ANALYSIS OF ETHOXYQUIN AND ITS OXIDATION PRODUCTS USING SUPERCRITICAL FLUID EXTRACTION AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH CHEMILUMINESCENT NITROGEN DETECTION

Daniel R. Brannegan

Thesis submitted to the Faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE
IN
CHEMISTRY

Larry T. Taylor, Chair
Harold M. McNair
James O. Glanville

March 10, 2000
Blacksburg, Virginia

Keywords: Supercritical, Extraction, Chromatography, Ethoxyquin, Oxidation, Beef, Beef Fat, HPLC, SFC, GC/MS, HPLC/CLND

ANALYSIS OF ETHOXYQUIN AND ITS OXIDATION PRODUCTS USING SUPERCRITICAL FLUID EXTRACTION AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH CHEMILUMINESCENT NITROGEN DETECTION

Daniel R. Brannegan

(ABSTRACT)

Ethoxyquin is an antioxidant commonly used to preserve vitamins and lipids in various food products and animal feeds. The extraction and determination of ethoxyquin is becoming increasingly important as products, which are labeled as "natural" are becoming more common. The present method of determination only ensures that ethoxyquin values are below 10-20 parts per million. Therefore, advances are needed in methods of extraction and analysis in order to lower the detection limits in various products.

The first part of this research investigates the use of supercritical fluids in the extraction of ethoxyquin from lean beef and beef fat. Supercritical fluids offer the advantages of safety, time, expense, and selectivity over liquid extractions. Three fluids were examined: carbon dioxide, trifluoromethane, and 1,1,1,2-tetrafluoroethane. Carbon dioxide appeared to react with ethoxyquin during the extraction. Methanol modified hydrofluorocarbons provided more complete extractions over pure hydrofluorocarbon

fluids. Methanol modified 1,1,1,2-tetrafluoroethane was used in the extraction of ethoxyquin from lean beef and beef fat, and provided a quantitative extraction at the 0.5 ppm level.

The second part of this research centered on the separation and quantitation of the oxidation products of ethoxyquin through the use of high pressure liquid chromatography with chemiluminescence nitrogen detection (HPLC/CLND). When ethoxyquin is oxidized, the resulting products also exhibit antioxidative properties. While these oxidation products are known, no effort has been made to separate and quantify them in real or clean samples. HPLC/CLND allows all nitrogen containing compounds to be quantified without a known standard. This method is of extreme interest in the case of ethoxyquin oxidation products, or other types of metabolites, where standards are difficult to obtain or are unstable. HPLC/CLND allowed a separation of ethoxyquin and four of its oxidation products to be detected, thus making future studies of the antioxidant behavior of ethoxyquin feasible.

This dissertation is dedicated to my parents Daniel P. and Jane Brannegan, and my

brother Dave.

Acknowledgments

I am sincerely indebted to many people for the level of support that I have received, and in doing so made this achievement possible. While, I cannot list all that have helped me, I would like to thank all of my friends who know nothing about chemistry, but have provided me with advice on topics more important. I would like to express my sincere gratitude to:

Daniel P. and Jane Brannegan, my parents, for their support and love; my brother, Dave, and my grandparents for their support and encouragement; the members of the Taylor group and the Virginia Tech chemistry department, especially, Mehdi Ashraf-Khorassani who provided unmatched knowledge and information in the laboratory.

Lastly, I would be remiss if I excluded my friends, both past (Craig Raposa, Chris Dziadozs, Charles Teeple, Jason Lachance), and new (Dwayne Johnson, Steve Austin, Scott Hall, Shawn Michaels, Steve and Julie Hunsucker, Amy Eichstadt, Lee Williams, Matt Thornberry, Dave and Shelly Porter, Jim Southers, Kevin Schug, Emmett O'Brien, Ron Difelice, Will Heard, Nori Yamaguchi), who have helped celebrate in my successes and rationalize my set backs.

Credits

      The author would like to thank the following persons and companies for their donations and technical assistance.

      Antek® Instruments, Inc. for the loan of the chemiluminescence nitrogen detector especially Jean Francois Borney and his lab for their technical support; Isco/Suprex Corporation for the loan of supercritical fluid extractors; Air Products and Chemicals Inc. for supplying supercritical fluids and; Hewlett Packard for the loan of their supercritical fluid chromatograph.

Table of Contents

List of Figures

List of Tables

Chapter 1

INTRODUCTION

Antioxidants in Food Products

Oxidation is the chemical conversion of one compound into another containing fewer electrons. In food products, the spontaneous or self-catalyzed reaction of a chemical substance with atmospheric oxygen is termed autooxidation.[1] While this transfer of electrons is a common phenomenon in chemistry and all life processes, the oxidation of food products is undesired. Commercial food products, and feedstuffs for animals, use artificially added antioxidants to inhibit oxidation. In doing so, antioxidants preserve the nutritional value of the food, increase the product's shelf life, preserve the food's taste, and delay the onset of rancidity.

The addition of these antioxidants must be carefully monitored, and their levels easily quantified. This monitoring must be undertaken upon processed food products, which contain levels of added preservatives. Consumer demand for products that are labeled as "natural" or containing no preservatives, require beef and fat be examined. This testing must be done because food-producing animals are nourished in many cases by animal feeds that contain added antioxidants. Therefore, seemingly "all natural" products must be tested to ensure that the antioxidants that were fed to the animals do not end up stored in edible muscle or fat tissue.

Mechanism of Action

Antioxidants act to stop the free radical damage done in food products by autooxidation. Autooxidation is a free radical chain reaction that occurs in three common steps: initiation, propagation, and termination. The first step in this process, initiation, is the formation of free radicals. However, the reaction of atmospheric oxygen with a lipid molecule does not occur directly, as the reaction of a singlet state lipid molecule and a triplet state oxygen molecule would contradict the conservation of spin angular momentum and would be highly improbable. Singlet oxygen molecules can be formed through various reactions with photosensitizers in the presence of light.[2] By absorbing light and transferring the excess energy, a singlet oxygen molecule can be formed.[3] The singlet state oxygen can now react with a lipid molecule to produce a hydroperoxide[3] (equation 1.1).

$$RH + {}^1O_2 \longrightarrow ROOH \qquad (1.1)$$

The hydroperoxides have weak oxygen oxygen bonds, and will readily form free radicals[3] (equation 1.2).

$$2ROOH \longrightarrow RO\bullet + 2OH + RO\bullet \quad (1.2)$$

Free radical damage can continue through the process of propagation[3] (equation 1.3.).

2

$$ROO\bullet \ + \ RH \ \longrightarrow \ ROOH \ + \ R\bullet \quad (1.3.)$$

The propagation step interacts with a lipid molecule (RH) and also forms more hydroperoxides that can generate more free radicals. The free radical chain reaction ends in the termination step where two free radicals join[3] (equations 1.4, 1.5 and 1.6).

$$R\bullet \ + \ R\bullet \ \longrightarrow \ R_2 \quad (1.4)$$

$$R\bullet \ + \ ROO\bullet \ \longrightarrow \ ROOR \quad (1.5)$$

$$ROO\bullet \ + \ ROO\bullet \ \longrightarrow \ ROOR \ + \ O_2 \quad (1.6)$$

While the termination step destroys free radicals, the number of lipid molecules available to react with a free radical as compared to another free radicals is very large. Therefore, antioxidants must be used to stop this free radical chain reaction. Antioxidants are classified into two groups, primary and secondary, dependent on the oxidation process. Primary antioxidants interact directly in the free radical chain reaction by scavenging free radicals. These antioxidants can donate hydrogen atoms, incorporate oxygen radicals into their structure, or change free radicals into more stable structures. Secondary antioxidants inhibit oxidation by retarding the onset of free radicals by scavenging oxygen, absorbing light, or decomposing hydroperoxides[4].

Ethoxyquin

Ethoxyquin (**Figure 1**) is a common primary antioxidant used to protect a variety of unsaturated hydrocarbon systems.[5] An antidegradant and antiozonant as well as an antioxidant in polymers,[6,7] ethoxyquin also is found in many food products. The most common use is in fishmeal and fish oils[8,9], but it is also found in other oils, fats, and meat meals. Its purpose is to protect lipids and preserve carotene and vitamins A and E[10,11]. Ethoxyquin also prevents the spontaneous combustion of stored food products by inhibiting the heat production caused by oxidation of lipids[12]. Ethoxyquin is used to preserve the red colors of many spices such as paprika and chili powder, and also as a post harvest dip for apples and pears to inhibit brown spots[13,14,15,16].

Sample Preparation and Analysis

Increasing consumer demand for products labeled as "natural" or those that contain no preservative has spawned investigations into the determination of ethoxyquin in a variety of matrices. Current methods of determination do not adequately provide for low levels of detection and are antiquated in terms of instrumentation. These methods use large amounts of organic solvents and require many time consuming sample preparation steps.

The current AOAC Official Method[17] for ethoxyquin has been in existence since 1963. This method, Ethoxyquin in Animal Feed, Fluorometric Method, uses liquid extraction. Samples (10 grams) are ground, weighed, and slurried with 50 mL of methanol. After stirring and standing for 10 minutes, the solvent is decanted through a
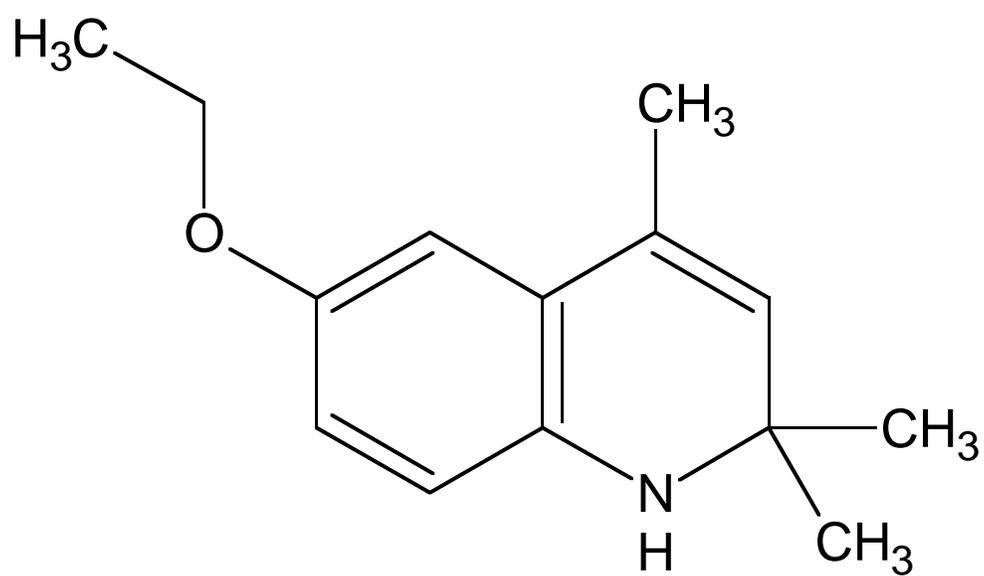
Figure 1: Chemical structure of ethoxyquin

plug of glass wool. The residue is re-slurried two more times with 50 mL of methanol

each and the extracts are combined and diluted to 250 mL in a volumetric flask. A 25

mL aliquot of the solution is then added with 100 mL of water to a separatory funnel.

Petroleum ether (50 mL) is then added and shaken for 1 minute. After 3 consecutive

extractions the combined ether extracts are then washed with water and diluted to 100 ml

in a volumetric flask. The above sample preparation steps consume 250 mL of methanol,

100 mL of water, and 100 mL of petroleum ether for every 10 gram sample. Analysis by

photofluorometer provides a detection limit of only 10-20 parts per million. This

detection limit is no longer considered low since a food product containing 5 ppm

ethoxyquin would pass as being all natural and containing no preservatives.

       The other method of ethoxyquin determination, Ethoxyquin Residues in Animal

Tissues, Photofluorometric Method[18], published by the AOAC in 1968, is applicable to

chicken tissues and eggs. This method also requires various liquid extractions and a

photofluorometer for detection. This method has not been adopted into official use at this

point.

       A new method of analysis using HPLC has recently been investigated[19] for use

with meat meal and extruded pet foods. This method uses liquid extraction but lower

quantities (7.5 mL) of solvent are used for every sample (0.5 gram). This method then

uses HPLC with fluorescence detection. The use of a separation process allows for fewer

sample preparation steps and fluorescence affords a lower detection limit (e.g. 0.5 ppm).

While determinations of ethoxyquin have been made in a variety of matrices, studies

done upon lean beef and beef fat have not been reported at this time.

Supercritical Fluid Extraction

Supercritical fluid extraction (SFE) provides many advantages over commonly used liquid extraction techniques. Firstly, the most common supercritical fluid used in extractions is CO_2, which is environmentally safe. SFE is less expensive than liquid extractions, as the purchase and disposal of expensive solvents is not necessary. Many times SFE is faster than liquid extraction techniques as it can be automated. The smaller number of sample handling steps in SFE also decreases the chances for human errors. Lastly, SFE allows for selective extractions since varying the temperature and pressure of the fluid changes the solvating power. This feature can allow for cleaner extracts and controlled fractionation.

Nitrogen Selective Detection

Nitrogen is an important and common element in many molecules. Food products contain many nitrogen containing molecules including proteins and vitamins, in addition to some artificial preservatives. Pesticides and herbicides commonly contain nitrogen. Amino acids are present in any biological sample, all containing nitrogen. The near omnipresence of nitrogen in important molecules of interest makes nitrogen detection and nitrogen selective detectors very interesting topics in analytical chemistry.

The Kjeldahl method[20] for total nitrogen determination has withstood the test of time since its development in 1883 and remains the most universal and accurate method of determining total nitrogen.[21] This method involves conversion of nitrogen containing molecules into ammonium sulfate. Sodium hydroxide is then added after the digestion is

complete to release nitrogen in the form of ammonia. Ammonia is quantified through a process of distillation and titration. While an effective and commonly used method, the Kjeldahl method has some major drawbacks including the use of concentrated sulfuric acid and use of large quantities of solvents plus numerous time consuming steps.

Nitrogen detection through the use of a chemiluminescence method offers many advantages over the Kjeldahl method. Chemiluminescence is the release of photons from vibrationally or electronically excited molecules formed though exoergic chemical reactions. This type of reaction can be conducted in both gas and liquid phases, making post-column chemiluminescence detection available to both liquid and gas chromatography. Chemiluminescence, like fluorescence, is a very sensitive detection process, as background noise from a light source is not involved in the detection. Selectivity can also be increased by using filters or monitoring only certain wavelengths of light, which result from a known reaction.

Chemiluminescence in the liquid phase, for example in detection with HPLC, requires either an analyte which will chemiluminescence or more commonly, an added nitrogen containing reagent which will form a derivative with the analyte of interest. In order for this derivative to be useful in HPLC detection, the reaction must be very efficient. This reaction usually takes place after the separation process and may require additional pumps and a mixing chamber prior to monitoring the chemiluminescence process. An example of HPLC chemiluminescence detection involving a chemical reaction is that of metal catalyzed reactions with luminol (5-amino-2,3-dihydro-1,4-

phthalazinedione).[22] This reaction finds application in quantitating transition metal ions after chromatography.[23]

While nitrogen chemiluminescence in the liquid phase is a way to improve detection in HPLC, chemiluminescence detection in chromatography commonly takes place in the gas phase. For nitrogen selective detection, the chemiluminescence reaction involves nitrogen monoxide and ozone (equation 1.7).

$$NO\ +\ O_3\ \longrightarrow\ NO_2^*\ +\ O_2 \qquad (1.7)$$

The use of this reaction as a chromatographic detector is based on the work of Fontijn et al.[24]. While the Thermal Energy Analyzer and the Redox Chemiluminescent Detectors are based upon the reaction of nitrogen oxide with ozone, the most powerful use of this reaction can be found in the chemiluminescence nitrogen detector (CLND). The detection process (**Figure 2**) is based upon the reaction of nitrogen oxide with ozone, but is more directly applicable to everyday chromatographic detection applications. The first step involves combusting nitrogen containing molecules in a high temperature furnace with an excess of oxygen. The products from this combustion include nitrogen oxide, water, carbon dioxide and other combustion products or oxides. Nitrogen dioxide is not formed, as its dissociation constant has a positive dissociation constant with temperature[25]. A combustion process that takes place at over 600 °C will ensure that all nitrogen dioxide will dissociate fully. Nitrogen oxide is then reacted in an

9

$$(R)_3\text{-N} \ + \ O_2 \ \longrightarrow \ NO \ + \ \text{other combustion products}$$

$$NO \ + \ O_3 \ \longrightarrow \ NO_2* \ + \ O_2$$

$$NO_2* \ \longrightarrow \ NO_2 \ + \ h\nu$$
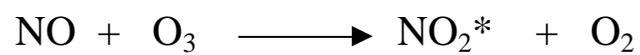
Figure 2: Reaction scheme for the CLND

excess of ozone, and the energy of the reaction produces nitrogen dioxide in an excited state, which provides the release of photons (**Figure 2**).

To provide total nitrogen determination the reaction above must meet certain criteria. Firstly, the reaction converting nitrogen-containing analytes to nitrogen oxide must be quantitative. Secondly, all nitrogen, regardless of their chemical environment, must provide the same signal for quantitation. Lastly, the chemiluminescence that takes place must be able to be monitored by a common detection process. These criteria were proved and the detection process was patented by Parks and Marietta[26] in 1977.

High Performance Liquid Chromatography Chemiluminescence Nitrogen Detection

The CLND was initially developed for gas chromatography[27,28,29,30,31,32,33,34,35,36] as the reaction of interest takes place in the gas phase, and it has also found use in SFC with unmodified and limited levels of methanol modifier[37,38,39]. However the basic design of the post-column reaction does not make the CLND used with GC and SFC directly amenable for use with HPLC. Obviously this gas phase reaction would have fewer drawbacks when a sample is introduced as a gas sample as compared to a liquid sample. While GC and SFC can simply introduce samples into the detector as a gas with a mixture of oxygen (required for combustion), liquid samples need special consideration. Firstly, a liquid sample must be nebulized for introduction to the high temperature furnace. Secondly, the rate of introduction must be optimized to allow proper combustion of the introduced liquid. Thirdly, water must be eliminated prior to

the reaction of nitrogen oxide with ozone. Water must not be allowed to deposit in the reaction chamber to ensure that both the reaction chamber volume stays constant, and no trapping of analytes occurs in the water inside the reaction chamber.

Antek® Instruments Inc. (Houston Texas) has spearheaded the drive to produce a CLND for use with HPLC. Prior to 1992, several HPLC/CLND methods had been reported[40], but they did not find wide application due to the problem of elimination of water which required long drying times for reaction chambers and inlet lines of the detector. The elimination of water is of importance as the chemiluminescence reaction takes place in the gas phase, and water cannot be allowed to deposit in the reaction chamber. In 1992 Antek® Instruments first reported a CLND for use with HPLC. Fujinari et al[41] reported determination of ammonium nitrogen in waste water through ion chromatography. The CLND exhibited a detection limit of 5 ng while demonstrating the quantitation of ammonium nitrogen in metropolitan waste water. The detector proved to have a linear response and seemed to be free of any interference.

In recent years the HPLC/CLND has gone through a limited growth period. Fujinari et al[42] continued to investigate the HPLC/CLND with a reversed phase separation of peptides. The research focused on the ability of the CLND to simplify complex mixtures by only giving a response to nitrogen containing compounds. Through the development of a reversed phase separation without acetonitrile and detection of the peptides by CLND, the everyday power of this detector was made evident. This topic was revisited by Fujinari et al[43] with similar results. Fujinari et al[44] also used the CLND with size exclusion chromatography to estimate the molecular mass distribution of food-

grade protein hydrolyzates. In this research the CLND proved able to provide an accurate peptide profile while the simultaneous UV detection could not.

In recent years two papers have discussed the use of HPLC/CLND for use with combinatorial libraries. Fitch et al.[45] briefly discusses the strengths of the CLND for use with HPLC in combinatorial chemistry. Taylor et al.[46] showed the utility of the HPLC/CLND in combinatorial chemistry by first evaluating the detector and then showing its real application to simplifying and quantitating chromatograms.

Overview

The focus of this research is to develop a method for extracting ethoxyquin from lean beef and beef fat using supercritical fluids, and to further investigate the chemistry of ethoxyquin by studying its oxidation products through the use of a chemiluminescence nitrogen detector. Chapter 2 deals with the supercritical fluid extraction of ethoxyquin from lean beef and beef fat samples. The objective of the study was to evaluate the extraction parameters of fluid type, pressure, temperature, and methanol modifier to develop a quantitative method for the determination of ethoxyquin from lean beef and beef fat matrices. CO_2, CHF_3, and CFH_2CF_3 were evaluated as fluids. The most effective method discovered via extractions from a sand/Hyromatrix™ mixture was applied to spiked lean beef and beef fat samples. Samples of lean beef and beef fat obtained from cows which had been fed ethoxyquin preserved feeds were then evaluated for levels of ethoxyquin

Chapter 3 concerns the isolation, and identification of the oxidation products of ethoxyquin. The objective of these experiments was to obtain samples of the oxidation products of ethoxyquin for the development of a general HPLC/CLND method. The availability of these oxidation products allowed a separation to be developed which could yield quantification of a total ethoxyquin concentration.

Chapter 4 deals with a chemiluminescence nitrogen detector for use with high performance liquid chromatography. The CLND was first evaluated for its detection limit, linear range, and equimolarity (equal response for all nitrogen). The HPLC/CLND was then used to develop a separation of the oxidation products of ethoxyquin, investigating both normal and reversed mobile phases.

Chapter 2

Comparison of Supercritical C$_2$OCHF$_3$, and CFH$_2$CF$_3$ for the Extraction of Ethoxyquin from Lean Beef and Beef Fat

INTRODUCTION

Carbon Dioxide

The use of carbon dioxide (CO$_2$) in SFE has found many uses in environmental, pharmaceutical, food, and polymeric applications. The effects of pressure, temperature, and modifier on extraction efficiency have been well studied. Increasing fluid density, by increasing the pressure at a fixed temperature, increases extraction efficiency in many cases.[47][48] Whereas, decreasing the fluid density by increasing the temperature at a fixed pressure has also been shown to increase recoveries for certain analytes and matrixes.[49][50] [51] The addition of a modifier during SFE is a common method for improving extraction efficiency by either increasing the solvating strength of the fluid or increasing desorption of the analyte from its matrix.[52][53][54]

Alternate Fluids

Supercritical CO$_2$ is the most popular fluid used in SFE and has found many applications. Other supercritical fluids have not found routine use in analytical SFE due to either their reactivity, poor solvating power, physical properties (i.e. high critical

parameters), or their incompatibility with instrumentation. However, some fluids other that carbon dioxide have found applications in SFE. Smith et al.,[55][56][57] despite the high reactivity of supercritical ammonia, have coupled SFE with mass spectrometry. In the extraction and analysis of diesel fuel they were able to extract most of the polar components in the fuel. Onuska et al.[58] compared nitrous oxide (N_2O) with pure CO_2, sulfur hexafluoride (SF_6), and mixtures of these fluids with different percentages of methanol modifier for the extraction of 2,3,7,8-tetrachlorodibenzo-p-dioxin and polynuclear aromatic hydrocarbons (PAHs) from aquatic sediment and soil. High recoveries (90%) were reported using pure, or methanol modified, N_2O. The lowest recoveries were obtained using SF_6 as a fluid, as it has poor solvating power. Levy et al.[59] also compared N_2O and CO_2 with methanol modifier and reported high recoveries of PAHs with pure of methanol modified N_2O. Ashraf-Khorassani et al.[60] compared N_2O with CO_2 through online SFE/GC despite the ability of N_2O to support oxidation. McNally et al.[61] showed acceptable recoveries of aromatic amines from different matrixes using CO_2 or N_2O as an extraction fluid. However, Raynie[62] concluded that for safety reasons, N_2O should not be used in SFE especially if the sample matrix is organic. Studies using subcritical water have been undertaken, but water's high critical temperature and pressure make it a less favorable alternative to CO_2. Hawthorne et al. [63] have used sub and supercritical water for the extraction of a variety of organic pollutants from environmental solids. Their work showed that by varying the temperature from 50 to 400 °C the polarity of water could be altered.

Hydrofluorocarbons

Two fluids that do not appear to have major drawbacks are the hydrofluorocarbons: trifluoromethane (CHF_3) and 1,1,1,2-tetrafluoroethane (CFH_2CF_3). CHF_3 has critical parameters (T_c 26°C, P_c 47 atm) very much similar to CO_2 (T_c 31°C, P_c 72 atm) while, CFH_2CF_3 has a much higher critical temperature (T_c 101°C, P_c 40 atm). CHF_3 has a dipole moment of 1.6 Debye, while CFH_2CF_3 has a higher dipole moment of 2.2 Debye[64]. Research involving hydrofluorocarbons, as supercritical fluid has been limited, giving most of the attention to CHF_3.

Stahl et al.[65] used N_2O, CO_2 and CHF_3 to determine the solubilities of different alkaloids, and found that CHF_3 was the fluid in which these analytes were the most soluble. Howard et al.[66] used CHF_3 to extract sulfonyl urea herbicides and polyaromatic hydrocarbons. Results of these experiments showed that CHF_3 was a better fluid for extracting polar herbicides as compared to CO_2, but was ineffective when extracting the less polar polyaromatic hydrocarbons. Combs et al.[67,68] investigated CO_2 and CHF_3 with and without methanol modifier to extract sulfonamides from sand and various food matrices. Results showed that CHF_3 and methanol modified CHF_3 yielded lower recoveries at higher pressures as compared to lower pressures. Results also showed that CHF_3 had higher extraction efficiency for two of three sulfonamides investigated as compared to CO_2.

While CFH_2CF_3 has received very little attention as an extraction fluid, Ashraf-Khorassani et al.[69] developed an apparatus to measure the solubility of analytes in supercritical fluids, and used CO_2, CHF_3 and CFH_2CF_3 to investigate sulfonamides

solubility. Results showed that, even though it was subcritical, the analytes were much

more soluble in the highly polar CFH_2CF_3 as compared to the supercritical CHF_3 and

CO_2. These results also showed that the sulfonamides have a higher solubility in CHF_3

than in CO_2 at every pressure investigated.

EXPERIMENTAL

Supercritical Fluid Extraction and Quantitation

A Suprex Autoprep 44™ (Pittsburgh, PA) supercritical fluid extractor was used

for method development extractions of ethoxyquin from a sand/Hydromatrix™ mixture,

and a Suprex Prepmaster™ supercritical fluid extractor was used for extractions of

ethoxyquin from lean beef and beef fat. Both systems are equipped with an Accutrap

electronically controlled variable restrictor and an in-line Suprex modifier pump. HPLC

grade methanol was purchased from EM Science (Gibbstown, NJ). SFC/SFE grade CO_2

and 1,1,1,2-tetrafluoroethane (pressurized with 200 psi of helium) were obtained from

Air Products and Chemicals, Inc. (Allentown, PA). CHF_3 was also obtained from Air

Products and Chemicals, Inc. but did not have helium head space or a dip stick, and

therefore it was inverted for use.

Extractions were conducted in 5 mL Suprex vessels when the Suprex Autoprep

44™ was used and in 50 mL vessels when the Suprex Prepmaster™ was used. A flow of

2 mL/min (liquid measured at the pump) was used for all extractions. Fluid, pressure,

temperature, and percent methanol modifier were varied throughout this study. Dynamic

time of 60 minutes was used for all extractions. Static time of 15 minutes was used for all extractions using methanol modifier. All extractions employed a solid sorbent trap of octadecyl silica (Applied Separations, Allentown, PA) at 0 °C using pure fluids. The trap temperature was set to 40 °C for 5% methanol modifier and 60 °C for 10% and 15 % methanol modifier. Each extraction was completed in triplicate and reported as the average of the three extractions. After extraction the solid phase trap was rinsed with 10 mL of methanol when pure fluid were used and 5 mL of methanol when methanol modified fluids were used.

Samples for method development were prepared by spiking 100 ug of ethoxyquin (methanol solution) directly onto a 50/50 Ottawa sand from Fischer Scientific (Fair Lawn, NJ)/Hydromatrix™ from Isco/Suprex (Lincoln, NE) mixture in the extraction vessel. Five minutes passed before sealing the vessel to allow methanol to evaporate. Samples for extraction from lean beef and beef fat were prepared by grinding 10 grams of sample, mixing with 10 grams of Hydromatrix™, spiking the appropriate level of ethoxyquin, allowing methanol to evaporate, and transferring to an extraction vessel. Lean beef and beef fat samples from cows fed ethoxyquin preserved feeds employed the same procedure.

A Hewlett-Packard (Little Falls, DE) series 1050 HPLC equipped with a variable wavelength UV detector was used to assay all extracts in the method development phase. A 250 x 4.6mm (5um dp) Prodigy ODS column from Phenomenex (Torrance, CA) was used throughout the study. The mobile phase employed was 75% methanol/25% water at a flow rate of 1mL/min with the column at 50 °C. An internal standard (2,6-

dinitrotoluene) method was used to determine percent recovery. Both ethoxyquin and

2,6-dinitrotoluene were detected at 358 nm. Samples were injected (20 ul) using a three

time overfilling method.

A model G1205A supercritical fluid chromatographic (SFC) system equipped

with both a variable restrictor which allowed independent control of pressure and flow

rate and a Model 1050 multiple wavelength UV detector (Hewlett-Packard, Little Falls,

DE) were used to assay all extracts of ethoxyquin spiked on lean beef and beef fat as well

as lean beef and beef fat samples from cows which were fed ethoxyquin preserved feeds.

An auxiliary-reciprocating pump in the SFC allowed methanol to be added on-line to the

carbon dioxide mobile phase. Samples were injected by a model 7673 auto injector

configured to an air actuated Rheodyne valve with 20 uL injection loop. Two 250 x 4.6

mm (5um dp) diol columns from Phenomenex were used in series. A pressure program

of 175 atm for 6 min., ramped to 210 atm at 5 atm/min was used at a temperature of 80

°C. A modifier program of 95%/5% (CO_2/methanol) for 4 minutes ramped to 92%/8%

(CO_2/methanol) at 0.5%/min was used at a liquid flow rate of 2.5 mL/min. An internal

standard, 4-nitroaniline, was used to determine percent recovery which was monitored

along with ethoxyquin at 358 nm.

RESULTS AND DISCUSSION

The objective of this Chapter was to develop a SFE method for the removal of

ethoxyquin from lean beef and beef fat. Extraction of ethoxyquin from a

sand/Hydromatrix™ mixture was first conducted. This was preformed in order to

evaluate the extraction behavior of ethoxyquin from an inert matrix and to optimize the extraction without the added complexity of matrix effects from beef or fat. Optimal extraction conditions were then applied to lean beef and beef fat spiked with ethoxyquin and lastly lean beef and beef fat from cows which were fed ethoxyquin preserved feeds.

Carbon Dioxide

CO_2 is the most commonly used supercritical fluid and has found many applications. However, for the extraction of ethoxyquin from beef or fat, CO_2 may not be the optimal fluid, as it is a non-polar fluid that extracts fat very well. Secondly, there have been reports of CO_2 reacting with certain amine analytes[70,71]. Regardless of these potential drawbacks of CO_2, its wide range of applicability and the amount of knowledge concerning the fluid made it the first choice for any supercritical extraction.

Figure 3 shows chromatograms of the standard methanol solution and a CO_2 extract of ethoxyquin. A constant third peak appeared in all CO_2 extracts of ethoxyquin. The extraction conditions used to obtain the extract seen in **Figure 3** were 400 atm, 60 °C, for 60 minutes. An average recovery of only 60% was achieved with these extraction conditions, and the lowness may be a direct result of the formation of the unknown analyte. Whether this analyte was formed during the extraction or during decompression of CO_2 in the solid phase trap is unknown. This suspected reactivity which takes place using CO_2 as an extraction fluid, and its ability to extract fat well, eliminated CO_2 as a suitable fluid for the extraction of ethoxyquin from beef and fat.
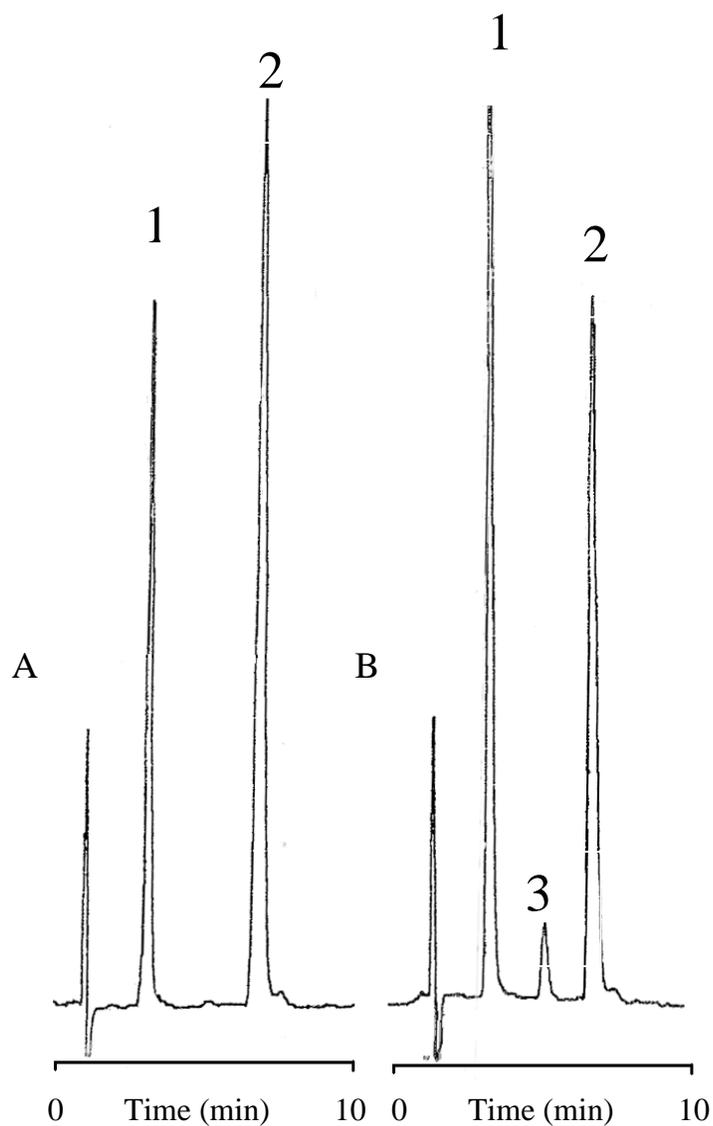
FIGURE 3: HPLC chromatogram of A) ethoxyquin (peak 2) and internal standard 2,6-dinitrotoleune (peak 1) and, B) extract of ethoxyquin from sand/Hydromatrix™ using CO_2 with 2,6-dinitrotoleune (peak 1), ethoxyquin (peak 2), and unknown product (peak 3).

Pure Trifluoromethane and Tetrafluoroethane

The most attractive alternative to CO_2 as a fluid for SFE of ethoxyquin from beef and fat are hydrofluorocarbons since they are more polar and they have little affinity for fat. **Figure 4** shows the percent recoveries of ethoxyquin extracted from sand/Hydromatrix™ with hydrofluorocarbons at various pressures and constant temperature. In these extractions CHF_3 was a supercritical fluid, while CFH_2CF_3 was used as a liquid due to its high critical temperature of 101 °C. Extractions with CHF_3 were performed at 60 °C for 60 minutes at pressures of 300, 350, 400, and 450 atm, and those with CFH_2CF_3 were performed at 50 °C for 60 minutes at pressures of 350, 400, 450, 500 atm. The first observation regarding these data is that quantitative recoveries are not attained for either fluid at any pressure investigated. Secondly, an unique optimized pressure appears to exist for each fluid (e.g. 500 atm for CHF_3 and 450 atm for CFH_2CF_3). This trend can be attributed to the highly polar nature of the two fluids. At higher pressures fluid-fluid interactions begin to dominate over any increase in fluid analyte interactions at high pressures.

Figure 5 shows the ethoxyquin extraction efficiencies with the two hydrofluorocarbons by changing temperature at constant pressure. The extractions with CHF_3 were done at 450 atm for 60 minutes at temperatures of 50°, 70°, and 90° C, and the extractions performed with CFH_2CF_3 were done at 400 atm for 60 minutes at temperatures of 60°, 80°, and 100° C. The pressures used corresponded to the highest recoveries in the previous study. CHF_3 showed very little change in percent recovery as the temperature was varied. CFH_2CF_3 extractions showed a trend similar to the pressure
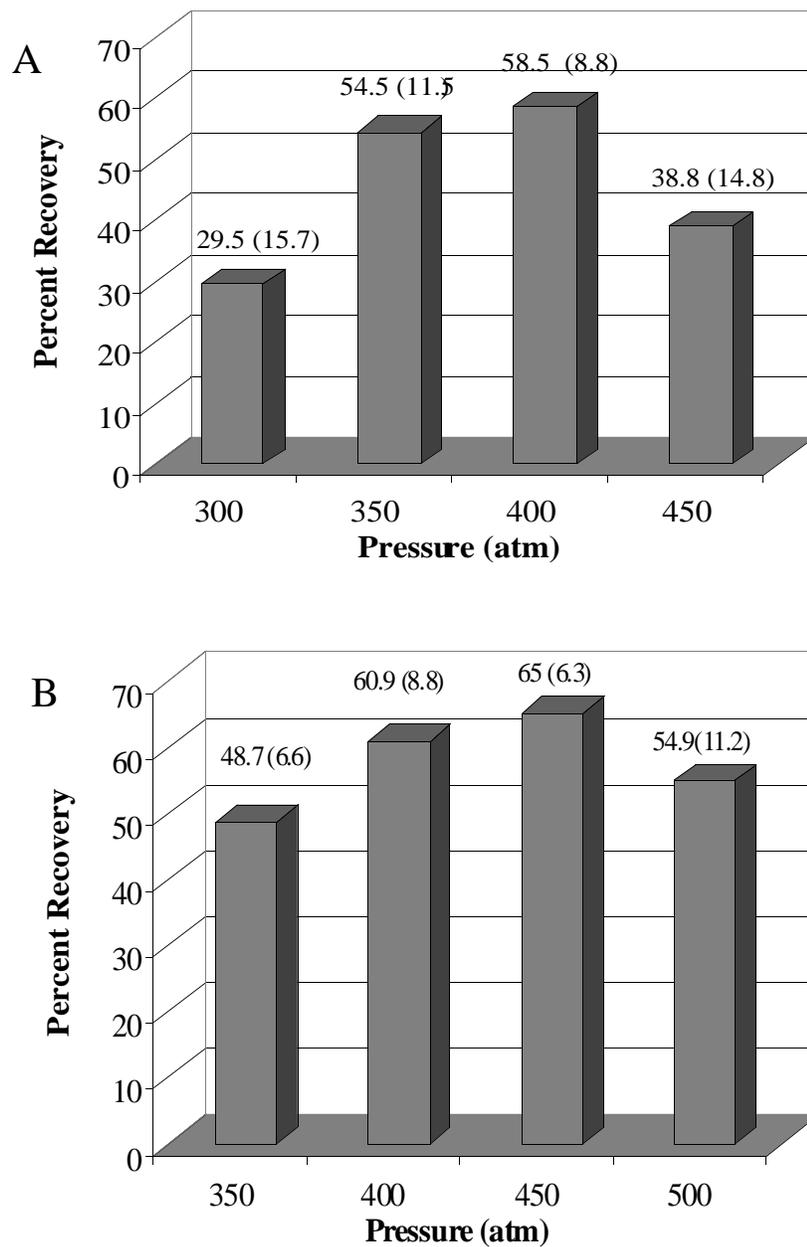
Figure 4: A) CHF$_3$ percent recoveries and percent RSD of ethoxyquin spiked on sand/Hydromatrix® (n=3), B) CFH$_2$CF$_3$ percent recoveries and percent RSD of ethoxyquin spiked on sand/Hydromatrix® (n=3) as a function of pressure.
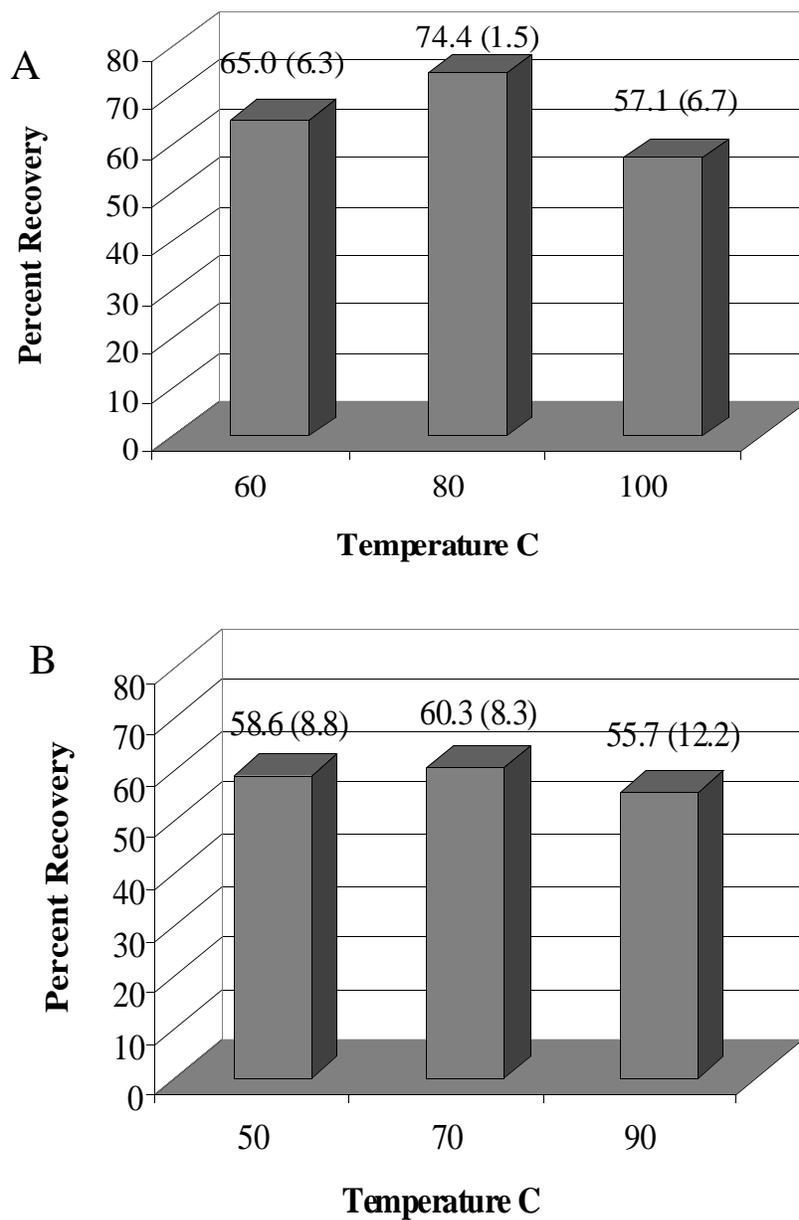
Figure 5:A) CHF$_3$ percent recoveries and percent RSD of ethoxyquin spiked on sand/Hydromatrix® (n=3), B) CFH$_2$CF$_3$ percent recoveries and percent RSD of ethoxyquin spiked on sand/Hydromatrix® (n=3) as a function of temperature.

profiles. The drop off at 100 °C can be explained by a suspected increase in fluid-fluid

interactions as the fluid approaches its supercritical point. However, at none of the

pressures and temperatures investigated did the pure hydrofluorocarbons provide

quantitative recoveries.

Methanol Modified Trifluoromethane and Tetrafluoroethane

Figure 6 shows the ethoxyquin extraction efficiencies using the

hydrofluorocarbons with the addition of 5, 10, and 15 percent methanol modifier. As

with the non-polar CO_2, the addition of a polar modifier increased recovery, and for both

hydrofluorocarbons quantitative recoveries were seen at the 5 percent methanol modifier

level. Above 5 percent methanol modifier, recoveries decreased. The lower recoveries

for both fluids at 10 and 15 percent modifier can be explained by the interactions of the

polar fluids with methanol. The higher levels of methanol modifier should enhance

fluid-methanol interactions thereby limiting fluid or methanol interactions with the

analyte. CFH_2CF_3 at 450 atm, 40 C, 5% methanol modifier, 15-minute static time, and

60-minute dynamic time will be used for the remaining extractions.

Lean Beef and Beef Fat Samples

Figure 7 shows the SFC chromatograms of a methanolic standard and CFH_2CF_3

extract of ethoxyquin spiked on lean beef. Comparison of the two chromatograms shows

that CFH_2CF_3 provides a very clean extract as compared to the standard. **Table 1** shows

the results of the extraction of ethoxyquin spiked at various concentration levels onto lean
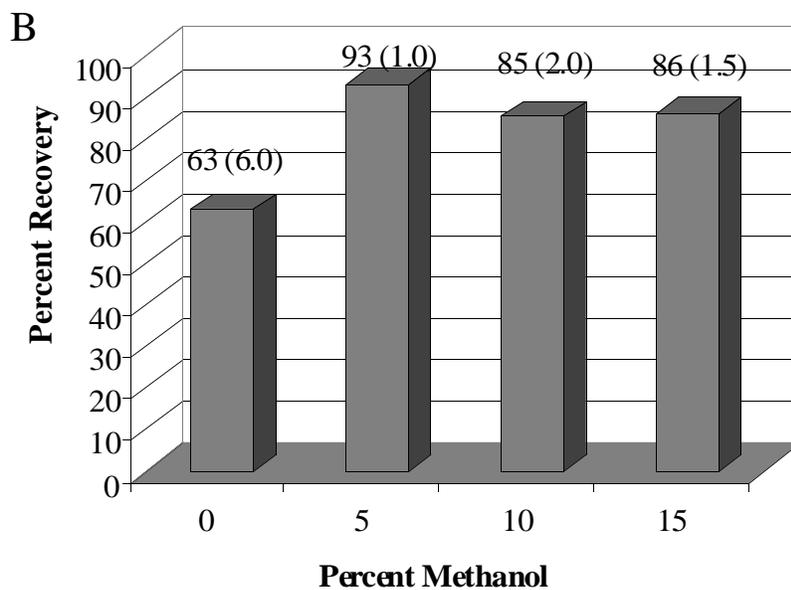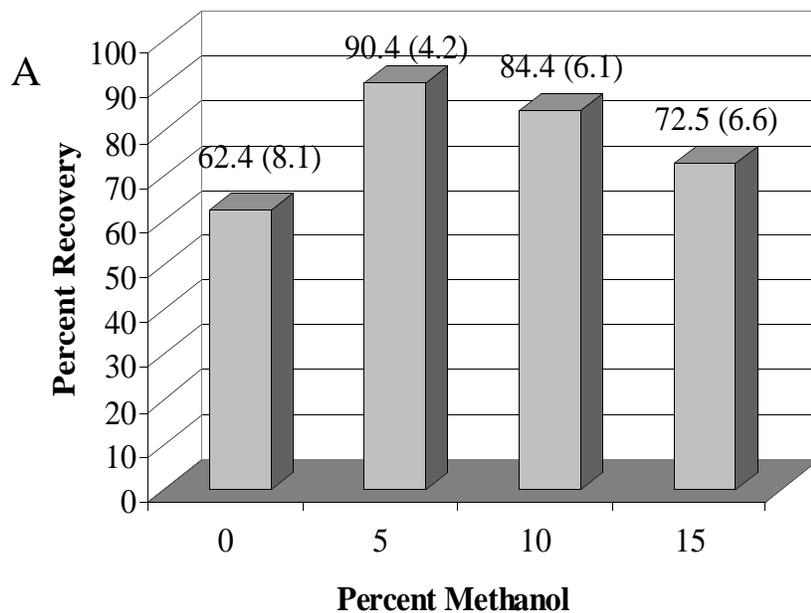
Figure 6: A) CHF$_3$ percent recoveries and percent RSD of ethoxyquin spiked on sand/Hydromatrix® (n=3), B) CFH$_2$CF$_3$ percent recoveries and percent RSD of ethoxyquin spiked on sand/Hydromatrix® (n=3) as a function of percent modifier.

Concentration of Ethoxyquin	Percent Recovery	% RSD (N=3)
10 ppm lean	66.0	4
10 ppm fat	81.0	2
5 pmm lean	70.0	9
5 pmm fat	94.0	5
0.5 ppm lean	99.7	1
0.5 ppm fat	94.5	2

Table 1: Percent recoveries of ethoxyquin spiked at various levels on lean beef and beef fat extracted with CFH_2CF_3 with 5 % methanol modifier.
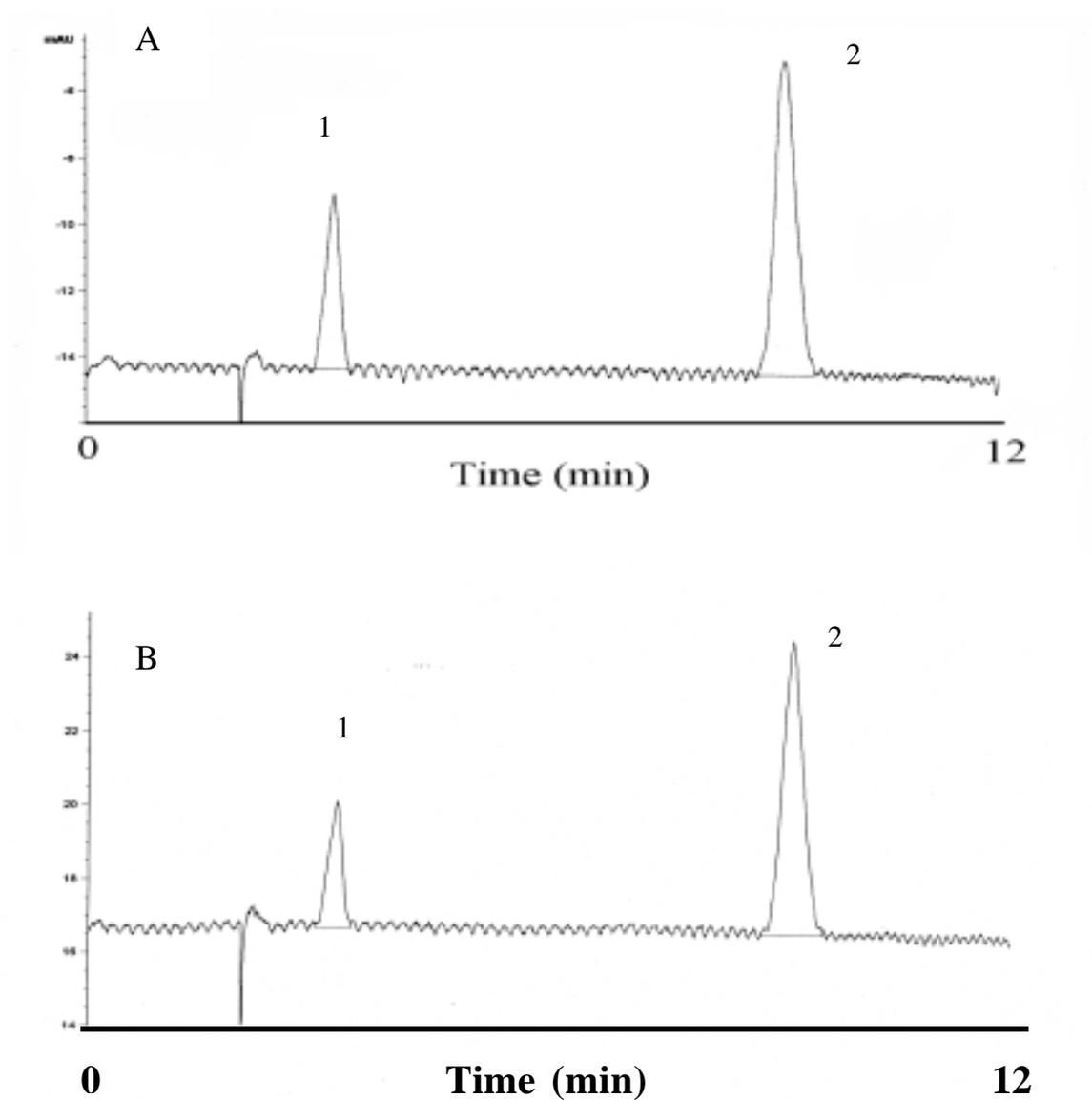
Figure 7:SFC chromatogram of A) ethoxyquin (peak 1) and internal standard 4-nitroaniline (peak 2) dissolved in methanol, B) extract of ethoxyquin from lean beef using CFH_2CF_3 with ethoxyquin (peak 1) and 4-nitroaniline (peak 2).

beef and beef fat. Extractions of lean beef and beef fat from cows which were fed

ethoxyquin preserved feeds did not show the presence of any ethoxyquin.

The fact that a quantitative recovery was only obtained at 0.5 ppm for the beef and fat

and 5 ppm for the fat is not readily explainable. However, a possible rationalization can

be tied to the preservative's mechanism of action and the effects of its oxidation products,

or the actual lean beef and beef fat samples which were spiked onto. A lean beef or beef

fat sample which has any level of oxidation in it, when spiked with ethoxyquin, will

convert ethoxyquin to one of its oxidation products. If this sample is then analyzed for

ethoxyquin, a lower level of the antioxidant will be present than was spiked, as the

ethoxyquin will have scavenged free radicals and have had its structure changed.

Therefore, in terms of detection, retention on the column or lack thereof, and the

wavelength being monitored may be different for the oxidation product than for the

parent ethoxyquin, thus providing the less than quantitative recoveries at higher spiking

levels. These topics make up the rest of the thesis as their oxidation products are

investigated in Chapter 3 and their detection and chromatographic behavior are

investigated in Chapter 4.

SUMMARY

Supercritical CO_2 extracts fat very well and appears to react with ethoxyquin

during extraction, and therefore was not considered to be a suitable fluid for the

extraction of ethoxyquin from lean beef and beef fat. While pure CHF_3 and CFH_2CF_3 did

not provide quantitative recoveries, methanol-modified CHF_3 and CFH_2CF_3 afforded

quantitative recovery of ethoxyquin at the 5% methanol modifier level. Quantitative

recoveries were achieved at 0.5 ppm level for both lean beef and beef fat with 5 percent

methanol modified CFH_2CF_3. No evidence of ethoxyquin was found in lean beef and

beef fat samples from cows that were fed ethoxyquin preserved feeds. These facts were

not able to be rationalized and thus provided the basis for further investigations into

ethoxyquin and its oxidation products.

Chapter 3

Isolation and Gas Chromatography/Mass Spectrometric Identification of Oxidation Products of Ethoxyquin

INTRODUCTION

The work in the previous Chapter investigated supercritical fluids for the extraction of ethoxyquin from lean beef and beef fat samples. However, low recoveries of ethoxyquin that was spiked on lean beef and beef fat, and the inability to detect ethoxyquin from lean beef and beef fat samples from cows which were fed ethoxyquin preserved feeds, required that a further investigation of ethoxyquin oxidation products be conducted. This Chapter deals with the preparation of ethoxyquin oxidation products through controlled oxidation and chemical reactions. The goal was to obtain common oxidation products of ethoxyquin so that a chromatographic method could later be developed for them.

Ethoxyquin Oxidation

Ethoxyquin is sacrificial in its antioxidant action[72], meaning that in scavenging free radicals ethoxyquin allows its chemical structure to be changed rather than that of unsaturated lipids. One of the main attributes of ethoxyquin which makes it such a good antioxidant is that some of its oxidation products also posses some antioxidant properties[72]. Therefore, ethoxyquin does not end its preservative nature after scavenging

one free radical, rather in an oxidized state it continues to inhibit oxidation. The four

oxidation products prepared were chosen as they were found to be present in the

autooxidzing system of fish meal[71]

The first product of oxidation of ethoxyquin is 2,4-dimethyl-6-ethoxyquinoline

which is formed in a high temperature reaction. This oxidation product is proposed to

first donate the amine hydrogen to free radicals. This is the proposed first reaction

intermediate for many of the oxidation products. The loss of a methyl radical then forms

2,4-dimethyl-6-ethoxyquinoline (**Figure 8**). The second oxidation product, quinone

imine N-oxide, also first loses the amine hydrogen allowing the attachment of oxygen.

After attachment of oxygen this intermediate loses an ethyl radical to stabilize the

oxidation product (**Figure 9**). The next oxidation product is the quinone imine, which

after loss of the amine hydrogen loses an ethyl radical (**Figure 10)** Lastly, 1,8-di(1,2-

dihydro-6-ethoxy-2,2,4-trimethyl-quinoline), or the 1,8 ethoxyquin dimer, is formed

when two ethoxyquin molecules that have lost their amine hydrogen react (**Figure 11**).

This is a direct termination of the free radical chain reaction as two radicals react to form

a stabile neutral dimer.

EXPERIMENTAL

GC-MS Instrumentation

A Hewlett Packard 5890 Gas Chromatograph was interfaced with a Hewlett

Packard 5972 model Mass Selective Detector. The inlet was set at 250 °C and the

Ethoxyquin

2,4-Dimethyl-6-
ethoxyquinoline
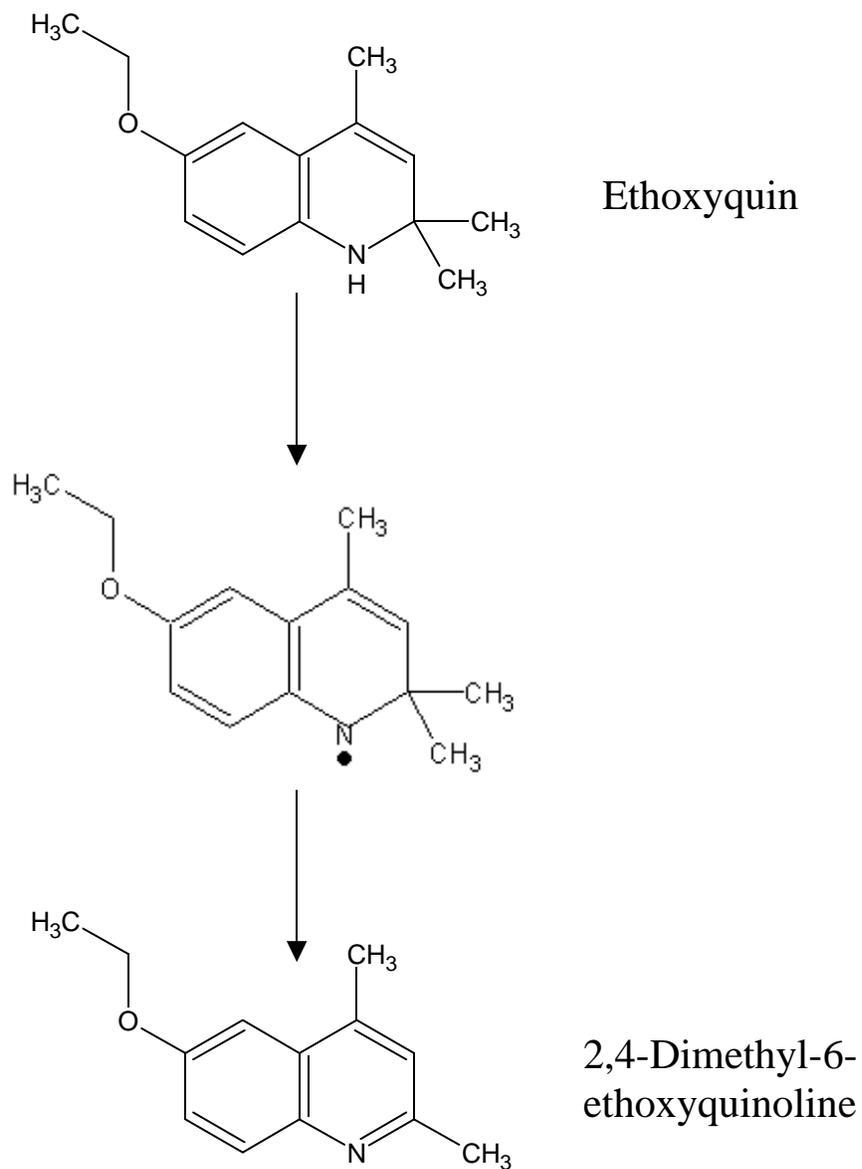
Figure 8: Proposed reaction scheme for the production of 2,4-dimethyl-6-
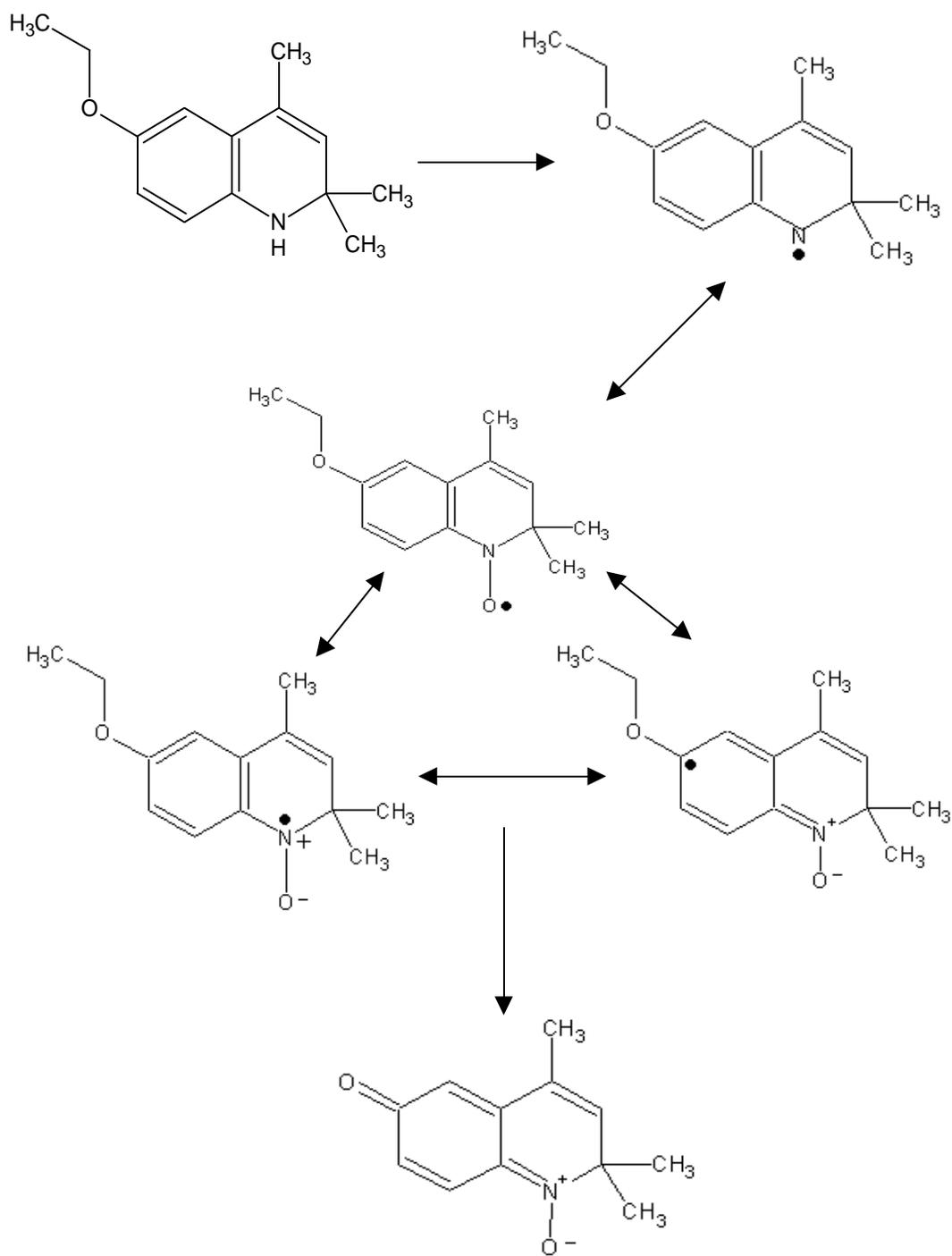
ethoxyquinoline

Figure 9: Proposed reaction mechanism for the production of quinone imine N-oxide
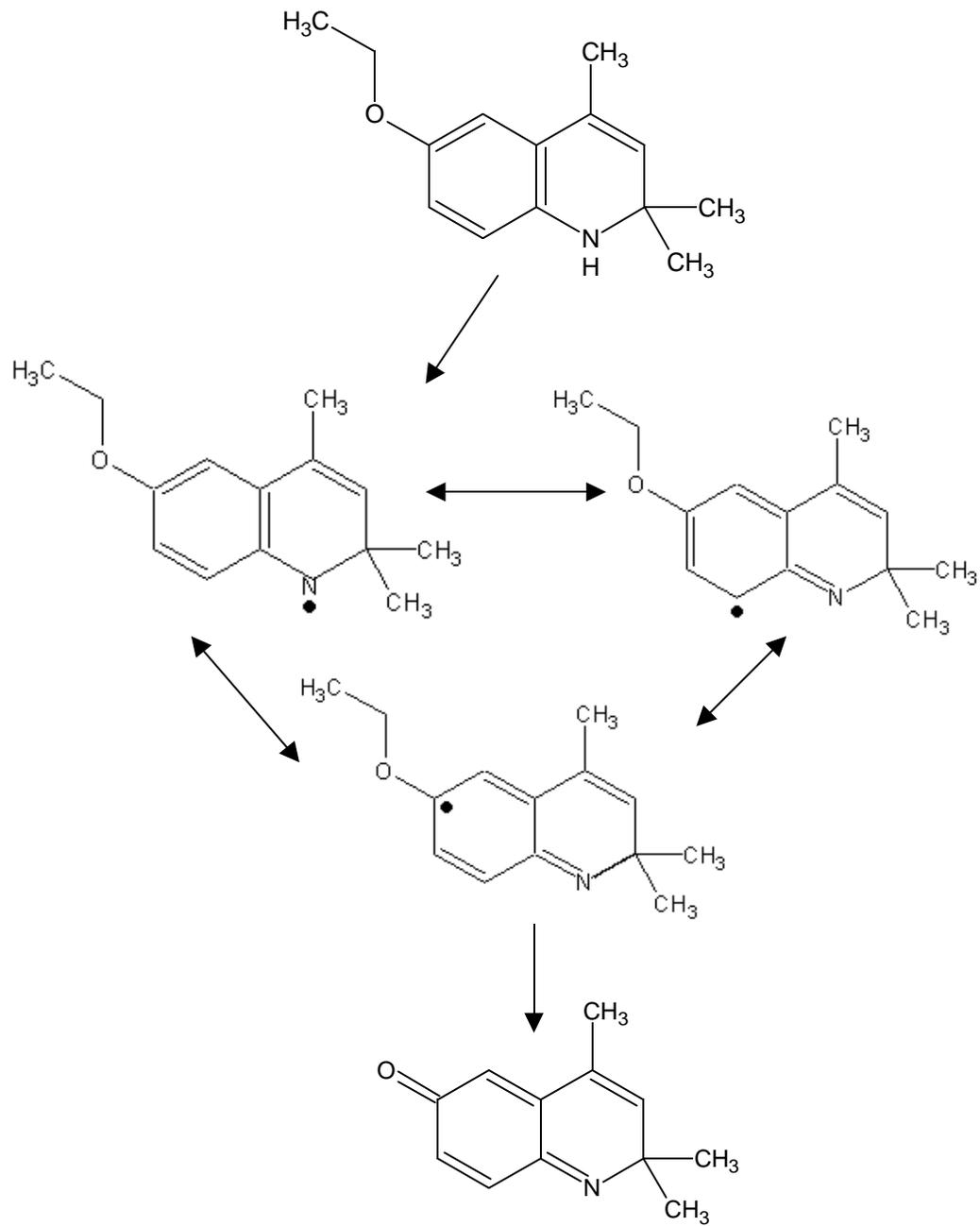
Figure 10: Proposed reaction scheme for production of quinone imine

Figure 11: Proposed reaction scheme for production of ethoxyquin 1,8 dimer

detector was kept at 280 °C. A DB5-MS (30m x 0.25 mm x 0.25 um film) column from

J. & W. Scientific (Folsom, CA) was used. Carrier Grade Helium was purchased from

Air Products and Chemicals, Inc. (Allentown, PA) to be used as the carrier gas. A split

ratio of 100:1 was used. Temperature programming was used with the initial temperature
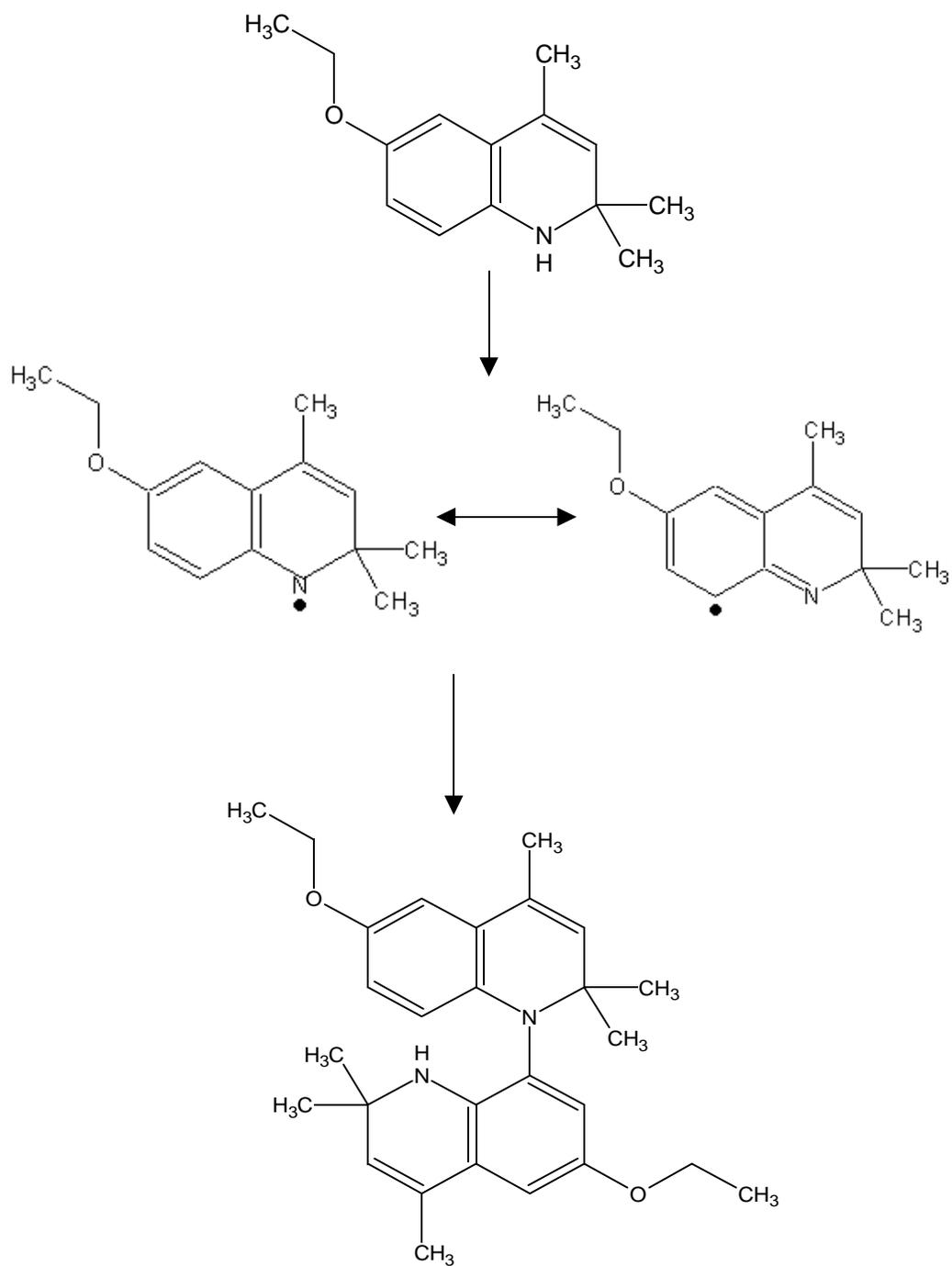
of 50 °C for 3 minutes with ramping of 10 °C/minute to a final temperature of 260 °C.

Total run time for each injection was 34 minutes. The mass selective detector scanned

from 33 to 500 mass units in each scan. A three minute solvent delay was used for all

injections. 0.5 uL of each sample was injected by autosampler with 1 sample wash with

methanol, 2 sample pumps, and no viscosity delay.

Chemicals, Reagents and Procedures

The four-oxidation products that were obtained have been reported before[73], and

have been studied for their antioxidant action[74]. The procedures described below are

based on the work of Thorisson et al.[71] with modification. The main modifications

concern extraction solvents, reaction scale, and the use of column chromatography rather

than prep scale normal phase chromatography.

2,4-Dimethyl-6-ethoxyquinoline

8.0 mL of ethoxyquin from Sigma Aldrich (St. Louis, MO)) was added to 100 mL

of xylene from EM Science (Gibbstown, NJ) in a 250 round bottom flask. Grade 4.3

oxygen purchased from Airco (Murray Hill, NJ) was bubbled into the solution though a

glass pipette as it was refluxed at 140 °C for 24 hours. The solution was cooled and

acidified (16 mL of 12 M HCL from EM Science in 200 mL of water from Mallinckrodt (Paris, KY)). The solution was extracted with chloroform from EM Science. The aqueous acidic layer was made basic with sodium hydroxide (NaOH) and the solution turned milky brown. The basic solution was extracted with chloroform, the chloroform was rotovapped off, brought up in petroleum ether, filtered by hot vacuum filtration, crystallization with petroleum ether. The resulting product was a brownish yellow powder and small amounts of it were diluted for GC/MS.

Quinone imine N-oxide

0.9981 grams of ethoxyquin was added to 40 mL of ethanol in a round bottom flask. Stirring began and 106 mg of sodium tungstate dihydrate purchased from Sigma Aldrich and 172 mg of EDTA was added in 12 mL of H2O. 10 ml of Hydrogen Peroxide (30%) was then added and the mixture was stirred at room temperature for 16 hours. 60 mL of water was added and the solution was saturated with sodium sulfate. The liquid was filtered off and the round bottom flask was washed with toluene (3 x 50 mL). The aqueous phase was extracted with the same toluene used to wash the flask (3 x 50 mL). The toluene was then washed with water (2 x 50 mL) and NaOH (50 mL) and saturated with sodium sulfate. Toluene was removed by rotovap and a red oil resulted. Column chromatography was performed upon the red oil using hexane and ethyl ether as elution solvents. 26 fractions were collected from column chromatography, ranging from 25 mL to 100 mL. The resulting fractions were blown down and 1 mL aliquots were diluted in hexane for GC/MS analysis

Quinine imine

1.02 grams of ethoxyquin was added to 100 mL of ethanol in a round bottom flask. 0.65 mL of t-butylhydroperoxide from Sigma Aldrich and 1.70 g of ammonium ferrous sulfate hexahydrate from Sigma Aldrich was added in 20 mL of water. The mixture was stirred for 28 hours at room temperature, acidified with 100 mL HCl (1 M), and extracted with hexane (4x 75 mL). Each extract was washed with acid (3x 75 mL) and all acid layers were combined. Acid solution was basified with NaOH and extracted with hexane (5 x 75mL). Hexane layers were combined and washed with NaOH (3x 50 mL) and saturated with sodium sulfate. Hexane was rotovapped off and brownish red oil resulted. Column chromatography was performed and the oil was eluted with hexane and isopropanol.

1,8-Di(1,2-dihydro-6-ethoxy-2,2,4-trimethyl-quinoline (ethoxyquin 1,8 dimer)

From the quinone imine reaction above, the hexane extracts remaining from the first set of acidic extractions were combined the hexane was rotovapped down. A deep purple solution resulted and column chromatography was performed on silica gel using hexane and isopropanol as elution solvents.   From column chromatography, 12 fractions were collected and run for GC/MS.

## RESULTS AND DISCUSSION

Ethoxyquin

A solution of ethoxyquin in methanol was injected for GC/MS analysis in order to obtain a baseline mass spectrum for comparison to the oxidation products. **Figure 12** shows the clean total ion chromatogram (TIC) for commercially available (>90 % pure by GC from Sigma Aldrich) ethoxyquin. The electron impact (EI) mass spectrum (**Figure 13**) shows the following peaks: 217 (M^+), 202 (M^+ - CH_3), 188 (M^+ - C_2H_5), 174 (202 –C_2H_4), 145 (174 – CHO).

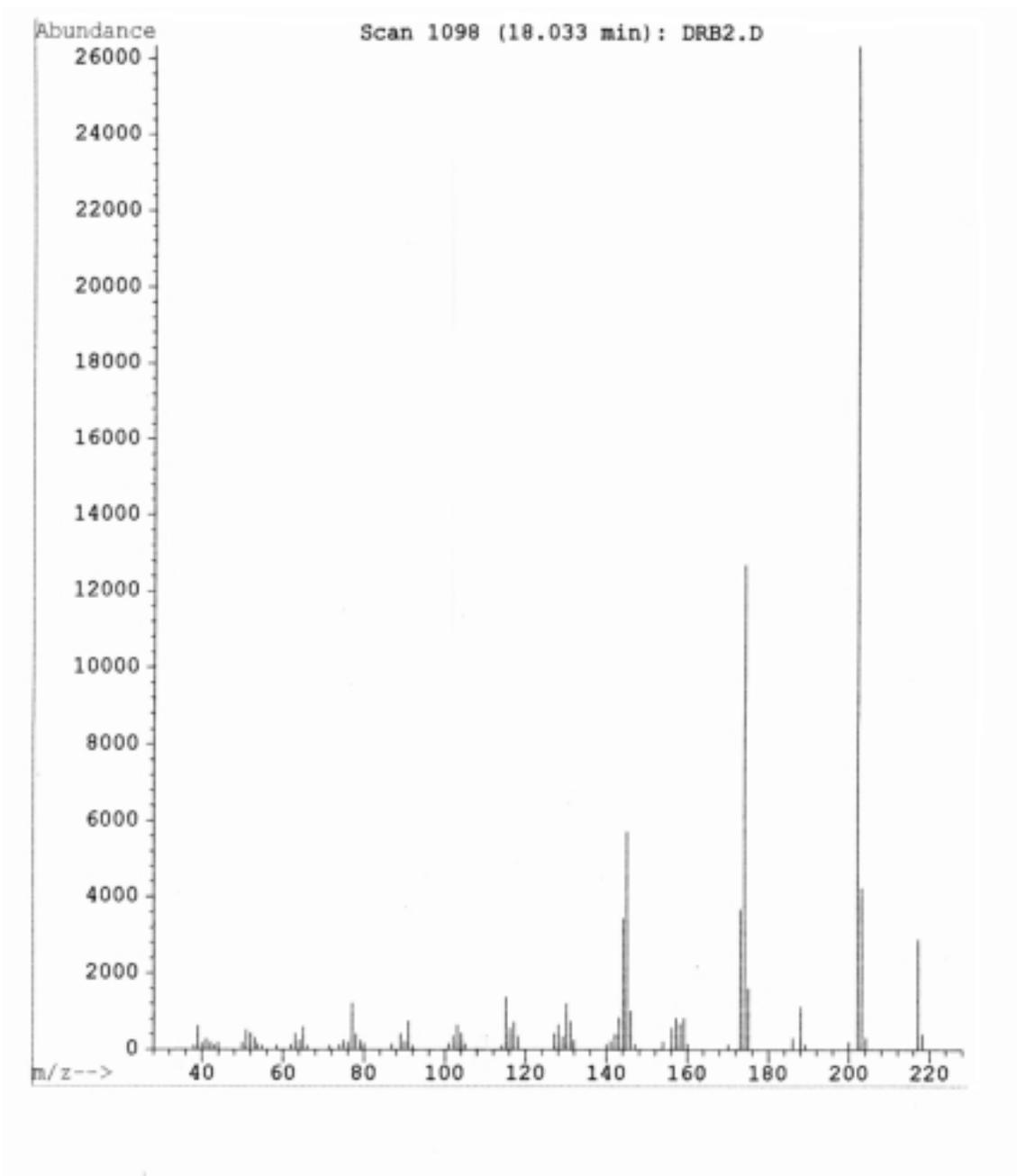## 2,4-Dimethyl-6-ethoxyquinoline

A solution of 2,4-dimethyl-6-ethoxyquinoline product in hexane was injected for GC/MS analysis. **Figure 14** shows the TIC for this product, with very few impurities present in very low levels as compared to the main product. The EI mass spectrum (**Figure 15**) shows the following peaks: 201 (M^+), 173 (M^+ - C_2H_4), 144 (173 – CHO). The M^+ peak corresponds to the predicted mass of 2,4-dimethyl-6-ethoxyquinoline, and the other peaks confirm the predicted structure. The absence of a peak that would indicate a loss of methane (M^+ - CH_3) also indicates that the molecule is 2,4-dimethyl-6-ethoxyquinoline.

Quinone imine N-oxide

Of the 26 fractions collected, 4 fractions (numbers 16 –19) showed clean chromatograms and mass spectra indicative of quinone imine N-oxide. **Figure 16** shows TIC of the product with minor impurity levels at less than 10%. **Figure 17** shows the EI mass spectrum with the main peaks: 203 (M^+), 187 (M^+ - O), 174 (M^+ - CHO), 159 (174
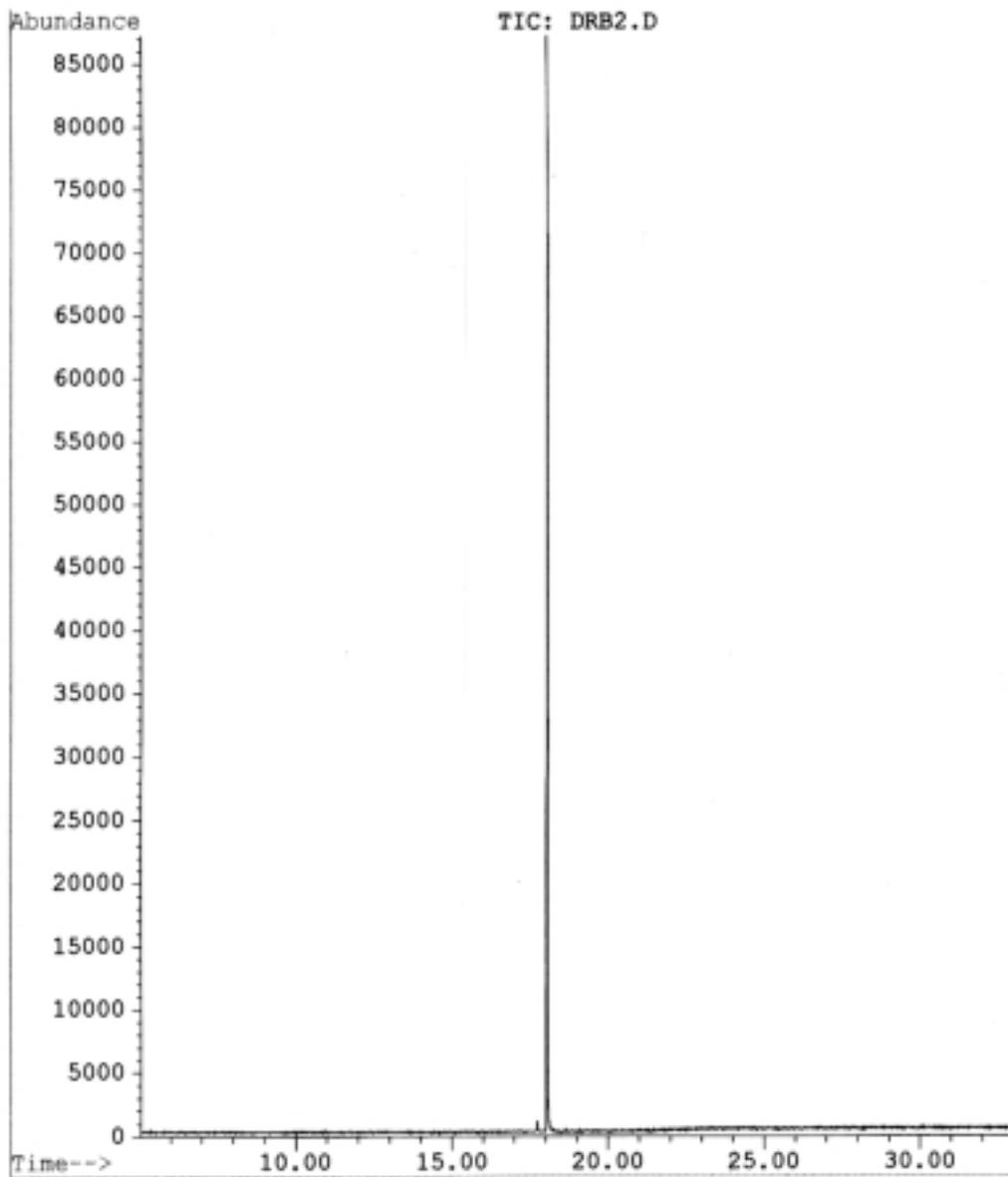
Figure 12: Total ion gas chromatogram of ethoxyquin

Figure 13: Electron impact mass spectrum of Ethoxyquin
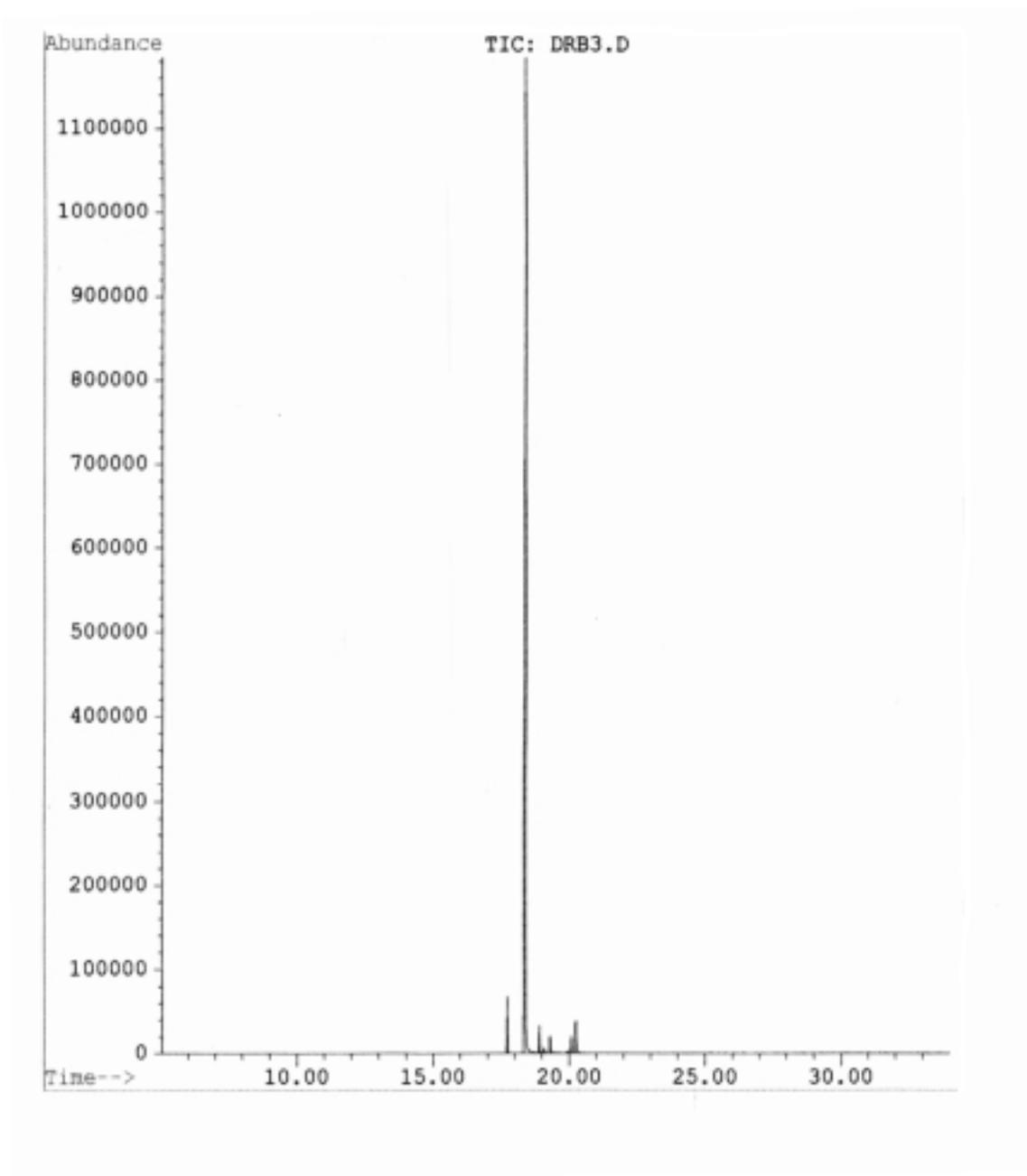
Figure 14: Total ion gas chromatogram of 2,4-dimethyl-6-ethoxyquinoline

Figure 15: Electron impact mass spectrum of 2,4-dimethyl-6-ethoxyquinoline

29

Figure 16: Total ion gas chromatogram of quinone imine N-oxide

Figure 17: Electron impact mass spectrum of quinone imine N-oxide

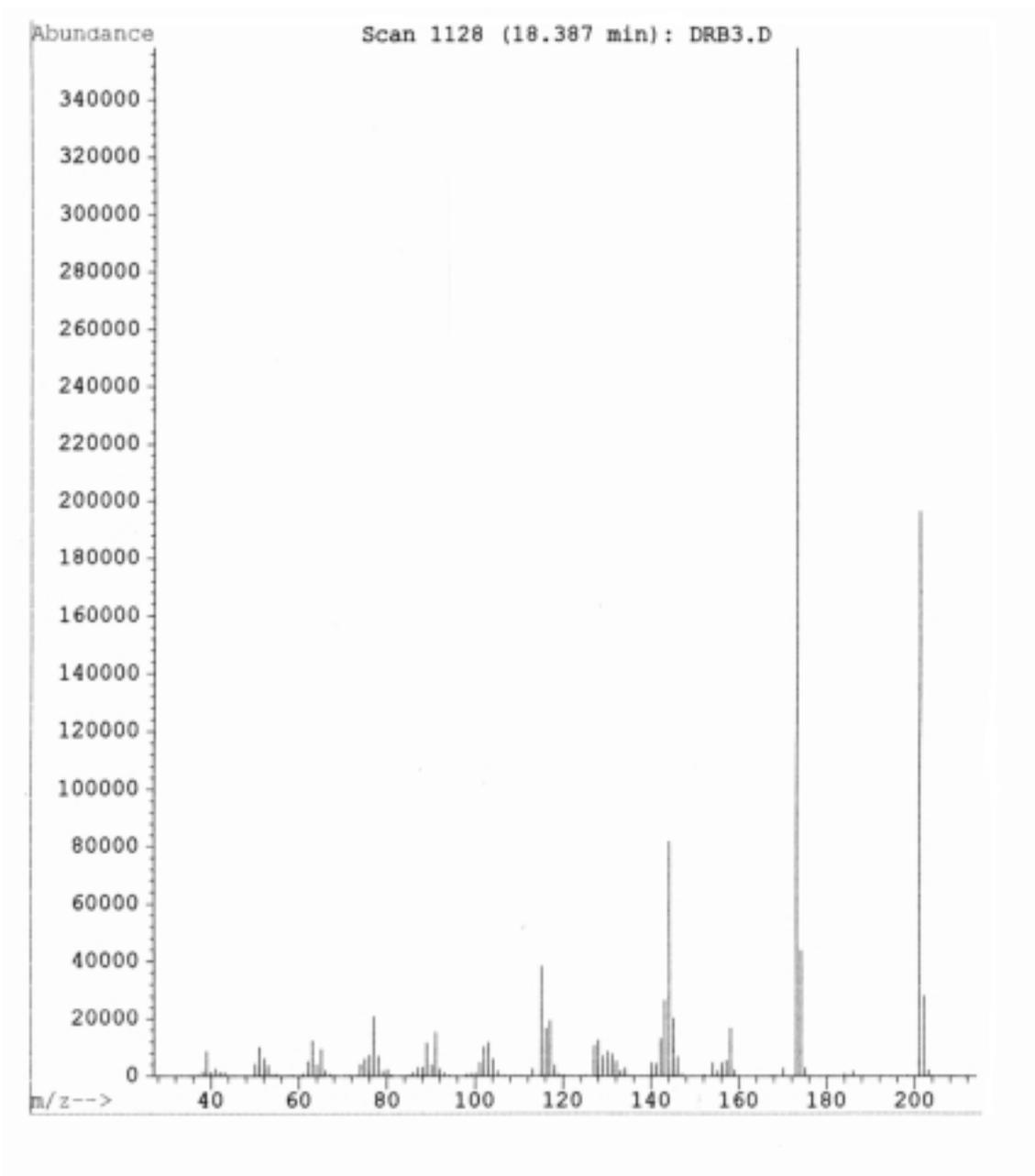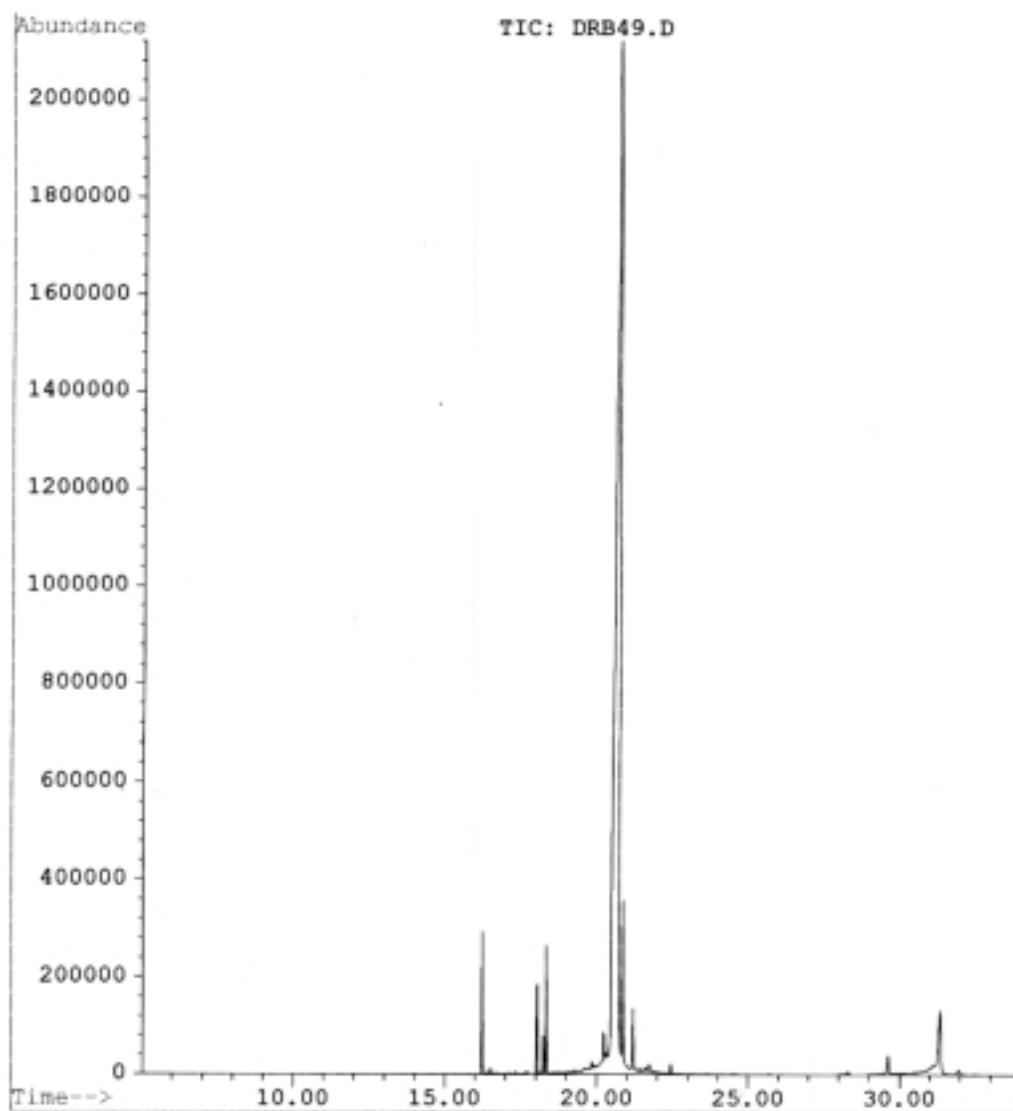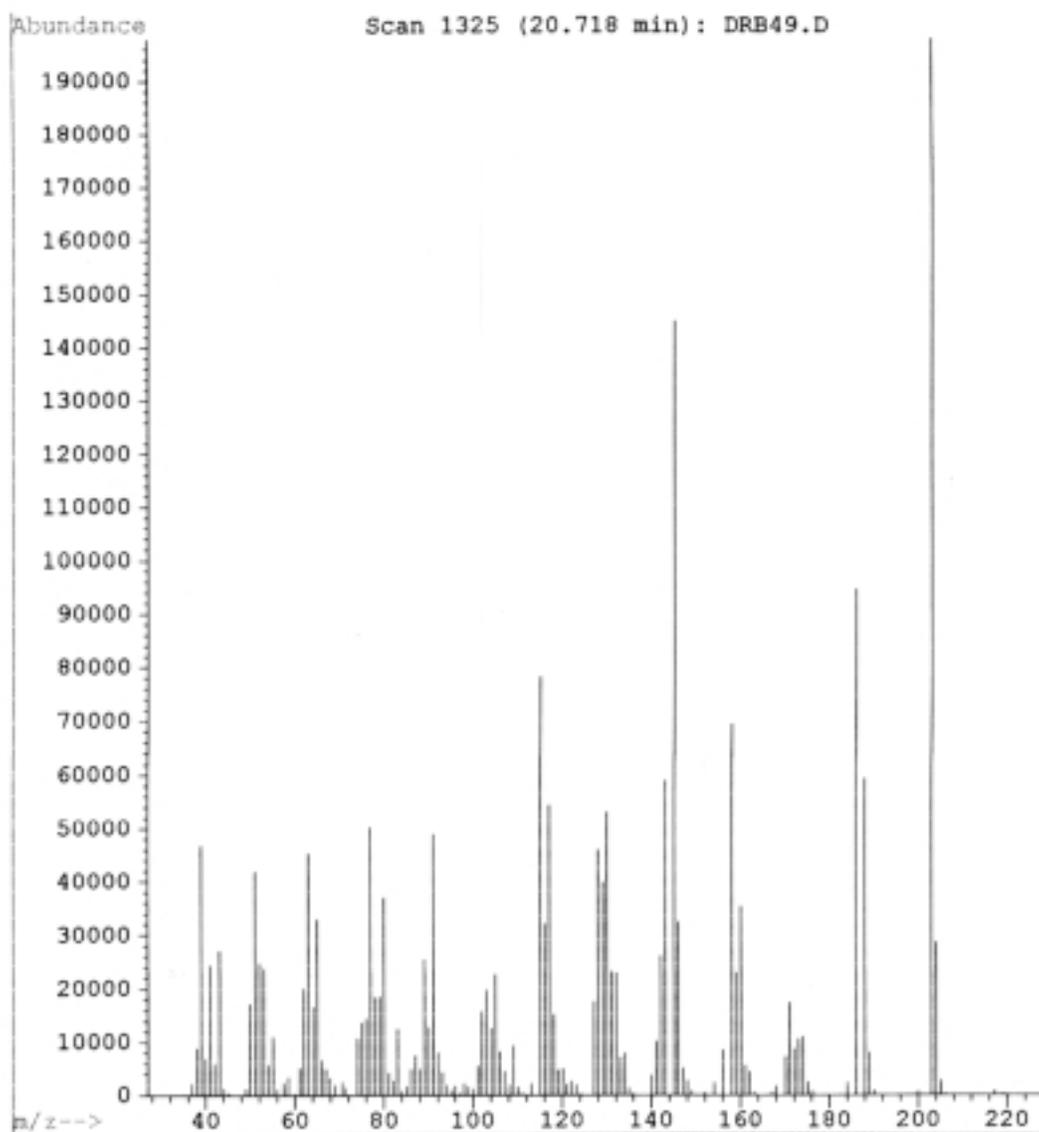– CH$_3$), 144 (159 – CH$_3$). The mass spectra indicate that the quinone imine N-oxide is present as the M$^+$ peak corresponds. Fragmentation also indicates the quinone imine N-oxide is the main products as there is an absence of a loss of the ethyl peak as seen in ethoxyquin. The high concentration of the injected sample may be the cause of the large M$^+$ peak and the abundance of other fragmentation peaks.

Quinine imine

The quinone imine proved to be the most difficult oxidation product to obtain. Column chromatography of the product produced only one fraction containing the proposed product. **Figure 18** shows the TIC of the proposed quinoneimine, and indiactes a very clean sample. **Figure 19** shows the EI mass spectra of the proposed quinone imine with the main peaks of: 187 (M$^+$), 172 (M$^+$ - CH$_3$), 159 (M$^+$ - CO), 144 (159 – CH$_3$). The mass spectra indicates that the quinone imine is present as the M$^+$ peak corresponds to the structures mass. The fragmentation also indicates the proposed structure.

1,8-Di(1,2-dihydro-6-ethoxy-2,2,4-trimethyl-quinoline (ethoxyquin dimer)

The first 4 fractions collected indicated that the dimer was present. **Figure 20** shows the TIC of the second fraction collected. **Figure 21** shows the EI mass spectrum of the second fraction collected and shows the main peaks of: 432 (M$^+$), 417 (M$^+$ - CH$_3$), 402 (417 – CH$_3$), 201 (402 – 201), 173 (201, C$_2$H$_4$). These peaks indicate that the
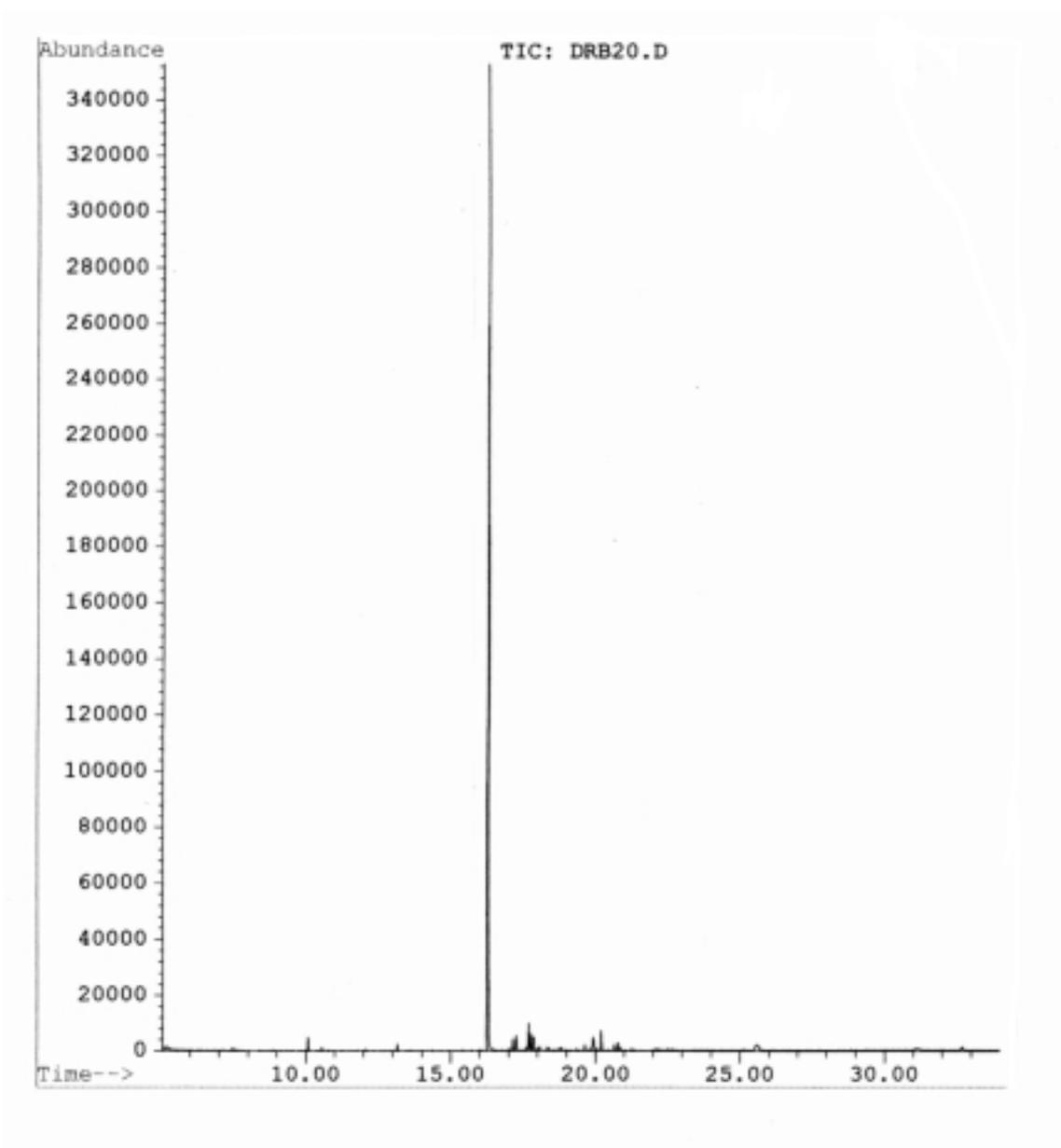
Figure 18: Total ion chromatogram of quinone imine
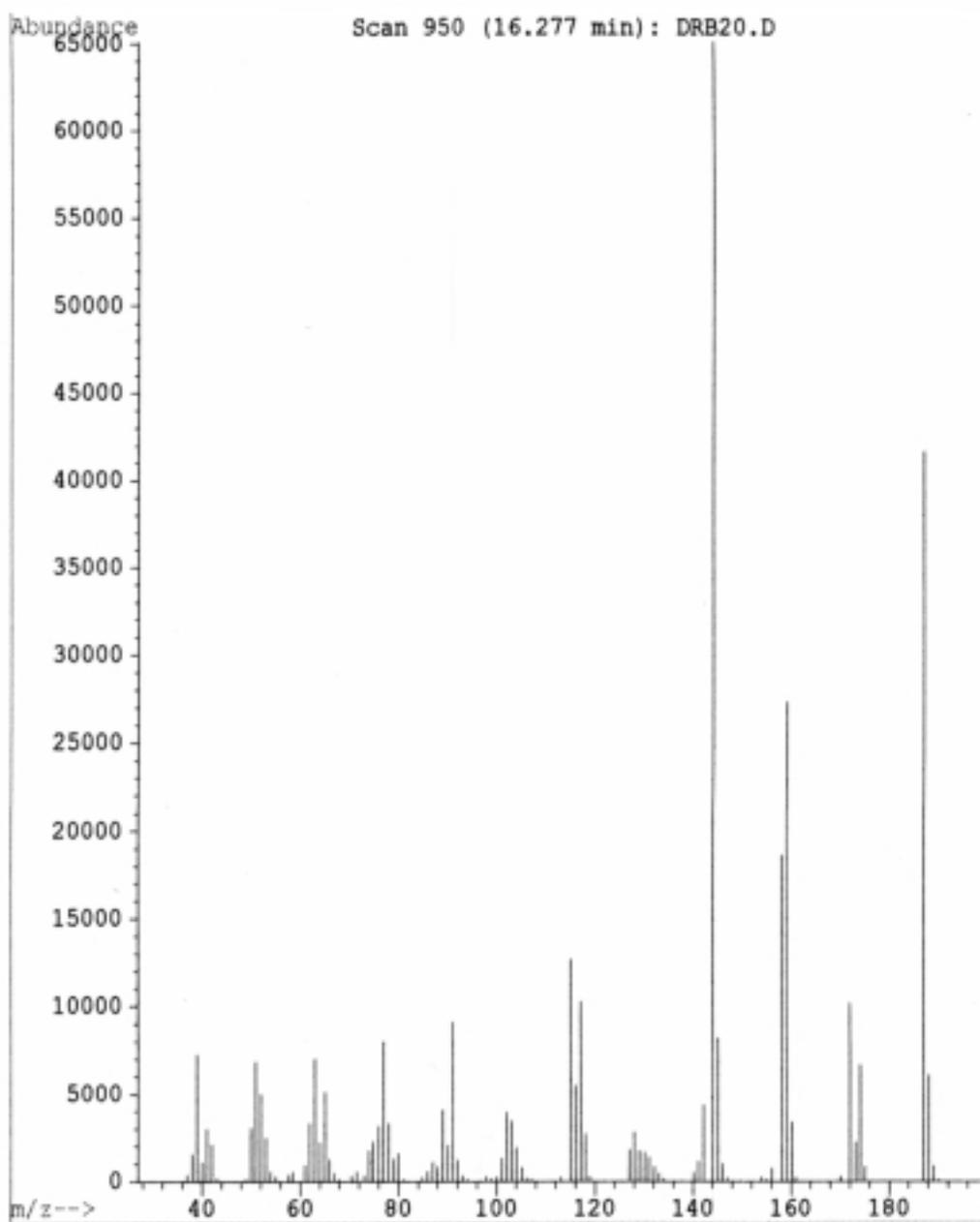
Figure 19: Electron impact mass spectrum of quinone imine
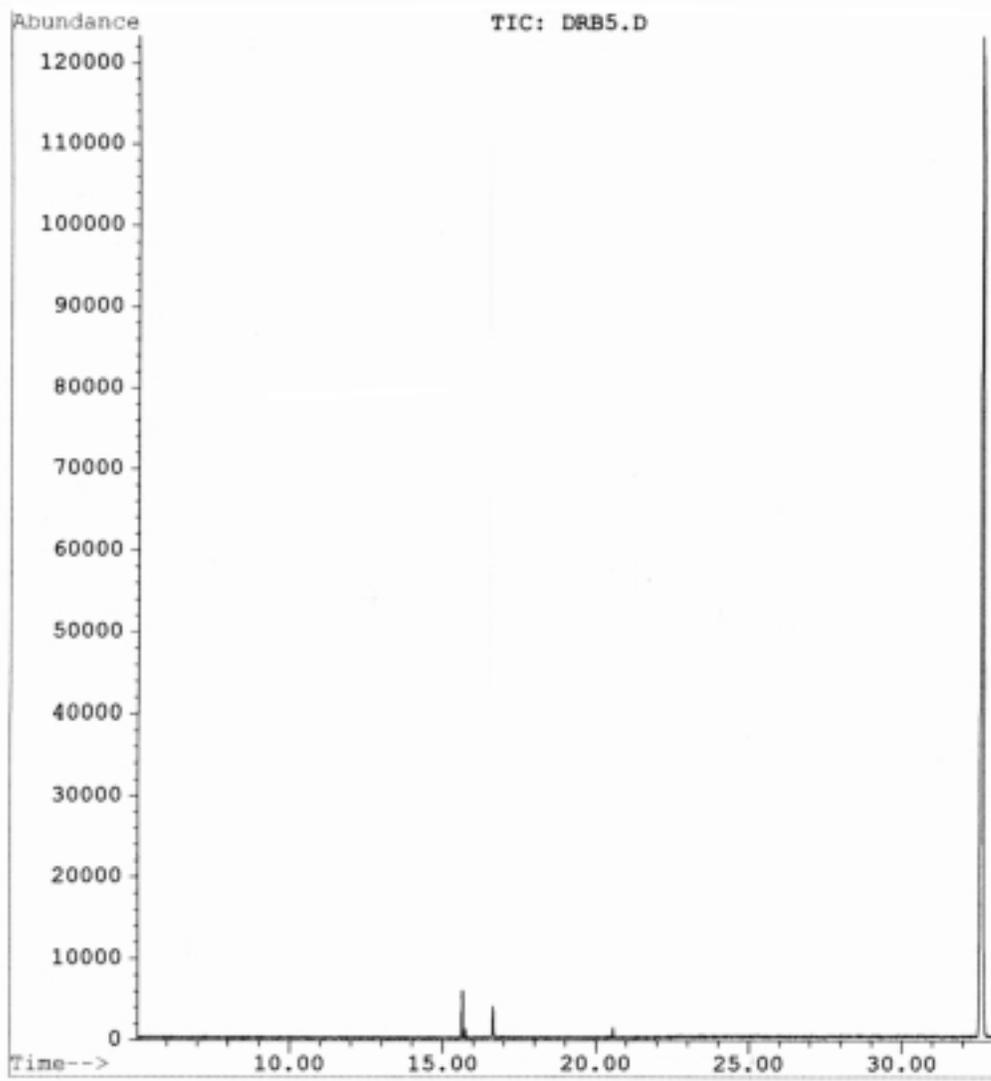
Figure 20: Total ion chromatogram of ethoxyquin 1,8 dimer
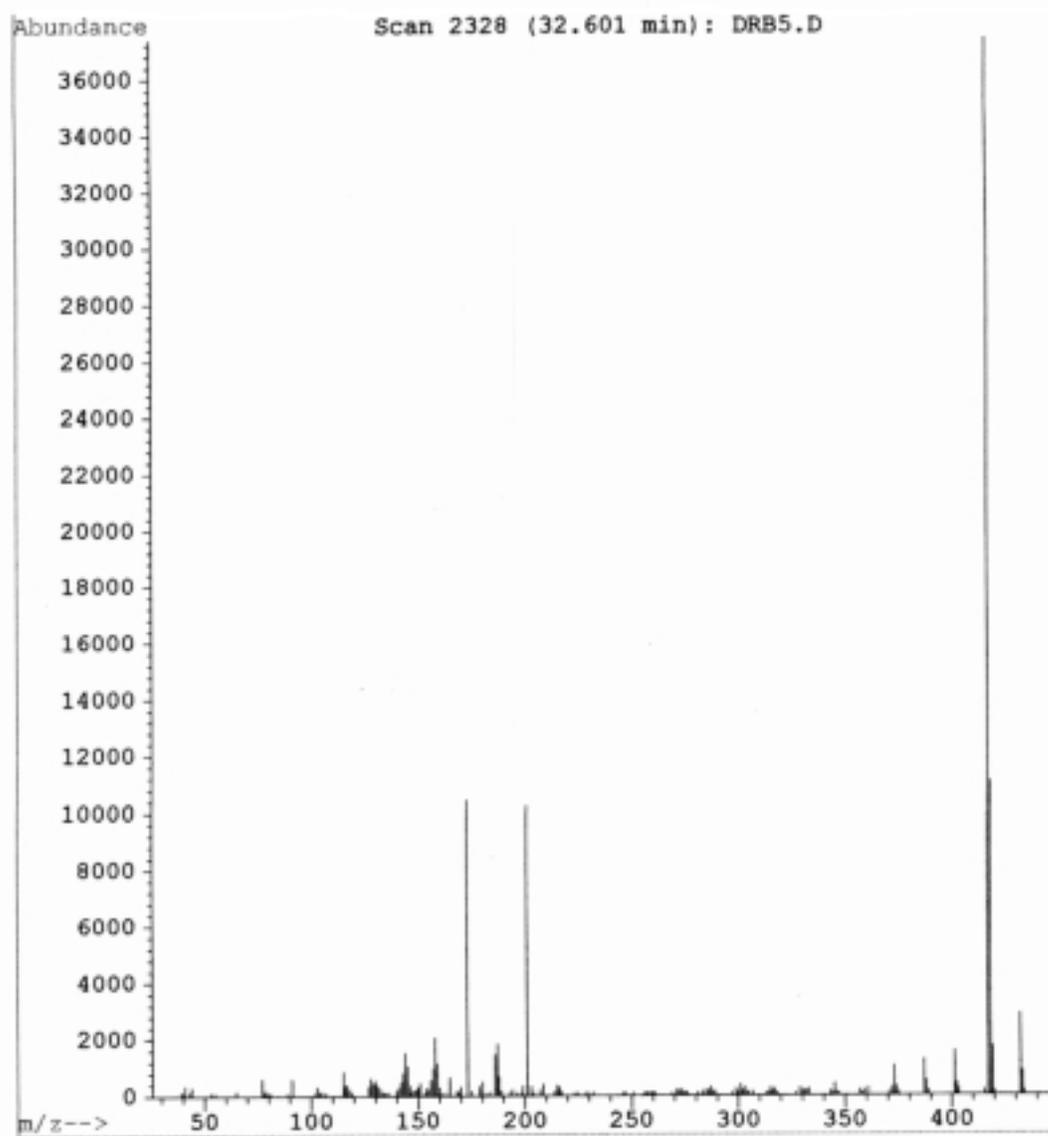
Figure 21: Electron impact mass spectrum of 1,8-ethoxyquin dimer

proposed dimer products are present as the M^+ peak matches the proposed structure, and fragmentation is similar to that of ethoxyquin.

Summary

The goal of the chapter, to synthesize oxidation products of ethoxyquin, was achieved through a series of chemical reactions, isolation through column chromatography, and identification by GC/MS. The oxidation products obtained were 2,4-dimethyl-6-ethoxyquinoline, quinone imine N-oxide, quinone imine, and 1,8-di(1,2-dihydro-6-ethoxy-2,2,4-trimethyl-quinoline (ethoxyquin dimer). These products can now be used to develop a HPLC method for ethoxyquin and it oxidation products.

Chapter 4

**Evaluation of High Performance Liquid Chromatography Chemiluminescence
Nitrogen Detector for Monitoring Oxidation Products of Ethoxyquin**

INTRODUCTION

In Chapter 2, a supercritical fluid extraction of ethoxyquin from spiked lean beef
and beef fat was developed. However, low recoveries at higher spiking levels and the
sacrificial preservative action of ethoxyquin led to a further investigation into the
oxidation products of ethoxyquin. In order to develop a total ethoxyquin determination,
some oxidation products of ethoxyquin were synthesized (Chapter 3). The first part of
this chapter evaluates the CLND, a novel selective nitrogen detector, for use with HPLC.
The second part of this chapter will use the HPLC/CLND to develop a method for
determination of ethoxyquin and some of its oxidation products.

HPLC/CLND

Elemental selective detection offers many advantages over other commonly used
detection processes.   The CLND allows a selective, sensitive, and simple analysis to be
performed as compared to other detection processes.[75] As compared to an UV or
fluorescence detector, the CLND does not require a molecule to have a chromophore or
the ability to fluoresce. Ultraviolet detectors, while the most common and useful in
HPLC, can only detect molecules that undergo an absorption process when irradiated

with a specific wavelength of light. Fluorescence detectors also are based on a quantum

process of absorption and release of energy. A nitrogen selective detector only requires

nitrogen be present, and does not require a specific process to take place. Secondly, in

complex mixtures, or those mixtures that can be partially separated, a nitrogen selective

detector will only detect nitrogen.

In addition, the CLND has also been reported to have an equimolar response[46].

This allows nitrogen-containing compounds to be quantified with any other nitrogen

containing compound. A known standard is required to accurately quantify an analyte

with UV, fluorescence, and MS, as analytes will have different molar absorptivities or

different ionization efficiencies. This fact makes the CLND an indispensable tool in

combinatorial chemistry and any situation where degradants, by products, oxidation

products, or metabolites must be detected and quantified in a timely manner. The

alternative with other detection methods is to isolate an analyte, take the sample to

dryness, accurately weighed, and generate calibration curves.

A flow diagram for the CLND is shown in **Figure 22**. The HPLC effluent mixes

with oxygen and argon to be nebulized into the quartz pyrotube. Oxygen is required for

combustion and argon is used to optimize the nebulization. The HPLC flow rate into the

CLND must be held relatively low as the CLND can eliminate water only at a rate no

faster than 500 uL/min. The quartz pyrotube is held at 1050°C and is where all organics

are combusted and nitrogen oxide is formed. Nitrogen oxide and the other products of

combustion are then taken into a membrane drier, consisting of a countercurrent vacuum
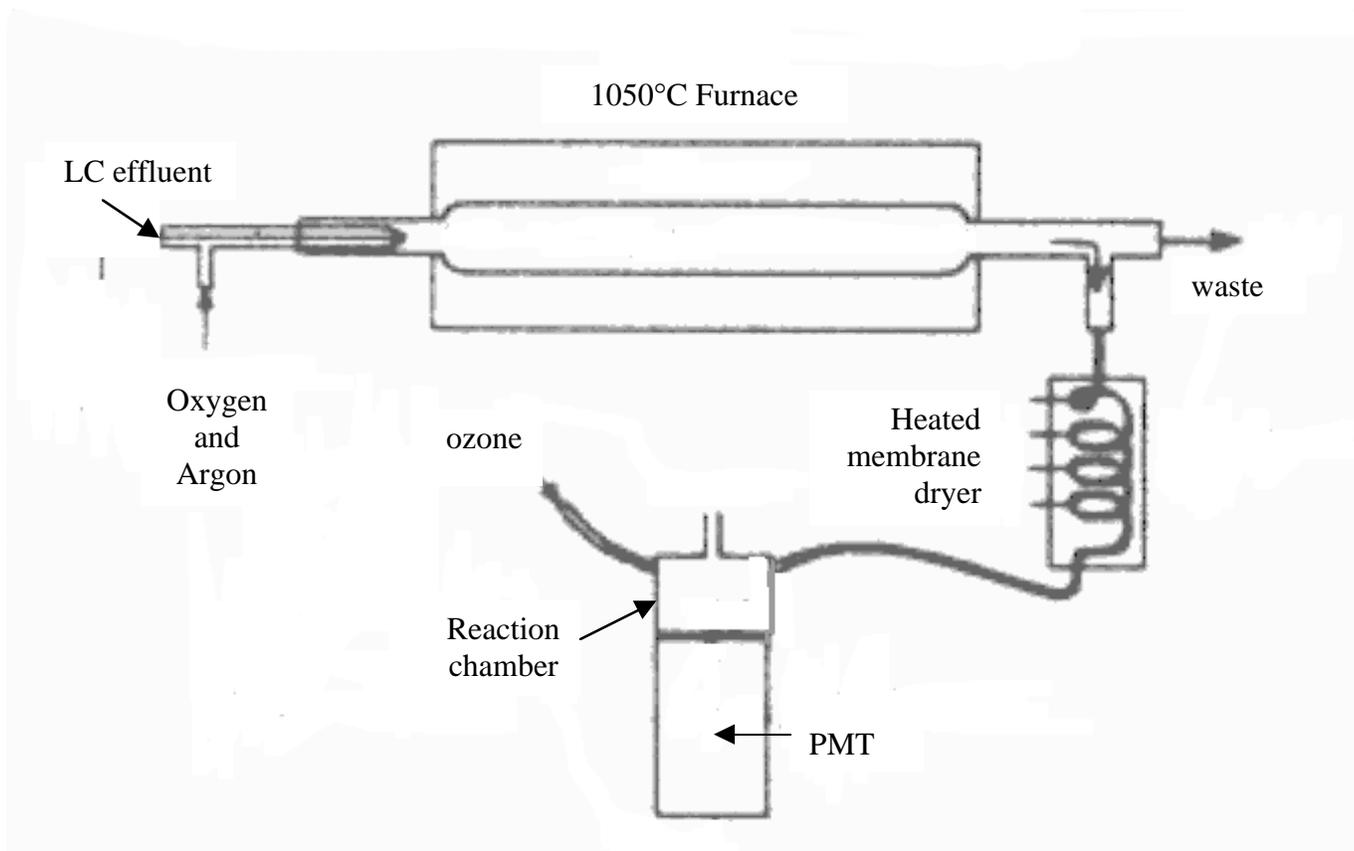
Figure 22: Flow diagram for the HPLC/CLND

on a Naphion membrane, which is enclosed in a heating jacket kept at 85°C. Water is eliminated throughout the membrane dryer and empties the nitrogen oxide into the reaction chamber. In the reaction chamber the nitrogen oxide is reacted with ozone to produce the exited state nitrogen dioxide, which upon relaxation will emit energy in the form of light. This light travels through a red UV filter and is monitored by photomultiplier tube in the 600 – 900 nm range.

The HPLC/CLND has been proven to be a powerful tool in chromatography by Antek® Instruments. The model 8060 model CLND is a new model specifically produced for use with HPLC. It includes patented pyro-chemiluminescence nitrogen detection, a patented sample introduction system, and electronic mass flow controllers. Specifically, there is no published research on this model detector. Previous research on the CLND has been limited to simple mobile phases of methanol and water. This research will first focus on evaluating this new CLND for use with reversed phase HPLC. The second part of the research will describe the use of the CLND in developing a separation of the oxidation products of ethoxyquin in both reversed and normal phase modes.

EXPERIMENTAL

Instrumentation

A model 8060 CLND nitrogen specific HPLC detector from Antek® Instruments Inc. (Houston TX) was interfaced to a Hewlett-Packard (Little Falls, DE) 1050 High

Performance Liquid Chromatograph. The HPLC system was plumbed with 0.005 inch

PEEK tubing from ChromoTech (Apple Valley, MN). Fused silica (50 um internal

diameter, 150 um outer diameter) from PolyMicro Technologies (Tucson, AZ) was used

in the Antek® nebulizer of the CLND as a replacement for the steel tubing which came

with the CLND. Data were recorded by a Hewlett-Packard 3390 Integrator.

Chromatographic separation was achieved using 3 um particle size C18 (2)

LUNA, cyano LUNA, or silica LUNA columns (150 x 1 mm) from Phenomenex

(Torrance CA). In the evaluation of the CLND the C18 column was eluted at 50 uL/min

with a linear gradient from 15 to 85 % buffer B in 30 minutes and holding until all

analytes eluted. Buffer A was 0.15% trifluoroacetic acid in water, and buffer B was 75%

methanol, 25 % isopropanol, 0.15% trifluoroacetic acid. A 2.5 uL sample was injected

onto the column using a 2.5 uL injection loop which was filled using a threefold

overfilling technique. In the normal phase separation of the oxidation products of

ethoxyquin, either a cyano or silica column was used. The column was eluted at 50

uL/min using an isocratic mobile phase made up of mixtures of hexane and isopropanol.

For the reversed phase separation of the oxidation products of ethoxyquin a C18 column

was used with a gradient of 55 to 95 buffer A in 20 minutes. Mobile phase component A

was water, and component B was 75% methanol, 25 % isopropanol.

Chemicals and Reagents

HPLC grade methanol, isopropanol (2-propanol), trifluoroacetic acid and hexane

were purchased from EM Science (Gibbstown, NJ). HPLC grade water was purchased

from Mallinckrodt (Paris, KY). Dibucaine, ethoxyquin, chlorpheniramine (citrate), triprolidine (hydrochloride), caffeine, diphenhydramine, diethylcarbamazine, 2,3 dimethylindole were purchased from Sigma (St. Louis MO). 2-Nitroaniline was purchased from Aldrich Chemical Co. (Milwaukee, WI). All chemicals were used without further purification. HPLC solvents were used in preparing standard solutions of the various nitrogen containing compounds. The compounds were diluted with 50 % water/50 % methanol/isopropanol (75/25%) to standard levels determined by nitrogen concentration. Argon for the CLND nebulizer, and grade 4.3 oxygen for the nebulizer, pyrolysis furnace, and ozone generator were purchased from Airco (Murray Hill, NJ). Ethoxyquin oxidation products were prepared per Chapter 3, taken to dryness, and then diluted with either isopropanol, for normal phase separations, or methanol for reversed phase separations.

RESULTS AND DISCUSSION

The objective of this Chapter was to evaluate the equimolarity, linearity, linear range, limit of quantitation, and limit of detection of HPLC/CLND. These characteristics are important for both the use of the CLND and the comparison to other HPLC detectors. These evaluations were performed using a variety of nitrogen containing compounds and gradient elution. Once the detector's characteristics were confirmed, the HPLC/CLND was used to separate and detect ethoxyquin and some of its oxidation products.

Chemiluminescence Nitrogen Detector Evaluation

The first goal of the Chapter is to evaluate the characteristics of the CLND for use with HPLC. This evaluation required the development of an HPLC method that separates different nitrogen containing compounds. These compounds needed to be varied in structure such that a variety of nitrogen chemical environments could be investigated. Investigating these different nitrogen containing compounds would also allow the response of the detector to be evaluated. Eight compounds containing nitrogen were investigated for a reversed phase separation (**Figure 23**).

The separation of these compounds on a C18 column required a gradient be used . The gradient used in this method used two buffer solutions made at 0.15% with TFA. The TFA is added to buffer the mobile phase to a pH level of 2, to insure that all compounds are being separated in one form. A low pH was chosen instead of a high pH to inhibit column dissolution. The gradient is made up of solution A, which is water, and solution B, which is a 75/25 methanol/isopropanol mixture. The nebulization process was optimized for proper combustion and to minimize sample loss on the sides of the pyrotube. Changes in viscosity will affect the nebulization process and may deteriorate detector response and affect the equimolarity response. The mixture of methanol/isopropanol allowed the viscosity of the mobile phase to remain very constant as compared to a gradient using only water and methanol which would gradually decrease in viscosity as the percent methanol increases.

The separation of the 8 nitrogen containing compounds, (**Figure 24**) with the gradient conditions noted above, yields baseline resolution of all compounds. The
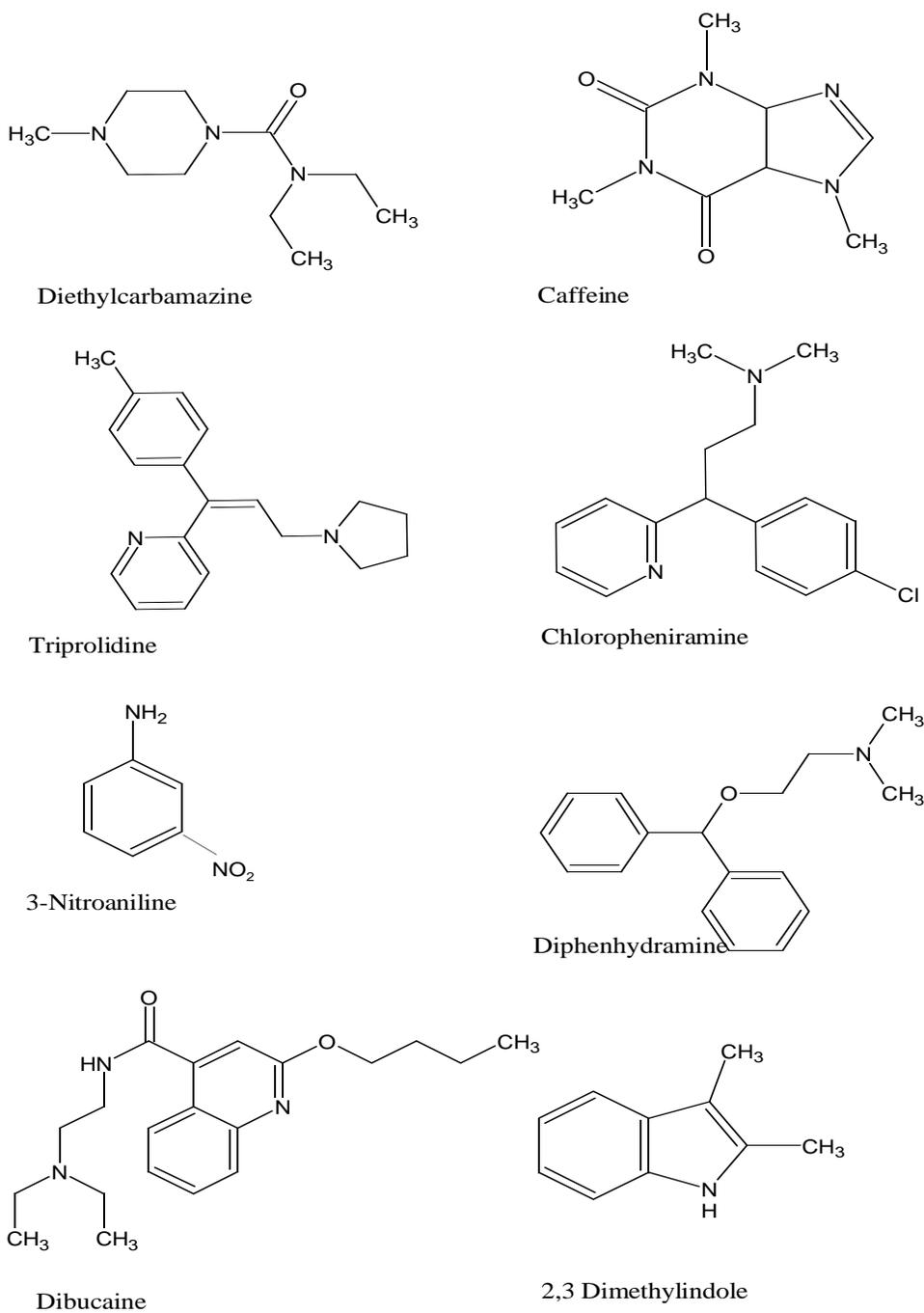
Figure 23: Chemical structure of the 8 nitrogen containing compounds used in the HPLC/CLND evaluation
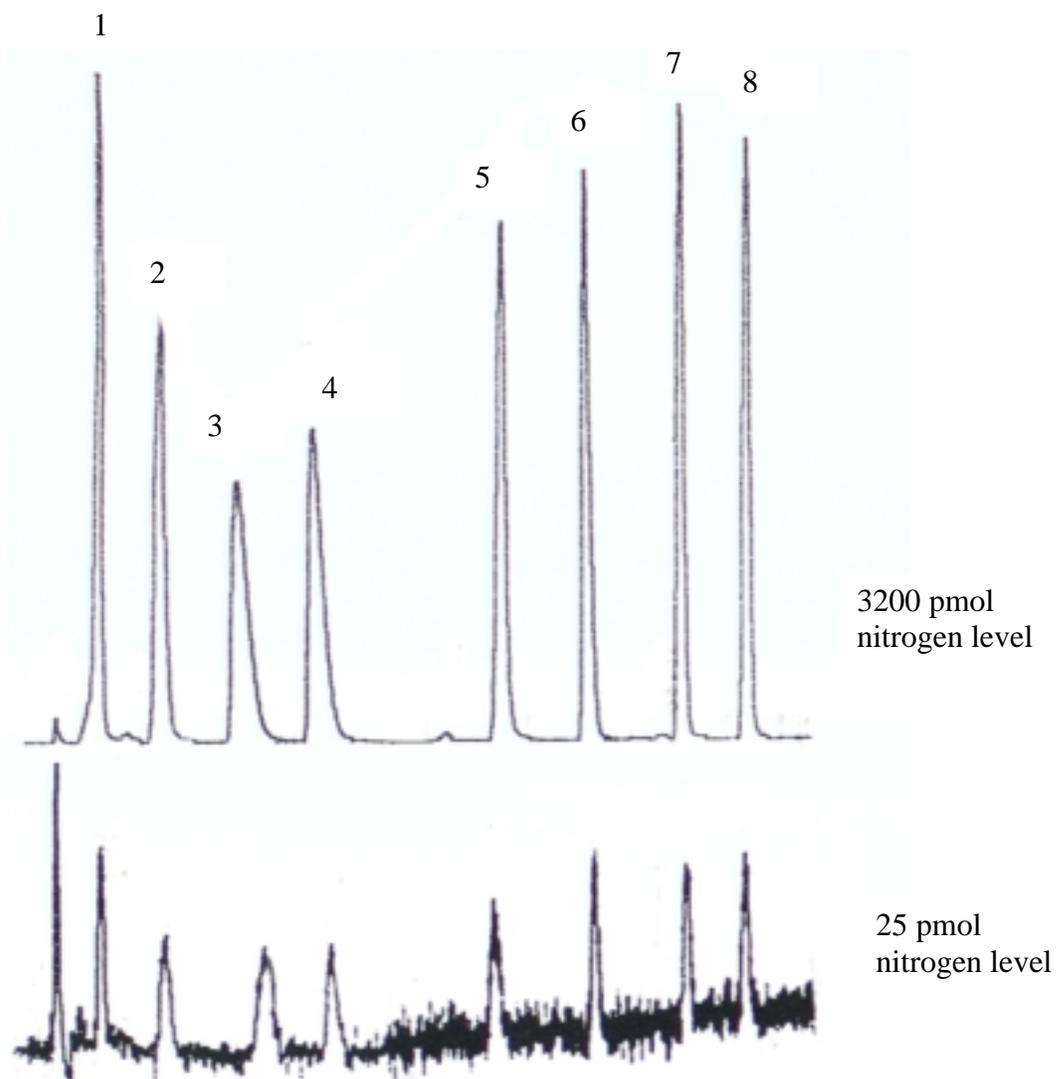
Figure 24: HPLC/CLND separation of the 8 nitrogen containing compounds at 3200 and 25 pmol nitrogen levels. 1. Diethylcarbamazine, 2. Caffeine, 3. Triprolidine, 4. Chlorpheniramine, 5. 3-Nitroaniline, 6. Diphenhydramine, 7. Dibucaine, 8. 2,3-Dimethylindole. Mobile phase components A: water/0.15 % TFA and B: 75% methanol/25% isopropanol/0.15% TFA. Gradient of 85/15 A/B to 15/85 A/B in 30 minutes on Phenomonex C18 Luna column (150 x 1 mm, 3um).

gradient was started at a low level to get a proper retention of peak 1. Peaks 3 and 4 provided poorer peak shapes as compared to other peaks. This can be attributed to the gradient conditions themselves and the fact that these compounds were analyzed as citrate salts. Each compound was diluted to a common level of nitrogen concentration, which yielded equal peak areas for all compounds, as the CLND is a mass type detector.

Analysis of the peak areas of these compounds at concentration levels of 25, 50, 100, 400, 800, 1600, 3200, and 6400 pmol of nitrogen provided calibration curves (**Figure 25**) and detector response data (**Table 2**). The calibration curves show the equal CLND response for all of the different compounds investigated. Triprolidine is the only real outlier as seen in the slope data in **Table 2**. This is due to a detector bias that was not corrected for caused by tripolidine's tailing peak shape. However the other slope data indicate that no other slopes differ more than a few percent from the total average. **Table 2** also shows the linearity, limit of detection and limit of quantitation for all compounds. Limit of detection and limit of quantitation were determined by measuring peak height and noise height (average of 1 min increments throughout run) and are defined as 3:1 and 5:1 signal to noise ratio respectively. As indicated, although each compound has a different number of nitrogen atoms in various environments, dilution to a similar level of nitrogen content provided the same detector limits and response for all compounds. These data prove the equimolarity of the HPLC/CLND and also provide the detector characteristics for comparison to other HPLC detectors. In summary, the HPLC/CLND
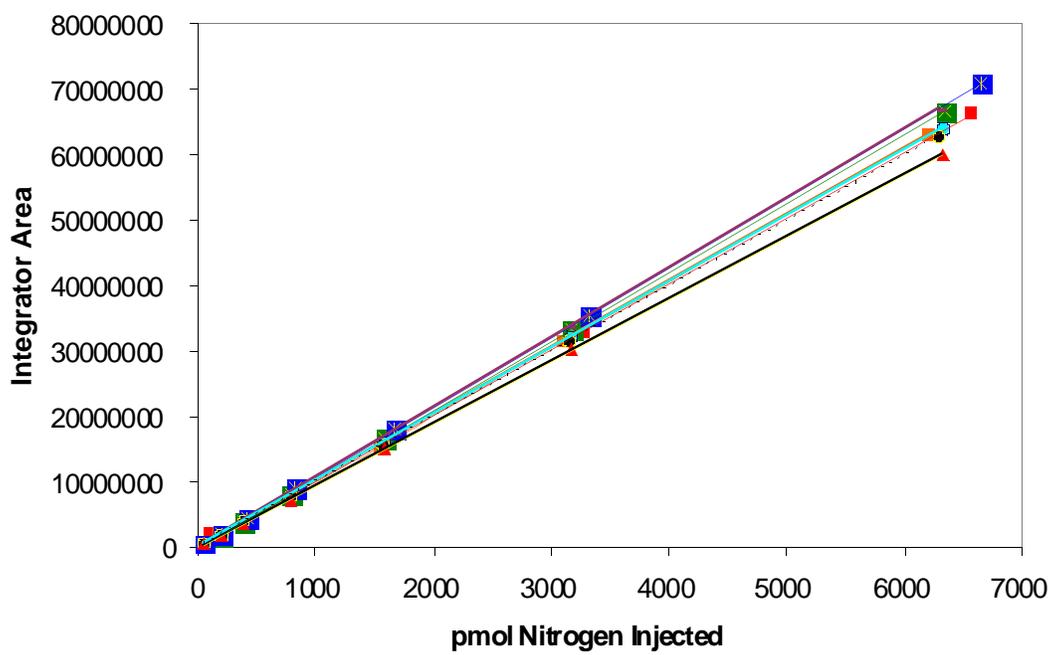
Figure 25: Calibration curves for the 8 nitrogen containing compounds

compound	MW	# N	LOD (pmol N)	LOQ (pmol N)	slope	R^2
Diethylcarbamazine	391.4	3	25.6	51.2	10057	0.9996
Caffeine	194.2	4	24.2	48.5	10157	0.9999
Triprolidine	390.9	2	24.7	49.4	9513	0.9995
Chloropheniramine	314.9	2	24.8	49.5	10552	0.9997
3 Nitroaniline	138.1	2	26.0	52.0	10639	0.9997
Diphenhydramine	291.8	1	24.6	49.2	10613	0.9998
Dibucaine	343.5	3	24.7	49.4	10105	0.9999
2,3-Dimethylindole	145.2	1	24.6	49.2	9964	0.9999

Table 2: Calibration data of the 8 nitrogen containing compounds used in the

HPLC/CLND evaluation

showed a response independent of nitrogen type, linearity from 50 pmol to 6400 pmol of nitrogen, and sensitivity of 25 pmol of nitrogen or 0.35 ng nitrogen.

However, the HPLC/CLND is not without its drawbacks. Firstly, the CLND is limited by the type of mobile phase entering the detector. The CLND, as a nitrogen specific detector, will not function if any nitrogen containing solvents or buffers are used in the mobile phase. Common HPLC solvents such as acetonitrile and ammonium based buffers will flood the detector's reaction chamber with chemiluminescence and not allow any detection to take place. A second limitation is the type of buffers that can be used. The CLND's plumbing becomes easily clogged if salt based buffers are used, as they deposit on the internal restrictors of the detector. Lastly, the flow rate into the detector must be sufficiently low in order that water can be efficiently eliminated.

Application to Oxidation Products of Ethoxyquin

The second goal of this Chapter was to use the evaluated CLND to investigate a separation of ethoxyquin's oxidation products. While the chromatographic behavior of ethoxyquin has been well studied by a variety of chromatographic techniques[76,77,78], the oxidation products of ethoxyquin have only been given few studies. Kato et al.[79] used HPLC and GC to study the chromatographic behavior of ethoxyquin and the ethoxyquin dimer separately. Tamir et al.[80] attempted separations of mixtures of ethoxyquin oxidation products through thin layer chromatography and normal phase HPLC. These authors found separation of the oxidation products of interest through TLC, but were not able to separate these oxidation products using normal phase HPLC.

Normal Phase

The positive results of the TLC experiments, and improved column technology available, led the method development to start in the normal phase. The CLND has not been reported to have been used in the normal phase to date. A separation using normal phase conditions expands the utility of the CLND.

The only change made to the CLND for normal phase conditions was an increase of oxygen flow to 300 mL/min and turning off the argon flow. This is done because the normal mobile phase, of 100 % organics, may require a higher oxygen environment to fully combust all of the mobile phase and analytes in the pyrotube. The argon flow is also turned off so that nebulization will not be affected by the increase in oxygen flow. After method development the best chromatograms achieved are seen in **Figure 26** and **Figure 27**. Both separations were achieved with 100% hexane but neither ethoxyquin or the 1,8 dimer were properly retained on the column. Therefore, the normal phase separation will not be used to separate ethoxyquin and its oxidation products as ethoxyquin and its dimer are not retained or fully resolved. However, the HPLC/CLND was able to operate in 100% organic mobile phases, which expands the utility of the detector.
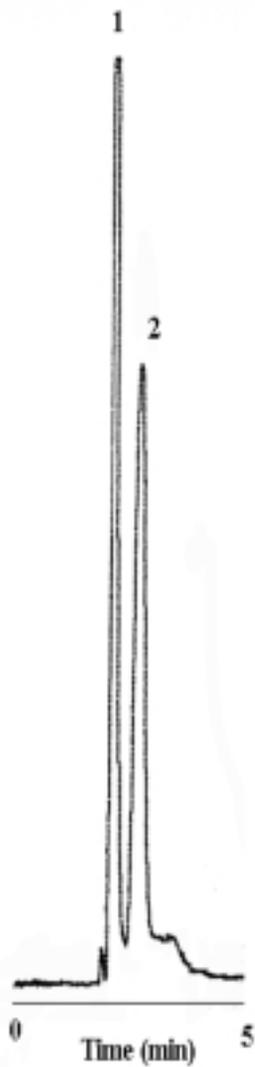
Figure 26: HPLC/CLND normal phase separation of a binary solution on acyano

column of 1. ethoxyquin 1,8 dimer, and 2. ethoxyquin using 100% hexane.
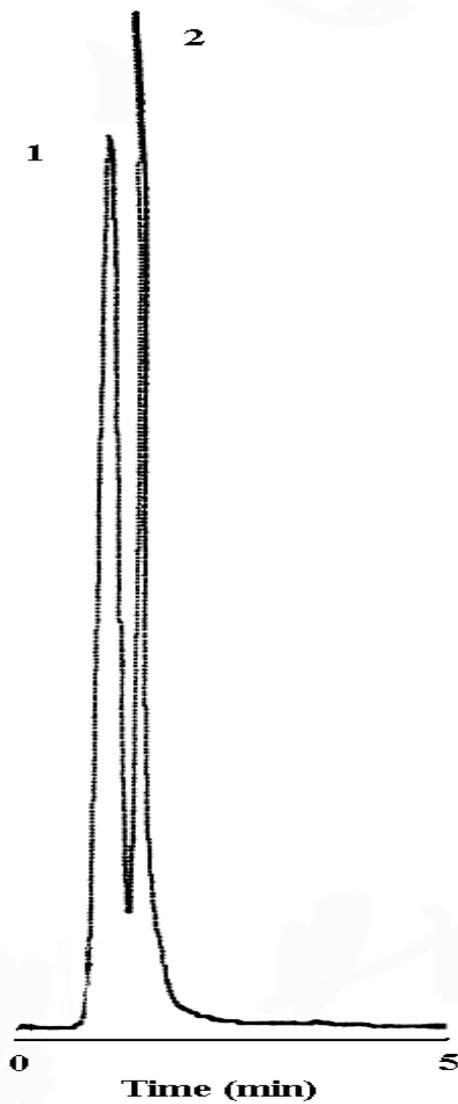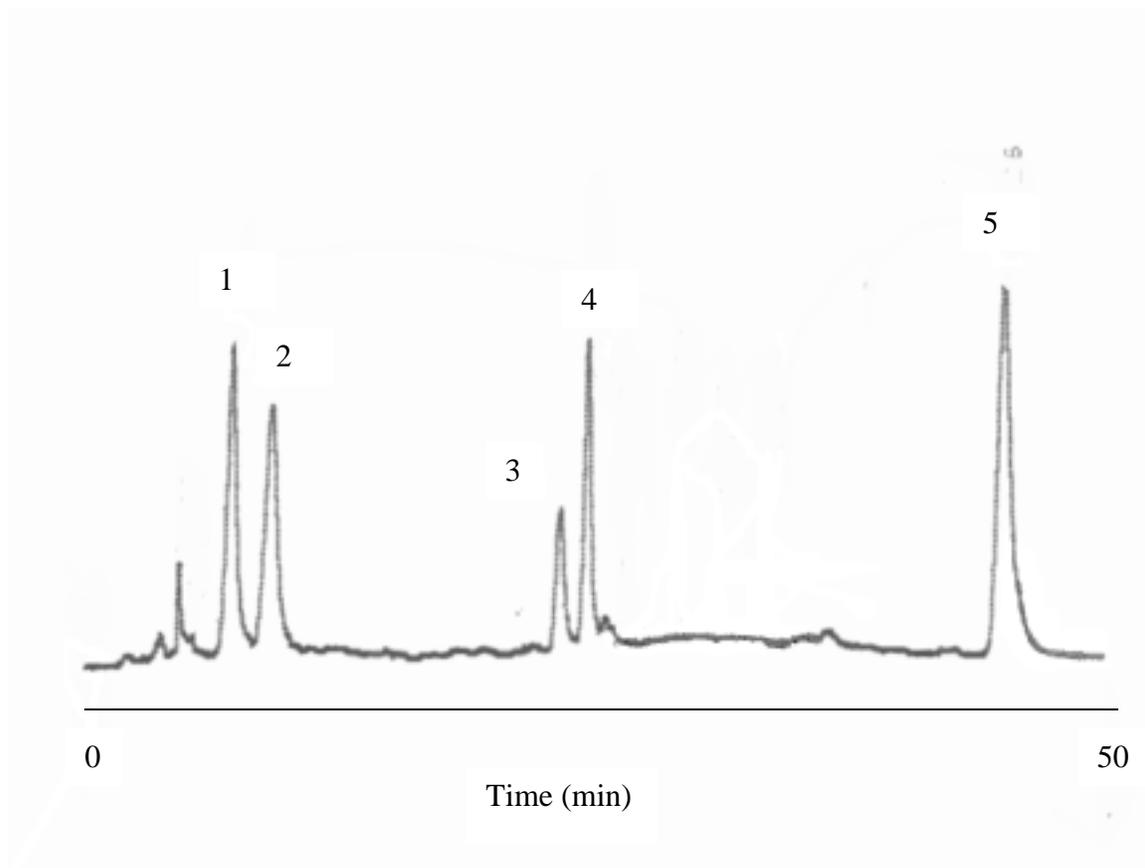
Figure 27: HPLC/CLND normal phase separation of a binary solution on a silica column

of 1. ethoxyquin 1,8 dimer, and 2. ethoxyquin using 100% hexane.

Reversed Phase

A reversed phase separation was next investigated for these compounds. Using mobile phase components of A: 75/25 methanol/isopropanol and B: water, with the gradient of 55/45 A/B to 95/5 A/B in 20 minutes, the separation in **Figure 28** was achieved. In this separation, peaks 1 and 2 and peaks 3 and 4 are over 90% resolved. In this case a total ethoxyquin determination can be made as the unresolved peaks each contain the same number of nitrogens. Further attempts to fully separate this mixture on a longer C18 column failed as the oxidation products degraded.

The separation of ethoxyquin and some of its oxidation products and the HPLC/CLND evaluation, provide the groundwork for future studies concerning oxidation and ethoxyquin. Primarily, the appearance of these oxidation products over time can provide mechanisms for ethoxyquin's antioxidant action for a variety of systems. This work is also important concerning the monitoring of oxidation. Oxidation is monitored by the unsaturations in lipids and by measuring oxygen uptake. However, oxidation has not been monitored by the quantitiation of oxidation products of ethoxyquin. The separation described and the evaluation of the CLND provide an easy method for the measurement of oxidation.

Figure 28: HPLC/CLND reversed phase separation of ethoxyquin and its oxidation products. 1. Quinone imine N-oxide, 2. Quinone imine, 3. Dimethylethoxyquinoline, 4. Ethoxyquin, 5. Ethoxyquin 1,8 dimer. Mobile phase components of A: 75/25 methanol/isopropanol and B: water, using a gradient of 55/45 A/B to 95/5 A/B in 20 minutes on a Phenomenex Luna C18 column (150 x 1 mm, 3 um)

SUMMARY

The objective of the work reported in this chapter was to evaluate the CLND and use it for a detector in the separation of the oxidation products of ethoxyquin. The CLND was evaluated using a variety of nitrogen containing compounds to ensure that the detector gives the same response to all nitrogens. A gradient was used to test the detectors response to a changing mobile phase. Results of these experiments showed the HPLC/CLND gave equal response to all nitrogen species independent of environment, linearity of 50 pmol to 6400 pmol, sensitivity of 25 pmol nitrogen, and its response was not affected by a changing mobile phase. The HPLC/CLND was then used to separate ethoxyquin and some of its oxidation products. A normal phase separation was attempted first. On both a silica and cyano column with 100% hexane mobile phase, the dimer was poorly retained. While unsuccessful in separating the analytes, the CLND, with an increased level of oxygen, worked with a normal phase system. This is a significant result as the CLND has only been used with methanol water mobile phases. The oxidation products could be separated using a C18 column and a reversed phase gradient. The ability to separate and detect these oxidation products with HPLC/CLND has laid the ground work for future studies into the oxidation products of ethoxyquin.

Chapter 5

<u>CONCLUSIONS</u>

The focus of this research was to develop an efficient extraction method for ethoxyquin from lean beef and beef fat and a detection method for some ethoxyquin oxidation products. The research investigated supercritical fluids for the extraction and HPLC/CLND for the detection.

The first goal of the research was to develop an extraction method of ethoxyquin from lean beef and beef fat. This goal was achieved by evaluating the pressure, temperature, percent methanol modifier, and fluid type in the extraction of ethoxyquin spiked on sand/Hydromatrix® mixture. CO_2 was the first fluid investigated but because it extracts fat very well, and the appearance of an unknown extraction product, eliminated it as a suitable fluid for the extraction. Hydrofluorocarbons, CHF_3 and CFH_2CF_3, were then investigated. The pressure effects of both of these fluids showed an increase in recovery as pressure was increased up to a 450 atm for CFH_2CF_3 and 400 atm for CHF_3. At this point recoveries dropped off as an increase in fluid-fluid interactions through dipole-dipole interactions overtake any increase in fluid analyte interaction at these higher pressures. The temperature effects of these two fluids had less of an effect on the extraction efficiency. The only effect of temperature was seen with CFH_2CF_3 which at 100°C showed a decrease in recovery. This can be rationalized again by an increase in fluid-fluid interactions as the fluid approaches its critical temperature of 101°C. The

addition of methanol modifier increased recoveries for both fluids. Quantitative

recoveries were attained at 5 percent methanol modifier for both fluids. Another

interesting trend appeared at increased levels of methanol modifier at the 10 and 15

percent levels. These levels showed a decrease in recovery due to the polar

hydrofluorocarbon's ability to hydrogen bond and have dipole-dipole interactions with

methanol. CFH_2CF_3 was chosen as the fluid to be used for the extractions of ethoxyquin

from lean beef and beef fat for the rest of the experiments. The extractions with

CFH_2CF_3 of ethoxyquin spiked on lean beef and beef fat showed quantitative recoveries

at the 0.5 ppm levels for both lean beef and beef fat and at the 5 ppm level for fat.

Quantitative recoveries were not attained at the 5 ppm level for beef fat nor the 10 ppm

levels for the lean beef and beef fat. No evidence of ethoxyquin was found in the lean

beef and beef fat from cows that were fed ethoxyquin preserved feeds. This set of

experiments showed the hydrofluorocarbons ability to cleanly extract ethoxyquin from

lean beef and beef fat, but also raised questions concerning the oxidation products of

ethoxyquin.

The second portion of the work focussed on obtaining samples of some of the

oxidation products of ethoxyquin. The oxidation products which were obtained through

controlled oxidation reactions, isolation by column chromatography, and identification by

GC/MS were: 2,4-dimethyl-6-ethoxyquinoline, quinone imine, quinone imine N-oxide,

and ethoxyquin 1,8 dimer.

The second goal of the research was to evaluate the CLND and use it for detection

of the oxidation products of ethoxyquin. The CLND was evaluated using a variety of

nitrogen containing compounds to ensure that the detector gave the same response to all nitrogens. A gradient was used to test the detector's response to a changing mobile phase. Results of these experiments showed the HPLC/CLND gave equal response to all nitrogens independent of environment, linearity of 50 pmol to 6400 pmol, sensitivity of 25 pmol nitrogen, and no effect of response with changing mobile phase. The HPLC/CLND was then used to separate ethoxyquin and some of its oxidation products. A normal phase separation was attempted first. On both a silica and cyano column with 100% hexane mobile phase the dimer was not retained or separated from ethoxyquin. While unsuccessful in the separation, the CLND, with an increased level of oxygen, worked with a normal phase system. The oxidation products were separated using a C18 column and a reversed phase gradient. The ability to separate and detect these compounds with the CLND has laid the ground work for future studies into the oxidation products of ethoxyquin from food products.

References

[1] R. A. Larson, "Naturally Occurring Antioxidants", Lewis Publishers, New York, 1997, 1-46

[2] M. B. Korycka-Dahl, T. Richardson, *CRC Crit. Rev. Food. Sci. Nutr.,* **10**, 1978, 209-241.

[3] "Food Antioxidants", ed. B. J. F. Hudson, Elsevier, 1990, 1-15.

[4] "Food Antioxidants , Technological, Toxicological, and Health Perspectives," Ed. D. L. Madhavi, S. S. Deshpande, D. K. Salunkhe, Marcal Dekker, Inc., New York, 1996, 41.

[5] W. A. Pryor, T. Strickland, D. F. Church, *J. Amer. Chem. Soc.* **110,** 1998,2224.

[6] M. Braden, A. N. Gent, *Rubber Chem. Technol.* **35, 1962**, 200.

[7] L. Tamir, M. Prusikova, J. Pospisil, *Angew. Makromol. Chem.* **190**, 1991, 53.

[8] A. A. Spark, *J. Am. Oil Chem. Soc.* **, 59,** 1982, 185.

[9] A. J. Koning, T. H. Mol, *J. Sci. Food Agric.,* **46**, 1989, 259.

[10] C. H. Van Peteghem, D. A. Dekeyser, *J. Assoc. Off. Anal. Chem.,* **64,** 1981, 1331.

[11] A. J. Jackson, A. K. Kerr, C. B. Cowey, *Aquaculture,* **38,** 1984, 211.

[12] A. A. Spark, *J. Am. Oil Chem. Soc.* **59,** 1982, 185.

[13] D. S. Johnson, *Chem. Ind.,*77, 1985.

[14] G. F. Ernst, S. Y. Verveld-Roder, *J. Chromatogr.,* **174,** 1979, 269.

[15] B. Winnel, *Analyst,* **101,** 1976, 883.

[16] G. A. Perfetti, C. R. Warner, T. Fazio, *J. Assoc. Off. Anal. Chem.,* **64,** 1981, 1453.

[17] *Official Methods of Analysis* (1995), 16th Ed., AOAC INTERNATIONAL, Arlington, VA, sec. **963.07**

[18] *Official Methods of Analysis* (1990), 15th Ed., AOAC INTERNATIONAL, Arlington, VA, sec **968.48**

[19] C. J. Schreier, R. J. Greene. *J. AOAC Int.,* **80,** 1997, 4.

[20] R. B. Broadstreet, The Kjeldahl Method for Organic Nitrogen, Academic Press, New York, 1965

[21]"Bioluminescence and Chemiluminescence: Instruments and Applications v.2", K. Van Dyke, Ed. CRC Press, 1985, p77-93.

[22] E. H. White, D. F. Roswell, "Chemi- and Bioluminescence" J. Burr, Ed. Dekker, 1985, 215-244, 469-488.

[23] W. R. Seitz, *CRC Crit. Rev. Anal. Chem.,* **13,**1981, 1.

[24] A. Fontijn, A. J. Sabadell, R. J. Ronco, *Anal. Chem.*, **42,** 1970, 575.

[25] M. A. A. Clyne, B. A. Thrush, R. P. Wayne, *Trans. Faraday Soc.,* **60**, 1967, 915.

[26] R. E. Parks, R. L. Marietta, U. S. Patent 4,018,562, 1977.

[27] A. J. Britten, *R&D Magazine,* **31**, 1989 76.

[28] L. O. Courthaudon, E. M. Fujinari, *LC-GC*, **9**, 1991, 732.

[29] E. M. Fujinari, L. O. Courthaudon, *J. Chromatogr.*, **592**, 1992, 209.

[30] S. M. Benn, K. Myung, E. M. Fujinari, "Food Flavors, Ingredients and Compositions", G. Charalambous (Ed.), Elsevier Science Publishers, Amsterdam, 1993, 56.

[31] E. M. Fujinari, J. D. Manes, *J. Chromatogr. A,* **676,**1994, 113.

[32] R. Bizanek, J. D. Manes, E. M. Fujinari, *Peptide Res.,* **9**, 1996, 40.

[33] E. M. Fujinari, J. D. Manes, R. Bizanek, *J. Chromatogr. A,***743,** 1996, 85

[34] J. Truchan, H. T. Rasmussen, N. Omelczenko, B. P. Mcpherson, *J. Liq. Chrom. & Rel. Technol.,* **19,** 1996, 1795.

[35] B. A. Tompkins, W. H. Griest, *Anal. Chem.,* **68,** 1996, 2533.

[36] B. A. Tompkins, W. H. Griest, C. E. Higgins, *Anal. Chem.,* **67,** 1996, 4387.

[37] H. Shi, L T. Taylor, E. M. Fujinari, *J. High Resolut. Chromatogr.,* **19**, 1996, 213.

[38] H. Shi, L. T. Taylor, E. M. Fujinari, *J. Chromatogr. A,* **757,** 1997, 183.

[39] M. T. Combs, M. Ashraf-Khorassani, L. T. Taylor, *Anal.Chem.,* **69,** 1997, 3044.

[40] A. Robbat, N. P. Corso, T. Y. Liu, *Anal. Chem.,* **60,** 1988, 173.

[41] E. M. Fujinari, L. O. Courthaudon, *J. Chromatogr.,* **592,** 1992, 209.

[42] E. M. Fujinari, J. D. Manes, *J. Chromatogr. A,* **676,** 1994, 113.

[43] E. M. Fujinari, J. D. Manes, R. Bizanek, *J. Chromatogr. A,* **743,** 1996, 85.

[44] E. M. Fujinari, J. D. Manes, *J. Chromatogr. A,* **763,** 1997, 323.

[45] W. L. Fitch, A. K. Szardenings, E. M. Fujinari, *Tetra. Lett.,* **38,** 1997, 1689.

[46] E. W. Taylor, M. G. Qian, G. D. Dollinger, *Anal. Chem.,* **70**, 1998, 3339.

[47] M. Ashraf-Khorassani, S. Gidanian, Y. Yamini, *J. Chromatogr. Sci.*, **33**, 1995, 658

[48] A. Daneshfar, M. Barzegar, M. Ashraf-Khorassani, J. L. Levy, *J. High Resolut. Chromatogr.*, **18**, 1995, 446.

[49] E. Stahl, D. Gerard, *Perfum. Flavor.*, **10**, 1985, 29

[50] T. A. Berger, *J. Chromatogr.*, **478**, 1989, 311.

[51] A. Hutz., F. P. Schmidt, D. Leyendescker, E. Klesper, *J. Supercrit. Fluids*, 3, 1990, 1.

[52] J. R. Wheeler, M. E. McNally, *J. Chromatogr. Sci.*, **27** 1989,534.

[53] B. W. Wright, C. W. Wright, R. W. Gale, R. D. Smith, *Anal. Chem.*, **59**, 1989, 38.

[54] F. I. Onuska, K. A. Terry, *J. High Resolut. Chromatogr.*, **12**, 1989, 357.

[55] R. D. Smith, H. R. Udseth, *Fuel*, **62**, 1983 466.

[56] R. D. Smith, H. R. Udseth, R.N. Hazlett, *Fuel*, **64**, 1985, 810.

[57] R. D. Smith, H. R. Udseth, B. W. Wright, "Supercritical Fluid Technology", J. M Penninger, M. Radosz, M. A. McHugh, V. J. Krukonis, Eds, Elsevier Science Publisher, 1985, 191.

[58] F. I. Onsuka, K. A. Terry, *J. High Resolut. Chromatogr*., **12**, 1989, 357.

[59] J. M. Levy, E. Storozynsky, R. M. Ravey, *J. High Resolut. Chromatogr*., **14**, 1991, 661.

[60] M. Ashraf-Khorassani, L. T. Taylor, P. Zimmerman, *Anal. Chem*., **62**, 1990, 1177.

[61] T. S Oostdyk, R. L. Grob, J. L Snyder, M. E. McNally, *Anal. Chem*., **65**, 1993, 596.

[62] D. E. Raynie, *Anal Chem*., **65**, 1993, 3127.

[63] S. B. Hawthorne, Y. Yang, D. J. Miller, *Anal Chem*., **66**, 1994, 2912.

[64] R. C. Reid, J. M. Prausnitz, T. K. Sherwood, "The Properties of Gases and Liquids" 3rd ed., Mcgraw-Hill, New York, 1977.

[65] E. Stahl, E. Willings, *Mikrochimica Acta,* 1981, 465.

[66] A. L. Howard, W. J. Yoo, L. T. Taylor, F. K. Schweighardt, A. P. Emery, S. N. Chester, W. A. MacCrehan, *J. Chromatogr. Sci.,* **40**, 1993, 401.

[67] M. T. Combs, M. Ashraf-Khorassani, L. T. Taylor, *Anal. Chem.,* **68**, 1996, 4507.

[68] M. T. Combs, M. Gandee, M. Ashraf-Khorassani, L. T. Taylor, *Anal. Chimi. Acta,* **341,** 1997, 285.

[69] M. Ashraf-Khorassani, M. T. Combs, L. T. Taylor, F. K. Schweighardt, P. S. Mathias, *J. Chem. Eng. Data,* **42,** 1997, 636.

[70] L. T. Taylor "Supercritical Fluid Extraction", John Wiley & Sons, Inc., New York, 1996, 18.

[71] K. P. Battjes, A. M. Barolo, P. Dreyfuss, *J. Adhesion Sci. and Tech.,* **5,** 1991, 785.

[72] L. Tamir, M. Prusikova, J. Pospisil, *Die Angewandte Makromol. Chem.,* **190,** 1991, 53.

[73] S. Thorisson, F. D. Gunstone, R. Hardy, *Chemistry and Physics of Lipids,* **60,** 1992, 263.

[74] L. Tamir, *Die Angewandte Makromol. Chem.,* **217,** 1994, 119.

[75] R. S. Hutte, R. E. Sievers, J. W. Birks, *J. Chromatogr. Sci.,* **24,** 1986, 499.

[76] G. F. Ernst, S. Y. Verveld-Roder, *J. Chromatogr.,* **174,** 1974, 269.

[77] M. Vega, R. Saelzer, N. Mendoza, J. Katalinic, *J. High Resolut. Chromatogr.,* **13,** 1990, 854.

[78] H. K. Dahle, J. U. Skaare, *J. Agric. Food Chem.,* **23,** 1975, 1093.

[79] S. Kato, K. Kanohta, *J. Chromatogr.,* **324,** 1985, 462.

[80] L. Tamir, M. Prusikova, *J. Chromatogr.,* **587,** 1991, 355.

VITA

Daniel Robert Brannegan was born in Westerly, Rhode Island on December 8, 1973. He received his Bachelor of Arts with Honors in Chemistry from the College of the Holy Cross in June 1996. During his undergraduate work he undertook organic synthesis at Holy Cross under Dr. McMaster though the Chemistry Honors Program. He also held summer internships at Pfizer Central Research in Groton, CT and at Merck and Co. Inc., in West Point, PA. After graduating, he spent time at Oregon State University before coming to Virginia Polytechnic Institute and State University.

He began his graduate studies in Blacksburg in July 1997 in the research group of Dr. Larry T. Taylor. His work focused on supercritical fluid extraction and chemiluminescence nitrogen detection for high performance liquid chromatography.

Presentations:

- **D. R. Brannegan**; A. M. Pinto, L. T. Taylor, D. L.Vassilaros "Sensitivity Determination of Various Make-Up Gases Utilizing Flame Ionization Detection" The Pittsburgh Conference, March 1998.

- **D. R. Brannegan;** M. Ashraf-Khorassani, L. T. Taylor "Extraction of Ethoxyquin from Various Matrices using Different Supercritical Fluids" The 8th International Symposium on Supercritical Fluid Chromatography and Extraction, July 1998.

Publications:

- Sekulic, S. S.; Ward, H. W.; **Brannegan, D. R.;** Stanley, E. D.' Evans, C. L.; Sciavolino, S. T.; Hailey, P. A.; Aldridge, P. K. "On-Line Monitoring of Powder Blend Homogeneity by Near-Infrared Spectroscopy" *Analytical Chemistry,* **68,** 1996, 509-513.