioxin congeners. The final source of high bias given, the contribution of high amounts of dlPCBs, is unlikely. As discovered in the work by a Master of Applied Science candidate at Ryerson University (Zhang, 2008, thesis submitted), extremely high levels of dlPCBs are transparent in the assay.

It has been concluded that the acid silica/carbon-ELISA method used for this study cannot achieve the goal of this research. When presented in one manner, the linear relationship between the ELISA and GC-HRMS methods was weak and the poor recovery by ELISA indicated that false negative results were possible with this method. When presented in another manner, there was high bias below 9 ppt TEQ, which has to be resolved before the method can be considered for screening purposes.

C Florisil®-ELISA

Three of the 18 fish samples cleaned using Florisil® columns were lost due to laboratory accidents. Thus, only 15 fish samples were analyzed by ELISA. Pearson correlation of the results revealed that there was a strong linear relationship between ELISA and GC-HRMS detection methods ($r = 0.85$, $n = 15$). A simple linear regression of the data showed that ELISA was slightly under-reporting (slope = 0.77, or 77% recovery) (Figure 4.9) and that 72% of the variation could be explained using the model.

False negative results will still occur due to the slight under-reporting using the Florisil®-ELISA technique. To correct for this, a response factor, given by the inverse value of the slope, can be applied. In this case, a response factor of 1.3 can be applied. This is possible since congener patterns in fish are fairly constant as a result of metabolic action (Nording et al., 2005). This response factor would tie in the relationship between ELISA and GC-HRMS data and thus make the screening of dioxins in fish possible.

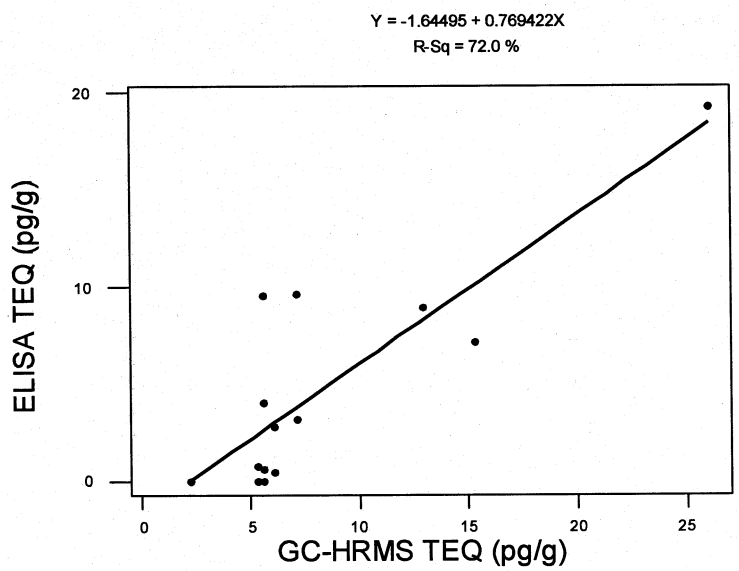


Figure 4.9 – Correlation of Florisil®-ELISA and GC-HRMS results.

The results of this study are encouraging and indicate that this Florisil®-ELISA method would be suitable as a screening technique. Similarly, the preliminary results by Lo et al. (2005), using the same method, were also encouraging. In their analysis of CRMs, the ELISA results were comparable to GC-HRMS results and the CRM result (see section 2.2.5). For the international QC, the ELISA results were 28% higher than the reference value (7.4 pg TEQ/g vs. 5.8 pg TEQ/g, respectively) (Lo et al., 2005). Thus, the encouraging results obtained from this

study using real fish samples support the results from the preliminary study by Lo et al. (2005) whereby analyses of two QC samples performed in replicates indicated that Florisil®-ELISA is suitable for screening fish samples.

This approach satisfied the goal of this research, which was to develop a screening method for dioxins in fish, and do so with increased sample throughput and at reduced costs and time. However, more fish samples need to be tested in order to ascertain the precision and accuracy of the results. More samples in the higher dioxin concentration ranges should also be tested as this study only examined fish with GC-HRMS results of no more than 25 pg TEQ/g fish. The implementation of this method would also serve an additional advantage at the OMOE in that the leftover cleaned extracts from PCB and OC pesticide analyses would be used rather than discarded. Thus, even more time and cost savings associated with dioxin analysis would be realized since no separate cleanup is necessary and only ELISA need be performed.

4.2 Discussions

Of the 3 methods attempted to achieve the ultimate goal of this research, Florisil®-ELISA was the most successful. This is not surprising when compared to acid silica/carbon-ELISA. Although both acid silica and Florisil® adsorbents are used extensively to remove lipids for dioxin analysis in fish, Florisil® is a more polar adsorbent (see section 2.3.2 and Appendix E, Table A5) and thus would be more effective in separating dioxins from lipids in the fish samples. GPC-ELISA was the least successful. Unlike the latter two cleanup techniques that used disposable columns, the polymer beads which comprise the GPC column are reusable. Although the GPC column was flushed with solvent to decontaminate the beads in between runs, stronger decontamination solvents could not be used; the use of a solvent different from that used for fish sample cleanup alters the properties (Focant et al., 2001), such as the amount of swelling, of the

beads. While not the ultimate goal of this research, it can be concluded that GPC and the acid silica and carbon cleanup combination are not compatible with ELISA.

A reason for differences in TEQ amounts obtained by ELISA and GC-HRMS may be due to the fact that the cross-reactivities relative to 2,3,7,8-TCDD do not mirror the TEF values of each of the 17 toxic dioxin congeners (as given by the WHO), which are used in the calculation of TEQ for GC-HRMS analysis. As mentioned by the manufacturer in the ELISA kit insert IN-DF1 (CAPE Technologies), the specificity of the test only approximates the TEF values of each of the 17 congeners. That is, selectivity for the 17 toxic congeners is not quite the same. The cross-reactivity data is given in Appendix C. This may have been the reasoning for high bias of some ELISA results for the acid silica/carbon-ELISA and in the study by Kolic et al. (1998).

The linear regression for the acid silica/carbon-ELISA study (Figure 4.8) showed that ELISA was under-reporting. The recovery was 46%, suggesting that more than half of the dioxin analyte was lost in the cleanup phase.

Generally, a linear relationship for the plot of ELISA versus GC-HRMS results would imply that ELISA and GC-HRMS are comparable detection methods. Data points under the line of idealism ($x = y$) where a strong linear relationship still exists would indicate that ELISA is under-reporting and that losses occurred during the cleanup stage (Nording et al., 2005). If this line of $x = y$ was drawn for Figure 4.9 (Florisil®-ELISA), it would show that most points lie below the line and thus implies that losses occurred during Florisil® cleanup. The actual linear regression of ELISA versus GC-HRMS (Figure 4.9) showed that ELISA was under-reporting and thus was caused by Florisil® cleanup.

As previously emphasized, the cost to analyze one sample by ELISA is considerably less than the cost per sample for GC-HRMS ($300 USD/sample versus $1900 USD/sample,

respectively, according to the US EPA SITE Program) (see section 2.2.3). The cost estimates above are for soil samples but are assumed to be similar for fish samples.

Moreover, according to OMOE method E3418 (OMOE, 2006b), it takes 8 working days to prepare/analyze 10 fish samples under optimum conditions. In this research, it was found that sample preparation and analysis by ELISA could be performed comfortably in batches of 20 tubes (14 fish samples + 6 standards) in 5 working days. Assuming 249 working days per year, the workload capacity for GC-HRMS would be 311 fish samples costing $590,900 USD, and the capacity for ELISA would be 697 fish samples costing $209,100 USD.

According to the data accessed from LIMS, in 2006 roughly half of the approximately 300 fish samples analyzed for dioxins by GC-HRMS were below the concern level of 2.7 ppt (dioxin-negative). Thus, the money spent on analyzing these negative samples by GC-HRMS would have been saved if an ELISA screening method were in place.

Table 4.4 summarizes the estimated cost for 2006 whereby $570,000 was spent to provide GC-HRMS results on only 150 dioxin-positive samples.

Table 4.4 – Number and cost of fish samples analyzed by GC-HRMS at OMOE in 2006

	GC-HRMS
# of dioxin-positive samples	150
# of dioxin-negative samples	150
Total # of samples	300
Cost per sample ($)*	1,900
Total cost ($)	570,000

*Cost based on US EPA SITE Program value.

Assuming the budget allocated for dioxin analysis of fish samples is fixed, then Table 4.5 illustrates the scenario if ELISA screening were in place. On a current budget of $570,000, ELISA would be able to screen 456 fish samples, and would flag 228 positive samples for GC-

HRMS analysis. Thus, ELISA would have increased sample throughput by 156 samples, or 152%, compared to no ELISA screening (Table 4.4). The number of dioxin-positive samples quantified by GC-HRMS would also be increased from 150 fish samples (Table 4.4) to 228 fish samples (Table 4.5). This translates to an extra 78 fish samples, or a 152% increase, in the number of dioxin-positive fish samples analyzed and included in the *Guide to Eating Ontario Sport Fish.*

Table 4.5 – Implementation of ELISA screening with fixed budget

	ELISA	GC-HRMS	
# of dioxin-positive samples	228	228	
# of dioxin-negative samples	228	0	
Total # of samples	456	228	
Cost per sample ($)*	300	1,900	
Total cost ($)	136,800	433,200	570,000

*Cost based on US EPA SITE Program values.

Assuming the current GC-HRMS workload of 300 fish samples per year is the limiting factor whereas the budget is flexible, then Table 4.6 illustrates the scenario if ELISA screening were in place. With the fixed GC-HRMS workload, ELISA would first screen 600 fish samples and flag 300 of these samples for GC-HRMS analysis. In this scenario, ELISA would have increased sample throughput by 300 samples, or 200%, when compared to no ELISA screening (Table 4.4). The number of dioxin-positive samples quantified by GC-HRMS would also be increased from 150 fish samples (Table 4.4) to 300 fish samples (Table 4.6), doubling the number of dioxin-positive fish samples analyzed and included in the *Guide.*

Table 4.6 – Implementation of ELISA screening with fixed GC-HRMS workload

	ELISA	GC-HRMS	
# of dioxin-positive samples	300	300	
# of dioxin-negative samples	300	0	
Total # of samples	600	300	
Cost per sample ($)*	300	1,900	
Total cost ($)	180,000	570,000	750,000

* Cost based on US EPA SITE Program values.

The low cost of ELISA per sample ($300) relative to GC-HRMS ($1,900), the simplicity of the test, its ability for high throughput, its minimal instrumentation costs, the lack of need for high level (PhD) staff to run the test and expensive specialized laboratory facilities, and ease of transferability makes ELISA a strong and attractive candidate for use in private laboratories and underdeveloped countries.

Although the Florisil®-ELISA method could be used for dioxin analysis in fish, it may be worthwhile to explore fish sample cleanup using the Fluid Management System's (FMS) automated Power-Prep™ instrument, followed by ELISA detection. This instrument uses classical adsorbents such as silica, alumina and carbon for sample cleanup. The Power-Prep™ system used by Focant et al. (2001) was able to clean 10 biota samples simultaneously and quickly. Though the detection method used in the study by Focant et al. (2001) was GC-HRMS, it is highly probable that detection by ELISA after Power-Prep™ cleanup would be just as successful since analysis by GC-HRMS requires a more exhaustive cleanup than what is required for ELISA. Even though instrumentation costs are high ($140,000 for 6 parallel modules), the money invested would be recaptured in one-year's saving of 150 negative GC-HRMS samples or $285,000 (Table 4.4).

This thesis did not include a hands-on investigation of the Power-Prep™ system due to its high cost. Instead, a request was made to FMS to provide a small number of fish samples

cleaned with the Power-Prep™. This request, however, was declined. FMS stated that it did not have any fish samples.

This research had the support of several section managers, the senior scientist, and the director of the LSB at the OMOE. Initially, a phase of this research involved the Dioxin and Toxic Organics (DTO) section carrying out manual sample preparation using conventional extraction and cleanup methods. This step, however, could not be fulfilled due to priority samples that needed to be analyzed by the DTO section. Currently, the DTO section of the OMOE possesses a Power-Prep™ system. Due to the constant heavy workload of the DTO section, a request was not made to provide cleaned fish samples with this automated method. The complete FMS Power-Prep™ system costs $222,000, which includes a 6-module sample extraction apparatus at $82,000 and a 6-module cleanup apparatus at $140,000., This total cost would be recaptured within half a year of operation (Table 4.4).

4.3 Summary

Three different approaches were investigated to develop an ELISA method to screen for dioxins in fish samples. Results showed that the GPC-ELISA approach did not work; the cleanup was insufficient and was evident when ELISA was carried out. Results of acid silica/carbon-ELISA demonstrated that this approach was not feasible; ELISA and GC-HRMS methods did not correlate well and the reporting of false-negative results was a possibility due to the under-reporting of data. The Florisil®-ELISA approach revealed that this methodology was acceptable for screening of dioxins in fish samples; there was a strong linear relationship between ELISA and GC-HRMS results and a response factor could be applied to correct for the slight under-reporting of data by ELISA. However, more samples should be tested to confirm the model.

5 Conclusions and Recommendations

5.1 Conclusions

To protect public health from exposure to the bioaccumulative and potentially toxic effects of dioxins, guidelines and limits exist on their concentrations in various environmental matrices. In regards to dioxins in fish, the Sport Fish Contaminant Monitoring Program exists to protect Ontarians.

The current and conventionally-used method for dioxin analysis, GC-HRMS, is expensive and time-consuming. The preparation of samples for GC-HRMS detection, which includes sample cleanup, is especially difficult and cumbersome. Thus, there is a need to find alternative methods that are less expensive and more time-efficient. ELISA is one such alternative method. However, more research is needed to find sample cleanup methods that are compatible with ELISA from the outset.

A method has been developed to screen for dioxins in fish. In this method, Florisil®-ELISA, fish samples are cleaned using Florisil® and dioxins are detected using ELISA. The under-reporting of ELISA can be corrected using a response factor. Two other approaches – GPC cleanup followed by ELISA detection (GPC-ELISA) and acid silica/carbon cleanup followed by ELISA detection (acid silica/carbon-ELISA) – that were investigated for the same purpose were unsuccessful.

GPC cleanup was insufficient for the purpose of ELISA. The interference of absorbance readings for most samples indicated that the ELISA wash steps were ineffective at removing the remainder of contaminants, including lipids. Despite the presence of detergent in the wash solution, the lipid remnants after GPC cleanup suggested that there was a contaminant overload. This contaminant overload could be theoretically explained by the following hypothesis. If it

were assumed that the GPC system was functioning at 99.99% efficiency and 1 g of a very lean fish sample (1% lipid) was applied, there would still be 1,000 ng of lipid remaining, translating into 1,000,000 pg of contaminants, which is overwhelming when detecting several pg of dioxins.

Using the acid silica/carbon-ELISA method, the results for fish samples showed only a weak linear relationship when compared to results from GC-HRMS. Poor recovery was also noted, which could lead to false-negative reporting. Furthermore, this study confirmed the findings of a previous similar study. When both datasets were displayed in the same manner, a trend was apparent. Analysis results of fish samples with dioxin concentrations greater than 9 ppt TEQ were comparable to those of GC-HRMS while the analysis results of fish samples below 9 ppt TEQ of dioxins were highly biased. Based on this trend, it was concluded that more work is needed to improve upon the acid silica/carbon-ELISA screening method before it can be acceptable.

The implementation of the Florisil®-ELISA method would increase sample throughput, and would considerably cut down on turnaround time and costs of dioxin-negative samples by GC-HRMS. At the OMOE, implementation of this method would increase efficiency, even if the budget allocated for dioxin analysis was fixed.. Thus, the increase in the number of dioxin-positive fish samples analyzed by GC-HRMS would allow for better protection of public health. As a result, there would be a better representation of dioxin concentrations in lake fish as published in the *Guide to Eating Ontario Sport Fish*.

In this research, a total of 84 fish samples – 47 by GPC-ELISA, 22 by acid silica/carbon-ELISA, and 15 by Florisil®-ELISA were analyzed for dioxins. If none of the fish samples had been lost, then a total of 127 fish samples – 49 by GPC-ELISA, 60 by acid silica/carbon-ELISA, and 18 by Florisil®-ELISA – would have undergone ELISA determination. The number of

successful ELISA determinations (84) performed for this thesis research is more than the number of determinations performed for any of the three reviewed publications for dioxin analysis in fish samples. Furthermore, each of those publications used only one cleanup method coupled with ELISA detection. In this thesis research, three different cleanup methods were employed, prior to ELISA detection, to arrive at the ultimate goal. As a result, this thesis exceeds all previous published work by performing 84 ELISA determinations using three combinations of methods.

Data not shown in this thesis include numerous ELISA determinations on checking cleanliness of labware, dioxin background in solvents, various method blanks, and various QC samples. These amount to hundreds of dioxin ELISA determinations on top of the 127 fish samples attempted within a span of 2 years Master of Applied Science program duration. Based on this extensive testing program, it can be concluded that ELISA can process many more folds of samples than GC-HRMS, which is in general agreement with the US EPA SITE Program report assessing the efficacy of ELISA and GC-HRMS.

5.2 Recommendations

Before implementation of the Florisil®-ELISA method, more experiments should be performed to ensure the validity of this approach. Included in this should be fish samples in which high concentrations of dioxins were found by GC-HRMS.

Another promising method worth researching as a screening technique for dioxins in fish is the use of the automated Power-Prep™ instrumentation for sample cleanup, followed by ELISA. Studies of fatty biological matrices cleaned using the Power-Prep™ and analyzed by GC-HRMS for dioxins reveal that this cleanup technique is efficient and effective. The Power-Prep™ manufacturer provides leasing opportunities for both the extraction and the cleanup modules. The minimum leasing term would cost $30,000 for 6-months plus a separate service

contract. This option is recommended as a cost-effective means of research for the purpose of proof of concept.

List of Abbreviations

2,3,7,8-TCDD – 2,3,7,8-tetrachlorodibenzo-*p*-dioxin
AhR – Aryl Hydrocarbon Receptor
CALUX – Chemically Activated Luciferase Gene Expression
CRM – Certified Reference Material
CSeQG – Canadian Sediment Quality Guideline
CSoQG – Canadian Soil Quality Guideline
CTRG – Canadian Tissue Residue Guideline
DCM – Dichloromethane
DDE – Dichlorodiphenyldichloroethylene
DDW – Deionized Distilled Water
dlPCB – Dioxin-like Polychlorinated Biphenyl
DTO – Dioxin and Toxic Organics
ELISA – Enzyme-Linked Immunosorbent Assay
EMRB – Environmental Monitoring and Reporting Branch
FMS – Fluid Management System
GC-HRMS – Gas Chromatography-High Resolution Mass Spectrometry
GC-MS – Gas Chromatography-Mass Spectrometry
GPC – Gel Permeation Chromatography
HPLC – High Performance Liquid Chromatography
LIMS – Laboratory Information Management System
LSB – Laboratory Services Branch
OC - Organochlorine
OMNR – Ontario Ministry of Natural Resources
OMOE – Ontario Ministry of the Environment
PAH – Polyaromatic Hydrocarbon
PBDE – Polybrominated Diphenyl Ether
PCB – Polychlorinated Biphenyl
PCDD – Polychlorinated Dibenzodioxin
PCDF – Polychlorinated Dibenzofuran
PCDPE – Polychlorinated Diphenylether
PCR – Polymerase Chain Reaction
QC – Quality Control
SITE – Superfund Innovative Technology Evaluation
TEF – Toxic Equivalency Factor
TEQ – Toxic Equivalent
WHO – World Health Organization
ww – Wet Weight

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Appendix

Appendix A

Table A1 – Mass Units used in Dioxin Analysis

Mass units	Terminology	Factor (relative to grams)	Alternate terminology	Symbol for alternate terminology
µg/g	Micrograms per gram	10^{-6}	Parts per million	ppm
ng/g	Nanograms per gram	10^{-9}	Parts per billion	ppb
pg/g	Picograms per gram	10^{-12}	Parts per trillion	ppt
fg/g	Femtograms per gram	10^{-15}	Parts per quadrillion	ppq

Appendix B

Table A2 – World Health Organization (WHO) toxic equivalency factors (TEFs) for dioxins (Adapted from Environment Canada, 2003)

Congener	2005 Human/Mammalian TEF[1]	1998 Human/Mammalian TEF[1,2]	Fish TEF[2]	Avian TEF[2]
2,3,7,8-TCDD	1	1	1	1
1,2,3,7,8-PeCDD	1	1	1	1
1,2,3,4,7,8-HxCDD	0.1	0.1	0.5	0.05
1,2,3,6,7,8-HxCDD	0.1	0.1	0.01	0.01
1,2,3,7,8,9-HxCDD	0.1	0.1	0.01	0.1
1,2,3,4,6,7,8-HpCDD	0.01	0.01	0.001	<0.01
OCDD	0.0003	0.0001	<0.0001	0.0001
2,3,7,8-TCDF	0.1	0.1	0.05	1
1,2,3,7,8-PeCDF	0.03	0.05	0.05	0.1
2,3,4,7,8-PeCDF	0.3	0.5	0.5	1
1,2,3,4,7,8-HxCDF	0.1	0.1	0.1	0.1
1,2,3,6,7,8-HxCDF	0.1	0.1	0.1	0.1
1,2,3,7,8,9-HxCDF	0.1	0.1	0.1	0.1
2,3,4,6,7,8-HxCDF	0.1	0.1	0.1	0.1
1,2,3,4,6,7,8-HpCDF	0.01	0.01	0.01	0.01
1,2,3,4,7,8,9-HpCDF	0.01	0.01	0.01	0.01
OCDF	0.0003	0.0001	<0.0001	0.0001

[1]Adapted from WHO, 2008.
[2]Adapted from Environment Canada, 2003.

Appendix C

Table A3. Cross-reactivity of dioxin congeners relative to 2,3,7,8-TCDD (adapted from ELISA kit insert, CAPE Technologies, available at www.cape-tech.com).

Compound		Compound	
Toxic Dioxin Congeners	Percent Cross-reactivity	Other PCDD/F Congeners	Percent Cross-reactivity
2,3,7,8-TCDD	100	2,3-dichlorodibenzo-*p*-dioxin	0.13
1,2,3,7,8-PeCDD	105	2,7-dichlorodibenzo-*p*-dioxin	0.003
1,2,3,4,7,8-HxCDD	1.6	2,3-dichlorodibenzofuran	0.02
1,2,3,6,7,8-HxCDD	7.9	2,7-dichlorodibenzofuran	<0.002
1,2,3,7,8,9-HxCDD	39	2,3,7-trichlorodibenzo-*p*-dioxin	24
1,2,3,4,6,7,8-HpCDD	0.7	2,3,8-trichlorodibenzofuran	0.26
OCDD	<0.001	1,2,3,4-TCDD	<0.001
		1,2,3,4-TCDF	<0.001
		1,3,6,8-TCDD	0.05
		1,3,6,8-TCDF	0.007
Toxic Furan Congeners			
2,3,7,8-TCDF	20		
1,2,3,7,8-PeCDF	4.6		
2,3,4,7,8-PeCDF	17	PolyChlorinated Biphenyls	
1,2,3,4,7,8-HxCDF	0.4	3,3',4,4' (PCB 77)	0.4
1,2,3,6,7,8-HxCDF	1.0	3,3',4,4',5 (PCB 126)	0.5
1,2,3,7,8,9-HxCDF	3.3	2,2',4,4',5 (PCB 153)	<0.1
2,3,4,6,7,8-HxCDF	4.9	3,3',4,4',5,5' (PCB 169)	<0.1
1,2,3,4,6,7,8-HpCDF	0.02	Aroclor 1254	<0.1
1,2,3,4,7,8,9-HpCDF	0.9		
OCDF	<0.001		

Appendix D

Table A4 – Commercially-available ELISA kits for environmental contaminants

Analyte	**Usage**	**Bioaccumulative?**	**Commercial ELISA source**	**Type of fish matrix**
Acetochlor	Herbicide	No	Abraxis LLC, PA, USA	Fish plasma
DDE	Insecticide	Yes	Abraxis LLC, PA, USA	Fish plasma
DDT	Insecticide	Yes	Abraxis LLC, PA, USA	Fish plasma
Diuron	Herbicide	Somewhat	Abraxis LLC, PA, USA	Fish plasma
Glyphosate	Herbicide	No	Abraxis LLC, PA, USA	Fish plasma
Pyrethroids	Pesticide	No?	Abraxis LLC, PA, USA	Fish plasma
Cyclodienes (dieldrin)	Insecticide	Yes	Abraxis LLC, PA, USA; Beacon Analytical Systems, ME, USA	Fish plasma; soil
Alachlor	Herbicide	No	Abraxis LLC, PA, USA; Beacon Analytical Systems, ME, USA	Fish plasma; water
Atrazine	Herbicide	No	Abraxis LLC, PA, USA; Beacon Analytical Systems, ME, USA	Fish plasma; water
Metolachlor	Pesticide	No	Abraxis LLC, PA, USA; Beacon Analytical Systems, ME, USA	Fish plasma; water
Dioxin-like PCBs	Electrical Insulator	Yes	Abraxis LLC, PA, USA	Fish tissue
PBDEs	Flame retardants	Yes	Abraxis LLC, PA, USA	Fish tissue
PCBs	Electrical Insulator	Yes	Abraxis LLC, PA, USA	Fish tissue
Toxaphene	Insecticide	Yes	Beacon Analytical Systems, ME, USA	Soil
Carbendazim/Benomyl	Fungicide		Abraxis LLC, PA, USA	water
Spinosyn	Insecticide	No	Abraxis LLC, PA, USA	water
Abamectin	Pesticide	No	Beacon Analytical Systems, ME, USA	water
Thiamethoxam	Pesticide	No	Beacon Analytical Systems, ME, USA	water
2,4-D	Herbicide	No	Abraxis LLC, PA, USA; Beacon Analytical Systems, ME, USA	water; water

Abraxis LLC, PA, USA; reference: http://www.abraxiskits.com/
Beacon Analytical Systems, ME, USA; reference: http://www.beaconkits.com/products.cfm

Appendix E

Table A5 – Adsorbents in increasing order of adsorptivity
(Vogel, 1989)

Adsorptivity	Adsorbent
Lowest adsorptivity ↓	Sucrose, starch
	Inulin
	Talc
	Sodium carbonate
	Calcium carbonate
	Calcium phosphate
	Magnesium carbonate
	Magnesium hydroxide
	Calcium hydroxide
	Silica gel
	Magnesium silicate (Florisil®)
	Alumina
Highest adsorptivity	Fuller's earth

Table A6 – Eluatropic series
(Vogel, 1989)

Polarity	Solvent
Least polar ↓	Hexane
	Cyclohexane
	Carbon tetrachloride
	Trichloroethylene
	Toluene
	Dichloromethane
	Chloroform
	Diethyl ether
	Ethyl acetate
	Acetone
	Propanol
	Ethanol
Most polar	Methanol

Appendix F

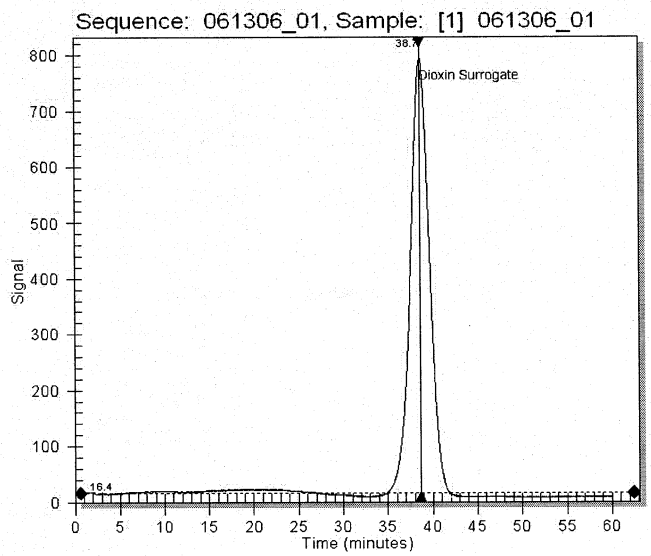
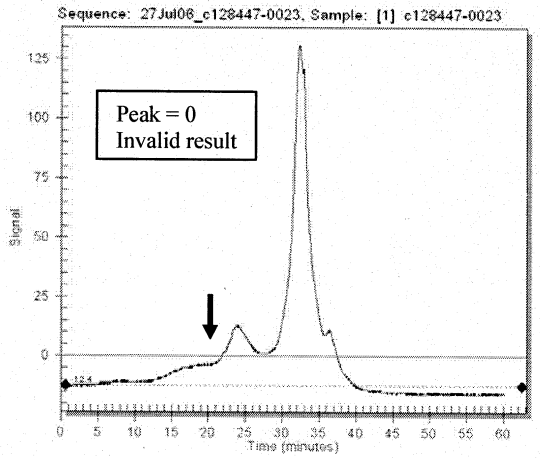
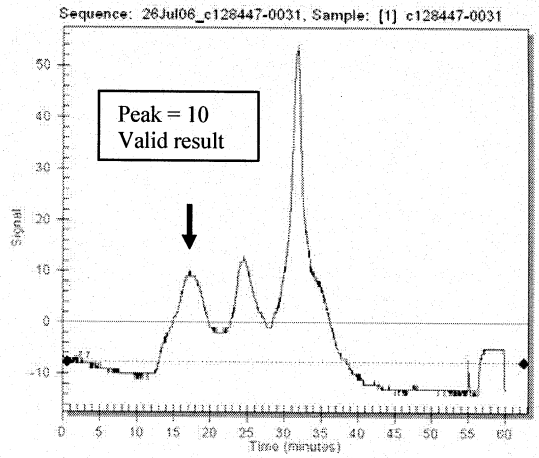
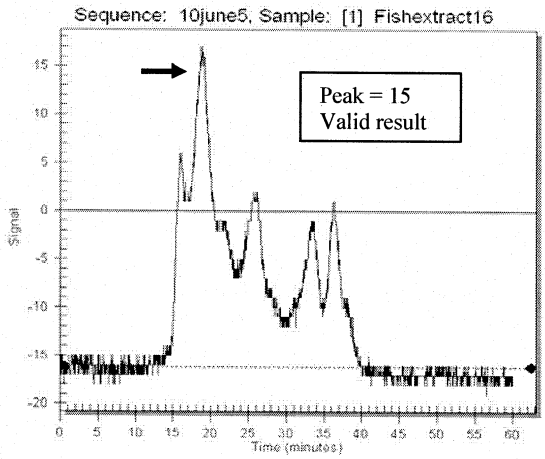


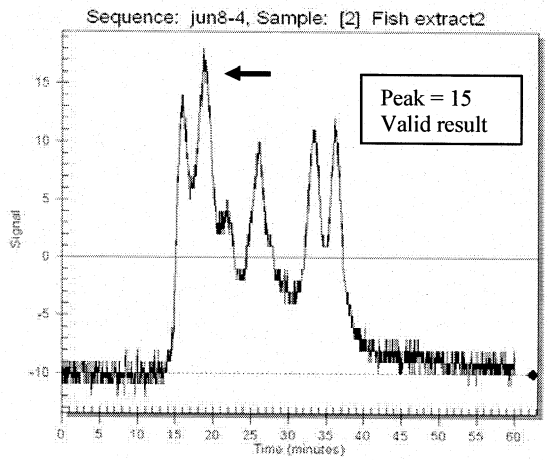
Figure A1 – UV chromatogram of the dioxin surrogate obtained from GPC. The time of elution of the dioxin surrogate determined the collection time for the dioxin fraction.

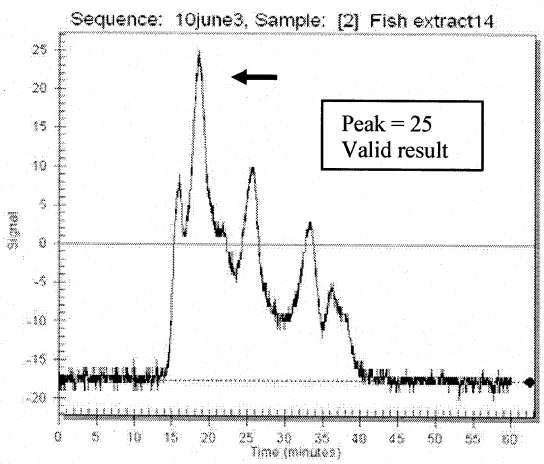
Appendix G. UV chromatograms of fish extracts. The magnitude of UV contaminant peaks (UV units; in the first 25 minutes) (black arrows) and whether or not the ELISA result was valid (in the 0-100 pg ELISA range) are indicated for each.

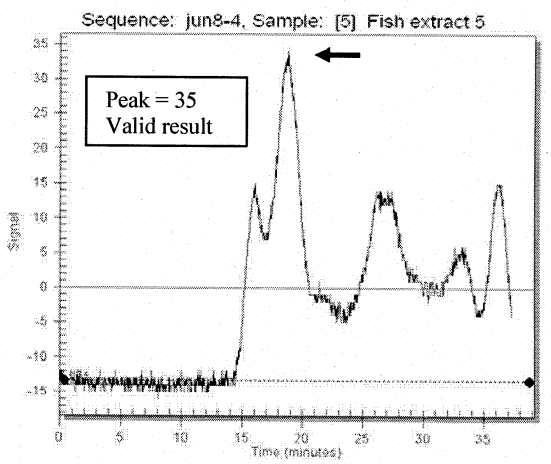


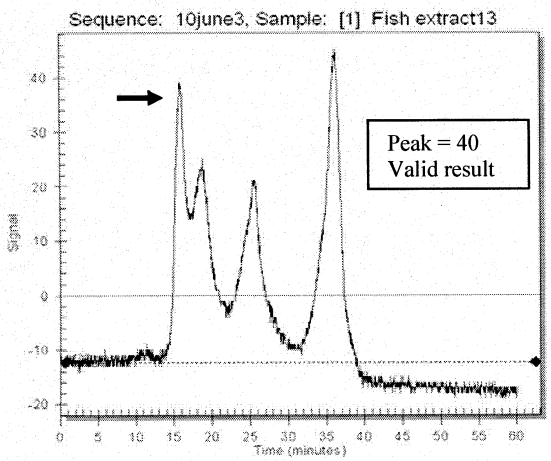


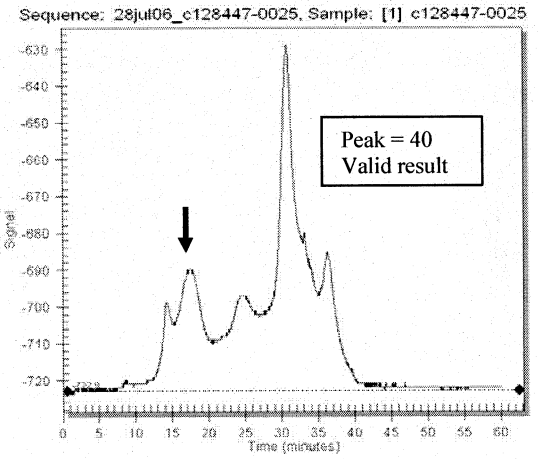


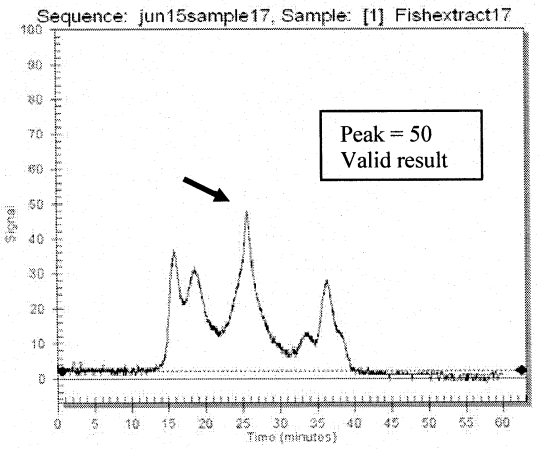


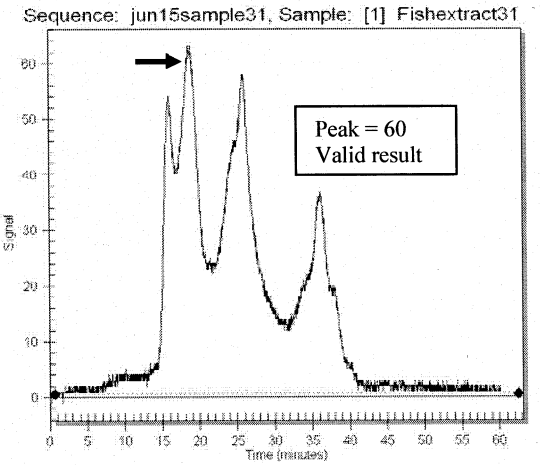


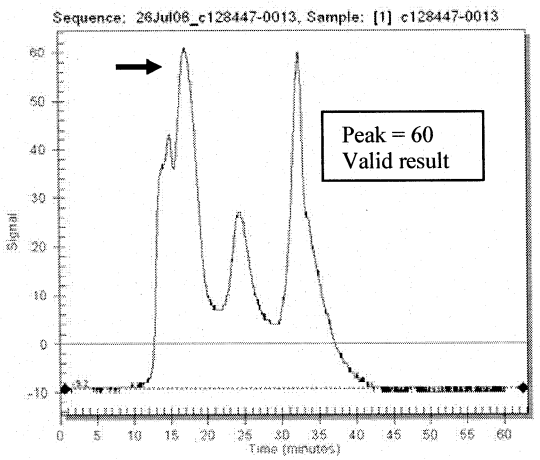



Sequence: 10june3, Sample: [1] Fish extract13
Peak = 40
Valid result
Signal
Time (minutes)


Sequence: 28jul06_c128447-0025, Sample: [1] c128447-0025
Peak = 40
Valid result
Signal
Time (minutes)


Sequence: jun15sample17, Sample: [1] Fishextract17
Peak = 50
Valid result
Signal
Time (minutes)


Sequence: jun15sample31, Sample: [1] Fishextract31
Peak = 60
Valid result
Signal
Time (minutes)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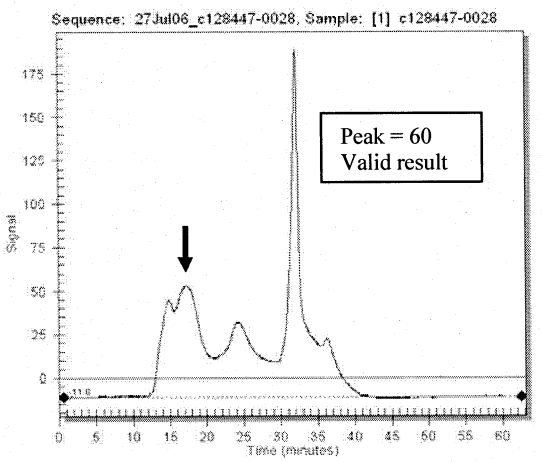
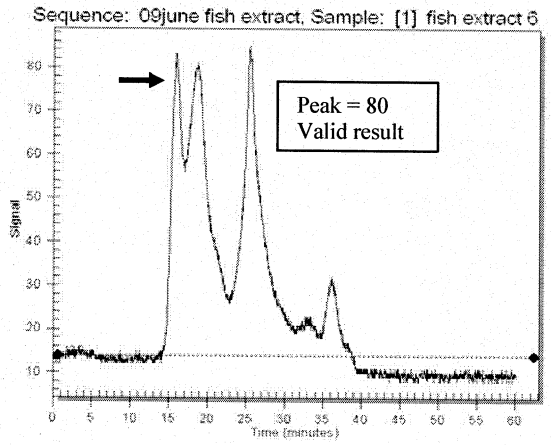
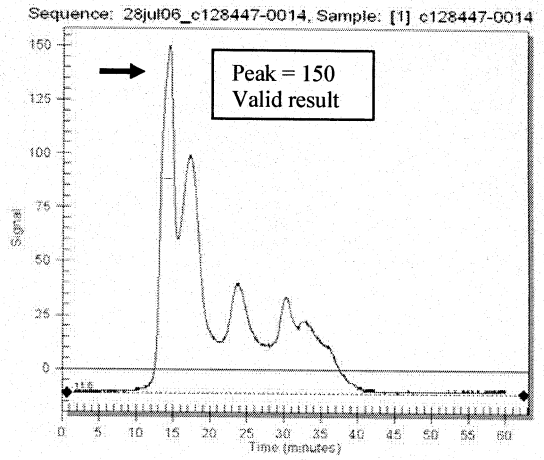
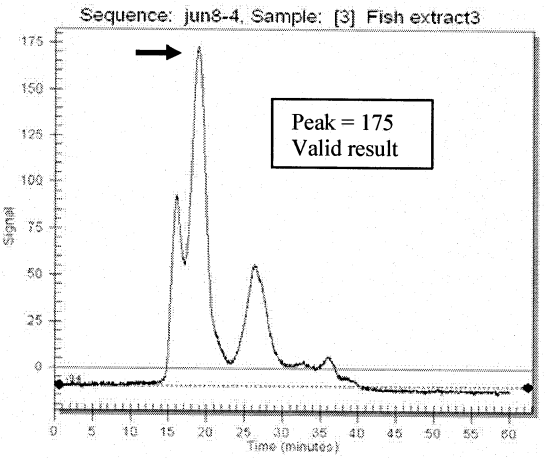
Peak = 60
Valid result
Signal
Time (minutes)

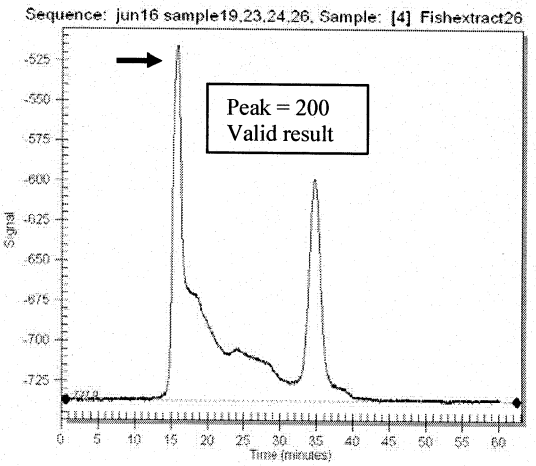

Sequence: 27Jul06_c128447-0028, Sample: [1] c128447-0028
Peak = 60
Valid result
Signal
Time (minutes)

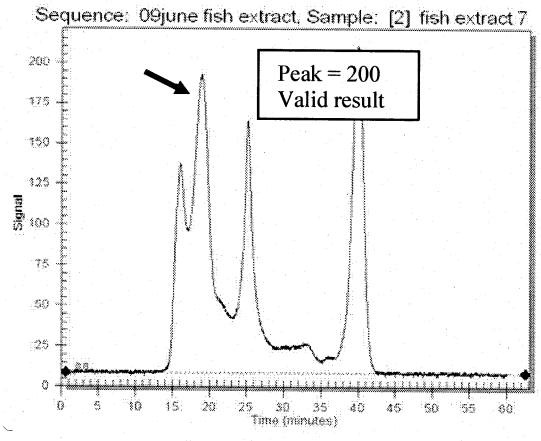
APPENDIX

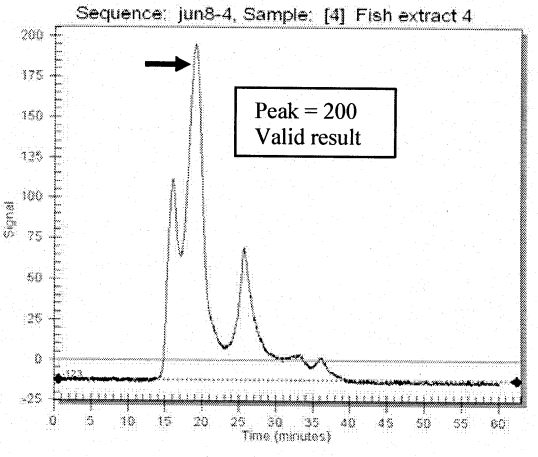

Sequence: 09june fish extract, Sample: [1] fish extract 6
Peak = 80
Valid result
Signal
Time (minutes)

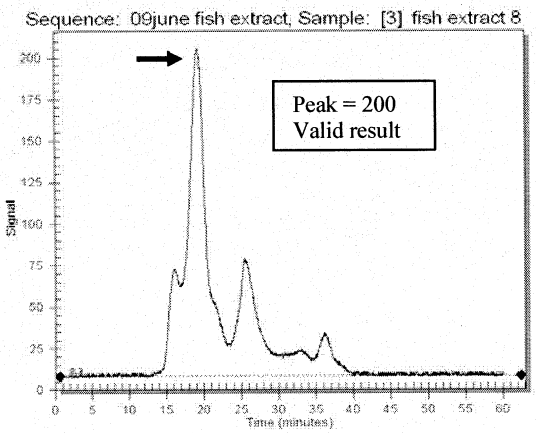

Sequence: 28jul06_c128447-0014, Sample: [1] c128447-0014
Peak = 150
Valid result
Signal
Time (minutes)

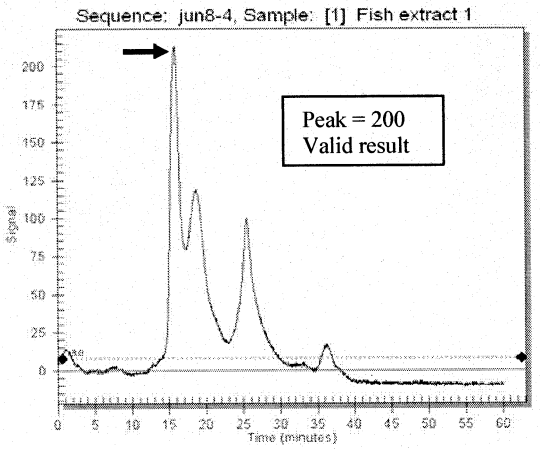

Sequence: jun8-4, Sample: [3] Fish extract3
Peak = 175
Valid result
Signal
Time (minutes)


Sequence: jun16 sample19,23,24,26, Sample: [4] Fishextract26
Peak = 200
Valid result
Signal
Time (minutes)


Sequence: 09june fish extract, Sample: [2] fish extract 7
Peak = 200
Valid result
Signal
Time (minutes)


Sequence: jun8-4, Sample: [4] Fish extract 4
Peak = 200
Valid result
Signal
Time (minutes)


Sequence: 09june fish extract, Sample: [3] fish extract 8
Peak = 200
Valid result
Signal
Time (minutes)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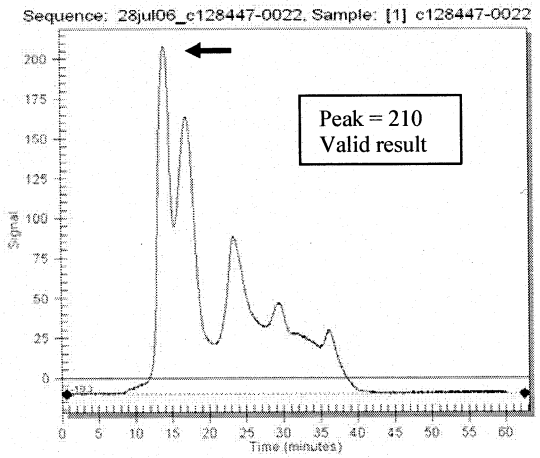
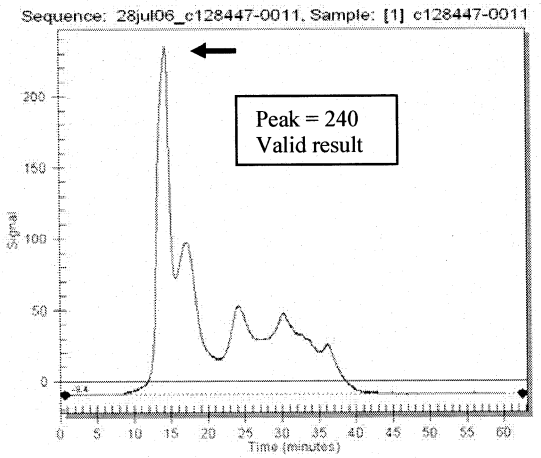
Peak = 200
Valid result
Signal
Time (minutes)


Sequence: 28jul06_c128447-0022, Sample: [1] c128447-0022
Peak = 210
Valid result
Signal
Time (minutes)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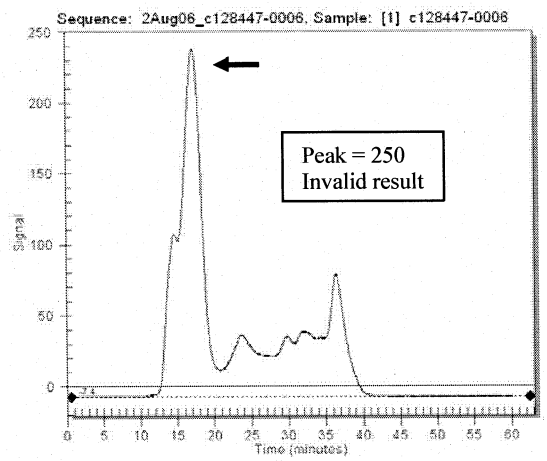
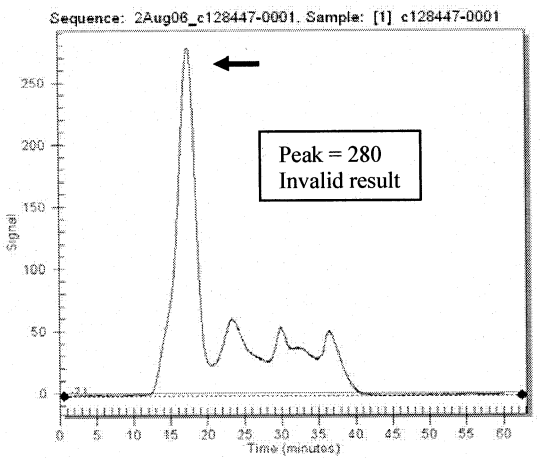
Peak = 240
Valid result
Signal
Time (minutes)


Sequence: 2Aug06_c128447-0006, Sample: [1] c128447-0006
Peak = 250
Invalid result
Signal
Time (minutes)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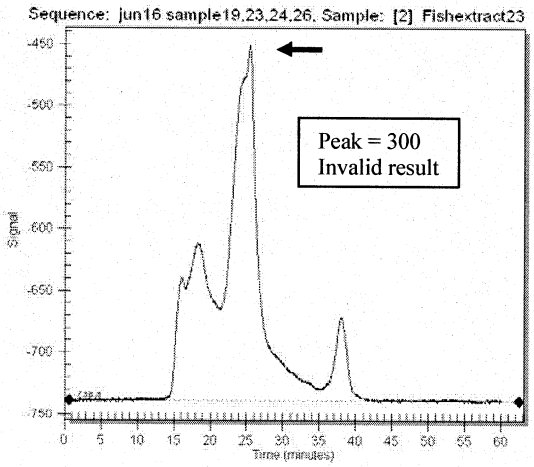
Peak = 280
Invalid result
Signal
Time (minutes)


Sequence: jun16 sample19,23,24,26, Sample: [2] Fishextract23
Peak = 300
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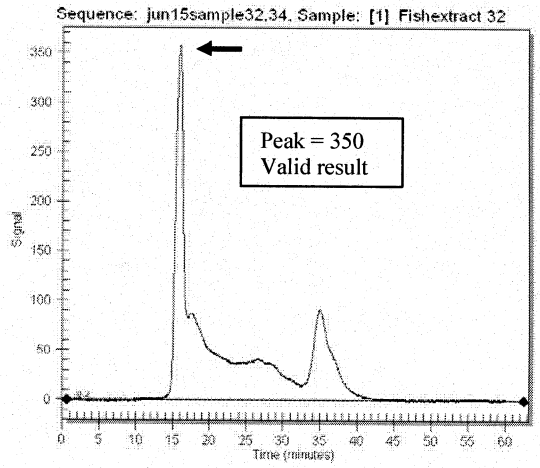
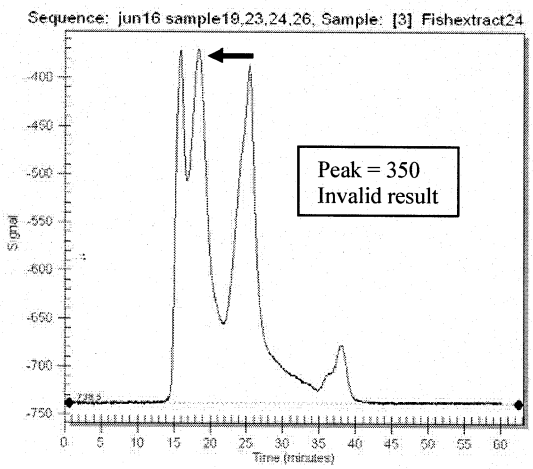
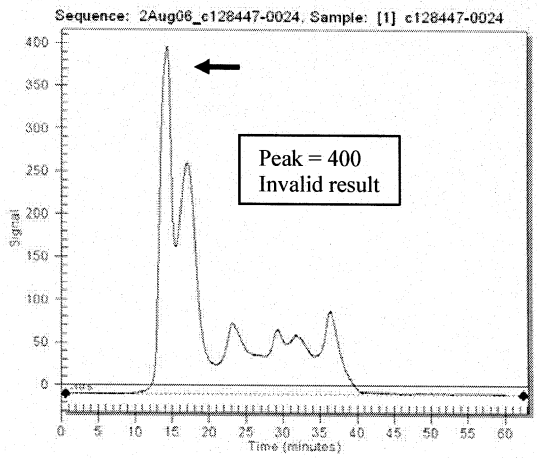
Signal
Time (minutes)


Sequence: jun15sample32,34, Sample: [1] Fishextract 32
Peak = 350
Valid result
Signal
Time (minutes)


Sequence: jun16 sample19,23,24,26, Sample: [3] Fishextract24
Peak = 350
Invalid result
Signal
Time (minutes)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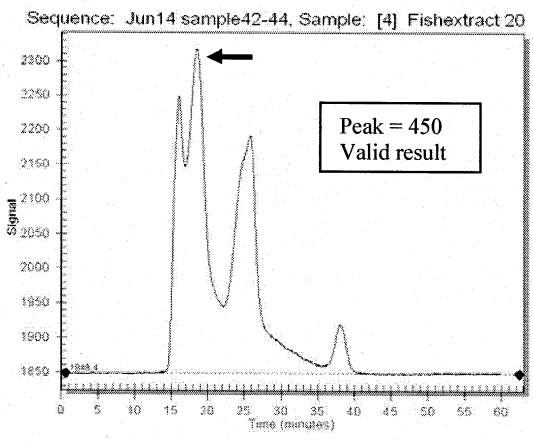
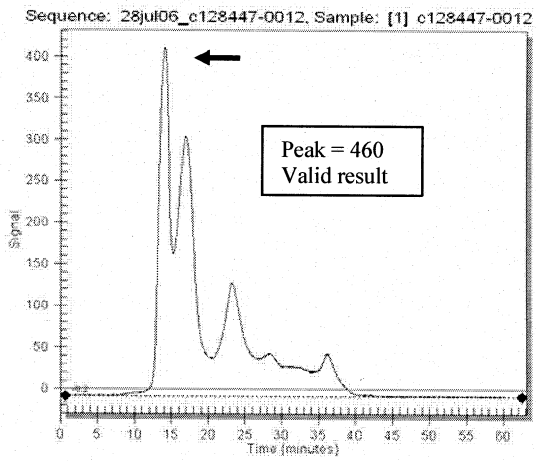
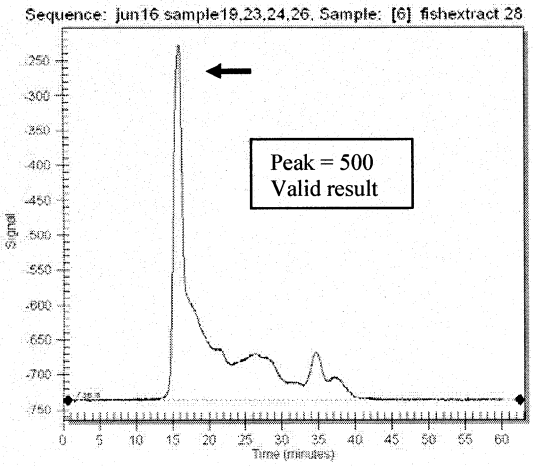
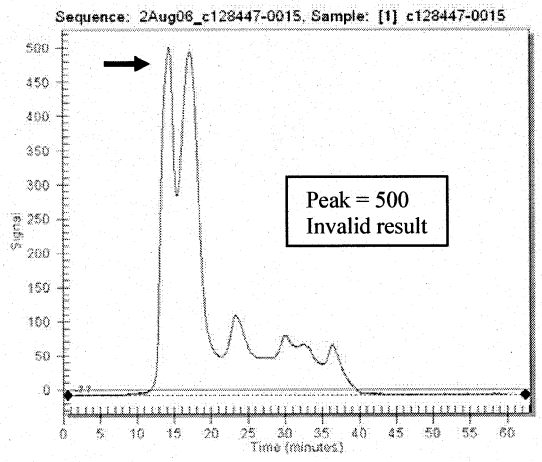
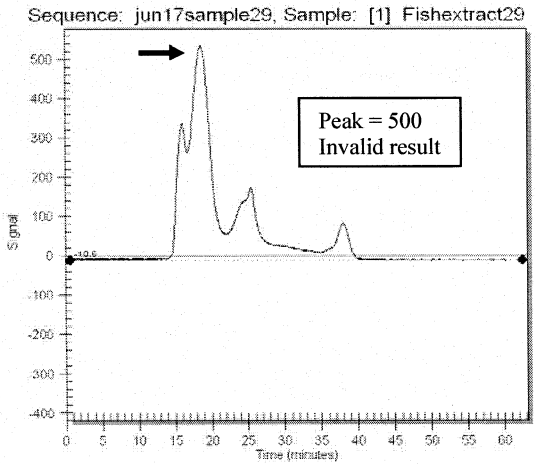
Peak = 400
Invalid result
Signal
Time (minutes)

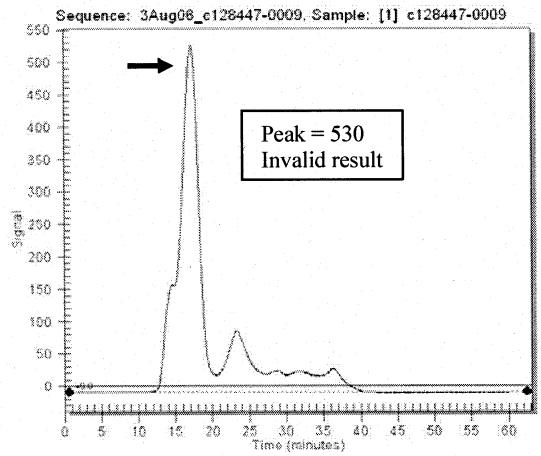

Sequence: Jun14 sample42-44, Sample: [4] Fishextract 20
Peak = 450
Valid result
Signal
Time (minutes)

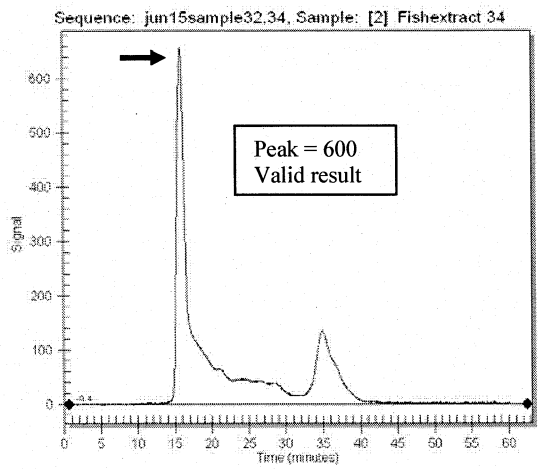

Sequence: 28jul06_c128447-0012, Sample: [1] c128447-0012
Peak = 460
Valid result
Signal
Time (minutes)

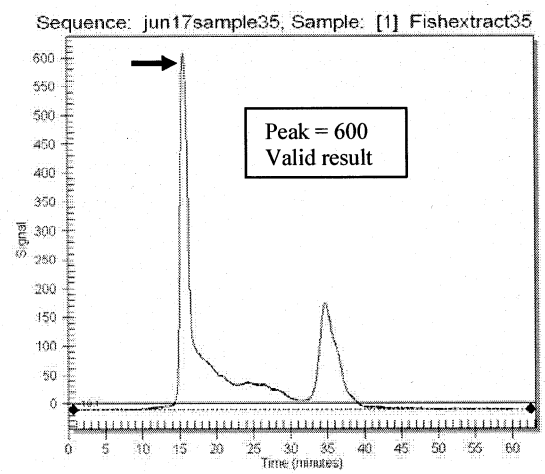

Sequence: jun16 sample18,23,24,26, Sample: [6] fishextract 28
Peak = 500
Valid result
Signal
Time (minutes)


Sequence: 2Aug06_c128447-0015, Sample: [1] c128447-0015
Peak = 500
Invalid result
Signal
Time (minutes)

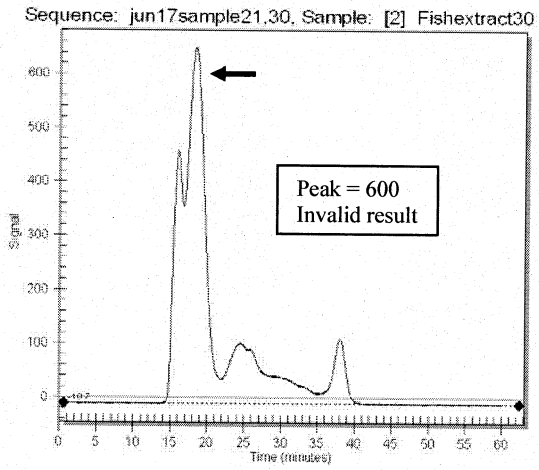

Sequence: jun17sample29, Sample: [1] Fishextract29
Peak = 500
Invalid result
Signal
Time (minutes)

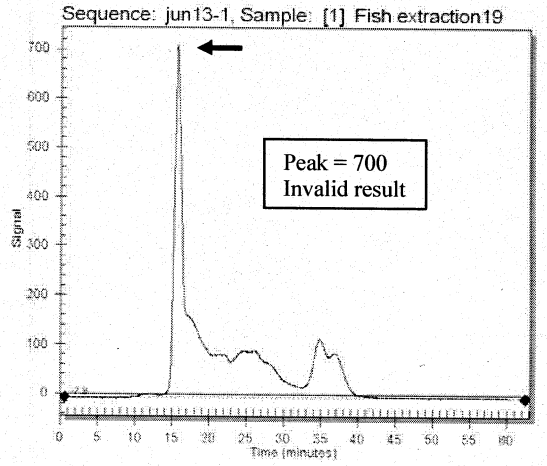

Sequence: 3Aug06_c128447-0009, Sample: [1] c128447-0009
Peak = 530
Invalid result
Signal
Time (minutes)

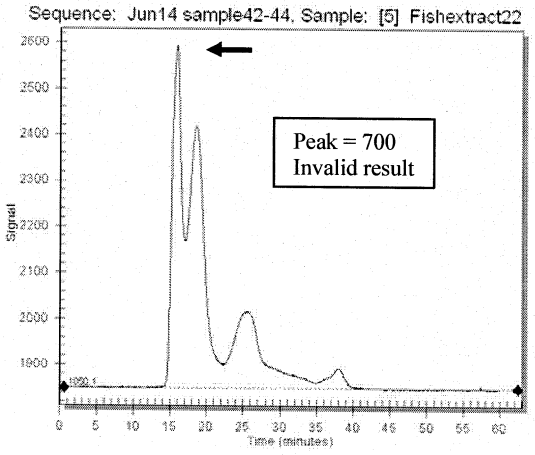

Sequence: jun15sample32,34, Sample: [2] Fishextract 34
Peak = 600
Valid result
Signal
Time (minutes)


Sequence: jun17sample35, Sample: [1] Fishextract35
Peak = 600
Valid result
Signal
Time (minutes)

APPENDIX


Sequence: jun17sample21,30, Sample: [2] Fishextract30
Peak = 600
Invalid result
Signal
Time (minutes)


Sequence: jun13-1, Sample: [1] Fish extraction19
Peak = 700
Invalid result
Signal
Time (minutes)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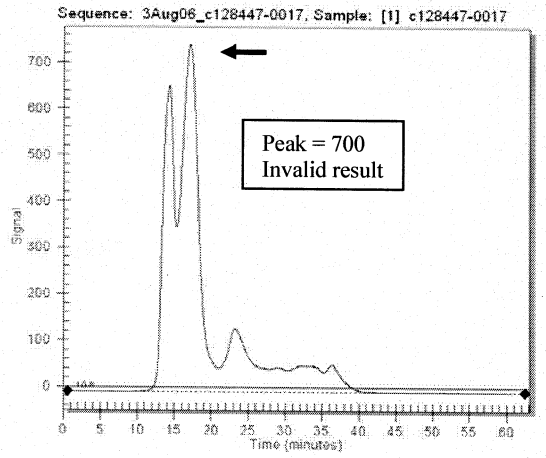
Peak = 700
Invalid result
Signal
Time (minutes)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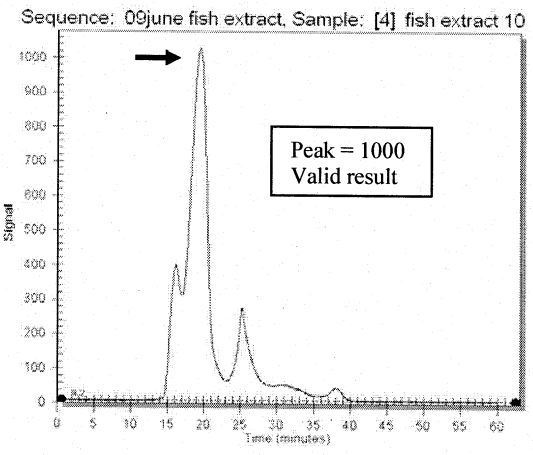
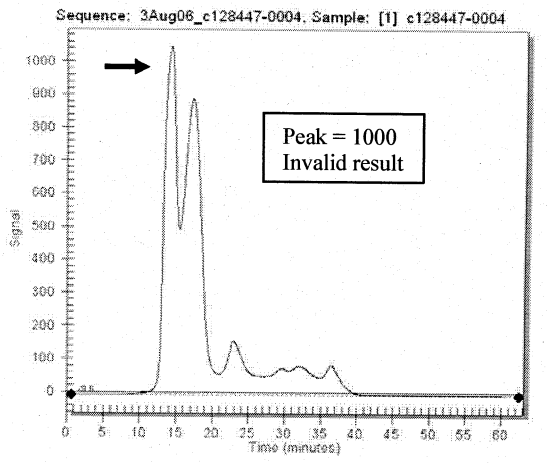
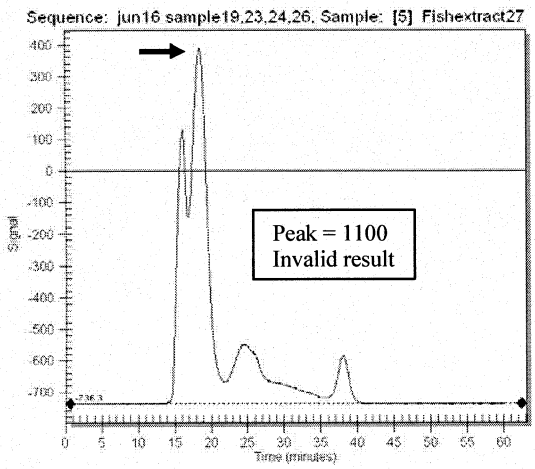
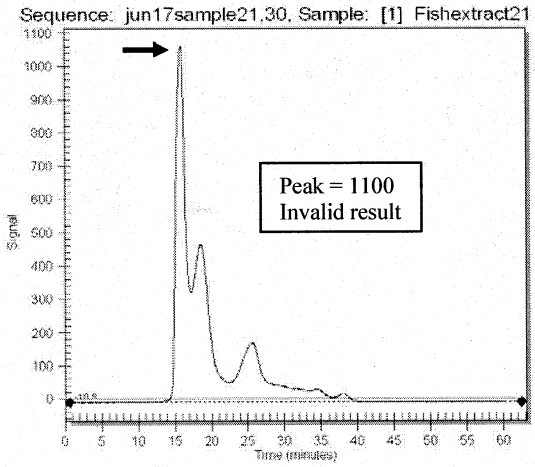
Peak = 700
Invalid result
Signal
Time (minutes)

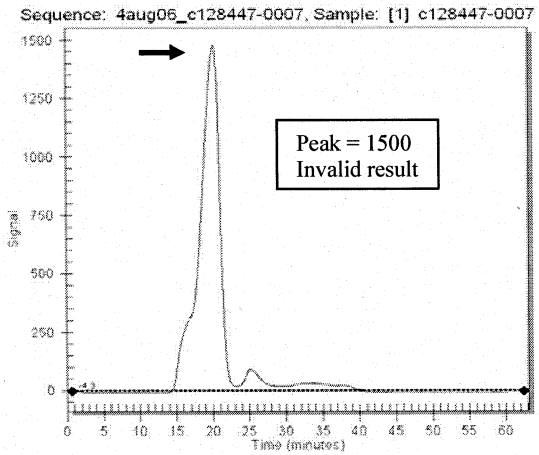

Sequence: 09june fish extract, Sample: [4] fish extract 10
Peak = 1000
Valid result
Signal
Time (minutes)

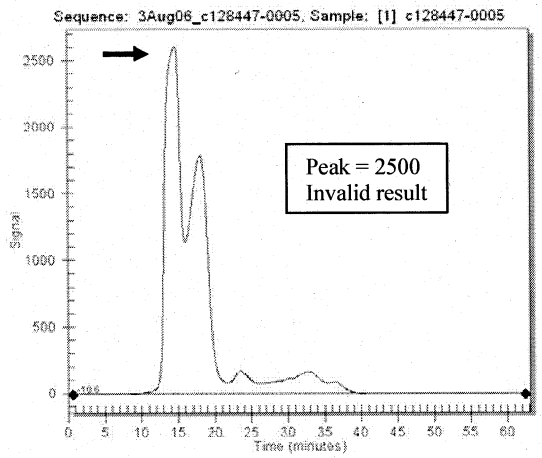

Sequence: 3Aug06_c128447-0004, Sample: [1] c128447-0004
Peak = 1000
Invalid result
Signal
Time (minutes)

APPENDIX


Sequence: jun16 sample19,23,24,26, Sample: [5] Fishextract27
Peak = 1100
Invalid result
Signal
Time (minutes)


Sequence: jun17sample21,30, Sample: [1] Fishextract21
Peak = 1100
Invalid result
Signal
Time (minutes)


Sequence: 4aug06_c128447-0007, Sample: [1] c128447-0007
Peak = 1500
Invalid result
Signal
Time (minutes)


Sequence: 3Aug06_c128447-0005, Sample: [1] c128447-0005
Peak = 2500
Invalid result
Signal
Time (minutes)