Effects of Air Frying and Enzymatic Oxidation on the Phenolic Content and Antioxidant Properties of Lower Grade Yellow Mustard Seeds

by

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ABSTRACT

Lower grade yellow mustard meal (LGYMM), a by-product of the mustard seed milling process has potential as a value added product in food, feed, and agro-allied industries. The under-utilized by-product of mustard can be used for the efficient release and extraction of bioactive phenolic compounds. Heat treatment and enzymatic assisted extraction have been reported to efficiently extract the bioactive compounds from oilseeds. This thesis was focused on the effect of air frying at different temperature/ time combinations (120 -180 °C from 5 to 20 min) and enzymatic oxidation using horseradish peroxidase (HRP) and tyrosinase at different incubation times (0, 1, 1.5, 2, 5 and 18 h) on the phenolic profile of LGYMM and its antioxidant activities. For air frying, sinapine was the major phenolic compound and its concentration decreased significantly ($p<0.05$) with prolonged time. The concentration of most of the phenolic compounds significantly ($p<0.05$) decreased with increased temperature-time with an exception at 180 °C where three thermogenerative compounds were detected. The highest total flavonoid content TFC (3.96 ± 0.19 QE µmol/g) and antioxidant activity (57.13 ± 8.81% for DPPH and 58.14 ± 7.91% for ABTS) for air frying was observed for the extracts at 180 °C for 15 and 20 min. For enzymatic oxidation, sinapine and SA were the major sinapates in untreated (UT) and alkaline treated (AT) mustard meal, which decreased significantly ($p<0.05$) with prolonged incubation time and generated some unknown end products. The maximum total phenolic content, TFC and antioxidant potential of both HRP and tyrosinase were evaluated at 1 and 2 h of incubation for UT and AT meal with HRP and tyrosinase, respectively. The results of this study found SA and sinapine were efficiently converted to enzymatic and thermogenerative end products that have high antioxidant activity. Enzymatic oxidation proved to be a better extraction technique for the extraction of polyphenolic

compounds as the extracts possess higher antioxidant activity than the air fried extracts (an increase of 28- 30% for DPPH and 20- 22% for ABTS assay).

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

1. INTRODUCTION

Mustard has been an important crop worldwide, belonging to the *Brassicae* family. Different varieties of mustard seeds are cultivated and used as sources of edible oil, spice, and medicine since ancient times (Ildiko et al., 2006). Canada is among one of the largest producers of mustard seeds with 160,000 tonnes of annual input. Yellow, brown and oriental mustard seed varieties are grown in Canada with 57%, 22% and 16% of the production area, respectively. In 2018-2019 Canada exported 112, 000 tonnes of mustard seeds (www.agcanada.com). According to the Canadian Grain Commission (CGC), annual production of mustard in 2019 in Manitoba, Saskatchewan, Alberta, and Western Canada was 0.8, 106.2, 27.6 and 134.6 thousand tonnes respectively. According to the CGC, weed seeds and foreign materials are a major factor in lowering the grade of yellow mustard seeds (YMS). Yellow mustard (*Sinapis alba*) and its by-products e.g. lower grade varieties, bran, hulls, etc can be used as a source of a wide range of bioactive compounds, which include isothiocyanates, phenolics, dithiolthiones and dietary fiber (Hendrix et al., 2012). Waste obtained from the oilseed compress (rapeseed, mustard, canola) is also rich in proteins and fiber (Zago et al., 2015).

Endogenous phenolic compounds of plants and oilseeds have received positive attention over the last twenty years. This is mainly due to their role as antioxidants in human health. Canola and rapeseed have bioactive phenolic compounds that exhibit therapeutic potential and can be efficiently incorporated into various food products as natural antioxidants resulting in improving the nutritional value of the food products. Various bioactive molecules in the oilseeds such as phenolics and glucosinolates are retained in the by-products (waste) of oilseed processing like press cakes, hulls, and defatted meal. Sinapic acid (SA) in its free and esterified form is present in

high concentration in press cake and protein of mustard. Many recent in-vitro assays have reported SA and 4-vinylsyringol as the major antioxidants in various food products (Mayengbam et al., 2014). Canolol, also known as 4-vinyl syringol is a strong antioxidant and a decarboxylated product of SA (Niu et al., 2013). Due to low polarity, the antioxidant activity of canolol is 15% more than that of SA. Owing to the abundance and respective bioactive attributes, researchers have suggested the use of phenolics from the rapeseed and mustard in the development of functional food products (Vuorela, 2005, Vuorela et al., 2004). The use of both waste and by-products is addressed by the re-use of these products (waste of oilseed processing). The value addition perspective of these bioactive molecules from the by-products of mustard can be increased by efficient extraction techniques.

Thermal treatment of rapeseed and mustard before extraction can lead to the efficient release of phenolic molecules. Roasting in the presence of dry air is one of the easiest and simplest methods of heat treatment, manifesting low operational costs. In dry air treatment, by convection, energy from the heat is transferred to the sample and then it is conveyed by thermal conduction internally (Mazaheri et al., 2019). In recent times, air-frying is emerging as an alternative to deep fat frying. Many domestic air-frying equipments are available with all of them providing direct contact between food and hot air. Air-fryer is a kind of countertop convection oven. It consists of a heating element and a fan that transfers heat around food in the cooking chamber with the air inside circulating very quickly in a circular motion. Thus, it does a better job of transferring air through all the surfaces of food when compared to deep oil frying (Arafat, 2014). Moreover, it has temperature and time adjustments and this domestic equipment can be used to roast the mustard seeds and to release the phenolic compounds from their matrixes by the application of hot circulating air. As opposed to the conventional extraction methods, enzymatic extraction methods

are gaining huge popularity, which is due to the disadvantages of conventional methods including environmental hazards, unusable waste produced alongside the extracted bioactive compounds, as well as industrial processes requiring a large number of steps and installations. Cell walls of plants consist of a number of complex structural polysaccharides including cellulose, hemicellulose and pectin, as well as lignin (an aromatic polymer) and even proteins. The stability and resistance to the extraction of the intracellular components depend on this structure. Thus, in order to gain access to the bioactive components from within the cytosolic spaces and even those bound to the cellular walls, enzymes with specific hydrolytic properties are used to rupture this matrix (Garcia, 2018; Panja, 2017).

According to the literature available, mustard seeds are rich sources of endogenous phenolic compounds with antioxidant potential (Mayengbam et al., 2014; Khattab et al., 2010; Thiyam et al., 2006). However, few studies have been done on the extraction of enzymatic end products from mustard seeds and no study was found on using air frying on extraction of polyphenolic compounds from mustard seeds.

This study included the two experimental chapters. The experimental design of this study is included in the Appendix. The main objectives of this study were as follow:

1. To investigate the effect of hot-air treatment (air-frying) and enzymatic oxidation on the SA derivatives of lower grade yellow mustard seeds.
2. To develop an integrated and scalable process for the extraction of sinapic acid derivatives and determine the feasibility of the developed process on large scale.
3. To identify and quantify the phenolic compounds in air fried and enzymatic extracts of LGYMS.

4. In-vitro assessment and comparison of the antioxidant potential of obtained extracts from air frying and enzymatic oxidation of LGYMS.

CHAPTER 2

2 LITERATURE REVIEW

2.1 Mustard seeds

For more than 5000 years, mustard seeds have been used as a condiment in ancient cultures including Romans, Chinese and Egyptians. In international trade, the mustard crop is considered the major spice crop with almost the production of around 529, 900 tonnes per annum (Clancey, 2013). The mustard plant belongs to the *Brassicaceae* family which is also known as the crucifer/cabbage family. The members of this family have four petal yellow flowers and have small edible seeds. Mustard is categorized into three varieties including white/yellow mustard (*Sinapis alba*), oriental/brown mustard (*Brassica juncea*) and black mustard (*Brassica nigra).* The member crops of the *Brassicaceae* family also includes rapeseed, canola, broccoli, turnip, radish etc (Cartea et al., 2010). Mustard seedlings tolerate harsh conditions such as drought, frost, and heat. Mustard seeds are mainly produced under the irrigation system in Canada, but they can also be cultivated under the rain fed systems (Benfey et al., 2005).

Canada is the major producer of mustard seeds all over the world but its production only accounts for 3% of all crop production in Canada. The three varieties of mustard including yellow, oriental, and black account for 57%, 22% and 16% of the total cultivated area respectively (Clancey, 2013). Canada's primary mustard seeds growers are the prairie provinces of Saskatchewan and Alberta. The seeded and production area for the Western Canadian mustard is shown in Table 2.1. It is estimated that 118.2 thousand hectares (more than 75%) of the area belongs to Saskatchewan with an average annual production of 122 thousand tonnes while Alberta accounts for the mean annual production of about 42.4 thousand tonnes (Canadian Grain Commission, 2018).

Table 2.1 Seeded area (thousand hectares) and production (thousand tonnes) area of mustard in Western Canada from 2009-2018.

Region	**Seeded area in 2019 (thousand hectares)**	**Seeded area in 2018 (thousand hectares)**	**Production in 2019 (thousand tonnes)**	**Production in 2018 (thousand tonnes)**	**Average production in 2009-2018 (thousand tonnes)**
Manitoba	0.7	2.0	0.8	2.2	0.2
Saskatchewan	118.2	152.0	106.2	134.7	122.0
Alberta	42.4	49.4	27.6	36.7	42.4
Western Canada	161.1	203.4	134.6	173.6	164.4

Data source: Canadian Grain Commission (2019)

2.2 Processing of Mustard

The production process flow chart for the commercial mustard products is shown in Figure 2.1. Mustard seeds are dried to a suitable moisture range of around 9% and are stored at a temperature of 20 ℃ or lower. To prevent mold growth and enhance the shelf and storage life, drying mustard is an essential step. The drying temperature should not exceed 43 ℃, otherwise, it may cause destruction and damage to the seeds (Benfey et al., 2005; Cui & Eskin, 1998). Mustard seeds are processed into different products before milling and processing which includes mustard flour, ground mustard and mustard bran. Mustard seeds are also used for oil extraction which ranges in their content from 29% -36%. The press cake is another product which is the residue left from the extracted seed kernels. Mustard seed is primarily used as a condiment in European and North American countries. In Asian countries such as India, cooking oil is the main product produced from mustard seeds (Cui & Eskin, 1998).

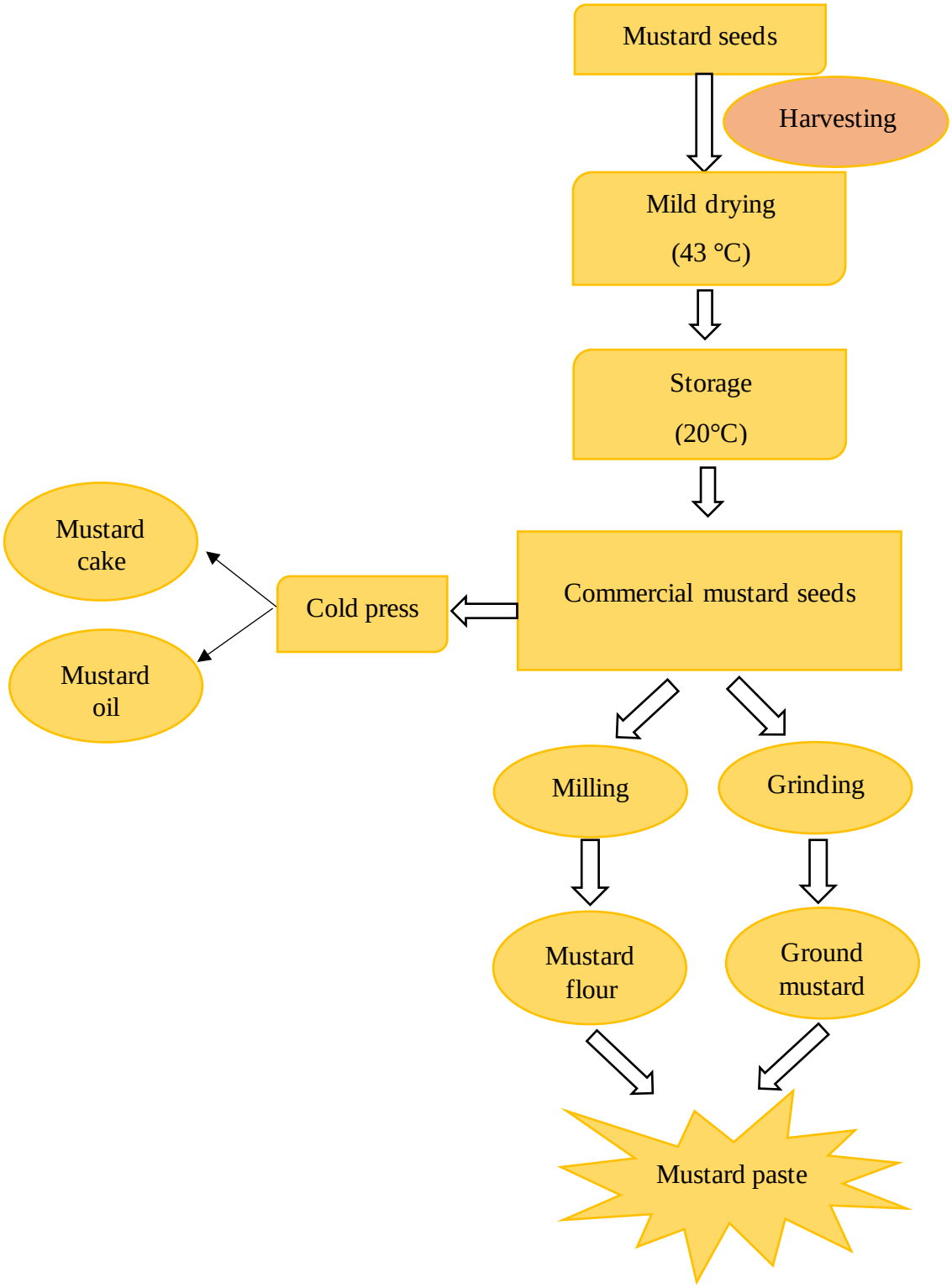


Figure 2. 1 Processing of mustard for commercial products (Adapted from Cui & Eskin, 1998)

2.3 Components of mustard

The chemical composition of the mustard crop differs depending on the variety of mustard, cultivation area and chemical composition. Mustard seeds contain approximately 30% of oil and 25% of very high-quality protein (Diosady et al., 2005; Xu & Diosady, 2002). In traditional mustard varieties, the main fatty components are erucic acid (30% of total oil content) and oleic acid (23% of total oil) (Diosady et al., 2005). In total, 11% of fatty acids in mustard oil are saturated while 89% are unsaturated (Anuradha et al., 2012). Oriental and brown mustard seeds usually contain a higher amount of fixed oil while white/yellow contains higher protein content and lower oil percentage (Cui & Eskin, 1998). The chemical composition of mustard products also differ such as mustard flour contains higher oil and protein concentration (30-42% and 30-35%, respectively). On the other hand, products of mustard such as bran, contain a rich amount of fiber (15%) and low oil percentage (7%) and protein (13-16 %) (Cui & Eskin, 1998). The main bioactive components of mustard that are reported include glucosinolates, phenolic compounds and tocopherols. Mustard is also a rich source of antioxidants such as phenolics and tocopherols which prevent the edible oil from oxidation (Amarowicz et al., 1996). Mustard contains the highest amount of phenolics than other oilseeds. The phenolic components remain in the meal after the pressing treatment. Phenolic compounds are naturally occurring phytochemicals in plants and consist of at least one aromatic ring and hydroxyl substituents. In plants, phenolic compounds play physiological and ecological roles that include controlling growth hormones, and protection against herbivores, insects and pathogens such as fungi and viruses (Cartea et al., 2010). Phenolic compounds are further classified as flavonoids, lignans, tannins and phenolic acids. Phenolic acids are further classified into two categories that include hydroxycinnamic acids and hydroxybenzoic acids. The hydroxycinnamic acids in mustard include caffeic acid, ferulic acid, *p*-coumaric acid

and sinapic acid derivatives. Phenolic compounds exhibit numerous health benefits including anti-inflammatory, anti-bacterial and anti-carcinogenic activity (Cartea et al., 2010). Antioxidant activity is the key function of the dietary phenolics which relates to their redox properties as well as metal chelating and chain-breaking properties (Shahidi et al., 1992). Tocopherol is another rich component found in mustard oil that contributes to the longer shelf life of finished food products when used as an additive in the form of fat containing mustard flour. 1-2% addition of mustard flour to meat helps in improving the slicing and cooking characteristics of the product. The defatted mustard meal is also used to develop edible films, a by-product of the biofuel industry (Cui & Eskin, 1998).

2.4 Mustard allergy

Despite the presence of rich contents of dietary bioactive nutrients, mustard seeds are among the important food allergens in countries where they are highly consumed such as France. Mustard seeds are the fourth most common source of food allergens among young children. Storage proteins of 2S albumin classes known as Sin a 1 and Bra j 1 are principal allergens found in YMS and Oriental Mustard seeds. These proteins are reported to be resistant to digestion by digestive enzymes and are highly heat stable (Monsalve et al., 2001).

2.5 Sinapates in mustard

Brassicae species such as rapeseed, canola (*Brassicae nupus* L.) and mustard (*Brassicae juncea, Sinapis alba)* contain SA derivatives as the characteristic phenolic compounds (Nićiforović & Abramovič, 2014). Sinapic acid (3, 5-dimethoxy-4-hydroxycinnamic acid) is present in both free and esterified form in mustard. Two common sinapoyl ester in oilseeds are sinapine (choline ester of SA) and sinapoyl glucose (sugar ester of SA). 4-vinylsyringol commonly known as canolol is

also a derivative of SA which is produced by decarboxylation of SA under high temperature (Nićiforović & Abramovič, 2014).

According to Khattab et al. (2010), 70-80% of total phenolics in canola seeds and canola press cake comprise sinapine with SA reported to be present in trace amounts. Sinapine and SA in rapeseed cake were present at 55-70% and 66-74% levels, respectively (Thiyam et al., 2014). SA represented about 73% of free phenolics and 80% of total phenolic acids in mustard (Das et al., 2009). This is in agreement with results reported by Thiyam et al. (2006) with sinapine as a major SA derivative in mustard constituting more than 90% of total phenolics in the mustard meal. SA has been reported as a strong antioxidant by many researchers (Nithya & Subramanina, 2017; Nićiforović & Abramovič, 2014). SA has higher antioxidant activity than other hydroxycinnamic acids like ferulic acid and *p*-coumaric acid, but lower than caffeic acid in the following order: caffeic acid > SA > ferulic acid > *p*- coumaric acid (Jin et al., 2010).

2.6 Application of heat (preheat treatment) before phenolic extraction

The application of thermal and mechanical treatment is necessary for the phenolic extraction from various oilseeds to initiate the rupture of cells and enhance the efficient extractability of these compounds. Cell membranes and cell walls are disrupted by thermal processing which results in the cleavage of the covalently bound phenolics and their efficient release from bound forms. Thermal treatment also exhibits its importance in terms of the nutritional value of roasted seeds (Terpinc et al., 2006). Results from previous work have shown that the oxidative stability of oils can be possibly increased by roasting before the oil pressing process (Różańska et al., 2019). Roasting rapeseed may increase the decarboxylation of sinapic acid for the formation of canolol. Canolol is a powerful and highly effective DPPH radical scavenger that can prevent the oxidative degradation of lipids and proteins. Sinapine was found to be the major phenolic in rapeseed meal

and free SA was quantified only in trace amounts (Vuorela, 2005). The contents of SA derivatives in rapeseed meal differ significantly (6-18 mg/g) due to factors such as the process of oil extraction and genetics of the rapeseeds. Among rapeseed and mustard antioxidants, sinapine is the major contributor to the attenuation of oxidation of liposome low-density lipid particles (Shahidi et al., 1992). Oxidation of low-density lipoprotein (LDL) particles by 90% were reported for rapeseed phenolics (Vuorela et al., 2004). The highest canolol content (707.69 µg/g) was achieved after roasting rapeseeds at 180°C for 10 min followed by petroleum ether extraction (Shrestha & De Meulenaer, 2014).

2.6.1 Microwave pre-treatment

Previous works indicated that microwave pre-treatment results in the release of non- extractable phenolics from the canola meal (Khattab et al., 2014; Yang et al., 2014). By applying microwave with superheated steam, 19% more sinapic acid was formed from conversion of the initial sinapine content in the meal. Moreover, microwave roasting resulted in an 80% increase in canolol with the same process at 180°C using superheated steam. Time of exposure during thermal treatment was found to be the most important factor in canolol formation (Zago et al., 2015). Microwave treatment (2450 MHz, from 1-8 min) with 1 min interval time was performed and 0.89 mg/g of canolol was obtained in 7 min, while 8 min of treatment time resulted in the decrease of canolol content to 0.83 mg/g (Yang et al., 2014). In addition to the power of the oven and intensity, exposure time is the major and determining factors in the production of canolol since this compound is sensitive to high temperatures (Khattab et al., 2014; Yang et al., 2014; Niu et al., 2013).

Another study conducted by Rękas et al. (2017) investigated the effect of microwave pretreatment of rapeseed using different time and temperature range. Canolol formation was the most

pronounced effect with a 63-fold increase (1807.63 ± 0.352 µg/g) after 10 min of exposure to microwave treatment as compared to the control (28.66 ± 0.062 µg/g). The hydrolyzed extract of canola meal phenolics was subjected to the canolol formation by microwave-induced decarboxylation. HPLC identification showed that via alkaline hydrolysis of canola meal and methanolic extracts of canola meal, 81% and 94.8% of total phenolics were converted to sinapic acid, respectively. Using microwave (360 W for 12 min) resulted in the highest (58.3%) conversion of sinapic acid to canolol, yielding 4.2 mg/g of canola meal (Khattab et al., 2014).

2.6.2 Preheat treatment using the conventional oven

By increasing the temperature and roasting time, canolol content was increased significantly. The highest canolol content was recorded with the roasting of rapeseed at 180 °C for 15 min which was reported to be 768.26 µg/g. It showed 90 times increase than the control sample. Authors also concluded that roasting before the cold pressing method of oil extraction results in a higher content of bioactive compounds. A given value of canolol can be achieved in two ways, either by increasing the roasting temperature or by increasing the length of exposure time at low temperatures. Canolol concentration was found to be similar to seeds roasted at 140°C for 15 min (132.28 ± 0.65 µg/g) and roasted seeds at 160 °C for 5 min (147.23 ± 0.85 µg/g) (Siger et al., 2016).

According to Thiyam et al. (2014), at temperatures up to 126 °C, the SA content in defatted canola meal increased with an increase in the toasting time but above 180 °C, it started to decrease suddenly. An increase in the phenolics in oil and reduction in total phenolics of defatted meal suggested that during roasting at high temperature, a significant amount of phenolics is converted to canolol and these are easily soluble in oil. As canolol is less hydrophobic, it is easily soluble in oil and this can be the reason for the increase in the total phenolic content of oil during toasting.

2.7 Extraction of phenolic compounds

Extraction of bioactive compounds can be defined as a procedure that separates, recover and purify the plant materials thus making them useful in a wide range of applications. The conventional extraction methods for plant phenolics are based on extracting the power of different solvents and the application of heat/mixing. Some classical conventional techniques for phenolic extraction from plants include Soxhlet, maceration, and hydrodistillation. But these techniques have some major drawbacks such as longer extraction time, costly and high purity solvents, low extract selectivity and thermal decomposition of heat sensitive compounds. These problems led to the development of new and improved techniques of phenolic extraction (Azmir et al., 2013; de Castro & Priego-Capote, 2010). Thus, the use of novel and improved extraction technologies has been promoted such as Accelerated Solvent Extraction (ASE), Supercritical Fluid Extraction (SFE), Enzyme Assisted Extraction (EAE), and Ultrasound Assisted Extraction (UAE). The major objective of these technologies is to reduce energy consumption and extraction time, which helps in lowering the final cost. Moreover, these extraction methods are also sustainable as they protect the environment and consumer health as well as enhance the economic and innovative competitiveness of industries (Medina-Torres et al., 2017).

2.7.1 Supercritical fluid extraction (SCFE)

SCFE extraction technique is one of the novel techniques that has successfully proven itself in the pharmaceutical, polymer and food industry. Supercritical fluid possesses several gases like properties (diffusion, viscosity and surface tension) and liquid like properties (density) which are responsible for extracting the phenolic compounds with higher yields in a shorter time period (Ahmad et al., 2019). Much research data is available on using SCFE with CO_2 for the extraction of plant phenolics. Santos et al. (2012) reported that the best results of phenolic extraction from

Eucalyptus bark were obtained by using ethanol as a solvent and much higher quantities of phenolics than conventional solid/liquid extraction methods. This strategy (SCFE) can be used to achieve increased extraction efficiency that cannot be achieved with conventional techniques (Shahid & Yusuf, 2015).

2.7.2 High voltage electric discharge (HVED) technology

HVED technology is an emerging technology that has been used by different researchers for the recovery of phenolics and proteins. It is based on the process of electric break down in the water. HVED technology is tested for its applicability in obtaining high added-value compounds (proteins, polyphenols, and isothiocyanates) from rapeseed and rapeseed press cake. The energy inputs of 80 KJ/Kg and 240 KJ/Kg were found to be optimal for the extraction of phenolics from rapeseed and rapeseed press cake respectively (Barba et al., 2015).

2.7.3 Accelerated Solvent Extraction (ASE)

This method is also known by a variety of names such as pressurized fluid extraction (PFE), enhanced solvent extraction (ESE) and high pressure solvent extraction (HPSE). Elevated temperature and pressure are used to extract the phenolic compounds from solid and semi solid samples. Pressure helps in increasing the contact between extractant and sample while temperature aids in breaking the analyte matrix bonds to release phenolic compounds (Shahid &Yusuf, 2015). According to a recent study by Nandasiri et al. (2019), the highest yield of SA derivatives was obtained from canola meal with ASE by using 70% methanol (20.72 ± 1.47 mg SAE/g DM) and 70% ethanol (24.71 ± 2.77 mg SAE/g DM). This method proved to be a useful method for the extraction of phenolics from underutilized agricultural by-products.

2.7.4 Ultrasound assisted extraction (UAE)

This method has been used for the extraction of phenolic compounds in food applications. UAE is the cheapest method and has the lowest instrumental requirement than other novel methods of extraction such as EAE, MAE, and SCFE. The passage of ultrasound ultrasonic waves into the solvent introduces the cavitation phenomenon which contributes towards the enhanced extraction efficiency of phenolics. Moreover, as this technique reduces the working time and increases the quality of the obtained extracts, it is recognized as an efficient extraction technique. Khattab et al. (2010) has reported this method as an efficient method of extracting phenolics from plants.

2.7.5 Enzyme assisted extraction (EAE)

There are some inherent drawbacks of solvent extraction e.g. safety hazards, high energy input, environmental risks and toxicological effects that require alternative methods. EAE is one of these alternative methods as they have an inherent ability to catalyze a reaction with exquisite specificity and their ability to function properly under mild processing conditions (Shahid & Yusuf, 2015). EAE for bioactive compounds has been studied as a green technology. Enzymes such as cellulase, pectinases, xylanase, and proteases have been used that aid in the disruption of plant cell walls and improve the extraction yield of phenolic compounds (Heemann et al., 2019).

Enzymes are biocatalysts that increase the rate of biochemical reactions in living organisms. They are widely used for catalyzing many commercially important processes. They play a vital role in several processes including the production of sweetening agents, moderation of antibiotics, washing powders, and analytical assays in laboratories that conduct experiments on clinical, forensic, and environmental applications. When we talk about enzymes as catalysts, they require very low concentrations for speeding up the reactions and are unconsumed during the reaction. Enzymes are capable to catalyze the substrate molecules into the product molecule. Enzymes are

also specific catalysts i.e, they generally catalyze a specific substrate molecule into end products. Group specificity is also demonstrated by some enzymes e.g., alkaline phosphatase is an enzyme that is widely used for enzymatic assays in the laboratory and can remove the phosphate group from different substrates. However, some enzymes show high specificity in terms of the substrate. For example, glucose oxidase only catalyzes the reaction with a specific substrate, β-D-glucose and it does not react with other monosaccharides. This specificity of an enzyme is of utmost importance in the analytical assays where there is a requirement of measurement for a specific substrate in a complicated mixture (Robinson, 2015).

2.7.5.1 Classification of enzymes

Enzymes usually have names that are related to the type of reaction they undergo with a specific substrate. The name of the enzyme usually ends with the suffix -ase (e.g., peroxidase, carboxylase, dehydrogenase). However, some proteolytic enzymes have the suffix - in their names including trypsin, chymotrypsin. There are also some names for the enzymes that do not provide much information about the substrate or the end products such as invertase and diastase. Due to these uncertainties in the trivial names of enzymes, the International Union of Biochemistry systematically addressed this issue. The enzymes are described by four part enzyme commission (EC) number within this system. The EC number consists of four parts. The first part relates to the reaction that is catalyzed by the enzyme. The remaining three digits have other meaning which relates to the reaction identified by the first digit. For example, lactase dehydrogenase has an EC number 1.1.1.27. It is an oxidoreductase (indicated by the first digit with the hydrogen donor (which is lactate molecule of alcohol group and a hydrogen acceptor which is NAD+ (third digit), and the 27th enzyme categorized within the group (fourth digit) (Robinson, 2015).

2.7.5.2 Structure and substrate binding

Enzymes are globular proteins that have consist of 100-2000 amino acids as building blocks. The specific three dimensional enzymatic structure is formed by the arrangement of these amino acids into one or more polypeptide chains. This three dimensional structure has a tiny area which is known as an active site where the substrate bind. The general reaction of enzyme with its substrate is demonstrated in Figure (2.2).

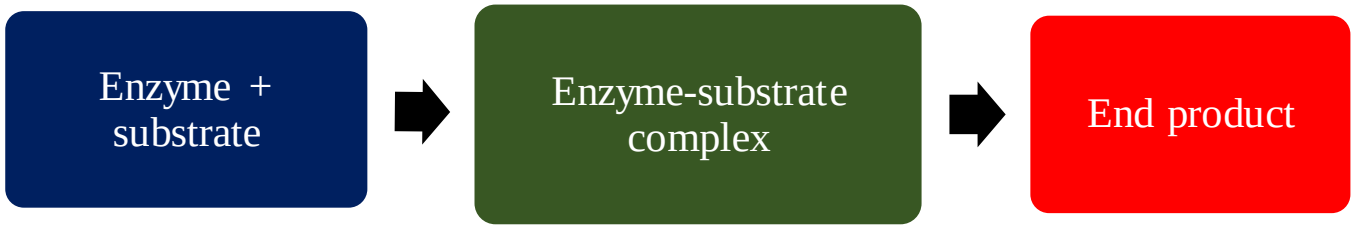


Figure 2. 2 General principle of enzyme activity on the substrate

Adapted from https://www.slideshare.net/mncwish/enzymes-press

To demonstrate the specificity of the enzyme in its catalysis, the shape and charge properties of the active site plays a key role in binding the substrate molecule. Some enzymes also consist of a non-protein part, which is known as a co-factor and it is necessary for the proper catalytic activity (Robinson, 2015).

2.7.5.3 Enzyme kinetics

The factors with which the speed of enzyme catalyzed reactions is measured is termed enzyme kinetics. The speed of enzymatic reaction rates is performed in both a continuous or discontinuous fashion. In the discontinuous method, substrate and enzyme are mixed and the product is measured after a set time period. This method is easy and quick to perform. The discontinuous method is usually used while performing some preliminary experiments (when little is known about the system) or when we are sure that the appropriate time interval has been chosen (when maximum information is available about the system). In the case of continuous enzymatic assays, we usually study the reaction rate of the enzyme by mixing the enzyme and substrate and the end product is continuously measured over time. For enzymatic kinetics, a substrate solution is mixed with enzymes and this reaction mixture is scanned via a spectrophotometer. The color produced is measured, which enables the reaction rate monitoring that can slow down after a few seconds/minutes. The reduction in reaction rate can be described as the substrate being fully consumed by the enzyme and thus become unavailable in the reaction mixtures. In another way, it is also possible that the enzyme is destabilized or denatured during the experiment. There can be other reasons for slowing down of reaction rate. So, the rate of reaction of an enzyme is specified before using it in the main experiment (Robinson, 2015).

There is mostly a simple direct relationship between the concentration of enzyme and reaction rate. For example, if 10% more enzyme is added to a control reaction, the rate of reaction will be 10 times faster as compared with the control. So, there is a linear relationship between enzymatic concentration and reaction rate. On the other hand, when a series of enzymatic assays are performed with different substrate concentration and uniform enzyme concentration, the relationship becomes a bit more complex. Initially, as the substrate concentration increases, the

rate of reaction also increases substantively. However, a point reaches where the increase in substrate concentration results in a decrease in the reaction rate until a stage reaches where there is a little effect on the reaction rate. This is the point where the enzyme comes close to saturation with the substrate and it exhibits the maximum velocity (V_{max}). There are some detailed mathematical steps to generate the Michaelis-Menten equation to check the reaction rate of specific enzymes. The main equation for calculating the reaction rate is as follow:

Initial reaction rate (V_o) = V_{max} * Substrate concentration/ Substrate concentration + K_m

K_m is the value that explains the concentration of substrate at which half the maximum velocity is achieved.

It is worth noting that an enzyme derived from different sources and can catalyze the same reaction, can have a different K_m value. Furthermore, the K_m value can be quite different for the enzyme with multiple substrates. A low K_m value demonstrates that for saturation, the enzyme requires a small substrate amount. Therefore, the maximum velocity is achieved at a low substrate concentration. On the other hand, high K_m values show that maximum velocity is achieved at relatively high substrate concentration. Thus, K_m is generally referred to as measuring the affinity of the enzyme for its specific substrate. Most of the times it is not possible to calculate K_m values from the plot against velocity and substrate concentration as we do not use a high enough concentration of the substrate to come close for the estimation of V_{max} and it makes it difficult to evaluate the K_m. To overcome this situation, we can plot the data in another way to obtain these values. The Lineweaver-Burk plot is the most used method. This plot produces a line that is easy to familiarize with and makes it easy to calculate the V_{max} and K_m. Some other kinetic plots are also used that include the Eadie-Hofstee plot, the Eisenthal-Cornish- Bowdey plots. Lineweaver-

Burk plot is somehow widely used in determining the kinetics of enzyme-catalyzed reactions (Robinson, 2015).

2.7.5.4 Factors that influence enzyme activity

Temperature and pH are the most crucial factors that affect enzyme activity. The rate of enzymatic reaction increases as the temperature increases but up to a certain point where enzymes are denatured. This temperature is termed optimum temperature and differs from one enzyme to another (Robinson, 2015). The optimal temperature is more evident above 40 °C. On the other hand, if the temperature is lower than the optimum temperature, enzyme activity gradually reduces until it reduces to a point where enzymatic activity stops. According to Robinson (2015) at 0 °C, the activity of most enzymes stops but a temperature increase can reactivate the enzyme. In the case of thermal stability, enzymes derived from microbes have much higher stability than those from mammals. Some enzymes e.g., thermolysin (a protease derived from *Bacillus thermoproteolyticus*) and Taq-polymerase (a polymerase from *Thermus aquanticus*) are stable up to 70 °C and can remain active even at 100 °C.

Enzymes are proteins composed of amino acids with acidic carboxyl groups (COOH-) and basic amino groups (NH_2) and are significantly affected by pH. There is an optimum pH for the maximum rate of an enzymatic reaction above and below the optimum pH. Several factors determine the pH profile of a particular enzyme. The rate of enzymatic reaction at the active site is greatly influenced by the ionization groups present, though it could be reversible. For example, if the enzyme with an optimal pH of 7 is placed in the reaction environment with pH 8 or pH 6, it is highly possible that binding and reaction is altered. However, the maximal reaction rate of the enzyme is restored when pH is readjusted to 7. On the other hand, if there is a drastic change in

pH e.g., it is lowered to 1 or is increased to 14, these adverse acidic and alkaline conditions will result in irreversible changes leading to the complete denaturation of the enzyme.

2.7.5.5 Enzymatic oxidation

One of the plant's defense mechanisms against herbivores includes the oxidation of plant phenolic compounds (Appel, 1993; Salminen, 2014). When oxidation of plant phenolics takes place, reactive quinones are produced, which covalently binds to the proteins and produces colored melanins. The oxidation of phenolics by enzymes has been extensively studied to produce enzymatic end products which might be further converted to produce compounds that can have higher antioxidant potential than the original compounds (Cao et al., 2019). There are various methods reported to measure the oxidative activity of phenolics. One includes the electron paramagnetic resonance (EPR) spectroscopy where the production and decay rate of primary products of one electron oxidation of phenolics can be measured (Barbehenn et al., 2006), or the two electron oxidation end products, which include the yellowish and brownish quinones that can be measured by UV-Visible spectroscopy (Barbehenn et al., 2006; Moilanen & Salminen, 2008). Another strategy that can be used for measuring the enzymatic oxidation is the measurement of the decrease in the phenolic compounds as the oxidation takes place (Salminen & Karonen, 2011; Vihakas et al., 2014). In all the processes mentioned above, the oxidative potential of the phenolic compounds can be defined as their potential to participate in the redox reaction in such a way that quinones are formed. Enzymatic oxidation is initiated by oxidizing enzymes such as polyphenol oxidase (PPO), peroxidase and laccase.

PPOs (polyphenol oxidases) are oxidative enzymes that are widely studied in the literature. PPOs consist of copper in their active site and vary in size and substrate specifications (Yoruk & Marshall, 2003). PPOs can oxidize *o*-dihydroxysubstituted phenolics to *o*-quinones. Some of the

PPOs cause the oxidation of monohydroxysubstituted phenols to *o*-phenols, which immediately oxidizes to the *o*-quinones. Tyrosinase also falls under the PPOs that catalyze the oxidation of monophenols and diphenols without any additional co-factors. Tyrosinases are type-3 (dinuclear) copper proteins that are widely distributed in bacteria and eukaryotes and are primarily involved in the mechanism of protection against UV radiations by participating in the initial stages of melanin biosynthesis and other important functions.

Another category of oxidative enzymes are peroxidases that contain the heme group and act as electron donors. Peroxidase reduces hydrogen peroxide and oxidizes a wide range of compounds that include phenolics, aromatic amines and fatty acids (Azevedo et al., 2003; Dunford, 1999). Horseradish peroxidase (HRP) is an extensively studied enzyme due to its high reaction activity, simple detection of end products and stability of the immobilized applications (Vojinović et al., 2007). Oxidation of peroxidases is a complex procedure as it includes two substrates, i.e hydrogen peroxide and phenol. This process involves the oxidation of two electrons of HRP to form the oxidized end products and a molecule of water.

2.7.5.6 Extraction of polyphenols using enzymes

Cell walls of plants comprise complex polysaccharides such as hemicellulose, cellulose, pectin, and proteins. These are the components that resist extraction of the intracellular components. Enzymes can be used to increase the extraction efficiency of bioactive compounds. EAE is also combined with other chemical substances and instrumentations to increase the extraction yield and efficiency of polyphenols. These treatments can be applied before, after or during the EAE. The most used techniques include ionic liquids, ultrasound, microwave, supercritical fluids and accelerated solvents. The common features of these techniques include shortened extraction time period, usage of non-toxic and recyclable solvents, simplified experimental procedures and steps.

Thus, when these instrumentations are combined with EAE, a better contact is established with enzymes and substrates and cell wall disruption is increased which in turn is effective in the maximum release of polyphenols (Azwanida, 2015; Barba, et al., 2014; Barba et al., 2016; Brusotti et al., 2014; Flórez-Fernándezet al., 2017; Joana Gil-Chávez et al., 2013). Enzymatic hydrolysis can also be used in combination with these processes, which can provide solvents with better access to the bioactive molecules present in the sample matrix (Gligor et al., 2019). The enzymatic treatment with cellulase, pectinase and proteases increased the yield of the phenolic compound and antioxidant activities in the pomegranate peel (Mushtaq et al., 2015). Miron & Herrero. (2013) also reported an increase in the antioxidant activity of phenolic extracts in lemon balm when treated with an enzymatic mixture containing cellulase, β-xylanase and pectinase in combination with pressurized liquid.

Odinot et al. (2017) established a process for the generation of canolol via enzymes by using free sinapic acid from rapeseed meal. Rapeseed meal is a rich source of phenolic compounds accounting from 5-18 mg/kg. Major phenolics present in rapeseed meal are SA derivatives like mustard with sinapine as the main phenolic compound (Vuorela et al., 2004; Morley et al., 2013). The enzyme from fungi *Aspergillus niger* naming feruloyl esterase type-A (AnFaeA) was used in the studies which allowed the release of free sinapic acid from sinapine. Sinapine was completely (100%) hydrolyzed by AnFaeA and free SA (6.6 to 7.4 mg/g of raw material) was recovered. This free SA was treated with a strain of fungi named *Neolentinus lepideus* to initiate the bioconversion of free SA into canolol. This study resulted in the formation of about 1.3 g/L of canolol. Other phenolic compounds, e.g. kaempferol derivatives and SA esters were also detected in the enzymatic mixture of rapeseed meal. Another study done by Laguna et al. (2019) used different carboxylic ester hydrolases from the *Aspergillus niger* for the release of phenolic compounds from

sunflower meal. AnFaeB was used on the industrial and nonindustrial meals of sunflower meal. Industrial meal (IM) was processed by the method that included heat treatment at 95-100 °C and desolventizing with steam at 105-107 °C. On the other hand, nonindustrial meal (NIM) was obtained from a pilot scale with the mild heat treatment. AnFaeB treated enzymatic extract of the sunflower meal resulted in the release of canolol. The highest yield of canolol obtained in IM was around 59.8 ± 2.1 µmol/g of defatted dry matter (DDM) after 3 h of incubation and it corresponded to 100% hydrolysis yield. In the case of sunflower NIM, the highest amount of canolol (52.3 ± 0.2 µmol/g DDM) was generated after 30 min of incubation time when treated with AnFaeB. Morley et al. (2013) studied canolol production from the canola meal when treated with laboratory evolved enzyme termed sinapic acid decarboxylase (SAD). SAD was able to generate 3 mg/g canolol in the canola meal with a 97% purity level.

Overall, the use of enzymes for phenolic extraction offers many advantages such as mild temperature treatments (rarely exceeds 50°C), complete exploitation of plant materials, the substrate specificity, which enables the extraction of bioactive compounds that are otherwise cannot be extracted, higher oxidation yields and enhanced bioavailability (Gligor et al., 2019). Although EAE has advantages, it has a limitation that includes high cost and expenditure when used. Thus, further optimizations are required to use this process on a large scale.

2.8 Effect of solvent in the extraction of phenolics

The extraction efficiency of phenolics is highly affected by the type of solvents used in the extraction procedure. Commonly used solvents are polar such as methanol, ethanol, acetone, and ethyl acetate as well as the mixture of these solvents in appropriate ratios. Organic solvents in their aqueous form are reported to be more effective in extracting phenolics when compared to anhydrous solvents. The use of aqueous solvents comes with many benefits as they provide cleaner

extracts that are rich in phenolic compounds with less interfering compounds. The efficiency of phenolic extraction from plant materials is determined by the difference in the polarity of these solvents (Liang et al., 2018). Nandasiri et al. (2019) reported that 70% methanol and 70% ethanol resulted in the best extraction yield of phenolic compounds from canola meal in ASE.

2.9 Identification and quantification

Several methods are used for the isolation and quantification of SA derivatives including High Performance Liquid Chromatography (HPLC), Ultra Performance Liquid Chromatography and Nuclear Magnetic Resonance (Shahid & Yusuf, 2015). The most commonly used method of identification found in the literature is HPLC with a diode array detector using a reversed phase C18 column. Researches have shown that during performing HPLC runs, extracts are filtered and injected onto the column. UV (ultraviolet) absorbance of SA derivatives is measured at 270-330 nm and quantification is done at 270 nm for canolol and 320-330 nm for sinapine and SA and then it is compared with the standard calibration curve to get the final results of HPLC analysis (Thiyam et al., 2006; Khattab et al., 2010).

2.10 Antioxidant potential of phenolic compounds

2.10.1 Lipid oxidation

Lipid oxidation also known as lipid peroxidation is the main risk factor of deterioration of food quality and harms the biological system. During harvesting, processing and storage, oxidation of food may take place. The oxidation of foods results in the onset of off odors and off flavor compounds, deprivation of essential fatty acids and other bioactive compounds, and development of toxic compounds (Zhong, 2010; Shahidi, 2000). Oxidation leads to a decrease in the nutritional potential and shelf life of foods as well as changes in the texture and color (Albishi et al., 2013;

Alamed et al., 2009). Oxidation of food products has been reported to cause harmful effects in biological systems and may cause several harmful diseases and health effects such as inflammatory cancer and ageing including others (Kruidenier & Verspayet, 2002; Floyd & Hensley, 2002; Davies, 2000; Dalton et al., 1999). As lipid oxidation directly relates to the nutritional and sensory attributes of food products, it is well studied. As lipids are composed of unsaturated fatty acids, they are susceptible to oxidation. Lipid oxidation is a difficult and complex process that can be further comprised of thermal oxidation, autooxidation, or enzymatic oxidation (Shahidi, 2000; Vercellotti et al., 1992). The oxidation process is initiated when unsaturated fatty acids lose a hydrogen atom and form a free radical and the reaction is further sped up by other factors including light, heat, and enzymes. These generated free radicals react with the oxygen which further generates the peroxyl radicals. These peroxy radicals attack the new lipid molecules as the chain carriers of the progressing reaction.

2.10.2 Antioxidant mechanism of phenolic compounds

Antioxidants are bioactive compounds that reduce or stop the rate of lipid oxidation or other reactive molecules by slowing down or inhibiting the onset of oxidation chain reactions (Sang et al., 2002; Velioglu et al., 1998). Food manufacturers have been using antioxidants globally for stabilizing lipid molecules in foods. Antioxidants play a variety of roles when added to foods, including rancidity reduction, inhibiting formation or activity of toxic free radicals, maintaining nutritional quality, and extending shelf life (Sang et al., 2002; Jadhav et al., 1995). Based on their mode of action, antioxidants are classified into primary antioxidants and secondary antioxidants. Primary antioxidants comprise molecules that break the chain reaction of oxygen by reacting with scavenging free radical intermediates while secondary antioxidants retard the oxidation process by stopping the initiators that regenerate primary antioxidants. Phenolic compounds can act both as

primary and secondary antioxidants depending on their chemical structure. They can act as a secondary antioxidant because they possess redox properties, which help to neutralize free radicals or decompose and scavenge peroxidases and other reactive oxygen species (Shahidi & Zhong 2007; Sang et al., 2002). The number and arrangements of the hydroxyl groups on the phenolics are responsible for the antioxidant potency (Sang et al., 2002). The processes involved in the antioxidant phenomena of phenolic compounds are shown in Figure (2.6). As an antioxidant, phenolic species donate their hydrogen atom to the lipid radicals, which leads to the formation of antioxidant radicals (known as a reaction 1) that are less prone to oxidation (Kiokias et al., 2008; Shahidi et al., 1992). The free radical of the antioxidant can further react with the propagation reactor in the chain (Reaction 2 and 3). The phenolic compounds trap the two lipid radicals after donating a hydrogen atom to one radical and receive another electron from another radical and form a stable non-radical product (Young & Woodside, 2001).

$L^{\circ}/LO^{\circ}/LOO^{\circ} + AH \longrightarrow A^{\circ} + LH/LOH/LOOH$ ..... **(Reaction 1)**

$LO^{\circ}/LOO^{\circ} + AH^{\circ} \longrightarrow LOA/LOOA$ ..... **(Reaction 2)**

$LOO^{\circ} + LH \longrightarrow LOOH + L^{\circ}$ ..... **(Reaction 3)**

Figure 2. 3 Reaction involved in antioxidant action of phenolic compounds (Adapted from Rehman, 2018)

2.11 Health benefits of sinapic acid (SA) derivatives

2.11.1 Sinapic acid

SA has demonstrated a variety of health promoting effects. Many in-vivo and in-vitro assays have reported its antimicrobial, antiinflammatory, antioxidant, antithrombotic and anticarcinogenic

effects (Chen, 2016; Nićiforović & Abramovič, 2014; Kim et al., 2011). Studies have shown that sinapic acid is a powerful antioxidant that helps in the prevention of cell damage caused by the free radicals which are also known as reactive oxygen species (ROS) (Chen, 2016).

According to a recent study, the antioxidant potential of SA was tested in the development of functional yogurt. Results showed that SA encapsulated in gelatin Arabic gum was released ranging from 6-20 % and 2.5-25 % in the gastric and intestinal fluid system, respectively. In treatments with gelatin cashew gum, the encapsulated material released from 7-16 % and 2.5-7 % SA in the gastric and intestinal fluid system, respectively. This study showed that the encapsulation of SA along with echium oil and phytosterol prevents lipid oxidation due to its efficient release in the intestinal and gastric fluids (Comunian et al., 2017).

According to a study conducted by a group of researchers in Korea in 2011, the neuroprotective effect of SA was examined on global cerebral ischemia. The result of this study indicated that the group of rats injected with SA at concentrations of 1,3 and 10 mg/kg of body weight showed the measurement of viable cells to be 26.9, 49.4 and 226.4 cells/mm^2, respectively. The results showed that SA showed its neuroprotective effects best at 10 mg/kg as it was able to rescue 72.7 % of ischemic neurons (Kim et al., 2015).

An in-vitro study investigated the growth-inhibiting effect of SA on cancer cells. The results of studies showed that SA decreased cell proliferation by 20% with an IC_{50} value of $7x10^{-11}$ M (Kampa et al., 2004). In-vivo studies on mice showed that treatment with SA at a concentration of 30 mg/kg body weight showed a reduction in the edema by 36.9 % at 24 min whereas treatment with 30 mg/kg led to a reduction in the edema by 40.4 % at 3 h. This study showed that SA can reduce active-phase inflammation in animal models.

2.11.2 Canolol

Canolol is reported to be a strong antioxidant, antimutagenic and anticancer molecule. It is reported to have the highest antioxidant activity when compared to other phenolic compounds like tocopherol, β -carotene, rutin and quercetin (Siger et al., 2016). The generation of free radicals in the human body is associated with oxidative stress that leads to chronic diseases. Canolol as a strong antioxidant can prevent oxidative stress. In a recent study, the effect of canolol on hydrogen peroxide (H_2O_2)-induced oxidative stress was determined in atypical glandular cells (AGS) cells. AGS cells were pre-treated with canolol that resulted in a reduction in reactive oxygen species (ROS). This study proved that canolol effectively prevented against H_2O_2 induced oxidative stress as its antioxidant effect was significant (Han et al., 2017).

Cao et al. (2008) reported canolol to be effective against gastric carcinogens in an animal model. This study showed that chronic active gastritis and gastritis carcinogenesis associated with *H. pylori* infection was suppressed by orally injected canolol in a 0.1 % concentration in the diet. A dose of canolol in 200 µM resulted in the reduction of ROS in oxidative stress induced cells in human retinal pigment epithelial cells (Dong et al., 2011).

Kuwahara et al. (2004) investigated the antimutagenic effect of canolol and it was evident from their studies that at concentration> 8 µM, canolol exhibited a potent antimutagenic effect in dose dependent manner. The antimutagenic property of canolol is possibly due to its peroxynitrite scavenging properties.

CHAPTER 3

3. EFFECT OF AIR FRYING AS HEAT PRE-TREATMENT ON THE PHENOLIC COMPOUNDS OF DE-OILED LOWER-GRADE YELLOW MUSTARD SEEDS

3.1 Abstract

Lower grade yellow mustard seeds (LGYMS), a by-product of the mustard milling process have potential as value-added products in food, feed, and agro-allied industries. Roasting in the presence of dry air is one of the easiest and simplest methods of heat treatment manifesting low operational costs. The under-utilized by-products of mustard, in addition to hot air treatment, can be used for the efficient release and extraction of bioactive phenolic compounds. This study investigated the effects of hot air treatment with different extraction temperatures (120, 140, 160 and 180 °C) and time (5, 10, 15 and 20 min) on phenolic compounds in defatted LGYMS. In lower grade yellow mustard meal fraction (LGYMM), sinapine was the major phenolic compound and its concentration decreased significantly ($p<0.05$) with prolonged time. The concentration of majority of the phenolic compounds significantly ($p<0.05$) decreased with increased temperature-time with exception at 180 °C where three thermogenerative compounds were detected. SA was present in trace amounts from 120° C to 160° C and was absent in all the time ranges at 180 °C. The highest antioxidant activity was observed for the extracts at 180 °C for 15 and 20 min. Interestingly, canolol was absent in the meal fractions of LGYMM and suggests the role of an oil-based medium as necessary for canolol formation. This study demonstrated that air frying (preheating) of LGYMM at 180 °C for 15 and 20 min proved to be the optimum condition to obtain the maximum antioxidant potential.

Keywords Lower grade yellow mustard seeds (LGYMS), sinapic acid derivatives, temperature, time, air frying.

3.2 Introduction

Mustard has been an important crop worldwide, belonging to the *Brassicae* family. Different varieties of Mustard seeds are cultivated and used as a source of edible oil, spice, and medicine since ancient times (Ildiko et al., 2006). Canada is among one of the largest producers of mustard seeds with 160,000 tonnes of annual input. Yellow, brown, and oriental mustard seeds are grown in Canada with 57%, 22% and 16% of the production area respectively. In 2018-2019 Canada exported 112, 000 tonnes of mustard seeds (www.agcanada.com). According to the Canadian Grain Commission, the annual production of mustard in 2019 in Manitoba, Saskatchewan, Alberta, and Western Canada was 0.8, 106.2, 27.6 and 134.6 thousand tonnes, respectively. According to the Canadian Grain Commission, weed seeds and foreign materials are a major factor in lowering the grade of yellow mustard seeds (YMS). Yellow mustard (*Sinapis alba*) and its by-products e.g. lower grade varieties, bran, hulls, etc can be used as a source of a wide range of bioactive compounds, which include isothiocyanates, phenolics, dithiolthiones and dietary fiber (Hendrix et al., 2012). Waste obtained from the oilseed compress (rapeseed, mustard, canola) is also rich in proteins and fiber (Zago et al., 2015).

Thermal treatment of rapeseed and mustard seeds before extraction can lead to the efficient release of phenolic molecules. The application of thermal and mechanical treatment is necessary for the phenolic extraction from various oilseeds to initiate the rupture of cells and enhance the efficient extractability of these compounds. Cell membranes and cell walls are disrupted by thermal processing which results in the cleavage of the covalently bound phenolics and their efficient release from bound forms. Thermal treatment also exhibits its importance in terms of the

nutritional value of roasted seeds (Terpinc et al., 2006). Results from the research have shown that the oxidative stability of oils can be possibly increased by roasting before the oil pressing process (Różańska et al., 2019). Roasting in the presence of dry air is one of the easiest and simplest methods of heat treatment manifesting low operational costs. In dry air treatment, by convection, energy from the heat is transferred to the sample and then it is conveyed by thermal conduction internally (Mazaheri et al., 2019). In recent times, air-frying is emerging as an alternative to deep fat frying. Many domestic air-frying equipments are available with all of them providing direct contact between food and hot air. Air-fryer is a kind of countertop convection oven. It consists of a heating element and a fan that transfers heat around food in the cooking chamber. The air in the air fryer is circulated very quickly in a circular motion. Thus, it does a better job of transferring air through all the surfaces of food (Arafat, 2014). Moreover, it has temperature and time adjustments, and this domestic equipment can be used to roast the mustard seeds and release the phenolic compounds from their matrixes by the application of hot circulating air.

Various bioactive molecules in the oilseeds such as phenolics and glucosinolates are retained in the by-products (waste) of oilseed processing like press cakes, hulls, and meal. Brassicae species such as rapeseed, canola (*Brassicae nupus* L.) and mustard (*Brassicae juncea, Sinapis alba)* contain SA derivatives as the characteristic phenolic compounds (Nićiforović & Abramovič, 2014). SA is present in both free and esterified form in mustard. Two common sinapoyl esters in oilseeds are sinapine (choline ester of SA) and sinapoyl glucose (sugar ester of SA). 4-Vinylsyringol commonly known as canolol is also a derivative of SA which is produced by decarboxylation of SA under high temperature (Nićiforović & Abramovič, 2014). Sinapine and SA in rapeseed cake were present in 55-70% and 66-74% respectively (Thiyam et al., 2006). SA represented about 73% of free phenolics and 80% of total phenolic acids in mustard (Das et al.,

2009). This was in agreement with results reported by Thiyam et al. (2006) with sinapine as a major SA derivative in mustard constituting more than 90% of total phenolics in the mustard meal. SA has been reported as a strong antioxidant by many researchers (Nithya & Subramanina., 2017; Nićiforović & Abramovič, 2014). SA has higher antioxidant activity than other hydroxycinnamic acids e.g., ferulic acid and p-coumaric acid, but lower than caffeic acid in the following order: caffeic acid > SA > ferulic acid > p- coumaric acid (Jin et al., 2010).

Results from the studies have shown that the oxidative stability of oils can be possibly increased by roasting before the oil pressing process (Różańska et al., 2019). Roasting of rapeseed may increase the decarboxylation of SA for the formation of canolol. Oxidation of LDL particles by 90% was reported for rapeseed phenolics (Vuorela et al., 2004). Previous works indicated that microwave pre-treatment results in the release of non-extractable phenolics from the canola meal (Khattab et al., 2014; Yang et al., 2014). Authors also concluded that roasting before the cold pressing method of oil extraction results in a higher content of bioactive compounds. Lower grade yellow mustard seeds are important by product of the mustard industry. For an efficient evaluation of the impact of air frying on SA derivatives in lower grade yellow mustard seeds (LGYMS), quantitative and qualitative research data is needed. The main research questions of this study are:

1. What is the effect of air frying on phenolic compounds in lower grade yellow mustard seeds?
2. What is the antioxidant potential of lower grade yellow mustard seeds LGYMM extracts from seeds pretreated with air frying?
3. What are the optimum conditions for the extraction of phenolic compounds from seeds pretreated with air frying?

3.3 Materials and methods

3.3.1 Chemicals and samples

Yellow mustard seeds were supplied by GS Dunn Ltd., Hamilton, Ontario, Canada. Samples were kept and stored at 4 °C using polythene bags until used. Sinapine (98%) and methyl sinapate (98%) were purchased from Chem Faces (Wuhan, Hubei, China). Folin–Ciocalteu's (FC) reagent, acetate, 2,4,6-tris-(2-pyridyl)-s-triazine (TPTZ>98%), and 2,2-diphenyl-1-picrylhydrazyl (DPPH, 97%) were purchased from Fisher Scientific Canada Ltd. (Oakville, ON, Canada). Quercetin hydrate (>95%) and 2-amino-ethyl-diphenyl borate (98%) were purchased from Acros (Mississauga, ON, Canada). The SA (98%), syringic acid (98%), potassium monosulfate (98%), sodium monobasic (99%), sodium dibasic (99%), 2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS>98%), and potassium persulfate (98%) were purchased from MilliporeSigma Canada Co. (Oakville, ON, Canada). Hexane, ethanol and methanol were purchased from Fisher Scientific Canada Ltd. All the chemicals used were of analytical grade.

3.3.2 Sample defatting

LG-YMS were cleaned and grounded for 5sec before extraction. Samples were defatted using hexane in a Soxtec 2050 (Foss-Tecator, Foss North America, Eden Prairie, MN, United States) to obtain lower grade yellow mustard meal (LGYMM). Moisture content of the LGYMM was analyzed using a moisture analyzer for 4 min at 130°C. The moisture content was calculated based on the initial and final weight of each sample.

3.3.3 Roasting of LGYMS using air fryer

Pre-heat treatment of LGYMS was done in an air fryer at four different temperatures (120, 140, 160 and 180 °C) and time ranges (5, 10, 15, 20 min) respectively. Roasted samples were placed in

aluminium dishes and were covered with aluminium foil. Holes were pierced in aluminium foil to have an equal and efficient flow of hot and pressurized air. Aluminium dishes were placed in the basket of the air fryer and individual samples were subjected to air frying at the specified temperatures and time periods.

3.3.4 Extraction of SA derivatives from defatted mustard samples

Phenolics from roasted and defatted LGYMS were extracted using the Ultrasound Assisted Extraction procedure as described by Khattab et al. (2010). One gram of each sample was extracted using 70% methanol in a 1:9 sample: solvent ratio. This was assisted by sonication at 1 min followed by centrifugation at 5000 rpm at 4 ºC for 15 min. The same procedure was repeated three times for each sample. The supernatant from each extraction was combined and filtered with Whatman No. 1 filter paper. The total volume of supernatant collected from each sample was made up to 25 ml using 70 % methanol. All the extractions were carried out in 4 replicates.

3.3.5 Total phenolic content (TPC)

The TPC of extracts was measured using the method explained by Thiyam et al. (2006), with slight modifications. Each extract (50 µl) was mixed with Folin–Ciocalteu's reagent (0.5 ml) in a 10-ml conical flask. The reaction mixture was vortexed for 30 s followed by the addition of 1.0 mL of 19% (w/v) sodium carbonate solution and the total volume made up to 10 ml. The reaction mixture was placed in the dark cabinet for 60 min with intermittent shaking at the 30 min interval. Absorbance of the blue-colored complex formed was measured in a FL 6500 Fluorescence Spectrophotometer (Perkin Elmer Inc., Waltham, MA, USA) at 750 nm. For blank, the sample was replaced with methanol and for calculations, the standard curve of SA was prepared in methanol (100%) at 1 mg/ml concentration (R^2 = 0.9946).

3.3.6 Total flavonoid content (TFC)

The TFC was measured using the method described by Nandasiri et al. (2019) with small modifications. Briefly, the methanolic extract (1.0 ml) was mixed with 3.0 ml of distilled water, followed by the addition of 100 µl of 1% (v/v) diphenylboric acid 2-aminoethyl ester solution. Absorbance of the reaction mixture was measured at 404 nm. Extracts were replaced by methanol for the blank and the standard curve generated using quercetin stock solution (1 mM) in 100% methanol ($R^2 = 0.9972$). The TFC was expressed as micromolar quercetin equivalents per gram of LGYMM (µM QE/g).

3.3.7 Determination of ferric reducing antioxidant power assay (FRAP)

FRAP was measured using the method described by Nandasiri et al. (2019), with slight modifications. The working solution of FRAP was prepared using a mixture of 200.0 mL of acetate buffer (300 mM, pH 3.6), 20 ml of TPTZ solution (10 mM), 20 ml of ferric chloride solution (20 mM), and 24 ml of distilled water. Briefly, 50 µl of methanolic extract of LGYMM was added to 900 µl of water. The mixture was vortexed followed by the addition of FRAP reagent (2.0 ml) and kept in the dark at 37 °C for 30 min. The absorbance of the colored complex was measured at 593 nm. Methanol was used to replace with sample extracts as the blank and the standard curve was prepared using different concentrations of ferrous sulfate stock solution (1 mM) in distilled water ($R^2 = 0.9988$). FRAP was expressed as ferrous sulfate (µmol/g).

3.3.8 Determination of DPPH radical scavenging activity

Antioxidant activity based on free radical–scavenging effect was determined using the DPPH assay according to the method described by Thiyam et al. (2006) with some modifications. In brief,

50 ul of methanolic extract of LGYMM was mixed with 2.95 ml of DPPH solution (0.1 mM, 100% methanol). The absorbance of the blue-colored complex was measured at 516 nm after 10 min.

Radical-scavenging activity was calculated using the following equation:

Scavenging Effect (%) = [A_c (562 nm) − A_s (562 nm)] / A_c (562 nm) × 100

where

A_c = absorbance of control at 562 nm

A_s = absorbance of sample at 562 nm

3.3.9 ABTS radical scavenging assay

The ABTS assay was performed according to the method described by Kosakowska et al. (2018) with slight modifications. The radical cation of ABTS (working solution) was prepared using the mixture of ABTS (7 mM) and potassium persulphate (2.45 mM) in potassium buffer saline (pH 7.4) followed by mixing of both solutions in equal (1/1) volumes. The mixture was allowed to stand for 14-16 h in the dark until the $ABTS^{\circ+}$ radical was generated, and stable absorbance was noted. The $ABTS^{\circ+}$ solution was further diluted with potassium buffered saline to reach an absorbance of 0.7 at 734 nm. For determining the scavenging effect of compounds present in LGYMM, 20 µl of methanolic extract was mixed with 2980 µl of $ABTS^{\circ+}$ solution. Absorbance of the reaction mixture was measured at 0 min and then after 10 min of incubation.

The radical scavenging effect was calculated using the following equation:

Radical scavenging activity (%) = $A_{control} - A_{(t0-t10)} / A_{control} \times 100$

where

$A_{control}$ = absorbance of blank at 734 nm

$A_{(t0-t10)}$ = absorbance of the sample at 0 min subtracted by absorbance of the sample after 10 min

3.3.10 HPLC- DAD identification of SA derivatives

Changes in the phenolic profile of LGYMS at different temperature and time were recorded using the method as described by Harbaum-Piayda et al. (2010) with some modifications. Phenolic compounds were analyzed using reversed phase HPLC Diode Array Detection (HPLC-DAD) (Ultimate 3000, Dionex, Sunnyvale, Torrance, CA, United States). For separation, KinetexR Biphenyl C18 reverse-phase (RP) column (2.6 mm, 150 × 4.6 mm, Phenomenex, Torrance, CA, United States) was used. Other analysis conditions were maintained as follows: flow rate (0.4ml/ min), column oven temperature (30 °C), injection volume (10 µl) and wavelength (270 nm and 330 nm). Solvent A (Milli Q water containing 0.1% formic acid) and solvent B (100% methanol containing 0.1% formic acid) were used as mobile phase for the separation of main sinapates using gradient elution. The gradient elution system operation was as follows; 25% B (0-3 min), 25 to 40% B (3-8 min), 40% B (8-13 min), 40 to 60% B (13-25 min), 60 to 70% B (25-38 min), 70% to 100% B (38- 41 min), 100% B (41-44 min), 100% to 25% B (44- 47 min), 25% B (47- 57 min). SA and its derivatives were quantified on the basis of a standard curve of SA, sinapine, and canolol in appropriate dilutions. Chromatograms were obtained at specific wavelengths and quantified using Chromeleon software Version 7.2 SR4 (Dionex Canada Ltd., Oakville, ON, Canada).

3.4 Statistical analysis

All the experiments were carried out in duplicates. Results are presented as the mean ± standard deviation of the duplicate analysis. Data analysis was carried out using one way analysis of

variance (ANOVA) and multiple mean comparisons were calculated by using Tukey's test with a significance level of 5% ($p < 0.05$). All the data analysis were performed using SPSS statistical software version 22 (IBM, New York, NY, United States).

3.5 Results and discussions

3.5.1 Effect of air frying on the moisture content of LGYMM

The moisture content of LGYMM at various temperature and time combination is shown in Table 3.1. Analysis of the experimental data showed that a decrease in the moisture content was observed as the temperature/ time was increased for air frying. There was a significant decrease observed in the moisture content of LGYMM when heated at 120 °C for 5 min (1.70 ± 0.01%) when compared to the control (6.28 ± 0.00%). The lowest moisture content for LGYMM from air fried seeds was recorded at 180 °C (5-20 min) ranging from 1.70 ± 0.01% to 1.20 ± 0.01%. The drying temperature of 60 °C and 90 °C also decreased the moisture content to 2.72% and 1.148% respectively. Hot air drying can contribute to the decrease in the moisture content of food materials when placed for prolonged exposure to drying temperatures (Kumar et al., 2019). Due to the differential permeability of the cellular membranes, water is removed from the food matrix. A decrease in the moisture content of food product comes with several advantages including a reduction in microbiological activities and long storage life. Dry heat is reported to reduce the content of moisture more efficiently than moist heat treatment. The moisture content of wheat flour was reduced by 47.16% when heated at 100 °C, which is somehow comparable to our study where moisture content of LGYMM prepared from seeds pretreated at 120 °C was reduced by 55% (Sudha et al., 2016). Microwave exposure was also reported to reduce the moisture content in rapeseed meal whereby 58.4% was lost after 7 min (Niu et al., 2013). In our study, the moisture content was reduced by 80% in LGYMM prepared from seeds exposed to 180 °C air frying pre-

treatment. Overall, an increase in the temperature and time resulted in the reduction of moisture content after air frying.

Table 3.1 Effect of air frying pre-treatment of lower grade yellow mustard seeds on moisture content of the defatted meal

Temperature/Time	Moisture content (%)
RT	6.28 ± 0.01^{a}
120 °C for 5 min	2.79 ± 0.01^{b}
120 °C for 10 min	2.70 ± 0.01^{b}
120 °C for 15 min	2.65 ± 0.04^{b}
120 °C for 20 min	2.24 ± 0.05^{b}
140 °C for 5 min	2.53 ± 0.04^{b}
140 °C for 10 min	2.29 ± 0.01^{b}
140 °C for 15 min	2.24 ± 0.01^{b}
140 °C for 20 min	2.10 ± 0.03^{bc}
160 °C for 5 min	2.49 ± 0.02^{b}
160 °C for 10 min	2.36 ± 0.03^{b}
160 °C for 15 min	2.30 ± 0.02^{b}
160 °C for 20 min	2.20 ± 0.05^{b}
180 °C for 5 min	1.70 ± 1.51^{bc}
180 °C for 10 min	1.51 ± 0.01^{bc}
180 °C for 15 min	1.39 ± 0.04^{c}
180 °C for 20 min	1.20 ± 0.03^{c}

All data are expressed as a mean of three replicates with standard deviation (n=3). Values with different superscripts are significantly different ($p \leq 0.05$)

3.5.2 Effect of air frying on major identified phenolics in lower grade yellow mustard meal (LGYMM)

In the LGYMM, four phenolic compounds were identified based on their retention times and UV-spectra when compared to the standard compounds after HPLC separation. As SA derivatives are the characteristic phenolic compounds present in mustard, sinapine, sinapic acid and methyl

sinapate and syringic acid were determined in the methanolic extracts of LGYMM from air fried seeds. HPLC chromatogram of LGYMM of the control sample is shown in Appendix II where the chromatograms of the extracts were acquired at 270 nm and 320 nm. Sinapine, sinapic acid and methyl sinapate were quantified at 320 nm while syringic acid was quantified at 270 nm. The effect of air frying as a heat pre-treatment on the concentration of identified phenolics is shown in Table 2.2. Following the air frying, the amount of sinapine decreased significantly at 120 ℃. There was no significant decrease observed in the sinapine content at 140 ℃ and 160 ℃ as the concentration ranged between 3.93 ± 0.11 to 3.01 ± 0.05 mg/g SAE. At 180 ℃, a maximum decrease (60%) in the concentration of sinapine was observed when compared to the control sample. The reduction in the sinapine content was also reported by Niu et al. (2013), where a 16.7% decrease was observed in rapeseed meal after 7 min of exposure to microwave treatment. Zago et al. (2015) also observed a decrease in the sinapine content (24.2%) of rapeseed meal prepared from seeds subjected to microwave roasting. Our study showed a greater decrease in sinapine content when compared with the reported studies above. The reduction in the sinapine amount could be due to the drastic hot airflow in the air fryer, which is in direct contact with seeds. Liazid et al. (2007) have reported that many phenolic compounds such as benzoic acids and cinnamic acids are chemically stable above 100 ℃ but they are significantly degraded when the temperature is increased above 125 ℃. Another study demonstrated the decrease in the sinapine content in rapeseed meal during the first 2 h of drying, which was accompanied by the loss of moisture during the drying process (Niu et al., 2013). Moreover, the decomposition point of sinapine is reported to be at 127 ℃, so it is also possible that the sinapine in LGYMM decreased significantly when the temperature is increased above 120 ℃. Enhanced temperature above 100 ℃ and time exposure led to the chemical degradation of sinapine during air frying. Sinapine is a choline ester of SA and

an increase in the thermal treatment can result in the breakage of this ester bond which could decrease its concentration. The exact phenomenon responsible for sinapine degradation still needs to be elaborated in future studies.

SA was present in a very low detectable amount in LGYMM (0.14 ± 0.01 mg/g SAE) prepared from seeds not treated with air frying. Mayengbam et al. (2016) have also reported a similar amount of SA (0.19 ± 0.02 mg/g SAE) in mustard seeds. Mustard overall contains a very low content of SA when compared to rapeseed and canola meal. Another study reported a comparatively less amount of SA in white mustard seeds (0.06 mg/g). As the thermal treatment was applied to the seeds, the SA content decreased and it was present in only trace amounts (not quantifiable) in air extracts of seeds air fried at 120 °C to 160 °C as shown in Table 3.2. However, at 180 °C, the complete degradation of SA was noted in the LGYMM extracts. The decrease in the concentration of SA is reported in many studies. SA was degraded by 31% in rapeseed meal during the microwave treatment which suggests its chemical instability during thermal treatment. Mizani et al. (2016) reported a decrease in SA concentration during hot air treatment at 100 °C when compared with control (0.04 mg/g and 0.06 mg/g, respectively). Superheated steam treatment of rapeseed meal for 2 h also resulted in the reduction of SA content by 80% (Zago et al., 2015).

Another interesting phenolic compound detected and identified in LGYMM was syringic acid. Syringic acid is reported to have a therapeutic potential including antioxidant, antimicrobial and anti-inflammatory activities (Srinivasulu et al., 2018). It is a benzoic acid that is found in different vegetables, wine and honey. The highest amount of syringic acid was calculated in the control sample (non-heat treated) with the concentration of 0.59 ± 0.01 mg/g CAE and its content decreased significantly to 0.47 ± 0.03 mg/g CAE when the seeds were exposed to 120 °C for 5 min. There was no significant difference observed in the amount of syringic acid from 120 °C to

160 °C as the concentration remained in the range of 0.45 to 0.43 mg/g CAE. At 180 °C, from 15 to 20 min, a significant decrease of 31.1% was observed when compared to lower temperature ranges. The trend for decreases in syringic acid concentration was comparable with another study where the content in the white mustard seeds was decreased as the heating time increased to 100 °C and maximum degradation was observed at 16.25 min of hot air drying (Mizani et al., 2016). A decreasing pattern for the antioxidant activity was observed for pure syringic acid from 90 °C to 150 °C (Réblová, 2012).

Methyl sinapate is another methyl ester that was identified in the extracts of LGYMM. It is also reported in rapeseed and its concentration depends on the method used for its processing (Quinn et al., 2017). This phenolic is also found in radish sprout and brown mustard. Chung et al. (1997) revealed that methyl sinapate in the methanolic extracts of brown mustard exhibited the best scavenging activity against hydroxyl radicals. The antioxidant activity of methyl sinapate is due to the dehydrogenation of a hydroxyl group present at the top of the ring or by the electron transfer mechanism (Gaspar et al., 2008). Methyl sinapate is also being used in making sunscreen as it has good power of absorbing UV-visible radiations and it is reported to have a similar photoprotection ability as a natural sunscreen sinapoyl malate (Zhao et al., 2019; Luo et al., 2017). According to our knowledge, there is no study available on the effect of high temperature treatment on methyl sinapate. In our study, the increase in temperature resulted in a significant decrease of methyl sinapate at 120 °C for 5 min (0.07 ± 0.01 mg/g SAE) when compared to the control sample (0.11 ± 0.01 mg/g SAE). After 120 °C for 5 min, the concentration of methyl sinapate in LGYMM remained constant throughout the heat treatments.

Table 3.2 Effect of air frying on major phenolics identified defatted meals prepared from air fried lower grade yellow mustard seeds

Temperature/Time	Sinapine (mg/g SAE)	Methyl sinapate (mg/g SAE)	Sinapic acid (mg/g SAE)	Syringic acid (mg/g CAE)
RT	5.88 ± 0.05^{a}	0.11 ± 0.00^{a}	0.14 ± 0.00	0.59 ± 0.01^{a}
120 °C for 5 min	4.33 ± 0.18^{b}	0.07 ± 0.00^{b}	nq	0.47 ± 0.03^{b}
120 °C for 10 min	3.89 ± 0.08^{b}	0.07 ± 0.00^{b}	nq	0.45 ± 0.02^{b}
120 °C for 15 min	3.81 ± 0.13^{b}	0.06 ± 0.00^{b}	nq	0.45 ± 0.02^{b}
120 °C for 20 min	3.23 ± 0.07^{bc}	0.06 ± 0.00^{b}	nq	0.41 ± 0.02^{bc}
140 °C for 5 min	3.93 ± 0.11^{b}	0.07 ± 0.00^{b}	nq	0.45 ± 0.00^{b}
140 °Cfor 10 min	3.61 ± 0.28^{bc}	0.07 ± 0.00^{b}	nq	0.46 ± 0.01^{b}
140 °C for 15 min	3.34 ± 0.06^{bc}	0.06 ± 0.00^{b}	nq	0.45 ± 0.00^{b}
140 °C for 20 min	3.02 ± 0.12^{bc}	0.06 ± 0.00^{b}	nq	0.43 ± 0.01^{b}
160 °C for 5 min	3.2 ± 0.16^{bc}	0.06 ± 0.00^{b}	nq	0.45 ± 0.01^{b}
160 °C for 10 min	3.33 ± 0.1^{bc}	0.06 ± 0.00^{b}	nq	0.46 ± 0.02^{b}
160 °C for 15 min	3.32 ± 0.09^{bc}	0.05 ± 0.00^{b}	nq	0.45 ± 0.01^{b}
160 °C for 20 min	3.01 ± 0.05^{bc}	0.06 ± 0.00^{b}	nq	0.43 ± 0.01^{b}
180 °C for 5 min	2.57 ± 0.21^{c}	0.06 ± 0.00^{b}	nd	0.43 ± 0.01^{b}
180°C for 10 min	2.49 ± 0.06^{c}	0.06 ± 0.00^{b}	nd	0.39 ± 0.01^{c}
180 °C for 15 min	2.34 ± 0.05^{c}	0.06 ± 0.00^{b}	nd	0.36 ± 0.02^{c}
180 °C for 20 min	2.29 ± 0.04^{c}	0.06 ± 0.00^{b}	nd	0.32 ± 0.01^{c}

Note nd= not detectable

nq= not quantifiable

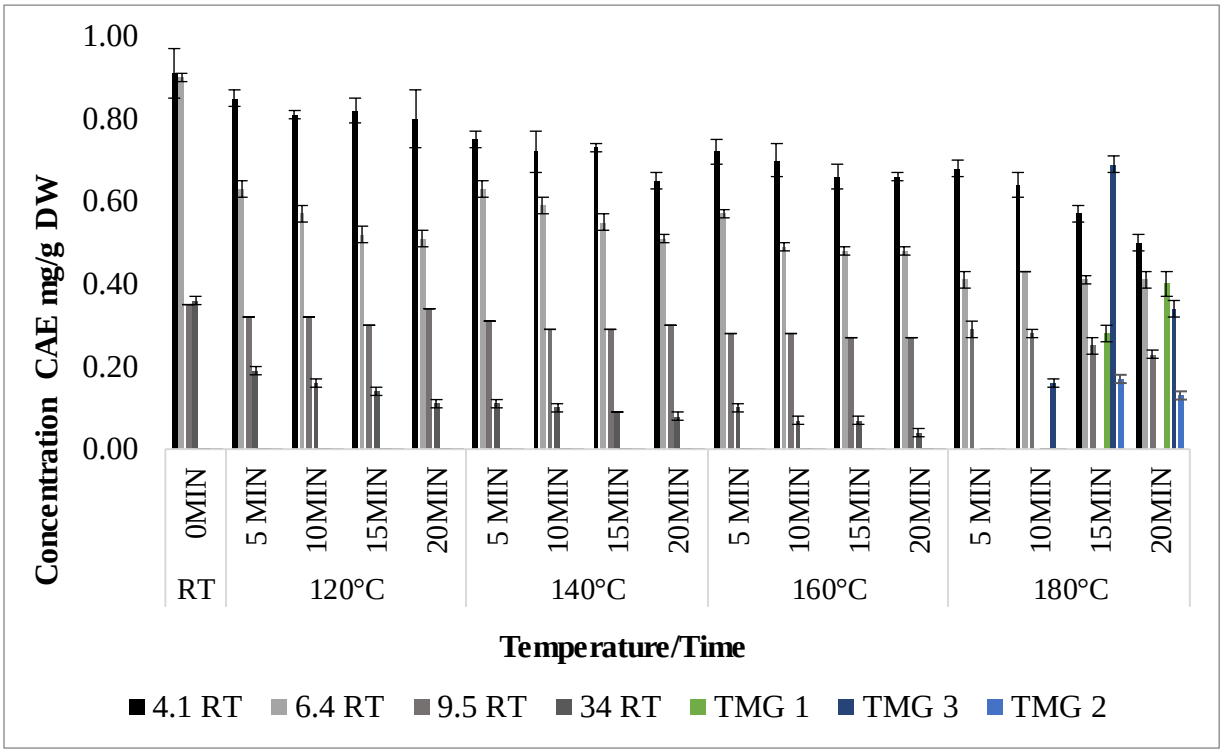
*All data are expressed as a mean of four replicates with standard deviation (n=4). Values with different superscripts in the same column are significantly different ($p \leq 0.05$)

3.5.3 Effect of air frying of seeds on unidentified phenolic compounds in LGYMM

The effect of hot air pre-treatment of mustard seeds on unidentified phenolic compounds is shown in Figure 3.1. For LGYMM, most of the phenolic compounds were detected at 270 nm as shown in Figure 3.1. Unknown compounds with retention time (RT) of 4.1 min (4.1RT), 6.4 min (6.4RT), 9.5 min (9.5RT) and 34 min (34RT) were detected in the extracts of LGYMM. A decreasing pattern was observed for 4.1RT and 6.4RT as the heat treatment time increased. The maximum concentration of 4.1RT and 6.4RT was observed in control (0.91 ± 0.06 and 0.90 ± 0.01 mg/g CAE), respectively while minimum concentration was observed at 180 ℃ for 20 min (0.50 ± 0.02 mg/g CAE for 4.1 RT and 0.41 ± 0.02 mg/g CAE for 6.4RT). A decrease in 9.1RT (34%) was observed with the increase in temperature/time of air frying but the decreasing percentage was lower than the percentage of 4.1RT (45.05%) and 6.4 RT (54.4%). The compound with 34RT detected in LGYMM was thermosensitive which significantly decreased to 0.19 ± 0.01 mg/g CAE at 120 ℃ for 5 min when compared with control (0.36 ± 0.01 mg/g CAE). The 34RT was reduced by 88.88% at 160 ℃ for 20 min and became completely degraded at 180 ℃. The decrease in concentration of phenolic compounds in this study can be related to the research done by Mizani et al. (2016) who reported that at 100 ℃, sinapic acid derivatives were reduced by 40% and 47% when yellow mustard seeds were treated with hot air and steam heating, respectively. Recently, another study has also reported a decrease in the content of phenolic compounds (catechin, chlorogenic acid, *p*-coumaric acid, rutin and quercetin) in roasted samples when compared with an unroasted sample of quince fruit and an overall 94.4 % in total phenolic content (Maghsoudlou et al., 2019).

The overall decline in the concentration of phenolic compounds can be attributed to the various reactions that are happening during thermal exposure such as oxidization or polymerization of phenolic compounds and generation of phenolic complexes with carbohydrates and proteins (Waszkowiak et al., 2020; Pelvan et al., 2012; Hečimović et al., 2011; Wang et al., 2000). Moreover, heat treatment can also result in the breakage of the ester bond and degradation at higher temperature ranges (Liu et al., 2020). Hydroxycinnamic acids such as sinapic acid, ferulic acid and p-coumaric acids are reported to contain an aromatic ring where a three carbon chain is attached. The roasting treatment can cause the sensitivity of the glycosidic bond present and can cause thermal degradation after their release from their bound forms (Lemos et al., 2012; Pelvan et al., 2012). The retention time and spectra wavelength of unknown compounds are shown in Appendix I. According to a previous research, most of the volatile phenols and flavour compounds are present at 270 nm and research has shown that flavanols are also thermoliable and are degraded when subjected to high temperatures (Wojdyło et al., 2013). These unidentified phenolic compounds can be volatile phenols, but they need further research for their identification.

Figure 3.1 Effect of air frying of mustard seeds on the contents of unidentified phenolic compounds in LGYMM.



* Data is represented as mean ± SD where n=4

3.5.4 Detection of thermogenerative phenolic compounds in LGYMM

An increase in heat treatment can result in the breakage of esterified bond of the phenolic compounds and they can be efficiently released from the cell matrix. Changes in the concentration of phenolic compounds (their increase or decrease) depend on the structure and type of heat treatment. In this study, interestingly, three thermogenerative (TMG) compounds were identified at RT of 6.1 min (TMG 1), 7.7 min (TMG 2) and 16.01 min (TMG 3) when air frying temperature reached 180 °C as shown in Figure 3.1. The TMG 1 and TMG 3 were formed at 180 °C for 15 and 20 min. The content of TMG 1 increased from 0.28 ± 0.02 to 0.40 ± 0.03 mg/g CAE as the time duration was increased from 15 to 20 min at 180 °C. On the other hand, TMG 2 exhibited a decreasing pattern when the time was increased to 20 min at 180 °C (0.17 ± 0.01 to 0.13 ± 0.01 mg/g CAE). The TMG 3 was formed at 180 °C for 10 min and increased to

its maximum by 76% at 180 °C for 15 min but decreased by 50% at 180 for 20 min when compared with control. The decrease in the other phenolic compounds as discussed above can be related to the formation of these TMG compounds at a higher temperature. These compounds could be degradation products of sinapine, syringic acid or other phenolic compounds with an observed decreasing pattern in LGYMM as many researchers have indicated the formation of canolol in rapeseed and canola by the degradation of sinapine and sinapic acid during heat treatment (Nandasiri et al., 2019; Zago et al., 2015; Niu et al., 2013; Khattab et al., 2014). But in this study, no canolol was detected in LGYMM extracts and it is not certain the nature of compounds that were degraded to form these new TMG compounds. The absence of canolol could be related to the explanation from the study done by Thiyam et al. (2014), which elaborated that an increase in the phenolics in oil and reduction in total phenolics of defatted meal suggests that during roasting at high temperature, a significant amount of phenolics are converted to canolol and these are easily soluble in oil. As canolol is also hydrophobic, it is easily soluble in oil and this can be the reason for the increase in the total phenolic content of oil during toasting. The presence of canolol in the lipophilic medium was further confirmed in another study where the oil fraction of LGYMM was studied at the same temperature/ time combinations as used in this study.

3.5.5 Effect of air frying of seeds on total phenolic (TPC) content and total flavonoid content (TFC) of LGYMM

According to the literature data available, an increase in temperature and time affects antioxidant activity (either decrease or increase). Gaspar et al. (2008) reported that the antioxidant activity of hydroxycinnamic acids is favoured by thermal treatment. The presence of a hydroxyl group or a

high number of moieties attached to the phenolic structure is linked with the lower redox potential, which is due to the formation of *o*-quinones.

The TPC in LGYMM was decreased with the increase in temperature and time as shown in Table 3.4. The highest TPC was recorded at room temperature (21.35 ± 0.21 mg/g SAE) and the lowest was recorded at 180 °C for 20 min (12.16 ± 0.36 mg/g SAE). Zago et al. (2015) also reported a decrease in TPC when rapeseed meal was subjected to superheated steam for 2 h. The decrease in TPC in this study could be possible due to the degradation of phenolic compounds, as described in sections 3.5.2 and 3.5.3. Shen et al. (2019) observed the decrease in TPC of pickled mustard after 5 min of steaming, microwaving and boiling when compared to the non-heat treated sample. The drastic airflow in the air fryer could be responsible for the lowering of TPC at higher temperatures in LGYMM. Mizani et al. (2016) also observed that untreated samples had the highest TPC when compared to heat-treated samples of white mustard during hot air drying. Flavonoids are the class of naturally occurring polyphenols in nuts, fruits, and beverages such as coffee and wine. The highest TFC value was observed at 180 °C for 10 min (4.1 ± 0.27 µmol QE/g DW) with no significant difference at 15 and 20 min. These results are comparable with the study done by Nandasiri et al. (2019) where the maximum TFC in canola meal was obtained at 180 °C using accelerated solvent extraction and the reported value (5.45 ± 0.27 µmol QE/g DM) is close to the results from current study. The lowest TFC was observed at 140 °C and 160 °C for the mustard meal. The decrease in TFC could be related to the degradation of phenolic compounds at 140 °C and 160 °C and an increase at 180 °C could be explained with the formation of thermogenerative compounds as discussed above. Other studies have also shown the same trend with higher TFC values as temperature and time increased (Ildiko et al., 2006; Sun et al., 2018). The results also suggest that TMG 1, TMG 2, and TMG 3 could be flavonoids because of the

significant increase in TFC at 180 °C for 10, 15 and 20 min. Flavonoids mostly exist as dimers and oligomers and heating can cause their breakage leading to the formation of monomers as a result of glycosidic bond hydrolysis (Manach et al., 2004).

3.5.6 Effect of air frying of seeds on antioxidant activity (FRAP, DPPH and ABTS assay) of LGYMM

To identify the reducing power of phenolic compounds, FRAP has been used extensively. With FRAP, the electron donating ability of the extract is measured which is important in determining the antioxidant ability of the phenolic compounds. In the presence of TPTZ, the reducing activity is measure based on the transformation of ferrous ions (Fe^{2+}). The FRAP is measured on the principle of antioxidant power which is required to reduce ferric- triphenyltriazine complex to the blue ferrous form. The intensity of the blue-colored complex formed is directly related to the concentration of ferrous ion as antioxidants may act as reductants in the presence of Fe^{3+}. In this study, there was no significant difference observed in FRAP values of LGYMM except at 140 and 160 °C as shown in Table 3.4. In other studies, FRAP has been reported to increase with the increase in temperature (Nandasiri et al., 2019; Thiyam et al., 2014; Gaspar et al., 2008). However, in this study, no significant difference was observed in control and heat-treated samples at 180 °C. The decrease in FRAP value at 140 °C and 160 °C and then again increase at 180 °C can be related to the formation of TMG compounds formed at 180 °C.

DPPH radical shows its characteristic absorption at 517 nm. This assay is sensitive and can measure the samples in a short time duration and measure the active ingredient at low concentrations in the sample matrix (Hseu et al., 2008). DPPH assay relies on the ability of antioxidants to perform a scavenging effect by electron donating mechanism. A decrease in the absorbance of the sample extract corresponds to higher antioxidant activity. In this study, sample

extracts at 180 °C exhibited the highest DPPH activity (Table 3.4). The maximum radical scavenging effect was observed at 180 °C for 15 min (57.13 ± 6.64 %) with no significant difference for the 20 min treatment (56.99 ± 1.67%). The lowest DPPH activity was calculated for the extracts at 160 °C ranging from 37.22 ± 1.55% to 36.2 ± 2.68 %. Terpinc et al. (2012) and Li and Guo (2016) also reported a similar radical scavenging activity of 50-60 % in oilseed cakes and meal. The results at 180 °C are also comparable with Nandasiri et al. (2019), where maximum DPPH activity of 62.53 ± 1.64% was observed at 180 °C for 70% methanolic extract of canola meal. This increase can be related to the synergistic effect between the TMG compounds generated and phospholipids as described by Li and Guo (2016). Another widely used method to access the antioxidant activity is the ABTS assay. It is also reported that ABTS assay is accurate and sensitive for the screening of complex antioxidant mixtures including plant extracts and beverages etc. ABTS radical solution is blue-green but the intensity can be reduced by hydrogen donating antioxidants which is reflected as lower absorbance reading at 734 nm. In this study, the pattem for the ABTS assay was similar to that of the DPPH assay (Figure 3.4). The maximum antioxidant activity for ABTS was observed at 180 °C for 15 min with no significant difference noted for the 20 min treatment (58.14 ± 7.91 % and 52.02 ± 5.1 %), respectively. On the other hand, the lowest radical scavenging effect was observed at 160 °C for LGYMM (41.71 ± 2.86% to 42.87 ± 0.34%). Shen et al. (2019) reported a similar trend for ABTS assay where it was increased by an increase in the steaming of pickles at 5, 15 and 25 min. According to Lim & Kim (2018), ABTS activity was significantly increased at 180 °C and 210 °C for Ginkgo biloba seed extracts.

The increase in the antioxidant activity by DPPH and ABTS assays suggest that TMG compounds generated at 180 °C in LGYMM during air frying possess strong radical scavenging activity. The increase in antioxidant activity was not directly related to the TPC in our study. Although a

decrease in TPC was observed during air frying, that total antioxidant activity was increased at 180 °C as reported in some of the previous studies done (Maghsoudlou et al., 2019; Satoh et al., 2005; del Castillo et al., 2002). The increase in the antioxidant activity can be explained by the formation of intermediate products at a higher temperature range that is stable and posses a strong antioxidant potential. These intermediate products can be generated due to the various reactions during thermal treatment such as pyrolysis or carbohydrate caramelization (Kim et al., 2011; Silva et al., 2004a, b; Turkmen et al., 2005). It has also been reported that newly generated compounds at higher temperatures could be intermediate oxidation products that have strong antioxidant power (Nicoli et al., 1999). The antioxidant activity and TPC depend on the media and type of heat treatment and can have a different relationship in different heat treatments (Lemos et al., 2012; Choi et al., 2012; Kim et al., 2011).

Table 3.3 Effect of air frying on total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activity of lower grade yellow mustard meal

Temperature/Time	TPC (SAE mg/g)	TFC (QE µmol/g)	FRAP (Fe^{+2} µmol/g)	DPPH (%)	ABTS (%)
RT	21.35±0.21^{a}	3.1±0.19^{ab}	85.5±0.14^{a}	50.56±0.19^{ab}	46.99±6.01^{b}
120 °C for 5 min	17.7±0.78^{b}	2.43±0.38^{b}	76.05±3.22^{b}	47.26±0.22^{ab}	45.94±0.58^{b}
120 °C for 10 min	16.57±0.34^{b}	2.85±0.28^{b}	81.35±6.34^{a}	44.03±4.43^{b}	46.47±0.59^{b}
120 °C for 15 min	13.06±0.31^{bc}	3.02±0.27^{b}	83.77±7.88^{a}	40.93±0.57^{bc}	49.57±0.51^{ab}
120 °C for 20 min	14.15±0.02^{bc}	2.8±0.26^{b}	77.03±10.54^{b}	42.77±0.18^{b}	44.73±4.2^{b}
140 °C for 5 min	12.38±0.42^{c}	2.87±0.44^{b}	71.43±7.71^{b}	42.64±0.52^{b}	48.89±2.55^{ab}
140 °C for 10 min	12.16±1.01^{c}	2.86±0.24^{b}	64.13±0.99^{c}	42.02±1.81^{b}	47.21±1.59^{ab}
140 °C for 15 min	11.27±1.01^{c}	2.97±0.40^{b}	60.63±2.85^{c}	40.95±0.24^{bc}	43.31±3.53^{b}
140 °C for 20 min	10.46±0.93^{cd}	2.85±0.48^{b}	57.65±4.98^{c}	41.79±0.62^{bc}	42.79±0.98^{b}
160 °C for 5 min	12.31±0.06^{c}	2.77±0.26^{b}	58.9±3.89^{c}	37.22±1.55^{c}	42.87±0.34^{b}
160 °C for 10 min	12.56±0.16^{c}	2.87±0.11^{b}	62.75±0.87^{c}	38.81±0.20^{c}	43.83±2.39^{b}
160 °C for 15 min	12.09±0.03^{c}	2.92±0.16^{b}	67.01±8.36^{bc}	36.81±1.52^{c}	40.9±4.32^{b}
160 °C for 20 min	11.51±0.07^{c}	3.38±0.24^{ab}	68.97±5.28^{bc}	36.2±2.68^{c}	41.71±2.86^{b}
180 °C for 5 min	10.54±0.98^{c}	3.45±0.2^{2}	66.89±7.28^{bc}	41.88±7.30^{b}	43.56±0.94^{b}
180 °C for 10 min	11.34±0.09^{c}	4.1±0.27^{a}	73.65±4.52^{b}	42.17±6.92^{b}	52.02±5.10^{a}
180 °C for 15 min	12.95±0.02^{bc}	3.96±0.19^{a}	76.7±3.94^{b}	57.13±8.81^{a}	58.14±7.91^{a}
180 °C for 20 min	12.16±0.36^{c}	4.00±0.33^{a}	75.85±4.52^{b}	56.99±1.67^{a}	54.69±8.40^{a}

*All data is expressed as mean of four replicates with standard deviation (n=4). Values with different superscripts in same column are significantly different ($p \leq 0.05$)

3.6 Conclusions

This study evaluated the effect of air frying of yellow mustard seeds as a preheating treatment on the phenolic compounds and antioxidant activities of LGYMM. Based on the external standard and UV spectra, sinapine, sinapic acid, methyl sinapate and syringic acid were identified in the methanolic extracts of LGYMM. The results showed that most of the phenolic compounds decreased with the increase in temperature and time of air frying treatment. However, at 180 °C, three thermogenerative compounds were formed which were supposed to be the degraded products of the phenolic compounds that showed decreasing trend in concentration. The optimum seed pre-treatment condition for the extraction of antioxidants was 180 °C for 15 and 20 min as the maximum antioxidant activity was observed at this temperature. An increase in TFC at 180 °C also suggest that the thermogenerative compounds formed could be the antioxidant flavonoids that are generated when mustard seeds are heated at 180 °C. These antioxidant compounds can be readily extracted from the LGYMM and can add value to the by-products of mustard processing.

CHAPTER 4

4. POLYPHENOLS FROM DE-OILED LOWER GRADE YELLOW MUSTARD MEAL: EFFECT OF ENZYMATIC OXIDATION

4.1 Abstract

Lower grade yellow mustard meal (LGYMM), a by-product of the mustard milling process**,** has potential as a value-added product in the food, feed, and agro-allied industries. Enzyme-assisted extraction has several benefits including higher product yields, reduction in extraction time, lower energy utilization, and minimization of solvent use. The under-utilized by-products of mustard can also be treated with enzymes for the production of oxidized phenolic compounds. The enzymatic oxidation of sinapic acid (SA) in the mustard meal was investigated by treating with horseradish peroxidase (HRP) and tyrosinase (TR) using an in vitro system. HPLC analysis of pure SA treated with both enzymes resulted in the formation of two main compounds (1 and 2). Interestingly, sinapine present in a methanolic extract of LGYMM was catalyzed significantly ($p \leq 0.05$) by HRP and TR into several unidentified oxidized end products. SA released after the alkaline hydrolysis of LGYMM was also converted into compound 1 and compound 2 by HRP and TR. The kinetics of enzymatic reaction showed that HRP has a significantly higher catalytic efficiency (CE = 0.348), reaction velocity (Vmax = 3.48) and Michaelis-Menten constant (Km = 604.56) than TR (CE = 0.044, Vmax = 0.44, Km = 201.60). The results of this study found SA and sinapine were efficiently converted to oxidized enzymatic end products with potential as precursors for canolol (a strong antioxidant). Therefore, in-situ enzymatic oxidation has the potential for adding value to the processing of LGYMM.

Keywords: Sinapine, sinapic acid, mustard, horseradish peroxidase, tyrosinase

4.2 Introduction

Mustard has been an important crop worldwide, belonging to the *Brassicae* family. Different varieties of mustard seeds are cultivated and used as a source of edible oil, spice, and medicine since ancient times (Ildiko et al., 2006). In recent years, the development of extraction procedures of bioactive compounds from natural resources has increased. Yellow mustard (*Sinapis alba*) and its by-products e.g. lower grade varieties, bran, hulls, etc can be used as a source of a wide range of bioactive compounds, which include isothiocyanates, phenolics, dithiolthiones and dietary fiber (Hendrix et al., 2012). Phenolic compounds present in the oilseeds are beneficial to human health. They help in the inhibition of oxidative damage-induced diseases such as stroke and cancer, which is linked to their high antioxidant activity (Fang et al., 2008). During oil processing, naturally occurring antioxidants are lost from oilseeds. To increase the antioxidant activity of edible oils and other food products, antioxidants from the mustard seed by -products can be used to avoid rancidity and oxidative damage. The by products of mustard contain a wide range of SA derivatives which can be further converted to canolol (4-vinyl syringol), a strong antioxidant and a decarboxylated product of SA (Niu et al., 2013). Many recent in-vitro assays have reported SA and 4-vinylsyringol as the major antioxidants in various food products (Mayengbam et al., 2014). It exceeds 15% of its antioxidant activity as compared to SA due to its low polarity. Owing to the abundance and respective bioactive attributes, previous researchers have suggested the use of phenolics from rapeseed and mustard in the development of functional food products (Vuorela et al., 2004; Vuorela, 2005). These antioxidant end products can be used to increase the stability and shelf life of edible oils and other foods.

The value addition perspective of these bioactive molecules from the by-products of mustard can be increased by efficient extraction techniques. Previously, microwave or conventional oven

pretreatment resulted in the conversion of SA to its decarboxylated product commonly known as canolol. (Khattab et al., 2010). Recently, procedures such as enzymatic assisted extraction that are considered as green technologies are gaining attention as they require less harsh conditions and are environment friendly (Morley et al., 2013). Moreover, the enzymatic end products are highly reproducible and specific with less chance of formation of undesirable end products.

Tyrosinase from mushroom and peroxidase from horseradish are reported to cause the oxidation of phenolic compounds (Cao et al., 2019). Phenoxy radicals are generated when HRP reacts with the phenols in the presence of H_2O_2 which is required to establish an oxidation cross-linking. (Cooper & Nicell, 1996; Elstner & Heupel, 1976). Other phenolic compounds including sinapic acid, ferulic acid and p-coumaric acid are reported to be oxidized by HRP (Cao et al., 2019; Takahama, 1995). HRP and tyrosinase was used in a previous study to convert canola meal SA to oxidized products (Cao et al., 2019). This current study extends the previous study through the change in the substrate from canola to lower grade yellow mustard seeds. Moreover, antioxidant assays were also performed for the enzymatic extracts to check the antioxidant potential of the end products formed during enzymatic oxidation. This work investigated the optimum conditions for the generation of oxidized end products of sinapine and SA after treatment of LGYMM with HRP and tyrosinase.

4.3 Materials and methods

4.3.1 Chemicals and samples

Lower grade yellow mustard seeds were obtained from G.S. Dunn Limited (North Hamilton, ON, Canada). The sinapic acid (98%), syringic acid (98%), potassium monosulfate (98%), sodium phosphate monobasic (99%), sodium phosphate dibasic (99%), 2,2′-Azino-bis (3-

ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS>98%), potassium persulfate (98%), horseradish peroxidase type II (200 U/mg) ,tyrosinase from mushrooms (7164 U/mg), hydrogen peroxide, sodium hydroxide, citric acid, methanol (100%), and HCl (95%) were purchased from Sigma Aldrich (Oakville, ON, Canada) and Fisher Scientific (Ottawa, ON, Canada). Sinapine (98%) and methyl sinapate (98%) was purchased from Chem Faces (Wuhan, Hubei, China). Folin–Ciocalteu's (FC) reagent, acetate, 2,4,6-tris-(2-pyridyl)-s-triazine (TPTZ>98%), and 2,2-diphenyl-1-picrylhydrazyl (DPPH, 97%) were purchased from Fisher Scientific Canada Ltd. (Ottawa, ON, Canada). Quercetin hydrate (>95%) and 2-amino-ethyl-diphenyl borate (98%) were purchased from Acros (Mississauga, ON, Canada). All the chemicals used were of analytical grade.

4.3.2 Enzymatic oxidation of pure sinapic acid

The oxidation of pure SA was carried out as described by Cao et al. (2019). The reaction mixture (2 ml) for HRP-catalyzed oxidation of pure sinapic acid was made using 50 µM of SA, 900 µM hydrogen peroxide, and 0.2U HRP in sodium phosphate buffer (0.1M, pH 6.0). In the same way, tyrosinase catalyzed oxidation was measured using 50 µM SA, 40.3 U of tyrosinase, and 0.1 M phosphate citrate buffer (pH 6.0) to bring the total volume up to 2 ml. These reaction mixtures were incubated for 60, 90, and 120 min in the water bath at 25 °C. Further analysis of the incubated sample mixture was carried out using HPLC.

4.3.3 Enzymatic oxidation of untreated mustard meal

The defatted LGYMM from non-heat treated seeds was extracted using an ultrasound assisted solvent method. One gram of defatted sample was extracted using 70% (v/v) methanol in a 1:9 sample to solvent ratio. This was assisted by sonication at 1 min followed by centrifugation (5000 rpm at 4 ºC for 15 min). The same procedure was repeated three times for each sample. The

supernatant from each extraction was combined and the total volume made up to 25 ml using 70% methanol (Nandasiri et al., 2019). Reaction mixtures (2 ml) containing 1 ml of methanolic extract were prepared according to the same procedure as described in section 2.2. HRP (2 U) and tyrosinase (40.3 U) were then added to the reaction mixtures. These mixtures were then incubated for 60, 90, and 120 min in the water bath at 150 C. The reaction mixtures were mixed with 70% methanol (v/v) in 1:1 and centrifuged at 4000 rpm for 10 min. The supernatant was filtered and injected in HPLC for further analysis.

4.3.4 Enzymatic oxidation of alkaline treated mustard meal

Defatted mustard meal (10 g) was mixed with 70% (v/v) methanol (1:9) and this mixture was sonicated for 20 min followed by centrifugation (5000 rpm, 4 °C,15 min). The supernatant was collected after centrifugation and the process was repeated 3 times. The supernatant after every extraction was combined and alkaline treatment was done according to the procedure described by Cao et al. (2019), with some modifications. An equal volume of NaOH was added to the methanolic extract followed by nitrogen flushing. The NaOH-treated extract was stirred for 2 h at 150 rpm. After stirring, the mixture was acidified to pH 5 and extracted using ethyl acetate (1:1). The ethyl acetate extract was evaporated and redissolved in 70% (v/v) methanol. The reaction mixture for enzymatic oxidation with HR was prepared using 20 µl of alkaline treated redissolved extract, 900 or 9000 µM H_2O_2, 0.2 U, or 2 U HRP. The total volume of the solution was made up to 2 ml using 0.1M sodium phosphate buffer. In the same way, the reaction mixture of tyrosinase contained 20 µl of neutralized extract, 900 0r 9000 µM of H_2O_2, and 40.3 U or 403.0 U of tyrosinase. The total volume of the mixture was made up to 2 ml using 0.1M sodium citrate buffer. The reaction mixtures of 0.2 U HRP and 40.3 U tyrosinase were incubated for 0, 1, 1.5, 2, and 18 h at 25 °C in the water bath. On the other hand, the reaction mixtures of 2 U HRP and 403 U

tyrosinase were incubated at 25 °C for 0, 1, 2, and 5 h. All the reaction mixtures were mixed with 70% methanol (1:1) and centrifuged at 4000 rpm for 10 min. The extracts were then filtered through a 0.45 µm syringe filter and injected into the HPLC.

4.3.5 Determination of total phenolic content (TPC) of enzymatic extracts of LGYMM

The TPC of the untreated and alkaline extracts of LGYMM was calculated as described by Nandasiri et al. (2019). In a 10 ml conical flask, 50 µl (untreated extract) and 20 µl (alkaline treated extract) were mixed with 0.5 ml of FC reagent. The sample mixture was vortexed followed by the addition of 1 ml of 19% of sodium carbonate solution. The total volume of the solution was made up to 10 ml using distilled water. The solution was kept in the dark for 60 min with intermittent shaking after 30 min. The colored blue complex formed was measured at 750 nm using the absorbance mode in an FL 6500 Fluorescence Spectrophotometer (Perkin Elmer Inc., Waltham, MA, USA). For blank, methanol was used in replacement with the sample and the standard curve of SA was prepared with 1 mg/ml of stock solution in methanol (100%). The results were expressed as sinapic acid equivalent in milligram per gram of LGYMM.

4.3.6 Determination of total flavonoid content of enzymatic extracts of LGYMM

For the calculation of TFC, the procedure described by Nandasiri et al. (2019) was used with slight modifications. Briefly, untreated extracts (1 ml and alkaline treated extract (100 µl) were mixed with 3 ml and 3.9 ml of distilled water, which was followed by the addition of 100 µl of diphenylboric acid 2-aminoethyl ester solution (1% v/v). Absorbance of the reaction mixture was measured at 404 nm. Methanol was used to replace the sample and used as a blank. The standard curve of quercetin was prepared using the stock solution of quercetin (1 mM) in 100% methanol. The TFC of the extracts was expressed in micromolar quercetin per gram of LGYMM.

4.3.7 Determination of Ferric reducing antioxidant power (FRAP)

FRAP for the enzymatic extracts of LGYMM was measured as described by Nandasiri et al. (1996) with minor modifications. The working FRAP solution was prepared by adding 200 ml of acetate buffer solution (pH 3.6), 20 ml of TPTZ solution (10 mM), and 20 ml of ferric chloride solution followed by 24 ml of distilled water. For the untreated and alkaline treated enzymatic extracts, 100 µl and 20 µl of the extracts were mixed with 900 µl and 980 µl of distilled water, respectively. The mixture was vortexed and followed by the addition of 2 ml of working FRAP reagent. The samples were kept in dark for 30 min and absorbance measured at 593 nm. For blank, methanol was used in place of sample extract and a standard curve was prepared using ferrous sulphate stock solution (1 mM). The values for FRAP was expressed as ferrous sulfate equivalent in micromole per gram of LGYMM.

4.3.8 Determination of DPPH-radical scavenging effect

The radical scavenging effect of the enzymatic extracts using DPPH was measured according to the procedure described by Thiyam et al. (2006) with slight changes. The untreated extract (50 µl) and alkaline treated extract (20 µl) was mixed with 2.95 ml and 2.98 ml of DPPH solution, respectively. The reaction mixture was stirred with plastic stirrers and allowed to stand in dark for 10 min. Absorbance of the blue-colored complex formed was measured at 517 nm. For blank, methanol was substituted as sample and scavenging effect of the samples were measured using the formula as follows:

$$\textbf{Scavenging Effect (\%)} = [A_c\,(562\ nm) - A_s\,(562\ nm)] / A_c\,(562\ nm) \times 100$$

where

A_c = absorbance of control at 562 nm

A_s = absorbance of sample at 562 nm

4.3.9 Determination of ABTS-radical scavenging effect

The ABTS assay was performed according to a method described by Kosakowska et al. (2018). For the generation of $ABTS^{\circ+}$ radical solution, ABTS (7 mM) and potassium persulfate (2.45 mM) solutions were prepared in phosphate buffered saline (pH 7.4) and both the solution were mixed in a 1:1 ratio. This solution was allowed to stand in dark for 14-16 h to generate the $ABTS^{\circ+}$ radical. The $ABTS^{\circ+}$ solution was diluted with phosphate buffer saline and was equilibrated to an absorbance of 0.7 at 734 nm. The radical scavenging effect was measured by adding 20 µl (untreated extract) and 10 µl (alkaline treated) in 3.8 and 3.9 ml of $ABTS^{\circ+}$ solution. Absorbance of the complex formed was measured at 0 min and after incubation at 10 min. The radical scavenging percentage was measured by using the formula described below.

$$\textbf{Radical scavenging activity (\%)} = A_{control} - A_{(t0\text{-}t10)} / A_{control} \times 100$$

where

$A_{control}$ = absorbance of blank at 734nm

$A_{(t0\text{-}t10)}$ = absorbance of the sample at 0 min subtracted by absorbance of the sample after 10 min

4.3.10 HPLC analysis

The end products of sinapic acid oxidation in LGYMM treated with HRP or tyrosinase were analyzed using the gradient HPLC method as described by Nandasiri et al. (2019). Oxidized end products were analyzed using reversed-phase High Performance Liquid Chromatography Diode Array Detection (HPLC-DAD) (Ultimate 3000, Dionex, Sunnyvale, Torrance, CA, United States).

For separation, KinetexR Biphenyl C18 100 °A RP column (2.6 mm, 150 × 4.6 mm, Phenomenex, Torrance, CA, United States) was used. Other analysis conditions were maintained as flow rate (0.4 ml/ min), column oven temperature (30 ºC), injection volume (10 µl), and wavelength for analysis of SA-derived enzymatic end products (270 nm and 330 nm). Solvent A (100% milli-Q water containing 0.1% formic acid) and solvent B (100% methanol containing 0.1% formic acid) were used as mobile phases for the separation of main enzymatic end products using gradient elution. The gradient elution system operation was as follows; 25% B (0-3 min), 25 to 40% B (3-8 min), 40% B (8-13 min), 40 to 60% B (13-25min), 60 to 70% B (25-38 min), 70% to 100% B (38- 41 min), 100% B (41-44 min), 100% to 25% B (44- 47 min), to 25% B (47- 57 min). SA and its derivatives were quantified based on a standard curve of SA. Chromatograms were obtained at specific wavelengths and were quantified using Chromeleon software Version 7.2 SR4 (Dionex Canada Ltd., Oakville, ON, Canada).

4.3.11 Enzymatic kinetics assay

The kinetics for enzymes were performed as previously reported by Cao et al. (2019). Enzymatic reaction mixtures (2 ml) with different substrate concentrations (50, 100, 150, 200, and 250) were prepared by adding HRP (0.2 U) or tyrosinase (40.3 U). These reaction mixtures were incubated at 25 °C for 2 h and a decrease in the absorbance was measured at 304 nm using a Du 800 UV/Vis spectrophotometer (Beckman Coulter Inc., Mississauga, ON, Canada). The velocity of the enzymatic reaction mixtures was expressed as the ΔA/h which is absorbance per hour. To obtain the Lineweaver-Burk plot, the reciprocal of substrate concentration was plotted against the reciprocal of reaction velocity. The maximum reaction velocity (V_{max}) and Michaelis-Menten constant (Km) were calculated by taking the y- and x-intercept, respectively. The catalytic efficiency for both the enzymes was calculated using the equation ($K_{cat}/K_m = V_{max}/K_m$).

4.4 Statistical analysis

All the experiments were carried out in duplicates. Results are presented as the mean ± standard deviation of the duplicate analysis. Data analysis was carried out using one way analysis of variance (ANOVA) and multiple mean comparisons were calculated with Tukey's test at 5% ($p < 0.05$) significance level. All the data analysis were performed using SPSS statistical software version 22 (IBM, New York, NY, United States).

4.5 Results and discussions

4.5.1 Effect of enzymatic oxidation on polyphenolic content of pure sinapic acid

The phenolic profile of reaction mixtures was measured by HPLC analysis to confirm the oxidation of SA by HRP and tyrosinase. Both enzymes are responsible for the catalytic degradation of SA. The changes in end products of SA were monitored after 0, 1, 1.5, and 2 h of incubation with each enzyme. In Table 4.1, compound 1 was the main oxidized form with a retention time of 31.2 min and UV-Visible spectra ranged from 212 to 340 nm. The concentration of SA and compound 1 was expressed in sinapic acid equivalent by using the standard SA curve ($R^2 = 0.9994$), which was generated with different concentrations of SA against the peak area at 320 nm. There was a significant increase in compound 1 from 0 to 1.5 h of incubation (1.96 ± 0.0 to 6.72 ± 0.03 mg/g SAE and 0.86± 0.00 to 4.42 ± 0.02 mg/g SAE for HRP and tyrosinase respectively). The increase in the concentration of compound 1 was related to the decrease in the SA concentration as it reduced significantly from 12.58 ± 0.12 to 2.76 ± 0.09 mg/g SAE for HRP catalyzed reaction and from 17.91 ± 0.38 to 10.77 ± 0.26 mg/g SAE for tyrosinase catalyzed reaction. No significant difference was observed in the reduction of SA and formation of compound 1 after 1.5 h of incubation as shown in Table 4.1.

Table 4.1 Effect of enzymatic oxidation on pure sinapic acid with horseradish peroxidase (HRP) and tyrosinase (TR)

Sample	Concentration (µg)	
	Sinapic acid	Compound 1
0.2U HRP (0 h)	12.58 ± 0.12^{a}	1.96 ± 0.00^{c}
0.2U HRP (1 h)	4.62 ± 0.16^{b}	4.93 ± 0.23^{b}
0.2U HRP (1.5 h)	2.76 ± 0.09^{c}	6.72 ± 0.03^{a}
0.2U HRP (2 h)	1.66 ± 0.11^{c}	4.61 ± 0.45^{b}
40.3U TR (0 h)	17.91 ± 0.38^{A}	0.86 ± 0.00^{B}
40.3U TR (1 h)	10.77 ± 0.26^{B}	3.55 ± 0.02^{B}
40.3U TR (1.5 h)	10.56 ± 0.05^{B}	4.42 ± 0.02^{B}
40.3U TR (2 h)	9.64 ± 0.15^{B}	3.35 ± 0.01^{B}

*For each column and enzyme, values with different superscript letters (lowercase or upper case) are significantly different ($p < 0.05$).

4.5.2 Effect of enzymatic oxidation on polyphenolic content of untreated mustard meal

Mustard meal contains a variety of SA derivatives with the main compound being sinapine, which is the choline ester. In Table 4.2, sinapine is the major phenolic compound detected in the reaction mixtures of HRP and tyrosinase treated mustard fraction. However, SA was detected only in unquantifiable trace amounts. In the reaction mixture of HRP with untreated (UT) mustard fraction, sinapine was the major phenolic detected and it decreased significantly from 0.94 ± 0.03 to 0.03 ± 0.0 mg/g SAE. As sinapine decreased, two unknown compounds were formed at a retention time of 7.7 min (Unknown 1) and 22.5 min (Unknown 2), respectively. UV-Visible spectra of compounds generated by sinapine degradation using HRP are shown in the Appendix. A significant increase was observed in the amount of Unknown 1 (0.03 ± 0.0 to 0.43 ± 0.02mg/g SAE) after 1 h of incubation. Unknown 2 was undetectable in the control sample and it reached its highest concentration (0.21 ± 0.02 mg/g SAE) after 2 h of incubation. In the tyrosinase-catalyzed

reaction mixture of UT mustard fraction, sinapine was significantly decreased from 4.61 ± 0.14 to 1.37 ± 0.10 after 1.5 h of incubation. The decrease in sinapine was related to the formation of the other two oxidized end products at a retention time of 11.4 min (Unknown 3) and 16.4 min (*p*-coumaric acid) as shown in Table 4.2. As sinapine decreased, Unknown 3 significantly increased from 0.06 ± 0.01 to 1.27 ± 0.08 mg/g SAE after 2 h. The results of this study demonstrate that HRP and tyrosinase can oxidize the pure SA as well as those present in the mustard meal. Another interesting aspect of this study was that HRP and tyrosinase also degraded the sinapine in the methanolic extract of mustard meal. The catalytic efficiency of enzymes was related to reaction time and enzyme concentration.

The results generated by this study are somewhat different from our previous study where there was no significant change observed in sinapine concentration when treated with HRP and tyrosinase. This could be possible due to the difference in the extraction procedure. However, the exact phenomena through which sinapine is degraded by enzymes is still not fully elaborated enough. A recent study showed that bacterial strains from the digestive tract of hen can degrade sinapine. There are very few studies involved with enzymatic degradation of sinapine. Bacteria and yeast are also reported to degrade sinapine. Lacasse that belongs to polyphenol oxidase is reported to oxidize phenolic acids. Laccase, β-glucosidase, and lipase are reported to initiate sinapine degradation (Yu et al., 2016). According to Qiao (2002), tyrosinase and tannase are responsible for sinapine degradation. As laccases are oxidoreductases, they help in the catalysis of mono electric oxidation of various substrates including plant phenolics. Enzymatic hydrolysis of carbohydrates in rapeseed meals resulted in an 81.93% decrease in sinapine (Yu et al., 2016). Our study showed there was a 96.1% and 70.7% decrease in sinapine in LGYMM when treated with HRP and tyrosinase. As the exact mechanism involved in sinapine degradation is not known yet,

more studies need to be done to fully elaborate. The *p*-coumaric acid was present in trace amounts in the control sample (0 min) of TR-catalyzed reaction mixture and it also increased significantly to 0.14 ± 0.01 mg/g SAE after 2 h of incubation. The p-coumaric acid has also been previously reported in mustard and its concentration increased from 0.013 to 0.025 mg/g after acidic hydrolysis (Fang et al., 2007). The current study generated a relatively higher amount of p-coumaric acid (0.14 mg/g SAE) after 2 h of incubation of untreated mustard meal with tyrosinase (Table 4.2). There was no significant difference observed in the concentrations of other phenolic compounds present in enzymatic reaction mixtures of UT mustard meal fraction.

Table 4.2 Effect of horseradish peroxidase (HRP) and tyrosinase (TR) oxidation of lower grade yellow mustard meal prepared from untreated mustard seeds

Sample	Concentration of phenolic compounds (mg/g SAE DW)				
	Sinapine	Unknown 1	Unknown 2	Unknown 3	p-coumaric acid
0.2U HRP (0 h)	0.9 ± 0.03^{a}	0.03 ± 0.00^{c}	nd	nd	nd
0.2U HRP (1 h)	$0.04\ 0.00^{c}$	0.43 ± 0.02^{a}	0.17 ± 0.02^{b}	nd	nd
0.2U HRP (1.5 h)	0.0 ± 0.00^{c}	0.28 ± 0.01^{b}	0.21 ± 0.01^{a}	nd	nd
0.2U HRP (2 h)	0.03 ± 0.00^{c}	0.16 ± 0.01^{b}	0.21 ± 0.02^{a}	nd	nd
40.3U TR (0 h)	4.61 ± 0.14^{A}	nd	nd	0.06 ± 0.01^{C}	nd
40.3U TR (1 h)	2.31 ± 0.09^{B}	nd	nd	0.76 ± 0.08^{B}	0.10 ± 0.01^{AB}
40.3U TR (1.5 h)	1.37 ± 0.10^{B}	nd	nd	1.14 ± 0.09^{A}	0.12 ± 0.01^{A}
40.3U TR (2 h)	1.3 ± 0.04^{B}	nd	nd	1.27 ± 0.08^{A}	0.14 ± 0.01^{A}

Note: nd, not detected

* For each column and enzyme, values with different superscript letters (lowercase or upper case) are significantly different ($p < 0.05$).

4.5.3 Effect of enzymatic oxidation on the polyphenolic profile of alkaline treated fraction

As SA was detected only in trace amounts in UT meals, NaOH treatment (alkaline hydrolysis) was used to release the bound form of sinapine to free sinapic acid. To evaluate the efficiency of tyrosinase and HRP on SA present in the mustard meal, the untreated extract was hydrolyzed using NaOH to liberate free SA. After the alkaline hydrolysis, SA was the major phenolic compound present the meal, which was then reacted with HRP or tyrosinase. SA was degraded and converted to compound 1 with increase in incubation time for both the enzymes. However, the conversion of SA was not as efficient as pure SA treated with HRP and tyrosinase. In the case of the alkaline treated (AT) mustard meal, HRP (0.2 U) catalyzed SA from 2.59 ± 0.02 to 0.04 ± 0.00 mg/g SAE after 1.5 h of incubation. No SA was detected after 1.5 h of incubation which showed the complete degradation of SA (Table 4.3). Moreover, the maximum amount of compound 1 was generated after 1.5 h of incubation when compared to control (0.25 ± 0.0 to 1.27 ± 0.10 mg/g SAE) and it decreased significantly afterwards. The amount of compound 1 (1.27 ± 0.01mg/g SAE) obtained in this study is comparable to the (1.56 ± 0.02 mg/g SAE) reported by Cao et al. (2019). However, in AT mustard fraction, the maximum amount of compound 1 was generated after incubation at 1.5 h by using 0.2 U HRP as compared to canola meal where the maximum amount was formed after 18 h of incubation. On the other hand, tyrosinase (40.3 U) was able to degrade sinapic acid from 4.52 ± 0.22 to 0.06 ± 0.01 mg/g SAE after 2 h of incubation. A maximum formation of compound 1 was observed after 18 h of tyrosinase-catalyzed reaction mixture when compared to control (0.06 ± 0.01 to 0.09 ± 0.00 mg/g SAE). The 40.3 U tyrosinase generated the maximum amount of compound 1 in the AT mustard whereas a higher enzyme concentration (403.0 U) was used to achieve similar level in canola meal. In the current study, increasing enzyme concentration resulted in the degradation of oxidized products in AT mustard meal extract. This could be possible

due to the interference from other phenolic compounds present in the extract. However, this study showed that enzymes reacted slower with the SA present in the mustard meal when compared to the pure SA where HRP and tyrosinase acted almost 5 and 3 times faster, respectively to generate compound 1. These results are consistent with those reported for the canola meal study (Cao et al., 2019). Another interesting end product with a retention time of 22.5 min (compound 2) as described by Cao et al. (2019), was also formed after 1h of incubation and its maximum concentration (0.06 ± 0.00 to 0.09 ± 0.00 mg/g SAE) was observed after 18 h of incubation.

To check the effect of enhanced enzyme concentration on the formation of compounds 1 and compound 2, AT extracts were treated with 10 times increase in the concentration of either HRP (2.0 U) or tyrosinase (403.0 U) using the same reaction mixture (2 ml) as described above. A sudden change in the color of the reaction mixture was observed when it was treated with 2 U of HRP, which indicated a very fast enzymatic reaction. The HPLC analysis of reaction mixtures with 2U HRP resulted in a blank chromatogram (no peaks observed) as SA was completely degraded. On the other hand, 403.0U of tyrosinase showed the presence of both SA and compound 1. However, decreases in the levels of compound 1 and compound 2 was observed when compared with the reaction mixtures of 40.3 U tyrosinase. (Table 4.3).

In previous studies, thermal treatments (conventional oven, pressurized heating using ASE, and pressurized steam heating) have been used to generate strong antioxidant compounds mainly canolol (Nandasiri et al., 2019; Zago et al., 2015; Khattab et al., 2010). The major drawback of these treatments is harsh environmental conditions and the cost to utilize energy in the form of heat and pressure. The formation of decarboxylated products using enzymes has been studied in beers and whiskey (Steinke & Paulson, 1964). In our study, no canolol formation was observed after the enzymatic oxidation. However, the main oxidized compounds were formed by the

degradation of sinapine and SA. The polyphenols from the plants are degraded into their respective quinones when treated with peroxidase. Compound 1 that was formed by treating AT fraction with HRP and tyrosinase is the same as reported by Cao et al. (2019) and is believed to be a precursor of canolol. Similarly, another interesting compound 2 was only formed in tyrosinase catalyzed meal fraction which decreased significantly as the enzyme concentration was increased. This could be possible because as *o*-quinones are formed after tyrosinase oxidation, they are quite unstable and degrade quickly at high pH. Another phenolic compound, caffeic acid is also reported to form oligomers when treated with tyrosinase (Cheynier & Moutonet, 1992). Thus, compound 1 and compound 2 need further investigation for their structural and antioxidant properties.

Table 4.3 Effect of horseradish peroxidase (HRP) and tyrosinase (TR) oxidation on phenolic compounds present in alkaline treated fraction of LGYMM from non-heat treated mustard seeds

	Concentration of phenolic compounds (mg/g SAE DW)		
	Sinapic acid	**Compound 1**	**Compound 2**
0.2U HRP (0 h)	2.59 ± 0.02^{a}	0.25 ± 0.01^{c}	nd
0.2U HRP (1 h)	1.77 ± 0.10^{b}	0.65 ± 0.10^{bc}	nd
0.2U HRP (1.5 h)	0.04 ± 0.00^{c}	1.27 ± 0.10^{a}	nd
0.2U HRP (2 h)	nd	0.21 ± 0.01^{c}	nd
0.2U HRP (18 h)	nd	0.03 ± 0.00^{d}	nd
40.3U TR (0 h)	4.52 ± 0.22^{A}	0.27 ± 0.02^{D}	nd
40.3U TR (1 h)	3.28 ± 0.22^{A}	0.50 ± 0.02^{C}	0.06 ± 0.00^{AB}
40.3U TR (1.5 h)	1.42 ± 0.22^{B}	0.98 ± 0.08^{B}	0.07 ± 0.00^{AB}
40.3U TR (2 h)	0.06 ± 0.01^{C}	1.03 ± 0.03^{AB}	0.07 ± 0.00^{AB}
40.3U TR (18 h)	0.09 ± 0.01^{C}	1.20 ± 0.09^{A}	0.09 ± 0.00^{A}
2U HRP (0 h)	nd	nd	nd
2U HRP (1 h)	nd	nd	nd
2U HRP (2 h)	nd	nd	nd
2U HRP (5 h)	nd	nd	nd
403.0U TR (0 h)	0.24 ± 0.02^{x}	0.20 ± 0.01^{z}	0.05 ± 0.00^{xy}
403.0U TR (1 h)	0.04 ± 0.00^{y}	0.72 ± 0.00^{x}	0.05 ± 0.00^{xy}
403.0U TR (2 h)	0.05 ± 0.00^{y}	0.64 ± 0.01^{y}	0.05 ± 0.00^{xy}
403.0U TR (5 h)	0.03 ± 0.00^{y}	0.73 ± 0.01^{y}	0.03 ± 0.00^{y}

Note: nd, not detected

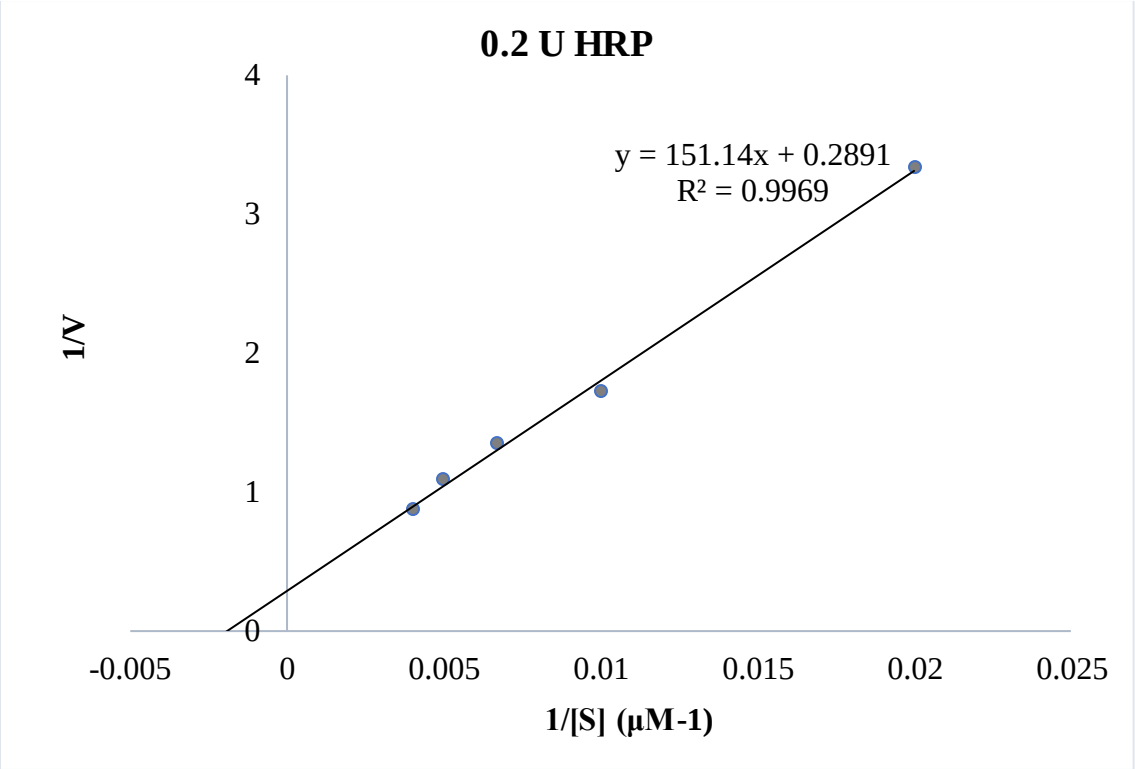
*For each column and enzyme, values with different superscript letters (lowercase or upper case) are significantly different ($p < 0.05$).

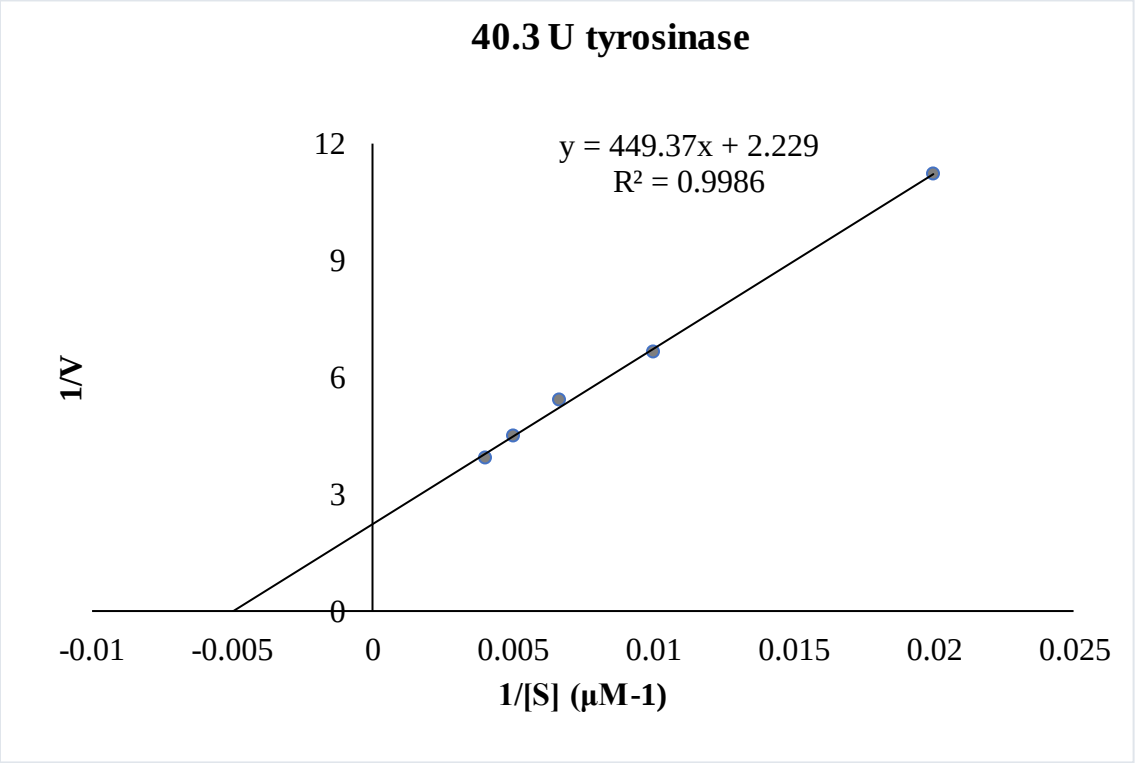
4.5.4 Kinetics of HRP and TR-catalyzed reactions

To demonstrate the enzymatic kinetics, Lineweaver Burk plots were created (Figure 4.1). The K_m value calculated was 604.56 µM and 201.60 µM for HRP and tyrosinase, respectively. The higher K_m value of HRP indicates that to reach the half-maximum concentration, higher substrate content

is needed when compared to tyrosinase. The Vmax values of HRP and tyrosinase are 3.48 ΔA/h and 0.44 ΔA/h, respectively. The catalytic efficiency of both HRP and TR was calculated to demonstrate the ability of both the enzymes to convert SA and sinapine into end products. The CE of HRP was 0.348 which is 8 times higher than that CE of tyrosinase (CE= 0.448). There is a direct relation of higher CE of HRP to the higher V_{max} and lower initial enzyme concentration as reported by Cao et al. (2019).

Figure 4.1 Lineweaver-Burk plots of 0.2 U HRP and 40.3 U TR at various concentration of pure sinapic acid solution





4.5.5 Evaluation of the antioxidant potential of enzymatic extracts

According to our knowledge, very few studies are done on determining the antioxidant activity of enzymatic extracts of oilseeds. Overall, the TPC of UT and AT extracts with 0.2 U HRP and 40.3 U tyrosinase remained non-significant with some exceptions (Table 4.4, 4.5). Increase in incubation time of untreated extract with 0.2 U HRP produced, no significant change in the TPC at 0, 1 and 1.5 h. However, there was a significant decrease observed in UT enzymatic mixture of 0.2 U HRP after 2 h of incubation when compared to control at 0 min (19.19 ± 0.16 and 26.03 ± 0.32 mg/g SAE), respectively. In the case of the UT sample with 40.3 U tyrosinase, TPC remained non-significant at all the incubation times ranging from 29.09 ± 0.22 to 25.21 ± 0.15 mg/SAE (Figure 4.2a). In the case of AT mustard extracts with the enzymes, the same pattern for TPC was observed; no significant changes were observed (Table 4.5). However, the maximum TPC in the case of UT meal fraction was present at 1.5 h for 0.2 U HRP and 2 h for 40.3 U tyrosinase and there was no significant difference observed for the maximum content of UT meal with 0.2 U HRP and 40.3 U tyrosinase as shown in Table 4.4. The same trend of TPC was observed in the study done by Puspita et al. (2017) where there was no significant difference observed in the enzymatic extracts of *S.muticum* when treated with neutrase and Ultraflo after 3 h of incubation. Another study reported a significant increase with the increase in incubation time for the enzymatic extracts of yerba mate and yellow lupine and maximum TPC was observed after 2 h (Krakowska-Sieprawska et al., 2020). The decrease in TPC was observed when enzyme concentration was increased to 2 U HRP and 403 U tyrosinase for AT fraction (Table 4.5). As expected, TPC for the AT extracts of 0.2 U HRP was unquantifiable as all the phenolics were degraded, while for 403 U tyrosinase, a significant decrease was observed when compared to 40.3 U. In the case of TFC, there was a significant increase observed in UT mustard samples with 0.2 U HRP and 40.3 U

tyrosinase. The maximum TFC observed for UT mustard meal with 0.2 U HRP and 40.3 U tyrosinase was observed at 1 h (34.25 ± 0.52 QE/g DW) and 2 h (30.15 ± 0.57 QE/g DW) when compared to the control, respectively. In the case of AT samples with 0.2 U HRP and 40.3 U tyrosinase, a significant increase was observed at 1 hr of incubation time but afterwards there a non-significant change in TFC as shown in Table 4.5. The highest TFC for the untreated sample with 0.2 U HRP was observed at 1.5 h of incubation with an amount of 15.27 ± 0.29 QE/g DW whereas, for 40.3 U tyrosinase, the maximum was observed at 18 h of incubation with an amount of 10.27 ± 0.07 QE/g DW. These results are comparable with the study by Krakowska-Sieprawska et al. (2020) where a significant increase of 10 % was observed in the TFC content of enzymatic extract of green yerba mate after 1 and 2 h of incubation. An increase in the TFC of the enzymatic extracts of non treated and alkaline treated samples could be possible due to the generation of new compounds (enzymatic end products) in the extracts by the degradation of sinapine and sinapic acid.

In the case of FRAP, extracts from HRP and tyrosinase treated UT meal exhibited rapid increases in the reducing power as the incubation time was increased (Table 4.4 and Figure 4.2c). For HRP-catalyzed UT extract, the highest activity was observed at 1.5 h of incubation (127.82 ± 0.47 Fe II µmol/g) when compared to the control at 0 min (87.37 ± 0.14 Fe II µmol/g). There was no significant difference observed at 1.5 and 2 h of incubation for the HR- catalyzed reaction mixture of the UT mustard meal. In the case of the tyrosinase-catalyzed UT mustard meal, there was a rapid increase observed in FRAP value at 1 h of incubation when compared to the control (91.61 ± 0.15 and 74.93 ± 0.18 Fe II µmol/g, respectively). Regarding, AT mustard meal fraction, HRP showed the maximum reducing value of 63.9 ± 0.07 Fe II µmol/g at 1 hr with no significant change at 1.5 h of incubation, but it rapidly reduced to 64.1 ± 0.39 Fe II µmol/g after 18 h of incubation

as shown in Table 4.5 and Figure 4.4c. The increase in FRAP with the increase in time can be related to the study where a significant increase was observed after the 3 h of incubation of enzymatic extracts of *S. muticum*. Increasing the enzymatic concentration had an inverse effect on FRAP values as there was a reduction of almost 84% when tyrosinase was increase to 403 U and compared to control of 40.3 U. The radical scavenging effect of the enzymatic extracts was determined based on two assays that included DPPH assay and ABTS assay. For the DPPH assay, the maximum activity in UT mustard fraction was observed at 1.5 h (78.23 ± 0.16%) for HRP and 18 h (72.32 ± 0.24%) for tyrosinase. There was no significant change observed in UT meal at 1 hr and 1.5 hr for HRP and 2 h and 18 h for tyrosinase. A similar trend was observed for AT meal fraction where maximum radical scavenging activity of DPPH was observed at 1.5 h for HRP and 18 h for tyrosinase (42.78 ± 0.98% and 43.09 ± 0.92%), respectively (Figure 4.2d). This trend is comparable with the study done by Puspita et al. (2017), where 3 h of incubation increased DPPH radical inhibition in enzymatic extracts of brown seaweed. In the case of the ABTS assay, the same trend was observed as the DPPH assay. In UT meal fractions, a rapid increase in radical scavenging effect was observed at 1.5 h (73.64 ± 0.67%) and 18 h (75.54 ± 0.96%) for HRP and tyrosinase, respectively. The ABTS value was increased to 45.78 ± 0.76% at 1.5 h as compared to the control (35.59 ± 0.65%) in the HRP-catalyzed reaction of AT mustard meal. For TR-catalyzed reaction of AT meal, the maximum percentage of 45.62 ± 0.51 was observed at 18 h of incubation. Casas et al. (2019) reported the same trend in ABTS radical scavenging activity of enzymatically extracted fractions of *S. muticum* after an increase in the incubation time.

The increase in TFC and antioxidant activity of enzymes can be explained as the sinapine and sinapic acid degraded they formed new oxidized end products that possess high antioxidant potential. According to Siriwardhana et al. (2008), the increase in phenolic content of the

enzymatic extract was due to the reaction of other molecules including proteins and polysaccharides. These molecules were a reason for the increase in antioxidant activity of *H. fusiform* extracts. Moreover, a relation between TPC and antioxidant activity is reported in the literature which indicates the possibility of neutralization of free radicals after the formation of phenoxy radicals that are very stable and can contribute to the high antioxidant potential of the extracts.

Table 4.4 Effect of enzymatic oxidation on antioxidant activity of extracts of untreated mustard meal after catalysis by horseradish peroxidase (HRP) and tyrosinase (TR)

Sample	TPC (mg/g SAE)	TFC (QE μmol/g)	FRAP (Fe II μmol/g)	DPPH (%)	ABTS (%)
0.2U HRP (0 h)	26.03±0.32[a]	13.59±0.31[c]	87.37±0.14[b]	60.66±0.92[b]	50.24±0.27[b]
0.2U HRP (1 h)	24.55±1.56[a]	34.25±0.52[a]	101.45±0.23[ab]	69.72±0.11[a b]	68.16±0.19[a]
0.2U HRP (1.5 h)	26.77±0.76[a]	26.7±0.52[b]	127.82±0.47[a]	78.23±0.16[a]	73.64±0.37[a]
0.2U HRP (2 h)	19.19±0.16[b]	28.56±0.27[b]	114.73±1.58[a]	75.89±0.54[a]	64.64±0.13[ab]
40.3U TR (0 h)	26.92±0.71[a]	12.12±0.30[b]	74.93±0.18[b]	60.58±0.91[b]	52.52±0.56[b]
40.3U TR (1 h)	25.21±0.15[a]	13.08±0.31[b]	91.61±0.15[a]	66.67±0.90[a b]	63.29±0.12[ab]
40.3U TR (1.5 h)	25.98±0.15[a]	16.57±0.45[b]	88.17±1.39[a]	67.81±0.13[a b]	70.18±0.51[a]
40.3U TR (2 h)	29.09±0.15[a]	30.75±0.57[a]	89.75±1.21[a]	72.32±0.24[a]	75.54±0.96[a]

Note: nd, not detected

*Values in the same column for each enzyme with no superscript letters in common are significantly different.

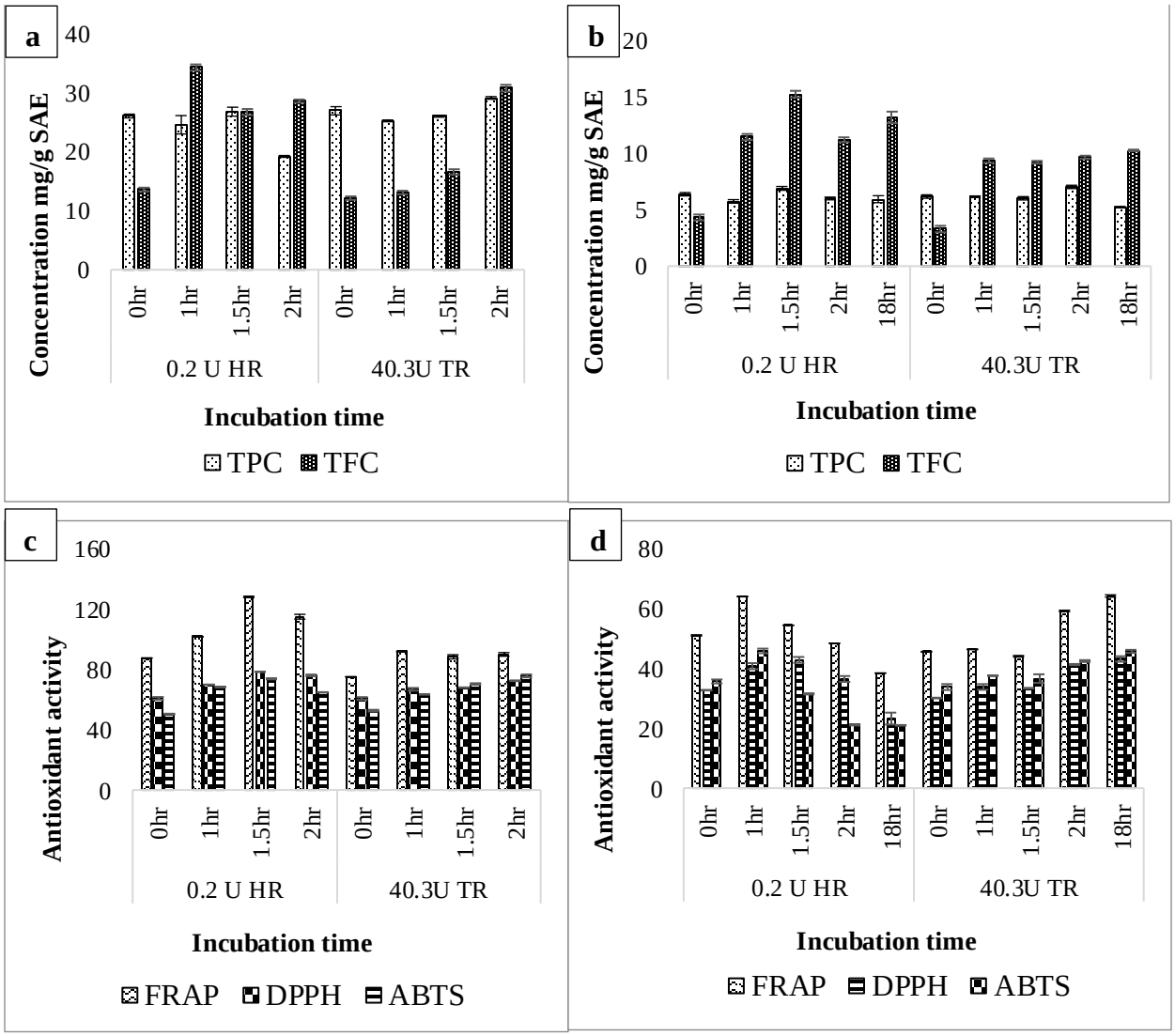
Table 4.5 Effect of enzymatic oxidation on antioxidant activity of alkaline treated LGYMM after catalysis by horseradish peroxidase (HRP) and tyrosinase (TR)

Sample	TPC (mg/g SAE)	TFC (QE μmol/g)	FRAP (Fe II μmol/g)	DPPH (%)	ABTS (%)
0.2U HRP (0 h)	6.38±0.15^{ab}	4.32±0.26^{c}	50.94±0.20^{by}	32.68±0.10^{b}	35.59±0.65^{b}
0.2U HRP (1 h)	5.74±0.16^{b}	11.46±0.27^{a}	63.9±0.07^{a}	40.68±1.02^{a}	45.78±0.76^{a}
0.2U HRP (1.5 h)	6.9±0.17^{a}	15.27±0.29^{ab}	54.33±0.02^{b}	42.78±0.98^{a}	31.45±0.20^{b}
0.2U HRP (2 h)	6±0.17^{ab}	11.22±0.21^{b}	48.33±0.02^{c}	36.41±0.98^{ab}	21.11±0.20^{c}
0.2U HRP (18 h)	5.94±0.12^{ab}	13.2±0.51^{ab}	38.36±0.08^{d}	23.21±1.98^{c}	20.94±0.10
40.3U TR (0 h)	6.18±0.31^{ab}	3.29±0.29	45.48±0.14^{b}	30.06±0.11^{b}	33.72±0.98^{b}
40.3U TR (1 h)	6.16±0.15^{ab}	9.36±0.19^{ab}	46.3±0.11^{b}	33.75±0.95^{b}	37.5±0.11^{b}
40.3U TR (1.5 h)	6.02±0.07^{ab}	9.22±0.12^{ab}	43.98±0.24^{b}	33.35±0.09^{b}	36.54±1.43^{b}
40.3U TR (2 h)	7.03±0.15^{a}	9.66±0.14^{a}	59.11±0.24^{ab}	41.22±0.19^{a}	42.39±0.31^{a}
40.3U TR (18 h)	5.26±0.15^{b}	10.27±0.07^{a}	64.1±0.39^{a}	43.09±0.92^{a}	45.62±0.51^{a}
2.0U HRP (0 h)	nd	nd	nd	nd	nd
2.0U HRP (1 h)	nd	nd	nd	nd	nd
2.0U HRP (1.5 h)	nd	nd	nd	nd	nd
2.0U HRP (2 h)	nd	nd	nd	nd	nd
403.0U TR (0 h)	4.21±0.01^{a}	6.21±0.05^{a}	7.66±0.11^{a}	39.21±0.21^{a}	45.11±0.32^{a}
403.0U TR (1 h)	3.22±0.07^{a}	5.44±0.10^{a}	7.01±0.14^{a}	34.21±0.24^{a}	42.34±0.41^{a}
403.0U TR (2 h)	3.11±0.05^{ab}	5.12±0.02^{a}	6.23±0.04^{a}	30.21±0.11^{ab}	30.21±0.11^{b}
403.0U TR (5 h)	2.11±0.01^{b}	3.22±0.01^{b}	4.01±0.06^{b}	19.01±0.31^{b}	22.87±0.32^{c}

Note: nd, not detected

*Values in the same column for each enzyme with no superscript letters in common are significantly different.

Figure 4.2 Effect of enzymatic oxidation on antioxidant activity of untreated and alkaline treated LGYMM after catalysis by horseradish peroxidase (HRP) and tyrosinase (TR)



*Data is expressed as mean ± SD where n=3

a: TPC and TFC of untreated LGYMM, b: TPC and TFC of alkaline treated mustard meal, c: antioxidant activity of untreated LGYMM, d: antioxidant activity of alkaline treated LGYMM

4.6 Conclusion

This study mainly focused on the effect of oxidation by HRP and tyrosinase on the degradation of major SA derivatives in LGYMM and their antioxidant activity. Increasing enzyme concentration

did not have a positive effect in this study and the maximum generation of phenolic compounds and antioxidant activity was calculated for the extracts catalyzed with low enzymatic concentration. HRP exhibited higher CE, which can be further used for the conversion of SA and sinapine into their by-products in less incubation time. HRP-catalyzed extracts also showed their maximum phenolic extraction and antioxidant activity in less incubation time than the reaction mixture of tyrosinase. This method could be an efficient strategy to use the by-products of mustard to extract high antioxidant property bearing end products, which can be further investigated for the formation of decarboxylated end-products including canolol a very powerful antioxidant.

CHAPTER 5

5. GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

5.1 Conclusions

Mustard is a crop grown with considerable economic value to Canada. Mustard species are rich sources of phenolic compounds. Like canola and rapeseed, SA derivatives are the most important phenolic compounds in mustard. However, no documentation on the quantitative profile of sinapates and related antioxidant from lower grade yellow mustard seeds is available. Therefore, the current research was carried out to fill the mentioned gaps with the following major outcomes:

1. Air frying at 180 °C is an efficient and economic method for the extraction of phenolics from mustard seeds that exhibit high antioxidant properties.

2. 180 °C proved to be the best pretreatment temperature to obtain maximum antioxidant potential.

3. The extracts at 180 °C displayed significant radical scavenging activity due to the presence of thermogenerative compounds which were the major contributor to the high antioxidant activity.

4. The major identified phenolic in all extracts during air frying was sinapine followed by syringic acid and methyl sinapate. Sinapic acid was present in relatively very less amount only in the control sample.

5. Enzymatic extraction of untreated LGYMM with 0.2 U HRP and 40.3 U tyrosinase proved to be the best technique for the extraction of end products with high antioxidant potential.

6. Increasing the enzyme concentration resulted in a decrease in phenolic content and antioxidant activity.

Comparison of air frying and enzymatic oxidation gave the major outcomes as follow:

1. The enzymatic extracts exhibited higher antioxidant potential than the air fried extract.
2. TPC, TFC, FRAP, DPPH and ABTS of enzymatic extracts was higher than the of maximum recorded for air fried extracts.
3. Moreover, in the case of air frying, most of the phenolic compounds decreased with the increase in temperature while in enzymatic extracts, only sinapine and SA showed a decreasing behavior.
4. Most compounds in enzymatically treated mustard meal remained unchanged except for the main compounds that are mentioned.
5. Enzymatic oxidation proved to be a better extraction technique for the extraction of polyphenolic compounds.
6. Enzymatic end products exhibited higher antioxidant potential than the thermo-generative end products in the mustard meal.

5.2 Future perspectives

The research reported in this thesis investigated many novel aspects including the effect of two innovative extraction methods (air frying and enzymatic oxidation) on phenolic contents lower grade yellow mustard seeds and their corresponding antioxidant potential. However, many questions remain to be addressed in future studies. The suggestions listed below are those which should be given priority in the future research effort.

1. Results of this study indicated air frying and enzymatic oxidation as an efficient technique for extracting phenolic compounds from mustard seeds. However, from the industrial viewpoint, further research work is needed to expand the findings for food-extraction and processing applications.

2. Replacing or combining other enzymes with HRP and tyrosinase should be explored in future studies.

3. In vitro, antioxidant assays confirmed mustard extracts as natural antioxidants. Further in vivo and food system studies are also needed to confirm the antioxidant nature of mustard phenolic extracts.

4. The enzymatic end products and thermo-generative compounds can further be studied for the generation of canolol which is a compound with high antioxidant potential.

5. As sinapic acid and its derivatives exhibit beneficial health properties, mustard extracts need to be considered attractive ingredients in the development of functional foods with health benefits.

6. Further study should be done to examine the sensory properties and consumer acceptability of food products prepared with mustard extracts as a natural antioxidant or antimicrobial agents.

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APPENDIX 1

Figure 1 Experimental design of Study 1

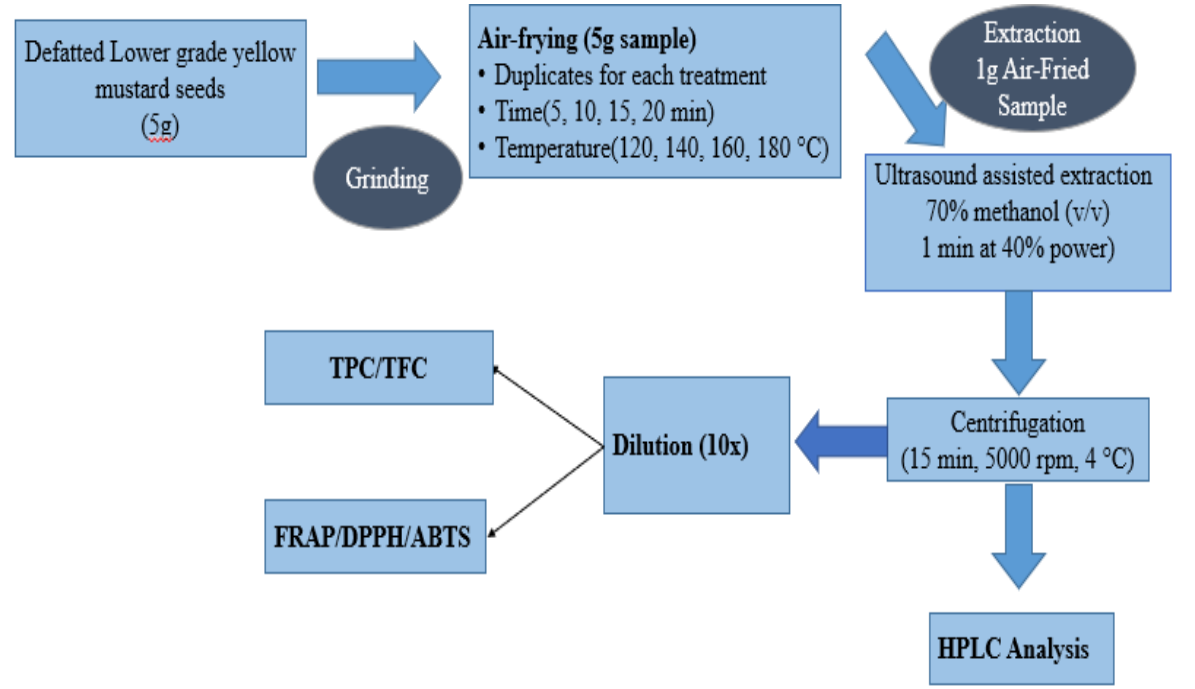
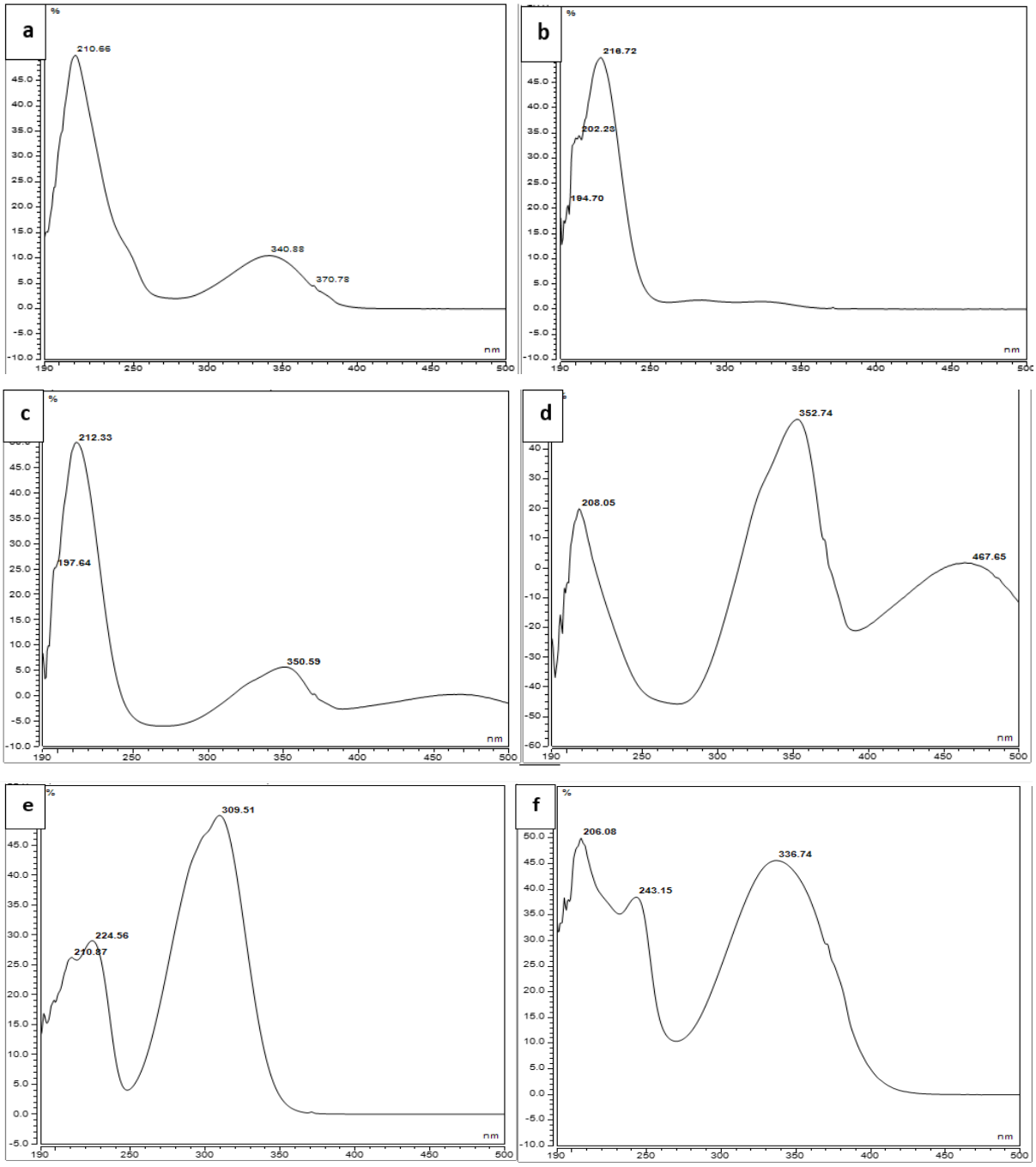


Table 1 Retention time and detection wavelength of major peaks in LGYMM during air frying

Peak	Retention time (min)	Detected Wavelength (nm)	Identity
1	4.1	270	unidentified
2	6.1	270	unidentified
3	6.4	270	unidentified
4	7.7	270	unidentified
5	9.5	270 and 330	unidentified
6	12.6	270 and 330	**Sinapine**
7	15.8	270	**Syringic acid**
8	16.01	270	unidentified
9	21.6	330	**Sinapic acid**
10	32.6	330	**Methyl sinapate**
11	34.01	270	Unidentified

APPENDIX 2

Figure 2 UV-Vis spectra of unknown compounds for enzymatic oxidation of LGYMM



a= Compound 1, b= Compound 2, c= Unknown 1, d= Unknown 2, e= Unknown 3, f= p-coumaric acid