Determination of Peroxide Value and Anisidine Value using Fourier Transform Infrared Spectroscopy

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Abstract

Lipid oxidation has important consequences in the edible oil industry, producing compounds with sensory impact and thus reducing the economic value of the products. This work focused on the development of two Fourier transform infrared (FTIR) spectroscopy methods for the measurement of peroxide value (PV) and anisidine value (AV), representing the primary and secondary oxidation products of edible oils.

The infrared method developed for PV determination was based on a mathematical treatment by the partial least squares method of the information contained in the spectral region between 3750 and 3150 cm^{-1}. The sources of interference present in that region (water, alcohols, free fatty acids, mono- and diglycerides) were included in the calibration matrix to generalize its application to the measurement of PV of any oxidized oil. The method allows the measure of PV within ±1.31PV.

The second method developed considered aldehyde content and anisidine value, a measure of secondary oxidation products. PLS was applied to the spectral regions between 2800 and 2670 cm^{-1} and between 1725 and 1670 cm^{-1}. Two calibrations were developed, one using synthetic calibration standards and a second using oils thermally stressed at 120, 155 and 200°C. The sources of interference (water, free fatty acids, hydroperoxides, alcohols and ketones) and the hydrogen bonding effect between alcohols and hydroperoxides and the triglyceride ester linkage were included in the synthetic calibration. The first approach was capable of measuring individual classes of aldehydes and AV within ±1.65 AV units. The PLS calibration derived by using thermally stressed samples as calibration standards provided similar predictive accuracy. As such, the quantitative determination of AV was shown to be feasible, the synthetic calibration approach providing additional information on the aldehyde types present in a sample and allowing the use of a simple gravimetric approach for calibrating an FTIR spectrometer.

The two methods developed are rapid (~2 min/sample) and have the advantage of being automatable. An infrared system coupled to a computer can collect the spectrum of an oil, analyze it and present a report without the need for personnel trained in FTIR spectroscopy. The cost of such a system would rapidly be absorbed through savings on personnel cost, time and chemical reagents required for conventional chemical methods and as such provides a useful advance in quality control methodology for the edible oils sector.

Résumé

L'oxydation des lipides est un phénomène d'importance pour l'industrie de l'huile comestible parce qu'elle engendre des sous-produits ayant un impact sensoriel qui réduisent la valeur économique des huiles. Ce travail était centré sur le développement de deux méthodes utilisant la spectroscopie infrarouge transformée de Fourier pour mesurer l'Indice de Peroxide (PV) et la Valeur d'Anisidine (AV) représentant respectivement les produits d'oxydation primaires et secondaires des huiles végétales.

La méthode infrarouge développée pour mesurer le PV est basée sur le traitement mathématique par méthode des moindres carrés partiels des informations contenues dans la région située entre 3750 et 3150 cm^{-1}. Les sources d'interférence présentes dans cette région (eau, alcools, acides gras libres, mono- et diglycérides) sont incluses dans la matrice de calibration afin de généraliser son application à tout système d'huile oxydée. La méthode permet de mesurer l'Indice de Peroxide avec une erreur de ±1.31 PV.

La seconde méthode développée permet de mesurer les aldéhydes, produits secondaires de l'oxydation et la Valeur d'Anisidine. La méthode des moindres carrés partiels est appliqué aux régions situées entre 2800 et 2670 cm^{-1} et entre 1725-1670 cm^{-1}. Deux calibrations ont été développées, l'une utilisant des standards de calibration synthétiques et la seconde utilisant des huiles ayant subi un traitement thermique à 120, 155 and 200°C. Les sources d'interférence (eau, acides gras libres, hydroperoxides, alcools et cétones) et l'effet des liens hydrogène entre le lien ester des triglycérides et les alcools et hydroperoxides ont été inclus dans la calibration. La première approche s'est montrée capable de mesurer les classes d'aldéhydes individuellement et la valeur d'anisidine à ±1.65 AV. La seconde approche permettant de produire une calibration à partir d'huiles ayant subi un traitement thermique a démontré la même justesse de prédiction.

Les deux méthodes sont rapides (~2 min/échantillon) et possèdent l'avantage de pouvoir être automatisées. Ainsi, un système infrarouge couplé à un ordinateur peut recueillir le spectre de l'échantillon, l'analyser et imprimer un rapport sans l'assistance de personnel spécialisé en spectroscopie FTIR. Les coûts d'un tel système seraient rapidements amortis grâce aux épargnes effectuées sur les coûts du personnel, du temps et des réactifs chimiques requis par les méthodes chimiques traditionnelles, fournissant ainsi un outil d'avangarde pour le controle de la qualité dans le secteur des huiles comestibles.

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Chapter 1

Introduction

Lipid oxidation is an important phenomenon in the food industry, being responsible for the deterioration of the quality of fats and oils and in foods containing fat. It is one of the four common deterioration mechanisms occurring in fats and oils along with hydrolysis, cross-contamination and contamination with foreign substances (Idris et al., 1992). Not only does oxidation affect the economic value of pure fat and oil systems like butter, margarine, edible oils and shortenings, but it also alters a large number of complex foods, including meats. The oxidation of lipids in foods produces rancidity and spoilage, extensive oxidation possibly giving rise to toxic compounds and overall, having negative economic consequences for the food industry. In order to minimize the negative effects of lipid oxidation on food products, the best strategy is to understand the state of oil degradation and react accordingly, be it by appropriate packaging or addition of antioxidants. A number of chemical methods are available to the industry to measure the state of oxidation but none alone is representative or rapid enough to be efficient in characterizing the extent of oxidation and the oxidative history of the product. Moreover, most standard methods of analysis make use of chemical reagents harmful to the environment and often need the skills of a qualified technician. With these factors in mind, the McGill IR group has endeavored to develop methods making use of infrared spectroscopy to rapidly and accurately analyze the oxidative state of oils and fats.

A context for the work carried out is presented via a literature review focusing on lipid oxidation, the mechanisms involved, the consequences and the methods most widely used to measure the oxidative state of lipids. An overview of the principles of IR/FTIR spectroscopy, the description of the instruments and related publications on IR spectroscopy follow. Subsequently, the general concepts of methodology development for the determination of hydroperoxide and aldehyde content is presented, followed by the results of the determination of peroxide value and anisidine value by FTIR. The work concludes with a general summary and analysis of the pros and cons of the methodology developed and its applicability in the industry.

Chapter 2

Literature review

2.1 Lipid Autoxidation

2.1.1 Introduction to Lipid Autoxidation

Rancidity is defined as a sensory defect resulting from oxidation or hydrolysis of lipids. In general terms, oxidation involves an attack of unsaturated fatty acids by oxygen with different combinations of factors such as heat, prooxidants, lipoxygenases and light acting as initiators or catalysts (Frankel, 1984). In the case of the present study, autoxidation alone is considered, excluding any enzymatic involvement. Lipid autoxidation is a rather complex chain of reactions whereby unsaturated fatty acids are attacked by molecular oxygen, via a free radical chain mechanism, to form fatty acyl hydroperoxides, the primary product of lipid autoxidation (Gray, 1978; Fennema, 1985). A series of secondary reactions follows the initial formation of hydroperoxides. These reactions lead to the degradation of the lipids and the development of oxidative rancidity. The first stage of the reaction produces hydroperoxides which will undergo further modifications to finally produce secondary products with sensory impact. In fact, the degradation of hydroperoxides leads to the formation of low molecular weight compounds such as aldehydes, ketones, alcohols, hydrocarbons, furans, esters, free fatty acids and

lactones which influence the taste and smell of the food product (Frankel, 1984; Ladikos and Lougovois, 1990).

The oxidation chain reaction is initiated when a labile atom of hydrogen leaves its site on the fatty acid chain, producing a free lipid radical which reacts with available oxygen to form a peroxy radical. This radical then abstracts an atom of hydrogen from another fatty acid chain to produce an hydroperoxide and a new peroxy radical, initiating a chain reaction. Figure 1 gives a general overview of the reactions typical to lipid autoxidation.

Initiation

$RH + O_2 \rightarrow$ free radicals (R., ROO.)

Propagation

$R. + O_2 \rightarrow ROO.$

$ROO. + RH \rightarrow ROOH + R.$

Termination

$R. + R. \rightarrow R\text{-}R$

$R. + ROO. \rightarrow RO_2R$

$ROO. + ROO. \rightarrow R{=}O + ROH$

Figure 1: Simplified Free Radical Mechanism of Oxidation.
(Source: Fennema, 1985)

As mentioned previously, autoxidation mainly affects unsaturated fatty acids due to the susceptibility of their double bonds to attack by oxygen. Accordingly, the fatty acid composition of oils and fats is an important factor in predicting the susceptibility of a product to oxidation. The more unsaturated a lipid is, the more susceptible it is to oxidation. The oils considered in this work are commercial vegetable oils which have varying composition depending on their source. The chain length, the degree of unsaturation of the fatty acids and their distribution on the triglyceride are all related to the physical state of the product at a determined temperature and the susceptibility to oxidation. Despite their high degree of unsaturation, vegetable oils benefit from the protection of natural antioxidants such as vitamin E. Table 1 presents the composition of some common vegetable oils.

Lipid	16:0	16:1	18:0	18:1	18:2	18:3	20:1
Sunflower oil	6 %		3 %	27 %	64 %		
Soya bean oil	12 %		4 %	24 %	51 %	9 %	
Rapeseed oil	3 %		2 %	22 %	15 %	14 %	15 %
Olive oil	14 %	2 %	2 %	64 %	16 %		

Table 1: Fatty Acids Composition of Selected Vegetable Oils (Hamilton, 1986).

In the last decade, greater attention has been given to the health hazards associated with lipid oxidation products, as aside from playing an important role in flavor and odor development, oxidation has a noticeable impact on the nutritional and functional value of

foods. This is mostly due to the fact that hydroperoxide radicals are very reactive with sulfur and amines, the functional groups of amino acids, whereas aldehydes and epoxides, which are secondary products of lipid oxidation, also react with thiols from cysteine. These affinities lead to a radical-induced cross-linking or scission of proteins (Gardner, 1979). Besides affecting their nutritional properties, the interactions with proteins can modify their solubility, water-binding and emulsification capacities, thus producing changes in the texture and other rheological properties of the protein systems. Moreover, it has been recognized that peroxides and free radicals destroy fat-soluble vitamins A and E. Lipid peroxides accelerate the rate of turnover of vitamin E in the body and thus increase the requirement for this vitamin. It is also known that damage caused to proteins may affect membranes and biological components, thus being implicated in modifications of vital cell functions (Frankel, 1984). Questions concerning possible carcinogenicity and relations with atherosclerosis, tissue congestion, fatty degeneration and necrosis in mice are also under investigation and enhance the importance of monitoring and controlling the oxidation process (Fennema, 1985).

2.1.2 Primary Oxidation Products

The primary products of oxidation are hydroperoxides, which are odorless and tasteless compounds which will further degrade into products with sensory impact (Frankel, 1984). The first step of the oxidation process is the production of free radicals either by thermal dissociation (in the case of thermal oxidation) or catalyzed by light, metal

ions (heme iron in the case of meat) with or without the presence of photosensitizers such as chlorophyll. Figure 2 presents the formation of primary oxidation products from linolenate.

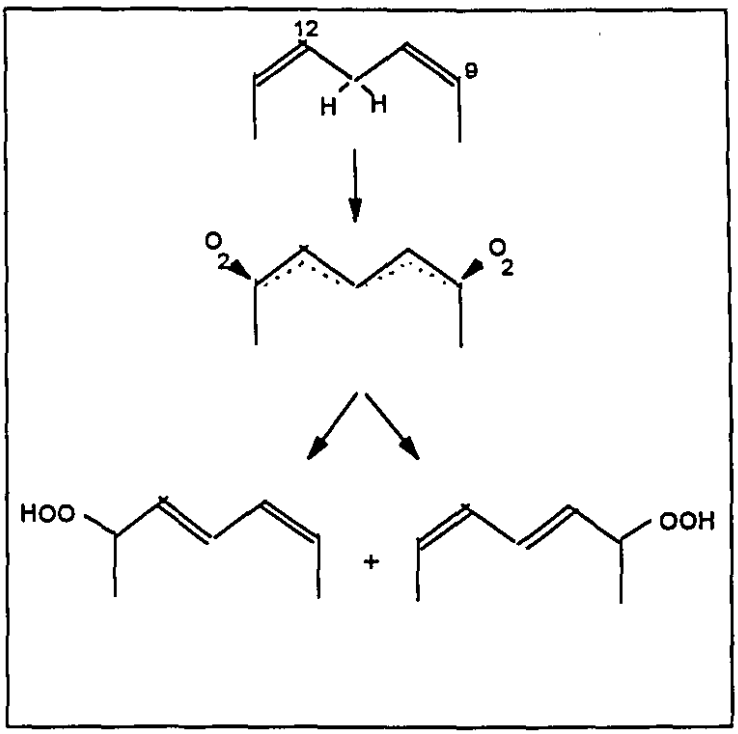

Figure 2: Production of Hydroperoxides from Linolenate (adapted from Frankel et al., 1977).

The reaction is favored because the abstraction of the hydrogen molecules on the carbon adjacent to the double bonds allows the formation of a very stable allyl radical where electrons are delocalized over the five carbon atoms (Paquette et al., 1985a).

Monitoring the formation of hydroperoxides provides an indication of the progress of the oxidation reaction which is indirectly related to the quality of the product. As such, hydroperoxides are indicators of future sensory defects justifying the need for their quantification in order to allow the prediction or prevention of further deterioration.

2.1.3 Secondary Oxidation Products

Following their formation, hydroperoxides can react with hydroxy or alkoxy radicals and a further cleavage of the fatty acid chain adjacent to the alkoxy radical will produce low molecular weight volatiles. As the reactions proceed, rancidity develops caused by the accumulation of volatile secondary products. Among the resulting products, carbonyl compounds and alcohols are in large part responsible for the rancid off-flavor of fats and oils due to their very low threshold values. Generally speaking, saturated aldehydes give a feeling of power, warmth, depth, roundness and freshness to products while unsaturated aldehydes are characterized by a sweet, fruity, oily and fatty note and alcohols by a solventy, grassy, green and fatty flavor (Hamilton, 1989). Table 2 presents the flavor thresholds and contributions in paraffin oil of some common aldehydes found in oxidized lipids.

Aldehydes can further be oxidized to produce tertiary oxidation products including short chain free fatty acids. Hydroperoxides can also condense into dimers and polymers

which may in turn oxidize and decompose into volatile breakdown products. These secondary oxidation products are also susceptible to break down and form volatile compounds and dialdehydes contributing to flavor deterioration (Ladikos and Lougovois, 1990). Measurement of the termination stage of the oxidation process, e.g. the measurement of the amount of secondary oxidation products, gives an indication of the quality of a fat or an oil and its past history but generally it is an inadequate indication of lipid flavor stability.

Aldehyde	Threshold (ppm)	Contribution
Hexanal	0.60	fresh, green
cis-3-Hexenal	0.09	green bean
trans-2-Hexenal	0.60	green
trans-2,*trans*-4-Decadienal	0.10	stale frying oil

Table 2: Flavor Threshold in Paraffin Oil and Contribution of Selected Aldehydes (adapted from Hudson, 1989).

2.2 Monitoring the Oxidation Process

A number of chemical methods are available to monitor oxidation processes in foods. The oxidation of food lipids can be measured at different stages including the initiation stage where there is a loss of unsaturation as measured by a decrease in the Iodine Value

and modification of the unsaturation by the appearance of isolated *trans* isomers (AOCS, 1989). The propagation step can be followed by measurement of the accumulation of primary peroxidation products via the determination of peroxide value (AOCS, 1989). The termination stage can be studied by measurement of general secondary carbonyl and hydrocarbon compounds (ultraviolet absorption of dinitrophenylhydrazones, hydroxylamine-hydrochloride reaction) or presence of specific types of products such as aldehydes (anisidine value), malonaldehyde (TBA number) and Free Fatty Acid Content (Henick et al., 1954; Bhalerao et al., 1961; AOCS, 1989; Gray, 1992;). On the other hand, susceptibility tests are often used to measure the stability of a lipid under specific conditions and include tests such as the Schaal Oven Test, Active Oxygen Method (Swift test) and Oxygen Adsorption Methods (Gray, 1978; Hamilton, 1989). Sensory evaluation is used as a subjective means of measuring taste and odor changes, but it has the disadvantages of being time consuming at all stages of the process, from the training of the panelists to the interpretation of the results. Moreover, the reproducibility of taste panels is often poor when the panelists training is inappropriate and because it is based on personal perceptions which vary according to the age, race, eating and smoking habits of the panelists.

Considering that the goal of this study is to develop rapid spectroscopic methods adaptable for the edible oil industry to monitor the oxidative state of vegetable oils, the focus has to be on oxidative measures familiar to the industry. It was determined that the chemical methods most utilized were peroxide value and anisidine value.

2.2.1 Peroxide value

The peroxide value (PV) is an official standard method of analysis and it is widely used around the world (AOCS, 1989). In the iodometric method, PV corresponds to the quantity of peroxides in a sample, expressed in terms of milliequivalents of active oxygen per kg, which oxidizes potassium iodide under the operating conditions (International Union of Pure and Applied Chemistry, 1986). The reactions are summarized in Figure 3.

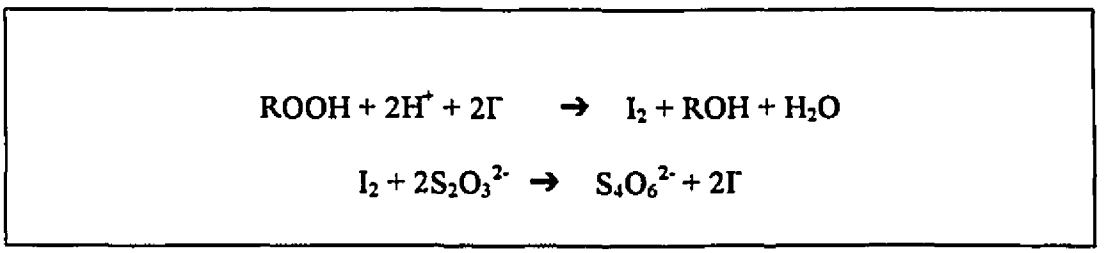

Figure 3: Mechanism of the Determination of Peroxide Value (adapted from AOCS, 1989).

The results and suitability of the test are highly dependent on the conditions of the experiment, namely temperature, time and the reducing agent used and the possible addition of iodine to double bonds of unsaturated fatty acids and liberation of iodine by air oxidation of the iodide (Robards et al., 1988, Gray, 1992). Consequently, whenever the quality of an oil is expressed in terms of PV, the analytical method chosen needs to be specified. The measurement of peroxide formation has the advantages of being useful for bulk lipids found in foodstuffs and applicable to all normal fats and oils. However, it is not a suitable method to determine the level of oxidation in frying oils typically maintained

at 170-225°C due to the rapid decomposition of the hydroperoxides at these temperatures. Moreover, the method fails to adequately measure low PV because of difficulties with the determination of the endpoint and the relative solubility of fats in the solvent suggested (Robards et al., 1988). The sensitivity of the method for peroxide value determination has been improved by replacing the titration step with an automated routine which would not require the visual evaluation of the endpoint and avoiding dilutions, the main sources of errors attributable to the operator in the chemical technique. Modifications of the method have been proposed which replace the titration step by an electrochemical technique in which the liberated iodine is reduced at a platinum electrode maintained at a constant potential, however deaeration is required to avoid experiment-induced peroxide formation (Fiedler, 1974). A spectrophotometric method based on the peroxide oxidation of iron(II) to iron(III) and a spectrophotometric measurement based on the reduction of lipid hydroperoxides with titanium(III) (Eskin and Frenkel, 1976) have also been introduced. The suggested improvements of the method increase its accuracy and reduce the human error factor, but in return, the time required per analysis is extended and the methods loses applicability for the industry.

2.2.2 Aldehyde content

A variety of methods are available to determine the carbonyl content of lipids, a number of them making use of visible/UV spectrophotometry. One is the official thiobarbituric acid test (TBA) (AOCS, 1989), which measures malonaldehyde, however,

there is no evidence that malonaldehyde is found in all oxidized systems. In fact, a mechanism postulated by Dahle et al. (1962) indicates that only peroxides which possess unsaturation β,γ to the peroxide group are capable of undergoing cyclisation to form malonaldehyde, such peroxides only being produced from fatty acids containing three or more double bonds. As a result, despite the fact that the TBA measurement is appealing for quality control purposes because it specifically quantifies a product with sensory impact, it is only meaningful for comparison of results obtained from a single material at different stages of oxidation, limiting its use to repetitive processes.

Another method widely used in research is the measurement of ultraviolet absorption by dinitrophenylhydrazone derivatives of carbonyl compounds. This measure is favored for certain analyses because it gives an answer exclusively related to the amount of carbonyls, regardless of their specific types. The method, however, has been criticized because hydroperoxides are decomposed at the temperature set for the analytical conditions, potentially increasing the level of carbonyl compounds. In order to avoid this problem, it has been suggested to carry out the reaction at $5^{o}C$, however completion of the reaction takes twenty hours and is inappropriate for routine analysis (Gray, 1978).

Another chemical method used to measure carbonyl compounds involves a reaction with hydroxylamine and hydrochloride. For that measurement, it was found that hydroperoxides interfere but the major disadvantages are the instability of the

hydroxylamine and the interference from conjugated unsaturation and hydrogen bonding very often present in oxidized systems (Bhalerao et al., 1961; Gray, 1978).

Other chemical and physical tests such as chromatographic methods using GC and HPLC have also been used. The HPLC has stayed in the early stages of utilization for actual rancidity measurements mainly because of the need for pre-separation of the oxidation secondary products from the triglyceride matrix. Moreover, the need for high purity of the mobile phase for the use of low wavelength UV detection may contribute to the reluctance to accept HPLC for the routine analysis of rancidity. In the case of GC, earlier work made use of solid adsorbents for separation and restricted the measure to hydrocarbons since the column irreversibly absorbed the more polar substances such as aldehydes, ketones and esters. The restricted life of alumina columns, the regular need to regenerate them and the thermal degradation of peroxides in the injection port along with the formation of additional secondary products were other problems encountered when trying to use gas chromatography to monitor lipid oxidation. With adapted methods, GCMS has been used to measure and identify hydroperoxides and secondary oxidation products from autoxidized fatty acid esters (Frankel et al., 1977; Selke et al., 1978). Gas liquid chromatography was used in the mid-1960's to correlate flavor scores and pentane formation in potato chips (Scholz et al., 1966) and soybean oil. However, the instrumentation required is expensive and the methods are inapplicable in smaller industries with limited access to trained technicians.

The determination of the anisidine value is a method adapted from the original Benzidine Method developed by Holm et al. (1957) to measure the extent of oxidation of fats. The reagent benzidine has been replaced due to its known carcinogenicity and the method is now an official AOCS method (AOCS, 1989). Anisidine value is defined by convention as one hundred times the optical density measured at 350 nm in a one centimeter cell of a solution containing one gram of the oil in one hundred milliliters of acetic acid and p-anisidine. The results are obtained from equation 1 (International Union of Pure and Applied Chemistry, 1986).

$$p\text{-AV} = 25\ (1.2As - Ab)/\ m \qquad [1]$$

Where:

p-AV= anisidine value

As= the absorbance of the fat solution after reaction with the p-anisidine reagent

Ab= the absorbance of the fat solution

m= mass of fat used, in grams.

Figure 4 illustrates the concept of the measurement.

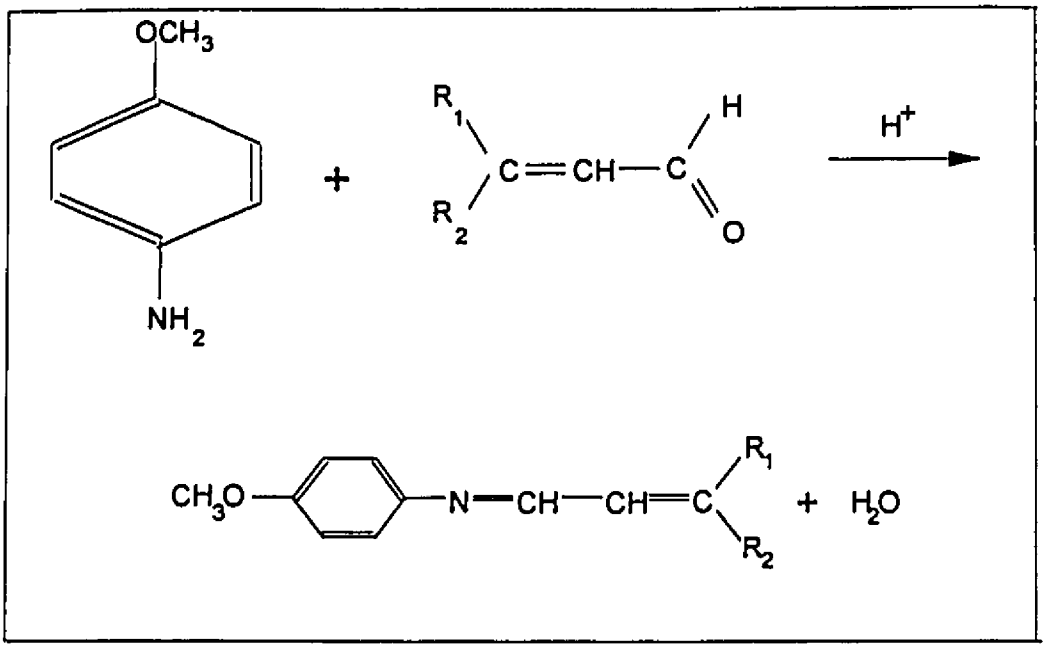

Figure 4: Reaction involved in the determination of anisidine value (Robards et al., 1988).

The reaction is time-dependent and the precise weighing of the samples and mixing of the solutions has an enormous impact on the results. Moreover, the method doesn't respond equally to the different types of aldehydes present, being more sensitive to alkenals and dienals. The latter have a molar absorbance four to five times greater when a double bond in the chain is conjugated with the carbonyl double bond (AOCS, 1989). The extinction coefficients have been estimated for the different classes of carbonyl compounds making it possible to make correlations between the measurement of single classes of aldehydes in relation to the overall anisidine value. The AV method is widely used and it has gained the confidence of the oil industry as an index of oil quality.

2.2.3 Potential Use of Infrared Spectroscopy

According to O'Connor (1956), infrared spectroscopy had its beginning with Sir William Herschel's discovery of the existence of radiation beyond the red limit of visible light, termed infrared radiation, in 1800. Almost one hundred years later, Julius (1892) determined that molecular infrared absorptions were related to the chemical groups present in a molecule by demonstrating that molecules containing the same groups all showed the same absorption maxima. The beginning of the use of infrared spectroscopy for fat analysis was marked by the publication by Coblentz (1905) of a library of 131 substances, including a number of spectra of fatty acids and vegetable oils. The first publication devoted to infrared analysis of fatty material was published fifteen years later by Gibson (1920).

Spectroscopy in general deals with the interactions between electromagnetic radiation at particular wavelengths and matter. Infrared radiation corresponds to wavelengths between 0.78 and 1000 microns. The part of this region of the spectrum which is mostly used in infrared spectroscopy is the vibrational portion, corresponding to wavelengths between 2.5 and 15 μm (4600-700 cm^{-1}) (Pavia et al., 1979), providing energies in the range of 2 to 12 kcal/mole (Hart and Conia, 1987). The molecules present in a sample can only absorb particular frequencies of radiation which correspond to the stretching and bending vibrational frequencies of their covalent bonds. A bond must have an electric dipole which is changing at the same frequency as the incoming radiation in order for

energy to be transferred. This added energy will serve to increase the amplitude of the vibrational and rotational motions of the bond (Skoog and Leary, 1992; Tinoco et al., 1985).

Complex molecules exhibit a large number of normal vibrations. Two categories of normal modes are currently defined. The skeletal vibrations involve only a small portion of the molecule and correspond to the functional groups. These vibrations are characteristic of each molecule and permit a comparison of spectra in order to determine the similarity or difference between two molecules with the same functional groups (Hart and Conia, 1987). Skeletal frequencies usually fall in the range of 4000-1600 cm^{-1} and are due to linear or branched chain structures in the molecule. Thus, the whole complex of bands produced by several skeletal modes of vibration is highly descriptive of the molecular structure of the compound under examination. When a portion of the molecule is changed structurally, it affects the pattern of the absorption spectrum in the fingerprint region. This region is located around 1600-700 cm^{-1}, below the skeletal mode region (Skoog and Leary, 1992; Hart and Conia, 1987).

The use of infrared spectroscopy for analysis of fats and oils is a relatively recent development. Many reviews on the capabilities of conventional IR spectroscopy have been published, in relation to its potential for qualitative analysis and oil identification, with the present emphasis being on more powerful instruments such as FTIR spectrometers. Morris, in 1954, reviewed the mechanisms of lipid autoxidation and the development of

rancidity and included a discussion of the infrared spectra of such oxidizing systems. In 1949, Honn and co-workers used the band centered at 2.9 μm (3448 cm^{-1}) to study the oxidation of linseed oil. They attributed the increase of the intensity of that particular band to the formation of carboxyl groups, hydroperoxides, alcohols and water. During studies of autoxidation done by Chang and Kummerow (1953), it was discovered that following the increase in the 2.9 μm band, two bands appeared at longer wavelength believed to be due to decomposition products. Moreover, many papers were published at the time dealing with infrared spectra showing a relationship between the conversion of *cis* double bonds to their *trans* isomers and autoxidation (O'Connor, 1956).

Despite the promising future infrared spectroscopy was showing in the 1950's, quantitative applications were not very successfully implemented largely due to limitations of dispersive instrumentation. Basically, the low energy throughput associated with dispersion of the infrared beam via a grating through a narrow slit, poor wavelength accuracy and the time required to collect information over the mid infrared range all contributed to the slow implementation of infrared spectroscopy for the routine analysis of fats and oils. Other limitations included limited chemometric development due to the lack of computing power.

2.3 Infrared spectroscopy

2.3.1 FTIR spectroscopy

Fourier transform infrared spectroscopy (FTIR) is based on the use of interferometry to study interactions occurring between electromagnetic waves and matter (Banwell, 1983). It uses a multiplex analytical instrument, a single-channel device in which all elements of the signal emitted by the source of infrared radiation are observed simultaneously. The Fourier transform is therefore used to decode this simultaneous information (Pavia et al, 1979) leading to a system which has three major advantages over dispersive instruments. First, the Jacquinot advantage or throughput advantage which states that the power of radiation reaching the detector is greater because the FTIR system has fewer optical elements and no slit to attenuate the radiation. Thus, much greater signal-to-noise ratio is obtained. The second major advantage is the extremely high wavelength accuracy and precision obtained through the use of a reference laser. Finally, the Felgett or multiplex advantage allows one to obtain an entire spectrum in a brief period of time. This is again achieved because all elements from the source reach the detector simultaneously, reducing analysis time (Anonymous 1). The superior performance of an interferometer based IR instrument is particularly relevant when dealing with samples with high absorbance like biological systems containing water (Lamba et al., 1991).

2.3.1.1 FTIR Instrumentation

A typical FTIR spectrometer is composed of a source of infrared radiation, an interferometer, an optical path, a detector and a sample holder. It is also equipped with an internal laser. The instrument is normally purged with dry air or N_2 when high performance analyses are required in order to minimize interference from water vapor and carbon dioxide. A typical spectrometer is illustrated on Figure 5.

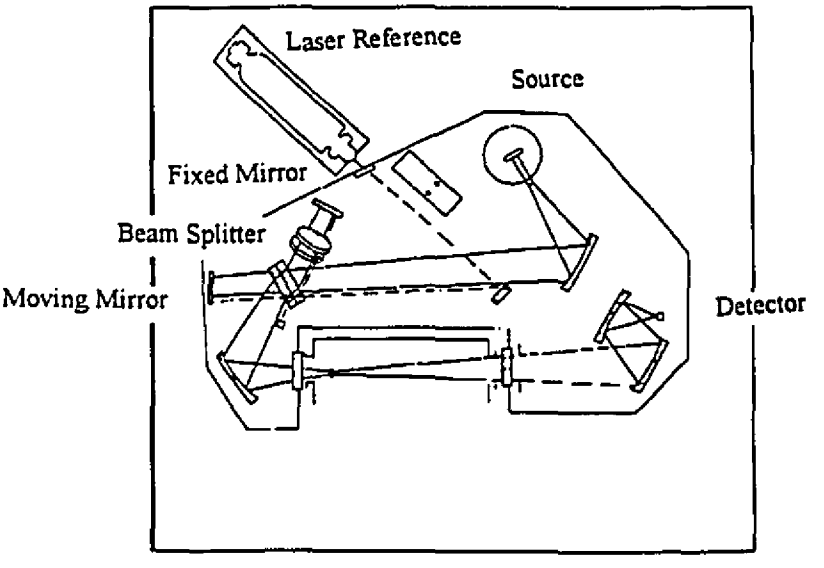

Figure 5: Single Beam FTIR Spectrometer (Source: Skoog and Leary, 1992).

The source is commonly a Nernst filament consisting of a spindle of rare-earth oxides about one inch long and a tenth of an inch in diameter. Platinum leads are sealed to the end of this cylinder to permit the passage of electricity which will maintain the temperature between 1200 and 2200 Kelvin. The filament has to be pre-heated in order to start

conducting electricity and the electric current is used to maintain its temperature at red or white heat (Skoog and Leary, 1992; Pavia et al, 1979).

The interferometer is the heart of the instrument, replacing the conventional monochromator, having the property of preserving both frequency and intensity information (Anonymous 1). The interferometer is made of three major parts: a beam splitter, a moving mirror and a fixed mirror. The beam splitter is basically a mirror coated with a very thin film of germanium which separates the incoming beam into two identical beams, one going to the fixed mirror and the other towards the moving mirror, both of which have silvered surfaces. All the windows used in the spectrometer have to be made of a mineral salt which is transparent to infrared radiation because glass absorbs over most frequencies used. A requirement for obtaining satisfactory interferograms is that the speed of the moving mirror be constant and its position known exactly at any instant. This function is achieved by the internal reference laser (HeNe), which monitors the position of the moving mirror during the scan. As a laser is monochromatic, the resulting interferogram is a cosine wave which can be accurately monitored. Thus, if the velocity of the mirror varies during a scan, the laser cosine wave elongates and the data collecting system waits before taking another point, leading to precise scanning and very accurate data collection in relation to wavelength position (Skoog and Leary, 1992; Pavia et al, 1987).

Once the beam has gone through the determined optical path, it is focused on the sample holder. The sample holder needs to be transparent to infrared radiation in the region of interest. Salts such as NaCl, BaF_2, CaF_2 and ZnS are commonly used as they have reasonably good transmission properties. When the sample contains moisture, the window materials are selected as a function of their insolubility in water to avoid ongoing decomposition of the cell windows. The pathlength of the cell is determined by the distance between the two windows where the radiation will be in interaction with the molecules of the sample (Anonymous 1).

The energy exiting from the cell is directed to a detector. Most of the common detectors are photoconductivity based. A semiconductor material, such as lead sulfide for example, absorbs the energy coming from the incident radiation. The conductivity of the semiconductor can then be measured continuously, and, after a fast Fourier transform, when plotted against the frequency of the radiation, give a direct transmittance spectrum of the sample.

The purge system noted previously is designed to introduce dry air in the optical cavity and body of the spectrometer to reduce the ubiquitous presence of water vapor and carbon dioxide, two normal constituents of ambient air. It is necessary to insure an environment minimizing these two gasses, especially water vapor as it absorbs infrared radiation strongly over most frequencies and can interfere. For example, water molecules absorb in the region of 3420-3250 cm^{-1} and around 1650 cm^{-1}, encompassing the range of

absorption of C=O bonds in aldehydes and O-H bonds in hydroperoxides formed during lipid oxidation (Lamba et al., 1991; Pavia et al., 1979).

When quantitative results are expected from the FTIR analysis, the transmission spectrum obtained must be converted to an absorbance spectrum in order for the Beer-Lambert law to apply. This law states that a linear relationship exists between the measurement of the absorbance of a sample and the number of molecules the light goes through as given by equations 2 and 3 (Tinoco, 1985).

$$T=Elc \qquad [2]$$

$$A = -\log_{10} T \qquad [3]$$

Where:
T= transmittance
l= pathlength
E= molar extinction coefficient
A= absorbance
c= concentration

Deviations from the Beer-Lambert law can be observed for a variety of reasons caused either by the properties of the sample or the conditions of analysis. Non-homogeneous samples, light scattering by the sample, dimerization or other aggregation at high concentrations or changes in equilibrium involving dissociable absorbing solutes are mentioned by Tinoco (1985). Moreover, Tyndal scattering, reflection losses, refractive index, temperature and detector nonlinearity can also affect FTIR results (van de Voort and Ismail, 1991). These factors can be minimized by a control of the absorbance using a

standard compound as reference, ensuring the reproducibility of analytical conditions and carrying out frequent calibration checks (Griffiths et al., 1986).

Finally, the Fourier transform processing of the interferometric data provides access to more readily interpretable spectra than obtained with conventional dispersive spectrometers. It provides the capacity of deconvolving overlapping bands from complex samples which give rise to instrumentally unresolvable multicomponent band contours. Under controlled conditions, the degree of improvement of spectral resolution may be sufficient to directly determine the proportions of each component in the sample using the integrated band intensities and reference values from the spectra of pure compound samples (Kauppinen et al., 1981).

FTIR technology has been applied to a wide range of research fields in the last decade, generating hundreds of publications. In the restricted field of food analysis, FTIR has been applied to analyze proteins in meat products (Bjarno et al., 1982), characterize oils, butter and margarine (Safar et al., 1994), to determine fat and moisture content in high-fat products (van de Voort et al., 1993), to study the isomerization of sugars (Yaylayan and Ismail, 1992; Yaylayan and Ismail, 1993) and to analyze milk for the concentration of its major components (van de Voort et al., 1991; van de Voort et al., 1992, Nathier-Dufour et al., 1995), to cite only a few examples. In the field of fats and oils, methodologies have been developed to measure saponification number, iodine value or degree of unsaturation (Ismail et al., 1993, van de Voort et al., 1992b; Arnold and

Hartuk, 1971), moisture content of fat products (van de Voort et al., 1992a), and *cis/trans* isomers ratios (van de Voort et al., 1995). With the contribution of new accessories such as temperature controlled sampling devices, flow-through cells, purging systems and computer automation, analyses have become rapid and very effective, providing opportunities to replace chemical methods of analysis by time-saving, environmental friendly and automated FTIR methods. Moreover, with the advent of sophisticated mathematical processing software, quantitative analysis has become feasible even in complex systems comprising overlapping bands and non-linearity between peak heights and concentrations of specific components due to interfering substances. Classical least-squares, partial least-squares and multiple linear regression are some of the chemometric techniques available (Anonymous, 1992) to assist in developing quantitative methods, spurred on by the development and availability of personal computers.

2.4 Objectives

The objective of this work centers on the development of rapid spectroscopic methods to follow oxidation in vegetable oils, specifically methods for the determination of peroxide value (PV) and aldehyde content (anisidine value) as they are well accepted measures used in the edible oil industry to characterize the oxidative state of bulk lipids and their quality. The aim is to develop spectroscopic methods designed for quality control purposes by benefiting from the numerous advantages FTIR spectroscopic instruments provide, including enhanced sensitivity and quantitative accuracy, coupled

with computerized routines and data processing software which provide capabilities for repetitive analysis. Most crucial for the food industry is the ability to develop automated routines which allow personnel without extensive knowledge of spectroscopy to make use of this technology.

Chapter 3

Methodology Development

3.1 General Considerations

FTIR spectroscopy is a secondary method, implying that it requires a calibration in order to be able to predict quantitative results for unknown samples. The general experience in the meat and milk industries has shown that the preparation of the standards and the constant calibration of the spectroscopic instruments can be as onerous as conventional chemical analysis. As a consequence, the IR spectroscopic analysis of milk was not applied extensively in the industry to an appreciable degree before pre-analyzed calibration milks were commercially available (van de Voort and Ismail, 1991). This experience demonstrates the importance of the preparation of stable and non-perishable calibration standards when developing a spectroscopic method for the analysis of fat and oil systems.

In the development of FTIR-based methods for the determination of characteristics of fats and oils such as iodine value and saponification number, the approach taken consisted of locating the peaks (regions) related to the specific parameter of interest using pure triglycerides (van de Voort et al., 1992a). For the determination of minor components that may be present in oils, such as free fatty acids, calibration standards may be prepared by adding known amounts of each of the components of interest to mimic the

spectrum of the final product. The single-beam spectra of both the pure oil and the oil spiked with the component of interest ratioed one against the other would then give an absorption spectrum of the added components (Ismail et al., 1993; Arnold and Hartuk, 1971). With the spectrum of the components of interest available, one attempts to establish a relationship between the amounts of the compound added relative to the recorded peak heights or areas. However, due to the presence of interfering components, overlapping bands and effects of chemical interactions of components present in oils, direct measurements of peak heights or areas do not always correlate linearly to individual concentrations. An example of such a system was investigated by van de Voort et al. (1992a) in the assessment of standards for milk analysis. Three multivariate methods of analysis were assessed for their ability to account for cross-interferences in the determination of protein, lactose and solids in milk: multiple linear regression (MLR) based on the conventional dual-wavelength ratio method normally used in filter-based IR milk analyzers, a classical least-squares (CLS) method and a partial least-squares (PLS) method. Both the classical least-squares and the partial least-squares programs take as input the concentrations of the reference standards and the spectral regions selected for the analysis on the basis of their information content with respect to the concentrations of the components. Although CLS and PLS are theoretically whole-spectrum methods, in practice the analysis is restricted to regions of the spectrum which exhibit variations with changes in the concentrations of the components of interest (van de Voort et al., 1992a). Results showed that predictions of chemically analyzed validation samples were well within AOAC specifications using multiple linear regression, conventional least-squares

and partial least-squares approaches. One disadvantage of the CLS method is that due to the fact that it is based on the Beer-Lambert law, which assumes that the absorbance is linearly related to concentration, all species which absorb in the range of interest have to be identified and all the components showing an absorption have to be included in known proportions in the calibration. PLS, however, was found to be able to compensate for unidentified sources of interference as long as they are present both in the calibration standards and the samples (van de Voort et al., 1992a). A fourth multivariate method, the principal component regression (PCR) method (Safar et al., 1994), was investigated in comparison with PLS and MLR by Nathier-Dufour et al. (1995) for the FTIR determination of fat and solids in sweetened condensed milk. It was found that PLS and PCR were equivalent in terms of accuracy and reproducibility of the results. However, because PCR does not use the concentration data in the determination of the principal components, it is a less efficient method. From these results, it was concluded that PLS was the most appropriate multivariate analysis method available for the present work.

In order to be suitable for treatment with PLS, the composition of the standards used for calibration has to mimic that of the samples (van de Voort et al., 1992a; Anonymous, 1992). In an infrared spectrum, the major components responsible for interference can be identified, making it possible to prepare standards representative of general vegetable oil systems (van de Voort et al.,1993). Two requirements need to be fulfilled in order for the mathematical processing of the PLS package to give satisfactory results in predicting unknowns. First, there can be no correlation between the concentrations of the

component of interest and those of any of the interfering substances, and secondly the concentration range of the components of interest and the interfering components must be adequately spanned (Anonymous, 1992).

Finally, in order to widen the field of application to fats and oils in general, the analysis must be carried out at a temperature high enough to melt fats so that all samples are in a liquid form. The presence of any crystalline forms influences the infrared spectra due to changes in the index of refraction of the resulting mixture and causes problems in quantitation (Banwell, 1983).

Chapter 4

Determination of Peroxide Value

4.1 Introduction

As was noted earlier, hydroperoxides are the primary products arising from oxidation of lipids. Consequently, monitoring the amount of hydroperoxides present in lipids provides a direct measure of the progress of oxidation over a variable period of time influenced by the conditions of conservation (Fennema, 1985). The American Oil Chemists' Society (AOCS) peroxide value (PV) determination is the standard method used to determine hydroperoxides in the oil industry, either directly or in a controlled fashion via the active oxygen method, to determine the oxidative stability of an oil.

It has been recognized for some time that hydroperoxide functional groups can be quantitatively determined by infrared spectroscopy. As early as in 1949, Honn and co-workers used the -O-H stretching band at 2.9 microns (3550 cm^{-1}) to study autoxidation in linseed oil and concluded that the increase in the intensity of that band was attributable to the formation of hydroperoxides (OOH), water, alcohols and ROH groups. Fukuzumi and Kobayashi (1972) reported later that a linear relationship exists between the intensity of the hydroperoxide absorption band at 3550 cm^{-1} in CCl_4 and the iodometric PV for fatty acid methyl ester hydroperoxides. This absorption was attributed to the O-H stretch in the peroxide molecule (Pavia et al., 1979). This basic information has not been

exploited in a practical way because of the limitations associated with dispersive instrumentation. The major advantages previously mentioned for the FTIR system provide the sensitivity and quantitative accuracy required for this type of studies.

From the standpoint of general oil analysis methodology development, van de Voort et al.(1993) observed that hydroperoxide absorption bands overlap with a number of other O-H absorptions such as those found in water, free fatty acids and alcohols, all possible components of fat and oil systems, especially when they have undergone oxidation processes. Moreover, some structural characteristics of the triglycerides making up the oils such as the distribution of the fatty acids on the triglyceride are also recognized as possible sources of variations in the spectra that can interfere with the measurement of the hydroperoxide absorption band. With so many potential sources of interference and variability affecting the PV determination, sophisticated chemometric techniques are called for. As mentioned in the general considerations of the methodology development, PLS is a powerful tool in such circumstances because it is capable of accounting for interactions, underlying absorptions, overlapping bands and other factors which may affect the spectra as the concentrations of all components change. This chapter describes the development of a practical, automatable FTIR method to determine PV.

4.2 Experimental Procedures

4.2.1 Instrumentation and Sample Handling

In the development of the method for the determination of peroxide value using FTIR spectroscopy, preliminary work was carried out using an Impact 400 (Nicolet, Madison, WI) which was not equipped with a heated sampling device. The calibration under development was originally targeted for oils only. The spectrometer was interfaced to a 486/33MHz PC operated under Windows-based Nicolet Omnic 1.1 software. The optical cavity of the instrument was purged with dry air from a Balston dryer (Balston, Lexington, MA) to minimize water vapor and CO_2 interferences. Sample handling involved the aspiration of oil samples through a 903μm pathlength CaF_2 flow cell via 1/16" silicone tubing. The cell was built with the two windows separated by a Teflon ring cut at both ends to allow the circulation of the sample. A valve was used to control the vacuum, thereby regulating the filling of the cell and facilitating the sequential analysis of samples. The temperature was not monitored and it was found after substantial study that this basic system contained too many uncontrolled parameters, and it was subsequently replaced with a special sample handling system developed in collaboration with Dwight Analytical Solutions Ltd.(Toronto, Ontario) (Figure 6 and 7). The new system was developed to provide a better control of the operating variables and to expand the analytical method to both liquid oil and solid fat samples. The sample holder consists of a temperature control block and a removable cell insert. The insert allows for ready removal of the cell to take

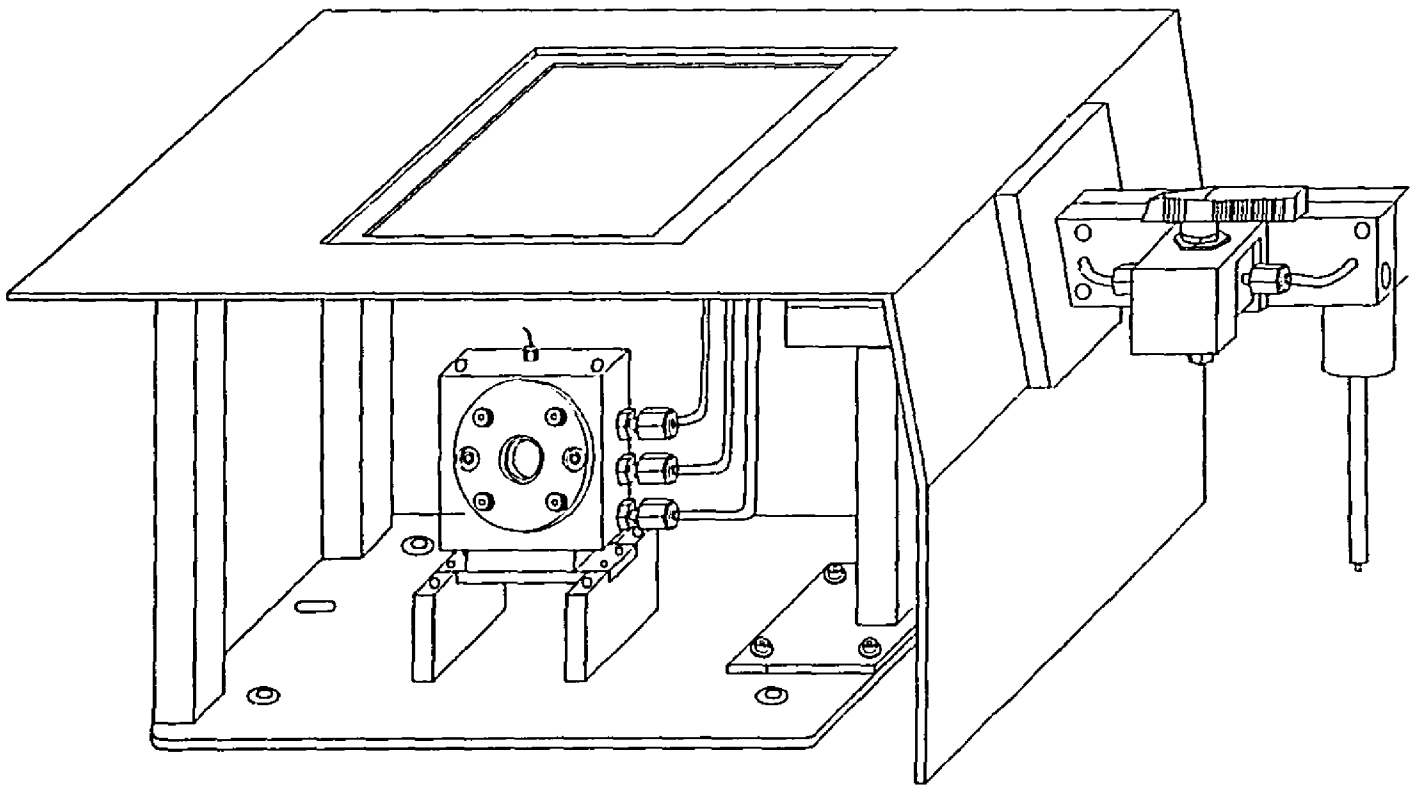

Figure 6: Sample Handling Accessory.

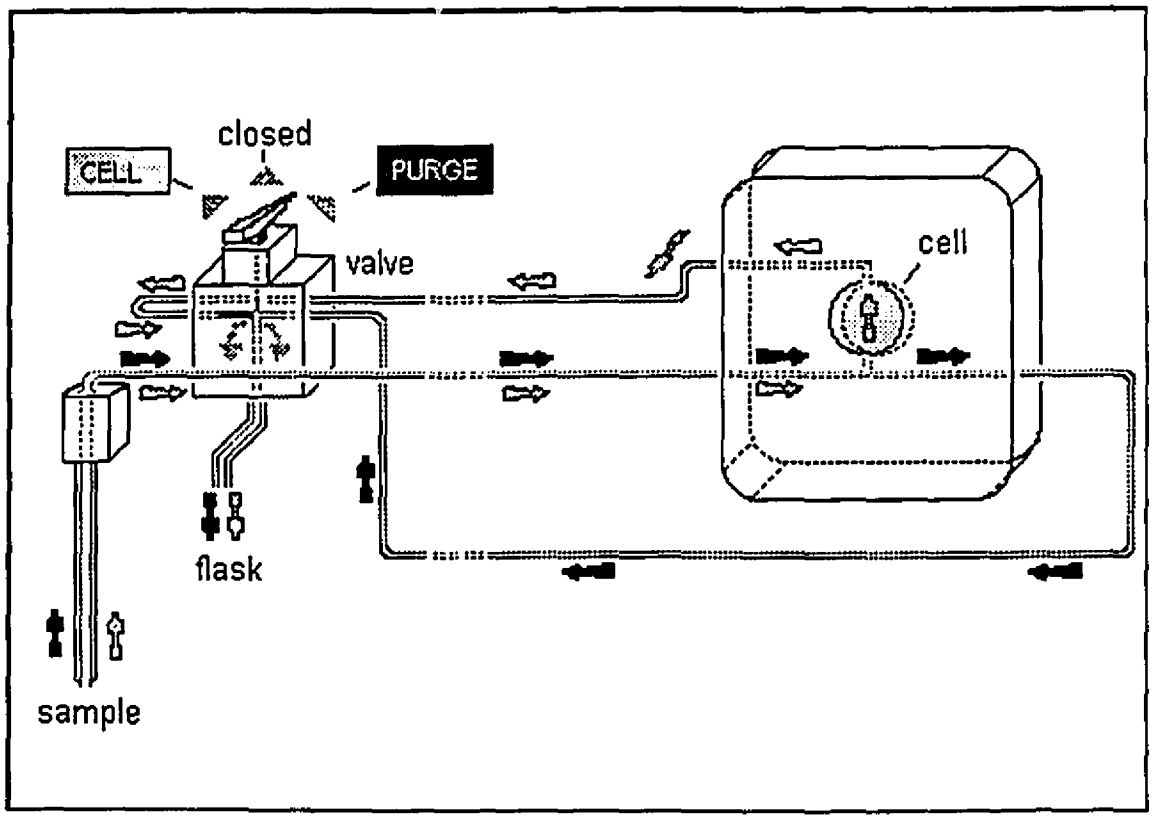

Figure 7: A Schematic Diagram of the Cell and Flow Pattern through the System.

an open beam background or to interchange cells if a change in cell configuration (i.e., windows, pathlength) is required for a particular analysis. For this system, a cell made with ZnS windows was prepared using one drilled and one plain window, spaced by a 906μm thick diamond shaped aluminum spacer, providing a better support against deformation of the cell due to the vacuum aspiration. The flow of the sample through the system is demonstrated on Figure 7. The temperature of the cell-holder and the arm through which the sample flows was precisely controlled and maintained at 70°C. During the scanning procedures, the pathlength stability of the cell and the tilt of the instrument's response were routinely monitored using methyl myristate as standard. This methylated fatty acid was chosen for its stability to oxidation and its favorable transmission characteristics in a long pathlength cell.

The cell was rinsed once a day with a 1% solution of Triton X-100 and regularly every ~5 samples with isooctane. All spectra were collected by co-adding 256 scans at a resolution of 4 cm^{-1} using a triangular apodization. All spectra were ratioed against a background spectrum of the clean empty cell, collected by coadding 256 scans. All samples were preheated for a short period of time in a water bath set at 70°C in a closed container to avoid infiltration of water into the samples.

The chemical determination of peroxide value was performed as specified by the Association of Pure and Applied Chemistry (1989) which corresponds to the AOCS iodometric method (AOCS, 1989).

4.2.2 Reagents and Standard Preparation

For the chemical determination of PV according to the AOCS method, samples which were oils at room temperature were used without further treatment. Solid fats were melted prior to reagent addition and analysis. All the chemicals used were reagent grade and the saturated potassium iodide solution was prepared fresh every day. No other treatment was applied and the reaction between the iodine and the fat was limited to one minute.

A three molar solution of *t*-butylhydroperoxide in isooctane was purchased from Aldrich Chemical Company (Milwaukee, WI) and used to represent the hydroperoxides in the preparation of the calibration standards. The peroxide value contribution of this reagent was measured in triplicate with the AOCS iodometric method, the TBHP having been diluted in isooctane. Due to this step, the accuracy of the new spectroscopic method is inherently limited by the reproducibility of the AOCS iodometric peroxide value determination, the method it is calibrated against.

Six commercial vegetable oils (corn, soybean, canola, sunflower, peanut and olive) were purchased from a local supermarket. The six oils were deodorized by vacuum distillation at 260°C for two hours. This procedure eliminates the peroxides present by thermal decomposition and the vacuum pumps out the secondary products. A food grade

antioxidant, Tenox-BHA (Eastman Chemicals Co, Kingston, Tennessee), was added in a proportion of 0.1% to stabilise the peroxide-free oils. This process is meant to insure that all the oils used as bases for the formulation of calibration standards are free of hydroperoxides (PV<1 measured by chemical PV) and thus that the contributions to the peroxide value are provided only by the TBHP added.

Oleic acid was purchased from Aldrich Chemical Company (Milwaukee, WI.) and its peroxide value measured with the AOCS iodometric method. No further treatment was imposed to the free fatty acid. Distilled water, monoglycerides fractionally distilled from vegetable oils obtained from Eastman Chemicals Co. (Rochester, NY.) and diglycerides (1,3-dilinolein, 1,3-dilinolenin and distearin) obtained from Sigma Chemical Co. (St-Louis, MO) were used without further treatment to complete the formulation of the calibration standards.

In preliminary development work carried out on the use of FTIR spectroscopy as a method for the determination of PV, a relatively simple calibration approach was attempted. It was based on the use of *t*-butylhydroperoxide (TBHP) as a standard, added to a peroxide-free canola oil, and ratioing the spectrum of the spiked oil against that of the peroxide-free oil to obtain a clearly resolved hydroperoxide band. This approach with a simple dual-wavelength calibration (3444-2225 cm^{-1}) produced excellent calibration curves and predictions for that base oil (Figure 8). However, predictions for oils other than the base oil did not always correspond well with the chemical PV. Upon assessment

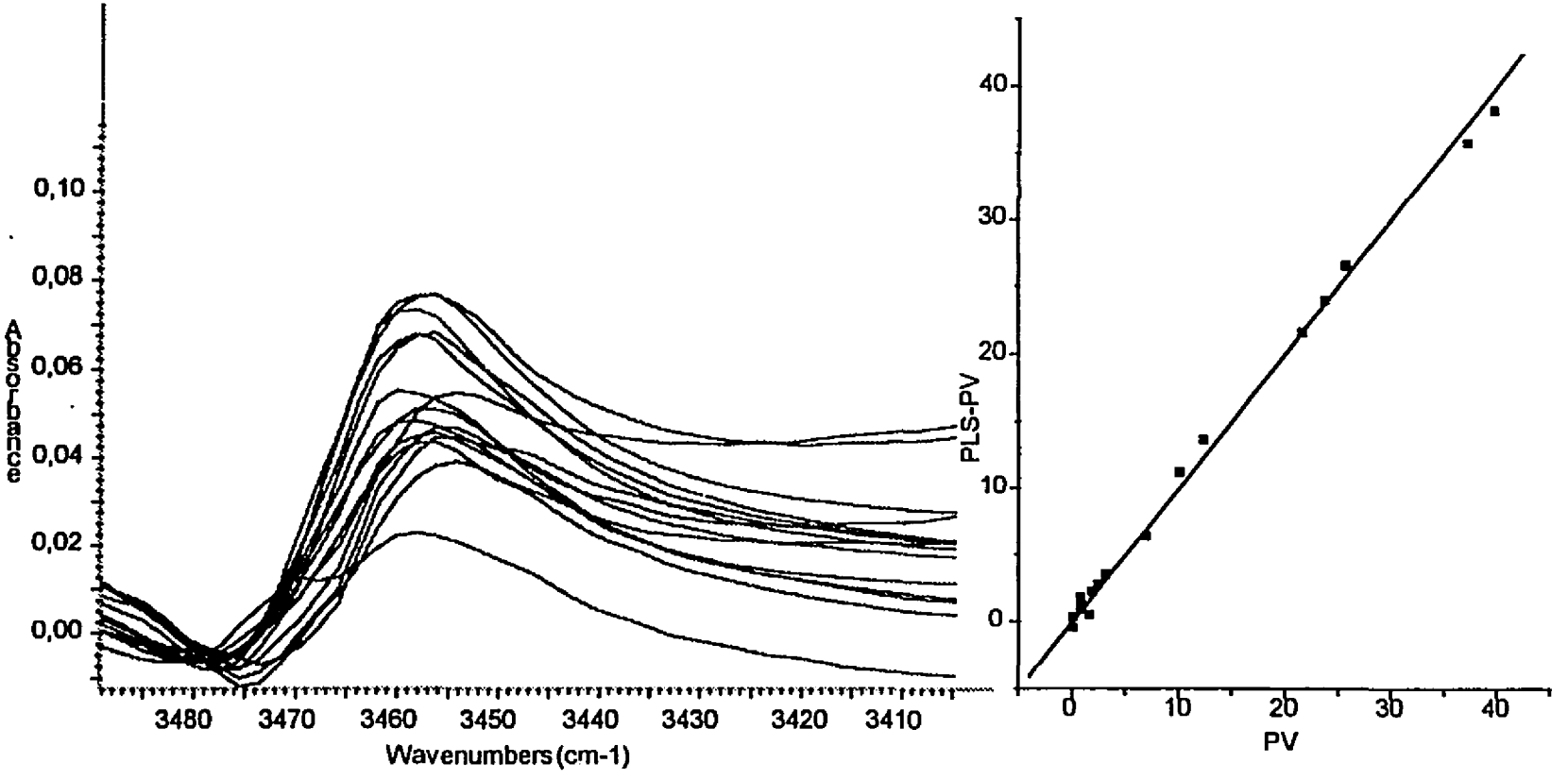

Figure 8: Selected Differential Spectra of Calibration Standards and Cross-Validation Results for the Basic PV Calibration.

of the spectra, it was determined that the overtone band at 3473 cm^{-1} was varying as a function of the average molecular weight of the samples (saponification number). This problem was overcome by using a variety of oils to account for this variable. The standard solutions required to produce the calibration were prepared by addition of random amounts of water, free fatty acids, mono- and diglycerides and hydroperoxides to the six vegetable oils. The randomness of the component addition is crucial in this procedure because one of the requirements of the partial least squares approach is that the variables be independent of each other and show no correlation. The quantities to be added were simply determined using the « random function » in Excel 2.0 (Microsoft, WA). The proportion of free fatty acids added was kept below 2%, mono- and diglycerides were added at a level of 1%, and water was maintained below 1% by means of filtration through a Whatman filter paper to eliminate free drops of water. A set of sixty-three primary calibration standards was prepared by physically mixing the reagents with the six deodorized oils in order to obtain a range of peroxide values between 0 and 45, with a relatively more concentrated distribution of values below PV of 15. Emphasis was given to the PV region below 15 because the low peroxide value oils present a higher proportion of interferences hiding the weaker hydroperoxide bands.

The primary set of calibration standards was expanded through the technique of spectral addition and subtraction (van de Voort et al., 1992c), incorporating contributions of the mono- and diglycerides and overtone bands representative of the six base oils. The spectral features representative of mono- and diglycerides, free fatty acids and water were

obtained by ratioing the spectrum of an oil spiked with the substance of interest with the spectrum of the unspiked oil. . This procedure ensures that the spectrum of the added component represents all hydrogen bonding effects and other interactions which could influence the spectrum. Moreover, overtone bands representative of the six vegetable oils were included. These overtone bands have an impact on the final spectrum because they produce interfering bands in the region of interest. The overtone spectrum of each oil was obtained by moving the absorption bands of water, free fatty acids, alcohol and hydroperoxides (all containing -OH bands) to lower frequencies by addition of D_2O in a proportion of 0.01% which resulted in the replacement of the hydrogen in the OH bond by deuterium (Figure 9 shows the overtone band in the spectrum of soya oil). The excess deuterium oxide was removed by centrifugation of the samples. The spectra of the overtone bands were multiplied by a scaling factor between 0.8 and 1.2 to represent oils containing different chain lengths. The final calibration set contained 80 spectra. Table 3 summarizes the 80 calibration standards prepared and used to develop the PLS calibration. Standards #1 to #63 are the primary calibration standards and were prepared by physical addition of TBHP, water, mono- and diglycerides and free fatty acids in varying amounts as specified earlier to the six base oils. Standards #64 to #80 were generated by spectral addition in various proportions of overtone vibrations or consisted simply in the overtone band of one of the base oils.

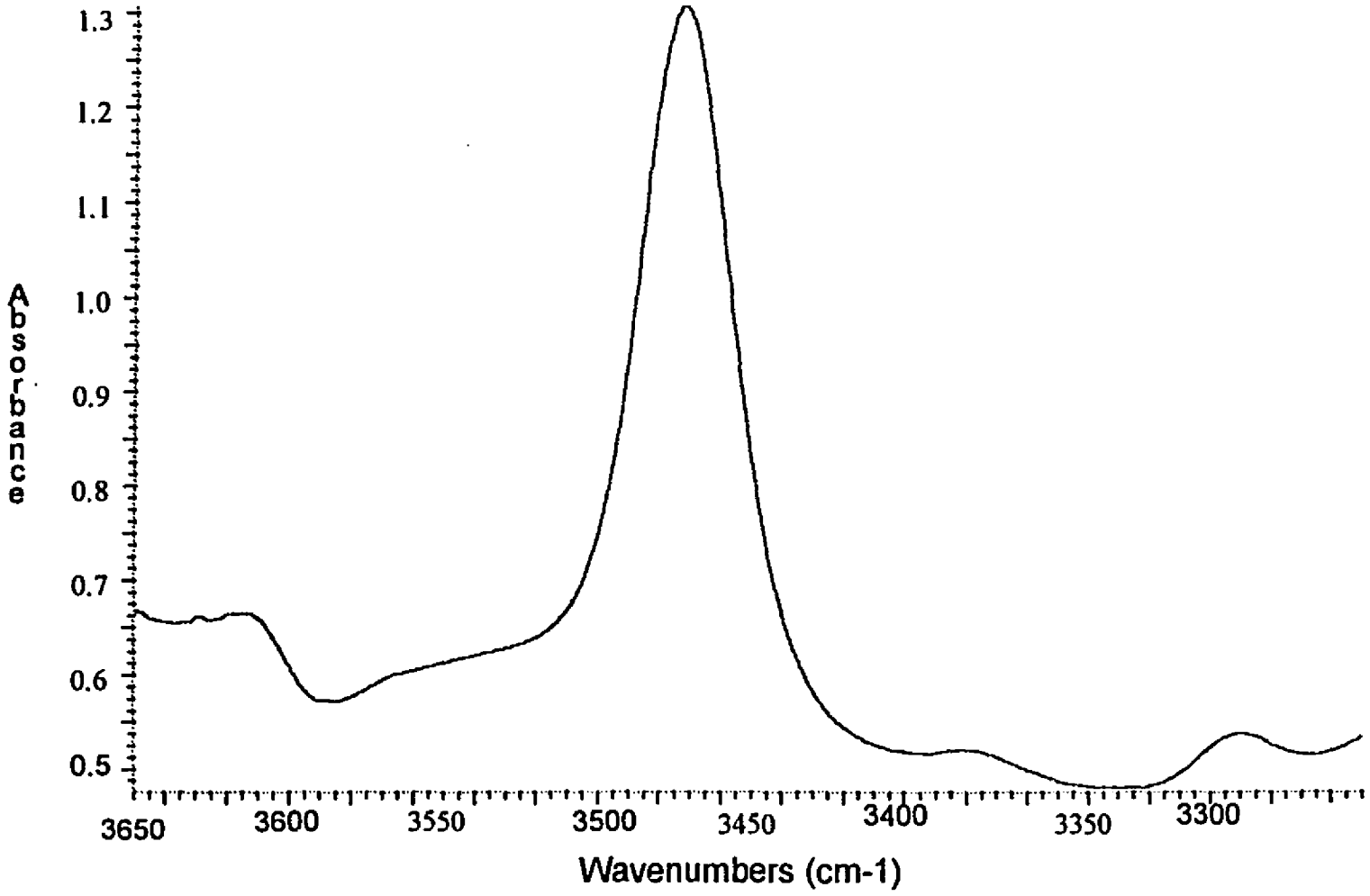

Figure 9: Spectrum of the Overtone Band of theTriglyceride Ester Linkage in Soya Oil

Sample	Oil	Added	PV	Sample	Oil	Added	PV
1	Can	W + FFA	0.85	41	Olive	D1	0
2	Can	W + FFA + P	10.38	42	Olive	D2	0
3	Can	P	20.32	43	Olive	D3	0
4	Can	P	29.60	44	Can	FFA + P	1.53
5	Can	FFA + P	39.61	45	Can	FFA + P	3.23
6	Corn	FFA	0	46	Can	FFA + P	0.93
7	Corn	P	3.20	47	Corn	FFA + P	2.38
8	Corn	P	13.08	48	Corn	W + FFA + P	0.04
9	Corn	W + FFA + P	23.45	49	Corn	W + FFA + P	0.82
10	Corn	W + FFA + P	32.58	50	Olive	W + FFA + P	5.90
11	Olive	W + FFA	0	51	Olive	FFA + P	10.21
12	Olive	FFA + P	11.50	52	Olive	FFA + P	1.95
13	Olive	FFA + P	20.67	53	Soya	FFA + P	0.89
14	Olive	FFA + P	29.09	54	Soya	W + FFA + P	1.71
15	Olive	FFA + P	40.34	55	Soya	FFA + P	4.96
16	Pnt	W + FFA	0	56	Sun	W + FFA + P	3.22
17	Pnt	P	10.56	57	Sun	FFA + P	1.69
18	Pnt	P	20.37	58	Sun	FFA + P	4.27
19	Pnt	FFA + P	29.22	59	Sun	FFA + P	8.11
20	Pnt	FFA + P	41.53	60	Pnt	W + FFA + P	1.40
21	Soya	W + FFA	0.18	61	Pnt	FFA + P	2.36
22	Soya	FFA + P	10.32	62	Pnt	FFA + P	5.86
23	Soya	FFA + P	20.42	63	Pnt	FFA + P	3.15
24	Soya	FFA + P	29.76	64	Can ovt		0
25	Soya	W + P	39.59	65	Corn ovt		0
26	Sun	FFA	0.78	66	Sun ovt		0
27	Sun	W + P	5.65	67	Soya ovt		0
28	Sun	FFA + P	14.89	68	Pnt ovt		0
29	Sun	P	25.30	69	Olive ovt		0
30	Sun	W + FFA + P	35.92	70	Can	Vovt	0
31	Can		0.85	71	Can	Vovt	0
32	Corn		0	72	Can	Vovt + M	0
33	Olive		0	73	Corn	Vovt	0
34	Pnt		0	74	Corn	Vovt + D1	0
35	Soya		0.18	75	Olive	Vovt	0
36	Sun		0.78	76	Olive	Vovt	0
37	Olive	M	0	77	Sun	Vovt	0
38	Olive	M	0	78	Sun	Vovt + D2	0
39	Olive	M	0	79	Pnt	Vovt	0
40	Olive	M	0	80	Pnt	Vovt	0

[a]The abbreviations for the base oils are canola (Can), corn, sunflower (sun), soybean (soya), olive and peanut (pnt). Other abbreviations are W for water, P for t-butylhydroperoxide, FFA for free fatty acids, ovt for overtone band, Vovt for vibrations of overtone, D1 is diglyceride 1 (diolein), D2 is diglyceride2 (dilinolenin), D3 is diglyceride 3(distearin), M is monoglyceride and PV stands for peroxide value.

Table 3: Calibration Matrix for peroxide value Calibration[a]

All the interfering substances and overtone contributions were taken in account in the development of the calibration standards in order for them to be representative of any vegetable oil one could need to analyze and thus enlarging the range of application of this new method.

4.2.3 Calibration and Validation

The calibration was developed using the Quant-IR ® Calibration Prediction Package from Nicolet Instrument (Anonymous, 1993). This package includes a partial least squares calibration routine. Despite the fact that the PLS package can treat the whole spectrum, a narrower spectral region was selected based on using the variance spectrum obtained from the calibration set. Selecting regions accelerates the prediction process and enhances its accuracy by narrowing the range where the program has to look for significant information. The region selected started at 3750 cm^{-1} and ended at 3150 cm^{-1} with the baseline drawn at 3750 cm^{-1}. The optimum number of spectral factors to be included in the calibration model was determined on the basis of significant changes based on the F-statistic in the predicted residual error sum of squares (PRESS) test. The "leave one out" cross-validation routine was used to assess the predictive accuracy of the calibration model. In this procedure, each spectrum is taken out of the calibration set and used as an unknown, its peroxide value being predicted using the remaining 79 spectra. Finally, the reproducibility was assessed by rescanning the 63 standard solutions and

predicting their PV. Moreover, the calibration model was tested by comparison between the chemically determined peroxide value of 23 oils at varying states of oxidation and the predicted PV.

4.2.4 Standardization of the instrument

Spectra collected on a FTIR spectrometer are totally dependent on the stability of the instrument and any change in the alignment of the optics or in the purge of the optical cavity results in a direct modification of the spectrum being collected. In order to verify the stability of the spectrometer and to be able to account for any day-to-day variability in the spectra, a stable reference standard was selected. Methyl myristate was chosen for its stability to oxidation and its spectral resemblance with the oils under investigation. The use of a standard allows the detection and quantification of any change in the pathlength of the cell, reflected by a change in the intensity of the absorption bands, and measures any tilt or drift of the spectral baseline. The use of a reference standard also provides an opportunity to determine the transferability of a calibration to another instrument. Methyl myristate was scanned at the beginning of the scanning process and after the collection of five consecutive spectra. Each spectrum of methyl myristate was compared to that collected at the beginning of the scanning process and the ratio of the peak height at 3467 cm^{-1} measured relative to a baseline drawn at 3746 cm^{-1} determined any change in the pathlength. The peak located at 937 cm^{-1} was used to check for any contamination of the methyl myristate in the cell with oil. If no contamination was detected, a pathlength

correction factor was derived from that ratio. The tilt correction was determined using the regions between 3100 and 2700 cm^{-1} and between 1850 and 1340 cm^{-1}, blanking the fingerprint region, CH and ester features which are off-scale in the spectrum of methyl myristate. The tilt correction spectrum was obtained by subtracting the spectrum of the first methyl myristate scanned from that of each spectrum of methyl myristate recorded. Finally, all the corrections determined from the spectrum of the standard were applied to the next five spectra collected. This technique was automated by a routine of macro commands written in Visual Basic (Microsoft Corporation, Redmond, WA) using Omnic Macros\Pro (Anonymous, 1993).

4.3 Results

4.3.1 General spectroscopy

Hydroperoxide moieties exhibit a characteristic absorption band between 3600 and 3400 cm^{-1} due to their -OO-H stretching vibrations. The peak maximum in this case is a function of the polarity of the medium and the extent of hydrogen bonding. As observed in Figure 9, oils exhibit a band centered at 3473 cm^{-1}, which is the overtone band of the strong triglyceride ester carbonyl band, being approximately double its frequency (1748 cm^{-1}). The overtone of the ester carbonyl linkage hides the band attributable to the hydroperoxide moieties, but when the features of the fresh or peroxide-free oil are ratioed out, the spectral changes due to the oxidation process are clearly visible. Figure 10

presents the stacked spectra of oxidized canola oil and the hydroperoxide band obtained by ratioing out the features of the fresh oil. The two sets of spectra shown in Figure 10 are not on the same absorbance scale, the hydroperoxide band having an absorbance of approximately 0.07 while the overtone band of the ester linkage shows an absorbance around 1.4. In Figure 10, the hydroperoxides are represented by TBHP, which produces an absorption band similar to the "natural" hydroperoxide band in the spectrum of an oxidized oil. The TBHP band is centered at 3444 cm^{-1}, confirming the assignment of this band in the spectra of oils to the -OO-H stretching vibration of hydroperoxides. Although TBHP is not representative of lipid hydroperoxides in its chemical behavior, being quite stable, its spectral behavior is similar to that of hydroperoxides formed in oils. In addition, the extinction coefficient determined for the hydroperoxide band of TBHP by serial dilution in oil was not significantly different from the extinction coefficient of hydroperoxides formed under accelerated oxidative conditions in any of the six base oils. As such, TBHP appears to be spectroscopically representative of the lipid hydroperoxides in general and was therefore considered to be a convenient stable standard. Preparing calibration standards by adding known weights of TBHP to oils avoids the extensive analytical effort usually required in calibrating a secondary method, such as FTIR, against a chemical method.

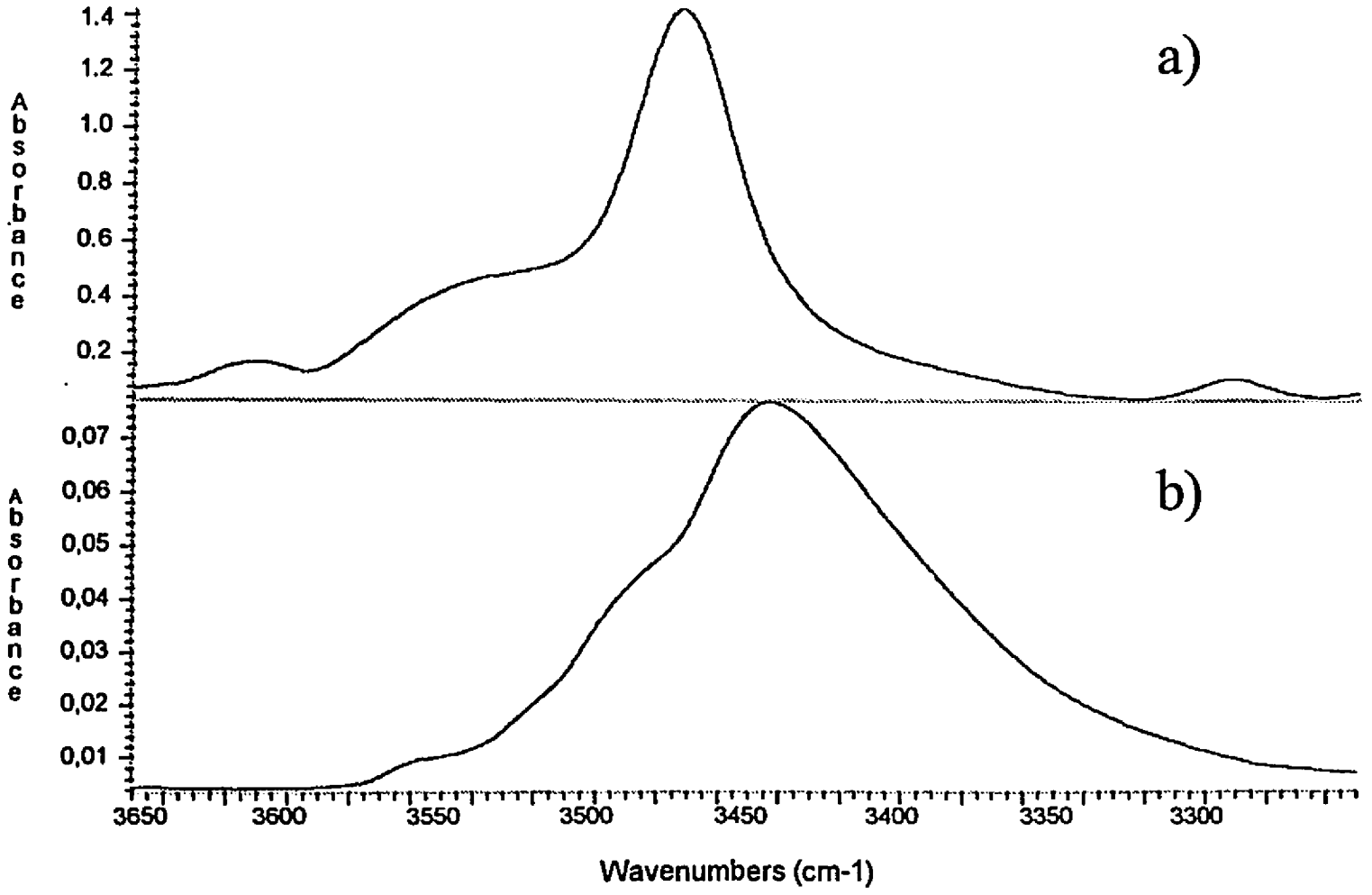

Figure 10: Spectra showing a) Overtone Band, b) Hydroperoxide Band from TBHP in Soya Oil.

4.3.2 PLS Calibration.

The power of PLS is based on its ability to mathematically correlate spectral changes to changes in the concentration of the component of interest while simultaneously accounting for all other significant spectral factors which perturb the spectrum (Anonymous, 1992). It was previously mentioned that all interfering substances should be incorporated in the calibration standards in order to account for their contributions to the spectra. Studies of the spectra of oils undergoing oxidation performed by van de Voort et al. (1994b) demonstrated that the main sources of perturbation of the spectra of hydroperoxides were moisture, free fatty acids, alcohols and the variability in the overtone band due to different saponification numbers of the oils being analyzed. Accordingly, the calibration set was designed to include all the known sources of interference in varying amounts representative of the natural composition of fresh and oxidized vegetable oils.

An optimal PLS calibration was developed with the calibration standards listed in Table 3. The spectral range chosen was 3750-3150 cm^{-1} with the baseline set at a single point (3750 cm^{-1}) due to the greater stability of the absorbance at that point compared to any other point in a close range from the region of interest. The number of spectral factors used to predict was set to 11 according to the results of the F-statistic in the PRESS test. Figure 11 illustrates the "leave one out" cross-validation plot from the PLS calibration for the 80 standards. Two standards were identified as outliers and removed from the validation plot. An overall standard deviation of 0.86 PV was obtained with a

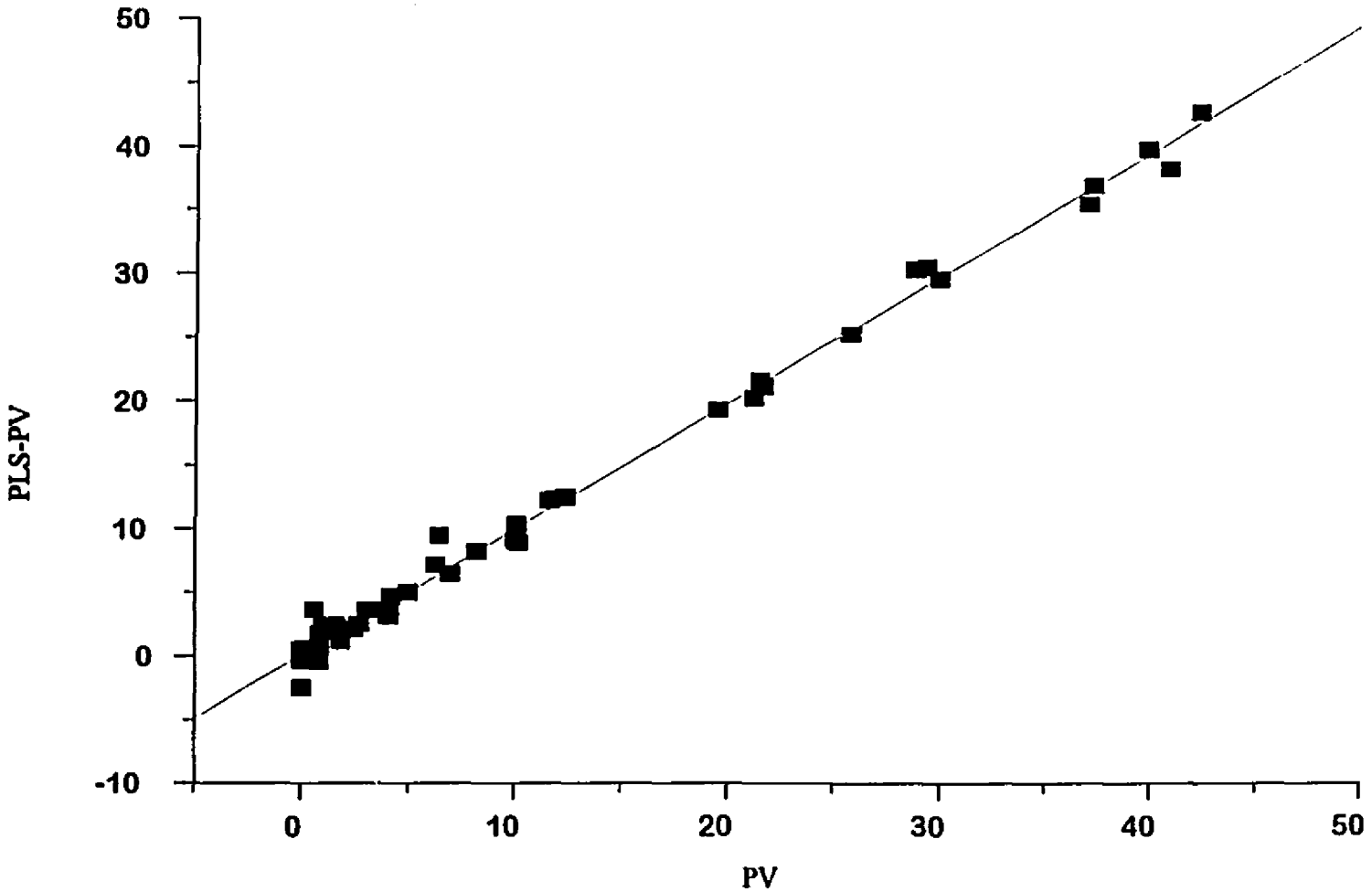

Figure 11: Cross-Validation Plot for the 80 PV Calibration Standards.

correlation factor of 0.997. Once the calibration was developed and the cross-validation results indicated good predictive accuracy, the PLS calibration was tested and validated for its ability to predict the peroxide value of real unknown mixtures. The first approach was to rescan the 63 physically available calibration standards and predict their peroxide value using the PLS calibration. It was found that the predictions for the duplicate run showed a standard deviation of 1.73 PV and a correlation coefficient (R^2) of 0.9826.

4.3.3 Analysis of Oils for PV

To verify the ability of the calibration to perform adequately with real unknowns, oxidized vegetable oil samples were analyzed for their PV and predicted using the calibration developed. Figure 12 shows a plot of the predicted PV vs the Chemical PV. An overall standard deviation of 1.31 based on an average PV of 12.6 was obtained with a correlation coefficient (R^2) of 0.9677.

4.3.4 Transferability of the calibration

It was thought at first that the transfer standard, methyl myristate, was an adequate means to insure the transferability of the calibration from one instrument to another. However, a limitation was found when trying to actually transfer the PV calibration from the Magna 500 to a Nicolet Impact 400. The difference in the energy provided by both instruments was so great that it resulted not only in differing peak heights, but also in

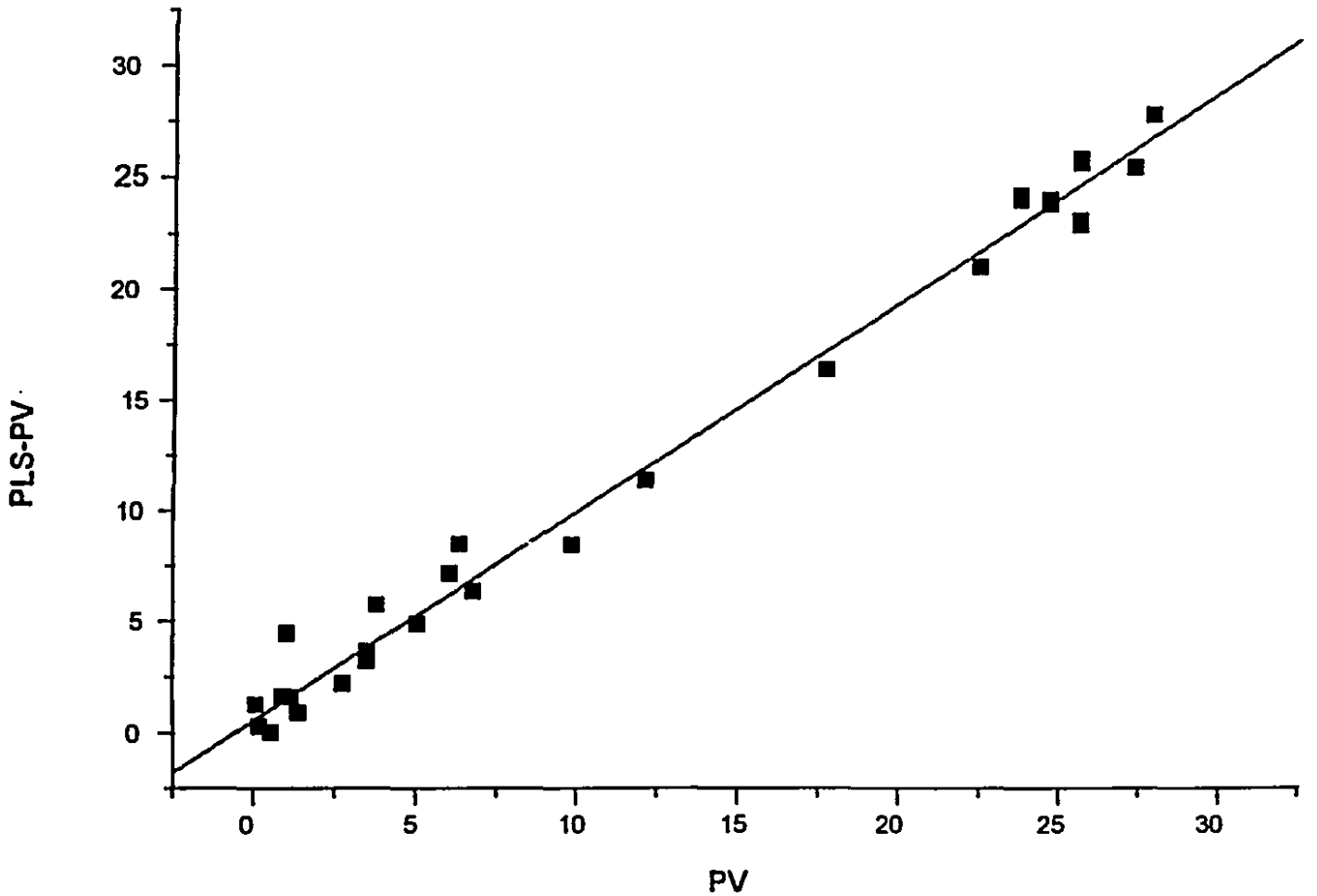

Figure 12: Plot of the Predicted PV (PLS-PV) vs the Chemical PV (PV) for Thermally Stressed Oils.

variable peak widths as illustrated on Figure 13. This observation demonstrates that the transfer standard can account for minor variations in the pathlength or the energy of the instrument, however, it cannot insure a good accuracy if the calibration is transferred to an instrumental system too different. At this point in time, calibrations can be made transferable within one series of instruments but recalibration would be required for different models. Further investigation needs to be done before any conclusion about the transferability can be made.

4.4 Discussion

The spectra of oxidizing oils are rather complex and influenced by a number of interfering substances. In the region of absorption of hydroperoxides, the main sources of variations have been attributed to alcohols, water, free fatty acids and the overtone band of the triglyceride ester carbonyl bond. With knowledge of the interfering substances and the magnitude of their influence, a calibration was developed using a partial least squares mathematical treatment of the spectral data. PLS provided a means of accounting for the variability and produced a reliable calibration which allows the prediction of the peroxide value of an oil by analysis of its FTIR spectrum. In order to apply this technique to routine analysis in the oil industry, the many steps of the procedure were programmed in the form of macro-commands with the use of Microsoft Visual Basic as an integrated part of the Nicolet Omnic (Anonymous, 1993) software which drives the spectrometer. A semi-automated procedure was achieved by which the operator needs only to pump the

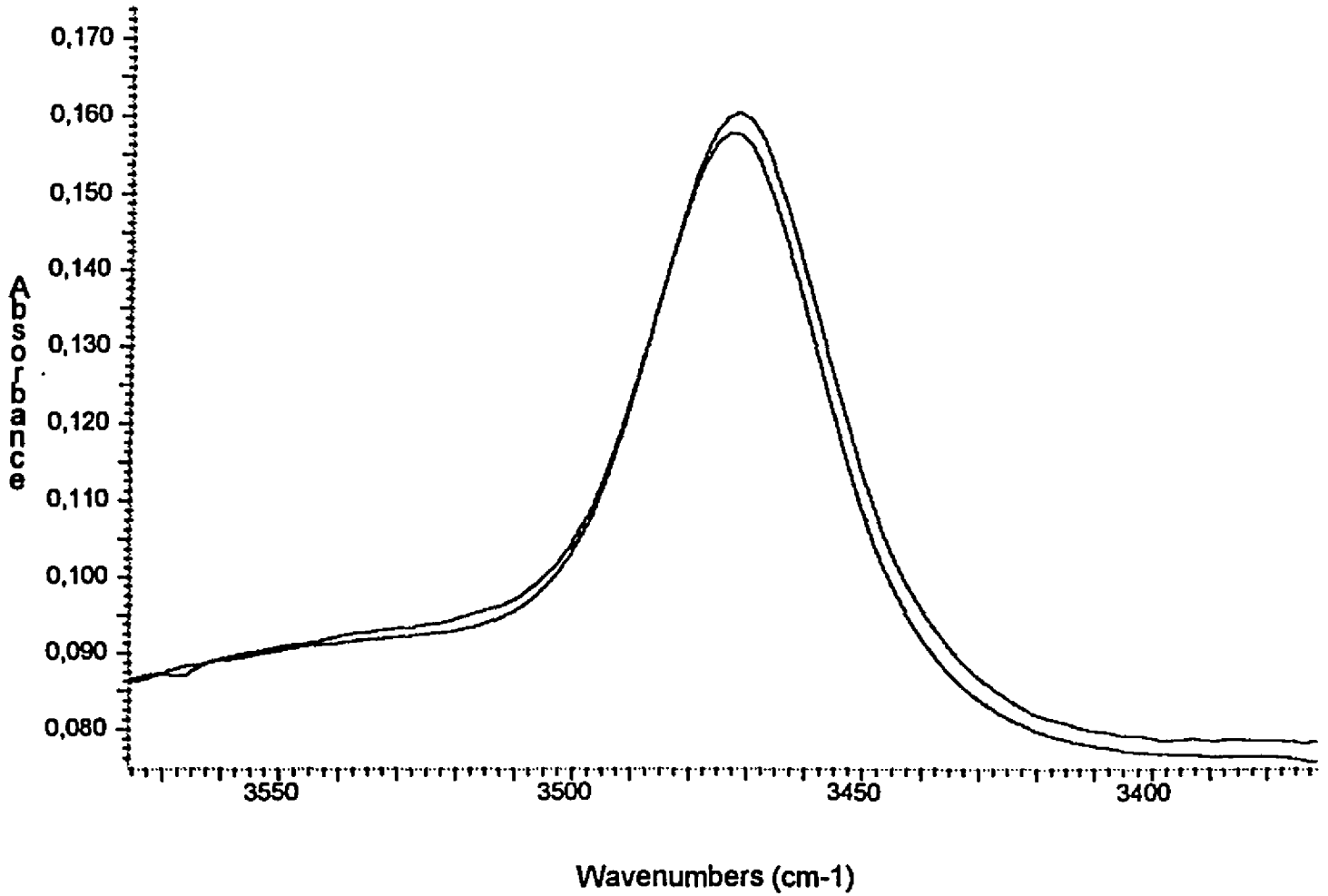

Figure 13: Variation of the Peak Height and Width due to Instrument Energy.

sample into the cell, give it a name and press enter. The computer-driven routine then scans the sample, applies the corrections required according to the methyl myristate run and predicts the peroxide value through the PLS calibration.

Chapter 5

Determination of Aldehyde Content and Correlation with AV.

5.1 Introduction

A variety of chemical methods are available which attempt to monitor secondary oxidation products, including the thiobarbituric acid test (TBA), the Kreis Test and various other methods which attempt to address both total and volatile carbonyl compounds, all of which have been reviewed by Gray (1992) and discussed in Chapter 2. Some of the methods developed for carbonyl compounds have been shown to be quite sensitive, quantitative and well correlated to compounds associated with the development of rancidity. A modification of the original method developed by Holm et al (1957), which uses *para*-anisidine instead of the carcinogenic benzidine acetate as the reactive reagent, is a widely accepted AOCS method, commonly known as the anisidine value (AV) test (AOCS, 1989). Although the method is relatively simple, the procedure requires substantial precision, analytical time, and uses relatively noxious reagents. AV is a combined measure of mostly 2-alkenals and 2,4-dienals, and to a more limited degree saturated aldehydes. The UV absorption of the *p*-anisidine/aldehyde reaction products varies with the aldehyde type, with a double bond in the carbon chain conjugated with the carbonyl double bond increasing the molar absorbance by a factor of 4-5 (AOCS, 1989). AV is commonly used to follow the formation of aldehydic compounds in edible oils, correlating well with the development of off flavors in lipids undergoing oxidation.

In our laboratory, work is ongoing to develop rapid automatable methods for the analysis of edible fats and oils based on Fourier Transform Infrared (FTIR) spectroscopy (van de Voort, 1994). To date, FTIR methods have been developed to measure iodine value, saponification number (van de Voort et al, 1992a), *cis-trans* isomers (van de Voort et al., 1995), free fatty acids (Ismail et al., 1993) and peroxide value (van de Voort et al., 1994a). Basic spectroscopic work has been carried out on secondary oxidation products which may be present in fats and oils as well as conceptual considerations related to the chemometric approaches which might be applied to developing appropriate quantitative FTIR methods (van de Voort et al., 1994b). In this chapter, we investigate the development of an FTIR method capable of measuring saturated, monounsaturated and conjugated unsaturated aldehydes and their relation to AV measurements of an oil undergoing thermal stress.

5.2 Experimental Procedures

5.2.1 Instrumentation/Sample Handling

The instrument used for this work was a Nicolet Impact 400 FTIR spectrometer (Nicolet Instruments Inc., Madison, WI) controlled by a 486 MHz PC run under OMNIC 1.2. The instrument was equipped with a heated sample handling accessory (van de Voort et al., 1995) set to 80°C, capable of handling both fats and oils using a ~100μm NaCl

transmission flow cell loaded by vacuum aspiration. Prior to starting any analysis, isooctane was passed through the system to clean the cell and transfer lines, and all samples were preheated for ~1 minute to 80°C in a water bath to minimize temperature perturbations in the cell. To limit water vapor and carbon dioxide interferences, the instrument was continuously purged with CO_2 free dry air supplied by a Balston dryer (Balston, Lexington, MA).

5.2.2 Calibration Standards/Chemometrics

The same approach as used in the development of the calibration for the determination of peroxide value detailed in the preceding chapter was taken. The calibration is based on taking a fresh oil, or an oil made fresh by cleaning to remove secondary oxidation products, and physically adding representative components and interfering substances. Commercial canola oil was obtained locally and passed twice through a column of activated silica gel to remove any carbonyl compounds or other partially polar molecules which might be present. The cleaned oil was analyzed for its AV (AOCS, 1989) to ensure that the AV was < 0.3 and that the aldehyde spectral contributions in the standards formulated were a direct result of the aldehydes added. Hexanal, *t*-2-hexenal and *t,t*-2,4-decadienal, *t*-4-hexen-3-one, hexanol, and *tert*-butylhydroperoxide (TBHP) were purchased from Aldrich Chemicals (Milwaukee, WI). A synthetic calibration matrix (Table 4) was developed by spiking the cleaned canola oil with known, random amounts of hexanal (0-26 μM/g), hexenal (0-12 μM/g), decadienal (0-4 μ

M/g), plus random variable amounts of hexenone, TBHP, hexanol, water and oleic acid to produce thirty randomized representative calibration standards (Anonymous, 1992). These standards were analyzed for their corresponding AV and served as a basis by which Partial Least Squares (PLS) calibration models could be derived to quantitate for both individual aldehydes and AV based on the FTIR spectra obtained for the standards. Multiple regression was used to relate the known concentrations of the individual aldehydes added to the base oil to the chemically determined AV.

For all standards and samples, single-beam spectra were collected using 256 scans at a resolution of 4 cm^{-1}, using triangular apodization at a gain of 1.0 and ratioed against an air emittance background (without the cell) to produce an absorbance spectrum and stored to hard disk for subsequent chemometric analysis. In order to accentuate spectral changes, the single-beam spectra were also ratioed against the single-beam spectrum of the starting oil to produce "differential spectra", a technique which allows one to more readily visualize subtle changes which otherwise might be difficult to detect (van de Voort et al., 1994b). For the development of FTIR calibrations, PLS was the chemometric method of choice (van de Voort et al., 1992b), variance spectra being used to determine regions correlating with the changes in component concentration and the calibrations developed using the Nicolet Quant-IR PLS package (Anonymous, 1992). The quality of the calibration was evaluated and optimized using a combination of partial residual error sum of squares (PRESS) test and the "leave-one-out" cross-validation approach.

Sample	Hexanal	Hexenal	Decadienal	Wat	Ket	FFA	ROOH	ROH	AV
1	1.320	0.190	0.003	+[1]	+	+	+	+	0.88
2	2.090	2.080	0.570		+	+	+		13.80
3	1.000	0.990	0.270				+	+	7.48
4	0.035	0.051	0.002		+		+		0.15
5	3.670	2.890	0.150		+		+		9.75
6	25.600	5.200	0.312	+			+	+	23.52
7	0.490	1.170	0.014		+		+		1.91
8	0.000	0.000	0.000						0.29
9	0.000	0.000	0.000	+		+	+		1.00
10	1.310	0.500	0.320	+	+		+		7.82
11	1.680	5.380	0.720		+	+	+		26.09
12	1.300	0.750	1.220	+		+	+		23.93
13	1.490	4.710	2.380		+	+	+	+	48.92
14	1.230	9.910	3.020		+	+	+	+	65.57
15	10.900	11.100	0.170		+	+	+	+	31.99
16	1.400	3.210	1.100	+	+	+	+	+	25.42
17	8.690	5.070	0.110		+	+	+	+	15.44
18	1.290	0.160	0.068					+	2.20
19	0.990	0.657	0.076	+			+	+	2.61
20	3.720	8.170	0.317	+		+	+	+	23.69
21	9.550	1.560	0.145		+	+	+	+	8.88
22	0.087	1.340	0.062	+		+	+		4.95
23	5.310	5.830	0.197		+		+		8.83
24	6.000	6.330	0.378		+	+	+		21.37
25	13.500	4.920	3.470	+	+		+	+	68.04
26	1.100	0.411	0.104	+	+	+	+		2.89
27	0.000	0.000	0.000				+	+	0.00
28	0.615	4.960	1.510		+	+	+	+	32.79
29	3.380	1.230	0.868	+	+		+	+	17.01
30	0.990	0.370	0.094	+	+	+	+		2.60
31	0.861	6.940	2.110		+	+	+	+	45.90
32	1.420	4.470	2.260		+	+	+	+	46.47

Table 4. Calibration Matrix for PLS Aldehyde Calibration (μmol/g). [1] (+) indicates component added to oil (Wat= water, Ket= ketone, FFA= free fatty acid, ROOH= hydroperoxide and ROH= alcohol).

The efficacy of the synthetic calibration to monitor the formation of aldehydes and predict AV was assessed by analyzing the spectra of canola oil thermally stressed at 120, 155 and 200°C. The oxidation reaction was promoted by bubbling dry air through the oil which was under continuous agitation. Aliquots were collected as a function of time, cooled, flushed with nitrogen and stored at -20°C for subsequent FTIR analysis and chemical AV determinations. A portion of these samples were also used to develop alternate PLS calibration models for AV determination.

5.3 Results

5.3.1 Spectral Analysis of Standards

Previous work on the monitoring of the oxidation of edible oils by FTIR spectroscopy (van de Voort et al., 1994b) indicated that it may be possible to develop and make use of synthetic calibration standards as a means of developing quantitative methods for the analysis of secondary oxidation products. Such standards are prepared by spiking a clean oil with compounds which are spectroscopically representative of oxidation products. From the point of view of developing an FTIR method which may substitute for the chemical AV method, saturated, α, β-unsaturated aldehydes and α, β, δ, γ-unsaturated aldehydes are the components of interest, and these can be represented by hexanal, 2-hexenal and 2,4-decadienal, respectively. The objective of this work was to use these aldehydes as a basis for developing a synthetic calibration matrix from which a

PLS calibration model could be derived to quantitatively predict both the AV and aldehyde content of thermally stressed oils. In a real system undergoing oxidation, products other than aldehydes are formed which may have an effect on quantitation and must be included in the calibration model to account for their contributions and/or interferences. Such compounds include hydroperoxides, alcohols, free fatty acids, water and possibly ketones. The basic IR absorptions associated with compounds representative of oxidation products in oil have been determined in a previous study and are summarized in Table 5.

In general terms, the aldehydes show a typical band at 1730-1680 cm^{-1} arising from the C=O stretch and weaker CH stretching bands between 2820 and 2700 cm^{-1}. For α,β unsaturated aldehydes, the C=O stretch band is shifted to a lower frequency and the C=C stretching band at 1640 cm^{-1} appears. Finally, in the case of *trans* conjugated aldehydes, a characteristic C=C-H bending absorption is observed at 987 cm^{-1}; the corresponding band is at 974 cm^{-1} when there is an isolated double bond. In the same study, it was established that saturated and unsaturated ketones are difficult to distinguish from α,β-unsaturated aldehydes because the absorption bands overlap with the C=O stretching of *trans* conjugated aldehydes and the C=C stretch and C=C-H bending of *trans*-2-hexenal.

COMPOUND	VIBRATION	PEAK MAXIMUM
Water	ν *OH*	3650 & 3550 cm^{-1}
Water	δ *HOH*	1625 cm^{-1}
Hexanol	ν R*OH*	3569 cm^{-1}
t-Butyl hydroperoxide	ν RO*OH*	3447 cm^{-1}
Hexanal	ν R*HC*=O	2810 & 2712 cm^{-1}
Hexanal	ν RH*C*=*O*	1727 cm^{-1}
Hexenal[a]	ν R*HC*=O	2805 & 2725 cm^{-1}
Hexenal	ν RH*C*=*O*	1697 cm^{-1}
Hexenal	ν R*C*=*C*H-HC=O	1640 cm^{-1}
Hexenal	δ R*C*=*C*H-HC=O	974 cm^{-1}
2,4-Decadienal[a]	ν R*HC*=O	2805 & 2734 cm^{-1}
2,4-Decadienal	ν RH*C*=*O*	1689 cm^{-1}
2,4-Decadienal	ν R*C*=*C*H-HC=O	1642 cm^{-1}
2,4-Decadienal	δ R*C*=*CH*-HC=O	987 cm^{-1}
4-Hexen-3-one[a]	ν R*C*(=*O*)HC=CHR	1703 & 1679 cm^{-1}
4-Hexen-3-one	ν RC(=O)H*C*=*C*HR	1635 cm^{-1}
4-Hexen-3-one	δ RC(=O)H*C*=*CH*-R	972 cm^{-1}
Oleic acid	ν RCO*OH*	3310 cm^{-1}
Oleic acid	ν R*C*(=*O*)OH	1711 cm^{-1}

[a] All double bonds in the *trans* form. (adapted from van de Voort et al., 1994b)

Table 5: Peak position of the functional group absorptions of reference compounds representative of products formed in oxidized oils.

To determine the influence of other components present in oxidized oils on the spectral contribution of the aldehydes of interest, TBHP, hexanol, water, oleic acid, and hexenone were added individually to a clean oil sample and to a bulk oil sample spiked with the three aldehydes. Figure 14 presents the resulting spectra of selected standards considering the four regions affected (OH, CH, C=O and *trans*). Analysis of these spectra indicated that hydroperoxides and alcohols caused band broadening and a shift toward lower frequencies of the strong triglyceride ester linkage absorption centered at ~ 1748 cm^{-1}, manifesting itself as a band around 1725 cm-1 in the differential spectra of the aldehyde free oil. This band appears at slightly lower frequencies in the case of hydroperoxides as compared to alcohols and was concentration dependent, shifting further to lower frequencies (1723 cm^{-1}) as hydroperoxide and alcohol concentration was increased. The appearance of this band was determined to be due to hydrogen bonding of alcohol and hydroperoxides with the triglyceride ester carbonyl. This observation was of consequence as the shifted band superimposes directly over the location of the carbonyl absorption of saturated aldehydes, indicating that this wavelength could only be used for quantitative analysis with a calibration including the entire range of intensity of possible hydrogen bonding. Fortunately, saturated aldehydes have a unique, but weak signal in the CH region, which provides an alternate means of making a measurement of this aldehyde contribution. Hexenal and decadienal (1697 and 1689-1642 cm^{-1}), although further from the hydrogen bond broadening effect, still appeared to be affected by the trailing end of this strong absorption, potentially limiting quantitation of these aldehydes at lower concentrations. Having considered these limitations, a calibration matrix was devised by

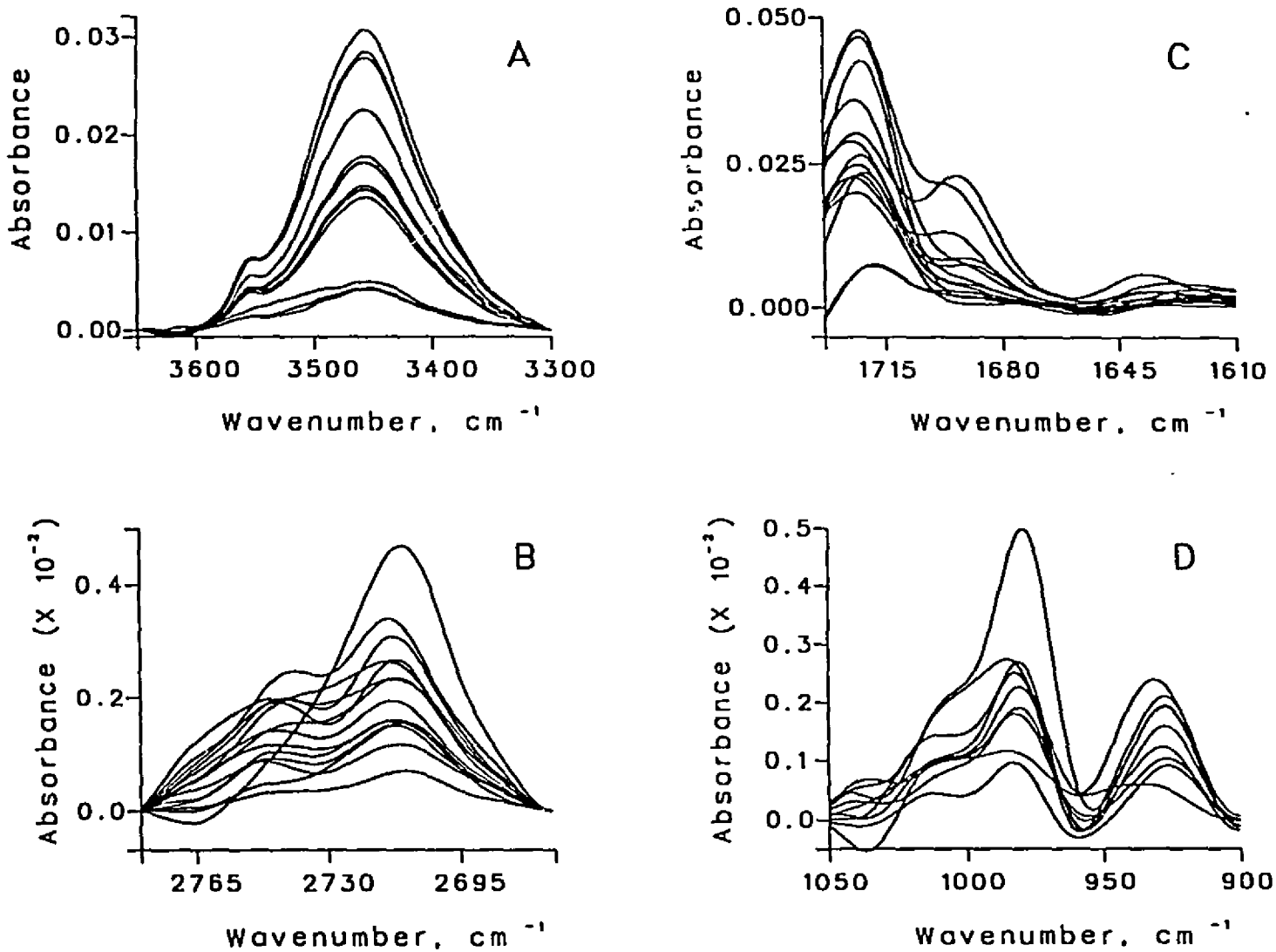

Figure 14: Differential Spectra of Selected Calibration Standards in the a) OH, b) CH, c) C=O and d) trans Regions.

adding random amounts of the three aldehydes (hexanal, hexenal and decadienal) to a clean canola oil plus random amounts of TBHP, hexanol, water, oleic acid, and hexenone as per Table 4.

5.3.2 Spectroscopy of Thermally Stressed Oils

FTIR spectroscopy is capable to measure the spectral changes associated with appearance or loss of a variety of diverse functional groups (van de Voort et al., 1994b) and as such may serve as a means of following the changes in aldehydes and provide an alternate means of determining AV. As an oil undergoes oxidation, the general reaction process involves the uptake of oxygen by the fat through the formation of hydroperoxides, which subsequently break down by various mechanisms to produce a variety of shorter chain aldehydes, fatty acids and alcohols (Frankel, 1984). Figure 15 provides a time course of canola oil undergoing oxidation at 155°C, the regions examined paralleling Figure 14.

In the OH region, it is apparent that hydroperoxides and alcohols are being formed, alcohols being formed later on in the reaction. In the CH region, changes indicating the formation of saturated aldehydes are apparent, while in the C=O region, unsaturated and conjugated unsaturated aldehyde bands appear. In this spectral region the loss of the *cis* double bond is also apparent, while in the *trans* region isolated and conjugated *trans* bands appear with time. Comparing these spectra with those of the calibration standards (Figure 14), many commonalties are evident in the OH and C=O

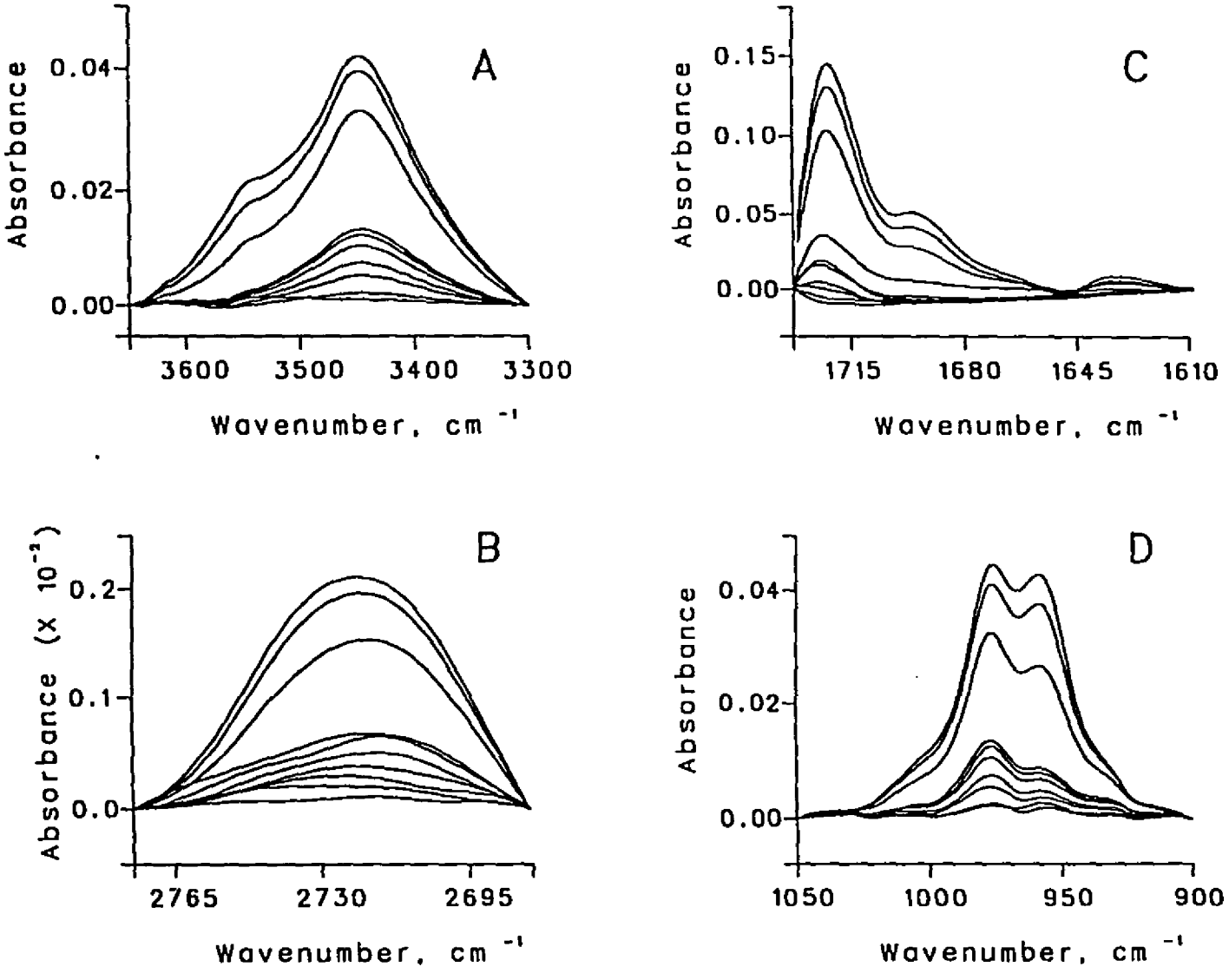

Figure 15:Differential Spectra of Canola Oil unergoimg Oxidation at 155°C in the A) OH, B) CH, C) C=O and D) *trans* Regions.

regions. In the C=O region (1710-1680 cm-1), unsaturated and conjugated unsaturated aldehyde contributions can be measured and the relatively weak CH bands can be used to determine the saturated aldehyde contributions. The major differences between these spectra lie in the *cis* double bond and *trans* region, as in the process of oxidation, *cis* double bonds are converted to isolated *trans*, while the appearance of conjugated *trans* double bonds are indicative of unconjugated, unsaturated aldehydes. Because the changes in the *cis* double bond and *trans* region cannot be mimicked by the addition of standards, these regions are not available for quantitation.

To confirm that the strong 1725 cm^{-1} band in the thermally stressed oil was in fact due to hydrogen bonding effects, a sample containing hydroperoxides was reacted with triphenyl phosphine (Aldrich Chemical Co., Milwaukee, WIS) to convert the hydroperoxide groups to alcohol groups. The efficacy of the reaction was demonstrated by a reduction in the intensity of the hydroperoxide band and the appearance of an alcohol band, this shift being accompanied by a corresponding shift of the 1725 cm^{-1} band to 1727 cm^{-1} (Figure 16) indicating that this band is due to hydrogen bonding as demonstrated earlier in the synthetic samples. The shoulder developing between 1705 and 1680 cm-1 shows the formation of unsaturated aldehydes, while the peak on the far right is due to the formation of α, β, δ, γ-unsaturated aldehydes (decadienal type).

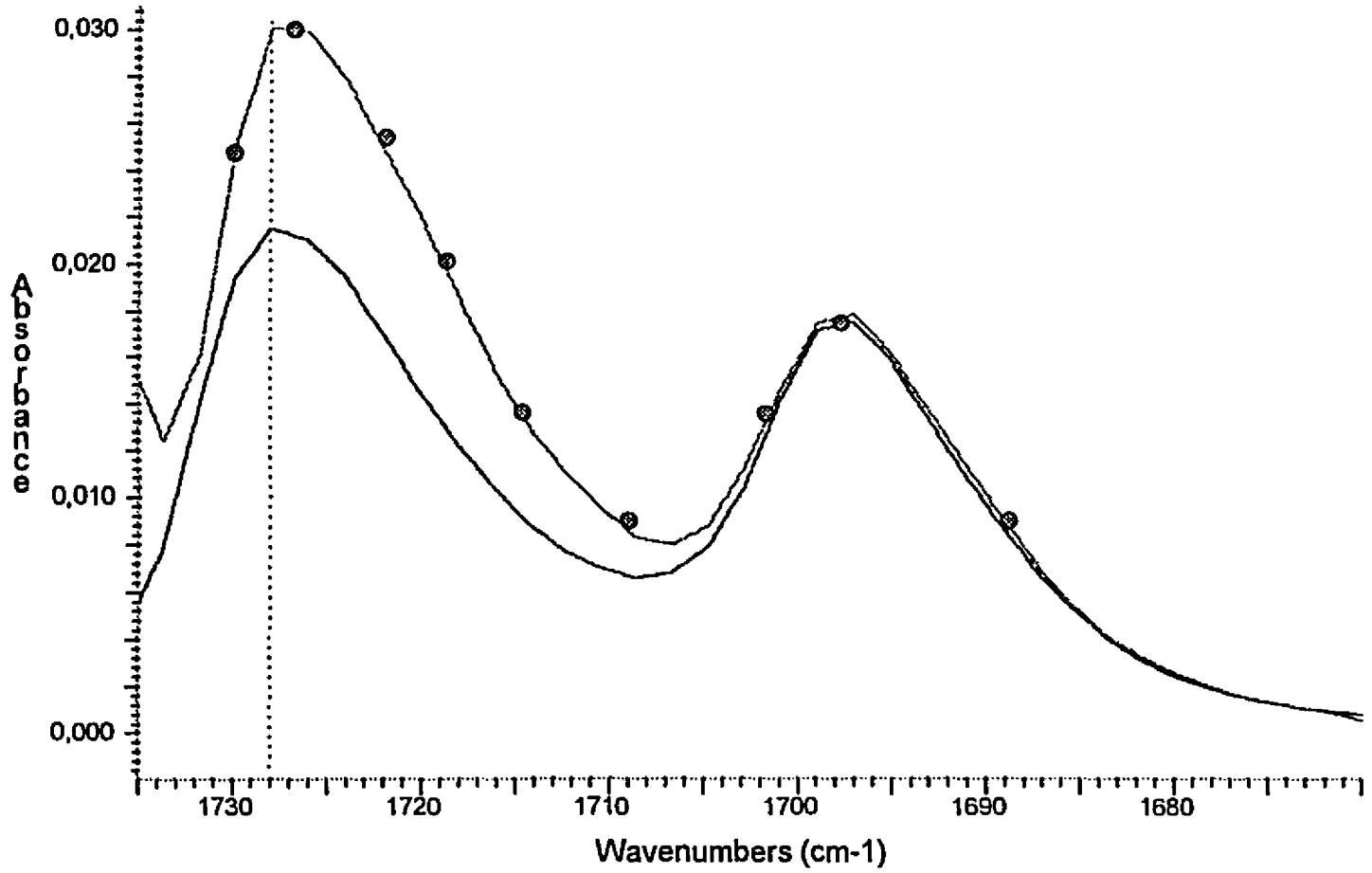

Figure 16: Hydrogen bonding effect killed with TPP. (- ⊙H-bonding present, ⊣ bonding absent)

5.3.3 Synthetic Calibration Assessment

The determination of AV is predicated on the reaction of *p*-anisidine with aldehydes, the reaction products absorbing in the UV portion of the spectrum, albeit the extinction coefficient being variable depending on the aldehyde type. The basic premise in developing a synthetic calibration is that the aldehydes contributing to the chemical AV can be mimicked using selected aldehydes, taking into account the interferences of other products such as moisture, free fatty acids, alcohols and hydroperoxides which are also produced as autoxidation proceeds (van de Voort et al., 1994b). The chemometric method of choice for developing a multicomponent calibration is PLS, as it is capable of accounting for interfering compounds which are external to the measure in question as long as the standards are a reasonable facsimile of the variation found in the samples to be analyzed. In addition, one has to determine the spectral regions associated with the variability of the components of interest and subsequently develop and optimize a calibration model which will quantitate the components of interest. One way of comparing the variability of the calibration set to the samples to be analyzed and guide the selection of appropriate wavelength regions is by examining the variance spectrum, which is indicative of the spectral regions that vary the most relative to the mean spectrum. Figure 17 compares the variance spectra of the calibration standards to the spectra of canola oil thermally stressed at 120, 155 and 200°C in the four spectral regions of interest. As can be seen, the relative variation in the thermally stressed oils is similar to that of the calibration standards, although it is apparent that the relative degree of variation is

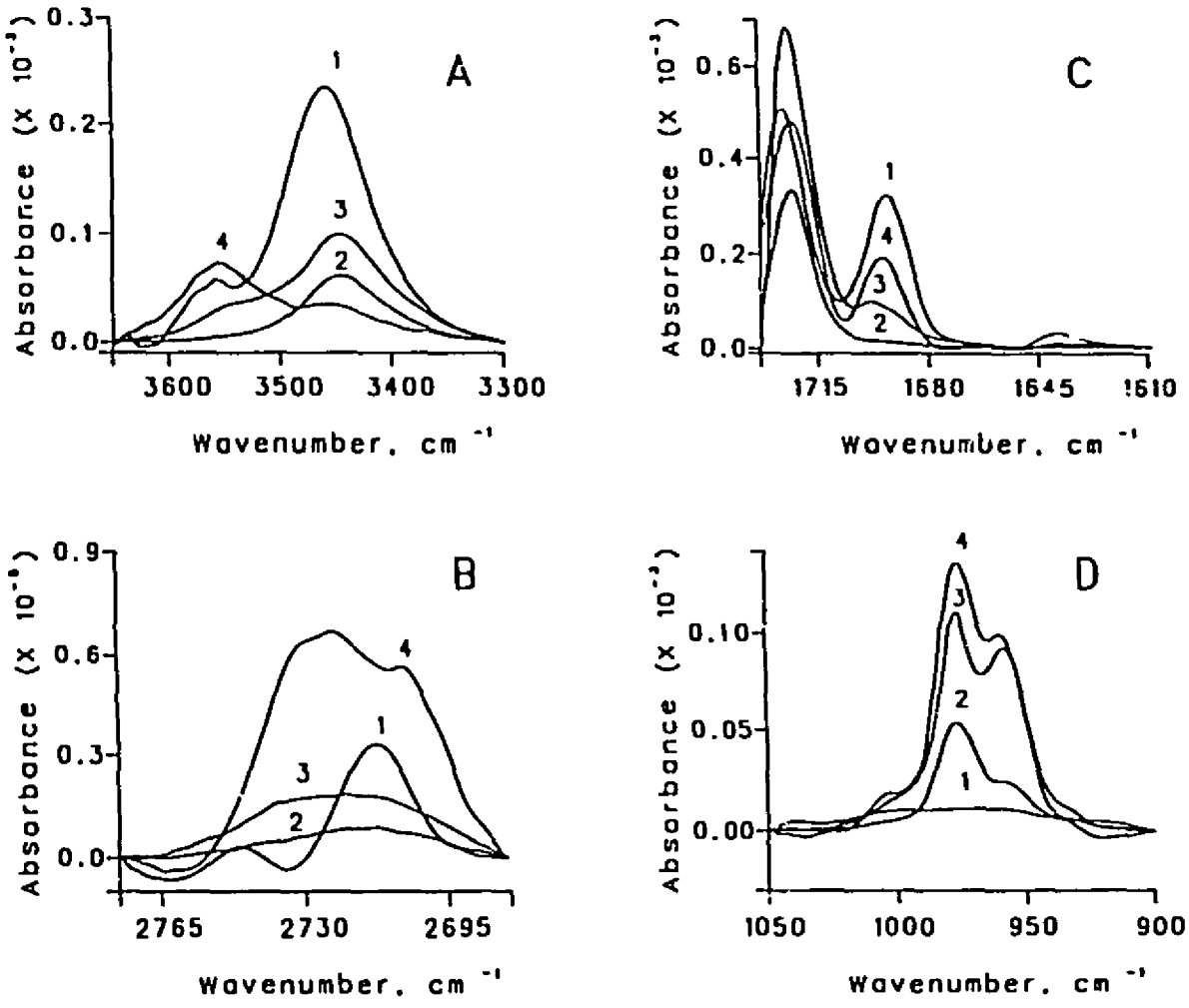

Figure 17: Variance spectra of (-1) the calibration set and the three temperature runs (-2=120°C, -3= 155°C and -4= 200°C).

different for each temperature condition, the 120°C run forming mostly hydroperoxides, the 155°C run forming hydroperoxides, some alcohol and aldehydes, while the 200°C run forms largely alcohols and aldehydes. In the carbonyl region, the correlation between the hydrogen bonding band and the hydroperoxide/alcohol formation is apparent in these variance spectra as there is a shift of this band to lower frequencies when hydroperoxides and alcohols are present. These variance spectra indicate that the unsaturated aldehyde carbonyl region (1710-1680 cm^{-1}) is showing increased variability as temperature rises while the saturated aldehyde region (CH) only shows evidence of substantive change at 200°C.

Two PLS calibration models were developed based on the synthetic calibration set, one based on the concentrations of hexanal, hexenal and decadienal and the other based on the chemical AV. Both calibrations were optimized using the "leave one out" cross validation procedure to minimize the prediction error and provide an estimate of the overall accuracy of the predictions. Linear regressions of the optimized cross validations of the individual aldehyde and AV predictions vs their corresponding added amounts and chemical AV values, respectively produced the equations presented in Table 6.

Component	Region (cm^{-1})	Slope	Intercept	R^2	SE (μM)
Hexanal	2790-2670 cm^{-1}	0.926	-0.104	0.890	1.76
Hexenal	1730-1670 cm^{-1}	1.000	0.004	0.996	0.18
Decadienal	1730-1670 cm^{-1}	1.005	-0.005	0.996	0.06
AV	2790-2670 cm^{-1} 1730-1670 cm^{-1}	1.006	0.417	0.998	0.99

Table 6: Regression coefficients obtained for aldehyde PLS cross validation plots.

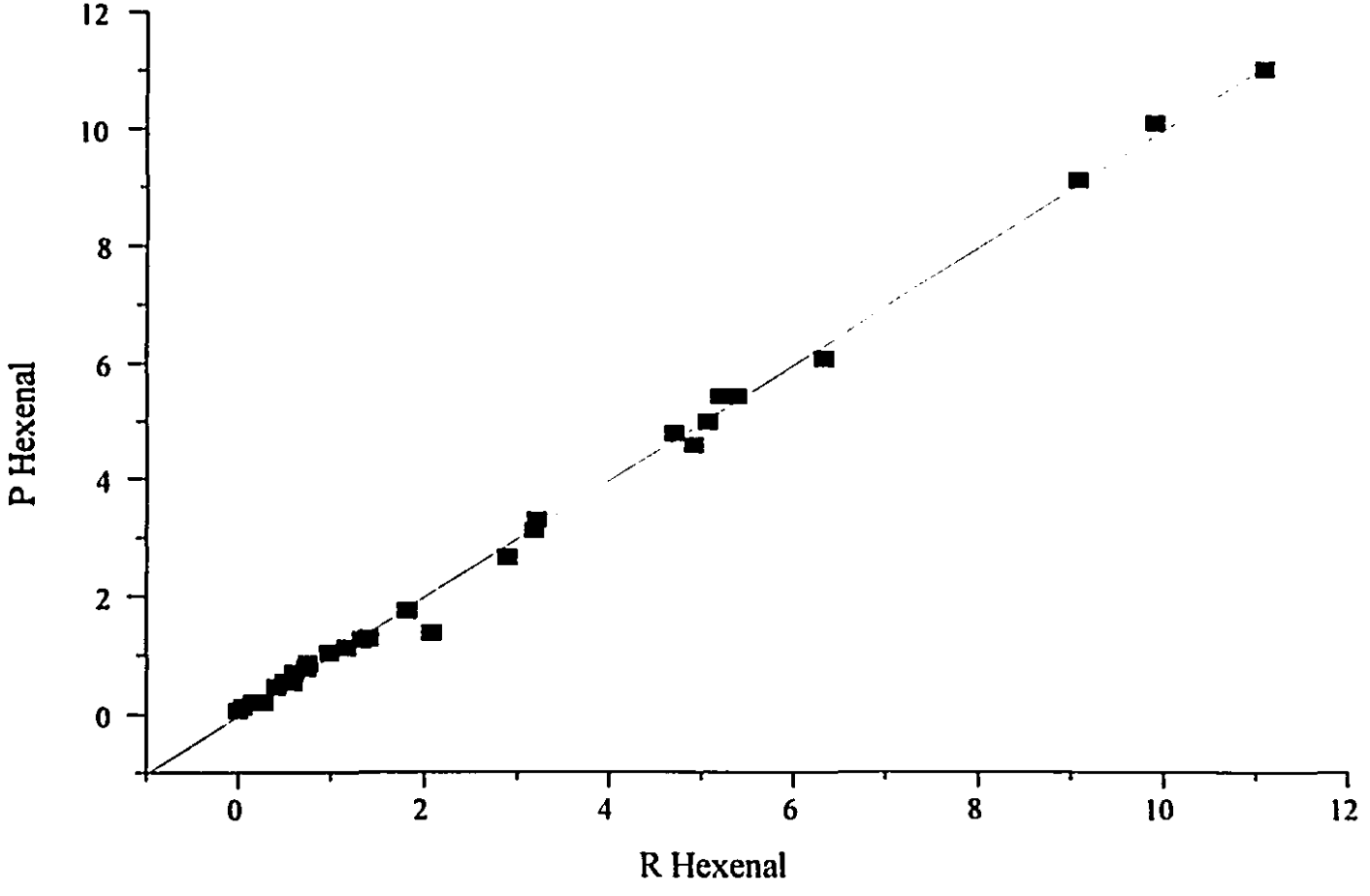

Figure 18: Plot of the Predicted Hexenal (P) vs the Real (R) Hexenal obtained from the Cross Validation.

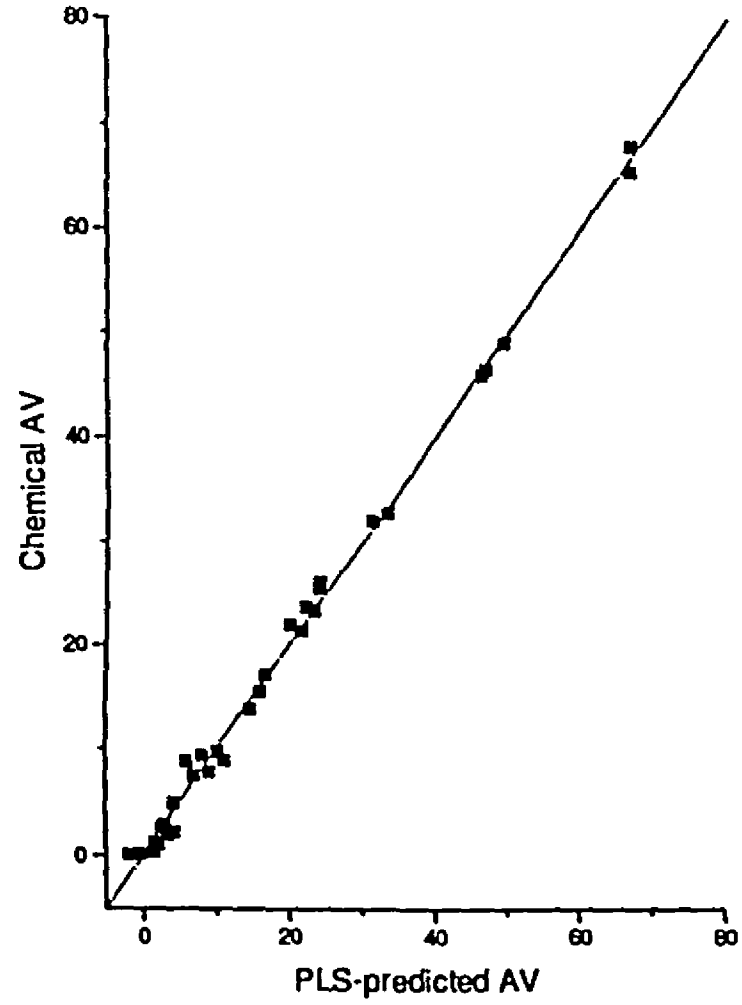

Figure 19: Plot of the Predicted AV (PAV) vs the Chemical AV (CAV) obtained from the cross validation.

Figure 18 and 19 show the plots obtained from the cross-validation. As structured, the PLS calibration models provide one with access to measures of either individual aldehyde content in an oil or its AV. Of particular interest is the relationship of individual aldehyde predictions relative to the chemical AV. It is well known that the AV test measures predominantly unsaturated aldehydes and is particularly sensitive to conjugated unsaturated aldehydes, especially dienals (AOCS, 1989). Therefore, the AV test is considered an indirect measure of carbonyl compounds in general, its overall response being a function of the types of aldehydes present (Robards et al., 1988). As the spiked aldehyde concentrations added to the clean canola oil are known along with their corresponding AV, it is possible to determine the relative contribution of each aldehyde to the AV using multiple regression analysis. The equation derived relating AV to each aldehyde class after forcing the regression through the origin was:

$$AV = 0.34S + 2.16U + 15.36C \qquad R^2 = 0.997 \quad SE = 1.37 \quad [4]$$

Where: S= saturated aldehydes (μmol/g)
U= monounsaturated aldehydes (μmol/g)
C= conjugated diunsaturated aldehydes (μmol/g)

The coefficients in equation [4] indicate that the relative UV response of the three aldehyde types (S, U and C) is approximately 1:6:45 for the AOCS AV method vs. 1:6:11 reported by Holm et al. (1957), who used benzidine acetate as the reagent and based their measurements on heptanal, 2-nonenal, and 2,4-hexadienal. The SE obtained from the

multiple regression can be considered a measure of the accuracy of the chemical AV method as it relates AV to gravimetrically added aldehyde concentrations. Equation [4] implies that one can convert PLS predicted aldehyde concentration data into "apparent" AV values, the plot of the predicted AV vs the chemical AV being shown in Figure 20. The benefit of such an approach is that one would have a means of obtaining both AV predictions and information relating the aldehyde types contributing to the measure of AV. The predicted aldehyde concentrations of the calibration standards obtained from cross-validation were transformed to "apparent" AV values and regressed against the chemical AV, yielding the following equation:

$$CAV = -0.70 + 1.031\ PAV \qquad R^2 = 0.994 \quad SE = 1.48 \qquad [5]$$

Where:
CAV= chemical AV
PAV= predicted AV

5.3.4 Quantitative Assessment of Thermally Stressed Oils

Aldehyde Predictions using the Synthetic Calibration

In thermally stressed oils the actual content of various aldehyde forms is unknown and a chemical AV determination is only a general indicator of the carbonyl compounds formed. Applying the aldehyde PLS calibration to canola oil thermally stressed at 120, 155 and 200°C, the individual "apparent" concentrations of hexanal, hexenal and decadienal were determined. Using the multiple regression equation [4] derived earlier,

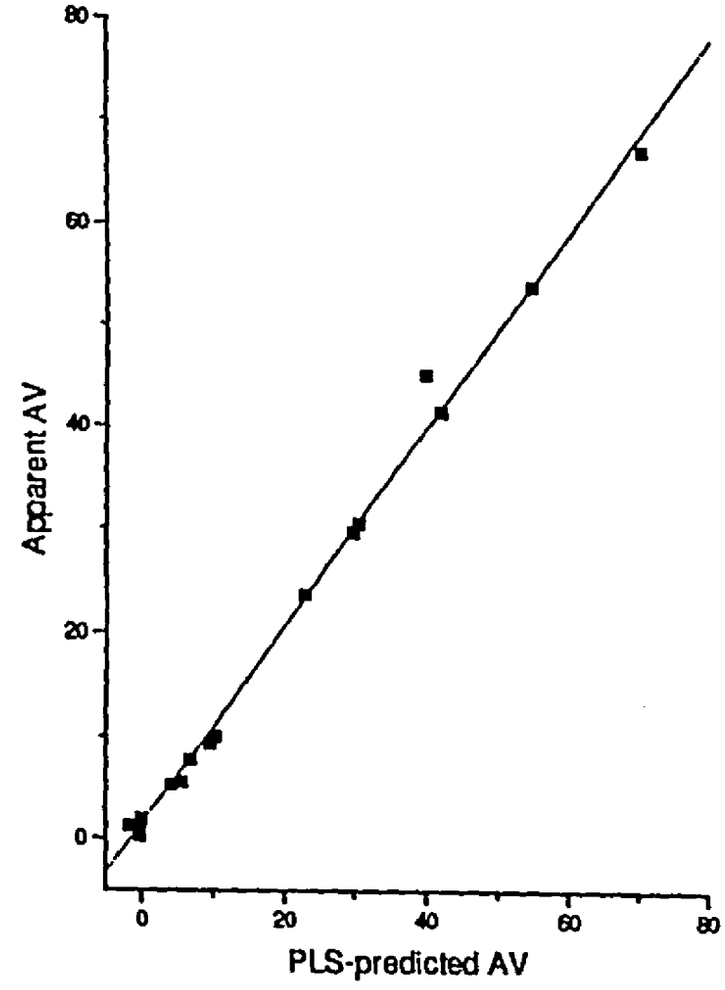

Figure 20: Plot of the AV obtained through Multiple Linear Regression Equation (MLR AV) vs the Chemical AV (CAV)

the aldehyde data was transformed into "apparent" AV values and regressed against the chemical values obtained for these samples, yielding the following equation:

$$CAV = 1.08 + 0.953\ PAV \qquad R^2 = 0.994 \quad SE = 1.65 \qquad [6]$$

Where:
CAV= chemical AV
PAV= predicted AV

The "apparent" and chemical AV values show a linear relationship, indicating that a generalized synthetic PLS calibration model is capable of predicting the AV of thermally stressed oils at temperatures ranging from 120 to 200°C. Using the PLS calibration based solely on the chemical AV measurements (Table 6), as opposed to the individual aldehyde concentrations, produced similar results:

$$CAV = 1.48 + 0.961\ PAV \qquad R^2 = 0.994 \quad SE = 1.67 \qquad [7]$$

Where:
CAV= chemical AV
PAV= predicted AV

In both equations [6] and [7], the SE is only slightly higher than that of equation [4], which is indicative of the accuracy of the chemical method. These results imply that a generalized synthetic calibration is a satisfactory model of the wide compositional variability of the "real" systems undergoing oxidation under different temperature conditions.

Calibrations based on the Thermally Stressed Samples

Another approach which can be taken to develop a calibration is to use the thermally stressed samples from the three temperature runs. This procedure has the benefit of including all the sources of variability encountered in the oxidation process, but this type of approach also limits the applicability to the same set of conditions since the variability included is representative of these conditions only. A calibration was developed using the same spectral regions as used for the aldehyde PLS model. To avoid building in any time correlations, the oxidized samples from the three temperature runs were physically mixed in known random proportions. A set of samples from the time runs was kept to validate the predictive accuracy of the calibration based on oxidized samples. The cross validation results of the AV PLS model produced equation [8]:

$$CAV = 1.33 + 0.961\ PAV \qquad R^2 = 0.998 \qquad SE = 1.25 \qquad [8]$$

The samples set aside for validation were analyzed and predicted using the PLS calibration based on the oxidized oils. Excellent results were obtained, described by equation [9].

$$CAV = 0.55 + 0.983\ PAV \qquad R^2 = 0.998 \qquad SE = 1.21 \qquad [9]$$

These results demonstrate the ability of PLS to predict the anisidine value of an oxidized sample with a calibration based on either synthetic calibration standards or

representative oxidized samples which have been mixed randomly to break any time correlations.

5.4 Discussion

The objective of this study was to develop a "universal" synthetic calibration for AV determination in thermally stressed oils, including the ability to quantitate individual aldehyde types contributing to the overall AV. This objective was considered reasonable as the concept has been successfully applied to the determination of peroxide value. The AV determination differs somewhat in that one is considering three components rather than one with the additional complication of hydrogen bonding obscuring one of the main quantitative regions (hexanal carbonyl @ 1725 cm^{-1}). The results indicate that the synthetic calibration approach works well as does a more conventional calibration approach using oxidized samples. A key advantage of the synthetic calibration approach is that one has the ability to monitor the changes in individual aldehyde types as well as AV with good accuracy. Furthermore, as the relationship between the concentrations of the aldehyde standards and AV has been established, one can calibrate an FTIR spectrometer for the determination of AV without carrying out chemical analyses of the calibration standards; i.e., with the availability of the relationship, the primary method against which the FTIR spectrometer is calibrated becomes gravimetrically based. A drawback of the synthetic calibration approach is the need to prepare a relatively large number of

calibration standards (30) in order to insure that the synthetic calibration matrix adequately models the concentrations and ranges of the interfering components that may be present in oxidized oils (specifically hydroperoxides and alcohols). Accordingly, in some quality control situations, where only AV is of interest, without the benefit of following the formation of individual aldehyde types, it may be more convenient to develop an AV calibration based on samples representative of the oxidation process being monitored, eliminating the need for the laboratory preparation of synthetic calibration standards. Using a frying oil as an example, relatively large samples (50 ml) can be taken over a time course and inspected using differential spectroscopy to select samples for AV analysis, using the changes in the OH, CH and C=O regions as a basis for selecting samples which have undergone significant spectral changes. Subsequently, the samples selected are analyzed for AV by the chemical method and then mixed quantitatively in a random fashion so that any correlations between aldehydes and other oxidation products are not built into the calibration set. A PLS calibration model is then developed to predict AV directly. Approached in this manner, the development of a calibration requires a minimum amount of chemical analysis, and the calibration model reflects the AV response for the oil system in question under the oxidation conditions of interest.

The AOCS AV method is predicted on the measurement of aldehydes reacting with *p*-anisidine to provide an empirical indication of the carbonyl content of the oil. This method is limited to being a general indicator of overall aldehyde content due to the variability in the UV absorptivity of saturated, mono-unsaturated and conjugated di-

unsaturated aldehyde adducts formed with *p*-anisidine. The FTIR approach is capable of measuring the contributions of the individual aldehyde types directly and theoretically does not face this limitation. However, accounting for all the potential interferences (water, free fatty acids, alcohols and hydroperoxides), their interactions with aldehydes and spanning the ranges and types of interfering compounds which might be encountered in any set of conditions requires a substantial expansion of the base calibration matrix to generalize the PLS calibration. This is especially true if one wishes to consider various oil types in the calibration and a wide range of temperatures of oxidation which produce totally different secondary products. This substantive task can be simplified by using one of the two approaches; (a) to calibrate on samples representative of an ongoing process, as described above, or (b), developing a general calibration matrix of the type employed in this study to predict individual aldehydes and adjusting it to the specific process conditions by adding a limited set of prepared samples spectrally representative of the oil in the conditions studied. In either case, FTIR spectroscopy can be used as a means of rapidly tracking AV in thermally stressed oils, an operation which can be carried out at line. The methodology and calibrations developed can be integrated and automated using Visual Basic programming to provide a user-friendly analytical quality control package which can be used at-line. Such a system would be particularly useful in snack food frying oil monitoring, where oil quality has a major effect on flavor of the end product, reducing analytical time and disposal problems associated with the reagents used in the chemical method.

Chapter 6

Conclusion

In this work, two rapid methods using FTIR spectroscopy have been developed to monitor oxidation in vegetable oils. The determination of peroxide value based on the use of *t*-butyl hydroperoxide and the inclusion of all interfering substances in the spectral region of interest gave good results. The method is applicable to different vegetable oils at different stages of oxidation. A single spectral region is used for this measure which has the advantage of having relatively few interferences, but the low intensity of the absorbance in that same region requires the use of a cell with a long pathlength. On the other hand, the measure of aldehydes in oxidized oils was complicated by a large number of interfering substances and their interactions with the triglyceride ester linkage in the carbonyl region of the spectrum. It was demonstrated that one can monitor the amount of three different classes of aldehydes and AV using the calibration developed and obtain very good results. The rationale for investigating the development of an FTIR method for AV determinations is that it allows for the rapid and routine determination of this parameter and provides for the possibility of monitoring the relative amount of various aldehyde types being formed, if of interest.

One of the major advantages of using the spectroscopic methods developed to monitor the oxidative reactions in oils is that they can both be automated through the use of Visual Basic programming and used at-line in the oil industry or in a food frying operation. A computer-driven system can be made totally independent and programmed to collect the samples, scan and print the results as a report. The automation step is relatively simple and the system does not require recalibration because the standardization step accounts for variance in the conditions compared to the conditions at the time of calibration through the use of a calibration reference standard (methyl myristate or mineral oil). The results of this work and other methods developed by the McGill IR research group provide a growing body of evidence that FTIR spectroscopy has potential and will develop into a useful rapid quality control method for fats and oils. With its benefits of speed, accuracy and simplicity of operation, the cost of the FTIR system can easily be absorbed through savings made on trained technicians, time and chemical reagents, factors all attached to the conventional chemical methods.

The FTIR methods serve as means of reducing both analytical time and reagent use and disposal problems associated with the chemical PV and AV methods and would be particularly useful in monitoring oil quality in snack food frying operations, with other potential applications including the evaluation of the oxidative stability of vegetable oil based lubricants and hydraulic fluids.

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