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DEVELOPMENT OF CHICKEN POLYCLONAL AND MOUSE MONOCLONAL-BASED ENZYME IMMUNOASSAYS FOR THE DETECTION OF β-CYCLOCITRAL IN CATFISH POND WATER

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Food Science

by

Cate Nakaweesa Munene
B.Sc. Makerere University, Uganda, 1998
M.S. Louisiana State University, 2000
December, 2004
DEDICATION

To my Lord Jesus Christ who has been my shield, my strength, my portion, my deliverer, my strong tower, my miracle and my very present help in time of need. Thank you my Lord for giving me a hope beyond hopes, for seeing me through this dissertation and everything so miraculously, for placing your angels along the way that my way may be smooth, for blessing me so abundantly and most of all for drawing me to yourself.
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My sincere and deepest appreciation goes to my major Professor Dr. Jack N. Losso. I wanted to do immunochemistry, he gave me a chance to do immunochemistry and provided all the resources as well as the guidance through out this project. I am grateful for all that I have learned from him. I did the organic chemistry work in Dr. Hammer’s laboratory (Department of Chemistry) under his supervision, this period was one of my greatest learning experiences and I am so grateful to Dr. Hammer for his guidance and support and making the working environment in his lab a wonderful one. I wish to especially acknowledge the contribution of Dr. Guifa Su and Dr. Serhi Pakhomiv. Dr. Pakhomiv translated my German reference and offered vital advice through out the project. Dr. Su put aside all his work, taught me my first practical organic chemistry, helped and guided me through the synthesis of the initial and most critical product. Dr. Su was always there to offer advice through out the entire project, I can’t thank him enough. My sincere appreciation goes to all the members of the Hammer group especially Arsham Sheybani who worked very long hours helping out with the experiments, especially I could not have managed without his expertise in setting up chromatography columns.

Most of the immunochemistry work was done in Dr. Truax laboratory in School of Veterinary Medicine. Dr. Truax did not only guide me, he helped me out, he immunized the mice, taught me the tough and tricky immunochemistry science. Through out this period Dr. Truax worked with a backache, this was such a sacrifice and I pray that the God Almighty will heal him completely of this back problem because he is indeed a valuable resource. He even let me use his multi-tip pipette through out the entire
study. This made such a difference and made my life a lot easier. I am so grateful for the contribution of Dr. Truax. The last part of my research was done in Dr. Grimm’s laboratory (USDA, New Orleans), with his help and support. His guidance was so crucial to this project that I can’t thank him enough and I look forward to working with him to refine the developed assays.

When I started venturing into my dissertation project, I worked with Dr. Portier in his laboratory. He guided me, made the working environment so favorable and his sense of humor always made a difference. Through this program, I have been blessed with a great team, my committee has been wonderful, I would not have asked for any more. Dr. Godber, has been so valuable throughout. When things got tough, he was there to listen and advise. This achievement would have been impossible without his support and constructive criticism and technical guidance. I am indeed indebted to him for his very essential contribution. Through this period I have not only been blessed with a dream committee, but with so many other angels that God has placed in my way to make my work a lot easier. I have run into Dr. Dale Treleaven, when there is a challenge and he has offered wonderful suggestions that enabled me to move forward in my research. I also acknowledge the valuable contribution of Mr. Frank Canfield; his editing and constructive criticism was very essential to this work.

Mr. Gary Foster of USDA-ARS willingly and happily supplied the enormous amount of ultra pure water that I needed through this project. Amanda Pittman did not only help with the immunization of hens but she became a wonderful friend, who checked on me and encouraged me. My colleagues, Rishipal, who has made the working environment in our lab very fun to be in and was always more than willing to lend a
helping hand. Sonja, who has encouraged me and physically helped with lab work and watched out for me and truly cared and made the lovely life of a Ph. D. student more bearable, I am so grateful to God for sending Sonja into my life. Shreya and Sajida, who I called upon and they left all that they were doing to come and give me a helping hand. I have been so blessed with wonderful and supportive colleagues, Alfredo, Hee-jung, Aisha, somehow each has helped me and made my work a lot easier.

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For my husband, Daniel, all I can say is I could never have done this without his love and support. He became everything, my student worker in every aspect. I can’t thank him enough for all the time he invested in my life, for the nights he spent in the lab with me even the night he defended his dissertation. What has amazed me about Daniel is where he gets the energy to just keep going, even when I gave up, he could still go five
steps further. This man of God is one of God’s greatest gifts to me and I will forever praise God for bringing him into my life.

I am very grateful to my grandmother who raised me and instilled values in me. I am grateful for my Dad and Mum, for that soft side of Mummy that was always so encouraging and made life so much easier. An e-mail from Mummy always put a smile on my face. For Daddy, I thank God for your life. For encouraging me to keep going; when things got really tough, I kept going for Daddy’s sake. Your dedication to work has inspired me greatly and has indeed propelled me forward. Being there whenever I have needed you has made such a different and I count myself blessed to have a father like you. My sisters and brothers and cousins and nephews, you guys have contributed so much to this journey, reading from Jackie in the middle of a frustrating experiment was so refreshing. Kizito has forced me to mature because he has looked up to me sought my advise and I strive to be a good example and model for my brother.

All in all I have been blessed of God. God has been so good to me and has brought me from far and has provided all that I have needed to walk this journey. I praise my God, the father of my Lord Jesus Christ for showing me favor and I will live to glorify Him and sing of His mercy and faithfulness for He has been a good God.
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<th>Full Form</th>
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<tbody>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacillus Calmette-Guerin</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CDCl₃</td>
<td>Deuterated chloroform</td>
</tr>
<tr>
<td>CD₂Cl₂</td>
<td>Deuterated dichloro methane</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CH₂Cl₂</td>
<td>Dichloro methane</td>
</tr>
<tr>
<td>CLSA</td>
<td>Closed loop stripping analysis</td>
</tr>
<tr>
<td>CLS</td>
<td>Closed loop stripping</td>
</tr>
<tr>
<td>Da</td>
<td>Daltons</td>
</tr>
<tr>
<td>DMAP</td>
<td>Dimethylaminopyridine</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's Modified Eagle Medium</td>
</tr>
<tr>
<td>DMF</td>
<td>Dimethylformamide</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>DPPA</td>
<td>Diphenyl phosphoryl azide</td>
</tr>
<tr>
<td>DTNB</td>
<td>5,5'-Dithiobis (2-nitrobenzoic acid)</td>
</tr>
<tr>
<td>EDC</td>
<td>1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELIFA</td>
<td>Enzyme-linked Immunoflow Assay</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked Immunosorbent Assay</td>
</tr>
<tr>
<td>Et₃N</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>FAB-MS</td>
<td>Fast atom bombardment-Mass spectrometry</td>
</tr>
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<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GC-FID</td>
<td>Gas chromatography-Flame ionization detector</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas chromatography-Mass spectrometry</td>
</tr>
<tr>
<td>HAT</td>
<td>Hypoxanthine, aminopterin, thymidine</td>
</tr>
<tr>
<td>HCl</td>
<td>Hydrochloric acid</td>
</tr>
<tr>
<td>¹H NMR</td>
<td>Proton Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>HPRT</td>
<td>Hypoxanthine-guanine phosphoribosyl transferase</td>
</tr>
<tr>
<td>HT</td>
<td>Hypothanthine-Thymidine</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IgY</td>
<td>Immunoglobulin Y</td>
</tr>
<tr>
<td>KCl</td>
<td>Potassium chloride</td>
</tr>
<tr>
<td>KI</td>
<td>Potassium iodide</td>
</tr>
<tr>
<td>KLH</td>
<td>Keyhole limpet hemocyanin</td>
</tr>
<tr>
<td>MD-SE</td>
<td>Microwave distillation-Solid phase micro-extraction</td>
</tr>
<tr>
<td>MD-SPME</td>
<td>Microwave distillation-Solvent extraction</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>Magnesium sulfate</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MIB</td>
<td>2-Methylisorborneol</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
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<tr>
<td>Na₂CO₃</td>
<td>Sodium carbonate</td>
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<td>NaHCO₃</td>
<td>Sodium bicarbonate</td>
</tr>
<tr>
<td>NEAA</td>
<td>Non-essential amino acids</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>Ammonium chloride</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NH$_2$OH</td>
<td>Hydroxylamine hydrochloride</td>
</tr>
<tr>
<td>NHS</td>
<td>$N$-hydroxy-succinimide</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$</td>
<td>Ammonium sulfate</td>
</tr>
<tr>
<td>Na$_2$SO$_4$</td>
<td>Sodium sulfate</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PBST</td>
<td>Phosphate buffered saline with tween</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PMPI</td>
<td>$p$-Maleimidophenyl isocyanate</td>
</tr>
<tr>
<td>PNPP</td>
<td>$p$-Nitrophenyl phosphate</td>
</tr>
<tr>
<td>PPD</td>
<td>Purified protein derivative of tuberculin</td>
</tr>
<tr>
<td>P&amp;T-SE</td>
<td>Purge and trap-Solvent elution</td>
</tr>
<tr>
<td>R$_f$</td>
<td>Retention factor</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SATA</td>
<td>$N$-succinimidyl S-acetylthioacetate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SPDP</td>
<td>$N$-Succinimidyl 3-(2-pyridyldithio) propionate</td>
</tr>
<tr>
<td>Sulfo-NHS</td>
<td>Sulfo $N$-hydroxysuccinimide</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>USDA-SRRC</td>
<td>United States Department of Agriculture-Southern Regional Research Center</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet</td>
</tr>
<tr>
<td>WTP</td>
<td>Water treatment plant</td>
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ABSTRACT

The Catfish industry faces a problem of off-flavors due to odorous compounds produced by cyanobacteria and blue-green algae. β-Cyclocitral imparts a hay-woody odor to pond water and fish tissues. At present there are no reliable pond treatment methods available to control these off-flavors. To monitor the levels of this compound for quality control, rapid, sensitive and inexpensive methods are needed. The major goal of this study was to develop enzyme-linked immunoflow assays based on monoclonal and polyclonal antibodies that are specific and sensitive enough to detect β-cyclocitral in catfish pond water.

β-Cyclocitral-PPD conjugate was prepared and used to immunize two chickens and two mice for the production of polyclonal and monoclonal antibodies against β-cyclocitral respectively. Monospecific polyclonal antibodies were purified from the eggs laid by the immunized chickens, using affinity chromatography. For the production of the monoclonal antibodies, hybridoma cells were made by fusion of myeloma cells and spleen cells of the mice that showed high antibody titer and specificity. Hybridoma cells that secreted high affinity monoclonal antibodies were cloned by the limiting dilution method and at least 10 hybridoma cell lines positive for anti-β-cyclocitral antibodies were established. Immunochemical methods based on anti-β-cyclocitral IgY and IgG were developed. The two ELISAs based on IgY and IgG had a limit of detection of 1.0 ng/mL and respective I$_{50}$ values of 3.93 and 7.98 ng/mL. Two enzyme-linked-immunoflow (ELIFA) assays were developed based on the ELISAs. The ELIFAs were very easy to perform but were less sensitive than the ELISAs as shown by their I$_{50}$ of 46 μg/mL for the IgY-based ELIFA and 93 μg/mL for the IgG-based ELIFA. Further investigations are
required for the more efficient recovery of the monoclonal antibodies, to validate the
ELISAs, to improve the sensitivity of the ELIFAs and to determine the potential to adapt
the developed assays to a kit format for use by the catfish industry and water treatment
industries as well as other aquaculture industries.
CHAPTER I. INTRODUCTION

Environmentally-derived off-flavor is a major problem for the channel catfish (Ictalurus punctatus) industry, as a consequence adequate quality control to guarantee that off-flavor fish are not marketed is essential. Farm raised catfish are evaluated for the absence of off-flavors, detection of which causes rejection of the consignment. The rejected fish are returned and held in ponds for costly periods until the off-flavors are eliminated from their bodies (Schrader, 2003). This method, referred to as depuration is the most common purification method used in dealing with this problem and it requires fish producers to maintain the market-weight fish until the problem subsides. Delays in harvesting result in higher production costs. Schrader (2003) reported that off-flavor problems cost the catfish farming industry as much as $50 million in losses annually.

A variety of off-flavors have been encountered in pond-raised catfish but the most commonly experienced are musty, muddy, or earthy (Martin et al., 1990). The two compounds known to impart an earthy/musty taste and odor to water and fish are 2-methylisoborneol (MIB) and geosmin (Malleret et al., 2001), which are metabolites of actinomycetes and blue-green algae. Blooms of blue-green algae occur during the summer months and are usually followed by the appearance of the characteristic musty odor. But even after this odor disappears an unpalatable flavor persists in the water into the winter. This is probably due to other odor-contributing compounds, some of which have been reported as major excretion products of cyanobacteria and algae (Ikawa et al., 2001).

Some of the other terms used to describe off-flavors in catfish are: rotten plants, woody, moldy, weedy, rancid, stale and sewage ((Johnsen et al., 1987; Lovell, 1983; Martin and Suffet, 1992). The chemical identity and etiology of most of these off-flavors is not yet
known but off-flavors due to \( \beta \)-cyclocitrinal have been known to affect water supply systems (Jüttner, 1976; Slater and Blok, 1983, Suffet et al., 1999). \( \beta \)-Cyclocitrinal (2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde) is produced in large amounts by *Microcystis* species (Jüttner, 1976) and has various odors at different concentrations. Young et al. (1999) reported three different descriptors of \( \beta \)-cyclocitrinal; fresh grass, hay-woody and tobacco-like, at concentrations in the range of 0.5-80 \( \mu \text{g/l} \).

Catfish flavor descriptors that implicate \( \beta \)-cyclocitrinal are very commonly reported in literature. Martin and Suffet (1992) reported a hay character in fish taste. Weedy (plant-like, grassy and algae-like) and “moldy hay” flavors were also reported as dominant in some pond-cultured channel catfish (Lovell, 1983). Additionally, a decaying vegetation flavor (Chambers and Robel., 1993) and a commonly encountered off-flavor described as rotten plants, characterized by a sense of decaying and fermenting vegetable matter, are probably due to \( \beta \)-cyclocitrinal. Woody is yet another off-flavor that has been reported to undermine the acceptability of fish (Grimm et al., 2000).

The off-flavor compounds are rapidly absorbed from water into the lipid tissue of fish (Lloyd and Grimm, 1999). Concentrations as low as 0.01 ppb can impart off-flavors to a variety of food and water resources (Dionigi et al., 1993; Malleret et al., 2001) and even brief exposures to off-flavor metabolites can be problematic (Johnsen et al., 1996).

At present, there are no consistently reliable pond treatment methods available to control off-flavor and the hope of finding these methods does not appear to be an immediate reality, meanwhile the industry and researchers need more reliable methods for evaluating off-flavor (Lovell, 1985; Lloyd and Grimm, 1999). Sensory evaluation, the current analysis method of choice in the catfish industry, is subjective and may show large variations between
tasters and between samples. Individual panelists may perceive flavor intensity differently because of adaptation, fatigue, and variations in detection thresholds (Bett and Johnsen, 1996).

Gas Chromatographic (GC) methods have been developed and are available for the determination of off-flavor metabolites. GC-flame ionization detector (FID), GC-Mass Spectrometry (MS) and sensory GC coupled with different methods to isolate and concentrate trace organics in water, are being used to analyze for low levels of odorants in water supply systems (Malleret et al., 2001). The methods available for the extraction and concentration of off-flavor metabolites include: closed-loop stripping analysis (CLSA), solvent extraction, purge and trap-solvent elution (P&T-SE), microwave distillation-solvent extraction (MD-SE) and microwave distillation-solid phase micro-extraction (MD-SPME) (Grimm et al., 2000; Lloyd and Grimm, 1999). These methods are, however, labor intensive, expensive, require technical training, and extensive sample preparation, and cannot be used for routine analysis.

Regular monitoring of off-flavor problems would act as an early warning procedure permitting the farmers to implement preventative or corrective action in a timely manner and thus improve pond management. If fish are routinely checked for flavor quality, pond harvest schedules can be adjusted to account for potential flavor problems and the success of treatments to remove the off-flavor can be monitored. The off-flavor compounds are, however, detected by humans at very low levels, for instance MIB and geosmin can be detected at concentrations as low as 20 ppt (20 pg/L) in water and 0.7 ppb (0.7 ng/g) in fish (Grimm et al., 2000) and β-cyclocitral can be detected in water at a concentration of less than 0.5 μg/L (Young et al., 1999). Therefore, efforts to monitor these off-flavors require sensitive
analytical tools capable of detecting these compounds at similar or lower concentrations. Hence there is a need for a sensitive, accurate, but simple, inexpensive analytical method for the detection of the off-flavor compounds.

Immunochemical methods can be sensitive, accurate, simple, inexpensive, and relatively fast and they are considered as a good alternative to the expensive classical chromatographic analysis (Sardinha et al., 2002). These methods can be adapted to be utilized by farmers and industries for the routine analysis of off-flavors in ponds and in catfish.

The main goal of this study was to develop an enzyme-linked immunoflow assay (ELIFA) based on polyclonal and monoclonal antibodies that are specific and sensitive enough to detect β-cyclocitrinal in water and fish without the need for extensive sample clean-up. The specific objectives of the study included: preparing β-cyclocitrinal-PPD (purified protein derivative of tuberculin) conjugate (immunogen), producing chicken-based polyclonal antibodies, producing mouse-based monoclonal antibodies and, developing an enzyme-linked-immunoflow (ELIFA) assay which would be correlated with the GC/MS analyses of catfish pond water samples.
CHAPTER II. LITERATURE REVIEW

2.1 Off-flavors in Water and Catfish Industries

Much attention has been focused on volatile, low-molecular-weight compounds liberated by cyanobacteria and algae into surface waters (Ikawa et al., 2001). These substances are regarded as the causative agents of odor and taste problems arising during water purification and fish breeding (Jüttner, 1984). Tastes and odors in water from algal metabolites have long been recognized as a significant problem to water supply authorities (Cotsaris et al., 1995) and in particular blooms of cyanobacteria have been reported as responsible for many incidents of earthy musty odors in potable water. Channel catfish (Ictalurus punctatus) are frequently afflicted with serious musty/muddy off-flavors as well, the incidence and severity of which is greatest when water temperatures, stocking densities and feeding rates are high (Martin and Suffet, 1992).

Many water supplies are subject to extensive growth of odor-producing algae and cyanobacteria, particularly during the hot summer months, when water temperatures are consistently above 70 °F. The association between algal blooms in water and episodes of unpleasant taste and odors has long been established (Hayes and Burch, 1989). During their growth cycles, algae produce many organic compounds some of which are odorous and others non-odorous (Dietrich et al., 1995). The most problematic organisms are the cyanobacteria Anabaena sp. and Microcystis sp. (Jones and Korth, 1995).

The two compounds most frequently identified as being responsible for unpleasant odors are geosmin produced by Anabaena sp (Jones and Korth, 1995) and 2-methylisoborneol (MIB). These compounds are responsible for the muddy and musty odors
in water and fish and they have very low odor thresholds, 15 and 42 ng/kg of water respectively (Jüttner, 1984). The quality of raw water is significantly affected by these two compounds since they are poorly removed by conventional water treatment procedures (Cotsaris et al., 1995). The compounds are accumulated in the food web and thus concentrated in the flesh of fish. According to Martin and Suffet (1992), the off-flavor compounds are rapidly absorbed by the fish and bio-concentrated in the adipose tissue making the fish off-flavor. The difficulty in selling the off-flavor fish leads to serious consequences for commercial fishery (Jüttner, 1984) since depuration of off-flavor compounds requires 3-14 days in clean water, which is an additional expense.

Controlling the off-flavor compounds is very difficult since different organisms are responsible for their production. Originally, actinomycetes were thought responsible for the occurrence of geosmin in lake water, but several studies have shown the importance of cyanobacteria (blue-green-algae) and likewise myxobacterales have been implicated (Hayes and Burch, 1989; Jüttner, 1992). The numerous odorous compounds that contribute to the taste and odor problems in water and fish make the management of off-flavor even more complex. Offensive odors have been noticed during the mass development of many different cyanobacteria and algae and a wide spectrum of different substances are responsible for the overall odor of water (Jüttner, 1984). Diverse descriptions such as geranium, grassy, cucumber have been reported and compounds including geosmin, 2-methylisoborneol, sesquiterpenes, 3-methyl-1-butanol, methyl-5-hepten-2-one and β-cyclocitral have been isolated from cyanobacteria. (Hayes and Burch, 1989).

Other odor-contributing compounds are non carotenoids resulting from the degradation of carotenoids. These compounds have been reported as major excretion
products of cyanobacteria and algae (Ikawa et al., 2001). They include β-cyclocitral, a non-carotenoid that derives from carotenes. β-Cyclocitral exhibited the highest concentration of all the volatile compounds found in the eutrophic shallow lake studied by Jüttner in 1984. β-cyclocitral along with geosmin were identified as the chemicals responsible for causing hay/woody and earthy odors in Lake Winnebago, the source of supply for the Appleton Water Treatment Plant in Appleton, Wisconsin (Young et al., 1999).

2.2 β-Cyclocitral

\[
\text{CH}_2\text{O} \\
\text{2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde}
\]

2.2.1 Occurrence of β-Cyclocitral

β-Cyclocitral is produced by the cyanobacterium, Microcystis (Young et al., 1999, Ikawa et al., 2001). High concentrations of β-cyclocitral were correlated with Microcystis abundance at Carcoar Dam (Jones and Korth, 1995). Jones and Korth (1995) found that β-cyclocitral was produced by M. aeruginosa at a cellular concentration of 10 fg cell\(^{-1}\). The maximum concentration of β-cyclocitral was also found to coincide with the maximum development of M. wesenbergii in a study of a eutrophic shallow lake (Jüttner, 1987). Laboratory experiments on M. wesenbergii indicated β-cyclocitral as its major volatile component (Jüttner, 1987).

β-Cyclocitral may be formed by a carotene-oxygenase or by photooxidative degradation of β-carotene in addition to other cleavage products of β-apo-carotenal series.
The dynamics of β-cyclocitral observed by Jüttner (1987) during his study of a eutrophic shallow lake was characterized by a steady increase of β-cyclocitral to a maximum followed by a steady decrease. Cotsaris et al., (1995) reported an odor threshold concentration for β-cyclocitral of 19 µg/l (19 ng/ml) with a sweet/fruity odor descriptor by flavor profile analysis (FPA). However Young et al. (1999) found the odor threshold concentration to be 0.5 µg/l.

2.2.2 Chemical and Biological Aspects of β-Cyclocitral

β-Cyclocitral, an alkylated cyclic enol, is reported to be an oxidation product of β-carotene (Hayes and Burch, 1989; Young et al., 1999). Cleavage of β-carotene by β-carotene 7,8 (7',8') oxygenase stoichiometrically yields two molecules of β-cyclocitral and one molecule of crocetindial (Walsh et al., 1998). β-Cyclocitral was found resistant to oxidation by chlorine, potassium permanganate and chlorine dioxide which are oxidants commonly used for water treatment (Dietrich et al., 1995). Oxidation of β-cyclocitral with any of the above three oxidants resulted in little change in the odor characteristics of this compound and little or no reduction in the flavor profile analysis odor intensity.

β-Cyclocitral is known to be an important factor in off-flavored fish and was isolated from pond water containing off-flavor fish but not from ponds containing on-flavor fish (Martin and Suffet, 1992). The mechanism by which β-cyclocitral contributes to off-flavor in catfish is still unclear since it is rarely isolated from off-flavor fish. According to Martin and Suffet (1992), β-cyclocitral may be degraded in the water to compounds that are odorous and more easily bio-concentrated in catfish, or it may be metabolized in fish to odorous products that are more highly bio-concentrated.
2.2.3 Impacts of β-Cyclocitrinal

After a Microcystis algal bloom in Lake Winnebago, the source of supply for the Appleton Water Treatment Plant (WTP) in Wisconsin, the Appleton WTP experienced the worst taste and odor event in several decades (Young et al., 1999). β-cyclocitrinal and geosmin were identified as the chemicals responsible for causing the hay/woody and earthy odors experienced in this lake. Slater and Blok (1983) also found that geosmin and β-cyclocitrinal occurred in water during a taste and odor incident in Buffalo Pound Lake (Saskatchewan, Canada). Jones and Korth (1995) identified β-cyclocitrinal and associated it with Microcystis blooms in Australian rivers. It is now known that β-cyclocitrinal contributes to the off-flavor of channel catfish, although it was rarely isolated from fish, probably due to its metabolism in the fish tissue (Martin and Suffet, 1992).

β-cyclocitrinal imparts odors that have been described as grape, sweet tobacco (Dietrich et al., 1995), smoky/tobacco (Hayes and Burch, 1989) and hay/woody (Crozes et al., 1999). β-Cyclocitrinal has been reported to have three odor descriptors at different concentration ranges (Young et al., 1999). Fresh grass and sweet descriptors were reported in the concentration range of 0.5-1 μg/l. A hay/woody descriptor was observed in the 2-20 μg/l concentration range. A tobacco-like descriptor was observed in the concentration range of 20 μg/l and beyond. β-Cyclocitrinal therefore contributes to the hay-woody odor and at lower concentrations, the grassy odor and, a tobacco-like odor at higher concentrations in water and fish samples.

2.2.4 Control of Off-flavor Compounds

There are large numbers of factors that affect algae growth and production of off-flavor compounds, which has made controlling off-flavor in ponds very difficult. According
to Tucker and Martin (1991), three approaches have been used to deal with off-flavor in pond-raised fish; managing around off-flavor episodes, preventing off-flavor episodes whenever possible and removing the off-flavor from the fish once they have developed it. Currently, depuration is the only practical method for removal of off-flavor compounds from off-flavor fish (Tucker and van der Ploeg, 1999). Lovell and Sackey (1973) held channel catfish having a distinct earthy-musty flavor in a clean water tank for 14 days. After 3 days in clean water, the flavor of fish had significantly improved and after 10 days the flavor was not significantly different from that of the control fish that had never been exposed to algae. The depuration of off-flavor compounds presumably results from gill excretion. All the approaches used to deal with off-flavor require the ability to detect the presence of the odorous compounds.

2.2.5 Detection/Analysis of Odorous Compounds

Identification of the odorous compounds has usually been carried out using gas chromatography/mass spectrometry (GC/MS) after isolation and concentration of the volatiles by liquid-liquid extraction, solid-liquid extraction, resin adsorption, steam distillation purge and trap extraction or a stripping method (Hayes and Burch, 1989).

The closed loop stripping (CLS) method involves activated carbon adsorption of organics purged from the sample by a re-circulating headspace air in a closed circuit (Hayes et al., 1991). The samples are stripped (odorous volatiles removed from the gas phase) in CLS apparatus and the carbon filters are extracted with an organic solvent (e.g. dichloromethane). With closed-loop stripping analysis, trace organics of odorous compounds can be detected at nanogram-per-liter levels (Hu and Chiang, 1996).
In the purge trapping method developed by Johnsen et al. (1992), the sample was sparged with nitrogen and trapped under vacuum. Carbopack and Carbosieve III were used as trapping materials. With the help of the purging gas and vacuum, the volatile compounds in the sample are released, carried through a condenser and trapped by an adsorbent. To desorb trapped compounds, washing solvents such as dichloromethane can be used. If necessary, the extract can be concentrated using nitrogen gas to evaporate solvent before injection into the GC.

The microwave distillation method is similar to the purge and trap but the heat source is from a microwave. The volatile compounds in a sample are released and carried by nitrogen gas without vacuum, and then condensed in a cooling system. In some cases the cooling system could be glassware immersed in a cold bath filled with acetone cooled with dry ice (-80 °C) (Martin et al., 1987) or water/ethylene glycol (0 °C) (Lloyd and Grimm, 1999). Solid-phase microextraction (SPME) is based on the partitioning of analytes from a water matrix to a solid phase. The solid phase is typically polymeric (e.g. poly(acrylate)), which adsorbs molecules relatively selectively. The solid phase is coated on a short length of fused silica fiber installed on a plunger of a modified syringe assembly. The plunger is retracted into a needle to protect the delicate fiber. The needle is used to pierce the septum of a sealed vial containing the sample. The fiber can be immersed in a water sample (liquid sampling) or exposed to the headspace above a sample (headspace sampling). After equilibration, the solid phase is retracted into the needle and inserted into the heated injection port of a gas chromatograph. The analytes adsorbed on the solid phase are thermally desorbed and subsequently separated by a GC column.
A microwave cooking method was developed for the isolation of off-flavor volatiles from the catfish tissue (Martin et al., 1987). This method involves microwave cooking of fish under a nitrogen stream and trapping the condensate at –80 °C. A method using microwave distillation-SPME with GC/MS to analyze volatiles in catfish was also developed (Lloyd and Grimm, 1999). The advantages of the SPME over the purge and trap extraction are relatively low cost, no use of solvent extraction, no dilution of volatiles and shorter extraction time.

Yasuhara and Fuwa (1979) reported determination of geosmin in water by GC-MS after direct extraction of water with dichloromethane. However, according to Grob and Grob (1975), accumulation of solvent impurities and severe loss of extracted substances during the concentration step makes this method impractical for the detection of trace concentrations of organic compounds in water. Direct solvent extraction can be liquid-liquid extraction or solid-liquid extraction.

Sensory analysis is currently the method of choice for the industry because of its technical ease and relatively low cost. Sensory testing is a relatively rapid method that can detect off-flavor in water and fish tissues (Martin and Suffet, 1992). Flavor profile analysis is performed by a trained panel that describes each odor as a specific flavor with a numeric value for intensity (Crozes et al., 1999). This method however suffers from being a subjective method with a large degree of variation between tasters and between tastes of the same taster (Bett and Johnsen, 1996). Individual panelists perceive flavor intensity differently because of variations in detection thresholds, adaptation, fatigue and enhancement or suppression. This makes detection of β-cyclocitral by this method even more challenging due to its concentration-dependent off-flavors.
Currently, \(\beta\)-cyclocitral is detected by SPME-GC/MS. Briefly, excess sodium chloride is added to the water sample in a glass vessel that is tightly sealed. The NaCl drives the volatiles out of the solution into the headspace. A solid phase micro extraction cartridge is then inserted into the headspace to equilibrate for about 15 minutes before the volatiles are thermally desorbed off the cartridge in the heated GC injection port. The volatiles are then separated and analyzed by GC/MS.

Analytical methods to detect off-flavor in catfish and water continue to be developed (Martin and Suffet, 1992). Polyclonal antibodies and an ELISA test for MIB were developed by Chung et al. (1991), however the test lacks the sensitivity to detect MIB at threshold odor concentrations. Analytical methods are currently labor and time intensive, require expensive analytical equipment or are not sensitive enough to detect off-flavor at significant concentrations (Martin and Suffet, 1992). “A faster and less expensive method could find broad application in catfish flavor research, the catfish processing industry and other aquaculture industries plagued by this problem” (Lloyd and Grimm, 1999).

### 2.2.6 Immunochemical Methods

Immunochemical methods make use of the specificity of antibody-antigen interaction. Antibodies are produced in an animal in response to an antigen introduced into the animal. All immunochemical procedures require a suitable antiserum or monoclonal antibody raised against the antigen of interest. Immunoassays currently play an important role in routine quantitation of analytes in test samples, such as drug levels in blood (Laurie et al., 1989), pesticide concentration in water or foods (Hottenstein et al., 1996), or hormone in human specimens (Rajkowski et al., 1989). Some immunoassays use labeled reagents such as radioisotope, enzyme or fluorescein. Immunoassays using enzymes are popular due to their
sensitivity and safety. In these immunoassays, the enzyme label can be conjugated to either an antigen or antibody, allowing antigen-antibody interaction to be monitored by measuring enzyme activity.

2.2.6.1 Enzyme Immunoassays

An enzyme immunoassay uses the catalytic properties of enzymes to detect and quantify immunological reactions. One of the most commonly used enzyme immunoassays is enzyme-linked immunosorbent assay (ELISA) in which one of the reaction components is adsorbed or covalently bound to the surface of a solid phase. High specificity and sensitivity is usually achievable in the ELISA but may take one working day to complete the assay.

Enzyme-linked immuno-flow (immuno-filtration) assay (ELIFA) is a modification of the ELISA (Shields et al., 1991). It has been reported to be less sensitive than the ELISA but very easy to perform, fast, and simple to read routinely (Dupont et al., 1990; Shields et al., 1991). Shields et al. (1991) also reported that the ELIFA system could be advantageous when large volumes of small concentrations need to be assayed. This would make the ELIFA technique appropriate for the catfish farmer who needs to monitor minute concentrations of off-flavor compounds in the pond water.

The steps and reagents for the ELIFA can be identical to those of the ELISA however filtration across the membrane reduces the time required for each step and thus the time taken by the entire ELIFA operation (Aubert et al., 1997). Figure 2.1 compares an ELIFA with an antibody-sandwich ELISA. The flow rates of the reagents across the membrane must be controlled and this determines the sensitivity of the assay. The sensitivity of the ELIFA can be increased by slowing the flow rate and therefore slowing the antigen adsorption or binding.
Antibody-sandwich ELISA
- Polystyrene microtiter plates
  - Incubate 2h at 37°C
  - Rinse 3x by filling with water and flicking water into the sink

2. To block
- Incubate 30min at RT
- Rinse as above

3. Antigen
- Incubate ≥2h at RT
- Rinse as above
- Block with BSA, 10min, RT
- Rinse as above

4. Antibody enzyme conjugate
- Incubate 2h at RT

5. Wash as in 3 above
6. Add substrate
- Incubate 1h, RT
7. Read plate on a microtiter plate reader
Time: 6-8h

ELIFA
1. Nitrocellulose membranes
   - Filter (10μl/min) antibody solution across membrane

2. Filter (50μl/min) BSA solution to block
3. Filter antigen solution
   - Slow the flow rate to increase sensitivity or,
   - Filter sample several times
   - Filter (50μl/min) BSA solution to block
4. Filter (20μl/min) antibody enzyme conjugate
5. Filter (50μl/min) PBS to wash
6. Filter (20μl/min) substrate to produce the color reaction
7. Collect substrate in ELISA plate and read
Time: 1-2h

Figure 2.1. Comparison of ELISA and ELIFA
step (Shields et al., 1991), but too slow a flow rate would reduce time savings. Dupont et al. (1990) also reported that it is possible to increase the sensitivity of detection by filtering the same sample several times, thus allowing more antigen to adsorb or be captured by the adsorbed antibody. In addition to the ELIFA being an easy-to-use technique, it can also be automated and would therefore require no handling of membranes (Aubert et al., 1997).

In addition to the Enzyme-Linked Immunosorbent Assay (ELISA) and ELIFA other examples of experimental applications which use antibodies are Western Blot, Immunohistochemistry and Immunocytochemistry, and Immunoprecipitation (Harlow and Lane, 1988).

2.3 Antibody Production

Antibodies have become important research tools for many applications including the detection and purification of desired targets. Antibodies are host proteins produced in response to the presence of foreign molecules in the body (Harlow and Lane, 1988). They are synthesized primarily by plasma cells, a terminally differentiated cell of the B-lymphocyte lineage, and circulate throughout the blood and lymph where they bind to foreign antigens. An antibody response however is the culmination of a series of interactions between macrophages, T lymphocytes and B lymphocytes all reacting to the presence of a foreign material (antigen). The extreme specificity at the molecular level of each immunoglobulin for its antigen has made antibodies valuable therapeutic and diagnostic tools.

Monoclonal and polyclonal antibodies are useful in a variety of circumstances for their different and unique properties. Monoclonal antibodies by their nature react only to a single epitope and are becoming increasingly popular as reagents for all sorts of research and
analytical assays because of their high specificity. However their creation and production are typically more complicated, thus they are more expensive and take longer to develop.

In contrast to monoclonal antibodies, polyclonal antibodies recognize multiple epitopes, making them more tolerant of small changes in the nature of the antigen they recognize and offering a more robust reactivity profile (Harlow and Lane, 1988). In addition, polyclonal antibodies may be generated in a variety of species, including rabbit, goat, sheep, donkey, chicken and others, offering many options in experimental design. They are also quicker and less expensive to produce.

2.3.1 Polyclonal Antibodies

Polyclonal antibodies are raised by injecting an immunogen into an animal and after an appropriate time, collecting the blood fraction containing the antibodies of interest. Polyclonal antibodies have been traditionally produced in mammals and purified from blood (Carlander et al., 2003). The principal animals that have been used as polyclonal antibody sources are rabbits, sheep, goat and donkey; however, there has been a surge of interest in the utilization of chicken egg-yolk immunoglobulin in recent years (Losso et al., 1998). Chicken antibody, or 7S immunoglobulin, is referred to as chicken IgG or IgY (Losso et al., 1997). The antibody in egg yolk differs in molecular weight and isoelectric point from mammalian IgG (Hansen et al., 1998).

IgY originates from the serum and is transferred preferentially by the follicular epithelium of the ovary to the developing ova via specific receptors 4-5 days before ovulation; it ranges from 9 to 25mg/ml of egg yolk (Losso et al., 1997). IgY is transported from the hen to the egg to protect the offspring against infections until the chick’s immune system has matured so that it can provide the young animal with sufficient amounts of
antibodies. The active transport of IgY from the serum to the egg results in a higher concentration in the yolk than in the serum and therefore more antibodies can be produced per month in a laying hen than in a rabbit (Larsson and Sjöquist, 1990).

Immunoglobulins (IgY) from egg yolk can be a valuable source of antibodies when obtained from hens immunized against the target antigen. The advantages that IgY offers over conventional sources of antibody include (1) the potential to produce gram quantities (2) low cost of production, the cost of feeding and handling is considerably lower for a hen than for a rabbit (Larsson et al., 1993), (3) purification of IgY is relatively simple, and (4) the convenience of collecting antibodies from eggs rather than bleeding animals, which facilitates compliance with animal welfare considerations (Losso et al., 1998 and Losso et al., 1997). Chicken antibodies also have biochemical advantages over mammalian antibodies (e.g., rabbit antibodies) that can be used to improve immunoassays where antibodies are used. Due to great evolutionary distance between birds and mammals, a chicken is able to produce antibodies against more epitopes on a human antigen than a rabbit, which will give a stronger signal in immunological assays (Carlander et al., 2003).

Chicken egg-yolk is an inexpensive source of polyclonal antibodies. And since one egg can easily provide 100mg of antibody, large quantities of antibodies can be isolated from a few hens per year (Losso et al., 1998). Even with all the advantages that IgY antibodies offer over mammalian antibodies, IgY has not been used extensively probably due to unsatisfactory purification procedures (Hassl and Aspöck, 1988).

Antibodies are purified from the yolk usually by separating the lipid fraction from the water soluble faction. Several methods have been used for the purification of IgY. Polson et al. (1985) used polyethylene glycol precipitation, Jensenius et al. (1981) used dextran sulfate
precipitation, Hassl and Aspöck (1988) used hydrophobic interaction chromatography and gel filtration to obtain pure egg yolk immunoglobulins. Hatta et al. (1990) used xanthan gum precipitation.

Akita and Nakai (1992) used a water dilution method in which water-soluble plasma proteins were separated from the granular proteins as the egg yolk granules aggregated with dilution. Factors that affect this purification method are extent of dilution, incubation time, pH of diluted egg yolk and addition of sodium chloride. This method was employed in this study.

Akita and Nakai (1993) compared the water dilution method with the polyethylene glycol, dextran sulfate and xanthan gum methods in terms of yield, purity, ease of use, potential scaling up and immunoactivity of IgY. They reported that the water dilution method gave the highest yield, followed by dextran sulfate, xanthan gum and polyethylene glycol method in that order. The underlying principle for the purification of IgY by the four methods compared is the precipitation of granules leaving the IgY in the water-soluble fraction or supernatant. Akita and Nakai (1993) also found the water dilution method superior in terms of ease of use and large scale production of IgY and was most efficient for the purification of IgY from egg yolk.

### 2.3.2 Monoclonal Antibodies

Presence of an immunogenic molecule in an animal stimulates B-lymphocytes (B-cells), which undergo proliferation, differentiation, and maturation such that numerous B-cells produce antibodies to combat the invasion. Each B-cell produces a single type of antibody molecule (monoclonal) such that the overall response involves different antibody molecules (polyclonal) from different B-cells (Liddell and Cryer, 1991).
Monoclonal antibody production aims at selecting a B-cell producing a single type of antibody that is specific to the antigen and proliferating it in vitro to produce the desired amount of antibody. The cells that are isolated from the immunized animal, however, do not continue to grow in tissue culture on their own. Kohler and Milstein (1975) showed that the property of permanent proliferation could be added to the B-lymphocyte by fusing it with a tumorigenic plasma cell (myeloma) from the same animal species. The myeloma cells provide the correct genes for continued cell division and the antibody secreting cells provide the functional antibody genes. The hybrid cell or hybridoma can then be maintained in vitro and will continue to secrete antibodies with a defined specificity.

Myeloma can be induced in a few strains of mice by injecting mineral oil into the peritoneum (Harlow and Lane 1988). Potter (1972) isolated myelomas from BALB/C mice and they are the most commonly used partners for fusion. Polyethylene glycol (PEG) is the most common fusogen for hybridoma production. It fuses the plasma membranes of adjacent myeloma and/or antibody-secreting cells, forming a single cell with two or more nuclei (Harlow and Lane, 1988). This transformation is a permanent alteration of the cell phenotype and some fusions may have unstable cell lines, which are eventually selected out leaving only those with the desired properties. Drug selection is used to remove unfused myeloma cells and probably the defective fusions. Usually, myeloma has a mutation in one of the enzymes of the salvage pathway of purine nucleotide biosynthesis (Liddell and Cryer, 1991). Selection with 8-azaguanine yields a cell line harboring a mutated hypoxanthine-guanine phosphoribosyl transferase gene (HPRT). The addition of any compounds (azaserine, aminopterin, etc) that block the de novo nucleotide synthesis pathway will force the cells to use the salvage pathway. Cells containing nonfunctional HPRT protein will die in these
conditions, while the hybrids between myelomas with a nonfunctional HPRT and antibody-secreting cells (B-cells) with a functional HPRT, will be able to grow. Figure 2.2 gives a summary of the steps that will be followed in the production of monoclonal antibodies against β-cyclocitral.

2.3.2.1 Bulk Monoclonal Antibody Production

Hybridoma cells (hybrid cells of myeloma cells and normal B-lymphocytes) can be grown in tissue cultures. However, only a low cell density with a corresponding low concentration of monoclonal antibodies is achievable in this in vitro method. In vivo, the hybridomas are grown in the peritoneum of the live mice and since the cells are tumorigenic, they grow invasively and aggressively causing the accumulation of large quantities of ascitic fluid in the peritoneum (Falkenberg, 1995). The cells secrete very high concentrations of antibodies (up to 20 mg/ml; 100-1000 times more concentrated than in vitro) in the ascitic fluid. To obtain the monoclonal antibodies, about 5ml of the ascitic fluid is drawn from the animals but most of the mice die in great pain and discomfort as a result of the growth of the cancerous cells. Falkenberg et al. (1995) described an easy-to-handle and reusable minifermenter (a miniPERM Bioreactor VIVAScience, Germany), which can allow for high density culture of hybridomas and production of antibodies in concentrations comparable to those produced in the form of ascitic fluid in live mice.

2.3.3 Preparing Protein Conjugates

Some antigens are intrinsically immunogenic and others have to be conjugated to a carrier to elicit an immune response (De Silva et al. 1999). Immunogens contain a B-cell epitope and a T-cell epitope, both of which are necessary for the production of antibodies against a particular antigen of interest. Non-immunogenic antigens, called haptens, lack the
Immunization of mice

Myeloma cell preparation

Immune spleen cell preparation

Fusion

Selection of hybrids

Screening for specific antibody production

Cloning of hybrid cell mixtures

Propagation of monoclonal cell lines

Bulk antibody production

Figure 2.2 Overall strategy for the production of monoclonal antibodies (Liddell and Cryer, 1991)

T-cell epitope, which can be introduced by conjugating to a protein. When hapten-labeled proteins are introduced into a suitable host animal, they generate hapten-specific antibodies (Brinkley, 1992).

Proteins, which are amino acid polymers, contain a number of reactive side chains that serve as handles for attaching a wide variety of molecules (Brinkley, 1992). In addition to these intrinsic groups, specific reactive groups may be introduced into the protein by chemical modification. Some of the reactive groups found on proteins are amines, thiols, phenols, and carboxylic acids.

2.3.3.1 Protein Modification Reagents

There are different reagents that are available for the purpose of protein modification. These reagents are designed to have specific reactivity with functional groups contained in each reactant (Annunziato et al. 1993) and may be grouped according to the reactive groups
that they target on the protein to be modified (Brinkley, 1992). These may be grouped as follows:

a) Amine-Reactive Reagents are those that react primarily with lysines and α-amino groups of proteins to give amide products. N-hydroxy-succinimide (NHS) ester of S-acetylthioacetic acid (SATA) is one of the commercially available amine-reactive reagents. These reagents have intermediate reactivity toward amines, with a high selectivity toward aliphatic amines. Other reagents that have been used to modify amines of proteins are acid anhydrides (Brinkley, 1992). Succinic anhydride is commonly used to succinylate amine groups for the purpose of changing their isoelectric point and other charge-related properties (Shiao et al. 1972).

b) Thiol-Reactive Reagents are those that will couple to thiol groups on proteins to give thioether-coupled products (Brinkley, 1992). Maleimides react with thiols resulting in formation of a thioether bond.

c) Carboxylic Acid-Reactive Reagents. Amines can be coupled to carboxylic acids of proteins via activation of the carboxyl group by a water-soluble carbodiimide followed by reaction with the amine (Brinkley, 1992) and resulting in formation of stable amide bonds.

d) Bi-functional reagents or cross-linking reagents are specialized reagents that will form a bond between two different groups, either on the same molecule or two different molecules (Brinkley, 1992). Those with the same reactive group at each end of the molecule are called homobifunctional while those with different reactive groups at each end of the molecule are called heterobifunctional. Succinimidyl (acetylthio)acetate (SATA) contains both an amine reactive and a protected thiol group. After de-protection, the thio-containing protein is reacted with a thiol-reactive group on the other protein. \( p \)-Maleimidophenyl isocyanate
(PMPI) is another heterobifunctional cross-linking agent containing a thiol reactive
(maleimide) and a hydroxyl reactive group (Annunziato et al. 1993). The isocyanate group
reacts with amines to form ureas and with alcohols to form carbamates. The
heterobifunctional reagents allow coupling to be carried out in a stepwise manner and result
in better control of the conjugation chemistry (Annunziato et al. 1993).

The hapten-protein conjugate once administered to an animal will elicit antibodies
that are specific to the haptenic group as well as the carrier protein (De Silva et al., 1999).
However, the successful coupling of haptens onto carrier protein molecules is of paramount
importance to a successful immune response that would result in the production of hapten-
specific antibodies in the host animal.

2.3.4 Carrier Proteins

A carrier protein is a large molecule capable of stimulating its own immune response
(Harlow and Lane, 1988) and it must come from a species different from the animal to be
immunized. The carrier molecule provides the T-cell epitope for the production of a
successful immune response (De Silva et al., 1999). The proteins to be used as carriers must
possess functional groups that can easily be substituted (Brinkley, 1992). Many different
carrier proteins have been used for coupling with peptides to create immunogens. The choice
of the carrier to use is based on immunogenicity, solubility, and whether adequate
conjugation with the hapten can be achieved.

Keyhole limpet hemocyanin (KLH) is one of the commonly used carriers, however
due to its large molecular weight (8,000,000Da) the serum antibody response to the carrier
often obscures that of the antigen of interest (De Silva et al., 1999) resulting in a reduced
antigen-specific antibody concentration in the IgG fraction.
According to Lachmann (1988), purified protein derivative of tuberculin (PPD) is an excellent carrier for generating antibody responses to weak antigens. PPD however has not been extensively used in the preparation of protein-hapten conjugates. Its principle practical use for the last half century has been as an in vivo diagnostic test for previous encounters with *Mycobacterium tuberculosis* (Lachman, 1988). Therefore, the immunogenic activity of PPD, which is made from cultures of *Mycobacteria*, is seen only in animals previously exposed to *M. tuberculosi*s or immunized with Bacillus Calmette-Guerin (BCG). BCG is an attenuated vaccine derived from *Mycobacterium bovis* and it is used as a vaccine against tuberculosis, an infectious disease caused by *Mycobacterium tuberculosis*. De Silva et al. (1999) described the exceptional ability of PPD as a carrier in the production of hapten-specific antibodies. Their results showed a weak antibody response to PPD compared to other carriers and an enhanced antibody response to the attached hapten. The reduction of carrier specific antibody response and a subsequent increase in antigen-specific antibodies makes the identification of the desired antibodies much simpler.

2.3.5 Screening

Out of a pool of antibodies, the desired antibodies are identified by screening of the serum or hybridoma supernatants for the antibodies of interest. When a hapten-carrier conjugate is administered to an animal, it will elicit production of antibodies that have specificity to the haptenic group, to the carrier and to the whole conjugate (De Silva et al., 1999). In order to identify hapten-specific antibodies, serum and hybridoma supernatants have to be screened against a hapten-protein conjugate, where the protein is different from that used in the immunization.
2.3.5.1 Solid Phase Conjugate

The hapten-protein conjugate with a protein different from that used in the immunization is needed to immobilize non-protein haptens onto a solid support for the purpose of identifying anti-hapten antibodies. Using a different protein from that used in the immunization ensures that only antibodies produced against the hapten of interest and not the carrier molecule are selected (De Silva et al., 1999). Identification of antigen specific antibodies is usually done using ELISA.
CHAPTER III: COUPLING OF \( \beta \)-CYCLOCITRAL TO PURIFIED PROTEIN DERIVATIVE OF TUBERCULIN AND BOVINE SERUM ALBUMIN

3.1 Introduction

Bioconjugation involves the linking of two or more molecules to form a novel complex having the combined properties of its individual components (Hermanson, 1996). Raising antibodies against \( \beta \)-cyclocitral, which is an off-flavor compound posing a problem in water supply systems and the fish industry, requires conjugating it to a carrier molecule. An antibody response is triggered in an animal following injection of an immunogenic molecule; however, substances of molecular weight less than 1000 Da (haptens, like \( \beta \)-cyclocitral) are not immunogenic by themselves and need to be conjugated or coupled to highly immunogenic macromolecules (Pauillac et al., 1998), referred to as carrier molecules.

Purified protein derivative (PPD) of tuberculin has been reported as the best carrier molecule for the production of both polyclonal and monoclonal antibodies (De Silva et al., 1999) due to its important immunological properties (Lachmann, 1988). It provides very powerful T cell help and does not elicit an antibody response against itself (Lachmann et al., 1988). Small molecules (haptens) may posses an epitope that can be recognized by surface immunoglobulins on B cells and hence can bind an antibody. But they do not posses a class II MHC T-cell receptor site that enables the cascade of events resulting in the production of antibodies. This T-cell component can be introduced to a hapten by coupling the hapten to an immunogenic (able to elicit production of an antibody) carrier such as PPD.

Coupling should be done to keep the antigen of interest in as native a condition as possible (Harlow and Lane, 1988). De Silva et al. (1999) pointed out that during preparation of hapten-carrier conjugates, one must strive to preserve the integrity of the antigen structure since many applications involving the antibodies require discrimination of analogues with...
very small conformational differences. Chung et al. (1991) used argosmin (structurally similar to geosmin)-BSA conjugate as an immunogen to make antibodies to be used in developing an ELISA for detection of geosmin (an off-flavor contributing metabolite of actinomycetes and blue green algae). The antibodies developed had a higher affinity for argosmin than for geosmin and consequently resulted in a less efficient analytical technique for the detection of geosmin.

In this study, hydroxycyclocitrinal 2 (3-Hydroxy-2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde), an analog of β-cyclocitrinal 1 (2,6,6-trimethyl-1-cyclohexene-1-carboxaldehyde), was synthesized in order to incorporate an extra functional group through which the coupling could be done so as to preserve the integrity of the hapten (β-cyclocitrinal). A hydroxyl- and sulfhydryl-reactive heterobifunctional cross-linker, p-maleimidophenyl isocyanate (PMPI) (Annunziato et al., 1993), was used to link the hydroxyl containing hydroxycyclocitrinal to thiolated PPD 4. The isocyanate group on PMPI 10 reacts with hydroxyl groups to form a carbamate linkage. PPD, which contains four lysines was thiolated using N-succinimidyl S-acetylthioacetate (SATA, Pierce Biotechnology, Rockford, IL), an amine-reactive heterobifunctional cross-linker, which introduces protected sulfhydryl groups. The protected sulfhydryl groups were deprotected using a reaction with hydroxylamine. The
free thiols on PPD were subsequently reacted with the thiol-reactive group (maleimide) on the PMPI-activated hydroxycyclocitral 3, to give hydroxycyclocitral-PPD conjugate 5, containing an intact \( \beta \)-cyclocitral 1 structure as the hapten.

3.2 Experimental Section

Reagents. All reagents were obtained from commercial sources and were of analytical grade or higher. Citral, aniline, \( m \)-chloroperbenzoic acid, pyrrolidine, 4-aminobenzoic acid, maleic anhydride, and triethylamine were obtained from Sigma Chemical Co. (St. Louis, MO). Diphenyl phosphoryl azide (DPPA) was obtained from Aldrich (Milwaukee, WI). DMSO (dimethylsulfoxide) (silylation grade), SATA (\( N \)-succinimidyl S-acetylthioacetate), hydroxylamine hydrochloride, and Ellman’s reagent were obtained from Pierce Biotechnology (Rockford, IL). Purified protein derivative of tuberculin (PPD) was obtained from Animal and Plant Health Inspection Service National Veterinary Services Laboratories (Ames, IA). Sodium phosphate, phosphoric acid, EDTA, cysteine, dimethylformamide (DMF), succinic anhydride, and pyridine were obtained from Sigma Chemical Co. (St. Louis, MO). Sulfo \( N \)-hydroxysuccinimide (Sulfo-NHS), bovine serum albumin (BSA), and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) were obtained from Pierce Biotechnology.
(Rockford, IL). PD-10 columns (Sephadex G-25) were purchased from Amersham Biosciences (Piscataway, NJ).

**Drying of Solvents.** DMSO was stored under argon in a sealed container. Acetonitrile and pyridine were dried by refluxing (2h), distilling from calcium hydride (CaH$_2$) under argon, and stored over 3-Å molecular sieves.

**Thin-Layer Chromatography.** Organic reactions were monitored using glass-supported silica gel plates and the spots were visualized under a Spectroline UV lamp (Model ENF-240C) and/ or by treatment with iodine vapors.

**Column Chromatography.** Purification of the organic crude organic products was done by flash chromatography techniques. Silica gel of 32-63μm particle size and 60Å pore size was used.

**Gel Permeation Chromatography.** PD-10 columns were equilibrated with the eluting buffer by passing at least five column volumes of the buffer through the column prior to sample application.

**Melting Points.** Melting points were obtained on Fisher-Johns Melting Point Apparatus.

**NMR Spectroscopy.** Proton nuclear magnetic resonance (¹H NMR) spectra were measured using a Bruker AC250 (250 MHz). Samples were dissolved in CDCl$_3$, DMSO or (CD$_2$Cl$_2$).

**Mass Spectroscopy.** Gas Chromatography-Mass Spectrometry (GC-MS) and Fast Atom Bombardment-Mass Spectrometry (FAB-MS) were run on Hewlett Packard 5971A GC/MS and Finnigan MAT 900 spectrometers respectively.

**Analyses.** Elemental analyses (C, H, N) were performed by M-H-W Laboratories (Phoenix, AZ).
**Ultraviolet Spectroscopy.** UV absorbance spectra of aqueous solutions were measured using a SpectraMax Plus Spectrophotometer (Molecular Devices, Sunnyvale CA).

### 3.2.1 Synthesis of 3-Hydroxycyclocitral 2

The transformation of citral 6 into hydroxycyclocitral 2, was performed through a 4-step sequence of reactions.

#### 3.2.1.1 Citral Anil 7

![Chemical structure of citral anil 7](image)

Citral anil was prepared with modification of the procedures of Colombi *et al.* (1951) and Frank (1981). A solution of aniline (4.65 g, 50 mmol) in 5 mL ether was added in a portion-wise manner to a stirred solution of citral 6 (7.6 g, 50 mmol) in ether (7.5 mL). After water droplets appeared, the reaction was left to stand for 1h at room temperature while monitoring the reaction with TLC (Hexane-ethyl acetate, 6:4, \( R_f = 0.63 \)). Anhydrous sodium sulfate (1.58 g) was added and the stirring continued. The solution was decanted from the solid and the solvent evaporated to give citral anil 7 (12.2g). \(^1\)H NMR (CDCl\(_3\)) (Appendix A): \( \delta \) 8.4-8.3 (m, 1H, CH=N), 6.25-6.21 (m, 1H, CH), 7.3-7.1 (m, 5H, Ar-H), 2.02-1.97 (m, 4H, CH\(_2\)), 1.69-1.61 (m, 6H, CH\(_3\)), 5.13-5.12 (m, 1H, CH), 2.24-2.16 (m, 3H, CH\(_3\)).

#### 3.2.1.2 \( \alpha \)-Cyclocitral 8

Citral anil 7 was cyclized to \( \alpha(8) \) - and \( \beta \)-cyclocitral 1 according to the method of Gedye *et al.* (1971) with some modification. A solution of anil 7 (12.2 g) in ether (7.5 ml)
was added drop-wise over 45 min to 50 g of sulfuric acid at –25 °C in an argon atmosphere and stirred for a further 45 min at –15 °C. The resulting dark brown sticky substance was added to ice (150 g) and the icy mixture extracted 6 times with ether. TLC (Hexane:ethyl acetate, 6:4) showed 2 spots ($R_f = 0.66, 0.52$) in a ratio of about 10:1. Under an inert atmosphere the ratio of 8:1 has been reported as 10:1 (Heather et al., 1976) and 15:1 (Gedye et al., 1971). The ether extract was washed with brine, dried over Na$_2$SO$_4$ and the solvent evaporated to obtain 5.09 g of yellowish brown product. This product was used without further purification.

$^1$H NMR (CDCl$_3$) (Appendix B): $\delta$ 9.45 (d, 1H, CHO), 7.33-7.30 (m, 1H, CH), 1.61-1.59 (m, 3H, CH$_3$), 1.69 (m, 2H, CH$_2$), 5.73 (m, 1H, CH), 1.19 (m, 2H, CH$_2$).

### 3.2.1.3 2,3-Epoxy-2,6,6-trimethylcyclohexane-1-carboxyaldehyde 9

Epoxidation of $\alpha$-cyclocitral 8 according to the procedure of Heather et al. (1976) gave the epoxide 9. A solution of 8.65 g (50 mmol) of m-chloroperbenzoic acid in 57 mL of dichloromethane was added to a stirred solution of 5 g (38 mmol) of crude compound 8 in
15 mL of CH$_2$Cl$_2$ while maintaining a temperature of 20-30 $^\circ$C with a water bath. After stirring for 2h, 18 mL of 10% aqueous Na$_2$SO$_3$ solution was added to the red, viscous reaction mixture in order to destroy the excess peracid. After determining (using KI and starch) that the reaction mixture had no excess peracid, a saturated solution of Na$_2$CO$_3$ was added to make the solution alkaline (translation monitored by the use of pH paper). The denser methylene chloride layer was separated from the aqueous layer, washed with water and brine and dried over Na$_2$SO$_4$. Evaporation of the solvent from the solution decanted from Na$_2$SO$_4$ gave the crude product (2.6g). TLC (Hexane: ethyl acetate, 6:4) showed presence of some starting material ($\alpha$-cyclotiral, $R_f$ = 0.66), thus purification was done using flash chromatography (eluant: Hexane: ethyl acetate, 6:4). Evaporation of the solvent yielded epoxide 9 (90% yield) with $R_f$ = 0.59. $^1$H NMR (CDCl$_3$) (Appendix C): $\delta$ 9.67 (d, 1H, CHO), 3.08 (t, 1H, CH), 0.92 (s, 6H, CH$_3$), 0.92-1.33 (m, 2H, CH$_2$), 1.33 (m, 3H, CH$_3$), 1.33-2.04 (m, 2H, CH$_2$), 2.17 (s, 1H, CH).

3.2.1.4 3-Hydroxycyclotiral 2
Treatment of the epoxide 9 with pyrrolidine, according to Heather et al. (1976), resulted in the epoxide opening to hydroxycyclocital. Pyrrolidine (0.32 mL) was added to a solution of the epoxide (2.6 g, 15.45 mmol) in 15 mL ether. The mixture was stirred under argon at room temperature for 3 hours. The reaction was quenched by addition of 10 mL water. The ether layer was washed with brine, dried over Na$_2$SO$_4$ and the crude product purified by flash chromatography (eluant: ether). Upon evaporation of solvent, pure product 2 (1.65 g) was obtained. $^1$H NMR (CDCl$_3$) (Appendix D): $\delta$ 10.15 (s, 1H, CHO), 1.18 (s, 3H, CH$_3$), 1.23 (s, 3H, CH$_3$), 2.17 (s, 3H, CH$_3$), 2.20 (s, 1H, OH), 4.07 (t, 1H, CH), 1.10 – 1.75 (m, 4H, CH$_2$). MS (GC): m/e 168.

3.2.2 PMPI-Activated Hydroxycyclocital 3

Hydroxycyclocital 2 was coupled to PMPI 10 through a 4-step reaction process.

3.2.2.1 p-Maleimidobenzoic Acid 12

This synthesis was done according to the procedure of Annunziato et al. (1993). 4-aminobenzoic acid 11 (4.26 g, 31 mmol) was suspended in 30 mL of acetone and solubilized by addition of methanol (5 mL). A solution of maleic anhydride (3.66 g, 37 mmol) in 10 mL of acetone was added. The resulting precipitate was stirred for 20 min, suction filtered, washed with acetone and vacuum dried to obtain a yellow powder (6.7 g). This powder was
dissolved in 13 mL of acetic anhydride, treated with 1.08 g of sodium acetate, and heated while stirring at 50 °C for 2h. After removal of the volatiles (in vacuo), 150 mL of water was added to the resulting residue and heated at 70 °C for 2.5h. The resulting white precipitate was suction filtered, washed with water, and vacuum dried to yield the acid 12 (4.9 g). \( ^1\)H NMR (DMSO-\(d_6\)) (Appendix E): \(\delta\) 3.54 or beyond 10 (s, 1H, \(\text{CO}_2\text{H}\)), 8.06 (d, 2H, Ar-H ortho to maleimide), 7.51 (d, 2H, Ar-H ortho to carboxylic acid), 7.22 (s, 2H, maleimide vinyl).

3.2.2.2 \(p\)-Maleimidobenzoyl Azide 13

The azide 13 was synthesized by modification of the procedures of Annunziato et al. (1993) and Gierasch et al. (2000). Triethylamine (3.04 mL, 22 mmol) was added to a stirred suspension of acid 12 (4.3 g, 20 mmol) in 20 mL dry acetonitrile. Addition of DPPA (4.7 mL, 22 mmol) changed the yellow solution to a more viscous reddish brown mixture. The reaction was stirred at room temperature under argon, protected from light, and the TLC (methylene chloride) after 30min revealed that the reaction was complete (\(R_f = 0.80\)). The volatiles were removed in vacuo and from the resulting residue, product 13 was recovered as a yellow band by flash chromatography (eluent: methylene chloride). Evaporation of the solvent yielded a pale yellow fluffy mass (1.95 g). \( ^1\)H NMR (CDCL\(_3\)) (Appendix F): \(\delta\) 8.13
(d, 2H, Ar-H ortho to maleimide), 7.55 (d, 2H, Ar-H ortho to carboxylic acid), 6.90 (s, 2H, maleimide vinyl).

3.2.2.3 PMPI-Activated Hydroxycyclocitral 3

Hydroxycyclocitral 2 was co-evaporated 3x with dry acetonitrile. Azide 13 (722 mg, 2.97 mmoles) was added to 250 mg (1.49 mmol) of compound 2 in a dry flask. Acetonitrile (20 mL) was added to the reaction vessel, refluxed under argon and protected from light for 2h. TLC (Hexanes: ethyl acetate, 1:1) showed two product spots ($R_f = 0.70, 0.61$) after the 2h. Flash chromatography was used to purify the product after evaporation of the solvent (pyridine) under high vacuum. Conjugate 3 eluted as the first band and the second band was identified as urea 18 ($R_f = 0.61$) by $^1$H NMR (CDCl$_3$). The pure product 3 was recovered as a yellow powder upon evaporation of the solvent. Melting point: 219-221°C. $^1$H NMR (CDCl$_3$) (Appendix G): δ 7.52 (d, 2H, Ar-H ortho to maleimide), 7.32 (d, 2H, Ar-H ortho to carbamate linkage), 6.85 (s, 2H, maleimide vinyl), 6.80 (s, 1H, NH), 5.31 (t, 1H, CH), 10.17 (s, 1H, CHO), 1.21 (s, 3H, CH$_3$), 1.26 (s, 3H, CH$_3$), 2.12 (s, 3H, CH$_3$), 1.47 – 1.88 (m, 4H,
CH₂). Elemental analysis: Calculated for C₂₁H₂₂N₂O₅: C, 65.96; H, 5.80; N, 7.33; Found: C, 66.13; H, 5.71; N, 7.50.

3.2.3 Thiolated PPD 4

Purified protein derivative of tuberculin (PPD) was thiolated using SATA 14 following the procedure described by Pierce Biotechnology (Rockford, IL).

PPD (10,000 Da) was dissolved in 50 mM sodium phosphate (pH 7.5, containing 1 mM EDTA) to a concentration of 100 μM (1 mg/mL, 100 nmoles). The SATA reagent was dissolved in DMSO to a concentration of 65 mM (15 mg/mL; 64.87 μmoles). Ten μl (648.7 nmoles) was added to each mL of the PPD solution in a test tube to yield a 6.5 molar
excess of SATA over the PPD. The two solutions were mixed and allowed to react at room temperature for 30 minutes. The modified protein was separated from un-reacted SATA and reaction by-products by gel filtration on a Sephadex G-25 (PD-10) column using the same buffer. The acetylated –SH groups were de-protected (de-acetylated) by adding 100 µl of 0.5 M hydroxylamine hydrochloride in 50 mM sodium phosphate, 25 mM EDTA, pH 7.5 to each mL of the SATA-modified protein solution and allowed to react for 2 hours at room temperature, after mixing. The sulfhydryl-modified protein was purified by gel filtration on a Sephadex G-25 column using the same buffer.

The amount of –SH (thiol) groups formed were determined by performing an Ellman’s assay. Briefly, 250 µl of thiolated-PPD sample and 50 µl of Ellman’s reagent solution (4 mg/mL) were added to 2.5 mL of 0.1 M sodium phosphate (pH 8.0), mixed, and allowed to react at room temperature for 15 minutes. Sulfhydryl standards (15, 10, 5, 2.5, 1.25 and 0.625 mg cysteine in 100 mL distilled water) were treated in the same manner. The absorbance at 412 nm was determined and the sulfhydryl content of the PPD calculated via a standard curve. The analysis for free thiol groups revealed 5.6 thiols per mole of PPD. Ellman’s assay is based on the production of a colored anion (3-carboxylato-4-nitrothiophenolate) which has an absorbance maxima at 412 nm (Gething and Davidson, 1972) after a reduction of 5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB) by sulfhydryl groups.

3.2.4 Hydroxycyclocitral-PPD Conjugate (5)

The immunogen 5 was prepared according to the above scheme. The SATA-activated PPD 4 with de-protected sulfhydryl groups was treated immediately with compound 3 dissolved in phosphate buffer (pH 7.5). The maleimide end of compound 3 and the –SH groups on compound 4 were allowed to react at room temperature for 2 hours to form a
stable thioether linkage. The maleimide group combines with a thiol group via its double bond (Kitagawa et al., 1981). Gel filtration on a PD-10 column, using the same buffer as an eluent, was used to separate the conjugate from the lower molecular weight reactants. The extent of incorporation of PMPI-activated cyclocitral (conjugate ratio) into PPD was determined using differential spectrum (Figure 3.1). To obtain the differential spectrum, absorption spectra of compounds 3 and 4 were recorded between 200 and 360nm on a SpectraMax Plus UV Spectrophotometer. The optical spectrum of the modified PPD, compound 5 was also determined and superimposed on the spectrum of compound 4. The difference between the two spectra was determined and plotted out to determine if it matched the spectrum for compound 3. Using the absorbance values at $\lambda_{\text{max}}$, the conjugation ratio was determined as number of molecules of PMPI-activated cyclocitral on one molecule of PPD. The immunogen 5 was dialyzed against ultra pure water and lyophilized.
3.2.5 Hydroxycyclocitral-BSA Conjugate 17

The solid phase conjugate was made with a different protein (BSA) and a different chemical linker (succinic anhydride). β-cyclocitral was coupled to bovine serum albumin (BSA) using a modified procedure of De Silva et al. (1999).

3.2.5.1 Hydroxycyocitral Hemisuccinate 15.

![Reaction Scheme]

Succinic anhydride 16 (20 mg, 0.197 mmol), DMAP (26 mg, 0.215 mmol) and hydroxycyclocitral 2 (30 mg, 0.179 mmol) were allowed to mix in dry pyridine (1.0 mL) for 16 h and the reaction monitored by TLC. The pyridine was removed by co-evaporation with toluene (2 x 4.0 ml) and the resulting product taken up in CH₂Cl₂ (10 mL) and washed with cold 10% aqueous citric acid solution (2 x 10 mL) and H₂O (2 x 10 mL). The aqueous layers were re-extracted with CH₂Cl₂ and the organic extracts combined, dried over MgSO₄ and rotor-evaporated to yield compound 15.¹H NMR (CDCl₃) (Appendix H): δ 1.25-1.18 (m, 6H, CH₃), 10.15 (s, 1H, CHO), 2.05 (d, 3H, CH₃), 2.72-2.65 (m, 4H, CH₂), 5.34 (m, 1H, CH), 2.03-1.26 (m, 4H, CH₂).
The succinylated hydrocyclocitrinal 15 was coupled to BSA through the –COOH group by first activating it with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), using sulfo N-hydroxsuccinimide (Sulfo-NHS, Pierce Biotechnology, IL) as enhancer, according to the method used by De Silva et al. (1999) and Staros et al. (1986). A hapten:protein molar ratio of 10:1 was used. One mg (3.73 μmol) of compound 15 (mw 268) was dissolved in 1 mL of 0.01 M phosphate buffer, pH 7.4. 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC, mw 191.7) was added to a final concentration of 0.1 M (19.2 mg added to 1 mL). After stirring for a few minutes, sulfo N-hydroxsuccinimide (Sulfo-NHS, mw 217.14) was added drop-wise (1.01 mg, 4.66 μmol; 1.25 times antigen concentration). Finally, 25 mg (0.373 μmol) of BSA was added, the solution was made up to 2 mL, and the reaction was allowed to proceed for 24 h at room temperature. Gel filtration was used to separate the unreacted reactants from the modified BSA.
3.3 Results and Discussion

3.3.1 β-Cyclocitral-PPD Conjugate 5

The primary goal of this project was to prepare a β-cyclocitral-PPD conjugate with minimal changes to the β-cyclocitral structure on the final conjugate. β-cyclocitral 1 could be conjugated through its inherent double bond or the aldehyde group but this would cause a major change to the structure. In the immunoassay system of Chung et al. (1991), antibodies were raised against argosmin conjugate, and as a consequence the affinity of the antibodies for this compound was greater than that for the intended compound (geosmin). By synthesizing hydroxycyclocitral 2 and coupling through the extra functional group (hydroxyl), we maintained the integrity of β-cyclocitral in the final conjugate 5. Hydroxycyclocitral was synthesized through a 4-step sequence of reactions as shown in Scheme 1.
Isocyanates are unstable and prone to hydrolysis, which results in the formation of urea. All the attempts to attach PMPI 10 to hydroxycycloctiral 2 in order to produce PMPI-activated hydroxycycloctiral 3 using Scheme 2 were unsuccessful. Scheme 3 was used to couple 2 to 10 through a 4-step reaction process. Using Scheme 3 it was possible to obtain a PMPI-hydroxycycloctiral conjugate 3 without using a large molar excess of PMPI. In this scheme, \( p \)-maleimidoazide was combined with the hydroxycycloctiral before refluxing. This ensured that as the Curtius rearrangement of the azide to the isocyanate occurred, the hydroxyl group on the hydroxycycloctiral would be ready to react with it to form the carbamate. This also eliminated an extra step of combining reactants that would probably result into introduction of water into the reaction. In this reaction, urea 18, which is a product of water and PMPI, was formed but all the hydroxycycloctiral present in the reaction mixture was converted to the carbamate with only a 2 molar excess of PMPI. It was more efficient to carry out the rearrangement reaction and the carbamate formation reaction together. Urea 18
and conjugate 3 were purified but no free PMPI was recovered, probably because it was all used up in the reaction and any possible excess was probably degraded during the flash chromatography. The PMPI-activated hydroxycyclocitral 3 was characterized, protected from light, and stored in the freezer for later use.

After thiolation of PPD and determining the number of sulfhydryls introduced to the PPD molecule, the carbamate 3 was coupled to PPD through the thiol-maleimide reaction. With about 5 thiol groups introduced per mole of PPD, coupling of about three hapten groups was achieved. This is in agreement with Lachmann et al. (1986) who thiolated PPD using N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) and obtained 5 thiol groups per mole of PPD which allowed for coupling of between two and three haptenic groups.

The conjugation ratio was determined using differential spectrum (Figure 3.1). The absorption spectra of the PMPI-activated cyclocitral 3 and the absorption spectra of PPD as well as that of PPD-cyclocitral (immunogen 5) were used. Difference spectroscopy according to Robyt and White (1990) was used to determine the level of incorporation of conjugate 3 into PPD. In difference spectroscopy, the absorption spectra of two samples are compared. Common features in the spectra cancel and the differences are recorded. In this study the difference between the spectra of PPD and PPD-β-cyclocitral conjugate was determined. The difference gave a spectrum similar to the spectrum of β-cyclocitral with the same λ_max.
implying that it is the same product. Using this spectrum, the conjugation ratio was determined as 3 molecules of cyclocitral-PMPI on one molecule of PPD.

3.3.2 Hydroxycyclocitral-BSA Conjugate 17

This conjugate was synthesized for the purpose of detecting β-cyclocitral specific antibodies produced as a result of immunizing host animals with β-cyclocitral-PPD conjugates (immunogen 5). Using a different protein from that used in the immunization ensures that only antibodies produced against the hapten of interest (β-cyclocitral) and not the carrier molecule are selected (DE Silva et al., 1999). Identification of antigen specific antibodies is usually done using ELISA, which requires immobilization of the antigen of interest on to a plastic support. In order to immobilize β-cyclocitral on an ELISA plate it must be attached to a protein. Bovine serum albumin (BSA) was the protein used in this study. Scheme 5 was used to attach hydroxycyclocitral to BSA resulting in an intact β-cyclocitral 1 molecule on the final conjugate 17.

The conjugation ratio (groups of β-cyclocitral attached to BSA) was determined using differential spectrum (Figure 3.2). Figure 3.2a shows the spectrum of hydroxycyclocitral hemisuccinate with a \( \lambda_{\text{max}} \) of 240nm whereas Figure 3.2b shows the spectrum of BSA superimposed with the spectrum of BSA-β-cyclocitral conjugate 17. The difference between
BSA and BSA-β-cyclocitral conjugate was determined and plotted as shown in Figure 3.2c. This difference gave a spectrum similar to that of hydroxycyclocitral hemisuccinate 15 with the same $\lambda_{\text{max}}$ implying that it is the same product. Using this spectrum, the conjugation ratio was determined as 33 molecules of hydroxycyclocitral hemisuccinate 15 on one molecule of BSA. BSA has a total of 59 lysine groups of which about 30-35 are available for coupling (Pierce Biotechnology, Rockford, IL). BSA was coupled to hydroxycyclocitral according to the enhanced coupling method of Staros et al (1986). After succinylating hydroxycyclocitral, the resulting carboxylic acid was activated by the carbodiimide (EDC) to form an O-acylurea which then reacted with the amines (lysine groups on BSA) to form BSA-cyclocitral. However this reaction is sensitive to water since the activated carboxylates are subject to hydrolysis; hence, $N$-hydroxysuccinimide (Sulfo-NHS) was used to enhance the reaction by formation of $N$-hydroxysulfosuccinimide active esters that hydrolyze very slowly. The reaction was successful as almost 100% of the lysine groups available for coupling were modified.

3.4 Conclusion

In this study, β-cyclocitral-PPD (immunogen) and β-cyclocitral-BSA (solid phase conjugate) were prepared. These conjugates maintained the integrity of β-cyclocitral on the final conjugate, which will be essential in developing and selecting antibodies that are specific for β-cyclocitral. In order to maintain the integrity of β-cyclocitral on the final conjugates, an analog of β-cyclocitral, hydroxycyclocitral was synthesized and linked to the respective molecules through its hydroxyl group. Four and 33 molecules of β-cyclocitral were successfully attached to PPD and BSA respectively.
Figure 3.2a. UV Spectrum of β-Cyclocitral Succinate (Conc = 0.032mg/ml)

Figure 3.2b. UV Spectrum of BSA (0.096mg/ml) and BSA-β-Cyclocitral Conjugate

Figure 3.2c. Differential Spectrum: Conjugate - BSA
CHAPTER IV. PRODUCTION OF CHICKEN MONOSPECIFIC ANTIBODIES (IgY) AGAINST β-CYCLOCITRAL

4.1 Introduction

β-cyclocitral is known to contribute to the off-flavor in channel catfish (Martin and Suffet, 1992). β-cyclocitral and other odorous compounds like geosmin and 2-methylisoborneol (MIB) have posed a threat to water treatment facilities and aquaculture industries. These compounds cause taste and odor problems that have financial implications. In addition to a better understanding of the chemical causes of tastes and odors in drinking water supplies and fish samples, the ability to regularly monitor the odor-causing compounds would help in the control of taste and odor problems in the affected industries. Analytical methods to detect off-flavor in catfish continue to be developed. Polyclonal antibodies and an ELISA test for MIB was developed (Chung et al., 1991) although the test was not sensitive enough to detect MIB at threshold levels (20 ppt in water and 0.7 ppb in fish, Grimm et al, 2000).

The demand for antibodies is increasing not only for immunological purposes but also as therapeutics (Carlander et al., 2003). Antigen-specific antibodies raised in hen have found increased applications in diagnosis and basic research for the detection, estimation and isolation of different molecules in biological systems (Losso et al., 1997). Hens, which have been immunized with an antigen, produce specific antibodies against the antigen. The chicken antibodies, also referred to as IgY, are preferentially transferred from the serum to the egg. Large amounts of antibody (IgY) can be extracted from the eggs of immunized chicken without the need for bleeding or euthanizing the animals, which would be preferred with regards to animal welfare. Compared to a rabbit, which is one of the traditional sources of polyclonal antibody, a laying hen produces more antibodies at a lower cost (Larsoon et al.,
Accordingly, egg yolk antibodies should be an inexpensive way of producing large amounts of specific antibodies.

In this study, polyclonal antibodies against β-cyclocitral were developed in chicken injected with β-cyclocitral-PPD (Purified Protein Derivative of tuberculin) conjugates and anti-β-cyclocitral antibodies were purified from the eggs of the immunized chickens.

4.2 Materials and Methods

β-cyclocitral-PPD and the solid phase (β-cyclocitral-BSA) conjugates were synthesized in Dr. Hammer’s laboratory (Department of Chemistry, Louisiana State University). All buffer components used were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO). BCG was kindly provided by Dr. Tom Gillis (Hansen's Disease Laboratory, School of Veterinary Medicine, Louisiana State University). Freund’s incomplete adjuvant and alkaline phosphatase labeled rabbit-anti chicken antibodies were obtained from Jackson ImmunoResearch Laboratories, Inc (West Grove, PA). Rabbit serum was obtained from Sigma Chemical Co. (St. Louis, MO). Diethanolamine substrate buffer and p-nitrophenyl phosphate (PNPP) substrate for alkaline phosphatase were obtained from Pierce Biotechnology (Rockford, IL). Costar 3590-96-well microtiter plates were from Fischer Scientific (Fairlawn, NJ). Tris-Acetate gels for SDS-PAGE were obtained from Invitrogen (Carlsbad, CA). Epoxy-activated affinity columns were obtained from Waters Division of Millipore Corporation (Milford, MA).

4.2.1 Immunization of Birds

Non-immune serum samples were taken from two white Leghorn chicken and were used as negative controls in the antibody-capture enzyme linked immunosorbent assays (ELISAs) used to test for antibody activity. Since PPD is reported to be a powerful carrier
whose activity is seen only in animals previously exposed to *Myobacterium tuberculosis*, or immunized with Bacillus Calmette-Guerin (BCG) (Lachmann, 1988), one week prior to the first immunization, the animals were sensitized with $10^9$ colony forming units (CFU) of heat-killed BCG per bird, via the intramuscular route. This was equivalent to 2 mg of BCG dissolved in 1 mL of sterile phosphate buffered saline.

The BCG-sensitized chicken were then immunized with 0.5 mg of immunogen ($\beta$-cyclocitral-PPD) in 1 ml of sterile PBS emulsified with Freund’s incomplete adjuvant at a ratio of 1:3 v/v PBS:adjuvant. Injections were intramuscular (pectoral muscle) and boosts were given biweekly until a desired circulating antibody concentration specific to $\beta$-cyclocitral was attained in the serum. Eggs were collected and marked everyday for identification during the immunization period, and for two weeks after the immunization period. The eggs were stored at 4 °C for at least one month until used. Pre-immunization eggs were also collected for 7 days before BCG sensitization.

### 4.2.2 Preparation of Serum

Blood samples (500 µl) were collected bi-weekly from the wing vein (Shimizu *et al.*, 1998) to assess the antibody response. After collection, blood was allowed to clot for 1h at 37 °C. The clot was then separated from the sides of the collection vessel and allowed to contract at 4 °C. The serum was removed from the clot and the remaining insoluble material removed by centrifugation at 8500 RPM for 10 min at 4 °C. The serum was tested for anti-$\beta$-cyclocitral antibody titer using enzyme-linked immunosorbent assay (ELISA). Pre-immune sera were used as negative control, and the antibody response was monitored by comparing the titers of antibodies isolated after successive injections.
4.2.3 Antibody Capture ELISA for Screening Sera

An antibody capture assay in which the antigen is attached to a solid support and antibody allowed to bind was used to monitor the presence of anti-β-cyclocitral antibodies in chicken sera and IgY. To enable the β-cyclocitral to be immobilized on the ELISA plates, it was coupled to BSA. In order to eliminate the selection of antibodies produced against PPD and the linking group, BSA and a different linking group (succinic anhydride) were used to prepare the solid phase conjugate as described in chapter III.

The ELISA plates were coated with 100 μl of β-cyclocitral-BSA conjugate, 50 μg/mL in 0.05 M carbonate buffer, pH 9.35 (plate coating buffer) and incubated overnight at 4 °C. Plates were washed (by flooding and emptying) four times with wash buffer (0.01M PBS containing 0.05 % Tween 20) and blocked (1h, 37 °C) with 200 μl/well of 5 % rabbit serum in wash buffer to block the remaining active sites on the plastic surface. The plates were washed, patted dry, and 50 μl/ well of serum (1:50) dilution or appropriately diluted IgY samples were added and the plates incubated for 1h at 37 °C. After washing and drying the plates, alkaline phosphatase labeled rabbit-anti-chicken antibodies (100 μl) was added and incubated for 2 h at 37 °C. Plates were again washed as above and 100 μl of p-nitrophenyl phosphate (PNPP) substrate solution in diethanolamine buffer was added. Absorbance at 405nm was measured after 30 min incubation at 37 °C using a SpectraMax Plus ELISA plate reader (Molecular Devices, Sunnyvale CA).

4.2.4 Isolation and Purification of IgY

Eggs were collected from the immunized chickens and stored for at least one month at 4 °C. IgY was isolated from egg-yolks using the water dilution method (Akita and Nakayi, 1992, Losso et al., 1998). Egg yolk was separated from egg white and washed with distilled
water to remove as much albumin as possible. The egg-yolk was rolled on paper towels to remove adhering egg white, before puncturing the membrane with a needle and allowing the yolk to pour into a beaker, without the membrane. The separated yolk was diluted 10 times with distilled water acidified with 100 mM HCL, to pH 2.5-2.8. The final yolk solution was stirred for a few minutes to homogenize and the pH adjusted to 5.0.

The diluted egg yolk solution was held overnight at 4 °C then centrifuged at 10,000 x g for 30 min at 4 °C. The supernatant, which contained IgY and other water-soluble proteins, was separated from the precipitate. To the supernatant was added 19% (w/v) of (NH₄)₂SO₄ - ammonium sulphate and the solution was left overnight at 4 °C. After centrifuging at 10,000 x g for 20 min at 4 °C, a precipitate containing IgY was obtained. The purified IgY pellet was dissolved in saline (0.15 M) and spun for 10 min at 10,000 x g at 4 °C to remove undissolved particles. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 10 % acrylamide tris-acetate gels according to the manufacturer’s instructions to determine the purity of the IgY and the effectiveness of the IgY isolation procedure. The protein concentration of the IgY was determined using the BCA assay (Pierce, Rockford, IL) and the activity of anti-β-cyclocitral IgY was determined by ELISA.

**4.2.5 Production of Monospecific Antibodies Against β-cyclocitral**

IgY against β-cyclocitral was purified by affinity chromatography. Epoxy-activated affinity microcolumns (Waters, Milford, MA) were used according to modified manufacturer's instructions. The micro-column was allowed to warm to room temperature, washed with nanopure water followed by equilibration with coupling buffer (0.1 M phosphate buffer with 25 % dimethylformamide, pH 11). Hydroxycyclocitral (100 μmoles) in 1.5 mL of the coupling buffer was added to the column which was stoppered and placed
on a rotator at room temperature for 17 h. β-cyclocitral was covalently attached to the epoxy-activated solid-phase matrix through a hydroxyl group reaction.

The coupled packing was washed with coupling buffer until a spot of the eluent on a thin layer chromatography plate was clear under UV. To block the unreacted epoxy groups, the column was equilibrated with 1 M ethanolamine solution at pH 9.5 in coupling buffer and rotated for 24 h. The column was washed and equilibrated with loading buffer (0.15 M saline).

One μmole (0.18 g) of IgY in 2.5 mL of loading buffer was added to the column, which was then stoppered and rotated for 20 minutes to ensure maximum binding of the β-cyclocitral-specific IgY to the immobilized β-cyclocitral. After the antibodies specific to β-cyclocitral were allowed to bind, the rest of the IgY was removed by washing. The unbound material was washed off the column with saline (0.15 M) until zero absorbance was read for the eluent at 280 nm. The bound protein (monospecific IgY) was eluted with 0.5 M glycine-HCl, pH 2.8 until zero absorbance was read for the eluent at 280 nm. The pH of the eluent containing the monospecific IgY was immediately adjusted to pH 7.0 by adding 1.0 M Tris HCl, pH 9.0 (Konecny et al. 1996). The column was re-equilibrated in loading buffer (0.15 M saline) and a second purification was performed. Electrophoresis was done to determine the purity of the isolated IgY and the activity of the IgY was determined by ELISA.

The monospecific IgY fractions were pooled together and dialysed against ultrapure water to remove glycine and other salts before lyophilizing. The concentration of the IgY was determined by BCA protein assay (Pierce, Rockford, IL) and the solution was lyophilized and stored at -20 °C. The lyophilized IgY was reconstituted with PBS, aliquoted and stored at -20 °C for future use.
4.2.6 Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 10% acrylamide tris-acetate gels according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). The protein was stained using the coomassie blue procedure also according to the manufacturer’s instructions.

4.3 Results and Discussion

4.3.1 Antibody Response in the Chickens

Chickens were immunized with \(\beta\)-cyclocitral-PPD and the anti-\(\beta\)-cyclocitral antibody activity in the chicken serum was monitored using ELISA. Figure 4.1 shows the antibody response in the two birds during the immunization period. Throughout the immunization period, chicken 2 had a superior anti-\(\beta\)-cyclocitral antibody response as compared to chicken 1, which showed a sufficient response in the serum after four immunizations with the immunogen. Until the fourth immunization, chicken 1 gave a poor response to \(\beta\)-cyclocitral. Levels of anti-\(\beta\)-cyclocitral antibodies were enhanced in the two birds after the fourth immunization, i.e. 60-75 days after the initial injection. No anti-\(\beta\)-cyclocitral antibodies were detected in the serum collected from the chickens prior to immunization.

4.3.2 Affinity Chromatography Purification of IgY

Eggs were collected each day through the immunization period and as reported by other authors (Larsson et al., 1993) a yolk volume of 10-15 mL per egg was obtained. IgY was extracted from eggs by the water dilution method (Akita and Nakai, 1992) followed by the ammonium sulfate precipitation method (Losso et al., 1998). Electrophoresis (Appendix I) showed that almost all the IgY was precipitated out of the egg yolk solution. The combined method of Akita and Nakayi (1992) and Losso et al. (1998) was found effective in isolating
IgY out of egg yolk. Appendix J shows the anti-β-cyclocitrinal activity of the IgY precipitated by ammonium sulfate. Compared to the positive control, the activity of the

Figure 4.1. Antibody response in serum during the immunization period

precipitated IgY was low, probably due to the fact that the monospecific IgY is only a small percentage of the overall IgY pool.

The IgY was purified by affinity chromatography and the activity detected by ELISA (Figure 4.2). The figure shows increased activity after affinity purification as compared to before purification (Appendix 4.2). Conversely, the activity of the purified IgY was less than that of the positive serum, which was used as the positive control. This decreased activity was probably due to the purification method of IgY. According to Jensenius et al. (1981), the
immunoglobulin content of the yolk is supposed to be higher than that of the hen’s serum, but this is usually not realized among immunologists.

![Activity of IgY purified from eggs by affinity chromatography](image)

Figure 4.2. Activity of IgY purified from eggs by affinity chromatography. NC is the negative control (serum from unimmunized chicken); PC is the positive control (serum from positive mice); 1-Feb is the antibody isolated from the pooled egg yolks from eggs laid by chicken 1 in the month of February.

A probable reason is the difficulties in purification. In this study, the monospecific antibodies were eluted off the affinity column using a pH of 2.8, which was adjusted to neutral immediately after elution. In spite of this, some activity may have been lost during this process and consequently the activity of the affinity-purified IgY was lower than the activity of the IgY in serum that was not subjected to this harsh elution process. Antibodies are prone to loss of reactivity due to partial denaturation at acidic conditions (Konecny et al., 1996). Affinity chromatography has nevertheless been used by other authors to purify specific IgY
(Gassman et al. 1990) and according to Nakano et al. (1997), affinity chromatography appears to be an appropriate technique to be used to isolate antigen-specific IgY.

Figure 4.3 Affinity Purified IgY. Feb1,2-April1,2 represent the pooled antibodies obtained from the pooled eggs laid by bird 1 or 2 during that month. SDS-PAGE was run on tris-acetate gels (Invitrogen, Carlsbad, CA).

Adjusting the pH of the eluant to neutral shortly after the elution maintained sufficient antibody activity as shown by the Figure (4.2) and further analyses. Figure 4.3 shows that the affinity chromatography yielded relatively pure β-cyclocitral-monospecific antibodies. There are some visible traces of contaminants, probably α- and β-livetins, with molecular weight of 70 kDa and 42 kDa, respectively though, these appear to be in insignificant concentrations. The protein band in the region of 180 kDa (Figure 4.3) is not visible for the control indicating that IgY from the negative control was not able to recognize and bind the β-cyclocitral immobilized on the affinity column. Table 4.1 shows the average amount of antibody recovered per egg laid each day for a specific month. Forty mg-100 mg
of IgY was obtained per egg through the immunization period. The variation may have been dependent on purification techniques and actual antibody content variation in each egg. In the study of Akita and Nakai (1992), over 100 mg of pure IgY was obtained from one egg. Of the 40-100 mg of antibody, 0.60-3.27% was specific to β-cyclocitral, which agrees with the findings of other authors. Hansen et al. (1998) determined that the amount of specific antibody present in egg yolk from an immunized chicken was about 1% (on average) of the total IgY. In this study, one chicken laid six eggs in one week, which is equivalent to 3.3 mg-17.4 mg of anti-β-cyclocitral antibodies. This, compared to an immunized rabbit that yields approximately 20 mL whole blood/week when repeatedly bled (Larsson et al., 1993), is much greater than any amounts of specific antibody that could be produced by a rabbit. Rabbit is the most popular species for polyclonal antibody production (Svendsen et al., 1995).

Table 4.1: Egg Yolk and Antibody Recovery

<table>
<thead>
<tr>
<th>Samples</th>
<th>Average yolk Recovered per egg (g)</th>
<th>Average IgY Recovered per egg (g)</th>
<th>IgY (% Yolk)</th>
<th>Specific IgY per yolk (mg)</th>
<th>Specific IgY (%IgY)</th>
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<tr>
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<td>Bird #</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>14.4</td>
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<td>0.5</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
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<td>14.4</td>
<td>0.04</td>
<td>0.3</td>
<td>0.54</td>
</tr>
<tr>
<td>March</td>
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<td>0.07</td>
<td>0.7</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>2</td>
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<td>0.09</td>
<td>0.7</td>
<td>0.70</td>
</tr>
<tr>
<td>April</td>
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<td>0.10</td>
<td>0.9</td>
<td>1.36</td>
</tr>
<tr>
<td></td>
<td>2</td>
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<td>0.10</td>
<td>0.8</td>
<td>0.60</td>
</tr>
<tr>
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<td>14.2</td>
<td>0.05</td>
<td>0.4</td>
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</tr>
</tbody>
</table>

4.4 Conclusion

Immunization of chickens with β-cyclocitral resulted in production of anti-β-cyclocitral antibodies in chicken serum. Since chickens transport IgY from the serum to the egg as a way of protecting their offspring, anti-β-cyclocitral antibodies transported from the
serum to the eggs were isolated. This study showed that eggs are indeed a convenient source of antibodies. The water dilution method followed by ammonium sulfate precipitation method, were efficient in isolating IgY from the egg yolk. The antibodies purified from the eggs by affinity chromatography showed slightly less activity than the serum antibodies that were not subjected to this purification process. This implies that the affinity chromatography method used in this study would need to be improved in order to reduce loss of antibody activity possibly due to the harsh acidic elution environment. Nevertheless, as determined by ELISA, the anti-β-cyclocitrals antibodies were functional and pure, as determined by SDS PAGE. The low pH elution was a slight impediment to the purification process but this can be improved by minimizing the time the antibodies are in a low pH environment. Affinity chromatography has potential for the production of monospecific polyclonal antibodies since it resulted in a large (0.6-3.3 % IgY) amount of anti-β-cyclocitrals antibodies with sufficient activity.

Laying hens are highly cost effective as producers of antibodies. Polyclonal antibodies produced in chicken against β-cyclocitrals and isolated from eggs in this study can be employed in diagnostic assays. Antibody titers in eggs seemed low compared to the serum. However, eggs, unlike serum, are available in large quantities and the overall antibody produced per chicken in this study was more than would be produced from one rabbit sera. Optimization of the purification process, nonetheless, would result in higher antibody titers. Hyper-immune eggs could become a valuable, inexpensive source of monospecific polyclonal antibodies against selected antigens. Overall, hens were found to be good hosts in which to raise polyclonal antibodies and did not require euthanasia at termination of the project.
CHAPTER V. PRODUCTION OF MOUSE MONOCLONAL ANTIBODIES AGAINST \( \beta \)-CYCLOCITRAL

5.1 Introduction

There are numerous hydrophobic compounds such as toxins, pesticides, off-flavor compounds and others in our environment that need to be measured. However the measurement of these compounds is complicated by extraction and purification steps (Matsuura et al., 1993). \( \beta \)-cyclocitrals is an alkylated cyclic enol that contributes to off-flavor problems in channel catfish (Martin and Suffet, 1992; Zimba et al., 2003).

Off-flavor due to compounds like \( \beta \)-cyclocitrals, MIB and geosmin is a worldwide problem to the aquaculture industry and water treatment and potable water facilities. In order to detect off-flavor compounds in fish and water samples, gas chromatography-mass spectrometry, which is costly, and sensory evaluation, which is subjective, have been used. The ability to routinely monitor these compounds may facilitate management of the aquaculture and water facilities and the development of methods for their control. However the currently available methods do not allow for regular monitoring of the off-flavor compounds.

Monoclonal antibodies are important tools for immunological diagnostics since they are able to distinguish individual antigens with sufficient precision because they possess a unique specificity and an extremely high selectivity for the epitope (Peters and Boron, 1992). Monoclonal antibodies are now standard tools and reagents in numerous scientific disciplines and their availability has expanded applications in diagnostics and therapeutics. Since they are produced by a single clone of B cells, they offer particular advantages such as uniform affinity and specificity of binding, homogeneity, and the ability to be produced in unlimited
quantities (Deshpande, 1996). With the monoclonal antibody methodology, many analytical and clinical applications have relied on the highly specific antigen antibody interaction made possible by this methodology (De Silva et al., 1999) and, monoclonal antibodies could be made to any molecule.

Compounds that are not intrinsically immunogenic (small molecules called haptens) however, need to be conjugated to a carrier in order to elicit an immune response. Keyhole limpet hemocyanin (KLH) and Purified Protein Derivative of tuberculin (PPD), which was reported by De Silva et al. (1999) as having an exceptional ability to elicit a specific antibody response to the antigen of interest without itself eliciting an immune response, were used as the carrier protein for the hapten β-cyclocitral. In this study, monoclonal antibodies against β-cyclocitral were developed in mice injected with PPD-β-cyclocitral conjugates.

5.2 Materials and Methods

PPD-β-cyclocitral, KLH-β-cyclocitral (immunogens) and BSA-β-cyclocitral (solid phase conjugate were synthesized in Dr. Hammer’s laboratory (Department of Chemistry, Louisiana State University). All buffer components used were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO). *Bacillus Calmette-Guérin* (BCG) was kindly provided by Dr. Tom Gillis (Assoc Prof, Hansen’s Disease Lab, School of Veterinary Medicine, Louisiana State University). Alkaline phosphatase labeled rabbit-anti mouse antibodies and Freund’s incomplete adjuvant were obtained from Jackson ImmunoResearch Laboratories, Inc (West Grove, PA). Rabbit serum and mouse serum were obtained from Sigma Chemical Co. (St. Louis, MO). Diethanolamine substrate buffer, desalting columns and *p*-nitrophenyl phosphate (PNPP) substrate for alkaline phosphatase were obtained from Pierce Biotechnology (Rockford, IL). Costar 3590-96-well microtiter plates were from
Fischer Scientific (Fairlawn, NJ). The following buffers were used throughout ELISA experiments: phosphate-buffered saline with Tween (0.01 M PBS containing 0.05 % Tween 20) for washing, 5 % rabbit serum in wash buffer as blocking buffer. Polyethylene glycol 1450 was used for fusion of myeloma and B-cells. The myeloma cell line was SP20 (Ag 14, American Type Culture Collection, Rockville, MD). The BALB/C mice were housed and cared for by the School of Veterinary Medicine (LSU) and all the immunochemistry work was done in Dr. Truax’s laboratory (Pathobiological Sciences, School of Veterinary Medicine, Louisiana State University).

Media and additives

The media used for the fusion process was a complete HT (Hypoxanthine-Thymidine) media with the following composition:

- DMEM + 4.5 g glucose + NaHCO₃ + 15mM Herpes (HyClone, SH 30317)
- 20 % FBS (Fetal bovine serum) (HyClone, SH 30070.3)
- L-Glutamine (Glutamax I), 200 mM (100x) (Invitrogen 35050)
- NEAA (non-essential amino acids, 100x) (HyClone, SH 30238.01)
- Sodium Pyruvate, 100 mM (100x) (HyClone, SH 30239.01)
- Hypoxanthine-Thymidine (Sigma, H-0137)
- 2x Gentamicin (100 µg/ml) (Sigma, G-1397)
- 2x Pen-Strep (200u/ 200 µg/ml) (Sigma, P-0781)
- 10 % conditioned media from J77A. 1 cells and 10 % unconditioned media from Sp 2/m IL-6 cells (American Type Culture Collection)
- OPI (Sigma, O-5003)
- 2ME (2-Hydroxyethylmercaptan) M.W. 78.13 at 1.12 g/ml
Use at 0.05 M concentration (3.48 ml/l)

5.2.1 Immunization

One week prior to the first immunization, two 6-8-weeks old BALB/C female mice were sensitized with $10^8$ colony forming units (CFU) of heat-killed *Bacillus Calmette-Guerin* (BCG) (Orme, 1988) via the intramuscular route. This was equivalent to 0.2 mg of BCG dissolved in 100μl of sterile phosphate buffered saline. The two mice were then given the first injection interperitoneally (ip) with 50 μg of immunogen (PPD-β-cycloctiral) dissolved in 100μl of sterile PBS/ Freund’s incomplete adjuvant (1:3v/v). Two other mice were injected ip with 50 μg/100ml of KLH-β-cycloctiral in PBS/ Freund’s incomplete adjuvant. After 2 weeks, the second immunization was ip with 250μg of immunogen in 500 μl of sterile saline. Two weeks after the second injection a sufficient antigen response was achieved in the serum and the mice were given a final boost of 50 μg of antigen in 100 μl of sterile saline. Three days after the final challenge, the spleens were harvested from the mice.

5.2.2 Sampling and Preparation of Serum

Blood samples were taken two weeks after each injection, allowed to clot for half an hour at room temperature and centrifuged at 10,000 x g for 10 minutes at 4 °C. The sera were collected and tested for anti-β-cycloctiral antibodies using ELISA. Comparing the titers of antibodies isolated after successive injections allowed for the antibody response to be monitored (Harlow and Lane, 1988).

5.2.3 Screening Procedures

Elisa procedures were designed to screen for antibodies that were produced against the antigen (β-cycloctiral) and not PPD or the linking arm (PMPI-SATA). An antibody capture assay in which the antigen is attached to a solid support and antibody allowed to bind
was used. To immobilize β-cyclocitral on the ELISA plates, it was coupled to BSA using a
different linking group (succinic anhydride) in order to eliminate the selection of antibodies
produced against PPD and the linking group (PMPI-SATA).

The ELISA plates were coated with 100 μl of β-cyclocitral-BSA conjugate, 50 μg/ml
in 0.05M carbonate buffer, pH 9.35 (plate coating buffer) and incubated overnight at 4 °C.
The plates were washed (by flooding and emptying) four times with wash buffer (0.01M PBS
containing 0.05% Tween 20) and blocked overnight at 4 °C with 200 μl/well of 5% rabbit
serum in wash buffer to block the remaining active sites on the plastic surface. The plates
were washed, patted dry, and 50 μl / well of serum (1:100) dilution or culture supernatant
was added and incubated for 2h at 37 °C. After washing and patting the plates dry, Alkaline
Phosphatase-labeled rabbit anti-mouse antibodies (100 μl) at 1/5,000 dilution were added and
incubated for 2 h at 37 °C. After a final washing, PNPP (100 μl of 1 mg/ml PNPP substrate
solution), a substrate for Alkaline Phosphatase was added, followed by a 30 min incubation
and the plate read at 405 nm using an ELISA SpectraMax Plus plate reader (Molecular
Devices, Sunnyvale CA). Commercial mouse serum from Sigma served as the negative
control while the positive control was serum obtained from the positive mice (by heart
puncture) during harvesting of the spleen. The plates were incubated stationary.

5.2.4 Myeloma Cell Culture

SP20 myeloma cell line was used for the production of hybridomas in this study. The
cell line was grown in media containing 8-azaguanine to ensure that the cells were HPRT-
negative, in which none of the cells should survive in HAT media. 8-Azaguanine was
removed 2 weeks before fusion of the myeloma cells with the B-lymphocytes.
5.2.5 Recovery of Splenocytes

After two immunizations, the two mice exhibiting high titer of anti-β-cyclocitral antibodies were immunized again. Three days later, the mice were sacrificed by cervical dislocation and the spleen was aseptically removed. Briefly, the mouse was anesthetized, and the heart was punctured to obtain about 500 μl of blood. Serum obtained from this blood was used as the positive control. The mouse was thoroughly wetted with 70 % ethanol and placed under a sterile hood. Splenectomy was performed under sterile conditions. The skin was cut and pulled out of the way being careful not to rupture the peritoneal cavity membrane. The peritoneal cavity was cut open, and the spleen removed. The harvested spleen was placed in an EC collector (50 mesh) on a petri dish containing 20 mL media. The spleen was cut into several pieces and using a plunger from a sterile 10 mL disposable syringe, the spleen was carefully ground and the connective tissue discarded. After removal of the collector from the petri dish, media with cells was drawn up from the petri dish and placed into a 50 mL centrifuge tube. After the large chunks had settled down, the cell suspension was drawn off, placed in another centrifuge tube and centrifuged at 950 x g for 5 min. To remove the red blood cells, the cell pellet was re-suspended in red blood cell lyses buffer (NH₄CL) for 10 minutes after which the remaining cells (splenocytes) were spun at 950 x g for 5 min to remove the lyses buffer. Cells were re-suspended in 10 mL media and a viability count performed on spleen cells to ensure at least 80 % viability. The spleen cells were used immediately for fusion with myeloma cells.

5.2.6 Production of Hybridomas (Cell Fusion Protocol)

To remove the serum (interferes with PEG) from the cells, cells were spun at 950 x g for 5 min and re-suspended in 10 mL media without additives. Spleen cells (B lymphocytes)
and myeloma cells (at least 90 % viable) were mixed at a ratio of 1 to 5 times more spleen cells than myeloma cells and spun at 950 x g for 5 min. One mL of PEG (50%PEG to 50% media) at 37 °C was added to the cell pellet over a thirty-second time period and left to stand in a 37 °C water bath for 1.5 minutes with occasional stirring. The cell mixture was diluted with 37 °C media (no additives), centrifuged (5min, 700 x g) and re-suspended in complete media containing HT (Hypoxanthine-Thymidine). Twenty 96-well plates of the fused cells were prepared and labeled with fusion number, plate number and date. One hundred μl of cell suspension was added per well, and the plates incubated in a 37 °C humidified incubator. Cells were re-fed with 100 μl per well of growth media containing HAT (aminopterin) on days 1, 2, 3, and 7 and observed daily for pH, color change and confluency. To re-feed the cells, 100 μl were removed from the culture plates using a sterile Pasteur pipette and vacuum aspirator.

5.2.7 Culture, Screening, and Selection of Hybridomas

Feeding of the cells was continued with growth media + HT without aminopterin, conditioned media and 2 ME, being careful not to allow the wells to become too acidic (bright yellow). The supernatants from the wells that were 25 % to 50 % confluent were tested for antibody production using the ELISA described above. Fifty μl of each supernatant was incubated for 2 h at 37 °C on microtiter plates that were previously coated (100 μl of 50 μg/ml of β-cyclocitral-BSA), blocked (200 μl of 5 % rabbit serum in wash buffer) and washed (4 x). After testing, the wells that were positive for anti-β-cyclocitral antibodies were pulled and expanded to 24 well plates containing 1 to 2 ml growth media. The 96 well plates were re-fed as a backup, in case of plate contamination.
5.2.8 Cell Cloning

The expansion wells were re-tested for anti-β-cyclocitrinal antibody production and the positive cells were cloned using limiting dilution method. Positive wells were diluted by taking 25 µl from the expansion wells into 75 µl of cloning media (20 % conditioned media). The cells were serial diluted (1:2) in row A to G and from column 1 to column 12. Plates were checked for growth on day 7. Single clusters in wells would indicate one-cell colonies. Clones were tested for anti-β-cyclocitrinal antibodies and the positive wells were expanded to 24-well plates and then to 2 mL wells. Growth was observed and once a heavy concentration of cells was reached, one well was frozen and the others transferred to 25-cm² flasks.

5.2.9 In Vitro Production of Monoclonal Antibodies

Monoclonal antibodies against β-cyclocitrinal were produced using 75-cm² flasks. Media that was low in serum (10 %) was used for this purpose. Monoclonals were grown to heavy concentration in 50 mL of complete DMEM media until 80 % death.

5.2.10 Purification of Monoclonal Antibodies

Antibodies were purified using caprylic acid precipitation method according to Harlow and Lane (1988), Steinbuch and Audran (1969) and Russ et al. (1983). The addition of short-chain fatty acids such as caprylic acid precipitates most serum proteins with the exception of IgG molecules (Harlow and Lane, 1988). Since the culture supernatant has mainly the serum proteins from the serum added to the growth media and the monoclonal antibody produced by the hybridoma as the main proteins, this method of purification was appropriate for this study.

The antibody-containing supernatant was pooled and transferred into a beaker. Two volumes of 0.06M sodium acetate buffer, pH 4.0 were added and the pH adjusted to 4.8 using
sodium hydroxide. Ten mls of caprylic acid (3.9 g) were added drop-wise with vigorous mixing and the mixture was stirred for a further 30 min before centrifuging (10,000 g, 30 min, 4 °C). The supernatant containing the IgG was transferred to a dialysis tubing and dialyzed against PBS. The dialyzed solution was concentrated, protein determined and gel electrophoresis performed.

5.2.11 Freezing, Storage and Thawing of Cell line

Healthy cells, approximately 10⁶ to 10⁷ cells, were centrifuged at 250 x g for 5 min, re-suspended in complete media with 10 % dimethylsulfoxide (DMSO), placed into 2 mL cryogenic vials and stored at –80 °C in liquid nitrogen.

Cells to be used were thawed quickly after removal from liquid nitrogen in a 37 °C water bath until the ice just melted. The cells were aseptically transferred into 10ml of complete media and mixed thoroughly, and then centrifuged at 250 x g for 5 min. Cells were re-suspended in complete medium and cultured.

5.3 Results and Discussion

5.3.1 Serum Antibody Response to β-Cyclocitral and Production and Selection of Hybridomas

The antibody-capture ELISA was aimed at evaluating the antibody responses to β-cyclocitral. Figure 5.1 shows the increase in antibody over the immunization period for the two mice that were immunized with β-cyclocitral-PPD conjugate. The serum from both mice showed high titers after two immunizations. Mouse #2 showed the highest titer of antibody; however, both mice were used as spleen cell donors for fusion with myeloma cells. This fusion resulted in hybridoma cultures equivalent to twenty 96-microtiter plates. After fusion, the plates were examined daily for pH and colony growth. After 10-15 days, the supernatant in wells showing large colonies were tested for anti-β-cyclocitral antibody production using
ELISA. About 70 wells were positive for anti-β-cyclocitral antibodies out of 1900 wells containing growing hybridoma culture.

![Graph showing absorbance 405nm over immunizations for Mouse 1 and Mouse 2.](image)

**Figure 5.1** Anti-β-cyclocitral antibody during the immunization period

Figure 5.2 shows the results of some of the hybridoma cell lines that were positive for production of antibodies against β-cyclocitral. The positive cells were selected for cloning using the limiting dilution method. Hybridoma line one was cloned once and cells from the highest dilution were tested for anti-β-cyclocitral antibodies. This cloning resulted in strong and healthy clones, indicating the possibility that the cells growing in each of the 32 wells
were derived from a single parent cell (Zola, 1987). Appendix K shows the anti-β-cyclocitral activity of the clones of hybridoma cell line 1 (1-85).

![Graph showing absorbance at 405 nm for different samples: NC, PC, HB line 1, HB line 2, HB line 3, and HB line 4.]

Figure 5.2. Results of Hybridoma screening process

The highest producers of anti-β-cyclocitral antibodies were cloned in order to obtain stable positive producers. At least 10 stable hybridoma cell lines secreting monoclonal antibodies to β-cyclocitral were established.

This study resulted in production of high antibody response against β-cyclocitral probably due to the use of PPD as a carrier protein for the preparation of the immunogen. According to De Silva et al. (1999), PPD-coupled antigen immunized mice generated a higher percentage of antigen-specific hybridomas compared to the other carrier proteins. De Silva et al. (1999) compared the carrier properties of KLH (Kehole limpet hemocyanin),
OVA (Ovalbumin) and PPD and their results showed that when KLH was used as the carrier, the immune response was far superior to that of the attached antigen while the immune response to PPD was significantly lower compared to that for the attached antigen. As a consequence, more hapten-specific antibodies are produce when PPD is used as a carrier, as compared to when KLH is used. PPD is a T cell antigen and does not elicit a favorable immune response (against it self). However this makes PPD a good carrier molecule that adds the T-cell component to the hapten and results in production of hapten specific antibodies (Lachman et al., 1986). This study also showed an inferior antibody response to β-cyclocitral by the mice immunized with β-cyclocitral-KLH conjugates as compared to the mice immunized with β-cyclocitral-PPD conjugates (Appendix L).

5.3.2 Antibody Purification

Once stable hybrid clones secreting anti-β-cyclocitral antibodies were obtained, a batch of monoclonal antibody was produced and semi-purified. The cell lines were maintained in DMEM medium and sub-cultured to keep the cell density within the range of $10^5$-$10^6$ cells/ml. The collected supernatants were combined and the monoclonal antibody purified using caprylic acid precipitation, dialyzed and stored at $-20$ °C. Figure 5.3 shows the gel electrophoresis of the mouse-generated antibodies against β-cyclocitral. About 1.3 mg/ml of antibodies were produced using the tissue culture flask method regardless of the extremely small size of the hapten (β-cyclocitral). The gel electrophoresis however shows that the caprylic acid precipitation method was not able to yield a completely pure antibody. Although the antibodies need further purification, the anti-β-cyclocitral antibody did not bind protein G. Protein G, a bacterial cell wall protein isolated from group G streptococci, binds to mammalian and mouse IgG. According to Harlow and Lane (1988), a successful antibody
puriﬁcation method will also depend on the type and sub-type of antibody. Therefore, isotyping the anti-β-cyclocitrinal antibody will aid in determining an optimal puriﬁcation method especially in the case of bulk production of this antibody.

Figure 5.3 Gel electrophoresis of the mouse-generated antibodies against β-cyclocitrinal

5.4 Conclusion

Monoclonal antibodies against β-cyclocitrinal, a compound posing off-ﬂavor problems in water, catﬁsh and other aquaculture industries, were developed. At least 10 stable cell lines are available that can be used anytime for bulk production of the antibody in order to develop and establish immunoassays for the detection of β-cyclocitrinal in catﬁsh and water environments. A miniPERM Bioreactor (VIVAScience, Germany) which is a reusable minifermenter can be used for the large scale production of anti-β-cyclocitrinal monoclonal antibodies using the established cell lines. Isotyping the monoclonal antibody will aid in the development of more efﬁcient puriﬁcation methods for the developed anti-β-cyclocitrinal
antibodies. Using PPD as the carrier molecule probably made production of anti-β-cyclocitral antibodies much easier. According to De Silva et al. (1999), using PPD as a carrier molecule resulted in reduction of carrier-specific hybridomas and increased hapten-specific antibodies, thus making monoclonal antibody production much easier.
CHAPTER VI. DEVELOPMENT OF CHICKEN MONOSPECIFIC AND MOUSE MONOCLONAL-BASED ENZYME IMMUNOFLOW ASSAYS FOR THE DETECTION OF β-CYCLOCITRAL IN CATFISH POND WATER

6.1 Introduction

There is an interest in the identification of volatile odor compounds produced by cyanobacteria and algae (Ikawa et al., 2001). Some of the known compounds are geosmin and 2-methyllisoborneol (MIB), which impart musty odors, and β-cyclocitral, which imparts different off odors (hay, grassy, smoky, woody) depending on the concentration (Young et al., 1999). In catfish aquaculture, off-flavor is a complex problem with multiple chemical etiologies. Of recent interest is the role β-cyclocitral plays in off-flavor within catfish aquaculture (Martin and Suffet, 1992). In their study, Martin and Suffet (1992) isolated β-cyclocitral from all the ponds producing off-flavored fish and not from ponds producing on-flavored fish. Other authors have reported off-flavors in catfish (e.g. woody and decaying vegetation) that implicate β-cyclocitral (Tucker and van der Ploeg, 1999). Zimba et al. (2003) identified β-cyclocitral in over 80% of the 485 catfish production ponds surveyed.

The difficulty in selling the off-flavor fish leads to serious consequences for commercial fishery and depuration of off-flavor compounds occurs in varying periods of time depending on the type of off-flavor. Tucker and van der Ploeg (1999) reported a case in which channel catfish with woody off-flavor were held in clean flowing well water for 21 days with only a modest improvement in flavor quality. However, the intense MIB off-flavor was completely purged from the fish in only 4 days under the same conditions. Effective control of the taste and odor problems has been difficult due to the numerous algal blooms and different species of algal monocultures as well as the many factors responsible for odors in water (Cotsaris et al., 1995). Meanwhile there is a need for constant monitoring of the
water for off-flavor compounds, which would enable timely and cost effective management of the off-flavors. According to Tucker and van der Ploeg (1999), a routine program for monitoring fish off-flavor could aid in finding a period in which flavor quality is acceptable and fish may be sold, and also aid in knowing when the problems might be treated with the greatest possibility of success. Lack of access, however, to appropriate laboratory facilities and the high cost of analyses has been prohibitive to routine analysis (Jones and Korth, 1995).

The need for a continuous monitoring of several pollutants has increased the development of new techniques for the detection and control of these compounds (Sardinha et al., 2002). Analytical methods to detect off-flavor in catfish continue to be developed and great progress has been made in instrumental determination of the muddy/musty off-flavors, for both MIB and geosmin (Grimm et al., 2000). The current analytical methods are however, labor intensive, require expensive analytical equipment or are not sensitive enough to detect off-flavor at meaningful concentrations (Malleret et al., 2001). Sensory evaluation, the only rapid and somewhat cost effective method for detection of off-flavors, is somewhat subjective. In the case of β-cyclocitrinal, whose odors are concentration-dependent (between 2 and 20 µg/l it has a hay/woody odor and a tobacco type of odor at higher concentrations), this method may not be effective.

Faster and less expensive methods would find broad application in catfish flavor research, within the catfish processing industry as well as other aquaculture industries plagued by this problem (Lloyd and Grimm, 1999). Immunoassays are considered an alternative to the expensive classical chromatographic analysis since they have gained acceptance as fast, sensitive and cost-effective alternatives (Park et al., 2002).
An ELISA test for MIB was developed by Chung et al. (1991) although the test currently lacks the sensitivity to detect MIB at threshold odor concentrations. The goal of this study was to develop a simple method that meets the requirements of the catfish producers and processors, in the form of an enzyme-linked immuno-flow assay (ELIFA) that can be used for the detection of β-cyclocitrinal in catfish pond water.

6.2 Materials and Methods

β-Cyclocitrinal was purchased from Sigma Chemical Co., St Louis, MO. Pond water samples were obtained from ten ponds at Ben Hur, Aquaculture Research Station, Louisiana State University Agricultural Center in Baton Rouge, LA. Chicken polyclonal antibodies against β-cyclocitrinal were isolated from eggs laid by hens immunized with β-cyclocitrinal-PPD (purified protein derivative of tuberculin) conjugate. Mouse monoclonal antibodies were produced from fusion of splenocytes of BALB/c mice immunized with β-cyclocitrinal-PPD conjugate. Ultra pure water purified by Milli-Q system (Millipore Corp., Milford, MA) was used in all experiments. All chemicals and solvents were of analytical grade. Costar 3590-96-well microtiter plates were from Fischer Scientific (Fairlawn, NJ). Phosphate buffered saline (PBS) solution was prepared by dissolving NaCl (8.064 g), disodium hydrogen phosphate (0.227 g), monobasic sodium phosphate (1.15 g) and KCl (0.201 g) in 1 L of ultra pure water and the pH adjusted to 7.4 with 1 N NaOH. To prepare PBS with Tween (PBST), 0.5 mL of Tween 20 was added. Five percent rabbit serum in PBST was used as the dilution buffer. The ELIFA device was acquired from Pierce Biotechnology (Rockford, IL). The flow-rate was controlled by a Masterflex pump (Cole-Parmer Instrument Co., Vernon Hills, IL).
6.2.1 Development of a Competitive Enzyme-Linked Immunosorbent Assay (ELISA)

To test the ability of the anti-β-cyclocitrail IgY to bind free β-cyclocitrail, an indirect competitive ELISA was performed. β-Cyclocitrail standards ranging from 1 pg/ml to 2000 μg/ml were prepared. Microtiter plates were coated with 50 μg/mL of β-cyclocitrail-BSA in 0.05 M carbonate buffer, pH 9.35 (plate coating buffer) and held overnight at 4 °C. The wells were blocked overnight at 4 °C with 200 μl of 5 % rabbit serum in PBS with Tween 20. After washing four times with 0.01M PBS containing 0.05 % Tween 20 (wash buffer), 100 μl of β-cyclocitrail-IgY or β-cyclocitrail-IgG mixtures were added to the plates and incubated for 2h at 37 °C. The β-cyclocitrail solutions at different concentrations were previously incubated with an equal volume of 25 μg/mL IgY or IgG for an hour. One hundred μl of rabbit anti-chicken or rabbit anti-mouse alkaline phosphatase conjugate at 1:10,000 dilution was added to each well of the corresponding incubated and washed plate. The plates were again incubated for 2 h at 37 °C, washed and the alkaline phosphatase substrate, PNPP (100 μl/ well; 1 mg/mL) in diethanolamine buffer was added. After 30-min incubation at 37 °C, the plates were read at 405 nm using ELISA SpectraMax Plus plate reader (Molecular Devices, Sunnyvale CA). Figure 6.1 shows the schematic representation of the competitive ELISA used in this study.

A sandwich assay was unsuccessfully attempted. In this assay, both chicken IgY and mouse IgG against β-cyclocitrail were tried as antigen capture antibodies and sandwicching antibodies in alternate assays; however, no quantifiable reaction was obtained.
A) Coating of the solid phase with antigen (β-cyclocitral-BSA)

B) Blocking of unoccupied adsorption sites with rabbit serum

C) Incubation with antibody ( ) and competitive antigen (free β-cyclocitral in solution)

D) Washing of the unbound antibody and incubating with rabbit anti-species (chicken or mouse) enzyme-labelled (alkaline phosphatase) antibody

E) Washing of the unbound enzyme-labelled antibody and incubation with substrate ( ) and determination of the colored product ( )

Figure 6.1 Schematic representation of the competitive ELISA procedure used in this study

6.2.2 Development of Enzyme-Linked-Immuno-Flow-Assay (ELIFA)

An ELIFA unit was purchased from Pierce Biotechnology (Pierce, Rockford, IL). Figure 6.2 shows the ELIFA set up. To immobilize the antigen, β-cyclocitral-BSA, on a wet nitrocellulose membrane embedded in the ELIFA unit, 200 µl of a 50 µg/mL antigen was placed into all of the 96 wells of the sample application plate. The solution was then filtered
Figure 6.2 Easy-Titer™ ELISA (Pierce Biotechnology, Rockford, IL) Setup. It utilizes a sheet of nitrocellulose sealed between two gaskets as the adsorptive surface. A sample application plate in the typical 8x12 microtiter plate format is clamped on top of the membrane under which 96 individual canulae empty into an enclosed lower chamber. A peristaltic pump is attached to this chamber to create a vacuum which pulls the reagents past the membrane at a given flow rate. In the final step the substrate/chromogen solution is pulled through the membrane and the product is collected into a microtiter plate held in the lower chamber. The plate is then removed and read on a plate reader.
through the unit at 40 μl/minute. The remaining protein binding sites on the nitrocellulose membrane were blocked by filtering a solution of 5% rabbit serum in PBST through the ELIFA unit also at a 40 μl/minute flow rate. The rest of the solutions were filtered through the membrane as follows: (a) 200 μl of β-cyclocitral-IgY and β-cyclocitral-IgG pre-incubated as discussed in the ELISA above were filtered through at a 20 μl/minute flow rate; (b) 200 μl of a 1:10,000 dilution of rabbit anti-chicken or rabbit anti-mouse alkaline phosphatase-labeled antibodies were filtered through at 20 μl/minute; (c) Three 30-second washes with 200 μl each of PBS were performed in order to clean any labeled secondary antibody from cannulas and tips; (d) 200 μl of a 1 mg/mL substrate (p-nitrophenyl phosphate [PNPP]) solution was added to the wells and filtered through the unit at 40 μl/minute. The color produced was quantitatively transferred via the canulas to the microtiter plate which was read at 405 nm in an automatic ELISA plate reader (SpectraMax Plus plate reader, Molecular Devices, Sunnyvale, CA). The ELIFAs took about 1 h to perform and the procedure was a modification of the manufacturer’s instructions.

6.2.3 Water Samples

Water samples were collected from 10 ponds at BenHur Aquaculture research Station (Louisiana State University). The samples were placed into acid-washed and base-washed glass bottles and covered with glass stoppers. Six ponds out of the 10 contained catfish. Samples were stored in a cooler containing chilled water to protect from degradation prior to analysis.

6.2.4 Quantification of β-cyclocitral in Raw Pond Water Samples Using the Competitive ELISA

Calibration curves were developed using 0.1ng/ml to 2 mg/mL β-cyclocitral in ultra pure water. Optimum concentrations of antibody and solid phase conjugate were determined
by using 50, 25, 10 and 1 μg/ml concentrations of both antibody and solid phase conjugate in the above ELISA. Twenty-five μg/mL and 50 μg/mL of antibody and solid phase conjugate respectively were adapted in this study. The calibration curve was constructed with $A_{405}$ (Absorbance at 405 nm) as the y-axis and logarithm of β-cyclocitral concentration on the x-axis. The equations for the calibration curves were determined using regression analysis. Standards and raw water samples were always analyzed on the same plate using triplicates within each plate and replicate plates.

**6.2.5 Correlations with GC/MS**

In order to test whether the developed immunoassays were giving the correct values, the same standards and pondwater samples that were analyzed by the IgY-based ELISA were also analyzed by SPME-GC/MS (USDA-SRRC). Water analysis by solid-phase microextraction and gas chromatography with mass spectrometry was done in Dr. Grimm’s laboratory (United States Department of Agriculture-Southern Regional Research Center, New Orleans, Louisiana). Standards and pond-water samples were processed in the laboratory about 4 h after sampling. Six mL of each pond water sample was treated with sodium chloride and sealed before GC analysis. The NaCl drives the volatiles out of solution into the head space of the sealed vial. The solid-phase probe was allowed to equilibrate with the volatiles in the head space for 15 minutes with agitation at 65 °C. The solid-phase probe was then desorbed in a 250 °C injection port of the GC/MS for 1 minute. The GC/MS operating conditions are shown in the Table 6.1 below.

**6.2.6 Data Analysis**

Calibration curves were constructed both for the standards analyzed by GC and those analyzed by the immunoassays. Since ELISA data spanned a large concentration
Table 6.1 GC/MS operating conditions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC Column</td>
<td>DB-5 (J&amp;W Sci.) 30m x 0.250 mm dia., 0.5 μm film</td>
</tr>
<tr>
<td>Injection size</td>
<td>SPME - 1 cm fiber possessing a Carboxen/DVB/PDMS (Supelco, Inc. Bellefonte, Pennsylvania)</td>
</tr>
<tr>
<td>Injection mode</td>
<td>Slitless 1 min</td>
</tr>
<tr>
<td>Carrier gas</td>
<td>Helium at 1 mL/min</td>
</tr>
<tr>
<td>Column program</td>
<td>1 min at 80 °C - 120 °C/10 °C/min – 280 °C/25 °C/min hold 3 min</td>
</tr>
<tr>
<td>Transfer line</td>
<td>280 °C</td>
</tr>
<tr>
<td>Ionisation source</td>
<td>150 °C</td>
</tr>
<tr>
<td>Ionisation energy</td>
<td>70 eV</td>
</tr>
<tr>
<td>Scan program</td>
<td>50-300 Da</td>
</tr>
</tbody>
</table>

range, the concentrations were plotted as their logarithms. The absorbance readings of the titrated β-cyclocitral revealed a sigmoidal dose response curve in the competitive ELISA. Sigmoidal curves were fitted to a four-parameter logistic equation using GraphPad Prism Version 4.0 (GraphPad Software Inc., San Diego, CA). The four parameters were obtained by the method of least squares and resulted in the following equation that was used to calculate the IC₅₀ values. The IC₅₀ values, which are the concentrations of β-cyclocitral that inhibited 50% of maximum signal, were considered an estimate of sensitivity of each assay.

\[
y = B + \frac{(T-B)}{\left(1 + 10^{(L-x)*H}\right)}
\]

Where \( y \) is absorbance at 405 nm, \( B \) is the baseline response, the \( y \) value at the bottom plateau of the curve, \( T \) is the maximum response, the \( y \) value at the top plateau of the curve, \( y \) starts at the bottom and goes to top with a sigmoid shape. \( L \) is LogIC₅₀ which is the
concentration giving 50% reduction in y, H is the slope factor which quantifies the steepness, and x is logarithm of concentration. A standard slope factor of 1.0 was used.

6.3 Results and Discussion

6.3.1 ELISA

An ELISA using anti β-cyclocitral IgY and IgG as sandwiching antibodies in the detection of β-cyclocitral in solution was unsuccessful. In order to develop a successful sandwich assay, the two antibodies should bind to non-overlapping epitopes on the antigen (Harlow and lane, 1988). This means that both types of antibodies (IgY and IgG) developed against β-cyclocitral recognize the same site on β-cyclocitral and cannot yield a successful sandwich assay. This is possibly due to the size of the β-cyclocitral molecule which would probably not allow for the presence of more than one epitope.

![β-cyclocitral](image)

**Figure 6.3 β-cyclocitral**

Indirect competitive ELISAs were developed using anti- β-cyclocitral IgY and IgG antibodies and β-cyclocitral-BSA as the coating antigen. Figure 6.4 shows a typical calibration curve obtained from the IgG-based competitive ELISA. Appendix M shows the curve fit parameters and the R-squares of the sigmoidal curves obtained for the immunoassays. The sigmoidal curves that were a result of replications within and between plates resulted in I₅₀ values of 3.93ng/mL and 7.98ng/mL for the anti-β-cyclocitral IgY and IgG based ELISAs respectively (Table 6.2).
Figure 6.4. Calibration graph for β-cyclocitrin based on an indirect competitive IgG-based ELISA. Free β-cyclocitrin in solution was allowed to compete with β-cyclocitrin (β-cyclocitrin-BSA) immobilized on polystyrene for anti-β-cyclocitrin IgG. Addition of alkaline phosphatase conjugated-rabbit-anti-mouse IgG followed by a chromogenic substrate revealed the antigen-antibody reaction, which was quantified by spectrophotometric determination of the resulting color. A high amount of free β-cyclocitrin results in less antibody binding the immobilized β-cyclocitrin and leads to less color development.

Table 6.2 Comparison of $I_{50}$ values for ELISAs

<table>
<thead>
<tr>
<th>Assay</th>
<th>$I_{50}$ (ng/mL)</th>
<th>Standard deviation (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgY-based ELISA</td>
<td>3.93</td>
<td>1.31</td>
</tr>
<tr>
<td>IgG-based ELISA</td>
<td>7.98</td>
<td>2.66</td>
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</table>

These low $I_{50}$ values are an indicator of the sensitivity of the developed ELISAs. They represent the amount of β-cyclocitrin in solution that would reduce the binding of the antibody to the immobilized β-cyclocitrin by 50%. Both ELISAs were sensitive in that less than 0.5 μg/mL of β-cyclocitrin in solution was needed to reduce binding of the respective antibody to the immobilized β-cyclocitrin by 50%. The ELISAs showed a detection limit of 1 ng/mL which is below the human threshold (5 μg/L, 5 ng/mL). The monoclonal antibody seemed less sensitive than the IgY probably due to impurities since the antibody was used
without further purification after caprylic acid precipitation of the serum proteins. Both ELISAs could be optimized to obtain better sensitivity.

6.3.2 ELIFA

Enzyme-linked immunoflow assays were developed basing on the ELISAs that had already been developed. The ELIFAs took about 1 h to complete each assay as compared to 7 h of actual assay time needed to perform each ELISA. Figure 6.5 shows a typical calibration curve obtained from the IgY-based competitive ELIFA and Appendix M shows the curve fit parameters.

![Calibration Curve](image)

Figure 6.5 Calibration graph for β-cyclocitrinal based on an indirect competitive IgY-based ELIFA. Free β-cyclocitrinal in solution was allowed to compete with β-cyclocitrinal (β-cyclocitrinal-BSA), immobilized on nitrocellulose membranes, for anti-β-cyclocitrinal IgY. Filtration of alkaline phosphatase conjugated- rabbit-anti-chicken IgG solution followed by filtration of a chromogenic substrate revealed the antigen-antibody reaction, which was quantified by spectrophotometric determination of the resulting color. A high amount of free β-cyclocitrinal results in less antibody binding the immobilized β-cyclocitrinal and leads to less color development.

The results indicated much higher \( I_{50} \) values for both IgY- and IgG-based ELIFAs (Table 6.3) as compared to the corresponding ELISAs (Table 6.2). \( I_{50} \) values of 46 and 93
μg/mL for IgY and IgG respectively, indicate that as much as 46 and 93 μg/ml of β-
cyclocitrinal per ml of solution is needed to inhibit the binding of the respective anti-β-
cyclocitrinal antibody to the immobilized β-cyclocitrinal by 50%.

Table 6.3 Comparison of $I_{50}$ values for ELIFA assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>$I_{50}$ (μg/mL)</th>
<th>Standard deviation (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgY-based ELIFA</td>
<td>46.32</td>
<td>0.0014</td>
</tr>
<tr>
<td>IgG-based ELIFA</td>
<td>93.07</td>
<td>0.0015</td>
</tr>
</tbody>
</table>

These values compared to the 3.9 ng/mL and 9.0 ng/mL for IgY and IgG-based ELISAs
indicate that ELISA was much more sensitive than the ELIFA under the conditions of this
study. This reduced sensitivity in the ELIFA compared to the ELISAs has been reported by
other authors (Shields et al., 1991; Dupont et al., 1990) and it arises due to various factors.

Some of the reasons reported to affect the sensitivity of the ELIFA are; lower binding
capacity of proteins on nitrocellulose membrane as compared to the polystyrene, desorption
of proteins from the nitrocellulose membrane during the various filtration steps, the flow of
substrate passing the enzyme in the final step of the ELIFA may not allow for the most
efficient conversion to colorimetric product, and the speed of flow for the coating step may
not allow sufficient binding of antigen to the nitrocellulose (Shields et al., 1991). The lower
sensitivity shown by the IgG-based ELIFA compared to the IgY-based ELIFA was probably
due to the purity of the monoclonal antibody.

The sensitivity of the ELIFA may be increased by optimizing each of these factors,
for example, slowing the antigen adsorption step may increase the amount of antigen
adsorbed on the nitrocellulose membrane since the amount of protein, that binds to the
nitrocellulose membrane is dependent on the rate of flow of molecules past the reaction
surface. Optimization of the ELIFA has been reported by Pinon et al. (1990), who obtained an ELIFA with a sensitivity of 1-5 ng. The incentive for optimizing the ELIFA for use in the catfish industry is that it can be advantageous when large volumes of small concentration need to be assayed and rapid assay completion is desired, which would be the case with determination and monitoring of seasonally varying off-flavor metabolites in catfish pond water. Although the ELIFA method is semi-quantitative (Dupont et al., 1990), sensitivity can be increased by filtering larger volumes of dilute samples several times through the unit. Allowing the antigen solution to incubate on the membrane for a few minutes before it is sucked through the membrane could also increase the sensitivity, although care should be taken not to lose the time-saving advantage.

6.3.3 Comparison of GC and ELISAs for Pond Water Analysis

Table 6.4 compares β-cyclotiral concentrations obtained by ELISA and GC/MS-SPME. Gas chromatography-mass spectrometry coupled with solid phase microextraction (GC/MS-SPME) was run for comparison to validate the immunoassays.

<table>
<thead>
<tr>
<th>Sample/Pond #</th>
<th>IgY-based ELISA</th>
<th>GC/MS-SPME</th>
<th>IgG-based ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Beta-cyclotiral concentration (ng/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td>Stdev</td>
<td>Average</td>
</tr>
<tr>
<td>B3</td>
<td>4.37</td>
<td>0.38</td>
<td>46.55</td>
</tr>
<tr>
<td>B4</td>
<td>2.73</td>
<td>1.25</td>
<td>2.64</td>
</tr>
<tr>
<td>B5</td>
<td>3.17</td>
<td>0.47</td>
<td>1.35</td>
</tr>
<tr>
<td>B6</td>
<td>2.37</td>
<td>0.32</td>
<td>33.01</td>
</tr>
<tr>
<td>B7</td>
<td>2.54</td>
<td>0.23</td>
<td>3.17</td>
</tr>
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<td>0.17</td>
<td>4.65</td>
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<td>0.43</td>
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</tr>
<tr>
<td>R1</td>
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<tr>
<td>R7</td>
<td>1.29</td>
<td>0.19</td>
<td>1.35</td>
</tr>
</tbody>
</table>
The pond water samples were analyzed for β-cyclocitral by both GC/MS-SPME and the IgY- and IgG-based ELISAs developed in this study. The β-cyclocitral concentrations of the unknowns (pond water samples) were determined from the calibration curves developed from each method. The results (Table 6.4) showed that the β-cyclocitral concentrations predicted in the unknowns by the ELISAs and the GC-MS were not significantly different (P>0.05) when the GC-MS predicted low concentrations (1-4 ng/mL). For the three pond water samples that the GC-MS predicted as having high β-cyclocitral concentrations (46.55, 33.01 and 19.45 ng/mL), both IgY- and IgG-based ELISAs predicted very low values. This indicated a correlation of about 70 % between the GC-MS and the developed ELISAs.

The failure of the ELISAs to predict the high β-cyclocitral concentrations predicted by the GC-MS is a concern that needs further attention. It could be due to some interfering substances in the pond water matrix since the GC-MS/SPME uses a sample of volatiles separated from the pond water matrix (by the NaCl) while the ELISAs make use of the pond water sample as is. Another possible reason could be due to the difference in the time of analysis. The GC-MS samples were prepared for analysis about 4 h after sampling whereas the ELISAs were performed about 24 h after sampling. All these factors, including other factors that would inherently affect ELISA procedures e.g. incubation times, the need to be investigated in order to improve these assays that have a potential for revolutionizing the management of the off-flavor problem in the catfish industry.

6.3.4 Pond Water Analysis by ELISA and ELIFA

Both of the ELIFAs developed in this study were unable to determine the concentration of β-cyclocitral in the unknowns. This was probably due to the high I_{50} values of the IgY and IgG based ELIFAs (46 and 93 μg/mL respectively) obtained. These I_{50} values
indicate that as much as 46 μg/mL (in case of IgY) and 93 μg/mL (in case of IgG) of β-cyclocitril is required in the test solution to reduce the binding of the respective antibody to the immobilized β-cyclocitril by 50%. This is a huge amount of β-cyclocitril needed in the solution before these ELIFAs can be effective. Table 6.4 shows that the highest concentration of β-cyclocitril in the pond water samples obtained was about 46 ng/mL, which must have been outside the detection range of the ELIFAs which were not that sensitive. The pond water samples were also directly applied to the ELISA method without an apparent need for sample cleanup; however, these same samples seemed to have interfering substances that made filtering through the ELIFA unit, which had a 0.45 μm nitrocellulose membrane as the adsorptive surface, a very difficult task. In order to improve the ELIFA, pond water samples would need to be cleaned or diluted before filtration across the nitrocellulose membrane.

6.4 Conclusions and Recommendations

The lowest detectable concentration of β-cyclocitril by the developed ELISAs was 1 ng/mL, which was less than the human threshold of β-cyclocitril in water. The correlation between GC and the developed ELISAs was relatively good at low concentrations. However the ELISA procedures seemed unable to predict β-cyclocitril concentrations greater than 4 ng/ml. The cause for this outcome needs to be investigated and more GC/ELISA tests need to be carried out before conclusive results can be made about the methods developed. The immunoassays need to be correlated with GC using a larger number and variety of samples than used in this study.

Comparing the ELISA and ELIFA methods for their ability to detect β-cyclocitril in pond water samples revealed that ELISA was more sensitive than the ELIFA but ELIFA was easier and quicker to perform. It is, however, too early to conclude that ELIFA cannot be
adapted for monitoring of $\beta$-cyclocitral in the catfish industries. Instead more work needs to
be done to improve the sensitivity of the assay and to optimize it regarding the conditions.
ELIFA is a very easy procedure that can be used for routine monitoring while the ELISAs
based on IgY and IgG would be preferable for specific and quantitative determination of the
$\beta$-cyclocitral. The filtration across the membrane reduces the time required for each ELIFA
step and reduces the time taken by the entire operation (Aubert et al., 1997). The qualities of
speed with which the ELIFA assay can be completed, ease of performance and simplicity, act
as the incentives to improve and adapt this assay to the catsfish industry. ELISA is used in
many basic research laboratories due to specificity and sensitivity (Shields et al., 1991), but it
may take somewhere between 6-8 h to complete as compared to 1 h or less required to
complete an ELIFA.

Specificity of the assays developed was not determined in this study and is
recommended as more refinement of these assays is done. In order to determine whether
these assays detect only the compound of interest, $\beta$-cyclocitral, cross-reactivity studies to
determine whether the developed antibodies bind molecules similar to $\beta$-cyclocitral would be
needed. An important part of the cross-reactivity studies is to determine if the compounds of
related structure are actually found within the same environment for which the assay is going
to be used. From literature, $\beta$- and $\alpha$-ionone, which are also products of cyanobacteria and
algae (Ikawa et al., 2001), are the only compounds with a close enough structure to $\beta$-
cyclocitral (Appendix N) and which could probably be found in the same environment as $\beta$-
cyclocitral. The cross-reactivity of the anti-$\beta$-cyclocitral antibodies with $\beta$- and $\alpha$-ionone was
not done in this study because of lack of a commercial source of these compounds. If a
source of these compounds can be identified, it is recommended that the specificity of the
developed assays be determined as a part of the refinement process. Good GC/MS, ELISA
correlations could also be used as an indirect measure of specificity especially in cases where
the different methods predict the exact same concentrations of β-cyclocitrinal in the unknowns.

After refinement, the developed immunoassays could potentially be adapted to a kit
format for use by the catfish and other aquaculture industries as well as water treatment
industries to regularly monitor the presence of β-cyclocitrinal.
CHAPTER VII. SUMMARY AND CONCLUSIONS

In order to produce monoclonal and polyclonal antibodies against β-cyclocitril, β-cyclocitril-PPD and β-cyclocitril-BSA were prepared as immunogen and solid phase conjugate respectively. The conjugates were prepared in such a way as to maintain the integrity of β-cyclocitril on the final conjugate. To attain this objective, hydroxycyclocitril, an analog of β-cyclocitril was synthesized and conjugated to the respective proteins through its hydroxyl group. Four β-cyclocitril molecules were attached to each molecule of PPD, while 33 molecules were attached to BSA. Immunization of chicken and mice with β-cyclocitril-PPD produced polyclonal antibodies after four immunizations of the birds and two immunizations of the mice. Screening of sera using β-cyclocitril-BSA as the solid phase conjugate allowed selection of only those antibodies that were produced against β-cyclocitril and not against the carrier or chemical linker.

Polyclonal anti-β-cyclocitril antibodies raised in chicken were extracted from the eggs by the water dilution method, precipitated out of solution by ammonium sulfate and affinity purified to obtain the monospecific IgY. Between 40-100 mg of antibody was obtained per egg, of which 0.6-3.27 % was specific to β-cyclocitril. The monospecific polyclonal antibodies were used for development of IgY-based immunoassays for the purpose of detecting β-cyclocitril in catfish pond water. Overall, hens were found to be good hosts in which to raise polyclonal antibodies.

Fusion of splenocytes from mice immunized with β-cyclocitril-PPD, produced at least 10 stable cell lines that were positive for anti-β-cyclocitril antibodies. After one cloning, the cell lines were established and used as the primary antibody source for the
development of IgG-based immunoassays that would detect β-cyclocitral in catfish pond water and other related industries.

A sandwich ELISA was attempted without any success probably due to the size of the β-cyclocitral molecule. Competitive ELISAs and ELIFAs were instead developed. In these assays, free β-cyclocitral in solution is allowed to compete with immobilized β-cyclocitral for the anti-β-cyclocitral antibody. A high concentration of β-cyclocitral in solution binds up more antibody, and leads to fewer antibodies being captured by the immobilized β-cyclocitral. Known concentrations of β-cyclocitral were prepared and standard curves were constructed to aid in the quantification of β-cyclocitral in the unknown samples. The sigmoidal dose-response curves were fitted to a four parameter logistic equation. Pond water samples were obtained from 10 ponds at BenHur Aquaculture Research Station (Louisiana State University) and analyzed for β-cyclocitral using polyclonal and monoclonal competitive ELISAs and ELIFAs as well GC/MS.

The results showed that the chicken and mice antibodies could detect β-cyclocitral down to 1 ng/mL when used in the ELISAs. The sensitivity of the assays was determined by their individual $I_{50}$, which is the amount of free β-cyclocitral in solution that would inhibit binding of the antibody to the immobilized β-cyclocitral by 50 %. The smaller this value, the more sensitive the assay. $I_{50}$ values for IgY-based ELISA and the IgG-based ELISA were 3.93 and 7.98 ng/mL respectively, while the IgY-based ELIFA and IgG-based ELIFA showed high $I_{50}$ values of 46 and 93 µg/mL respectively. These high values imply that a high concentration of β-cyclocitral is needed in solution in order to inhibit the binding of the respective antibodies to the immobilized β-cyclocitral by 50 %. The sensitivity of the ELIFAs could be improved by optimizing parameters such as the rate of flow of analytical
reagents through the ELIFA unit. The ELISA’s displayed a sensitivity that is able to detect β-cyclocitral in solutions at concentrations lower than the human threshold (5 ng/mL). These assays, which need more refinement, have a potential to provide a rapid and accurate method for the analysis of β-cyclocitral. The ability to monitor off-flavor compounds will improve the management of the off-flavor problem in the catfish and other aquaculture and water treatment industries.

Future efforts should be directed at validating the information reported in this manuscript and modifying the important parameters in order to develop more sensitive and more reliable assays that could be adapted to bulk production followed by dissemination to the end user. Other future efforts should be directed at optimal recovery of the monoclonal antibody and finding a purification procedure that would improve the purity and sensitivity of the final product. The methods developed in this study, have the potential to be adapted to more user friendly kits.
REFERENCES


APPENDIX A. NMR SPECTRUM OF CITRAL ANIL
APPENDIX B. NMR SPECTRUM OF α-CYCLOCITRAL
APPENDIX C. NMR SPECTRUM OF 2,3-EPOXY-2,6,6-TRIMETHYLCYCLOHEXANE-1-CARBOXALDEHYDE
APPENDIX D. NMR SPECTRUM OF HYDROXYCYCLOCITRAL
APPENDIX E NMR SPECTRUM OF $p$-MALEIMIDOBENZOIC ACID
APPENDIX F. NMR SPECTRUM OF p-MALEIMIDOBENZOYL AZIDE
APPENDIX G. NMR SPECTRUM OF PMPI-ACTIVATED HYDROXYCYCLOCITRAL
APPENDIX H. NMR SPECTRUM OF HYDROXYCYCLOCITRAL HEMISUCCINATE
APPENDIX I. RESULTS OF AMMONIUM SULFATE PRECIPITATION OF IgY

Feb1, 2-April 1, 2 represent the IgY sediment obtained for the pooled yolks of a particular month after ammonium sulfate precipitation. Feb1S, 2S-April 1S, 2S represent the corresponding IgY-free supernatant obtained after ammonium sulfate precipitation.
APPENDIX J. ANTI-β-CYCLOCITRAL ACTIVITY OF AMMONIUM SULFATE-PRECIPITATED IGY BEFORE AFFINITY PURIFICATION

![Graph showing absorbance at 405 nm for different samples]

Absorbance at 405 (nm)

Samples:
- NC
- PC
- 1-Feb
- 2-Feb
- 1-Mar
- 2-Mar
- 1-Apr
- 2-Apr
## APPENDIX K. ANTI-β-CYCLOCITRAL ACTIVITY OF CLONED HYBRIDOMA LINE

<table>
<thead>
<tr>
<th>Samples</th>
<th>Absorbance at 405 (nm)</th>
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<tbody>
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<td>NC</td>
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</tr>
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</tr>
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</tr>
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APPENDIX L. COMPARISON OF ANTI-β-CYCLOCITRAL ANTIBODIES IN MICE IMMUNIZED WITH KLH AND PPD CONJUGATES

![Graph showing comparison of absorbance 405 (nm) for KLH-M1, KLH-M2, PPD-M1, and PPD-M2 immunizations. The graph indicates an increase in absorbance over immunizations 0, 1, and 2, with KLH-M2 showing the highest absorbance at 2 immunizations.]
### APPENDIX M. TABLE OF CURVE FIT PARAMETERS

<table>
<thead>
<tr>
<th>Assay</th>
<th>B</th>
<th>T</th>
<th>L</th>
<th>R²</th>
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<td>0.40</td>
<td>1.04</td>
<td>-2.41</td>
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<td>IgG-based ELISA</td>
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<td>1.04</td>
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<td>IgY-based ELIFA</td>
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<td>IgG-based ELIFA</td>
<td>0.75</td>
<td>1.04</td>
<td>4.97</td>
<td>0.94</td>
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</tbody>
</table>

\[ y = B + \frac{(T-B)}{(1+10^{(L-x)\times H})} \]

\( y \) is the absorbance at 405 nm, \( B \) is the baseline response, \( T \) is the maximum response, \( L \) is \( \text{LogIC}_{50} \), \( H \) is the slope factor (1.0) and \( x \) is log of concentration (ng/ml).
APPENDIX N. THE STRUCTURES OF \( \beta \)-CYCLOCITRAL, \( \beta \)- AND \( \alpha \)-IONONE

(IKAWA ET AL., 2001)

\( \beta \)-cyclocitral  \( \beta \)-ionone  \( \alpha \)-ionone
VITA

The author was born in Kampala, Uganda, on December 13, 1972, and had the privilege of being raised by her grandmother, a very wise and tough old lady. The author attended St Thereza Gayaza Girls Primary School and attained the Primary Leaving Examination Certificate in 1987. In 1990 she obtained a first division Uganda Certificate of Education at Mt. St. Mary’s College Namagunga, and in 1993 a Uganda Advanced Certificate of Education at Nabisunsa Girls’ School. For the advanced level education, the author majored in physics, chemistry, biology and subsidiary mathematics. She joined Makerere University and obtained the degree of Bachelor of Science in food science in January 1998 and joined the Department of Food Science, Louisiana State University, in August 1998 to pursue a master’s degree. She worked as a graduate assistant and obtained the degree of master of science in December 2000. She enrolled in a doctoral program in the same department, worked as a graduate assistant and she will be awarded the degree of Doctor of Philosophy in December 2004. The author married Daniel Moriasi on the 22nd of December 2001 and she is expecting her first born son in November 2004.