Rapid Assessment of Acrylamide and Its Precursors in Potato Tubers and Snacks by Infrared Spectroscopy

Dissertation

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Abstract

Acrylamide is a chemical compound naturally formed during processes such as frying, roasting and baking in a wide variety of foods including potato chips and French fries and it has been reported to be a human neurotoxin, a rodent carcinogen and a "probable carcinogen to humans." Maillard reaction between sugars and amino acids is considered to be the main mechanism for acrylamide formation, with asparagine and reducing sugars as the major reactants present in foods. Since potatoes are high in both asparagine and reducing sugars such as glucose and fructose, frying potato chips generates optimum conditions for acrylamide formation.

Currently, the most popular methods used to determine the level of acrylamide precursors in raw potato tubers and acrylamide in finished cooked products requires intensive sample preparations and chromatographic separations with liquid or gas chromatographies. As an alternative, infrared spectroscopy is simple, fast and specific technique with minimal or no sample preparation and it is commonly used in food analysis. Additionally, instead of more sophisticated and costly benchtop spectrometers, portable and handheld devices are now becoming popular since they are more practical and economical. Therefore, overall objective of this study was to develop simpler, low-cost and sensitive methods for routine monitoring of precursor levels in raw tubers and acrylamide levels in fried chips. A comprehensive literature review and three experimental studies on acrylamide are presented in this dissertation.

The main objective of the first study was to develop models for simultaneous quantification of acrylamide precursors in raw potato tubers using a portable FT-IR system with single-bounce Attenuated Total Reflectance (ATR) and dial path accessories. Eighty-four experimental and commercial varieties from the years of 2012 and 2013 were analyzed for their sugar and main amino acid levels (asparagine and glutamine). Excellent quantitative infrared models were developed for prediction of acrylamide precursor levels in tubers, which can significantly benefit potato breeding, certain aspects of crop management, production and research.

In the second study, 58 different commercial potato chips were used to evaluate the feasibility of using ATR mid-infrared microscopy (IRMS) as a rapid method for quantification of acrylamide in potato chips. Acrylamide content of potato chips was determined using LC-MS/MS as reference method. Our results indicated that IRMS could be used as a simple, rapid and high-throughput screening tool for acrylamide analysis in potato chips.

In the third study, portable Mid-IR and handheld Near IR spectrometers were evaluated as rapid methods for quantification of acrylamide in 64 commercial potato chips and their performances were compared to those of benchtop systems. The results obtained revealed that portable and handheld systems showed similar performance to the benchtop units tested. These systems can provide food producers with increased flexibility and potential for in-field applications compared to bench-top systems which can only be used in a laboratory setting. Overall, infrared spectrometers along with pattern recognition techniques were shown to be efficient alternatives to traditional

methods for measuring acrylamide precursors in raw potatoes and acrylamide in potato chips, saving time and cost.

Dedication

To my beloved father who unexpectedly passed away during my Ph.D. study

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Fields of Study

Major Field: Food Science and Technology

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CHAPTER 1

LITERATURE REVIEW

1.1. What is acrylamide?

Acrylamide (CH_2=CH-CO-NH_2, 2-propenamide, CAS Registry No. 79-06-1) is a chemical compound commonly used as a monomer in the production of polyacrylamide which has been utilized in several industries since the 1950's (Claeys, De Vleeschouwer & Hendrickx, 2005). This compound is commercially produced from the hydration of acrylonitrile and has a wide range of applications including roles in the production of polyacrylamide gels, treatment of waste water, ore processing, soil conditioning, papermaking, printing ink emulsion stabilizers, grouting agent in construction, filler in cosmetics and a superabsorbent in disposable diapers (Claeys et al., 2005; Mannsville, 1993; USFDA, 2013).

Polyacrylamide was shown to degrade to acrylamide monomer in the presence of heat and light while pH had no effect on depolymerization process (Smith, Prues & Oehme, 1996). The Food and Drug Administration regulates the allowance of maximum levels of acrylamide and polyacrylamide contamination in foods in the Unites States. The level of acrylamide monomers allowed in packaging related materials and food processing aids such as washing and peeling of fruits and vegetables can be found in

detail in CFR- Code of Federal Regulations Title 21 (Parts 172, 173, 175, 176, 573 and 872) (USFDA, 2014).

Acrylamide monomer has a melting point of 84.5 ºC, high boiling point (136 ºC at 3.3 kPa/25 mmHg) and low volatility (vapor pressure of 0.007 mmHg at 25 ºC) (Norris, 1967; American Cyanamid, 1969; Habermann, 1991). Additionally, the solubility of acrylamide in different solvents is highly variable, being highly soluble in water, methanol, ethanol, acetone, acetonitrile, ethyl acetate and chloroform (in descending order) and very low in benzene and heptane (Habermann, 1991; American Cyanamid, 1969). Due to its low molecular weight (71.08 g/mol) and high solubility in water, this white and odorless flake-like crystalline substance can penetrate and contaminate ground and drinking water as well as pass through various biological membranes (Besaratinia & Pfeifer, 2007; Singh et al., 2014).

As shown in **Figure 1.1**, in addition to an amide group, acrylamide contains an electrophilic reactive double bond. This compound does not have a strong chromophore and it does not fluoresce (Eriksson, 2005).

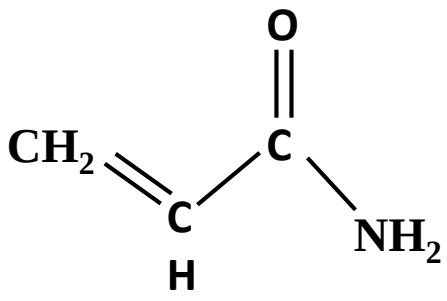

Figure 1.1. Structure of acrylamide

1.2. How did acrylamide become a concern in foods?

During the construction of a tunnel located in the south west of Sweden in 1997, an industrial sealant, Rhoca-Gil, produced by the Rhone-Poulenc was used to block the passage of groundwater into the tunnel at an amount of 1400 tons within the period of two months. This sealant contained monomeric acrylamide and methylolacrylamide. Then, a large leak of this sealant into the streams and ground water occurred, which resulted in dead fish and paralyzed cows around the tunnel. The Department of Environmental Chemistry at Stockholm University revealed that the tunnel workers had been exposed to high levels of acrylamide and some of them had showed symptoms of peripheral nerve damage (Eriksson, 2005; Hagmar, Wirfält, Paulsson & Törnqvist, 2005).

Previously, it was known that there was no acrylamide product (hemoglobin adducts) in wild animals since their diet was uncooked, while acrylamide products were found in smokers (Bergmark, 1977) originated from tobacco smoke (Schumacher, Green, Best & Newell, 1977). This information led to the idea that acrylamide could be formed during heating of organic matters. After the leakage of the sealant, surprisingly, acrylamide was also detected in the blood of a subject group of non-smokers, who did not get exposed to the leakage. All this information indicated that acrylamide could be present due to dietary exposure and Tareke, Rydberg, Karlsson, Eriksson & Törnqvist (2000) proved this hypothesis through their experiments by observing that the increased level of acrylamide products in tested animals was due to the fried food.

Later in April 2002, Swedish scientists surprised the world by announcing the discovery of significant quantities of the chemical acrylamide in a variety of cooked

foods (Tareke, Rydberg, Karlsson, Eriksson & Törnqvist, 2002). These researchers at the Swedish National Food Administration and Stockholm University reported to the press that they had found acrylamide in fried and oven-baked foods. They reported moderate levels (5-50 µg/kg) and high levels (150-4000 µg/kg) of acrylamide in heated carbohydrate and protein-rich foods, respectively. This report was later supported by Svensson et al. (2003), who confirmed the significant level of acrylamide in fried and baked potato-based products along with cereal products, including crisp bread, breakfast cereals and cookies.

1.3. Why is acrylamide a concern?

By cooking foods, many benefits can be obtained such as microbial inactivation, destruction of toxins, improvement of nutrient bioavailability and formation of desired colors, textures and flavor compounds (Van Boekel et al., 2010). However, cooking also results in the formation of undesirable compounds such as acrylamide (**Table 1.1)**.

Acrylamide is naturally formed in a wide variety of foods, including French fries, potato chips, cereal, bread and coffee and has been found to cause cancer in laboratory animals when consumed in excessive amounts (LD_{50} values are in the range of 107-203 mg/kg bw in rats).

Table 1.1. Some chemicals formed in processing of foods including heat treatment (Modified from Robin, 2007).

Contaminant	Source of foods	Mean intake (µg/day)
Acrylamide	Heated potato and cereal products (e.g., French fries, potato chips, cookies, crackers), coffee	21-60
Furan	Canned soups, stews, and sauces; jarred baby foods; coffee	15
Heterocyclic amines	Cooked meat or fish	≤ 1
3-monochloro-propane-1,2-diol	Acid-hydrolyzed vegetable protein, soy sauce and related sauces, heated cereal products (e.g., cookies, crackers)	3.5-25
N-nitroso compounds	Meats cured with sodium nitrite or nitrate	≤ 1
Polycyclic aromatic hydrocarbons	Grilled and smoked foods, vegetable oils and also fruits, vegetables, and mollusks, through environmental deposition	2-3
Urethane	Fermented foods and beverages	1-4

Although polymerized acrylamide is known to be non-toxic, the monomeric form of it is a neurotoxin and can cause damage to the nervous system in both humans and animals (LeQuesne, 1980; Lopachin & Lehning 1994; Tilson, 1980). Additionally, the study conducted by Johnson et al. (1986) using acrylamide incorporated in drinking water of rats revealed that acrylamide was carcinogenic in rodents. Dearfield, Douglas, Ehling, Moore, Sega & Brusick (1995) showed that acrylamide was a reproductive toxin with mutagenic and carcinogenic properties via *in vitro* and *in vivo* mammalian studies. Based

on the carcinogenicity evidences in animals, acrylamide was later classified as "probably carcinogenic to humans" by the International Agency for Research on Cancer (IARC, 1994).

The studies conducted by Friedman, Dulak & Stedham (1995) and Johnson et al. (1986) regarding the cancer risk assessment of acrylamide in rats based on tumor incidences were recently redone by Beland (2010) and Beland, Mellick, Olson, Mendoza, Marques & Doerge (2013). The studies were used by The Joint Expert Committee on Food Additives (JECFA) of the World Health Organization (WHO) (Viswanath, 2012) for acrylamide risk assessment. Hogervost, Schouten, Konings, Goldbohm & van den Brandt (2007 and 2008) showed the carcinogenic effects of acrylamide also in humans, reporting positive relationships between dietary acrylamide intake and the risk of several types of cancers namely endometrial, ovarian and kidney.

Acrylamide is metabolized to glycidamide in the liver and becomes more reactive toward DNA and proteins (Pedreschi, Mariotti & Granby, 2014). As of now, the greatest risk from acrylamide seems to be the neurotoxic effects in humans due to the changes that occur in the central nervous system after exposure to high doses of acrylamide (Serrano-Niño, Cavazos-Garduño, González-Córdova, Vallejo-Cordoba, Hernández-Mendoza & García, 2014). Even though the actual pathway is not clearly known as of now, recombination induced by DNA can lead to malignancies by interfering with genetic replication process (García-Lopez and Alfaro-Macedo, 2007).

Acrylamide has the ability to be absorbed by the skin, inhaled by mucosa or by ingestion through the oral route (Friedman, 2003). Upon consumption through the diet,

acrylamide is quickly absorbed from the gastrointestinal tract and distributed to the tissues (Fennell et al., 2005). Sörgel et al. (2003) reported that up to 50% of the acrylamide obtained from the diet in pregnant woman can be transferred to the fetus via blood through the placenta.

A major international evaluation of acrylamide by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in 2005 concluded that the levels of acrylamide in food were of concern, given the carcinogenicity rates seen in rodents (FAO/WHO, 2005). According to the WHO, the daily intake of dietary acrylamide is between 0.3 – 2.0 μg/kg/body wt and in the case of children; it was estimated as 2-3 times more than that of adults due to average body weight ratios (Becalski, Lau, Lewis & Seaman, 2003). Similarly, FDA scientists estimate that the average U.S. consumer intake of acrylamide is 0.4 μg/kg body weight/day with a 90^{th} percentile of 0.95 μg/kg body weight (USFDA, 2006a), while international estimates for the average consumer range from 0.2 to 1.4 μg/kg-bw/d as of 2005. For drinking water, permissible acrylamide levels were set as 1 μg/L by the WHO (World Health Organization, 2006) and European Union (Directive, 1998) whereas it was set to 0.5 μg/L by the US Environmental Protection Agency (EPA) (Tekkeli, Önal & Önal, 2012).

Even though acrylamide levels needed to cause cancer is not clear, there has been an agreement among scientific committees and governmental organizations that acrylamide levels should be kept as minimum as possible (Pedreschi et al., 2014).

Although, there is still not a threshold dose assigned yet, the U.S. EPA set a level which is the concentration that acrylamide is a cancer risk to one person in one million people and it is a low value of 0.00077 and it is lower than some other well-known

toxins including benzene (0.12), carbon tetrachloride (0.067), chloroform (0.043) and lead (0.013) (Arvanitoyannis & Dionisopoulou, 2014).

Taeymans et al. (2004) claimed that even some staple foods such as bread can contribute to total acrylamide intake even though they have very small amounts compared to some other fried foods. Similarly, it is known that genetoxic carcinogens are usually accepted with no threshold limit of exposure since even one molecule of this compound has the possibility to start a process leading to cancer (FAO/WHO, 2005).

Being a known animal carcinogen and human neurotoxicant, the discovery of acrylamide in food prompted immediate reaction from governments worldwide, as well as intense media coverage. Acrylamide in food has become a significant issue for the FDA, appearing on the FDA Center for Food Safety and Applied Nutrition (CFSAN) Program Priority list (Tareke et al., 2002). Acrylamide was also added to the list of substances of very high concern by The European Chemical Agency (ECHA) in March, 2010 (ECHA, 2010).

Additionally, acrylamide has been included in California's Proposition 65 list of chemicals that can potentially cause cancer or reproductive harm resulting in lawsuits against a number of U.S. food manufacturers and restaurant chains, alleging that food sold by these companies contains a known carcinogen (acrylamide) without warning (California OEHHA, 2011). Proposition 65, or the Safe Drinking Water and Toxic Enforcement Act of 1986, is a state law that requires businesses in California to warn citizens of significant exposure to chemicals that can potentially cause cancer or reproductive harm. Businesses can either reformulate products to lower exposures below the level deemed significant by the state, or else they should warn citizens of the

exposures by labeling their products or posting a warning sign. The California Attorney General and private interest groups filed lawsuits against a number of U.S. food manufacturers and restaurant chains, declaring that food sold by these companies violates Proposition 65 guidelines (Robin, 2007; Baylen, 2010) and the companies were fined with millions of dollars in penalties and costs. Consequently, the food industry is modifying processing operations to limit acrylamide formation as consumer concerns increase as a result of new scientific evidence or federal/state regulations.

1.4. How is acrylamide formed in foods?

Acrylamide in foods is formed during either cooking for home preparation, catering or commercial production in the food industry. Even though the exact pathways for formation of acrylamide in foods have not been totally elucidated yet, some ways have been proposed. According to one of the proposed ways, acrylamide can be formed through the heating of decarboxylated asparagine (3-aminipropioamide) by deamination without the presence of reducing sugars (Granvogl & Schieberle, 2006). However, the authors reported this pathway as inefficient with very low yields. Another pathway proposed for acrylamide formation was related to the lipid oxidation, with acrylic acid being the precursor formed from acrolein when lipids are degraded oxidatively (Gertz & Klostermann, 2002). Similarly, Weisshaar (2004) highlighted that acrylamide formation through lipid oxidation is not that significant either. Additionally, acrylamide can be generated by heating peptides (Granvogl et al., 2006) and by thermolytic release from gluten in wheat grain (Claus, Weisz, Schiever & Carle, 2006). However, it is generally

accepted that acrylamide is predominantly formed through Maillard reaction (**Figure 1.2**).

The Maillard reaction is a very complex reaction between amino compounds (usually amino acids) and reducing sugars, and it has been studied extensively for a long time since it is the main route for the formation of flavors and browning in cooked foods (Kawamura, 1983). The Maillard reaction occurs especially at temperatures above 100 °C during processes such as frying, roasting and baking (Becalski et al., 2003).

Through some labelling studies (Stadler et al., 2004a; Zyzak et al., 2003) including 15N-labeled asparagine and 13C-labeled glucose, it was shown that the three carbon atoms and the nitrogen of the amide group of the acrylamide (backbone of the acrylamide molecule) was derived from asparagine. This is further confirmed by the reaction involving the condensation of asparagine and 13C-labeled glucose. Acrylamide produced from this reaction did not contain any of the Carbon atoms from the labelled glucose (Stadler et al., 2004a). However, reducing sugars such as glucose have a role of promoting the acrylamide formation. Considering these findings, acrylamide is accepted to originate mainly from heat-induced reactions between the α-amino group of the free amino acid asparagine and carbonyl group(s) of reducing sugars such as glucose (Friedman, 2003). A simple version of major pathway for acrylamide formation in foods is shown in **Figure 1.2**.

When asparagine and reducing sugars react, the corresponding N-glycosyl derivative of the asparagine is formed. By releasing the water under high temperatures, a Schiff base is formed and further rearranged to produce Amadori products. This is followed by the β-elimination and the subsequent acrylamide formation (Yaylayan,

Wnorowski & Perez Locas, 2003). This pathway is considered to be the main mechanism for acrylamide formation (Stadler et al., 2002) and it is enhanced at high temperature and low moisture conditions.

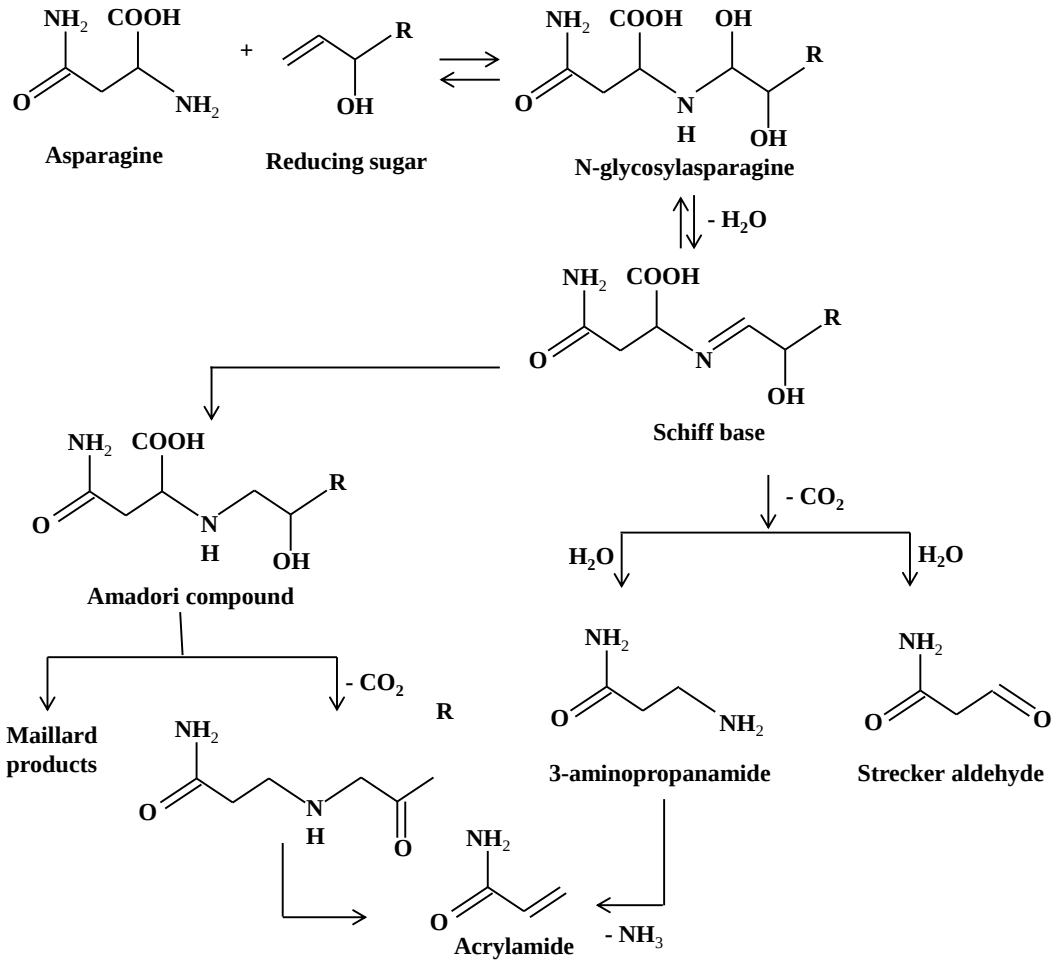

Figure 1.2. Simplified version of acrylamide formation in foods (modified from Zyzak et al., 2003)

Weisshaar (2004) simply summarized the requirements for acrylamide formation in heated foods as free asparagine, free reducing sugar, low water activity at the surface

of the food, and product temperature above 100^0C. The highest acrylamide levels have been found in French fries, potato chips, and deep-fried or oven-baked potato products, as well as some crisp bread, biscuits, crackers and breakfast cereals (Tareke et al., 2002). However, based on the USFDA survey on food products on the market until 2004, no or very low amounts of acrylamide were found in products such as cooked infant formulas, fish, chicken or meat (USFDA, 2004). The low or undetectable levels of acrylamide in meat products are explained by a few scenarios including low level of asparagine and high moisture content of the meats and other constituents in meat inhibiting the reaction or binding the acrylamide (Taeymans et al., 2004).

There are several factors affecting the acrylamide formation in heated potato products including the level of precursors and their ratios in the raw potato tubers, temperature and time of the processing, surface to volume ratio of the food, pH and water activity (Friedman, 2003). Different cooking practices dramatically affect the acrylamide content of foods (Ahn, Castle, Clarke, Lloyd, Philo & Speck, 2002; Medeiros Vinci, Mestdagh & De Meulenaer, 2012). Other factors such as potato cultivar, oil type, presoaking and frying temperature and time have also been tested. Williams (2005) reported frying time and temperature as the most important variables for acrylamide formation in foods. Ahn et al. (2002) reported the presence of very high levels of acrylamide (more than 10 mg/kg) in overcooked fried chips demonstrating the influence of cooking temperature and duration on acrylamide formation. Statistics about acrylamide levels found in some types of food products analyzed by FDA are presented in **Table 1.2** below.

Table 1.2. Statistics for the acrylamide values in food product samples collected from the market between November 20, 2003, and October 7, 2004 (modified from USFDA, 2004)

Category	Number of samples tested	The lowest acrylamide level (ppb)	The highest acrylamide level (ppb)	Mean value of acrylamide levels (ppb)
Chocolate candies	9	ND	74	32
Coffee	17	27	609	191
Breads and Bakery Products	48	ND	59	20
Cereals	36	ND	534	131
Cookies	25	ND	955	115
Crackers	29	39	1540	271
Fruits and Vegetable Products	9	ND	239	101
Potato chips	16	462	1970	798
Snack foods other than potato chips	32	ND	1340	264
Tortilla chips	8	29	794	178
French fries	15	109	1250	544
Infant formulas	25	ND	ND	-
Nuts and nut butters	13	ND	457	137

ND = nondetectable, Limit of quantification is 20 ppb

*The level ND is assumed as 0 for the calculation of mean value within the each category

Acrylamide formation in heat treated foods is known to be a surface phenomenon. For instance, during the frying of French fries at 170^0C, there was no acrylamide

observed in the beginning of the frying. However, as the surface heats up to the temperature levels above 120^0C due to the drying effect, acrylamide started to form on both the surface and near surface areas (Palazoğlu & Gökmen, 2008a). Due to the different heat transfer and evaporation of the water from the chips, the center of the French fries usually does not exceed 100^0C and therefore acrylamide formation in the core and on the surface of the French fries are not the same (Gökmen, Palazoğlu & Şenyuva, 2006). Gökmen and his co-workers also emphasized that acrylamide is generated more at the end of the frying process. However, if frying continues for longer times, there may be acrylamide formed in the inner part of the foods as well due to high internal temperatures. Carrieri, De Bonis, Pacella, Pucciarelli & Ruacco (2009) reported that acrylamide formation approximately doubles when the processing temperature is increased by 10%.

In a study conducted by Gökmen et al. (2006), French fries were fried at different temperatures between 150^0C and 190^0C and acrylamide formation was evaluated. The authors reported a linear relationship between frying time and acrylamide formation up to 9 minutes. Additionally, Gökmen, Şenyuva, Dülek & Çetin (2007c) fried potato chips and reported that after 8 minutes of frying in the conditions they set, acrylamide concentration started to decrease exponentially as opposed to a vacuum frying study conducted by Granda & Moreira (2005), where exponential increase of acrylamide formation was observed at all frying times even though the levels were lower compared to those of traditional frying.

Information from the literature on acrylamide formation of coffee revealed that acrylamide formation during the roasting process (210-250^{0}C) reaches its maximum earlier than when the roasting is completed and therefore dark-roasted coffee may contain lower amount of acrylamide than the medium roasted coffee (Lantz, Ternité, Wilkens, Hoenicke, Guenther & van der Stegen, 2006: Granby & Fagt, 2004). According to Bagdonaite & Murkovic (2004), roasting the coffee for longer than 5 minutes at temperatures above 240^{0}C caused a decrease in acrylamide formation. These findings summarized above indicate that acrylamide is an intermediate product formed during the Maillard reaction and it is the net amount of acrylamide as a result of both formation and degradation that occurs during heating. However, the reaction causing degradation of acrylamide in foods is not clear, yet (Keramat, LeBail, Prost & Jafari, 2011).

1.5. Precursors of acrylamide

Among food products, potato products present higher acrylamide levels since potato tubers are high in both asparagine and reducing sugars such as glucose and fructose, causing the formation of acrylamide in French fries and potato chips. Potato and bakery products are the sources of 50 and 20% of acrylamide consumed by people, respectively (Keramat et al., 2011). Therefore, potato products and in particular potato chips and French fries will be emphasized for the rest of this literature review.

Among the free amino acids in potato tubers, asparagine and glutamine are found at the highest concentrations (Brunton et al., 2007), with asparagine being the most abundant free amino acid accounting for about one-third of the total free amino acids

(Eppendorfer & Bille, 1996; Amrein et al., 2003; Oruna-Concha, Duckham & Ames, 2001). As a response to some events occurred during the growing stages of the plants including seed germination, nitrogen transport and stressed caused by environment such as drought, mineral deficiency or pathogens, asparagine is accumulated (Lea, Sodek, Parry, Shewry & Halford, 2007).

Direct correlation between the level of asparagine as a precursor and the acrylamide level formed in the heated food products has been previously reported (Becalski et al., 2003, Matsuura-Endo et al., 2006). Additionally, Claeys et al. (2005) added several amino acids into the asparagine-glucose model system and found that glutamine increased the acrylamide formation considerably and gave a higher reaction rate constant for acrylamide formation in the presence of asparagine.

Compared to levels of sugars in potatoes, asparagine is found at higher levels making reducing sugars the most important factor for acrylamide formation (Gökmen & Palazoğlu, 2008). Reducing sugars (glucose and fructose) in particular are the main factors showing linear relationships with the generated acrylamide levels upon heat treatment in potato products as opposed to other food products such as cereals and coffee where the limiting substance needed for acrylamide formation is free asparagine (Pedreschi et al., 2014). As an example, cooked whole grain products may contain higher amounts of acrylamide than those of sifted fractions due to the higher levels of asparagine present in whole grain raw flour (Brathen & Knutsen, 2005). Unlike regular potatoes, sweet potatoes contain low asparagine and high reducing sugars, making asparagine the limiting factor for acrylamide formation (Truang et al., 2013).

Some studies emphasized sugar levels as limiting factor directly affecting the acrylamide formation (Amrein et al., 2003; Oruna-Concha et al., 2001). It has been generally reported that the level of reducing sugars changes more compared to asparagine among different varieties and during the storage (Amrein et al., 2003). Halford et al. (2012) reported a change in the content of free amino acid during storage, which could be explained by protein degradation caused by sprout formation (Brierley, Bonner & Cobb, 1997) or tubers mobilizing the nitrogen sources in the last stages of the storage (Halford et al., 2012). Choosing the varieties with low level of acrylamide precursors and finding the genotypes with low acrylamide risk could be evaluated for plant breeding programs.

Although it is not a reducing sugar, sucrose, which is the predominant sugar in potato tubers, can go through decomposition under the frying and baking conditions. According to Taeymans et al. (2004), sucrose has two reducing sugars (glucose and fructose) so it can provide two hexoses. Furthermore, in a model study at high temperature (350^0C), sucrose was found to be three times more reactive with asparagine in forming acrylamide (Yaylayan et al., 2003). Stadler et al. (2002) reported relatively similar reactivities of sucrose with glucose and fructose in model reactions with asparagine at 180 ºC. However, Claeys et al. (2005) reported that sucrose was only 50% as reactive with asparagine compared to glucose on molar basis in the temperature range of 140 ºC – 200 ºC. These findings suggest that apart from the glucose and fructose, sucrose should also be considered for acrylamide formation in heated potato products.

Upon the experiment on the influence of sugars on acrylamide formation with asparagine in washed whole wheat matrix, glucose, fructose and sucrose showed similar

effect, with fructose being more efficient (Taeymans et al., 2004). Similarly, Biedermann, Noti, Biedermann-Brem, Mozzetti & Grob (2002a) and Pollien, Lindinger, Yeretzian & Blank (2003) also reported that the fructose was more efficient than glucose for acrylamide formation. Similarly, Robert et al. (2004) observed higher acrylamide levels formed with fructose and asparagine than glucose and asparagine. The authors attributed this finding to the different melting points of the sugars measured using differential scanning calorimetry. Fructose melted at 126^0C, whereas glucose needed 157^0C, which confirmed that fructose generated acrylamide earlier than glucose.

Several factors including variety (Heuser, Gerendas & Sattekmacher, 2005; Silva & Simon, 2005), storage temperature and time and the level of nitrogen and phosphorous in the soil can alter the level of precursors (Heuser, Gerendas & Sattekmacher 2005; Silva & Simon 2005) and therefore the concentration of acrylamide upon heating. Gerendás, Heuser & Sattekmacher (2007) reported that the highest level of precursors was found in the tubers grown with high N and low K supply.

Storing potato tubers at temperatures lower than 8^0C to avoid sprouting and not re-conditioning properly before their sale can cause accumulation of sugars rapidly (cold sweetening) (Sowokinos, Vayda & Park, 1990) since part of the starch in the tuber is consumed for respiration through conversion to sugars (Hertog, Tijskens & Hak, 1997). Moreover, Olsson, Svensson & Roslund, (2004) has shown that this increase in sugar content was dependent on the genotype, which was in accordance with Burch et al. (2008), who showed that the changes in the level of acrylamide precursors during storage were cultivar-dependent. Biedermann, Biedermann-Brem, Noti & Grob (2002b) has

stored potatoes of cultivar Erntestolz at 4^0C for 15 days and observed a dramatic increase in the level of reducing sugars from 80 to 2250 mg/kg fresh weight, which is expected to increase the acrylamide formation 28 times at 120^0C.

Viklund, Olsson, Sjöholm, & Skog (2008) evaluated the impact of harvest year and weather conditions on five potato clones and found that free amino acid and sugar contents of potato clones changed from clone to clone as well as from year to year for the same clone. The authors attributed the highest level of precursors in one year to the extreme weather conditions (a long period of drought and subsequent heavy rain). Similarly, Brunton et al. (2007) analyzed reducing sugar content of potato samples bought from market over an 11-month length and reported variations as high as 80 folds. Dry and warm weathers usually lowers the sugar level (Davies, Jefferies & Scobie, 1989) while cold climate slows down the maturity and therefore results in a higher content of reducing sugars (Knutsen, Dimitrijevic, Molteberg, Segtnan, Kaaber & Wicklund, 2009). Similarly, since sugars have not been converted to starch yet, small and immature tubers tend to have a higher level of sugars (De Wilde et al., 2006). However, in industrial manufacturing, potato tubers smaller than 50 mm are eliminated in the beginning of the process (De Wilde et al., 2006).

1.6. How to reduce acrylamide in foods?

Even though there have been some recommendations on acrylamide levels allowed in foods such as maximum of 100 µg/kg in French fries by Grob et al. (2003), currently there is no legal limit set for acrylamide allowance in foods. Hence, strategies to

lower the acrylamide in high acrylamide containing foods need to be employed voluntarily due to the negative health association of acrylamide (Pedreschi et al., 2013). Food Business Operators expected to obey to the concept of "ALARA" which is the acronym of "As Low As Reasonably Achievable" meaning that every reasonable precautions need to be taken to reduce the acrylamide level in a final product (CIAA, 2014).

Considerable variations observed in acrylamide levels among the food products indicate the possibility of reducing the levels by changing the methods of production and preparation (Claus, Carle & Schieber, 2008) and therefore there has been many research studies focusing on the strategies for acrylamide mitigating in foods in the last decade (Anese, Manzocco, Calligaris & Nicoli, 2013). The Confederation of Food and Drink Industries of the European Union (CIAA) has published the "Acrylamide Toolbox" and continuously updates it (updated for the 13th time as of October, 2014). This toolbox contains the work done by the industry and academia on acrylamide reduction and helps manufacturers to find the optimum ways to mitigate acrylamide in their products (CIAA, 2014).

Overall, potential strategies to prevent acrylamide formation include (1) removal of the acrylamide precursors, (2) interference with the Maillard reaction and changing processing conditions, or (3) interventions to remove or decompose the already formed acrylamide in the final food products. However, since the Maillard reaction is essential for the desired and characteristic flavor and color formation in potato products, this constitutes the first challenge for food scientists on how to reduce acrylamide formation without affecting final product specifications and quality (Medeiros Vinci et al., 2012).

1.6.1. Variety Selection

Since the amount of acrylamide formed is directly related to the level of reducing sugars in raw potatoes (especially when fructose/asparagine < 2 (CIAA 2014)), it is very important to choose the varieties suitable for the processing. De Wilde et al. (2006) recommends that the tubers sold at the market should be indicated as suitable for frying or not since potatoes may be stored at low temperatures to avoid sprouting which may cause the cold sweetening. Monitoring tuber composition is critical for effectively planning breeding lines for the purpose of producing tubers suitable for their further processing (Haase, 2011).

Fiselier & Grob (2005) suggested the maximum level of reducing sugars in potato tubers to be lower than 0.7 g/kg. They categorized this group as low reducing sugar, while the levels between 0.7 and 3 g/kg and above 3 g/kg as normal and high reducing sugars, respectively. Similarly, Palazoğlu and Gökmen (2008b) mentioned the level of reducing sugars as normal if it is about 1 g/kg and maximum if it is above 3 g/kg.

1.6.2. Maturity

Normally, dry mass of the tubers increases during maturation since nutrients are transferred from leaves to the tuber. When potato tubers are not mature yet, the amount of sugar moved from the leaves to tuber exceeds the level of sugar converted into starch (De Wilde et al., 2006). Hence, potato cultivars with large, long and oval shapes are known to contain lower reducing sugars and higher dry matter content than others (Torres & Parreno, 2009). Accordingly, choosing the correct size of the tubers may play an important role in lowering the acrylamide levels upon subsequent heating. Additionally,

pieces made out of the outer sphere of the potato tubers are better to be removed since these little parts contains more reducing sugars and they overheat during the frying and forms excessive amounts of acrylamide compared to other bigger size parts (Foot, Haase, Grob & Gonde, 2007).

1.6.3. Asparaginase treatment

Using asparaginase enzyme is another way of reducing acrylamide levels in heated foods. Asparaginase is an enzyme obtained from *Aspergillus oryzae with* GRAS status (Generally Recognized As Safe) (USFDA, 2006b) and is allowed to be used in many countries to lower the acrylamide levels in foods. This enzyme breaks down the precursor asparagine amino acid into aspartic acid and ammonia (Amrein, Schöbächler, Escher & Amadó, 2004) and is claimed to lower the acrylamide without altering the taste and appearance of the final product as long as it is used at proper concentrations and incorporated well with the food (Medeiros Vinci et al., 2012). The optimum pH range for asparaginase is between pH 6 and 7. However, it is still good to use this enzyme at pH levels between 5 and 8; therefore it is compatible with potato products such as French fries and potato chips (Pedreschi, Mariotti, Granby & Risum, 2011). The optimum temperature for a commercial asparaginase (Acrylaway launched by Novozymes) was tested as 60^0C at pH 7 and enzyme activity was found to decrease significantly above 60^0C (Novozymes Switzerland, 2007).

Acrylamide reduction obtained in model systems using the asparaginase enzyme was overall higher than using the real food matrixes (Anese et al., 2013). Penetration of

asparaginase into potato is difficult, but dipping potato strips and slices in asparaginase containing solutions resulted in 30% and 15% reductions in French fries (Pedreschi, Kaack and Granby 2008) and potato chips (Pedreschi et al., 2011) respectively.

1.6.4. Blanching / Soaking

Another technique for acrylamide mitigation is blanching. Blanching is commonly used in the industry for several reasons such as removing reducing sugars (therefore reducing browning), inactivating enzymes and gelatinization of starch which in return reduces the oil uptake (Shojaee-Alibadi et al., 2013). Mestdagh et al. (2008a) studied the optimization of blanching process for potato products and reported that acrylamide could be more efficiently reduced for potato chips than the French fries due to higher amounts of sugar removal during the blanching. According to the authors, the most important parameter effecting the sugar removal as well as following acrylamide reduction was the blanching temperature with 70^0C for about 10 minutes being the most effective. They also found that reducing sugar removal in water which was already used for blanching previously was over 10% lower, which could be important for industrial processing where the blanching water usually contains the extracted reducing sugars.

Pedreschi et al. (2011) tested using the commercial asparaginase enzyme with blanching in hot water and concluded that blanching in hot water was almost as efficient as dipping the potatoes into the asparaginase solution. They also found that blanching the potato slices prior to dipping into asparaginase solution led to almost 90% acrylamide reduction in potato chips and recommended the blanching and asparaginase combination

as a more efficient treatment for the acrylamide mitigation compared to asparaginase immersion without blanching. Similarly, Pedreschi, Granby & Risum (2010a) blanched potato slices in 1% NaCl solution and obtained nearly 62% acrylamide reduction in the end, reporting that blanching enhanced the penetration of the NaCl into potato tissues. This reduction in acrylamide content caused by NaCl was attributed to the system trying to equilibrate the changes in osmatic potential and reducing sugars being leached out during this process. Similarly, Pedreschi, Kaack & Granby (2008) treated the French fries with asparaginase solution at 40^0C for 20 minutes and obtained a reduction of 30% in acrylamide content. When they combined the enzyme treatment with the blanching, the reduction was further improved to 60% lower than the blanched only French fries. This could be similar enhancing effect of blanching for the efficiency of subsequent treatment as reported by Pedreschi et al., (2011) for the 1% NaCl solution.

Truong et al. (2013) water blanched and soaked sweet potato strips in 0.5% sodium acid pyrophosphate prior to frying and obtained about 7 times reduction in acrylamide formation. They also tried an additional soaking in 0.4% calcium chloride and obtained further remarkable reductions in acrylamide concentrations. By combining all water blanching, sodium acid pyrophosphate and calcium chloride treatments, the author obtained sweet potato French fries with acrylamide levels much lower than 100 ng/g (proposed concentration threshold by Grob et al., 2003), which is a lot lower than the typical acrylamide concentration of regular French fries. On the other hand, Pedreschi et al. (2007) blanched the potato slices in hot water (at 85^0C for 3.5 minutes) and then air

dried to final moisture content of 60%. The author obtained up to 44% acrylamide reduction compared to blanched only samples upon frying at 180^{0}C.

1.6.5. Storage

In order to supply potatoes throughout the year, potatoes need to be stored through the fall, winter and spring in temperature and humidity controlled rooms equipped with ventilation (USFDA, 2013). When potatoes are stored for long times, sprouting can occur, which causes the starch to convert into sugars. This may be avoided either by using sprout suppressants or cold storage. Keeping the potato tubers at low temperature has some advantages including lower weight loss, inhibition of sprouting, excluding the use of sprout inhibitors (Pinhero, Pazhekattu, Whitfield, Marangoni, Lie & Yada, 2012). However, low temperature storage results in increase of reducing sugars and therefore acrylamide formation upon heat treatment. Moreover, Olsson et al. (2004) has shown that this increase in sugar content was dependent on the genotype, which was in accordance with Burch et al. (2008), who showed that the changes in the level of acrylamide precursors during storage were cultivar-dependent.

Potatoes exposed to cold sweetening can be reconditioned at higher temperatures which leads to reducing sugars being converted back to starch. Biedermann, Biedermann-Brem, Noti & Grob (2002b) has stored potatoes of cultivar Erntestolz at 4^{0}C for 15 days and observed a dramatic increase in the level of reducing sugars from 80 to 2250 mg/kg fresh weight, which is expected to increase the acrylamide formation 28 times at 120^{0}C. Pinhero et al. (2012) produced potato chips using a transgenic potato variety they

developed with low temperature tolerance and stored at 5^0C. They obtained 69% less acrylamide formed compared to the control variety.

1.6.6. Irradiation

Gökmen, Akbudak, Serpen, Acar, Turan and Eris (2007a) evaluated the efficacy of controlled atmosphere storage and low-dose irradiation (50 Gy) on potato tubers and observed lower rate of sweetening in the tubers and no change in asparagine levels. Similarly, Mulla, Bharadwaj, Annapure, Variyar, Sharma & Singhal (2011) made potato chips using the irradiated and stored (at 14^0C for 6 months) potatoes and obtained 10.7% lower reducing sugar and 8.4% corresponding acrylamide upon frying compared to non-irradiated potatoes since gamma irradiation avoided the sprouting which is known to increase the reducing sugar content in potato tubers.

1.6.7. Lowering the pH

Since the major pathway leading to acrylamide formation between asparagine and reducing sugars are related to Maillard reaction and this reaction is slowed down by lowering the pH when it is undesirable, decreasing the pH could be a possible mitigating strategy as suggested by Jung, Choi & Ju (2003). The authors evaluated the effect of citric acid on acrylamide formation on potato and reported significant reductions due to low pH of the food matrix. However, dipping in organic acid solutions such as acetic acid and citric acid has a negative effect on the taste of foods (Mestdagh, Maertens, Cucu, Delporte, Van Peteghem & Meulenaer, 2008b).

1.6.8. Treatment with cations

Kalita & Jayanty (2013a) added vanadium salt at the concentrations of 1, 3 and 6 mM into the model system of asparagine and glucose pyrolyzed for 30 minutes and obtained 40.7, 83.3 and 97.6% reductions in acrylamide levels, respectively. It was previously reported that metal ions may react with asparagine and avoid the formation of Schiff base and therefore the following acrylamide (Gökmen & Şenyuva, 2007b). Gökmen & Şenyuva (2007b) tested divalent cations such as calcium and observed complete prevention of acrylamide formation, whereas when they used monovalent cations such as Na, 50% reduction was observed. Sensory qualities of the potato products tested were not adversely effected either. Açar, Pollio, Di Monaco, Fogliano & Gökmen (2010) added calcium chloride and calcium lactate to dough at a concentration of 0.5% and obtained 70% reduction in acrylamide level with no negative impact on sensory attributes. Kolek, Šimko, & Simon (2006) added different levels of NaCl to the model system consisting of asparagine and glucose and reported 32% decrease with the addition of 1% salt. This level was found practical since increasing the salt level to 10% only resulted in additional 8% acrylamide reduction.

1.6.9. Antioxidant addition

Morales, Jimenez, Garcia, Mendoza & Beristain (2014) tried several natural aqueous antioxidant extracts sourced from oregano, thyme, cinnamon, bougainvillea and green tea at a concentration level of 1 g/L for 1 minute against the acrylamide formation in fried potatoes and achieved 62, 39 and 17% reduction for the extracts obtained from green tea, cinnamon and oregano, respectively, without affecting the sensorial characteristics of the

fried potatoes. The authors recommended this method to be used at home preparations as well since it is quick to apply and it does not require either any special instrument. Similarly, Zhang, Chen, Zhang, Wu & Zhang (2007) used the extract from bamboo leaves to avoid acrylamide formation and obtained 74% reduction in fried potatoes.

1.6.10. Addition of amino acids and vitamins:

Another strategy to lower the acrylamide formation could be the addition of other amino acids such as glycine (other than asparagine) or protein containing foods to favor the competing reactions with reducing sugars or by binding the acrylamide formed (Lindsay & Jang, 2005). Shin, Kim, Lee, Choi, Na & Lee (2010) evaluated the effect of a free-non protein amino acid, taurine, for reducing the acrylamide formation in potato chip model and significant reductions were obtained and this reduction was attributed to the amino group of taurine which competes with asparagine for acrylamide formation. Salazar, Arámbula-Villa, Vázquez-Landaverde, Hidalgo, & Zamora (2012) used amaranth flour (16.45% protein) and amaranth protein isolate to reduce the acrylamide levels in model systems and food products. Although amaranth flour did not help in reducing the acrylamide levels, the protein isolate version reduced the acrylamide levels by 40% in the model system, 89% in cookies, 51% in fried tortilla chips and 62% in baked tortilla chips. Even though the authors needed further sensory analysis for amaranth protein, they mentioned this approach as a way to improve the nutritional characteristics of foods too.

On the other hand, Zeng et al. (2009) tried 15 different vitamins as acrylamide mitigating strategies and found that water-soluble vitamins were effective for the

purpose, while fat soluble vitamins had only minor contributions. Nicotinic acid and pyridoxal lowered the acrylamide formation in potato strips by 51 and 34%, respectively.

1.6.11. Thermal Input:

As previously mentioned, frying conditions dramatically affect acrylamide formation, color, texture and flavor development in potato products. Intense frying conditions (time and temperature) lead to darker fries and higher acrylamide contents (Medeiros Vinci et al., 2012) but lowering frying temperatures (<160 °C) results in increased frying time and enhanced fat uptake (Foot et al., 2007). Additionally, cooking the product in the industry to the maximum moisture level with acceptable product is important for the acrylamide formation since it forms faster when moisture content is low (CIAA, 2014).

Decreasing the amount of heat supplied during the heating of foods can be an easy way of slowing the acrylamide formation. This could be done either by longer heat treatment at lower temperatures, vacuum frying or modifying the temperature and time profile (lowering the temperature of the heating medium in the final stage of the heating when the water content of food is low) (Anese et al., 2013). Additionally, high temperature flash frying and rapid cooling used by manufacturers reduce the acrylamide formation (CIAA, 2014). Finishing the frying on time is crucial since the acrylamide formation occurs exponentially through the end of the process. Grob et al., (2003) described the ideal French fries to be crispy enough with typical flavor and light browning at the tips without general browning.

When frying oil temperature of 190^0C was reduced to 170^0C and 150^0C, acrylamide formation was cut by 68% and 88% in potato slices, respectively (Pedreschi, Kaack & Granby, 2004). Using thawed potato strips compared to their frozen counterparts shorten the frying time needed and therefore the acrylamide generated was reduced by 89% (Tuta, Palazoğlu & Gökmen, 2010). Palazoğlu & Gökmen (2008b) employed different time and temperature combinations throughout the frying process of potato strips and reduced the acrylamide levels in French fries by 50% when the final part of the frying process was done at lower oil temperatures. Granda, Moreira & Tichy (2004) tried frying potato chips under vacuum and obtained 94% reduction in acrylamide content compared to traditional frying when reached to the same final moisture content. As opposed to 165^0C for traditional frying at atmospheric conditions, the authors used 118^0C for frying under vacuum and obtained potato chips with desired color and texture and low acrylamide content. However, vacuum frying has limited availability and throughput capacity (CIAA, 2014).

Romani, Bacchiocca, Rocculi & Dalla Rosa (2009) tested the effects of several factors such as domestic and catering type fryers, and different potato to oil ratios. They observed faster increase in oil and potato surface temperatures in domestic fryers and which produced higher acrylamide values. Similarly, when smaller quantity of potatoes was fried in the oil, oil temperature kept higher leading to higher amount of acrylamide formed as well. This study shows how critical the potato to oil ratio and performance (efficient heating power) of the fryer is in order to overcome the initial drop in oil temperature due to potato addition and shorter overall frying time. Palazoğlu, Savran and

Gökmen (2010) compared baking and frying on acrylamide formation in potato chips. They observed that an increase in temperature from 170^0C to 190^0C also increased the acrylamide levels formed. However, the highest level of acrylamide in baking was formed at the lowest temperature tested (170^0C) (more than double amount of acrylamide) compared to 190^0C.

1.6.12. Slicing

One of the other recommendations to lower the acrylamide content is to cut the potatoes thicker to increase the volume-to-surface area and therefore lower the acrylamide formation (FAO, 2007). The interior of the thicker pieces cooks slower and acrylamide is formed mostly on the surface unless the cooking time is extended to cook the inside, which may lead to the surface to be overcooked and contain higher acrylamide levels. This was further confirmed by the experiment conducted by Palazoğlu and Gökmen (2008a), who used the same potato stripes at identical frying temperature and time (170^0C for 5 minutes) and obtained 980 and 1502 ppb acrylamide for 10 x 10 mm and 8.5 x 8.5 mm strips upon frying.

1.6.13. Removing already formed acrylamide from the foods

Banchero, Pellegrino & Manna (2013) tried to remove the already formed acrylamide in coffee beans using supercritical CO2 extraction and reported that it was possible (79% acrylamide extraction efficiency obtained) without altering the caffeine content. Similarly, Serrano-Nino et al. (2014) used 14 different strains of lactic acid

bacteria to remove the acrylamide in vitro. They reported up to 29% binding ability of the strains to acrylamide. They proposed the use of lactic acid strains for the detoxification of the acrylamide in foods by binding and reducing the bioavailability of it in the body.

1.6.14. Additional notes on mitigating strategies:

Medeiros Vinci et al. (2012) mentioned that none of the agents used in the lab scale could have been conveyed to the industrial processing lines and have become successful so far, indicating that further methods are needed for the industry.

On the other hand, some of the acrylamide mitigation strategies may have negative consequences other than sensorial properties. Using the Ca^{2+} and glycine was reported to cause higher levels of hydroxymethylfurfural (HMF) with Ca^{2+} preventing the Schiff base formation and diverting the reaction to dehydration of glucose whereas addition of glycine speeding up the Maillard reaction in general (Pedreschi et al. 2014).

In one interesting study (Grob et al., 2003), researchers worked in collaboration with the cooking experts in order to find the optimum conditions which could provide both the culinary quality and minimum acrylamide levels in the final product. The authors obtained up to 10 times lower acrylamide concentrations (40-70 mg/kg) in French fries compared to normal fries and attributed this reduction to following conditions;

- ✓ Minimum amount of reducing sugars in the raw potato by selecting the cultivar and not storing the potato tubers at temperatures below 10^0C.
- ✓ Removing the fine pieces of potatoes following the cuttings.

- ✓ Immersing the potato strips in cold water or warm water for 15 minutes to help asparagine and reducing sugars leach out without removing the starch.
- ✓ Pre-frying in oil at about 140^0C for 2.5 minutes to facilitate the crispiness
- ✓ Choosing the frying oil temperature, potato to oil ratio and end point of frying adequately.

1.7. Analytical methods to measure acrylamide

Since acrylamide became an issue, there have been numerous studies performed to develop new methods or improve the existing methods for acrylamide quantification in foods. Scientists focus on sample preparation techniques for the purpose of developing an analytical technique which could be implemented for different types of foods. The goal is also to make this technique simpler, faster, cleaner and cheaper with lower limit of detection (LOD) and limit of quantification (LOQ) values and minimum solvent use (Tekkeli et al., 2012).

As previously mentioned, acrylamide is highly reactive and soluble in water and it does not either contain a strong chromophore or it fluoresces (Eriksson, 2005), which makes it difficult to be detected. Even though, there is not an official method established for acrylamide determination in foods, there have been several analytical methods developed so far including Liquid and Gas Chromatographies based methods, Enzyme Linked Immunosorbent Assay (ELISA), Capillary Electrophoresis (CE) and Infrared systems.

Acrylamide concentration shows large variability even in a given product brand. This variation could be originated from composition of the raw material, process conditions, processing equipment and such (CIAA, 2014). Therefore, careful procedures and statistically representative sampling needs to be applied prior to acrylamide analysis (Taeymans et al., 2004) no matter what method is used for the subsequent quantification.

Among the alternative methods, GC and LC coupled with mass detectors are commonly used sensitive equipment even if the methods are expensive and require labor intensive sample preparation and skilled operators. However, because of the strong polarity and low molecular weight of acrylamide, GC–MS and LC-MS/MS methods brings some difficulties and therefore analysis of this compound in food matrices are challenging in regards to its extraction and determination (Fernandes & Soares, 2007). Labor intensive and time consuming extraction processes for acrylamide from foodstuff usually requires multiple extractions, centrifugation and separating the supernatants (Fernandes et al., 2007). As an extraction solvent, water is usually preferred due to the strong polar characteristics of the acrylamide molecule. However, other water soluble compounds such as sugars, proteins and organic acids are co-extracted with water along with the acrylamide (Fernandes et al., 2007). To avoid the co-extracts, either a different organic solvent should be used instead of water as recommended by Dunovská, Čajka, Hajšlová, & Holadová (2006) or additional clean-up procedures need to be applied for acrylamide to be separated from the other components. Further details on the type of SPE columns and stationary phases for analytical columns as well as optimum settings for the instruments parameters used for acrylamide analysis can be found at the dissertation written by Eriksson (2005).

For both LC and GC based methods, internal standards are commonly used. As opposed to previously used internal standards such as methacrylamide or N, N-dimethylacrylamide which required overnight incubation at refrigerator conditions, isotopic substituted acrylamides (e.g. $13C_3$ or deuterium-labelled acylamide-d_3) used as internal standards today shorten the reaction times to 1-2 hours (Eriksson, 2005).

1.7.1. Liquid Chromatography Based Methods

After the discovery of acrylamide in foods in 2002, Liquid Chromatography-Tandem Mass spectrometry (LC-MS/MS) was used for the first time for quantification of acrylamide in different heat treated foods (Rosen & Hellenas, 2002) to verify the initial results of acrylamide levels obtained in Sweden using GC-MS so the results from the GC-MS were not artefacts formed during the analytical procedure. In this experiment, Rosen et al. (2002) monitored the transitions m/z 72 > 55, 72>54, 72 >44, 72>27, 72 > 72 and 75>58 for identification and quantification. The method included using deuterium-labelled acylamide-d_3 as internal standard and water as extraction solvent as well as SPE and ultrafiltration prior to LC-MS/MS analysis.

Acrylamide is a very polar molecule and therefore it has very low retention on LC reversed-phase sorbents. For this reason, effective clean-up steps are still required to avoid interferences from co-extracts even if tandem mass spectrometers are used (Ahn et al., 2002). Acrylamide usually elutes with a lot of impurities in reversed phase HPLC and that is why a good separation of acrylamide from the remaining compounds is critical to lower the noise, minimize the ion suppression and achieve low LOD values (Dunovská et al., 2006). The main advantage of the SPE cleaning for acrylamide analysis is the

retention of the other interfering compounds since binding of acrylamide to any sorbent is difficult.

HPLC with single stage MS is challenging due to the interferences from food matrices (Wenzl et al., 2006). When HPLC-MS/MS is used instead of a single stage mass detector, usually a two stage SPE cleaning is applied for cleaning and enrichment purposed to minimize the ion suppression effects in the ion source (Wenzl et al., 2006). Even though, triple-quadrupole mass spectrometers (LC-MS/MS) are costly, they provide better sensitivity and are more applicable to be used for acrylamide analysis in foods since they do not require a prior derivitization. The method developed by Roach, Andrzejewski, Gay, Nortrup & Musser (2003) using LC-MS/MS was tested in more than 450 food products and 35 types of foods. The main ions monitored at ESI+ are m/z 72, 55 and 27 corresponding to protonated ion, acrylamide with loss of amino group and CO, respectively. High-performance liquid chromatography with detection by mass spectrometry has shown excellent analytical performances for the detection of acrylamide in foods with limits of quantitation of 10 ppb (µg/kg), response linearity over a range of 8-3600 µg/kg and recoveries > 95% (Roach et al, 2003).

However, LC-MS/MS may not be present in most of the laboratories while LC-MS is more frequently used. Weisshaar (2004) recommended derivitazing the acrylamide (e.g. using mercaptobenzoic acid) to get a less polar and higher mass derivative which may show better retention in the column and separate better from the interfering compounds. Şenyuva & Gökmen (2006) extracted acrylamide with 0.01 mM acetic acid from potato and cereal based foods and analyzed with LC-MS. The authors identified valine as major interfering co-extract with the ions of m/z 118 and 72 produced in

positive mode. They reported that the presence of valine increased the baseline signal and avoided accurate and precise quantifications. To avoid this interference, the authors either diverted the first 6.5 minutes of the run to the waste using the software or applied a SPE clean-up with strong cation exchanger and accuracy, precision and sensitivity of the method was improved.

To compare the performance of LC-MS/MS to GC-MS, several studies were conducted. Tareke et al. (2002) measured the acrylamide levels in foods by both GC-MS after bromination and LC-MS/MS. They reported close to 100% recoveries for both methods with LOD values of 5 and 10 µg/kg for GC-MS and LC-MS/MS, respectively. However, they recommended using LC-MS/MS since it is simpler and more preferable for routine analysis of acrylamide. Twenty five laboratories (mostly from Europe) joined in a study to validate GC-MS and LC-MS/MS based methods for acrylamide quantification in bakery and potato products (Wenzl et al., 2006). Within the concentration range of products changing between 20 and 9000 µg/kg and HPLC-MS/MS was reported as superior to GC-MS, with higher precision parameters.

Apart from the mass detectors, there have been some other HPLC methods developed as well. Paleologos & Kontominas (2005) used a normal phase HPLC with a UV detector for acrylamide determination in commercial foods samples using a polar HPLC column (Aminex HPX-87H) design for separation of organic acids using 0.01 M sulfuric acid as mobile phase. Under the conditions set, acrylamide was converted into a cationic, protonated ammonium product and detection and quantification was made at 200 nm and LOD of 10 µg/L and a recovery of 95% were obtained. Similarly, Wang, Lee, Shuang & Choi (2008) developed an HPLC method using UV detector for

acrylamide quantification in fried dough at UV wavelengths of 210 and 225 nm, with higher than 78% recoveries. Their sample preparation included water extraction, centrifugation at low temperatures and SPE clean-up steps. The authors used a gradient elution program with acetonitrile and water for higher resolution of acrylamide. Gökmen, Şenyuva, Acar & Sarıoğlu (2005) utilized LC coupled to diode array detection (DAD) for quantification of low levels of acrylamide in heated potato products, setting up the DAD at 226 nm. They extracted acrylamide with methanol, purified with Carez I and II solutions. Then, solvent was evaporated and changed to water before SPE cleaning. Final extracts was analyzed using LC-DAD (LOQ was 20 µg/kg) and results were confirmed by LC-MS.

1.7.2. Gas Chromatography Based Methods

Since GC-MS instruments are cheaper and used more commonly in laboratories, the method of acrylamide analysis with bromination and subsequent GC-MS measurement may be preferred over LC-MS/MS (Weisshaar, 2004). The presence of a concentration step in GC-MS sample preparation also makes a higher sensitivity and lower LOD possible.

Acrylamide analysis using GC is conducted through either derivitization (usually with potassium bromate and potassium bromide with acidification to pH 1-3) or direct analysis without derivitization. Most of the GC-based acrylamide methods require bromination to produce a less polar and more volatile analyte with more specific ions (higher m/z). Along with the advantages, derivitization using bromine may also carry

some disadvantages since bromine is a hazardous chemical and the process of derivitization is labor intensive and lengthy (Dunovská et al., 2006).

Addition of bromine to acrylamide under acidified conditions is illustrated in **Figure 1.3**. Although it is more labor intensive, acrylamide methods including derivitization through the bromination to 2,3-dibromopropionamide have several advantages including better selectivity, higher volatility, less co-extracts, better sensitivity and possibility of using relatively simpler GC-MS instead of GC-MS/MS for low detection limits (Taeymans et al., 2004). The ions monitored for identification of the analyte, 2,3-dibromopropionamide, were m/z 152 [C3H581BrON]+ (100%), 150 [C3H579BrON]+ (100%) and 106 [C2H379Br]+ (65-70%) using m/z 150 for quantification. The ions monitored for identification of the internal standard, brominated to 2,3-dibromo($13C_3$)propionamide, were m/z 155 [13C3H581BrON]+ (100%) and 110 [13C2H381Br]+ (65 - 70%) using m/z 155 for quantification (Eriksson, 2005).

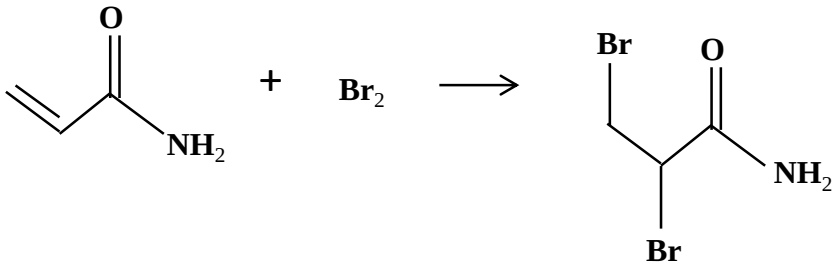

Figure 1.3. Addition of bromine to acrylamide at acidic conditions (Adapted from Weisshaar, 2004)

Acrylamide analysis was developed for the very first time using bromination and GC for drinking and discharge-water (Habermann, 1991). For the derivitization involving method, once the acrylamide is extracted from the food and derivatized to dibromopropanamide, extraction from aqueous phase to ethyl acetate is applied. This helps concentrating the analyte, reducing the polarity of acrylamide and increasing the detection of the analyte by mass detector due to the switch from low molecular mass to a higher molecular mass (Wenzl et al., 2006).

Analysis of acrylamide by GC-MS without derivitization is also possible since acrylamide is slightly volatile but artifact formation of acrylamide can occur in the injection port when acrylamide precursors are still present in the final analyte (Stadler & Scholz, 2004b). Acrylamide extracted from complex food matrices are difficult to be determined by GC-MS without derivitization since there are still some interfering compounds with low m/z ions co-extracted, which makes achieving low LOD impossible (Dunovská et al., 2006). However, Dunovská et al. (2006) developed and validated a GC method with a high-resolution time-of-flight mass analyzer without the need of derivitization in foods. As extraction solvent, the authors used n-propanol and later exchanged it to acetonitrile to avoid co-elution of acrylamide precursors. If water is used as extracting solvent, sugars and asparagine can also be extracted and further acrylamide formation in the hot GC injector can cause false quantifications. The authors reported LOQ ranging between 15 and 40 µg/kg while the recoveries changed between 97 and 108% based on the type of the food analyzed.

A solid phase dispersion technique using C18 as dispersive agent and water as extraction solvent was also studied for acrylamide determination in potato chips to

achieve a faster and easier sample preparation prior to GC-MS analysis by Fernandes et al., (2007) (LOD and LOQ values were 12.8 and 38.8 µg/kg). They validated their method by analyzing 17 different potato chips with another GC-MS method using hot water as the extraction solvent. They obtained a very high correlation factor (r) of 0.9985 between the results of the two methods.

Recently, Russo, Avino, Centola, Notardonato & Cinelli (2014) introduced a GC method coupled with electron capture and ion trap mass spectrometry detectors (GC-ECD and GC-IT/MS) for acrylamide quantification in foods. After the extraction of acrylamide from cereal based foods and potato chips using acetonitrile, they used trifluoroacetic acid anhydride for derivitization instead of bromination prior to reconstituting in toluene and injection. The method gave LOD values of 1and 2 µg/kg for GC-ECD and GC-IT/MS, respectively with higher than 90% recoveries.

1.7.3. Other Methods

Apart from the LC and GC based methods, there are other assays applied for acrylamide analysis in foods.

Enzyme-linked Immunosorbent assay (ELISA), based on the affinity between antibodies and antigens is one of the techniques applied for acrylamide analysis in foods. Singh et al. (2014) developed an indirect competitive enzyme-linked immunosorbent assay for quick determination of acrylamide in potato chips, French fries and bagel. By producing more immune-sensitive derivative of acrylamide, which is a small molecule, a fast, specific and sensitive immunological method can be developed (Singh et al., 2014). The authors reported the assay as very sensitive with LOD of 5 µg/kg and the results

were comparable to those obtained using LC-MS/MS. Similarly, Sun, Xu, Ma, Qiao & Xu (2014) developed a direct competitive biomimetic enzyme-linked immunosorbent assay (BELISA) to determine acrylamide levels in French fries and crackers. The authors developed a hydrophilic imprinted artificial membrane which has a high affinity and specificity towards acrylamide. The BELISA method had a good sensitivity and low LOD values. Results obtained using this technique was compared to a GC-based method and no statistical difference was found between the results. There have been also some other studies published on developing immunoassays for acrylamide determination as Preston, Fodey & Elliott (2008) in water samples with LOD of 65.7 µg/kg and Zhou, Zhang, Wang & Zhao (2008) in French fries and biscuits with LOD of 6 µg/kg.

Near-Infrared spectroscopy was also used for acrylamide analysis in foods. Pedreschi, Segtnan & Knutsen (2010b), used an online NIR interactance imaging to predict acrylamide content along with some other parameters in potato chips. The authors developed a Partial least squares regression (PLSR) mode using NIR interactance and VIS reflectance. The model gave a prediction error of 266 µg/kg with correlation of 0.83 between predicted and measured reference values. The authors reported this prediction error as high and recommended the model to be used for screening rather than prediction. Similarly, Segtnan, Kita, Mielnik, Jørgensen & Knutsen (2006) used VIS and NIR spectra for acrylamide determination in potato crisps. The model developed for prediction gave an error of 247 µg/kg with correlation of 0.952 between predicted and measured values. The author suggested that the model could be used for screening acrylamide levels in potato crisps too.

Measuring color is another approach for predicting acrylamide levels in foods. Pedreschi, Kaack & Granby (2006) evaluated the color changes in French fries fried at different temperatures and reported linear relationships (high correlations) between acrylamide content and L*(r^2 of 0.79) and A* (r^2 of 0.83). Similarly, a linear correlation between acrylamide level and red color of potato chips was reported by Pedreschi, Moyano, Kaack & Granby (2005). Brunton et al. (2007) also reported a negative relationship between the lightness value (L*) and acrylamide concentration of French fries, suggesting that lightness value could be used as indicator of acrylamide formation. Gökmen et al. (2007c) used digital color images of French fries and potato chips to estimate acrylamide levels using LC-MS as a reference method. The authors assigned bright yellow, yellowish brown and darker brown regions in fried potato images and classified the image in three sets. The authors reported a strong relationship between the ratio of the number of pixels in set 2 to total number of pixels in the entire image (NA2 values) and acrylamide concentrations. The linear regression equation developed was further used to predict the acrylamide levels in home-made French fries and commercial potato chips and -14 ± 24% and +4 ± 14% mean differences were obtained between measured and predicted acrylamide levels in home-made French fries and commercial potato chips, respectively. The authors recommended that NA2 values higher than critical value indicated high acrylamide content and therefore these fried potatoes can be eliminated from the processing line.

Pollien et al. (2003) monitored the formation of acrylamide in real time during the thermal treatment in model systems and thermally treated potatoes using proton transfer reaction mass spectroscopy (PRT-MS) by monitoring the volatile compounds in

headspace. Additionally, Capillary Electrophoresis (CE) coupled to mass spectrometry was used for acrylamide analysis in foods after derivitization with 2-mercaptobenzoic acid and field amplified sample injection in negative electrospray mode (Bermudo, Nunez, Moyano, Puignou, & Galceran, 2007). With this approach, a detection limit which is comparable to LC-MS/MS results and low enough (8 µg/kg) to implement in analysis of acrylamide in foods was achieved.

Zargar, Sahraie & Khoshnam (2009) used square-wave voltammetry for determination of acrylamide in potato chips. When cobalt (II) ions were added, a new catalytic peak appeared and this peak was proportional to the acrylamide concentration. However, this method involved a lengthy sample preparation, including grounding, defatting with hexane, extraction with acetone and water, filtering and drying. Once the sample preparation was done, measurement was quite short (less than a minute). Results obtained were similar to those obtained by the HPLC method.

CHAPTER 2

APPLICATION OF A PORTABLE INFRARED INSTRUMENT FOR SIMULTANEOUS ANALYSIS OF SUGARS, ASPARAGINE AND GLUTAMINE LEVELS IN RAW POTATO TUBERS

2.1. ABSTRACT

The level of reducing sugars and asparagine in raw potatoes is critical for potato breeders and the food industry for the production of commonly consumed food products including potato chips and French fries. Our objective was to evaluate the use of a portable infrared instrument for the rapid quantitation of major sugars and amino acids in raw potato tubers using single-bounce Attenuated Total Reflectance (ATR) and dial path accessories as alternatives to time-consuming chromatographic techniques. Samples representing a total of 84 experimental and commercial potato varieties harvested in two consecutive growing seasons (2012 and 2013) were obtained from the Ohio Agricultural Research and Development Center (OARDC) in Wooster, OH, USA. The levels of sugars and two main free amino acids in samples (asparagine and glutamine) were determined with reference HPLC-RID and GC-FID methods, respectively. Samples had wide ranges of sugars (Non-Detectable (ND)-7.7 mg glucose, ND-9.4 mg fructose and 0.4-5.4 mg sucrose per 1g fresh weight), and asparagine and glutamine levels (0.7-2.9 mg and 0.3-

1.7 mg per 1 g fresh weight, respectively). Infrared spectra collected from 64 varieties were used to create Partial Least Squares Regression (PLSR) calibration models that predicted the sugar and amino acid levels in an independent set of 16 validation potato varieties. Excellent linear correlations between infrared predicted and reference values were obtained. PLSR models had a high correlation coefficient of prediction (rPred >0.95) and Residual Predictive Deviation (RPD) values ranging between 3.1 and 5.5. Overall, the results indicate that the models can be used to simultaneously predict sugars, free asparagine and glutamine levels in the raw tubers, significantly benefiting potato breeding, certain aspects of crop management, crop production and research.

2.2. INTRODUCTION

The potato is the fifth most produced crop worldwide after sugar cane, maize, wheat and rice with a production surpassing 368 million tons in 2012 (FAOSTAT, 2014). Among the many potato products available, potato chips and French fries are the most popular. The quality of these products are highly dependent on the composition of the raw potatoes, which makes the monitoring of tuber composition a critical step for effectively planning breeding lines to produce tubers suitable for adqueate processing in the food industry (Haase, 2011).

Factors such as variety, storage temperature and time, as well as the level of nitrogen and phosphorous in the soil can alter the level of tubers`reducing sugars and free amino acids (Heuser, Gerendas & Sattekmacher, 2005; Silva & Simon, 2005), the key components for the browning reactions that occur during cooking. Previous labelling studies have shown that asparagine provides the backbone of the acrylamide molecule (2-

propenamide, CAS Registry No. 79-06-1) (Stadler et al., 2004a; Zyzak et al., 2003), a known human neurotoxin, rodent carcinogen and "probable carcinogen to humans" (IARC, 1994). Even though there are several pathways proposed for acrylamide formation (Granvogl, Jezussek, Koehler & Schieberle, 2004; Claus, Weisz, Schieber & Carle, 2006), it is generally accepted that acrylamide is predominantly formed through the Maillard reaction pathway, with free asparagine and reducing sugars (e.g. glucose and fructose) as the major reactants present in foods (Friedman, 2003). Gerendás, Heuser & Sattekmacher (2007) reported that these major reactants in potatoes were found at their highest levels when grown with high N and low K supply. Additionally, storing potato tubers at temperatures lower than 8^0C can cause rapid accumulation of sugars (cold sweetening) (Sowokinos, Vayda and Park, 1990) in a genotype-specific manner (Olsson, Svensson & Roslund, 2004). Among the amino acids in potato tubers, asparagine and glutamine are found at the highest concentrations (Brunton et al., 2007), with asparagine being the most abundant and accounting for about one-third of the total free amino acids (Amrein et al., 2003). Compared to the typical level of sugars in potatoes, asparagine is usually present at higher concentrations; therefore reducing sugars are the most important elements needed for Maillard reactions (Gökmen & Palazoğlu, 2008).

The most popular methods used to determine the levels of sugars and amino acids in raw potatoes require intensive sample preparations and chromotographic separations with HPLC or GC. Unlike many traditional methods used in food quality testing, infrared spectroscopy provides valuable information about the biochemical composition of the samples, quickly and with minimal or no sample preparation needed (Rodriguez-Saona &

Allendorf, 2011). Along with improvements in optical technology and modern computing technology for chemometric analysis of infrared spectra, portable and handheld spectrophotometers are now available as practical and economical alternatives to their benchtop counterparts (Rodriguez-Saona et al., 2011). Although these miniaturized systems have not been available until recently, they have already been successfully used in many different food applications, including the quantitation of trans fat in edible oils (Birkel & Rodriguez-Saona, 2011), mineral fortification in whole grain cornmeal (Hassel & Rodriguez-Saona, 2012) and sucrose levels in infant cereals (Lin, Ayvaz & Rodriguez-Saona, 2014).

The objective of this study was to develop simple and robust methods for simultaneous quantification of sugars (glucose, fructose and sucrose), free asparagine and glutamine levels in raw potatoes using a portable FTIR system equipped with dial path and ATR accessories. The methods would be developed based on highly specific MIR spectroscopic signature profiles and supervised pattern recognition techniques.

2.3. MATERIALS AND METHODS

Potato tubers from 2012 (47 varieties) and 2013 harvests (37 varieties) representing a total of 84 experimental clones or commercial varieties (some used or in development for chip production) were obtained from the Ohio Agricultural Research and Development Center in Wooster, Ohio, USA.

Five potato tubers of each clone were washed under running tap water and dried using paper towels. Tubers were then cut into quarters and frozen immediately at -40ºC

until used. Potato quartes were blended with liquid nitrogen and the fine powder obtained was used for all further experiments.

2.3.1. Reference Methods

2.3.1.1. Determination of Sugar Levels in the Tubers by HPLC

About 5 g of potato powder was weighted into 50 ml screw-top tubes in duplicate for each clone and mixed with 40 mL of 80% (v/v) ethanol. The mixture was vortexed briefly, placed in a rotating mixer inside a 50 °C oven for 1 h and then centrifuged at 4 °C at 12,000 g for 20 minutes. After centrifugation, the supernatant was transferred to a round-bottom flask and placed on a Buchi R110 rotary evaporator (Brinkmann Instruments, Rexdale, Ontario) to remove the ethanol solvent. The residual left in the round bottom flask was transferred to a volumetric flask using Pasteur pipettes and filled up to 5 ml volume with water. A 5 ml extract was then passed through an activated C_{18} cartridge to remove phenolics and the elute was passed through a syringe filter with 0.45 μm pore size (Thermo Fisher Scientific Inc, Waltham, MA, USA) into transparent HPLC vials.

The sucrose, glucose and fructose levels were determined using a high-performance liquid chromatography system (HPLC, Shimadzu Scientific Instruments, Inc., Columbia, MD) equipped with LC-6AD pumps, SIL-20AHT autosampler, CTO-20A column oven and RID-10A refractive index detector. Twenty-five microliter of each filtrate was injected into the system. Glucose, fructose and sucrose were separated using a Rezex RCM-monosaccharide column (300 x 7.8 mm; Phenomenex, Torrance, CA, USA). Isocratic separation of the sugars was achieved at 80 °C using HPLC grade water with a

flow rate of 0.6 mL/min for 25 min. LC Solutions software version 3.0 (Shimadzu Scientific Instruments, Inc. Columbia, MD) was used to analyze the chromatograms.

2.3.1.2. Determination of Free Asparagine and Glutamine by Gas Chromatography

About 2 g of potato powder was weighted into 50 ml screw-top tubes in duplicate for each clone. Then, 25 ml 0.01 M HCl was added into each tube and vortexed briefly. The tubes were stirred for 15 minutes at room temperature and then allowed to settle for 45 minutes. From the supernatant, 2 ml aliquots were transferred into the microcentrifuge tubes and centrifuged at 4 ºC and 10000 g for 30 minutes. The top 1 ml was retained and used for the commercially available EZ:faast amino acid kit (Phenomenex, Torrance, CA:catalog no. 90501-1430). One hundred µL of the retained samples was transferred into sample preparation vials included in the EZ:faast GC-FID free amino acid kit. Potato sample extracts were prepared using the manual provided by the manufacturer and quantified using GC-FID (Model HP 6890, Hewlett-Packard) equipped with an automatic injector (Model HP 7683). A Zebron ZBAAA capillary column (10 x 0.25 mm) was used for the separation of amino acids. The oven was set to start at 110 ºC and increased 32 ºC per minute up to 320 ºC. An aliquot of the derivatized sample (1 µL) was injected at 250 ºC in split mode (15:1). The FID detector was set to 320 ºC and the carrier helium gas flow rate was kept at 1.5 mL/min during the run.

2.3.2. Infrared Spectroscopy

Approximately 1 g of potato powder was weighed into a 2 ml capacity microcentrifuge tube and centrifuged at 3000 g for 15 minutes. The supernatant obtained after centrifugation (**Figure 2.1a**) was used for infrared spectra collection. Two replicates of 1 g powder were centrifuged per each potato clone.

A Cary 630 FTIR spectrometer (Agilent Technologies Inc., Danbury, CT, USA) equipped with ZnSe beam splitter and deuterated triglycine sulfate (DTGS) detector was used to collect the MIR spectra of the potato tubers. Two different accessories of the Cary 630 FTIR spectrometers were used for infrared spectra collection; a 30 µm dial path (**Figure 2.1b**) and ATR accessories (**Figure 2.1c**).

For the application with dial path accessory, 10 µL of the supernatant was directly placed onto the transmission cell of the instrument with 30 µm path length as shown in **Figure 2.1b**. One spectrum for each of the centrifuged potato powders (total of 2 spectra per each potato clone) was collected. For the ATR accessory, 1 µL of the supernatant was placed onto the ATR crystal of the instrument and vacuum dried for 1 minute as displayed in **Figure 2.1c**. Then, one spectrum from each centrifuged potato powder was collected (total of 2 spectra per each potato clone).

The spectral resolution was 4 cm^{-1} and 64 spectra were co-added to improve the signal to noise ratio over the frequency range 4000-700 cm^{-1}. Spectral backgrounds were collected before each sample. The absorbance spectrum was obtained by dividing the sample spectrum to that of a blank optical path (background spectrum). The collected spectra were recorded using Agilent MicroLab PC software (Agilent Technologies Inc., Danbury, CT, USA) on a personal laptop.

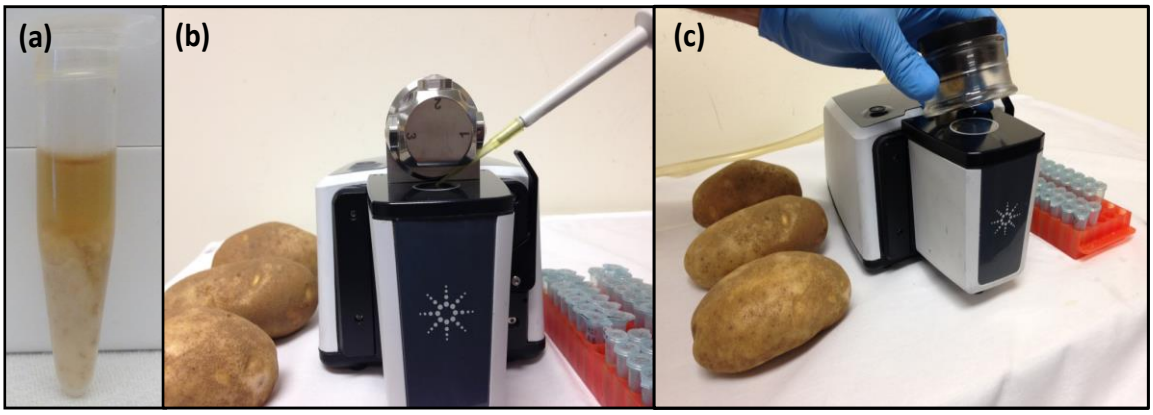

Figure 2.1. (a) The supernatant obtained after the centrifugation of 1 gram potato powder at 3000 g for 15 minutes **(b)** The application of the potato powder supernatant to the dial path cell **(c)** The application of the potato powder supernatant to the ATR accessory with vacuum attachment

2.3.3. Partial Least Squares Regression (PLSR)

The spectra collected using both dial path and ATR accessories of the Cary 630 system were imported into Pirouette software (Infometrix, Bothell, WA, USA). The data was normalized, second derivative transformed (Savitzky-Golay second order polynomial filter with a 25-point window) and smoothed (Savitzky-Golay second order polynomial filter with a 25-point window) prior to PLSR analysis.

PLSR models were developed using the infrared spectra and reference values obtained for glucose, fructose, reducing sugars (sum of the glucose and fructose), sucrose (from HPLC-RID) and free asparagine and glutamine (from GC-FID). Separate PLSR models were developed for the dial path and ATR accessories of the system for each compound of interest. Full cross-validation (leave-one-out approach) was used to internally validate the calibration models, and an independent sample set consisting of 16

potato clones (out of the 84 potato clones used in the study) was used for their external validation. Samples were randomly assigned into either calibration or independent validation sets ensuring that both replicates belonging to the same clone were allocated into the same category.

PLSR with the features adapted from both Principal Component Analysis (PCA) and Multi Linear Regression (MLR), uses not the thousands of wavenumbers collected in the spectra, but the extracted important latent variables (PLS-factors) to explain the variation in the samples (Moseholm, 1988). PLS regression takes into consideration that measured values obtained from the reference analyses such as sugar levels by HPLC-RID and free amino acid levels by GC-FID may contain errors associated with sample preparation (Brereton, 2000).

Using the measured values from the reference method and the corresponding spectra collected, the program predicts a value for the compound being analyzed in the model. To evaluate the performance of a model, loading vectors, standard error of cross validation, prediction correlation coefficient (r) and outlier diagnostics (Standard Residual of Sample vs. Leverage) were used. The SEP shows the magnitude of error expected when independent samples are introduced into the calibration models and the compound concentrations in the unknown samples are predicted. During the model development, samples with large residuals, unusual pattern and high leverage are considered outliers and removed from the models. To further evaluate the strength of a model and/or compare the models to each other, the residual predictive deviation (RPD) values were calculated by dividing the standard deviation of the reference (measured) values for the samples in the independent set to the standard error of the prediction

obtained. RPD classification reported by Saeys, Mouazen & Ramon (2005) were used to analyze the strength of the PLSR models. According to the authors, models with RPD values above 2.5 are considered "good" and models with vallues above 3 are considered "excellent". However, models with RPD values under 1.5 indicate a very poor model that would not be suitable for use. Models with RPD values between 1.5 and 2.0 are only valid to distinguish among high and low constituent concentration levels, while models with RPD of 2-2.5 can be used to make approximate predictions.

2.3.4. T-test

In order to analyze means differences between the clones from the year of 2012 and 2013 for each reference analysis, unequal variances and unbalance *T-test* was used. P-values were calculated for each comparison, and significant levels were assigned to probabilities lower than 0.05 (α=0.05) (Microsoft Excel software, Redmond, WA, USA).

2.4. RESULTS AND DISCUSSION

2.4.1. Characterization of the Potato Tubers

Representative chromatograms obtained from the reference methods are displayed in **Figure 2.2**. **Figure 2.2A** and **2.2B** illustrate chromatograms for amino acids using GC-FID and for sugars using HPLC-RID, respectively. A summary of the reference values obtained for free asparagine, glutamine, glucose, fructose, reducing sugars (sum of glucose and fructose) and sucrose levels in the potato tubers analyzed in this study is presented in **Table 2.1** based on their harvest year.

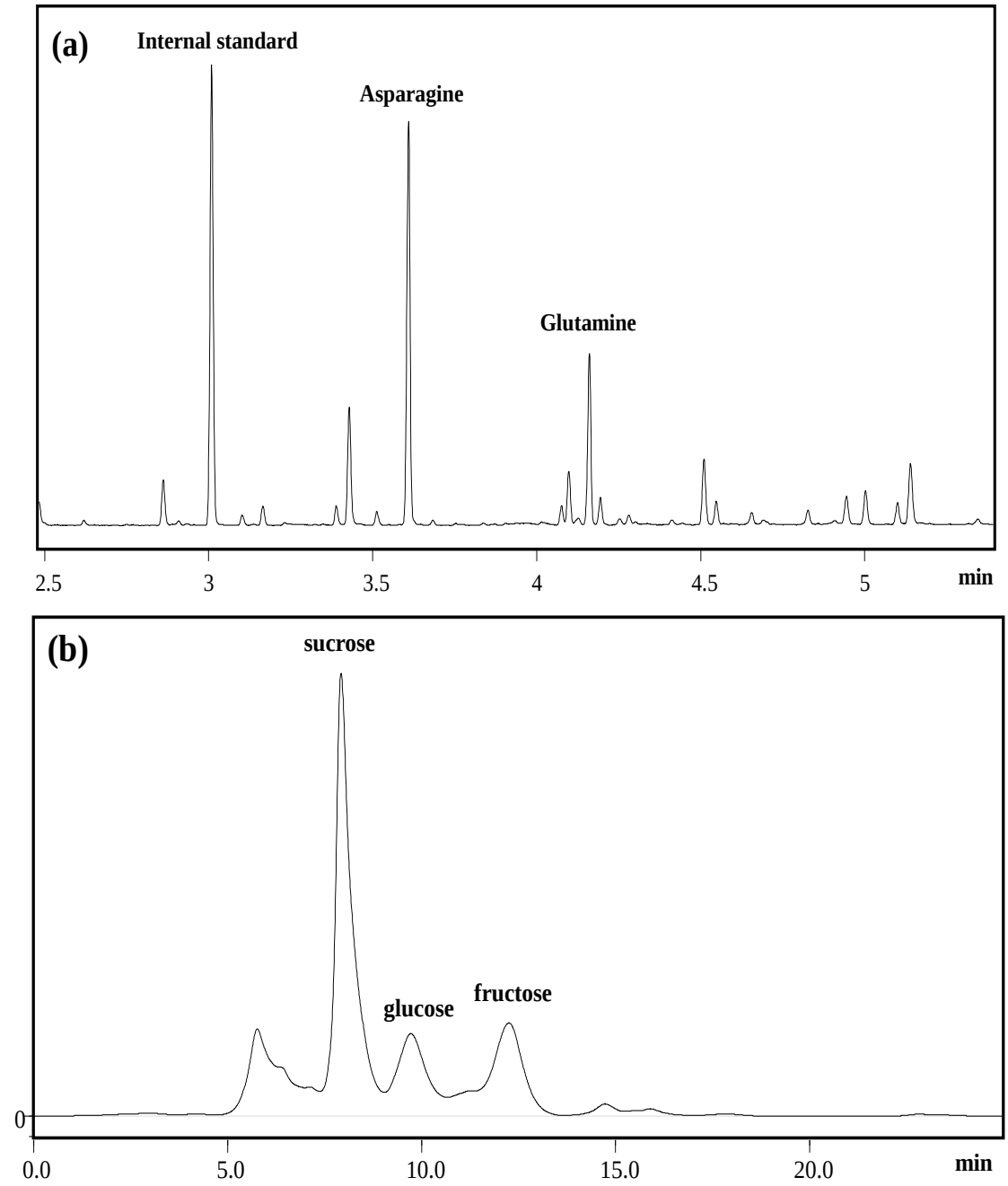

Figure 2.2. Representative chromatograms obtained from reference methods for **a)** asparagine and glutamine using GC-FID and **b)** sugars using HPLC-RID

Table 2.1. Reference concentration levels for the compounds measured in the tubers based on the harvest year and their comparison to the previously reported literature data

Parameters	Harvest Year	n	Min.	Max.	Mean	Standard Deviation	Literature range (mg/g fresh weight)
Asparagine (mg/g fresh weight)	**2012**	47	0.7	2.9	1.9	0.5	1.5 - 11.4[a]; 1.8 - 9.0[b]
	2013	37	0.8	2.0	1.3	0.4	1.7 - 2.5[c]; 2.0 - 4.3[d]; 0.8 - 2.5[e*];
Glutamine (mg/g fresh weight)	**2012**	47	0.4	1.7	0.8	0.3	1.7 - 2.0[c]; ND - 2.1[d]
	2013	37	0.3	1.1	0.7	0.2	1.3 - 6.3[g*]
Glucose (mg/g fresh weight)	**2012**	47	0.7	7.7	3.5	1.7	0.1 - 6.3[a]; 1.5 - 1.9[c]
	2013	37	ND	3.2	0.3	0.5	0.1 - 2.6[d]
Fructose (mg/g fresh weight)	**2012**	47	0.5	9.4	5.2	2.2	0.1 - 6.1[a]; 1.5 - 1.7[c]
	2013	37	ND	0.7	0.1	0.1	0.0 - 1.5[d]
Reducing sugars (mg/g fresh weight)	**2012**	47	1.7	17	8.9	3.8	0.6 - 10.6[a]; 1.4 -11.7[b]
	2013	37	ND	4	0.4	0.7	
Sucrose (mg/g fresh weight)	**2012**	47	1.1	5.4	2.7	1.1	0.4 - 1.6[d]; 0.7 - 11.1[f*]
	2013	37	0.4	2.3	1.2	0.4	

*shows that the unit is based on dry weight

[a] Becalski et al., 2004; [b] Kalita, Holm, & Jayanty, 2013b; [c] Brunton et al., 2007; [d] Amrein et al., 2003; [e] Knutsen, Dimitrijevic, Molteberg, Segtnan, Kaaber, & Wicklund, 2009; [f] Zhu, Cai, Ke, & Corke, 2010; [g] Viklund, Olsson, Sjohol, & Skog, 2008

The range and the mean value for asparagine levels were higher for the tubers harvested in 2012 compared to 2013 ($p<0.05$). In 2012, the level of free asparagine in potato tubers ranged between 0.7 and 2.9 mg/g fresh weight, averaging at 1.9 mg/g fresh weight; while in 2013 the range was between 0.8 and 2.0 mg/g fresh weight with the mean value of 1.3 mg/g fresh weight. A similar trend was seen for free glutamine levels. The level of free glutamine in the tubers was between 0.4 and 1.7 mg/g fresh weight (mean value was 0.8 mg/g fresh weight) in 2012, which was higher than the values obtained in 2013 (0.3 to 1.1 mg/g fresh weight with a mean value of 0.7 mg/g fresh weight). Although variety, preharvest and postharvest conditions can affect the levels of free asparagine and glutamine in tubers, our data showed reasonably similar levels to those previously reported, as shown in **Table 2.1**.

The higher analyte concentrations observed in 2012 compared to 2013 ($p<0.05$) were more pronounced for the potato tuber sugars with levels for reducing sugars and sucrose being 52 and 3 times higher, respectively, in 2012 compared to 2013. Among the other factors, this could be primarily due to the difference in postharvest storage temperature. The pronounced cold sweetening was expected to occur in tubers from 2012 which were stored at lower temperatures and analyzed in December in our laboratories. After harvesting and prior to analysis, the tubers in 2012 were kept in a humidified refrigerated storage at 3 ºC whereas the tubers from 2013 were kept in a humidified refrigerated storage at 9 ºC (Kleinhenz, Moyseenko, Walker, & Williams, 2013a; Kleinhenz, Moyseenko, Walker, & Williams, 2013b). Similar findings were reported by Biedermann, Biedermann-Brem, Noti & Grob (2002) stored potatoes of cultivar

Erntestolz at 4^0C for 15 days and observed an increase in reducing sugars from 80 mg to 2250 mg per kg fresh weight. Fortunately, the resulting variation provided a wider range of sugar levels, which we considered an advantage for this infrared study.

2.4.2. Regression Analysis

Average transmission and single bounce ATR spectra obtained from potato powder supernatants showed strong water absorption bands as shown in **Figure 2.3**. In transmission spectra collected by the dial path accessory, the strong infrared absorptivity of the water saturated the signal between the region of 3750 and 2800 cm^{-1}. Additional effect of water absorption due to the OH stretching vibrations was seen in the region of 1800 – 1500 cm^{-1}. Therefore, these regions were excluded during the development of the PLSR models and only the 1500 – 900 cm^{-1} fingerprint region was used. The application of 1 minute quick vacuum drying on the ATR accessory of the Cary 630 system helped reduce the strong effect of water in the spectra upon drying and allowed the use of the spectral region from 1800 to 1500 cm^{-1} in the free asparagine and glutamine models.

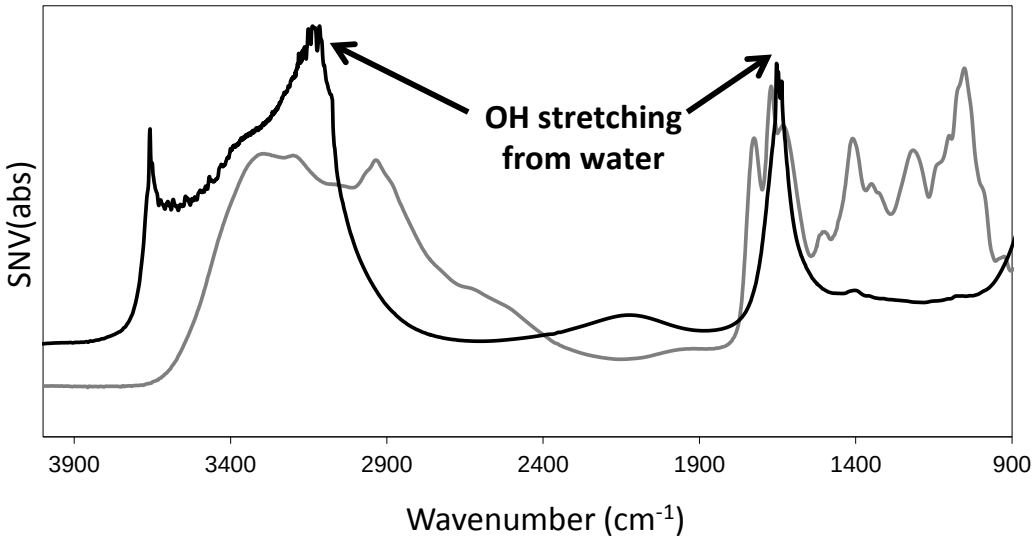

Figure 2.3. Average of all spectra collected using dial path (black line) and ATR (gray line) accessories of the portable Cary 630 IR system after normalization and second derivative transformed (Savitzky-Golay second order polynomial filter with a 25-point window)

The most important bands in the regression vectors of the PLSR models for the main soluble potato components (asparagine and reducing sugars) are shown in **Figure 2.4**. Even though the regression vectors of the PLSR models for other compounds (glucose, fructose, sucrose and glutamine) are not illustrated, they are discussed as well.

The most important bands in the regression vectors for asparagine (**Figure 2.4a**) and glutamine models using the ATR accessory were related to 1677, 1627, 1401 and 1345 cm^{-1}, which were assigned as C=O stretch vibration, NH_2 deformation, C-N stretches and C-H deformation, respectively (Barth, 2000). Vacuum drying the potato supernatant revealed the amide vibration bands of Asn and Gln side chains that were masked by the strong water stretching vibration in the 1800-1600 cm^{-1} for measurements

using the dial-path accessory. Nevertheless, models for asparagine and glutamine using the dial path showed important bands centered at 1429, 1400 and 1357 cm^{-1} (**Figure 2.4a**) corresponding to C-N stretches and C-H deformation, respectively (Barth, 2000).

The regression vectors of the PLSR models developed for sugars were dominated by the region of 1200-1000 cm^{-1} which is associated with C-C ring vibrations, overlapped with the stretching vibrations of C-OH side groups and the C-O-C glycosidic band vibrations of carbohydrates. Loading vectors for glucose were associated with bands at 1015, 1034 and 1060 cm^{-1} associated with C-O-H deformation and C-O stretch vibration, respectively (Cadet, Robert & Offman, 1997; Max & Chapados, 2007). Fructose models were dominated by bands related to C-O-H deformation (1428 and 1358 cm^{-1}) and C-O stretching vibrations (1051 cm^{-1}) (Max & Chapados, 2007). Major bands in the regression models of the reducing sugars (combined glucose and fructose) (**Figure 2.4b**) were in the 1030-1070 cm^{-1} region corresponding to C-O stretch vibrations (Max & Chapados, 2007). Sucrose models showed a predominant band centered at 997 cm^{-1} which is associated with the disaccharide linkage between α-D-glucopyranosyl and β-D-fructofuranosyl groups (Lin et al., 2014). However, there was an additional band at 1062 cm^{-1} in the regression vector of the sucrose model with dial path accessory and it was characteristic of the C-O stretch vibration (Wilkerson, Anthon, Barrett, Sayajon, Santos & Rodriguez-Saona, 2013)

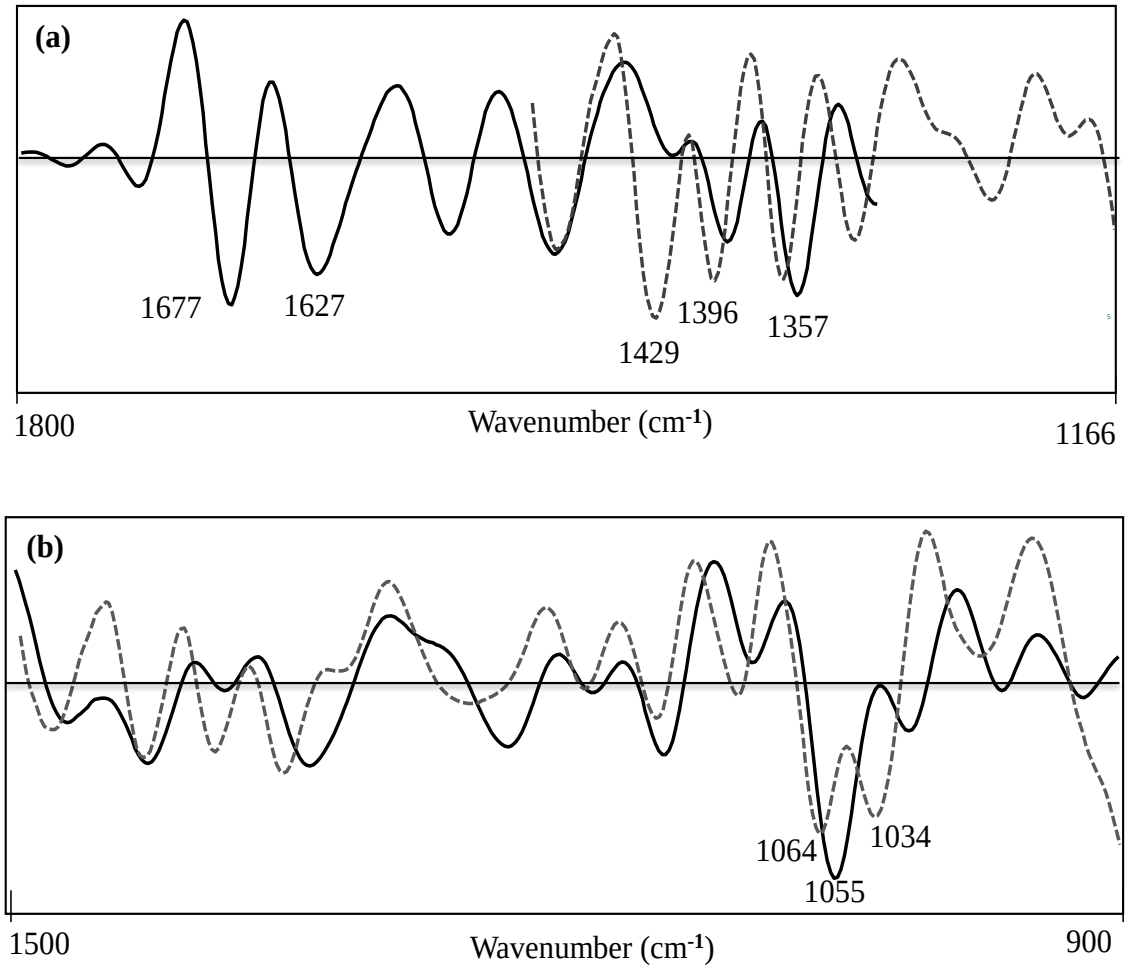

Figure 2.4. Regression vectors obtained for **a)** asparagine using ATR and Dial Path and **b)** reducing sugars (glucose and fructose combined) using ATR and Dial Path (straight and dashed lines represent ATR and Dial Path accessories, respectively)

The sample statistics for calibration and external validation sets used in the development of PLSR models for each parameter measured in this study are presented in **Table 2.2.** For all the PLSR models, the same 16 potato tuber genotypes randomly chosen from both 2012 and 2013 harvests were used as an independent validation sets. Even though there were 68 tuber genotypes in the calibration sets, the numbers in **Table 2.2** are different due to a few outliers taken out while developing the model.

Performance statistics obtained for each of the PLSR models are shown in **Table 2.3**. Similar number of factors ranging between 4 and 6 were used in the models avoiding the potential overfitting problem, which could weaken the ability of the models to predict the concentrations in the unknown samples. In general, the dial path and ATR accessories of the Cary 630 showed similar performances in terms of SECV, SEP, rCV, rPred and RPD values.

Table 2.2. Comparison of some statistical parameters of the sample sets used to develop calibration and prediction models in potatoes using transmission and ATR accessories of Cary 630 Mid- nInfrared systems

Analyte	Accessory	Sample Set	Number of samples	Min.	Max.	Mean	Standard Deviation
Asparagine (mg/g fresh weight)	**ATR**	**Calibration**	66	0.7	2.9	1.7	0.5
		Validation	16	0.8	2.6	1.5	0.5
	Dial path	**Calibration**	64	0.7	2.9	1.6	0.5
		Validation	16	0.8	2.6	1.5	0.6
Glutamine (mg/g fresh weight)	**ATR**	**Calibration**	66	0.3	1.7	0.7	0.2
		Validation	16	0.4	1.4	0.7	0.3
	Dial path	**Calibration**	65	0.3	1.7	0.7	0.2
		Validation	16	0.4	1.4	0.7	0.3
Glucose (mg/g fresh weight)	**ATR**	**Calibration**	66	0.0	6.9	2.0	1.9
		Validation	16	0.0	5.8	2.3	2.1
	Dial path	**Calibration**	65	0.0	6.9	1.9	1.8
		Validation	16	0.0	5.8	2.3	2.1
Fructose (mg/g fresh weight)	**ATR**	**Calibration**	65	0.0	9.4	2.8	3.0
		Validation	16	0.0	8.3	2.9	2.8
	Dial path	**Calibration**	66	0.0	9.4	3.0	3.1
		Validation	16	0.0	7.5	2.8	2.9
Reducing sugars (mg/g fresh weight)	**ATR**	**Calibration**	66	0.0	15.0	4.8	4.7
		Validation	16	0.1	11.9	4.7	4.4
	Dial path	**Calibration**	66	0.0	15.0	4.4	4.6
		Validation	16	0.1	14.1	4.8	4.8
Sucrose (mg/g fresh weight)	**ATR**	**Calibration**	64	0.4	5.4	2.0	1.2
		Validation	16	0.9	4.6	2.2	1.1
	Dial path	**Calibration**	63	0.4	5.4	1.9	1.2
		Validation	16	0.9	4.3	2.2	1.1

Table 2.3. Cross-validation and prediction results of PLSR models developed by using transmission and ATR accessories of Cary 630 Mid-Infrared systems

Analyte	Accessory	Region used[a]	Factors[b]	SECV[c]	SEP[d]	rCV[e]	rPred[f]	RPD[g]
Asparagine	ATR	1299 - 1800	5	0.17	0.15	0.95	0.95	3.4
(mg/g fresh weight)	Dial Path	1166 - 1500	5	0.15	0.16	0.96	0.97	3.4
Glutamine	ATR	1299 - 1800	5	0.12	0.09	0.88	0.95	3.1
(mg/g fresh weight)	Dial Path	1166 - 1500	5	0.11	0.08	0.86	0.96	3.4
Glucose	ATR	900 - 1500	6	0.44	0.46	0.97	0.98	4.6
(mg/g fresh weight)	Dial Path	900 - 1500	6	0.43	0.38	0.97	0.98	5.5
Fructose	ATR	900 - 1500	6	0.81	0.73	0.96	0.96	3.9
(mg/g fresh weight)	Dial Path	900 - 1414	6	0.95	0.70	0.95	0.97	4.1
Reducing Sugar	ATR	900 - 1500	6	1.06	1.15	0.97	0.96	3.9
(mg/g fresh weight)	Dial Path	900 - 1500	6	1.09	1.15	0.97	0.97	4.2
Sucrose	ATR	900 - 1500	4	0.34	0.32	0.96	0.96	3.5
(mg/g fresh weight)	Dial Path	900 - 1500	5	0.31	0.34	0.97	0.95	3.2

[a] The part of the Mid-IR region used for the model (cm^{-1})
[b] Factors: set of orthogonal factors that account for most of the variation in the response
[c] SECV: standard error of cross-validation
[d] SEP: standard error of prediction
[e] *r*CV: correlation coefficient of cross-validation
[f] *r*Pred: correlation coefficient of prediction
[g] RPD (Standard Deviation of Reference Data)/(Standard Error of Prediction)

High correlations between reference values obtained from GC-FID and the infrared predicted levels for the two major free amino acids (asparagine and glutamine) in the calibration and independent validation sets are shown in **Figure 2.5**. The amino acid models with dial path and ATR accessories both used 5 factors. SEP for asparagine was about 0.15 mg/g fresh weight yielding to an RPD value of 3.4 for both of the accessories in the asparagine models. SEP values for glutamine were about 0.08 mg/g fresh weight for both accessories and the model with dial path yielded a slightly higher RPD value than the model with the ATR (3.4 versus 3.1).

The optimum number of factors used for the glucose, fructose and reducing sugar (sum of glucose and fructose) models was 6. Glucose models gave SEP of 0.46 and 0.38 mg/g fresh weight for ATR and dial path accessories respectively, and RPred of 0.98 in both cases. RPD value for glucose using dial path was slightly higher than the ATR (5.5 versus 4.6). For fructose, SEP was about 0.7 mg/g fresh weight and RPD values were 3.9 for ATR and 4.1 for dial path. PLS regression plots for reducing sugars and sucrose content between reference values and infrared predicted levels are shown in **Figure 2.6**. The SEP obtained for reducing sugars was 1.15 mg/g fresh weight for both applications and RPD values were again slightly higher for the dial path with values quite similar to those from fructose models (4.2 versus 3.9). Lastly, sucrose models using 4 and 5 factors with ATR and dial path, respectively resulted in SEP of about 0.32 mg/g fresh weight and RPD of 3.5 for ATR and 3.2 for dial path applications. Overall, good correlations (rPred 0.95 – 0.98) were obtained for all the models. Based on the scale reported by Saeys et al. (2005), all models gave RPD values above 3 (between 3.1 and 5.5) indicating that they

are excellent models and can be used for predicting concentration levels in unknown samples. Even though the dial path accessory was generally yielding higher RPD values in comparison to those of ATR models, both accessories gave excellent performances for these applications since the SEP and RPD values obtained for the independent validation sets were quite similar between the models.

Similar PLSR model performances were obtained by analyzing the supernatant after centrifuging potato powders at 3,000 and 12,000 g indicating that simple portable fixed speed centrifuge units can be used increasing the practical applicability of the proposed techniques. To our knowledge, this is the first study done with raw potatoes to predict the level of sugars and amino acids by using an MIR spectrophotometer and particularly a portable MIR device. Previous related research in the literature used Near-Infrared Spectroscopy for online measurement of reducing sugars in potatoes, which reported very low accuracy of predictions compared to the acceptable standards (Mehrubeoglu and Cote, 1997). For instance, Haase (2011) used NIR spectroscopy to predict the quality of ground raw potatoes. The author developed calibration models for reducing sugar and sucrose and obtained (r^2) of 0.43 for reducing sugar and 0.71 for sucrose. The RPD values for the models were unacceptably low (1.7) and low (2.4) for reducing sugar and sucrose, respectively.

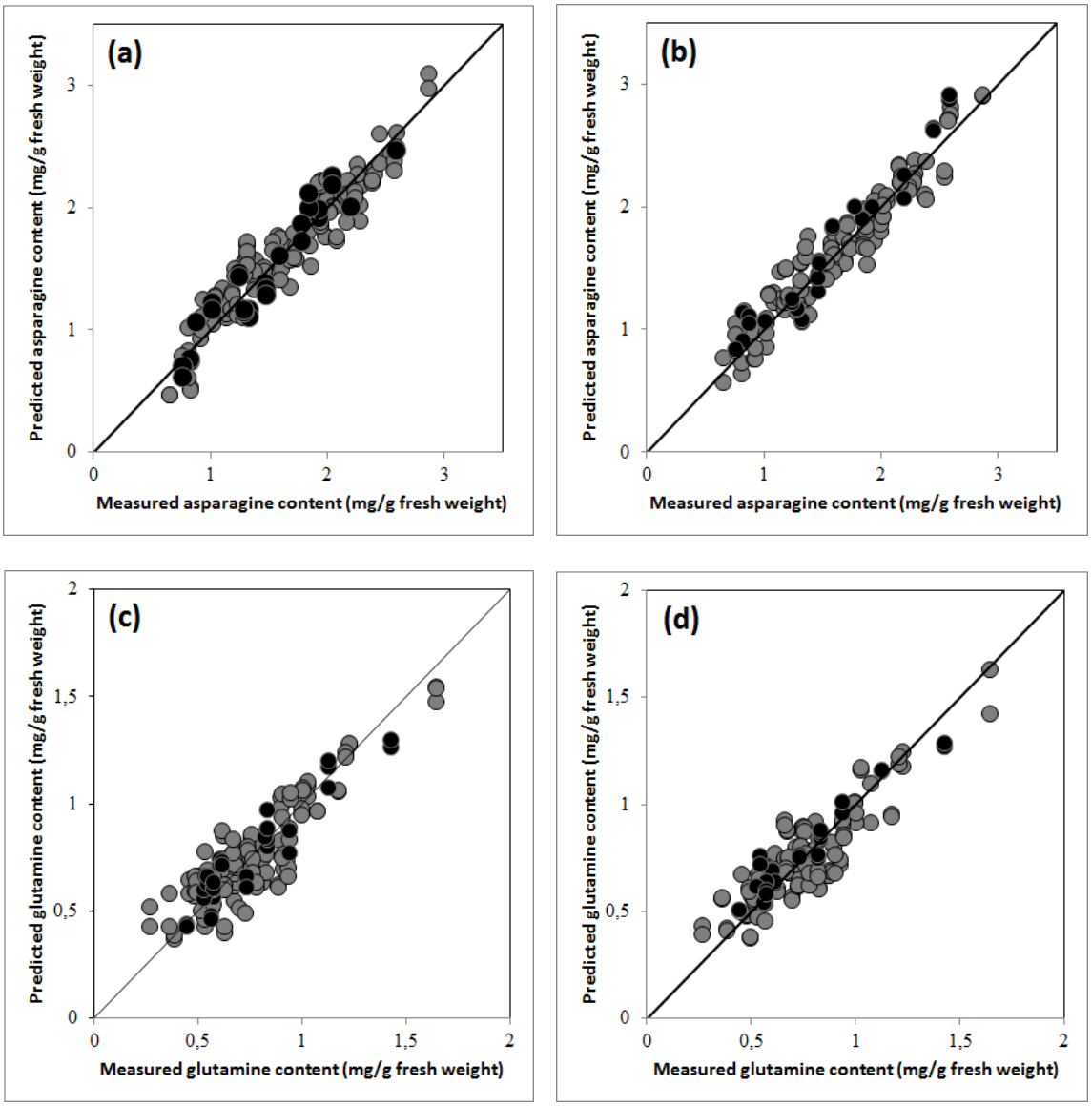

Figure 2.5. Partial least squares regression (PLSR) plots for asparagine content in raw potatoes using **(a)** ATR accessory **(b)** Dial path accessory and glutamine content **(c)** ATR accessory **(d)** Dial path accessory (● and ● represent samples in calibration and validation groups, respectively)

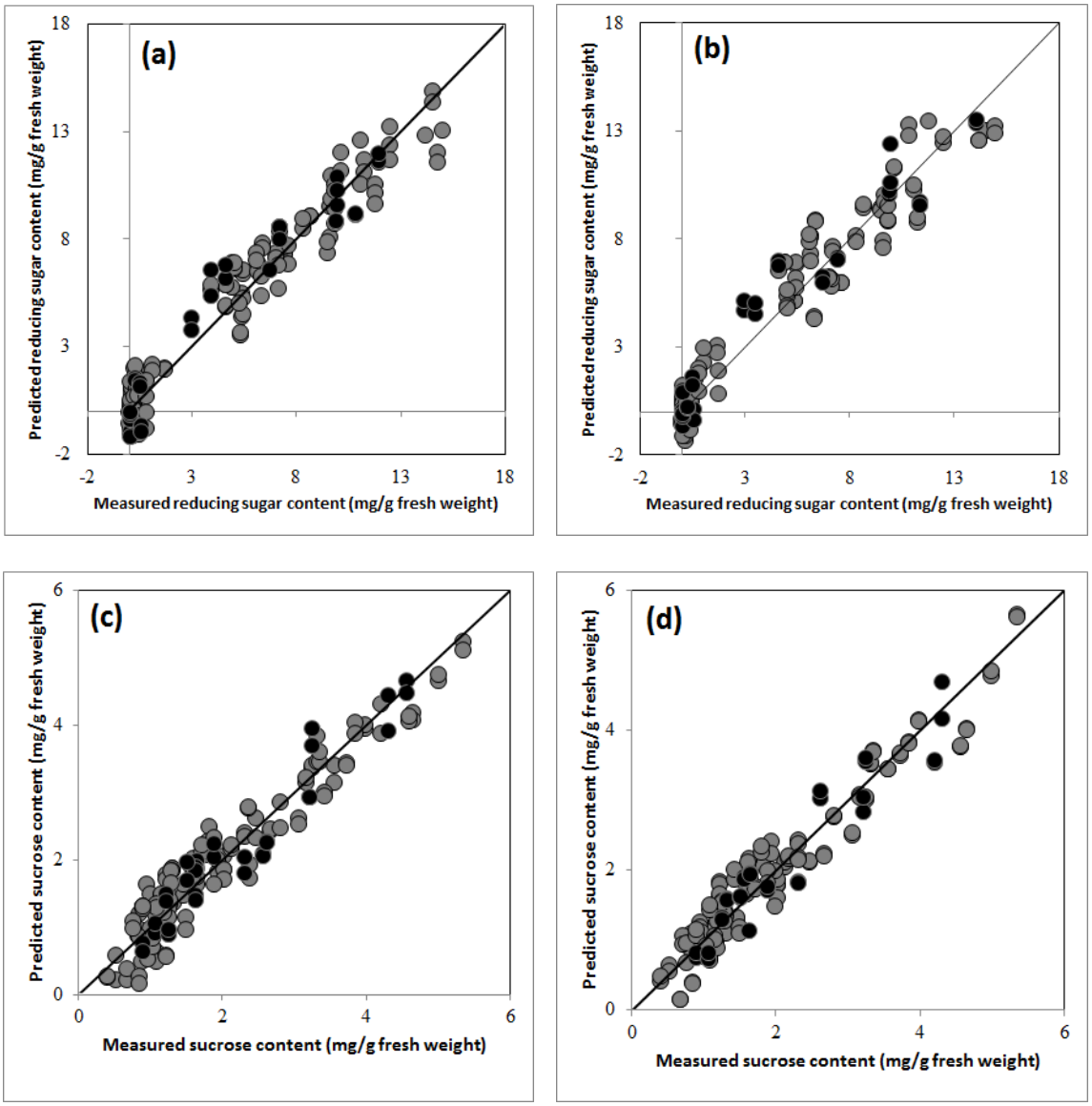

Figure 2.6. Partial least squares regression (PLSR) plots for reducing sugar and sucrose content in raw potatoes using **(a)** ATR accessory **(b)** Dial path accessory and glutamine content **(c)** ATR accessory **(d)** Dial path accessory (and represent samples in calibration and validation groups, respectively)

2.5. CONCLUSION

Based on the data obtained from the PLSR models developed, it can be said that the spectra obtained by using both ATR and Dial Path accessories of the modern Cary 630 portable FT-IR system paired with multivariate analysis, yielded very strong models, providing low SEP and high RPD values. Using the same spectra, separate free asparagine, free glutamine, glucose, fructose, reducing sugars and sucrose PLSR models were successfully developed. Using the portable systems is simple, cost-effective and requires low sample volume; once the instrument is purchased, there are minimal operational costs involved to perform the tests. Additionally, portable systems provide increased flexibility and great potential for in-field applications compared to bench-top IR systems or chromatographic systems such as HPLC and GC, which can only be used in a laboratory setting. Therefore, PIRT can significantly benefit potato breeding and certain aspects of crop management, crop production and research.

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CHAPTER 3

APPLICATION OF INFRARED MICROSPECTROSCOPY AND CHEMOMETRIC ANALYSIS FOR SCREENING ACRYLAMIDE CONTENT IN POTATO CHIPS

Ayvaz, H., Plans, M., Riedl, K. M., Schwartz, S. J., & Rodriguez-Saona, L. E. (2013). *Analytical Methods*, 5, 2020-2027

3.1. ABSTRACT

Currently, LC-MS/MS and GC-MS are the most common techniques used for acrylamide analysis in foods. However, these techniques require extensive sample preparation, highly specialized machinery, and are time consuming. Our objective was to evaluate the feasibility of using attenuated total reflectance (ATR) mid-infrared microspectroscopy (IRMS) as a rapid method for detection and quantification of acrylamide in potato chips. The acrylamide content of 58 commercially available potato chips (110-1876 µg/kg) was determined by LC-MS/MS. Spectra for aqueous extracts of potato chips with and without solid phase extraction (SPE) clean-up were collected using ATR-IRMS. Partial Least Squares Regression (PLSR) was applied to generate calibration models to predict acrylamide levels. Samples were grouped into three categories; regular potato chips (n=46), potato chips with seasonings (n=12) and combination of regular and

seasoned potato chips (n=58). Overall, good linear correlation was found between the predicted acrylamide levels of independent sample sets and actual measured acrylamide concentrations by LC-MS/MS (rPred was between 0.91 and 0.92). Standard error of prediction (SEP) for the models ranged between 22.1 and 28.9 µg/L. Models developed without SPE cleaning provided similar or better results than those with SPE treatment, which makes IRMS technology even more promising for acrylamide analysis since the SPE step can be avoided. Our results indicate that IRMS can be used as simple, rapid and high-throughput screening tool for acrylamide detection and quantification in potato chips and help addressing risk management, assessing safety and brand equity.

3.2. INTRODUCTION

Acrylamide (2-propenamide, CAS Registry No. 79-06-1) is a chemical compound which has been used in industries such as paper, textile and construction since the 1950s for the production of polyacrylamides (Claeys, Vleeschouwer & Hendrickx, 2005). Although polymerized acrylamide is known to be non-toxic, the acrylamide monomer was reported as a human neurotoxin, a rodent carcinogen and it is classified as "probable carcinogen to humans" (Becalski, Lau, Lewis & Seaman, 2003). The neurotoxicity of acrylamide in humans is well-known (Claeys et al., 2005). This compound has a low molecular weight and is very soluble in water, which help it pass through various biological membranes (Besaratinia, 2007). Acrylamide was recently added to California's Proposition 65 list and is of concern to the snack and fried food industries (California OEHHA, 2011).

In 2002, significant amounts of acrylamide were detected in some foods processed at high temperatures (Tareke, Rydberg, Karlsson, Eriksson & Törnqvist, 2002). Researchers at the Swedish National Food Administration and Stockholm University reported to the press that they had found acrylamide in fried and oven-baked foods. They reported moderate levels (5-50 μg/kg) in heated protein-rich foods and higher levels (150-4000 μg/kg) in carbohydrate-rich foods. Different cooking practices dramatically affect the acrylamide content of foods (Ahn, Castle, Clarke, Lloyd Philo & Speck, 2002; Medeiros Vinci, Mestdagh & De Meulenaer, 2012). Ahn et al. (2002) reported the presence of very high levels of acrylamide (more than 10 mg/kg) in overcooked fried chips demonstrating the influence of cooking temperature and duration on acrylamide formation. The highest acrylamide levels have been found in French fries, potato chips, and deep-fried or oven-baked potato products, as well as some crisp bread, biscuits, crackers and breakfast cereals (Tareke et al., 2002). Acrylamide is generally formed at temperatures above 100 °C during processes such as frying, roasting and baking (Becalski et al., 2003; Mucci & Wilson, 2008), predominantly through the Maillard reaction pathway (Andrzejewski, Roach, Gay & Musser, 2004; Friedman, 2003). This reaction, between sugars and amino acids, is considered to be the main mechanism for acrylamide formation, with asparagine and reducing sugars as the major reactants present in foods (Hedegaard, Frandsen, Granby, Apostolopoulou & Skibsted, 2007; Zhu, Cai, Ke & Corke, 2010). The frying of potato chips creates ideal conditions for acrylamide formation, since potatoes are high in both asparagine and reducing sugars such as glucose and fructose (Zhang, Ren & Zhang, 2009). According to the WHO, daily intake of dietary

acrylamide is between 0.3 – 2.0 µg/kg/body wt and in the case of children; it was estimated as 2-3 times more than that of adults due to average body weight ratios (Becalski et al., 2003).

Current assays for acrylamide rely on GC-MS or LC-MS/MS to identify and quantitate acrylamide in foods (Wenzl et al., 2006; Clarke, Kelly & Wilson, 2002). These methods have shown excellent analytical performances for the detection of acrylamide in foods with limits of quantitation of 10 (µg/kg), response linearity over a range of 8-3600 µg/kg and recoveries > 95% (Roach, Andrzejewski, Gay, Nortrup & Musser, 2003). However, their use requires extensive sample preparation (extraction and SPE cleanup steps before analysis), is expensive and time consuming, which limits their application for monitoring acrylamide content during processing or finished products (Andrzejewski et al., 2004). Generally, this method requires food producers to send samples out rather than performing in-house acrylamide testing. Considering all these factors, there is a need for a rapid method that could be used for routine analysis and real-time monitoring during processing to identify samples exceeding a preset value (Segtnan, Kita, Mielnik, Jorgensen & Knutsen, 2006).

Fourier-transform infrared spectroscopy (FT-IR) provides a rapid alternative to traditional analytical techniques and is extensively used in the food industry (Reid, O`Donnell & Downey, 2006). Advances in FT-IR spectroscopic instrumentation combined with multivariate data analysis have made this technology ideal for rapid screening and identification of various analytes. Modern computing technology has allowed FT-IR to become more practical by allowing for complex chemometric analysis

of spectra (Rodriguez-Saona & Allendorf, 2011). Positive results from past studies indicate that NIR are promising tools for acrylamide analysis (Segtnan et al., 2006; Pedreschi et al., 2010b). Infrared spectroscopy provides valuable information about the biochemical composition of the samples, especially in the fingerprint region, which has been used for numerous food applications related to quality control and safety of our food supply (Segtnan et al., 2006; Reid et al., 2006; Rodriguez-Saona et al., 2011; Pedreschi et al., 2010; Shiroma & Rodriguez-Saona, 2009). This analytical technique is simple, fast and highly specific, and requires minimal sample preparation. ATR-IRMS has been effectively used to detect subtle differences among samples (Männig, Baldauf, Rodriguez-Romo, Yousef & Rodriguez-Saona, 2008; Grasso, Yousef, De Lamo Castellvi & Rodriguez-Saona, 2009; Prabhakar, Kocaoglu-Vurma, Harper & Rodriguez-Saona, 2011; De Lamo-Castellvi and Rodriguez-Saona, 2011), which indicated that IRMS technique could deliver a rapid and sensitive alternative to currently used traditional methods.

The objective of this research was to evaluate the feasibility of IRMS for rapid, sensitive and high-throughput screening of acrylamide content in a variety of commercial potato chips.

3.3. MATERIALS AND METHODS

3.3.1. Chemicals and Consumables

Acrylamide (99+ %) was purchased from Acros Organics (NJ, USA). $^{13}C_3$-Labeled acrylamide (98%) (Internal standard) was obtained from CIL (Andover, MA).

LC/MS grade acetonitrile, methanol, water, formic acid, glacial acetic acid (99%), certified disposable pipet tips and 50 mL polypropylene conical tubes with caps were purchased from Fisher Scientific (Pittsburg, PA). PVDF maxi spin filtration tubes (0.45 µm and 50 mL) were purchased from Grace Davison Discovery Sciences (Deerfield, IL). Oasis HLB (6 mL, 200 mg) SPE cartridges were obtained from Waters Corp (Milford, MA) and Bond Elut-Accucat (200 mg, 3 mL) SPE cartridges were obtained from Varian Inc. (Chicago, IL). Amber glass autosampler vials with septum screw caps were obtained from Waters Corp (Milford, MA). Reversed phase Hypercarb LC column (50 mm x 2.1 mm) and guard column were acquired from Thermo Scientific (Waltman, MA). Microarray slides were purchased from Arrayit Corporation (Sunnyvale, CA).

3.3.2. Preparation of Standards

Acrylamide is stable in acid and is sensitive to light. Therefore, accurately weighted native acrylamide and $^{13}C_3$-labelled acrylamide (internal standard) stock solutions were prepared by dissolving them in 0.1% formic acid using separate amber glass volumetric flasks. Aliquots of the native and labeled acrylamide were used for preparation of working solutions. By diluting the samples in 0.1% formic acid solution, five different concentrations of native acrylamide (ranging from 0 to 720 µg/L) and fixed concentration of labeled acrylamide (2000 µg/kg) were prepared and used to obtain standard curve for LC-MS/MS and to verify instrument daily performance. Internal standard was used to account for any possible loss during sample preparation or any changes in LC-MS/MS sensitivity and performance. Roach et al. (2003) reported that

acrylamide stock solutions were stable up to 6 months in amber glasses even at room temperatures.

3.3.3. Analysis for Acrylamide in Potato Chips by LC-MS/MS

Different commercial potato chip (n=58) samples were purchased at local grocery stores (Columbus, OH, USA) and evaluated for their acrylamide levels using a method modified from Roach et al. (2003). Three independent extractions were conducted from each potato chip sample (bag). About 100 g per sample was blended using a Waring blender. For each extraction, sub-sample (1 g) was mixed with 9 mL water. Along with potato chips and water, 1 ml of 2000 µg/L $^{13}C_3$-Labeled acrylamide was added as internal standard. The samples were mixed for 20 min, centrifuged at 9681 x g for 15 min (Sorvall RC 5C Plus with SS-34 rotor), then 5 mL of the clarified aqueous layer (avoiding oil layer on top and solid layer on the bottom of the tube) was removed and transferred into a spin filtration tube and further centrifuged at 9681 x g for 4 min. The extract passed through spin filtration tube was centrifuged again for 10 min at 13400 x g before the SPE cleaning. SPE cleanup was performed using Oasis HLB SPE and Accucat SPE cartridges.

LC-MS/MS was used to measure levels of acrylamide in aqueous extracts. Analytes were separated using Waters Acquity Ultra Performance LC system (Milford MA, US) on reversed phase Hypercarb column (50 mm x 2.1 mm, Thermo Scientific, Waltham, MA) by running isocratically with acidified water (0.1% formic acid) as the mobile phase. Mass spectrometry was conducted by using positive-ion electrospray

ionization on a triple quadrupole mass spectrometer. The transitions m/z 72>55 (for native acrylamide) and 75>58 (for internal standard) were monitored and response curve plotted daily for the ratio of m/z 55/58 versus injected acrylamide concentration including constant amount of labeled acrylamide. Additionally, LOD and LOQ for LC-MS/MS were calculated. The LOD concentration (μg/L) was calculated as 3.3 times ratio of standard deviation of the response (SD) and the slope of the calibration curve while LOQ concentration (in μg/L) was 10 times the ratio of standard deviation of the response (SD) and the slope of the calibration curve.

3.3.4. Infrared spectroscopy measurements

Since three independent extractions were done for each potato chip bag to determine acrylamide levels, three aqueous extracts were also obtained. These aqueous extracts (200 μL) from the 58 commercial potato chips (58 x 3 = 174 in total), with and without SPE clean-up, were evaporated to dryness in a SpeedVac Concentrator (Thermo Fisher Scientific, Waltham, MA) and re-suspended in 5 μL water. Then, 1 μL of the sample was placed on a 192-well microarray slide (Arrayit Corporation, Sunnyvale, CA), air-dried and spectra were collected. For each independent aqueous extract conducted, two IRMS spectra were collected. IRMS were performed on a FT-IR microscope (UMA 600 series IR microscope interfaced with a FTS Excalibur 3100GX FT-IR spectrometer; Agilent, Walnut Creek, CA) equipped with a motorized x–y stage, a broadband mercury cadmium telluride detector, and slide-on ATR germanium objective (Varian 600 UMA, Palo Alto, CA). The spectra were collected from 4,000 to 700 cm^{-1} with a resolution of 4

cm^{-1} co-adding 128 scans to improve the signal-to-noise ratio. The spectrometer was controlled using Win-IR Pro control software (Version 3.4.2, Varian Inc., Palo Alto, CA). Spectra were displayed in terms of absorbance obtained by rationing the single beam spectrum against that of the air background.

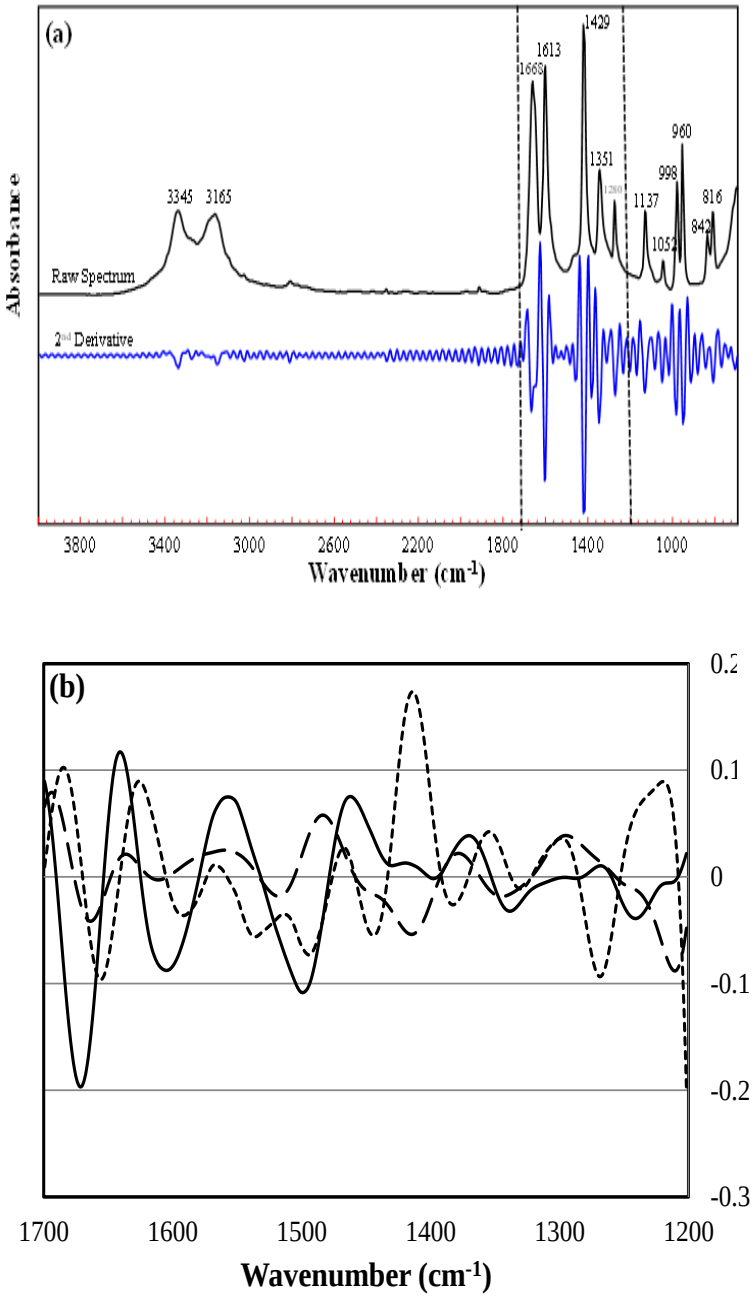
3.3.5. Multivariate data analysis

Collected IRMS spectra were imported into Pirouette software (Infometrix, Bothell, WA, USA). Spectra were mean-centered and processed using normalization and the second derivative transformation (Savitzky-Golay second order polynomial filter with a 25-point window) prior to partial least squares regression (PLSR) analysis. PLSR combines the features of Principal Component Analysis (PCA) and Multi Linear Regression (MLR) to compress a large number of variables into a few latent variables (PLS-Factors). It is particularly useful when the size of independent variables (spectra) is much larger than that of dependent variables (acrylamide content). A very important advantage of PLSR in the case of acrylamide measurement is that it takes into account errors both in the concentration measured by LC-MS/MS and the spectra collected by IRMS. It does not assume that the concentrations of acrylamide used as reference numbers (obtained from LC-MS/MS) are error free since there could be errors in sample preparations, dilutions, weighing and such (Brereton, 2000). Thus, PLSR is becoming a standard tool for modeling correlated relationships between multivariate measurements (Jong, 1993). Using the spectra obtained from IRMS and reference acrylamide concentrations from LC-MS/MS (dependent variable), quantitative models were

generated with PLSR. Calibration models were internally validated using full cross-validation (leave-one-out approach) and externally validated with an independent set. Cross-validation was used for every calibration model while independent validation was conducted for all models except the seasoned potato chip's due to limited number of samples (n=12). Independent validation studies were conducted for regular and combination of regular and seasoned potato chip groups using approximately 80% of the whole set to generate calibration models and about 20% to serve as independent validation set. Splitting the samples between calibration and independent validation sets was done so that all replicates belonging to the same potato chip bag were either used only in a calibration or a validation set. The performance of the models was evaluated in terms of loading vectors, standard error of cross validation (SECV), standard error of prediction (SEP), correlation coefficient (r) and outlier diagnostics (Standard Residual of Sample vs. Leverage). The SEP is an estimate of the standard error of prediction (magnitude of error expected when independent samples are predicted using the model). X residuals and leverage were used for the evaluation of outliers. Observations with large residuals or an unusual residual pattern normally indicate an outlier. The leverage of a calibration sample was used to determine its potential contribution to the estimated calibration model. Any observation with abnormal standard residual (>2) with high leverage was reanalyzed and eliminated if necessary, after which the calibration model was repeated. However, no outliers were removed from the independent validation sets.

3.4. RESULTS AND DISCUSSION

Figure 3.1a shows the raw and 2nd derivative of pure acrylamide spectra (99+ %) with related range (1201 to 1699 cm^{-1}) used for PLSR models highlighted. Additionally, assignment of important mid-infrared bands to functional groups in this region was done as reported by Jonathan (1961). Important infrared bands obtained with pure acrylamide (99+ %) were associated with the strong infrared vibrations of C=C, NH_2, CH_2, CH and C-N functional groups, consistent with the structure of acrylamide (C_3H_5NO, prop-2-enamide). A representative loading plot of SPE cleaned-up seasoned potato chip samples (**Figure 3.1b**) provided information on bands of the spectrum associated with the highest variation in the calibration set that correlated with combinations of different chemical phenomena. Loading plots of the first three factors, accounting for 84% of the X-variance, showed that the highest relevant variation in the calibration set for acrylamide content was in the 1201 to 1699 cm^{-1} region mainly associated to ν_s(C=C) and $\delta(NH_2)$ vibrations (Jonathan, 1961). The loading plots of other models were not presented since they had very similar behavior.



Band Position (cm^{-1})	Assignments
3345	v_a (NH_2)
3165	v_s(NH_2)
1668	v (C=C)
1613	δ (NH_2)
1429	δ (CH_2)
1351	δ (CH)
1280	v (C-N)
1137	γ (NH_2)
1052	γ (CH_2)
998	v (C-C)
960	w (CH_2=C)
842	w (NH_2)
816	w (HRC=C)

* v: stretching, v_a *and* v_s: asymmetric and symmetric stretching, δ: in plane bending, γ: rocking, w: wagging

*****Band assignments were done according to Jonathan, 1961

Figure 3.1. (a) Raw and 2nd derivative of pure acrylamide spectra (99+ %) **(b)** Loadings of the first three factor (second derivative) for calibration model of SPE treated potato chips with seasonings from 1201 to 1699 cm^{-1} region **(▬ ▬ :first factor, ▬ ▬ ▬ : second factor, ▬▬▬ :third factor) (rCal for the first three latent variables are 0.64, 0.72 and 0.84, respectively)**

Using the ratio of transitions m/z 72>55 (for native acrylamide) and 75>58 (for internal standard) versus injected acrylamide concentration including constant amount of labeled acrylamide, the standard curve was obtained with LC-MS/MS (ratio of mz 55/58 = 0.00264 x [acrylamide concentration] + 0.0215) and used to calculate acrylamide levels in potato chips. Our results showed that acrylamide content of selected potato chips (regular and with seasonings) ranged between 110 and 1876 µg/kg, which were within levels reported in the literature (Becalski et al., 2003; Zhu et a., 2010; Roach et al., 2003). LOD and LOQ concentrations for LC-MS/MS were calculated using the standard curve and reported as 18 and 55 µg/L, respectively.

The aqueous solutions used in LC-MS/MS served as reference acrylamide concentration for the IRMS study. In addition to the native acrylamide from potato chips in the aqueous extract, the labeled acrylamide (internal standard) added at 200 (µg/L) (after 10 times dilution of 2000 µg/L) was accounted for IRMS. Although the labeled acrylamide could be easily detected and quantified using the difference in mass by LC-MS/MS, it was not possible for IRMS to distinguish the native and labeled acrylamide due to their identical structures. Therefore, concentration of total acrylamide in aqueous extracts (sum of native acrylamide and amount of internal standard) used as reference values in developing PLSR models ranged from 206 to 510 µg/L (**Table 3.1**).

Table 3.1. Comparison of some statistical parameters of the sample sets used to develop calibration and prediction models [-1]

SPE	Type of Chips		Number of brands (n)	Minimum	Maximum	Mean	Standard Deviation
No	Regular [a]	Calibration	30	206	510	367	66
		Prediction	7	276	457	352	50
	Seasoned [b]	Calibration	11	262	381	340	36
	All samples [c]	Calibration	36	219	510	359	52
		Prediction	10	232	457	346	59
Yes	Regular [a]	Calibration	32	206	489	378	68
		Prediction	9	276	457	362	53
	Seasoned [b]	Calibration	11	262	381	336	38
	All samples [c]	Calibration	41	206	489	366	59
		Prediction	10	276	457	373	49

[a]: Potato chips were referred to the ones contained only potatoes, vegetable oils and salt as ingredients
[b]: Seasoned potato chips were referred to the ones contained different ingredients than that of regular potato chips
[c]: All samples were referred to the combination of regular potato chips and seasoned potato chips
[n]: Number of potato chip brands used to form calibration and validation models excluding outliers
***:** Results are for the samples which were prepared with 1 g subsamples of potato chips in 10 mL water and run by LC-MS/MS(µg/L)

Aqueous extracts both with and without further SPE clean-up were used to generate PLSR models. The average of all regular and seasoned potato chip sample spectra with and without SPE cleaning is shown in **Figure 3.2** along with the spectra of pure acrylamide to illustrate the difference in the spectral pattern. Average spectra without SPE clean-up showed three main bands at 1672, 1573 and 1394 cm^{-1}, attributed to C=C, NH_2 and CH_2. However, average spectra of SPE treated samples showed bands with reduced signal intensity absorbing at 1662 (C=C) and 1406 cm^{-1} (CH_2). The 58 potato chip brands were divided into three different categories: regular potato chips (containing only potatoes, oils and salt), seasoned potato chips (containing potatoes, oils, salt and other seasonings) and combination of these two groups. Some of the ingredients listed in the labels of seasoned potato chips included maltodextrin, yeast extract, cheddar cheese, sour cream, whey powder, beer, honey, cane juice, onion and garlic powder, vinegar, natural flavors among others, which could potentially interfere with the acrylamide signal. The SPE protocol involved the use of two types of cartridges (Oasis HLB and Accucat SPE cartridges) and intensive centrifugation (20 min at 9681 x g and 10 min at 13400 x g) steps during sample preparation targeted at removing potential interfering compounds for LC-MS/MS runs. The lower spectral intensity observed for the SPE treated samples in **Figure 3.2c** could be attributed to the removal of infrared absorbing compounds during sample preparation resulting in lower intensities for SPE treated samples.

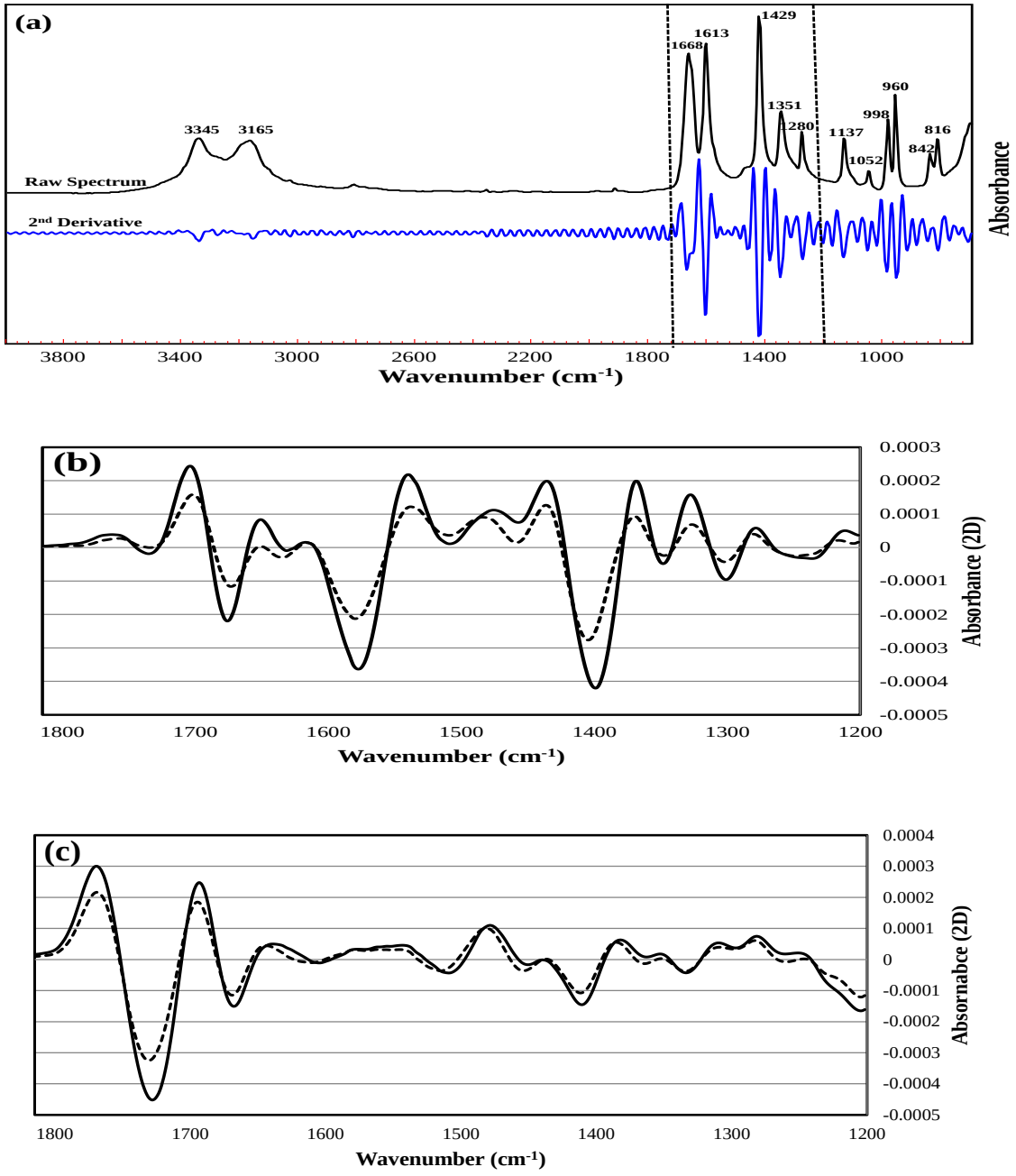


Figure 3.2. Effect of food matrixes and SPE treatment on spectral pattern (1699-1201 cm^{-1} region) collected with IRMS (Second derivative, $\partial A/\partial \lambda^2$) (Savitzky-Golay 25-point window) (a) Raw and 2nd derivative of pure acrylamide spectra (99+ %) (b) Spectra without SPE treatment (c) Spectra with SPE treatment (——— : Regular chips, – – – : Seasoned chips)

The PLSR regression graph (**Figure 3.3**) shows the correlation between the infrared estimated levels and reference acrylamide values for calibration and validation models. Models developed for regular and seasoned potato chips regardless of SPE treatment showed better prediction abilities compared to the models for "all sample categories (regular and seasoned chips combined)", which presented worse predictions. **Table 3.2** shows the performance statistics of the PLSR models developed for each sample group with and without SPE cleaning. Overall, models for both SPE and non-SPE cleaned-up samples gave high correlation coefficients (r values) and low standard errors (better goodness of fit) indicating that the centrifugation (20 min at 9681 x g and 10 min at 13400 x g) step during sample preparation removed interfering components from ingredients in seasoned potato chips regardless of SPE cleaning.

(A) Solid Phase Extraction clean-up (B) No Solid Phase Extraction clean-up

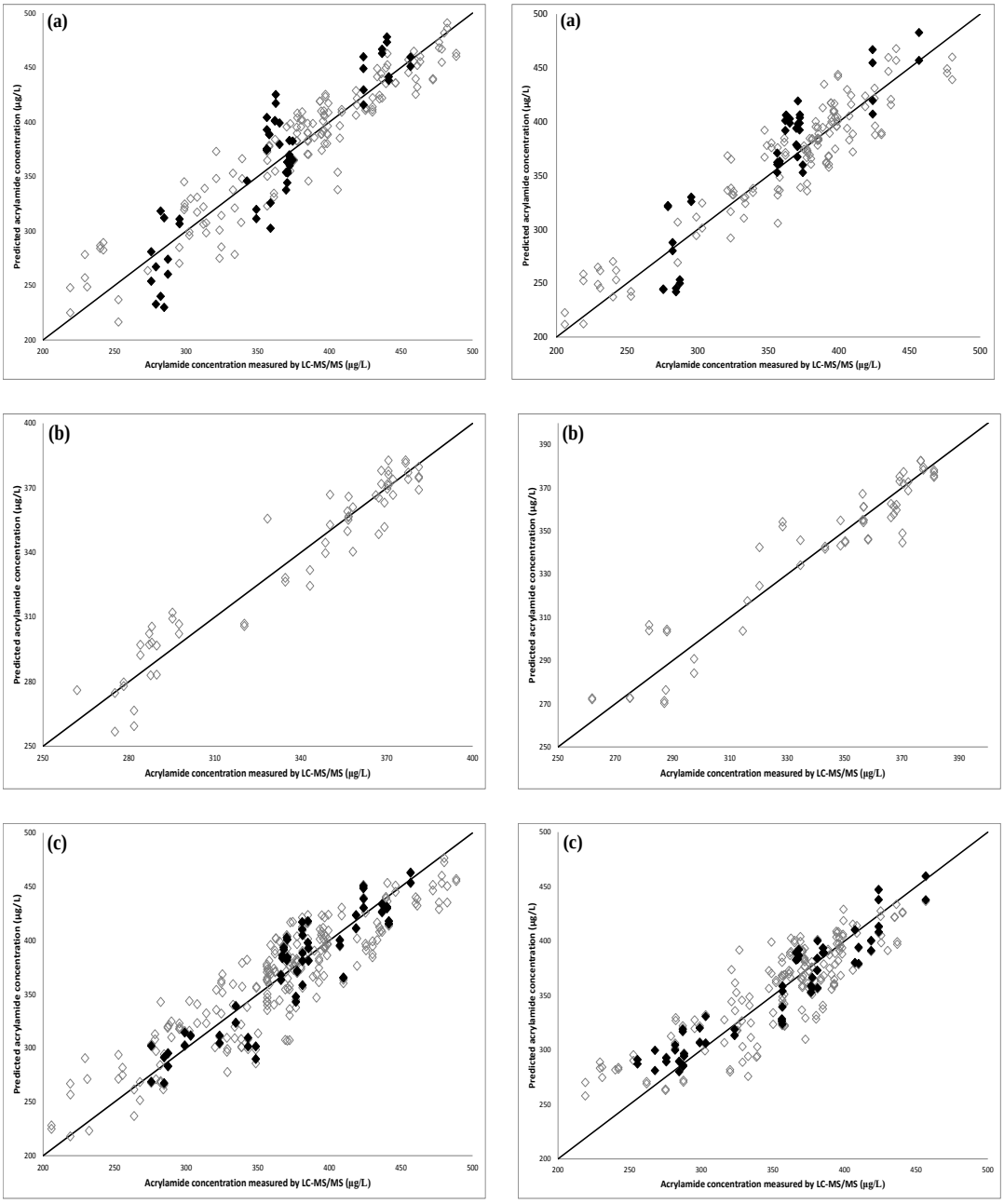


Figure 3.3. Partial least squares regression (PLSR) plots for acrylamide content in SPE cleaned-up (A) and no SPE cleaned-up (B) potato chip aqueous extracts for (a) Regular potato chips (b) Seasoned potato chips and (c) all samples (regular and seasoned samples combined) (acrylamide levels are expresed as µg/L) (◇, ◆ present samples in calibration and validation groups, respectivelys)

Table 3.2. Calibration, cross-validation and prediction results of PLSR models developed by using mid-infrared microspectroscopy from 1201 to 1699 cm^{-1} region

SPE	Type of Potato Chips		Factors	SE (µg/L)	r
No	Regular	Calibration	6	20.2	0.95
		Cross Validation	6	21.8	0.94
		Prediction	6	28.5	0.92
	Seasoned	Calibration	6	9.3	0.97
		Cross Validation	6	11.1	0.95
	All samples	Calibration	6	24.2	0.89
		Cross Validation	6	25.1	0.88
		Prediction	6	24.9	0.91
Yes	Regular	Calibration	7	20.6	0.96
		Cross Validation	7	22.1	0.95
		Prediction	7	28.9	0.91
	Seasoned	Calibration	8	8.6	0.98
		Cross Validation	8	10.6	0.96
	All samples	Calibration	9	23.5	0.92
		Cross Validation	9	25.1	0.90
		Prediction	9	22.1	0.91

[a]**:** Potato chips were referred to the ones contained only potatoes, vegetable oils and salt as ingredients

[b]**:** Seasoned potato chips were referred to the ones contained different ingredients than that of regular potato chips

[c]: All samples were referred to the combination of regular potato chips and seasoned potato chips

***:** Results are for the samples which were prepared with 1 g subsamples of potato chips in 10 mL water and run by LC-MS/MS (µg/L)

Models developed using regular potato chips had acrylamide range of approximately 200 to 500 µg/L in the aqueous extracts. Performance statistics for regular potato chip models gave r-value >0.91, SECV of ~ 22 µg/L and SEP of ~ 28 µg/L regardless of the use of SPE treatment. In addition, similar SECV and SEP in the models indicate that the models are reliable and robust in predicting acrylamide levels in potato chips. Seasoned potato chip samples had a smaller range of acrylamide (262-381 µg/L) compared to regular potato chips (**Table 3.1**), therefore, the standard error values were also lower for this group. SECV for seasoned potato chips with and without SPE cleaning were 10.6 and 11.1 µg/L, respectively. An independent sample set was not used for seasoned potato chip group due to the limited number of samples compared to that of regular potato chips. On the other hand, methods developed for combined groups (regular and seasoned potato chips) gave similar results to regular potato chip models. The combined group had an acrylamide range of approximately 200 to 500 µg/L in the aqueous extracts and PLSR models gave r-value ~ 0.9, SECV of 25 µg/L and SEP of ~24.9 µg/L, regardless of SPE cleaning. **Table 3.2** shows that a lower number of factors was required for non-SPE models (e.g. 6 factors used for non-SPE complete model versus 9 factors for SPE cleaned complete model). The selection of the optimal number of factors was done by a cross-validation approach yielding the minimum error of prediction. Increased number of factors could make the model less robust by augmenting the interference of noise signals (Hawkins, 2004). The spectrum collected without SPE yielded higher signal intensity of bands compared to that of SPE treated samples, reducing inclusion of spectral noise (**Figure 3.2**).

Our data supports the application of mid-infrared microspectroscopy for acrylamide analysis in potato chips, reporting prediction errors (SEP) ranging from 22.1 to 28.9 µg/L. Our results gave lower SEP as compared to research studies conducted using NIR spectroscopy, predicting acrylamide levels with SECV of ~270 µg/L (Segtnan et al., 2006; Pedreschi et al., 2010b). Previous research on screening acrylamide in potato chips using NIR had focused on evaluating a single cultivar of potato tubers (Saturna) and palm oil (as frying medium) with no other ingredients and manipulating frying conditions (time and temperature) to generate varying levels of acrylamide ranging from 40 to 2987 µg/kg (Segtnan et al., 2006; Pedreschi et al., 2010b). As opposed to previous research, commercially available potato chip brands with large spectrum of potato cultivars, oil types, seasonings and processing conditions were used in this research. Additionally, the similar results obtained for the non-SPE models compared to SPE models would allow for exclusion of the SPE cleaning step, increasing efficiency and reducing the length of the analysis.

3.5. CONCLUSION

This study explored the feasibility of using ATR-IRMS for acrylamide detection and quantification in potato chips. A simple, sensitive, high-throughput and robust method for screening acrylamide content in potato chips was developed by combining attenuated total reflectance mid-infrared microspectroscopy (IRMS) and chemometrics. Overall, the performance of the PLSR models showed low prediction errors (SEP ranging from 22.1 to 28.9 µg/L) and good linear correlation (rPred ranging from 0.91 to 0.92)

between the acrylamide levels estimated using spectral information and reference acrylamide values from LC-MS/MS for regular, seasoned and combined (regular and seasoned) potato chip categories. Furthermore, models developed using extensive SPE cleaning gave comparable performance statistics as models without SPE cleaning, making IRMS technology promising for rapid, robust and sensitive acrylamide detection and quantification since SPE treatment step can be avoided. Our results indicate that IRMS would allow detection and quantification of acrylamide levels in potato chip products through spectral signature profiles enabling high-throughput measurements for controlling the product stream, addressing risk management, assessing safety and brand equity.

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CHAPTER 4

APPLICATION OF HANDHELD AND PORTABLE SPECTROMETERS FOR SCREENING ACRYLAMIDE CONTENT IN COMMERCIAL POTATO CHIPS

4.1. ABSTRACT

Current techniques for acrylamide analysis in foods rely on LC-MS/MS and GC-MS that require extensive sample preparation, highly specialized instrumentation, and are time consuming. In this study, portable and handheld infrared (IR) spectrometers were evaluated as rapid methods for detection and quantification of acrylamide in potato chips and their performances were compared to those of benchtop IR systems. The acrylamide content of 64 commercial potato chips (169-2453 µg/kg) was determined by LC-MS/MS. Spectral data were collected using mid-infrared (MIR) and near-infrared (NIR) spectrometers. Partial Least Squares Regression (PLSR) calibration models were developed to predict acrylamide levels. Overall, good linear correlation was found between the predicted acrylamide levels of independent sample sets and actual measured acrylamide concentrations by LC-MS/MS (rPred > 0.90 and SEP < 100 µg/kg). Our results indicate that portable and handheld spectrometers can be used as simple and rapid alternatives for acrylamide analysis in potato chips.

4.2. INTRODUCTION

Acrylamide (2-propenamide, CAS Registry No. 79-06-1) is a chemical compound naturally formed during processes such as frying, roasting and baking in a wide variety of foods including french fries, potato chips, cereal, bread and coffee (Ahn, Castle, Clarke, Lloyd, Philo & Speck, 2002). It has been reported to be a human neurotoxin, a rodent carcinogen and a "probable carcinogen to humans" (Becalski, Lau, Lewis & Seaman, 2003).

In 2002, significant levels of acrylamide in some foods processed at high temperatures were found (Tareke, Rydberg, Karlsson, Eriksson & Tornqvist, 2002). Moderate levels of acrylamide (5-50 μg/kg) in heated protein-rich foods and higher levels of acrylamide (150-4000 μg/kg) in carbohydrate-rich foods were reported. Ahn et al. (2003) overcooked fried chips and exhibited the presence of very high levels of acrylamide (more than 10 mg/kg) indicating the influence of cooking temperature and duration on acrylamide formation. As stated by the World Health Organization (WHO), daily intake of dietary acrylamide ranges between 0.3 – 2.0 μg/kg/body wt and, it is estimated to be 2-3 times higher in children than that of adults because of average body weight ratios (Becalski et al., 2003).

Maillard reaction between sugars and amino acids is considered to be the main mechanism for acrylamide formation, with asparagine and reducing sugars as the major reactants present in foods (Friedman, 2003). Since potatoes are high in both asparagine and reducing sugars such as glucose and fructose, frying potato chips generates optimum conditions for acrylamide formation (Zhang, Ren & Zhang, 2009). Acrylamide has also

been included in California`s Proposition 65 list of chemicals. Proposition 65 requires businesses in California to warn citizens of significant exposures to chemicals that can potentially cause cancer or reproductive harm (California OEHHA, 2011). Business in California can either reformulate their products to lower the acrylamide levels below the level deemed by the state or they can post a warning sign on the label to warn citizens. The California Attorney General and private interest groups filed lawsuits against a number of U.S. food manufacturers and restaurant chains, declaring that food sold by these companies violates Proposition 65 guidelines (United States Food and Drug Administration, 2007; Baylen, 2010) and the companies were fined with millions in penalties and costs. Additionally, the companies needed to post acrylamide warnings on their products as needed or work to reduce the acrylamide content of their foods to acceptable levels (State of California Department of Justice, 2008).

Currently, the most popular methods for acrylamide measurements in foods rely on GC-MS or LC-MS/MS (Wenzl et al., 2006). However, their use requires extensive sample preparation, highly specialized instrumentation and skilled operators. They are also expensive and time consuming, which restricts their use for monitoring acrylamide content during processing or on finished products (Andrzejewski, Roach, Gay & Musser, 2004). As a consequence, food producers generally send samples out for acrylamide testing instead of performing acrylamide analysis within their companies. Therefore, there is a need for a rapid and simple method for routine analysis of acrylamide.

Infrared spectroscopy has been used for numerous food applications related to quality control and safety of our food supply providing valuable information about the

biochemical composition of the samples, especially in the fingerprint region (Segtnan, Kita, Mielnik, Jorgensen & Knutsen, 2006; Reid, O`Donnell & Downey, 2006; Rodriguez-Saona & Allendorf, 2011). This analytical technique is simple, fast, highly specific, requires minimal or no sample preparation and it has been widely used in food analysis (Reid et al., 2006). Improvements in modern computing technology have led infrared spectroscopy to become more practical by allowing complex chemometric analysis of the spectra (Rodriguez-Saona et al., 2011). Additionally, optical technology is rapidly developing and instruments are now offered commercially as portable, handheld and micro-devices. Miniaturization of vibrational spectroscopy components has allowed the development of portable and handheld systems that offer simplicity, speed, selectivity and performance similar to that of benchtop instruments used in laboratories. Instead of more sophisticated and costly benchtop spectrometers, portable and handheld devices can be used since they are more practical and economical. There are research studies published employing these devices including the quantitation of trans fat in edible oils (Birkel & Rodriguez-Saona, 2011; Mossoba et al., 2012), monitoring of oil oxidative stability (Allendorf, Subramaniam & Rodriguez-Saona, 2012) and mineral fortification in whole grain cornmeal (Hassel & Rodriguez-Saona, 2012).

The objective of this study was to develop simple, sensitive and robust methods for detection and quantification of acrylamide in potato chips using handheld and portable IR spectrometers based on highly specific MIR/NIR spectroscopic signature profiles in combination with supervised pattern recognition techniques and compare the performances of these systems to those of benchtop IR systems.

4.3. MATERIALS AND METHODS

4.3.1. Chemicals and Consumables

Native acrylamide (99+ %) was acquired from Acros Organics (Fairlawn, NJ). Internal standard ($^{13}C_3$-Labeled acrylamide (98%) was purchased from CIL (Andover, MA). LC/MS grade water, methanol, acetonitrile, formic acid and 50 mL polypropylene conical tubes with caps were purchased from Fisher Scientific (Pittsburg, PA). Additional supplies included: 50 mL maxi spin filtration tubes (0.45 µm, PVDF) from Grace Davison Discovery Sciences (Deerfield, IL), Oasis HLB (6 mL, 200 mg) SPE cartridges and amber glass autosampler vials with septum screw caps from Waters Corp (Milford, MA), Bond Elut-Accucat (200 mg, 3 mL) SPE cartridge from Varian Inc. (Chicago, IL), and reversed phase Hypercarb LC column (50 mm x 2.1 mm) with guard column from Thermo Scientific (Waltman, MA).

4.3.2. Preparation of Standards

Stock solutions of native acrylamide and $^{13}C_3$-labeled acrylamide (internal standard) were prepared by accurately weighing native acrylamide and $^{13}C_3$-labeled acrylamide in separate amber glass volumetric flasks and dissolving them in 0.1% formic acid. Aliquots of the stock solutions were used to prepare working solutions. By diluting the working solutions in 0.1% formic acid, five different concentrations of native acrylamide (from 0 to 720 µg/L) including fixed concentration of internal standard (2000 µg/kg) were prepared and used to obtain standard curve using LC-MS/MS. Using an internal standard helped account for any losses during sample preparation or fluctuations

in LC-MS/MS sensitivity. Acrylamide stock solutions were reported as stable for up to 6 months in amber glass containers even at room temperatures (Roach, Andrzejewski, Gay, Nortrup & Musser, 2003).

4.3.3. Reference Analysis: Analysis for Acrylamide in Potato Chips by LC-MS/MS

Sixty-four different commercial potato chips were purchased at local grocery stores (Columbus, OH) and their acrylamide levels were determined using a method modified from Roach and others (Roach et al., 2003). Based on the ingredients listed on the labels of the packages, 42 of the potato chip bags were regular potato chips (including only potatoes, oil and salt), 12 were seasoned potato chips and 10 were sweet potato chips. Approximately 100 g potato chip per sample bag was blended using a Waring blender. Three independent extractions were conducted from each potato chip bag. A sub-sample (1 g) was mixed with 9 mL water for each extraction. In addition to blended potato chips and water, 1 ml of 2000 µg/L $^{13}C_3$-labeled acrylamide was added as internal standard. The samples were mixed for 20 min and centrifuged at 9681 x g for 15 min (Sorvall RC 5C Plus with SS-34 rotor, DuPont Co., Newtown, CT). Five mL of the clarified aqueous layer was removed avoiding oil layer on top and solid layer on the bottom of the tube and transferred into a spin filtration tube for further centrifugation at 9681 x g for 4 min. The extract passed through a spin filtration tube was centrifuged again for 10 min at 13400 x g prior to the SPE cleaning. Using Oasis HLB SPE and Accucat SPE cartridges, SPE cleaning was completed.

LC-MS/MS was used to measure acrylamide levels in aqueous extracts. Waters Acquity Ultra Performance LC system (Milford, MA) was used to separate the analytes on reversed phase Hypercarb column (50 mm x 2.1 mm, Thermo Scientific, Waltham, MA) by running acidified water (0.1% formic acid) isocratically as the mobile phase. Mass spectrometry was conducted by using positive-ion electrospray ionization on a triple quadrupole mass spectrometer and monitoring the transitions m/z 72>55 (for native acrylamide) and 75>58 (for internal standard). Response curve was plotted daily for the ratio of m/z 55/58 versus injected acrylamide concentration as well as constant amount of labeled acrylamide. LOD and LOQ for LC-MS/MS were calculated. The LOD (μg/kg) was calculated as 3.3 times ratio of standard deviation of the response (SD) and the slope of the calibration curve whereas LOQ (μg/kg) was 10 times the ratio of standard deviation of the response (SD) and the slope of the calibration curve.

4.3.4. Infrared spectroscopy measurements

For all the IR measurements, about 10 g blended potato chip was weighed out and oil was expelled using a hydraulic press (Carver Inc., Wabash, IN). This was done to minimize strong infrared absorption from oils in the collected spectra. The resulting "chip cake" was further used for IR measurements. For every potato chip sample, three chip cakes were produced using the hydraulic press and one spectrum for each cake (total of three spectra per every potato chip bag) was collected.

4.3.4.1. Near Infrared spectroscopy measurements

Benchtop system: NIR spectra of each "chip cake" were collected using an Excalibur 3500 Fourier-Transform IR spectrometer (Varian, Palo Alto, CA) with a NIR integrating sphere diffuse reflectance accessory (Integrat IR^{TM}, Pike Technologies, Madison, WI). It was equipped with a quartz beam splitter and Lead Selenide (PbSe) detector. Spectra were collected over a range of 10,000-4,000 cm^{-1} by co-adding 64 scans at a resolution of 4 cm^{-1} and displayed in terms of absorbance. The absorbance spectrum was obtained by rationing the sample spectrum against that of a blank optical path (reference spectrum). The instrument was continuously purged with CO_2-free, dry air from a CO_2RP140 dryer (Domnick Hunter, Charlotte, NC).

Handheld system: NIR spectra of the "chip cakes" were collected using a dispersive handheld NIR (microMICROPHAZIR, Thermo Fisher Scientific Inc., Wilmington, MA) equipped with a single Indium Gallium Arsenide (InGaAs) detector. All diffuse reflectance spectra were computed at an optical resolution of 11 nm, spectral range from 1,600 to 2,400 nm (6,250-4,170 cm^{-1}) and 64 scans were co-added to increase the signal to noise ratio.

Fiber Optic Accessory: The chip cakes were crushed and the powder used to collect spectra using the NIR Fiber Optic Accessory (Pike Technologies, Madison, WI). The accessory had a 2 meter long fiber optic cable for remote and speedy analysis. The cable was directly coupled to an indium gallium arsenide (InGaAs) detector. The fiber optic accessory was connected to an Excalibur 3500 Fourier-Transform IR spectrometer (Varian, Palo Alto, CA). The instrument was continuously purged with CO_2-free, dry air

from a CO_2RP140 dryer (Domnick Hunter, Charlotte, NC). Each potato chip cake was crushed and filled in the drum of the accessory separately. The probe's tip was touched onto potato chips for spectra collection over a range of 10,000-4,000 cm^{-1} by co-adding 64 scans at a resolution of 4 cm^{-1} and displayed in terms of absorbance.

4.3.4.2. Mid-Infrared spectroscopy measurements

Benchtop system: Mid-infrared (MIR) spectra of the chip cake powders were collected using an Excalibur 3500 Fourier-Transform IR spectrometer (Varian, Palo Alto, CA) with triple bounce ATR accessory (Pike Technologies, Madison WI) equipped with a KBr beamsplitter and deuterated triglycine sulfate (DTGS) detector. Spectra were collected over a range of 4,000-700 cm^{-1} by co-adding 64 scans at a resolution of 4 cm^{-1} using a pressure clamp to apply pressure on the sample. The absorbance spectrum was obtained by rationing the sample spectrum against that of a blank optical path (reference spectrum). The instrument was continuously purged with CO_2 free dry air from a CO_2RP140 dryer (Domnick Hunter, Charlotte, NC).

Portable system: A Cary 630 FTIR spectrometer (Agilent Technologies Inc., Danbury, CT) equipped with ZnSe beamsplitter and DTGS detector was used to analyze the chip cake powders. The powder was placed on the crystal and pressed by the pressure clamp of the instrument. The MIR spectra were collected in the reflectance mode. The spectral resolution was 4 cm^{-1} and 64 spectra were co-added to improve the signal to noise ratio over the frequency range 4,000-700 cm^{-1}. The infrared spectra of

background and samples were recorded on a personal computer using Agilent MicroLab PC software (Agilent Technologies Inc., Danbury, CT).

4.3.5. Multivariate data analysis

Collected spectra were imported into Pirouette software (version 4.0, Infometrix Inc., Woodville, WA) for multivariate analysis. Spectra from every instrument were mean centered. For MIR systems and benchtop NIR system, data was normalized and the second derivative transformed (Savitzky-Golay second order polynomial filter with a 25-point window) prior to partial least squares regression (PLSR) analysis. For handheld NIR and fiber optic accessory (NIR), the spectra were mean-centered and transformed by multiplicative scatter correction (MSC). PLSR has been utilized to avoid overfitting during quantitative spectroscopic analysis (Haaland & Thomas, 1988). PLS regression extracts the "latent variables (PLS-Factors)" and only these variables which are important to explain the variation in the model (usually less than 10) are used instead of thousands of wavenumbers (Moseholm, 1988). PLSR contains the features from both Principal Component Analysis (PCA) and Multi Linear Regression (MLR), taking the error into account both in the acrylamide levels measured by LC-MS/MS and the spectra collected using infrared spectrometers. PLSR does not assume that the acrylamide levels obtained from LC-MS/MS and used as reference values are error free since there could be errors involved in sample preparations and such (Brereton, 2000). Therefore, PLSR is becoming a standard tool for modeling correlated relationships between multivariate measurements (Jong, 1993) and commonly used by both academia and industry.

Quantitative models were generated with PLSR using the IR spectra and reference acrylamide concentrations from LC-MS/MS (dependent variable). Full cross-validation (leave-one-out approach) was used to internally validate the calibration models while an independent sample set was included for external validation. Cross-validation was used for every calibration model while independent validation was conducted only for the regular potato chip model due to limited number of samples in seasoned and sweet potato chip categories. For independent validation studies, approximately 75% of the sample set was used to generate calibration models and about 25% to serve as an independent validation set. Samples were divided into calibration and independent validation sets in a way that all replicates belonging to the same potato chip bag were either used only in a calibration or a validation set. Loading vectors, standard error of cross validation (SECV), standard error of prediction (SEP), correlation coefficient (r) and outlier diagnostics (Standard Residual of Sample vs. Leverage) were used to evaluate the performance of the models. The SEP shows the magnitude of error expected when independent samples are introduced and predicted using the model. Outliers were evaluated using X residuals and leverage and observations with large residuals or an unusual residual pattern were considered as outliers. The leverage of a sample in the calibration model shows its potential contribution to the estimated calibration model. Samples containing abnormal standard residual (>2) and high leverage were reanalyzed and excluded if necessary, after which the calibration model was repeated.

Additionally, Soft Independent Modeling of Class Analogy (SIMCA) was conducted using benchtop MIR spectra for regular, seasoned and sweet potato chips to

evaluate the dissimilarity among these groups. Spectra were normalized and then the second derivative (Savitzky-Golay second order polynomial filter with a 45-point window) transformed prior to SIMCA analysis. SIMCA is a supervised chemometric method for sample classification which requires assigning training data sets to classes (Wold, Sjostrom & Eriksson, 2001). In this study, 3 classes were designed: regular potato chips (class 1), seasoned potato chips (class 2) and sweet potato chips (class 3). Performances of the SIMCA models were evaluated through the interclass distance and the three-dimensional principal component analysis scores plot. SIMCA`s interclass distance (ICD) describes quantitatively the similarity or dissimilarity of the different classes. Generally, if ICD > 3, classes can be differentiated (Vogt & Knutsen, 1985).

4.4. RESULTS AND DISCUSSION

4.4.1. Classification Analysis

Acrylamide content of selected potato chips was determined using the ratio of transitions m/z 72>55 (for native acrylamide) and 75>58 (for internal standard) by LC-MS/MS (Roach et al., 2003) (ratio of mz 55/58 = 0.00264 x [acrylamide concentration] + 0.0215) and found to be ranging between 169 and 2453 µg/kg, which were within the levels reported in the literature (Becalski et al., 2003; Roach et al., 2006). LOD and LOQ concentrations for LC-MS/MS were calculated using the standard curve and reported as 18 and 55 µg/L, respectively.

Our spectra collection technique included pressing the oil out from the potato chips with a hydraulic press and using the pellet directly. This sample preparation

minimized the strong signal from the oil and allowed to resolve unique spectral vibrations associated with acrylamide. Initially, the influence of the matrix was evaluated using MIR fingerprinting capabilities for the 64 commercial (regular, seasoned and sweet potato) chips. Regular potato chip ingredients were comprised only of potatoes, oil and salt while the ingredients listed on the labels of seasoned potato chips included maltodextrin, yeast extract, cheddar cheese, sour cream, whey powder, beer, honey, cane juice, vinegar, natural flavors, onion and garlic powder among others. Sweet potato chip ingredients included sweet potato, oil and salt except for a commercial brand that had additional ingredients such as evaporated cane juice, spices & flavors, and milk. A SIMCA analysis was conducted to evaluate if different potato chip groups (regular, seasoned and sweet potato chips) showed distinctive clustering patterns (**Figure 4.1**). SIMCA's Coomans plot (**Figure 4.1**) was used to determine class membership in terms of distance from the boundaries (95% confidence limits) of the categories generated by the classification model. In a Coomans plot, the two internal axes represent the distance of each sample from a specific class (e.g., regular, seasoned or sweet potato), so that each class model is drawn as a rectangle corresponding to the critical distance (p=0.05) from the class. Any sample having a distance to the corresponding class rectangle greater than the critical distance is considered as being outside the class model and, as a consequence, rejected as an outlier from the specific class (graphically, it is plotted outside the rectangle defining the class model). Any sample in the lower left square of the diagram is assigned to both classes. The Coomans plot showed clear discrimination of the regular vs. seasoned potato chip classes (p=0.05), while samples belonging to sweet potato chips

were modeled as a different class. The cross-validation analysis showed that all samples were correctly predicted into their assigned classes with zero miss-classifications. Interestingly, the clustering of sweet potato chip samples showed 2 marked groupings associated with brands containing only sweet potatoes, oil and salt and those including the additional ingredients (evaporated cane juice, flavors and milk). ICD`s for SIMCA analysis of benchtop MIR proved the groups clustering. Statistically significant ($p<0.05$) interclass distances were obtained among the all three groups, with ICD values between regular and sweet potato chips, seasoned and sweet potato chips and regular and seasoned potato chip of 12.7, 10.8 and 3.3, respectively. Thus, separate PLSR models were developed for regular, seasoned and sweet potato chips. There is also an option for food producers to make measurements right after frying and prior to addition of the seasonings. This practice will allow using one single model for acrylamide determination in potato chips.

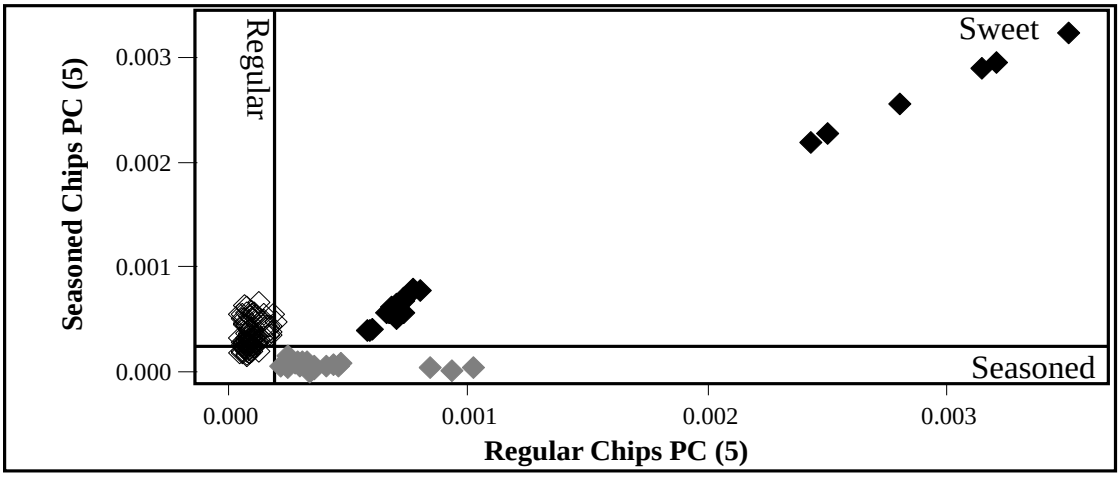

Figure 4.1. Coomans` plot of regular, seasoned and sweet potato chips spectra collected using benchtop MIR (900-1699 cm^{-1} region) (normalized and second derivative transformed (Savitzky-Golay second order polynomial filter with a 45-point window), $\partial A/\partial \lambda^2$) (◇: Regular chips, ◆: Seasoned chips, ◆ : Sweet chips)

4.4.2. Development of PLSR models using Near Infrared spectroscopy

Table 4.1 summarizes the sample diversity with regard to acrylamide levels for regular, seasoned or sweet potato chips used to develop calibration and validation models for NIR systems. Models developed using regular potato chips had an acrylamide range of approximately 200 to 1100 µg/kg while seasoned potato chip samples had smaller range of acrylamide levels (ca. 225-850 µg/kg). On the other hand, sweet potato chips had the highest acrylamide contents ranging from 920 to 2450 µg/kg, possibly due to the higher amount of reducing sugars present in sweet potato cultivars compared to that of regular potatoes (United States Department of Agriculture, 2013).

Table 4.1. Comparison of some statistical parameters of the sample sets used to develop calibration and prediction models for Near and Mid-Infrared systems used

Sensor	Type of Chips		Number of brands (n)	Min.	Max.	Mean	Std. Dev
Benchtop NIR	Regular [a]	Calibration	29	178	1084	543	223
		Prediction	8	240	831	559	189
	Seasoned [b]	Calibration	12	223	850	473	185
	Sweet [c]	Calibration	10	921	2453	1555	438
microPHAZIR Handheld NIR	Regular [a]	Calibration	30	196	1109	537	215
		Prediction	8	240	831	564	194
	Seasoned [b]	Calibration	12	223	850	435	176
	Sweet [c]	Calibration	10	921	2453	1493	429
Fiber Optic Accessory	Regular [a]	Calibration	32	178	1085	546	214
		Prediction	8	240	831	580	185
	Seasoned [b]	Calibration	13	226	850	451	173
	Sweet [c]	Calibration	10	960	2453	1561	471
Benchtop MIR	Regular [a]	Calibration	32	169	1109	554	226
		Prediction	8	240	831	594	191
	Seasoned [b]	Calibration	12	223	850	430	177
	Sweet [c]	Calibration	9	921	2453	1569	456
Portable Cary 630 MIR	Regular [a]	Calibration	29	196	1084	506	202
		Prediction	8	240	831	544	167
	Seasoned [b]	Calibration	13	223	850	466	193
	Sweet [c]	Calibration	9	921	2453	1569	456

[a]: Regular was referred to the potato chips containing only potatoes, vegetable oils and salt as ingredients
[b]: Seasoned was referred to the potato chips containing different ingredients than that of regular potato chips
[c]: Sweet was referred to the potato chips made of sweet potatoes
[n]: Number of potato chip brands used to form calibration and validation models excluding outliers
Acrylamide levels shown are for 1 g subsamples of potato chips run by LC-MS/MS (µg/kg)

Table 4.2 shows the performance statistics of the PLSR models developed for each sample category using NIR systems; benchtop, handheld and fiber optic accessory. All instruments provided very high correlation coefficients (r) values and low standard errors of prediction for regular, seasoned and sweet potato chip groups (r>0.91 and SEP<100 µg/L). To compare the models developed using different equipment, the ratios of prediction to validation (RPD) values were also reported in **Table 4.2**. RPD is the ratio of standard deviation of the acrylamide concentrations for the samples in calibration models found by LC-MS/MS to SECV. Since regular models also included an independent sample set, additional RPD values were calculated for predictions by dividing the standard deviation of the acrylamide concentrations among independent prediction sets found by LC-MS/MS to SEP. The criteria for RPD levels adopted from Saeys, Mouazen & Ramon (2005) who reported five levels of RPD classification. According to the authors, an RPD value below 1.5 indicates very poor model/predictions and that model would not be useful; an RPD value between 1.5 and 2.0 indicates that the model could distinguish between high and low values, while a value between 2.0 and 2.5 makes approximate quantitative predictions possible. RPD values between 2.5 and 3.0 is considered as good and above RPD value of 3.0, the prediction is classified as excellent. As can be seen in **Table 4.2**, RPD values for regular, seasoned and sweet potato chip models using benchtop NIR system ranged between 2.6 to 4.4 which indicates that models can be used for quantitative measurements. RPD values for the models developed using fiber optic NIR accessory was the lowest compared to those of benchtop and handheld NIR instrument ranging between 1.9 and 3.1. The reason of lower performance

by the fiber optic accessory could be due to the signal lost in the 2- meter long fiber optic cable resulting in higher spectral noise. Representative PLSR loading plots of regular potato chips analyzed with benchtop NIR instrument (**Figure 4.2a)** showed that the highest relevant variation in the calibration set for acrylamide content was in the range of 4501 to 5406 cm^{-1} associated with 2^{nd} stretching overtone of C=O (5208 cm^{-1}), N-H stretching / N-H bending combination (5025 cm^{-1}), symmetrical N-H stretch/amide I combination (4866 cm^{-1}), N-H 2^{nd} overtone bending (4587 and 4854 cm^{-1}) and C-H stretch/C=O stretching combination (4546 and 4673 cm^{-1}) (Shenk, Workman & Westerhaus, 2001). The loadings plot for the MicroPHAZIR handheld NIR unit was not informative regarding the functional groups in the potato chip samples responsible for acrylamide quantitation (data not shown) because of the broad band signals obtained with this dispersive system (resolution-pixel 8 nm) as opposed to the FT-NIR unit (4 cm^{-1} resolution). Nevertheless, the handheld NIR system performed similarly to that of benchtop units, providing RPD values between 1.9 and 4.5. Strong performance of handheld NIR system is very promising since it increases the flexibility and potential of its in-field applications.

Although the determination of acrylamide by NIR spectroscopy has been investigated in the past (Segtnan et al., 2006; Pedreschi, Segtnan & Knutsen, 2010b), no information is available on the use of handheld systems. Previous research had focused on evaluating the frying of a single potato cultivar (Saturna) in palm oil without the addition of any other ingredients. Instead, we evaluated samples of commercially available potato chips brands which were produced using a wide variety of potato

cultivars, oils, seasonings and manufacturing conditions. Improved results were obtained in this research with SEP<100 µg/kg and RPD values between 1.9 (NIR fiber optic) to 4.5 (benchtop NIR and microPhazir handheld NIR), as opposed to previous research reporting SECV of 270 µg/kg and RPD value of 1.85 (Segtnan et al., 2006; Pedreschi et al., 2010b).

Table 4.2. Calibration, cross-validation and prediction results of PLSR models developed by using Near-Infrared Instruments

Sensor	Potato Chip Type		Factors	SE (µg/L)	r	RPD
Benchtop NIR	Regular [a]	Calibration	8	68	0.96	
		Cross Val.	8	87	0.92	2.6
		Prediction	8	78	0.91	2.4
	Seasoned [b]	Calibration	7	32	0.99	
		Cross Val.	7	47	0.97	3.9
	Sweet [c]	Calibration	8	38	1.00	
		Cross Val.	8	99	0.98	4.4
Handheld NIR	Regular [a]	Calibration	8	70	0.95	
		Cross Val.	8	81	0.93	2.6
		Prediction	8	100	0.94	1.9
	Seasoned [b]	Calibration	6	55	0.96	
		Cross Val.	6	66	0.92	2.7
	Sweet [c]	Calibration	8	60	0.99	
		Cross Val.	8	96	0.97	4.5
Fiber	Regular [a]	Calibration	8	40	0.98	
		Cross Val.	8	109	0.86	2.0
		Prediction	8	97	0.92	1.9
	Seasoned [b]	Calibration	6	32	0.99	
		Cross Val.	6	56	0.95	3.1
	Sweet [c]	Calibration	7	12	1.00	
		Cross Val.	7	185	0.92	2.6

[a]: Regular was referred to the potato chips containing only potatoes, vegetable oils and salt as ingredients
[b]: Seasoned was referred to the potato chips containing different ingredients than that of regular potato chips
[c]: Sweet was referred to the potato chips made of sweet potatoes
Acrylamide levels shown are for 1 g subsamples of potato chips run by LC-MS/MS (µg/kg)

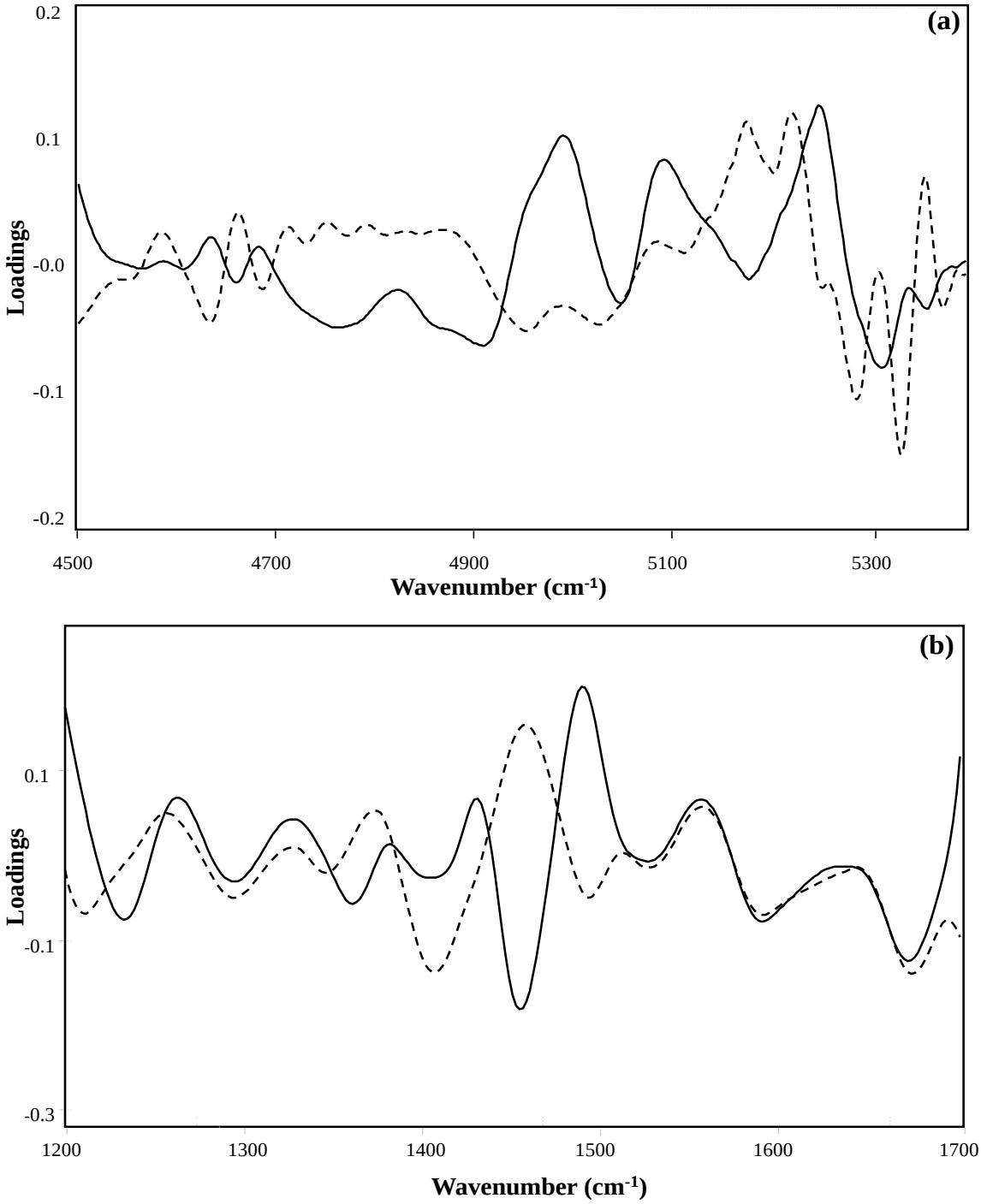

Figure 4.2. Loadings of the first two factors for calibration model of regular potato chips using **(a)** Benchtop NIR equipment (Savitzky-Golay second order polynomial filter with a 35-point window), $\partial A/\partial \lambda^2$) **(b)** Benchtop MIR equipment (Savitzky-Golay second order polynomial filter with a 25-point window), $\partial A/\partial \lambda^2$) (**——— : first factor, – – – : second factor)**

4.4.3. Development of PLSR models using Mid-Infrared (MIR) spectroscopy

Table 4.1 shows the statistical parameters for the different chip groups used to develop calibration models for MIR systems. The statistical parameters for the different chip groups used for MIR measurements were quite similar to those of NIR instruments (**Table 4.1**) since same commercial potato chips were used for both MIR and NIR applications.

Table 4.3 shows the performance statistics of the PLSR models developed for each chip sample group using benchtop and portable Cary 630 ATR-MIR systems. Benchtop MIR instrument gave RPD values between 2.0 and 3.9. SEP value for regular potato chips using benchtop MIR was 83 µg/kg. Seasoned and sweet potato chip groups had SECV of 45 and 159 µg/kg, respectively. The portable Cary 630 ATR-MIR system performed exceptionally well providing RPD values for the models ranged between 2.2 to 4.7. SEP value for regular potato chips using portable Cary 630 ATR-MIR was 75 µg/kg. Seasoned and sweet potato chip groups had SECV of 75 and 98 µg/kg, respectively. Representative loading plot of regular potato chips analyzed with benchtop MIR instrument is shown in **Figure 4.2b**. Loading plots of the first two factors showed that the highest relevant variation in the calibration set for acrylamide content was in the range of 1201 to 1699 cm^{-1} for MIR. In the region of 1201 to 1699 cm^{-1}, C=C stretching (1675 cm^{-1}), NH_2 in plane bending (1613 cm^{-1}), CH_2 stretching (1429 cm^{-1}), CH in plane bending (1351 cm^{-1}) and C-N stretching (1280 cm^{-1}) were reported previously (Jong, 1993). The region around 1730 cm^{-1} associated with fat in MIR was excluded in the

PLSR model development. Similar loadings were obtained for the Cary 630 ATR-MIR system (data not shown).

This is the first research reporting on acrylamide testing in foods using MIR spectrometers, with the portable Cary 630 MIR system performing slightly better than the benchtop MIR that indicates that rapid and robust acrylamide analysis can be done using the regression models developed with a portable system. The use of the portable system can provide food producers with increased flexibility and great potential for in-field applications compared to bench-top systems which can only be used in a laboratory setting.

Figure 4.3 shows the correlation between the infrared estimated levels and reference acrylamide values for calibration and validation models obtained for regular potato chips using all five instruments utilized in this research. When the benchtop (**Figure 4.3a**) and portable Cary 630 MIR systems (**Figure 4.3b**) were compared, the portable system showed a slightly better performance, providing improved predictions. As for NIR systems, both the benchtop (**Figure 4.3c**) and handheld instruments (**Figure 4.3d**) showed similar performances, while the fiber optic NIR accessory resulted in less accurate predictions (**Figure 4.3e**).

Overall, the optimum number of PLS-factors used for method development was very similar (either 7 or 8 PLS factors) for all the models **(Table 4.2 and Table 4.3)**. The selection of the optimal number of factors was done by a cross-validation approach yielding the minimum error of prediction. Increased number of factors could make the model less robust by augmenting the interference of noise signals (Hawkins, 2004).

Additionally, performance statistics for cross-validation and prediction in the same chip category were similar, which also indicates that the models are reliable and robust in predicting acrylamide levels in these potato chips.

Table 4.3. Calibration, cross-validation and prediction results of PLSR models developed by using Mid-Infrared Instruments

Sensor	Potato Chip Type		Factors	SE (µg/L)	r	RPD
Benchtop MIR	Regular [a]	Calibration	7	96.1	0.91	
		Cross Val.	7	112	0.87	2.0
		Prediction	7	83	0.90	2.3
	Seasoned [b]	Calibration	7	38	0.98	
		Cross Val.	7	45	0.97	3.9
	Sweet [c]	Calibration	7	100	0.98	
		Cross Val.	7	159	0.94	2.9
Portable Cary 630 MIR	Regular [a]	Calibration	7	65	0.95	
		Cross Val.	7	74	0.93	2.7
		Prediction	7	75	0.90	2.2
	Seasoned [b]	Calibration	7	59	0.96	
		Cross Val.	7	75	0.92	2.6
	Sweet [c]	Calibration	7	74	0.99	
		Cross Val.	7	98	0.98	4.7

[a]**:** Regular was referred to the potato chips containing only potatoes, vegetable oils and salt as ingredients
[b]**:** Seasoned was referred to the potato chips containing different ingredients than that of regular potato chips
[c]: Sweet was referred to the potato chips made of sweet potatoes
Acrylamide levels shown are for 1 g subsamples of potato chips run by LC-MS/MS (µg/kg)

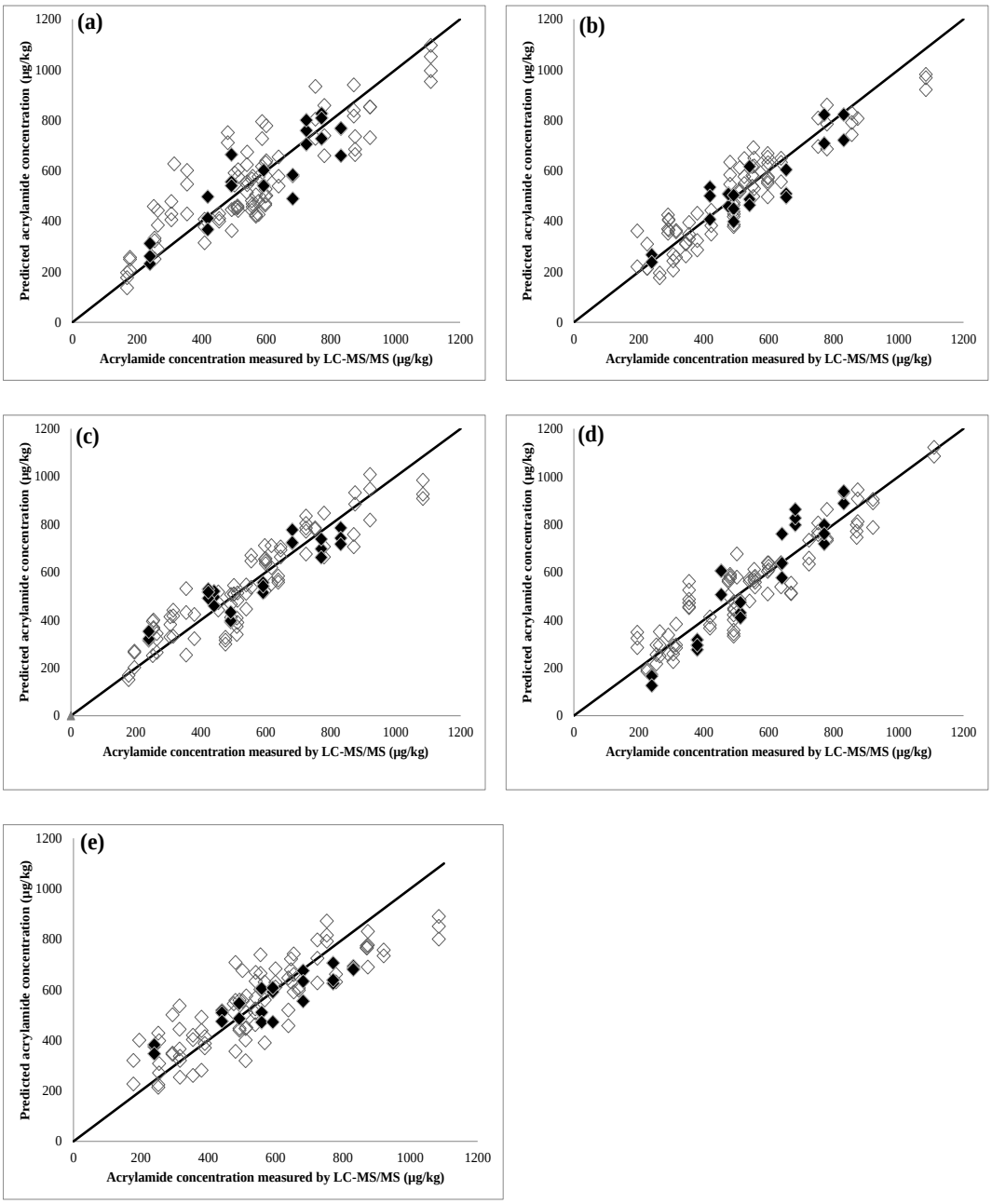

Figure 4.3. Partial least squares regression (PLSR) plots for acrylamide content in regular potato chips using **(a)** Benchtop MIR **(b)** Portable Cary 630 MIR (Cary 630) **(c)** Benchtop NIR **(d)** Handheld NIR (Phazar NIR) **(e)** Fiber Optic Accessory (NIR) (acrylamide levels are expressed as µg/kg) (◇, ◆ represent samples in calibration and validation groups, respectively)

4.5. CONCLUSION

Our data supports the application of both MIR and NIR instruments for acrylamide analysis in commercial potato chips, reporting RPD values between 2.0 to 4.7 for MIR instruments and 1.9 to 4.5 for NIR instruments used in this research. The portable and handheld systems performed similarly to the benchtop units tested, which indicates that acrylamide tests can be done using the models developed with the portable and handheld systems rapidly (only sample preparation involves blending the potato chips and pressing the oil out using a hydraulic press). Additionally, the possible use of large volumes of solvents, other chemicals, cartridges and such, which are required for traditional LC-MS/MS and GC-MS based methods, were avoided. Using handheld and portable systems is simple, cost-effective and requires low sample volume; once the instruments are purchased, there are minimal operational costs involved on performing the acrylamide test. Moreover, the use of the portable and handheld systems provides food producers with increased flexibility and great potential for in-field applications compared to bench-top systems which can only be used in a laboratory setting.

4.6. REFERENCES

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CHAPTER 5

CONCLUSION

In this study, developing quick and easy methods utilizing infrared spectrometers and multivariate analysis as alternatives to traditional methods for screening acrylamide precursor`s level in potato tubers **(Chapter 2**) and quantification of acrylamide level in commercial potato chips collected from market (**Chapter 3 and 4**) were aimed.

In the first study (**Chapter 2**), performance of a modern portable FT-IR unit with ATR and dial path accessories were tested. Reference levels of sugars and free amino acids were determined using methods based on HPLC-RID and GC-FID. By using the same spectra, strong quantitative infrared methods for prediction of main acrylamide precursors (free asparagine, glucose, fructose and sucrose) as well as the second most common free amino acid (glutamine) in potato tubers were developed and externally calibrated using an independent validation set. These models can help potato breeders, industrial manufacturers and research facilities by providing fast and simple ways of measuring these compounds.

In the second study (**Chapter 3**), a benchtop ATR-IRMS was tested for acrylamide quantification in commercial potato chips collected from market. Reference levels of acrylamide in chips were determined by using LC-MS/MS. Using the spectra

collected without the need of prior SPE cleaning, rapid, robust and sensitive method for acrylamide quantification was possible.

In the last study (**Chapter 4**), handheld NIR and portable MIR units were evaluated for acrylamide quantification in commercial potato chips. Acrylamide levels obtained using LC-MS/MS were used as reference values. The performances of handheld and portable units were also compared against the benchtop NIR and MIR units. Results revealed that by just blending the chips and pressing by hydraulic press to release the excessive oil as sample preparation, infrared models with high predictive ability could be achieved. Both portable and handheld units performed similar to their benchtop counterparts providing increased flexibility and in-field use. By using the infrared methods for acrylamide tests, materials used during the sample preparation of traditional LC and GC based methods such as solvents, chemicals and cartridges can be avoided. Once the infrared spectrometers are purchased, there is minimum cost needed for routine acrylamide test.

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