APPLICATION OF INFRARED SPECTROSCOPY AND CHEMOMETRICS FOR THE AUTHENTICATION OF ORGANIC BUTTER AND DETERMINATION OF SUGARS IN TOMATOES (*SOLANUM LYCOPERSICUM*)

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By

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ABSTRACT

Fourier-Transform infrared (FT-IR) spectroscopy is a simple, fast and highly specific technology that can provide valuable insights into the complex chemical make-up of foods. Infrared provides tools, especially in the fingerprint region of the spectrum, to detect specific compounds in biological systems without the use of time-consuming methods or the use of hazardous organic solvents. Advances in FT-IR instrumentation and pattern recognition techniques have made it possible to extract information related to composition and conformation of food components from the spectra. We have evaluated the capability of infrared spectroscopy in classification and quantification of chemical compounds of interest for the dairy (butter) and tomato industries.

Authentication is a critical quality issue for organic products since consumers are willing to pay 10-40% price premiums. There is a need for rapid and reliable analytical tools for determination of authenticity since traditional methods often involve time-consuming and laborious processes. Our objective was to evaluate the application of infrared spectroscopy combined with pattern recognition techniques to discriminate among organically and conventionally-produced butter in relation to quality and authenticity. Spectra from butter purchased from a local market (Columbus, OH) were collected by using Attenuated total reflectance (ATR) spectroscopy and analyzed using soft independent modeling of class analogy (SIMCA), a multivariate classification

technique. This simple protocol generated unique mid-infrared signature profiles that permitted the chemically-based classification of butter samples based on manufacturer and production practice (organic *vs.* conventional). By using the spectral region from 1400-800 cm^{-1}, multivariate (SIMCA) modeling showed well-separated clusters that discriminated among butter samples according to manufacturer, due to -HC=CH- *trans* bending out of plane vibration modes, (966 cm^{-1}) presumably attributed to conjugated fatty acids. Infrared spectroscopy combined with multivariate analysis provides a simple and efficient tool for monitoring butter authenticity with minimal sample preparation.

The objective of the second study was to develop a simple, accurate and cost effective protocol using ATR-IR spectroscopy and multivariate analysis to determine sugars in tomatoes. Tomatoes, the second most produced and consumed vegetable in the United States, are classified for use as fresh or processed tomato products based on their sugar and acid profile. Current methods to analyze sugar content of tomatoes are time and labor intensive making efficient assays for quantification desirable. Samples were obtained from genetically diverse tomato varieties that encompassed hybrids and elite parents used in the processing and fresh market industry. Samples were centrifuged, the supernatant vacuumed dried on a ZnSe crystal and infrared spectra collected. Enzymatic kits for glucose and fructose were used as reference methods. Multivariate models (PLSR) accurately predicted glucose and fructose using the supernatant with R-values > 0.98 and SECV <0.25g/100g, using the fingerprint infrared region of 1200-900cm^{-1} for sugars. Vacuum drying of the sample onto the ATR crystal caused spectral artifacts in some samples. ATR-IR combined with chemometrics could provide the tomato industry with a

simple and high throughput method for determination of sugars in tomatoes that could lead to improved varieties with enhanced characteristics for industry and consumer demands.

DEDICATION

Dedicated to my parents

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LITERATURE REVIEW

1.1 THE TOMATO

The tomato (*Solanum lycopersicum*) is an important agricultural commodity worldwide and the United States is one of the world's leading producers of tomatoes, second only to China (Lucier and Detteman 2009). Production of fresh and processing tomatoes has increased steadily for the past 20 years, and the farm value of the tomato crop is around $800 million (Lucier and Detteman 2009). In the United States tomatoes are bred specifically to serve the requirements of either fresh or processing markets. A tomato's composition classifies it for use as a fresh market or processing tomato. Factors such as soluble sugars, amino acids, acids, minerals and carotenoids, as well as the overall nutrient value are important in determining overall quality and best use of a tomato crop.

1.1.2 Tomato Composition

Soluble sugars are well known for contributing to the overall flavor of tomatoes and tomato products (Lenucci and others 2008). Sugars make up about 4-6% of tomato composition (Gould 1974). It has been demonstrated that the characteristic sweet taste of tomatoes is mainly due the reducing sugars glucose and fructose. Sucrose is also present

in tomatoes but at negligible levels (Petro-Turza 1987). It is also worth noting that fructose is found in higher concentrations than glucose (Hernández, Rodríguez-Rodríguez and Díaz-Romero 2008). Sugar concentration in tomatoes varies based on several factors such as genetics, growing conditions, environment and water availability (Davies, Hobson and Davies 1981). In addition to this previous studies have also shown that fruits picked earlier in development showed lower soluble sugar than fruits that were allowed to continue to develop on the vine (Davies, Hobson and Davies 1981).

Amino acid composition of tomatoes is not widely reported because it varies considerably mostly due to variety and different growing conditions (Hobson and Davies 1971). It is generally noted that the prominent amino acid in ripe tomatoes is glutamic acid which has been found to have a concentration as high as 270mg/100g fw (Hobson and Davies 1971). During ripening, amino acid contents remain stable however, glutamic acid concentrations rise sharply during maturation (Hobson and Davies 1971).

Tomatoes also contain a variety of organic acids. The presence of these acids imparts the sour or acid taste many associate with tomatoes and can vary widely from one variety to another (Hobson and Davies 1971). The ratio of sugars to acids has also been studied as a tool to determine flavor acceptability (Malundo, Shewfelt and Scott 1995). The major organic acids in tomatoes are citric, malic and oxalic acids; also present, although in much lower concentrations are fumaric and pyruvic acids (Hernández, Rodríguez-Rodríguez and Díaz-Romero 2008). Other studies on tomatoes have shown that of the acids present in tomatoes, citrate makes the greatest contribution to sourness because it is present in high concentrations ranging from 321mg/100g fw to 389mg/100g fw while malic and oxalic levels range from 71mg/100g fw to 92mg/100g fw and

24.9mg/100 to 29.3mg/100g, respectively (Hernández, Rodríguez-Rodríguez and Díaz-Romero 2008). Similar to sugars, organic acid levels vary between varieties, as a function of ripening and the stage at which the fruit is picked. Generally, the acidity in tomatoes peaks during development, and then decreases during ripening (Hobson and Davies 1971)

Large variations in vitamin C levels have been reported in tomatoes of different varieties. While one study by Abushita (2000) reports vitamin C contents of 210-480mg/kg other studies showed vitamin C contents of 10 varieties ranging from 84-324mg/kg fw (George and others 2004). During thermal processing, vitamin C is labile to degradation and in the case of tomato paste has been found to degrade by as much as 55% (Abushita, Daood and Biacs 2000).

While there is much data on the sugar and acid contents of tomatoes, there is limited information about the mineral content. Tomatoes are good sources of minerals such as potassium, sodium, calcium, magnesium and phosphorus (Souci and others 2008). The most prominent mineral in tomatoes is potassium with 0.126g/100g fw (Hobson and Davies 1971). Some studies have shown a relationship between the amount of potassium and overall acidity (Hobson and Davies 1971). It is suggested that the potassium acts as a buffer for the weak acids of the tomato fruit (Hobson and Davies 1971). Thus, any action that results in increased acid levels will also increase potassium to maintain a constant pH around 4.5 (Hobson and Davies 1971).

1.1.3 Carotenoids in Tomato

Carotenoids are a class of fat soluble pigments found in tomatoes. Carotenoids are becoming increasingly recognized for their health benefits and plant breeders are looking to maximize the carotenoid contents of their plants. They have been linked to

disease prevention in the case of certain cancers, macular degeneration, athersclerosis and other degenerative diseases (Inbaraj and Chen 2008). Daily consumption of carotenoids is highly desirable because the human body cannot synthesize carotenoids so they must be obtained from dietary sources (Krinsky, Mayne and Sies 2005). In plants, carotenoids are responsible for harvesting light energy and this is made possible by their extensive conjugated double bond system. It is also what imparts the characteristic yellow, orange and red color of tomatoes (Inbaraj and Chen 2008). The predominant carotenoid in tomatoes is lycopene with levels ranging from 72-200mg/100g fw, and it comprises 90% of the total carotenoid content (Inbaraj and Chen 2008). The concentration of lycopene will vary based on variety and also on environmental conditions. It has been shown that lycopene content is reduced at extreme temperatures, both low and high, as well as in times of moisture stress (Inbaraj and Chen 2008). In nature, lycopene occurs in the all *trans* position but it is labile to light, heat, oxygen and the presence of pro-oxidant ions such as Cu^{2+} and Fe^{2+} (Shi and Le Maguer 2000). In the presence of these materials lycopene will isomerize to its mono and poly *cis* forms mainly mono-5-*cis*, mono-9-*cis*, and mono-15-*cis* (Shi and Le Maguer 2000). Thus the *trans* form of lycopene is prevalent in fresh tomatoes and the *cis* isomers are found in processed tomato products (Shi and Le Maguer 2000). It has been found that thermal processing increases the total lycopene content of processed tomato products (Abushita, Daood and Biacs 2000).

Another carotenoid found in tomatoes is β-carotene. β-carotene is of particular interest because of its pro-vitamin A properties. It can be converted by enzymes in the intestinal mucosa to vitamin A (retinol) (Krinsky, Mayne and Sies 2005). Like lycopene, β-carotene is subject to degradation, however when exposed to high heat the levels of β-

carotene decrease as the isomers are far less bioactive than the naturally occurring all trans-β-carotene (Inbaraj and Chen 2008).

Lycopene and β-carotene have both been shown to have many health benefits, as have other carotenoids that are present at lower concentrations in tomatoes (Krinsky, Mayne and Sies 2005). While β-carotene is a precursor for vitamin A, lutein and zeaxanthin may be linked to the lower risk of cataracts by filtering harmful blue light and scavenging singlet oxygen in retinal tissues (Krinsky, Mayne and Sies 2005).

1.2 ANALYTICAL TECHNIQUES FOR TOMATO CONSTITUENTS

1.2.1 High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) is the most traditional method for analysis of sugars. HPLC is a form of chromatography where there is a separation between the mobile phase and the stationary phase. It has gained acceptance for use in food analysis because it can be used with a variety of compounds and it is highly automated.

An HPLC is comprised of four main components (Figure 1.1): a pump, a column, an injector, and a detector (Bélanger and Pare 1997). The pump is electronically controlled to regulate the pressure, flow and delivery rate of the mobile phase (Bélanger and Pare 1997). Samples must be soluble in the mobile phase, therefore it is important to keep the polarity of the sample in mind when choosing the mobile phase. The injector is used to inject the sample onto the mobile phase so that it can be carried to the column. The column is where the actual separation of the compounds occurs (Bélanger and Pare 1997). The most widely used type of chromatography is adsorption and can be classified as normal phase and reverse phase (Bélanger and Pare 1997). During normal phase

chromatography the stationary phase is polar and the mobile phase is non polar. Reverse phase is the opposite with a non-polar stationary phase and a polar mobile phase. Other types of chromatography include partition chromatography, ion exchange chromatography and size exclusion (Bélanger and Pare 1997). The retention of solutes depends on the mobile phase ionic strength, pH, and flow rate (Bélanger and Pare 1997). Generally the flow rate of the mobile phase is kept low to maximize separation (Bélanger and Pare 1997). Using an HPLC system requires a lot of trial and error because it is sensitive and the slightest change will create drastically different results.

Once an anylate passes through a column it goes on a detector. There is no universal detector for HPLC so it also important to use the correct detector for the analyte (Bélanger and Pare 1997). There are four commonly used detectors for HPLC, these include UV-absorption, fluorescence, refractive index, and mass detectors (Bélanger and Pare 1997). The most commonly used detector is a UV/Vis because it has good selectivity, high sensitivity for most compounds, and is easy to operate and non destructive (Bélanger and Pare 1997). The last step is presenting the results to the analyst for further investigation.

Sugars are often analyzed using HPLC utilizing a refractive index detector (Table 1.1). Hernández (2008) used HPLC to determine sugars in tomatoes and Porretta (1992) examined the sugars in tomato paste.

While HPLC is a sensitive and accurate tool for analysis it does have drawbacks. Sample preparation and method development is lengthy and depends mostly on trial and error before a suitable technique is found. The end results depend greatly on the stability of the compounds, as well as the ability of the analyst to properly extract the compounds

of interest and prepare the sample for analysis properly. In addition, HPLC is an expensive instrument that requires the use and disposal of hazardous organic solvents (Halim and others 2006).

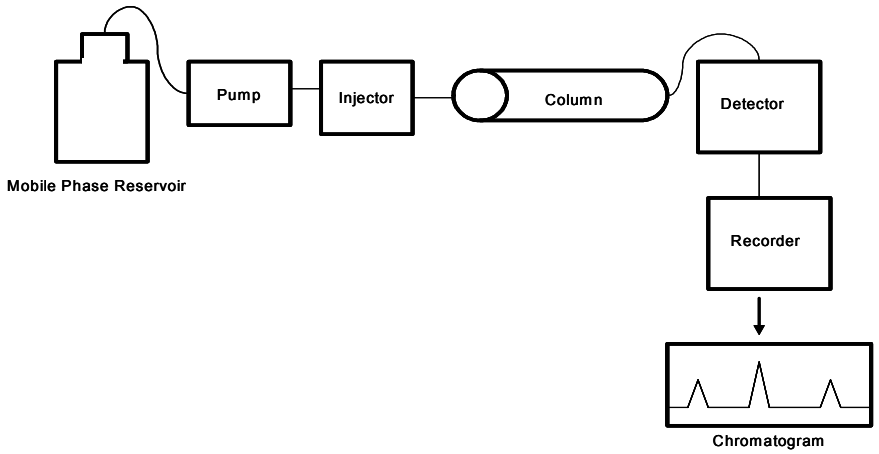

Figure 1.1 HPLC Schematic

	Column	Detector	Solvent	Conditions	Source
Sugar	C_{18}	Refractive index	Acetonitrile	Isocratic	(Irudayaraj and Tewari 2003)
Organic Acid	C_{18}	Photo Diode array	Triflouracetic	Isocratic	(Marconi, Floridi and Montanari 2007)
Carotenoids	C_{18}	Photo Diode array	Methanol: Methyl tert-butyl ether	Gradient 0-20% MTBE	(Halim and others 2006)

Table 1.1 HPLC analysis of different tomato components

1.2.2 Ultra-Violet/Visible Spectroscopy

Ultra Violet/Visible Spectroscopy (UV/Vis) is a common tool for food analysts. UV/Vis uses wavelengths from 200-700. The colorless UV section ranges from 200-

350nm while the Visible section ranges from 350-700nm. Characteristic colors from violet to red represent the different wavelengths (Penner 2003). UV/Vis is most commonly used for quantitative work but can be used for qualitative techniques as well (Penner 2003). Quantitative assays are based on measuring the absorbance of a sample at one wavelength, because the absorbance of the test solution will be a function of the concentration (Penner 2003).

The objective of quantitative absorption spectroscopy is to determine the concentration of analyte in a sample and is based on the amount of light that is absorbed as it passes through the sample (Penner 2003). In some cases, the analyte may naturally absorb in the UV/Vis range and in other cases the analyte must be altered by chemically converting the analyte into a species that can readily absorb radiation (Penner 2003). In cases like this, the absorbance is used as an indicator of the analyte concentration.

Absorbance is a unitless expression that is directly proportional to the concentration of a specific analyte (Penner 2003). The relationship between the absorbance of a solution and the concentration is known as Beer's Law.

$A = \varepsilon lc$……[Equation 1]

A is the absorbance of a sample. The concentration variable, c, can be expressed in any terms and the path length, l, is in centimeters. The molar absorptivity (ε), is expressed in (1/(cm x M)). This value is wavelength dependent and is determined by the molecular properties of a molecule and can vary based on the pH and solvent (Penner 2003).

UV/Vis is almost entirely dependent on the use of a reference cell. This is because quantitative spectroscopy is based on the amount of light that is absorbed by the sample, so any other decrease in the incident beam must be accounted for (Penner 2003).

This is accomplished with the use of a reference cell. A reference cell is one that exactly matches the sample cell except that it contains no analyte. Most often this is accomplished with an absorbance cell filled with distilled water and the absorbance of this cell is used as $P_{solvent}$ for the sample cell. And the following equation is used in the laboratory to determine absorbance.

$$A=\log \frac{P_{solvent}}{P_{analyte\ solution}} \quad \text{[Equation 2]}$$

There are three essential components of all spectrophotometers: a light source, a monochromator, and a detector. The light source used in spectrophotometer must be capable of emitting strong bands of radiation that will encompass the entire wavelength range (Penner 2003). The two common radiation sources are a tungsten filament lamp for Vis and a deuterium electrical-discharge lamp for UV (Penner 2003). Tungsten filament lamps can cover from 350nm to 2500nm while the deuterium lamps can cover 160nm to 375nm (Penner 2003).

Light emitted from a light source is polychromatic and a monochromator isolates the specific group of wavelengths to be used for analysis (Penner 2003). The polychromatic light enters the monochromator and is dispersed according to wavelength, with only a single wavelength exiting. Once the light exits the monochromator it continues on to interact with the sample.

When the light passes through the sample it is quantified by means of a detector. There a several different types of detectors but the two most popular are phototubes and photomultiplier tubes (Penner 2003). Both detectors work by converting the energy from

incoming photons into electrical current. The signal from a detector is amplified and then displayed to the analyst in the form of absorbance.

The determination of sugars in food samples is complicated by the fact that sugar does not absorb UV radiation. Thus, enzymatic kits are used to determine the sugar content of a sample by measuring the amount of NADH formed during a chemical reaction. During this chemical reaction, fructose is phosphorylated by adenosine triphosphate (ATP) to fructose-6-phosphate via hexokinase (Bergmeyer 1974). Fructose-6-phosphate is converted to glucose-6-phosphate by phosphoglucose isomerase (PGI) (Bergmeyer 1974). The glucose-6-phosphate is oxidized to 6-phosphogluconate in the presence of nicotinamide adenine dinucleotide (NAD) (Bergmeyer 1974). This reaction is catalyzed by glucose-6-phosphate dehydrogenase. During this oxidation an equimolar amount of NAD is reduced to NADH (Bergmeyer 1974). This increase in NADH causes an increase in absorbance at 340nm and it is directly proportional to the concentration of fructose (Bergmeyer 1974). A similar reaction is used to quantify the amount of glucose in a sample. In this reaction, glucose is phosphorylated directly to glucose-6-phosphate by hexokinase at which point the reaction proceeds in the same manner as fructose (Bergmeyer 1974).

Figure 1.2 Enzymatic determination of Fructose

Figure 1.3 Enzymatic determination of Glucose

Enzymes have been used as analytical tools with great success in a variety of applications including food, pharmaceutical and biochemical industries. Porretta (1992) compared the accuracy of HPLC and enzymatic kits for measuring fructose and glucose in tomato paste and found that there was no significant difference between the methods. Steegmans (2004) also used an enzymatic method on a variety of food matrices with good results. While enzymatic kits are reproducible and rapid they take some laboratory fines and can be very costly.

1.2.3 Infrared Spectroscopy

Currently, the quantification of sugars is still heavily reliant on HPLC and UV/Vis, but infrared spectroscopy (IR) is emerging as a new technique that can easily, rapidly, and accurately determine sugar concentrations in a sample.

Infrared spectroscopy (IR) is an analytical technique that measures the absorption of different infrared radiation frequencies of a sample. As a molecule absorbs infrared radiation it vibrates via a stretching and bending motion. This vibrational energy is measured and is directly proportional to the strength of the bond. Different functional groups absorb IR radiation in a distinct wavelength region, making it possible to identify unknown molecules based on the infrared spectrum. IR can be classified into three regions, near-infrared (NIR), mid-infrared (MID), and far-infrared. The near and mid IR

regions are most commonly used in the food industry as many organic molecules show absorbance in this region.

Mid-infrared spectroscopy uses light in the 4,000-650 cm^{-1} region. The most popular spectrophotometer in mid-IR is Fourier transform (Wehling 1994). Unlike UV/Vis which uses a monochromator to separate light into individual wavelengths, a Fourier transfer uses an interferometer (Wehling 1994) which splits the beam into two parts (Wehling 1994, Ismail, Van de Vort and Sedman 1997). One beam is reflected onto the stationary mirror and the other is transmitted to a moving mirror whose motion varies with time (Jaggi and Vij 2006). Once reflected back they recombine and pass through the beam splitter again and undergo interference. The combined beams are then passed through the sample and the signal is detected (Jaggi and Vij 2006). This signal, created by the absorbance of the radiation by functional groups, results in a series of peaks in the spectrum (Wehling 1994). The signal is converted by Fourier transform, a series a mathematical equations, which converts a time domain to a frequency domain (Ismail, Van de Vort and Sedman 1997).

Almost all functional groups show absorption in the mid infrared region, therefore absorption bands are well defined. Mid-IR also utilizes the region between 1200-900 cm^{-1}, which is known as the fingerprint region, and produces distinct and reproducible chemical fingerprints that reflect the total composition of the sample. The fingerprint region shows bands that represent lipids, proteins, carotenoids and polysaccharides (Halim and others 2006, Ismail, Van de Vort and Sedman 1997).

Near infrared spectroscopy utilizes light in the 700-2500nm. Unlike mid-IR, which is characterized by well defined peaks, absorption bands in the near-IR region are

mainly overtones and absorption is weak (Wehling 1994). This causes the bands to be broad and overlap making analysis difficult (Wehling 1994). Bands that have enough intensity to be observed in this region are mostly due to hydrogen atoms attached to carbon, nitrogen, or oxygen (Wehling 1994). This makes near-IR an ideal tool for measuring water, proteins, lipids, and carbohydrates (Wehling 1994).

Depending on the sample, either reflectance or transmittance measurements can be made with near-IR (Wehling 1994). Solid samples are generally measured with reflectance because this method measures the light that bounces off the sample (Wehling 1994). To do this, detectors are set up at a 45° angle to measure the light that bounces off the sample (Wehling 1994). Transmittance is used for liquid samples where the light can pass through the sample (Wehling 1994). The sample is placed in a quartz cuvette, and the radiation light passes through the entire sample, measuring the absorbance at the wavelength of interest (Wehling 1994). Transmission measurements are more desirable because they have higher signal-to-noise ratios, use inexpensive tools for sample preparation, and reduce the sample preparation time by not requiring a homogenous sample surface since the radiation passes through the whole sample (Wehling 1994).

Attenuated total reflectance (ATR) is a technique widely used in mid-IR spectroscopy, because it is one of the easiest and most convenient ways of handling samples for IR spectroscopy (Ismail, Van de Vort and Sedman 1997). Traditional infrared sampling, called transmission, is based on the samples absorption of infrared radiation as the beam propagates through the sample; thus measurements in this mode have severe limitations on thickness (Sedman, van de Vort and Ismail 1999). ATR was developed as a solution to this problem. ATR-IR measures the total amount of energy

reflected from the portion of the sample in direct contact with the crystal (Sedman, van de Vort and Ismail 1999). ATR-IR uses a highly refractive index material, called the internal reflection element. When light from the source strikes the element at one end it is internally reflected at the top and bottom faces of the crystal before it exits (Ismail, Van de Vort and Sedman 1997). The radiation only penetrates a short distance into the sample before it is reflected back onto the crystal (Sedman, van de Vort and Ismail 1999). A spectrum is produced as the sample absorbs radiance (Ismail, Van de Vort and Sedman 1997).

FTIR/ATR spectroscopy is catching on in popularity as they are able to analyze large amounts of data in a very short time. It has been proven a fast, cost effective tool for routine monitoring of sugars in fruit and fruit juices (Davies, Hobson and Davies 1981, Irudayaraj and Tewari 2003, Beullens and others 2006, Bureau and others 2009).

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CHAPTER 2

CLASSIFICATION OF ORGANICALLY AND CONVENTIONALLY PRODUCED CREAM BUTTER BY INFRARED SPECTROSCOPY AND MULTIVARIATE ANALYSIS

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2.1 ABSTRACT

Organic foods constitute more than 2% of all U.S. food; sales are estimated to have increased nearly 20% annually since 1990, reaching $13.8 billion in 2005. This rapid growth may be traced to increased consumer confidence in organic foods as well as concern about possible health risks and environmental impacts of conventional food production methods. Authentication is a critical quality issue for organic products since consumers are willing to pay 10-40% price premiums. There is a need for rapid and reliable analytical tools for determination of authenticity since traditional methods often involve time-consuming and laborious processes. Our objective was to evaluate the application of infrared spectroscopy combined with pattern recognition techniques to discriminate among organically and conventionally-produced butter in relation to quality and authenticity. Butter produced by different manufacturers from different production lots were purchased from a local market (Columbus, OH). Samples were filtered at 65°C and the collected fat samples were directly applied onto a temperature-controlled single bounce ZnSe crystal for attenuated total reflectance measurements. The ZnSe crystal was heated at 65°C and spectra analyzed using soft independent modeling of class analogy (SIMCA), a multivariate classification technique. This simple protocol generated unique mid-infrared signature profiles that permitted the chemically-based classification of butter samples based on manufacturer and production practice (organic *vs.* conventional). By using the spectral region from 1400-800 cm^{-1}, multivariate (SIMCA) modeling showed well-separated clusters that discriminated among butter samples according to manufacturer, due to -HC=CH- *trans* bending out of plane vibration modes (966 cm^{-1})

presumably attributed to conjugated fatty acids. SIMCA effectively classified between organically and conventionally-produced butter (interclass distance of 3.2) with major discrimination due to -C-O asymmetric stretching vibrations of esters. Infrared spectroscopy combined with multivariate analysis provides a simple and efficient tool for monitoring butter authenticity with minimal sample preparation

2.2 INTRODUCTION

Sales of organic products in the U.S. topped $14.6 billion last year (Winter CK and Davis SF 2006). Despite higher production costs, the increased consumer demand and high profitability are convincing more producers to switch to organic (Winter CK and Davis SF 2006). Consumers site avoidance of pesticides, freshness and health and nutrition as the main reasons they purchased organic foods. These consumers are willing to pay 10%-40% more for organically produced butter (Winter CK and Davis SF 2006).

Adulteration of food goes back centuries and involves using inferior or cheaper ingredients to cheat consumers (Kurtzweil P 1999). It rarely presents a health hazard but it cheats the consumer out of hundreds of thousands of dollars each year and undercuts the competition (Kurtzweil P 1999). High value products like organic butter must maintain a strong image with consumers in order to maintain sales and profit margins (Fairchild GF, Nichols JP and Capps O 2003).

Methods of adulteration have become more sophisticated in recent years, making it more difficult to identify (Kurtzweil P 1999). Traditional methods of fat analysis involve hydrolysis and methylation of the fatty acids for gas chromotography (GC). Although GC is sensitive and can detect adulteration of butter fat with a detection

limit of 1-2%, it is time and labor intensive and requires the use of hazardous organic solvents (Dupuy and others 1996). FTIR has also been proven as an effective tool to determine adulteration of butter samples with a detection limit of 3% (Sato 1990). FTIR is also able to classify fats and oils. The most important spectral region in the analysis of fats and oils occurs between 1400-1800cm^{-1} which represents C-H bending, C=O stretching, and C=C stretching (Yang, Irudayaraj and Paradkar 2005). Fourier transform infrared spectroscopy (FT-IR) is a rapid and non-destructive authentication tool that is capable of detecting butter adulteration with minimal sample preparation.

FTIR has also been used to detect fat and oil adulteration in a variety of products. One study was able to predict the lard adulteration in chocolate and chocolate products with great success (Che Man and others 2005). FTIR combined with principal component analysis (PCA) has been shown as an effective method to discriminate between oils of different plant species, detect adulteration of extra virgin olive oil and discriminate between butter and margarine (Dupuy and others 1996, Lai, Kemsley and Wilson 1995).

The industry is looking for a fast way to detect butter adulteration, and infrared technology is ideal for rapid screening and identification of target analytes in foods with minimal sample preparation. Our objective was to evaluate the application of infrared spectroscopy combined with pattern recognition techniques to discriminate among organically and conventionally-produced butter in relation to authenticity.

2.3 MATERIALS AND METHODS

2.3.1 Butter Samples

A total of 12 butter samples were purchased from local markets (Columbus, OH). There were 6 traditional and 6 organic butters each with 3 salted and unsalted products. The butter was analyzed in triplicate with repetitions from different lots and production dates. Samples were melted in a 60° C oven and filtered through Whatman filter paper (Kent, England). The fat (top) layer was extracted and analyzed on a single bounce fatIR with a ZnSe crystal (Harrick, Plesantville, NY) attached to a temperature controller (Harrick, Pleasentville, NY). A FTS 3500GX Fourier-Transform (FT) infrared spectrophotometer (Varian, Palo Alto, California) was used with a potassium bromide beam splitter and Deuterated Triglycine Sulfate (DTGS) detector for all readings, operating at 4 cm^{-1} resolution. Spectra were collected over the frequency region from 4000-600 cm^{-1} and interferogram of 32 scans were co-added according to Beer-Norton apodization. Spectra were displayed in terms of absorbance and viewed using Win-IR Pro Software (Varian, Palo Alto, California). Each sample was analyzed in triplicate. To prevent interference in the spectra, the instrument was continuously purged with CO_2 – free dry air from CO_2RP140 dryer (Dominick Hunter, Charlotte, NC, USA).

2.3.2 Multivariate Analysis

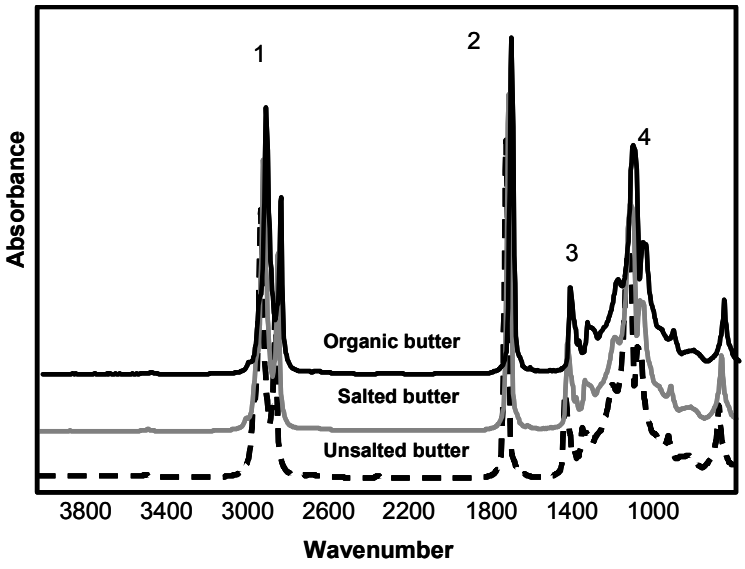
The spectra were exported as GRAMS.spc file format and imported into Pirouette®, for Windows Comprehensive Chemometrics Modeling Software, version 3.11 (Infometrix, Inc. Bothell, WA). The spectra were then analyzed by soft independent modeling of class analogy (SIMCA) to generate clustering groups. SIMCA is a multivariate analysis technique based on principal component analysis (PCA). The

program allows for the visualization of clustering among samples. This was used to evaluate the ability of the ATR-IR spectra to discriminate butters based on production method and manufacturer.

In SIMCA, training sets are assigned to classes and a principle component model is generated for each class with distinct confidence regions within them (Naumann, Schultz and Helm 1996). The performance of this method depends not only on the difference between classes, but also strongly on the training set for each class (Naumann, Schultz and Helm 1996). The scores plot allows the visualization of clustering among samples (sample patterns, groupings or outliers). Between-class distances were calculated using interclass distances and Mahalanobis distances were used for outlier diagnostics. The clusters can be defined using the discriminating power, which identifies the wavenumbers that have a predominant effect on sample classification by minimizing the difference between samples within clusters and maximizing those from different clusters (Dunn and Wold 1995). If a sample falls outside the class border, it is considered an outlier. For this reason, class-modeling techniques can be regarded as outlier detection methods (Candolfi and others 1999). Therefore, the identity of unknown samples can be predicted using the training models with three possible outcomes: (i) the unknown is part of one class, (ii) the unknown is part of more than one class, or (iii) the unknown does not belong to one class.

2.4 RESULTS AND DISCUSSION

The typical ATR-IR spectrum for the butter samples showed characteristic bands associated with fats between 1200-700 cm^{-1} (Figure 2.1).



No.	*Frequency cm^{-1}*	*Funtional Group*
1	**3000-2800**	C-H Streching
2	**1800-1600**	C=O Stretching
3	**1400-8000**	C=H *cis* and *trans* bending

Figure 2.1 Attenuated total reflectance (ATR) spectra of butter samples

Visual analysis of the ATR-IR spectrum of the lipid phase for organic and conventional salted and unsalted samples showed no unique IR spectral peaks. Major bands in the spectra occur between 3000-2800 cm^{-1}, 1800-1600 cm^{-1}, and 1400-8000 cm^{-1}. These regions correspond to C-H stretching, C=O stretching, C=H *cis* and *trans* bending out of

plane. Mathematical processing of the raw spectra using a Savitzky-Golay second derivative algorithm (5-pt gap size) resulted in removal of baseline shifts, resolved overlapping peaks, and reduced variability between replicates (Kansiz and others 1999).

Class projections are used to illustrate the ability of SIMCA to differentiate IR data based on the first three principal components (Figure 2.2A). This model offered good class separation, tight clustering among butter samples, zero misclassifications, and an interclass distance of 3.2. Generally, interclass distances above 3 are considered good for discrimination with larger interclass distances indicating well separated classes (Dunn and Wold 1995). The major discrimination bands were between 1300 and 800 cm^{-1} with strong absorption bands at 966 cm^{-1}, as shown by the discrimination power (Figure 1.1B) Wavelength 966 cm^{-1} is characteristic of C=H *trans* bending out of plane (Stuart 2004). This is presumably due to the presence of conjugated fatty acids in butter and other dairy products (Guillén and Cabo 1997).

Separation based on manufacturer also showed tight clustering without any misclassifications (Figure 2.3A). The interclass distances ranged from 2-16 (Table 2.1). Some of the class distances obtained in the experiment were lower than three, due to the subtle differences between manufactures. It is interesting to note that the lowest interclass distance occurs between two organic manufacturers. The discriminating bands for the separation of butter samples based on manufacturer showed the peak at 966 cm^{-1} as well as an additional peak at 1109 cm^{-1} (Figure 1.2B). This band is attributed to C-O stretching (Sato 1990, Guillén and Cabo 1997).

We were not able to separate between salted and unsalted products. Salt exhibits no absorption in the infrared region, however monitoring the change in the water

component of spectra gives information about the salt in a sample (Begley and others 1984). This method only utilizes the lipid fraction of the sample, making it impossible to monitor salt using this method.

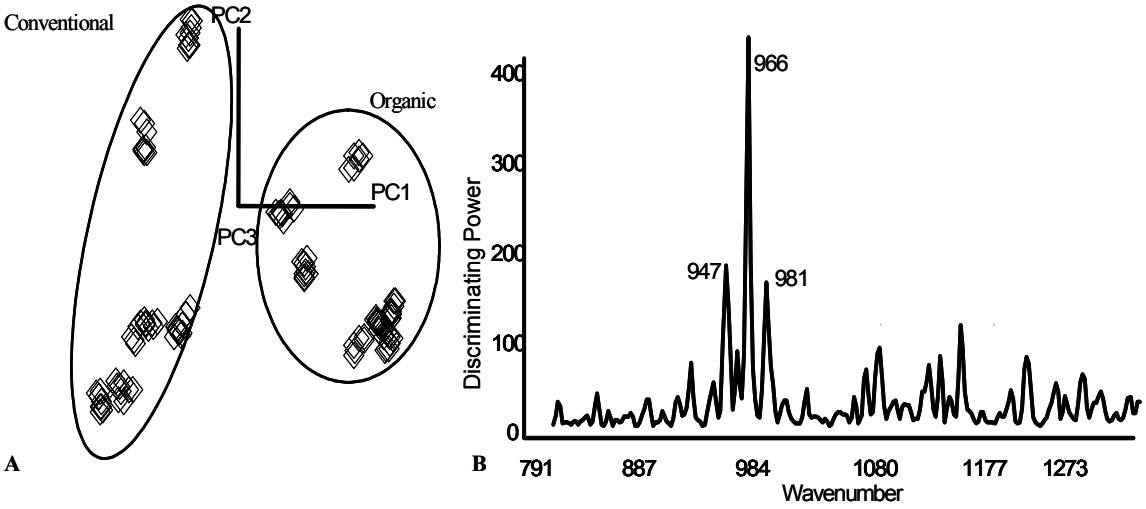


Figure 2.2 SIMCA classification of organic and conventional butter (A) and discriminating bands (B).

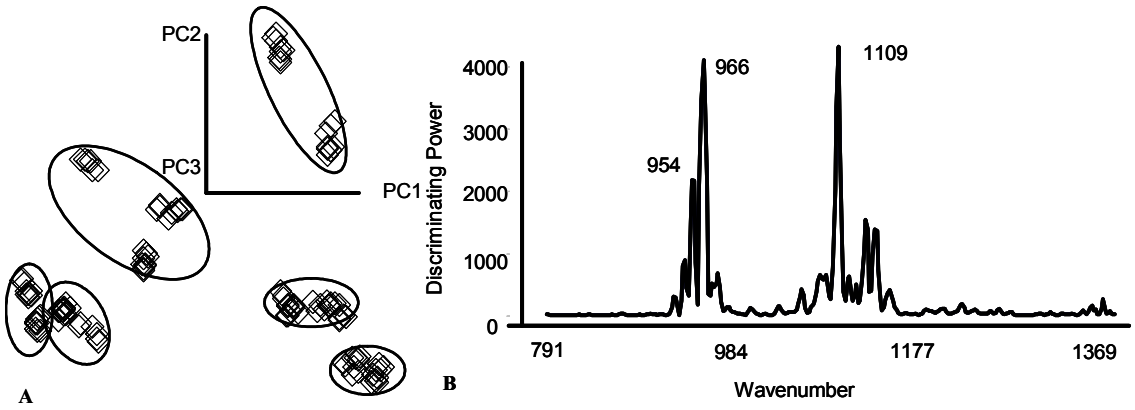


Figure 2.3 SIMCA classification of butter by manufacturer (A) and discriminating bands (B).

Manufacturer	A	B	C	D	E	F
A	0					
B	13.32324	0				
C	10.15078	6.612761	0			
D	8.005266	6.307381	3.263797	0		
E	4.279833	8.303251	6.282442	4.287127	0	
F	2.180727	16.08013	13.99808	11.11726	5.252298	0

Table 2.1 Interclass distances for the separation of butter based on manufacturer

2.5 CONCLUSIONS

The ATR-IR technique allowed the development of SIMCA models for the qualitative analysis of organic and conventional butters. The determination of organic and conventional butters occurs mostly at wavenumber 966cm^{-1} and is presumably attributed to a difference in conjugated acids. These differences in conjugated acids could be due to differences in feed between organically and conventionally raised dairy cattle. This method also shows promise for the discrimination of butter based on manufacturer as well as butter origin. The same band at 966cm^{-1} is also important in discriminating butter based on manufacturer in addition to a band at 1109cm^{-1} which is attributed to C-O stretching.

These results show that FTIR is a rapid, accurate, and cost-effective assay that is less time consuming than current analytical practices, such as gas chromatography, for the authentication of butter. IR spectroscopy can resolve the unique information of samples accurately and discriminate between conventional and organic products as well as manufacturer.

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CHAPTER 3

MONITERING GLUCOSE AND FRUCTOSE IN GENETICALLY-DIVERSE TOMATOES BY INFRARED SPECTROSCOPY

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3.1 ABSTRACT

Tomatoes are the second most produced and consumed vegetable in the United States with a production value in the U.S. over $2 billion every year since 2005. Tomatoes are classified for use as fresh or processed tomato products based on their sugar and acid profile and the presence of carotenoids which provide health benefits. Current methods to analyze sugar content of tomatoes are time and labor intensive making efficient assays for detection and quantification desirable. Our objective was to develop a simple, accurate and cost effective protocol to determine sugars using Attenuated Total Reflectance Infrared (ATR-IR) spectroscopy and multivariate analysis.

Samples were obtained from genetically diverse tomato varieties that encompassed commercial hybrids, experimental hybrids, and elite parents used in tomato processing and fresh market industry. Fresh tomatoes were blended, aliquots (5 mL) centrifuged and infrared spectra collected from supernatant (2 μL) vacuumed dried onto a ZnSe crystal. Enzymatic kits for glucose and fructose were used as the reference methods. Partial least squares regression (PLSR) was used to create calibration models that correlated the sugar concentration in tomatoes with infrared spectra. Multivariate models accurately predicted reducing sugars (glucose, fructose) using the supernatant with R-values > 0.98 and SECV <0.25 g/100g using the fingerprint infrared region of 1200-900cm^{-1} for sugars. Vacuum drying of the tomato soluble extracts onto the ATR crystal caused spectral artifacts in selected samples. ATR-IR combined with chemometrics could provide the tomato industry with a simple and high throughput

method for determination of sugar in tomatoes that could lead to improved varieties with enhanced characteristics for industry and consumer demands.

3.2 INTRODUCTION

The United States is one of the world's leading producers of tomatoes, second only to China (Lucier and Detteman 2009). Production of fresh and processing tomatoes has increased steadily for the past 20 years and the farm value of the tomato crop is around $800 million (Lucier and Detteman 2009). In the United States, tomatoes are bred specifically to serve the requirements of either fresh or processing markets. A tomato's composition classifies it for use as a fresh market or processing tomato. Factors such as soluble sugars, amino acids, acids, minerals and carotenoids, as well as the overall nutrient value are important in determining overall quality and best use of a tomato crop.

The market price for tomatoes depends on a variety of factors including the intended use (fresh market or processing), as well as composition. The most important aspect in flavor acceptability is the sugar to acid ratio (Malundo, Shewfelt and Scott 1995).

Current methods to analyze tomato composition include high performance liquid chromatography (HPLC) and enzymatic kits. While these methods are accurate and widely accepted they do have limitations. HPLC requires extensive sample preparation, method development is lengthy and depends mostly on trial and error before a suitable technique is found (Halim and others 2006). To monitor the total composition of tomatoes would require the development of a new method of extraction and analysis for

each compound. The end results depend greatly on the stability of the compounds as well as the ability of the analyst to properly extract the compounds of interest and prepare the sample for analysis properly (Halim and others 2006). Enzymatic kits are another option for monitoring tomato composition. Enzymatic kits require the use of a spectrophotometer and this method has a high cost per test. As opposed to HPLC or spectroscopy, which requires an initial investment and upkeep, enzymatic kits are an ongoing financial burden. Enzymatic kits are very sensitive and require much practice on the part of the analyst before accurate results are obtained (Bergmeyer 1974).

Attenuated total reflectance infrared spectroscopy (ATR-IR) combined with multivariate analysis offers the industry and plant breeders a rapid and accurate test to monitor tomato composition with little sample preparation. Spectral bands arising from functional group vibrations of organic molecules in the mid-infrared region (4000-700cm^{-1}) may be associated to specific functional groups with known wavelength assignments in most cases. Halim (2006) used ATR-IR to quantify lycopene in tomatoes. ATR-IR has also been used to monitor organic acid and sugars in apple and mango juice (Irudayaraj and Tewari 2003, Duarte and others 2002). Sugar production is often an indicator of tomato ripeness and by picking at the right time producers have fewer spoiled fruits and are able to obtain a higher price for their tomatoes (Hulme 1970). Using IR, tomato producers can track the development of tomatoes in the field to determine the optimum picking time. ATR-IR is the ideal method for monitoring tomato composition because it is rapid, easy to use, and less costly than other techniques.

3.3 MATERIALS AND METHODS

3.3.1 Plant material and Sample Preparation

A total of 17 tomato varieties were grown and harvested in Fremont, Ohio, at the north central agricultural research center. The homogenized frozen samples were obtained from varieties and breeds that encompass a wide range of sugar, pigment, and acid levels. Plant materials included hybrids, experimental hybrids, and elite parents used in the tomato processing industry.

Aliquots (1mL) of the homogenized tomato sample were centrifuged for five minutes at 3000rpm and the supernatant withdrawn. Each tomato variety was analyzed in triplicate.

3.3.2 Sample preparation: enzymatic determination of sugar

Glucose and fructose were quantified using a fructose assay kit from Sigma Aldrich (Saint Louis, MO). Quantification of glucose and fructose is based on the phosphorylation of fructose and glucose catalyzed by hexokinase. During the reaction NAD is reduced to NADH causing an increase in absorbance. Samples were prepared in a 1:20 dilution with distilled water and were analyzed according to the method by Sigma/Bergmyer with the addition of a cuvette of 2.02 mL of distilled deionized water and 0.1 mL of diluted tomato sample. Absorbencies were monitored at 340nm using a UV/Visible spectrophotometer 2450 (Shimadzu, Columbia, Md.) with 1cm pathlength disposable cells.

3.3.3 Infrared spectroscopy

To obtain spectra, the samples were dried directly on an ATR-IR ZnSe crystal before the spectra were collected. An FTS 3500GX Fourier-Transform infrared spectrophotometer (Varian, Palo Alto, California) was used with a potassium bromide beam splitter and Deuterated Triglycine Sulfate (DTGS) detector for all readings, operating at 8 cm^{-1} resolution. A three-reflection ZnSe ATR accessory was used with a refractive index of 2.5 that permitted a triple reflection within the sample at an incidence angle of 45°, for the highest infrared sample throughput (Pike Technologies, Madison, WI). Spectra were collected over the frequency region from 4000-600 cm^{-1} and interferogram of 32 scans were co-added according to Beer-Norton apodization. Spectra were displayed in terms of absorbance and viewed using Win-IR Pro Software (Varian, Palo Alto, California). Each sample was analyzed in duplicate. To prevent interference in the spectra, the instrument was continuously purged with CO_2-free dry air from CO_2RP140 dryer (Dominick Hunter, Charlotte, NC, USA).

3.3.4 Multivariate Analysis

The spectra were exported as GRAMS.spc file format and imported into Pirouette®, for Windows Comprehensive Chemometrics Modeling Software, version 3.11 (Infometrix, Inc. Bothell, WA). Partial least squares regression (PLSR) was used to analyze the spectra using a cross validated (leave-one-out approach). PLSR is a bi-linear regression model that reduces a large number of variables into a small number of latent variables that are linear combinations of the spectral variables and uses these to

determine the analyte's concentration (Wold, Sjostrom and Eriksson 2001). These latent variables explain much of the co-variance of X and Y (Martens and Martens 2001). PLSR is a versatile analytical approach because of its ability to analyze large, complex, and noisy data sets (Wold, Sjostrom and Eriksson 2001, Wold and others 2001). This technique is widely used in spectroscopy because it uses the concentration information (Y) to determine how regression factors are computed from the data set (X); this reduces the impact of irrelevant variations in the calibration model (Martens and Martens 2001). PLSR models are evaluated in terms of standard error of calibration (SEC), standard error of cross validation (SECV), and coefficient of determination (r-values).

3.4 RESULTS AND DISCUSSION

3.4.1 Enzymatic Kits

The glucose and fructose levels for 17 varieties of tomatoes were determined by enzymatic methods, used as a reference method in developing multivariate calibration models for ATR-IR spectroscopy. Based on enzymatic kits, fructose and glucose levels in tomatoes ranged from 0.395-1.114g/100 g and 0.194-0.882g/100g, respectively (Table 3.1). Levels of sugars in tomatoes have been reported to range from 0.9g -1.62g/100g for glucose and 1.25-1.70g/100g for fructose, based on HPLC analysis (Souci and others 2008). The values reported in this study are close to those found in literature, taking into consideration that sugar levels may be affected by factors such as variety, maturity, temperature, and soil nutrients among others (Gould 1974, Jones and Scott 1983). The lower levels of sugars reported in our study could be attributed to the use of enzymatic kits instead of the more accurate HPLC analysis. Also, our results showed higher values for fructose than glucose which is consistent with literature (Hernández, Rodríguez-Rodríguez and Díaz-Romero 2008). The sugar concentration of tomatoes has also been evaluated as a tool to determine flavor acceptability (Malundo, Shewfelt and Scott 1995)

Our precision (%CV) for replicated enzymatic analysis was <16% and <11% for fructose and glucose, respectively. The variable %CV indicates the limitations of the enzymatic method for the determination of sugars. CV reflects the cumulative effect of the sample's in homogeneity among replicated samples, due to natural variability of the tomatoes, signal noise, the skill of the analyst, experimental error (mainly dilution effects, reaction times, assay temperature), day-to-day environmental variations (such as

temperature, humidity, air, etc.), and many more (Halim and others 2006). For instance, the detection range of the kits required a concentration range from 100-1000µg/ml fructose/glucose, resulting in sample dilutions of 20 to 40 fold to be within the acceptable concentrations for analysis. Consequently, %CV can be lowered as the skill of the experimentalist improves and assay variations can be minimized. Enzymatic kits have been found to have higher %CV in sugar determination in tomato products as compared to HPLC and Fehling reagent methods (Porretta and others 1992).

Tomato Variety	Code	Fructose	CV	Glucose	CV
		mg/100g	%	mg/100g	%
Vintage Cultivar	**6407**	0.876	4.561	0.700	4.711
Vintage Cultivar	**6409**	0.794	6.537	0.626	7.448
Fresh Market	**6414**	0.664	15.754	0.569	7.243
Vintage Cultivar	**6420**	0.737	12.620	0.655	10.701
Fresh Market	**6423**	0.803	6.093	0.638	8.227
Vintage Cultivar	**6435**	0.637	5.596	0.479	4.700
Latin American Land Race	**6451**	0.910	6.520	0.736	5.644
Processing	**6453**	0.395	6.837	0.194	8.383
Wild Cherry	**6455**	0.909	8.819	0.729	10.298
Vintage Cultivar	**6467**	0.794	6.388	0.639	7.683
Processing	**6480**	0.747	6.810	0.576	8.718
Latin American Land Race	**6485**	0.968	0.788	0.821	6.705
Unimproved breeding line	**6495**	0.843	8.998	0.549	10.749
Processing	**6498**	0.599	2.327	0.436	2.200
Processing	**6501**	0.557	1.274	0.394	1.558
Vintage Cultivar	**6511**	0.999	6.255	0.828	8.848
Processing	**6547**	0.593	0.000	0.436	2.153

Table 3.1 Sugar concentrations determined by enzymatic kits

3.4.2 Infrared Spectroscopy

Infrared analysis was first carried out using an infrared microscope because of its sensitivity and high throughput capabilities. The ATR-IR microspectroscopic technique uses a slide on an ATR with a germanium crystal that is lowered onto the sample surfaces to generate the spectra. Samples were vacuum dried to minimize the effect of the strong water absorption bands centered at 3400 cm^{-1} and 1700 cm^{-1} that possibly overlapped the analyte spectral signal. Elimination of the solvent and analysis of the dried sugar extract resolved several spectral features (Figure 3.1). However, upon vacuum drying, the high sugar levels of the tomato samples resulted in syrup instead of a homogeneous film which caused inconsistency in the spectra because the ATR probe was disturbing the sample's surface, thus, resulting in irreproducible results (Fig. 3.1).

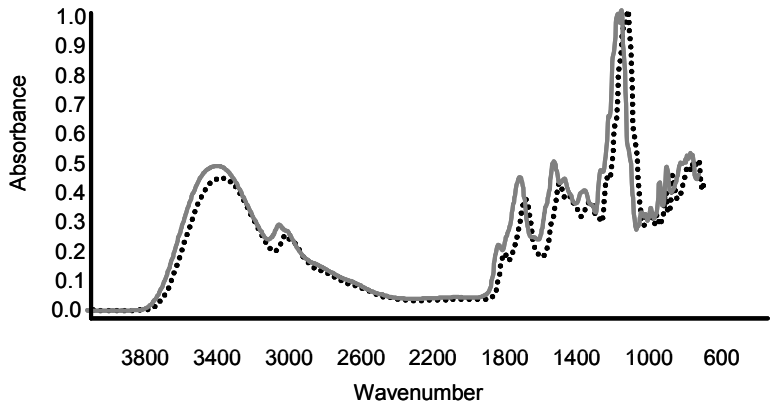

Figure 3.1 Attenuated Total Reflectance (ATR) infrared absorption spectrum of one tomato sample

The analysis protocol was modified by using the standard ATR-IR benchtop equipped with a 3-bounce ZnSe crystal onto which the sample was vacuum dried, minimizing disruption of the sample. The spectra were reproducible within a sample with no difference in spectral bands among replicates (Figure 3.2 A), while spectral differences were noticed when comparing tomato varieties with extreme sugar concentrations (Figure 3.2 B). However, spectra from tomato samples (6409, 6420, 6480) having comparable levels of fructose and glucose, with average values of 0.764 mg/100g and 0.596mg/100g, respectively, produced spectra with vastly different spectral intensities and profiles (Figure 3.2C). This is even more evident when comparing the second derivative (Figure 3.3). This mathematical transformation of the spectral measurements resolves overlapping bands and allows for easier comparisons of the differences between samples. This indicates that there might be components in the samples, other than sugar, that are influencing the spectra.

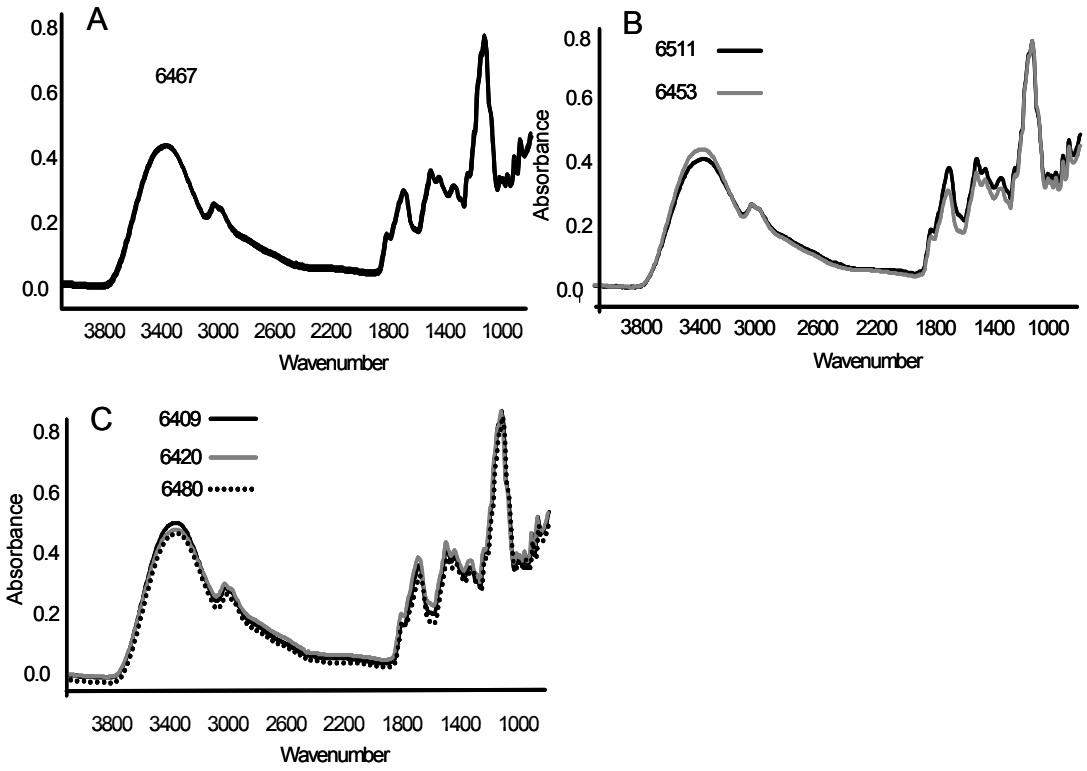

Figure 3.2 Attenuated Total Reflectance (ATR) infrared absorption spectrum of tomato samples: repetition of the same sample (A), comparison of a high and low sugar variety (B), and comparison of three different samples with similar sugar concentrations (C).

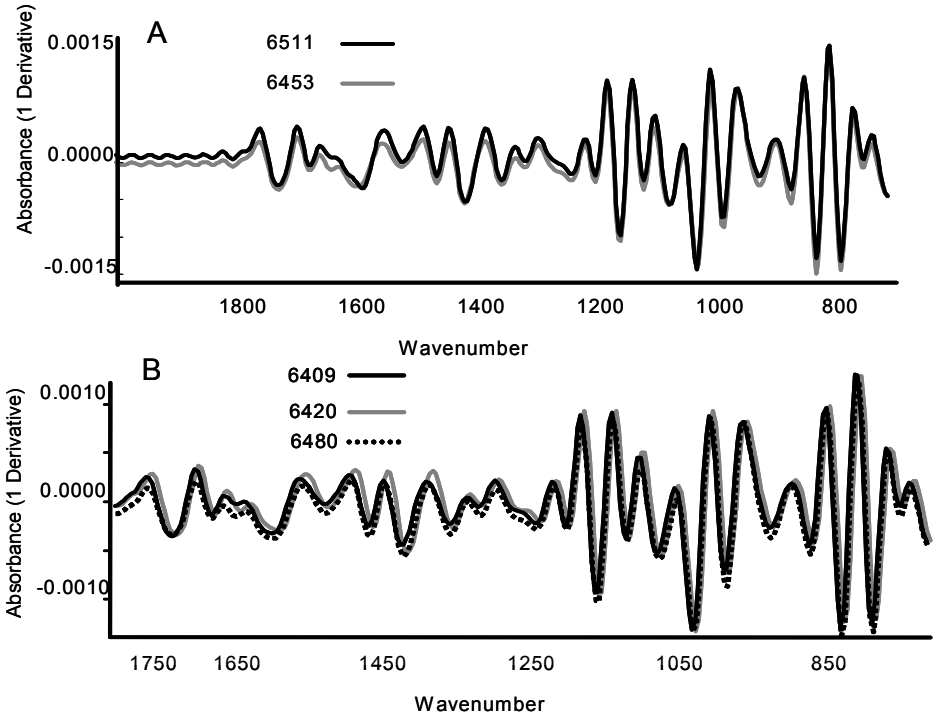

Figure 3.3 Second derivative transformation of ATR spectrum of tomato samples: comparison of a high and low sugar variety (A), and comparison of three different samples with similar sugar concentrations (B).

The cross validated leave one out PLSR model results are shown in Figure 3.4. The estimated glucose and fructose content measured by ATR-IR spectroscopy showed good correlation with the enzymatic analysis (Figure 3.4). The PLSR models (Table 3.2) for fructose (r value >0.98, and SECV of 0.027 g fructose) were comparable to that for glucose (r value >0.97, and SECV of 0.028 g glucose). Most of the variance was explained by the first five latent variables (>91%). The high number of factors could introduce spectral noise into the model, resulting in overfitting which could impair its ability to estimate sugar levels in unknown samples. In addition, the high %CV values

obtained for the reference method can impact our model, limiting the precision of the PLSR prediction.

Examination of these loading spectra indicates which regions of the spectrum are associated with the most sample variation. The PLSR loading spectra (Fig. 3.5) show absorption features for glucose and fructose. The regions with the highest variation were similar in fructose and glucose, and are associated with C-O and C-O-H functional groups. Frequencies from 1000-1300 cm^{-1} are characteristic of C-O stretching and the bands at 930 cm^{-1} and 1430 cm^{-1} are due to C-O-H in plane bending (Stuart 2004).

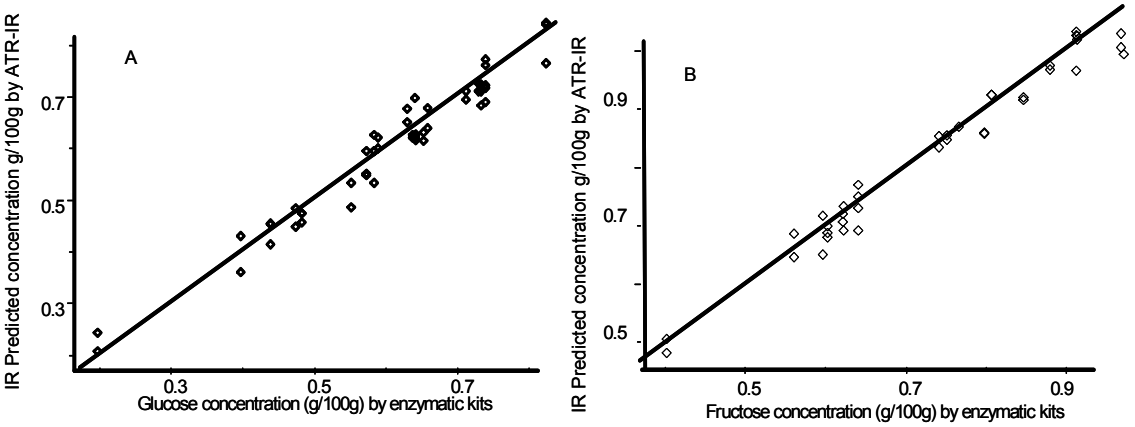

Figure 3.4 Cross-validated (leave one out) PLSR plots for glucose (A) and fructose (B)

	SECV	r-value	factors
Fructose	0.027	0.98	11
Glucose	0.028	0.97	7

Table 3.2 Comparison of SECV, r-values and factors for partial least squares regression

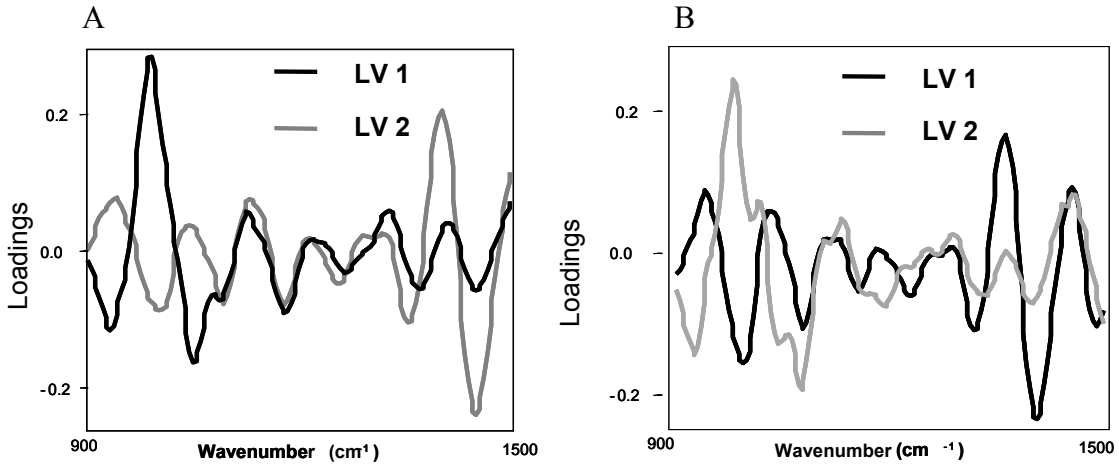

Figure 3.5 Partial least squared loadings plot for cross validated models for the determination of fructose (A), glucose (B).

3.5 CONCLUSIONS

The ATR-IR technique allowed the development of PLSR models for the quantitative analysis of sugars in tomatoes. The PLSR models in this study showed correlation coefficients (r-value) of 0.98 and 0.97 for fructose and glucose, respectively and with standard error of cross validation (SECV) of 0.027g/100g and 0.028g/100g between the ATR-IR predicted and enzymatic kits. The drying procedure had an effect on the spectral reproducibility within samples, probably due to changes in the crystallization

or sugar-water interactions. This could impact the spectral profiling, limiting the ability of PLSR to accurately estimate sugar levels in unknown samples. In summary, ATR-IR coupled with multivariate analysis has shown promise as a fast and reliable technique for determination of sugars in tomato samples. This spectroscopic technique could provide a valuable tool for the rapid screening of tomato sugars for the industry.

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