Rapid Analyses of Carotenoids in Tomato Paste Using Handheld Raman Spectroscopy

THESIS

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By

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Abstract

The objective of this study was to develop reliable, fast and non-destructive protocols for analyses of carotenoids in tomato paste by Raman spectroscopy combined with pattern recognition analyses. Tomato paste samples were kindly provided by tomato processing companies in California and different carotenoid varieties of tomatoes were obtained from the OSU Tomato Genetics and Breeding Program at Wooster, Ohio. Spectra of tomato paste were directly collected, without any sample preparation steps, with a handheld Raman spectrometer equipped with a 1064nm laser. Lycopene concentration in red tomato paste was determined by UV-Vis spectrophotometry at 503nm. HPLC equipped with a photodiode array detector was utilized for carotenoid profiling. Raman spectra were evaluated by pattern recognition analyses, including quantification by partial least square regression (PLSR) and classification by soft independent modeling of class analogy (SIMCA). Raman spectra of red tomatoes showed unique marker bands at 1510 cm^{-1} (C=C stretching), 1156cm^{-1} (C-C stretching), and 1002cm^{-1} ($C-CH_3$ in-plane rocking) that are characteristic of the long conjugated polyene chain of lycopene. Cross-validated (leave-one-out) PLSR model (3 latent variables) predicted lycopene content with a standard error of cross-validation (SECV) of 4.7 mg/100g and a coefficient of determination of 0.96. Screening of tomato paste from varieties that accumulate a diverse carotenoid profile showed a unique region associated with C=C stretching that changed according to the dominant carotenoid: all-*trans*-lycopene at 1511 cm^{-1}, β-carotene at 1521 cm^{-1}, and tetra-*cis*-lycopene at 1525 cm^{-1}. The unique Raman spectral signatures allowed SIMCA to classify the tomato paste into four classes based on their carotenoid profile without any misclassification, producing tight and well-separated clusters that can be used for predicting unknown samples. Raman spectroscopy is positioning as an attractive fingerprinting technique for tomato industry because it offers portable and ruggedized instrumentation for field deployment and requires little to no sample preparation to rapidly and accurately assess carotenoid quality and quantity in tomato-based products.

Acknowledgments

First and most importantly, I would like to thank my advisor Dr. Luis Rodriguez-Saona for recognizing my potential and guided me tremendously during my research. Dr. Rodriguez not only taught me to how to succeed in graduate study, but also encouraged me to explore the food industry. I am also very grateful for my committee members Dr.Christopher Simons and Dr. Rafael Jimenez-Flores for giving me advice on statistics and taught me the importance of critical thinking. In addition, I would like to thank all of my lab mates (Peren, Mei-ling, Hacer, Ahmed, Amanda and Luju) for helping me with experiments, being patient with me, and making lab times so much more fun. And lastly, I would like to thank Californian tomato paste producers and Dr. Francis' lab at Wooster, Ohio for generously providing the tomato materials for my research.

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Chapter 1 Literature Review

1.1 The Tomato

The tomato (*Lycopersicon esculentum*) is one of the most important vegetable of the family Solanaceae (Thakur et al. 1996). Botanically, the tomato is a fruit, consisting of the ovary and the seeds (Harlan 1928). Nevertheless, it is cooked as vegetables and by law, the tomato is classified as a vegetable for tariff purposes (United States Supreme Court 1893). Tomato is an essential ingredient in American diet and provides important nutrients: approximately 94% water, 2.5% total sugars, 2% total fibers, 1% protein, and other micronutrients such as acids, amino acids, vitamin C, provitamin A and carotenoids (Klunklin and Savage 2017). Now, the tomato is the second most commonly consumed vegetables and the second largest tomato producer globally (USDA Economic Research Service 2017). In 2016, approximately 35 billion pounds of tomatoes were produced, accounted for more than $2 billion in annual farm cash receipts (USDA Economic Research Service 2017). Tomato varieties are bred in different ways in order to serve certain requirements that are needed for either the fresh or processing markets (Economic Research Service 2012)

1.1.1 Fresh versus Processing Tomatoes

The processing and fresh tomato industries target different markets. In the U.S., 75% of total tomato consumptions comes from processed tomato products, such as ketchup, pizza sauce, and canned tomatoes. California accounts for 94% of the area harvested for processing tomatoes in the U.S. and the majority of the processing tomatoes are thermally processed into tomato paste, which is the main raw material for tomato processing firms.

(USDA Economic Research Service 2017). Processing tomatoes are grown under contract with tomato processing companies (USDA Economic Research Service 2017). Due to high demand, processing tomatoes are machine harvested and the quality is closely monitored in order to produce high consistency tomato paste. The variety of processing tomatoes is not as diverse as fresh tomatoes; the majority are red, blocky or oval-shaped fruits with a higher percentage of soluble solids (five to nine percent), good internal color, and thick walls (García-Valverde et al. 2013). On the other hand, fresh tomatoes are hand-picked and sold largely on the open market. Due to the uncertainty in market demands and larger production costs, fresh tomatoes are priced higher than processing tomatoes (USDA Economic Research Service 2017). In addition, fresh tomatoes are bred to have different shapes, sizes, and colors. The five major groups of fresh tomatoes are classic round tomatoes, cherry tomatoes, plum and baby plum tomatoes (grape tomatoes), beefsteak tomatoes, and vine or truss tomatoes (Costa and Heuvelink 2005). Different colored variety of tomatoes exist on the market to satisfy consumer preferences and the increased attention in the health benefits of different types of carotenoids, which are the major pigments in tomatoes (Capanoglu et al.; Johnson 2000; Khachik et al. 2002).

1.1.2 Tomato Pastes

Processed tomato products can be broadly categorized into four main category: tomato pulp, tomato juice, tomato paste, and tomato puree (Hayes et al. 1998). Tomato paste is concentrated tomato pulp without skins and seeds and has to contain more than 24% natural tomato soluble solids (NTSS).

Tomato paste processing steps are as followed (Gould 1992a; Macrae et al. 1993; Hayes et al. 1998):

(1) Wash and tomatoes to remove soil, dirt, molds, and microorganism. Then, the washed tomatoes that are off-color or defected are sorted out (Gould 1992b).

(2) Hot or cold break depending on the intended use of the concentrated paste. Breaking refers to the heat treatments where tomatoes are heated to 90-95 °C to inactivate pectin degrading enzymes pectin methylesterase and polygalacturonase for hot break or 65 °C where the enzymes are still active. Hot break process is more suitable for ketchup and pizza sauce processing and cold break process is for tomato juice or cocktails products (Koh et al. 2012).

(3) Skins and seeds are then removed and filter through difference sized-screens to control the texture of the final products (Hayes et al. 1998).

(4) Evaporate moisture by multi-stage evaporators based on the required viscosity of final products (Hayes et al. 1998).

(5) Lastly, the pastes are sterilized at 100°C for three to five minutes and rapidly cooled to 35°C. The pastes are then pumped into feed tank for aseptic filling. Sterilization, cooling and filling (Gould 1992b; Koh et al. 2012).

Because tomato paste is the major raw material for further processing of ketchup, pizza sauce, pasta sauce, and more, the quality has to be closely monitor. The principal quality parameters for tomato paste include sugars and organic acids that can affect the flavor; vitamin C content for nutritional value; soluble solids that influence consistency; titratable acidity and pH that impact flavor the microbial safety; serum viscosity and consistency that can affect the texture, application, and appearance.

1.2 Carotenoids

Carotenoids (Figure 1-1) are 40-carbon isoprenoid derivatives and lipid-soluble pigments with colors ranging from yellow to orange to red (Barrett, Diane M., Anthon 2008). Carotenoids can be chemically divided into two groups: carotene, containing only polyunsaturated hydrocarbons, and xanthophylls, which contain polyunsaturated hydrocarbons with oxygen (Kopec et al. 2012; Sigurdson et al. 2017). Carotenoids can also be acyclic, monocyclic, or dicyclic (lycopene, δ-carotene, and β-carotene, respectively). The long conjugated double bonds create π-electrons delocalized along the polyene chain, which are highly absorbed at orange to red (~450 to 500nm) visible spectrum region. The greater the number of conjugated double bonds, the redder the color (i.e. lycopene, having 11 conjugated double bonds, is red, whereas, phytofluene, which has five, doesn't exhibit color hue) (Sigurdson et al. 2017). Furthermore, cyclization can also influence the color of the carotenoids; lycopene and β-carotene, having the same number of conjugated double bonds, exhibit red and orange color, respectively (Sigurdson et al. 2017). Because of the polyunsaturated hydrocarbon nature, a carotenoid can have several *cis/trans* isomers and can provide different chemical and biological activity (Rodriguez 2001; Kopec et al. 2012).

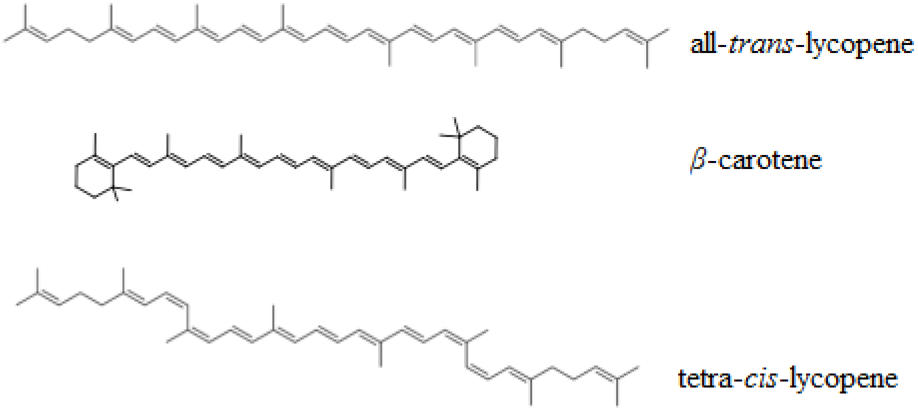

Figure 1-1 Chemical structure of carotenoids commonly found in tomatoes.

The functions of carotenoid in plants are to protect against photosensitization and aid in production of a plant hormone that can promote growth (Kopec et al. 2012).

1.2.1 Carotenoids and Health

Unlike plants, human cannot synthesize carotenoids but can modify some of them by consuming plant foods. Carotenoids are not only found in tomatoes but also in several fruits and vegetables, such as peppers, watermelons, mangoes, carrots, sweet potatoes and pink grape (Xu 2012). Dominantly, carotenoids occur in the *trans* form in raw vegetables (Clevidence et al. 2000). Among all the fruits containing carotenoids, the best sources of lycopene come from tomatoes and processed tomato products, which is where approximately 80 percent of lycopene consumption in the average American diet comes from (Story et al. 2010). Research have found that lycopene, a non-provitamin A carotenoid, can act as antioxidants to prevent cardiovascular diseases, some cancers, and improve immune system (Clinton 1998; Johnson 2000; Rodriguez-Amayal et al. 2006; Story et al. 2010). A study suggested that consuming 6-15mg of lycopene on a daily basis could potentially build immune system and inhibit prostate cancer (Dehghan-Shoar et al. 2011). More recent clinical studies investigated and found that compared to all-*trans*-lycopene, tetra-*cis*-lycopene (traditionally presented in processed tomato products) found in a new breeding line of tangerine tomato was more bioavailable (Unlu et al. 2007a, b; Burri et al. 2009; Cooperstone et al. 2015). Another beneficial carotenoid is *β*-carotene which possess both antioxidant capacity and provitamin A activity. Comparing to tomatoes, only carrots are a richer source of vitamin A. However, advancing genetic breeding strategies have allowed development of tomatoes with higher amount of *β*-carotene, and other unique lines with different carotenoids that are not presented in conventional red tomatoes due to increased evidence on different carotenoid's bioavailability and the possible health benefits (Unlu et al. 2007a; Yoo et al. 2017).

1.2.2 Carotenoids in Tomatoes

The characteristic red color of tomatoes are due to the carotenoids, specifically due to the lycopene content. In nature, carotenoids exist in small globules and outer pericarp of the fruit has the highest amount of total carotenoids, followed by industrial waste, whole tomato and pulp (Thakur et al. 1996; Amorim-Carrilho et al. 2014b). The different carotenoid profile defines the diverse pigmentation of the tomato fruit colors. Carotenoid profiles differ based on the genetics (Figure 1-2), maturity, cultivation techniques and growing condition of tomato plants (Shi and Le Maguer 2000).

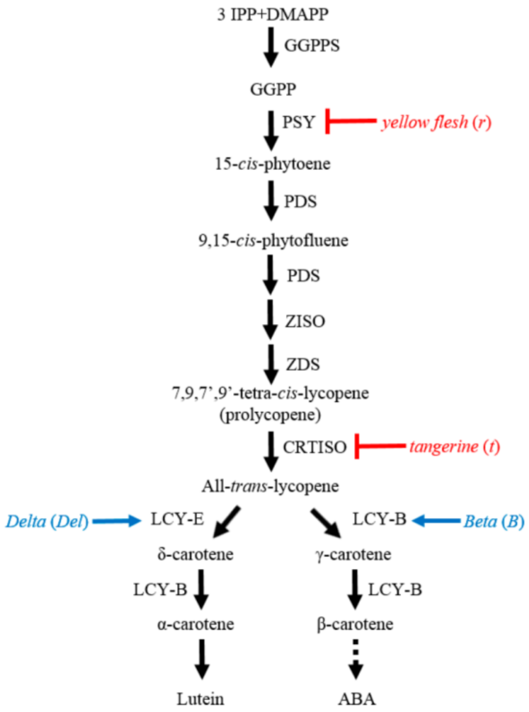

Figure 1-2 Carotenoid biosynthetic pathway in tomatoes (Yoo et al., 2017)

The biosynthesis of carotenoids starts out two carotenoid precursors, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), and convert to a C20 terpenoid geranylgeranyl diphosphate (GGPP). Next, 15-cis-phytoene is condensed from two GGPPs. 15-cis-phytoene is then converted to tetra-cis-lycopene via two desaturation and one isomerization reactions by three enzymes. Subsequently, an isomerase converts tetra-cis-lycopene to all-trans-lycopene. Later, all-trans-lycopene is cyclized through two routes and branched to form δ-carotene or *β*-carotene, and ultimately results in lutein or neoxanthin (Vijayalakshmi et al. 2015; Yoo et al. 2017).

In red tomatoes, the most predominant carotenoid is *trans*-lycopene (83%), and *β*-carotene is the minor component, accounted for around three to seven percent of total carotenoids (Hayes et al. 1998). Lutein, γ-carotene, *cis*-lycopene, prolycopene, δ-carotene also exist in trace amount in red tomatoes (García-Valverde et al. 2013; Yoo et al. 2017). For tangerine tomatoes, it could be *β*-carotene, tetra-*cis*-lycopene or δ-carotene content is higher and all-*trans*-lycopene content is reduced (Ronen et al. 1999; Yoo et al. 2017). Yellow tomatoes have lower in both lycopene and *β*-carotene, and all other trace carotenoids (Yoo et al. 2017). Black tomatoes exhibited blackish red skin because the chlorophyll content was retained and possess higher lycopene content compared to red tomatoes, and *β*-carotene and lutein are the minor carotenoids (Li et al. 2011; Park et al. 2018).

1.2.3 Analysis of Carotenoids

Many analysis methods of carotenoids have been developed to extract, quantify and identify carotenoids from foods. Carotenoids are typically extracted by a mixture of organic solvents that are suitable for the relative polarity of the target carotenoids. To extract carotenoids from fruits of vegetables, water-miscible solvents, such as tetrahydrofuran or acetone, are used to penetrate the samples. Typical solvents that have been used are hexane, acetone, methanol, ethanol, ethyl ether, tetrahydrofuran (THF), petroleum ether, or dichloroethane (Kopec et al. 2012; Amorim-Carrilho et al. 2014b). Agitation and repeated extraction steps are generally needed to ensure disruption of tissues and complete removal of the carotenoid pigments. Later, removal of unwanted components is followed (Kopec et al. 2012). Other than liquid-liquid and solid-liquid extraction methods, supercritical-fluid extraction, ultrasound-assisted extraction, and pressurized liquid extraction have also been used as a more environmentally-friendly alternative methods (Amorim-Carrilho et al. 2014a; Saini and Keum 2018).

UV-Visible spectrophotometry is a useful tool in identifying major carotenoids because the long conjugated polyene chain absorbs light in the range of 400 to 500nm (Rivera and Canela-Garayoa 2012). It is important to note that the absorption spectra only allow for distinguishing different chromophore of the carotenoids (Kopec et al. 2012). For example, the absorption spectrum of *β*-carotene will be indistinguishable from its oxygenated derivative zeaxanthin (Figure 1-3).

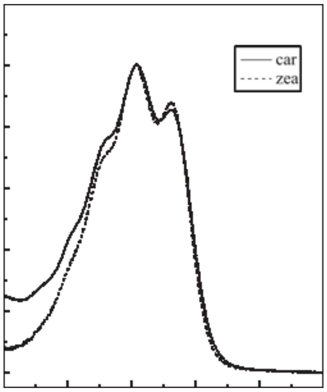

Figure 1-3 Absorbance spectra of β-carotene (bold line) and zeaxanthin (dashed line), dissolved in ethanol (Vidolova et al. 2017).

Most importantly, spectrophotometic method permits quantification analysis because of Beer-Lambert's law where the absorbance intensity is linearly correlated with carotenoid concentration (Fish et al. 2002; Anthon and Barrett 2007a). Another commonly used analytical technique is normal or reverse HPLC (Hart and Scott 1995; Bicanic et al. 2010;

Rubio-Diaz et al. 2010a; Kopec et al. 2012; Amorim-Carrilho et al. 2014b). C18 or C30 columns are typicall used, with C30 phases providing better separations of carotenoid geometric isomers than C18 columns (Rivera and Canela-Garayoa 2012). In addition, to obtain well resolved carotenoids peaks, polarity, viscosity, and volatility of mobile phase solvents are important factors to consider. Typical mobile phase solvents are acetonitrile, methanol, dichloromethane, acetone, hexane, THF, MTBE, ethyl acetate or mixtures of them (Rivera and Canela-Garayoa 2012). Methanol has shown to provide higher recovery of carotenoids and a less polar solvent such as MTBE is typically added to assist on increasing solubility of the analytes, obtaining the desired retention, and improving resolution (Amorim-Carrilho et al. 2014a). Elution order can be useful in the identification of different carotenoids and their isomers, for example when using C30 column, more polar xanthophylls elute before carotenes, and central *cis* isomers elute first then *trans* isomer, due to former being more polar (Saini et al. 2015). Most common HPLC detectors for carotenoid analysis are photodiode array (PDA) or UV-Vis detector. However, UV-Vis and PDA systems cannot provide molecular sturcture information and speficfic bond locations, plus, absorption spectra of several carotenoids are similar (Rivera and Canela-Garayoa 2012). Thus, MS and/or tandem MS detectors are complementary to obtain molecular information based on molecular mass and fragmentation pattern (Amorim-Carrilho et al. 2014a). Nevertheless, to definitively obtain exact molecular structure of bond locations, HPLC in combination with nuclear magnetic resonance (NMR) experiments will be the most powerful tool for identification purposes (Kopec et al. 2012).

The above analytical techniques described are often not possible for tomato growers and processors, as they skilled personnel to conduct the measurement, very time-consuming and produces large amount of environmentally-unfriendly organic solvent wastes that are costly to dispose of. Here is where vibrational spectroscopy comes in and gaining attention as a simple, fast, and environmentally-friendly technique to analyze lycopene and other carotenoids.

1.3 Vibrational Spectroscopy

Vibrational spectroscopy is a method based on the absorption and scattering of electromagnetic radiation associated with molecules (Mengshi Lin , Barbara A Rasco , Anna G Cavinato 2009). It measures vibrational energy levels which associate with the chemical bonds in sample. Vibrational spectroscopy, especially infrared and Raman spectroscopy have been evaluated and shown advantages of obtaining fingerprint regions for fast, precise, and direct measurement for quality control purposes, adulteration detection, and the study of molecular structures (Baranska et al. 2006; De Nardo et al. 2009).

1.3.1 Mid-infrared Spectroscopy

Infrared spectrum provides chemical information based on the interaction of infrared radiation with matter. Infrared (IR) radiation can be divided into near IR (14,000 to 4,000 cm^{-1}), mid IR (4,000 to 400 cm^{-1}), and far IR (400 to 4 cm^{-1}) (Guillén and Cabo 1997). Mid IR is the main region of vibrational spectroscopy because it is a very reproducible and robust region that even slight compositional differences of samples can be detected (Subramanian and Rodriguez-Saona 2009a). A net change of dipole moments is required in infrared spectroscopy. If the frequency of the infrared radiation matches the natural frequency of the molecular vibration (stretching, bending, rocking, wagging, or twisting), the infrared phonon is absorbed (Subramanian and Rodriguez-Saona 2009a) . Different chemical bonds absorb different infrared frequencies depending on the surrounding molecules and the atoms connected (Thygesen et al. 2003). The absorption of the energy produces characteristic and reproducible spectrum that can be regarded as a fingerprint of the compound's functional groups, which can be used to analyze the composition of food sample and determine chemical structure of the target analyte (Subramanian and Rodriguez-Saona 2009b; Lin et al. 2014).

The fundamental law governs absorption is the Beer-Lambert law,

$$A = \varepsilon c l \qquad (1)$$

where A=absorbance, ε=absorptivity of the target analyte, c=concentration of target analyte, and l=pathlength (Schrader and Keller 2003). Thus, mid-infrared is a practical quantitative tool that's been utilized in the food industry (Ellis et al. 2015).

1.3.2 Fourier-Transform Infrared Spectroscopy (FTIR)

Among diverse instrumentations, Fourier transformed mid IR (FTIR) equipped with attenuated total reflectance sampling accessory (ATR) has become the predominant device for its speed and accuracy (Subramanian and Rodriguez-Saona 2009a). FTIR improves signal-to-noise ratio by utilizing an interferometer to generate controlled interference patterns (interferograms) and Fourier transformed algorithm is used to obtain a spectrum in frequency domain (Mengshi Lin , Barbara A Rasco , Anna G Cavinato 2009; Subramanian and Rodriguez-Saona 2009a). An interferometer (Figure 1-4) is an optical device that is composed of a beam splitter, a stationary mirror and a moving mirror. The two mirrors are aligned so that the recombination of the two light beams focuses at the beam splitter.

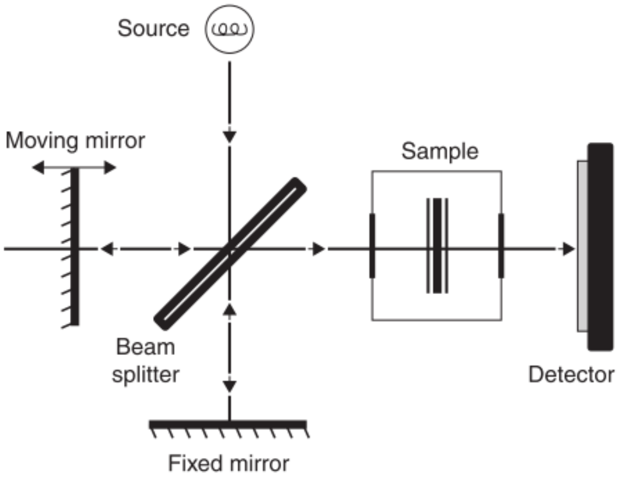

Figure 1-4 Schematic diagram of an interferometer (Subramanian and Rodriguez-Saona 2009).

As the moving mirror moves, differences in distances traveled by the two light beams that pass through the sample and eventually reaching the detector create an interferogram. Fourier transform algorithm is later used to convert the interferogram to a spectrum (Subramanian and Rodriguez-Saona 2009a). FTIR allows measurement of all wavelength at the same time, as opposed to a typical dispersive device where the spectrum is recorded one wavelength at a time. In addition, an interferometer allows all infrared radiation passes through the sample, which gives higher intensity and higher spectral resolution than using a monochromator (Subramanian and Rodriguez-Saona 2009a).

1.3.3 Attenuated Total Reflectance (ATR) Sampling Accessory

Attenuated Total Reflectance (ATR) is today the most widely used FTIR sampling technique (De Nardo et al. 2009). ATR is a sampling technique (Figure 1-5) utilizes internal reflection, instead of the conventional IR spectroscopy of measuring transmitted light from the sample (Khoshhesab 2012). For the conventional transmission IR spectroscopy, the effective pathlength of the infrared beam is dependent on the sample's thickness, which adds preparation steps (e.g. weighing) and lengthens analysis time. ATR occurs when the light source enters from a high refractive index element (ATR crystal made of diamond, zinc selenide, or germanium) into a closely-contacted lower refractive index medium (less dense food sample). An evanescent wave is created at the interface between the two media and penetrates into the less dense food sample where only the top 2-3 microns are analyzed. (Rodriguez-Saona and Allendorf 2011; Khoshhesab 2012; Agilent Technologies 2017). The evanescent wave is absorbed by the food sample and less intensity is reflected because the intensity is attenuated in the regions of the IR spectrum where the sample absorbs. The resulted attenuated radiation as a function of the spectrum region specific to the chemical bonds in food produces an ATR spectrum (Khoshhesab 2012). Thus, ATR provides advantages of little to no sampling preparation on solids, semisolids, pastes, liquids and thin films with high reproducibility and speed while minimzing user-to-user spectral variation. (Halim et al. 2006; Rodriguez-Saona and

Allendorf 2011; Lu and Rasco 2012; Wilkerson et al. 2013; Ayvaz et al. 2016a). In addition, as the number of reflections increases, the intensity of the absorbed wavelength improves; thus, multi-reflection ATR is recommended for analysis of minor component in the sample with improved signal to noise ratio in most cases (Mojet et al. 2010; Rodriguez-Saona and Allendorf 2011; Technologies 2011).

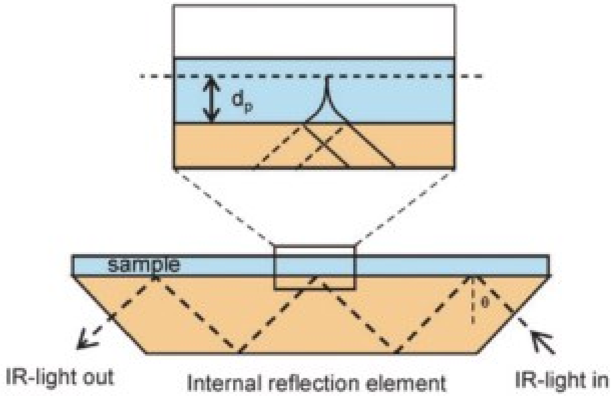

Figure 1-5 Schematic representation of total internal refection with (top) Single reflection, and (bottom) Multiple reflections (Mojet et al. 2010).

1.3.4 Raman Spectroscopy

Another vibrational technique utilized in food analysis is Raman spectroscopy. When a monochromatic laser light interacts with the sample, light scattered in all directions (Schrader 1994). The majority of the scattered light has the same frequency (energy) as the incident light, which is called Rayleigh scattering (Figure 1-6). Only about 1 in 10^7 of the incident photons undergo Raman scattering, or exhibit a shift in the frequency (energy) (McCreery 2000).

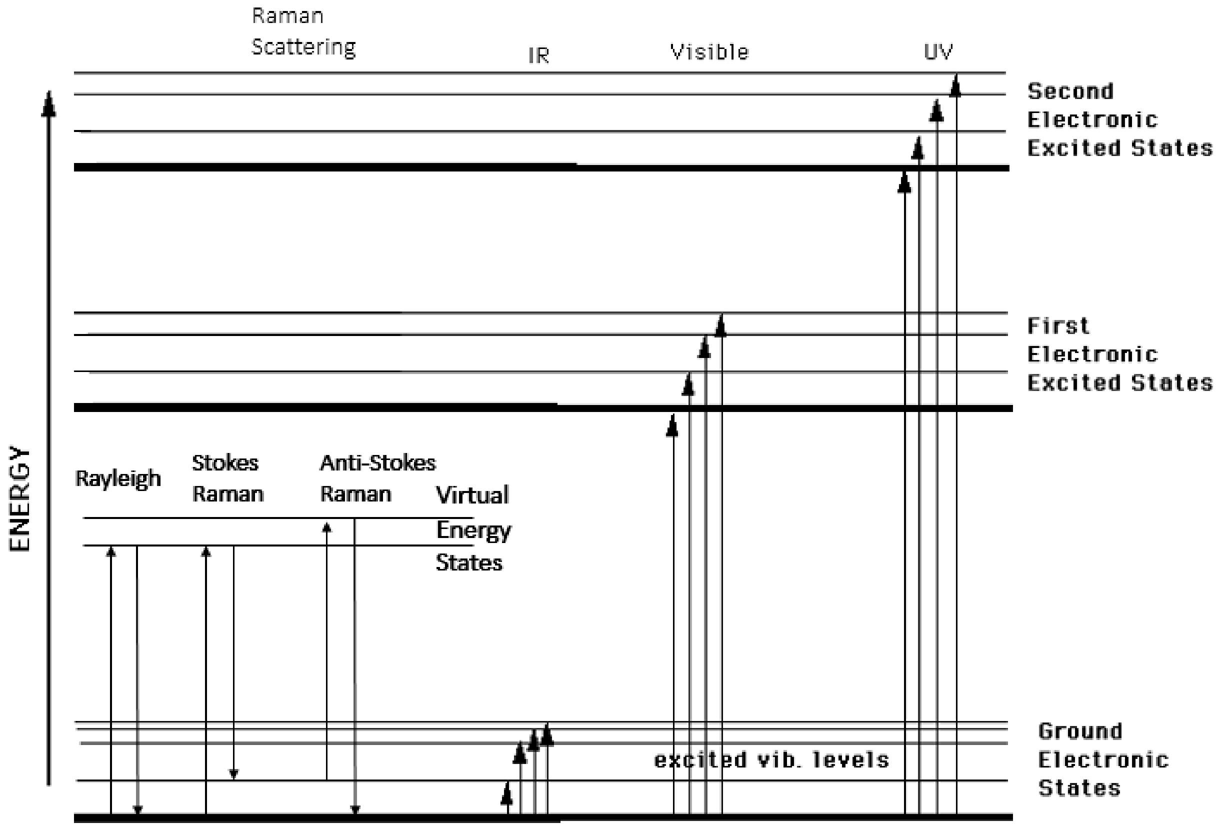

Figure 1-6 Energy level diagram showing the states involved in Raman, Infrared, UV and Visible signal. Adapted from Schwartz et al. 1994.

The frequency (energy) shift is resulted from an inelastic collision between the transfer of energy from the incident light photon to the molecule, corresponding to the vibrational or rotational level energy difference in molecule (Skoulika et al. 2000; Moreira et al. 2008). If the molecule gains energy, scattered photon is shifted to lower frequency, so that the energy remains the same, and give rise to Stokes Raman shift; when the molecule loses energy, higher frequency scattered light occurs, and this is anti-Stokes Raman shift

(Moreira et al. 2008; Nunes 2014). A change in molecular polarizability is required to obtain Raman shifts, and various frequency shifts associated with different molecular vibrations can then be processed and presented as spectra, providing qualitative information about the molecular bonds and the vibrational modes in the sample (Larkin 2011). In addition, Raman spectrum can also perform quantitative analysis as the intensity of an analyte's Raman band is linearly proportional to the analyte concentration (McCreery 2000; Jancke et al. 2005). One advantage of using Raman spectroscopy is its good compatibility with aqueous systems. Because O-H bonds are weakly polarizable, water is an ideal solvent to dissolve samples without interfering important chemical information (Bumbrah and Sharma 2016). In food application, it has been utilized for both solid and aqueous samples, offering chemical changes during processing, classification of different products, and identification of the internal components (Yang and Ying 2011).

1.3.5 Raman Instrumentation

A typical Raman spectrometer consists of four major components: an excitation laser source, sampling illumination system and light collection optics, a wavelength selector, and a detector (Chalmers et al. 2012). A sample is exposed with a monochromatic laser beam in the UV, visible or near infrared range. The scattered light is collected with an optic system and is passed through an interference filter to obtain a Raman spectrum (Princeton Instruments 2012). The laser source can be one of the Argon ion laser (488nm), Krypton ion laser (530.9nm), Helium-Neon (632.8nm), Near infrared diode laser (785 and 830nm) or Neodymium–Yttrium Aluminum Garnet (Nd:YAG) (1064nm), depending on the needs for the application (Bumbrah and Sharma 2016). Raman scattering intensity is proportional to laser wavelength to the negative fourth power (λ^{-4}), meaning that longer wavelength laser will result in a decrease in Raman intensity, vice versa. However, Raman spectrophotometers that use near IR laser (1064nm) source can minimize the fluorescence interference that would normally mask Raman signal, because NIR excitation is lower in energy that most of the electronic transitions responsible for fluorescence are not excited (McCreery 2000) (Figure 1-7).

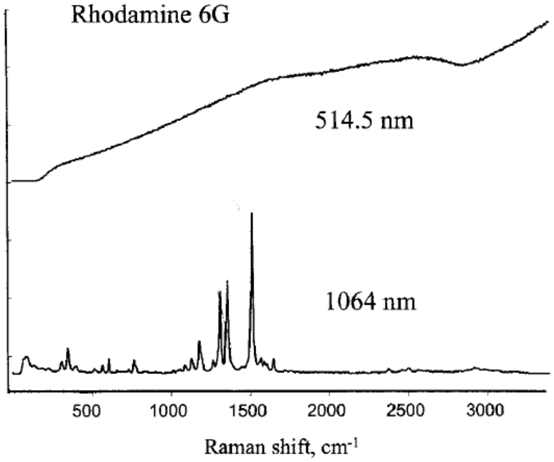

Figure 1-7 Spectra of rhodamine 6G dye obtained with a 514.5nm laser (upper) or an FT-Raman spectrometer and 1064 nm laser (lower) (McCerry 2000)

In addition, Raman spectroscopy equipped with a 1064nm laser can decrease incidents of photolytic degradation of samples, allowing the usage of higher powered laser, nevertheless, the spectral resolution would be poorer, and samples could self-absorb energy and overheat (Baranska et al. 2006; Moreira et al. 2008).

To combat these downfalls, several techniques have been proposed, such as increasing laser excitation power, using a tight focus, performing multiple passes through the sample, or improving detector quantum yield (Schwab and McCreery 1987).

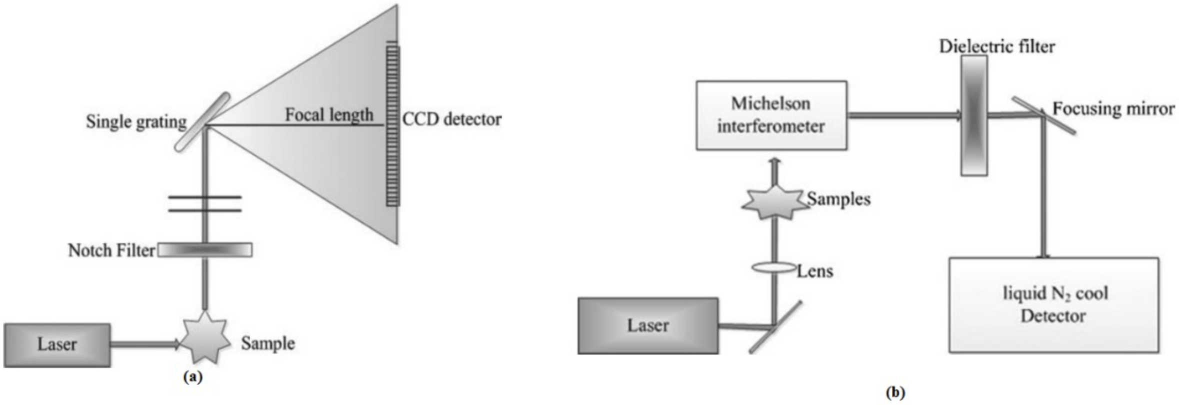

Figure 1-8 Schematic diagram of a typical (a) dispersive Raman spectrometer and (b) FT-Raman spectrometer (Yang and Ying 2011).

A dispersive Raman spectrometer (Figure 1-8a) uses a grating to disperses light to a detector and a notch filter is typically used to reduce intense Rayleigh scattering and the stray light (created upon light dispersion on grating) that can mask Raman scattering, while a FT-Raman spectrometer (Figure 1-8b) uses an interferometer as the light collection optics. For the detector, a charge-coupled devices (CCD) is typically used for laser light source less than 1000nm and an Indium–Gallium–Arsenic (InGaAs) detector is used with a 1064nm laser source (Bumbrah and Sharma 2016). To enhance the inherently weak Raman scattering, Resonance Raman Spectroscopy (RRS), Surface-Enhanced Raman Spectroscopy (SERS), Surface-Enhanced Resonance Spectroscopy (SERRS), and Coherent Anti-Stokes Raman Spectroscopy (CARS) were developed to improve the sensitivity (Princeton Instruments 2012; Bumbrah and Sharma 2016). More intense Raman spectrum is obtained in RRS because the frequency of incident radiation matches with an electronic transition of molecule (Bumbrah and Sharma 2016). SERS utilizes a colloidal metallic surface (silver, gold or copper) to enhance the polarizability and electrical field (Walia et al. 2015). In CARS, two strong collinear lasers shine the sample, instead of one laser, and the Raman signal is enhanced when the frequency difference between the two lasers equals the frequency of the Raman active vibrational mode (Version 2015).

1.3.6 Comparison of Infrared and Raman Spectroscopy

Both IR and Raman spectroscopy techniques allow rapid and close-to-real-time analysis of sample (Aykas et al.; Moreira et al. 2008; De Nardo et al. 2009; Fu et al. 2016). IR and Raman spectroscopy are used as complementary techniques, in that vibrations that are strong in an infrared spectrum are typically weak in a Raman spectrum, vice versa (Figure 1-9). IR shows intense signals with polar functional groups, while Raman is more sensitive to backbone structures and symmetric bonds that involve changes in molecular polarizability (Schrader 1994; Thygesen et al. 2003). Both IR and Raman techniques provide important chemical information useful for food industry because both show characteristics absorbance bands (O-H, C-H, C-O, and N-H, etc.) representing important food constituent such as water, fat, protein and carbohydrates, albeit different intensities. Raman spectroscopy gives advantage of better spatial resolution ($\geq 1 \mu m^2$) compared to infrared ($\geq 10 \mu m^2$) (Moreira et al. 2008). In addition, Raman spectroscopy allows confocality, meaning it can focus on different planes below surface (Thygesen et al. 2003).

Thus, it provides the convenience of taking measurement of sample within the original container without contamination.

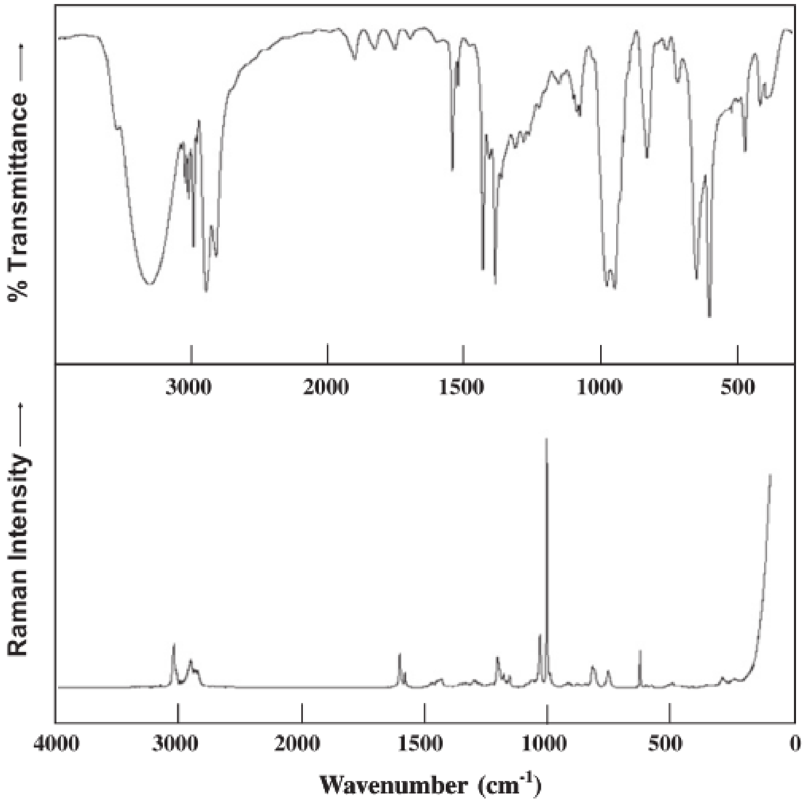

Figure 1-9 Infrared (upper) and Raman (lower) spectra of liquid 3-phenyl-1-propanol (Badawi and Forner 2011).

Furthermore, because water has low polarizability, Raman technique eliminates sample drying preparation that's needed for infrared spectroscopy measurement, where artifact can be generated during drying (Li-Chan 1996). However, Infrared spectroscopy provides better signal-to-noise ratio and fluorescence would not be an issue, while if the Raman instrument utilizes lower wavelength laser source (e.g. 785nm or 830nm), fluorescence can be detrimental when obtaining Raman spectrum (Thygesen et al. 2003; Kerr et al. 2015).

1.3.7 Portable Instrumentations

Recently, with advancement in micro-electro-mechanical system (MEMS) technology, it became possible to miniaturize hardware size of large, stationary spectroscopic instruments to portable and/or handheld units that perform comparable with benchtop devices (Kenda et al. 2009). MEMS is the integration of mechanical elements, sensors, actuators, and electronics on a common silicon substrate through micro-fabrication technology (Li-Chan et al. 2010). These portable systems are able to measure spectra at millisecond time resolution or co-add several scans to one spectrum, generating good signal-to-noise ratio (Kenda et al. 2009). Furthermore, portable and handheld devices make rapid and precise on-line food analysis at a production plant or on the field possible for food industry (De Nardo et al. 2009; Lin et al. 2014; Ayvaz et al. 2016a).

The Agilent 4500 Series Portable FTIR Spectrometer (Figure 1-10) is designed by Agilent Technologies to support on-site analysis of materials and finished products in the chemical, food, and polymer industry (Agilent Technologies 2017). The device is compact, lightweight (~6.8kg), and rugged for high quality and fast analysis in non-lab environments.

Figure 1-10 The Agilent 4500 series portal FTIR spectrometer (Agilent Technologies, 2017).

The 4500 series can be configured with different sampling interfaces: DialPhath, TumblIR transmission (4500t), and Spherical Diamond ATR (4500a), which was utilized in this study. The 4500a device uses a diamond-ATR crystal with options of multiple-bounce-reflections to allow for the analysis of solids, liquids, pastes, gels and powders with minimal or no sample preparation required.

The Progeny™ Analyzer (Figure 1-11) is the world's first completely handheld Raman spectrometer designed for incoming material receiving, laboratory analysis and non-lab environment to rapidly conduct quality assurance and control analysis for food, pharmaceutical, nutraceutical, and cosmetic ingredients industry (Rigaku Corporation 2017). The Progeny analyzer utilizes a unique 1064nm excitation laser to avoid fluorescence interferences and it allows for analysis through the container or packaging material. The handheld spectrometer possess a user-friendly interface with a touchscreen and large buttons, which delivers one-handed operation and allows for the possibility of 100% inspection.

Figure 1-11 The handheld Progeny™ Raman analyzer (Rigaku Corporation 2017).

1.3.8 Rapid Analysis of Carotenoids in Food

De Nardo *et al.* conducted a study using a benchtop ATR-IR combined with PLSR to create models that were able to directly and rapidly determine lycopene and β-carotene in tomato juice with good prediction performance (R values >0.9) and low standard error of cross validation (0.4 and 0.05 mg/100g for lycopene and β-carotene, respectively). However, lipid extraction step was needed to provide improved carotenoid qualitative analysis, as the region between 1770 and 1720 cm^{-1} associated with carbonyl ester (C=O) stretching vibration of triglycerides gave the most discriminating power in classification (De Nardo et al. 2009). Rubio-Diaz *et al.* profiled carotenoids from genetically-diverse tomatoes and intensively investigated how processing stages and storage time affect carotenoid content in juices processed from different variety of tomatoes using a benchtop ATR-IR. The intensity of the IR band at ~960 cm^{-1} representing carotenoid increased after hot-break (93°for 5 min), decreased after canning, and increased again during one-year storage. In addition, SIMCA model was able to discriminate different varieties with tight and well-separated clusters. Nevertheless, to obtained better results, extraction of lipid fractions was done for quantification and qualification (Rubio-Diaz et al. 2010a, b). For analysis using

Raman spectroscopy, Oliveira *et al.* summarized and directly collected spectra using a benchtop Raman spectrometer using a 1064nm laser without any sample preparation on 43 samples of biological samples that contain carotenoids. The researchers observed characteristic carotenoid bands representing v_1(C=C), v_1(C-C), and p(C-CH_3) with varying wavenumbers in papaya, tomato, strawberry pulp, papaya, mango, orange rind, yellow passion fruit pulp, maize, nectarine, sweet potato, etc. It was found C=C vibration shows greater sensitivity and variation toward identifying the kind of carotenoids, ranging from 1510 cm^{-1} to 1536 cm^{-1}, indicating the complexity and the interaction between organic food matrix and cellulosic moieties (de Oliveira et al. 2010). Schulz et al. observed gradually increasing carotenoid content from periderm toward the core in carrot roots by using a benchtop NIR-FT-Raman (1064nm laser) spectroscopy. The group reported Raman bands assignment at 1520 cm^{-1} to β-carotene, 1510 cm^{-1} to lycopene, and 1527 cm^{-1} to α-carotene (Schulz et al. 2005). Qin *et al.* used a benchtop spatially-offset Raman with 785nm laser Raman system to evaluate internal maturity of tomatoes and found the peaks at 1001 cm^{-1} and 1151 cm^{-1} remained consistent and the third peak associated with v (C=C) gradually shifted from 1525 cm^{-1} (lutein at mature green stage) to 1513 cm^{-1} (lycopene at red stage) due to the loss of lutein and β-carotene and the accumulation of lycopene during tomato ripening (Qin et al. 2012). A study from Baranska et al. identified characteristic market bands and determined the content of lycopene and β-carotene in tomato products using benchtop FT-Raman, ATR-IR and NIR systems spectroscopy combined with multivariate analysis (Baranska et al. 2006). A recent study conducted by Trebolazabala et al. used a portable Raman spectroscopy using a 785nm laser to monitor the ripening of tomatoes where they found an increased intensity of lycopene and decreased presence of chlorophyll and cuticular waxes during ripening. Moreover, phytofluene which is a transition pigment in carotenoid biosynthetic pathway, was higher in middle ripening stage (Trebolazabala et al. 2017). Some studies regarding quantification analysis of carotenoids using Raman spectroscopy were done on 31 lines of carrots and PLSR model created from Raman band at 1520 cm^{-1} gave R value of 0.87 (Killeen et al. 2013). Another quantification study was conducted on processed sweet potatoes (n=30) using a benchtop Raman spectrometer with a 532 nm laser system and obtained a PLSR model with R value of 0.93 (Sebben et al. 2018). A preliminary study done by Bhosale et al. used a benchtop Raman spectrometer with a 488nm laser system and found positive correlation in Raman signals and carotenoid content measured by HPLC in different varieteis of tomatoes (n=10) at different ripening stages; nevertheless, a quantitative model was not created (Bhosale et al. 2004). Another study by Fu et al. utilized a Raman spectroscopy containing a 785 nm laser source and obtained a PLSR model with R values of only 0.50 (Fu et al. 2016). In another word, there is limited research on quantitative and profiling of carotenoids in tomatoes and tomato based products and Raman spectroscopy has shown promising potential in identifying different carotenoids and quantifying lycopene levels in tomatoes without solvent extraction. This study will be one of the first research to provide quanlitative and quantitative results by utilizing a handheld Raman spectrometer with a 1064 laser source in rapid and nondestructive carotenoid analysis without extraction steps.

1.4 Chemometrics

Chemometrics uses statistical methods to design or select optimum procedures in order to provide the maximum chemical information by analyzing chemical data (Varmuza and Filzmoser 2009). Food exists as a complex matrix, so the IR or Raman spectra contain complicated peaks or variables about all compounds in food with overlapping peaks and noises, meaning they are inherently multivariate. Hence, more complicated solutions to the Beer-Lambert relationship are required. Because traditional statistical analysis techniques cannot handle this great amount of information, multivariate analysis is then necessary to efficiently extract the maximum useful information from data.

Multivariate analysis can be supervised or unsupervised. Unsupervised pattern recognition such as clustering is often used in exploratory phase where information about the samples and target analytes is limited. While, supervised methods include regression and classification where prior information about the samples exist. Supervised pattern recognition techniques use the known class membership of the samples to in order to quantatively predict and classify new unknown samples on the basis of its pattern measurements (Berrueta et al. 2007). The general steps (Figure 1-12) are as followed: (1) select calibration and validation samples where the calibration set is to train and optimize the parameters of the model; (2) select variables that contain the aim of the model and exclude noises with low discrimination power; (3) build a model using the calibration set by analyzing multivariate correlations between the spectra collected and the known class or quantitative parameter; (4) validate the model using the validation set to evaluate the reliability of the regression or classification (Berrueta et al. 2007).

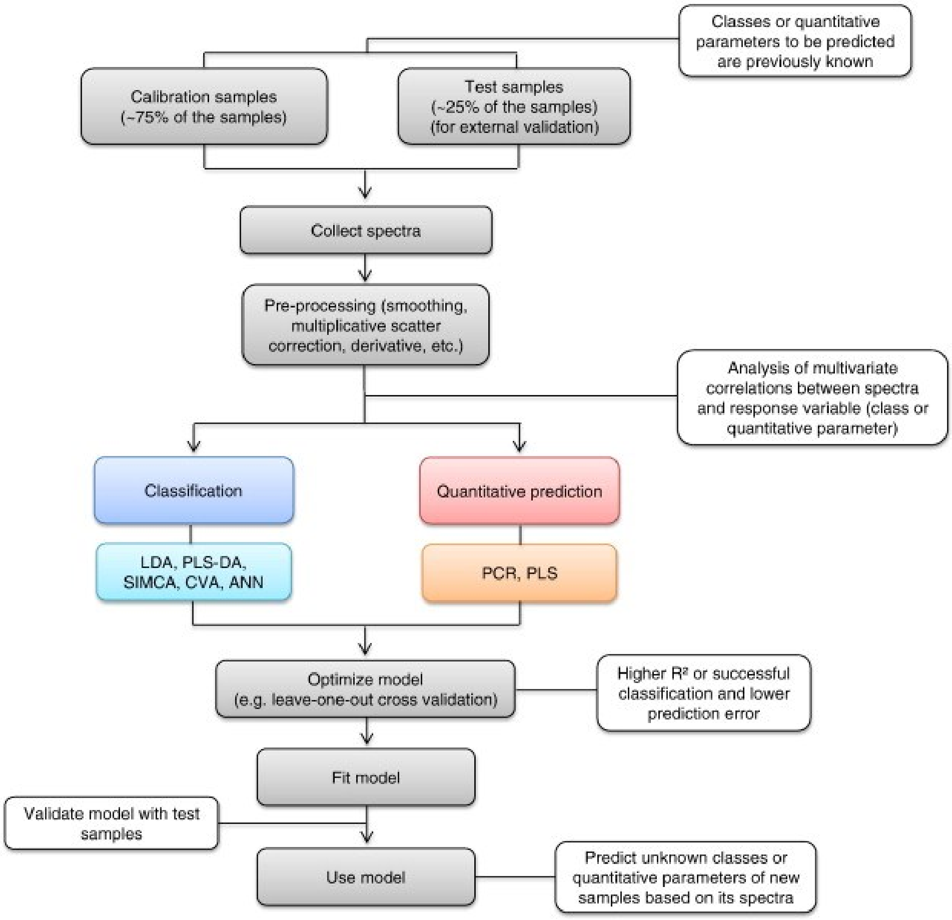

Figure 1-12 Scheme of a chemometric method to predict quantitative parameters or assess class based on spectroscopic data (Nunes 2013).

For quantitative prediction purpose, Principal Component Regression (PCR) and Partial Least Square Regression (PLSR) have been applied in food analytical research. Both techniques model a response variable (y-variable) by reducing numbers of predictor variables (x-variable) and construct a new set of predictor variables, known latent variables or component, as linear combinations of the original predictor variables. The difference lies in how the method construct those components. PCR creates components to explain the variances solely in the predictor values, without considering the response variable. While, PLSR accounts for the relation to the response variables, and is able to develop models that utilizes fewer component (Varmuza and Filzmoser 2008a).

For classification purposes such as detecting adulterated verses unadulterated samples, Linear Discriminant Analysis (LDA), Partial Least Squares Discriminant Analysis (PLS-DA), K-Nearest Neighbors (KNN) or Soft independent modeling of class analogy (SIMCA) were previously used in food analysis. Partial least-squares regression (PLSR) and Soft independent modeling of class analogy (SIMCA) are two commonly used supervised classification methods utilized in food analysis and were used in this study (Nunes 2014).

1.4.1 Partial Lease Square Regression (PLSR)

Partial least-squares regression (PLSR) is widely used in chemometrics to generate mathematical models that are capable of predicting the values of important properties that cannot be directly measured (Varmuza and Filzmoser 2009). This technique takes information-rich spectrum data and reduces the dimensionality and eliminate data noise, resulting in more accurate and reproducible calibration models (Wold et al. 2001).

PLSR is a bilinear regression method that compresses a large number of independent variables, which account for variation in the data, into smaller number of uncorrelated latent variables that are linear combinations of the spectral frequencies and use these factors to predict the analyte’s concentration (Varmuza and Filzmoser 2008a). Researcher have used this technique to quantify soybean oil, palm oil and hydrogenated vegetable fat in waste frying oils based on ATR-IR spectra and obtained R-value of 0.99 and standard error of 2.8-5.5% (Hocevar et al. 2012). Aykas and Rodriguez-Saona also used this statistical method to monitor oil quality parameters such as peroxide value, p-anisidine value and free fatty acids by using a portable FT-IR spectrometer with R values > 0.93 and low standard error (Aykas and Rodriguez-Saona 2016b). Another study used a visible-NIR spectrometer to measure °Brix in navel orange fruit with R value of 0.91 and standard error of 0.71 °Brix (Liu et al. 2010). Lin et. al quantified sugar levels in 88 different infant cereals samples by using portable mid- and near-infrared spectrometers and obtained R values of 0.96 and standard error of 3.7% (Lin et al. 2014). PLSR has shown to be a great statistical tool to analyze complex spectral data to assess and quantify quality parameters in food.

1.4.2 Soft Independent Modeling Classification (SIMCA)

Soft independent modeling of class analogy (SIMCA) is the most used class-modeling technique (Berrueta et al. 2007). SIMCA can be used as a discriminant technique to evaluate the ability of the spectroscopic spectra to discriminate among different groups (De Nardo et al. 2009). In SIMCA, each class is independently modeled using principal components, which are determined by cross-validation (Berrueta et al. 2007). SIMCA creates a three-dimensional model over the first three principle components that explain the greatest amount of variation within each class (Subramanian and Rodriguez-Saona 2009b) and enables clustering of samples based on sample similarity and differences while reducing noises. The interclass distances can be calculated as the geometric distances between PC of each class. In addition, each class is bounded by a region, which represents

95% of confidence level. The discriminating power measures how well a variable (spectral wavelength) discriminates between two classes. Moreover Coomans plots can also be used to discriminate between two classes and identify outliers. The distance from the model for class 1 is plotted against that from model 2. The plot divides samples into four quadrants: class 1, class 2, overlap of both classes, and the outlier zone (Varmuza and Filzmoser 2008a; Nunes 2014). The ultimate goal of SIMCA is to create a reliable model to correctly classify new samples into their corresponding category. SIMCA has been used in detecting adulteration in sesame oil with vegetable oils based on FT-IR spectral information (Deng et al. 2012); classifying ten different types of frying oils extracted from 95 commercial potato chips samples (Aykas and Rodriguez-Saona 2016b); characterizing and differentiating between organic and conventional bovine butter (Plans Pujolras et al. 2015).

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Chapter 2 : Rapid Analyses of Carotenoids in Tomato Paste Using Handheld Raman Spectroscopy

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2.1 Abstract

The objective of this study was to develop reliable, fast and non-destructive protocols for analyses of carotenoids in tomato paste by Raman spectroscopy combined with pattern recognition analyses. Tomato paste samples were kindly provided by tomato processing companies in California and different carotenoid varieties of tomatoes were obtained from the OSU Tomato Genetics and Breeding Program at Wooster, Ohio. Spectra of tomato paste were directly collected, without any sample preparation steps, with a handheld Raman spectrometer equipped with a 1064nm laser. Lycopene concentration in red tomato paste was determined by UV-Vis spectrophotometry at 503nm. HPLC equipped with a photodiode array detector was utilized for carotenoid profiling. Raman spectra were evaluated by pattern recognition analyses, including quantification by partial least square regression (PLSR) and classification by soft independent modeling of class analogy (SIMCA). Raman spectra of red tomatoes showed unique marker bands at 1510 cm^{-1} (C=C stretching), 1156cm^{-1} (C-C stretching), and 1002cm^{-1} ($C\text{-}CH_3$ in-plane rocking) that are characteristic of the long conjugated polyene chain of lycopene. Cross-validated (leave-one-out) PLSR model (3 latent variables) predicted lycopene content with a standard error of cross-validation (SECV) of 4.7 mg/100g and a coefficient of determination of 0.96. Screening of tomato paste from varieties that accumulate a diverse carotenoid profile showed a unique region associated with C=C stretching that changed according to the dominant carotenoid: all-*trans*-lycopene at 1511 cm^{-1}, β-carotene at 1521 cm^{-1}, and tetra-*cis*-lycopene at 1525 cm^{-1}. The unique Raman spectral signatures allowed SIMCA to classify the tomato paste into four classes based on their carotenoid profile without any

misclassification, producing tight and well-separated clusters that can be used for predicting unknown samples. Raman spectroscopy is positioning as an attractive fingerprinting technique for tomato industry because it offers portable and ruggedized instrumentation for field deployment and requires little to no sample preparation to rapidly and accurately assess carotenoid quality and quantity in tomato-based products.

2.2 Introduction

The United States is the second world’s largest producers of tomatoes (*Lycopersicon esculentum*), producing approximately 35 billion pounds of tomatoes that accounted for over $2 billion in annual farm cash receipts in 2016 (USDA Economic Research Service 2017). Tomatoes are attracting public’s attention as “superfoods” because more research have found the possible health benefits that the major pigments carotenoids can offer to reduce the risk of chronic diseases, such as cardiovascular diseases and prostate cancer, due to their antioxidant capacity (Johnson 2000; Khachik et al. 2002; Çapanoğlu 2008; Story et al. 2010; Saini et al. 2015). Carotenoids are 40-carbon isoprenoid derivatives and lipid-soluble pigments with colors ranging from yellow to orange to red due to their long conjugated polyene backbone (Sigurdson et al. 2017). The characteristic red color of traditional tomatoes is the result of *trans*-lycopene, accounting for 83% of total carotenoids (Shi and Le Maguer 2000). Moreover, the minor and trace carotenoids are also gaining attention: *β*-carotene, which is the minor carotenoid in red tomatoes, can be converted to vitamin A in human body; tetra-*cis*-lycopene has found to be more bioavailable than the predominant all-*trans*-lycopene in red tomatoes (Burri et al. 2009; Cooperstone et al. 2015; Luterotti et al. 2015). Thus, there has been an increasing interest in breeding unique lines of tangerine (orange colored) tomatoes: *β*-carotene dominant, tetra-*cis*-lycopene dominant, and δ-carotene dominant tomatoes (Unlu et al. 2007a, b; Rubio-Diaz et al. 2010a; Yoo et al. 2017). Current methods to accurately monitor carotenoids in tomatoes rely on UV-Vis spectrophotometer or by HPLC (Rivera and Canela-Garayoa 2012; Amorim-Carrilho et al. 2014a; Saini and Keum 2018). However, these techniques require highly trained personnel and substantial amount of sample preparation that needs the use of hazardous organic solvents. Most importantly, the traditional methods are time-consuming and are not suitable for routine “in-situ” quality control purpose.

Thus, there is a need to develop simple, fast, robust and more economical methods to analyze carotenoids in tomato-based products. Vibrational spectroscopy have shown promising potential in profiling chemical components without the need of time-consuming preparation steps (Wilkerson et al. 2013; Ayvaz et al. 2016). Studies regarding rapid detection and quantification of lycopene in tomato-based products using benchtop mid-infrared equipped with an attenuated total reflectance accessory (ATR-IR) and benchtop Raman instruments have been evaluated. However, lipid extraction step was needed to create classification models from unique ATR-IR signature marker bands associated with carotenoids without the interferences of carbohydrates and moisture (De Nardo et al. 2009; Rubio-Diaz et al. 2010a). In terms of rapid carotenoid analysis in tomatoes using Raman spectroscopy, the research is limited. Studies have used benchtop Raman spectrometers to identify signature Raman bands associated with different carotenoids in tomatoes (Schulz et al. 2005; Qin et al. 2012; Vijayalakshmi et al. 2015), and correlateed changes in carotenoid contents with Raman signals during tomato fruits ripening (Fu et al. 2016; Trebolazabala et al. 2017). Another study created a partial least square regression (PLSR) model to quantify β-carotene and lycopene content in tomato based products using a benchtop FT-Raman system (Baranska et al. 2006); however, only a limited number of samples (n=28) were employed in developing a PLSR model that required 11 factors to explain the variance which can result in over-fitting problems (Tobias 1995); in the addition, the standard error of the model was large.

Recently, with advancement in micro-electro-mechanical system (MEMS) technology, it became possible to miniaturize hardware size of large, stationary spectroscopic instruments to portable and handheld units without compromising performance (O'Brien et al. 2012). MEMS is the integration of mechanical elements, sensors, actuators, and electronics on a common silicon substrate through micro-fabrication technology (Li-Chan et al. 2010). These portable systems are able to measure spectra at millisecond time resolution or co-add several scans to one spectrum, generating good signal-to-noise ratio (Kenda et al. 2009). In addition, these portable vibrational spectrometers can make rapid, precise and cost-effective in-line food analysis at a production plant or on the field possible for food industry (Lin et al. 2014; Ayvaz et al. 2016b; Trebolazabala et al. 2017); nevertheless, no study has been published to quantify carotenoid content and classify different carotenoid varieties tomatoes using a portable/handheld Raman unit.

To our best knowledge, this research will be one of the first studies utilizing a handheld Raman spectrometer to conduct extensive quantitative and classification analysis on a large number of tomato paste samples. The hypothesis is that Raman spectroscopy combined with multivariate analyses can achieve quantification and classification of carotenoids because its sensitivity toward molecular structure with strong polarizability which provides sensitive and specific spectral information regarding carotenoids in tomatoes (Bumbrah and Sharma 2016). Moreover, the Raman technique will be a rapid and non-destructive method because Raman spectroscopy poses advantages of moisture being Raman insensitive, the near infrared laser equipped with the handheld Raman spectrometer minimizes fluorescence interferences, and Raman spectroscopy has the ability to measure through containers (Li-Chan 1996; Bhosale et al. 2004; Moreira et al. 2008). The significance of the study is that rapid analysis of carotenoids by Raman spectroscopy can expand the market and provide added values of health benefit to carotenoid-rich agricultural commodities. For the tomato growers and breeders, the technique will provide an efficient tool to aid in selection of tomato varieties of specific carotenoid traits. The study will also be a first step to provide a real-time assessment regarding the processing parameters critical of retaining these bioactive components carotenoids in tomato-based products.

The objective was to develop robust and rapid methods for carotenoid quantification and classification analyses in tomato paste using handheld Raman technology combined with multivariate analyses.

2.3 Materials and Methods

2.3.1 Tomato Paste Samples

A total of 123 concentrated red tomato paste samples were generously provided by several major tomato processors in California. They were stored in vacuum sealed packaging at -18°C before analysis. In addition, fresh tomato fruits (n=50) with a varied carotenoid profile were generously provided by the OSU Tomato Genetics and Breeding Program (n=39) at Wooster, Ohio and supplemented (n=11) by purchasing assorted variety packs from groceries stores (Columbus, OH). These varieties were developed by selective breeding to combine specific naturally occurring alleles of genes affecting the biosynthesis of carotenoids. The fruits were blended into juices and centrifuged at 4,000rpm for 20 minutes (Sorvall ST 8 Centrifuge, Thermo Scientific, MA) to obtain the paste at the bottom of the centrifuge tubes.

2.3.2 Carotenoids Extraction from Tomato Pastes

Carotenoids extraction in concentrated tomato pastes and fresh tomatoes was done according to Anthon and Barret and Fish et.al with some modifications (Fish et al. 2002; Anthon and Barrett 2007b). Tomato paste (~0.02g of concentrated tomato paste or ~0.1g of paste from different carotenoid varieties tomatoes) was transferred in a 15mL centrifuge tube. 500μL of water was added and the tube's contents were vortex for 30 sec. Then, 8mL of hexane/ethanol/acetone solvent (HEA) 2:1:1 (v/v/v) was added and immediately vortex for 30 seconds. Acetone is important in the extraction solvent because its water-miscibility allows for complete penetration of high water content of tomatoes. Then, the sample was stored in the dark for 20 minutes to allow for complete extraction (the residue would appear to be white.) Next, 500μL of deionized water was added to induce phase separation and immediately vortex for 30 seconds. The sample was left to rest for 10 minutes. Finally, the upper yellow carotenoid lipid fraction was collected for UV-Vis spectrophotometry and HPLC reference analyses.

2.3.3 UV-Visible Spectrophotometric Reference Analysis of Lycopene Content in Concentrated Tomato Paste

Lycopene concentration in tomato paste samples was determined according to Anthon and Barrett (Anthon and Barrett 2007a) with some modification. A Cary 50 UV-Vis spectrophotometer (Agilent Technologies Inc., Santa Clara, CA) was used to measure the absorbance of lycopene extract fraction (see section 2.3.2). Absorbance readings at a single wavelength were taken using SimpleRead Cary WinUV software. Approximately 3mL of HEA solvent was added to a 3.5mL, 1cm path length quartz cuvette (Agilent Technologies Inc., Santa Clara, CA) to zero the instrument, then discarded. ~3mL of lycopene extract was transferred to the cuvette and absorbance measurement was taken at 503 nm. The cuvette was washed with pure acetone three times in between measurements to prevent cross-contamination. Calculation of lycopene concentration was based on

$$Lycopene\left(\frac{mg}{100g}\right) = A503 \times 537 \times 8 \times 0.55/(wt\ of\ sample \times 172) \div 10$$

Where 537g/mole is the molecular weight of lycopene, 8mL is the volume of HEA solvent, 0.55 is the volume ratio of the upper layer to the mixed solvents, and 172 mM^{-1} is the extinction coefficient for lycopene in hexane (Anthon and Barrett 2007b). Triplicate measurements were collected for each sample.

2.3.4 HPLC Reference Analysis for Fresh Tomatoes

Carotenoid profiles of different carotenoid varieties tomatoes were determined by a reverse phase HP1050 HPLC system equipped with a photodiode array detector (Agilent Tech, Palo Alto, CA). A C30 YMC column (5μm particle size, 250×4.6 mm) (Waters

Corp., Milford, MA) was used for all separation. 3mL of the upper carotenoid extract fraction (see section 2.3.2) was transferred into an amber glass tube and dried under nitrogen flush. The extract fraction aliquots were redissolved in 3mL mixture of 1:1 methanol/methyl tert-butyl ether (MTBE) solvent, and filtered using a PTFE membrane filter (30 mm diameter, 0.45µm pore size, ThermoFisher, MA) into an amber HPLC vial (Thermo Fisher Scientific, MA). The HPLC conditions reported by Rubio-Diaz et. al (Rubio-Diaz et al. 2010b) were used. Elution solvents were (A) 81% methanol, 15% MTBE, and 4% water and (B) 90% MTBD, 7% methanol, and 3% water. All solvents were HPLC grade. Separation was done with a 75 min linear gradient (0-88% B). The flow rate was 1.0 mL/min with detections at 450nm (*β*-carotene, δ-carotene, tetra-*cis*-lycopene, *cis*-lycopene isomers), and 470 (all-*trans*-lycopene). Carotenoids were tentatively identified using retention times, order of elution, and UV-Vis absorption spectra. Duplicate analyses were performed.

2.3.5 Direct Determination of Lycopene content in Tomato Paste Samples Using Mid-infrared Spectroscopy as Complementary Technique

The spectrum of concentrated tomato paste sample was directly collected without prior sample preparation step using an Agilent 4500a portable Fourier transform mid-infrared (FTIR) system coupled with a triple bounce diamond attenuated total reflectance (ATR) sampling accessory (Agilent Technologies Inc., Santa Clara, CA). A zinc selenide (ZnSe) beam splitter and a thermoelectrically-cooled deuterated triglycine sulfate (dTGS) detector were used in the instrument. The spectrum was collected from 4000 to 650 cm^{-1}

wavenumbers with a 4cm^{-1} spectral resolution and 64 scans of interferograms were co-added to increase the signal-to-noise ratio. A background spectrum was collected each scan to correct for the collection variations. Approximately 0.02 grams of each tomato paste samples was directly applied onto and covered the ATR diamond crystal. The crystal was cleaned with 70% ethanol and dried with Kimwipe tissue in between every scan. For each sample, triplicate scans were taken and each scan took one minute. Agilent MicroLab PC software (Agilent Technologies Inc., Danbury, CT) was used to execute the scans.

2.3.6 Direct Carotenoid Analysis in Tomato Pastes by Handheld Raman Spectroscopy

A handheld ProgenyTM Raman spectrometer (Rigaku Analytical Devices Inc., Wilmington, MA) was used to directly collect Raman spectra of concentrated tomato paste samples and paste samples from different carotenoid varieties of fresh tomatoes. The Raman spectra of concentrated tomato paste were collected through quartz glass. The paste made from different carotenoid varieties of tomatoes were patted dried using filter papers and placed on a small rounded filter paper, flattened and wrapped with polyethylene plastic wrap. The sample was placed tightly against the laser opening of the Raman spectrometer. The handheld Raman spectrometer has a near infrared neodymium-doped yttrium aluminum garnet (Nd:YAG) laser light source at 1064nm was used to avoid florescence interferences and the laser power output is adjustable from 3mW to 490mW. The laser focuses at a distance of 11mm with the focused spot of 25 microns in diameter. Spectrum was collected from 2500 to 200 cm^{-1} wavenumbers with a 8-11cm^{-1} spectral resolution. A transmissive volume phase grating was used to diffract different wavelengths of light from the monochromatic laser light source. The detector was a 512 pixel thermoeletrically cooled indium gallium arsenide (InGaAs) detector. The laser power, exposure time, averages (number of times the collections take place during one scan), and the focal position can be adjusted to optimize Raman spectra to reduce noise and generate optimal peak intensity. For concentrated tomato paste samples, a power of 490mW, exposure time of 2000ms, averages of 30 collections per scan, and focal position of 3 were used to collect Raman spectra. For paste made from varieties of tomatoes, the laser power was 490mW, exposure time was 1000ms, the averaged collections per scan was 30, and the focal position was at 2.5. Triplicate measurements were taken. A background scan was taken every scan to minimize the effect of possible instrument drift. Each scan took four minutes.

2.3.7 Multivariate analysis: Partial Least Square Regression (PLSR) and Soft Independent Modeling of Class Analogy (SIMCA)

Multivariate analysis software Pirouette for Windows Chemometrics Modeling Software, version 3.11 (Infometrix, Inc. Bothell, WA) was used to analyze spectral data. Two multivariate analytical techniques were used in this study: Partial Least Square Regression (PLSR) and Soft Independent Modeling of Class Analogy (SIMCA). PLSR was used to predict lycopene concentration in concentrated tomato paste samples by generating a calibration model to correlate reference lycopene content values obtained from UV-Vis spectrophotometry with spectral data from the spectroscopic (ATR-IR or Raman) method.

PLSR is a bilinear regression method that compresses a large number of independent variables, which account for variation in the data, into smaller number of uncorrelated latent variables that are linear combinations of the spectral frequencies. Then these latent variables are used to predict the analyte's concentration (Varmuza and Filzmoser 2008a). The Raman spectra data were processed by smoothing and second derivative at 35 points window and three factors were utilized to explain the maximum variances in creating the model. The infrared spectra were also processed by smoothing and second derivative at 35 points windows but six factors were used to create a PLSR model. Models were validated using a leave-one-out approach. The optimal number of latent variables (factors) that explained the most of the variance in the model were selected by choosing the smallest number of factors that gave the least amount of standard error of cross-validation (SECV). Performance was evaluated by outlier diagnostics, the coefficient of determination (R value), and the standard error of cross-validation (SECV). Samples with high leverage and studentized residual were flagged from the models as outliers. R value tells the goodness of fit of the model and SECV is a criterion for the distribution of the prediction errors (Jank 2011). An external validation set, which included an independent set of twenty percent of total samples, was selected to assess the ability the calibration model to withstand unknown variability.

For classification, soft independent modeling of class analogy (SIMCA) is a supervised classification technique based on principle component analysis (PCA) and the most used class-modeling technique to discriminate among different groups (Berrueta et al. 2007). SIMCA creates a three-dimensional model over the first three principle components that explain the greatest amount of variation within each class (Varmuza and Filzmoser 2008a) and discriminate among classes. Calibration sets were assigned to classes and a PCA was done on each class with a confidence region (95%). Normalizing statistical processing was used to create a model. Sample residuals and Mahalanobis distance were used for outlier diagnostics (De Maesschalck et al. 2000) . Discriminating power, interclass distances, Coomans plots, and class projections were used to evaluate performance. The higher the discriminating power a variable (spectral frequency) has, the better the variable is for differentiating samples. An interclass distance greater than three is considered good separation (Kvalheim and Karstang 1992). A Coomans plot is a scatter plot to display the residual distances of samples in one class to the other class (Kvalheim and Karstang 1992). In Coomans plot, four quadrants were divided based on the critical sample residual thresholds (95% confidence level). A sample in the northwest quadrant is a member only of the X-axis class because its distance to that class is small enough for it to be considered a member of the class. A sample falling in the southeast quadrant is a member only of the Y-axis class. A sample in the southwest quadrant could belong to either class and northeast quadrant belongs to neither (Infometrix Inc 2014). The class projection plot uses the first three principal components to visualize clustering among samples (Rubio-Diaz et al. 2010b).

2.4 Results and Discussion

2.4.1 Quantification of Lycopene in Tomato Paste

Because of the long conjugated polyene chain structure, carotenoids absorb light strongly at visible region of the spectrum, between 400 and 500nm, allowing for quantification based on Lambert-Beer's law by using a UV-Vis spectrophotometer. Anthon and Barrett (2007a) developed a spectrophotometric assay that utilizes a mixture of hexane/ethanol/acetone (2:1:1 v/v) for extraction, followed by 20 minutes incubation, the hexane layer is removed and lycopene is determined by measuring absorbance at 503nm. This method has been adopted by some major tomato processors in California (personal communication). A typical absorption spectrum of tomato paste extracted in hexane showed three absorbance bands centered at 445, 470, and 503nm (Figure 2-1). The absorbance band at 503 nm is unique for lycopene because the β-carotene and other trace carotenoids have low absorbance (Anthon and Barrett 2007a). Lycopene concentration in tomato paste samples (Table 2-1) using the spectrophotometric assay ranged from 23 to 97.4 mg lycopene/100g paste and had good precision (RSD~5%) for all replicated samples. The lycopene levels in tomato paste were similar to those reported in the literature, ranging from 5.4 to 150 mg/100g of paste with variation coming from different variety, cultivation conditions, processing conditions, soil conditions, etc. (Clinton 1998; Shi and Le Maguer 2000; Baranska et al. 2006; Schulz et al. 2006; Alda et al. 2009).

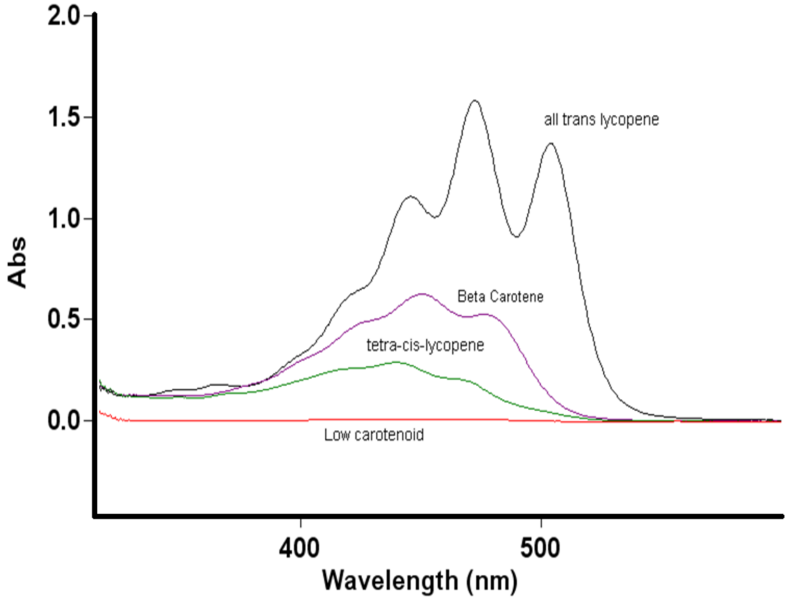

Figure 2-1 Absorption spectrum (300-600nm) of carotenoids extract from tomato paste in hexane based on method adapted from Anthon and Barrett 2007.

Table 2-1 UV-Vis spectrometry reference analysis result of lycopene content in concentrated tomato paste samples

	Sample Number	Range	Mean	STD	RSD%
Lycopene (mg/100g)	123	23.00-97.36	65.15	3.32	5.19

Attenuated total reflectance Fourier transformed mid-infrared (ATR-IR) has been shown to provide good prediction of lycopene content in tomato juices and tomato puree (Halim et al. 2006; De Nardo et al. 2009). Infrared radiation entering a prism made of a high refractive index infrared transmitting material (ATR crystal) will be totally internally reflected creating an evanescent wave extending beyond the surface of the crystal (13μ effective penetration depth for a 3 reflection diamond crystal) into the sample held in tight contact with the crystal, making ATR insensitive to sample thickness, which allows for the analysis of thick or strongly absorbing samples (Subramanian and Rodriguez-saona 2009a). Thus, ATR-IR allows for little to no sampling preparation because the spectra are not defined by the physical state or quantity of the sample but governed by the nature of the ATR crystal (Halim et al. 2006; Rodriguez-Saona and Allendorf 2011; Lu and Rasco 2012; Wilkerson et al. 2013; Ayvaz et al. 2016a). A representative ATR-IR spectrum (Figure 2-2) of tomato paste dominated by the strong OH stretching (3600-3000 cm^{-1}) and OH bending (1700-1500 cm^{-1}) of water (Sinelli et al. 2009). The weak absorbing bands in the 2970-2950 cm^{-1} range are associated with trace lipid content (Coates 2006; Rohman and Che Man 2011), while the 1460-1180cm^{-1} region provides information related to carboxylic groups (COO^-) in organic acids and the 1200-1000 cm^{-1} region is dominated by C-OH and C-O-C stretching of sugars (Ayvaz et al. 2016b). Most importantly, the absorbance band at 956 cm^{-1} corresponds to *trans* –CH=HC- bending out of plane deformation band is the unique IR marker band specific to lycopene (Halim et al. 2006; De Nardo et al. 2009).

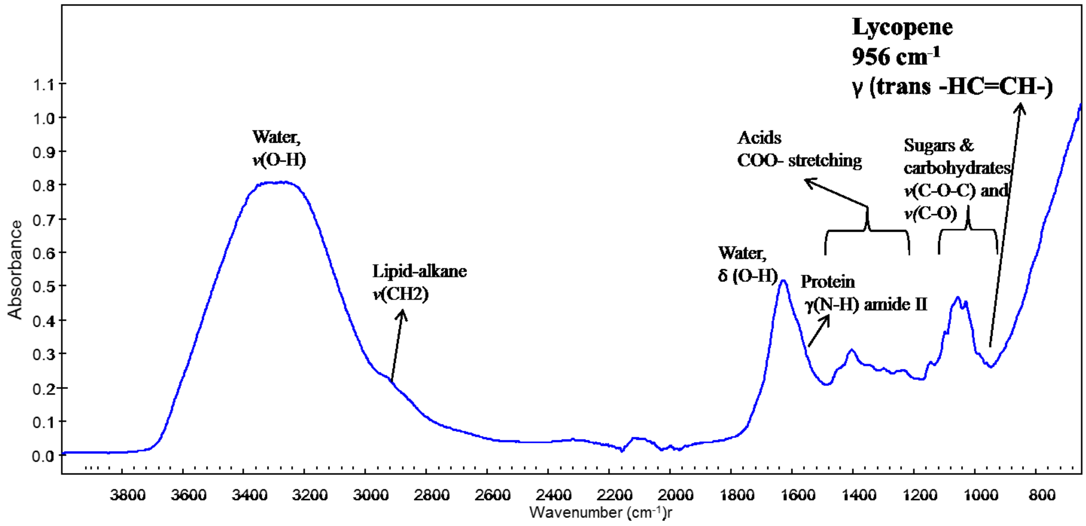

Figure 2-2 Mid-IR absorption spectrum of tomato paste using an ATR accessory equipped with a 3-bounce diamond plate.

ATR provides sampling versatility but requires intimate contact of the sample with the crystal (Kazarian and Chan 2013). On the other hand, Raman spectroscopy offers the flexibility of measuring through films and glass containers (Kazarian and Chan 2013) which provides an alternative method that can further reduce sample preparation compared to ATR-IR. However, variation in sample placement relative to the Raman analyzer affects the Raman intensity because the light scattering intensity depends on the distance from the point of photon entry, particle size and distribution, and the density of packing (Sil and Umapathy 2014). This effect can be reduced by using collection optics having a larger depth of field or by using special apparatus to more precisely position the sample such as flow cells that provide precise sample placement (Pelletier 2003). Thus, we stabilize the Raman signal intensity by using a quartz cuvette and focusing the laser near the sample cell window to minimize laser path penetration differences (Pelletier 2003). The quartz cuvette was not devoid of fluorescence (Figure 2-3) but the 1064 nm laser lessened its spectral impact, limiting to the region below 500 cm^{-1}, allowing resolving characteristic marker bands associated with carotenoids. The Raman spectra (Figure 2-3) of tomato paste samples showed strong bands at 1156 and 1510 cm^{-1} corresponding to C-C stretching and C=C stretching modes, respectively (Baranska et al. 2006; Schulz and Baranska 2007; de Oliveira et al. 2010). Moreover, the spectrum also had low intensity at 1002 cm^{-1} representing C-CH_3 in plane rocking (Baranska et al., 2006). The small shoulder at 1520 cm^{-1} (Figure 2-2, insert) is attributed to *β*-carotene, which is the minor carotenoid in tomato pastes (Baranska et al. 2006; de Oliveira et al. 2010).

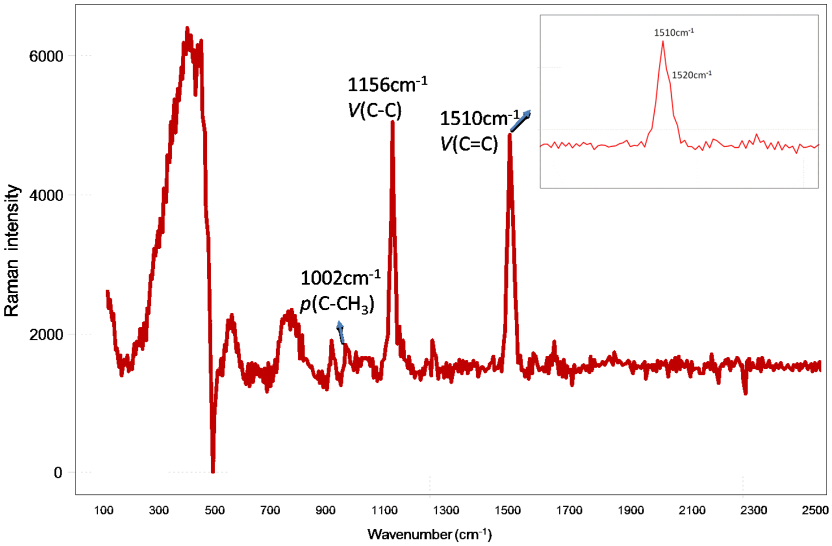

Figure 2-3 Raman spectrum of tomato paste collected on a handheld Raman device equipped with a 1064 nm laser. The insert shows the shoulder associated with β-carotene.

In creating PLSR prediction models, both ATR-IR and Raman spectra were pre-processed by second derivative to reduce baseline and background shift (Nunes 2014) and smoothing (35 point window) to minimize spectral noise without much distortion of the original data (Savitzky and Golay 1964). The PLSR loading vectors were used to identifying frequencies associated with the highest variation in the spectra that reflect to lycopene. Signal to noise could be strengthened and more effective spectral signals could be identified and distinguished by carefully choosing the optimal number of factors with the lowest standard error that explains most of the variance (Huang et al. 2014). The cross-validation (leave-one-out) PLSR model generated by using ATR-IR spectra with the region between 800 and 1632 cm^{-1} and using six factors explained most of the variance (99.95 %) associated with lycopene content (Figure 2-4A). The predicted contents of lycopene measured by portable ATR-IR spectroscopy showed good correlation (R=0.94) with the reference values obtained from UV-Vis spectrophotometry (Figure 2-5A). The standard error of cross-validation (SECV) was small at 3.7mg/100g (Table 2-2). SECV is the standard deviation of the prediction errors (Varmuza and Filzmoser 2008a). A small SECV is also an indicator of a good model showing the distribution of the error is narrow and the standard deviation is small (Varmuza and Filzmoser 2008a). A set of samples accounting for 20 percent of the entire tomato paste samples (n=22) was used to predict the lycopene content and validate the statistical performance of the model. The performance was also good with R value of 0.93 and standard error of prediction (SEP) of 3.59 mg/100g. The similarity between the value of SECV in calibration set and the value of SEP in the validation set evidencing the robustness of the prediction models (Aykas and Rodriguez-Saona 2016a). In addition, the statistical model was able to predict wide range of lycopene concentration in tomato paste samples (23.0 to 97.4 mg/100g paste), showing ATR-IR has the capability of providing reproducible and good quality lycopene quantification in tomato based products. The result from this study was also comparable with previous studies on lycopene quantification analysis in tomato juices using a benchtop ATR-IR where R value of 0.97 and SECV of 0.40 mg/100g juice (De Nardo et al. 2009) and performed superior compared to using a benchtop near infrared spectrometer with R value of 0.75 and SECV of 0.688g/100g juice (Deák et al. 2015).

Table 2-2 Performance of calibration and prediction models generated by portable MIR and Raman spectrometers for quantifying lycopene content in concentrated tomato pastes samples

Technique	Lycopene range (mg/100g)	Calibration				Validation			
		n	Factor[a]	R value[b]	SECV[c] (mg/100g)	n	Factor	R value	SEP[d] (mg/100g)
ATR-IR	23.00-97.36	88	6	0.94	3.70	22	6	0.93	3.59
Raman		48	3	0.96	4.70	15	3	0.90	5.79

[a] Latent variable: set of orthogonal factors that account for most of the variation in spectra

[b] Correlation coefficient

[c] Standard error of cross-validation in calibration set.

[d] Standard error of prediction in validation set

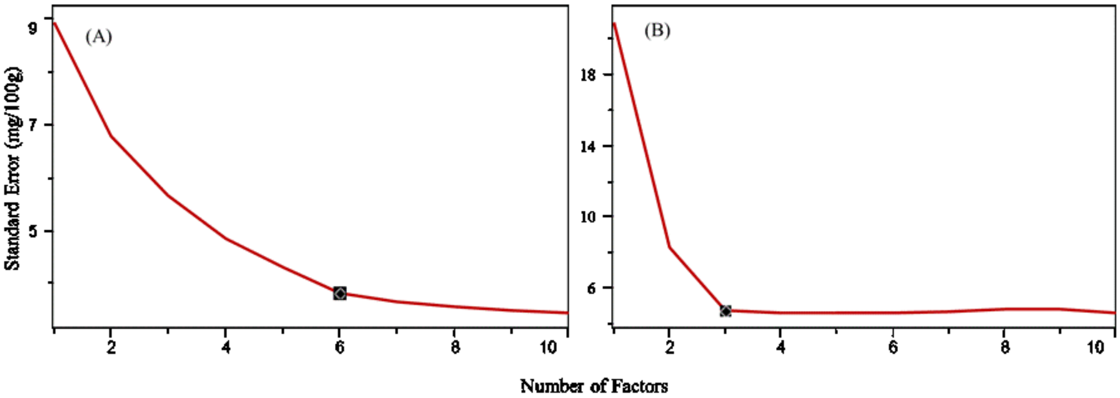

Figure 2-4 Scree plots determining optimal number of latent variables (factors) in creating Soft Independent Modeling of Class Analogy (SIMCA) classification models. (A) Based on infrared spectra; (B) based on Raman spectra.

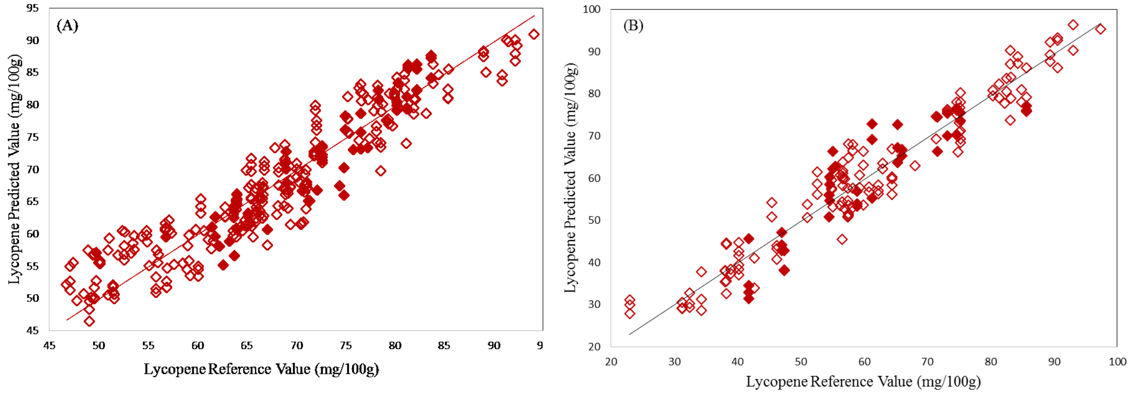

Figure 2-5 Partial least square regression (PLSR) plot for lycopene content prediction in concentrated tomato paste samples based on (A) ATR-IR and (B) Raman spectra

Using Raman spectra, the cross-validation (leave-one-out) PLSR model (Table 2-2 and Figure 2-5B) gave correlation coefficient (R value) of 0.96 and standard errors of cross-validation (SECV) of 4.7 mg/100g. The Raman model were developed by selecting the spectral regions of 850-1230 cm^{-1} and 1360-1680 cm^{-1}, which includes C-CH$_3$ in-plane-rocking (1002 cm^{-1}), C-C stretching (1156 cm^{-1}) and C=C stretching (1510 cm^{-1}). The first three latent variables (factors) (Figure 2-4B) explained most of the variance (98.14%) in the PLSR model for determination of lycopene content in tomato paste. This result also showed good correlation (R value=0.96) between the predicted lycopene content based on Raman spectra and the reference values obtained by UV-Vis spectrophotometric method. Validation set (n=15) was used to predict the lycopene content and validate the statistical performance of the model. The prediction performance was also good in the validation set, only slightly worse comparing against the calibration set, with R value of 0.90 and standard error of prediction (SEP) of 5.79 mg/100g (Table 2-2); nevertheless, the model was accepted as a good-fit model because SEP was within the recommended range of 1.3 times the SECV (Cantor et al. 2011). The Raman result from this study performed superior compared to a previous study using a benchtop Raman spectrometer combined with PLSR where R value= 0.91 and SECV=74.34mg/100g and only 28 samples were analyzed (Baranska et al. 2006). Both portable ATR-IR and handheld Raman spectrometer allowed for reliable prediction of lycopene content in tomato paste but Raman spectroscopy allows for rapid and non-destructive analysis of wet samples because water has weak Raman scattering properties (Li-Chan 1996). In addition, Raman spectroscopy provides the flexibility of sampling through container (Pelletier 2003) compared to using ATR-IR where close, intimate contact of the sample with the ATR crystal is needed (Kazarian and Chan 2013). Overall, the results from this study presented good performance, and the direct determination technique allowed prediction of lycopene content in tomato paste in a few minutes (~1 min for ATR-IR and ~4 min for Raman) without sample preparation.

2.4.2 Carotenoid Classification Analysis among Tomato Paste of Different Carotenoid Profile

Now that Raman spectroscopy has shown to be able to provide quantitative analysis, we further investigated its ability to screen for carotenoid profiles in tomato paste. First, tomato paste obtained from a diverse germplasm that accumulated different carotenoids were profiled by HPLC to characterize the major carotenoids and their isomers present in the samples. Based on reported literature information (Rubio-Diaz et al. 2010b; Yoo et al. 2017), the major carotenoids were tentatively identified by order of elution, absorption spectra and standards (when available) (Table 2-3 and Figure 2-6).

All-*trans* lycopene, *β*-carotene, tetra-*cis*-lycopene, δ-carotene, and lycopene isomers (Figure 2-7) were tentatively identified in the paste samples. A variety containing a mutation in gene r prevents accumulation of carotenoids in the flesh of the fruit by inhibiting phytoene synthase (PSY1) was also included as “control” (Lewinsohn et al. 2005). The tomato paste samples were sub-divided into 4 classes based on their dominant carotenoid profile, including all-*trans*-lycopene (class 1; n=35), *β*-carotene (class 2; n=4), tetra-*cis*-lycopene (class 3; n=7) and low carotenoid (class 4; n=4).

Table 2-3 Tentative identification of carotenoids in tomatoes based on HPLC retention time and absorption peaks

Carotenoid	Retention Time (min)	Observed Wavelength (nm)	Absorption Bands (nm)		
tetra-*cis*-lycopene	29.8	450			438
β-carotene	30.3	450	425	452	480
δ-carotene	43.9	450	436	465	496
all-*trans*-lycopene	58.0	470	442	472	502

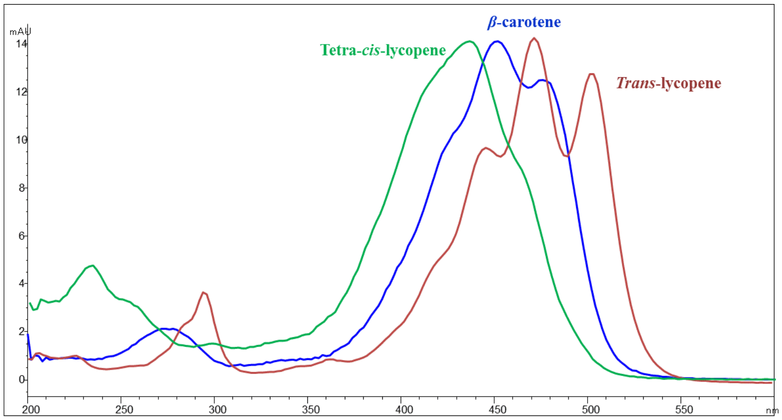

Figure 2-6 Absorption spectra of different major carotenoids in tomatoes obtained from photodiode array detector in HPLC reference analysis.

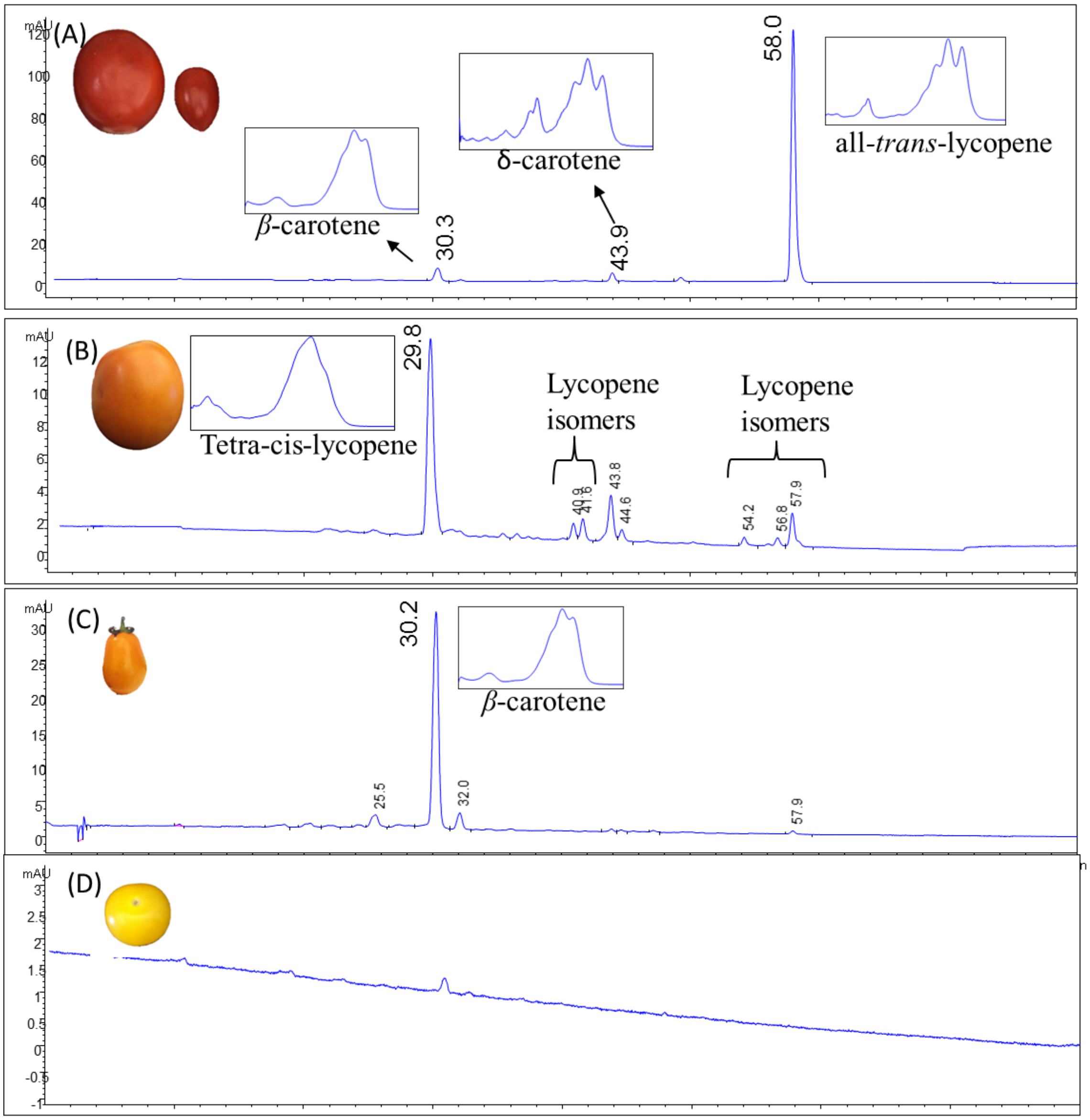

Figure 2-7 HPLC chromatogram of carotenoids in tomatoes as determined by their absorption at 450nm. (A) red tomatoes; (B)tangerine tomatoes high in tetra-*cis*-lycopene; (c) tangerine tomatoes high in *β*-carotene; (D) yellow tomatoes low in all carotenoid.

The Raman spectra of selected tomato paste made from different variety of tomatoes are shown in Figure 2-8. The bands representing C-CH$_3$ in-plane-rocking and C-C stretching remained at 1002 cm^{-1} and 1158 cm^{-1}, respectively for all different major carotenoids, while the variation in the location of C=C stretching band shifted according to the different major carotenoid presented among different the carotenoid-variety tomatoes. A typical Raman spectrum of a red tomato high in all-*trans*-lycopene (Figure 2-8A) had v(C=C) at 1511 cm^{-1}, while β-carotene dominant tangerine tomatoes (Figure 2-8B) had v(C=C) at 1521 cm^{-1}, and high tetra-*cis*-lycopene tangerine tomatoes had v(C=C) at 1525 cm^{-1} (Figure 2-8C).

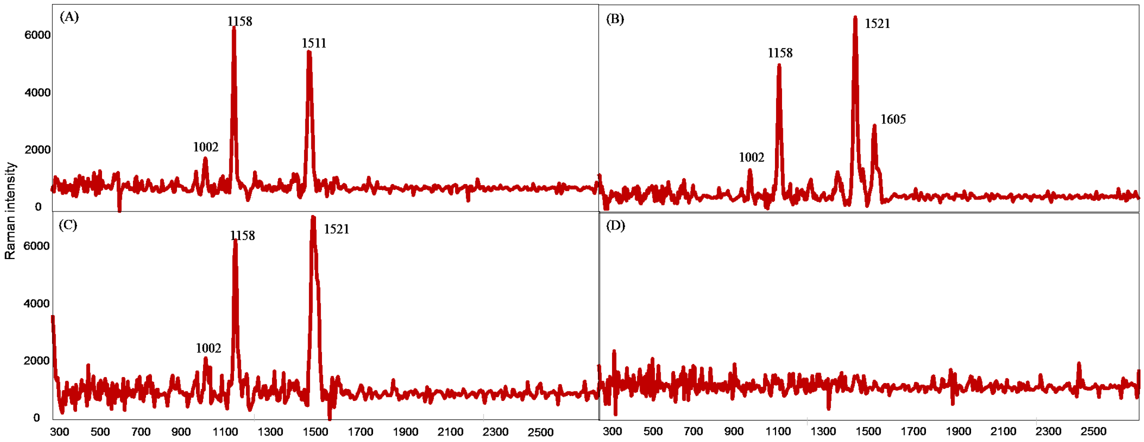

Figure 2-8 Raman spectra of tomato pastes containing different carotenoid profile with excitation laser at 1064nm: (A) red tomatoes high in all-trans-lycopene; (B) tangerine tomatoes high in beta-carotene; (C) tangerine tomatoes high in tetra-cis-lycopene; (D) yellow tomatoes low in all carotenoids.

The C=C stretching Raman band position has an inverse effect of the length of the conjugated polyene chain and is depended on the molecular configuration (*trans-cis* isomerization) of the carotenoid (Vijayalakshmi et al. 2015). The shift of v(C=C) band is due to electron-phonon coupling where the increased length of conjugated polyene chain, the less the energy and the wavenumber of the transition (de Oliveira et al. 2010). Even though tetra-*cis*-lycopene and all-*trans*-lycopene have the same number of conjugated double bonds, C=C stretching Raman shift located differently. Vibration is sensitive to the configuration of the isomer and the effective conjugation length (accounted for the electronic and vibrational properties) for tetra-*cis*-lycopene is actually 9.1; thus, the shift of the Raman band to higher wavenumber (Macernis et al. 2014; Llansola-Portoles et al. 2017). In addition, a strong Raman band at 1605 cm^{-1} assigned to C-C ring chain vibration (Schrader 1994) was associated with the distinguishable structure of β-rings at the both ends of the β-carotene molecule (Figure 2-7B). For yellow tomatoes low in all carotenoids (Figure 2-7D), the spectrum did not exhibit the distinct signature marker bands associated with carotenoids.

Raman spectroscopy has shown to be able to detect characteristic marker bands associated with the different major carotenoid presented based on HPLC reference analysis in tomato paste sample, a supervised classification statistical method called soft independent modeling of class analogy (SIMCA) was used to classify them. Normalization statistical transformation was used to pre-process the spectral data. When normalization is processed on spectra, the variables are divided by the square root of the sum of the variable squares in order to minimize the relative influences of the variables caused by day-to-day data collection and other variability (Berrueta et al. 2007). The region between 1450cm^{-1} and 1600cm^{-1}, which included the band of the highest discriminating power associated with C=C stretching vibration, was used in creating the SIMCA model (Figure 2-9). The discriminating power provides important information regarding the chemical vibrations responsible for the separation of tomato paste and higher the discriminating power means greater the influence the Raman shift in classifying the tomato paste samples (Berrueta et al. 2007).

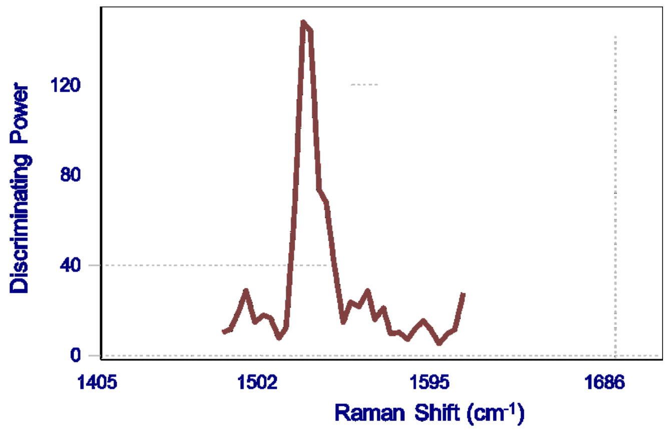

Figure 2-9 Discriminating power plot of region between 1450 and 1600 cm^{-1} showed C=C stretching vibration Raman shift explained the greatest amount of variants in discrimination of tomato paste based on carotenoid profile

The SIMCA class projection plot of the samples (Figure 2-10) exhibited well-separated and tight clusters with interclass distances (Table 2-4) ranging from 2.6 to 4.0. An interclass distance is a Euclidian distance between the center of a cluster to the other and is one of the important indicators of class separation in a SIMCA model (Kvalheim and Karstang 1992). Interclass distances greater than three are considered one group significantly different from another (Wold 1976).

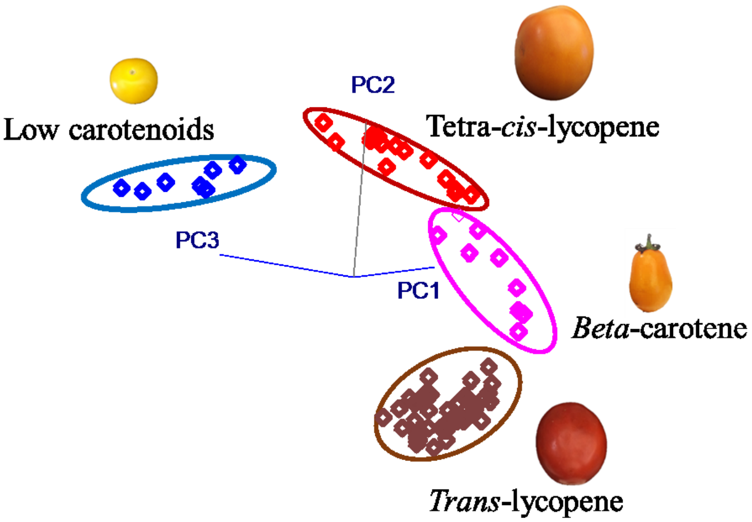

Figure 2-10 Soft independent modeling of class analogy (SIMCA) 3D project plots, transformed using Normalization, for different variety of tomato paste based on Raman spectra

Even though the interclass distance between class "low carotenoids" and class "tetra-*cis*-lycopene" was only 2.6 (Table 2-4), the Coomans plot showed they belong to its own class and well separated (Figure 2-11). A Coomans plot is a scatter plot to display the residual distances of samples in one class to the other class (Kvalheim and Karstang 1992). In Coomans plot, four quadrants were divided based on the critical sample residual thresholds (95% confidence level). A sample in the northwest quadrant is a member only of the X-axis class because its distance to that class is small enough for it to be considered a member of the class. A sample falling in the southeast quadrant is a member only of the Y-axis class. A sample in the southwest quadrant could belong to either class and northeast quadrant belongs to neither (Varmuza and Filzmoser 2008b; Infometrix Inc 2014). The plot further demonstrated the ability of the model to differentiate between different carotenoid variety of tomatoes. The SIMCA model built from Raman spectra was able to correctly discriminate tomato paste of different carotenoid profiles into four classes, which corresponded to HPLC reference analysis results.

Table 2-4 Interclass distances between different variety of tomato pastes based on the soft independent modeling of class analogy (SIMCA) class projections of the Raman spectra

Class	*Trans*-lycopene	Tetra-*cis*-lycopene	*Beta*-carotene	Low carotenoids
Trans-lycopene	0			
Tetra-*cis*-lycopene	4.2	0		
Beta-carotene	3.7	3.5	0	
Low carotenoids	3.6	2.6	4.0	0

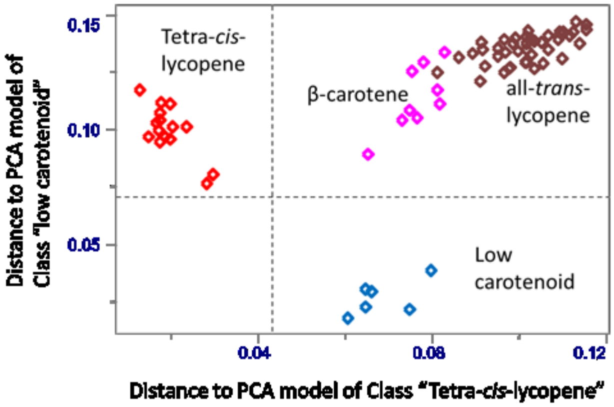

Figure 2-11 SIMCA Coomans plot based on normalized Raman spectra of tomato paste made from different carotenoid varieties of tomatoes. Dotted lines indicate critical sample residual thresholds.

2.5 Conclusion

Raman spectroscopy combined with multivariate analyses was able to provide simple and rapid quantitative and classification analysis of carotenoids in tomato pastes. Carotenoids showed strong Raman scattering at 1158 cm^{-1} (C-C stretching) and between 1500 and 1550 cm^{-1} (C=C stretching), and were used to generate models. The intensity of these unique Raman marker bands combined with PLSR allowed good performance and low prediction error quantification analysis of lycopene concentration in tomato paste. In addition, SIMCA based on Raman spectroscopy was effective to give successful classification of different carotenoid variety tomatoes without misclassification. In conclusion, portable Raman spectroscopy combined with multivariate analysis is able to provide tomato industry a fast, reliable, and high throughput alternative technology to analyze carotenoids in the field or in lab setting without producing solvent wastes. In addition, these user-friendly and automatic spectrometers would only require minimal training of quality control personnel. Most importantly, once the device is purchased, cost-minimization related to operational cost can be achieved.

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