IMPROVING LIPID OXIDATION MEASUREMENTS: INVESTIGATING A ^{1}H NMR ALTERNATIVE TO AND EVALUATING THE ASSUMPTIONS OF STANDARD CHEMICAL METHODS

by

Emily Harris

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ABSTRACT

Commonly used measures of lipid oxidation, such as the peroxide value (PV), conjugated diene value (CDV), and *p*-anisidine value (*p*AV) tests, are labourious and reagent-intensive. The objective of this thesis was to improve upon these methods. To assess the suitability of proton nuclear magnetic resonance (^{1}H NMR) as an alternative method of measuring lipid oxidation, correlations between ^{1}H NMR spectra and the PV, CDV, and *p*AV were evaluated by determining these parameters in soybean and canola oils oxidized to different extents. At high levels of oxidation, relationships were found between the measured PV and CDV and the ^{1}H NMR signal responses of hydroperoxidic and conjugated dienic signals, respectively. A relationship was observed between the measured *p*AV and aldehydic signal responses only over a limited range. All relationships were hindered by poor sensitivity of the ^{1}H NMR method. In the case of the *p*AV, sensitivity issues were compounded by deviation of the test from linearity at high levels of oxidation. These findings suggest that without additional time spent on sample preparation, instrument optimizations, or other sensitivity enhancements, ^{1}H NMR cannot be applied to the quantitative assessment of oxidation in edible plant oils at low levels of oxidation.

The *p*AV test can also be inaccurate due to contributions from aldehydic flavour compounds. For this reason, the Global Organization for EPA and DHA Omega-3s (GOED) recommends a modified method to measure the *p*AV of flavoured marine oils that is predicated on the assumption that the flavour compounds themselves do not oxidize or degrade as the oil oxidizes. To test the null hypothesis that flavour oxidation and/or degradation is not occurring, I performed stability studies to compare the *p*AV of fish oil samples to which flavour had been added before and after oxidation, respectively. The *p*AV and the ^{1}H NMR signal responses of the aldehydic flavour signals in these oils were evaluated to determine the extent of flavour degradation over the course of oxidation. For several sampling points in both chocolate-vanilla and lemon flavoured oils, the *p*AV of oils to which flavour was added before oxidation were significantly lower than those of oils to which flavour was added after oxidation, suggesting that these flavours degrade during oxidation. These findings suggest that the GOED recommendation may not consistently provide accurate estimates of the *p*AV in all flavoured marine oils.

LIST OF ABBREVIATIONS AND SYMBOLS USED

ALA	Alpha-linolenic acid
AOCS	American Oil Chemists' Society
$CDCl_3$	Deuterated chloroform
CDV	Conjugated diene value
d	Doublet
DHA	Docosahexaenoic acid
DMSO-*d6*	Deuterated dimethyl sulfoxide-d6
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EPA	Eicosapentaenoic acid
FAO	Flavour added after oxidation
FBO	Flavour added before oxidation
FID	Free induction decay
GC	Gas chromatography
GOED	Global Organization for EPA and DHA Omega-3s
^{1}H NMR	Proton nuclear magnetic resonance spectroscopy
HPLC	High-performance liquid chromatography
m	Multiplet
meq	Milliequivalents
n-3	Omega-3
*p*AV	*p*-Anisidine value
PUFA	Polyunsaturated fatty acids
PV	Peroxide value

RF	Radiofrequency
t	Triplet
TAV	True Anisidine Value
UV-Vis	Ultraviolet-visible
β_0	Applied magnetic field
γ	Gyromagnetic ratio
ω_0	Larmor frequency

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CHAPTER 1: INTRODUCTION

1.1 POLYUNSATURATED FATTY ACIDS

Consumption of polyunsaturated fatty acids (PUFA) has been associated with a wide range of health benefits (Riediger et al., 2009; Simopoulos, 1999). For example, marine omega-3 (*n*-3) fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been shown to reduce inflammation (Allaire et al., 2016) and lower blood triglycerides (Zulyniak et al., 2016) among other positive effects (Arterburn et al., 2006; Calder, 2012). Similarly, some studies have suggested that alpha-linolenic acid (ALA), an *n*-3 PUFA found in soybean and canola oils, may offer some cardiovascular benefit (Bassett et al., 2009; Fleming & Kris-Etherton, 2014).

Consequently, consumers are choosing to supplement their diets with marine oil products in order to increase their daily intake of EPA and DHA (Langlois & Ratnayake, 2015). Similarly, consumption of plant oils containing PUFA is increasing (Kojima et al., 2016). This is likely due in part to a consumer shift away from products, such as partially hydrogenated vegetable oils, that are no longer recognized as safe by the United States Food and Drug Administration (2015), and towards alternatives, such as soybean and canola oils, that contain PUFA (Kojima et al., 2016). Unfortunately, due to their unsaturated structure, EPA, DHA, and ALA, as well as supplements and products containing these fatty acids, are highly susceptible to lipid oxidation (Shahidi & Zhong, 2010).

1.2 LIPID OXIDATION

Lipid oxidation is a deteriorative process that occurs when oxygen reacts with unsaturated fatty acid radicals. Lipid oxidation is a free radical process that proceeds via a chain reaction involving three stages: initiation, propagation, and termination (Fig. 1.1). In the initiation stage, lipid radicals are formed when, in the presence of an initiator, hydrogen atoms are abstracted from

unsaturated fatty acids to create lipid radicals. In the propagation stage, lipid radicals react with oxygen to form peroxyl radicals. These peroxyl radicals then abstract labile hydrogen atoms from unsaturated fatty acids to form hydroperoxides and lipid radicals. The final stage of lipid oxidation, termination, occurs when peroxyl and other radicals accumulate and react with one another to form non-radical products (Frankel, 2005).

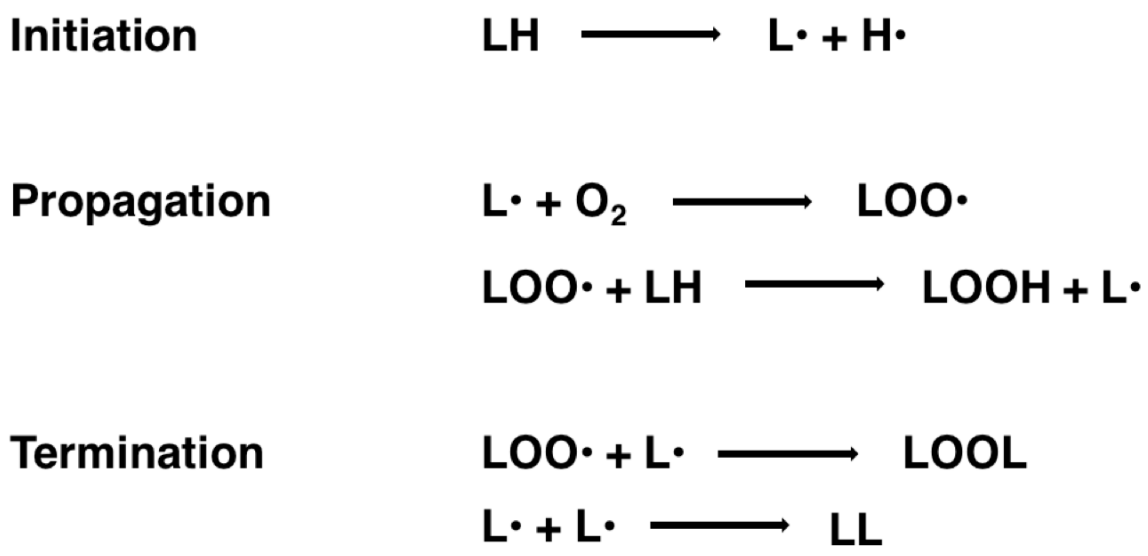


FIG. 1.1 The chain-reaction mechanism of lipid oxidation (adapted from Choe & Min, 2006 and Frankel, 2005).

The products that form during oxidation can be divided into two classes: primary and secondary oxidation products. Primary oxidation products, including hydroperoxides and conjugated dienes, are the first oxidation products formed during the propagation stage of oxidation. These products accumulate rapidly but are unstable and may decompose to give rise to alkoxy and hydroxyl radicals. These radicals can participate in further reactions to form secondary oxidation products, such as alcohols, ketones, and aldehydes (Choe & Min, 2006). For example, consider the oxidation of linoleic acid (Fig. 1.2). In the initiation stage of oxidation, the bis-allylic

hydrogen on the eleventh carbon is abstracted. Oxygen reacts with the resulting carbon radical to from conjugated diene hydroperoxides at the ninth and thirteenth positions. As oxidation progresses, hydroperoxide decomposition generates peroxyl radicals that can undergo β-scission, generating aldehydes as well as alkyl radicals that will participate in reactions to generate further secondary oxidation products.

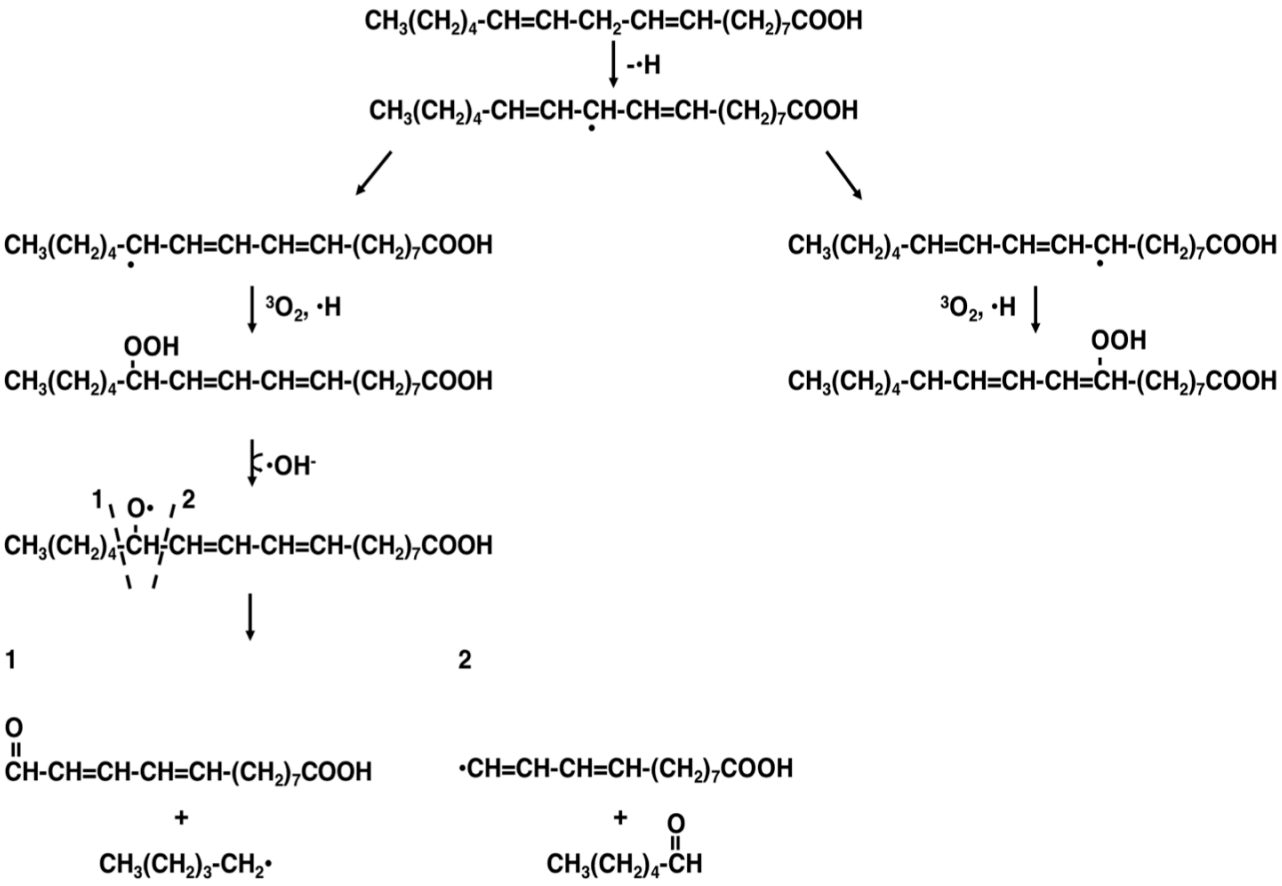


FIG. 1.2 The oxidation of linoleic acid to generate conjugated diene hydroperoxides, aldehydes, and lipid alkyl radicals (adapted from Choe & Min, 2006 and Frankel, 2005).

1.3 MEASURING LIPID OXIDATION

Lipid oxidation reduces product shelf-life and results in the formation of off-flavours and off-odours. Furthermore, the consumption of oxidized fatty acids may have negative health consequences (Esterbauer, 1993; Staprans et al., 2005). For these reasons, it is common practice to monitor the level of oxidation in edible oils. This is typically achieved by measuring the concentrations of various compounds that form over the course of oxidation, including hydroperoxides, conjugated dienes, and aldehydes. These tests are so routine that several organizations, including the American Oil Chemists' Society (AOCS) and the International Union of Pure and Applied Chemistry, have developed standard protocols for their measurement.

Hydroperoxides in edible oil samples are measured via iodometric titration using the peroxide value (PV) test. In this test, hydroperoxides in the sample oxidize potassium iodide, yielding iodine. A starch indicator is added to the solution, forming a purple chromophore with the iodine. The solution is then titrated with sodium thiosulfate until a colorimetric endpoint is reached (Frankel, 2005). The volume of sodium thiosulfate required to reach the endpoint is proportional to the concentration of hydroperoxides in the sample (Equation 1.1):

$$\mathrm{PV\ (meq/kg)} = \frac{(\mathrm{S} - \mathrm{B}) * \mathrm{N} * 1000}{\mathrm{m}} \qquad 1.1$$

where S and B are the volumes of sodium thiosulfate required to titrate the sample and the blank, respectively (mL), N is the normality of the sodium thiosulfate solution, and m is the mass of the oil sample (g). Note that the PV is measured in milliequivalents (meq) of oxygen per kilogram (AOCS, 2013b).

Conjugated dienes have absorbance maxima at 233 nm and can be measured spectrophotometrically using the conjugated diene value (CDV) test. The measured absorbance of

an oil sample dissolved in isooctane is related to the concentration of conjugated dienoic acids in the sample (Equation 1.2):

$$\text{CDV (\%)} = \frac{A_s}{bc} - k_o \qquad 1.2$$

where A_s is the absorbance of the sample solution at 233 nm, b is the cell length (cm), c is the concentration of the final dilution used for the absorption measurement (g/L), and k_o is a molar absorptivity factor (AOCS, 2013c).

Hydroperoxides and conjugated dienes may decompose during oxidation (Frankel, 2005); in this case, obtaining low PV or CDV measurements may lead to an underestimation of the extent of oxidation (Gray, 1978). Consequently, it is necessary to measure the concentration of secondary oxidation products to get a thorough estimate of the extent to which a sample is oxidized. The *p*-anisidine value (*p*AV), which measures high molecular weight carbonyl compounds, is often used as a measure of the amount of secondary oxidation products in an oil sample. In this protocol, a *p*-ansidine reagent reacts with aldehydes, forming an imine chromophore with an absorbance maximum at 350 nm. The measured absorbance of an oil sample dissolved in isooctane is related to the *p*AV (Equation 1.3):

$$p\text{AV} = \frac{\left(25(1.2A_s - A_b)\right)}{m} \qquad 1.3$$

where A_s is the absorbance of the sample solution after the addition of *p*-anisidine solution, A_b is the absorbance of the sample solution prior to the addition of *p*-anisidine solution, and m is the mass of the oil in the sample solution (g) (AOCS, 2013a).

Despite widespread academic and industrial use, there are several disadvantages to these standard methods. These tests are costly, laborious, and time-consuming. Furthermore, they are destructive and require large amounts of glassware and potentially toxic reagents (Yildiz et al.,

2001). Additionally, the spectrophotometric *p*AV and CDV tests may deviate from linearity at high sample concentration (Higson, 2004). Finally, the *p*AV test is more sensitive to unsaturated than saturated aldehydes (Gordon, 2004) and is affected by the contribution of additives, such as flavours (Jackowski et al., 2015).

This latter point is a major issue for producers of marine oil products, who often add flavours to their products to improve their palatability and consumer acceptability. However, aldehydes and aldehyde-like compounds in the flavours react with the *p*-ansidine reagent and contribute to the formation of the imine chromophore that forms the basis of the *p*AV test. This leads to inaccurate results (Jackowski et al., 2015; Semb, 2012), causing incorrect conclusions about the shelf-life and quality of the supplement in question. To combat this, the Global Organization for EPA and DHA Omega-3s (GOED) (2016) recommends a modified protocol to assess the level of secondary oxidation in flavoured marine oil products (Fig 1.3). This recommendation is predicated on the assumption that the flavours themselves do not oxidize, degrade, or in any way change their contribution to the measured *p*AV over time (GOED, 2016). If this assumption is violated, estimates of the extent of oxidation will be inaccurate.

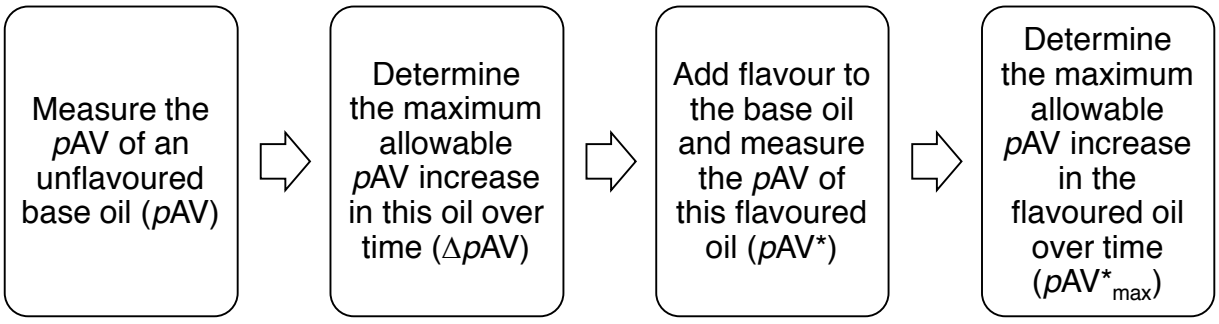


FIG. 1.3 The GOED recommendation for measuring the *p*AV of flavoured marine oils. Δ*p*AV is calculated by subtracting the *p*AV of the unflavoured base oil from the maximum acceptable *p*AV suggested by GOED (i.e., 20). pAV^*_{max} is calculated by adding Δ*p*AV to *p*AV*.

1.4 PROTON NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

Alternative methods of measuring oxidation in edible oils have been developed, but their use is not as widespread as that of the standard methods. These alternatives include chromatographic methods, such as gas chromatography (GC) and high-performance liquid chromatography (HPLC), as well as spectroscopic methods, such as nuclear magnetic resonance spectroscopy. Chromatographic methods often require laborious sample preparation protocols; therefore, proton nuclear magnetic resonance spectroscopy (^{1}H NMR) is the focus of this work because it is an inherently efficient method of analysis. ^{1}H NMR is based on the principle that ^{1}H nuclei are charged, spinning particles and as such have a magnetic dipole moment. Normally, the magnetic moments of all such nuclei will be randomly oriented in space. When a magnetic field (β_0) is applied, the magnetic moments of the nuclei will be polarized (Fig. 1.4). Magnetic moments that are aligned with β_0 (called the α spin state) are in a lower energy conformation than those aligned against β_0 (called the β spin state); therefore, at thermodynamic equilibrium, there are more nuclei in the lower energy α spin state. The energy difference between the α and β spin states, the "resonance energy", is proportional to the strength of β_0 (Fig. 1.5). During a ^{1}H NMR experiment, radiofrequency (RF) energy is applied that is equal to the resonance energy. This causes nuclei in the less energetic α spin state to absorb energy and flip into the higher energy β spin state. When these excited nuclei release their absorbed radiation, a signal is recorded by the spectrometer (Balci, 2005).

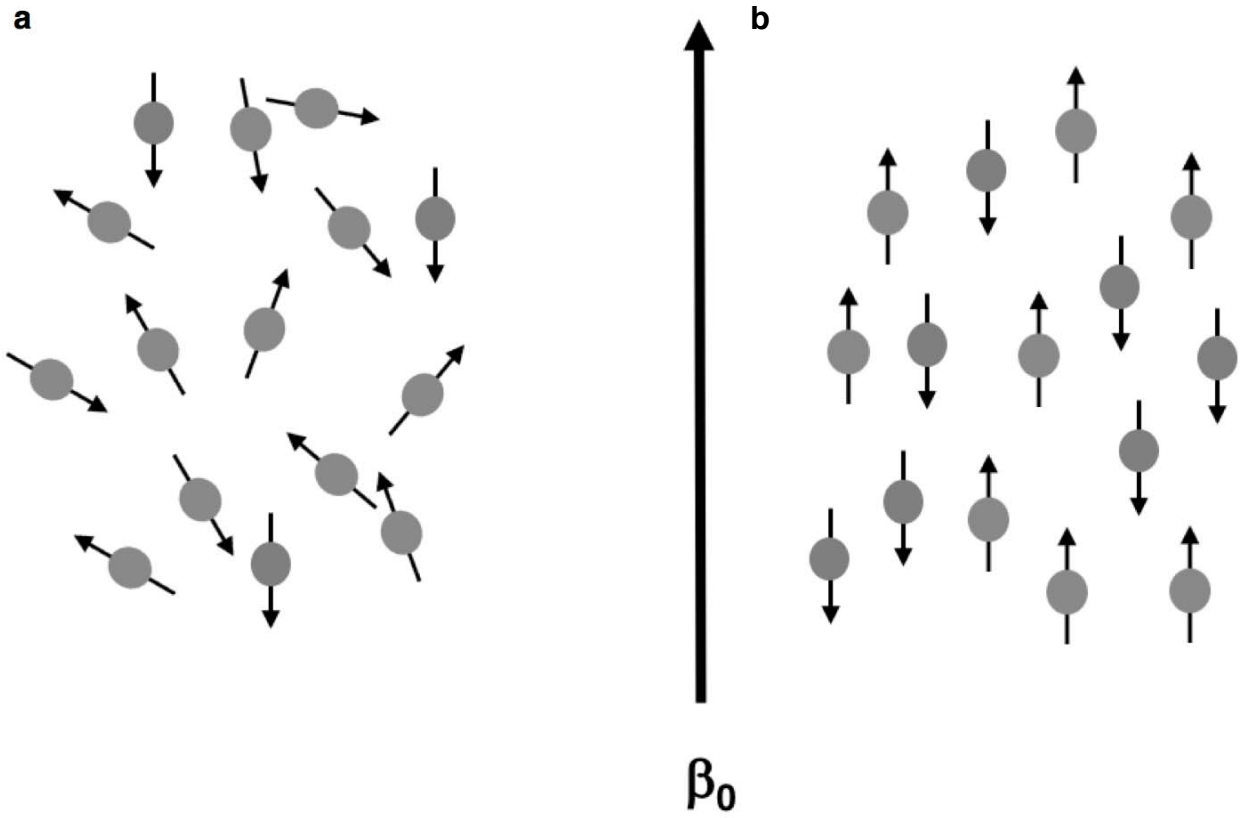


FIG. 1.4 The orientation of the magnetic moments of hydrogen nuclei in (a) the absence and (b) the presence of an applied magnetic field (β_0) (adapted from Balci, 2005).

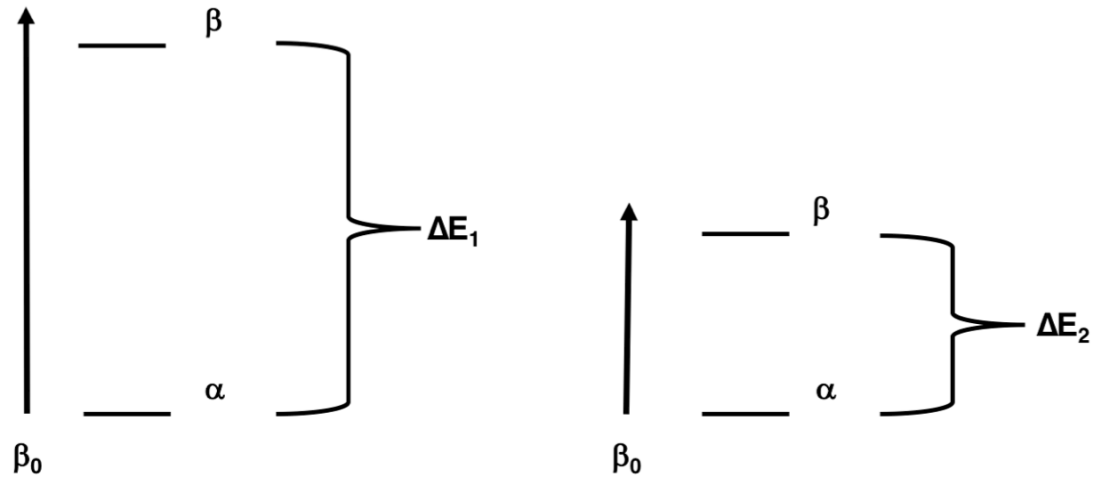


FIG. 1.5 The dependence of the resonance energy (ΔE) on the magnetic field strength (β_0).

For a more thorough understanding of ^{1}H NMR, the polarized alignment of magnetic moments in an applied magnetic field can be conceptualized as vectors precessing around an axis with a positive component (+*z*) aligned with β_0 and a negative component (-*z*) aligned against β_0. The frequency at which the magnetic moments precess is called the Larmor frequency (ω_0). Because there is an energy difference between the positive and negative components of the *z* axis, there are more magnetic moments precessing in the +*z* direction; thus, there is a net magnetization in this direction (Fig. 1.6).

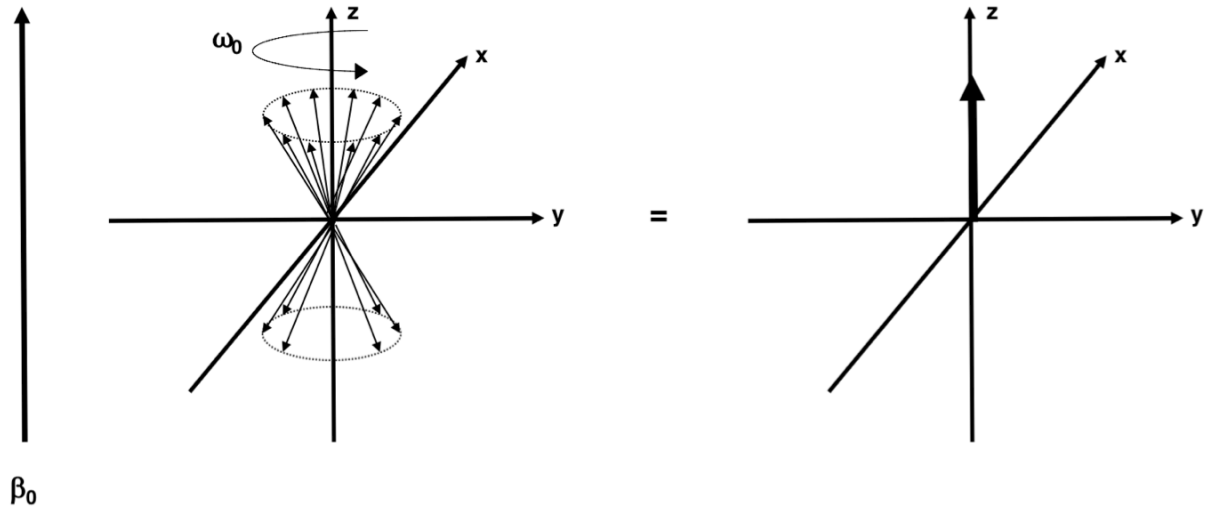


FIG. 1.6 The conceptualization of magnetic moments as vectors that precess around an axis parallel to the applied magnetic field (β_0). This leads to a net magnetization in the +*z* axis (adapted from Balci, 2005).

During a ^{1}H NMR experiment, energy equal to the Larmor frequency is applied perpendicular to the *z* axis. This causes the magnetization to tip into the *xy*-plane where it is detected by a receiver, generating an NMR signal (Fig. 1.7). Over time, the magnetization in the *xy*-plane decays and the magnetization slowly returns to the *z* axis in a process known as relaxation. The decaying signal is measured by the spectrometer to generate a free induction decay (FID)

signal. Fourier transformation is used to change this time-dependent FID spectrum into a frequency-dependent NMR spectrum (Fig. 1.8) (Balci, 2005; Keeler, 2010).

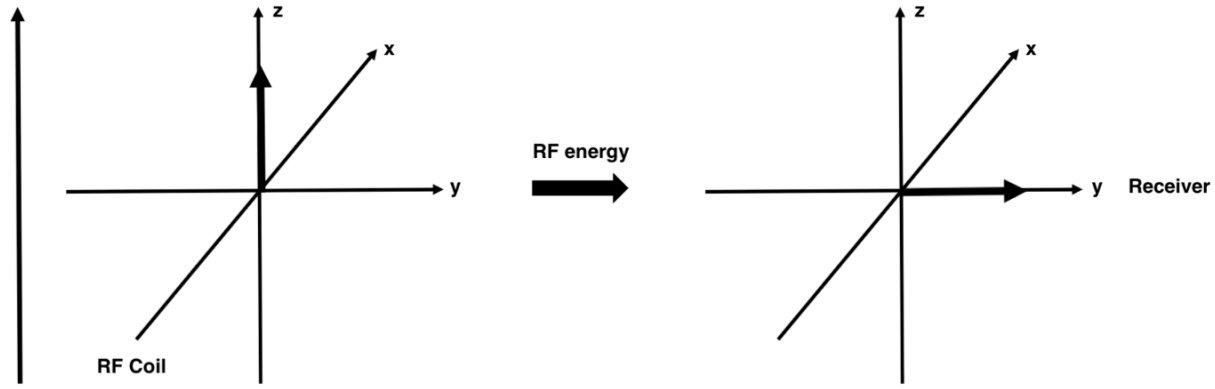


FIG. 1.7 The application of RF energy to an NMR sample tips the net magnetization into the horizontal plane, generating a signal that can be detected by the spectrometer's receiver.

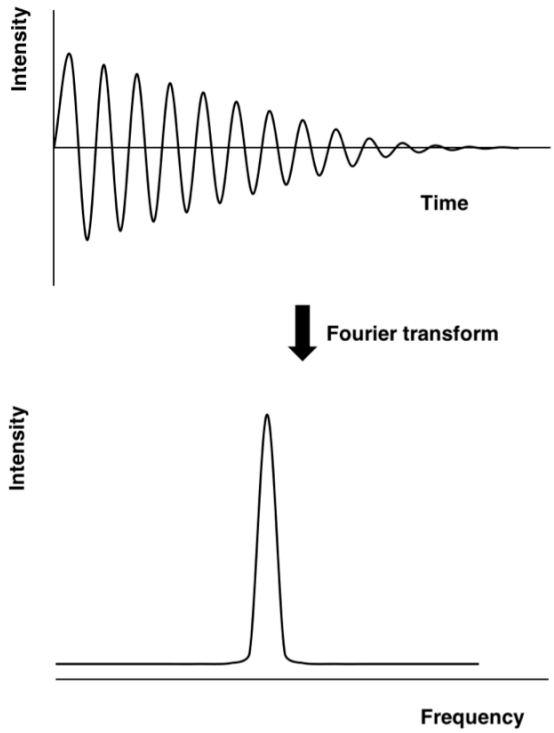


FIG. 1.8 Fourier transform turns the time-dependent FID spectrum into the frequency-dependent NMR spectrum (adapted from Balci, 2005).

Many NMR signals occurring at different frequencies are observed in a typical NMR spectrum. This is possible because the Larmor frequency of a given nucleus depends on the strength of β_0 (Equation 1.4):

$$\omega_0 = \frac{\gamma \beta_0}{2\pi} \qquad 1.4$$

where ω_0 is the Larmor frequency, γ is the gyromagnetic ratio, a constant specific to each nucleus, and β_0 is the applied magnetic field. β_0 depends on the chemical environment in which the nucleus resides. For example, electron density around a nucleus generates a magnetic field that opposes β_0. Nuclei in regions of high electron density are said to be 'shielded' from β_0 and therefore have lower Larmor frequencies. Conversely, nuclei lacking electron density, such as nuclei in close proximity to electronegative atoms, are 'deshielded' and thus have higher Larmor frequencies. Nuclei of unique Larmor frequencies generate unique FIDs that are Fourier transformed to generate a typical NMR spectrum comprised of many signals (Balci, 2005; Keeler, 2010).

1.5 APPLICATION OF ^{1}H NMR TO THE STUDY OF EDIBLE OILS

There are many advantages of ^{1}H NMR over traditional methods of measuring oxidation in edible oils. ^{1}H NMR analyses require very little reagent and sample, are rapid, and allow for sample recovery after analysis. Furthermore, NMR analyses can be automated, reducing labour requirements while allowing for high sample throughput. Finally, information about a number of parameters can be obtained simultaneously during the acquisition of a single NMR spectrum. Because of its many advantages, several research groups have applied ^{1}H NMR to the study of lipid oxidation in edible oils, including fish oils (Mozuraityte et al. 2016; Nieva-Echevarría et al., 2016; Tyl et al. 2008; Vidal et al., 2015), as well as plant oils submitted to deep frying conditions (Guillén & Ruiz, 2008; Guillén & Uriarte, 2009, 2012; Martínez-Yusta & Guillén, 2014a, 2014b; Sacchi et al., 2006) and moderate thermo-oxidative conditions (Goicoechea & Guillén, 2010;

Guillén & Goicoechea, 2009; Guillén & Ruiz, 2004, 2005a, 2005b, 2005c). These studies have demonstrated the utility of ^{1}H NMR as a qualitative method of monitoring lipid oxidation in edible oils. The ^{1}H NMR signals of various oxidation products have been identified and are well-characterized. Furthermore, these studies have provided information on the nature and timing of oxidation product formation over the course of oxidation, demonstrating the variation that occurred under different oxidative conditions and in different types of oils.

1.6 OBJECTIVES

The objective of this thesis was to improve upon current methods of measuring lipid oxidation in edible oils. Specifically, this involved:

1. Developing a ^{1}H NMR method capable of simultaneously measuring the PV, CDV, and *p*AV in oxidized soybean and canola oils. This was done by testing the hypothesis that the PV, CDV, and *p*AV are related to the ^{1}H NMR signal responses of hydroperoxidic, conjugated dienic, and aldehydic signals, respectively, with the anticipation that a relationship would exist between these parameters, allowing ^{1}H NMR to be used for the direct quantification of oxidation products in edible oils.
2. Assessing the suitability of the GOED recommended method for measuring the *p*AV in flavoured marine oils by investigating the stability of flavour compounds during oxidation. This was done by testing the null hypothesis, as followed by GOED, that flavour compounds do not oxidize or otherwise degrade over the course of oxidation, with the anticipation that changing signal responses of aldehydic flavour signals could be calibrated to the change in the flavour's contribution to the measured *p*AV.

CHAPTER 2: DEVELOPING A ^{1}H NMR METHOD TO QUANTIFY LIPID OXIDATION PRODUCTS IN OXIDIZED VEGETABLE OILS

2.1 INTRODUCTION

Vegetable oils, such as canola and soybean oil, that contain PUFA are prone to lipid oxidation (Shahidi & Zhong, 2010). Oxidation reduces the palatability and shelf-life of these oils and may have negative health consequences (Esterbauer, 1993; Staprans et al., 2005). For these reasons, it is common practice to monitor the level of oxidation in edible oils. Lipid oxidation is typically assessed using chemical tests that measure the concentrations of certain oxidative markers in a sample. These include the PV test, which measures the hydroperoxides in the sample, the CDV test, which measures the conjugated dienes in the sample, and the *p*AV test, which measures the aldehydes in the sample. Despite their widespread use, these tests suffer from several disadvantages; they are time-consuming, costly, and resource-intensive. For this reason, alternative methods of analysis are being developed. One such alternative is ^{1}H NMR spectroscopy, which has been successfully applied to the qualitative study of oxidized vegetable oils (see work by the group of Maria Guillén; Sacchi et al., 2006).

Building on these qualitative studies, Skiera et al. (2012a, 2012b) used ^{1}H NMR to measure the concentration of hydroperoxides and aldehydes in plant oils, demonstrating the potential for quantitative applications of ^{1}H NMR to the study of edible oils. However, the successful development and application of these methods required several sensitivity-enhancing techniques that presumably increased the cost, labour, and time required for the ^{1}H NMR analyses. Experimental protocols that fail to capitalize on the inherent advantages of ^{1}H NMR reduce its acceptability as an alternative method for measuring oxidation in edible oils; therefore, it is necessary that an NMR-based method of analysis be developed that does not sacrifice these advantages.

Here, I proposed to build upon the work done by Skiera et al. by developing a simple and efficient ^{1}H NMR-based method to measure oxidation products in edible oils. This involved testing the hypothesis that the hydroperoxidic, conjugated dienic, and aldehydic ^{1}H NMR signal responses of oxidized soybean and canola oils were related to their PV, CDV, and *p*AV, respectively. Upon validation of this hypothesis, I intended to exploit these relationships to create a ^{1}H NMR method capable of measuring oxidation directly. To test my hypothesis, I first oxidized a series of soybean and canola oils and measured their PV, CDV, and *p*AV using standard tests. Then, I acquired the ^{1}H NMR spectra of these same oils and integrated the signals of the corresponding oxidation products. Finally, I compared these two parameters using weighted linear regression, anticipating that a relationship would exist that would allow ^{1}H NMR to be used for the direct quantification of oxidation products in edible oils.

2.2 MATERIALS AND METHODS

The following oxidation procedures were performed twice, once with soybean oil (President's Choice, Toronto, ON) and once with canola oil (Clic International Incorporated, Montreal, QC).

2.2.1 Sample Preparation and Oxidation

Accurately weighed aliquots of oil (~9 g) were added to 15 mL round-bottomed test tubes and heated at 100 ± 2°C in an oven (Fisher Scientific, Ottawa, ON) for 20 hours. Following removal from the oven, the test tubes were covered in aluminium foil, placed on a heating block (Fisher Scientific, Ottawa, ON) at 100°C, and aerated with a steady stream of compressed air (Air Liquide, Montreal, QC). Triplicate soybean oil samples were removed from the heating block after 7, 8, 9, 10, 12, 14, 16, 18, and 20 hours. Triplicate canola oil samples were removed from the

heating block after 22, 26, 28, 30, 32, 38, 42, 44, and 46 hours. After heating, the sample tubes were purged with nitrogen, capped, and stored in the dark at -30°C for several days until analysis.

2.2.2 Measurement of Hydroperoxides

Hydroperoxides in the oils were measured following a modified version of AOCS Official Method Cd 8b-90, the Peroxide Value (AOCS, 2013b). Here, the method was modified to use a sample mass of 2.5 g rather than the 5.0 g described in the Official Method. Reducing the sample mass to account for high expected levels of oxidation is routine (Pegg, 2005) and modification of the sample size does not significantly affect the measured PV (Crowe and White, 2001). Aliquots of oil (2.5 g) were added to 250 mL Erlenmeyer flasks. Following this, 30 mL of a 3:2 (v/v) mixture of acetic acid (Fisher Scientific, Ottawa, ON) and chloroform (Fisher Scientific, Ottawa, ON) was used to dissolve the oil, and 0.5 mL potassium iodide solution (Fisher Scientific, Ottawa, ON) was then added to the sample. After one minute, 30 mL distilled water and 1 mL starch indicator solution (Ricca Chemical Company, Arlington, TX) were added to the flask. The sample was then titrated with 0.01 N sodium thiosulfate solution (Fisher Scientific, Ottawa, ON) until the titration's endpoint. For highly oxidized samples, 0.1 N sodium thiosulfate solution was used. The resulting PV was calculated using Equation 1.1.

2.2.3 Measurement of Conjugated Dienoic Acids

Conjugated dienes in the oils were measured following a modified version of AOCS Official Method Ti 1a-64, the Spectrophotometric Determination of Conjugated Dienoic Acid (AOCS, 2013c). A modified method was necessary because the original method is applicable to dehydrated castor oil or fatty acids and their methyl or ethyl esters rather than the bulk liquid oils used in this study. The official method recommends a target sample solution concentration of 0.01 g L^{-1}; instead, a concentration of 0.5 g L^{-1} was used for this work. This modified concentration is

recommended for most edible oils (White, 1995) and has been successfully applied to the determination of conjugated dienoic acids in soybean oil in the past (Bachari-Saleh et al., 2013). Aliquots of oil (0.25 g) were added to 50 mL volumetric flasks, which were then made to volume with isooctane (Fisher Scientific, Ottawa, ON) and diluted ten-fold. The absorbance of each sample solution was measured at 233 nm against an isooctane blank using an ultraviolet-visible (UV-Vis) spectrophotometer (Amersham Pharmacia Biotech, Amersham, UK). Sample solutions that gave absorbance readings outside the recommended linear range of 0.2 to 0.8 were diluted until the absorbance readings fell within this range. The resulting CDV was calculated using Equation 1.2.

2.2.4 Measurement of Aldehydes

Aldehydes in the oils were measured following AOCS Official Method Cd 18-90, the *p*-Anisidine Value (AOCS, 2013a). Aliquots of oil (0.7 g) were added to 25 mL volumetric flasks and diluted with isooctane (Fisher Scientific, Ottawa, ON). The absorbance of each sample solution was measured at 350 nm on a UV-Vis spectrophotometer (Biochrom, Holliston, MA). Following this, 1 mL of *p*-anisidine solution (0.25 g *p*-anisidine (Sigma-Aldrich, Oakville, ON) in 100 mL glacial acetic acid (Fisher Scientific, Ottawa, ON)) was added to 5 mL aliquots of each sample solution. After 10 minutes, the absorbance of each solution was again measured at 350 nm. The resulting *p*AV was calculated using Equation 1.3.

2.2.5 ^{1}H NMR Spectral Acquisition and Processing

To prepare samples for ^{1}H NMR analysis, 0.05 g aliquots of oil were dissolved in 1000 µL deuterated chloroform ($CDCl_3$) with 1% TMS as a chemical shift reference (Sigma-Aldrich, Oakville, ON). The samples were placed in 5 mm NMR tubes (Wilmad Lab Glass, Vineland, NJ) and subjected to analysis on a 500 MHz NMR spectrometer (Bruker, East Milton, ON) operating

at 500.13 MHz. The acquisition parameters were as follows: time domain 65536, number of scans 128, spectral width 20.1559 ppm, and a relaxation delay of 15 s, with a total recovery time of 39 minutes. The spectra were acquired at 300K using a flip angle of 90°.

All spectra were processed using Topspin Version 3.5 (Bruker, East Milton, ON). Exponential multiplication with a line-broadening factor of 0.3 Hz was applied before Fourier transformation of the raw FID data. Phasing and baseline correction were done manually. The signals of the *sn*-3 and *sn*-1 glycerol protons on the glycerol backbone resonating at 4.29 and 4.14 ppm were used to calibrate the integrals of the hydroperoxidic, conjugated dienic, and aldehydic signals. Signal integration was done manually and in triplicate; signal response values used in subsequent calculations are the mean of triplicate integrations of each signal.

2.2.6 Statistical Analyses

Weighted linear regression (Minitab 17, State College, PA) was used to fit a model to the data describing the relationship between hydroperoxidic signal responses and the PV. Because several conjugated dienic signals were observed in the spectra of the oils tested here, weighted multiple linear regression (Minitab 17, State College, PA) was used to fit a model to the data comparing the conjugated dienic signal responses to the CDV.

2.3 RESULTS

As the PV, CDV, and *p*AV of the oil samples increased during oxidation at 100ºC, hydroperoxidic, conjugated dienic, and aldehydic signals became visible in the ^{1}H NMR spectra of these oils (Table 2.1; Fig. 2.1). For each sample, the PV, CDV, and *p*AV were plotted against the hydroperoxidic, conjugated dienic, and aldehydic signal responses, respectively, to determine if a relationship existed between the results of the standard chemical tests and the magnitude of the signal responses of the corresponding ^{1}H NMR signals (Figs. 2.2-4).

Table 2.1 The [1]H NMR signals generated by various oxidation markers that were observed in the spectra of the soybean and canola oils tested in this work. Proposed signal assignments are based the results of previously published literature.

Signal Letter	Chemical Shift (ppm)	Signal Type[1]	Proposed Signal Assignment	Reference
A	5.74	m	Protons associated with the double bonds of hydroperoxide groups (-CHOOH-CH=C**H**-)	Martínez-Yusta et al., 2014
B	6.04	ddtd	*cis, trans* conjugated diene system (-C**H**=C**H**-C**H**=C**H**-)	Goicoechea & Guillén, 2010; Guillén & Ruiz, 2004, 2005a
C	6.27	ddm	*trans, trans* conjugated diene system (-C**H**=C**H**-C**H**=C**H**-)	Goicoechea & Guillén, 2010; Guillén & Ruiz, 2004, 2005a
D	6.57	dddd	*cis, trans* conjugated diene system (-C**H**=C**H**-C**H**=C**H**-)	Goicoechea & Guillén, 2010; Guillén & Ruiz, 2004, 2005a
E	9.50	d	(*E*)-2-alkenals (-C**H**O)	Goicoechea & Guillén, 2010; Guillén & Ruiz, 2004, 2005a; Skiera et al., 2012a
F	9.52	d	(*E,E*)-2,4-alkadienals (-C**H**O)	Goicoechea & Guillén, 2010; Guillén & Ruiz, 2004, 2005a; Skiera et al., 2012a
G	9.75	t	*n*-alkanals (-C**H**O)	Goicoechea & Guillén, 2010; Guillén & Ruiz, 2004, 2005a; Skiera et al., 2012a

[1]d: doublet, t: triplet, m: multiplet

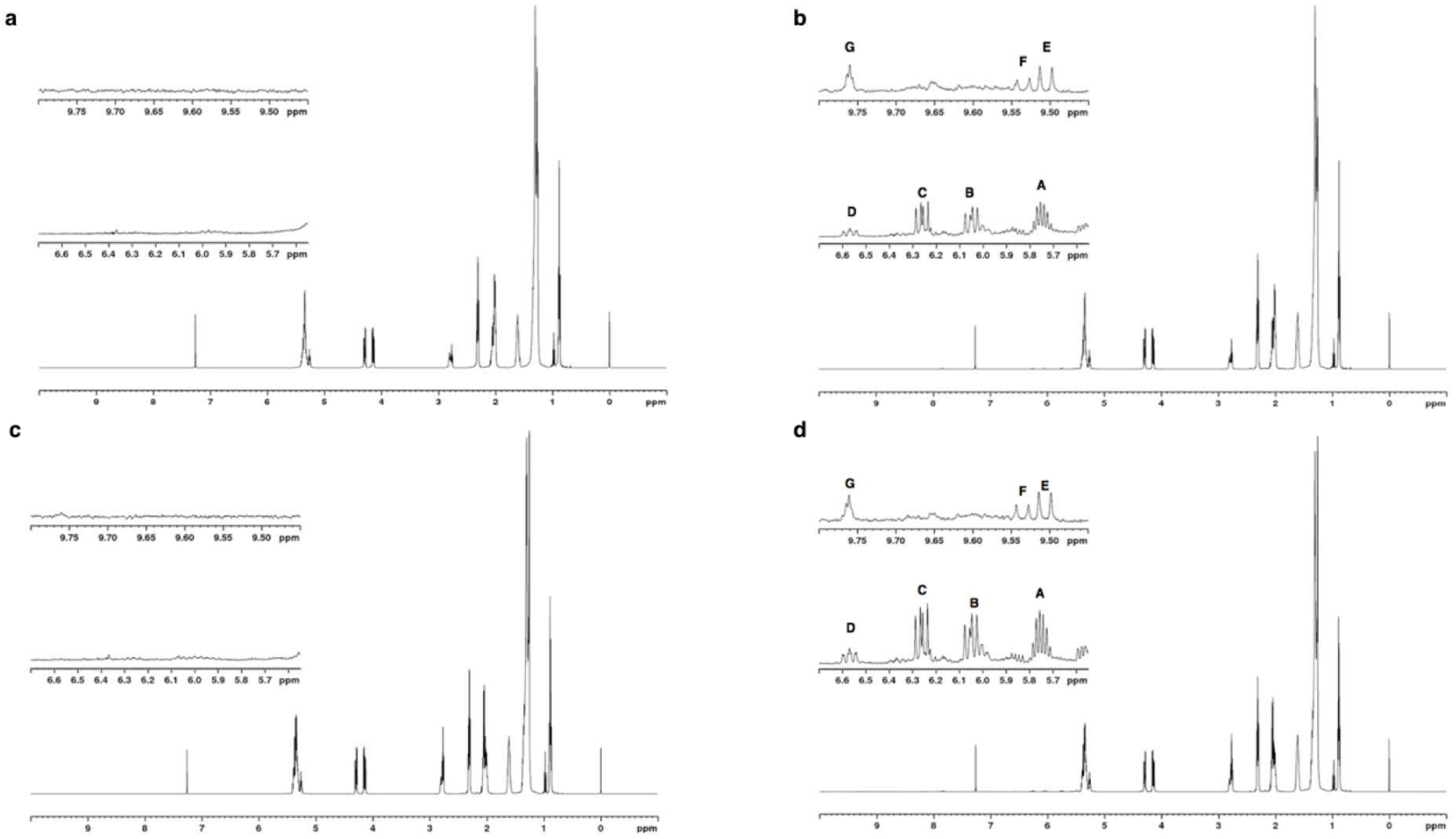


FIG. 2.1 The ^{1}H NMR spectra of (a) fresh canola oil, (b) canola oil oxidized at 100ºC for 66 hours, (c) fresh soybean oil, and (d) soybean oil oxidized at 100ºC for 38 hours. The spectral region between 6.70 and 5.55 ppm was enlarged 16 times to show the signals of conjugated dienic protons and protons associated with the double bonds of hydroperoxide groups. The spectral region between 9.80 and 9.45 ppm was enlarged 20 times to show the signals of aldehydic protons. Proposed signal assignments are shown in Table 2.1.

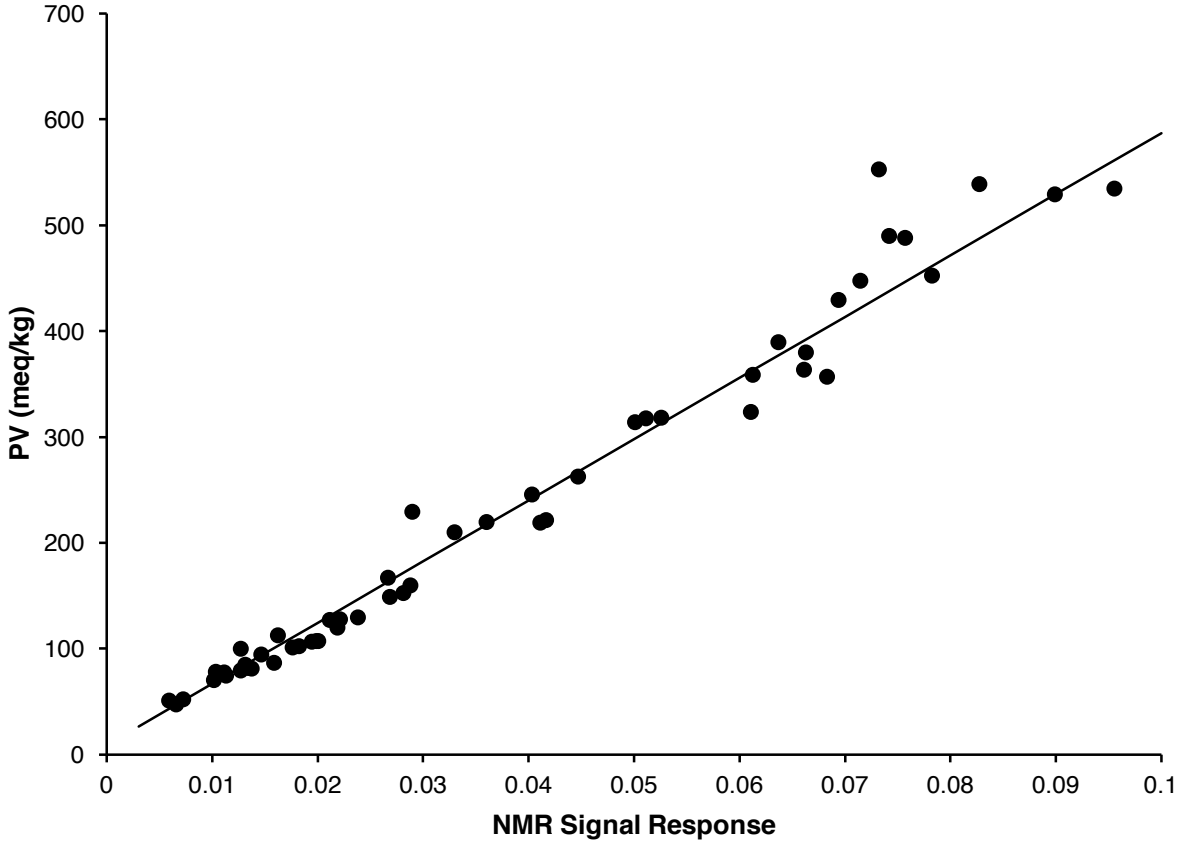


FIG. 2.2 The relationship between the PV and the NMR signal response of the multiplet signal at 5.74 ppm corresponding to protons associated with the double bonds of hydroperoxide groups in canola and soybean oils oxidized at 100ºC. Weighted linear regression was used to fit the following model to the data (indicated by the black line): PV = 8.72 + Signal Response$_{5.74\ ppm}$ (R^2 = 0.9719). Each sampling point represents one sample (n=1). Error bars are too small to be visible in the figure.

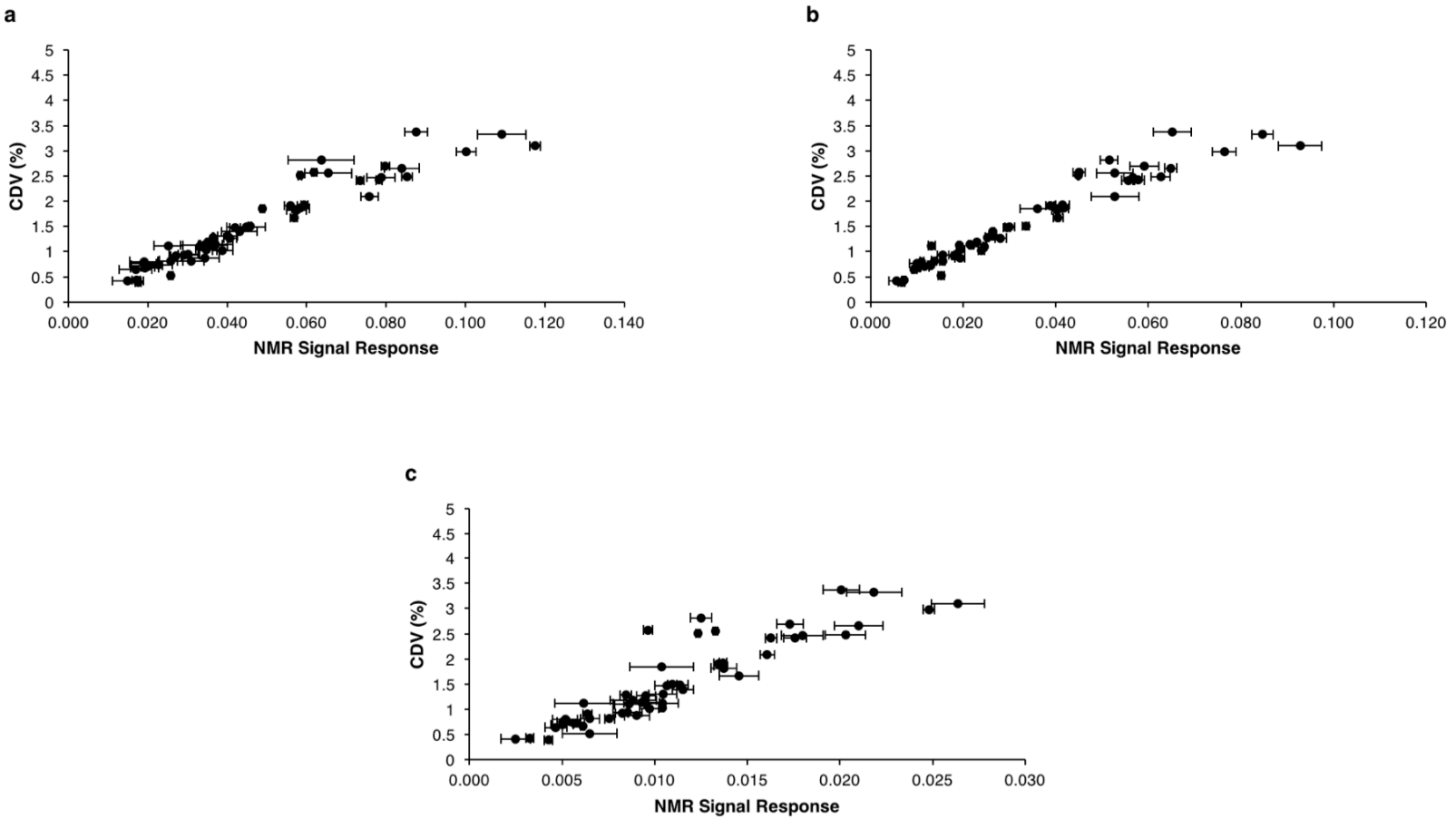


FIG. 2.3 The relationship between the CDV and the NMR signal response of (a) the ddtd signal at 6.04 ppm, (b) the ddm signal at 6.27 ppm, and (c) the dddd signal at 6.57 ppm corresponding to protons in conjugated dienic systems in canola and soybean oils oxidized at 100ºC. Weighted multiple linear regression was used to fit the following model to the data: CDV = 0.3057 + 0.2 * Signal $Response_{6.04\ ppm}$ + 44.1 * Signal $Response_{6.27\ ppm}$ − 18.5 * Signal $Response_{6.57\ ppm}$ (R^2_{adj} = 0.9338). Each sampling point represents one sample (n=1) with error bars indicating the standard deviation of the three integrations performed on the corresponding ^{1}H NMR signals.

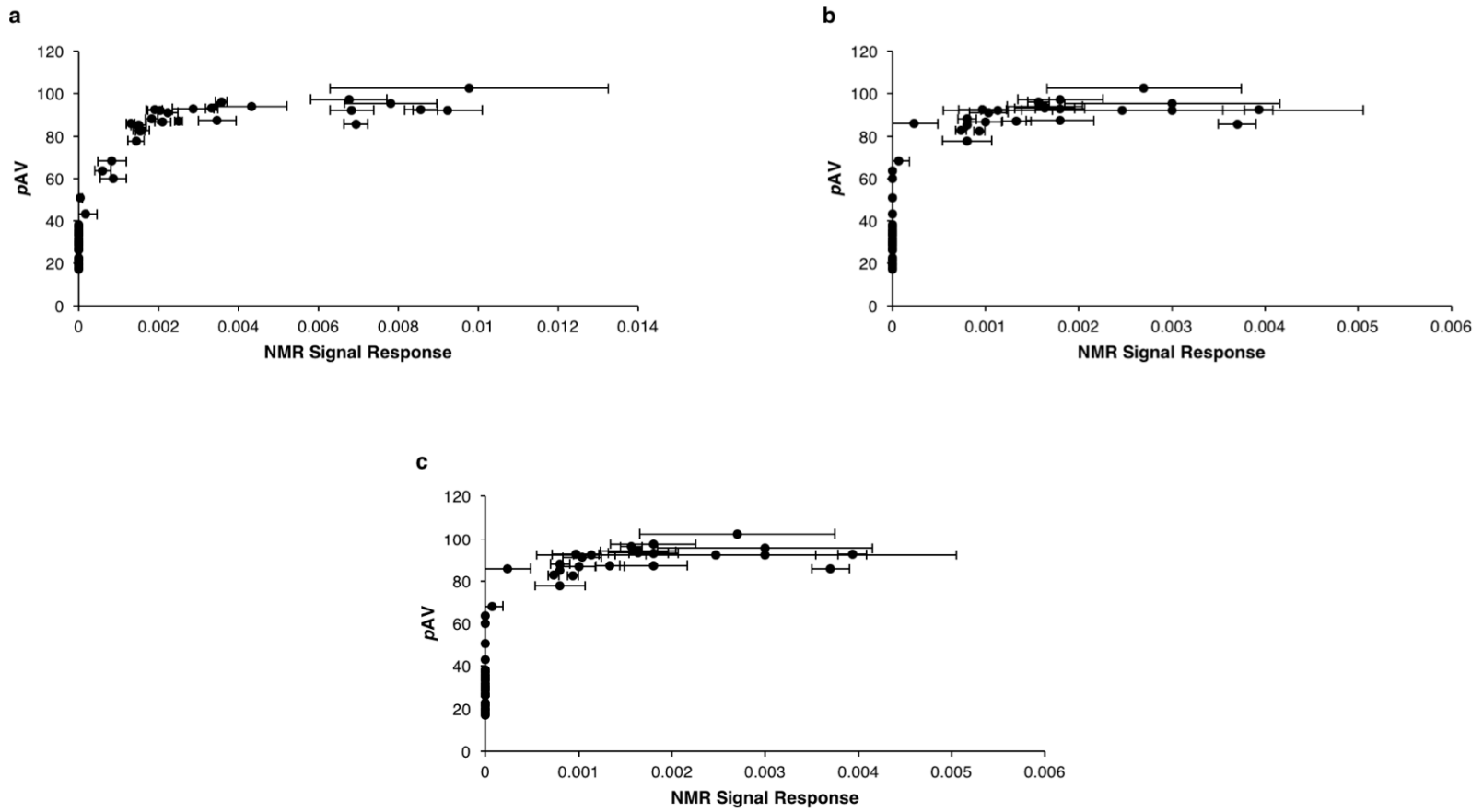


FIG. 2.4 The relationship between the *p*AV and the NMR signal response of (a) the doublet signal at 9.50 ppm, (b) the doublet signal at 9.52 ppm, and (c) the triplet signal at 9.75 ppm corresponding to aldehydic protons in canola and soybean oils oxidized at 100ºC. Each sampling point represents one data point (n=1) with error bars indicating the standard deviation of the three integrations performed on the corresponding ^{1}H NMR signals.

2.4 DISCUSSION

2.4.1 ^{1}H NMR Spectra of Oxidized Plant Oils

Signals attributable to the protons of primary oxidation products, i.e. conjugated dienes and hydroperoxides, and to the protons of secondary oxidation products, i.e. aldehydes, were observed in the ^{1}H NMR spectra of the oxidized soybean and canola oils tested here (Fig. 2.1). These signals are routinely observed in the ^{1}H NMR spectra of plant oils oxidized at moderate temperatures, including sesame (Guillén & Ruiz, 2004), corn, sunflower (Guillén & Ruiz, 2005b), linseed, and walnut (Guillén & Ruiz, 2005a) oils. In agreement with these studies, aldehydic signals did not appear in the spectra of the oils tested here until the later stages of oxidation, well after the time at which the signals of primary oxidation products had been observed. This is to be expected; as previously discussed, hydroperoxides and conjugated dienes form during the propagation stage of lipid oxidation, while aldehydes do not form until subsequent stages of the reaction (Frankel, 2005). Indeed, Guillén and Ruiz (2005c) demonstrated that, while hydroperoxidic and conjugated dienic signals were observed in the ^{1}H NMR spectra of extra virgin olive, hazelnut, and peanut oils after seven, four, and two days of oxidation at 70ºC, respectively, aldehydic signals were not observed until 28, 9, and 12 days, respectively, had passed.

The ^{1}H NMR spectra of the oxidized soybean and canola oils tested in this work (Fig. 2.1), and the spectra of the oxidized plant oils studied by Guillén and Ruiz (2004, 2005a, 2005b, 2005c) demonstrated that the same products are formed during the oxidation of a variety of vegetable oils; however, there are differing rates of oxidation among these oils because they contain different concentrations of PUFA (Frankel, 2005). In this work, for example, soybean oil, which contains a higher proportion of PUFA (~61%), oxidized more rapdily than the comparatively less polyunsaturated canola oil (~36%) (Frankel, 2005; Gunstone, 1996). The relationship between the

degree of unsaturation of an oil and the rate at which oxidation progresses was also demonstrated in extra virgin olive, hazelnut, and peanut oils by Guillén and Ruiz (2005c).

2.4.2 ^{1}H NMR Signal Detection and Integration

The goal of this work was to relate the results of the PV, CDV, and *p*AV tests to the ^{1}H NMR signal responses of hydroperoxidic, conjugated dienic, and aldehydic signals, respectively. Signals corresponding to each of these oxidation products were chosen for integration (Table 2.1), including the multiplet signal resonating at 5.74 ppm used as a measure of hydroperoxides in the oxidized oils. Previous qualitative ^{1}H NMR studies of hydroperoxides in oxidized plant oils have focused on signals resonating between 8.0 and 8.9 ppm; however, these signals are typically broad (Goicoechea & Guillén, 2010; Guillén & Ruiz, 2004, 2005a, 2005b, 2005c). This broadness is due to proton exchange, which occurs when protons bonded to an oxygen atom, such as the protons in hydroperoxides (-OO**H**-), are transferred between oxygen atoms in the sample, sample impurities, or residual water (Bruice, 2011; Skiera et al., 2012b). The resulting signal represents an average of the various chemical environments occupied by the proton during exchange, and is therefore broad (Bruice, 2011). Because narrow signals are a requirement for accurate integration (Holzgrabe, 2010), the multiplet signal resonating at 5.74 ppm was chosen for analysis here.

In addition to being narrow, signals must be well-separated and well-resolved to be integrated accurately (Holzgrabe, 2010). In this work, incipient signals of primary oxidation products became visible at early stages of oxidation but were too small for accurate integration; signals of an appropriate size and resolution for integration were not visible until PV were beyond acceptable limits for vegetable oils (<10 meq/kg). Similarly, because aldehydic signals were not observed until the later stages of oxidation, aldehydic signal integration was not possible in the spectra of the oils tested here until the *p*AV, too, were beyond acceptable limits (<20). Compared

to other analytical techniques, ^{1}H NMR spectroscopy is insensitive (Holzgrabe, 2010; Kovacs et al., 2005; Molinski, 2010; Pauli et al., 2012; Tsiafoulis et al., 2014; Webb, 2005); therefore, it is difficult to detect small concentrations of oxidation products in ^{1}H NMR spectra. This finding also agrees with Guillén and Ruiz' (2005b) ^{1}H NMR study of hydroperoxides and aldehydes in oxidized corn and sunflower oils, in which accurately integratable signals were not observed until the PV and *p*AV of these oils were greater than 100 meq/kg and 50, respectively. This finding is also in agreement with the results of the qualitative studies discussed above, in which easily integrated hydroperoxidic, conjugated dienic, and aldehydic signals were not observed until oxidation was advanced (Goicoechea & Guillén, 2010; Guillén & Ruiz, 2004, 2005a, 2005b, 2005c).

Skiera et al. (2012a, 2012b), however, were able to observe and integrate hydroperoxidic and aldehydic signals in unoxidized plant oils. The group was able to maximize the sensitivity of their method by using a selective inverse probe (Skiera, 2013). The probe is the part of the spectrometer in which the sample resides during spectral acquisition. Inside the probe are two RF coils that generate and detect the NMR signal (Kovacs et al., 2005). The inner coil is situated closest to the sample; typically, the nucleus to which the inner coil is tuned is detected with the highest sensitivity. For example, in standard broadband probes, the inner coil is tuned to nuclei other than ^{1}H, such as ^{13}C, while in inverse probes, the inner coil is tuned to ^{1}H nuclei. Therefore, inverse probes provide the best sensitivity for ^{1}H nuclei (Claridge, 2016). It should be noted that not all research groups have access to inverse probes; typically, many research groups make use of a single spectrometer that is equipped with a standard broadband probe to satisfy the varied needs of all users. The group was also able to increase the sensitivity of their method by using thin wall NMR tubes, which intensify signals by concentrating the sample volume into the area of the

RF coils, maximizing the amount of RF energy to which the sample is exposed (Claridge, 2016). While Skiera et al. (2012a, 2012b) did achieve excellent sensitivity with their ^{1}H NMR method, they employed several additional techniques that limited the efficiency and scope of their method.

First, to enhance the sensitivity of their method towards hydroperoxidic signals, the group used a solvent mixture of 5:1 (v:v) $CDCl_3$:deuterated dimethyl sulfoxide-d6 (DMSO-*d6*) (Skiera et al., 2012b). Typically, pure $CDCl_3$ is the solvent of choice for ^{1}H NMR studies of lipid oxidation in edible oils (see research by Falch et al., 2004; the group of Maria Guillén; Tyl et al., 2008). DMSO-*d6* can be added to the solvent mixture to reduce rate of proton exchange, narrowing the signals of otherwise rapidly exchanging protons (Abraham et al., 2005). Additionally, DMSO-*d6* solubilizes minor sample components to a greater degree than does pure $CDCl_3$ (Mannina et al., 2012; Sacchi et al., 1996). However, the addition of DMSO-*d6* to solvent mixtures will change the chemical shifts at which the signals of exchanging protons are observed. This was demonstrated by Abraham et al. (2005), who compared the ^{1}H NMR spectra of alcohols obtained using three different ^{1}H NMR solvents, $CDCl_3$, deuterated water, and DMSO-*d6*. The chemical shifts of hydroxyl protons in samples solubilized in DMSO-*d6* differed considerably from those in samples solubilized in $CDCl_3$ or deuterated water. This phenomenon can also be seen when comparing the work of Skiera et al. to that of other authors. Skiera et al. (2012b) observed hydroperoxidic signals between 10.1 to 11.0 ppm, while other groups using pure $CDCl_3$ as their solvent have reported that hydroperoxidic signals resonate between 8.0 to 8.9 ppm (Goicoechea & Guillén, 2010; Guillén & Ruiz, 2004; 2005a; 2005b; 2005c). Therefore, adding DMSO-*d6* to solvent mixtures may make comparison among studies difficult.

To further enhance the resolution of the hydroperoxidic signals in their spectra, Skiera et al. (2012b) elected to dry their NMR samples over a molecular sieve. Molecular sieves remove

any residual water in a sample that could participate in proton exchange with hydroperoxidic protons, leading to signal broadening (Skiera, 2013). By adding this drying step, the group increased the resolution of their hydroperoxidic signals; however, this increased the time required for sample preparation.

Finally, the group used two different protocols to prepare samples for the analysis of hydroperoxides and aldehydes in their samples, respectively (Skiera et al., 2012a, 2012b), effectively doubling the time, labour, and resource requirements of the ^{1}H NMR analysis. Compared to other methods of analysis, such as wet chemical tests or GC, ^{1}H NMR is an inherently rapid method that requires minimal sample preparation. In order to capitalize on these advantages, the work described in this thesis focused on developing a method of ^{1}H NMR analysis that used a single sample preparation protocol, widely available instrumentation, and a solvent that would allow comparison with the results of other research groups.

2.4.3 The Relationship Between Standard Test Results and ^{1}H NMR Signal Responses

Weighted linear regression models were fit to both the hydroperoxidic and conjugated dienic data to describe the relationship between the results of the standard chemical tests and the signal responses of the corresponding ^{1}H NMR signals (Fig. 2.2 and 2.3, respectively), demonstrating that significant relationships exist between these parameters in highly oxidized oils. Few quantitative studies exist that attempt to relate ^{1}H NMR spectral information to data obtained via standard chemical tests. This is probably due to the aforementioned limited sensitivity of the NMR method. However, Skiera et al. (2012a, 2012b) demonstrated that linear relationships exist between the NMR-determined concentration of hydroperoxides and aldehydes in commercial plant oils and the results of the PV and *p*AV tests, respectively. Furthermore, several groups have fit linear models to data relating the signal intensities of oxidation indices to the heating time of edible

oils submitted to oxidative conditions (Guillén & Uriarte, 2012; Martínez-Yusta & Guillén, 2014a, 2014b; Tyl et al., 2008).

Ideally, multiple regression would have been used to fit a model to the data comparing the results of the *p*AV test to aldehydic signal responses. Unfortunately, this was not possible for the data presented here (Fig. 2.4). Because aldehydic signals are not observed in the ^{1}H NMR spectra of oxidized plant oils until oxidation is advanced, the data set contained many data points with high *p*AV, but ^{1}H NMR signal responses of zero. As aldehydic signals became visible, there was a period during which the responses of these signals appeared to increase linearly with *p*AV. However, this period of linearity was brief and was followed by a plateau. On this plateau, aldehydic signal responses increased while *p*AV remained constant, suggesting that the *p*AV test was no longer accurately measuring the increasing concentration of aldehydes in the samples.

The observed inaccuracy of the *p*AV test at high *p*AV is likely due to an inherent limitation of all spectrophotometric methods, which is that they tend to deviate from linearity at high concentrations. Spectrophotometric methods, including the *p*AV test, are governed by the following principle (Equation 2.1):

$$A = \log_{10} \frac{I_O}{I} \qquad 2.1$$

where A is the absorbance, I_O is the incident light to which the sample is exposed, and I is the light transmitted through the sample. There is a linear relationship between the absorbance of the sample solution and its concentration; as the concentration of analyte in the sample solution increases, less light is transmitted through the sample and thus the absorbance increases. However, when the concentration of the sample solution becomes too great, interactions between analyte molecules and the solvent will cause deviations from linearity. Additionally, if the sample solution is so concentrated or darkly coloured that the incident light is completely or nearly completely absorbed,

the ratio of I_O to I will be large. In this situation, further increases in sample concentration have very little effect on the ratio and thus are difficult to measure. Therefore, there is no longer a linear relationship between absorbance and concentration and the measured absorbance will appear to plateau (Higson, 2004).

This latter phenomenon was likely the cause of the non-linearity observed at high *p*AV in the data presented here. As the concentration of aldehydes in the oils increased, so too did the concentration of the imine chromophore and thus the colour intensity of the sample solution. In this experiment, some of the oils were so highly oxidized that the colour of the sample solution was extremely dark; likely, very little light was transmitted through the sample solution. Therefore, the absorbance, and thus the measured *p*AV, appeared to plateau.

2.5 CONCLUSION

This work confirms findings obtained in other ^{1}H NMR studies of oxidized plant oils and extends these findings to soybean and canola oil. First, the ^{1}H NMR analyses performed here demonstrate that the oxidation products that form in canola and soybean oils oxidized at 100°C are the same as those that form in other plant oils, such as sesame (Guillén & Ruiz, 2004), corn, sunflower (Guillén & Ruiz, 2005b), linseed, and walnut (Guillén & Ruiz, 2005a) oils. Second, this work confirms that, similar to other plant oils oxidized at moderate temperatures, incipient hydroperoxidic and conjugated dienic signals were visible in the early stages of oxidation of canola and soybean oil, but that aldehydic signals were not observed until much later.

This work also demonstrates the suitability of the multiplet signal resonating at 5.74 ppm as an indicator of hydroperoxides in oxidized plant oils. This signal is better resolved and is, therefore, easier to integrate than the broad hydroperoxide signals resonating between 8.0 – 8.9 ppm that are typically used as hydroperoxide indicators. Additionally, this work provides evidence

that a relationship exists between the ^{1}H NMR signal responses of hydroperoxidic and conjugated dienic signals and the measured PV and CDV, respectively, in highly oxidized oils. Unfortunately, when using standard experimental conditions and instrumentation, it was not possible to demonstrate a relationship between these two measures under oxidative conditions that would be observed during regular storage and use of such oils. However, future work should focus on making use of technological advances in the field and on further optimizations of the experimental protocol to increase the sensitivity of this method in ways that are attainable for all researchers.

CHAPTER 3: EVALUATING THE STABILITY OF FLAVOUR COMPOUNDS IN FLAVOURED FISH OILS DURING OXIDATION

3.1 INTRODUCTION

It is necessary to measure the concentrations of both primary and secondary oxidation products when assessing the extent of lipid oxidation in edible oils. Secondary oxidation is typically measured using the pAV test. However, this test suffers from interference from aldehydic flavour compounds added to marine oil supplements by their producers to enhance their palatability. There are examples in the literature in which the *p*AV test has been applied to measure the level of secondary oxidation in flavoured marine products (Albert et al., 2015; Jackowski et al., 2015) and unexpectedly high *p*AV are reported in these studies. If the contribution of flavour to these inflated *p*AV is not acknowledged, it will be erroneously concluded that such products are highly oxidized. This may lead to consumers' rejection of marine oil supplements as sources of EPA and DHA.

Necessarily, there must be an alternative method to measure the *p*AV in flavoured oils. In addition to GOED's (2016) recommendation for measuring the *p*AV of flavoured marine oils (Section 1.3), GOED also recommends using the True Anisidine Value (TAV) Test. The TAV Test is an HPLC-based method created by Nutrasource Diagnostics (2017) that claims to be able to accurately measure secondary oxidation without interference from flavours. It does so by measuring the amount of *p*-anisidine remaining in a sample solution of oil after sufficient reaction time, with the assumption that the amount of *p*-anisidine consumed by reaction with aldehydes in the oil sample will be proportional to the *p*AV (U.S. Patent No. 9,506,902, 2014).

While solutions exist that are designed to circumvent the interference of flavour compounds to the measured *p*AV, these solutions are not without their disadvantages. The TAV Test, for example, is a proprietary method and as such requires out-of-lab testing for products,

making it a costly alternative (Nutrasource Diagnostics, 2016). The GOED recommendation for measuring the *p*AV of flavoured marine oils is predicated on the assumption that the flavours themselves do not oxidize, degrade, or in any way change their contribution to the measured *p*AV over time (GOED, 2016). It is, in fact, likely that this is occurring; a number of flavour compounds have been shown to degrade or oxidize under a variety of oxidative conditions. For example, citral is degraded in acidic conditions (Liang et al., 2004; Schieberle & Grosch, 1988). This degradation is also seen in neutral citral emulsions and is exacerbated at increased temperatures (Djordjevic et al., 2008b). These same effects were seen with the flavour compounds limonene (Djordjevic et al., 2008a) and vanillin, which have been shown to oxidize during storage at elevated temperatures (Mourtzinos et al., 2009).

I proposed to test the null hypothesis, as followed by GOED, that flavour compounds do not oxidize or otherwise degrade over the course of oxidation. To test this, I first evaluated a number of flavours to determine those with the greatest contribution to the measured *p*AV, and thus would be the most likely to change in observable ways over the course of oxidation. Following this, I performed a series of stability studies to compare oil samples to which flavour had been added before and after oxidation, respectively. The *p*AV and the ^{1}H NMR signal responses of the aldehydic flavour signals were evaluated to determine the extent of flavour degradation over the course of oxidation. I anticipated that the two measurements would be related in such a way that the change in the aldehydic ^{1}H NMR flavour signal could be calibrated to the change in the flavour's contribution to the measured *p*AV.

3.2 METHODS AND MATERIALS

3.2.1 Screening Flavoured Fish Oils

3.2.1.1 Sample Preparation

Accurately weighed aliquots of fish oil (~ 2.5 g) (18% EPA and 12% DHA with 3 mg/g mixed natural tocopherols) (DSM Nutritional Products, Dartmouth, NS) were added to fourteen 4 mL screw top vials (Thermo Scientific, Waltham, MA). To each of these vials, 2% flavour (by weight) was added. The flavours tested were apple, blackcurrant, bubblegum, chocolate-vanilla, citrus punch, coconut, cranberry, grapefruit-tangerine, lemon, mandarin, mango, meat, orange, and peanut butter (FONA International, Geneva, IL). All vials were then purged with nitrogen, capped, and vortexed for 30 seconds. Samples were stored in the dark at -30°C for several days until analysis.

3.2.1.2 Measurement of Aldehydes

Aldehydes in the oils were measured following AOCS Official Method Cd 18-90, the *p*-Anisidine Value, described in Section 2.2.4 of this thesis. The resulting *p*AV was calculated using Equation 1.3. Reported *p*AV are the mean of duplicate *p*AV measurements.

3.2.1.3 ^{1}H NMR Spectral Acquisition and Processing

To prepare the samples for ^{1}H NMR analysis, 0.1 g aliquots of oil were dissolved in 650 µL $CDCl_3$ with 1% TMS as a chemical shift reference (Sigma-Aldrich, Oakville, ON). The samples were placed in 5 mm NMR tubes (Wilmad Lab Glass, Vineland, NJ) and subjected to NMR analysis on a 500 MHz NMR spectrometer (Bruker, East Milton, ON) operating at the conditions specified in Section 2.2.5 of this thesis. All spectra were processed following the protocol outlined in Section 2.2.5. Signal integration was done manually and in triplicate; reported signal response values are the average of triplicate analyses.

3.2.2 Oxidation of Chocolate-vanilla and Lemon Flavoured Fish Oil

The following experiment and subsequent analyses were performed twice, once with chocolate-vanilla flavour and once with lemon flavour.

3.2.2.1 Sample Preparation and Oxidation

Accurately weighed aliquots of fish oil (~ 3.5 g) were added to fifty-one 4 mL screw top vials. To eighteen of these vials, 2% flavour (by weight) was added. All vials were then purged with nitrogen, capped, and vortexed for 30 seconds. Following vortexing, six sample vials were put aside for analysis. These six reserved samples served as unoxidized baseline samples and consisted of triplicate unflavoured fish oil samples and triplicate fish oil samples to which 2% flavour had been added. The remainder of the sample vials were placed uncapped in a 40 ± 2°C oven (Lab-line Instruments, Melrose Park, IL) for oxidation. Every four days for a period of 20 days, three triplicate sets of oil samples (two triplicate sets of unflavoured oils, and one triplicate set of oil to which flavour had been added) were removed from the oven and cooled to room temperature. To one triplicate set of unflavoured oil samples, 2% flavour (by weight) was added, thus creating three streams of oxidized oil: unflavoured oil, oil to which flavour had been added *before* oxidation (FBO), and oil to which flavour was added *after* oxidation (FAO). A scheme of this experiment can be seen in Figure 3.1. All three triplicate sets of oil samples were then purged with nitrogen, capped, and vortexed for 30 seconds. Samples were stored in the dark for several days at -30°C until analysis.

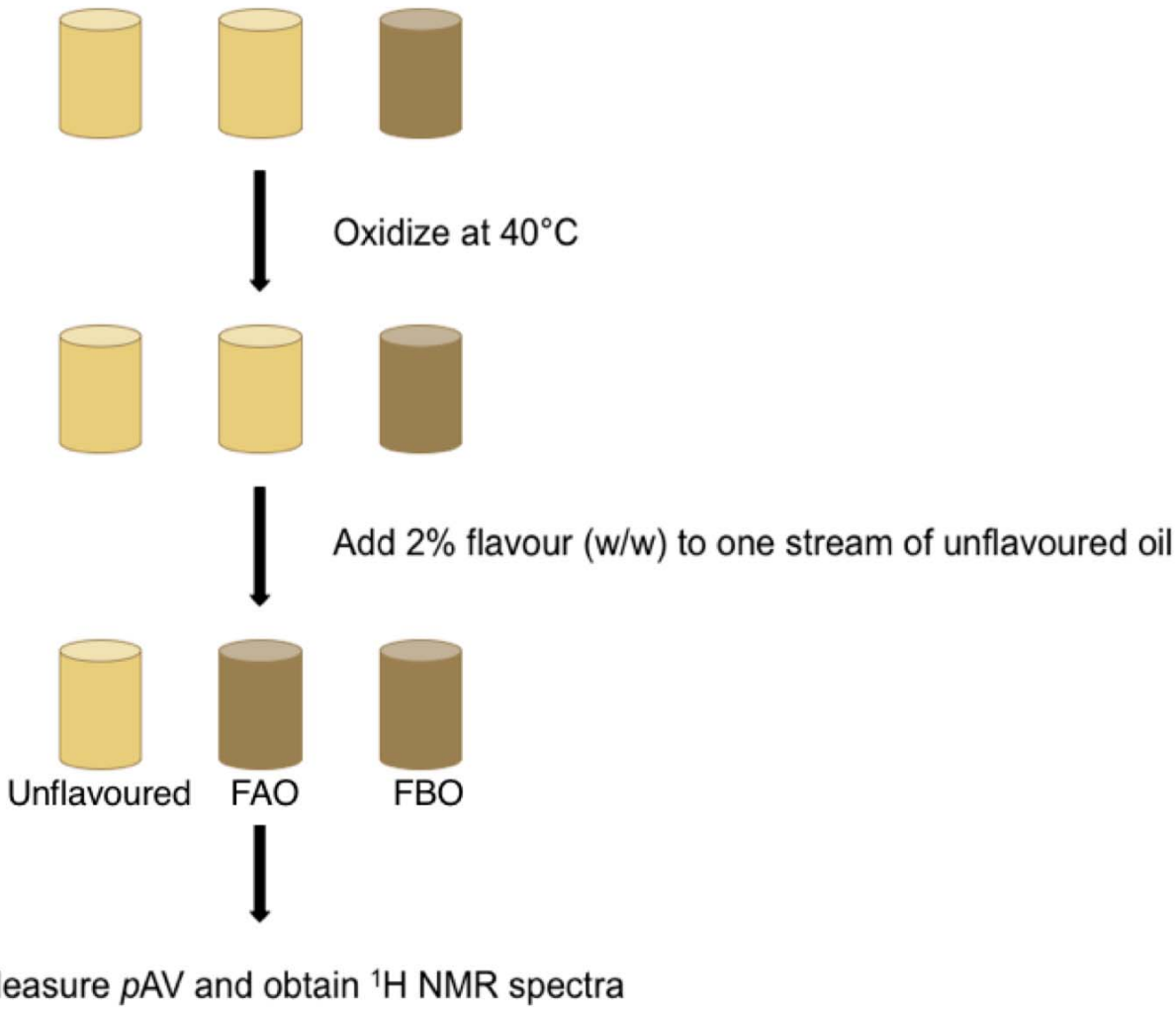


FIG. 3.1. The three streams of oil prepared and analyzed over the course of this experiment. Lighter cylinders represent unflavoured oil while darker cylinders represent oil to which flavour was added.

Aldehydes in the oils were measured following the protocol described in Section 2.2.4 of this thesis. ^{1}H NMR spectral acquisition and processing were done following the protocol described in Section 2.2.5 of this thesis.

3.2.2.2 Confirming the Identity of Flavour Signals with Standard Compounds

A vanillin pharmaceutical secondary standard (Sigma-Aldrich, Oakville, ON) and a citral standard (95%) (Sigma-Aldrich, Oakville, ON) were used to confirm the identity of vanillin and

citral in the ^{1}H NMR spectra of chocolate-vanilla and lemon flavoured fish oils, respectively. Following a protocol similar to the one described in Mannina et al. (2012), a small amount of each standard was added to 0.1 g of flavoured oil and dissolved in 650 µL $CDCl_3$ with 1% TMS. Samples of 10 mg of each standard dissolved in 650 µL $CDCl_3$ with 1% TMS were also prepared. The NMR spectra of these samples were acquired and processed using the protocols outlined in Section 2.2.5.

3.2.2.3 Statistical Analyses

The following equation was used to model the oxidation data (Minitab, State College, PA):

$$Y_f(t) = p\text{AV}_{\text{initial}} + \alpha^{*}\exp^{kt} \quad 3.1$$

where $Y_f(t)$ is the *p*AV on a given day, a is a modulation factor that considers the magnitude of the *p*AV, k is the rate of oxidation, and t is the time. Two-sample t-tests (Minitab 17, State College, PA) with p-values < 0.05 indicating significant differences were used to compare the *p*AV between FBO and FAO samples, as well as the oxidation rate (k) among sample types.

3.3 RESULTS

3.3.1 Flavoured Fish Oil

The *p*AV of fish oil samples to which flavour was added were consistently higher than the *p*AV of the unflavoured control oil; however, the magnitude of this *p*AV difference differed among flavours (Fig. 3.2). Of the flavours tested, chocolate-vanilla, lemon, citrus punch, orange, bubblegum, cranberry, blackcurrant, and mandarin gave rise to distinct signals in the aldehydic region of the ^{1}H NMR spectrum (Fig. 3.3). The remainder of the flavours tested (apple, coconut, mango, meat, and peanut butter) did not give rise to signals in the aldehydic region of the ^{1}H NMR spectrum.

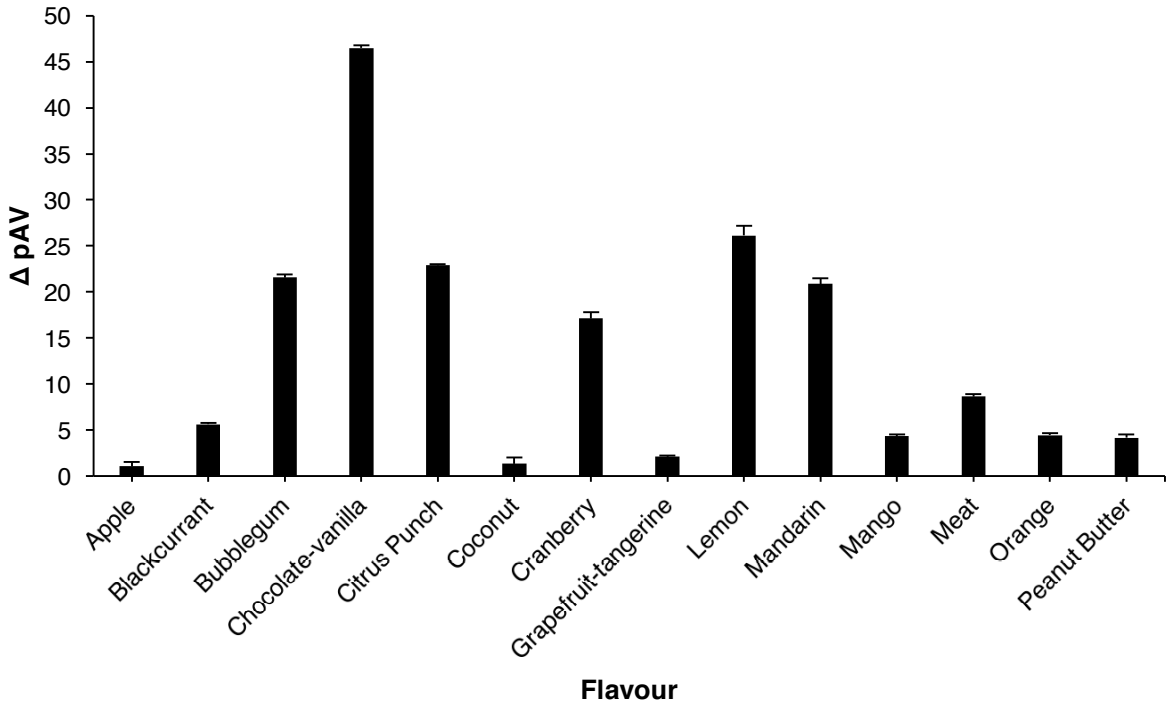


FIG. 3.2 The difference in *p*AV caused by the addition of various flavours (2% by weight) to unflavoured fish oil. The values reported are the means of duplicate measurements with error bars indicating the propagated standard deviation.

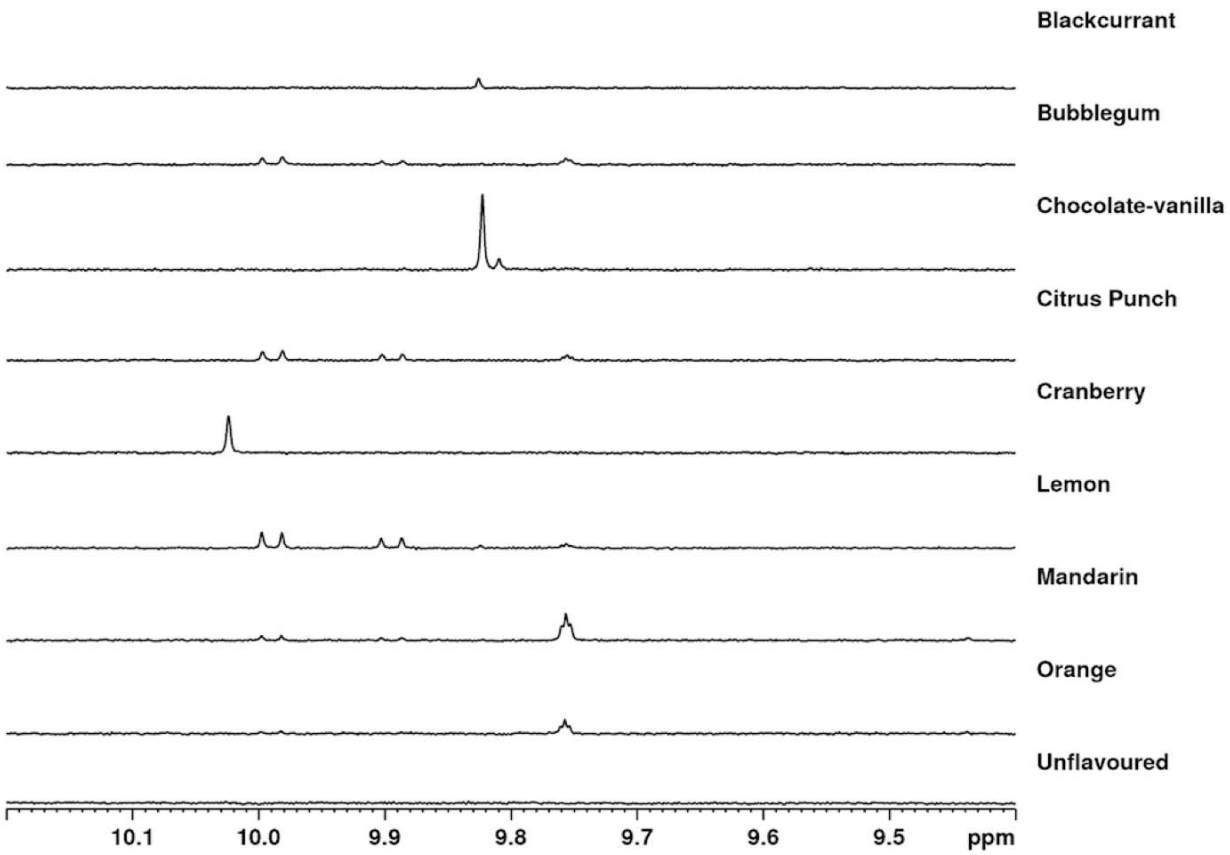


FIG. 3.3 The aldehydic region between 10.20 and 9.40 ppm in the ^{1}H NMR spectra of various flavoured fish oils, as well as an unflavoured fish oil for comparison.

3.3.2 Oxidation of Chocolate-vanilla and Lemon Flavoured Fish Oil

Of all of the flavours tested in this experiment, chocolate-vanilla and lemon flavour caused the greatest change in *p*AV when added to unflavoured fish oil (Fig. 3.2). Therefore, these flavours were selected for further analysis.

3.3.2.1 Effect of Oxidation on the *p*AV

The *p*AV of all of the fish oil samples tested in this study increased over the course of 20 days of oxidation at 40°C (Figs. 3.4 & 3.5). Two-sample t-tests showed statistically significant differences ($p < 0.05$) between the measured *p*AV of FBO and FAO samples for the chocolate-

vanilla flavour experiment at days 4, 16, and 20 (Fig. 3.4). In the lemon flavour experiment, significant differences were found in samples at days 8, 12, 16, and 20 (Fig. 3.5). The oxidation data were modelled empirically (Equation 3.1) and nonlinear regression was used to estimate the oxidation rate (k) for each sample type (Table 3.1). Two-sample t-tests did not provide evidence of significant differences in the k values among sample types ($p > 0.05$).

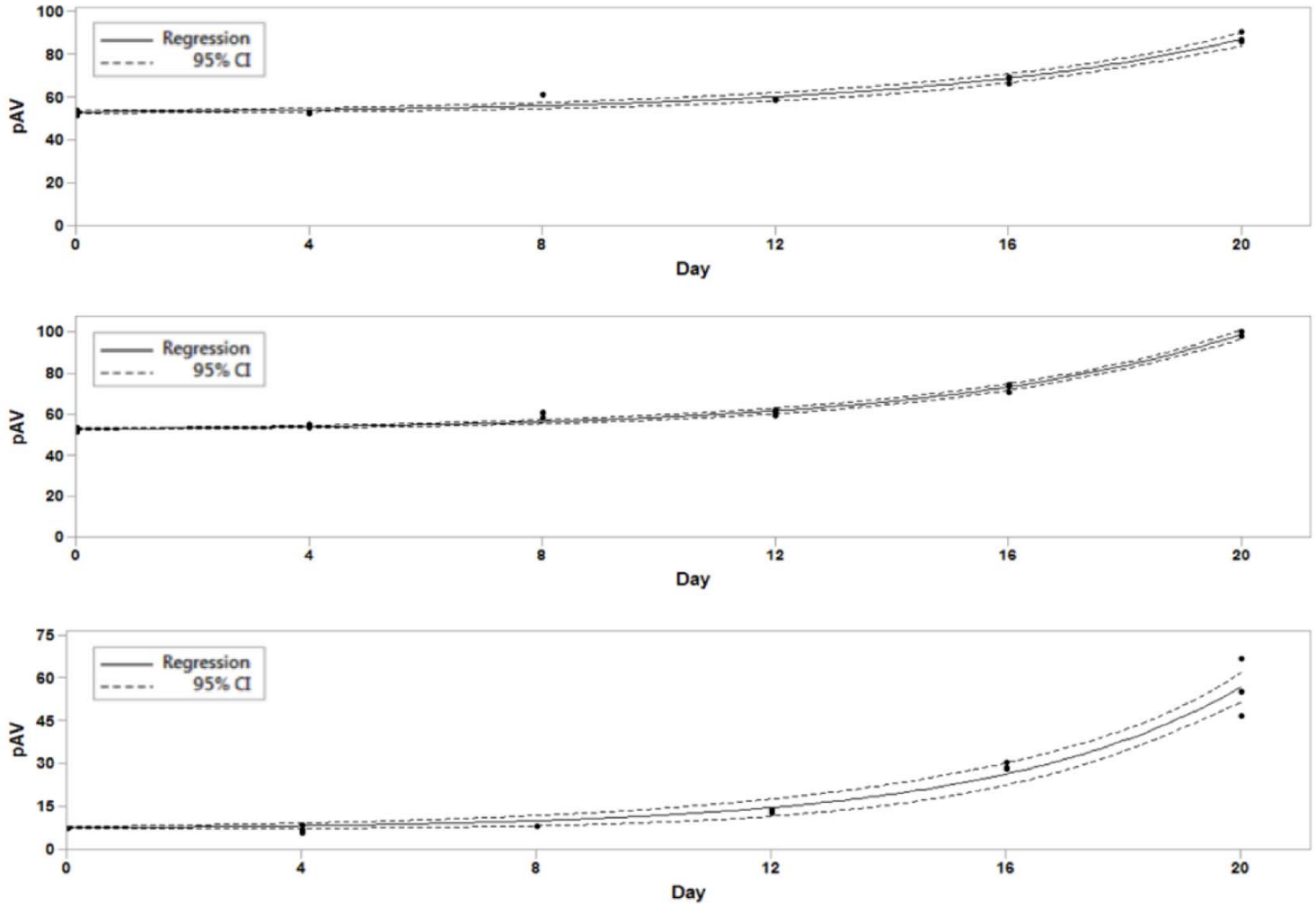


FIG. 3.4 The *p*AV of FBO (top panel), FAO (center panel), and unflavoured (bottom panel) samples during oxidation at 40°C for 20 days in chocolate-vanilla flavoured fish oil. Data are fitted with Equation 3.1 as a model (indicated by the black line).

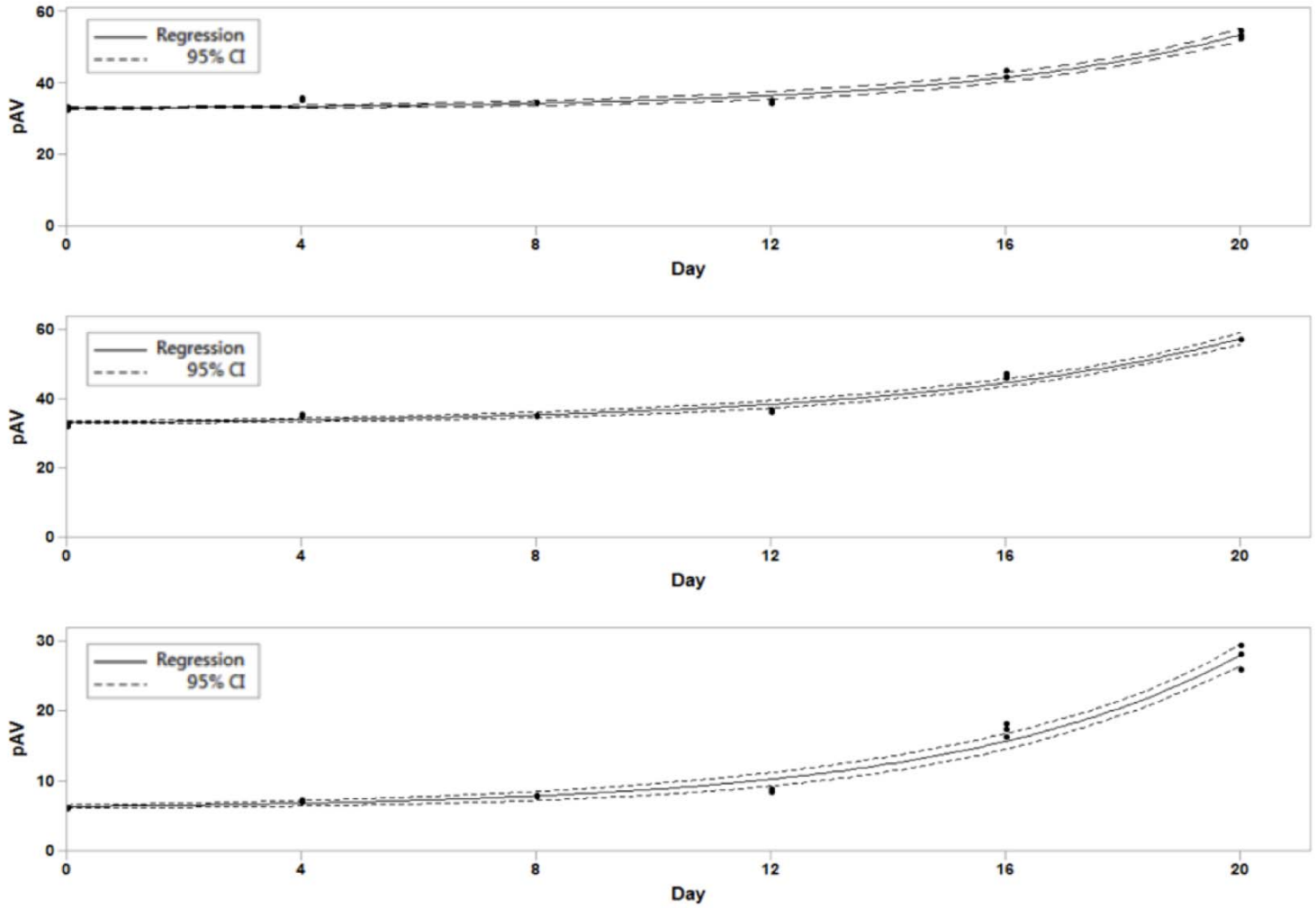


FIG. 3.5 The *p*AV of FBO (top panel), FAO (center panel), and unflavoured (bottom panel) samples during oxidation at 40°C for 20 days in lemon flavoured fish oil. Data are fitted with Equation 3.1 as a model (indicated by the black line).

Table 3.1 Estimates of the oxidation rates (k) of FBO, FAO, and unflavoured samples derived from the exponential models fit to the data obtained during 20 days of oxidation at 40°C.

Sample		k ($days^{-1}$)	RMSE (*p*AV)
Chocolate-vanilla	FBO	0.1847	2.54
	FAO	0.1981	1.81
	Unflavoured	0.2364	3.99
Lemon	FBO	0.2123	1.41
	FAO	0.1791	1.38
	Unflavoured	0.2052	1.23

3.3.2.2 ^{1}H NMR Signal Responses

The identities of the aldehydic flavour signals in chocolate-vanilla and lemon flavoured oils were confirmed using standard compounds (Figs. 3.6 & 3.7, respectively). The ^{1}H NMR signal responses of these signals changed only slightly over the course of oxidation (Fig. 3.8). Similarly, there was almost no difference in signal response between FBO and FAO samples; the magnitude of their integrals differed by 0.0002 at most (Fig. 3.9).

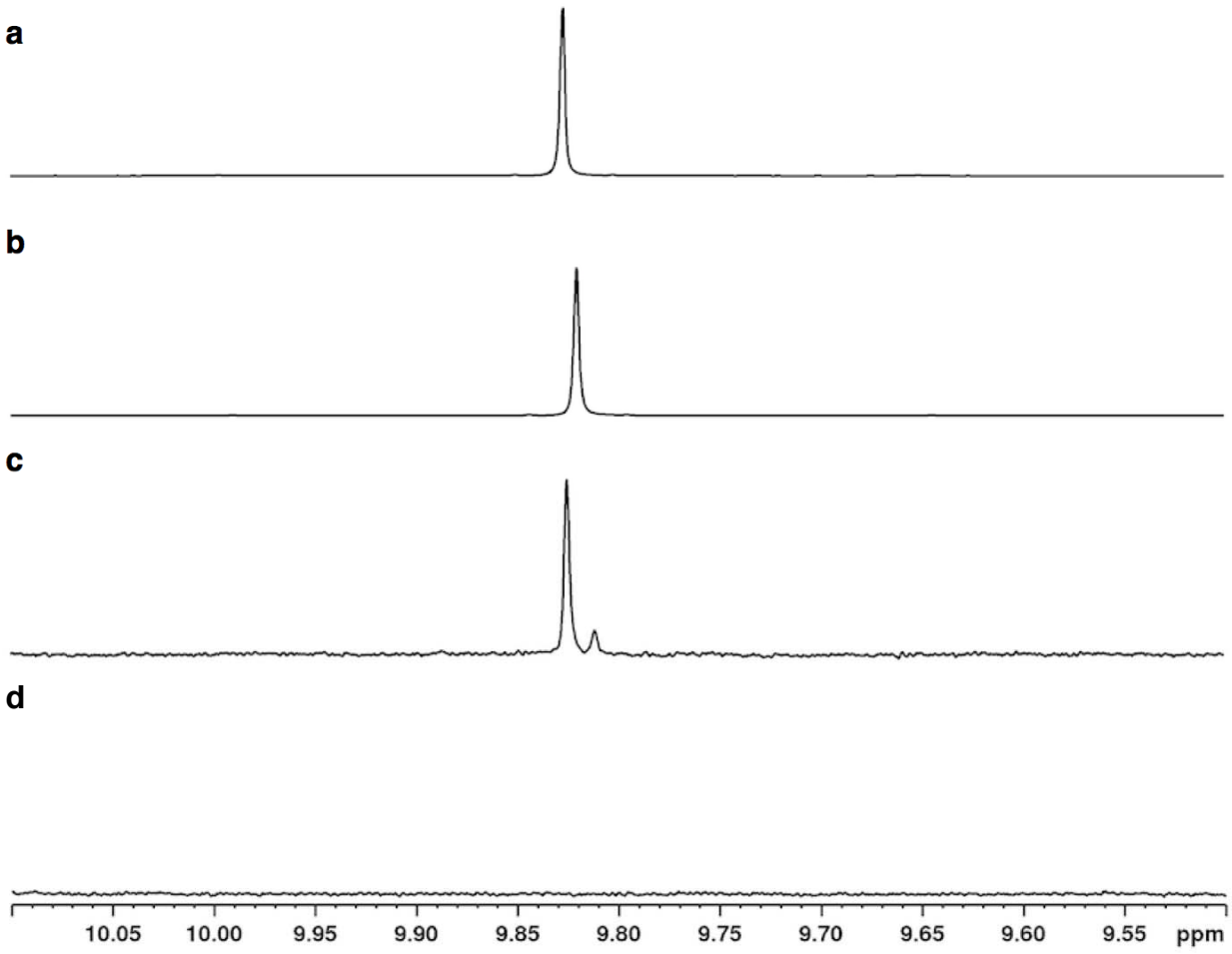


FIG. 3.6 The region between 10.10 and 9.50 ppm in the ^{1}H NMR spectra of (a) a vanillin standard, (b) fish oil flavoured with 2% (by weight) chocolate-vanilla flavour to which a vanillin standard was added, (c) fish oil flavoured with 2% (by weight) chocolate-vanilla flavour, and (d) unflavoured fish oil. The spectra (a), (b), (c), and (d) were not enlarged to the same scale; spectrum (a) was enlarged two times, spectrum (b) was enlarged 4 times, and spectrum (c) and (d) were enlarged 20 times.

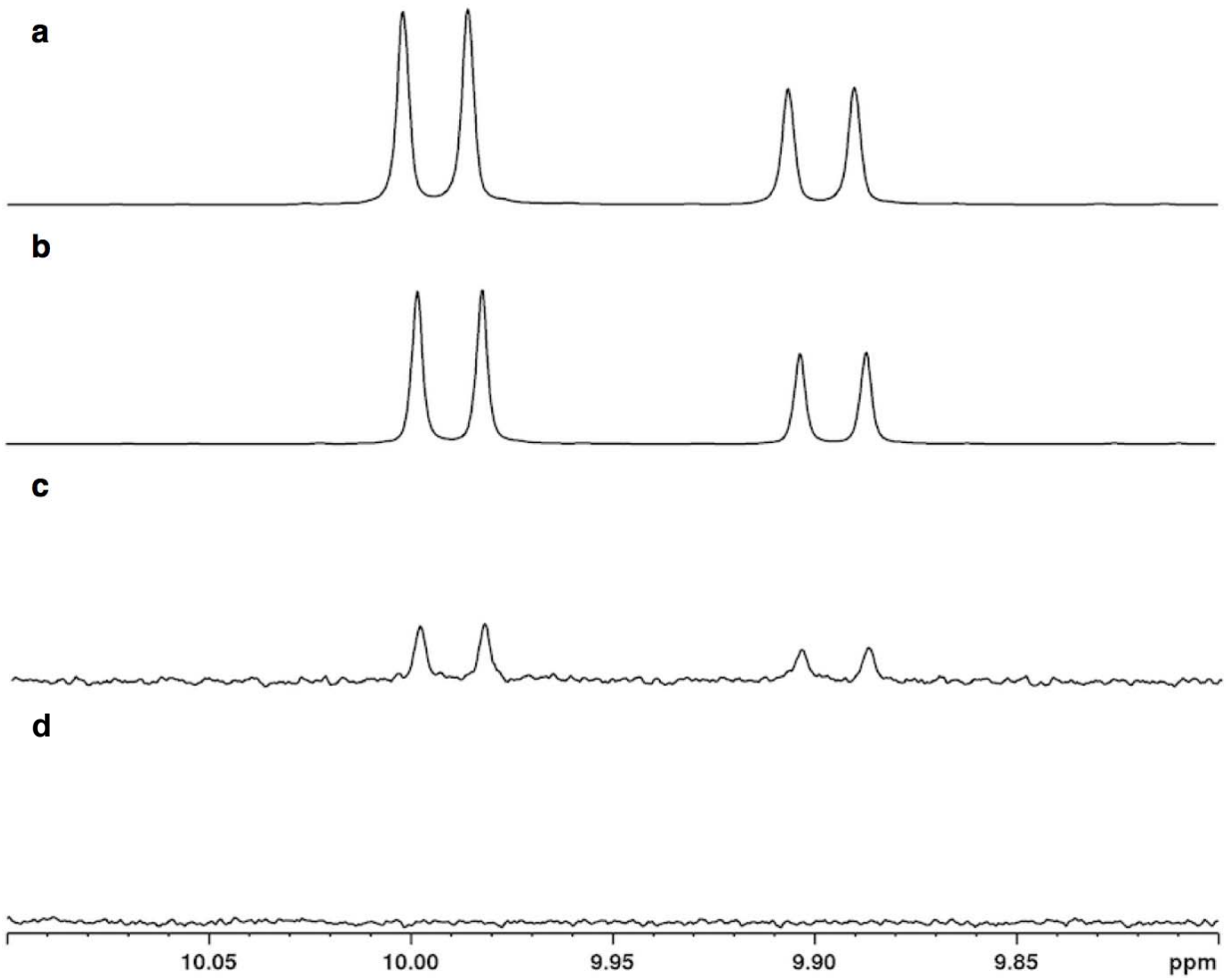


FIG. 3.7 The region between 10.10 and 9.80 ppm in the ^{1}H NMR spectra of (a) a citral standard, (b) fish oil flavoured with 2% (by weight) lemon flavour to which a citral standard was added, (c) fish oil flavoured with 2% (by weight) lemon flavour, and (d) unflavoured fish oil. The spectra (a), (b), (c) and (d) were not enlarged to the same scale; spectrum (a) and (b) were enlarged 8 times, while spectrum (c) and (d) were enlarged 22 times.

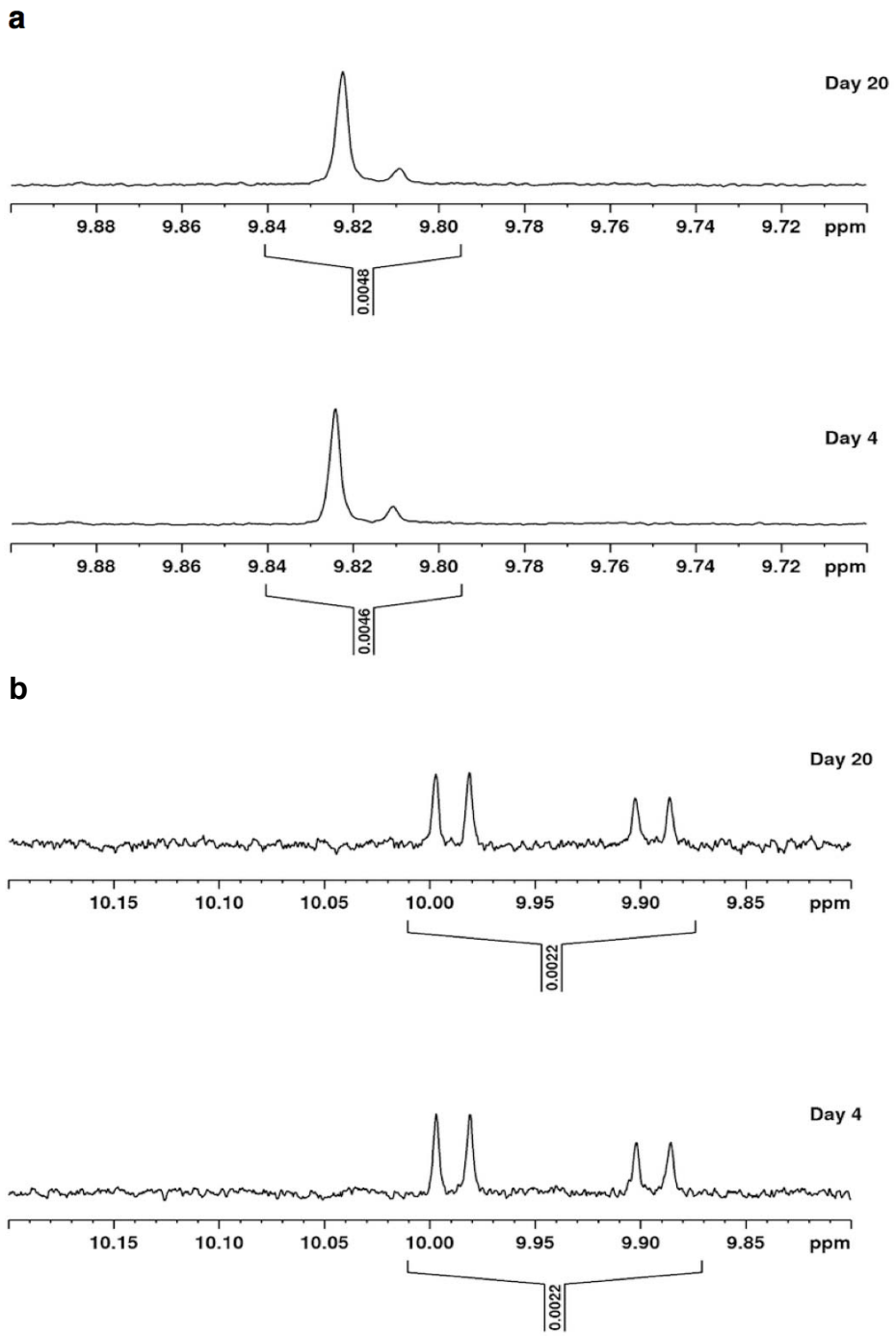


FIG. 3.8 The regions between 9.90 and 9.70 ppm and 10.20 and 9.80 ppm in the ^{1}H NMR spectra of (a) chocolate-vanilla and (b) lemon flavoured FAO samples, respectively, on days 4 and 20 of oxidation at 40°C. The aldehydic flavour signals were integrated relative to the signals of the *sn*-3 and *sn*-1 glycerol protons resonating at 4.29 and 4.14 ppm. The signals of these glycerol protons were calibrated to 4.000, as there are four such protons on the glycerol backbone.

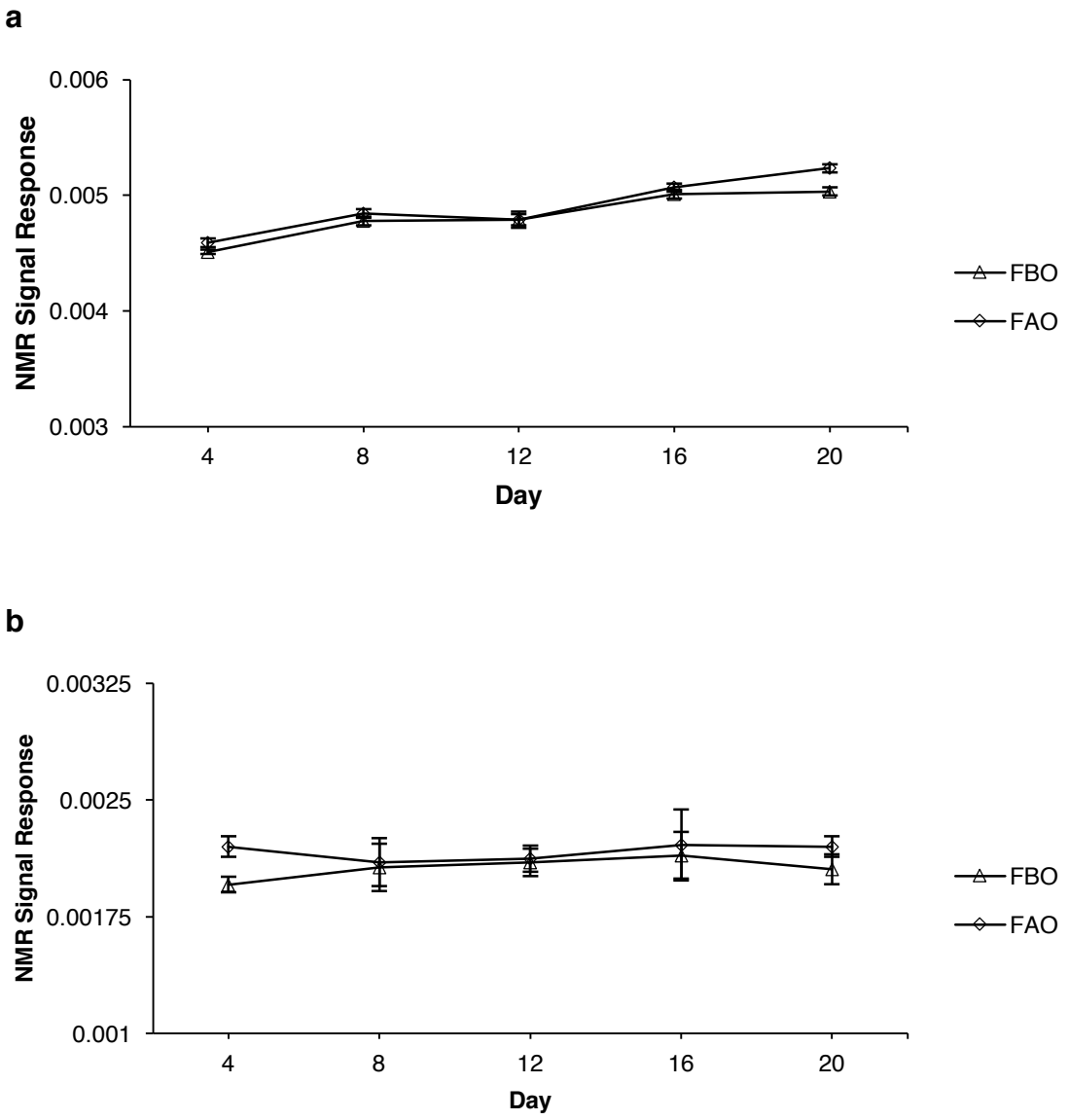


FIG. 3.9 The ^{1}H NMR signal responses of the aldehydic flavour signals of FBO and FAO samples on days 4, 8, 12, 16, and 20 of oxidation at 40°C for (a) chocolate-vanilla flavoured samples and (b) lemon flavoured samples. Each sampling point represents the mean value (n=3) with error bars indicating the standard deviation. Lines between data points are meant only to guide the eye.

3.4 DISCUSSION

3.4.1 Flavoured Fish Oil

3.4.1.1 Flavour-Induced *p*AV Changes

This experiment began with an investigation into the effects of flavour addition on the *p*AV of fish oils. It was anticipated that flavour addition would cause an elevation in the measured *p*AV of the oils, as informal studies in this laboratory have demonstrated this very occurrence. Similarly, research by Jackowski et al. (2015) has shown that the *p*AV of flavoured *n*-3 PUFA supplements are higher than those of unflavoured products. As expected, when added to unflavoured fish oil at a level of 2% (by weight), all flavours tested during this experiment caused an elevation in the measured *p*AV (Fig. 3.2). This is likely due to aldehydes and aldehyde-like compounds in the flavours reacting with the *p*-ansidine reagent, thereby contributing to the formation of the imine chromophore that forms the basis of the spectrophotometric *p*AV test (GOED, 2016).

The magnitude of a flavour's contribution to the measured *p*AV is flavour-dependent (Fig. 3.2); indeed, different flavours are known to contribute to the *p*AV to a different degree (GOED, 2016). In this study, the change in *p*AV with added flavour ranged from 1.06 (apple flavour) to 46.48 (chocolate-vanilla flavour). Likely, the various compounds and aldehydes composing each flavour react with the *p*-ansidine reagent in unique ways, giving rise to structurally unique chromophores. Indeed, it has been shown that the molar absorptivity of the imine chromophore that forms between an aldehyde and the *p*-anisidine reagent differs considerably among aldehydes (Szabó et al., 2010), with the *p*AV method being more sensitive to unsaturated than saturated aldehydes (Gordon, 2004). For this reason, the AOCS (2013a) acknowledges that unsaturated aldehydes with a conjugated double-bond structure, like 2-alkenals and dienals, will have higher

molar absorptivities and therefore contribute more to the absorbance measurement than will aldehydes without these conjugated functionalities.

3.4.1.2 ^{1}H NMR Spectra of Flavoured Fish Oils

To further explore the idea that aldehydes in the flavour compounds contribute to the measured *p*AV to different degrees, the aldehydic regions of the ^{1}H NMR spectra of the flavoured oils evaluated during this experiment were examined (Fig. 3.3). Signals were not observable in the aldehydic region of the ^{1}H NMR spectra of apple, coconut, mango, meat, or peanut butter flavoured oils. In the spectrum of grapefruit-tangerine flavour, only a small signal located at approximately 9.76 ppm was visible. With the exception of meat flavour, which had a *p*AV contribution of 8.66, these flavours had *p*AV contributions of less than 4.5. Presumably, these flavours do not contain aldehyde or aldehyde-like flavour compounds in concentrations detectable by ^{1}H NMR. These flavours are likely composed of other flavour compounds, such as alcohols and ketones (Mannina et al., 2012). Therefore, their contribution to the measured *p*AV is minimal and there are no observable signals in the aldehydic region of the ^{1}H NMR spectrum. Distinct signals were observed in the aldehydic region of the ^{1}H NMR spectra for the remainder of the flavoured oils (blackcurrant, bubblegum, chocolate-vanilla, citrus punch, cranberry, lemon, mandarin, and orange). With the exception of orange and blackcurrant flavours, which had *p*AV contributions of 4.43 and 5.63, respectively, these flavours all had *p*AV contributions of more than 16. These findings demonstrate a relationship between the magnitude of each flavour's contribution to the measured *p*AV and the presence of aldehydic signals in the ^{1}H NMR spectrum.

A triplet resonating at 9.76 ppm is one of several signals present in the ^{1}H NMR spectra of citrus punch, lemon, bubblegum, and mandarin. It is also the only aldehydic signal that can be seen in the spectrum of orange flavoured oil. Orange flavour was unusual in that it made a minimal

contribution to the measured *p*AV (4.43 (Fig. 3.2)) yet still showed the presence of a signal in the aldehydic region of the ^{1}H NMR spectrum. Therefore, it can be assumed that the aldehyde giving rise to this triplet signal contributes little to the measured *p*AV. Indeed, this triplet is frequently identified in the ^{1}H NMR spectra of edible oils and is attributed to *n*-alkanals (Guillén & Ruiz, 2004; Nieva-Echevarría et al., 2016; Sacchi et al., 2006). As previously discussed, saturated aldehydes, like *n*-alkanals, contribute less to the measured *p*AV than do unsaturated aldehydes (Gordon, 2004) particularly those with conjugated double bond functionalities (AOCS, 2013a).

While orange flavour had a *p*AV contribution of only 4.43, citrus punch, lemon, mandarin, and bubblegum flavours contributed 22.90, 26.14, 21.58, and 20.89 to the *p*AV, respectively (Fig. 3.3). Presumably, the large *p*AV contributions of these flavours compared to that of orange flavour are due to the aldehydes that generated the other signals visible in the spectra of these flavoured oils, specifically the doublet signals located at 9.99 and 9.89 (Fig. 3.3). Based on the results of previous work, it was assumed that these signals were attributable to geranial and neral (Fig. 3.10), isomers of the commonly used flavour compound citral (Mannina et al., 2012; Marti et al., 2014). Here, this assumption was confirmed by comparing the aldehydic region of the ^{1}H NMR spectrum of lemon flavoured oil to that of lemon flavoured oil to which a citral standard had been added, as well as to that of a pure citral standard (Fig. 3.7).

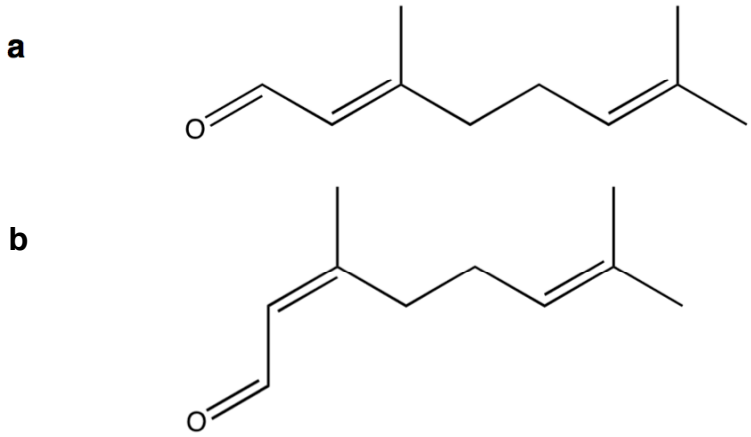


FIG. 3.10 The chemical structures of citral isomers (a) geranial and (b) neral.

Geranial and neral contain a double bond conjugated to the aldehyde group, making their contribution to the measured *p*AV more significant than other non-conjugated aldehydes (AOCS, 2013a). Presumably, geranial and neral in the citrus punch, mandarin, lemon, and bubblegum flavours contributed substantially to the measured *p*AV. This is in agreement with the findings of Jackowski et al. (2015) who found that *n*-3 PUFA products flavoured with citrus flavours, as well as products marketed towards children (e.g. bubblegum-flavoured products) often have higher *p*AV than other flavoured *n*-3 PUFA products.

Similarly, blackcurrant and chocolate-vanilla flavours can be examined together. The aldehydic regions of the ^{1}H NMR spectra of chocolate-vanilla and blackcurrant flavoured oil both contain a singlet located at approximately 9.82 ppm (Fig. 3.3). An additional, smaller singlet located at 9.81 ppm can also be seen in the aldehydic region of the spectrum of chocolate-vanilla flavoured oil. Kayaci and Uyar (2011) and Mukonyi and Ndiege (2001) both obtained the ^{1}H NMR spectra of vanillin (4-hydroxy-3-methoxybenzaldehyde), a phenolic aldehyde commonly used in flavour. On the spectra presented in Kayaci and Uyar (2011) and Mukonyi and Ndiege (2001), signals resonating at approximately 9.8, 7.4, 6.9, and 3.8 ppm were observed. These same signals are present in the spectra of blackcurrant and chocolate-vanilla flavoured oils presented here;

therefore, in this work, the singlet located at approximately 9.82 ppm was attributed to vanillin (Fig. 3.11). This identification was confirmed by comparing the aldehydic region of the ^{1}H NMR spectrum of chocolate-vanilla flavoured oil to that of chocolate-vanilla flavoured oil to which a vanillin standard had been added, as well as to that of a pure vanillin standard (Fig. 3.6).

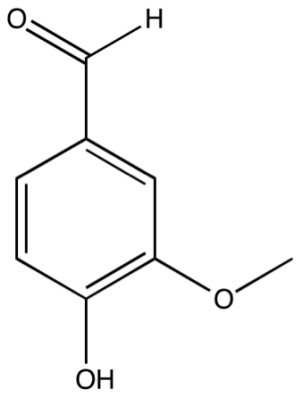


FIG. 3.11 The chemical structure of vanillin.

Despite the fact that both blackcurrant and chocolate-vanilla flavour contain vanillin, their contribution to the measured *p*AV was quite different; blackcurrant flavour had a *p*AV contribution of 5.63, while chocolate-vanilla flavour had a *p*AV contribution of 46.48, the largest found in this study (Fig. 3.2). The difference in contribution to the measured *p*AV between these two flavours may be due to the difference in concentration of vanillin in each of these flavours, evidenced by the greater magnitude of the vanillin signals in the spectrum of chocolate-vanilla than blackcurrant flavoured oil. This is to be expected; a flavour's contribution to the measured *p*AV is concentration-dependent (GOED, 2016). This difference may also be due to the unidentified aldehyde giving rise to the singlet located at 9.81 ppm.

Finally, a singlet at 10.02 ppm is observable on the ^{1}H NMR spectrum of cranberry flavoured oil (Fig. 3.3). Cranberry flavour had a *p*AV contribution of 17.13 (Fig. 3.2). Unfortunately, it was not possible to confidently assign an identity to this signal. It is possible that this signal is attributable to an aromatic aldehyde such as benzaldehyde. Benzaldehyde is often

used in cranberry flavour (U.S. Patent No. 7,097,872, 2011) and gives rise to signals on the ^{1}H NMR spectrum at approximately 10.02 ppm (Abraham et al., 2003). Therefore, this signal may be attributable to benzaldehyde or another aromatic aldehyde; analysis of standards would be necessary to confirm this.

3.4.2 Oxidation of Chocolate-vanilla and Lemon Flavoured Fish Oils

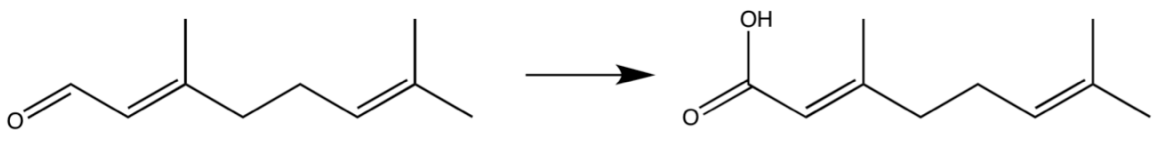
After confirming the contribution of flavour to the measured pAV, the next goal of this project was to determine if flavour compounds were oxidized or modified when stored under oxidative conditions. Previous research has demonstrated that flavour compounds including citral (Djordjevic et al., 2008b; Liang et al., 2004; Schieberle & Grosch, 1988) limonene (Djordjevic et al., 2008a), and vanillin (Mourtzinos et al., 2009) may degrade and oxidize over the course of storage and in a variety of oxidative conditions. This degradation leads to a loss of the desired flavour, as well as to the development of off-flavours (Djordjevic et al., 2008a; Yang et al., 2011). For the scope of this work, the most important ramification of flavour degradation is the resulting inaccuracy of the GOED recommendation for measuring the pAV of flavoured oils, which is predicated on the assumption that the contribution of a flavour to the measured pAV remains constant over the course of oxidation (GOED, 2016).

3.4.2.1 Comparison of FBO and FAO Sample Oxidation

Chocolate-vanilla and lemon flavours were the highest contributors to the *p*AV, with contributions of 46.48 and 26.14, respectively (Fig. 3.2). For this reason, the oxidation experiments discussed in this section were carried out using oils containing these flavours. As expected, the *p*AV of all flavoured and unflavoured fish oil samples increased over the course of oxidation at 40°C (Figs. 3.4 & 3.5). As is typically seen in lipid oxidation, all samples went through an induction period, during which time the rate of oxidation was low and the measured *p*AV increased

slowly (Frankel, 2005). At some point between the eighth and twelfth day of oxidation, the rate of oxidation increased and, thus, the measured *p*AV of the oil samples increased more rapidly until the final day of oxidation.

More interestingly, the *p*AV of FAO samples were significantly higher than those of FBO samples for lemon flavour days 8 to 20 and for chocolate-vanilla flavour on days 4 and 16 to 20 (Figs. 3.4 & 3.5). Because the sole difference between FAO and FBO samples is the time at which flavour is added to the sample, this phenomenon suggests that, during oxidation, flavours are oxidized or degraded in such a way as to decrease their contribution to the measured *p*AV. There are several potential explanations for this phenomenon. Firstly, degradation of the flavour compounds over time could lower their contribution to the development of the imine chromophore if their degradation products did not include similarly unsaturated aldehydes. Another explanation of this occurrence could be that, as the flavours oxidize, non-aldehydic oxidation products are formed that do not contribute to the *p*AV. Indeed, Nguyen et al. (2009) demonstrated that, during storage of lemon oil at 40ºC, the flavour aldehydes geranial and neral were oxidized to geranic and nerolic acid, respectively (Fig. 3.12). This likely occurs following the mechanism discussed by Marteau et al. (2013). In this mechanism, a peracid formed during the autooxidation of an aldehyde reacts with further aldehydes in the Baeyer-Villiger oxidation reaction, generating two molecules of carboxylic acid (Fig. 3.13).



Geranial Geranic acid

FIG. 3.12 The oxidation of the flavour aldehyde geranial to the carboxylic acid geranic acid.

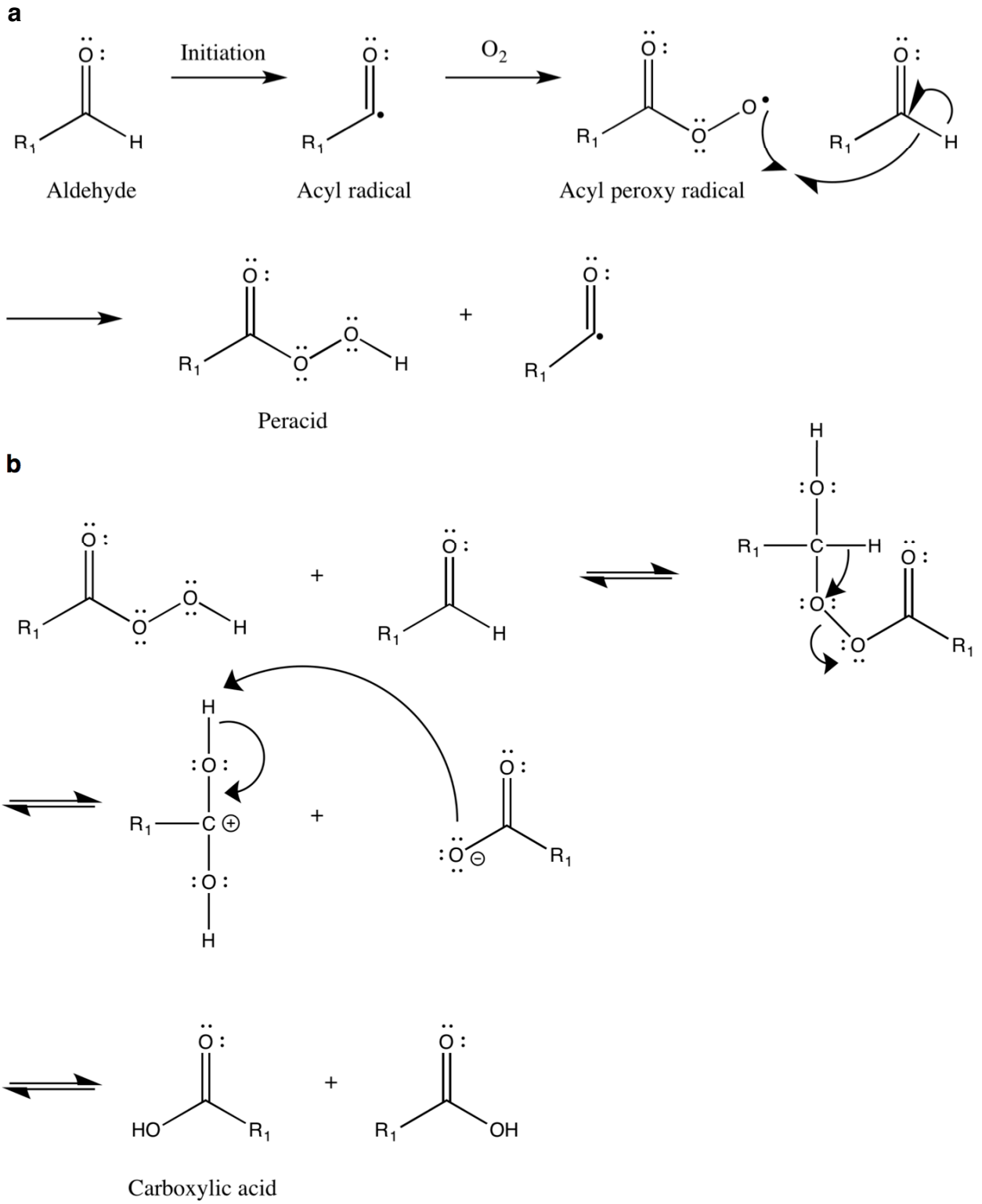


FIG. 3.13 The mechanism for the oxidation of an aldehyde to a carboxylic acid beginning with (a) the autooxidation of an aldehyde to form a peracid and (b) the subsequent formation of two molecules of carboxylic acid via the Baeyer-Villiger oxidation reaction.

In an attempt to determine the source of the observed *p*AV difference between FBO and FAO samples over the course of oxidation, the ^{1}H NMR spectra of these two sample types were compared. Ideally, if flavour degradation during oxidation were occurring, the observed *p*AV differences between sample types would manifest themselves on the ^{1}H NMR spectrum as diminished signal responses of the aldehydic flavour signals of FBO samples relative to those of FAO samples. Unfortunately, the aldehydic signal responses of neither chocolate-vanilla nor lemon flavoured oil changed in a measurable way over the course of oxidation; therefore, it was not possible to draw a conclusion about the nature of the observed *p*AV difference (Figs. 3.8 and 3.9). In the chocolate-vanilla flavour experiment, there did appear to be a small difference in signal response between FBO and FAO samples; however, this difference was miniscule and the measured signal responses did not correlate with measured *p*AV.

The lack of observable difference between the measured signal responses of FBO and FAO samples is likely due to the lack of sensitivity of the ^{1}H NMR method. The sensitivity of ^{1}H NMR is dependent on several factors, including the strength of the instrument's magnetic field, the parameters used during spectral acquisition, and the temperature at which spectral acquisition occurs (Holzgrabe, 2010). Efforts to increase the sensitivity of the ^{1}H NMR method may come at the expense of the time saved by using ^{1}H NMR analysis for this application.

3.4.2.2 Consequences of Flavour Oxidation and Degradation

The fact that the *p*AV of FBO samples are lower than those of FAO samples has ramifications for the GOED recommended method for measuring the *p*AV of flavoured oils. This work has shown that flavours have a diminished influence on the measured *p*AV over time, i.e., that the contribution of flavour to the *p*AV is not constant. During oxidation, as the contribution of flavour to the measured *p*AV is decreasing, the concentration of aldehydes formed through

oxidation of the oil itself is increasing. Therefore, more oxidation occurs before the maximum target *p*AV allowable by the GOED recommendation is reached. In other words, the GOED recommendation for measuring the *p*AV of flavoured oils underestimates the amount of oxidation that is occurring in the oil. This underestimation of the extent of oxidation may lead to overly optimistic inferences about the quality and shelf-life of the flavoured oil in question. Furthermore, underestimating the level of aldehydes in an oil can have negative consequences; in some cases, toxic oxygenated α,β-unsaturated aldehydes, such as 4-hydroxy-*trans*-2-nonenal (Fig. 3.14) form over the course of oxidation. These oxygenated aldehydes can be harmful to health and have been implicated in a number of chronic diseases (Alary et al., 2003). Finally, consuming oxidized *n*-3 PUFA supplements has been shown to have a negative impact on circulating blood lipid profiles (García-Hernández et al., 2013) and has been associated with atherosclerosis and tumor formation (Esterbauer, 1993).

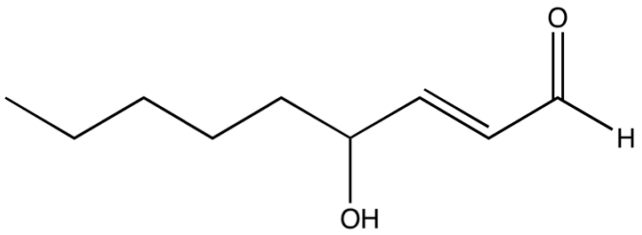


FIG. 3.14 The chemical structure of 4-hydroxy-*trans*-2-nonenal.

3.4.2.3 Flavours as Antioxidants

An alternative interpretation of the data is that compounds in the flavours are acting as antioxidants. To investigate this, it was necessary to first fit a model to the data in order to then compare the rate of *p*AV increase between FBO and unflavoured samples. An exponential model was chosen for this purpose, showing an acceptable fit to the oxidation data obtained in both the chocolate-vanilla and lemon flavour experiments (Table 3.1). Using nonlinear regression, the rates of oxidation (k) were estimated from the model and compared for each sample type (Table 3.1).

In the chocolate-vanilla flavour experiment, statistically significant differences were not observed in the rates of oxidation among sample types. Based on this data, it is not possible to conclude that chocolate-vanilla flavour had antioxidant activity. However, visual inspection of the data would suggest that the rate of oxidation is faster in unflavoured than FBO samples. Perhaps, had the experiment been continued beyond 20 days, antioxidant activity would have been observed. Such antioxidant activity could have been attributed to vanillin. Vanillin is a phenolic aldehyde and as such should be capable of scavenging radicals and thus having antioxidant activity; nevertheless, there are conflicting reports in the literature of the antioxidant effect of vanillin. One of the first studies into this topic shows that vanillin can act as an antioxidant in food systems containing PUFA (Burri et al., 1989). Other studies have shown that, while vanillin is a contributor to the total phenolic content, it shows weak antioxidant activity in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay (Cassani et al., 2016; Silveira et al., 2015), which assess the radical scavenging ability of potential antioxidants (Molyneux, 2004). However, results based on the DPPH assay may not be accurate, as the structure of vanillin results in its poor reactivity with DPPH (Brand-Williams et al., 1995). For this reason, Tai et al. (2011) performed a systematic, multi-assay study of the antioxidant activity of vanillin that included the DPPH test as well as several others. The results of these assays suggest that vanillin does indeed act as an antioxidant.

The research discussed above pertained to food systems other than bulk oils. However, antioxidants can have dramatically different effects in different food systems, such as aqueous systems and emulsions (Jacobsen et al., 2008). No studies have investigated the effect of vanillin as an antioxidant in bulk oils. Therefore, it is difficult to determine if the results of these studies are applicable to the study of bulk fish oil. However, research does exist on the antioxidant activity of vanillic acid (Fig. 3.15) in bulk oil systems. Mourtzinos et al. (2009) demonstrated that vanillin

itself may be oxidized to vanillic acid during heating, and that vanillic acid is 3.3 times more effective an antioxidant than vanillin. Indeed, vanillic acid has been shown to exert antioxidant effects in corn oil subjected to deep-frying conditions (Naz et al., 2005) and, more importantly, in fish oil at temperatures between 35 to 55ºC (Farhoosh et al., 2016). Here, it is possible that vanillin was oxidized to vanillic acid. Vanillic acid would have contributed less to the measured *p*AV than vanillin; thus, this could be one possible explanation for the observed difference in *p*AV between FBO and FAO samples.

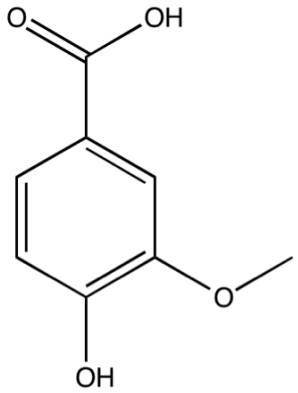


FIG. 3.15 The chemical structure of vanillic acid.

There were also no significant differences observed in the rates of oxidation among sample types in the lemon flavour experiment. To my knowledge, the antioxidant activity of lemon flavouring has not been investigated; however, citral, one of the compounds identified in the lemon flavour in this experiment, does not exert strong antioxidant effects when included in edible fruit coatings (Guerreiro et al., 2015, 2016) or when tested in model systems (Misharina et al., 2011). Compounding this, citral has been shown to degrade in a variety of conditions, such as during storage in acidic conditions (Liang et al., 2004; Schieberle & Grosch, 1988) as well as in neutral citral emulsions (Djordjevic et al., 2008b). Additionally, GC analysis of cold-pressed lemon oil stored in the dark for two months at 30°C showed significant losses of citral (Nguyen et al., 2009).

This effect is exacerbated at increased temperatures (Djordjevic et al., 2008b; Nguyen et al., 2009). Therefore, due to citral's limited ability to function as an antioxidant and the potential for citral loss during storage, it is unlikely that the observed differences between the *p*AV of FAO and FBO samples in the lemon flavour experiment were due to antioxidant activity by citral.

3.5 CONCLUSION

This work has demonstrated that flavours added to increase the palatability of *n*-3 PUFA products contribute to the measured *p*AV, leading to inaccurate results. Additionally, data from this study would suggest that flavour compounds suffer oxidation and/or degradation during storage at elevated temperatures. This has ramifications for GOED's current recommendation for measuring the *p*AV of flavoured oils; flavour degradation and oxidation will lead to an underestimation of the extent of oxidation in the flavoured product. This has negative consequences not only for the quality and shelf-life of the product, but for its health and safety as well.

Unfortunately, a lack of sensitivity of the ^{1}H NMR method developed here limits its applicability to the measurement of the *p*AV in flavoured oils. However, this ^{1}H NMR method is an excellent qualitative screening method for assessing the potential contribution of a given flavour to the measured *p*AV. It is a simple and rapid method that requires less labour, fewer resources, and less sample material than the *p*AV test. Additionally, it avoids the use of *p*-anisidine, which is a highly toxic compound (Schaich, 2016). Industrially, this method could be used to assess flavour formulations on the basis of their contribution to the measured *p*AV. Specifically, potential applications of this screening method could include assessing the suitability of previously untested flavours, assessing the impact of changes to existing flavour formulations or ingredients, or assessing the potential contribution of a new flavour brand or source.

Future work in this topic should focus on several points. First, this experiment should be repeated with a longer duration to assess the antioxidant activity of chocolate-vanilla flavour over longer periods. Second, this experiment could be repeated with additional flavours to determine if flavour degradation and/or antioxidant activity are universal phenomena. Finally, efforts could be made to develop a more sensitive ^{1}H NMR method capable of measuring the degradation of flavour signals over the course of oxidation. In this way, the cause of the decrease in *p*AV between FAO and FBO samples could be definitively assessed. Ideally, this information would be applied to the creation of an ^{1}H NMR-based alternative to the current GOED recommendation for measuring the *p*AV of flavoured oils.

CHAPTER 4: CONCLUSION

4.1 CLOSING STATEMENTS

This thesis demonstrates that the inherent insensitivity of ^{1}H NMR limits its application to the quantitative assessment of lipid oxidation in edible oils. While relationships were observed between ^{1}H NMR spectra and traditional measures of oxidation, including the PV and CDV, these relationships only occurred at high levels of oxidation. Currently, the successful application of ^{1}H NMR for these aims requires sensitivity enhancements, such as instrument optimizations and additional time invested in sample preparation, that increase the cost, time, and labour required for the ^{1}H NMR analysis and thus limit its scope.

Although it was not possible to apply ^{1}H NMR to the quantitative assessment of lipid oxidation, this work confirmed qualitative results obtained by other researchers who used ^{1}H NMR to monitor the formation of oxidation products in edible plant oils and extended these findings to soybean and canola oil. For example, incipient hydroperoxidic and conjugated dienic signals were visible in the ^{1}H NMR spectra of these oils in the early stages of oxidation, and were followed by aldehydic signals much later. This data also provided evidence for the utility of the signal resonating at 5.74 ppm as an indicator of hydroperoxides in oxidized plant oils.

Several useful findings unrelated to ^{1}H NMR analyses were also demonstrated. First, the data confirmed that flavours contribute to the measured *p*AV and demonstrated that their contributions vary with flavour. Second, by demonstrating that chocolate-vanilla and lemon flavours may degrade or oxidize during storage at elevated temperatures, this study also provided evidence that a key assumption of the GOED recommended method for measuring *p*AV in flavoured marine oils is not always met. This suggests that the GOED recommendation may not consistently provide accurate estimates of the *p*AV in flavoured marine oils.

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