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Principles and techniques towards successful development of enzyme-linked immunosorbent assay (ELISA) for dioxin analysis

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**PRINCIPLES AND TECHNIQUES TOWARDS SUCCESSFUL DEVELOPMENT OF
ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR DIOXIN ANALYSIS**

By

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A thesis presented to Ryerson University

In partial fulfillment of the requirements for the degree of Master of Applied Science in
the Program of Environmental Applied Science and Management

Toronto, Ontario, Canada, 2008

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Abstract

Principles and Techniques towards Successful Development of Enzyme-Linked Immunosorbent Assay (ELISA) for Dioxin Analysis

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Toronto, 2008

Dioxins are highly toxic, persistent and bio-accumulative compounds. Laboratory detection of dioxins in various environmental matrices is one of the most technically demanding and expensive tasks in analytical chemistry. The cost to analyze a soil sample by conventional gas chromatography-high resolution mass spectrometry (GC-HRMS) is approximately \$1900 USD according to the United States Environmental Protection Agency (US EPA) (Billets, 2005). As an alternative, enzyme-linked immunosorbent assay (ELISA) for dioxin analysis has been commercially available for over a decade and recognized as US EPA Method 4025. However, assay attributes need to be examined, especially at trace level detection. In this study, sources of error in ELISA, such as background contamination and dioxin-like polychlorinated biphenyl (dl-PCB) cross-reactions have been investigated. Quality assurance data on spikes have been reviewed and the recovery was estimated to be 70%. Technical details that are crucial for the performance of dioxin ELISA were also identified and addressed.

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Table of Content

AUTHOR'S DECLARATION	II
ABSTRACT	III
ACKNOWLEDGEMENTS	IV
LIST OF TABLES	VII
LIST OF FIGURES.....	VIII
NOMENCLATURE.....	IX
 CHAPTER 1. INTRODUCTION.....	 1
CHAPTER 2. LITERATURE REVIEW.....	6
2.1 DIOXINS, FURANS AND DIOXIN-LIKE PCBs	6
2.1.1 <i>Chemical properties</i>	6
2.1.2 <i>Environmental fate</i>	7
2.1.3 <i>Toxicity</i>	8
2.1.4 <i>Exposure Pathways</i>	9
2.2 ANALYTICAL METHODS	10
2.2.1 <i>Sample preparation</i>	10
2.2.2 <i>Detection methods</i>	11
CHAPTER 3. COMPETITIVE ELISA.....	17
3.1 COMPARISON OF ELISA KITS FROM TWO SUPPLIERS	17
3.2 CAPE ELISA KIT	17
3.3 CAPE ELISA PRINCIPLE	18
3.4 CURVE FITTING METHODS.....	19
3.5 US EPA SITE PROGRAM EVALUATION	23
3.6 ACCURACY AND SENSITIVITY AT LOW DETECTION LEVELS.....	23
CHAPTER 4. SOURCES OF BACKGROUND CONTAMINATION.....	25
4.1 PREAMBLE.....	25
4.2 MATERIALS AND METHODS	25
4.2.1 <i>Reagents and Equipment</i>	25
4.2.2 <i>Lab ware cleaning</i>	26
4.2.3 <i>Detection methods</i>	28
4.2.4 <i>Data analysis</i>	30
4.3 RESULTS AND DISCUSSION	30
4.3.1 <i>Background testing</i>	30
4.3.2 <i>Comparison between ELISA and GC-HRMS</i>	35
4.3.3 <i>Dust contamination</i>	36
4.4 SUMMARY	37

CHAPTER 5. THE CROSS-REACTIVITY OF PCBS IN DIOXIN ELISA	38
5.1 PREAMBLE.....	38
5.2 MATERIALS AND METHODS	38
5.3 RESULTS AND DISCUSSIONS.....	39
CHAPTER 6. SPIKE RECOVERY	40
6.1 PREAMBLE.....	40
6.2 MATERIALS AND METHODS	40
6.3 RESULTS	41
6.4 DISCUSSIONS	44
CHAPTER 7. OPTIMIZATION OF THE PERFORMANCE OF ELISA	46
7.1 CONSUMPTION OF ONE NEW POUCH OF ALL 20 ELISA TUBES PER ASSAY.....	46
7.1.1 <i>Hypothesis under investigation</i>	46
7.1.2 <i>Materials and Methods</i>	46
7.1.3 <i>Results and Discussions</i>	46
7.2 DENATURATION OF ANTIBODIES.....	48
7.2.1 <i>Chemistry of solvent exchange</i>	48
7.2.2 <i>Materials and Methods</i>	50
7.2.3 <i>Results and Discussions</i>	52
7.3 SLOWLY VORTEX THE ELISA TUBE AFTER SAMPLE ADDITION	54
7.3.1 <i>Hypothesis</i>	54
7.3.2 <i>Materials and Methods</i>	55
7.3.3 <i>Results and Discussions</i>	55
7.4 REAGENT SHELF LIFE.....	57
7.4.1 <i>Conjugate Shelf life</i>	57
7.4.2 <i>Substrate Shelf life</i>	64
CHAPTER 8. CONCLUSIONS AND RECOMMENDATIONS	70
8.1 CONCLUSIONS	70
8.2 RECOMMENDATIONS	74
REFERENCES	76

List of Tables

TABLE 1-1. SUMMARY OF DATA QUALITY OBJECTIVES OF DIOXIN AND FURAN STANDARDS FROM DIFFERENT ENVIRONMENTAL MATRICES	2
TABLE 1-2. SUMMARY OF EXPERIMENTAL TESTS CONDUCTED IN THIS THESIS	4
TABLE 4-1. FOUR DIFFERENT CLEANING PROCEDURES FOR 16×100 MM GLASS TUBES	27
TABLE 4-2. THREE DIFFERENT CLEANING PROCEDURES FOR PASTEUR PIPETTES	27
TABLE 4-3. DIOXIN BACKGROUND IN 16×100 MM GLASS TUBES BY ELISA	31
TABLE 4-4. DIOXIN BACKGROUND TEQ IN GLASS PASTEUR PIPETTES BY ELISA	32
TABLE 4-5. DIOXIN BACKGROUND IN SOLVENTS BY ELISA	33
TABLE 4-6. DIOXIN-LIKE PCB (DL-PCB) BACKGROUND IN 16×100MM GLASS TUBES BY GC-HRMS	35
TABLE 4-7. COMPARISON OF ELISA TEQ PER TUBE WITH GC-HRMS TEQ	36
TABLE 5-1. CROSS-REACTION OF PCBs IN DIOXIN ELISA	39
TABLE 7-1. DIOXIN STANDARDS BIND MORE TO TUBES FROM UNOPENED THAN OPENED POUCH	47
TABLE 7-2. READINGS OF DIOXIN VALUES IN VORTEX ELISA TUBES VERSUS TAPPED TUBES	56
TABLE 7-3. ABSORBANCE (OD UNIT) OBTAINED BY TITRATION OF CONJUGATE FROM DIFFERENT LOTS	61
TABLE 7-4. ABSORBANCE (OD UNITS) OF CONJUGATE TITRATION WITH DIFFERENT LOTS OF SUBSTRATE	64
TABLE 7-5. ABSORBANCE (OD UNITS) AT 450 NM (650 NM AS REFERENCE)	66
TABLE 7-6. PERFORMANCE OF FRESH SUBSTRATE (Lot# A0696) BEFORE AND AFTER FORTIFICATION WITH THREE CONCENTRATIONS OF H ₂ O ₂ IN A MICROTITER PLATE	67
TABLE 7-7. FAILURE OF ELISA IN THE PRESENCE OF 0.1% H ₂ O ₂ ADDED TO THE SUBSTRATE SOLUTION	68

List of Figures

FIGURE 2-1. GENERAL STRUCTURE OF DIOXIN, FURAN AND DIOXIN-LIKE PCB MOLECULES (US EPA, 2004).....	7
FIGURE 3-1. PRINCIPLE OF COMPETITIVE ELISA	18
FIGURE 3-2. ELISA ORIGINAL CALIBRATION CURVE PROVIDED BY CAPE TECHNOLOGIES AND WELLINGTON STANDARD DATA POINTS BEFORE CURVE FITTING BY THE SOLVER FUNCTION.....	20
FIGURE 3-3. ELISA CALIBRATION CURVE BY FOUR-PARAMETER FITTING AFTER SOLVER FUNCTION	21
FIGURE 4-1. WHITE DUST COLLECTED OVERNIGHT ON THE FUME HOOD SASH (LEFT) AND ON THE BEAKER TIN FOIL COVER (RIGHT).....	37
FIGURE 6-1. DOSE-RESPONSE LINEARITY OF ELISA BY SPIKING 10 mL TOLUENE.....	42
FIGURE 6-2. DOSE-RESPONSE LINEARITY OF ELISA BY SPIKING 10 mL DCM/HEXANE/ISOCTANE	42
FIGURE 6-3. DOSE-RESPONSE LINEARITY OF ELISA BY SPIKING 10 mL DCM	43
FIGURE 6-4. DOSE-RESPONSE LINEARITY OF ELISA BY SPIKING 62.5 μ L OF PEG-TRITON KEEPER	43
FIGURE 6-5. SPIKE RECOVERY BASED ON COMBINED SPIKE SAMPLES (N=66)	44
FIGURE 6-6. NITROGEN GENERATOR (LEFT) AND DRY BATH WITH A HOMEMADE PLASTIC UDDER TO DISTRIBUTE NITROGEN (RIGHT)	45
FIGURE 7-1. MOLECULAR STRUCTURE OF TRITON X-100	51
FIGURE 7-2. MOLECULAR STRUCTURES OF PEG AND WATER	51
FIGURE 7-3. ANTIBODY DETERIORATION DURING INCUBATION	53
FIGURE 7-4. TUBE BEING VORTEX (LEFT); TUBE BEING TAPPED (RIGHT)	55
FIGURE 7-5. CHEMICAL REACTIONS IN CONJUGATE SYNTHESIS.....	59
FIGURE 7-6. CONJUGATE SHELF LIFE AS MEASURED BY REACTION INTENSITY AT 1:64 DILUTION....	62
FIGURE 7-7. SUBSTRATE SHELF LIFE AS MEASURED BY REACTION INTENSITY USING 1:32 CONJUGATE DILUTION	65

Nomenclature

% v/v	volume/volume percentage
$\geq$	greater than or equal to
1 psi	1 pound per square inch, a unit of pressure
2,3,7,8-TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
CV	Coefficient of Variation
DCM	dichloromethane
DDW	Deionized distilled water
dl-PCBs	dioxin-like polychlorinated biphenyls
ELISA	Enzyme-linked Immunosorbent Assay
GC-HRMS	Gas Chromatography-High Resolution Mass Spectrometry
mM	millimole
MDL	method detection limit
NC	negative control
OD	optical density
PBS	Phosphate Buffered Saline
PCDDs	Polychlorinated dibenzo-p-dioxin
PCDFs	Polychlorinated dibenzofurans
PEG	Polyethylene glycol
pg	picogram (1×10^{-12} of one gram)
ppb	parts per billion (1 part in 10^9)
ppt	parts per trillion (1 part in 10^{12})
ppq	parts per quadrillion (1 part in 10^{15})
QA	Quality Assurance
QC	Quality Control
R^2	Square of Spearman's rank correlation (r)
SD	Standard Deviation
SOPs	Standard Operation Procedures
TEF	Toxic equivalency factor
TEQ	Toxicity equivalent

Chapter 1. Introduction

Polychlorinated dibenzo-*p*-dioxin (dioxins or PCDDs), polychlorinated dibenzofurans (furans or PCDFs) and dioxin-like polychlorinated biphenyls (dl-PCBs) are well-known harmful chemicals. These chemicals are mainly released from combustion in the presence of chlorine donors (Mukerjee, 1998). Effective detection of these chemicals is vital for public health.

The conventional method of Gas Chromatography-High Resolution Mass Spectrometry (GC-HRMS) has been employed to detect dioxin, furan and dl-PCBs for decades. It is regarded as the standard method worldwide, due to its sensitive and accurate capability of detecting and quantifying each congener of these compounds in any sample matrix (Reiner *et al.*, 2006). However, as a detection tool, this method is very expensive and time-consuming, which leads to huge backlogs of samples to be tested. New detection methods with lower costs and less processing time are required to serve the purpose of dioxin detection in various environmental matrices, especially in food and drinking water.

Enzyme-linked Immunosorbent Assay (ELISA) was developed more than ten years ago as an alternative detection method. Immunological assays in general have the advantages of saving both time and money, and the capacity to process more samples than GC-HRMS. ELISA is a promising method to be applied to dioxin detection.

The ELISA method has been officially approved by the United States Environmental Protection Agency (US EPA) as Method 4025 (US EPA, 2002b) for measuring dioxin in soil samples at the parts per billion (ppb) level. The unit of measurement is defined in terms of toxicity equivalent (TEQ) to the most toxic congener 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8- TCDD). This level of detection is sufficient for soil sample analysis, because the upper

TEQ concentration limit in Ontario is 10 ng TEQ/g soil (10 ppb) (OMEE, 1996).

In contrast, the dioxin detection level is three and six orders of magnitudes lower in food and drinking water, respectively. For example, the Ontario Sport Fish Program provides a guideline to restrict daily consumption of fish containing TEQ ≥ 2.7 parts per trillion (ppt) (MOE, 2007). TEQ detection at 15 parts per quadrillion (ppq) is required for drinking water according to the *Safe Drinking Water Act* 2002, Ontario Regulation 169/03 (Table 1-1).

This thesis identifies factors influencing the performance of the ELISA kit as per US EPA Method 4025 and remedies to improve its sensitivity in order to meet detection limits of dioxin in fish samples at ppt level and in water samples at ppq level. The needed research is conducted at the Bioassay laboratory, Laboratory Services Branch, Ontario Ministry of the Environment, where these assay improvements will be implemented.

Table 1-1. Summary of data quality objectives of dioxin and furan standards from different environmental matrices

<i>Environmental matrices</i>	<i>Data quality level</i>	<i>Standards established</i>	<i>Reference/Legislation</i>
Soil	ppb	10 ng TEQ/g soil	Guidance on Site Specific Risk Assessment for use at contaminated sites in Ontario. Appendix E: Upper Concentrations Limits for Soil and Groundwater (OMEE, 1996)
Fish	ppt	2.7 pg TEQ/g fish	Guide to Eating Ontario Sport Fish, 2007-2008 Edition (MOE, 2007)
Drinking Water	ppq	15 pg TEQ /L drinking water	<i>Safe Drinking Water Act</i> 2002, Ontario Regulation 169/03.

The Ontario Ministry of the Environment, Laboratory Services Branch has a quality control system for producing a product or service that meets the quality standards and needs of customers (MOE, 2006b). It requires the Quality Control (QC) and Quality Assurance (QA) in the routine analysis of environmental samples (MOE, 2006b). The practices include: identifying

the stability, bias and precision of equipment and instrumentation; ensuring the continuing purity of essential reagents and solvents; and assessing the overall performance of the analytical system (MOE, 2006b).

According to this quality control system, a series of tests have been conducted in this research study (Table 1-2) to validate this ELISA method at ultra-trace level detection. Four aspects have been investigated. Firstly, the bias and the possible errors have been examined, including the background contamination of both the glassware and the solvents, and the cross-reaction of dl-PCBs in ELISA that is designed to detect dioxin and furan. Secondly, the quality control samples, including calibration standards and spikes, have been performed to determine the recovery of the ELISA method. Thirdly, the essential reagents (Triton X-100 Polyethylene Glycol (PEG)-keeper, conjugate and substrate) from the commercially available ELISA kit have been studied. Lastly, some techniques and certain standard operating procedures (SOPs) that are critical to achieve the successful performance have been optimized. The results of this study will contribute to a further improvement of an effective and economical detection method for dioxin analysis.

Table 1-2. Summary of experimental tests conducted in this thesis

<i>No.</i>	<i>Chapter</i>	<i>Tests conducted in this study</i>	<i>Purposes</i>	<i>Rationale</i>
1	Four	ELISA and GC-HRMS	To check the background contamination in 16×100 mm sample tubes due to dioxin, furan and dl-PCBs, and to compare the effects of different cleaning procedures	The background contamination should be minimized to match the low detection level (ppt); the agreement between the ELISA and the reference method of GC-HRMS has to be determined.
2	Four	ELISA	To check dioxin background contamination in Pasteur pipettes as well as in beakers	The background contamination should be minimized to match the low detection level (ppt)
3	Five	ELISA	To check the cross-reactivity of dl-PCBs in dioxin ELISA	DL-PCBs have similar chemical structures to dioxins. The cross-reactivity of dl-PCBs has to be determined
4	Six	ELISA by other technologists	To check the recovery of the ELISA method using a sample or a blank with known concentrations (spikes)	According to the requirement of the Ministry of the Environment, the QC samples should be performed to validate an analytical method
5	Seven	ELISA	To check integrity of antibody-coated tubes in an unopened vacuum-sealed pouch from an opened pouch	Antibody-coated tubes from an opened pouch may deteriorate during storage over time. Thus, they have lower reactivity and may underestimate the dioxin concentration in the samples
6	Seven	ELISA	To test antibody deterioration during ELISA operation	The antibody may deteriorate during overnight incubation; the methanol added to each ELISA tube may accelerate the deterioration
7	Seven	ELISA	To optimize the Operating Procedure	Vortex the ELISA tube after addition of methanol produced better mixing effects than tapping the tube, and can minimize the harmful effects to antibodies due to the localized methanol
8	Seven	ELISA	To trouble shoot the expired reagents (conjugate and substrate) and to learn the principles and chemistry of the reagents	Expired reagents may cause inaccurate results due to the reduced activity or the declining of other components

Eight chapters are presented in this thesis. Chapter Two describes the chemical properties of dioxin, furan and dioxin-like PCBs (dl-PCBs) and the analytical techniques. Chapter Three introduces the dioxin ELISA kit manufactured by Cape Technologies. Chapter Four examines the sources of contamination due to the presence of dioxins and dl-PCBs in the background of the laboratory environment. Chapter Five reports the cross-reaction of dl-PCBs in dioxin ELISA. Chapter Six evaluates the recovery of known concentrations of control materials added to the method blank in order to validate the assay. Chapter Seven describes important observations made during ELISA performances, and some technical details that would affect the ELISA results. Finally, conclusions and recommendations are highlighted in Chapter Eight.

Chapter 2. Literature Review

2.1 Dioxins, Furans and Dioxin-like PCBs

2.1.1 Chemical properties

Dioxins and furans are highly toxic organic chemicals that share similar chemical structures (US EPA, 2004). Overall there are 210 congeners, including 75 dioxin congeners and 135 furan congeners (Health Canada, 1990). Out of 210 congeners, 17 congeners, including seven dioxins and ten furans have been considered to be toxic (Environment Canada, 2003). Moreover, 12 out of 209 forms of polychlorinated biphenyls (PCBs) are referred to as dioxin-like PCBs (dl-PCBs), which have the same toxicological properties (MOE, 2007; Tsutsumi *et al.*, 2006). All these toxic congeners of dioxin, furan and dl-PCBs cause a major concern for public health. In this thesis dioxin and furan are referred to as dioxins.

The dioxin molecule consists of two benzene rings joined by two oxygen atoms (Figure 2-1). The furan molecule has the same structure as dioxin, except the benzene rings are joined by one oxygen atom (Figure 2-1). The toxicity depends on the allocation of chlorine atoms on the two benzene rings. The 17 toxic congeners share the characteristic of having chlorine atoms at the 2, 3, 7 and 8 positions on the molecular framework (Environment Canada, 2003). The most toxic form of dioxin is 2,3,7,8-tetrachlorodibenzo-p-dioxin (2,3,7,8-TCDD) (Health Canada, 1990; Health Canada, 2006).

Figure 2-1. General structure of dioxin, furan and dioxin-like PCB molecules (US EPA, 2004)

2.1.2 Environmental fate

Dioxins are primarily released as undesirable by-products by several industrial sources, including combustion of municipal, sewage-sludge, hospital and hazardous wastes, pulp and paper industry, metallurgical industries, accidental fires and spills involving PCBs (Health Canada, 1990; Mukerjee, 1998). Recent studies also show that household garbage burning has become one of the most important dioxin sources in Ontario, Canada (Environment Canada, 2005, Reiner *et al.*, 2006) and in USA (Neurath, 2004).

So far, dioxins and PCBs have been detected in air (Lohmann & Jones, 1998), water, soil (Lorber *et al.*, 2000) and sediment (Custer *et al.*, 2002; Mukerjee, 1998). Furthermore, these chemicals have bioaccumulated in the food chain because of their fat-solubility, including

accumulation in fish, meat, dairy products, and other foods that humans consume (Huwe, 2002).

Dioxins are also long-lived chemicals that degrade very slowly. The half-life of the most toxic form of dioxin, 2,3,7,8-TCDD, is 10-12 years in soil and about 7-11 years in humans (Mukerjee, 1998).

2.1.3 Toxicity

Dioxins and PCBs are hydrophobic (Environment Canada, 2003) and persistent chemicals. They can bioaccumulate in the fatty tissues of animals or humans (Health Canada, 1990), and cause damage due to their toxicity. Generally, the health effects include certain types of cancers, liver problems, disorders involving endocrine, developmental, reproductive and immune systems, as well as the skin disease chloracne (Health Canada, 2006).

Historically, there are two well-known events that indicate the adverse health impacts of dioxin poisoning. One is the dioxin poisoning of Ukrainian President Viktor Yushchenko in 2004. His blood samples were found to contain dioxins at concentrations 1,000 times higher than the acceptable level (Rosenthal, 2004). Consequently, he developed chloracne on his face shortly after being poisoned (Weir, 2005). The other event is the application of Agent Orange, which contains considerable amounts of dioxins, by US troops during the 16-year Vietnam War. Twenty million gallons of Agent Orange were sprayed to remove forest cover, crops and vegetation (Fawthrop, 2004). Decades later, children in Vietnam are still suffering dermatitis and multiple disorders (Fawthrop, 2004).

In the environment, dioxins are typically found as a mixture of congeners (Stahl *et al.*, 1992). The toxicity of the mixed compounds is quantitatively measured in toxic equivalency factors (TEFs) and total toxicity equivalent (TEQ). The TEF of each congener is derived from the

relative potency compared to the most toxic dioxin congener 2,3,7,8-TCDD, which is assigned a TEF of 1 (Environment Canada, 2003). This concept is based on the biochemical and toxic responses of the aryl hydrocarbon receptor (AhR), a cytosolic receptor protein with high affinity towards 2,3,7,8-substituted dioxins (Van den Berg *et al.*, 2006). Based on the TEFs, the total toxicity of a mixture is expressed in TEQ. The TEQ is calculated by summing up each congener concentration multiplied by its TEF value (Van den Berg *et al.*, 2006; Reiner *et al.*, 2006). It is an estimate of the total 2,3,7,8-TCDD-like toxicity of the mixture (Van den Berg *et al.*, 2006). Thus, TEFs and TEQ are applied to quantify the harm due to dioxin exposure in multiple matrices.

Currently, there are two schemes of TEFs and TEQ that are commonly used. One is the international scheme set up by the North Atlantic Treaty Organization (NATO) in 1988 (NATO, 1988a; NATO, 1988b), the other is established by the World Health Organization (WHO). The WHO scheme is better accepted than the NATO scheme because it is based on the studies on mammal system in vivo, chronic effects and toxicological outcomes (Reiner *et al.*, 2006). Thus, the WHO scheme takes into account multiple biochemical and toxicological endpoints whereas the NATO scheme only considers biochemical responses (Reiner *et al.*, 2006). The WHO scheme has assigned TEFs to 7 dioxins, 10 furans and 12 PCBs, ranging from 0.00001 to 1 (Van Overmeire *et al.*, 2001). At this time, Canada mainly uses the WHO scheme.

2.1.4 Exposure Pathways

Humans are exposed to these toxic compounds mostly by ingestion and inhalation (US EPA, 2000). It is reported that food consumption accounts for 90% of dioxin exposure for the general population (Environment Canada, 2007). Thus dioxins in food such as meat, eggs, fish and dairy products should be quantified. Intake of contaminated food should be restricted, especially for

children and pregnant mothers. For example, the dioxin level in fish is 100,000 times that of its surrounding aqueous environment (Otles & Yildiz, 2003). According to the MOE (2007) the consumption restriction for sport fish begins when dioxin levels exceed 2.7 parts per trillion (ppt). That is, the total toxicity of each gram of fish fillet digested, is equivalent to 2.7 picograms (pg) of 2,3,7,8-TCDD, the most toxic form of dioxin.

2.2 Analytical methods

2.2.1 Sample preparation

Dioxins from various environmental matrices should be extracted and cleaned up before detection. Sample preparation includes sampling, extraction, and sample cleanup. Techniques to extract dioxins have been significantly improved in the last decade (Lopez-Avila, 1999; Giergielewicz-Możajska *et al.*, 2001; Nichkova *et al.*, 2004; Reiner *et al.*, 2006), and many fast, safe and environmentally friendly methods have been developed. Following extraction, the cleanup procedure is conducted to remove co-extractable and interfering compounds (Reiner *et al.*, 2006). Effective cleanup is a significant and challenging step. So far, many techniques, such as Gel Permeation Chromatography (GPC) (US EPA, 1994; Reiner *et al.*, 2006), High Performance Liquid Chromatography (HPLC) (US EPA, 1994), and multiple-column (Shan *et al.*, 2001; Sugawara *et al.*, 2002; Nichkova *et al.*, 2004), have been used for sample cleanup. However, the removal of fatty tissue and other interfering compounds for the ELISA method still remains to be resolved (Chen, E., Master Thesis, Submitted, 2008).

2.2.2 Detection methods

Dioxins are detected at three levels for different matrices in the environment. For soil samples, the detection level is at parts per billion (ppb) (1 part in 10^9), for food samples it is at parts per trillion (ppt) (1 part in 10^{12}), and for water samples it is at parts per quadrillion (ppq) (1 part in 10^{15}). The detection methods, including the reference method of GC-HRMS and alternative biological methods are described below.

2.2.2.1 Reference method of GC-HRMS

GC-HRMS has been used for dioxin detection since the 1970s (Crummett & Stehl, 1973; Reiner *et al.*, 2006), and was approved for use as US EPA Method 1613. This method can accurately quantify the amount of each congener at ppt to ppq concentrations in any sample matrix (Reiner *et al.*, 2006).

However, this method requires complicated and time-consuming sample preparation, expensive equipment (up to \$1 million each) with high associated maintenance costs (Nichkova *et al.*, 2004; Sugawara *et al.*, 2002). Furthermore, analysts trained at a PhD level are needed to operate the equipment (Nichkova *et al.*, 2004). Therefore only a few laboratories in the world can afford to perform such analyses (Schechter, 1998).

Thus the increasing demand for large-scale dioxin determination has promoted the development of alternative techniques. These new alternatives are primarily bioanalytical methods that cost less and have quicker processing times.

2.2.2.2 Alternative methods

These techniques include Ah Receptor-based assays and immunoassays (Billets, 2005). The two major Ah receptor-based assays are Chemically Activated Luciferase Gene Expression (CALUX) and Aryl hydroxylase Receptor Complex Polymerase Chain Reaction (AhRC PCRTM).

➤ CALUX

Chemical-Activated Luciferase Expression (CALUX) is a reporter gene assay that uses genetically modified cell cultures that respond to chemicals that activate the aryl hydrocarbon receptor (AhR) (Windal *et al.*, 2005). This method uses a recombinant mouse hepatoma (H1L6.1c3) cell line that has been inserted with a firefly luciferase reporter gene under the control of the AhR gene (Xenobiotic Detection Systems, Inc., 2004). This cell line is used to detect dioxins with high sensitivity, and to quantify AhR agonists and agonist activity of complex mixtures (Windal *et al.*, 2005). The compound 2,3,7,8-TCDD is used as one of the most potent activators of the AhR effects (Xenobiotic Detection Systems, Inc., 2004).

The measurement of CALUX is based on the luciferase activity, which is proportional to the amount of dioxin-like compounds in the environmental sample (Clark *et al.*, 2001). The bioluminescence emitted is measured by a luminometer (US EPA, 2002a). The dioxin or dioxin-like TEQ activity can be estimated based on the standard curve of luciferase activity versus dioxin concentration.

CALUX method has three modes for sample analysis: (1) screening mode with historical recovery by processing only one aliquot, (2) screening mode with surrogate recovery by processing two aliquot, and (3) semi-quantitative mode, which required processing four aliquots (US EPA, 2008). The analytical costs depend on which mode is chosen for analyses, which in

turn depends upon different levels of sample contamination (US EPA, 2008).

So far CALUX has been employed to analyze various experimental samples such as soil, milk, food and feed (Clark *et al.*, 2001; Van Overmeire, *et al.*, 2004; Hoogenboom *et al.*, 2006). Recently, this method has been accepted as US EPA method 4435 (US EPA, 2008).

➤ Real-time PCR

Real-time PCR is another biological technique. Commercially available kits have been developed, such as Aryl hydroxylase Receptor Complex PCR (AhRC PCRTM) from Hybrizyme Corporation and percept® Rapid Dioxin Assay from Eichrom Technologies, Inc. This technique can investigate the interaction between the dioxin molecules and the Ah receptors in the cell lines, and can measure an actual biological response (US EPA, 2005a).

According to the US EPA (2007), the general principle is as follows. Dioxin molecules are used to activate the AhR proteins extracted from the mammalian cell lines. The activated AhR forms an activated receptor complex with a specific DNA response element. The DNA element along with the activated complex is bound to the surface of a well. The bound DNA was then amplified and measured by the technique of real-time PCR. It is also indirectly measuring the amount and the toxic potency of the dioxins (US EPA, 2007).

By December 2007, the method developed by Eichrom Technologies Inc. has been approved by US EPA as Method 4430 (US EPA, 2007)

➤ Immunoassay

Immunoassay is a biological technique based on the reaction between an antibody and its antigen. In this technique, the antibody can recognize the specific site on the antigen and bind to it. Both the presence and quantity of the antibodies or the antigens can be measured. Immunoassay has several features, such as selectivity, sensitivity, portability, rapid turnaround time and very low detection limits (Lesnik, 2000), which are all desirable attributes in environmental applications. Thus it has also developed as a useful tool for environmental monitoring (Plaza *et al.*, 2000).

In terms of dioxin detection, immunoassay has been designed to have a correlation between TEF and congener recognition. The antibody has the strongest preference towards 2,3,7,8-TCDD, the most toxic congener with a TEF of 1, and has less affinity to other less toxic congeners (US EPA, 2004). Thus immunoassay is an ideal method to test the overall toxicity of mixed compounds. It can also be used as an ideal method for preliminary sample screening and as a semi-quantitative technique prior to the GC-HRMS analysis on the same sample (Roy *et al.*, 2002).

So far, radioimmunoassay (Albro *et al.*, 1979; Kennel *et al.*, 1986) and enzyme-labeled immunoassay (Harrison & Carlson, 1999; Shan *et al.*, 2001; Roy *et al.*, 2002; Sugawara *et al.*, 2002) have been reported to detect dioxins. Radioimmunoassay uses radiolabeled isotope and detect the analyte concentration by measuring the radioactivity (Harrison & Eduljee, 1999). But this method would generate radioactive wastes, which may cause concern in term of potential health hazards to people, the problem of disposal, and release of radioactivity into the environment (Maggio, 1983; Harrison & Eduljee, 1999). On the other hand, using the enzyme as the label is much simpler and safer, and the enzyme activity can be related to the concentration of

the analytes (Maggio, 1983). Thus enzyme-labeled immunoassay is more popular (Maggio, 1983).

There are two types of enzyme-labeled immunoassay depending on whether the antigen is soluble or insoluble (Maggio, 1983). When detecting insoluble antigen present on the cell surface, the cell can be fixed on a microscope slide or plastic surfaces. Then the antigen can be detected directly by an enzyme-labeled antibody or indirectly by first a unlabelled antibody followed by a labeled anti-antibody (Roitt, 1973). The enzyme-labeled antibody is referred to as an enzyme-linked conjugate (Maggio, 1983). In the case of soluble antigen, the antigen must be captured and immobilized first. This is usually accomplished by a first antibody adsorbed to a plastic surface (Roitt, 1973). After capture, the antigen is detected directly or indirectly as described for the insoluble antigen. The first capturing antibody is referred to as an Immunosorbent (Maggio, 1983). Thus, the antigen capture immunoassay is generally referred to as enzyme-linked Immunosorbent assay (ELISA).

When the antigen is a large molecule, such as a virus or a protein, the first and the second antibody can bind at the same time (David, 1997). This is called as a sandwich ELISA because the antigen is sandwiched between two antibodies (Roitt, 1973). However, small antigens, such as dioxins, are entirely engulfed by the first antibody, and there is no room for the second antibody to bind (David, 1997). In this case, detection is possible only by a competitive ELISA. The principle of competitive ELISA will be elaborated in the next chapter.

ELISA is a powerful immunological method and the most commonly used immunochemical technology for environmental analyses (Lesnik, 2000). This technique can process samples in batches. More than 10 samples can be analyzed per batch each day. Thus, in comparison to GC-HRMS, which analyzes one sample at a time, ELISA is much more time effective. ELISA was

first validated for dioxin analysis in 1999 in soil samples (Harrison & Carlson, 1999). It became an official method three years later (US EPA, 2002b).

In this study, the rationale for choosing immunoassay among these biological methods is as follows. Both CALUX and AhRC PCRTM are bioassays based on the Aryl hydroxylase receptor. Both methods require tissue cultures to provide active cell lines, which are inherently difficult to maintain and work with. Firstly, the technique of cell line cultivation is time-consuming, labour-intensive, and demands high quality culture media, cultivation equipments and daily attention. Secondly, it is not easy to control the timing of the culture to attain a growth phase suitable for sample testing. Thirdly, mycoplasma contamination of the cell line is a frequent problem. On the other hand, immunoassay technology, such as ELISA, is developed based on the principle of antibody and antigen. It is much simpler and more economical compared to cell line cultivation.

In this study competitive ELISA was chosen as the tool to measure dioxin TEQ. It can quantify dioxins based on the competition between target analyte (dioxin in the sample) and an artificial conjugate consisting of dioxin-linked enzyme.

Chapter 3. Competitive ELISA

The competitive ELISA employed in this study utilized the test kit from Cape Technologies, which has been granted official status as US EPA Method 4025 "Screening for Polychlorinated Dibenzodioxins and Polychlorinated Dibenzofurans (PCDD/Fs) by Immunoassay" in October 2002 (US EPA, 2002). This method specification was to analyze dioxin and furan in soil samples, with detection levels of 100 pg/g soil and greater (US EPA, 2002).

3.1 Comparison of ELISA kits from two suppliers

Two corporations supply commercial dioxin ELISA kits. They are Wako Pure Chemical Industries, Ltd and Cape Technologies LLC. Both methods can significantly reduce the analytical costs and are time-effective. In this study, Cape Technologies' dioxin ELISA kit was selected over Wako's because the former has been approved as an official US EPA method (Method 4025) but not the latter.

3.2 Cape ELISA kit

"High Performance Dioxin/Furan (D/F) Immunoassay Kit" catalogue # DF1-60 manufactured by Cape Technologies was employed for this study. This kit provides Antibody-coated tubes, as well as the reagents, including Sample Dilution, Competitor-Horseradish Peroxidase (HRP) Conjugate, HRP Substrate Solution and Stop Solution, one vial of neat Triton X-100, and two vials of "PEG-Triton Keeper Stock" solution (Triton X-100 in polyethylene glycol).

The vial of Triton X-100 was diluted 10,000 times in deionized distilled water (DDW) and used as a wash solution. PEG-Triton keeper stock was diluted 5 times in methanol and added to

the samples to trap dioxin molecules.

3.3 Cape ELISA Principle

Figure 3-1. Principle of competitive ELISA

The principle is that the target analyte (dioxin in the sample) and an artificial conjugate made up by linking an enzyme to the analyte (dioxin-enzyme conjugate) compete for binding sites on antibodies that are immobilized on the surface of the bottom portion of a test tube (Figure 3-1). More target analyte molecules occupying the binding sites of the antibodies will lead to less binding sites available for dioxin-enzyme conjugate (US EPA, 2005b). A solution of peroxide substrate and colorless chromogens is then added. The enzyme on the conjugate cleaves the peroxide into H_2O and O^- . The chromogens are oxidized by the O^- ion to a colored form (Meiattini, 1977). This color change allows the spectrophotometric measurement of absorbance, which is inversely proportional to the analyte concentration.

3.4 Curve fitting methods

For the purpose of constructing a calibration curve, calibrators consisting of various concentrations of 2,3,7,8-TCDD standards are purchased from Wellington Laboratory and henceforth referred to as Wellington Standards. CAPE Technologies uses a four-parameter equation to fit the calibration curve (Harrison & Carlson, 1999). Values for the four parameters are calculated with the “Solver” function available in the MS Excel software. The empirical four-parameter equation is as follows:

$$\text{Equation [1]. } Y_{\text{pred}} = \frac{(A - D)}{\left[1 + \left(\frac{X}{C} \right)^B \right] + D}, \text{ where}$$

X = pg per ELISA tube (2,3,7,8-TCDD for Wellington Standards, TEQ for samples)

Y_{pred} = normalized ELISA response (OD as % of NC OD)

A = Y value of upper asymptote

B = relative slope of middle region

C = X value at midpoint of curve

D = Y value of lower asymptote

The original A, B, C, D values are given based on empirical knowledge or previous experiments. The curve based on the original A, B, C, D values is shown as the dashed curve in Figure 3-2. The concentration of dioxin in pg is plotted on the x-axis. Plotted on the y-axis is the percent inhibition of each calibrator concentration, which is the optical density (OD) of that calibrator, divided by the OD of the negative control (NC) and multiplied by 100. This dose-response calibration curve is reverse-sigmoidal in shape by nature of competitive ELISA.

Figure 3-2. ELISA original calibration curve provided by CAPE Technologies and Wellington Standard data points before curve fitting by the solver function

After absorbance of the 6 calibration points is put in, the Y values, which are the percentage of the negative control for each Wellington Standard, can be calculated. The “Solver” function in MS Excel (Billo, 2001) can then be utilized. The “Solver” is an iterative procedure that minimizes the “sum of squares of the differences” ($\sum[y-y_{\text{pred}}]^2$) between Y and Y_{pred} by changing the A, B, C and D values. Thus, a new set of values for the four parameters (A', B', C' and D') with the smallest Solver value is calculated automatically.

Figure 3-3. ELISA calibration curve by four-parameter fitting after solver function

The four new parameters (A', B', C' and D') allow for the plotting of a new calibration curve (Figure 3-3) based on the following equation

$$\text{Equation [2]. } Y = \frac{(A' - D')}{\left[1 + \left(\frac{X}{C'}\right)^{B'}\right]} + D', \text{ where,}$$

X = pg per ELISA tube (2,3,7,8-TCDD for Wellington Standards, TEQ for samples)

Y = new normalized ELISA response (OD as % of NC OD)

A' = new Y value of upper asymptote

B' = new relative slope of middle region

C' = new X value at midpoint of curve

D' = new Y value of lower asymptote

The quality of the calibration curve is assessed by three indicators, namely, (1) the sum of squares of differences ($\sum[y - y_{\text{pred}}]^2$), (2) the 50% inhibition point (I_{50}) and (3) the 85% inhibition point (I_{85}). First, the value of $\sum[y - y_{\text{pred}}]^2$ indicates how good the calibration curve (observed Y) fits to the predicted curve by the four parameter formula. Hence, $\sum[y - y_{\text{pred}}]^2$ is a measure of the experimental fit of the calibration curve obtained from each routine ELISA run. Second, the I_{50} is the midpoint of the standard curve and is defined as the concentration that gives 50% of the OD of the NC (CAPE Technologies, 2003a). The I_{50} varies for each different ELISA experiment. The range of I_{50} values provided by the manufacturer is 21.9 ± 7.4 pg/tube (mean $\pm$ SD). When $I_{50} < 14.5$ pg, the ELISA is oversensitive because a small amount of dioxins brings about 50% inhibition of the NC. When $I_{50} > 29.3$ pg, the ELISA is under-sensitive because a higher amount of dioxins is required to bring about 50% inhibition of the NC. When the I_{50} value falls within the range of 14.5-29.3 pg, the ELISA is considered accurate as the sensitivity is within the correct range. Therefore, I_{50} is a measure of the accuracy of the ELISA. The third indicator is I_{85} , which is the concentration giving 85% of the negative control OD. This is a measure of the

capability to respond to a small concentration of dioxin with signal strength at least 15% above baseline. The range of I_{85} is 3.9 ± 1.4 pg/tube (Cape Technologies, 2003a).

3.5 US EPA SITE Program evaluation

In 2004, the US EPA launched the Superfund Innovative Technology Evaluation (SITE) Program to evaluate several biological methods for dioxin detection, by using GC-HRMS results as a reference (Billets, 2005). Dioxin ELISA from Cape Technologies was evaluated in this program. In total, 209 soil, sediment and extract samples were analyzed for dioxin. The reference laboratory spent 8 months analyzing all the samples at a cost of \$398,029 USD (Billets, 2005), which equals approximately \$1900 USD per sample. By using dioxin ELISA kits from CAPE Technologies, all 209 samples were analyzed within 3 weeks by a single operator at a cost of \$59,234 USD (Billets, 2005). This amounts to \$283 USD per sample. In terms of accuracy, the mean recovery was from 160% to 236%, with mean relative standard deviation of 71% to 123% (Billets, 2005). Additionally, ELISA is field portable and it requires less skill to perform compared to GC-HRMS (Billets, 2005).

3.6 Accuracy and Sensitivity at low detection levels

The detection limit of this kit is around 4 pg 2,3,7,8-TCDD per ELISA tube (Cape Technologies, 2003a). Using Cape dioxin ELISA, the calibration curve ranges from 0 to 100 picograms (pg).

The assay is capable of detecting dioxins at all 3 logs (ppb, ppt and ppq) of detection necessary for environmental matrices. This is achieved by adjusting the sample size used for detection. For example, 1 g of soil, 10 – 20 g of fish, and 10 – 100 L of water would be used. By using the appropriate sample amount, the final dioxin extract can be assayed by the same ELISA

with a standard curve ranging from 0 – 100 pg 2,3,7,8-TCDD. The accuracy of the assay at all 3 logs of detection levels is therefore entirely dependent on the accurate construct of one and the same calibration curve. In other words, lowering the detection level is not associated with the need to increase the sensitivity of the assay. Regarding assay development, this is an important principle not found in the literature review.

Chapter 4. Sources of background contamination

4.1 Preamble

Trace detection levels of ppt and ppq are required for food or drinking water samples. Therefore the laboratory background contamination should be low enough to meet such requirements. Meeting such demanding requirements is no trivial matter. Various cleaning procedures have been developed for the glassware that is most frequently used in laboratory analysis, and the background level of the solvents used for sample extraction, cleanup and glassware cleaning have also been tested.

4.2 Materials and Methods

4.2.1 Reagents and Equipment

Solvents used in this research included dichloromethane (DCM), toluene and hexane (Caledon Laboratories Ltd., Ontario, Canada).

Lab ware included 14mL borosilicate glass, culture tubes (16×100 mm disposable, Catalog No. 47729-576, VWR International Inc. Mississauga, Ontario, Canada), disposable borosilicate glass Pasteur pipettes with short tip (Catalog No. 14673-010, VWR International Inc. Mississauga, Ontario, Canada) and beakers (Catalog No. 89000-208VWR, Griffin low-form beakers with double-capacity scale, borosilicate glass, and Catalog No. 89003-788, KIMAX brand Griffin Beakers, borosilicate glass, VWR International Inc. Mississauga, Ontario, Canada).

Equipment included a Digital Dry Bath (Models D1100 and D1200, Catalog No. NLD 1200, Labnet International Inc., Woodbridge, New Jersey, USA); a Nitrovap Nitrogen Generator (Model NITROVAP-2LV, Catalog No. 195936, Fisher Scientific, Markham, Ontario, Canada); a

Sorvall Tabletop Centrifuge (Model Legend RT, Catalog No. SVH-75004377, Kendro Laboratory Products, USA); a Vortex Mixer (Model VX-100, Catalog No. NLS0100CAN, Labnet International Inc., Windsor, Berkshire, United Kingdom); a MicroChemTM Photometric Analyzer (Abraxis LLC, Warminster, PA, USA); Repeater[®] plus pipettes (Catalog No. 213809, Brinkmann Instruments Inc. Westbury, New York, USA) and Combitips Plus 10 mL (Catalog No. 2226650-1, Brinkmann Instruments Ltd. Canada), and Glass capillary microdispensers (Drummond series 500 Digital Microdispensers: 10 μ L capacity, Catalog No. 3-000-510; 25 μ L capacity, Catalog No. 3-000-525; 50 μ L capacity, Catalog No. 3-000-550; 100 μ L capacity, variable, Catalog No. 3-000-575, Drummond Scientific Company, Broomall, PA, USA).

ELISA kits (High Performance Dioxin/Furan Immunoassay Kit, Product Number DF1-60) were manufactured by CAPE Technologies (South Portland, ME, USA). Sets of 2,3,7,8-TCDD calibration standards (DF1-SK2) were purchased from Wellington Laboratories Inc. (Lot No. 071800; Guelph, Ontario, Canada). These standards were referred to as Negative Control (NC), S₁, S₂, S₃ and S₄, with concentrations of 0, 0.064, 0.2, 0.64 and 2.0 ppb respectively.

4.2.2 Lab ware cleaning

The lab ware tested included 16×100 mm borosilicate glass tubes, Pasteur pipettes, and beakers. Various procedures were employed to clean glass tubes (Table 4-1) and Pasteur pipettes (Table 4-2), either by using DCM to rinse or using toluene to soak. Glass tubes that were used for dioxin-like PCBs detection were cleaned by procedure C described in Table 4-1.

Glass beakers were detergent washed in the dishwasher and autoclaved to dry. Then 20 mL DCM was used to rinse each dried beaker and the rinses were collected and tested by ELISA. To evaluate manual cleaning, two beakers were detergent cleaned by hand, after which 20 mL of

DCM was used to rinse each beaker.

Table 4-1. Four different cleaning procedures for 16×100 mm glass tubes

<i>Procedure</i>	<i>Solvent</i>	<i>Cleaning procedure</i>
A	Dichloromethane	1mL DCM was added to each glass tube taken directly from its original package. The collection of DCM rinses was tested by ELISA.
B	Dichloromethane	A second 1mL of DCM was added to each glass tube to rinse them for a second time. These DCM rinses were also collected and tested by ELISA.
C	Toluene	Toluene was used to soak tubes in a glass beaker overnight, after which they were dried in a dry bath at 60°C. 1 mL DCM was used to rinse each tube. The collected DCM rinses were tested by ELISA.
D	Toluene	Toluene was used to soak for a second time overnight. Then 1 mL DCM was used to rinse each of the tubes. The DCM rinses were collected and tested by ELISA.

Table 4-2. Three different cleaning procedures for Pasteur pipettes

<i>Procedure</i>	<i>Solvents</i>	<i>Cleaning Procedure</i>
E	Dichloromethane	Each 16×100 mm tube was filled with 10mL DCM. New Pasteur pipettes were taken straight out of the original package, and were not pre-washed. A rubber bulb was attached to a pipette. The pipette was immersed in the tube and the bulb was squeezed 10 times for each pipette. Ten pipettes were cleaned in each batch in the same 10 mL of DCM, which was subsequently tested by ELISA.
F	Dichloromethane	After dust was removed by the first DCM rinse in procedure E, the batch of 10 pipettes were washed for the second time by repeating procedure E and evaluated by ELISA.
G	Toluene	Pasteur pipettes were soaked overnight in a beaker filled with toluene, and were dried in a dry bath. A 16×100 mm tube was filled with 10mL DCM. A rubber bulb was attached to a pipette. The dried pipette was immersed in the tube and the bulb was squeezed 10 times. 10 pipettes were cleaned in a batch. The 10 mL DCM were tested by ELISA.

The DCM used for cleaning glassware was collected and 62.5 μL of PEG-Triton keeper was added. The solutions were mixed by gently vortex and then evaporated on the dry bath. Since the boiling point of DCM is 40°C (CIS, 2000), the dry bath temperature was set at 35°C for DCM evaporation. The nitrogen gas from a nitrogen generator was used to help the solvents evaporate. The nitrogen gas was blown on top of the tube containing the DCM, in order to blow away the evaporated solvent vapor. The solutions were evaporated in the dry bath until no solvent odor remained. The efficiency of the cleaning procedures was determined by ELISA and/or GC-HRMS by testing the DCM rinses from the cleaned tubes.

The dioxin background of the various chemical solvents used through out the study was also tested. Fourteen mL of solvent (Table 4-5) was poured from its original amber bottle directly into a 16×100 mm glass tube. Several amber bottles for each type of solvent were checked. The solvent in each glass tube was mixed with 62.5 μL of PEG-Triton keeper and evaporated on the dry bath with nitrogen gas blowing until no odor remained. The evaporation was followed by ELISA detection.

4.2.3 Detection methods

➤ ELISA method

The competitive ELISA is performed according to the manufacturer's protocol (Cape Technologies, 2003a). The procedure is described briefly below.

A series of Wellington Standards were used to gage the ELISA performance. Fifty μL of each standard (NC-S4) were added into corresponding antibody-coated tubes containing 500 μL of sample diluent. Antibody incubation lasted between 2 hours and overnight. Tubes were washed four times with wash solution (0.01% v/v, Triton X-100) in order to remove unbound analytes.

Then 500 μL of conjugate solution were added into each tube for incubation. After 15 minutes of conjugate incubation, the tubes were washed four times with DDW to remove excess, unbound conjugate. The wash was followed by adding 500 μL of substrate into each tube and the substrate incubation occurred in the dark for 30 minutes. Finally, a stop solution (1N hydrochloric acid) was added to halt color development before taking absorbance readings (OD units at 450 nm) with a spectrophotometer (Cape Technologies, 2003a). All the reagents were dispensed directly from the reagent bottles into ELISA tubes by using the Repeater[®] plus pipettes with dedicated tips. The whole immunoassay procedure was done at room temperature.

All the ELISA tests were performed in the bioassay laboratory at the Laboratory Services Branch, MOE.

➤ GC-HRMS method

After ELISA detection, the background levels of dioxins and dl-PCBs in the 16×100 mm glass tubes were further confirmed by GC-HRMS method.

GC-HRMS can determine the concentrations of dioxins and PCBs using isotope dilution. All samples are fortified with known amounts of $^{13}\text{C}_{12}$ - isotopically labeled dioxins or PCBs (MOE, 2006a). The difference in mass distinguishes the spectrum of the labeled standards from the spectrum of the unknowns. The dioxins or PCBs in the sample are quantified based on the recovery of the labeled standards (MOE, 2006a).

The instrumental analysis by GC-HRMS was performed according to US EPA method 1613 (US EPA, 1994) and MOE method 3418 (MOE, 2006a). The entire analysis was performed by staffs in the Dioxin and Toxic Organics Section, Laboratory Services Branch, MOE.

4.2.4 Data analysis

The software Minitab Student Release 12 for Windows (Minitab, Inc.) was used for the data analysis. The assumption that data points were normally distributed was tested by Ryan-Joiner analysis. When normal distribution held true, the difference between the arithmetic means was evaluated by a one-tail Student's t-test. When data were not normally distributed, nonparametric statistics were employed to assess the difference between the population medians. The difference between the two medians was assessed by a one-tail Mann-Whitney test.

4.3 Results and Discussion

4.3.1 Background testing

4.3.1.1 Dioxin background contamination level

➤ **Glass tubes 16×100 mm**

Glass tubes (16×100 mm) were the most frequently used lab ware in dioxin ELISA preparation. They were used either to hold sample extracts or to hold solvents. Results (Table 4-3) show that the background was approximately 0.33 pg per tube after the first DCM rinse, which was easily reduced to an average of 0.05 pg per tube by a second DCM rinse. Also, the standard deviation (SD) became lower in the second rinse indicating better repeatability. The reduction occurs because the first rinse of sample tubes may contain dust, which can be a major contributor to the dioxin background. The results of the second DCM rinse suggest that most of the dust has been removed by the first DCM rinse. Surprisingly, the toluene-washed tubes, which were expected to have lower background noise, were a lot dirtier than the DCM rinsed tubes (0.6~1 pg/tube). ELISA results for solutions soaked either once or twice in toluene indicate that toluene

introduced more background contamination, which was not acceptable for low-level detection.

Both parametric and nonparametric statistics were employed to assess the difference between the results obtained from the four cleaning procedures A, B, C and D. The background level obtained by the second DCM rinse (Procedure B) reduced the contamination level obtained by the first DCM rinse (Procedure A). A one-tail Mann-Whitney test demonstrated that the difference between procedures A and B was significant ($P=0.0003$). Furthermore, a one-tail Student's T test showed that the differences between the background levels obtained by rinsing with DCM twice (procedure B) and by soaking in toluene (Procedure C and D) were significant ($P=0.0000$ and $P=0.0007$ respectively).

Therefore, the cleaning procedure for the 16×100 mm glass tubes can be simplified. ELISA only requires glass tubes to be rinsed with DCM twice, whereas GC-HRMS always requires toluene washed glassware, which is time-consuming and generates a large amount of environmentally unfriendly solvent wastes.

Table 4-3. Dioxin background in 16×100 mm glass tubes by ELISA

<i>Number of Batches</i>	<i>Number of Tubes per Batch</i>	<i>16×100 mm Tube Cleaning Procedure*</i>	<i>ELISA Results pg TEQ per Unit [mean ± 1 SD (%CV)]</i>
15	25	A	0.33±0.75 (227.62%)
15	24	B	0.05±0.04 (91.65%)
8	20	C	1.03 ± 0.35 (34%)
4	25	D	0.60 ± 0.11 (18%)

*A, B, C, D stand for the cleaning procedures described in Table 4-1

➤ Glass Pasteur pipettes

In the same manner, the three cleaning procedures described in Table 4-2 were employed to clean the glass Pasteur pipettes. Table 4-4 shows the results of the background level of Pasteur pipettes by the three different cleaning procedures. The Pasteur pipettes cleaned by procedure E and G were both approximately 0.2 pg TEQ individually, while the results obtained by the second DCM rinse (procedure F) were 0.05 pg less than the other two. A Mann-Whitney test demonstrated that this difference (0.05 pg on average) is not statistically significant ($P=0.3238$). The three procedures did not significantly affect the background level of Pasteur pipettes. However, the method of DCM rinsing is more simple and time-efficient. Therefore, for practical benefits, procedure F is preferred.

Table 4-4. Dioxin background TEQ in glass Pasteur pipettes by ELISA

<i>Number of Batches</i>	<i>Number of Pipettes per Batch</i>	<i>Lab ware Cleaning Procedure*</i>	<i>ELISA Results pg TEQ per unit [mean $\pm$ 1 SD (%CV)]</i>
12	10	E	0.20 $\pm$ 0.18 (87.87%)
12	10	F	0.15 $\pm$ 0.11 (77.48%)
3	10	G	0.205 $\pm$ 0.02 (11%)

*E, F, G stand for the cleaning procedures described in Table 4-2

➤ Glass beakers

Glass beakers were also tested for background contamination. The dishwasher could clean the beaker to less than 1 pg per beaker in most cases. The background level of the two hand-washed beakers was 0.05 pg on average, which was cleaner than the other beakers. However the hand washing work was very time-consuming. Therefore, the dishwasher cleaning was chosen as

the procedure to clean all of the beakers.

➤ Solvents

Solvents including hexane, toluene and DCM taken directly from their original amber bottles were tested by ELISA. The results (Table 4-5) show that the average background level was approximately 0.1 pg per mL of solvent. If 40 mL of organic solvent were used for sample extraction or sample cleanup, the total background would be around 4 pg, which was comparable to the detection limit. Therefore the acceptable range of the solvent background should be less than 0.1 pg/mL. Results also showed about 50% - 60% of hexane and toluene, and only 17% of DCM tested was within the acceptable range. The solvents represent different bottles from the same brand. The solvents that did not pass the test could only be used to test samples with high dioxin levels, such as soils or sediments.

Since the background contamination derived from chemical solvents cannot be eliminated from the method, this problem should be taken into account. In future, it is recommended that solvents from other chemical manufacturers should be evaluated in order to find the cleanest supplier or chemical lots for our purposes.

Table 4-5. Dioxin background in solvents by ELISA

<i>Solvents</i>	<i>Number of Batches</i>	<i>Mean $\pm$ SD (%CV) (pg TEQ/mL)</i>	<i>Percentage within Acceptable Range (<0.1 pg/mL)</i>
Hexane	13	0.123 $\pm$ 0.094 (76.5%)	66%
Toluene	13	0.085 $\pm$ 0.04 (47.1%)	50%
DCM	12	0.147 $\pm$ 0.189 (128.8%)	17%

4.3.1.2 Dioxin-like PCB (dl-PCB) background in 16×100mm glass tubes

The background level of dioxin and dl-PCBs in four batches of 16×100mm glass tubes, 25 tubes per batch, cleaned by procedure C (Table 4-1) were determined by GC-HRMS as well as by ELISA. This was accomplished by splitting the final DCM rinses into two equal portions, one portion for GC-HRMS, the other for ELISA. GC-HRMS detected large amount of PCBs background contamination in all four batches of tubes (Table 4-6). The background levels of dl-PCB congeners PCB123 and PCB 81 in each batch were up to thousands of picograms. Obviously tubes with such high levels of PCB background could not be used for GC-HRMS purpose.

The high concentrations of PCBs in these tubes were further translated to TEQs. The TEQs of PCB backgrounds were less than 0.1 (last row in Table 4-6). These dl-PCB TEQs were negligible compare to the TEQs due to dioxin as determined by GC-HRMS (Table 4-7). Based on this observation, it can be hypothesized that tubes contaminated with high levels of PCBs, unacceptable for GC-HRMS use, are still acceptable for ELISA use. This hypothesis would be valid assuming that the PCBs are transparent to the ELISA specifically designed for dioxin detection. This assumption was proven correct (Section 4.3.2), and subsequently confirmed in other experiments (Section 5.3).

Table 4-6. Dioxin-like PCB (dl-PCB) background in 16×100mm glass tubes by GC-HRMS

<i>PCB congener</i>	<i>TEF</i>	<i>PCB (pg) in 25 Glass Tube Batch</i>			
		<i>Batch I</i>	<i>Batch II</i>	<i>Batch III</i>	<i>Batch IV</i>
PCB 167	0.00001	510	418	442	553
PCB 118	0.001	0	0	0	0
PCB 105	0.001	0	0	0	0
PCB 126	0.1	0	0	0	0
PCB 123	0.0001	3231	2520	2601	3283
PCB 81	0.0001	5662	0	4530	5882
PCB 77	0.0001	0	4381	0	0
Total PCB TEQ per tube		0.07	0.06	0.06	0.08

4.3.2 Comparison between ELISA and GC-HRMS

Dioxin TEQ results from both GC-HRMS and ELISA methods were compared (Table 4-7). Paired T-test indicates that the two methods agree well at the 99% confidence level ($t = -0.89$; $P = 0.424$), although the sample size was small. It is remarkable that TEQ determined by both methods are in significant agreement at the ultra-trace level of a fraction of 1 TEQ.

The desire to increase the sample size was prohibited by two factors. Firstly, each GC-HRMS run costs upwards of a thousand dollars. It was very expensive to test empty tubes rather than a real sample. Secondly, tubes containing high levels of PCBs, although transparent to ELISA, created major problems for the GC-HRMS system since the system required a thorough flushing of the PCB contaminants.

At this point, the hypothesis that dl-PCBs are transparent to dioxin ELISA has been validated, and it was confirmed further by spiked experiments described in Chapter Five. That is to say, test tubes contaminated with high levels of PCBs background did not result in false dioxin TEQs.

Thus, ELISA method could tolerate “dirty” glassware that is intolerable by GC-HRMS. In practice, this means ELISA method drastically reduced the elaborate cleaning requirements and chemical solvents demanded by GC-HRMS. Henceforth, the cleanliness of all glassware for ELISA purpose was checked by ELISA only. GC-HRMS confirmation of cleanliness is unnecessary and irrelevant.

Table 4-7. Comparison of ELISA TEQ per tube with GC-HRMS TEQ

<i>Tube Batch</i>	<i>ELISA TEQ</i>	<i>GC-HRMS dioxin TEQ</i>	<i>GC-HRMS PCB TEQ</i>
<i>I</i>	0.6	0.86	0.07
<i>II</i>	0.8	0.65	0.06
<i>III</i>	0.5	0.64	0.06
<i>IV</i>	0.7	0.88	0.08
<i>V</i>	0.08	0	0

4.3.3 Dust contamination

During glassware background checking, it was discovered that dust contamination originated from the dirty air in the fume hood. Specks of dust particles were observed to have collected overnight on the fume hood sash (Figure 4-1 Left) and on the tin foil covering the beakers (Figure 4-1 Right). Repair work on the air system throughout the building might have caused this dust contamination.

Dust contamination was consistent with the observation that the background became 10~100 times higher after prolonged exposure to dirty air during experiments. Tubes rinsed with DCM outside of the fume hood were cleaner than tubes washed inside of the fume hood at that time. Furthermore, during solvent evaporation, the samples were unprotected from falling dust, so

there was no guarantee of background levels of contamination even after the glassware had been proven clean.

Figure 4-1. White dust collected overnight on the fume hood sash (Left) and on the beaker tin foil cover (Right)

4.4 Summary

Background contamination levels of dioxin and dl-PCBs were investigated. The results showed that all unwashed lab ware tested had measurable dioxin backgrounds. Compared to the detection limit, the unclean glassware was not acceptable for trace level detection for dioxins. This indicated that all lab ware must be washed before use.

Various cleaning procedures were developed to reduce the background contamination. Statistically significant differences between those procedures were observed. Consequently, simple but effective procedures were chosen for each type of glassware. Dust contamination was also considered as a contributing factor to the background contamination. A further comparison between the lab ware test results from ELISA and GC-HRMS showed that the TEQ values of the two methods agreed.

Chapter 5. The cross-reactivity of PCBs in dioxin ELISA

5.1 Preamble

Twelve out of the possible 209 congeners of PCBs are assumed to be dioxin like (Tsutsumi *et al.*, 2006). Their molecular structures are similar to dioxin when chlorine atoms occupy both para positions and at least two meta positions; usually there is no more than one chlorine atom in the ortho positions (Winters, 2003).

Due to this similarity in chemical structure, dioxin-like PCBs (dl-PCBs) may potentially bind to the antibody coated on the tube and cross-react in dioxin ELISA. In this chapter the question of whether or not PCBs cross-react or contribute to the dioxin immunoassay signal was investigated. An overload of PCB mixtures or pure dl-PCB congeners was used to challenge dioxin ELISA.

5.2 Materials and Methods

PCB standards were purchased from Wellington Laboratories Inc. (Guelph, Ontario, Canada). Both PCB mixtures and pure congeners were tested (Table 5-1). The PCB mixtures included BP-MS and BP-EC. BP-MS was a mixture of 62 PCB congeners each at a concentration of 2,000 pg/uL. BP-EC was a mixture of 62 PCB congeners but the two monochlorobiphenyls (PCB 1 and 3) were at a concentration of 50,000 pg/uL and the six trichlorobiphenyls (PCB 18, 19, 22, 28, 33 and 37) were at 5,000 pg/uL; the remaining 54 congeners were at 1,000 pg/uL. Pure congeners were represented by using PCB77 (3,3',4,4'-tetrachlorobiphenyl) and PCB118 (2,3',4,4',5-pentachlorobiphenyl). All the PCB spikes were performed in duplicate. The cross-reactivity was calculated by using the TEQs obtained by ELISA divided by the picograms of PCBs.

Competitive ELISA was performed, which was described in section 4.2.3.

5.3 Results and Discussions

The results (Table 5-1) show that the PCB backgrounds range from 20 to 30 pg TEQ. That is, more than 1,000,000 pg of PCBs produced ELISA signal equal to only 20 to 30 pg TEQ, which was five logs lower than the PCB's picogram concentration.

Table 5-1. Cross-reaction of PCBs in dioxin ELISA

<i>PCB Standards</i>	<i>pg Added to ELISA tube</i>	<i>Mean ELISA pg TEQ</i>	<i>% Cross-reaction</i>
<i>10 μL of BP-MS</i>	1,120,000	27.5	0.0025
<i>20 μL of BP-EC</i>	1,600,000	28.3	0.0018
<i>10 μL of PCB77</i>	1,000,000	17.8	0.0018
<i>20 μL of PCB118</i>	1,000,000	4.8	0.0005

The amount of PCBs added to the ELISA tube was 10 to 100 times higher than what is normally observed in fish samples. Thus it was assumed that under normal conditions, the PCBs from the sample should be negligible for ELISA detection. This cross-reactive signal was very small when expressed as a percentage of >1 million pg of PCB input.

Hence, it was concluded that PCBs tested were transparent in dioxin ELISA. This ELISA was specific to dioxin and furan. The dl-PCBs do not cross-react in dioxin ELISA.

Chapter 6. Spike Recovery

6.1 Preamble

Spike refers to adding a known concentration of 2,3,7,8-TCDD to a sample or a blank. Spike recovery refers to the amount reported by ELISA relative to the spike concentration.

Determination of spike recovery is an important aspect to validate an assay, and to determine the precision and accuracy of the assay (Smolec *et al.*, n.d.). Cape Technologies has performed quality assurance on soil samples using a nominal level of spikes from 30 to 195 pg/g of sample. The recovery from spiked method blank extracts was $79\pm 27\%$ [(mean $\pm$ SD)%]. The recovery from spiked soil sample extracts was $67\pm 28\%$ [(mean $\pm$ SD)%] (Cape Technologies, 2003b). In this chapter, spikes from 0 to 30 pg were tested. Four years of data (between 2004 and 2007) from our lab were analyzed.

6.2 Materials and Methods

Check Samples (CS) of DF1-CS1, DF1-CS2, DF1-CS3 and DF1-CS4, containing 2,3,7,8-TCDD in toluene were purchased from Wellington Laboratories Inc. (Guelph, Ontario, Canada). The concentrations of CS1, CS2, CS3 and CS4 were 0.18, 0.6, 3 and 12 pg/ μ L of TCDD respectively. Spikes ranging from 0 pg to 30 pg per ELISA tube were tested by ELISA. Different spike concentrations were adjusted by adding different volumes of check samples. Spikes at each concentration were performed in triplicate.

Four technologists have been working on testing spike recovery previously. Technologist A prepared spikes using 10 mL toluene along with DF1-CS, and 62.5 μ L of PEG-triton keeper. The spikes were mixed using a vortex, and evaporated at 85°C on the dry bath with nitrogen blowing

above the tubes to remove solvent vapor. Technologist B used 10 mL DCM/Hexane/Isooctane to replace toluene. The spikes were mixed and evaporated on the dry bath at 45°C. Technologist C spiked DF1-CS into 62.5 µL of PEG-triton keeper, along with 10 mL DCM. The mixture was evaporated on the dry bath at 35°C with nitrogen. Technologist D spiked DF1-CS directly into the keeper without adding other solvents and then evaporated the sample.

The sample evaporation was followed by ELISA detection using Wellington Standards (NC to S4) as calibrators. Recovery was calculated by using observed pg of TCDD in ELISA divided by the expected pg of TCDD in the spike.

6.3 Results

Results obtained by technologists A, B, C and D were plotted in Figure 6-1, Figure 6-2, Figure 6-3 and Figure 6-4 respectively. Under four variable conditions, three types of solvents (toluene, DCM/hexane/isooctane, DCM) were evaporated along with DF-CS and PEG-triton keeper. Reproducible results of recovery (slope) (0.76, 0.57, 0.77, 0.70) and correlation R^2 (0.94, 0.83, 0.88, 0.92) were observed. Finally all of the data were combined (Figure 6-5), the recovery was $70 \pm 36\%$ [(mean $\pm$ SD)%]. For the purpose of calculating the SD, data points with zero values on the abscissa were excluded because ELISA can not return TEQ values less than zero on the ordinate. This recovery is comparable to the recovery of the spiked soil sample extracts ($67 \pm 28\%$) by Cape Technologies.

Figure 6-1. Dose-response linearity of ELISA by spiking 10 mL toluene

Figure 6-2. Dose-response linearity of ELISA by spiking 10 mL DCM/Hexane/Isooctane

Figure 6-3. Dose-response linearity of ELISA by spiking 10 mL DCM

Figure 6-4. Dose-response linearity of ELISA by spiking 62.5 µL of PEG-triton keeper

Figure 6-5. Spike recovery based on combined spike samples (N=66)

6.4 Discussions

An obvious loss of dioxins was observed during the experimental operation. The 70% spike recovery indicated that a portion of the dioxins was lost either by solvent evaporation or from the nitrogen blowing. This occurred even though the dioxins were supposed to remain in the sample tube while the solvent was being evaporated.

The solvent evaporation apparatus included a nitrogen generator and a dry bath. The generator produced clean, dry (to -58°F) dewpoint evaporator grade nitrogen gas (Parker Hannifin Corporation, 2005). The dry bath was purchased with tall heating blocks, and in order to complete an apparatus for proper solvent evaporation, a nitrogen distributor was built over the heating block (Figure 6-6). Solvents, such as toluene and isooctane, required temperatures of up to 80°C to evaporate under the nitrogen stream. The nitrogen gas was produced by a generator

and was distributed by Pasteur pipettes to the top of the sample tubes. The nitrogen blowing at the mouth of the tube helps to dissipate the solvent vapor, which otherwise would reflux back down into the tube.

Figure 6-6. Nitrogen generator (Left) and Dry bath with a homemade plastic udder to distribute nitrogen (Right)

An average of 30% of analyte was lost despite using a very gentle nitrogen pressure of 1 psi or less. This intricate problem of analyte loss at the solvent evaporation step will require further study. Variables that require investigation also include the efficiency of detergent micelles to function as keeper (see section 7.2.1), and the effect of the water-mimicking capacity of various ethylene glycols.

Chapter 7. Optimization of the performance of ELISA

During this research, it was observed that some techniques and procedures needed to be modified in order to succeed in dioxin ELISA.

7.1 Consumption of one new pouch of all 20 ELISA tubes per assay

7.1.1 Hypothesis under investigation

Twenty antibody-coated tubes are vacuum-packed in each resealable foil pouch. Once the tin foil pouch is opened, the vacuum seal is broken. The integrity of the ELISA tubes stored in their original zip-lock tin foil pouch with desiccant could deteriorate, even stored refrigerated.

7.1.2 Materials and Methods

Tubes from a vacuum-sealed pouch (I) were compared to tubes in a zip-lock pouch (II) that were opened and refrigerated for 28 days. Two series of five Wellington Standards (NC, S1, S2, S3 and S4) were used to assess the reactivity of the tubes from the two pouches. ELISA was performed according to the manufacturer's protocol.

Two sets of data were used to plot two standard curves by four-parameter fit. The standard curve based on tubes from pouch II was used to quantify the readings in the tubes from pouch I, and *vice versa*. The results are shown in Table 7-1.

7.1.3 Results and Discussions

The absorbance values of S1 to S4 using the tubes from pouch I (or new tubes) were lower than those of the tubes from pouch II (or old tubes) (Table 7-1). In competitive inhibition ELISA,

a lower absorbance indicates that the analytes are more successful in out-competing the conjugate for binding sites on the antibodies. Based on the calibration curve produced by the standards using the old tubes, the readings of standards from the new tubes were higher than expected. This indicates that antibodies in the new tubes bind dioxins more readily than those in the old tubes. Conversely, based on the calibration curve produced by the standards using the new tubes, the readings of dioxin from the old tubes were dramatically lower than expected, especially at high dioxin concentrations of 50 and 100 pg per tube. Again, this indicates that the old tubes were less able to bind dioxins.

Table 7-1. Dioxin Standards bind more to tubes from unopened than opened pouch

	<i>Pouch</i>	<i>Wellington Standards</i>				
		<i>0 pg</i>	<i>3.2 pg</i>	<i>10 pg</i>	<i>32 pg</i>	<i>100 pg</i>
<i>Absorbance of Tubes from Pouch (OD units)</i>	<i>I</i>	1.269	1.055	0.78	0.541	0.339
	<i>II</i>	1.253	1.057	0.838	0.578	0.444
<i>Observed pg by Calibration Curve Based on:</i>	<i>I</i>	0.3	2.9	8.5	25.1	49.3
	<i>II</i>	<0	3.3	12.5	41.6	>100

The antibody-coated tubes from the opened pouch bind dioxin molecules poorly. A plausible explanation could be moisture contamination after opening the vacuum seal. The antibody-coated tubes from the opened pouch need to be warmed to room temperature. Cold temperature retards diffusion thereby causing slower and inconsistent molecular interaction between antigen and antibody. The pouch is opened to retrieve the tubes. At this moment warm air enters the pouch, and contacts all the tubes. Then the zip-lock pouch is put back into the refrigerator in which the moisture from the warm air could condense. If the moisture content exceeds the desiccant capacity to absorb it all, or if the zip-lock is not airtight, excess moisture would

accumulate. Moisture would encourage microbes to grow. Most microbes would produce protease to digest proteins in their surrounding for food. Microbial proteases would digest the antibody polypeptides, which leads to deterioration of antibodies.

The hypothesis that antibody-coated tubes deteriorated after the vacuumed pouch was opened was experimentally substantiated. Mixing new and old tubes in the same ELISA is not recommended. Each ELISA should be designed to consume all 20 tubes from an unopened new pouch. Other recommendations include (1) double check the color of the desiccants; (2) place several desiccant capsules in each opened pouch; (3) roll up the pouch of tubes and place inside another pouch with desiccants (double-bag); (4) keep extra desiccants dry and ready in a 43°C incubator.

7.2 Denaturation of antibodies

7.2.1 Chemistry of solvent exchange

Samples from different environmental matrices are typically extracted and cleaned up with organic solvents (toluene or DCM/hexane), in order to eliminate fatty tissues or other interfering substances that will affect detection. These organic solvents are incompatible with ELISA aqueous buffer. Therefore, samples in organic solvents need to be exchanged to a water-miscible solution for ELISA analysis. At the solvent exchange step, dioxins are retained in a PEG-Triton keeper, formulated by Cape Technologies.

The keeper formulation is 100 ppm Triton X-100 detergent dissolved in 80% methanol and 20% PEG. The solubility of dioxins in methanol is augmented significantly by the addition of PEG and Triton X-100 (Cape Technologies, 2003a). Each sample received 62.5 µL of this keeper,

which amounts to 2.5 μL Triton X-100, 50 μL methanol and 10 μL PEG. The chemistry of the solvent exchange step is described below.

Triton X-100 is a nonionic, mild detergent (Figure 7-1). The number “100” relates only indirectly to the number of ethylene oxide units in the structure (Sigma-Aldrich Corp., 1999). Triton X-100 is soluble at 25°C in water, benzene, toluene, xylene, trichloroethylene, ethylene glycol, ethyl ether, ethanol, isopropanol and ethylene dichloride (Sigma-Aldrich Corp., 1999). The critical micelle concentration (CMC) is 0.22-0.24 mM (Tiller *et al.*, 1984), which is equal to 1.32×10^{20} molecules per Litre to 1.445×10^{20} molecules per Litre respectively. The keeper formulation is 100 ppm of Triton X-100 in 80:20 methanol:PEG. One hundred ppm of Triton X-100 with average molecular weight of 625 equals 0.963×10^{20} molecules per Litre of keeper. This concentration is just below the CMC. Therefore, micelles will not form in the keeper, and the dioxins are not protected inside micelles. Only when the toluene is evaporated down to dryness, 2.5 μL of Triton X-100 will reach its CMC in 10 μL PEG, and form micelles with hydrophilic tails outside and hydrophobic heads inside (Figure 7-1). PEG and Triton X-100 have high boiling points of 250°C (IPCS INCHEM, 2004) and 200°C (Oxford University, 2008), respectively. They will remain as liquid keepers while toluene is evaporated at 80C (US EPA 2002b).

At the end of the evaporation, the dioxin molecules would be trapped inside the micelles that appear as detergent droplets adhering to the wall of the test tube. These droplets settle to the bottom of the tube by centrifuging at 1000g for 30 minutes. The Triton micelles are stable in PEG because the structure of PEG (Figure 7-2A PEG) mimics the structure of water molecules (Figure 7-2B Water). Lastly, the solvent exchange step is completed when the detergent pellets are re-dissolved in 50 μL methanol. The addition of 50 μL methanol returns the detergent pellets back into their original formulation of 2.5 μL Triton X-100, 50 μL methanol and 10 μL PEG, in

which the triton X-100 is above its CMC. At this point, it becomes possible to pipette the sample in keeper directly into the aqueous ELISA buffer, as it is impossible to pipette a dried pellet. At the end of solvent exchange, 50 μL of the mixed solution is transferred into an ELISA tube.

During the process of solvent exchange, some methanol may evaporate. Therefore instead of the theoretical total volume of 62.5 μL , usually only 50 ± 2 μL remains. A recovery of 50 μL out of 62 μL represents approximately a 20% loss of methanol rendering the methanol concentration at this point to be approximately 64% v/v. This results in 6.4% (v/v) final methanol concentration in the actual ELISA tube instead of the theoretical 8% final concentration. The methanol concentration may vary for each sample depending on how long it takes to finish exchanging the solvents.

In summary, 62.5 μL of PEG-Triton keeper must be added to the sample for solvent evaporation, after which 50 μL of methanol is added to dissolve the keeper pellet. Since 80% of the Triton keeper is methanol, there is a concern that methanol may cause denaturation of antibodies. Therefore, the effect of methanol contained in keeper was examined.

7.2.2 Materials and Methods

Using the manufacturer's protocol, three sets of Negative Controls, which consist of only PEG-Triton keeper and sample diluent, were performed in the ELISA tubes. Each set was incubated over a different time period: 2 hours, 5 hours and 24 hours respectively. All three sets were performed in triplicate. All the Negative Controls were treated as regular samples for ELISA detection. The manufacturer recommends antibody incubation from 2 to 24 hours, but prefers overnight incubation.

Hydrophilic tail

Hydrophobic head

Figure 7-1. Molecular structure of Triton X-100

Figure 7-2A. PEG

Figure 7-2B. Water

Figure 7-2. Molecular structures of PEG and Water

7.2.3 Results and Discussions

A spontaneous decrease in the absorbance was detected as the antibody incubation time increases (Figure 7-3). The average absorbance decreased by 0.178 OD units in a span of 24 hours, which amounts to a 12% decrease.

The negative effects caused by the methanol were observed through the decreasing absorbance. Only sample dilute along with keeper were sitting in the ELISA tube with no dioxin molecules binding to the antibodies. This drop in OD units suggests that methanol in the keeper was denaturing the antibodies, so that fewer antibodies were available for the conjugate to bind. This resulted in less color after substrate incubation and lower absorbance. The mechanism of antibody deterioration could be due to the dehydration effect of methanol disrupting certain hydrogen bonds in the antibody molecule. Disrupted antibodies would be unable to bind dioxin or dioxin-conjugate. Reduced conjugate binding means less color development. The net result would be reduced assay sensitivity.

The results also indicate that the longer the antibody incubation period, the lower the absorbance. It would appear that methanol could exert a stronger effect on an increasingly larger amount of antibodies during an overnight incubation.

Figure 7-3. Antibody deterioration during incubation

Furthermore, the observation that approximately 12% of the antibody denatured during overnight incubation suggests that the antibody incubation should be minimized within a period, during which it can both guarantee enough time for the antibody-antigen reaction, and to minimize the antibody deterioration. Therefore, two hours of antibody incubation is preferred in this research. Moreover, methanol concentration should be constant in the samples, especially during solvent exchange, in order to keep this deterioration rate constant for each sample.

The process of solvent exchange involves approximately 3 to 4 minutes of manipulation for each sample, during which the methanol evaporation could result in concentration variation. Thus, after dissolving the sample residue with 50 μL methanol, one should work quickly to avoid excessive evaporation. The manufacturer suggests performing this procedure in batches of four

or fewer samples (Cape Technologies, 2003a). In this study, two tubes were processed at each time, including the addition of methanol, vortex, centrifugation, and transfer of up to 50 μL of mixed solution into each ELISA tube, to ensure that the experimental conditions were equal for all of the sample tubes and to maintain a more or less even sample volume.

The prescribed procedure is considered as the best practice, because it normalizes the variability between samples. This practice allows the solvent recovery to stay within the range of $50 \pm 5 \mu\text{L}$ in most instances. The delivery of 50 μL of sample in its entirety will circumvent a dilution factor correction.

The final sample volume recovered after solvent exchange should be recorded immediately after the mixture is transferred into an ELISA tube. If the final sample volume was less than or equal to 50 μL , then 100% of the sample would be introduced into the ELISA tube and no correction factor is necessary. Alternatively, if the sample volume was more than 50 μL then there may be a need to correct for having tested less than 100% of the sample.

7.3 Slowly vortex the ELISA tube after sample addition

7.3.1 Hypothesis

After solvent exchange, 50 μL of mixed solution was added into the ELISA tube. The manufacturer's procedure recommends immediately mixing each tube briefly and shaking the test tube rack to swirl the liquid when all of the tubes are completed.

Vortex is available and more consistent than mixing by hand. Vortex immediately can mitigate antibody denaturation due to localized methanol. The hypothesis that vortex (Figure 7-4 Left) is better than swirling the liquid by briefly tapping the tube (Figure 7-4 Right) has been

investigated. The movement of the solution inside of the tubes would be different by the two mixing methods.

Figure 7-4. Tube being vortex (Left); Tube being tapped (Right)

7.3.2 Materials and Methods

Two sets of Wellington Standards (NC, S1, S2, S3 and S4) were added to ELISA tubes. One set of standards was mixed by vortex at low speed. The other set of standards was mixed by gently tapping the ELISA tube. Both sets of standards were tested by the ELISA method described previously. A calibration curve based on vortex values was used to read the tapped tubes results and *vice versa*.

7.3.3 Results and Discussions

Results are shown in Table 7-2. Standards in the vortex ELISA tubes produced a calibration curve with a better fit to the 4-parameter curve fitting formula, because the sum of squares of

differences was smaller than that of the calibration curve based on tapped tubes. Additionally, I_{50} of the calibration curve based on vortex tubes was within the correct range, which indicates an accurate curve. Conversely, the I_{50} of the calibration curve based on tapping tubes was greater than the upper limit of the range, indicating that the ELISA was under-sensitive.

Table 7-2. Readings of dioxin values in vortex ELISA tubes versus tapped tubes

<i>Standards & Values</i>	<i>Expected pg</i>	<i>Calibration Curve Based on Vortex Standards*</i>		<i>Calibration Curve Based on Tapped Standards*</i>	
		<i>Vortex</i>	<i>Tapping</i>	<i>Vortex</i>	<i>Tapping</i>
NC	0	0.1	<0	0.2	0.1
Wellington-S1	3.2	3.3	2.7	4.7	3.7
Wellington-S2	10	9.6	5.5	16.1	8.5
Wellington-S3	32	34.2	20.6	59.2	36.4
Wellington-S4	100	92.7	59.8	124.1	93.3
Calibration curve QA	Sum of squares of differences	1.29		13.95	
	I_{50} (14.5 to 29.3)	20		34.2	
	I_{85} (2.5 to 5.3)	2.6		3.2	

* 'Vortex Standards' means that the calibration curve is built based on the standards from vortex tubes, the observed standards from tapped tubes can be obtained based on this calibration curve. Similarly, 'Tapped Standards' means that calibration curve is built based on the standards from tapped tubes.

Furthermore, the tapped tubes produced lower readings of observed picograms of dioxin. This demonstrates that mixing by gently tapping the ELISA tube was inadequate to disperse the dioxin homogeneously. Even after overnight antibody incubation, the diffusion at room temperature did not allow tapped tubes to reach reaction equilibrium compared to vortex tubes. Standard curves constructed using tapped tubes would return erroneously high dioxin readings in well-mixed tubes. Consequently, if both samples and standards are tapped, the tapped calibration

curve will likely return erroneous results based on the standards. This is because a poor quality curve would result from the tabbed standards, judging by a high sum of square of differences, and with I_{50} and I_{85} deviated from ideal norm (Table 7-2).

Thus after 50 μ L of a standard or a sample was added to an ELISA tube, vortex was recommended. However, vortex speed should be properly controlled. Vortex at high-speed may splash droplets up the tube walls, which could mean that dioxin molecules in the droplets would not react to the antibodies that were only coated on the bottom 500 μ L portion of the ELISA tube.

7.4 Reagent Shelf Life

The three most expensive components in the kits are the antibody-coated tubes, the conjugate, and the substrate in that order. Conjugate and substrate are essential components of the ELISA kit. Together they produce a color change, which allows for the visualization of molecular reactions invisible to the naked eye. Study of the shelf life of these reagents is relevant in the context of cost savings and quality control. The experiments designed below constitute standard operating procedures for the quality assurance of ELISA reagents and for trouble shooting. Both conjugate and substrate reagents from the expired kits (2004, 2005, 2006) were studied.

7.4.1 Conjugate Shelf life

The manufacturer formulated the conjugate by linking modified Horseradish peroxidase enzyme to TCDD haptens. A hapten is a small molecule which by itself cannot stimulate antibody synthesis but will combine with pre-formed antibody (Roitt, 1973).

Horseradish peroxidase (HRP) has four enzymatic sites (Lebedeva & Ugarova, 1996). The native enzyme has a molecular weight of 40,000 (McKie *et al.*, 1999) and a pH optima around

6.0 (Sun *et al.*, 1998). The manufacturer has modified the enzyme such that the final enzyme hetero-complex contains an average of 14 enzymatic sites (Figure 7-5, Equation [1]). The manufacturer then linked TCDD haptens (Denison *et al.*, 2007) to the HRP hetero-enzyme complexes to make the conjugate. This is accomplished by cross-linking the acidic groups (-COOH) of the TCDD hapten to the amino group (-NH₂) of the HRP enzyme molecules, with carbodiimides as the catalyst (Robert Harrison, personal communication) (Figure 7-5, Equation [2]).

Equation [1]: Synthesis of hetero HRP complexes using glutaraldehyde:

Equation [2]: Synthesis of conjugate with the catalyst of carbodiimides:

Figure 7-5. Chemical reactions in conjugate synthesis

7.4.1.1 Materials and Methods

A 96-well microplate was used for conjugate titration. Nine bottles of conjugate representing four lots of expired kits from the past three years (2004-2006) were tested. The lot numbers and expiry dates are described in Table 7-3. A two-fold titration series in Phosphate Buffered Saline (PBS) (0.01mM) was performed for each conjugate in duplicate. Each conjugate dilution from 1:8 to 1:128 (v/v) was tested. Fifty μL of each conjugate dilution was transferred into corresponding test wells. And 50 μL of substrate was added. The microtiter plate was shaken on a plate shaker (LAB-LINE® Titer Plate shakers, Model No. 4625, Catalog No. 57019-600, Barnstead Lab-Line Instruments Inc., Melrose Park, Illinois, USA) for 30 minutes at room temperature. The reaction was stopped by adding 100 μL of stop solution. The absorbance at 450 nm was read from a plate reader (SpectraMax Plus 384, Molecular Devices Corporation, Sunnyvale, California, USA) using 650 nm as a reference wavelength. Absorbance values were captured using Softmax software program (SOFTmax® Pro Version 4.3.0 for Windows and Macintosh, Molecular Devices Corporation, Sunnyvale, California, USA). The reagents from an unexpired 2007 kit (lot# D1227) were used as reference for comparisons.

7.4.1.2 Observations

The titration results (Table 7-3) indicated that conjugate dilution at 1:64 was the end-point concentration that allowed for a clear discrimination of performances among the conjugate lots under these experimental conditions. Therefore, the absorbance at 1:64 concentration was plotted in Figure 7-6.

Figure 7-6 illustrates that conjugate deteriorates over time upon storage at 4°C after the bottles have been opened. Compared to conjugate from 2007, conjugate that expired in 2006 (four bottles) retained about 50% enzyme activity. Two out of three bottles of conjugate that expired in 2005 retained 50% enzyme activity while the third bottle retained only 25% activity. Only one bottle that expired in 2004 was available for testing and it retained 15% activity.

Table 7-3. Absorbance (OD unit) obtained by titration of conjugate from different lots

<i>Conjugate Dilutions</i>		<i>1:8</i>		<i>1:16</i>		<i>1:32</i>		<i>1:64</i>		<i>1:128</i>	
<i>Lot #</i>	<i>Expired Date</i>	<i>1</i>	<i>2</i>	<i>3</i>	<i>4</i>	<i>5</i>	<i>6</i>	<i>7</i>	<i>8</i>	<i>9</i>	<i>10</i>
<i>C04-1 lot# C1963</i>	15-Jan-04	2.52	2.50	1.35	1.34	0.70	0.68	0.34	0.34	0.17	0.16
<i>C05-1 lot# D0825</i>	23-Dec-05	2.40	2.42	1.81	1.60	2.00	1.90	0.97	0.97	0.48	0.48
<i>C05-3 lot# D0825</i>	23-Dec-05	2.31	2.46	1.83	2.30	2.16	2.09	1.06	1.06	0.51	0.49
<i>C05-4 lot# D0825</i>	23-Dec-05	2.69	2.54	1.86	1.86	0.9	0.97	0.49	0.49	0.24	0.26
<i>C06-5 lot# B0686</i>	09-Dec-06	2.34	2.42	2.32	2.91	2.03	2.03	0.99	0.96	0.45	0.45
<i>C06-3 lot# B0686</i>	09-Dec-06	2.41	2.64	1.86	1.76	2.30	2.29	1.11	1.10	0.51	0.51
<i>C06-1 lot# B0686</i>	09-Dec-06	2.40	2.66	2.85	1.80	2.11	2.06	1.03	1.04	0.48	0.47
<i>C06-2 lot# B0686</i>	09-Dec-06	2.43	2.38	2.53	1.79	2.11	2.08	1.04	1.02	0.49	0.48
<i>Reference lot# D1227</i>	02-Feb-08	2.28	2.17	2.44	2.38	1.79	1.78	1.98	1.93	1.08	1.10

Figure 7-6. Conjugate shelf life as measured by reaction intensity at 1:64 Dilution

7.4.1.3 Discussions

As previously mentioned, the HRP has been modified to be a hetero-complex with 14 enzymatic sites. Consequently, the catalytic activity of the final enzyme conjugate is much enhanced (Robert Harrison, personal communication). However, the pH optima of the hetero-complex are unknown. The well-known bi-functional reagent glutaraldehyde ($\text{CHOCH}_2\text{CH}_2\text{CH}_2\text{CHO}$) could have been used to covalently bond individual enzyme molecules to form such hetero-complexes. This is accomplished by glutaraldehyde linkage of amino groups of the enzymes, resulting in the formation of $\text{C}=\text{N}$ (imine) bonds through condensation reactions (removal of water molecules) (Figure 7-5, Equation [1]). After the HRP modification, the manufacturer linked TCDD haptens to HRP hetero-enzyme complexes with carbodiimides as the catalyst (Figure 7-5, Equation [2]). Unlike glutaraldehyde, carbodiimide does not act as a bridge

between the hapten and the HRP complex.

It could be speculated that the chemical modification of HRP to enzyme hetero-complex might have destabilized the enzyme. For example, if the hetero-complex disintegrates back into its individual enzyme molecules or subunits, the conjugate signal will decrease – the scattered subunits would be washed away. This is one plausible explanation for the limited shelf life (less than 1 year) of the conjugate.

The conjugate is also susceptible to degradation by bacteria or fungi. Up to 2004, expired conjugate occasionally exhibited microbial colonies visibly flocculating in unopened bottles. The manufacturer was informed of this problem. In 2005, the manufacturer incorporated a new anti-fungal formulation into their conjugate. After that, flocculating contaminants are no longer seen and shelf life appears to have stabilized up to one year. Beyond one year, conjugate activity deteriorated (Figure 7-6). Although the exact mechanism causing conjugate deterioration is unknown, several plausible explanations were given in the preceding paragraphs.

7.4.2 Substrate Shelf life

7.4.2.1 Preamble

The dioxin ELISA manufacturer purchased the substrate from another supplier. Although the exact patented chemical is unknown, it is believed that the substrate is probably organic peroxide, such as urea-peroxide rather than inorganic hydrogen peroxide (Robert Harrison, Personal communication).

7.4.2.2 Materials and Methods

Three bottles of substrate were tested with varying conjugate dilutions from 1:16 to 1:256 (Table 7-4), in a microplate under experimental conditions as previously described for conjugate titration (Table 7-3). The absorbance was compared to a reference lot of unexpired substrate (Lot A2846).

7.4.2.3 Observations

Table 7-4. Absorbance (OD units) of conjugate titration with different lots of substrate

<i>Conjugate Dilution</i>		<i>1:16</i>		<i>1:32</i>		<i>1:64</i>		<i>1:128</i>		<i>1:256</i>	
<i>Substrate Lot#</i>	<i>Expiry Date</i>	<i>1</i>	<i>2</i>	<i>3</i>	<i>4</i>	<i>5</i>	<i>6</i>	<i>7</i>	<i>8</i>	<i>9</i>	<i>10</i>
<i>A3321</i>	15-Jan-04	2.41	2.50	1.33	1.32	0.65	0.66	0.30	0.31	0.15	0.14
<i>A0696 (unopened)</i>	09-Dec-06	2.45	2.75	1.73	1.72	0.85	0.86	0.39	0.39	0.21	0.17
<i>A0696 (opened)</i>	09-Dec-06	2.92	2.62	1.68	1.68	0.83	0.81	0.38	0.37	0.17	0.17
<i>A2846 (N=8)</i>	2-Feb-08	2.20±0.52		2.04±0.07		1.00±0.03		0.47±0.01		N/A

Figure 7-7. Substrate shelf life as measured by reaction intensity using 1:32 conjugate dilution

Conjugate dilution at 1:32 (v/v) exhibited absorbance that are the most descriptive and are therefore plotted in Figure 7-7. Results show that substrate deteriorated over time whether the bottle has been opened or not (Figure 7-7). Compared to the reference substrate (lot # A2846), substrate that expired in 2006 (Lot# A0696) deteriorated about 17%. Substrate that expired in 2004 (Lot# A3321) has deteriorated around 35% (Table 7-4).

The ELISA manufacturer claimed that the substrate was stable in their hands for over 3 to 4 years (Robert Harrison, personal communication). However, the titration experiment shows 17-35% deterioration of the reagent reactivity within four years. It should be noted that the manufacture did not carry out thorough QC procedures as described here.

7.4.2.4 Additional proof and Discussions

Similar observations were identified in previous experiments conducted in 2006 (Table7-5).

Table 7-5. Absorbance (OD units) at 450 nm (650 nm as reference)

<i>Conjugate Dilution</i>		<i>1:8</i>		<i>1:16</i>		<i>1:32</i>		<i>1:64</i>		<i>1:128</i>		<i>1:256</i>	
<i>Substrate Lot#</i>	<i>Expiry Date</i>	<i>1</i>	<i>2</i>	<i>3</i>	<i>4</i>	<i>5</i>	<i>6</i>	<i>7</i>	<i>8</i>	<i>9</i>	<i>10</i>	<i>11</i>	<i>12</i>
A0615	09-Dec-06	2.44	2.49	1.61	1.53	0.64	0.60	0.23	0.24	0.08	0.08	0.03	0.03
A0696 (unopened)	09-Dec-06	2.27	2.12	2.96	2.80	1.41	1.39	0.59	0.60	0.21	0.21	0.07	0.07

The new substrate Lot# A0696 (unopened) was twice as potent as the old Lot# A0615, which was opened for three weeks. The new substrate Lot# A0696 was so strong that the color complex precipitated out of the solution at a 1:8 conjugate dilution. The manufacturer prepared the two lots on different dates, thus the two aliquots had different lot numbers. However, the lots were dispensed from the same bottle, which was purchased from another substrate supplier. Therefore, the two lots of substrate were assigned the same expiry date (Robert Harrison, personal communication). Cape Technologies has been using the same product for eight years (Robert Harrison, personal communication). Cape Technologies has also investigated substrate deterioration by adding 20 μ L of conjugate directly to 0.5 mL of substrate, and observing the color with the naked eye (Robert Harrison, personal communication). However they did not identify this problem of substrate deterioration over time (Robert Harrison, personal communication).

One of the questions that remained unanswered was why the substrate supplier formulated the pH of the substrate to 3.5-3.7, when the pH optimum of the native HRP enzyme was 6.0-6.5. Another unanswered question was the exact cause of the substrate deterioration. The following

investigation was conducted.

One hypothesis is that peroxide might have leached from the plastic substrate bottle over time. This will lead to decreased peroxide concentration. To test this assumption, the substrate was fortified with three concentrations of hydrogen peroxide (H_2O_2) (Table 7-6) to test if the substrate solution contained a sufficient amount of peroxide.

Table 7-6. Performance of fresh substrate (Lot# A0696) before and after fortification with three concentrations of H_2O_2 in a microtiter plate

	<i>Color Reaction Intensity</i>			
<i>H_2O_2 (V/V) Added to the Substrate Solution</i>	0 %	0.01 %	0.1 %	1 %
<i>Absorbance Value at 450 nm Average (N=8)</i>	0.82	1.58	2.80	1.87
<i>SD</i>	0.01	0.07	0.06	0.08
<i>CV (%)</i>	1.67	4.12	2.08	4.29

Experimental conditions: 50 μL conjugate (Lot # B0686) at 1:16 in PBS plus 50 μL substrate solution, 5 minutes of incubation at room temperature, 100 μL stop solution.

For optimal enzyme reaction, substrate should always be in excess and should not be a limiting factor. The results in Table 7-6 suggest that substrate (hydrogen peroxide) was limiting for the enzyme (horseradish peroxidase) in the ELISA kit. Thus, the color reaction signal was doubled when the concentration of H_2O_2 was increased by 0.01%, and was more than tripled with additional H_2O_2 at 0.1%. This observation demonstrates that the manufacturer's substrate solution was formulated incorrectly; the amount of peroxide was insufficient to produce maximum color.

Another observation is that a further increase of H_2O_2 from 0.1% to 1% results in a decreased signal from 2.8 to 1.87 OD units, which itself is more than double the original substrate signal of 0.82 OD units. This unexpected decrease in signal intensity in the presence of excessive peroxide substrate was puzzling. A couple of possible explanations could be discounted below. Firstly,

some enzymes are susceptible to feedback inhibition when too much product accumulates due to excess substrate availability. This feedback mechanism was not known to occur for horseradish peroxidase enzyme. Secondly, a pH change due to excessive substrate might affect the enzyme activity. This explanation is unlikely because the reaction was buffered with phosphate buffered saline (PBS pH 7.2). Since H_2O_2 is a strong oxidizing agent, the enzyme activity might have been affected at 1% concentration.

Table 7-7. Failure of ELISA in the presence of 0.1% H_2O_2 added to the substrate solution

<i>Concentration of 2,3,7,8-TCDD Standards Added to ELISA Tubes (pg)</i>	<i>Absorbance (450 nm) of Original Substrate</i>	<i>2,3,7,8-TCDD Back Calculated from Calibration Curve (pg)</i>	<i>Absorbance (450 nm) of Substrate Fortified with 0.1% H_2O_2</i>	<i>2,3,7,8-TCDD Back Calculated from Calibration Curve (pg)</i>
0	1.043	0.07	0.254	Out of range
3.2	0.874	3.5	0.232	Out of range
10	0.734	9.1	0.198	Out of range
32	0.502	34.7	0.156	Out of range
100	0.373	95.4	0.129	Out of range

The experiment involving liquid-liquid titration in PBS, as described in Table 7-6, suggested that the manufacturer's substrate solution could be optimized by adding excess H_2O_2 (0.1% v/v). This new formulation was tried out in dioxin ELISA, using 2,3,7,8-TCDD standards in antibody-coated tubes (solid-phase competition chemistry). Surprisingly, the results between the solid-phase system and the liquid-liquid system disagreed. Additional H_2O_2 (0.1%) abolished the enzyme reaction in the solid-phase ELISA (Table 7-7), that is, color development was inhibited across the board. The excessive H_2O_2 could not have oxidized the enzyme proteins, because the same H_2O_2 concentration did not denature the enzyme in the liquid-liquid titration. Furthermore,

pH change could not explain the loss of enzyme activity. Although this series of investigations were insightful, the exact source of this error could not be identified.

Chapter 8. Conclusions and Recommendations

8.1 Conclusions

Dioxin analysis by the conventional method of GC-HRMS is perhaps the most expensive method, with a cost of approximately \$1900 USD per sample. In contrast, ELISA provides TEQ values with dramatic improvement in turnaround time, sample throughput and cost. For the same budget, ELISA can process several times the amount of samples, thereby providing a greater amount of statistically meaningful data to policy decision makers in a much shorter time.

US EPA Method 4025 is based on a commercially available ELISA kit designed for the specific purpose of dioxin detection in soil samples at ppb levels of concern. According to the ELISA manufacturer, the method detection limit (MDL) is 4 pg TEQ per gram of soil. Therefore, the state-of-the-art of dioxin ELISA falls short for the purpose of dioxin detection in food at ppt level of concern. For example, the Ontario guideline to monitor sport fish requires MDL at 2.7 pg TEQ per gram of fish (MOE, 2007), which is below the MDL of the current ELISA version.

The bioassay laboratory at the Ontario Ministry of the Environment intends to introduce the US EPA Method 4025 to Canada for soil samples. Furthermore, the ministry has funded the university to research and develop an upgrade version of the ELISA suitable for dioxin detection at ppt and ppq levels of concern, which is the subject of this thesis.

The contributions of this research can be illustrated in three categories: (1) the advancement of immunochemical principles, including identification of the sources of error, (2) the development of new techniques to control for these errors and enhance assay performance, and (3) confirmation of certain valid principles as suggested by the manufacturer. These contributions in principles and techniques together have elevated the performance of this ELISA to meet the

challenge of ultra-trace MDL suitable for ppt level of detection. Each category of contribution is described below.

(1) Advancement of immunochemical principles:

The first and foremost immunochemical principle advanced by this study is “one ELISA three detection levels”. In the case of GC-MS, the MDL can be improved only by employing a new generation of high resolution instrumentation (GC-HRMS) at substantial added costs. In contrast, this study advanced the principle that ELISA MDL can be pushed to the level of 1 ppt, three orders of magnitude lower than US EPA Method 4025. At this level, the results from the two methods — ELISA and GC-HRMS — were comparable and statistically agreed well. Therefore, ELISA has a strong potential to be useful and accurate for ultra-trace analysis of dioxin at the 1 ppt level. As added proof of this principle, recent results demonstrated that both soil and food samples can be analyzed by using the improved ELISA technique (Chen, *et al.*, 2008). In the future, the principle of using the same ELISA could be advanced to analyze water samples at ppq level by concentrating dioxins from large sample volume to ppt.

The second principle discovered involves antibody integrity. Methanol used for solvent exchange has been found to cause antibody deterioration over time, which in turn would affect the assay sensitivity. To avoid this undesirable effect, 2-hour antibody incubation is recommended.

The third principle validated is that dioxin background in lab wares will be an important source of error at the MDL of 1 ppt.

Fourthly, the principle of potential cross-reaction with PCBs was investigated and resolved. The anti-dioxin antibodies only recognize the shape of the dioxin core structure and the

chlorination pattern. When millions of picogram of PCBs were added to the ELISA test, the cross-reaction was negligibly small. Furthermore, both pure dl-PCBs congeners and mixtures of other PCB congeners that would reasonably expected to be found in the environmental samples have been tested in this research. Two mixtures of PCBs, each containing 62 PCB congeners, were used. In contrast, the manufacturer only tested 4 pure dl-PCB congeners.

Finally, the principle of antibody specificity has technical advantages. PCB contamination has been proven to be transparent to the Dioxin ELISA. On the one hand, when the GC-HRMS system detected high levels of PCBs in glassware, the whole system must go offline to flush away the contaminants. On the other hand, ELISA ignores PCB contaminants thus simplifying glassware washing while eliminating the associated chemical solvent waste.

(2) New techniques developed:

Firstly, a new method for cleaning glassware has been developed to control background contamination. The procedure of using DCM to rinse each glassware twice proved to be a simple but efficient cleaning method. This technique saves both time and organic solvents, requiring only 1 mL of DCM per tube and 10 seconds of vortex. In contrast, the GC-HRMS glassware cleaning procedure involves two changes of alternating toluene and DCM soaking over a three-day period.

Secondly, using up all the tubes from a vacuum-sealed pouch is recommended. Experiments should be designed to consume all 20 tubes contained in one pouch. After contacting the moisture in the air, the integrity of the remaining tubes may deteriorate when stored in the zip-lock pouch even with desiccants. Mixing tubes from an opened and an unopened pouch will introduce variability.

Thirdly, a critical procedure of slowly vortex the tube after sample addition is recommended. This procedure can mix the solutions homogenously, and can guarantee the diffusion of the dioxin molecules in order to reach the binding sites on the antibodies.

Lastly, a new titration technique has been developed to quality control the essential reagents. This technique involves a box-titration of conjugate versus substrate, and the exact titers of both reagents are obtained.

(3) Principles confirmation

Firstly, the manufacturer stressed under Quality Assurance to use “evaporation controls to verify proper solvent exchange” (Cape Technologies, 2003a). Using spiked samples, a 30% analyte loss during solvent evaporation has been identified in this study. A combination of equipments, including a nitrogen generator, a dry bath and a homemade plastic udder to distribute the nitrogen, were used at this step. It is important to note that solvent exchange apparatus is not commercially available, and equipment manufacturers contacted were not interested in custom-making one or two modules to specification. Consequently, all ELISA users must cope with this problem of homemade apparatus with variable recovery efficiency.

Secondly, the four-parameter curve fitting method augmented by the solver function in Microsoft Excel has been confirmed to be the best curve fitting method. Linear regression was found inappropriate due to the sigmoidal nature of competitive ELISA. The four-parameter equation for curve fitting is widely used by many immunoassay softwares. However, the solver function was found to improve the four-parameter equation by minimizing the difference between daily results with empirical results. Consequently, calibration curve with better quality can be constructed, and more accurate sample results can thus be obtained.

Thirdly, essential components of the ELISA kit, such as antibody-coated tubes and PEG-Triton keeper have been studied, and their properties have been characterized.

Lastly, reactivity deterioration of the conjugate and substrate after expiry date has been confirmed. Explanations are based on the chemical structure and the properties of the components in the reagents.

The present study showed that the performance of this ELISA does not depend on a single dominant factor but a number of influential factors. Each of these factors has small impacts on the performance, but in combination, they will effect major improvements. The performance based on the manufacturer's procedures was acceptable for soil analysis. All the principles and techniques that have been developed in this research have resulted in a successful ELISA method suitable for dioxin determination in food samples at ppt level.

8.2 Recommendations

This study demonstrated that ELISA generated accurate TEQ values but without the cost, labor and complexity associated with the GC-HRMS method.

The principles and techniques expounded in the conclusion section above should be observed and followed. Firstly, a clean lab that is free of dust is required. Secondly, most of the lab ware has background contamination that must be cleaned. These two requirements must be fulfilled in order to perform ELISA successfully. Finally, meticulous work habits are required during ELISA operation, including designing experiments to consume exactly one sealed pouch of ELISA tubes, vortex at low speed after reagent addition to the ELISA tube, performing solvent exchange in minimal time, and observing expiry dates of kit reagents.

In the future, ELISA should be automated in order to circumvent many of the variables

described in this study. Random or human errors are difficult to avoid even with good SOPs. ELISA automation will process each sample under identical conditions. Incubation time, temperature, reagent volumes, and mixing speed are the critical parameters to be addressed by automation. Although analysts can be trained to become proficient, automation is even more cost-effective offering wider user appeal worldwide.

References

- Albro, P.W., Luster, M.I., Chae, K., Chaudhary, S.K., Clark, G., Lawson, L.D. *et al.*, 1979. A radioimmunoassay for Chlorinated Dibenzo-p-Dioxins. *Toxicology and Applied Pharmacology*, 50, 137-146
- Billets, S., 2005. United States Environment Protection Agency (US EPA) SITE Program Monitoring and Measurement Technologies Trace Elements by XRF Dioxin and Dioxin-like Compounds in Soil and Sediment, Technology Support Project [Online]. Available from http://www.epa.gov/tio/tsp/download/2005_fall/ [cited 20 April 2007]
- Billo, E.J., 2001. Excel® for Chemists. 2nd edition. John Wiley & Sons, Toronto, p. 483
- Cape Technologies, 2003b. High Performance Dioxin/Furan Immunoassay Kit. Application Note AN-008. Analysis of ppt (pg/g) range PCDD/Fs in soil and sediment using rapid extraction and rapid cleanup (modified EPA Method 4025 or Method 4025m)
- Cape Technologies, 2003a. High Performance Dioxin/Furan Immunoassay Kit. DF1 Kit Insert (IN-DF1). Available from: [www. Cape-tech.com](http://www.Cape-tech.com) [cited 29 October 2007]
- Chen, E., Zhang, W., Buan, E., Li, J., Lo, C., 2008. The screening of dioxins by ELISA. Poster for Best in Science Symposium in Ontario Ministry of the Environment
- CIS (International Occupational Safety and Health Information Center), 2000. Dichloromethane [Online]. Available from: http://www.ilo.org/public/english/protection/safework/cis/products/icsc/dtasht/_icsc00/icsc0058.htm [cited 8 February 2008]
- Clark G. C., Brown D. J., Van Overmeire I., Goeyens L. en Denison M. S., 2001. Analysis of brominated flame retardants and brominated dibenzodioxins and biphenyls for Ah receptor activation using the CALUX® bioassay. *Organohalogen Compounds*, 54, 12
- Crummett, W.B. & Stehl, R.H., 1973. Determination of chlorinated dibenzo-p-dioxins and dibenzofurans in various materials. *Environmental Health Perspectives*, 5, 15-25

- Custer, T.W., Custer, C.M., and Hines, R.K., 2002. Dioxins and congener-specific polychlorinated biphenyls in three avian species from the Wisconsin River, Wisconsin. *Environmental Pollution*, 119(3), 323-332
- David, P. H., 1997. Immunoassays for environmental contaminations (pesticides) in food and water [Online]. Available from: <http://www.sdix.com/TechSupport/bulletins/t00037.pdf> [cited 08 April 2008]
- Denison, M.S., Baston, D.S., Sorrentino, C., He, G., Hayashi, A. and Zhao, B., 2007. Bioanalytical approaches for the detection and relative quantitation of halogenated Dibenzo-p-dioxins and related chemicals. Presented on 27th International Symposium on Halogenated Persistent Organic Pollutants, DIOXIN 2007. Tokyo, Japan.
- Environment Canada, 2003. National science assessment on dioxins and furans in the Canadian aquatic environment. Ecosystem health: Science-based solutions report no. 1-5. National Guidelines and Standards Office, Water Policy and Coordination Directorate, Environment Canada, Ottawa, ON
- Environment Canada, 2005. The Canada-Ontario agreement respecting the Great Lakes basin ecosystem, Tackling backyard burning [Online]. Available from <http://www.on.ec.gc.ca/greatlakes/default.asp?lang=En&n=BBBE2C55-1> [cited 19 April 2007]
- Environment Canada, 2007. National Pollutant Release Inventory. Data and Reports. The Four New Groupings of ATH Substances - Substance-Specific Context Dioxins and Furans [Online]. Available from http://www.ec.gc.ca/pdb/npri/npri_dioxins_e.cfm [cited 19 April 2007]
- Fawthrop, T., 2004. Vietnam's war against Agent Orange. BBC News [Online]. Available from: <http://news.bbc.co.uk/2/hi/health/3798581.stm> [cited April 26 2007]
- Giergielewicz-Możajska H., Dąbrowski L., Namieśnik J., 2001. Accelerated solvent extraction (ASE) in the analysis of environmental solid samples- some aspects of theory and practice. *Critical Reviews in Analytical Chemistry*, 31, 149-165
- Harrison, R.O. & Carlson, R.E., 1999. Immunoassay analysis of dioxin in soil: validation of TEQ screening at 500 ppt using rapid extraction and cleanup. *Organohalogen Compound*, 40
- Harrison, R.O. & Eduljee, G.H., 1999. Immunochemical analysis for dioxins - progress and prospects. *The Science of the Total Environment*, 239, 1-18

- Health Canada, 1990. Canadian Environmental Protection Act: Priority substances list assessment report No. 1. Polychlorinated dibenzodioxins and polychlorinated dibenzofurans. Environment Canada and Health Canada, ISBN: 0-662-17644-8 [Online]. Available from: http://www.hc-sc.gc.ca/ewh-semt/pubs/contaminants/psl1-lsp1/dioxins_furans_dioxines_furannes/index_e.html [cited March 26 2007]
- Health Canada, 2006. It's your health: dioxins and furans [online]. Available from http://www.hc-sc.gc.ca/iyh-vsv/environ/dioxin_e.html [cited 7 June 2007]
- Hoogenboom, L., Goeyens, L., Carbonnelle, S., Loco, J.V., Beernaert, H., Baeyens, W., *et al.*, 2006. The CALUX bioassay: Current status of its application to screening food and feed. *Trends in Analytical Chemistry*, 25(4), 410-420
- Huwe, J.K., 2002. Dioxins in food: a modern agricultural perspective. *Journal of Agricultural and Food Chemistry*, 50(7): 1739-1750
- IPCS INCHEM (International Programme on Chemical Safety, Chemical Safety Information from Intergovernmental Organizations), 2004. Polyethylene Glycol (200-600) [online]. Available from: <http://www.inchem.org/documents/icsc/icsc/eics1517.htm>
- Kennel, S.J., Jason, C. Albro, P.W., Mason, G. and Safe, S. H., 1986. Monoclonal antibodies to chlorinated dibenzo-p-dioxins. *Toxicology and Applied Pharmacology*, 82, 256-263
- Lebedeva, O.V. & Ugarova, N.N., 1996. Mechanism of peroxidase-catalyzed oxidation. Substrate-substrate activation in horseradish peroxidase-catalyzed reactions. *Russian Chemical Bulletin*, 45 (1), 18-25
- Lesnik, B., 2000. Immunoassay techniques in environmental analysis. *Encyclopedia of Analytical Chemistry*, 2653-2673
- Lohmann, R., & Jones, K.C., 1998. Dioxins and furans in air and deposition: A review of levels, behaviour and processes. *Science of the Total Environment*, 219(1), 53-81
- Lopez-Avila, V., 1999. Sample preparation for environmental analysis. *Critical Reviews in Analytical Chemistry*, 29(3), 195-230

- Lorber, M., Eschenroeder, A. and Robinson, R., 2000. Testing the USA EPA's ISCST-Version 3 model on dioxins: a comparison of predicted and observed air and soil concentrations. *Atmospheric Environment*, 34(23), 3995 - 4010
- Maggio, E.T., 1983. *Enzyme-Immunoassay*. 3rd edition. CRC Press, Inc. Boca Raton, Florida, United States.
- McKie, A. T., Zammit, P.S. and Naftalin, R.J., 1999. Comparison of cattle and sheep colonic permeabilities to horseradish peroxidase and hamster scrapie prion protein in vitro. *Gut*, 45, 879-888
- Meiattini, F., 1977. United States Patent: Process for the enzymatic determination of glucose with a glucose-oxydized/peroxidized enzyme system [Online]. Available from: <http://patft.uspto.gov/netacgi/nph-Parser?Sect2=PTO1&Sect2=HITOFF&p=1&u=%2Fmetahtml%2FPTO%2Fsearch-bool.html&r=1&f=G&l=50&d=PALL&RefSrch=yes&Query=PN%2FRE29498> [cited 10 April 2008]
- MOE (Ontario Ministry of the Environment), 2006a. DFPCB-E3418, The detremination of polychlorinated dibenzo-p-dioxins, polychlorinated dibenzofurans and dioxin-like polychlorinated biphenys (dlPCBs) in environmental matrices by gas chromatography-high resolution mass spectrometry (GC-HRMS). Laboratory Services Branch, Quality Management Unit.
- MOE (Ontario Ministry of the Environment), 2006b. *Quality Assurance Manual*, Chapter 2: Quality system. Laboratory Services Branch, Revision 6.0
- MOE (Ontario Ministry of the Environment), 2007, *Guide to Eating Ontario Sport Fish*, 2007-2008 edition, Industrial Chemicals, dioxins, furans and dioxin-like PCBs. Queen's Printer for Ontario, Toronto, p.17
- Mukerjee, D., 1998. Health impact of polychlorinated dibenzo-p-dioxins: A critical review. *The Journal of the Air & Waste Management Association*, 48, 157-165
- NATO (North Atlantic Treaty Organization), 1988a. North Atlantic Treaty Organization, Committee on the Challenges of Modern Society. International toxicity equivalency factor (I-TEF) method of risk assessment for complex mixtures of dioxins and related compounds. Pilot Study on International Information Exchange on Dioxins and Related Compounds. Report No. 176, 1-26
- NATO (North Atlantic Treaty Organization), 1988b. North Atlantic Treaty Organization, Committee on the Challenges of Modern Society. Scientific basis for the development of the international toxicity

- equivalency factor (I-TEF) method of risk assessment for complex mixtures of dioxins and related compounds. Pilot Study on International Information Exchange on Dioxins and Related Compounds. Report No. 178, 1-56
- Neurath, C., 2004. PVC's role in dioxin emissions from open burning: New analysis of US EPA data. *Organohalogen Compounds*, 66, 1146-1152
- Nichkova, M., Park, E-K., Koivunen, M.E., Kamita, S.G., Gee, S.J., Chuang, J., *et al.*, 2004. Immunochemical determination of dioxins in sediment and serum samples. *Talanta*, 63, 1213-1223
- OMEE (Ontario Ministry of Environment and Energy), 1996. Guidance on Site Specific Risk Assessment for use at contaminated Sites in Ontario. Appendix E: Upper Concentrations Limits for Soil and Groundwater. Standards Development Branch. ISBN-0-7778-4058-03 (disk copy), Version 1.0
- Otles, S. & Yildiz, H., 2003. Dioxin in food and human health. *Electronic Journal of Environment, Agriculture and Food Chemistry*. ISSN: 1549-4377, 2(5), 593-608
- Oxford University, 2008. The Physical and Theoretical Chemistry Laboratory, Safety data for Triton X-100 [online]. Available from: http://msds.chem.ox.ac.uk/TR/triton_X-100.html
- Parker Hannifin Corporation, 2005. Analytical Gas Systems, NitroVap Gas Generators [Online]. Available from: <http://www.parker.com/balston/AGS/cat/english/BulletinNitrovap.pdf> [cited 02 January 2008]
- Plaza, G., Ulfig, K. and Tien, A.J., 2000. Immunoassays and Environmental Studies. *Polish Journal of Environmental Studies*, 9(4), 231-236
- Reiner, E.J., Clement, R.E., Okey, A.B. and Marvin, C. H., 2006. Advances in analytical techniques for polychlorinated dibenzo-p-dioxins, polychlorinated dibenzofurans and dioxin-like PCBs. *Analytical Bioanalytical Chemistry*, 386(4), 791-806
- Roitt, I.M., 1973. *Essential Immunology*. 3rd edition. William Clowes & Sons, Limited, London, Beccles and Colchester
- Rosenthal, E., 2004. Liberal leader from Ukraine was poisoned. *The New York Times* [online]. Available: <http://www.nytimes.com/2004/12/12/international/europe/12ukraine.html> [cited 06 April 2008]
- Roy, S., Mysior, P. and Brzezinski, R., 2002. Comparison of dioxin and furan TEQ determination in

- contaminated soil using chemical, micro-EROD, and immunoassay analysis. *Chemosphere*, 48, 833-842
- Schechter, A., 1998. A selective historical review of congener-specific human tissue measurements as sensitive and specific biomarkers of exposure to dioxins and related compounds. *Environmental Health Perspectives*, 106 (Suppl 2), 737-742
- Shan, G.M., Leeman, W.R., Gee, S.J., Sanborn, J.R., Jones, A.D., Chang, D.P.Y., et al., 2001. Highly sensitive dioxin immunoassay and its application to soil and biota samples. *Analytica Chimica Acta* 444, 169-178
- Sigma-Aldrich Corp., 1999. Triton™ X-100 (T8532) - Product Information Sheet [Online]. Available from: <http://www.sigmaaldrich.com/sigma/product%20information%20sheet/t8532pis.pdf> [cited 5 December 2007]
- Smolec, J.M., Cornacchia, J.W., Serl S.R., Tabani, I.D., Chan, A. and Amchislavsky, M., n.d. ALIS-IC 2000 Quantitative Module and Its Use in Facilitating Studies Utilizing Multiple Biomarkers
- Stahl, B.U., Kettrup, A. and Rozman, K., 1992. Comparative toxicity of four chlorinated dibenzo-p-dioxins (CDDs) and their mixture. Part I: Acute toxicity and toxic equivalency factors (TEFs). *Archives of Toxicology*, 66 (7), 471-477
- Sugawara, Y., Saito, K., Ogawa, M., Kobayashi, S., Shan, G.M., Sanborn, J.R., *et al.*, 2002. Development of dioxin toxicity evaluation method in human milk by enzyme-linked immunosorbent assay-validation for human milk. *Chemosphere*, 46(9-10), 1471-1476
- Sun, J.J., Fang, H.Q. and Chen, H.Y., 1998. Immobilization of horseradish peroxidase on a self-assembled monolayer modified gold electrode for the detection of hydrogen peroxide. *Analyst*, 123, 1365-1366
- Tiller, G.E., Mueller, T.J., Dockter, M.E. and Struve, W.G., 1984. Hydrogenation of Triton X-100 eliminates its fluorescence and ultraviolet light absorption while preserving its detergent properties. *Analytical Biochemistry*, 141, 262-266
- Tsutsumi, T., Amakura, Y., Okuyama, A., Tanioka, Y., Sakata, K., Sasaki, K., *et al.*, 2006. Application of an ELISA for PCB 118 to the screening of dioxin-like PCBs in retail fish. *Chemosphere*, 65, 467-473

- US EPA (United States Environment Protection Agency), 1994. Method 1613: Tetra- through octa-chlorinated dioxins and furans by isotope dilution HRGC-HRMS. Revision B. Office of Water, US Environmental Protection Agency, Washington, D.C.
- US EPA (United States Environment Protection Agency), 2000. Exposure and Human Health Reassessment of 2,3,7,8-Tetrachlorodibenzo-p-Dioxin (TCDD) and Related Compounds. Draft Final. Exposure Assessment and Risk Characterization. Group National Center for Environmental Assessment - Washington Office of Research and Development US Environmental Protection Agency. Washington, DC. EPA/600/P-00-001
- US EPA (United States Environment Protection Agency), 2002a. Report on the comparison of cell-based assays with mass spectrometry methods for the analysis of PCDDs/PCDFs and PCBs in biosolids. Officer of Water, Office of Science and Technology, Engineering and Analysis Division
- US EPA (United States Environment Protection Agency), 2004. Demonstration and Quality Assurance Project Plan. Technologies for the monitoring and measurement of dioxin and dioxin-like compounds in soil and sediment. Office of Research and development
- US EPA (United States Environmental Protection Agency), 2002b. Method 4025: Screening for polychlorinated dibenzodioxins and polychlorinated dibenzofurans (PCDD/Fs) by immunoassay. Revision 0. US Environmental Protection Agency, Washington, D.C.
- US EPA (United States Environmental Protection Agency), 2005a. Innovative technology verification report, Technologies for monitoring and measurement of dioxin and dioxin-like compounds in soil and sediment, Hybrizyme Corporation AhRC PCR™ Kit [Online]. Available from <http://www.epa.gov/ORD/SITE/reports/540r05005/540r05005.pdf> [cited 12March 2007]
- US EPA (United States, Environmental Protection Agency), 2005b. Citing online sources: Innovative Technology Verification Report, Technologies for Monitoring and Measurement of Dioxin and Dioxin-like Compounds in Soil and Sediment [Online]. Available from http://www.trwnews.net/Documents/Cleanup/SITE_report.pdf [cited April 26 2007]
- US EPA (United States, Environmental Protection Agency), 2007. Method 4430, Screening for polychlorinated dibenzo-p-dioxins and furans (PCDD/Fs) by aryl hydrocarbon-receptor PCR assay. Revision 0 [Online]. Available from: <http://www.epa.gov/epaoswer/hazwaste/test/pdfs/4430.pdf> [cited 03 March 2008]

- US EPA (United States, Environmental Protection Agency), 2008. Method 4435: Method for toxic equivalents (TEQs) determinations for dioxin-like chemical activity with the CALUX Bioassay [Online]. Available from: <http://www.epa.gov/SW-846/pdfs/4435.pdf> [cited 29 February 2008]
- Van den Berg, M., Birnbaum, L.S., Denison, M., De Vito, M., Farland, W., Feeley, M., *et al.*, 2006. Review--The 2005 World Health Organization reevaluation of human and mammalian toxic equivalency factors for Dioxins and Dioxin-like compounds. *Toxicological Sciences*, 93 (2), 223-241
- Van Overmeire, I., Clark, G.C., Brown, D.J., Chu, M.D., Cooke, W.M., Denison, M. S., *et al.*, 2001. Trace contamination with dioxin-like chemicals: evaluation of bioassay-based TEQ determination for hazard assessment and regulatory responses. *Environmental Science and Policy*, 4 (6), 345-357
- Van Overmeire, I., Loco, J.V., Roos, P., Carbonnelle, S. and Goeyens, L., 2004. Interpretation of CALIX results in view of the EU maximal TEQ level in milk. *Talanta*, 63(5), 1241-1247
- Weir, E., 2005. Public Health, Dioxin contamination and poisoning. *Canadian medical Association Journal*, 172 (7), 873
- Windal, I., Denison, M.S., Birnbaum, L.S., Wouwe, N.V., Baeyens, W. and Goeyens, L., 2005. Chemically Activated Luciferase Gene Expression (CALUX) Cell Bioassay Analysis for the Estimation of Dioxin-Like Activity: Critical Parameters of the CALUX Procedure that Impact Assay Results. *Environmental Science & Technology*, 39, 7357-7364
- Winters, D., 2003. Dioxin-like PCBs [Online]. United States Environment Protection Agency (US EPA), office of Pollution Prevention and Toxics. Available from: http://www.epa.gov/glnpo/bns/dioxin/pres/winters_dec2003.pdf [cited 27 November 2007]
- Xenobiotic Detection Systems Inc., 2004. Draft Method 44XX, Method for Toxic Equivalents (TEQs) Determinations for Dioxin-Like Chemical Activity with the CALUX by XDS Bioassay