Application of a Portable Handheld Infrared Spectrometer for Quantitation of *trans* Fat in
Edible Oils

THESIS

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Abstract

Trans fat has been linked to increased risk of heart disease, and consumption poses a risk to consumers. FDA has mandated inclusion of *trans* fat on nutritional labeling, which has created a need for accurate, rapid analytical techniques. FTIR spectroscopy has been successfully applied to *trans* measurement, and advances in multivariate chemometric analysis and handheld FTIR spectrometers are promising for industrial applications. The objective of this study was to develop a methodology for the application of a portable handheld infrared spectrometer for the accurate measurement of *trans* fat in edible oils.

Pure triglycerides were spiked with known levels of *trans*-trielaidin and spectra of heated samples were collected on a handheld FTIR spectrometer and benchtop FTIR as a reference method. Calibration models were created by measuring height of the second derivative band at 966 cm^{-1} and by partial least squares regression (PLSR). Predictive accuracy of the models was validated with an independent test set of commercial edible oils also spiked with trielaidin. Calibration models developed using PLSR and linear regression of band heights gave correlation coefficients $R^2 > 0.98$.

The handheld spectrometer coupled with the second derivative method did not produce accurate predictions. However, the handheld unit coupled with PLSR multivariate analysis gave standard error of prediction (SEP) of approximately 1%, comparable to

values obtained with benchtop systems. This study demonstrates that handheld FTIR spectroscopy coupled with chemometrics is a suitable method for quantitation of *trans* fat. This combination is promising for *in situ* analysis of *trans* fat in edible oils.

Dedicated to my family and friends.

I couldn't have done this without your love and support.

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Chapter 1: Literature Review

1.1 *Trans* fat

1.1.1 *Definition and general information*

The term *trans* fat refers to any unsaturated lipid that contains one or more double bond in the *trans* isomeric configuration (Mossoba and others 2009). The FDA adds to the definition that the double bonds must be isolated and non-conjugated (Ledoux and others 2007, Mossoba and others 2009a). Codex Alimentarius defines *trans* fatty acids (TFAs) as “all the geometrical isomers of monounsaturated and polyunsaturated fatty acids having non-conjugated, interrupted by at least one methylene group, carbon-carbon double bonds in the *trans* configuration” (2010). In TFAs, the two moieties of the fatty acid chain are on opposite sides of the double bond, while in a *cis* fatty acid the moieties are on the same side of the double bond. Therefore, as shown in Figure 1, *cis* fatty acids are bent, while *trans* fatty acids have straight chains similar to saturated fatty acids (Ghotra and others 2002). The difference in the geometry of the unsaturation gives *trans* fats not only different physical and structural properties from *cis* fats, but also leads to different functional behavior in foods and in human health.

Figure 1. Geometric configuration of *cis* and *trans* fatty acids (Mensink and Katan 1990).

1.1.2 Origins of *trans* fats

Trans fat is both a natural and industrially created compound. Small amounts of *trans* fatty acids are formed naturally by ruminant biohydrogenation, which consists of conversion of *cis* unsaturated fatty acids to saturated fatty acids and *trans* fatty acids by bacterial isomerase enzymes in the rumen (Precht and others 2000, Juaneda and others 2007, Ledoux and others 2007). TFAs are also formed during frying above 180°C and during oil deodorization (Richter and others 2009, Bansal and others 2009, Ledoux and others 2007). However, most dietary *trans* fat is from inclusion of partially hydrogenated oils in food, where TFAs are formed during industrial oil hydrogenation (Mossoba and others 2009). Analytical methods cannot distinguish between natural and artificial *trans* isomers because they are chemically identical (Juaneda and others 2007), although industrially produced and ruminant produced TFAs have distinct fatty acid profiles (Richter and others 2009).

Industrial hydrogenation is a process that saturates oils by adding hydrogen across the double bonds of unsaturated fatty acids, which converts an oil to a fat (Ghotra and others 2002). It was first introduced around 1900 by Sabatier and Senderens, who used nickel catalysts to hydrogenate fats (Ackman 2000). Although the process is primarily intended to saturate pi-bonds, geometric and positional isomers are formed as a consequence of an incomplete reaction (Musavi and others 2008). While fully hydrogenating an oil creates a saturated fat, partial hydrogenation causes between 1 and 60% of double bonds to be isomerized into *trans* fatty acids (Ledoux and others 2007, Ghotra and others 2002). Oils are partially and fully hydrogenated in order to give them desirable functional characteristics, such as oxidative stability, longer shelf life, higher melting point, and more desirable texture (Ghotra and others 2002, Ledoux and others 2007, Christy and others 2003, Kim and others 2007). The amount of TFA formed during hydrogenation is strongly dependent on the starting oil and the conditions employed. TFA content can be decreased by using higher pressure, higher agitation, lower temperature and lower catalyst concentration, although temperature has the greatest effect (Drozowski and Szukalska 2000).

1.1.3 Dietary sources of trans fat

Industrial hydrogenated TFAs can make up a large portion of human dietary fat intake. The US Department of Health and Human Services' Continuing Survey of Food Intakes by Individuals estimated that in 1994-1996 the average *trans* fat intake was 2.6% of total energy, 80% of which was from processed food and oils; the other 20% was from natural

animal sources (U.S. Food and Drug Administration 2003). A 1995-1996 study of foods consumed in Europe estimated that TFA accounted for 0.5-2% of total energy intake and out of 119 foods analyzed, 45 contained more than 2% *trans*, and 8 contained more than 20% *trans*. Breakfast cereals had the lowest content with a mean of 0.35% of total fat, and bakery products had the highest mean, 6.1% of total fat (Richter and others 2009). The amount of ruminant TFA in the human diet is generally small compared to industrially-derived *trans* fat, although one study found it can account for up to 9% of total fatty acids in butter (Ghotra and others 2002).

1.1.4 Health effects

The health effects of *trans* fat have been under examination since the 1940s (Richter and others 2009), although the first significant report of negative health effects was published by Mensink and Katan in 1990. This study showed the high consumption of TFAs led to lower high density lipoprotein (HDL) and higher low density lipoprotein (LDL) levels, which are both factors for coronary heart disease (CHD). Additionally, the authors demonstrated that *trans* fat increased levels of plasma apolipoprotein B, increased the ratio of LDL to HDL, increased the total cholesterol to HDL ratio, and increased serum triglycerides. Another important result of this initial report was in demonstrating that the negative effects of TFA persisted across gender, age and ethnicity (Mensink and Katan 1990). Since 1990, numerous reports have confirmed these findings. Mozaffarian and others reported that a 2g increase in TFA intake can increase risk of CHD by 25% (2009). Additionally, *trans* fat is associated with weight gain, adiposity, inflammation and higher

risk of coronary heart disease (Mozaffarian and others 2009). According to the Mayo Clinic, not only does TFA intake raise LDL and lower HDL, it also increases triglycerides, increases Lp(a) lipoprotein and raises levels of inflammation, which raises one's risk for heart attack, stroke, diabetes, and heart disease (2009). *Trans* fat may also increase insulin resistance in overweight and diabetic individuals, increasing risk of diabetes (Mozaffarian and others 2009).

According to the 2010 Dietary Guidelines for Americans, adults should consume roughly 20-35% of daily calories as fat while the amount of *trans* fat should be minimized. One of four key recommendations in the Dietary Guidelines urged Americans to “consume less than 10 percent of calories from saturated fatty acids and less than 300 mg/day of cholesterol, and keep *trans* fatty acid consumption as low as possible” (United States Department of Agriculture 2010). The American Heart Association recommends that less than 1% of total calories should be obtained from TFA, which is less than 2g for a 2000 calorie per day diet (Mayo Clinic 2009).

1.1.5 Labeling and industry response

Due to the weight of evidence indicating negative effects of TFAs on human health as well as consumer petitioning, the FDA ruled in 2003 that nutritional labels must declare *trans* fat levels (U.S. Food and Drug Administration 2003). The law went into effect on January 1, 2006 and covers any product containing more than 0.5g TFA per serving (U.S. Food and Drug Administration 2003). The aim of declaring the *trans* fat content of foods and dietary supplements was to help the consumer make decisions based on sound dietary

recommendations (Mossoba and others 2009). *Trans* fat is labeled in grams per serving, with increments of 0.5g below 5g and 1 gram increments about 5g (U.S. Food and Drug Administration 2003). When a product contains 0.5 or more grams of *trans* fatty acid per serving; the minimum corresponding *trans* fat content is estimated to be approximately 2% of total fat by weight (Mossoba and others 2009). Therefore, an analytical method accurate at or below 2% (w/w) *trans* is necessary for labeling compliance. Consistent with previous labeling laws, non-packaged foods such as restaurant food are not mandated for nutritional labeling (Mossoba and others 2007). The FDA considers a food misbranded if it contains a 20% greater amount than is listed on the label (Mossoba and others 2009a). Thus, accurate analytical techniques for determining *trans* fat levels in food are necessary to today's food industry.

TFA labeling requirements and regulations vary internationally, with a lower labeling limit of 0.2g in Canada, and an upper limit of 2g TFA per 100 g oil or fat, regardless of serving size (Mossoba and others 2009). Some governments are taking more drastic action to decrease TFA intake; in January 2008, New York banned *trans* fat from restaurants entirely (Mossoba and others 2007, Mayo Clinic 2009). Additionally, Denmark has a legal upper limit on TFA content in food set at 2% (Richter and others 2009).

After *trans* fat labeling was mandated, the food industry began efforts to decrease it in formulations. Reformulation is one strategy, with companies seeking to implement alternatives including naturally stable oils, interesterified fats, customized fats and oils,

and partially hydrogenated products with reduced *trans* levels (Mossoba and others 2007). Specialty oils have been created that exhibit similar functional properties to *trans*-containing fats (Ghotra and others 2002). Replacement with palm oil is also an option, and some research shows that saturated fats can mimic some functionality of *trans* fats. Some authors believe that using an alternative type of oil in frying and food preparation is the easiest and cheapest alternative to lower TFA levels (Butt and Sultan 2009).

Another method of controlling *trans* fat levels is to change the hydrogenation process itself. Hydrogenation is dependent on temperature, hydrogen pressure, the concentration and activity of the catalyst used, the amount of agitation used, and length of the process (Ghotra and others 2002). Musavi and others (2008) have shown that the amount of *trans* fat created during the hydrogenation process can be altered by changing parameters such as temperature, stirring rate, catalyst, and pressure of the hydrogenation reaction all affect the outcome. In fact, using a low temperature helps in lowering the amount of *trans* generated by up to 20% (Musavi and others 2008).

With so much attention on the negative health effects of TFA and efforts in place to reduce levels in food products, it is no wonder that *trans* fat content in foods has been shown to have decreased since labeling was mandated. A comparison of levels found in various food categories before and after mandatory labeling was implemented showed that in most categories significant decreases in *trans* fat levels did occur (Table 1). However, *trans* levels in biscuits showed an increase, while no significant change was found for potato chips (Mossoba and others 2010). Albers and others also found a

decrease in *trans* fat content in some foods in a survey of several food categories (2008). Developing countries have also shown trends of decreasing *trans* levels in foods, as most governments in developed nations have taken action through policy or law. However, in developing countries such legislation, as well as general nutrition education, is lacking, making *trans* fat consumption a pressing issue (Butt and Sultan 2009). While efforts are currently in place to decrease the levels of TFA intake worldwide, the labeling mandate has created an increased need for rapid, simple analytical techniques. Such techniques would allow food producers to accurately and quickly assess *trans* fat content of their products in order to comply with government regulations (Mossoba and others 2009).

<u>Food</u>	<u>2004</u>	<u>2006-2007</u>
Cakes		
Mean trans fat levels	n=10	n=11
g/serving (SE) ^a	1.75 (0.74)	0.59(0.26)
g/100g (SE)	2.85 (1.03)	0.98 (0.47) ^b
Biscuits		
Mean trans fat levels	n=5	n=5
g/serving (SE)	1.9 (0.29)	3.3 (0.8)
g/100g (SE)	4.40 (0.25)	5.41 (0.70) ^c
Margarines and spreads		
Mean trans fat levels	n=7	n=9
g/serving (SE)	1.71 (0.15)	0.61 (0.33)
g/100g (SE)	12.24 (1.06)	4.37 (2.36) ^b
Cookies		
Mean trans fat levels	n=12	n=14
g/serving (SE)	1.33 (0.19)	0.5 (0.23)
g/100g (SE)	4.5 (0.62)	1.9 (0.84) ^b
Crackers		
Mean trans fat levels	n=11	n=17
g/serving (SE)	1.41 (0.20)	0.18 (0.12)
g/100g (SE)	5.20 (0.51)	0.71 (0.39) ^b
Potato chips		
Mean trans fat levels	n=8	n=10
g/serving (SE)	0.13 (0.13)	0.0 (0)
g/100g (SE)	0.45 (0.45)	0.0 (0) NS ^a
Tortilla chips		
Mean trans fat levels	n=8	n=9
g/serving (SE)	0.56 (0.18)	0.0 (0)
g/100g (SE)	1.76 (0.6)	0.0 (0) ^b
Frozen potato products		
Mean trans fat levels	n=6	n=7
g/serving (SE)	1.67 (0.4)	0.79 (0.21)
g/100g (SE)	1.97 (0.48)	0.74 (0.24) ^b

Continued

Table 1. Results of studies before and after mandatory *trans* fat labeling showing composition changes in various foods (Mossoba and others 2010)

Table 1 Continued

<u>Food</u>	<u>2004</u>	<u>2006-2007</u>
Cereal and Granola bars		
Mean trans fat levels	n=8	n=9
g/serving (SE)	0.50 (0.19)	0.0 (0)
g/100g (SE)	1.70 (0.8)	0.0 (0) ^b
Tortillas		
Mean trans fat levels	n=6	n=7
g/serving (SE)	0.33 (0.17)	0.14 (0.14)
g/100g (SE)	0.76 (0.39)	0.22 (0.22) ^b
^a NS, not significant; SE, standard error ^b Significant decrease $p \leq 0.05$ ^c Significant increase $p \leq 0.05$		

1.2 Analysis of *trans* fat

There are currently several analytical techniques that are used for the qualitative and quantitative analysis of *trans* fats. Gas chromatography and FTIR are the two most common methods used (Christy and others 2003, Mossoba and others 2007). Other methods which are less widely used include HPLC coupled with UV detection, GC-FTIR, reverse-phase HPLC, silver nitrate chromatography (including TLC, HPLC and SPE), and argentation TLC (Juaneda and others 2007).

1.2.1 Gas chromatographic methods

Gas chromatography is a separation technique used for volatile compounds. Compounds are eluted based on their affinity for the stationary phase present in the column. In *trans*

fat analysis, a 100m fused capillary column coated in 100% cyanopropyl polysiloxane is generally considered necessary to achieve resolution between *cis* and *trans* isomers of the same fatty acid (Mossoba and others 2009a, Delmonte and Rader 2007) and flame ionization detector (FID) is used as a detector (Bansal and others 2009). Retention time of fatty acids depends on chain length, branching, and degree, position and geometry of unsaturation (Delmonte and Rader 2007). Use of GC allows 0.1% accuracy in *trans* quantitation to be achieved (Mossoba and others 2009a, Delmonte and Rader 2007).

Several official GC methods exist. AOCS Official Method Ce 1h-05 was approved in 2005 as the official method for “determination of *cis*- and *trans*-, saturated, monounsaturated and polyunsaturated fatty acids in vegetable or non-ruminant animal oils and fats by capillary GLC (Ratnayake and others 2006). While labeling regulations do not specify the methods that should be used for *trans* fat determination, the official method used by the FDA is AOAC Method 996.06, which is approved for all types of products. AOAC Method 996.06 requires either hydrolysis or saponification of samples followed by methylation with boron trifluoride, followed by separation and analysis on a 100m polar biscyanopropyl siloxane column (Mossoba and others 2009a). As shown in Figure 2, this GC method can adequately resolve even very similar isomers and thus provide a highly accurate picture of both fatty acid profile and *trans* fat content. GC official methods do not specify lower limits of quantitation, however, accurate determination for levels as low as 0.06% has been reported using these two methods (Mossoba and Kramer 2009).

Figure 2. *Cis* and *trans* isomers in butter separated using AOAC method 996.06 (Rozema and others 2008).

While GC is an excellent analytical technique that allows for identification and quantitation of fatty acid composition, it also presents challenges. Since fatty acids found in foods are longer chain and are not volatile, they must be derivitized to volatile fatty acid methyl esters (FAMES) prior to GC analysis (Bansal and others 2009).

Additionally, GC methods call for use of internal standard, use of a specific column, and manual calculations (Mossoba and others 2009a). Additionally, variability and age of columns has been shown to affect elution time for FAMES (Delmonte and Rader 2007).

So, while GC can identify and quantify individual fatty acids to a high level of accuracy, they do have several drawbacks such as slow analysis, use of harsh chemicals, and extensive sample preparation. One proposed alternative is Fourier transform infrared spectroscopy (FTIR).

1.2.2 Infrared spectroscopy

Electromagnetic (EM) radiation is divided into many regions according to frequency and wavelength of the radiation from radio waves to gamma waves. EM waves can be measured using frequency, wavelength or wavenumber. Frequency, ν , is the number of oscillations per unit time, and is usually measured in Hertz, s^{-1} . Wavelength, λ , measures the distance between wave minima or maxima and is expressed in μm or nm . More commonly used in spectroscopy is the unit wavenumber, $\tilde{\nu}$, which measures the number of waves per unit length and is proportional to both frequency and energy. Wavenumber is calculated by dividing the frequency by the speed of light, and is generally in units cm^{-1} (Gunzler and Gremlich 2002).

Each form of radiation corresponds to a type of atomic, molecular or electronic transition (Baeten and Dardenne 2002). As Figure 3 shows, each of these EM regions and transitions corresponds to a different type of spectroscopy. The general principle behind spectroscopic measurement is that the energy ($E=h*\nu$) of the radiation corresponds to the energy change between electronic, vibrational, or rotational states, that is, $E_1-E_2 = h* \nu$ (Gunzler and Gremlich 2002). For infrared, radiation absorbed must cause vibrational excitation and a corresponding change in dipole moment (Ismail and others 1997,

Gunzler and Gremlich 2002). These vibrational excitations have many forms, some of which are shown in Figure 4.

Figure 3. Electromagnetic regions, corresponding vibrations and associated spectroscopic methods (Baeten and Dardenne 2002).

Figure 4. Various modes of molecular vibration. Part (a) shows a C-H stretch, part (b) shows a C-C-H in-plane deformation and part (c) shows a C-C-H out-of-plane deformation (Gunzler and Gremlich 2002).

Infrared (IR) spectroscopy is an analytical technique that measures the interaction of infrared radiation with bonds in a chemical structure. Infrared radiation is divided into three regions: far-IR ($400\text{--}50\text{ cm}^{-1}$), mid-IR ($4000\text{--}400\text{ cm}^{-1}$), and near-IR ($14000\text{--}4000\text{ cm}^{-1}$) (Guillen and Cabo 1997). Mid-IR is useful for structural and qualitative determination, since functional groups have characteristic absorbances at this region (Guillen and Cabo 1997, Perkin Elmer 2005). A portion of the mid-infrared region, between $650\text{--}1500\text{ cm}^{-1}$ is known as the fingerprint region, in which even similar molecules give characteristic absorbances (Gunzler and Gremlich 2002). The fingerprint region is useful for identification rather than individual band assignment (van de Voort and others 2001, Gunzler and Gremlich 2002).

Peaks seen in a spectrum correspond to various vibrational modes of chemical bonds and functional groups (Ismail and others 1997, van de Voort and others 2001). This allows for correlation of certain peaks to functional groups and molecular bonds, as shown in

Figure 5. This can aid in structural determination, substance identification, and component quantification.

Figure 5. Infrared band assignments for a typical lipid spectrum (van de Voort and others 2001).

1.2.3 The FTIR spectrometer

Figure 6. General setup of an infrared spectrometer and design of a Michelson Interferometer (Baeten and Dardenne 2002).

An FTIR spectrometer has three main components: a continuous IR radiation source, a spectral apparatus to resolve each wavelength, and a detector (Figure 6). There is also a laser signal that stays at a constant wavelength for reference (Gunzler and Gremlich 2002). The radiation source is commonly a Globar (resistively heated silicon carbide) for Mid-IR (Griffiths and de Haseth 2007). The spectral apparatus is necessary in order to isolate the radiation to certain spectral regions, and is almost always a Michelson interferometer (Gunzler and Gremlich 2002). The interferometer splits the beam using a KBr/Ge beamsplitter, and the beam is then reflected by both a stationary and moving

mirror. The beams then recombine by either constructively or destructively interfering, and absorption by the sample changes the intensity (Ismail and others 1997, Gunzler and Gremlich 2002, Griffiths and de Haseth 2007). A detector converts optical signal into voltage, which is a measureable electrical signal recorded as an interferogram (Gunzler and Gremlich 2002). The most commonly used detector is a pyroelectric deuterated triglycine sulfate, because it is useable at room temperature. Semiconductor detectors such as Mercury Cadmium Telluride (MCT) are up to 50 times more sensitive than DTGS, but require cooling with liquid nitrogen (Gunzler and Gremlich 2002, Griffiths and de Haseth 2007). Several interferograms are co-added to improve signal-to-noise ratio (Gunzler and Gremlich 2002). The interferogram is cosine Fourier transformed from time to frequency domain to yield an absorption spectrum (Griffiths and de Haseth 2007).

FTIR has been gaining acceptance as a legitimate analytical tool since the 1980s, as instruments became higher quality, smaller, less expensive, and easier to use.

Spectroscopic methods are rapid, non-destructive and require no reagents (Baeten and Dardenne 2002). FTIR is advantageous in that it offers simpler data handling, better signal to noise ratio, quick scan time, good resolution, and cleaner spectra (Ismail and others 1997, Juaneda and others 2007). The emergence of personal computing made operation easier, by allowing for automated computations and data processing (Juaneda and others 2007), while the development of sampling technologies such as ATR, diffuse reflectance, and FTIR microscopy made sample preparation much quicker and easier by shifting from the traditional pellet or Nujol mull to neat samples (Rein 2008). FTIR gives

results which are precise and accurate, and it is accepted by AOCS and AOAC in many official methods (Juaneda and others 2007).

FTIR is useful for three major reasons. First, it is much quicker than separation techniques such as GC, and does not require harmful solvents. Second, it enables one instrument to perform many types of analyses, such as oil quality assessment and *trans* fat determination. Third, chemometrics allows more sophisticated and well-calibrated analysis (van de Voort and others 2001).

The most common historic uses of IR spectroscopy for studying oils have been *trans* analysis and qualitative analysis such as detection of adulteration and studying oil stability (van de Voort and others 2001). Mid-IR is commonly used in the dairy industry for determination of total fat, as well as more minor components and attributes such as iodine value (a measure of unsaturation), *cis* and *trans* isomeric content, saponification number, and peroxide and anisidine values. It also has applications in authentication by using a spectrum as a fingerprint of a product (Baeten and Dardenne 2002). The development of ATR technology allowed quantitative and qualitative methods to be more widely used, as it solved many problems of traditional IR.

1.2.4 Attenuated total reflectance (ATR)

Throughout its history infrared spectroscopy has undergone many changes that have allowed it to evolve into a useful modern analytical method (van de Voort and others 2008). In the last 50 years, attenuated total reflectance (ATR) has been developed to

augment IR spectroscopy (Gunzler and Gremlich 2002). ATR works by providing a fixed pathlength for radiation to travel through the sample. When radiation exits the spectrometer, it enters the ATR crystal, which has a high refractive index and is usually made of zinc selenide (ZnSe), germanium, or diamond (Guillen and Cabo 1997, Mossoba and others 2009a, Juaneda 2007, Perkin Elmer 2005). Diamond and ZnSe have almost identical indices of refraction, so diamond coating can be used on ZnSe crystals to minimize the possibility of damage to the crystal, which can be a problem with other crystal materials (Griffiths and de Haseth 2007).

The radiation passes between materials of different refractive indices (RI). Above a critical angle ($=\text{inv. sin (RI}_2\text{/RI}_1\text{)}$) of incidence, total internal reflection (*i.e.* no refraction) occurs (Mossoba and others 2009a, Juaneda 2007, Griffiths and de Haseth 2007). The radiation bounces either 1 time (single-bounce) or 3 times (triple-bounce) in the crystal. While single bounce crystals have weaker signal than triple bounce crystals, they require a smaller amount of sample and still have acceptable signal-to-noise ratio (Griffiths and de Haseth 2007). The bounce or bounces create an evanescent wave, which travels out of the crystal and penetrates 5-10 micrometers into the sample (Figure 7). This pathlength is uniform, fixed by the amount of total internal reflection, angle of incidence and refractive indices of the crystal and sample. The effective pathlength increases marginally as wavelength increases, which is corrected after spectral collection. The wave decays exponentially and is attenuated by absorption of the sample. Measurement of the attenuation by the detector yields an interferogram which is Fourier transformed,

and the background is subtracted to yield an absorption spectrum (Ismail and others 1997, Mossoba and others 2007).

Figure 7. Three bounce ATR crystal depicting the evanescent wave and depth of sample penetration (Martín-Gil and others 2007).

Because it has a fixed pathlength, a cuvette is not required with ATR, but rather a sample can be placed directly onto the crystal for analysis (Ismail and others 1997). This makes sample preparation much simpler than traditional transmission IR (Mossoba and others 2007), and FTIR-ATR is useful for analysis of liquid, solid, paste and powder analytes (Ismail and others 1997, Sedman and others 1999). Analysis of neat, or undiluted, samples is possible (Ismail and others 1997) and sample thickness is irrelevant, so thick or strongly absorbing samples can be analyzed (Gunzler and Gremlich 2002). This enables ATR users to take advantage of quick sampling and higher reproducibility than

other methods, while minimizing user-to-user variation (Perkin Elmer 2005, Griffiths and de Haseth 2007).

The design of ATR leads to a few inherent concerns. First, the fixed path length does not allow for high sensitivity. Second, since the path length is so short, the sample must be homogenous to ensure that a representative spectrum is collected (Sedman and others 1999). The sample must be in good contact with the ATR crystal because the evanescent wave only travels $\sim 5 \mu\text{m}$ beyond the crystal surface (Perkin Elmer 2005). If the sample area (about 2mm usually) is not entirely filled, stray light can interfere to give incorrect signal (Griffiths and de Haseth 2007). Finally, temperature must be carefully controlled. Change in temperature affects the refractive index of the sample (which changes the effective path length), so maintaining constant temperature during sampling is crucial (Sedman and others 1999).

Since ATR has significantly simplified sample preparation and measurement using FTIR, researchers are now interested in implementing IR techniques in fat and oil quality control in industrial applications. To be transferred from the laboratory to industry, systems would need to be simplified and made more integrated and user-friendly (van de Voort and others 2001). One solution that has been proposed to this challenge is the portable FTIR system.

1.2.5 Handheld FTIR systems

Handheld infrared systems, Figure 8, operate using the same components and principles as benchtop FTIR spectrometers, but are a unique tool in chemical analysis. Handheld systems can be employed when a sample cannot be brought to the laboratory for size or other logistical reasons where sampling is not an option (Rein 2008).

Figure 8. Example of a handheld portable infrared spectrometer (Thermo Fisher Scientific, Inc. 2011).

The first portable FTIR systems were developed in 2000. These systems were originally employed by the government for the identification of illegal drugs, weapons materials, and hazardous substances. Results were promising, and development of FTIR technology continued with the introduction of handheld systems. These systems have

been implemented for identification of surface metals, polymers, contaminants, coatings, and components in chemical manufacturing (Rein 2008).

To be useful, a handheld system must operate well at varying temperature and humidity, at any orientation, and be stable to vibration. The application will determine what is prioritized in the model, smaller size or better performance. Systems can use either an external reflectance probe for hard, reflective surfaces or a diamond internal reflectance probe for soft, nonreflective samples. Handheld systems must be compatible with creation of varying methods by scientists, as well as ease of operation by non-scientists (Rein 2008).

Handheld spectroscopy also shows promise as a tool to be implemented in the food and agriculture industry. Research has been published showing that this technology is viable for use in several aspects of the fruit industry (Perez-Marin and others 2009), and further investigation into applications is being conducted by the Rodriguez-Saona research group.

1.2.6 Statistical analysis

While IR spectroscopy has been used since the 1940s, improvements in technology, sample handling, and data analysis have transformed it. Collection of more information is now possible and has created a need for sophisticated data handling techniques such as chemometrics. Chemometrics encompasses partial least squares regression (PLSR), principal component analysis (PCA), soft independent modeling of class analogy

(SIMCA), as well as many others. Since spectra represent the overall vibrational response of a compound to IR radiation, it can be difficult or impossible to directly correlate a specific component with a particular wavenumber. Chemometrics allows the user to examine variations in the overall system, rather than one particular wavenumber, as concentration is varied (Christy and others 2003). Chemometric analysis methods, including partial least squares regression (PLSR) are well-suited to use with FTIR because they can accurately analyze data that is strongly collinear, noisy, and has numerous x-variables (Wold and others 2001).

Chemometric methods such as PLSR don't use specific peak heights; instead they use what are known as loading spectra, or factors, determined from the training set (Ismail and others 1997, Griffiths and de Haseth 2007). Each calibration standard is analyzed as a weighted collection of factors, and each weight is regressed against the concentration to build a standard curve. The analyst can use the entire spectrum, although more heavily weighting important regions allows better precision. Chemometrics also allows for the presence of interference and can compensate as long as it is present in both standards and samples. Models should be cross-validated, and a leave one out approach helps avoid overfitting (Ismail and others 1997).

Chemometrics can be used for two purposes: to model known characteristic of data and to predict unknown aspects. Analysis can be either quantitative or qualitative.

Chemometric methods predict unknowns by the same method as they create models –

reconstructing the spectrum as weighted loading factors and relating these scores to the aspect of the system to be predicted (Ismail and others 1997).

When using FTIR, it is important to recognize that not only do multiple components interact, but the matrix also affects the spectrum. It is therefore necessary when using chemometrics for calibration standards to match as closely as possible the system to be analyzed in order to achieve best accuracy (Christy and others 2003). In making a PLSR calibration model, design of the training set is crucial, as spectra are matrix-dependant. It is important to employ some type of discriminant analysis in order to identify if outliers do not fit in the training population. It is therefore essential for the calibration set to include a variety of representative samples that encompass a broad range of the characteristic of interest (van de Voort and others 2001).

Chemometrics has enabled use of Raman, IR and NIR spectroscopy for determining oil quality parameters such as iodine value, peroxide value, free fatty acid value, and anisidine value (van de Voort and others 2001). However, researchers have also investigated to uses of chemometrics coupled with FTIR spectroscopy for detecting *trans* fat in oils.

1.3 FTIR and *trans* fat

1.3.1 Trans fat analysis using FTIR

FTIR-ATR has successfully been applied to the analysis of fats for many years, however *trans* fat analysis is the only officially recognized application of mid-IR spectroscopy to

fats or oils (American Oil Chemists' Society 2010). *Trans* fat is an ideal candidate for IR analysis because it presents a very clear, somewhat isolated spectral peak. As shown in Figure 5, the peak at 966cm^{-1} corresponds to the C-H out of plane deformation (Figure 3), which is unique to isolated *trans* double bonds regardless of chain length or double bond position (Mossoba and others 1996, van de Voort and others 2001). Thus, measurement is possible based on the CH out-of-plane deformation vibration present at 966cm^{-1} , which effectively sums each of the fatty acid containing isolated *trans* double bonds (van de Voort and others 2001, Juaneda 2007, Mossoba and others 2009a). FTIR is a novel alternative to GC for *trans* analysis because it skips the separation and analysis of individual fatty acids, in favor of measuring absorbance of this bond (Delmonte and Rader 2007, Mossoba and others 2009).

Trans fat in edible oils and fats has been analyzed by IR spectroscopy as far back as the 1950s (Mossoba and others 2009a, van de Voort and others 2001). IR was later used to measure changes in the height of the 966cm^{-1} peak during catalytic hydrogenation reactions (Dutton 1974), which showed that such continuous measurement of unfiltered oil was possible (Sedman and others 1999).

FTIR was not employed in analytical testing of *trans* fats until the early 2000s, when advances in instrumentation, sampling technology, and computing made it a viable alternative to traditional GC analysis. Investigation of new methodologies lead to a rapid progression. With all methods, quantitation was carried out using a standard calibration curve method (Mossoba and others 2009, Ismail and others 1997, Mossoba and Kramer

2009). Initially, *trans*-trielaiddin (TE, 18:1 *trans*-9) spiked into triolein (TO, 18:1 *cis*-9) was used, but TO was found to contain 0.5% *trans*, so other triglycerides were evaluated and tripalmitin (TP, 16:0) was found to be a suitable alternative (Mossoba and others 2007, Mossoba and others 2009, Mossoba and others 2009a). Since standard curves are created using TE as the *trans* fat, measurements are expressed in FTIR as % *trans* as trielaiddin (Juaneda and others 2007). This is reasonable since trielaiddin, or simply elaidic acid, is the most prevalent fatty acid in hydrogenated oils (Sherazi and others 2009).

A method for analysis using transmission IR spectroscopy was investigated, and led to the creation of two official methods: AOCS Cd 14-95 and AOAC 965.34. In these methods, samples are first converted to FAMES, dissolved in carbon disulfide (CS₂), measured in a KBr or NaCl cell, and quantitated using the height of the peak at 966cm⁻¹ (Ismail and others 1997, Mossoba and Kramer 2009). This method has many drawbacks: the 966 cm⁻¹ band is broad and has a sloping baseline due to both triglyceride and conjugated linoleic acid absorption it requires sample preparation with hazardous solvents and a methylation step converting fatty acids to FAMES, and results vary depending if the TFAs are present as glycerides or methyl derivatives (Christy and others 2003). Additionally, interference because of overlapping peaks cause issues at low levels, and the method calls for saponification and methylation of samples prior to analysis at *trans* levels below 15% (Ismail and others 1997).

To correct these deficiencies, ATR sampling was applied to the problem of *trans* fat measurement. ATR methods call for a heated crystal (65° C) in order to keep fats melted

during analysis (Mossoba and others 2009a). Official Methods AOCS Cd 14d-99 and AOAC 2000.10 were created measuring band area rather than height (Mossoba and Kramer 2009). These methods require a reference fat that is *trans* free and has a similar fatty acid profile to the sample to be analyzed rather than CS₂ solvent (Mossoba and others 2009a). This method eliminated the sloping baseline of the transmission IR method, which eliminated the need to convert fatty acids to FAMES prior to analysis (Mossoba and others 1996, Mossoba and Kramer 2009, Juaneda 2007). Instead, the spectrum is simply a symmetrical *trans* band on a flat, horizontal baseline since the background absorbance has been subtracted (Mossoba and others 2009, Mossoba and Kramer 2009, Juaneda 2007). However, this introduced the difficulty of finding an appropriate reference oil (Bansal and others 2009). This method was problematic for samples containing less than 5% *trans* fat, which was a severe limitation of the method (Mossoba and others 2009, Mossoba and others 2009a).

A method was needed that both required no reference oil and corrected the sloping baseline. In 2004, Milosevic and others proposed a novel method using air as the background material and taking the negative second derivative of the spectra, improving the detection of *trans* levels and also the presence of interference (Bansal and others 2009). This method proposed measuring peak height of the spectral band at 966cm⁻¹. Second derivative has been implemented in IR spectroscopy because it can remove the sloping baseline that had hindered *trans* identification, allow for more bands to be identified than in a raw spectrum and help minimize day-to-day baseline variation (Christy and others 2003). Figure 9 shows a portion of IR spectra of *trans* fat containing

samples which have been doubly derived. This illustrates that the band at 966 cm^{-1} is isolated from surrounding interferences, on a horizontal baseline, and easily measurable. This isolation is due to a narrow bandwidth, and eliminates interferences from saturated fat and conjugation (Mossoba and others 2007, Mossoba and others 2009).

Figure 9. Portion of second derivative spectra of samples containing various levels of TFA (Mossoba and others 2009).

Using the second derivative to measure *trans* fat has since undergone several iterations in an attempt to simplify and improve the method and has resulted in Official Method AOCS 14e-09 (Mossoba and others 2011). This method measures peak height and has a limit of detection of 0.5% (Mossoba and others 2009). Below 0.5%, the TE band appears as a shoulder on the edge of the adjacent TP band, which prevents accurate measurement

of the height of the *trans* band at 966 cm^{-1} (Mossoba and others 2009). Another concern with this method is that in saturated fats with very low concentrations of TFAs, it is difficult not to misinterpret the saturated fat band just below 966cm^{-1} as the *trans* band, since this would lead to erroneous values (Mossoba and others 2009a).

The second derivative method has many advantages over previous methods. It increases resolution of IR bands by decreasing bandwidth, which allows for identification of interferences and band shifts (Mossoba and others 2007, Mossoba and others 2009). Taking the second derivative solves the problems associated with the baseline offset and slope, and the height of the second derivative is directly proportional to the amount of total *trans* fat in a sample. It completely eliminates the need for any *trans*-free reference oil (Mossoba and others 2009). Earlier research showed discrepancies between GC and IR results for low *trans* levels (Mossoba and others 2007), however with the implementation of the second derivative procedure, good agreement was found between FTIR-ATR and GC determinations (Mossoba and others 2009). Indeed, a comparative study showed that the second derivative method gave results that were less statistically different from GC results than AOCS Official Method Cd 14d-99 (Bansal and others 2009).

1.3.2 Spectral interferences

One of the main difficulties in using FTIR for determination of *trans* fat is potential interferences from surrounding spectral bands. Older FTIR methods have strongly sloping baselines due to overlaps with other spectral bands (Adam and others 2000,

Mossoba and others 2009a, Juaneda 2007), which makes accuracy at low levels difficult and has necessitated development of second derivative methods (Mossoba and others 2007). Use of underived FTIR determination was limited to products with <5% conjugated unsaturation, because of band overlap of *trans/trans* or *cis/trans* bonds. Another potential interference is due to OH out of plane deformation of carboxylic acids, which can interfere with *trans* <15% (Mossoba and others 1996).

The presence of 1% conjugated fatty acids has been reported to interfere with low levels of *trans* fat (Mossoba and others 2007, Mossoba and others 2009a). However, the narrow bandwidth of the second derivative bands once again allowed the spectral resolution of the 966 cm⁻¹ band from weak diene absorption bands at 990 cm⁻¹ (*trans/trans*) and the doublet at 985 and 945 cm⁻¹ (*cis/trans*) due to conjugated fatty acids (Mossoba and others 2009). High levels of saturated fat also cause interference with the *trans* band at *trans* levels <1% (Mossoba and others 2007, Mossoba and others 2009a). Saturated samples have maxima at lower frequencies, roughly 962–956 cm⁻¹. This signal could be erroneously attributed to isolated *trans* fatty acids. However, the narrower bandwidth of the second derivative method allows for discrimination between adjacent bands (Mossoba and others 2009).

Although many issues have been resolved, there are still concerns with FTIR methodologies. In the most recent second derivative method, there was a limit of detection of 0.5%. This is because below 0.5%, the *trans* band appears as a shoulder on the edge of the triglyceride band, which prevented the measurement of the height of the

trans trielaidin band at 966 cm^{-1} (Mossoba and others 2009). Additionally, FTIR spectroscopy measures monoenes and does not compensate for spectral contributions from dienes and trienes, and *trans* levels will be inaccurate if samples contain significant amounts of them. However, most partially hydrogenated oils do not contain enough to matter (Mossoba and others 2009a).

Research is currently investigating methods to correct for these interferences, in an attempt to continue refining and improving IR detection of *trans* fats. In preliminary experiments, 2-dimensional correlation spectroscopy has shown potential application in correcting models for underlying absorbance, which could allow for higher precision in models (Van de Voort and others 2008). Chemometric PLSR modeling is also capable of correcting for the underlying absorbances that can confound measurement of the 966cm^{-1} peak height, by incorporating it into calibration models.

1.3.3 Advantages of FTIR

As with any comparison of analytical techniques, each has distinct benefits and drawbacks. It is generally acknowledged that GC is still a more accurate method at low *trans* levels (Mossoba and others 1996), and is considered the standard technique in industry since it can also elucidate fatty acid composition. However, GC methods are time consuming, and require conversion of fat test samples into volatile fatty acid methyl esters before separation using 100 m capillary columns (Mossoba and others 2009).

However, FTIR-ATR is “the tool of choice for the rapid determination of total isolated *trans* double bonds in oils and fats” (Mossoba and others 2007, Bansal and others 2009). One collaborative study found that ATR measurements of *trans* fat were consistently accurate even among analysts who had no experience using this method (Adam and others 2000), and Mossoba and others conclude that FTIR-ATR coupled with second derivative analysis is better suited to industrial application, where less accuracy is needed and individual fatty acid analysis is not necessary (Mossoba and others 2009a). The ATR technique is advantageous because it has a precise effective pathlength, allows for the measurement of a single microliter of oil, and requires no sample preparation. FTIR-ATR measurement is also rapid, with measurement time of about 5 minutes per sample.

1.3.4 PLSR and FTIR-ATR

Chemometrics, especially PLSR, has been utilized in several studies in combination with fat analysis, although some analysts consider it impractical for routine measurements (Van de Voort and others 2008). Van de Voort and others used PLSR to determine iodine value as well as *cis* and *trans* isomer content (1995). Authors determined that an FTIR-ATR system coupled with chemometrics could be of high value to online lipid analysis, and found that it was accurate to a limit of 1% *trans* fat. Christy and others also combined FTIR-ATR analysis with PLSR, and reported that at levels less than 0.3% the *trans* peak was not clear, although upon taking the second derivative it became clear at lower levels. They found an error limit of 0.1%, and an error of prediction of 0.9%, and concluded that using multivariate modeling is better than measuring peak height at 966

cm⁻¹ (Christy and others 2003). Azizian and Kramer used Near-IR to quantify *trans* fats using chemometrics and three separate models for low, medium and high levels of *trans*, which allowed for standard error of prediction as low as 0.2% (2005). Kim and others (2007) used FTIR-ATR in combination with PLSR for screening *trans* content of ground cereal products, giving SECV values around 1.1% and SEP values around 1%. Some studies have found that chemometric analysis coupled with FTIR can determine not only *trans* fat, but also can facilitate rapid determination of fatty acid composition (Mossoba and others 2007, Azizian and Kramer 2005).

In conclusion, FTIR-ATR is a well-established and accurate method for measurement of *trans* fats, and PLSR chemometrics can be used to minimize error from interferences. The combination of handheld FTIR-ATR with PLSR for measurement of *trans* fat is promising for *in situ* and in-process measurement of *trans* fat.

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Chapter 2: Application of a Portable Handheld Infrared Spectrometer for Quantitation of *trans*
Fat in Edible Oils

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2.1 Abstract

Trans fat poses serious health risks to consumers. In order to meet the FDA labeling requirements for *trans* fatty acids, development of fast, accurate, easy-to-use analytical methods for oils, fats and related products is desirable. Fourier transform infrared spectroscopy (FTIR) is a well-established analytical technique for quantifying *trans* fats, and the development of handheld FTIR units over the past decade presents new application opportunities. Our objective was to evaluate the performance of a handheld FTIR sensor for measuring *trans* fat content in edible saturated and unsaturated oils. Calibration models were built by measuring height of the second derivative band at 966 cm^{-1} and by partial least squares regression (PLSR) using benchtop FTIR as a reference method. Predictive accuracy of the models was validated with an independent test set of commercial edible oils. Calibration models developed using PLSR and linear regression of band heights gave correlation coefficients $R^2 > 0.98$. Multivariate analysis for the handheld unit gave standard error of prediction (SEP) of approximately 1%, comparable to values obtained with benchtop systems. For the handheld spectrometer, chemometrics was more accurate than the second derivative method. This study demonstrates that handheld FTIR spectroscopy coupled with chemometrics is a suitable method for quantitation of *trans* fat content.

2.2 Introduction

Dietary *trans* fat has been shown to have adverse effects on blood lipoprotein profiles and coronary heart disease risk impacting individuals and populations. It is recommended by

the World Health Organization (WHO) that mean intake of *trans* fat should be less than 1% of daily energy intake and worldwide efforts from consumers and regulators are in effect to reduce TFA intake (Uauy and others 2009). Since 2006 the FDA has required *trans* content greater than 0.5 g/serving to be included on nutrition labeling (U.S. Food and Drug Administration 2003).

Gas chromatographic and infrared spectroscopic methods for determining *trans* fat levels to meet FDA regulatory standards were recently reviewed by Mossoba and others (Mossoba and others 2009). Established IR methods such as AOCS Cd 14d-99 and AOAC 2000.10 use band area at 966 cm^{-1} to measure *trans* fatty acid content, although these methods have limits of quantitation around 5% *trans*. This limitation has been overcome by a new methodology which measures the height of the negative second derivative of the *trans* absorbance band (Mossoba and others 2007) which is sensitive below 2% *trans*.

Another current approach to quantitation of components in complex food matrices is chemometrics. Chemometrics offers a more robust method of spectral analysis which takes into account multiple spectral bands. FTIR-attenuated total reflectance (ATR) combined with multivariate analysis has been successfully implemented as a reliable, quick, and simple technique for analyzing food products, edible oils and lipids (Reid and others 2006, van de Voort and others 2001). Chemometric analysis methods, including partial least squares regression (PLSR) are well-suited to use with FTIR because they can accurately analyze data that is strongly collinear, noisy, and has numerous x-variables

(Wold and others 2001). PLSR has been used in combination with FTIR-ATR in one study to quantify *trans* fat and conjugated linoleic acid in fats and oils (Christy and others 2003).

Development of handheld portable infrared units has allowed for high quality in-field and on-site analysis of quality parameters of plums (Perez-Marin and others 2010). Handheld FTIR-ATR technology has also been applied to threat screening, anti-counterfeiting and toxin identification, and application in the food industry is under current investigation (Rein 2008). The compact nature of this technology could enable in-process or on-site measurements, allowing food producers to obtain real-time, *in situ* information, rather than performing post-production quality control tests.

Our objective was to evaluate the performance of a handheld FTIR-ATR sensor coupled with second derivative linear regression and chemometric analysis in measuring *trans* fat content of edible fats and oils.

2.3 Materials and methods

2.3.1 Sample preparation

Pure trielaidin (TE) was weighed gravimetrically into tripalmitin (TP) and triolein (TO) (>99%, Nu-Chek Prep, Elysian, MN) to create calibration standards of 0.1-20% *trans* (w/w). Validation samples were prepared in the same manner by weighing TE into edible oils (peanut, safflower, corn, or coconut) obtained from local grocery stores.

2.3.2 Infrared spectroscopy

Samples were heated in an oven to 65° C prior to measurement. Spectra of all standards and test samples were measured in duplicate with the TruDefenderTM FT infrared handheld spectrometer (Ahura Scientific, Wilmington, MA) equipped with a diamond ATR crystal. The crystal was heated by heat gun to 65±5°C to prevent sample solidification during measurement. Spectra were collected by co-adding 4 scans at a resolution of 4cm⁻¹. Resolution was a fixed parameter on this instrument. Reference spectra were also collected using an FTS 3500GX Fourier-transform infrared spectrometer (Varian, Palo Alto, CA) in combination with a KBr beamsplitter and deuterated triglycine sulphate (DTGS) detector. Samples were pipetted directly onto a Pike MIRacleTM triple-bounce ZnSe ATR crystal (Pike Technologies, Madison, WI) that had been heated with a heat gun to 65±5°C to prevent fat solidification and a FatIRTM temperature-controlled single bounce ZnSe crystal (Harrick Scientific, Pleasantville, NY) set to 65±1°C. Spectra were collected over a range of 4000-700 cm⁻¹ at 4 cm⁻¹ resolution, and an interferogram of 64 scans was co-added.

2.3.3 Data analysis

Second Derivative Linear Regression: Spectra were analyzed in Resolutions Pro software (Varian Inc., Palo Alto, CA). Regression models were constructed using trielaidin in tripalmitin standards due to higher purity than triolein (Mossoba and others 2009). The second derivative of the spectrum was taken and height of the band at 966 cm⁻¹, associated with the *trans* CH out-of-plane deformation vibration, was measured

(Milosevic and others 2004). Taking the second derivative of the spectrum allows for resolution of overlapping bands and extraction of useful spectral information (Hruschka 1987, Geise and French 1955). A standard curve relating band height to percent *trans* content was constructed.

Partial Least Squares Regression (PLSR): Spectra were imported into Pirouette software (Infometrix, Bothell, WA). The region of 930-1400 cm^{-1} was analyzed by PLSR by mean-centering, normalizing, and taking the second derivative of each spectrum. Models were constructed using both tripalmitin and triolein standards in order to account for spectral differences of saturated and unsaturated matrices. Models were cross-validated using a leave one out approach. Key bands were identified and standard error of cross-validation (SECV) was calculated in order to evaluate model fitness.

The predictive accuracy of models was validated with an independent test set of oils spiked with known amounts of trielaidin. Oils had been evaluated for *trans* fat content prior to spiking. Spectra were collected in duplicate with the temperature-controlled ATR unit, and levels were calculated using the second derivative methodology and previously established calibration models. Predictive ability of second derivative and PLSR models was evaluated by calculating mean and standard deviation for the predictions of the test sets. Standard error of prediction (SEP) was also calculated for the PLSR models.

2.4 Results and discussion

Determination of *trans* fat level in oils, fats, and food products by infrared spectroscopy has been extensively investigated (Mossoba and others 2009, Milosevic and others 2004). In this study, we evaluated the feasibility of using a portable handheld FTIR-ATR unit to quantify *trans* levels with no sample preparation except heating to 65°C. The handheld spectrometer collected a spectrum that was highly similar to benchtop instruments, although it showed higher noise levels (Figure 10). Noise was due to co-adding of fewer scans per sample due to the need to maintain a constant sample temperature throughout measurement and to keep a uniform effective path length (Sedman and others 1999).

Figure 10. Comparison of spectral quality and shape of handheld and benchtop spectrometers.

The spectral profiles collected (Figure 10) show characteristic bands of lipids. The region from 900-1200 cm^{-1} is considered to be the “fingerprint region” and is rich with spectral information. It contains the band at 966 cm^{-1} that is associated with CH out-of-plane deformation vibration of *trans* double bonds (Guillen and Cabo 1998) and the region 1050-1200 cm^{-1} , associated with several C-O stretching vibrations (Koca and others 2010).

Overlaying doubly derived sample spectra at varying *trans* levels from the calibration set (Figure 11) shows that the intensity of the band at 966 cm^{-1} in the second derivative spectra varies with *trans* content. A comparison of all three figures reveals that the triple bounce and temperature-controlled accessories show equally good separation of bands for different *trans* levels. All three systems have bands which are indistinguishable between 0-0.5%. Figure 11 begins to suggest that the limit of quantification is around 1%, since separation of bands cannot be easily distinguished for levels below 1% *trans*. This is most likely due to interferences from surrounding bands such as saturated fat (Mossoba and others 2009), which are most evident at low *trans* concentrations.

Figure 11. Overlaid spectra after mean-centering, normalization and second derivative pre-processing shows the change in absorbance at 966 cm^{-1} with change in *trans* fat level.

Figure 12. Second derivative *trans* band for 4.1% TE in TP collected using (a) temperature controlled ATR accessory, (b) triple bounce ATR and (c) handheld spectrometer.

Figure 12 shows that when the second derivative bands at 966 cm^{-1} are overlaid, the handheld spectrum has a wider band than the benchtop instruments. This again shows that the handheld cannot achieve as high a level of band separation and may include noise in the *trans* band, making accurate measurement more challenging.

2.4.1 Models

Second derivative linear regression models showed excellent linear correlation between 1-20% *trans* content in tripalmitin standards and band height at 966 cm^{-1} (Figure 13). Infrared calibration models developed using linear regression of band height gave $R^2 = 0.995$ for benchtop triple bounce and temperature-controlled systems. The triple bounce accessory regressions had much higher slope due to additional internal reflections in the crystal, which yields a stronger signal. Signal is improved by an increased effective path length, which is caused by a higher number of reflections within the ATR crystal (Pike Technologies 2010). Handheld correlation coefficient was $R^2 = 0.992$, indicating a strong linear relationship between band height and *trans* fat content.

Figure 13. Comparison of linear regression calibration models for tripalmitin spiked with trielaidin.

PLSR calibration models are shown in Figure 14. Models were constructed using only standards between 0-5%, in order to focus on model sensitivity at lower *trans* levels. It was necessary to build PLSR models with both tripalmitin and triolein calibration standards in order to obtain accurate predictions. A PLSR model was originally constructed using only tripalmitin standards due to concerns over the purity of triolein (Milosevic and others 2004). While the model showed good linearity (SECV ~0.5) and could accurately predict saturated validation samples (SEP ~1%), error was unacceptably

high for the unsaturated validation set (SEP ~5%). Spectral information regarding differences between saturated and unsaturated fats needed to be built into the models in order to accurately predict both types of samples (Christy and other 2003). Models were then created using both tripalmitin and triolein standards. Although this introduced some error based on the inherent *trans* fat content on triolein, it was necessary for construction of models robust enough to predict the validation oils. Model improvements are reflected in the lower SEP values presented in Table 4.

It can be clearly seen that the benchtop systems yielded the best calibration and that the handheld spectrometer showed a lower degree of linearity. PLSR multivariate analysis gave standard error of cross-validation (SECV) of less than or equal to 1% for the handheld and benchtop systems (Table 2). R of validation (R_{val}) was >0.96 for all models, indicating good model linearity. Loadings (Figure 15) indicated which spectral bands were important in model construction and prediction. The *trans* marker band at 966cm^{-1} explained ~80% of the variance in the models, although other spectral factors were used to increase model robustness and performance. These additional bands were distinct for saturated and unsaturated fat. Bands at 1084 cm^{-1} (unsaturated), 1144 cm^{-1} and 1182 cm^{-1} (saturated), related to C-O stretching were the most important loadings (Guillen and Cabo 1998).

Figure 14. PLSR calibration models for trielaidin in saturated tripalmitin using (a) triple bounce ATR, (b) temperature controlled ATR accessory and (c) handheld spectrometer.

	Temperature-controlled	Triple Bounce	Handheld
Factors	3	4	4
SECV ^a	0.19	0.22	0.54
Rval ^b	0.992	0.991	0.968
^a Standard error of cross-validation ^b R of validation			

Table 2. Comparison of PLSR model performance evaluation statistics for calibration curves.

Figure 15. Loadings of the first two factors for PLSR calibration model using the handheld spectrometer.

2.4.2 Initial oil study

Prior to spiking validation samples with trielaidin, potential oils to be used were analyzed for *trans* content. *Trans* fat can be present at levels up to 3% in commercially available oils that are not partially hydrogenated due to isomerization caused by high temperatures of the industrial deodorization process (Mossoba and others 2010). The *trans* fat content of oils used in validation samples was evaluated using the temperature-controlled system and the second derivative procedure prior to spiking with trielaidin.

Low levels of *trans* fat were found in all oils evaluated (Table 3). Levels were highest in sesame and vegetable oil. Oils containing less than 0.5% *trans* were chosen for spiked validation samples: coconut for a saturated oil and corn, safflower, and peanut for unsaturated oils. The values given for the *trans* fat levels in validation samples are the

sum of values from both the oil, determined using FTIR, and the trielaidin, determined gravimetrically.

Oil Type	Average <i>trans</i> content (% w/w)
Coconut	0.50
Sesame	1.18
Corn	0.27
Safflower	0.27
Vegetable	0.73
Peanut	0.27

Table 3. *Trans* fat content of oils to be used in validation study.

2.4.3 Predictions

Predictions were made using the second derivative and PLSR methods on an independent test set of unsaturated and saturated oils spiked with trielaidin measured with all three FTIR-ATR systems. Although calibration models were made from spiked triglycerides, models were validated using commercial oils spiked with TE. Since infrared spectra are highly matrix-dependent, it is expected that predictions will lose some accuracy due to spectral differences caused by switching matrices for validation (Christy and others 2003). Thus, models were validated on varying matrices (peanut, safflower, corn, or coconut oils) in order to account for the matrices' effects on model performance. Validation samples were constrained to *trans* content less than 5% in order focus on model sensitivity at lower levels.

Standard error of prediction (SEP) is an estimate of the predictive ability of a chemometric model. SEP was calculated for each prediction set using PLSR prediction analysis (Table 4). The benchtop systems showed lower SEP than the handheld system, which is expected due to differences in system components and performance. SEP was ~0.6% for the handheld spectrometer, therefore the limit of quantification for this technology was estimated at ~1% *trans* fat by weight. In a high fat product, 1% *trans* (w/w) is equal to 0.25g per serving (Mossoba and others 2009). It is hypothesized that this limit is due to interference from saturated fat, which absorbs in a similar region as *trans* fat (Milosevic and others 2004).

	Temperature-controlled	Triple Bounce	Handheld
SEP ^a	0.56	0.28	0.62
^a Standard Error of Prediction calculated from predictions with an independent test set			

Table 4. Comparison of standard errors of prediction for PLSR models.

Using band height measurement, the handheld unit showed poor predictive ability (Table 5). Predictions were inaccurate by as much as 6% (w/w), indicating spectral differences between triglyceride calibration standards and commercial oil validation samples were too great and spectra were too noisy. However, these results confirmed what has been shown in numerous studies, that benchtop systems were able to accurately predict validation samples (Mossoba and others 2009, Milosevic and others 2004).

Using PLSR analysis, the handheld performed on par with the benchtop systems for oil predictions (Table 5). Samples with *trans* content >1% were accurately predicted by the handheld system to within 0.3% of gravimetrically determined values, although lower level sample predictions were less accurate. Samples less than 1% *trans* (0.62, 0.68, 0.52 and 0.75%) were not accurately predicted, but samples above 1% could be accurately predicted by the handheld spectrometer combined with PLSR analysis. It should be noted that predictions made using both benchtop systems had a lower standard deviation than the handheld spectrometer. This indicates that while the handheld was able to predict *trans* levels to a suitable level, it lacks some of the precision and consistency of the benchtop systems.

PLSR Predicted Values								Second Derivative Predicted Values							
Actual		Handheld (SD)		Temperature – controlled (SD)		Triple Bounce (SD)		Actual		Handheld (SD)		Temperature –controlled (SD)		Triple Bounce (SD)	
Unsaturated								Unsaturated							
1	0.82	0.25	(0.39)	0.86	(0.00)	0.65	(0.01)	1	0.82	3.13	(0.31)	0.73	(0.00)	0.82	(0.00)
2	0.88	0.04	(0.02)	0.94	(0.03)	0.78	(0.08)	2	0.88	2.48	(0.00)	0.73	(0.00)	0.95	(0.19)
3	1.30	0.73	(0.74)	1.70	(0.00)	0.90	(0.05)	3	1.30	4.87	(1.54)	0.95	(0.32)	1.21	(0.19)
4	1.59	1.00	(0.31)	1.89	(0.03)	1.57	(0.17)	4	1.59	6.39	(1.84)	1.18	(0.00)	1.61	(0.00)
5	2.05	1.74	(0.37)	2.12	(0.06)	1.37	(0.01)	5	2.05	5.96	(0.61)	1.64	(0.00)	1.87	(0.00)
6	2.12	1.65	(0.03)	1.58	(0.08)	2.31	(0.06)	6	2.12	6.17	(2.15)	1.18	(0.00)	2.00	(0.19)
7	4.38	3.81	(0.28)	4.01	(0.00)	4.16	(0.03)	7	4.38	9.87	(2.46)	3.00	(0.00)	4.11	(0.19)
Saturated								Saturated							
8	1.02	1.27	(0.44)	1.33	(0.01)	1.03	(0.04)	8	1.02	1.83	(0.31)	0.73	(0.00)	0.68	(0.19)
9	1.25	1.38	(0.02)	1.17	(0.02)	1.03	(0.08)	9	1.25	2.70	(0.92)	0.95	(0.32)	0.68	(0.19)
10	1.48	0.94	(0.01)	1.45	(0.03)	1.38	(0.06)	10	1.48	2.70	(0.31)	0.73	(0.00)	1.08	(0.00)
11	2.18	1.58	(0.06)	2.27	(0.05)	1.88	(0.01)	11	2.18	2.48	(1.84)	1.41	(0.32)	1.61	(0.00)
12	2.79	2.35	(0.45)	2.82	(0.00)	2.58	(0.01)	12	2.79	5.52	(0.61)	1.64	(0.00)	2.26	(0.19)
13	4.20	2.68	(0.28)	3.68	(0.00)	3.62	(0.01)	13	4.20	5.09	(0.61)	2.55	(0.00)	3.32	(0.19)
SD = Standard Deviation															

SD = Standard Deviation

Table 5. Validation set sample predictions using peak height and linear models.

Overall, the handheld spectrometer coupled with the second derivative method greatly over-predicted the level of *trans* fat in this study. PLSR analysis, however, generally gave *trans* values closer to the actual amount present in the sample. Handheld FTIR-ATR has been demonstrated in this study to be useful for the quantitation of *trans* fat at levels >1% with an SEP of 0.6%.

A variety of spectroscopic methods have been used in combination with chemometric analysis for determining *trans* fat with varying levels of accuracy (Table 6). FTIR has been extensively studied, and a limit of quantitation for the band height measurement

technique has been set at 1% (Mossoba and others 2009). FTIR coupled with PLSR analysis gave an SEP of 0.9% (Christy and others 2003). Other forms of spectroscopy have also been investigated for application to *trans* quantitation. A study using Raman spectroscopy coupled with PCA (Johnson and others 2002) to investigate partially hydrogenated oils could predict levels with an SEP ~2%, while SEP ~0.2% was found using NIR coupled with PLSR (Azizian and Kramer 2005) on fats and oils. Each method provides unique information and detection method selected depends on available resources and analytical needs. Raman, NIR and handheld spectroscopy need to be validated via collaborative study to assess repeatability, reproducibility and established limits of quantitation.

Instrument	LoQ ^a or SEP ^b
FTIR-ATR	1% ^a ,
FTIR-ATR with Chemometrics	0.9% ^b
NIR	0.2% ^b
Raman	2.1% ^b
Handheld FTIR-ATR	0.6% ^b
^a Limit of Quantitation	
^b Standard Error of Prediction	

Table 6. Error estimation of spectroscopic studies on *trans* fat determination.

Combining the portable handheld FTIR spectrometer with PLSR analysis offers three distinct benefits for *trans* fat analysis. First, a handheld unit is portable, which allows for flexible sampling. Spectra can be collected when samples are available and analyzed at a later time. Second, PLSR analysis is advantageous because it can simultaneously use

several spectral bands to quantify the component. Multiple loadings are useful in creating robust models that give reproducible results. While increasing the number of loadings offers greater model calibration, it is important to evaluate the loadings so as not to include noise into calibration models (Haaland and Thomas 1988). PLSR also allows for quantitation of components in a more complex matrix and can be useful when a component is not correlated with a specific wavenumber. Third, as this study has demonstrated, analysis can be semi matrix-independent. This aids in ease of analysis, as a new calibration model would not necessarily need to be created for each new oil matrix. Overall, the combination of portable FTIR and PLSR offers greater flexibility and ease of analysis in measuring *trans* fat.

The implementation of handheld FTIR technology has both benefits and tradeoffs. FTIR-ATR technology allows for minimal sample preparation, measurements are very quick, and the portable nature of handheld devices would allow use *in situ*, in receiving and processing of edible oils. However, the compact and portable nature of the handheld spectrometer necessitates some compromises and limitations in signal strength, spectral quality, and interface features. Modifications such as the addition of heat control and a triple bounce crystal could improve the performance and applicability of the handheld portable unit. So while the handheld FTIR unit was found suitable for quantifying levels >1% of *trans* fat in edible oils, it may be advantageous to use benchtop FTIR or GC analysis in other situations depending on the precision and information required.

2.5 Conclusion

This study found that a portable handheld infrared spectrometer is a suitable method for the detection and quantitation of trans fat. When used in combination with the handheld spectrometer, chemometrics was determined as a more accurate and robust technique than measuring height of the *trans* band at 966cm^{-1} . Using chemometrics, levels greater than 1% *trans* were accurately measured in edible oils with no sample preparation. In summary, FTIR-ATR coupled with PLSR has shown to be a simple, quick and robust technique for *trans* fat quantitation that will give food manufacturers real-time, field-based measurements to assess the nutritional quality of foods.

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