Development of Immunoassays for the Detection of 2-Methylisoborneol and Monensin in Water Samples

by

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ABSTRACT

DEVELOPMENT OF IMMUNOASSAYS FOR THE DEVELOPMENT OF 2-METHYLISOBORNEOEL AND MONENSIN IN WATER SAMPLES

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Immunosassays for 2-methylisoborneol (MIB) and monensin in water were developed, devised and tested to see if the sensitivity could be established and improved. MIB and monensin are hydrophobic haptens with molecular weights of 168 and 671 Da, respectively. Rabbits were immunized with (-) camphor-BSA and (-) borneol-BSA for the production of polyclonal antibodies (pAbs) to MIB. Monoclonal antibodies (mAbs) were produced in Mus musculus using (-) camphor-BSA as immunogen. (+) Bornylamine-thyroglobulin (TG) and MIB-TG were synthesized and used as plate coatings. For the monensin immunoassay, monensin was conjugated to BSA and OVA for immunogen and plate coating, respectively. Several physical parameters that affect the sensitivity of immunoassays including pre-incubation of antibody and antigen, incubation time and temperature, detergent, organic solvents, and ionic strength were evaluated. Improvement of immunoassay sensitivity was also performed by reducing the concentrations of coating antigen and antibodies and using alternative reporter systems such as chemiluminescence (CICL-ELISA), tyramide signal amplification (TSA) and biotin-streptavidin. Different assay formats, i.e., competitive indirect and competitive direct were also compared. Usability of both pAb-based immunoassays for MIB and monensin was evaluated in fortified water samples.

A polyclonal-based (pAb) ELISA for MIB had a detection limit of 4.8 ng mL\(^{-1}\) and an IC\(_{50}\) of 105 ng mL\(^{-1}\). Rabbits immunized with (-) camphor-BSA showed a higher
immune response than rabbits immunized with (−) borneol-BSA. One clone (i.e., 4F11) of fourteen characterized clones was used to create the monoclonal antibody (mAb)-based ELISA, which had an IC$_{50}$ of 100.2 ng mL$^{-1}$ and an LOD of 1.9 ng mL$^{-1}$. The pAb- and mAb-based CI-ELISA were not specific to MIB alone and cross reacted with camphor and camphor-like compounds. Meanwhile, a pAb-based ELISA for monensin produced a detection limit of 0.1 ng mL$^{-1}$ and had an IC$_{50}$ of 1.056-1.090 ng mL$^{-1}$ with high specificity to monensin. Other reporter systems did not improve the sensitivity of the immunoassays significantly. MIB and monensin polyclonal-based assays showed good correlation to analytical instrumental methods (i.e., GC-MS and LC-MS) in fortified water samples.

With a detection limit of ca. 5 ng mL$^{-1}$ and 0.1 ng mL$^{-1}$ for MIB and monensin, respectively, both polyclonal-based assays can be used for detection of these analytes in water from different sources and employed as screening tools to complement GC/HPLC-MS instrument methods.

Keywords: immunoassay, polyclonal antibodies, monoclonal antibodies, 2-methylisoborneol, monensin
This thesis (Chapter 3, 4 and 5) is organized as manuscript to be submitted for publication in peer reviewed journals. Hence, a repetition of some information is unavoidable. All chapters were written by Rashidah Sukor as primary author and J. C. Hall as corresponding author. Editorial comments in Chapter 3 were contributed by Dr. J.C. Hall and Gabrielle Richard; Chapter 4 by Dr. J.C. Hall and Dr. Jyothi Kumaran and Chapter 5 by Dr. J.C. Hall, Gabrielle Richard and Shokouh Makvandi-Nejad. The Chapters are listed below:

**Chapter 3**: Sukor, R., Hall, J.C. 2013. Development of a polyclonal-based immunoassay for the detection of 2-methylisoborneol in water. (To be submitted to Journal of Agricultural and Food Chemistry)


**Chapter 5**: Sukor, R., Makvandi-Nejad, S., Veldhuis, L., Hall, J.C. 2013. Development and comparison of a chemiluminescence and direct immunoassays for the detection of monensin. (To be submitted to Journal of Environmental Quality)
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<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>ABA-atrazine</td>
<td>2-Aminobutylamino-4-ethylamino-6-isopropylamino-1,3,5-triazine</td>
</tr>
<tr>
<td>ACN</td>
<td>Acetonitrile</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>A&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximum absorbance in the absence of analyte</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>AP</td>
<td>Alkaline phosphatase</td>
</tr>
<tr>
<td>BA</td>
<td>(+) Bornylamine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CAFO</td>
<td>Concentrated animal feeding operations</td>
</tr>
<tr>
<td>CAP</td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>CARD</td>
<td>Catalyzed reporter deposition</td>
</tr>
<tr>
<td>CDI</td>
<td>Carbonyldiimidazole</td>
</tr>
<tr>
<td>CDR</td>
<td>Complementary determining region</td>
</tr>
<tr>
<td>C&lt;sub&gt;H&lt;/sub&gt;</td>
<td>Constant domain of heavy chain of IgG</td>
</tr>
<tr>
<td>C&lt;sub&gt;L&lt;/sub&gt;</td>
<td>Constant domain of light chain of IgG</td>
</tr>
<tr>
<td>CL</td>
<td>Chemiluminescent</td>
</tr>
<tr>
<td>CI-ELISA</td>
<td>Competitive indirect enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>CICL-ELISA</td>
<td>Competitive indirect chemiluminescence enzyme-linked immunosorbent assay</td>
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<tr>
<td>CLIA</td>
<td>Chemiluminescent immunoassay</td>
</tr>
<tr>
<td>CMC</td>
<td>1-Cyclohexyl-3-(2-morpholinoethyl) carbodiimide</td>
</tr>
<tr>
<td>CMO</td>
<td>O-(Carboxymethoxy) oxime</td>
</tr>
<tr>
<td>CR</td>
<td>Cross reactivity</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>CV</td>
<td>Co-efficient of variation</td>
</tr>
<tr>
<td>2,4-D</td>
<td>2,4-Dichlorophenoxy acetic acid</td>
</tr>
<tr>
<td>2,5-DHB</td>
<td>2,5-Dihydroxybenzoic acid</td>
</tr>
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<td>DCC</td>
<td>N,N'-Dicyclohexylcarbodiimide</td>
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<td>DDT</td>
<td>Dichlorodiphenyltrichloro-ethane</td>
</tr>
<tr>
<td>DES</td>
<td>Diethylstilbestrol</td>
</tr>
<tr>
<td>DIC</td>
<td>Diisopropyl carbodiimide</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
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<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<td>DON</td>
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<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>Fab</td>
<td>Antigen binding fragment of antibody</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FIA</td>
<td>Fluorescent immunoassay</td>
</tr>
<tr>
<td>FPIA</td>
<td>Fluorescence polarization immunoassay</td>
</tr>
<tr>
<td>FPLC</td>
<td>Fast protein liquid chromatography</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>4PL</td>
<td>Four-parameter log model</td>
</tr>
<tr>
<td>Fv</td>
<td>Variable region fragment</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography mass spectrometry</td>
</tr>
<tr>
<td>GSM</td>
<td>Geosmin</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>$^3$H</td>
<td>Tritium</td>
</tr>
<tr>
<td>HT</td>
<td>Hypoxanthine-thymidine</td>
</tr>
<tr>
<td>HAT</td>
<td>Hypoxanthine aminopterin thymidine</td>
</tr>
<tr>
<td>HS-SPME</td>
<td>Headspace solid phase microextraction</td>
</tr>
<tr>
<td>HQC</td>
<td>High quality control</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>$^{125}$I</td>
<td>Iodine-125</td>
</tr>
<tr>
<td>IC$_{50}$</td>
<td>Concentration of analyte that inhibit binding of an antibody by 50%</td>
</tr>
<tr>
<td>i.d.</td>
<td>Internal diamater</td>
</tr>
<tr>
<td>IPCR</td>
<td>immune polymerase chain reaction</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
</tr>
<tr>
<td>IgY</td>
<td>Immunoglobulin Y</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneally</td>
</tr>
<tr>
<td>IV</td>
<td>Intravenous</td>
</tr>
<tr>
<td>$K_A$</td>
<td>Affinity association constant</td>
</tr>
<tr>
<td>$K_D$</td>
<td>Affinity dissociation constant</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo dalton</td>
</tr>
<tr>
<td>KLH</td>
<td>Keyhole limpet hemocyanin</td>
</tr>
<tr>
<td>$K_{OW}$</td>
<td>Octanol/water partition coefficient</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography mass spectrometry</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>LC-MS/MS</td>
<td>Liquid chromatography coupled with tandem mass spectrometry</td>
</tr>
<tr>
<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Lethal dose</td>
</tr>
<tr>
<td>LLOQ</td>
<td>Lowest limit of quantification</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit of quantification</td>
</tr>
<tr>
<td>LPH</td>
<td><em>Limulus polyphemus</em> hemocyanin</td>
</tr>
<tr>
<td>LPME</td>
<td>Liquid-phase microextraction</td>
</tr>
<tr>
<td>LQC</td>
<td>Low quality control</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>MALDI-TOF</td>
<td>Matrix-assisted laser desorption/ionisation-time of flight</td>
</tr>
<tr>
<td>MDL</td>
<td>Method detection limit</td>
</tr>
<tr>
<td>MIB</td>
<td>2-methylisoborneol</td>
</tr>
<tr>
<td>MQC</td>
<td>Medium quality control</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cut-off</td>
</tr>
<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>NSB</td>
<td>Non-specific background</td>
</tr>
<tr>
<td>NZW</td>
<td>New Zealand white</td>
</tr>
<tr>
<td>OCP</td>
<td>Organochlorine pesticide</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>pAb</td>
<td>Polyclonal antibody</td>
</tr>
<tr>
<td>PAC</td>
<td>Powdered activated carbon</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffer saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffer saline with Tween 20 (0.05%)</td>
</tr>
<tr>
<td>PCB</td>
<td>3,3',4,4'-Tetrachlorobiphenyl</td>
</tr>
<tr>
<td>ppb</td>
<td>Part-per-billion</td>
</tr>
</tbody>
</table>
ppt  Part-per-trillion  
RI A  Radioimmunoassay  
R GR  Relative growth rates  
R SD  Relative standard deviation  
RT  Room temperature  
SB SE  Stir bar sorptive extraction  
sc F v  Single chain variable fragment  
SD  Standard deviation  
SDS-PAGE  Sodium dodecyl sulfate polyacrylamide gel electrophoresis  
S/N  Signal to noise ratio  
SPE  Solid phase extraction  
SP ME  Solid phase microextraction  
SPR  Surface plasma resonance  
TCP  3,5,6-trichloro-2-pyridinol  
TLC  Thin layer chromatography  
TMB  3,3',5,5'-tetramethylbenzidine  
TG  Thyroglobulin  
T & O  Taste and odor  
TSA  Tyramide signal amplification  
ULOQ  Upper limit of quantification  
US EPA  United States Environmental Protection Agency  
USERIA  Ultra-sensitive enzymatic radioimmunoassay  
V H  Variable domain of heavy chain of IgG  
V L  Variable domain of light chain of IgG  
v/v  Volume per volume  
WHO  World Health Organization
1 GENERAL INTRODUCTION AND RESEARCH OBJECTIVES

1.1 Introduction

Since the development of immunoassays in the 1960s, these assays are now being widely used as diagnostic and analytical tools for monitoring of organic chemicals as well as peptides and proteins. Immunoassays often provide reliable, accurate and timely data to regulatory agencies and general public, akin to that provided by analytical instrument-based methods, to assist in the decision-making process regarding environmental fate and persistence. Consequently, the demand for high quality antibodies for successful development of immunoassays with superior sensitivity and selectivity, wider measuring range, and increased precision is growing. Billions of dollars have been allocated in diagnostic industries to boost research and development in aiming for lower detection limits and better technologies for detection of complex molecules in intricate sample matrices. A Scopus search using ELISA as the keyword produced more than 200,000 references from 1970 to 2013 with largest number of articles being published in the last 10 years. Thus, although more than 40 years have passed since it was conceptualized, ELISA and immunochemistry still has its charm and holds a strong position as a subject of interest.

Most contaminants in food and the environment consist of small molecular weight organic compounds. Some of these contaminants may have adverse effects on flora and fauna as well as human health. The greater use of chemicals in households and industry has led to more frequent occurrence of these contaminants in water, soil and agricultural products (Buser, 1990; Dankwardt et al., 1995; Dejonckheere et al., 1996; Thurman et al., 1990). Therefore, it is crucial that the fate and persistence of these contaminants be monitored to determine whether they pose a risk to non-target organisms and humans.
In developing immunoassays, especially for hydrophobic haptens, challenges are often encountered. Hence, the research presented in this thesis explores these challenges.

The secondary metabolite of cyanobacteria, 2-methylisoborneol (MIB), is an off-flavor and off-odor compound. It causes major problems in channel catfish industry and large scale water supplies by causing unpleasant odor and taste. Tackling the issues of taste and odor (T&O) triggered by MIB has cost millions of dollars. At present, many producers and municipal councils turn to sensory analyses and analytical instrument-based methods to monitor the level of MIB in water supplies. Although these methods are sensitive, they are time consuming, costly and require sophisticated instruments that must be operated by highly trained personnel. A simple and rapid method such as ELISA to detect MIB in environmental and food samples at low concentrations is crucial to reduce the need for more costly analytical methods. This effort will be helpful and of immense benefit in terms of economy and productivity to the aquaculture industry and government to circumvent the problems associated with MIB.

Monensin is a polyether antibiotic used to treat coccidiosis infection in animals. There is a possibility of chronic exposure to monensin by aquatic organisms and adverse effects on terrestrial plant species. It has been detected in the environment especially in surface waters at trace levels. A rapid and simple technique such as ELISA may complement and offer advantages over conventional analytical techniques in detection of monensin.

Chapter two (Literature Review) offers background information regarding the hapten used in this study, i.e., 2-methylisoborneol and monensin, and describes the production of antibodies and challenges anticipated while working with them. Basic principles and important concepts in immunoassay development are addressed. Development and application of polyclonal and monoclonal antibodies against different haptens in food and the environment are given as examples. Different immunoassay
formats and importance of hapten heterology and homology with regard to improving the specificity and sensitivity of ELISA are discussed. Physical factors which are vital to sensitivity of an immunoassay are also addressed. This chapter also describes brief examples of some alternative reporter systems, which can be used to improve the sensitivity of immunoassays.

Development of polyclonal-based ELISA for MIB is presented in Chapter 3. This assay employs different haptens as immunogens and coatings. Performance of the ELISA under different physical conditions is presented. Different approaches are used to improve assay sensitivity. Utility of this assay was shown by conducting a precision and validation study. Extension of work on the development of immunoassay for MIB is presented in Chapter 4 using a monoclonal-based antibody. This work was done to improve the sensitivity and specificity of the polyclonal-based ELISA by finding clones with high affinity and specificity for MIB.

Polyclonal antisera for monensin have been previously prepared in Dr. Hall’s laboratory by Makvandi-Nejad et al. (2010). Using the antisera, we evaluated different ELISA formats and signal amplification systems to try and improve the sensitivity and performance of monensin ELISA (Chapter 5). In addition, development of polyclonal-based ELISA was also studied by testing different physical parameters, assay precision and validation in real water samples.
1.2 Research objectives

Immunochemical methods have proven to be important and effective screening and/or confirmation tools when coupled with other analytical methods for detection of various analytes such as organic contaminants, pesticide and drugs in many disciplines of science including therapeutic, food and environment industries.

The study aimed to achieve two main objectives. Firstly our goal was to develop a highly sensitive immunoassay for detection and quantification of 2-methylisoborneol using polyclonal and monoclonal antibodies. Once the immunoassays were optimized and the desired sensitivities of antibodies were achieved, the usability of the immunoassays was compared through validation using real samples and correlation studies with instrument-based methods. These studies were conducted with anticipation that the specific antibodies for MIB would assist and provide a more precise water-quality test in aquaculture industries and for regulatory authorities. With regards to MIB, the specific objectives were to:

1. Prepare hapten protein conjugates by crosslinking (-) camphor and (-) borneol to BSA and (+) bornylamine and MIB to TG and to use them to raise antibodies in animals and for plate coatings in ELISA.

2. Raise and produce polyclonal antibodies against MIB by immunizing rabbits with (-) camphor-BSA and (-) borneol-BSA as immunogens

3. Produce a more sensitive and specific assay through production of monoclonal antibodies.

Secondly, we wanted to examine the practical ability of different antibody-based detection systems that were adapted to analyze haptens in real sample matrices. This work was conducted using monensin immunoassays. With regards to monensin, the specific objectives were to:
1. Compare different immunoassay formats, i.e., indirect competitive versus direct competitive

2. Characterize, optimize and validate the assays through correlation with analytical instrument-based methods

3. Improve the sensitivity of ELISA using a chemiluminescence assay (CICL-ELISA)

In an immunoassay, sensitivity is much affected by the concentrations of coating antigen and the amount of antibody during the competition with the free hapten. Therefore, we hypothesized that lowering the concentrations of coating conjugate and antibody would increase the sensitivity of an ELISA. In addition, physical parameters such as time and temperature of incubation, pH, ionic strength, and detergent also impact the sensitivity of an ELISA. Many researchers have shown that detection of hapten at the part-per-billion (ppt) or part-per-trillion (ppt) level is possible using monoclonal antibodies (Knopp et al., 1999; Li et al., 2012; Matschulat et al., 2005). Hence, we hypothesized that monoclonal antibodies might increase the sensitivity of the ELISAs for 2-methylisoborneol when compared to ELISAs using the polyclonal antiserum. In addition, since sensitivity is also affected by the assay format, we also hypothesized that the competitive direct ELISA format would be more sensitive than the competitive indirect ELISA format. Furthermore, it was hypothesized that signal amplification system using different detector labels (i.e., chemiluminescence, biotin-streptavidin, and tyramide) would improve the sensitivity of an ELISA.
2 LITERATURE REVIEW

2.1 Introduction

With increased human population and activities, environmental contaminants, such as pesticides, fertilizers, drugs, hormones, antibiotics and heavy metals are being released into the environment. Concerns over the occurrence of these contaminants in environmental matrices especially in food and water supplies, are on the rise due to their reported adverse effects. In surface and groundwater, these contaminants are being monitored in response to increasing environmental and safety concerns such as the possibility of estrogenic or androgenic effects both to wildlife and human health (Richardson, 2007). Many of these contaminants are small molecules which are also known as haptens. For example, dichlorodiphenyltrichloro-ethane (DDT), an organochlorine pesticide (OCP), was successfully used in 1940 to control malaria, typhus and other insect-borne human diseases (US Environmental Protection Agency, 2011). With the publishing of Rachel Carson’s Silent Spring in 1962 and the accruing evidence of DDT’s environmental fate and toxicological properties, its application was banned in 1972 (US Environmental Protection Agency, 2011). Apart from chemicals which have been recognized for their adverse effects, some chemicals are not known to cause any detrimental aftermath to the environment or living organisms. However their presence in the environment may still be undesirable. For example, contamination of water supplies with geosmin and 2-methylisoborneol (MIB) are undesired because of the off-flavor and off-odor they cause. Therefore, such contaminants are also monitored to improve the palatability of drinking water.

The most common methods for identifying contaminants in food and environmental samples are gas and liquid chromatography (Malik et al., 2010). Over the years, these methods have allowed reliable and quantitative measurements of environmental contaminants with considerable sensitivity. However, conventional
methods are tedious, expensive, require long analyses time and a limited number of samples can be analyzed at one time. To overcome the shortcomings of conventional analytical methods, immunoassays have gained popularity for the detection of food and environmental contaminants. Immunoassays utilize the unique properties of antibodies to capture antigens and can be used to develop assays with high affinity and specificity. In principle, an antibody binding to an antigen works analogously to the key and lock concept, recognizing only their corresponding target with precise attachment. Since binding is based on the biophysicochemical properties of the antigen, antibodies will theoretically not bind to molecules that have different structures from the target analyte. In enzyme-linked immunosorbent assay (ELISA), the exclusive sensitivity of an antibody towards its antigen is detected using an enzyme-labeled antibody which is quantified colorimetrically. To date, several commercial immunoassay kits have been developed for the detection of contaminants, mainly in food and environmental matrices. Examples include kits for the detection of mycotoxins, veterinary drugs, algal toxins, biogenic amines, antibiotics, pesticides residues, and surfactants. Although immunoassays cannot compete with the fast progress and advances in modern instrumental analysis, the strength of immunoassays lies in their ability to screen a large number of samples for a single analyte within a short period of time (Hock et al., 1995). Furthermore, it allows the possibility of performing simple tests on site while keeping sensitivity and specificity at par with, or in some cases better than conventional methods.

This literature review will provide a brief overview of three main topic areas. In the first section, basic information regarding polyclonal and monoclonal antibody production for immunoassay development is presented. The second section will give the reader some basic principles and concepts of immunoassay development, while focusing on hapten monitoring. This topic includes some details about the characterization, optimization, and validation of immunoassays after the crucial reagents have been
produced. This section will also provide an insight into the production of reagents that are necessary for immunoassay development. The application of immunochemical techniques and immunoassay development for hapten detection in food and environment will be reviewed (e.g. Bonwick and Smith, 2004; Franek and Hruska, 2005; Jung et al., 1989; Morozova et al., 2005; Plaza et al., 2000; Rebe Raz and Haasnoot, 2011; Ricci et al., 2007; Shan et al., 2002; Spinks, 2000; Van Emon, 2010).

The third section provides background information on the two haptens, i.e., 2-methylisoborneol and monensin, which were used to develop immunoassays. Their mode of action, chemical properties, occurrence, method of detection and the importance of detecting them in the environment are discussed. The fourth section of this review discusses the different types of reporter systems that can be used to improve immunoassay sensitivity. Finally, a summary of importance and future perspectives of immunoassay development conclude the review chapter.

### 2.2 Production of antibody

#### 2.2.1 Basic antibody structure

Antibodies, also known as immunoglobulin G (IgGs, molecular weight of 150 kDa), are heterodimer proteins which are produced by B lymphocytes of the immune system of vertebrates as a defense mechanism against antigens. The basic structure of an antibody is composed of two identical heavy (H) chains and two identical light (L) chains that form a Y-shape. Heavy chains are composed of three constant domains (C\textsubscript{H}1, C\textsubscript{H}2 and C\textsubscript{H}3) and one variable domain (V\textsubscript{H}), while light chains are composed of only one constant domain (C\textsubscript{L}) and one variable domain (V\textsubscript{L}). The antigen-binding site is mostly formed by the combination of the complementarity-determining regions (CDRs, also known as hypervariable regions) of the V\textsubscript{H} and V\textsubscript{L} domains which account for most
of the variability and differences among antibodies. The part of the antigen that is recognized by the antibody is called the epitope (Kindt et al., 2006).

2.2.2. Generation of an immune response

In theory, any vertebrate could be used to produce antibodies. Animals such as sheep, goats, horses, and cows offer large volumes of antisera while smaller animals such as rabbits, mice, and rats are often used because they are easy to handle and can produce sufficient quantities of antisera for many applications. Rabbits and guinea pigs are often used because they are relatively inexpensive with regard to animal care and can provide 10 to 30 mL of total blood volume, which is often sufficient for most projects (Adrian, 2002).

Immunization schemes involve preliminary priming of animals subcutaneously, intramuscularly, or intradermally (main routes for rabbits) with an immunogen emulsified in adjuvant (normally Freund complete adjuvant), which helps to enhance the immune system to produce a higher amount of antibodies. Subsequent injections or boosts (usually with Freund incomplete adjuvant) are administered at two to five week intervals, depending on the animal species, until the antibody titer is stationary (Marco, 1995). Freund adjuvants are paraffin oil which contains manniode mono-oleate as surfactant and used as water-in-oil emulsion. In complete adjuvant, it contains heat-killed Mycobacterium tuberculosis which provides persistent release of antigens needed to stimulate high and strong immune responses in vertebrates (Freund and McDermott, 1942; Freund, 1947; 1956).

When the immune system of a vertebrate is exposed to a non-self antigen, it produces an immune response against the antigen(s). Circulating B-cells are normally dormant and express surface IgM receptors that have relatively low affinity for most types of antigen. These cells become activated when foreign antigen binds to their
surface antibody receptors and when co-stimulated with T-helper cells located in peripheral lymphatic organs. Once activated, B-cells can differentiate into plasma cells to produce soluble circulating antibodies or can undergo a process called class-switching to replace their heavy-chain isotypes, which alters the antibody’s effector functions. Furthermore, antibodies can undergo somatic hypermutation of their CDR regions to produce slightly mutated antibodies with heterogeneous affinities towards the antigen. As B-cells are clonal, each B-cell clone will produce one unique antibody sequence. Since different epitopic sites on the antigen will be recognized by different B-cells, the immune system will produce antibodies with heterogeneous specificity and affinity towards the antigen.

Antibodies are the principal reagent used in ELISAs. The initial step in immunoassay development involves the immunization of animals with the antigen(s) of interest for the production of specific antibodies. High quality antibodies are required to develop a useful immunoassay as they affect the overall quality and specificity of the assay. As discussed below, immunoassays can be developed which use either polyclonal or monoclonal antibodies.

2.2.3 Polyclonal antibodies

Animals can be stimulated to produce many different antibodies to different epitopes on a specific antigen and are known as polyclonal antibodies (pAbs) (Russo et al., 1988). Different epitopic sites on the antigen can activate different B-cell clones thus producing this heterogeneous immune response in terms of specificity and affinity. Moreover, polyclonal antibodies can include all of the IgG classes and IgM (Crowther, 1995; Hall et al., 1990). Polyclonal antibodies are contained in antiserum of the immunized animal.
Because of the heterogeneity of polyclonal antibodies, it is not possible for two animals of the same species to produce antibodies with identical specificity (Franek and Hruska, 2005). It is simple to obtain polyclonal antibodies, but the supply of antiserum is limited. Nevertheless, antiserum from a group of two to three rabbits, for instance, are often sufficient for the development of an assay for both research and development (Marco, 1995). Although there is a limited time during which antibodies are produced by immunized animals (i.e., animals life span; Hall et al., 1990), the assay can be used for years after the animal has died, as antiserum maintains its activity even after being frozen for many years (Lee and Kennedy, 2007).

2.2.4 Monoclonal antibodies

Advances in immunochemistry have allowed the generation of cloned immortalized cell lines from B lymphocytes expressing and secreting a single type of monoclonal antibody (mAb) by hybridoma technology (Kohler and Milstein, 1975). Hybridomas are created by fusing antibody-producing plasma cells isolated from spleenocytes of immunized animal with myeloma (cancerous B cells) that have lost their ability to produce antibodies. Since plasma cells grow poorly on their own, this cell fusion allows them to rapidly propagate, thereby secreting large quantities of a mAb into the growth medium. Monoclonal antibodies offer great advantages over polyclonal antibodies as they are homogeneous, i.e., they have uniform affinity and specificity. Furthermore, since they are grown in tissue-culture laboratories, they offer an unlimited supply of a specific antibody. In addition, the combination of multiple mAbs allows simultaneous detection of numerous analytes (Deshpande, 1996a; Plhak and Sporns, 1994). However, the process of obtaining mAbs is expensive and requires more time than preparing pAbs because it involves tedious tissue-culture work. Comprehensive
reviews and monographs on the production of mAbs have been published earlier (Chapney et al., 1992; Clementi et al., 1991; Zola, 1987).

Mice are typically used to produce hybridoma cell lines because they are easy to handle and have low housing cost. Hybridomas can also be grown in vivo in the peritoneum of live mice to produce ascites fluid containing large quantities of mAbs, up to 20 mg mL⁻¹, which is 100 to 1000-fold higher than *in vitro* methods (Munene, 2004). Since the research breakthrough of hybridoma work by Kohler and Milstein in 1975, mAbs from mice have been widely used in various fields of biology, especially in diagnostics and therapeutics. Other than mice, development of plasmacytoma from transgenic rabbits as fusion partner to generate rabbit mAbs has been reported (Spieker-Polet et al., 1995). Monoclonal antibodies from rabbit are advantageous in producing responses towards antigens which are not immunogenic in mice (Bystryn et al., 1982; Krause, 1970; Norby et al., 1987; Raybould and Takahashi, 1988; Weller et al., 1987). However, the production cost can be greater and the generation of a more stable rabbit fusion partner which is absent in both endogenous heavy and light chain genes is still in progress (Feng et al., 2011).

Several important factors are required for the successful generation of an immune response to generate good quality polyclonal or monoclonal antibodies against a hapten. They include the immunogenicity of the conjugate; the size, solubility, and composition of the hapten; the immunization protocol employed; the type of adjuvant used; the dose, mode and time constraints used; and the animal species employed (Egorov et al., 1991). No single method is the most practical and efficient for antibody production as many different types of immunogens and animals have been used (Marco, 1995). Polyclonal antibodies are often sufficient and suit research purposes and commercialization. Advantages and disadvantages for both antibody systems have been previously compared (Hall et al., 1990).
2.2.5 Anti-hapten antibodies

Haptens are small molecules with low molecular weight (usually less than 10 kDa). While they are considered antigenic they often lack immunogenicity and thus are incapable of inducing a specific immune response on their own (Kindt et al., 2006). Owing to the pioneer work of Landsteiner (1945) who was the first scientist to study immune response to a hapten, i.e., dinitophenol, the detection and quantitation of haptens has progressed rapidly. To generate a strong immune response, both B-cell and T-cell responses are required. However, due to the smaller molecular size of haptens, they lack the T-cell epitopes. Therefore, to generate an immune response against haptens, conjugation of a hapten to larger protein molecules may introduce a more complex epitope for generation and antibody recognition (Brinkley, 1992; Hall et al., 1990). Consequently, various types of antibodies against each target site of the hapten-carrier protein complex are produced in serum of the immunized animal. Three types of antigenic determinant will be produced from animals immunized with hapten-carrier conjugate: (1) unchanged epitopes on the carrier protein; (2) the hapten determinant, and (3) new epitopes formed by combination of both regions of the hapten and the carrier protein (Figure 2.1).

It is known that conformational changes occur when antibodies bind to antigens (Conway-Jacobs et al., 1970; Crumpton and Small, 1967; Schechter et al., 1971). Sagawa et al. (2005) showed that the binding stimulates conformational changes in the CH domains and hypothesized that hapten-binding induces a large change in spatial configurations between two Fab arms or between Fab arms and the Fc region. Hapten and short peptides tend to bind in a pocket or groove between heavy and light chain of variable regions through non-covalent interactions between 1-2 amino acids (KPL, 2005).
2.2.6 Kinetics of antibody-antigen interaction

The affinity (binding strength) and specificity of an antibody are critical factors when developing an immunoassay. The interaction of an antibody to its antigen is a reversible process that involves non-covalent interactions. An affinity constant (expressed as $K_a$) provides an insight on how satisfactory an antibody may function in an immunoassay (Lee and Kennedy, 2007). In a 1:1 binding interaction, formation of antibody and antigen complex is governed by the law of mass action (Harlow and Lane, 1988), and is represented in Equation 2-1:

$$
\frac{k_d}{k_a} \frac{[Ab] + [Ag]}{[Ab-Ag]} \quad (2.1)
$$
where [Ab] and [Ag] are the antibody and antigen concentration, respectively; [Ab-Ag] is the concentration of antibody-antigen complex; $k_a$ is the association rate constant and $k_d$ is the dissociation rate constant. The binding strength of an antibody can be expressed as a binding constant ($K_A$ or $K_D$). The affinity association constant $K_A$ is the ratio of $k_a/k_d$, while the dissociation constant $K_D$ is the ration of $k_d/k_a$ or $1/K_A$. The specificity of an antibody can be determined by comparing the binding constants at equilibrium which describes the ability of an antibody to preferentially form a complex with one molecule over another.

The interaction of antibody and antigen are often described as affinity and avidity. It is important to distinguish the difference between these two terms. Affinity of an antibody is the strength of the interaction between a single antigenic determinant and the homologous antibody binding site through several chemical bonds (Steward, 1984), which can be expressed in thermodynamic terms (Rudnick and Adams, 2009). Avidity, on the other hand, designates the binding force which exists between multivalent antigens and antibodies (Paraf and Peltre, 1991). Therefore, avidity is the collective strength of multiple affinities from several binding interactions, and is frequently referred to as functional affinity (Rudnick and Adams, 2009).

### 2.3 Enzyme immunoassay

The concept of ELISAs was introduced and developed by Peter Perlmann and Eva Engvall at Stockholm University (Sweden) while enzyme immunoassay (EIA) technique was brought forward by Anton Schuurs and Bauke van Weemen at the Research Laboratories of NV Organon, Oss, The Netherlands (Lequin, 2005). Although differing in assay designs, both ELISA and EIA use an enzyme as reporter system, as opposed to radiolabelled ligands pioneered in 1960 by Yalow and Berson (Lequin, 2005). Earlier, the term ELISA usually applied to antibody assays while EIA was the
nomenclature used exclusively to describe homogenous enzyme immunoassays (Schuurs and Van Weemen, 1977). An immunoassay is an analytical method or immunochemical technique that is based on the interaction of antibodies to antigens. The most commonly used type of immunochemical methods is enzyme-linked immunosorbent assay (ELISA). Immunoassays rely on the unique binding of antibodies with high specificity to its antigen according to the ‘key-lock’ model. Obeying the law of mass action, the antibody-antigen complex is joined together by the summation of weak interactions composed of electrostatic forces, hydrogen bonds and hydrophobic and Van der Waals interactions. The combination of these individual bonds leads to the stabilization of antigen-antibody complex (Deshpande, 1996a). ELISAs or EIA utilize enzymes such as horseradish peroxidase or alkaline phosphatase as markers attached to an antibody or antigen for the sensitive detection of analyte. In the early 1970s, ELISAs were adopted in environmental studies for the sensitive detection of pesticides including dithiothreitol (DDT), malathion and aminotriazole in ecosystems (Ercegovich, 1971). Over the next years, equipped with the possibility of synthesizing hapten-protein conjugates as immunogens for producing pAbs (Hammock and Mumma, 1980), immunoassays were developed for the detection of numerous contaminants in water, soil, and sediment (Bull et al., 1998; Gerlach et al., 1997; Knopp et al., 1999; Oubina et al., 1999; Roda et al., 1999). In fact, ELISA is the method of choice for the analysis of about 90% of pesticide samples (Gabaldon et al., 1999). Examples of polyclonal- and monoclonal-based immunoassays for determination of food and environmental contaminants are shown below (Table 2.1).
Table 2.1. Examples of immunoassay for the detection of hapten in food and environmental contaminants using polyclonal and monoclonal antibodies

<table>
<thead>
<tr>
<th>Compound</th>
<th>Sample</th>
<th>Type of antibody</th>
<th>Sensitivity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aflatoxin B1</td>
<td>Peanut, corn, pistachio, soybeans</td>
<td>pAb</td>
<td>$IC_{50} = 12 \pm 1.5 \mu g \text{kg}^{-1}$</td>
<td>(Lee et al., 2004)</td>
</tr>
</tbody>
</table>
| Atrazine              | Soil, corn shoot, cow milk             | pAb              | $IC_{50} = 17,500 \text{ng mL}^{-1}$  
LOD = 0.1 ng mL$^{-1}$ | (El-Gendy et al., 2011) |
| Azo dye               | Chili powder, chili oil, braised pork   | pAb              | LOD = 0.22 – 0.97 ng g$^{-1}$  
LOQ = 0.91 – 1.48 ng g$^{-1}$ | (Xue et al., 2012)         |
| Benzo[a]pyrene        | Potable water                          | mAb              | $IC_{50} = 0.065 \text{ng mL}^{-1}$  
LOD = 0.024 ng mL$^{-1}$ | (Matschulat et al., 2005) |
| Bococald              | Fruit juice                            | pAb              | LOD < 0.1 ng mL$^{-1}$  
LOQ = 10 ng mL$^{-1}$ | (Abad-Fuentes et al., 2012) |
| Bromoxynil            | Water samples                          | pAb              | LOD = 5 ng mL$^{-1}$ | (Cao et al., 2005) |
| Carbofuran            | Water, soil, food samples              | mAb              | $IC_{50} = 18.5 \text{ng mL}^{-1}$  
LOD = 0.11 ng mL$^{-1}$ | (Yang et al., 2008)         |
| Chloramphenicol       | Milk, meat, eggs                       | pAb              | LOD = 0.08 µg kg$^{-1}$ | (Kolosova et al., 2000)       |
| Chlopyrifos-methyl    | Grape, Chinese cabbage, water, soil    | mAb              | $IC_{50} = 75.2 \text{ng mL}^{-1}$  
LOD = 0.32 ng mL$^{-1}$ | (Qian et al., 2009)         |
| Cyhalothrin           | Water samples                          | pAb              | $IC_{50} = 37.2 \text{ng mL}^{-1}$  
LOD = 4.7 ng mL$^{-1}$ | (Gao et al., 2006)          |
| Cypermethrin          | Water samples                          | pAb              | $IC_{50} = 13.5 \text{ng mL}^{-1}$  
LOD = 1.3 ng mL$^{-1}$ | (Lee et al., 2004)          |
| Deltamethrin          | River water                            | pAb              | $IC_{50} = 17.5 \text{ng mL}^{-1}$  
LOD = 1.1 ng mL$^{-1}$ | (Lee et al., 2002)          |
| Deoxynivalenol (DON)  | Cereal                                 | mAb              | $IC_{50} = 61.1 \text{ng mL}^{-1}$  
LOD = 6.1 ng mL$^{-1}$ | (Li et al., 2012)           |
<table>
<thead>
<tr>
<th>Compound</th>
<th>Sample</th>
<th>Type of antibody</th>
<th>Sensitivity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dicamba</td>
<td>Water samples</td>
<td>pAb</td>
<td>IC$_{50}$ = 195 ng mL$^{-1}$; LOD = 2.3 ng mL$^{-1}$</td>
<td>(Clegg et al., 2001)</td>
</tr>
<tr>
<td>2,4-dichlorophenoxy acetic acid (2,4-D)</td>
<td>nd$^a$</td>
<td>mAb</td>
<td>LOD = 1000 ng mL$^{-1}$</td>
<td>(Tanaka et al., 2003)</td>
</tr>
<tr>
<td>2,4-dinitroaniline (2,4-DNA); 2,6-dinitroaniline (2,6-DNA)</td>
<td>Water, soil</td>
<td>mAb</td>
<td>IC$<em>{50}$ = 0.24 ± 0.06 ng mL$^{-1}$; IC$</em>{50}$ = 0.61 ± 0.08 ng mL$^{-1}$</td>
<td>(Kramer et al., 2008)</td>
</tr>
<tr>
<td>Fenthion</td>
<td>nd</td>
<td>pAb</td>
<td>IC$_{50}$ = 0.08 ng mL$^{-1}$</td>
<td>(Zhang et al., 2007)</td>
</tr>
<tr>
<td>Fluoroquinolone</td>
<td>Honey, eggs, chicken, shrimp</td>
<td>mAb</td>
<td>IC$_{50}$ = 3.9 – 23 ng mL$^{-1}$</td>
<td>(Wang et al., 2007)</td>
</tr>
<tr>
<td>Fluoroxypyr</td>
<td>Water, soil</td>
<td>pAb</td>
<td>IC$_{50}$ = 0.1 ng mL$^{-1}$; LOD = 1.6 ng mL$^{-1}$</td>
<td>(Johnson and Hall, 1996)</td>
</tr>
<tr>
<td>HT-2; T-2 toxin</td>
<td>Cereal, maize-based baby food</td>
<td>mAb</td>
<td>LOD = 25 - 26 µg kg$^{-1}$</td>
<td>(Meneely et al., 2010)</td>
</tr>
<tr>
<td>Imidacloprid</td>
<td>Water samples</td>
<td>pAb</td>
<td>IC$_{50}$ = 17.3 ng mL$^{-1}$</td>
<td>(Lee et al., 2001)</td>
</tr>
<tr>
<td>Metolcarb</td>
<td>Rice seed, tap water, soil</td>
<td>pAb</td>
<td>IC$_{50}$ = 40.7 ng mL$^{-1}$; LOD = 1.4 ng mL$^{-1}$</td>
<td>(Zhang et al., 2008)</td>
</tr>
<tr>
<td>Metsulfuron-methyl</td>
<td>Water samples</td>
<td>pAb</td>
<td>IC$_{50}$ = 1.4 ng mL$^{-1}$; LOD = 0.04 ng mL$^{-1}$</td>
<td>(Knopp et al., 1999)</td>
</tr>
<tr>
<td>Microcystin; nodularins</td>
<td>Water samples</td>
<td>pAb</td>
<td>LOQ = 0.02- 0.07 ng mL$^{-1}$</td>
<td>(Fischer et al., 2001)</td>
</tr>
<tr>
<td>Nisin A</td>
<td>nd</td>
<td>pAb</td>
<td>IC$_{50}$ = 300-500 ng mL$^{-1}$; LOD = 5 – 100 ng mL$^{-1}$</td>
<td>(Suarez et al., 1996)</td>
</tr>
<tr>
<td>Nitrofuran</td>
<td>Animal feed</td>
<td>pAb</td>
<td>LOD = 5 - 16 µg kg$^{-1}$</td>
<td>(Li et al., 2010)</td>
</tr>
<tr>
<td>Ochratoxin A</td>
<td>Chicken meat, wheat fluor, porcine plasma, bovine serum</td>
<td>mAb</td>
<td>LOD = 0.1 – 1 ng g$^{-1}$</td>
<td>(Kawamura et al., 1989)</td>
</tr>
</tbody>
</table>

$^a$ not detected
<table>
<thead>
<tr>
<th>Compound</th>
<th>Sample</th>
<th>Type of antibody</th>
<th>Sensitivity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organophosphorus pesticide</td>
<td>nd</td>
<td>pAb</td>
<td>IC₅₀ = 70-7000 ng mL⁻¹</td>
<td>(Kim et al., 2003)</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>Yew tree</td>
<td>mAb</td>
<td>LOD = 98-31,200 ng mL⁻¹</td>
<td>(Chao et al., 2013)</td>
</tr>
<tr>
<td>Picloram</td>
<td>River water, soil, plant, urine</td>
<td>mAb; pAb</td>
<td>IC₅₀ = 10 ng mL⁻¹; LOD = 1 ng mL⁻¹; IC₅₀ = 140 ng mL⁻¹; LOD = 5 ng mL⁻¹</td>
<td>(Deschamps et al., 1990)</td>
</tr>
<tr>
<td>Ractopamine</td>
<td>Swine feed</td>
<td>mAb</td>
<td>LOD = 0.24 µg g⁻¹</td>
<td>(Wang et al., 2006)</td>
</tr>
<tr>
<td>Sudan I</td>
<td>Food samples</td>
<td>pAb</td>
<td>IC₅₀ = 0.3 - 2 ng mL⁻¹; LOD = 0.02 - 0.1 ng mL⁻¹</td>
<td>(Han et al., 2007)</td>
</tr>
<tr>
<td>Sudan II</td>
<td>Egg</td>
<td>mAb</td>
<td>LOD = 0.2 - 0.5 ng g⁻¹</td>
<td>(Liu et al., 2012)</td>
</tr>
<tr>
<td>Sulfonamides</td>
<td>Milk</td>
<td>pAb</td>
<td>LOD = 1.3 - 90 ng mL⁻¹</td>
<td>(Zhang et al., 2006)</td>
</tr>
<tr>
<td>Tetrabromobisphenol A</td>
<td>Soil, sediment</td>
<td>pAb</td>
<td>LOD = 0.05 ng mL⁻¹</td>
<td>(Xu et al., 2012)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>Honey</td>
<td>pAb</td>
<td>LOD = 0.4 ng mL⁻¹</td>
<td>(Pastor-Navarro et al., 2007)</td>
</tr>
<tr>
<td>Triazoles</td>
<td>Fruit juice</td>
<td>mAb</td>
<td>LOD = 0.3 ng mL⁻¹</td>
<td>(Manclus et al., 2008)</td>
</tr>
<tr>
<td>Triclopyr</td>
<td>Water, soil</td>
<td>pAb</td>
<td>IC₅₀ = 0.1 ng mL⁻¹</td>
<td>(Johnson and Hall, 1996)</td>
</tr>
<tr>
<td>Triclosan</td>
<td>Surface water, wastewater</td>
<td>pAb</td>
<td>IC₅₀ = 3.85 ng mL⁻¹</td>
<td>(Brun et al., 2008)</td>
</tr>
<tr>
<td>Trifloxystrobin</td>
<td>nd</td>
<td>mAb</td>
<td>LOD = 0.1 - 0.21 ng mL⁻¹</td>
<td>(Mercader et al., 2008a)</td>
</tr>
<tr>
<td>Zearalenone</td>
<td>Wheat</td>
<td>mAb</td>
<td>IC₅₀ = 0.8 ng mL⁻¹; LOD = 0.1 ng mL⁻¹</td>
<td>(Burmistrova et al., 2009)</td>
</tr>
</tbody>
</table>
2.3.1 Immunoassay format

The two basic types in immunoassay are competitive and non-competitive formats. In the scope of this thesis, we will focus on the competitive format as it is the most prevalent format used for the quantification of hapten; i.e., direct competitive and indirect competitive ELISA formats. The third format which is known as sandwich ELISA will be briefly described.

2.3.1.1 Direct competitive ELISA

Direct competitive format necessitates the immobilization of antibody to the microtiter plate. The signal is generated based on the competitive binding of free and enzyme-labelled antigen in solution towards the immobilized antibody. The competition signal yields the total binding of hapten-enzyme conjugate to the antibody, thereby producing a signal which is indirectly proportional to the amount of free antigen added (Johnson and Hall, 1996; Parnell and Hall, 1998). Normally, a specific antibody towards the antigen is purified since other serum proteins may interfere with the binding to the plate surface thus reducing the signal. The antibody can either be bound directly on microtiter plates or immobilized via pre-coated protein A or protein G microtiter plates. Johnson and Hall (1996) demonstrated that by pre-coating the wells with protein A, the herbicides fluoroxypyr and triclopyr were detected at 0.1 ng mL\(^{-1}\) with an IC\(_{50}\) of 1.6 and 1.7 ng mL\(^{-1}\), respectively. This strategy assists in proper orientation of IgG to the plate surface thus exhibiting greater sensitivity. Direct competitive ELISA formats are simple, rapid and use fewer reagents; however, this system requires the availability of haptens labeled with enzymes for detection.

Direct ELISA formats can also be performed by directly crosslinking haptens through their functional group(s) to the surface of the microtiter plate. This approach has been shown to improve the assay sensitivity by 10-fold for atrazine (IC\(_{50}\) = 0.8 ng mL\(^{-1}\))
and 2,4-dichlorophenoxyacetic acid (IC$_{50}$ = 7 ng mL$^{-1}$) herbicides. Through modification of the polystyrene surface using acid and 3-aminopropyltriethoxysilane (APTES), amino groups are presented on the plate to be linked covalently with the carboxyl group of the haptens (Kaur et al., 2008). By applying the same direct hapten coating concept, a sensitive assay (IC$_{50}$ of 0.130 ng mL$^{-1}$) for terbutryn was achieved when a glutaraldehyde treated plate was covalently linked to 2-aminobutylamino-4-ethylamino-6-isopropylamino-1,3,5-triazine (ABA-atrazine) (Holthues et al., 2001).

2.3.1.2 Indirect competitive ELISA

For indirect competitive immunoassays, either the antigen or the antibody is immobilized on the microtiter plate. For small molecular weight molecules or haptens, direct immobilization is not possible because of the small size and limited number of epitopes on the surface. Therefore, haptens must be conjugated to carrier proteins before being passively adsorbed to microtiter plates. A pre-incubation step (which we call inhibition ELISA) may be incorporated to increase the sensitivity of the immunoassay. In this step, the antibody is pre-incubated with free antigen for a certain period of time prior to being added to the coated plate. Competition can also be performed by addition of free antigen in solution and antibody simultaneously. This will allow competitive binding of free antigen in the solution with the immobilized antigen on the plate surface towards antibody. A secondary antibody with an enzyme label that is specific to the animal species of the primary antibody is then added to produce a detectable signal. After the addition of the chromogenic substrate, the color intensity is inversely proportional to the concentration of the free antigen. This format is widely used to detect environmental and food contaminants due to its versatility. In addition, it is easier to control or manipulate the coating antigen and primary antibody to increase
immunoassay sensitivity without affecting the enzyme-labelled secondary antibody. Furthermore, for haptens, there is no requirement to purify the antibody.

### 2.3.1.3 Sandwich ELISA

A third format, which is called sandwich immunoassay, is performed by ‘sandwiching’ the antigen between an immobilized antibody and a free antibody in solution. In sandwich immunoassays, a specific antibody is first immobilized on the microtiter plate and then antigen is added and allowed to bind. Thereafter, a second antibody, usually enzyme labelled, which is specific to a different epitope on the antigen is added for signal generation. This format is normally used for the detection of larger molecules such as proteins that have a minimum of two distinct epitopes where no antibody competition would be required. However, this format is not suitable for haptens because of their limited number of epitopes and the difficulty in exposing more than one epitope on the hapten surface.

### 2.3.2. Heterogeneous and homogeneous assays

Competitive assays can either be heterogeneous or homogeneous. In homogeneous assays, no physical separation of free and bound hapten is needed and the system can be adjusted to allow automation. Fluorescent polarization immunoassay (FPIA) is an example of a homogeneous assay where free hapten and tracer are added simultaneously within the same solution to compete for antibody binding. On the other hand, in heterogeneous assay, the free and bound haptens are physically separated before the signal can be measured. Examples of heterogeneous assays are radioimmunoassay and an ELISA. Both large and small molecules can be detected using a heterogeneous system, while homogeneous assays are better suited for the detection of small molecules (Kumar, 2010).
2.4 Development of enzyme immunoassay

There are a number of critical steps involved in the development of a robust immunoassay. They comprise the preparation of the immunogen, which includes hapten synthesis and characterization, the immunization schedule, and the isolation and characterization of high affinity antibodies. Immunoassays also need to be validated to evaluate the robustness of the system for detecting the analyte of interest in the given matrices. Validation includes the overall assay performance, the comparison with established analytical methods, the stability of the assay, the ability to transfer the technology to the end user, and the ability to follow-up on assay performance (Lee and Kennedy, 2007). Some of the important features of developing an immunoassay are discussed below.

2.4.1 Preparation of hapten

2.4.1.1 Homology and heterology of hapten

As stated earlier, haptens are unable to function as an immunogenic epitope and conjugation to larger proteins is required. Here, we discuss the importance of hapten design for producing a sensitive immunoassay. To design hapten conjugates that elicit the production of high affinity and specific antibodies in animals, many crucial factors need to be considered. Two different schemes, called homology and heterology, can be used when designing haptens for use in an immunoassay.

Hapten homology is the scheme when the same hapten is employed to make the immunogen and coating antigen. This system is considered when the molecular structure of the hapten is unique and when cross reactivity with other structurally related molecules is not an issue. Sensitive immunoassays for diazinon (Ferguson et al., 1993), chlorpyrifos (LOD = 0.2-0.6 ng mL\(^{-1}\); Hill et al., 1994), aflatoxin B\(_1\) (LOD = 5-100 ng mL\(^{-1}\);
Chu et al., 1987; IC$_{50}$ = 9-12 µg kg$^{-1}$; Lee et al., 2004b) and ochratoxin A (LOD= 50 pg mL$^{-1}$; Kawamura et al., 1989) have been developed using homologous systems. In contrast, a heterologous system is when the hapten used for immunization is different from the target analyte or coating antigen. This is not limited to the hapten structure, but also applies to different bridges, spacer arm length, and attachment site. Many researchers opt for this scheme as variation in hapten design has a better chance of producing more sensitive immunoassays (Kim et al., 2003a; 2003b; Kolosova et al., 2000; Liang et al., 2008). For example, for the detection of chloramphenicol (CAP) in food, the influence of immunogen structure and composition (CAP succinate and diazo derivative of CAP) including the variation of molar ratio of CAP into coating conjugate was evaluated (Kolosova et al., 2000). By using this approach, it was possible to achieve a detection limit in the range of 0.08–100 ng mL$^{-1}$ for CAP. Furthermore, when a shorter spacer arm was used versus a longer one for the immunogen, the sensitivity for thiocarbamate immunoassay was improved by 100-fold (Gee et al., 1990). Valuable references are available (Hermanson, 2008; Thermo Scientific, 2009; Wong and Wong, 1992) which discuss the technical aspects, techniques and fitness of each system.

2.4.1.2 Coupling of hapten

The design of an appropriate hapten to a carrier protein is a crucial step in immunoassay development. The most appropriate hapten is the one that preserves steric and electronic characteristics of the molecule and structurally mimics the target analyte as closely as possible (Lee and Kennedy, 2007). Coupling usually involves the activation of functional groups on the hapten, the most common ones being –COOH, -NH$_2$,-OH, and -SH (Clegg, 2002), and their linkages to Lys residues of the carrier protein (as immunogen) or enzyme (as detector) through covalent bonding (Schneider et al., 1995). In cases where functional groups are not available on the hapten molecule,
such groups can be introduced followed by activation and subsequent (Fasciglione et al., 1996) coupling to the carrier protein (Erlanger, 1980). The procedure selected depends on the nature of functional groups present.

Technical challenges are often encountered for coupling lipophilic haptens due to their hydrophobic nature. These haptens may react and hidden within the hydrophobic hidden domains of the carrier protein, thereby not being exposed to B-cells in the animal for antibody production (Fasciglione et al., 1996). Normally, addition of water miscible organic solvent is vital; however, this introduces additional difficulties which lead to a low yield of conjugates (Erlanger et al., 1957; 1959).

To enhance greater antibody recognition, a linker or spacer arm is normally attached to offset the hapten from the protein surface. Employment of a spacer arm has been shown to increase antibody titers in mice by three times (Clementi et al., 1991). Nonetheless, hapten-protein conjugate without a linker has been described with success (Ercegovich et al., 1981; Hammock and Mumma, 1980; Jung et al., 1989; Kawamura et al., 1989; Newsome and Collins, 1990). Three to six carbon alkyl chains are commonly used in hapten design (Lee and Kennedy, 2007). Eight or more carbons spacer arms are not generally used as they minimize antibody recognition; hence there is less probability of producing useful antibodies especially in hydrophobic environments (Harlow and Lane, 1988). A brief summary of commonly used spacer arms and chemical groups for hapten-carrier conjugates is shown in Table 2.2.
### Table 2.2. Bifunctional cross-linkers and chemical groups commonly used for conjugation of haptens

<table>
<thead>
<tr>
<th>Types of cross-linker</th>
<th>Target functional group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bis (sulfosuccinimidyl) suberate</td>
<td>Amine</td>
</tr>
<tr>
<td>Bis(diazobenzidine)</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>Dimethyl Adipimidate</td>
<td>Amine</td>
</tr>
<tr>
<td>Dimethyl Pimelimidate</td>
<td>Amine</td>
</tr>
<tr>
<td>Dimethyl Suberimidate</td>
<td>Amine</td>
</tr>
<tr>
<td>Disssuccinimidyl Suberate (DSS)</td>
<td>Amine</td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td>Amine</td>
</tr>
<tr>
<td>(m)-Maleimidobenzoyl-(N)-Hydroxysuccinimide</td>
<td>Amine to thiol</td>
</tr>
<tr>
<td>Sulfo-(m)-maleimidobenzoyl-(N)-hydroyxysuccinimide</td>
<td>Amine to thiol</td>
</tr>
<tr>
<td>Sulfo succinidyl 4-((N)-Maleimidomethyl) Cycloheane-1-carboxylate</td>
<td>Amine to thiol</td>
</tr>
<tr>
<td>Sulfo succinimidyl 4-((p)-Maleimido-Phenyl) Butyrate</td>
<td>Amine to thiol</td>
</tr>
<tr>
<td>(N)-hydroxysuccinimide (NHS)</td>
<td>Carboxyl</td>
</tr>
<tr>
<td>Dicyclohexyl carbodiimide (DCC)</td>
<td>Carboxyl</td>
</tr>
<tr>
<td>Diisopropyl carbodiimide (DIC)</td>
<td>Carboxyl</td>
</tr>
<tr>
<td>1-Cyclohexyl-3-(2-morpholinoethyl) carbodiimide (CMC)</td>
<td>Carboxyl</td>
</tr>
<tr>
<td>1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC)</td>
<td>Carboxyl</td>
</tr>
<tr>
<td>Diazomethane or other diazoalkyl derivaties</td>
<td>Carboxyl</td>
</tr>
<tr>
<td>(N,N)-Carbonyldiimidazole (CDI)</td>
<td>Carboxyl or hydroxyl</td>
</tr>
</tbody>
</table>

Source: (Harlow and Lane, 1988; Shan et al., 2002)
2.4.1.3 Carrier protein

Most proteins selected are usually large molecules, highly immunogenic, hydrophilic, easily available and possess an abundance of functional groups to allow multiple site cross-linking (Adrian, 2002; Hermanson, 2008). Keyhole limpet hemocyanin (KLH; MW 4.5 x 10^5 to 1.3 x 10^7 kDa), bovine serum albumin (BSA; MW 67 kDa), aminoethylated (or cationized) BSA (cBSA), thyroglobulin (TG; MW 660 kDa), ovalbumin (OVA; MW 43 kDa) and various toxoid proteins, including tetanus toxoid and diphtheria toxoid are the most common carrier proteins used for hapten conjugation (Hermanson, 2008). Epitope density or hapten to protein ratio is an important criterion and should be determined before immunization. This can be performed through several protocols which include using radiolabeled haptens, spectrophotometrically measuring free amino groups with trinitrobenzenesulphonic acid (Habeeb, 1966) before and after the conjugation, and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry.

With respect to epitope density, Landsteiner (1945) concluded that too much or too little hapten to protein density leads to poor antibody response against the hapten. It was suggested that 10 hapten molecules are optimal for each serum albumin molecule. A 4:1 molar excess of epitope density has been proposed to achieve better antigenicity. Higher epitope density may result in a lower affinity antibody, although some have reported success with a ratio as high as 40:1 (Adrian, 2002). In addition, higher epitope density may also lead to inter- and intra-molecular cross linking during conjugate preparation (Jin and Karol, 1988). Using steroid protein conjugates, Niswender and Midgley Jr. (1970) suggested that at least a 20:1 hapten to BSA ratio is sufficient for a good antibody response. Good antibody titers can usually be obtained with epitope densities anywhere between 8 and 25 (Erlanger, 1980). Thus, it is clear that the epitope
density that leads to a successful immune response is subjective since other factors such as the nature of the hapten also play an important role.

2.4.1.4 Selection of animal species

Most of the information on the species of animals to use has been mentioned earlier in Section 2.2.2. The most important criteria for the selection of animals should be based on the amount of antibody required, whether polyclonal, monoclonal or recombinant antibodies are desired, the proposed plan on how the antibody will be used (i.e., direct vs. indirect assay), the cost of purchase and care of animals, and the ease of animal handling (Australian Animal Welfare Committee, 2005; Sheedy and Yau, 2011). Other than rabbits and mice, which are normally used for antibody production, several other animal species have been used to raise antibodies including sheep, goat, cow, horse, chicken, and species of Camelidae family.

2.4.2 Characterization and optimization of immunoassay

2.4.2.1 Sensitivity of the immunoassay

The strict regulations to minimize contaminants in food and the environment demand that immunoassays have superior sensitivity. For instance, the European Community (2000) has regulated that the maximum residue level (MRL) for individual pesticides in drinking water be below 0.10 μg L⁻¹ (Lee and Kennedy, 2007). Sensitivity is commonly described or reported as an IC₅₀, which is the concentration of free analyte that is able to inhibit antibody binding by 50%. The limit of detection (LOD) is the lowest concentration of free analyte which is able to compete with the analyte for antibody binding. It is statistically calculated as the mean absorbance of solution in wells that do not receive any free analyte (i.e., zero competition), (A₀) ± 3 standard deviations (SD), while the limit of quantification (LOQ) is expressed by the mean (A₀) ± 10 SD (Long and
Some researchers have also reported their limit of detection by IC\textsubscript{15} or IC\textsubscript{20} values from their standard curves (Fang et al., 2011; Jiang et al., 2011; Li and Jiang, 2012).

To obtain the most sensitive assays, several important factors must be determined. The most crucial factor is the affinity of an antibody. With a biochemically unchanged antibody at an optimal physiological condition, the affinity constant has a fundamental value which cannot be changed or improved and is ultimately responsible for the sensitivity of the immunoassay (Bonenberger and Doumanas, 2006). However, affinity of an antibody may be altered or reduced due to modification of the antibody molecule, such as labeling, which may lead to steric hindrance resulting in loss of affinity (Davies et al., 1988).

Decreasing background noise by optimizing the blocking and washing procedures may directly diminish non-specific binding, thus improving the sensitivity of the immunoassay. Improvement of low signal in ELISA can be achieved through prolonging incubation times to achieve binding equilibrium and incorporation of direct labeling of antibody by using chromogenic to chemiluminescent agents (Hammock and Mumma, 1980; Van Emon et al., 1986). However, this method could also lead to substantial background noise due to the high sensitivity of the label. Improvement of immunoassay sensitivity can also be achieved using different reporter system other than enzymes. Some of these systems are described in detail in Section 2.5. In addition, testing different types of plate materials or modification of the plate surface through covalent linking of analyte may also improve the signal to noise ratio (Hammock and Mumma, 1980; Van Emon et al., 1986).

The sensitivity of immunoassays can also vary widely depending on the quality and type of antibody used, the tagged reagent(s), and the format employed. Generally, direct ELISA formats provide a greater sensitivity than indirect formats. This is mainly
due to the higher non-specific binding of the antibody to the reagents used in the indirect format thus reducing immunoassay sensitivity (Lee and Kennedy, 2007). Schneider and Hammock (1992) compared four different immunoassay formats for s-triazine herbicide and observed that the most sensitive format was using pre-coating steps with either goat anti-mouse antibodies or protein A prior to the addition of the antibody. Another approach named ultra-sensitive enzymatic radioimmunoassay (USERIA), which combined the elements of ELISA and radioimmunoassay achieved a detection limit as low as $10^{-21}$ M of cholera toxin as compared to ELISA with detection limit of $10^{-18}$ M of the toxin (Harris et al., 1979). This technique was also found to improve the sensitivity of ELISA by 100-fold and RIA by 1000-fold for the detection of rotavirus (Harris et al., 1979).

The reaction between solid phase and proteins also plays a role in determining assay sensitivity. Immobilization of antibody onto a microtitre plate will significantly reduce the rate of equilibrium due to the limited surface area compared to the relatively large reaction volume in the microtitre wells (Newman and Price, 1997). Moreover, this also changes the intrinsic $K_A$ of the reaction (Karlsson et al., 1994; Olson et al., 1989; Spitznagel and Clark, 1993). As proteins are not indefinitely stable on solid surfaces, adsorption of either antibody or antigen to polystyrene surfaces will vary depending on the mechanism of attachment. Adsorbed proteins may denature or orient improperly which can greatly influence the sensitivity. Both antigen and the antibody and the rate of denaturation on the surface are crucial factors, which determine the binding of both molecules to a solid surface, qualitatively and quantitatively (Pesce et al., 1978).

2.4.2.2 Specificity of the immunoassay

Although the binding of antibody to an antigen is regarded as highly specific, an antibody that is raised for a particular antigen might cross-react with an unrelated
antigen that shares an identical epitope or a similar structure. Specificity of an assay is defined as the ability of an antibody to recognize and distinguish only the analyte of interest. It is usually represented by percentage of cross reactivity, which measures the antibody binding to other structurally related compounds. Specificity or cross-reactivity analyses are crucial steps in assay evaluation to provide an insight into the performance and the uniqueness of an antibody recognizing a single epitope or molecule.

Many proteins have structurally related molecules with highly conserved epitopes, while haptens, such as steroid hormones, have many structurally related compounds (i.e., different epitopes) that are similar to each other. This is also the case for many drugs and their metabolites (Davies, 2005). Antibody specificity may also be influenced by different congeners and side chains of an analyte. For example, congener-independent immunoassays have been developed for microcystins and nodularins by Fischer et al. (2001). Previously it has been shown that pAbs (An and Carmichael, 1994; Chu et al., 1989; McDermott et al., 1995) and mAbs (Nagata et al., 1995; Saito et al., 1994; Tsutsumi et al., 1998; Ueno et al., 1996) cross-reacted with other microcystin and/or nodularin analogues, which may have contributed to false negatives when toxin congeners were present. For analytes with similar structure to their parent and associated metabolites, the utilization of cross-related analytes in the intended matrix is crucial for determining the assay specificity where detection of both parent analyte and their metabolites are equally important (Lee and Kennedy, 2007).

It is important to note that the specificity of an antibody is directed towards sites on a hapten which are distal to the point of linker attachment (Hammock and Mumma, 1980; Landsteiner, 1945; Skeritt and Lee, 1996). The chemistry of the hapten-carrier protein conjugation, thus, has a major influence on the specificity of an immunoassay. Cross-reactivity can be reduced by careful preparation of pAbs or utilization of mAbs (Allen and Smith, 1987).
2.4.2.3 Characterization of the immunoassay

Characteristics which are important for the performance of an immunoassay include: sensitivity, specificity, accuracy, precision and reproducibility, dynamic behavior, matrix interference, and stability of the antibody (Bansal and DeStefano, 2007; Clark and Engvall, 1980; Food and Drug Administration, 2001; Lee and Kennedy, 2007). Calibration curves or dose-response curves are plotted to present the competitive binding of antigen to antibody, which corresponds to the concentrations of analyte present. These curves are in sigmoidal in shape and are best described using nonlinear curve fitting algorithms for experimental data (Findlay et al., 2000).

The accuracy and precision of an immunoassay should be more lenient than an analytical method since the core of the assay is based on the antigen-antibody reaction (Findlay et al., 2000). Literature on the validation of bioanalytical methods of micro and macromolecules are available (Bansal and DeStefano, 2007; European Medicine Agency, 2007; Findlay et al., 2000; Gee et al., 1994; Geng et al., 2005; Lipton et al., 2000; Mire-Sluis et al., 2004; Shankar et al., 2008). However, thorough and extensive guidelines for the development and validation of immunoassays specific for haptens are limited to date (Lee and Kennedy, 2007). Despite this fact, many immunoassay test kits have been available for various food and environmental contaminants such as pesticides, since the 1980s (Gabaldon et al., 1999).

2.5 Alternative reporter systems

Immunoassays require the use of labelled material or a reporter to produce a signal that allows the measurement of antigen or antibody. Many types of reporter systems or labels have been used for detection of analyte in immunochemistry. The detection system selected is based on the objectives of the study, sample matrices,
availability of instruments, length of time of the method and ease of use. Some of the systems are briefly discussed below.

2.5.1 Radioimmunoassay

The first radioimmunoassays (RIA) were introduced in 1960 using $^{131}$I as a label for the detection of insulin (Yalow and Berson, 1960) and thyroxine (Ekins, 1960). Since 1960 radioimmunoassays remain one of the most sensitive detection methods for analytes, especially in clinical applications, and are capable of measuring hormones, serum proteins, drugs and vitamins at 1 ng mL$^{-1}$ or less (Kindt et al., 2006).

Radioimmunoassays use radioactive isotopes, usually tritium (${}^{3}$H) or iodine-125 (${}^{125}$I) as labels to detect either the antigen or antibody. Miles and Hales (1968) published their first results about an “immuno-radiometric” technique for measuring insulin in human plasma using radiolabeled antibodies rather than labeled antigen. For hapten RIA, a radiolabeled antigen competes with an unlabeled antigen for binding with the antibody and is measured through beta or gamma ray emission by these isotopes usually using a liquid scintillation counter. RIA instrumentation is expensive, and their application is limited due to the strict regulation of radioactive waste, which has potential health and safety risks. Due to these drawbacks, ELISAs have largely replaced radioimmunoassay techniques in most laboratories (Schuurs and Van Weemen, 1977).

2.5.2 Fluorescent immunoassay

Antibodies labelled with fluorophores have been used since the 1940s, first being described by Coons and co-workers (1941) for histological immunofixation techniques. In fluorescent immunoassays (FIA), substituting the enzyme for a fluorophore as a detector has been proven to have greater analytical sensitivity. Fluorescent molecules or fluorophores absorb light (excitation) of one wavelength and emit light (emission) at
another wavelength (Kindt et al., 2006). Fluorescence polarization immunoassay (FPIA) is one of methods of FIA which is homogenous or based on non-separation technology without the need to incorporate washing steps. The theory was first described by Perrin (1926) and expanded with the development of the instrumentation by Weber (1952). It is based on the principle that in solution, all molecules rotate with a rate inversely proportional to its size. The excitation and emission cycle are assisted by an intramolecular electronic process or oscillating dipole with a defined planar orientation (Wood and Barnard, 1997). When molecules are excited by polarized light, they will release energy, which will be absorbed by molecules possessing oscillating dipoles parallel to the plane of the polarized light (Dandliker et al., 1973; Rhys Williams, 1988). A small fluorescent-labelled antigen (tracer) will increase in fluorescence when bound by a specific antibody. In a competition assay, non-labeled antigen competes with a tracer for antibody binding. The presence of a higher concentration of non-labeled antigen will result in less binding of antibody to the tracer, thus lowering the fluorescence measured.

One major drawback of this system is the high background of fluorescence from the sample and the large variation among replicates, which recurrently leads to low sensitivity (Soini and Hemmit, 1979). In addition, non-specific binding of other serum proteins which potentially increase the level of polarization may limit the sensitivity of this method. These drawbacks can be overcome by sample dilution, or pre-treatment using chaotrophic ions, proteolytic enzymes, protein-precipitating reagents or solvents (Wood and Barnard, 1997). Due to these disadvantages, FPIA has not gained widespread popularity (Wood and Barnard, 1997). Nevertheless, FPIA has been used to detect furazolidone in feed with a LOD range of 0.5 – 0.9 ng mL\(^{-1}\) (Zhang et al., 2010); chlorsulfuron in water samples with a LOD of 10 ng mL\(^{-1}\) (Eremin and Smith, 2003); simultaneous detection of organophosphorus pesticides in vegetable and environmental water samples below 10 ng mL\(^{-1}\) (Xu et al., 2011); and melamine in milk and milk powder
with a LOD of 9.3 ng mL\(^{-1}\) (Wang et al., 2011). Furzer et al. (2006) observed that FPIA (IC\(_{50}\) = 253 ng mL\(^{-1}\)) was more sensitive than ELISA (IC\(_{50}\) = 306 ng mL\(^{-1}\)) and requires less time and is less labor-intensive.

2.5.3 Chemiluminescent immunoassay (CLIA)

Chemiluminescent immunoassays measure the signal or response from the emission of chemiluminescent (CL) light produced by molecules that are excited by chemical energy and detected by a light detector. The most widely used system among CL reactions is the luminol-H\(_2\)O\(_2\)-horseradish peroxidase, with a phenol or derivative compound acting as an enhancer of the CL reaction (Díaz et al., 1996).

Luminol-based substrate for HRP acts as an enhancer, which results in rapid kinetic light output and elevated signal intensity (Thermo Scientific, 2011). CLIA have emerged as an alternative technique for sensitive immunoassays due to high quantum yield and chemiluminescent reaction rate, efficiency of the light detector, and they require fewer reagents (Arefyev et al., 1990; Dzgoev et al., 1999). Researchers have successfully improved assay sensitivity using luminescence probes (Ahn et al., 2007; Lin et al., 2005; Zhang et al., 2006). Detection of 2,4-dichlorophenoxyacetic acid (2,4-D) in water samples at 3 ng mL\(^{-1}\) was possible using a combination of a gold nanoparticle-labeled antibody with luminol-silver nitrate (Boro et al., 2011). Enhanced chemiluminescence (ECL) reaction adopted by Quan et al. (2011) offers the possibility of improving the sensitivity of immunoassays by at least 2-3 orders of magnitude when compared to conventional assays that use colorimetric detection. Using this system, detection of acrylamide in food products was reported at an IC\(_{50}\) of 60.6 ng mL\(^{-1}\) and a LOD of 18.6 ng mL\(^{-1}\) (Quan et al., 2011). However, as with any sensitive detection method, this system also suffers from considerable background issues. For example, crosstalk between wells on the same plate contributes to high background signal.
2.5.4 Biotin-streptavidin

For many years, researchers have exploited the versatility and the high affinity of streptavidin or avidin for biotin ($K_A = 10^{15} \text{M}^{-1}$) in a wide variety of bioanalytical applications especially in immunoassay. Each molecule of avidin has four binding sites for biotin. The specific binding between avidin, an egg white protein, and biotin, a water-soluble vitamin, is unaffected by salt, pH, and chaotropic agents such as guanidine hydrochloride (up to 3 M) (Crowther, 2009). Biotin molecules can easily be linked to an antibody or antigen, while avidin can be conjugated to enzymes, usually to HRP or AP. The main principle of this system is that avidin can recognize biotinylated antigen or antibody even at very low concentrations (Deshpande, 1996b).

Many biotinylated reagents that are designed for ELISA applications are commercially available. This system allows the flexibility to control the hapten or antibody concentration without affecting the detection signal; furthermore, the signal may be enhanced by using higher dilutions with insignificant background. Kim et al. (2001) compared an ELISA to a biotin-avidin system and demonstrated that the latter system was able to detect a concentration of atrazine one order of magnitude lower (ELISA: $IC_{90} = 0.5 \text{ng mL}^{-1}$ vs. biotin-avidin: $IC_{90} = 0.05 \text{ng mL}^{-1}$). In another example, Zhang et al. (2010) showed that biotin-avidin immunoassay improved sensitivity almost 20-fold (ELISA: $LOD = 0.58 \mu \text{g mL}^{-1}$ vs. biotin-avidin: $LOD = 0.03 \mu \text{g mL}^{-1}$) for detection of ketamine in biological samples.

2.5.5 Tyramide signal amplification (TSA)

Tyramide signal amplification, sometimes called Catalyzed Reporter Deposition (CARD), was pioneered by Bobrow et al. (1989; 1991; 1992) for immunoblotting and ELISA. It is an enzyme-mediated detection method that uses the catalytic activity of HRP to generate a high signal. It is based on the deposition of a large amount of tyramine
molecules by HRP activity. When tyramine is present at a high concentration, its dimerization can be catalyzed by HRP, likely by the generation of free radicals (Zaitsu and Ohkura, 1980). In signal amplification reactions where tyramine is applied at low concentrations, dimerization is reduced and this will favor the binding of the highly reactive intermediates to tyrosine residues (in proteins) of the peroxidase binding site (Speel et al., 1999). Biotin molecules on tyramide act as further binding sites for streptavidin conjugates, and thus augment the signal (Speel et al., 1999). TSA has been reported to increase sensitivity by up to 100-fold when compared to avidin-biotinylated enzyme complexes (Adams, 1992; Berghorn et al., 1994; Speel et al., 1999; Uchihara et al., 2000; van Heusden et al., 1997; von Wasielewski et al., 1997).

Figure 2.2. ELISA amplification system (ELAST) using tyramide as signal enhancer (modified from Perkin Elmer™ LAS, 2007)
2.5.6 Immuno-PCR (IPCR)

Immuno-PCR combines the elements and specificity of antibody-antigen interaction and powerful signal amplification of PCR. Instead of using an enzyme as a reporter in ELISA, IPCR employs DNA that is linked to the detection antibody and serves as template for PCR (Lind and Kubista, 2002). First described by Sano et al. (1992) for the detection of protein, IPCR has been getting much attention due to the real-time detection with ultimate sensitivity. IPCR can improve the sensitivity in the range of 100-10,000-fold over conventional ELISAs that use enzymes as reporters (Adler et al., 2003; Crowther, 1995). Since the system offers tremendous sensitivity, one of the main challenges of this system is the non-specific background (NSB) that results from the amplification of single DNA strand with non-specific binding that leads to false positive results. To tackle this issue, employment of specific primers such as TaqMan probe or minimization of nonspecific reagents is crucial. Elimination of non-specific reagents can be achieved by using a specific antibody which is directly linked to DNA, for example the Imperacer system commercialized by Chimera Biotech (Dortmund, Germany) (Adler et al., 2005). Conjugation of antibody to DNA can also be performed using commercial kit made available by Solulink Inc. (San Diego, CA) or other methods (Kazane et al., 2012; Kozlov et al., 2004). IPCR has been described for monitoring environmental and food contaminants; for example, detection of 3,3',4,4'-tetrachlorobiphenyl (PCB 77) at 1.5 fg mL\(^{-1}\) and PCBs in soil samples at 5 fg mL\(^{-1}\); anthracene and fluoranthene at 0.5 fg mL\(^{-1}\) and 5 fg mL\(^{-1}\), respectively (Zhuang and Zhou, 2009); ricin at 10-100 pg mL\(^{-1}\) in ground beef, chicken egg and milk (He et al., 2010); dopamine at 1.5 pg mL\(^{-1}\) and adrenaline at 0.04 ng mL\(^{-1}\) (Adler et al., 2005).
2.6 Occurrence and properties of 2-methylisoborneol and monensin and their detection in the environment

2.6.1 2-methylisoborneol (MIB)

2-methylisoborneol (MIB) is widely known for its odor and volatity. MIB is a tertiary alcohol that exists as a (+) or (-) enantiomer, with the (-) isomer being 10 times more potent (Watson et al., 2007). The chemical structure and characteristics of MIB are shown below (Table 2.3). MIB is a naturally occurring organic compound and a secondary metabolite of several genera of cyanobacteria, for example Oscillatoria, Phormodium, Anabaena, and Lyngbya spp. and actinomycetes (Izaguirre and Taylor, 2004; Johnsen and Bett, 1996; Stahl and Parkin, 1994; Wnorowski and Scott, 1992). Lists of MIB-producing species are given elsewhere (Juttner and Watson, 2007; Krishnani et al., 2008; Smith et al., 2008).
<table>
<thead>
<tr>
<th>Characteristics</th>
<th>2-methylisoborneol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecule structure</td>
<td><img src="image" alt="Molecule structure" /></td>
</tr>
<tr>
<td>Chemical name</td>
<td>1,2,7,7-tetramethyl-exo-bicyclo-heptan-2-ol</td>
</tr>
<tr>
<td>Molecular formula</td>
<td>C₁₁H₂₀O</td>
</tr>
<tr>
<td>Formula weight</td>
<td>168.28</td>
</tr>
<tr>
<td>Appearance</td>
<td>White solid</td>
</tr>
<tr>
<td>Boiling point</td>
<td>210°C</td>
</tr>
<tr>
<td>Solubility in water (mg L⁻¹)</td>
<td>194.5</td>
</tr>
<tr>
<td>Henry Law constant</td>
<td>10⁻⁵ m³ atm/mol</td>
</tr>
<tr>
<td>Organoleptic properties</td>
<td>Musty</td>
</tr>
<tr>
<td>Odor threshold concentration (ng L⁻¹)</td>
<td>29</td>
</tr>
<tr>
<td>Human olfactory sense (ng L⁻¹)</td>
<td>7-15</td>
</tr>
<tr>
<td>Toxicity (mg L⁻¹)</td>
<td>10 (rainbow trout)</td>
</tr>
<tr>
<td>LC₅₀ (mg L⁻¹)</td>
<td>69 (sea urchin embryo)</td>
</tr>
</tbody>
</table>

Sources: (Cees et al., 1974; Gagne et al., 1999; Gerber, 1969; Nakajima et al., 1996; Persson, 1979; Pirbazari et al., 1992; Rosen et al., 1970; Schlenk, 1994; Watson et al., 2000; Young et al., 1999)
2.6.1.1 Occurrence and impact of MIB

Cyanobacteria are common inhabitants of freshwater lakes, running waters and reservoirs throughout the world. Because of the ubiquituousness of these organisms, MIB can cause off-flavor problems in potable water and aquaculture industries especially in channel catfish raised both in farms and the wild (Dionigi et al., 1998; Farmer et al., 1995; Lazur, 2004; Lovell and Broce, 1985; Persson, 1981; Piet et al., 1972; Rosen et al., 1970). Off-flavor problems cost catfish producers nearly $60 million annually due to operational costs to remove or reduce its effects, and loss of sales (Engle et al., 1995; Tucker, 2000). More than 30% of potential revenue was estimated to be lost by the US channel catfish industry (Engle et al., 1995). Off-flavor has also been claimed to reduce the quality of other commercially important species such as Nile tilapia (Yanprayoon and Noomhorm, 2000), shrimp (Whitfield et al., 1988), Atlantic salmon (Farmer et al., 1995), rainbow trout (From and Horlyck, 1984), cultured largemouth bass, and white sturgeon (Schrader et al., 2005). In fish, MIB is absorbed rapidly from water, accumulating in lipid tissues (Tucker and Martin, 1991). Off-flavor by MIB does not only affect potable water and aquaculture industries; for example, in food and beverage industries, MIB was found to contaminate wheat grains (Jelen et al., 2003; Wasowica et al., 1988), Arabica and Robusta coffee (Rouge et al., 1993; Vitzthum et al., 1990) before and after roasting, Brie and Camembert cheese (Karahadian et al., 1985), and apple juice (Siegmund and Pollinger-Zierler, 2006). MIB has also been detected in soil (Davies et al., 2004) and indoor air (Omur-Ozbek et al., 2007).

Over fifteen million Canadians and Americans consume water from the Laurentian Great Lakes of North America, which hold approximately 20% of global fresh water (US Environmental Protection Agency, 2012). Incidents of odor outbreaks caused by geosmin and MIB have occurred on several areas of the Lakes and their recurrence is increasing with a variety of relative and absolute abundances (Watson and Ridal,
Such off-flavor outbreaks can have extensive socio-economic impact, affecting millions of water consumers (Moore and Watson, 2007; Davies et al., 2004).

Currently, MIB is considered non-toxic; however, toxicological studies on MIB are limited. MIB was not found to induce mutagenic response in *Salmonella Typhimurium* (TA98 and TA100 strains) at concentrations as high as six times more than the odor threshold (Dionigi et al., 1993; Masaki et al., 1987). Using the same range of concentrations, the IC$_{50}$ (50 % inhibitory concentration) for MIB was observed at 69 mg L$^{-1}$ in sea urchin embryos (Nakajima et al., 1996), which is relatively non-toxic. The possibility of mutagenicity in cyanobacteria cultures (Huang et al., 2007) and hepatotoxicity in rainbow trout (Gagne et al., 1999) has also been hypothesized. Moreover, MIB has been observed to upregulate or downregulate various cytochrome P450 isoforms in the liver and kidney of channel catfish (Schlenk, 1994). Overall, the cytotoxicity of MIB toward higher organisms remains undetermined. More toxicological studies and data are needed to confirm if MIB has an adverse effect on aquatic or higher organisms.

### 2.6.1.2 Control of MIB

To allow depuration of MIB from flesh of fish, the most common practice used in aquaculture, specifically the catfish industry, is to delay the harvest until the odor-producing algae naturally disappear from the pond (Dionigi et al., 1998; Tucker and Martin, 1991). However, this method is not feasible for smaller farms. Instead, many producers apply algicides, e.g., copper sulphate and diuron to eliminate odor-producing algae. However, the use of algicides can be toxic to fish and/or result in toxic residue in the fish (Tucker, 2000). Since MIB is fairly stable to chemical and biological degradation (Peter and von Gunten., 2007; Westerhoff et al., 2006), it remains in the open water in...
the dissolved form for long periods of time. Therefore, chemical and photooxidative
techniques are widely used to remove MIB through various filtration media such as sand,
activated carbon, and membrane systems (Cook and Newcombe, 2004; Hung and Lin,
2006; Reiss et al., 2006). Other methods employed include powdered activated carbon
(PAC) (Cook et al., 2001), ozonation (Sangehashi et al., 2005), and polymers such as
cyclodextrin-based nanoporous polyurethanes (Mamba et al., 2007). Phosphorus has
also been found to effectively control the growth of blue-green algae in tropical fish
ponds, thus preventing deterioration of water quality. Furthermore, shortening the culture
season prevents the development of eutrophic conditions by limiting the accumulation of
organic matter from decayed plant and fecal matter, thus restricting the growth of MIB
producing organisms (Yusoff and McNabb, 1997).

Control of off-flavor events are challenging and necessitate a multi-level
approach. Long-term, proactive management actions are crucial and may consist of
identifying the source of contamination and using environmental and biological agents to
control the organisms that produce MIB (Watson, 2004; Watson et al., 2007).

2.6.1.3 Methods of detection for MIB

MIB is generally present at exceptionally low concentrations (at part-per-trillion
level) making measurement of this metabolite extremely challenging. Sensory evaluation
is commonly used to detect MIB in water and fish samples. In addition, gas
chromatography is also used for MIB detection and quantification. Table 2.4 shows the
odor threshold and the detection limit for MIB in different sample matrices using various
methods of analyses.

Sensory evaluation is an analytical method using human senses as a
measurement tool to detect quality of food and food products. For MIB, it is based on the
ability of assessors, who are trained to detect the presence of MIB through their sense of
smell, generally conducted following the ISO 13301 guidelines. Through these methods, a safety margin for off-flavor can be provided since the assessors will have higher sensitivities than the general consumers (Flick Jr., 2011). Humans are able to detect MIB at levels close to 10 ng L\(^{-1}\) (Persson, 1980) in pure water and approximately 0.7 µg L\(^{-1}\) in fish tissue (Johnsen and Kelly, 1990). The gustatory (i.e., sense of taste) thresholds for MIB in humans are reported in the range of 0.004 µg L\(^{-1}\) to 0.2 µg L\(^{-1}\) (Arganosa and Flick Jr., 1992; Buttery et al., 1976; Johnsen and Kuan, 1987; Mallevialle and Suffet, 1987; Persson, 1980; Saffereman et al., 1967). MIB threshold concentration is defined as the concentration at which 75% of the judges agree there is a muddy taste in the sample (Persson, 1979); it was found that threshold concentrations of MIB in water range from 0.018 to 0.042 µg L\(^{-1}\). Although sensory evaluation offers sensitive detection of MIB, it suffers from being subjective with a high degree of variation (Johnsen and Bett, 1996; Persson, 1979; 1980).

Since MIB is a volatile compound, gas chromatography is suitable for the analyses. Detection of MIB at ng L\(^{-1}\) is possible using various extraction techniques coupled with gas chromatography-mass spectrometry (GC-MS). To date, the most sensitive analytical method for detection of MIB was reported at 0.22 ng L\(^{-1}\) in water using dynamic headspace coupled with gas chromatography-mass spectrometry with simultaneous olfactory detection (Ochiai et al., 2011).

Another approach to detect MIB is by using antibody-based techniques. For example, ELISAs can be used to quantify MIB by employing pAbs (Chung et al., 1990) or mAbs (Miyamoto et al., 1997; Plhak and Park, 2003) against MIB. By using this type of assay, detection limits of 1000 µg L\(^{-1}\) (Chung et al., 1990), 0.6 µg L\(^{-1}\) (mAb; Plhak and Park, 2003) and 630 µg L\(^{-1}\) (mAb; Miyamoto et al., 1997), respectively, were possible. No commercial antibody-based test kit for detection of MIB is currently available.
### Table 2.4. Methods of detection for MIB reported in literature

<table>
<thead>
<tr>
<th>Sample</th>
<th>Odor threshold/ Detection limit</th>
<th>Method of detection</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rainbow trout</td>
<td>0.55 µg kg⁻¹</td>
<td>Sensory analyses</td>
<td>(Persson, 1980)</td>
</tr>
<tr>
<td>Bream</td>
<td>0.095 µg kg⁻¹</td>
<td>Sensory analyses</td>
<td>(Persson, 1980)</td>
</tr>
<tr>
<td>Catfish</td>
<td>100-200 µg kg⁻¹</td>
<td>Sensory analyses</td>
<td>(Martin et al., 1988)</td>
</tr>
<tr>
<td>Water</td>
<td>0.1 µg L⁻¹</td>
<td>Sensory analyses</td>
<td>(Medsker et al., 1969; Wood and Snoeyink, 1977)</td>
</tr>
<tr>
<td>Water</td>
<td>10 µg L⁻¹</td>
<td>Sensory analyses</td>
<td>(Martin et al., 1988)</td>
</tr>
<tr>
<td>Water</td>
<td>42 ng L⁻¹</td>
<td>Sensory analyses</td>
<td>(Persson, 1980)</td>
</tr>
<tr>
<td>Water</td>
<td>2 ng L⁻¹</td>
<td>GC/MS⁴; closed loop stripping analysis</td>
<td>(Izaguirre et al., 1982; Krasner et al., 1981)</td>
</tr>
<tr>
<td>Water</td>
<td>0.8 ng L⁻¹</td>
<td>GC/MS; closed loop stripping analysis</td>
<td>(Hwang et al., 1984)</td>
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<tr>
<td>Fish</td>
<td>5 ng g⁻¹</td>
<td>GC/MS</td>
<td>(Martin et al., 1987)</td>
</tr>
<tr>
<td>Water</td>
<td>19.2 ng L⁻¹</td>
<td>GC/MS; liquid extraction</td>
<td>(Johnsen and Kuan, 1987)</td>
</tr>
<tr>
<td>Catfish</td>
<td>0.1 µg kg⁻¹</td>
<td>GC/MS; purge and trap extraction</td>
<td>(Johnsen and Lloyd, 1992)</td>
</tr>
<tr>
<td>Soil</td>
<td>1 µg kg⁻¹</td>
<td>GC/MS; purge and trap extraction</td>
<td>(Stahl and Parkin, 1994)</td>
</tr>
<tr>
<td>Catfish</td>
<td>1.7 µg kg⁻¹</td>
<td>GC/MS; Microwave assisted distillation-solid phase adsorbent trapping</td>
<td>(Conte et al., 1996)</td>
</tr>
<tr>
<td>Algal samples</td>
<td>20 ng L⁻¹</td>
<td>GC/MS; MASE⁵-PTV⁶</td>
<td>(Hurlburt et al., 2009)</td>
</tr>
<tr>
<td>Water</td>
<td>1 ng L⁻¹</td>
<td>GC/MS; Stir bar sorptive extraction</td>
<td>(Benanou et al., 2003)</td>
</tr>
<tr>
<td>Water</td>
<td>1 ng L⁻¹</td>
<td>GC/MS; HS-LPME⁷</td>
<td>(Ma et al., 2011)</td>
</tr>
<tr>
<td>Water</td>
<td>0.6 ng L⁻¹</td>
<td>GC/MS; SPME⁸</td>
<td>(Nakamura and Daishima, 2005; K. Saito et al., 2008; Sung et al., 2005)</td>
</tr>
<tr>
<td>Drinking water</td>
<td>0.36 ng L⁻¹</td>
<td>GC/MS; SPME-PTV</td>
<td>(Chang et al., 2008)</td>
</tr>
<tr>
<td>Drinking water</td>
<td>0.22 ng L⁻¹</td>
<td>DHS-1D/2D GC-O/MS⁹</td>
<td>(Ochiai et al., 2011)</td>
</tr>
<tr>
<td>Water</td>
<td>0.34 ng L⁻¹</td>
<td>GC/MS; LVI-PTV-LLE⁸</td>
<td>(Zhang et al., 2006)</td>
</tr>
<tr>
<td>Water</td>
<td>0.33 ng L⁻¹</td>
<td>GC/CI-MS; SPME⁹</td>
<td>(Raab, 2009)</td>
</tr>
<tr>
<td>Water</td>
<td>1 µg L⁻¹</td>
<td>GC/MS; SPE</td>
<td>(Ikai et al., 2003)</td>
</tr>
</tbody>
</table>
Table 2.4. Continued

<table>
<thead>
<tr>
<th>Sample</th>
<th>Odor threshold/ Detection limit</th>
<th>Detection method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na</td>
<td>1000 µg L⁻¹</td>
<td>ELISA; pAb</td>
<td>(Chung et al., 1990)</td>
</tr>
<tr>
<td>Na</td>
<td>630 µg L⁻¹</td>
<td>ELISA; mAb</td>
<td>(Miyamoto et al., 1997)</td>
</tr>
<tr>
<td>Na</td>
<td>0.6 µg L⁻¹</td>
<td>ELISA; mAb</td>
<td>(Plhak and Park, 2003)</td>
</tr>
</tbody>
</table>

*a* Gas chromatography-mass spectrometry  
*b* Membrane-assisted solvent extraction  
*c* Programmable temperature vaporizer  
*d* Headspace liquid phase microextraction  
*e* Solid phase microextraction  
*f* Dynamic headspace coupled one dimensional or two-dimensional gas chromatography-mass spectrometry with simultaneous olfactory detection  
*g* Large volume injection programmable temperature vaporizer liquid-liquid extraction  
*h* Chemical ionization tandem mass spectrometry solid phase microextraction  
*i* Solid phase extraction  
*j* Not available
2.6.2 Monensin

2.6.2.1 Uses, mode of action and occurrence

Antibiotics are chemotherapeutic agents that impede or eradicate the growth of microorganisms including bacteria, fungi, or protozoa (Kummerer, 2009). There are approximately 250 different antibiotics that are registered for application in human and veterinary medicine (Kummerer and Henninger, 2003). In 1999, it was estimated that 4700 tons of antibiotics were used as feed supplements in the European Union (Song et al., 2007). In 2007, 87% of antibiotics were used to treat specific pathogenic infections while the remaining 13% were used to increase animal productivity (United States Institute of Animal Agriculture, 2011). In fact, in 2011, ionophore antibiotics sales comprised the second largest category for food-animal production purposes, which was over 4 million kilograms (Food and Drug Administration, 2011). In the USA, approximately 1.5 million kilograms of monensin is used annually for subtherapeutic purposes in cattle and poultry production accounting for about 13% of the total antibiotic usage (Mellon et al., 2001).

Monensin (Figure 2.3.), an antibiotic naturally produced by Streptomyces cinnamonensis, has a molecular weight of 671 Da and is usually referred to by its trade name, Rumensin. Monensin is widely used in animal feed as monensin sodium (Figure 2.2; MW 693 Da) in North America, Australia and New Zealand (Ellis et al., 2012). It is used for the treatment of coccidiosis, which is caused by protozoan parasites of the genus Éimeria, in the poultry, beef and dairy industries (Butaye et al., 2003; Matsuoka et al., 1996). This parasitic disease can rapidly spread through contaminated feces and results in poor performance and productivity loss in farm animals, especially poultry (Chapman et al., 2010). In the beef and dairy industries, monensin is also used to increase the production of propionic acid at the expense of lactic acid production (Matsuoka et al., 1996) and thus increase weight gain, while minimizing loss of muscle
tone, prevent bloating, and increasing the rate of milk production in lactating dairy cows (Canadian Food Inspection Agency, 2011). Furthermore, in cattle, monensin can increase feed efficiency by as much as 9% and reduce methane emissions by almost 20% (Tedeschi et al., 2003). The use of monensin as a feed additive is found to be safe and effective at levels up to 40 mg kg\(^{-1}\) (European Food Safety Authority, 2006). However, toxicity of monensin has been reported in horse, sheep, swine, dog, goat, rat, cattle, mouse, and chicken at LD\(_{50}\) values of 2-3, 12, 17, 20, 26, 35, 50-80, 135, and 200 mg kg\(^{-1}\), respectively (Gonzales et al., 2005; MacDonald, 2012; Matsuoka et al., 1996; Osweiler et al., 1985; Pinkerton and Steinrauf, 1970).

![Chemical structure of monensin sodium salt](image)

**Figure 2.3.** Chemical structure of monensin sodium salt (Sigma-Aldrich Co., St. Louis, MO).

Monensin is a member of polyether monocarboxylic ionophores, which refers to molecules comprised of oxygenated heterocyclic rings and a single terminal carboxyl group (Chapman et al., 2010; Westley, 1982). It acts as an ion bearer indicating its dynamic ion transport mechanism (Pressman, 1976). Monensin readily forms complexes with polar cations, such as K\(^+\), Na\(^+\), Ca\(^{2+}\), and Mg\(^{2+}\), and, due to its hydrophobic exterior, these cations are easily transported with monensin across lipid cell membranes of *Eimeria* spp. This action causes osmotic distress to *Eimeria* spp., which leads to the
depletion of its stored energy levels (Matabudul et al., 2001) and a decrease in the intracellular pH, making monensin an effective antibacterial agent (Mollenhauer et al., 1986; Pressman and Fahim, 1982).

Various pharmaceuticals contaminate diverse environmental settings, including surface water, ground water, soil and sediments (Hamscher et al., 2002; Kolpin et al., 2002; Schlusener et al., 2003). This contamination originates from their wide application in human and veterinary medicine and their use in animal feed (Watanabe et al., 2008). Concerns about the use of veterinary antibiotics in concentrated animal feeding operations (CAFO) are increasing (Burkholder et al., 2007; Gilchrist et al., 2007; Thorne, 2007) because of their deleterious effects on aquatic and terrestrial organisms. More importantly, their broad presence in the environment could progress to the development of antibiotic-resistant strains of microorganism (Watanabe et al., 2008), which could in turn lead to microbial-based disease epidemics in humans and animals. Fifty percent of monensin administered in animal feed is absorbed, rapidly metabolized and excreted in bile, while the remainder is eliminated in feces as unmetabolized parent molecule (Donoho et al., 1978; Donoho, 1984; Herberg and Van Duyn, 1969; Herberg et al., 1978). When released in the environment, monensin may reach aquifers because it remains unchanged due to low hydrolysis and slow photolysis (Elanco Products Company, 1989). Occurrence of monensin has been observed in river water and sediments on agricultural sites in Colorado (Cha et al., 2005; Kim and Carlson, 2006), in surface water near heavily farmed areas of southern Ontario, Canada (i.e., range of 0.006 to 1.17 µg L⁻¹; Hao et al., 2006; Lissemore et al., 2006), and in ground water in California (i.e., range of 0.04 to 0.39 µg L⁻¹; Watanabe et al., 2008). Because of its stability in surface water (Lissemore et al., 2006), there is a possibility of chronic exposure to aquatic organisms (McGregor et al., 2007). Previous studies showed frequent foliar and root exposure to monensin had an adverse effect on the growth rates.
of several terrestrial plant species (Hoagland, 1996; Mollenhauer et al., 1986). In contrast, McGregor et al. (2007) found that environmental concentrations (up to 100 µg L⁻¹) of monensin are unlikely to cause toxicity in freshwater macrophytes. However, characterization of toxicity was based on the partial relative growth rates (RGRs) of these plants, which could underestimate toxicity under simulated field study (McGregor et al., 2007). Consequently, the US Environmental Protection Agency (US EPA) and World Health Organization (WHO) have encouraged agency networking to study and better understand the fate and impact of pharmaceuticals, including monensin, on environment and human health (Waters, 2008).

2.6.2.2 Methods of detection for monensin

Earlier methods used to detect monensin include bioautographic techniques (Donoho and Kline, 1968; Kline and Golab, 1965), cylinder plate method (Kline et al., 1970), turbidimetric method (Kavanagh and Willis, 1972), microbiological assays (Breunig et al., 1972; Breunig et al., 1977; Donoho and Kline, 1968; Martinez and Shimoda, 1983), colorimetric method (Golab et al., 1973), fluorodensitometric method (Asukabe et al., 1984), and thin layer chromatography (Koufidis, 1976; Owles, 1984). Each method was developed according to the specific needs of the end user, who was using the available technologies at that time (Elliott et al., 1998).

The most common method used to detect the presence of monensin is UV-liquid chromatography. However, due to the limited UV-vis absorption of monensin, its detection is more recently measured by mass spectrometry (LC-MS). To overcome low UV absorbance, derivatization methods have been developed (Dusi and Gamba, 1999; Moran et al., 1994); however, these methods are not applicable for trace analysis of monensin (< 20 µg L⁻¹ or kg⁻¹) (Dolliver et al., 2008). Therefore, monensin is extracted and concentrated from numerous matrices using solid phase extraction (SPE) followed
by liquid chromatography tandem mass spectrometry (LC-MS/MS) (Song et al., 2007). Using LC-MS/MS, a limit of quantification (LOQ) of monensin of 2.5 ng g\(^{-1}\) in animal tissue and eggs (Matabudul et al., 2001), a limit of detection (LOD) of 100 ng g\(^{-1}\) in feed samples (Hormazabal et al., 2005), and a method detection limit (MDL) of 0.03 µg L\(^{-1}\) in surface water (Cha et al., 2005) have been reported. Although LC-MS is a sensitive analysis method, it often requires sample cleanup, is costly and time consuming, and not applicable for field settings. Therefore, there is a demand for a simple, rapid and sensitive detection method for monensin in surface waters, which could be performed on-site.

As with MIB, immunoassays for the sensitive detection of monensin have been developed. Early immunoassay development was described by Heitzman et al. (1986) for the detection of monensin in bovine plasma with sensitivity of 1 ng mL\(^{-1}\) using a pAb. A year later, Mount and Failla (1987) described another anti-monensin immunassay using rabbit pAb, which had a detection limit of 5 ng mL\(^{-1}\). Thereafter, using specific mAbs, immunoassays were developed with similar detection limits (Pauillac et al., 1993; Watanabe et al., 1998). Currently, ELISA kits for monensin are commercially available from Immuno-Diagnostic Reagents (Vista, CA) and Abraxis Bioscience, LLC (Westminster, PA) with reported limit of quantifications of 3.0 and 0.176 ng mL\(^{-1}\), respectively. Technical details regarding these kits are available in Dolliver et al. (2008) and from Abraxis Bioscience LLC website.
2.7 Future perspectives

Since the introduction of radioimmunoassays (Yalow and Berson, 1960), immunoassays continue to progress with new and improved techniques complemented with state-of-the-art technology. Analytical detection systems are moving towards miniaturization using antibody-based techniques. The employment of DNA as an antibody label which amplifies the signal tremendously through polymerase chain reaction is a promising technique giving the possibility of detecting attomole ($1 \times 10^{-18}$) concentrations. Advances in micro- and nanotechnology are on the verge of making a significant impact especially in clinical applications. The high demand for better sensitivity and ease of application in the shortest time have driven new technologies such as immunosensors, DNA probes, nanotechnology, and simultaneous multianalyte techniques to emerge. New advancement in immunodiagnostics is evolving towards novel diagnostic markers for disease monitoring and determination of food and environmental contaminants. The past few decades have witnessed the expansion of immunoassays from the development of new test kits in a wide range of scientific disciplines including food industry, environmental sciences, endocrinology, biomedical and clinical chemistry, assisted by the simplification of the test formats and complemented with automation and electronic instrumentation. For clinical application, the world market for immunoassays continues to grow exponentially; with sales increasing from an estimated $6.7 billion in 2007 to $8.9 billion by 2012 with 6% growth annually (CardioGenics Inc.). For analytical chemistry applications, particularly in food and environmental contaminant analysis, immunoassays have been increasingly acknowledged as important and indispensable tools.

The generation and exploitation of tailored recombinant antibodies by creating high quality anti-hapten libraries will continue to mature and may supersede hybridoma technology in the near future. Nevertheless, until the complexity of the techniques is
resolved, polyclonal and monoclonal antibodies will persevere and dominate this field.
In short, immunoassays have been shown to be a reliable method for detection of
contaminants in environmental and food analysis. The possibility to screen a large
number of samples within a short time serves as a valuable supplement to conventional
methods in reducing sample load. Further advancements in antibody-antigen
development will continue to progress and opens the world of opportunity for many to
venture in advance technologies in realization of their goals.
3 DEVELOPMENT OF A POLYCLONAL-BASED IMMUNOASSAY FOR THE DETECTION OF 2-METHYLISOBORNEOL IN WATER

3.1 Abstract

2-Methylisoborneol (MIB) is a secondary metabolite produced by several species of cyanobacteria or blue-green algae causing musty/earthy off-flavor in fish and water. MIB is difficult to remove by conventional water treatment methods. Detection methods for MIB include sensory evaluation, which suffers from high variation among panelists, and gas chromatography, which is costly, time consuming and requires high-end instrumentation and trained operators. The objective of this research was to develop a simple and rapid method for detection of MIB that can be used for on-site analyses with minimal use of instruments. This study describes the development of sensitive enzyme immunoassays (ELISA) for the detection and quantification of MIB in water samples. Rabbits were immunized with structurally related compounds of MIB; (-) camphor and (-) borneol conjugated to bovine serum albumin (BSA). Polyclonal antisera from rabbits immunized with (-) camphor-BSA (rabbits CR1 and CR2) showed higher immune response compared to antisera from rabbits when (-) borneol-BSA (rabbits BR1 and BR2) was used as immunogen. Competitive-indirect ELISAs (CI-ELISAs) were carried out with MIB and (+) bornylamine conjugated to thyroglobulin (TG) as coating conjugate. This assay had a limit of detection (LOD) of 4.8 ng mL\(^{-1}\), limit of quantification (LOQ) of 13.2 ng mL\(^{-1}\) and IC\(_{50}\) value of 105 ng mL\(^{-1}\). Furthermore, the polyclonal antisera showed cross reactivity relative to MIB (100%) of 151.9, 57.3, and 75.3 for (-) camphor, (+) camphor and isoborneol, respectively. The effects of various assay conditions, including incubation time, temperature, pH, organic solvent, detergent and salt concentration on assay sensitivity were evaluated. The ELISA was used to detect MIB in various water...
samples, which were analyzed directly without sample treatment. A correlation study was done to compare the ELISA with headspace, solid-phase microextraction (HS-SPME) coupled with gas chromatography–mass spectrometry (GC-MS). Good correlation ($r^2 = 0.997, 0.998, 0.995, 0.993$ for nanopure, tap, river and lake water, respectively) was observed between ELISA and GC-MS when fortified water samples were analyzed. The ELISA offers a clear alternative or complementary method to conventional analysis to determine the presence of MIB in water samples.

3.2 Introduction

2-Methylisoborneol (MIB; 1-R-exo-1,2,7,7-tetramethyl bicyclo-[2-2-1]-heptan-2-ol; Figure 3.1a) is a naturally occurring organic compound and secondary metabolite produced by a variety of organisms including cyanobacteria, actinomycetes, and fungi (Izaguirre and Taylor, 2004; Stahl and Parkin, 1996; Whitfield, 1999; Wnorowski, 1992). Ubiquitous in nature, MIB is often co-produced with geosmin, another earthy-smelling, organic compound and both have been implicated as the cause of earthy-musty odor (taste and odor) problems in water and freshwater fish, especially catfish (Piet et al., 1972; Rosen et al., 1970). Off-flavor problems may cost catfish producers nearly $60 million annually due to operational costs and income deficit. Delaying catfish harvest for depuration of off-flavor resulted in deterioration of water quality and loss of fish to disease and bird predation (Engle et al., 1995; Tucker, 2000). In natural waters, off-flavor is a common and worldwide problem, which results in dissatisfaction among consumers, substantial costs for water treatment and degradation in aesthetic value of recreational areas (Persson, 1983).

MIB also contaminates soil (Davies et al., 2004), indoor air (Omur-Ozbek et al., 2007), and several foods and beverages such as mushrooms (Davies et al., 2004), fish (Llyod and Grimm, 1999; Papp et al., 2007), red beets (Lu et al., 2003), wheat grains
(Jelen et al., 2003; Wasowica et al., 1988), cheese (Karahadian et al., 1985), coffee (Rouge et al., 1993; Vitzthum et al., 1990), wine (Boutou and Chatonnet, 2007), and apple juice (Siegmund and Pollinger-Zierler, 2006). Humans can detect MIB by smell at levels close to 10 ng L\(^{-1}\) (Persson, 1980) in pure water and approximately 0.7 µg L\(^{-1}\) in fish tissue (Johnsen and Kelly, 1990) and by taste in the range of 0.004 to 0.2 µg L\(^{-1}\) (Arganosa and Flick Jr., 1992; Buttery et al., 1976; Johnsen and Kuan, 1987; Mallevialle and Suffet, 1987; Persson, 1980; Safferman et al., 1967). MIB exist as (+) and (-) enantiomers, with the (-) MIB being 10 times more potent than the (+) isomer (Watson et al., 2007). Negative health effects of MIB in public water supplies have not been shown and no toxicity has been reported, although possibility of mutagenicity and hepatotoxicity has been hypothesized to occur (Gagne et al., 1999; Huang et al., 2007).

Currently, MIB contaminants in food and water samples are detected by sensory evaluation and gas chromatography-mass spectrometry (GC-MS) (Jelen et al., 2003; Johnsen and Bett, 1996; Persson and York, 1978; Petersen et al., 2011). For GC-MS analyses, a pre-concentration step is usually necessary since MIB occurs in minute concentrations in samples especially in environmental matrices. Various extraction or enrichment techniques including closed-loop stripping and purge-and-trap methods combined with GC-MS (Salemi et al., 2006), solid-phase micro-extraction (SPME) with GC-MS (American Water Works Association, 1988; Boutou and Chatonnet, 2007; Ikai et al., 2003; Jelen et al., 2003; Lin et al., 2003; Lloyd et al., 1998; Lloyd and Grimm, 1999; Lu et al., 2003; McCallum et al., 1998; Papp et al., 2007; Zhang et al., 2005; Zimmerman et al., 2002), stir bar sorptive extraction (SBSE) (Benanou et al., 2003; Ochiai et al., 2001) and liquid-phase microextraction (LPME) (Xie et al., 2007) have been previously described. Chang et al. (2008) reported a detection limit of MIB at 1 pg mL\(^{-1}\) employing GC-MS coupled with selected ion monitoring with a single quadrupole mass spectrometer.
Although chromatographic techniques allow highly sensitive detection of MIB, these methods are generally arduous, time consuming, involve high cost of analyses and require highly technical equipment that is not available in many facilities. Sensory evaluation suffers from high variation among sensory panelists and requires tedious construction of calibration curves for every panel session (Persson, 1979). Conversely, immunoassays may offer a simpler, more rapid and cost-effective analysis tool for the detection of MIB in food and water samples. These characteristics will greatly assist municipal officials in monitoring water and be highly advantageous to catfish producers enabling them to take prompt action when MIB is present in ponds.

An ELISA for MIB with a moderate sensitivity of 1 µg mL\(^{-1}\) was previously developed by cross-linking (+) camphor, a structurally related compound to MIB, to BSA and to OVA as coating conjugate (Chung et al., 1990). Using a similar immunogen and coating antigen, Miyamoto et al. (1997) developed monoclonal antibodies (mAbs) with sensitivity of 0.63 µg mL\(^{-1}\). However, poor sensitivities of these pAb- and mAb- based immunoassays did not permit for quantitative detection of MIB in water and food (Chung et al., 1990). A sensitive mAb for detection of MIB was developed by Plhak and Park (2003), by crosslinking the hydroxyl group of (-) borneol to Limulus polyphemus hemocyanin (LPH), and using it as immunogen, and MIB-BSA as coating antigen. This mAb had a detection limit of 0.6 ng mL\(^{-1}\) and an IC\(_{50}\) of 5 ng mL\(^{-1}\) and 20% cross-reactivity with borneol or isoborneol and 4-5% with camphor. Although this mAb-based assay has a low detection limit, no validation data were available to demonstrate the usability of the assay to detect MIB in real water sample. In addition, since human olfactory threshold to MIB is at ng L\(^{-1}\), the detection limit of this assay may not be sensitive enough to be used for routine analyses.

The research describes herein, the production and development of specific and sensitive ELISA for detection of MIB using rabbit pAb. The ELISA utilized both (-)
camphor-BSA and (-) borneol-BSA as immunogen and (+) bornylamine and MIB conjugated to TG as coating antigens. The combination of (-) camphor-BSA as immunogen and MIB-TG as coating antigen, allowed the detection of MIB at 4.8 ng mL\(^{-1}\) level. After optimizing and validating the assay, several water samples were tested for MIB contamination.

### 3.3 Materials and methods

#### 3.3.1 Materials, reagents and equipment

2-Methylisoborneol (MIB, 99.9%, 20 mg) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). MIB (20 mg) was dissolved in methanol (HPLC Grade, Fisher Chemicals, Nepean, Canada) to prepare a 1 mg mL\(^{-1}\) stock solution and stored at -20\(^\circ\)C. Geosmin (GSM, \(\geq 98\%\), 2 mg mL\(^{-1}\) in methanol), (-) borneol, isoborneol, bornylamine, (-) camphor, (+) camphor, camphorquinone, norcamphor, norborne, norberneol, (+)-endo-2-norborneol, norbornylamine, fenchol, bovine serum albumin (BSA), porcine thyroglobulin (TG), Titermax and Freund’s incomplete adjuvant were purchased from Sigma Aldrich Co. (Oakville, Canada). Materials used for conjugation, which include carbonyldiimidazole, disuccinimidylsuberimidate, \(O\)-carboxymethoxylamine hemihydrochloride, triethylamine, and isobutyl chloroformate, were purchased from Sigma Aldrich Co. (Oakville, Canada).

Standard solutions of MIB for ELISA were prepared in volumetric flasks from the stock at 0.001 to 100,000 ng mL\(^{-1}\) in 10% methanol/water (v/v) and stored at 4\(^\circ\)C. Goat anti-rabbit IgG-horseradish peroxidase conjugate was purchased from Jackson ImmunoResearch (West Grove, PA). Superblock in PBS was obtained from Thermo Scientific (Nepean, Canada), while TMB liquid substrate system was from Sigma Aldrich Co. (Oakville, Canada). All other chemicals were from Sigma Aldrich Co. (Oakville,
Canada) unless otherwise stated. All chemicals and reagents were of analytical grade unless stated otherwise.

Phosphate buffered saline (PBS) was prepared at a 10X stock concentration and diluted to 1X with Nanopure® water (1XPBS; 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na2HPO4, and 0.24 g of KH2PO4 per liter of water, pH 7.4). For PBST, Tween 20 was added to 1X PBS at 0.05% (v/v). Carbonate bicarbonate buffer (0.05 M; 1.59 g of Na2CO3, 2.93 g of NaHCO3, 10H2O, 0.2 g of NaN3 per liter of water, pH 9.6) was used as coating buffer in ELISA.

ELISAs were conducted using 96-well microplates (high-binding; Corning Inc. Life Sciences, Lowell, MA). Plates were analyzed at absorbance 450 nm with a microplate reader from Bio-Rad Laboratories (Model 3350-UV, Hercules, CA).

Conjugates were analyzed using Micromass Global Q-TOF Ultima (MALDI/CapLC-ESI Quadrupole Time of Flight) Mass Spectrometer located in the Department of Chemistry, McMaster University, ON. MIB in water samples was analyzed by gas chromatography mass spectrometry (GC-MS; Varian Model Saturn 2000R) equipped with ion trap mass spectrometer located in the Department of Plant Agriculture, University of Guelph, ON.

3.3.2 Preparation of immunogens and coating conjugates

The hydroxyl group of (−) borneol (Figure 3.1b) was activated and directly coupled with bovine serum albumin (BSA) using carbonyldiimidazole (CDI) as described by Xiao et al. (1995) and as outlined in Figure 3.2. Briefly, 5 mg (30 μmol) of (−) borneol were dissolved in 500 μL dimethyl sulfoxide (DMSO) and reacted with 7.5 mg (50 μmol) of CDI at 25 °C for 5 min in a 5-mL glass tube. The mixture was added drop wise to 50 mg (0.72 μmol) of BSA dissolved in 5 mL of 0.1 M sodium carbonate and stirred for 12 h at 4°C. Upon completion of the reaction, the mixture (5 mL) was precipitate with 50 mL
of 80% (v/v) cold acetone (-20°C) and allowed to stand for 3 h at 4°C. Half of the solution (25 mL) was transferred into another tube containing 25 mL of cold acetone and allowed to stand for 30 min. The solution was centrifuged at 4°C for 10 min at 1500 x g and the supernatant was discarded. The pellet was re-dissolved with 1X PBS, pH 7.4 and re-precipitated with acetone as described above. The final conjugate (borneol-BSA) was dissolved in 1 X PBS to a concentration of 1 mg mL⁻¹ and stored at -20°C.

(-) Camphor O-(carboxymethyl)oxime (CMO) was prepared according to the method of Langone and Van Vunakis (1976) with minor modifications as featured in Figure 3.3. Briefly, 1 g of (-) camphor (6.5 mmol) (Figure 3.1c) and 2.84 g (13 mmol) of O-carboxymethylamine hemihydrochloride were refluxed for 6 h in a mixture of 14 mL of absolute ethanol and 16.5 mL of 2 M NaOH (33 mmol). The mixture was allowed to stand at 25°C overnight followed by the addition of water (200 mL) and adjustment of the pH to 9.5 with 2 M NaOH. The solution was extracted three times with 100 mL of ethyl acetate in a separatory funnel. The aqueous layer was acidified to pH 3.0 with 1 M HCl and stored at 0°C overnight to yield (-) camphor-CMO as a white precipitate. The precipitate was collected following centrifugation (1500 x g, 5 min, 4°C), washed with 500 mL of water and lyophilized. Preparation of (-) camphor-CMO to BSA was done by the mixed-anhydride method (Dean et al., 1972). Specifically, 18.9 mg (84 µmol) of (-) camphor-CMO were mixed with 12 µL (85 µmol) of triethylamine and cooled to -5°C, after which 12 µL (85 µmol) of isobutyl chloroformate were added and the solution was stirred for 30 min at -5°C. The reaction mixture was added drop wise to a cold solution (5 mL) of BSA (20 mg mL⁻¹, 1.5 µmol) in water: dioxane (7:3; v/v), pH 9.0. Sodium hydroxide (0.1 M) was added to maintain the pH at 9.0 and the mixture was stirred continuously at 4°C overnight, dialyzed against water, and stored at -20°C.
(+) Bornylamine (Figure 3.1d) and MIB were used for solid phase coatings. Conjugation of (+) bornylamine to porcine thyroglobulin (TG) was done following the method of Paek et al. (1993) and Skeritt et al. (2000). (+) Bornylamine was coupled through its amine group using disuccinimidylsuberimidate (DSS). Briefly, (+) bornylamine (11.6 mg, 75.6 μmol) was added to DSS in anhydrous DMSO (55.25 mg mL⁻¹, 150 μmol). The mixture was stirred for 30 min at room temperature (RT) and added to 20 mg of TG (0.03 μmol) in 5 mL of 10 mM sodium phosphate buffer, pH 7.0. After incubating for 2 h at RT, the mixture was dialyzed (MWCO 12-14 kDa) against 1X PBS, pH 7.4 at 4°C for 24 h with four buffer changes. The solution was aliquoted into another tube and kept at -20°C until required. MIB-TG conjugate was produced and kindly provided by Abraxis LLC (Warminster, PA).

Molar ratios of the immunogen conjugates were determined using MALDI-TOF mass spectrometry (Micromass Global Q-TOF Ultima MALDI/CapLC-ESI Quadrupole Time of Flight). 2, 5-Dihydroxybenzoic acid (2, 5-DHB) was used as matrix solution and BSA as reference. The conjugate samples and matrix solution were mixed in equal amounts (1 μL each) and placed on the stainless steel probe. The samples were allowed to dry at room temperature. The data were acquired with 50 shots per sample in the linear mode at 30 kV and analyzed using the software provided with the system.
Figure 3.1. Structures of (a) 2-methylisoborneol, (b) (-) borneol, (c) (-) camphor, and (d) (+) bornylamine. Crosslinking to protein were done through the hydroxyl group of (-) borneol and MIB, the carbonyl group of (-) camphor, and the amine group of (+) bornylamine.
3.3.3 Immunization of rabbits

Four female New Zealand white rabbits (2.5 – 3 kg) were immunized subcutaneously with 500 \( \mu \)g of the immunogen (-) borneol-BSA (rabbits BR1 and BR2) or (-) camphor-BSA (rabbits CR1 and CR2) for the primary immunization. Immunogens were emulsified with TiterMax® adjuvant and 1X PBS (1:1, v/v), in a total volume of 1.5 mL. For subsequent immunizations, rabbits were injected with 200 \( \mu \)g of the respective immunogens emulsified with Freund’s incomplete adjuvant and 1X PBS (1:1, v/v, total volume of 1.5 mL). Rabbits were injected monthly, followed by a 2-week rest over twenty nine weeks. Blood was collected from the ear vein (ca. 1-2 mL) 7 days after each injection, stored at 4°C overnight prior to centrifugation at 15,000 \( x \) g to separate the serum from blood cells. Sera were aliquoted (20-50 \( \mu \)L) into 0.5 mL tubes and kept at -20°C until required for further use. Negative control serum (pre-immune bleed) was collected prior to the first injection. Upon termination of the immunization, all the blood from each rabbit was collected via cardiac puncture.

3.3.4 Confirmation of titer

Confirmation of antibody response and titer were done by indirect non-competitive ELISA according to standard ELISA protocols. Polyclonal antisera from each rabbit were tested against (+) bornylamine (BA) and MIB conjugated to TG. Briefly, 96-well microtiter plates were coated with MIB-TG or BA-TG at 1 \( \mu \)g mL\(^{-1}\) (100 \( \mu \)L well\(^{-1}\)) in 0.05M carbonate bicarbonate buffer, pH 9.6. For negative control, wells were coated (1 \( \mu \)g mL\(^{-1}\); 100 \( \mu \)L well\(^{-1}\)) with the same protein used to make the coating conjugates (TG). Pre-immune serum was used as another negative control. Plates were covered and sealed to prevent evaporation and incubated for 16 h at 4°C. After overnight incubation, plates were washed with PBST and blocked (250 \( \mu \)L well\(^{-1}\)) with Superblock for 1 h at RT
with orbital shaking. After washing with PBST, pre-immune serum and antisera ranging from bleed 1 to the terminal bleed (day 43, 71, 99, 127, 155, 181, and 225) were diluted (1:100 v/v) in PBS and added to the wells. Subsequently, antisera were further diluted serially (1:2 v/v) across the plate and incubated at RT for 1 h with orbital shaking. Plates were then washed thoroughly (five times) with PBST. Horseradish peroxidase (HRP) conjugated with goat anti-rabbit IgG was diluted (1:6000 v/v) in PBST and was used as the secondary antibody (100 µL well⁻¹). After incubation and subsequent washing, TMB substrate (100 µL well⁻¹) was added and plates were developed in the dark for 15 min. The reaction was quenched by adding 1N H₂SO₄ (100 µL well⁻¹). Absorbance values were measured at 450 nm using a microtiter plate reader. To determine antibody titer, antisera (bleed 5) from rabbits immunized with both immunogen were compared using MIB-TG as coating conjugate in an indirect ELISA. The best antiserum was selected based on the highest sample dilution giving an ELISA signal three times that of the negative control value (Szurdoki et al., 2002).

3.3.5  Development and optimization of assay

3.3.5.1 Indirect checkerboard ELISA

Ninety-six well polystyrene microtiter plates were coated with (+) bornylamine-TG or MIB-TG at concentrations of 1 µg mL⁻¹ (100 µL well⁻¹) in 0.05M carbonate bicarbonate buffer, pH 9.6. A concentration of coating conjugate was placed in the first row (12 wells) and diluted by half down each column. TG was used as negative control (1 µg mL⁻¹; 100 µL well⁻¹). Plates were incubated for 16 h at 4°C. Plates were washed with 1X PBS and blocked with Superblock for 1 h at RT with gentle shaking. Test serum from a rabbit was added at 1:1000 (v/v) dilution in 1X PBS to the wells (8) of the first column of the plate and serially diluted across the plate. Antibodies were allowed to react for 1 h at RT with
gentle shaking. Horseradish peroxidase (HRP) conjugated with goat anti-rabbit IgG (1:6000 v/v dilution) was used as secondary antibody at 100 μL well⁻¹ in PBST and plates were further incubated using the same conditions described above. Plates were washed three to five times with PBST after each step. TMB substrate was added (100 μL well⁻¹) and assays were developed for 30 min in the dark at RT. H₂SO₄ (1N; 100 μL well⁻¹) was added to quench the reaction and resulting color intensity was determined at 450 nm.

3.3.5.2 Competitive indirect ELISA (CI-ELISA)

Coating and blocking buffers, the secondary antibody and washing steps were as described in Section 3.3.5.1. Microtiter plates were coated with MIB-TG or BA-TG at 1/27,000 (v/v) dilutions, which were pre-determined from checkerboard ELISAs. Plates were incubated for 16 h at 4°C, washed with 1X PBS and blocked with Superblock in PBS for 1 h at RT with continuous shaking. Plates were washed three times with PBST and MIB standards ranging from 0.001 to 100,000 ng mL⁻¹ in 10% methanol, and antiserum was added at a 1/54,000 (v/v) final dilution into the wells. Free MIB was allowed to compete with the coated antigen for antibody binding for 1.5 h at RT with continuous shaking. Plates were washed five times with PBST, the secondary antibody was added, and plates were further incubated for another hour at RT with continuous shaking. After final washing, TMB substrate was added and incubated for 30 min in the dark at RT. The reaction was stopped by adding 1N H₂SO₄ and absorbance was read at 450 nm.

For inhibition ELISAs, a pre-incubation step of antisera and free MIB was incorporated before the solution was added to the coated wells. Inhibition was done by mixing MIB standards at different concentrations with antiserum at a final concentration
of 1/54,000 (v/v) in 2-mL glass vials for 1 h with continuous rotation (rotary cell culture system, Synthecon Inc., Houston, TX). The inhibition solution (100 μL) from each tube was subsequently transferred to triplicate wells of a pre-coated and pre-washed microtiter plate and further incubated for 1.5 h at RT with continuous shaking. Color development was inversely proportional to the concentration of free MIB.

3.3.5.3 Optimization of assay conditions

Effect of time and temperature was determined by performing CI-ELISA for 0.5 h, 1 h and 1.5 h at RT and 37ºC. For evaluation of pH effect, pHs of assay buffers (1X PBS) were adjusted to pH 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0 and were used in CI-ELISA. For detergent effect, PBST (Tween 20 at 0.05%) was used as assay buffer and compared to 1X PBS. Effect of salt concentration was evaluated by preparing the assay buffer at different concentrations of PBS, i.e., 1X, 2X, 4X, and 10X PBS from the same stock of 10X PBS. For the effect of solvent, MIB standards were prepared in 2.5, 5, 10, and 20 % final concentrations of methanol or acetonitrile.

3.3.6 Cross-reactivity (CR)

Specificity of the polyclonal antisera from rabbit CR1 was evaluated with compounds of closely related molecular structures, which are listed in Table 3.2. CI-ELISAs were performed using MIB-TG as coating conjugate and their respective IC$_{50}$ values were determined. Each compound was prepared at concentrations ranging from 0.001 to 100,000 ng mL$^{-1}$ in 10% methanol (v/v). Percentage of cross reactivity was calculated as IC$_{50}$ value of MIB/IC$_{50}$ value of test compound X 100.
3.3.7 Analyses of water samples

Groundwater samples were collected from Speed River and Guelph Lake, Guelph, Ontario, Canada. Tap water was obtained from the laboratory. Nanopure water was obtained from a Mili-Q water purification system (18 MΩ, Milipore, Bedford, MA). Water samples were collected in 1-L amber glass bottles and filtered through a 0.45 µm nylon membrane filter in a Milipore filtration system (Bedford, MA). Water samples were stored in the dark at 4°C until required. Assay precision was calculated with MIB standards on three different plates to determine intraassay variability on three different days and by two different analysts to determine interassay variability. ELISA was performed at conditions described in Section 3.3.5.2.

3.3.8 SPME extraction and GC-MS analyses

Water samples for GC-MS analyses were prepared according to the procedure of Saito et al. (2008). The MIB stock standard (1 mg mL⁻¹ in methanol) was used to create standard concentrations of 1, 10, 50, and 100 ng mL⁻¹. Water (10 mL) was aliquoted into 500 mL conical flask and geosmin (GSM) was spiked as internal standard at 10 ng mL⁻¹. Water samples were freshly prepared prior to analysis to prevent volatilization losses (Ma et al., 2011). Five percent (v/v) methanol was used in all samples. An SPME fiber assembly consisting of a divinylbenzene/carboxen on a polydimethylsiloxane fiber (DVB/CAR/PDMS; 50/30 µm coating; Supelco, Oakville, Canada) was placed above the headspace of each sealed flask for 30 min at 50°C (Precision oven Model STM 135, Precision Scientific, Chicago, IL). The fiber was then retracted into the needle and injected immediately into the GC injection port of the GC-MS instrument with ion trap mass spectrometer and held in the port for 5 min. After removal, the needle and fiber was used for the next sample. Analyses were performed in triplicate.
GC-MS analyses were executed with a Varian 2000R Model 3800 (Walnut Creek, CA) equipped with Varian CP-SIL 8 CB Low Bleed/MS fused silica column (Crawford Scientific, Scotland, UK; 30 m x 0.25 mm i.d., 0.25 µm film thickness) coupled to a Saturn GC-MS workstation. Instrument conditions were as follows: injector and detector temperature, 250°C, held for 28 min; helium carrier gas flow rate, 1 mL min\(^{-1}\) with constant flow; oven temperature, 40°C held for 4 min, increased to 250°C at 10°C min\(^{-1}\), held for 3 min. The electron impact (EI)-MS was run with an ion source temperature of 200°C and ionizing voltage of 70 eV. Ion masses were scanned at full scan mode at \(m/z\) = 40 to 399. GSM and MIB peaks were monitored at \(m/z\) =112 and \(m/z\) = 95, respectively. Relative peak area, which is the peak ratio of MIB to GSM was used to determine the concentrations of MIB in water samples.

3.3.9 Data analyses

All standards and samples were replicated three times (\(n=3\)). Maximum response or blank (\(A_o\)), is the absorbance value with no free MIB added to the wells, i.e. zero competition. All data were analyzed using Microsoft Excel (Microsoft Office ver. 2007, Microsoft Corporation, Redmond, WA) and four parameter log graph (4PL) to represent standard curve of absorbance or response (\(A/A_o\)) versus log concentration (\(y\)-axis) of free MIB (\(x\)-axis). \(IC_{50}\) values were obtained at 50% inhibition of the curve. Statistical analysis and four parameter log graph were performed using GraphPad Prism ver. 5.0 (GraphPad Software Inc., San Diego, CA). Limit of detection (LOD) and limit of quantification (LOQ) were calculated as the mean absorbance of blanks or zero competition well (\(A_o\)) ± 3SD for LOD and ± 10SD for LOQ, respectively. Each value was interpolated from the standard curve using GraphPad Prism.
3.4 Results and discussion

3.4.1 Production of immunogens and coating conjugates

With a molecular weight of 168 Da, MIB is a hapten that is unable to elicit an immune response on its own. Therefore, attachment to a carrier protein such as BSA or KLH is necessary to make this molecule immunogenic. In order to achieve this, (-) borneol and (-) camphor were conjugated to BSA (Figure 3.1) and used as immunogens. Both compounds have structural similarities to MIB with a hydroxy reactive group for (-) borneol but an absence of methyl group at the C2 position (Phak and Park, 2003) and a carbonyl reactive group for (-) camphor, respectively. These compounds were selected on the basis that they possess functional groups, on the opposite side of MIB hydroxyl group. The hydroxyl group of (-) borneol is readily converted to an imidazole carbamate active intermediate by CDI (Figure 3.2). Further reaction with amine groups in BSA resulted in one-carbon spacer forming a stable urethane (N-alkyl carbamate) linkage (Hermanson, 2008). This work supports previous suggestion by Xiao et al. (1995) that a CDI-based reaction is able to produce linkages and orientations similar to those obtained using succinic anhydride. The imidazole carbamate intermediates which are formed by the CDI reactions are short-lived and quickly decompose in water to CO₂, imidazole, and the original hapten (Hermanson et al., 1992; Hermanson, 2008). This reduces the formation of non-specific and undesired epitopes being introduced onto the protein during conjugation (Clarke et al., 1993; Gendloff et al., 1986).

For the camphor-BSA conjugate, carbonyl group of (-) camphor was derivatized to camphor O-(carboxymethylloxime) with the formation of carboxyl group which was then further crosslinked to amine groups of BSA through isobutyl chloroformate (Figure 3.3). Samokhin and Filimonov (1985) emphasized the advantage of the mixed anhydride method over that of the carbodiimide method, which was used for camphor conjugate to avoid protein-protein crosslinking. We employed a heterologous ELISA system,
incorporating structurally similar but different hapten for immunogen than the ones used in the assay thereby increasing assay sensitivity (Harrison et al., 1989; Wie and Hammock, 1984).

From MALDI-TOF analysis, a (-) borneol-BSA peak was shown at ca. 67,000 m/z, while the chromatogram for (-) camphor-BSA displayed a peak at ca. 70,420 m/z (data not shown; see Appendix I; Figure A1). Comparing with the molecular weight of BSA at ca. 66,200 m/z, the molar ratios of (-) borneol and (-) camphor to BSA were calculated to be 5:1 and 18:1 hapten:BSA, respectively. While the ratio of (-) camphor to BSA was in what is considered a good range for antigenicity, the ratio of hapten to protein for (-) borneol was lower and may have reduced the antigenicity towards the hapten.

For the coating conjugates, conjugation of MIB to proteins is somewhat technically difficult since the tertiary alcohol is sterically hindered by the methyl group. Attempts to conjugate the carrier protein through hydroxyl group at C2 of MIB directly through CDI activation and coupling was not successful. MIB-TG was prepared and kindly provided by Abraxis LLC. (+) Bornylamine was conjugated to TG using DSS following the method by Paek et al. (1993) and Skerritt et al. (1992). DSS is an amine-reactive, NHS ester, homobifunctional cross-linker. Reaction with DSS produces an 8-atom bridge between conjugated molecules (Pierce).

It is also crucial that anhydrous solvent is used for the conjugation work to prevent hydrolysis of the activation agent or subsequent reactive groups in the reaction (Hermanson, 2008), which will result in low conjugate yield. In a different attempt, conjugation of isoborneol, an exo isomer of borneol to TG as coating conjugate through its hydroxyl group was prepared by Dr. Karsunke (Institute of Hydrochemistry, Technical University of Munich, Germany). However, the sensitivity of the antibody was not favorable towards MIB (IC\textsubscript{50} = 139.4 ng mL\textsuperscript{-1}).
Figure 3.2. Schematic of conjugation of (-) borneol to BSA through interaction of CDI with (-) borneol, which forms an imidazole carbamate intermediate. Upon interaction of the intermediate with amines of BSA, a stable peptide linkage is formed (modified from Hermanson, 2008 and Xiao et al., 1995).
(-) Camphor

\[
\text{NH}_2\text{OCH}_2\text{COOH}
\]
Carboxymethoxyamine

Camphor O-(carboxymethoxyl)oxime

Isobutyl chloroformate

Mixed anhydride

(-) Camphor-BSA conjugate

**Figure 3.3.** Schematic of (-) camphor conjugation with BSA by derivatization with carboxymethoxylamine forming camphor-O-(carboxymethyl)oxime (CMO) after which the free carboxyl group of the CMO derivative was crosslinked to amine groups of BSA after formation of the mixed anhydride derivative using isobutyl chloroformate (adapted from Chung et al, 1990).
3.4.2 Serum analyses

Rabbits were immunized with (-) borneol-BSA (rabbits BR1 and BR2) and (-) camphor-BSA (rabbits CR1 and CR2). Antisera collected over the course of immunization were tested for polyclonal immune responses by indirect non-competitive ELISA using (+) bornylamine or MIB conjugated to TG as solid phase coatings as described in Section 3.3.4. Rabbits immunized with (-) camphor-BSA showed a good response as early as the first boost, which was maintained until the last boost, while rabbits immunized with (-) borneol-BSA had a poorer response towards the immunogen even after the fifth and sixth boosts (Figure 3.4). Our results are in agreement with a previous study (Chung et al., 1990) which showed that (-) camphor-BSA is an effective immunogen to produce high and constant titers after only a few injections. Perhaps the dissimilarity of binding of antisera from rabbits immunized with different immunogens could be due to a lower hapten load of BSA conjugated with (-) borneol (ca. 5:1) as compared to (-) camphor (ca. 18:1). In fact, some studies have shown that immunization with higher hapten density conjugates leads to higher antibody titers as they present many haptens as epitopes for antibody recognition (Landsteiner, 1945).

The antisera titers were determined by doing an end-point titration ELISA. By definition, the value of the antisera titer corresponds to the antisera dilution, resulting in uninhibited assay signal three times the background signal; in this case, pre-immune sera under the given assay conditions (Szurdoki et al., 2002). The antisera titers were compared for rabbits from bleed 5 using plates coated with MIB-TG. The titer values were 1/409,600 for rabbits CR1, and CR2, and 1/204,800 for rabbits BR1 and BR2, respectively (See Appendix II, Figure A2). Because antisera from rabbits immunized with (-) camphor-BSA (CR1 and CR2) displayed higher titers than rabbits immunized with (-) borneol-BSA, we carried out subsequent experiments with CR1 and CR2 antisera.
Figure 3.4. Indirect non-competitive ELISA showing the immune responses of rabbits immunized with (-) borneol-BSA (BR1 and BR2) and (-) camphor-BSA (CR1 and CR2). Antisera from days 0 (pre-immune), 43, 71, 99, 127, 155, 181 and 225 (terminal bleed) were diluted at 1/800 (v/v) and assayed against (A) MIB-TG and (B) (+) bornylamine-TG at 1 µg mL\(^{-1}\). Values represent mean ± SD of three replicates.
For competitive-inhibition ELISAs (CI-ELISA), rabbit antisera were first assayed by checkerboard ELISAs as described in Section 3.3.5.1. Checkerboard ELISAs help to select optimal dilutions for both coating antigen and antiserum in order to produce the lowest IC₅₀ values. Terminal bleed antisera from both rabbits (CR1 and CR2) immunized with (-) camphor-BSA were tested against both coating conjugates, (+) bornylamine-TG (BA-TG) and MIB-TG in CI-ELISA and their respective curves are shown in Figure 3.5. The best inhibition curve, i.e., having the lowest IC₅₀ value (115 ng mL⁻¹) was obtained from rabbit CR1 with plates coated with MIB-TG. In comparison, the IC₅₀ value for CR1 against BA-TG was 304 ng mL⁻¹. For rabbit CR2, the IC₅₀ values were at 693 and 2902 ng mL⁻¹ for MIB-TG and BA-TG coated plates, respectively. In addition, a CI-ELISA against free TG carrier protein showed a flat curve with no inhibition indicating that rabbit antisera immunized with (-) camphor-BSA did not cross react with TG on the coating conjugates (data not shown). Further optimization and validation of assays were carried out with antisera from rabbit CR1 using MIB-TG as the coating conjugate.
Figure 3.5. Inhibition curves from CI-ELISA for rabbits (A) CR1 and (B) CR2 which were immunized with (-) camphor-BSA; plates were coated with (+) bornylamine-TG (BA-TG) (●) or MIB-TG (■). Coating conjugates and antisera were used at 1/9,000 (v/v) and 1/3,000 (v/v) dilutions, respectively. CI-ELISAs were done as described in Section 3.3.5.2. Values represent mean ± SD of three replicates.
Using the combination of (-) camphor-BSA as immunogen and MIB-TG as coating conjugate, a more sensitive assay \( (IC_{50} = 115 \, \text{ng mL}^{-1}) \) than that of Chung et al. (1990) \( (IC_{50} = 10 \, \mu\text{g mL}^{-1}) \) was developed. However, a more sensitive CI-ELISA \( (IC_{50} = 5 \, \text{ng mL}^{-1}) \) was developed by Phlak and Park (2003) using a mAb with (-) borneol-LPH as immunogen and MIB-BSA as coating conjugate.

3.4.3 Optimization of assay conditions

The assay was further optimized and validated using terminal bleed antisera from rabbit CR1 and MIB-TG as the coating conjugate using the four-parameter log model (4PL) where \( A/A_0 \) data was plotted against log of analyte concentrations. The \( IC_{50} \) value at the central point of the curve is the free analyte concentration inhibiting the absorbance of the control by 50%. It signifies the sensitivity of an assay at the linear portion which represents the analytical range where smaller \( IC_{50} \) denotes higher sensitivity (Lee et al., 2001).

Optimization of the ELISA was conducted by varying different parameters as described by other researchers (Kim et al., 2007; Lee et al., 2001). A competitive indirect immunoassay was chosen as the immunoassay format to optimize the ELISA because MIB only has one epitopic site on the molecule, and thus, it is not possible to use a sandwich ELISA format since it requires two specific antibodies to bind to different epitopes. As for a direct ELISA format, purification of antibodies against hapten is necessary as coating of diluted antisera directly onto plate surface would result in a low signal as other proteins in antisera would bind unspecifically to the plate exterior. Furthermore, the use of direct competitive ELISA requires the synthesis of hapten labeled enzyme (e.g. MIB-HRP or MIB-AP) for competition with free hapten (e.g. MIB) in the test sample.
Finally, although several researchers have shown that the direct competitive ELISA format is more sensitive than indirect competitive format, other researchers have shown otherwise. For example, detection of permethrin was found to be more sensitive using direct competitive ELISA (Skerritt et al., 1992). In contrast, indirect competitive ELISAs were found to be more sensitive for detection of metachlor in soil samples (Schlaeppi et al., 1991) and imidacloprid in agricultural and environmental matrices (Lee et al., 2001). On the basis of these facts, it was decided to proceed with a competitive indirect ELISA format.

3.4.3.1 Optimal concentrations of antisera and coating conjugate

For CI-ELISA format, it is crucial that the concentrations of antibody and coating conjugate should be high enough to produce an acceptable signal for antibody-antigen binding yet low enough to optimize competition with free analyte. Thus, the effect of antisera concentrations and coating conjugate dilutions on the sensitivity of MIB assay were investigated. From checkerboard ELISA, a few combinations of coating conjugate and antisera were used in the CI-ELISA as presented in Table 3.1. Lowering the concentration of both coating antigen and antibody may provide better sensitivity. As compared to the IC$_{50}$ value determined for dilution A (Table 3.1), which was 115 ng mL$^{-1}$, the IC$_{50}$ value for dilution B (Table 3.1) was significantly higher ($p<0.05$) at 145 ng mL$^{-1}$. However, the IC$_{50}$ value for dilution C (Table 3.1) was slightly lower than dilution A, but not significantly different ($p>0.05$). As expected, the absorbance reading in the maximum (zero competition) wells was decreased as lower concentrations of coating conjugate and antisera were used. As shown by dilution C, the lowering of both concentrations allowed the antibody to bind with the lesser analyte on the plate. As a result, the antibody will better recognize the analyte in the solution, thus the sensitivity will increase. Dilution C has the lowest IC$_{50}$ and the highest $A/D$ value as compared to dilution A and
B. Thus, the combination in dilution C was chosen for assay optimization. The CI-ELISA curve for dilution C is shown in Figure A3 (Appendix III), with an IC$_{50}$ of 105 ng mL$^{-1}$, a LOD of 4.8 ng mL$^{-1}$, and a LOQ of 13.2 ng mL$^{-1}$, respectively (Table 3.1). The sensitivity of an ELISA is very dependent on the optimal concentrations of the antibody and the coating antigen. Furthermore, as shown by the comparison of these three dilutions, the sensitivity of ELISA is dependent on the antibody’s recognition of the free antigen, rather than the bound antigen.

### Table 3.1. Effect of concentrations of MIB-TG as coating conjugate and antisera dilution from rabbit anti (-) camphor-BSA (CR1) on MIB ELISA

<table>
<thead>
<tr>
<th>Coating conjugate dilution</th>
<th>Anti-serum dilution</th>
<th>$A^a$</th>
<th>Slope ($B$)</th>
<th>IC$_{50}$ (ng mL$^{-1}$)</th>
<th>$D^b$</th>
<th>A/D</th>
<th>A$_{max}^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1/9,000</td>
<td>1/3,000</td>
<td>1.018</td>
<td>-0.468</td>
<td>115</td>
<td>-0.030</td>
<td>33.9</td>
</tr>
<tr>
<td>B</td>
<td>1/27,000</td>
<td>1/27,000</td>
<td>0.997</td>
<td>-0.648</td>
<td>145</td>
<td>-0.005</td>
<td>199.4</td>
</tr>
<tr>
<td>C</td>
<td>1/27,000</td>
<td>1/54,000</td>
<td>1.014</td>
<td>-0.518</td>
<td>105</td>
<td>-0.002</td>
<td>507.0</td>
</tr>
</tbody>
</table>

$^a$A and D are top and bottom value on y-axis derived from four-parameter log graph plotted for $A/A_0$ versus log MIB concentrations

$^b$Absorbance for maximum (zero competition) well

3.4.3.2 Pre-incubation of the analyte and antisera mixture

In CI-ELISAs, the analyte and antisera are added simultaneously to the wells, while in inhibition indirect ELISAs they are allowed to pre-incubate before being added to the wells. The effect of pre-incubation (i.e., inhibition ELISA) for 1 h at RT with constant rotating was evaluated. We observed that this pre-incubation step did not improve the sensitivity of the MIB ELISA. In fact, the absorbance reading was reduced by more than 50% (data not shown) when compared to CI-ELISA. Therefore, CI-ELISAs were carried out throughout the remainder of the study.
3.4.4 Cross reactivity (CR)

The specificity of antisera from rabbit CR1 immunized with (-) camphor-BSA was evaluated by testing its cross reactivity with compounds that are related to MIB. The cross-reactivity of MIB was defined as 100%, while cross reactivity was defined as the percentage of the ratio of IC\(_{50}\) of MIB over IC\(_{50}\) of the related compound. As shown in Table 3.2, the antisera had specificity for some compounds that are structurally related to MIB. The antisera were most specific for (-) camphor, MIB, (+) camphor, and isoborneol with cross-reactivities of 151.9, 100, 57.3, and 75.3, respectively. Although these compounds may be present in the environment, they are not known to contribute to taste and odor (T&O) problems in drinking waters. Both camphor and isoborneol, an isomer of borneol, are unlikely to be found with MIB. Since (-) camphor was used as immunogen, the polyclonal antisera seem to recognize the presence of the methyl group on MIB and camphor-like compounds. Compounds that lack methyl groups, including norcamphor, norborneol, norbornylamine, and norbornene, had significantly lower cross reactivity to the polyclonal antisera. Interestingly, the presence of an amine group in (+) bornylamine inhibited antibody recognition despite the presence of methyl groups. The polyclonal antisera also showed some cross-reactivity (ca. 14.2%) with camphorquinone, which has an additional carbonyl group, when compared to camphor. While isoborneol and (-) borneol are epimers, the polyclonal antibody showed better recognition to isoborneol than (-) borneol, suggesting that the position of hydroxyl group is important for antibody binding. Geosmin, which is another off-flavor causing compound that often co-occurs with MIB, showed low cross reactivity (ca. 14.9%) since its structure is different from MIB and camphor. However, it could contribute to false positives in the ELISA for MIB. Dehydration products of MIB, 2-methylenebornane and 2-methyl-2-bornene were not tested because commercial standards were not available. These two
compounds can be present in fish flesh but they do not cause off-flavor (Korth et al., 1992; Martin et al., 1988).
Table 3.2. Cross-reactivities (CR) of rabbit (CR1) polyclonal serum (anti (-) camphor-BSA) to MIB and structurally related compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>MW</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (ng mL&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>% CR&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIB</td>
<td>[Image]</td>
<td>168.28</td>
<td>105</td>
<td>100</td>
</tr>
<tr>
<td>(+) Camphor</td>
<td>[Image]</td>
<td>152.23</td>
<td>183.3</td>
<td>57.3</td>
</tr>
<tr>
<td>(-) Camphor</td>
<td>[Image]</td>
<td>152.23</td>
<td>69.1</td>
<td>151.9</td>
</tr>
<tr>
<td>Camphorquinone</td>
<td>[Image]</td>
<td>166.22</td>
<td>738.6</td>
<td>14.2</td>
</tr>
<tr>
<td>(-) Borneol</td>
<td>[Image]</td>
<td>154.25</td>
<td>364</td>
<td>28.8</td>
</tr>
<tr>
<td>Isoborneol</td>
<td>[Image]</td>
<td>154.25</td>
<td>139.4</td>
<td>75.3</td>
</tr>
<tr>
<td>Norcamphor</td>
<td>[Image]</td>
<td>110.16</td>
<td>&gt;10000</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>(+) Endo-2-norborneol</td>
<td>[Image]</td>
<td>112.17</td>
<td>&gt;10000</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>(+) Bornylamine</td>
<td>[Image]</td>
<td>152.26</td>
<td>&gt;10000</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Norbornylamine</td>
<td>[Image]</td>
<td>111.6</td>
<td>&gt;10000</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Fenchol</td>
<td>[Image]</td>
<td>154.25</td>
<td>354.8</td>
<td>29.6</td>
</tr>
<tr>
<td>Geosmin</td>
<td>[Image]</td>
<td>182.30</td>
<td>706.3</td>
<td>14.9</td>
</tr>
</tbody>
</table>

<sup>a</sup>% CR is calculated as IC<sub>50</sub> value of MIB/IC<sub>50</sub> value of structurally related compound x 100
3.4.5 Physicochemical effects on assay performance

3.4.5.1 Effect of incubation time and temperature

Incubation time (0.5, 1.0, 1.5 h) and temperature (22°C and 37°C) were shown to affect the sensitivity of the ELISA. At 22°C (i.e., RT) the IC\textsubscript{50} values for 1.0 and 1.5 h were not significantly different ($p>0.05$) whereas IC\textsubscript{50} for 0.5 h was significantly greater ($p<0.05$). This may be due to the insufficient time for free MIB to compete with immobilized MIB for antibody binding. Regardless of the incubation time, all IC\textsubscript{50}s at 37°C were significantly greater ($p<0.05$) than at 22°C. Furthermore, at 37°C the IC\textsubscript{50}s increased as incubation time increased. Thus, a 1.5 h incubation time at 22°C was chosen as the optimal for the CI-ELISA.
**Figure 3.6.** Effect of incubation time and temperature on the ELISA for MIB. CI-ELISAs were compared at three different incubation times (0.5, 1.0 and 1.5 h) in two different temperatures; (A) 22ºC (RT) and (B) 37ºC. Longer incubation time (1.5 h) at 22ºC increased the assay sensitivity. Values represent mean ± SD of three replicates.

3.4.5.2 Effect of solvent and detergent
The effects of organic solvents, which are commonly used for analyte extraction from various matrices or for solid phase extraction (SPE) cartridge elution, were examined. Methanol and acetonitrile were added to the MIB standards at 2.5, 5, 10, and 20% (v/v). CI-ELISA was carried out according to Section 3.3.5.2 and free MIB at different percentages of organic solvent were allowed to compete with bound MIB for antibody binding. As shown in Figure 3.7, IC₅₀ values increased as the percentage of both organic solvents increased. Statistical analysis (F test to compare log IC₅₀ values) demonstrated that the IC₅₀ values obtained for 0 - 5% methanol were not different (p>0.05). The addition of methanol and acetonitrile at 2.5% and 5% had less effect on the sensitivity of the MIB ELISA, than did the addition of 10% and 20%. Furthermore, acetonitrile had a greater negative effect on the ELISA than methanol. The work of Lee et al. (2001) also showed that the presence of organic solvents, such as DMSO and DMF, increased the sensitivity of imidacloprid ELISA significantly, i.e., the IC₅₀ values were reduced. This result indicates that no more than 5% methanol should be used to dissolve MIB standards. MIB is insoluble in water with a solubility reported at 194.5 mg L⁻¹ (Pirbazari et al., 1992; Young et al., 1996). When CI-ELISAs were performed with MIB standard prepared in 1X PBS, we found that the standards were unstable producing inconsistent results. Standard series of MIB were prepared from 1 mg mL⁻¹ stock in 10% methanol (v/v) with the final concentration of methanol in all CI-ELISAs being 5% (v/v).
Figure 3.7. Effect of organic solvents; (A) methanol and (B) acetonitrile on the ELISA for MIB. Respective IC$_{50}$ values (ng mL$^{-1}$) are given adjacent to each concentration of solvent. Addition of organic solvents at 10 and 20% increased the IC$_{50}$ values significantly ($p<0.05$). Means sharing different superscript are significant different ($p<0.05$) between each other. Values represent mean ± SD of three replicates.
Some researchers have reported that low concentrations of detergent improve the assay sensitivity especially for haptens such as chlorpyrifos (Manclus and Montoya, 1996a), permethrin (Stanker et al., 1989) and endosulfan (Lee et al., 1995). Detergent molecules are made up of distinct hydrophobic and hydrophilic regions. In ELISA, detergents are used to remove unstable and unspecific hydrophobic bonds between the polystyrene surface and coating antigen molecules, and between reactants on the surface (Esser, 2010). The effect of Tween 20 at 0.05% on the sensitivity of MIB assay was evaluated by diluting the antisera in 1X PBST versus 1X PBS as diluent. As shown in Figure 3.8, it was observed that Tween 20 decreases assay sensitivity by reducing the mean of $A_{\text{max}}$ reading by ca. 36% and increasing IC$_{50}$ values by ca. 30%. This might be due to the interference of hydrophobic group of Tween 20 with the binding sites of the antibody. Several groups have also found that adding detergent to the assay buffer decreases assay sensitivity. For example, Xu et al. (2006) observed the same trend in their ELISA for detection of diethylstilbestrol (DES) residue in chicken and liver tissues where $A_{\text{max}}$ values were lowered and IC$_{50}$ values were increased as the concentration of Tween 20 increased. They demonstrated that the addition of Tween 20 at 0.05% was optimal for their assay. Another report demonstrated that IC$_{50}$ values increased considerably while the $A_{\text{max}}$ decreased when detergent concentration reached values around 0.5% (Galve et al., 2002). In fact, this group found that assay buffer containing Tween 20 at concentrations ranging from 0.05 to 0.025% provided the best results (IC$_{50}$ 1.53–1.51 g L$^{-1}$) for trichlorophenol ELISA. Finally, Lee et al. (2001) demonstrated that sensitivity (in terms of IC$_{50}$ values) of imidaclopid ELISA was reduced by 42.7 % when 1X PBS containing 0.05% Tween 20 (v/v) was used as assay buffer. It was suggested that non-specific hydrophobic interaction may occur between the detergent and very water soluble analytes, such as imidaclopid (0.61 g L$^{-1}$, at 20 °C). In contrast, the
addition of Tween 20 to buffer did not affect the very polar compound, 3,5,6-trichloro-2-pyridinol (TCP) (Manclus and Montoya, 1996b).

![Graph showing IC50 values for MIB with and without Tween 20.](image)

**Figure 3.8.** Effect of Tween 20 (0.05%) on the ELISA for MIB. Tween 20 increased the IC50 significantly (p<0.05) and decreased the A_max. Values represent mean ± SD of three replicates.

3.4.5.3 Effect of pH

Matrix effects such as pH and solvent concentrations have been reported to influence assay sensitivity for a number of immunoassays, especially for pesticide analysis (Goh et al., 1990; Kido et al., 1997; Koppatschek et al., 1990). The effect of pH on the MIB ELISA was evaluated by adjusting the pH value of assay buffer from pH 4.0 to pH 9.0. It was observed that the IC50 values decreased as the pH increased (Figure 3.9). However, there was no significant effect (p>0.05) of pH ranging from 6.0 to 8.0 on IC50 values (F test to compare log IC50 values). Although pH 9.0 had a lower IC50 as compared to other pH, the A_max value also decreased. Subsequently, 1X PBS at pH 7.4 was used as antibody diluent in all MIB ELISAs.
Figure 3.9. Effect of pH (4.0, 5.0, 6.0, 7.0, 7.4, 8.0, and 9.0) on the ELISA for MIB. Antiserum from rabbit CR1 was diluted in 1X PBS at different pH values. IC\textsubscript{50} values are shown adjacent to the respective pH values. IC\textsubscript{50} values and A\textsubscript{max} decreased as pH increased. Values represent means ± SD of three replicates.

3.4.5.4 Effect of concentration of salt

The effect of salt concentration (i.e., ionic strength, I) on MIB assay sensitivity was studied by utilizing four different concentrations of PBS (1X, 2X, 4X, and 10X; I = 0.167, 0.333, 0.668, and 1.67 respectively) as assay buffer for antibody diluent. As shown in Figure 3.8, it was apparent that salt concentration influenced ELISA performance. As ionic strength increased from 1X PBS (ionic strength, I = 0.167) to 10X PBS (I = 1.67) the IC\textsubscript{50} was lowered, and A\textsubscript{max} values decreased. Compared to 1X PBS, IC\textsubscript{50} values decreased by 59.1, 66.1, and 68.9 %, while the A\textsubscript{max} decreased by 36.2, 74.5, and 81.8 % for 2X (I = 0.333), 4X (I = 0.668), and 10X PBS, respectively. Lee et al. (2001) speculated that salt interferes with antibody binding to the coating conjugate, as the presence of salt as high as 10X almost diminishes the binding. Other reports have
also noted the same effect of salt on ELISAs for pyrethroid deltamethrin (Lee et al., 2002) and O-O-dimethyl organophosphorus pesticides (Liang et al., 2008).

![Graph of MIB concentration vs. absorbance](image)

**Figure 3.10.** Effect of concentration of salt on the ELISA for MIB. IC₅₀ values are shown adjacent to the respective PBS concentrations accordingly. IC₅₀ values and A_max decreased as higher salt concentration was used. Values represent mean ± SD of three replicates.

In conclusion, after evaluations of physicochemical factors that may affect the sensitivity of CI-ELISA, the optimal conditions for the assay are: coating conjugate (MIB-TG) at 1/27,000 (v/v); 1/54,000 (v/v) dilution of antiserum in 1X PBS, pH 7.4; methanol 5% (v/v); 22°C; 1.5 h incubation time for competitive indirect (CI-ELISA) and the assay was stable at pH ranging from 6.0-8.0.
The reproducibility and precision of the CI-ELISA were determined using MIB standards on three different plates (intraassay) and on three different days (interassay). The results of this precision assay are presented in Table 3.3. The co-efficient of variation of the intra- and inter-assay are expressed as: \( \% \text{CV} = \frac{\text{standard deviation}}{\text{mean}} \times 100 \). Standard concentrations between 0.01 to 10,000 ng mL\(^{-1}\) showed intraassay CV ranged from 1.50% to 10.08%. As for the interassay precision, CV was shown to be in the 2.01% to 10.94% range. Both intraassay and interassay CV were within the acceptable range of ± 20% (Krotzky and Zeeh, 1995). It was observed that the highest CV was in the mid-range of MIB standards which may affect the characteristic of the inhibition curve and sensitivity in terms of IC\(_{50}\) values. Therefore, it is crucial that the standard curve and determination of unknown sample concentrations be performed within the same day and using the same plate. Findlay et al. (2000) suggested that the criteria for accuracy and precision for immunoassays should be more lenient than analytical methods since the core of the assay is based on the antigen-antibody reaction. When compared to chromatographic methods, immunoassays may require more validation runs to obtain the same level of confidence in assay performance (Kringle and Khan-Malek, 1994). Since immunoassays are expected to be less precise than chemical analysis, a less restrictive acceptance limit (e.g. 25%) has been recommended (Braggio et al., 1996; Findlay and Das, 1998).
Table 3.3. Intraassay and interassay precision of MIB standards

<table>
<thead>
<tr>
<th>MIB (ng mL(^{-1}))</th>
<th>Intraassay variation</th>
<th>Interassay variation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A/Ao</td>
<td>SD</td>
</tr>
<tr>
<td>0.01</td>
<td>0.986</td>
<td>0.150</td>
</tr>
<tr>
<td>1</td>
<td>0.904</td>
<td>0.031</td>
</tr>
<tr>
<td>10</td>
<td>0.827</td>
<td>0.083</td>
</tr>
<tr>
<td>50</td>
<td>0.729</td>
<td>0.067</td>
</tr>
<tr>
<td>100</td>
<td>0.556</td>
<td>0.057</td>
</tr>
<tr>
<td>500</td>
<td>0.364</td>
<td>0.017</td>
</tr>
<tr>
<td>1000</td>
<td>0.262</td>
<td>0.010</td>
</tr>
<tr>
<td>10,000</td>
<td>0.171</td>
<td>0.009</td>
</tr>
</tbody>
</table>

Assays were performed on three different plates (intraassay) on three different days (interassay).

3.4.7 Comparison to GC-MS analyses

The results obtained from our optimized MIB assay were compared to those obtained by GC-MS analyses. For GC-MS analyses, different water samples (10 mL) were fortified with MIB at 1, 10, 50, and 100 ng mL\(^{-1}\) prior to analysis using SPME fiber diffusion at 50\(^{\circ}\)C. Geosmin (10 ng mL\(^{-1}\)) was used as an internal standard. Analyses were carried out in triplicate. The proper sorption onto SPME fibers depends on the methanol concentration in the sample (Chang et al., 2008) because it affects the vapor pressure of the analyte. Therefore, we standardized the percentage to 5% methanol in all the samples. Furthermore, we also found that heating the samples to 50\(^{\circ}\)C assisted with the adsorption of both compounds to the SPME fiber as volatiles have better diffusion rate at a higher temperature. Similar peak area and height were observed when sodium chloride (4%) was added to the water samples and stirred using magnetic stirrer bar at 50\(^{\circ}\)C (data not shown). Thus, salt did not aid with the absorption of MIB onto the fiber as has been reported previously (Saito et al., 2008). Changing the SPME fiber from PDMS to DVB/CAR/PDMS increased the peak area of MIB by 16-fold (data not shown).
This is in agreement with Saito et al. (2008) who reported that the additional organic material in the packaging of the fiber of the latter SPME fiber enhances the absorption. We also found that the polarity and thickness of the stationary phase coating on the SPME fiber influenced the quantity of volatiles (i.e., MIB and GSM) adsorbed. Using this method, the detection limit of GC-MS was found to be at 1 ng mL\(^{-1}\) (signal to noise ratio; S/N = 3) (data not shown).

The same samples used for GC-MS analyses without internal standard were analyzed by CI-ELISA. The concentration of MIB recovered from the fortified water samples were interpolated from a standard curve performed within the same day. Recovery of MIB from fortified water samples by CI-ELISA and GC-MS methods is presented in Table 3.4. In addition, comparison of results for fortified water samples obtained by ELISA (y-axis) and by GC-MS method (x-axis) is presented in Figure 3.11. Good correlations \((r^2 > 0.990)\) of the two methods were obtained for all water samples (Figure 3.11) when values recovered were plotted using linear regression. The MIB concentrations in water samples determined by ELISA overestimated MIB levels as compared to GC-MS, as indicated by the slope of the regression equation. Hence, this assay can be considered as a qualitative assay. The overestimation of MIB using ELISA could be due to the instability or insolubility of MIB in sample matrix as less than 10% of methanol was used. The other reason is the presence of other compounds in the water sample, or the overall composition of the sample may interfere with the antibody binding (Brown, 2011). GC-MS analyses were not affected because MIB is extracted through volatization (pre-concentration step) and captured by SPME fiber and thus there was little or no matrix interference. In ELISA, the recovery of fortified water samples at 1 ng mL\(^{-1}\) was more than 200% (Table 3.4). Therefore, the ELISA could be performed better within a working range of ca. 10-1000 ng mL\(^{-1}\) as opposed to 1-1000 ng mL\(^{-1}\).
The GC-MS method was found to be suitable for MIB concentrations in the range of 5 - 500 ng mL\(^{-1}\). Above 500 ng mL\(^{-1}\), non-symmetrical (overloaded) peaks were seen (data not shown). Hence, it is suggested that split injection or sample dilution be performed at concentrations above 500 ng mL\(^{-1}\). Recovery form fortified MIB samples at 50 and 100 ng mL\(^{-1}\) falls within the range of generally acceptable quantitative ELISA (80-120%; Brown, 2011) and 10-100 ng mL\(^{-1}\) for GC-MS analyses. Furthermore, recovery as determined by GC-MS was less than 80% of MIB at 1 ng mL\(^{-1}\) because this concentration is close to the method detection limit (MDL). One key advantage of ELISA over GC-MS analyses was the shorter analysis time; where GC-MS required about 1 h for each sample run; while multiple samples (ca. 20-24 samples) could be performed within a single microtiter plate with the standards in triplicate within 4-5 h using ELISA.
Table 3.4. Recovery of MiB from fortified water samples as measured by ELISA and GC-MS. Data are means of three replicates

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fortified (ng mL(^{-1}))</th>
<th>Detected (ng mL(^{-1}))</th>
<th>Recovery (%)</th>
<th>CV (%)</th>
<th>Detected (ng mL(^{-1}))</th>
<th>Recovery (%)</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nanopure water</td>
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<td>50.0</td>
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</tr>
<tr>
<td></td>
<td>10</td>
<td>11.0</td>
<td>110.0</td>
<td>18.5</td>
<td>10.6</td>
<td>106.3</td>
<td>4.5</td>
</tr>
<tr>
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<td>50</td>
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<td>50.6</td>
<td>101.2</td>
<td>1.1</td>
</tr>
<tr>
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<td>100</td>
<td>105.3</td>
<td>105.3</td>
<td>3.7</td>
<td>99.6</td>
<td>99.6</td>
<td>3.1</td>
</tr>
<tr>
<td>Tap water</td>
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<td>250.0</td>
<td>29.5</td>
<td>0.4</td>
<td>40.0</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>14.3</td>
<td>143.0</td>
<td>25.6</td>
<td>10.2</td>
<td>102.0</td>
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<tr>
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<td>50</td>
<td>52.8</td>
<td>105.6</td>
<td>12.8</td>
<td>50.6</td>
<td>101.2</td>
<td>4.1</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>109.8</td>
<td>109.8</td>
<td>1.5</td>
<td>101.8</td>
<td>101.8</td>
<td>5.5</td>
</tr>
<tr>
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<td>60.2</td>
<td>0.5</td>
<td>50.0</td>
<td>6.4</td>
</tr>
<tr>
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<td>10</td>
<td>14.7</td>
<td>147.0</td>
<td>36.2</td>
<td>13.5</td>
<td>135.0</td>
<td>9.6</td>
</tr>
<tr>
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<td>21.8</td>
<td>47.1</td>
<td>94.2</td>
<td>5.4</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>106.3</td>
<td>106.3</td>
<td>11.9</td>
<td>92.8</td>
<td>92.8</td>
<td>4.4</td>
</tr>
<tr>
<td>Lake water</td>
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<td>80.0</td>
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<td>111.0</td>
<td>4.0</td>
<td>111.1</td>
<td>111.1</td>
<td>8.4</td>
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Figure 3.11. Correlation of MIB concentrations in four types of fortified water samples as measured by ELISA and GC-MS.
3.4.8 Improvement of assay sensitivity through signal amplification

Several attempts were made to further increase the sensitivity of the MIB assay. In a first trial, the ELISA substrate for HRP was changed from a chromogenic (TMB substrate) to a chemiluminescent (Super Signal ELISA Femto substrate; Thermo Scientific, Nepean, Canada) substrate while keeping all other parameters constant. Several studies reported an improved sensitivity using luminescence probes (Ahn et al., 2007; Lin et al., 2005; Zhang et al., 2006). Specifically, chemiluminescent immunoassays have emerged as sensitive assays due to their high quantum yield and chemiluminescent reaction rate, high efficiency of modern light detectors, and fewer reagents are required (Arefyev et al., 1990; Dzgoev et al., 1999). Although this method was highly sensitive, the background of the negative control was substantial, partly due to the non-specific binding of primary and secondary antibodies to the plate. Furthermore, crosstalk between wells also contributed to the high background signal. Hence, we found that this method did not improve the sensitivity of the ELISA.

Many researchers have also successfully exploited the remarkable binding of streptavidin or avidin to biotin and used it to improve the sensitivity of their assay (Kendall et al., 1983; Sai et al., 2010; Zhang et al., 2012) to the femtomolar level (Green, 1990). In a second trial, we therefore used mouse anti-rabbit biotin conjugate (Jackson ImmunoResearch, West Grove, PA) and streptavidin-HRP (Pierce Thermo Scientific; Nepean, Canada) at 1/10,000 (v/v) and 1/100 (v/v), respectively, as secondary and tertiary antibodies in the MIB ELISA. We employed a streptavidin-biotin system as signal amplification strategy to lower the concentrations of coating conjugate and antisera to achieve better sensitivity. Applying coating conjugate concentrations at 1/54,000 (v/v) and varying antisera dilutions, i.e., 1/54,000, 1/81,000, and 1/108,000, maximum absorbance for wells \( A_{\text{max}} \) was observed to be at ca. 1.0, 0.8, and 0.3, respectively.
Further tests at 1/54,000 and 1/81,000 antisera dilutions produced IC₅₀ values of assay at 119.5 and 127 ng mL⁻¹, respectively (data not shown).

Finally, another signal amplification scheme called ELAST ELISA amplification system (Perkin Elmer Inc. Waltham, MA) was attempted to further improve assay sensitivity. This system involves the incorporation of a biotinyl tyramide molecule which will bind to tyrosine and tryptophan residues on the protein molecules immobilized on the plate by catalyzed reaction of HRP on secondary antibody. Subsequent reaction with streptavidin-HRP results in the binding of additional HRP (on streptavidin molecule) to the biotinyl tyramide on the solid phase resulting in signal amplification (Perkin Elmer Inc., 2007).

Concentrations of reagents were initially optimized and selected based on OD values > 0.3 of non-competitive wells. Inhibition curves for various dilutions (1/54,000, 1/81,000, 1/108,000, and 1/162,000 v/v) of MIB-TG as coating conjugate with two different dilutions (1/10,000 and 1/20,000 v/v) of antisera from rabbit CR1 are shown in Figure 3.12. As shown, lowering the concentration of both coating conjugate and antisera decreased the IC₅₀ values significantly (p<0.05); however, the sensitivity of the ELAST MIB ELISA was lower than that of indirect ELISA when HRP was used as the reporter system.
Figure 3.12. ELAST ELISA amplification system using MIB-TG as coating conjugate at various concentrations (1/54,000, 1/81,000, 1/108,000 and 1/162,000 v/v) and antisera from CR1 at (A) 1/10,000 and (B) 1/20,000 (v/v) dilutions. Horseradish peroxidase goat anti-rabbit IgG conjugate and biotinylated tyramine were used at 1/10,000 and 1/100 (v/v) dilutions, respectively. Values represent mean ± SD of three replicates.
These experiments showed that lowering the concentrations of coating conjugate and antibody does not necessarily result in better immunoassay sensitivity. Although favorable signals are produced using more sensitive signaling systems or probes, the affinity of antibody towards the intended antigen is the single most important factor in determining the sensitivity of an assay. Chung (1992) took several approaches to improve the sensitivity of their pAb to MIB by: (1) immunizing different rabbits with camphor-ethylenediamine-BSA conjugate; (2) employing camphor-alkaline phosphatase as a detection system; and (3) adjustment of ELISA conditions using five different blocking agents, two types of microtiter plates and longer incubation time. Despite these approaches, Chung found that the sensitivity of the ELISA remained at 1 µg mL\(^{-1}\).

3.5 Conclusion

In this study, a heterologous system was employed for antibody production against MIB. Two structurally related compounds to MIB; i.e., (\(-\)) camphor and (\(-\)) borneol, were conjugated to BSA and used as immunogen to produce polyclonal antibodies in rabbits. Polyclonal antibodies recognized MIB in the ELISA, with antibodies raised against (\(-\)) camphor-BSA showing higher titer than antibodies raised against (\(-\)) borneol-BSA. A cross-reactivity study showed that the pAb serum is specific to MIB and camphor-like compounds with recognition of the methyl group in these molecules. The fact that our pAb serum was able to cross-react with other compounds is not of major concern because these compounds do not tend to co-occur where MIB is found. However, cross-reactivity of geosmin at 14.9% could be problematic as it often occurs with MIB and is also responsible for off-flavor.

Various factors which may affect the performance of an immunoassay were evaluated and presented. The CI-ELISA was best performed with 1.5 h incubation time.
at 22°C. The assay was found to be stable and most sensitive at pHs near 7.0 in the presence of 5% methanol. Addition of Tween 20 at 0.05% decreased the absorbance and sensitivity of the assay. Increasing the salt concentration decreased the IC_{50} and A_{max} values. Intraassay and interassay CV were less than 20% which falls within the recommended level of precision at ± 25% for bioanalysis validation (Braggio et al., 1996; Findlay and Das, 1998). Good correlation (r^2 > 0.990) was exhibited when the assay was compared with GC-MS analysis.

Using the combination of (-) camphor-BSA as the immunogen and MIB-TG as the coating conjugate, a more sensitive assay (IC_{50} = 105 ng mL^{-1}) was developed than that of Chung et al. (1990) (IC_{50} = 10 µg mL^{-1}). However, a more sensitive CI-ELISA (IC_{50} = 5 ng mL^{-1} vs. IC_{50} = 105 ng mL^{-1}) was developed by Plhak and Park (2003) using a monoclonal antibody with (-) borneol-LPH as immunogen and MIB-BSA as coating conjugate. However, the polyclonal antibody produced in this study could possibly be used to detect MIB in water samples providing the concentration present in the sample is above the limit of quantification (ca.13.2 ng mL^{-1}).

Several efforts to improve the assay sensitivity by lowering the concentrations of coating conjugate and antibody while producing an acceptable signal were attempted using chemiluminescence, streptavidin-biotin and ELAST ELISA amplification systems. However, none of these methods were successful in improving the sensitivity of the ELISA. We concluded that affinity of an antibody is the ultimate factor which determines the sensitivity on an immunoassay.
DEVELOPMENT OF MONOCLONAL-BASED IMMUNOASSAY FOR THE DETECTION OF 2-METHYLISOBORNEOL

4.1 Abstract

This study describes the development of a sensitive enzyme-linked immunosorbent assay (ELISA) for the detection of 2-methylisoborneol (MIB) using monoclonal antibodies (mAb). Four BALB/c mice were immunized with (-) camphor, a structural relative of MIB, conjugated to bovine serum albumin (BSA). Mouse monoclonal antibodies were raised and splenocytes of the two mice showing highest antibody titer and sensitivity in competitive ELISA were fused with SP2/0 Mus musculus myeloma cells for hybridoma production. Fourteen of more than a thousand recovered colonies were shown to exhibit high affinity to MIB-TG (thyroglobulin) through screenings by ELISA. Eleven clones were of IgG isotype and three of IgM. All 14 clones possessed a κ-light chain. Nine clones had a higher affinity to MIB-TG as compared to (+) bornylamine-TG, while four clones had similar affinities to both coating conjugates and one clone showed a higher binding to the latter conjugate.

Ten of the fourteen clones showed a higher sensitivity towards free (-) camphor than to MIB while one was not inhibited by (-) camphor. Clone 4F11 was the most sensitive mAb when tested against free MIB with an IC$_{50}$ of 100.2 ng mL$^{-1}$ and limit of detection (LOD) of 1.9 ng mL$^{-1}$ when MIB-TG was used as coating conjugate. Further optimization by lowering the concentration of coating conjugate and tissue-culture supernatant improved the sensitivity of the immunoassay, i.e., an IC$_{50}$ of 0.9 ng mL$^{-1}$ using MIB-TG as coating conjugate at 0.63 µg mL$^{-1}$ and supernatant dilution at 1/8. Pre-incubation of antibody and free analyte prior to addition to coated plates was shown to have better sensitivity (IC$_{50}$ = 100.2 ng mL$^{-1}$) compared to CI-ELISA where antibody and analyte were added simultaneously to the coated plate (IC$_{50}$ = 231.6 ng mL$^{-1}$). 4F11 was
shown to cross react with camphor-like compounds with % cross reactivity above 10,000, and to other structurally related compounds containing a methyl group, such as (-) borneol, isoborneol, fenchol with % cross reactivity of 24.1, 444.1, and 344.7, respectively. Low cross reactivity to geosmin was observed.

4.2 Introduction

2-methylisoborneol (MIB) is a hapten with molecular weight of 168 Da. It is a naturally occurring organic compound and secondary metabolite of several genera of cyanobacteria and actinomycetes (Izaguirre and Taylor, 2004; Stahl and Parkin, 1996; Whitfield, 1999; Wnorowski, 1992; Wood et al., 2001). MIB is often present in public water supply reservoirs and freshwater fish, especially catfish. The presence of MIB results in an undesirable off-flavor and odor due to its strong musty and earthy odor. Humans are able to detect MIB at level as low as 10 pg mL$^{-1}$ or less (Davies et al., 2004; Mallevialle and Suffet, 1987). Detection methods for MIB are normally performed through sensory analyses and gas chromatography with mass spectrometry (GC-MS) (Jelen et al., 2003; Johnsen and Bett, 1996; Persson, 1979; Petersen et al., 2011). A detection limit of 1 pg mL$^{-1}$ has been previously reported by utilizing solid-phase microextraction (SPME) coupled with selected ion monitoring with a single quadrupole mass spectrometer (GC-MS) (Chang et al., 2008).

Although sensory analysis is routinely used by catfish producers, this method suffers from variation of sensory panellists (Persson, 1979). In addition, SPME is limited to qualitative work as various factors may introduce large variations from sample to sample (Hurlburt et al., 2009). Alternatively, GC-MS offers high detection limits, but it is expensive, time-consuming and not applicable for field use. Inconsistent results using SPME coupled with GC-MS have been observed on routine analysis for MIB in catfish pond water (Grimm, unpublished data). However, immunoassay, which is based on
antibody and antigen interaction, may offer a simpler, rapid and cost-effective detection method for routine monitoring which may be advantageous to catfish producers and municipal officials.

Polyclonal-based enzyme linked immunosorbent assay (ELISA) for MIB was developed by Chung et al. (1990) by cross-linking (+) camphor, a structurally related compound to MIB, to carrier proteins BSA and OVA to use in solid phase coating; the detection limit of the assay is 1 µg mL\(^{-1}\). Monoclonal antibodies for MIB were produced by Miyamoto et al. (1997) and Plhak and Park (2003) using (+) camphor conjugated to BSA and (-) borneol crosslinked to \textit{Limulus polyphemus} hemocyanin (LPH) as immunogens, respectively. Indirect competitive immunoassay of these antibodies had detection limits of 0.63 µg mL\(^{-1}\) and 0.6 ng mL\(^{-1}\), respectively. The pAb produced by Chung et al. (1990) was ineffective due to its low sensitivity and high non-specificity, which was not useful for MIB since it occurs in small concentrations in potable water and food. Although the mAbs produced by Phlak and Park (2003) were of high sensitivity for MIB detection, there were no further characterization and validation data indicating that these antibodies were suitable for detection of MIB in different matrices. We have previously described the development of polyclonal-based ELISA for the detection of MIB in water (Chapter 3). The objective of this study was to produce mAbs for MIB detection using the same immunogen, i.e., (-) camphor-BSA, used to develop the polyclonal-based ELISA. A new immunoassay for MIB was developed aiming to produce, characterize and validate the antibodies selected with the ultimate goal of producing a commercial immunoassay kit to detect the presence of MIB at concentrations lower than human detection threshold level. The production of the commercial kit will be of great benefit to catfish producers and municipal officials assessing potable water.
4.3 Materials and methods

4.3.1 Materials, reagents and equipment

2-Methylisoborneol (MIB, 99.9%, 20 mg) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). MIB (20 mg) was dissolved in methanol (HPLC Grade, Fisher Chemicals, Nepean, Canada) to prepare a 1 mg mL\(^{-1}\) stock solution; it was stored at \(-20\,^\circ\text{C}\). (+) Camphor, (-) camphor, camphorquinone, (-) borneol, isoborneol, (+) bornylamine, (+) endo-2-nornorneol, norbornylamine, fenchol, geosmin, carbonyldiimidazole, disuccinimidylsuberimidate, O-carboxymethoxylamine hemihydrochloride, triethylamine, and isobutyl chloroformate, bovine serum albumin (BSA), porcine thyroglobulin (TG) were purchased from Sigma Aldrich (Oakville, Canada).

Standard solutions of MIB for use in ELISA were prepared in volumetric flasks from the stock at 0.001 to 100,000 ng mL\(^{-1}\) in 10% methanol/water (v/v) and stored at 4 \(\circ\text{C}\). Goat anti-mouse IgG-horseradish peroxidase conjugate (GaM-HRP) and IgG/IgM trapping antibody were purchased from Thermo Scientific (Nepean, Canada). Skim milk was from EMD Biosciences (Newark, NJ). Superblock in PBS was obtained from Thermo Scientific (Nepean, Canada). ELISA substrates used were BioFix TMB One Component HRP Microwell substrate (SurModics, Eden Prairie, MN) and 1-Step™ Turbo TMB-ELISA (Thermo Scientific, Nepean, Canada). All other chemicals were from Sigma Aldrich (Oakville, Canada) unless otherwise stated. All chemicals and reagents were of analytical grade unless stated otherwise.

Mice immunizations and hybridoma production were conducted at ImmunoPrecise Laboratories, Victoria, British Columbia, Canada. Freund’s complete and incomplete adjuvants were purchased from Sigma Aldrich Co. (St. Louis, MO). Media and supplements used in hybridoma development were from Invitrogen (Life
Technologies Inc., Burlington, Canada): Dulbecco’s Modified Eagle Medium (DMEM), 2-mercaptoethanol (0.05 M, 1000X), sodium pyruvate (0.1 M, 100X), penicillin/streptomycin (100X), dipeptide glutamine (100X), HT supplement (50X). All supplements were added to DMEM medium at 1% final concentration (v/v). Serum-free medium and fetal bovine serum (FBS, ultralow IgG, < 5 µg mL⁻¹, US origin) were from Invitrogen. PEG 1500 was from Roche Diagnostics GmbH (Manheim, Germany). All solutions and media for tissue-culture work were filtered through sterile-top filter (0.22 µm) and stored at 4 °C or -20 °C. Monoclonal clones were stored in liquid nitrogen (-200 °C) until required for use.

Phosphate buffered saline (PBS) was prepared at 10X concentration (1X PBS: 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄, and 0.24 g of KH₂PO₄ per liter of water, pH 7.4) and diluted to 1X with nanopure water. For PBST, Tween 20 was added to 0.05% (v/v). Carbonate bicarbonate buffer (0.05 M; 1.59 g of Na₂CO₃, 2.93 g of NaHCO₃, 0.2 g of NaN₃ per liter of water, pH 9.6) was used as coating buffer in ELISA.

ELISAs were conducted using 96-well microtiter plates (high-binding; Corning Inc. Life Sciences, Lowell, MA). Plates were analysed at absorbance 450 nm with a multiplate reader from Bio-Rad Laboratories (Model 3350-UV, Hercules, CA). Conjugates were analysed using Micromass Global Q-TOF Ultima (MALDI/CapLC-ESI Quadrupole Time of Flight) Mass Spectrometer located in the Department of Chemistry, McMaster University, ON.

4.3.2 Production of immunogen and coating conjugates

(·) Camphor O-(carboxymethyl)oxime (CMO) was prepared according to the method of Langone and Van Vunakis (1976) with minor modifications. Briefly, 1 g of (·) camphor (6.5 mmol) and 2.84 g (13 mmol) of O-carboxymethylamine hemihydrochloride were refluxed for 6 h in a mixture of 14 mL of absolute ethanol and 16.5 mL of 2 M
NaOH (33 mmol). The mixture was allowed to stand at 25 °C overnight followed by the addition of water (200 mL) and adjustment of the pH to 9.5 with 2 M NaOH. The solution was extracted three times with 100 mL ethyl acetate in a separatory funnel. The aqueous layer was acidified to pH 3.0 with 1 M HCl and stored at 0 °C overnight to yield (-) camphor-CMO as a white precipitate. The precipitate was collected following centrifugation (1500 x g, 5 min, 4°C), washed with 500 mL of water and lyophilized.

Conjugation of (-) camphor-CMO to BSA was done by the mixed-anhydride method (Dean et al., 1972). Specifically, 18.9 mg (84 μmol) of (-) camphor-CMO were mixed with 12 μL (85 μmol) of triethylamine and cooled to -5°C, after which, 12 μL (85 μmol) isobutyl chloroformate were added and the solution was stirred for 30 min at -5 °C. The reaction mixture was added drop wise to a cold solution of BSA (5 mL, 20 mg mL⁻¹, 1.5 μmol) in water: dioxane (7:3; v/v), pH 9.0. Sodium hydroxide (0.1 M) was added to maintain the pH at 9.0 and the mixture was stirred continuously at 4°C overnight, dialyzed against water, and stored at -20 °C.

Another MIB structurally related compound, (+) bornylamine (Figure 4.1c) and MIB (Figure 4.1a) were used as solid phase coatings. Conjugation of (+) bornylamine to porcine thyroglobulin (TG) was carried out following the method of Paek et al. (1993). (+) Bornylamine was coupled through its amine group using disuccinimidylsuberimidate (DSS). (+) Bornylamine (11.6 mg, 75.6 μmol) was added to DSS in anhydrous DMSO (55.25 mg mL⁻¹, 150 μmol). The mixture was stirred for 30 min at room temperature (RT) and added to 20 mg of TG (0.03 μmol) in 5 mL of 10 mM sodium phosphate buffer, pH 7.0. After incubating for 2 h at room temperature, the mixture was dialyzed overnight against 1X PBS, pH 7.4 at 4 °C with 4X buffer changes. The solution was aliquoted into another tube and kept at -20°C until required for use. MIB-TG conjugate was produced and kindly provided by Dr. Rubio from Abraxis LLC. (Warminster, PA).
The molar ratio of the (-) camphor-BSA was determined using a Micromass Global Q-TOF Ultima (MALDI/CapLC-ESI Quadrupole Time of Flight) Mass Spectrometer. 2,5-Dihydroxybenzoic acid (2,5-DHB) was used as matrix solution and BSA as reference. The conjugate sample and matrix solution were mixed in equal amounts (1 µL each) and placed on the stainless steel probe. The samples were allowed to dry at RT. The data were acquired with 50 shots per sample in the linear mode at 30 kV and analysed using the software provided with the system.

Figure 4.1. Molecular structures of (a) 2-methylisoborneol, (b) (-) camphor and (c) (+) bornylamine. Crosslinking to protein was done through the carbonyl group of (-) camphor for immunogen and the hydroxyl group of MIB and the amine group of (+) bornylamine for solid phase coatings.

4.3.3 Immunization of mice and generation of hybridoma

4.3.3.1 Immunization of mice

Production of anti-(-) camphor-BSA monoclonal antibody clones was conducted at ImmunoPrecise Antibodies Ltd. (IPA; Victoria, BC, Canada). Four female BALB/c mice were immunized intraperitoneally (IP) with 25 µg (-) camphor-BSA per mouse in an equal volume (1:1) of Freund’s complete adjuvant and sterile 1X PBS and subsequent boosters given at three weeks intervals with 25 µg (-) camphor-BSA in an equal volume (1:1) of Freund’s incomplete adjuvant and 1X PBS. Mice were given six booster
injections (on day 21, 42, 63, 84, 105 and 126) and a final boost (day 158) of 20 μg per mouse was administered through intravenous injection (IV) in 100 μL sterile 1X PBS via the lateral tail vein (intravenous) three days prior to fusion (day 161).

4.3.3.2 Fusion

Hybridomas were produced by fusing splenocytes (B cells) from mouse 2 and 3 with SP2/0 *Mus musculus* myeloma cells using PEG (Roche Diagnostics GmbH, Manheim, Germany) and methylcellulose semi-solid medium according to the ClonaCell®-HY Hybridoma Cloning Kit procedures manual with minor modifications (IPA fusion protocol IPASOP-G-020). In brief, 1 x 10^8 viable splenocytes were mixed with 2 x 10^7 myeloma cells (in 25 mL) in serum-free medium in a 50-mL conical tube. The cells were centrifuged at 400 x g for 10 min at RT. The supernatant was removed with a Pasteur pipette, and the pellet was disrupted gently by tapping the bottom of the tube. For fusion, 1 mL aliquot of 50% (w/v) PEG (MW 1500), pre-warmed to 37 °C was added drop wise to the pellet using a 1-mL pipette over a period of 1 min without stirring. The mixture was gently and continuously stirred with the pipette tip over the next minute. One millilitre of medium B (fusion: DMEM containing gentamycin), pre-warmed to 37 °C was added to the fusion mixture and stirred continuously for 1 min. Three millilitres of medium B were then added and the mixture was stirred continuously over a period of 3 min. Ten millilitres of medium B were then added slowly while the tube was being continuously rotated and the mixture was incubated in a 37°C water bath for 15 min. Thirty millilitres of medium A (pre-fusion: DMEM containing pre-selected serum, gentamycin and supplements) were added slowly and the cells were centrifuged at 400 x g for 7 min. The supernatant was discarded and the cells were washed with 40 mL of the same medium to ensure PEG removal. The cell pellet was slowly resuspended in 10 mL
of medium C (recovery: DMEM containing pre-selected serum, gentamycin and supplements) and transferred to a 75-cm² tissue-culture flask containing 20 mL of medium C and incubated for 16-24 h at 37 °C in 5% CO₂ (Forma Series II water jacket, Model 3110; Thermo Scientific, Nepean, Canada).

4.3.3.3 Selection and cloning

The cell suspension from the 75-cm² tissue-culture flask was transferred into a 50-mL conical tube and centrifuged at 400 x g for 10 min and the supernatant was discarded. The cells were resuspended in medium C to a total volume of 10 mL and transferred into 90 mL of medium D (selection and cloning: DMEM containing methylcellulose, pre-selected serum, HAT, gentamycin and supplements). The suspension was mixed gently by inverting the tube several times. The tube was allowed to sit for 15 min at RT to allow the bubbles to rise. Using a 12-mL syringe and a 16-gauge blunt needle, 9.5 mL of cell-suspension medium were aseptically plated into ten 100 mm diameter petri dishes. Plates were tilted to distribute the medium to cover the bottom of the plate evenly while avoiding bubbles during plating. The plates were incubated undisturbed for 10-14 days at 37 °C in 5% CO₂.

4.3.3.4 Cell harvest

After 10-14 days, plates were examined by eye for the presence of visible colonies. The formed colonies were removed using sterile pipette tips and a pipette set to 10 µL and placed into individual wells of a 96-well tissue-culture plate containing 200 µL of medium E (growth; DMEM containing pre-selected serum, HT, gentamycin and supplements). The colonies were resuspended by mixing the entire contents of the wells by pipetting up and down (using 150 µL) several times. The plates were then incubated
at 37 °C in 5% CO₂ for 4 days without feeding. On the fourth day, the supernatant (150 µL) was transferred to a new 96-well ELISA microtiter plate and screened against MIB-TG. Fresh medium E (150 µL) was placed into the tissue culture plates containing hybridoma colonies. Hybridomas showing positive results in ELISA screening were gently resuspended and transferred (100 µL) to each of 2 wells of a 24-well tissue-culture plate containing 1 mL of medium E. The cells were further grown to a suitable density (~ 4 x 10⁵ cells mL⁻¹). After the cells were stable, the cells from one well were frozen and cells from another well were expanded in a 75-cm² tissue-culture flask containing 5 mL of medium A and 5 mL of medium E (1:1) and grown to suitable density. Subsequently, 5-10 mL of cell culture was transferred into 20 mL of medium A in a 75-cm² tissue-culture flask. The cells were maintained in 100% of medium A at a concentration of ~ 5 x 10⁴ – 5 x 10⁵ cells mL⁻¹.

4.3.4 Screening of positive clones

Screening of positive clones was performed by ImmunoPrecise Antibodies Ltd. (Victoria, BC, Canada). Primary screening and secondary screening were conducted on day 15 and day 18 after fusion. For hybridoma screening, 10 µg mL⁻¹ (100 µL well⁻¹) of MIB-TG was coated onto microtiter plates in 1X PBS and incubated for 16 h at 4 °C. For negative control antigen, human transferrin, anatoxin-BSA and anatoxin-OVA were used at 0.5 µg in distilled water (50 µL well⁻¹) followed by drying at 37 °C. Plates were blocked with 3% (w/v) skimmed milk in 1X PBS (100 µL well⁻¹) and incubated at RT for 1 h. Undiluted tissue-culture supernatants (100 µL well⁻¹) were added. Mouse anti-(−) camphor-BSA antisera and pre-immune serum were used as positive and negative controls at 1:500 (v/v) diluted in SP2/0 tissue culture supernatant (100 µL well⁻¹). Plates were incubated at 37 °C for 1 h with continuous shaking. Three to five washes in 1X
PBST were incorporated between each step. Goat anti-mouse HRP conjugated IgG Fc (Jackson ImmunoResearch, West Grove, PA) and goat anti-mouse HRP conjugated IgMµ were used as secondary antibodies at 1/10,000 (v/v) and 1/25,000 (v/v) dilutions, respectively, in PBST (100 µL well⁻¹) and plates were incubated at the same condition described above. After three to five PBST washes, TMB substrate (SurModics, Eden Prairie, MN) was added at 50 µL well⁻¹ and plates were developed in the dark at RT. Reaction was quenched by addition of 1 N HCl (50 µL well⁻¹) after 30 min. Absorbances were read at 450 nm (BioRad, Hercules, CA; Model 3350-UV).

4.3.5 Isotyping of monoclonal antibody

The antibody isotype of each of the fourteen monoclonal antibody clones was determined using a monoclonal antibody isotyping kit (HRP/ABTS; Pierce, Rockford, IL) according to the manufacturer’s instructions. In brief, microtiter plates were coated with 25 µg (50 µL well⁻¹) of goat anti-mouse Ig (G +A +M), supplied at 0.5 mg mL⁻¹ in 1X PBS, pH 7.4 containing 10% (v/v) glycerol and 0.05 % (w/v) sodium azide. Coating antibody was prepared in 0.01 M sodium bicarbonate buffer, pH 9.6. Plates were sealed and incubated overnight at 4 ºC. Microtiter plates were washed and blocked for 1 h at 37 ºC with 1X blocking buffer solution (125 µL well⁻¹; 0.5 % bovine serum in 1X PBS containing 0.05% (w/v) sodium azide. After repeated washing, monoclonal antibody from tissue-culture supernatant of each clone was added at 50 µL well⁻¹. Mouse mAb IgG1 in RPMI-1640 tissue-culture medium containing 10% (v/v) FBS and 0.05% (w/v) sodium azide was used as positive control and added at 50 µL well⁻¹. Plates were then incubated at 37 ºC for 1 h. After subsequent washes, each mAb clone was probed with each of the following antibodies (IgA, IgM, IgG1, IgG2a, IgG2b, IgG3, κ and λ) to determine its isotype. Rabbit anti-mouse IgG1 antibody and normal rabbit serum were added (50 µL
well) in positive and negative control wells, respectively. Plates were incubated as described earlier. After subsequent washes, HRP conjugated goat anti-rabbit IgG was added (50 µl well) and further incubated at the same conditions. Plates were washed and substrate solution (1X ABTS) was added at 100 µL well. Color development was monitored for 30 min. Absorbance was measured at 405 nm using a microtitre plate reader (BioRad Laboratories, Model 3350-UV, Hercules, CA). All incubations, unless stated otherwise, were performed at 37 °C for 1 h with continuous shaking. Washing steps were incorporated in each procedure, four times with 125 µL of 1X PBST (0.05% (v/v) Tween-20 in 1X PBS).

4.3.6 Indirect ELISA

4.3.6.1 Confirmation of antibody titer

Polyclonal antisera from mice were tested against MIB conjugated to thyroglobulin (MIB-TG) for titer confirmation in indirect ELISA using a standard ELISA protocol (Crowther, 1995). Briefly, wells of a 96-well microtiter plate were coated with MIB-TG at 1 µg mL (100 µL well), in 0.05 M carbonate-bicarbonate buffer, pH 9.6. Plates were covered and incubated for 16 h at 4 °C. After overnight incubation, plates were washed with 1X PBS and blocked with 3% (w/v) skimmed milk in 1X PBS (MPBS; 200 µL well) for 1 h at RT with orbital shaking. After washing in PBST, pre-immune serum (negative control) and antisera from mice 1-4 from the fourth (day 48) and the sixth boost (day 126) were added at 1:100 (v/v) dilution and serially diluted (1:2 v/v) in the plate. Plates were incubated at 37 °C for 1 h with orbital shaking. Plates were then washed thoroughly (five times) with 1X PBST (0.05% v/v Tween 20) and HRP conjugated goat anti-mouse IgG (GaM-HRP) in PBST was added as secondary antibody at 1/10,000 (100 µL well) and incubated at the same condition. After subsequent
washing, TMB substrate (SurModics, Eden Prairie, MN) was added (50 µL well\(^{-1}\)) and plates were developed in the dark for 15 min. The reaction was quenched with 1 M HCl (50 µL well\(^{-1}\)). Absorbance was measured at 450 nm using a microtiter plate reader.

4.3.6.2 Indirect binding of monoclonal clones and checkerboard ELISA

To test the binding of tissue culture supernatant of 14 positive hybridoma clones, two different coating conjugates were selected, i.e., (+) bornylamine-TG and MIB-TG, and tested using indirect ELISA. Both conjugates were coated in wells of a 96-well microtiter plate at 1 µg mL\(^{-1}\) (100 µL well\(^{-1}\)) and ELISA was performed as described in Section 4.3.6.1 with minor modifications. Briefly, a 96-well microtiter plate was coated with MIB-TG or (+) bornylamine-TG at 1 µg mL\(^{-1}\) (100 µL well\(^{-1}\)), in 0.05M carbonate-bicarbonate buffer, pH 9.6. For negative control, wells were coated with TG at 0.1 µg mL\(^{-1}\) (100 µL well\(^{-1}\)). Plates were covered and incubated for 16 h at 4 °C. After overnight incubation, plates were washed with 1X PBS and blocked with 3% (w/v) skimmed milk in 1X PBS (MPBS; 200 µL well\(^{-1}\)) for 2 h at RT with orbital shaking. Tissue-culture supernatants from 14 hybridoma clones were added (without dilution) at 100 µL well\(^{-1}\) and plates were incubated at 37 °C for 1 h with orbital shaking. Plates were then washed thoroughly (five times) with 1X PBST (0.05% v/v Tween 20). HRP-conjugated goat anti-mouse IgG (GaM-HRP; Thermo Scientific, Nepean, Canada) in MPBS was added as secondary antibody at 1/2,500 (v/v) (100 µL well\(^{-1}\)) and incubated as described above. After subsequent washing, 1-Step™ Turbo TMB substrate (Thermo Scientific) was added at 100 µL well\(^{-1}\) and plates were developed in the dark for 45 min. The reaction was quenched using 1 N H\(_2\)SO\(_4\) (100 µL well\(^{-1}\)) and absorbances were measured at 450 nm using a microtiter plate reader.
For indirect checkerboard ELISA, (±) bornylamine-TG and MIB-TG were coated in the wells of a 96-well microtiter plate at a concentration of 10 μg mL\(^{-1}\) (100 μL well\(^{-1}\)) in 0.05M carbonate bicarbonate buffer, pH 9.6. A concentration of coating conjugate was placed in the first row (12 wells) and diluted 2-fold across the plate. TG at 1 μg mL\(^{-1}\) was used as negative control (100 μL well\(^{-1}\)). Undiluted tissue culture supernatants were added to the wells (8) of the first column of the plate and serially diluted across the plate. ELISA protocols and conditions were performed as previously described.

**4.3.6.3 Inhibition (competitive indirect) ELISA**

To evaluate if mice antisera have affinity towards free MIB in solution, inhibition ELISAs were conducted with all four mice antisera against MIB standards using MIB-TG to coat wells. In brief, wells of a 96-well microtiter plate were coated with MIB-TG at 1 μg mL\(^{-1}\) (100 μL well\(^{-1}\)), in 0.05 M carbonate-bicarbonate buffer, pH 9.6. For negative controls, wells were coated with TG at 0.1 μg mL\(^{-1}\) (100 μL well\(^{-1}\)). Plates were incubated for 16 h at 4 °C. The next day, plates were washed with 1X PBS and blocked with 3% (w/v) skimmed milk in 1X PBS (MPBS; 200 μL well\(^{-1}\)) for 2 h at RT with orbital shaking. For competitive indirect ELISA, inhibition steps were executed where antibody (tissue-culture supernatant) and MIB standards were pre-incubated for 1 h at RT prior to addition to the coated and blocked plates. In this step, 0.5 mL of mice antisera at 1/1,000 (v/v) for mouse 1, 2, and 4 and 1/4,000 for mouse 4, were selected from the checker board ELISA and mixed with an equal volume of an MIB standard; concentrations of standards ranged from 5 to 10,000 ng mL\(^{-1}\) in 2-mL glass vials. The mixtures were allowed to react (i.e., inhibit) for 1 h at RT with continuous rotation (rotary cell culture system, Synthecon Inc., Houston, TX). After a 1-h incubation, the mixtures were added to coated and washed wells (100 μL well\(^{-1}\)) and further incubated for another hour at 37
°C with orbital shaking. Plates were washed thoroughly (five times) with 1X PBST (0.05% (v/v) Tween 20). HRP-conjugated goat anti-mouse IgG (GaM-HRP; Thermo Scientific, Nepean, Canada) in MPBS was added as secondary antibody at 1/2,500 (v/v) (100 µL well⁻¹) and incubated under the same conditions. After subsequent washing, 1-Step™ Turbo TMB substrate (Thermo Scientific) was added at 100 µL well⁻¹ and plates were developed in the dark for 30 min. The reaction was quenched using 1 N H₂SO₄ (100 µL well⁻¹) and absorbances were measured at 450 nm. Inhibition ELISAs for tissue-culture supernatants against free MIB and (−) camphor standards (concentrations of standards ranged from 0.001 to 100,000 ng mL⁻¹) were performed using similar protocols and conditions as described for mice antisera.

4.3.7 Cross reactivity

Specificity of the monoclonal antibodies was tested against compounds structurally related to MIB (see Table 4.5). Compounds were prepared at concentrations of 0.001 to 10,000 ng mL⁻¹ in 10 % (v/v) methanol. CI-ELISA was performed as described in Section 4.3.6 and respective IC₅₀ values were determined for each compound. Percentage of cross reactivity for each compound was calculated as the IC₅₀ value of MIB divided by the IC₅₀ value of test compound multiplied by 100.

4.3.8 Data analyses

Statistical analyses of data were carried out using GraphPad Prism ver. 5.0 (GraphPad Software Inc., San Diego CA). Inhibition curves of response (A/A₀) versus log concentrations of MIB were fitted through a four parameter log graph (4PL). IC₅₀ values were obtained at 50% inhibition of response. Limit of detection (LOD) was calculated based on 3 times the standard deviation of the mean at zero inhibition (A₀).
4.4 Results and discussion

4.4.1 Selection of immunogen

Chemistry of hapten conjugation was previously discussed in Chapter 3, section 3.4.1. Utilization of (-) camphor as an immunogen introduced hapten heterology to the system because MIB or (+) bornylamine were used to coat plates in an attempt to improve immunoassay sensitivity. (-) Camphor was coupled to BSA via the carbonyl by derivatization to camphor O-carboxymethylxoxime (CMO) which was then crosslinked to Lys groups of BSA using isobutyl chloroformate. From previous experiments, higher immune responses from rabbit sera were observed to (-) camphor-BSA than (-) borneol-BSA using rabbit polyclonal antibodies (Chapter 3). Based on this result, we immunized four BALB/c mice with (-) camphor-BSA to isolate monoclonal antibodies. From MALDI-TOF analysis, hapten to protein ratio for (-) camphor-BSA was 18:1. A moderate number of hapten per protein molecule is vital to stimulate a good antibody response towards a hapten. Good antibody titers can usually be obtained with epitope densities anywhere between 8 and 25 (Erlanger, 1980).

4.4.2 Antibody titer

Sera collected from immunized mice were tested for antibody titer and immune response against MIB-TG using an indirect ELISA as described in Section 4.3.6.1. As shown in Figure 4.2, there was a considerable difference between the titration curves of sera from pre-immune and immunized mice, indicating that antibodies against (-) camphor-BSA were present in the antisera. A good antibody response was observed in all four mice after the fourth boost (day 84). Two additional boosts improved the immune response substantially in all four mice against (-) camphor-BSA (Figure 4.2). Statistical analysis using Tukey’s multiple comparison test (following an ANOVA) showed that after the sixth boost, mice 2 and 3 had significantly higher immune response ($p<0.05$) as
compared to mice 1 and 4 at 1/100, 1/200, 1/400 and 1/800 (v/v) serum dilution.
Antibody titers for all four mice were observed at the highest dilution of antiserum at 1/6,400 (v/v) which produced ELISA signals significantly higher ($p<0.05$) than negative control value (pre-immune serum).
Figure 4.2. Titration of mouse antisera using MIB-TG coated (1 µg mL⁻¹) microtiter plates. The immune response of mice after (A) fourth (day 84) and (B) sixth boosts (day 126), were tested. ELISAs were carried out as described in Section 4.3.6.1. The immune response for all four mice increased significantly (p<0.05) from fourth to sixth boosts. Mouse 2 and 3 showed the highest response.
Affinity of mice antisera towards free MIB was tested in inhibition ELISAs with MIB-TG as the coating conjugate. Competition was seen in all of the four mice antisera when inhibited using free MIB in solution (Figure 4.3). In addition, no inhibition was seen when TG was used, indicating that sera from mice immunized with (-) camphor-BSA did not recognize TG (result not shown). Mice 2 and 3 showed the lowest IC$_{50}$ values of 158.9 and 284.8 ng mL$^{-1}$, respectively, as compared to mice 1 and 4 with IC$_{50}$ values of 520.4 and 383.7 ng mL$^{-1}$, respectively. The antisera from mice 2 and 3 also showed the highest percentage inhibition (72.1% and 81.4 %, respectively) at an inhibition level of 10,000 ng mL$^{-1}$ free MIB while mice 1 and 4 were inhibited at 64.5% and 68.2 %, respectively, at the same concentration of MIB. With the highest immune response and lowest IC$_{50}$ values, mice 2 and 3 were selected for hybridoma fusion for monoclonal antibody production.

For inhibition ELISAs, the optimal concentrations of coating conjugate and antisera were selected based on results from checkerboard ELISAs (see Section 4.3.6.2). Optimal dilutions of antisera and coating conjugate for use in inhibition ELISAs were selected at an absorbance value close to or above 1.0. However, no inhibition was seen at these concentrations. This may be due to the higher affinity of the antibodies towards the immobilized antigen than to the free antigen in the solution. Inhibition was observed when lower concentrations of antisera were selected, which corresponded to absorbance values of ~ 0.3 - 0.7. This suggests that the antibody binds strongly to immobilized analyte (MIB) and the binding was not easily disrupted with addition of free MIB in the solution. When lower amounts of coating conjugate were immobilized, the chances of free analyte in the solution binding with the antibody increased, resulting in competition for antibody binding between immobilized and free analyte. Hence, for competition ELISAs, and especially for haptens, a compromise between obtaining a significant ELISA signal and the sensitivity of the immunoassay should be considered.
Figure 4.3. Inhibition ELISA of four mice immunized with (-) camphor-BSA before fusion. Microtiter plates were coated with MIB-TG at 1 µg mL$^{-1}$. Antisera were used at 1/1,000 (v/v) dilution for (A) mouse 1, (B) mouse 2, and (D) mouse 4 and 1/4,000 (v/v) dilution for (C) mouse 3, respectively. All mice showed inhibition when exposed to free MIB. Mouse 2 and mouse 3 showed lower IC$_{50}$ and higher percentage of inhibition compared to mouse 1 and mouse 4. B cells from mouse 2 and 3 were used for hybridoma production. ELISAs were performed as described in Section 4.3.6.3. Values are mean ± SD of three replicates.
4.4.3 Production of hybridoma

Spleen cells from mice 2 and 3 immunized with (−) camphor-BSA were fused with SP2/0 myeloma cells and the forming hybridomas were individually selected on a methylcellulose semi-solid medium, transferred into individual 24-well microtiter plates and grown in the presence of hypoxanthine-aminopterin-thymidine (HAT). Using HAT as selection medium prevents the growth of unfused myeloma or spleen cells. Cloning and selection of hybridoma cells were carried out using a semi-solid medium containing methylcellulose, as opposed to using the traditional limiting dilution method, which is more tedious. This step saves a significant amount of time and tissue-culture work. HAT selection and cloning and selection of hybridomas can be done simultaneously in a single step thus reducing contamination (StemCell Technologies, 2009). After fusion, cells cohered and formed distinct colonies, which were harvested and screened individually in separate wells as described in Wognum and Lee (2013). Separate isolation and screening can also be performed for fast and slow growing clones because they remain physically separated from each other. This increases clone diversity and prevents overgrowth of faster growing clones over slow growers (Wognum and Lee, 2013). Faster growing clones frequently do not synthesize antibodies (Goding, 1980) and result in fewer chances of rescuing potentially valuable hybridomas (Goding, 1980; Lemke et al., 1979).

Approximately one thousand hybridoma clones were formed after fusion. A total of 948 individual colonies were picked and transferred from the 100 mm diameter Petri plates into wells of 96-well tissue-culture plates and grown. Smaller colonies, i.e., slow growers, were preferentially picked as they are often better antibody producers (StemCell Technologies, 2009). Four days after colony transfer from Petri plates to wells of 96-well tissue-culture plates, the medium turned yellow as a result of high cell density. The supernatants were then screened against bound MIB-TG (10 µg mL⁻¹) as described
in Section 4.3.4. After the first round of screening, 66 wells with OD higher than 0.125 were selected and grown. The supernatants were subjected to a second screening against bound MIB-TG at 10 µg mL$^{-1}$, three days following the first screening. The supernatants were also tested against bound anatoxin-BSA, anatoxin-OVA and human transferrin as negative controls and mouse anti (−) camphor-BSA serum as positive control at 1/500 (v/v) dilution. Screening of the hybridoma clones was performed with MIB conjugates rather than with the original immunogen, (−) camphor, because this should result in selection of clones with higher affinity and specificity towards MIB. After the second screening, a total of 14 clones were selected on the basis of their reactivity with MIB-TG and the absence or minimal cross-reactivity with non-related controls. Antibodies which are directed towards the BSA carrier or chemical spacers were potentially precluded through screening against non-related hapten-protein conjugates (Pauillac et al., 1993).

The result of screenings of the final 14 positive hybridoma clones is presented in Table 4.1. Using a monoclonal isotyping kit (Pierce, Rockford, IL), 10 of the 14 clones were determined to be IgG1, one was IgG2a (clone 6G8) and three were IgM (clone 3H9, 6D3 and 9G7). All 14 clones possessed a κ-light chain.
### Table 4.1 Screening of hybridoma clones

<table>
<thead>
<tr>
<th>Clone</th>
<th>First screening</th>
<th>Second screening</th>
<th>Negative control&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Final stability test&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Isotype&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>3H9</td>
<td>2.588</td>
<td>1.848</td>
<td>0.145</td>
<td>0.941</td>
<td>IgM</td>
</tr>
<tr>
<td>4A12</td>
<td>0.147</td>
<td>0.246</td>
<td>0.084</td>
<td>1.357</td>
<td>IgG1</td>
</tr>
<tr>
<td>4C3</td>
<td>0.707</td>
<td>0.913</td>
<td>0.083</td>
<td>1.279</td>
<td>IgG1</td>
</tr>
<tr>
<td>4F11</td>
<td>2.780</td>
<td>2.075</td>
<td>0.097</td>
<td>1.702</td>
<td>IgG1</td>
</tr>
<tr>
<td>5G3</td>
<td>0.671</td>
<td>0.713</td>
<td>0.078</td>
<td>1.332</td>
<td>IgG1</td>
</tr>
<tr>
<td>6C4</td>
<td>0.251</td>
<td>0.195</td>
<td>0.151</td>
<td>0.945</td>
<td>IgG1</td>
</tr>
<tr>
<td>6D3</td>
<td>0.185</td>
<td>0.342</td>
<td>0.163</td>
<td>1.690</td>
<td>IgM</td>
</tr>
<tr>
<td>6G8</td>
<td>2.690</td>
<td>2.700</td>
<td>0.110</td>
<td>1.744</td>
<td>IgG2a</td>
</tr>
<tr>
<td>7C10</td>
<td>0.291</td>
<td>0.440</td>
<td>0.079</td>
<td>0.687</td>
<td>IgG1</td>
</tr>
<tr>
<td>8C12</td>
<td>0.332</td>
<td>0.426</td>
<td>0.072</td>
<td>0.899</td>
<td>IgG1</td>
</tr>
<tr>
<td>8E1</td>
<td>0.629</td>
<td>0.715</td>
<td>0.075</td>
<td>1.200</td>
<td>IgG1</td>
</tr>
<tr>
<td>9E10</td>
<td>1.676</td>
<td>1.719</td>
<td>0.078</td>
<td>1.570</td>
<td>IgG1</td>
</tr>
<tr>
<td>9F4</td>
<td>2.416</td>
<td>2.301</td>
<td>0.088</td>
<td>1.355</td>
<td>IgG1</td>
</tr>
<tr>
<td>9G7</td>
<td>0.264</td>
<td>1.007</td>
<td>0.094</td>
<td>0.781</td>
<td>IgM</td>
</tr>
<tr>
<td>SP2/0 TC sup&lt;sup&gt;d&lt;/sup&gt;</td>
<td>0.103</td>
<td>0.066</td>
<td>0.073</td>
<td>0.069</td>
<td>Non-Ig Secretor</td>
</tr>
<tr>
<td>Mouse 5F12 mAb TC sup</td>
<td>0.138</td>
<td>0.066</td>
<td>0.068</td>
<td>1.287</td>
<td>IgG2a</td>
</tr>
<tr>
<td>Mouse 2B4.4 mAb TC sup</td>
<td>0.625</td>
<td>0.103</td>
<td>0.108</td>
<td>0.982</td>
<td>IgG3</td>
</tr>
<tr>
<td>Mouse anti-HT mAb TC sup</td>
<td>0.098</td>
<td>0.068</td>
<td>2.812</td>
<td>0.991</td>
<td>IgG1</td>
</tr>
<tr>
<td>Mouse anti-(-) camphor-BSA immune serum&lt;sup&gt;e&lt;/sup&gt;</td>
<td>2.941</td>
<td>2.938</td>
<td>0.149</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>Mouse pre-immune serum&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0.278</td>
<td>0.127</td>
<td>0.125</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

<sup>a</sup>Human transferrin (HT) was used as negative control antigen at 0.5 mg mL<sup>-1</sup>

<sup>b</sup>After the two screenings, cells were cultured and tested after one month, for their ability to produce mAb

<sup>c</sup>mAb isotyping was performed by ELISA as described in Section 4.3.5

<sup>d</sup>Tissue-culture supernatant

<sup>e</sup>Mouse anti-(−) camphor-BSA and pre-immune sera were used at 1/500 dilution (v/v)

<sup>f</sup>n/a: not available

First and second screenings were performed three days apart
4.4.4  Characterization of the mAb clones

Fourteen clones were isolated using MIB-TG as the screening antigen and binding of the clones to MIB-TG was compared to (+) bornylamine-TG; mAbs from nine clones had a higher affinity to MIB-TG (Figure 4.4). This could be due to a higher hapten to protein ratio of MIB-TG than (+) bornylamine-TG (not determined). Four clones (3H9, 6D3, 7C10, 8C12) had almost similar affinity ($p>0.05$) to both coating conjugates while clone 6C4 had higher affinity for (+) bornylamine-TG.

Only 11 clones were used in the inhibition CI-ELISA because 3 were IgMs and hence not useful. When MIB-TG was used as coating conjugate, 11 of the 14 clones clones were first inhibited with (-) camphor, the compound used as immunogen. IC$_{50}$ values for inhibition curves against (-) camphor are listed in Table 4.2. Clone 4F11 showed the highest sensitivity with an IC$_{50}$ value of 0.38 ng mL$^{-1}$, while lowest sensitivity was observed for clone 6G8 with an IC$_{50}$ value of 1886 ng mL$^{-1}$. No inhibition curve was observed for clone 6C4. The clones were further inhibited with MIB in separate experiments and the results are shown in Table 4.3. Clone 4F11 was the most sensitive with an IC$_{50}$ value of 100.2 ng mL$^{-1}$ and limit of detection (LOD) of 1.9 ng mL$^{-1}$ (Figure 4.5). This clone was selected for further characterization.
**Figure 4.4.** Affinity of 14 mAb clones to two different coating conjugates; (+) bornylamine-TG (BA-TG) (checkered bars) and MIB-TG (vertical bars) at 1 µg mL\(^{-1}\). Tissue-culture supernatants were added without dilution (neat). Clones 3H9, 6D3 and 9G7 are IgM. All other clones (i.e., 11 clones) are IgG. ELISA was carried out as described in Section 4.3.6.2. Values are mean ± SD of three replicates.

**Table 4.2.** IC\(_{50}\) values of 11 mAb IgG clones in inhibition CI-ELISA with (-) camphor

<table>
<thead>
<tr>
<th>Clone</th>
<th>IC(_{50}) (ng mL(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>4A12</td>
<td>679.9</td>
</tr>
<tr>
<td>4C3</td>
<td>30.8</td>
</tr>
<tr>
<td>4F11</td>
<td>0.38</td>
</tr>
<tr>
<td>5G3</td>
<td>179.8</td>
</tr>
<tr>
<td>6C4</td>
<td>n/a</td>
</tr>
<tr>
<td>6G8</td>
<td>1886</td>
</tr>
<tr>
<td>7C10</td>
<td>499.7</td>
</tr>
<tr>
<td>8C12</td>
<td>666.6</td>
</tr>
<tr>
<td>8E1</td>
<td>334.3</td>
</tr>
<tr>
<td>9E10</td>
<td>111</td>
</tr>
<tr>
<td>9F4</td>
<td>362.7</td>
</tr>
</tbody>
</table>

n/a; no inhibition curve observed
Table 4.3. IC$_{50}$ values and other statistics for the 11 mAb IgG clones using MIB-TG as coating conjugate in an inhibition CI-ELISA with MIB as analyte

<table>
<thead>
<tr>
<th>Clone</th>
<th>IC$_{50}$ (ng mL$^{-1}$)</th>
<th>Linear range (ng mL$^{-1}$)</th>
<th>MIB-TG dilution (µg mL$^{-1}$)</th>
<th>Supernatant dilution</th>
<th>$A_{\text{max}}$</th>
<th>Slope</th>
<th>$R^2$</th>
<th>% inhibition$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4A12</td>
<td>335.6</td>
<td>10 - 4,000</td>
<td>1.25</td>
<td>1/2</td>
<td>0.779</td>
<td>0.594</td>
<td>0.927</td>
<td>59.5</td>
</tr>
<tr>
<td>4C3</td>
<td>323.5</td>
<td>20 - 2,000</td>
<td>1.25</td>
<td>1/2</td>
<td>0.467</td>
<td>-0.492</td>
<td>0.925</td>
<td>75.3</td>
</tr>
<tr>
<td>4F11</td>
<td>100.2</td>
<td>1-1,000</td>
<td>1.25</td>
<td>1/2</td>
<td>1.012</td>
<td>-0.404</td>
<td>0.990</td>
<td>89.1</td>
</tr>
<tr>
<td>5G3</td>
<td>5541</td>
<td>200 -10,000</td>
<td>0.625</td>
<td>1/2</td>
<td>0.643</td>
<td>-1.020</td>
<td>0.976</td>
<td>61.0</td>
</tr>
<tr>
<td>6C4</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>6G8</td>
<td>1259</td>
<td>100-10,000</td>
<td>2.5</td>
<td>1/2</td>
<td>0.614</td>
<td>-0.587</td>
<td>0.989</td>
<td>83.6</td>
</tr>
<tr>
<td>7C10</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>8C12</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>8E1</td>
<td>4303</td>
<td>400 -20,000</td>
<td>1.25</td>
<td>1/2</td>
<td>0.733</td>
<td>-0.959</td>
<td>0.977</td>
<td>64.7</td>
</tr>
<tr>
<td>9E10</td>
<td>2395</td>
<td>100-10,000</td>
<td>0.32</td>
<td>1/16</td>
<td>0.961</td>
<td>-0.595</td>
<td>0.974</td>
<td>93.3</td>
</tr>
<tr>
<td>9F4</td>
<td>502.5</td>
<td>20 - 6,000</td>
<td>0.32</td>
<td>1/16</td>
<td>0.713</td>
<td>-0.399</td>
<td>0.965</td>
<td>85.6</td>
</tr>
</tbody>
</table>

$^a$ % inhibition at 100,000 ng mL$^{-1}$ of MIB standard
n/a; No inhibition curve observed
IgM clones were not tested
4.4.4.1 Effect of supernatant and coating conjugate concentrations on ELISA sensitivity

Clone 4F11 showed the best inhibition when tested against MIB as analyte based on comparison of IC₅₀ values (see Table 4.3) thus providing the highest sensitivity compared to other clones. Therefore, further characterization and optimization were performed using this clone. The effect of coating conjugate and tissue-culture supernatant dilution on the sensitivity of an MIB immunoassay was evaluated for clone 4F11. We tested the hypothesis that in a competition ELISA, higher affinity of the antibody to MIB in solution than to the immobilized conjugate produces greater immunoassay sensitivity. This can be achieved through lowering the concentration of either the immobilized conjugate or the antibodies. The effect of coating conjugate and supernatant concentration on the sensitivity of the MIB immunoassay is summarized in
Table 4.4. Lowering both MIB-TG and mAb concentrations greatly enhanced the sensitivity of the assay, with the lowest IC_{50} value being 0.9 ng mL^{-1} for MIB-TG at 0.63 µg mL^{-1} and a supernatant dilution of 1/8. However, this action also resulted in lower absorbance values as binding of antibody to the coating antigen was reduced (lower A_{max} values); consequently it was not practical to use the assay with these parameter concentrations.

Table 4.4. Effect of coating conjugate concentration and tissue-culture supernatant dilution on attributes of MIB dose response curves

<table>
<thead>
<tr>
<th>MIB-TG dilution (µg mL^{-1})</th>
<th>Supernatant dilution</th>
<th>IC_{50} (ng mL^{-1})</th>
<th>A_{max}</th>
<th>Slope</th>
<th>R^2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.25</td>
<td>1/2</td>
<td>100.2</td>
<td>1.012</td>
<td>-0.404</td>
<td>0.990</td>
</tr>
<tr>
<td></td>
<td>1/4</td>
<td>26.6</td>
<td>0.796</td>
<td>-0.692</td>
<td>0.983</td>
</tr>
<tr>
<td></td>
<td>1/8</td>
<td>19.5</td>
<td>0.595</td>
<td>-0.490</td>
<td>0.994</td>
</tr>
<tr>
<td>0.63</td>
<td>1/2</td>
<td>5.9</td>
<td>0.719</td>
<td>-0.319</td>
<td>0.985</td>
</tr>
<tr>
<td></td>
<td>1/4</td>
<td>1.7</td>
<td>0.554</td>
<td>-0.369</td>
<td>0.975</td>
</tr>
<tr>
<td></td>
<td>1/8</td>
<td>0.9</td>
<td>0.328</td>
<td>-0.476</td>
<td>0.982</td>
</tr>
</tbody>
</table>

4.4.4.2 Pre-incubation of the analyte and supernatant mixture

The effect of pre-incubation (i.e., inhibition ELISA) of antibody and free analyte (MIB) in solution on sensitivity of MIB ELISA was evaluated. Pre-incubations were performed as described in Section 4.3.6.3 where mAb tissue culture supernatants were pre-incubated with MIB standards (0.001 to 100,000 ng mL^{-1}). These solutions were then transferred to wells coated with MIB-TG at 1 µg mL^{-1}. In competition ELISA (CI-ELISA), the analyte and mAb supernatant were added simultaneously to the coated wells. In contrast to rabbit pAb immunized with the same antigen (i.e., (-) camphor-BSA), which was discussed in Chapter 3, it was shown that for mAb clone 4F11, the pre-incubation step improved sensitivity of the MIB ELISA. By comparing the difference in IC_{50} values,
the sensitivity of inhibition ELISA was improved by 56.7 % ($p<0.05$) when compared to CI-ELISA (Figure 4.6). It was also noticed that the percentage inhibition was higher in inhibition ELISA than in CI-ELISA. This result is in agreement with Lee et al. (2001) who observed that pre-incubation of imidacloprid and antiserum for 2 h resulted in a lower IC$_{50}$ value compared to control, i.e. no pre-incubation step. Therefore, a competitive indirect inhibition ELISA was used from this point.
Figure 4.6. Effect of pre-incubation on MIB ELISA sensitivity using mAb clone 4F11. Pre-incubation of supernatant and MIB in solution increased the sensitivity of the ELISA ($p<0.05$). IC$_{50}$ values were observed at 100.2 and 231.6 ng mL$^{-1}$ for inhibition and competition (CI-ELISA), respectively. Values are mean ± SD of three replicates.
4.4.5 Cross reactivity of clone 4F11 to compounds structurally related to MIB

Specificity of mAb clone 4F11 was evaluated through inhibition ELISA with compounds structurally related to MIB. The results are summarized in Table 4.5. Cross reactivity for MIB is regarded as 100%. Clone 4F11 was most specific for camphor-like compounds with percentage of cross-reactivity above 10,000. This result was not surprising as (-) camphor was used as the immunogen; it is expected that the antibody will have higher affinity to the original compound. 4F11 was also shown to recognize molecules having a methyl group at C8 and C9. The percentage cross-reactivity towards (-) borneol, isoborneol, fenchol was found to be 24.1%, 445.3%, and 344.3 %, respectively. Compounds lacking methyl groups were recognized less well by clone 4F11 with cross-reactivity towards (+) endo-2-norborneol and norbornylamine being less than 0.1%. These results suggest that the presence of methyl groups in the hapten is important for recognition by 4F11. However, although camphorquinone has these important methyl groups, the presence of a second carbonyl group seems to significantly reduce the specificity of the antibody; the cross-reactivity was only 0.94%. The low cross reactivity to geosmin was expected since its structure is very different than that of MIB. Dehydration products of MIB, 2-methylenbornane and 2-methyl-2-bornene were not tested because commercial standards were not available, and, although they may be present in fish flesh, they do not cause off-flavor (Korth et al., 1992; Martin et al., 1988; Mills et al., 1993). In conclusion, the cross-reactivities of clone 4F11 to MIB and other structurally related compounds indicate that the monoclonal antibody produced is not highly specific for MIB.
**Table 4.5.** Specificity of mAb clone 4F11 to compounds structurally related to MIB

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure</th>
<th>MW</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (ng mL⁻¹)</th>
<th>% CR&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIB</td>
<td><img src="image1.png" alt="Structure" /></td>
<td>168.28</td>
<td>100.2</td>
<td>100</td>
</tr>
<tr>
<td>(+) Camphor</td>
<td><img src="image2.png" alt="Structure" /></td>
<td>152.23</td>
<td>0.96</td>
<td>10480</td>
</tr>
<tr>
<td>(-) Camphor</td>
<td><img src="image3.png" alt="Structure" /></td>
<td>152.23</td>
<td>0.38</td>
<td>26200</td>
</tr>
<tr>
<td>Camphorquinone</td>
<td><img src="image4.png" alt="Structure" /></td>
<td>166.22</td>
<td>&gt;10,000</td>
<td>0.94</td>
</tr>
<tr>
<td>(-) Borneol</td>
<td><img src="image5.png" alt="Structure" /></td>
<td>154.25</td>
<td>414.9</td>
<td>24.1</td>
</tr>
<tr>
<td>Isoborneol</td>
<td><img src="image6.png" alt="Structure" /></td>
<td>154.25</td>
<td>22.5</td>
<td>444.1</td>
</tr>
<tr>
<td>(+) Bornylamine</td>
<td><img src="image7.png" alt="Structure" /></td>
<td>152.26</td>
<td>607.4</td>
<td>16.5</td>
</tr>
<tr>
<td>(+) Endo-2-norborneol</td>
<td><img src="image8.png" alt="Structure" /></td>
<td>112.17</td>
<td>&gt;10,000</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Norbornylamine</td>
<td><img src="image9.png" alt="Structure" /></td>
<td>111.6</td>
<td>&gt;10,000</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Fenchol</td>
<td><img src="image10.png" alt="Structure" /></td>
<td>154.25</td>
<td>29.1</td>
<td>344.7</td>
</tr>
<tr>
<td>Geosmin</td>
<td><img src="image11.png" alt="Structure" /></td>
<td>182.30</td>
<td>1132</td>
<td>8.8</td>
</tr>
</tbody>
</table>

<sup>a</sup>Percentage of cross reactivity
4.5 Conclusion

The purpose of this chapter was to improve the sensitivity and specificity of an MIB immunoassay through production of hybridoma clones. Monoclonal antibodies supersede polyclonal antibodies in terms of their unlimited supplies and since they are individually selected, the selection of the clones can be tailored towards meeting the objectives. To achieve this, (-) camphor was crosslinked to BSA and used as immunogen to produce hybridomas. Four BALB/c mice were immunized over 126 days (six boosts) and tested for their antibody titer. Mice 2 and 3, which produced the highest serum titer and greatest percentage of inhibition against free MIB were utilized to produce monoclonal antibodies. Splenocytes from these mice were fused with SP 2/0 Mus musculus fusion partner and hybridoma clones were established. Supernatants collected from these clones were screened against MIB-TG. Fourteen of over a thousand clones showed high affinity towards MIB-TG; eleven were IgGs and three were IgMs. (+) Bornylamine and MIB were crosslinked to TG and used as plate coatings in ELISAs. Affinity studies on both coating conjugates showed that out of 14 clones, 9 clones had a higher affinity to MIB-TG; 4 clones had similar affinities to both coating conjugates and 1 clone showed a higher affinity to (+) bornylamine-TG. Ten of the 14 clones showed higher sensitivity when inhibition ELISAs were performed against (-) camphor than with MIB with one clone showing no inhibition. Clone 4F11, displaying the highest sensitivity, was selected for further characterization in ELISAs. The sensitivity of ELISA was improved by lowering the concentrations of both coating conjugates and supernatant. As a comparison, pre-incubation of antibody and free analyte was shown to have better sensitivity than adding both simultaneously to the coated plates. Clone 4F11 showed significant cross reactivities to camphor than to MIB; while some cross reactivity was observed to isoborneol and fenchol. Other compounds showed little or no cross reactivity.
Using the same immunogen described by Chung et al. (1990) and MIB-TG as coating conjugate, we have shown that the monoclonal clones produced had a higher affinity and better sensitivity than those previously reported (Chung et al., 1990; Miyamoto et al., 1997). Nonetheless, Phlak and Park (2003) have reported a highly sensitive mAb-based assay using with an LOD of 0.1 ng mL$^{-1}$. We conclude that (-) camphor did not produce a high sensitivity ELISA as compared to (-) borneol as immunogen, as shown in Phlak and Park (2003). Although we had a fairly sensitive assay with an LOD of 1.9 ng mL$^{-1}$, we could not proceed with the work because in order to utilize MIB ELISA as screening kit, especially for application by catfish producers, an LOD below that detectable by smell, i.e., 10 pg mL$^{-1}$, is necessary.
DEVELOPMENT AND COMPARISON OF A CHEMILUMINESCENCE AND DIRECT IMMUNOASSAYS FOR THE DETECTION OF MONENSIN

5.1 Abstract

This study describes the development of a polyclonal-based immunoassay for the detection of monensin in water. Rabbits were immunized with monensin conjugated to bovine serum albumin (BSA) to produce polyclonal antisera for the development of a competitive indirect ELISA (CI-ELISA) using goat anti-rabbit HRP as the secondary antibody. CI-ELISA IC$_{50}$ values ranged from 1.056-1.090 ng mL$^{-1}$ with a limit of detection (LOD) of 0.1 ng mL$^{-1}$. The polyclonal antiserum was specific to monensin with little or no cross reactivity to other coccidiostats, i.e., narasin, nigericin, maduramicin, and lasalocid A, which had % cross reactivities of 0.1, 7.6, 1.1, and 0 %, respectively. The ELISA was optimal after a 1-h incubation at RT, within a pH range of 6 to 7.4, 1-4X PBS, in the presence of 5% of methanol. To improve the sensitivity of the CI-ELISA, it was reformatted into chemiluminescence ELISA (CICL-ELISA) using a goat anti-rabbit HRP to convert luminol to a chemiluminescent product. Although the CICL-ELISA was found to be more sensitive with IC$_{50}$ values ranging from 0.545 to 0.959 ng mL$^{-1}$, both assays had a similar detection limit of 0.1 ng mL$^{-1}$. For the competitive direct ELISA, polyclonal IgGs were purified from rabbit antiserum by using protein G affinity chromatography. Direct competitive ELISA did not improve the sensitivity of the immunoassay significantly with IC$_{50}$ values ranging from 1.128 to 1.505 ng mL$^{-1}$.

Correlation studies were done with liquid chromatography–mass spectrometry (LC-MS) using solid phase extraction (SPE) cartridges. Good correlations were observed between indirect ELISA and LC-MS ($r^2 = 0.998, 0.966, 0.997, 0.99$) and direct ELISA and LC-MS ($r^2 = 0.930, 0.997, 0.823, 0.979$)
for nanopure, tap, river and lake water, respectively. Both direct and indirect competitive ELISAs developed in this work may offer a reliable, simple, cheap and quick alternative or a complement to conventional analysis to determine the presence of monensin in water samples.

5.2. Introduction

In 1999, it was estimated that 4700 tons of antibiotics were used as feed supplements in the European Union for veterinary health (Song et al., 2007). One of these antibiotics was monensin (trade name Rumensin; MW 671 Da), which is used in animal feed to treat coccidiosis in poultry, beef and dairy industries (Butaye et al., 2003; Matsuoka et al., 1996). Monensin is commonly used as a feed additive in North America, Australia and New Zealand (Ellis et al., 2012). In the USA, approximately 1.5 million kilograms of monensin are used annually for subtherapeutic purposes in cattle and poultry production accounting for about 13% of the total antibiotic usage (Mellon et al., 2001).

Administration of monensin to control coccidiosis (i.e., protozoan parasite of the genus *Eimeria*) in farm animals, especially poultry, has been shown to be successful to prevent productivity losses (Chapman et al., 2010). Furthermore, the development of resistance to monensin is slow (Chapman, 1984). In the beef and dairy industries, monensin increases the production of propionic acid in the rumen (Matsuoka et al., 1996), which increases weight gain (Russell and Strobel, 1989) while minimizing loss of muscle tone, preventing bloating, and increasing the rate of milk production in lactating dairy cows (Canadian Food Inspection Agency, 2011). Tedeschi et al. (2003) have also shown that monensin increases feed efficiency in cattle by as much as 9% and reduces methane emissions from cattle by almost 20%. Monensin is composed of analogues, i.e., monensin-A, -B, -C, and -D, with monensin-A being the major component of the
mixture (98%); it is used primarily as a sodium salt or free acid (European Food Safety Authority, 2008). The sodium salt of monensin-A has a molecular weight of 693 Da and the molecular formula \( \text{C}_{36}\text{H}_{61}\text{O}_{11}\text{Na} \). Monensin is a pentacyclic molecule with seven methyl groups, and an ethyl, a carboxyl and four hydroxyl groups (Figure 5.1 Agtarap and Chamberlin, 1967). It has a water solubility of 8.78 mg L\(^{-1}\) and a log octanol/water partition coefficient (log \( K_{\text{ow}} \)) of > 6.3 (European Food Safety Authority, 2005). Monensin is considered a polyether monocarboxylic antibiotic, which refers to complex molecules with antimicrobial activity that is composed of oxygenated heterocyclic rings and a single terminal carboxyl group (Chapman et al., 2010; Westley, 1982). Monensin readily forms complexes with polar cations, such as K\(^+\), Na\(^+\), Ca\(^{2+}\), and Mg\(^{2+}\) and because of its hydrophobicity, these cations are easily transported across the lipid cell membrane and out of the microbes thus decreasing intracellular pH, which results in cell death (Mollenhauer et al., 1986; Pressman and Fahim, 1982).

![Chemical structure of monensin sodium salt](Sigma-Aldrich Co., St. Louis, MO).

**Figure 5.1.** Chemical structure of monensin sodium salt (Sigma-Aldrich Co., St. Louis, MO).
Veterinary pharmaceuticals in animal feed can be released to the environment from animal manure (Watanabe et al., 2010), resulting in surface and ground water contamination (Kolpin et al., 2002; Song et al., 2010). Therefore, there is concern about the effect of these pharmaceuticals on terrestrial and aquatic ecosystems. In addition, the development of antibiotic-resistant strains of microorganisms is also rising at an alarming rate (Watanabe et al., 2008). The use of monensin as a feed additive is safe and effective at concentrations as great as 40 mg kg\(^{-1}\) (European Food Safety Authority, 2006). Toxicity of monensin is dose-dependent and may affect a number of animal species. The LD50 for mice was reported as 44 mg kg\(^{-1}\) (Pinkerton and Steinrauf, 1970), while for cattle it was estimated to range from 21.9 to 80 mg kg\(^{-1}\) body weight (Gonzalez et al., 2005). The toxicity of monensin has also been reported in other animals with an LD\(_{50}\) of 2.3 mg kg\(^{-1}\) body weight for horses; 12 mg kg\(^{-1}\) for sheep; 16 mg kg\(^{-1}\) for pigs; 20 mg kg\(^{-1}\) for dogs, and 200 mg kg\(^{-1}\) for chickens (MacDonald, 2012). Frequent exposures to monensin can have adverse effects on the roots of terrestrial plants, aquatic animals and microorganisms (McGregor et al., 2007). Lethal concentrations of monensin have been reported above 9 mg L\(^{-1}\) for rainbow trout and fathead minnows and less than 1 mg L\(^{-1}\) in water or 1 mg kg\(^{-1}\) in sediment for aquatic microorganisms, such as green algae and amphipods (Elanco Products Company, 1989; Ramsdell, 2003). Adverse effects on plant growth have also been reported at concentrations above 1 mg kg\(^{-1}\) (Brain et al., 2004). Monensin is not used as a human antibiotic because it is not well tolerated (Russell and Houlihan, 2003); hence, there may be a negative impact on human health by exposure to contaminated drinking water and food. Based on two cases of death from direct ingestion of monensin by humans (Caldeira et al., 2001; Kouyoumdjian et al., 2001), it appeared that monensin targets skeletal and heart muscles in humans (World Health Organization, 2009).
Approximately 50% of monensin administered in animal feed is absorbed, rapidly metabolized and excreted in bile, while the remainder is eliminated in feces as unmetabolized monensin (Donoho et al., 1978; Donoho, 1984; Herberg and Van Duyn, 1969; Herberg et al., 1978). Due to its slow hydrolysis and photolysis when released in the environment, monensin may reach aquifers (Elanco Products Company, 1989). In fact, the occurrence of monensin has been observed in river water and sediments on agricultural sites in Colorado (Cha et al., 2005; Kim and Carlson, 2006) and in surface waters of heavily farmed areas of southern Ontario, Canada in the range of 6.2 – 1172 ng L⁻¹ (Hao et al., 2006; Lissemore et al., 2006). Hence, the demand for a simple and rapid method for the sensitive detection of monensin in surface waters that can be used on-site is warranted.

Over the years, numerous methods have been developed for the detection of monensin such as microbiological assays (Breunig et al., 1972; 1977), thin layer chromatography (Owles, 1984), bioautographic method (Donoho and Kline, 1968; Kline and Golab, 1965; Martinez and Shimoda, 1983), cylinder plate method (Kline et al., 1970), turbidimetric method (Kavanagh and Willis, 1972), colorimetric method (Golab et al., 1973), and fluorodensitometric method (Asukabe et al., 1984). Some of these methods are not able to separate monensin from other minor components, which leads to inaccuracy, while other methods such as the bioautographic method are time consuming and cannot be used for quantitative purposes (Macy and Loh, 1983). Liquid chromatography has been employed to overcome these shortcomings (Macy and Loh, 1983). Normally, monensin is first extracted from contaminated samples using a C18 solid phase extraction (SPE) cartridge, and then analysed using high performance liquid chromatography (C18 column) coupled to a mass spectrometer. Because monensin is non-volatile and does not exhibit UV-vis absorption, a high pressure liquid chromatography equipped with mass spectrometry is used for its detection (Cha et al.,
However, LC-MS instrumentation is expensive, time-consuming, often involves extensive sample preparation and is not suitable for on-site analysis. A simple, rapid, low cost tool applicable in field settings, such as immunoassay, is therefore needed.

Preliminary immunoassay development for the detection of monensin was described by Heitzman et al. (1986) in bovine plasma using a pAb with a sensitivity of less than 1 µg L\textsuperscript{-1}. Mount et al. (1987) reported another pAb-based assay for biological samples, which had a detection limit of 5 ng mL\textsuperscript{-1}. A specific monoclonal antibody (mAb)-based assay with a detection limit of 5 ng mL\textsuperscript{-1} in a microELISA format was described by Pauillac et al. (1993). Another mAb-based assay with detection limits of 40 ng mL\textsuperscript{-1} in bovine milk and plasma and 160 ng mL\textsuperscript{-1} in chicken plasma (Watanabe et al., 1998) were also reported. Currently, there are monensin ELISA kits commercially available. For example, the monensin ELISA kit from Immuno-Diagnostic Reagents (Vista, CA) has a sensitivity of 1.5 ng mL\textsuperscript{-1} and a limit of quantification (LOQ) of 3.0 ng mL\textsuperscript{-1} while the kit available from Abraxis Bioscience, LLC (Westminster, PA) claimed a limit of quantitation (LOQ) of 0.176 ng mL\textsuperscript{-1} and IC\textsubscript{50} of 0.889 ng mL\textsuperscript{-1}, respectively.

The purpose of this study was to develop a sensitive polyclonal-based immunoassay for the detection of monensin. To achieve this, we evaluated factors that can affect the sensitivity of an immunoassay. We evaluated the sensitivities obtained using two immunoassay formats, competitive indirect ELISAs and competitive direct ELISAs. Both formats were compared and validated through a correlation study in real water samples with an analytical method. We also tested the hypothesis that signal amplification using a chemiluminescence system that uses a luminol substrate will improve the sensitivity of an ELISA when compared to a conventional colorimetric substrate.
5.3. Materials and methods

5.3.1 Materials, reagents and equipment

Monensin sodium salt (90-95%, TLC), narasin, nigericin, maduramicin, and lasalocid A were purchased from Sigma Aldrich (Oakville, Canada). Their molecular structures are shown in Table 5.3. Stock solutions of monensin and other related compounds were prepared in methanol at 1 mg mL\(^{-1}\) and stored at -20\(^\circ\)C. Standards were prepared in volumetric flasks, at concentrations ranging from 0.001 to 100,000 ng mL\(^{-1}\) in 10% methanol/water (v/v) and stored at 4\(^\circ\)C.

Horseradish peroxidase (HRP) and biotin conjugated to goat anti-rabbit IgG were purchased from Jackson ImmunoResearch (West Grove, PA). HRP labelled streptavidin was obtained from Pierce (Thermo Scientific, Nepean, Canada), while HRP labelled monensin was purchased from USBiological (Swampscott, MA). Superblock in PBS was obtained from Thermo Scientific (Nepean, Canada). 1-Step\textsuperscript{TM} Turbo TMB ELISA and ELISA Femto SuperSignal used for CL substrate were purchased from Pierce (Thermo Scientific, Nepean, Canada) while TMB liquid substrate system was from Sigma Aldrich (Oakville, Canada). All other chemicals were from Sigma Aldrich (Oakville, Canada) unless otherwise stated. All chemicals and reagents were of analytical grade unless stated otherwise.

ELISAs were conducted using 96-well flat bottom transparent microtiter plates (high-binding; Corning Inc. Life Sciences, Lowell, MA). Plates were measured at 450 nm with a microplate reader from Bio-Rad Laboratories (Model 3350-UV, Hercules, CA). Chemiluminescence (CL) ELISAs were conducted using 96-well flat bottom white polystyrene microtiter plates (high-binding, Greiner Bio-One, VWR International, Mississauga, Canada) and were measured with an EnVision 2100 Microplate Luminometer (Perkin-Elmer) equipped with EnVision software (Wallac, Gaithersburg,
A HiTrap Protein G HP column which was used for IgG purification was purchased from GE Healthcare (Baie d'Urfe, Canada). Monensin in fortified water samples were analyzed by liquid chromatography mass spectrometry (LC-MS, Agilent 1100/1200 LC) coupled to an Agilent 6430 ion trap mass spectrometer located in the Department of Chemistry, McMaster University, ON.

All aqueous solutions and buffers were prepared with water purified on a Milli-Q system (Millipore, Bedford, MA). The following buffers were used: (A) coating buffer, 0.05 M carbonate-bicarbonate buffer, pH 9.6 (1.59 g of Na₂CO₃, 2.93 g of NaHCO₃, 0.2 g of NaN₃ per liter of water); (B) blocking buffer, Superblock in PBS (Thermo Scientific, Nepean, Canada); (C) assay buffer, 1X PBS, pH 7.4 diluted from 10X stock concentration (8 g of NaCl, 0.2 g of KCl, 1.44 g of Na₂HPO₄, and 0.24 g of KH₂PO₄ per liter of water, pH 7.4); and (D) Washing buffer, PBST (0.05% Tween 20 in 1X PBST). Buffers for IgG purification by protein G affinity chromatography were prepared according to the manufacturer’s instructions.

5.3.2 Preparation of monensin conjugates

Conjugates of monensin were made by attaching the carboxyl group of monensin to a Lys group of the carrier proteins using methods described by Fleeker (1987). BSA and OVA were used as the carrier proteins to prepare the immunogen and coating conjugates, respectively, as previously described (Makvandi-Nejad et al., 2010). Briefly, monensin (150 mg, 0.124 mM) was mixed with N-hydroxysuccinimide (NHS; 50 mg, 0.124 mM) in 2 mL of methanol in a 5-mL glass vial. Dicyclohexylcarbodiimide (DCC; 39 mg, 0.124 mM) in 1 mL of methanol was added to the solution, mixed gently and incubated overnight at 22 °C in the dark. BSA (200 mg) or OVA (ovalbumin; 200 mg) in 3 mL of borate buffer (0.1 M, pH 9.0) was added drop-wise to the vial with constant
stirring. The reaction mixture was stirred for 2 h at 22 °C. The resulting conjugates were dialyzed against 1X PBS at 4 °C for 24 h.

5.3.3 **Immunization of rabbits**

Immunization of rabbits was carried out by Makvandi-Nejad et al. (2010). In brief, a pair of New Zealand White female rabbits was immunized with monensin-BSA. The conjugate was diluted in sterile 1X PBS and emulsified with an equal volume of TiterMax® Classic Adjuvant (Sigma-Aldrich, St. Louis, MO). Rabbits were injected subcutaneously with 100 μg mL\(^{-1}\) for the primary immunization. Two weeks after the primary injection, animals were boosted at monthly intervals with 50 μg mL\(^{-1}\) of a monensin conjugate mixed with an equal volume of Freund's incomplete adjuvant (ca. 100 μL; Sigma-Aldrich). Sera (ca. 500 μL) were collected a week after each immunization and stored at 4°C overnight prior to centrifugation at 15,000 x g to separate the serum from blood cells. The immune response was monitored by ELISA. The final boost was administered three days before sacrificing the animals and the terminal bleeds were collected by cardiac puncture.

5.3.4 **Competitive indirect ELISA (CI-ELISA)**

Optimal concentrations for the coating conjugate and antiserum were selected by performing a checkerboard and two-dimensional CI-ELISA as described by Cervino et al. (2008) (Table 5.1; See also Appendix IV, Table A4). The optimal concentrations were chosen based on the lowest IC\(_{50}\) values giving a detectable signal above the background maximum (zero competition) well. Since the two-dimensional ELISA only provides values for single well, measurements were validated afterwards by performing CI-ELISA in triplicate (n=3).
For CI-ELISA, microtiter plates were coated with monensin-OVA at 1/45,000 (v/v) dilution in 0.05 M carbonate bicarbonate buffer, pH 9.6. OVA was coated at 0.1 µg mL⁻¹ and served as a negative control. Plates were incubated for 16 h at 4 °C, washed with 1X PBS and blocked with Superblock (250 µL well⁻¹) for 1 h at 22 °C with gentle shaking. Plates were washed three times with PBST. Monensin standards of different concentrations (0.001 to 100,000 ng mL⁻¹ in 10% methanol v/v, 50 µL well⁻¹) were added followed by antisera at 1/45,000 (v/v) final dilution (50 µL well⁻¹). Monensin was allowed to compete with the coated antigen for antibody binding for 1 h at 22 °C with gentle shaking. Plates were washed five times with PBST. Goat anti-rabbit IgG conjugated to horseradish peroxidase (GaR-HRP) was added at 1:6000 (v/v) dilution (100 µL well⁻¹) in PBST and plates were further incubated for another hour at 22 °C with gentle shaking. After the final wash step, TMB substrate was added and color development was observed after 30 min incubation in dark at 22 °C. The reaction was quenched by adding 1N H₂SO₄ (100 µL well⁻¹) and the resulting color intensity was determined at 450 nm.

For the inhibition ELISA, antiserum and free monensin were pre-incubated before being added to the coated wells. Inhibition was performed by mixing the monensin standards with antiserum at a final concentration of 1/45,000 (v/v) in 2-mL glass vials for 1 h at 22 °C with gentle rotation (rotary cell culture system, Synthecon Inc., Houston, TX). The inhibition solution from each vial was subsequently transferred (100 µL well⁻¹) to three wells of a pre-coated and pre-washed microtiter plate and further incubated for 1 h at 22 °C with gentle shaking. Subsequent ELISA steps were carried out as described previously.
Table 5.1. Two-dimensional CI-ELISA to determine the optimal dilutions for the coating conjugate and antiserum (Cervino et al., 2008).

<table>
<thead>
<tr>
<th>Column&lt;sup&gt;a,c&lt;/sup&gt;</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coating conjugate dilutions&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1/x</td>
<td>1/(nx)</td>
<td>1/x</td>
<td>1/(nx)</td>
<td>1/(n^2x)</td>
<td>1/(nx)</td>
<td>1/(n^2x)</td>
<td>1/(n^2x)</td>
<td>1/(n^2x)</td>
<td>1/(n^2x)</td>
</tr>
<tr>
<td>Antisera dilutions&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1/y</td>
<td>1/y</td>
<td>1/(my)</td>
<td>1/(my)</td>
<td>1/(m^2y)</td>
<td>1/(m^2y)</td>
<td>1/(m^2y)</td>
<td>1/(m^2y)</td>
<td>1/(m^2y)</td>
<td>1/(m^2y)</td>
</tr>
<tr>
<td>Row&lt;sup&gt;c&lt;/sup&gt;</td>
<td>A</td>
<td>B</td>
<td>C</td>
<td>D</td>
<td>E</td>
<td>F</td>
<td>G</td>
<td>H</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monensin (ng mL&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>0</td>
<td>0.01</td>
<td>0.1</td>
<td>1.0</td>
<td>10</td>
<td>100</td>
<td>1,000</td>
<td>10,000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Positive and negative controls were added in columns 11 and 12 (not shown).

<sup>b</sup>The assays were carried out at dilutions of x=1,000 and y=1,000. \(n=3\) and \(m=3\) were used as dilution factors.

<sup>c</sup>The dilutions of coating conjugate (monensin-OVA) and antiserum were added in column 1 to 10. For competition, monensin standards (0 to 10,000 ng mL<sup>-1</sup>) were added to rows A to H.

### 5.3.5 Optimization of ELISA conditions

The effect of time and temperature on CI-ELISA sensitivity was done by performing the assay for 0.5 h, 1 h and 1.5 h at 22 °C and 37° C. To evaluate the effect of pH on sensitivity, the assay buffer (1X PBS) was adjusted to pH 4.0, 5.0, 6.0, 7.0, 7.4, 8.0, and 9.0 by adding 0.1 M HCl or 0.1 M NaOH. For detergent effect, PBST (0.05% Tween 20) was compared to the assay buffer, 1X PBS. The effect of salt concentration (i.e., ionic strength) was determined by preparing the assay buffer at different concentrations of PBS, i.e., 1X, 2X, 4X, and 10X PBS from the same stock of 10X PBS.

For the effect of solvent, MIB standards were reconstituted in 5, 10, 20, and 40% methanol/water or acetonitrile/water (v/v). The final concentrations of solvents were reduced by half in CI-ELISA (i.e., 2.5, 5, 10, and 20%, respectively).
5.3.6 Cross reactivity (CR)

The specificity of the rabbit polyclonal antiserum was investigated by comparing its cross reactivities (CR) to monensin and closely related molecular structures listed in Table 5.3. Four ionophore antibiotics used to treat coccidiosis were selected, i.e., narasin, nigericin, maduramicin, and lasalosid. Having similar molecular structures, narasin has been reported as a growth stimulant, while monensin and lasalocid have been shown to improve the efficiency of feed utilization (Martinez and Shimoda, 1986).

Standard solutions were prepared in the concentration range of 0.001 to 100,000 ng mL$^{-1}$ in 10% methanol (v/v) and used in CI-ELISA. CR was expressed as percent IC$_{50}$ values based on 100% response of monensin and according to the following equation:

\[
\% \text{ CR} = \frac{\text{IC}_{50} \text{(monensin)}}{\text{IC}_{50} \text{(structurally related compounds)}} \times 100.
\]

The IC$_{50}$ can be considered a measure (inverse) of the affinity of an antibody for a given analyte (Han et al., 2007).

5.3.7 Chemiluminescence ELISA

Competitive indirect chemiluminescent ELISAs (CICL-ELISA) were performed according to the standard ELISA procedures previously described in Section 5.3.4. Optimizations of monensin-OVA and antisera dilutions were determined by checkerboard ELISA. In brief, 96-well microtiter plates (white, high-binding, Greiner Bio-One, VWR International, Mississauga, Canada) were coated with monensin-OVA by putting a $1/1000$ (v/v) dilution in the first row (Row A; 200 µL well$^{-1}$) and serially diluting by half down the plate. Final volumes were 100 µL well$^{-1}$. OVA was used as the negative control and was added in the last row of the plates (Row H; 100 µL well$^{-1}$). Plates were covered and sealed to prevent evaporation and incubated for 16 h at 4°C. After overnight incubation, plates were washed with PBST and blocked (250 µL well$^{-1}$) with Superblock for 1 h at 22 °C with gentle shaking. After subsequent washes, rabbit
antiserum from the final bleed was added at 1/200 (v/v) dilution in 1X PBS and serially
diluted by half from column to column across the plate. Plates were incubated at 37°C
for 30 min. Plates were washed three times with PBST. After several washes, GaR-HRP
was added at 1/500,000 (v/v) and further incubated using the same conditions. After
subsequent washes, SuperSignal ELISA Femto substrate solution (luminol and
peroxidase pre-mixed at equal volume) was added at 100 µL well⁻¹ and
chemiluminescence was measured immediately at 700 nm (after 1 min of gently shaking
the plate). As determined by checkerboard ELISA, the optimal concentration of
monensin-OVA was 1/16,000 and rabbit antiserum was 1/6400 (v/v). Using these
concentrations, the concentration of secondary antibody (GaR-HRP) was optimized
using 1/10,000 to 1/10,240,000 (v/v) dilutions. Four different dilutions of GaR-HRP
(1/40,000, 1/80,000, 1/160,000 and 1/320,000 v/v) were then selected and employed in
competitive indirect chemiluminescent ELISA (CICL-ELISA)

For CICL-ELISA, wells of microtiter plates were coated with 100 µL well⁻¹ of
monensin-OVA solution dissolved in coating buffer (1/16,000 v/v) for 16 h at 4 °C. The
wells were washed three times with 1X PBS and blocked with Superblock (250 µL well⁻¹)
for 1 h at 22 °C with gentle shaking. After the plates were washed five times with PBST,
monensin standards (0.001 to 10,000 ng mL⁻¹; 50 µL well⁻¹) and antiserum (1/6400 final
dilution; 50 µL well⁻¹) were added simultaneously into the wells. Following incubation at
37°C for 30 min and plate washing (five times with PBST), GaR-HRP diluted in PBST
(100 µL well⁻¹) was added at concentrations mentioned previously and further incubated
at 37°C for 30 min. Addition of substrate and CL measurements were performed as
previously described.
5.3.8 Direct ELISA

5.3.8.1 Isolation of monensin polyclonal antibody (IgG)

IgGs were purified from rabbit polyclonal serum according to the manufacturer’s (GE Healthcare) protocol. Briefly, 5-10 mL of polyclonal rabbit serum was mixed with an equal volume of 1X PBS and the pH was adjusted to 7.4. The solution was filtered through a 0.45 μm membrane filter (Whatman, Piscataway, NJ) and passed through a 5 ml HiTrap protein G HP column (pre-equilibrate with binding buffer: 20mM phosphate buffer, pH 7.0) connected to an AKTA-FPLC (GE Healthcare, Uppsala, Sweden). Unbound serum proteins were removed by washing the column with binding buffer until the UV spectrum reached a stable baseline. Polyclonal IgGs were eluted using 0.1 M glycine-HCl, pH 2.5. The pH of eluant was immediately neutralized by adding neutralizing buffer (1M Tris-HCl, pH 9.0) to the collection tubes prior to elution, and then dialyzed for 16 h against 1X PBS at 4°C. Following dialysis, purified IgGs were concentrated using Amicon Ultra-4 size exclusion membrane, 10K MWCO (Millipore, Billerica, MA). The concentration of purified IgGs was determined at A280 using PBS as a reference and the molar extinction coefficient of IgG according to the formula of Stoscheck (1990):

\[
\text{Antibody concentration (mg mL}^{-1}) = \frac{A \times d}{\varepsilon}
\]

where \( A \) = absorbance at 280 nm; \( d \) = dilution factor; and \( \varepsilon \) = rabbit IgG extinction coefficient, which is 1.35 (Nikolayenko et al., 2005). The concentration of purified polyclonal IgG was determined as ca. 7.6 mg mL\(^{-1}\).
5.3.8.2 Competitive direct ELISA

The optimal binding of polyclonal IgG to monensin-HRP was determined by coating the microtiter plates with a serial dilution of purified IgG (10, 5, 2.5, and 1 µg mL\(^{-1}\); 100 µL well\(^{-1}\)) in carbonate bicarbonate buffer, pH 9.6 and incubated overnight at 4°C. After several washes and blocking, monensin-HRP was added at different dilutions (1/150 to 1/9600 v/v in PBS; 100 µL well\(^{-1}\)) and further incubated for 1 h at 22 °C. After final washes, TMB substrate was added (100 µL well\(^{-1}\)) and the plate was allowed to develop for 30 min in dark. The reactions were stopped by adding 1N H\(_2\)SO\(_4\) (100 µL well\(^{-1}\)) and the absorbances were measured at 450 nm.

A competitive direct ELISA was carried out with the determined optimal concentrations as follows; briefly, microtiter plates were coated with protein G purified rabbit polyclonal IgG at 5 µg mL\(^{-1}\) in coating buffer and incubated for 16 h at 4°C. Plates were washed with 1X PBS, blocked with blocking buffer (200 µL well\(^{-1}\)) for 1 h at 22 °C and washed with PBST (200 µL well\(^{-1}\)). Competition was carried out by adding monensin standards and monensin-HRP at a final dilution of 1/600 (v/v) in equal volume (total volume of 100 µL well\(^{-1}\)). The plates were incubated at 22 °C for another hour with gentle shaking. After final washing, TMB substrate was added (100 uL well\(^{-1}\)) and the reactions were allowed to develop in the dark. Stop solution (1N H\(_2\)SO\(_4\), 100 uL well\(^{-1}\)) was then added to quench the reactions and absorbances were measured at 450 nm.

5.3.9 Preparation of water samples and solid phase extraction

Ground water samples were collected from Speed River and Guelph Lake, Guelph, Ontario, Canada. Tap water was obtained from the laboratory. Nanopure® water was obtained from a Mili-Q water purification system (18 MΩ, Milipore, Bedford, MA). Water samples were collected in 1-L amber glass bottles and filtered through a
0.45 µm nylon membrane filter housed in a Milipore filtration system (Bedford, MA). Water samples were stored in the dark at 4°C until required. Assay precision was performed with fortified monensin at 0, 0.01, 0.3, 300, 3000, and 10,000 ng mL⁻¹. The assays were performed on three different days with three different plates to determine intra-assay variability. For inter-assay variability, the assays were performed by two different analysts. The remaining ELISA steps were performed as described in Section 5.3.4.

For the correlation study, water samples were fortified with monensin at 0.1, 0.5, 1, 5 and 10 ng mL⁻¹ and CI-ELISAs were carried as described in Section 5.3.4. Analyses of fortified water samples were also compared with competitive direct ELISA and performed as described in Section 5.3.8.2.

For LC-MS analyses, fortified water samples were prepared as described earlier and extraction of monensin was performed according to Song et al. (2007) with some modifications. An internal standard (salinomycin; 5 ng mL⁻¹) was added to the fortified water samples prior to extraction. A hydrophilic-lipophilic balanced cartridge (Oasis HLB, 30µm, 6cc, 150 mg; Waters Corporation, Milford, MA) on a vacuum manifold (JT Baker Inc., Phillipsburg, NJ) was used to extract the analytes from water, which also served as a purification and concentration step. The cartridge was preconditioned by using 4 mL of methanol followed by 4 mL of nanopure water at a flow rate of 3 mL min⁻¹. The water samples (10 mL) were passed through the preconditioned HLB cartridge at a rate of 2 mL min⁻¹. The analyte-loaded cartridge was washed with 4 mL of nanopure water and the analytes were eluted from the cartridge with 1.5 mL of methanol containing 2% formic acid. The eluted analytes were collected in a vial and evaporated to dryness under a stream of nitrogen. Samples were reconstituted by adding 1 mL methanol and subjected to LC-MS analyses.
5.3.10 LC-MS analyses

LC-MS was performed using an Agilent 1100/1200 LC coupled to an Agilent 6340 ion trap mass spectrometer. The capillary voltage was set at 4500 V, and desolvation gas temperature was set at 325°C with a flow rate of 12 L min\(^{-1}\). Fragmentation amplitude was set to 1.00 for both ions. Monensin was detected by monitoring the m/z 675 fragment ion produced by fragmentation of the [M+Na]\(^+\) ion at m/z 693. Under these conditions the detection limit was below 1 ng mL\(^{-1}\). With an LC method using isocratic elution (H\(_2\)O/ACN, 97% ACN) on a Luna C18 (2), 2x150 mm column, monensin eluted at 11 min. The internal standard, salinomycin, was detected by monitoring the m/z 755 fragment ion produced by fragmentation of the [M+Na]\(^+\) at m/z 755. Under these conditions, it eluted at 9 min. The relative area is the ratio of peak area for monensin to the peak area of an internal standard (i.e., salinomycin) for each point.

5.3.11 Data analyses

Standards and samples were analyzed in triplicates. Dose response curves were fitted to a four-parameter logistic equation: \( Y = [(A-D)/1+ (x/C)^{B}] + D \), where \( A \) is the upper asymptote (maximum absorbance in the absence of analyte, \( A_{\text{max}} \)), \( B \) the curve slope at the inflection point, \( C \) the x value at the inflection point (corresponding to the analyte concentration giving 50% inhibition of \( A_{\text{max}}, IC_{50} \)) and \( D \) is the minimum asymptote (background signal) (Noguera et al., 2002). The y-axis represents the normalized absorbance value of each standard divided by the absorbance value of a zero competition well (well containing 0 ng mL\(^{-1}\) of monensin; \( A/A_0 \)), while x-axis represents the log value of monensin standard concentrations. Statistical analysis and four-parameter logistic graphs were performed using GraphPad Prism ver. 5.0 (GraphPad Software Inc., San Diego, CA). Limit of detection (LOD) was calculated as
mean absorbance of maximum or zero competition well \( (A_{\text{max}} \text{ or } A_0) \pm 3\text{SD} \), interpolated from the inhibition curve using GraphPad Prism.

### 5.4 Results and discussion

#### 5.4.1 Synthesis of monensin conjugates

Due to its low molecular weight, monensin is not immunogenic and therefore needs to be covalently linked to a larger molecule such as a protein to be recognized by B cells (Erlanger, 1980). Monensin conjugates were previously crosslinked to BSA as immunogen and to OVA for coating ELISA plates (Makvandi-Nejad et al., 2010). Monensin-BSA was used to produce polyclonal antibodies in rabbits (Makvandi-Nejad et al., 2010). This serum and coating conjugate were used in this study. Monensin was crosslinked to BSA and OVA for the synthesis of immunogen and coating antigen, respectively, using the Fleeker method (1987). In this scheme, carboxylic acid is reacted with N-hydroxysuccinimide (NHS) in the presence of dicyclohexyl carbodiimide (DCC) forming an activated NHS ester of carboxylic acid, which is stable under anhydrous and slightly acidic conditions. This ester reacts rapidly with amino groups to form an amide bond (Shan et al., 2002). This mixed anyhride method is highly advantageous because it is direct (Greenstein and Winitz, 1961; Vaughan and Osato, 1951), simple and the preparation and isolation of an active derivative is not necessary (Erlanger, 1980). DCC, a zero-length cross linker, was used instead of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) for the conjugation because it is more suitable for an organic soluble compound (Hermanson, 2008) such as monensin. Watanabe et al. (1998) employed a mixed anhydride method for preparation of monensin conjugates to human serum albumin (HSA), OVA and alkaline phosphatase (ALP) as immunogen, coating antigen and detection label. Using these conjugates, a monoclonal antibody-based assay with a detection limit of 1 ng mL\(^{-1}\) was achieved.
MALDI-TOF analysis using BSA as standard had a 5:1 ratio of monensin: BSA (See Appendix V; Figure A5). This ratio is at the lower range of what is an acceptable ratio and may have resulted in poor antibody response to monensin (Landsteiner, 1945). However, it was shown that this ratio of monensin to BSA was successful in producing high immune responses in rabbits (Makvandi-Nejad et al., 2010). It is likely that the complexity of monensin molecular structure led to a reasonably good B cell response. Good antibody titers normally can be obtained with hapten to protein ratio between 8 and 25 (Erlanger, 1980). Nonetheless, other factors, for example the nature of hapten also play a critical role in antibody recognition; therefore the optimal hapten to protein ratio which leads to high antibody response remains subjective.

5.4.2 Optimization of ELISA conditions

ELISA data are normally analyzed with non-linear sigmoidal curves. These curves plot the normalized response (A/A₀) or absorbance (y-axis) versus the logarithm of the competing analyte concentration (x-axis), called four-parameter logistics or 4PL. The sensitivity of an immunoassay is expressed by an IC₅₀ value, i.e., the concentration of free monensin that inhibits 50% binding of the anti-monensin antibodies. This is determined from the analytical range (linear range) of the sigmoidal curve. The lower the IC₅₀ value, the more sensitive the immunoassay is. It is therefore crucial to optimize the conditions of an immunoassay to obtain the most sensitive assay.

The immune response of rabbits immunized with monensin-BSA was previously evaluated using an ELISA plate coated with monensin-OVA (Makvandi-Nejad et al., 2010). Since monensin has a low water solubility, the addition of organic solvents are necessary to ensure monensin is completely dissolved (i.e., solubility of monensin in methanol is ~ 50 mg mL⁻¹; Merck Index, 1996). Therefore, the stock standard of monensin was dissolved in methanol at 1 mg mL⁻¹. To ensure the monensin was
completely dissolved, the standards were first prepared in methanol up to the water solubility level of monensin (i.e., 8.78 µg mL⁻¹; European Food Safety Authority, 2005), and then the working standards used in the ELISA were subsequently prepared at concentrations ranging from 0.001 to 100,000 ng mL⁻¹ in 10% methanol from those standards. Since the presence of organic solvents can affect the performance of an immunoassay, we minimized the percentage of methanol to a final concentration of 5% methanol in the ELISA, since the antibody and analyte were in the CI-ELISA at an equal volume.

5.4.2.1 Effect of the concentrations of coating conjugate and antiserum

In competitive ELISA, the sensitivity is determined by the relative affinities of an antibody to both antigen immobilized on the plate and free antigen in solution. In general, the highest sensitivity can be achieved if the antibody has lower affinity for the immobilized antigen than the same unbound analyte in the solution (Matschulat et al., 2005). We hypothesized that by lowering the coating antigen concentration, a more sensitive immunoassay could be achieved provided that the ELISA signal is sufficient for the range of free antigen to be determined. As there is less binding of antibody to immobilized antigen, the sensitivity of the assay should be lower. However, when a very low concentration of coating antigen is used, the signal is too low to determine the differences among antigen concentrations (Fouad et al., 2006). Therefore, we tested several combinations of coating conjugate and antiserum to find the optimal concentrations to provide the highest reproducible sensitivity.

Several combinations of coating conjugate and antisera dilutions were selected based on a checkerboard ELISA, and CI-ELISA were performed using these concentrations (row 1-3; Table 5.2). Another set of CI-ELISAs was conducted using two-dimensional CI-ELISA (row 4-10; Table 5.2). The attributes of the dose response curves
from both checkerboard and two-dimensional CI-ELISAs are presented in Table 5.2. For two-dimensional CI-ELISA, the selected values were validated in triplicate to obtain reliable dose response curves. For the last two rows, a more sensitive TMB substrate was used which resulted in a greater $A_{\text{max}}$. Coating conjugate and antiserum at 1/45,000 (v/v) dilutions were found to have a wider linear range, low IC$_{50}$ value, high $A/D$ ratio, highest percentage of inhibition and reasonable signal (i.e., $A_{\text{max}}$ close to 1.0). Therefore, this combination was selected for further use.
Table 5.2. Effect of the concentration of coating conjugate and antiserum, as determined by CI-ELISAs from checkerboard analysis and two-dimensional CI-ELISA, on monensin dose response curves

<table>
<thead>
<tr>
<th>Coating conjugate dilution&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Anti serum dilution&lt;sup&gt;b&lt;/sup&gt;</th>
<th>IC&lt;sub&gt;50&lt;/sub&gt; (ng mL&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>LOD (ng mL&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Linear range (ng mL&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>% maximum inhibition&lt;sup&gt;c&lt;/sup&gt;</th>
<th>slope</th>
<th>A&lt;sup&gt;d&lt;/sup&gt;</th>
<th>D&lt;sup&gt;d&lt;/sup&gt;</th>
<th>A/D</th>
<th>A&lt;sub&gt;max&lt;/sub&gt;&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/16,000</td>
<td>1/3,200</td>
<td>0.9</td>
<td>0.1</td>
<td>0.1-10</td>
<td>65</td>
<td>-0.810</td>
<td>1.022</td>
<td>0.353</td>
<td>2.89</td>
<td>0.515</td>
</tr>
<tr>
<td>1/32,000</td>
<td>1/1,600</td>
<td>1.3</td>
<td>0.1</td>
<td>0.1-10</td>
<td>59</td>
<td>-0.702</td>
<td>1.119</td>
<td>0.405</td>
<td>2.76</td>
<td>0.584</td>
</tr>
<tr>
<td>1/8,000</td>
<td>1/2,000</td>
<td>2.1</td>
<td>0.1</td>
<td>0.1-30</td>
<td>76</td>
<td>-0.602</td>
<td>1.004</td>
<td>0.216</td>
<td>4.65</td>
<td>0.640</td>
</tr>
<tr>
<td>1/1,000</td>
<td>1/1,000</td>
<td>100.5</td>
<td>10</td>
<td>10-1000</td>
<td>34</td>
<td>-0.852</td>
<td>0.975</td>
<td>0.652</td>
<td>1.49</td>
<td>1.050</td>
</tr>
<tr>
<td>1/3,000</td>
<td>1/3,000</td>
<td>14.8</td>
<td>1</td>
<td>1-500</td>
<td>48.2</td>
<td>-0.426</td>
<td>0.992</td>
<td>0.476</td>
<td>2.08</td>
<td>0.959</td>
</tr>
<tr>
<td>1/3,000</td>
<td>1/9,000</td>
<td>9.37</td>
<td>0.1</td>
<td>0.1-100</td>
<td>64.8</td>
<td>-0.508</td>
<td>0.982</td>
<td>0.323</td>
<td>3.04</td>
<td>0.693</td>
</tr>
<tr>
<td>1/9,000</td>
<td>1/9,000</td>
<td>2.61</td>
<td>0.1</td>
<td>0.1-100</td>
<td>64.1</td>
<td>-0.448</td>
<td>0.999</td>
<td>0.334</td>
<td>2.99</td>
<td>0.672</td>
</tr>
<tr>
<td>1/9,000</td>
<td>1/27,000</td>
<td>2.10</td>
<td>0.1</td>
<td>0.01-100</td>
<td>67.2</td>
<td>-0.310</td>
<td>0.353</td>
<td>0.090</td>
<td>3.89</td>
<td>0.335</td>
</tr>
<tr>
<td>1/45,000</td>
<td>1/15,000</td>
<td>1.04</td>
<td>0.1</td>
<td>0.1-10</td>
<td>71.1</td>
<td>-0.619</td>
<td>1.042</td>
<td>0.303</td>
<td>3.43</td>
<td>1.214</td>
</tr>
<tr>
<td>1/45,000</td>
<td>1/45,000</td>
<td>1.07</td>
<td>0.1</td>
<td>0.01-10</td>
<td>76.2</td>
<td>-0.420</td>
<td>1.017</td>
<td>0.292</td>
<td>3.67</td>
<td>0.826</td>
</tr>
</tbody>
</table>

<sup>a</sup>monensin-OVA was used as coating conjugate
<sup>b</sup>antiserum was collected from the final bleed of a rabbit producing anti-monensin IgG
<sup>c</sup>percentage of inhibition by competition with 10,000 ng mL<sup>-1</sup> monensin calculated as (1-A/A<sub>o</sub>) x 100
<sup>d</sup>A and D are top and bottom asymptote values on y-axis derived from 4PL graph
<sup>e</sup>Absorbance for maximum (zero competition) well
5.4.2.2. Pre-incubation of monensin and antiserum

In CI-ELISAs, free monensin and antiserum are added simultaneously to the wells, while in competitive indirect inhibition ELISAs, they are allowed to pre-incubate prior to being added to wells. It was found that pre-incubating the antibody and free monensin significantly decreased assay sensitivity. As shown in Figure 5.2, pre-incubation increased the IC₅₀ significantly (F test; \( p<0.05 \)) from 1.08 to 24.1 ng mL⁻¹. We also observed that the \( A_{\text{max}} \) was lowered in competitive indirect inhibition ELISA. It is likely that during pre-incubation the antibody binding site was occupied by free monensin thus reducing antibody binding to the plate to the level where the signal was too small to generate a response curve. This is in agreement with Galve et al. (2002) who observed that a pre-incubation step and length of competitive step did not cause a significant improvement to the sensitivity of an ELISA for trichlorophenol. In contrast, Lee et al. (2001) reported that pre-incubation for 1-2 h increased the sensitivity (IC₅₀ = 32.9 and 29.5 ng mL⁻¹ for 1 h and 2 h, respectively) of imidacloprid ELISA while prolonging the incubation to 4 h decreased the sensitivity (IC₅₀ = 842 ng mL⁻¹). They also found that the \( A_{\text{max}} \) decreased as incubation time increased. Therefore, all ensuing experiments were conducted using a CI-ELISA without the pre-incubation step.
Figure 5.2. Comparing the effect of pre-incubation (competitive indirect inhibition ELISA) and no incubation (competitive indirect ELISA) of monensin and antiserum on an ELISA for monensin. For the indirect inhibition ELISA, monensin standards were incubated with antiserum for 1 h at 22 °C prior to adding to coated plates. Data are compared with CI-ELISA where monensin standard and antiserum were added simultaneously to the coated plates. The IC$_{50}$ value was significantly higher ($F$ test; $p<0.05$) for the competitive indirect inhibition ELISA as opposed to CI-ELISA. $A_{max}$ value was also decreased. ELISA conditions are described in Section 5.3.4. Values represent mean ± SD of three replicates.
5.4.2.3 Effect of incubation time and temperature

It has been reported that time and temperature affects the antibody-antigen interaction, and hence assay sensitivity (Ballesteros et al., 1997; Gascon et al., 1997). Therefore, we studied the effect of time (0.5, 1 and 1.5 h) and temperature (22 and 37°C) on the sensitivity of CI-ELISA. As shown in the dose-response curves of Figure 5.3, the CI-ELISA performed for 0.5 and 1 h at 22°C had the greatest sensitivity, i.e., the IC$_{50}$s for 0.5 and 1 h at 22 °C was lower than after 1.5 h ($p<0.05$). At 37°C the 1.5 h incubation assay had the lowest sensitivity ($p<0.05$) compared to the two other curves. Although results achieved after incubation for 0.5 h and 1.5 h at 22°C were not statistically different ($p>0.05$), when experiments were repeated several times, results for 0.5 h incubation time did not show good reproducibility, probably due to insufficient antibody-antigen binding. Furthermore, incubation at 22°C is easier because an incubator is not required. Consequently, all subsequent experiments were conducted for 1 h at 22°C.
Figure 5.3. Effect of incubation time and temperature on the ELISA for monensin. CI-ELISA conducted with three different incubation times (0.5, 1 and 1.5 h) at two different temperatures; (A) 22°C and (B) 37°C. Means sharing different superscript are significantly different from each other (p<0.05). Values represent mean ± SD of three replicates.
5.4.2.4 Effect of solvent and detergent

In order to solubilize monensin at concentrations required for spiking water, an organic solvent had to be used. Consequently, the effect of organic solvent on the Cl-ELISA was evaluated by preparing monensin standards in various percentages of methanol and acetonitrile (i.e., 0, 5, 10, 20 and 40%). An equal volume of the monensin standard and antiserum was added simultaneously to each well. Therefore, the final percentages of organic solvents were reduced to half, i.e., 2.5, 5, 10, 20% (Figure 5.4). It was observed that as the concentration of the solvents was reduced, the sensitivity of the assay increased as determined by comparing IC$_{50}$ values. The negative effect of acetonitrile was greater. Furthermore, A$_{max}$ was reduced as the percentage of solvent increased (Figure 5.4). Other researchers have also found that a percentage of organic solvent above 5% generally reduces ELISA sensitivity (Bushway et al., 1989; Cao et al., 2005; Gee et al., 1988; Goh et al., 1990; Johnson and Hall, 1996; Lee et al., 2001). However, the addition of a low percentage of co-solvent may assist the immunoassay to be more resilient to matrix effects, especially for ELISAs to detect lipophilic compounds (Jung et al., 1989). Furthermore, adding co-solvents prevents adsorption to surfaces, especially for hydrophobic analytes, thereby maintaining their presence in solution (Matschulat et al., 2005). On the other hand, depending on the concentration of the co-solvent, their presence may alter the organization of water molecules which may disrupt the antibody-antigen complex (Johnson and Hall, 1996). Association of antibody-antigen generates holes and channels which can be occupied by water molecules thus contributing to the stabilization of the complex (Bhat et al., 1994). Organic solvents may also alter the conformational structure of the antibody; hence reducing its binding (Johnson and Hall, 1996). Our findings also suggest that organic solvents as diluents for a hydrophobic hapten in a competitive assay for monensin should be limited to 10 % or less (Figure. 5.4). This is supported by Lee et al. (2001) who suggested that ELISA
should be conducted in a buffer with little or no organic solvents. They found that 2-4% methanol had no detectable effect on an imidacloporid ELISA. Generally, it is worth noting that since the binding of an antibody is exclusive towards its antigen, a concentration of organic solvent that has no effect on one immunoassay can dramatically affect the sensitivity of other assays (Gee et al., 1988).
Figure 5.4. Effect of (A) methanol and (B) acetonitrile on the ELISA for monensin. Sensitivity of ELISA decreased as the percentage of organic solvent increased, (i.e., see IC$_{50}$ values), with the exception of acetonitrile and methanol at 2.5 and 5%. Addition of higher percentage of solvents also reduced the A$_{max}$. Means sharing different superscript are significantly different from each other ($p<0.05$). Values represent mean ± SD of three replicates.
The effect of detergent on monensin ELISA was evaluated by comparing the use of 1X PBST (0.05% Tween 20) with 1X PBS as antibody diluents. As shown in Figure 5.5, addition of Tween 20 at 0.05% to the assay buffer reduced \((p<0.05)\) the sensitivity (i.e., \(IC_{50}\) increased) of the monensin ELISA. In addition, \(A_{\text{max}}\) was also increased. Other researchers have found that reducing the concentration of Tween 20 from \(10^{-1}\) to \(10^{-4}\) (%) significantly increases the sensitivity of an immunoassay against chlorpyrifos, a nonpolar compound (\(IC_{50}\) of 7.5 to 0.2 nM) (Manclus and Montoya, 1996a). Manclus and Montoya (1996b) speculated that detergent may have a hydrophobic interaction with nonpolar haptens in an aqueous environment, thereby enhancing the antibody-antigen interaction. In contrast, detergent did not have any significant effect on 3,5,6-trichloro-2-pyridinol (TCP) immunoassay, a polar degradation product of chlorpyrifos (Manclus and Montoya, 1996b). Meanwhile, in other reports, no competition was observed in ELISAs for kresoxim-methyl (Mercader et al., 2008b) and trifloxystrobin (Mercader et al., 2008a) when Tween 20 was not present. Lower concentrations of detergent are often required to reduce non-specific adsorption of antibody to plate wells (Mercader et al., 2008a). However, in this study we did not examine the effect of further lowering the concentration (< 0.05%) of detergent on monensin ELISA; hence Tween 20 was not added to the buffer.
Figure 5.5 Effect of detergent (Tween 20 at 0.05 %) on the ELISA for monensin. Addition of Tween 20 at 0.05% reduced ($p<0.05$) the sensitivity of monensin ELISA as the $IC_{50}$ increased. Values represent mean ± SD of three replicates.
5.4.2.5 Effect of pH

Matrix effects caused by pH and salt are reported to influence a number of immunoassays, especially for those used for pesticide analysis (Goh et al., 1990; Koppatschek et al., 1990). In general, the pHs of water and soil lie within the range of 6 to 8. Depending on the nature of the analyte being tested, the concentration of H⁺ and OH⁻ in water and soil matrices may dramatically influence the antibody-antigen interactions thus affecting the sensitivity of an immunoassay. Seven different pH values ranging from 4.0 to 9.0 were evaluated using the CI-ELISA. The pH of PBS was adjusted accordingly and used as the antiserum diluent. It was observed that IC₅₀ values were lowest at pHs close to physiological pH, i.e., pH 6 to 7.4 (Figure 5.4); no statistical difference (F-test; \( p > 0.05 \)) was observed between pH 6 and 7 and between pH 7 and 7.4. At pHs lower than 6 and above 7.4, the sensitivity of the monensin immunoassay was negatively affected. There have only been a few immunoassays previously reported to be stable at a wide pH range. For example, the ELISA for benzo(a)pyrene showed a good tolerance at pHs ranging from 6.5 to 9.5, owing to the molecular structure of benzo(a)pyrene which is absent in ionizable group (Matschulat et al., 2005; Scharnweber et al., 2001). Nevertheless, extreme pH values should be avoided since they can irreversibly denature proteins and thus be detrimental to the antigen-antibody binding (Matschulat et al., 2005). In fact, many researchers have reported extreme pH as a major contributor to reducing sensitivity of an immunoassay (Galve et al., 2002; Johnson and Hall, 1996; Lee et al., 2001; Li et al., 1991).
Figure 5.6. Effect of pH on the ELISA for monensin. IC₅₀ values are the lowest at pHs ranging from pH 5 to 7.4. Means sharing different superscript are significantly different from each other (p<0.05). Values represent mean ± SD of three replicates.
5.4.2.6 Effect of concentration of salt

Four different concentrations of PBS, i.e., 1X (I = 0.167), 2X (I = 0.333), 4X (I = 0.668), and 10X (I = 1.67) were used as antiserum diluents and evaluated for their effect of salt concentration (i.e., ionic strength, I) on assay sensitivity. As shown in Figure 5.7, only 10X PBS decreased the IC$_{50}$. Increment of ionic strength to 4X PBS did not change the sensitivity of ELISA as the IC$_{50}$ values were not statistically different (F-test; $p>0.05$). It was also observed that $A_{\text{max}}$ was reduced as ionic strength of the buffer increased. The high ionic strength of the buffer (10X PBS; I = 1.67) may interfere with antibody-antigen binding (Lee et al., 2001; 2002). These results agree with those found by Lee et al. (2002), for deltamethrin; Lee et al. (2004), for cypermethrin; and Liang et al. (2008), for O-O-dimethyl organophosphorus pesticide. In contrast, no significant variations on the effect of ionic strength were observed for microcystin-LR ELISA (Long et al., 2009).
Figure 5.7. Effect of concentration of salt on the ELISA for monensin. 10X salt reduced the $A_{\text{max}}$ and IC$_{50}$ values of the ELISA. Means sharing different superscript are significantly different from each other ($p<0.05$). Values represent mean ± SD of three replicates.

5.4.3 Cross reactivity (CR)

The specificity of the rabbit polyclonal antiserum was evaluated by performing CI-ELISA with compounds structurally related to monensin, i.e., narasin, nigericin, maduramicin, and lasalocid A. The IC$_{50}$ values and % CR of each compound tested are shown in Table 5.3. The polyclonal antiserum had little or no cross reactivity with any of the compounds tested except nigericin (CR of 7.6%). These related compounds are often found along with monensin in waste water. Hence, our monensin ELISA can be used to specifically test for monensin.
Table 5.3. Specificity of anti-monensin-BSA polyclonal antiserum to monensin and structurally related compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>MW (Da)</th>
<th>Structure</th>
<th>IC$_{50}$ (ng mL$^{-1}$)</th>
<th>% CR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monensin</td>
<td>671</td>
<td><img src="image" alt="Monensin structure" /></td>
<td>1.2</td>
<td>100</td>
</tr>
<tr>
<td>Narasin</td>
<td>765</td>
<td><img src="image" alt="Narasin structure" /></td>
<td>1000</td>
<td>0.1</td>
</tr>
<tr>
<td>Nigericin</td>
<td>725</td>
<td><img src="image" alt="Nigericin structure" /></td>
<td>15.8</td>
<td>7.6</td>
</tr>
<tr>
<td>Maduramicin</td>
<td>917</td>
<td><img src="image" alt="Maduramicin structure" /></td>
<td>109</td>
<td>1.1</td>
</tr>
<tr>
<td>Lasalocid A</td>
<td>591</td>
<td><img src="image" alt="Lasalocid A structure" /></td>
<td>Nil</td>
<td>0</td>
</tr>
</tbody>
</table>
5.4.4 Assay precision

Precision of a method is defined as the closeness of agreement between independent test results obtained under prescribed conditions, that can be measured quantitatively (Environmental Protection Agency, 2009; Shankar et al., 2008). It is usually expressed as a relative standard deviation (RSD) or the percentage of coefficient variation (CV) of the determined concentrations among the replicates; it is calculated as:

\[
\% \text{CV} = \frac{\text{standard deviation}}{\text{mean}} \times 100.
\]

The reproducibility and assay precision of the CI-ELISA were determined using monensin standards on three different plates (intraassay) on three different days (interassay) as determined by two different analysts. Water samples from Guelph Lake were fortified with monensin at 0, 0.01, 0.3, 300, 3000, and 10,000 ng mL\(^{-1}\).

The reproducibility and assay precision data are presented in Table 5.4. The intraassay and interassay variations (CV) ranged from 0.75 to 4.77\% and 2.16 to 10.78\%, respectively. Both intraassay and interassay CV were within the acceptable range of ± 20\% proposed by Krotzky and Zeeh (1995). A higher acceptance limit of ± 25\% has been recommended for ELISAs (Braggio et al., 1996; Findlay and Das, 1998), presumably because immunoassays are subject to more sources of error. In addition, standard curves of immunoassays are based on non-linear regression as opposed to linear regression, which is used for conventional analytical methods (Braggio et al., 1996). With % CV or RSD less than 20\%, the monensin ELISA protocol which we developed is considered robust. It was also observed that the highest CV was due to difference between analysts. For quantitative measurement in ELISA, it is crucial that the design of validation experiments minimize the confounding effects due to analysts, assay runs, lots or batches etc. without affecting other important parameters such as incubation time, temperature, concentration, pH, ionic strength, matrix effects etc. (Shankar et al., 2008).
Table 5.4. Intraassay and interassay precision for estimation of concentration of monensin standards in fortified lake water\(^a\)

<table>
<thead>
<tr>
<th>Monensin (ng mL(^{-1}))</th>
<th>Intraassay variation</th>
<th>Interassay variation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>0</td>
<td>1.571</td>
<td>0.042</td>
</tr>
<tr>
<td>0.01</td>
<td>1.458</td>
<td>0.017</td>
</tr>
<tr>
<td>0.3</td>
<td>0.917</td>
<td>0.009</td>
</tr>
<tr>
<td>300</td>
<td>0.560</td>
<td>0.027</td>
</tr>
<tr>
<td>3,000</td>
<td>0.525</td>
<td>0.012</td>
</tr>
<tr>
<td>10,000</td>
<td>0.496</td>
<td>0.004</td>
</tr>
</tbody>
</table>

\(^a\)Data are mean absorbance values determined on three different plates (intraassay) on three different days by two different analysts (interassay).

5.4.5 Competitive direct ELISA

The sensitivity obtained with competitive direct ELISAs in comparison to competitive indirect ELISAs is often debated. For example, detection of permethrin, an insecticide, was found to be more sensitive using a competitive direct ELISA (Skerritt et al., 1992). In contrast, competitive indirect ELISAs have been found to be more sensitive for the detection of metachlor, a herbicide, in soil samples (Schlaeppi et al., 1991) and imidacloprid, an insecticide, in agricultural and environmental matrices (Lee et al., 2001). With this in mind, we evaluated and compared both formats for the detection of monensin. The use of each format for the analysis of real water samples is further discussed in Section 5.4.6.

For competitive direct ELISA, we first attempted to coat the plate with monensin antisera; however, this resulted in low color development. This could be due to the random orientation of antibodies on the plate, which may have limited the availability of the variable regions for antibody-hapten interaction. Alternatively, the low binding could have been caused by the denaturation of the antibody upon passive adsorption
In fact, 90% of monoclonal antibodies and 75% of polyclonal antibodies denature upon passive adsorption (Butler et al., 1993). Incorporation of protein A pre-coating on the plate surface to bind to the Fc region of IgG has been shown to improve color development (Deschamps and Hall, 1991; Johnson and Hall, 1996). Conversely, previous reports have shown the successful quantification of atrazine in soil and water by directly immobilizing antibodies to microtiter plates without protein A pre-coating (Wittmann and Hock, 1989; 1991). We found that directly coating the wells with purified IgG, without protein A, and subsequent addition of monensin-HRP produced a high ELISA signal. We then optimized this scheme for the competitive direct assay. However, we did not compare the results with protein A pre-coated microtiter plates.

The standard curve for monensin was optimized by coating microtiter plates with different concentrations of protein G purified IgGs (1, 2.5, 5, and 10 µg mL\(^{-1}\)) and by performing a competitive direct assay. For all four coating concentrations, it was observed that the addition of 100 ng mL\(^{-1}\) of free monensin fully inhibited the binding of antibody to monensin-HRP (i.e., % inhibition almost 100%) and as the concentration of coating IgGs increased so did \(A_{\text{max}}\) (Figure 5.8). An IgG concentration of 5 µg mL\(^{-1}\) resulted in lowest IC\(_{50}\) (1.128 ng mL\(^{-1}\)), produced a good ELISA signal (i.e., \(A_{\text{max}}\) above 0.8), and had a linear range from 0.1 to 10 ng mL\(^{-1}\). Therefore, this concentration was used for further experiments to quantify monensin in fortified water samples.

The characteristics of standard or competitive curves are greatly affected by the different assay formats employed. When a competitive direct and competitive indirect ELISA were compared, the competitive direct had a steeper slope with higher % inhibition (above 90% vs. 80%) at lower concentrations of monensin (i.e., at 100 vs. > 1000 ng mL\(^{-1}\)) (Figure 5.8 vs. Figure 5.5). In addition, a competitive direct ELISA format is more advantageous than the competitive indirect because it requires less time per
assay and the setup is simpler because it does not require the use of a secondary antibody. However, since the HRP enzyme is directly attached to the analyte there is one less step in assay, consequently there are fewer parameters to manipulate to improve assay sensitivity. Thus, we did not observe any significant improvement (p>0.05) in sensitivity for monensin immunoassay by employing the direct ELISA format as opposed to the indirect ELISA format (compare Figure 5.2; competitive indirect ELISA vs. Figure 5.8; competitive direct ELISA).
**Figure 5.8.** Effect of IgG coating concentrations on a monensin competitive direct ELISA. $A_{\text{max}}$ values increased as IgG coating concentrations increased. The assay was most sensitive when 2.5 and 5 µg mL$^{-1}$ of IgGs were used for coatings. Means sharing different superscript are significantly different from each other ($p<0.05$). Values represent mean ± SD of three replicates.
In a separate experiment, an attempt was made to isolate the IgG fraction with monensin activity by cross-linking monensin to an affinity column using the Carboxylink immobilizing kit (Thermo Scientific, Nepean, Canada). Thereafter, we compared the functionality of IgGs, purified either using protein G or the monensin affinity column, by direct competitive ELISA. Proteins concentrations eluted from the affinity column were calculated using Bradford assay. The elutions and washes were also subjected to SDS-PAGE and Western blotting. Protein concentrations were higher in the elutions than in washes. The concentration of monensin-specific IgG obtained from the affinity column was ca. 90% less than the concentration of IgG purified using the Protein G column. SDS-PAGE (reducing conditions) and Western blotting showed two distinctive heavy and light chains bands (data not shown). Competitive direct ELISA using affinity purified IgG did not yield a good inhibition curve suggesting that the affinity purification was not successful (data not shown). A possible explanation for this might be that the high organic solvent (at 50%) applied to fully dissolve monensin may have interfered with the stability of the resin binding to monensin. We used 2 mg mL\(^{-1}\) of monensin in 50% of organic solvent and prepared the affinity column by gradually increasing the organic solvent from 5% to 50%.

5.4.6 Chemiluminescence ELISA

Chemiluminescence and fluorescence assays, as part of a signal amplification system, provide excellent sensitivity for the detection of analyte in different matrices. Choosing a sensitive detection system may allow one to enhance the sensitivity of the assay by lowering the concentrations of coating conjugate bound to the plate. This in turn allows the concentrations of free analyte in the sample to be lowered thus providing an increase in assay sensitivity. Many have reported improvement of ELISA sensitivity by using chemiluminescence for the detection of pesticides and antibiotics in various
sample matrices. For example, detection limits of acrylamide in food products (Quan et al., 2011), chloramphenicol in chicken muscle (Zhang et al., 2006), 3-phenoxybenzoic acid in urine (Ahn et al., 2007), *Clostridium botulinum* neurotoxin (Guglielmo-Viret et al., 2005), the herbicide triclopyr in drinking water (Díaz et al., 2012), 2,4-dichlorophenoxyacetic acid (2,4-D) in water (Boro et al., 2011), ochratoxin A in green coffee (Sauceda-Friebe et al., 2011) and microcystin-LR in water samples (Long et al., 2009), all were improved using chemiluminescence assays.

To improve the sensitivity of our monensin immunoassay, a competitive indirect chemiluminescent ELISA (CICL-ELISA) was developed and its applicability for real sample analyses was evaluated. We hypothesized that lowering the concentration of coating conjugate would result in a more sensitive immunoassay provided the signal was sufficient for the range of concentrations of free monensin to be determined. By doing a checkerboard ELISA, we determined the optimal dilution for monensin-OVA (i.e., the coating conjugate) as 1/16,000 (v/v) and for antiserum (rabbit anti-monensin-BSA from final bleed) as 1/6400 (v/v). In addition, increasing the dilution of GaR-HRP, the secondary antibody, from 1/40,000 to 1/160,000 (v/v) resulted in reduction of IC$_{50}$ from 0.960 to 0.545 ng mL$^{-1}$ (Figure 5.9), thus, improving the sensitivity of the assay. However, further dilutions of GaR-HRP to 1/320,000 (v/v) did not improve the sensitivity of the assay.

The chemiluminescent system is a simple method and the only change required from the standard ELISA protocol was the use of a different substrate. Although this system is very sensitive for the detection of analyte, we found it to result in high background and high variation between wells. In addition, cross talk readings between adjacent wells were also observed. We tried several approaches to minimize the background. For example, we compared the effect on the background signal of two
schemes: GaR-HRP versus GaR-biotin followed by probing with neutravidin-HRP. It was found that using GaR-biotin and neutravidin-HRP increased the background signal.

Various negative controls were also used to evaluate which component of the CICL-ELISA was the main contributor to the increased background signal. It was observed that background signal was at its lowest when the secondary antibody (GaR-HRP) was used as control without the addition of rabbit antiserum. Hence, rabbit antiserum was the main contributor to the high background signal. This is likely because other proteins e.g. albumin in the serum, may bind non-specifically to the microtiter plate surface. In addition, high concentrations of neutravidin-HRP bound non-specifically to the plate. Furthermore, incubation of antiserum for longer than 30 min, i.e., for 1 h and 1.5 h, resulted in higher background. Therefore, an incubation time of 30 min was used in these experiments. As sensitivity of ELISA is very much affected by proper blocking, the effect of several common blocking agents was evaluated and included 1% casein, 4% skimmed milk in PBS, a proprietary blocking solution from CANDOR Biosciences GmbH (Wangen, Germany) and Superblock from Pierce (Thermo Scientific, Nepean, Canada). Plates were blocked and ELISAs were performed according to standard procedures. It was found that of the five common blocking agents tested, Superblock provided the lowest background (data not shown). Nevertheless, since we were not able to decrease the background signal substantially, the CICL-ELISA was not used to evaluate water samples.
Figure 5.9. Competitive indirect chemiluminescent ELISA (CICL-ELISA) using four different concentrations of the secondary antibody goat anti-rabbit horseradish peroxidase (GaR-HRP), i.e., (A) 1/40,000; (B) 1/80,000; (C) 1/160,000 and (D) 1/320,000. Greater dilutions of GaR-HRP resulted in a trend towards higher sensitivity; however, the IC₅₀ values were not statistically different (F-test; \( p > 0.05 \)). The IC₅₀ for the inhibition curve using GaR-HRP at 1/320,000 could not be determined (nd) as the data did not fit the 4PL model. CLCI-ELISA were performed as described in Section 5.3.7 using monensin-OVA as coating conjugate at 1/16,000 (v/v) and antiserum at 1/6,400 (v/v) dilutions. Each point represents the mean ± SD of three replicates.
5.4.7 Comparison of ELISA to LC-MS analyses

To study the recovery of monensin from fortified water, samples were collected from Speed River (Guelph, Ontario) and Guelph Lake (Guelph, Ontario); tap water and nanopure water were obtained from the Hall laboratory. All samples were spiked with five different concentrations of monensin (0.1, 0.5, 1, 5, and 10 ng mL\(^{-1}\); \(n=3\)) and analyzed using the competitive direct and competitive indirect ELISAs. For LC-MS analyses, salinomycin (5 ng mL\(^{-1}\)) was used as an internal standard and analytes were extracted from the water samples using Oasis HLB SPE cartridges as described in Section 5.3.9.

The correlation between monensin values determined from four types of water using LC-MS versus the direct and indirect competitive ELISAs are shown in Table 5.5 and Figure 5.10. As indicated by the slopes (higher than 1) in Figure 5.10, quantification by indirect competitive ELISA was slightly greater relative to the LC-MS method. This is likely due to the matrix effect, which is more significant in ELISA than LC-MS. Since a SPE cartridge was used prior to LC-MS analyses the matrix effects were reduced. For competitive direct ELISA format, the slopes for most water samples were lower than 1 indicating that this format slightly underestimated monensin concentrations as compared to LC-MS method.

For fortified monensin samples at 0.1, 0.5, 1, 5, and 10 ng mL\(^{-1}\), recoveries were in the range of 50-157 %, 133-153 %, 80-178 %, 118-127 % and 104-112%, respectively, for the indirect ELISA; while for the direct ELISA, the recoveries ranged from 50-130 %, 78-154 %, 76-183 %, 101-146 % and 55-90 %, respectively. For LC-MS analyses, all recoveries were in the acceptable range of 80-120% (Brown, 2011) except for the 0.1 ng mL\(^{-1}\) concentration of monensin for nanopure and lake water, which had negative values. The inconsistency of the results could be due to monensin concentration, which was below the detection limit.
A good correlation was obtained between competitive indirect ELISA and LC-MS measurements for monensin for all four water types with \( r^2 = 0.998, 0.966, 0.997, \) and 0.998 for nanopure, tap, river and lake water, respectively. It was also observed that direct competitive ELISA showed a good correlation with LC-MS with \( r^2 = 0.930, 0.997, 0.823, \) and 0.979 for the same four water samples. These results indicate that the immunoassays we developed were suitable for the detection of monensin in water samples from different sources.
Table 5.5. Recovery of monensin from four types of fortified water samples as measured by competitive direct and indirect ELISA and LC-MS

| Sample         | Fortified (ng mL\(^{-1}\)) | Indirect ELISA |  | Direct ELISA |  | LC-MS |  |
|----------------|-----------------------------|----------------|-----------------------------|----------------|-----------------------------|
|                | Detected (ng mL\(^{-1}\)) | Recovery (%)   | CV (%)                     | Detected (ng mL\(^{-1}\)) | Recovery (%) | CV (%)          | Detected (ng mL\(^{-1}\)) | Recovery (%) | CV (%) |
| Nanopure       | 0.1                         | 0.13           | 130.9                      | 46.1                        | 0.13          | 130.0          | 48.2                        | -0.45     | -452.0 | -83.8 |
| water          | 0.5                         | 0.67           | 133.3                      | 76.9                        | 0.39          | 78.0           | 68.1                        | 0.45      | 89.5   | 21.2  |
|                | 1                           | 1.78           | 178.0                      | 98.3                        | 1.03          | 103.0          | 27.2                        | 1.45      | 145.3  | 26.6  |
|                | 5                           | 5.89           | 117.7                      | 60.1                        | 7.30          | 146.0          | 7.9                         | 5.08      | 101.5  | 4.3   |
|                | 10                          | 10.39          | 103.9                      | 60.8                        | 9.02          | 90.2           | 1.8                         | 9.52      | 95.2   | 6.8   |
| Tap water      | 0.1                         | 0.05           | 50.0                       | 38.5                        | 0.14          | 140            | 16.4                        | 0.10      | 100.9  | 68.8  |
|                | 0.5                         | 0.72           | 144.0                      | 40.5                        | 0.77          | 154            | 22.1                        | 0.60      | 120.2  | 8.6   |
|                | 1                           | 0.8            | 80.0                       | 12.1                        | 1.83          | 183            | 6.8                         | 1.18      | 117.9  | 20.7  |
|                | 5                           | 6.1            | 122.0                      | 13.8                        | 7.14          | 142.8          | 17.9                        | 4.96      | 99.2   | 1.4   |
|                | 10                          | 11.2           | 112.0                      | 4.9                         | 9.03          | 90.3           | 9.9                         | 6.75      | 67.5   | 6.9   |
| River water    | 0.1                         | 0.16           | 157.2                      | 23.9                        | 0.05          | 50.0           | 1.6                         | 0.10      | 102.0  | 66.5  |
|                | 0.5                         | 0.72           | 144.0                      | 56.1                        | 0.50          | 100.0          | 12.3                        | 0.54      | 108.3  | 10.8  |
|                | 1                           | 1.69           | 169.0                      | 31.0                        | 0.76          | 76.0           | 8.9                         | 0.95      | 94.7   | 2.5   |
|                | 5                           | 5.97           | 119.4                      | 5.8                         | 6.22          | 124.4          | 1.5                         | 4.70      | 93.9   | 9.5   |
|                | 10                          | 10.81          | 108.1                      | 17.5                        | 5.52          | 55.2           | 7.4                         | 8.01      | 80.1   | 31.6  |
| Lake water     | 0.1                         | 0.13           | 125.5                      | 55.7                        | 0.08          | 80.0           | 48.6                        | -0.19     | -193.3 | -26.0 |
|                | 0.5                         | 0.77           | 153.5                      | 62.5                        | 0.48          | 96.0           | 4.9                         | 0.51      | 101.9  | 27.6  |
|                | 1                           | 1.26           | 126.0                      | 53.2                        | 0.76          | 76.0           | 11.8                        | 1.25      | 124.6  | 18.7  |
|                | 5                           | 6.37           | 127.4                      | 20.7                        | 5.08          | 101.6          | 2.8                         | 5.63      | 112.6  | 11.8  |
|                | 10                          | 10.37          | 103.7                      | 17.9                        | 6.95          | 69.5           | 8.1                         | 9.71      | 97.1   | 3.1   |
Figure 5.10. Correlation of monensin concentrations from four types of fortified water samples as measured by (A) competitive direct ELISA, (B) competitive indirect ELISA versus LC-MS.
5.5 Conclusion

A homologous system utilizing the same hapten (i.e., monensin) as the immunogen and plate coating was used in this study. BSA and OVA were conjugated to monensin through its carboxyl groups using the Fleeker method. We developed a sensitive monensin CI-ELISA, with IC$_{50}$ values of 1.056 - 1.090 ng mL$^{-1}$ and limit of detection of 0.1 ng mL$^{-1}$ using antiserum from the terminal bleed of a rabbit. Our results showed that the polyclonal antiserum was specific to monensin having minimal cross reactivity to other coccidiostats tested, i.e., with the highest cross reactivity was 7.6% for nigericin.

Various factors, which may affect the sensitivity of an immunoassay, were evaluated and presented. The assay performed best at 22 °C with a 1-h incubation time with both monensin-OVA and antiserum concentrations of 1/45,000 (v/v). Pre-incubation (competitive inhibition ELISA) did not improve the sensitivity as indicated by an increased IC$_{50}$ and a decreased A$_{max}$. Addition of both acetonitrile and methanol (0 to 10%) did not affect the immunoassay curve. However, addition of both solvents (0-10%) did reduce the A$_{max}$ values. Addition of Tween 20 at 0.05% increased both the A$_{max}$ and IC$_{50}$ of the assay. Ionic strength of the assay buffer decreased the A$_{max}$ but did not significantly affect the IC$_{50}$ values, except for 10X PBS. The assay was found to be stable within a pH range of 5 to 7.4. Intraassay and interassay CVs were less than 10%.

CI-ELISA was compared to competitive direct ELISA by coating the microtiter plate with protein G purified IgG from rabbit antiserum. Competition was performed by incubating monensin standards and horseradish peroxidase conjugated monensin simultaneously. The competitive direct ELISA format had an IC$_{50}$ of 1.128 ng mL$^{-1}$ with a linear range from 0.1 to 10 ng mL$^{-1}$ using a coating concentration of IgG at 5 µg mL$^{-1}$. Competitive direct ELISA did not significantly improve the assay sensitivity. A CICL-ELISA format using luminol as the HRP substrate was also compared to the CI-ELISA.
The CLCI-ELISA had a higher sensitivity for monensin but was too variable because of a background signal. Recovery of monensin in fortified water samples ranged from 50 to 178% using competitive indirect ELISA and 50 to 146% using competitive direct ELISA. Good correlation ($r^2 = 0.823$ to 0.998) was exhibited when the competitive indirect and direct ELISAs were compared to the LC-MS.

In conclusion, our monensin polyclonal antibody-based assay can be used to detect monensin in water samples with minimal sample treatment. The direct and indirect assays were comparable to LC-MS. At least, this assay could be used as screening method for determining monensin in water samples before confirmation by LC-MS.
CONCLUSION AND FUTURE RECOMMENDATIONS

This thesis describes the process of developing immunoassays for two hydrophobic haptens and the challenges encountered. Optimization of assay parameters and validation using real water samples was also described.

Chapter 3 reports on the development of immunoassays using polyclonal sera for the detection of 2-methylisoborneol (MIB), a secondary metabolite produced by cyanobacteria and actinomycetes causing off-flavor and off-odor episodes in food and water. A heterologous format was employed to develop this assay where (-) camphor and (-) borneol, compounds, which are structurally related to MIB, were used as immunogen, while (+) bornylamine and MIB were used to create the plate coatings after conjugation to proteins (e.g. BSA, TG). Through this approach, the optimized assay had a detection limit of 4.8 ng mL\(^{-1}\) and an IC\(_{50}\) of 105 ng mL\(^{-1}\). When the pAb assay with a heterologous format using (-) camphor-BSA as the immunogen was developed, it was found that the assay recognized (-) camphor better than MIB when MIB-TG was used as coating conjugate, hence the pAb assay did not achieve the desired sensitivity. Since human odor threshold for MIB is at part-per-trillion (ppt) level, our main focus was to achieve this level of sensitivity to make the assay useful.

Many successful immunoassays have been previously developed and reported in the literature using monoclonal antibodies against haptens. To improve the sensitivity and specificity of the antibodies to MIB, hybridomas were produced by immunizing BALB/c mice with (-) camphor-BSA as immunogen. One of the fourteen mAb clones was characterized. The mAb clone (i.e., clone 4F11) did not show a significant improvement in sensitivity or specificity to MIB as compared to the pAb-based CI-ELISA. Test of Ab and coating conjugate concentrations showed that sensitivity of ELISA could be improved by lowering concentrations of Ab and coating conjugate; however, it was not possible to reproduce the clone to maintain good affinity.
The development of immunoassay for the second hydrophobic hapten, monensin, an ionophore antibiotic used to treat coccidiosis in poultry, beef and dairy industries (Butaye et al., 2003; Matsuoka et al., 1996) is described in Chapter 5. Previous work, which was initially done in the Hall lab by Makvandi-Nejad et al. (2010), produced several good binders using recombinant antibody technology. Taking advantage of the availability of quality polyclonal serum (pAb) to monensin from Makvandi-Nejad’s research, a study was undertaken to evaluate and compare different assay formats in terms of their application and reliability for quantification of monensin in real samples. In contrast to the heterologous hapten system used in MIB assay, a homologous system was used to develop ELISAs for monensin.

Both the pAb-based ELISAs were best when performed at 22°C for 1.5 h and 1 h, for MIB and monensin, respectively. The ELISAs were shown to be stable at low salt concentration, absence of detergent, less than 10% organic solvents and at pH close to 7. Both ELISAs also showed good intra- and interassay precision and correlated well with analytical instrument-based methods when fortified water from different sources were used.

For the next objective, we attempted to improve the sensitivity of the monensin ELISA through a signal amplification system. By using the HRP substrate luminol, a chemiluminescence-based CI-ELISA (known as CICL-ELSA) was created. The chemiluminescent method was found to improve the sensitivity of the ELISA for monensin; however, the system suffered from a large background signal and was not further used. Two different assay formats, i.e., competitive direct and competitive indirect, were then compared for detection of monensin in different type of waters. Although the competitive direct assay format was faster, simpler and had one less step, it was found that this format did not appreciably improve the sensitivity of the ELISA for monensin.
On the basis of the previous work, future work could involve a diverse hapten heterology approach by comparing the same hapten and different system used for immunogen and coating conjugates directly. Other immunogens and coating conjugates that are structurally related to MIB with different hapten to protein ratios, different carrier proteins and/or addition of linker arms should also be prepared and tested. This would allow the best combination of these molecules which may produce a sensitive assay for MIB to be found. Subsequently, production of monoclonal antibodies can be performed based on the outcomes of the pAb work since it is not practical to produce hybridomas using many different immunogens.

Further work on the immunoassay optimizations can be addressed. For example, minimization or elimination of matrix effects is necessary to convert the assays from semi-quantitative to being quantitative. Thus, more complex matrices such as food samples should also be tested. Non-specific binding (NSB) is always a major drawback and challenge in immunoassay development and is detrimental to its sensitivity and specificity. Further work can be performed to reduce or minimize the background or NSB for example by selecting a more appropriate blocking system, optimization of washing steps and testing different plate materials. The experimental design for assay optimization could also be improved to produce more accurate results in less time. This approach is particularly applicable for immunoassays that involve a multiplicity of interactions between reagent components, environmental conditions and other factors.

Improvement of assay sensitivity may also be achieved through affinity purification of specific antibody against MIB or monensin from the polyclonal antisera. This can be done through monensin or MIB attachment to an affinity support through covalent immobilization. Activated resins for preparation of immobilized hapten via a variety of chemistries are commercially available (Pierce, Thermo Scientific).
Adaptation of a different reporter system may also assist in improvement of sensitivity of these immunoassays. One of these systems is called immuno-PCR, a method that combines the specificity and versatility of ELISA and the amplification power of PCR. First described by Sano in 1992, this method has been shown to increase the detection limit of ELISA by 100- to 1000-fold (Niemeyer et al., 2005; Malou and Raoult, 2011). Nevertheless, as with other highly sensitive detection systems, it often comes with a formidable challenge of background issues leading to false positives. When this has been addressed and solved, immuno-PCR offers a promising future for detection of low amounts of antigens.

Recombinant antibody engineering has been developing rapidly and has found its application in food and environmental diagnostics (Alcocer et al., 2000; Brichta et al., 2005; Cho et al., 2008; Daly et al., 2002; Dunne et al., 2005; Grennan et al., 2003; Moghaddam et al., 2001). It offers advantages over pAb and mAb through the use of antibody libraries, thus by-passing the need for immunization. Furthermore, antibodies and their binding fragment (scFv, Fv, Fab) can be manipulated in vitro and tailored to match specific needs (Kramer and Hock, 2003; Lee and Kennedy, 2007). No rAb for MIB has been reported to date. rAb for monensin has been developed by Makvandi-Nejad et al. (2010) with scFv clones having lowest IC_{50} of 0.03 µM (ca. 20 ng mL^{-1}). Improvement of antibody affinities through affinity maturation and mutagenesis, for example through chain shuffling (Cramer et al., 1996; Figini et al., 1994; Marks et al., 1992), error prone PCR (Hawkins et al., 1992), DNA shuffling (Cramer et al., 1996), propagation of phage in mutator strains of Escherichia coli (Low et al., 1996) or by using combinatorial libraries (Rajpal et al., 2005) is possible. Large increases (up to 300-fold) in affinity for hapten using these approaches has been shown (Low et al., 1996; Marks et al., 1992).
Most food and environmental contaminants are small molecular weight compounds or haptens. Consequently, the production of high affinity antibodies for haptens, especially of the hydrophobic type is more challenging. However, despite the daunting and arduous process, many have reported the successful development of immunoassays for hydrophobic haptens with exceptional sensitivity and specificity (Brun et al., 2005; 2008; Matschulat et al., 2005; Spier et al., 2009). Immunoassay, which is based on the supreme specificity of an antibody towards its antigen, will continue to expand in diagnostics industry. Although it may take from several months to many years to develop one immunoassay, the advantage of having a technology, which is cheap, simple and rapid surpass its shortcomings in providing a way to determine and quantify emerging contaminants in food and environment.


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Figure A1. Matrix assisted desorption ionization (MALDI) spectrums for (A) BSA; (B) (-) borneol-BSA and (C) (-) camphor-BSA. Molar ratio of conjugates were calculated as 5:1 and 18:1 for (-) borneol-BSA and (-) camphor-BSA, respectively.
Figure A2. Titer of polyclonal sera (bleed 5; day 155) from rabbits immunized with (-) borneol-BSA (BR1 and BR2) and (-) camphor-BSA (CR1 and CR2) as compared to pre-immune bleed (day 0)
**APPENDIX III**

![Inhibition curve](image)

**IC\textsubscript{50} = 105 ng/mL**

**slope = -0.518**

**R\textsuperscript{2} = 0.989**

**Figure A3.** Inhibition curve for antisera from rabbit anti-(\(-\)) camphor-BSA (CR1) against MIB-TG as coating conjugates. MIB-TG and anti-sera from CR1 were used at 1/27,000 and 1/54,000 (v/v) dilutions, respectively. The inhibition curve has an IC\textsubscript{50} of 105 ng mL\(^{-1}\), LOD of 4.8 ng mL\(^{-1}\) and LOQ of 13.2 ng mL\(^{-1}\). Data are means ± SD of three replicates.
APPENDIX IV

Table A4. Two-dimensional CI-ELISA template for optimization of coating conjugate and antisera dilutions in minimizing IC\textsubscript{50} values (adapted from Cervino et al., 2008). Monensin-OVA as coating conjugate and antisera dilutions were added vertically according to the respective concentrations in column 1 to 10. Monensin standards were added horizontally in row A to H accordingly. ELISA was performed as described in Section 5.3.4

<table>
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<tr>
<th>Monensin-OVA concentrations</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
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<tr>
<td>Monensin (ng mL\textsuperscript{-1})</td>
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<td>1/3,000</td>
<td>1/1,000</td>
<td>1/3,000</td>
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<tr>
<td>Antisera dilutions</td>
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<td>1/1,000</td>
<td>1/3,000</td>
<td>1/3,000</td>
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<td>1/9,000</td>
<td>1/27,000</td>
<td>1/27,000</td>
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</table>
APPENDIX V

Figure A5. MALDI-TOF spectrums of monensin conjugates used as immunogen in ELISA. Monensin was conjugated to BSA as described in Section 5.3.2. (A) BSA and the resulting conjugate; (B) monensin-BSA peaks are shown above. Monensin to BSA ratio was calculated as ~5:1.