Investigations of the Application of Fourier Transform Infrared Spectroscopy in Lipid Analysis

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A Thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Master of Science

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LIST OF ABBREVIATIONS

AOCS	Americam oil chemists society
ATR	Attenuated Total Reflectance
BHA	Butylated Hydroxy-Anisole
C=O	Carbonyl stretch
$CaCO_3$	Calcium Carbonate
CaF_2	Calcium Flouride
CO_2	Carbon Dioxide
E(a)	Activation Energy
FTIR (or FT-IR)	Fourier Transform Infrared Spectroscopy
Io	Background infrared intensity
IR	Infrared
Is	Sample infrared intensity
LPO	Lipid Peroxide
μ	micron
-OH	Hydroxyl stretch
-OOH	Hydroperoxide stretch
PC	Personal Computer
PG	Propyl Gallate
PLS	Partial Least Squares
PRESS	Predicted Residual Error Sum of Squares
PV	Peroxide Value
R*	Free lipid radical
ROO*	Free lipid peroxide radical
TBA	Thiobarbituric acid

TBARS	**Thiobarbituric Acid Reacting Substance**
TBHQ	**Tert-Butyl Hydroxy Quinone**
TGS	**Triglycine Sulfate**
ZnSe	**Zinc Selenide**

ABSTRACT

Fourier transform infrared (FTIR) spectroscopy was employed to monitor the oxidation of edible oils in the absence and presence of antioxidants. Three synthetic antioxidants (butylated hydroxyanisole, propyl gallate and tert-butylhydroxyquinone) were added to menhaden oil at three different concentrations (1%, 0.1%, and 0.01%), and the FTIR spectra of the oils under conditions of oxidative stress were recorded as a function of time. The efficacy of each antioxidant was assessed by monitoring bands at 3444 cm^{-1}, characteristic of hydroperoxide formation, 971 cm^{-1} for trans double bond formation, and 3008 cm^{-1} for cis depletion. An FTIR spectroscopic method was also developed for the determination of the peroxide value of edible oils undergoing oxidation, based on the measurement of the 3444-cm^{-1} band. The results of the FTIR method were compared to those from both the iodometric chemical method and the enzymatic hemoglobin-methylene blue assay for the determination of hydroperoxide content. The FTIR predictions were within ±3% of the values obtained by both the chemical and the enzymatic method. Finally, the determination of the hydroxyl value of emulsifiers by FTIR spectroscopy was investigated. Both attenuated total reflectance (ATR) and transmission flow cell techniques were used to record the spectra of commercial monoglycerides. Calibration models for the prediction of hydroxyl value from the FTIR spectra were developed using partial-least-squares (PLS) regression for both the ATR and transmission spectra. Cross-validation of the calibration models yielded an overall average error in the predicted hydroxyl values of ~3% for both the ATR and transmission flow cell methodologies. Linear regression of the FTIR-predicted versus the hydroxyl values determined by the reference chemical method yielded $r = 0.998$ for the ATR and $r = 0.997$ for the transmission flow cell method.

RESUME

La spectroscopie infra-rouge à Transformée de Fourier a été employé comme un moyen de contrôle de l'oxydation des huiles de consommation et d'évaluation de l'efficacité des antioxydants à retarder cette oxydation. On a examiné cette efficacité pour trois antioxydants synthétiques (BHA, PG et TBHQ) en analysant plusieurs pics connus dans les spectres infra-rouge d'huiles de poisson en cours d'oxydation. On a chauffé trois solutions de cette huile contenant chacune une concentration différente en antioxydant (1.00%, 0.1% et 0.01%) et l'on a mesuré ces concentrations en étudiant trois bandes d'oxydation prédéterminées à 3444 cm^{-1}, caractéristique de la formation d'hydroperoxyde, 971 cm-1 pour la formation de double-liaisons trans et 3008 cm^{-1} pour la diminution des double-liaisons cis. Une méthode infra-rouge a été développée pour la détermination de la teneur en peroxyde dans les huiles de consommation subissant une oxydation. Cette méthode a donné une précision comparable pour la détermination de la teneur en hydroperoxyde, à celle de la technique iodimétrique chimique et à celle du test enzymatique hémoglobine - bleu de méthylène. On a opéré une régression linéaire entre les prédictions par la spectroscopie infra-rouge et les valeurs obtenues par les deux méthodes chimiques et enzymatiques.

Finalement, la spectroscopie infra-rouge a été employée pour déterminer la teneur en hydroxyle des émulsifiants. Les deux types de techniques à cellule à Réflexion Totale Atténuée (ATR) et à Transmission de flux ont été utilisés pour analyser la teneur en hydroxyle de monoglycérides commercialisés. Ces techniques couplées à la technologie de la spectroscopie infra-rouge à Transformée de Fourier ont permis d'effectuer les analyses souhaitées avec succès et les deux méthodes ont montré une précision et une reproductibilité comparables. Pour chacune des deux techniques, on a obtenu une erreur globale d'environ 3%. Les coefficients de corrélation avec la méthode chimique de détermination sont de 0.998 pour la technique ATR et de 0.997 pour la technique par Transmission.

CHAPTER I

INTRODUCTION

Food or nutrient intake is essential for the survival of all biological systems. The term food encompasses a multitude of various compounds both organic and inorganic in nature. Historically, most nutrients were fresh and picked for daily consumption. Today's consumer does not grow his / her food, as a result, most modern society ingest processed and or packaged foods. Because of this increase need of food packaging and processing, there seems to also be a need for food preservation and food quality control. Due to this increased requirement of optimal quality control for food over the years, a variety of different physical and chemical methods have been developed to extend the shelf life of food products. Various methods have been described in the literature (ref.1-5) to assess the deteriorative condition of food products.

Oxidation of lipids in food matrices is one of the major problems in food preservation. The fact that molecular oxygen is ubiquitous amongst all living and non-living systems makes the problem of lipid oxidation many times more difficult to correct for. The oxidation of food systems, in particular, food matrices including high levels of fat constituents, results in the formation of various hydroperoxides along with secondary end products of oxidation such as malondialdehyde (ref. 6-7). Many chemical methods were developed (ref. 8-12) for the purpose of monitoring and quantitative determination of oxidation by products within a food. Along with the analysis of the deteriorative processes occurring in foods, there are various chemical methods available to analyze food components and food make-up (ref. 13-15), such as the determination of the hydroxyl number of emulsifiers. Emulsifiers have been increasingly used over the years in food processing to allow for the miscibility of a water phase into a lipid phase or vice-versa.

Processes of food systems such as oxidation of lipids or the determination of the hydroxyl values of a food, all involve the evolution or diminishment as well as the

involvement of some functional molecular entity within the food matrix. Because of the various molecular entities existing in food systems, different corresponding molecular vibrations are known to occur within the molecular make-up of the diverse components found within a food. Vibrational spectroscopy (i.e., infrared spectroscopy) is particularly useful in determining both the presence and or absence of certain functional groups of a food system. The characteristic mode frequencies of the various functional groups are detected and quantitated. Infrared radiation is known to absorb at a particular frequency if that IR frequency matches the vibrational frequency of a particular molecular entity. The basis of this absorption phenomenon is what the following thesis work is about.

Infrared spectroscopy has been increasingly used over the years as a possible tool to measure both qualitatively and quantitatively the various molecular entities making up biological systems and foods (ref.16-26). Although, IR-based methods are increasingly cited within the literature for analysis of food products, sample preparation and the presence of water still posses serious limitations. Water is known to be problematic in IR spectroscopy because of the fact that infrared radiation strongly absorbs water. Hence, due to the strong absorption of IR radiation by water molecules, samples must either be dessicated or studied by special IR techniques such as ATR spectroscopy. The problem with drying the sample is two fold; 1) a large amount of sample and sample handling is required and 2) the matrix of the sample may be altered significantly resulting in errors in analysis. In today's technology, because of the increase in computer availability and speed coupled to analytical instruments (i.e., Fourier Transform infrared spectrometers), and the developement of new sampling methods, problems encountered previously with the analysis of biological systems are becoming more manageable.

The Fourier transform infrared or FTIR spectroscopic method has been increasingly used since the early 1970's (ref. 27) in most infrared analysis. This instrument does not consist of the same optical layout as the previous infrared instruments allowing

for various additional advantages over conventional infrared analysis such as, greater sensitivity and a decrease in analysis time. Because of these advantages, FTIR spectroscopy was used to monitor the degradation processes occurring in fat / oil systems along with monitoring antioxidative efficacy of various synthetic antioxidants such as BHA, PG, and TBHQ. The determination of the hydroxyl values of emulsifiers was also investigated by FTIR spectroscopy.

In this thesis, attenuated total reflectance (ATR) based sampling accessory along with the transmission flow-through IR cell were used to study lipid oxidation. Four separate chapters are included in this thesis and all four deal with some aspect of infrared spectroscopy in lipid analysis. Chapter 2, describes the theory of vibrational spectroscopy and includes a brief survey of the historical development of infrared spectroscopy and its use in fats and oil analysis. Chapter 3, describes how FTIR spectroscopy is used to monitor antioxidant efficiency in retarding the oxidation of edible oils at various concentration levels. Chapter 4, describes a method for hydroperoxide determination in fresh and oxidized edible oils by FTIR spectroscopy. This chapter compares the FTIR protocol with two standard methods used in peroxide value determination; one is the peroxide value known as the iodometric chemical determination of peroxides and the second is the enzymatic hemoglobin-methylene blue assay, both methods are currently employed in measuring the hydroperoxide content of lipids. Finally, chapter 5 describes an FTIR-based method for the determination of the hydroxyl value of acetylated monoglycerides.

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CHAPTER II

LITERATURE REVIEW

I. A Historical Survey on the Early Development of Infrared (IR) Spectroscopy : (Reminiscences of early IR Pioneers)

Spectroscopy in all its different variations has probably been the most valuable of all the experimental techniques in the development of both physics and chemistry. The complete history of spectroscopy is of a fascinating nature which may be well traced into the mid to late part of the 17^{th} century. Sir Isaac Newton was the first investigator known to study the visible spectrum in a scientific manner. Newton had retired to the family home from Cambridge University in the fall of 1665 due to the epidemic of the Great Plague. For the next year or so, Newton had come up with astonishing and monumental discoveries of which one was the composition of white light and the nature of color. In the year 1666, Newton had admitted a narrow beam of sunlight into a darkened room and placed a glass prism into the beam's projection. Newton to his dismay had discovered a band of colors on the wall, and by the simultaneous addition of a lens, he had spread the white beam band of colors into a rainbow of various colors 25 cm long. In 1672 he had published his finding as his first scientific paper, and is noted to be one of the most famous scientific paper of all time. In this paper, Newton had first introduced the word "Spectrum" to the scientific community. The word 'Spectrum" was acquired from Newton as he noted that as a prism was moved ever so slightly, the colored image jumped around in such a manner that made him think of "spectres" meaning essentially ghosts (ref.1). Hence, the birth of spectroscopy had begun .

Today, the electromagnetic spectrum is known to extend over 55 octaves, from gamma radiation to radio waves a factor of 10^{17} in wavelength or frequency. The infrared region of the electromagnetic spectrum will be of the main focus and concern in this review. The infrared region encompasses radiation with wavenumbers ranging from approximately

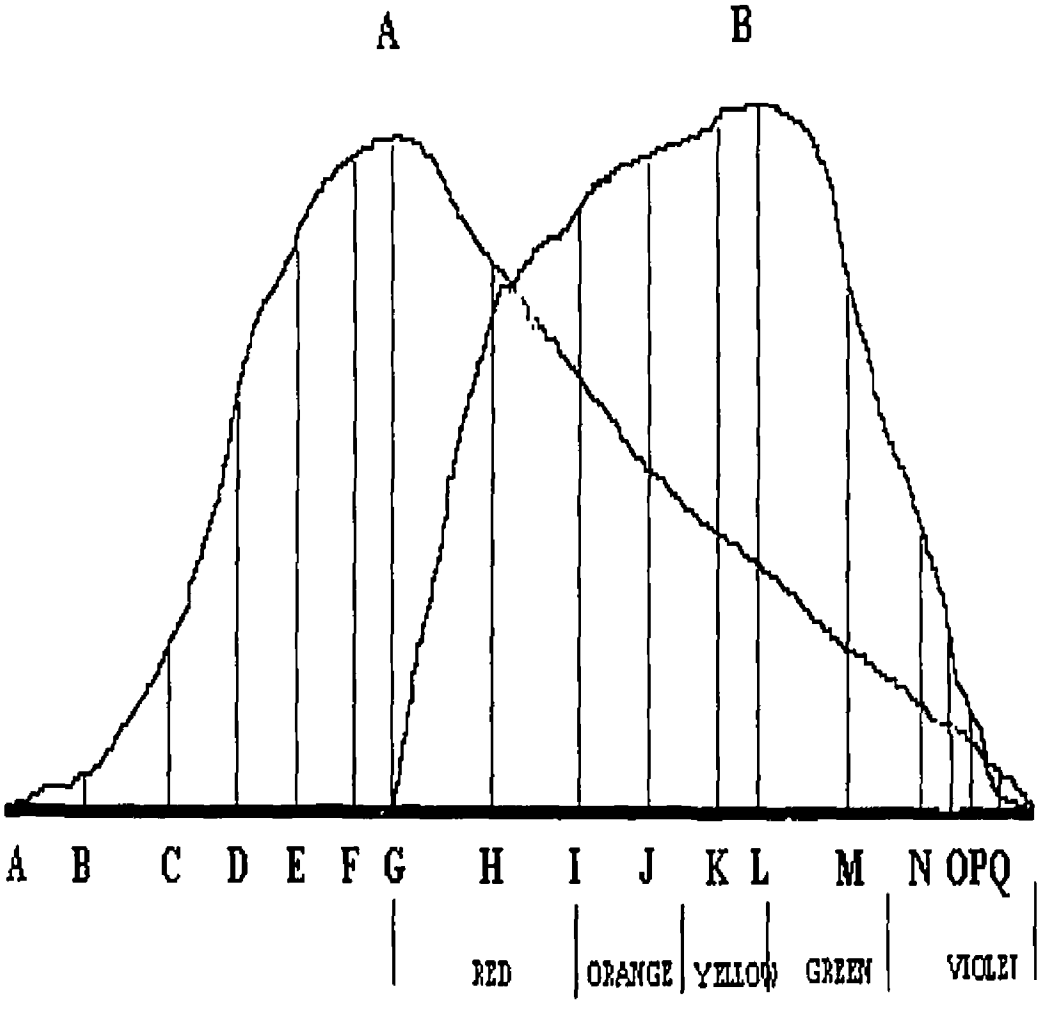
12800 to 10 cm^{-1}. It is conveniently divided into the near, mid and far infrared regions. The main focus of this review as well as the complete thesis as a whole, is on the mid portion (~ 4000 - 400 cm^{-1}) of the infrared (IR) region.

The preliminary findings of the existence of the infrared region may be credited to the discovery of Landriani, who seems to have been the first pioneer to examine a solar spectrum by passing a thermometer through it around some time before 1777. Although, similar investigations were also made by Rochon in 1776 and Senebier in 1785 allowing for some sort of thermal peak mapping both in the orange and yellow regions of the spectrum. All of these discoveries unfortunately have passed without any general notice for several years (ref.2).

The actual discovery of the infrared (IR) region was finally made in the spring of 1800 by a famous German-born English astronomer named Sir Frederick William Herschel (1738-1822). At the age of 19 , Sir Herschel left a musical career in the army band as an organist to pursue a new life as an organist and later as an astronomer in England. Astronomy was initially a kind of hobby for Sir William Herschel which later turned into a passion and life time persuasion and accomplishment. The observation made in 1800 by Sir Herschel of invisible solar energy was quoted as follows in one of his preliminary publications ; " *In a variety of experiments I have occasionally made relating to the method of viewing the sun, with large telescopes, to the best advantage, I used various combinations of differently colored darkening glasses. What appeared remarkable was, that when I used some of them I felt a sensation of heat, though I had but little light; while others gave me much light , with scarce sensation of heat (ref.3)* ". This observation had determined the starting point of a set of experiments to verify the hypothesis that "the power of heating and illuminating objects, might not be equally distributed among the variously colored rays" which had been primarily determined by Landriani by a set of preliminary

experiments and measurements of the temperature of the components of the prismatic spectrum around 1778. By use of a filtered prism, Sir Herschel had studied the relative heating effect indicated by a thermometer passed through the spectrum of the sun light and observed that, to quote Sir Herschel, " *It appears that the maximum of the full red falls still short of the maximum of heat; which perhaps lies even a little beyond visible refraction. In this case, radiant heat will at least partly , if not chiefly, consist, if I may be permitted the expression , of invisible light.*" In a subsequent paper Sir Herschel expressed the opinion that the invisible radiation (i.e. infrared) was of the same nature as visible light. Sir Herschel had then divided the visible spectrum into the domains proposed by Sir Newton and drew ordinates representing his opinion of the relative brightness, obtaining a peak curve maximum of brightness intensity in the yellow-green of Sir Newton's visible spectrum. Next to the illuminating spectrum, Sir Herschel constructed the invisible radiation spectrum showing some considerable overlap with the complete visible spectrum varying in relative heat intensities within the visible spectrum shown in **Figure 2.1**. As a consequence of this finding, Sir Herschel had located the peak maximum of heat radiation far off the sun's visible spectrum with heat maximum peaking immediately next to the red portion of the visible spectrum. Obviously, the peak heat intensity was proposed to not coincide directly with the visible peak brightness and was determined to be shifted to the left of the red end of the visible spectrum. And as a consequence of this discovery the two spectra (i.e. heat and visible) were located one next to the other but with separate maxima and several overlapping spectral regions. This lead to the discovery of infrared radiation by Sir Herschel as being separate from visible radiation with a different spectrum although with some overlapping spectral regions. Over the following few years there had been many contradictory opinions as to the validity of Sir Herschel's actual findings and many conflicting reports were then presented.

Figure 2.1: Herschel's curves for relative luminosity [2].



NOTE : These are the overlapping heat radiation (**A**) and light radiation (**B**) curves depicted by Herschel in 1800. Note the overlapping regions of both spectra.

Definitive experiments were then forced to await for improvements in technology to confirm various believes and opinions by many well known experimentalists such as Thomas Johann Seebeck (1770-1831) and Leopoldo Nobili (1784-1835) who invented among other things a highly sensitized thermometer known then as the "*thermo-multiplicateur*". Along with another famous inventor, Macedonio Melloni (1798-1854) , Leopoldo Nobili improved on the "thermo-multiplicatuer" increasing its sensitivity significantly and this detection system had proved to be one of the key elements to which unlocked many of the mysteries of thermal radiation (i.e. Sir Herschel's infrared). As a result of these inventions and discoveries, it was then accepted that infrared (i.e. thermal) radiation was separate from visible radiation with certain overlapping common regions. Macedonio Melloni was a great inventor of that era and much of his work was concerned with undispersed radiation. The variety of instrumental sources for experimentation used by Melloni such as; copper heated to 400° C and platinum heated to incandescence and a Locatelli lamp, resulted in his observation of some of the effects associated with a "change in wavelength" (ref. 4). Melloni's work included measurements of reflection and absorption of many substances and he introduced the widely used terms " *diathermanous*" (as the parallel of transparent) and " *thermochrose*" (color in the infrared). Among his discoveries of paramount importance was that rock salt was almost perfectly transparent to radiation from all sources experimented on. His discovery resulted in the introduction of rock salt prisms and lenses. Melloni had also undertaken an extensive study of dispersed infrared radiation and therefore, it may be said that he laid the foundation for infrared spectroscopy. Melloni as early as 1843, stressed the opinion that, " *light as being merely a series of calorific indications sensible to the organs of sight, or vise versa, the radiation's of obscure heat are veritable INVISIBLE RADIATION'S of light*" . Therefore, with this progression in improved instrumental analysis over several decades of scientific insights (along with determined solutions to paradoxical questions as spectral diversity), the IR region was shown to be separate from the spectral visible region and

over the proceeding years other spectral regions came to existence. From this point onwards, infrared was considered to consist of its own spectral region separated by three regions of wavelengths known as the far, mid, and near IR regions.

What may be considered one of the last fundamental tests of the nature of infrared radiation were those carried out by Louise Fizeau (1819-1896) and Jean Bernard Foucault (1819-1868). By means of a tiny thermometer, read by a microscope, both investigators successfully observed interference fringes with IR radiation (ref.5). They had also made some measurements of the solar spectrum with sufficient resolution to show the existence of narrow absorption regions just beyond the red of the visible region, verifying one of Melloni's speculations that the atmosphere of the earth had absorption bands. In this connection, John Tyndall (1820-1893) in 1859, was the first to study the absorption of gases (ref.6). William Draper (1811-1882) who came from England to the USA at the age of 22, and became professor at the University of New York at the age of 27, had studied thermal emission from various substances. His studies were of particular importance in establishing the *"Identity of infrared radiation"*. Along with his thermal blackbody radiation work as a result of some crude work with a grating device, Draper was the first to point out the advantage of the grating spectrum over that of the conventional prism, in that the former made possible the description of radiation (i.e. infrared amongst others) in terms of wavelengths. Along with these discoveries Draper is known to be possibly the first person to acquire a photograph of a person (his sister). This new technique was then used by Draper to make the first photograph of the moon and a photograph of the solar spectrum far enough to record the atmospheric absorption bands in the near infrared region. The photographic infrared was also explored by Lieutenant William de Wiveleslie Abney(1843-1920) and Captain Festing and in 1881 the first photographic spectra in the infrared was obtained. Unfortunately, however, they had both incorrectly ascribed the observed absorption's to atoms rather than molecules. Around 1889, the fact that infrared

absorption arise from molecular, not atomic, activity was established by Knut Johan Angstrom. Angstrom had demonstrated that carbon monoxide and carbon dioxide had different spectra and that carbon dioxide intensities did not demonstrate a double intensity effect as postulated if IR absorption was of atomic basis. This work demonstrated that infrared absorption was not of atomic nature and therefore due to molecular vibrational phenomenon. The investigations undertaken by Abney and Festing on the selective absorption of radiant energy by various organic bodies by infrared, was also related to other research work in spectroscopy carried out by Tyndall on the IR absorption of some organic compounds around 1866. The results obtained for approximately 52 compounds by Abney and Festing, clearly indicated that these substances could be grouped according to their absorption spectra. More importantly, it was established that this grouping " agrees on the whole with that adopted by chemists groupings via various chemical testings". Both investigators classified compounds according to the spectra obtained and any meaningful feature was put into relation with atoms or groups of atoms in the molecules. This predetermined the nature of infrared spectroscopy to be used as a *"correlating tool"*, offering new perspectives both as to the constitution of molecular species and their identification. Hence, both Abney and Festing can be accredited with correlating various spectral regularities to atomic groupings and thus can be considered as the first pioneers of the correlation tables of infrared spectroscopy. Regularities observed were so convincing that they made the 'first assignments" : a line at 740 millimicrons and a band at 892 and 920 millimicrons were diagnostic for an ethyl radical; and a set of lines between 900 and 972 millimicrons indicated the presence of hydrogen atoms in the molecule. It is clear that a correlation of lines to microscopic entities (i.e. atoms and groupings) were made; however, they did not take into account the bonding situation of the molecules. Only names and molecular formulas were reported and not their structural formulas as became usual in later works by other authors. In the following years much of the experimental data had clarified the nature of the interaction of radiation with matter in

the IR region. The main achievement was that absorption had to be connected to the structure of the molecules and not only atomic groupings. This idea was founded on the basis carried out by work perceived by various researchers in this period of development. In 1890 Angstrom, had obtained spectra of various molecular species in different physical phases. Spectral entities were appreciably alike in these physical states (i.e. liquid versus gas) apart from the absorption intensities of the atomic groupings. This was then taken as an indication that absorption in the IR region, had to be connected to an "*intra-molecular*" property of the molecules. As he obtained spectra from carbon-monoxide and carbon-dioxide in the gaseous phase, he concluded that absorption did not depend on the number of atoms present since the band intensities were not a direct relationship to the amount of oxygen present. Angstrom also demonstrated the importance of not only atomic groupings present for IR absorption but also their molecular interactions and structural linkages of various molecular entities.

Several other influential investigators arrived at similar conclusions and one of the latest well known infrared authors of the twentieth century (i.e. Coblentz) had furthered the spectral correlations of some 150 organic compounds in 1905. Coblentz was looking at intramolecular phenomenon and his main goal was to record absorption spectra in a region never reached before, where significant peaks may be detected instead of large characteristic bands. Coblentz chose isomeric compounds for such a study. Coblentz was well aware that although spectra might appear to be similar, significant differences may be found far into the IR . Thus, different conclusions may be drawn with regard to the effect of the structure of the molecule on the absorption curve, depending on the investigated region. Coblentz demonstrated conclusively that a different arrangement of atoms is responsible for the different IR curves of a series of isomers and so he had made the definite conclusion that "*structural differences*" in molecules could be detected in other ways than by chemical behavior of substances. Hence, Coblentz made use of spectroscopic

data in a rather modern way: as an independent route to molecular structure assignment or as a validation of a given structure. Hence, from 1881 onwards, the need to collect empirical data predominated IR spectroscopy. Selective absorption and intramolecular vibrations were correlated through a comparative analysis of the spectra of well characterized compounds. The selection of these known compounds was a key factor as it allowed the possibility of interpreting IR data in a conclusive manner.

By 1940 there was a large body of knowledge concerning infrared spectroscopy. At this point of IR history, there was virtually no IR instruments commercially available except for a small and unsatisfactory one that Adam Hilger Ltd. had first marketed in 1913. The optical layout was of the constant-deviation Wadsworth type and used a Nernst filament as a source, a thermopile as the detector, a 60 ^{o} natural rock salt prism, and spherical mirrors made of nickel and steel (ref. 7). By 1938 approximately 15 IR instruments were operational in the United States of which each was probably homemade (Ref. 8). The impetus of World War II and the synthetic rubber program stimulated the introduction of commercial IR spectrometers. This was the beginning of IR spectroscopy in a commercial sense for organic molecular analysis.

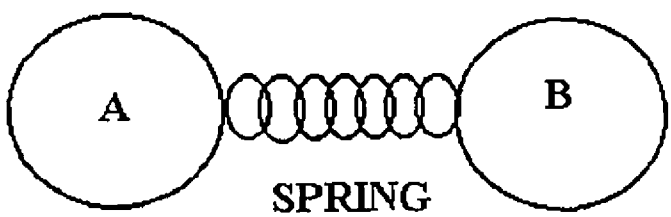
This historical perspective to infrared spectroscopy was brief in nature, covering the contributions of the many well known IR pioneers over approximately a 250 year period. We will now backtrack into the "*instrumental*" developments of IR spectroscopy from its initial growth employing dispersive technology to todays well known Fourier Transform Infrared spectroscopy (FT-IR).

II. PRINCIPLES AND INSTRUMENTATION OF IR SPECTROSCOPY. DISPERSIVE VERSUS FT-IR SPECTROSCOPY (A COMPARATIVE LOOK AT BOTH METHODOLOGIES)

a) *THEORETICAL PERSPECTIVE ON INFRARED SPECTROSCOPY:*

At an ambient temperature as well as elevated or decreased temperatures, molecules are in a constant state of vibration. Every bond has a characteristic *stretching* and *bending* frequency hence being capable of absorbing light at that particular frequency. An analogous mechanical model representing two atoms joined by a chemical bond would be that of the vibrations of two balls joined by a spring. The vibrational frequency of any bond can be calculated with reasonable accuracy for a molecular system, in the same way as the vibrational frequency of two balls connected to a spring system. The equation employed in this calculation is known as ***"Hooke's Law"***

Schematic 2.1: Hooke's Law of Force.



The law correlates frequency with bond strength and atomic masses as follows;

ν = sq. root [(bond strength) / (reduced mass)] ; where ν = frequency

$\nu = 1/2\,\pi$ * sq. root [$k/m_A m_B/(m_A + m_B)$] ; where k = a constant related to the spring (bond) strength &

m_A & m_B = mass (atomic) of the two atoms.

Hence, it is possible to predict the approximate vibrational frequency of a bond providing that both atomic masses and an approximate force constant is known. We can therefore state that the absorption of infrared radiation is due to a "*vibrational*" phenomenon known to occur in existing molecular entities. One other very important factor required for a vibrating molecule to absorb IR radiation, is the presence of a dipole moment. The particular vibration should produce a fluctuating dipole (thus producing a fluctuating electric field); otherwise it cannot interact with the fluctuating electric fields of the infrared light. It is important to realize that absorption occurs only by quantized energy levels. The energies associated with bonds are quantized in much the same way that electrons in atoms are quantized; and only certain energies are absorbed by certain bonds. A dispersive infrared spectrometer changes the frequency (i.e. the energy) of the IR beam continuously. When the energy of the beam passing through the sample is exactly equal to the energy needed to either stretch or bend a certain bond, the sample absorbs that particular energy and at this instant the amplitude of that particular vibration increases suddenly by a certain amount. Since virtually all functional groups within molecular structures show specific and characteristic absorptions in the IR region (Ref.9), infrared spectroscopy can be employed effectively in the elucidation of molecular structures.

b) ***INFRARED INSTRUMENTATION:***

i) DISPERSIVE INFRARED SPECTROMETER:

Currently, there are two types of infrared spectrometers, characterized by the manner in which the infrared frequencies are measured. In the first type, a dispersive spectrometer separates IR radiation into its individual frequencies by dispersion (dispersive-IR), using a grating-prism monochromator. In the second type of spectrometers, all the IR frequencies are constructively and destructively combined to produce a modulated "interference pattern". The pattern is then analyzed mathematically, through a Fourier Transformation, to determine the individual frequencies and intensities. **Figure 2.2** is a simplified diagram of a typical optical layout of a dispersive infrared spectrometer. One important factor is

that a double beam instrument may be of two different types: 1) Optical null ; and 2) ratio recording.

Figure 2.2: A DOUBLE - BEAM (DISPERSIVE) INFRARED SPECTROMETER:

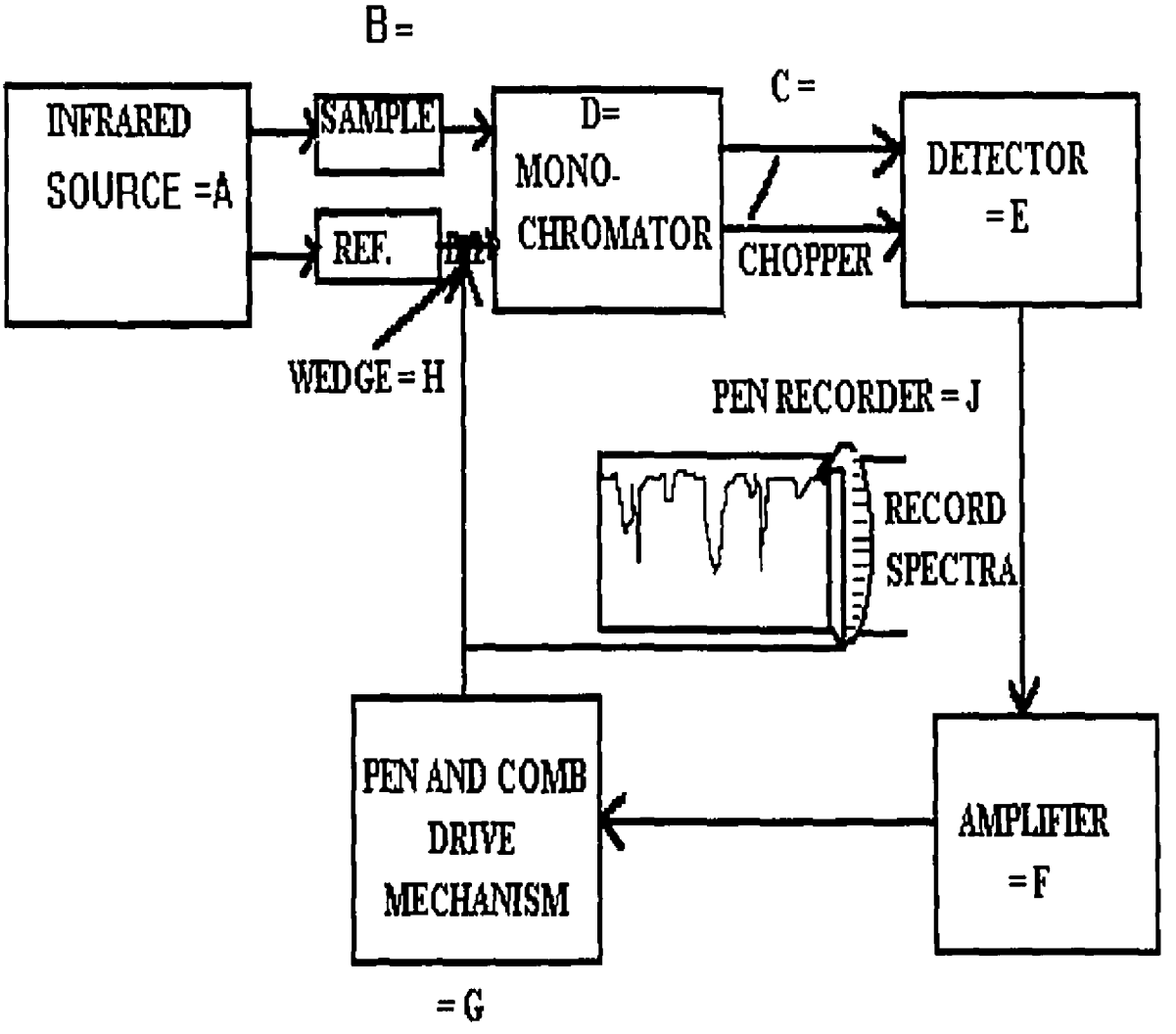


Figure 2.2 shows a schematic representation of a double beam spectrometer. Light from the IR source (A) is split into two equal beams, one passes through the sample (B) and the

other through the reference cell, and a measure of the difference in intensities between the two beams at each wavelength is recorded.

The two beams are reflected to a chopper (C) which consists of a rotating segmented mirror. As the chopper rotates it causes the sample and reference beams to be reflected alternately to the monochromator grating (D). As the grating rotates slowly, it sends individual frequencies to the detector (E), which converts IR energy to electrical energy. When the sample absorbs light of a particular frequency, the detector receives alternately from the chopper an intense beam (i.e. reference beam) and weak beam (i.e. sample beam) leading to a pulsating alternative current (AC) flowing from the detector to the amplifier (F). If no absorption in the sample occurred, both sample and reference beams would have equal intensities causing a direct current (DC) to occur from the detector.

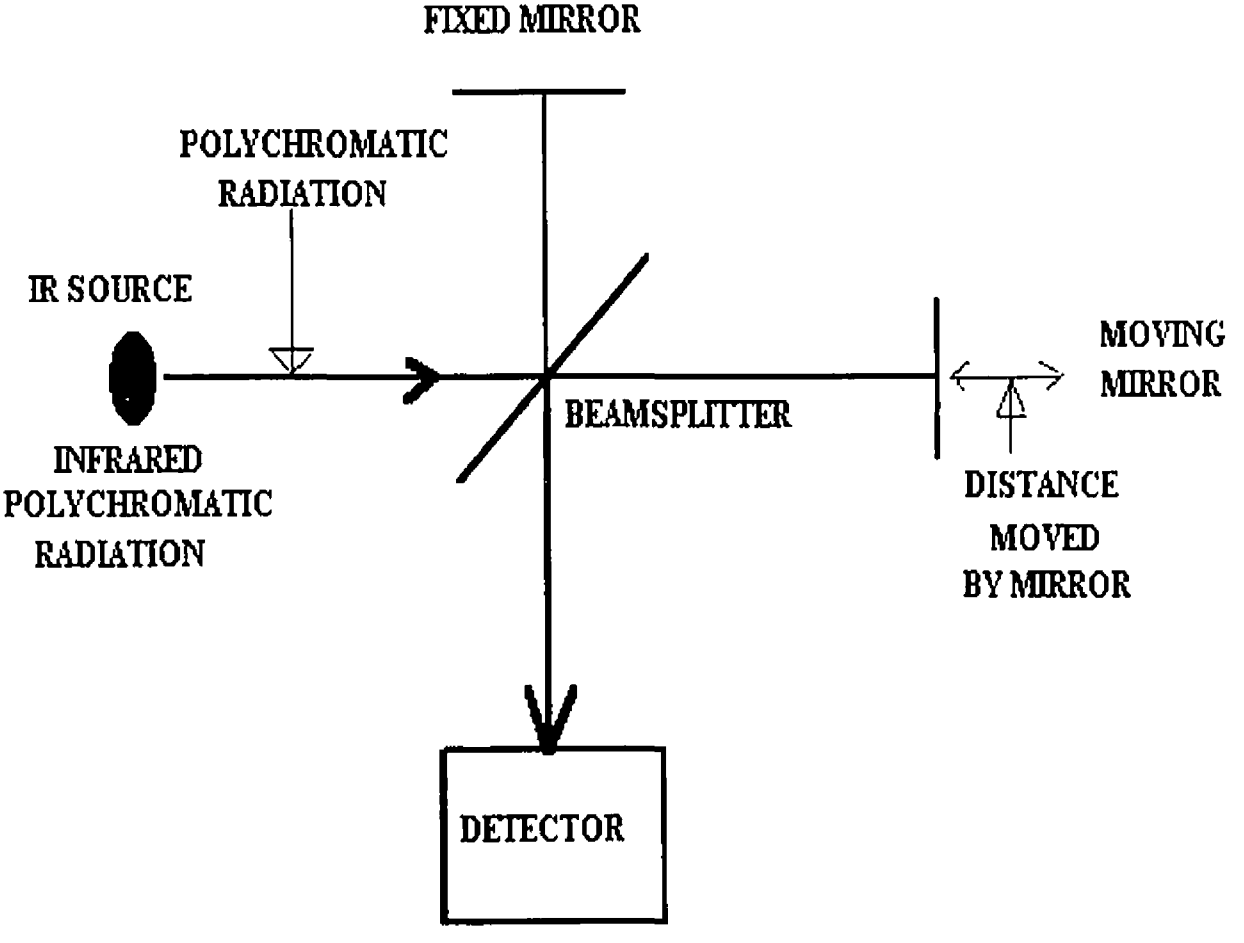
The amplifier, receiving the out-of-balance signal, is coupled to a small servo-motor (G), which then drives an optical wedge (H) into the reference beam until the detector receives light of equal intensity from both sample and reference beams. The wedge movement is in turn coupled to a pen recorder (J), so that movement of the wedge in and out of the reference beam shows as characteristic absorption bands on the printed spectrum of the sample. The instrument therefore is depicted to balance itself by optical means by differentiating between two separate beams and is called the *double-beam optical null* spectrometer.

In *ratio recording* mode, the intensity of the sample and reference beams can both be individually measured and ratioed. This aspect of recording circumvents a major deficiency in *optical null* methods in which the comb attenuator blocks off much of the light beam. This does not only lead to reduced sensitivity, but also poor analytical performance and slow scan speeds. These instruments however, were known to be fairly expensive in comparison to double-beam instruments.

ii) FOURIER TRANSFORM - IR (FTIR) SPECTROMETER:

An entirely different principle is involved in FTIR spectroscopy, which is based on the Michelson interferometer. In 1887, Albert Michelson had perfected the interferometer and used it for several measurements in his study of light and relativity. The basic component of the ***Michelson interferometer*** is shown in **Figure 2.3:**

Figure 2.3: The Michelson Interferometer.



The interferometer consists of two mirrors whose planes are perpendicular to each other. One of the mirrors is fixed while the other moves usually at a constant velocity. Between both mirrors is a *beamsplitter*, at which 50% of the incident radiation is ideally transmitted to one mirror and the other 50% to the other mirror. The radiation after reflection from the mirrors then recombines at the splitter resulting in an interference pattern detected at the detector. This combined interference beam is usually then passed through the sample compartment to a detector.

Constructive and destructive interference occurs between the two light beams dependent on the path difference of both moving and fixed mirrors. Constructive interference will take place each time the path difference is an integral number of wavelengths. On the other hand, if the path difference is equal to a half fraction of the wavelength, the waves will interfere destructively and no signal will be observed at that frequency. For all the polychromatic infrared signals reaching at the detector, the interference pattern of all waves (i.e.,constructive and destructive) produces an "interferogram" as shown in **figure 2.4 (a).** The interferogram is then mathematically converted into a spectrum by a Fast Fourier Transform Algorithm. A single beam spectrum obtained after Fourier transformation of an interferogram is shown in **figure 2.4 (b)**.Upon ratioing two single beam spectra a transmittance spectrum is then obtained.

The FTIR spectrometer also includes within its optical cavity a reference He-Ne laser for calibration purposes. The radiation from the laser is passed through the interferometer, and effectively allows all infrared wavenumbers to be referenced directly to the laser frequency. Due to the reproducibility and accuracy of FTIR spectrometers, many data manipulation are possible compared to dispersive IR instruments. Data manipulations may include: subtraction, derivative spectroscopy, Fourier self-deconvolution, curve-fitting, smoothing, spectral searching.

Figure 2.4 (a): FTIR interferogram of an edible oil.

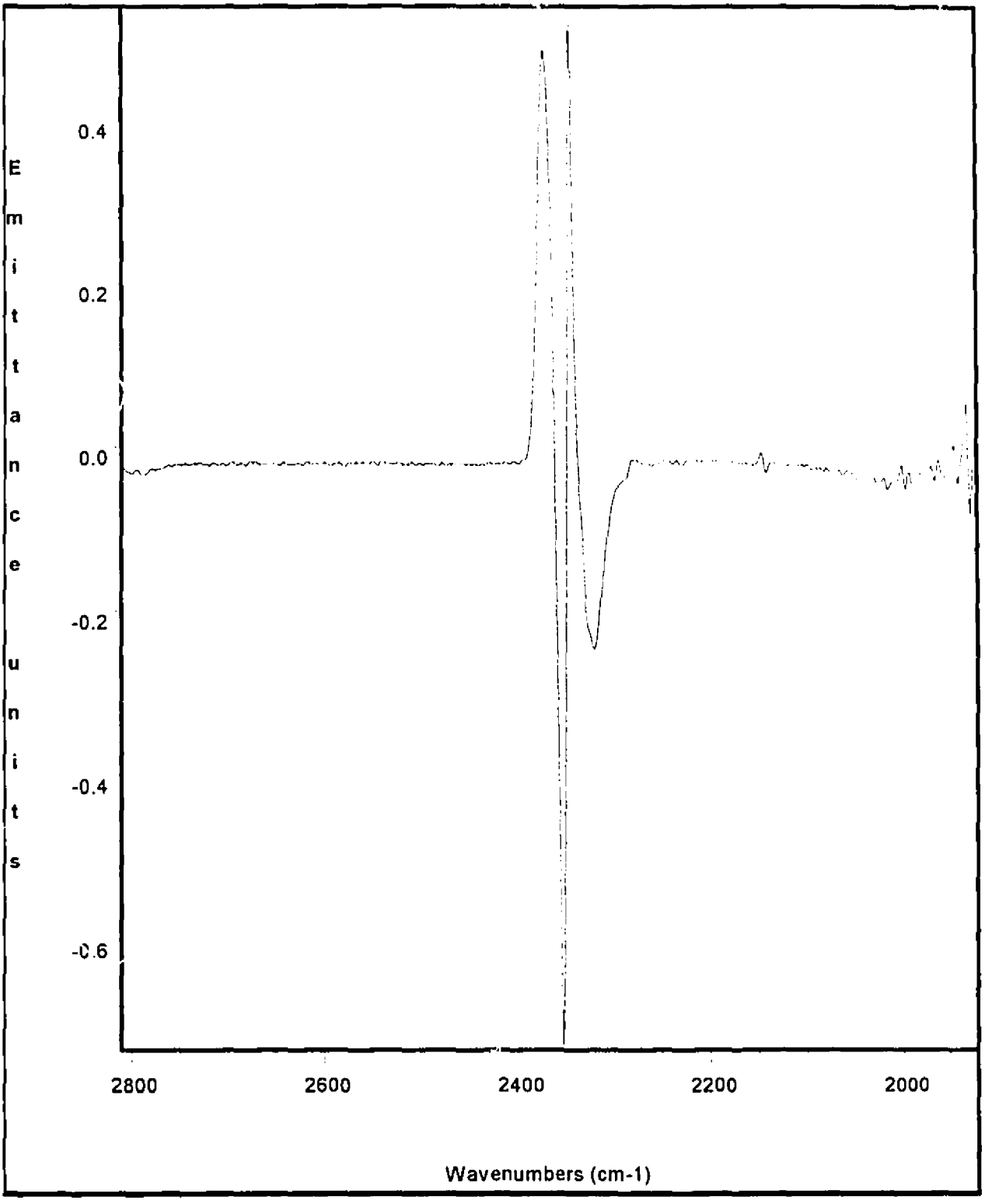
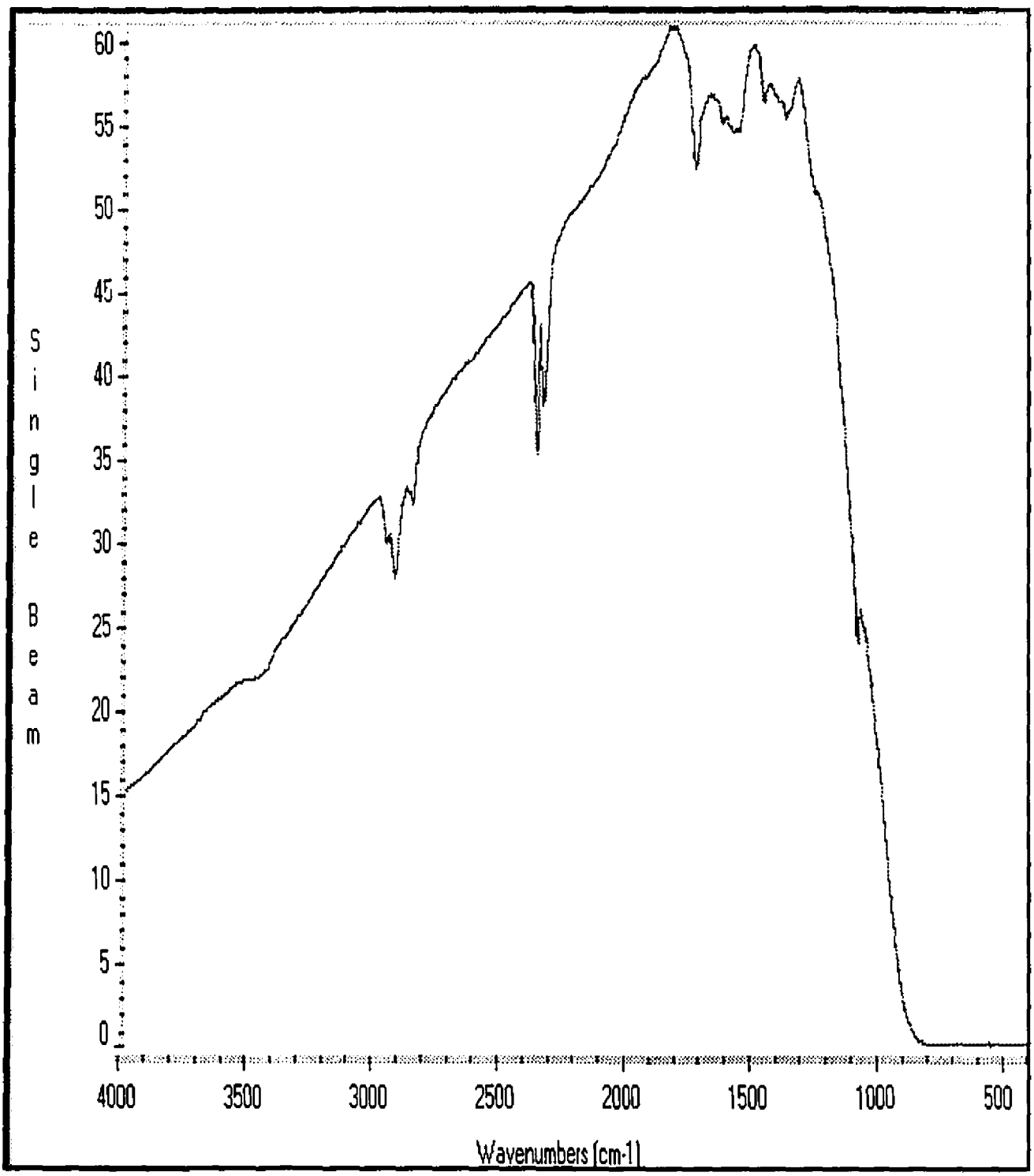


Figure 2.4 (b): Single Beam FTIR Spectrum.



There are three primary advantages of Fourier transform spectrometers over grating monochromators. The first advantage known as the *Fellgett advantage*, or *multiplex* advantage, where all resolution elements in the spectrum are acquired simultaneously, rather than sequentially as occurs in dispersive IR. Thus, the measurement time for a spectrum is drastically reduced to seconds rather then minutes in FTIR spectroscopy. The second advantage of FT-IR spectroscopy is the *Jacquinot*, or *throughput* advantage, and relates to the optical throughput being greater for FTIR spectrometers than for monochromators for operation in any given spectral range and resolution. The third advantage of FTIR interferometers is that of *Connes'* advantage, due to the usage of a He-Ne laser to trigger data acquisition resulting in exceptionally good wavelength repeatability. Hence, due to the three characteristic advantages of FTIR spectroscopy, data acquisition, and manipulation are significantly improved dispersive IR spectroscopy (ref. 10, 11,& 12).

A brief overview of the use of ***infrared spectroscopy in fatty-acid analysis*** over the past 70 or so years will be presented.

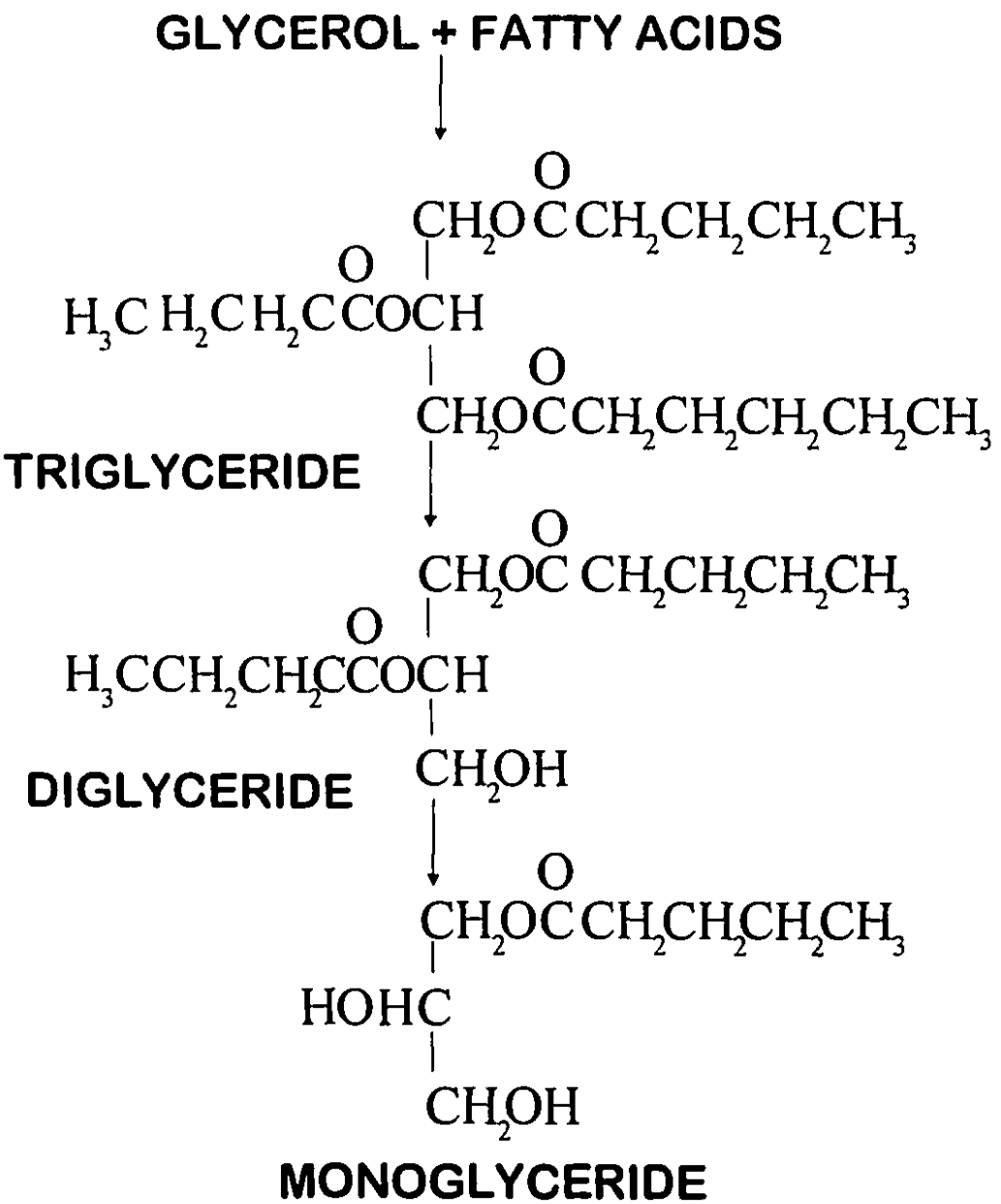
Lipid and Fatty-acid Analysis by Infrared Spectroscopy:

The following literature review on the analysis of triglycerides and fatty-acids by infrared spectroscopy will cover a chronological time frame from the first IR analysis of lipids and fatty acids. Preliminary results from the IR analysis of lipids and fatty acids will be discussed with a subsequent follow up on fatty-acid analysis in the postwar era (i.e. 1940's to 50's), and finally a discussion of work carried out in the 60's to the latest work done in the 90's.

Lipids consist of a broad group of components of adipose tissue and cellular membrane structures found to be soluble in organic solvents. The term *lipids* encompasses several molecular entities such as *triglycerides, phospholipids, sphingolipids and acylglycerols.*

Triacylglycerides are made up of a glycerol moiety forming ester linkages with three different fatty acid molecules as shown in **scheme 2.2**. The chain length of the fatty acids characterizes the physical property of a triglyceride (i.e. whether it is a solid fat or liquid oil).

Scheme2.2: Formation of tri-, di-, and monoglycerides.



During the past forty years, infrared spectroscopy has been employed as a tool for qualitative and quantitative determinations and for elucidation of the chemical structures of fatty acid materials. The first paper known to of been published exclusively on the infrared spectra of fatty acids was that of K.S. Gibson in 1920 entitled, " The Infrared Spectra of Vegetable Oils" (Ref.15). The first evidence of the actual applications of IR spectroscopy to fat and oil chemistry was in the 1940-1950 World-War epoch. One of the most useful application of infrared spectroscopy to fatty acid analysis developed in the late forties, entailed a method distinguishing between *cis* and *trans* unsaturation and the determination of the *trans* bonds in the presence of *cis* double bonds. In 1947, R.S Rasmussen, R. Robert Brattain , and P.S. Zucco (Ref.16) of the Shell Development company, studied the IR absorption spectra of olefins and reported the spectra of seven octenes. What was of significant importance for future endeavors in the use of IR in fatty acid analysis was a small footnote remarked by the authors on the possible correlation of a *trans*-configuration about a double bond of an olefin. Quoted directly from the footnote was the following statement; "*From work on compounds studied subsequent to the writing of this paper, it appears that the strong 10.3 micron infra-red band is characteristic of the trans-form of the CHR=CHR system. The cis form shows a band of much lower intensity and more variable position in the 10 to 11 micron region, and also a very strong band in the 14 to 16 micron region. On this basis, the 14.2 micron band observed in the 2-octene would denote a few percent of cis-form, while the very strong 10.3 micron band indicates the trans-form as the principle constituent*". Hence, this initial observation resulted in the subsequent assignment of 10.3 micron to the *trans configuration*. The 10.3 micron peak has since been assigned to the C-H deformation about a *trans* C=C bond as denoted by Sheppard and Sutherland (ref.17) two years after Rasmussen et al. initial observations. McCutcheon, Crawford, and Welsh as early as 1941 (ref.18), had also used infrared spectra to distinguish between the *cis* and *trans* bonds. They had measured the spectra from 5 to 6.5 microns and used the very weak 6.0 micron band assigned to the C=C stretching vibration. Although, there had been some

complication in interpretation due to band overlaps at 6.5 microns. The absorbance of the *trans* wavelength has since been employed for both the qualitative and quantitative analysis of a variety of materials (ref. 19,20,21). In 1950, Shreve, Heether, and Swern (ref.22), developed an infrared spectrophotometric method for the determination of ***isolated trans*** bond concentration in saturated and monounsaturated octadecenoic acids and related compounds based on the measurements of the 10.36 micron band. In developing the method, the extinction coefficients were determined from pure compounds (i.e., for saturated and for *cis* and *trans* unsaturated free acids, esters, triglycerides, and derived alcohol) and the subsequent *trans* content was then calculated from a given formula. This was the first protocol directly applicable to the determination of *trans* components of esters , acids, and alcohol mixtures. Hence, the three authors were the first pioneers in the ***quantitative analysis of isolated trans double bond*** by infrared spectroscopy. From this point to 1953, basically four papers (ref. 23,24,25,26) had described techniques that used the 10.3 micron band as a means for the quantitative determination of the internal, *isolated* (i.e., nonconjugated) *trans* isomer. The published methods for the determination of the *isolated trans* content have since been the subject of extensive study by the spectroscopy committee of ***the American Oil Chemists' Society***. As a final result of a long collaborative testing the committee had then recommended a procedure in 1959 (ref. 27) which has since been adopted as a ***tentative official method*** of the society. The recommended procedure provided for the determination of *isolated trans* content of long chain fatty acid esters and their triglycerides. The long chain fatty acids were analyzed directly if the *trans* content was 15% or more. If the *trans* content was suspected to be below 15%, the recommended procedure required a conversion of the fatty acid to its methyl ester prior to the determination of the trans content.

Paschke, Jackson, and Wheeler (ref.28), studied the polymerization of isomers of methyl linoleate and reported that, the absorption band at 10.36 microns in the spectra of methyl *cis*-9, *trans*-12-linoleate was nearly of the same intensity as in the spectra of methyl elaidate and that absorptivity of the band in the spectra of methyl linolelaidate (*trans*-9, *trans*-12) was twice this value. The data indicated that the absorptivities of the *isolated*

trans band at 10.36 microns are ***additive*** in nonconjugated compounds and that the method of Shreve et al. can therefore be extended to the analysis of polyunsaturated, nonconjugated olefinic acids and related compounds. Bickford et al. (ref.29), found that in chloroform solutions, the absorptivity of the 10.1 micron band in *alpha*-eleostetaric acid (*cis*-9, *trans*-11, *trans*-13-octadecatrienoic) was 0.8 , nearly twice that of elaidic acid, and that of beta-eleostearic (*trans*-9, *trans*-11, *trans*-13-octadecatrienoic) acid was 1.2, almost three times that of elaidic acid. Hence, these data indicated that the C-H deformations at approximately 10.3 microns are ***additive*** in conjugated as well as nonconjugated compounds. Ahlers, Brett, and McTaggart (ref. 30) accounted for the position of the peak maxima in spectra of conjugated acids containing *trans* bonds as a *hypsochromic shift* from the position of the isolated trans band, consistent with the effect of conjugation on the C-H deformation frequency.

Correlations of *cis* and *trans* infrared absorption data about the C=C deformation bonds have been found to be very useful in *selective hydrogenation studies* in the early 50's. Lemon and Cross (ref. 31) had demonstrated that hydrogenation of oils is accompanied by a *cis* to *trans* change in some of the double bonds of the unsaturated fatty acids, by the appearance and increase in intensity of the band at 10.3 microns in the hydrogenated oil spectra. Lemon (ref. 32) also showed graphically the formation and disappearance of *trans* double bonds during vegetable oil hydrogenation. Feuge et al. (ref. 33), studied the hydrogenation of methyl linoleate and cottonseed oil and calculated the concentration of isolated *trans*-isomers from the IR spectra.

Infrared spectroscopy was also employed in the study of ***autoxidation process of lipid compounds***. From 1942 to 1946, Farmer and co-workers (ref. 34-36) demonstrated that hydroperoxides were formed by the attack of molecular oxygen on the carbon alpha to a double bond. They also indicated that hydroperoxides undergo further reaction with a carbon-carbon double bond to form epoxy and several hydroxyl derivatives. In 1949, J. Honn, I.I. Bezman and B.F. Daubert (ref. 37), devised a method which combined infrared spectroscopy and chemical methods to analyze the various molecular -OH containing

oxidation species. Of the three types of hydroxyl compounds believed to be formed due to oxidation, two may be estimated by chemical means. Hydroperoxides were determined iodometrically and carboxylic acids by acid-base titration. By means of the spectroscopic-chemical method of analysis, the so called ROH fraction was calculated by subtracting the sum of the hydroperoxide and carboxylic acid contributions, chemically measured, from the total hydroxyl content, measured by IR. The paper by Honn et al. described a scenario for the examination of raw linseed oil by infrared spectroscopy before and after vigorous oxidation of the oil. Two absorption bands were noted to increase significantly as the oxidation process proceeded. The first band was a rather broad band at 10.2 microns with a subsequent increase in a far more distinct band at 2.9 microns characteristic of the bonded hydroxyl group. Along with the described combined spectroscopic chemical methods for the analysis of various hydroxyl species formed during the oxidation of linseed oil, the induction period of the oil was also depicted by infrared spectroscopy. The increase in the intensity of the band at 2.9 microns (i.e. hydroxyl species) was followed as a function of time. The intensity of the 2.9 micron -OH band of an oil undergoing oxidation was found to increase slowly in the first four hours. Subsequently, the band quickly increased in intensity as the various oxidation reactions proceeded. Hence, Honn et al. as early as 1949, appears to be one of the first forerunners to use infrared spectroscopy as a method for determining the induction period of an oil. In 1949, L.R. Dugan et al. published an article on the study of autoxidation of methyl linoleate by IR spectroscopy. Gamble and Barnett (ref.38), examined the IR absorption of methyl oleate and methyl elaeostearate before and after exposure to ultraviolet (UV) radiation. They found that the strong IR absorptions at 2.9 microns, shifted toward higher frequencies after UV irradiation. This shift was attributed to the introduction of hydroxyl groups. Dugan et al. prepared a series of autoxidized methyl linoleate samples ranging in peroxide value from 1 to 940 meq/kg sample. For comparative purposes, spectra were obtained from oxidized methyl linoleate and on a portion of the same sample reduced to a peroxide value (PV) of 0 by treatment with a KI reagent. Earlier infrared measurements made by the same authors on autoxidized fatty acid esters showed that two principal changes occurred in the IR regions: at 3400-3550 cm^{-1} where -OH groups absorbed and 1650-

1775 cm^{-1} where C=O groups were known to absorb. Accordingly, the measurements made in the autoxidized methyl linoleate study were made in only the two reported IR regions. The authors had observed that a common absorption band existed for all samples in the 3467-3470 cm^{-1} region with the lower frequency predominating in the sample most highly oxidized. They attributed this band to the hydroxyl groups. The authors had observed other samples in which the band was missing or much less intense than the methyl linoleate sample with as low a PV as 1. From this evidence, they then concluded that "It would appear that infrared absorption in this region should serve as a criterion of the purity of a fat with respect to oxidation effects". This provided the incentive to use the hydroxyl absorption as a means for fat oxidative rancidity/spoilage measurements (i.e. PV). The authors then made observations of band shifts in the hydroxyl band in the IR spectrum and attributed the initial frequency shift to an increase in PV of the sample. The shift from approximately 3445 cm^{-1} to 3430 cm^{-1} was believed to be due to an "association" state of both -OOH and -OH groups (i.e. hydrogen bonding) in the oxidized molecules as was pointed out by Barnes, Liddel, and Wilson in 1943 (ref. 39). The peak at 3430 cm^{-1} was related to the hydroperoxides formed via oxidation. **Figure 2.5** below demonstrates the evidence that the authors gave for assigning of the band at 3470 cm^{-1} to -OH groups and the band at 3430-45cm^{-1} to -OOH groups. The Log I/Io vs. frequency curves are shown to shift towards the lower frequency as the autoxidized methyl linoleate is reduced. Hence, demonstrating the strong evidence of two molecular entities occurring during autoxidation of an oil.

The carbonyl region between 1690 and 1780 cm^{-1} of the oxidized fatty acid ester was also examined . In order to obtain an idea of what the absorption bands due to other carbonyl groups were like, a plot of the log of the intensity of the least oxidized sample as Io (PV~1) versus that for each oxidized sample as I is shown in **Figure 2.6**.

Figure 2.5: Absorption by -OH and -OOH of substances eluted from an adsorption column.

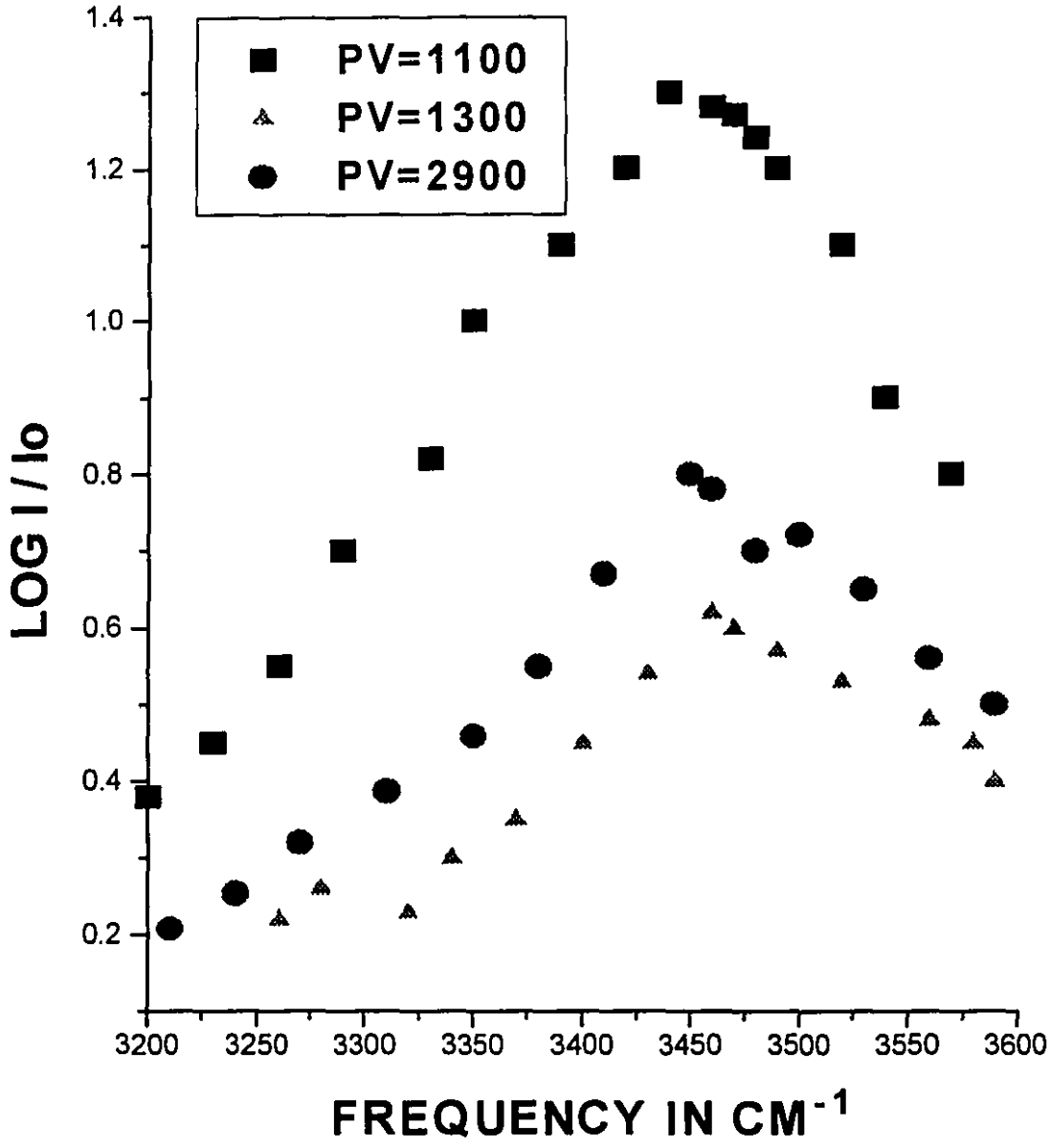
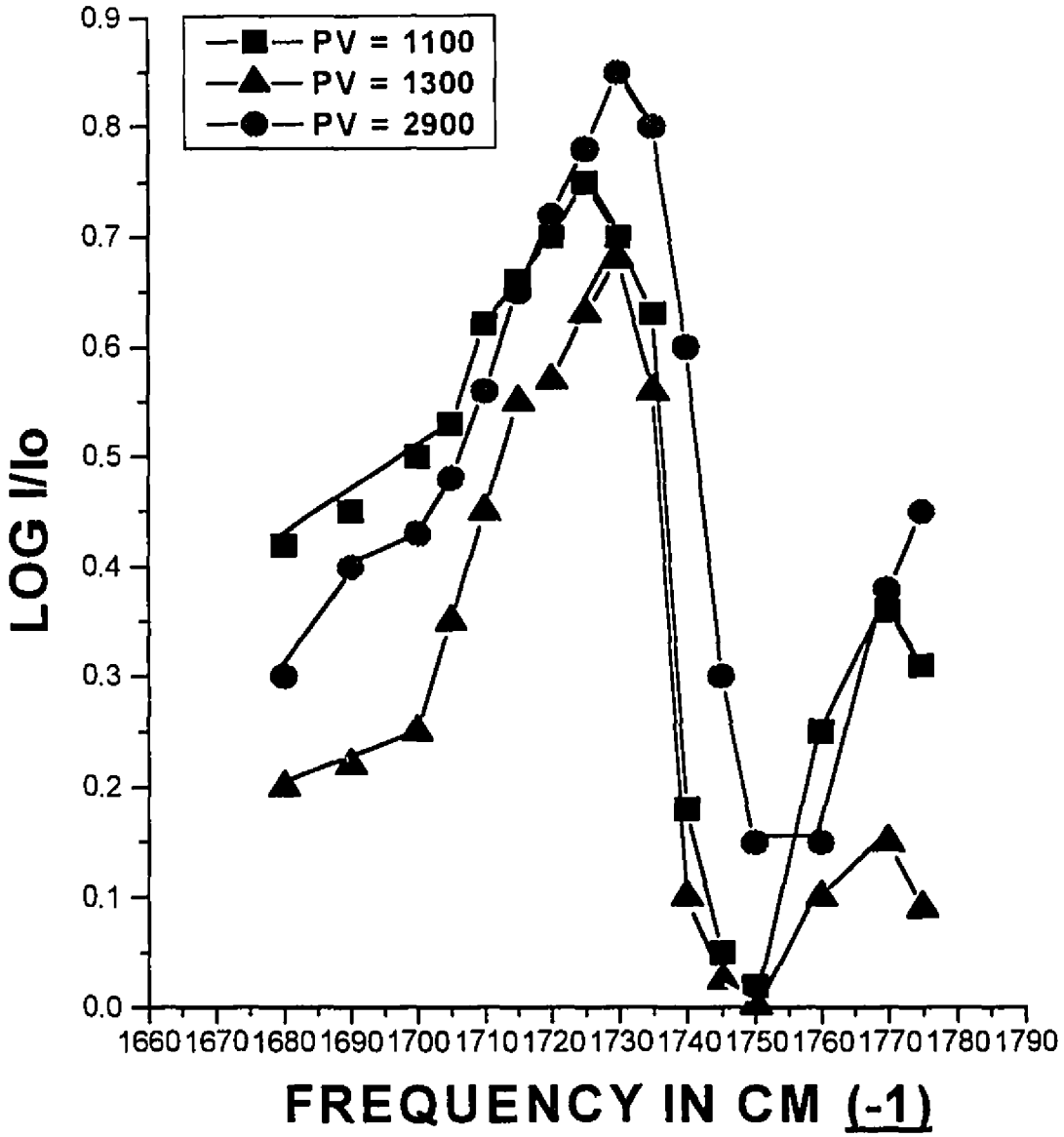


Figure 2.6: **Absorption by Carbonyl groups other than ester Carbonyl in substances eluted from adsorption column.**



The plot illustrates a double maximum for all ratioed samples at 1724 cm^{-1} and 1733 cm^{-1} with an additional small maxima in only some samples at 1755 cm^{-1}. The authors related the two maxima to the presence in the autoxidized samples of at least two separate carbonyl containing compounds formed in different quantities. The band at the lower frequency was attributed to ketones conjugated to a double bond and the higher frequency band to unconjugated ketones. The weaker 1755 cm^{-1} band was attributed to aldehydes via scission products of autoxidation. Hence, the above plot illustrated the presence of other carbonyl C=O containing species aside from the ester carbonyl . This preliminary study on the autoxidation of a fatty ester demonstrated the potential utility of IR spectroscopy in the characterisation of oxidation species. Dugan et al. may therefore be regarded as pioneers in correlating certain characteristic IR absorptions to molecular functional groups (i.e., that of -OOH, -OH and carbonyl C=O containing entities) found in oxidized fatty acid esters. In 1950, A.S. Henick (ref.40) published the first IR study ever done on a food system (i.e., since the work of K.S. Gibson in 1920), that of the "Detection of Deterioration Products of Autoxidizing Milk Fat by IR spectroscopy". The loss of flavor was correlated with a specific absorption band, and the growth of off-flavors was attributed to other IR bands. The authors suggested that " infrared examination of autoxidizing milk fat suggested itself as a possible means for detecting products of deterioration before they had accumulated in quantities sufficient for chemical estimation". Absorption bands in the hydroperoxide absorption region (i.e. ~ 2.9 μ) and the carbonyl absorption region (i.e. ~ 5.75-5.80 μ) were measured. Formation of new compounds during the oxidation of milk fat was followed by infrared spectroscopy and a sensory evaluation of the milk fat was also determined simultaneously by a chosen panel. The suspected carbonyl compounds were collected by steam distillation in vacuo. The changes in the carbonyl absorption region was followed as a function of time of oxidation of milk fat. The occurrence of several peaks in the carbonyl region were observed upon long-term storage of the milk fat. The presence of a sharp peak at 5.75 microns was used as an indicator of the freshness in the fat, and a peak at 5.70 microns as an indicator of flavor loss. The authors demonstrated that the infrared spectrum of milk fat in the region of 5.5 to 6.20 microns was more sensitive as a test of freshness than an actual taste panel. The

authors then concluded with a suggestion that " a quantitative method may be developed whereby the flavor and acceptability of fats and food products containing fats can be predicted from infrared absorption measurements, and perhaps, storage life may be estimated". Thus, Henick as early as 1950 had demonstrated the utility of infrared spectroscopy as an analytical tool in the food industry, reducing the time required for chemical measures and eliminating much of the guess work present in panel testing. In the early part of the 1950's, numerous infrared band assignments were described in the literature. In 1950, Shreve, Heether, Knight and Swern (ref. 41) , reported the assignments of IR bands of long chain fatty acids and glycerides. Common spectral features of all compounds were described as well as the spectral differences between each class of the compounds studied. Band assignments were confirmed with previous work done in fatty acid IR analysis by various authors as well as some unpublished work carried out by the authors. The authors concluded the article by stating that " the spectra presented should prove useful in assessing the potentialities of the infrared method as applied to studies of fat systems; they are primarily intended to serve as a guide in the development of spectroscopic or combined spectroscopic-chemical methods for the analysis of mixtures of fatty materials". As a result of this study, the authors had developed an infrared spectrophotometric method for the determination of the trans components in mixtures of long-chain compounds, utilizing the strong 10.36 μ band . In 1951, Knight, Eddy and Swern (ref. 42), studied the reaction of several fatty materials with oxygen by infrared spectroscopy. They concluded that the trans content of methyl oleate increased as oxidation proceeded, and it was evident that cis to trans isomerization had occurred during the initial stages of autoxidation. The data suggested that most, if not all, of the peroxides produced during the autoxidation of methyl oleate, are trans peroxides and not methyl oleate peroxides. A mechanism for the formation of trans peroxides from allylic free radicals was proposed. From 1951 to 1960, confirmation of IR band assignments were made by various groups. O'Conner et al. (ref. 43) presented data to show which bands of seven fatty acids and their methyl and ethyl esters in the infrared spectra follow Beer's law and which IR bands do not follow Beer's law. These studies were clearly an important factor for future application of IR spectroscopy in the

quantitative analysis of fatty compounds. In 1951, peak assignments epoxy groups (ref. 44) were made along with some common absorption bands of hydroperoxides (ref. 45). In the same year, H.W. Lemon et al, published an article in the Canadian Journal of Technology (ref. 46) on the effects of temperature and added iron on the infrared spectrum of methyl esters of peanut oil. They reported significant changes in three regions of the IR spectrum, I) the 3730 to 3005 cm^{-1}, II) 1840 to 1630 cm^{-1} and III) the 1130 to 740 cm^{-1} regions. Peroxide values, iodine values and free fatty acid values were determined according to the official standard methods of the American Oil Chemists' Society. In the oxidation studies, the major band assignments were that of the hydroperoxides and formed alcohol's in the 3700-3000 cm^{-1} region. The increase in the carbonyl intensity with subsequent band splitting due to the presence of other carbonyl containing compounds in the 1840-1630 cm^{-1} region II was also reported.In the 1130-740 cm^{-1} region certain bands were assigned to conjugated dienes and the well known trans groups. Preliminary band assignments of free fatty acids were also carried out in all three regions. In the hydroxyl absorption region (3730-3005 cm^{-1}), unoxidized methyl esters of peanut oil spiked with 5% free fatty acids showed increased absorption between 3400 and 3000 cm^{-1} . The IR spectrum of the unoxidized oil spiked with 5% added free fatty acids also showed a new band with maximum at 1710 cm^{-1} along with the strong ester carbonyl band. In the IR spectrum between 1130 - 740 cm^{-1} an increase in absorption between 980 and 880 cm^{-1} caused by the addition of free fatty acids was also reported. In 1952, Sinclair et al. recorded the spectra of saturated fatty acids and esters (ref. 47). In a subsequent publication of the same year (ref. 48), the same authors published an article on the possibility of determining the chain length of fatty acids in the solid state from the band progressions in the infrared spectrum region between 1180 to 1350 cm^{-1}. It was suggested that the band progressions arose from interactions among the rocking and /or twisting vibrations of the methylene groups. In the same year, a third publication in the same journal by the same authors (ref.49) described the band assignment of *unsaturated fatty acids and esters* complimenting the first paper published on saturated fatty acids and esters. The comparative study of the infrared spectra of unsaturated fatty acids and esters had been undertaken in order to evaluate the extent to which IR spectroscopy may be

applied to the problems of identification and quantitation of unsaturated fatty acids. In 1953, Ahlers et al. (ref. 50) studied the effect of cis to trans isomerisation of fats by infrared spectroscopy. The work had been extended to a wider series of unsaturated fatty acids, including those containing two, three and four double bonds in comparison to earlier IR studies of *cis* to *trans* isomers. A significant amount was published from this point onwards to the early sixties on IR band assignments of various fatty acids, and oils (ref. 51-53), including a publication on the differentiation of mono-,di-,and triglycerides by infrared spectroscopy by O'Conner et al. (ref. 54) In 1960, Chapman (ref. 55), elaborated on the work done in the previous year by O'Connor et al. on the characterization of the mono-, di-, and triglycerides. The main differences between both publications was that O'Conner had investigated the IR spectra of the glycerides in chloroform solution and Chapman investigated the glycerides as pure melts. Throughout the 60's several publications (ref. 56-68) were published on the use of infrared spectroscopy in lipid analysis. Most of the publications were studies done on determining the average carbon chain length of solid state lipids (ref. 56) and saturated fatty acid esters (ref. 59) and further elaboration's on the AOCS CD 14-61 method on the analysis of isolated trans unsaturation in lipids by infrared spectroscopy (ref. 58, 62 & 66). Between 1960 to 1970, three excellent reviews (ref.56, 62 & 67) on lipid analysis by infrared spectroscopy were published. Up to this point , the instruments employed were all of dispersive infrared systems which incorporated prism and diffraction gratings. In the late 60's to early 70's a new type of infrared instrument became commercially available in the market that of the Fourier Transform Infrared Spectrometer employing the principle of interferometry as described earlier. Computer technology had also advanced significantly in its use in the scientific milieu from its early beginning in the sixties. Although, the cited literature on lipid analysis by infrared spectroscopy in the 70's, in general, did not routinely employ FT-IR spectrometers in lipid analysis mainly due to high cost of computer technology and instrumentation, even though its advantages over dispersive IR spectroscopy were well known. Throughout the early to late 70's, the published literature (ref. 68 - 76) on lipid analysis by infrared spectroscopy mainly focused on the optimization of the standard AOCS method to measure isolated trans moieties. Many of the

publications in the 70's, described various alterations to the standard method such as the use of the Attenuated Total Reflectance (ATR) infrared spectroscopy in place of transmission-based infrared spectroscopy (ref. 73). In 1972, Fukuzumi and Kobayashi (ref. 71) developed a method for the quantitative determination of methyl octadecadienoate hydroperoxides by infrared spectroscopy. Within this time frame (i.e. 1970-1980) new FT-IR methods of analysis of lipid unsaturation was also being developed (ref. 69,72 & 74). In 1970, Low et al. (ref. 68) published one of the first uses of FT-IR spectroscopy in food oil analysis entitled "Infrared Fourier Transform Spectroscopy in Flavor Analysis" analyzing a series of commercial orange oils. It was not until the mid to late 80's that FT-IR instrumentation became widely used. In 1988, Lanser and Emken (ref. 77), published a paper in the Journal of the American Oil Chemists' Society describing a method of analysis of trans unsaturation by FTIR spectroscopy. The paper compared the FTIR computer assisted technology to a capillary gas chromatographic method for the quantitation of trans unsaturation in fatty acid methyl esters. The agreement between both methods of analysis was found to be good and the authors concluded that "it is reasonable to assume that both methods give accurate results". A subsequent article in '89 by Sleeter and Matlock (ref . 78), described an automated quantitative method of analysis of isolated trans isomers using FTIR spectroscopy with further refinements in the previous AOCS method of analysis.

As we come to the 1990's, the use of FTIR spectroscopy in lipid analysis increased quite substantially. Not only did the instrument cost decrease significantly to an affordable level, computer technology was advancing enormously on a daily basis. Due to the advancement in computer technology , computer hardware and software costs decreased drastically to a level where even a small quality control laboratory was able to purchase a new FTIR spectrometer for as little as $ 20,000 - 30,000. Due to this decrease in cost and advancement in computer technology, much of the cited literature (if not all) on oil and fat analysis by infrared spectroscopy employed FTIR spectroscopy. Numerous authors active in oil and fat research were publishing articles on the usage of FTIR spectroscopy in lipid analysis such as the use of FTIR mid-IR spectroscopy for food analysis by Wilson in 1990

(ref. 79), or the article by Lanser et al. on FTIR estimation of free fatty acid content in crude oils extracted from damaged soybeans (ref. 80) and the use of FTIR in determining low levels of trans unsaturation in fats (ref. 81). Various other uses of FTIR spectroscopy in lipid analysis were cited with the most recent article published in 1994 by Safar, Bertrand, Robert, Devaux and Genot on the characterization of edible oils by ATR / FTIR spectroscopy (ref. 82). The utility of FTIR spectroscopy is greatly enhanced by its ability to handle multicomponent analysis through the use of sophisticated software analysis packages and computer hardware. Quantitative multicomponent analysis based on chemometrics (such as the Partial Least Squares (PLS) or K and P matrix) is now possible. Due to these capabilities and advantages brought forth by the FTIR spectroscopy and ATR-based accessories, a number of analytical protocols for lipid and edible oil analysis are currently under development (The McGill University FT-IR group) (ref. 84-90). The impetus of this IR work is based on the replacement of time consuming chemical-based methods which require tedious titrations on the part of the analyst as well as one of various toxic chemical reagents. Preliminary papers (ref. 83 & 84) by Van de Voort and Ismail in 1991 and 1992, describe the potential application of FTIR / ATR spectroscopy in food analysis. In 1992, two papers were published by Van de Voort et al. (ref. 85 & 86) on the determination of fat and moisture content of high fat products; butter and milk. A third article (ref. 87) in the same year by the same authors, was published on the use of FTIR spectroscopy in the rapid determination of the iodine value and saponification number of fats and edible oils. In 1993, Ismail et al. had published an article on the use of FTIR spectroscopy in the rapid determination of free fatty acids in fats and oils (ref. 88). The rapid determination of fat and moisture in high fat products such as mayonnaise and peanut butter (Van de Voort et al., ref. 89) was also reported. Finally in 1994, one article was published on the real time monitoring of oxidation in edible oils by ATR / FTIR spectroscopy (ref. 90). These articles demonstrate the tremendous potential of FTIR spectroscopy in lipid and edible oil analysis both for research and commercial use. This concludes the literature review on lipid oil and fat analysis by IR spectroscopy, briefly covering over 50 years of research done in this subject matter. The following three

chapters will describe in some detail more recent application of FT-IR in lipid analysis carried out in fulfillment of an M.Sc. thesis by the author.

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CHAPTER III

Application of ATR / FTIR spectroscopy in the evaluation of Antioxidant Efficiency

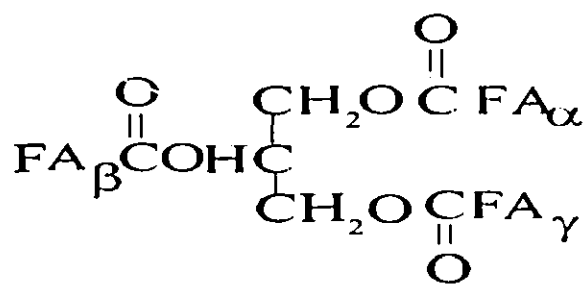
ABSTRACT

FTIR spectroscopy was employed in following the degradation process of an oil undergoing oxidative stress. Menhaden oil was oxidized in open air on a heated Attenuated Total Reflectance (ATR) crystal. Changes in infrared absorption bands were monitored as a function of time. Changes in the peak height of the band attributed to hydroperoxides at 3444 cm^{-1}, as well as changes in the bands attributed to the cis and trans double bonds at 3008 cm^{-1} and 971 cm^{-1} respectively, gave a very good measure of the oxidative status of the oil. Selected antioxidants, butylated hydroxyanisole (BHA) , propyl gallate (PG) and tert-butylhydroxyquinone (TBHQ) were then added separately to the menhaden oil at concentrations of 1.00%, 0.1% and 0.01%, respectively. The efficacy of each antioxidant was measured by its effect on the intensity of the three bands at 3444cm^{-1} , 971cm^{-1} , and 3008cm^{-1} as a function of the duration of heating over a heated 65^{o} C ATR crystal. The antioxidant efficacy was found to be dependent on both the type and concentration of the antioxidant. At the 1.00% concentration level, all three antioxidants exhibited an increase in the induction period as compared to the other concentrations. BHA was the most effective antioxidant at 0.01% but not at 0.1%. These studies show that FTIR spectroscopy provides a simple and rapid means of evaluating antioxidant efficacy as well as monitoring changes in oils undergoing thermal and oxidative stress.

Introduction

The spontaneous reaction of lipids with oxygen causes oxidative rancidity; this process is known as "autoxidation". During autoxidation, lipid molecules primarily undergo a three-stage chain reaction. In the first stage, a free radical (primarily oxygen) abstracts a hydrogen radical from a lipid molecule, forming a lipid radical. This radical, in turn, reacts readily with molecular oxygen to form peroxides. The formed peroxides then react with other unsaturated lipids forming hydroperoxides and new fatty acid radicals. At this point the rate of reaction accelerates through propagation forming a continuous amount of hydroperoxides and reactive lipid free radicals. The propagation will continue unless it is quenched by addition of an antioxidant. Otherwise, it is terminated when two free peroxide radicals combine to form stable products thus terminating autoxidation (ref.1). The decomposition products of hydroperoxides include aldehydes and ketones which are responsible for the rancidity of an oil . Fig. 3.1 is a representative general structure of a lipid triglyceride molecule which undergoes the autoxidation process with molecular oxygen as depicted in figure 3.2 (Note: R = lipid molecule and R* = lipid free radical molecule formed by autoxidation).

Figure 3.1 A representative triglyceride molecule.



Where FA – A FATTY ACID

Figure 3.2 The three steps of the autoxidation process.

Step 1. ***Initiation of oxidation:***

$$RH \xrightarrow{\text{initiator}} R^* \text{ (i.e. free radical formation where } R^* = \text{ lipid free radical).}$$

Step 2. ***Propagation step:***

$$R^* + O_2 \leftrightarrows ROO^*$$
$$ROO^* + RH \leftrightarrows ROOH + R^*$$

Step 3. ***Termination step:***

$$ROO^* + ROO^* \leftrightarrows \text{nonradical products}$$

The stability of an oil (i.e., the elapsed interval until the lipid constituents become rancid), depends on intrinsic factors such as degree of unsaturation of the oil, antioxidant content, and environmental conditions such as storage temperature, air exposure and metal contamination (ref.2).

Antioxidants are the most common compounds used by manufacturers in the food industry to retard autoxidation. Although there are a wide variety of antioxidants, antioxidants are primarily classified into two main groups: (1) chain-breaking antioxidants; and (2) preventative antioxidants (see ref.3 for more details on antioxidants). The great majority of synthetic antioxidants as well as certain natural antioxidants presently in commercial use are chain-breaking antioxidants.These antioxidants retard lipid oxidation by donating a hydrogen radical to a lipid free radical and forms an antioxidant with a free radical. The

antioxidant and free radical form a resonance-stabilized antioxidant radical and a non-radical lipid. The life-time of the antioxidant radical is lengthened to a point where it meets a second free radical and then becomes quenched. This results in a prolonging of the induction period of an oil (i.e., the period of time prior to the actual hydroperoxide formation and degradation).

Antioxidants vary in their efficacy on retarding the autooxidation of an oil. In most circumstances many of the synthetic antioxidants increase in antioxidative potency as concentration levels of the antioxidant increase within a food system. On the other hand, certain antioxidants can act as pro-oxidants within a food system at certain concentration levels thereby increasing deterioration of a food product. The efficiency of antioxidants with respect to type and concentration is of importance to the food industry for optimal processing, storage and consumer acceptance of lipid-containing foods. Various methods have been developed for the measurement of antioxidant performance; however, most of the measurements are time intensive requiring the use of various types of wet chemical analysis.

The present work reports a new FT-IR method for the determination of the efficiency of three synthetic primary chain-breaking antioxidants namely butylated hydroxy anisole (BHA), tert butyl hydroxy quinone (TBHQ), and propyl gallate (PG). This chapter describes the application of FTIR spectroscopy to follow the main chemical events occurring during the oxidation of oils, as well as the effect of various antioxidants on retarding the oxidation of menhaden oil. A comparison of the capability of each antioxidant to retard autoxidation of menhaden oil was also assessed at various concentration levels. These antioxidants were added separately at concentration levels of 1%, 0.1% and 0.01% to menhaden oil. Menhaden oil was chosen due to the high unsaturated content of the oil which increases the oxidative instability of the oil. The

FTIR-based method monitors the change in the infrared bands which correspond to hydroperoxide and double bond formation as well as cis double bond depletion as a function of time.

MATERIALS AND METHOD

INSTRUMENTATION

A Nicolet 8210 FTIR spectrometer (Nicolet Instrument Corp. Madison, WI) shown in Figure 3.3 was used for this study. The spectrometer operated under a Nicolet DX operating system. To minimize the interference of water vapor and CO_2, the instrument was purged with a continuous flow of dry air from a Balston dryer (Balston, Lexington, MA). A 45 ° ZnSe ATR horizontal accessory (Figure 3.4) was heated to 65°C using an Omega rheostat. All spectra were collected using 256 scans with an initial 512 scans collected for the background emmitance spectrum. A resolution of 4 cm^{-1} with a gain of 2 was used.

To monitor the efficiency of the antioxidants over time, the Nicolet spectrometer was programmed in macro-command language (ref.28) to automatically record and save spectra at specified time intervals for subsequent analysis.

Figure 3.3 A Nicolet 8210 FTIR Spectrometer

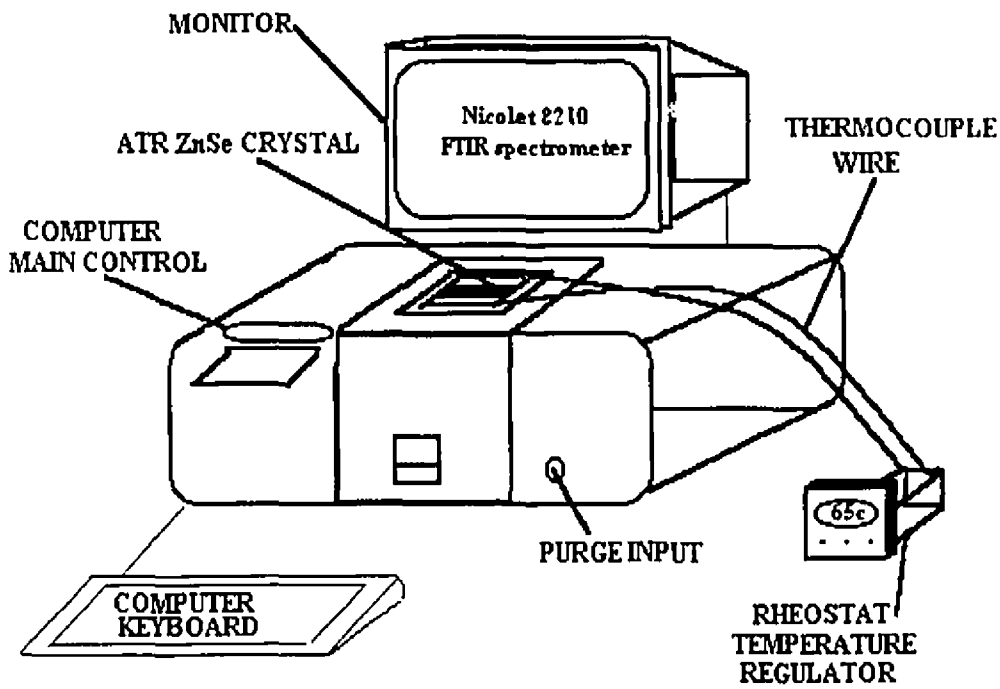
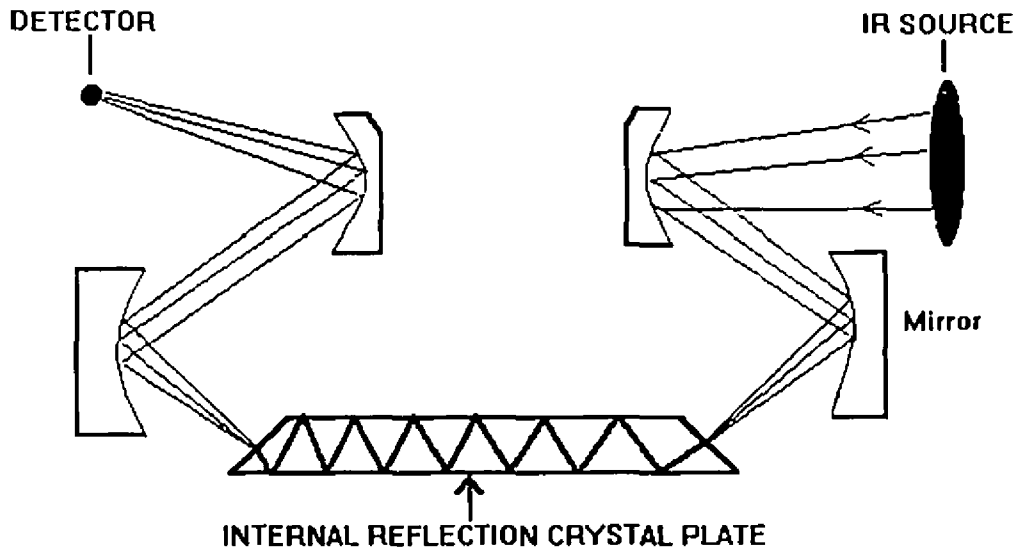


Figure 3.4 A 45° ZnSe ATR Horizontal Plate



A Visual-Basic program was written to measure peak heights at specified wave numbers as a function of time for each antioxidant. The program is shown in Appendix A.

Assessment Trials of Antioxidant Efficiency

Menhaden fish oil was provided by Zephta Inc. and used as the base oil for the antioxidant studies. Three synthetic antioxidants (BHA, TBHQ, PG) were supplied by Eastman Chemical Company (Rochester, N.Y.) and added to menhaden oil at concentrations of 1.00%, 0.1% and 0.01%. In a first oxidation study, menhaden oil was heated at 65 ^{o}C for approximately 28 hours on an ATR accessory in the absence of antioxidant. In subsequent studies, antioxidants were added at concentrations of 0.01%, 0.1% and 1.00% to menhaden oil. Approximately 250 µl of oil was placed onto a 65 ^{o}C ATR plate (~ 8 cm^{2}) and the FTIR spectrum was recorded at 40 minute time intervals and saved for further spectral analysis. All samples were left to oxidize on the ATR plate over several days and the single beam spectra of each sample were automatically ratioed against the spectrum of fresh Menhaden oil recorded at t = 0 (for the various antioxidant concentrations).

RESULTS AND DISCUSSION

SPECTRAL RATIOING :

Due to the capability of the FTIR spectroscopic method to accurately ratio one spectrum against another, spectral peak d[illegible]ion can be made from small and normally undetectable differences not apparent in a raw spectrum. The ratioing of one spectrum onto another is feasible because most FTIR spectrometers are single-beam instruments allowing for the acquisition of single-beam spectra. A single-beam spectrum recorded for a sample (Is) consists of the emmitance spectrum of the source on which are superimposed both the

absorptions of the sample and air in the optical path (Fig.3.5). Digital ratioing of the single-beam spectrum of the sample plus source and air against a single-beam spectrum of only the source plus air (i.e., background spectrum = Io), allows one to obtain the absorbance spectrum (Fig. 3.6) of the sample in absorbance units {Abs.(sample) = -Log (Is/Io)}(ref.29).

FIGURE 3.5 Single-Beam spectrum of fresh menhaden oil.

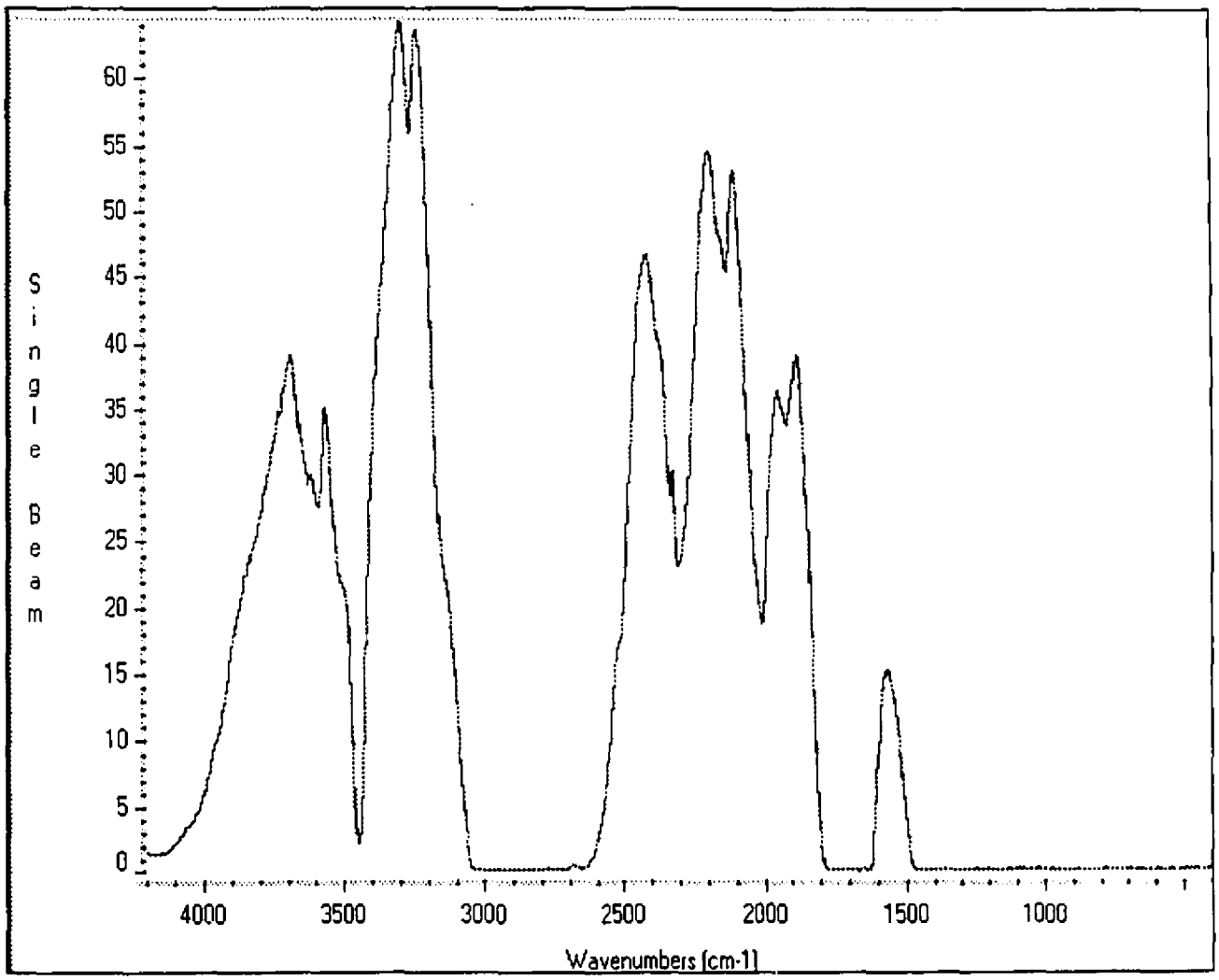
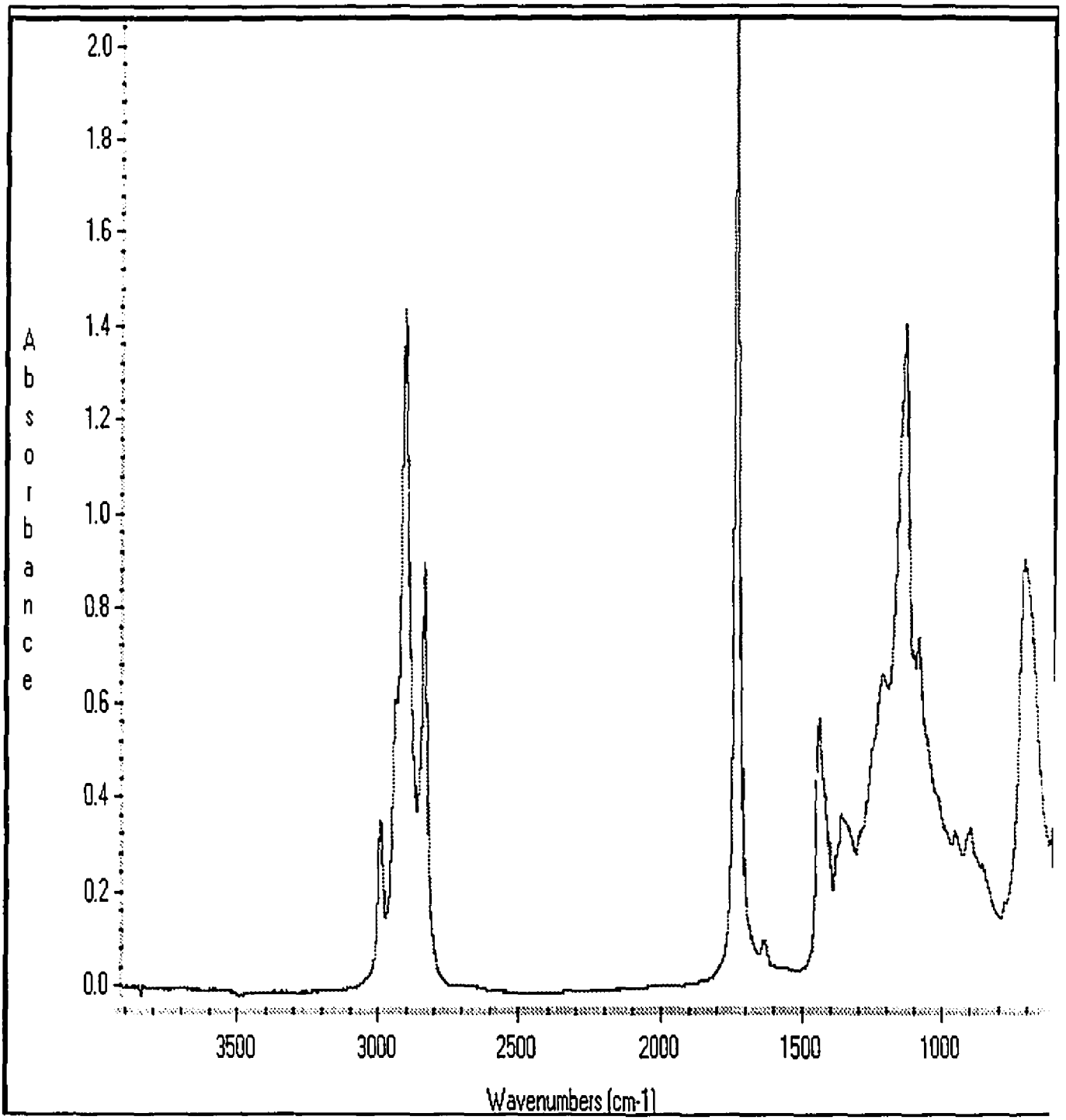


Figure 3.6 Absorbance spectrum of non-oxidized Menhaden oil.



MENHADEN OIL OXIDATION:

Figure 3.6 and 3.7a illustrates the infrared spectrum obtained from menhaden oil in its neat form on a ATR horizontal crystal and heated at 65 ^{o}C. The infrared spectrum was obtained by ratioing the single-beam spectrum of the oil against that of air, and illustrates all of the dominant infrared bands of menhaden oil. The primary peaks are those of the CH stretching absorptions observed in the region from 3100 to 2800 cm $^{-1}$ (i.e., CH_3, CH_2/ CH_3 and cis-C=CH stretching absorption), the carbonyl C=O absorption of the ester linkage at approximately 1745 cm^{-1} and the bands associated with the 1500-1000 cm $^{-1}$ fingerprint region. Figure 3.7b demonstrates the spectrum of neat menhaden oil oxidized in open air for approximately 350 minutes. The changes of band shapes and the presence of new peaks are not easily apparent. Figure 3.7c shows the spectrum of menhaden oil after 1400 minutes of oxidation on the heated ATR plate. This spectrum illustrates spectral band shifts and band width changes in the 3500 cm $^{-1}$ spectral region (i.e. -OOH or -OH stretch) although these changes in band spectra are difficult to discern.

Upon ratioing the single beam spectra of figure 3.7c or figure 3.7b against figure 3.7a, the spectral features of the oxidized menhaden oil became apparent as illustrated in figure 3.8 and figure 3.9 respectively. The spectral contributions of the oil itself was ratioed out (i.e., peaks in the 3700 - 3300 cm^{-1} region, negative peaks in the 3100 - 3000 cm^{-1} region and 1800 - 1700 cm^{-1} and a band at ~ 1000 -900cm^{-1} region) allowing for the differences between the two oil samples (i.e., neat and oxidized oil samples) to be seen more clearly. As the oxidation process proceeds over time, the spectral features of oxidation becomes more clear as illustrated in figures 3.8 and 3.9. In these figures, negative peaks are depicted in the CH stretching region and at the position of the triglyceride ester linkage (~ 1760 - 1740 cm^{-1}). In addition, these spectra show the appearance of a sharp rise in

positive peaks in the trans double bond 1000 - 900 cm^{-1} region and the appearance of a broad band in the OH stretching region (~3800 - 3200 cm^{-1}). All positive bands are attributed to the production of new molecular species. Whereas, the negative peaks are due to the ratioing of both the oxidized and neat menhaden oils but are not necessarily attributed to the loss of functional groups (ref.29). The negative peak at approximately 3010 cm^{-1} is attributed to the decrease in cis C=CH stretching conformation as the oil becomes oxidized over time. The band at ~965 cm^{-1} increases in height as the cis C=C-H decreases. The negative peak at the ~1745 cm^{-1} is attributed to oxygen incorporation into the menhaden oil as oxidation products are formed. This oxygen uptake in effect will dilute the sample relative to the reference and as a consequence, bands common to both the sample and reference oils do not ratio out perfectly, but instead will exhibit negative intensities in the ratioed spectrum (ref.29). Thus, the ratio technique is a very sensitive means of detecting changes occurring within a spectrum as long as a strong enough signal reaches the detector. If there is a strong absorption taking place in a particular region of a spectrum, not enough of the source rays will be detected and so this causes a randomization of signal detection and an increase in noise levels. The signal reaching the detector at the CH stretching 3100 - 3000 cm^{-1} region was too small to be sampled properly , leading to the randomized digitization noise in the ratioed spectrum as illustrated in Figure 3.8 at ~ 3000 - 2800 cm^{-1}.

With all these considerations, it will be useful to examine each of the major spectral regions chosen for monitoring the various antioxidants in some detail.

Figure 3.7 a, b, c: IR spectra of Menhaden oil oxidation over time.

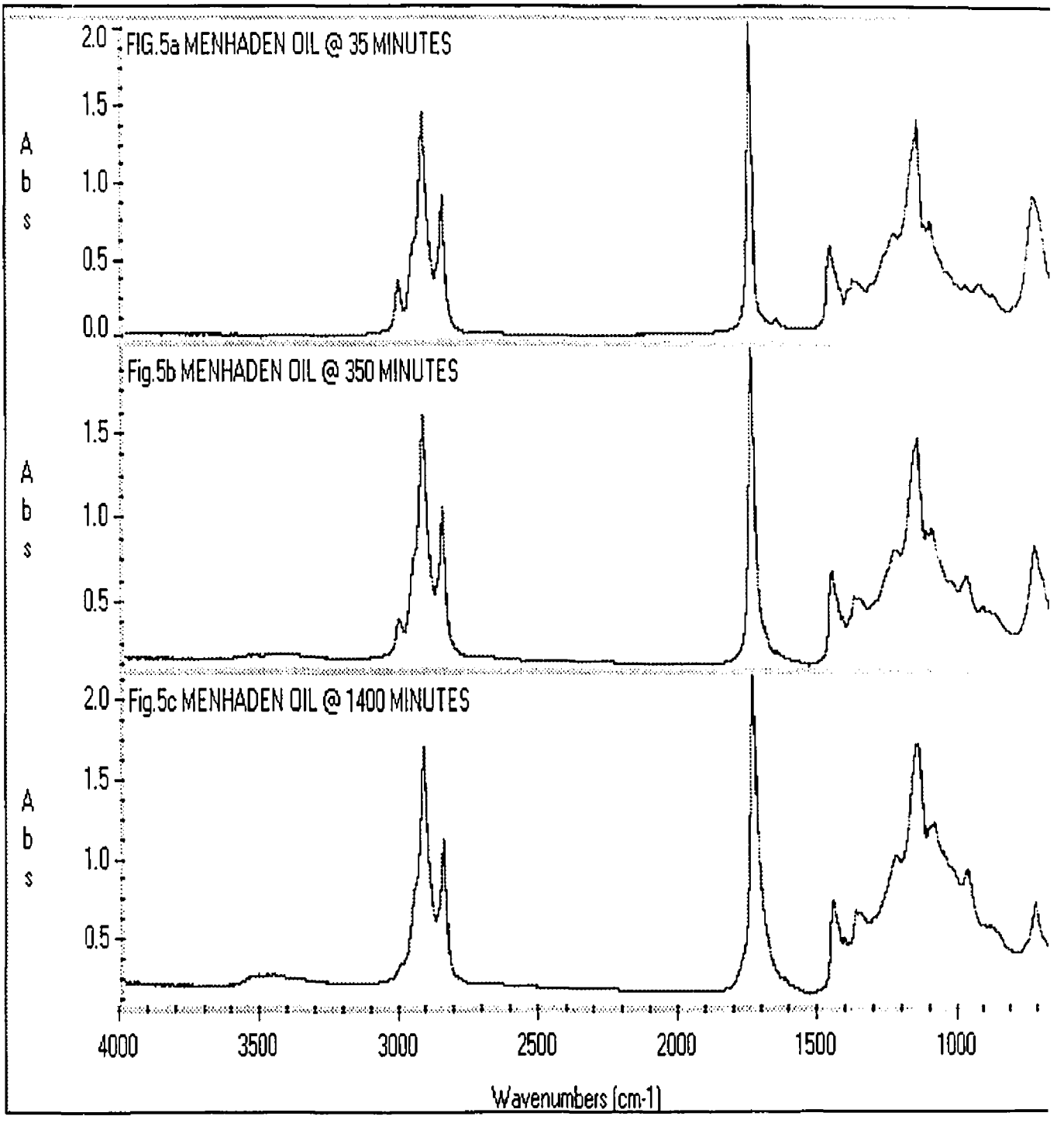
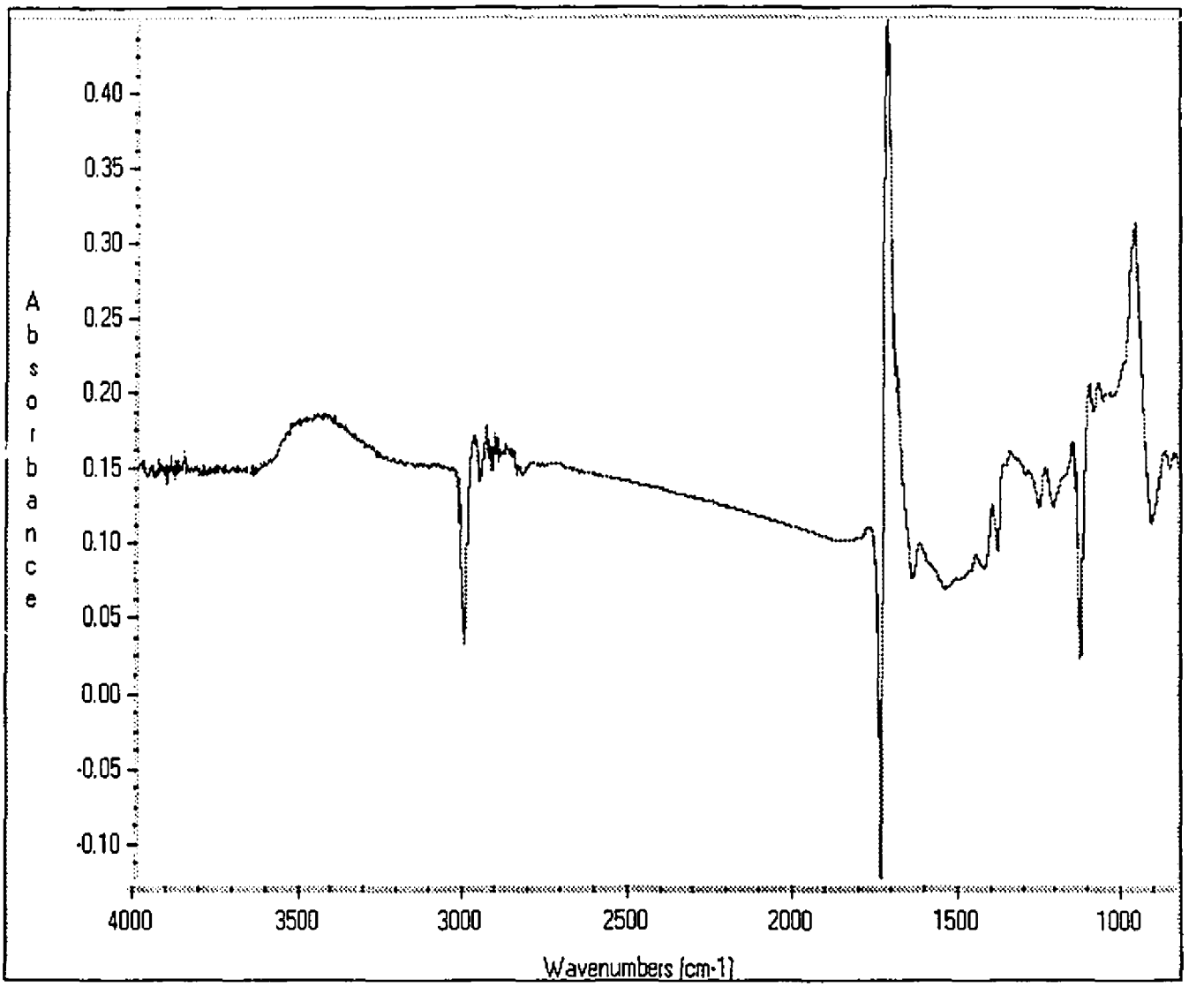
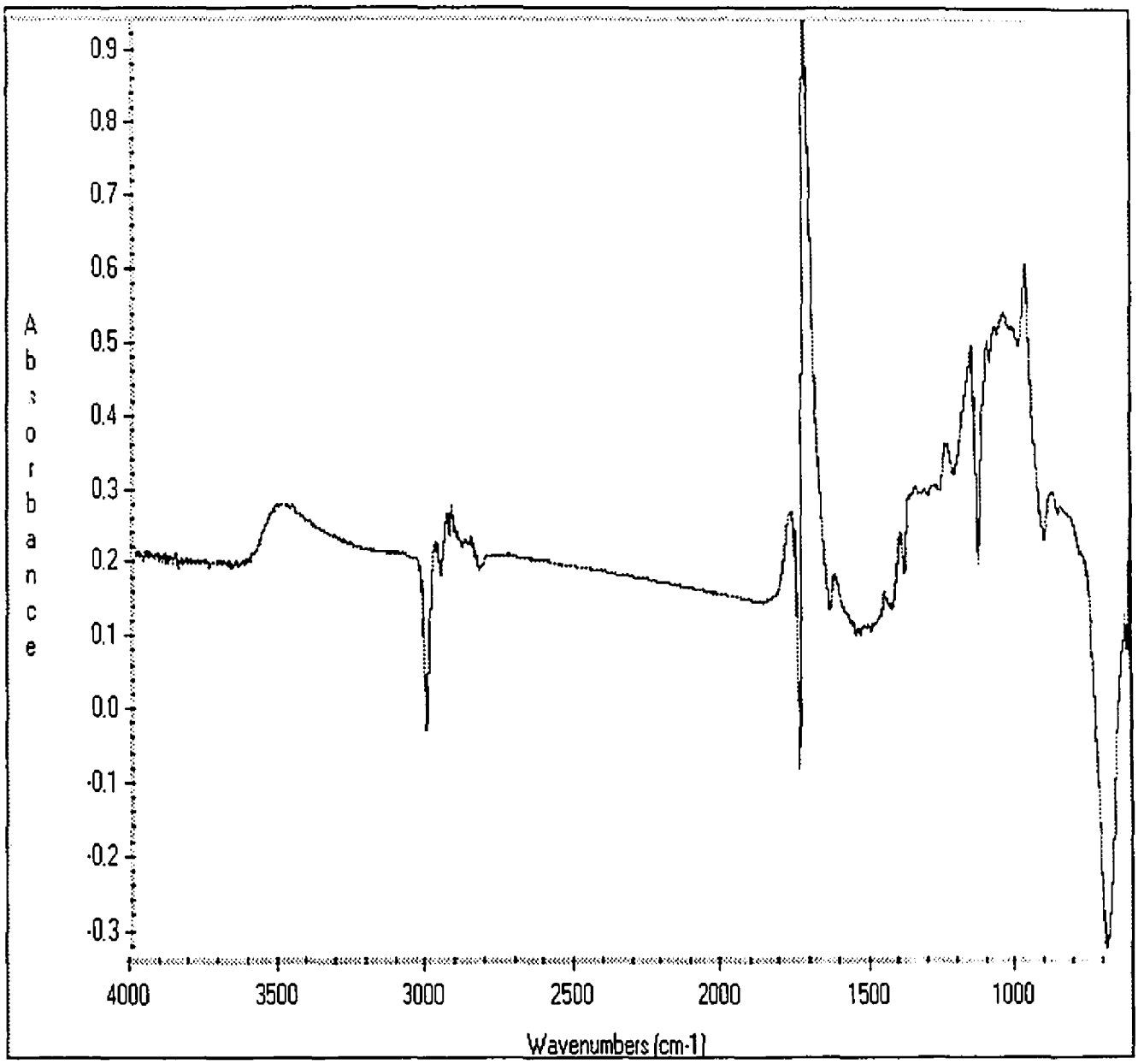


Figure 3.8: Difference Spectrum of Menhaden oil at 350 min.



NOTE: This is the difference spectrum obtained by subtracting non-oxidized menhaden oil at time = 0 minutes with oxidized menhaden oil at time = 350 minutes on ATR .

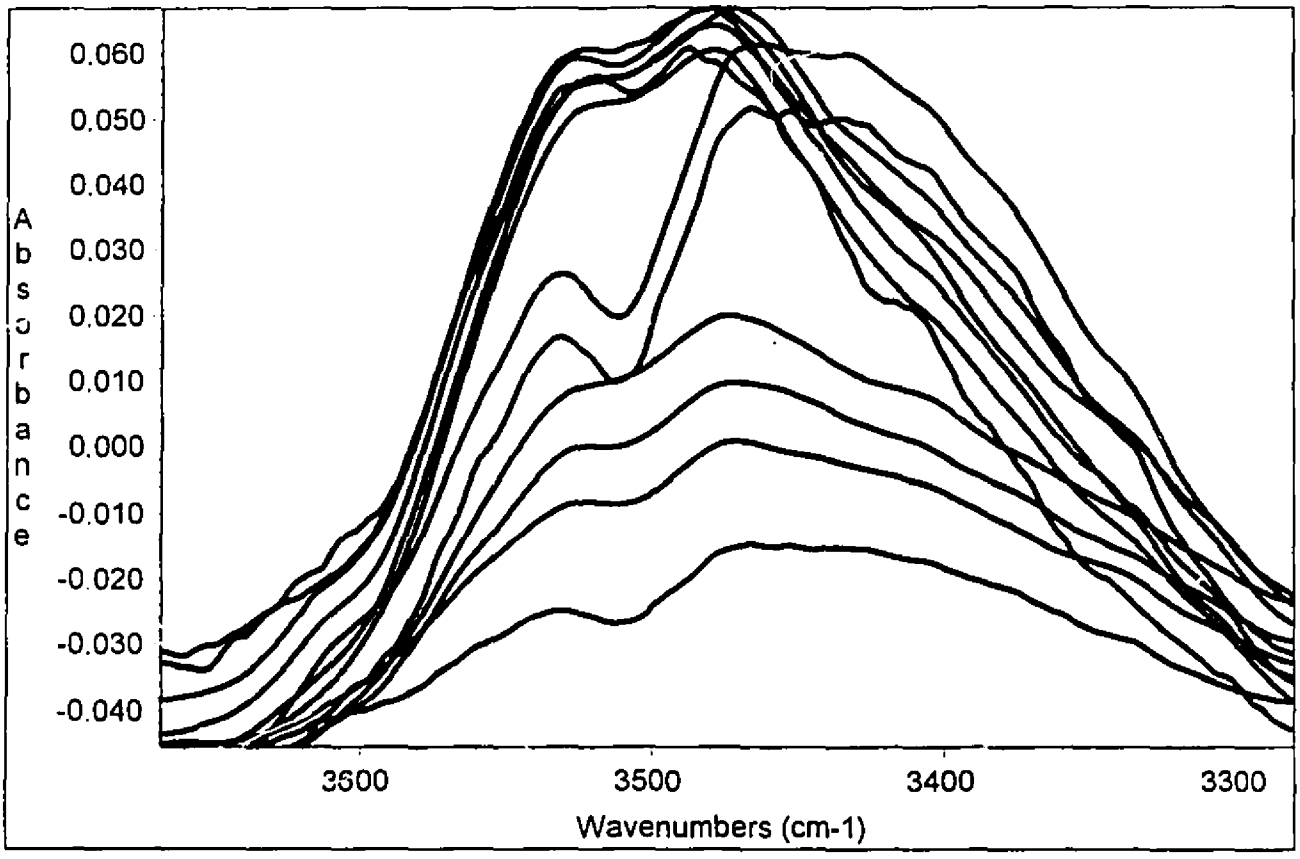
Figure 3.9: Difference spectrum of Menhaden oil at 1400 min.



NOTE: This is the difference spectrum obtained by subtracting non-oxidized menhaden oil at time = 0 by oxidized menhaden oil at time = 1400 minutes on ATR plate.

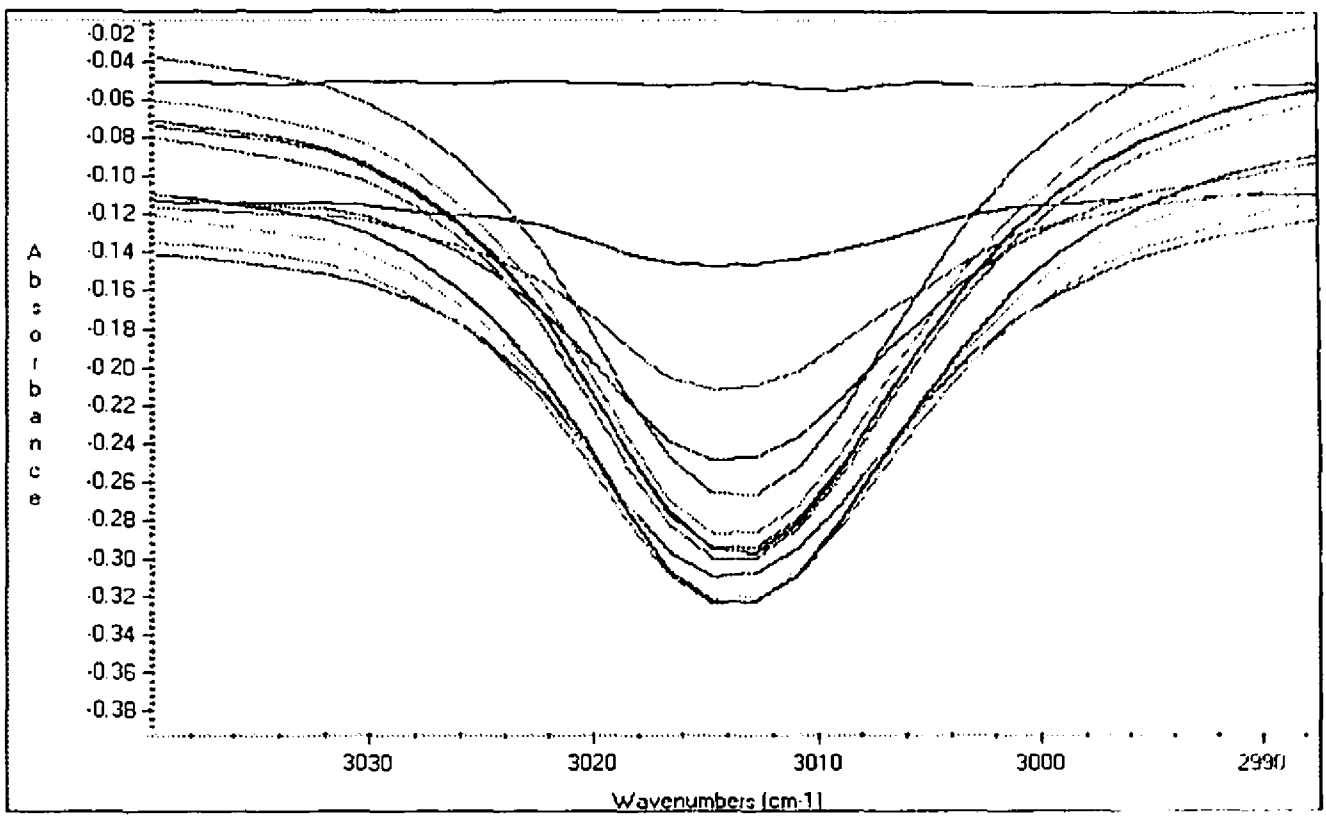
(1) ***The OH region:*** Figure 3.10 shows a stacked plot of infrared spectra between 3600-3200 cm^{-1} of menhaden oil undergoing autooxidation in the presence of 0.01% propyl gallate. The broad peak at approximately 3444 cm^{-1} is that of the O-H stretching vibration of the hydroperoxides. There is no indication of alcohol formation or any release of free fatty acids as illustrated by the symmetrical increase in 3444 cm^{-1} band. The noise level associated with each individual peak had been digitally smoothed by use of the Omnic software (Nicolet Instruments Corp.). This is one of the primary regions chosen for the study of antioxidant efficacy as it provides qualitative and quantitative infrared analysis of primary oxidation products (ref. 29 - 34).

Figure 3.10 HYDROPEROXIDE PEAK FORMATION.



(2) ***The C-H cis peak region:*** Figure 3.11 illustrates the changes taking place in the C-H cis double bond region in the infrared spectrum of menhaden oil in the presence of 0.01% propyl gallate. Initially, no change in intensity of the C-H stretch at 3008 cm^{-1} is seen due to the antioxidant effect of PG. This is then followed by a sudden decrease in peak height of the ~3008 cm^{-1} band. The sudden decrease in the cis C-H conformation in the lipid molecules is accompanied by a simultaneous increase in trans C-H as shown in figure 3.12

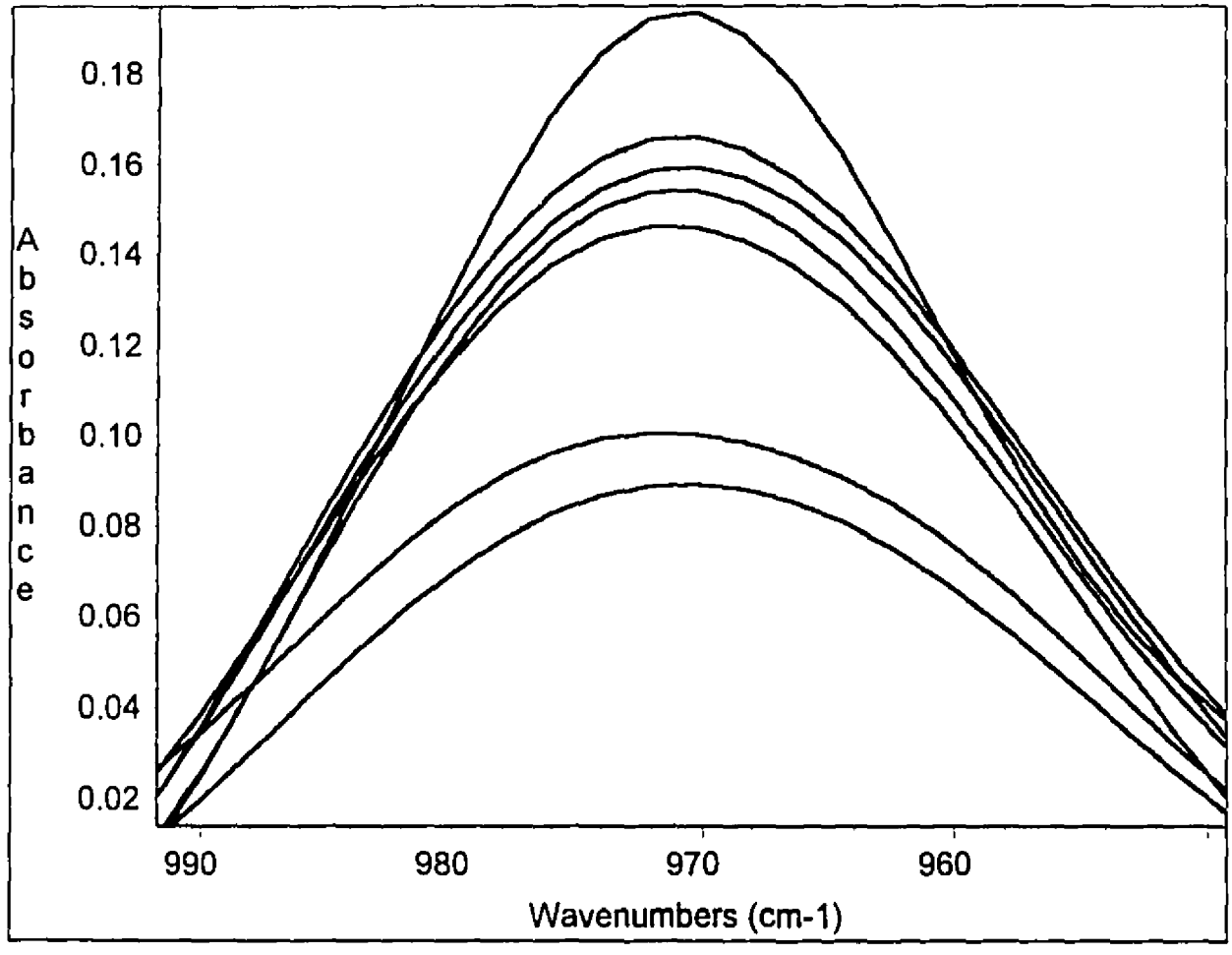
Figure 3.11 Cis depletion at ~ 3008 cm^{-1} of menhaden oil oxidation



NOTE: These are difference spectra of menhaden oil oxidation over time minus first non-oxidized menhaden oil.

(3) ***The trans C-H peak region***: Figure 3.12 illustrates the changes occurring in the ~971 cm^{-1} trans band of the infrared spectrum attributed to the trans C=C-H stretch as the menhaden oil oxidizes. A resistance to oxidation by the oil in the presence of PG is noted initially with a subsequent increase in peak height at 971 cm^{-1} due to the isolated trans formation induced by lipid oxidation.

Figure 3.12 Trans C-H formation at ~ 971 cm^{-1} for Menhaden oil oxidation.



NOTE: These are difference spectra of menhaden oil oxidized over time minus first non-oxidized spectrum.

INTERPRETATION OF REAL-TIME PLOTS OF OXIDATION :

(1) ***Butylated HydroxyAnisole (BHA)*** : Figure 3.13 depicts three plots of the increase in the band at 3444 cm^{-1} attributed to hydroperoxide formation at three different BHA concentrations in menhaden oil. At 1.00% BHA, the induction period was increased significantly in comparison to the other two lower concentrations of BHA. BHA at a concentration of 0.01% was noted to be least effective in prolonging the induction period since the rapid increase in hydroperoxide band formation was observed upon heating of menhaden oil. The induction period was approximately 10 hr for 0.1% BHA as compared to 35 hr for the 1.00% and ~ 2 hr for 0.01% BHA. Hence, at 1.00% BHA this concentration of antioxidant was most effective in menhaden oil as demonstrated by the significant increase in induction period.

Figure 3.14 depicts the effect of BHA at three different concentrations on the trans formation. At 0.01% BHA, a rapid increase in the absorbance of the trans band is observed. The peak height of the trans band for the 0.01% BHA is noted to plateau at approximately 30 hours oxidation and then descends slowly around 10 hours after the initial plateau effect. The descending curve appears to be due to the appearance of secondary oxidation products at the expense of the trans moieties. At 1.00% BHA, the trans band showed an increase in sigmoidal fashion whereby there was an initial increase in the induction period and a diminishment in trans formation until a final plateau was reached at approximately 60 hrs from the time of initial oxidation. At approximately 70-75 hr of oxidation, the 1.00% BHA trans curve demonstrated an apparent decrease. It is notable that all three oxidation curves showed a plateau effect in terms of trans formation. After several hours the trans content decreased as demonstrated by the fall in the trans peak. It should be noted that the time at which the plateau in peak height occurred was dependent on the concentration level of the antioxidant.

Figure 3.15 shows the impact of the oxidation of the oil on the cis double bond moiety. At 1.00%, the BHA had the longest induction with respect to a decrease in peak height of the cis-double bond at 3011 cm^{-1}. Depletion of the cis-double bond content was most noticeable for the 0.01% BHA in menhaden oil in comparison to the other two concentrations and the 1.00% BHA antioxidant system showed the best protection against oil oxidation. As demonstrated for trans formation, cis depletion leveled over time for each antioxidant concentration.

Figure 3.16 shows the increase in the trans peak at 971 cm^{-1} accompanied by the simultaneous decrease in cis at 3008 cm^{-1} for the 1.00% BHA concentration over an 80 hour time period. Both the increase of trans peaks and the decrease of the cis peaks occurred almost at equivalent points in time. Such a phenomenon would be expected during oxidation of lipids since unsaturated lipids are predominantly in the cis configuration which is converted to trans during oxidation. Finally, the formation of oxidation products was slowed by the addition of antioxidants particularly at the concentration of 1.00% as depicted in Figures 3.16 and 3.17. There was a noticeable decrease in time for the initiation of trans formation and cis depletion with the lower antioxidant concentration (Fig.3.17) as compared to higher levels of antioxidant (Fig.3.16). These figures also show that a plateau effect occurred at approximately 18-20 hrs of oxidation suggesting an equilibrium between trans formation and trans breakdown.

Figure 3.13 BHA added to Menhaden Oil at 65^0 C on ATR ZnSe Crystal.

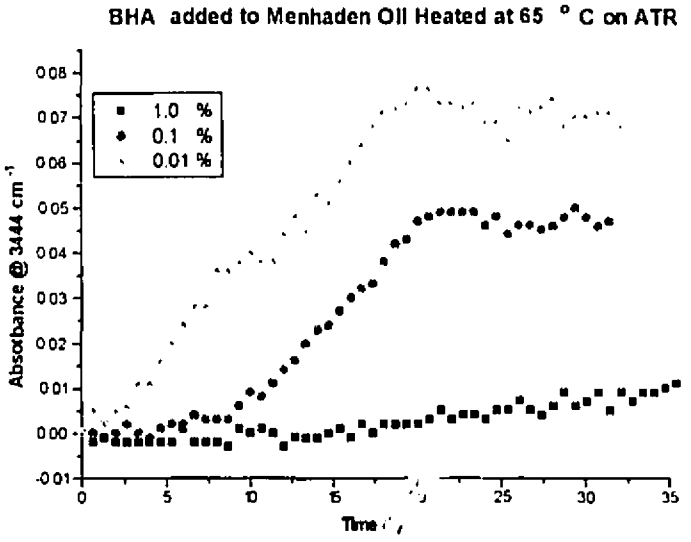


Figure 3.14 BHA added to Menhaden Oil at 65^0 C on ATR ZnSe crystal.

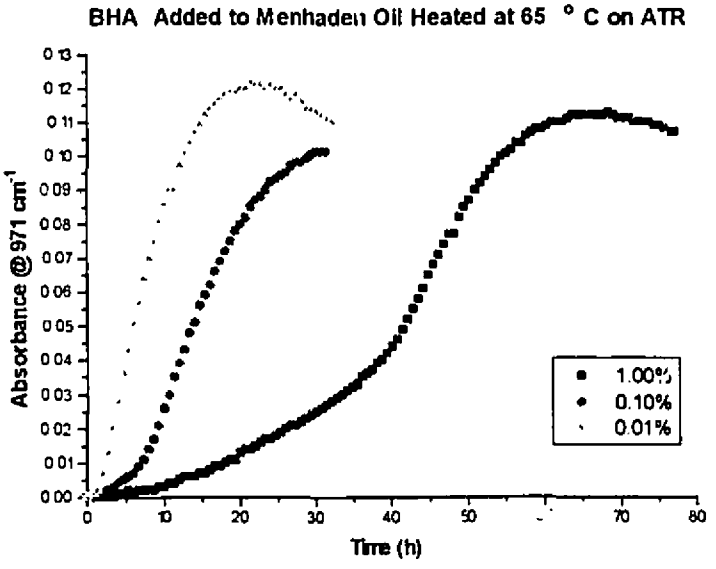


Figure 3.15 BHA added to Menhaden Oil at 65° C on ATR ZnSe crystal.

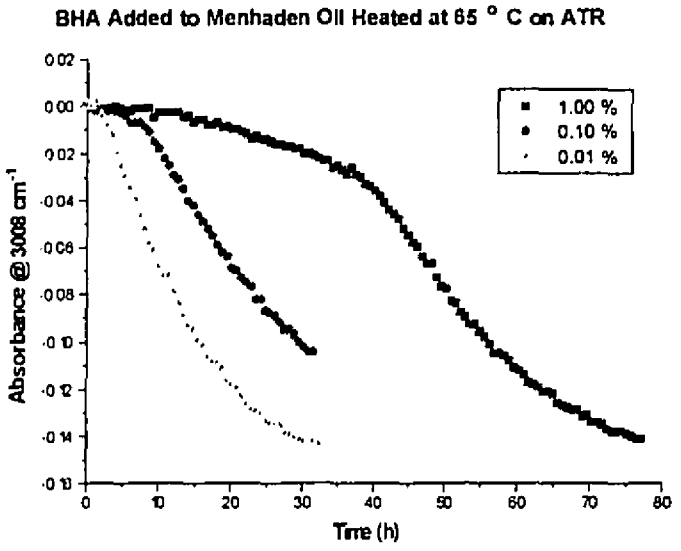


Figure 3.16 1.00% BHA added to Menhaden Oil at 65° C on ATR ZnSe crystal.

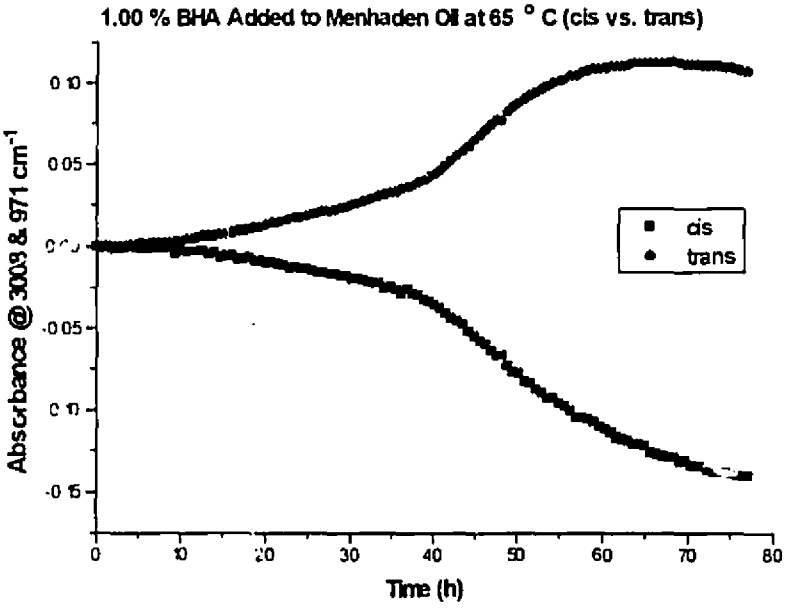
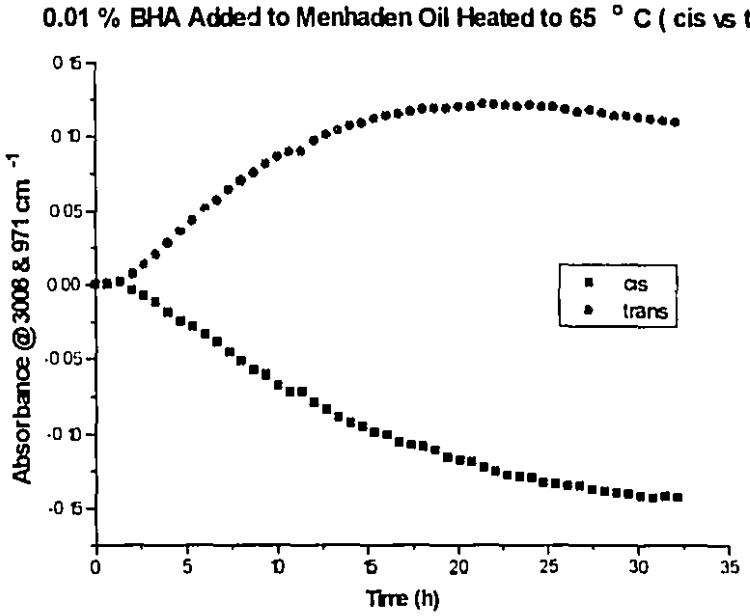


Figure 3.17 0.01% BHA added to Menhaden Oil on 65° C ATR ZnSe crystal.



(2) *PROPYL GALLATE (PG)* : Figure 3.18 depicts the effect of two different concentrations of PG on hydroperoxide formation. At 1.00%, PG caused a complete resistance to hydroperoxide formation over the first 50 hours of oxidation. On the other hand, 0.01%, PG appeared to cause a slight increase in the induction period followed by a sudden increase which reached a plateau of 0.08 absorbance at approximately 28 hrs of oxidation Figure 3.19 illustrates the effect of 1.00% PG on trans formation compared to 0.01% PG added to the menhaden oil. The rapid decrease in trans formation is likely due to degradation of the trans double bond leading to the formation of secondary oxidation products.

Figure 3.20 demonstrates the strong resistance to loss of cis-double bond in the presence of 1.00% concentration PG for the complete time interval of the oil oxidation experiment. Whereas cis depletion was noted after five hrs of oil oxidation when 0.01% PG was added. Comparing the effect of both the 0.01% and the 1.00% PG effects, the 1.00% PG

was shown to be very effective in increasing the induction period, in comparison with 0.01% PG. Figure 3.21 demonstrates the inverse relationship between the cis and trans peaks. The level of antioxidant is noted to be fairly low (0.01% PG). The trans curve showed a decrease after the plateau effect at approximately 20 - 25 hrs. of oxidation probably due to the formation of secondary products and a decrease in trans formation. Figure 3.22 depicts the significant effect that occurs in menhaden oil oxidation over 80 hrs when 1.00 % PG was added.

Figure 3.18 Propyl Gallate added to Menhaden Oil at 65° C on ATR ZnSe crystal.

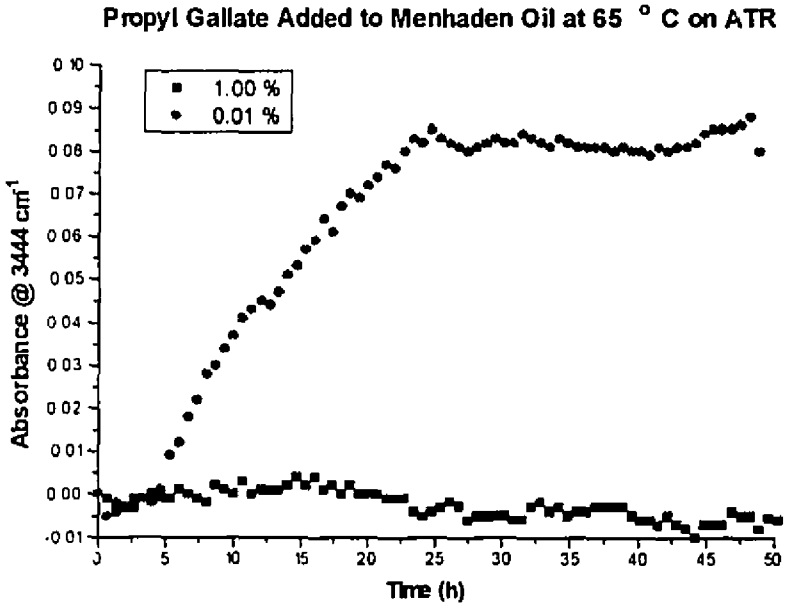


Figure 3.19 Propyl Gallate added to Menhaden oil at 65° C on ATR ZnSe crystal.

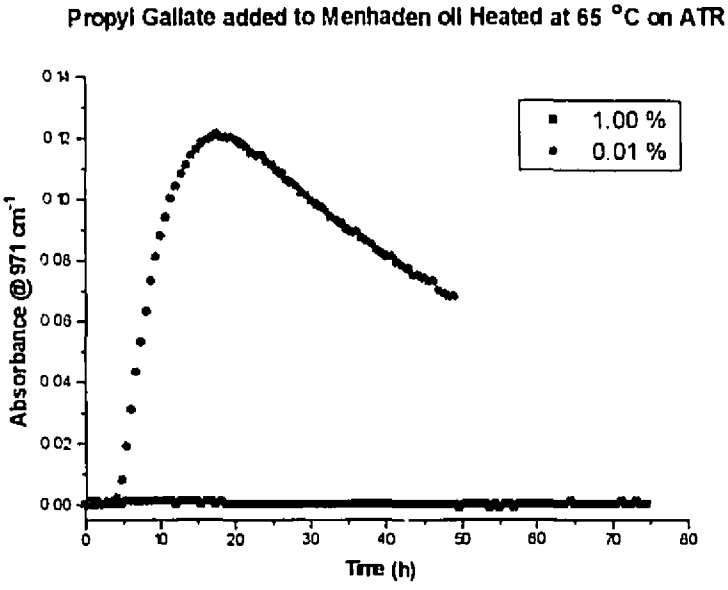


Figure 3.20 Propyl Gallate added to Menhaden Oil at 65° C on ATR ZnSe crystal.

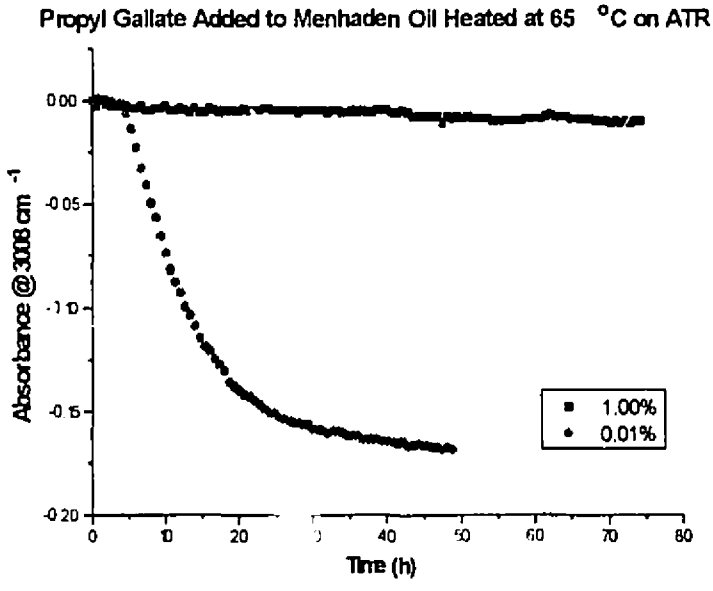


Figure 3.21 0.01% Propyl Gallate added to Menhaden Oil heated to 65°C on ATR.

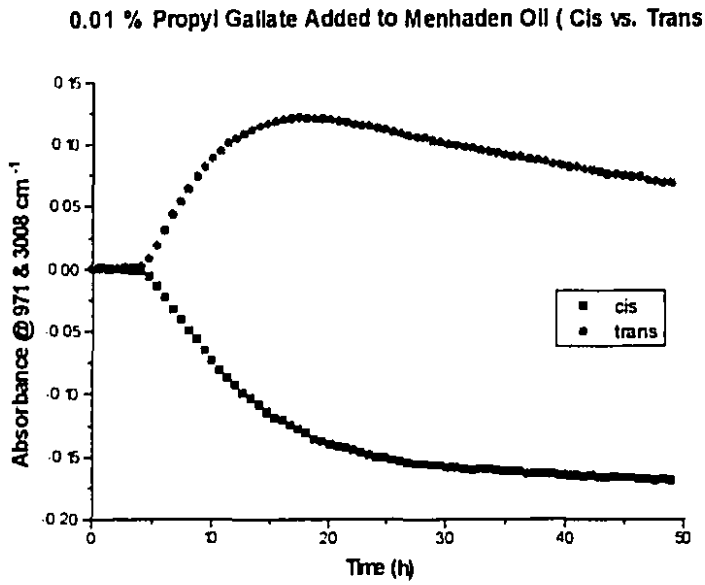
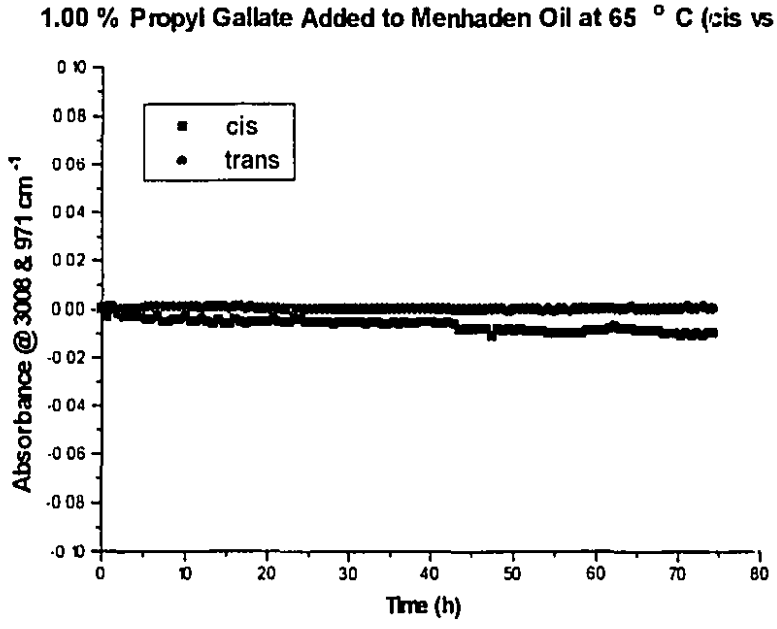


Figure 3.22 1.00% Propyl Gallate added to Menhaden Oil heated at 65° C on ATR.



(3) ***Tert-Butyl Hydroquinone (TBHQ)*** : Figure 3.23 shows the effect of TBHQ at increasing concentration on hydroperoxide formation in menhaden oil undergoing autooxidation. TBHQ at the highest concentration level of 1.00% examined causes the greatest resistance to oxidation as evidenced by an increase in induction period. At 0.01%, TBHQ was least effective with an almost immediate increase in hydroperoxide formation whereas the 0.1% exerted an intermediate effect. Figure 3.24 illustrates the formation of the trans double bond as a function of time for over 110 hrs. in open air oxidation. As was observed with the other antioxidants, the 0.1% TBHQ appeared to plateau for a brief time period and then showed a decrease in trans peak formation. Figure 3.25 shows the effect of TBHQ at three different concentrations on the absorbance of the cis double bond band. As expected, there was an immediate decrease in peak height in the presence of 0.01% TBHQ antioxidant added, while at 1.00% TBHQ a significant increase in the induction period for the menhaden oil oxidation was observed. Although the 0.1% level increased the induction period to approximately 15-20 hours, this concentration was not as effective as the 1.00% TBHQ level.

Through the application of infrared spectroscopy, the efficacy of three different antioxidants, at three different antioxidant concentrations was determined.Increasing the concentration of the antioxidant , resulted in a significant increase in the induction period. Furthermore, there appeared to be no pro-oxidant effect for all three antioxidants upto the 1.00% concentration levels. Although government regulations allow for a maximal amount of 0.02% antioxidant per kg of oil or fat containing food.The efficiency of the regulated maximum level of 0.02% antioxidant addition was demonstrated to be poor.

Figure 3.23 TBHQ added to Menhaden Oil at 65° C on ATR crystal.

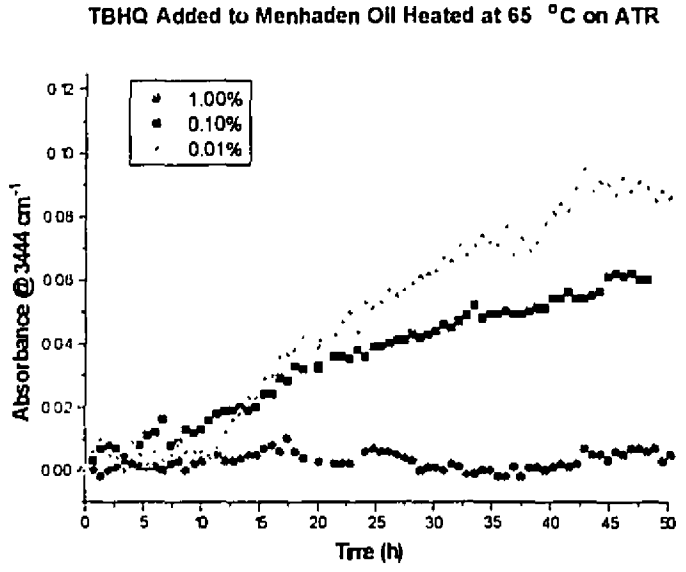


Figure 3.24 TBHQ added to Menhaden oil at 65° C on ATR crystal.

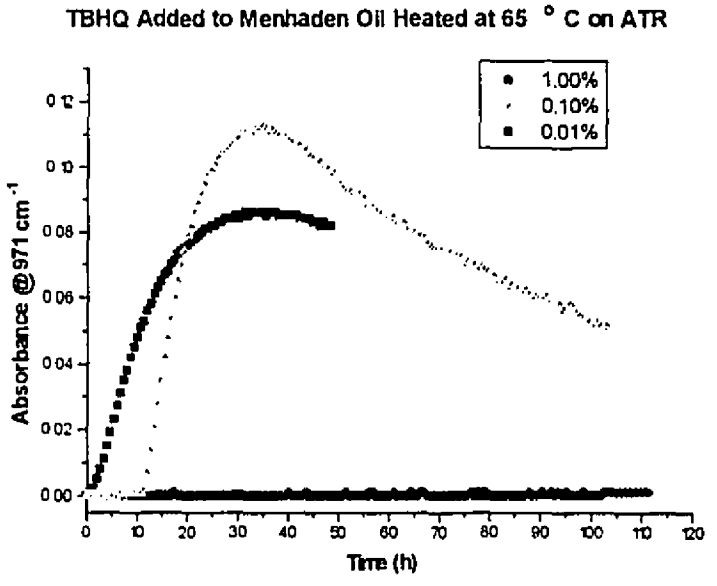


Figure 3.25 TBHQ added to Menhaden oil at 65° C on ATR crystal.

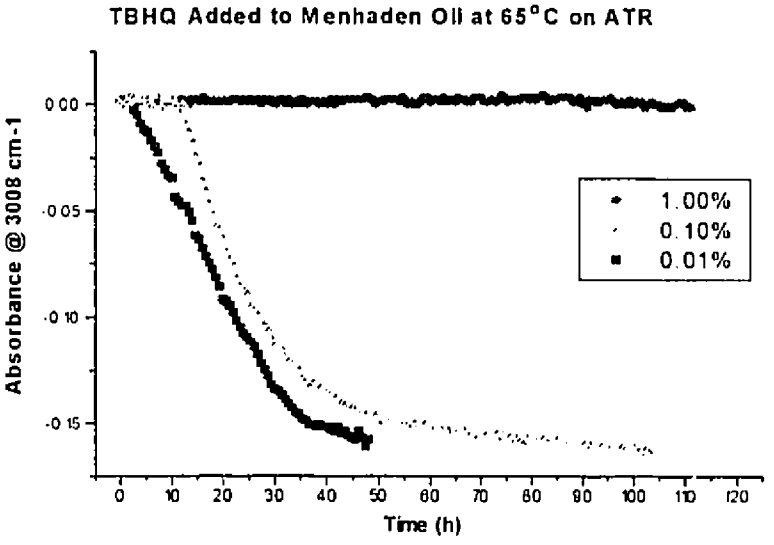
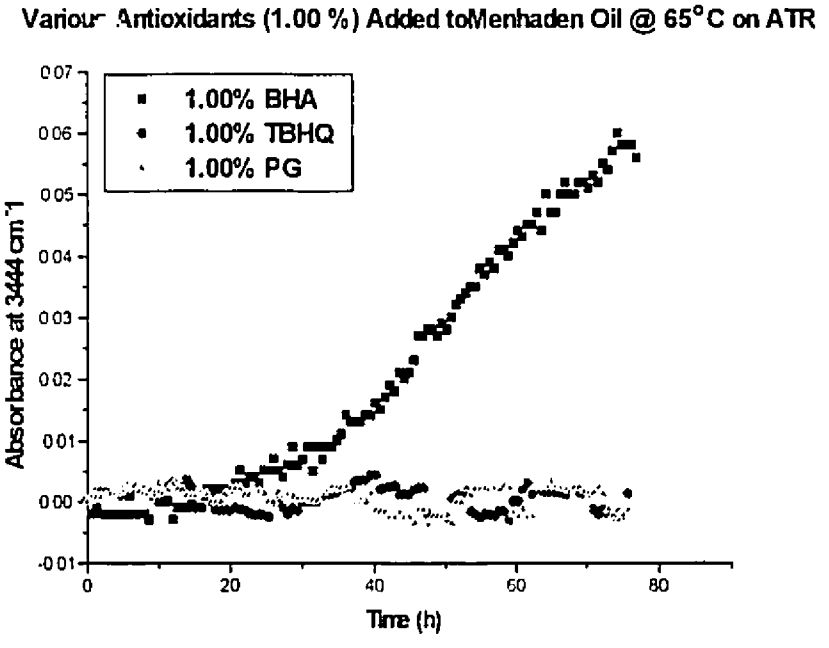


Figure 3.26 Various Antioxidants (1.00%) added to Menhaden oil at 65°C on ATR crystal.



(4) *Comparison of the three antioxidant efficiencies* :

Figure 3.26 illustrates the effect of the three different antioxidant types at 1.00% concentration on lipid oxidation at 65°C in menhaden oil. Hydroperoxide (-OOH) formation was followed by the increase in the IR band at ~ 3444 cm^{-1} over approximately 80 hrs. All three antioxidants induced a substantial increase in the induction period of oxidation. After approximately 35-40 hrs of oxidation, in the presence of 1.00% BHA an increase in hydroperoxide formation was observed. There are likely two explanations for this decrease in effectiveness of BHA. Firstly, BHA is known to be fairly volatile at elevated temperatures above ~ 50 - 60°C. The oil was heated to 65°C on a horizontal ATR plate exposed to the air with a small amount of oil added to the plate (i.e., this allowed for a large oxygen distribution and a faster and more efficient heating effect). Thus, BHA may have partially evaporated out of the oil after the initial 35 hour oxidation period thereby decreasing its effectiveness. On the other hand, propyl gallate and TBHQ resisted oxidation during the 80 hour period. Secondly, the difference in the three antioxidant efficiencies could also be due to the chemical structures of the three antioxidants (Fig. 3.27). The ability of primary antioxidant compounds to act as oxidation inhibitors is dependent on their phenolic configuration within their molecular structure. As seen in Figure 3.28, the primary antioxidant or phenolic substance functions as a free radical acceptor, thereby terminating the oxidation at the initiation step (ref.35). The free radical product formed is resonant stabilized and so will not propagate oxidation. The hydroxyl group plays a crucial role in inhibiting free radical oxidation as hydroxyl moieties are attached to the phenolic structures (Fig.3.27). Thus, propyl gallate which consists of three hydroxyl groups provides a higher antioxidant potency as indicated by its potent antioxidative effect in menhaden oil (Fig. 3.26). Butylated hydroxyanisole (BHA) is a mixture of 2- and 3- isomers of tertiary butyl-4-methoxy phenol (Fig. 3.27). The tertiary butyl group either ortho or meta to the hydroxyl group causes steric hindrance which may

also be partially responsible for the relative ineffectiveness of BHA in menhaden oil. The tertiary butyl group can interfere with the antioxidant activity of the phenolic structure by decreasing the availability of the phenolic hydrogen proton to free radical species.

As demonstrated in Figure 3.26, TBHQ is a highly effective antioxidant as depicted by the large increase in induction period of menhaden oil. As TBHQ contains two ortho hydroxyl groups (Fig. 3.27), these -OH moieties permit the compound to act as a very effective antioxidant.

Figures 3.29 and 3.30 illustrate the oxidation of menhaden oil and canola oil without added antioxidant by measuring hydroperoxide peak height as a function of time. It is evident from these plots, that menhaden oil oxidizes at a faster rate than canola oil as shown by the immediate increase in hydroperoxide peak height in the menhaden oil. In contrast, the canola oil demonstrated a resistance to oxidation as shown by its large induction period. It is likely that menhaden oil oxidizes more rapidly due to the higher polyunsaturated fatty acid composition of the fish oil triglyceride lipid structures. These plots also demonstrate the case by which FTIR spectroscopy can be employed to track the oxidation of edible oils in general in addition to evaluating antioxidant efficiancy.

Figure 3.27 CHEMICAL STRUCTURES OF THE THREE ANTIOXIDANTS.

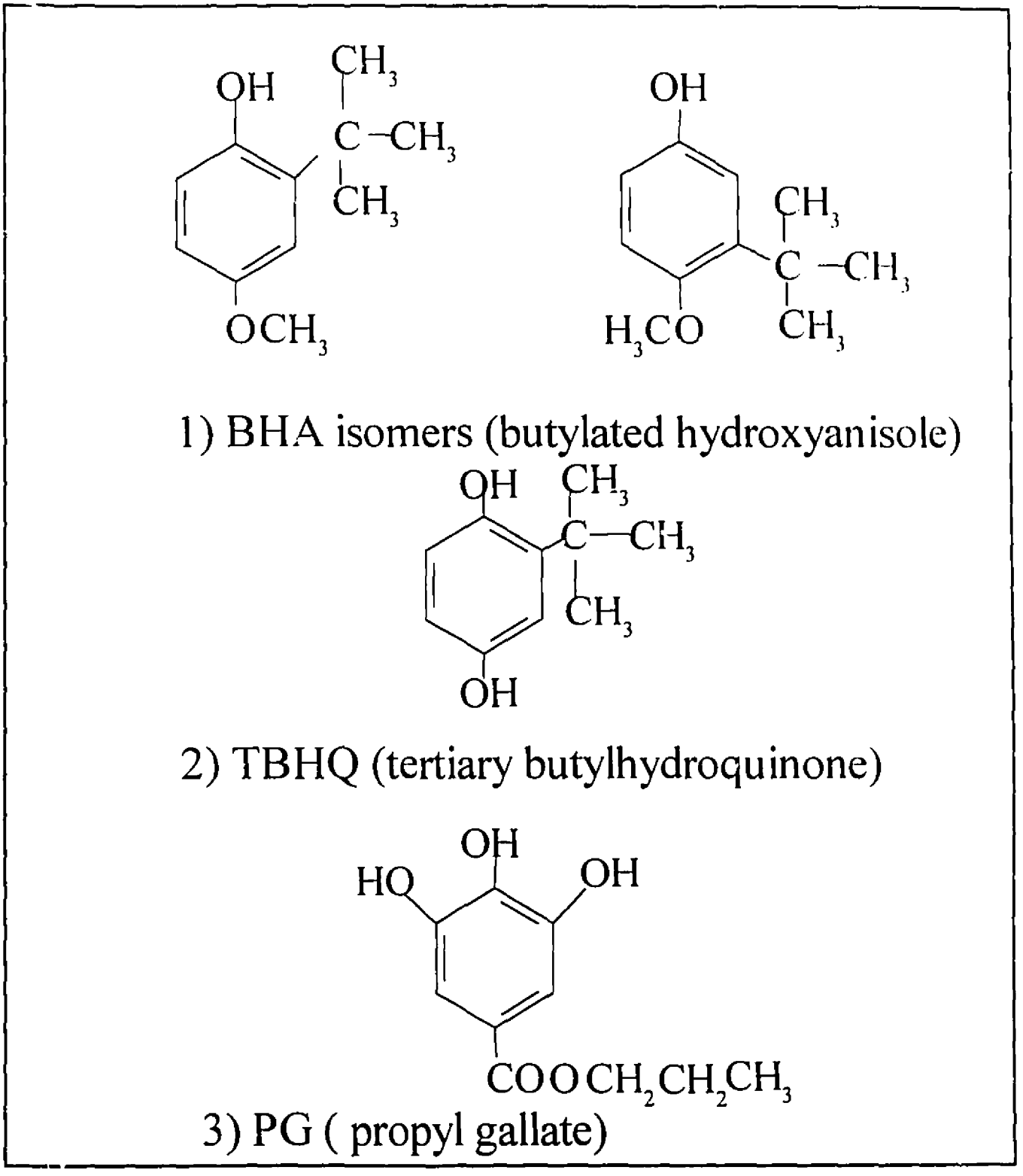
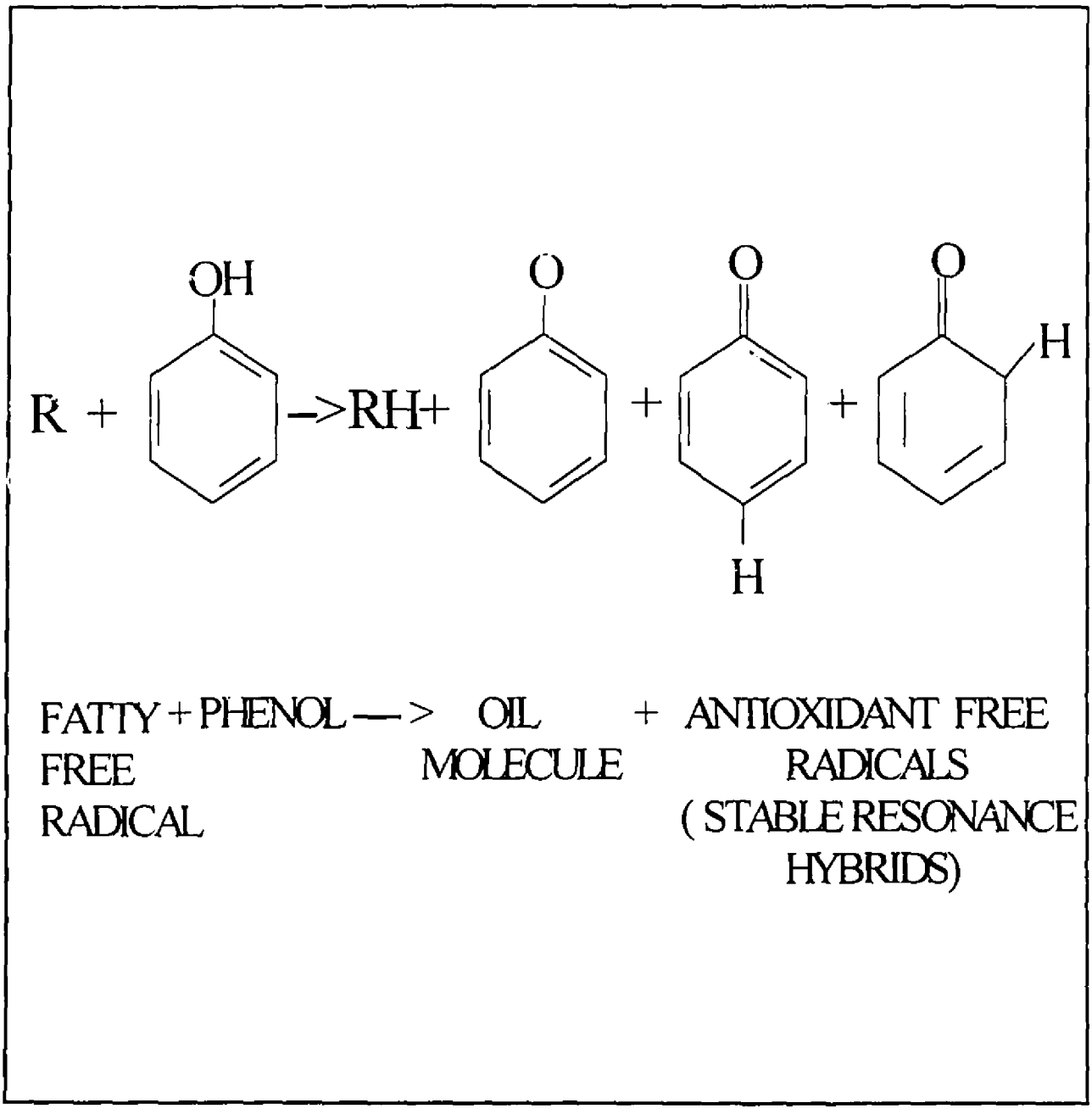


Figure 3.28 Functions of the three Antioxidants.



Note : The above figure demonstrates the phenolic antioxidant mechanism of the three antioxidants in fish or vegetable oils.

Figure 3.29 Menhaden oil oxidation at 65° C on ATR ZnSe crystal.

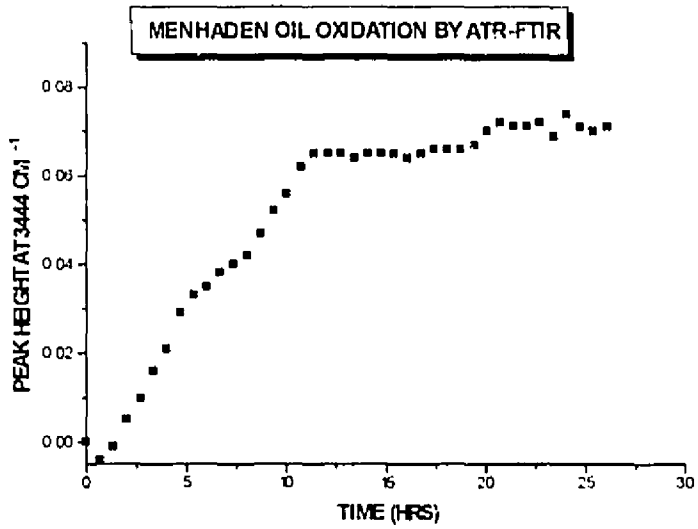
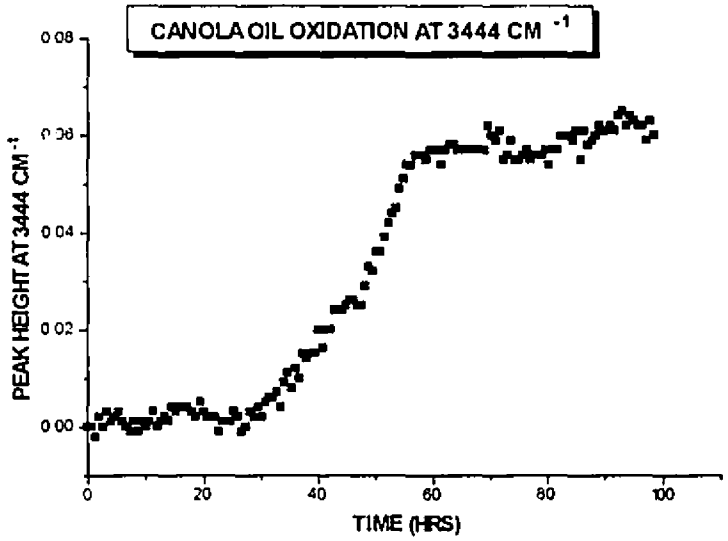


Figure 3.30 Canola oil oxidation at 65 o C on ATR ZnSe crystal.



CONCLUSION

The present study demonstrated the capability of FTIR spectroscopy to monitor the oil oxidation in the presence of varying levels of antioxidants in real time, by monitoring changes in band intensity of selected IR bands of the fresh and oxidized oil. Three antioxidants (BHA, TBHQ and PG), were compared in terms of their efficacy in prolonging the induction period. The greatest increase in the induction period was proportional to the increase in the concentration of the antioxidant. This study demonstrated the capability of FTIR spectroscopy to measure the efficacy of antioxidants in relation to their structure, demonstrating that TBHQ and PG to be more effective than BHA at prolonging the induction period at the same concentration levels. In comparison to other methods used to measure antioxidant efficiency such as the active oxygen method (AOM) or the rancimat test, FTIR spectroscopy has a number of advantage, including: greater ease of use; real-time monitoring capability and the elimination of the need for labourious wet chemical methods (for hydroperoxide determination), and chromatographic separation methods (for separation of cis and trans products).

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CHAPTER IV

A COMPARATIVE STUDY BETWEEN FOURIER TRANSFORM INFRARED SPECTROSCOPY AND TWO STANDARD METHODS [(1) THE IODOMETRIC CHEMICAL AND (2) HEMOGLOBIN-METHYLENE BLUE ASSAY] FOR HYDROPEROXIDE DETERMINATION

ABSTRACT

A Fourier Transform Infrared (FTIR) spectroscopy based method for the determination of the hydroperoxide content of sunflower oil was developed. The FTIR method was compared to both the standard iodometric chemical method and enzymatic hemoglobin-methylene blue assay for peroxide value (PV) determination. The PV values predicted by FTIR spectroscopy were within 3% of chemical and enzyme PV values. The FTIR method based on the correlation of the peak height at 3444 cm^{-1} and the PV value determined from the chemical method had a R= 0.999 with SD= 0.001. A plot of PV determined by the enzymatic method versus the peak height of the hydroperoxide FTIR band at 3444 cm^{-1} was also linear with r = 0.999 and SD = 0.001. A second set of experiments were performed with corn oil to test the precision of the iodometric method versus the enzymatic method. The iodometric chemical method was found to be more precise.

INTRODUCTION

Lipid oxidation is a major deteriorative mechanism affecting various fat containing foods and edible oils. Modern distribution and technology practices developed in various countries have significantly improved the food supply in terms of variety and freshness. Prolonged storage, shipping, and shelf life of many food products, however, has resulted in an increased exposure of food components to conditions that initiate lipid peroxidation (ref. 1). The stability and the oxidative state of fats and oils has many economical and nutritional ramifications due to food spoilage by lipid oxidation, and is of great concern to processors and consumers. Initiation of the oxidation process of polyunsaturated lipids may occur via either a free radical autoxidation or photosensitized oxidation . Free radical autoxidation involves the reaction of oxygen with the unsaturated lipids via a free radical initiation step which is followed by propagation and termination reactions. Photosensitized oxidation involves the exposure of unsaturated lipids to light and a sensitizer such as chlorophyll. In this non-free radical process, oxygen becomes activated to a singlet state by a transfer of energy from the photosensitizer (ref.2). The highly reactive singlet oxygen can react with the unsaturated fatty-acids at the double bonds in a concerted "ene" addition pathway.

Rancidity of fat foods and oils is mainly due to the peroxidation of the polyunsaturated lipid constituents. There is a wide spectrum of end products which are associated with the oxidative deterioration of fats and oils (ref. 3-6). The three main end products thought to be most significant in food oxidation are: (1) hydroperoxides; (2) alcohols; and (3) aldehydes. Other minor end products of lipid oxidation are produced but are not as abundant as these three major components. Additionally, cis to trans isomerization occurs in oxidized lipids as well as the conversion of unconjugated to conjugated double bonds

due to hydroperoxide formation in the presence of double (=) bonds. Significant off-flavors and smells associated with rancidity are caused primarily by the secondary oxidation products of hydroperoxides. Lipid oxidation has been a central area of study by biochemists and food scientists for many years. Consequently, many chemical and physical methodologies have been developed to measure the primary and secondary end products of lipid oxidation. The three major chemical methods developed for the study of lipid oxidation are: (1) the thiobarbaturic acid (TBA) assay ; (2) the peroxide-value for hydroperoxide detection; and (3) the anisidine value. These methods are highly empirical and consist of various sources of error in measurement such as titration errors and non-precise volumetric readings amongst other possible errors. A variety of other analytical methods based on chromatography have also been perfected over the years to assess lipid oxidation.

Infrared spectroscopy has been employed as a means of characterizing lipids since 1920 (ref. 7) . The first application of infrared spectroscopy to fat and oil analysis was first observed in the 1940's (ref. 8). From the early fifties to late sixties a large amount of literature on the application of infrared to fat and oil (i.e., fatty acid analysis) analysis had been published (ref. 9-15). A significant number of functional group assignments were undertaken in the fifties with respect to fatty-acid oxidation products. The primary hydroperoxide absorption in the infrared spectrum was the hydroxyl stretch vibration which occurred between 3400 - 3700 cm^{-1} .

Due to the limited sensitivity of the early dispersive infrared spectrometers, quantitative determination of the lipid oxidation end products was difficult. Since the early seventies, FT-IR spectroscopy proved to be a successful technique for both qualitative and quantitative analysis due to the inherent advantageous parameters of FTIR as compared

to conventional dispersive IR techniques. The main advantages of FTIR spectroscopy are include greater sensitivity and precision of the technique.

The 1990's, witnessed the development of rapid, general-purpose FTIR based quality control methods for the food industry (ref. 16-20). Most of the work focused on the development of simple, rapid methods for edible lipid analysis. To date, analytical methods have been developed for the analysis of free fatty acids (ref. 21) , the determination of saponification number and iodine value (ref. 22) and the peroxide value (ref.23). Oils in their neat form are relatively simple molecular systems and thus provide relatively uncomplicated spectra. From a practical point of view, since FTIR methods are generally rapid (i.e., an approximate run time of 1 - 3 min.), are automatable, reduce the need for toxic solvents and reagents associated with wet chemical methods , they could be of major interest to the fats and edible oil industry. At this point, however, there has been little investigation of FTIR spectroscopy in the analysis of edible oil that undergo oxidation in a quantitative fashion.

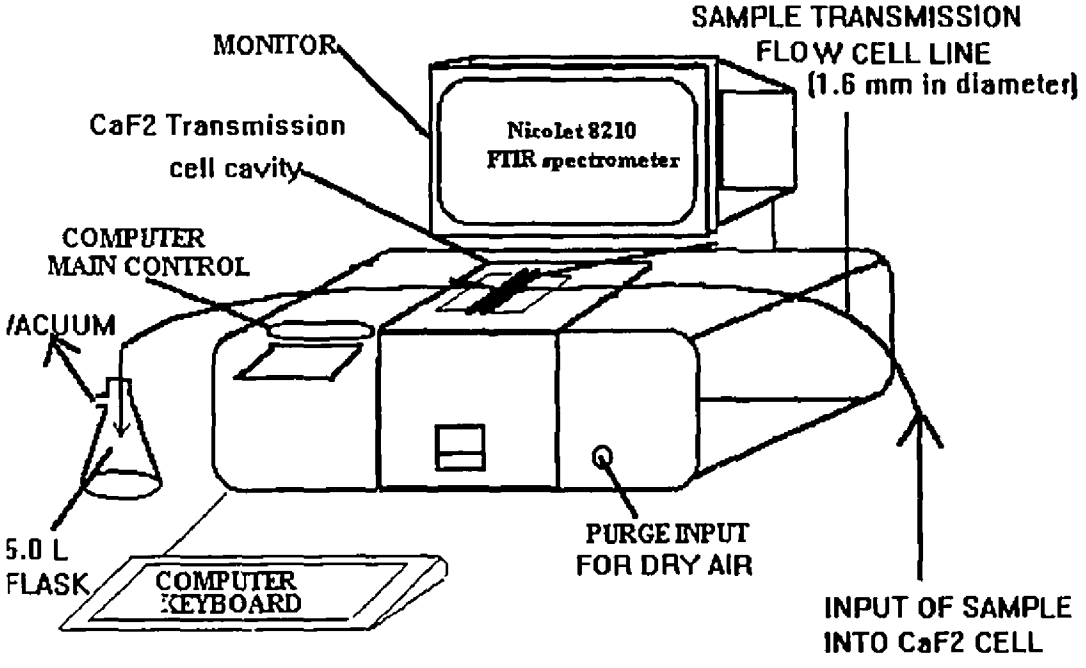
This paper describes the evaluation of the hemoglobin enzyme assay for peroxides, and the AOAC peroxide value to a Fourier transform infrared spectroscopic method, for the quantitation of the primary hydroperoxide products of sunflower and corn oil.

MATERIALS AND METHODS

INSTRUMENTATION

A Nicolet 8210 FT-IR spectrometer (Nicolet Instrument Corp.,Madison, WI) equipped with a CaF_2 transmission flow through cell with a 500 μ teflon spacer was used for this study (Fig. 4.1).

Figure 4.1: A Nicolet 8210 FT-IR spectrometer.



The IR CaF_2 cell was connected to a sipper accessory using a 1.6 mm (ID) input and output tubing to allow the sample to be automatically aspirated through the 500 μ flow cell. The complete tubing accessory was assembled and attached to a standard laboratory vacuum line and each sample had been aspirated into a 2.0 L flask after analysis. The spectrometer was purged with dry air to remove water vapor and CO_2 using a Balston air dryer (Lexington , MA) and the IR cell was employed at ambient temperature. Spectral acquisition were carried out at a resolution of 4 cm^{-1}, using 256 co-added scans for each sample. The background spectrum consisted of 256 scans of the empty transmission cell. All sample emmitance spectra were then ratioed against the same background of the empty cell. The spectrometer was operated under Nicolet DX-FTIR software (Anon , 1988). A program was written to automate sample analysis using a Nicolet Macro command language for spectral acquisition for both calibration and analysis. The FT-IR spectroscopic method is based on mid-infrared absorption of the hydroperoxide moiety (C-O-O-H) at approximately 3444 cm^{-1}.

Materials and methods:

Acetic acid and iso-octane solvents were used for the iodometric determination. A starch indicator (Fisher chemicals), potassium iodide and sodium thiosulfate (0.0999 N standard grade) titrant were also purchased for the chemical iodometric peroxide value analysis. The peroxide assay is known as an iodometric assay. Essentially, iodide is a weak reducing agent and will reduce strong oxidizing agents such as peroxides. When an excess of iodide is added to a solution of an oxidizing agent , iodine is produced in an amount equivalent to the oxidizing agent present. The iodine is titrated with a reducing agent (i.e., sodium thiosulfate) to determine the concentration of lipid peroxides present.

Isopropanol was used to dissolve and dilute all oil samples for analysis. Cumene Hydroperoxide was included in the enzyme assay kit and used as an external standard for

calibration . A Hemoglobin-methylene blue based assay kit was purchased from Kamiya Biomedical Co. (Thousand Oaks, CA). The kit included all the required chemicals and enzymes for adequate peroxide determination (i.e., a reagent containing ascorbate oxidase and lipoprotein lipase and a second reagent containing the hemoglobin and methylene blue derivative). The enzyme assay is based on a sensitive colorimetric method. A reaction of lipid hydroperoxides with a leucomethylene blue derivative in the presence of hemoglobin is the chemical basis of the assay. The amount of methylene blue formed via the reaction is measured by its absorbance at 666 - 667 nm and is proportional to the amount of lipid hydroperoxide present.

The only chemical required for FT-IR spectroscopy was that of iso-octane which was used to clean the transmission cell prior to sample analysis.

FT-IR spectroscopy versus Iodometric PV assay (sunflower oil):

Approximately 4 L of sunflower oil was purchased from a local supermarket. One liter of the oil was added to a 2 L Erlenmeyer flask and the flask was then set on top of a heating pad . A thermometer was placed into the oil and the oil was heated at 96 °C on a hot plate. Dry air (i.e., air stripped of moisture by passing through anhydrous $CaCO_3$ pellets) was bubbled into the oil at a rate of 1 cm^3 / min. The oil was heated for approximately 8 days while continuously being purged with dry air over the hot plate at ~ 96 °C. Every second day a 30 ml aliquot was removed and the FTIR spectrum was recorded. After 8 days, a value of approximately 270 PV was obtained by the iodometric method. Ten standards were prepared for calibration purposes as follows; (1) the greatest oxidized sunflower oil (i.e., ~270 PV) was first diluted with fresh sunflower oil to obtain a 5:1 ratio of fresh oil to oxidized oil to decrease the PV by five folds. The most oxidized oil diluted at a 5:1 ratio with fresh oil was considered as the oil with the greatest oxidation state or as 100% oxidized for experimental purposes; and (2) a 9:1 ratio of

oxidized oil was prepared by accurately diluting 9 grams 100% oxidized oil with 1 g fresh oil, then a 8:2 ratio was prepared by diluting 8 grams oxidized oil with 2 grams fresh, and so forth up to 100% fresh oil . Three subsequent samples were prepared as unknown samples. The FTIR spectra of all the samples were then recorded.

Results and Discussion:

A COMPARISON OF THE HEMOGLOBIN-METHYLENE BLUE ASSAY VERSUS IODOMETRIC ASSAY AND FTIR METHOD WITH CORNOIL (STANDARD AND SAMPLE PREPARATION):

The same protocol as that of the sunflower oil was followed except for the following alterations: 1) the corn oil had been left over a hot plate for 6 days ; 2) the oxidized oil was not diluted with fresh oil since the oxidation state was not as high as that of the sunflower oil (ie., ~ 130 PV). All other subsequent standard dilutions were prepared in a fashion as similar to the sunflower oil standards for FTIR calibration purposes. An FTIR calibration was also performed.

Corn oil oxidation:

Oxidized and unoxidized corn oil was used for further comparative purposes between the iodometric analysis and the enzymatic assay. The oxidation of corn oil was followed by FTIR spectroscopy to compare the difference between both the sunflower and corn oil natural capability to resist auto-oxidation. The general oxidation of the oil and subsequent dilution protocol for the preparations of standards were identical to the protocol used for sunflower oil. Table 4.1 depicts the actual values obtained for both the chemical iodometric and enzymatic assays.

Table 4.1 CORN OIL CHEMICAL PV vs. ENZYME CALCULATED PV

SAMPLE	*CHEMICAL PV*	*ENZYME PV*
CLEAN 1	1.96	4.03
CLEAN 2	1.85	1.96
CLEAN 3	1.85	-----
CLEAN 4	1.97	-----
50:50 / 1	69.00	64.71
50:50 / 2	68.55	71.89
50:50 / 3	68.27	55.34
OXIDIZED 1	130.04	115.03
OXIDIZED 2	129.18	129.41
OXIDIZED 3	130.19	-----
OXIDIZED 4	129.65	-----

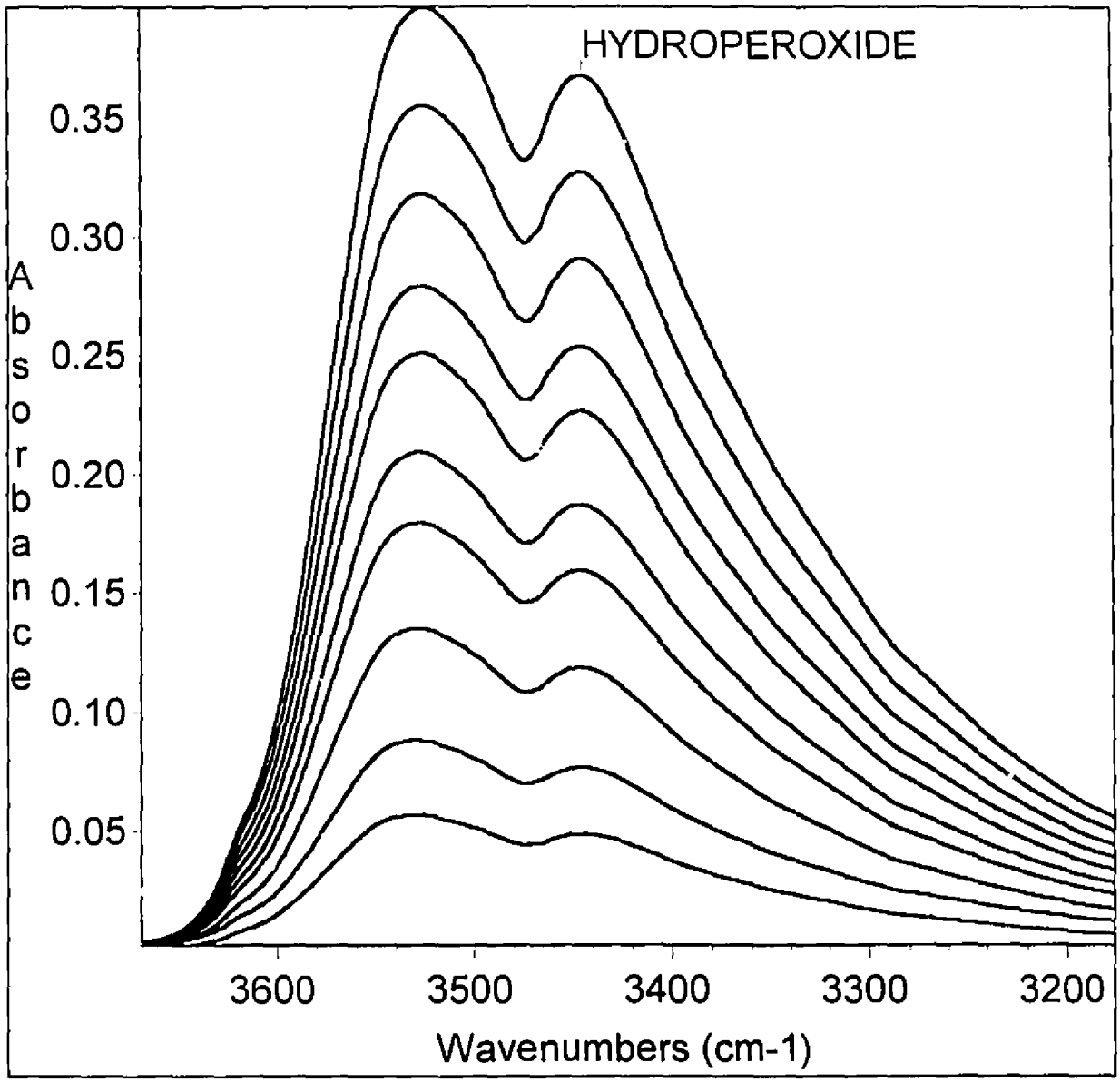
The greater precision obtained by the chemical method as compared to the enzymatic assay is shown in Table 4.1. The higher precision of the iodometric method could be due to: (1) a greater dilution of the sample prior to analysis by the enzymatic method ; (2) the instability of enzymes in the hemoglobin- methylene blue assay ; and (3) the need for precise temperature control in the enzyme assay. The main disadvantage of the iodometric method is the expertise required for end-point detection in the titrations and the large sample size required (i.e., 5.00 g per analysis).

Development of an FTIR method for the determination of the Peroxide Value:

The emmitance FTIR spectra of all ten sunflower standards were recorded and ratioed against an empty transmission cell background using 256 scans at 4 cm^{-1} resolution. Prior to recording the FTIR spectra, the cell was cleaned with anhydrous iso-octane and then allowed to air-dry for approximately 30 - 40 sec. It was important to keep the iso-octane solvent stored over molecular sieves in order to make sure that the solvent was maintained in an adequately anhydrous state. The completely oxidized oil had been initially diluted with fresh sunflower oil. The reason for this dilution is due to the fact that chemical peroxide values are not reliable above ~90 - 100 PV. Hence, to calibrate the FT-IR against the iodometric method, the peroxide value of the sunflower oil had to be reduced to a value below 100 PV by dilution with fresh oil to a peroxide value of 75. The diluted oil was then used for subsequent serial dilutions.

Each of the sunflower oil calibration standards were aspirated into a 500 μ CaF_2 cell (requiring approximately 3 - 5 ml) , and the emmitance spectrum was recorded for each standard and ratioed against an empty cell background producing the absorbance spectrum of each standard. The peak heights of each standard was obtained by calculating the difference between the height at 3444 cm^{-1} (νOOH group frequency) from the height at 3200 cm^{-1} used as a reference point for analysis after subtracting the spectrum of the clean oil. Figure 4.2 shows the various peaks obtained for the ten separate sunflower oil standards.

FIGURE 4.2: Infrared spectra between 3650 and 3200 cm-^{1} of ten sunflower oil standards.



Along with the uniform increase of the hydroperoxide peak at ~ 3444 cm^{-1} , another peak also increases simultaneously with the 3444 cm^{-1} peak. This peak located at approximately 3540 - 3550 cm^{-1} is attributed to the hydroxyl -OH stretch of alcohols and because of its simultaneous and uniform increase with that of the hydroperoxide -OH peak, the alcohol hydroxyl peak does not effect the quantitation of the hydroperoxide levels . Hence, since both the hydroperoxide -OH and alcohol hydroxyl peaks increase in a linear fashion with respect to one another, the calibration model is not affected by this overlapping hydroxyl peak nor is the predictive ability of the calibration model. The peak heights of the 3444 cm^{-1} band were regressed against the calculated peroxide values for each standard.

The sample handling for the unknowns was similar to that of the standards. Three unknown samples were prepared prior to analysis with each one consisting of a random amount (wt. / wt.) of oxidized sunflower oil diluted with fresh oil. The FTIR spectrum of each sample was recorded and the PV determined from the regression equation obtained above. The peak heights of the 3444 cm^{-1} band were obtained as were those for the standards (i.e., peak height at 3444 cm^{-1} minus reference peak height at 3200 cm^{-1}) and the calibration equation was used to obtain the respective concentrations of hydroperoxides for each unknown sample.

The spectral operations and calculations were done interactively although the Nicolet macro programming language may have been used to automate the spectral analysis. After recording the FTIR spectrum of the sample, it is then possible to obtain the hydroperoxide values immediately once the FTIR is calibrated.

HEMOGLOBIN - METHYLENE BLUE ASSAY :

Fifty μL of partially oxidized corn oil and sunflower oil, were dissolved in isopropanol in varying dilution (1:500 to 1:2000) to allow for a measure of absorbance by the colorimetric system . A 10 μL aliquot of each oil in the isopropanol was assayed for lipid hydroperoxide content using the hemoglobin-methylene blue-based assay kit (Kamiya Biochemical Company). The assay involved incubation of the sample at 30 °C with a reagent containing ascorbate oxidase and lipoprotein lipase for 5 min followed by a 10 min incubation with a second reagent containing hemoglobin and a methylene blue derivative. The lipid hydroperoxide content of the reaction mixture was measured by recording the absorbance of methylene blue at 675 nm using a microtiter plate reader (Model 7000, Cambridge Technologies) for corn oil, or a colorimetric spectrometer for the sunflower oil. A calibration curve was generated with serial dilutions of a cumene hydroperoxide standard solution supplied with the kit and the results were expressed in terms of lipid peroxides (LPO) (micromoles of hydroperoxide/ml of oil). For comparison purposes , the LPO values were converted to their corresponding AOCS PV values using the following equation : PV = 2.0 (LPO)/density; where PV = peroxide value; LPO = enzyme assay lipid peroxide value mM / mL and density = density of the oil.

Determination of PV by the Iodometric method:

Hydroperoxides in both the sunflower and corn oil samples were determined by the AOAC method (Cd 8b-90) . The peroxide values, were determined for the clean , 50:50 mixture (of clean and oxidized) and extensively oxidized oil. Approximately 5 g of oil and 50 ml acetic acid / iso-octane (3:2) were added to the oil sample to be analyzed along with 0.5 ml of a saturated KI solution. Sodium thiosulfate titrant was subsequently

diluted at a ratio of 10:1 with water to obtain a final concentration of 0.01N to allow for accurate titration of oil samples containing low amounts of hydroperoxides. After 1 min, 30 ml of distilled water was added. The mixture was titrated with 0.1N or diluted 0.01N sodium thiosulfate in the presence of a starch indicator until the blue color associated with the starch-iodine complex disappeared. The results are expressed as peroxide value or PV (milli-equivalents of peroxide / 1000 g of sample).

RESULTS

Infrared absorption is known to follow Beer's law which states that, "absorbance at a particular reference wavenumber equals to the molar absorptivity at that wavenumber × pathlength × concentration". This law holds true for linear increases of a particular functional group absorption as a function of concentration. Hydroperoxides do not necessarily follow such a linear trend above a particular concentration value due to intermolecular interactions such as H-bonding within the solvent matrix, which may cause the band to shift or become broad. For the purpose of this experiment, the increase in peak height was followed as a function of calculated peroxide value.

Table 4.2 depicts the LPO enzyme values obtained from the enzyme assay and the corresponding peroxide values (PV) for sunflower oil.

Table 4.2 Enzyme LPO values & calculated peroxide values of oxidizedsunflower oil samples.

Oil type	*Replicates*	*LPO*	*PV equivalence*	*Average*
Clean oil	1	1226.3	2.67	2.74
	2	1241.4	2.71	
	3	1305.8	2.85	
50:50 mixture	1	11175.3	24.35	24.98
	2	11754.1	25.61	
Oxidized oil	1	29296.5	63.83	68.79
	2	33541.2	73.07	
	3	31895.2	69.49	
Unknown #1	1	13297.6	28.97	27.79
	2	12317.0	26.83	
	3	12644.8	27.55	
Unknown #2	1	15404.5	33.56	32.74
	2	14647.5	31.91	
Unknown #3	1	5194.0	11.32	11.04
	2	4938.4	10.76	

Replicates had been obtained so to allow for a more representative mean value of the PV for each sample. As shown by the LPO and calculated peroxide values obtained, the precision of the enzyme method is not as good as the chemical method of analysis (Table 4.2).

Table 4.3 **Peroxide values determined by iodometric analysis for clean and oxidized Sunflower samples.**

	PEROXIDE VALUE	*AVERAGE*
CLEAN SAMPLE	5.18	5.38
	5.57	
	5.21	
	5.55	
50:50	39.52	40.09
	39.84	
	40.92	
OXIDIZED	71.86	73.18
	73.27	
	74.40	
UNKNOWN #1	37.30	37.71
	37.90	
	37.92	
UNKNOWN #2	60.00	59.63
	59.12	
	59.76	
UNKNOWN #3	19.16	19.75
	19.84	
	20.24	

The lack of precision of the enzyme method may be due to tedious manipulations required for sampling; precise temperature control required; and the handling errors of the enzyme solutions.

TABLE 4.4 **Calibration of FTIR spectrometer with chemical peroxide values of oxidized sunflower oil.**

STANDARD #	*CALCULATED PV*	*PK. HT. @ 3444 CM $^{-1}$*
1	9.23	0.040
2	14.09	0.064
3	22.08	0.097
4	29.47	0.132
5	34.56	0.153
6	41.93	0.185
7	46.56	0.207
8	53.57	0.237
9	59.97	0.267
10	67.81	0.300

Table 4.4 shows a list of the calculated chemical PV for all diluted standards by using the iodometrically obtained peroxide values of the clean and oxidized sunflower samples and the corresponding peak heights @ 3444 cm-1 from the infrared spectra of the samples. The formula used to calculate the PV is as follows:

Calculated PV =(wt.of non-ox oil × PVnon-ox) + (wt.of oxidized oil × PVoxid)

---------- - PVclean.

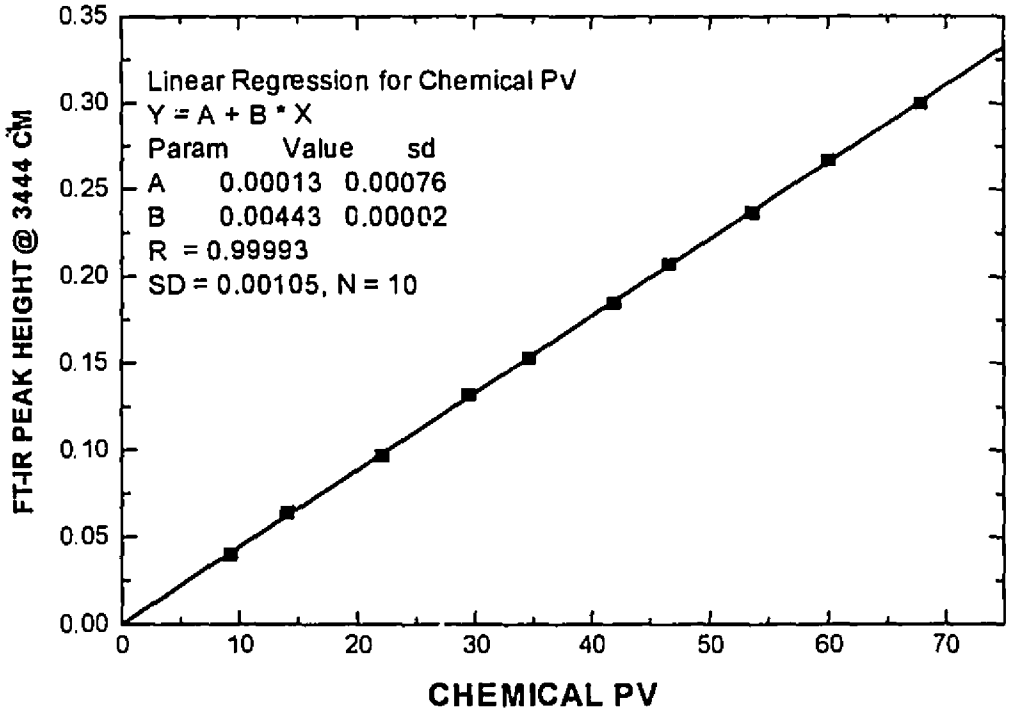
Total weight (non-oxid + oxid. oil)

Figure 4.2 depicted the spectra of all 10 sunflower oil standards prepared for the calibration. The chosen frequency range was between 3700 and approximately 3150 cm^{-1}. The range encompasses several molecular entities such as hydroperoxides and hydroxyl groups as well as the overtone absorption of the ester carbonyl. The peaks shown in the spectral range are mainly due to the absorption of two functional groups. The peak at approximately 3444 cm^{-1} is due to hydroperoxide -OOH absorption (ref.25) and the ~3550 cm^{-1} peak is due to the hydroxyl -OH stretch of alcohol's. A constant linear increase in IR absorbance of both moieties can be observed as the concentration of oxidized oil added to the standards is increased. Since the spectral bands overlap this would cause problems in the use of such oil standards as "universal calibrants". As prediction of the hydroperoxide content is from the same oil as the standards, the overlap will not effect the prediction significantly. Moreover, the hydroxyl peak should not effect the hydroperoxide predictions since the 3550 cm^{-1} band increased linearly with the 3444 cm^{-1} band thereby providing a constant effect on the hydroperoxide predictions. If the alcohol content in a particular oil increased at a different rate than hydroperoxides, then some error would be introduced in the prediction unless this effect is factored through the use of multiple linear regression or multivariate analysis method. What would be required would be a multicomponent calibration with standards consisting of all the molecular entities found within oxidized oils (i.e., such as free-fatty acids, alcohols, aldehydes and so on) if a global calibration is desired. A PLS model could then be obtained taking into

consideration all overlapping bands and molecular interactions between all the oxidation by products. Such a calibration approach was recently performed (ref. 24).

A simple linear regression model was obtained for all ten sunflower oil standards. Figure 4.3 shows the regression of peak height at 3444 cm^{-1} against calculated PV (by chemical values).

Figure 4.3 **Regression of FT-IR peak heights of hydroperoxide vs chemical peroxide value of sunflower oil samples.**

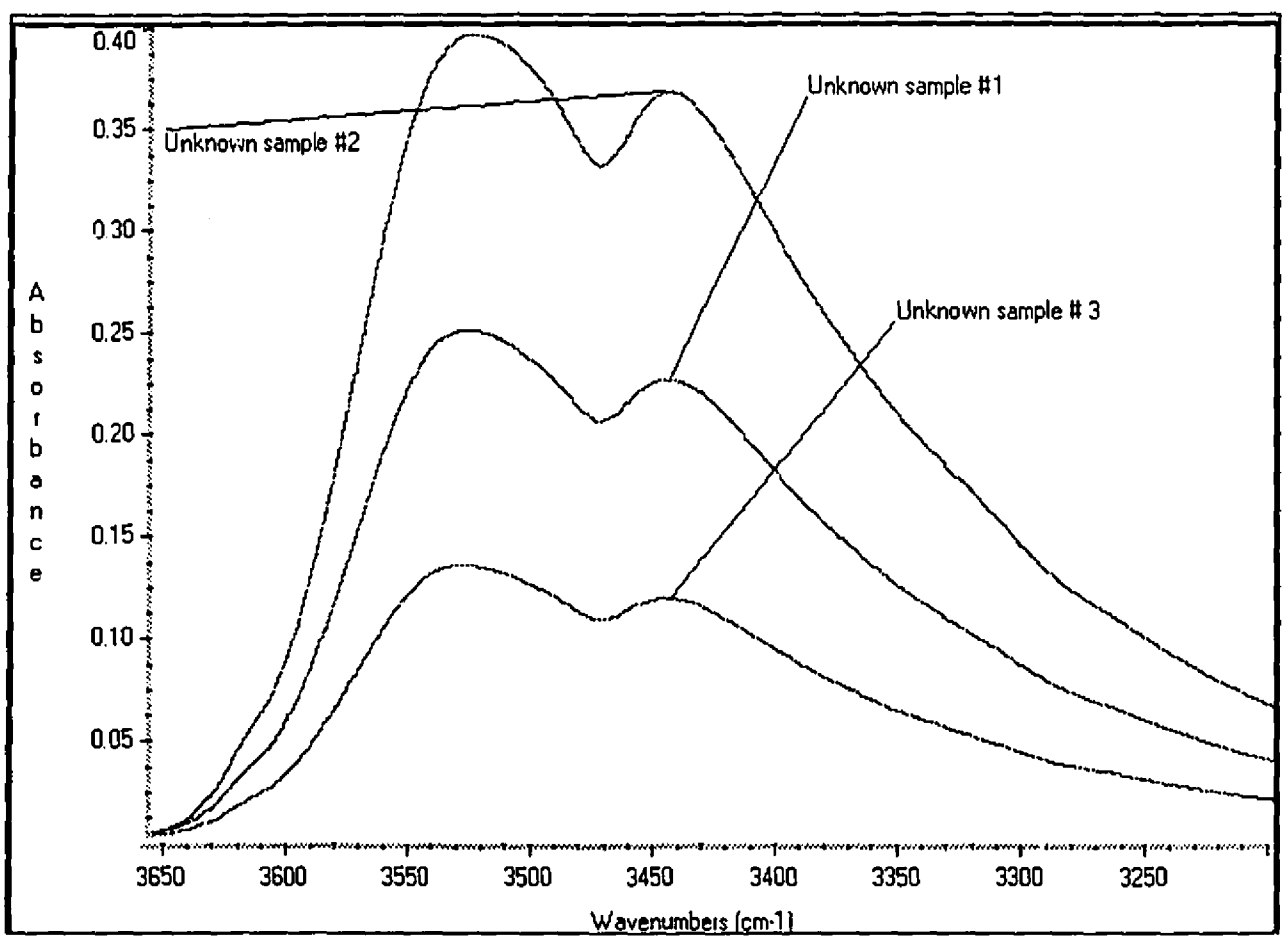


An optimal correlation coefficient of 0.99993 with a SD = 0.00105 was obtained from the regression. The regressed equation was then used to obtain the peroxide values of the unknown samples. Three unknown samples were prepared by varying the amounts of non-oxidized oil to oxidized sunflower oil for predictive purposes. Figure 4.4 depicts the actual spectra of all three unknown samples. The peak height at 3444 cm^{-1} was obtained for each unknown and the PV predicted from equation 1.

PV = (PEAK HEIGHT) / 0.00443 - 0.00013 eq. (1)

Figure 4.4

Infrared spectra between 3650-3200 cm-1 of three oxidized sunflower oil samples.



A calibration of the FTIR spectrometer was also performed with the peroxide values calculated from the hemoglobin-methylene blue enzyme assay of oxidized and non-oxidized oils (Table 4.5).

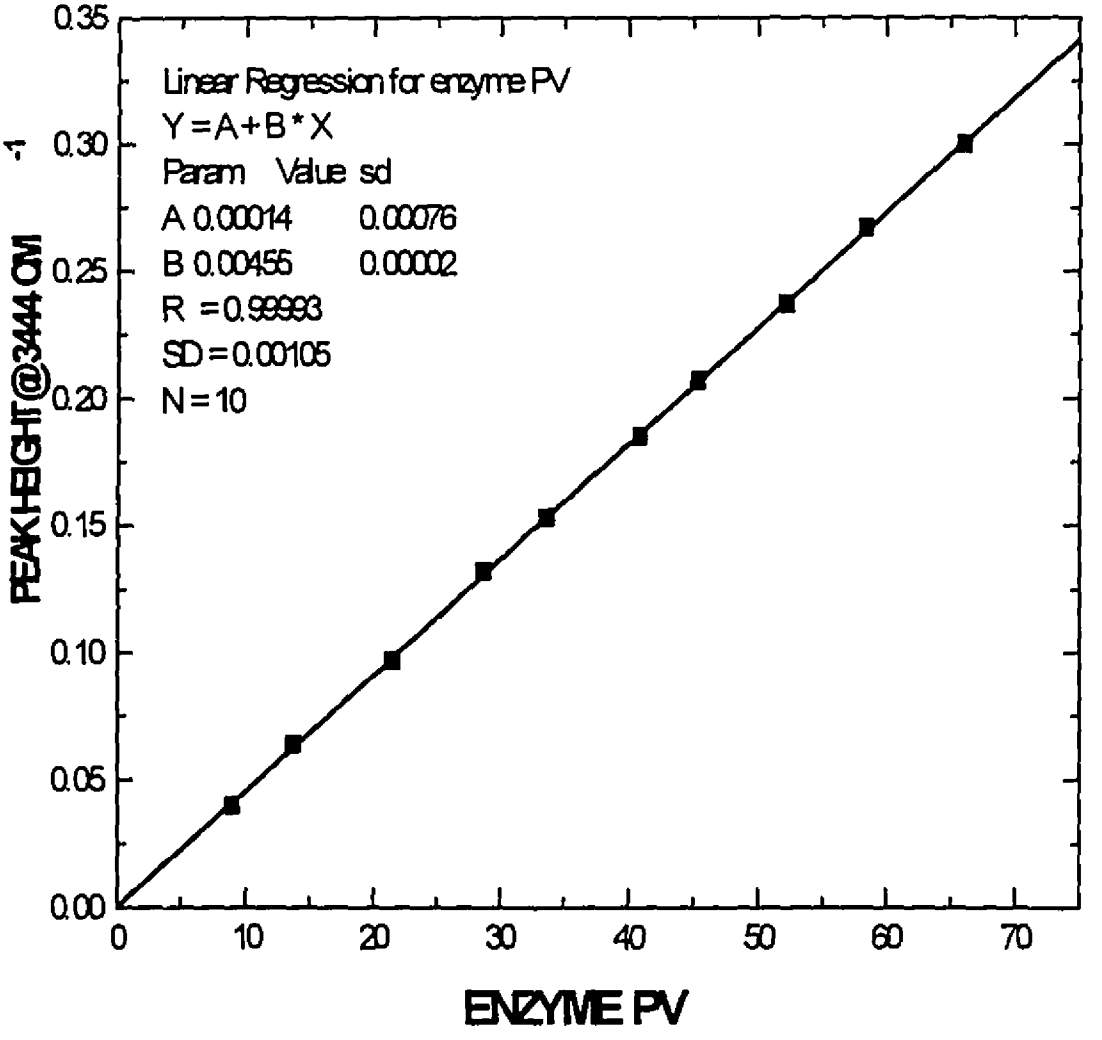
Table 4.5 Calibration of FTIR by hemoglobin-methylene blue enzyme assay.

STANDARD	*PV by enzyme*	*PK.HT.@3444cm^{-1}*
1	8.992	0.04
2	13.724	0.064
3	21.512	0.097
4	28.712	0.132
5	33.668	0.153
6	40.845	0.185
7	45.366	0.207
8	52.194	0.237
9	58.423	0.267
10	66.062	0.300

The regression plot obtained for the theoretical PV as a function of peak height at 3444 cm^{-1} is shown in Figure 4.5. A correlation coefficient of r = 0.99993 was obtained with a SD = 0.00105. The regression equation was also used to predict the PVs for the unknown samples (eq. 2).

PV (enzyme) = Peak Height / 0.00455 - 0.00014 eq. (2)

Figure 4.5 Regression of FTIR peak height at 3444 cm^{-1} against the PV from hemoglobin methylene-blue enzyme assay.



The two methods of calculations for theoretical peroxide values are nearly equivalent, although a better predictive power was obtained via the chemically acquired peroxide

values in the theoretical PV formula. Table 4.6 demonstrates the differences between the calculated peroxide values acquired from the enzyme assay and the iodometric method.

Table 4.6 **ENZYME PV vs. CHEMICAL PV**

SAMPLE	*ENZ. PV*	*CHEM. PV*	*DIFFERENCES*
CLEAN	2.741	5.375	2.634
50:50	------	40.09	------
OXIDIZED	68.787	73.18	4.393
UNKNOWN 1	27.785	37.71	9.925
UNKNOWN 2	------	59.63	------
UNKNOWN 3	11.038	19.75	8.712

Shown in Table 4.7, are the actual predictions obtained for all three unknowns by the linear equation along with other comparative values. As depicted in Table 4.4, the FTIR predictions are comparitively close to the actual standardized chemical iodometric method.

Table 4.7

Comparison of predicted FTIR values with chemical PV and enzyme calculated PV.

UNKNOWN	*FTIR Chem prediction*	*FTIR ENZ.prediction*	*CHEM. PV*	*ENZ. PV*
1	38.08	34.58	37.71	27.79
2	57.95	53.92	59.63	53.74
3	19.30	16.25	19.75	11.038

Table 4.8

DIFFERENCES BETWEEN FTIR (CHEM. & ENZYME VALUES) & CHEMICAL PV

UNKNOWN	*FTIR chem- CHEM*	*FTIR enz- CHEM.*
1	0.365	6.79
2	1.685	0.18
3	0.455	5.21

Table 4.8 shows the differences between the FTIR predicted values obtained from calibration against the chemical and enzymatic methods. These differences take into consideration a bias value of 5.375 for the calculation of unkowns by FTIR. This is due to the fact that for the regression of the chemical peroxide values calculated versus peak height, a subtraction of the initial PV of the non-oxidized oil from each standard was performed. Hence, when the bias value of 5.375 was added to each prediction, the predictions were nearly identical to the standard iodometric values obtained for each unknown (Table 4.8). Thus, FTIR may be calibrated by an oil and regressed linearly to predict unknowns with a high level of accuracy.

The FTIR spectrometric method of analysis is preferable in comparison to the iodometric method of analysis due to a significant decrease in analysis time, the elimination of chemical reagents needed to develope expertise and experience in titration. As shown by Table 4.8, the FTIR method of analysis is very accurate in prediction. One must also keep in mind that the FTIR method is a secondary method of analysis. Hence, the spectrometric mode of analysis relies on the accuracy and precision of a primary method

for calibration. Therefore, the FTIR based method is as accurate as the primary method used for calibration purposes.

The reproducibility of the FTIR method was also tested. Table 4.9 depicts the actual PV's obtained for the three unknowns within an 8 day interval.

Table 4.9 REPRODUCIBILITY OF FTIR SPECTROSCOPY

UNKNOWN	*DAY 1*	*DAY 8*	*% REPRODUCIBLE*
1	19.295	19.345	99.74%
2	38.075	33.15	98.83%
3	57.945	53.02	99.23%

Footnote : Reproducibility followed over a one week period.

The calibration of the spectrometer was done only on the first day with subsequent predictions of unknowns on the first and eight day. The reproducibility of the method was very good (Table 4.9) demonstrating the stability of the calibration.

The present study has demonstrated the successful application of FTIR spectroscopy in the determination of hydroperoxides in oxidized oils. FTIR is recognized as a secondary method of analysis, Hence, the calibrations performed of a single oil type would not be successful in the application of predictions of unknown oils of various types, due to the wide spectrum of various molecular lipid moieties found within different oxidized oils. The FTIR spectrometer must be calibrated with respect to a primary method such as the chemical or enzymatic methods. The calibration of FTIR with the chemical method (i.e., iodometric values) gave a regression comparable to that of the calibration with the enzyme method (i.e., enzymatic values). The need for large amounts of sample, the use of chemical reagents and the difficulty in end-point determination for the chemical method and the required stringent conditions (i.e., temperature control, time control,and enzyme

stability) and repetative dilutions for the enzyme method make these assays less attractive than FTIR spectroscopy for analysis of oxidized oils. A properly calibrated FTIR system would be the best mode of analysis for predictions of hydroperoxide content. Moreover, the FTIR method, required approximately 2-4 min per sample after calibration of the instrument as compared to 10-15 min for the chemical iodometric method and approximately 30 min or more for the determination of PV by the enzyme assay. Hence, the high level of precision demonstrated by FTIR spectroscopy as well as the elimination of several negative factors associated with chemical and enzymatic methods indicate that FTIR spectroscopy can provide an optimal means of measuring hydroperoxides in oxidizing oils.

CONCLUSION

The advent of FTIR spectroscopy along with improved sampling methodologies, makes FTIR spectroscopy a new tool for the measurement of primary lipid degradation products with minimal sample preparation. The infrared method affords a very rapid and quantitative mode of analysis for monitoring hydroperoxide formation. The accuracy of the infrared method is governed by the accuracy of the respective primary standard method. The primary methods in the present study were the iodometric assay and hemoglobin - methylene blue enzymatic method. As FTIR requires little sample analysis time (~ 2-4 min.), infrared spectroscopy serves as a very attractive mode of quantitative analysis of hydroperoxides in oxidized oils. The final predictions from the FTIR calibration model calibrated against the chemical PV method or enzymatic PV assay were acceptable and within reasonable error (i.e. +/- 3.00%).

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CHAPTER V

Determination of the Hydroxyl Value of Acetylated Monoglycerides by FT-IR Spectroscopy

ABSTRACT

A simple and rapid method was developed to determine the hydroxyl value of acetylated monoglycerides. Two infrared techniques for the analysis of commercial monoglyceride products were investigated . The first technique is based on the principle of attenuated total reflectance (ATR), and the second technique employs a flow through transmission cell. Both techniques were successful in providing comparable accuracy and reproducibility with an overall error of approximately 3.00%. Forty-five monoglyceride samples were pre-analyzed by the standard AOCS method Cd 13-60 and their FT-IR spectra were subsequently recorded. For calibration purposes, approximately half of these samples were randomly chosen as standards (hydroxyl values from 7-190). The calibration was based on the partial least squares (PLS) method which utilized selected IR spectral regions and the chemically-derived hydroxyl number to generate a calibration model for prediction of hydroxyl number from the FTIR spectrum of monoglyceride samples. The calibration model was tested by prediction of the other half of the monoglyceride samples. The PLS predicted hydroxyl number was compared to the actual values determined from the standard AOCS chemical method. The predicted vs. chemical values were regressed against each other. The correlation coefficients for both ATR and transmission based methods were highly comparable with a value of $r = 0.998$ for ATR and $r = 0.997$ for transmission based methods. The standard errors were 3.45 and 3.86, respectively. Hence, both IR-based methods of analysis employing PLS calibration models were comparable. Once the FT-IR spectrometer was calibrated, hydroxyl values could be obtained within 3 to 5 minutes. This can be regarded as a major improvement over the

conventional standard AOCS wet chemical methods, not only due to the significant decrease in analysis time but also the decrease in the usage of chemical reagents and the need for skilled personnel to carry out the analysis.

INTRODUCTION

A growing interest in the food industry for rapid and accurate methods in the analysis of food components is evidenced from various publications in recent years (ref.1-6). Chemical methods are generally satisfactory , although require tedious manipulations and an abundance of chemicals which require special disposal protocols. The increase in the sensitivity of instruments and their availability at low cost has resulted in the rapid development of spectroscopically-based methods of analysis. Mid-infrared spectroscopy based methods appear to be gaining wide spread acceptance by both analytical and food chemists.

The general components that make up a food are lipids, proteins, carbohydrates and moisture with a minor amount of various minerals. Lipids is a term used by many to signify the fat components of a food system. The term lipid, however, encompasses much more than the fat constituent of a food . Lipids also include components such as the sterols (i.e., cholesterol) , fat soluble vitamins , and membrane associated lipids such as phospholipids and sphingolipids (ref. 7) . Monoglycerides consist of a glycerol backbone esterified at one hydroxyl group by a fatty acid with two free hydroxyl groups . This particular chemical structure allows monoglycerides to act as emulsifiers or surfactants. These entities are also known as amphiphilic consisting of both a lipophilic and hydrophilic end thereby, allowing monoglycerides to act as emulsifying agents. Monoglycerides were first characterized by the French chemist Berthelot around 1853 and their industrial usage dates to the 1930's (ref.8). Monoglycerides were first used in the margarine industry on a

large scale around the 1930's and gained increased popularity as a general surfactant. Monoglycerides are polymorphic and can therefore exist in different crystal forms depending on temperature. This chapter describes a quantitative method for the analysis of the hydroxyl content of monoglycerides in their liquid state by FT-IR spectroscopy.

The chemometric model used for the quantitation of the hydroxyl content (hydroxyl value) of monoglycerides from their infrared spectra is based on the PLS (partial least squares) method. PLS is based on a spectral decomposition technique which uses concentration information during the decomposition process. The spectral data is separated into two sets, one set of scores and another set of loading vectors for calibrations. Hence, the spectral decomposition produces a series of "mathematical spectra " also known as loading vectors or factors (ref. 9). These factors are then correlated and multiplied by scores (i.e., scalar fractions such as concentration) . PLS-based calibration have many more advantages compared with the inverse and classical least squares based-methods. While complete description of the mathematical basis of PLS is beyond the scope of this chapter, PLS-based methods are capable of extrapolating information from overlapping bands within a spectral region as well as molecular interactions in a chemical model. The application of PLS to mid-infrared spectroscopy is investigated for determination of the hydroxyl value of acetylated monoglycerides.

Two separate infrared sampling methods (i.e., ATR and flow through transmission) was also assessed for the quantitative analysis of the hydroxyl value of the acetylated monoglycerides. Flow through transmission cell consisted of two CaF_2 windows with a 38 micron teflon spacer (Fig.5.1). The flow-through transmission-cell is equipped with a by-pass valve for rapid cleaning and a temperature controlled block heater to maintain the temperature of the IR cell above the melting point of the monoglycerides during analysis.

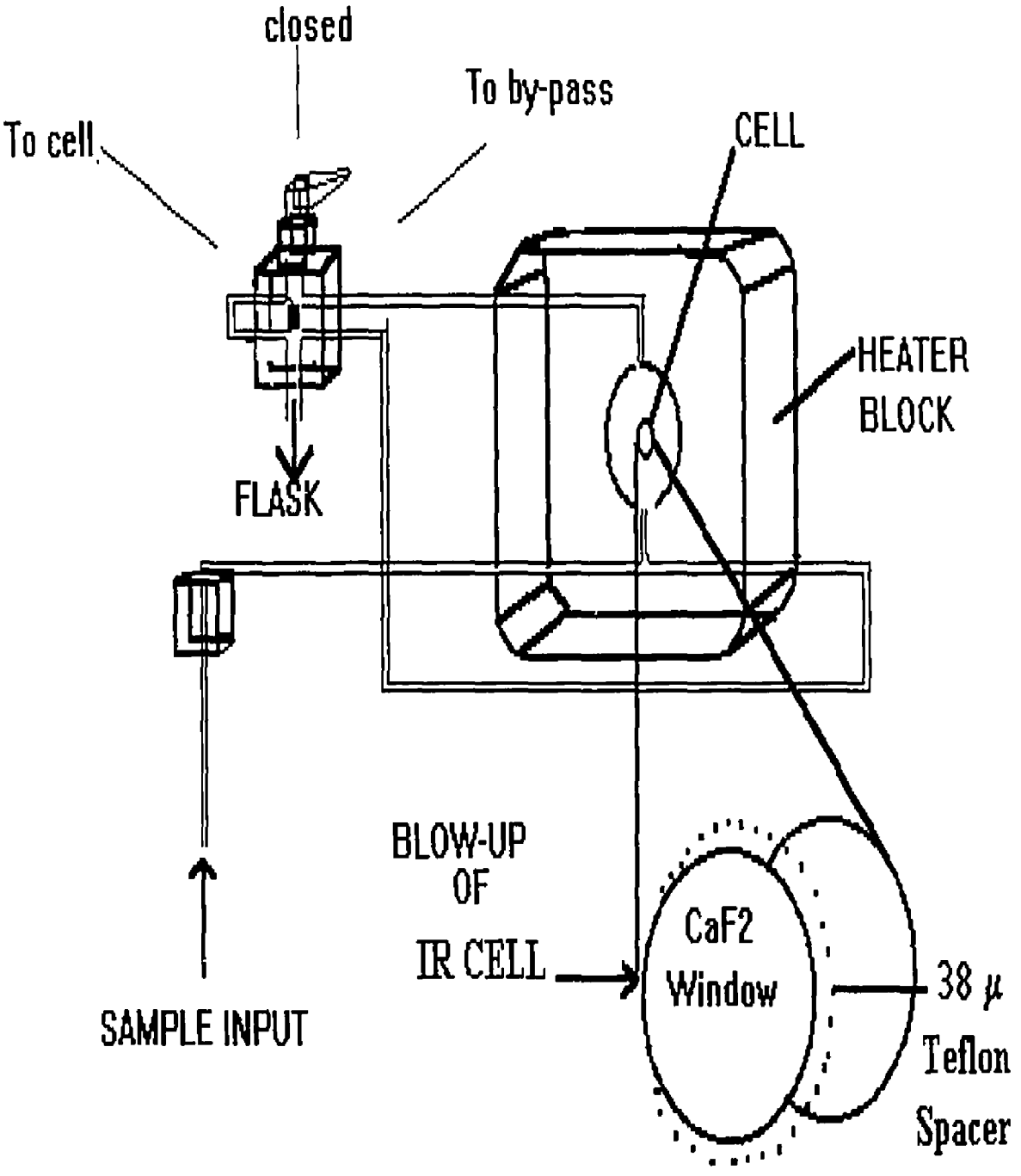


Figure 5.1 A schematic diagram of a CaF_2 IR cell and flow pattern of monoglyceride through the IR assembly.

An infrared beam is transmitted through the cell from an initial entrance point through to a final exit of the IR beam (Fig. 5.2).

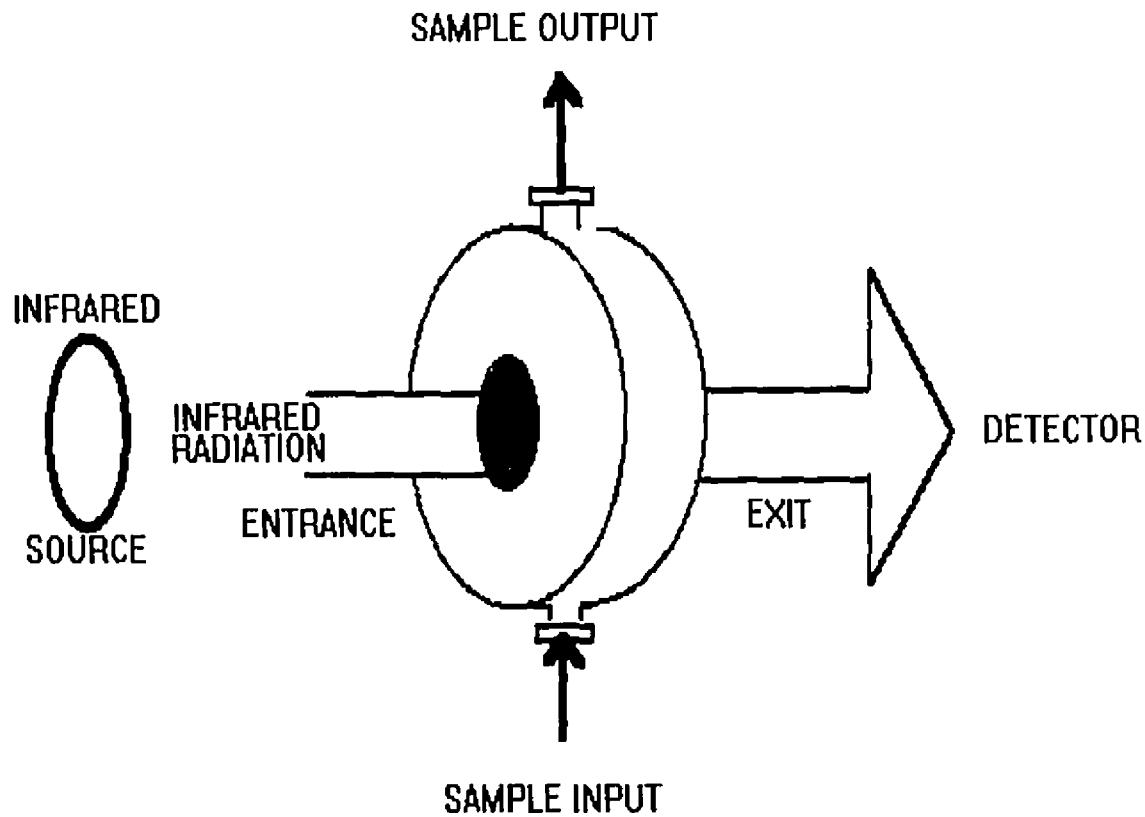


Figure 5.2 **A schematic of an infrared beam penetrating a transmission CaF_2 cell.**

The second experimental protocol employed a horizontal ATR accessory consisting of a ZnSe crystal. The infrared beam is launched at a fixed angle (45°) into the ZnSe crystal where it undergoes multiple internal reflectance as it travels down the crystal (Fig. 5.3), resulting in the generation of an evanescent wave perpendicular to the surface of the crystal.

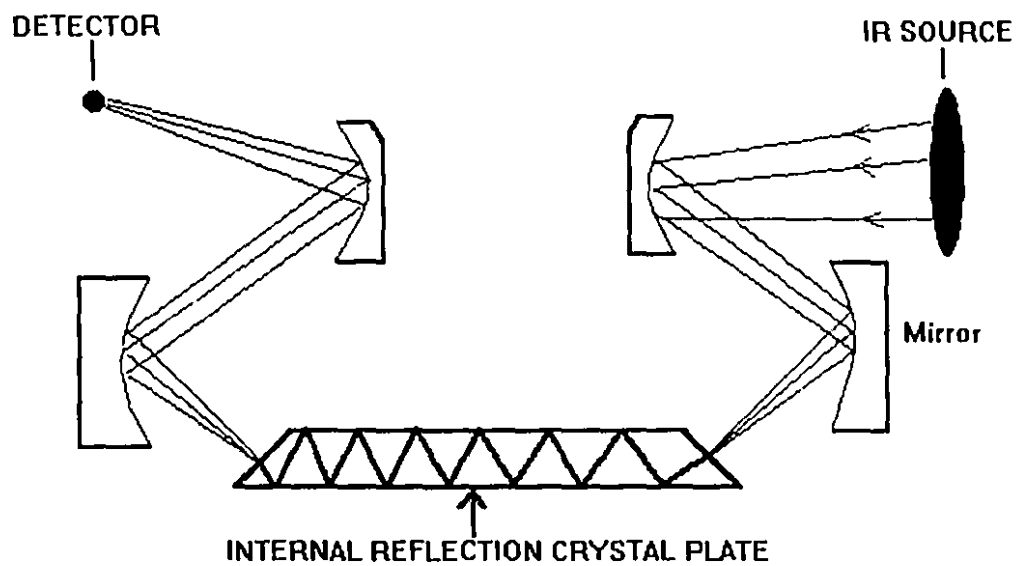


Figure 5.3 **A schematic of a typical attenuated total reflectance (ATR) crystal.**

The penetration depth of the evanescent wave is noted to be a function of the refractive index of both the crystal and sample . The depth of penetration of the evanescent wave is dependant on both the launch angle and the characteristic wavelength propagating through the crystal.

$$d_p = \lambda / \{2\pi n_1^2[\sin(\theta) - (n_2/n_1)]\}^{1/2}$$

Where; dp = depth of penetration of the evanescent wave into the sample.

λ = wavelenght of the infrared light.

n_1 = refractive index of crystal.

n_2 = refractive index of sample.

θ = launge angle of infrared light onto the ATR crystal plate.

A comparison of the analytical performance of both techniques was made, employing both the calibration and predictions data from the two sampling methods.

The AOCS method for the hydroxyl value determination is fairly cumbersome and lengthy. It requires skill to be properly executed. The analyst must be skilled in such analytical techniques as titration which involves a great deal of expertise to obtain adequate analytical results. There is also the cumbersome setup of a reflux apparatus which requires lengthy refluxing time. Along with these requirements is the required use of chemical reagents such as pyridine and acetic anhydride among others. Hence, due to these factors there is a current need from the industry for the development of a fast and accurate method of analysis that can alleviate these limitations. The possible implementation of the FT-IR to the analysis of the hydroxyl value is investigated. A further investigation was also performed on the merit of the two different infrared sampling techniques for acquiring infrared spectra of the sample.

MATERIALS AND METHODS

Instrumentation:

A Nicolet Impact 400 FT-IR spectrometer (Nicolet instrument Corp. , Madison , WI) was used for the transmission study , equipped with a CaF_2 transmission flow cell and a 38 μ teflon spacer. The cell was connected to a sipper accessory using a 1.6 mm ID input and output tubing to allow the sample to be automatically aspirated through the 38 μ flow cell . The accessory was attached to a standard laboratory vacuum line where each sample is aspirated into a 2000 ml trap flask after recording the FTIR spectrum (Fig. 5.1). The complete cell assembly was heated to 80 ^{o}C. The sample was placed into a heated sample holder and allowed to equilibrate to 80 +/- 0.5 ^{o} C. Alternatively, samples were melted using a micro-wave and aspirated into the IR cell. Approximately 1 ml of the sample passed through the transmission IR cell (to flush the cell) prior to recording the IR spectrum of the sample. The sample was then left in the transmission cell for several seconds (~ 20 - 30) to equilibrate to the temperature of the IR cell. Finally , the sample was discarded into the vacuum trap and the next samples were then aspirated into the IR cell for subsequent analysis. The FTIR spectrometer was interfaced to a PC computer

which operated the spectrometer using Omnic software (Nicolet Inc., Madison). The spectrometer was purged with dry air using a Balston dryer (Lexington , MA) . An initial single-beam infrared spectrum was collected of an empty sample compartment and saved as the background spectrum, a total of 512 scans were co-added at a resolution of 4 cm^{-1}. All subsequent infrared spectra of the samples were then automatically ratioed against the initial background spectrum to obtain a final absorbance spectrum for each sample . The absorbance spectra were then saved for subsequent analysis.

FTIR / ATR spectra was recorded employing a horizontal ZnSe accessory heated to 65 +/- 0.5 ° C by heating strips and an Omega controller (Omega , New Jersey). The spectrometer was purged with dry air by using a Balston dryer to expel the water and CO_2 components out of the optical cell cavity . An initial single-beam background infrared spectrum was recorded at 65 °C of the empty ATR ZnSe plate. Subsequently, the single-beam spectrum of the monoglycerides was recorded and ratioed against the background spectrum. Between each sample a cleaning protocol of the ATR plate was essential to remove any residual sample adhering to the ZnSe crystal. This was done to avoid buildup of hydrophobic compounds onto the ATR crystal. The cleaning was carried out by wiping the monoglycerides off the ATR plate and then adding an organic solvent such as iso-octane or xylenes, leaving it on for approximately 30 seconds and then wiping it off, this procedure was repeated twice prior to the introduction of the next sample for analysis.

Standards and Samples :

Approximately 45 pre-analyzed acetylated monoglycerides were obtained from the Eastman Kodak corporation. Each sample was of a thick and viscous butter-like texture. All 45 samples were pre-analyzed for their hydroxyl and saponification numbers using the recommended AOCS methods.

Development of a PLS calibration employing FTIR spectra recorded from the transmission flow-through cell:

The FT-IR spectra of the acetylated monoglyceride samples were recorded and automatically ratioed against the background spectrum. Prior to recording the IR spectra of the samples, the flow cell was cleaned with an organic solvent (i.e., such as xylenes) followed by iso-octane then allowed to dry for several seconds , and the temperature of the cell assembly was then equilibrated to 80 °C. The monoglyceride standards were completely melted prior to their introduction into the cell. Approximately 5 ml were used to flush the tubing leading to the cell and discarded through the by pass valve, subsequently ~ 1 ml was passed through the CaF_2 cell prior to recording the FTIR spectrum of the sample.

The monoglyceride samples were chosen to span the chemically determined hydroxyl values. The chemical hydroxyl values were correlated to the infrared spectral regions between 3700 and 3200 cm^{-1} , and, 1192 to 1135 cm^{-1}. A second PLS model was developed employing only the infrared spectral region between 3700 and 3200 cm^{-1} .

The PLS calibration model was developed using Nicolet Quant-IR calibration and prediction software package. The calibration routine involves a primary calibration of the standard compounds followed by a " Leave One out Validation" process which provides for a means of testing the predictive accuracy of the PLS calibration model. In the development of the PLS calibration mode the variance spectrum was generated from the differences in the infrared spectra of the standards so as to identify the location of the regions that correlated well with the chemical data. Two calibration models developed were obtained, the first employed a single infrared region between 3700 and 3200 cm^{-1} and the other included a second region between 1193 and 1135 cm^{-1}. A PRESS (Predicted Residual Error Sum of Squares) was calculated to determine the number of factors in the Leave-One-Out Validation of the calibration model.

The development of an FTIR / ATR method employing a PLS calibration model for the determination of Hydroxyl Number:

Prior to the scanning of the samples, the ZnSe crystal was cleaned with 1% triton solution. An organic solvent (i.e., iso-octane) was then used to further wipe the ZnSe crystal clean of any hydrophobic matter . An initial 512 scans was then recorded of the empty ZnSe crystal and stored as the background emmitance spectrum. This was then followed by recording the FTIR spectra of all the subsequent samples using 256 scans at 65 °C. In between each sample the plate was wiped clean twice with iso- octane and then let dry in open air . An aliquot of each monoglyceride solid standard was melted onto the heated ZnSe crystal and the IR spectrum recorded when the sample was completely melted.

The known chemical values were also correlated to the spectral -OH regions of 3700-3200 cm^{-1} and 1192 - 1135 cm^{-1} as described for the calibration based on the flow through transmission cell sampling method.

Analysis of Samples :

The infrared spectra of twenty-two monoglyceride samples were chosen for the development of a PLS both for the calibration model for transmission based and ATR based infrared sampling methods. To facilitate the routine analysis of the standards and samples, the PC-FT-IR spectrometer was pre-programmed employing Visual Basic programming language .

RESULTS

Spectra :

Figure 5.4 shows a plot of selected transmission FTIR spectra of monoglyceride standards in the region between 3700 and 3200 cm^{-1}. It can be seen that as the hydroxyl value increases for each monoglyceride sample, the peak area also increases proportionately. As the hydroxyl number increases, the peak (assigned to the -OH stretch) becomes broader

mainly due to the increase in hydrogen bonding in the sample. It can also be seen that the peak height increases as concentration increases. Although, the peak height increase does not necessarily increase proportionately with increasing concentration.

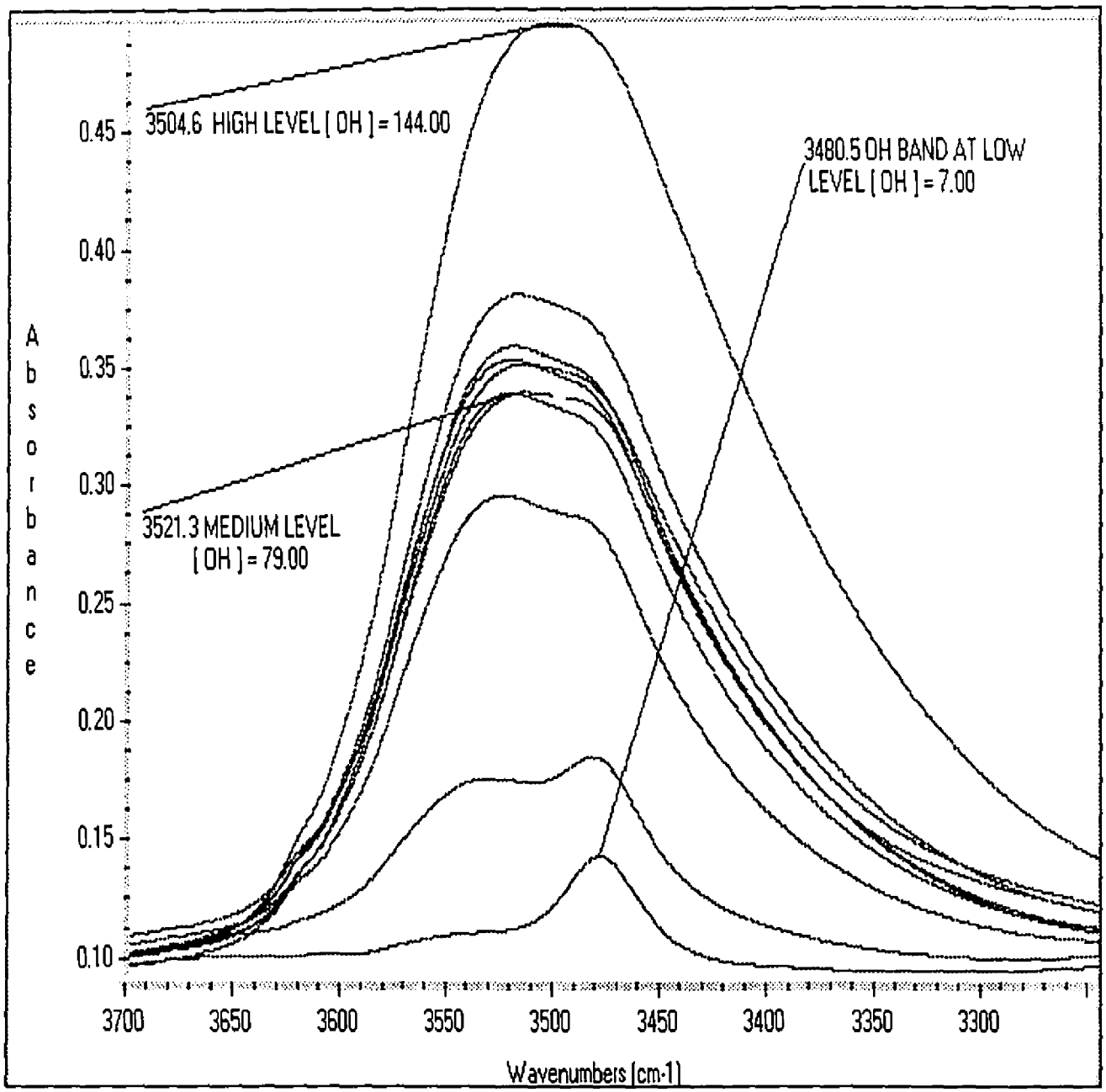


Figure 5.4 Infrared spectra of several monoglyceride standards varying in hydroxyl number for FT-IR calibration [spectral range between 3250 - 3700 cm^{-1}].

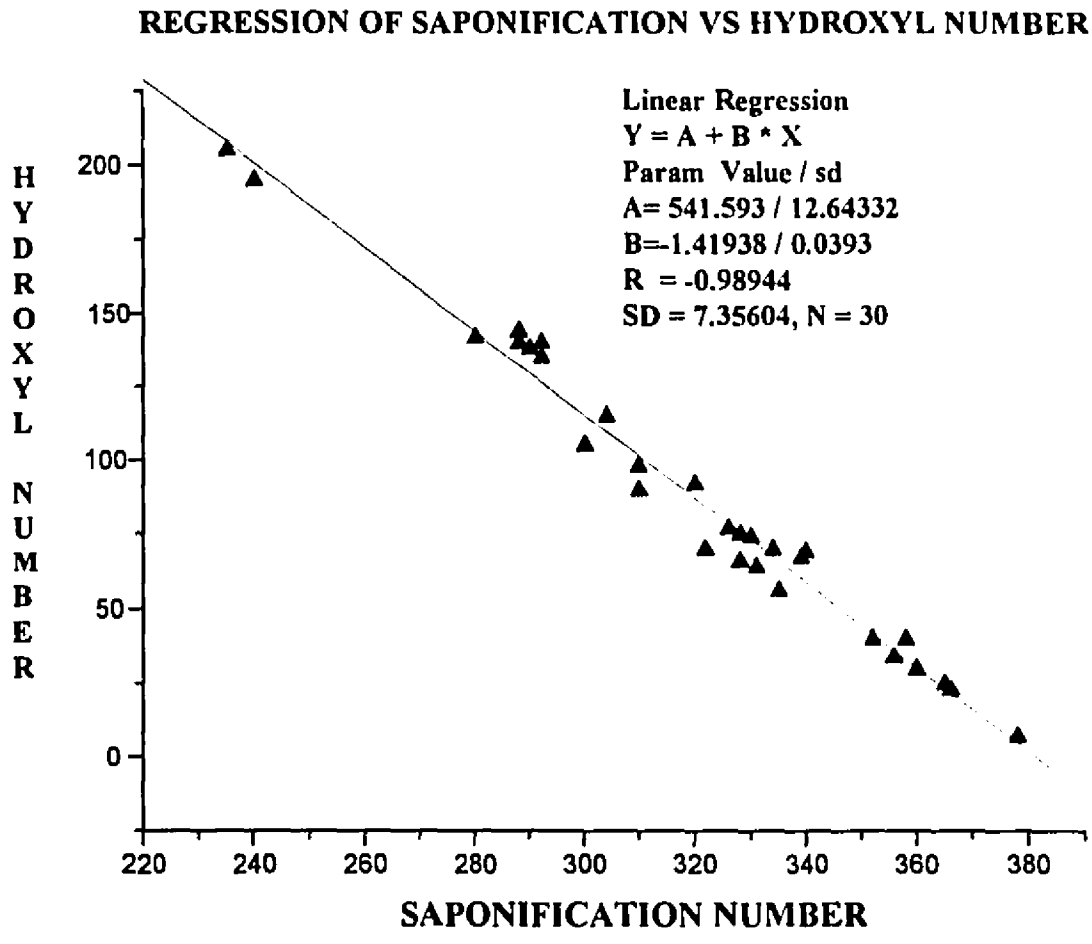


Figure 5.5 **A plot of the linear regression between the saponification number and hydroxyl number of 30 monoglyceride samples.**

Figure 5.5 depicts an inverse correlation between the hydroxyl values and saponification numbers. The chemical hydroxyl values were regressed against the chemical saponification

numbers and an inverse linear correlation was obtained. This is due to the fact that the two values are inversely related and this in turn would explain the non-proportional peak heights obtained by the standards for the hydroxyl value (Fig. 5.1a) along with the hydrogen bonding phenomenon. Thus an increase in the saponification number causes an underestimation of the hydroxyl value . The second spectral region used between 1193 and 1136 cm^{-1} due to the C-O-R bend was also affected by changes in the saponification number, resulting in non-linearity between peak height and the chemical hydroxyl value of the standard.

Because of the inverse relationship between hydroxyl value and saponification number and the hydrogen bonding effects, a calibration based on the peak height method would not be applicable. Therefore, the partial least squares model was considered. The partial least squares methodology is an alternative calibration approach which allows for the complete spectrum to be investigated.

Comparison Between (a) ATR and (b) Transmission sampling methods in developing a PLS calibration Mode:

a) FTIR / ATR sampling approach:

Approximately half of the supplied 45 monoglyceride samples were randomly chosen for calibration purposes.A PRESS plot was generated employing two spectral regions (3700-3200 cm^{-1}) as shown in figure 5.6. From inspection of the PRESS plot, the total number of factors for the calibration was approximately 2. Figure 5.7, shows a " leave-One-Out" validation plot of the PLS predicted hydroxyl number versus the hydroxyl number determined by the AOCS method. As shown in figure 5.7, the validation of the calibration model was successfully performed. The curve depicts the regression of the actual (i.e., the chemically derived hydroxyl values) versus predicted values (i.e., the IR predicted hydroxylvalues).

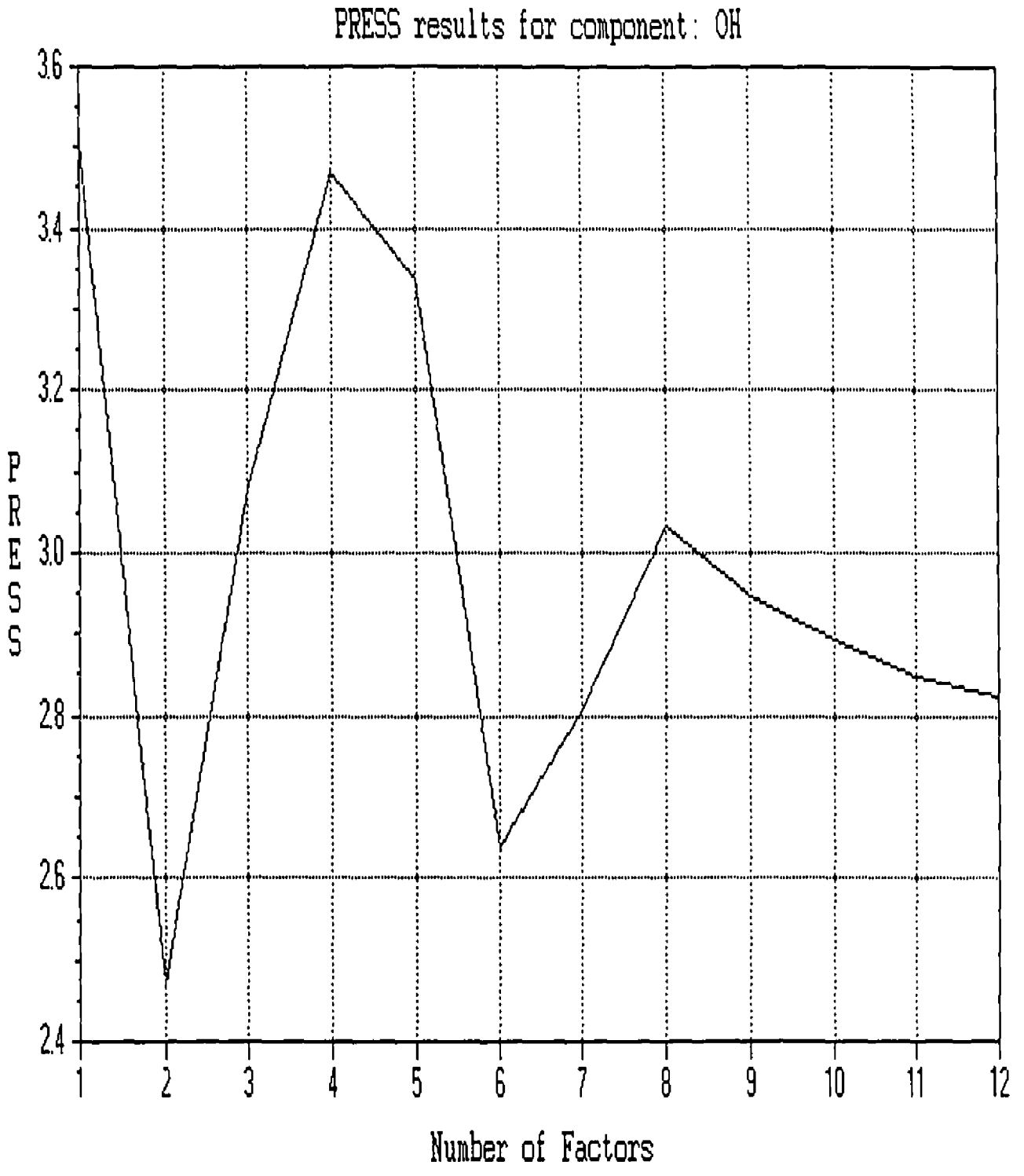


Figure 5.6 A plot of The predicted residual error sums of squares (PRESS) of the monoglyceride standards.

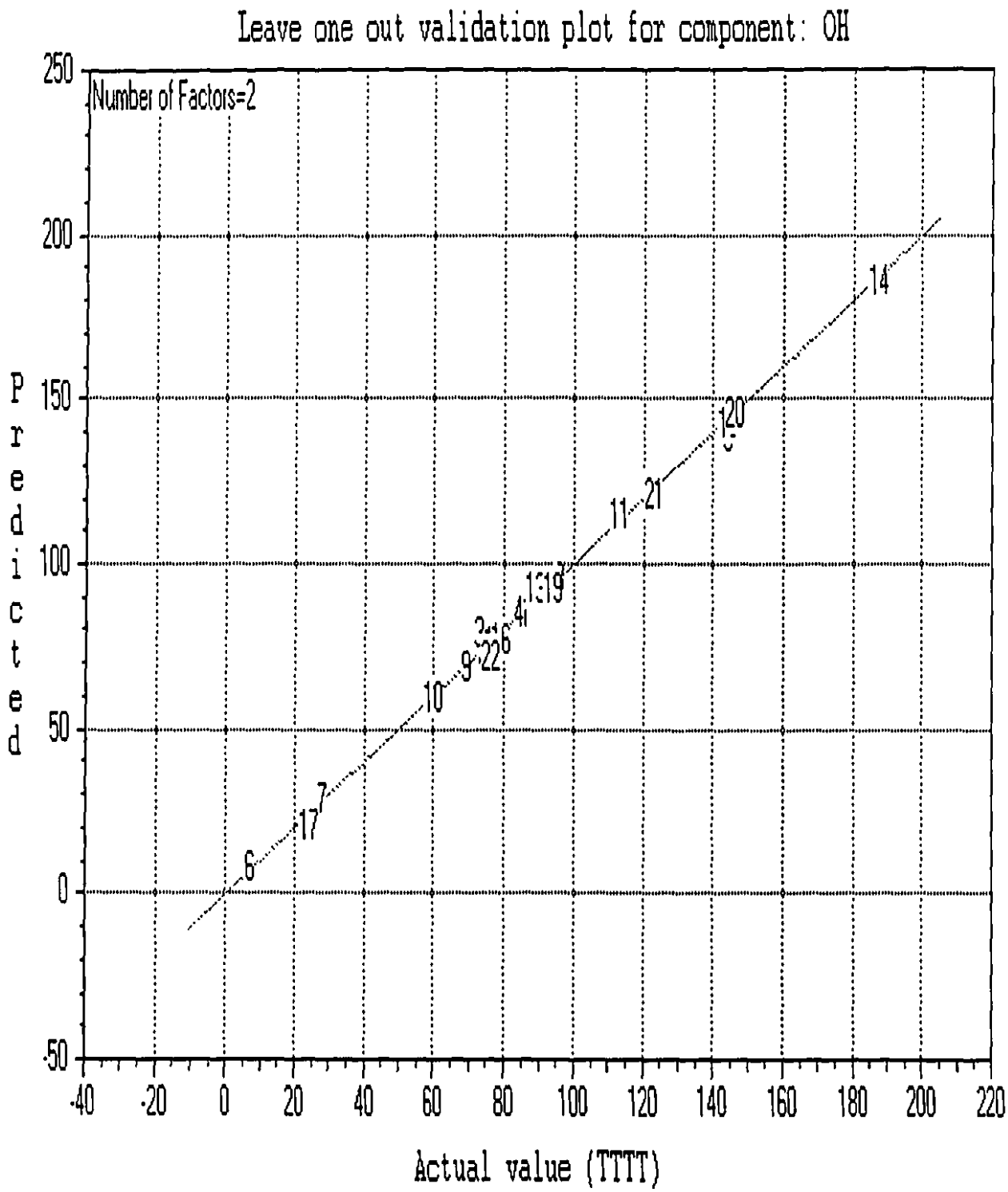


Figure 5.7 A plot of PLS predicted hydroxyl number versus the hydroxyl number determined by the AOCS method.

Table 5.1 contains the chemical and PLS predicted hydroxyl number of the standards along with the error difference between the two values. The summary of the errors for all the standards gives a value of 1.84. This is the overall average error of all the standards and is usually indicative of the successfully validated calibration model. Hence, it may be stated that the ATR calibration model obtained is satisfactory.

Table 5.1 Leave one out Cross Validation by Attenuated Total Reflectance spectroscopy values (i.e.actual chemical vs. calculated IR values).

STANDARD	ACTUAL	CALC.	ERROR
311169	86	86	0.5
310869	74	74	0.4
311251	73	79	6.9
311114	84	86	1.5
314656	144	139	5.2
310771	7	8	1.6
310871	28	29	0.8
311230	96	96	0.3
310885	69	69	0.4
310960	60	59	-0.4
310989	113	115	2.4
311193	77	77	-0.1
310868	89	93	4.3
314861	187	187	-0.3
314820	144	143	0.9
311014	79	77	-2.0
310725	24	21	-3.1
314518	146	148	2.1
311012	94	93	1.3
314540	146	146	-0.0
314806	123	122	-1.0
311017	76	72	4.1
			Av. error = 1.84

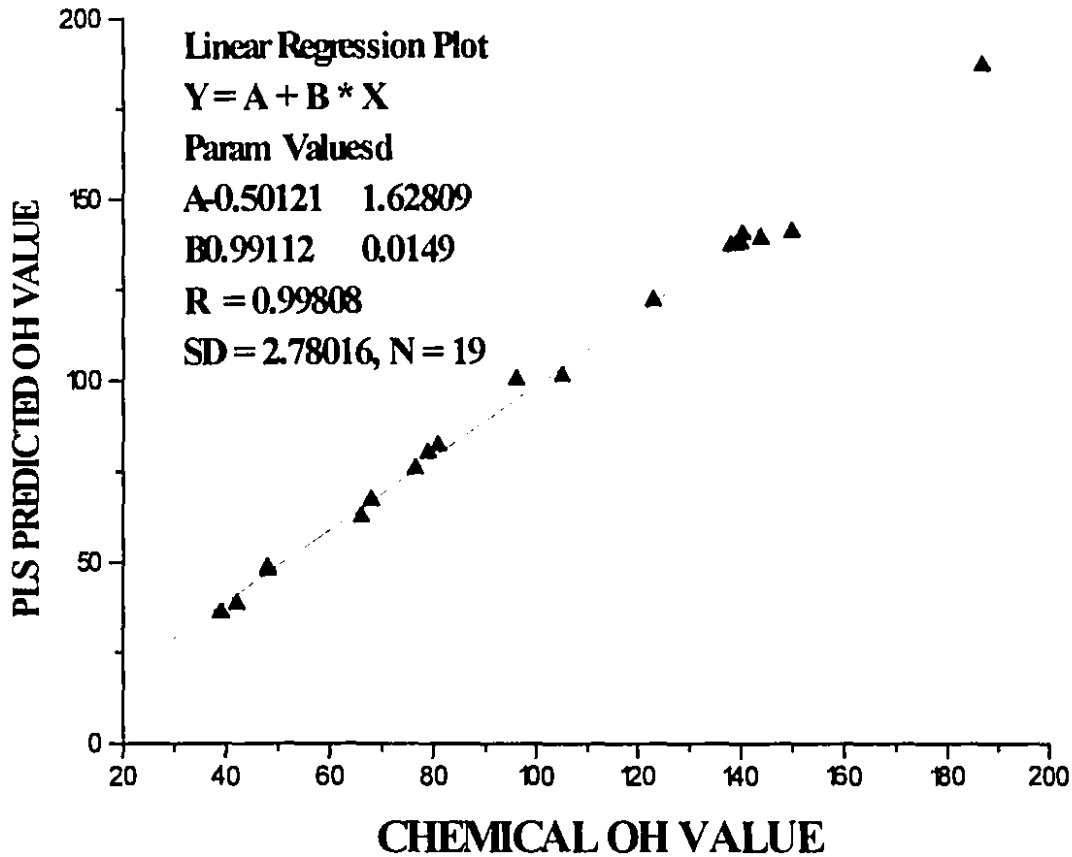
Table 5.2 depicts the PLS predicted hydroxyl number employing the above calibration model and chemical values. Each IR predicted value differed from its respective chemical value by approximately 3%.

Table 5.2 Predicted IR values by ATR / PLS.

STANDARD	ACTUAL	PREDICTED
310870	96.00	100.23
310872	48.00	48.62
311011	105.00	101.26
311202	66.00	62.61
311229	79.00	80.01
311238	68.00	66.99
314617	139.00	137.58
314656	144.00	139.30
314657	150.00	141.38
314686	140.50	140.46
314687	138.20	137.55
314806	123.00	122.04
314822	140.00	138.15
314861	187.00	187.52
310726	42.00	38.64
310873	48.00	47.81
310988	39.00	36.05
311161	76.50	75.56
311258	81.00	81.95

A plot of the PLS predicted versus actual chemical values, obtained from a linear regression model is presented in Figure 5.8 .

Figure 5.8 Regression of Chemical vs Predicted Hydroxyl values of Monoglycerides.



Shown along with the plot of the predicted values are the regression equation and the associated statistics. The plot is linear with a slope close to unity and a standard error of +/- 2.78 units. This plot illustrates that a linear relationship exists between the ATR / FT-IR determined hydroxyl values and the chemically determined values. Hence, considering all the time and savings derived by using the ATR /FT-IR protocol , it would be very advantageous to use the FT-IR-based method as an alternative to the chemical analysis without sacrificing accuracy.

b) Hydroxyl Value Determination Employing a Flow-Through Transmission IR cell:

The same samples employed for developing a calibration for the ATR method were also used for testing the transmission based method to allow for a direct comparison between the two techniques. The cell windows used were that of CaF_2 separated by a pathlength of approximately 38 microns. The cell compartment had been pre-heated to 80 °C as stated previously in this paper. Figure 5.9 shows the plot of PLS predicted hydroxyl number versus the hydroxyl number determined by the AOCS methods demonstrating that a calibration model was successfully developed.

Figure 5.9 Leave one out Validation plot by Transmission IR.

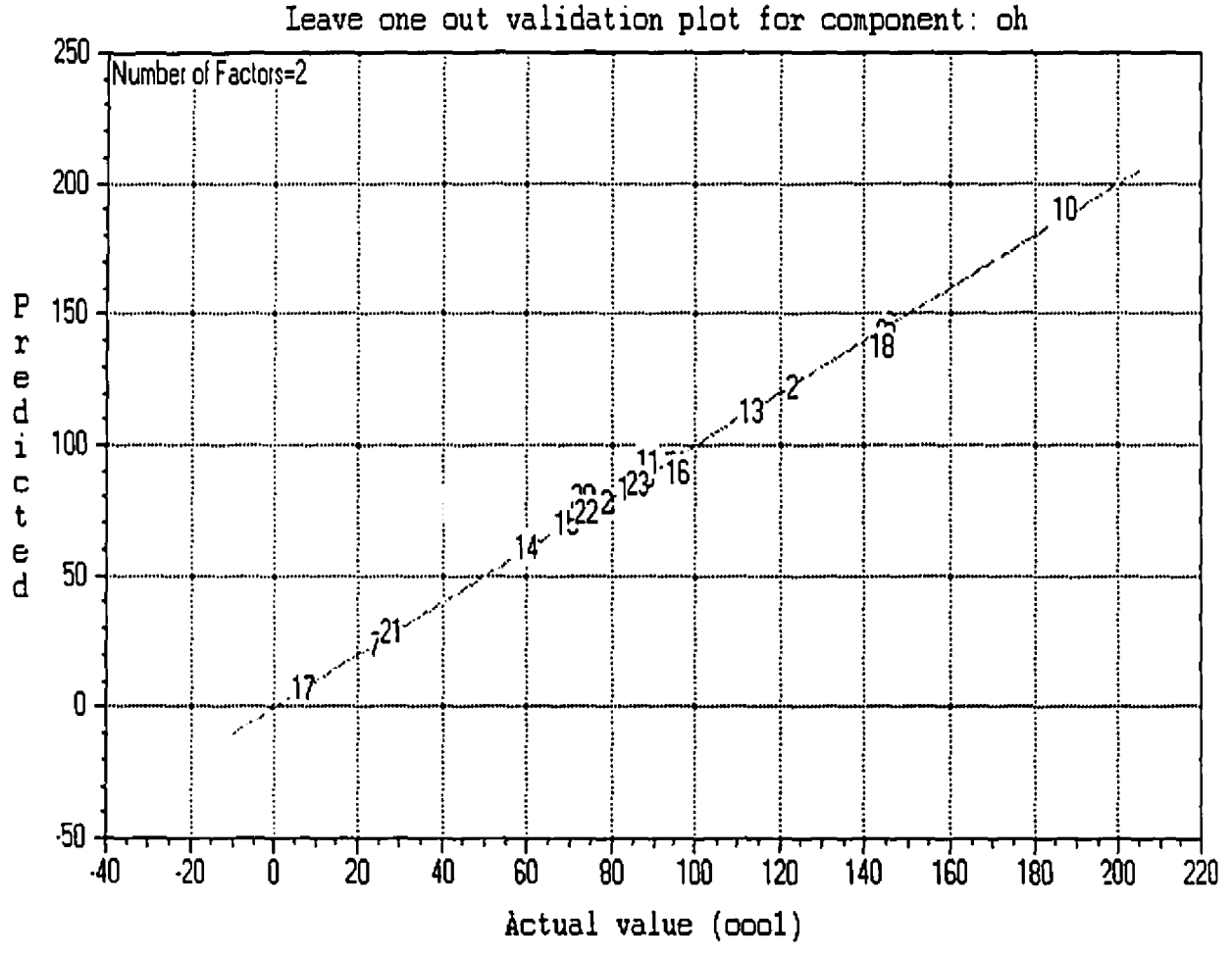


Table 5.3 Leave one out Validation by Transmission IR spectroscopy.

STANDARD	AOCS METHOD	PLS Predicted	ERROR
311017#2	76	72.3	-3.7
314806#2	123	122.6	-0.3
310868#2	89	88.7	-0.3
314540	146	146.6	0.5
311012	94	92.7	-1.3
314518	146	146.7	0.8
310725	24	23.8	-0.2
311014	79	76.9	-2.1
314820	144	142.9	-1.0
314861	187	190.6	3.7
310868	89	93.9	4.9
311193	77	78.2	1.2
310989	113	113.5	0.5
310960	60	61.3	1.3
310885	69	69.8	0.8
311230	96	89.5	-6.4
310771	7	7.8	0.8
314656	144	138.4	-5.5
311114	84	83.1	-0.9
311251	73	80.2	7.2
310871	28	29.4	1.4
310869	74	74.7	0.7
311169	86	86.1	0.1
ERROR	2.0	PCT. ERROR	2.8

Table 5.3 shows a list of hydroxyl numbers from the chemical method and those predicted by the PLS-calibration method. The mean error for all standards was approximately +/- 2 units. The slight increase in error as compared to ATR may be due to 1 or 2 standards inappropriately sampled (i.e., possible cross contamination or improper filling of the IR transmission cell). Hence, the overall error for this technique demonstrates that the

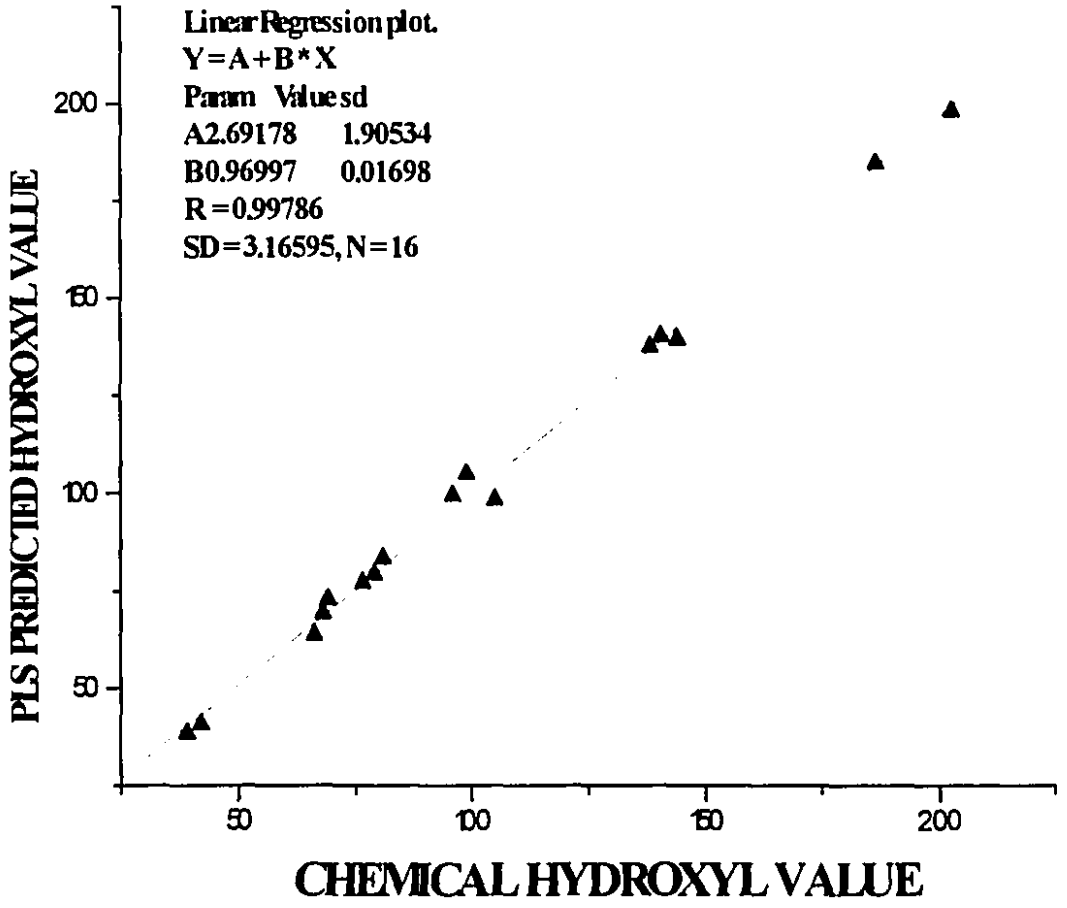
transmission mode is just as workable as is the ATR-based sampling method. Table 5.4 shows the AOCS hydroxyl values of a selected set of monoglycerides and those obtained from the PLS model . Most IR predictions come within a 5% range of the chemical values. In general, the overall predictive ability of the transmission model is comparable to the ATR based PLS model.

Table 5.4 Predicted Hydroxyl values by Transmission IR spectroscopy.

SAMPLE	HYDROXYL # FROM AOCS	PLS-PREDICTED
314861	187	185
314887	203	198
311249	69	73
311238	68	69
311258	81	83
311011	105	99
311229	79	79
314686	141	140
311161	77	77
314687	138	138
310726	42	41
310988	39	39
314656	144	139
311202	66	64
310870	96	99
311009	99	104

Figure 5.10 shows a plot of the data presented in table 5.4 . Along with the plot is also the regression equation for the curve and the correlation coefficient of 0.998 and a standard error of ~ 3.17. This also demonstrates the good predictive ability of the IR Transmission-based method.

Figure 5.10 Regression of chemical vs. PLS predicted hydroxyl number from transmission spectra of monoglycerides.



DISCUSSION

The results obtained in this study demonstrate a successful application of both the ATR and transmission / FT-IR techniques for the determination of the hydroxyl value of monoglycerides. The ATR sampling method was somewhat more troublesome to use than the flow cell irrespective of the calibration performance. The transmission mode of

analysis was fairly trivial although it required the need for a customized heated sampling accessory. The transmission cell was heated to 80°C allowing the monoglyceride samples to remain in their melted state. If the transfer line had not been heated to the same temperature, the monoglycerides would of solidified causing blockage in the transfer line leading to the IR cell. The increased pathlength in the flow cell provided a stronger signal and was easier to clean. For the ATR method, a cleaning step is required between each sample and is thus cumbersome and time consuming. This was necessary to avoid monoglyceride buildup on the ATR crystal. Even though the ATR technique has some drawbacks , it is still fairly simple to use as an alternative method .

The PLS calibration developed is based on a multivariate analysis approach and makes use of a broad frequency range rather than the peak height measurements at selected frequencies. The multivariate approach of analysis has been cited in the literature to provide a superior precision in FT-IR quantitative analysis (ref.10-12) . The chemometric software package (i.e., Quant-IR), basically correlates regions within a spectrum to the measure of interest (i.e., such as concentration or physical attributes). The spectral region of interest to be included in the PLS calibration are those that account for the major variations in the measure of interest. These regions of the spectrum should correspond to the characteristic vibrations of the hydroxyl absorption's within the monoglycerides. Thus, the partial least squares (PLS) model automatically incorporates the contribution from these spectral regions singling out any contribution at a specific wavelength as would be the case for peak height vs. concentration calibration. For these reasons the PLS was chosen to be the optimal method of calibration. Based on the analysis of the acetylated monoglycerides, the optimized spectral correlation regions for the determination of hydroxyl values was determined to be between 3730 and 3200 cm^{-1} for the OH stretch and 1193 to 1136 cm^{-1} for the ester linkage. The multivariate analysis also allows for the full information content of the infrared spectrum to be exploited. The PLS results obtained with both sampling methods demonstrated that ATR and transmission were fairly comparable.

Both ATR and transmission based protocols required little skill compared to the chemical analysis method. Due to these factors, it would be safe to state that the reproducibility between analysis is increased significantly by replacing the chemical method by an FT-IR analysis. The coupling of the FT-IR spectrometer to a PC also facilitated the data acquisition and spectral analysis.

The IR method developed provides for a practical procedure that may be implemented in a straightforward manner for the hydroxyl value determination of monoglycerides. The average time of analysis to acquire data employing either method varied to some extent. The ATR required approximately 6-7 minutes between sample analysis including the cleaning protocol. The transmission mode required ~ 5-6 minutes between samples, since the sample had to be melted down prior to analysis and a pre-sipping routine was required to rinse the IR cell with the new sample. Both methods required less time for analysis compared to the AOCS chemical method. Thus, the ATR/ FTIR and transmission / FTIR methods provide for a practical, simple , cost efficient and reagent - free means of rapidly analyzing a large number of monoglycerides.

The above discussion outlined significant advantages of the ATR and flow-through FTIR based methods over the standard chemical method used for hydroxyl value determination. The accuracy of both ATR and flow cell methodologies is related to the accuracy of the primary method (i.e., chemical method). The FT-IR is regarded as a secondary method and the degree of concurrence one can obtain with respect to the primary chemical method is depended on the accuracy of the primary method used. The precision and accuracy of primary methods are affected by many technical as well as experimental errors. These factors are incorporated into chemical values obtained and in turn affect the secondary method of calibration. Therefore, the calibration errors obtained in the experimental protocol incorporates two sources of errors. The first is that of the primary chemical method and the second from the sampling and instrumental errors. Despite these limitations both the ATR and transmission-based techniques provided results close to the

chemically derived hydroxyl values and therefore can be considered adequate for quality control purposes.

CONCLUSION

The results obtained from this study indicate that mid-FTIR spectroscopy can be employed in the determination of the hydroxyl number of monoglycerides. Both ATR and transmission flow cell-based sampling techniques provided comparable performance. The main disadvantage of the ATR method was due to the need to employ organic solvents for cleaning the ATR crystal. In the case of the transmission flow cell-based method , the main disadvantage to the method itself was the requirement for construction of a customized heated cell assembly. The transmission flow-through cell required less cleaning making this approach much more attractive. Another advantage with the transmission technique is the signal to noise level in the transmission spectra which is much higher than that obtained by ATR. All sampling operations required approximately 5-7 minutes. Along with the advantage in decreased analysis time, simplicity and the elimination of the need for chemical reagents makes this method more attractive to use. In conclusion, once the FT-IR spectrometer is calibrated against a primary chemical method , further hydroxyl value determinations may be performed rapidly.

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APPENDIX A

```
Sub Command1_Click ( )
' This macro is used to generate a CSV file (for ORIGIN)
' containing peak heights vs. time for oil monitoring
' experiments
' This version is written for .spa files ( Nicolet Omnic Software )
' Dimension array variables to fit maximum number of spectra
' to be processed
ReDim itemx(170)
ReDim itemy(170)
heading = "BASE FILE NAME"
msg = "INPUT BASE FILE NAME FOR THIS SET OF SAMPLES"
defval = 1
FirstSpectrum = InputBox(msg, heading, defval)

heading = "LAST SPECTRUM NUMBER"
msg = "INPUT NUMBER OF LAST SPECTRUM"
defval = ""
LastSpectrum = InputBox(msg, heading, defval)

heading = "FIRST BASELINE POINT"
msg = "INPUT FREQUENCY OF FIRST BASELINE POINT"
defval = 3750
base1 = InputBox(msg, heading, defval)
heading = "LAST BASELINE POINT"
defval = 3750
```

```
base2 = InputBox(msg, heading, defval)

heading = "FREQUENCY CODE"
msg = "INPUT A FREQUENCY CODE (CSV FILE NAME WILL BE BASE FILE
NAME + CODE  - CANNOT EXCEED 8 CHARACTERS)"
defval = 3444
freqcode = mInputBox(msg, heading, defval)

    form1.Hide
    cmdtxt$ = "import" + name1 + Format$(FirstSpectrum, "000") + ".spa"
    executeomnic cmdtxt$
    executeomnic "Smooth 15"
    cmdtxt$ = "CorrectedPeakHeight " + Format$(base1) + "" + Format$Peak + "" +
Format(base2)
   executeomnic cmdtxt$
   ht$ = getomnic("result current")
   baseht = getval(ht$, "HEIGHT")
   itemx(1) = 0
   itemy(1) = 0
   y = 1
  NextFreq = 1
  NumSpec = (LastSpectrum - Firstspectrum) +1
For numb = 2 To NumSpec
   numb1 = numb + (FirstSpectrum - 1)
   prevnumb = numb -1
   cmdtxt$ = "import " + name1 + Format$(numb1,"000") + ".spa"
   executeomnic cmdtxt$
```

```
    executeomnic "Smooth 15"
     cmdtext$ = "CorrectedPeakHeight" + Format$(base1) + "" + Format$(peak) + "" +
Format$(base2)
     executeomniuc cmdtext$
     ht$ =  getomnic ("result current")
     htnext = getval (ht$, "Height")
     htoil = htnext - baseht
"Array itemx contains time variable (spectra scanned every 40 min)
     itemx(numb) = itemx(prevnumb) + .67
     itemy(numb) = htoil
    executeomnic "select all"
    executeomnic "deletespectrum"
Next numb
outfile = name1 + Format$(freqcode) + ".csv"
Open outfile For Output As #1
For numb = 1 To NumSpec
    x = itemx(numb)
    y = itemy(numb)
    write #1, x, y
Next numb
form1.Show
End Sub

Sub Form Load ( )
Load OMTALK
executeomnic "MAXIMIZEWINDOW"
End Sub
```