Antioxidant and antibacterial properties of endogenous phenolic compounds from commercial mustard products

By

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Abstract

This study investigated the antioxidant and antimicrobial properties of endogenous phenolic compounds in Oriental (*Brassica junceae*) and yellow (*Sinapis alba*) mustard seeds. Phenolics in selected Canadian mustard products (seeds/ powder/ flour) were extracted using Accelerated Solvent Extraction (ASE) and their corresponding sinapate profiles were established through HPLC-DAD analysis. The antioxidant capacity of each extract was assessed by DPPH assay and correlated with the total phenolic content (TPC) measured using the Folin–Ciocalteau method. Sinapine was the major phenolic compound in all the samples analysed, with negligible amounts of sinapic acid. The sinapine content, expressed as sinapic acid equivalents (SAE), ranged from $5.36 \times 10^3 \pm 0.66$ to $14.44 \pm 0.43 \times 10^3$ µg SAE/g dry weight of the samples, with the highest in the yellow mustard seed extract and lowest in Oriental mustard powder. The level decreased in the following order: yellow mustard seed > Oriental mustard seed > yellow mustard bran > Oriental mustard bran > yellow mustard powder > Oriental mustard powder. Extracts from yellow mustard seeds had the highest TPC ($17.61\times 10^3 \pm 1.01$ µg SAE/g), while Oriental mustard powder showed the lowest TPC with $4.14 \times 10^3 \pm 0.92$ µg SAE/g. The DPPH radical scavenging activity of mustard methanolic extracts ranged between 36% and 69%, with the following order for both varieties: ground mustard seed > mustard bran > mustard powder. The antioxidant activities of the extracts correlated with their TPC (correlation coefficients were ≥ 0.72).This study confirmed that Canadian yellow and Oriental mustard varieties and their products are rich sources of endogenous phenolic compounds.

The antimicrobial effectiveness of Oriental (1071 ppm sinapine) and yellow (1200 ppm sinapine) mustard seed phenolic extracts, and of sinapic acid standard in two different concentrations (1200, 3000 ppm) against five strains of *E. coli* O157:H7 (02-0627, 02-0628, 02-

0304, 00-3581and non motile 02-1840) and three strains of *L. monocytogenes* (GLM-3, GLM-4, 2–243) were investigated using minimum inhibitory concentration (MIC) assay. The MICs were determined with a microdilution method using 96-well microplate platforms. The tested concentrations of sinapine and sinapic acid standard had no antibacterial activity against all *E. coli* O157:H7 and *L. monocytogenes* strains at 35 ºC and pH 7.

The effect of various pre-treatments such as microwave irradiation (20 min, 300 W) and also 2 h soaking with 70% methanol (with and without acidification) on the sinapates profile and contents of selected defatted mustard products were investigated. Microwave irradiation did not affect the phenolic profile significantly as the sinapine content of yellow mustard bran (6.87×10^3 ±0.47 µg SAE/g), yellow mustard powder (19.31×10^3 ±0.01 µg SAE/g), Oriental mustard bran (7.28×10^3 ±0.06 µg SAE/g), and Oriental mustard powder (12.19×10^3 ±2.07 µg SAE/g) were similar to their corresponding untreated samples. However, the soaking process, irrespective of its pH, significantly reduced the sinapine content in all investigated samples. Soaking in acidified 70% methanol resulted in further decreases in the sinapine values to reach 4.73×10^3, 10.82×10^3, 3.25×10^3 and 10.01×10^3 µg SAE/g in yellow mustard bran, yellow mustard powder, Oriental mustard bran and Oriental mustard powder, respectively.

Keywords: Mustard, ASE, phenolics, sinapates, *E. coli* O157:H7, *L. monocytogenes*.

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Most of all, my deepest love and gratitude goes to my parents for their unflagging love and support throughout my life. This work would not have been accomplished without their encouragement, endless patience and continuous support.

Dedication

It is an honour to dedicate this thesis to my lovely parents

Thesis Format

The current thesis is comprised of five chapters including an overall introduction to the subjects of the study (Chapter 1), a literature review (Chapter 2), two manuscripts (Chapters 3 and 4), and one short report (Chapter 5) prepared for publication in peer reviewed scientific journals. Manuscripts were standardized for presentation in the thesis format and they are identified as follows:

Chapter 3 entitled "*In vitro* antioxidant activity of endogenous phenolic compounds from commercial mustard products and wasabi" was prepared for submission to the journal of European Food Research and Technology with authorship by Fahmi, R., Eskin, N.A.M, Eck, P., and Thiyam-Hollander, U.

Chapter 4 "Antimicrobial activity of phenolic compounds from yellow and Oriental mustard seed" was prepared for submission to the Journal of Food Science with authorship by Fahmi, R., Cordeiro, R.P., Eck, P., Thiyam-Hollander, U., and Holley, R.A.

Chapter 5 entitled "Impact of pre-treatments on the sinapate profile of mustard products" by Fahmi, R., Aachary, A.A., Eck, P., and Thiyam-Hollander, U. was prepared for submission to the Journal of Food Science and Technology.

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Chapter 1

General introduction

Mustard seeds have been used as condiments for thousands of years. Canada is one of the most important producers and exporters of the crop worldwide (Clancey, 2013). The average value of Canadian mustard seed exports is around $128 million dollars per year (Benfey et al., 2005) and accounts for more than 57% of the international spice market share. The three major types of condiment mustard seeds are yellow, brown and Oriental all currently cultivated in Canada (Clancey, 2013). Oriental and brown varieties belong to the *Brassica junceae* species of *Brassicacea* family and contain higher amounts of fixed oil, whereas the yellow type belongs to the *Sinapis alba* species which normally has lower concentrations of oil, but higher protein content and stronger mucilage properties (Cui and Eskin, 1998). Though mustard seeds are mainly used as condiments, various products including cooking oil, ground seeds, powder/flour, bran and prepared mustard pastes are commercially available in the market.

Endogenous phenolic compounds from plant materials have received considerable attention due to their biological activities and health benefits over the past few years. Phenolic compounds are an extensive group of plant secondary metabolites which function as antioxidants, defensive or signaling compounds (Balasundram et al., 2006; Parr and Bolwell, 2000). Sinapic acid and its derivatives (SADs) are the predominant phenolic compounds in *Brassicacea* species. Various health benefits such as antioxidant, antimicrobial, anticancer, anti-anxiety, and anti-inflammatory activities have been attributed to the SADs (Nićiforović and Abramovič, 2014). The antioxidant and antimicrobial properties of sinapic acids and its

derivatives are of specific interest in this research. Sinapic acid has been reported to be an effective antioxidant (Kikuzaki et al., 2002; Natella et al., 1999; Nićiforović and Abramovič, 2014; Zou et al., 2002) with antimicrobial activity against various Gram-negative (*Escherichia coli*, *Enterobacter aerogenes*, and *Pseudomonas fluorescens*) and Gram-positive (*Bacillus subtilis*, *Bacillus cereus*, *Streptococcus lactis*, and *Streptococcus cremoris*) bacteria (Barber et al., 2000; Engels et al., 2012; Lyon and McGill, 1988; Nowak et al., 1992; Tesaki et al., 1998).

Traditional techniques such as solid/liquid extraction have been utilized for extracting phenolic compounds from mustard seed products and other members of the *Brassicacea* family. However, conventional methods of extraction have many drawbacks such as long extraction times and high solvent consumption (Ajila et al., 2011; Luthria et al., 2004). To overcome the limitations of traditional methods of extraction, various techniques have been developed over the past few years (Ajila et al., 2011; Carabias-Martínez et al., 2005; Richter et al., 1996). One such technique is Accelerated Solvent Extraction (ASE), an automated extraction technique that rapidly performs solvent extraction using a combination of high temperature (50–200°C) and high pressure (1450–2175 psi). Maintaining the extraction solvent in its liquid state at high temperature, results in a more efficient extraction procedure, shortened extraction time, and a lower solvent consumption by increasing the solvent diffusion rate, mass transfer and the solubility of target compounds (Ajila et al., 2011; Co et al., 2009; Luthria et al., 2004; Mustafa and Turner, 2011). ASE technique has been successfully used for extracting polyphenols from a variety of plant materials (Bonoli et al., 2004; Cacace and Mazza, 2006; Co et al., 2009; Hossain et al., 2011; Okuda et al., 2009).

Based on the available literature, mustard seeds are rich sources of endogenous phenolic compounds with antioxidant (Mayengbam et al., 2014, Khattab et al., 2010; Thiyam et al., 2006) and antimicrobial activities that could be used as a natural preservative in the food industry (Nićiforović and Abramovič, 2014; Dubie et al., 2013; Engels et al., 2012). However, few studies have investigated the individual identity, quantity and antioxidant activity of phenolics derived from mustard seed commercial products. Also, no study has reported the extraction of mustard phenolics using ASE technique. In the above context, the major objectives of this research were to:

- Identify and quantify the phenolic compounds in Oriental (*Brassica junceae*) and yellow (*Sinapis alba*) mustard seed commercial products including ground seed, powder and bran fractions using high performance liquid chromatography (HPLC-DAD) technique.
- Develop an improved Accelerated Solvent Extraction (ASE) process for the extraction of endogenous phenolic compounds from the studied products.
- Evaluate the antioxidant potential of mustard phenolic extracts *in vitro.*
- Assess the antimicrobial potential of the yellow and Oriental mustard seed phenolic extracts against pathogenic food microbes including *Escherichia coli* and *Listeria monocytogenes* (Figure 1.1).
- Investigate the effect of different pre-treatments (microwave irradiation and soaking) on the phenolic profile of mustard commercial products.

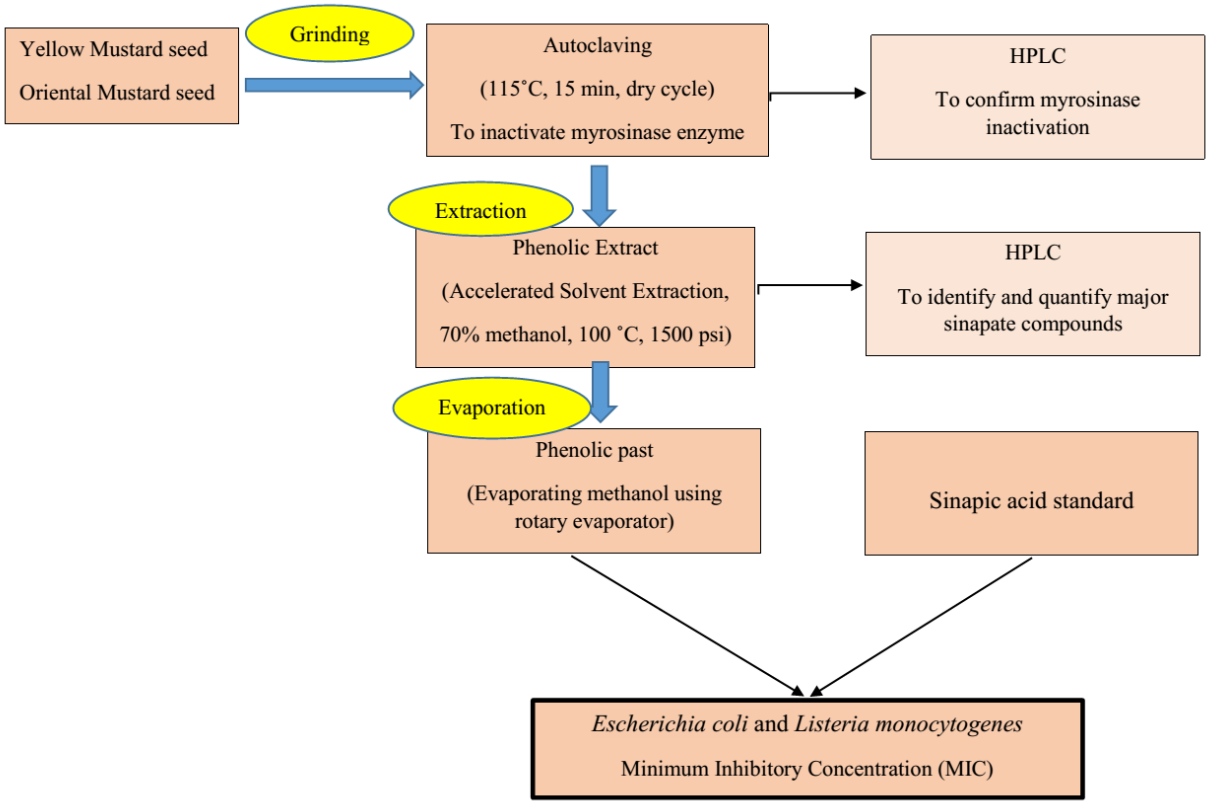

Figure 1-1 Experimental design for assessing the antimicrobial activity in yellow and Oriental mustard seed phenolic extracts

Chapter 2

Literature Review: Mustard Seeds and their Phenolic Compounds

2.1 Mustard seed

Mustard seeds have been used as condiments for more than 5,000 years in ancient cultures such as Romans, Egyptian, Sumerian and Chinese. With almost 529,000 tons production per year, mustard is considered as the major spice in international trade (Clancey, 2013).

The mustard plant is a member of the *Brassicaceae* family, also known as crucifers or the cabbage family, which bears characteristic four petal yellow flowers as well as tiny round edible seeds. Different varieties of mustard include white or yellow mustard (*Sinapis alba*); Oriental, brown or Indian mustard (*Brassica juncea*), and black mustard (*Brassica nigra*). Food crops such as rapeseed, canola, cabbage, broccoli, turnip, cauliflower, radish, horseradish and wasabi are also members of the *Brassicaceae* family (Cartea et al., 2010a).

Mustard is an annual cool season plant that requires long days and a relatively short growing season. Depending on the seeding time and growing condition, the yellow variety of mustard seeds mature in 85 to 95 days, whereas Oriental and brown types require 95 to 105 days. Mustard is usually cultivated in rotation with small grains such as cereal crops. In comparison to other *Brassicaceae* members such as canola, mustard seedlings have a higher tolerance to harsh growing conditions such as drought, frost and heat. However, excessive moisture and heat stress while flowering can result in a lower seed yield in *Brassica* crops. Mustard seeds can grow under both rain-fed and irrigation systems but are mainly produced under irrigated conditions in western Canada (Benfey et al., 2005).

Considering the favorable growing condition, mustard is mainly cultivated in the northern hemisphere. Canada is the most important producer of mustard with 28% of world production, followed mainly by countries such as Nepal, Myanmar, Ukraine and Russia (Figure 2.1). Canada is also the dominant exporter of mustard seed with an average of 57% of the international spice market share (Clancey, 2013) and almost 128 million dollars annual economic value. Canadian mustard seed is mainly exported to the United States with an average of 43%, followed by Germany and Belgium at 19% each, and also Japan and the Netherlands at 4% each (Benfey et al., 2005). Asian countries use mustard seed mainly as oilseed crops, while European countries, Canada and the United States use it primarily for the condiment and spice trade (Clancey, 2013).

Although Canada is a major producer of pure mustard seed globally, it is a minor crop accounting for only 3% of all specialty crop production in Canada with an average of 160,000 tonnes annual production (Clancey, 2013). Yellow, brown and Oriental are three types of condiment mustard that are mainly grown in Canada and comprise almost 57%, 22% and 16% of the total production area, respectively (Clancey, 2013). The Prairie Provinces of Saskatchewan and Alberta are Canada's primary mustard seed producers. Table 2.1 shows the seeded area (hectares) and production (metric tonnes) for western Canadian mustard from 2005 to 2015. It is estimated that more than 75% of seeded area (131,827 ha) belong to Saskatchewan, with an average of almost 120,000 metric tons annual production. Alberta also supplies about 38,000 tonnes of mustard seed per year, using 37,691 ha of its prairie regions (Government of Canada, 2015). Yellow, brown and Oriental are three types of condiment mustard that are mainly grown in Canada and account for almost 57%, 22% and 16% of the total production area, respectively (Clancey, 2013).

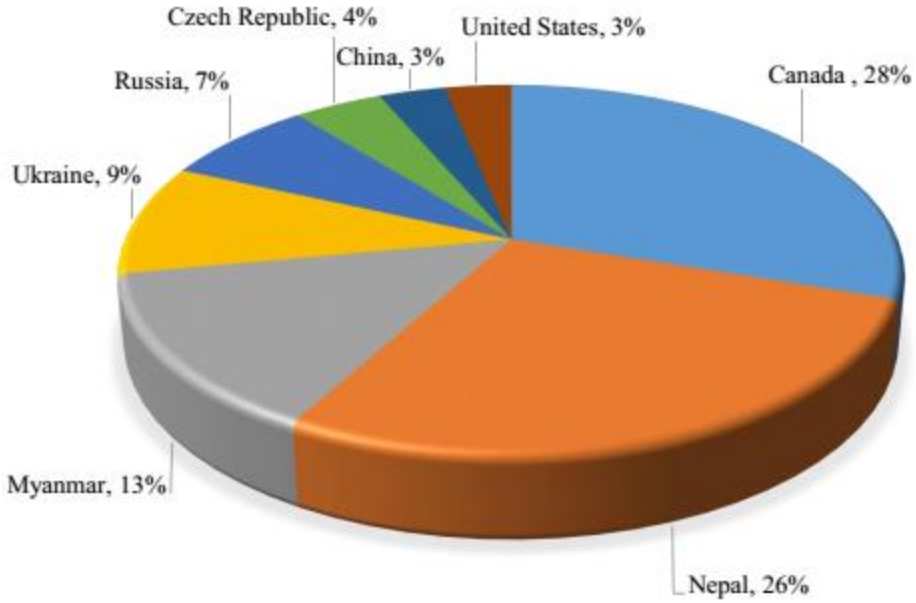

Figure 2-1 World's top ten mustard seed producers (Clancy, 2013).

Table 0-1 Seeded area (hectares) and production (metric tonnes) for western Canadian mustard (from 2005 to 2015).

Year	Alberta		Saskatchewan		Prairie provinces	
	Seeded area (ha)	Production (tonnes)	Seeded area (ha)	Production (tonnes)	Seeded area (ha)	Production (tonnes)
2005	32,300	31,100	161,800	152,700	194,100	183,800
2006	25,200	25,600	108,600	82,600	133,800	108,200
2007	34,400	29,500	151,800	95,300	186,200	124,800
2008	40,400	37,200	149,700	123,900	190,100	161,100
2009	48,500	47,700	163,900	160,600	212,400	208,300
2010	40,500	47,700	149,700	134,300	190,200	182,000
2011	25,400	26,800	107,300	103,200	132,700	130,000
2012	38,400	35,900	97,100	82,700	135,500	118,600
2013	38,500	37,200	109,300	117,300	147,800	154,500
2014	54,600	59,400	147,700	138,600	202,300	198,000
2015	36,400	31,900	103,200	915,00	139,600	123,400
Average	37,691	37,273	131,827	119,120	169,518	153,882

Data source: Statistics Canada (Government of Canada, 2015).

2.1.1 Processing of mustard and mustard seed products

Figure 2.2 shows the production process of commercial mustard seed products. After harvest, mustard seeds should be dried to an appropriate moisture content of 9% and then stored at temperatures lower than 20 °C. The drying process is essential to prevent mold growth, increase shelf life and guarantee long-time storage. Partial drying of the crop starts immediately on the farm and then is completed at the processing plants. Drying temperature should not be higher than 43 °C, otherwise it might damage the seed (Benfey et al., 2005; Cui and Eskin, 1998).

Prior to milling and processing the mustard seeds into various products such as flour, bran and ground mustard, the crop is usually partially deoleated to facilitate the crushing process. Mustard flour is a fine powder obtained from the seed kernel (endosperm). It is prepared by successive milling and sifting to remove the bran (testa and aleurone layer) from the interior part (embryo and cotyledons) of the seed. Mustard flours are used primarily to give mustard flavour to a product and for their functionality as emulsifiers. They are commonly used as binding agent in dressings, sauces, pickles and processed meat products, especially sausages. Mustard bran is the by-product from flour production, which is widely used as a natural thickener in sauces and dressings. The majority of the functional properties of mustard bran such as water-banding, emulsifying, and stabilizing are mainly attributed to the present of water-soluble polysaccharides in its structure. Ground mustard is another commercial product that is produced from grinding the whole mustard seed including the kernel and bran parts. It is widely used in processed meat products such as, salami, bologna, and frankfurters for flavoring, emulsifying, water-binding and also as a bulking agent for the inexpensive replacement of meat with vegetable protein sources. Ground mustard is also used in the production of pickles, sauces and dressings. Altogether, the

mucilaginous compounds of the bran part play an important role in the functional properties of ground mustard (Cui and Eskin, 1998).

Mustard oil is cold extracted from mustard seeds, which depending on the mustard variety, contain between 29% and 36% fixed oil. Residues from the extracted seed kernels are known as "press cake". Well refined mustard oil has pleasurable flavour with a brownish yellow colour. In the European countries and North America where mustard seed is primary used as condiment, only small portions are cold pressed for extracting the oil. However, in Asian countries such as India, mustard seed is more likely used to produce mustard cooking oil (Cui and Eskin, 1998).

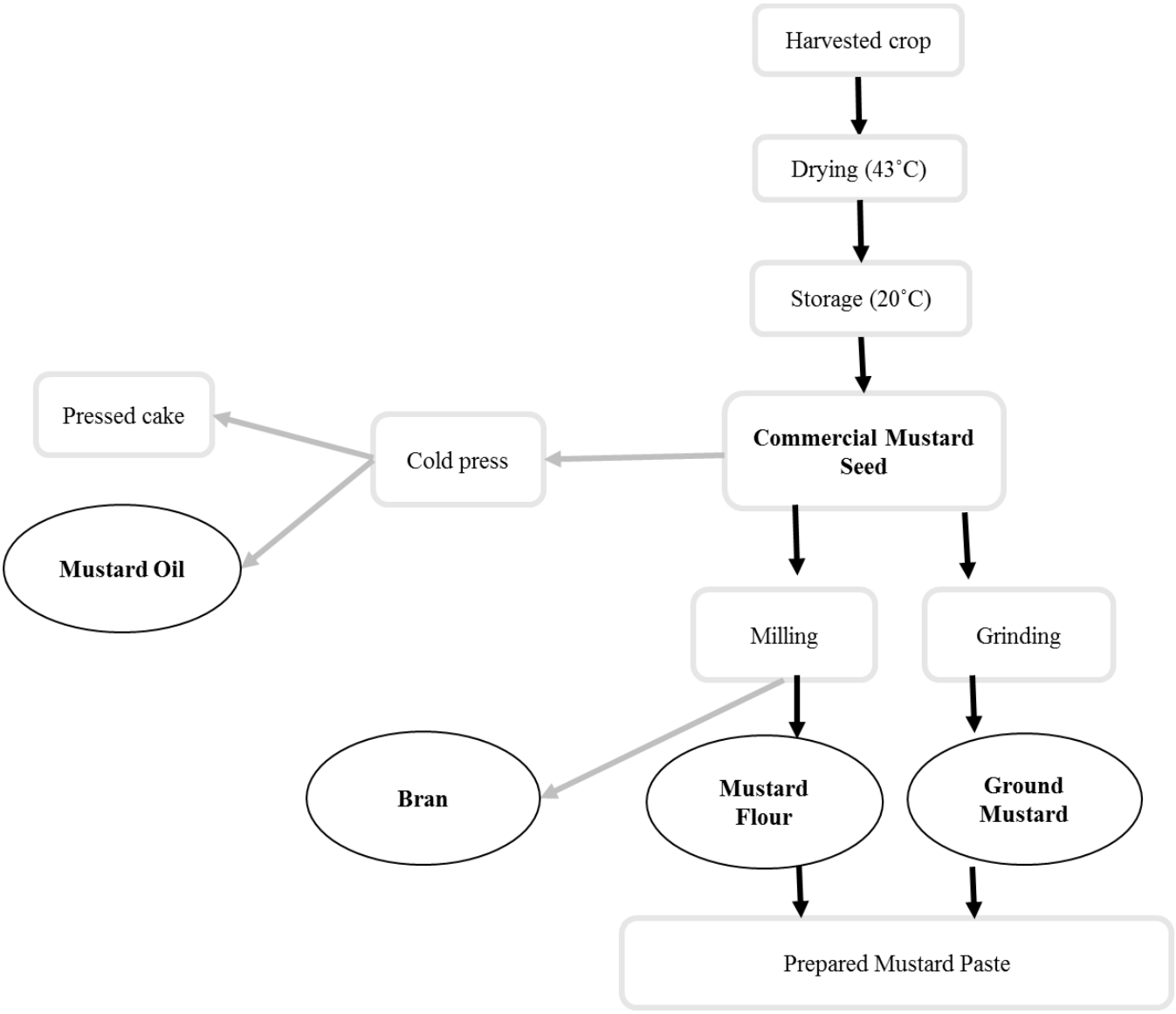

Figure 2-2 Processing diagram of mustard seed's commercial products (Cui and Eskin, 1998).

2.1.2 Mustard Components

Mustard seeds are rich sources of energy, having on average 23-30% fixed oil, 29-36% protein and 12-18% carbohydrate content. Minor compounds such as minerals, isothiocyanates, and phytin are also present in mustard seeds (Table 2.2). Depending on the variety of mustard, area of cultivation and condition of growth, the chemical composition of the crop varies considerably. Usually, Oriental and brown varieties of mustard seeds contain higher amounts of fixed oil, whereas, yellow varieties have lower concentrations of oil and higher protein and mucilage. (Cui and Eskin, 1998). The average oil and protein contents for Oriental, brown and yellow mustard crops harvested between 2002 and 2011 are summarized in Table 2.3 (Siemens, 2012). Also, the chemical composition varies significantly according to the type of mustard products. For instance, flour product contains higher concentration of oil and protein (30-42% and 30-35%, respectively), whereas, the bran fraction is considered a rich source of fibre (15%) with lower amounts of oil (7%) and protein (13-16%) (Cui and Eskin, 1998).

Mustard seeds, like other oilseed crops, are also rich sources of natural antioxidants that prevent the oxidation of the oil such as phenolic compounds and tocopherols (Amarowicz et al., 1996).

2.1.3 Mustard allergy

In spite of being a rich source of dietary bioactive nutrients (Rudrappa, 2009), mustard seed and its products are among the most important food allergens, especially in countries where the consumption of mustard seed is high, such as France. In young children mustard seed products are the fourth most common food allergen source. The principle allergens from yellow and oriental mustard seeds are storage proteins of the 2S albumin class known as Sin a 1 and Bra

j 1, respectively. These proteins are highly heat stable and resistant to digestion by trypsin and other digestive enzymes (Monsalve et al., 2001; Rance, 2003).

Table 0-2 Mustard seed components (Cui and Eskin, 1998).

Components	Percentage
Protein	23-30%
Fixed Oil	29-36%
Carbohydrate	12-18%
Minerals	4%
Phytin	2-3%
Isothiocyanates	0.8-2.3%

Table 2-3 Mean oil and protein content of western Canadian mustard seeds, 2002-2011 (Siemens, 2012).

Mustard Product	Oil%	Protein%
Oriental Mustard Seed	41.9%	26.8%
Brown Mustard Seed	39.3%	26.8%
Yellow Mustard Seed	30.3%	32.0%

2.2. Phenolics

Phenolic compounds are a large group of phytochemicals that occur naturally in a wide variety of plants and contain at least one aromatic ring and different numbers of hydroxyl substituents. They are produced from phenylalanine as secondary metabolite derivatives through the shikimate pathway with phenylalanine ammonia-lyase as the first key enzyme. Their structure can vary from a single-ringed phenolic compound to a very complicated polyphenol with high degree of polymerization. Phenolic compounds play various physiological and ecological roles in the growth and reproduction of plants such as controlling growth hormones, facilitating pollination by attracting insects, and providing protection against herbivores, insects, and pathogens such as fungi, bacteria and viruses (Cartea et al., 2010). In epidemiological studies phenolic compounds were associated with many health benefits such as anti-inflammatory, anti-bacterial, anti-allergic, cardio protective, and anti-carcinogenic activity (Cartea et al., 2010; Crozier et al., 2009). One of the key actions of dietary phenolics is their antioxidant activity (Fukumoto and Mazza, 2000; Podsędek, 2007) which is attributed to their redox properties, free radical scavenging, chain breaking, metal chelating, and other biological activities (Rice-Evans et al., 1996; Shahidi et al., 1992).

Polyphenols are classified into various categories as displayed in Figure 2.3. Flavonoids, lignans, tannins, and phenolic acids are the four dominant phenolic compounds in the plant kingdom. Phenolic acids are divided into two groups: hydroxycinnamic acids and hydroxybenzoic acids. Hydroxycinnamates are phenylpropanoid compounds characterized by the C6-C3 structure. The major derivatives of cinnamic acid which occur widely in various crops including fruits, vegetables, cereals, legumes and oilseeds are caffeic, ferulic, *p*-coumaric, and

sinapic acids, which mainly exist as conjugates of sugar or other hydroxycinnamic acids (Shahidi and Chandrasekara, 2009; Shahidi and Naczk, 2004).

2.2.1 Sinapates

Sinapic acid and its derivatives (sinapates) have been reported to be characteristic phenolic compounds in oilseed crops belonging to *Brassicacea* species such as rapeseed, canola (*Brassica napus L*.) and mustard (*Brassica juncea/ Sinapis alba*) (Nićiforović and Abramovič, 2014). Sinapic acid (3, 5-dimethoxy-4-hydroxycinnamic acid) can be found in both free and esterified forms. Sinapine, the choline ester of sinapic acid, and sinapoyl glucose (1-O-β-D glucopyranosyl sinapate), the sugar ester of sinapic acid, are two common sinapoyl esters in oil seeds. Canolol (4-vinylsyringol) is also another derivative of sinapic acid which is produced mainly as a result of the decarboxylation of sinapic acid under the combination of high temperature and pressure in the oil extraction process. Figure 2.4 shows the chemical structure of some sinapic acid derivatives (Nićiforović and Abramovič, 2014).

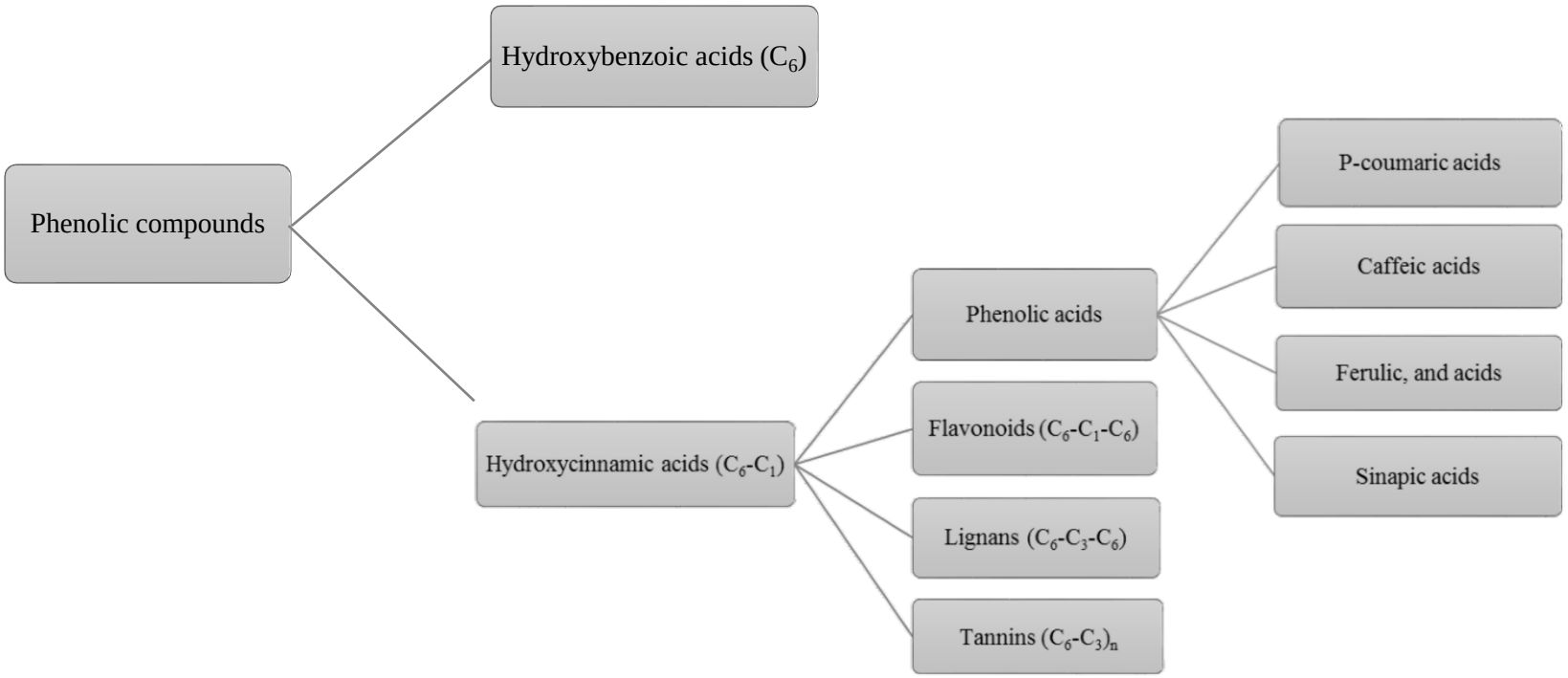

Figure 0-3 Phenolic compound classes (Shahidi and Chandrasekara, 2009; Shahidi and Naczk, 2004).

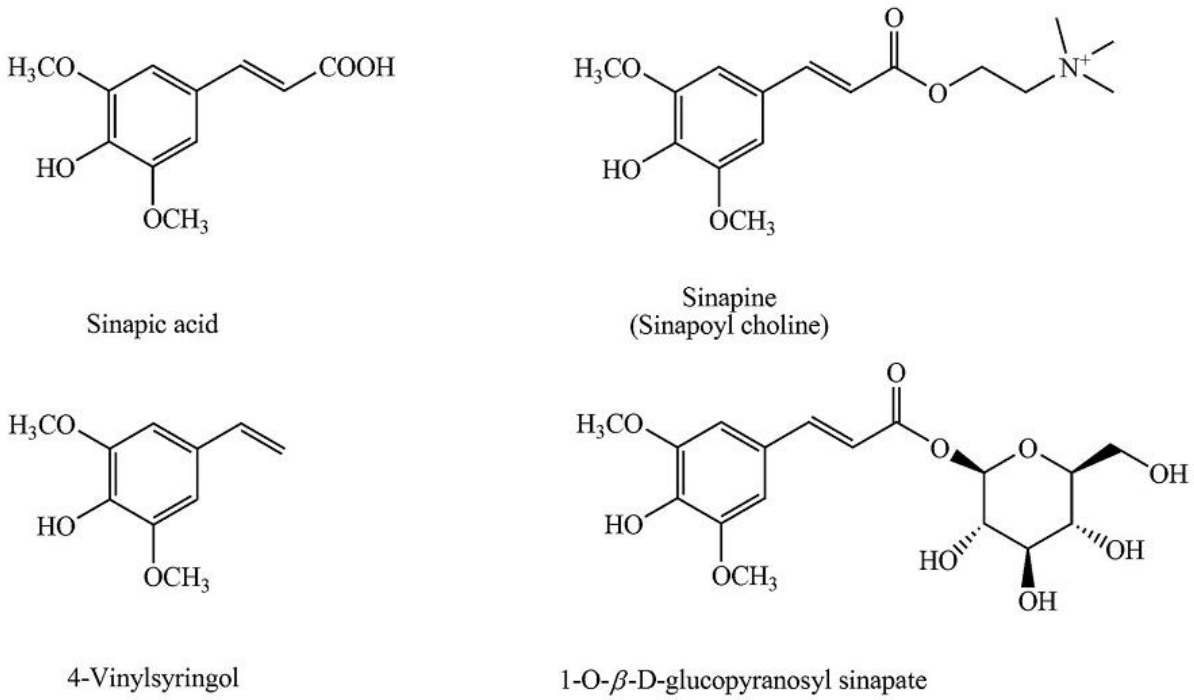

Figure 0-4 Structure of sinapic acid and related compounds.

Sinapine ester was reported to be the major form of sinapate, accounting for almost 80% of the total phenolic content in canola seeds (Kozlowska et al., 1990). Similar results were noted by Khattab et al. (2010) where sinapine constituted more than 70% and 87% of the total phenolics in canola seeds and canola press cakes, respectively. Sinapic acid was detected only in trace amounts (Khattab et al., 2010). Table 2.4 shows the amounts of sinapic acid and its derivatives in different *Brassicaceae* oilseed crops.

The contribution of sinapine, sinapoyl glucose and sinapic acid to the total phenolic content of rapeseed press cake was estimated to be 55% to 70%, 14% to 27% and 6% to 14%, respectively (Thiyam et al., 2006).

In mustard meal, sinapic acid represented over 73% of free phenolic acids and about 80-99% of the total phenolic acids (Das et al., 2009). This was in agreement with the results from Thiyam and others (2006) who identified sinapine as the main sinapic acid derivative accounting for more than 90% of the total phenolic compounds in mustard meal extracts.

Table 0-4 Contents of sinapic acid and its derivatives in some *Brassicaceae* oilseed crops.

Source	Sinapine (mg/g)	Sinapic acid (mg/g)	Sinapoyl glucose (mg/g)	Reference
Canola flour (*Brassica campestris)*	12.03	0.39	ND[a]	(Cai and Arntfield, 2001)
Canola meal (*Brassica campestris)*	11.38	0.24	ND	
Rapeseed meal (*Brassica rapa*)	5.04	0.45	ND	(Vuorela et al., 2003)
Canola seeds (*Brassica napus L.*)	8.59-11.89	0.09-0.28	1.13-6.65	(Khattab et al., 2010)
Canola press cakes	9.90-11.31	0.29-0.44	2.37-4.01	
Canola meals	6.11-10.11	0.32-0.41	1.35-1.99	
Rapeseed seeds (*Brassica napus L.*)	10.70	0.59	5.82	
Indian mustard seeds (*Brassica juncea*)	10.94	0.15	0.5	
Oriental mustard seed meal (*Brassica juncea*)	NR[b]	2.66[C]	ND	(Engels et al., 2012)
Canola seeds (*Brassica napus)*	8.35	0.15	5.45	(Mayengbam et al., 2014)
Mustard seeds (*Brassica juncea)*	10.17	0.19	0.66	

[a] Not detected.

[b] Not reported: sinapine was detected as the dominant phenolic compound. However, the exact amount was not reported.

[c] the amount of sinapic acid in crude extract was reported after alkaline hydrolysis.

Sinapic acid has been reported to be an effective antioxidant (Kikuzaki et al., 2002; Natella et al., 1999; Nićiforović and Abramovič, 2014; Zou et al., 2002). In comparison to other hydroxycinnamic acids, the antioxidant activity of sinapic acid was reported higher than ferulic acid and *p*-coumaric, but lower than caffeic acid with the following order: caffeic acid > sinapic acid > ferulic acid > *p*-coumaric acid (Hotta et al., 2002; Jin et al., 2010). Kikuzaki et al. (2002) evaluated the DPPH radical scavenging activity of sinapic acid (33.2%) to be close to butylated hydroxytoluene (BHT) (29.2%) and comparable to α-tocopherol (41.8%) and caffeic acid (49.6%).

Thiyam et al. (2006) found that the DPPH˙ scavenging activity of sinapic acid was higher than its derivatives with the following order: sinapic acid >sinapoyl glucose>sinapine. This reduction in the antioxidant activity was mainly attributed to the addition of the glucose moiety to sinapic acid in sinapoyl glucose and the further esterification of sinapic acid in sinapine. These were in accordance to previous studies where the DPPH˙ radical scavenging activity of 6-O-sinapoyl sucrose, a sinapoyl glycoside with sucrose as its sugar moiety, were reported to be lower than sinapic acid (Fabre et al., 2000). However, a later study showed no significant differences between the radical scavenging activity of free sinapic acid (89%) and its sinapoyl glucosides, methyl 6-O-sinapoyl-α-D-glucose (96%) (Kylli et al., 2008).

The antimicrobial activity of sinapic acid against various Gram-negative (*Escherichia coli, Enterobacter aerogenes, Pseudomonas fluorescens*) and Gram-positive (*Bacillus subtilis, Bacillus cereus, Listeria monocytogenes, Streptococcus lactis, Streptococcus cremoris, Staphylococcus aureus*) bacteria have been reported for a range of concentrations (Barber et al., 2000; Engels et al., 2012; Lyon and McGill, 1988; Nowak et al., 1992; Salih et al., 2000; Tesaki et al., 1998). Table 2.5 summarizes the minimal inhibitory concentrations (MICs) of mustard

phenolic extracts and sinapic acid standard against various food spoilage and pathogenic bacteria in related studies.

Table 0-5 Reported minimal inhibitory concentrations (MICs) of mustard phenolic extracts and sinapic acid.

Bacterial strains	MIC	Source	Reference
Erwinia carotovora	1 mg/mL (or 1000 ppm)	Sinapic acid standard	(Lyon and McGill, 1988)
Escherichia coli	2.2 mM (or 493.26 ppm)	Yellow mustard seed phenolic extracts	(Tesaki et al., 1998)
Salmonella enteritidis	2.0 mM (or 896.84 ppm)		
Staphylococcus aureus	1.9 mM (or 425.99 ppm)		
Bacillussubtilis	2 mM (or 448.42 ppm)	Synthesised sinapic acid	(Barber et al., 2000)
Escherichia coli	4 mM (or 896.84 ppm)		
Pseudomonas syringae	8 mM (or 1796.68 ppm)		
Bacillus subtilis	0.3 g/L (or 300 ppm)	Sinapic acid standard	(Engels et al., 2012)
Escherichia coli	0.7 g/L (or 700 ppm)		
Staphylococcus aureus	0.3 g/L (or 300 ppm)		
Listeria innocua	0.3 g/L (or 300 ppm)		
Listeria monocytogenes	0.2 g/L (or 200 ppm)		
Pseudomonas fluorescens	0.6 g/L (or 600 ppm)		
Bacillus subtilis,	0.1 g/L (or 100 ppm)	Oriental mustard seed phenolic extracts	(Engels et al., 2012)
Escherichia coli	0.1 g/L (or 100 ppm)		
Staphylococcus aureus	0.1 g/L (or 100 ppm)		
Listeria innocua	0.1 g/L (or 100 ppm)		
Listeria monocytogenes	0.1 g/L (or 100 ppm)		
Pseudomonas fluorescens	> 0.1 g/L (or 100 ppm)		

2.3 Extraction of phenolic compounds

Polyphenols can be applied in various products such as functional foods, nutraceuticals, cosmetics or pharmaceuticals for different purposes. The first step to utilize phenolic compounds in any industry is to extract the targeted compound from its original matrix and then analyse, and characterize it. Therefore, developing effective methods with optimized protocols for higher efficiency in extraction is a crucial step (Ajila et al., 2011).

Solvent extraction is the most common technique used to extract phenolic compounds from various food, plant and biological samples. It is applicable for solid, semi solid and liquid materials and has an appropriate recovery for phenolic compounds. However, traditional methods for extraction usually require long extraction times with laborious work, large amounts of samples, high quantities of extraction solvents, along with increased financial costs and adverse effects on human and environmental health. Also, sometimes the targeted phenolic compound might degrade as a result of exposure to high temperature, light and oxygen (Ajila et al., 2011; Luthriaa et al., 2004). Soxhlet extraction is the major conventional technique used for the extraction of phenolic compounds from a wide range of sources (Carabias-Martínez et al., 2005). In an attempt to overcome the limitations of traditional methods of extraction, various modern techniques such as automated Soxhlet extraction, microwave-assisted extraction (MAE), ultrasonic-assisted extraction (UAE), supercritical fluids extraction (SFE) and accelerated solvent extraction (ASE) have been developed. (Ajila et al., 2011; Carabias-Martínez et al., 2005; Richter et al., 1996). Among them, ASE and SFE, especially when water is used as extraction solvent, are considered ‘green’ technologies that are more environmentally friendly because in addition to shortening the extraction time, they significantly reduce the amount of

solvent consumption and thereby lessen the negative side effect of sorbents and organic solvents on the environment and also human health (Mustafa and Turner, 2011).

2.3.1 Accelerated solvent extraction (ASE)

ASE is an automated extraction technique that rapidly performs solvent extraction using a combination of high temperature (50–200°C) and pressure (1450–2175 psi). The technology was first introduced at the Pittcon Conference by Dionex Corporation in 1995 (Luthria et al., 2004; Mustafa and Turner, 2011). It is also categorized as pressurized liquid extraction (PLE), pressurized fluid extraction (PFE) and high-pressure solvent extraction (HPSE) in the literature. Additionally, ASE is referred to as high-temperature water extraction (HTWE), pressurized hot water extraction (PHWE) and sub-critical water extraction (SWE), and because water is used as the extraction solvent the result tends to be more "environmentally friendly" (Carabias-Martínez et al., 2005; Luthria et al., 2004; Mustafa and Turner, 2011).

ASE has many advantages over conventional methods of extraction, including higher efficiency in extraction, shortened extraction time and reduced solvent consumption. For instance, 10 g of sample can be extracted in about 12 min using ASE, whereas it takes up to 24 h using traditional soxhlet (Luthria et al., 2004). Table 2.6 shows the required amount of time, solvent, and costs to extract a 10 g sample using ASE compared to other extraction methods.

Table 2-6 Amount of time, solvent and expenses per extraction using various extraction techniques (based on 10 g sample).

Extraction method	Time	Solvent (mL)	Cost ($)
Soxhlet	4-48 hr	200-500	27
Automated Soxhlet	1-4 hr	50-100	16
Sonication	30 min-2hr	100-300	24
Super Critical Extraction	30 min-2 hr	8-50	23
Accelerated Solvent Extraction	12-18 min	15-40	14

Data source: (Dionex, 2016)

Sample preparation in ASE systems usually includes size reduction and drying. Samples with particle sizes larger than 1 mm should be fined using sieving or grinding to enlarge surface interaction and thereby facilitate solvent penetration into the sample matrix and also enhance analyte diffusion into the extraction solvent. Wet samples should also be dried as moisture may result in a significant reduction in the extraction efficiency, particularly when solvents used for extraction are non-polar. All samples were either mixed with drying agents such as diatomaceous earth and sodium sulphate, or dispersed with Ottawa Sand, or dried using vacuum ovens, lyophilisation or freeze-drying (Carabias-Martínez et al., 2005; Luthria et al., 2004).

Usually in ASE, stainless steel extraction cells are filled with 1-50 g solid or semisolid sample which are subjected to extraction under high pressure and temperature conditions using appropriate aqueous or organic solvent. Afterwards, the extracted samples were flushed with clean solvent, and finally purged and passed into a glass collection vial via compressed nitrogen gas. Figure 2.5 shows a diagram of how an ASE system works (Ajila et al., 2011; Luthria et al., 2004).

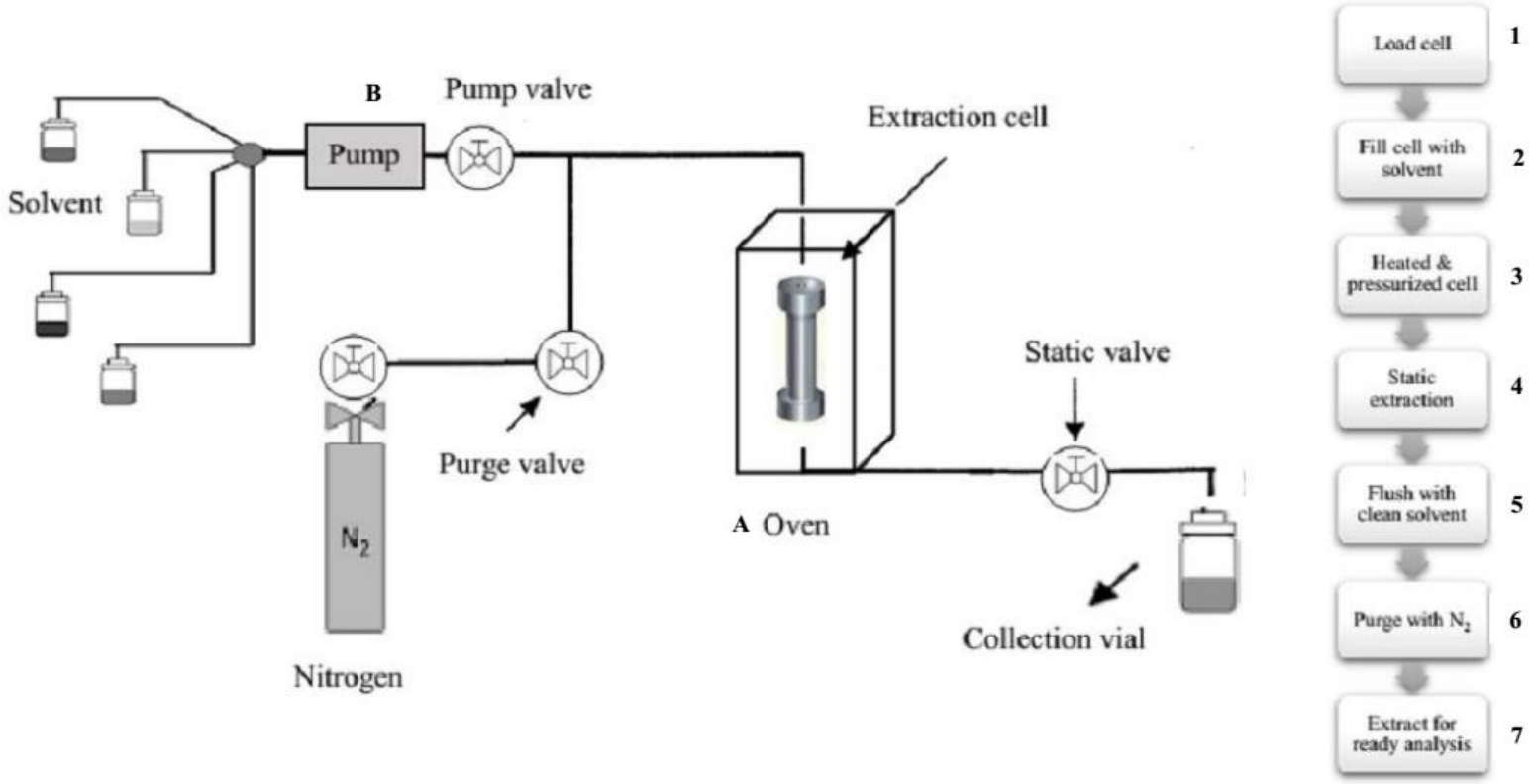

Figure 0-5 Schematic diagram of an ASE system. Adopted from (Luthria et al., 2004; Richter et al., 1996).

ASE automatically operates the extraction as follows: 1- loading the extraction cell 2-Filling the cell with extraction solvent 3- Heating and pressurizing: heating the cell by direct contact with the oven (A) and maintaining the extraction solvents in their liquid state by applying high pressure (1500 psi) via the pump (B). 4- Static extraction: by holding the sample at appropriate pressure and temperature for 5-10 min. Analyte diffusion from the matrix into the solvent occur mainly during this phase. 5- Flushing: by flushing the fresh solvent over the sample and entire lines, the previous volume exhausts into the collection vial. 6- Purging: the whole system is purged with compressed nitrogen gas to force all of the solvent from the cell into the collection vessel under an inert nitrogen layer. 7- Extracted samples are ready for analysis.

Temperature and pressure are among the most important parameters that affect the efficiency of extraction in ASE. Elevated temperature facilitates the release of the targeted analytes by providing the required thermal energy to overcome and disrupt the adhesive interactions (hydrogen bonding, van der Waals forces, and dipole attraction) between the analyte and sample matrix. Furthermore, at higher temperature, the solvent penetration into the sample matrix is easier due to the lower viscosity of the extraction solvent. Also, increased temperature reduces the surface tension of the solvent and matrix and consequently improves the solubility of both targeted analyte and extraction solvent, which leads to a considerable increase in the rates of mass transfer and diffusion. By increasing the pressure, ASE is able to maintain extraction solvents in a liquid state at temperatures higher than their atmospheric boiling points and thereby enhances the extraction efficiency significantly (Luthria et al., 2004; Mustafa and Turner, 2011; Richter et al., 1996).

Accelerated solvent extraction was initially used for the fast extraction of environmental pollutants in sewage sludge, water sediments and soil (Carabias-Martínez et al., 2005). Nowadays, it is successfully used for laboratory extractions of many products in the polymer, food, nutraceutical, cosmetic and pharmaceutical industries (Luthria et al., 2004; Mustafa and Turner, 2011). In terms of food and nutraceuticals, the ASE approach has proven useful for extracting polyphenols and antioxidants from various natural products. For example, the efficiency of extracting phenolic antioxidants from canola meal using pressurized liquid extraction was reported to be considerably higher than hot water or ethanolic extraction (Hassasroudsari et al., 2009). Similarly, pressurized liquid extraction was able to recover higher amounts of isoflavones from soybeans compared to conventional methods of extraction such as Soxhlet, stirring, shaking, vortexing and sonication (Luthria et al., 2007). ASE was also used

successfully in extracting carotenoids from micro algae (Herrero et al., 2004), phenolics from barley flour (Bonoli et al., 2004), lignans from flaxseed (Cacace and Mazza, 2006), polyphenols from apple and peach pomaces (Adil et al., 2007), Betulin and antioxidants from birch bark (Co et al., 2009), quercetin from yellow onions (Lindahl et al., 2010), as well as antioxidants from rosemary, oregano and marjoram (Hossain et al., 2011).

Therefore, considering the advantages of ASE over the conventional methods of extraction in terms of analyte recovery, time and solvent consumption, phenolic compounds from yellow and Oriental mustard seeds were extracted using ASE in this study.

2.4 Foodborne pathogens

Producing safe food products for consumers has been a great challenge for the food industry and health authorities over the past few decades. According to a report from the United States Centres for Disease Control and Prevention (CDC), foodborne pathogens cause approximately 48 million illnesses, 128,000 hospitalizations, and 3000 deaths in the United States every year (Scallan et al., 2011). Similarly, in the European Union more than 5600 foodborne outbreaks with approximately 70,000 illnesses, 7200 hospitalisations and more than 90 deaths were reported in 2011 (ECDC, 2013).

In Canada, it has been estimated that 4 million people (1 in 8 Canadians) suffer from foodborne illnesses every year (Thomas and Murray, 2014). The annual economic burden of acute bacterial foodborne diseases was reported to be more than 1.1 billion dollars for 1 million cases in Canada and 7 billion dollars for about 5.5 million cases in the United States (Todd, 1989). Contaminated food supplies are responsible for the transmission of more than 200 types of known foodborne illness. A variety of pathogenic microorganisms such as viruses, bacteria, toxins, parasites, and prions are the main causes of foodborne diseases. Food poisoning symptoms vary with the source of contamination from mild and self-limiting vomiting and diarrhea to severe and life-threatening neurological conditions (Bryan, 1982).

Escherichia coli O157:H7 and *Listeria monocytogenes* are among the most important pathogenic microorganisms that cause foodborne illnesses worldwide (Pragalaki et al., 2013). *Escherichia coli* O157:H7 and *Listeria monocytogenes* can be found in a wide variety of foods.

2.4.1. *Escherichia coli* O157:H7

Escherichia coli (E. coli) defined as a Gram negative, facultative anaerobic, rod-shaped bacteria belonging to the *Enterobacteriaceae* family and the genus *Escherichia.* The microorganism is usually found as a part of the common microflora in the large intestine of human and animals (Singleton, 1999). The optimal growth temperature and pH for *E. coli* are 37 ˚C and pH between 6.4 and 7.2 (Holt et al., 1994). However, *E. coli* is acid resistant, and has the ability to survive in the extreme low pH (pH<3) environment of the gastric system (Lim et al., 2010).

Most *E. coli* strains are harmless and considered an important part of a healthy human gastrointestinal tract. However, some serotypes, like enterohaemorrhagic *Escherichia coli* (EHEC), are pathogenic and can cause a wide range of clinical illnesses, from food poisoning and gastrointestinal diseases to meningitis, urinary tract infections, and septicaemia in humans (Piérard et al., 2012). *E. coli* O157:H7 is the most frequently isolated serotype of EHEC that causes severe illnesses in humans (Lim et al., 2010). EHEC serotype O157:H7 was first described as a foodborne pathogen after the bloody diarrhea outbreaks in two US states, Michigan and Oregon, in 1982, which affected more than 48 persons as a result of the consumption of contaminated hamburgers (Riley et al., 1983).

Nowadays, *E. coli* O157:H7 is well recognized as one of the most common foodborne pathogens worldwide. The overall incidence of *E. coli* O157:H7 infections is less than other common enteric bacterial pathogens such as *Campylobacter* and *Salmonella* species. However, the mortality and hospitalization rates of *E. coli* O157:H7 illnesses are considerably higher (Mead et al., 1999). According to a report from the United States Centres for Disease Control and Prevention (CDC), *E. coli* O157:H7 infections cause nearly 73,500 illnesses, 2,200

hospitalizations, and 60 cases of death in the United States every year (Mead et al., 1999). However, due to the higher concern and awareness of consumers and food service operators towards food safety, and better detection and investigation of the pathogen outbreaks, the incidence of *E. coli* O157:H7 witnessed a reducing pattern in the United States in recent years (Centers for Disease Control and Prevention (CDC), 2011). Also, the Canadian National Enteric Surveillance Program (NESP) reported that the number of *E. coli* O157:H7 infections had declined significantly from 3 cases per 100,000 in 2006 to 1.18 cases per 100,000 in 2010 (NESP, 2012). Although the prevalence of *E. coli* O157: H7 infection has decreased considerably in North America over the past few years, the high economic cost of foodborne illness needs extra attention to control this pathogen. According to the reported studies, the annual cost of *E. coli* O157: H7 infections, including the medical care and unproductivity, was estimated to be approximately 400 and 21 million dollars in the United States (Frenzen et al., 2005) and Canada (Grier and Schmidt, 2013), respectively.

E. coli O157: H7 is not only the emerging cause of food borne diseases, but also the major cause of haemorrhagic colitis, post diarrhea haemolytic uremic syndrome, and acute renal failure in children. The pathogen has a low infectious dose. Depending on the individual susceptibility to disease, 10 to 100 organisms are sufficient to develop clinical infections. Young children, elderly, and immunocompromised patients are more vulnerable to *E. coli* O157:H7 infections (Peacock et al., 2001).

E. coli O157:H7 has the ability to survive the low pH of the stomach (pH 1.5 to 3.0) while crossing the gastric system (Page and Liles, 2013), colonize the epithelial cells of the gastric surface, pass mucosal layers, enter the blood flow, secret toxins, attack the tissues causing illnesses and dysfunctions in the targeted organs of the host (Piérard et al., 2012). Like other

foodborne pathogens, symptoms of *E. coli* O157: H7 infection may vary from simple stomach cramps and diarrhea to severe clinical symptoms such as severe abdominal pain, fever, vomiting**,** and bloody diarrhea in haemorrhagic colitis and post diarrhea haemolytic uremic syndrome (Su and Brandt, 1995).

E. coli O157: H7 has various virulence factors, including the ability to produce Shiga toxins, the presence of a locus of enterocyte effacement, and the possession of the pO157 plasmid, that plays an important role in its pathogenicity (Lim et al., 2010). The locus of enterocyte effacement encodes proteins that cause attaching and effacing lesions which are responsible for the suppression of microvilli and the adherence of bacteria to the intestinal epithelial cell membrane (Page and Liles, 2013).

E. coli O157:H7 is a zoonotic pathogen whose predominant reservoirs are healthy cattle and other ruminants (Piérard et al., 2012). *E. coli* is excreted into the environment via fecal shedding. Consumption of contaminated food products and water is the predominant source for *E. coli* O157:H7 infection in humans. However, the pathogen can occasionally be transferred by direct contact from person to person (Lim et al., 2010).

Undercooked ground beef and raw milk are the most common vehicles that have been associated with *E. coli* O157:H7 outbreaks (Doyle, 1991). However, a variety of contaminated food products such as beef jerky, salami, yogurt, unpasteurized apple juice, spinach and lettuce have also been implicated in *E. coli* O157:H7 occurrence (Lim et al., 2010; Rhee et al., 2003).

2.4.2 *Listeria monocytogenes*

L. monocytogenes was first identified by Murray et al. (1926). It is a small (length: 1-2 μm, width: 0.5 μm), rod-shaped, Gram positive, facultative anaerobic bacterium that belongs to the *Listeriaceae* family containing the *Listeria genus* (Gray and Killinger, 1966). Among various species of *Listeria*, two, including *L. ivanovii* and *L. monocytogenes*, are pathogenic for animals. However, *L. monocytogenes* is the only one which causes disease in humans (Allerberger, 2003). *L. monocytogenes* is well adapted to the natural environment and can tolerate a wide spectrum of pH (4.3 to 9.6), temperatures (0.5 to 45 °C), and even high concentrations of NaCl (10%). However, pH 7.0 and a temperature ranging from 30 to 37 °C are the optimum condition for the growth of organism. The organism grows parallel to each other and produces small milky colonies that are smooth, almost flattened and glistening (Gray and Killinger, 1966; Low and Donachi, 1997)

L. monocytogenes was described as a potential foodborne pathogen that causes severe infection in humans in the early 1980s, when a significant listeriosis outbreak involving 41 individuals and 18 cases of death, mainly among pregnant women and new born children, occurred in Nova Scotia, Canada. Consumption of coleslaw containing cabbage contaminated with *L. monocytogenes* was identified as the main source for the outbreak (Schlech et al., 1983). Since then, several listeriosis outbreaks have been reported worldwide, and *L. monocytogenes* have been officially recognized as one of the most virulent foodborne pathogens. Although rare, the case fatality rate of listeriosis is very high with approximately 20 to 30% of cases resulting in death (Allerberger, 2003)

Listeria monocytogenes was reported to cause approximately 23,000 cases of illnesses and 5500 deaths in the world in 2010 (de Noordhout et al., 2014). According to the United States

Centres for Disease Control and Prevention, foodborne listeriosis results in more than 2,500 illnesses, 2,300 hospitalizations, and 500 deaths annually in the United States (Mead et al., 1999). In Canada, the prevalence of listeriosis infection increased from 1.8 per 1,000,000 cases in 1996 to 4.2 cases per 1,000,000 in 2007. Several major outbreaks of listeriosis were recorded in Canada during this time. However, one of the most important outbreaks of this pathogen occurred in 2008 in 7 different provinces including Alberta, British Colombia, Manitoba, New Brunswick, Ontario, Quebec and Saskatchewan, as a result of consuming contaminated deli-meat. The later outbreak resulted in 57 cases of illness and 23 deaths increasing the incidence of foodborne listeriosis to 7.2 cases per 1,000,000 in 2008 (Clark et al., 2010; Farber et al., 2011).

Symptoms of listeriosis infection can vary from mild self-limiting gastrointestinal signs (fever, cramps, vomiting, and diarrhea) and/or influenza-like symptoms to severe classical listeriosis such as meningitis, endocarditis, abortion, sepsis, and encephalitis, which can result in death in some cases. Older individuals, immunocompromised patients, pregnant women, unborn children and neonates are at higher risk for severe listeriosis infection (Allerberger and Wagner, 2010; de Noordhout et al., 2014).

Consumption of contaminated food products is the predominant route for *L. monocytogenes* transmission. However, the pathogen can rarely be transferred by direct contact from infected animals to humans or from person to person (Allerberger, 2007). In contrast to many foodborne pathogens, *L. monocytogenes* is able to tolerate and continue growth in high concentrations of salt, low water activity, acidic conditions and at low temperatures. Therefore, it can be isolated from wide variety of food products, especially processed and refrigerated ones (Allerberger and Wagner, 2010). Most listeriosis outbreaks have been associated with ready-to-

eat foods such as soft cheese, crab meat, sausage, jellied pork, and other meat and poultry products (Clark et al., 2010; Eleimat, 2015; Farber et al., 2011).

L. monocytogenes is an intracellular pathogen that is capable of multiplication within phagocytic cells (macrophages) and also some non-phagocytic (epithelial and endothelial) cells of the host. Listeriosis infection usually starts in the gastrointestinal tract of the host. After passing through the stomach, the organism is able to cross the intestinal mucosal layers, enter the lymph and the blood stream, and invade cells of the targeted tissues. Once entering the cell, the pathogen escapes the vacuole by lysing the phagosomal membrane via secreting pore-forming listeriolysin O proteins. After leaving the phagocytic vacuole, *L. monocytogenes* replicates and grows in the cytoplasm, and eventually spreads intracellularly between cells. Various virulence factors play roles in the pathogenicity of *L. monocytogenes*. However, its ability secrete listeriolysin O proteins, which facilitate both pore formation and also escaping from the vacuole, and its ability to escape the intracellular lethality of the immune system by cell-to-cell spread are among the most important virulence features (Allerberger, 2007; Low and Donachi, 1997). .

The antimicrobial activity of mustard products and their isothiocyanates (non-phenolic bioactive) against important foodborne pathogens such as *E. coli* O157:H7 and *L. monocytogenes* was reported previously (Cordeiro et al., 2014; Lin et al., 2000; Olaimat and Holley, 2013), whereas corresponding reports on the mustard phenolic compounds are lacking. Therefore, this study investigated the antimicrobial properties of yellow and Oriental mustard seed phenolic extracts against different strains of these two microorganisms.

Chapter 3

Manuscript 1: *In vitro* antioxidant activity of endogenous phenolic compounds from commercial mustard products and wasabi

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3.1 Abstract

Sinapic acid derivatives (SADs) and their antioxidant activity were investigated in two different varieties of western Canadian mustards, Oriental (*Brassica junceae*) and yellow (*Sinapis alba*), and compared to wasabi powder. Phenolic extracts from two different mustard seeds, bran and flour and wasabi powder were obtained by Accelerated Solvent Extraction (ASE) and the SADs quantified by HPLC-DAD. The antioxidant capacity of each extract was assessed by a modified DPPH assay and correlated with the total phenolic content (TPC) measured using the Folin–Ciocalteau method. Sinapine was the major phenolic compound in all the samples analysed, with negligible amounts of sinapic acid and its other glycosides. The sinapine content, expressed as sinapic acid equivalents (SAE), ranged from $2.67 \times 10^3 \pm 0.33$ to $14.44 \pm 0.43 \times 10^3$ µg SAE/g dry weight of the samples, with the highest in the yellow mustard seed extract and lowest in wasabi powder. The level decreased in the following order: yellow mustard seed > Oriental mustard seed > yellow mustard bran > Oriental mustard bran > yellow mustard powder > Oriental mustard powder > wasabi powder. Extracts from yellow mustard seeds had the highest TPC ($17.61\times 10^3 \pm 1.01$ µg SAE/g), while Oriental mustard and wasabi powder showed the lowest TPC with $4.14 \times 10^3 \pm 0.92$ and $2.7 \times 10^3 \pm 0.33$ µg SAE/g, respectively. The

antioxidant activities of the extracts correlated positively with their TPC with a correlation coefficient ≥ 0.72. This study confirmed that Canadian yellow and Oriental mustard varieties and their products are rich sources of endogenous phenolic compounds.

3.2 Introduction

Canada is one of the most important producers and exporters of mustard seeds worldwide (Clancey, 2013). Both yellow (*Sinapis alba*) and Oriental (*Brassica junceae*) varieties of mustard belong to the *Brassicacea* family (Oram et al., 2005) and are sources of bioactive substances such as glucosinolates and phenolic compounds. Phenolics are an extensive group of plant secondary metabolites which function as antioxidants, defensive or signalling compounds (Balasundram et al., 2006; Parr and Bolwell, 2000). Sinapic acid and its derivatives (SADs) are predominant phenolics in *Brassicacea* species. Various health benefits such as antioxidant, antimicrobial, anticancer, anti-anxiety, and anti-inflammatory activities have been attributed to the SADs (Nićiforović and Abramovič, 2014). Extraction, identification and quantification of these constituents from residual processing materials could add agronomic value. Other products of mustard, such as mustard flour, have also been reported to possess antioxidant activity (Shahidi et al., 1994). Sinapic acid derivatives including sinapine and sinapic acid (Figure 3.1) were reported as the major water-soluble phenolics in mustard meal and flour extraction (Dabrowski and Sosulski, 1984; Dubie et al., 2013). Sinapic acid was reported to comprise more than 73% of free phenolic acids and about 80-99% of the total phenolic acids in mustard meal (Das et al., 2009).

Wasabi (*Wasabia japonica* Matsum) or Japanese horseradish, a perennial plant belonging to the *Brassicaceae* family (Depree et al., 1999) was also included in this study. The crop is

mainly used as a condiment, either in fresh form or as a dry powder, in Japanese cuisine (Sultana and Savage, 2008). Reports on the endogenous phenolic compounds in wasabi products are very limited. However, few studies have reported the presence of sinapic acid esters in wasabi leaves. (Depree et al., 1999; Hosoya et al., 2005; Yoshida et al., 2015). The current study is the first to identify and quantify the sinapic acid constituents in wasabi powder.

Conventional methods of extraction such as solid/liquid extraction have been used extensively for extracting phenolic constituents from various food and plant sources. However, drawbacks such as long extraction times, laborious work, consuming large quantities of solvents and high expense (Ajila et al., 2011; Luthria et al., 2004) have drawn the attention to utilising innovative techniques such as Accelerated Solvent Extraction (ASE). ASE is an automated extraction technique that rapidly performs solvent extraction using a combination of high temperature (50–200°C) and pressure (1450–2175 psi) (Ajila et al., 2011; Kaufmann and Christen, 2002). By maintaining the solvents in their liquid state at high temperature, the target compounds solubility, mass transfer and solvent diffusion rate increase, while solvent viscosity and surface tension decrease. This results in a more efficient extraction procedure, shortened extraction time, and a substantial reduction in solvent and sample volumes (Co et al., 2009; Denery et al., 2004; Hossain et al., 2011; Luthria et al., 2004). ASE has been successfully used for extracting polyphenols from a variety of plant materials such as rosemary, oregano and marjoram (Hossain et al., 2011), betulin (Co et al., 2009) and barley flour (Bonoli et al., 2004) as well as phenolic antioxidants from canola meal (Hassasroudsari et al., 2009).

Here we report a comparative profile of sinapic acid derivatives in yellow and Oriental mustard seeds, their commercial byproducts (bran and powder), and wasabi powder. An

improved extraction method using an ASE technique was developed. In addition, the total phenolic content and *in vitro* antioxidant potential of the extracts using Folin–Ciocalteau and DPPH methods are reported.

3.3 Materials and Methods

Materials

Yellow mustard seeds (YMS), yellow mustard powder (YMP), yellow mustard bran (YMB), Oriental mustard seeds (OMS), Oriental mustard powder (OMP), Oriental mustard bran (OMB) and Wasabi powder were supplied by Sakai Spice (Canada) Corp., Lethbridge, AB. All samples were stored at 4°C in polyethylene bags until used. The standard sinapic acid ≥98%, 2, 2-di (4-tert-octylphenyl)-1-picrylhydrazyl free radical (DPPH), and Folin-Ciocalteau's phenol reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade.

Oil and moisture contents

The oil content in different yellow and Oriental mustard commercial products and wasabi powder were measured using the Soxtec 2050 (Foss Tecator, Foss North America, Eaden Prairie, MN, USA) according to FOSFA method (FOSFA, 1998). Samples (3 g each) were put in the thimbles which were then loaded in the Soxtec (unit at 135°C). The pre-dried aluminum cups were then inserted into the extraction unit and 35 mL of *n*-hexane added to each sample. The system was programmed as follows: boiling (15 min), rinsing (60 min) and recovery (20 min). Samples were extracted in two cycles. To determine the moisture content in studied products 1 g

of each mustard sample was placed in the moisture analyzer for 4 min at 130 °C. Based on the initial and final weight of the sample, the moisture content was calculated as percentage. All analyses were done in triplicate.

HPLC-DAD analysis of sinapates

The phenolic profiles of the different extracts were determined by a reversed-phase HPLC-DAD analysis using an HPLC system (Ultimate 3000; Dionex, Sunnyvale, CA, USA), consisting of a diode array detector and other accessories. Solvent A, 90% methanol (aqueous) acidified with *o*-phosphoric acid (1.2%) and solvent B, 100% methanol acidified with *o*-phosphoric acid (0.1%) were used as mobile phases in a gradient elution, in where the concentration of mobile phase B (%, indicated in brackets) changed in the following sequences at specified time periods (min) 0 (10), 7 (20), 20 (45), 25 (70), 28 (100), 31 (100) and 40 (10) (Khattab et al., 2010). C18 column; Synergi 4_ Fusion-RP 80 Å; 150 x 4.0 mm 4 _m (Phenomenex, Torrance CA, USA) was used for the separation of SADs. Both the mobile phases and phenolic extracts were filtered through 0.45 μm syringe filters prior to use. Other conditions of analysis were strictly maintained which included: flow rate (1 ml/min), column compartment temperature (25 °C) and wavelengths of analysis (270 nm and 330 nm). The contents of sinapic acid and its derivatives were quantified based on a calibration curve with the sinapic acid standard in appropriate dilutions (0.05 – 0.5 mg/ml) by plotting each concentration against the obtained area. All samples were injected in duplicate and results were expressed as μg sinapic acid equivalents (μg SAE) based on calculation of its standard curve.

Extraction of Phenolic Compounds

i) Accelerated Solvent Extraction (ASE)

Phenolics in mustard products were extracted using an Accelerated Solvent Extraction (ASE 300, Dionex, Sunnyvale, CA, USA). Mustard seeds were sieved and ground for 30 sec in a coffee grinder. Fifteen grams of ground samples were mixed carefully with 15 g of Ottawa sand using a spatula. Two filter papers were placed at the bottom of each sample cell followed by filling it with sample up to top level of the cell. Cell caps were hand tightened securely for both sides and each cell was placed in the ASE cell holder. Samples were defatted using n-hexane (100%) at 23˚C, followed by the extraction of phenolic compounds using analytical grade methanol (100 %) at 23˚C, 100, 120 and 160ºC. The final extraction temperature for comparison between different products was adjusted according to the optimized extraction temperature from ground mustard seeds. Other extraction conditions were kept constant as following: 1500 psi pressure, two static cycles (5 min for each), 60% flush volume and 1 min purging. Approximately 100 ml extract were obtained from each 15 g sample.

ii) Conventional extraction

Phenolics of defatted yellow and Oriental mustard seeds were also extracted using a conventional method of extraction according to Thiyam et al. (2006). In brief, 1 g of each sample was extracted three times in aqueous methanol (70%) assisted by ultra-sonication (1 min) followed by centrifugation at 5000 × g under refrigerated conditions for 10 min. The supernatants from all three extractions were combined and filtered using Whatman No. 1 filter paper. The pooled extracts were made up to a total volume of 30 mL with 70% methanol. All the extractions were conducted in triplicate.

Total content of phenolic compounds

All samples were analyzed for total phenolic content (TPC) using Folin–Ciocalteau's phenol reagent according to the procedure outlined by Swain and Hillis (1959), with slight modifications. Briefly, aliquots (0.2 ml) of each extract were diluted to 0.5 ml with distilled water, and mixed with Folin–Ciocalteau's phenol reagent (0.5 ml), followed by addition of 1 mL 19% sodium carbonate after 3 min. The absorption was recorded at 750 nm using a DU 800 UV/Visible Spectrophotometer (Beckman Coulter Inc., Mississauga, ON, Canada) after 60 min incubation in the dark. Sinapic acid was used for calibration, and all results were expressed as μg sinapic acid equivalents (μg SAE).

Free-radical-scavenging activity

All samples were analyzed for antioxidant activity using the DPPH assay. The DPPH radical scavenging activity of extracts was determined following the procedure described by Schwarz and others (2001) with slight modifications. Briefly, 50 μL of phenolic extract was added to ethanolic DPPH (2.95 mL, 0.1 mM) solution and vortexed thoroughly for 30 sec in a covered test tube. The content was allowed to stand at room temperature for 10 min before measuring the absorbance at 516 nm using the DU 800 UV/Visible Spectrophotometer. The absorbance of control (A_c) and sample (A_s) was used to calculate scavenging effect (%), which is the percentage change in absorbance (A_c-A_s) with respect to A_c (Schwarz et al., 2001).

Inhibition % = $[1- (A_s / A_c)] \times 100$

Data expression and analysis

Means and standard deviations were based on duplicate values. Data on phenolic content of mustard fractions and their antioxidant activity were statistically interpreted using one factor ANOVA. For multiple comparisons, Tukey mean separation was followed using SPSS for Windows version 18.0 (2010). Significance level was defined using the value $P<0.05$.

3.4 Results and Discussion

Oil and moisture contents of different yellow and Oriental mustard commercial products

Table 3.1 summarizes the oil content for the different yellow and Oriental mustard products and wasabi powder. The percentage of oil in yellow and Oriental mustard seeds was 26.70% ± 0.14 and 32.22% ± 0.26, respectively. In both varieties, the oil content for powder products was significantly lower than the corresponding seeds but higher than the bran fraction. Data clearly indicated a higher percentage of oil in the Oriental mustard products compared to that of yellow mustard. The lowest amount of oil was observed for wasabi powder (< 5.77% ± 0.24).

Identification and quantification of phenolic constituents from commercial mustard products using the HPLC-DAD

Phenolic components were identified based on their retention times, UV spectra and comparison with the sinapic acid reference compound. Both mustard products and wasabi powder contained significant amounts of sinapates. Figure 3.2 shows the HPLC-DAD chromatogram of phenolic extracts from yellow (A) and Oriental mustard seeds (B) at two

different wave lengths (330 and 270 nm). Sinapine was the major phenolic compound in all the samples analysed, with only negligible amounts of sinapic acid and its glycosides. These results are in agreement with previous studies (Dubie et al., 2013; Mayengbam et al., 2014; Thiyam et al., 2006). Table 3.2 shows the phenolic content ($\times 10^3$ µg SAE/g samples) of different yellow and Oriental mustard products and wasabi extracts at 330 nm. In addition to the sinapine peak, several unknown peaks were detected at wavelength 270 and 330 nm in the above mentioned products. Table 3.3 summarizes the retention time and spectra for some of the unknown peaks in the phenolic extract for the different samples. Absorbance spectra of each detected peak is included in Appendix I (a-h).

Sinapine content, expressed as sinapic acid equivalents (SAE), ranged from $2.67\times10^3 \pm 0.33$ to $14.44 \times10^3 \pm 0.43$ µg SAE/g dry weight of the samples, with the following decreasing order of yellow mustard seed > Oriental mustard seed > yellow mustard bran > Oriental mustard bran > yellow mustard powder > Oriental mustard powder > wasabi powder (Table 3.2). Overall, it can be concluded that ground seed contained a higher content of phenolics than the corresponding bran or powder. These results showed that yellow mustard is a much better source of endogenous phenolic compounds than Oriental mustard in terms of the presence of sinapates. In the case of wasabi, however, this is the first study to report the presence of sinapic acid derivatives, which had the lowest level among all samples analysed.

ASE of phenolic compounds from mustard products

The automated pressurized liquid extraction, the ASE 300, Dionex system was used to extract phenolic compounds from yellow and Oriental mustard seeds with methanol. In order to optimize the ASE method, the effect of different temperatures at stable pressure of extraction

process (1500 psi) was investigated (Figure 3.3) for ground yellow and Oriental mustard seeds. Significant increases in the sinapine yield from both yellow (from 4.73×10^3 to 14.44×10^3 µg SAE/g) and Oriental (from 3.43×10^3 to 9.80×10^3 µg SAE/g) mustard seed extracts by increasing the temperature from room temperature (23˚C) to 100 ˚C were observed. This might be attributed to the enhanced solubility of phenolic compounds as a result of easier solvent penetration of the plant matrix and higher rates of mass transport (Hossain et al., 2011; Luthria et al., 2004). Sinapine content, the major phenolic compound of both yellow and Oriental mustard seed extracts reached a plateau at 100 ^{o}C with no change with increased temperature of 160 ˚C. Therefore, 100 ˚C was considered the optimal temperature for conducting the ASE extraction for obtaining extracts with higher phenolic capacity for all studied products including yellow and Oriental bran, powder and ground seeds. .

A comparison between the conventional method of extraction (23 ˚C) and ASE at an optimised temperature of 100 ˚C for yellow and Oriental mustard seeds is shown in Figure 3.4. A significant increase in the amount of extracted sinapine was observed using ASE for both varieties. The higher efficiency of ASE in extracting targeted phenolic compounds was mainly attributed to the increased mass transfer, higher solvent diffusion rate, decreased solvent viscosity and lower surface tension (Ajila et al., 2011).

Total phenolic content of various mustard product extracts

The Folin-Ciocalteau method was used to evaluate the total phenolic content (TPC) in methanolic extracts of all the mustard products and wasabi powder (Figure 3.5). TPC values, expressed as sinapic acid equivalents (SAE), ranged from $2.71 \times 10^3 \pm 0.33$ to $17.61 \times 10^3 \pm 1.01$ µg SAE/g dry weight of the samples, with the following decreasing order of yellow

mustard seed > Oriental mustard seed > yellow mustard bran > Oriental mustard bran > yellow mustard powder > Oriental mustard powder > wasabi powder. As mentioned, sinapine was the principle phenolic compound in all studied products. Strong positive correlation between the concentration of sinapine and obtained TPC (r = 0.93) in Appendix I (Table 1), Overall, yellow mustard products were higher in total phenolics than the Oriental products. Also, the bran was considerably higher in total phenolics than both types of powder.

According to Table 3.2, the amount of total phenolics estimated by HPLC-DAD were lower than that determined by the Folin-Ciocalteau method (Figure 3.5) in yellow mustard products. In fact, Folin–Ciocalteau assay is a general method that measures sample's reducing capacity and detects not only phenolics, but also other non-phenolic compounds that are naturally present in mustard seed, such as glucosinolates, sulfur dioxide, carbohydrates, amino acids. Such compounds can participate in the oxidative–reduction reaction of the Folin–Ciocalteau assay and affect the total phenolics measured (Szydlowska-Czerniak et al., 2015). On the other hand, the Folin–Ciocalteau assay indicated lower total phenolic contents (Figure. 3-5) in comparison to the sinapine (Table 3.2) values in the Oriental mustard seed and bran extracts. That might be attributed to the presence of other oxidants in the Oriental mustard products that had the ability to compete with the Folin-Ciocalteau reagent and to interfere with measuring the extracts's reducing capacity in an inhibitory manner.

Antioxidant activity of extracts from commercial mustard products

One of the most common and reliable assays for measuring the antioxidant activity of phenolic compounds in plant extracts is the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging method (Co et al., 2009). DPPH is a stable free radical, which is reduced to α, α-

diphenyl-β-picrylhydrazine by reacting with an antioxidant (hydrogen donor) and changes from purple to yellow; the reaction progress is conveniently monitored by a spectrophotometer at a wavelength of 516 nm. The results were expressed as percentage of DPPH radical elimination. Decreasing of the DPPH solution absorbance indicates an increase of the DPPH radical scavenging activity and antioxidant potential of studied extracts. The antioxidant activity of the sinapic acid standard was first compared to other stablished antioxidants such as ascorbic acid, gallic acid and quercetin at a concentration of 1×10^3 μg/ml (Figure 3.6). The radical scavenging activity of the sinapic acid standard (88%) was higher than quercetin (65.99%) and comparable to gallic acid (88.54%) and ascorbic acid (96.93%). Figure 3.7 shows the radical-scavenging activity of various mustard products and wasabi powder in comparison to the sinapic acid standard (88%). The percentage of radical scavenging activity of different extracts ranged between 27% and 69% with the following order: yellow mustard seed > Oriental mustard seed > Oriental mustard bran > yellow mustard bran > yellow mustard powder > Oriental mustard powder> wasabi powder. Extracts of mustard ground seeds were the most effective DPPH radical scavengers, with 69.49% and 66.74% inhibition, respectively. Phenolic extracts of Oriental (54.98%) and yellow bran (47.67%) products were more effective in radical scavengers compared to the corresponding powder products with almost 36.15% inhibition. Wasabi's methanolic extract resulted in lower radical scavenging activity (26.70%) compared with other tested compounds. Positive Pearson's linear correlations were found between TPC and antioxidant activity ($r = 0.72$), as shown in Appendix I (Table 1), indicating that antioxidant activity might be directly correlated with phenolic compounds due to the presence of their hydroxyl groups (Jun et al., 2014).

3. 5 Conclusions

Accelerated solvent extraction using methanol provides an efficient method for the extraction of phenolics of mustard seed products and wasabi. An optimum temperature of 100˚C was recorded as the best extraction temperature to recover sinapic acid derivatives from these products. Both yellow and Oriental varieties of mustard are rich sources of endogenous phenolic compounds. Sinapine (sinapoyl choline) was the major identified sinapate in all mustard extracts, with significant variation between the two seed varieties and their products. It was also the predominant phenolic compound in wasabi powder which was reported for the first time in this paper. The extracts rich in sinapine also displayed significant radical-scavenging activity. Strong positive correlation between the sinapine concentration and antioxidant activity of various extracts, indicated sinapine was the major contributor to the antioxidant potential of these products. In addition to antioxidant activity, the inhibitory effects of sinapine on activity of acetylcholinesterase has various health benefits, biological and therapeutic applications (Nićiforović and Abramovič, 2014). It is evident that extracts from mustard seed products could be useful ingredients in the food industry and for the development of functional foods. In the same context, future work will be focused on examining the antimicrobial activity of mustard extracts from various food products as a natural preservative to inhibit spoilage and prolong shelf life of food products.

Acknowledgment

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extraction experiment in 2014. The ASE extraction project was funded by Growing Innovation – Agri-Food Research & Development Initiative (GF2 GI-ARDI, Project # 1000108059) and NSERC Discovery Grant (RG PIN-2015-03809).

Table 3-1 Oil and moisture (w/w) contents of different yellow and Oriental mustard commercial products and wasabi powder.

Products	**Oil content %** (Dry weight basis)	**Moisture %**
Yellow mustard seed	26.70 ± 0.14	6.92 ± 0.16
Yellow mustard powder	15.18 ± 0.01	7.24 ± 0.47
Yellow mustard bran	8.73 ± 0.10	8.56 ± 0.37
Oriental mustard seed	32.22 ± 0.26	6.71 ± 0.36
Oriental mustard powder	16.31 ± 0.19	7.97 ± 0.64
Oriental mustard bran	10.51 ± 0.16	8.21 ± 0.41
Wasabi powder	5.77 ± 0.24	7.66 ± 0.28

All data are expressed as mean ± standard deviation (n=3).

Table 0-2 Phenolic contents ($\times 10^3$ μg SAE*/g dry weight basis) of different yellow and Oriental mustard products and wasabi extracts obtained using ASE at 100 ˚C.

Products	Sinapine	Sinapic Acid	Unidentified
Yellow mustard seed	14.44 ± 0.43 [a]	0.05 ± 0.01[d]	1.26 ± 0.04 [c]
Yellow mustard bran	10.99 ± 1.81[b]	0.14 ± 0.02 [b]	1.79 ± 0.30 [b]
Yellow mustard powder	6.41 ± 0.75 [c]	0.07 ± 0.01 [d]	0.56 ± 0.07 [d]
Oriental mustard seed	9.8 ± 0.17 [b]	0.21 ± 0.01 [a]	3.27 ± 0.06 [a]
Oriental mustard bran	9.57 ± 0.12 [b]	0.11 ± 0.00 [b c]	3.46 ± 0.04 [a]
Oriental mustard powder	5.36 ± 0.66 [c]	0.10 ± 0.01 [c]	1.98 ± 0.24 [b]
Wasabi powder	2.67 ± 0.33 [d]	0.04 ± 0.01 [d]	0.8 ± 0.10 [d]

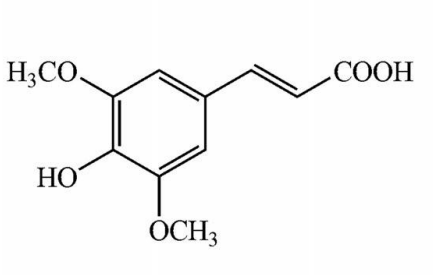
All data are expressed as mean ± standard deviation. Values with different superscripts were significantly different at $p \leq 0.05$.

* Sinapic acid equivalents (SAE).

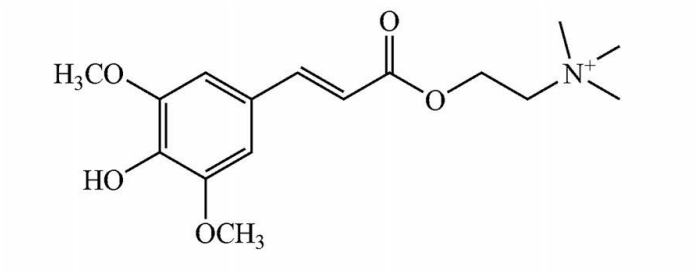
Table 0-3 Retention time, area, and UV spectra of detected peaks in various mustard phenolic extracts using HPLC-DAD at wavelengths of 270 nm and 330nm.

Peak	Retention Time (min)	Peak Area (mAU*min)	λ_{max} (nm)	Detection wavelength (nm)	Identity	Product*
1	5.32	74.33	201-207-258 (Appendix I, a)	270	Unidentified	YMS
2	9.02	28.91	208-259 (Appendix I, b)	270	Unidentified	YMS
3	11.81	117.86 - 472.48	200-238-328 (Appendix I, c)	330, 270	Sinapine	YMS, OMS, WP
4	14.53	12.75	213-242-330 (Appendix I, d)	330, 270	Unidentified	YMS
5	13.32	28.29	200-239-329 (Appendix I, e)	330, 270	Unidentified	OMS
6	17.09	1.43 - 6.14	200-236-324 (Appendix I, f)	330	Sinapic acid	YMS, OMS, WP
7	22.43	11.94	200-239-329 (Appendix I, g)	330	Unidentified	OMS
8	24.56	10.78 - 29.02	202-232-322 (Appendix I, h)	330	Unidentified	OMS, WP

*Yellow mustard seed (YMS), yellow mustard powder (YMP), yellow mustard bran (YMB), Oriental mustard seed (OMS), Oriental mustard powder (OMP), Oriental mustard bran (OMB) and wasabi powder (WP).



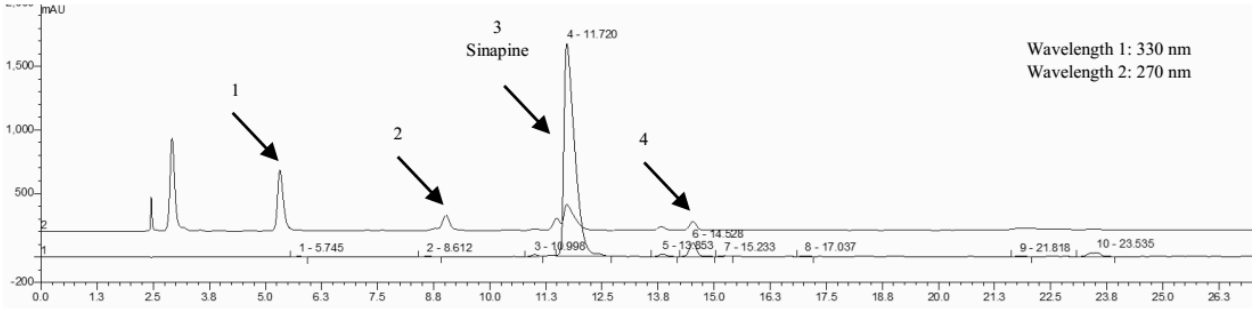
Sinapic acid



Sinapine

Figure 0-1 Chemical structures of sinapic acid and its choline ester, sinapine.

(A)



(B)

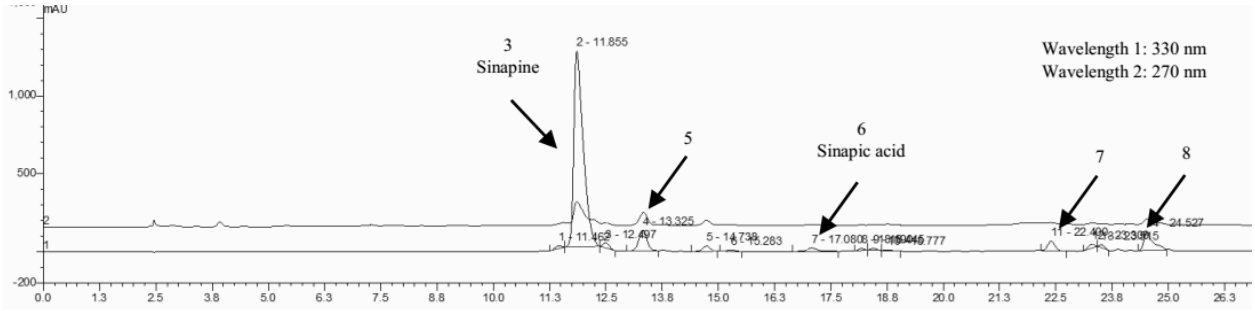


Figure 0-2 HPLC-DAD chromatograms (330 & 270 nm) of phenolic extracts obtained from yellow (A) and Oriental mustard seeds (B).

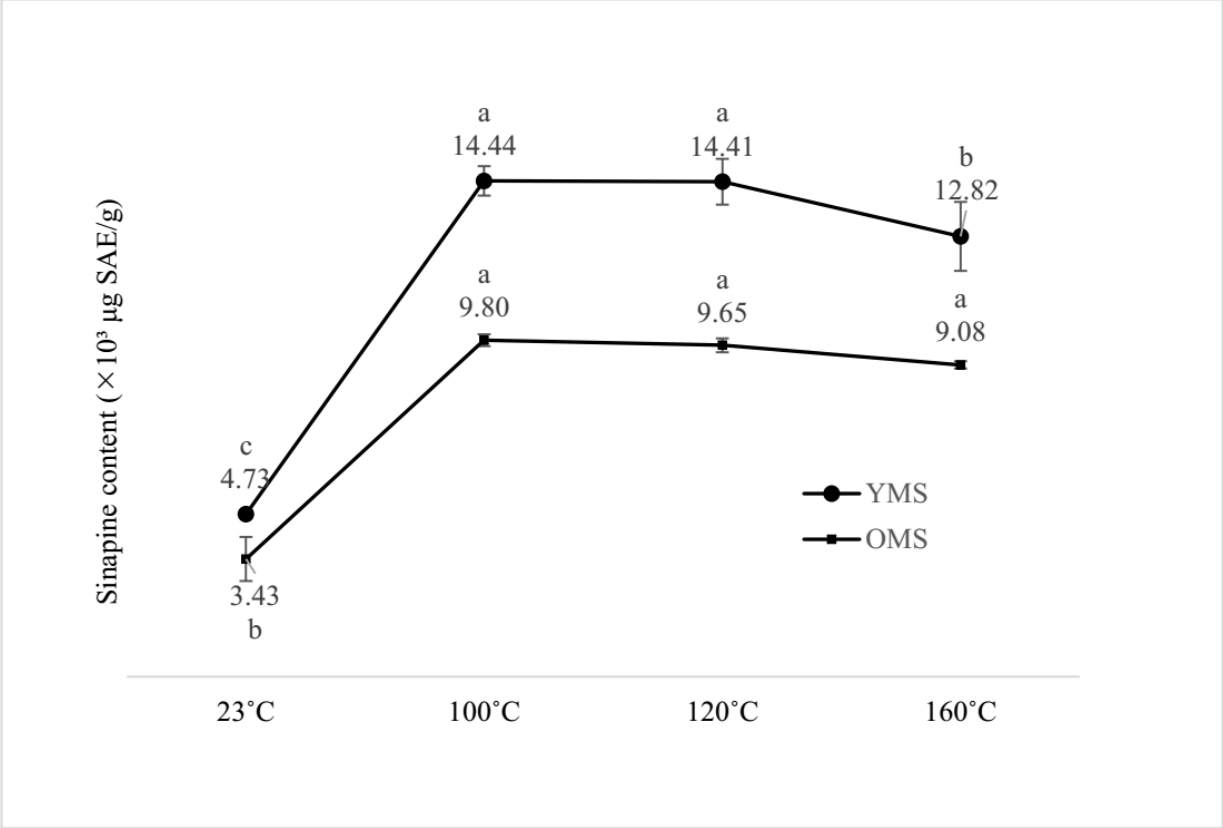


Figure 0-3 Effect of extraction temperature on sinapine content (×10³ μg SAE*/ g dry weight basis), the major phenolic compound, in ASE extracts from yellow (YMS) and Oriental mustard seeds (OMS).

All data are expressed as mean ± standard deviation (n=3). Values with different superscripts were significantly different at $p \leq 0.05$.

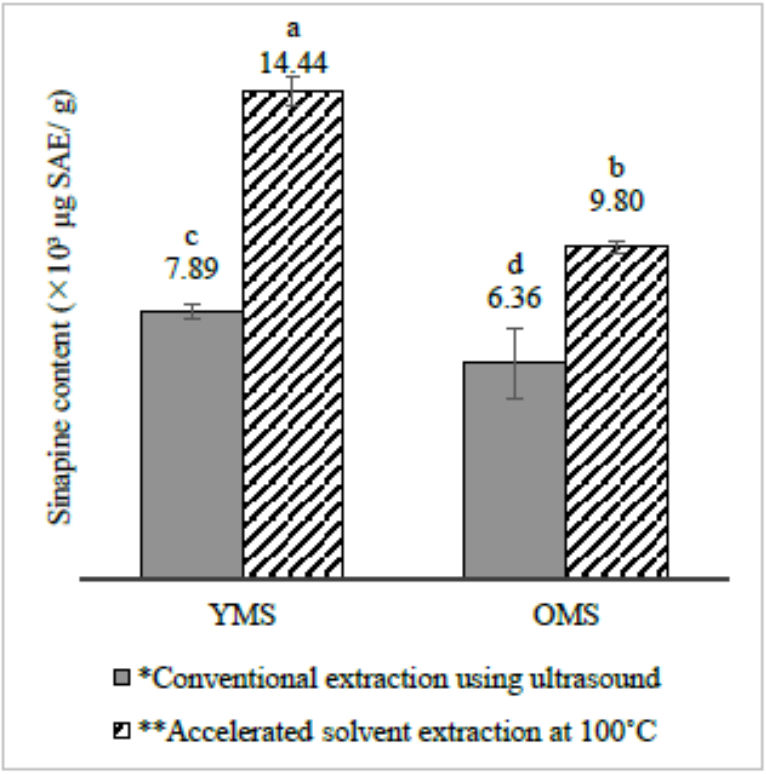


Figure 0-4 Sinapine content (×10³ µg SAE*/ g dry weight basis) of yellow (YMS) and Oriental mustard seeds (OMS): Conventional extraction*vs. ASE** at 100˚C and 1500 psi.

All data are expressed as mean ± standard deviation (n=3). Values with different superscripts were significantly different at $p \leq 0.05$. * Sinapic acid equivalents (SAE).

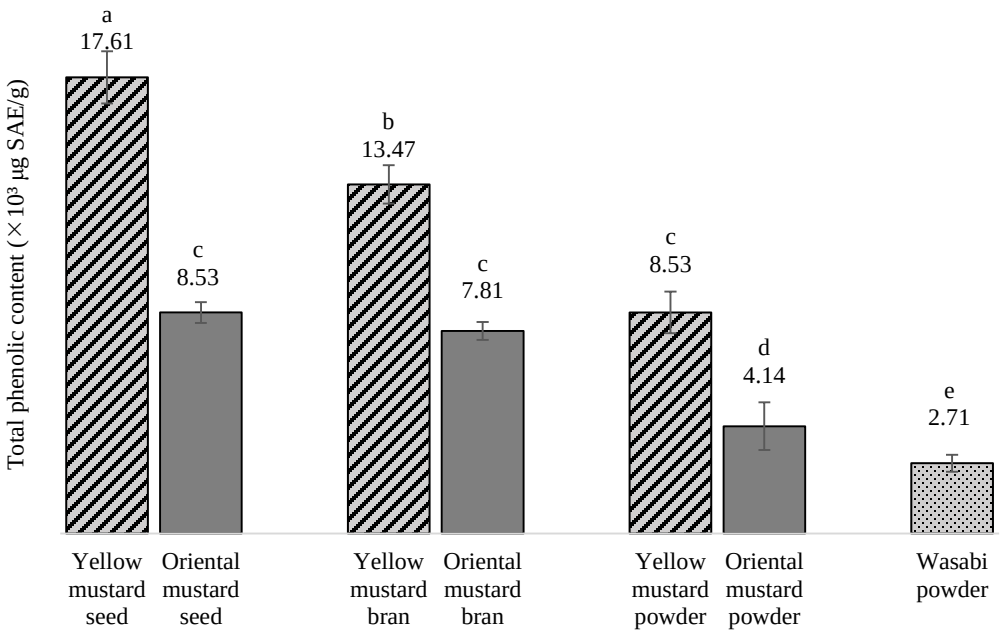


Figure 0-5 Total phenolic content in different yellow and Oriental mustard products and wasabi extracts. Results were expressed as μg sinapic acid equivalents (μg SAE*/ g dry weight basis). All data are expressed as mean ± standard deviation (n=3). Values with different superscripts were significantly different at $p \leqslant 0.05$.

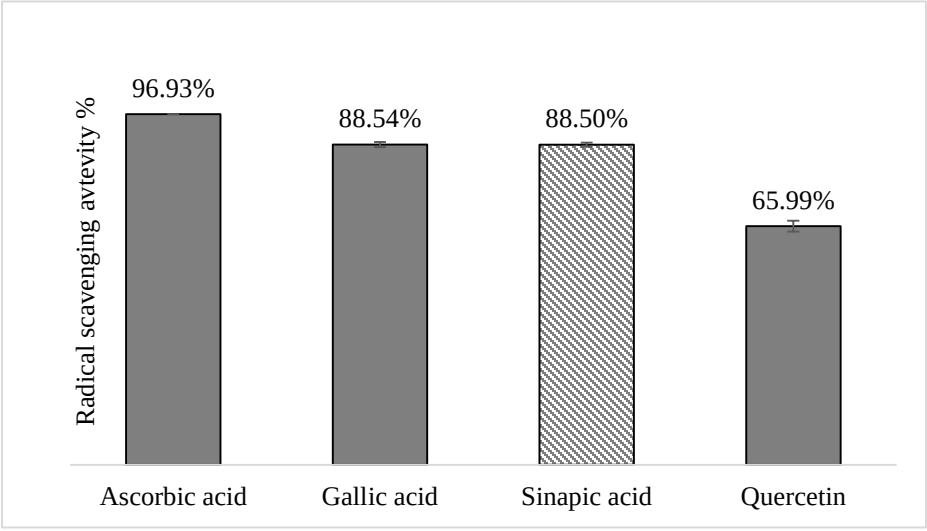


Figure 0-6 Radical scavenging activity of sinapic acid as compared to other phenolic standards at a concentration of 1×10^3 μg/ml.

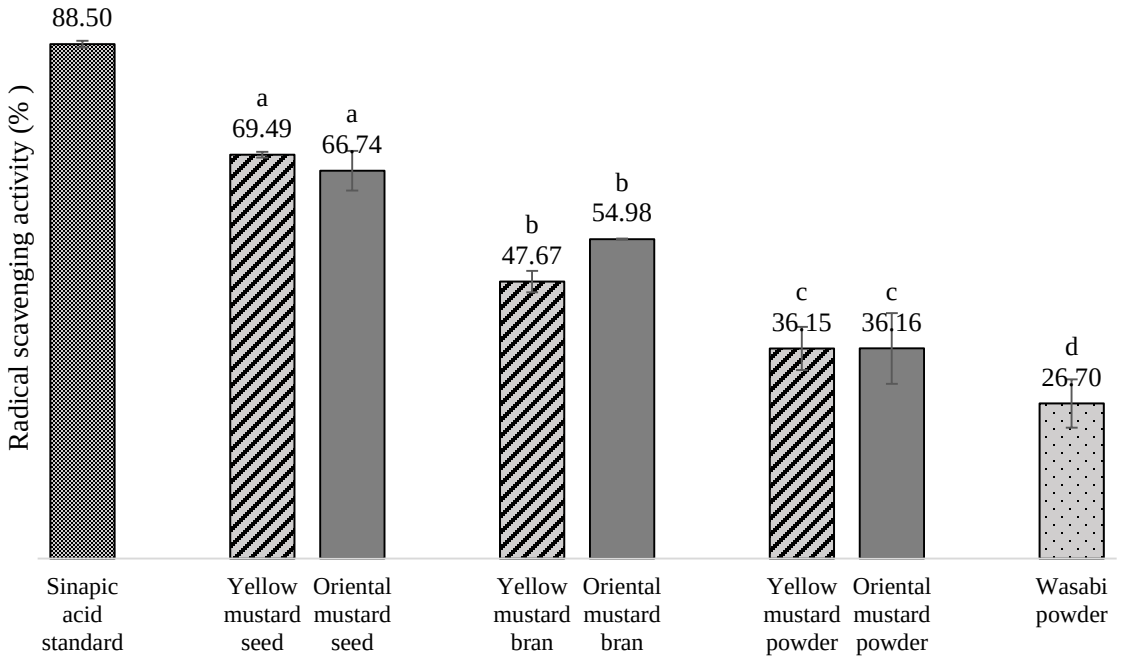


Figure 0-7 Antioxidant activity in different yellow and Oriental mustard products and wasabi extracts in comparison to sinapic acid standard at a concentration of 1×10^3 μg/ml. All data are expressed as mean ± standard deviation (n=3). Values with different superscripts were significantly different at $p \leqslant 0.05$.

Chapter 4

Manuscript 2: Antimicrobial activity of phenolic compounds from yellow and Oriental mustard seed

Fahmi R., Cordeiro R.P., Eck, P., Thiyam-Hollander, U., and Holley R.A.

4.1 Abstract

Antimicrobial properties of extracts from Oriental (*Brassica junceae*) and yellow (*Sinapis alba*) mustard seed have been attributed to their glucosinolates and isothiocyanates content. However, plant phenolics had also been reported as bactericidal. We therefore determined the identity and antimicrobial activity of endogenous phenolic compounds extracted from mustard against strains of *Escherichia coli* O157:H7 and *Listeria monocytogenes*. Mustard powders were deodorized by autoclaving at 115 ºC for 15 min to inactivate plant myrosinase and prevent the interference of glucosinolates and bactericidal isothiocyanates in assessing any antimicrobial activity. Phenolics were extracted using the Accelerated Solvent Extraction (ASE) technique and characterized using reverse-high-performance liquid chromatography (HPLC-DAD). Antimicrobial efficacy was investigated using a minimum inhibitory concentration (MIC) assay.

Sinapine was the major phenolic compound in all the samples analysed, with trace amounts of sinapic acid. Sinapic acid inhibited the growth of E. coli O157:H7 (02:0628) at a concentration of 1500 ppm, and the growth of E. coli O157:H7 (00:3581) at 750 ppm. The growth of L. monocytogenes GLM4 was inhibited by sinapic acid at a concentration of 750 ppm. No inhibition was observed for all mustard sees extracts.

Keywords: Mustard, phenolics, sinapine, *E. coli* O157:H7, *L. monocytogenes*.

4.2 Introduction

Foodborne illness caused by pathogenic bacteria, their associated morbidity, and economic impacts are a global public health concern. Every year, nearly 48 million people suffer from food related infections in the United States (Scallan et al., 2011). *Escherichia coli* O157:H7 is one of the major foodborne pathogens causing haemorrhagic colitis leading to life-threatening hemolytic uremic syndrome in humans (Peacock et al., 2001). Cattle and other ruminants are the major reservoirs of *E. coli* O157:H7 (Piérard et al., 2012). Bovine food products such as undercooked ground beef, raw milk and other contaminated bovine food products have been most commonly associated with *E. coli* O157:H7 outbreaks (Doyle, 1991).

Another major foodborne pathogen, *L. monocytogenes* causes listeriosis infection with a mortality rate of almost 20-30% (Allerberger, 2003). This pathogen is widely spread in the environment and present in soil, water, vegetation and animals. Generally, *L. monocytogenes* is able to grow in extreme environmental conditions such as high concentration of salt, low water activity, acidic conditions and low temperatures. Contaminated ready-to-eat, processed and refrigerated food products are the most common vehicles that have been implicated in *L. monocytogenes* outbreaks (Allerberger and Wagner, 2010).

With the growing consumer demand for natural and safe food preservatives to replace synthetic chemical compounds and their potential health risks, the use of natural plant extracts as antibacterial compounds seems to be an attractive alternative to control the presence of foodborne pathogens and to extend the shelf life of food products (Cetin-Karaca, 2011; Hintz et al., 2015). Plant-derived phenolic compounds in particular, have received considerable attention as natural antimicrobial agents for use in food preservation over the past few years. The antimicrobial activity of phenolic compounds was mainly attributed to their ability to penetrate

the organism cell membrane and inactivate its functional enzymes which consequently damage the cellular integrity and leads to cell dysfunction and death (Cetin-Karaca, 2011; Moreno et al., 2006).

Mustard seed is a rich source of bioactive substances such as glucosinolates and phenolic compounds. Sinigrin and sinalbin are two major glucosinolates found in Oriental and yellow mustards, respectively. As a result of mechanical damage, plant endogenous myrosinase react with stored glucosinolates to hydrolyse them to bactericidal isothiocyanates. Allyl isothiocyanate and benzyl isothiocyanate are the main hydrolysis by-products derived from sinigrin and sinalbin, respectively (Herzallah and Holley, 2012). The antimicrobial properties of mustard glucosinolates and their metabolite isothiocyanates against various foodborne pathogens including *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* have been documented (Aires et al., 2009; Cordeiro et al., 2014; Lin et al., 2000; Olaimat and Holley, 2013; Turgis et al., 2009), However, little information is available on the antimicrobial activity of phenolic compounds in mustard seed extracts.

Sinapic acid and its derivatives (sinapates) are characteristic phenolics in *Brassicacea* species including mustard seeds (*Brassica juncea* and *Sinapis alba*) (Nićiforović and Abramovič, 2014). Sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) belongs to the hydroxycinnamic acids and exists in both free and esterified forms such as sinapine and sinapoyl glucose (Cartea et al., 2010). The antimicrobial activity of sinapic acid against various Gram negative and positive bacteria has been reported previously (Barber et al., 2000; Engels et al., 2012; Johnson et al., 2008; Nowak et al., 1992; Tesaki et al., 1998). Therefore, the main objectives of this study were i) to characterize the phenolic profile in deodorized yellow and Oriental mustard seeds using (HPLC-DAD), and ii) to determine the antimicrobial activity of the

obtained phenolic extracts against five strains of *E. coli* O157:H7 (00:3581, 02:0304, 02:0627, 02:0628 and 02:1840) and three strains of *L. monocytogenes* (2-243, GLM-3,GLM-4).

4.3 Materials and Methods

Materials

Yellow and Oriental mustard seeds were supplied by Sakai Spice Canada Corp. (Lethbridge, AB, Canada). All samples were stored at 4°C in polyethylene bags until used. The standard sinapic acid ≥98%, was purchased from Sigma-Aldrich (St. Louis, MO., USA). Sinigrin hydrate was from Sigma-Aldrich and sinalbin hydrate was from AppliChem Inc (St Louis, MO, USA). High-performance liquid chromatography (HPLC) grade acetonitrile was purchased from Fisher Scientific Co. (Fair Lawn, N.J., U.S.A.). All other chemicals used were of analytical grade.

Bacterial strains

Five strains of *E. coli* O157:H7, including 02-0627, 02-0628, 02-0304, 00-3581and non motile 02-1840 (nonpathogenic human clinical isolate) and three strains of *L. monocytogenes* (GLM-3, GLM-4, 2–243) were used in this study. *E. coli* O157:H7 strains were obtained from Rafiq Ahmed, National Microbiology Laboratory, Public Health Agency, Canadian Centre for Human and Animal Health. The three *L. monocytogenes* strains used were from the culture collection of the Food Science Department, University of Manitoba.

Mustard heat treatment and inactivation of plant endogenous myrosinase

Prior to extraction, mustard samples were heat treated (deodorized) in order to inactivate the plant myrosinase and prevent the degradation of glucosinolate to isothiocyanates according to a procedure used by Cordeiro et al., 2014 with slight modification. Mustard seeds were sieved and ground to fine powder using a coffee grinder for 30 sec. Then, a 1 cm thick layer of obtained powder was placed in a metal tray, covered with aluminum foil and autoclaved at 115 ºC for 15 min. Heat-treated mustard samples were added to distilled water (20%), kept for 3 h at room temperature (23˚C), and centrifuged for 20 min at 12,000 ×g and 4 °C. The absence of myrosinase enzyme in deodorized samples was confirmed by evaluating the stability of glucosinolate (sinigrin and sinalbin in Oriental and yellow mustard, respectively) levels using reversed phase-liquid chromatography (HPLC-DAD). Therefore, resultant supernatant was collected, filtered using a 0.22 μm PES syringe filter (VWR Scientific, Toronto, ON, Canada) and injected to a HPLC-DAD Waters 2695 unit (Waters Corporation, Milford, MA, USA) equipped with a C18 column (Gemini-NX, 150×4.60 mm, 5μ; Phenomenex, Torrance, CA, USA) for the separation of glucosinolates. The system was running with isocratic elution for 10 min at a flow rate of 1 ml/min and an injection volume of 5 μl. The solvent system included 20% (v/v) acetonitrile and 80% water which contained tetrabutylammonium hydrogen sulfate (0.02 M, pH 5.5) as an ion-pair agent. The wavelength used for analysis of glucosinolate was set at 227 nm (Cordeiro et al., 2014; Herrero et al., 2004; Luciano et al., 2011).

Extraction of phenolic compounds from mustard seeds

Phenolic extracts of deodorized mustard samples were obtained using an Accelerated Solvent Extraction (ASE 300, Dionex, Sunnyvale, CA, USA) system. Heat-treated samples were mixed and homogenized well with Ottawa sand in a ratio of 50% (w/w). ASE stainless steel extraction cells were filled with 30 g of prepared mixture and went through extraction. Two cellulose filter papers were placed at the bottom of each extraction cell and the cell caps were hand tightened securely for both sides of the cells prior to the extraction. Extraction was conducted under the condition of high pressure (1500 psi) with two statistic cycles (5 min static time for each cycle), 60% flush volume and 1 min purging time using the appropriate extraction solvent. In this trail, samples were first defatted using *n*-hexane (100%) at room temperature (23°C), followed by the extraction of phenolic compounds using analytical grade methanol (100 %) at 100^{o}C. Approximately 15 g of deodorized mustard powder in each extraction cell yielded about 100 ml methanolic extract of phenolic compounds. In order to prepare the compounds for inoculation, the final concentration of methanol solvent in the pooled extracts was reduced to 2% using a vacuum rotary evaporator (R-205 Buchi, Flawil, Switzerland) at 50°C for 2 h.

Characterization of phenolic compounds in deodorized mustard extracts

Mustard extracts were analyzed by a reversed-phase HPLC-DAD system (Ultimate 3000; Dionex, Sunnyvale, CA, USA) according to Khattab et al., (2010). A gradient elution was performed using water/methanol (90 : 10) with 1.25% *o*-phosphoric acid as solvent A, and methanol (100%) with 0.1% *o*-phosphoric acid as solvent B, using a C18 column (Synergi 4µ Fusion-RP 80 Å; 150 × 4.0 mm- 4 micron (Phenomenex) at 0, 7, 20, 25, 28, 31 and 40 min with 10, 20, 45, 70, 100, 100 and 10% B. Mobile phases A and B as well as mustard extracts were

filtered through 0.45 μm syringe filters prior to injection. The mobile phase flow rate was adjusted to 1 ml/min and the column temperature was maintained at 25˚C. Peaks were analysed at 330 nm and identified based on retention time, UV spectra and by comparison with the authentic sinapic acid standard. Results of duplicate analyses from each extract were expressed as μg sinapic acid equivalents (μg SAE) based on sinapic acid reference substance calibration curve in a dilution range of 0.05 to 0.5 mg/ml.

Bacterial culture preparation

All bacterial cultures were stored at −80°C in Brain Heart Infusion (BHI) broth (Oxoid Ltd., Basingstoke, England) containing 25% glycerol. Frozen cultures were activated by streaking one loopful from each strain on Tryptone Soy Agar (TSA, Oxoid) which was incubated at 35 ºC for 24 h to 48 h. One colony of each *E. coli* strain was streaked on Violet Red Bile Agar (VRB, Oxoid) and *L. monocytogenes* strains were streaked on Listeria Selective Agar Base with Listeria Selective Supplement (LSA, Oxoid), and incubated at 35 ºC for 24 h to 48 h. A single colony of *E. coli* and *L. monocytogenes* strains from the selective agars was transferred to Mueller Hinton (MH, Oxoid) broth and cultured overnight at 35°C. Then 0.1% (v/v) of this culture was transferred individually to fresh MH broth and incubated as before. In order to prepare a bacterial suspension with a density equivalent to 10^6 CFU/ml for *E. coli* strains *and* 10^8 CFU/ml for *L. monocytogenes* strains, 0.1% (v/v) of the later culture resuspended in MH broth was incubated for 3-6 h until it reached an absorbance of 0.5 in 600 nm using a spectrophotometer (Ultrospec 2000; Pharmacia Biotech, Baie d'Urfe, QC, Canada). After adjusting the turbidity, to prevent the change in the cell number of bacteria, the inoculum was used within 30 min to determine the MIC of phenolic compounds as described below.

Minimum Inhibitory Concentration assay of yellow and Oriental mustard extracts against *E. coli* O157:H7 and *L. monocytogenes*

The antimicrobial action of phenolic extracts of deodorized yellow (1200 sinapine) and Oriental (1071 ppm sinapine) mustard seed, and of sinapic acid standard in two different concentrations (1200 ppm and 3000 ppm) against Gram positive (*L. monocytogenes*) and Gram negative (*E. coli* O157:H7) bacteria were investigated using the minimum inhibitory concentration (MIC) assay. The MICs were determined with a microdilution method. Twelve two-fold serial dilutions were performed using sterile 96-well microplates with lids (Corning Inc., Corning, NY) as follows: 50 µl of sterile MH broth were placed into each well of row A to C (Figure 4.1). Then, 50 µl of the challenging compound (mustard phenolic extracts or sinapic acid standard) was used for downstream serial dilutions in 11 consecutive wells and the last 50 µl were discarded from the last column. Then, 50 µl of inoculum was added to each well, which already contained 50 µl of mixed MH broth and phenolic extracts with different concentrations, to reach a final volume of 100 µl in each well. A positive control (containing inoculum but no phenolic compound) and negative control (containing phenolic compound but no inoculum) were included on each microplate. The contents of the wells were thoroughly mixed and the microplates were incubated at 35°C for 24 h. Three replicates of each assay were made and the experiment was carried out twice. MICs were defined as the lowest concentration of the compound that was able to inhibit the visible growth of the organism (Mann and Markham, 1998). Turbidity caused by bacterial growth in the 96-wells was determined at 630 nm using an FL×800 microplate reader (Bio-Tek Instruments, Inc., Winooski, VT). The plate reader was controlled by KC4 3.0 software (version 29).

Data expression and analysis

Data on phenolic content of mustard extractions and their antimicrobial activity were statistically interpreted using one factor ANOVA. For multiple comparisons, Tukey mean separation was followed using SPSS for Windows version 18.0 (2010). Significance level was defined using the value $p<0.05$.

4.4 Results and Discussion

Verification of myrosinase inactivation in autoclaved mustards

As members of the *Brassicaceae* family, both yellow and Oriental varieties of mustard contain high levels of glucosinolates which can be degraded in the presence of moisture by endogenous myrosinase (E 3.2.1.147) in plant tissue to yield bactericidal isothiocyanates. The antimicrobial activity of isothiocyanates is attributed to their ability in penetrating the bacteria cell membrane, inactivating the functional enzymes, and disrupting the cellular metabolic reactions (Luciano et al., 2011; Olaimat and Holley, 2014). To inactivate the plant myrosinase and prevent the interference of glucosinolates and corresponding isothiocyanates in assessing the antimicrobial activity of phenolic compounds in yellow and Oriental mustard seeds, samples were deodorized by autoclaving at 115 ºC for 15 min. Myrosinase absence was confirmed by the constant measurement of sinalbin and sinigrin concentrations over 72 h using reversed-phase HPLC-DAD (Figure 4.2). Results demonstrated that the levels of glucosinolate were constant in both yellow and Oriental mustards over a time period of three days, which indicated the full inactivation of myrosinase enzyme. Results were well in accordance with previous studies conducted by Cordeiro et al. (2014) and Olaimat and Holley (2014) who found that myrosinase

was completely inactivated after deodorization of mustard powder and meal at 115 °C for 15 min.

Identification and quantification of phenolic constituents

Figure 4.3 displays the HPLC-DAD chromatogram of deodorized yellow (A) and Oriental mustard (B) extracts at 330 nm. Sinapine, the choline ester of sinapic acid was the principal phenolic compound in Oriental and yellow mustard seeds, with 6.64×10^3 and 11.51×10^3 µg /g, respectively. Sinapic acid was detected in trace amounts ($\leq 0.1 \times 10^3$ µg /g) for all samples. The corresponding values of the sum of phenolic acids estimated by HPLC-DAD were 8.1×10^3 and 12.37×10^3 µg /g, respectively. This was well in accordance with the results from Thiyam et al., (2006) who identified sinapine as the main sinapic acid derivative accounting for more than 90% of the total phenolic compounds in mustard meal extracts. Similarly, Khattab et al. (2010) and Mayengbam et al. (2014) found sinapic acid in negligible amounts and reported the presence of sinapine (10.17×10^3 to 10.94×10^3 µg /g) as the predominant phenolic compound in mustard seeds (*Brassica juncea*) with negligible amounts of free sinapic acid.

Antimicrobial activity as determined with the minimum inhibitory concentrations assay

The phenolic extracts of yellow and Oriental mustard seeds had no antimicrobial activity against investigated bacterial strains at 35 ºC and pH 7 (Table 4.2-4.6), and against *E. coli* O157:H7 strains at 21 ºC and pH 5 (results not shown). However, a dose dependent bacterial growth stimulation was observed for all investigated *E. coli* O157:H7 strains, except *E. coli* O157:H7 (02:1840), and all L. monocytogenes strains, when incubated with the yellow mustard seeds extract. Similarly, Orient mustard seed extracts stimulated growth of all bacterial strains, except *L. monocytogenes* (2-243). This indicates that mustard extracts from both varieties contained compounds that promoted the growth of the investigated bacteria.

In contrast, sinapic acid showed antimicrobial activity against *E. coli* O157:H7 02-0628 and 00-3581 and *L. monocytogenes* (GLM-4) with MICs of 1500, 750 and 750 ppm respectively.

Our findings do not confirm an antimicrobial activity of phenolic compounds, mainly constituted of sinapine, extracted from yellow or Oriental mustard seeds. This conforms with the report of Nowak et al. (1992), which did not observe an antimicrobial activity of sinapine, the main compound of the mustard extracts, against various Gram-negative (*E. coli*, *Enterobacter aerogenes*, and *Pseudomonas fluorescens*) and Gram-positive (*Bacillus subtilis*, *B. cereus*, *Streptococcus lactis*, and *S. cremoris*) food spoilage bacteria. However, they also reported that a fraction of rapeseed extract containing sinapic acid, the hydrolysis product of sinapine, had antimicrobial activity against the tested microorganisms, a finding we also observed for pure sinapic acid. Similarly, Engels et al. (2012) reported sinapine as the major phenolic compound in Oriental mustard meal crude extracts, which after alkaline hydrolysis to sinapic acid (2.6 mg/g) showed antibacterial activity against *Bacillus subtilis, Escherichia coli, L. monocytogenes, Pseudomonas flourescens, and S. aureus* (MICs $\leq$ 0.1 g/L). However, lactic acid bacteria showed resistance to sinapic acid, most likely due to the metabolic activity of their decarboxylases and reductase enzymes (Engels et al., 2012).

The inhibitory activity of sinapic acid (2.2 mM) against *E. coli* was attributed to the presence of methoxy and two hydroxyl groups on the benzene ring of sinapic acid (Tesaki et al., 1998). The conversion of sinapic acid in plant extracts to sinapine seems to be essential for the antibacterial activity. Consequently, mustard and rapeseed extracts previously reported to be bactericidal (Nowak et al., 1992; Tesaki et al., 1998; Engels et al., 2012), must have either undergone significant hydrolysis, or had not been properly treated to eliminate isothiocyanates.

Significantly, we show here that sinapine containing mustard extracts did increase bacterial growth, contrasting the antimicrobial action of it derivate sinapic acid in plant extracts. At present there are no reports of enhanced bacterial growth for sinapine containing plant extracts (PubMed search on August 11th 2016). However, if sinapine could selectively stimulate the growth of beneficial commensal bacterial species and strains, mustard extracts could very well be developed into prebiotic products. Future research should address this.

4. 5 Conclusions

The present study identified sinapine as the major phenolic compound present in deodorized yellow and Oriental mustard seed. Sinapine containing mustard extracts stimulated bacterial growth and could be developed into prebiotic products. Sinapic acid, the hydrolysis product of sinapine, exhibits selective antibacterial activity, and we conclude that this conversion must be necessary for a natural extract to exhibit the antibacterial effect.

Acknowledgment

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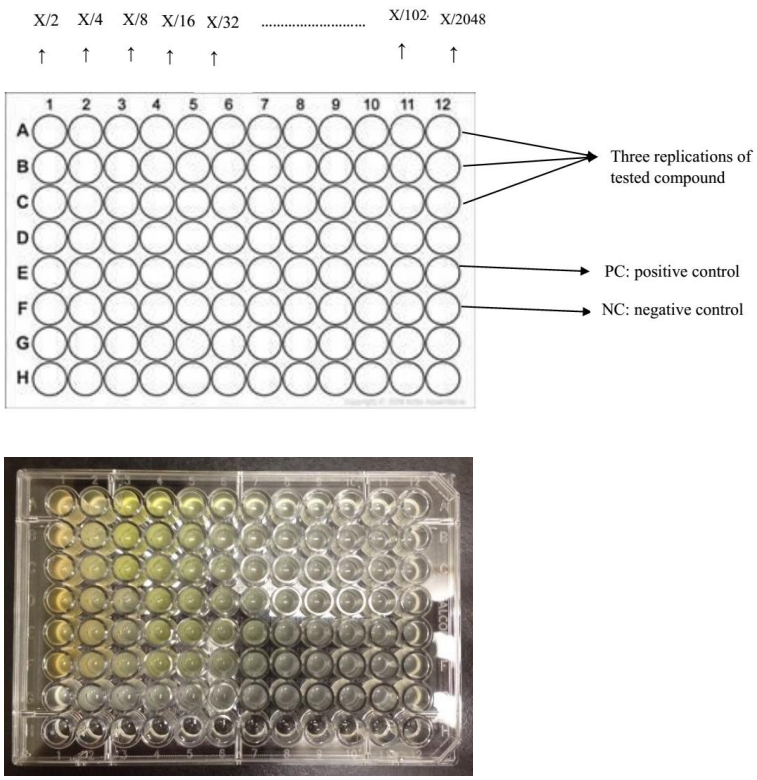


Figure 0-1 Performing two-fold serial dilutions to assess minimum inhibitory concentration of the tested compound using 96-well microplate. X, the highest concentrations of tested (ppm) compound; PC, positive control (broth with bacterial inoculum); NC, negative control (broth and tested compound only).

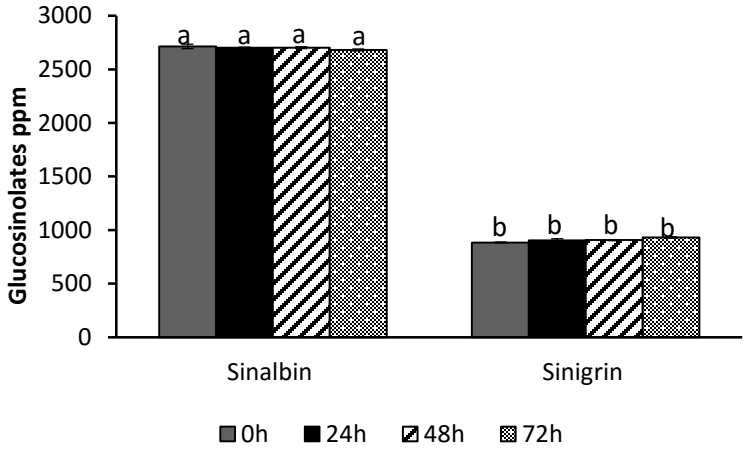
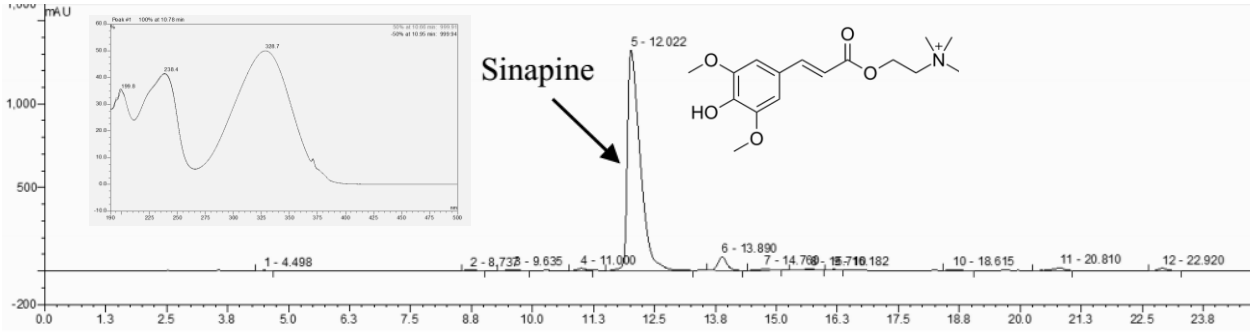


Figure 0-2 Measurement of myrosinase inactivation by glucosinolate substrates, sinalbin and sinigrin, stability in deodorized yellow and Oriental mustard seeds, respectively. All data are expressed as mean ± standard deviation (n=3). Values with different superscripts were significantly different at $p \leq 0.05$.

A)



B)

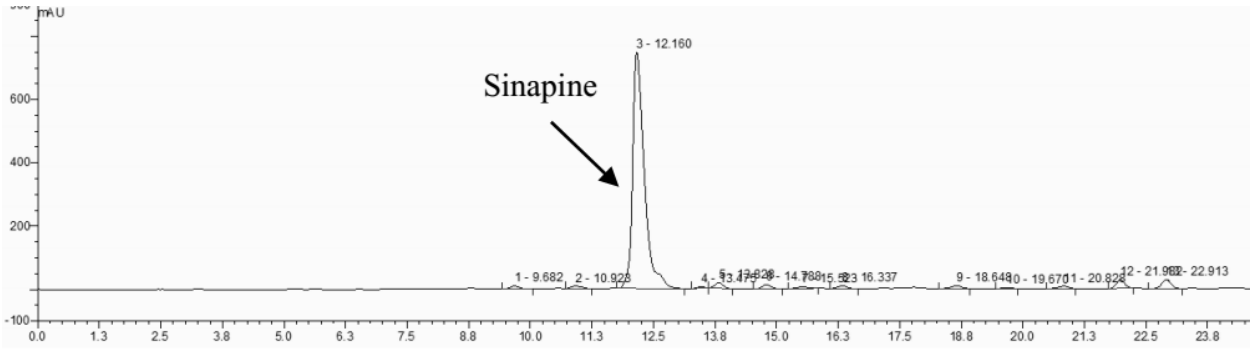


Figure 4-3 HPLC-DAD chromatograms (330 nm) of methanolic extracts obtained from deodorized yellow (A) and Oriental mustard seeds (B) showing sinapine as the predominant phenolic compound. Absorption spectra and chemical structure of sinapine are also included.

Table 0-1 Phenolic contents ($\times 10^3$ µg SAE/g samples) of deodorized yellow and Oriental mustard seed extracts.

Sample	Sinapine	Sinapic Acid	Unidentified
Yellow mustard seeds	11.51± 0.98*	0.09± 0.01	≈ 1.13
Oriental mustard seeds	6.64± 0.42**	0.10± 0.00	≈ 1.36

*sinapine concentration in crude extract of autoclaved yellow mustard seeds: 0.33 mg/ml

**sinapine concentration in crude extract of autoclaved Oriental mustard seeds: 0.17 mg/ml

Table 4-2 Thresholds of growth inhibition for sinapic acid and phenolic extract from deodorized yellow (YMS) and Oriental mustard seed against *E. coli* O157:H7 and *L. monocytogenes* strains. Minimum inhibition assays were performed at 35˚C and pH=7.

Bacterial (strain)	Minimum inhibitory concentration (ppm)			
	Yellow mustard seed	Oriental mustard seed	Sinapic Acid	
	(1200 ppm)	(1071 ppm)	1200 ppm	3000 ppm
E. coli O157:H7 (02-0304)	NA*	NA*	NA**	NA**
E. coli O157:H7 (02:0627)	NA*	NA*	NA**	NA**
E. coli O157:H7 (02:0628)	NA*	NA*	NA**	1500 ppm
E. coli O157:H7 (02:1840)	NA*	NA*	NA**	NA**
E. coli O157:H7 (00:3581)	NA*	NA*	750 ppm	750 ppm
L. monocytogenes 2-243	NA*	NA*	NA**	NA**
L. monocytogenes GLM3	NA*	NA*	NA**	NA**
L. monocytogenes GLM4	NA*	NA*	750 ppm	750 ppm

*NA, not active at the highest test concentration, 1200 ppm and 1071 ppm of sinapine in yellow and Oriental mustard seed phenolic extracts, respectively.

**NA, not active at the highest test concentration, 1200 ppm and 3000 ppm of sinapic acid standard.

Table 4-3 Effect of yellow mustard seed phenolic extract on the growth of E. coli O157:H7 and L. monocytogenes in the MIC assay.

Bacterial Strain	Optical Density (OD)											
	Concentration of Sinapine (ppm)											
	1200	**600**	**300**	**150**	**75**	**37.5**	**18.75**	**9.38**	**4.69**	**2.35**	**1.17**	**0.59**
E. coli O157:H7 (02-0304)	1.13^{a}	0.98^{ab}	0.84^{bc}	0.77^{bcd}	0.66^{cd}	0.63^{cd}	0.72^{bcd}	0.62^{cd}	0.60^{d}	0.61^{cd}	0.62^{cd}	0.75^{bcd}
E. coli O157:H7 (02:0627)	1.21^{a}	1.08^{ab}	0.95^{bc}	0.83^{cd}	0.75^{d}	0.72^{d}	0.73^{d}	0.71^{d}	0.74^{d}	0.76^{cd}	0.76^{cd}	0.82^{cd}
E. coli O157:H7 (02:0628)	1.16^{a}	0.94^{b}	$0.81b^{c}$	0.72^{cd}	0.70^{cde}	0.68^{cde}	0.62^{de}	0.62^{de}	0.63^{de}	0.65^{cde}	0.55^{e}	0.72^{cd}
E. coli O157:H7 (02:1840)	0.92^{a}	0.97^{a}	0.88^{a}	0.81^{a}	0.70^{a}	0.70^{a}	0.69^{a}	0.70^{a}	0.69^{a}	0.71^{a}	0.72^{a}	0.82^{a}
E. coli O157:H7 (00:3581)	1.18^{a}	1.00^{ab}	0.84^{bc}	0.75^{cd}	0.69^{cd}	0.67^{cd}	0.67^{cd}	0.66^{cd}	0.66^{cd}	0.66^{cd}	0.66^{d}	0.76^{cd}
L. monocytogenes (2-243)	0.82^{a}	0.76^{a}	0.48^{b}	0.31^{c}	0.26^{c}	0.23^{c}	0.20^{c}	0.20^{c}	0.20^{c}	0.21^{c}	0.22^{c}	0.24^{c}
L. monocytogenes (GLM3)	0.87^{a}	0.79^{a}	0.45^{b}	0.35^{bc}	$0.32b^{c}$	0.32^{c}	0.30^{c}	0.28^{c}	0.28^{c}	0.29^{c}	0.30^{c}	0.39^{bc}
L. monocytogenes (GLM4)	0.85^{a}	0.75^{a}	0.44^{b}	0.29^{c}	0.23^{c}	0.22^{c}	0.19^{c}	0.19^{c}	0.19^{c}	0.19^{c}	0.19^{c}	0.26^{c}
Negative Control*	**0.15**	**0.10**	**0.07**	**0.06**	**0.05**	**0.05**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**

* Negative control includes no bacterial inoculation (only broth and tested compound). All data are expressed as mean of three replications with standard deviation < 0.09 (n=3). Values with different superscripts were significantly different at $p \leq 0.05$.

Table 0-4 Effect of Oriental mustard seed phenolic extract on the growth of E. coli O157:H7 and L. monocytogenes in the MIC assay.

Bacterial Strain	Optical Density (OD)											
	Concentration of Sinapine (ppm)											
	1071	**534.5**	**267.75**	**133.88**	**66.93**	**33.47**	**16.73**	**8.36**	**4.2**	**2.1**	**1.04**	**0.52**
E. coli O157:H7 (02-0304)	1.20^{a}	1.10^{a}	0.95^{b}	0.7^{4c}	0.64^{cde}	0.58^{def}	0.68^{ef}	0.55^{ef}	0.55^{f}	0.58^{def}	0.54^{ef}	0.68^{cd}
E. coli O157:H7 (02:0627)	1.26^{a}	1.19^{a}	1.11^{a}	0.95^{c}	0.82^{d}	0.74^{ef}	0.72^{fg}	$0.70f^{g}$	0.69^{g}	0.73^{ef}	0.75^{e}	0.82^{d}
E. coli O157:H7 (02:0628)	1.29^{a}	1.16^{b}	1.00^{c}	0.82^{d}	0.71^{e}	0.69^{ef}	0.68^{gh}	0.66^{fg}	0.65^{h}	0.63^{gh}	0.63^{gh}	0.71^{e}
E. coli O157:H7 (02:1840)	0.57^{f}	0.60^{e}	0.88^{ab}	0.91^{a}	0.81^{b}	0.69^{c}	0.60^{cd}	0.62^{cd}	0.63^{de}	0.66^{c}	0.6^{7c}	0.80^{b}
E. coli O157:H7 (00:3581)	1.04^{ab}	1.16^{a}	0.99^{b}	0.82^{ab}	0.73^{ab}	0.66^{b}	0.64^{b}	0.65^{b}	0.65^{b}	0.62^{b}	0.61^{b}	0.71^{e}
L. monocytogenes (2-243)	0.96^{a}	0.86^{b}	0.60^{c}	0.43^{d}	0.34^{e}	0.26^{f}	0.21^{h}	0.23^{g}	0.21^{i}	0.21^{h}	0.20^{hi}	0.21^{ab}
L. monocytogenes (GLM3)	0.93^{a}	0.86^{a}	0.63^{b}	0.51^{c}	0.39^{d}	0.30^{e}	0.28^{e}	0.29^{e}	0.27^{e}	0.27^{e}	0.27^{e}	0.37^{d}
L. monocytogenes (GLM4)	0.88^{a}	0.85^{a}	0.57^{b}	0.44^{c}	0.34^{d}	0.25^{e}	0.21^{f}	0.20^{f}	0.19^{g}	0.19^{fg}	0.19^{f}	0.23^{e}
Negative Control*	**0.17**	**0.12**	**0.09**	**0.07**	**0.05**	**0.05**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**

* Negative control includes no bacterial inoculation (only broth and tested compound). All data are expressed as mean of three replications with standard deviation < 0.09 (n=3). Values with different superscripts were significantly different at $p \leq 0.05$.

Table 0-5 Effect of sinapic acid on the growth of E. coli O157:H7 and L. monocytogenes in the MIC assay.

Bacterial Strain	Optical Density (OD)											
	Concentration of Sinapic Acid (ppm)											
	1200	**600**	**300**	**150**	**75**	**37.5**	**18.75**	**9.38**	**4.69**	**2.34**	**1.2**	**0.58**
E. coli O157:H7 (02-0304)	0.68^{ab}	0.59^{ab}	0.60^{ab}	0.60^{ab}	0.60^{ab}	0.62^{ab}	0.63^{ab}	0.61^{ab}	0.62^{ab}	0.63^{ab}	0.61^{ab}	0.72^{a}
E. coli O157:H7 (02:0627)	0.69^{ab}	0.62^{bc}	0.64^{bc}	0.63^{bc}	0.64^{c}	0.66^{abc}	0.68^{abc}	0.72^{abc}	0.74^{a}	0.67^{abc}	0.65^{abc}	0.71^{ab}
E. coli O157:H7 (02:0628)	0.63^{ab}	0.58^{ab}	0.60^{ab}	0.60^{ab}	0.60^{ab}	0.61^{ab}	0.60^{b}	0.63^{ab}	0.67^{ab}	0.62^{ab}	0.62^{ab}	0.74^{a}
E. coli O157:H7 (02:1840)	0.70^{a}	0.59^{a}	0.58^{a}	0.61^{a}	0.57^{a}	0.62^{a}	0.62^{a}	0.62^{a}	0.63^{a}	0.65^{a}	0.62^{a}	0.74^{a}
E. coli O157:H7 (00:3581)	0.53^{cde}	0.48^{e}	0.51^{de}	0.55^{cd}	0.55^{de}	0.57^{cd}	0.58^{cd}	0.60^{bcd}	0.60^{bcd}	0.74^{a}	0.61^{bc}	0.70^{ab}
L. monocytogenes (2-243)	0.10^{cd}	0.11^{cd}	0.14^{bcd}	0.16^{abcd}	0.17^{bcd}	0.20^{a}	0.18^{ab}	0.18^{ab}	0.20^{a}	0.18^{ab}	0.16^{abcd}	0.17^{abc}
L. monocytogenes (GLM3)	0.23^{a}	0.23^{a}	0.24^{a}	0.23^{a}	0.23^{a}	0.23^{a}	0.22^{a}	0.23^{a}	0.22^{a}	0.24^{a}	0.23^{a}	0.26^{a}
L. monocytogenes (GLM4)	0.13^{bc}	0.14^{bc}	0.16^{ab}c	0.17^{abc}	0.15^{bc}	0.18^{abc}	0.18^{abc}	0.18^{abc}	0.18^{abc}	0.19^{ab}	0.18^{ab}	0.20^{a}
Negative Control*	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**

*

Negative control includes no bacterial inoculation (only broth and tested compound). All data are expressed as mean of three replications with standard deviation < 0.09 (n=3). Values with different superscripts were significantly different at $p \leq 0.05$.

Table 0-6 Effect of sinapic acid on the growth of E. coli O157:H7 and L. monocytogenes in the MIC assay.

Bacterial Strain	Optical Density (OD)											
	Concentration of Sinapic Acid (ppm)											
	3000	**1500**	**750**	**375**	**187.5**	**93.75**	**46.87**	**23.43**	**11.72**	**5.85**	**2.93**	**1.47**
E. coli O157:H7 (02-0304)	0.60^{b}	0.57^{b}	0.56^{b}	0.56^{b}	0.59^{b}	0.57^{b}	0.57^{b}	0.60^{b}	0.57^{b}	0.57^{b}	0.57^{b}	0.67^{ab}
E. coli O157:H7 (02:0627)	0.62^{a}	0.58^{a}	0.63^{a}	0.63^{a}	0.65^{a}	0.64^{a}	0.66^{a}	0.64^{b}	0.74^{a}	0.64^{a}	0.62^{a}	0.69^{a}
E. coli O157:H7 (02:0628)	0.59^{d}	0.57^{e}	0.59^{d}	0.60^{bcd}	0.61^{bcd}	0.61^{bcd}	0.62^{b}	0.63^{b}	0.62^{b}	0.62^{b}	0.62^{bc}	0.67^{a}
E. coli O157:H7 (02:1840)	0.63^{b}	0.55^{b}	0.57^{b}	0.60^{b}	0.62^{b}	0.58^{b}	0.60b	0.61^{b}	0.62^{b}	0.63^{b}	0.63^{b}	0.75^{a}
E. coli O157:H7 (00:3581)	0.51^{d}	0.51^{d}	0.54^{cd}	0.56^{bcd}	0.57^{bcd}	0.58^{bcd}	0.60^{bcd}	0.61^{bc}	0.61^{bc}	0.70^{a}	0.63^{b}	0.71^{a}
L. monocytogenes (2-243)	0.15^{def}	0.13^{f}	0.15^{ef}	0.16^{cde}	0.16^{bcd}	0.20^{a}	0.18^{bc}	0.18^{b}	0.21^{a}	0.17^{bc}	0.17^{bc}	0.16^{bcd}
L. monocytogenes (GLM3)	0.25^{a}	0.21^{a}	0.21^{a}	0.21^{a}	0.22^{a}	0.23^{a}	0.22^{a}	0.22^{a}	0.22^{a}	0.24^{a}	0.22^{a}	0.22^{a}
L. monocytogenes (GLM4)	0.12^{e}	0.13^{de}	0.14^{d}	0.16^{c}	0.18^{ab}	0.17^{abc}	0.18^{ab}	0.18^{a}	0.18^{ab}	0.18^{a}	0.17^{abc}	0.17^{bc}
Negative Control*	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**	**0.04**

* Negative control includes no bacterial inoculation (only broth and tested compound). All data are expressed as mean of three replications with standard deviation < 0.09 (n=3). Values with different superscripts were significantly different at $p \leq 0.05$.

Chapter 5

Short report: Impact of pre-treatments on the sinapate profile of mustard products

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5.1 Abstract

Pre-treatments such as soaking and microwaving can affect the extractability, contents and the composition of the main phenolic compounds in mustard extracts. This study investigated the effect of various pre-treatments on the sinapates profile of selected defatted mustard products. The pre-treatments included 2 h soaking with 70% methanol (with and without acidification) and also microwave irradiation for 20 min at a power level of 300 W. Phenolics were extracted with 70% aqueous methanol and analysed using a high-performance liquid chromatography with diode array detection (HPLC-DAD. Microwave irradiation did not affect the sinapate profile of defatted mustard extracts significantly as the sinapine content of yellow mustard bran ($6.87\times 10^3 \pm 0.47$ µg SAE/g), yellow mustard powder ($19.31\times 10^3 \pm 0.01$ µg SAE/g), Oriental mustard bran ($7.28\times 10^3 \pm 0.06$ µg SAE/g), and Oriental mustard powder ($12.19\times 10^3 \pm 2.07$ µg SAE/g) were very similar to their corresponding untreated samples. However, the soaking process, irrespective of its pH, significantly reduced the sinapine content in all investigated samples. Soaking in acidified 70% methanol resulted in further decreases in the sinapine values to reach 4.73×10^3, 10.82×10^3, 3.25×10^3 and 10.01×10^3 µg SAE/g in yellow mustard bran, yellow mustard powder, Oriental mustard bran and Oriental mustard powder,

respectively. In another set of experiments, the formation of canolol was reported during microwave treatment of non-defatted Oriental mustard seeds ($0.42 \times 10^3 \pm 0.13$ µg SAE/g), yellow mustard seeds ($0.20 \times 10^3 \pm 0.05$ µg SAE/g) and canola seeds ($0.37 \times 10^3 \pm 0.04$ µg SAE/g). Formation of canolol was attributed to the presence of oil in the substrate of defatted samples.

Keywords: Mustard, Phenolics, Sinapine, Soaking, Canolol, Microwave treatment.

5.2 Introduction

Mustards varieties (*Brassica juncea/ Sinapis alba*) belong to the *Brassicacea* family and are considered good sources of bioactive phenolics, with the main compounds derived from the sinapate family. (Nićiforović and Abramovič, 2014). Sinapine, the choline ester of sinapic acid, is the main phenol in mustard meal and flour (Dabrowski and Sosulski, 1984; Dubie et al., 2013), and is also found in canola (Khattab et al., 2010; Thiyam et al., 2006).

Sinapine can be decarboxylated into canolol during processing. The formation of canolol in the food matrix can be increased as a result of roasting or microwaving. Significant amounts of canolol have been reported in roasted rapeseed (Spielmeyer et al., 2009), canola (Wijesundera et al., 2008), crude canola oil (Koski et al., 2002; Wakamatsu et al., 2005), high erucic mustard oils (Shrestha et al., 2013), oil extracted from microwaved canola seeds (Mayengbam et al., 2014), and in commercial mustard oils (Mayengbam et al., 2014). However, the presence of canolol in mustard seed commercial products such as powder and bran had not been reported.

Canolol was identified as a potent antioxidant in crude canola oil, exhibiting more potent anti-alkylperoxyl [ROO] radical activity than established antioxidants such as α-tocopherol, vitamin C, β-carotene, rutin and quercetin (Wakamatsu et al., 2005).

We investigated the influence of different pre-treatments on the sinapic acid contents and its conversion into its derivatives. Defatted mustard products were subjected to microwave irradiation with steaming and soaking. The latter method involved soaking of defatted samples in acidified (pH 2) 70% methanol for 2 h, followed by phenolic recovery with 70% methanol, assisted by ultrasound extraction. Soaking was also carried out using 70% methanol, without any pH adjustment to determine the effect of acidification. The canolol formation upon microwave irradiation in non-defatted yellow mustard, Oriental mustard seeds, and canola seeds were also assessed.

5.3 Materials and Methods

Materials

All chemicals were of analytical grade (≥ 99.9%) and were purchased from Sigma Aldrich, Canada. Standard sinapic acid was procured from Sigma-Aldrich (St. Louis, MO, USA). All mustard products including yellow and Oriental mustard seeds, brans and flours were obtained from Sakai Spice, Lethbridge, AB, Canada. Also, canola seeds were obtained from Viterra Canola (Ste. Agathe, MB, Canada).

Defatting samples prior to pre-treatments

Defatting of mustard products (seed, bran and powder) and also canola seed was conducted using a Soxtec 2050 (Foss Tecator, Foss North America, MN, USA) following the manufacturer's application guidelines (FOSFA, 1998). Samples were extracted using n-hexane.

Pre-treatment prior to phenolic extraction.

The following pre-treatments were applied:

Soaking in 70% methanol: defatted samples, 1 g of each, were soaked in 9 ml of 70% methanol, with (pH 2) and without acidification (pH 7). The pH of the acidified solvents was adjusted to pH 2 with Ortho-phosphoric acid. Samples were mixed well for 30 sec and kept in a shaking water bath (23˚C, 150 RPM) for 2 h.

Microwaving: 1 g of each defatted sample was heated for 20 min at 300 W power (with steam) using a Panasonic microwave oven (Model NN-CS597S, 1000 Watt; Secaucus, NJ, USA) according to Mayengbam et al. (2014). Conditions were applied to original and defatted samples.

Extraction of phenolic compounds

Phenolics were extracted with aqueous methanol (70%) according to Thiyam et al. (2006). Briefly, 1 g of each product was extracted with 9 ml of solvent, using ultrasound probe (40% power) (Sonopuls HD 2070, Bandelin Electronic, Berlin) for 60s followed by centrifugation at 5000 x *g* for 10 min at refrigerated condition (4˚C).The supernatant was collected and the residue was again extracted following the same procedure and repeated twice more. The supernatants were pooled, filtered using Whatman filter paper (No: 1) and the mixture was adjusted to 30 ml with 70% methanol. These extractions were carried out in duplicate. All extracted samples were stored at -20^{o}C until used for further experiments and analysis.

HPLC-DAD analysis of sinapic acid derivatives and canolol

The phenolics contents of defatted and non-defatted samples were analyzed by reversed phase HPLC - DAD (Ultimate 3000; Dionex, Sunnyvale, CA, USA) according to Khattab et al.,

(2010). A gradient elution was performed using water/methanol (90 : 10) with 1.25% *o*-phosphoric acid as solvent A, and methanol (100%) with 0.1% *o*-phosphoric acid as solvent B, using C18 column; Synergi 4_ Fusion-RP 80 Å; 150 x 4.0 mm 4 _m (Phenomenex) at 0, 7, 20, 25, 28, 31 and 40 min with 10, 20, 45, 70, 100, 100 and 10 % solvent B. Chromatograms were acquired at 330 nm for sinapic acid and sinapine, and 270 nm for canolol. Sinapine and canolol were identified based on absorption spectrum and retention time of 11 min (at 330 nm) and 18 min (at 270 nm), respectively. The retention time for sinapic acid was 15 min. The contents of sinapic acid, sinapate esters and canolol were quantified based on a calibration curve with the sinapic acid standard using appropriate dilutions ($0.05 – 0.5 \times 10^3$ µg /ml). Results were expressed as sinapic acid equivalents (µg SAE/g dry-base) by plotting the concentration of each compound against the obtained area.

Statistical Analysis

All measurements were carried out in triplicate and results presented as mean ± SD. One way analysis of variance (ANOVA) was estimated using SPSS for Windows version 18.0 (2010). Statistical significance was determined using Tukey mean separation with statistical significance established at $p<0.05$.

5.4 Results and Discussion

Sinapine and sinapic acid in mustard products.

Sinapine was the predominant component in the crude extract of all mustard products analyzed. The highest amount of sinapine (19.76 ±1.36) was found in the yellow mustard

powder, followed by Oriental Mustard powder (14.75±0.37), Oriental Mustard bran (8.36±0.61), and yellow mustard bran (7.53±0.34) (all values in 10^3 µg SAE/g dry weight).

Sinapic acid was detected for all mustard products, however the levels did not differ (yellow mustard powder: 1.41±0.04, Oriental Mustard powder: 1.26±0.03, Oriental Mustard bran 1.13±0.00, and yellow mustard bran 1.21±0.01 [all values in 10^3 µg SAE/g dry weight]).

Effects of soaking on the sinapates profile of mustard products.

Table 5-1 shows the effect of various pre-treatments on the contents of sinapine and its derivatives in defatted mustard products. The soaking process, irrespective of its pH, significantly reduced the extractable sinapine contents in all samples. The highest reduction was seen after soaking in acidified 70% methanol, which decreased the sinapine content of extracts from yellow mustard bran (4.73±0.97 × 10^3 µg SAE/g), yellow mustard powder (10.82±0.47 × 10^3 µg SAE/g), Oriental mustard bran (3.25±0.02 × 10^3 µg SAE/g), and Oriental mustard powder (10.01±1.38 × 10^3 µg SAE/g). The reduction of extractable sinapine was less pronounced after soaking in non-acidified 70% methanol; sinapine content of extracts from yellow mustard bran: 5.03±0.11 × 10^3 µg SAE/g, yellow mustard powder: 15.58±0.72 × 10^3 µg SAE/g, Oriental mustard bran: 5.28±0.30 × 10^3 µg SAE/g, and Oriental mustard powder: 11.1±0.64 × 10^3 µg SAE/g. The observed reductions after soaking could be attributed to the hydrolysis of sinapine into its derivatives (A. Sabra, personal communication, July 2016).

The soaking in acidified 70% methanol had no influence on the sinapic acid content of yellow mustard bran (1.23±0.09 × 10^3 µg SAE/g), yellow mustard powder (1.23±0.26 × 10^3 µg SAE/g), and Oriental mustard powder (1.28±0.05 × 10^3 µg SAE/g), but eliminated it from

Oriental mustard bran. The soaking in non-acidified 70% methanol had no influence on the sinapic acid content of yellow mustard bran (1.05±0.00 × 10^3 µg SAE/g), Oriental mustard powder (1.3±0.02 × 10^3 µg SAE/g), and Oriental mustard bran (1.08±0.05 × 10^3 µg SAE/g), but eliminated it from yellow mustard powder. The elimination of sinapic acid after non acidified methanol treatment in yellow mustard powder and the acidified methanol treatment in Oriental mustard bran are inconsistent with all other results. However, at current we cannot explain a possible mechanism for this phenomenon in these two food matrices. In spite of this, it appears that sinapic acid levels remain fairly stable during soaking in most food matrices. However, considering the hydrolysis of sinapine we would have expected to observe an increase in sinapic acid or canolol (the product of further hydrolysis). The lack of this might be attributed to a constant degradation rate to a non-detected downstream derivate.

Effects of microwaving on the sinapates profile of mustard products.

Microwaving under steam did not affect the extractable sinapine levels in any food matrix (yellow mustard bran: 6.87±0.27, yellow mustard powder 19.31±0.01, Oriental mustard bran 7.28±0.04, and Oriental mustard powder 12.19±1.20 [all values in 10^3 µg SAE/g dry weight]). However, free sinapic acid was completely eliminated in all matrices as a result of microwave irradiation under steam.

No canolol was present under any conditions.

No canolol was detected in any of the analysed food matrices and treatment conditions. This was in contrast with results from previous studies by Mayengbam et al. (2014) who found

microwave toasting of canola seeds at 300 W for 20 min with steam significantly increased canolol content in oil by more than 1700 fold. The lack of canolol formation in the mustard products could be attributed to the fact that the microwave irradiation was carried out on defatted samples and not the whole seeds or oil fractions. The presence of a lipid component seems to be essential for canolol formation (A. A. Aachary, personal communication, September 2015). We therefore designed experiments to validate this and describe the results below.

Effect of defatting prior to microwave treatment on the phenolic content:

Previously, microwave treatment of sinapate rich oil seeds, specifically canola and mustard, produced canolol at high temperatures (> 180℃), a finding the could not be repeated in this study. However, in the present study the mustard products were defatted before microwave treatment and the presence of a lipid component may be necessary for canolol formation. Therefore, the effect of microwave treatment on non-defatted yellow and Oriental mustard seeds as well as canola seeds was investigated.

Table 5.2 shows the levels of sinapine and its derivatives in the extracts of the meal and oil fractions of untreated and microwave-treated canola and mustard seeds. In the meal fraction of all food matrices, sinapine levels did not change upon microwave irradiation. In contrast, sinapic acid levels increased in the canola seeds meal fraction (untreated: 0.48 ± 0.03; microwaved: 0.60 ± 0.04 [values expressed as $\times 10^3$ µg SAE*/g dry-base]). However, sinapic acid was undetectable in the mustard meal fractions. Canolol was absent in the meal fraction of both treated and untreated samples for canola, yellow and Oriental mustard seeds.

Neither sinapine and sinapic acid was detectable in the oil fractions of all food matrices. However, canolol was detected as the sole phenolic compound in the oil fraction of the microwave-treated canola and mustard seeds (Table 5.2). It was evident that canolol was produced as a result of microwave irradiation and that the presence of oil in the matrix was necessary for its formation.

Figure 5.2 shows the HPLC-DAD chromatogram of phenolic extracts obtained from oil fraction of microwave-treated mustard seed. Similar results were also noted in the case of canola seeds.

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Table 0-1 Effects of various pretreatments on the sinapate profile (× 10^3 μg SAE*/g dry-base) of defatted mustard products.

Pre-treatments	Sinapine	Sinapic acid	Canolol
YELLOW MUSTARD BRAN			
Untreated	7.53±0.20[a]	1.21±0.01[ab]	ND*
Soaking in methanol (pH 7)[1]	5.03±0.11[b]	1.05±0.00[b]	ND
Soaking in methanol (pH 2)[2]	4.73±0.97[b]	1.23±0.09[a]	ND
Microwave[3]	6.87±0.27[a]	0.00±0.00[c]	ND
YELLOW MUSTARD POWDER			
Untreated	19.76±0.79[a]	1.41±0.04[a]	ND
Soaking in methanol (pH 7)	15.58±0.72[b]	0.00±0.00[b]	ND
Soaking in methanol (pH 2)	10.82±0.47[c]	1.23±0.26[a]	ND
Microwave	19.31±0.01[a]	0.00±0.00[b]	ND
ORIENTAL MUSTARD BRAN			
Untreated	8.36±0.35[a]	1.13±0.00[a]	ND
Soaking in methanol (pH 7)	5.82±0.30[c]	1.08±0.05[a]	ND
Soaking in methanol (pH 2)	3.25±0.02[d]	0.00±0.00[b]	ND
Microwave	7.28±0.04[b]	0.00±0.00[b]	ND
ORIENTAL MUSTARD POWDER			
Untreated	14.75±0.21[a]	1.26±0.03[a]	ND
Soaking in methanol (pH 7)	11.10±0.64[b]	1.30±0.02[a]	ND
Soaking in methanol (pH 2)	10.01±1.38[b]	1.28±0.05[a]	ND
Microwave	12.19±1.20[ab]	0.00±0.00[b]	ND

[1] Soaking pre-treatment in 70% methanol (pH 7) for 2 h at room temperature (23˚C).
[2] Soaking pre-treatment in 70% methanol with adjustment (pH 7) for 2 h at room temperature (23˚C).
[3]Microwave pre-treatment with steam at 300W for 20 min.

*Not detected (ND). All data are expressed as mean ± standard deviation (n=3). Values with different superscripts for the given analyte in each column were significantly different at $p \leq 0.05$.

Table 5-2 Contents of sinapic acid and its derivatives (× 10^3 µg SAE*/g dry-base) in the phenolic extracts of the meal and oil fractions of untreated and microwave-treated canola and mustard seeds.

Samples	Sinapine (330 nm)	Sinapic Acid (330 nm)	Canolol (270 nm)	Unidentified phenolics (330 nm)
Meal Fraction				
Canola seed, untreated	7.31± 0.70 ^{d}	0.48 ± 0.03 ^{b}	ND	5.64 ± 0.53 ^{a}
Yellow mustard seed untreated	14.32± 1.57 ^{ab}	ND**	ND	1.08 ± 0.12 ^{c}
Oriental mustard seed untreated	10.96± 1.30 ^{c}	ND	ND	2.74 ± 0.33 ^{b}
Canola seed, microwaved	8.27± 0.84 ^{d}	0.60 ± 0.04 ^{a}	ND	5.91 ± 0.59 ^{a}
Yellow mustard seed, microwaved	14.73± 1.28 ^{a}	ND	ND	1.28 ± 0.11 ^{c}
Oriental mustard seed, microwaved	11.89± 1.08 ^{bc}	ND	ND	2.97 ± 0.27 ^{b}
Oil Fraction				
Canola seed, untreated	ND	ND	ND	ND
Yellow mustard seed untreated	ND	ND	ND	ND
Oriental mustard seed untreated	ND	ND	ND	ND
Canola seed, microwaved	ND	ND	0.37 ± 0.04 ^{a}	ND
Yellow mustard seed, microwaved	ND	ND	0.20 ± 0.05 ^{b}	ND
Oriental mustard seed, microwaved	ND	ND	0.42 ± 0.13 ^{a}	ND

All data are expressed as mean ± standard deviation. Values with different superscripts were significantly different at $p \leq 0.05$.
*Sinapic acid equivalents (SAE). **Not detected (ND).

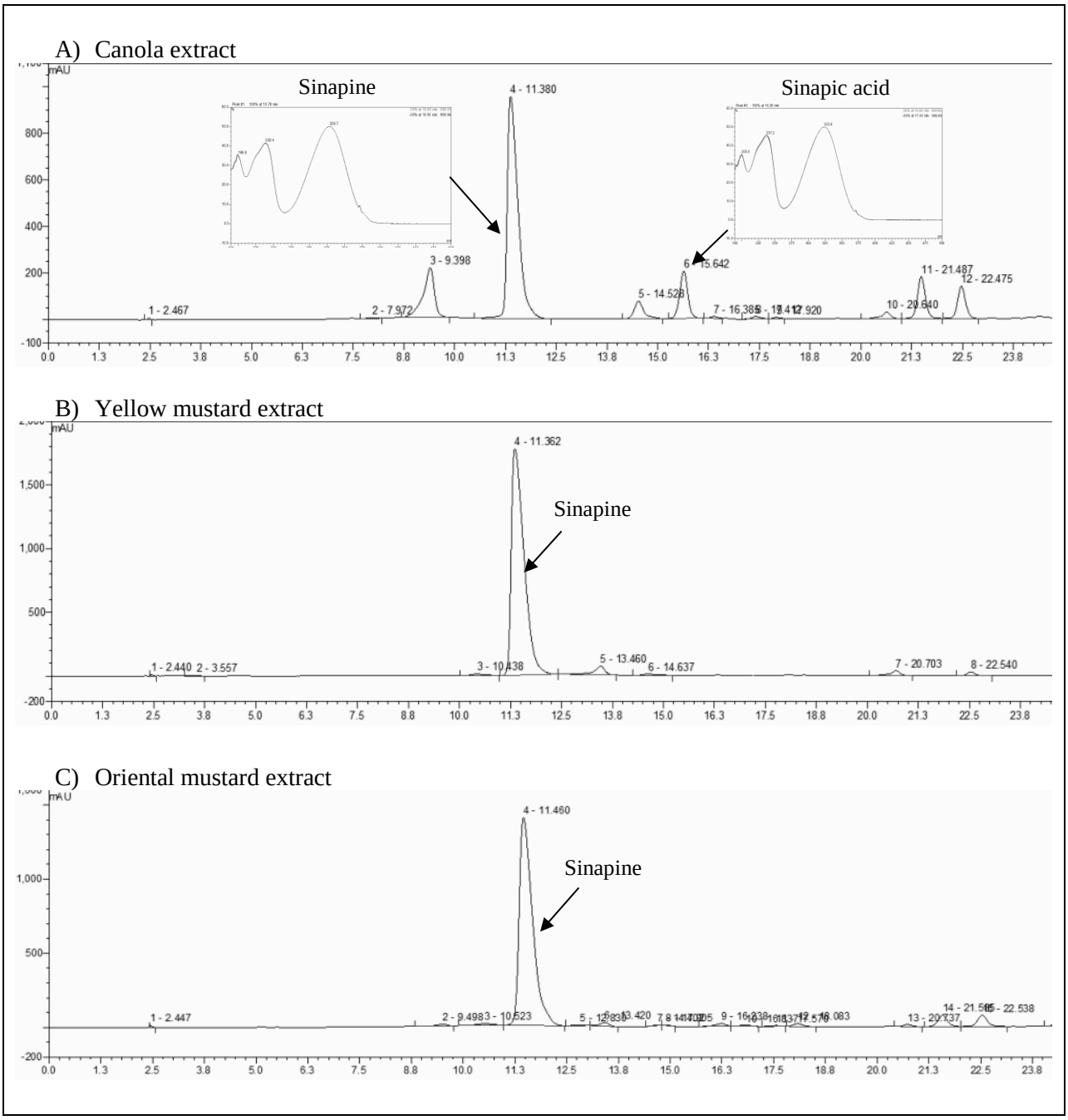

Figure 5-1 HPLC-DAD chromatograms (330nm) of phenolic extracts obtained from defatted meals of microwave treated canola seeds (A), yellow mustard seeds (B) and Oriental mustard seeds (C).

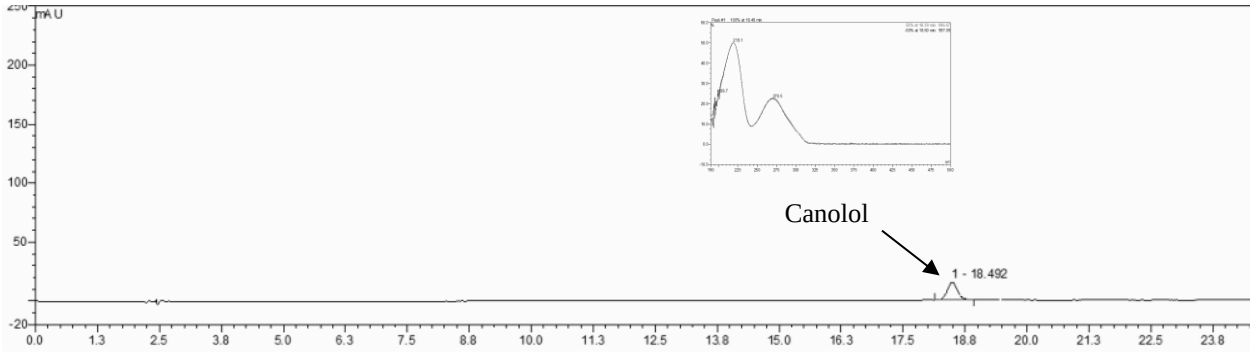

Figure 0-2 A representative HPLC-DAD chromatogram of phenolic extracts (270 nm) from oil fraction of microwave treated mustard seeds.

Chapter 6

General conclusions and future perspectives

6.1 General conclusions

Mustard is a crop grown on the Canadian prairies that comes with considerable economic value. As a part of the *Brassicacea* family, mustard species are rich sources of phenolic compounds. Like canola and rapeseed, sinapic acid derivatives, belonging to the hydroxycinnamate group, are the most important phenolic compounds in mustard. However, no documentation on the quantitative profile of sinapates and related antioxidant and antibacterial properties from commercial Canadian mustard products is available. Therefore, the current research was an attempt to fill the mentioned gaps with the following major outcomes:

- Accelerated solvent extraction using methanol is an efficient and fast method for the extraction of phenolics from mustard seed products.
- 100◦C was the best extraction temperature to recover phenolics especially sinapic acid derivatives.
- The major identified phenolic in all extracts was sinapine with relatively negligible amount of sinapic acid, with significant variation between the two seed varieties and their products.
- The extracts rich in sinapine also displayed significant radical-scavenging activity.

- Positive correlations between the sinapine concentration and antioxidant activity of various extracts, indicated sinapine as the major contributor to the antioxidant potential of the mustard products examined.
- The tested concentrations of sinapine in yellow (1200 ppm) and Oriental (1071 ppm) mustard extracts, showed no substantial antibacterial activity against all investigated strains of *E. coli* O157:H7 (02-0627, 02-0628, 02-0304, 00-3581and 02-1840) and *L. monocytogenes* (GLM-3, GLM-4, 2–243) at 35 ºC and pH 7.

6.2 Future perspectives

The research reported in this thesis investigated many novel aspects concerning the phenolic contents of two varieties of Canadian mustard seeds and their corresponding antioxidant and antimicrobial potential. However, many questions still remain to be addressed in future studies. The suggestions listed below are those which should be given priority in future research effort.

- Results of this study indicated ASE as an efficient technique for extracting phenolic compounds from mustard seed products. However, further research work, from the industrial viewpoint, is needed in order to expand the findings for food-extraction and processing applications.
- Further studies are suggested to assess the extraction efficiency, recovery yield, energy consumption and overall production cost of ASE in comparison to other novel extraction techniques such as supercritical fluid extraction.

- Further work should be done to optimise the extraction of phenolic compounds from mustard seed products using ASE with regard to various variables including extraction time, temperature and solvent concentration.
- Replacing or combining methanol with other environmentally friendly extraction solvents such as ethanol or water using ASE should be explored in future studies.
- *In vitro* assay systems confirmed mustard extracts as natural antioxidants with free radical scavenging activity. Further *in vivo* assessment is also needed to confirm the antioxidant nature of mustard phenolic extracts.
- Further work should be done to investigate the effect of mustard phenolic extracts against other food pathogens with different conditions of growth (pH, nutrient and temperature).
- Investigating the effect of chemical or enzymatic hydrolysis on the phenolic composition and subsequent alterations in antibacterial activity of mustard seed extracts is of interest.
- Considering the beneficial health properties of sinapic acid and its derivatives, mustard extracts represent attractive ingredients in the development of functional foods with health benefits.
- Further study should be done to examine the sensory properties and consumer acceptability of food products prepared with mustard extracts as natural antioxidant or antimicrobial agents.

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Appendix I

Table 1. Pearson correlation matrix (r) for Sinapine concentration (SP concn), total phenolic content (TPC) and antioxidant activity (AA) in various mustard product extracts.

Variables	SP concn	TPC	AA
SP concn	1		
TPC	0.93	1	
AA	0.90	0.72	1

Table 2 Response of five different strains of E. coli O157:H7 to phenolic extract from yellow and Oriental mustard seeds and sinapic acid standard at 21˚C (pH=5).

Bacterial (strain)	Minimum inhibitory concentration (ppm)				
			SAS		
	YMS (1200 ppm)	OMS (1071 ppm)	1200 ppm	3000 ppm	5000 ppm
E. coli O157:H7 (00:3581)	*R**	*R*	*R*	*R*	*R*
E. coli O157:H7 (02-0304)	*R*	*R*	*R*	*R*	*R*
E. coli O157:H7 (02:0627)	*R*	*R*	*R*	*R*	*R*
E. coli O157:H7 (02:0628)	*R*	*R*	*R*	*R*	*R*
E. coli O157:H7 (02:1840)	*R*	*R*	*R*	*R*	*R*

*Resistance at highest investigated concentration.

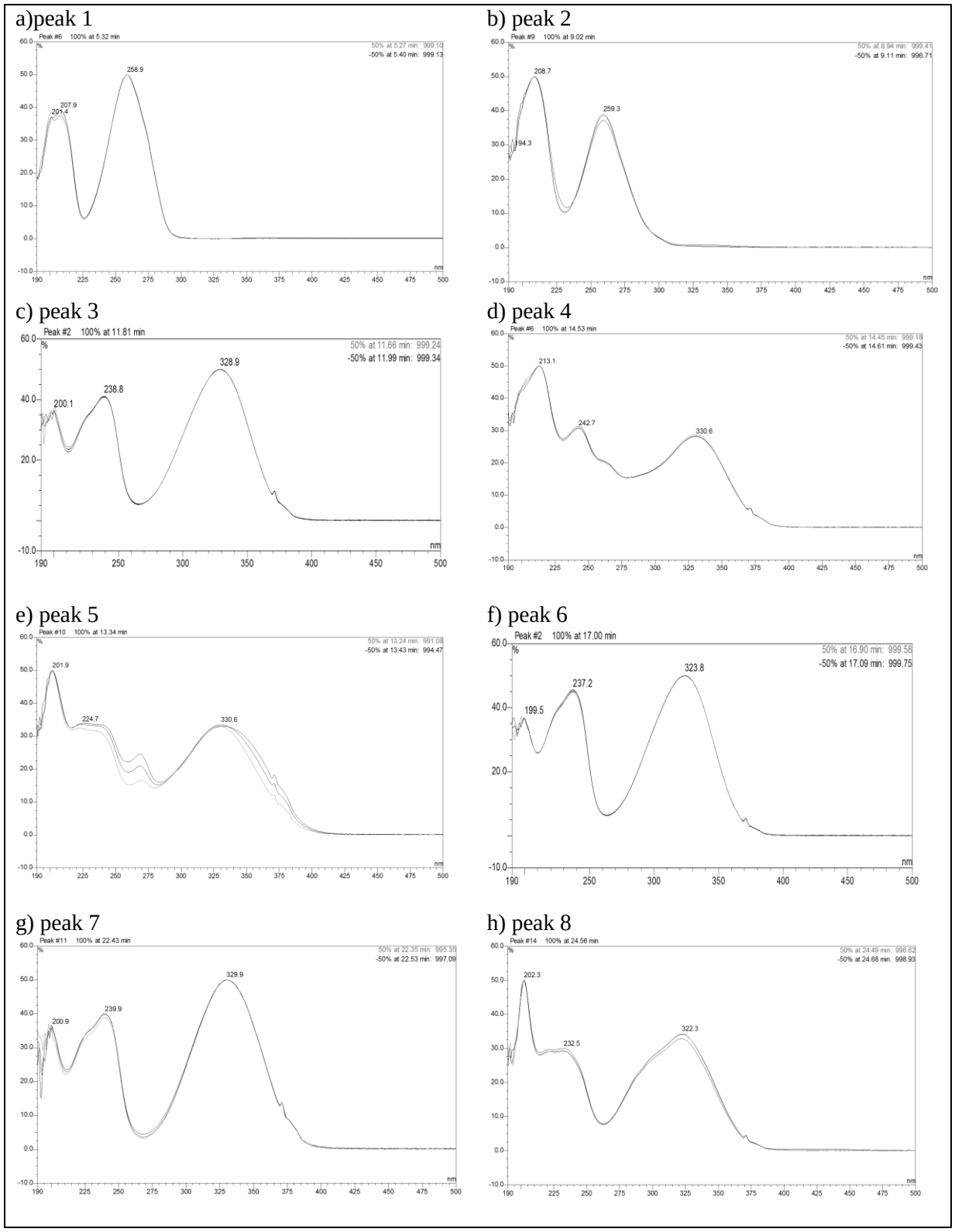

Figure 1 Absorption spectra of individual peaks showing maximum wavelength of absorption (λ_{max}).